BIOSYNTHESIS OF FUNGAL METABOLITES

by

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Dedication

To M, L, D and A

"Forgive our foolish ways"
Acknowledgments

The work described in this thesis has been carried out at the Universities of Bristol, Liverpool and Edinburgh, the Australian National University, and the National Chemical Research Laboratory, Pretoria, between 1970 and 1985. I wish to record my gratitude to all the colleagues too many to mention who over the years have contributed help, advice, ideas and support. However, special thanks must go to some particular people who have provided guidance and friendship. First to Professor Jake MacMillan who introduced me to the chemistry of fungi and for continual help and advice since. Secondly to Stan Holker with whom I took the first faltering and fondly remembered steps along the $^{13}$C pathway and who has been a constant source of ideas and provider of essential scientific critical rigor. He also encouraged me to push out the boat on my own and this process was furthered by Professor Arthur Birch who provided the environment for myself and many others to find our scientific feet. He also provided the original scientific and intellectual background for an area of research to which much of this thesis is devoted. I gratefully acknowledge stimulating collaborations with Pieter Steyn, and John Vederas and the deep friendships which have developed during these.

I must also thank the research students who have
been involved in much of this work;— Esi Bardshiri, Des Stenzel, Mike Chandler, Rupert McIntyre, Fiona Scott and Graeme Stevenson. Final and special thanks go to Suzan Kasperek for keeping the fungi growing and particularly for her invaluable help in keeping the ship afloat through many troubled waters.
This thesis describes researches carried out between 1970 and 1985 on the structural elucidation and biosynthesis of fungal metabolites. The predominant theme is the application of $^{13}$C n.m.r. spectroscopy, in conjunction with precursors containing the stable isotopes $^{13}$C, $^2$H and $^{18}$O to elucidating the details of the biosynthetic pathways responsible for the formation of metabolites of polyketide and mixed polyketide-terpenoid (meroterpenoid) origins. In addition $^{13}$C n.m.r. has been used extensively as an aid to structural elucidation; and a significant proportion of the biosynthetic studies involves the use of $^2$H n.m.r. While the earlier part of the work described concentrates on structural and stereochemical problems, the main part describes work involving (a) polyketide-derived molecules whose biosynthesis involves significant modification, either by ring cleavage or rearrangement processes, of the parent polyketide-derived precursor; and (b) a group of highly modified meroterpenoid metabolites. The latter part of the thesis describes work aimed at obtaining information on the processes involved in the early stages of polyketide chain-assembly processes.

A number of other relevant publications including reviews on biosynthetic methodology and polyketide biosynthesis are included.
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Biosynthesis of Fungal Metabolites
Introduction

The study of biosynthetic pathways received a major new impetus in the early 1970's with the advent of pulsed Fourier-transform n.m.r. spectrometers which greatly facilitated the routine determination of \(^{13}\text{C}\) n.m.r. spectra of realistically available amounts of natural products. In addition, precursors highly enriched with \(^{13}\text{C}\) and other stable isotopes were becoming more widely available. These were particularly timely developments because structures were increasingly being determined mainly by spectroscopic and other physical methods with little or no recourse to degradative chemistry, so that classical biosynthetic studies with radioisotopes necessitating extensive degradative schemes to locate the positions of incorporation of isotopic labels were becoming increasingly difficult. The increasing complexity of molecules that were targets for study meant that at best only a partial labelling pattern might be determined. These problems were to be largely overcome by \(^{13}\text{C}\)-labelling methods which again provided a biosynthetic technique which was complementary to the methods used for structure elucidation (C2).

It should be noted that the early studies using singly \(^{13}\text{C}\)-labelled precursors did not provide any information which in principle at least, could not be obtained by classical radioisotope methods: they merely (!) facilitated the determination of information such as complete labelling patterns by observation of enhancements
of individual $^{13}$C resonances. However these studies soon led to the use of precursors doubly labelled with $^{13}$C which inter alia enabled the mode of incorporation of intact biosynthetic units, and the integrity of particular carbon-carbon bonds to be established by observation of $^{13}$C-$^{13}$C spin couplings, and bond fragmentation and rearrangement processes to be detected by the loss of $^{13}$C-$^{13}$C couplings.$^1$ This in fact represented the real advance offered by $^{13}$C-labelling techniques as this type of information could not be obtained, even in principle, by classical radioisotope methods.

Subsequent and equally important developments have been the use of precursors doubly labelled with eq $^{13}$C and $^{18}$O, and $^{13}$C and $^2$H which enable the biosynthetic origins of hydrogen$^2$ and oxygen$^3$ to be determined by observation of isotope-induced shifts in $^{13}$C n.m.r. spectra; and direct $^2$H n.m.r. observation of the incorporation of label from $^2$H-enriched precursors. Besides these essentially biosynthetic techniques, n.m.r. methodology has developed to permit the rigorous assignment of spectra and of structures, both of which are essential prerequisites of biosynthetic studies.

The author has been closely involved in this area since its earliest days and the work presented in this thesis is intended to describe his involvement in the growth and development of biosynthetic studies using stable isotope methodology. For this reason the work is
presented in a more or less chronological order which means that certain molecules will be revisited as developments in methodology which permitted further information on their biosynthesis to be elucidated are discussed. The compounds studied are nearly all metabolites of the lower fungi as the requirements of high and reliable precursor incorporation rates are more easily, but not it must be emphasised always, achieved with microorganisms. The major biosynthetic pathway studied is the polyketide pathway first described by Birch and Donovan. Compounds of terpenoid origin also feature and a large part of the work concerns metabolites of mixed polyketide-terpenoid origins — the so-called meroterpenoids.

Discussion

The papers listed in section A describe work carried out during the course of PhD studies in Bristol. These studies involved the elucidation of the structure and stereochemistries of a number of diverse fungal metabolites, wortmannin (1), a highly oxygenated steroid from *Penicillium wortmannii* (A1,2), colletodiol (2)* and related macrocyclic dilactones from *Colletotrichum capsici* (A3); viopurpurin (3)* and related naphthalenoid pigments from *Aspergillus sulphureus* and *A. melleus* (A4); and colletotrichin (4), a

* As a result of more recent work the structures of colletodiol and viopurpurin have been revised to those shown.
co-metabolite of colletodiol in *C. capsici* (A5). This metabolite which is of mixed polyketide-terpenoid origins was to provide the first encounter with a group that was to provide a major theme for later work. These studies involved much $^1$H n.m.r. spectroscopy, degradative chemistry and biosynthetic studies using precursor labelled with the radioisotopes $^{14}$C and $^3$H.

In 1973 the author moved to Liverpool to carry out postdoctoral studies with J.S.E. Holker. The first problem tackled was the elucidation of the structures of a group of xanthone metabolites of *Aspergillus variecolor*, the major one being tajixanthone (5). This provided our first experience of the use of $^{13}$C n.m.r. to identify the types of carbon present in a molecule (B1). The $^{13}$C n.m.r. spectrum of tajixanthone was fully assigned on the basis of simple chemical shift and multiplicity considerations and by shift comparison studies amongst a number of closely related derivatives. After much thought and experimentation to work out the feeding protocols and the other requirements necessary to obtain significant $^{13}$C enrichment values, incorporation of [1-$^{13}$C]- and [2-$^{13}$C]acetate into tajixanthone was achieved and from the enrichments observed in the $^{13}$C n.m.r. spectra of the enriched metabolite, the origins of all the carbons in tajixanthone were deduced. This enabled a biosynthetic pathway via oxidative ring cleavage of an anthraquinone to produce an intermediate benzophenone to be proposed (B2). Further support for
Scheme 1

Me\(\text{CO}_2\text{Na}\) $\rightarrow$ \(\text{HO CO}_2\text{H}\)

Scheme 2

Me\(\text{CO}_2\text{Na}\) $\rightarrow$ \(\text{HO}_2\text{C}\)

(7)
this pathway was provided by the isolation of the varie-coxanthones, arugosins and other related co-metabolites from a number of strains of A. variecolor (B3, B4).

$^{13}$C N.m.r. was also used in the structural elucidation of multicolic acid (6) and related tetronic acid metabolites of Penicillium multicolor (B5). Their biosynthesis was studied by incorporations of singly and doubly $^{13}$C-labelled acetate to provide one of the earliest applications of this then recently described technique.\textsuperscript{1}

The observation of $^{13}$C-$^{13}$C couplings and more significantly their absence on the $^{13}$C resonances of certain carbons led to the proposal that the tetronic acids were biosynthesised via oxidative cleavage of an intermediate containing a benzenoid ring (Scheme 1). These proposals were subsequently confirmed by the incorporation of 6-pentyl-resorcylic acid (B23) and from $^{18}$O-labelling studies.\textsuperscript{9}

Incorporation of $[^{13}$C$_2$]acetate was next applied to the biosynthesis of aspyrone (7) a metabolite of Aspergillus melleus. The resultant labelling pattern (Scheme 2) suggested that its biosynthesis was either via a ring cleavage pathway or by rearrangement (B6). The rearrangement pathway was supported by the observation of a two-bond $^{13}$C-$^{13}$C coupling of 6.2 Hz between C-2 and C-7 in the $^{13}$C n.m.r. spectrum of $[^{13}$C$_2$]acetate enriched aspyrone (B7, B24). This was the first observation of such a coupling in biosynthetic studies.

At about this time the idea was conceived that the
Scheme 3

(8)

MeCO₂Na

(9)
involvement of a symmetrical intermediate at any stage in a biosynthetic pathway would result in a randomisation of labelling, e.g., of $^{13}\text{C}$$-^{13}\text{C}$ spin couplings if a double $^{13}\text{C}$-labelled precursor were used. Griseofulvin (8) was chosen as a model to test this hypothesis and $[^{13}\text{C}_2]\text{acetate}$ was incorporated using a high-yielding commercial strain of *Penicillium patulum*. However, the results obtained in this study turned out to be very complicated. Rapid metabolic turnover of exogenous acetate resulted in multiple labelling of individual molecules and therefore the observation of extensive inter-acetate and long range $^{13}\text{C}-^{13}\text{C}$ couplings in addition to the desired intra-acetate couplings. In fact, the most efficient route for incorporation of label from acetate was via the C$_1$-pool into the methoxyl carbons (B13).

However, the hypothesis was soon proved to be correct. On moving to a Research Fellowship in Canberra, a study on the xanthone metabolite ravenelin (9) was carried out. Incorporation of $[^{13}\text{C}_2]\text{acetate}$ into ravenelin in cultures of *Helmenthosporium ravenelii* resulted in the predicted randomisation of $^{13}\text{C}-^{13}\text{C}$ couplings in ring C consistent with the intermediacy of a symmetrical benzophenone intermediate (Scheme 3), itself derived from cleavage of an anthraquinone (B8). This type of information has subsequently found extensive use in biosynthetic studies (B26, B45, see also C3, C4, C5, C6, C7, C9, C10).

In this study the $^{13}\text{C}$ n.m.r. spectrum of ravenelin was
Scheme 4

(12)
partially assigned by analysis of long-range $^1$H-$^{13}$C couplings in the fully $^1$H-coupled $^{13}$C n.m.r. spectrum (B10), a technique to which the author was introduced by P.W. Westerman, and which was to form the basis of many future studies. It was and remains one of the best but underutilised methods for both structural elucidation and spectral assignment studies. This was exemplified by a study of deoxyherqueinone (10) and herqueichrysin (11), phenalenone metabolites of *Penicillium herquei* (B21). The aromatic ring system of these metabolites could be derived *a priori* by any one of three foldings of a heptaketide precursor or by numerous possible multichain condensations. Deoxyherqueinone was isolated and purified as its diacetate derivative. Due to the tautomeric possibilities in these hydroxyphenalenone structures the actual structure of this diacetate was uncertain. However, analysis of the long range $^1$H-$^{13}$C couplings by selective $^1$H irradiation and D$_2$O exchange experiments simultaneously defined the precise structure and produced an unambiguous spectral assignment. Similar studies defined the structure of herqueichrysin. Subsequent incorporation experiments with singly and doubly $^{13}$C-labelled acetates and malonate and analysis of the resultant enrichments and $^{13}$C-$^{13}$C couplings indicated formation of the phenalenone ring system as shown in Scheme 4 by a specific folding of a single heptaketide precursor (B9).
Another major study completed in Canberra finally established the structure of phomazarin (12), an aza-anthraquinone, produced by the plant pathogen Phoma terrestris, for which numerous structures had been proposed since its original isolation in 1940. The exact structures of phomazarin and its co-metabolite iso-phomazarin, including the tautomeric form of the 4-hydroxypyridine ring were established using $^{1}H-^{13}C$ couplings and in particular $^{15}N$ chemical shifts (INDOR), $^{1}H-^{15}N$, and $^{15}N-^{13}C$ couplings in derivatives of biosynthetically $^{15}N$-enriched phomazarin (B11, B17, B19). Subsequent labelling studies with $^{13}C$-labelled acetates and malonate showed that phomazarin was biosynthesised via oxidative ring fission of an anthraquinonoid intermediate itself formed by a specific folding and condensation of a single nonaketide precursor rather than the two chain pathways that had been previously proposed (B18). This study helped to confirm a suspicion that whenever a two-chain pathway is proposed in polyketide biosynthesis the possibility, however remote, of formation via a single chain modification should not be excluded. Studies on multicolic acid, aspyrone, ravenelin and tajixanthone all exemplify this.

Further work completed while in Canberra included a biosynthetic study on xanthomegnin (B14) and a systematic chemical shift assignment study on some naturally occurring naphthoquinones (B12) which made extensive use of $^{1}H-^{13}C$
Scheme 5
couplings and L.I.S. studies. The incorporation of $[^{13}\text{C}_2]$acetate into wortmannin was also studied in collaboration with Professor Jake MacMillan (B20). The results were consistent with its biosynthesis via lanosterol. In addition to the expected intra-acetate $^{13}\text{C}^{13}\text{C}$ couplings, this study also revealed readily distinguishable inter-acetate and inter-mevalonate unit couplings.

On returning to Liverpool in 1977 studies were initiated in what was to become a consuming interest: the biosynthesis of andibenin and related meroterpenoids. Andibenin (13) had been isolated from Aspergillus variecolor by R.A.W. Johnstone and its structure which was elucidated by X-ray crystallography strongly suggested a sesterterpenoid origin. However the results of incorporation experiments with $^{13}\text{C}$-labelled acetates and methionine showed this hypothesis to be incorrect. The labelling pattern which resulted was consistent with a biosynthetic pathway in which the key step was alkylation of a bis-C-methylated tetraketide derived phenolic precursor (18) by farnesyl pyrophosphate followed by further cyclisations and oxidative modifications as shown in Scheme 5 (B15). A number of closely related co-metabolites eg andilesin C (14) were isolated in the course of these studies and their structures were assigned by $^1\text{H}$ and $^{13}\text{C}$ spectral comparisons and chemical correlations (B22). Two further, structurally unrelated but biosynthetically relevant, metabolites were isolated from mutant strains of A. variecolor. These were
the bis-C-methylated tetraketide metabolite, stellatin (15) whose structure was defined almost entirely from analysis of the fully $^1$H-coupled $^{13}$C n.m.r. spectrum (B16) and the sesquiterpenoid astellolide A (16) whose structure was confirmed by X-ray crystallography (in collaboration with R.O. Gould and M.A. Walkinshaw) after moving to Edinburgh in late 1978 (B25).

A further significant metabolite was isolated along with andilesin C from another strain of A. variecolor. This was anditomin (17) whose structure was deduced by spectroscopic methods and confirmed by X-ray (B28) and whose biosynthesis was confirmed by $^{13}$C-labelling experiments (B29). It represented an important modification of the meroterpenoid pathway as it was the first metabolite in which the carbocyclic ring of the tetraketide-derived moiety had been fragmented. While this work was in progress, attention was drawn to two further metabolites whose structures could be rationalised by extensions, albeit drastic of the meroterpenoid pathway. These metabolites were austin (19) and terretonin (20) which had been isolated as toxic metabolites of Aspergillus ustus$^{11}$ and Aspergillus terreus$^{12}$ respectively. Modified terpenoid origins had been suggested for both metabolites. However, incorporation of $^{13}$C-labelled acetates and methionine gave results which supported the hypothesis that these were further examples of the meroterpenoid pathway (B30, B31).

The conclusive evidence for the meroterpenoid origins
Scheme 6

(21)  (22)
of these metabolites was provided by the synthesis (B40) of labelled 3,5-dimethylorsellinic acid (18) and its specific incorporation into andibenin (B32), and austin and terretonin (B36). This was established by $^2$H n.m.r. analysis of the metabolites enriched from feeding experiments with (18) specifically labelled with $^2$H in the 5-methyl. $^2$H N.m.r. is particularly suitable for studying the incorporation of advanced intermediates due to its low natural abundance (0.012%). The mode of incorporation of the carbon skeleton of 3,5-dimethylorsellinate into these metabolites is summarised in Scheme 6. Whereas the skeleton is incorporated intact into andibenin B and andilesin C, and suffers one bond cleavage only on incorporation into anditomin, it is fragmented to an unprecedented degree on incorporation into austin and terretonin. This was the subject of further studies described below. Further evidence for the biosynthetic relationship of austin and andibenin B was provided by the isolation of both metabolites from A. variecolor (B33). The $^{13}$C n.m.r. spectral assignment of austin and the structures of dehydroaustin and iso-austin, related metabolites isolated from A. ustus and Penicillium diversum were largely established by detailed analysis of fully $^1$H-coupled $^{13}$C spectra and $^1$H-$^{13}$C correlation experiments (B33). P. diversum produces an amazing range of metabolites Apart from iso-austin, it produced the known polyketides lichenxanthone, alternariol monomethyl ether and two new
structural types. These were the diversonolic esters (21) whose structures were elucidated by extensive $^1$H and $^{13}$C n.m.r. studies (B41), and a novel isocoumarin (22) the structure of which was confirmed by X-ray studies. This metabolite is probably biosynthesised by a novel aromatic ring contraction mechanism from the known alternariol co-metabolite.

The studies carried out up till 1981 had mainly concerned determining the origins of the carbon skeletons of metabolites. However in studying the nature of the intermediates on a biosynthetic pathway and in particular elucidating the detailed mechanisms of their interconversions, it is essential to determine the biosynthetic origins and fate of the hydrogen and oxygen atoms. The studies carried out from about 1981 onwards were more concerned with these aspects. Deuterium incorporation can be monitored directly by $^2$H n.m.r. or indirectly from isotope-induced shifts in $^{13}$C n.m.r. (C10). These two methods are essentially complementary. The isotope-induced shift techniques allow the number of deuteriums incorporated at a particular site to be monitored. Up till 1981 the $\alpha$-isotope shift method had been used notably by McInnes, but this technique requires a spectrometer capable of simultaneous $^1$H and $^2$H noise decoupling to be usefully applied. In 1981, Abell and Staunton advocated the use of $\beta$-isotope induced shifts. These are small and require high field spectrometers to resolve them.
satisfactorily but they overcome the instrumental requirements of the α-shift method. However neither of these methods provides reliable information on the stereospecificity of deuterium labelling. Although $^2$H n.m.r. spectra are disadvantaged by their inherently low dispersion and broad lines, they do have the advantage of providing information on the stereospecificity as well as regiospecificity of labelling. $^2$H N.m.r. however does not prove the number of deuteriums incorporated. At about the same time, the first biosynthetic application of $^{18}$O isotope-induced shifts in $^{13}$C n.m.r. was reported. These techniques provided the basis of much of the biosynthetic work described below.

Incorporations of $[^{13}$C$_2$]acetate and $[^2$H$_3$]acetate into tajixanthone were carried out (B26). The absence of $^2$H label on C-25 and C-5 indicated that cleavage of an anthraquinone rather than an anthrone intermediate occurred and that decarboxylation of the octaketide precursor occurs after cyclisation and aromatisation. The observed scrambling of $^{13}$C-$^{13}$C couplings in ring C implies the involvement of a symmetrical benzophenone intermediate which in turn means that ring cleavage of the anthraquinone precursor must precede introduction of the C-prenyl residue, cf ravenelin. The stereospecificity of labelling in the dihydropyran ring, however, suggests its formation from an O-prenylaldehyde intermediate by a concerted "ene" reaction (Scheme 7).
Incorporations of singly and doubly $^{13}\text{C}$-labelled acetates confirmed the formation of O-methylasparvenone (23) from specific folding and condensation of a hexaketide precursor in Aspergillus parvulus. $^2\text{H}$ N.m.r. analysis of the $[^2\text{H}_3]$acetate-enriched metabolite showed that $^2\text{H}$ label was incorporated specifically into the 10-methyl, 5-, 2-axial, and 3-axial hydrogens with none at C-4. This indicated that oxygen is introduced at C-4 by an aromatic hydroxylation process with an accompanying NIH shift of hydrogen from C-4 to C-3 and that reduction from the naphthalene to the dihydroaromatic level occurs with stereospecific introduction of hydrogen at C-2 and C-3 (B27). In a further study, incorporation of $[1-^{13}\text{C}, ^2\text{H}_3]$acetate and analysis of the $^2\text{H}$ β-isotope shifts in the resultant $^{13}\text{C}$ n.m.r. spectrum showed that one hydrogen was lost from the C-10 methyl to indicate formation of the ethyl moiety by a reduction-elimination-reduction sequence on the corresponding acetyl group. The magnitude and direction of the β-isotope shifts were observed to depend markedly on the functionality of the reporter $^{13}\text{C}$ nucleus and surprisingly on the stereospecificity of $^2\text{H}$ incorporation (B38). For carbonyl groups the observed shift could be downfield or even zero in contrast to the usually observed upfield shifts, thus indicating the necessity for caution in the interpretation of results when carbonyl groups are involved. The incorporation of $^2\text{H}$ label from $[^2\text{H}_3]$acetate into the similar dehydro-
naphthalene scytalone (24) in Phialaphora lagerbergii was also studied. In contrast to 9-methylasparvenone, reduction of the aromatic ring was not stereospecific (B42).

In 1981, three months were spent working with Pieter Steyn at the National Chemical Research Laboratory in Pretoria on the biosynthesis of aflatoxin B₁ and other mycotoxins. Although averufin (25) was generally held to be an early intermediate on the biosynthetic pathway to aflatoxin B₁ (27), this had never been rigorously established. Accordingly, [4'-2H₂]averufin was prepared and incorporated into aflatoxin B₁ by cultures of Aspergillus flavus. ²H N.m.r. analysis showed that the ²H label was incorporated specifically at C-16 of aflatoxin B₁ (B34). The incorporation of ²H from [1-¹³C,²H₃]acetate into averufin, sterigmatocystin (26) and aflatoxin B₁ was studied both by direct ²H n.m.r. analysis and by observation of β-isotope shifts. The results showed that one ²H was incorporated stereospecifically into the C-2' and C-4' positions of the side chain of averufin (B35) and that these ar retained on conversion of the C₆ side chain into the C₄-bisfuranoid side chain of the aflatoxins (B39). Other important observations included the retention of ²H label at C-6 of sterigmatocystin so ruling out mechanisms for xanthone ring formation requiring introduction of a phenolic hydroxyl group on this carbon (B37). However the appearance of ²H at C-4 of aflatoxin B₁ shows that such a hydroxylation with accompanying NIH shift does occur in the
biosynthesis of aflatoxin B_1 (B39). Further work carried out in Pretoria involved the structural elucidation (B47) and biosynthesis (B48) of the asticolorins eg (28) novel mycotoxins produced by *Aspergillus multicolor* whose biosynthesis proceeds via oxidative coupling of orcinol moieties with introduction of a highly modified prenyl substituent.

At this same time a major collaborative programme was initiated with John Vederas to apply ^18_0-labelling studies to problems in polyketide biosynthesis. The first compound studied was tajixanthone due to a joint interest in the mechanisms of xanthone ring closure. ^18_0 Isotope shifts observed in the ^13_ C n.m.r. spectrum of tajixanthone isolated from *A variecolor* grown under an atmosphere containing ^18_0_2 were consistent with the intermediacy of a symmetrical benzophenone and a ring closure mechanism via a Michael addition-elimination process in which a ring C hydroxyl attacks ring A. Mass spectral analysis of tajixanthone produced in a mixture of ^18_0_2 and ^16_0_2 showed that each aerobically-derived oxygen atom was derived separately by mono-oxygenation so that ring cleavage mechanisms involving dioxygenases could be ruled out (B50).

The origins of the oxygen atoms in the meroterpenoid metabolites were then studied by incorporation experiments in the presence of ^18_0_2 and [1-^13_ C, ^18_0_2]acetate, to try to elucidate information on the mechanisms by which the extensive modifications observed for the orsellinate-derived
Scheme 8 - The Fungal Meroterpenoid Pathway
moiety in austin and terretonin occurred and also for the formation of the spiro-δ-lactone systems in andibenin B and austin. Whereas 18O label from acetate was successfully incorporated into andibenin B (B51), the low level of incorporation obtained precluded the observation of the necessary isotope shifts for andilesin A and austin (B49). Nonetheless the results from incorporation of label from 18O2 into austin were consistent with a modification scheme in which the orsellinate-moiety undergoes a ring contraction via an α-ketol rearrangement followed by biological Baeyer-Villiger type of oxygen insertions to form the δ-lactone moieties found in both the polyketide and terpenoid derived portions of the molecule (B49). The problem of low incorporation of labelled acetate was overcome by synthesising 3,5-dimethylorsellinate doubly labelled with 13C and 18O in both the carboxyl and at the C-6 position. This was incorporated with high efficiency into austin (B54) to confirm the 18O2 results, and also into andilesin A to rule out the possible involvement of deoxyorsellinate intermediates in the biosynthesis of the andibenins and andilesins (B55).

Interestingly, further metabolites related to austin have been isolated from Emericella dentata,17 and two unrelated metabolites which are almost certainly further products of the meroterpenoid pathway, fumigatonin (29) and paraherquonin (30) have been isolated from Aspergillus fumigatus18 and Penicillium paraherquei.19 It is of
interest to note that studies which were initiated on the mistaken assumption of a sesterterpenoid origin for andibenin B have led to the unravelling of a complex, novel and now apparently wide spread biosynthetic pathway. The meroterpenoid pathway as it stands at the present time is summarised in Scheme 8. This will clearly be an area in which much biosynthetic and synthetic work will be carried out in the future.

The work described above on O-methylasparvenone was initiated due to an interest in the biosynthesis of polyketide-derived molecules containing an ethyl side chain. This also prompted an investigation of LL-D253α (32), one of several closely related chromanones produced by Phoma spp and other plant pathogens. In the course of $^{13}$C assignment studies it became apparent that the previously assigned structure (31) was incorrect and analysis of the fully $^1$H-coupled $^{13}$C spectrum effectively defined structure (32), which was confirmed by synthesis (B44). Incorporation of singly and doubly $^{13}$C-labelled acetates revealed that while the hydroxyethyl side chain was derived from an intact acetate unit, label from C-1 or C-2 of acetate was partially randomised between the two carbons. Subsequent incorporation experiments with [1-$^{13}$C, $^2$H$_3$]- and [1-$^{13}$C, $^{18}$O$_2$]acetates and $^{18}$O$_2$ gave results (B45) that were consistent with derivation of LL-D253α by a two-chain condensation and indicated that the randomisation of label occurred via alternate hydrolytic ring openings of a cyclopropyl inter-
mediate (33).

The success of these methods in revealing subtle biosynthetic information encouraged a re-examination of the formation of aspyrone (B52). Experiments with \([1-^{13}C,^{18}O_2]\)acetate and \(^{18}O_2\) revealed the surprising result that none of the oxygens were derived from acetate, three being derived from the atmosphere and one from the medium. A pathway involving epoxide-mediated rearrangement and ring closure reactions was proposed to explain these results, thus providing a relatively simple model for similar processes which appear to be involved in the formation of the much more complex polyether and ionophore antibiotics.

A longstanding problem is the exact relationship between polyketide biosynthesis and the corresponding pathway in primary metabolism viz fatty acid biosynthesis. \(^{18}O\) and \(^2H\) labelling studies on appropriate polyketide-derived molecules permit indirect information on the processes which must be occurring on the polyketide synthetase enzymes to be obtained and compared with the much better understood processes catalysed by fatty acid synthetases. These studies required molecules with intermediate oxidation levels between the highly oxygenated fully aromatic polyketides and fatty acids to be examined. Two ideal molecules for this were monocerin (34) and colletodiol (2). These have been studied in Dreschera ravenelii and a Cytospora sp which is a more reliable colletodiol producer than C. capsici used in earlier work.
Scheme 9
The results of these studies (B46, B53) have provided an insight into the stepwise manner in which the enzyme-bound polyketide precursors are elaborated by the polyketide synthetases and necessitate a revision of the classical picture of polyketide chain assembly (C11). At its simplest, poly-β-ketide intermediates are built up by a cyclic process analogous to fatty acid biosynthesis but omitting the reduction-elimination-reduction sequence responsible for the loss of acetate oxygen. While some aromatic metabolites do retain the full oxidation level of a poly-β-ketide, most metabolites show varying degrees of reduction and/or deoxygenation and an increasing body of evidence suggests that this occurs by processes analogous to fatty acid biosynthesis before the initial release of metabolites or intermediates from the chain-assembly enzymes. Thus path (a) in Scheme 9 would simply produce poly-β-ketides but by invoking paths (b), (c) and (d) intermediates with varying degrees of reduction may be formed. The studies described above enable valuable indirect information on these processes to be obtained and while studies with stable isotopes in whole cells will certainly continue to make a major contribution for many years to come, it is clear that the way forward to obtaining a complete understanding of the mechanisms of polyketide biosynthesis and the nature of the control mechanisms which regulate the cycling process around the component enzymes of the polyketide synthetase complexes will require recourse to
cell-free systems and purified enzymes. This is the challenge for the future.

References
List of Publications

The publications are listed in three sections. Section A contains research publications arising from work carried out during PhD studies. Section B contains the main body of research papers arising from subsequent work. Section C lists a number of other publications including reviews and published conference proceedings. Attention is drawn to the substantial reviews on biosynthetic applications of $^{13}$C n.m.r. (C2, C10) and the series of reviews on polyketide biosynthesis (C3, C4, C6, C7, C9).
Papers arising from postgraduate researches


B Research papers describing studies relating to the biosynthesis of fungal metabolites


42. $^{13}$C and $^2$H Labelling Studies on the Biosynthesis of Scytalone in Phialaphora lagerbergii, E. Bardshiri and T.J. Simpson, Tetrahedron, 1983, 39, 3539.


53. Studies of Polyketide Chain Assembly Processes: 
Origins of the Hydrogen and Oxygen Atoms in Colletodiol, 

54. Biosynthesis of the Meroterpenoid Austin, by Aspergillus 
ustus. Synthesis and Incorporation of $^{13}$C, $^{18}$O-
labelled 3,5-Dimethylorsellinate, F.E. Scott, T.J. 

55. Biosynthesis of the Meroterpenoid Metabolite, Andilesin A 
by Aspergillus variecolor: origins of the Oxygen Atoms, 
C.R. McIntyre, F.E. Scott, T.J. Simpson, L.A. Trimble 
C Reviews, Conference proceedings etc


9. Biosynthesis of Polyketides, T.J. Simpson, 
    *Natural Product Reports*, 1985, 2, 321.

10. "$^{13}$C N.m.r. in Metabolic Studies", T.J. Simpson, 
in "Modern Methods of Plant Analysis", Volume 2, 
    eds H.F. Linskens and J.F. Jackson, Springer-Verlag, 
    1986, pp 1-42.

11. "Studies of Polyketide Chain-Assembly Processes", 
    T.J. Simpson in "Mycotoxins and Phycotoxins", 
    eds P.S. Steyn and R. Vleggaar, Elsevier, 1986, 
    pp 85-96.
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1972

Fungal Products. Part II. Structure and Stereochemistry of the Acid C_{18}H_{18}O_{5}, a Degradation Product of Wortmannin

Fungal Products. Part II.1 Structure and Stereochemistry of the Acid C_{18}H_{16}O_{5}, a Degradation Product of Wortmannin


Degradation of the fungal metabolite, wortmannin, by dilute mineral acid yields, inter alia, an acid C_{18}H_{16}O_{5} to which structure (Ia) is assigned from the spectroscopic properties of its derivatives. The assigned structure (Ia) is supported by dehydrogenation of the methyl ether methyl ester (Ia) to methyl 2,3-dihydro-10-methoxy-6-methyl-3-oxo-1H-cyclopenta[7,8]naphtho[2,3-b]furan-7-carboxylate (VII), a previously described degradation product of the fungal metabolite, viridin (VI; X = O).

Wortmannin was first isolated from culture filtrates of Penicillium wortmannii Klocke by Brian et al.2 but not characterized by them. In our hands wortmannin gave inconsistent combustion analyses but the molecular formula, C_{23}H_{20}O_{8}, was established by high resolution mass spectrometry. On treatment with boiling 2N-hydrochloric or -sulphuric acid, the metabolite yielded acetic acid (1 mol. equiv.), methoxyacetaldehyde (0.5 mol. equiv.), an acid C_{18}H_{16}O_{5} (0.5 mol. equiv.) and an acid C_{21}H_{22}O_{7} (0.5 mol. equiv.). This paper presents evidence3 that the acid C_{18}H_{16}O_{5} has the structure (Ia). Anticipating subsequent papers showing that wortmannin has the structure (IV) and that it has a steroidal biogenesis, we use the numbering system shown in formula (I) for the acid (Ia) and its derivatives.

From the reaction of wortmannin and 2N-mineral acid, the acid C_{18}H_{16}O_{5} was obtained as the free acid (Ta), the methyl ester (Ib), or the ethyl ester (Ic) depending upon whether the reaction was performed in aqueous, methanolic, or ethanolic acid. For convenience the methyl ester (Ib) was used for the structure determination. Functional derivatives of the methyl ester [compounds (I)], of the dihydro-methyl ester [compounds (II)], and of the tetrahydro-methyl ester [compounds (III)] were prepared as outlined in Scheme 1. The spectroscopic data are shown in Tables 1—5.

The i.r. spectra (Table 1) indicated the presence of benzenoid and furanoid rings in derivatives of types (I) and (II) and of a benzenoid, but no furanoid ring, in the tetrahydro-derivatives (III). These data further indicated the presence of one hydroxy- and two carbonyl groups in the methyl ester (Ib) itself. The hydroxy-group exhibited phenolic properties and the acetates (Ig, k, and n), (IIg), and (IIIg) showed carbonyl absorption typical of phenyl acetates. The presence of a benzenoid ring was also evident from the n.m.r. spectra (Tables 2—4), which contained typical aryl methyl absorption (10-Me) at 7.1—7.5 for all derivatives and typical aryl methyl ether singlets (7-OMe) around 6.0.


TABLE 1
I.r. absorption data (cm⁻¹) for the acid (Ia) and derivatives (Nujol mulls)

<table>
<thead>
<tr>
<th>Compd.</th>
<th>v(OH)</th>
<th>v(C-H)</th>
<th>v(C=C)</th>
<th>v(CC)</th>
<th>v(C=O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ia)</td>
<td>3480</td>
<td>* 1530</td>
<td>1629, 1590</td>
<td>1726, 1710</td>
<td></td>
</tr>
<tr>
<td>3300-2600</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ib)</td>
<td>3450-3200</td>
<td>3150</td>
<td>1550</td>
<td>1620, 1590</td>
<td>1725</td>
</tr>
<tr>
<td>(Ic)</td>
<td>3600</td>
<td>3150</td>
<td>1520</td>
<td>1620, 1590</td>
<td>1730br</td>
</tr>
<tr>
<td>(Id)</td>
<td>3400-3200</td>
<td>3150</td>
<td>1550</td>
<td>1605</td>
<td>1740, 1720</td>
</tr>
<tr>
<td>(If)</td>
<td>3400-2500</td>
<td>3160s</td>
<td>1550s</td>
<td>1620, 1580</td>
<td>1768, 1741, 1728</td>
</tr>
<tr>
<td>(Ig)</td>
<td>3400, 3200</td>
<td>3125</td>
<td>1540</td>
<td>1620, 1590</td>
<td>1710</td>
</tr>
<tr>
<td>(Ih)</td>
<td>3500</td>
<td>3140</td>
<td>1600</td>
<td>1710</td>
<td>1720</td>
</tr>
<tr>
<td>(Ii)</td>
<td>3400</td>
<td>3140</td>
<td>1550</td>
<td>1700</td>
<td>1720</td>
</tr>
<tr>
<td>(Ik)</td>
<td>3150s</td>
<td>1555s</td>
<td>1620w, 1590w</td>
<td>1770, 1742, 1725</td>
<td></td>
</tr>
<tr>
<td>(II)</td>
<td>3500-3200</td>
<td>3050</td>
<td>1580w</td>
<td>1630w, 1600w</td>
<td>1720w, 1595w, 1770, 1740</td>
</tr>
<tr>
<td>(Ih)</td>
<td>3500</td>
<td>* 1570</td>
<td>1610, 1590</td>
<td>1715</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 derivatives showed that the benzene ring was fully substituted. The n.m.r. spectra of the derivatives (I) indicated a cis-disubstituted double bond. The vinylic AB-system (11-H and 12-H) had a $J_{AB}$ value typical of a cyclohexene, showed no other coupling, and was not present after hydrogenation to the derivatives (II) and (III). Conjugation of this double bond to the aromatic system was revealed by the differences between the u.v. spectra (Table 5) of the derivatives (I) and those of their dihydro-counterparts (II). The u.v. data also showed that the furan ring was conjugated to the benzene ring; the dihydro-triol (III), in which both the methoxycarbonyl and olefinic functions were reduced, possessed the absorption of a highly substituted benzofuran. Also the tetrahydro-derivatives in which the furan ring was reduced (see later) showed the absorption expected of a fully substituted catechol.

The low-field one-proton singlet below 2 ppm in the n.m.r. spectra of compounds (Ia—k) and (IIb, d, g, and h) could be assigned to the furanoid 2-proton, deshielded by the adjacent methoxycarbonyl group. Warburgin (V) contains a similar proton absorbing at $\tau$ 1.96. In agreement with this assignment, reduction of the methyl ester grouping with lithium aluminium hydride ($\text{LiAlH}_4$) caused an upfield shift ($\Delta \delta$ 0.5 p.p.m.)

of this singlet to higher field (e.g., \( \tau 2-23 \)); the similar proton in viridiol (VI; \( X = H, \beta-\text{OH} \)) absorbs at \( \tau 2-23 \). In the tetrahydro-derivatives (III) the low-field singlet of the 20-proton is replaced by an AB-system of the 20-protons further coupled to the 4-proton. Thus the furan ring is reduced in the tetrahydro-derivatives (III).

Reduction of the methoxy carbonyl and olefinic functions caused upfield shifts of 0.14-0.20 and 0.12-0.4 p.p.m., respectively, in the n.m.r. signal of the arylethyl group, indicating the proximity of these two groups. The proximity of the tertiary methyl group in the methyl ester (Ib) to both the olefinic double bond and the second carbonyl group was likewise inferred from the n.m.r. data. Thus, in \( \text{C}_6 \text{D}_5 \text{N} \) solution, the tertiary methyl signal moved upfield by about 0.10 p.p.m. on hydrogenation of the olefinic double bond in compounds (Ib, h, and e) and it moved downfield by 0.25 p.p.m. on reduction of the carbonyl group in compounds (Ib) and (Iib). The new one-proton triplet in the n.m.r. spectra of the borohydride reduction products (Iib—n), (IIIb, l) was assigned.

### Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>20-H</th>
<th>11-H</th>
<th>12-H</th>
<th>16-H</th>
<th>17-H</th>
<th>3-OMe</th>
<th>7-OMe</th>
<th>13-Me</th>
<th>10-Me</th>
<th>Other signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ia) ( f )</td>
<td>1.85</td>
<td>3.23</td>
<td>5.55</td>
<td>6.90</td>
<td>7.0-7.2</td>
<td>6.12</td>
<td>9.26</td>
<td>7.34</td>
<td>5.6 (OH)</td>
<td></td>
</tr>
<tr>
<td>(Ib) ( f )</td>
<td>1.83</td>
<td>3.20</td>
<td>5.66</td>
<td>6.74</td>
<td>6.12</td>
<td>9.18</td>
<td>7.30</td>
<td>5.6 (OH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ib) ( f )</td>
<td>1.81</td>
<td>3.15</td>
<td>5.44</td>
<td>6.60</td>
<td>6.27</td>
<td>9.05</td>
<td>7.18</td>
<td>5.66 and 8.61 (OEt); 4.56 (OH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ic)</td>
<td>1.86</td>
<td>3.25</td>
<td>5.27</td>
<td>6.60</td>
<td>6.10</td>
<td>5.98</td>
<td>9.20</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Id)</td>
<td>1.80</td>
<td>3.20</td>
<td>5.60</td>
<td>6.80</td>
<td>6.10</td>
<td>5.90</td>
<td>9.15</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ie)</td>
<td>1.88</td>
<td>3.25</td>
<td>5.60</td>
<td>6.80</td>
<td>6.10</td>
<td>5.90</td>
<td>9.15</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(If)</td>
<td>1.88</td>
<td>3.25</td>
<td>5.60</td>
<td>6.80</td>
<td>6.10</td>
<td>5.90</td>
<td>9.15</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(If)</td>
<td>1.88</td>
<td>3.25</td>
<td>5.60</td>
<td>6.80</td>
<td>6.10</td>
<td>5.90</td>
<td>9.15</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ig)</td>
<td>1.88</td>
<td>3.25</td>
<td>5.60</td>
<td>6.80</td>
<td>6.10</td>
<td>5.90</td>
<td>9.15</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ih)</td>
<td>1.88</td>
<td>3.25</td>
<td>5.60</td>
<td>6.80</td>
<td>6.10</td>
<td>5.90</td>
<td>9.15</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Ii)</td>
<td>1.88</td>
<td>3.25</td>
<td>5.60</td>
<td>6.80</td>
<td>6.10</td>
<td>5.90</td>
<td>9.15</td>
<td>7.30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For \( \text{CDCl}_3 \) solutions except where stated otherwise. \( f \) Singlet. \( d \) Triplet \( (f 9-0-9) \). \( a \) For \( [\text{H}_4] \text{acetone} \) solution. \( b \) For \( [\text{H}_4] \text{pyridine} \) solution.

### Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>20-( H )</th>
<th>3-( \text{OMe} )</th>
<th>13-( \text{Me} )</th>
<th>16-( \text{Me} )</th>
<th>Other signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Iib)</td>
<td>1.88</td>
<td>6.12</td>
<td>9.19</td>
<td>7.41</td>
<td></td>
</tr>
<tr>
<td>(Iib) ( f )</td>
<td>1.88</td>
<td>6.27</td>
<td>9.18</td>
<td>7.30</td>
<td></td>
</tr>
<tr>
<td>(Iid)</td>
<td>1.83</td>
<td>6.10</td>
<td>9.20</td>
<td>7.37</td>
<td>6.00 ( (7-\text{OMe}) )</td>
</tr>
<tr>
<td>(Iig)</td>
<td>1.85</td>
<td>6.10</td>
<td>9.19</td>
<td>7.30</td>
<td>7.59 ( (7-\text{OMe}) )</td>
</tr>
<tr>
<td>(Iii)</td>
<td>1.82</td>
<td>6.28</td>
<td>9.12</td>
<td>7.26</td>
<td>5.90 ( (17-H) )</td>
</tr>
<tr>
<td>(IIIb)</td>
<td>2.23</td>
<td>6.02</td>
<td>9.22</td>
<td>5.90</td>
<td>7.50 ( (17-H) )</td>
</tr>
</tbody>
</table>

* For \( \text{CDCl}_3 \) solutions except where otherwise indicated. \( a \) In \( \text{C}_6 \text{D}_5 \text{N} \). \( b \) Singlet. \( c \) Triplet \( (f 9-6) \).

### Table 4

<table>
<thead>
<tr>
<th>Compound</th>
<th>20-( H )</th>
<th>4-( H )</th>
<th>3-( \text{OMe} )</th>
<th>13-( \text{Me} )</th>
<th>17-( \text{Me} )</th>
<th>Other signals</th>
</tr>
</thead>
<tbody>
<tr>
<td>(IIIb)</td>
<td>5.14 ( (J 4 \text{and} 9) )</td>
<td>5.74 ( (J 4 \text{and} 9) )</td>
<td>6.20</td>
<td>9.18</td>
<td>7.85</td>
<td>7.69 ( (7-\text{OMe}) )</td>
</tr>
<tr>
<td>(IIIg)</td>
<td>5.14 ( (J 4 \text{and} 9) )</td>
<td>5.72 ( (J 4 \text{and} 9) )</td>
<td>6.25</td>
<td>9.18</td>
<td>7.85</td>
<td>7.69 ( (7-\text{OMe}) )</td>
</tr>
<tr>
<td>(IIIf)</td>
<td>5.39 ( (J 9 \text{and} 9) )</td>
<td>6.14</td>
<td>9.09</td>
<td>7.68</td>
<td>7.65</td>
<td>7.69 ( (7-\text{OMe}) )</td>
</tr>
<tr>
<td>(IIIh)</td>
<td>5.12 ( (J 4 \text{and} 9) )</td>
<td>5.74 ( (J 4 \text{and} 9) )</td>
<td>6.25</td>
<td>9.18</td>
<td>7.85</td>
<td>7.69 ( (7-\text{OMe}) )</td>
</tr>
<tr>
<td>(IIIi)</td>
<td>5.40 ( (J 9 \text{and} 9) )</td>
<td>5.90</td>
<td>7.84</td>
<td>7.69 ( (7-\text{OMe}) )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* For \( \text{CDCl}_3 \) solutions except for (IIIh) \( (\text{in} \ [\text{H}_4] \text{pyridine}) \). \( b \) In overlapping multiplets.

The environment of the 14-hydrogen atom was established from the n.m.r. spectra of compounds (Ia—n) (see Table 2). The chemical shift was typical of a benzylic carbon.

### References


methine proton and its triplet or quartet multiplicity revealed the presence of two vicinal hydrogen atoms, the resulting AMX system being clearly identified in the 220 MHz spectrum (Figure) of the dideuterio-compound (Ie) in benzene solution. A comparison of this spectrum with the 220 MHz spectrum of the undeuteriated compound (Id) showed that the 15-hydrogen atoms were further coupled to the exchangeable 16-hydrogen atoms in the methyl ether methyl ester (Id). This information provided final proof for the five-membered ring.

<table>
<thead>
<tr>
<th>Compound</th>
<th>λ_max, nm (a)</th>
<th>(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ib)</td>
<td>233.5</td>
<td>262 (17,280)</td>
</tr>
<tr>
<td>(IIb)</td>
<td>208 (41,430)</td>
<td>235 (17,270)</td>
</tr>
<tr>
<td>(IIIb)</td>
<td>208 (28,600)</td>
<td>234 * (8510)</td>
</tr>
<tr>
<td>(Id)</td>
<td>236 (34,000)</td>
<td>256 (18,700)</td>
</tr>
<tr>
<td>(IId)</td>
<td>208 (44,400)</td>
<td>233 (21,540)</td>
</tr>
<tr>
<td>(Ig)</td>
<td>235.5</td>
<td>273.5</td>
</tr>
<tr>
<td>(IIg)</td>
<td>210 (23,240)</td>
<td>225 (12,000)</td>
</tr>
<tr>
<td>(IIIg)</td>
<td>206 (30,500)</td>
<td>232 * (6580)</td>
</tr>
<tr>
<td>(Ih)</td>
<td>233 (33,000)</td>
<td>262.5</td>
</tr>
<tr>
<td>(IIh)</td>
<td>210 (40,150)</td>
<td>238 (15,950)</td>
</tr>
<tr>
<td>(IIIh)</td>
<td>209.5</td>
<td>232 * (8250)</td>
</tr>
<tr>
<td>(II)</td>
<td>246 (25,000)</td>
<td>273 (15,000)</td>
</tr>
<tr>
<td>(III)</td>
<td>221 (37,500)</td>
<td>261 (11,000)</td>
</tr>
<tr>
<td>(III)</td>
<td>209.5</td>
<td>236 * (8080)</td>
</tr>
</tbody>
</table>

* Shoulder.

The absolute stereochemistry, shown in structure (I) for C/D ring junction was established by the o.r.d. behaviour of the dihydro-derivative (Ida). The molecular amplitude (+134°) was typical for trans-14α-17-oxosteroids, such as 3β-hydroxy-5α-androstan-17-one (+138°); that for cis-14β-17-oxosteroids is much lower (ca. +35°).

The foregoing chemical and spectroscopic data provided conclusive evidence for structure (Ib) for the methyl ester obtained by degradation of wortmannin. This structure (Ib) was, however, confirmed by dehydrogenation of the methyl ester methyl ether (Id) to the naphthofuran (VII), identical with the methyl ether methyl ester obtained by methylation of the hydrogen peroxide oxidation product of the fungal metabolite, viridin (VI; X = O).

The mass spectral fragmentation of ring D in steroids has been studied in detail by Tokes et al. As shown in Scheme 2 (path a) the ion \( M^+ - (41 + R) \) involves two hydrogen transfers (18 → 17 and 16 → 18), followed by fission of the 14,15-bond. The ion \( M^+ - (42 + R) \) arises by hydrogen transfer to the uncharged fragment in a random process from the 8β-, 12β-, 14α-, and 18-positions but mainly from the 14α-position (path b) to give either the unarranged (path c) or rearranged (path d) ions shown in the Scheme. In the case of the wortmannin derivative (Id), formation of the \( M^+ - (41 + R) \) (i.e. \( M^+ - 56 \) ion does not involve transfer of hydrogen from the 16-position, since both deuterium atoms in the derivative (Ie) remain with the unchanged fragment. Also in the formation of the \( M^+ - 57 \) (i.e. \( M^+ - (42 + R) \)) ions from the derivatives (I) hydrogen must be exclusively transferred from position 18. The high abundance of the \( M^+ - 56 \) and \( M^+ - 57 \) ions in the spectra of the derivatives (I) is ascribed both to the

alylic nature of positions 13 and 14 which facilitate fission of the 13,17- and 14,15-bonds and to the aromaticity of the resulting ion. Thus these ions are of insignificant intensity in the spectra of the corresponding dihydro-derivatives (II).

Other notable fragmentations of the derivatives (I) - (III) are listed but, in the absence of definitive evidence, speculative fragmentation pathways are not presented. Of the derivatives (I), the 17-ketones are distinguished from their 17-hydroxy-analogues by the presence of $M^+ - 43$ ions, shown by mass-matching to occur by loss of $C_2H_3O$. Since this loss is also shown by the dideuterio-compound (Ie) and by the acid (Ia), this fragmentation probably represents a rearrangement involving the elimination of the 17-carbonyl and 18-methyl groups. The 17-hydroxy-derivatives (Ii, i, and m) and (IIh and I) show intense $M^+ - 44$ ions with accompanying intense ions at m/e 43 in the case of compounds (Ii, i, and m). Metastable ions for the transition $M^+ \rightarrow M^+ - 44$ were observed in each case. The $M^+ - 44$ ions show metastable ions for their fragmentation to $M^+ - 59$ ions and their formation apparently involves the 17-hydroxy-group since the 17-methoxy-compounds show an intense $M^+ - 58$ ion in place of the $M^+ - 44$ ion. The dihydro-ketones show substantial $M^+ - 69$ and $M^+ - 71$ ions while the corresponding dihydro-17-hydroxy-derivatives show $M^+ - 71$ and $M^+ - 73$ ions. The four tetrahydro-derivatives (IIIh, i, l, and g) gave intense ions at m/e 161 (C$_{10}$H$_{14}$O$_2$), 85, and 83. Their spectra also contained ions corresponding to $M^+ - R'$. 

**EXPERIMENTAL**

M.p.s were determined on a Kofler hot-stage apparatus and are corrected. N.m.r. spectra were determined with tetramethylsilane as internal standard on Varian HA100 and Varian HA220 instruments. U.v. spectra were obtained for solutions in ethanol on a Unicam SP 800 spectrometer. I.r. spectra were obtained for probe samples on an A.E.I. MS9 instrument. G.l.c. was performed on a Pye 104 chromatograph with glass columns (5 ft x 0.25 in) packed with 2% SE-33, 2% QF-1 or 2% OV-1 coatings on acid-washed and silanised Gas-Chrom A.

**Acid Hydrolyses of Wortmannin.**—(a) With aqueous hydrochloric acid in methanol. Wortmannin (20 g), methanol (30 ml), and 1-5% hydrochloric acid (25 ml) were boiled for 5 h in a stream of nitrogen. No volatile carbonyl compounds, 2,4-dinitrophenylhydrazine trap and no carbon dioxide (barium hydroxide trap) were detected. After 18 h, the crystalline methyl ester (Ib) was collected; it recrystallised from methanol in needles (730 mg), m.p. 219-220° (Found: C, 69-9; H, 5-5%; $M^+$, 326-115. C$_{18}$H$_{10}$O$_5$ requires C, 69-9; H, 5-5%; $M^+$, m/e 326[100%], 284[20], 283[24], 270[58], 269[51], 251[25], 237[66], 210[16], 182[9], 181[11], 153[15], 76[12], 69[15], 144[27], and 45[11].

The filtrate was extracted with chloroform which, in turn, was extracted with aqueous sodium carbonate. The recovered neutral oil (130 mg) yielded the methyl ester (Ib) in needles (60 mg). The recovered acidic material yielded a yellow foam (830 mg), crystallised from ether to yield the acid (C$_{21}$H$_{22}$O$_7$) as pale yellow needles (827 mg), m.p. 236-238° (Found: $M^+$, 346-134. Calc. for C$_{21}$H$_{22}$O$_7$: $M^+$, 386-136).

(b) With aqueous hydrochloric acid in ethanol. Wortmannin (560 mg), ethanol (30 ml), and 1-5% hydrochloric acid (30 ml) were boiled for 5 h under nitrogen; work-up as in (a) gave the acid C$_{21}$H$_{22}$O$_7$ and the ethyl ester (Ic), needles (210 mg), m.p. 231-235° (from methanol) (Found: $M^+$, 340-130. C$_{19}$H$_{16}$O$_5$ requires $M^+$, 340-131).

(c) With aqueous sulphuric acid: identification of acetic acid. Wortmannin (425 mg) and 1-5% sulphuric acid were heated on a water bath for 5 h under nitrogen. The acid (Ic) was collected; m.p. 258-256° (from methanol) (Found: $M^+$, 312-099. C$_{18}$H$_{10}$O$_5$ requires $M^+$, 312-099). The filtrate was steam-distilled. Titration of the steam distillate with standard potassium hydroxide indicated 0-91 mol. equiv. of a monobasic acid. The alkaline distillate was evaporated to dryness and the residue in aqueous ethanol was boiled for 1 h with p-bromophenacyl bromide to give p-bromophenacyl acetate, plates (from ethanol), m.p. and mixed m.p. 82-83°.

The residue from steam-distillation was extracted with chloroform. The acidic portion of the extract gave a yellow gum, crystallising from ether in rosettes, m.p. 232-234° identical (mixed m.p. and i.r. spectrum) with the acid C$_{21}$H$_{22}$O$_7$ obtained in (a).

(d) Identification of methoxyacetaldehyde. As in experiment (a), the methyl ester (Ib) was filtered off. The filtrate was steam-distilled and the distillate treated with 2,4-dinitrophenylhydrazine sulphate in ethanol. The precipitated methoxyacetaldehyde 2,4-dinitrophenylhydrazone was crystallised from ethanol in orange plates, m.p. 123.5-124.5°, identified by mixed m.p. and i.r. and n.m.r. spectroscopy.

**Derivatives of the Acid (Ia).**—(i) The methyl ether methyl ester (Id). Prepared from the methyl ester (Ib) and diazomethane, it crystallised from ethanol in needles, m.p. 156-158° (Found: $M^+$, 340-131. C$_{30}$H$_{32}$O$_7$ requires $M^+$, 340-131; v$_{max}$ (CHC$_3$), 3150, 1740, 1620, 1580, and 1550 cm$^{-1}$ (340(100%), 326(47), 325(44), 297(34), 284(67), 283(60), 282(34), 281(31), 270(47), 269(55), 268(30), 267(25), 237(66), 181(31), 163(32), 153(30), 142(36), 139(21), and 44(60). The methyl ether methyl ester was also prepared (a) from the acid (Ia) and diazomethane, and (b) by boiling the methyl ester (Ib) with dimethyl sulphate and 2-n-sodium hydroxide for 2 h.

(ii) The 16,16-dideuterio-methyl ether methyl ester (Ie). To the foregoing methyl ether methyl ester (116 mg) in acetonitrile (7 ml) was added potassium hydroxide (5-8 mg) in deuterium oxide (3-5 ml) and the mixture was stirred under nitrogen at 45-50° for 4 days. Extraction of the purple solution with ether, and recovery, afforded the 16,16-dideuterio-ether (Ie) as pink needles, m.p. 144-150° (from methanol) (Found: $M^+$, 342-142. C$_{20}$H$_{22}$D$_{12}$O$_7$ requires $M^+$, 342-142; $m/e$ 342[100%], 341[23], 327[27], 284[62], C$_{19}$H$_{17}$D$_{10}$O$_5$ and/or C$_{19}$H$_{17}$D$_{10}$O$_5$, 283[63, C$_{19}$H$_{17}$O$_5$ and/or C$_{19}$H$_{17}$D$_{10}$O$_5$, 288[23], and 237[4]).

(iii) The methyl ether (If). The methyl ether methyl ester (Id) (90 mg), methanol (10 ml) and 2-n-sodium hydroxide (16 ml) were heated under nitrogen at 60° for 4-5 h. After extraction with methylene dichloride, the solution was acidified and the acidic fraction was recovered in methylene dichloride as a foam (90 mg). Preparative
t.l.c. on pre-washed silica gel with 3% acetic acid in methylene dichloride gave the methyl ether (I), needles, m.p. 170—173° (from ether) (Found: M⁺, 342.149). C₁₀H₂₂O₅ requires M⁺, 342.147. When the reaction was performed at 20° for 20 min a mixture of the diol monomethyl ether and the triol monomethyl ether (Im) (see later) was obtained.

(vii) The diol dimethyl ether (Iv). Dry silver oxide (400 mg) was added in portions during 1 h to a boiling solution of the diol monomethyl ether (I) (35 mg) in methylene chloride. After stirring under reflux for 17 h, the silver oxide was filtered off and washed with methylene chloride. Recovery from the filtrate and washings gave the diol dimethyl ether (Iv) (28 mg), m.p. 102—104° (from ether) (Found: M⁺, 356.164. C₁₉H₂₄O₅ requires M⁺, 356.162; m/e 356(100%), 225(27), 324(29), 309(30), 298(50), 285(34), 283(94), 270(52), 263(22), 181(20), 165(24), 153(24), 152(22), 44(28), 43(20), and 41(30).

(viii) The diol diacetate (Iv). Prepared by heating the diol (Iv) in acetic anhydride and pyridine at 100° for 1 h, the diacetate (Iv) was obtained as rods, m.p. 180—183° (from methanol) (Found: M⁺, 412.153. C₂₀H₂₄O₇ requires M⁺, 412-152; m/e 412, 381, 370, 352, 328, 327, 310(100%), and 295.

(ix) The triol (Iv). Lithium aluminium hydride (500 mg), suspended in tetrahydrofuran (100 ml) was added in portions to a stirred solution of the methyl ester (Iv) (500 mg) in tetrahydrofuran (50 ml). After 1 h at 20° then 1 h under reflux, ethyl acetate, water, and 2N-sulphuric acid were added. The usual work-up gave a yellow gum (380 mg) which crystallised from ethyl acetate to give the triol (Iv), needles, m.p. 211—213° (Found: M⁺, 300.136. C₁₉H₂₂O₅ requires M⁺, 300-136; m/e 300(92%), 282(59), 267(29), 259(100), 244(40), 243(24), 241(68), 228(49), and 223(67).

(x) The triol monomethyl ether (Iv). The methyl ester (Iv) (20 mg) in ether (6 ml) was added slowly to a stirred suspension of lithium aluminium hydride (14 mg) in ether. After 1 h at 20°, ethyl acetate and ethanol were added with vigorous stirring. The mixture was then poured over 2N-sulphuric acid and ice. Recovery in ether gave the triol monomethyl ether (Iv) (17 mg), m.p. 208—210° (from ether) (Found: M⁺, 314-151. C₂₀H₂₄O₇ requires M⁺, 314-152.)

(xi) The triol triacetate (Iv). Prepared from the triol (Iv), acetic anhydride, and pyridine at 100° for 30 min, it was obtained as an intractable yellow oil (Found: M⁺, 426-167. C₂₀H₂₄O₇ requires M⁺, 426-167; m/e 324, 250, 270, and 281 nm (e 45,900, 16,120, 8380, and 6800); m/e 426(35%); 384(60), 343(10), 324(100), 309(35), 282(27), 281(13), 265(20), 263(11), 249(40), 237(15), 225(12), and 233(12).

(xii) The dihydro-methyl ether (Iv). The methyl ester (Iv) (220 mg), 10° palladium—barium carbonate (75 mg), and ethyl acetate (50 ml) were shaken at 20° for 10 min with hydrogen. The usual work-up gave needles (220 mg), shown by g.l.c. (2% OV-1) 239° to be a mixture of the dihydro (Iv) (80%) and tetrahydro—(Iv) (20%) compounds. Recrystallisation from methanol gave the dihydro—compound (Iv) as needles m.p. 196—198° [containing no tetrahydro—compound (Iv) by g.l.c.] (Found: M⁺, 328-133. C₁₉H₂₄O₅ requires M⁺, 328-131; m/e 316(100%), 326(2-5), 313(21), 297(10), 285(56), 273(40), 271(48), 259(38), 257(22), and 45(38).

(xiii) The dihydro-methyl ether (Iv). The methyl ether (Iv) (35 mg), in ethyl acetate (5 ml) was shaken at 18° for 2 h with hydrogen and 5% palladium—barium carbonate (30 mg). The usual work-up gave the dihydro—compound (Iv), needles (50 mg), m.p. 146—148° (from methanol) (Found: M⁺, 342-147. C₂₀H₂₄O₇ requires M⁺, 342-147; m/e 358(100%), 330(100%), 326(27), 311(15), 297(11), 285(56), 273(40), 271(48), and 43(17).

(xiv) The monocacetate (Iv). Prepared from the dihydro—methyl ether (Iv) (40 mg), acetic anhydride (1 ml), and pyridine (2 drops), the acetate (Iv) crystallised from methanol in prisms (36 mg), m.p. 201—203° (Found: M⁺, 370-143. C₁₉H₂₄O₅ requires M⁺, 370-141; m/e 370(14%), 328(100%), 313(11), 285(30), 272(19), 271(21), 259(18), 257(8), and 43(17).

(xv) The dihydro—diol (Iv). The dihydro—compound (Iv) (50 mg), in methanol (10 ml), was stirred at 20° for 10 min with sodium borohydride (50 mg). The usual work-up gave the dihydro—diol (Iv) as needles (40 mg), m.p. 215—217° (from methanol) (Found: M⁺, 330-149. C₁₉H₂₄O₇ requires M⁺, 330-147; m/e 330(100%), 316(27), 297(30), 286(31), 271(83), 259(30), 257(18), 243(19), and 219(19).

(xvi) The dihydro—trio! (Iv). To a suspension of lithium aluminium hydride (46 mg) in tetrahydrofuran (10 ml) was added the dihydro—methyl ester (Iv) (54 mg). The mixture was stirred at 20° for 45 min then under reflux for 45 min. The usual work-up gave the dihydro—trio! (Iv), needles (40 mg) m.p. 219—221° (from ethyl acetate) (Found: M⁺, 302-155. C₁₉H₂₄O₇ requires M⁺, 302-152; m/e 302(100%), 287(26), 269(27), 258(17), 244(16), 243(53), 237(18), 229(9), 191(10), and 43(52).

(xvii) The tetrahydro—methyl ester (Iv). The methyl ester (Iv) (120 mg), in ethyl acetate (25 ml), was shaken with hydrogen and 10% palladium—barium sulphate (125 mg) at 20° for 1 h. The usual work-up gave the tetrahydro—derivative (Iv) as a gum (110 mg) showing a single g.l.c. peak on a 2% OV-1 column at 240° (Found: M⁺, 330-147. C₂₀H₂₄O₇ requires M⁺, 330-147; m/e 330(100%), 328(27), 326(16), 301(11), 300(18), 299(6), 298(21), 287(19), 285(10), 283(18), 281(11), 279(6), and 43(17).
The acetate (IIIg), prepared with acetic anhydride in pyridine, was also an intractable gum (Found: $M^+$, 372.157. $\text{C}_{25}\text{H}_{34}\text{O}_6$ requires $M^*$, 372.155); $m/e$ 372(18%), 330(100), 287(11), 271(30), 161(67), 85(10), 83(16), and 43(>100).

(xviii) *The tetrahydro-diol* (IIIh). The tetrahydro-methyl ester (IIIib) (30 mg) in methanol (3 ml) was stirred at 20$^\circ$ for 15 min with sodium borohydride (30 mg). The usual work-up gave the *tetrahydro-diol* (IIIh) as an intractable gum (33 mg) (Found: $M^*$, 332.162. $\text{C}_{19}\text{H}_{24}\text{O}_5$ requires $M^*$, 332.161); $m/e$ 332(100%), 330(12), 273(40), 258(74), 257(23), 256(32), and 161(64).

(xix) *The tetrahydro-triol* (IIIi). The triol (II) (20 mg), in ethyl acetate (12 ml), was hydrogenated as before to give a gum (18 mg) which, on trituration with chloroform, gave the *tetrahydro-triol* (IIIi) as an amorphous solid (Found: $M^*$, 304.166. $\text{C}_{18}\text{H}_{24}\text{O}_4$ requires $M^*$, 304.161); $m/e$ 304(53%), 302(5), 273(74), 161(100), 85(37), 83(58), 47(19), and 43(14%).

*Alkaline Hydrolysis of the Methyl Ester* (Ib).—The methyl ester (200 mg), methanol (15 ml) and 2N-potassium hydroxide (20 ml), were heated at 90$^\circ$ for 3 h. The usual work-up gave an acidic solid (117 mg), identical (m.p. and i.r. spectrum) with the acid (Ia) already described and obtained from wortmannin by aqueous acidic hydrolysis.

*Dehydrogenation of the Methyl Ester Methyl Ester* (Id).—An intimate mixture of the methyl ether methyl ester (62 mg) and 20% palladium-charcoal (72 mg) was heated at 190$^\circ$ for 40 min under nitrogen. Heating was continued at 196—200$^\circ$ for 1 h. Extraction of the cooled mixture with ether, then recovery, gave a pink gum (44 mg). A portion (16 mg) of this product was subjected to preparative t.l.c. on silica gel G, pre-washed with benzene-ethyl acetate (3:1) and re-activated at 100$^\circ$ for 0.5 h, and developed with benzene-ethyl acetate (3:1). The band, fluorescing blue and corresponding in $R_f$ value to the authentic derivative (VII) from viridin, was collected and extracted with ether. Recovery gave methyl 2,3-dihydro-10-methoxy-6-methyl-1H-cyclopenta[7,8]naptho[2,3-b]furan-7-carboxylate (VII), as yellow needles (1 mg), m.p. 236—238$^\circ$ (sublimation from 190$^\circ$) (from ethanol), not depressed on admixture with the viridin derivative (m.p. 235.5—237$^\circ$). The i.r. spectrum (Nujol) $\nu$ 3150, 2990, 2970, 2850, 1730, 1735, 1730, 1710—1690, 1625, 1604, 1510, 1465, 1440, 1405, 1390, 1370, 1330, 1305, 1272, 1250, 1152, 1136, 1090, 1075, 1024, 990, 918, and 900 cm$^{-1}$ and g.l.c. retention times (10.5 min on 2% SE-33 at 238$^\circ$ and 75 ml N$_2$ min$^{-1}$; 11.8 min on 2% QF-1 at 238$^\circ$ and 60 ml N$_2$ min$^{-1}$) were identical with those of the viridin derivative (VII).

Financial support from the A.R.C. (A. E. V.), UNESCO (S. K. Y.), and the S.R.C. (T. J. S.) is gratefully acknowledged. We also thank the University of Cape Coast, Ghana, for leave of absence to S. K. Y., and Dr. J. S. Moffat for samples of viridin and its degradation product.

[Received, 30th June, 1972]
Absolute Stereochemistry of the Fungal Product, Wortmannin


(Received, 31st July 1972; Comm. 1331)

Summary The absolute stereochemistry at C-10, C-11, C-13, and C-14 in the fungal product, wortmannin, has been deduced from biogenetic and spectroscopic evidence. A X-ray study, described in the accompanying communication, confirms the structure, proposed by us,* for the fungal metabolite, wortmannin, and it defines the absolute stereochemistry (I). We have independently deduced the same absolute stereochemistry (I) except for C-1 which we were unable to determine. The 13β-Me,14α-H configuration was indicated by the n.d. (a + 138) of the 11,12-dihydromethyl ether of the hydrolysis product (III).² The normal stereoidal stereochemistry at C-10, C-13, and C-14 was established by the enantiomeric (0-2–0-6%) of 13-[H]-lanosterol² into [13]-wortmannin (I) and -11-desacetoxywortmannin (II) in shake-cultures of Penicillium wortmannii. The specific configuration of tritium was demonstrated by acidic hydrolysis² into methoxycetaldehyde which contained all of the label. The 11α-acetoxy-configuration was indicated by the down-field shift (0-07 p.p.m.) of the 18-protons in the m.n.r. spectrum of wortmannin (I) compared to 11α-desacetoxywortmannin (II). Similar downfield shifts (0-06 p.p.m.) have been observed for the 18-protons in steroids benz substituted by an 11α-acetoxy-group. Similar n.m.r. evidence for an 11α-acetoxy-group in wortmannin was obtained from the products of mild alkaline hydrolysis. 1n-K₂CO₃ gave the triol (IV) (enolic-OH at T 274 and —3-60, the 13 Hz coupling of the latter to 20-H removed by addition of D₂O) which formed a triacetate (V) and a diacetate (VI). Hydrolysis of wortmannin with KHCO₃ afforded the diol (VII) with enolic hydroxy-signals in the n.m.r. spectrum at T 282 and —3-75, the latter being coupled (13 Hz) to 20-H. The negligible changes in the chemical shift of the 18-protons in going from the mono-

TABLE

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<th>Compound</th>
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<th>2S-Isomer</th>
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<td>Methoxyacetaldehyde</td>
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² Incorporations of 1-6% based upon the [1-4C] ratios shown in the Table. These ratios clearly showed substantial loss of the [1H]-label from both the 2S- and 2R-isomers and, unexpectedly, a considerable interchange between the 2S- and 2R-[1H] atoms. These results will be discussed in detail in the full paper but it is salutary to note that the greater retention of the 2R-[1H] compared to the 2S-[1H] at C-1 suggests the opposite C-1 stereochemistry to that established by the accompanying X-ray determination.

*(Received, 31st July 1972; Comm. 1331.)

References

Fungal Products. Part V. The Absolute Stereochemistry of Colletodiol and the Structures of Related Metabolites of *Colletotrichum capsici*

By Jake MacMillan* and Thomas J. Simpson, School of Chemistry, The University, Bristol
Fungal Products. Part V: The Absolute Stereochemistry of Colletodiol and the Structures of Related Metabolites of Colletotrichum capsici

By Jake MacMillan * and Thomas J. Simpson, School of Chemistry, The University, Bristol

Colletoketol (2), colletol (3), and colletallol (4), three new co-metabolites of colletodiol (1) (10,11-dihydroxy-2,8-dimethyl-1,7-dioxacyclotetradeca-4,12-diene-6,14-dione), have been isolated from culture filtrates of Colletotrichum capsici and shown to be the 11-oxo-analogues of colletodiol, 11-deoxycolletodiol, and 10-deoxycolletodiol, respectively. The absolute stereochemistry for colletodiol [i.e. (2R,8R,10R,11S)] has been assigned from chemical and optical rotatory evidence, and that for colletoketol [i.e. (2R,8R,10R)] has been established by chemical correlation with colletodiol.

From the culture filtrates of the plant pathogen, Colletotrichum capsici, Grove et al.2 isolated the metabolite colletodiol, which was subsequently shown to be the 14-membered cyclic dilactone (1)3a (cf. ref. 3b). The absolute stereochemistry (5) has now been assigned to this metabolite (see later). In the course of isolating colletodiol for this stereochemical investigation, three closely related dilactones, colletoketol (2), colletol (3), and colletallol (4), have been isolated from the culture filtrates of C. capsici together with 2-phenylethanol.

Structure (1) for colletodiol was deduced mainly from spectroscopic data3a which is not discussed in detail here. Part IV, R. J. Crane, P. Hedden, J. MacMillan, and W. B. Turner, J.C.S. Perkin I, 1973, 194.

To facilitate comparison with the new dilactones, i.r. and n.m.r. data for colletodiol are included in Tables 1 and 2, respectively. In the mass spectrum of the bistrimethylsilyl (TMS) ether of colletodiol, it was suggested3a that the base peak at m/e 143 (C₇H₁₅OSi) arose by McLafferty rearrangement to the ion (6) followed by cleavage a to the ion (8). The presence of an intense m/e 143 ion in the mass spectrum of the TMS ethers of colletodiol and its analogues is therefore diagnostic of the partial structure (9) and this correlation proved useful (see later) in the determination of the structures of colletoketol (2), colletol (3), and colletallol (4).

Colletodiol, which formed a dibenzoate and a diacetate, was hydrolysed by dilute aqueous sodium hydroxide to 5-hydroxyhex-2-enoic acid and, in low yield, to 4,5,7-trihydroxyoct-2-enoic acid. Hydrogenation of the former acid gave 5-hydroxyhexanoic acid, which was lactonised by heating in chloroform. The yield, to 4,5,7-trihydroxyoct-2-enoic acid. Hydrogenation of the lactone (10) from colletodiol had the sign opposite to that of the lactone (14), whereas the lactone (15), with the opposite configuration, gives a negative Cotton effect. The 11S-configuration is therefore assigned to colletodiol.

To determine the C-8 stereochemistry the 4,5,7-trihydroxyoctenoic acid was oxidised by periodate to give a neutral product which, without characterisation, was oxidised with silver oxide to yield -hydroxybutyric acid with \([\alpha]_D^{20} = -21^\circ\). Since \((R)-\) -hydroxybutyric acid has \([\alpha]_D^{20} = -24^\circ\), colletodiol possesses the 8R-stereochemistry.

The R-configuration was assigned to the remaining asymmetric centre (position 10) in colletodiol in two ways. The first utilised the findings of Harada and Nakanishi that the chirality of α-glycols can be determined from the c.d. of their dibenzoates. The interacting chromophores of the dibenzoates give rise to two strong Cotton effects of the same amplitude (Δε ca. 9-18); the first occurs at ca. 233 nm and its sign is determined by the chirality of the acid, \([\alpha]_D^{20} = -7.6^\circ\), to the lactone, \([\alpha]_D^{20} = +18.4^\circ\), indicated the R-configuration. Secondly, the specific rotation of the lactone (10) from colletodiol had the sign opposite to that, \([\alpha]_D^{20} = -39^\circ\), reported for the lactone obtained from sorbin oil and assigned the S-configuration. Thirdly, the c.d. \([\alpha]_D^{20} = +408^\circ\), of the lactone from colletodiol agrees with the positive Cotton effect predicted for the R-configuration from the lactone sector rule. Seven from these data, the 2R-configuration is assigned to colletodiol.

Mild alkaline hydrolysis of the tetrahydro-derivative (11), obtained by hydrogenation of colletodiol, gave 5-hydroxyhexanoic acid and 5,7-dihydroxyoct-4-olide (13). The structure of the lactone (13) was established by high resolution mass spectroscopy and by the i.r. (1768 cm\(^{-1}\)) and n.m.r. \([\delta]_H = 5-6\) (1H, sextet, /4, 7, and 7 Hz) spectra. Like that of the lactone (10), the mass spectrum of the lactone (13) showed a strong M + 1 ion. The lactone (13) had \([\alpha]_D^{20} = -42^\circ\) in ethanol and \(+14^\circ\) in ethanol containing 2% potassium hydroxide. From application of the Hudson-Klyne lactone rules, \([\Delta\alpha]_D^{20} = -43-4^\circ\) indicated the S-configuration (13), which was supported by the positive c.d. curve, predicted from the lactone sector rule. The magnitude (+528\(^\circ\)) and sign of the molecular ellipticity of the lactone (13) agree with the data, \([\alpha]_D^{190} + 440^\circ\), reported for the lactone (14), whereas the lactone (15), with the opposite configuration, gives a negative Cotton effect. The 11S-configuration is therefore assigned to colletodiol.

The 10R-configuration was also inferred from the reduction of colletoketol, shown later to be the ketol. This reduction with metal hydrides was complete.

\(\text{J.C.S. Perkin I}\)

**TABLE 1**

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Measured chemical shifts (\(\gamma\)) of protons in the 100 MHz n.m.r. spectra of metabolites from *C. capsici*

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The 10,11-erythro-configuration was also supported by the formation of an acetonide from colletodiol without conformational change as judged by the similarity of the coupling constants in this derivative and in colletodiol; for example, $J_{10,11}$ values for colletodiol and its acetonide were 9.0 and 10 Hz, respectively.

The foregoing data define the absolute stereochemistry (5) for colletodiol. The structures of the three dilactone metabolites of *C. capsici* were determined as follows.

Colletoketol (2) was the major metabolite (ca. 20 mg $^{-1}$) from one fermentation; otherwise it was obtained in yields of ca. 5 mg $^{-1}$. By g.l.c. monitoring of the fermentations it was shown that colletodiol (5) was formed initially, then reabsorbed from the medium to be replaced by colletoketol. The ketol structure (2) for colletoketol was indicated by the molecular formula, C$_{14}$H$_{18}$O$_6$, determined by high resolution mass spectrometry and by the spectroscopic properties. The i.r. spectrum (Table 1) was similar to that of colletodiol and the presence of a single hydroxy-group was shown by the formation of a monobenzoate and a mono-TMS ether. The mass spectrum of the TMS ether, like that of the bis-TMS ether of colletodiol, had a base peak at m/e 143 indicative of the partial structure (9). The n.m.r. spectrum of colletoketol was fully analysed by comprehensive decoupling experiments. The chemical shifts (Table 2) and coupling constants (Figure 2) are in complete agreement with structure (2); in particular the observed multiplicity of the 9- and 10-proton signals confirms the mass spectroscopic evidence that the hydroxy-group is positioned at C-10, excluding the alternative 11-hydroxy-10-oxo-structure. Thus the reduction of colletoketol, described earlier, to give colletodiol provided final proof of structure (2) for colletoketol. Oxidation of colletodiol (5) with either Jones reagent or silver carbonate on Celite gave colletoketol (2). These chemical effects were completed stereospecific indicating the approach of hydride at C-10 *trans* to the 11-hydroxy-group, probably via the complex (16).

![Figure 1](image_url)  
**Figure 1** C.d. curves for (a) colletodiol, (b) the diacetate, and (c) the dibenzoate; curve (d) is curve (c) minus curve (b).
correlations with colletodiol (5) establish the absolute stereochemistry (17) for colletoketol.

Like colletodiol (5), mild alkaline hydrolysis of colletoketol gave 5-hydroxyhex-2-enoic acid. Mild alkaline hydrolysis of the tetrahydro-derivative (12), obtained by hydrogenation of colletoketol, gave 5-hydroxyhexanoic acid and succinic acid but no 5,7-dihydroxy-4-oxo-octanoic acid. The succinic acid may be formed of colletoketol (2) with sodium borohydride in methanol gave colletodiol (5) and, in one experiment only, a methanolysis product to which structure (19), rather than that of the alternative lactone cleavage product, was assigned from the base peak at $m/e$ 143 (8) in the mass spectrum of the mono-TMS ether. An analogous methanolysis of colletodiol (5) was observed on treatment with methanolic sodium hydroxide. Formation of a monobenzoate by treatment of colletoketol (2) with benzoyl chloride and pyridine gave a minor product which was formulated as $C_{29}H_{29}O_{7}$ on the basis of a high resolution mass measurement on the parent ion; the presence of one chlorine atom was also indicated by the $^{35}Cl$:$^{37}Cl$ isotopic ratio and by ions at $m/e$ 35 and 37. The n.m.r. spectrum of this minor product showed that the vinylic protons at C-12 and C-13 of colletoketol (2) were not present and a new ABX system was present at $\tau$ 7.99, 7.73, and 5.22, respectively, with $J_{AB}$ 16, $J_{AX}$ 5, and $J_{MX}$ 4 Hz, diagnostic of the 13-, 12-, and 11-protons of structure (4). Secondly, the 13-proton was deshielded by 0.03 p.p.m. with respect to the 5-proton by the 11-hydroxy-group. Thirdly, in the absence of the 10-hydroxy-group, the 8-methyl signal occurs at 0.17 p.p.m. to higher field than in colletol (3) (Table 2).

The macrocyclic dilactones from C. capsici are closely related to pyrenophorin (21), isolated from both Pyrenophora avenae and Stemphlygium radicinum, and to pyrenophorol (22), isolated from Byssoclamis nivae. They are presumably derived from the hydroxy-$C_{8}$- and -$C_{6}$-acids of which the following $C_{8}$-derivatives are known fungal metabolites: the 6-lactone (23) and the 8-lactone (24), both from a Nigrospora species; and the tetracentic acid lactone (25) from Penicillium stipitatum. 19

**EXPERIMENTAL**

M.p.s were determined on a Kofler hot-stage apparatus and are corrected. Silica gel M.F.C. (Hopkin and Williams) and Kieselgel G and HF (Merck) were used for column and thin-layer chromatography (t.l.c.), respectively. Analytical t.l.c. plates were developed by spraying with 2% ceric sulphate in 40% sulphuric acid and heating. Silica HF plates were viewed under u.v. light (254 and 366 nm). T.L.

13 Z. Kiss, P. Forger, and H. P. Sigg, Experientia, 1969, 25, 123.
spectra were obtained on a Perkin-Elmer 257 spectrometer for chloroform solutions unless otherwise stated. N.m.r. spectra (2 values; $J$ in Hz) were obtained with a Varian HA100 spectrometer for deuteriochloroform solutions with tetramethylsilane as internal standard. Mass spectrometry was carried out with an A.E.I. MS9 spectrometer and the data were processed by an on-line Linc 8 computer for probe samples. Combined gas chromatography–mass spectrometry (g.c.–m.s.) was carried out with a G.E.C.–A.E.I. MS90 spectrometer. U.v. spectra were taken for solutions in methanol on a Unicam SP 800 spectrophotometer. Specific rotations were measured on a Perkin-Elmer 141 polarimeter. N.d.d. data were determined for methanolic solutions by Dr. P. M. Scoles, Westfield College, University of London. For gas chromatography (g.c.), a Pye 104 instrument was used with columns ($5 \times 0.25$ in o.d.), packed with 2% SE33, 2% QF1, 2% OV1, or 2% OV210 on Gaschrom Q. Light petroleum had b.p. 60–80°.

**Culture Methods.**—Master cultures of *Colletotrichum capsici* Syd. Butler and Bisby were stored on slopes of 2% malt agar under liquid paraffin. Sub-cultures, grown for 7 days on slopes of the same medium in medicine bottles, were used to prepare a spore suspension for the inoculation of cultures on a preparative scale. For the latter the following liquid medium was used (% in parentheses): Dextrose (5), potassium dihydrogen phosphate (1), ammonium nitrate (0.24), magnesium sulphate heptahydrate (0.2), trace elements (4), and 2% Casamino acids. Encoding of the cultures was carried out with an A.E.I. MS30 spectrometer. U.v. spectra were taken for solutions in methanol containing 1% methanol; and (v) colletodiol (diethyl ether containing 50% benzene); (ii) colletoketol (diethyl ether containing 20% benzene), (iii) colletol (diethyl ether containing 20% benzene); and (iv) colletol (diethyl ether containing 10%, benzene), (iv) colletol (diethyl ether containing 20%, benzene); and (v) colletol (diethyl ether containing 1%, methanol); and (v) colletol (diethyl ether containing 2%, methanol).

(a) Colletodiol, obtained as a waxy solid in fraction (i), crystallised from acetone–light petroleum as needles (yield ca. 10 mg 1-1), m.p. 162–164° (lit. 163–164°; $J_{c}=183(2), 122(10), 113(2), 105(100), 77(37),$ and 68(3). The diacetate prepared from colletodiol (100 mg), acetic anhydride (2 ml), and pyridine (1 ml) was obtained as a gum which, after preparative t.l.c. (silica gel; ethyl acetate–light petroleum–acetic acid (45: 50: 5); $R_F$ 0.62, crystallised from ethyl acetate–light petroleum as needles (68 mg), m.p. 132–133° (lit. 130–131° (Found: $M^+=368-147$. $\Delta_{2850}=295(45), M^+_{2850}=183(1), 160(5), 143(100), 120(10), 113(2), 105(100), 77(37),$ and 68(3)).

(b) Colletoketol (2) (10-hydroxy-2,8-dimethyl-1,7-dioxacyclooctadeca-4,12-diene-6,11-dione), obtained as a semi-crystalline solid from fraction (ii), crystallised from acetone–light petroleum as prisms (yield 5–20 mg 1-1), m.p. 138–139° (Found: $M^+=282-111$. $C_{14}H_{18}O_5$ requires $M^+=282-110$; $\Delta_{2850}=295(45), M^+_{2850}=183(1), 160(5), 143(100), 120(10), 113(2), 105(100), 77(37),$ and 68(3)).

(c) Colletol (3) (10-hydroxy-2,8-dimethyl-1,7-dioxacyclooctadeca-4,12-diene-6,11-dione) was obtained as an oil from fraction (iii), crystallised from ethyl acetate–light petroleum as needles (yield 1 mg 1-1), m.p. 101–104° (Found: $M^+=268-131$. $C_{14}H_{18}O_5$ requires $M^+=268-131$; $\Delta_{2850}=295(45), M^+_{2850}=183(1), 160(5), 143(100), 120(10), 113(2), 105(100), 77(37),$ and 68(3)).

(d) Colletodiol (4) (11-hydroxy-2,8-dimethyl-1,7-dioxacyclooctadeca-4,12-diene-6,11-dione) was obtained as an oil from fraction (iv), admixed with colletol (3). The mixture was separated on silica gel HF plates by multiple development with ethyl acetate–light petroleum–acetic acid (20: 75: 5). The less polar band (colletol) was separated from the slower moving colletodiol (5), which was obtained as an intractable gum (10 mg) (Found: $M^+=310-142$. $C_{14}H_{18}O_5$ requires $M^+=310-142$; $\Delta_{2850}=295(45), M^+_{2850}=183(1), 160(5), 143(100), 120(10), 113(2), 105(100), 77(37),$ and 68(3)).

(e) Alkaline Hydrolysis of Colletodiol.—Colletodiol (100 mg) in 0.05N-sodium hydroxide (25 ml) was stirred at 20° for 24 h. Continuous extraction of the acidified solution with ether for 48 h and recovery from the ether gave a dark gum (95 mg) which was subjected to preparative t.l.c. in silica gel.
Development with ethyl acetate-light petroleum-acetic acid (45 : 50 : 5). The more polar product, obtained as an intractable gum (25 mg), showed no parent ion in the mass spectrum but was presumed to be the methyl ester of 5,7-dihydroxyhept-l-enoic acid (Found: M+ = 209, 208; C13H20O6 requires M+ = 208). Continuous extraction of the filtered reaction mixture with ether gave (R)-5-hydroxybutyric acid (1 mg), which ran concurrently with an authentic sample on a silica gel layer (developed with acetone, then sprayed with Methyl Orange).

Tetrahydrocolletodiol.-Colletoketol (100 mg) in ethyl acetate (15 ml) was hydrogenated for 2 h over 10% palladium-charcoal (25 mg) to give tetrahydrocolletoketol, crystallised from ethyl acetate-light petroleum as needles (85 mg), m.p. 129-132° (lit., 133°), 135-139°, 138-140°, 145-146°, 148-150°, 150-155°, 157-160°, and 172°). The more polar product, obtained as an intractable gum (Found: M+ = 209, 208; C13H20O6 requires M+ = 208). Continuous extraction of the filtered reaction mixture with ether gave (R)-5-hydroxybutyric acid (1 mg), which ran concurrently with an authentic sample on a silica gel layer (developed with acetone, then sprayed with Methyl Orange).

Oxidation of Colletodiol.-a) Jones reagent. Colletodiol (50 mg) in acetonitrile (3 ml) was treated with Jones reagent (5 drops) at 0° for 5 min. After the addition of water the gummy product was recovered in chloroform. It gave only one peak on g.l.c., corresponding to colletoketol, and recrystallisation from ethyl acetate-light petroleum gave colletoketol as needles, m.p. 129-132° (lit., 133-134°). Prolonged oxidation gave an intractable mixture of products.

Silver carbonate-Celite. Colletodiol (20 mg) in chloroform (10 ml) was stirred for 24 h with silver carbonate-Celite reagent. G.L.C. indicated 20% conversion into colletoketol.

Oxidation of 1,5,7-Trihydroxy-2-enoic Acid to (R)-3-Hydroxybutyric Acid.-The trihydroxy-acid (15 mg), sodium periodate (60 mg), and water (4 ml) were stirred at 25° overnight. After basification with N-sodium hydroxide, the neutral product was recovered in ether and stirred with silver oxide (200 mg) and water (4 ml) for 48 h. Continuous extraction of the filtered reaction mixture with ether gave (R)-3-hydroxybutyric acid (1 mg), which ran concurrently with an authentic sample on a silica gel layer (developed with acetone, then sprayed with Methyl Orange).

Alkaline Hydrolysis of Tetrahydrocolletodiol.-Tetrahydrocolletodiol (98 mg) in 0.02N-hydrochloric acid (25 ml) was stirred at room temperature for 24 h. Work-up as for the colletodiol hydrolysis gave a brown gum (85 mg), which was intractable, showed no parent ion in the mass spectrum but was presumed to be the methyl ester of 5,7-dihydroxyhept-l-enoic acid as needles, m.p. 90-91° (Found: M+ = 209, 208; C13H20O6 requires M+ = 208). Continuous extraction of the filtered reaction mixture with ether gave (R)-5-hydroxybutyric acid (1 mg), which ran concurrently with an authentic sample on a silica gel layer (developed with acetone, then sprayed with Methyl Orange).

Reduction of Colletoketol to Colletodiol.-a) Sodium borohydride (108 mg) was added in portions to an ice-cold, stirred solution of colletoketol (48 mg) in methanol (50 ml). After 40 min, the mixture was worked up in the usual manner. The gummy product, recovered in ethyl acetate, was crystallised from ethyl acetate-light petroleum to give colletodiol as needles (38 mg), m.p. 161-163°.

(b) From an identical experiment, the gummy product (40 mg) showed a g.l.c. retention time of 4.25 min on an OV-1 column at 190°; cf. 4.25 min for colletodiol. This gum, which was intractable, showed no parent ion in the mass spectrum but was presumed to be the methyl ester (19): m/z = 984, 1175, 1260, 1490, 1575, and 3410 cm-1; r = 3.02 (2H, m), 3.08 (1H, J 4 and 8), 5.91 (1H, m), 6.10 (1H, m), 6.50 (2H, exchanged with D2O), 7.4-7.6 (2H, m), 7.8-8.0 (2H, m), and 8.7-8.9 (3H, J 6). The tri-TMS ether, prepared as already described, showed a single peak on g.l.c.-m.s.; m/z = 552, 480, 408, 320, and 148.

Tetrahydrocolletodiol.-Colletoketol (48 mg) in ethyl acetate (10 ml) was hydrogenated for 3 h over 10% palladium-charcoal. The usual work-up gave the tetrahydrocolletodiol (12), which crystallised from ethyl acetate-light petroleum as needles, m.p. 106-108° (Found: M+ = 250. C13H22O6 requires M+ = 250). Continuous extraction of the filtered reaction mixture with ether gave (R)-3-hydroxybutyric acid (1 mg), which ran concurrently with an authentic sample on a silica gel layer (developed with acetone, then sprayed with Methyl Orange).
1973

m/e (%) 286(1), 271(3), 258(4), 185(23), 155(12), 155(100), 97(39), 15(24), and 69(27).

**Tetrahydrocolletoketol Monobenzoate.**—Colletoketol monobenzoate (9 mg) in ethyl acetate was hydrogenated over 10% palladium-charcoal in the usual way to give tetrahydrocolletoketol monobenzoate, needles (6 mg), m.p. 121—124° (from ethyl acetate—light petroleum) (Found: $M^+$, 390.142. $C_{21}H_{26}O_7$ requires $M^+$, 390.142); $[\alpha]_{D}^{2°}$ -58.5° (c 0.53 in CHCl$_3$); $\nu_{max}$ 3000, 1725 cm$^{-1}$; $\delta$ 1.94 (2H, m), 2.68 (3H, m), 4.43 (1H, t, J 6), 6.74 (2H, m), 6-93 (1H, m), 6-25 (1H, m), 7.0—8.5 (11H, m), 8-67 (3H, d, J 6), and 8-76 (3H, d, J 6); m/e (%) 390(4), 362(8), 277(15), 219(100), 185(78), 134(23), 115(98), 105(100), 95(21), 77(100), 69(59), and 55(23).

**Alkaline Hydrolysis of Tetrahydrocolletoketol.**—Tetrahydrocolletoketol (100 mg) and 0.05N-sodium hydroxide (30 ml) were stirred at 20° for 21 h. The usual work-up gave a brown gum (73 mg) which was subjected to preparative t.l.c. on silica HF with ethyl acetate—light petroleum—acetic acid (45: 50: 5). Recovery from the bands at $R_f$ 0.5 and 0.2 gave, respectively, 5-hydroxyhexanoic acid (25 mg), identical with that obtained from tetrahydrocolletol, and succinic acid (20 mg), identified by comparison (m.p., mixed m.p., and i.r.) with an authentic sample.

**Benzoylation of Colletoketol.**—Colletoketol (32 mg), benzoyl chloride (2 ml), and pyridine (1 ml) were stirred at 20° for 8 h. Work-up as for colletodiol dibenzoate gave a gum which was subjected to preparative t.l.c. on silica HF plates, which were successfully developed for four times with ethyl acetate—light petroleum—acetic acid (25: 75: 2) to give two closely running bands. The upper band gave colletoketol monobenzoate, prisms (13 mg), m.p. 133—135° (from ethyl acetate—light petroleum) (Found: $M^+$, 386.138. $C_{21}H_{24}O_7$ requires $M^+$, 386.137); $[\alpha]_{D}^{2°}$ -60.0° (c 0.94 in CHCl$_3$); $\nu_{max}$ 987, 1100, 1600, 1725 cm$^{-1}$; $\delta$ 1.90 (2H, m), 2.44 (3H, m), 2.94 (1H, d, J 16), 3.18 (1H, d, J 16), 3.24 (1H, dt, J 5 and 16), 4.30 (1H, d, J 16), 4.47 (1H, t, J 5), 4.72 (2H, m), 7.24 (4H, m), 8-59 (3H, d, J 6), and 8-71 (3H, d, J 6); m/e (%) 386(2), 342(3), 288(9), 183(75), 122(14), 113(13), 105(98), 95(17), 77(100), 68(55), and 44(23).

The lower band gave the chloro-monobenzoate (20), needles (10 mg), m.p. 122—124° (from ethyl acetate—light petroleum) (Found: $M^+$, 422.114. $C_{21}H_{24}O_7Cl$ requires $M^+$, 422.113); $\nu_{max}$ 900, 1120, 1603, 1655, and 1725 cm$^{-1}$; $\delta$ 1.92 (2H, m), 2.44 (3H, m), 3.20 (1H, d, J 8 and 16), 4.19 (1H, d, J 16), 4.46 (1H, dd, J 1 and 7), 4.71 (1H, m), 5.04 (1H, m), 5.22 (1H, dd, J 6 and 7), 7.73 (1H, dd, J 4 and 20), 7-98 (1H, dd, J 6 and 20), 7-60 (4H, m), 8-60 (3H, d, J 6), and 8-71 (3H, d, J 6); m/e (%) 422(4), 394(6), 387(3), 359(38), 342(5), 289(10), 183(48), 167(10), 113(15), 105(100), 95(24), 82(20), 77(64), 68(76), and 55(23).

We thank the S.R.C. for a Research Studentship and for a Research Grant towards the purchase of a G.E.C.—A.E.I. MS30 mass spectrometer. We also thank I.C.I. Ltd. for a culture of *Colletotrichum capsici*; Dr. P. M. Scopes, Westfield College, University of London, for the c.d. measurements; and Mr. D. Morgan for technical assistance.

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Fungal Products. Part XIII. \(^1\) Xanthomegnin, Viomellin, Rubrosulphin, and Viopurpurin, Pigments from *Aspergillus sulphureus* and *Aspergillus melleus*

By Richard C. Durley, Jake MacMillan,\(^*\) and Thomas J. Simpson, School of Chemistry, The University, Bristol BS8 1TS

(In part) Alasdair T. Glen and W. Brian Turner, Imperial Chemical Industries Ltd., Pharmaceutical Division, Alderley Park, Macclesfield, Cheshire

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*PERKIN TRANSACTIONS I*

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Fungal Products. Part XIII. Xanthomeglin, Viomellin, Rubrosulphin, and Viopurpurin, Pigments from Aspergillus sulphureus and Aspergillus melleus

By Richard C. Durley, Jake MacMillan,* and Thomas J. Simpson, School of Chemistry, The University, Bristol BS8 1TS

The known fungal metabolites xanthomeglin (1) and viopurpurin (19) have been isolated from Aspergillus sulphureus together with the new pigments rubrosulphin (3,4,11,12-tetrahydro-9,17-dihydroxy-7-methoxy-3,12-dimethyl-2,13,16-trioxanaphth[1',2'-5,6]indenacene-1,8,14,15-tetrone) (16) and viomellien (8-(3,4-dihydro-9,10-dihydroxy-7-methoxy-3-methyl-1-oxo-1H-naphtho[2,3-c]pyran-8-y1)-3,4-dihydro-9-hydroxy-8-methoxy-3-methylnaphtho[1,2-c]pyran-1,7,10-trione) (10). The pigments (1), (10), and (19) were also isolated from Aspergillus melleus. Chemical and spectroscopic evidence for the proposed structures is presented. On this evidence structure (19) for viopurpurin is preferred to the alternative pyranobisnaphthopyran structures (22) or (23) previously suggested for this pigment.

N.m.r. evidence is presented showing that xanthomeglin (1) and viomellin (10) exist in solutions at room temperature as diastereoisomeric mixtures owing to restricted rotation about the dimeric linkage.

Four pigments have been isolated from the mycelium of Aspergillus sulphureus in yields which varied with the conditions of culture. These pigments were: xanthomeglin, previously isolated from Trichophyton rubrum,2 T. megzini,3 and T. violaceum4 and assigned structure (1);5,6 viomellin and rubrosulphin, two new metabolites for which structures (10) and (16) are respectively proposed; and viopurpurin, previously isolated from T. violaceum4 and tentatively assigned structures (22)4 or (23),6 for which the revised structure (19) is now proposed. Xanthomeglin (1), viomellin (10), and viopurpurin (19) were also isolated from the mycelium of A. melleus.

Despite some minor differences in the n.m.r. data (Table 1) and a different m.p. for the diacetate (2), the identity of xanthomeglin (1) was established by direct comparison with an authentic sample supplied by Dr. G. Just. An interesting feature of the n.m.r. spectrum, not noted previously,6 in which the signal for the strongly hydrogen-bonded hydroxy-group at $\tau - 3.1$ occurred as a double signal, is discussed below.

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* In CDCl$_3$ at 100 MHz. | Double signals.

* T. violaceum4 and tentatively assigned structures (22)4 or (23),6 for which the revised structure (19) is now proposed. Xanthomeglin (1), viomellin (10), and viopurpurin (19) were also isolated from the mycelium of A. melleus.

The n.m.r. (Table 1) and i.r. data indicated that the other three pigments had naphthoquinone–naphthalene structures in which the naphthaquinone portion was similar to that in xanthomeglin (1). This was confirmed by oxidation with alkaline hydrogen peroxide.

Like xanthomegnin (1), viomellein (10) and rubrosulphin (16) were oxidised to the phthalic acid (5) which was characterised as the anhydride (8). Similarly rubrosulphin dimethyl ether (18) and viopurpurin trimethyl ether (21) were oxidised to the methyl ether (6), $M^+ + 2\gamma$ ions after flushing the mass spectrometer source with deuterium oxide.

The naphthalene portion of viomellein (10) was shown to include a fused δ-lactone ring similar to that in the naphthoquinone portion by the n.m.r. and i.r. spectra.

The naphthalene portion of viomellein (10) was shown to include a fused δ-lactone ring similar to that in the naphthoquinone portion by the n.m.r. and i.r. spectra.
The carbonyl absorption at 1660 cm$^{-1}$ in viomellein was therefore assigned to the 5-lactone of the naphthalene portion. This assignment was supported by the i.r. spectrum of the triacetate (12) which showed strong carbonyl absorption at 1730-1725 but none at 1660 cm$^{-1}$. One of the strongly bonded hydroxy-groups ($\tau = -3.88$ or $-3.44$) was therefore vicinal to the lactone carbonyl group in the naphthalene portion, the other being at C-5 in the naphthaquinone portion.* The less strongly hydrogen-bonded hydroxy-group ($\tau = 0.21$) was also shown to be intramolecularly bonded by its concentration-independent i.r. absorption and was placed vicinal to the strongly-bonded hydroxy-group in the naphthalene portion. This vicinal arrangement was supported by the rapid formation of the diacetate (11) and slow formation of the triacetate (12).

The two aromatic protons in the naphthalene portion of viomellein (10) gave singlets and each was shown to be ortho or para to one of the hydroxy-groups by the changes in their chemical shift upon acetylation.\(^5,9\) The proton appearing at $\tau = 3.34$ was deshielded by 0.27 p.p.m. on formation of the diacetate (11) and assigned to C-1', and the proton at $\tau = 3.04$ was deshielded by 0.52 p.p.m. in the triacetate (12) and assigned to C-8'. The substitution pattern deduced from the spectroscopic pattern was finally established by base-catalysed conversion of viomellein (10) into the cyclic ether, rubrosulphin (16) (see below).

Rubrosulphin (16) was too insoluble for useful n.m.r. data to be obtained but its i.r. spectrum and the n.m.r. (Table 1) and i.r. spectra of the diacetate (17) and of the dimethyl ether (18) were similar to those of the corresponding derivatives (11)—(13) of viomellein (10). These data also showed that rubrosulphin contained one methoxy- and one hydroxy-group less than viomellein (10) and one additional oxygen-containing ring. Otherwise the substitution pattern in the two pigments appeared to be analogous. Structure (16) was therefore deduced for rubrosulphin, the position of the ether bridge in the naphthalene ring being supported by the higher chemical shift of the 1'-proton in the diacetate (17) compared with that of the 1'-proton in viomellein diacetate (11). The relationship between rubrosulphin (16) and viomellein (10) was established when, from the methylation of viomellein (10) with dimethyl sulphate and base, there was obtained the rubrosulphin dimethyl ether (18) in addition to viomellein trimethyl ether (13). Treatment of viomellein (10) with base in the absence of dimethyl sulphate afforded rubrosulphin (16). This conversion presumably involves attack of the C-4' alkoxide ion on C-2 of the quinone followed by elimination of methoxide ion (cf. ref. 10).

The deep purple quinone from A. sulphureus and A. melleus was identified as viopurpurin, originally isolated from T. violaceum by Just et al.\(^4\) from the spectroscopic data published by these authors for the quinone, its triacetate, and trimethyl ether. In particular the i.r. spectrum of the triacetate was identical with that reproduced\(^4\) for viopurpurin triacetate. Like rubrosulphin (16), viopurpurin (19) was very insoluble but the i.r. spectra of the two pigments were very similar as were those of the acetates and methyl ethers of the two pigments. The spectroscopic data indicated that viopurpurin was a 1'- or 8'-hydroxy-derivative of rubrosulphin (16). Comparing the chemical shifts (Table 1) of the aromatic protons in the acetates and methyl ethers of rubrosulphin and viopurpurin, the lower field proton in both sets is assigned as 6-H. The proton which is absent from the spectra of the viopurpurin derivatives is that which occurs at highest field, i.e. 1'-H, in the rubrosulphin derivatives. The 1'-hydroxy-rubrosulphin structure (19) is therefore proposed for viopurpurin. The deshielding of the 8'-proton in viopurpurin trimethyl ether (21) by 0.5 p.p.m. is precisely that expected from the presence of a peri(1')-methoxy-group.\(^11\) The observed deshielding of the 8'-proton by the peri(1')-acetoxy-group is smaller; no literature data are available but the electron-withdrawing acetyl group would be expected to reduce the effect.

The two alternative structures (22) and (23), previously suggested\(^4,6\) for viopurpurin, were based partly on the isolation of the phthalic acid (24) from the hydrogen peroxide oxidation of viopurpurin trimethyl ether. This acid (24) may come from the naphthalene portion of structure (21) although, in our hands, the isomeric phthalic acid (6) from the naphthaquinone portion was the only isolable product. Similarly we have obtained only the phthalic acids (5) and (6) from analogous oxidations of viomellein (10), rubrosulphin (16), and rubrosulphin dimethyl ether (18); no evidence for the formation of the phthalic acid (25) or its methyl ether (24) from the pigments or their methyl ethers was obtained. Further evidence which is incompatible with the structures (22) or (23) for viopurpurin, but consistent with our proposed structure (19), includes:


### Table 2

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<th>Pigment</th>
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<td>100</td>
<td>52</td>
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</table>

* The numbering system based on two naphthalene rings [see e.g. (1) and (10)] is used throughout the Discussion section.
(a) violpurpurin shows lactonic carbonyl absorption in the i.r. spectrum at 1725 cm\(^{-1}\) whereas structures (22) and (23) should show lower (1660 cm\(^{-1}\)) absorption due to strongly hydrogen-bonded carbonyl groups; (b) the chemical shift (\(\tau 2.29\)) of the naphthalenic proton in the trimethyl ether is too low for structure (23) in which the chemical shift should be similar to that (\(\tau 2.70\)) of the \(1'\)-proton in viomellein trimethyl ether (13); and (c) the vinylic or quinonoid proton of structure (22) would be expected to occur at higher field than \(\tau 2.23\) in the trimethyl ether and \(\tau 2.42\) in the triacetate; for example, the corresponding proton in 2-methoxy-1,4-naphthoquinone absorbs \(^{12}\) at \(\tau 3.8\).

The absolute stereochemistry of the asymmetric carbon in the lactone rings of xanthomegnin (1) was established by Just et al.\(^{12}\) by degradation to \((-\cdot)(R)\)-3-hydroxybutyric acid. The asymmetric centre in the naphthaquinone portion of viomellein (10), rubrosulphin (16), and violpurpurin (19) was shown to have the same absolute configuration as in xanthomegnin (1) by c.d. The phthalic acid (6), the dimethyl ester (6), and the methyl ether dimethyl ester (7) from all four pigments showed a negative Cotton effect at \(ca. 260 \text{ nm}\).\(^{15}\) The closely related \((-\cdot)(R)\)-mellein (26), another metabolite of \(A. melleus\), also shows \(^{16}\) a negative c.d. curve. Asperentin (27), a metabolite of \(A. flavus\) has the opposite lactone configuration and shows \(^{18}\) a positive Cotton effect at 260 nm. The c.d. curves of xanthomegnin (1) and viomellein (10) and of their acetates (2) and (12) were complex.

An interesting feature of the n.m.r. spectrum of xanthomegnin (1), viomellein (10), and their derivatives was the doubling of the singlets for some of the hydroxy-, acetyl, and methoxy-signals. The signals showing this doubling at 100 Hz are indicated in Table I. At 220 MHz doubling of other signals was observed in addition to increased separation of the doublets observed at 100 MHz. Similar doubling of signals was observed in the methyl ether acetates of procyanidin dimers by Weinges et al.\(^{19}\) who attributed the phenomenon to restricted rotation about the dimeric linkage and provided evidence for this explanation from the observed temperature dependence of the doublets. Similar evidence for restricted rotation in dimeric and trimeric derivatives of flavans has been reported by du Preez et al.\(^{17}\) and in the natural procyanidins by Thompson et al.\(^{17}\) In the present cases, the doublets coalesced on raising the temperature; for example, the doublets in the 100 MHz spectra of xanthomegnin (1) coalesce at 60°. Further evidence that this phenomenon is due to the mixture of diastereoisomers arising from the chirality at the 3,3’-dimeric linkage is provided by the absence of double signals of rubrosulphin (16) and violpurpurin (19) even at 220 MHz. The c.d. of xanthomegnin (1) and violpurpurin (10) did not show a large Cotton effect associated \(^{18}\) with the chirality of the dimeric linkage. However, the small amplitude at 300 nm is probably due to the slight excess of one of the diastereoisomers, since the doublets observed in the n.m.r. spectrum at room temperature were not of equal intensity.

The mass spectra of the pigments show similar fragmentation patterns consistent with the proposed structures. Xanthomegnin (1) and viomellein (10) show intense ions at \(m/e 85\) and 83, assigned structures (28) and (29) and derived from the quinone ring.

Rubrosulphin (16) and violpurpurin (19), which contain the ether bridge, do not show this fragmentation. Apart from these ions, the mass spectra of all four pigments show no major fragmentations after initial losses from the molecular ion. These high mass fragmentation ions which were mass-matched are rationalised for xanthomegnin (1) as follows. An initial loss of formaldehyde from the 2-methoxy-group, followed by the successive loss of carbon dioxide and a methyl radical from the lactone, gives the base peak which may be formulated as (30). The mass spectrum of violpurpurin (19) is analogous to that of xanthomegnin (1) except for the absence of the ions \(m/e 85\) and 83 and of the initial loss of formaldehyde from the molecular ion. The mass spectrum of viomellein (10) is dominated


\(^{13}\) A. Arakawa, N. Torimoto, and Y. Matsui, Annalen, 1969, 789, 162.

\(^{14}\) J. F. Grove, J.C.S. Perkin I, 1972, 240.


by the fragmentation to the ions m/e 85 and 83 but
after the initial loss of methanol it is very similar to
that of rubrosulphin (16) (cf. the chemical conversion
of viomellein into rubrosulphin involving the elimination
of methanol) and both spectra show a similar fragmenta-
tion sequence from the molecular ion, analogous to
that of xanthomegnin (I) after the initial loss of form-
alddehyde.

EXPERIMENTAL

For general experimental details see Part V.18

Production of Pigments from Aspergillus sulphureus.—
(a) A. sulphureus (CM1 128,939) was grown under stirred
aerated conditions in Raulin-Thom medium (30 l) containing
Celulose (5%). After 7 days the mycelium was collected and
extracted with acetone (3 x 6 l). The acetone was removed in vacuo
and the aqueous residue was extracted with chloroform to give a brown oily solid (661 g)
which was washed with light petroleum. The resultant
brown powder (21-4 g) contained, by p.i.c. (see later),
rubrosulphin (40%) and viopurpurin (10%).
(b) A. sulphureus was grown as surface cultures in
Thompson bottles each containing 11 of the medium
containing (see later) viomellein (19%), rubrosulphin (7%)
and xantomelogenin (28%), and viopurpurin (9%).
(c) A. sulphureus was grown as in (b) except that each
bottle contained 380 ml medium. Extraction as before
gave a brown solid (11 g) containing viomellein (19%),
rubrosulphin (7%), xantomelogenin (11%), and viopurpurin
(5%).

Production of Pigments by Aspergillus melleus.—A.
melleus was grown as previously described by Mills and
Turner 20 and the dried mycelium from 48 flasks was
extracted as described for A. sulphureus cultures. The brown
solid (8 g) contained viomellein (11%), xantomelogenin
(50%), and viopurpurin (1%) but no rubrosulphin.

Isolation of Pigments.—The following is typical of the
pigments from A. sulphureus. The crude mixture of
pigments (800 mg) in chloroform was applied to eight silica
gel plates (40 x 20 x 0.05 cm). Multiple elution with
methanol was added to give a brown solid (3 g) containing (see later) viomellein (19%),
rubrosulphin (7%), xantomelogenin (28%), and viopurpurin
(9%).

Isolation of Pigments from Aspergillus melleus.—A.
melleus was grown as previously described by Mills and
Turner 20 and the dried mycelium from 48 flasks was
extracted as described for A. sulphureus cultures. The brown
solid (8 g) contained viomellein (11%), xantomelogenin
(50%), and viopurpurin (1%) but no rubrosulphin.

Isolation of Pigments.—The following is typical of the
pigments from A. sulphureus. The crude mixture of
pigments (800 mg) in chloroform was applied to eight silica
gel plates (40 x 20 x 0.05 cm). Multiple elution with
methanol was added to give a brown solid (3 g) containing (see later) viomellein (19%),
rubrosulphin (7%), xantomelogenin (28%), and viopurpurin
(9%).
which was purified by p.l.c. on silica gel with ethyl acetate-light petroleum-acetic acid (200: 40: 1). The fluorescent band at Rf 0.06 was extracted with acetone to give the acid (9), which crystallised from acetone-light petroleum in yellow rods (3 mg) identical (m.p., mixed m.p., i.r., and 
\[\delta^1 H_{0.3} \]) with the acid (5) obtained in (a).

Bands from the p.l.c. at Rf 0.3, 0.56, and 0.69 gave intractable gums when extracted with chloroform.

(c) Rubrosulphin. The quinone (200 mg) in methanol (20 ml) and 3N-sodium hydroxide (30 ml) was oxidised with 30% hydrogen peroxide (30 ml) as in (a) to give uncleaned quinone (11 mg) and the acid (9) (90 mg). The latter was purified as in (b) to give rods (28 mg), identified by m.p., n.m.r., i.r., and 
\[\delta^1 H_{0.3} \] as an orange prisms (14 mg), m.p. 252-253° (lit., 5 227-228°) which was purified by p.l.c. on silica gel with ethyl acetate-light petroleum as needles, m.p. 104-105°, \[
\delta^1 H_{0.3} \].

Xanthomagnin Diacetate (2).—Prepared by treatment of the quinone (25 mg) with acetic anhydride (1.5 ml) and pyridine (8 drops) at room temperature in the dark for 24 h, the diacetate was purified by p.l.c. on silica gel with chloroform. The orange oil recovered from the band at Rf 0.35 was crystallised from chloroform-benzene as needles, m.p. 197-198° (Found: C, 606; H, 4.4%, C 40 H 36 0 16 requires C, 603; H, 4.7%); v., 1776, 1720, 1630, and 1575 cm'. Acetylation in pyridine with acetic anhydride at room temperature for 39 h gave dihydro-viomellein penta-acetate (15) which was purified by p.l.c. on silica gel with chloroform-formic acid (2000: 1) and crystallised from carbon tetrachloride in pale yellow beads (11 mg), m.p. 195-198° (Found: C, 60.6; H, 4.4%. C 34 H 29 0 13 requires C, 60.3; H, 4.4%); \[\delta^1 H_{0.3} \].

Acetylation of Viomellein (10).—Viomellein (48 mg), acetic anhydride (3 ml), and pyridine (12 drops) were stirred for 18 h at room temperature in the dark. The usual work-up gave a yellow solid which was fractionated by p.l.c. on silica gel with chloroform-formic acid (400: 1). Recovery from the band at Rf 0.70 in chloroform gave the diacetate (11), which crystallised from benzene-light petroleum as yellow beads (27 mg), m.p. 189-190° (Found: C, 63.8; H, 4.4. C 34 H 29 0 13 requires C, 63.8; H, 4.15%); \[\delta^1 H_{0.3} \].

Recovery from the band at Rf 0.40 gave the triacetate (12), crystallising from benzene-light petroleum in yellow beads, m.p. 105-106° (Found: C, 60.3; H, 4.5. C 34 H 29 0 13 requires C, 60.3; H, 4.4%); \[\delta^1 H_{0.3} \].

Recovery from the band at Rf 0.35 gave the trimethyl ether (21). The trimethyl ether (64 mg) was oxidised as in (d) for 22 h. Continuous extraction of the reaction mixture for 48 h with chloroform gave a yellow solution was extracted for 48 h to give 3,4-dihydro-6-methoxy-3-methyl-1-oxo-2-benzopyran-7,8-dicarboxylic acid (6), crystallised from methanol as needles, m.p. 200-208°, \[\delta^1 H_{0.3} \].

Sublimation of the diacid (6) at 180° and 4 mmHg gave anhydride (9), needles, m.p. 243-246° (Found: M, 308.090. C 15 H 16 0 7 requires C, 638; H, 44. C 34 11 25 0 14 requires C, 635; H, 4.15%)

viomellein trimethyl ether (13).—Prepared by hydrogenation of viomellein diacetate (20 mg) in ethyl acetate (18 ml) in the presence of pre-reduced palladium oxide (8 mg) at room temperature and pressure, the dihydro-derivative (14) which was purified by p.l.c. (Rf 0.27) on silica gel with chloroform-formic acid (2000: 1) and crystallised from carbon tetrachloride in pale yellow beads (11 mg), m.p. 195-198° (Found: C, 60.6; H, 4.4%. C 34 H 29 0 13 requires C, 60.3; H, 4.4%); \[\delta^1 H_{0.3} \].

Acetylation of viomellein diacetate (20 mg) in ethyl acetate (18 ml) in the presence of pre-reduced palladium oxide (8 mg) at room temperature and pressure, the dihydro-derivative (14) which was purified by p.l.c. (Rf 0.27) on silica gel with chloroform-formic acid (2000: 1) and crystallised from carbon tetrachloride in pale yellow beads (11 mg), m.p. 195-198° (Found: C, 60.6; H, 4.4%. C 34 H 29 0 13 requires C, 60.3; H, 4.4%); \[\delta^1 H_{0.3} \].

Xanthomagnin Diacetate (2).—Prepared by treatment of the quinone (25 mg) with acetic anhydride (1.5 ml) and pyridine (8 drops) at room temperature in the dark for 24 h, the diacetate was purified by p.l.c. on silica gel with chloroform. The orange oil recovered from the band at Rf 0.35 was crystallised from chloroform-benzene as orange prisms (14 mg), m.p. 252-253° (lit. 8 227-228°) (Found: C, 62.1; H, 4.1%, C 62.0; H, 4.0%); \[\delta^1 H_{0.3} \].

Tetrahydroxanthomagnin Diacetate (4).—Xanthomagnin diacetate (14 mg) in ethyl acetate (15 ml) was hydrogenated at room temperature and pressure in the presence of pre-reduced palladium oxide. The usual work-up gave the tetrahydro-derivative (4), which crystallised from carbon tetrachloride as beads (7 mg), m.p. 191-192° (lit., 8 192-193°). \[\delta^1 H_{0.3} \].

Dihydroviomellein Diacetate (14).—Prepared by hydrogenation of viomellein diacetate (20 mg) in ethyl acetate (18 ml) in the presence of pre-reduced palladium oxide (8 mg) at room temperature and pressure, the dihydro-derivative (14) which was purified by p.l.c. (Rf 0.27) on silica gel with chloroform-formic acid (2000: 1) and crystallised from carbon tetrachloride in pale yellow beads (11 mg), m.p. 195-198° (Found: C, 60.6; H, 4.4%. C 34 H 29 0 13 requires C, 60.3; H, 4.4%); \[\delta^1 H_{0.3} \].

Acetylation of viomellein diacetate (100: 1) (Rf 0.22) and crystallised from benzene-light petroleum as needles, m.p. 197.199° (Found: C, 62.4; H, 4.8. C 34 H 29 O requires C, 62.2; H, 4.7%); \[\delta^1 H_{0.3} \].

 Conversion of Viomellein (10) into Rubrosulphin (16).—Viomellein (65 mg) was added to anhydrous potassium carbonate (3 g) in aceton (30 ml) and the mixture was refluxed with stirring for 15 h. After removal of the
acetone under vacuum, water (20 ml) was added to the dark blue residue which was then acidified slowly with concentrated hydrochloric acid. Recovery of the resulting red precipitate gave a semi-solid (61 mg) which was fractionated by p.l.c. on silica gel after two elutions with chloroform-methanol-formic acid (90:5:5). Recovery from the band at $R_F$ 0.8 gave unchanged viomellein (40 mg). Recovery from the red zone at $R_F$ 0.7 gave rubrosulphin (10), identified by i.r., n.m.r., and mass spectra.

Rubrosulphin Diacetate (17).—Prepared by treatment of the quinone (250 mg) with acetic anhydride (4 ml) and pyridine (10 drops) at room temperature for 18 h in the dark, the diacetate (17) was obtained as an orange microcrystalline powder, m.p. 200—205° (Found: C, 64.3; H, 3.8; $C_{31}H_{24}O_{12}$ requires C, 64.7; H, 3.9%); $\nu_{\text{max}}$ 1780, 1730, 1687, 1635, 1605, 1575, and 890 cm$^{-1}$.

Rubrosulphin Dimethyl Ether (18).—Rubrosulphin (91 mg) in acetone (40 ml) was treated with dimethyl sulphate (10 ml) and potassium carbonate (4 g) as described earlier for xanthomegnin. The crude gummy product was purified by p.l.c. on silica gel with dichloromethane-formic acid (50:1) to give the dimethyl ether (18) as a red microcrystalline powder (70 mg), m.p. 183—187° (Found: $M^+$, 556.138; $C_{31}H_{24}O_{12}$ requires $M^+$, 556.137); $\nu_{\text{max}}$ (Nujol) 1720, 1675, 1621, and 1565 cm$^{-1}$, m/e 558 (52%, $M^+ + 2$), 556 (100, $M^+$), 541 (53), 538 (53), 523 (42), 495 (26), 485 (26), and 438 (19).

Viopurpurin Triacetate (20).—Prepared from viopurpurin (150 mg), acetic anhydride (3 ml), and pyridine (15 drops), the triacetate (120 mg) was purified by p.l.c. on silica gel with dichloromethane–formic acid (50:1) and obtained as a microcrystalline orange powder, m.p. 200—205° (lit., 280—285°); i.r. spectrum identical with that reproduced by Blank et al.

Viopurpurin Trimethyl Ether (21).—Prepared from viopurpurin (200 mg), anhydrous potassium carbonate (10 mg), and dimethyl sulphate (25 ml) in acetone (100 ml) as for xanthomegnin dimethyl ether, the trimethyl ether was purified by p.l.c. on silica gel with dichloromethane–formic acid (50:1) and obtained as a microcrystalline red powder, m.p. 170—175° (lit., 173—174°), $\nu_{\text{max}}$ (Nujol) 1722, 1678, 1619, and 1574 cm$^{-1}$.

We thank the S.R.C. for Student Scholarships for R. C. D. and T. J. S.
Fungal Products. Part 22. X-Ray and Molecular Structure of the Mono-acetate of Colletotrichin

By Richard Goddard, Ian K. Hatton, Judith A. K. Howard, Jake MacMillan, and Thomas J. Simpson, School of Chemistry, The University, Bristol BS8 1TS

Christopher J. Gilmore, Chemistry Department, The University, Glasgow G12 8QQ

The structure and relative stereochemistry (1) for colletotrichin, a metabolite of Colletotrichum capsici, previously isolated under the name acetylcolletotrichin, is deduced from an X-ray crystallographic study of the mono-acetate (2). The crystals are orthorhombic P2₁2₁2₁, \(a = 16.370(12), b = 11.919(3), c = 15.506(9)\) Å. Using 1554 independent 'observed' intensities, the refinement converged to \(R = 0.058\) \((R' = 0.074)\). The metabolite consists of a tetra-substituted \(\gamma\)-pyrone, containing all non-hydroxylic oxygens, and linked to an unusual bicyclic terpene.

In the crystal structure the molecules of the monocacetate (2) (see Figure 1) have both alicyclic rings in chair-like conformations in which the angular C(10)-methyl, the C(4)-methyl, the C(3)-acetoxy, and the C(9)-pyronylmethyl groups are axial while the C(4) isopentanyl group is equatorial. The C-C and C-O single-bond lengths (Table 2) are consistent with the values predicted from covalent radii of 0.77, 0.74, and 0.66 Å for C(sp³), C(sp²), and O respectively. The values for the C=C bonds between C(2') and C(3'), C(5') and C(6'), and C(8)-C(12), and the C=O bonds between C(3'-1) and O(3'-1) and C(3-1) and O(3-2) fall within the usual ranges. The carbon–oxygen bond at C(4')-O(4'-1) (1.248 Å) is longer than a normal C=O bond but is part of the planar \(\gamma\)-pyrone ring (mean deviation from the least-square
plane through O'(1), C(2'), C(3'), C(4'), O(4'-1), C(5'), and C(6') equals 0.02 Å. The elongation of the C-O bond length at C(4')-O(4'-1) is thus a measure of the aromatic character of the pyrone ring, and is in agreement with the ω = α at 1.662 and 1.663 cm⁻¹ in the acetate (2) and compound (3) which also indicates considerable single-bond character. The plane of the C(3')-methoxy-carbonyl group makes an angle of 14.2° with the pyrone

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<td>0.314(7)</td>
<td>1.077(5)</td>
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<td>C(2)</td>
<td>0.488(6)</td>
<td>0.235(6)</td>
<td>1.140(5)</td>
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<tr>
<td>C(3)</td>
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<td>0.114(7)</td>
<td>0.960(5)</td>
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<tr>
<td>C(4)</td>
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<td>0.087(6)</td>
<td>1.106(5)</td>
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<td>C(5)</td>
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<td>0.175(6)</td>
<td>0.960(5)</td>
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<td>C(6)</td>
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<td>0.235(6)</td>
<td>0.804(5)</td>
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<td>C(7)</td>
<td>0.490(6)</td>
<td>0.356(6)</td>
<td>0.831(5)</td>
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<td>C(8)</td>
<td>0.532(8)</td>
<td>0.374(7)</td>
<td>0.914(5)</td>
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<td>0.201(6)</td>
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<td>C(10)</td>
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<td>0.914(5)</td>
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<td>C(11)</td>
<td>0.463(6)</td>
<td>0.440(7)</td>
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<td>C(12)</td>
<td>0.480(6)</td>
<td>0.329(6)</td>
<td>0.899(5)</td>
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<td>C(13)</td>
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<td>C(14)</td>
<td>0.395(8)</td>
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<td>O(11)</td>
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<td>0.321(9)</td>
<td>0.660(2)</td>
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**Table 2**

<table>
<thead>
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<th>Bond lengths (Å) and angles (°)</th>
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<tr>
<td>(a) Distances</td>
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<tr>
<td>C(10)-C(1)</td>
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<td>C(11)-C(1)</td>
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<tr>
<td>C(12)-C(2)</td>
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<td>O(3)-C(2)</td>
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<td>O(3)-C(2)</td>
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<tr>
<td>C(3)-C(4)</td>
</tr>
<tr>
<td>C(4)-C(5)</td>
</tr>
<tr>
<td>C(5)-C(6)</td>
</tr>
<tr>
<td>C(6)-C(7)</td>
</tr>
<tr>
<td>C(7)-C(8)</td>
</tr>
</tbody>
</table>

The sign convention is such that the sign is positive if clockwise rotation is required of atom (1) to eclipse atom (4) whilst looking down the (2)-(3) bond.

ring, and there is little multiple-bond character in the C(3')-C(3')-1 bond length which approximates to twice the covalent radius of an sp²-carbon.
In the crystal packing (Figure 2) all the intermolecular distances are larger than the sums of the relevant van der Waals radii but there is a slight interaction between the C(17) tertiary hydroxy-group and the C(4'-1) carboxyl oxygen of the \( \gamma \)-pyrone in molecules which are related by a translation of \( y \) \( O(4'-1) \cdots O*(17-1) = 2.87(1) \) Å where * denotes the symmetry operation \( x, 1 + y, z \).

The derived structure (1) for the parent metabolite contains no acetyl group. Hence it is suggested that the name acetylcolletotrichin be replaced by colletotrichin. The \( ^1 \)H n.m.r. signal at \( \tau 7.65 \) (s, 3 H), originally assigned \( ^2 \) to an acetyl group, can now be assigned to the C(2')-methyl group on the \( \gamma \)-pyrone \( ^{11} \) and the C-2 and C-6 methyl signals of compounds (3) \( ^3 \) which occur at \( \tau 7.61 \) and 7.77 respectively and the C-methyl signal of compound (4) \( ^{11} \) at \( \tau 7.81 \). The \( \nu_{CO} \) band at 1 736 cm\(^{-1} \), also assigned \( ^2 \) to an acetate carbonyl, can now be reassigned to the methoxycarbonyl group at C(3') of the \( \gamma \)-pyrone ring. The i.r. absorption at 1 736 cm\(^{-1} \) agrees with that observed \( ^4 \) (1 682 cm\(^{-1} \)) for the carbonyl of the \( \gamma \)-pyrone (4) and the bands at 1 607 and 1 588 cm\(^{-1} \) are the \( \nu_{C=O} \) absorptions of the pyrone. The u.v. spectrum, \( \lambda_{max} \) 261 (\( \epsilon 7 000 \)), agrees with the data \( ^5 \) for the \( \gamma \)-pyrone chromophore in compound (5). All other spectroscopic assignments made by Grove et al. \( ^3 \) fit structure (1). Structure (1) also explains the observed \( ^2 \) instability of colletotrichin to basic conditions and the alkaline hydrogen peroxide oxidation to an acid for which structure (6) can now be considered.

The mass spectrum of colletotrichin can be rationalised by cleavage of the C(9)-C(11) bond to give the base peak \( m/e \) 212 (C\(_{18}\)H\(_{26}\)O\(_5\)) containing the pyrone ring and \( m/e \) 243 (C\(_{18}\)H\(_{27}\)O\(_5\)) containing the terpenoid portion. In the mass spectrum of the bis-TMSI ether, the three most intense peaks at \( m/e \) 212, 199, and 131 probably arise by cleavage of the C(9)-C(11), C(11)-C(5'), and C(18)-C(17) bonds respectively.

Colletotrichin (1) is one of the few natural \( \gamma \)-pyrones and the first which is polypropenylated. Another notable feature is the novel terpenoid ring system though since our preliminary announcement \( ^6 \) of structure (1) for colletotrichin, the dialdehydes (7) and (8) have been isolated from liverworts.\( ^7 \) \( ^8 \) The interesting biochemical properties \( ^9 \) of colletotrichin may be primarily associated with the pyrone ring; the \( \gamma \)-pyrone, aureothin,\( ^{10} \) shows mammalian toxicity and the aurovertins\( ^{11} \) are potent inhibitors of ATP-synthesis and ATP-hydrolysis catalysed by mitochondrial enzymes.

Since the publication of a preliminary account \( ^6 \) of this work, the single crystal X-ray structure of colletotrichin has been reported in preliminary form \( ^{12} \) and serves to confirm structure (1). More recently the preliminary account of \( ^{[13]} \) biosynthetic studies has been published \( ^{13} \) indicating that colletotrichin is a mero-norditerpene.

**EXPERIMENTAL**

General details are given in refs. 1 and 14; i.r. spectra were determined for Nujol mulls except where stated otherwise.

**Isolation of Colletotrichin (1).—Colletotrichum capsici Syd. Butler and Bisby strain 1287, obtained from I.C.I. (Pharmaceuticals) Ltd., was stored on slopes of 2% malt agar under liquid paraffin. For inoculation a spore suspension was prepared from agar slopes, grown for 7 days in medicine bottles. The spore suspension was dispensed (1 ml per
to penicillin flasks (9 in diameter) each containing 200 ml of a medium consisting of dextrose (5%), potassium dihydrogen phosphate (1%), ammonium nitrate (0.25%), magnesium sulphate heptahydrate (0.02%), and trace elements solution (1 ml/l). The fermentations, at pH 4.6, were monitored by g.l.c. (TMSi derivative; 2% OV-17, glass column (162.5 x 0.64 cm); isothermal, 265 °C; Rf 28 min) and t.l.c. [silica gel; ethyl acetate-light petroleum-acetic acid (60: 35: 5); Rf 0.2]; yields of colletortichin reached a maximum at 25-32 days.

From a large-scale fermentation, carried out at M.R.E. Forton Down, the culture filtrates (50 l) were extracted with chloroform to yield a crude product (12 g) which was adsorbed on silica gel (25 g) and placed on top of a column (70 x 5 cm) of silica gel (300 g). The column was eluted with 500 ml portions of 2, 5, 10, 25, 50, and 75% diethyl ether in light petroleum, then with ether (1 l) followed by 1 l fractions of 1, 2, 5, 10, and 25% methanol in ether. The last fraction yielded 1.27 g of material which was crystallised from ethyl acetate-light petroleum to give colletortichin (1.0 g).

Further purification by p.l.c. on silica gel layers (0.8 mm), developed with ethyl acetate-light petroleum-acetic acid (9: 10: 1) gave colletortichin (Rf 0.5) which crystallised from ethyl acetate-light petroleum as small needles, m.p. 181-184 °C (lit. 186-187 °C) (Found: C 49.0, H 4.2, N 3.9, requires C 49.2, H 4.0, N 3.8; R(CDCl) 1746, 1672, and 1591 cm').

Crystal Data.—C 4.90 H 5.29 O 7 requires Mo-K 3 X-radiation (λ = 0.7096 Å; μ(Mo-K) = 0.78 cm'). The structure was determined by the multi-solution method of crystal structure solution, deduced by Germain and Woolfson. The origin and enantiomorph definitions were chosen and their phases fixed according to normal criteria. It was decided to include two reflections (i.e., those whose phases were restricted to one of two values by the space group symmetry) and two general reflections, and these were chosen by the MULTAN program. Finally, for each of the 64 sets of phases generated by the bipyramid formula, figures of merit were calculated. One set of phases had a combined figure of merit 3.0; this was selected from the subsequent E map based on these phases. 25 atom were located at chemically reasonable positions. After 4 cycles of isotropic least-squares refinement, R = 0.26 for the remaining 10 non-hydrogen atoms were located from successive electron-density difference syntheses.

The data proved insensitive to atom type and so it was only after the final cycles of refinement that the chemical nature of the molecule was confirmed (Figure 1). The hydrogen atoms were set at calculated positions (C-H = 0.98 Å) except for the methyl and hydroxyl-hydrogens, which were located from difference-Fourier maps. All the parameters for the hydrogen atoms were fixed during refinement (U(eq) = 0.07 Å). In the final cycles, refinement was carried out with block-matrix least-squares with anisotropic thermal parameters for all non-hydrogen atoms. The weighting scheme used for refinement was w = (2.25 - 0.410F0) + 0.022F02 - 0.003 F02, adjusted to minimise the variation of <(ω/ω)2> with |F0|.

The refinement converged at R = 0.058 (R' = 0.074) with a mean shift/error value in
The final cycle of 0.05. A final electron-density difference-Fourier synthesis showed no peaks > 0.3 e Å⁻³ or < -0.4 e Å⁻³. Atomic scattering factors for carbon and oxygen were taken from ref. 19, and for hydrogen from ref. 20. Final atomic co-ordinates are listed in Table 1; temperature factors and observed and calculated structure factors are listed in Supplementary Publication No. 22484 (12 pp.).

We thank the S.R.C. for Research Studentships (R. G., C. K. H., and T. J. S.); Dr. R. J. Pryce for preliminary exploratory experiments; and Dr. J. Melling for his expert help with the large-scale fermentation.

[Received, 27th July, 1978]

For details of the Supplementary publications scheme see Notice to Authors No. 7, J.C.S. Perkin I, 1978, Index issue.

REFERENCES


The Biosynthesis of Fungal Metabolites. Part III. Structure of Shami-xanthone and Tajixanthone, Metabolites of Aspergillus variecolor

By Kuldip K. Chexal, Christopher Fouweather, John S. E. Holker,* Thomas J. Simpson, and Kenneth Young, Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

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1974
The Biosynthesis of Fungal Metabolites. Part III. Structure of Shamilxanthone and Tajixanthone, Metabolites of Aspergillus variecolor

By Kuldip K. Cheval, Christopher Fouweather, John S. E. Holker,* Thomas J. Simpson, and Kenneth Young, Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

The structures of shamixanthone and tajixanthone, optically active metabolites of Aspergillus variecolor, are shown to be (1R,2S)-1,11-dihydroxy-2-isopropenyl-5-methyl-8-(3-methylbut-2-enyl)- (IV) and (1R,2S)-8-[(2S)-2,3-epoxy-3-methylbutyl]-1,11-dihydroxy-2-isopropenyl-5-methyl-2,3-dihydro-1H-pyran[3,2-a]xanthene-12-one (V) respectively. These structures were established by detailed analyses of the 1H and 13C n.m.r. spectra of the metabolites and their derived compounds as well as by chemical degradation of the substituted dihydrobenzopyran system. The absolute configurations were assigned by application of the Horeau asymmetric synthesis.

The isolation of six optically active xanthones from cultures of Aspergillus variecolor (Curzi) (A. stellatus) has been reported and structures (I), (II), and (III) have been proposed for three of these; shamixanthone (C_{25}H_{26}O_{5}), tajixanthone (C_{25}H_{26}O_{5}), and ajaryxanthone (C_{25}H_{24}O_{4}) respectively. In view of the apparent novelty of these structures we have carried out a re-examination of the same strain of A. variecolor and have isolated a number of metabolites, two of which are clearly identical with shamixanthone and tajixanthone. We now report investigations leading to new structures (IV) and (V) respectively for these metabolites.

* We are grateful to Dr. A. Kamal for supplying this strain of A. variecolor.


The presence of a peri-hydroxyxanthone chromophore in these compounds is indicated by the identical u.v. spectra, \( \lambda_{\text{max}} \) 392, 292, 278, 270sh, 256, and 242 nm (c 5100, 7400, 27,400, 25,400, 18,800, and 20,800), i.r. bands at 3240-3100 and 1642 cm\(^{-1}\), and a singlet at \( \delta -2.58 \) (1H, exchangeable with D\(_2\)O), in their 1H n.m.r. spectra. Furthermore, both compounds give typical iron(III) chelate colourations. This structural conclusion represents the limit of our agreement with previous workers.

The presence of a secondary alcoholic function in both


(a) In addition to the usual vic- and allylic couplings in the C-prenyl residue which serve to confirm this structural feature, the methylene protons of this residue show an allylic coupling ($J = 0.5$ Hz) with the lower field ortho-coupled aromatic proton. Hence the prenyl group is located in a tetrasubstituted aromatic ring.

(b) The protons of the aromatic methyl substituent are coupled ($J = 0.9$ Hz) with the aromatic proton, 2-75, referred to above. Since this proton shows no further coupling, the methyl substituent is located in a penta-substituted aromatic ring.

tively), they correspond to 

gaucho-conformations of the individual protons, and the substituent R in this system must therefore have a pseudo-axial conformation.

(d) In the spectrum of shamixanthone the isopropenyl group shows the usual gem-coupling of the $H_2C\equivC\equiv$ protons ($J = 1.4$ Hz) and allylic couplings between these and the methyl group ($J$ ca. 0.8 and 1.4 Hz respectively). However, each of the $H_2C\equivC\equiv$ protons is also coupled with the high field proton of the $-OCH_2CH(R)CH(OH)$-fragment ($J = 1.4$ and ca. 0.6 Hz respectively). Hence, the substituent R in this fragment is defined as the isopropenyl group in shamixanthone and as isopropyl in the tetrahydro-derivative (Table 2b).

At this stage the partial structure (XVII) can be advanced for shamixanthone, the only features to be decided, apart from stereochemical considerations, being the positions of the aromatic methyl and prenyl substituents in rings A and C respectively. The latter was established from the following considerations. (a) Tajixanthone and tetrahydroshamixanthone gave negative Gibb's tests under the modified conditions of King et al.\(^6\) The 4-position of the 1-hydroxyxanthone system must therefore be substituted by the prenyl

residue. It is interesting that shamixanthone slowly developed a positive reaction with the Gibb’s reagent. Although the reason for this is not clear it does not invalidate the general conclusion.

(b) No loss of 56 mass units was seen in the mass spectrum of shamixanthone. α-Prenyl phenols characteristically show this loss. 6

c) The 1H n.m.r. spectrum of O-methyl tajixanthone (VII) in 75% C6D6-DCl3 showed an upfield shift of 0.36 p.p.m. for the methoxy-protons, compared with the spectrum in DCl3. This indicates that at least one of the positions ortho to the methoxy-group is unsubstituted. 6

The position of the aromatic methyl substituent in ring A of the metabolites was established by the following reaction sequence, which also provides confirmatory evidence for the substituted dihydrobenzopyran ring. Oxidation of tajixanthone with osmium tetroxide and sodium periodate gave the methyl ketone (XVIII) (+ 7.69 and v max. 1710 cm⁻¹). Since this compound is a β-hydroxyketone it was readily dehydrated with potassium hydroxide in methanol to the αβ-unsaturated ketone (XIX) (v max. 1660–1640 cm⁻¹) which had 1H n.m.r. signals at + 7.59 (Me-CO) and 1.69 (t, J 1-5 Hz, -CH-C=CO-), allylic coupling to ring methylene group). The very low field chemical shift of this vinyl proton is due to the deshielding effect of the xanthone carbonyl group. Reaction of compound (XIX) with osmium tetraoxide, decomposition of the resultant osmate ester and subsequent addition of methanol, gave the vic-diol (XXI), in which the elements of methanol had added across the epoxide ring. Oxidation of this compound with periodic acid in ether 8 gave the formyl-acid (XXIII), formed by cleavage of the vic-diol followed by oxidative fission of the resultant α,α, diketone. The structure of this compound was established by its 1H n.m.r. spectrum in (CD3)2CO which showed signals at + 5.37 (O-CH2-CO2H) and -0.61 (Ar-CHO). The corresponding methyl ester, prepared with diazomethane, was decarboxylated with chlorotri triphosphinephosphorohydrid(m) in benzene 10 to give the product (XXIV), in which the chemical shifts of the two ring A aromatic protons were readily differentiated since the signal at + 2.08 showed the usual 0.9 Hz coupling with the aromatic methyl substituent and that at + 2.76, which must be due to the newly introduced proton, showed no coupling greater than ca. 0.3 Hz which represented the spectral resolution obtained [spectrum in (CD3)2CO]. The absence of m-coupling between these two protons defines the position of the methyl substituent in ring A and provides the final piece of evidence which establishes structures (IV) and (V) for shamixanthone and tajixanthone respectively, apart from stereochemical assignments.

The mass spectra of the metabolites accord with the proposed structures and the principal fragmentation patterns of structural interest are summarised in Schemes 1 and 2. Since both shamixanthone and tajixanthone show relatively abundant ions due to losses of C5H8 and C6H9, these must occur principally from the substituted dihydrobenzopyran residues. It is probable that rearrangement of the parent ions occur leading to O-prenyl aldehydes, as illustrated for shamixanthone in Scheme 1. Subsequent losses would then be as expected for prenyl ethers, leading to ions a and b from shamixanthone and a' and b' from tajixanthone. Ions a and a' further fragment by loss of CO, presumably from the aldehyde groups, giving ions c and e' respectively. Other fragmentations of both parent ions are similar and include losses of H2O, presumably from the secondary alcohol portions, giving ions d and d' respectively. A series of fragmentations of particular interest in tajixanthone is represented by ions k', i', and j' due to successive losses of CH2=CO, C5H8, and H2O respectively from the parent. Ion j' represents the base peak of this spectrum. This fragmentation pattern is characteristic of C-prenyl epoxides 11 and is thought to arise by initial pinacol type rearrangement to give ion g', followed by fragmentations as shown in Scheme 2. In the case of shamixanthone the interesting loss of MeO from ions c and d to give ions e and f respectively is typical of an aromatic prenyl substituent with ether oxygen in the α-position. 11 The mass spectra of the methyl ethers (VI) and (VII) are very similar and include losses of C5H9 giving base peaks at m/e 351 and 367 respectively.

The relative and absolute stereochemistry of shamixanthone and tajixanthone must now be considered. In shamixanthone (IV) there are two chiral centres, C(20) and C(25), and in tajixanthone the additional centre at C(15). 12 Furthermore, since tajixanthone has been converted into shamixanthone both compounds have the same absolute stereochemistry at C(15). 13 The absolute configuration at C(15) in tajixanthone was readily established by the method of Boar and Damps. 14 Thus, methylation of the αβ-unsaturated ketone (XIX) gave the methyl ether (XX) which was converted into the dimethoxy-alcohol (XXI) by acid-catalysed methanolysis. This compound was treated with (+)-x-phenylbutyric anhydride, as in the Horeau asymmetric synthesis, 15 to give an excess of (–)-x-phenylbutyric acid (optical yield 33%). Hence, tajixanthone has the (1S,2S)-configuration, as shown in structure (V). The absolute configuration at C(25) was also established by the same method. Thus, 10 Y. Shimizu, H. Misumashi, and E. Caspi, Tetrahedron Letters, 1966, 4113.

11 J. K. MacLeod, Org. Mass Spectrometry, 1972, 6, 1011.
metabolites is established by the $^1$H n.m.r. spectra which show a hydroxyl proton, exchanged with D$_2$O, at $\tau$ 4.88 (d, $J$ 3.9 Hz) with the corresponding CHO-H signal at $\tau$ 4.61 (q, $J$ 3.9 and 2.8 Hz, collapsing to d, $J$ 2.8 Hz after D$_2$O exchange). The O-H stretching at 3615 cm$^{-1}$ in the i.r. spectrum of shamixanthone is independent of concentration and this high frequency suggests that the group is not hydrogen bonded. It is unusual to find a secondary alcohol which does not form an intermolecular hydrogen bond in concentrated solution and indicates that the hydroxy-group must be in a sterically crowded environment. Methylation (VIII) which had typical $^1$H n.m.r. signals at $\tau$ 7.93 (Me-CO) and 3.02 (methine proton shifted 1.55 p.p.m. to low field compared with parent alcohol).

Two isolated olefinic double bonds were shown to be present in shamixanthone by oxidation with one mol. equiv. of monoperphthalic acid to give a monoepoxide, and with excess of reagent, a diepoxide. The $^1$H n.m.r. spectra and m.p.s of the monoepoxide and tajixanthone were identical, establishing the structural relationship between the two metabolites. This was confirmed by conversion of tajixanthone into shamixanthone by reaction with triphenylphosphine selenide of shamixanthone and tajixanthone with methyl iodide and potassium carbonate in acetone gave the mono-methyl ethers (VI) and (VII) respectively, and the secondary alcoholic O-H stretch was now at 3470 cm$^{-1}$ in the i.r. spectrum of (VI). Since this is at lower frequency than in the parent (IV) and is unchanged on dilution, the hydroxy-group must now be intramolecularly hydrogen bonded. The change from a non-bonded hydroxy-group in the parent metabolites to an intramolecular hydrogen bonded group suggests that the OH group is relatively close to the xanthone carbonyl and becomes hydrogen bonded when the original strong hydrogen bond of the peri-phenolic hydroxy-group is removed by methylation. Acetylation of tajixanthone methyl ether gave the acetate and trifluoracetic acid, a reaction which effects the conversion of an epoxide into an alkene.$^4$

Hydrogenation of shamixanthone with chlorotristriphenylphosphinerrhodium(I) catalyst in benzene$^6$ gave the dihydro-derivative (IX), with palladium-carbon in ethyl acetate, the tetrahydro-derivative (X), and with platinum in ethyl acetate, a mixture of the tetrahydro-derivative (X) and the tetrahydrodeoxy-derivative (XI). The formation of the latter compound indicates that the secondary alcoholic hydroxy-group is benzylic and this is confirmed by the position of the signal of the methine proton ($\tau$ 4.61) in the $^1$H n.m.r. spectrum of shamixanthone. Hydrogenation of tajixanthone with a platinum catalyst in ethyl acetate gave a mixture of the dihydro-derivative (XII) and the


tetrahydro-derivative (XIII) in which the epoxide ring had been cleaved to a secondary alcohol, shown by oxidation with chromic oxide to the ketone (XIV). It is noteworthy that neither in this oxidation nor in similar attempted oxidations of shamixanthone (IV) and its tetrahydro-derivative (X) has it been possible to oxidise the benzyl alcohol group. These observations, and the hydrogen bonding studies discussed earlier, suggest that the benzyl alcohol group must be in a ring system and substituted ortho to the xanthone carbonyl group. Oxidation would then be difficult since the hypothetical derived ketones would have strong steric and electronic repulsions between the introduced and xanthone carbonyl groups. Hydrogenation of tajixanthone with palladium-carbon in methanol gave compound (XV), in which saturation of the isopropenyl double bond was accompanied by addition of the elements of methanol to the epoxide ring. Presumably a trace of acid in the catalyst facilitates this addition. Oxidation of compound (XV) with chromic oxide gave the methoxy-ketone (XVI).

The principal structural features in shamixanthone and tajixanthone were deduced initially from correlations of 100 MHz 1H and proton-noise decoupled 25-2 MHz 13C n.m.r. spectral data summarised in Table 1. The assignments of individual signals in the spectra were made from expected chemical shift values, comparisons between the 1H and 13C spectra of the metabolites and their derivatives, and off-resonance decoupling of the 13C spectra.

The main conclusions from these studies are summarised as follows. (a) The spectra of shamixanthone (IV) and tajixanthone (V) differ only in the signals which must be assigned to an aromatic prenyl substituent in the former compound and the corresponding epoxide in the latter.

(b) The signals due to the isopropenyl residue in both metabolites are replaced in dihydrotajixanthone (XII) by the characteristic signals of an isopropyl group with non-equivalent methyls. i.e. 8 9-02 and 8-98 (J 6-5 Hz) in the 1H spectrum and at 8 20-4 and 21-2 in the 13C spectrum. The CH fragment of the isopropyl group occurs at 8 8-32 (1H) and 8 25-2 (13C) in the corresponding spectra.

(c) The aromatic methyl substituents have the same chemical shifts in all the above derivatives, i.e. 8 7-68 (1H) and 8 17-3 (13C).

(d) The 13C spectra show twelve aromatic carbon atoms of which three only are C-H carbons. In the 1H spectra the corresponding three protons occur as an ortho-coupled pair and another showing a small coupling (J 0-9 Hz) which is discussed below. 13C Chemical shifts show that four of the remaining nine aromatic carbon atoms carry oxygen substituents.

(e) The signal at lowest field in the 13C spectra, i.e. 8 183-6 is assigned to the xanthone carbonyl group.

The many couplings in the 1H spectrum of shamixanthone were elucidated by extensive decoupling experiments using a degassed solution in acid free CDCl3. These couplings, which are summarised in Table 2a, were vital in the following structural deductions.

The many couplings in the 1H spectrum of shamixanthone were elucidated by extensive decoupling experiments using a degassed solution in acid free CDCl3. These couplings, which are summarised in Table 2a, were vital in the following structural deductions.
treatment of o-methylshamixanthone with (±)-α-phenylbutyric anhydride gave an excess of (±)-α-phenylbutyric acid (optical yield 20%). On the assumption that the phenyl group is the largest substituent at C(25) in this chiral alcohol, then shamixanthone, and corresponding tajixanthone, have the (25R)-configuration as substituent and the adjacent hydroxy-group at C(25). If this assumption holds, butyric acid (optical yield 20%). On the assumption that butyric anhydride gave an excess of (+)-α-phenylbutyric anhydride, the isopropyl substituent in the hydroxy-substituents are trans-related there would seem to be no valid reason for this conformational preference. On the other hand, trans-relationships of these two groups would be better accommodated in a diaxial rather than a disequatorial conformation with its attendant vicinal bonded interactions. Hence, it seems probable that the isopropyl and hydroxy-substituents are trans-related in the dihydropyran rings of shamixanthone and tajixanthone as shown in the structures (IV) and (V) respectively.

The structures of minor metabolites of Aspergillus variecolor and the biogenesis of all these compounds are being investigated and will be reported subsequently.

EXPERIMENTAL

Unless otherwise stated, i.r. absorption spectra were measured with a Perkin-Elmer model 125 instrument for KBr discs, u.v. spectra with a Unicam SP 800 instrument for solutions in ethanol, 1H n.m.r. spectra with a Varian HA-100 instrument for solutions in acid-free deuteriochloroform containing tetramethylsilane as internal standard, 13C n.m.r. spectra with a Varian XL-100-15 FT spectrometer for similar solutions, and optical rotations with an ETL-NPL automatic polarimeter for solutions in chloroform. Mass spectra were measured at 70 eV with an A.E.I. MS12 spectrometer and accurate masses with an MS8 instrument. Thin-layer chromatography (t.l.c.) was performed using silica gel G.F. (Merck). M.p.s were determined with a Kofler hot-stage instrument. Spectral data marked with an asterisk are listed in Supplementary Publication No. SUP 20692 (12 pp., 1 microfiche).

Isolation of Shamixanthone (IV) and Tajixanthone (V).—Aspergillus variecolor, I.M.I. strain 112543, was grown from a spore suspension in static culture for 15 days at 25° in flat vessels (ca. 1 l capacity), each containing Czapek-Dox medium (500 ml). The dried mycelium (ca. 7-8 g) was ground and continuously extracted with light petroleum (b.p. 40-60°). The resulting dark oil was triturated with warm methanol and the methanol-soluble fraction evaporated to give a yellow solid which was fractionated by preparative t.l.c. using benzene-ether (95:5 v/v) as developing solvent. Shamixanthone (IV) was eluted with ethyl acetate from the band with Rf 0.96, and crystallised from methanol to give yellow needles (60-80 mg), m.p. 158-159°, [x]_D^24 -5-6° (c 2.44), vmax*, * (Found: C, 71-1; H, 6.2. Calc. for C_{25}H_{26}O_6: C, 71.3; H, 6.6%).

Methylation of Shamixanthone and Tajixanthone.—On reaction with methyl iodide and anhydrous potassium carbonate in acetone, shamixanthone (60 mg) gave (1R,2S)-1-hydroxy-3-isopropenyl-5-methoxy-5-methyl-2,3-dihydro-1H-pyran-3,2-a-xanthone-12-one (VI), needles (45 mg) from acetone-light petroleum (b.p. 60-80°), m.p. 170-171°, [x]_D^24 +22.3° (c 1.34), vmax*, * (Found: C, 74.2; H, 6.8. C_{26}H_{28}O_7 requires C, 74.3; H, 6.7%).

Under similar conditions, tajixanthone (100 mg) gave (1R,2S)-8-(2S,3S)-1-hydroxy-3-isopropenyl-5-methoxy-5-methyl-2,3-dihydro-1H-pyran-3,2-a-xanthone-12-one (VII), needles (72 mg) from methanol-ether, m.p. 176-177°, [x]_D^24 +18.7° (c 2.0), vmax*, * (Found: C, 71.3; H, 6.2. C_{26}H_{28}O_7 requires C, 71.6; H, 6.4%). Accretion of O-methyltajixanthone (VIII) (100 mg) with acetic anhydride-pyridine under the usual conditions gave the monocacetate (VIII) as a gum (86 mg), vmax*, * (m/e).

Exposition of Shamixanthone.—Shamixanthone (50 mg) was treated with 1 mol. equiv. of monoperphthalic acid (23 mg) in ether (60 ml) at 0° for 4 days. The ether solution was then extracted with saturated sodium hydrogen carbonate solution (3 x 20 ml), washed with water (3 x 20 ml), and dried (MgSO_4). After removal of the solvent, the residue was recrystallised from methanol to give tajixanthone (42 mg), m.p. and mixed m.p. 158-159°, with identical i.r. and 1H n.m.r. spectra.

Hydrogenation of Shamixanthone.—(a) Shamixanthone (148 mg) in dry benzene (20 ml) was stirred under hydrogen for 24 h with chlorotristriphenylphosphinerhodium(x) (148 mg) in dry benzene (20 ml). After hydrogenation at room temperature and atmospheric pressure for 3 days in the presence of Adams catalyst (100 mg) to give a mixture of two products which were separated by t.l.c. using ether-benzene (5:9 v/v) as developing solvent. The band, Rf 0.60, was removed and extracted with ethyl acetate to give shamixanthone, yellow needles (70 mg) from methanol, m.p. and mixed m.p. 154-156°, with identical i.r. and 1H n.m.r. spectra.

(b) Shamixanthone (150 mg) in ethyl acetate (50 ml) was hydrogenated at room temperature and atmospheric pressure for 3 days in the presence of Adams catalyst (100 mg) to give a mixture of two products which were separated by t.l.c. using ether-benzene (5:9 v/v) as eluting solvent. The band, Rf 0.96, gave tajixanthone (X), as yellow needles (80 mg) from methanol, m.p. 141-142°, [x]_D^24 +11.6° (c 1.07), vmax*, * (Found: C, 73.1; H, 6.3%).

* For details of Supplementary Publications, see Notice to Authors No. 7 in J.C.S. Perkin 1, 1972, Index issue.
H, 7-4%. C_{25}H_{25}O requires C, 73-3; H, 7-4%). The band, \( R_f \) 0-80, gave tetrahydrodioxyshamixanthone (XI), yellow prisms (36 mg) from light petroleum (b.p. 40-60°), m.p. 78-82°, \([x]_{D}^{25} = -90.7 \pm 4.3^\circ (c 1.2)\). \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* \) (Found: \( R_f \) 0-72; C, 76-2; H, 7-6. C_{25}H_{25}O requires C, 76-1; H, 7-7%).

(c) Shamilxanthone (140 mg) in ethyl acetate (60 ml) was hydrogenated at room temperature and atmospheric pressure for 24 h in the presence of palladium-carbon (10%); 32 mg) to give tetrahydrosamixanthone (130 mg), yellow needles from methanol, m.p. and mixed m.p. 141-145° with identical i.r. and \( \lambda_{\text{m.r.}} \) spectra.

Hydroxylation of Tajixanthone.-(a) Tajixanthone (120 mg) in ethyl acetate (100 ml) was hydrogenated at room temperature and atmospheric pressure for 24 h in the presence of Adams catalyst (100 mg) to give a mixture of two products which were separated by t.l.c., using ether-benzene (1:9 v/v) as eluting solvent. The band, \( R_f \) 0-24, gave dihydrotajixanthone (XIII) as yellow hexagonal prisms (40 mg) from acetone-light petroleum (b.p. 60-80°), m.p. 179-180°, \([x]_{D}^{25} = -18.4° (c 2-8)\), \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* \) (Found: C, 70-5; H, 6-6. C_{25}H_{24}O requires C, 70-7; H, 6-6%).

Oxidation of dihydrotajixanthone (60 mg) with Jones reagent at 0° gave (1R,2S)-1,1-dihydroxy-2-isopropyl-5-methyl-8-(3-methyl-3-oxobutyl)-2,3-dihydro-1H-pyrano[3,2-\( \alpha \)]xanthone-12-one (XIV), yellow needles (20 mg) from methanol, m.p. 163-164°, \([x]_{D}^{25} = +18.4° (c 1-29)\), \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* \) (Found: M, 456-196. C_{25}H_{24}O requires M, 456-216).

Oxidation of this compound (100 mg) with Jones reagent at 0° gave (1R,2S)-1,1-dihydroxy-2-isopropyl-8-(3-methoxy-3-methyl-2-oxobutyl)-5-methyl-2,3-dihydro-1H-pyrano[3,2-\( \alpha \)]xanthone-12-one (XV), yellow needles, m.p. 156-158°, \([x]_{D}^{25} = -86° (c 0-70)\), \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* m^*/e^* \) (Found: M', 439-196. C_{25}H_{24}O requires M', 439-216).

Osmonium Tetrahydroxide-Periodate Cleavage of Tajixanthone. - Tajixanthone (100 mg) in 25% aqueous dioxan (50 ml) was treated with osmonium tetrahydroxide (20 mg) in dioxan (5 ml) and sodium metaperiodate (150 mg). After stirring for 24 h the mixture was poured into water and the product isolated in ethyl acetate. (1R,2S)-2-Acetyl-8-[(2S)-2,3-epoxy-3-methylbutyl]-11-hydroxy-5-methyl-3H-pyrano[3,2-\( \alpha \)]xanthone-12-one (XVIII) separated from methanol in yellow needles (90 mg), m.p. 177-179°, \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* \) (Found: C, 67-8; H, 6-0. C_{25}H_{24}O requires C, 67-9; H, 5-7%).

Depolymerization of the Ketol (XVIII).-The ketol (100 mg) in methanol (50 ml) was heated under reflux for 10 min with 10% methanolic potassium hydroxide (30 ml) and then poured into water (200 ml). The mixture was extracted with ethyl acetate and the extract washed successively with water, 2% sulphuric acid, water, and then dried. Evaporation of the solvent gave 2-acetyl-8-[(2S)-2,3-epoxy-3-methylbutyl]-11-hydroxy-5-methyl-3H-pyrano[3,2-\( \alpha \)]xanthone-12-one (XIX) (60 mg), yellow needles, m.p. 192-194° (from methanol), \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* \) (Found: C, 70-5; H, 5-8. C_{25}H_{24}O requires C, 70-9; H, 5-5%).

Prepared with methyl iodide and anhydrous potassium carbonate, the methyl ether (XX) formed pale yellow needles, m.p. 205-207° (from methanol), \( \tau^* \) (Found: C, 71-1; H, 5-7. C_{25}H_{24}O requires C, 71-4; H, 5-7%).

Hydroxylation of the \( \alpha, \beta \)-Unsaturated Ketone (XIX).—This compound (400 mg) in dry tetrahydrofuran (40 ml) was treated overnight with osmium tetroxide (300 mg) in tetrahydrofuran (10 ml). After saturating the suspension with hydrogen sulphide the solid was removed and the clear solution evaporated. The residue gave 2-acetyl-1,2,11-trihydroxy-8-[(2S)-2,3-epoxy-3-methylbutyl]-5-methyl-2,3-dihydro-1H-pyrano[3,2-\( \alpha \)]xanthone-12-one (XX), yellow rods (300 mg), m.p. 185-186°, from methanol, \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* m^*/e^* \) (Found: C, 63-0; H, 6-0. M', 472-171. C_{25}H_{24}O requires C, 63-5; H, 6-0% M, 472-173).

Periodate Cleavage of the Diol (XXI).—50% Periodic acid in water (0-7 ml) was added to a vigorously stirred solution of the diol (139 mg) in ether (25 ml). After 90 min the pale yellow ether layer was separated, washed with water, dried (MgSO_{4}), and evaporated to a yellow oil (120 mg) which was crystallised from acetone-light petroleum (b.p. 60-80°) to give the 2-carboxymethoxy-1-formyl-8-hydroxy-5-(2S)-2,3-hydroxy-3-methylbutyl-5-methyl-2,3-dihydro-1H-pyrano[3,2-\( \alpha \)]xanthone (XXII) as yellow needles (85 mg), m.p. 112-115°, \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* m^*/e^* \) (Found: C, 61-5; H, 6-0%. M', 444-144. C_{25}H_{24}O requires C, 62-2; H, 5-4%. M, 444-142).

The Decarboxylation Product (XXIV).—The formyl-acid (XXIII) (75 mg) was treated with diazomethane in ether and the resultant crude methyl ester in benzene (25 ml) heated under reflux for 4 h in an atmosphere of nitrogen with chlorotriphenylphosphinephorodihium(l) (100 mg). After removal of the solvent the crude oil was purified by preparative t.l.c. the band at \( R_f \) 0-40 (benzene-ether, 90:50) being eluted to give an oil (20 mg) which separated from methanol in pale yellow needles of 8-hydroxy-5-(2S)-2,3-hydroxy-3-methylbutyl-2,3-dihydro-1H-carboxymethoxy-3-methylxanthone (XXIV), m.p. 136-138°, \( v_{\text{max}}^* \lambda_{\text{max}}^* \tau^* m^*/e^* \) (Found: M, 430-159. C_{25}H_{24}O requires M, 430-150).

Methanalysis of the Methyl Ether (XX).—This compound (100 mg) in hot methanol (60 ml) was treated with 72% perchloric acid (2 ml) on a steam-bath for 5 min. The solution was then poured into water (200 ml), and the product isolated in ethyl acetate and purified by t.l.c. The band, \( R_f \) 0-3 (benzene-acetone 3:1), separated from methanol in pale yellow needles (85 mg) of 2-acetyl-8-[(2S)-2,3-hydroxy-3-methylbutyl]-5-methyl-3H-pyrano[3,2-\( \alpha \)]xanthone-12-one (XXI), m.p. 229-231°, \( \tau^* \) (Found: C, 68-5; H, 6-2. C_{25}H_{24}O requires C, 69-0; H, 6-2%).

Determinations of Absolute Stereochemistry.—(a) The methanolate (XXI) (90 mg, 0-2 mmol) was treated with (\pm)-\( \alpha \)-phenylbutyric anhydride (187 mg, 0-6 mmol) in pyridine (3 ml) for 3 days at room temperature. Water (20 ml) was added and after warming to 100° for 20 min
Scheme 1  Principal mass spectral fragmentations of shamixanthone; relative abundance of ions in parentheses. Metastable ions are indicated.
Scheme 2. Principal mass spectral fragmentations of tajixanthone
the resultant α-phenylbutyric acid (99 mg) was isolated (in ethyl acetate), [α]_D = -6.4° (c 0.98), optical yield, 33%.

(b) O-Methylshamixanthone (VI) (82 mg, 0.2 mmol) was treated with (+)-α-phenylbutyric anhydride (187 mg, 0.6 mmol) as above for 10 days at room temperature to give α-phenylbutyric acid (115 mg), [α]_D = +3.8° (c 0.90), optical yield 20%. We acknowledge technical assistance from Mrs. A. Spencer (H n.m.r., decoupling studies), Miss G. Littler (microbiological work), and Dr. R. D. Lapper (13C n.m.r. studies). We also thank the S.R.C. for a maintenance award (to K. Y.).

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The Biosynthesis of Fungal Metabolites. Part IV. Tajixanthone: $^{13}$C Nuclear Magnetic Resonance Spectrum and Feedings with [1-$^{13}$C]- and [2-$^{13}$C]-Acetate

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The Biosynthesis of Fungal Metabolites. Part IV. \[ ^{13}C \] N.m.r. Spectra and Feedings with [1-\(^{13}C\)]- and [2-\(^{13}C\)]-Acetate

By John S. E. Holker, Roy D. Lapper, and Thomas J. Simpson, Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

The \(^{13}C\) n.m.r. spectra of tajixanthone (I) and related compounds (VI)–(XV) have been completely assigned. Spectra of tajixanthone derived by incorporation of [1-\(^{13}C\)]- and [2-\(^{13}C\)]-acetate show patterns of enrichment consistent with a biosynthetic pathway in which the xanthone arises by ring scission of 1,8-dihydroxy-3-methyl-9-anthrone (chrysophanol anthrone), derived on a \( \delta \)-ketide pathway, with introduction of \( ^{13}C \) and \( ^{13}C \)-O-prenyl units from mevalonate.

In a recent paper 1 we established the structures of tajixanthone (I) and related compounds (II)–(XV) have been completely assigned. Further studies 2 have shown that this organism contains a number of structurally related minor metabolites, together with the known compounds arugosin A (II) and arugosin B (III). The structures of these compounds (IV)–(XV) suggest that they constitute a biogenetically related group of compounds, together with arugosin C (IV). The structures of these compounds (IV)–(XV) were confirmed by comparison of their \( ^{13}C \) n.m.r. spectra with those of model compounds prepared by incorporation of [1-\(^{13}C\)]- and [2-\(^{13}C\)]-acetate.
group: the xanthone system in tajixanthone (I) and shamixanthone (VI) could be formed by cyclodehydration of the oo'-dihydroxybenzophenone system present genetically derived from an anthraquinone, such as islandicin (V), through oxidative cleavage of the quinone ring, by a pathway similar to those established for the biosynthesis of the benzophenone sulochrin from questin 6 and the ergot pigments from emodin.7 A similar pathway has also been suggested for the biosynthesis of the fungal xanthone sterigmatocystin.8 In the Scheme, we suggest that the actual precursor of the A. variecolor pigments may be chrysophanol anthrone, the probable precursor of chrysophanol 9 and islandicin.

In the present study we set out to obtain biogenetic evidence for the above proposals. Since it had been established that fungal anthraquinones, e.g. islandicin (V), are derived on the 3-ketide pathway,10 it appeared that incorporations of acetate into tajixanthone would provide such evidence. However, in view of the predicted difficulties of degrading the xanthone system in tajixanthone, necessary to establish labelling patterns from 14C-labelled precursors, it was decided to use [1-13C]- and [2-13C]-acetate and to establish the specificity of incorporations by 13C n.m.r. spectroscopy. Hence, the investigation required (i) the establishment of 13C assignments for tajixanthone and (ii) studies to determine the necessary conditions for suitable incorporations of precursors into the pigment.

Assignments of 13C Resonances.—The assignments were made by comparisons between resonances of tajixanthone (I) and the derived compounds (VI)—(XV), by use of off-resonance decoupling to determine the number of attached protons at each carbon atom, and by carrying out lanthanide-induced shift (LIS) studies on compounds (I), (VI), (VII), and (VIII), and were confirmed by comparison with literature values.11-13 The assignments of resonances due to sp³-hybridised carbon atoms, with chemical shifts less than 80 p.p.m. downfield from tetramethylsilane, were straightforward and the chemical shifts are listed in Table 1.

The assignment of resonances due to sp²-hybridised atoms (δ0 > 100 p.p.m.) was more difficult. The xanthone carbonyl (C-13) resonance in each compound is assigned in the range 177—184 p.p.m., and the high-field shift of this resonance on methylation of the 1-hydroxy-group is consistent with a loss of intramolecular hydrogen bonding.14-15 The resonance due to the ester carbonyl group (C-20) in compound (XV) was identified by its typical shift of 168J ppm., and the a-unsaturated carbonyl (C-21) resonances of compounds (XIII) and (XIV) were also typical.

Resonances associated with protonated olefinic and aromatic carbon atoms were identified by their high...
intensities in comparison with those of the quaternary carbon atoms, and assignments were confirmed by off-resonance decoupling. The C-22 methylene resonance was thus assigned in compounds (I), (VI), (VIII), and (X)—(XII); the absence of this resonance in the spectra of dihydrotaixanthone (IX) and tetrahydroshamixanthone (VII) provided confirmatory evidence. The similar lack of the \( \gamma = \) resonance at 142.0 p.p.m. in the spectra of compounds (IX) and (VII) enabled the assignment of the C-21 signals. By similar reasoning the C-15 and C-16 olefinic resonances of shamixanthone (VI) were assigned.

The assignment of the signal due to the protonated aromatic carbon atom (C-5) in taixanthone (I) was based on comparison with the 2-bromo-derivative (XI) in which this was the only unaffected protonated aromatic carbon atom. The signals due to the two remaining aromatic CH systems in taixanthone are at 109.7 and 136.2 p.p.m.; the corresponding resonance for the 2-bromo-derivative is at 139.0 p.p.m. Since the substituent chemical shift (SCS) effects of a bromo-substituent on an aromatic ring are expected to be ca. -6 p.p.m. at the attached carbon atom and ca. +3 p.p.m. at the ortho-positions, C-3 must correspond to the 136.4 and 139.0 p.p.m. signals and C-2 in (I) to the resonance at 109.7 p.p.m. By comparison C-2 in (XI) is assigned the signal at 101.9 p.p.m.

The nine \( \gamma = \) resonances of the aromatic rings present the greatest assignment difficulties. The use of additivity of SCS effects on aromatic rings requires caution in extrapolating from single ring to multi-ring aromatic systems, but does provide the following groupings: the four oxygen-substituted atoms at positions 1, 7, 10, and 11 correspond to the four signals always observed in the 148–160 p.p.m. range, and the carbon atoms at positions 4, 6, 8, 9, and 12 correspond to the remaining five resonances in the range 108–138 ppm. Comparisons of these latter five shifts in compounds (I) and (XIII), where the only change is in the function attached to C-8, show that the values of 108.7 and 114.8 p.p.m. in (I) are not altered significantly in (XIII). These two shifts must correspond to C-4 and C-9, which are the furthest from the point of change. Comparison of compounds (I), (VI), and (XII), in which the C-4 substituent is varied, shows that the 108.7 p.p.m. resonance is unchanged and so this may be assigned to C-9 (meta to the point of change). The 114.8 p.p.m. signal in (I), becoming 118.4 in (VI) and 115.9 p.p.m. in (XII), can then be assigned to C-4. The assignment of these resonances in the other derivatives then follows by comparison and has been confirmed by LIS studies for compound (VIII) (see Table 2) and by observing the SCS effect of 2-bromination in (XI).

Comparison of the spectra of compound (VI) and the derived methyl ether (VIII) shows that two of the four signals due to oxygen-bearing aromatic carbon atoms are significantly different, whereas the other two are relatively unaffected. The former two must be due to C-1 and C-10 and the latter to C-7 and C-11. LIS Studies (Table 2) of compounds (VI) and (VII) show a large down-field shift of the C-25 signal due to co-ordination of Eu(fod)₃ at the secondary hydroxy-group whereas in the methyl ether (VIII) this signal is relatively unaffected and the site of co-ordination is the oxygen atom at C-1. The LIS of 13.3 and 3.6 p.p.m. (downfield) in the signals at 157.9 and 153.9 p.p.m., respectively, for compound (VIII) establish that these signals are due to C-1 and
C-10, respectively. These latter assignments can then be applied to the other molecules in conjunction with SCS effects of 2-bromination [compound (VII)] and comparison of shifts.

For the remaining five resonances, associated with carbon atoms 6, 7, 8, 11, and 12, comparisons between chemical shifts of compounds (I), (VI), and (XIII) and their respective methyl ethers (VIII), (X), and (XIV) allow self-consistent groupings of shifts across the series but do not permit unambiguous assignments. However, the LIS effects in shammixanthone (VI) clearly define the C-8 and C-12 signals as a pair in the high-field region of the $\gamma'$-band, leaving only the $186$ p.p.m. resonance which must therefore be assigned to C-6. Distinction between signals due to C-7 and C-11 is based on comparison of compounds with structural changes at C-8. Thus, comparison between tajixanthone (I) and its methyl ether (X) on the one hand with compound (VIII), (X), and (XIV) on the other, shows larger changes in chemical shift for one of the oxygen-bearing carbon atoms than the other. Since C-11 is $\beta$ to the point of change, it should be less affected than C-7, and hence the resonances for tajixanthone are assigned as those at 151-4 and 149-0 p.p.m., respectively.

Incorporation Studies and Discussion.—In order to obtain incorporations of $^{13}$C-labelled precursors sufficiently large to be readily observed in $^{13}$C n.m.r. spectra it is necessary to use much larger quantities of precursor than acetate-derived samples, respectively, then intensities normalised to the natural abundance spectrum are given by $Y = (\rho/\rho')y$ for the [1-$^{13}$C]-acetate-derived sample and $Z = (\rho/\rho')z$ for the [2-$^{13}$C]-acetate-derived sample, where $y'$ and $z'$ are the resonance intensities for the expected unlabelled atoms in the $^{14}$C studies for which radioactivity was used as an assay method. In the present investigation the amounts of [1-$^{14}$C]- and [2-$^{14}$C]-acetate necessary for the feedings were calculated by determining the overall dilution of $^{14}$C label in experiments with [1-$^{14}$C]acetate. Under our culture conditions the required enrichment was obtained by using 10 g of acetate for every 25 g of sucrose in the medium.

The major disadvantages of loading the biological system with relatively large quantities of acetate precursor are: (i) the possible metabolic effects on the organism and (ii) the much greater opportunity for randomisation of label from [2-$^{13}$C]acetate via the tricarboxylic acid cycle than would occur in $^{13}$C studies with small amounts of precursor. In the present investigation the yields of tajixanthone were not materially affected by the acetate additions and it is therefore unlikely that any major metabolic changes occur.

From the $^{13}$C n.m.r. spectra of the [1-$^{13}$C]- and [2-$^{13}$C]-acetate-derived samples of tajixanthone the $^{13}$C enrichments at individual positions were calculated as follows:

\[ X = \frac{\rho'}{\rho}X \]

\[ Y = \frac{\rho}{\rho'}Y \]

\[ Z = \frac{\rho}{\rho'}Z \]

\[ Y' = \frac{\rho'}{\rho}Y' \]

\[ Z' = \frac{\rho}{\rho'}Z' \]

Table 1 is based on their different biogenetic origins as revealed in the enrichment studies.

Incorporation Studies and Discussion.—In order to obtain incorporations of $^{13}$C-labelled precursors sufficiently large to be readily observed in $^{13}$C n.m.r. spectra it is necessary to use much larger quantities of precursor than acetate-derived samples, respectively, then intensities normalised to the natural abundance spectrum are given by $Y = (\rho/\rho')y$ for the [1-$^{13}$C]-acetate-derived sample and $Z = (\rho/\rho')z$ for the [2-$^{13}$C]-acetate-derived sample, where $y'$ and $z'$ are the resonance intensities for the expected unlabelled atoms in the [1-$^{14}$C]- and [2-$^{14}$C]-acetate-derived samples respectively. This is a valid operation since all resonances in the spectrum of each enriched

<table>
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<tr>
<th>Carbon</th>
<th>(I)</th>
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*Assignments may be reversed. † For numbering system see formula (I).
sample are normalised by the same factor; the prior assumption of which carbon atoms are not expected to be enriched does not affect the overall operation. The method merely allows the spectrum of each enriched sample to be compared directly with the natural abundance spectrum, with automatic compensation for any randomisation of [2-\(^{13}\)C]acetate label. The enrichment for each carbon atom is given by 
\[ F_i(Y - Z)/Z \] for [1-\(^{13}\)C]acetate-enriched positions and 
\[ 1.1(Z - Y)/Y \] for [2-\(^{13}\)C]acetate-enriched positions.

<table>
<thead>
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<th>TABLE 2</th>
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\(^{13}\)C Resonance shifts induced by addition of Eu(fod)\(_3\) in p.p.m. downfield; the values quoted are the sum from four experiments with 1: 20, 1: 10, 3: 20, and 1: 5 ratios of Eu(fod)\(_3\) to compound, i.e. the effective Eu(fod)\(_3\)-to-compound ratio in each case is 1: 2.

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* Overall enrichment for C-15 and C-25.

The results thus calculated from the data in Table 3 are summarised in Table 4, and indicate that each carbon atom in tajixanthone is derived from either C-1 or C-2 of acetate. The wide range of values for each label is due to the relatively low enrichment obtained and the limited number of instrumental plot data points. Although individual differences have no biogenetic significance, it is clear that overall enrichment at positions derived from C-1 of acetate is larger than at those derived from C-2, despite the fact that the feedings of the two labelled precursors were carried out under identical conditions. This is probably a result of randomisation of C-2 label which would lead to an overall increase in \(^{13}\)C abundance at 'unlabelled' positions, thereby reducing the apparent enrichment at labelled positions.

Since the coincidence of the C-15 and C-25 resonances at 630 p.p.m. in the spectrum of tajixanthone might have cast doubt on the origin of these two atoms, the spectrum of the metabolite obtained from the [2-\(^{13}\)C]-acetate experiment was redetermined after addition of a lanthanide shift reagent, which caused separation of the two resonances (Table 2). The spectrum clearly indicated that the two positions were enriched to approximately the same extent.

| TABLE 3 |

Computer-listed relative intensities of individual signals in the \(^{13}\)C n.m.r. spectra of tajixanthone

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* Overall enrichment for C-15 and C-25.

The observed labelling pattern in tajixanthone is identical with that predicted in the Scheme, which shows the derivation of the xanthone system from an anthrone or anthraquinone precursor with introduction of two...
prenyl units from mevalonate. In particular, the derivation of C-25 from the methyl group of acetate strongly suggests that this atom corresponds to the methylene group of the anthrone system in the precursor. Furthermore, the formation of the 20,25-bond in tajixanthone by linkage between two carbon atoms derived from the methyl groups of acetate is consistent with the proposed cyclisation of the o-prenyloxyaldehyde system in the precursor xanthone.

**Experimental**

The isolation of tajixanthone (I) from cultures of *A. variegatus* and the preparation of all derived compounds except (XI) and (XII) was effected as previously described. All compounds were crystallised to constant molar radioactivity, which was determined with a Packard 3003 Tricarb Scintillation Spectrometer operating at 25-197 MHz; 12 mm tubes were used, with 5 mm coaxial tubes containing D2O to provide the locking signal. The use of 13C-enriched samples of tajixanthone, 5 mm tubes were used, because of the small quantities available, and CDCl3 was used to provide the locking signal. Sweep widths of 5120 Hz with 2048 data points were used throughout, to give chemical shift values accurate to within ±0.5 Hz, i.e. ±0.1 p.p.m. A pulse width of 80 μs corresponding to a `tilt angle' of the nuclear magnetisation vector of ca. 20° was used throughout and in the computer data memory size (4096 addresses) limited the data acquisition time to 0.4 s. Both proton noise-decoupled and single-frequency off-resonance decoupled spectra were obtained for determining the number of protons attached to each carbon atom. Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionato)europium(III) ([Eu(fod)3]) was used as shift reagent and in each case spectra were run with the following molar ratios of Eu(fod)3 to compound: 1:20, 1:10, 3:20, and 1:5.

13C-n.m.r. Determinations.—The 13C n.m.r. spectra were obtained for samples in acid-free deuteriochloroform (<=0.3 M depending on sample availability and solubility) with Me4Si (2-5%) as internal reference, with a Varian XL-100-15FT spectrometer operating at 25-197 MHz; 12 mm tubes were used, with 5 mm coaxial tubes containing D2O to provide the locking signal. In the case of 13C-enriched samples of tajixanthone, 5 mm tubes were used, because of the small quantities available, and CDCl3 was used to provide the locking signal. Sweep widths of 5120 Hz with 2048 data points were used throughout, to give chemical shift values accurate to within ±0.4 Hz, i.e. ±0.1 p.p.m. A pulse width of 80 μs corresponding to a `tilt angle' of the nuclear magnetisation vector of ca. 20° was used throughout and in the computer data memory size (4096 addresses) limited the data acquisition time to 0.4 s. Both proton noise-decoupled and single-frequency off-resonance decoupled spectra were obtained for determining the number of protons attached to each carbon atom. Tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyloctane-4,6-dionato)europium(III) ([Eu(fod)3]) was used as shift reagent and in each case spectra were run with the following molar ratios of Eu(fod)3 to compound: 1:20, 1:10, 3:20, and 1:5.

13C-Radiochemical Assays.—13C-labelled tajixanthone was crystallised to constant molar radioactivity, which was determined with a Packard 3003 Tricarb Scintillation Spectrometer. Counting efficiencies were measured with [14C]hexadecane as internal standard and using aqueous scintillator solution, prepared from 5-(biphenyl-4-yl)-2-toluene (500 ml) and methanol (500 ml). The samples for counting were decolourised by reduction with di-borane, as previously described for rubropunctatin derivatives.

**Incorporations of Sodium [1-14C]- and [2-13C]-Acetate.—**To each of two culture vessels containing a 5-day growth of *A. variegatus* was added [1-14C]- or [2-13C]-acetate (1 g; 92.1 and 90.2% enriched, respectively). After a further 9 days the mycelium was harvested and the tajixanthone isolated (24 mg from [1-14C]-acetate and 20 mg from [2-13C]-acetate feedings, after purification). These samples were used for 13C n.m.r. studies.

**2-Bromotajixanthone (XI) (with K. Young).—**A solution of tajixanthone (100 mg) and N-bromosuccinimide (50 mg, 1.2 mol equiv.) in carbon tetrachloride (30 ml) was heated under reflux for 4 h. The precipitated succinimide was filtered off and the filtrate evaporated to dryness. The residue was purified by t.l.c. (silica gel GF (Merck)) with ether-benzene (5:95 v/v) as developing solvent. The band corresponding to 2-bromotajixanthone (ca. 20 mg; 0.13 cEi mmol⁻¹) was obtained.

**Tajixanthone 'Hydrate' (XII) (with K. Young).—**Tajixanthone (50 mg in dioxan (24 ml) and oxalic acid dihydrate (6 mg) in water (6 ml) were mixed and heated under reflux for 15 h. After dilution with water (100 ml), the product was isolated in ethyl acetate (3 x 25 ml); the solution washed with water, dried (Na2SO4), and evaporated. The residue was purified by t.l.c. (silica gel GF (Merck)) with ether-benzene (19:81 v/v) as developing solvent. The band of Rf 0.40 was separated and crystallised from ethanol-chloroform to give fine yellow needles (84 mg) of 2-bromo-tajixanthone, m.p. 181-183°C. v max (KBr) 3490, 1639, 1598, 1570, 1475, and 1240 cm⁻¹. Mercedes was obtained. The residue was purified by t.l.c. (silica gel GF (Merck)) with ether-benzene (20:80 v/v) as developing solvent. The band corresponding to 2-bromotajixanthone (50 mg) in dioxan (24 ml) and oxalic acid dihydrate (6 mg) in water (6 ml) were mixed and heated under reflux for 15 h. After dilution with water (100 ml), the product was isolated in ethyl acetate (3 x 25 ml); the solution washed with water, dried (Na2SO4), and evaporated. The residue was purified by t.l.c. (silica gel GF (Merck)) with ether-benzene (19:81 v/v) as developing solvent. The band of Rf 0.40 was separated and crystallised from ethanol-chloroform to give fine yellow needles (84 mg) of 2-bromo-tajixanthone, m.p. 181-183°C. v max (KBr) 3490 br, 1645, 1600, 1580, 1475, 1245, and 1050 cm⁻¹. M, 502.082/500.081; C, 59.9% H, 5.0%. C25H25BrO6 requires M, 502.082/500.081; C, 59.9% H, 5.0%.

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We acknowledge a grant from the S.R.C. for purchase of the Varian XL-100 spectrometer and financial support (to R. D. L.). We also thank Mrs. J. Pearson for assistance in the microbiological experiments.
The Biosynthesis of Fungal Metabolites. Part V: Structure of Variecoxanthones A, B, and C, Metabolites of Aspergillus variecolor; Conversion of Variecoxanthone A into (±)-De-C-prenylepishamixanthone

By Kuldip K. Chexal, John S. E. Holker, Thomas J. Simpson, and Kenneth Young, Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

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The Biosynthesis of Fungal Metabolites. Part V. Structure of Variecoxanthones A, B, and C, Metabolites of Aspergillus variecolor; Conversion of Variecoxanthone A into (+)-De-C-prenylepishamixanthone

By Kuldip K. Chexal, John S. E. Holker, Thomas J. Simpson, and Kenneth Young, Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

Variecoxanthones A, B, and C, metabolites of a variant strain of Aspergillus variecolor are shown to be 8-hydroxy-1-hydroxymethyl-3-methyl-2-(3-methylbut-2-en-2-yl)-xanthone (IV), 8-hydroxy-1-hydroxymethyl-3-methyl-5-(3-methylbut-2-enyl)-2-(3-methylbut-2-en-2-yl)-xanthone (V), and 5-(2,3-epoxy-3-methylbutyl)-8-hydroxy-1-hydroxymethyl-2-(3-methylbut-2-en-2-yl)-xanthone (VI), respectively. These structures were established by detailed spectroscopic comparisons with shamixanthone (I) and tajixanthone (II) and by hydrogenolysis of variecoxanthone A to 2,8-dihydroxy-1,3-dimethylxanthone (X). Acid-catalysed cyclisation of the variecoxanthone-A-derived compound, 1-formyl-8-hydroxy-3-methyl-2-(3-methylbut-2-en-2-yl)xanthone (XII), gave 1,2-cis-2,3-dihydro-1,11-dihydroxy-2-isopropenyl-5-methylpyran-3,2-axanthen-12(1H)-one (XIII). The cis-relationship of the 1- and 2-substituents in this compound and the corresponding trans-relationship in shamixanthone are confirmed by comparison of 1H couplings in the hydrogenated derivatives (XIV) and (III), respectively, with those in the synthetic 1,2-cis- and 1,2-trans-2,3-dihydro-1-hydroxy-2-isopropyl-7-methyl-1H-benzopyrans (XVI) and (XVII), respectively, prepared by reduction of the corresponding chromone (XVIII) with sodium borohydride. The relative stereochemistry of compounds (XVI) and (XVII) was determined by lanthanide-induced shift studies on their respective 1H and 13C n.m.r. spectra. The mechanistic and biogenetic implications of the acid-catalysed cyclisation of compound (XI) are discussed.

We have recently 1,2 established the structures of shami-xanthone (I) and tajixanthone (II), metabolites of Aspergillus variecolor (IMI 112543), and have shown by C-labelelling studies that these compounds probably arise by oxidative fission of a polyketide-derived anthrone precursor from two prenyl residues from the polyketide. We have also suggested that these metabolites, together with arugosins A, B, and C, 3,4 constitute a biogenetically related group. As part of a search for related compounds we have investigated a number of variant strains of A. variecolor and from one, CBS 13555, we have isolated three new compounds: variecoxan-thones A (C22H20O5), B (C26H20O5), and C (C25H28O6), together with shamixanthone (I), arugosins A and B, and erigmatocystin. We now report investigations leading to the assignment of the structures (IV)—(VI), respectively, for the new compounds respectively.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(IV)</th>
<th>(V)</th>
<th>(VI)</th>
<th>(X)</th>
<th>(XIII)</th>
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</table>

* Exchangeable in D2O. † In dimethyl sulphoxide.

1H Chemical shifts of principal metabolites and their transformation products (τ values for solutions in CDCl3)

The spectroscopic properties of variecoxanthones A—C reveal their structural relationships to each other and to shamixanthone and tajixanthone. Thus, the presence of a peri-hydroxv/xanthone chromophore is indicated by its closely similar u.v. spectra, e.g. (IV) has λmax. 374, 229, and 260 nm (ε 5500, 11,100, and 26,000), i.r. bands at 500—3100 cm⁻¹, and an n.m.r. singlet at ν ca. 2-6 to 2-9, showing exchange with D2O. Furthermore all the compounds give typical iron(III) chelate colourations.

Comparisons between the 1H n.m.r. spectra of compounds (IV)—(VI) (Table I) and those of shamixanthone 1 and tajixanthone 2 (cf. the following paper (Table I) enable structural assignments to be made as follows.

1 All compounds (IV)—(VI) show an aromatic methyl signal at ν 7-6, coupled to an aromatic proton signal at ν ca. 2-8 (J 0-9 Hz) which has no further coupling.

2 The signals due to the dihydropyran residue in shamixanthone and tajixanthone are replaced in the three new compounds by signals due to an O-prenyl residue and a benzyl alcohol group in which the hydroxylic proton is coupled to the methylene protons (J 8 Hz).

3 Variecoxanthone B (V) has signals due to an aromatic prenyl substituent and variecoxanthone C (VI) shows signals for the corresponding epoxide, whereas variecoxanthone A (IV) lacks this C5 residue (cf. 14-, 15-, 17-, and 18-protons in (V) and (VI)). Furthermore, whereas the
2- and 3-protons in (V) and (VI) are an ortho-coupled pair (J 9 Hz), the 2-proton in (IV) shows ortho- and meta-couplings (J 9 Hz and 1 Hz), the 3-proton shows two ortho-couplings (J 9 and 9 Hz), and the additional 4-proton shows ortho- and meta-couplings (J 9 and 1 Hz).

Hydrogenolysis of variecoxanthone A (IV) over palladium-carbon in ethyl acetate gave the dihydro-deoxy-derivative (X). The structure of compound (X) was confirmed by comparison with 2,8-dihydroxy-1,3-dimethylxanthone (I) and the chemical shift differences of the methoxyl protons in the derived methyl ethers (VII) and (VIII) for solutions in C₆D₆ and CDCl₃ [δ(C₆D₆) δ(CDCl₃)] 0-49 p.p.m., respectively] are consistent with the proposed structures.

The principal mass spectral fragmentation patterns of the variecoxanthones, summarised in the Scheme, accords with the proposed structures. In each case the initial loss of C₉H₈O from the O-prenyl residue is followed by loss of water. In variecoxanthone A there is also a competitive loss of CH₂O from the initial fragment. In variecoxanthones B and C subsequent fragmentations of the compound was indicated by its 'H n.m.r. spectrum; the introduced aromatic proton in this compound shows no coupling greater than ca. 0 Hz, which represents the spectral resolution obtained.

The positions of the C-prenyl residue in variecoxanthone B (V) and the corresponding epoxide in variecoxanthone C (VI) have been confirmed by the method previously described for shamixanthone (I) and tajixanthone (II), respectively. Thus, the absence of a M⁺ - 56 ion in the mass spectrum of the metabolite (V) and the chemical shift differences of the methoxyl protons in the derived methyl ethers (VII) and (VIII) for solutions in C₆D₆ and CDCl₃ [δ(C₆D₆) - δ(CDCl₃)] 0-49 p.p.m., respectively] are consistent with the proposed structures.

Variecoxanthone C is chiral, having an asymmetric centre at C-15, whereas variecoxanthones A and B are achiral. Unfortunately, the small amounts of variecoxanthone C available for investigation have precluded determination of the specific rotation and the absolute configuration at C-15.

A reaction of the aldehyde (XI) was of particular interest. Under very mild acidic conditions (0-02% HCl in CHCl₃) it was isomerised at room temperature to (+)-de-C-prenylepishamixanthone (XIII). The absence of a substituted dihydrobenzopyran ring in the compound was indicated by its ¹H n.m.r. spectrum, which showed chemical shifts close to those of the corresponding ring in shamixanthone, cf. proton 19-23 and 25 and 25-OH for compounds (XIII) and (I) in Table 1. Furthermore, compound (XIII) did not show signals corresponding to those of the O-prenyl aldehyde groups in the precursor (XI). Hydrogenation of compound (XIII) over palladium-carbon in ethyl acetate gave a mixture of the dihydro-derivative (XIX) and the dihydro-deoxy-derivative (XV). This closed parallels the hydrogenation of shamixanthone and tajixanthone respectively.

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**Figure 1:**

- **(I)** R=CH₂, R=Me
- **(II)** R=CH₂CH=CHMe₂, R'=H
- **(III)** R=CH₂CH₂CH₂Me₂, R=Me
- **(IV)** R=Me, R=H
- **(V)** R=CH₂, R=Me
- **(VI)** R=CH₂, R=H
- **(VII)** R=CH₂, R=Me
- **(VIII)** R=CH₂, R=H
- **(IX)** R=H, R=Me
- **(X)** R=H, R=Me
- **(XI)** R=H, R=Me
- **(XII)** R=H, R=Me
- **(XIII)** R=H, R=Me
- **(XIV)** R=H, R=Me
- **(XV)** R=H, R=Me
- **(XVI)** R=H, R=Me
- **(XVII)** R=H, R=Me
- **(XVIII)** R=H, R=Me

---

**Table 1:**

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<tr>
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<tr>
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<td>(VI)</td>
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des confirmation for the similar residue in both compounds. However, despite similar chemical shift values, significant differences in vic-couplings are apparent in the... demonstrated by asterisks. Metastable ions are indicated by asterisks.

**TABLE 2**

<table>
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<th>Assignment</th>
<th>cis-Isomer (XVI)</th>
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<td>14</td>
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and conformations of the two isomers (XVI) and (XVII) as shown in Figure 1. The small vicinal couplings, J₁, J₃, and J₅ in the trans-isomer are consistent with gauche conformations of the individual protons and hence a pseudodiadial relationship of the isopropyl and hydroxy-substituents. In the cis-isomer the large vicinal couplings J₃ and small gauche coupling J₅ indicate anti- and gauche-conformations of the respective hydrogen atoms and hence a pseudoaxial conformation of the isopropyl group. From the LIS studies it is clear that in both isomers the co-ordination site for the shift reagent, Eu(fod)₃, is the C-1 hydroxy-group, i.e., the largest shifts are observed at C-1 and H-1. The larger shifts in the atoms of the isopropyl group, i.e., 11, 12, and 13 of the cis-isomer, as compared with the trans-isomer, confirm the...
The gauche relationship of this group with the hydroxy-group in the former compound and the diaxial relationship in

**TABLE 4**

<table>
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<tr>
<th>cis-Isomer (XVI)</th>
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<td>14</td>
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</table>

* For 1 : 20 and 1 : 10 ratios only.

the latter. In further agreement, all the other atoms in the cis-isomer show smaller shifts than the corresponding ones in the trans-isomer owing to the greater steric hindrance of the hydroxy-group co-ordination site by the isopropyl group in the former compound.

The close correspondence in $^1$H couplings between the cis-compound (XVI) and dihydrode-C-prenylepisamixanthone (XIV) and between the trans-compound (XVII) and tetrahydroshamixanthone (III) provides clear evidence for the stereochemical assignments and conformations of the compounds derived from the natural products.

The formation of the cis-product (XIII) from the O-prenyloxy-aldehyde (XI) under very mild acid-catalysed conditions could formally be regarded either as an electrophilic addition of the protonated aldehyde species

\[
\begin{align*}
\text{cis} & \rightarrow \text{trans} \\
\text{trans} & \rightarrow \text{cis}
\end{align*}
\]

FIGURE 1 Conformations of the dihydrobenzopyran ring substituents in: (a) tetrahydroshamixanthone (III) and trans-4-hydroxy-3-isopropylchroman (XVII), and (b) (±)-dihydrode-prenylepisamixanthone (XIV) and cis-4-hydroxy-3-isopropylchroman (XVI)

or an acid-catalysed 'ene-reaction.' Although analogous reactions between alkenes and carbonyl compounds are relatively well known\(^{10,11}\) the detailed mechanisms

\[^{10}\text{H. M. R. Hoffmann, Angew. Chem. Internat. Edn., 1969, 8, 556.}\]

\[^{11}\text{G. Ohloff, Tetrahedron Letters, 1960, 11, 10.}\]

do not appear to be clearly understood. However, in the present case the stereospecific formation of the cis-product (XIII) is difficult to rationalise on the basis of mechanism involving electrophilic addition. It is more likely that this is a synchronous 'ene-reaction' involving a transition state of the type shown in Figure 2(A). The alternative transition state [Figure 2(B)] which would lead to a trans-product, is unlikely under the reaction conditions in chloroform owing to unfavourable electrostatic interactions between the aldehyde and xanthone carbonyl groups.

We have already suggested that the corresponding dihydropyran ring in shamixanthone (I) arises biogenetically by cyclisation of an O-prenylaldehyde residue.\(^1\) Indeed the co-occurrence of the variecoxanthones supports this hypothesis. However, shamixanthone is the trans product. Although the mechanism of an enzyme-controlled reaction is difficult to predict, an 'ene-reaction' mechanism would require a transition state of the type shown in Figure 2(B). It is possible that in vivo this could be stabilised by hydrogen-bonding of the xanthone and aldehyde carbonyl groups. Alternatively this could be favoured if dihydroxyran ring formation precede xanthone formation, in which case the carbonyl group of the precursor benzophenone could rotate away from the aldehyde group.

EXPERIMENTAL

Unless otherwise stated, i.r. spectra were measured for solutions in chloroform with a Perkin-Elmer 125 instrument u.v. spectra for solutions in ethanol with a Unicam SP 80 instrument, $^1$H n.m.r. spectra with a Varian HA-100 or XL-100 instrument for solutions in acid-free deuteriochloroform.

\[^{10}\text{O. Achmatowicz and B. Szczepański, J. Org. Chem., 1972, 37, 964.}\]


\[^{12}\text{J.C.S. Perkin}\]
containing tetramethylsilane as internal standard, 1H m.r. spectra with a Varian XL-100-15 FT spectrometer for nolar solutions, mass spectra with an A.E.I. MS 12 instrument at 70 eV, and accurate masses with an A.E.I. MS9 instrument. T.l.c. was performed on silica gel GF (Merck). Isolation of Variecoxianthones A–C ([IV]–[VI]).—Spergillus variecolor, strain CBS 135-55, was grown in statu- turfo development for 15 days at 25°, as previously described for strain II 112543. The dried mycelium (ca. 6 g 1−1) was ground continuously extracted with light petroleum (b.p. 60–80°) and the resulting semi-solid was triturated with warm ethanol and the solution evaporated to give a yellow solid which was fractionated by preparative t.l.c. with silica gel GF (Merck). T.l.c. was performed on silica gel GF (Merck). Per- 86 to 62 was eluted from the band of 58–62 was eluted and the material re-chromatographed (24 mg) from methanol, m.p. 143–145°, requiring (i.r., u.v., and 1H n.m.r. spectra) with an authentic sample. Variecoxanthone A was eluted from the band of RF 0.55 gave yellow needles (11.5 mg) from methanol, m.p. 212–215°. Which separated from chloroform-methanol in yellow needles (17 mg), m.p. 154–156°, v 3470 and 1648 cm−1, A, 212–215°). 8-Dihydroxy-1,3-dimethyl-2-(3-methy1but-2-eny1oxy)xanthone (X) was eluted from the band at RF 0.4, affording yellow needles (26 mg) from methanol. m.p. 211°, v3490 and 1643 cm−1, λmax 238, 247, 259, 267, 290, 312sh. and 380 nm (ε 21,700, 22,400, 25,000, 22,550, 9850, 3700, and 8700), identical (i.p., mixed m.p., i.r., u.v., and 1H n.m.r. spectra) with the compound prepared as described above giving RF 0.2–0.4, giving yellow needles (95 mg), m.p. 192–195°, requiring (1H, m. 0.58 [toluene-ether (97:3 v/v)], 8-Hydroxy-1,3-dimethyl-2-(3-methy1butyloxy)xanthone (IX) was eluted from the band at RF 0.8, giving pale yellow prisms (0.8 mg 1−1) from hexane, m.p. 135°, requiring (i.r., u.v., and 1H n.m.r. spectra) with an authentic sample. Variecoxon- thone A was eluted from the band of RF 0.55 and afforded pale yellow needles (6.0 mg 1−1) from methanol, m.p. 113–115°, v3300–3100 and 1643 cm−1; C, 70.5; H, 5.9%; M, 340–130. C21H2806 requires C, 70.6; H, 5.6%; M, 340–131. The band of RF 58–62 was eluted and the material re-crchromatographed with benzene-ether (RF 0.2–0.4), requiring (1H, s), 5.01 (2H, s), 5.57 (2H, d, J 5.0 Hz), 3.30 (1H, d, J 5.0 Hz), 7.64 (3H, s), and 8.28 (3H, s) (Found: M+, 438–204). Variecoxanthone A.—(a) This compound (200 mg) in acetone (50 ml) was titrated with Jones reagent in the usual way. After adding ethanol (2 ml), the mixture was poured into water and the product isolated in ether and purified by preparative t.l.c. [toluene-ether (97:3 v/v)]. The band at RF 0.45 gave starting material (35 mg) and the band at RF 0.55 gave 1-formyl-8-hydroxy-3-methyl-2-(3-methy1but-2-enoxy)xanthone (XI), yellow needles (95 mg) m.p. 143–145°, vmax 3470, 1708, and 1644 cm−1, υ 2–06 (1H, s), −0.01 (1H, d), −2.42 (1H, t, J 8–6 and 8–6 Hz), 2.6 (1H, s), 3–1 (1H, dd, J 8–6 and 1–4 Hz), 2–33 (1H, dd, J 8–6 and 1–4 Hz), 4–49 (1H, t, J 7–6 Hz), 5–25 (2H, d, J 7–6 Hz), 7–53 (3H, s), 8–22 (3H, s), and 8–28 (3H, s) (Found: M+, 770–77, H, 5.4%; M, 338–116). Variecoxanthone A (80 mg) in dichloromethane (15 ml) was treated with Collins reagent (400 mg; 6:1 molar ratio) for 1 h at room temperature. Isolated in the usual way, the product was purified as described above giving starting material (25 mg) and the aldehyde (XI) (20 mg), m.p. and mixed m.p. 143–145°.

The Decarbonylation Product (XI).—The aldehyde (XI) (88 mg) in benzene (20 ml) was heated under reflux for 4 h in an atmosphere of nitrogen with chlorotriphenylphosphorine (80 mg). Removal of the solvent left a crude oil which was purified by preparative t.l.c., the band at RF 0.08 [toluene-ether (97:3 v/v)] being eluted to give 8-hydroxy-3-methyl-2-(3-methy1but-2-enoxy)xanthone (XII), which separated from chloroform-methanol in yellow needles (17 mg), m.p. 164–165°, v3470 and 1648 cm−1, υ 2–76 (1H, s), 2–49 (1H, t, J 8–6 and 8–6 Hz), 2–51 (1H, s), ca. 2–8 (1H, s), 3–14 (1H, dd, J 8–6 and 1–4 Hz), 2–38 (1H, dd, J 8–6 and 1–4 Hz), 4–51 (1H, t, J 7–4 Hz), 5–41 (2H, d, J 7–4 Hz), 7–64 (3H, s), and 8–2 (6H, s) (Found: M+, 310–120. C21H28O6 requires C, 71.0; H, 5.4%; M, 338–116). Acid-catalysed Cyclisation of the Aldehyde (XI).—This
compound (90 mg) was dissolved in a solution of hydrogen chloride (0.002M) in chloroform (60 ml) at room temperature. After 1 h the solution was washed with 2M-sodium hydrogen carbonate and water, dried (MgSO₄), and evaporated. The residue was purified by preparative t.l.c., the band at Rₚ 0.65 (light petroleum-acetone (4:1 v/v)) being eluted to give 1,2-cis-3-dihydroxy-2-isopropyl-5-methylpyrano[3,2-a]xanthen-12(1H)-one (XIII), which separated from benzene-hexane in yellow needles (56 mg), m.p. 141°, vmax 3500 and 1648 cm⁻¹, λmax 239, 249, 265, 294, and 384 nm (ε 24,500, 25,700, 30,900, 12,600, and 9500) (Found: C, 70.3; H, 5.3%; M⁺, 338-116. C₁₂H₁₂O₄ requires C, 71.0; H, 5.3%; M⁺, 338-116).

Hydrogenation of Compound (XIII).—This compound (60 mg) in ethyl acetate (40 ml) was hydrogenated at room temperature and 1 atm over 10% palladium-carbon (20 mg) for 2 h to give a mixture of two products which were separated by preparative t.l.c. (light petroleum (b.p. 40–60°)-acetone (4:1 v/v)). The band at Rₚ 0.72 gave 1,2-cis, 2,3-dihydro-1,1-dihydroxy-2-isopropyl-5-methylpyran (3,2-a)-xanthen-12(1H)-one (XIV) as fine yellow needles (38 mg) from methanol, m.p. 173–174°, vmax 3480 and 1640 cm⁻¹, λmax 253, 249, 265, 270, 292, and 384 nm (ε 18,200, 18,600, 22,400, 21,900, 8900, and 5400), τ = 2-59 (IH, s), 2-50 (IH, t, J = 8-4 and 8-4 Hz), 2-85 (IH, s), 3-20 (IH, dd, J = 1-3 Hz and 8-4 Hz), 3-28 (IH, d, J = 8-4 and 1-3 Hz), 8-24 (3H, d, J = 6-8 Hz) (Found: C, 70.8; H, 6.0%; M⁺, 340-130). The band at Rₚ 0.80 gave 2,3-dihydro-11-hydroxy-2-isopropyl-5-methylpyran (3,2-a)-xanthen-12(1H)-one (XV) as yellow needles (2 mg) from methanol, m.p. 95–96°, vmax 3420 and 1665 cm⁻¹, λmax 223, 225, 232, and 328 nm (ε 7490, 930, and 2300), τ = 2-45 (IH, d, J = 2-2 Hz), 2-7 (IH, dd, J = 2-2 and 8-1 Hz), 2-17 (IH, d, J = 8-1 Hz), 2-7 (IH, d, J = 8-1 Hz), 4-28 (IH, dd, J = 2-3 and 8-1 Hz), 6-27 (IH, d, J = 4-2 Hz), 7-68 (IH, dd, J = 2-3 and 8-7 Hz), 7-80 (3H, s), 8-04 (1H, m), 8-97 (3H, d, J = 7-5 Hz), and 9-0 (3H, d, J = 7-5 Hz) (Found: C, 70-9; H, 7-3. C₁₂H₁₂O₄ requires C, 70-9; H, 7-3%).

This compound (200 mg) was dehydrated by heating under reflux for 3 h with concentrated hydrochloric acid (1:15 v/v; 8 ml). Isolated in ether, t-chromone (XVIII) formed needles (160 mg) from hexane and the product was isolated in ethyl acetate (4:1 v/v) being eluted to give 1,2-cis-3-dihydroxy-2-isopropyl-5-methylpyran (3,2-a)-xanthen-12(1H)-one (XIX) (2-hydroxy-4-methylphenyl)-2-methylbutan-2-one (XVIII) formed needles (160 mg) from hexane, m.p. 106-108°, vmax 3480 and 1640 cm⁻¹, λmax 239, 249, 265, 294, and 384 nm (ε 18,200, 18,600, 22,400, 21,900, 8900, and 5400), τ = 2-59 (IH, s), 2-50 (IH, t, J = 8-4 and 8-4 Hz), 2-85 (IH, s) 3-20 (IH, dd, J = 1-3 Hz and 8-4 Hz), 3-28 (IH, d, J = 8-4 and 1-3 Hz), 8-24 (3H, d, J = 6-8 Hz) (Found: C, 70.8; H, 6.0%; M⁺, 340-130). The band at Rₚ 0.80 gave 2,3-dihydro-11-hydroxy-2-isopropyl-5-methylpyran (3,2-a)-xanthen-12(1H)-one (XV) as yellow needles (2 mg) from methanol, m.p. 95–96°, vmax 3420 and 1665 cm⁻¹, λmax 223, 225, 232, and 328 nm (ε 7490, 930, and 2300), τ = 2-45 (IH, d, J = 2-2 Hz), 2-7 (IH, dd, J = 2-2 and 8-1 Hz), 2-17 (IH, d, J = 8-1 Hz), 2-7 (IH, d, J = 8-1 Hz), 4-28 (IH, dd, J = 2-3 and 8-1 Hz), 6-27 (IH, d, J = 4-2 Hz), 7-68 (IH, dd, J = 2-3 and 8-7 Hz), 7-80 (3H, s), 8-04 (1H, m), 8-97 (3H, d, J = 7-5 Hz), and 9-0 (3H, d, J = 7-5 Hz) (Found: C, 70-9; H, 7-3. C₁₂H₁₂O₄ requires C, 70-9; H, 7-3%).

We acknowledge technical assistance from Mrs. A. Le [4/1943 Received, 23rd September, 197]
The Biosynthesis of Fungal Metabolites. Part VI. Structures and Biosynthesis of Some Minor Metabolites from Variant Strains of Aspergillus variecolor

By Kuldip K. Chexal, John S. E. Holker, and Thomas J. Simpson, Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

New minor metabolites from variant strains of A. variecolor are shown to be (1R,2S)-8-(2S)-2,3-dihydroxy-3-methylbutyl)-2,3-dihydro-1,11-dihydroxy-2-isopropenyl-5-methyl- (III), (1R,2S)-2,3-dihydro-1,11-dihydroxy-8-(2S)-2,3-dihydro-3-methoxy-3-methylbutyl)-2-isopropenyl-5-methyl- (IV), 1,2-trans-1-acetoxy-8-(2,3-epoxy-1-hydroxy-3-methylbutyl)-2,3-dihydro-11-hydroxy-2-isopropenyl-5-methyl- (V), 1,2-trans-1-acetoxy-8-(2,3-epoxy-1-methoxy-3-methylbutyl)-2,3-dihydro-11-hydroxy-2-isopropenyl-5-methyl- (VI), 1,2-cis-2,3-dihydro-1,11-dihydroxy-2-isopropenyl-5-methyl-10-(3-methylbut-2-enyl)-pyran-3,2(2H)-one (XVIII), and 1,12a-dihydro-6,8-dihydroxy-11-(1-hydroxy-1-methylthyl)-4-methyl-2,2'-dihydro-4'-methoxy-1-0-(3-methylbut-2-enyl)-PyranO[3.2-b]xanthen-12(1H)-one (XXI). A biogenetic relationship is suggested which explains the co-occurrence of these compounds with other metabolites of known structures.

Fungal metabolism it is sometimes possible to obtain insight into the biosynthesis of the major secondary abalites by examining the co-occurrence of minor abalites, which may either be biogenetic interlates or derived from them on 'shunt' pathways, 

During already established the structures of shamixan-

The small quantities of these compounds available for estimation necessitated their identification by spectro-

The illustrated numbering of the carbon atoms in these compounds occurs with retention of configuration at C-15, the relative and absolute stereochemistry of the natural products is established.

Part V, K. K. Chexal, J. S. E. Holker, T. J. Simpson, and K. me, preceding paper.

The methyl acetal, 25-O-methylarugosin A (XVIII), was prepared from the known racemic hemiacetal mixture of arugosins A and B ([XVI] and [XVII], respectively) with methanol and hydrochloric acid, giving an orange gum identical with the natural product. The hemiacetals arugosins A and B exist as a 2 : 1 equilibrium mixture but, on formation of the methyl acetal, only one isomer is obtained. Structure (XVIII) is assigned on the basis of the following evidence. Methylating with methyl iodide and potassium carbonate in acetic acid gave the dimethyl ether (XIX) in which the introduced O-methyl groups have $^1H$ chemical shifts of $\tau$ 6.20 at 6.24 in CDCl$_3$ and 6.08 and 6.72 in C$_6$H$_5$O$_2$. The upfield shift of only one of these two signals in C$_6$H$_5$O$_2$ indicates that only one of the two introduced methoxy-groups is at a free ortho-position.$^2$ Since this must be the methoxy-group, that at the 1-position must be flanked by two ortho-substituents, i.e. the C-prenyl residue must be at C-2. We also observe similar chemical shifts differences with arugosin C dimethyl ether$^8$ (XXIV), $\tau_{619}$ 6.19 and 6.27 in CDCl$_3$ and 6.13 and 6.65 in C$_6$H$_5$O$_2$. Presumably the formation of only one acetal from the equilibrium mixture of arugosins A and B is due to an increase in steric crowding on replacing the C-25 hydroxyl group by OMe, sufficient to displace the equilibrium towards isomer (XVIII) in which there is no steric interaction between the C-25 methoxy- and C-prenyl groups.

The methyl acetal (XVIII) does not appear to be an artifact formed by using methanol in the isolation of the natural product, since when this was replaced by ethanol we were unable to detect the corresponding ethyl acetal.

The original tentative assignment of structure (XV) to the predominant isomer, arugosin A,$^4$ in the equilibrium mixture of A and B is supported by the following evidence. Although the $^1H$ n.m.r. spectrum of the arugosin A and B mixture in CDCl$_3$ shows the 14-protons of both isomers with the same chemical shift, $\tau$ 6.74 (J 8 Hz); in C$_6$H$_5$O$_2$ the signals separate and occur at $\tau$ 6.64 ± 6.70 (J 8 Hz in each case) in the ratio 2 : 1, respectively.

Since the methyl acetal (XVIII) has this signal at $\tau$ 6 in C$_6$H$_5$O$_2$, then the predominant isomer in the arugosin A and B mixture must also have the C-prenyl group at C-2. In the original publication on arugosin C (XX),$^5$ it was reported that the small quantity of material prevented confirmation of the structure by conventional degradative techniques. Evidence for the presence of the benz ether grouping at C-25 has now been obtained by reductive fission of the C(25)-O bond. Thus, although attempted hydrogenolysis experiments arugosin C gave only the dihydro-derivative (XXIII), fission of the methyl ether (XXII) occurred on reduction with sodium in liquid ammonia giving in low yield a compound which contains both a chelated phenolic and a tertiary hydroxy group ($\tau$ -2.62 and 8.70, respectively). Hence, the compound is the benzophenone (XXV) and correspondingly, arugosin C must be (XX).

The $^1H$ n.m.r. spectrum of arugosin D shows that it is closely related to arugosin C, the only differences being associated with the five carbon atoms of the C-prenyl residue, cf. (XX) and (XXI) in Table 1. However, since very small amounts of arugosin D were available for study, no conclusion was possible.

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tigation, an accumulated spectrum only was obtained although this strongly suggested that the compound tained a 2-hydroxy-3-methylbut-3-enyl residue, the splings were not sufficiently well resolved to confirm s feature. However, this residue was also present in

D and compound (XXVII) show similar direct losses of C,H,O, supported by the requisite metastable ions. Since this loss has not been observed in any other compound in the series, it presumably arises from this new structural feature which must therefore be present in

### Table 1

N.m.r. chemical shifts of metabolites in CDCl₃, including values for the known compounds (I), (II), and (XX) to facilitate comparisons [couplings in Hz where the values are different, or additional to those in (I), (II), and (XX)]

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<th>(I)</th>
<th>(II)</th>
<th>(III)</th>
<th>(IV)</th>
<th>(V)</th>
<th>(VI)</th>
<th>(VII)</th>
<th>(X)</th>
<th>(XVIII)</th>
<th>(XXI)</th>
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<td>3.31</td>
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</table>

**Note:** 15- and 21- OH signals not seen in accumulated spectrum.

The compound (XXVII), which we obtained by isomerisation of the previously reported u.p.-unsaturated ketone LXVI) 2 by a trace of acetic acid in hot ethyl acetate, and in this case the H n.m.r. spectrum clearly confirmed the structure, i.e. -CH₂CH(OH)₃ shows an ABX pattern with Jₓ=7-16, Jₙ=6-88, Jₜ=6-66 (Jₓ=14, Jₙ=25, Jₜ=8 Hz) and the isopropenyl group has Jₜ=5-10, J₉=5-18 (methylene protons), and 6-14 (vinyl methyl). Since arugosin D has closely similar chemical shifts for this five-carbon residue, it also contains the 2-hydroxy-3-methylbut-3-enyl group. Furthermore the mass spectra of both arugosin D and compound (XXVII) show similar direct losses of C₄H₇O, supported by the requisite metastable ions. Since this loss has not been observed in any other compound in the series, it presumably arises from this new structural feature which must therefore be present in the compound (XXVII), hence, we propose structure (XXI) for this compound, in which, by analogy with arugosin C, the modified prenyl group is located at C-2 of the dibenzoxepin ring.

14-Hydroxy- (V) and 14-methoxy-tajixanthone 25-acetate (VI) are two further metabolites with modified C-prenyl residues. The 14-hydroxy-group in the former compound is indicated by the H n.m.r. spectrum which shows a single proton at C-14, with sharpening on addition of D₂O; the equivalent proton in the latter compound resonates at τ=5.40 (J₁₄,₁₅=7 Hz) with the methoxy-signal at τ=6.66. In both compounds the 15-, 17-, and 18-protons have chemical shifts closely similar to those in tajixanthone (II). The close similarities between the chemical shifts and couplings of the 19-, 20-, and 25-protons in both these new compounds and the known Omethoxy-tajixanthone 25-acetate (VII) confirm the presence of the 1,2-trans-1-acetoxy-2-isopropenylidenehydropyran residue, although the absolute configuration in this residue and at C-14 has not been determined owing to shortage of material. The structure of the 2,3-epoxy-1-methoxy-3-methylbutyl residue in compound (VI) was...
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confirmed by acid-catalysed hydration leading to compound (VIII), containing the 2,3-dihydroxy-1-methoxy3-methvlbutyl residue. Oxidation of this with lead
tetra-acetate gave the -methoxy-aldehyde (IX).
Epi-isoshamixanthone (X) was isolated in minor
amounts from one strain only of A. variecolor (IMI
53749). Its i.r., u.v., and tH n.ni.r. spectra were closely
similar to those of shamixanthone (I). Indeed the only
marked differences occurred in the 19-, 20-, and 25-1H
couplings, i.e. J19 , 11, J,9. ,, 5-5 and 10, and f0 36 Hz
for (X). These values are identical with those for (+)de-C-prenylepishamixanthone (XII), which has been
shown to contain a I,2-cis-1-hydroxy-2-isopropenyldihydropyran residue.' Hence, compound (X) must also
have the corresponding cis-relationship. Unlike that of
shamixanthone (I), the mass spectrum of epi-isoshamixanthone showed an ion due to loss of 56 mass units.
Since this is indicative of an ortho-hydroxyprenyl function,4 it seemed likely that compound (X) contained the

A and B are derived by hemiacetal formation, and sh
xanthone by further transformations. The co-occurr
of these compounds in several strains of A. variec
(Table 2) tends to support this suggestion. Furtherm
equivalent oxidative fission has been shown to occu
the biosynthesis of the benzophenone su.lochrin from
anthraquinone 5-0-methylemodin.78 Although we l
not been able to detect either anthrone or anthraquiii
precursors in strains of A. variecolor, it is possible that
biological oxidation of such a precursor to arugosin
and B is so efficient as to prevent its accumulation.
The biosynthesis of shamixanthone (I) from arugo
A and B, (XVI) and (XVII), requires (a) cyclodeh:
ation to give the xanthone ring and (b) cyclisation of
o-prenyloxy-aldehyde unit to the substituted dihy
pyran ring, but it is not clear which of these react
occurs first. Isolation of the variecoxanthones (XII.
(XV) ' tended to suggest that prior formation of
xanthone ring occurred and that these compounds :

TABLE 2
Mycolium
(g l)
150
IMI 112543
CBS 134-56
5-5
80
CBS 135-65 1
(MI 53749
76

Ca,

Distribution of metabolites in strains of A. variecolor (in
(XVI) and
(XVIII)
(XVII)
(XLV)
(XIII)
(LI)
(I)
10
15-0
100
Ca. 70
30
11-0
8'9
95
6-0
205
07
11.5
145
06
23
20

mg 1'
Others
(III). 10; (IV), 15; (V) 5
(XVI, 0-8; sterigmatocystin, 5-0
(X). 0-45; (XX), 38; (XXI), 0-0
(XXVIII), 100;t sterigmatocystii
09

45
TM! 77894
7-0
(V) 4-2; (VI), 15-0
8-6
16-0
80
(MI 136778
49
110
6-1
11-0
7•3
IMI 146-289
8-9
A. variecolor IMI 60318 and 75129 produced mycelium (4-9 and 8-1 g1') but no metabolites could be detected.
ing paper.

C-prenyl residue at C-2. This was confirmed by comparison of the 'H n.m.r. spectra of the derived methyl ether
(XI) in C.D. and CDCI3, which showed an upfield shift of
the methoxy-signal [(C6D6 ) — t(CDCI3) 020 ppm.]
smaller than is required for a compound containing a free
position ortho to the methoxy-group,7 cf. 036-050
p.p.m. for O-methyltajixanthone 2 (XIX) and (XXIV).
Hence epi-isoshamixanthone has structure (X), in which
the absolute configuration has not been determined
owing to lack of material.
The pattern of occurrence and relative quantities of the
biogenetically related dibenzoxepins and xanthones from
the various strains of A. variecolor are summarised in
Table 2, which also lists the major metabolite, 4,7-dimethoxy-5-methylcoumarin (XXVIII) from strain IMI
53749. This biogenetically unrelated compound is discussed separately in the following paper.
We have suggested ' that shamixanthone (I), tajixanthone (II), and arugosins A—C, (XVI), (XVII), and
(XX), respectively, are derived biogenetically from
chryosphanol anthrone by oxidative ring fission and
introduction of 0- and C-prenyl units from mevalonate,
to give the 0- form ylbenzophenone from which arugosins

then formed by subsequent reduction of the benza
hyde groups to the corresponding benzyl alcol
However, although the aldehyde corresponding to va
coxanthone A readily cyclises in vitro to give a compo
containing the requisite dihydropyran ring, this ste
specific isomerisation gives only the product in which
isopropenyl and hydroxy groups are cis-related I whei
in shamixanthone they are trans-related.2 If the e
valent in vivo cyclisation follows the same ste
chemical course it seems unlikely that shamixanth
arises in this way, although the minor metabolite
isoshamixanthone (X) may well do so. We h
suggested that the trans-relationship in shamixanth
could arise if the dihydropyran ring is formed before
xanthone ring.' However, none of the metabol
isolated contains the dihydropyranobenzophenone sti
ture which would support this hypothesis. On the ot
hand, it is probable that arugosin C (XX) arises from s
an intermediate by the alternative cyclodehydral
between C(25)-OH and C(10)-OH to the dibenzox€
system.
The metabolites tajixanthone (II), the hydrate ('
and methanolate (IV), and the hyclroxy- and metho

S. Gatenbeck and L. MalmstrOrn, Acta Chem. Scand., 1969,
23, 3493.

1970, 1512.


derivatives (V) and (VI), respectively, appear to be derived biogenetically from shamiloxanthone by oxidations of the C-prenyl residue. Presumably epoxidation to tajixanthone occurs first and this is the parent of the other compounds. Arugosin D (XXI) would be formed similarly from arugosin C (22), by rearrangement of a hypothetical epoxide.

EXPERIMENTAL

The general methods used for spectral measurements are identical to those outlined in Part V.† Spectral data indicated with an asterisk are listed in Supplementary Publication No. SUP 21245 (9 pp.).†

Isolation of Metabolites.—The cultures of the individual strains of A. variicolor were made and the isolations of the mycelial metabolites from the methanol-soluble fractions of the light petroleum extracts were carried out as reported for shamiloxanthone and tajixanthone in Part III. The individual metabolites were separated by preparative t.l.c. on silica gel GF (Merck) and known metabolites were characterised by i.r., u.v., and H n.m.r. spectra (also by m.p. and mixed m.p. for crystalline compounds). In the case of arugosin C final purification was effected by chromatography on Sephadex LH-20 as previously described.§ The amounts of the purified metabolites from each strain are summarised in Table 2, which also contains comparative data for strain CBS 1355, previously reported in Part V.

Tajixanthone Hydrate (III) (with K. Young).—Isolated from mycelium, this compound ((1R,2S)-8-(2S)-2,3-dihydroxy-3-methylbutyl)-2,3-dihydroxy-2-isopropenyl-5-methylpyran-3,2-α-xanthene-12(1H-one) formed yellow needles from acetone, m.p. and mixed m.p. 195°-196° with a sample prepared by acidic hydrolysis of tajixanthone (III) as previously described.

Tajixanthone Methanolate (IV).—(a) Isolated from mycelium, this compound ((1R,2S)-2,3-dihydro-1,11-dihydroxy-8-(2S)-2-hydroxy-3-methoxy-3-methylbutyl)-2-isopropenyl-5-methylpyran-3,2-α-xanthene-12(1H-one) formed yellow needles from acetone, m.p. 190°-191°, [α]D -56.8° (c 5.6 in CHCl₃), ν_max \( \text{max} \) m/e (Found: C, 68.6; H, 6.8%; M⁺, 454.196. \( \text{C}_{25} \text{H}_{30} \text{O}_{11} \) requires C, 68.7; H, 6.7%; M⁺, 454.198).†

(b) Tajixanthone (100 mg) in boiling methanol was treated with 72% perchloric acid (2 ml) for 5 min. After cooling, the mixture was diluted with water (200 ml) and the product isolated in ethyl acetate (3 x 50 ml) and purified by t.i.c. The band at \( R_f 0.20 \) (benzene-ether (4:1 v/v)) gave tajixanthone methanolate as yellow needles (80 mg) from methanol, m.p. and mixed m.p. 169°-191°, identical (spectroscopic properties) with the natural product.

14-Hydroxytajixanthone 25-Acetate (V).—Isolated from mycelium, this compound \((1,2-trans-1-acetoxy-8-(2S)-2,3-epoxy-1-hydroxy-3-methylbutyl)-2,3-dihydro-11-hydroxy-2-isopropenyl-5-methylpyran-3,2-α-xanthene-12(1H-one)\) formed yellow needles from light petroleum (b.p. 60°-80°), m.p. 224°-227°, [α]D +45.0° (c 0.5 in CHCl₃), ν_max \( \text{max} \) m/e (Found: C, 67.7; H, 6.9%; M⁺, 480.176. \( \text{C}_{25} \text{H}_{29} \text{O}_{10} \) requires C, 67.5; H, 5.9%; M⁺, 480.176).

14-Methoxytajixanthone 25-Acetate (VI).—Isolated from mycelium, this compound \((1,2-trans-1-acetoxy-8-(2S)-2,3-epoxy-1-methoxy-3-methylbutyl)-2,3-dihydro-11-hydroxy-2-isopropenyl-5-methylpyran-3,2-α-xanthene-12(1H-one)\) formed yellow needles from acetic-light petroleum (b.p. 60°-80°), m.p. 198°-200°, [α]D -19.6° (c 1.0 in CHCl₃), ν_max \( \text{max} \) m/e (Found: C, 67.7; H, 6.3%; M⁺, 494.195. \( \text{C}_{25} \text{H}_{29} \text{O}_{10} \) requires C, 68.0; H, 6.1%; M⁺, 494.193).

Epo-isoshamiloxanthone (X).—Isolated from mycelium, this compound \((1,2-trans-2,3-dihydro-1,11-dihydroxy-2-isopropenyl-5-methyl-10-[3-methylbutyl-2-enyl]pyran-3,2-α-xanthene-12(1H-one)\) formed yellow needles from methanol, m.p. 114°-115°, [α]D -51.6° (c 0.8 in CHCl₃), ν_max \( \text{max} \) m/e (Found: M⁺, 408.176. \( \text{C}_{24} \text{H}_{27} \text{O}_{10} \) requires M⁺, 408.176).

Prepared from this compound (10 mg) with methyl iodide and potassium carbonate in acetone, the O-methyl derivative (XI) formed needles (6 mg) from benzene-hexane, m.p. 121°-122°, [α]D -42.4° (c 0.4 in CHCl₃), ν_max \( \text{max} \) m/e (Found: M⁺, 420.194. \( \text{C}_{26} \text{H}_{31} \text{O}_{10} \) requires M⁺, 420.194).

25-O-Methylarugosin A (XVIII).—(a) Isolated from mycelium, this compound \((1,11-dihydroxy-6-methoxy-5-methyl-2-(3-methylbutyl-2-enyl)-7-(3-methylbutyl-2-enolyl)di-benz[e,exo-pen]-11[6H]one\) was a viscous orange oil, \( R_f 0.60 \) (benzene), ν_max \( \text{max} \) m/e (Found: M⁺, 438.207. \( \text{C}_{25} \text{H}_{29} \text{O}_{11} \) requires M⁺, 438.204).

Prepared from the above compound (100 mg) with methyl iodide and potassium carbonate in acetone, and purified by t.l.c., the di-O-methyl derivative (XIX) was obtained as a viscous yellow oil (78 mg), \( R_f 0.60 \) [benzene-acetone (4:1)], ν_max \( \text{max} \) m/e (Found: M⁺, 466.235. \( \text{C}_{26} \text{H}_{31} \text{O}_{11} \) requires M⁺, 466.235).

Arugosin D (XXI).—Isolated from mycelium, this compound \((1,12a-dihydro-6,8-dihydro-1-[1-hydroxy-1-methyl-ethyl]-4-methyl-9-[2-hydroxy-3-methylbutyl-3-enyl](1)-benzo-pyran-4,5,6-tric(1-1)-benzoxepin-7(2H)-one)\) was a viscous yellow oil, \( R_f 0.40 \) [ether-benzene (1:1)], [α]D -31.2° (c 0.95 in CHCl₃), ν_max \( \text{max} \) m/e (Found: M⁺, 440.182. \( \text{C}_{25} \text{H}_{27} \text{O}_{9} \) requires M⁺, 440.182).

Dihydroarugosin C (XXIII).—Arugosin C (110 mg) in ethanol (25 ml) was shaken at room temperature with palladium-carbon (20 mg; 10%) for 24 h under hydrogen at 1 atm. After removal of the catalyst and solvent, the residue was purified by preparative t.l.c. [benzene-ether (95:5)]. Dihydroarugosin C (XXIII) \((1,12a-dihydro-6,8-dihydro-1-[1-hydroxy-1-methyl-ethyl]-4-methyl-9-[3-methylbutyl-5-(3-methylbutyl)-1-benzo-pyran-4,5,6-tric(1-1)-benzoxepin-7(2H)-one)\) was obtained as a yellow oil (80 mg), \( R_f 0.3 \) [benzene-ether (95:5)], [α]D -5.2° (c 2.2 in CHCl₃), ν_max \( \text{max} \) m/e (Found: M⁺, 428.204. \( \text{C}_{25} \text{H}_{29} \text{O}_{11} \) requires M⁺, 428.204).

Similar hydrogenations with platinum oxide catalyst under various conditions of temperature and pressure gave essentially similar results.

Reduction of Arugosin C Dimethyl Ether (XXII).—Prepared as previously described† from this compound (200 mg) in ethanol (5 ml) and liquid ammonia (80 ml) at -80° was treated with sodium (55 mg). After 10 min the solution was treated with ammonium chloride (400 mg) and allowed to evaporate at room temperature, and the residue was diluted with 2m-hydrochloric acid (2 ml) in water (25 ml). After
isolation in chloroform the product was purified by preparative t.l.c. [benzene-acetone (4:1)]. The band at $R_p$ 0.7 gave the benzophenone (XXV) as a yellow oil (19 mg), [o]$_D$ -94° (c 0.63 in CHCl$_3$). $v_{max}$ * * * m/e * * (Found: M*, 434-235. C$_{27}$H$_{30}$O$_4$ requires M, 454-250).

**Isomerisation of the 3a-Unsaturated Ketone (XXVI).**—Prepared as previously described, this compound (34 mg) was heated under reflux for 15 min in ethyl acetate (10 ml) containing acetic acid (0-2 ml). The product was separated by preparative t.l.c. [benzene-ether (95:5)]. Eluted with ethyl acetate, 2-acetyl-5-fluro-2(3H)-isopropenylpyran-3-one (XXVII).—separated from acetone-light petroleum (b.p. 60-80) in yellow needles (21 mg), m.p. 200-202°, [o]$_D$ -33-3° (c 0.27 in CHCl$_3$). $v_{max}$ * * * m/e * (Found: C, 70.5; H, 5.8. C$_{27}$H$_{30}$O$_4$ requires C, 70.9; H, 5.3%).

**Hydrolysis of 14-Methoxyxanthone 25-Acetate (VI).**—This compound (70 mg) in 50% aqueous tetrahydrofuran (30 ml) was heated to boiling and treated with 72% perchloric acid (0.5 ml). After 5 min the mixture was cooled and poured into water and the product isolated in chloroform (3 x 50 ml). After washing with 2N-sodium hydrogen carbonate (2 x 25 ml) and water (2 x 25 ml) and drying (Na$_2$SO$_4$), the solvent was evaporated off and the residue purified by preparative t.l.c. [ether-benzene (1:4)]. The band at $R_p$ 0.23 was eluted and the product crystallized from aqueous ethanol, giving yellow needles (40 mg) of trans-1-acetoxy-8-(2,3-dihydroxy-1-methoxy-3-methylbut-3-enyl)-5-methylpyran-3-one (VIII), m.p. 180-182°, [o]$_D$ -30° (c 1.4 in CHCl$_3$). $v_{max}$ * * * m/e * * (Found: C, 65.2; H, 6.3; M*, 512-205. C$_{32}$H$_{30}$O$_5$ requires C, 65.6; H, 6.3%; M, 512-204).

**Lead Tetra-acetate Cleavage of the Diol (VIII).**—tetra-acetate (60 mg) was added to a stirred solution of the diol (VIII) (45 mg) in acetic acid (2 ml) at room temperature. After 1.5 h the mixture was diluted with water (50 ml) and extracted with chloroform (2 x 25 ml). After washing with water, the extract was dried (MgSO$_4$). The yellow oily residue (40 mg) was purified by preparative t.l.c. [ether-benzene (3:7)]. The band eluted at $R_f$ 0.10 was extracted with acetone to give 12-acetoxy-8-fluro-2,3-dihydro-5-methylpyran-3,2-a]xanthene-25(1H)-one as pale yellow needles (14 mg) from acetone-light petroleum (b.p. 60-80°), m.p. 145-146°, $v_{max}$ * * * m/e * (Found: C, 65.2; H, 6.3; M*, 492-151. C$_{32}$H$_{30}$O$_5$ requires C, 65.6; H, 6.3%; M, 492-145).

We acknowledge the help of Mrs. A. Lewis in the biological work.

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Use of Singly and Doubly Labelled $^{13}$C-Acetate in the Elucidation of the Structures and Biosynthesis of Multicolic and Multicolosic Acids, New Tetronic Acids from *Penicillium multicolor*

By Julian A. Gudgeon, John S. E. Holker,* and Thomas J. Simpson

(Robert Robinson Laboratories, The University of Liverpool, P.O. Box 147, Liverpool L69 3BX)

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Use of Singly and Doubly Labelled $^{13}$C-Acetate in the Elucidation of the Structures and Biosynthesis of Multicolic and Multicolosic Acids, New Tetronic Acids from *Penicillium multicolor*

By JULIAN A. GUDGEON, JOHN S. E. HOLKER,* and THOMAS J. SIMPSON

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**Summary** Multicolic and multicolosic acid, isolated from cultures of *Penicillium multicolor*, are shown to have structures (I) and (II) respectively by $^1$H- and $^{13}$C-n.m.r., and u.v. spectroscopic studies. $^{13}$C-couplings observed in the spectrum of methyl O-methylmulticolate (III), prepared from multicolic acid enriched with $[1,2-^{13}$C]$^-$acetate establishing the substitution pattern in the tetronic acid chromophore; the biosynthesis of these metabolites from acetate, *via* oxidative fission of a pre-formed aromatic precursor, e.g., 6-pentylresorcylic acid, is suggested by the $^{13}$C-n.m.r. spectra of derivatives, enriched with $[1-^{13}$C$^-]$-, $[2-{^{13}$C}$^-]$-, and $[1,2-{^{13}$C}$^-]$-acetate.

Two new optically inactive metabolites, multicolic and multicolosic acids, $C_{11}H_{12}O_6$, m.p. 129-131°, and $C_{11}H_{11}O_7$, m.p. 150-153°, respectively† have been isolated from the fermentation liquors of *P. multicolor* (IMI 104602), which had previously been reported to produce pencolide.¹ The structures (I) and (II) respectively have been established, for these compounds on the basis of the following evidence.

(a) Multicolic acid (I) gave methyl O-methylmulticolate (III) with diazomethane. Oxidation of this derivative with chromic oxide gave the acid (IV) which was converted in the methyl ester (V) with diazomethane, identical with the product obtained by similar methylation of multicolosic acid (II).

(b) Hydrogenation of multicolic acid with Pd-C in ethyl acetate gave the dihydro-derivative (VI) which showed spectral properties characteristic of a tetronic acid chromophore: $\lambda_{\text{max}}$ (EtOH) 234 nm (ε 7000); $\lambda_{\text{max}}$ (EtOH–KOH)...

---

*All compounds described gave satisfactory elemental analyses.*
The presence of the residue \(-\text{CH}_2\text{CH}_2\text{OH}\) in multicolic acid was clearly demonstrated by the \(^1H\) n.m.r. spectrum of methyl \(\beta\)-methylmulticolate (III); \(\tau\) (CDCl\(_3\)) = 41 (2H, t, \(J = 7\) Hz), 7-52 (2H, t, \(J = 7\) Hz) 7-75 (1H, exchangeable in D\(_4\)O), and 8-50 (6H, m). Lanthanide induced shift L.I.S. studies with Eu(fod)\(_3\) showed that the principal \(\alpha\)-ordination site was the primary alcoholic hydroxy-group and separate resolution of the individual methylene groups of the pentyl side-chain was achieved.

The residue \(>\text{C}=\text{CH}-\text{CO}_2\text{H}\) in both metabolites was demonstrated by the presence of a singlet proton, \(\tau = 4-10\) in the \(\^1H\) n.m.r. spectra of the methylated compounds (III) and (V), which was replaced in the dihydro-derivatives VII and (VIII) by typical ABX patterns \((\tau_A = 7-15, \tau_B = 7-50, \tau_C = 8-01; f_{AB} = 16\) Hz, \(f_{AX} = 4\) Hz, and \(f_{XX} = 8\) Hz). The conjugation of this residue with the tetronic acid chromophore was shown by the u.v. spectra of the parent metabolites; \(A_{\text{max}} (\text{EtOH}) = 265\) and 295 nm (\(\epsilon = 15,000\) and 8000).

The \(^13C\)-n.m.r. spectrum (Figure 2) of compound (III), derived from multicolic acid enriched by feeding the organism with [\(1,2\-{}^{13}\text{C}\)]acetate, shows \(13C\)-couplings of 48 and 90 Hz between C(2)-C(5) and C(4)-C(10) respectively. These values, which are typical of those for \(sp^2\)-\(sp^2\) and \(sp^2\)-\(sp^2\) hybridised coupled carbon atoms respectively, establish the positions of the substituent groups in the tetronic acid chromophore and complete the structure elucidation, apart from the stereochemistry of the 4,10 double bond, which has not been determined.

Two separate biosynthetic pathways have been established for fungal tetronic acids. (a) Oxidative cleavage of an aromatic or quinonoid precursor, as in the formation of penicillic acid from orsellinic acid, and (b) condensation of a polyketide derived \(\beta\)-ketoacid with a Kreb's cycle acid, e.g. succinic acid, as in the formation of carolic and carlosic acids. In the present case, the \(^13C\)-n.m.r. spectra (Figure 2) of the derivative (III), prepared from multicolic acid enriched with [\(1,2\-{}^{13}\text{C}\)]acetate respectively, establish the labelling patterns indicated in Figure 1. These are compatible only with a biosynthetic origin by oxidative cleavage of a polyketide derived aromatic precursor, e.g. fission between C(4) and C(5) in 6-pentylresorcylic acid (IX). The \(^13C\)-n.m.r. spectrum of derivative (III), derived from [\(1,2\-{}^{13}\text{C}\)]acetate enriched multicolic acid, (Figure 2), confirms this postulate since the observed couplings C(8)-C(9), C(6)-C(7), C(2)-C(5), and C(4)-C(10), are those between pairs of atoms derived from the same acetate residues in the aromatic precursor. The absence of couplings at C(1), C(3), and C(11) rules out the possibility of 1,2-cleavage in (IX) and precludes the biosynthetic intermediacy of any symmetrical aromatic intermediate, e.g. 5-pentyresorcinol. This route which is summarised in the Scheme, appears to be the first example of the use of \(^13C\)-doubly labelled acetate to establish the intermediacy of an aromatic precursor in the biosynthesis of a fungal metabolite. This technique, due to Seto and his co-workers is finding increasing application in fungal biosynthesis, where the necessary high incorporations can be obtained relatively easily. Similar findings have
recently been reported for the biosynthesis of penicillic acid, where it has been shown by tritium labelling studies that orsellinic acid undergoes specific 4,5-cleavage.\textsuperscript{7}

We thank Mrs. J. Pearson and Mrs. A. Lewis for the microbiological work and Dr. R. D. Lapper for \textsuperscript{13}C-spectroscopic determinations.

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THE $^{13}$C NMR SPECTRUM OF A PYRONE METABOLITE OF ASPERGILLUS MELLEUS.
BIOSYNTHETIC INCORPORATION OF SINGLY AND DOUBLY LABELLED $[^{13}$C$]$-ACETATE.

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Fermentations of Aspergillus melleus provide a variety of metabolites; the mycelium is a rich source of naphthaquinones whereas the principal metabolite in the liquors is the pyrone (1). Incorporation of $[^{14}$C$]$-acetate into pyrone (1) is reported to give the labelling pattern shown (Scheme 1). This distribution of label, particularly the linkage of two carbons derived from the methyl of acetate, is difficult to rationalise in terms of the normal pathways of polyketide biosynthesis. Studies based on $^{13}$C-labelling especially the use of doubly labelled $[^{13}$C$]$-acetate have facilitated the elucidation of unusual biosynthetic pathways thus samples of pyrone (I) enriched biosynthetically with $[^{1}$C$]$-, $[^{2}$C$]$- and $[^{1,2}$C$]$-acetate have been prepared and their $^{13}$C spectra determined.

A full assignment of the $^{13}$C n.m.r. spectra of pyrone (I) and its acetate (II) (table) has been made as follows: $C_1$, $C_2$, $C_3$ and the $C_4$-acetate group were readily assigned from known chemical shift data and their multiplicities in the off-resonance-decoupled spectra. $C_4$, $C_5$, $C_7$, and $C_8$ all have chemical shifts characteristic of oxygen-bearing aliphatic carbons and give doublets in the off-resonance spectra. They were distinguished in the acetate (II) by plotting the peak frequencies in the off-resonance spectra against the $^1$H irradiating frequency as it is stepped through the $^1$H spectral region (figure 1). The $^1$H resonance Scheme 1. Incorporation of $[^{1}$C$]$-acetate into A. melleus pyrone.
frequency corresponding to the carbon shift is determined when the residual coupling goes to zero. The method is only applicable in this case as the $^1$H frequencies in acetate (II) are well separated and have been unambiguously assigned.\(^3\)

The C\(_6\) and C\(_9\) methyl signals could not be readily distinguished in the natural abundance spectra. However, in the spectrum of (I) enriched with [1,2-\(^13\)C]-acetate, C\(_9\) shows a strong C-\(^1\)C coupling with C\(_8\) (see below).

<table>
<thead>
<tr>
<th>Carbon</th>
<th>(I)</th>
<th>(II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>163.0</td>
<td>161.7</td>
</tr>
<tr>
<td>2</td>
<td>129.0</td>
<td>131.2</td>
</tr>
<tr>
<td>3</td>
<td>141.2</td>
<td>135.5</td>
</tr>
<tr>
<td>4</td>
<td>67.6</td>
<td>67.8</td>
</tr>
<tr>
<td>5</td>
<td>79.3</td>
<td>76.5</td>
</tr>
<tr>
<td>6</td>
<td>18.0</td>
<td>18.1</td>
</tr>
<tr>
<td>7</td>
<td>54.6</td>
<td>54.3</td>
</tr>
<tr>
<td>8</td>
<td>59.1</td>
<td>58.7</td>
</tr>
<tr>
<td>9</td>
<td>17.6</td>
<td>17.5</td>
</tr>
</tbody>
</table>

CH\(_3\)CO  | - | 20.6 |
CH\(_3\)CO  | - | 169.4 |

In the \(^{13}\)C n.m.r. spectrum of (I) labelled from [1-\(^13\)C]-acetate (fig 2a) C\(_1\), C\(_3\), C\(_5\) and C\(_8\) exhibit strong enhancement relative to natural abundance, whereas the [2-\(^13\)C]-acetate enriched spectrum (fig 2b) shows enhancement of C\(_2\), C\(_4\), C\(_6\), C\(_7\), and C\(_9\) and a \(^{13}\)C-\(^1\)C coupling of 61Hz between C\(_2\) and C\(_7\) indicative of a head-to-head linkage of acetate groups. This confirms the previously reported labelling pattern.\(^4\) In addition, the [1,2-\(^13\)C]-acetate enriched spectrum (fig 2c) shows intense couplings between C\(_2\)-C\(_3\), C\(_4\)-C\(_5\), and C\(_8\)-C\(_9\) of 68,41 and 44Hz, respectively indicating their origin from intact acetate units. Due to the high enrichment, couplings of 61, 42, 40, and 32Hz between C\(_2\)-C\(_7\), C\(_3\)-C\(_4\), C\(_5\)-C\(_6\) and C\(_7\)-C\(_8\) respectively were seen as weak intensity lines indicating their origin from adjacent acetate units. This is believed to be the first assignment of the low intensity couplings in a [1,2-\(^13\)C]-acetate enriched spectrum.

The absence of an intense coupling clearly indicates that the C\(_8\) methyl cannot be part
of a "starter" unit in the biosynthesis of pyrone (I). A pathway which would account for the observed incorporation of acetate is given in Scheme 2. In this context it is notable that mellein which represents an alternate folding of the pentaketide chain is a co-metabolite of (I) and on repeated culture of \textit{A. melleus}, yields of pyrone (I) decreased with concomitant increase in mellein production\textsuperscript{3}. Penicillic acid, known to be formed by ring cleavage of orsellinic acid in \textit{Penicillium patulum} is also a co-metabolite. A Favorskii-type rearrangement would account for the head-to-head linkage of acetate units. A head-to-head linkage in polyketide metabolites has previously only been observed in the aflatoxins and related compounds\textsuperscript{10,11}. The mechanism proposed to account for this\textsuperscript{10} is without precedent amongst known chemical rearrangements and must clearly be in doubt with the recent incorporation\textsuperscript{12} of averufin into aflatoxin B\textsubscript{1}.

Further studies to provide evidence for the above postulates are in progress.

The author thanks Dr J.S.E. Holker for helpful discussions, Mrs A Lewis for microbiological work and Dr R.D. Lapper for C spectra.

Scheme 2. Postulated biosynthesis of pyrone (I) via a pentaketide precursor.

\textbf{REFERENCES}

1. Present address: Research School of Chemistry, Australian National University, Box 4 P.O., Canberra. A.C.T. 2600 Australia.

Figure 2. $^1$H noise-de-coupled $^{13}$C n.m.r. spectra of pyrone (I) from (a) CH$_3$$^{13}$COONa, (b) $^{13}$CH$_3$COONa, and (c) $^{13}$CH$_2^{13}$COONa.
The pyrone (1), a weak broad-spectrum antibiotic, has been isolated from Aspergillus species. 

$^{13}$C-labeling studies suggest a polyketide origin, and incorporation of [1-$^{13}$C]-, [2-$^{13}$C]-, and [1,2-$^{13}$C] acetate into the pyrone by static cultures of A. melleus indicated its formation from three intact acetate units and three carbons derived from cleaved acetate units. A biosynthetic pathway involving cyclisation and rearrangement of a pentaketide precursor followed by ring cleavage, was suggested to account for this most unusual labelling pattern. 

An alternative pathway, involving rearrangement of a pentaetide precursor followed by loss of the terminal carboxyl as shown in the scheme, would also account for the observed labelling pattern. In the course of this rearrangement, an originally intact acetate unit is cleaved and so the 1,2 coupling should be lost, as is observed in the $^{13}$C n.m.r. spectrum of the [1,2-$^{13}$C]acetate enriched pyrone (Table). However, the respective carbons are now in a 1,3 relationship and so, if this is the correct pathway, there should be a two-bond $^{13}$C-$^{13}$C coupling.
SCHEME. Proposed biosynthesis of pyrone (1) via a pentaketide precursor.

FIGURE. $^{13}$C N.m.r. spectrum of [1,2-$^{13}$C]acetate enriched pyrone (1).

Determined on Varian XL-100 operating at 25.2 MHz, 500 Hz total sweep widths.
coupling between C-1 and C-7. Two-bond $^{13}\text{C}$-$^{13}\text{C}$ couplings are small, typically 0-10 Hz, and can be difficult to resolve, but should provide a method for differentiating between the two possible biosynthetic pathways.

A closer examination of the [1,2-$^{13}\text{C}$]acetate enriched spectrum revealed a significant broadening of the C-1 and C-7 resonances, relative both to the other carbon resonances in the spectrum and to the C-1 and C-7 resonances in unenriched spectra. Thus the $^{13}\text{C}$ n.m.r. spectrum of the [1,2-$^{13}\text{C}$]acetate enriched pyrone was redetermined using 500 Hz total sweep widths. A $^{13}\text{C}$-$^{13}\text{C}$ coupling of 6.2 Hz (see Figure) between C-1 and C-7 is apparent, so providing conclusive evidence for the biosynthetic pathway shown in the scheme. There is no established biosynthetic precedent for this pathway, but a similar rearrangement can be postulated to account for the formation of the fused difuran ring system, found in the aflatoxins and related metabolites, whose origin has been a subject of much speculation.

This is believed to be the first example of detection of a long-range $^{13}\text{C}$-$^{13}\text{C}$ coupling arising from biosynthetic rearrangement of a doubly $^{13}\text{C}$-labelled precursor. Two-bond $^{13}\text{C}$-$^{13}\text{C}$ couplings have been detected due to very high incorporations of [1-$^{13}\text{C}$]-acetate into aflatoxin B$_1$, and intramolecular rearrangement of [2,11-$^{13}\text{C}$]porphobilinogen during the course of

TABLE. $^{13}\text{C}$ N.m.r. chemical shifts (p.p.m.) and coupling constants (Hz) in [1,2-$^{13}\text{C}$]acetate enriched pyrone (1)

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protoporphyrin biosynthesis gives rise to a one-bond $^{13}\text{C}-^{13}\text{C}$ coupling. The method has potential application to the terpenoid field where the path of methyl and other bond migrations may be followed, as well as to the study of molecular rearrangements in general.

The authors thank Mrs M. Anderson and Mrs J. Rothschild for microbiological work and Mr. D.J. Birch for $^{13}\text{C}$ spectral determinations.

References

BIOSYNTHESIS OF RAVENELIN FROM [1-13C]-AND [1,2-13C]-ACETATE

Arthur J. Birch, Thomas J. Simpson and Philip W. Westerman*
Research School of Chemistry, Australian National University,
P.O. Box 4, Canberra ACT 2600, Australia
(Received in UK 30 September 1975; accepted for publication 9 October 1975)

Ravenelin (1) is a fungal metabolite from some Helminthosporium species.1,2

An early suggestion3 was that compounds with a benzophenone skeleton are biosynthesised by two types of route: wholly acetate-polyketide, or shikimate-polyketide (C6C1 plus 3-C2 or C6C3 plus 2-C2). The former route is probable in fungi and the latter in higher plants,4 a conclusion supported by biosynthetic studies.5,6

A preliminary investigation of ravenelin using [14C]-acetate supported a complete acetate-polyketide origin but was not definitive of detail.7

A culture of H. ravenelli has now been fed with sodium [1-13C]-acetate (57%) or with sodium [1,2-13C]-acetate (91.6% and 93.1% respectively) and the resulting ravenelin, which was enriched in 13C- abundance about twofold (mass spectrometry), was examined by 13C-nmr spectroscopy.

The results from feeding experiments with [1-13C]-acetate are in complete agreement with the origin shown in (2) (see Table 1)
The proton-noise-decoupled $^{13}$C-n.m.r. spectrum of ravennin enriched with [1,2-$^{13}$C]-acetate showed the presence of intense satellite resonances due to $^{13}$C-$^{13}$C spin-spin coupling between C$_{11}$-C$_3$, C$_4$-C$_{9a}$, C$_5$-C$_8$ and C$_{1}$-C$_9$ indicating their origin from four intact acetate units. C$_5$ also shows intense satellites indicating origin from an intact acetate unit. However these satellites are very broad, due to coupling of C$_5$ to both C$_6$ and C$_{10a}$ to the same extent. Similarly C$_7$ is coupled to both C$_6$ and C$_8$. These observations indicate that C$_{10a}$, C$_5$, C$_6$, C$_7$ and C$_8$ are derived from two intact acetate units distributed in equal amounts as shown in (3) and (4). In agreement with this, the relative intensities of the C$_3$ and C$_{10a}$ satellites are only half those of the remaining nuclei derived from intact acetate units (Table 2). C$_2$ shows no intense satellites indicating its origin from a cleaved acetate unit.

The high value for the satellite intensities of C$_{11}$ in Table 2 arises because the satellite signals are not further split by $^{13}$C-$^{13}$C spin-spin coupling with adjacent intact acetate units. Such coupling is observed for other signals because of a relatively high level of acetate incorporation. Satellites of the C$_2$ signal arise from spin-spin coupling of the C$_2$ nucleus with adjacent acetate units (C$_{11}$-C$_3$ and C$_1$-C$_9$) so a low value is anticipated.
The above results indicate that an oxygenated benzophenone derivative (5),

![Diagram of compound (5)](image)

of polyketide origin, is an intermediate in the biogenesis of ravenelin. The observed labelling distribution results from the equal probability of cyclization between positions 2 and 2', or 2 and 6' to give the xanthone skeleton.

We thank Mrs. M. Anderson for assistance in the microbiological experiments.

References

### TABLE 1

$^{13}$C-Chemical shifts of ravenelin, excess $^{13}$C-abundance at individual positions in [1-$^{13}$C]-acetate enriched ravenelin, and coupling constants ($^{1}J_{^{13}C^{13}C}$/Hz) of [1,2-$^{13}$C]-acetate enriched ravenelin.

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* Determined on 0.09M solutions of unenriched ravenelin at ambient probe temperature (37°C); in p.p.m. downfield from Me$_4$Si; measured from internal d$_6$-DMSO and corrected by using the expression $\delta_{\text{TMS}} = \delta_{\text{DMSO}} + 39.6$. Multiplicities are indicated from the proton off-resonance decoupled spectrum.

# For method of calculation see reference 6.

+ Broad unresolved signals.
**TABLE 2**

Ratio of satellite intensities to natural-abundance peak intensities in [1,2-$^{13}$C]-acetate enriched ravenelin.

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Biosynthesis of Deoxyherqueinone in *Penicillium herquei* from \[^{13}\text{C}]\text{Acetate} \text{ and}\ \[^{13}\text{C}]\text{Malonate. Assembly Pattern of Acetate into the Phenalenone Ring System}

BY THOMAS J. SIMPSON
(Research School of Chemistry, Australian National University, P.O. Box 4, Canberra, A.C.T. 2600, Australia)

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1976

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Biosynthesis of Deoxyherqueinone in *Penicillium herquei* from $^{13}$C-Acetate and $^{13}$C-Malonate. Assembly Pattern of Acetate into the Phenalenone Ring System

By THOMAS J. SIMPSON

(Research School of Chemistry, Australian National University, P.O. Box 4, Canberra, A.C.T. 2600, Australia)

Summary The $^{13}$C-n.m.r. spectra of deoxyherqueinone (I) enriched with sodium $[1^{13}C]$, $[2^{13}C]$, and $[1,2^{-13}C]$-acetate, and diethyl $[2^{13}C]$malonate by the fungus *Penicillium herquei* indicate formation of the phenalenone ring system from seven intact acetate units and the acetate 'starter' effect is apparent in the $[2^{13}C]$malonate enriched $^{13}$C-n.m.r. spectrum.
Deoxyherqueinone (I) is one of a group of antibiotics based on the phenalenone ring system, produced by *Penicillium herquei*. 1C-Tracer studies have indicated the polyketide origin of the nucleus and the mevalonate origin of the C₅ side chain; the inter-relationships among this group of phenalenones have been elucidated by labelling studies with *P. herquei*. However, the nature of the intermediates leading from acetate and malonate to the phenalenone ring system, in common with the majority of phenolic polyketides, is unknown. Three alternate foldings of a single heptaketide chain, as well as multi-chain condensations, could account for the formation of the phenalenone ring system. With the advent of 13C-n.m.r. spectroscopy in biosynthetic studies it has become possible to obtain direct information on these intermediates.

Table. 13C-Chemical shift (δ, relative to Me₄Si) of deoxyherqueinone diacetate (II); coupling constants (Hz) of [1,2-13C]-acetate-enriched (II); and enrichments observed in [2-13C]-malonate-enriched (II).

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Deoxyherqueinone was isolated, along with major amounts of what is believed to be herqueichrysin, a phenalenone of uncertain structure, from the mycelium of *P. herquei* (CML 113950). The 13C-n.m.r. spectrum of the diacetate (II) was assigned (Table) from literature values and detailed analysis of the fully proton-coupled spectrum. In order to facilitate comparison of incorporation efficiencies into the polyketide- and mevalonate-derived portions of the molecule, proton-noise-decoupled (p.n.d.) spectra were determined in the presence of 0.1M [Cr(acac)₃] under GATED-2 decoupling conditions, whereupon the wide range of line intensities due to variable T₁ and N.O.E. factors was removed and almost integral intensities for all resonances in the natural abundance spectrum were obtained (Figure).

The p.n.d. 13C-n.m.r. spectra of the [1-13C]- and [2-13C]-acetate-enriched samples showed the enhancements anticipated for the acetate origin of the molecule (Scheme). The observed enrichments were very high with ca. 9% excess 13C-abundance at each labelled position, and with equal incorporation into the polyketide- and mevalonate-derived parts of the molecule. The [2-13C]-malonate-enriched spectrum showed high enrichment of six positions in the phenalenone nucleus: C(2), C(4), C(6), C(8), C(10), and C(12). The C(14) methyl, together with C(5'), C(4'), and C(5') are also enriched but to less than half the extent (Table). Thus a clear acetate 'starter' effect is observed, indicating that the phenalenone ring system is formed from a single heptaketide chain.

## Table

<table>
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</table>


The p.n.d. 13C-n.m.r. spectrum of the [1,2-13C]acetate-derived sample showed nine pairs of 13C-13C couplings.
indicating that C(14)—C(13), C(12)—C(11), C(10)—C(9), C(2)—C(3), C(4)—C(5), C(6)—C(7), C(8)—C(9), C(5')—C(3'), and C(2')—C(1') originate from intact acetate units. Thus the phenalenone ring system is formed by condensation of a heptaketide chain folded as shown (Scheme).

Other fungal phenalenones and their related metabolites have been shown to be polyketide in origin and a similar assembly pattern of acetate units in their formation is likely. In the only previous biosynthetic study using [13C]malonate, the malonate-derived carbon atoms in the ‘ansa’ chain of rifamycin S were enriched, with no significant enrichment of the acetoxy-substituent being observed.7

The author thanks Mrs. M. Anderson and Mrs. J. Rothschild for the microbiological work.

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Biosynthesis of the Fungal Xanthone Ravenelin

By Arthur J. Birch, John Baldas, Joseph R. Hlubucak, Thomas J. Simpson, and Philip W. Westerman,*
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Biosynthesis of the Fungal Xanthone Ravenelin

By Arthur J. Birch, John Baldas, Joseph R. Hlubucek, Thomas J. Simpson, and Philip W. Westerman,* Research School of Chemistry, Australian National University, P.O. Box 4, Canberra ACT 2600, Australia

Carbon-13 and carbon-14 labelling experiments have shown that the fungal xanthone ravenelin (1) is acetate-derived. Enrichment studies with [1,2-13C]acetate have demonstrated that an oxygenated benzophenone derivative is an intermediate in the biosynthetic pathway.

It has been suggested that compounds with a benzophenone skeleton are biosynthesised by two types of route: wholly acetate-polyketide and shikimate-polyketide (C6C1 plus 3C2 and C6C3 plus 2C3). In the case of naturally occurring xanthones, it has been shown in biosynthetic studies that the former route is probable in fungi and the latter in higher plants. 1

Scheme 1

The detailed mechanism of the biogenesis of xanthones has been studied in several cases. Labelling studies have established that a benzophenone derivative is a precursor to gentisin, and that anthrone and/or anthraquinone intermediates most likely are involved as intermediates in the formation of several fungal xanthones. 4

In contrast, Vining and his co-workers have shown that the fungal xanthone, bikaverin is derived via the folding of a single polyketide chain without an anthraquinone intermediate.

Ravenelin (1), another naturally occurring xanthone, has been isolated from the mycelium of two phytopathologically active members of the lower fungi, Helminthosporium ravenelii Curtis and H. turcicum.
skeleton (2) is involved. Alternative routes involve a branched-chain polyketide such as that leading to citromycetin, and the production of the carbocyclic rings in separate stages. In the latter case, a monocyclic precursor derived from a polyketide route could add either directly to another independently formed rings in separate stages. In the latter case, a monocyclic precursor derived from a polyketide aromatic ring or to the malonate units required to produce such a ring (Scheme 1).

Incorporation of acetate into ravenelin was studied to distinguish between the alternative biosynthetic pathways. [14C]Ravenelin, isolated from the mycelium of cultures of *H. ravenelii* fed with [14C]-labelled sodium acetate or diethyl malonate, was degraded to establish labelling patterns. These results prompted a thorough biosynthetic study with [1-14C]- and [1,2-14C]-acetate precursors.

**14C Incorporation Studies.**—Acetate incorporation into ravenelin (1) by *H. ravenelii* was studied by degradation of [14C]ravenelin, obtained from cultures grown with [1-14C]acetate and with [2-14C]acetate. The reactions are summarised in Scheme 2 and the results are collected in Table 1.

Sodium [1-14C]acetate was incorporated into ravenelin (1) by cultures of *H. ravenelii* to the extent of 3.3%.

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Radioactivity of degradation products of [14C]ravenelin trimethyl ether</strong></td>
</tr>
<tr>
<td><strong>Labelled with</strong></td>
</tr>
<tr>
<td><strong>Compound</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>(4)</td>
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<tr>
<td>(6)</td>
</tr>
<tr>
<td>(11)</td>
</tr>
<tr>
<td>(12)</td>
</tr>
<tr>
<td>(9)</td>
</tr>
<tr>
<td>(10)</td>
</tr>
</tbody>
</table>

Kuhn—Roth oxidation of the labelled xanthone (1) gave carbon atoms 3 and 11 as acetic acid which was further degraded (Scheme 2) to show that carbon atom 3 of the xanthone (1) is shown in Table 2. Acetate incorporation into the xanthone trimethyl ether (3) gave the diphenyl ether (6), with concomitant loss of carbon atom 9 from (1) and 1/7th of the total radioactivity of the xanthone trimethyl ether (3).

These results support a complete polyketide origin for ravenelin (1). This xanthone cannot be derived from only one polyketide chain, however, unless it is produced by cleavage of an intermediate such as an anthrone and/or anthraquinone derivative.

If ravenelin (1) is derived from a single polyketide chain then [1-14C]acetate should be incorporated to an equal extent into rings A and C of the xanthone (1).

---

10. Preliminary work provided evidence of expected acetate incorporation, but a number of details were incomplete.
decoupling of H-2; consequently this is assigned to carbon atom 2. In the proton-coupled $^{13}$C n.m.r. spectrum the resonance at 107.4 p.p.m. appeared as a doublet of doublets \([J(13\text{C}-\text{H}) 168; 3J(13\text{C}-\text{C}-\text{CH}) 7.4\text{ Hz}]\) and is assigned to carbon atom 5; the signal at 110.3 p.p.m. showed additional splitting probably arising from coupling with the proton of the C-8 hydroxy-group.

The eight resonances due to the quaternary carbon atoms of the aromatic rings present the greatest assignment difficulties. The use of additivity of substituent-chemical-shift effects on aromatic rings requires caution in extrapolating from single ring to multi-ring aromatic systems, but does provide the following groupings, based on the \(ipso\)-hydroxy- (aryloxy-) deshielding and the \(ortho\)-hydroxy- (aryloxy-) shielding effects: the three resonances at 105.9 and 107.2 p.p.m.; and the carbon atoms at positions 3, 4, and 4a, subject to one shielding \(ortho\)-hydroxy- (aryloxy-) substituent effect and one deshielding \(ipso\)-hydroxy- (aryloxy-) or one deshielding \(ipso\)-alkyl substituent effect, correspond to the remaining three resonances in the range 135.0—143.7 p.p.m.

The multiplicities for this last group of signals, at 135.0, 138.9, and 143.7 p.p.m., in the proton-coupled carbon-13 n.m.r. spectrum, were a doublet of quartets \([J(13\text{C}-\text{C}) 7.4\text{ Hz}]\) and is assigned to carbon atom 7.

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CH) 9.0; \( \delta \) \((C-C-CH_2) 4.0 \text{ Hz} \), a quartet \( \delta \)(\(^{13}C-CH_2\) 6.7 Hz), and a singlet, \( \delta \) were assigned, on the basis of the known magnitudes of \( \delta \)/(\(^{13}C-CH_2\) and \( \delta \)(\(^{13}C-CH_2\)) in aromatic systems and the observation that \( \delta \)(\(^{13}C-CH_2\) > \( \delta \)(\(^{13}C-CH_2\)) in monosubstituted benzenes,\textsuperscript{15} to carbon atoms 4, 3, and 4a, respectively. The first group of carbon resonances, at 151.7, 155.7, and 160.4 p.p.m., were a doublet \( \delta \)(\(^{13}C-CH_2\) 2.3 Hz), a doublet of doublets \( \delta \)(\(^{13}C-CH_2\) 11.5; \( \delta \)(\(^{13}C-CH_2\) 8.0 Hz), and another doublet of doublets \( \delta \)(\(^{13}C-CH_2\) 11.2; \( \delta \)(\(^{13}C-CH_2\) 2 Hz), and were assigned to carbon atoms 1, 10a, and 8, respectively. Differentiation between the C-10a and C-8 signals was made on the basis of chemical shift data for a large number of substituted xanthone derivatives.\textsuperscript{16}

The carbon signal at 105.9 p.p.m. is a doublet \( \delta \)/\(^{13}C\)-\((C-C-CH_2\) 6.0 Hz) in the proton-coupled \(^{13}C\) n.m.r. spectrum and is assigned to carbon atom 9a; the signal at 107.2 appears as a triplet, coupled to protons 5 and 7, and is assigned to carbon atom 8a.

The following biosynthetic arguments are not altered if the doubtful assignments (C-5 and C-7) are reversed.

In order to obtain incorporations of \(^{13}C\)-labelled precursors sufficiently large to be readily observed in \(^{13}C\) n.m.r. spectra it is necessary to use much larger quantities of precursor than are usual in equivalent \(^{13}C\) studies for which radioactivity is used as an assay method. In the present investigation the amounts of [\(^{13}C\)] and [\(^{13}C\)]-acetate necessary for the feedings were calculated by determining the overall dilution of \(^{13}C\) label in experiments with [\(^{13}C\)]acetate. Under our culture conditions the required enrichment was obtained by using 0.5 g of sodium acetate for every 50 g of sucrose in the medium.

The major disadvantage of loading the biological system with relatively large quantities of acetate precursor is the possible effect on the metabolism of the organism. Attempts to grow cultures of \(H. ravenelii\), in either Czapek-Dox media supplemented with relatively large quantities of sodium acetate, or by replacing the Czapek-Dox medium with a solution of sodium acetate, sucrose, and peptone after 5 days of growth, gave mycelium, on further growth, much darker in colour than normal. Solvent extraction of these mycelia according to described procedures yielded little or no ravenelin. Optimum incorporation of sodium acetate was obtained by growing the mycelium in modified Czapek-Dox medium, removing two-fifths of the medium, and feeding the remainder with 1 ml samples of an aqueous solution of sodium acetate every 48 h.

Ravenelin was extracted from the mycelium according to described procedures and used without further purification for \(^{13}C\) n.m.r. spectral studies. An isotopic enrichment in ravenelin of approximately twofold above the natural \(^{13}C\) abundance, as established by mass spectrometry, was found when the medium was supple-

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**TABLE 2**

\[^{13}C\] Chemical shifts of ravenelin, excess \(^{13}C\) abundance at individual positions in \([1-^{13}C]\)acetate-enriched ravenelin, and coupling constants \(^{2}J(\text{^{13}C-^{13}C})\)/[Hz] of \([1,2-^{13}C]\)acetate-enriched ravenelin.

<table>
<thead>
<tr>
<th>Carbon no.</th>
<th>Signal</th>
<th>( E )</th>
<th>( ^{2}J(\text{^{13}C-^{13}C}))</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>120.7</td>
<td>9.0</td>
<td>64.1</td>
</tr>
<tr>
<td>2</td>
<td>111.1d</td>
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</tr>
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<td>3</td>
<td>136.9</td>
<td>14.1</td>
<td>42.1</td>
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<tr>
<td>4</td>
<td>135.0</td>
<td>1.9</td>
<td>76.9</td>
</tr>
<tr>
<td>4a</td>
<td>143.9</td>
<td>6.8</td>
<td>78.7</td>
</tr>
<tr>
<td>5</td>
<td>107.4</td>
<td>1.1</td>
<td>62#</td>
</tr>
<tr>
<td>6</td>
<td>187.7d</td>
<td>4.4</td>
<td>55.8</td>
</tr>
<tr>
<td>7</td>
<td>110.3d</td>
<td>0.5</td>
<td>62#</td>
</tr>
<tr>
<td>8</td>
<td>160.4d</td>
<td>10.7</td>
<td>69.6</td>
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<td>107.2</td>
<td>1.0</td>
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</tr>
<tr>
<td>9</td>
<td>185.2</td>
<td>9.8</td>
<td>54.9</td>
</tr>
<tr>
<td>9a</td>
<td>105.9</td>
<td>1.2</td>
<td>64.1</td>
</tr>
<tr>
<td>10a</td>
<td>155.7</td>
<td>10.8</td>
<td>67.7</td>
</tr>
<tr>
<td>11</td>
<td>17.0q</td>
<td>0.4</td>
<td>42.1</td>
</tr>
</tbody>
</table>

\# Determined on 0.09M-solutions of unenriched ravenelin at ambient probe temperature (37°C); in p.p.m. downfield from Me\(_2\)SO; measured from internal Me\(_2\)SO and corrected by using the expression \(8(Me\(_2\)SO) = 8(Me\(_2\)SO) + 39.6\). Multiplicities are indicated from the proton off-resonance decoupled spectrum. \# Determined as described in reference 5. § Broad unresolved signals.

indicate that carbon atoms 1, 3, 4a, 10a, 6, 8, and 9 in ravenelin are derived from the carboxy-carbon atom of acetate. The range of values is wide owing to the limited number of instrumental plot data points; the individual differences probably have no biogenetic significance.

The observed labelling pattern in ravenelin enriched with \([1-^{13}C]\)acetate is shown in structure (13).

The proton noise-decoupled \(^{13}C\) n.m.r. spectrum of ravenelin enriched with \([1,2-^{13}C]\)acetate showed intense satellite resonances due to \(^{13}C-^{13}C\) spin–spin couplings \( [C(11)-C(3)], [C(4)-C(4a)], [C(9)-C(8a)], and [C(1)-C(9a)] \) indicating their origin from four intact acetate units. The C(9) signal also showed intense satellites, indicating origin from an intact acetate unit. However these

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\textsuperscript{14} P. W. Westerman, M. U. S. Sultanbawa, S. P. Gunasekera, and R. Kazlauskas, unpublished data.
satellites were very broad, due to couplings of C(6) to both C(6) and C(10a) to the same extent. Similarly C(7) is coupled to both C(8) and C(8).

These observations indicate that C(10a), C(5), C(6), C(7), and C(8) are derived from two intact acetate units, distributed in equal amounts as shown in (14) and (15). In agreement with this, the relative intensities of the C(8) and C(10a) satellites are only half those of the remaining nuclei derived from intact acetate units (Table 3). The C(2) signal shows no intense satellites, indicating its origin from a cleaved acetate unit.

The high value for the satellite intensities of C(11) in Table 3 arises because the satellite signals are not further split by $^{13}$C-$^{13}$C spin–spin coupling with adjacent intact acetate units. Such coupling is observed for most other signals because of the relatively high level of acetate incorporation. The weak satellites of the C(2) signal arise from spin–spin coupling of the C(2) nucleus with adjacent acetate units C(11)-C(3) and C(1)-C(9a), so a low value in Table 3 is anticipated.

The above results indicate that an oxygenated benzophenone derivative (16), of polyketide origin, is an intermediate in the biogenesis of ravennelin. The observed labelling distribution results from the equal probability of cyclisation between positions 2 and 2', or 2 and 6', to give the xanthone skeleton. There are several in vitro precedents for such a cyclisation. It has been established from tritium incorporation experiments in the plant Gentiana lutea, that 2,3,4,6-tetrahydroxybenzophenone is a precursor of the xanthone gentisin. The coexistence of a benzophenone with related xanthones has also been observed in the plants Symphoria globulifera and Chlorophora tinctoria and the fungus Penicillium patulum.

The biosynthetic origin of naturally occurring benzophenones is usually discussed in terms of an acetate-malonate or a polyketide-shikimate pathway; our results show that the former occurs for ravennelin. Acetate-derived benzophenone (16) may be formed from two distinct units each separately derived from acetate and malonate, or through an anthrone and/or anthraquinone intermediate derived from a single C$_8$ polyketide chain. The latter process has been established for biosynthesis of the benzophenone sulochrin. The even distribution of the $^{14}$C label and $^{13}$C label (Table 3) favours a similar biosynthetic pathway in the formation of the intermediate (16). If an anthraquinone derivative is an intermediate our results rule out a concerted oxidation mechanism for the replacement of the carbonyl group by an oxygen atom.

### EXPERIMENTAL

#### $^{14}$C Radioactivity Assays.

$^{14}$C-labelled organic compounds were recrystallised to constant radioactivity, measured (1 mg sample in a mixture of toluene (1 ml) and the scintillator solution [0.5% 2,5-diphenyloxazole in toluene (9 ml)]) on a Beckman LS-150 liquid scintillation system, calibrated by the external standard channel-ratio method. The product of counts min$^{-1}$ mg$^{-1}$ and the molecular weight gave the relative molar activity (r.m.a.).

Barium [${}^{14}$C]carbonate was decomposed as described later and a sample (1 ml) of the ethanamine absorbent was used in place of the toluene solvent (1 ml) in the counting solution. [${}^{14}$C]Ravennelin trimethyl ether (3) and [${}^{14}$C]-2,3,5-trimethoxy-3-methyldiphenyl ether (6) were best purified as their respective tribromo- and tetrabromo-derivatives, (4) and (6). Radioactivity assay of the labelled bromo-compound (4) necessitated recounting of samples with an added $^{13}$C standard to correct for scintillator quenching by the bromo-derivative. A convenient standard was a sample (1.0 ml) of a solution of [$^{13}$C]-l-tryptophan benzyl ester in 20% ethanolic toluene of activity 3 700 counts min$^{-1}$ ml$^{-1}$. Merck adsorbents were used for column chromatography.

#### $^{13}$C n.m.r. Determinations.

The $^{13}$C n.m.r. spectra were obtained from samples in hexadeuteriodimethyl sulfoxide ($\leqslant$0.09m depending on sample availability). Chemical shifts were measured from the centre peak of the Me$_2$SO signal, corrected by use of the expression $8$(Me$_4$Si) =

#### Table 3

<table>
<thead>
<tr>
<th>Carbon no.</th>
<th>Ratio</th>
<th>Carbon no.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.2</td>
<td>7</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>0.8</td>
<td>8a</td>
<td>0.7</td>
</tr>
<tr>
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</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>11</td>
<td>2.5</td>
</tr>
</tbody>
</table>

#### Diagram

- 

intermediate in the biogenesis of ravennelin. The observed labelling distribution results from the equal

#### References

solution, and saturated sodium chloride solution, dried single product. The mixture was diluted with benzene and hydrogen sulphite solution, 5% sodium hydrogen carbonate solution was maintained until t.l.c. activity. An excess of bromine was maintained until t.l.c. activity. mg) in glacial acetic acid (7 ml) was treated with an excess of bromine. An excess of bromine was maintained until t.l.c. conversion into a single product. The mixture was diluted with benzene and ethyl acetate, and washed with water, saturated sodium hydrogen sulphite solution, 8% sodium hydrogen carbonate solution, and saturated sodium chloride solution, dried (MgSO₄), and evaporated to leave a crystalline residue (120 mg). Percolation through alumina in 50% light petroleum (b.p. 60–80°C)–benzene and recrystallisation from ethanol–chloroform gave the pure product (100 mg). m.p. 240–241°C (Found: C, 38.1; H, 2.6; Br, 44.3. C₁₁H₁₂Br₂O₅ requires C, 38.0; H, 2.4; Br, 44.6%).

[¹⁴C]-2,3',5-Trimethoxy-3-methylidiphenyl Ether (5) and its Tetrabromo-derivative. —[¹⁴C]Ravenelin trimethyl ether (120 mg) was added to a suspension of sodamide (1 g) in dry toluene (100 ml) and the mixture was refluxed under nitrogen for 5 h. The crude mixture was diluted with water, and the organic layer was separated, dried, and evaporated under reduced pressure. The dark oily residue (113 mg) was purified by column chromatography on alumina [light petroleum (b.p. 60–80°C)–benzene mixtures] to yield the pure [¹⁴C]diphenyl ether (72 mg) as a colourless oil, which was characterised as the tetrabromo-derivative (6). The [¹⁴C]diphenyl ether (50 mg) was heated on a steam-bath in glacial acetic acid solution (1 ml) with an excess of bromine for 0.5 h. The cooled mixture was diluted with ethyl acetate and washed with water, dilute sodium hydrogen sulphite solution, 5% sodium hydrogen carbonate solution, and saturated sodium chloride solution, dried, and evaporated to leave a crystalline residue (120 mg), which was recrystallised from methanol to yield the [¹⁴C]tetrabromo-derivative (59 mg), m.p. 156–157°C (lit., 157°C).

Fission and Reduction of [¹⁴C]-2,3',5-Trimethoxy-3-methylidiphenyl Ether (5). —The [¹⁴C]diphenyl ether (5) (0.4 g) in ether (50 ml) was added to liquid ammonia (380 ml). Sodium (0.1 g) was added and then, after stirring for 1 h, ethanol (8 ml) was added, followed by more sodium (1 g). After disappearance of the metal, the ammonia was evaporated off and water (30 ml) was added. The alkaline solution was extracted with ether and the combined extracts were washed with water, dried, and evaporated. The residual oil was treated with Brady’s reagent to give a yellow 2,4-dinitrophenylhydrazone which was purified by chromatography on Bentonite–Kieselguhr (3:1) with 30% ethanol–chloroform as eluant. Recrystallisation from ethanol–chloroform gave [¹⁴C]-2-methylcyclohexa-2,5-diene-1,4-dione bis-2,4-dinitrophenylhydrazone (0.16 g), m.p. and mixed m.p. 235–236°C (Found: C, 46.5; H, 3.75). Calc. for C₁₃H₁₀N₂O₄: C, 46.0; H, 3.75%.

The alkaline solution from the above reduction of the diphenyl ether was acidified and extracted with ether. The extract was suspended in 0.1M-hydrochloric acid (10 ml) and bromine was added until a colour persisted. The gum that precipitated on addition of sodium hydrogen sulphite solution was purified by chromatography on silica [elution with 5% ether–light petroleum (b.p. 60–80°C)]. Recrystallisation from light petroleum gave colourless crystals of [¹⁴C]-2,4,6-trimethyl-3-methoxyphenol (89 mg), m.p. 106–107°C, identical with an authentic sample.

Kuhn–Roth Oxidation of [¹⁴C]Ravenelin Trimethyl Ether and Schmidt Degradation of the Resulting [¹⁴C]Acetic Acid. — [¹⁴C]Ravenelin trimethyl ether (72 mg) was oxidised under standard conditions[1] to acetic acid (12 mg), which was subjected to Schmidt degradation.[1] The [¹⁴C]carbon dioxide was trapped as barium [¹⁴C]carbonate (23.8 mg), and the [¹⁴C]methyldiene was assayed as [¹⁴C]N-methyl-2,4-dinitroaniline (21.8 mg). An accurately weighed sample of the barium [¹⁴C]carbonate was decomposed in concentrated sulphuric acid in a slow stream of nitrogen and the [¹⁴C]-carbon dioxide liberated was passed into ethanoloamine (5 ml). After 0.5 h a sample (1 ml) of the ethanoloamine solution was removed for radioactivity assay.
Incorporation of Sodium [1-13C]- and [1,2-13C]-Acetate.—
The optimum yield of 13C-enriched ravenelin was obtained under the following conditions. A penicillin flask containing Czapek-Dox medium (500 ml) and mycological peptone (1 g) was inoculated with a macerate of *H. ravenelii* grown on 3 plates over 9 days. After 5 days incubation, 200 ml of the medium was removed and the remainder fed with a solution of sodium [1-13C]acetate (0.1 g; 57% enriched) or sodium [1,2-13C]acetate (0.1 g; 91.6 and 93.1% enriched, respectively) in water (1.0 ml). Similar feedings were repeated on alternate days until a total of 500 mg of 13C-enriched sodium acetate had been added. Three days after the last feeding, the mycelium was harvested by filtration, washed with water, and dried in a current of air. It was further dried *in vacuo* over phosphorus pentaoxide. Powdered mycelium was extracted (Soxhlet) with light petroleum (500 ml; b.p. 60—80%) for 24 h. Concentration of the solution to 50 ml and cooling to 0 °C gave a crystalline precipitate (0.061 g with sodium [1-13C]acetate and 0.036 g with sodium [1,2-13C]acetate) which was removed by filtration. The 13C-enriched samples showed no signals arising from impurities in either the 1H or the 13C n.m.r. spectrum.

The powdered mycelium was further extracted with chloroform (500 ml) to yield a crop of less pure ravenelin on concentration of the extract to 15 ml (0.076 g with [1-13C]-acetate and 0.014 g with [1,2-13C]acetate). Samples of all the above products on recrystallisation from acetone-chloroform gave intensely yellow crystals, m.p. 268—269°, alone or mixed with authentic ravenelin.

Isotope incorporation levels on 13C-enriched samples were determined mass spectrometrically by analysis of the molecular ion region. Ravenelin enriched with singly and doubly labelled acetate showed enrichment factors of 2.2 and 3.7, respectively.

1H N.m.r. Spectrum of Ravenelin.—Ravenelin showed $\delta$(CD$_3$)SO 2.30 (CH$_3$, s), 3.30 (4-OH and 1- or 8-OH, s, exchangeable), 6.54 (2-H, s), 6.76 (5- or 7-H, d, $J_{5,6}$ or $J_{7,8}$ 8.2 Hz), 7.02 (7- or 5-H, d, $J_{5,6}$ or $J_{7,8}$ 8.2 Hz), 7.72 (6-H, dd, $J_{6,7} = J_{7,8} = 8.2$ Hz), and 9.04 (1- or 8-OH, s, exchangeable).

We thank Mrs M. Anderson for assistance in the microbiological experiments.

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THE STRUCTURE OF PHOMAZARIN, A POLYKETIDE AZAANTHRAQUINONE FROM PYRENOCHAETA TERRESTRIS HANSEN

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Phomazarin, C_{19}H_{17}N_{08}, from the fungus P. terrestris Hansen, was suggested by Kogl\(^1\) to have one of the two structures (1). The substitution pattern of the heterocyclic ring was later modified,\(^2\) and this together with a consideration of infra-red evidence and biogenetic studies, which confirmed a polyketide origin and the derivation of CO\(_2\)H from Me of acetate, suggested (2) as a possible structure.

Assignment of the structure of the benzenoid nucleus depended on Kogl's conclusion\(^3\) that (3a) is the structure of an oxidation product, which was isolated but further converted into (3b), m.p. 173-174\(^\circ\). The isolation of the free phthalic acid led us to question this structure since experience\(^3\),\(^4\) with such acids substituted adjacent to both CO\(_2\)H with groups other than OH, is that the anhydride is produced spontaneously. The acid (3b) was therefore synthesised by Alder-Rickert reaction of 1,5-di-n-butyl-2,4-dimethoxycyclohexa-1,3-diene with dimethyl acetylenedicarboxylate\(^4\) followed by hydrolysis. As expected, the free acid could not be isolated but became converted into an anhydride m.p. 134\(^\circ\), clearly differing from Kogl's anhydride for which he quotes m.p. 170\(^\circ\). Thus Kogl's evidence for the benzenoid substitution is invalid.

We now present evidence for the revised structure (4a), which is also biogenetically acceptable, for phomazarin.

The \(^1\)H n.m.r. resonance of the aromatic proton at the low value of 2.10 \(\tau\) in trimethylphomazarin methyl ester (4b)\(^2\) is inconsistent with structure (2) as in 1,3-dimethoxyanthraquinones, the 2-proton resonates at ca. 3.5 \(\tau\).\(^5\) In anthraquinone with 2-methyl-3,4,5,7-tetramethoxy or 3-hexyl-2,4,5,7-tetramethoxy substituents, the 1-proton, peri to a carbonyl function resonates at 2.13 \(\tau\) and 2.47 \(\tau\) respectively,\(^6\),\(^7\) indicating probably a similar proton in the phomazarin derivative.

Irradiation of the benzylic methylene triplet at 7.23 \(\tau\) in (4b) caused a nuclear Overhauser enhancement of 26% in the intensity of the signal at 2.10 \(\tau\), indicating an ortho-relationship between this aromatic proton and the butyl group,\(^8\) also in accord with (4a).

Purdie methylation of phomazarin gave, inter alia, a di-O-methylphomazarin methyl ester (4c), C_{22}H_{23}N_{05}, m.p. 136-138\(^\circ\), which on heating with sulphuric acid produced an O-methyl-decarboxyphomazarin (5a), C_{19}H_{13}N_{05}, m.p. 228-230\(^\circ\). This with POCI\(_3\) gave (5b), C_{18}H_{18}CINO\(_5\), m.p. 197-199\(^\circ\). Similar treatment of tri-O-methylphomazarin methyl ester (4b) gave the chloro-compound (5c), C_{20}H_{20}ClNO\(_5\), m.p. 160-161\(^\circ\), exhibiting only one absorption band at \(\nu_{max} = 1675\) cm\(^{-1}\).
(1)

(2)

(3a) R = H
(3b) R = Me

(4a) R₁ = R₂ = R₃ = R₄ = H
(4b) R₁ = R₂ = R₃ = R₄ = Me
(4c) R₁ = H, R₂ = R₃ = R₄ = Me
(4d) R₂ = H, R₁ = R₃ = R₄ = Me
(4e) R₁ = R₂ = H, R₃ = R₄ = Me

(5a) R₁ = H, R₂ = OH, R₃ = Me
(5b) R₁ = H, R₂ = Cl, R₃ = Me
(5c) R₁ = R₃ = Me, R₂ = Cl
(5d) R₁ = R₃ = Me, R₂ = OH
(5e) R₁ = R₂ = H, R₃ = OH

(6a) R₁ = CO-Me, R₂ = CO₂H, R₃ = H
(6b) R₁ = R₂ = Me, R₃ = H

(7)
despite the presence of two quinone carbonyls. In contrast, compound (5b) had absorptions at 1640 and 1670 cm⁻¹ indicating one hydrogen-bonded and one unbonded carbonyl. The former was supported by definition of an exchangeable proton at low field, -3.6 τ, and it can only be on the peri-position of the benzenoid ring since the pyridol OH has been replaced.

Confirmation of the general conclusion was obtained by repetition of Kögl's degradation, the initial acetoxy-n-butylmethoxyphthalic acid (6a) being converted by hydrolysis and removal of CO₂H ortho to OH, followed by methylation, to produce (6b) m.p. 77°, identical with an authentic synthetic specimen.

2- And 4- hydroxypyridines and quinolines usually exist as pyridone or quinolone tautomers. ¹³C n.m.r. studies of phomazarin and its derivatives, to be discussed in detail elsewhere, suggest that the unusual 4-pyridol form predominates. Addition of Na¹⁵NO₃ (95 atom %) as sole nitrogen source to cultures gave [¹⁵N]phomazarin. The derived di-O-methylphomazarin methyl ester (4d) had a low field exchangeable proton, -3.23 τ, showing no ¹⁵N-¹H coupling (typical J¹⁵N-H is 93Hz), indicating its presence in a pyridol OH rather than pyridone NH. A possibility that this result is due to exchange rather than lack of coupling was ruled out by examination of di-O-methyldecarboxyphomazarin (5d), C₂₀H₂₁¹⁵NO₆. The proton on the heterocyclic ring, resonance 1.53 τ, exhibits a two-bond ¹⁵N-¹H coupling of 10 Hz and the ¹⁵N chemical shift of 55 p.p.m. determined by double irradiation, clearly establishes the pyridol structure shown.

The remaining problem is the relative orientation of the unsymmetrical heterocyclic and aromatic rings. The origin of CO₂H in Me of acetate is in accord with structure (4a) only, and excludes (7) if the polyketide chain has the usual head-to-tail linkage. This structure is supported by the infra-red spectra of the O-methyldecarboxyphomazarin (5a) and the O-methylphomazarin methyl ester (4e), C₂₁H₂₁NO₈, m.p. 180-182° (obtainable by selective methylation of phomazarin) neither of which show absorption above 1640 cm⁻¹ due to unchelated quinone carbonyl. Also in the proton-coupled ¹³C n.m.r. spectrum of (4b) the quinone carbonyl resonance at 182 p.p.m. shows a coupling to the C-5 proton of 3.9 Hz, whereas in the proton-noise-decoupled spectrum of ¹⁵N-enriched (4b) the quinone carbonyl resonance at 179 p.p.m. shows a 2 bond ¹³C-¹⁵N coupling of 7.8 Hz. A similar coupling, 8.8 Hz, is observed on the carbomethoxy resonance at 164 p.p.m. In [¹⁵N]quinoline a 2J¹³C-¹⁵N coupling of 9.3 Hz is observed for the C-8 resonance. Structure (7) reversing the heterocyclic ring of (4a), would require that both of the above couplings involve the same quinone carbonyl resonance. The infra-red spectrum of decarboxyphomazarin (5a), shows absorptions at 1665 and 1630 cm⁻¹, the former suggesting the presence of a quinone carbonyl not involved in hydrogen-bonding.² The origin of this absorption is uncertain.

Alternative modes of biosynthesis of phomazarin (4a) would be by the condensation of two polyketide chains, or by cleavage of an anthrone or anthraquinone derived from one chain. The secalonic acids A and E known to be derived from anthraquinones by quinonoid ring-cleavage have been isolated from P. terrestris.¹³ Further studies are in progress on phomazarin biosynthesis.


$^{13}$C N.M.R. Spectral Studies of Some Naturally Occurring Quinones and Related Compounds

Ian A. McDonald, Thomas J. Simpson and Andrew F. Sierakowski

Abstract

The $^{13}$C n.m.r. spectra of quinones (1)-(14) have been recorded and fully interpreted. Analysis of the fully $^1$H-coupled spectra has enabled the assignment of substituents in the benzene ring and has allowed the relative orientation of the benzene and quinone rings to be established.

Although $^{13}$C n.m.r. spectroscopy has been a valuable aid in elucidating the structures of many naturally occurring substances, there have been few systematic studies of quinones. So far, Berger has studied the carbon chemical shifts in a number of benzoquinones, a report has appeared recently on the $^{13}$C n.m.r. spectra of juglone and naphthazarin derivatives, and some individual quinones have been studied, mostly in conjunction with $^{13}$C-biosynthetic studies. These include the benzoquinones perezone and helicobasidin, the naphthaquinones mollisin and the rifamycins, the anthraquinones islandicin and daunoniycin, and the extended quinone cercosporin.

We now wish to report some $^{13}$C n.m.r. studies on the quinones (4), (9), (10) and (11) isolated recently from the roots of two Conospermum species. The study has been extended to include several related naturally occurring quinones (13) and synthetic analogues (1)-(3), (5)-(8), (12) and (14).

The assignment of $^{13}$C resonances was facilitated by the use of $^1$H noise, broadband off-resonance, single-frequency off-resonance, selective $^1$H decoupling

methods, known substituent effects, use of lanthanide shift reagents, and, in particular, analysis of gated-proton-decoupled spectra. Long-range C–H coupling was analysed by selective low-power decoupling experiments and assignments were in accord with the recorded values of JCC and JCCCH, and the knowledge that the magnitude of JCCCH coupling is greater than JCC in aromatic systems. All spectra were measured in CDCl₃ solutions except in the case of (11) which was dissolved in (CD₃)₂SO. Spectra of (4) and (8) were determined in both solvents and only slight differences in chemical shifts were observed when these were corrected (see Experimental) to compensate for solvent influence.

The multiplicities observed in the single-frequency off-resonance decoupled and fully H-coupled C n.m.r. spectra readily allowed the identification of the protonated carbons and the number of protons attached to each. Selective proton decoupling then confirmed the assignment of these carbons, particularly those of the y,y-dimethylallyl-derived substituents.

**Quinone Ring Substituents**

The chemical shifts (Table I) of the carbons in the quinone ring can be consistently interpreted in relation to the nature of the substituents in this ring. Substitution of C2 and C3 resulted in shifts in the resonances of these carbons in line with the shifts observed for simple olefins. Thus, a hydroxyl or ether substituent on C3 resulted in a downfield shift of approximately 20 ppm at the site of substitution and an upfield shift of approximately 30 ppm of the adjacent C2 resonance. Similarly, alkyl substitution at C2 caused a downfield shift of approximately 10 ppm at C2 and an upfield shift of approximately 8 ppm at C3. In agreement with observations on simple olefins, the downfield shift of C2 increased with the size of the substituent whereas the upfield shift of C3 was not increased greatly by groups larger than methyl. A methyl substituent at C2 did not noticeably affect the chemical shift of the carbonyl carbons C1 or C4. However, the effect of hydroxyl substitution at C3 was to shield C4 by 3–4 ppm, while C1 was not significantly influenced.

In fully H-coupled C n.m.r. spectra, both C2 and C3 showed multiplicities in accord with the nature of the C2 substituent. In (11), in which C2 is unsubstituted, the C2 resonance appeared as a doublet due to one-bond C–H coupling (JCH 164 Hz) to H2, whereas the C3 resonance showed a two-bond C–H coupling (JCH 4 Hz). Substitution of a methyl or alkyl group at C2 revealed that both C2 and C3 couple to the x protons of the substituent. For example, in lapachol (4), the H11 methylene protons are coupled through two bonds to C2 (JCH 7 Hz) and through three bonds to C3 (JCH 5 Hz). Selective low-power irradiation of the H11 protons (3:38 ppm) collapsed both C2 and C3 triplets to sharp singlets. In compounds with 2-methyl substituents, C2 and C3 appeared as quartets in the fully H-coupled spectrum (see Fig. 1), although in several compounds these were obscured.

Table 1. $^{13}$C n.m.r. chemical shifts of compounds (1)–(14)

For carbon numbering, see formulae on p. 1728

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OCH$_3$

56-3

56-4

56-9

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A In (CD$_3$)SO.

B,C These assignments may be reversed.

Table 2. Lanthanide-induced shifts observed for quinones (3) and (6)

2:5 molar ratio of shift reagent; quinone shifts given in ppm

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tially integral $^{13}$C intensities, the resonance at 128.6 ppm had approximately double the intensity of the other resonances. This indicated the presence of two coincident quaternary carbon resonances and these were assigned to C2 and C10.

**Benzene Ring Substituents**

The effect of substitution in the benzene ring can be seen in the spectra of quinones (7)-(13) (Table I). A detailed study of one of these [(7), a derivative of the naturally occurring quinone$^9$] enabled the spectra of the others to be readily interpreted. In the original structural studies of the quinones from *C. teretifolium* R. Br., this substance proved to be the most difficult to analyse and its structure was eventually proved conclusively by synthesis.$^9$ The low-field region of the fully $^1$H-coupled spectrum of (7) (Fig. I) can be interpreted as follows:

(i) C2 appeared as a quartet ($^2J_{CH}$ 7 Hz) due to coupling to the methyl group (see above).

(ii) The signal due to C3 overlapped that of C7 and it was not possible to make an accurate assignment.

(iii) C5 resonated as a triplet ($^2J_{CH}$ 6 Hz) due to coupling to the H11 protons.

(iv) C6 was observed as a broad multiplet which collapsed to a doublet ($^3J_{CCCH}$ 8 Hz) on selective low-power irradiation of the H11 protons; the remaining coupling was to H8. This irradiation also collapsed C5 and C10 to a singlet and doublet ($^3J_{CCCH}$ 6 Hz), respectively.

(v) C8 appeared as a well defined doublet ($^1J_{CH}$ 162 Hz) due to coupling to H8.

(vi) C9 resonated as a singlet which indicated there were no protons in a three-bond coupling situation.

(vii) C10 was revealed as a doublet of partially resolved triplets which was caused by three-bond couplings with H8 and the H11 protons [see (ii) above].

Consideration of the two carbonyl carbon (C1, C4) signals was of great value in the assignment of the methoxyl group to C7 rather than C8, and in the assignment of the relative orientation of the two rings in the naphthaquinone nucleus. The low-field resonance (184.9 ppm), due to C1, appeared as a quintet which could be analysed as a doublet of quartets due to three-bond couplings to H8 ($^3J_{CCCH}$ 4 Hz) and H16 ($^3J_{CCCH}$ 4 Hz). In contrast, the higher field (181.7 ppm) C4 carbonyl carbon appeared as a singlet.

With an understanding of the spectrum of (7), the $^{13}$C n.m.r. spectra of the related quinones (8)-(12) can be interpreted accordingly. In (8), the methoxyl substituent at C7 in (7) has been replaced by hydrogen and this gives rise to predictable changes in chemical shift and modes of coupling. The signals due to C6, C8, and C10 moved downfield by 11.4, 19.3, and 3.8 ppm, respectively, and C7 moved upfield by 30.3 ppm relative to the corresponding shifts in (7). In the fully coupled spectrum, C7 and C8 appeared as doublets with $^1J_{CH}$ 163 and 166 Hz, respectively; C9 resonated as a doublet ($^3J_{CCCH}$ 9 Hz) being coupled to H7, and C6 gave rise to a doublet of triplets ($^3J_{CCCH}$ 10 and 2 Hz) due to coupling to H8 and H11, respectively. It was not possible to resolve the signals due to C5 and C10 which appeared as a broad multiplet at 124.1 ppm. In accord with the observations in the spectrum of (7), the C1 carbonyl carbon exhibited coupling to both H8 and H16. As expected this feature is also present in the spectra of (9) and (10).

Introduction of a double bond in the dihydropyran ring caused some changes to the spectrum. This was evident in the spectrum of (9) in which the only significant
by other resonances. Coupling of the \( \alpha \) protons to the adjacent carbonyl carbon (C1) also was observed, but the resolution was often poor.

Although compounds (3)–(6) are unsubstituted in the benzene ring, the presence of an ether or hydroxyl substituent at C3 has an effect on the chemical shifts of carbons C5 to C10 in this ring. It was expected that this could be accounted for by resonance contributions which have been postulated to account for differences in the \(^1\text{H n.m.r.} \) spectra of some substituted naphthaquinones.\(^{15} \) Thus, C6, C8, and C10 were expected to resonate at lower fields than C7, C5, and C9, respectively.

However, when an accurate assignment of the relevant carbons, by means of lanthanide-induced shift studies with Pr(fod)\(_3\) and Yb(fod)\(_3\) (Table 2), was carried out, it became clear that this premise was incorrect and that C7, C5, and C9 actually resonated at a lower field than C6, C8, and C10, respectively. The data in Table 2 indicate that the primary coordination site of the shift reagent was the C1 carbonyl for Pr(fod)\(_3\) and Yb(fod)\(_3\) but not for Eu(fod)\(_3\) which appeared to coordinate at the ether oxygen. In addition, whereas Pr(fod)\(_3\) and Yb(fod)\(_3\) caused a linear shift behaviour, the shifts caused by Eu(fod)\(_3\) were anomalous and difficult to interpret satisfactorily. This anomalous behaviour with europium has been noted previously in \(^{13}\text{C n.m.r.} \) studies and is attributable to large Fermi-contact contributions to \(^{13}\text{C n.m.r.} \) chemical shifts. Praseodymium and ytterbium, on the other hand, have predominantly pseudo-contact shifts and, therefore, are more suitable in \(^{13}\text{C n.m.r.} \) studies.\(^{16} \)

Difficulty was encountered in identifying all the expected lower field quaternary carbons in the \(^1\text{H n.m.r.} \) spectra of (5). Instead of the expected six resonances, only five were immediately evident. Moreover, the single-frequency off-resonance decoupled spectrum revealed that no quaternary resonance was coincident with any of the protonated carbons. However, on retesting the spectrum under gated-2 decoupling\(^{17} \) in the presence of Cr(acac)\(_3\),\(^{18} \) conditions known to give essen-

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change was in the C5 resonance which moved upfield by 2·9 ppm. The olefinic carbon resonances (C11 and C12) appeared at 119-5 and 135-7 ppm and were readily distinguished by the multiplicity of each signal. The assignments of the protonated carbon resonances in (9) were confirmed by specific decoupling experiments.

In the substances which contain a γ,γ-dimethylallyl moiety significant changes in the chemical shifts of some signals occurred. These were an upfield shift (8-10 ppm) of C7 and a downfield shift (8-10 ppm) of C5 relative to the shifts in the quinones (8) and (9). Only ten low-field resonances were observed in the 1H-noise decoupled spectrum of (10), but on determining the broad-band off-resonance decoupled spectrum in which only quaternary resonances were observed, it became apparent that two closely resonating quaternary carbons had been obscured by the strong protonated carbon resonance of C8. In the fully coupled spectrum C12 appeared as a doublet of broad resonances due to coupling to H12 and long-range couplings to H11, H14, and H15; C7 and C8 appeared as sharp doublets (J_CCH 166 and 162 Hz, respectively). As before, analysis of long-range couplings enabled the assignment of quaternary carbons: C9 was observed as a doublet (J_CCH 7 Hz) due to coupling with H7 and this was superimposed on the broad resonance of C10. Irradiation of 3·7 ppm (H11) caused this resonance to collapse to a doublet (J_CCH 4 Hz), the residual coupling being to H8. This irradiation also caused the broad resonance due to C13 to collapse to a singlet and C5 to collapse to a doublet (J_CCH 5 Hz), still showing coupling to H7. In addition, the broad, poorly resolved C6 multiplet collapsed to a doublet (J_CCH 10 Hz), presumably due to coupling to H8.

The shifts observed in the spectra of compounds (11) and (12) were very similar to those of (10). The assignments of C5 and C13, and C9 and C10 in (11) are tentative as overlapping signals prevent analysis of the long-range couplings and, in (12), C9 and C10 are coincident. The coupling patterns in (11) differed significantly from that of the other substances because there was no substituent at C2. This results in both C1 and C4 carbonyl resonances appearing as doublets due to three-bond couplings to H8 (J_CCH 4 Hz) and H2 (J_CCH 7 Hz). If the hydroxyl group had been located on C2 instead of C3, both couplings would have been to C1, leaving C4 as a sharp singlet. These observations, therefore, fixed the relative orientation of the benzene and quinone rings. These modes of coupling can be seen in the 1H-coupled spectra of 2-hydroxy-5-methyl-1,4-naphthaquinone and 3-hydroxy-5-methyl-1,4-naphthaquinone.

In hydroxydroserone (13), the effect of hydrogen bonding of the C5 and C8 hydroxyl groups to the carbonyl centres was to shift the resonances of both C1 (particularly) and C4 downfield. It was not possible to assign individually C3, C5, and C8 because of the similarity of the chemical shifts. While C6 and C7 both displayed one-bond couplings (J_CCH 165 and 166 Hz, respectively), C7 showed an additional three-bond coupling (J 7 Hz), presumably to the 8-OH proton. Previous work by Wehrli has shown that strongly chelated phenolic protons will couple in this manner. A noticeable feature of the 13C n.m.r. of naphthazarin was that the chemical shifts of C1, C4, C5, and C8 were identical (172-9 ppm) which confirmed the fact that this substance exists as tautomeric isomers. However, in (13), in which substitution at C2 and C3 is known to stabilize the quinone ring,21 the spectrum

19 Simpson, T. J., unpublished data.
confirmed that tautomerism is not a significant factor. The assignment of chemical shifts in \(\beta\)-lapachone (14) was based on the shift values given for 1,2-naphthaquinone\(^\text{22}\) and the expected substituent effects of the dihydropyran ring.

This study has shown that \(^{13}\text{C}\) n.m.r. can provide valuable structural information on naphthaquinones. In particular, analysis of fully \(^1\text{H}\)-coupled spectra has enabled the assignment of substituents in the benzene ring and has allowed the relative orientation of the benzene and quinone rings to be established.

**Experimental**

The \(^{13}\text{C}\) n.m.r. spectra were determined as 0.2-0.6 molar solutions in CDCl\(_3\) with Me\(_4\)Si as an internal reference, or in (CD\(_3\))\(_2\)SO, the centre peak of the (CD\(_3\))\(_2\)SO signal being used as the internal reference. In the latter case, chemical shifts were corrected by the expression: \(\delta(\text{Me}_4\text{Si}) = \delta(\text{Me}_2\text{SO}) + 39.6\). Chemical shifts are reported as \(\delta\) (ppm) downfield from Me\(_4\)Si and spectra were recorded on a JEOL JNM FX60 spectrometer operating at 15.04 MHz. Fully coupled spectra were determined under gated decoupling conditions with a 0.5 KHz noise modulated proton irradiating frequency at a power level of approximately 5 W. Specific decoupling experiments used a single \(^1\text{H}\)-irradiating frequency 40 dB below this level. Single-frequency off-resonance decoupled and broad-band off-resonance decoupled spectra were determined with a \(^1\text{H}\) irradiating frequency at a power level of approximately 5 W, 600 Hz upfield from Me\(_4\)Si with noise modulation when necessary. 0.1 molar chromium tris(acetoacetonate) [Cr(acac)\(_3\)] was used as a relaxation agent, with gated decoupling conditions, to eliminate the nuclear Overhauser effect. Pr(fod)\(_3\), Yb(fod)\(_3\), and Eu(fod)\(_3\) were used as lanthanide shift reagents and in each case the spectra were run with the following molar ratios of shift reagent to quinone: 1:20, 1:10, 1:5, and 2:5.

The quinones were either natural products,\(^9\) or were prepared by standard methods.\(^{23}\)

**Acknowledgment**

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\(^{23}\) Cannon, J. R., unpublished data.


\textbf{13C-NMR STUDIES ON GRISEOFULVIN BIOSYNTHESIS AND ACETATE METABOLISM IN PENICILLIUM PATULUM}

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Key Word Index—Penicilliumpatulum; Ascomycetes: biosynthesis; fungal polyketide; griseofulvin.

Abstract—Incorporations of singly and doubly-labelled acetate-[13C] into griseofulvin by a mutant strain of Penicilliumpatulum confirm its origin from simple folding of a single heptaketide chain. An acetate 'starter' effect is observed in the 13C-NMR spectra of griseofulvin enriched from acetate-[13C], and analysis of the 13C—13C spin—spin couplings observed indicate a rapid metabolic turnover of added acetate. Methyl, but not carboxyl, of acetate is efficiently metabolised into the C\textsubscript{1} pool.

\section*{INTRODUCTION}

Griseofulvin (I), an important antifungal antibiotic, was first isolated from the mycelium of Penicilliurn griseofulvum, and has since been recognised as a metabolic product of many species of Penicilliurn [1]. Its biosynthesis has been a subject of several studies and much speculation. The polyketide origin of griseofulvin was indicated by incorporation studies with acetate-[1,14C] by Birch and co-workers [2], who suggested derivation of griseofulvin from a single chain of seven acetate units as shown in Scheme 1. Studies by Rhodes et al. [3] have established that griseophenone C(2), and griseophenone B(3) but not griseophenone A(4) are likely intermediates between acetate and griseofulvin and they have proposed biogenesis which aim to account for the formation of all fungal heptaketides from common intermediates, which would be formed either by condensation of two shorter polyketide chains [4, 5], or by ring closure and subsequent ring fission of a single heptaketide chain [6]. As 13C-NMR has proved useful for providing information on the nature of the biosynthetic intermediates between acetate and malonate and the final polyketide metabolites [7], incorporations of singly and doubly-labelled acetate-[13C], as well as malonate-[14C] into griseofulvin using a high-yielding mutant strain of Penicilliurn patulum have been carried out in an attempt to solve some of the uncertainties in griseofulvin biosynthesis. The 13C-NMR spectrum of griseofulvin has recently been fully assigned [8].

\section*{RESULTS AND DISCUSSION}

Preliminary incorporation experiments were carried out with sodium acetate-[14C] in order to determine the minimum amount of acetate-[13C] required to give sufficiently low dilution values for significant enrichments to be observed in the resultant 13C-NMR spectra.

Scheme 1. Alternate foldings of the precursor heptaketide chain in griseofulvin.

that the final stage in griseofulvin biosynthesis involves the binding of griseophenone B to a multi-enzyme complex which can effect oxidation, reduction, and methylation of enzyme bound intermediates. The co-occurrence of griseofulvin and fulvic acid (5) in the same organism, as well as the co-occurrence of other heptaketides in other fungi, has led to the proposal of several theories of
Table 1. Dilution of sodium acetate-[1-14C] on incorporation into griseofulvin by cultures of *P. patulum*

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Acetate (mg/ml)</th>
<th>μCi</th>
<th>mg</th>
<th>dpm/mg</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>176</td>
<td>4569</td>
<td>53.6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.5</td>
<td>10</td>
<td>209</td>
<td>3768</td>
<td>32.5</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>10</td>
<td>168</td>
<td>4054</td>
<td>15.1</td>
</tr>
</tbody>
</table>

[7]. These results, summarized in Table 1, indicated that at least 2 g/l of acetate was required to give a doubling of 13C-abundance at each labelled position. Accordingly fermentations supplemented with 4 g/l of sodium acetate-[1-13C], [2-13C], and [1,2-13C] respectively were carried out. The 13C-NMR spectra of the acetate-[1-13C], and acetate-[2-13C] enriched griseofulvin samples are summarized in Table 2.

The alternate labelling pattern anticipated for the polyketide origin of griseofulvin was generally observed but, the apparent enrichment of labelled sites is very much lower with acetate-[2,13C] than with acetate-[1-13C] particularly C-7 which does not show the anticipated enrichment from acetate-[2-13C]. On the other hand, MS studies (see below) indicate that the total incorporation of acetate-[2,13C] is at least as high as that of acetate-[1-13C]. The most likely explanation of this apparent discrepancy is that the label of acetate-[2-13C] becomes randomised into the 1-position via operation of the Krebs' Cycle. The method of calculating enrichments [9] automatically compensates for any excess 13C-abundance arising in this way, therefore an apparently low enrichment of acetate-[2-13C] is observed.

The degree of enrichment at positions C-1 and C-2 is significantly higher than in the remaining acetate-derived carbons. Differential labelling of a polyketide has not been observed previously in 13C-biosynthetic studies. However, Birch et al. [10] have observed a small preferential incorporation of acetate-[14C] in tracer experiments into the methyl terminal acetate unit of both griseofulvin and curvularin and on feeding sodium acetate-[1-14C], along with unlabelled diethyl malonate, to cultures of *Penicillium urticae* the resultant 2-hydroxy-6-methylbenzoic acid showed a 12-15% excess isotopic abundance in the 'starter' acetate unit, no difference being observed in the absence of added malonate. This suggests that in *P. patulum* the acetate-[13C] derived malonate is diluted by an endogenous metabolic pool of malonate with no corresponding dilution of the acetate-[13C] so causing a clear acetate 'starter' effect to be observed. No other acetate unit shows this enhanced incorporation, so establishing that griseofulvin is formed from a single heptaketide chain and ruling out the ring-fission pathway which requires that the methyl group at position C-1 is not part of the chain initiating acetate being formed in the ring-fission process (Scheme 2).

Kuhn–Roth oxidation of griseofulvin-[14C] obtained...
from fermentations of \( P. \) patulum supplemented with diethyl malonate-[2-\textsuperscript{14}C], produces acetic acid with only 9.8\% of the total activity of griseofulvin (even labelling of a heptaketide would require 14.3\% so confirming that the methyl at position C-1 is part of a chain initiating acetate unit, the observed incorporation into this position being due to decarboxylation of malonate to acetate.

It may be seen, Table 2, that incorporation of acetate-[2-\textsuperscript{13}C], but not acetate-[1-\textsuperscript{13}C] results in substantial enrichment of the methoxyl groups of griseofulvin. These have previously been shown to arise from the usual biochemical C\textsubscript{1} donor systems \[11\]. Enrichment of carbons derived from the C\textsubscript{1} pool has been observed in biosynthetic studies with both acetate-[1-\textsuperscript{14}C] and acetate-[2-\textsuperscript{14}C] but usually at a low level and possibly it occurs \textit{via} catabolism of acetate to CO\textsubscript{2} and subsequent re-incorporation. The specific enrichment from the methyl of acetate observed in this case could arise from conversion of acetate \textit{via} the Krebs Cycle and pyruvate into serine. It has been shown \textit{inter alia} in \textit{Torulopsis} yeast grown on acetate that the \( \alpha \) and \( \beta \) carbons of serine are derived from the methyl carbon only of acetate \[12\]. Subsequent dehydroxymethylation of serine would give rise to glycine-[2-\textsuperscript{13}C] and a [\textsuperscript{13}C]-enriched C\textsubscript{1} pool (Scheme 3). Incorporations of serine-[3-\textsuperscript{13}C] and glycine-[2-\textsuperscript{13}C] in \textit{Serratia marcescens} \[13\] and \textit{Streptomyces longisporus rubber} \[14\] result in substantial enrichments of the methoxyl group in the prodigiosins, though no enrichment of this group was observed from acetate-[\textsuperscript{13}C].

The \textsuperscript{13}C-NMR spectrum of griseofulvin enriched from acetate-[1,2,\textsuperscript{13}C] is shown in part in Fig. 1. The anticipated characteristic triplets normally observed in doubly labelled spectra arising from the natural abundance signal with satellites on either side due to \textsuperscript{13}C-\textsuperscript{13}C coupling between carbons derived from acetate units incorporated intact are seen only for C-1 and C-2. C-3, C-5 and C-11 show more complex patterns of satellites and the remaining carbons are of such low intensity that no satellites can be readily distinguished above the spectral noise level. The methoxyl carbons are again significantly enriched, presumably from the acetate methyl carbon only. The relatively high intensity of the satellites on the resonances due to C-1 and C-2 again indicate the preferential incorporation of acetate into this unit. The complex satellite patterns observed for the

![Fig. 1. \textsuperscript{13}C NMR spectrum (0-110 ppm) of acetate-[1,2,\textsuperscript{13}C] enriched griseofulvin.](image-url)
remaining carbons can only be accounted for by substantial $^{13}$C-$^{13}$C coupling between adjacent doubly-labelled acetate units. This further coupling reduces the intensity of the satellites arising from incorporation of intact acetate units and further satellites appear due to one-bond $^{13}$C-$^{13}$C coupling between adjacent acetate units as is indicated for C-11 in Fig. 1. The innermost satellites in this case are obscured by the large natural abundance resonance. An additional complicating factor is the possibility of two-bond $^{13}$C-$^{13}$C couplings, which owing to the size of doubly-labelled acetate in which there is greater than 90% $^{13}$C at both positions, is almost certainly one-bond $^{13}$C-$^{13}$C coupling between adjacent units. Two-bond $^{13}$C-$^{13}$C couplings are usually small, typically 0-15 Hz, so are not always resolvable. However, the satellite pattern for C-5 shows an additional small splitting. 9.4 Hz, due to the two-bond coupling between C-5 and C-7, or possibly C-3. The usual shape of the C-3 signal is partly explained by the fact that the one-bond couplings between the aliphatic C-3 and C-2 are comparatively smaller than those for the aromatic carbons above. However, no significant satellites corresponding to the one bond coupling between C-3 and C-4 is observable. It has already been established that acetate is incorporated into C-1 and C-2 to a greater extent than in the remainder of the molecule, so it is likely that for every griseofulvin molecule in which C-3 and C-4 are derived biosynthetically from added precursor acetate, so will be C-1 and C-2, though the reverse obviously is not true. The result of this is that essentially all the carbons at position C-3 which are coupled to C-4 are further split by both C-2 and C-1 to give the observed pattern. The reciprocal couplings on C-1 and C-2 are not readily observable due to the high proportion of these atoms not further coupled in this way. Confirmation of the two-bond coupling between C-1 and C-3 is found in the acetate-[2-$^{13}$C] NMR spectrum where the C-3 signal appears to be broader than the remaining signals. Redetermination of the spectrum on a narrow sweep width, readily allows a coupling of 12.2 Hz to be resolved. Two-bond couplings arising from incorporation of singly-labelled acetate-$^{13}$C into aflatoxin B have been reported by Heevel et al.[15], but in this case, very high overall enrichments (20-30%) of the labelled carbones were obtained.

Intensive acetate-unit coupling of this sort can only occur when there is a high probability of two or more acetate units being incorporated into adjacent positions in any one molecule. This has been recognised as a problem with organisms where the incorporation of acetate is very efficient, giving very low dilution values, and dilution of the labelled acetate with unlabelled acetate before feeding has been used to reduce the possibility of two labelled units coming together [16], although the actual validity of doing this has been questioned by a recent detailed statistical treatment [17]. This problem was not anticipated in P. patulum, however, due to the comparatively high dilution values observed in the preliminary $^{14}$C experiments and indeed the overall average $^{13}$C-enrichment observed is much too low to allow this and suggests there is only an approx. 4% probability of adjacent units being labelled.

MS studies can be used to estimate the excess $^{13}$C abundance, and Table 3 shows the ion intensities in the molecular ion region for unlabelled, and both acetate-[1-$^{13}$C] and acetate-[2-$^{13}$C] enriched griseofulvin. Although accurate estimations are complicated by the isotope pattern exper so that, until this pool falls below a threshold level and endogenous acetate production recommences, most of the metabolite is produced from endogenous precursor. Subsequently, predominantly unenriched griseofulvin is produced to give the low average enrichment observed. The unexpected pattern of acetate metabolism observed in P. patulum is probably due to two factors. Firstly a highly developed, mutant strain was used, which is probably capable of rapid utilisation of any readily available carbon source. Secondly, it is due to the use of elevated amounts of precursor, in contrast to the trace

Table 3. Ion intensities in the molecular ion region of the mass spectra of natural abundance, and acetate-[1-$^{13}$C] and acetate-[2-$^{13}$C] enriched griseofulvin

<table>
<thead>
<tr>
<th>Sample</th>
<th>M+ (352)</th>
<th>M+1</th>
<th>M+2</th>
<th>M+3</th>
<th>M+4</th>
<th>M+5</th>
<th>M+6</th>
<th>M+7</th>
<th>M+8</th>
<th>M+9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural abundance</td>
<td>100</td>
<td>19</td>
<td>37</td>
<td>7</td>
<td>0.4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Acetate-[1-$^{13}$C]</td>
<td>100</td>
<td>21</td>
<td>37</td>
<td>7</td>
<td>0.5</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>Acetate-[2-$^{13}$C]</td>
<td>100</td>
<td>25</td>
<td>39</td>
<td>9</td>
<td>0.7</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>
amounts used in classical radioisotope studies, and these results serve to emphasise that this factor must always be taken account of in interpretation of 13C-biosynthetic studies. The effects observed in this study provide an excellent illustration of the problems and factors that can arise through using non-tracer amounts of precursor, and will probably be encountered more frequently as 13C-biosynthetic studies become more commonplace. Great care must be exercised when, for example, using unenriched carbons of different biosynthetic origin for normalisation of spectra and calibration of enrichments. However, it also serves to illustrate the power of 13C methods in elucidating the overall metabolic patterns in an organism.

**EXPERIMENTAL**

13C-NMR spectra were determined for saturated solns in CDCl3 with Me3Si as internal reference on either a Varian XL-100-15FT spectrometer operating at 25.197 MHz or a Jeol JNM FX60 spectrometer operating at 15.04 MHz. The enrichment values quoted in Table 2 were calculated from spectra determined in the presence of 0.1 molar chromium Tris-acetate [19] as relaxation agent under GATED-2 decoupling conditions [20]. These conditions eliminate the wide range of resonance intensities arising from variable relaxation times and nuclear Overhauser enhancements so facilitating measurement of intensities. The natural abundance and enriched spectra were each determined three times, and the average intensities after normalisation were used to calculate the enrichments using the method reported previously [9]. The actual values are the ratio of the intensities in the enriched spectrum to the same intensities in the natural abundance spectrum and are consistent within a range of ±20%. 13C-Radiochemical assays were carried out by scintillation counting. Counting efficiencies were measured with [1-13C]hexadecane as internal standard using Butyl-PBD (10 g) in toluene (111) as scintillator solution.

**Culture conditions.** A Glaxo mutant strain of *P. patulum* was grown in 100 ml shake flasks each containing 30 ml of Glaxo standard shake flask medium with 10% lactose. Each flask was inoculated with 1.5 ml of pre-grown mycelium. A time study indicated that griseofulvin production commenced at about day 2 and had reached a conc. of approx. 200 mg per flask after 7 days growth.

**Incorporation studies.** (a) Sodium acetate-[1-14C]. Increasing amounts of sodium acetate, as indicated in Table 1, each spiked with sodium acetate-[1-14C] (10 µCi), in 1 ml H2O were added to 2 day old cultures of *P. patulum*. After 7 days the mycelium was collected by filtration, stirred in Me2CO (75 ml) together with added lime (200 mg) for 10 min. The Me2CO extract was then filtered off and the Me2CO removed in vacuo at 61-70°C. The resulting hot aq. suspension was filtered and the crude product approx. 200 mg washed with H2O (60°C). Recrystallisation from Me2CO petrol (bp 60-80°C) gave essentially pure griseofulvin which was crystallised to constant specific radioactivity. The minimum dilution value obtained was 15. On this basis it would be anticipated that equivalent feedings of 0.2% acetate-[1-13C] (90%) would give griseofulvin with an excess of 0.8% 13C-label over natural abundance at each labelled position, assuming a total of 7 labelled positions in the molecule.

(b) Diethyl malonate-[2-14C]. Diethyl malonate-[2-14C] (25 µCi) was added as above to a 2 day old culture flask and the product isolated after 7 days growth. Recrystallization to constant specific radioactivity gave griseofulvin-[1-14C] (108 mg, 5.00 x 106 dpm/mmol). After dilution with unlabelled griseofulvin (150 mg, 8.34 x 106 dpm/mmol) it was subjected to Kuhn-Roth oxidation. The resultant HOAc was isolated and converted to the p-bromophenacyl derivative (35 mg) by standard procedures. Recrystallization to constant specific radioactivity gave p-bromophenacyl acetate with an activity of 8.21 x 106 dpm/mmol. Thus the terminal unit contains 9.8% of the total radioactivity of griseofulvin.

(c) Sodium acetate-[1-13C]. To each of 3 flasks containing a 2 day old culture of *P. patulum* was added sodium acetate-[1-13C], [2-13C], or [1,2-13C] (120 mg, 90% enriched). After a further 5 days growth, the enriched griseofulvin, 56, 65 and 82 mg respectively after recrystallization, was isolated as above.

**Acknowledgements**—We wish to thank Glaxo Laboratories Ltd., Uiverston, Cumberland for the use of cultures, culture facilities and technical assistance. We thank Dr R. D. Lapper for 13C-NMR spectral determinations.

**REFERENCES**

$^{13}$C Nuclear Magnetic Resonance Spectra and Biosynthetic Studies of Xanthomegnin and Related Pigments from Aspergillus sulphureus and melleus

By Thomas J. Simpson,* Research School of Chemistry, Australian National University, P.O. Box 4, Canberra, A.C.T. 2600, Australia
The $^{13}$C n.m.r. spectra of xanthomegnin (1) and related compounds [(2)—(5), (7), and (9)] have been assigned. Spectra of xanthomegnin and viomellein biosynthesised from singly and doubly $^{13}$C-labelled acetate confirm the polyketide origin of these metabolites and provide additional evidence for the structures proposed for the pigments.

The isolation of the fungal pigments xanthomegnin (1), viomellein (4), rubrosulphin (6), and viopurpurin (8) from the mycelium of *Aspergillus sulphureus* and *A. melleus* was described recently. Xanthomegnin had previously been isolated from *Trichophyton* spp. and assigned the dirneric naphthoquinone structure (1). The naphthoquinone–naphthalene structures for the remaining pigments were proposed on the basis of chemical and spectroscopic evidence. $^{13}$C N.m.r. structural studies of xanthomegnin and its related metabolites and biosynthetic studies in *A. melleus* are now reported which provide additional evidence for their structure and indicate their mode of biogenesis.

These metabolites are closely related to other groups of fungal pigments, e.g. the dimeric naphthoquinone aurofusarin, fuscofusarin, which has a naphthoquinone–naphthalene structure, and the dimeric naphthalenes vioxanthin, viriditoxin, and flavoinannin. Owing to the highly substituted nature of these structures, $^1$H n.m.r. is of limited applicability, but $^{13}$C n.m.r. was expected to prove a useful method of structure elucidation in this area. The $^{13}$C n.m.r. spectrum of viriditoxin has been reported but only partially assigned.

Assignments of $^{13}$C Resonances.—The $^{13}$C n.m.r. spectra of xanthomegnin (1), xanthomegnin acetate (3), viomellein (4), and the methyl ethers [(2), (5), (7), and (9)] of xanthomegnin, viomellein, rubrosulphin, and viopurpurin, respectively, are summarised in Table 1. The assignments are based on comparisons between resonances in the proton noise-decoupled (p.n.d.) spectra, use of single frequency off-resonance-decoupled (s.f.o.r.d.) spectra to determine the number of attached protons on each carbon atom, and comparisons with model compounds. The substituent-induced shifts tabulated by Wells *et al.* are useful in indicating the expected chemical shifts in the naphthalene portions of the molecules. Detailed analysis of the fully proton-coupled spectra, making use of D$_2$O exchange and low-power selective proton decoupling to identify the origin of long-range couplings, is of particular utility.

Only fourteen resonances are observed in the normal p.n.d. spectrum of xanthomegnin. The multiplicities...
observed in the s.f.o.r.d. spectrum readily allow the assignment of C-1, C-3, C-5, and OCH₃; the remaining carbon atoms all give singlets. The quionone carbonyl resonances at 186.0 and 179.8 p.p.m. are assigned to C-8 and C-11 respectively, the C-8 signal appearing at lower field owing to chelation with the C-6 OH, whereas the C-11 signal appears at higher field than in 1,4-naphthoquinone (184.6 p.p.m.).¹² owing to the shielding effect of the C-10 OMe. In 2-methoxy-1,4-naphthoquinone the C-1 carbonyl resonance appears at 180.0 p.p.m. On removal of the chelation, the C-8 carbonyl resonance moves upfield to 180.8 and 179.8 p.p.m. in the methyl ether (2) and the acetate (3), respectively. In addition, in the fully-coupled spectrum of (1), the C-8 resonance appears as a sharp singlet, whereas C-11 gives a doublet, J 5 Hz, due to a five-bond coupling to H-5 (low-power irradiation at the frequency of H-5 causes collapse of the resonance to a sharp singlet). This long-range coupling is valuable in distinguishing the quinone carbonyl resonances in all the compounds. The resonances at 157.9, 162.2, and 162.7 p.p.m. are assigned to C-10, C-14, and C-6, respectively: in the fully-coupled spectrum the broad resonance at 157.9 p.p.m. collapses to a sharp singlet on irradiation at the frequency of the methoxy-protons (5.85); the resonance at 162.7 p.p.m. appears as a doublet, J 5 Hz, owing to coupling to the C-6 OH, which collapses to a singlet on addition of D₂O with a small upfield deuterium isotope shift of 0.2 p.p.m.; and the 162.2 p.p.m. resonance appears as a singlet which greatly increases in intensity on irradiation at the frequency of the C-2 proton (5.39). The remaining resonances at 148.1, 134.7, 117.5, and 114.8 p.p.m. are assigned to C-4, C-12, C-13, and C-7, respectively. In the fully coupled spectrum the 148.1 p.p.m. resonance appears as a broad unresolved signal which collapses to a sharp singlet on irradiation at the frequency of the C-3 benzylic protons (6.90). This irradiation also causes the broad satellites of the C-5 doublet (J₁₋₀ 168 Hz) to sharpen, and the broad resonance at 117.5 p.p.m. to collapse to a doublet, J 4 Hz, the residual splitting being due to a three-bond coupling to H-5. Addition of D₂O causes the triplet at 114.8 p.p.m. to change to a doublet, J 7 Hz, owing to removal of the three-bond coupling to the chelated phenolic proton. The remaining three-bond coupling to H-5 is removed by irradiation at 5 Hz.² In di-O-methylxanthomegnin (2), the C-7 resonance has moved downfield to 124.0 p.p.m., owing to removal of chelation.° The remaining resonance at 134.7 p.p.m. is a sharp singlet in the fully coupled spectrum as anticipated for C-12; in 1,4-naphthoquinone, the corresponding carbon atom resonates at 131.7 p.p.m. Under normal conditions of spectral determination the C-9 resonance of xanthomegnin cannot be observed owing to its very long relaxation time. However, on addition of the relaxation agent, Cr(acac)₃, the C-9 resonance appears at 123.0 p.p.m. In the spectra of the remaining compounds, the resonance is visible, but usually as a very weak singlet, which aids its assignment. In viriditoxin, the signal for the equivalent carbon atom at the dimer linkage was not observed.³ Similar analysis of the gC n.m.r. spectra of the dimethyl ether (2) and the diacetate (3) readily allows the assignments given in Table 1.

### TABLE 1

| Table 1  |
|---|---|---|---|---|---|---|
| Carbon | (1) | (2) | (3) | (4) | (5) | (9) |
| 1 | 20.6q | 20.5q | 20.5q | 20.7q | 20.7 | 20.8q |
| 2 | 74.4q | 74.3q | 74.5q | 74.1q | 74.3 | 74.2q |
| 3 | 36.1q | 36.4q | 35.8q | 36.3q | 36.4 | 36.4q |
| 4 | 141.8 | 146.9 | 146.2 | 147.9 | 148.2 | 147.2 |
| 5 | 116.8q | 120.9q | 123.2d | 116.4d | 120.8 | 120.4d |
| 6 | 162.7 | 162.3 | 161.3 | 162.8 | 162.3 | 162.7 |
| 7 | 134.8 | 124.0 | 124.0 | 114.8 | 124.2 | 124.2 |
| 8 | 158.0 | 180.8 | 179.8 | 183.3 | 182.2 | 177.8 |
| 9 | 125.0 | 125.7 | 125.7 | 126.2 | 127.2 | 129.2 |
| 10 | 157.9 | 156.4 | 156.7 | 158.2 | 156.4 | 150.8 |
| 11 | 179.8 | 180.2 | 179.8 | 180.1 | 180.7 | 172.9 |
| 12 | 134.7 | 136.0 | 135.3 | 134.6 | 136.1 | 131.6 |
| 13 | 117.5 | 125.2 | 132.7 | 117.6 | 125.3 | 125.8 |
| 14 | 162.2 | 160.4 | 159.7 | 162.4 | 160.4 | 160.1 |

**Assignments may be reversed.**

Twenty-nine resonances are resolved in the p.n.d. 13C n.m.r. spectrum of viomellein (4), and the fifteen resonances due to the naphthoquinone moiety are readily assigned by comparison with those of xanthomegnin. In both viomellein and tri-O-methylviomellein (5), substitution of C-9 by a naphthalene rather than a naphthoquinone unit causes a ca. 2 p.p.m. downfield shift of the C-8 signal. The C-1 and C-1' resonances are coincident, but not those of C-2 and C-2', and of C-3 and C-3'. The C-14' lactone carbonyl resonance at 171.2 p.p.m. moves upfield to 160.6 p.p.m. on methylation

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T. J. Simpson, unpublished observations.

[to (5)], with concomitant shifts of the C-13' resonance from 107.9 to 113.9 p.p.m., and the C-2' and C-3' resonances become coincident with those of C-2 and C-3. These chemical shifts and their behaviour on methylation are almost identical with those observed in mellein (10), with concomitant shifts of the C-13' resonance. The C-7' and C-8' resonances become coincident with those of C-2 and C-3. These chemical shifts and their behaviour on methylation and O-methylmellein (11), and are due to removal of the chelation and resultant slight conformational changes in the lactone ring. The aromatic protonated carbon resonances at 116.0 and 97.8 p.p.m. are readily identified from the s.f.o.r.d. spectrum of viomellein and are assigned to C-6' and C-7', respectively, the C-7' signal being at higher field owing to the extra shielding from the C-8' OMe. The resonances at 155.3, 160.1, and 161.3 p.p.m. are assigned to C-12', C-8', and C-10', respectively: in the fully-coupled spectrum the broad resonance at 160.1 p.p.m. sharpens to a singlet on irradiation at the OCH$_3$ frequency; the resonance at 155.3 p.p.m. appears as a doublet, 7/3 Hz, collapsing to a singlet on addition of D$_2$O; the 161.3 p.p.m. resonance appears as a sharp singlet. Wehrli has shown that only strongly chelated phenolic protons normally couple to the phenolic carbon atom. The resonances at 134.0 and 140.5 p.p.m. can be assigned to C-4' and C-6', respectively, on the basis of calculated chemical shifts (136 and 140 p.p.m., respectively), and these assignments are confirmed in the fully-coupled spectrum where the broad resonance at 134.0 p.p.m. sharpens on irradiation at the frequency of the benzyl C-3' protons; the C-6' resonance appears as a sharp singlet, showing no coupling as expected. The remaining resonances at 99.9 and 101.5 p.p.m. are assigned to C-9' and C-11' (calculated shifts 98 and 112 p.p.m., respectively). No confirmation could be obtained in the fully-coupled spectrum, but in the p.n.d. $^{13}$C n.m.r. spectrum of [1,2-13C]acetate-derived viomellein (see below) a $^{13}$C-$^{13}$C coupling is apparent between C-11' and C-12'. The assignment of the $^{13}$C n.m.r. spectrum of tri-O-methylviomellein (5) follows from comparison with that of di-O-methylxanthomegnin and similar considerations, as above, of the fully-coupled spectrum. The large downfield shifts of the C-9' and C-11' signals are difficult to account for by simple change of substituent effects, but are almost certainly due to changes in hydrogen bonding and steric effects which are difficult to quantify as yet.

On formation of the furanoid ring in di-O-methylrubrosulphin (7), only the C-8-11 and C-8'-11' resonances move significantly relative to those in tri-O-methylviomellein. The quinone carbonyl resonances both move upfield from 182.2 and 180.7 to 176.8 and 172.5 p.p.m., respectively. The C-10 and C-10' resonances also move upfield, to 160.8 and 154.9 p.p.m., respectively, and can be distinguished from the COMe resonances by their lack of coupling in the fully-coupled spectrum, and from each other by the further upfield shift of C-10' in tri-O-methylviopurpurin (9) due to shielding by the C-7' OMe.

The structure of viopurpurin (8), a 7'-hydroxy-derivative of rubrosulphin, was based on the $^{13}$H n.m.r. shift of the 5'-proton. The $^{13}$C n.m.r. spectrum of tri-O-methylviopurpurin (9) is entirely consistent with this. The C-7' and C-8' resonances at 101.9 and 155.1 p.p.m. in di-O-methylrubrosulphin are replaced by resonances due to ortho-methoxy-substituted carbon atoms at 145.1 and 146.2 p.p.m. In addition the C-6', C-10', and C-5' signals move upfield owing to extra shielding by the introduced methoxy-group in an ortho-, para-, or peri-position, respectively. Thus the $^{13}$C n.m.r. data provide strong confirmatory evidence for the structure (8) proposed for viopurpurin.

$^{13}$C Enrichment Studies.—The amounts of [13C]acetate necessary for feedings were found by determining the dilution of $^{14}$C label in experiments with [1-14C]acetate. These conditions were optimised for study of metabolites found in the culture medium, but they also give high enrichment of the mycelial pigments. Accordingly, A. melleus was grown in the presence of sodium [1-13C]- and [1-2,13C]-acetate (0.8 and 0.5 g 1$^{-1}$, respectively) and the enriched xanthomegnin and viomellein were isolated. The enhancements observed in the p.n.d. $^{13}$C n.m.r. spectra of the [1-13C]acetate derived samples are summarised in Table 2. The enrichment of labelled sites is high so that only the enriched resonances are visible, seven for xanthomegnin and fourteen for viomellein, clearly showing the labelling of alternate carbons anticipated for a polyketide origin.

The yields of pigment in the [1,2-13C] acetate experiment were low; only viomellein was isolated in sufficient amounts of [13C]acetate

<table>
<thead>
<tr>
<th>Carbon</th>
<th>Xanthomegnin</th>
<th>Viomellein</th>
<th>$^{13}$C$_{\text{acetate}}$/Hz</th>
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<tbody>
<tr>
<td>1</td>
<td>74.4</td>
<td>74.1</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>74.4</td>
<td>74.1</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>148.0</td>
<td>147.9</td>
<td>41</td>
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<tr>
<td>4</td>
<td>162.7</td>
<td>162.9</td>
<td>64</td>
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<tr>
<td>5</td>
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<td>7</td>
<td>134.8</td>
<td>134.5</td>
<td>60</td>
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<td>8</td>
<td>162.2</td>
<td>162.6</td>
<td>68</td>
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<td>9</td>
<td>130.0</td>
<td>130.0</td>
<td>40</td>
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<td>10</td>
<td>140.6</td>
<td>140.6</td>
<td>50</td>
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<td>11</td>
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<td>155.5</td>
<td>77</td>
</tr>
<tr>
<td>14</td>
<td>171.2</td>
<td>171.2</td>
<td>67</td>
</tr>
</tbody>
</table>

amount for $^{13}$C spectral determination, and owing to the poor signal-to-noise ratio obtained and to overlapping of signals it was not possible to resolve all the $^{13}$C-$^{13}$C couplings. However, the results (Table 2) clearly indicate that viomellein is derived from fourteen intact acetate units, arranged as shown in the Scheme, suggesting formation of viomellein from two units, arising from alternate foldings of a common heptaketide chain, followed by introduction of $C_4$ units and oxidative coupling. Subsequent ring closure and hydroxylation would give rise to rubrosulphin and viopurpurin. The order and timing of these operations is not clear, though in this respect it is noteworthy that viomellein is readily converted into rubrosulphin \textit{in vitro}.

Coupling of two napthoquinone moieties would give rise to xanthomegnin. In the structural assignment of xanthomegnin, and hence of the remaining pigments, the 10-position for the methoxy-group was chosen in accord with the acetate hypothesis, and was not established from chemical evidence. The above spectral and biosynthetic evidence confirms this assignment, as the methoxy-group is shown to be adjacent to the C-11 carbonyl and on a carbon atom derived from a carboxy-group of acetate, as required by the acetate hypothesis.

**EXPERIMENTAL**

The isolation of pigments from cultures of \textit{A. melleus} and \textit{A. sulphureus} and the preparation of all derived compounds were effected as previously described.


$^{13}$C N.m.r. Determinations.—The $^{13}$C n.m.r. spectra were obtained for samples in acid-free deuteriochloroform with tetramethylsilane as internal reference. Proton noise-decoupled spectra, single frequency off-resonance decoupled spectra, and the spectra of $^{13}$C enriched samples were obtained with a Varian XL100-15FT spectrometer operating at 25.197 MHz as previously described; additional p.p.m. spectra and fully proton-coupled spectra were determined with a JEOL JNM FX-60 spectrometer operating at 15.04 MHz. Fully coupled spectra were determined under GATED-1 decoupling conditions, to retain nuclear Overhauser effects, by using a 0.5 kHz noise modulated proton irradiating frequency of 57 dB. For specific decoupling experiments a continuous wave irradiating frequency of 17 dB was used. Trisacetylacetonatocromium ($Cr(acac)_3$; 0.1M) was used as relaxation agent.

Incorporations of Sodium $[1-^{13}$C$]$- and $[1,2-^{13}$C$]$-Acetate.—To each of two culture vessels containing a 7-day growth of \textit{A. melleus} was added 90% $[1-^{13}$C$]$acetate (400 mg) or 90% $[1,2-^{13}$C$]$acetate (250 mg). After a further 4 days growth the mycelium was harvested and the pigments were isolated (viomellein (26 mg) and xanthomegnin (24 mg) from $[1-^{13}$C$]$ acetate and viomellein (20 mg) from $[1,2-^{13}$C$]$acetate feedings, after purification).

Part of this work was carried out in the Department of Organic Chemistry, University of Liverpool, and a grant from the S.R.C. for purchase of the Varian XL-100 spectrometer is acknowledged. I thank Dr. R. D. Lapper and Mr. D. J. Birch for $^{13}$C spectral determinations and Mrs. A. Lewis for microbiological work.

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Biosynthesis of Andibenin, a Novel Polyketide-terpenoid Metabolite of *Aspergillus variecolor*

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**Summary**
Incorporation of singly and doubly labelled [13C]acetates and [Me-13C]methionine into andibenin by cultures of *A. variecolor* indicates its biosynthesis via alkylation of a bis-C-methylated, polyketide-derived aromatic precursor by farnesyl pyrophosphate.

Andibenin, C25H31O6, was recently isolated from static cultures of *Aspergillus variecolor* and its structure (1), determined by X-ray crystallography, suggested a biosynthetic pathway via a polyisoprenoid, possibly sesterterpenoid, precursor. A preliminary report of 13C-incorporation studies which indicate that andibenin is in fact of mixed polyketide-terpenoid origin is now presented.

Although the original yields of andibenin were too low for biosynthetic studies, it was found that an irradiation-induced mutant of *A. variecolor*, produced in connection with previous studies,* gave yields of andibenin of ca. 50 mg/l along with smaller amounts of several closely related compounds, including andilesin. Preliminary 14C-studies indicated that the incorporation efficiency of acetate, though low, was sufficient for satisfactory 13C-enrichments to be obtained. The 13C-n.m.r. spectra of samples of andibenin, derived by incorporation of [1-13C]acetate, [2-13C]acetate, and [1,2-13C2]acetate in cultures of *A. variecolor* showed the enrichments of individual atoms and 13C-couplings summarised in the Table. The assignment of the resonances in the 13C-n.m.r. spectrum followed from standard shift data, off-resonance multiplets, and magnitudes of 13C-13C couplings in the doubly labelled sample. Significantly the 9' and 10' methyl carbons were enriched by acetate and so must originate from the C9-polyester.
This was confirmed by feeding [methyl-13C]methionine, which resulted in high enrichments being observed at these two positions.

The results clearly indicate that andibenin is derived from two precursors, one of which is a sesquiterpene, and the other a tetraketide containing two C-methyl groups derived from methionine. A plausible route, involving the alkylation of the precursor phenol with farnesyl pyrophosphate is shown in the Scheme, an interesting feature being the proposed formal 4 + 2 cycloaddition which gives the correct carbon skeleton. Studies to determine the actual sequence of precursors and reactions are in progress.

Although several triprenyl phenols have been isolated from fungal sources, andibenin is unique in the position of attachment of the sesquiterpene unit and the introduction of two alkyl substituents on the same carbon of the polyketide unit, and in having more than one carbon-carbon bond between the terpenoid and polyketide units. In addition the terpenoid spiro-lactone ring system has only been found in andibenin and in austin, a mycotoxin produced by *Aspergillus ustus*. A sesterterpenoid origin has been suggested for austin; however, a polyketide-terpenoid pathway analogous to that occurring in andibenin would also account for the biosynthesis of austin.

The support of the S.R.C. is gratefully acknowledged.

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Use of Long Range $^1$H–$^{13}$C Couplings in Structure Determination: Stellatin, a Novel Dihydroisocoumarin from *Aspergillus variecolor*

By Thomas J. Simpson

(The Robert Robinson Laboratories, The University, P.O. Box 147, Liverpool L69 3BX)

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By Thomas J. Simpson
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Summary Stellatin, a novel phenolic metabolite of Aspergillus variecolor is shown, by examination of its chemical and spectroscopic properties, in particular by analysis of the fully 'H-coupled 'C n.m.r. spectrum, to be 3,4-dihydro-8-hydroxy-7-hydroxymethyl-6-methoxy-isocoumarin.
w reported, and from examination of its chemical and spectroscopic properties, the dihydroisocoumarin structure is established.

Stellatin, C, H₂O₀, m.p. 126–128 °C, showed λmax (EtOH) 265 and 300 nm (ε 16,800 and 5900); λmax (MeOH-D₂O) 338 nm (ε 6000), [cf. 3,4-dihydro-8-hydroxy-6-thoxy-3-methylisocoumarin, 4 λmax (EtOH) 267 and 2 nm) (ε 14,800 and 6000)]; and νmax 3580, 3200 (br), 1629, and 1588 cm⁻¹. The 'H n.m.r. spectrum exhibited the presence of an ArCH₂CH₂OCO-unit (δ 3-03, both 2H, t, J 7 Hz), an aromatic proton (δ 6.32), aromatic hydroxymethyl (δ 4.76), a methoxy (δ 3.90), and two exchangeable protons (δ 2.38 and 11.50). Treatment of stellatin with acetic anhydride in pyridine gave the diacetate (2), C₁₆H₁₆O₄, m.p. 171–172 °C, which showed νmax 1775, 1722, and 1615 cm⁻¹. The 'H n.m.r. spectrum of (2) confirmed the presence of a phenolic (δ 2.39) and the acetylation of the hydroxymethylop unit (δ 2-03 and 516, 3H and 2H singlets, respectively), and the downfield shift of the aromatic proton, to δ 6.68, suggested the presence of a phenol with a free para-position.' The ortho-relationship between the phenolic hydroxy and lactone carbonyl groups was shown by the presence of the w-field exchangeable proton in the 'H n.m.r. spectrum of (1). The C-5 resonance, 101-2 p.p.m., appeared as a doublet of triplets (J 162 and 3 Hz), due to coupling to 5-H and 4-CH₃, respectively, collapsing to a simple doublet on selective low-power irradiation of 4-CH₃. This irradiation also changed the quintet (J 6 Hz) at 141-1 p.p.m. to a triplet. Irradiation of the 3-CH₃ also caused the quintet to collapse to a triplet so this resonance must be assigned to C-4a showing equal 2- and 3-bond couplings to the lactone ring methylene protons. The C-8 resonance appeared as a quartet (J 4 Hz) at 161-4 p.p.m. owing to coupling to the chelated phenolic proton and 9-CH₃, changed to a triplet on addition of D₂O, and further sharpened to a singlet on irradiation of 9-CH₃. This irradiation also sharpened the multiplet at 114-9 p.p.m. due to C-7 to a doublet (J 4 Hz) the residual coupling being the expected 3-bond coupling to the aromatic proton. Addition of D₂O also caused the multiplet due to C-8a at 102-4 p.p.m. to sharpen, and irradiation of 4-CH₃ sharpened it further to a doublet (J 7 Hz), the residual coupling again being a 3-bond coupling to the aromatic proton. C-6 appeared as a broad unresolved resonance at 163-0 p.p.m. which sharpened on irradiation of either 9-CH₃ or OCH₃. The remaining 1, 3, 4, 9, and OMe carbon atoms, were readily assigned to the resonances at 169-3, 67-5, 28-0, 55-9, and 53-9 p.p.m., respectively. These observations can only be accommodated by structure (1) for stellatin.

Although dihydroisocoumarins are comparatively common fungal metabolites, 7 stellatin is unique in being unsubstituted at both C-3 and C-4. The overall structure is consistent with a polyketide origin. However, if stellatin is a tetraketide, C-3 must be derived by introduction of a methyl group from the C₁-pool on the methyl carbon of the chain-initiating acetate unit. Alternatively, if it is of pentaketide origin, the methyl carbon of the chain-initiating acetate must be lost. Neither of these processes have any firmly established precedent in polyketide biosynthesis.

The support of the S.R.C. is gratefully acknowledged.

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The Structure of Phomazarin, an Aza-anthraquinone produced by Pyreno-
chaeta terrestris Hansen

By Arthur J. Birch, Douglas N. Butler, Reinhardt Effenberger, Rodney W. Rickards, and Thomas J. Simpson,∗† Research School of Chemistry, Australian National University, P.O. Box 4, Canberra, A.C.T. 2600, Australia

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The structure (4) has been established for phomazarin, 6-n-butyl-2-carboxy-3,4,8-trihydroxy-7-
methoxy-1-aza-anthraquinone, from n.m.r. and i.r. spectra, and degradative studies of phomazarin and its derivatives. Nitrogen-15 chemical shifts, and $^{15}N-\text{H}$ and $^{13}N-^{15}C$ coupling constants in derivatives of biosynthetically $^{15}N$-enriched phomazarin are of particular utility in differentiating between possible alternate structures.

In 1940 Kögl and his co-workers isolated two pigments from the mycelium of Phoma lerreslyis Hansen, the fungus responsible for 'pinkroot disease' of onions and since re-named Pyrenochaeta terrestris Hansen. One of these metabolites was shown to be the anthraquinone cyondontin (1), and the other, phomazarin, an orange pigment, $C_{19}H_{17}NO_8$, was suggested by Kögl to have the unique aza-anthraquinone structure (2), in which the orientation of the heterocyclic ring was not established. The redox properties and colour reactions displayed by phomazarin indicated the presence of a quinonoid system, necessarily a para-quinone on account of the extreme stability of phomazarin towards base. The presence of an n-butyl group was deduced from the isolation of n-butyric and valeric acids when phomazarin was oxidised with hydrogen peroxide in sulphuric acid, and larger fragments were obtained by degradation of the triacetate with chromic acid. The main product was thought to be 3-n-butyl-6-hydroxy-4-methoxyphthalic acid which established the benzenoid ring-substitution pattern in (2). The nitrogen function appeared to be tertiary but it could not be quaternised and since one derivative, the methyl ester triacetate, showed basic properties, it was concluded that the nitrogen atom must be in an aromatic ring. The structure of the hetero-
aromatic ring was based on mild treatment of tri-O-
methylphomazarin methyl ester, $C_{22}H_{25}NO_9$, with ethanolic alkali to give 'dimethylphomazarin hydrate', $C_{21}H_{23}NO_9$, which on melting lost the elements of water and carbon dioxide to afford di-O-methyldecarboxy-
phomazarin, $C_{19}H_{21}NO_6$, identical with material obtained (together with the corresponding tri-O-methyl derivative) by methylation of decarboxyphomazarin. To account for this behaviour, Kögl assigned to 'dimethylphomazarin hydrate' the ring-opened malonic acid structure (3) which would decarboxylate and recyclise on heating, and upon this based his formulation of phomazarin as a 3-carboxy-2,4-dihydroxy-1-aza-anthraquinone. We now report studies revising the substitution pattern of both the benzenoid and heterocyclic rings and leading to structure (4) for phomazarin.

RESULTS AND DISCUSSION

Initial work was hampered by the failure of the fungus to produce consistent yields of phomazarin. Previous attempts to culture P. terrestris as described by Kögl gave either low yields of phomazarin or the production of cyanodontin only. However, modifications of the medium and method of culture in these laboratories have produced, after incubation at 25 °C in shake culture, a good growth of thick filterable mycelium from which phomazarin can be isolated by chloroform extraction after treatment of the dried mycelium with acid, a procedure which avoids Kögl's cumbersome method of refluxing pyridine in vacuo.

Treatment of phomazarin (4) with silver oxide and methyl iodide in chloroform, either under reflux (2 h), or overnight (room temperature) gave a quantitative yield of tri-O-methylphomazarin methyl ester (8). Shorter reaction times at room temperature gave mixtures of the partial methylation products (5), (6), and (7) which were readily isolated by preparative-layer chromatography. The i.r. spectrum of phomazarin methyl ester

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(5) showed no carbonyl absorption above 1685 cm$^{-1}$ (Table 1), whereas its mono-O-methyl derivative (6) showed an ester carbonyl at 1748 cm$^{-1}$, indicating the presence of a hydroxy adjacent to the carboxy group in phomazarin (4). Mild hydrolysis of the di-O-methylphomazarin methyl ester (7) cleaves the ester and the labile pyridinoid methoxy, re-esterification then affording the mono-O-methylphomazarin methyl ester (6). This establishes the relationship of (7) to (6), and in particular the presence of the free 8-hydroxy group in both compounds. Treatment of (8) to give 'dimethylphomazarin hydrate' as described by Kögl, and recrystallisation from non-aqueous solvents gave material, m.p. 123°C, analysing for C$_{21}$H$_{21}$NO$_{8}$. Thus Kögl's material, m.p. 116°C from aqueous methanol, is probably a hydride of di-O-methylphomazarin (9) arising by hydrolysis of the ester and labile pyridinoid methoxy groups. Consequently, his evidence for the structure of the heterocyclic ring of phomazarin is invalid. Esterification of (9) with methanolic hydrochloric acid gave the methyl ester (10) as expected. Similarly, mild hydrolysis of di-O-methylphomazarin methyl ester (7), followed by re-esterification, gave the mono-O-methylphomazarin methyl ester (6), also obtained as a minor product from direct methylation of phomazarin.

Hydrolysis and decarboxylation of tri-O-methylphomazarin methyl ester (8) with hot sulphuric acid gave directly di-O-methyldecarboxyphomazarin (13). Hydrogenation and decarboxylation of tri-O-methylphomazarin methyl ester (8) with hydrogenolysis mixture. Compound (17) failed to react with ortho-phenylenediamine, even under forcing conditions, confirming the presence of an anthraquinone rather than a phenantherquinone structure in phomazarin.

In contrast to the i.r. spectrum of (17) in which both quinonoid carbonyls absorb at 1673 cm$^{-1}$, the spectrum of the tetrahydro-compound (18) showed separate carbonyl bands at 1667 and 1614 cm$^{-1}$. This lowering of a quinone carbonyl absorption frequency can only be due to the presence of a vinylogous amide system,

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>ν(CO) bonded</th>
<th>ν(CO) non-bonded</th>
<th>ν(NH)</th>
<th>ν(OH)</th>
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<tr>
<td>(4)</td>
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<td>1670</td>
<td>1694</td>
<td>1637</td>
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<td>(5)</td>
<td>1637</td>
<td>1675</td>
<td>1685</td>
<td>1637</td>
</tr>
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<td>(6)</td>
<td>1637</td>
<td>1675</td>
<td>1641</td>
<td>1670</td>
</tr>
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<td>(7)</td>
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<td>1675</td>
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<td>1670</td>
</tr>
<tr>
<td>(8)</td>
<td>1640</td>
<td>1675</td>
<td>1670</td>
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</tr>
<tr>
<td>(9)</td>
<td>1640</td>
<td>1675</td>
<td>1670</td>
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<td>(10)</td>
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<td>1675</td>
<td>1670</td>
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</tr>
<tr>
<td>(11)</td>
<td>1630</td>
<td>1675</td>
<td>1670</td>
<td>1670</td>
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<tr>
<td>(12)</td>
<td>1640</td>
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<td>(13)</td>
<td>1640</td>
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<td>(14)</td>
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<td>(15)</td>
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<tr>
<td>(21)</td>
<td>1640</td>
<td>1670</td>
<td>1670</td>
<td>3529</td>
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</tbody>
</table>

* ν(CO) vinylogous amide. Includes vinylogous amide.
so locates the nitrogen function adjacent to the quinonoid ring. In confirmation of the vinlylogous amide system, the tetrahydro-compound (18) is non-basic. That the remaining methoxy group in the heteroaromatic ring of (17) is meta to the ring-nitrogen was suggested by its resistance to acid hydrolysis and the failure of (17) to rearrange to an N-methylpyridone on heating with methyl iodide in a sealed tube. Thus the original hydroxy and carboxy substituents which have been removed during the formation of (17) must occupy the 2- and 4-positions of phomazarin itself. Their relative disposition is indicated by 1H n.m.r. spectroscopy. The 1H n.m.r. spectrum of tri-O-methyl-decarboxyphomazarin (14) contains inter alia an aromatic proton singlet at \( \tau \) 1.33 (Table 2), corresponding in chemical shift to a proton adjacent to a heteroaromatic nitrogen. In 15N-enriched (13), see below, this proton is coupled to the ring nitrogen, \( J(15N-'H) \) 10 Hz. In [15N]pyridine and with such acids, substituted adjacent to both carboxys with groups other than hydroxy, is that the anhydride is formed spontaneously in contrast to Kögl's reported isolation of (21), which only formed the anhydride, m.p. 170 °C, on vacuum sublimation.

**Table 2**

| Hydrogen-1 chemical shifts (\( \tau \)) and multiplicities (J/Hz) in the 100 MHz n.m.r. spectra \( ^a \) of phomazarin and derivatives |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Compound        | 2-H             | 3-H             | 4-H             | 5-H             | 6-H             |
| (20) \( R_1 = R_2 = CO_2H \), \( R_3 = H \) | \( \tau \) 1.34  | 5.85  | 5.85  | 5.85  | 5.85  |
| (21) \( R_1 = R_2 = CO_2H \), \( R_3 = Me \) | 1.34  | 2.30  | 5.85  | 5.85  | 5.85  |
| (22) \( R_1 = R_3 = H \), \( R_2 = CO_2H \) | 1.34  | 2.30  | 5.85  | 5.85  | 5.85  |
| (23) \( R_1 = CO_2H \), \( R_2 = R_3 = H \) | 1.34  | 2.30  | 5.85  | 5.85  | 5.85  |
| (24) \( R_1 = R_2 = R_3 = H \) | 1.34  | 2.30  | 5.85  | 5.85  | 5.85  |

For CDCl \(_3\) solutions except where stated otherwise. \( ^a \) Singlet. \( ^b \) Assignments may be interchanged. \( ^c \) Exchangeable with D\(_2\)O. \( ^d \) Triplet (J 7–8 Hz). \( ^e \) Broad multiplet. \( ^f \) Triplet (J 7 Hz). \( ^g \) For CF\(_3\)CO\(_2\)H solution. \( ^h \) Doublet (J 3 Hz).

15N]quinoline,\(^\text{10}\) the 2-proton shows coupling of 10.9 and 11.8 Hz, respectively, to the ring 15N atom. In the 1H n.m.r. spectrum of the deoxydecarboxy-compound (17), the proton at \( \tau \) 1.33 is meta-coupled (J 3 Hz) to the new proton signal at \( \tau \) 2.12. Thus phomazarin is a 2-carboxy-3,4-dihydroxy-1-aza-anthraquinone. In agreement, the tetrahydro-compound (18) shows resonances at \( \tau \) 6.2 (CHO\(_2\)Me), 6.56 (CHO\(_2\)H and NH\(_2\)H), and 7.3 (C-CHO) (Table 2).

The substitution pattern of the benzene ring in Kögl's formulation (2) followed from his conclusion that the phthalic acid isolated from degradation of phomazarin had structure (20). This structure was based on decarboxylation to a benzoic acid (22) and a phenol (24), which were compared with synthetic samples. However, the initial product of decarboxylation should have been the isomeric acid (23) due to preferential loss of the pseudo-\( \beta \)-ketoadic. Moreover, the phthalic acid (20) could not be crystallised but was converted to a crystalline methyl ether (21), m.p. 174 °C. The isolation of this methoxy-phthalic acid is unexpected as experience this conflict has been resolved by an unambiguous synthesis of the phthalic acids (20) and (21) via Diels–Alder reaction of dimethyl acetylenedicarboxylate with 1,5-di-n-butyl-2,4-dimethoxy-2,3,4,5-tetrahydro-1,3-diene, which was prepared by base-catalysed conjugation of the corresponding 1,4-diene obtained by Birch reduction of 1,5-di-n-butyl-2,4-dimethoxybenzene. The di-n-butyl functionality was required since base-catalysed conjugation of 1-n-butyl-2,4-dimethoxy-2,3,4,5-tetrahydro-1,3-diene led to the 1,3-diene with an allylic rather than vinylic butyl group. This diene then formed a Diels–Alder adduct which underwent an Alder–Rickert reaction on pyrolysis with the loss of hex-1-ene to give simply dimethyl 3,5-dimethoxypthalate. The di-n-butyl functionality overcame this difficulty. The adduct from this diene also lost hex-1-ene on pyrolysis but a butyl group remained on the resultant phthalate ester, saponification of which gave a mixture of acid (21) and the corresponding anhydride, from which no free acid could be isolated. On complete conversion, in refluxing acetic anhydride, the anhydride melted at 134 °C.
clearly differing from Kögl's anhydride and so phomazarin cannot have the benzenoid substitution pattern depicted in structure (2). Selective demethylation \(^1\) of the anhydride followed by hydrolysis gave the hydroxy-acid (20) which was crystalline, m.p. 156 °C, as expected.

The correct 6-n-butyl-8-hydroxy-7-methoxyzaanthraquinone structure was suggested by the \(^1\)H n.m.r. and i.r. spectra of phomazarin derivatives. In contrast to 1,3-dimethoxyanthraquinones \(^4\) where the 2-proton resonates at ca. + 3.5, in the \(^1\)H n.m.r. spectrum of tri-O-methylphomazarin methyl ester (8), the benzenoid proton resonates at a low value of \(\tau = 2.12\), a value clearly inconsistent with structure (2). However, in 2-methyl-3,4,5,7-tetramethoxy- and 3-hexyl-2,4,5,7-tetramethoxyantraquinones \(^5,6\) the 1-protons peri to a carbonyl resonate at \(\tau = 2.13\) and \(\tau = 2.47\), respectively, indicating that the aromatic proton in phomazarin occupies a peri position. Further, irradiation of the benzyl methylene triplet at \(\tau = 7.25\) in (8) caused a nuclear Overhauser enhancement of 26\% in the intensity of the aromatic proton signal, showing that the butyl group is ortho to the aromatic proton.\(^7\) Thus the methoxy and hydroxy substituents must occupy the remaining two benzenoid positions. That the hydroxyl occupies the remaining peri-position was proved by heating the di-O-methylphomazarin methyl ester (7) with hot sulphuric acid to give mono-O-methyldicarboxyphomazarin (12), which with POCl\(_3\) gave the chloro-compound (15). The corresponding compound (16) obtained from tri-O-methylphomazarin methyl ester (8), see above, has only one carbonyl absorption band at \(\nu_{\text{max}} = 1675\) cm\(^{-1}\), despite the presence of two quinone carbonyls. In contrast compound (15) has absorptions at 1640 and 1670 cm\(^{-1}\) indicating one chelated and one free carbonyl.

The former is supported in the \(^1\)H n.m.r. spectrum of (15) by the presence of a low-field exchangeable proton at \(\tau = -2.7\), which can only be due to a peri-hydroxy on the benzenoid ring since the heterocyclic peri-hydroxy has been replaced. Confirmation of this substitution pattern has been obtained by repetition of Kögl's chromic acid degradation of phomazarin triacetate. The initially obtained acetoxy-n-butylmethoxycphenylacetic acid (25) was converted by hydrolysis and removal of the carboxy ortho to the hydroxy to give the benzoic acid (26), which was methylated to give methyl 5-n-butylveratrurate (28), identical with a synthetic sample prepared as shown in the Scheme. The bromo-aromatic elaboration of the aldehyde to an n-butyl group by standard procedures. Attempts to carboxylate the Grignard reagent from (32) resulted in low yields due to solubility problems. However, the lithium derivative, formed from (32) with n-butyl-lithium, reacted smoothly with carbon dioxide to produce the acid (27) in good yield. The acid was readily methylated with ethereal diazomethane to the ester (28).

The remaining ambiguities in the structure of phomazarin were (a) the tautomeric form of the heterocyclic ring and (b) the relative orientation of the unsymmetrical benzenoid and heterocyclic rings. 2- and 4-hydroxy-pyridines and quinolines normally exist as the oxo-tautomers. However, \(^1\)H n.m.r. studies of phomazarin derivatives, discussed in the following paper, suggest that the unexpected hydroxy-tautomer predominates. Many methods have been used to investigate tautomerism in heterocyclic compounds, including \(\text{^1}H\) n.m.r. spectroscopy, \(\text{^13}C\) n.m.r. spectroscopy, X-ray crystallography, and dipole-moment studies.\(^8\) None of which are readily applicable in this case. Nuclear chemical shifts have been used to distinguish the tautomeric forms of hydroxy- and amino-pyridines and quinolines, being obtained either by direct measurements\(^9\) or by double irradiation (INDOR)\(^10\) techniques. Nitrogen chemical shifts in normal heteroaromatics range from about 60 to 100 p.p.m., e.g. 85 and 90 p.p.m. in 3-hydroxy- and 4-methoxy-pyridines respectively; whereas the amide nitrogen shift is ca. 200 p.p.m., e.g. 209 and 201 p.p.m. in 2- and 4-pyridone respectively.\(^11\) In di-O-methyldecarboxyphomazarin (13) the 2-proton appears to be broadened by coupling to the \(^15\)N atom. However an INDOR experiment failed to detect the corresponding \(^15\)N resonance frequency, presumably because it is very broad due to a large \(^15\)N quadrupole relaxation rate. With \(^1^5\)N, spin \(\hat{\gamma}\), this problem is removed, and in addition \(^1^5\)N-\(^1\)H coupling has been used to measure tautomer equilibria.\(^12\) Thus \(^15\)N-enriched phomazarin was prepared by addition of Na\(^{15}\)NO\(_3\), as the main nitrogen source, to cultures of \(P. terrestris\). The \(^1\)H n.m.r. spectrum of the derived \[^{15}\text{N}\,\text{di-O-methylphomazarin methyl ester}\) (10), has a low-field proton singlet, \(\tau = -3.23\), which shows no evidence of \(^15\)N-\(^1\)H coupling (typically 90 Hz) indicating that it is a pyridol-OH rather than a pyridone-NH, though the possibility that the lack of coupling is due to

\[
\begin{align*}
(25) & R^1 = H, R^2 = CO_2H, R^3 = COMe \\
(26) & R^1 = R^2 = R^3 = H \\
(27) & R^1 = R^2 = H, R^3 = Me \\
(28) & R^1 = R^2 = Me, R^3 = H
\end{align*}
\]
exchange processes could not be excluded. However, on further conversion to [15N]di-O-methyldecarboxyphomazarin (13), the 2-proton exhibits a two-bond 15N-'H coupling of 10 Hz, consistent with the value observed in aromatic heterocycles. In confirmation, the INDO method allows the 15N chemical shift of 79 p.p.m. to be determined. This value is clearly consistent only with an aromatic ring, and so established the predominance of the 4-hydroxy-tautomer in phomazarin. Recent studies on the protomeric equilibria of simple hydroxypyridines and related compounds show that, in contrast to observations in solution, the hydroxy tautomer is often the more stable form in the gas phase.

In solution the main factors influencing the equilibrium are the polarity of the solvent, the presence of strongly electron-withdrawing substituents to the nitrogen atom, which tend to shift the equilibrium to favour the pyridinium form by altering the basicity of the nitrogen atom, and the presence of substitutens which stabilise one or other of the tautomers by hydrogen bonding. The predominant influence in stabilising the hydroxy form in phomazarin derivatives would appear to be hydrogen bonding to the quinone carbonyl, as this form predominates even after removal of the electron-withdrawing 2-methoxycarbonyl group.

Incorporation studies with 11C acetate suggest that phomazarin is polyketide in origin, with the carboxy group originating from the methyl carbon of acetate.

Of the possible orientations of phomazarin, (4) and (33), only (4) would be consistent with formation of phomazarin via the normal processes of polyketide biosynthesis. In the 13C n.m.r. spectrum of tri-O-methylphomazarin methyl ester (8), the quinone carbonyls appear at lowest field at 178.9 and 181.3 p.p.m. In the fully 1H-coupled 13C n.m.r. spectrum of (8), the quinone carbonyl resonance at 181.3 p.p.m. appears as a doublet (J 4 Hz) which can only be due to a 3-bond coupling to the 5-proton. In the proton-noise-decoupled spectrum of 15N-enriched (8), the other quinone carbonyl resonance at 178.9 p.p.m. appears as a doublet (J 8 Hz) which must be due to a 2-bond coupling to 15N. A similar coupling is observed on the methoxycarbonyl resonance at 164.4 p.p.m. These couplings are discussed further in the following paper. The occurrence of the 1H and 15N couplings on the alternate quinone carbonyl resonances proves the orientation shown in structure (4) is the correct orientation in phomazarin. Reversing the heterocyclic ring, as in (33), would require that both the 1H and 15N couplings appear on the same quinone carbonyl resonance.

The i.r. spectra of phomazarin and its derivatives are summarised in Table 1. Previous attempts to interpret the carbonyl region were hampered by uncertainties due to pyridol-pyridone tautomerism, and the differing hydrogen-bonding possibilities offered by the different possible structures. However, with one exception, all the spectra are readily accounted for. In all compounds with 4-hydroxy and/or 8-hydroxy substituents the peri-carbonyl absorption is lowered by chelation, as is the ester carbonyl in phomazarin methyl ester (5). The corresponding hydroxys appear as a very broad band in the 3 300–3 000 cm⁻¹ region. In the 1H n.m.r. spectra of these compounds (Table 2) the bonded hydroxys can be seen as sharp singlets at very low field, ca. 3, and the effect of chelation on the carbonyl resonance in the 13C n.m.r. spectrum is discussed in the following paper. The lowering of the C-10 carbonyl absorption in the tetrahydro-compound (18) is in agreement with the i.r. spectrum of 2-amino-1,4-naphthoquinone which shows νmax 1 686 and 1 640 cm⁻¹. On demethylation of (18) to the 7,8-dihydroxy-derivative (19), chelation to the 8-hydroxy lowers the absorption of the other carbonyl to 1 636 cm⁻¹. Both (18) and (19) show strong N-H stretching bands at 3 405 cm⁻¹; and (19) also shows a strong O-H stretch at 3 525 cm⁻¹, and an exchangeable proton singlet at 4.50 due to the 7-hydroxy. Both these features due to a 7-hydroxy are also apparent in the spectra of the demethyl-derivative (34).

Decarboxyphomazarin (11) unexpectedly shows two bands at 1 665 and 1 630 cm⁻¹ and so would appear to
possess a quinone carbonyl not involved in either mesomerism with the nitrogen, or hydrogen-bonding to a peri-hydroxy, and this led to the wrong orientation being assigned to phomazarin in a previous study. However, 3-0-methyldecarboxyphomazarin (12), shows no carbonyl absorption above 1632 cm⁻¹. Decarboxyphomazarin is an extremely insoluble compound, so its anomalous i.r. behaviour may be due to some form of strong inter-molecular association.

Phomazarin is the only naturally occurring 1-azanaphthoquinone known. Bostrycoidin (35), is a 2-azanaphthoquinone, which co-occurs in Fusarium solani with fusarubin (36), and it seems likely that bostrycoidin is formed in vivo by condensation of the corresponding aldehyde with ammonia. Streptonigrin (37) is a complex 5-azanaphthoquinone isolated from Streptomyces flocculus.

**EXPERIMENTAL**

Melting points were taken with a Kofler hot-stage microscope. Unless otherwise stated i.r. spectra were measured for solutions in chloroform, u.v. spectra in methanol, and "H n.m.r. spectra at 100 MHz in deuteriochloroform with SiMe₄ as internal reference. Mass spectra were recorded at 70 eV with an A.E.I. MS9 high-resolution spectrometer. Light petroleum refers to the fraction boiling point 60–80 °C. Silica gel GF (Merck) was used for preparative layer chromatography (p.l.c.).

Cultivation of P. terrestris and Isolation of Metabolites.—The medium, consisting of starch (50 g), sodium nitrate (2 g), potassium dihydrogenphosphate (1 g), magnesium sulphate heptahydrate (0.5 g), calcium chloride (0.5 g), and ferrous sulphate heptahydrate (0.01 g), was made up to 11 1 with distilled water, divided amongst 10 250-ml conical flasks and sterilised at 120 °C for 20 min. Each flask was then inoculated with a mycelial suspension of Pyrenochaeta terrestris Hansen (CBS 37752) in distilled water and incubated on a rotary shaker for 5 d at 25 °C. This 5-d culture was used to inoculate 42 flasks of the above medium (10 ml of shake culture mycelial suspension per flask) and these were incubated at 25 °C for 1 month on the rotary shaker. The deep purple mycelium was then filtered off and dried. The dried mycelium (90 g) was finely powdered and extracted with ether, and the solvent removed to give an orange solid. Recrystallisation from methanol gave tri-O-methylphomazarin methyl ester (8), m.p. 136–138 °C. The lower band gave an orange solid (60 mg) which recrystallised from methanol to give tri-O-methylphomazarin methyl ester (8), m.p. 136–138 °C. The remaining mycelium was then filtered off and phomazarin isolated as before. Mass spectra showed the phomazarin to contain mainly [¹³C]phomazarin methyl ester, M⁺ 402 (100%).

Purdie Methylation of Phomazarin.—(a) Silver oxide (750 mg) and methyl iodide (2 ml) were added to a solution of phomazarin (120 mg) in chloroform (20 ml) and the reaction mixture was stirred at room temperature for 24 h. The solid residues were then filtered off, and the solvent removed to give an orange solid. Recrystallisation from methanol gave tri-0-methylphomazarin methyl ester (8) as orange rods, m.p. 136–138 °C (lit., 136–138 °C; "H n.m.r. spectrum showed the phomazarin to contain mainly [¹³C]phomazarin methyl ester, M⁺ 402 (100%).

Preparation of Phomazarin Hydrate.—This
A solution of sodium methoxide in methanol (3.0 ml, 0.4N) was heated at reflux for 2.5 h with di-O-methyldeoxy-
carboxyphomazarin (16) (10 mg). The solution was 
evaporated to dryness, taken up in benzene and washed 
with water. The dried benzene solution was then chro-
matographed on Florisil, the yellow chloroform eluate 
evaporated to dryness, and the residue recrystallised to 
afford tri-O-methyldioxyphomazarin (17) (mg), m.p. 
159—160 °C (lit., m.p. 160°C); \( \lambda_{\text{max}} \) 256 and 355 nm (log \( \varepsilon \) 4.38 
and 3.74 respectively).

**Catalytic Hydrogenation of Di-O-methylchlorodeoxy-
carboxyphomazarin (16).—Di-O-methylchlorodeoxy-
carboxyphomazarin (77 mg) was dissolved in warm methanol (10 
ml) and added to a pre-hydrogenated palladium—charcoal 
catalyst (5%, 70 mg) in methanol (25 ml) containing triethyl-
amine (0.3 ml). This mixture was hydrogenated at room 
temperature until 2 mol of hydrogen were absorbed, the 
colour changing from yellow to green. The catalyst was 
then filtered off and air passed through the filtrate for 30 
min. After evaporation of the solvent in vacuo, the residue 
was dissolved in benzene and chromatographed on Florisil. 
A trace of a red product was eluted with 20% chloroform in 
benzene followed by a yellow product eluted with 20% 
 methanol in chloroform. This was recrystallised from 
 methanol to give di-O-methyldeoxyphomazarin (17) 
(43 mg), m.p. 138—139 °C; \( \lambda_{\text{max}} \) 236, 295, and 355 nm 
(log \( \varepsilon \) 4.31, 4.30, and 3.65 respectively) (Found: C, 67.59; 
H, 5.71; N, 4.16. \( \text{C}_{20} \text{H}_{25} \text{N}_{17} \text{O}_{5} \) requires C, 66.83; H, 
7.01; N, 3.90; OMe, 25.9%). A repeat hydrogenation allowing 4 mol 
of hydrogen to be absorbed and working-up as above afforded 
di-O-methyltetrahydrodeoxyphomazarin (18), 
recrystallised from light petroleum (b.p. 30—40 °C), m.p. 
92—93 °C; \( \lambda_{\text{max}} \) 236, 291, 361, and 480 nm (log \( \varepsilon \) 4.23, 4.47, 
3.67, and 3.48 respectively) (Found: C, 66.68; H, 7.30; 
N, 4.54. OMe, 25.6. \( \text{C}_{20} \text{H}_{25} \text{N}_{17} \text{O}_{5} \) requires C, 66.83; H, 
7.01; N, 3.90; OMe, 25.9%). \( \text{m/e} \) 359 (100%), 335 (16), 
328 (93), 314 (10), and 229 (5).

**Demethylation of the Tetrahydro-compound (18).—The 
tetrahydro-compound (18) (32 mg) was dissolved in dry 
 methylene chloride (10 ml) and boron trichloride (0.5 ml) 
was added. After stirring for 45 min at room temperature, 
the reaction mixture was diluted with chloroform (40 ml) 
and washed with water (2 x 30 ml). Removal of solvent 
gave a dark gum which was purified by p.l.c. Elution with 
chloroform—methanol (96: 4), removal of the band at \( R_f 
0.44, 
and recrystallisation, gave the demethylated product 
(19) as purple crystals, m.p. 158—159 °C (from acetone— 
light petroleum); \( \lambda_{\text{max}} \) 277, 338, and 432 nm (log \( \varepsilon \) 4.32, 
3.74, and 3.50 respectively) (Found: \( M^n \), 331.141 9. 
\( \text{C}_{20} \text{H}_{25} \text{N}_{17} \text{O}_{5} \) requires M, 331.142 0); \( \text{m/e} \) 331 (91%), 316 (8), 
300 (100), 298 (21), and 286 (15).

**Demethylation of Di-O-methylchlorodeoxyphomazarin. —The 
chloro-compound (10) (20 mg) was dissolved in 
 methylene chloride and cooled to —10 °C. Boron 
trichloride in slight excess was added and the mixture was set 
aside for 5 h. Destruction of excess of reagent with water, 
followed by isolation of the product by chloroform 
 extraction, gave a red solid (15 mg) which crystallised from 
 methanol, to give the demethylated product (34), m.p. 
211—212°C.

**Preparation of Decarboxyphomazarin. —Phomazarin was 
decarboxylated as described by Kögl by subliming at 
220—230 °C under high vacuum. Recrystallisation from 
 methanol gave decarboxyphomazarin (11), m.p. 253—255 °C 
(lit., m.p. 254 °C); \( \lambda_{\text{max}} \) 229, 270, and 435 nm (log \( \varepsilon \) 4.34, 
respectively) (Found: C, 60.82; H, 5.16; N, 3.70; OMe, 
21.76. \( \text{C}_{20} \text{H}_{25} \text{N}_{17} \text{O}_{5} \) requires C, 60.70; H, 5.10; N, 3.37; 
O=C, 21.0%). Esterification of (9) by refluxing in 10% dry 
hydrogen chloride in methanol for 30 min gave di-O-methy-
lphomazarin methyl ester (10), as orange needles, m.p. 122— 
123 °C (with decarboxylation), recrystallises at 123 °C and re-
melts at 180.5 °C; \( \lambda_{\text{max}} \) 272 and 370 nm (log \( \varepsilon \) 4.53 and 
3.90 respectively) (Found: C, 60.82; H, 5.16; N, 3.70; OMe, 
21.76. \( \text{C}_{20} \text{H}_{25} \text{N}_{17} \text{O}_{5} \) requires C, 60.70; H, 5.10; N, 3.37; 
O=C, 21.0%). Esterification of (9) by refluxing in 10% dry 
hydrogen chloride in methanol for 30 min gave di-O-methy-
lphomazarin methyl ester (10), as orange needles, m.p. 122— 
123 °C (with decarboxylation), recrystallises at 123 °C and re-
melts at 180.5 °C; \( \lambda_{\text{max}} \) 272 and 370 nm (log \( \varepsilon \) 4.53 and 
3.90 respectively) (Found: C, 60.82; H, 5.16; N, 3.70; OMe, 
21.76. \( \text{C}_{20} \text{H}_{25} \text{N}_{17} \text{O}_{5} \) requires C, 60.70; H, 5.10; N, 3.37; 
O=C, 21.0%). Esterification of (9) by refluxing in 10% dry 
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3.90 respectively) (Found: C, 60.82; H, 5.16; N, 3.70; OMe, 
21.76. \( \text{C}_{20} \text{H}_{25} \text{N}_{17} \text{O}_{5} \) requires C, 60.70; H, 5.10; N, 3.37; 
O=C, 21.0%). Esterification of (9) by refluxing in 10% dry 
hydrogen chloride in methanol for 30 min gave di-O-methy-
lphomazarin methyl ester (10), as orange needles, m.p. 122—
Synthesis of 6-n-Butyl-3,4-dimethoxyphthalic Acid (21).—

(a) 2,4-Dimethoxybutyrophenone. Polyphosphoric acid was prepared by equilibrating a mixture of orthophosphoric acid (250 g), and phosphorus pentoxide (250 g) at 100 °C for 1 h. Butyric acid (50 g, 0.57 mol) was added and the mixture was equilibrated for a further 15 min. Resorcinol dimethyl ether (50 g, 0.36 mol) was then added and the difference mixture was reacted for 45 min, when it was quenched with ice and extracted with ether. The solid was washed with 2 M aqueous sodium hydroxide and water, dried, and evaporated to dryness to leave a red oil. Distillation gave a clear, colourless oil (80 g), b.p. 156-158 °C at 0.1 mmHg (44 g); vmax (film) 1 620s, 1 650s, and 1 595m cm-1.

(b) 1-n-Butyl-2,4-dimethoxybenzene. The phenone (above, 65.5 g, 0.32 mol) was treated with lithium aluminium hydride-aluminium chloride. The compound was obtained as a clear oil on distillation, b.p. 81-82 °C at 0.1 mmHg (44 g); vmax (film) 1 620s and 1 650s cm-1; r 3.12 (br d, J 7 Hz, H-6), 3.71 (br, J 3-H), 3.74 (dd, J 7 and 2 Hz, H-5), 6.25 and 6.30 (s, 2 ArOMe), 7.51 (br t, J 7 Hz, ArCH2CH3), 8.56 (m, ArCH2CH3), and 9.09 (br t, J 6 Hz, ArCH3) (Found: C 72.69; H, 9.15%).

(c) 5-n-Butyl-2,4-dimethoxybenzene. This was prepared by condensation of the butylresorcinol dimethyl ether (above, 394 g, 2.22 mol) with butyric acid (39.5 g, 0.57 mol) in polyphosphoric acid (500 g) as previously described. Reaction time was 2.5 h at 100 °C. The phenone was a pale yellow liquid, b.p. 139 °C at 0.05 mmHg (60 g), which solidified on cooling, forming yellow prisms, m.p. 34-35 °C; vmax (film) 1 670s, 1 618s, and 1 585s cm-1; r 3.25 (br, J 6 Hz, 3.70 (s, 3-H), 6.14 and 6.16 (s, 2 ArOMe), 7.22 (t, J 6 Hz, ArCOCH2Et), 7.50 (br t, J 8 Hz, ArCH2CH3), 8.42 (sextet, J 6 Hz, ArCOCH2CH3), 8.53 (m, ArCH2CH3), and 9.05 (br t, J 6 Hz, ArCOCH2Me and ArCH3Me) (Found: C 73.37; H, 7.84. C14H14O requires C 73.69; H, 7.91%).

(d) 1,5-Di-n-butyl-2,4-dimethoxybenzene. The phenone (above, 42.5 g, 0.16 mol) was reduced with lithium aluminium hydride-aluminium chloride reagent as previously described. Distillation of the crude dialkyl aromatic gave a clear oil (56 g), b.p. 102 °C at 0.05 mmHg; vmax (film) 1 618s and 1 590s cm-1; r 3.28 (s, 3-H), 3.74 (s, 3-H), 6.26 (s, 2 ArOMe), 7.53 (br t, J 7 Hz, 2 ArCH2CH3), 8.56 (m, 2 ArCH2CH3), and 9.08 (br t, J 6 Hz, 2 ArCH3) (Found: C 72.69; H, 9.15%).

(e) 1,5-Di-n-butyl-2,4-dimethoxycholehaxa-1,3-diene. The dimethoxydibutylbenzene (above, 12 g) was distilled in a mixture of tetrahydrofuran (40 ml) and t-butyl alcohol (30 ml). Ammonia (150 ml) was distilled off sodium and ferric nitrate into the reaction mixture. Lithium (2.8 g) was slowly added and the reaction was allowed to proceed for 3 h. Excess of lithium was destroyed by the addition of ethanol, and the reaction mixture was then diluted with water and extracted with light petroleum. The extracts were washed with water, dried (sodium sulphate), and evaporated to dryness to leave a pale yellow oil (11.5 g). This oil, without further purification, was added to a solution of potassium t-butoxide in dry DMSO (7 g, 40 ml) and maintained under a dry nitrogen atmosphere at 25 °C for 3 h. The reaction was then quenched with water and extracted with light petroleum. The extracts were washed with water and dried (sodium sulphate). Evaporation left a yellow oil which distilled as a clear, colourless liquid (10.7 g), b.p. 70-75 °C at 0.03 mmHg. This was shown to consist of the required conjugated diene (90%) by 1H n.m.r. and u.v. spectroscopy; rmax 276 nm (calc. 275); r (CCL4) 5.26 cm-1 (olefinic proton).

(f) 6-n-Butyl-3,5-dimethoxyphthalic anhydride. The conjugated diene mixture (above, 10.7 g, 0.04 mol) was treated with dimethyl acetylenedicarboxylate (8 g, 0.06 mol) at room temperature. The reaction temperature rose to 120 °C during 15 min with the formation of a red solution. The mixture was set aside overnight and then heated to 190 °C for 45 min and vacuum-distilled to remove excess of reagent. Crude dimethyl 6-n-butyl-3,5-dimethoxyphthalate distilled as a yellow viscous oil, b.p. 140-155 °C at 0.1 mmHg. Treatment of this material with ethanolic potassium hydroxide (6 g in 60 ml) at reflux for 1 h followed by acidification gave a mixture of 6-n-butyl-3,5-dimethoxyphthalic acid (21) and the anhydride. The mixture was distilled in acetic anhydride (20 ml) and maintained at 100 °C for 1 h. Removal of volatile material by vacuum distillation left a tacky residue which solidified on treating with ether. The solid crystallised from glacial acetic acid as plates, m.p. 153 °C, and was identified as the required anhydride (1.2 g); vmax 1 830s, 1 770s, 1 630s, and 1 596cm-1; r 3.34 (s, 4-H), 5.94 and 6.01 (s, ArOMe), 7.00 (br t, J 7 Hz, ArCH2CH3), 8.65 (m, ArCH2CH3), and 9.05 (br t, J 6 Hz, ArCH3Me); rmax 227, 246, and 346 nm (ε 25 000, 25 200, and 7 900 respectively) (Found: C 63.62; H, 6.30. C17H16O requires C 63.62; H, 6.10%).

6-n-Butyl-3-hydroxy-5-n-butoxyphthalic anhydride. The dimethoxy anhydride (above, 620 mg, 3 mmol) was dissolved in anhydrous dichloromethane (20 ml) and cooled to -10 °C. Boron trichloride was added in slight excess and the mixture was left to warm to room temperature overnight. Destruction of the excess of reagent, followed by extraction of the organic material with ethyl acetate in the usual manner, yielded a pale yellow solid which on washing with ether-light petroleum gave the white hydroxy-anhydride (530 mg). It crystallised from acetonitrile-ether as plates, m.p. 160-162 °C; vmax 2 300s, 1 830s, 1 745s, 1 645m, and 1 620s cm-1; r 3.32 (s, 4-H), 6.06 (s, ArOMe), 7.02 (br t, J 7 Hz, ArCH2CH3), 8.55 (m, ArCH2CH3), and 9.05 (br t, J 6 Hz, ArCH3Me); rmax 227, 246, and 346 nm (ε 25 400, 12 500, and 3 200 respectively)

5-Bromo-ortho-vanillin (31).—5-Bromo-ortho-vanillin was prepared by the method of Brink in 95% yield. The compound crystallised from dilute ethanol, m.p. 128 °C (lit. 128 °C); r 0.16 (s, ArCHO), 2.72 and 2.86 (s, J 2 Hz, ArH), and 6.10 (s, ArOMe).
was added and the mixture was set aside for 12 h and then poured into water. The mixture was extracted with ether, washed with 2M aqueous sodium hydroxide and water, dried, and evaporated to dryness to leave a white solid (1.5 g) which crystallised from dilute ethanol, m.p. 84 °C; \( v_\text{max} \) (film) 3400s and 1575s cm\(^{-1}\); \( r \) 2.95 and 3.12 (d, C\(_2\)H\(_4\)MC\(_\text{2}\)).

And 3.12 (d, C\(_2\)H\(_4\)MC\(_\text{2}\)).

To ortho-veratraldehyde (800 mg) was reacted with the Grignard reagent (in ether) formed from magnesium (2 equiv.) and n-propyl bromide (2 equiv.) in the usual manner to give the alcohol essentially quantitatively; a 14-h reaction time was required; \( v_\text{max} \) (film) 3400s and 1575s cm\(^{-1}\); \( r \) 2.95 and 3.18 (d, C\(_2\)H\(_4\)MC\(_\text{2}\)).

The alcohol was used in the next step without further purification.

1-(n-Butyl-5-bromo-2,3-dimethoxyphenyl)butan-1-ol. — 5-Bromoortho-veratraldehyde (800 mg) was reacted with the Grignard reagent (in ether) formed from magnesium (2 equiv.) and n-propyl bromide (2 equiv.) in the usual manner to give the alcohol essentially quantitatively; a 14-h reaction time was required; \( v_\text{max} \) (film) 3400s and 1575s cm\(^{-1}\); \( r \) 2.95 and 3.18 (d, C\(_2\)H\(_4\)MC\(_\text{2}\)).

The alcohol was used in the next step without further purification.

1-n-Butyl-5-bromo-2,3-dimethoxybenzene (32). — The alcohol was quantitatively dehydrated to the styrene by refluxing in benzene solution containing toluenesulphonic acid hydrate (0.2 equiv.); Water was removed by a Dean-Stark trap over a reaction time of 6 h; \( v_\text{max} \) (film) 1645w, 1586w, 1505s, and 970s cm\(^{-1}\); \( r \) 2.85 and 3.14 (coalesced AB quartet, C\(_2\)H\(_4\)MC\(_\text{2}\)).

The styrene was hydrogenated at 15 °C and 1 atm in ethanol using platinum oxide catalyst (0.05 equiv.) to yield the alkylbenzene quantitatively; \( v_\text{max} \) (film) 1585s and 1570s cm\(^{-1}\); \( r \) 5.14 (coalesced AB quartet, ArCH\(_2\)).

The alcohol was used in the next step without further purification.

Methyl 5-n-butylveratrate (28). — The bromo-aromatic (31) (1.09 g, 4 mmol) was dissolved in dry ether and cooled to 0 °C. n-Bulut-lithium (2.25M in hexane, 4.2 mmol) was added and the mixture was stirred at 0 °C for 45 min. Excess of solid carbon dioxide was added and the mixture was set aside until it had returned to room temperature. Dilute hydrochloric acid was added and the substituted benzoic acid was extracted with ether. The extract was separated into neutral and acid fractions and thus yielded the crude substituted benzoic acid (27) as a red gum (660 mg). The gum was sublimed at 120 °C at 0.2 mmHg as a white solid, m.p. 77 °C; \( r \) 2.39 and 2.49 (d, J 6 Hz, ArH\(_2\)), 6.24 (s, 2 ArOMe), 7.64 (br s, ArCH(OH)Pr), 8.05 (m, ArCH\(_2\)H\(_4\)Me), and 9.01 (br t, J 7 Hz, ArCH\(_3\)H\(_4\)Me).

Methyl 5-n-butylveratrate from Phomazarin. — Phomazarin (260 mg) was converted to the triacetate (150 mg) as described by Kög. The acetate was a yellow powder of indefinite m.p. Oxidation of this material by chromic acid as described gave an orange-red gum (20 mg) after hydrolysis of the acetoxy group. This gum was dissolved in hot water (10 ml), filtered, and the resulting clear solution was acidified to pH 2 with concentrated sulphuric acid (1 drop).
Studies in Relation to Biosynthesis. Part 48.\textsuperscript{1} Phomazarin. Part 2.\textsuperscript{1} 13C N.m.r. Spectra and Biosynthesis of Phomazarin

By Arthur J. Birch and Thomas J. Simpson,\textsuperscript{•} Research School of Chemistry, Australian National University, P.O. Box 4, Canberra, A.C.T. 2600, Australia

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Studies in Relation to Biosynthesis. Part 48. Phomazarin. Part 2. 13C N.m.r. Spectra and Biosynthesis of Phomazarin

By Arthur J. Birch and Thomas J. Simpson, Research School of Chemistry, Australian National University, P.O. Box 4, Canberra, A.C.T. 2600, Australia

The 13C n.m.r. spectra of phomazarin methyl ester and seven further derivatives of phomazarin have been assigned. Incorporations of [13C]acetates and [13C]- and [14C]-malonates by cultures of Pyrenochaeta terrestris indicate that phomazarin is biosynthesised by condensation, followed by oxidative ring-fission, of a single nonaketide precursor chain.

PHOMAZARIN, an orange pigment isolated from the mycelium of Pyrenochaeta terrestris has been shown to have the unique aza-anthraquinone structure (1). The origin of the branched C18 skeleton of phomazarin is of interest as previous studies have shown that [14C]-formate is incorporated mainly into the 7-O-methyl group only, so the C1-pool does not contribute to the branched structure; and the activities of the acetic acid and carbon dioxide obtained respectively from Kuhn-Roth oxidation and decarboxylation of phomazarin from feeding experiments with [2-14C]acetate are consistent with an entirely polyketide origin for phomazarin from nine acetate molecules. However, the biosynthesis of phomazarin cannot be accounted for by a normal polyketide pathway but must involve a condensation between two, or possibly more, preformed polyketide chains for which different possibilities exist (Scheme 1 indicates some of these), or by cleavage of a carbocyclic intermediate, which can itself be formed via either of the two possible condensations of a single polyketide chain shown in Scheme 1. This latter route would necessarily involve oxidation of a carbon derived from methyl of acetate to a carboxy, so accounting for the origin of the C=II carboxy function. However, it must be noted that oxidation of methyl to higher levels is not an uncommon biosynthetic process.

![Scheme 1 Possible polyketide derivations of phomazarin](image-url)
same acetic acid molecule, cleavage of an originally intact acetate derived unit during the biosynthetic pathway being revealed by a loss of the $^{13}C$-$^{13}C$ coupling. Studies of this type have been particularly useful in distinguishing between different foldings of polyketide chains, and in elucidating pathways involving a ring-intact acetate derived unit during the biosynthetic studies is an ambiguous assignment of the $^{13}C$ n.m.r. spectrum of the metabolite. The $^{13}C$ n.m.r. spectra of phomazarin methyl ester (2) and the further derivatives (3)—(9) have been assigned and are summarised in Table 1. These assignments are based on standard chemical-shift data; single frequency off-resonance decoupled (S.F.O.R.D.) spectra; comparison of chemical shifts in compounds (2)—(9); analysis of $^{13}C$-$^1H$ couplings in relative to the neighbouring resonances (due to non-protonated carbons) and from its multiplicity in S.F.O.R.D. spectra. In fully $^1H$-coupled spectra it appears as a doublet of triplets, $^1J(^{13}C-H)$ 164.6 and $^3J(^{13}C-C-H)$ 5.8 Hz, due to coupling to H-5 and the benzylic methylene protons of the adjacent n-buty1 group. The C-5 resonance appears at 121—122 p.p.m. in compounds (2)—(4) and (7) but moves downfield by ca. 3 p.p.m. in the 8-O-methyl derivatives (5), (6), (8), and (9). In the decarboxy-compounds (7)—(9) the additional protonated aromatic carbon resonance at 137 p.p.m. is readily assigned to C-2.

The quinonoid carbonyl carbons were expected to appear at lowest field and in phomazarin methyl ester (2), C-9 and C-10 appear at 184.7 and 187.6 p.p.m. respectively. They are readily distinguished by the three-bond coupling of C-10 to the aromatic proton, $^3J(^{13}C-C-H)$ 4.4 Hz, and in $^{15}N$-enriched phomazarin methyl ester by the coupling of C-9 to $^{15}N$. $^3J(^{13}C-C-^{15}N)$ 7.8 Hz. In the fully $^1H$-coupled spectrum of tri-O-methylphomazarin methyl ester (5) determined on a

### Table 1

Carbon-13 chemical shifts of a series of phomazarin derivatives (in p.p.m. downfield from SiMe$_4$, for CDCl$_3$ solutions)

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<thead>
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<th>Compound</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
<th>(7)</th>
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<tr>
<td>C-2</td>
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4-0-methyl derivatives. Replacing the 4-hydroxy group in the decarboxy-derivative (8) by chlorine to groups on either C-S or C-4, the C-9 and C-10 resonances shift of e.g. C-8a resonances, which show characteristic downfield of the deshielding chelation effect, to appear at give (9) likewise results in a carbonyl resonance, removal of the hydrogen-bonding shift, 4.3 p.p.m., by the carbonyl. In compounds methyl derivative (4), and C-8a moves downfield from C-10 resonance. As well as the shifts of the quinone carbonyl resonance, removal of the hydrogen-bonding by methylation also allows assignment of the C-4a and C-8a resonances, which show characteristic downfield shifts of ca. 10 p.p.m., e.g. C-4a moves downfield from 117.3 in (3) to 127.9 p.p.m. in the corresponding 4-O-methyl derivative (4), and C-8a moves downfield from fully 1H-coupled spectrum C-6 appears as a partially resolved multiplet due to coupling to the butyl methylene protons whereas C-10a appears as a sharp singlet, there being no protons in a three-bond relationship to C-10a.

In the p.n.d. spectrum of the tri-0-methylphomazarin methyl ester (5) only 12 of the 14 low-field resonances could be seen. On re-determining the spectrum on a 1000 Hz sweep width the intense resonance at 125 p.p.m. was resolved into three closely spaced signals and in the fully 1H-coupled spectrum, also determined on a 1000 Hz sweep, these three signals appear as a doublet of triplets (J 164.6 and 5.7 Hz), a doublet (J 7.3 Hz), and a singlet (see Figure 1) allowing their assignment to C-5, C-8a, and C-10a, respectively. In Figure 1, the C-4a singlet, at 130.3 p.p.m., lies under the low-field half of the C-5 resonance.

The resonances still to be assigned are those of the phenolic carbons and the heteroaromatic carbons, C-2 and C-9a; C-2 is readily assigned in compounds (2) and (7)—(9), but is not so readily distinguished from the resonances in the 146—161 p.p.m. region in the remaining compounds. The resonances of methoxy-bearing carbons can be identified by their multiplicities in fully 1H-coupled spectra; in the spectrum of (5) carbons 3, 4, 8, and 11 appear as quartets due to coupling to the methoxy protons, 3J(13C-O) ca. 4 Hz, further splitting being observed in the C-7 resonance due to three-bond coupling to H-5 and the 12-methylene protons, whereas C-2 and C-9a appear as sharp singlets (see Figure 1). In the p.n.d. spectrum of 15N-enriched (5), however, C-2, C-3, C-4, and C-9a all appear as doublets due to coupling (J 1.5, 2, 2, and 3.9 Hz, respectively) to 15N, thus allowing C-3 and C-4 to be distinguished from C-7 and C-8, which are themselves differentiated by the further couplings, discussed above, displayed by the C-7 resonance. In the partially methylated derivatives, the free phenolic carbons are identified as they appear as doublets due to coupling to the hydrogen proton, 3J(13C-OH) ca. 5 Hz; this coupling is commonly observed in strongly hydrogen-bonded phenols and can be removed by exchange with D2O.

On formation of the 8-O-methyl ether, the C-8 resonance is moved to lower field by 2—3 p.p.m. compared with the 8-hydroxy compounds. In contrast, formation of the 4-O-methyl ether has no effect on the C-4 resonance but does result in a downfield shift of the C-3 resonance from ca. 147 to ca. 151 p.p.m. On decarboxylation, the C-4 resonance is moved upfield by ca. 5 p.p.m. and in the chloro-compound (9), the C-4 resonance undergoes a large upfield shift to 131.8 p.p.m.

Of the remaining carbons only C-6 and C-10a are not attached to either oxygen or nitrogen. They are assigned in phomazarin methyl ester (2) to the resonances at 144.9 and 126.1 p.p.m., respectively; in the

![Figure 1](image_url)
derivative (10), C-4 resonates at 175.8 ppm and so must exist as the keto-tautomer. The authors experienced some difficulty in recognising the presence of the 4-quinolone moiety, but it would appear that 13C n.m.r. provides a good method for indicating the presence of the system. The relatively large two-bond 13C-15N couplings to C-9 and C-11 have been observed in 15N-enriched phomazarin methyl ester (2) and tri-O-methylphomazarin methyl ester (5) and these couplings are most useful in both assignment and structural studies.

The magnitude of the coupling depends on the inter-
action of the carbon with the nitrogen lone-pair of

electrons as 1/J(13C-15N) is much larger when the carbon is cis to the lone-pair than when it is trans, thus the two-bond coupling to C-3 is only 2 Hz. In 13C-quinoline the two-bond couplings to C-3 and C-8 are 2.7 and 9.3 Hz, respectively.17 The remaining 13C-15N couplings observed in phomazarin, particularly the small one-bond couplings, are consistent with observations on 15N-pyridine18 and 15N-quinoline.17 On protonation the large two-bond coupling in 13C-quinoline is reduced to 1.0 Hz, and the couplings to C-2 and C-9 increase from 1.4 and 0.6 Hz (CCl4) to 15.9 and 13.8 Hz (H2SO4 solution).17 Similarly in 15N-pyridine the coupling to C-2 increases from 0.6 Hz to 11.9 Hz on protonation and to 15.2 Hz on N-oxide formation.18

As previously described for other fungal metabolites,19 feedings of sodium 1-14C acetate to P. terrestris were used to establish conditions which would give a suitable abundance of 13C in the individual acetate-derived atoms of phomazarin on feeding of 13C acetate. Studies of the course of fermentation indicate that phomazarin production commences after 5 d growth maximising after a further 7 d, and optimum dilution values were obtained by separate additions of sodium acetate on days 5, 6, and 7 to a total level of 0.5 g l-1; feeding to four flasks, i.e. 400 ml of culture medium, gave sufficient phomazarin for spectral determinations. Despite its rather low solubility, ca. 30 mg ml-1 phomazarin methyl ester (2), was used for the study of 13C enrichments as the overlap of signals in its 13C n.m.r. spectrum makes the more soluble tri-O-methylphomazarin methyl ester (5) unsuitable. The natural abundance 13C n.m.r. spectrum of (2) determined under normal conditions displays an extremely wide range of resonance intensities which, despite high enrichments, makes identification and comparison of enriched resonances somewhat difficult. However, if the spectrum is determined in the presence of the relaxation agent20 [Cr(acac)3]3 these intensity differences are removed and all the non-protonated carbons display almost identical intensities.

The protonated carbons also giving similar intensities to one another but at a slightly higher overall intensity than the non-protonated carbons. It is suggested that, solvent considerations permitting, this procedure should be standard practice in 13C-enrichment studies, as it largely removes the uncertainties that can arise in 13C biosynthetic studies.

The 13C n.m.r. spectra of phomazarin methyl ester enriched from feedings of [1-13C]- and [2-13C]-acetate showed the alternate labelling pattern anticipated, carbons 2, 4, 5, 7, 8a, 9a, 10, 12, and 14 being derived from the carboxy of acetate and carbons 3, 4a, 6, 8, 9, 10a, 11, 13, and 15 being derived from the methyl carbon of acetate (Table 2) so confirming an entirely polyketide origin of the phomazarin skeleton. An approximately five-fold enrichment at each labelled site, denoted by an asterisk, * Feeding with [13C13C] acetate. ** See Experimental section for details of calculation; enriched (+) average 1.6, unenriched average 1.0.

** TABLE 2 **

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<td>63.5</td>
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* Approximately five-fold enrichment at each labelled site, denoted by an asterisk. ** See Experimental section for details of calculation; enriched (+) average 1.6, unenriched average 1.0.
possibilities e.g. (a), (c), and (f) in Scheme 1, but it is not at this stage possible to differentiate between a two-chain

\[ \text{MeCO}_2\text{Na} \]  

[FIGURE 2 Low-field region of the p.n.d. $^{13}$C n.m.r. spectrum of $[^{13}$C$]_{\text{acetate}}$ enriched spectra of phomazarin methyl ester (2) determined at (a) 15.04 MHz and (b) 67.89 MHz.

\[ \text{[e.g. (b), (d), or (e)] or a single chain [e.g. (g)] origin for phomazarin. However, if a two-chain pathway is}

operative then both C-15 and C-11 are derived from the methyl carbon of an acetate 'starter' unit, whereas if a

natural-abundance and enriched spectra to give average enrichment values, satisfactory results can be ob-
The enrichments observed in phomazarin methyl ester (Table 2) indicate that C-15, but not C-11, is labelled to a much lower extent than the remaining \( [2-^{13}C] \) malonate-labelled carbons and so confirm the \(^{13}C \) results. Thus phomazarin contains only one acetate starter molecule, and so must be formed from a single nonaketide chain folded as shown in path (a), Scheme 2, probably via an anthraquinone, e.g. (11), Scheme 3.

A large variety of natural products appear to be formed via oxidative fission of the quinonoid ring of anthraquinones, e.g. sulochrin, geodin, ravenelin, were obtained for samples in acid-free deuteriochloroform with tetrakis(trimethylsilyl)propionitrile as internal reference. Proton noise-decoupled spectra, single frequency off-resonance decoupled spectra and fully proton-coupled spectra were determined on a JEOL JNM FX-60 spectrometer operating at 16.44 MHz. Fully proton-coupled spectra were determined under gated decoupling conditions to retain nuclear Overhauser enhancements. The spectrum of \([^{13}C] \) acetate-enriched phomazarin methyl ester was determined on a Bruker WH-270 spectrometer operating at 67.89 MHz. Trisacetyl-acetonatochromium \([Cr(acac)\]) (0.1 m) was used as a relaxation agent.

Scheme 3 Proposed biosynthetic pathway to phomazarin from acetate via anthraquinone intermediates

Feeding Experiments.—(a) Sodium \([1-^{14}C] \) acetate (290 mg; 4.1 \( \mu Ci \) mmol\(^{-1} \)) was added in portions on days 5, 6, and 7 to four shake-culture flasks. After 11 d growth, the mycelium was filtered off and dried. The dried mycelium was exhaustively extracted with ether and methanol and then was refluxed with 10% dry hydrogen chloride in methanol for 5 h. After cooling to 0 °C, the mycelium was filtered off and extracted with chloroform. The resulting crude red solid was methylated and tri-O-methylphomazarin methyl ester (60 mg; 1.47 \( \mu Ci \) mmol\(^{-1} \)) isolated as previously described. On the basis of this dilution factor (2.8) it was anticipated that feedings of \([^{13}C] \) acetate (95 atom-%) would give phomazarin with an excess 3.8% of \(^{13}C \) label over natural abundance at each labelled position (assuming formation from nine acetate units).

(b) Accordingly sodium \([1-^{13}C]-[2-^{13}C]-\) and \([^{13}C] \) acetates were fed as above. The resultant labelled phomazarins were isolated as the methyl ester (2) and their p.m.d. \(^{13}C \) n.m.r. spectra determined. Comparison of the labelled and unlabelled resonance intensities indicated an approximately 4% excess \(^{13}C \) abundance at each labelled position.

(c) Diethyl \([2-^{13}C] \) malonate (250 mg) in ethanol (6 ml) was fed to four flasks on days 6, 7, and 8, and after 11 d growth phomazarin methyl ester (65 mg) was isolated and its \(^{13}C \) n.m.r. spectrum determined three times, as was the natural abundance spectrum, and the average intensities after normalisation were used to calculate the enrichment.
values given in Table 2, using the method reported previously.  

(d) Diethyl [2-14C]malonate (100 PCi) in ethanol (1 ml) was distributed among five flasks on day 6 along with inactive sodium acetate (100 mg) in water (5 ml). After 11 d growth tri-O-methylphomazarin methyl ester (100 mg; 2.403 Ci mmol⁻¹) was isolated as above. For degradation, the [14C]compound (100 mg) was diluted with unlabelled material (200 mg).

Tri-O-methylphomazarin methyl ester (80 mg; 0.801 μCi mmol⁻¹) was oxidised under standard conditions to acetic acid; this was then converted to p-bromophenacyl acetate, which was crystallised to constant activity (0.067 μCi mmol⁻¹). A second portion of tri-O-methylphomazarin methyl ester (100 mg; 0.801 μCi mmol⁻¹) was converted to di-O-methyldecarboxyphomazarin (8) as described previously.

Crystallisation to constant activity gave (8) with an activity of 0.710 μCi mmol⁻¹. Thus the activity of CO₂ (by difference) liberated in the decarboxylation is 0.091 μCi mmol⁻¹.

[8/630 Received, 21st March, 1978]

REFERENCES

11. Reference 10, p. 98.
Phomazarin. Part 3. The Structure of Isophomazarin

By Reinhardt Effenberger and Thomas J. Simpson, Research School of Chemistry, Australian National University, P.O. Box 4, Canberra, A.C.T. 2600, Australia

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PERKIN TRANSACTIONS I
1979
Isophomazarin, a minor pigment isolated from the mycelium of Pyrenochaeta terrestris has been assigned the structure 6-n-butyl-4,5,8-trihydroxy-7-methoxy-1-aza-anthraquinone-2-carboxylic acid from biogenetic considerations and a comparison of its chemical behaviour, and of spectral data of its derivatives with those of phomazarin.

FROM chromatography of the mycelial extracts of Pyrenochaeta terrestris, we have isolated, in low yield, a red pigment, C_{19}H_{27}NO_8, m.p. 215–216 °C, isomeric with phomazarin (9), whose structure determination and biosynthesis have been discussed in previous papers.1,2 We now report studies leading to structure (1) for this new metabolite, isophomazarin.

**RESULTS AND DISCUSSION**

The similarity of their physical properties and spectral data suggested a close structural relationship between phomazarin and isophomazarin. The 1H n.m.r. spectrum of iso-phomazarin (Table 1) showed the presence of an uncoupled aromatic proton, methoxy, and n-butyl groups as in phomazarin.2 On treatment with methanolic HCl, isophomazarin formed a methyl ester (2), m.p. 201–202 °C, whose i.r. spectrum (Table 2) indicated the presence of an unchelated ester carbonyl, v(CO) at 1730 cm⁻¹, in contrast to phomazarin methyl ester (10) in which the ester is chelated and has v(CO) at 1685 cm⁻¹, and chelated quinone carbonyls at v(CO) 1615 cm⁻¹. In addition to the butyl, methoxy, and aromatic proton resonances, the 1H n.m.r. spectrum of (2) showed three low-field exchangeable protons indicating the presence of three strongly hydrogen-bonded phenolic groups in isophomazarin. If isophomazarin has the same 1-aza-anthraquinone structure as phomazarin then, as none of these phenolic hydroxys can be adjacent to the ester function, they must occupy the three remaining peri-positions to the quinone carbonyls, the fourth being occupied by the heteroaromatic nitrogen, see below. Thus isophomazarin must have the partial structure (8).

The presence of three phenolic hydroxys was confirmed by Purdie methylation of isophomazarin to give both di-O-methylisophomazarin methyl ester (3), m.p. 197–201 °C and tri-O-methylisophomazarin methyl ester (6), m.p. 171–175 °C. The latter compound was clearly different from tri-O-methylphomazarin methyl ester (11), m.p. 136–138 °C, and so isophomazarin is not a simple methylation isomer of phomazarin, in accord with partial structure (8). The i.r. spectrum of the di-O-methyl compound (3) shows the presence of both chelated and non-chelated quinone carbonyls with v(CO) at 1625 and 1680 cm⁻¹, respectively, whereas in the tri-O-methyl derivative both quinone carbonyls absorb showing v(CO) at 1680 cm⁻¹. The di-O-methylisophomazarin methyl ester is assigned structure (3) by analogy with phomazarin, in which the last position to be methylated is the 8-OH. However, structure (4) is also a possibility for this compound and cannot be definitely excluded on the available evidence.

In a series of nuclear Overhauser experiments with tri-O-methylisophomazarin (6) it was found that irradiation of the benzylid methane protons at δ 7.21 caused no enhancement of the aromatic proton intensity and so they were not adjacent to each other as in phomazarin. However, irradiation of the methoxy resonance at δ 5.89 did result in an enhancement of ca. 15% in the intensity of the aromatic proton resonance, indicating the presence of a methoxy ortho to the aromatic proton. This behaviour, considered with the chemical shift of
the aromatic proton, is in accord with partial structure (8a) only and means that the remaining butyl and methoxy substituents must be on carbons 6 and 7.

in the formation of the corresponding decarboxy-derivatives. However on prolonged treatment of phomazarin methyl ester itself (10), with hot sulphuric acid, no decarboxylation occurred, the sole product being the 7-demethyl derivative (12). Thus it would appear that under acidic conditions, the participation of an ortho-methoxy function is necessary to effect decarboxylation.

The 13C n.m.r. spectra of isophomazarin methyl ester (2) and the tri-O-methyl derivative (6) (Table 3) are in full agreement with the above structure. In (2) the quinone carbonyl resonances appear at 188.3 and 183.6 p.p.m. and can be assigned to C-10 and C-9 respectively. In the fully 1H-coupled 13C spectrum, both these resonances appear as sharp singlets as there is no possibility of coupling to the aromatic proton, as is observed for phomazarin derivatives. The resonance at 134.0 p.p.m. appears as a partially resolved multiplet in the coupled

**Table 1**

Hydrogen-1 n.m.r. spectra of isophomazarin and related compounds

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<th>12-CH₃</th>
<th>13-CH₃</th>
<th>15-Me</th>
<th>OH</th>
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* For CDCl₃ solutions except where stated otherwise. † For CF₃CO₂H solution.

The chemical shift of the aromatic proton is much too low for it to be on C-6 or C-7.

**Table 2**

I.r. data (cm⁻¹) for isophomazarin derivatives (CHCl₃ solutions)

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* Vinylogous amide.

The presence of the heterocyclic ring in isophomazarin was confirmed by the ease of reduction of (6) over palladium-charcoal to give, after aerial re-oxidation of the quinone system, the tetrahydro-derivative (7), whose i.r. spectrum shows absorptions at 1740, 1670, and 1543 cm⁻¹. The latter absorption can only be due to a quinone carbonyl is evidenced by the lowering of the aromatic proton, is in accord with partial structure (8a) only and means that the remaining butyl and methoxy substituents must be on carbons 6 and 7.

For CF₃CO₂H solution.

**Table 3**

Carbon-13 n.m.r. spectra of isophomazarin derivatives (2) and (6), and the corresponding phomazarin derivatives (10) and (11)

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<th>(10)</th>
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* In p.p.m. relative to SiMe₄, CDCl₃ solutions. † Assignments may be interchanged.
the ortho-shielding effect of the 5-hydroxy. Similarly, see below, C-10a is moved upfield from 164.3 p.p.m. in (10) to 107.0 p.p.m. in (2). The resonance at 164.3 p.p.m. in (2) is assigned to C-5 as in the fully 1H-coupled spectrum it appears as a doublet of triplets \(2J(13C-O-H) 6 \text{ Hz and } 3J(13C-C-H) 4 \text{ Hz}\). On addition of D\(_2\)O, the doublet coupling to the chelated hydroxy proton was removed, the remaining coupling being to the benzylic methylene protons. In phomazarin methyl ester, the protonated aromatic carbon appears as a doublet of triplets due to coupling to the H-5 and H-12 protons respectively: however, in isophomazarin methyl ester, C-3 gives a doublet \(2J(13C-O-H) 171 \text{ Hz}\) of broad signals which sharpen considerably on addition of D\(_2\)O, consistent with C-3 being adjacent to the chelated 4-hydroxy. On the basis of expected chemical shifts of the adjacent chelated hydroxy protons. The highest field resonance (107.0 ppm.) was assigned to C-10a as it should appear at higher field than C-8a due to the extra shielding it experiences from the para 7-methoxy group. Of the remaining non-aliphatic resonances in (2), all showed coupling to either methoxy or hydroxy except the resonances at 160.1 and 153.2 p.p.m. and so these were assigned to C-9a and C-2. Both C-2 and C-4 appeared at much lower field than in phomazarin methyl ester (10), as the shielding effect of the 3-hydroxy in phomazarin methyl ester has been removed. The remaining resonances were readily assigned by comparison with phomazarin derivatives. The differences in chemical shifts in the trimethyl ester derivative (6), particularly those of C-4a, C-8a, and C-10a, were consistent with the previously observed effect of removal of chelation on methylation of the phenolic hydroxys. No significant change is observed in the C-4 resonance on methylation, consistent with isophomazarin having a pyridol rather than a pyridone ring structure, in agreement with previous observations with phomazarin. The co-occurrence of phomazarin and isophomazarin. Moreover structure (1) is fully consistent with a polyketide derivation whereas (13) is not. The shortage of material available precluded any further experiments to clarify this point.

The co-occurrence of phomazarin and isophomazarin is biosynthetically interesting as isophomazarin retains the oxygen from the terminal carboxy of the precursor polyketide chain which is lost in phomazarin itself but lacks the 'extra' 3-hydroxy function. Thus neither phomazarin nor isophomazarin is derived from the other but most likely represent different initial products, e.g. (14) and (15) (Scheme), after condensation of the precursors nonaketide which then both undergo an exactly parallel sequence of secondary modifications, including (not necessarily in this order) C-4 hydroxylation, O-methylation, oxidative ring-cleavage, transamination, and heterocyclic ring closure to give the final metabolites.

**EXPERIMENTAL**

General details have been described previously.\(^2\)

**Isophomazarin (I).**—Chromatography of the chloroform extract of the acidified mycelia of *P. terrestris* gave a red gum, which after several recrystallisations from acetic acid produced pure *isophomazarin (I)* as red needles (60 mg), m.p. 215–216 °C (Found: C, 59.2; H, 4.5; N, 3.9. \(\text{C}_{19}\text{H}_{17}\text{N}_{8}\text{O}_{8}\) requires C, 58.9; H, 4.4; N, 3.6%)

**Isophomazarin Methyl Ester (2).**—Isophomazarin (20 mg) was dissolved in methanol (2 ml) containing one drop of concentrated hydrochloric acid and the solution was refluxed for 3 h. A fluffy red precipitate formed which could be recrystallised from methanol as red needles (20 mg), m.p. 201–202 °C; \(\lambda_{\text{max}} 248\text{ and }263\text{ nm (log } e 4.53\text{ and }4.60\text{ respectively) (Found: C, 59.6; H, 4.9; N, 3.7. }\text{C}_{20}\text{H}_{19}\text{N}_{8}\text{O}_{8}\) requires C, 59.9; H, 4.7; N, 3.5%).

**Purdie Methylation of Isophomazarin Methyl Ester.**—The
methyl ester (24 mg) was dissolved in chloroform (15 ml) and silver oxide (200 mg) and methyl iodide (1 ml) were added and the mixture stirred at room temperature for 15 h. After filtering off solid residues and removal of solvent an orange gum was obtained. This was purified by preparative t.l.c. on a 20 x 20 Kieselgel GF plate (0.5 mm thick), eluent chloroform–methanol (96: 4); removal of the yellow band (Rf 0.53) and recrystallisation from methanol gave di-O-methylisophomazarin methyl ester (3) as orange crystals (10 mg), m.p. 197–201 °C; \( \lambda_{\max} 256, 276(\text{sh}) \), and 460 nm (log \( c \) 4.29, 4.10, and 3.63 respectively) (Found: C, 61.49; H, 5.41; N, 3.30. \( \text{C}_{22}\text{H}_{23}\text{N}_0_8 \) requires C, 61.53; H, 5.40; N, 3.28%). 

\( \text{m/e 428 (100%), 429 (100%), 414 (12), 400 (10), 398 (8), 386 (48), 378 (14), and 337 (9). Isophomazarin (30 mg) was treated as above, but the reaction was worked-up after 24 h. Recrystallisation of the resultant orange-red gum from methanol gave tri-O-methylisophomazarin methyl ester (6) as orange needles (12 mg), m.p. 171–175 °C; \( \lambda_{\max} 260 \) and 452 nm (log \( c \) 4.31 and 3.54 respectively) (Found: \( M^+ \), 443.158 0. \( \text{C}_{23}\text{H}_{25}\text{N}_0_8 \) requires \( M, 443.158 0 \)); \( \text{m/e 443 (100%), 429 (57), 427 (40), 414 (15), 400 (15), 386 (21), and 368 (17). \)

Di-O-methylisophomazarin (5).—Tri-O-methylisophomazarin was dissolved in methanol (1 ml), 6N sulphuric acid (4 ml) was added, and the mixture refluxed for 18 h. The reaction mixture was worked-up to give a red gum which was refluxed in 5% methanolic HCl for 2 h. Removal of solvent gave a red solid which was recrystallised from methanol to give di-O-methylisophomazarin methyl ester (5) as orange needles (2 mg), m.p. 190–191 °C; \( \lambda_{\max} 250, 265, \) and 440 nm (log \( c \) 4.32 and 3.60 respectively) (Found: \( M^+ \), 429.143 4. \( \text{C}_{22}\text{H}_{23}\text{N}_0_8 \) requires \( M, 429.142 4 \)); \( \text{m/e 429 (100%), 414 (4), 400 (11), 388 (44), 373 (15), and 357 (19). \)

Catalytic Hydrogenation of Tri-O-methylisophomazarin Methyl Ester (6).—Compound (6) (10 mg) was dissolved in methanol (10 ml). Triethylamine (0.2 ml), and 5% palladium–charcoal catalyst (20 mg) were added and the mixture was hydrogenated at room temperature. The catalyst was then filtered off and air passed through to remove any hydrogen. After removal of solvent, the orange residue was purified by t.l.c. on a 20 x 20 silica GF plate, eluent chloroform–methanol (96: 4). The yellow band (Rf 0.6), was removed and eluted to give tri-O-methyltetrahydroisophomazarin methyl ester (7) as an orange gum (4 mg) \( \lambda_{\max} 272, 313, \) and 374 nm (log \( c \) 4.34, 3.97, and 3.66 respectively) (Found: \( M^+ \), 447.189 1. \( \text{C}_{23}\text{H}_{21}\text{N}_0_8 \) requires \( M, 447.189 1 \)); \( \text{m/e 447 (14), 416 (45), 356 (100), 342 (21) and 328 (16). \)

Acid Hydrolysis of Phomazarin Methyl Ester (10).—A solution of phomazarin methyl ester (20 mg), 6N H\(_2\)SO\(_4\) (10 ml), and methanol (4 ml) was refluxed for 24 h. The resulting solution was cooled, extracted with chloroform and the red residue obtained on removal of solvent was recrystallised from methanol to give 7-demethylphomazarin (12) as red needles (10 mg), m.p. 192–196 °C; \( \text{m/e 37 (100%), 329 (40), 300 (67), 287 (50), and 286 (47) \tau(CF}_3\text{CO}_2\text{H}) 1.92 (1 H), 7.08 (2 H, t, J 7 Hz), 8.4 (4 H, m) and 9.00 (3 H, t, J 7 Hz). \)

[8/531 Received, 21st March, 1978

REFERENCES

1 Part 2; A. J. Birch and T. J. Simpson, preceding paper.
Fungal Products. Part 21.1 Biosynthesis of the Fungal Metabolite, Wortmannin, from [1,2-13C2]-Acetate

By Thomas J. Simpson,* Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

Martin W. Lunnon and Jake MacMillan,* School of Chemistry, University of Bristol, Bristol BS8 1TS

The 13C-n.m.r. spectrum of wortmannin, enriched with sodium [1,2-13C2]-acetate by cultures of Penicillium wortmannii, is consistent with the biogenesis of this metabolite via triterpenoid intermediates. Although the overall incorporation of 13C was low, 13C-13C coupling was observed between carbon atoms derived from adjacent acetate units in addition to the expected coupling between carbon atoms in intact acetate units.

Wortmannin, a metabolite isolated from culture of Penicillium wortmannii, was shown to have structure (1) except for the C3-stereochemistry by Millan et al.3 This structure was confirmed, and the complete stereochemistry (1) was established, by X-ray action studies by Pecher et al.4 Preliminary investigations of the incorporation of [14C]- and [3H]-labelled precursors were consistent with a triterpenoid genesis for wortmannin. To provide further evidence for this pathway the incorporation of [1,2-13C2]-acetate into wortmannin has been studied.

The anticipated incorporation pattern of acetate via malonic acid, squalene, and lanosterol is shown in the scheme. Thus the pairs of carbon atoms, 20 and 4, and 10, 5 and 6, 9 and 11, 12 and 13, 16 and 17, 14 and 18, and 21 and 22, were expected to be derived from acetate units and show 13C-13C spin couplings. The remaining carbon atoms, derived from acetate residues which have been cleaved at various stages along the biosynthetic pathway, should appear as uncoupled resonances.

The 13C n.m.r. spectrum of wortmannin is summarised in the Table and the region 70—10 p.p.m. downfield from internal tetramethylsilane is reproduced in the Figure. The assignments were based on (a) standard chemical shift data;6 (b) off-resonance decoupling experiments using the graphical method devised by Birdsall et al.7 which allowed the assignment of the protonated carbon resonances for wortmannin (1) and the degradation product (2); (c) comparison of the resonances observed for wortmannin (1) and the degradation product (2); and (d) the resonances published8 for furan derivatives.

The amount of [13C]-enriched precursor required to be fed to give satisfactory dilution values was determined by supplementing a series of shake cultures of P. wortmannii with sodium [1-14C]-acetate and varying both the concentration of precursor and the time of addition. Optimum (minimum) dilution values were obtained by feeding acetate at a level of 200 mg l−1. In this way dilution values of 13–21 were obtained and these values were not improved by adding higher concentrations of sodium acetate to the fermentations. These dilution values correspond to enrichments of 0.4 to 0.7% at each labelled site assuming 10 equally labelled sites (Scheme). Although such enrichment is low it was
anticipated that $^{13}$C-$^{13}$C coupling satellites, 20—35% of the intensity of the natural abundance resonance, would be observed for the carbons derived from intact [1,2-$^{13}$C$_2$]-acetate units.

In the event, the noise-decoupled $^{13}$C n.m.r. spectrum of wortmannin, derived from a [1,2-$^{13}$C$_2$]-acetate feed, was not as simple as anticipated (see Figure). Extensive spin coupling was observed between carbons derived from adjacent acetate units in addition to the expected coupling between carbons derived from intact acetate units.

\[
\text{MeCO}_2\text{Na} \rightarrow \begin{array}{c}
\text{HO} \\
\text{HO} \\
\text{OH}
\end{array} \rightarrow \begin{array}{c}
\text{HO} \\
\text{HO} \\
\text{OH}
\end{array} \rightarrow \begin{array}{c}
\text{MeO} \\
\text{Me} \\
\text{C}_2 \text{O} \\
\text{MeOCH}_2
\end{array}
\]

Scheme Incorporation of [1,2-$^{13}$C$_2$]-acetate into wortmannin via mevalonic acid, squalene, and lanosterol

Additional coupling of this type has previously been observed in several biosynthetic studies using [1,2-$^{13}$C$_3$]-acetate in which high incorporation efficiencies result in an increased probability of adjacent acetate-derived units being labelled. It should be noted, however, that the overall enrichments are not, per se, sufficient to account for the intensity of the observed additional couplings. Also, in some cases where the overall enrichment levels are much too low to allow for any significant inter-acetate coupling, additional couplings have been observed to a minor extent and, in one recent study, to a major extent. Considering the present case the additional couplings indicate that the wortmannin was being produced, over a limited period, mainly from the added [1,2-$^{13}$C$_3$]-acetate to give a small proportion of the total wortmannin with very high low overall level of enrichment. That there was dilution of the exogenous acetate (and consequently of the mevalonate) by endogenous metabolic pools was apparent from the relative intensities of the $^{13}$C-$^{13}$C satellites arising from intra-acetate, inter-acetate and mevalonate couplings (see Table and Figure). The consequence of the inter-acetate couplings, the intense primary satellites arising from intra-acetate couplings are reduced, the 'lost' intensities appear as secondary satellites to the primary satellites. Some quaternary carbons (e.g. C-5, C-8, and C-9) primary satellites were not observed due to the additional coupling to several attached carbon atoms and inherently low intensity of these quaternary resonances.
ary coupling constants of intact acetate units could be dis-

Table 1

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Values in parentheses observed as secondary coupling satellites. *No coupling observed due to low intensity of resonance. *Assignments may be reversed.

ment with the biogenetic pathway in the Scheme. There is no possibility of additional coupling of intact acetate units (C-19, C-20, C-21, and C-22), the sities of the primary satellites are ca. 30% of those of uncoupled resonances, as predicted from the dilution factors. For the remaining carbons, from the incorporation of intact acetate units C-6, C-10, C-11, C-12, C-13, C-16, and C-17, the sities of the primary satellites are lower due to endogenous metabolic pool of acetate units (C-14 with which it originally sited an intact acetate unit; however the biosynthetically significant two-bond 13C-13C coupling between and C-14 was not resolved.

In conclusion the results, though more complex than expected, are in full agreement with the biogenesis of wortmannin as outlined in the Scheme.

EXPERIMENTAL

13C N.M.R. Determinations.—Natural abundance 13C spectra were determined for solutions in CDCl3 or (CD3)2CO with Me3Si as internal standard on a JEOL PFT-100 instrument operating at 25.2 MHz in the Fourier transform mode. Sweep-widths of 6.25 KHz with 4 K data points were used to give chemical shifts with an accuracy of ±1.52 Hz (±0.06 p.p.m.). A proton-noise-decoupled spectrum of [1,2-13C2]-acetate enriched wortmannin was recorded with a Bruker WP-270 F.T. spectrometer operating at 67.89 MHz. A sweep width of 15 KHz with 16 K data points was used to give chemical shifts accurate to ±0.9 Hz (±0.01 p.p.m.).

13C-Enriched Wortmannin.—Forty 250 ml baffled conical flasks, each containing 50 ml Czapek-Dox medium were inoculated with mycelium from pre-grown 5-day old shake cultures of Penicillium wortmannii. Sodium [1-13C]-acetate (20 μCi) and unlabelled sodium acetate (80 mg) were distributed equally among 8 flasks in two portions on the third and fourth day after inoculation. After a further 4 days the flasks were removed and wortmannin isolated with a 1,2 migration from C-14 with which it originally sited an intact acetate unit; however the biosynthetically significant two-bond 13C-13C coupling between and C-14 was not resolved.

Incorporation of Sodium [1,2-13C2]-Acetate.—Sodium [1,2-13C2]-acetate (800 mg) was distributed among 80 flasks after 9 and 4 days' growth as above and the enriched [13C]-wortmannin (56 mg) isolated after a further 4 days' growth.

T. J. S. thanks the A.N.U. for a Fellowship under which a part of this work was performed. M. W. L. thanks the S.R.C. for a Research Studentship. We also thank Dr. M. Murray for determining 13C n.m.r. spectra.

REFERENCES


13 T. J. Simpson and J. S. E. Holker, Phytochem., 1977,
Carbon-13 Nuclear Magnetic Resonance Structural and Biosynthetic Studies on Deoxyherqueinone and Herqueichrysin, Phenalenone Metabolites of Penicillium herquei

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PERKIN TRANSACTIONS I

1979
**Carbon-13 Nuclear Magnetic Resonance Structural and Biosynthetic Studies on Deoxyherqueinone and Herqueichrysin, Phenalenone Metabolites of Penicillium herquei**

By Thomas J. Simpson, † Research School of Chemistry, Australian National University, Box 4, P.O., Canberra ACT 2600, Australia

A detailed analysis of the $^{13}$C-$^1$H couplings observed in the fully $^1$H-coupled $^{13}$C n.m.r. spectrum of deoxyherqueinone diacetate allows its structure to be defined as (3) and a full assignment of the $^{13}$C n.m.r. spectrum to be made. The structure of herqueichrysin triacetate (9) is established by analysis of $^{13}$C-$^{13}$C couplings in the fully $^1$H-coupled, natural abundance $^{13}$C n.m.r. spectrum, and $^{13}$C-$^{13}$C couplings in the [1,2-$^{13}$C]acetate enriched $^{13}$C n.m.r. spectrum. Further chemical and spectral studies allow structure (9) to be proposed for herqueichrysin.

Incorporations of sodium $[^1{\text{C}}]$acetate and $[^2\text{C}]$malonate and diethyl $[^2\text{C}]$malonate indicate formation of the phenalenone ring system via a specific folding of a single heptaketide chain.

Penicillium herquei and *P. atrovenetum* produce a group of closely related antibiotics based on the phenalenone nucleus fused to a 1,2,2-trimethylidihydrofuran ring.\(^1\) These are atrovenetin (1), deoxyherqueinone (2), herqueinone (4), and norherqueinone (5). \(^{13}$C Studies have shown that these phenalenones are derived via the acetate-polymalonate pathway, with methionine and mevalonate providing the carbons of the methoxy-group and the dihydrofuran ring respectively; \(^2\) and labelling studies with *P. herquei* have provided evidence in favour of a sequential biosynthetic relationship between atrovenetin (1), deoxyherqueinone (2), and herqueinone (4); atrovenetin was incorporated into norherqueinone (5) but the latter was not converted into herqueinone (4) by *P. herquei*.\(^3\) However, the nature of the intermediates leading from acetate and malonate to the phenalenone ring system, in common with the majority of aromatic metabolites, is unknown. The ring system could be formed by any one of three alternate foldings of a single heptaketide chain as shown in Scheme 1, and in addition numerous multichain condensations are possible. With the advent of $^{13}$C n.m.r. methods for biosynthetic studies, in particular the use of doubly labelled $^{13}$C acetate and analysis of the resultant $^{13}$C-$^{13}$C coupling patterns it has become possible to obtain direct information on these intermediates.\(^4\) *P. herquei* (CM1 112950) previously reported \(^5\) to produce mainly atrovenetin (1) was chosen for this study. However in our hands, this strain produced deoxyherqueinone (2), along with major amounts of herqueinone, a phenalenone of uncertain structure,\(^6\)\(^7\) only. Studies leading to the assembly pattern of acetate units in the phenalenone ring system and to structure (9) for herqueichrysin are now reported.

A prerequisite of any $^{13}$C study is an unambiguous assignment of the $^{13}$C n.m.r. spectrum. The metabolites were most conveniently isolated by acetylation and chromatography of the crude mycelial extracts. Thus, deoxyherqueinone was obtained as a diacetate, since chelation with the phenalenone carbonyl renders the peri-hydroxy very resistant to derivatisation. However, due to the tautomericism of the trihydroxyphenalenone system there are *a priori* four possible structures for this diacetate in which the ring carbonyl may be at C-5, C-7, C-9, or C-11. This uncertainty as to the precise structure makes assignment of the $^{13}$C spectrum particularly difficult. However, a detailed analysis of the fully proton-coupled $^{13}$C n.m.r. spectrum has enabled both the precise structure of the diacetate and unambiguous assignments to be determined.

In the proton-noise-decoupled (p.n.d.) $^{13}$C n.m.r. spectrum determined under normal conditions a very wide range of resonance intensities is observed, the protonated carbons are the most intense and these are readily assigned from their chemical shifts, multiplicities in single frequency off-resonance decoupled (s.f.o.r.d.) spectra, and by specific proton decoupling experiments.\(^8\) The remaining resonances of the phenalenone ring have been assigned by analysis of the low field portion of the fully proton-coupled $^{13}$C n.m.r. spectrum in which most of the resonances exhibit long range $^{13}$C-$^1$H couplings.

The origin of all of these couplings has been established by use of deuterium exchange and, in particular, specific proton decoupling experiments using very low decoupling

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\(\text{Scheme 1} \) Alternative polyketide derivations of the phenalenone ring system

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\[ \text{R}^1 = R^2 = H \\
\text{R}^1 = \text{Me}, R^2 = H \\
\text{R}^1 = \text{Me}, R^2 = \text{COCH}_3 \\
\text{R} = \text{Me} \\
\text{R} = H \]
ling powers so that only the long-range coupling due to the proton being irradiated is removed with little or no perturbation of the remainder of the spectrum. The results of these experiments are summarised in Table I.

| Table 1 |

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$\Delta$</th>
<th>$J_{13C-1H}$</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>125.2 s</td>
<td>55</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>112.4 dq</td>
<td>65</td>
<td>2.8</td>
</tr>
<tr>
<td>3</td>
<td>165.5 s</td>
<td>64</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>118.8 m</td>
<td>68</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>173.1 d</td>
<td>68</td>
<td>0.9</td>
</tr>
<tr>
<td>6</td>
<td>107.6 d</td>
<td>80</td>
<td>2.8</td>
</tr>
<tr>
<td>7</td>
<td>173.2 d</td>
<td>59</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>141.3 q</td>
<td>85</td>
<td>1.0</td>
</tr>
<tr>
<td>9</td>
<td>142.9 s</td>
<td>85</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>110.9 d</td>
<td>56</td>
<td>2.7</td>
</tr>
<tr>
<td>11</td>
<td>148.2 d</td>
<td>88</td>
<td>1.0</td>
</tr>
<tr>
<td>12</td>
<td>121.6 Dq</td>
<td>68</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Chemical shift relative to MeSi. Capital letters refer to the multiplicity resulting from directly bonded protons, and small letters to long range $13C-1H$ coupling. $S =$ singlet, $D =$ doublet, $Q =$ quartet. * Removed on irradiation of 14-Me, $\tau$ 7.11. * Removed on irradiation of 12-H, $\tau$ 3.00. * Removed on irradiation of 4', 5-Me's, $\tau$ 8.5. * Removed on irradiation of OMe, $\tau$ 5.95. * Resonance enhanced on irradiation of 2'-H, $\tau$ 5.27. * Removed on addition of D$_2$O.

The two resonances at lowest field, 173.9 and 173.1 p.p.m. must be due to the carboxyl and chelated phenolic carbons and these appear as doublets, 2 and 5 Hz respectively, which on addition of D$_2$O sharpen to singlets. It is well established that in strongly hydrogen-bonded carbonyl systems, two-, three- and four-bond $13C-1H$ couplings can be observed to the hydroxy-proton. In addition, on deuterium exchange the 173.1 p.p.m. resonance displays an upfield shift of 0.6 p.p.m. This is large for a deuterium geminal isotope effect. The other immediately noticeable change in the spectrum is the collapse of the doublet resonance at 107.8 p.p.m. to a singlet, and so this can be assigned to C-6, also displaying a three-bond coupling of 7 Hz to the phenolic proton. This coupling locates the chelated carbonyl system on carbons 5, 6, and 7. If it had been centred on carbons 0, 10, and 11, then C-11 would necessarily have appeared as a doublet on doublets, simplifying to a doublet on deuterium exchange, the remaining coupling being a three-bond coupling to the aromatic proton on C-12. Irradiation of the 12-proton at $\tau$ 3.00 (Table 2) in fact causes the doublet resonance at 110.9 p.p.m. ($J$ 5 Hz) to sharpen to a singlet and so it is assigned to C-10. This irradiation also causes the doublet at 148.2 p.p.m. ($J$ 3 Hz) to collapse to a singlet and so from its chemical shift it must be assigned to C-11, bearing an acetoxy group. Two-bond couplings of this type are not usually large enough to be resolved readily but they have been observed previously in phenolic acetates. The broad resonance at 112.4 p.p.m. also sharpens on irradiation of H-12, suggesting that it must be due to C-2 which also should exhibit a three-bond coupling to H-12. This is confirmed by irradiation of the 14-methyl protons at $\tau$ 7.11, whereupon the 112.4 p.p.m. resonance simplifies to a doublet ($J$ 6 Hz) as expected, the residual coupling on removal of the three-bond coupling to the methyl protons being to H-12. This irradiation also causes the C-12 resonance which appears as a doublet ($J$ 163 Hz, being the one-bond coupling to H-12) of quartets ($J$ 6 Hz) to sharpen to a simple doublet due to removal of the three-bond coupling to the methyl protons; and more importantly the quartet at 142.1 p.p.m. ($J$ 6 Hz) collapses to an intense singlet and so it is assigned to C-13. Irradiation of the methoxy-protons, $\tau$ 5.95, collapses the quartet ($J$ 4 Hz) at 141.3 p.p.m. to a singlet allowing its assignment to C-8, its rather high field chemical shift being due to the presence of shielding oxygen substituents on both adjacent carbons. The crucial decoupling experiment involved irradiation of the gem-dimethyl protons of the dihydrofuran ring, $\tau$ 8.5, resulting in the collapse of the very broad resonance at 119.8 p.p.m. to a doublet ($J$ 4 Hz) and so this resonance must be assigned to C-8, sharpening due to removal of the three-bond coupling to the methyl protons. The residual coupling is due to a three-bond coupling to the hydrogen-bonded hydroxy-proton since on repeating the irradiation in the presence of D$_2$O, the doublet splitting was also removed, and so C-5 must bear the phenolic hydroxy and thus C-7 the carbonyl function. Finally, irradiation of the dihydrofuranoid proton at $\tau$ 5.27 caused an approximately 50% increase in the intensity of the singlet resonance at 165.5 p.p.m. which was therefore assigned to C-3. The remaining very sharp singlets at 125.2 and 142.9 p.p.m. were assigned to C-1 and C-9 respectively, which showed
no long-range coupling as expected, there being no protons in a three-bond relationship to C-1 or C-9. These couplings allow the contiguous partial structures shown in Figure 1 to be deduced, and these define the precise structure of deoxyherqueinone diacetate as (3).

Two further major products were isolated from chromatography of the acetylated extract. $^1$H N.m.r. spectroscopy (Table 2) allowed these to be identified as a monoacetate and a triacetate. On further acetylation the monoacetate was converted into the triacetate and it was apparent from its physical and spectral properties that the triacetate was identical to herqueichrysin triacetate. Herqueichrysin, a phalenolone isomeric with deoxyherqueinone (2), has recently been isolated from P. herquei $^6$-$^7$ and on the basis of its physical and spectral properties, Vining et al. suggested structure (6). $^6$ This structure lacks peri-related carbonyl and hydroxy-functions as suggested by the formation of a triacetate in contrast to the diacetate obtained from deoxyherqueinone under identical conditions. On demethylation, demethylherqueichrysin (7) was obtained and this structure was subsequently confirmed by synthesis.$^{12}$ However the ready formation of a monoacetate is not consistent with structure (6) for herqueichrysin and the $^1$H n.m.r. of the monoacetate does, in fact, show a strongly chelated hydroxy-proton at $\tau$ - 6.8.

$^{13}$C N.m.r. studies allow structure (8) to be deduced for herqueichrysin triacetate. The $^{13}$C spectrum is summarised in Table 3. The aliphatic and protonated resonances were readily assigned as for deoxyherqueineone diacetate and examination of the fully proton-coupled spectrum allowed the remaining resonances to be assigned. The uncoupled resonance at lowest field, 182.5 p.p.m., must be assigned to the carbonyl carbon, less shielded than in deoxyherqueinone diacetate. Irradiation of the aromatic methyl protons caused the doublet of quartets due to C-12 centred on 127.0 p.p.m. ($J$ 159 and 6 Hz) to sharpen to a single doublet; the resonance at 125.1 p.p.m. to a sharp singlet, and irradiation of the dihydrofuranoid proton, $\tau$ 5.47, caused a large increase in the intensity of the singlet resonance at 123.5 p.p.m.

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^1$H N.m.r. chemical shifts (p.p.m.) of deoxyherqueinone and herqueichrysin and derivatives</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>12-H</td>
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<tr>
<td>(2)</td>
<td>3.15</td>
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<tr>
<td>(3)</td>
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<td>(10)</td>
<td>3.25</td>
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</tbody>
</table>

For CDCl$_3$ solutions, Me$_4$Si as internal standard.

Table 3

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$^1$H N.m.r. chemical shifts (p.p.m.) of deoxyherqueinone and herqueichrysin and derivatives</th>
</tr>
</thead>
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<tr>
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<td>125.7 dq</td>
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<tr>
<td>3</td>
<td>182.5 s</td>
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<tr>
<td>4</td>
<td>120.1 m</td>
</tr>
<tr>
<td>5</td>
<td>161.2 s</td>
</tr>
<tr>
<td>6</td>
<td>113.1 s</td>
</tr>
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<td>144.3 s</td>
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<td>24.8 Q</td>
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<tr>
<td>2'</td>
<td>90.9 D</td>
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<td>43.9 S</td>
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<td>4'</td>
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<td>168.7 q</td>
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<tr>
<td>CH$_3$CO</td>
<td>167.9 q</td>
</tr>
</tbody>
</table>

* See footnote to Table 1. * Removed on irradiation of 14-Me, $\tau$ 7.03. * Removed on irradiation of 12-H, $\tau$ 2.82.

161.2 p.p.m. Irradiation of the methoxy-protons, $\tau$ 6.10, collapsed the quartet ($J$ 4 Hz) at 143.4 p.p.m. to an intense singlet. The similarity of the chemical shift to the methoxylated carbon in deoxyherqueineone diacetate suggested a similar environment for it in herqueichrysin triacetate. Finally the singlet resonances at 126.4, 113.1, 144.3, and 142.1 p.p.m. can be assigned.
from their chemical shifts, to C-1, C-6, and the two remaining acetylated phenolic carbons. This data allows the partial structure shown in Figure 2(a) to be deduced. Incorporation of [(2,13C)acetate indicated that the methoxy-bearing carbon and the carbon resonating at 125.1 p.p.m. were derived from the methyl carbon of acetate and final evidence for structure (8) was provided by the spectrum of the triacetate enriched from [1,2-13C]acetate. This showed that C-2 which has been definitely assigned is coupled to the carbonyl carbon which must, therefore, be placed at C-3; also C-6 is coupled to the resonance at 144.3 p.p.m. and the methoxy-bearing carbon is coupled to the remaining acetylated phenolic carbon at 142.1 p.p.m. as shown in Figure 2(b), thus defining structure (8) for herqueichrysin triacetate.

Herqueichrysin itself was readily obtained by saponification of the triacetate and proved identical in all respects to an authentic specimen. The precise structures of herqueichrysin and the monoacetate are more difficult to define. Both show strong absorptions in their i.r. spectra at 3 600 cm⁻¹ characteristic of non-bonded hydroxy-group. The ¹H n.m.r. of the monoacetate shows the presence of a chelated hydroxy-proton, δ -6.84, and a non-bonded hydroxy, ca. δ 4.1. This chelated carbonyl system can only be accommodated by structure (10). The alternative structure (11) with C-3 to give a 9-hydroxy-group which will readily acetylate.

Preliminary experiments with [14C]acetate indicated that acetate was efficiently incorporated into the phenalenone metabolites. Thus on feeding [1-13C]- and [2-
metabolites (Scheme 2). In order to facilitate comparison of incorporation efficiencies into the polyketide and mevalonate derived portions of the molecules, p.n.d. spectra were determined in the presence of 0.1 M Cr(acac)₃ under Gated-2 decoupling conditions, whereupon the very wide range of line intensities due to variable T₂ and nuclear Overhauser factors was removed and essentially integral intensities for all resonances in the natural abundance spectra were obtained. The enhancements observed in the spectra of the enriched samples were high, with ca. 9% excess ¹³C-abundance at each labelled position, and with equal incorporation into the polyketide and mevalonate derived parts of the molecules indicating full equilibration of the added [¹³C]-acetate with the endogenous metabolic pools of acetate and mevalonate. The incorporation of [¹²¹³C]malonate is of more interest. As shown in Table 1 the overall enrichment was lower than with acetate. The values in Table 1 were calculated by multiple determination of both natural abundance and enriched spectra, which were averaged after normalisation to overcome the variation in resonance intensities arising from digitisation and transformation of data. The [²¹³C]malonate-enriched spectrum showed high enrichment of six positions in the phenalenone nucleus: C-2, C-4, C-6, C-8, C-10, and C-12. Partial decarboxylation of the [²¹³C]malonate results in the C-14 methyl together with C-14 and the mevalonate derived carbons is formed from a single heptaketide chain. The equal enrichment is consistent with the incorporation of malonate into mevalonate only after decarboxylation to acetate.

The p.n.d. ¹³C n.m.r. spectra of the [¹²¹³C₂]acetate enriched samples showed nine pairs of ¹³C—¹³C couplings, indicating that carbons 14 and 13, 12 and 11, 10 and 9, 8 and 7, 6 and 5, and 4 and 3, and 2 and 1' originate from intact acetate units. Due to severe overlap of the coupled ¹³C signals and second-order ¹³C—¹³C couplings arising from the similarity of chemical shift of some of the coupled carbons, the ¹³C n.m.r. spectra determined at the normal operating frequency of 15.04 MHz were extremely difficult to interpret. However on redetermining the spectra at 67.89 MHz the higher dispersion allowed the couplings to be resolved (see Figure 3). Thus the phenalenone ring system is formed by condensation of a heptaketide chain folded as shown in Scheme 2.

Herqueichrysin is the only member of the herqueinone group of metabolites to display the alternative orientation of the fused dihydrofuranoid ring. Their co-occurrence suggests that deoxyherqueinone and herqueichrysin are formed from a common prenylated phenalenone precursor, e.g. (14) which can cyclise to either of the adjacent phenolic hydroxy-groups. The ¹³C n.m.r. data clearly show that in both herqueichrysin and deoxyherqueinone, enriched from [¹²¹³C₂]acetate, it is C-5' trans to C-1' which is coupled to C-3' (C-4' is not coupled as it arises from C-2 of mevalonic acid from which C-1 is lost in the formation of dimethylallyl pyrophosphate, the presumed precursor of the dihydrofuranoid ring). Thus both deoxyherqueinone and herqueichrysin must have the same configuration at C-2'.

Other fungal phenalenones and their related metabolites are polyketide in origin and a similar assembly pattern of acetate units in their formation is likely.

**EXPERIMENTAL**

M.p.s were taken with a Kofler hot-stage microscope. Unless otherwise stated i.r. spectra were measured for solutions in chloroform, u.v. spectra in methanol, and ¹H n.m.r. spectra at 100 MHz, in deuteriochloroform with tetramethylsilane as internal reference. Mass spectra were recorded at 70 eV with an A.E.I. MS9 high-resolution spectrometer. Optical rotations were measured for solutions in chloroform at room temperature with an ETL-NPL automatic polarimeter. ¹³C n.m.r. spectra were obtained for samples in acid-free deuteriochloroform with tetramethylsilane as internal reference. Proton-noise-decoupled spectra, single frequency off-resonance decoupled spectra, and fully proton-coupled spectra were determined on a JEOL JNM FX60 spectrometer operating at 67.89 MHz. Specific decoupling experiments were determined on 2500 MHz sweep widths using a single ¹H irradiating frequency of 16 dB. The spectra of [¹²¹³C₂]acetate enriched samples were determined on a Bruker WH-270 spectrometer operating at 67.89 MHz. Trisacetylacetonatochromium, Cr(acac)₃, 0.1 molar, was used as relaxation agent.

**Isolation of Metabolites.**—Penicillium herquei. CMI 112 950, was grown from a spore suspension in shaken culture for ten days in 250-ml conical flasks, each containing 100-ml of culture medium composed of 5% sucrose, 0.3% NaN₃, 0.9% corn steep liquor, 0.1% KH₂PO₄, 0.1% KCl, 0.06% MgSO₄·7H₂O, 0.001% FeSO₄·7H₂O, 0.006% ZnSO₄·7H₂O, and 0.3% CaCO₃. The mycelium was filtered off and as much moisture as possible removed by pressing.
solvent. Deoxyherqueinone diacetate (3) was eluted with ether for 24 h. After drying, the ether was removed in vacuo to give a dark yellow solid which was acetylated by stirring overnight at room temperature with acetic anhydride in pyridine. The crystal product mixture was purified by preparative thin-layer chromatography using silica GF254 plates (20 x 20 x 0.1 cm) eluted several times with acetic–light petroleum (15:100) as developing solvent. Deoxyherqueinone diacetate (3) was eluted with ethyl acetate from the least-polar band and recrystallised from methanol to give yellow needles, m.p. 174–176 °C (lit. m.p. 174–176 °C).

Herqueichrysin triacetate (8) was similarly eluted from the yellow band of intermediate polarity and crystallised from methanol to give yellow rods, m.p. 183–185 °C (lit. m.p. 183–185 °C). Elution of the most-polar band gave herqueichrysin monoacetate (10) as yellow needles, m.p. 220–221 °C, from methanol (Found: C, 66.0; H, 5.6%. C22H20O7 requires C, 66.3; H, 5.6%); vmax 3590, 3400br, 1765, 1624, and 1592 cm⁻¹; λmax 235, 282, 325, and 432 nm (log ε 3.56, 3.56, 2.85, and 3.54). On further reaction with acetic anhydride in pyridine, herqueichrysin monoacetate was converted quantitatively into herqueichrysin triacetate.

Saponification of Herqueichrysin Triacete.—Herqueichrysin triacetate (300 mg) was stirred for 12 h in methanolic sodium hydroxide (5 ml). The solution was acidified, diluted with water, and extracted with ethyl acetate to give a yellow gum which crystallised from methanol to give herqueichrysin as yellow needles (120 mg), m.p. 174–175 °C (lit. m.p. 174 °C).

Incorporations of Sodium [1-13C]Acetate.—Preliminary experiments indicated that phenalenone production in cultures of P. herquei commenced on the fourth day after inoculation and reached a maximum on the eighth day. Further experiments indicated that maximum incorporation of [1-13C]acetate into the phenalenones occurred when the label was introduced in a series of additions on days 3, 4, 5, and 6 and the metabolites were isolated from 9-day-old cultures. Thus when sodium [1-13C]acetate (200 mg; 9.02 x 10⁶ μCi mmol⁻¹) was added to 2 culture flasks in equal portions on days 3, 4, 5, and 6 and the mycelium harvested after 9 days, herqueichrysin triacetate (30 mg, 2.90 x 10⁶ μCi mmol⁻¹) was obtained. This corresponds to a dilution factor of 3.1. On this basis it would be anticipated that equivalent feeding of [1-13C]acetate (95%) would give herqueichrysin triacetate with an excess of 3.4% of 13C-label over natural abundance at each labelled portion (assuming a total of 9 labelled portions in the molecule).

Incorporations of 13C Precursors.—Sodium [1-[13C]], [2-[13C]], and [1,2-[13C]₂]-acetate (200 mg) were added in aqueous solution to two shake cultures of P. herquei in equal portions on days 3, 4, 5, and 6 after inoculation; similarly with diethyl [2-[13C]₂]-malonate (250 mg) in ethanol. After 9 days growth the mycelium was harvested and the partially purified crude product mixture was transferred to a Soxhlet thimble and extracted with ethanolic sodium hydroxide (5 ml). The solution was acidified, diluted with water, and extracted with ethyl acetate to give a yellow gum which crystallised from methanol to give herqueichrysin as yellow needles (120 mg).

The assistance of Mrs. M. Anderson and Mrs. J. Rothschild in the mycological work is gratefully acknowledged. Professor L. C. Vining kindly supplied a sample of authentic herqueichrysin.

REFERENCES

17 J. S. E. Holker, R. D. Lapper, and T. J. Simpson, J.C.S. Perkin 1, 1974, 2135.

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PERKIN TRANSACTIONS I

1979

By Thomas J. Simpson.* The Robert Robinson Laboratories, The University, P.O. Box 147, Liverpool L69 3BX

Five C14 metabolites have been isolated from Aspergillus variecolor, and on the basis of their spectroscopic properties, in particular 1H and 13C n.m.r., and chemical correlations, structures (2)—(6) are proposed for these metabolites. They are thus closely related to the meroterpenoid andibenin-B (1), also isolated from A. variecolor and recently shown to have a mixed polyketide-terpenoid biosynthetic origin.

The fungal metabolite andibenin-B (previously named andibenum),† C25H32O6, was recently isolated in low yield from the liquors of static cultures of Aspergillus variecolor, and shown to have structure (1) by X-ray crystallography.1 This structure suggested a biogenesis from a polyisoprenoid, possibly sesterterpenoid precursor, so with a view to obtaining larger yields of andibenin for biosynthetic studies and biological testing, several wild-type and irradiation-induced mutant strains of A. variecolor available from previous studies2 were examined. One of the mutant strains, designated 212K-169, was found to produce yields of andibenin-B of ca. 50 mg l−1. Incorporation of singly and doubly labelled [13C]acetates and [methyl-13C]methionine into andibenin

<table>
<thead>
<tr>
<th>Compound</th>
<th>C-CH₃ *</th>
<th>1-H *</th>
<th>2-H *</th>
<th>6'-H *</th>
<th>7'-H *</th>
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<td>5.77</td>
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<td>2.87 *</td>
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</tbody>
</table>

* Singlet. † d, J 10 Hz. ‡ d, J 8 Hz. § d, J 12 Hz. ‴ s, exchangeable with D₂O. § dt. J 4 and 12 Hz.

The main features of their 1H n.m.r. spectra are summarised in Table 1, which shows that both andibenin-A (2) and andibenin-C (3) contain the cis-coupled vinylic protons (8 ca. 6.1 and 6.7) assigned to the spiro-δ-lactone ring in andibenin-B (1), five uncoupled methyls, and an AB coupled -CH₂-O- grouping (ca. 8 4.2) assigned to the δ-lactone ring in andibenin-B, but both lack the uncoupled vinylic proton assigned to the double bond exocyclic to the δ-lactone ring. This vinylic proton is replaced in (2) by mutually coupled doublets (J 8 Hz) at δ 2.88 and 4.15, and in (3) by a triplet at δ 2.88 (J 8 Hz). The 13C n.m.r. spectra indicate that the olefinic carbon resonances at δ 140.9 and 134.0 p.p.m. assigned to C-6 and -7, respectively, in (1) have been replaced by high-field aliphatic resonances in (3) and similarly the conjugated δ-lactone carbonyl resonance at δ 167.4 p.p.m. in (1) has moved downfield to δ 177.1 p.p.m. in (3), a shift indicative of loss of conjugation. These 1H and 13C n.m.r. data, together with the molecular formulae immediately suggest that andibenin-A and -C are related to andibenin-B by hydration and reduction respectively of the 6',7' double bond. This was readily confirmed.

Hydrogenation of andibenin-B (1) in ethyl acetate over Adams catalyst gave the tetrahydro-derivative (7). Similar reduction of andibenin-C (3) resulted in the uptake of one mole of hydrogen only, to give a product identical to (7) in all respects. Further, treatment of andibenin-B (1) with thionyl chloride in pyridine resulted in the loss of one molecule of water to give the tetrahydro-derivative (7). The 13C n.m.r. spectrum showed that the C-9 and -10 resonances at δ 52.7 and 77.2 p.p.m., respectively, in (1) were replaced by olefinic carbon resonances at δ 126.6 and 147.6 p.p.m. in (8), and the 1H

* Present address: Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland.
† Andibenins-A, -B, and -C are so named to conform with the corresponding andilesins, see preceding paper. Andibenin-A and andibenin-B correspond to the previously isolated dihydro-andibenin and deoxyandibenin.
n.m.r. spectrum of (8) showed the presence of an olefinic methyl (δ 1.55). Similar treatment of andibenin-A resulted in the loss of two molecules of water to give a product identical to (8), thus establishing structure (2) for andilesin-A.

Comparison of the molecular formulae and n.m.r. spectra of andilesin-A, -B, and -C suggested that the andilesins were related to one another in the same way as the andibenins and this was confirmed in an analogous manner. Catalytic hydrogenation in ethyl acetate over Adams catalyst converted andilesin-C (8) into the dihydro-product (9) identical to the product obtained from similar hydrogenation of andibenin-B (5). Treatment of andilesin-A (4) with thionyl chloride in pyridine resulted in dehydration of the secondary alcohol to give a good yield of a product identical to andilesin-B (5). Hydrogenation of andilesin-B or -C in methanol over palladium-charcoal resulted in quantitative yields of the ring-A seco-product (10) which will be discussed further below.

The relationship between the andibenins and andilesins was revealed by comparison of their molecular formulae and n.m.r. spectra. The andilesins all contain one less oxygen atom than the corresponding andibenins and the only significant difference in their 1H n.m.r. spectra is the upfield shift of the cis-vinylic protons from ca. δ 6.7 and 6.1 in the andibenins to ca. δ 5.8 and 5.9 in the andilesins. The 13C n.m.r. spectra of andibenin-B (1) and andilesin-B (5) are summarised in Table 2. Both compounds show resonances assignable to a ketonic carbonyl and two α,β-unsaturated lactone systems. The remaining resonances are all very similar in chemical shift and multiplicity, indicating the presence of identical carbon skeletons, except that the C-6 non-protonated and C-10 oxygen bearing non-protonated

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The relationship between the andibenins and andilesins was revealed by comparison of their molecular formulae and n.m.r. spectra. The andilesins all contain one less oxygen atom than the corresponding andibenins and the only significant difference in their 1H n.m.r. spectra is the upfield shift of the cis-vinylic protons from ca. δ 6.7 and 6.1 in the andibenins to ca. δ 5.8 and 5.9 in the andilesins. The 13C n.m.r. spectra of andibenin-B (1) and andilesin-B (5) are summarised in Table 2. Both compounds show resonances assignable to a ketonic carbonyl and two α,β-unsaturated lactone systems. The remaining resonances are all very similar in chemical shift and multiplicity, indicating the presence of identical carbon skeletons, except that the C-6 non-protonated and C-10 oxygen bearing non-protonated
carbon resonances observed at δ 46.7 and 77.2 p.p.m. respectively in andibenin-B have been replaced by a methyl carbon at δ 57.5 p.p.m. and a non-protonated carbon at δ 43.8 p.p.m. in andilesin-B. These differences can only be accommodated by replacing the 10-hydroxy-8-spiro-3-lactone A-B ring systems in the andibenins by the 6-lactone A-B ring system in the andilesins. Support for the presence of this 6-lactone in the andilesins is provided by the lack of hydroxy absorption in the i.r. spectrum of andilesin-B and comparison with the 13C chemical shifts observed for the corresponding carbons in the meliacoccic obacunone (12), shown in Table 2.

Chemical proof for the presence of the seven-membered lactone ring in the andilesins was given by the ready ring-opening of the lactone to the unsaturated acid (11), δ 1.74, 4.79, and 4.88 (Me=C-CH3), on treatment of dihydroandilesin-C (9) with mild acid. Treatment of 3a,4a,6a-dimethyl-4-oxa-a-homocholestan-8-one (13) with 10% sulphuric or hydrochloric acid in acetic acid gives a high yield of 4-methyl-4-methylene-3,4-secocholestan-3-olic acid (14). As stated above, attempted hydrogenation of andilen-C with palladium-charcoal in methanol resulted in formation of the seco-product (10), presumably due to ring-opening and esterification being catalysed by traces of acid in the catalyst followed by further reduction of the isopropylidene group.

The X-ray structure of andilesin-A (4) has been reported 7,8 and the above conclusions are in agreement with this. The stereochemistry shown in the above structures follows from the X-ray and c.d. work.

The andilesins may be seen as biosynthetic precursors, or branches from the biosynthetic pathway, to the andibenins. As stated above, preliminary 13C incorporation studies 5 indicate that andibenin-B is formed by prenylation of a tetraketide precursor, possible 3,5-dimethylorsellinic acid, by farnesyl pyrophosphate. In the light of this, an interesting feature of andibenin-B is the lack of an oxygen substituent on C-6' as inspection of known polyketides reveals that compounds which are in progress. Full details of the 13C assignments will be presented elsewhere.

EXPERIMENTAL

Unless otherwise stated, i.r. absorption spectra were measured with a Perkin-Elmer model 135 instrument for CHCl3 solutions, u.v. spectra with a Unicam SP 800 instrument for solutions in ethanol, 1H n.m.r. spectra with a Perkin-Elmer R34 instrument for solutions in deuteriochloroform containing tetramethylsilane as internal standard, 13C n.m.r. spectra with a Varian XL-100-15 Fourier-transform spectrometer for similar solutions, and optical rotations with an ETL-NPL automatic polarimeter for solutions in chloroform. Mass spectra were measured at 70 eV with an A.E.I. MS9 instrument. T.l.c. was performed using silica gel GF254 (Merck) in 0.5 mm thick layers on 20 cm x 20 cm plates eluted several times with methanol-chloroform (2:8 v/v). M.p.s were determined with a Kofler hot-stage instrument.

Isolation of Metabolites.—Aspergillus variecolor (strain 213K-169) was grown from a spore suspension in static culture for 15 days at 25°C in flat vessels (ca. 11 capacity), each containing Czapek-Dox medium (600 ml). The mycelium and liquors (ca. 5 l) were separated by filtration and the liquors were concentrated to ca. 1 l and extracted with ethyl acetate (3 x 500 ml). Evaporation of the solvent gave a brown semi-crystalline oil (700 mg) which on recrystallisation from ethyl acetate gave a crystalline solid (200 mg) which was further purified by preparative t.l.c. The least polar, strongly u.v. absorbing band was removed and recrystallised from ethyl acetate to give andilesin-B (15 mg), m.p. >310°C (lit., >310°C). The broad, weakly u.v. absorbing band of intermediate polarity was removed and recrystallised from ethyl acetate to give andilesin-C (24 mg), m.p. >300°C (lit., >290°C), δ 8.4 6.5 (c 0.8), δ 216.5 (s), 178.2 (s), 166.2 (s), 149.5 (d), 119.4 (d), 83.5 (s), 69.2 (t), 58.6 (s), 56.4 (d), 56.0 (s), 52.7 (t), 45.3 (s), 43.8 (s), 42.2 (d), 32.7 (t), 36.5 (d), 32.3 (t), 30.2 (d), 24.7 (t), 23.5 (q), 22.8 (q), 19.5 (q), and 16.8 (q) p.p.m. The broad, weakly u.v. absorbing band at highest polarity, was removed and recrystallised from ethyl acetate to give andilesin-A (78 mg), m.p. >300°C (lit., >310°C), δ 214.9 (s), 175.4 (s), 166.2 (s), 149.6 (d), 119.4 (d), 83.5 (s), 71.9 (d), 69.1 (t), 56.7 (s), 55.9 (d), 55.2 (s), 50.7 (d), 48.1 (s), 45.8 (s), 43.8 (s), 42.2 (t), 41.0 (d), 39.4 (t), 30.2 (d), 24.9 (t), 23.5 (q), 22.8 (q), 22.5 (q), 16.8 (q), and 16.1 (q) p.p.m. The mother-liquors (600 mg) were also chromatographed as above. The least polar, strongly u.v. absorbing band gave andilesin-B (33 mg), and the broad, strongly u.v. absorbing band of next highest polarity gave andibenin-B (210 mg), m.p. 218--220°C (lit., 219--220°C). A weakly u.v. absorbing band of only slightly higher polarity on removal and recrystallisation from ethyl acetate gave andilen-C (24 mg), m.p. 220--228°C (c 0.85), δ 86.5 (c 0.85), δ max 3450, 1770, 1720, and 1705 sh cm·1; [a]D26 1.1°, 3400, 1770, 1720, and 1705 sh cm·1; [a]D26 1.04, 1.15, 1.17, 1.40, 1.52 (all 3 H, s), 1.9--2.0 (12 H, m), 2.25 (2 H, m), 2.67 (2 H, m), 2.68 (1 H, t, J 8 Hz), 4.12 (1 H, d, J 11 Hz), 4.25 (1 H, d, J 11 Hz) (Found: C, 69.4; H, 7.7%); C25H34O2 requires C, 69.8; H, 7.5%. The band of highest polarity on removal and recrystallisation from ethyl acetate gave andilen-A (7 mg), m.p. 281--284°C, δ 816.3 (c 0.75), δ max 3600, 3400, 1770, 1720, and 1700 cm·1; [a]D26 0 (Found: C, 67.4; H, 7.2%); C25H34O2 requires C, 67.8; H, 7.3%).

Hydrogenation of Andibenin-B.—Andibenin-C (100 mg) in ethyl acetate (50 ml) was stirred at room temperature and atmospheric pressure in the presence of Adams catalyst (100 mg) for 16 h to give after removal of catalyst and solvent a gum which recrystallised from ethyl acetate to give tetrathydroandibanin-B, m.p. 187--200°C, δ 0--7.80 (c 1.10), 3400, 1770, and 1720 cm·1, δ 0.84, 1.04, 1.15, 1.17, 1.40, 1.52 (all 3 H, s), 1.9--2.0 (12 H, m), 2.25 (2 H, m), 2.67 (2 H, m), 2.68 (1 H, t, J 8 Hz), 4.12 (1 H, d, J 11 Hz), 4.25 (1 H, d, J 11 Hz) (Found: C, 69.4; H, 7.7%); C25H34O2 requires C, 69.7; H, 6.0%). Similar hydrogenation of andibenin-C, resulted in the uptake of 1 mole of...
hydrogen to give a quantitative yield of a product identical in all respects to tetrahydroandibenin-B.

Dehydration of Andibenin-B.—Andibenin-B (96 mg) was stirred in pyridine (5 ml), cooled in an ice-salt bath and thionyl chloride (1 ml) added, and stirring continued overnight. The mixture was poured into ice-water and extracted into chloroform. The chloroform layer was washed with water and the solvent removed to give an oily product which was purified by t.l.c. using two 20 cm x 20 cm plates eluted with chloroform-methanol (93 : 7 v/v). The broad band at Rf 0.7 was removed and eluted with ethyl acetate to give a gum (8 mg) which crystallised from ethyl acetate to give an almost quantitative yield of tetrahydroandilesin-C (100 mg) in ethyl acetate over Adams catalyst (100 mg) for 10 h gave a quantitative yield of the dehydration product (8), m.p. 277—279 °C, \( \delta^a_p 664.7 °C \), \( \lambda_{max} 250 \text{ and } 310 \text{ nm (c 3 700 and 300)} \), \( \delta_H 1.15, 1.27, 1.37, 1.39, 1.55 \text{ (all 3 H, s), 1.4—1.9 (6 H, m), 2.23 (1 H, d, } J 17 Hz \), 2.52 (1 H, d, } J 17 Hz \), 4.38 (2 H, s), 6.07 (1 H, d, } J 10 Hz \), 6.37 (1 H, d, } J 10 Hz \), and 7.04 (1 H, s); \( \delta_C 212.7 \text{ (s), 166.5 \text{ (s), 163.6 \text{ (s), 146.3 \text{ (d), 142.6 \text{ (s), 140.2 \text{ (d), 132.7 \text{ (s), 126.6 \text{ (s), 119.4 \text{ (d), 85.4 \text{ (s), 66.9 \text{ (t), 58.9 \text{ (s), 51.1 \text{ (s), 49.9 \text{ (s), 49.6 \text{ (s), 47.4 \text{ (s), 44.8 \text{ (s), 41.5 \text{ (t), 27.7 \text{ (t), 27.2 \text{ (t), 24.8 \text{ (q), 23.4 \text{ (q), 18.1 \text{ (q), 17.2 \text{ (q), and 15.3 \text{ (q) ppm. (Found: } C, 73.1; \text{ H, 6.7. } C_{25}H_{34}O_5 \text{ requires } C, 73.5; \text{ H, 8.9%.}} \)

Dehydration of Andibenin-A.—Andibenin-A (10 mg) was treated with thionyl chloride in pyridine as above to give dehydration product (8), m.p. 277—279 °C.

Hydrogenation of Andibenin-B.—Andibenin-B (100 mg) was stirred in ethyl acetate (50 ml) over Adams catalyst (100 mg) for 15 h. Removal of catalyst and solvent gave a solid which crystallised from ethyl acetate to give an almost quantitative yield of tetrahydroandibenin-B, m.p. 295—297 °C, \( \delta^a_p 7.8 °C \), \( \lambda_{max} 1770 \text{ and } 1200 \text{ cm}^1 \); \( \delta_H 1.02, 1.04, 1.20, 1.38, 1.42 \text{ (all 3 H, s), 1.2—2.0 (12 H, m), 2.63 (1 H, dd, } J 14 \text{ and } 5 \text{ Hz), 2.76 (1 H, d, } J 14 \text{ Hz), 2.87 (1 H, t, } J 8 \text{ Hz), and 4.21 (2 H, s) (Found: } C, 72.0; \text{ H, 8.2. } C_{24}H_{24}O_5 \text{ requires C, 72.4; H, 8.3%).}} \)

Hydrogenation of Andidesin-C.—(a) Hydrogenation of andidesin-C (100 mg) in ethyl acetate over Adams catalyst as above gave an almost quantitative yield of tetrahydroandidesin-C, m.p. 295—297 °C.

(b) Hydrogenation of andidesin-C (90 mg) in methanol (50 ml) over 10% palladium-charcoal catalyst (75 mg) for 10 h gave a quantitative yield of the methyl ester (10) as a gum which could not be crystallised; \( \lambda_{max} 1775 \text{ and } 1711 \text{ cm}^1 \); \( \delta_H 1.02 \text{ (3 H, s), 1.08 \text{ (3 H, s), 1.16 \text{ (6 H, d, } J 7 \text{ Hz), 1.27 (3 H, s), 1.2—2.0 (15 H, m), 2.24 \text{ (2 H, m), 2.74 (1 H, d, } J 8 \text{ Hz), 3.67 (3 H, s), 4.23 (1 H, d, } J 10 \text{ Hz), and 4.22 (1 H, d, } J 10 \text{ Hz) (Found: } M+, 430.2719. \text{ C}_{24}H_{29}O_5 \text{ requires } M, 430.2718 \).

Dehydration of Andilesin-A.—Andilesin-A (100 mg) was treated with thionyl chloride in pyridine as above. The usual work-up and purification by t.l.c. gave a gum (70 mg) which on recrystallisation gave andilesin-B.

Acid-catalysed Isomerisation of Tetrahydroandilesin-B.—Tetrahydroandidesin-B (100 mg) was treated with 20% hydrochloric acid in acetic acid (1 ml) and dichloromethane (2 ml) at room temperature for 24 h. After dilution with ether (20 ml), the solution was washed with water (2 x 20 ml), saturated sodium hydrogencarbonate solution (2 x 20 ml), and water (3 x 20 ml), and dried (MgSO₄). The ether was removed in vacuo to give the acid (11) as a gum (90 mg which could not be crystallised, \( \lambda_{max} 3.300—2.600, 1770 \text{ and } 1720 \text{ cm}^1 \); \( \delta_H 1.02 \text{ (6 H, s), 1.15 \text{ (3 H, s), 1.74 \text{ (3 H, s), 1.2—2.3 (ca. } 17 \text{ Hz, m), 2.87 (1 H, t, } J 8 \text{ Hz), 4.22 \text{ (2 H, s), 4.70 (1 H, s), and 4.88 (1 H, s) (Found: } M^+, 414.2415. \text{ C}_{23}H_{23}O_5 \text{ requires } M, 414.2460 \).

The support of the S.R.C. is gratefully acknowledged.

[8/1514 Received, 17th August, 1978.]

REFERENCES


The Structures and Biosynthesis of Multicolanic, Multicolic, and Multicolosic Acids, Novel Tetronic Acid Metabolites of Penicillium Multicolor

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Received February 1, 1979

Multicolanic, multicolic, and multicolosic acids, metabolites of Penicillium multicolor, are shown by chemical transformations and spectroscopic methods to be 4-ylidenetetronic acids with structures (I), (II), and (III), respectively. The biosynthesis of these metabolites from acetate, via oxidative fission of preformed 6-pentyresorcylic acid is established by incorporation studies with [1-13C]acetate and ethyl [2-13C]-6-pentyresorcylic.

Two new optically inactive crystalline metabolites multicolic acid, C_{11}H_{14}O_6 (II), and multicolosic acid, C_{11}H_{12}O_7 (III), have been isolated from the fermentation liquors of a strain of Penicillium multicolor (CM1 104602), which has previously been reported to produce pencolide (I). Evidence for the presence of a third compound, multicolanic acid, C_{11}H_{14}O_3 (I), has also been obtained. Spectral and chemical evidence leading to the structures of these compounds and the elucidation of their biosynthesis is now reported in full.

Three acidic groups in multicolosic acid (III) were demonstrated by potentiometric titration against sodium hydroxide when ionizations with pKs of 2.9, 9.8, and 11.4, respectively, were observed. Furthermore, methylation with diazomethane gave dimethyl O-methylmulticolosate (VI). Under similar conditions multicolic acid (II) gave methyl O-methylmulticolate (V) showing two acidic groups in the parent. When the crude fungal extract was similarly treated with diazomethane, prior to chromatography, a small amount of methyl O-methylmulticanolate (IV) was isolated, in addition to larger amounts of the two previous derivatives, (V) and (VI), respectively. Clearly, methyl O-methylmulticolate (IV) is derived from the parent multicolanic acid (I), but attempts to isolate the latter have been unsuccessful.

Hydrogenation of multicolic acid (II) gave a dihydro-derivative (VIII), which showed spectral properties characteristic of a tetronic acid chromophore, viz \( \lambda_{\max} (\text{EtOH}) = 234 \text{ nm (e 7000)} \); \( \lambda_{\max} (\text{EtOH–KOH}) = 262 \text{ nm (e 12 000)} \), cf \( \alpha \)-ethyltetronic acid, \( \lambda_{\max} (\text{EtOH}) = 233 \text{ nm (e 12 000)} \); \( \lambda_{\max} (\text{EtOH–KOH}) = 258 \text{ nm (e 18 000)} \) (J). Hydrogenation of methyl O-methylmulticolate (V) and dimethyl O-methylmulticolosate (VI) similarly

1 This work is part of a series, X, “The Biosynthesis of Fungal Metabolites.” This series of papers and many other related contributions are based on work carried out in Liverpool during the occupancy of the Heath Harrison Chair by the late Professor G. W. Kenner. Throughout this period of 21 years the senior author (J.S.E.H.) received enormous academic stimulation, great encouragement, and warm friendship from George Kenner, to whom this paper is gratefully dedicated.

2 To whom correspondence should be addressed.

3 A preliminary communication on part of this study has already been published (2).

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gave dihydro-derivatives, (IX) and (X), respectively. The residue $\text{C}=\text{CH} \cdot \text{CO}_2\text{Me}$ in compounds (IV), (V), and (VI) was shown by a vinylic proton, $\tau$ ca. 4.15, in their $^1\text{H}$ nmr spectra (Table 1) and resonances at ca. 164, 150, 101, and 52 ppm in their $^{13}\text{C}$ nmr spectra (Table 2). In the dihydro-derivatives (IX) and (X), these were replaced by the diagnostic ABX pattern ($\tau_\text{A} 7.15$, $\tau_\text{B} 7.50$, $\tau_\text{X} 5.01$; $J_{\text{AX}} 4$ Hz, $J_{\text{BX}} 8$ Hz, and $J_{\text{AB}} 16$ Hz) in the $^1\text{H}$ nmr spectra and resonances at 169, 73.5, 38, and 52 ppm in the $^{13}\text{C}$ nmr spectra. Conjugation of this residue with the tetronic acid chromophore was apparent from the change in u.v. spectra on hydrogenation. Thus methyl O-methylmulticolate (V) had $\lambda_{\text{max}}$ (EtOH) 266 nm ($\varepsilon$ 11 600), whereas the corresponding dihydro-derivative (IX) had $\lambda_{\text{max}}$ (EtOH) 227 nm ($\varepsilon$ 7100). No further reduction takes place, consistent with the known resistance of the tetronic acid chromophore to hydrogenation (3).
Singlet.

\( J_x, J_y \leq 16 \text{ Hz}, J_{xy} \leq 4 \text{ Hz}, J_{xz}, J_{yz} \leq 8 \text{ Hz} \).

Triplet (7 Hz).

Broad multiplet.

\( \text{CDCI}_3/\text{d}-\text{DMSO}. \)

The remainder of the multicolic acid structure is accounted for by the residue \(-(\text{CH}_2)_4 \cdot \text{CH}_2 \text{OH}. \) This was clearly demonstrated by the \( ^1\text{H} \) nmr spectrum of methyl \( O\)-methylmulticolate (V): \( \tau \ 6.41 \ (2\text{H}, \text{t}, J 7 \text{ Hz}), 7.52 \ (2\text{H}, \text{t}, J 7 \text{ Hz}), 7.75 \ (1\text{H}, \text{d}) \).

### TABLE 1

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<th>Compound</th>
<th>10-H (^a)</th>
<th>10-CH (_2) (^b)</th>
<th>5-CH (_2) (^e)</th>
<th>6-CH (_2) (^d)</th>
<th>7-CH (_2) (^d)</th>
<th>8-CH (_2)</th>
<th>4-H (^b)</th>
<th>Me-O (^a)</th>
<th>Others</th>
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<tr>
<td>(II)(^c)</td>
<td>4.10</td>
<td>7.66</td>
<td>8.5</td>
<td>8.5</td>
<td>8.5 (^d)</td>
<td></td>
<td></td>
<td>6.38 (6 Hz) 9-CH (_2)</td>
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<tr>
<td>(III)(^d)</td>
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<td>7.70</td>
<td>8.4</td>
<td>8.4</td>
<td>7.70 (^e)</td>
<td></td>
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<tr>
<td>(IV)(^e)</td>
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<td>7.52</td>
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<td>8.5</td>
<td>8.5 (^d)</td>
<td></td>
<td></td>
<td>5.93, 6.26 9.08 (7 Hz) 9-Me</td>
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<tr>
<td>(V)(^f)</td>
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<td>7.52</td>
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<td>8.5</td>
<td>8.5 (^d)</td>
<td></td>
<td></td>
<td>5.93, 6.28 6.41 (7 Hz) 9-CH (_2)</td>
<td>7.75 CH (_2)OH</td>
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<tr>
<td>(VI)(^g)</td>
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<td>8.4</td>
<td>7.66 (^f)</td>
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<td></td>
<td>5.93, 6.26 6.36</td>
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<tr>
<td>(VII)(^f)</td>
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<td>8.5 (^d)</td>
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<td>5.94, 6.26 5.97 (7 Hz) 9-CH (_2)</td>
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<td>2.5–4.0</td>
<td>(3H, exchangeable)</td>
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<td>(IX)(^i)</td>
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<td>8.4</td>
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<td>4.98</td>
<td></td>
<td>5.96, 6.30</td>
<td>6.36</td>
</tr>
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</table>

\(^a\) Singlet.

\(^b\) ABX system \( J_{xy} \leq 16 \text{ Hz}, J_{xx} \leq 4 \text{ Hz}, J_{yy} \leq 8 \text{ Hz} \).

\(^c\) Triplet (7 Hz).

\(^d\) Broad multiplet.

\(^e\) CDCl\(_3\)/\text{d}-\text{DMSO}.

\(^f\) CDCl\(_3\).

\(^g\) d\(_4\)-DMSO.

The remainder of the multicolic acid structure is accounted for by the residue \(-(\text{CH}_2)_4 \cdot \text{CH}_2 \text{OH}. \) This was clearly demonstrated by the \( ^1\text{H} \) nmr spectrum of methyl \( O\)-methylmulticolate (V): \( \tau \ 6.41 \ (2\text{H}, \text{t}, J 7 \text{ Hz}), 7.52 \ (2\text{H}, \text{t}, J 7 \text{ Hz}), 7.75 \ (1\text{H}, \text{d}) \).

### TABLE 2

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<th>Carbon</th>
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<th>(VI)</th>
<th>(IX)</th>
<th>(X)</th>
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<td>168.1s</td>
<td>173.2s</td>
<td>173.2s</td>
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<tr>
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<tr>
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<td>73.5d</td>
<td>73.5d</td>
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<td>101.0d</td>
<td>101.0d</td>
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<tr>
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<tr>
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<td>29.2t</td>
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<td>25.5t</td>
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<td>33.4t</td>
<td>32.3t</td>
<td>33.5t</td>
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<td>62.2t</td>
<td>172.9s</td>
<td>62.3t</td>
<td>173.2s</td>
</tr>
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<td>59.5q</td>
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<td>11 MeO</td>
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<td>52.0q</td>
<td>52.0q</td>
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<td>9 MeO</td>
<td>51.4q</td>
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</table>
exchangeable in D$_2$O, and 8.50 (6H, m). The presence of the primary alcoholic hydroxyl group was established by acetylation of methyl O-methylmulticolinate to give the acetate (VII) and by oxidation with chromic oxide to give the corresponding carboxylic acid, which was not characterized but converted directly to dimethyl O-methylmulticolosate (VI) with diazomethane. The latter experiment confirms the structural relationship between multicolic and multicolosic acids. Comparison of the $^1$H and $^{13}$C nmr spectra of methyl O-methylmulticolanolate (IV) and methyl O-methylmulticololate (V) (Tables 1 and 2, respectively) clearly demonstrates the presence of the pentyl side-chain in the former compound, compared with the pentanol side-chain in the latter.

**TABLE 3**

<table>
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<tr>
<th>Position</th>
<th>$^1$H</th>
<th>$^{13}$C</th>
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<tr>
<td>2</td>
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</tr>
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</tr>
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<tr>
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<td>0.40</td>
</tr>
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<tr>
<td>3-OMe</td>
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<td>0.20</td>
</tr>
<tr>
<td>11-OMe</td>
<td>0.18</td>
<td>0.12</td>
</tr>
</tbody>
</table>

*Values quoted are for ratios of Eu(fod)$_2$:compound of 2:5 and 1:10 for $^1$H- and $^{13}$C-spectra, respectively.*

The $^{13}$C assignments in Table 2 are based on comparisons of the proton noise decoupled (pnd) and off-resonance decoupled spectra, standard chemical shift data (4), and lanthanide induced shift (LIS) studies on methyl O-methylmulticolate (V) (Table 3). The latter showed that the principal site of coordination was the primary alcoholic hydroxyl group. The study permitted unambiguous assignments of the $^{13}$C-chemical shifts of the individual side-chain carbons, and separate resolution of the individual pairs of methylene protons in the $^1$H nmr spectrum. The $^{13}$C-assignments of C-1 and C-3 are made by analogy with the corresponding spectrum of penicillic acid (XI), which has been assigned unambiguously (5). These represent a reversal of our original assignments for compound (V), which were made (2) prior to publication of the work on penicillic acid.

The $^{13}$C nmr spectrum of compound (V), derived from multicolic acid enriched by feeding of [1,2-$^{13}$C]acetate to cultures of *F. multicolor* (see below), showed $^{13}$C-$^{13}$C couplings of 48 and 90 Hz between C-2 and C-5 and between C-4 and C-10,
respectively. These values, which are typical for those of sp$^2$–sp$^3$ and sp$^2$–sp$^2$ hybridized coupled carbons, respectively, confirmed the positions of the substituent groups in the tetronic acid chromophore. It is worthy of note that the alternative type of structure (XII), which would be consistent with most of the structural data, and also biogenetically reasonable (see below), is excluded by the observation of the ABX system (see above) in the $^1$H nmr spectra of the dihydro-derivatives (IX) and (X) and by the multiplicities observed for C-4 and C-10 in their off-resonance decoupled $^{13}$C nmr spectra.

The only remaining ambiguity in the structures of this group of tetronic acids is the stereochemistry around the 4,10-double bond. It was observed that on prolonged standing, samples of the methylated derivative (IV), (V), and (VI) which had been recovered from CDCl$_3$ solutions, but not further purified, were partially isomerized to compounds in which the vinyl proton resonated at ca. $\tau$ 4.4, compared with 4.10 in the original compounds. This equilibration is presumably due to traces of acid remaining after removal of the solvent. This observation suggested the E-stereochemistry for the natural products, as the vinyl proton in the corresponding Z-isomers would be expected to resonate at higher field due to the shielding effect of the tetronic acid enolic ether oxygen atom. This conclusion has been confirmed by recent synthetic studies. Methyl O-methylmulticolate (IV), the corresponding Z-isomer, together with the E- and Z-isomers of the 2-methyl analog (XIII), have been synthesized; X-ray crystallographic studies were carried out on the latter (6). It is found that the isomer in which the vinyl proton resonates at lower field does indeed have the E-stereochemistry. Furthermore, multicolane acid (I) has been synthesized and shown to give the bislactone (XIV) on dehydration with bicyclohexylcarbodiimide (7), which could only arise from the E-isomer.

Two separate biosynthetic pathways to tetronic acids have been established: (a) oxidative ring cleavage of an aromatic or quinonoid intermediate, which may itself be polyketide or shikimate derived, and (b) condensation of a poly-$\beta$-ketide derived $\beta$-ketoacid with a C$_4$-dicarboxylic acid from the Krebs’s Cycle (8). In the case of the new tetronic acids from P. multicolor, the biosynthesis was demonstrated by $^{13}$C-incorporation experiments. Extracts from this organism, to which [1-$^{13}$C]-, [2-$^{13}$C]-, and [1,2-$^{13}$C]acetate had been fed were methylated and separated in the usual way to give suitably enriched samples of methyl O-methylmulticolate (V) and dimethyl O-methylmulticolosate (VI). The pnd $^{13}$C nmr spectra of these compounds are summarized in Tables 4 and 5, respectively. The spectra from [1-$^{13}$C]- and [2-$^{13}$C]acetate-derived samples show that all the carbon atoms of the multicolanic and multicolosic acid skeleta are acetate derived. However, the spectra from the [1,2-$^{13}$C]acetate-derived samples show $^{13}$C–$^{13}$C couplings only in the carbon atoms 8–9, 6–7, 2–5, and 4–10, indicating that these are the only four intact acetate residues in the molecules. The complete absence of couplings in carbons 1, 3, and 11 indicate their origin from acetate units which have been cleaved during biosynthesis.

These results are best accommodated by the assumption that the poly-$\beta$-ketide derived 6-pentyresorcylic acid (XV) is a biosynthetic intermediate, as shown in Scheme 1. However, it is necessary to postulate that this intermediate is not converted at any stage to a symmetrical aromatic compound, e.g., 5-pentyresorcinol (XVI), since this would give rise to scrambling of $^{13}$C–$^{13}$C couplings in the compounds derived from [1,2-
### Table 4

**13C-Enrichment Data for Methyl O-Methylmulticolate (V)**

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</table>

* Average enrichment: 3.6 atom% 13C over natural abundance.

### Table 5

**13C-Enrichment Data for Dimethyl O-Methylmulticolate (VI)**

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</tr>
<tr>
<td>11</td>
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</table>

* Average enrichment: 1.2 atom% 13C over natural abundance.

### Notes

13C acetate. A possible intermediate is the trihydric phenol (XVIII), which could arise from (XV) via an arene oxide of type (XVII). Ring scission would have to occur at the position shown in (XVIII) to give the diacid (XIX). Enol lactone formation could then lead either to the observed metabolites (I), (II), and (III) or to a compound of type (XII). It is interesting that the latter has not been detected as a natural product, either because of enzyme mediation in the formation of tetronic acids (I), (II), and (III) or possibly because of a compound of type (XII) is much less thermodynamically stable than the observed compounds. This point is being investigated.
Scheme 1. Incorporations of [1-13C], [2-13C] and [1,2-13C]acetate into metabolites of *P. multicolor* (heavy bonds denote inact acetate residues).
SCHEME 2. Distribution of label from ethyl [2-14C]-6-pentylresorcylicate (RMA = relative molar activity).
To confirm the intermediacy of 6-pentyresorcylic acid on the biosynthetic pathway, ethyl [2-\(^{14}\)C]-6-pentyresorcylic acid was synthesized from ethyl [3-\(^{14}\)C]acetooacetate and methyl oct-2-enoate (9) and fed to cultures of \(P.\) multicolor. Methyl \(O\)-methylmulticololate and dimethyl \(O\)-methylmulticolosicate were isolated in the usual way, after methylation, and found to contain 0.25% of the fed radiolabel. To establish the specificity of labeling the former compound (4.01 \(\times\) 10\(^{-3}\) \(\mu\)C mmol\(^{-1}\)) was degraded by dilute sulfuric acid to the \(\alpha\)-diketone (XX), which was isolated as its quinoxaline derivative (XXI) (3.92 \(\times\) 10\(^{-3}\) \(\mu\)C mmol\(^{-1}\)) (see Scheme 2). Kuhn–Roth oxidation of this compound gave acetic acid, which was characterized as its \(p\)-bromophenacyl ester (3.63 \(\times\) 10\(^{-3}\) \(\mu\)C mmol\(^{-1}\)). Schmidt degradation of the acetate gave methylamine, which was isolated as the hydrochloride and found to contain negligible radioactivity. Hence essentially all of the radioactivity of the acetate is located in the carboxyl group. Since this accounts for 90% of the total activity of the methyl \(O\)-methylmulticololate, and corresponds to C-4 of this compound, it is clear that 6-pentyresorcylic acid is a specific precursor, which is incorporated with minimal randomization of label. It seems likely that o-oxidation of the pentyl side-chain to give multicolic and multicolosic acids (II) and (III), respectively, occurs at a late stage in the biosynthetic sequence, although this has not yet been established.

In many respects the biosynthesis of multicolic acid is analogous to that of the extensively investigated compound, penicillic acid (XI) (8). In this case the precursor has been shown to be orsellinic acid, which undergoes \(O\)-methylation at the 2-position, followed by exactly analogous oxidative decarboxylation and ring scission reactions to those observed above. In this case 2-methyl-6-methoxybenzoquinone has been shown to be an intermediate (10). The absence of symmetrical intermediates in penicillic and multicolic acid biosynthetic pathways is in direct contrast to ravenelin (11) and griseofulvin (12) pathways, where studies with [1,2-\(^{13}\)C]acetate have demonstrated scrambling of couplings, presumably arising from the participation of intermediates containing symmetrically substituted aromatic rings.

**EXPERIMENTAL SECTION**

Unless otherwise stated, ir spectra were measured for solutions in carbon tetrachloride with a Perkin–Elmer 125 instrument, uv spectra for solutions in ethanol with a Unicam SP800 instrument, \(^1H\) nmr spectra with a Varian HA-100 or XL-100 instrument, the latter also being used for \(^{13}\)C nmr spectra. Mass spectra were determined with an A.E.I. MS-12 instrument at 70 eV, and accurate masses with an A.E.I. MS-9 instrument. tlc was performed on silica gel GF.254 (Merck). mp’s were determined with a Kofer hot-stage instrument. For general details of \(^{13}\)C-incorporation studies, see Part IV (13).

**Isolation of metabolites.** Penicillium multicolor (CMI 104602) was grown from spore suspension in static culture for 8 days at 25°C in 16 flat vessels (ca. 1 liter capacity), each containing Raulin-Thom medium (500 ml). After filtration, the culture broth was basified with a saturated solution of potassium carbonate, extracted with ethyl acetate, until the organic phase was colorless, acidified with concentrated hydrochloric acid, and reextracted with ethyl acetate. After removal of the solvent, a solution of the residual
brown gum (10 g) in ether containing 5% methanol (200 ml) was filtered and silica gel (300 g, Grace 200–300 mesh) was stirred in. After evaporation of the solvent, benzene (500 ml) was added, and the resultant slurry added to the top of a column (90 × 12 cm) of silica gel (2 kg) in benzene. Gradient elution was then carried out using benzene, containing increasing proportions of ether:dichloromethane (1:1). Five hundred ml fractions were collected and after every fourth fraction the concentration of ether:dichloromethane was increased by 1%, so that after 200 fractions had been taken, a mixture of benzene:ether:dichloromethane (2:1:1) was being used. Slowing increasing proportions of methanol were then added until most of the color had been removed from the column.

**Multicosic acid (III)** was obtained in fractions 100–116 (ether:dichloromethane:benzene, 1:1:20) and separated from dichloromethane in needles (500 mg), mp 150–153°C, \(\nu_{\text{max}}\) (CH\(_2\)Cl\(_2\)) 3460, 3200, 2800–2400, 1785, 1640 cm\(^{-1}\), \(\lambda_{\text{max}}\) 262, 295 nm (e 15 000 and 8000), m/e 256 (0.08), 239 (0.23), 238 (1.00), 221 (0.15), 220 (0.88), 210 (0.40), 207 (0.20), 192 (0.45), 182 (0.82), 178 (0.45), 164 (0.55), m*/e 221.2 (256–238), 205.4 (238–221), 203.4 (238–220), 193.4 (221–207), 185.5 (238–210), 147.6 (182–164) (Found: C, 51.6; H, 4.7. C\(_{11}\)H\(_{12}\)O\(_7\) requires C, 51.5; H, 4.7%).

**Multicosic acid (II)** was obtained in fractions 190–215 (while the first trace of methanol was being added) and separated from dichloromethane in needles (1.2 g), mp 129–131°C, \(\nu_{\text{max}}\) (CH\(_2\)Cl\(_2\)) 3420, 2920, 2850, 1785, 1700, 1640 cm\(^{-1}\), \(\lambda_{\text{max}}\) 263, 295 nm (e 15 000 and 8000), m/e 242 (0.30), 224 (0.24), 207 (0.06), 206 (0.50), 196 (0.15), 182 (0.80), 178 (0.40), 164 (1.00), 157 (0.18), m*/e 207.3 (242–224), 189.4 (224–206), 161.7 (196–178), 147.8 (182–164) (Found: C, 54.0; H, 5.8. C\(_{11}\)H\(_{14}\)O\(_6\) requires C, 54.5; H, 5.8%).

**Methyl O-methylmulticolanate (IV), methyl O-methylmulticolate (V), and dimethyl O-methylmulticolosate (VI).** (a) Compounds (V) and (VI) were obtained as colorless gums from multicosic and multicosolic acids, respectively, by treatment with excess ethereal diazomethane in the usual way. Methyl O-methylmulticolate (V) had \(\nu_{\text{max}}\) 3605, 3500 (br), 1742, 1665, and 1185 cm\(^{-1}\), \(\lambda_{\text{max}}\) 266 nm (e 11 600) (Found: m/e, 270.110. C\(_{13}\)H\(_{18}\)O\(_5\) requires m/e, 270.110). Dimethyl O-methylmulticolosate (VI) had \(\nu_{\text{max}}\) 1793, 1739, 1718, 1672, and 1643 cm\(^{-1}\), \(\lambda_{\text{max}}\) 266 nm (e 11 500) (Found: m/e, 298.105. C\(_{14}\)H\(_{18}\)O\(_5\) requires m/e, 298.106). The acetate (VII) was prepared from methyl O-methylmulticotate (50 mg) with acetic anhydride/pyridine at room temperature for 4 hr, in the usual way. Purified by preparative tlc, with ether as developing solvent, it was obtained as an oil (48 mg), \(\nu_{\text{max}}\) 1786, 1738, 1635, and 1230 cm\(^{-1}\), \(\lambda_{\text{max}}\) 266 nm (e 10 500) (Found: m/e, 312.123. C\(_{14}\)H\(_{18}\)O\(_4\) requires m/e, 312.123). (b) The crude extract from the organism was methylated with diazomethane and the total product partitioned by preparative tlc with ether as the developing solvent. Methyl O-methylmulticotate (60 mg liter\(^{-1}\) of culture broth) was eluted with ethyl acetate from a band with \(R_f\) 0.3, and dimethyl O-methylmulticoslate (55 mg liter\(^{-1}\)) from a band with \(R_f\) 0.45. A third band, \(R_f\) 0.8, was isolated and further purified by multiple elution tlc, using light petroleum (bp 60–80°C):ether (1:1) as developing solvent. A band with \(R_f\) 0.2 was extracted in ethyl acetate to give methyl O-methylmulticoslate (IV) as an oil (3 mg liter\(^{-1}\)), \(\nu_{\text{max}}\) 1775, 1727, 1630, and 1138 cm\(^{-1}\), \(\lambda_{\text{max}}\) 256 nm (e 12 600) (Found: m/e, 254.116. C\(_{13}\)H\(_{18}\)O\(_4\) requires m/e, 254.116).
Oxidation of methyl-O-methylmulticolate. To a solution of this compound (60 mg) in acetone (5 ml) at 0°C, Jones reagent (three drops) was added, and after 5 min the reaction was quenched with ethanol (1 ml). The product was isolated in ethyl acetate (2 x 10 ml), after dilution of the reaction mixture with water (20 ml), and methylated with ethereal diazomethane. Purified by tlc dimethyl O-methylmulticolosate, with identical spectral properties to those above, was isolated as a gum (40 mg).

Dihydromulticolic acid (VIII), methyl O-methylidihydromulticololate (IX), and dimethyl O-methylidihydromulticolosate (X). Multicolic acid, methyl O-multicololate and dimethyl O-methylmulticolosate were each hydrogenated in ethyl acetate containing 10% palladium on carbon, with hydrogen gas at atmospheric pressure and room temperature, to give quantitative yields of the respective dihydro-derivatives. Thus obtained, dihydromulticolic acid (VIII) was an amorphous solid, \( \lambda_{\text{max}} \) 234 nm (e 6900), \( \lambda_{\text{max}} \) (EtOH–NaOH) 262 nm (e 12 400), methyl O-methylidihydromulticololate (IX), an oil, \( \lambda_{\text{max}} \) 227 (e 7100), \( \nu_{\text{max}} \) (CHCl₃) 1740 and 1665 cm⁻¹ (Found: m/e 272.126. C₁₃H₂₀O₆ requires m/e 272.126), and dimethyl O-methylidihydromulticolosate (X), an oil, \( \lambda_{\text{max}} \) 228 (e 7200), \( \nu_{\text{max}} \) (CHCl₃) 1735 and 1665 cm⁻¹ (Found: m/e 300.119. C₁₄H₂₀O₇ requires m/e 300.118).

Incorporations of [1-¹³C]-, [2-¹³C]-, and [1,2-¹³C]acetate into the metabolites. Sodium [1-¹³C] and [2-¹³C]acetate (200 mg, respectively) were each added to a 7-day-old growth culture pan of *P. multicolor*. After a further 3 days the culture liquors were extracted and methylated in the usual way to give [¹³C]methyl O-methylmulticololate (92 and 84 mg, respectively, from [1-¹³C]- and [2-¹³C]acetate) and [¹³C]dimethyl O-methylmulticolosate (63 and 67 mg, respectively). Similarly [1,2-¹³C]acetate (250 mg) was added to a 6-day-old culture pan of *P. multicolor* to give, after work-up and isolation, [¹³C]methyl O-methylmulticololate (70 mg) and [¹³C]dimethyl O-methylmulticolosate (30 mg).

Ethyl [2-¹⁴C]-6-pentyldihydroresorcylate. Prepared from ethyl [3-¹⁴C]acetoacetate (483 mg, 134.9 mCi mmol⁻¹) and methyl oct-2-enoate (478 mg) by the literature method (9), ethyl [2-¹⁴C]-6-pentyldihydroresorcylate was obtained as prisms (535 mg, 134.1 mCi mmol⁻¹) from light petroleum (bp 40–60°C), mp 64°C [lit. mp (14) 64-65°C]. Bromination of this compound (530 mg) with bromine (1 g) in acetic acid (1.3 ml), by the literature method (9) gave a mixture which was separated by chromatography on silica. Elution with light petroleum (bp 60–80°C):acetone (1:1) gave successively ethyl [2-¹⁴C]dibromo-6-pentyldihydroresorcylate, prisms (320 mg, 134.0 mCi mmol⁻¹), mp 66–67°C [lit. mp (14) 67°C]. From light petroleum (bp 40–60°C) and [1,3-¹⁴C]-2,4,6-tribromo-5-pentyldihydroresorcinol, needles (100 mg) from light petroleum (bp 40–60°C), mp 64–65°C, \( \nu_{\text{max}} \) 2475, 1520, 1358, 1110 cm⁻¹, \( \lambda_{\text{max}} \) 213 nm (e 13 000), r 3.91 (2H, s, exchangeable), 7.03 (2H, t, J 7.5 Hz), ca. 8.5 (6H, m) and 9.09 (3H, t, J 5.5 Hz) (Found: C, 31.7; H, 3.1; Br, 57.5. C₁₉H₁₄O₂Br₂ requires C, 31.7; H, 3.1; Br, 57.5%). Hydrogenolysis of ethyl [2-¹⁴C]dibromo-6-pentyldihydroresorcylate (300 mg) in 1 M sodium hydroxide (3.5 ml), containing 5% palladium on calcium carbonate (150 mg) with hydrogen at atmospheric pressure and room temperature gave, after isolation in ether (4 x 25 ml), ethyl [2-¹⁴C]-6-pentyldihydroresorcylate, which formed prisms (160 mg, 134.6 mCi mmol⁻¹) from light petroleum (bp 40–60°C), mp 69°C [lit. mp (14) 69°C] (Found: C, 66.7; H, 8.0. Calcd for C₁₉H₁₄O₂: C, 66.7; H, 8.0%).
methylmulticolate was diluted with inactive material to give a sample (90 mg, 4.01 × 10⁻³ μC mmol⁻¹) which was heated under reflux with 1.5 M sulfuric acid (10 ml), under nitrogen, for 48 hr. After neutralization of the mixture with sodium hydrogen carbonate, p-phenylenediamine (50 mg) in ethanol (3 ml) was added and the whole warmed to 100°C for 15 min. The product was isolated in chloroform (4 × 10 ml) and purified by preparative tlc, using acetone:light petroleum (bp 60–80°C) (1:3) as the developing solvent. The band with Rf 0.25 was eluted with ethyl acetate and the material crystallized from aqueous ethanol to constant radioactivity giving [¹¹C]-2-methyl-3-(6-hydroxyhexyl)-quinoxaline (XXI) as needles (55 mg, 3.92 × 10⁻³ μC mmol⁻¹), mp 139°C, νmax 3480 (br), 1563, 1592, 1340, 1328, 1315, 1155, 1140, and 1124 cm⁻¹, λmax 204, 237, and 315 nm (ε 13 200, 12 300, and 9700), τ 1.89–2.42 (4H), 6.40 (2H, t, J 7.5 Hz), 7.0 (2H, t, J 7.0 Hz), 7.33 (3H, s) and 8.2–8.8 (8H, m) [Found: C, 73.5; H, 8.3; N, 11.7; m/e, 244.156. C₁₅H₂₀N₂O requires C, 73.7; H, 8.3; N, 11.5; m/e, 244.158].

Degradation of quinoxaline (XXI). Kuhn–Roth oxidation of compound (XXI) was carried out by the standard procedure (15) and a portion of the acetic acid isolated was converted to its p-bromophenacyl ester. This was purified by tlc and crystallized by constant activity from light petroleum (bp 60–80°C), giving plates (3.63 × 10⁻³ μC mmol⁻¹), mp 86°C. The remainder of the acetic acid was subjected to Schmidt degradation, as previously described (16). Methylamine was isolated as its hydrochloride and found to be essentially radiochemically inactive.

REFERENCES

Studies on Fungal Metabolites. Part 2. Carbon-13 Nuclear Magnetic Resonance Biosynthetic Studies on Pentaketide Metabolites of Aspergillus melleus: 3-(1,2-Epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one and Mellein

By John S. E. Holkar and Thomas J. Simpson, Department of Organic Chemistry, The Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

The 13C n.m.r. spectra and incorporations of 13C-labelled precursors into pyrone and dihydroisocoumarin pentaketide metabolites from the culture liquors of Aspergillus melleus are reported. Detection of a two-bond 13C-13C coupling in a metabolite enriched from [1,2-13C2]acetate provides proof for an intramolecular rearrangement occurring during biosynthesis.

FERMENTATIONS of Aspergillus melleus provide a variety of metabolites: the mycelium is a rich source of polyketide-derived naphthoquinone pigments, e.g. xanthone (1). The culture liquors are the usual pyrone (2), a weak broad spectrum antibiotic which was first isolated along with mellein (4) and penicillic acid (6), and has since been isolated from other Aspergillus species. Incorporation of [14C]acetate into pyrone (2) was reported to give the labelling pattern shown in Scheme 1. The distribution of label, particularly the linkage of two carbons derived from the methyl carbon of acetate, and the branched structure of the pyrone are difficult to rationalise in terms of the normal pathways of polyketide biosynthesis. Studies based on 13C-labelling have facilitated the elucidation of unusual biosynthetic pathways.

and 13C n.m.r. studies of pyrone (2) and its co-metabolites are now reported.

Before undertaking 13C-enrichment studies it was necessary to have an unambiguous assignment of the 13C n.m.r. spectrum, and a full assignment of the 13C n.m.r. spectra of the pyrone (2) and its acetate (3) has been made as follows: C-1, C-2, C-3, and the 4-acetate carbons were readily assigned from standard chemical-shift data and their multiplicities in the single frequency off-resonance decoupled (s.f.o.r.d.) spectra. Carbons 1, 5, 7, and 8, however, all have similar chemical shifts, characteristic of oxygen-bearing aliphatic carbons, and give rise to doublets in the s.f.o.r.d. spectra. They were assigned for the acetate (3) by the Birdsall method, in which the peak frequencies in s.f.o.r.d. spectra are plotted against the 1H irradiating frequency which is stepped through the 1H spectral region. The corresponding 1H and 13C resonances are determined by extrapolating (Figure) the residual couplings to zero, and as the 1H frequencies have been unambiguously assigned, the corresponding 13C resonances can be assigned. The 6- and 9-methyl resonances could not be readily distinguished in the natural abundance 13C n.m.r. spectra. However in the spectrum of (2) enriched from [1,2-13C2]-acetate, the resonance at 17.6 p.p.m. shows a 13C-13C coupling of 44 Hz to the resonance at 59.1 p.p.m., which

[Diagram of Scheme 1 Incorporation pattern of acetate into A. melleus pyrone (2)]

has been unambiguously assigned to C-8, and so the former must be assigned to C-9.

The 13C n.m.r. spectra of (2) enriched from feedings of [1-13C]-, [2-13C]-, and [1,2-13C2]-acetate, and [2-13C]malonate are summarised in Table 1. High enrichment of carbons 1, 3, 5, and 8 from [1-13C]acetate, and carbons 2, 4, 6, 7, and 9 from [2-13C]acetate were appa-
rent, confirming the previously reported labelling pattern. The specific incorporation of [2,13C]acetate was sufficiently high for satellites due to a 13C-13C coupling of 61 Hz, corresponding to the head-to-head linkage of acetate units, to be observed on the resonances due to carbons 2 and 7. It should be noted that the overall enrichment, ca. 4.5 atom % 13C, is much too low to allow such satellites to be observed so a significant proportion of the enriched molecules must be multiply labelled, with the remaining molecules having a much lower level of enrichment to give the observed overall level of enrichment.10

The 13C n.m.r. spectrum of [1,2,13C2]acetate-enriched pyrone (2) shows 13C-13C couplings between the following pairs of carbons: 2 and 3 (68 Hz), 4 and 5 (41 Hz), and 8 and 9 (44 Hz) thus proving their origin from acetate units which have remained intact throughout the biosynthetic sequence to give the labelling pattern shown in Scheme 2. On redetermining the spectrum using 500 Hz sweep widths, an additional small coupling of 6 Hz was resolved on the resonances due to carbons 1 and 7. Thus carbons 1 and 7 must also be derived from an originally intact acetate molecule which has undergone an intramolecular rearrangement during the course of biosynthesis. The above enrichment data is consistent with the biosynthetic pathway shown in Scheme 2, and indicate that pyrone (2) is formed from a pentaketide precursor via a Favorskii-type rearrangement; generating both the observed head-to-head linkage of acetate units, and the 1,3-coupling between carbons 1 and 7, followed by loss of the terminal carboxy-group, a biosynthetically unexceptional step. A similar rearrangement can be postulated to account for the formation of the fused bis-furan ring system found in the aflatoxin and related metabolites, whose origin has been a subject of much research and speculation.11 Since our preliminary report, a similar rearrangement has been shown to occur in the biosynthesis of vulgagamycin,12 and a furanoid metabolite of Chaetomium coarcatum.13

**Scheme 2** Incorporation of [1,2,13C2]acetate into the pyrone (2), via intramolecular rearrangement of a pentaketide precursor

**TABLE 1**

<table>
<thead>
<tr>
<th>Carbon</th>
<th>13C Chemical shifts (δ, p.p.m. downfield from internal SiMe4) in pyrones (2) and (3), and enrichments observed in 13C incorporation experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(2)</td>
</tr>
<tr>
<td>2</td>
<td>129.0 (s)</td>
</tr>
<tr>
<td>3</td>
<td>141.2 (d)</td>
</tr>
<tr>
<td>4</td>
<td>67.6 (d)</td>
</tr>
<tr>
<td>5</td>
<td>79.3 (d)</td>
</tr>
<tr>
<td>6</td>
<td>18.0 (q)</td>
</tr>
<tr>
<td>7</td>
<td>54.8 (d)</td>
</tr>
<tr>
<td>8</td>
<td>50.1 (d)</td>
</tr>
<tr>
<td>9</td>
<td>17.6 (q)</td>
</tr>
<tr>
<td>CH₃CO</td>
<td>-</td>
</tr>
<tr>
<td>CH₃CO</td>
<td>-</td>
</tr>
</tbody>
</table>

* Multiplicities refer to off-resonance decoupled spectra. ^1,4 Average enrichments are +13, 4.5, and 0.3 atom % 13C respectively. ^J[13C-13C] 91 Hz observed.
Incorporation of [2-13C]malonate resulted in very low overall enrichment and no 'starter' effect was observed, C-9 being enriched to the same level as the other carbons derived from the methyl carbon of acetate.

In addition to the pyrone (2), variable yields of mellein (4) and penicillic acid (6), the previously reported coproducts of the fermentation liquors, were obtained from the A. melleus fermentations. However, a further metabolite was isolated from the fermentation liquors. From its spectral and physical properties it was identified as cis-4-hydroxymellein (7) which has been isolated from several fungi, including Lasiodiplodia theobromae, 14 and Cercospora taiwanensis. 15

The 13C n.m.r. spectra of mellein (4), O-methylmellein (5), and (7) are summarised in Table 2.

### Table 2

<table>
<thead>
<tr>
<th>Carbon</th>
<th>1H Couplings (J/Hz)</th>
<th>13C Chemical Shifts (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>102.7 (s)</td>
<td>36.1 (d)</td>
</tr>
<tr>
<td>3</td>
<td>74.0 (d)</td>
<td>117.6 (d)</td>
</tr>
<tr>
<td>4</td>
<td>36.1 (s)</td>
<td>178.7 (s)</td>
</tr>
<tr>
<td>5</td>
<td>116.1 (d)</td>
<td>116.2 (d)</td>
</tr>
<tr>
<td>6</td>
<td>116.1 (d)</td>
<td>116.2 (d)</td>
</tr>
<tr>
<td>7</td>
<td>116.1 (d)</td>
<td>116.2 (d)</td>
</tr>
<tr>
<td>8</td>
<td>116.1 (d)</td>
<td>116.2 (d)</td>
</tr>
<tr>
<td>9</td>
<td>116.1 (d)</td>
<td>116.2 (d)</td>
</tr>
<tr>
<td>10</td>
<td>116.1 (d)</td>
<td>116.2 (d)</td>
</tr>
<tr>
<td>11</td>
<td>116.1 (d)</td>
<td>116.2 (d)</td>
</tr>
<tr>
<td>MeO</td>
<td>56.1 (q)</td>
<td>116.2 (d)</td>
</tr>
</tbody>
</table>

Multiplicities refer to off-resonance decoupled spectra.

The 13C resonances were assigned from standard shift values, multiplicities in s.f.o.r.d. spectra, and by analysis of the long-range 1H-13C couplings of fully 1H-coupled 13C n.m.r. spectra. This is particularly useful for distinguishing the 5 and 7 carbons which are of similar chemical shift in all three compounds. C-6, as expected, appears as a simple doublet due to one-bond coupling to H-6, typically 102 Hz in (7). In all three compounds C-7 appears as a doublet of doublets due to one bond coupling to H-7 and a three bond coupling to H-5, typically 166 Hz and 7 Hz respectively in (7). However in both (4) and (5) C-5 appears as a doublet of quintets due to the large one bond coupling to H-5, with the 5-line pattern arising from a 3-bond coupling of ca. 7 Hz to H-7 and ca. 3.5 Hz coupling to the 4-methylene protons; selective low-power irradiation of the 4-protons collapses the quintets to doublets.

Similarly C-10 shows a 3-bond coupling of ca. 7 Hz to H-6 with 2- and 3-bond couplings of ca. 3.5 Hz to the 4- and 3-protons respectively so that, for example, irradiation of the 4-methylene protons in (5) causes the broad poorly resolved multiplet due to C-10 to sharpen to a doublet of doublets (7 and 3.5 Hz) and irradiation of H-4 causes it to sharpen to a doublet of triplets. In compound (7) on the other hand C-6 appears as a doublet of triplets due to the one-bond coupling (102 Hz) to H-5 and equal three bond coupling to H-7 and H-4 (7 Hz); C-10 appears as a doublet of poorly resolved triplets due to a 7 Hz coupling to H-6 and smaller couplings to H-4 and H-3.

On methylation of (4) to give (5), the removal of the peri-related hydroxyl group results in characteristic changes in chemical shift. 16 Sufficient mellein was isolated from the [1,2-13C]acetate fermentation for its doubly labelled 13C n.m.r. spectrum to be determined, and the observed 13C-13C couplings (Table 2) confirm that mellein is formed by the anticipated folding of a pentaketide chain (Scheme 3). A similar acetate assembly pattern has been found for the related dihydroisocoumarin (9), a metabolite of Sporaria cinerea. 17

It is noteworthy that both mellein (4) and the pyrone (2) lack the equivalent oxygen atom from the precursor pentaketide chain. Moreover, on repeated culture of A. melleus yields of the pyrone decreased with concomitant increase yields of mellein which was originally produced in very low yields. This suggests that both compounds are derived from a common deoxypentaketide which is diverted to mellein production when pyrone production is inhibited. Pyrone (2) has also been isolated from A. ochraceus 1c which also produces the ochratoxins [e.g. (8)], dihydroisocoumarins whose biosynthesis must also involve a deoxypentaketide precursor.

### EXPERIMENTAL

For general experimental details see part 1.

13C N.m.r. Determinations.—The 13C n.m.r. spectra were obtained for samples in acid-free CDCl3 with SiMe4 as internal reference. Proton noise-decoupled and single frequency off-resonance decoupled spectra were determined on a Varian XL100-15FT spectrometer operating at 25.197 MHz or on a JOEL JNMFX-60 spectrometer operating at 15.04 MHz. Fully coupled spectra were determined under gated-1 decoupling conditions to retain nuclear Overhauser effects.

Isolation of Metabolites.—Aspergillus melleus (CMI 49108) was grown at 25 °C in static culture in penicillin flasks each containing 500 ml of an aqueous medium made up from potassium dihydrogen phosphate (0.1%), magnesium sulphate heptahydrate (0.06%), potassium chloride...
(0.05%), urea (0.07%), and glucose (7.5%), the solution being adjusted to pH 5 before autoclaving. The fermentation was harvested after 18 days growth and the culture filtrate was extracted with ethyl acetate to give a brown gum (ca. 400 mg l⁻¹) which was purified by preparative t.l.c. on 20 × 20 cm silica-gel plates eluted with ether–light petroleum (60 : 40, v/v). The three u.v.-quenching bands were removed to give in order of decreasing polarity (a) 3-(1,2-epoxypropyl)-5,6-dihydro-5-hydroxy-6-methylpyran-2-one, m.p. 109–111 °C (lit., 4 m.p. 109–111 °C) (ca. 244 mg l⁻¹); (b) 4-hydroxymellein (ca. 20 mg l⁻¹), m.p. 114–117 °C (lit., 14 m.p. 112–117 °C); and (c) mellein (ca. 10 mg l⁻¹), m.p. 54–56 °C (lit., 15 m.p. 55–56 °C). In later fermentations yields of the pyrone decreased with concomitant increase in the yields of mellein.

**Incorporations of ¹³C Labelled Precursors.—**To each of three vessels containing a 7-day growth of *A. melleus* was added 90% sodium [1-¹³C]acetate (400 mg), sodium [2-¹³C]-acetate (250 mg), and sodium [1,2-¹³C₂]acetate (250 mg). After a further 4 days growth the liquors were extracted to give pyrone (2), in yields of 25, 39, and 37 mg from the [1-¹³C]-, [2-¹³C], and [1,2-¹³C₂]-acetate feeds respectively. In addition, mellein (15 mg) was isolated from the [1,2-¹³C₂]-acetate feed.

Diethyl [2-¹³C]malonate (250 mg) in ethanol (1 ml) was similarly added to a 7-day old culture of *A. melleus* and pyrone (2) (30 mg) was isolated after a further 4 days growth.

**Acetylation of the Pyrone (2).—**The pyrone (100 mg) in acetic anhydride (2 ml) and pyridine (0.5 ml) was stirred at room temperature for 2 h. Work-up gave an almost quantitative yield of the acetate (3), m.p. 65–87 °C (lit., 4 m.p. 65–67 °C).

**Methylation of Mellein.—**Mellein (100 mg) was stirred at room temperature overnight in the presence of chloroform (10 ml), methyl iodide (1 ml), and silver oxide (500 mg). Filtration of solids and removal of solvent gave an oil which crystallised from ethyl acetate to give O-methylmellein, m.p. 88–89 °C (lit., 15 m.p. 88–89 °C).

**References**

ISOLATION AND X-RAY CRYSTAL STRUCTURES OF ASTELLOLIDES A AND B, SESQUITERPENOID METABOLITES OF ASPERGILLUS VARIECOLOR

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Abstract. Two sesquiterpenoid metabolites, astellolides A and B, have been isolated from cultures of Aspergillus variecolor. Their structures have been elucidated by X-ray crystallography and spectroscopic methods.

In the course of biosynthetic and structural studies of andibenin and related metabolites of Aspergillus variecolor we obtained a mutant of the andibenin producing strain, 212KI69, which lacked the usual abundant polyketide derived mycelial pigments. We now wish to report the isolation and structures of two biosynthetically significant sesquiterpenoid metabolites from extraction and chromatography of the fermentation liquors of this mutant strain.

The major metabolite astellolide A, ${C_{26}H_{30}O_8}$ (mass spec. and analysis), formed colourless prisms from ethyl acetate with m.p. 213-214°C, showed $[α]_{D}^{25}$-8°, and u.v. maxima at 221 (log $E$ 4.30) and 275 (3.02) nm. The 360MHz $^1H$ n.m.r. spectrum showed signals at $61.15$, (tertiary methyl); $1.90$ and $2.11$ (both $3H$, s), $3.99$ and $4.38$ (both $1H$, d, $J$ 12Hz) and $4.88$ and $4.97$ (both $1H$, d, $J$ 11Hz) assignable to two tertiary acetoxy methyls; and $5.95$ (1H, m) $8.02$ (2H, d, $J$8Hz), $7.57$ (1H, t, $J$8Hz), $7.47$ (2H, t, $J$8Hz) assignable to the benzoate of a secondary alcohol. The corresponding carbon signals were readily identified in the 25MHz $^{13}C$ n.m.r. spectrum (Table). Extensive spin-decoupling experiments showed that the methine proton at $65.95$ which was coupled to a methine proton at $61.82$ (1H, d, $J$1.5Hz) was also coupled ($J$5.0 and $2.0Hz$) to allylic methylene protons at $2.72$ (2H, m). This allylic methylene in turn showed a homo-allylic coupling to two doublets of triplets at $64.81$ and $5.97$ (each $1H$, $J$17 and $2.5Hz$) corresponding to an oxygen bearing allylic methylene. No other allylic protons were present. From the chemical shifts and consideration of the remaining functionality, this oxymethylene and the tetrasubstituted double bond ($6c$ 122.5 and 165.6) must be linked with the remaining acyl carbon ($6c$173.0) to form an $α,β$-unsaturated $γ$-lactone and so partial structure (A) can be deduced. After allowing for the acetate and benzoate carbons, the remaining carbon skeleton is $C_{15}$ and tricyclic, and the carbons not accounted for in the above functions consist of three aliphatic
methylenes and two aliphatic quaternary carbons. These features can best be accommodated in a substituted confertifolin structure as indicated in (1).

The coupling constants of the 6-proton to the 5- and 7-protons indicate that H-6 is equatorial and thus the benzoate is in the axial position. The C-methyl can be placed at the 4-equatorial position, rather than the 4- or 10-axial positions by virtue of its high chemical shift value and comparison with the shifts observed for C-methyls and acetoxymethyls in a variety of terpenoid compounds e.g. in podocarpol acetate the 4-equatorial methyl and 4-axial acetoxymethylene carbons resonate at 27.4 and 67.0 p.p.m. respectively whereas in andalusol 6,18-diacetate the 4-axial methyl and 4-equatorial acetoxymethylene resonate at 17.9 and 74.1 p.p.m. respectively. Thus all the n.m.r. data are consistent with structure (1) for astellolide A. This has been confirmed by a single crystal X-ray diffraction study.

Crystal data: $C_{26}H_{30}O_8$, $M=470$, clear colourless orthorhombic crystals, space group $P2_12_12_1$ (No.19), $a=11.023$ (3), $b=14.426$ (4), $c=15.045$ (5) Å, $U=2392 Å^3$, $Z=4$, $D_c=1.31$ g cm$^{-3}$; MoKα radiation ($λ=0.71069 Å$, $μ=0.58$ cm$^{-1}$). Intensity data were collected on a Nonius CAD 4 diffractometer (to 2θ = 50°). Of 2436 unique reflections, 1631 had $I > 3σ(I)$. Multan 77 was used to solve the structure which was refined isotropically to a R value of 0.085 using SHELX. Hydrogen atoms were included in calculated positions. The structure of the molecule is illustrated in the Figure by a PLUTO drawing.

Astellolide B, C_{26}H_{30}O_9, gave colourless rods from ethyl acetate m.p. 251-253°C. Its spectroscopic properties differed from astellolide A, only in signals that were readily attributable to having a p-hydroxybenzoate function in place of the benzoate of astellolide A. Thus $^1H$ n.m.r. showed signals at 66.84 and 7.87 (each 2H, d, J=8Hz) and the aromatic ring carbons in the $^{13}C$ n.m.r. spectrum appeared at 154.3 (s), 150.0 (2xd), 119.8 (2xd), 120.9 (s); so astellolide B has structure (2).
Table  $^{13}$C n.m.r. chemical shifts (p.p.m. from Me$_4$Si) and multiplicities observed for astellolide A (1) and astellolide B (2).

<table>
<thead>
<tr>
<th>Carbon</th>
<th>(1)</th>
<th>(2)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>31.6t</td>
<td>31.7t</td>
</tr>
<tr>
<td>2</td>
<td>17.9t</td>
<td>17.9t</td>
</tr>
<tr>
<td>3</td>
<td>37.0t</td>
<td>37.0t</td>
</tr>
<tr>
<td>4</td>
<td>37.7s</td>
<td>37.7s</td>
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<tr>
<td>5</td>
<td>53.9d</td>
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<td>8</td>
<td>122.5s</td>
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<td>9</td>
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<tr>
<td>10</td>
<td>40.5s</td>
<td>40.5s</td>
</tr>
<tr>
<td>11</td>
<td>65.9t</td>
<td>66.0t</td>
</tr>
<tr>
<td>12</td>
<td>173.0s</td>
<td>173.4s</td>
</tr>
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<td>14</td>
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<tr>
<td>15</td>
<td>71.3t</td>
<td>71.4t</td>
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<tr>
<td>CH$_3$CO</td>
<td>170.4s</td>
<td>171.1s</td>
</tr>
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<td>170.3s</td>
</tr>
<tr>
<td>1'</td>
<td>165.9s</td>
<td>165.8s</td>
</tr>
<tr>
<td>2'</td>
<td>129.5s</td>
<td>120.6s</td>
</tr>
<tr>
<td>3'7'</td>
<td>129.5d</td>
<td>131.3d</td>
</tr>
<tr>
<td>4'6'</td>
<td>128.6d</td>
<td>115.6d</td>
</tr>
<tr>
<td>5'</td>
<td>133.3d</td>
<td>161.3s</td>
</tr>
</tbody>
</table>

Previous studies have indicated that andibenin is formed by alkylation of a bis-C-methylated tetraketide precursor by farnesylpyrophosphate. It is significant that this mutant strain which is apparently impaired in polyketide production should divert the farnesylpyrophosphate to sesquiterpenoid production. Mycophenolic acid, the important antitumour metabolite of *Penicillium breviformicompactum* is biosynthesised by a pathway analogous to that proposed for andibenin. It is thus interesting to note that the pebroliodes with similar structures to the astellolides have also been reported as metabolites of *P. breviformicompactum*.12
References

7. Crystallographic coordinates have been deposited with the Cambridge Crystallographic Data Centre.

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Biosynthesis of Tajixanthone in *Aspergillus variecolor*; Incorporation of \([\text{H}_3\text{]Acetate}}\) and \([1,2-{^13}\text{C}_2}\text{]Acetate}

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Biosynthesis of Tajixanthone in Aspergillus variecolor; Incorporation of [PH₃]Acetate and [1,2-¹³C₂]Acetate

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Summary The results of ¹³C and ¹H n.m.r. analyses of [PH₃]- and [1,2-¹³C₂]-acetate-enriched tajixanthone are reported, which indicate, inter alia, that ring cleavage of an anthraquinone, and not anthrone, precursor must precede C-prenylation, and that dihydropyran ring formation precedes xanthone ring formation during biosynthesis of tajixanthone.

Two major biosynthetic pathways are evident in Aspergillus variecolor, one leading to tajixanthone (1) and related mycelial pigments, and the other to andibenin and related compounds isolated from the culture liquors. Previous studies have indicated that biosynthesis of tajixanthone occurs via an octaketide-derived anthrone or anthraquinone with the introduction of two prenyl units from 3,3-dimethylallylypyrophosphate (DMAPP) to give an O-prenyloxyaldehyde intermediate (2), which then undergoes an intramolecular 'ene' reaction to form the substituted dihydropyran ring, and cyclodehydration to form the xanthone system. Further indirect evidence in support of this pathway was given by a detailed study of the metabolites of a number of variant strains of A. variecolor, but the sequence and mechanistic details of the required steps remained to be determined. We now report the results of incorporation studies with [1,2-¹³C₂]- and [²-¹H₃]-acetate which, in conjunction with ¹³C and ¹H n.m.r. data, allow some of these details to be elucidated.

The labelling patterns resulting from these incorporation studies are summarised in the Scheme and the following conclusions can be drawn from the ¹³C and ¹H n.m.r. data: (i) The acetate assembly pattern in the xanthone system is entirely consistent with an octaketide precursor folded as shown in the Scheme; cf. islandicin. (ii) The randomisation of labelling in ring c means that ring c must have been symmetrical and free to rotate on the enzyme surface at some stage in the biosynthesis of tajixanthone. This means that ring cleavage of the carbocyclic precursor must precede introduction of the C-prenyl residue; cf. ravenelin. (iii) The details of the ¹³C and ¹H n.m.r. data will be published elsewhere.
C-Prenylation and epoxidation, in agreement with recent studies on echinulin and flavoglaucin, occurs with retention of configuration about the O-prenylaldehyde moiety by a concerted ‘ene’ reaction. The transition state necessary for the observed 20,25-trans stereochemistry of (1) in a concerted reaction requires dihydropyran ring formation to occur before cyclodehydration to the xanthone system, as the transition state necessary for trans stereochemistry in an ‘ene’ reaction of the xanthone aldehyde (3) would have a highly unfavourable interaction between the aldehyde and xanthone carbonyls. Indeed, in vitro cyclisation of (3) gives the cis product.

2H N.m.r. spectroscopy has been successfully applied to the study of terpenoid biosynthesis but so far only to a few polyketide problems. The apparent intermediacy of the aldehyde (2) in tajixanthone biosynthesis suggests that ring cleavage might occur at the anthrone rather than anthraquinone oxidation level, in which case H-25 would be derived from the hydrogen of acetate. As the 25-hydroxy group of (1) is known to be resistant to reaction, e.g. oxidation, a 2H-labelling study seemed appropriate. The 2H n.m.r. spectrum of [2H3]acetate-enriched tajixanthone indicated the labelling pattern shown in the Scheme and permits the following conclusions. (i) There is no 4H label on C-25; this implies cleavage of an anthraquinone rather than an anthrone intermediate. (ii) The absence of 4H on C-5 indicates that decarboxylation of the octaketide precursor occurs after cyclisation and aromatisation. (iii) A differential incorporation of 4H is apparent. The DMAPP-derived positions are enriched to a greater extent than the polyketide methyl which appears to be more highly enriched than the polyketide methylene positions. Indeed it is reassuring to see the presence of label on C-2 as, in two recent studies, 4H was incorporated only into the acetyl coenzyme A-derived position and not those derived from malonyl coenzyme A. (iv) There appears to be some loss of 4H from the E methyl group relative to the Z methyl group of DMAPP (after allowing for the anticipated 2:3 ratio). This could be occurring from acetocoezyme A, or could be due to dimethylallyl-isopentenyl pyrophosphate equilibration.

The above results allow the sequence of steps shown in the Scheme to be proposed for the biosynthesis of tajixanthone. Further studies to delineate the pathway are in progress and will be reported in due course.

The support of the S.R.C. is gratefully acknowledged.

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$^{13}$C and $^1$H N.M.R. Studies on the Biosynthesis of O-Methylasparvenone, a Hexaketide Metabolite of Aspergillus parvulus

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3C and 1H N.M.R. Studies on the Biosynthesis of O-Methylasparvenone, a Hexaketide Metabolite of Aspergillus parvulus

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Summary

Incorporations of singly and doubly labelled 13C acetates and [2H3]acetate into O-methylasparvenone, a dihydronaphthalene metabolite of Aspergillus parvulus, and analysis of the resultant enriched samples by high-field 13C and 1H n.m.r. spectroscopy indicate a hexaketide biosynthesis and a novel acetate assembly pattern; the 1H-labelling pattern and levels of enrichment provide information on the sequence and mechanisms of the reduction, oxidation, and deoxygenation steps on the biosynthetic pathway.

The use of precursors doubly labelled with both 13C and 1H, combined with the use of 13C n.m.r. spectroscopy to determine the fate of acetate or mevalonate hydrogen during the course of a biosynthetic pathway, has been applied with success in polyketide and terpenoid studies.1 The alternative method of using 1H-labelled precursors and direct 1H n.m.r. spectroscopy determination of labelling has also been used successfully, particularly in the terpenoid area, notably by Cane and co-workers,2 but also in the polyketide field, a notable example being the studies of Sato on griseofulvin biosynthesis.3 With the availability of high-field n.m.r. spectrometers the direct use of 1H n.m.r. spectroscopy becomes an even more attractive option. We now report the results of incorporation studies using both 13C-labelled acetates and 1H-labelled acetate to investigate the biosynthesis of O-methylasparvenone (I), a metabolite of Aspergillus parvulus, a fungus found in the acid soil of pine or sweetgum forests.4 Preliminary 13C-labelling studies have suggested a polyketide origin for (I).

Before carrying out incorporation studies both the 1H and 13C n.m.r. spectra of O-methylasparvenone were rigorously assigned. These studies, which will be described in detail elsewhere, resulted in the assignments summarised in Tables 1 and 2. The [1-13C]-, [2-13C]-, and [1,2-13C2]acetates were efficiently incorporated into (I) by shaken cultures of

TABLE 1. 13C Chemical shifts (δ, relative to Me4Si) of O-methylasparvenone (I); coupling constants (Hz) of [1,2-13C2]acetate enriched (I); and enrichments observed in [1-13C]acetate- and [2-13C]acetate-enriched (I).

<table>
<thead>
<tr>
<th>Carbon</th>
<th>δ/ppm.</th>
<th>J(13C—13C)</th>
<th>Enrichment</th>
</tr>
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<tbody>
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<td>1</td>
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<td>42</td>
<td>•</td>
</tr>
<tr>
<td>2</td>
<td>34-5</td>
<td>41</td>
<td>•</td>
</tr>
<tr>
<td>3</td>
<td>31-7</td>
<td>37</td>
<td>•</td>
</tr>
<tr>
<td>4</td>
<td>68-2</td>
<td>37</td>
<td>•</td>
</tr>
<tr>
<td>4a</td>
<td>145-3</td>
<td>62</td>
<td>•</td>
</tr>
<tr>
<td>5</td>
<td>100-5</td>
<td>63</td>
<td>•</td>
</tr>
<tr>
<td>6</td>
<td>153-8</td>
<td>70</td>
<td>•</td>
</tr>
<tr>
<td>7</td>
<td>119-4</td>
<td>71</td>
<td>•</td>
</tr>
<tr>
<td>8</td>
<td>101-8</td>
<td>61</td>
<td>•</td>
</tr>
<tr>
<td>8a</td>
<td>109-8</td>
<td>62</td>
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<td>15-4</td>
<td>33</td>
<td>•</td>
</tr>
<tr>
<td>10</td>
<td>12-9</td>
<td>34</td>
<td>•</td>
</tr>
<tr>
<td>MeO</td>
<td>55-6</td>
<td>•</td>
<td>—</td>
</tr>
</tbody>
</table>

a Average enrichment for [1-13C]acetate is 2 atom %
b Average enrichment for [2-13C]acetate is 14 atom %
c 10 Hz observed.

TABLE 2. 1H Chemical shifts (δ, relative to Me4Si) of O-methylasparvenone (I); 1H chemical shifts and relative intensities observed in [1H2]acetate-enriched (I).

<table>
<thead>
<tr>
<th>Hydrogen</th>
<th>δ(1H)b</th>
<th>δ(1H)c</th>
<th>Relative intensityd</th>
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<tr>
<td>2ax</td>
<td>2-52</td>
<td>2-56</td>
<td>0-4</td>
</tr>
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<td>2eq</td>
<td>3-30</td>
<td>3-32</td>
<td>0-2</td>
</tr>
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<td>3ax</td>
<td>2-04</td>
<td>2-08</td>
<td>1-6</td>
</tr>
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<td>3eq</td>
<td>2-24</td>
<td>2-26</td>
<td>1-2</td>
</tr>
<tr>
<td>4ax</td>
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<td>4-80</td>
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<tr>
<td>10</td>
<td>1-08</td>
<td>1-06</td>
<td>1-8</td>
</tr>
<tr>
<td>OMe</td>
<td>3-85</td>
<td>3-85</td>
<td>3-1</td>
</tr>
</tbody>
</table>

a [1H2]Acetate (2g) was distributed among 12 shaken flasks (75 ml medium per 250 ml flask) 36 h after inoculation. After a further 24 h growth, (I), (65 mg) was isolated. b Measured at 360 MHz. c Measured at 55-3 MHz on a Bruker WH 360 spectrometer. d Normalised to H-5, which is itself enriched to ca. 5 atom % 1H.

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parvulus and the resultant 3H n.m.r. spectra indicated enrichments summarised in Table 1. The enrichment levels were essentially identical throughout the molecule and indicate its formation from a hexaketide with the acetate semiblly pattern shown in the Scheme.

Scheme. △ = 3H label.

To convert the hexaketide precursor into (1) it is necessary lose acetate-derived oxygen from C-3 and C-9, insert an extra oxygen atom at C-4, and reduce to the dihydrophthalene oxidation level. It is generally assumed that ketide oxygen is lost by reduction of the ketone and hydration of the resulting alcohol as in fatty-acid biosynthesis. The dehydration may be followed by reduction of the resultant 'H n.m.r. spectrum. This indicated enrichments summarised in Table 1. The enrichment levels were essentially identical throughout the molecule and indicate its formation from a hexaketide with the acetate semibly pattern shown in the Scheme.

medium can be ruled out by the absence of label on C-9). Thus the C-3 polyketide oxygen must be lost before aromatisation; introduction of the 'extra' oxygen on C-4, and reduction to the dihydrophthalene level must occur after condensation and aromatisation of the precursor polyketide. These results are interesting in relation to recent studies on vernolone [3,4-dihydro-3,8-dihydroxynaphthalene-1(2H)-one] and scytalone [3,4-dihydro-8-hydroxynaphthalene-1(2H)-one], pentaketide metabolites of Verticillium dahliae, which indicate that both reduction and loss of oxygen are post-aromatic processes, and in relation to aflatoxin biosynthesis where the apparent intermediacy of averufin had been questioned on the grounds of the required loss of phenolic oxygen.

While it is recognised that the enriched positions are probably subject to differing probabilities of loss of 3H through exchange, as their environments alter during the course of the biosynthetic pathway, so making interpretation of differing levels of enrichment subject to uncertainty, the following further observations can be made. (a) The 3-axial position is labelled to only 50% of the level of the 2-axial position. This is entirely consistent with the levels of enrichment of the 2- or 3-equatorial hydrogens so that the ring reduction is an entirely stereospecific process. (b) The 10-methyl is labelled to less than twice the level of H-5. This is particularly surprising as recent 3H labelling studies have shown preferential labelling of acetyl coenzyme A-derived 'starter' positions relative to positions derived from malonyl coenzyme A in polyketide metabolites. We interpret this observation as a strong indication that the C-9 ketide oxygen is not lost until after aromatisation. This would then allow loss of 3H label from C-10 relative to C-9 by exchange from an acetyl side chain and/or via reduction and dehydration of the resultant 1'-hydroxyethyl side chain. Some support for this hypothesis comes from the isolation of trace amounts of 9-oxygenated analogues of O-methylaspervone from A. parvulus fermentations.

In conclusion, the present studies demonstrate how 3H n.m.r. labelling studies can provide valuable information on the on often inaccessible intermediates and mechanisms of polyketide metabolism. The differing levels of 3H labelling and the nature of the intermediates on the biosynthetic pathway are the subject of further investigations which will be reported in due course.

The support of the S.R.C. is gratefully acknowledged.

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Anditomin, a new C$_3$ Metabolite from *Aspergillus variecolor*

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Anditomin, a new C\textsubscript{25} Metabolite from \textit{Aspergillus variecolor}

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\textbf{Summary} The structure of anditomin, C\textsubscript{25}H\textsubscript{30}O\textsubscript{5}, isolated from \textit{Aspergillus variecolor}, was deduced from a detailed analysis of its \textsuperscript{1}H and \textsuperscript{13}C n.m.r. spectra and was confirmed by X-ray crystallography.

We recently reported the isolation and structure determination of a number of novel C\textsubscript{26} metabolites\textsuperscript{1} related to andibenin,\textsuperscript{2} including andilesen C (1), C\textsubscript{26}H\textsubscript{48}O\textsubscript{8}, from \textit{Aspergillus variecolor} (strain 212K169). We now report the isolation of a further metabolite of this series, anditomin, for which we have deduced structure (2), along with similar amounts of andilesen C, from \textit{A. variecolor} (C.M.I. 60316).

Anditomin, C\textsubscript{25}H\textsubscript{30}O\textsubscript{5} (mass spectrum and analysis), crystallised from ethyl acetate as prisms, m.p. >300 °C and showed [\(\alpha\)]\textsubscript{D}\textsubscript{20} \(= -61^\circ\), and \(\nu\)\textsubscript{max} (CHCl\textsubscript{3}) 1786, 1705, and
Comparison of their 'H and 13C n.m.r. spectra indicated a close relationship between andilesen C and anditomin, which showed indicator 197 5.94 and 5.84 (CH=O-C=O, 8 8-8 Hz), 1.66 and 3.02 (CH=O-C=O, Jmax 14.6, Jmax 0 and Jmax 8-5 Hz), 1.36 and 1.97 (C=CH=O-C, Jmax 13.5 Hz), 1.14-2.1 (c, 6H). However, in contrast with the five tertiary C-methyl singlets shown by andilesen C, anditomin showed only four methyl singlets at δ 1.08, 1.26, 1.33, 1.42 and additional resonances at 5.09, 5.08, 3.34, and 3.77 (all 1H, singlets). Similarly, the only significant differences in their 13C n.m.r. spectra were the replacement of the methyl, methylene and quaternary carbon resonances δ 19.5, 38.7, and 76.8 p.p.m. assigned to C(9'), C(11), C(3') respectively, in andilesen C by olefinic quaternary and methylene, and aliphatic methine resonances at 148.0, 144, and 64.1 p.p.m., respectively, in anditomin. These differences in 'H and 13C resonances are best accommodated by structure (2) where the 'absent' methyl has come the exocyclic methylene with migration of the bond to the carbonyl carbon [C(4')] from C(3') to C(11). The chemical shift of C(11)-H (334 p.p.m.) is entirely consistent with a proton flanked by carbonyl and olefinic functions and was confirmed by sodium borohydride reduction of anditomin, to give (3) with δ 2.85 and 3.34 =C-CH-C=O, J 3.0 Hz). A Dreiding model of (2) shows a dihedral angle of ca. 90° between C(11)-H and 9-H (δ 6.77) consistent with the complete lack of coupling between them. However, in the 13C n.m.r. spectrum of anditomin enriched biosynthetically from [13C2] acetate, the C(9) and C(11) resonances at 615 and 611 ppm. respectively assigned to C(9)- and C(11)-H showed a mutual 13C-13C coupling of 3.7 Hz to confirm that they are indeed adjacent. Thus all observed spectroscopic properties are consistent with structure (2) for anditomin. As this structure represents a novel skeletal rearrangement in this group of compounds a X-ray crystal study was undertaken to provide final confirmation.\(^6\)

Crystal data: C25H30O4, M = 410, clear colourless tetragonal crystals, space group P41, (No. 76), a = 9.310(5), c = 24.899(13) Å, U = 2153 Å", Z = 4, \(D_x = 1.27 \text{ g cm}^{-3}\). Mo-K\(_\alpha\) radiation, \(\lambda = 0.71069 \text{ Å}, \mu = 0.94 \text{ cm}^{-1}\). A crystal of dimensions 0.3 x 0.3 x 0.4 mm was used to record layers 06/ through 56/ with 2\(\theta\)max = 50° (graphite monochromatised Mo-K\(_\alpha\) radiation) on a Stoe Stadi-2 diffractometer. Of the 1810 unique reflections 1271 had \(I > 3\sigma(I)\). The highest 230 E-values were phased using the MULTAN-77 system.\(^4\) Structure refinement was carried out using SHELX.\(^5\) All hydrogen atoms were included in their calculated positions. All non-hydrogen atoms were refined isotropically to give a final R factor of 0.086. The arbitrary assignment of space group P41, as opposed to P41, is in accord with the likely stereochemical similarity of anditomin and andilesin C. The structure of the molecule is illustrated in the Figure by a PLUTO drawing.\(^6\)

(Received, 22nd April 1981; Comm. 466.)

\(^{1}\) The atomic co-ordinates for this work are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW. Any request should be accompanied by the full literature reference for this communication.


BIOSYNTHESIS OF HIGHLY MODIFIED MEROTERPENOIDS IN ASPERGILLUS VARIECOLOR. INCORPORATION OF $^{13}$C-LABELLED ACETATES AND METHIONINE INTO ANDITOMIN AND ANDILESIN C

Thomas J. Simpson

(Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ)

Summary: Incorporations of [1-$^{13}$C], [2-$^{13}$C], [1,2-$^{13}$C$_2$]-acetates and [1,2-$^{13}$C]-methionine into anditomin, a metabolite of Aspergillus variecolor, indicate its formation by a mixed polyketide-terpenoid biosynthetic pathway similar to that elucidated for andibenin; observations are made on the possible biosynthetic relationship of the A. variecolor metabolites with austin and terretonin, mycotoxins recently isolated from A. ustus and A. terreus respectively.

We recently reported incorporation studies$^1$ on andibenin (1) a complex C$_{25}$ metabolite of Aspergillus variecolor for which a sesterterpenoid origin had been proposed.$^2$ These studies indicated biosynthesis of andibenin via alkylation of a bis-C-methylated tetraketide-derived phenolic precursor by farnesyl pyrophosphate, followed by drimane-type cyclisation of the farnesyl moiety, intramolecular 4 + 2 cycloaddition, and oxidative modifications and rearrangement. This pathway represented a unique and elaborate variation of the triprenylphenol pathway which is not uncommon among fungi and marine organisms. We now wish to report studies on anditomin (2) and andilesin (3), co-metabolites in A. variecolor (CMI 60316)$^3$, which parallel those on andibenin and which indicate a further elaboration of this pathway.
Scheme
The $^{13}$C nmr spectra of anditomin and andilesin C have been assigned from standard chemical shift data, multiplicities in s.f.o.r.d. spectra, $^1$H-$^{13}$C decoupling experiments and $^{13}$C-$^{13}$C coupling data and are summarised in the table. Incursions of $[^{13}$C] methionine, $[1-^{13}$C]-, $[2-^{13}$C]-, and $(1,2-^{13}$C) acetates into anditomin (2) by cultures of A. variecolor resulted in the enrichments and $^{13}$C-$^{13}$C couplings summarised in the table. In the case of andilesin C (3), low yields prevented determination of the $[^{13}$C] acetate enrichments but the efficient incorporation of methionine allowed the diagnostic enrichments of the 9' and 10' methyls to be ascertained. The resulting labelling pattern for anditomin is shown in the scheme and the important features are the lack of coupling between C-3' and C-4' indicating cleavage of the original polyketide-derived carbocyclic ring, and the derivation of the olefinic methylene carbon (C-9') from the C$_1$-pool. These results indicate the pathway shown in the scheme which proposes that anditomin is formed by a novel rearrangement of the andilesin skeleton or an immediate precursor. The actual timing and mechanism of the rearrangement relative to the other necessary modifications of the proposed intermediate (4) remain to be elucidated, and this together with other aspects of the mechanism of formation and biosynthetic inter-relationships among the andibenins and andilesins are being investigated. The recent isolation of the astellolides, a trimane-type sesquiterpenoids from a mutant of the andibenin producing strain which appears to be impaired in polyketide synthesis; and stellatin, a bis-C-methylated tetraketide from A. variecolor provide further indirect support for this biosynthetic pathway. The pathway may not be unique to A. variecolor as it is possible to account for the formation of both austin, and terretin, mycotoxins isolated from A. ustus and A. terreus respectively for which sesterterpenoid and triterpenoid origins have been proposed, from the common intermediate (5). Studies to test whether these structurally diverse metabolites are indeed formed by variations of a common pathway are in progress.

The support of the S.R.C. is gratefully acknowledged. Miss S Christie is thanked for microbiological work.

References

Table 13C-Chemical Shifts (δ, relative to Me₄Si) of anditomin (2) and andilesin C (3); coupling constants (Hz) of [1,13C]acetate-enriched (2) and enrichments observed in [13C]acetate (*), [2-13C]acetate (•) enriched (2), and [Me-13C] methionine (△) enriched (2) and (3).

<table>
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<th>Carbon</th>
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<th>δ(13C-13C)</th>
<th>δ/p.p.m. (3)</th>
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<td>149.5</td>
</tr>
<tr>
<td>2</td>
<td>120.1 * a</td>
<td>68</td>
<td>119.4</td>
</tr>
<tr>
<td>3</td>
<td>165.9 *</td>
<td>68</td>
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<td>83.6</td>
<td>37</td>
<td>83.5</td>
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<td>5</td>
<td>44.5 *</td>
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<tr>
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<td>34</td>
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</tr>
<tr>
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<td>-</td>
<td>24.7</td>
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<td>10'</td>
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<td>16.8△</td>
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</table>

a enrichment ca 1 atom %  b enrichment ca 10 atom %

(Received in UK 2 July 1981)
Biosynthesis of Austin, A Polyketide–Terpenoid Metabolite of
Aspergillus ustus

By Thomas J. Simpson* and Desmond J. Stenzel
(Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ)

Reprinted from the Journal of The Chemical Society
Chemical Communications 1981
Biosynthesis of Austen, A Polyketide—Terpenoid Metabolite of Aspergillus ustus

By THOMAS J. SIMPSON* and DESMOND J. STENZEL

(10.42 J.C.S. CHEM. COMM., 1989)

Summary


We have recently carried out studies on andibeni and anditomin, complex C25 metabolites of Aspergillus varicolor which indicated their formation by a novel and elaborate extension of the triprenylphenol biosynthetic pathway, in which a bis-C-methylated tetraketide-derived phenolic precursor is alkylated by farnesyl pyrophosphate to give the intermediate (2), Scheme, followed by further extensive modifications. We now report 13C-labelling studies which indicate that the mycotoxin austin (1), a metabolite of Aspergillus ustus for which a sesterterpenoid origin has been proposed, is also formed via (2) by a further variation of this pathway.

After considerable experimentation, conditions were obtained which gave much improved yields (ca. 100 mg 1) of austin and satisfactory incorporation of acetate. The 13C n.m.r. spectrum of austin was unambiguously assigned from chemical shift considerations, multiplicities in s.f.o.r.c. spectra, 1H and 13C chemical shift correlations, and analysis of long range couplings in fully 1H-coupled 13C spectra.

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<th>Enrichment</th>
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<tr>
<td>CH3CO</td>
<td>20-6</td>
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</tbody>
</table>

(1)

Scheme
xtensive low power selective decoupling experiments. These studies gave the assignments listed in the Table. Incorporation of \([1^{13}C]\), \([2^{13}C]\), and \([1,2^{13}C_2]\)-acetate, and \([Me-^{13}C]\) methionine gave the enrichments and \(^{13}C\) couplings summarised in the Table. The resultant labelling pattern in austen is entirely consistent with the pathway shown in the Scheme in which formation of the key intermediate (2) is followed by formation of the C(8)–C(7) bond, ring contraction, and oxidative cleavage of the phenolic ring, and elaboration of the farnesyl moiety to the terpenoid spiro-lactone ring system, although the sequence in which these processes occur is uncertain. The required degree of modification of the precursor tetra-ketide is quite exceptional.

The support of the S.R.C. is gratefully acknowledged.

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Biosynthesis of Terretonin, a Polyketide-terpenoid Metabolite of *Aspergillus terreus*

By C. Rupert McIntyre and Thomas J. Simpson*  
(Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland)

Reprinted from the Journal of The Chemical Society  
Chemical Communications 1981
Biosynthesis of Terretonin, a Polyketide-terpenoid Metabolite of *Aspergillus terreus*

By C. Rupert McIntyre and Thomas J. Simpson
(Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland)

**Summary**
Incorporations of [1,2-$^{13}$C$_2$]acetate and [Me-$^{13}$C]methionine into the mycotoxin terretonin, a metabolite of *Aspergillus terreus*, indicate its formation by a mixed polyketide-terpenoid pathway.

*ANDIBENIN* and *anditomin*, metabolites of *Aspergillus variecolor*, and *austin*, a metabolite of *Aspergillus ustus*, have been shown to be formed by a novel variation of the triprenyl-phenol biosynthetic pathway in which C

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Scheme. Biosynthesis of terretonin in *Aspergillus terreus*
alkylation of 3,5-dimethylorsellinate by farnesyl pyrophosphate gives the key intermediate (2) which is then subject to further elaboration to produce the above metabolites. We suggested that the mycotoxin terretonin (1) for which a triterpenoid origin has been proposed, could also be a product of this pathway. We now report studies which support this proposal.

Incorporation of \([\text{Me-}^{13}\text{C}]\)methionine and \([1,2-{^13}\text{C}_2]\)acetate by cultures of *A. terreus* (NRRL 6273) resulted in terretonin being labelled as indicated in the Scheme. This labelling pattern can be best accounted for by the pathway summarised in the Scheme in which cyclisation of (2) as in austin biosynthesis gives the intermediate (3), which by ring contraction, followed by retro-Claisen reaction, hydroxylation, and lactonisation as indicated to generate the ring D keto-lactone system, and oxidative modification of the bicyclogeranyl moiety, gives terretonin. Further studies to establish the timing and mechanisms of these processes are in progress.

The support of the S.R.C. and microbiological assistance by Miss S. Christie are gratefully acknowledged.

\(\text{(Received, 1st July 1981; Com. 772.)}\)

† Details of \(^{13}\text{C}\) n.m.r. assignments, enrichments, and \(^{13}\text{C}-^{13}\text{C}\) couplings will be reported in full elsewhere.


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Chemical Communications 1981
Biosynthesis of the Meroterpenoid Metabolite, Andibenin B: Aromatic Precursors

BY ALAN J. BARTLETT, JOHN S. E. HOLKER,* and EUGENE O'BRIEN

(Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX)

and THOMAS J. SIMPSON

(Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ)

Summary ¹⁴C- and ²H-Labeling experiments, together with ³H n.m.r. spectroscopy show that ethyl 2,4-dihydroxy-3,5,6-trimethylbenzoate (4) and ethyl 4-hydroxy-2,3,5,6-trimethylbenzoate (5), but not ethyl 2,4-dihydroxy-
Lethylbenzoate (2) or ethyl 4-hydroxy-2-methylbenzoate are efficiently and specifically incorporated into andibenin B (1) by the fungus Aspergillus variecolor, and the iogenetic significance of these results is discussed.

Andibenin B (1) is a major meroterpenoid metabolite produced by the fungus A. variecolor. Incorporation experiments with [l-14C]-, [2-13C]-, [l,2-14C]-acetates, and thyl-[14C]-methionine have shown that this compound is derived from a sesquiterpene and a tetraketide, in which C-methyl groups have been introduced from methionine. Structural analysis of andibenin B and related metabolites suggested a number of possible tetraketide-derived phenolic carboxylic acids which might be directly involved in the biosynthetic pathway. To provide information on this point a series of 14C-labelled compounds (2-5) and the trideuteriomethyl compound (10) have been synthesised and their incorporations studied.

Ethyl [carboxy,2-14C]-2,4-dihydroxy-6-methylbenzoate was prepared by condensation of pent-3-en-2-one with ethyl [1-14C]malonate in the presence of sodium ethoxide, followed by aromatisation of the ethyl dihydro-orsellinate with bromine to give ethyl dibromo-orsellinate, and subsequent hydrogenolysis. Ethyl [carboxy,2-14C]-2,4-dihydroxy-3,5,6-trimethylbenzoate (4) was similarly synthesised from 4-methylhex-4-en-3-one and diethyl [1-14C]malonate, followed by dehydrogenation with bromine.

The monohydric phenols (3) and (5) were synthesised from the respective dihydric phenols (2) and (4) by conversion into the monobenzyl ethers, (6) and (8), respectively, reaction of these with 5-chloro-1-phenyl-1H-tetrazole to give the 1'-phenyl-tetrazolyl derivatives, (7) and (9), respectively, and subsequent hydrogenolyses of these. This procedure for deoxygenation of phenols is an adaptation of a literature method and gave satisfactory overall yields. The tri-tertiometerethyl compound (10) was prepared by monoalkylation, with tri-tertiometerethyl iodide and sodium ethoxide, of the cyclohexanedione (11) (enol form shown), prepared as above from 3-methylpent-2-en-4-one and diethyl malonate, followed by aromatisation of the product (12) with bromine. Compound (10) showed only a singlet at δ 2.09 p.p.m. in the 1H n.m.r. spectrum and the absence of a corresponding signal in the 1H n.m.r. spectrum.

The aromatic compounds (2-5, and 10) were fed to cultures of the A. variecolor mutant described previously, and andibenin B was subsequently isolated. The incorporations of the 14C-labelled compounds are summarised in the Table. The poor incorporations of ethyl orsellinate (2) and its deoxy-derivative (3), relative to those of the dimethyl derivatives (4) and (5), strongly suggests that only the latter two compounds, or more likely the corresponding carboxylic acids, are true biological intermediates. In the feeding experiment with the deuterated compound (10) andlensin A (13) was isolated, in addition to andibenin B, and both were subjected to 1H n.m.r. spectroscopy. The only observed signals were at δ 1.25 and 1.00 p.p.m. respectively, corresponding to those of the 10'-trideuteriomethyl groups in}

<table>
<thead>
<tr>
<th>Precursor</th>
<th>Specific activity (μCi mmol⁻¹)</th>
<th>Specific activity of product (μCi mmol⁻¹ × 10⁴)</th>
<th>Specific incorporation ratio/× 10⁶</th>
<th>Total incorporation ratio/× 10⁴</th>
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<tr>
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<td>0-904 (43)</td>
<td>2-27</td>
<td>0-60</td>
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<tr>
<td>(3)</td>
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<td>0-860 (39)</td>
<td>2-16</td>
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<tr>
<td>(4)</td>
<td>40-7 (63)</td>
<td>16-7 (176)</td>
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<td>39-7 (65)</td>
<td>42-3 (173)</td>
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</table>
|           | Isolated by preparative layer chromatography and crystallised to constant activity. b Ratio of specific activities of metabolite precursor × 160. c Ratio of total activities of isolated metabolite and fed precursor × 100.

All new compounds have been fully characterised by elemental analysis and the usual range of physical methods. Details of their synthesis will be published elsewhere.
these compounds. Hence, it is clear that 2,4-dihydroxy-3,5,6-trimethylbenzoic acid is a specific precursor of this group of metabolites, as indicated in the Scheme.

It is known that C-9' and C-10' of andibenin-B (1) are derived from methionine, with C-1' to C-8' coming from a tetraketide. The differential between the incorporations of ethyl orsellinate (2) and the dimethyl derivative (4) provides clear evidence that biological C-methylation precedes aromatisation of the tetraketide, in contrast to the post-aromatic introduction of the sesquiterpene moiety. The feeding experiments described here, which had been designed to elucidate this point, are decisive, as both the dihydric phenol (4) and its deo-derivative (5) are incorporated with comparable efficiency. The result suggests that both (4) and (5) can be utilised in biosynthesis. It is intrinsically more attractive to consider that interconversion would follow introduction of the sesquiterpene residue, rather than direct biological interconversion of phenols (4) and (5).

(Received, 20th August 1981; Commun. 102)

Studies on Fungal Metabolites. Part 3. 13C N.m.r. Spectral and Structural Studies on Austin and New Related Meroterpenoids from Aspergillus ustus, Aspergillus variecolor, and Penicillium diversum

By Thomas J. Simpson * and Desmond J. Stenzel, Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland

Alan J. Bartlett, Eugene O'Brien, and John S. E. Holker, Department of Organic Chemistry, The Robert Robinson Laboratories, University of Liverpool, P.O. Box 147, Liverpool L69 3BX

The 13C n.m.r. spectrum of austin has been fully assigned by inter alia analysis of long range 1H-13C couplings in the fully 1H-coupled 13C n.m.r. spectrum. Further related metabolites, dehydroaustin, austinol, and isoaustin, have been isolated from Aspergillus ustus, Aspergillus variecolor, and Penicillium diversum. Their structures have been assigned by detailed analysis of their 1H and 13C n.m.r. spectra.

Endibenins, e.g. (1) and andilesins are a series of related compounds isolated from Aspergillus variecolor. They have been shown by 13C- and 2H-labelling studies to be of mixed polyketide-terpenoid origins and the suggestion was made that the mycotoxins austin (2) and retinin (3), metabolites of Aspergillus ustus and Penicillium terrus respectively, for which sesterterpenoid origins have been proposed, might be biosynthetically related to them. In this paper we report detailed 13C n.m.r. studies on austin and the application of these studies to assigning the structures of further closely related metabolites isolated from A. variecolor, A. ustus, and Penicillium diversum.

The chemical shifts observed in the proton noise decoupled (p.n.d.) 13C n.m.r. spectrum of austin, and the multiplicities observed in the single frequency off-resonance decoupled (s.f.o.r.d.) spectrum are summarised in Table 1. Chemical shift considerations and the observed multiplicities allowed the assignment of the resonances at 146.5, 120.2, and 118.0 p.p.m. to C-1, -2, and -1' respectively. The triplet at 27.0 p.p.m. in the s.f.o.r.d. spectrum could be assigned to C-6 as the remaining ethylene carbon C-7 gives a doublet of doublets at 26.5 p.m. due to the large difference in the chemical shifts of the 7-methylene protons (Table 2). However, due to the complexity of the structure and the lack of suitable models, the task of assigning the remaining resonances appeared formidable, with the main problems being differentiating the four carbonyl resonances, the five resonances due to oxygen-bearing sp3-hybridised carbons, and the ven resonances due to C-methyls. The problem was solved by analyses of long range 1H-13C couplings in the fully 1H-coupled 13C n.m.r. spectrum using low power specific 1H decouplings and 2H exchange. While these methods have been applied extensively to aromatic compounds, their application to complex alicyclic compounds is novel and is made possible by use of very high old spectrometers where, in addition to the higher sensitivity and dispersion of the 13C n.m.r. spectrum, the extra dispersion of the 1H spectrum allows specific irradiation of the protons to be carried out more easily.

Figure 1 shows the appearance of the fully 1H-coupled 13C n.m.r. spectrum in the carbonyl and oxygen-bearing aliphatic regions, and the effect on the spectrum of a number of specific decoupling and exchange experiments. Each of the four carbonyl resonances gives a distinctive
Figure 1 The 73—86 and 163—172 p.p.m. regions of the fully 1H-coupled $^1$H.n.m.r. spectrum of austin, and results of D$_2$O exchange and selective low power decoupling experiments.

### TABLE 1

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<th>Carbon atom</th>
<th>$\delta_c$ *</th>
<th>$\delta_{c,H}$</th>
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<td>115.9 (D)</td>
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<td>4</td>
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<td>CH$_3$CO</td>
<td>20.6 (Q)</td>
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* Capital letters refer to one-bond couplings observed in s.f.o.r.d. spectra; S, singlet; D, doublet; T, triplet; Q, quartet. Low case letters refer to long range couplings observed in fully 1H-coupled $^1$H.n.m.r. spectra: d, doublet; t, triplet; m, multiplet; q, quartet; sex, sextet. * Couplings not resolved.
of 11-H simplifies to a quartet, the remaining splitting being the two-bond coupling to the methyl groups. Irradiation of the hydroxy-proton at 4.19 p.p.m. also causes collapse of the doublet at 170.1 p.p.m., which appears as a sharp singlet allowing its assignment to C-4'. This doublet splitting is also removed by addition of D$_2$O and incommittant $\gamma$-deuterium isotope shift of $-$0.04 p.p.m. observed. The remaining carbonyl resonance at 8 p.p.m. which appears as a sharp singlet showing no $\gamma$-coupling to the methyl groups. Irradiation of the methyl protons at 1.61 p.p.m. causes the multiplet at 170.1 p.p.m. to collapse to a doublet, allowing its assignment to C-2'. The diffuse multiplet at 85.5 p.p.m. must, by elimination, be due to C-4, and this multiplet shows a strong intensity increase on irradiation of the methyl resonances at either 1.38 or 1.53 p.p.m., which are therefore assigned to the 14 and 15-methyls. Irradiation of the methyl resonance at 1.85 p.p.m. results in sharpening of the diffuse multiplets at 132.5 and 143.8 p.p.m. but the final

Table 2

<table>
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<td>Others</td>
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<td>1.5-1.8 (3 H, m)</td>
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<td>1.97</td>
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Observation of the effect on the $^{13}$C n.m.r. spectrum of irradiation of the methyl proton signals as described above results in an unambiguous assignment of the methyl region of the $^1$H spectrum. Correlation of the $^1$H and $^{13}$C resonances by carrying out a Feeney plot experiment indicated that the proton resonances at 1.19, 1.29, 1.38, 1.53, 1.61, 1.85, and 2.02 p.p.m. are coupled to the carbon resonances at 23.5, 11.3, 25.9, 22.4, 20.2, 15.4, and 20.6 p.p.m., respectively, and thus permits a rigorous assignment of the methyl signals in the $^{13}$C spectrum. The connectivity patterns revealed by detection of long range $^1$H-$^{13}$C couplings as described above are summarised in Figure 2(a) and, as well as giving invaluable spectral assignment data in a molecule of known structure, they can be a source of invaluable structural information in molecules of uncertain structure, particularly those where, as in austin, the protons are highly insulated and the structural information available from $^1$H-$^1$H coupling data is limited. This is illustrated below.

Two further compounds have been isolated from A. shows any coupling to 5'-H. However, see the following cussion of dehydroaustin.

Similar decoupling experiments permit the resonances the 74-85 p.p.m. region to be rigorously assigned. irradiation of either the hydroxy-proton at 4.19 p.p.m. 5'-H at 4.46 p.p.m. causes the sextet (J 4 Hz) at 80.6 p.p.m. to change to a pentet allowing its assignment to 6'. On addition of D$_2$O the sextet similarly changes to pentet with a concomitant upfield shift of 0.05 p.p.m. d on further irradiation of the 10'-methyl protons at 29 p.p.m., the pentet collapses to a doublet (J 4 Hz), residual coupling being to 5'-H. Irradiation of the 10'-methyl protons also changes the doublet of quartets (J 0 and 5 Hz) centred at 78.7 p.p.m., to a simple doublet, allowing its assignment to C-5'. C-11 Appears as a doublet poorly resolved quartets (J 153 and 4 Hz) centred at 7.7 p.p.m., and irradiation of the methyl protons at 1.61 p.p.m. removes the quartet splitting. This methyl signal must therefore be due to the 9'-methyl showing a tree-bond coupling to C-11. Irradiation at 1.61 p.p.m. so causes the complex multiplet at 84.1 p.p.m. to resolve to a 5 line multiplet, as indicated in Figure 1. Further specific decoupling experiments indicate that its multiplet arises from couplings of 10 and 5 Hz to the 1'-methylene protons and a further coupling of 5 Hz to 11-H giving in essence an overlapping doublet of unambiguous assignment of the C-9 and C-10 resonances comes from the $^{13}$C-$^{13}$C couplings of 46 and 43 Hz to C-13 and C-11 respectively, observed in the $^{13}$C spectrum of austin enriched biosynthetically from sodium $\left[1,2-{^{13}}C\right]$ acetate.

The connectivity patterns revealed by detection of long range $^1$H-$^{13}$C couplings as described above were summarised in Figure 2(a) and, as well as giving invaluable spectral assignment data in a molecule of known structure, they can be a source of invaluable structural information in molecules of uncertain structure, particularly those where, as in austin, the protons are highly insulated and the structural information available from $^1$H-$^1$H coupling data is limited. This is illustrated below.

Two further compounds have been isolated from A.
These are dehydroaustin, $C_{27}H_{30}O_6$, and austinol, $C_{25}H_{28}O_6$, for which structures (4) and (5) have been established. Dehydroaustin has also been isolated along with austin from a chance mutant of the andibenin-producing culture of $A. variecolor$ which no longer produced andibenin. This is a very significant observation in view of the proposed common biosynthetic origins of austin and andibenin B.

Comparison of the $^1H$ and $^{13}C$ n.m.r. spectra of austin and dehydroaustin indicated a close similarity between the two compounds. In the $^{13}C$ n.m.r. spectra, the signals assigned to the C-9 and C-10 olefinic and C-13 methyl carbons in austin are replaced by signals at 139.2 (S), 125.4 (T), and 90.5 (S) p.p.m. attributable to olefinic methylene and quaternary, and allylic oxygen-bearing sp$^3$-hybridised carbons. These differences are paralleled in the $^1H$ n.m.r. spectra. As both the $^1H$ n.m.r. and i.r. spectra indicate the absence of hydroxy-groups in dehydroaustin, the molecular formula indicates that dehydroaustin contains two less hydrogens than austin, the allylic ether structure (4) is indicated. The X-ray crystal structure of austin reveals that the 6'-hydroxy-oxygen is only 2.12 Å from the 7α-proton and so the proton is severely deshielded. Examination of Dreiding models shows that formation of the ether linkage to C-9 in dehydroaustin requires a conformational change in the D-ring which removes the oxygen atom from the vicinity of the 7α-proton and so it moves upward. The same conformational change takes 5'-H close to the C-4' carbonyl and this is evidenced by the large downfield shift of 0.82 p.p.m. observed for 5'-H in dehydroaustin compared with austin itself. In addition 11-H shows an upfield shift of 0.40 p.p.m. in dehydroaustin consistent with it no longer being allylic. Final confirmation of the structure comes from the long range $^1H-^{13}C$ couplings observed in dehydroaustin. Again these have been extensively analysed by decoupling experiments and relationships revealed are summarised in Figure 1.

With the exceptions noted below these are essentially identical with those observed in austin. The significant differences are: (a) C-6' now gives a pentet, which splits to either a quartet or doublet on irradiation of 5'- or 10'-protons; (b) C-4' again appears as a doublet but now the splitting is due to a 3-bond coupling of 13.2 Hz to 5'-H and is removed on irradiation of 5'-H; and C-9 appears as a broad multiplet which sharpens on irradiation of either 11-H or the 13-CH$_2$ protons. methyl signals in the $^1H$ and $^{13}C$ spectra were again related by a Feeney plot.

The $^1H$ and $^{13}C$ n.m.r. spectra and molecular formul compound (5) suggested that it was related to austin loss of the 11-acetyl group. In the $^1H$ n.m.r. spectra 11-H appears at higher field than in austin as a doublet (5.5 Hz) at 4.69 p.p.m. coupled to a hydroxy-proton at 5.77 p.p.m. which exchanges with D$_2$O with the rem. of the doublet splitting on 11-H. Confirmation of structure was given by conversion of compound (5) into austin on acetylation with acetic anhydride and pyridine.

We have been engaged in a study of polyketide derived metabolites of $Penicillium diversum$. In course of this work we isolated from the culture lip, two metabolites which were obviously related to austin. The major of the two metabolites was clearly identical with austinol isolated from $A. ustus$. The minor metabolite had the same molecular formula $C_{27}H_{30}O_6$ as austin and lip-protons occupy pseudo-equatorial positions respectively and the angu
endence for allylic coupling requires the proton to be
axially perpendicular to the plane of the olefinic
C-H. 9 Confirmation of the structure again comes
in the close similarity of the couplings in the fully 1H-
labelled 13C n.m.r. spectrum to those observed for austin,
only observable differences being fully consistent with
structure (6) for isoaisustin. Thus the doublet of dou-
bles in austin (J 7 50 Hz)
doubly doubles (J 7 5 and 6 Hz) due to C-6, coupling
sumably to the adjacent 7a- and 7b-protons. C-1, i,
ach appears as a doublet of doublets in austin
in the co-occurrence of andibenin B and austin in
the isolation of these closely related substances from a
mber of different organisms requires comment. We
recently shown, on the basis of 13C-labelling studies,
t austin and andibenin are probably formed via a
mon intermediate (7), itself formed by alklylation of
-dimethyloserrinate by farnesyl pyrophosphate, a
is the co-occurrence of andibenin B and austin in
variecolor lends extra support to this proposal. In
lition, the occurrence of related metabolites in P.
ersum and teretomin in A. terreus indicates that this
synthetic pathway is of relatively wide occurrence, a
recent isolation from A. usits of the austalides, e.g.
which are clearly biogenetically related to the above
apounds, should also be noted. 10

PERIMENTAL

For general experimental details, see part 2.1
13C N.m.r. Determinations.—Proton noise-decoupled and
gle frequency off-resonance decoupled spectra were de-
ined on a Bruker WH 360 spectrometer operating at
56 MHz. Fully coupled spectra were determined un-
der decoupling conditions to retain nuclear Overhauser
ffects. Specific H-decoupling experiments were carried
using a decoupling power of 30 dB below 0.2 W. De-
coupling frequencies were determined by observation of

Note added in proof. Austinol (6) has recently been isolated in Emericella dentata (Prof. Y. Maebayashi, personal communi-

REFERENCES

\[\text{References}\]

synthesis of Aflatoxins. Incorporation of $[4'-^2\text{H}_2]$Averufin into aflatoxin B$_1$ by Aspergillus flavus

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National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria 0001, Republic of South Africa

Regiospecific incorporation of $^2\text{H}$ from $[4'-^2\text{H}_2]$averufin into aflatoxin B$_1$, by cultures of Aspergillus flavus, demonstrated by $^2\text{H}$ n.m.r. spectroscopy, confirms the intermediacy of averufin in the biosynthesis of aflatoxin B$_1$. 

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*Chemical Communications 1982
Biosynthesis of Aflatoxins. Incorporation of [4'-2H₂]Averufin into Aflatoxin B₁ by Aspergillus flavus

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a Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland
b National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria 0001, Republic of South Africa

The regiospecific incorporation of ²H from [4'-²H₂]averufin into aflatoxin B₁ by cultures of Aspergillus flavus as demonstrated by ²H n.m.r. spectroscopy, confirms the intermediacy of averufin in the biosynthesis of aflatoxin B₁.

The biosynthesis of the aflatoxins, potent carcinogenic mycotoxins produced by the common fungi Aspergillus flavus and A. parasiticus, has been a subject of intensive and continuing study since their isolation. Averufin (1), a metabolite of decaketide origin, also produced by A. parasiticus, appears to be the first isolable intermediate on the aflatoxin biosynthetic pathway and several experiments have been described which indicate that averufin is incorporated into aflatoxin B₁ (2). However, all these experiments have used averufin labelled biosynthetically from ¹C or ¹C labelled acetate and so, despite the sometimes impressive incorporations reported, they are subject to the criticism that degradation of the side-chain could produce labelled acetate which on reincorporation would give identically labelled aflatoxin B₁. As the role of averufin as a key intermediate on the aflatoxin pathway is crucial to all current biosynthetic proposals we have prepared a specifically labelled averufin and examined its incorporation into aflatoxin B₁.

Averufin was subjected to an acid catalysed exchange reaction by stirring at room temperature for 6 days in a mixture prepared by addition of ²H₂O to acetyl chloride in dimethyl sulfoxide. This resulted in complete and specific exchange of the 4'-hydrogens only, as shown by inter alia the disappearance of the C-4' resonance from the proton noise decoupled ¹³C n.m.r. spectrum of (1) and ²H n.m.r. spectroscopy. [4'-²H₂]Averufin in acetone solution was fed to shaken cultures of A. flavus on low salts medium and the aflatoxin B₁ produced was analysed by ²H n.m.r. spectroscopy. The resultant spectrum, Figure 1(a), showed a strong signal at δ 6.45 corresponding to the anticipated signal for H-16.

δ₈ 3.89 is due to natural abundance deuterium in the methoxy-group.

For comparison the ²H spectrum of uniformly labelled aflatoxin B₁ is shown in Figure 1(b). This sample is produced by the simple expedient of growing A. flavus on a medium supplemented with 10% ²H₂O. As the spectrum shows signals due to H-16 and H-9 are not resolved and so to that labelling from [4'-²H₂]averufin has occurred at H-16. The labelled aflatoxin B₁ was converted into 3-deoxy-aflatoxin B₁ (3) by catalytic hydrogenation and its ²H n.m.r. spectrum determined. This, Figure 1(c), shows that the signal at δ 3.89 has disappeared to be replaced by 2 signals at 3.5 and 4.07 p.p.m. due to the 16-pro-S and 16-pro-R hydroxyl, respectively. This indicates that reduction of aflatoxin B₁ is not completely stereospecific but the greater intensity of

1 at 3.59 p.p.m. means that reduction from the less red β face i.e. the 15-re, 16-re face, predominates. Conclusion these results prove that averufin is indeed porated intact into aflatoxin B1 and so averufin is now established as an obligate intermediate on the aflatoxin synthetic pathway.

References
synthesis of Aflatoxins. Incorporation of [2-2H₃]Acetate and ³C₂,2-2H₃[Acetate into Averufin

Jas J. Simpson,* Amelia E. de Jesus, Pieter S. Steyn, and Robert Vleggar

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Regiospecificity of incorporation of ²H from [1-¹³C₂,2-²H₃]acetate into averufin by cultures of

Aspergillus toxicarius has been determined using the ²H-²H isotope shift effect in ¹³C n.m.r. and by ²H n.m.r.

n.m.r. spectroscopy; the results are discussed in relation to aflatoxin biosynthesis and polyketide biosynthesis in

natural.

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Biosynthesis of Aflatoxins. Incorporation of [2-\textsuperscript{2}H\textsubscript{3}]Acetate and [1-\textsuperscript{13}C,2-\textsuperscript{2}H\textsubscript{3}]Acetate into Averuﬁn

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The regiospeciﬁcity of incorporation of $\textsuperscript{2}H$ from [1-\textsuperscript{13}C,2-\textsuperscript{2}H\textsubscript{3}]acetate into averuﬁn by cultures of Aspergillus toxicarius has been determined using the $\beta$-$\textsuperscript{2}H$ isotope shift effect in $\textsuperscript{13}C$ n.m.r. and by $\textsuperscript{2}H$ n.m.r. spectroscopy; the results are discussed in relation to aflatoxin biosynthesis and polyketide biosynthesis in general.

Despite much notable work in recent years,\textsuperscript{1} the biosynthetic pathway leading to the aflatoxins, potent carcinogenic mycotoxins produced by Aspergillus flavus and Aspergillus parasiticus, is still poorly understood in its key steps. The intermediates and mechanisms involved in the conversion of the C\textsubscript{6} side-chain of averuﬁn (1) into the bisfuranoid moiety, the conversion of the anthraquinone system into the xanthone system, and the conversion of the xanthone system into the coumarin system are not at all clear. We are currently carrying out systematic studies to elucidate the details of these steps. This paper reports the results of incorporation of $\textsuperscript{2}H$-labelled acetates into averuﬁn. As averuﬁn is the earliest established intermediate on the aflatoxin biosynthetic pathway,\textsuperscript{2} it is important to determine the mode of incorporation of acetate-derived hydrogen as a basis for studies on later metabolites. In addition averuﬁn comprises an aromatic nucleus with a relatively large, highly reduced side-chain and so the results of $\textsuperscript{2}H$ incorporation are of intrinsic interest in relation to recent studies on both aromatic and highly saturated polyketide metabolites such as brefeldin A\textsuperscript{4} and the macrolide antibiotics.\textsuperscript{4}

Both direct ($\textsuperscript{2}H$ n.m.r.) and indirect methods ($\textsuperscript{13}C$ n.m.r.) have been used to trace the incorporation of $\textsuperscript{2}H$ into a metabolite.\textsuperscript{4} Staunton has recently suggested a technique in which

\[ \text{CD}_2\textsuperscript{13}\text{CO}_2\text{Na} \rightarrow \text{HO-CD}_3-\text{HO} \]

$\textsuperscript{2}H$ is attached $\beta$ to $\textsuperscript{13}C$ in a precursor; the incorporation into a metabolite can then be detected by $\beta$-$\textsuperscript{2}H$ is induced shifts in the $\textsuperscript{13}C$ n.m.r. spectrum of the en metabolite.\textsuperscript{4} This has proved particularly useful in the present study.

The $\textsuperscript{13}C$ n.m.r. spectrum resulting from incorporation of [1-\textsuperscript{13}C,2-\textsuperscript{2}H\textsubscript{3}]acetate into averuﬁn by static cultures mutant of A. toxicarius (ATCC 24551) is shown in Fig. The isotopically shifted signals accompanying the resonances due to C-6 (\(\Delta \delta = -0.04\)), C-8 (\(-0.04\)), C-1' (\(-0.04\)), C-5' (\(-0.11\)), and C-7 (\(-0.11\)) indicate that $\textsuperscript{2}H$ is incorporated at C-5, C-7, C-2', C-4', and C-6'.

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<td>100.76</td>
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| Intensity of isotopically shifted resonance relative to corresponding non-isotopically shifted resonance. | $\textsuperscript{13}C$-$\textsuperscript{13}C$ coupling of 13.3 Hz observed. |

Resolution enhanced proton noise decoupled $^{13}$C n.m.r. spectrum of $[1-^{13}$C,2-2H$_2$]acetate-enriched averufin determined at 4Hz in CDCl$_3$, [H$_2$]DMSO solution. Spectral width 20 kHz, 32 K data points, line broadening —2Hz, and gaussian multiplier incorporation of deuterium varies widely at the different $\delta$s, as indicated in Table 1. By far the highest level of incorporation of deuterium occurs into the 6'-methyl group: nows three isotopically shifted signals corresponding to incorporation of 1—3 $^1$H atoms into the 6'-methyl grouphe signals due to $^{13}$CH$_2$H$_2$ and $^{13}$CH$_3$ species beingintense. This confirms that the 6'-methyl is part of the r acetate unit of the polyketide chain. Only one $^1$H isorated at C-4' and one at C-2'. Studies on brefeldin Athat both hydrogens from the first malonate unit to beused with the acetyl coenzyme A starter unit are re-
3. The retention of only one of these hydrogens inin may be due either to a rapid stereospecific exchange ar malonyl or polyketide methylene hydrogen; or to thegment of the 4'-methylene in the loss of ketide oxygenC-3'. This latter possibility would in turn imply thation of the side-chain occurs after ring closure and nisation of the polyketide precursor. In the aromaticmolecule it can be seen that significantly more retainted at C-5 than at C-7 and that none at all is retainedto. These differing levels of $^1$H retention cannot be rationalised at this stage but it will be interesting to
ty they are reflected in the subsequent metabolites on the
thetic pathway.$^1$H n.m.r. spectrum of [2-2H$_2$]acetate enriched averufin, 2, is in agreement with the above results and confirms differential retention of $^1$H at C-6' and the differing levels
and C-14 show a relatively large 2-bond $^{13}$C—$^{13}$C coupling 3 Hz via the C-10 carbonyl since a degree of multipleg had occurred.

Figure 2. The 55.28 MHz $^1$H n.m.r. spectra of 6,8-di-O-methylaverufin in CHCl$_3$ solution (a) enriched with sodium [2-2H$_2$] acetate and (b) uniformly labelled by addition of 10% $^2$H$_2$O to cultures of A. toxicarius.
of retention on carbons 4, 5, and 7. It was hoped that $^2$H n.m.r.
spectroscopy would provide information on the stereospecificity of $^2$H incorporations into the side-chain but this is not possible owing to inadequate chemical shift dispersion. However specific deuteriation studies on averufin which are in progress may help to resolve this aspect of the problem.

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References
synthesis of the Meroterpenoid Metabolites, Austin and Terretin: Incorporation of 3,5-Dimethylorsellinate

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and 2H Labelling experiments, together with 2H n.m.r. spectroscopy show that 3,5-dimethylorsellinic acid is a specific precursor of austin and terretin in Aspergillus ustus and Aspergillus terreus, respectively, and so substantiate the mixed polyketide—terpenoid origin proposed for these metabolites from the incorporation of 13C-labelled simple precursors.

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Biosynthesis of the Meroterpenoid Metabolites, Austin and Terretonin: Incorporation of 3,5-Dimethylorsellinate

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C and H Labelling experiments, together with H n.m.r. spectroscopy show that 3,5-dimethylorsellinic acid is a specific precursor of austin and terretonin in Aspergillus ustus and Aspergillus terreus, respectively, and substantiate the mixed polyketide-terpenoid origin proposed for these metabolites from the incorporation of 13C-labelled simple precursors.

13C and 2H Labelling studies1–3 have shown that andibenin B (1), andilesin A (2), and anditomin (3), C25 metabolites of Aspergillus variecolor are formed by a mixed polyketide-terpenoid biosynthetic pathway in which the key step is alkylation of 3,5-dimethylorsellinic acid (4) by farnesyl pyrophosphate to give (5). We suggested that the mycotoxins austin (6) and terretonin (7), metabolites of Aspergillus ustus and Aspergillus terreus, respectively, could also be formed via intermediate (5), and incorporations of singly and doubly labelled [13C]acetates and [13C]methionine into austin and terretonin result in labelling patterns consistent with proposals.4,5 However, as the suggested pathways required precedent degrees of modification4 of the tetral derived phenolic precursor, we have carried out and now studies to show that 3,5-dimethylorsellinate is indeed a precursor of both austin and terretonin (Scheme I). Ethyl (carboxy,2-13C2)-3,5-dimethylorsellinate (8) (39 mmol–1) was fed to static cultures of A. ustus (24...
and A. terreus (33 mg to 1 l), respectively, to give austin (ng, 1.60 μCi mmol⁻¹) and terretion (34 mg, 1.01 μCi mmol⁻¹), specific incorporations of 4.0 and 2.5%, respectively. Importantly, the 2-deoxyorysellinate (9) which was incorporated into andibenin B with comparable efficiency to was not incorporated into austin to any significant extent (specific incorporation, 0.06%). As with andibenin B, the complexity of the metabolites precluded the degradative analyses essential to establish specificity of labelling, so the austeriomethyl analogue (10) was fed, and the resultant metabolites analysed by 55 MHz ¹H n.m.r. spectroscopy. Austin showed only one signal at δ 1.22 p.p.m.; terretion similarly showed only one signal at δ 1.68 p.p.m.,

chemical shifts corresponding in each metabolite to the 10'-methyl hydrogens,† in agreement with our proposed pathways. Thus it is clear that 3,5-dimethylorysellinate is a specific precursor to both austin and terretion and so their meroterpenoid origins are established beyond doubt.

Further evidence for the common biogenetic origins of these metabolites comes from the isolation of austin and dehydroaustin (11), a co-metabolite of austin in A. ustus, from a chance mutant of the andibenin producing culture of A. variecolor which no longer produced andibenin. Another observation to note is the recent isolation of the austalides [e.g. (12)] from a toxigenic strain of A. ustus. Structural analysis suggests they are biosynthesised via alkylation of 5-methylorysellinic acid by farnesyl pyrophosphate, cf. mycophenolic acid followed by cyclisation of the farnesyl moiety and oxidative modifications analogous to those occurring in the andibenins and andilesins.

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References

† Control experiments show that the 10'-methyl signals in the ¹H n.m.r. spectra of universally deuteriated austin and terretion are sufficiently well resolved from other signals to ensure specificity of labelling.
osynthesis of Aflatoxins: Incorporation of [1,2-\textsuperscript{13}C\textsubscript{2}]Acetate, [\textsubscript{1}H\textsubscript{3}]Acetate, and [1-\textsuperscript{13}C, \textsubscript{2}H\textsubscript{3}]Acetate into Sterigmatocystin in *P. versicolor*

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The complete \textsuperscript{13}C--\textsuperscript{13}C coupling pattern in sterigmatocystin enriched from [\textsuperscript{13}C\textsubscript{2}]acetate has been determined to confirm that no randomisation of labelling in ring-A occurs; \textsuperscript{2}H-labelling studies indicate that \textsuperscript{2}H from [\textsubscript{3}]acetate is retained at C-6 of sterigmatocystin to rule out mechanisms for xanthone ring formation, thereby introducing the phenolic hydroxy-group on this carbon.
Biosynthesis of Aflatoxins. Incorporation of [1,2-\(^{13}\)C\(^2\)]Acetate, [\(^{2}\)H\(_3\)]Acetate, and [1-\(^{13}\)C, \(^{2}\)H\(_3\)]Acetate into Sterigmatocystin in Aspergillus versicolor

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The complete \(^{13}\)C-\(^{13}\)C coupling pattern in sterigmatocystin enriched from [\(^{13}\)C\(_2\)]acetate has been determined and confirms that no randomisation of labelling in ring-A occurs; \(^2\)H-labelling studies indicate that \(^2\)H from [\(^{2}\)H\(_3\)]acetate is retained at C-6 of sterigmatocystin to rule out mechanisms for xanthone ring formation requiring introduction of the phenolic hydroxy-group on this carbon.

Sterigmatocystin (1), a carcinogenic mycotoxin produced by a number of Aspergillus species is believed to be an intermediate in the biosynthetic pathway to the even more potent aflatoxin B\(_1\) (2) in Aspergillus flavus and Aspergillus parasiticus.\(^5\) Versicolorin A (3) has in turn been proposed as an immediate precursor to sterigmatocystin and incorporation of label from versicolorin A into sterigmatocystin\(^5\) and aflatoxin B\(^5\) has been reported. However little is known of the biochemical steps responsible for the conversion of versicolorin A into sterigmatocystin, points of particular interest being the timing and mechanism of the required loss of the phenolic hydroxy-group on C-6 of versicolorin A, a process for which there is little biosynthetic precedent;\(^4\) and the mechanisms of conversion of the anthraquinone into the xanthone ring system. Holker has proposed\(^3\) a pathway similar to that shown in Scheme I which assumes loss of a hydroxy-group to give 6-deoxyversicolorin A (4) followed by para-hydroxylation either of (4)\(^\dag\) or the product (6) of ring cleavage to permit an oxidative coupling mechanism for xanthone ring formation. The intermediate (8) formed in this way could function as a common precursor to both sterigmatocystin (1) and 6-hydroxysterigmatocystin (10). This implies that (10) and not sterigmatocystin would lie on the direct aflatoxin pathway, and there is indeed some indirect evidence for this.\(^5\) Alternatively, a metabolic grid could account for the co-occurrence of sterigmatocystin, 6-methoxysterigmatocystin, and 5,6-dimethyl sterigmatocystin.\(^7\)

Vederas has recently reported\(^8\) incorporation studies with [1-\(^{13}\)C, \(^{18}\)O\(_2\)]acetate which indicate that the 1-hydroxy-group of versicolorin A becomes the xanthone ring oxygen of sterigmatocystin, and he proposed that xanthone formation occurs by an addition-elimination mechanism of enzyme-bound benzophenone intermediate (11). However precedent suggests that a symmetrical intermediate of type invariably results in randomisation of label whereas Tanabe has reported that [1,2-\(^{13}\)C\_]acetate incorporated into sterigmatocystin without randomisation. However poor spectral resolution and severe overlapping signals allowed only a partial analysis of the coupling pattern. We now report labelling studies with \(^{13}\)C- and \(^2\)H-labeled acetates which bear upon the above proposals.

Labelled acetates were incorporated into sterigmatocystin by cultures of Aspergillus versicolor (NRRL 5219) as previously described.\(^12\) To minimise multiple labeling individual molecules, sodium [1,2-\(^{13}\)C\_]acetate was three-fold with unlabelled acetate prior to addition to cultures. Similarly [1-\(^{13}\)C, \(^{2}\)H\(_3\)]acetate was diluted with acetate prior to addition. The resultant labelled steroid was analysed by \(^{13}\)C and \(^2\)H n.m.r. spectroscopy appropriate. In the proton noise-decoupled 90.6 MHz n.m.r. spectrum of [\(^{13}\)C\_]acetate-enriched sterigmatocyste all the anticipated couplings were observed (Table I the carbons in ring-A show only one coupling apart from which appears as a single resonance with no evidence of satellites, and so there is clearly no randomisation of labelling. Thus a symmetrical intermediate, e.g. would appear to be unlikely.

\(^\dag\) 6-Deoxyversicolorin A (4) is a known metabolite of \(A.\) versicolor (ref. 5b). Its hydroxy-analogue (5) has been isolated as bisdeoxyhydrodothistromin from the phytotoxic fungus Dothistroma pini (ref. 18).
The p.n.d. $^{13}$C n.m.r. spectrum of [1-$^{13}$C, $^{1}$H$_2$]acetate-labeled sterigmatocystin, the resonances due to carbons 5, 16 show $\beta$-$^{2}$H isotope shifts (Table 1) indicating the poration of $^{3}$H at C-6, C-15, and C-17 only. This was verified by the 55.3 MHz $^1$H n.m.r. spectra in which sterigmatocystin itself showed signals at $\delta$ 6.83 p.p.m. to 4-H and/or 6-H, 6.52 (11-H and/or 17-H), 4.77 (15-H). On conversion into 3-O-methylhydromatocystin the signals moved to $\delta$, 6.98, 4.18, and p.p.m. allowing their unambiguous assignment to 6-H, and 17-H pro-R, and 17-H pro-S, respectively. There evidence for incorporation of $^3$H at C-4 or C-11; reflecting the results obtained previously for incorporation of [PH$_4$]acetate into averufin.

The observed retention of acetate-derived hydrogen at C-6 is of crucial importance as it rules out all mechanisms for xanthone formation necessitating introduction of a phenolic hydroxy-group at this carbon during the biosynthesis of sterigmatocystin. On the basis of the existing information, three mechanisms appear to be feasible. These, outlined in Scheme 2, are Michael addition followed by oxidative decarboxylation, path (a), oxidative coupling to give a spiro-intermediate, cf. erdin, followed by rearrangement and decarboxylation, path (b), or addition to an epoxide,§ path

§ This or a very similar arene oxide would necessarily be involved in the formation of both (7) and (11) from (6).
Table 1. 90.6 MHz $^{13}$C n.m.r. spectral data of sterigmatocystin (1) enriched from labelled acetates.

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* Sterigmatocystin enriched from $[1,2-^{13}$C$_2$]acetate. * $^{2}$H-isotope shift for sterigmatocystin enriched from $[1-^{13}$C, $^{2}$H$_3$]acetate.

(c). The latter mechanism is the one we prefer as it has the merit of leading to the previously proposed intermediate (9) which by concerted decarboxylation and elimination would give sterigmatocystin, or by oxidation to (8) followed by decarboxylation would give 6-hydroxyxysterigmatocystin.

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References
4. For one example see, R. D. Stipanovic and A. A. Bell, Chem., 1976, 41, 2468.
7. J. S. E. Holker, personal communication.
Application of $^2\text{H}$ $\beta$-Isotopic Shifts in $^{13}\text{C}$ N.M.R. Spectra to Biosynthetic Studies. Incorporation of [1-$^{13}\text{C},^2\text{H}_3$]Acetate into O-Methylasparvenone

Aspergillus parvulus

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Incorporation of [1-$^{13}\text{C},^2\text{H}_3$]acetate into O-methylasparvenone (1) and analysis of the $^2\text{H}$ $\beta$-isotope shifts in resulting $^{13}\text{C}$ n.m.r. spectrum indicate that one hydrogen is lost from the methyl derived from the acetate unit; the magnitude of the observed $^2\text{H}$ $\beta$-isotope shifts showed a marked dependence on the ionality of the reporter $^{13}\text{C}$ nucleus and the stereospecificity of $^2\text{H}$ incorporation.

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Application of $^2$H $\beta$-Isotopic Shifts in $^{13}$C N.M.R. Spectra to Biosynthetic Studies. Incorporation of [1-$^{13}$C,$^2$H$_3$]Acetate into $O$-Methylasparvenone in Aspergillus parvulus

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Incorporation of [1-$^{13}$C,$^2$H$_3$]acetate into $O$-methylasparvenone (1) and analysis of the $^2$H $\beta$-isotope shifts in the resulting $^{13}$C n.m.r. spectrum indicate that one hydrogen is lost from the methyl derived from the acetate 'starter' unit; the magnitude of the observed $^2$H $\beta$-isotope shifts showed a marked dependence on the functionality of the reporter $^{13}$C nucleus and the stereospecificity of $^2$H incorporation.

Staunton recently suggested a novel method for detecting and quantifying the biosynthetic incorporation of deuterium. In this method the incorporation of $^2$H placed $\beta$ to a $^{13}$C atom in a precursor is detected by a small upfield $\beta$-isotope shift in the resonance of the $^{13}$C nucleus in the $^{13}$C n.m.r. spectrum of the enriched metabolite. We have found that this technique is indeed most useful, and we have applied it to studies of the aflatoxin biosynthetic pathway. We now report its application to $O$-methylasparvenone (1) and note some complicating features, both limiting and potentially useful, which this study has revealed.

In a previous study, we used $^1$H n.m.r. spectroscopy to show that $^2$H from [H$_3$]acetate was incorporated into (1) at the 10-methyl, 5-, 2-, and 3-axial hydrogens, and from

Scheme 1. $\bullet = ^{13}$C
P.n.d. $^{13}$C n.m.r. spectrum of [1-$^{13}$C, 2-$^{2}$H]-acetate-enriched O-methylasparvenone (1) determined at 90.56 MHz in CDCl$_3$ solution. Spectral width 20 kHz, 32 k data points, pulse width 4 μs, acquisition time 0.82 s, relaxation delay 0.18 s, line broadening 2 Hz, gaussian multiplier 0.3.

1. Comparison of the incorporation levels we suggested that one oxygen was lost from the methyl of the starter acetate, implying that the necessary loss of ketide oxygen from occurred after condensation and aromatisation of the hexaketide precursor. The $\beta$-isotope shift method ideal for verifying the number of $^2$H atoms retained -10. Thus [1-$^{13}$C, $^{2}$H]-acetate was incorporated into (1) products of Aspergillus parvulus (Scheme 1), and the reporter noise decoupled (p.n.d.) $^{13}$C n.m.r. spectrum, in Figure 1, confirmed our proposals as C-9 showed two isotopically shifted signals corresponding to the loss of one and two deuteriums on the adjacent methyl n. The $\beta$-shift is clearly additive (−0.08 p.p.m. per $^2$H). Also showed an isotopically shifted resonance (−0.06 p.p.m.) due to $^2$H on C-5. However C-1 showed an isotopically shifted resonance downfield (+0.06 p.p.m.) and the intensity was much lower than that expected from the observed level ($^2$H n.m.r. spectroscopy) of incorporation of $^2$H 2-axial position. In addition, as a consequence of a high of multiple labelling of individual molecules from the precursors, the $^4$H at the 2-axial position resulted ball isotopically shifted resonance (−0.13 p.p.m.) being +8 for C-3.$^\dagger$

In order to confirm that the observed isotope shifts at C-1-3 were reasonable [2-$^{2}$H]-O-methylasparvenone was provided by base-catalysed exchange (NaOMe in MeOH), p.n.d. $^{13}$C n.m.r. spectrum now showed isotopically shifted resonances at +0.08 and −0.17 p.p.m. for C-1 and C-3 respectively, suggesting either that the induced isotope shifts are additive or that equatorial $^2$H has a much

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**Figure 2.** Signals from the 90.56 MHz p.n.d. $^{13}$C n.m.r. spectrum of O-methylasparvenone deuteriated at C-2. Resonances are for (a) C-1, (b) C-3. Determined using 1 kHz spectral widths, 4 k data points acquired and transformed into 32 k data points, pulse width 4 μs, acquisition time 2.04 s, line broadening −1 Hz, and gaussian multiplier 0.25.
smaller effect than axial $^1$H. This was further examined by carrying out a second exchange experiment in equimolar MeOH and MeOH $^2$H. N.m.r. spectroscopy of the product confirmed that equal exchange of axial and equatorial hydrogens had occurred. The p.n.d. $^{13}$C n.m.r. spectrum, Figure 2(b), now showed three isotopically shifted resonances for C-3 at $-0.03$, $-0.13$, and $-0.17$ p.p.m. of similar intensities as expected for the statistical mixture of molecules labelled at the 2-equatorial and 2-axial positions separately, and both 2-equatorial and 2-axial positions. C-1 also showed, Figure 2(a), three isotopically shifted resonances at $+0.02$, $+0.06$, and $+0.08$ p.p.m. but their intensities decreased markedly as the number of adjacent deuterium atoms increased. These results show that the $\beta$-isotope shifts are additive and moreover are dependent on the stereospecificity of labelling. This has not to our knowledge been noted previously and should permit $\beta$-shifts to be used to determine stereospecificity as well as regiospecificity of labelling. The particular sensitivity of the C-1 resonance intensity to substitution of the adjacent hydrogens by $^2$H explains the misleading low intensity observed for the isotopically shifted C-1 resonance in the biosynthetic experiment described above. Although steps be taken to eliminate such intensity variations arising differences in relaxation times and nuclear Overhauser enhancements, these are often difficult or impossi
ditional reasons, such as in the above case where the amount of sample available precluded the use of long relaxation delays.

Details of long range shift effects observed in the chemical exchange experiments described above will be rep
ght elsewhere.

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References
Biosynthesis of Aflatoxins. Incorporation of [2-$^2$H$_3$]Acetate into Aflatoxin B$_1$ by Aspergillus flavus

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The observation of an NIH shift on incorporation of [2-$^2$H$_3$]acetate into aflatoxin B$_1$ in cultures of Aspergillus flavus provides evidence for the timing of deoxygenation and hydroxylation steps which are an essential part of the biosynthetic pathway.
Biosynthesis of Aflatoxins. Incorporation of [2-^{2}H_{3}]Acetate into Aflatoxin B, by *Aspergillus flavus*

Thomas J. Simpson,*a* Amelia E. de Jesus,*b* Pieter S. Steyn,*b* and Robert Vleggaar*b*

*a* Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ, Scotland

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The observation of an NIH shift on incorporation of [^{2}H_{3}]acetate into aflatoxin B, in cultures of *Aspergillus flavus* provides evidence for the timing of deoxygenation and hydroxylation steps which are an essential part of the biosynthetic pathway.

Despite extensive studies the exact sequence of intermediates on the biosynthetic pathway leading to aflatoxin B, (1), the carcinogenic mycotoxin produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus*, is uncertain.1 Even less...
Own of the detailed mechanisms responsible for the interconversion of the known intermediates. Accordingly we set out to elucidate the fate of acetate-derived hydrogen on incorporation into aflatoxin B1 and its precursors, with a view to obtaining mechanistic information. We have recently reported ¹H labelling studies on averufin (2) and sterigmatocystin (3) which along with versicolorin A (4) are believed to be key intermediates on the biosynthetic pathway. We now report the results of extension of these studies to aflatoxin B1 itself.

[²-¹H]Acetate was added to shaken cultures of A. flavus on low-salts medium and the aflatoxin B1 (4) produced examined by ¹H n.m.r. spectroscopy. The resulting spectrum showed signals at δH 6.45, 4.72, 3.35, and 2.58 p.p.m., indicating that acetate-derived hydrogen is incorporated on carbons C-9 and/or C-16, C-14, C-5, and C-4. As the signals due to H-16 and H-9 are not resolved in aflatoxin B1 itself, the labelled sample was converted into 3-deoxy-aflatoxin B1 (5). The ¹H n.m.r. spectrum of (5) [Figure 1(a)] confirmed the above observations and indicated that most of the label was on C-16 with possibly a trace of ¹H incorporation at C-9. The 360.13 MHz ¹H n.m.r. spectrum of (5) derived from universally labelled aflatoxin B1 [Figure 1(b)] is shown for comparison.

The crucial observation is the presence of acetate-derived hydrogen on C-5. The position is derived from the carboxy-carbon of acetate and so it must be assumed that the ¹H label has migrated to C-5 from the adjacent carbon during the course of biosynthesis. The most likely mechanism would be

† Note that all the signals of interest are resolved. There is no signal for the C-3 methylene hydrogens in Figure 1(b) as these are introduced in the hydrogenation step.
as a result of an NIH shift‡ indicating that hydroxylation
the carbon adjacent to that which becomes C-5 is an essen-
step in the cleavage and conversion of the aromatic ring wh
becomes the cyclopentenone moiety in aflatoxin B₁.

One of the outstanding problems concerned with the a-
toxin biosynthetic pathway is the timing of the required l
of the 6-hydroxy-function from versicolorin A (4). One of
more plausible proposals was that versicolorin A was e
verted first into 6-deoxyversicolorin A (6) which was th
hydroxylated to give (7), both of which are known metabol
(Scheme 1). However we have recently shown that (7) can
be an intermediate in the formation of sterigmatocystin (3)
H is retained at C-6. The present results indicate that
intermediate which has already lost the 6-hydroxy-funct
present in versicolorin A (4) is hydroxylated, otherwise no N
shift could be observed. Thus the most probable sequence
vants is that versicolorin A (4) is converted via (6), (3), i
(8) into aflatoxin B₁. A less likely sequence, (4) → (6) → (7)
(8) → (1), would mean that sterigmatocystin is not an obli-
tory intermediate and cannot yet be entirely ruled o
Cleavage of the aromatic ring and formation of the cyc
pentanone ring can occur by precedent procedures as o
lined in Scheme 2. Further studies to delineate the ex
sequence of events between versicolorin A and aflatoxin
are in progress.

Received, 16th November 1982; Comm. 1310

References

1 P. S. Steyn, R. Vleggaar, and P. L. Wessels, in 'The Biosynthesis
p. 105.
2 T. J. Simpson, A. E. de Jesus, P. S. Steyn, and R. Vlegga
3 T. J. Simpson and D. J. Stenzel, J. Chem. Soc., Chem. Com-
1982, 890.
4 T. Asao, G. Buchi, M. M. Abdel-Kadar, S. B. Chang, E.
5 T. J. Simpson, A. E. de Jesus, P. S. Steyn, and R. Vlegga
6 K. G. R. Pachler, P. S. Steyn, R. Vleggaar, P. L. Wessels, a
7 T. J. Simpson and D. J. Stenzel, J. Chem. Soc., Chem. Com-
1981, 239.

‡ We have observed a similar NIH shift occurring during po
detide biosynthesis in studies on O-methyl-asparvenone, a
metabolite of Aspergillus parvulus.
Synthesis and Regiospecific Deoxygenation of \( \beta \)-Resorcylic Ester Derivatives to 4-Hydroxybenzoates

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Department of Chemistry, University of Edinburgh, West Mains Road, Edinburgh EH9 3JJ

Ethyl \([\text{carboxy,6-}^{14}\text{C}_2]-4\text{-hydroxy-2-methyl-}
\text{hydroxy-2-methyl-(6a)}\) and \(-2,3,5\text{-trimethyl-benzoate (6b)}, \text{together with}
\text{ethyl 4-hydroxy-2,3-dimethylbenzoate (6c), have been prepared in satisfactory yields by deoxygenation of the}
\text{resorcylate esters, (5a—c), respectively, via hydrogenolysis of their 4-benzoyloxy-2-(1-phenyl-1H-tetrazolyl-)derivatives, (8a—c). Ethyl 2,4-dihydroxy-5,6-dimethyl-3-trideuteriomethylbenzoate (5d) has been synthesised by trideuteriomethylation of ethyl 2,3-dimethyl-4,6-dioxocyclohexanecarboxylate (10c), followed by dehydrogenation. Preparations of the requisite resorcylate derivatives are included.}

...
parent esters (5a) and (5c), respectively. Repetition of this work with diethyl [1-14C]malonate gave similar radiochemical yields of the corresponding [carboxy, 2-14C]-derivatives.

The required trideuteriomethyl derivative (5d) was prepared from the dihydroresorcylic ester (10c) by reaction with one equivalent of trideuteriomethyl iodide in the presence of sodium ethoxide, followed by dehydrogenation of the intermediate dihydroxyresorcylic derivative (10d) with bromine.

A new synthetic route to alkyl 3-resorcylicates, published since the completion of the present work, does not appear to offer significant advantages for the synthesis of the labelled compounds in the present study.

**Experimental**

Except where otherwise stated 1H n.m.r. spectra were measured in CDCl3 with SiMe4 as internal standard on a Perkin-Elmer R34 instrument operating at 220 MHz, 13C n.m.r. spectra with a Bruker WH 360 instrument operating at 55.26 MHz, u.v. spectra in ethanol on a Pye-Unicam SF8-100 instrument, and i.r. spectra in CCl4 on a Perkin-Elmer 257 instrument. Mass spectra were determined with an AEI MS-12 spectrometer, and i.r. spectra in Cd4 on a Perkin-Elmer 257 instrument. Preparative layer chromatography (p.l.c.) was performed with a 1-mm thick layer of Kieselgel 60FF 254 (Merck) on glass plates (20 x 20 cm), activated at 110 °C for 12 h after air drying. Light petroleum refers to that having a b.p. of 60-80 °C, and ether refers to diethyl ether.

**Ethyl 2,4-Dihydroxy-6-methylbenzoate (5a).—** Hydrolysis of the above product (1.03 g) in a solution of potas-
ethyl 4-Benzoyloxy-2-hydroxy-6-methyl-(7a), -5,6-dimethyl-6,5,3,3,6,5-trimethylbenzoate (5b) (814 mg) were effected
3; H, 7.2%;
6; H, 5.4; N, 12.6%;
7(1 H, d,
5 mg), m.p. 67-68 °C, \( \lambda_{max} 267 (c 8 900) \), and 310 nm
25 cm'; 6H 1.07 (3 H, t,
7.2 Hz, \( \lambda_{max} 258 \) (4600); \( \nu_{\text{carboxy}}, \lambda_{\text{carboxy}} \)). C 19 H 22 0 4  requires C, 72.6; H, 7.1;

3 For summary of these methods and literature references, see J.

**References**

11 O. Hesse, Ann., 1861, 117, 297.

Received 28th July 1982; Paper 2;
The Structures of Some Metabolites of *Penicillium diversum*: α- and β-Diversonolic Esters

John S. E. Holker* and Eugene O’Brien

The metabolites of *Penicillium diversum* include the meroterpenoids austinol (1) and isoaustin (2), together with the known poly-β-ketides lichenxanthone (3), alternariol monomethyl ether (4), and two new compounds, α- and β-diversonolic esters, (5) and (6) respectively. The structure elucidation of the latter compounds is based essentially on extensive 1H and 13C n.m.r. spectroscopic studies. The biogenetic significance of these compounds is discussed.

![Chem Soc Perkin Trans 1 1983](https://doi.org/10.1039/P19830000244)
1. **H N.m.r. chemical shifts of α- and β-diversonolic esters and derivatives (p.p.m. with couplings in Hz)**

<table>
<thead>
<tr>
<th>Proton(s)</th>
<th>Compound</th>
<th>(5)</th>
<th>(6)</th>
<th>(8)</th>
<th>(9)</th>
<th>(10)</th>
<th>(11)</th>
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<tr>
<td>1-OH</td>
<td>2.82</td>
<td>4.76 s</td>
<td>4.57 s</td>
<td>5.27 s</td>
<td>5.15 s</td>
<td>4.60 s</td>
<td>4.00 s</td>
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<tr>
<td>2</td>
<td>2.82 (J9a = 5.9) and 8.9, J9b = 18.7)</td>
<td>2.83 m</td>
<td>5.27 s</td>
<td>5.15 s</td>
<td>4.60 s</td>
<td>4.00 s</td>
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<tr>
<td>3</td>
<td>2.17 (J9a = 5.9 and 6.4 or 4.9, J9b = 3.9, J9c = 16.0)</td>
<td>2.11 m</td>
<td>2.82 m</td>
<td>2.80 m</td>
<td>2.83 m</td>
<td>2.73 m</td>
<td>4.00 s</td>
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<tr>
<td>4</td>
<td>4.08 (J9a = 3.9 and 10.8)</td>
<td>4.03 (J9a = 5 and 6.5)</td>
<td>4.11 (J9a = 5 and 6.5)</td>
<td>4.00 (J9a = 5 and 11)</td>
<td>5.19 (J9a = 5)</td>
<td>5.11 (J9a = 5 and 6.5)</td>
<td>4.00 (J9a = 5 and 6.5)</td>
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<tr>
<td>5</td>
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<td>5 and 7</td>
<td>6.67</td>
<td>6.66 d</td>
<td>6.79 d</td>
<td>6.78 d</td>
<td>7.12 d</td>
<td>7.10 d</td>
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<tr>
<td>6</td>
<td>2.38 s</td>
<td>2.39 s</td>
<td>2.43 s</td>
<td>2.40 s</td>
<td>2.45 s</td>
<td>2.35 s</td>
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<tr>
<td>6'-omethy</td>
<td>11.85 s</td>
<td>11.92 s</td>
<td>3.79 s</td>
<td>3.79 s</td>
<td>3.76 s</td>
<td>3.70 s</td>
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<tr>
<td>8</td>
<td>3.85 s</td>
<td>3.81 s</td>
<td></td>
<td></td>
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</tbody>
</table>
| 8'-OMe    | 3.96 s (8-OMe) | 3.91 s (8-OMe) | 2.03, 2.37 (8- and 2.10, 2.43 (8-)
| Others    | 4a'-OMe | 4a'-OMe | 4a'-OMe | 4a'-OMe |

* Measured at 360 MHz. † Measured at 220 MHz.

2. | 7-H N.m.r. chemical shifts of α- and β-diversonolic esters and derivatives (p.p.m. with multiplicities for off-resonance decoupled spectra, where obtained (spectra at 25.19 MHz)

<table>
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<th>Carbon</th>
<th>(5)</th>
<th>(6)</th>
<th>(8)</th>
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<tr>
<td>1</td>
<td>166.7 s</td>
<td>166.9 s</td>
<td>163.4 s</td>
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<td>2</td>
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<td>260 t</td>
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<tr>
<td>3</td>
<td>244 t</td>
<td>253 t</td>
<td>244 t</td>
</tr>
<tr>
<td>4</td>
<td>72.6 d</td>
<td>70.4 d</td>
<td>70.4 d</td>
</tr>
<tr>
<td>4a</td>
<td>76.3 s</td>
<td>72.8 s</td>
<td>73.0 s</td>
</tr>
<tr>
<td>4a'</td>
<td>172.7 s</td>
<td>173.9 s</td>
<td>172.5 s</td>
</tr>
<tr>
<td>5</td>
<td>107.1 d</td>
<td>107.2 d</td>
<td>107.6 d</td>
</tr>
<tr>
<td>6</td>
<td>147.5 s</td>
<td>147.6 s</td>
<td>145.7 s</td>
</tr>
<tr>
<td>6'</td>
<td>22.4 q</td>
<td>22.4 q</td>
<td>22.3 q</td>
</tr>
<tr>
<td>7</td>
<td>121.4 d</td>
<td>112.1 d</td>
<td>111.3 d</td>
</tr>
<tr>
<td>8</td>
<td>160.0 s</td>
<td>160.1 s</td>
<td>159.3 s</td>
</tr>
<tr>
<td>8a</td>
<td>108.0 s</td>
<td>108.0 s</td>
<td>109.8 s</td>
</tr>
<tr>
<td>9</td>
<td>181.8 s</td>
<td>181.1 s</td>
<td>177.6 s</td>
</tr>
<tr>
<td>9a</td>
<td>116.6 s</td>
<td>116.7 s</td>
<td>119.0 s</td>
</tr>
<tr>
<td>10a</td>
<td>155.9 s</td>
<td>155.8 s</td>
<td>157.8 s</td>
</tr>
<tr>
<td>OMe</td>
<td>53.2 q</td>
<td>53.5 q</td>
<td>52.8 q</td>
</tr>
<tr>
<td>8-OMe</td>
<td>56.3 s</td>
<td></td>
<td></td>
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</table>

2.82, J 5.9, 8.9, 18.7 Hz and 2.88, J 4.9, 6.4, 18.7 Hz (CHOH-CH2CH2CH2).

The 13C n.m.r. spectra of the two compounds are very similar (Table 2), the chemical shift values and multiplicities in the off-resonance decoupled spectra being entirely compatible with the proposed structures. The fully proton coupled 13C spectrum of the α-compound (5) measured at 90.6 MHz was particularly informative in establishing the substitution pattern of the aromatic ring. It showed the following features. The signal at δ 160.0 p.p.m. due to C-8, showed a large upfield shift (22 Hz, 0.24 p.p.m.) and sharpened on addition of D2O, confirming this as the carbon carrying the phenolic hydroxy group. The multiplet at δ 108 p.p.m. due to C-8a also moved upfield slightly with D2O (2.5 Hz, 0.03 p.p.m.) and sharpened to a triplet (J ca. 7 Hz) due to equal coupling to both 5-H and 7-H, as shown by irradiation of the aromatic protons when the triplet collapsed to a singlet. The doublet of multiplets centred at 112.0 p.p.m. also showed a small upfield shift and sharpened with D2O, allowing its assignment to C-7; and on irradiation of the protons of the aromatic methyl this resonance collapsed to a doublet of doublets (J 160 Hz, due to 1-bond coupling to 7-H) due to the removal of the 3-bond coupling to the methyl hydrogens, the residual coupling (J ca. 7 Hz) being the 3-b coupling to 5-H. Similarly the doublet of multiplets due to C-5 (δ 107.1 p.p.m.) collapsed to a doublet of doublets (J and 6 Hz) on irradiation of the 6'-methyl protons. Irradiation also sharpened the quartet (J 6 Hz) at 147.5 p.p.m. to an intense singlet and it must be due to C-6. Irradiation of the aromatic hydrogens also caused a sharpening and hancement of the C-8 resonance at 160 p.p.m., with removal of the 2-bond coupling (J 2.4 Hz) to 7-H and enhancement of resonance at 155.9 p.p.m. due to C-10a. Finally, the carbonyl singlet resonance at 181.8 p.p.m. also showed small upfield shift on addition of D2O (2.5 Hz, 0.03 p.p.m.) and sharpened. This evidence leaves no doubt that the orientation of the substituents around the aromatic ring is as defined by structure.

Apart from the carbon resonances due to the CH2OH and CH2CH2 fragment, which have the expected chemical shift values and multiplicities in the off-resonance decoupled spectrum, the only remaining 13C-resonances are singlets at δ 167 and 116.5 p.p.m. in the off-resonance decoupled spectrum. These chemical shift values are entirely consistent with those expected for the residue CO-C(OH). Furthermore, in the fully coupled spectrum of the α-compound the signal at 173 p.p.m. is broad presumably due to coupling to the methoxy hydrogens. Although specific decoupling was not carried out none of the other irradiations affected it, so by default the coupling must be to OMe.

The presence of a methoxy carbonyl group in these compounds is compatible with an i.r. absorption at ca. 1 740 cm⁻¹ and an OMe signal in the 1H n.m.r. spectra at δ ca. 3.8 p.p.m. 13C signals at δ ca. 173 and 53.5 p.p.m., due to the carbonyl and methoxy carbon atoms respectively. In the fully coupled spectrum of the α-compound the signal at 173 p.p.m. is broad presumably due to coupling to the methoxy hydrogens. The presence of a methoxy group is also consistent with the i.r. and n.m.r. spectra.
Although there seems little doubt that structures (5) and (6) rely satisfy the spectroscopic properties of the $2\beta$- and $2\beta$-sononic esters, there are some anomalies in the chemistry of the isomers of this region of the monocyclic structure. Thus the chemical shift of the exchangeable enolised $2\beta$-dicarboxyl compound at 4.76 and 4.57 p.p.m. in compounds (5) and (6) is considerably higher than the less stable cis-orientation of the hydroxy and acetoxy groups in these compounds.

In the case of an excess of diazomethane or acetylated with acetic acid, although the isolated yields of these derivatives are very similar and no stereochemical conclusions can be drawn from these evidence.

The determination of the relative configurations of the $2\alpha$-diversonolic esters is based on LIS studies on the parent compounds. It was anticipated by analogy with previous results that the principal site of co-ordination of Eu(fod)$_3$ would be the secondary alcoholic hydroxy group at C-4, although the isolated yields of these derivatives are very similar and no stereochemical conclusions can be drawn from these evidence. In the case of an excess of diazomethane or acetylated with acetic acid, although the isolated yields of these derivatives are very similar and no stereochemical conclusions can be drawn from these evidence.

The allicyclic ring of the diversonolic esters would be expected to be relatively conformationally mobile. In the case of the parent cis-$2\alpha$-compound (5) and its monomethyl ether (7) which would be removed on acetylation, the corresponding trans-$2\beta$-compound (6) and its monomethyl ether (9) the observed C-3 to C-4 proton couplings are both small (ca. 5 and 6.5 Hz), suggesting a pseudo-equatorial C-4 proton and hence a pseudo-axial hydroxy group. However, on acetylation the corresponding couplings are again changed (ca. 4 and 11 Hz), similarly suggesting a conformational change in the allicyclic ring. Although the reasons for this are not entirely clear, they are probably associated with intramolecular hydrogen bonding between the C-4 hydroxy and C-4a' methoxycarbonyl groups in the parent cis-$2\alpha$-alcohol (5), which would be removed on acetylation.

It has been pointed out that diversonol (7) appears to be related biosynthetically to the sulochrin group of fungal metabolites, which are derived by cleavage of an anthraquinone intermediate. In this case the 4a-$2\beta$-methyl group of diversonol must result from complete reduction of a carboxyl intermediate. Since in the diversonolic esters (5) and (6) the corresponding 4a-$2\beta$-carbon is unreduced and present as a methoxycarbonyl group, the anthraquinone derivation of these compounds is supported. It is interesting that such derivation would involve oxidative fission of an anthraquinone or anthrone precursor, e.g., chrysophanol (14) at (a) as shown in the Scheme. This corresponds with the biosynthesis of geodin, where emodin (15) has been shown to be an intermediate. On the other hand alternative oxidative fission at (b) has been demonstrated in the biosynthesis of the ergochromes and has also been proposed to account for the results of feeding experiments on the fungal xanthone, tajixanthone, and related compounds.16

**Scheme.**

Unless otherwise stated i.r. spectra were measured in Nujol with a Perkin-Elmer 257 instrument and u.v. spectra in ethanol (95%) with a Pye-Unicam SP 8-100 instrument. 1H N.m.r. spectra were measured in CDC$\text{I}_3$ with Me$_4$Si as internal standard either at 220 MHz with a Perkin-Elmer R34 instrument or at 360 MHz with a Bruker WH360 instrument.

Mass spectra were determined with an AE1 MS-12 instrument at 70 eV and accurate mass measurements with an AE1 MS-9 instrument. Preparative layer chromatography (p.l.c.) was performed on 1-mm thick layers of Kieselgel 60 PF$_{254}$ (Merck) on glass plates (20 x 20 cm.), activated at 110°C for 12 h.
Isolation of α- and β-Diversonic Ester (5) and (6).—

Pencillium diversum (no. 946 in I.C.I. collection) was grown from those suspensions in static cultures, for 29 days at 25 °C in 100 flat vessels (ca. 1 l capacity) each containing Raulin-Thom medium (500 ml). After removal of the mycelium the culture broth was exhaustively extracted with chloroform (15 l). After concentration, the resultant extract was washed repeatedly with 2m-sodium hydrogen carbonate, and then water. After drying (NaSO₄), the chloroform solution was evaporated and the residue (2.2 g) was subjected to p.l.c. using ethanol-chloroform (1:25) as developing solvent. The band, Rₚ 0.15, was isolated, extracted and further separated by p.l.c., developing with ether, to give two bands. That with a higher Rₚ was separated and extracted to give α-diversionic ester (5) which formed prisms (ca. 100 mg), m.p. 182 °C; [α]D-22.6° (0.23 in CHCl₃), v₉ max (CCL₃) 3.570, 3.490, 1.740, 1.655 and 1.625 cm⁻¹; λₚ max 281 (4 300), 261 (7 400), and 250 nm (5 000) (Found: C, 39.9; H, 5.2; M, 320.08). C₁₂H₂₀O₉ requires C, 40.0; H, 5.0%; M, 320.09. The lower Rₚ band from above was re-purified by p.l.c., developing with ethyl acetate, to give β-diversionic ester (6) as a gum (20 mg), [α]D+40.0° (0.12 in CHCl₃); v₉ max (CH₃Cl) 3.540, 2.480, 1.750, 1.660, 1.650, and 1.600 cm⁻¹; λₚ max 226 (2 500), 259 (10 500), 250 (10 600), 239 (17 300), and 228 nm (17 000). (Found: M*, 320.08). Both compounds gave intense red-brown colourations with ferric chloride solution.

Methylation of Diversionic Ester.—The parent compounds (5) and (6) (30 and 30 mg respectively) were separately methylated in chloroform with an excess of ethereal diazomethane at 0°C for 12 h. Methylated compound (5) was purified by p.l.c. with chloroform into two principal components. The major and one minor component on t.l.c. and were therefore purified by p.l.c. with ether as developing solvent. Di-O-acetylα-diversionic ester (10) was an amorphous solid (22 mg), [α]D-11.2° (0.16 in CHCl₃); v₉ max (CH₃Cl) 3.480br 1.740, and 1.640 cm⁻¹ (Found: M*, 404.113. C₂₀H₂₀O₁₂ requires M, 404.111). Di-O-acetylβ-diversionic ester (11) was an amorphous solid (16 mg), [α]D+30.0° (0.13 in CHCl₃); v₉ max (CH₃Cl) 3.480br, 1.780, 1.740, 1.715, and 1.640 cm⁻¹ (Found: C, 59.3; H, 4.7. C₂₀H₂₀O₁₂ requires C, 59.4; H, 5.0%). Neither compound gave a colouration with ferric chloride solution.

Isolation and Characterisation of Lichenanthone and Alternariol Monomethyl Ether.—A portion (40 g) of the dried mycelium (200 g) from the above growth was continuously extracted with chloroform for 24 h. The extract was separated by p.l.c. with chloroform into two principal components. The faster running band on isolation and extraction gave lichenanthone as cream needles (80 mg) from chloroform, m.p. 190 °C (lit.,² m.p. 186—187 °C), with identical i.r. and u.v. spectral bands to those published,⁴ [α]D+13.3° (s, 1 H, exchangeable with D,O, phenolic OH) 6.65 (s, 2 H, ArH), 6.2 H, ArH), 3.90 (s, 3 H, OMe), 3.88 (s, 3 H, OMe), and p.p.m. (s, 3 H, ArMe), (Found: C, 67.0; H, 4.85%; 286.083. Calc. for C₁₇H₁₇O₃: C, 67.1; H, 4.9%; M, 286. Prepared with methyl iodide and potassium acetone under reflux for 10 h the methyl ether separated from ether, m.p. 155 °C (lit.,⁵ m.p. 155—156 °C, 6.65 (s, 2 H, ArH), 6.41 (d, 1 H, J 2 Hz, ArH), 6.32 (d, 1 J 2 Hz, ArH), 3.97 (s, 3 H, OMe), 3.89 (s, 3 H, OMe), and 2.85 (s, 3 H, ArMe).

The slower moving band from isolation extraction gave alternariol monomethyl ether (4), m.p. (45 mg) from chloroform, m.p. 270—272 °C (dec [lit.,⁶ m.p. 267 °C (decomp.), with identical i.r. and u.v. to those published,⁷ [α]D+40.0° (H, acetone), 9.27 (s, 1 H, exchangeable with D,O, H-bonded OH), 7.31, 6.82, 6.73, 6.58, 6.30, (each 1 H, J 1.5 Hz, ArH), 3.98 (s, 3 H, OMe), and 2. H, ArMe) (Found: M*, 272.067. Calc. for C₁₄H₁₉O₂: 272.071). Methylation of this compound (50 mg) in chloroform with an excess of ethereal diazomethane at 0°C for 12 h a mixture of two components, separated by p.l.c. in chloroform in di-O-methylalternariol, needles (15 mg) from chloroform, m.p. 183 °C (lit.,⁷ m.p. 186 °C, [α]D+11.9° (1 H, exchange with D,O, H-bonded OH), 7.24 (d, 1 H, J 2 Hz, ArH), 6.33 (d, 1 H, J 2 Hz, ArH), 3.90 (s, 3 H, C₂₂₁, 3.85 (s, 3 H, OMe), and 2.78 (s, 3 H, ArMe) (Found: 286. Calc. for C₁₄H₁₉O₂: M, 286) and tri-O-methylalternariol needles (26 mg) from ethanolate, m.p. 142 °C, then 165 °C, cooling and re-melting (lit.,⁷ m.p. 140 °C, then 162.5—164 °C, 7.28 (s, 1 H, ArH), 6.70 (s, 1 H, ArH), 6.69 (d, 1 H, ArH), 6.51 (d, 1 H, J 1.5 Hz, ArH), 3.90 (3 H, 3.93 (s, 3 H, OMe), 3.82 (s, 3 H, OMe), and 2.78 p.p.m. (s, 3 H, ArMe). An authentic sample of alternariol, K supplied by Professor R. Thomas, University of Surrey similarly methylated to give di- and tri-O-methylalternariol with identical m.p.s and 'H n.m.r. spectra.

References

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Scytalone (1) is a dihydronaphthalene metabolite of a number of fungi including a Scytalidium sp., Phialaphora lagerbergii, and Verticillium dahliae in which it has been shown to be an intermediate on the pathway to fungal melanins. A number of biosynthetic studies using $^{13}$C and $^2$H labelled precursors have been reported. Incorporation of singly labelled $^{13}$C-acetates into scytalone has been shown to be an intermediate on the pathway to fungal melanins. Studies with [2-$^3$C] malonate have failed to reveal an acetate "starter" effect suggesting that scytalone may be derived from a hexaketide precursor rather than a pentaketide as previously proposed.

A necessary prerequisite of any stable isotope labelling study is an unambiguous assignment of the NMR spectrum. The $^1$H NMR spectrum of scytalone has been rigorously assigned and indeed at normal field strengths the signals due to the C-2 and C-4 methylenes appear as complex overlapping, non-first order multiplets. However at 360.13 MHz a full analysis of the spectrum and assignment of all the range coupling data. The $^1$H chemical shifts and couplings consistent with scytalone being biosynthesised via a symmetrical intermediate, 1,3,6,8-tetrahydroxynaphthalene (2), as indicated in the scheme, and this compound can indeed be converted in vitro to scytalone by sodium borohydride reduction. Incorporation of [2-$^{13}$C, $^2$H]acetate and examination of both proton noise-decoupled and $^2$H noise-decoupled spectra indicated that $^2$H was incorporated at C-4 and C-5 only. Surprisingly no $^2$H incorporation could be observed at C-2 or C-7. In order to obtain more information on the stereospecificity of labelling at C-4 and to study further the question of labelling at C-2 and C-7 we have used the more sensitive approach of direct $^2$H NMR. We have also studied the incorporation of $^{13}$C-malonate to try to obtain more information on the nature of the assembly pattern of the precursor polyketide chain.

The $^{13}$C AND $^2$H LABELLING STUDIES ON THE BIOSYNTHESIS OF SCYTALENONE IN PHIALAPHERA LAGERBERGII

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Abstract—The regio- and stereospecificity of incorporation of label from [F,H] acetate into scytalone, a dihydronaphthalene metabolite of Phialaphora lagerbergii, has been determined by high field $^1$H and $^2$H NMR studies. Incorporation studies with [2-$^{13}$C] malonate have failed to reveal an acetate "starter" effect suggesting that scytalone may be derived from a hexaketide precursor rather than a pentaketide as previously proposed.

Scytalone (1) is a dihydronaphthalene metabolite produced by a number of fungi including a Scytalidium sp., Phialaphora lagerbergii, and Verticillium dahliae in which it has been shown to be an intermediate on the pathway to fungal melanins. A number of biosynthetic studies using $^{13}$C and $^2$H labelled precursors have been reported. Incorporation of singly labelled $^{13}$C-acetates suggested a pentaketide origin for scytalone, and studies with [1,2-$^{13}$C]acetate showed a randomisation of $^3$C in which it has been shown to be an intermediate on the pathway to fungal melanins. A number of biosynthetic studies using $^{13}$C and $^2$H labelled precursors have been reported. Incorporation of singly labelled $^{13}$C-acetates showed a randomisation of $^3$C in which it has been shown to be an intermediate on the pathway to fungal melanins.
Fig. 1. 55.28 MHz NMR spectra of scytalone (a) [3H]-acetate enriched; (b) [U-2H]-labelled; (c) after reaction for 72 hr at room temperature in NaOME, MeO\textsubscript{2}H. All spectra were determined with proton noise-decoupling using 1000 Hz sweep widths, 2 K data points acquired and transformed into 16 K data points, pulse width 23 μs, acquisition time 1.0 s, line broadening -2.5 Hz, gaussian multiplier 0.40.
venone (3) where the corresponding reduction is clearly stereospecific. Thus there is no incorporation of H, even at a low level on C-2 or C-7 of scytalone.

In order to obtain information on the disposition of the "starter" acetate group of the polyketide precursor the incorporation of [2-13C]malonate into scytalone was also examined. This resulted in high enrichment (ca 5 at.%) of each of the five carbons enriched from [2-13C]acetate, i.e. C-2, C-4, C-5, C-7 and C-8a but there was no discernible difference in enrichment levels. On repeating the experiment with simultaneous addition of unlabelled sodium acetate, a technique commonly used to facilitate observation of malonate "starter" effects, the overall enrichments were lower (ca 3 at.%) but again no significant differences in enrichment levels were apparent.

These H and 13C labelling results are open to two possible interpretations. Although studies have been reported in which H from acetate has been incorporated only into the acetyl-CoA derived "starter" unit of a polyketide chain and not into the malonyl CoA derived chain extending units, we ourselves, in a reasonably large number of studies to date have always observed incorporation into both positions.22 However, this could imply that C-4 and C-5 were partially derived from the acetate "starter" unit of a polyketide precursor and so make assembly pattern (7) likely. However the absence of H on C-2 and C-7 can be partially explained along with the failure to observe a "starter" effect on incorporation of [2-13C]malonate by proposing that the 1,3,6,8-tetrahydroxynaphthalene (2) is formed by loss of an acetyl moiety from the corresponding 2-acetyl-derivative (9), which itself would be formed from a hexaketide precursor e.g. 8. Such hexaketide-derived naphthalene metabolites e.g. 3 and 4 have been isolated from a number of fungi, including Scytalidium species.23 There is also evidence that the naphthalene (5) is derived by loss of the acetyl moiety from nepodin (6) in Rumex alpinus.24

Studies with potential advanced intermediates to further test these proposals are in progress.

EXPERIMENTAL

NMR spectra were determined on a Bruker WH 360 spectrometer: H NMR spectra at 360-13 MHz in hexadeuterioacetone solns; 13C NMR spectra at 55.28 MHz in acetone solns, and 1H NMR spectra at 90.56 MHz in hexadeuterioacetone solns.

Incorporation of labelled precursors. Philalephora lagerbergii (CM 96745) was grown at 25° in shaken culture in 500 ml conical flasks each containing 200 ml Czapek-Dox medium containing 0.1°, yeast extract and 5% sucrose. Preliminary experiments indicated that scytalone production commenced after 4 days' growth and reached a maximum after 12-15 days' growth. Further experiments were carried out with [6-13C]acetate and [13C]malonate to determine the optimum regime for feeding 2H- and 13C-labelled precursors.

(a) Incorporation of [6-13C]acetate. Sodium [6-13C]acetate (2 g) was dissolved in distilled water (4 ml) and the sterilised soln was distributed among eight shake flask cultures of P. lagerbergii after 5 days' growth. After a further 4 days' growth the mycelium was separated from the culture liquors by filtration and the acidified filtrate was extracted with ethyl acetate (4 x 100 ml). Evaporation of the extract gave a brown solid (1.011 g) which was purified by preparative thin layer chromatography on 20 x 20 x 0.05 cm silica GF254 plates eluted with 50% acetone in light petroleum (b.p. 60-80°) to give pure scytalone (600 mg).

(b) Incorporation of [2-13C]malonate. Diethyl [2-13C]malonate (0.25 g) dissolved in EtOH (3 ml) was added in equal portions to one shake flask culture of P. lagerbergii after one, two and three days' growth. After a further 3 days' growth, the culture was worked up as above to give scytalone (100 mg).

(c) Incorporation of [13C]malonate in the presence of unlabelled sodium acetate. Diethyl [13C]malonate (0.2 g) dissolved in ethanol (2 ml) was added in two equal portions to one shake flask culture of P. lagerbergii after one and two days' growth along with sodium acetate (0.25 g) in water (2.5 ml) each day. Work up as above yielded scytalone (90 mg).

(d) Fermentation in D2O supplemented medium. Two shake flask cultures of P. lagerbergii were grown for one day, 10 ml of culture medium was then removed from each flask and replaced by 10 ml D2O. After a further 7 days' growth the cultures were worked up to give [U-13C]scytalone (150 mg). H exchange experiments. Scytalone (100 mg) was dissolved in d4-methanol (1.5 ml) and NaOMe (10 mg) was added. The mixture was left at room temp and the rate of exchange monitored by H NMR. After 3 days complete exchange of the 2-equatorial H and partial exchange of the 2-axial and 7-H's had occurred. After 5 days, exchange of the 2-axial H was complete and leucine and valine were isolated in almost complete exchange of the 5- and 7-H's. After acidification and preparative TLC, pure scytalone (62 mg) was isolated.

Exchange of [6-13C]acetate-enriched scytalone in [4-13C]mevalonate. The labelled scytalone (254 mg) was dissolved in d4-methanol (1.5 ml) and NaOMe (30 mg) was added and the mixture was left to stand for 5 days at room temp. The mixture was then poured into water (30 ml) and the soln acidified to pH 2 with dil HCl. Extraction with EtOAc (4 x 100 ml) followed by evaporation of the solvent gave the crude product (200 mg) which was purified by preparative TLC to give pure scytalone (154 mg).

REFERENCES


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**C and **H labelled studies

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**Table 1. **H NMR data for scytalane (1)**

Scheme 1. Alternative pentaketide and hexaketide biosynthetic pathways to scytalane.
dies on a Synthesis of (RS)-Mevalonic Acid Lactone

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Full details of a high yielding synthesis of mevalonic acid lactone (1) which is of particular value in the preparation of 3- and/or 3'-labelled compounds are described. The key step, conversion of 3-hydroxy-3-methylpentane-1,5-dioic acid (3) into 3-hydroxy-3-methylpentane-1,5-dioic anhydride (4) using acetic anhydride, has been fully investigated, and an additional method using acetyl chloride and triethylamine is described.

Through a number of methods for the synthesis of isotopically labelled mevalonic acid lactone (1) have been described in the past years, there are a few applicable to the synthesis of the diacid (3) and labelled compounds. A short, high yielding synthesis of mevalonic acid lactone which enabled isotopic labelling to be introduced into these positions was recently described. In this synthesis ethyl acetate was treated with magnesium bromide to give 4-hydroxy-4-methylhepta-1,6-dione (2) which was then converted by ozonolysis and further hydrogenation of the crude product with hydrogen peroxide in acetic acid to give mevalonic acid lactone (1). By starting with [2-13C]acetate, [3'-13C]mevalonic acid lactone could be prepared in essentially quantitative yield. In a typical experiment, a solution of the diacid (3) (405 mg, 2.5 mmol) in acetic anhydride (3.5 ml, 35 mmol) was stirred at 18 °C. The course of the reaction was monitored by withdrawing small aliquots of the reaction mixture and determining the 80 MHz 1H n.m.r. spectrum. As shown in Figure 1 the acid was converted smoothly into anhydride (4) with no trace of the acetate (5). After 24 h the initial suspension had turned into a clear solution. The excess of acetic anhydride was then removed to give a white solid shown by 1H n.m.r. spectroscopy [Figure 2(a)] to be essentially pure (4). On repeating the reaction using the same batch of acid, but on double the scale, approximately 48 h were required for complete reaction. However, the time taken for complete reaction varied with the batch of acid used [normally the diacid (3) obtained from the ozonolysis step is used without further purification].

If 25 or more equivalents of acetic anhydride are used, the product is indeed the acetate (5) [Figure 2(b)]. Increasing the temperature to 30 °C (14 equivalents of acetic anhydride) still resulted in the anhydride (4) as the major product, but at 40 °C the acetate (5) was formed along with (4) as a 2:1 mixture. Interestingly, heating the diacid (3) to 100 °C with only 1.2 equivalents of acetic anhydride again gave anhydride (4) as the sole product.

Although we find the use of acetic anhydride the most convenient method for the production of anhydride (4), an alternative method not subject to the same variability is to react the diacid with acetyl chloride and triethylamine in tetrahydrofuran (THF) at 0 °C. Reaction is again quantitative and is complete in only 2 h. The acetate (5) can be more conveniently prepared from the diacid (3) by heating in acetic chloride at 50 °C for 4 h. Removal of excess of acetyl chloride gives a quantitative yield of (5). Anhydride (4) can also be converted readily into the corresponding acetate (5) by being stirred at room temperature with a slight excess of acetyl chloride.

Treatment of the anhydride (4) with sodium borohydride in propan-2-ol, acidification, and continuous extraction with ether gave almost pure mevalonic acid lactone which was purified finally by column chromatography on silica using hexane–ether as eluant.

OH
Me
OH
Me
OH
Me
OR
(1)
(2)
(3)
(4) R = H
(5) R = Ac

OH
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OH
Me
OH
Me
OR
(1)
(2)
(3)
(4) R = H
(5) R = Ac

OH
Me
OH
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OH
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OR
(1)
(2)
(3)
(4) R = H
(5) R = Ac

Oh to whom enquiries should be addressed.
Figure 1. 80 MHz $^1$H n.m.r. spectra of the reaction mixture from 3-hydroxy-3-methylpentane-1,5-dioic acid (3) with acetic anhydride (14 eq) at room temperature. Samples were taken at (a) $t = 0$, (b) $t = 2$, (c) $t = 4$, and (d) $t = 6$ h. The excess of acetic anhydride was removed under reduced pressure and the resulting white solid was dissolved in $[^2]$H$_6$acetone and the spectra determined on a Bruker WP80 spectrometer.

Figure 2. 80 MHz $^1$H n.m.r. spectra in $[^2]$H$_6$acetone of (a) 3-hydroxy-3-methylpentane-1,5-dioic anhydride (4), and (b) 3-acetoxy-3-methylpentane-1,5-dioic anhydride (5).

Experimental

M.p.s. were determined on a Kofler hot-stage apparatus and are uncorrected. $^1$H N.m.r. spectra were determined on either Varian EM360, Bruker WP80, or Bruker WP200 spectrometers for deuteriochloroform or hexadeuteroacetone solution. I.r. spectra were determined on a Perkin-Elmer 257 spectrophotometer as KBr discs.

Conversion of Sodium Acetate into n-Butyl Acetate.—Sodium acetate (5 g, 60 mmol) was mixed with tri-n-butyl phosphine (5 ml) and the mixture was heated under reflux for 5 h on a water-bath at 200—220°C. The viscous mixture was cooled to room temperature, the upper end of the reflux condenser was passed through a liquid-nitrogen-cooled trap to a vacuum pump, and the product ester was distilled into the cold trap by heating the reaction flask to 100—160°C for 2.5 h at 1 mmHg pressure and cold water running in the reflux condenser. n-Butyl acetate (5.98 g, 96%) was obtained.

4-Hydroxy-4-methylhepta-1,6-diene (2).—A mixture of n-butyl acetate (0.5 g) and allyl bromide (2.06 g) in diethyl ether (10 ml) was added dropwise to a stirred mixture of sodium turnings (0.55 g) in diethyl ether—THF (1: 1; 2 ml). The mixture had been stirred overnight, crushed ice (7 g) added, and the mixture was acidified with 6M sulphuric acid. The resulting solution was extracted with diethyl ether, the extract was washed with saturated potassium hydroxide solution and dried over Na$_2$SO$_4$. Removal of the solvent gave a yellow oil which was distilled at water-pump pressure yield the dienol (2) as an oil (0.73 g), b.p. 90—92°C; $\delta$ (C$_6$D$_4$CO) 1.18 (3 H, s), 1.8 (1 H, br s, exchangeable), 2.23 (4 H, d, $J^\prime$ = 6 Hz, d, $J^\prime$ = 5.00—6.00 (6 H, m).

3-Hydroxy-3-methylpentane-1,5-dioic Acid (3).—Ozonized a mixture of methylene dichloride and acetic acid (10: 1; 78°C until a blue colour appeared. The reaction mixture was then allowed to warm up to room temperature and acetic anhydride (10 ml) was added. After concentration of the reaction mixture to about 5 ml, more acetic acid (10 ml) and a 30% solution of hydrogen peroxide (4 ml) were added and the mixture heated under a reflux for 13 h. Evaporation of the solvent and the diacid (3) as an oil (0.55 g) which slowly solidified crystallisation from diethyl ether gave the acid as needles 110—111°C (lit., 110—111°C); $\delta$(CD$_3$CO) 1.38—1.43; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$(CD$_3$CO) 1.80—1.84; $\delta$
Treatment with methane gave the dimethylester which showed $\delta$ (CDCl$_3$, $H$, s), 2.73 (4 $H$, s), and 3.75 (6 $H$, s).

14. Equivalents of acetic anhydride at 18 °C. A mixture of (3) (810 mg, 5 mmol) and acetic anhydride (6.6 ml, 70 °C) was stirred at room temperature. After 72 h the reaction had turned into a clear solution. The excess of acetic anhydride was removed under high vacuum to give a white solid which was recrystallised from diethyl ether–light petroleum (b.p. 30–40 °C) to give the anhydride (4) as needles (350 mg), m.p. 102–103 °C.

15. Equivalents of acetic anhydride at 100 °C. A solution of acid (3) (405 mg) in acetic anhydride (0.35 ml) was stirred at 100 °C for 1.5 h. After 45 min a clear solution was formed. Removal of excess of acetic anhydride gave a pale solid which was recrystallised as above to give the anhydride (4) as prisms (300 mg).

16. Equivalents of acetic anhydride at 18 °C. Reaction of the diacid (3) (162 mg, 1 mmol) and acetic anhydride (30 ml) and the mixture was cooled to 0 °C in an ice-bath. The reaction mixture was stirred overnight at room temperature. After removal of the solvent, water (10 ml) was added and the mixture was acidified to pH 2 in an ice-bath. The solution was extracted continuously with diethyl ether for 45 h. The extract was dried (Na$_2$SO$_4$) and the solvent removed on a rotary evaporator to give an oil which was shown by t.l.c. to have one component, corresponding to mevalonic acid lactone. Column chromatography on Malinckrodt silica AR-CC-7 gave pure mevalonic acid lactone (1) (300 mg).

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References

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Structural Revision and Synthesis of LL-D253α: a Chromanone Metabolite of Phoma pigmentivora

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D253α, a chromanone metabolite of Phoma pigmentivora, has been shown by analysis of the 1H-coupled 13C n.r. spectrum and by synthesis to be 7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methylchromanone.

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Structural Revision and Synthesis of LL-D253α: a Chromanone Metabolite of Phoma pigmentivora

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LL-D253α, a chromanone metabolite of Phoma pigmentivora, has been shown by analysis of the 1H-coupled 13C n.m.r. spectrum and by synthesis to be 7-hydroxy-8-(2-hydroxyethyl)-5-methoxy-2-methylchromanone.

LL-D253α was first isolated from Phoma pigmentivora and was assigned the chromanone structure (1) on the basis of spectroscopic and degradative studies. It has also been isolated along with a number of related co-metabolites from Phoma violacea and Sclerotinia fructigena. We now report 13C n.m.r. studies on LL-D253α, carried out as a preliminary to biosynthetic labelling experiments, which necessitate revision of the structure to (2) on the basis of a full analysis of the fully 1H-coupled 13C n.m.r. spectrum of LL-D253α diacetate (3). This has been confirmed by unambiguous syntheses of both structures (1) and (2).
The high-frequency region of the fully ¹H-coupled 200 MHz ¹³C n.m.r. spectrum of LL-D253α diacetate, and results of selective low-power ¹H decoupling experiments. All the aromatic carbons give characteristic triplet patterns. The hydroxyethyl side chain must be placed at C-8 rather than C-6 of the chromanone skeleton as irradiation of the benzylic methylene hydrogens (10-CH₂) respectively caused the multiplet resonance at δ 162.1 to sharpen to a broad singlet and a sharp triplet (Figure 1). These results, along with the chemical shift, indicate that this resonance must therefore be assigned to C-8α, showing 3-bond couplings to 10-CH₂ and H-2. Similarly the phenolic acetate must be placed at C-7 as irradiation of 10-CH₂ and H-6 respectively caused the quartet at δ 155.1 to a doublet and a triplet; and the methoxy substituent must be placed at C-5 as a quintet at δ 160.0 collapses to a quartet and doublet respectively on irradiation of H-6 and OCH₃. The complex multiplet at δ 110.0 is assigned to C-8 as it sharpens to a quartet on irradiation of either 10-CH₂ or 11-CH₂. It also opens slightly and shows an intensity increase on irradiation of H-6 due to removal of the expected 3-bond coupling. 4a also shows a 3-bond coupling to H-6. The carbon–hydrogen couplings revealed by these experiments are summarised in Figure 2. Although these effectively define structure (2) for LL-D253α, confirmatory evidence was provided by unambiguous syntheses of both (1) and (2) from 5,7-dihydroxy-2-methylchromanone (4) as outlined in Scheme 1. Allylation of the non-chelated hydroxy group on C-7 followed by methylation gave (5), which on heating to melting underwent a Claisen rearrangement to furnish (6).† In

† There is much precedent in the literature for Claisen rearrangement of 7-allyl ethers in chromone and chromanone systems to give mainly or exclusively the 8-allyl derivatives.⁸
the presence of the free 7-hydroxy group all attempts to cl
the double bond oxidatively gave intractable mixts. However, after protection as the benzyl ether (7), os
tetroxide oxidation to the vicinal diol, periodate cleavage
tor hydroxide reduction of the resultant aldehyde, compd
(2) was obtained in acceptable overall yield [16% from (4)
hydrogenolysis of the benzyl protecting group. This
identical in all respects to natural LL-D253α. Structure (1)
obtained from (4) by methylation followed by allylatic
give (8), which underwent Claisen rearrangement to (9).
allyl moiety was cleaved to the hydroxyl moiety as a
without protection of the chelated hydroxy group t
ecessary. The overall yield of (1) from (4) was 44%
contrast to LL-D253, compound (1) was readily solub
CDCl₃ and its ¹H n.m.r. spectrum showed the presence
chelated hydroxy proton at δ 12.17, whereas the ¹H n.
spectrum of LL-D253α in CDCl₃ showed that no chel
hydroxy group was present.

The ¹H-coupled ¹³C n.m.r. spectra of (1) and a third isc
have been obtained and will be reported in full elsewhei

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References
1 W. J. McGahren, G. A. Ellestad, G. O. Morton, and A
2 G. C. Crawley and C. J. Strawson, unpublished results quot
"Fungal Metabolites II," W. B. Turner and D. C. Aldh
3 C. R. McIntyre, T. J. Simpson, L. A. Trimble, and J. C. Vedt
Simpson, D. J. Stenzel, A. J. Bartlett, E. O'Brien, and J.
1981, 111, 103, and references therein.
synthesis of LL-D253α in *Phoma pigmentivora*. Incorporation of $^{13}$C, $^2$H, and $^{18}$O enriched Precursors

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Incorporation of $^{13}$C, $^2$H, and $^{18}$O labelled acetates and $^{18}$O$_2$ gas into LL-D253α (1), a chromanone metabolite of *ma pigmentivora*, and analyses of the enriched metabolites by $^{13}$C and $^2$H n.m.r. and mass spectroscopy indicate its formation from two preformed polyketide chains; evidence for the mechanism of formation of the chromanone ring is presented, and a cyclopropyl intermediate is proposed to account for the unique randomisation label observed in the hydroxyethyl side chain.

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Biosynthesis of LL-D253α in Phoma pigmentivora. Incorporation of $^{13}$C, $^2$H, and $^{18}$O Enriched Precursors

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The incorporation of $^{13}$C, $^2$H, and $^{18}$O labelled acetates and $^{18}$O gas into LL-D253α (1), a chromanone metabolite of Phoma pigmentivora, and analyses of the enriched metabolites by $^{13}$C and $^2$H n.m.r. and mass spectroscopy indicate its formation from two preformed polyketide chains; evidence for the mechanism of formation of the chromanone ring is presented, and a cyclopropyl intermediate is proposed to account for the unique randomisation of label observed in the hydroxyethyl side chain.

LL-D253α (1), a metabolite of Phoma pigmentivora, is a chromanone with an unusual hydroxyethyl substituent. Structural analysis strongly suggests a polyketide derivation for the chromanone nucleus but the origin of the C2-side chain is obscure. Possible routes include: (a) condensation of two preformed polyketide chains; or elaboration of the side chain onto a preformed pentaketide-derived precursor either by (b) C-acylation, or (c) stepwise introduction of two C1 equivalents, or (d) introduction and oxidative cleavage of a propanoic acid substituent. Since all of these represent unusual routes of fungal polyketide biosynthesis, we have carried out incorporation studies with $^{13}$C, $^2$H, and $^{18}$O labelled acetates and to identify the correct pathway. Incorporation of $[^{13}$C$_2$]acetate indicated that all the skel...
Incorporation of label from [1-14C]mevalonic acid was examined and that at C-2 being significantly higher than that at C-1, thereby rendering route (d), our initially favoured synthetic pathway. The results of incorporating [1-13C]acetate and detection of enriched C-2, C-4, C-5, C-7, C-8α, and C-10 were all highly significant, indicating its origin from an acetate starter unit; C-4 shows one downfield shifted resonance, corresponding to the incorporation of one deuterium atom at C-3; and finally both C-10 and C-11 each showed two isotopically shifted resonances corresponding to the incorporation of two deuterium atoms at C-11 and C-10 respectively. All these results are summarised in Scheme 1.

On incorporation of [1,13C,18O]acetate, the 13C n.m.r. spectrum of (2) showed isotopically shifted resonances for C-4, -5, -7, and -8α, indicating that the oxygens attached to these carbons are acetate-derived, and therefore that the corresponding carbon-oxygen bonds had remained intact throughout the course of the biosynthesis (Table 1). These results and the incorporation of only one deuterium at C-3, indicate that the chromanone ring is formed by conjugate addition of a phenolic hydroxy group to the corresponding α,β-unsaturated ketone. LL-D253α has the 2R configuration so the ring closure process is stereospecific with respect to C-2. To examine the stereospecificity with respect to C-3 the 1H n.m.r. spectrum of [1H]acetate-enriched LL-D253α diacetate was examined. The axial and equatorial hydrogens at C-3 have almost coincident chemical shifts and even at high field strengths form part of a complex ABX system. However on addition of Eu(fod)₃ (fod = i,i,i,2,2,3,3-heptahfluoro-7,7-dimethyloctane-4,6-dionate), the induced shifts in the 360 MHz 1H n.m.r. spectrum allow the resonances to be resolved and assigned from their coupling constants to the axial and equatorial hydrogens. Determining the 2H n.m.r. spectrum of [U-2H]LL-D253α under the same conditions confirmed that the corresponding 1H resonances could also be resolved. Finally repeating the experiment with the [1H]acetate-enriched compound showed that both positions were enriched to equal extents so that protonation of the intermediate enolate must proceed with equal facility from both sides of the molecule (Scheme 2). This contrasts with the corresponding chalcone to flavanone ring closure which has been shown to be completely stereospecific.

The observed randomisation of label between C-10 and C-11 indicates that these carbons may become equivalent during the biosynthesis. To account for this and the lack of randomisation of label in the phloroglucinol ring we propose that the randomisation of label can then be explained by reduction and elimination (cf. fatty acid biosynthesis) to give the vinyl

<table>
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<th>Carbon</th>
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<th>(ppm. x 100)</th>
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<td>4</td>
<td>190.1a</td>
<td>4.1</td>
<td>(90:10)</td>
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<td>8α</td>
<td>162.1a</td>
<td>1.6</td>
<td>(80:20)</td>
</tr>
<tr>
<td>5</td>
<td>160.0b</td>
<td>1.7</td>
<td>(75:25)</td>
</tr>
<tr>
<td>7</td>
<td>155.1a</td>
<td>2.1</td>
<td>(83:17)</td>
</tr>
<tr>
<td>11</td>
<td>62.8b</td>
<td>2.7</td>
<td>(85:15)</td>
</tr>
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</table>


The relative enrichments at C-10 and C-11 are consistent with label in C-1 or C-2 of acetate being randomised to the extent of ca. 90% between C-10 and C-11, e.g. in the [1-13C]acetate feed C-10 has 60% label and C-11 40%.

**Scheme 1**

The 100.6 MHz 13C n.m.r. spectrum of LL-D253α diacetate (2) showed isotopically shifted resonances for C-4, -5, -7, and -8α, indicating that the oxygens attached to these carbons are acetate-derived, and therefore that the corresponding carbon-oxygen bonds had remained intact throughout the course of the biosynthesis (Table 1). These results and the incorporation of only one deuterium at C-3, indicate that the chromanone ring is formed by conjugate addition of a phenolic hydroxy group to the corresponding α,β-unsaturated ketone. LL-D253α has the 2R configuration so the ring closure process is stereospecific with respect to C-2. To examine the stereospecificity with respect to C-3 the 1H n.m.r. spectrum of [1H]acetate-enriched LL-D253α diacetate was examined. The axial and equatorial hydrogens at C-3 have almost coincident chemical shifts and even at high field strengths form part of a complex ABX system. However on addition of Eu(fod)₃ (fod = i,i,i,2,2,3,3-heptahfluoro-7,7-dimethyloctane-4,6-dionate), the induced shifts in the 360 MHz 1H n.m.r. spectrum allow the resonances to be resolved and assigned from their coupling constants to the axial and equatorial hydrogens. Determining the 2H n.m.r. spectrum of [U-2H]LL-D253α under the same conditions confirmed that the corresponding 1H resonances could also be resolved. Finally repeating the experiment with the [1H]acetate-enriched compound showed that both positions were enriched to equal extents so that protonation of the intermediate enolate must proceed with equal facility from both sides of the molecule (Scheme 2). This contrasts with the corresponding chalcone to flavanone ring closure which has been shown to be completely stereospecific.

The observed randomisation of label between C-10 and C-11 indicates that these carbons may become equivalent during the biosynthesis. To account for this and the lack of randomisation of label in the phloroglucinol ring we propose the pathway shown in Scheme 3. Two preformed polyketide chains, either a C₄ plus C₈ as shown, or C₆ plus C₆, or C₃ plus C₆, probably condense before aromatisation of ring A. The transposition of oxygen in the C₂-side chain and randomisation of label can then be explained by reduction and elimination (cf. fatty acid biosynthesis) to give the vinyl...
intermediate (3). Epoxidation and reductive opening of the epoxide (4) would furnish the hydroxyethyl moiety directly. To account for the observed 80% randomisation of label between C-10 and C-11 we propose the involvement of a cyclopropyl intermediate (5) which would be formed by participation of the phloroglucinol ring in expulsion of the hydroxy group at C-11. Hydrolytic opening of the resulting cyclopropyl ring at either the α or β carbons would then produce the observed randomisation of labelling between C-10 and C-11 in (1). An analogy for this process is found in the mould metabolite mikrolin (6) which contains a cyclopropyl ring fused to a cyclohexadienone moiety. The observed degree of randomisation requires that 20% of the natural product is derived directly from reduction of the epoxide (4). Support for this was provided by carrying out a fermentation of P. pigmentivora under an atmosphere of ^18O2. The mass spectrum of the derived LL-D253α shows an M + 2 peak, corresponding to 15% of the metabolite, which mass matches for C13H15^16O4^18O. In addition, examination of the ^13C n.m.r. spectrum for isotope shifts determined that, within experimental error, all of the ^18O was located at C-11 (Table 1). At present we cannot say with certainty whether the randomisation is an in vitro or in vivo process. However [10-^2H2]-LL-D253α has been prepared and no randomisation
label between C-10 and C-11 is observed on either mild acid
mild base treatment.
We thank the S.E.R.C., NATO, and the Natural Sciences
& Engineering Research Council of Canada (NSERC) for
financial support.

References
synthesis of Monocerin. Incorporation of $^2$H-, $^{13}$C-, and $^{18}$O-Labelled Acetates by
Drechslera ravenelli

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Chemical Communications 1984
Biosynthesis of Monocerin. Incorporation of $^2$H-, $^{13}$C-, and $^{18}$O-Labelled Acetates by Drechslera ravenelii

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Incorporation of $^2$H-, $^{13}$C-, and $^{18}$O-labelled acetates into monocerin (1) by cultures of Drechslera ravenelii and analysis of the enriched metabolites by $^2$H and $^{13}$C n.m.r. spectroscopy indicate a heptaketide origin; observation of $^2$H and $^{18}$O isotope shifts in the $^{13}$C n.m.r. spectrum allows the fate of acetate-derived hydrogen and oxygen on incorporation into monocerin to be followed and conclusions on the mechanism of formation of the fused furobenzopyrone ring system to be drawn.

Monocerin (1) was first isolated as a compound active against powdery mildew of wheat from Helminthosporium monosporum.1 It was subsequently isolated, along with the fusarentins (e.g., (2)), a group of related compounds with insecticidal activity, from Fusarium larvarum;2 and from Readeriella mirabilis.3 We have also isolated monocerin from Drechslera ravenelii in the course of biosynthetic studies on ravenelin.4

Monocerin had previously been assigned the $8R,9R$ configuration but the configuration at C-11 was uncertain.5 Analysis of the 360.13 MHz $^1$H n.m.r. spectrum, Figure 1(a), and difference n.O.e. studies have allowed a full assignment of the spectrum and indicate the $S$ configuration at C-11.5 The structure of monocerin suggests a polyketide origin and now report incorporation studies with $^{13}$C-, $^2$H-, and $^{18}$O-labelled acetates which confirm a heptaketide origin, suggest a mechanism of formation of the fused furobenzopyrone ring system, and provide information of more fundamental significance on reduction and deoxygenation processes in polyketide biosynthesis.

* Full details of the $^1$H and $^{13}$C n.m.r. spectral assignments and isotope shifts will be given in the full paper.
Incorporation of [1-13C]- and [1,2-13C2]-acetates into monocerin by cultures of D. ravenelii and analysis of the 13C n.m.r. spectra of the enriched metabolites showed that acetate was incorporated with high efficiency to give the labelling pattern summarised in Scheme 1. The 55 MHz 1H n.m.r. spectrum of [U-2H]monocerin showed, Figure 1(b), that all the 1H resonances including the diastereotopic hydrogens on C-10, C-12, and C-13 were resolvable. Incorporation of [1-13C,2H3]acetate into monocerin and determination of the 2H n.m.r. spectrum showed, Figure 1(c), that 2H was incorporated into the 14-methyl group and at an essentially equal level at H-6, H-8, both H-10α and H-10β, and only one of the diastereotopic C-12 hydrogens. The equal incorporation at both diastereotopic positions on C-10 strongly suggests that the molecules are doubly labelled, but as retention of two acetate-derived hydrogens at a methylene is an unusual observation in polyketide biosynthesis5 we have used the 3-shift method6 to confirm this.

The 13C n.m.r. spectrum of the [1-13C,2H3]acetate-derived metabolite (Figure 2) shows isotopically shifted resonances on C-13, C-11, C-9, C-7, and C-5 indicating respectively the retention of up to three acetate-derived hydrogens on C-14 thereby confirming it as a 'starter' unit; it also shows two acetate-derived hydrogens on C-10, and one acetate-derived hydrogen on C-12, C-8, and C-6. Incorporation of [1-13C,18O]acetate and 13C n.m.r. analysis of the enriched metabolite showed isotopically shifted resonances8 for C-1, C-3, C-5, C-9, and C-11 (Δδ, 0.03, 0.01, 0.01, 0.03, and 0.03 p.p.m. respectively) indicating that the corresponding carbon-oxygen bonds had remained intact throughout the biosynthetic pathway. These 2H and 18O labelling patterns are summarised in Scheme 1.

The above results enable us to make the following conclusions about the main events occurring during the biosynthesis of monocerin. (a) The retention of two acetate-derived hydrogens at C-10 suggests that reduction of the β-ketoacyl intermediates to the corresponding β-hydroxyacyl intermediates takes place during chain assembly before significant loss through exchange processes can occur, i.e. the trihydroxy moiety (3) is a likely enzyme-bound polyketide precursor. In agreement with observations in fatty acid biosynthesis9 and prepared by producing the metabolite in a medium supplemented with 5% 18O2.12
mellein biosynthesis\textsuperscript{5} the carbon–carbon bond formation in the chain assembly process probably occurs with concomitant decarboxylation of the malonyl CoA unit which is added. (b) The loss of oxygen from C-13 presumably occurs by an elimination-reduction sequence analogous to fatty acid biosynthesis,\textsuperscript{6} Since only one of the diastereotopic hydrogens on C-12 is labelled the process is clearly stereospecific, but the absolute stereochemistry of the process is as yet uncertain. (c) The benzopyrone ring must be formed by nucleophilic attack at the terminal carboxy moiety by a hydroxy group on C-9. It is likely that the cyclisation takes place on the thioester (4), to give (5) as the first enzyme-free intermediate. (d) The retention of the carbon–oxygen bond at C-11 indicates that the tetrahydrofuran ring is formed by attack of a C-11 hydroxy function at C-8. A mechanism for this consistent with the observed \textsuperscript{1}H and \textsuperscript{18}O labelling would be nucleophilic addition onto a quinonemethide intermediate (7) formed (Scheme 2) by oxidation of (6), the hydroxylated derivative of (5). A similar ring closure mechanism has been proposed in granaticin biosynthesis,\textsuperscript{11} and is supported by the co-occurrence of monocerin and fusaretrin methyl ether (2) in \textit{F. larvarum}.\textsuperscript{2}

We thank the S.E.R.C., N.A.T.O., and the Natural Sciences and Engineering Research Council of Canada for financial support.

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References

structure and Absolute Configuration of the Asticolorins, Toxic Metabolites from
Aspergillus multicolor

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Aspergillus multicolor, a mycotoxin isolated from toxic extracts of Aspergillus multicolor, strain MRC, crystallised from acetone as monoclinic crystals, m.p. 0 °C, and showed [α]D20 = -120.5° (c 0.20, acetone) and (MeOH) 225 (ε 81 370), 263 (28 080), 290 (23 290), 304 (23 560), and 316 nm (20 480). Field desorption mass spec-
tructure and Absolute Configuration of the Asticolorins, Toxic Metabolites from
Aspergillus multicolor

Figure 1. Perspective view of the crystal structure of asticolorin A (1).

1. The asymmetric unit as shown in Figure 2 contains 93 non-hydrogen atoms present as two asticolorin A, one water, and three acetone molecules. The four solvent molecules are linked to the four phenolic hydroxy groups by intermolecular hydrogen bonds with interoxygen distances of less than 2.8 Å. Furthermore the oxygen atom of the water molecule is also 2.77 Å removed from the oxygen atom of the C-30 hydroxy group of an asticolorin A molecule in the other asymmetric unit.

With the relative configuration of asticolorin A (1) available, the chirality of the C-29 hydroxy group and thus the absolute configuration of the molecule was determined by the method of Horeau.3 Methylation of asticolorin A with methyl iodide and potassium carbonate in acetone gave the dimethyl ether (2), 2C15H44O7CH5. m.p. 258-260 °C. Esterification of (2) with racemic (S)-phenylbutyric anhydride and 4-di-
Methylaminopyridine proceeded smoothly, leading quantita-
tively to the 29-O-phenoxybutyrate. The recovered (S)-phenyl-
butyric acid had [α]D20 = -21.0° (c 7.4, benzene). Asticolorin A must therefore have the 29S conformation3-4 and consequently the absolute configuration as depicted in (1).

The structure elucidation of the related metabolite, asticolorin B (3), m.p. >320 °C (M+ m. 536; C21H22O7) is based on the high-field 1H and 13C n.m.r. data (Bruker WM-500 spec-
trometer) of these metabolites. It was evident that the C-29 secondary hydroxy function present in asticolorin A (1) (29H: δH 5.079d, δC 180.84) is replaced by a carbonyl group in asticolorin B (3) [νmax (KBr) 1665 cm⁻¹; δC 180.84].

The atomic co-ordinates for this work are available on request from the Director of the Cambridge Crystallographic Data Centre, University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW. Any request should be accompanied by the full literature citation for this communication.
metabolite, asticolorin C (4) \(2\text{C}_{27}\text{H}_{26}\text{O}_{13}\text{CHCl}_3\), \(>320 \, ^\circ\text{C} (M^+, 568)\), was produced. The n.m.r. data indicated that both the C-8 and C-24 methyl groups (\(\delta_H \, 2.343; \delta_C \, 21.60, 21.16\)) present in asticolorin B (3) replaced by hydroxymethyl functions (\(\delta_H \, 4.543, 4.57; \delta_C \, 62.73, 62.65\)) in asticolorin C (4).

The asticolorins, a new type of mycotoxin, are biosynthetically from mevalonate and inositol and orsellinic acid by oxidative phenol coupling.

The authors thank Dr. A. E. de Jesus for microbiological assistance.

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References


2 G. M. Sheldrick, SHELX program system 1976, University of Cambridge.


Isotope Labelling Studies on the Biosynthesis of Asticolorin C by Aspergillus multicolor. Evidence for a Symmetrical Intermediate

S. Steyn, Robert Vleggaar, and Thomas J. Simpson

Involvement of mevalonate and four molecules of a symmetrical intermediate, orcinol, in the biosynthetic way leading to asticolorin C is evident from incorporation studies with $^{13}$C and $^2$H labelled precursors.

Asticolorins, e.g. asticolorin C (1), are toxic metabolites produced by cultures of Aspergillus multicolor, strain MRC. The structures are characterized by the novel way in which a mevalonate-derived 3,3-dimethylallyl group is utilized to produce two dibenzofuran moieties. The possible involvement of aminic acid (3,9-dihydroxy-1,7-dimethylbenzofuran-2-carboxylic acid), a compound previously isolated from Lepraria membranacea, and of orsellinic acid, or pannarol (1,7-dihydroxy-3,9-dimethylbenzofuran) in the biosynthesis of the asticolorins investigated using primitive $^{13}$C and $^2$H labelled precursors.

Cultures of A. multicolor were grown on a yeast extract-sucrose medium. Preliminary feeding experiments with $[1^{-13}C]$acetate as precursor established that a good incorporation (0.3%) and satisfactory dilution values ($0.7$, assuming 14 labelled positions) were obtained for asticolorin A by pulse-feeding cultures of A. multicolor every 12 h from day 3 to day 14 with sodium acetate to a total amount of 1.0 g l$^{-1}$. 

[Diagram of asticolorin C (1)]
Table 1. $^1$H (500.13 MHz) and $^{13}$C (125.76 MHz) n.m.r. data for asticolorin C.

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<tr>
<th>Carbon atom</th>
<th>$\delta^{c,b}$</th>
<th>$J$(C,C)/Hz</th>
<th>$\Delta\delta$</th>
<th>$\delta^{d}$</th>
<th>$J$(H,H)/Hz</th>
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<td>1.641m</td>
<td></td>
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<tr>
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<td>38.9</td>
<td>0.069</td>
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<tr>
<td>19</td>
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<td>6.8, 2.0</td>
</tr>
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</tr>
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<td>56.2, 41.6</td>
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<td>1.829s</td>
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<tr>
<td>31</td>
<td>62.73 T</td>
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<td>4.8</td>
</tr>
<tr>
<td>32</td>
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<td>0.089</td>
<td>4.572d</td>
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<tr>
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</tr>
<tr>
<td>34</td>
<td>25.36 Q</td>
<td>35.2</td>
<td>0.089</td>
<td>4.572d</td>
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</tr>
<tr>
<td>35</td>
<td>62.65 T</td>
<td>46.3</td>
<td>0.089</td>
<td>4.572d</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Recorded on a Bruker WM-500 spectrometer; solvent (CD)2SO. Relative to Me4Si. Capital letters refer to the pattern resulting directly bonded (C,H) couplings. S = singlet, D = doublet, T = triplet, and Q = quartet. * = enriched by [1-13C]acetate, D = doublet. S = singlet, T = triplet, and Q = quartet. • = enriched by [2-13C]acetate. Value obtained from the broad-band proton-decoupled spectrum of asticolorin C derived from [1,2-13C]acetate. AB spin system obscured by overlapping resonances. Relative to internal Me4Si. s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. The chemical shifts of the protons of the different hydride groups are as follows: C-10, 6 10.26; C-22, 10.84; C-32, 5.193t br; C-36, 4.572t br (2.0 Hz).

The $^1$H and $^{13}$C n.m.r. data for asticolorin C (1) are collated in Table 1. The signals of the proton-bearing carbon atoms were correlated with specific proton resonances. Extensive heteronuclear $^{13}$C-$^1$H selective population inversion experiments, to be described in a subsequent publication, established the two- and three-bond (C,C) connectivity pattern for asticolorin C. The method, however, does not allow us to differentiate between the resonances of the ring A and H carbon atoms. This ambiguity was resolved by the observation of one-bond (C,C) couplings for C-11-C-12 (58.9 Hz) and C-20-C-21 (54.7 Hz) in the broad-band proton-decoupled $^{13}$C n.m.r. spectrum of asticolorin C derived from [2-13C]acetate (91.4 atom % 13C). In addition the spectrum showed enhancement of the signals of 19 carbon atoms (average enrichment factor 4.2) whereas that of asticolorin C derived from [1-13C]acetate (91.6 atom % 13C) showed 14 enhanced signals (average enrichment factor 5.0) (see Table 1) so that all the carbon atoms are acetate-derived.

![Figure 1. Arrangement of intact acetate units in asticolorin C derived from [1,2-13C]acetate.](image-url)
The arrangement of intact acetate units in asticolorin C was studied by addition of [1,2-\(^{13}\)C]acetate to the culture medium. The signals of a number of carbon atoms exhibited, as a result of one-bond (C=C) couplings, two pairs of satellite signals of equal intensity in the broad-band proton-decoupled n.m.r. spectrum of this enriched asticolorin C (see Table I). This phenomenon, observed for the corresponding carbon atoms in rings A, C, F, and H, indicates the existence of two different arrangements of intact acetate units in each of these rings (see Figure 1). Thus a symmetrical intermediate, orcinol, formed by enzymatic decarboxylation of orsellinic acid, must be involved in the biosynthesis of asticolorin C. Subsequent oxidative coupling of two molecules of orcinol would lead to the formation of pannarol.

The low-intensity satellite peaks observed for the C-34 signal are due to (C=C) coupling with C-17 and could be the result of multiple labelling as C-34 is derived from C-2 of mevalonate. However, although the C-34 signal is enhanced (enrichment factor 5.0) in the broad-band proton-decoupled \(^{13}\)C n.m.r. spectrum of asticolorin A\(^1\) derived from (3RS)-\(^{13}\)C\(^-\)mevalonolactone, an enhancement is also observed for the C-18 signal (enrichment factor 2.2). This result would imply that the stereochemical integrity of the two prochiral enantiomeric methyl groups in 3,9-dihydroxy-1,7-dimethyl-2-(3,3-dimethylallyl)dibenzofuran is lost in the subsequent oxidative coupling reactions leading to asticolorin C. This duality in the origin of C-34 and C-18 is under investigation.

The fate of the hydrogen atoms in the biosynthesis of asticolorin C was studied by incorporation of [1-\(^{13}\)C,2-\(^2\)H\(_3\)]acetate into the metabolite. The incorporation of 2H located to a \(^{13}\)C atom can be detected by the small characteristic upfield \(\beta\)-isotope shift in the resonance position of the \(^{13}\)C nucleus in the \(^{13}\)C n.m.r. spectrum. The number of \(\beta\) atoms located to a particular \(^{13}\)C atom can be deduced from the value of the \(\beta\)-isotope shift. The labelling pattern of asticolorin C enriched with [1-\(^{13}\)C,2-\(^2\)H\(_3\)]acetate is shown in Figure 2. The retention of 2H at C-31, evident from the \(\beta\)-isotope shift of \(-0.089\) p.p.m. for C-30 (see Table I), indicates that C-6 hydroxylation of pannarol occurs at the aromatic stage before oxidative coupling with 3,9-dihydroxy-1,7-dimethyl-2-(3,3-dimethylallyl)dibenzofuran. In the course of this coupling reaction the 4-pro-R proton of mevalonate is retained at C-16 in asticolorin C as a \(\beta\)-shift of \(-0.082\) p.p.m. is observed for the C-15 resonance. Significantly, the \(\beta\)-shift observed for the C-17 resonance (\(\Delta\beta = -0.072\) p.p.m.) indicates that only one 2H atom is present at C-18 in asticolorin C. A mechanism for the formation of the central rings D and E in asticolorins based on the above results is shown in Figure 3.

The authors thank Dr. A. E. de Jesus for microbiological assistance.

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References
synthesis of the Meroterpenoid, Austin, by *Aspergillus ustus*: Incorporation of $^{18}\text{O}_2$, dium $[1-^{13}\text{C},^{18}\text{O}_2]\text{Acetate, and } [\text{Me}-^{13}\text{C},^{2}\text{H}_3]\text{Methionine}$

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As spectral and $^{13}\text{C}$ n.m.r. analyses of austin (1) produced by fermentation of *Aspergillus ustus* in the presence of $^{18}\text{O}_2$ and $[\text{Me}-^{13}\text{C},^{2}\text{H}_3]\text{Methionine}$ showed that all labelled methionine-derived hydrogens were retained, determined five of nine oxygens were introduced by aerobic oxidation, and elucidated the general mechanism of late synthetic stages.

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Mass spectral and ¹³C n.m.r. analyses of austin (1) produced by fermentation of Aspergillus usus in the presence of ¹⁸O₂ and [Me-¹³C,²H₃]methionine showed that all labelled methionine-derived hydrogens were retained, determined that five of nine oxygens were introduced by aerobic oxidation, and elucidated the general mechanism of late biosynthetic stages.

Recent studies show that andibenin B,¹ andilesin C,² anditomin,¹ and austin (1),³ and terretonin³ may all be formed via a common biosynthetic precursor (3), which is the product of C-alkylation of dimethylorsellinic acid (2) by farnesyl pyrophosphate. Epoxidation of (3) followed by cyclisation could produce (4) which can serve as a common precursor to both austin (1) and terretonin (Scheme 1). The conversion of 3,5-dimethylorsellinic acid (2), a proven tetraketide precursor,³ into austin (1) requires an unusually extensive modification of the aromatic ring. A number of chemically reasonable pathways from (4) to (1) (Scheme 2) may be distinguished because they require different origins for the various oxygen and hydrogen atoms. We now report ¹⁸O and ²H lab studies which indicate that path a is correct and which provide information on other aspects of austin biosynthesis.

[Me-¹³C,²H₃]Methionine was added to cultures of Aspergillus usus NRRL 6017 and the resulting labelled austin analysed by fast atom bombardment (f.a.b.) mass spectrometry. Prominent M + 4 and M + 8 peaks demonstrate that the methionine-derived methyl groups of (2) are incorporated into austin (1) without loss of ²H label. This excludes the mechanism shown in path b.

A fermentation of A. usus in which the normal atmosphere was replaced with one containing ¹⁸O₂ (96 atom %) after onset of austin (1) production gave this metabolite labelled with up to five ¹⁸O atoms per molecule, as determined by mass spectrum. The positions of label incorporation located by observation of ¹⁸O isotope-induced shifts in 100.6 MHz proton noise decoupled ¹³C n.m.r. spectrum of ¹⁸O-enriched austin (1). An equal quantity of unlabelled austin had to be added to this sample as an internal reference because of the high level of ¹⁸O incorporation and the magnitude of such isotope shifts. Although all four carbon carbons showed ¹⁸O-isotopically shifted signals (Table 1), the 11-ac- and 3,5-dimethylorsellinic acid oxygen atoms of the spiro-lactone are added by oxidative processes, in agreement with observations for andibenin.

Table 1. ¹⁸O Isotopically shifted resonances observed in the MHz ¹³C n.m.r. spectrum of austin (1).³

<table>
<thead>
<tr>
<th>Carbon</th>
<th>δ (p.p.m.)</th>
<th>[p.p.m.×100]</th>
<th>Ratio ¹⁸O</th>
</tr>
</thead>
<tbody>
<tr>
<td>8'</td>
<td>170.8</td>
<td>1.2</td>
<td>55:45</td>
</tr>
<tr>
<td>4'</td>
<td>170.2</td>
<td>1.3</td>
<td>57:43</td>
</tr>
<tr>
<td>MeCO</td>
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<td>1.4</td>
<td>56:44</td>
</tr>
<tr>
<td></td>
<td>3.8</td>
<td></td>
<td>85:15</td>
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<tr>
<td>3'</td>
<td>163.6</td>
<td>3.7, 4.7</td>
<td>45:13:11</td>
</tr>
<tr>
<td>4'</td>
<td>85.6</td>
<td>4</td>
<td>67:33</td>
</tr>
<tr>
<td>6'</td>
<td>84.4</td>
<td>3.8</td>
<td>60:40</td>
</tr>
<tr>
<td>6'</td>
<td>80.8</td>
<td>0.8</td>
<td>63:37</td>
</tr>
<tr>
<td>5'</td>
<td>78.9</td>
<td>3.1</td>
<td>66:34</td>
</tr>
<tr>
<td>11</td>
<td>74.9</td>
<td>2.7</td>
<td>67:33</td>
</tr>
</tbody>
</table>

For experimental conditions see ref. 11. ¹³C Enriched by 100% [¹-¹³C,¹⁸O₂]acetate; all others enriched by ¹⁸O₂.
the aliphatic region the resonances due to C-4, C-11, and C-5' all show isotope shifts which complement those in the carbonyl region, thereby accounting for all $^{18}$O atoms incorporated into austin. Interestingly, C-6' has a shifted signal (Table 1). The shift magnitude is too low for a tertiary alcohol, and is due to a $\beta$-shift from the atom attached to C-3' or C-5' or a double $\beta$-shift from C-3. This is the first observation of a $\beta$-shift in a biosynthetic trace. Path c requires that the oxygen bridging C-5' and C-8' derivd from the medium, our results are consistent with an addition-reduction sequence like path a. The hydrogen, 5' and oxygen labelling patterns suggest hydroxylation -5' of (4), ring contraction via $\alpha$-ketol rearrangement, reduction of the resulting 5'-keto function to an alcohol, and anion formation by attack of the 5'-hydroxy group on the carboxy function. Since no $^{18}$O$_2$-derived label is observed at the 4'-carboxy carbonyl oxygen, this carbon cannot exist at any stage free carboxy group. Introduction of the 11-acetoxy function of austin (1) ably occurs at a late biosynthetic stage by allylic hydroxylation and acetylation. Incorporation of sodium [1-13C, 11$^{18}$O]-acetate into austin (1) followed by 13C n.m.r. analysis showed a large amount of 11$^{18}$O label was present only at the ole-bonded acetate oxygen in accord with its probable from acetyl coenzyme A. Incorporation of 11$^{18}$O at other radiodated sites (C-4', C-6', and C-8') was too low for sensitive determination of isotope shifts. The isolation of austinol (5)$^{10}$ as a co-metabolite of austin (1) also supports the notion that modification at C-11 is a late biosynthetic event.

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References
Synthesis of Tajixanthone and Shamixanthone by *Aspergillus variecolor*: Incorporation of Oxygen-18 Gas

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Spectral and 13C n.m.r. analyses of tajixanthone (1) and shamixanthone (2) formed during growth of *Aspergillus variecolor* under atmospheres containing [18O2] oxygen gas showed incorporation of four and three 18O atoms per molecule of (1) and (2), respectively, and provided information about the mode of xanthone ring formation.

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Biosynthesis of Tajixanthone and Shamixanthone by Aspergillus variecolor: Incorporation of Oxygen-18 Gas

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Mass spectral and 13C n.m.r. analyses of tajixanthone (1) and shamixanthone (2) formed during growth of Aspergillus variecolor under atmospheres containing [18O2] oxygen gas showed incorporation of four and three labels per molecule of (1) and (2), respectively, and provided information about the mode of xanthone ring formation.

Mycelial pigments like tajixanthone (1) and shamixanthone (2)1 as well as various meroterpenoids2 illustrate how Aspergillus species can combine polyketide and terpenoid precursors to form secondary metabolites which have often undergone extensive oxidative elaboration. The isolation of a number of closely related xanthones3–5 and 13C and 1H labelling studies6,7 on tajixanthone strongly support the biosynthetic pathway outlined in Scheme 1. Carbon labelling patterns present in equal amounts in ring C of (1) and (2) are shown in Table 1.

Table 1. 18O Isotopically-shifted resonances in the 13C n.m.r. spectraa of tajixanthone (1) and shamixanthone (2).

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<th>δ (1)</th>
<th>Δδ (1) (× 100)</th>
<th>18O : 13C δ (1)</th>
<th>δ (2)</th>
<th>Δδ (2) (× 100)</th>
<th>18O : 13C δ (2)</th>
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<td>13</td>
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<td>159.7</td>
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<td>1.0</td>
<td>152.8</td>
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<td>10</td>
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<tr>
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<td>2.4</td>
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<td>7</td>
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<td>19</td>
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<tr>
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<td>16</td>
<td>58.5</td>
<td>4.1</td>
<td>64.36</td>
<td></td>
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</tr>
</tbody>
</table>

a Spectra run at 100.6 and 90.6 MHz; for experimental conditions see ref. 9. b Enriched by sodium [1-13C,18O2]acetate only; all others enriched by 18O2. c These assignments were originally reversed in ref. 6. d Approximate (±5%) ratios from peak areas. e Not resolvable.
the intermediacy of a symmetrical dihydroxyphenyl moiety which is free to rotate prior to cyclization to a xanthone. Since the detection of $^{18}$O-induced isotope shifts in $^{13}$C n.m.r. has proved useful in determining the mode of xanthone ring formation in ravenelin and sterigmatocystin, we have studied the incorporation of $^{18}$O$_2$ gas into tajixanthone (1) and shamixanthone (2).

A fermentation of Aspergillus variecolor in which the normal atmosphere was replaced with one containing $^{18}$O$_2$ gas (98.7% isotopic purity) gave tajixanthone (1), the mass spectrum of which showed the presence of four $^{18}$O atoms per molecule. The 100.6 and 90.6 MHz $^{13}$C n.m.r. spectra of a mixture of this and unlabelled material displayed isotopically-shifted resonances for eight of the nine oxygen-bearing carbons (Table 1). Only the carbonyl oxygen at C-13 remained completely unlabelled in this experiment. Within experimental error, the relative amount of $^{18}$O incorporated at C-1 and at C-10 is half of that at the other labelled sites. Taken together with the mass spectral results, this shows that in a particular molecule of tajixanthone (1) either the oxygen at C-1 or the one at C-10 was labelled, but not both. This confirms the intermediacy and oxidative origin of a conformationally labile benzophenone which has an axis of symmetry in a dihydroxyphenyl ring. More importantly, the results demonstrate that xanthone ring closure must proceed almost exclusively by a Michael addition-elimination process in which the ring oxygen attacks the ring oxygen with ultimate loss of the ring oxygen at C-11 (paths a$_2$ and b$_2$). Cyclization in the opposite sense with retention of the ring oxygen (paths a$_1$ and b$_1$) is very minor if it occurs at all.

The presence of $^{18}$O at C-25 and the previously reported loss of $^2$H from acetate at that position suggest oxidative cleavage of an anthraquinone rather than anthrone precursor. Mass spectral analysis of the molecular ion region of (1) obtained from a fermentation utilizing a mixture of $^{18}$O$_2$ and $^{16}$O$_2$ shows that each aerobically-derived oxygen atom is introduced separately by mono-oxygenation. Thus the involvement of dioxygenase-derived dioxetanes or endoperoxides which have been proposed as intermediates in the cleavage mechanism can be ruled out. Presumably cleavage occurs via a biological Baeyer-Villiger type oxidation to give an intermediate lactone which can undergo direct reduction to the hemiacetal (cf. arugosin A/B) and thence to the benzophenone.

In a separate experiment sodium [1-$_{13}$C, $^{18}$O$_2$]acetate (90% $^{18}$O) was fed to cultures of A. variecolor grown in a normal atmosphere, and the resulting tajixanthone (1) was analysed by $^{13}$C n.m.r. Unfortunately the incorporation level was too low to detect isotope shifts at any carbons except C-13, the C-O bond of which was thereby shown to be acetate-derived.

As expected, shamixanthone (2) isolated in the same experiment with $^{18}$O$_2$ showed, by mass spectral analysis, the incorporation of three $^{18}$O atoms per molecule. Although the isotope shift in the $^{13}$C n.m.r. of (2) at C-1 could not be completely resolved for accurate determination of the $^{16}$O : $^{18}$O ratio, the presence of $^{18}$O at that site and the reduced $^{18}$O content of the xanthone ring oxygen relative to other sites (Table 1) confirm the operation of the same biosynthetic pathway as that of tajixanthone (1). It is interesting to note that in ravenelin biosynthesis the same type of xanthone ring closure (paths a$_2$ and b$_2$) occurs with retention of the oxygen of a symmetrical dihydroxyphenyl moiety. In contrast, retention of oxygen from the other ring and a single carbon labelling pattern during sterigmatocystin biosynthesis suggest an oxidative coupling mechanism rather than addition-elimination for xanthone formation in that case.

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References


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synthesis of the Meroterpenoid Metabolite, Andibenin B: Incorporation of Sodium $^{13}$C,$^{18}$O$_2$Acetate and $^{18}$O$_2$

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Incorporation of sodium [1-$^{13}$C,$^{18}$O$_2$]acetate and $^{18}$O$_2$ gas into andibenin B (1) by cultures of Aspergillus variecolor establishes the presence of all the oxygen atoms and provides mechanistic information on the biosynthetic pathway.

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Chemical Communications 1984
Biosynthesis of the Meroterpenoid Metabolite, Andibenin B: Incorporation of Sodium [1-13C,18O2]Acetate and 18O2

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Incorporation of sodium [1-13C,18O2]acetate and 18O2 gas into andibenin B (1) by cultures of Aspergillus variecolor and observation of 18O isotope-induced shifts in the 13C n.m.r. spectra of the enriched metabolites establish the origins of all the oxygen atoms and provide mechanistic information on the biosynthetic pathway.

We have recently shown that andibenin B (1), a metabolite of Aspergillus variecolor, is a member of a group of biosynthetically related metabolites formed by a mixed polyketide-terpenoid pathway.1,2 The key step is alkylation of 3,5-dimethylorsellinic acid (2), a bis-C-methylated tetraketide, with farnesyl pyrophosphate to give (3), which then cyclises to give product (4). Further condensations and oxidative modifications convert (4) into andibenin B (1). Since observation of isotopic isotope shifts induced in 13C n.m.r. spectra by 18O provides information on intermediate oxidation states and mechanisms,3 we have incorporated sodium [1-13C,18O2]acetate and 18O2 gas into andibenin B (1) to study these modifications.

The proton noise-decoupled 13C n.m.r. spectrum of (1) enriched by fermentation of A. variecolor with sodium [1-13C,18O2]acetate shows isotopically shifted signals (Table 1) due to incorporation of acetate-derived oxygen into the C-4' and C-8' carbonyl groups. In a separate experiment, growth of the cultures under an 18O2 atmosphere with unlabelled carbon sources produced andibenin B (1) whose 13C n.m.r. spectrum demonstrated the origin of both oxygen atoms on C-3 and of the single-bonded oxygen atoms on C-4, C-10, C-1', and C-8' from oxidative processes. The appearance of oxygen label in the γ-lactone ring oxygen in the experiment suggests that the pathway proceeds by hydration of the 6-methyl group of (2) followed by nucleophile attack of this hydroxy group on the carboxy group. It rest to be established whether ring closure occurs before or after alklylation with farnesyl pyrophosphate.

In accord with earlier carbon labelling studies,4 the present results show that the C-3 lactone function must be formed biological Baeyer-Villiger-type oxidation5 of a corresponding ketone precursor. Generation of the spiro ring system involves a ring contraction which requires development of a carbocation character at C-5. Similar ring contractions have been observed in steroid derivatives on acid treatment either 4β,5β- or 5α,6α-epoxides.6 Since the 10-hydroxy function of andibenin B (1) is derived from atmospheric oxygen, rearrangement cannot terminate by capture of an intermediate C-10 carbocation by water. Instead, intramolecular attack by the carboxy group or an elimination-epoxidation process...

Scheme 1

Scheme 2
1. $^{18}O$ Isotopically shifted resonances observed in the 100.6 $^3C$ n.m.r. spectrum of andibenin B (1).$^a$

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$\delta$ (p.p.m.)</th>
<th>$\Delta\delta$ (p.p.m. $\times$ 100)</th>
</tr>
</thead>
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<tr>
<td>4'</td>
<td>215.8</td>
<td>5.0$^b$</td>
</tr>
<tr>
<td>8'</td>
<td>169.0</td>
<td>3.5$^b$</td>
</tr>
<tr>
<td>3</td>
<td>165.5</td>
<td>4.7$^b$</td>
</tr>
<tr>
<td>4</td>
<td>86.9</td>
<td>4.2$^b$</td>
</tr>
<tr>
<td>10</td>
<td>79.3</td>
<td>3.2$^b$</td>
</tr>
<tr>
<td>1'</td>
<td>70.4</td>
<td>2.9$^b$</td>
</tr>
</tbody>
</table>

experimental conditions see ref. 9. $^a$ Enriched by sodium $^{18}O_2$acetate. $^b$ Enriched by $^{18}O_2$.

We thank the S.E.R.C., N.A.T.O., and the Natural Sciences and Engineering Research Council of Canada (N.S.E.R.C.) for financial support.

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References

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Incorporation of [1-13C,18O2] acetate and 18O2 gas into aspyrone (1) and asperlactone (2) by cultures of *Aspergillus Ieus* and observation of 18O isotope-induced shifts in the 13C n.m.r. spectra of the enriched metabolites establish origins of all the oxygen atoms and suggest a biosynthetic pathway involving epoxide-mediated rearrangement ring closure reactions.

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*Chemical Communications* 1985
Biosynthesis of Aspyrone and Asperlactone, Pentaketide Metabolites of Aspergillus melleus. Incorporation Studies with [1-\textsuperscript{13}C,\textsuperscript{18}O\textsubscript{2}] Acetate and \textsuperscript{18}O\textsubscript{2} Gas

Salman A. Ahmed,\textsuperscript{a} Thomas J. Simpson,\textsuperscript{a,*} James Staunton,\textsuperscript{b} Andrew C. Sutkowski,\textsuperscript{b} Laird A. Trimble,\textsuperscript{c} John C. Vederas\textsuperscript{a,*}

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\textsuperscript{b} University Chemistry Laboratory, Lensfield Road, Cambridge CB2 1EW, U.K.
\textsuperscript{c} Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Incorporation of [1-\textsuperscript{13}C,\textsuperscript{18}O\textsubscript{2}] acetate and \textsuperscript{18}O\textsubscript{2} gas into aspyrone (1) and asperlactone (2) by cultures of Aspergillus melleus and observation of \textsuperscript{18}O isotope-induced shifts in the \textsuperscript{13}C n.m.r. spectra of the enriched metabolites estimated the origins of all the oxygen atoms and suggest a biosynthetic pathway involving epoxide-mediated rearrangements and ring closure reactions.

Aspyrone (1)\textsuperscript{1} and asperlactone (2)\textsuperscript{2} are closely related metabolites of Aspergillus melleus. Their biosynthesis has been extensively studied,\textsuperscript{3} and as a result of incorporation studies with isotopically labelled acetates and malonate and potential advanced intermediates the intermediary of aromatic precursors could be ruled out; a pathway was proposed via decarboxylation and Favorinski-type rearrangement of a linear pentaketide intermediate as shown in Scheme 1. In support of this, incorporations of [1,2-\textsuperscript{13}C\textsubscript{2}] acetate resulted in two-bond \textsuperscript{13}C-\textsuperscript{13}C couplings being observed between C-2 and C-8 in aspyrone\textsuperscript{4} and asperlactone.\textsuperscript{2} The stereochemistry of (1) and (2) has been established\textsuperscript{5} and this is consistent with their derivation from a common intermediate, e.g. (3), via alternative ring openings of the epoxide by the carboxylic function. In order to obtain evidence for this proposal and to obtain information on the nature of intermediates on the pathway we have studied the incorporation of [1-\textsuperscript{13}C,\textsuperscript{18}O\textsubscript{2}] acetate and \textsuperscript{18}O\textsubscript{2} gas into aspyrone and asperlactone.

\footnotesize{$^{1\textsuperscript{13}C,\textsuperscript{18}O\textsubscript{2}}$}Acetate was fed to cultures of A. melleus the aspyrone and asperlactone isolated from separate fermentations were analysed by \textsuperscript{13}C n.m.r. spectroscopy at MHz. High levels of \textsuperscript{13}C-enrichment (ca. 5 atom %) observed at C-2, -4, -6, and -9 but surprisingly no isotope-induced shifts were apparent indicating the acetate-derived oxygen was incorporated into the metabolite.

On carrying out a fermentation in the presence of \textsuperscript{18}O\textsubscript{2} the \textsuperscript{13}C n.m.r. spectrum shown in Figure 1 was obtained. The isotope shifts observed (Table 1) for C-5 and C-9 indicated that the epoxide and alcohol oxygens both highly and equally enriched. In addition C-2 shows isotopically shifted signals and C-6 shows one. W

\footnotesize{$^{*}$} Fermentations were carried out in a closed system under an atmosphere composed of N\textsubscript{2}, \textsuperscript{18}O\textsubscript{2}, and \textsuperscript{16}O\textsubscript{2} (80:10:10) for aspyrone and (80:6:14) for asperlactone.
Me—O2NQ
-HO2C—MeCSCoA
Me

Scheme 1

Scheme 2

Table 1. \(^{18}O\) Isotopically shifted resonances observed in the 100.6 MHz \(^{13}C\) n.m.r. spectrum of aspyrone (1). Enriched from \(^{18}O\) gas.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>(\delta)</th>
<th>100 (\Delta\delta)/p.p.m.</th>
<th>(\text{MeO}^{18}O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>163.2</td>
<td>0.9, 3.7</td>
<td>61:20:19</td>
</tr>
<tr>
<td>5</td>
<td>67.7</td>
<td>1.6</td>
<td>63:37</td>
</tr>
<tr>
<td>6</td>
<td>79.3</td>
<td>3.1</td>
<td>78:22</td>
</tr>
<tr>
<td>8</td>
<td>54.6</td>
<td>3.1</td>
<td>62:38</td>
</tr>
<tr>
<td>9</td>
<td>59.0</td>
<td>3.4</td>
<td>63:37</td>
</tr>
</tbody>
</table>

* For experimental conditions see ref. 9. All values (±2%) were determined using a Du Pont curve analyser.

Experimental error, the intensities of these signals are essentially equal to one another but are half those observed at C-8, and C-9. The most reasonable interpretation of the results is that one oxygen-18 atom has been introduced into the atmosphere onto C-2 and that this labelled atom has incorporated equally into both the carbonyl and ether rings of the lactone moiety. Thus three of the oxygen atoms of aspyrone appear to be derived from the atmosphere and so remaining oxygen on C-2 must be derived from the medium. Asperlactone, produced in the presence of \(^{18}O\) gas in a separate experiment, gave essentially identical results.

Carbon-6 and 100 MHz \(^{13}C\) n.m.r. spectrum of aspyrone (1). Enriched from \(^{18}O\) gas. The most reasonable interpretation of the results is that one oxygen-18 atom has been introduced into the atmosphere onto C-2 and that this labelled atom has incorporated equally into both the carbonyl and ether rings of the lactone moiety. Thus three of the oxygen atoms of aspyrone appear to be derived from the atmosphere and so remaining oxygen on C-2 must be derived from the medium. Asperlactone, produced in the presence of \(^{18}O\) gas in a separate experiment, gave essentially identical results.

Table 1. \(^{18}O\) Isotopically shifted resonances observed in the 100.6 MHz \(^{13}C\) n.m.r. spectrum of aspyrone (1). Enriched from \(^{18}O\) gas.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>(\delta)</th>
<th>100 (\Delta\delta)/p.p.m.</th>
<th>(\text{MeO}^{18}O)</th>
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</thead>
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<tr>
<td>2</td>
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<td>0.9, 3.7</td>
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<td>1.6</td>
<td>63:37</td>
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<tr>
<td>6</td>
<td>79.3</td>
<td>3.1</td>
<td>78:22</td>
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<tr>
<td>8</td>
<td>54.6</td>
<td>3.1</td>
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</tr>
<tr>
<td>9</td>
<td>59.0</td>
<td>3.4</td>
<td>63:37</td>
</tr>
</tbody>
</table>

* For experimental conditions see ref. 9. All values (±2%) were determined using a Du Pont curve analyser.

...polyether antibiotics...
We thank the S.E.R.C., NATO, the Government of Iraq, and the Natural Sciences and Engineering Research Council of Canada for financial support.

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References

Thomas J. Simpson* and Graeme I. Stevenson
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The origins of all the oxygen and hydrogen atoms in colletodiol (2) have been elucidated by incorporation of labelled [1,1-13C,18O2] and [1,1-13C,2-13H2]-acetate and 18O2 gas into (2) in cultures of Cytospora sp. (ATCC 20502); from the resultant labelling pattern the structures of the enzyme-bound precursors can be deduced and information obtained on the processes occurring during the early stages of polyketide chain-assembly.

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Chemical Communications 1985

Thomas J. Simpson* and Graeme I. Stevenson
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The origins of all the oxygen and hydrogen atoms in colletodiol (2) have been elucidated by incorporation of label from $[^{13}\text{C},^{18}\text{O}_2]$- and $[^{1-13}\text{C},2^{13}\text{H}_2]$-acetate and $^{18}\text{O}_2$ gas into (2) in cultures of Cytospora sp. (ATCC 20502); from the resultant labelling pattern the structures of the enzyme-bound precursors can be deduced and information obtained on the processes occurring during the early stages of polyketide chain-assembly.

The polyketide pathway is one of the major pathways of secondary metabolism, but despite much effort over the last 30 years since the recognition of the pathway,¹ little is known of the exact nature of the intermediates involved in the early stages of polyketide chain-assembly. At its simplest, it is thought that poly-β-ketide intermediates (1) are built up by a cyclic process (Scheme 1) analogous to fatty acid biosynthesis² but omitting the reduction–elimination–reduction sequence responsible for the loss of acetate oxygen. While some aromatic metabolites do retain the full oxygen content of intermediate (1) most metabolites show varying degrees of reduction and/or deoxygenation and an increasing body of evidence suggests that this occurs by processes analogous to fatty acid biosynthesis before the initial release of metabolites or intermediates from the chain-assembly enzymes. Path a in Scheme 1 would simply produce poly-β-ketide intermediates by invoking paths b, c, and d intermediates with varying degrees of reduction may be formed. There has been progress in enzymatic or other direct methods of observing these early intermediates but recent developments in n.m.r.- and 2H n.m.r. spectroscopy (viz. $^3\text{H}$ and $^{18}\text{O}$ isotope-induced shifts) which facilitate determination of the biosynthetic origins of hydrogen and oxygen enable significant indirect evidence for the nature of

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**(Scheme 1)**

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**(Scheme 2)**

---
mediates to be obtained. We now report \(^2\)H and \(^18\)O labelling studies on colletodiol (2) designed to obtain information on the processes occurring during the early stages of tetide chain-assembly. 

Colletodiol (2) and colletoketol (3) are macrocyclic dilacon metabolites originally isolated from the plant pathogen, \textit{Cotylorhiza capsici}. More recently grahamimycin A was described as a broad spectrum antibiotic from a species of \textit{Sporo spora} and was subsequently shown to be identical to colletoketol. All four chiral centres in colletodiol have the configuration. Incorporation studies with singly labelled acetates have confirmed the acetate-origin of colletodiol in \textit{C. capsici}. These metabolites can be seen to be derived by combination of \(C_6\) and \(C_8\) moieties and \textit{a priori} one postulate a number of triketide- and tetraketide-derived triketides as the actual enzyme-bound precursors. Some of these are shown in Scheme 2. Depending on the nature of the actual intermediates a number of mechanisms can be proposed for the formation of the lactone functions. These are summarised in Scheme 3 along with the possible stereochemical outcome and the predicted origins of the associated oxygen and hydrogen atoms. Similarly a number of different mechanisms can be proposed for the formation of the \(1,2\)-diol and \(\alpha\)-ketol systems found in colletodiol and colletoketol respectively. These are shown in Scheme 4 and again they may be differentiated, as indicated, by appropriate \(^2\)H and \(^18\)O labelling experiments.

In our hands \textit{Cytospora sp.} (ATCC 20502) has produced colletodiol as the major metabolite and only minor amounts of grahamimycin A. Fermentations were carried out in the presence of \([1\text{-}^{13}\text{C},2\text{H}_3]\) acetate and \([1\text{-}^{13}\text{C},^{18}\text{O}_2]\) acetate and under an atmosphere of \(^18\)O. The \(^2\)H and \(^18\)O isotope shifts observed in the proton noise decoupled \(^{13}\text{C}\) n.m.r. spectra of colletodiol isolated in each case are summarised in Table 1. No \(^2\)H isotope-induced shifts could be observed for C-1 or C-1' in the \(^{13}\text{C}\) n.m.r. spectrum of [\(1\text{-}^{13}\text{C},2\text{H}_3\)]acetate-enriched colletodiol. However carbonyl groups are known to be poor reporter groups for \(^2\)H shifts and the presence of \(^2\)H label at both C-2 and C-2' was shown by \(^2\)H n.m.r. analysis of the

---

**Table 1.** \(^2\)H and \(^18\)O isotope-induced shifts observed in the 90.56 MHz \(^{13}\text{C}\) n.m.r. spectrum of colletodiol (2).

<table>
<thead>
<tr>
<th>(\delta_{\text{C}}) (ppm)</th>
<th>(\Delta\delta \times 10^4)</th>
<th>(^{18}\text{O}:^{18}\text{O})</th>
<th>(^{2}\text{H}:^{2}\text{H})</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-1</td>
<td>166.3</td>
<td>3.4a</td>
<td>69:31</td>
</tr>
<tr>
<td>C-1'</td>
<td>164.9</td>
<td>3.2a</td>
<td>70:30</td>
</tr>
<tr>
<td>C-3</td>
<td>146.4</td>
<td>9.0c</td>
<td>95:5</td>
</tr>
<tr>
<td>C-3'</td>
<td>143.9</td>
<td>4.4,4,4.5c</td>
<td>53:19:28</td>
</tr>
<tr>
<td>C-5</td>
<td>71.7</td>
<td>2.2b</td>
<td>54:46</td>
</tr>
<tr>
<td>C-5'</td>
<td>68.6</td>
<td>3.9b</td>
<td>53:20:27</td>
</tr>
<tr>
<td>C-7</td>
<td>67.9</td>
<td>3.7b</td>
<td>79:21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.2,4,2,4,3c</td>
<td>30:10:22:38</td>
</tr>
</tbody>
</table>

*a [\(1\text{-}^{13}\text{C},^{18}\text{O}_2\)]acetate-enriched. b \(^{18}\text{O}_2\)-enriched. c [\(1\text{-}^{13}\text{C},^{2}\text{H}_3\)]acetate-enriched.
enriched metabolite. The labelling pattern resulting from these experiments is summarised in Scheme 5.

The retention of acetate-derived oxygen on both the carbonyl and ether oxygens of the lactone functions indicates that ring closure must proceed by mechanism (a) in Scheme 3 and so the enzyme-bound intermediates must retain the oxygen of the acetate 'starter' units as hydroxy functions with the (R) configuration.

Considering the formation of the 1,2-diol system, a low but significant level of acetate-derived hydrogen is retained at C-4. This means that colletoketol cannot be the precursor of colletodiol, and route (c) in Scheme 4 is ruled out. The oxygen labelling results indicate that the 5-hydroxy group is derived from the atmosphere i.e. via an oxidative process, whereas the 4-hydroxy group must be derived from the medium of route (b), Scheme 4. A mechanism consistent with the observed labelling and the (R) configuration at both centres is shown in Scheme 6; epoxidation of a (Z)-alkene from the 3-face is followed by hydrolytic ring opening by attack of water from the α-face at C-4.

On the basis of these results, the thioesters (4) and (5) can be proposed as the actual enzyme-bound precursors for colletodiol. These may be built up by the sequence shown in Scheme 7 which the diol (6) in which the C-3 stereochemistry is uncertain, is proposed as a common intermediate, trans-elimination of water giving rise to the C₄ precursor diene whereas cis-elimination followed by addition of a further unit produces the C₅ precursor. The relative timing of the formation step is not yet known but it may occur concomitantly and release from the enzyme surface as cated.

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References
synthesis of the Meroterpenoid Austin, by *Aspergillus ustus*: Synthesis and Corporation of $^{13}$C,$^{18}$O-Labelled Ethyl 3,5-Dimethylorsellinate

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3,5-dimethylorsellinate (3) doubly labelled with $^{13}$C and $^{18}$O in the carbonyl of the carboxy group and at the C-6 position has been synthesised using a four-step procedure from sodium [$^{1-13}$C,$^{18}$O]acetate, and incorporated into Austin (1) by cultures of *Aspergillus ustus.*

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Biosynthesis of the Meroterpenoid Austin, by *Aspergillus ustus*: Synthesis and Incorporation of $^{13}$C,$^{18}$O-Labelled Ethyl 3,5-Dimethylorsellinate

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**Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

Ethyl 3,5-dimethylorsellinate (3) doubly labelled with $^{13}$C and $^{18}$O in the carbonyl of the carboxy group and at the position has been synthesised using a four-step procedure from sodium [1-$^{13}$C,$^{18}$O]acetate, and incorporated into austin (1) by cultures of *Aspergillus ustus*.

We have recently shown$^1$ that austin (1), a toxic metabolite of *Aspergillus ustus*, is biosynthesized by a mixed polyketide-terpenoid pathway in which C-alkylation of the tetraketide-derived 3,5-dimethylorsellinate (2) by farnesyl pyrophosphate is followed by cyclisation and oxidative modification (Scheme 1). From the results of incorporation of label from $^{18}$O into austin (1) by cultures of *Aspergillus ustus*, we propose the mechanism shown in Scheme 2 for the modification of the orsellinate-derived portion of the cyclised intermediate en route to austin (1). In agreement with these proposals the oxygen atoms attached to C-3' and were derived from the atmosphere. The C-4' and carbonyl and C-6' tertiary alcohol oxygens should be derived via 3,5-dimethylorsellinate (2) from acetate. However,

![Scheme 1](image1)

![Scheme 2](image2)
1. $^{18}O$-Isotopically shifted resonances observed in the 100.6 MHz $^{13}C$ n.m.r. spectra of austin (1), ethyl 3,5-dimethylorsellinate (3), acetate (4). *

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$\delta(1)$</th>
<th>$\Delta\delta(1)$</th>
<th>$^{18}O$ : $^{16}O$</th>
<th>$\delta(3)$</th>
<th>$\Delta\delta(3)$</th>
<th>$^{18}O$ : $^{16}O$</th>
<th>$\delta(4)$</th>
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</tr>
</thead>
<tbody>
<tr>
<td>6'</td>
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<td>1.0</td>
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<td>145.0</td>
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<tr>
<td>8'</td>
<td>172.3</td>
<td>3.3</td>
<td>41.59</td>
<td>180.0</td>
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<td>167.0</td>
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<tr>
<td>6</td>
<td>172.3</td>
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<td>1.0</td>
<td>45.55</td>
<td>167.0</td>
<td>3.7</td>
<td>58.42</td>
</tr>
</tbody>
</table>

Experimental conditions see ref. 5. * p.p.m. * p.p.m. *100. Unlabelled (3) or (4) added as internal reference.

Figure 1. $^{18}O$-Isotopically shifted resonances in the 100.6 MHz proton noise-decoupled $^{13}C$ n.m.r. spectrum of austin (1) enriched by ethyl $[^{13}C, ^{18}O]3,5$-dimethylorsellinate (3).

hydroxy group at C-5' on C-8' to form the y-lactone of austin (1), as indicated in Scheme 2.

We thank the S.E.R.C., N.A.T.O., and the Natural Sciences and Engineering Research Council of Canada (N.S.E.R.C.) for financial support.

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References
synthesis of the Meroterpenoid Metabolite, Andilesin A, by Aspergillus variecolor: origins of the Oxygen Atoms

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Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

The origins of all the oxygen atoms in andilesin A (1) have been determined by labelling studies with 13C, 18O2-acetate, 18O2, and ethyl 3,5-dimethylorsellinate doubly-labelled with 13C and 18O at the carbonyl group at C-6; the results suggest a biosynthetic pathway in which andilesin A (1) is dehydrated to give andilesin B (2) which is then reduced to andilesin C (3).

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Biosynthesis of the Meroterpenoid Metabolite, Andilesin A, by Aspergillus variecolor: Origins of the Oxygen Atoms

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The origins of all the oxygen atoms in andilesin A (1) have been determined by labelling studies with [1-13 C, 18O2]acetate, [18O2], and ethyl 3,5-dimethylorsellinate doubly-labelled with 13C and 18O at the carbonyl group and at C-6; the results suggest a biosynthetic pathway in which andilesin A (1) is dehydrated to give andilesin B which is then reduced to andilesin C (3).

Andilesins A (1), B (2), and C (3), together with andibenins A, B, and C and anditomin constitute a biogenetically homogeneous group of metabolites that have been isolated from Aspergillus variecolor.1 On the basis of labelling studies2 with 13C-labelled acetates and methionine, a novel triprenylphenol (meroterpenoid) biosynthetic pathway was proposed for these metabolites, the key step involving alkylation of a tetraketide derived phenolic intermediate (4) with farnesyl pyrophosphate to give (5) which can cyclise to (6). Further cyclisation and oxidative modifications convert (6) into the observed metabolites as outlined in Scheme 1. Further support for these proposals came from the efficient (1.1%) incorporation of 14C-labelled 3,5-dimethylorsellinate (4, R = OH) into andibenin B and the specific incorporation of 2H-labelled (4, R = OH) into both andibenin B and andilesin A.3 However, the 14C-labelled-deoxyorsellinate (4, R = H) was also incorporated efficiently (0.4%) into these metabolites. This surprising observation could be explained by (a) hydroxylation of deoxyorsellinate (4, R = H) to produce orsellinate (4, R = OH); (b) low specificity of the biosynthetic enzymes and operation of a metabolic grid; or (c) incorporation of 14C-label from deoxyorsellinate via prior degradation to e.g. acetyl CoA. In order to obtain more information to resolve this problem and in turn to define the biosynthetic inter-relationships among the A, B, and C metabolites, we have carried out oxygen-18 labelling studies on andilesin A to ascertain the origin of the oxygen atoms, in particular of the 6'-hydroxy.

Only low levels of enrichment were obtained on incorporation of [1-13C,18O2]acetate into andilesin A by A. variecolor. The proton noise-decoupled (p.n.d.) 13C n.m.r. spectrum of the enriched metabolite showed small isotopically shifted signals (Table 1) due to incorporation of acetate-derived oxygen into the C-4' and C-8' carbonyls. However, no resolvable shifted signal was observed for C-6', although a small shoulder was visible on the C-6' resonance. In a separate experiment, fermentation under an atmosphere enriched with 18O2 produced andilesin A which showed isotopically shifted signals (Table 1) due to incorporation of acetate-derived oxygen into the C-4' and C-8' carbonyls.

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Is for C-3, C-4, C-1', and C-8' consistent with the origin of the oxygens in the δ-lactone and the ether oxygen in the one from oxidative processes (cf. andibenin B). No unusually shifted signal was observed for C-6' so the hydroxyl is not derived from the atmosphere. This rules out ways in which andilesin A is formed by hydroxylation of sin C, or orsellinate (4, R = OH) is formed by xylation of deoxyorsellinate (4, R = H).

The ambiguity in the origin of the 6'-hydroxy function was studied by incorporation of ethyl 3,5-dimethylorsellinate (7)5 which was doubly-labelled with 13C and 18O at either the carboxyl or at C-6. The 13C n.m.r. spectrum (Figure 1) showing clear isotopically shifted signals for both C-6' and C-8'. Interestingly the ratio of 18O-label incorporated at C-8' is approximately 90:11 and at C-6' 72:28 suggesting that the δ-lactone is formed by dehydration of the hydroxy group on C-1' onto C-8' which has a free carboxylate at some stage during biosynthesis. The results of the incorporation experiments are summed in Scheme 2. The derivation of the 6'-hydroxy function from the 4, R = OH) confirms its role as an obligatory intermediate to andilesin A (1). The observed incorporation of label from deoxyorsellinate (4, R = H) is thus probably for degradation, path (c) above, as both paths (a) and (b) necessitate some labelling of the 6'-hydroxy by atmospheric oxygen. The results indicate a pathway in which andilesin A (1) is the first metabolite to be formed and that it is converted to andilesin B (2) which is reduced to andilesin C (3).

Figure 1. 100.6 MHz p.n.d. 13C n.m.r. spectrum of C-8' and C-6' of andilesin A (1) enriched from ethyl [13C,18O]-3,5-dimethylorsellinate (7).

Table 1. 18O Isotopically shifted resonances observed in the 100.6 MHz 13C n.m.r. spectrum of andilesin A (1). *

<table>
<thead>
<tr>
<th>Carbon</th>
<th>δ</th>
<th>Δδ p.p.m. × 100</th>
<th>Ratio 16O:18O</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-3</td>
<td>166.2</td>
<td>7.4a</td>
<td>83:15</td>
</tr>
<tr>
<td>C-4</td>
<td>83.5</td>
<td>4.8a</td>
<td>87:11</td>
</tr>
<tr>
<td>C-1'</td>
<td>69.1</td>
<td>2.9a</td>
<td>88:12</td>
</tr>
<tr>
<td>C-4'</td>
<td>214.9</td>
<td>5.3b</td>
<td>84:16</td>
</tr>
<tr>
<td>C-6'</td>
<td>71.9</td>
<td>1.2b</td>
<td>—</td>
</tr>
<tr>
<td>C-8'</td>
<td>174.5</td>
<td>3.9c</td>
<td>82:18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.3c</td>
<td>90:11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.8d</td>
<td>88:12</td>
</tr>
</tbody>
</table>

* For experimental conditions see ref. 6. a Enriched by sodium [1,13C, 18O2]acetate. b Enriched from 18O; unlabelled andilesin A added as internal reference. c Enriched from ethyl [13C,18O]-3,5-dimethylorsellinate (7).
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References
COMMUNICATIONS

Limitations of $^{12}$C Labeling in $^{13}$C NMR Studies

In two recent papers (1, 2) the use of $^{12}$C labeling in biosynthetic (1) and mechanistic (1, 2) applications of $^{13}$C NMR has been advocated: in a $^{12}$C-labeling study, the missing rather than the enhanced lines would be identified in the final $^{13}$C spectrum. The advantages of $^{12}$C over $^{13}$C labeling have been stressed, particularly the much reduced cost of $^{12}$C-enriched materials compared to $^{13}$C-enriched but also the lack of complications from $^{13}$C-$^{13}$C coupling and the potential hazard to man of elevated $^{13}$C concentrations in metabolic studies. However, these comparisons are not entirely valid.

In a mechanistic study, to obtain a significant result when using $^{12}$C labeling, complete, or nearly so, elimination of the $^{13}$C atoms at the position of interest must be achieved; thus a precursor with effectively 100% $^{12}$C at the requisite position is required. However to achieve the equivalent result with $^{13}$C labeling, i.e., 100% enhancement of a line intensity as opposed to 100% elimination of intensity, only an extra 1.1% $^{13}$C over natural abundance at that position is required. Therefore, the cost of material containing only 2.2% $^{13}$C is the relevant comparison and this must compare favorably with 100% $^{12}$C-labeled material. In addition the $^{13}$C-enrichment experiment is inherently more sensitive with a consequent saving in spectral accumulation costs, and, at this low concentration, $^{13}$C-$^{13}$C coupling complications do not arise.

In biosynthetic studies using $^{13}$C NMR, the important factor is the change in $^{13}$C abundance. For this we must consider the percentage of atoms at a given position in a metabolite that have been derived from or replaced by atoms from the added precursor. The table (Table 1) shows the change in $^{13}$C abundance for a range of incorporations of

<table>
<thead>
<tr>
<th>Replacement (%)</th>
<th>$^{12}$C added 1.11%</th>
<th>$^{13}$C added 1.11%</th>
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<td>0</td>
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<td>50.56</td>
</tr>
<tr>
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<td>0.00</td>
<td>100</td>
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</tbody>
</table>

* Natural abundance = 1.11%.
$^{13}$C- and $^{13}$C-labeled precursors. It is clear that only replacement values of greater than 50% will give really significant results for $^{13}$C, as against 1% for $^{12}$C. Incorporations of this order have been achieved in a few cases, e.g., proline incorporation into prodigiousin (3), but as a rule replacement values of less than 10% are much more typical due to metabolic pools, precursor uptake and product dilution factors. Thus apart from a few very favorable cases, the use of $^{13}$C labeling in biosynthetic studies is impractical and, even in these cases, very low concentrations of $^{13}$C will give equally significant results, thus negating the cost factor.

Similar arguments apply to metabolic studies: $^{12}$C labeling is likely to be useful in mass spectral studies but as regards $^{13}$C NMR studies it is unlikely that the extra 1.1% $^{13}$C required in a metabolite to obtain the result equivalent to that with $^{12}$C labeling will present a significant risk.

Nevertheless, $^{12}$C-enriched materials are available at an acceptable cost and will almost certainly find a use, particularly in mechanistic studies. But in these the cost factor need not be as great as has been suggested and should not be a deterrent to using $^{12}$C in mechanistic studies.

REFERENCES


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CARBON-13 NUCLEAR MAGNETIC RESONANCE IN BIOSYNTHETIC STUDIES

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Carbon-13 Nuclear Magnetic Resonance in Biosynthetic Studies

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1 Introduction

Recent years have seen $^{13}$C n.m.r. grow from a relatively obscure technique to one rivalling $^1$H n.m.r. in utility and scope. The theory of $^{13}$C n.m.r.,$^1,2$ biological$^3$ and biochemical$^4$ applications, and early biosynthetic work$^5,6$ have been reviewed, two general texts$^7,8$ and a compilation of spectra$^9$ have appeared, and the $^{13}$C n.m.r. spectra of a wide range of compounds have been assigned.$^{10}$

Although radio-isotopes, particularly $^{14}$C, have proved extremely useful in biosynthetic studies, they have one severe disadvantage in the necessity of carrying out extensive degradations to locate the incorporated isotope in a metabolite. This is often very difficult owing to formidable structural complexities or the presence of carbon atoms in an unreactive aromatic framework so that only a partial analysis is obtained. Furthermore, since in many cases chemical degradations are no longer necessary as structure proofs for natural products, their use for establishing $^{14}$C-labelling patterns becomes a tiresome exercise. However, since $^{13}$C n.m.r. is itself an integral component of structure elucidation, its use in biosynthetic studies is very attractive and allows the establishment of labelling patterns without recourse to extensive chemical degradations.

The aim of this review is to discuss the methodology of $^{13}$C n.m.r. biosynthetic studies, both as an aid to evaluating the fast-expanding literature and to the undertaking of these studies, with emphasis on recent developments and applications.

1 J. B. Stothers, Quart. Rev., 1965, 19, 144.
3 J. B. Grutzner, Lloydia, 1972, 35, 375.
Carbon-13 Nuclear Magnetic Resonance in Biosynthetic Studies

2 Proton Satellite Method
Before discussing $^{13}$C n.m.r., brief mention must be made of the proton-satellite method. Spin-spin coupling between a $^{13}$C nucleus and directly bound protons results in satellite bands appearing on either side of the main proton signal in the $^1$H n.m.r. spectrum, so providing an indirect probe for monitoring the $^{13}$C abundance at a given position in a molecule. Any incorporation of a $^{13}$C-enriched precursor can be detected by the increase in intensity of these satellites. Sepedonin, griseofulvin, fusaric acid, picrocidin A, mollisin, and variotin have all been studied by this method. However, despite its advantage of only requiring readily available instrumentation, the method has severe limitations as only carbons with attached protons can be studied, and often complex $^1$H spectra, traces of impurities, and spinning side-bands obscure the satellites. These limitations are overcome in the direct $^{13}$C n.m.r. method.

3 Carbon-13 Nuclear Magnetic Resonance
The theory of $^{13}$C n.m.r. has been thoroughly reviewed so a brief introduction only is given here, a working knowledge of $^1$H n.m.r. being sufficient to understand $^{13}$C n.m.r. Table 1 compares the relevant properties of $^{13}$C and $^1$H and it may be seen that $^{13}$C, a stable isotope, natural abundance 1.1% has a nuclear spin 1/2 and so is n.m.r. active and will show the same general splitting patterns as $^1$H. As may be deduced from Table 1, the main difficulties in obtaining a $^{13}$C n.m.r. spectrum are its low abundance and low nuclear sensitivity, so that for a given sample $^{13}$C is 6000 times less sensitive than $^1$H. It is the development of techniques to overcome this that has led to the huge growth in $^{13}$C studies. These include

(i) the use of large samples, which became possible with the development of high-stability spectrometers taking up to 15 mm diameter n.m.r. tubes;
(ii) multiscan techniques, initially multiple accumulation in the continuous-

| Comparison of nuclear properties of $^1$H and $^{13}$C |
|-----------------|-----------------|
| $^1$H            | $^{13}$C         |
| Nuclear spin     | 1/2             |
| Resonance frequency at 23.5 kG | 100 MHz | 25.2 MHz |
| Natural abundance (%) | 99.99 | 1.11   |
| Relative sensitivity | 1.00 | 0.016  |
| Normal chemical shift range for organic molecules | 10 p.p.m. | 200 p.p.m. |

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wave mode but subsequently pulsed Fourier transform (FT) n.m.r.\textsuperscript{15} which enables much faster spectral determination than conventional methods;

(iii) proton-noise-decoupling, providing two gains in sensitivity. By applying a wideband proton-decoupling frequency the diffuse multiplets arising from $^{13}$C–H spin–spin coupling are collapsed to a single sharp line; this also induces a nuclear overhauser effect (NOE) leading to an intensity enhancement up to three-fold, due to disturbance of the $^{13}$C energy-level populations for carbon atoms with attached protons.

Owing to the wide range of chemical shifts observed for $^{13}$C nuclei, even complex molecules generally give $^{13}$C n.m.r. spectra in which every carbon has a discrete resonance. Much $^{13}$C n.m.r. assignment data has been accumulated; nevertheless complete assignment of resonances to a new molecule requires great care, and often considerable effort, especially in biosynthetic studies where the conclusions can only be as good as the original spectral assignments. Several aids to assignment are available:

(a) **Known chemical shifts and substituent chemical shift effects.** The chemical shifts of different functional types fall into well-defined ranges:\textsuperscript{7,8} carbonyl carbons resonate at low field (ca. 200 p.p.m.), aromatic and olefinic carbons at 160–100 p.p.m., aliphatic carbons with electronegative substituents at 50–80 p.p.m., and simple aliphatics at highest field 10–30 p.p.m. Substituent effects are generally found to be additive and rules for predicting chemical shifts in hydrocarbons\textsuperscript{18} and benzenoid aromatics\textsuperscript{7} are available.

(b) **Off-resonance and specific proton decoupling.** In the proton noise-decoupled spectrum all $^{13}$C–H coupling information is lost. In the off-resonance decoupling experiment, the $^1$H irradiation is kept at high power levels but the centre frequency is moved ca. 500 Hz away from the protons being irradiated so that one-bond $^{13}$C–H coupling patterns return, and the non-protonated, methine, methylene, and methyl carbons are observed as singlets, doublets, triplets, and quartets respectively. The observed or residual couplings, $J_{rs}$, are smaller than the actual one-bond coupling and are a function of the actual coupling, $J$, the decoupling power, $H$, and the decoupler offset $\Delta \nu$:\textsuperscript{10}

$$ J_{rs} = J \Delta \nu / H $$

If $J$ is known the residual coupling can be an aid to assignment. In the study of asperlin (1), a metabolite of *Aspergillus nidulans*, C-4, C-5, C-6, and C-7 all have one attached proton and are oxygen-bearing and so appear in the range 55–80 p.p.m. and give doublets in the off-resonance spectrum. However, comparison of the observed and calculated residual couplings allowed an unambiguous assignment to be made and confirmed the incorporation pattern of sodium $[2-^{13}$C]acetate shown.\textsuperscript{20}
Carbon-13 Nuclear Magnetic Resonance in Biosynthetic Studies

If the $^1H$ n.m.r. spectrum has been fully or partially assigned, the $^1H$ and $^{13}C$ resonances can be interrelated by single-frequency decoupling with the decoupler set exactly on a specific $^1H$ frequency. The attached carbon appears as a singlet in the $^{13}C$ spectrum whereas the remaining carbons show off-resonance patterns. This process becomes tedious if several resonances require studying but can be overcome by plotting the line frequencies in the $^{13}C$ spectrum as the $^1H$ irradiating frequency is stepped through the $^1H$ n.m.r. spectrum. Where the lines cross gives the point where $J_{CH}$ is zero, and hence the $^1H$ and $^{13}C$ frequencies can be correlated. Figure 1 illustrates the results obtained for NAD$^+$ (2).

(c) **Lanthanide-induced shift studies.** These are not as useful in $^{13}C$ studies as in $^1H$ n.m.r. because the actual shifts are of the same absolute value in both and so are relatively small in $^{13}C$ n.m.r., as are solvent and anisotropy effects. However, they can be useful for separating overlapping resonances. In complicatic acid (3), the resonances due to C-3 and C-10 both occur at 46 p.p.m. However, addition of $[\text{Eu(fod)}_3]$ separated these resonances and showed that C-3 but not C-10 was enriched from $[1-^{13}C]$acetate-enriched cultures of *Stereum complicatum.*

Another important use of shift reagents is to resolve the $^1H$ n.m.r. spectrum prior to specific proton-decoupling studies on the $^{13}C$ n.m.r. spectrum.

(d) **Model and derivative studies.** Model compounds whose chemical shifts are known can be helpful in assigning the spectrum of a new compound, though they must be used with care. Quaternary carbons present the greatest difficulties in assignment, and studying the variation of chemical shifts in a series of closely related compounds may be the only method of reaching an unambiguous assignment. The $^{13}C$ n.m.r. spectrum of tajixanthone (5), a prenylated xanthone metabolite of *Aspergillus variecolor*, was fully assigned by a study of eleven derivatives. Subsequent incorporation of $[1-^{13}C]$- and $[2-^{13}C]$-acetates indicated its biogenesis by prenylation and cleavage of an anthrone precursor via the known co-metabolite arugosin (4).

(e) **Synthesis** of a compound enriched at a known site with $^{13}C$ has been used for


Figure 1  Plot of peak frequencies in the $^1$H off-resonance selectively decoupled $^{13}$C spectra of NAD$^+$ as a function of the position of irradiation in the $^1$H spectrum, expressed in p.p.m. to high frequency of internal dioxan. The position of the peaks in the $^1$H noise-decoupled $^{13}$C spectrum are shown by lines on the ordinate and the position of the proton peaks by lines of the abscissa. The arrows ↑ indicate the point of collapse of the $^{13}$C doublet and the connection between a given $^{13}$C peak and the assigned proton peak. Small doublet splittings are observed on some of the signals from long-range (C-H) spin couplings.

![Diagram](image-url)
Carbon-13 Nuclear Magnetic Resonance in Biosynthetic Studies

An exact assignment of the meso-carbons of protoporphyrin IX (6) was required prior to biosynthetic studies. This was accomplished by synthesis of protoporphyrins enriched specifically at the \( \beta \), \( \gamma \), and \( \delta \)-meso positions respectively.\(^\text{25}\)

(f) Partially relaxed Fourier transform (PRFT) n.m.r. The \( T_1 \) relaxation times for \( ^{13}\text{C} \) atoms generally increase in the sequence methylene, methine, methyl, and quaternary carbon, so as the pulse internal time, \( \tau \), is increased the negative peaks obtained in PRFT for short values invert in the sequence above. This can be of great value in assigning resonances, especially in congested spectra where off-resonance may be of limited value.\(^\text{26}\)

(g) Incorporation studies. \( ^{15}\text{N} \) is an isotope of spin \( \frac{1}{2} \) and so is n.m.r. active and will couple with \( ^{13}\text{C} \) nuclei. Thus a metabolite grown in the presence of, say, K\(^{15}\text{NO}_3 \) will exhibit \( ^{15}\text{N}-^{13}\text{C} \) couplings for any carbons bonded to nitrogen. Similarly, any compound with adjacent \( ^{13}\text{C} \) nuclei will exhibit a \( ^{13}\text{C}-^{13}\text{C} \) coupling between these nuclei. Both these points are illustrated below.

4 Biosynthetic Methodology

The availability of \( ^{13}\text{C} \)-enriched compounds has increased rapidly and a wide range, similar to that for \( ^{14}\text{C} \), is now available at enrichments of up to 95% and many others may be readily synthesized by standard methods from simple precursors such as \( ^{13}\text{CO}_2 \), \( ^{13}\text{CH}_3 \), and K\(^{13}\text{CN} \).

A. Precursor Incorporation.—Precursor efficiency may be assessed in several ways\(^\text{27} \) but for \( ^{13}\text{C} \) studies the important criterion is dilution of added label. For \( ^{14}\text{C} \), dilution per labelled site is given by:

\[ \text{dilution per labelled site} = \frac{\text{total activity}}{\text{activity in label}} \]


To obtain unequivocal results in $^{13}$C studies using 90% enriched precursors, dilutions per labelled site of ca. 100 or less are required. This is due to inherent errors in $^{13}$C n.m.r. resonance intensities (see below), requiring a two-fold increase in $^{13}$C abundance to be certain that enrichment has occurred. Relatively large amounts of precursor, typically 1–20 mmol l$^{-1}$, have to be used to obtain this, especially for low precursor efficiencies. This introduces problems of expense and interference with normal metabolism; in contrast to $^{13}$C studies, non-tracer amounts are now being used. A lowering of metabolite yields is common and cases of toxicity have been reported for elevated concentrations of acetate (0.2 g 1$^{-1}$) and propionate (0.2 g 1$^{-1}$), and mevalonate (0.1 g 1$^{-1}$). However, in other cases, higher concentrations have been used successfully, e.g. 2 g 1$^{-1}$ of acetate, and the problem can often be overcome by pulsed feedings of precursor.

Preliminary experiments with $^{14}$C-labelled precursors are generally carried out to ascertain the feasibility of $^{13}$C studies and to optimize conditions. Three main parameters require studying: time of precursor addition, incubation time, and amount of precursor. Maximum precursor incorporation usually occurs with addition of precursor at the start of maximum metabolite production, i.e. the start of the idiophase in microbial fermentations, necessitating the determination of growth and production curves. Incorporation may be very sensitive to time of addition. Figure 2 illustrates the marked variation of incorporation with day of addition of mevalonolactone into the sequiterpenoid trichothecins.

The period of growth after addition of precursor may also be critical. The variation in dilution of $[^{14}$C$]$acetate on incorporation into sepedonin (7) is shown in Figure 3, which illustrates that neither maximum yield of metabolite nor even maximum total incorporation of label is the important factor, the prime consideration being minimum dilution of label given a sufficient yield of metabolite for $^{13}$C spectral determination.

Finally, mass versus incorporation studies will determine the minimum amount of precursor that must be added to obtain a satisfactory enrichment. Table 2 shows the variation of dilution with amount of added $[^{14}$C$]$acetate during biosynthetic studies on shanorellin (8) in Shanorea spirotrichi. Incorporation of $[^{13}$C$]$acetate and $[^{13}$C$]$methionine indicated its origin from a tetraketide with the methyls derived from the C3-pool.

B. Interpretation and Presentation of Results.—Having obtained a $^{13}$C-enriched metabolite as above, the results from the $^{13}$C n.m.r. spectrum must be evaluated.

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89 J. R. Hanson, T. Marten, and M. Siverns, J.C.S. Perkin I, 1974, 1033.
Table 2 Incorporation of radioactivity from [1-14C]acetate into shanorellin

<table>
<thead>
<tr>
<th>mmol Acetate</th>
<th>μCi mmol⁻¹</th>
<th>mg</th>
<th>μCi mmol⁻¹</th>
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<td>0.5</td>
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<td>8.05</td>
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<td>21.1</td>
<td>27</td>
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<td>2.6</td>
</tr>
</tbody>
</table>

Figure 2 Variation of incorporation of [14C]mevalonic acid (MVA) into trichothecin with day of addition (Reproduced from J.C.S. Perkin I, 1974, 1033)
Figure 3  Yield and specific activity of sepedonin produced by cultures of S. chrysospernum administered [14C]acetate (9 mmol l⁻¹) on the 7th day after inoculation (Reproduced by permission from Canad. J. Biochem., 1969, 47, 945)

If high enrichments are obtained the labelled sites will be readily apparent by visual inspection of the spectra. In the case of radicimin (9), the enrichment was so high that only the labelled peaks were visible²² (Figure 4).

Incorporations are commonly given as percentage enrichments:

\[
\text{Percentage enrichment} = \left( \frac{\text{observed } ^{13}\text{C abundance}}{\text{natural } ^{13}\text{C abundance}} \right) - 1.1
\]

Very high enrichments, 5—60%, have been recorded but lower values, 0.5—5%, are more typical and can require more care in assessing, particularly at the lower values, because of the 'intensity problem'.

In \(^{13}\text{C}\) n.m.r. line intensities are non-integral owing to the variable NOE and widely varying relaxation times. With FT n.m.r. the interval between scanning pulses may be shorter than the relaxation times of individual \(^{13}\text{C}\) nuclei, resulting in differential amounts of saturation occurring and thus variable line intensities, particularly for quaternary carbons. Addition of a free radical or a paramagnetic species, e.g. chromium trisacetoacetonate, \([\text{Cr(acac})_3]\), can partly overcome the problem. This complex quenches the NOE and shortens the relaxation times to give more uniform line intensities and was used to advantage in

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studying the biosynthesis of helicobasidin (10) in *Helicobasidium mompa*. 

[2-13C]Mevalonate was expected to label C-4 and C-12, with the remaining label distributed equally between C-8 and C-10 owing to tautomerism of the dihydroxy-quinone system. However, in the resultant spectrum, the large variations in intensity made the labelling of C-8 and C-10 uncertain, but from the spectrum in the presence of 0.1 mol l⁻¹ [Cr(acac)₃] the equal labelling of C-8 and C-10 was apparent (Figure 5).

A second method uses gated decoupling. In this, the NOE is eliminated by switching off the ¹H noise decoupling frequency during the interval between scanning pulses. This method was used to study the incorporation of [2-¹³C]acetate into asperentin (11) by *Aspergillus flavus*. The saturation problem can be eliminated by a sufficiently long delay between the scanning pulses, but as some ¹³C relaxation times are very long a compromise has to be made in practice to maintain reasonable spectral acquisition times.

Ultimately the only reliable method is a direct comparison of the respective line intensities in the natural-abundance and enriched spectra; both sets of intensities are subject to identical NOEs and relaxation considerations which should cancel out provided both spectra are standardized. This can be achieved by using identical concentrations and instrument parameters but a more convenient technique is to normalize both spectra to a reliable standard. If the material is derivatized before spectral acquisition, as with asperentin (11) as the dimethyl ether or neomycin (see below) as the hexa-acetate, all the remaining line intensities can be normalized to the average value of the intensities of the introduced methyl groups in each spectrum. Correction for the difference of these averages in the respective spectra then allows direct comparison of indi-

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Figure 5. $^{13}$C N.m.r. spectra of helicebaxadin from (a) [2-$^{13}$C]mevalonate, (b) [2-$^{14}$C]-acetate, (c) [1-$^{13}$C] acetate, and (d) at natural abundance, all in the presence of 0.1 M-[Cr(acac)$_3$]$_x$, and (e) at natural abundance alone (Reproduced by permission from Tetrahedron Letters, 1973, 4723).

Individual intensities. However, derivatization is not always feasible. Incorporation of [2-$^{13}$C]mevalonate was used to distinguish between the two possible foldings of the farnesyl pyrophosphate precursor of trichothecalane (12) (Scheme 1) as $^{14}$C studies provided conflicting evidence.$^{29}$ Incorporation of label at C-4 and C-8 rather than at C-10 indicated biosynthesis via path (b). The natural-abundance and enriched spectra were normalized to the line intensity of C-12 which was assumed to be unlabelled. This was not an ideal choice as, being one of the least intense peaks in the spectrum, it is most sensitive to errors. A more general method using all the unlabelled peaks in the spectrum for normalization has been proposed.$^{24}$
However, despite these operations, there remains for FT n.m.r. spectra an uncertainty in line intensities due to the digitization of data during spectral accumulation and transformation so that a series of experiments run on the same sample under apparently identical spectrometer parameters can give intensities that vary by ±20%. This means that enrichments of less than 0.5% must be regarded with caution in the absence of supporting data such as multiple spectral determinations, proton-satellite enrichments, or $^{13}$C-$^{13}$C coupling. The problem is alleviated by the use of larger data-storage facilities but these are expensive and not always readily available.

5 Further Biosynthetic Studies

A large variety of metabolite types have been studied, several having been mentioned above. Further examples are discussed below.

Lasocolic acid (13) contains three unique C-ethyl groups. $^{14}$C Studies failed to establish the origin of these groups but addition of sodium $[^{1-13}$C]butyrate to the culture established their butyrate origin. Incorporation of $[^{1-13}$C]propionate confirmed the origin of the C-4, C-10, C-12, and C-16 methyls and $[^{1-13}$C]acetate that of the C-23 methyl.

Considerable attention has been given to the origin of the ANSA chain in the

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rifamycins and related antibiotics. Incorporation of \([1-^{13}C]\), \([2-^{13}C]\), and \([3-^{13}C]\)-propionates and of \([1-^{18}C]\) and \([2-^{18}C]\)-acetates into rifamycin S (14) by \textit{Nocadia mediterranei} confirmed the origin of the ANSA chain from eight propionate and two acetate units, linked in a clockwise manner\(^{18}\) (Scheme 2),

![Scheme 2]

since \([1-^{13}C]\)acetate enriches C-17 rather than C-19. The exact assignment of the C-17 and C-19 \(^{18}C\) resonances was crucial here and was achieved by specific proton decoupling.\(^{40}\) Similar findings are reported for the related streptovaricins.\(^{41}\) The origin of the \(	ext{C}_7\text{N}\) moiety, C-1 to C-4 and C-8 to C-10, is obscure, but the enrichment of C-1 and C-10 of rifamycin S on incorporation of \([1-^{13}C]\) glucose has been reported,\(^{42}\) providing the first direct evidence for the origin of this moiety, also found in the mitomycins, validamycins, and kinamycins. In this study the first use of \([2-^{13}C]\) malonate is reported; C-5 and C-18 are enriched but, interestingly, not the C-25 acetoxy-group.

Incorporations of both \(^{13}C\)-labelled acetate and mevalonate into terpenes have been reported. Studies on heliocobasidin, complicatic acid, and trichothecalone have been discussed above; incorporation of \([^{13}C]\)acetate into the virescenesides,\(^{43}\) fusidic acid,\(^{44}\) and \textit{Acrostolagmus} 5 lactone have also been reported.

The neomycins [e.g. (15)] are antibiotic metabolites of \textit{Streptomyces fradiae}. The lack of crystalline derivatives and satisfactory degradations hampered \(^{13}C\) biosynthetic studies but incorporation of \([1-^{13}C]\)glucosamine and \([6-^{13}C]\)glucose led to the labelling pattern shown in Scheme 3.\(^{45}\) The preferential incorporation

of glucose rather than glucosamine into the deoxystreptamine residue (b) was unexpected and necessitated the proposal of a new pathway for deoxystreptamine biosynthesis. It and related amino-cyclitols are important constituents of several antibiotics.\textsuperscript{47}

Incorporations of [\textsuperscript{12}C]-labelled acetates and valines into cephalosporin C (16) are as expected from \textsuperscript{14}C studies. The higher degree of labelling with [2-\textsuperscript{13}C] acetate of C-14 compared with C-11, C-12, and C-13, which were similar, suggested the formation of the \(\alpha\)-aminoadipyl side-chain from \(\alpha\)-ketoglutaric acid and acetyl coenzyme A.\textsuperscript{48} The stereoselective incorporation of \textsuperscript{13}C in (2RS,3R)-[4-\textsuperscript{13}C]valine into C-2\textsuperscript{49} and of (2RS,3S)-[4-\textsuperscript{12}C]valine into C-17 of (16)\textsuperscript{50} has been demonstrated. The (3R)-isomer specifically labels the \(\beta\)-methyl group of penicillin V (17) in \textit{Penicillium chrysogenum}\textsuperscript{51} and the (3S)-isomer labels the \(\alpha\)-methyl group.\textsuperscript{52}

\begin{equation}
\text{Scheme 3}
\end{equation}

\begin{equation}
\text{Scheme 4}
\end{equation}

\textsuperscript{47} W. T. Dobranzki, \textit{Chem. in Britain}, 1974, 10, 386.


Carbon-13 N.m.r. has been used extensively in biosynthetic studies of porphyrins and vitamin B₁₂ (18). Early work on the incorporation of ¹³C-labelled porphobilinogen, 8-amino-laevulinic acids, and Urogens I—IV has been reviewed. More recently, three groups have independently shown that seven of the eight methyl groups of vitamin B₁₂ are enriched by feeding [¹³C]methionine to Propionibacterium shermanii. The methyl group not enriched is one of those at C-12. Battersby has suggested this to be the 12β; degradation of vitamin B₁₂ gives the imide (19) in which the methyl ¹H n.m.r. resonances have been assigned.

Only the signal due to the 12α-methyl showed enhanced ¹³C satellites. Scott is in agreement with this, arguing that the C-2, C-7, C-12α, and C-17 methyls should have similar chemical shifts owing to the γ-effect of the syn-propionate side-chain whereas that at C-12β, lacking this, should be shifted downfield. Good support was obtained for this analysis from the ¹³C n.m.r. spectrum of the enriched vitamin B₁₂ after epimerization at C-13 when one of the enriched signals, presumably the C-12α, moved downfield by ca. 12 p.p.m. Shemin and Katz have reached the opposite conclusion using specific proton decoupling to correlate the enriched ¹³C resonances with the ¹H resonances. However, doubt has been cast on the crucial ¹H assignments in this case.


E. McDonald, Ann. Reports (B), 1974, 70, 597.
$^{13}$C Studies clearly indicate that the mechanism of pyrrole-ring formation in prodigiosin (20) is unrelated to that operative in the porphyrins and so is of special interest. A complete assignment of the $^{13}$C spectrum of prodigiosin has been made, enabling $^{13}$C biosynthetic studies to be carried out. $^{13}$C-Labelled acetates, alanine, proline, glycine, and serine have been incorporated by cultures of *Serratia marcescens* (Scheme 5), allowing a biosynthetic path to be proposed.

Recently metacycloprodigiosin and undecyiprodigiosin have been isolated, and $^{13}$C studies indicate a similar biosynthesis.

Specific proton decoupling and PRFT methods have been used to assign the $^{13}$C n.m.r. spectra of cytochalasin B (21) and cytochalasin D (22). Incorporation of sodium [1-$^{13}$C]- and [2-$^{13}$C]-acetate confirms previous proposals of biosynthesis of the cytochalasins from phenylalanine, methionine, and a C18 or C16 polyketide (Scheme 6).

$^{6}$ $^{13}$C-$^{13}$C Spin–Spin Coupling

In natural-abundance $^{13}$C n.m.r. spectra $^{13}$C-$^{13}$C spin–spin coupling is not observed as the probability of $^{13}$C nuclei being adjacent is equal to the square of the natural abundance, giving satellites of 0.55% of the intensity of the main signal. However, in enriched material the probability is much higher and the detection of a $^{13}$C-$^{13}$C coupling can provide conclusive evidence that two labels have been incorporated into adjacent positions in a molecule.

A. Singly Labelled Precursors.—A $^{13}$C-$^{13}$C coupling can arise from administration of singly labelled precursors in a variety of ways.

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Molecular rearrangement of a biosynthetic intermediate may give rise to a $^{13}$C-$^{13}$C coupling. The pyrone (23), a metabolite of *Aspergillus quercinus*, when enriched from $[2-^{13}$C$]$acetate shows a coupling of 61 Hz between C-2 and C-7 (Figure 6b). This coupling probably arises from an intramolecular rearrangement of the precursor polyketide chain (Scheme 7). A similar coupling from head-to-head linkage of acetate units is observed on incorporation of $[2-^{3}$C$]$acetate into sterigmatocystin (24).61

Folding of a pentaketide chain to give a five-membered ring results in a $^{13}$C-$^{13}$C coupling between C-5 and C4a of dihydroalticulidin (25) enriched from $[2-^{13}$C$]$acetate.62 Folding of a terpenoid chain enriched from $[1-^{13}$C$]$acetate gives rise to a coupling between C-1 and C-5 in heliobasidin, (Figure 5), and between C-8 and C-14 in fusidic acid (26). The head-to-head linkage of farnesyl units, via squalene, gives rise to the coupling observed between C-11 and C-12.44

Conversion of $[2-^{13}$C$]$acetate into succinate in the Krebs cycle results in a

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Figure 6 ¹H Noise-decoupled ¹³C n.m.r. spectra of pyrone (23) from (a) CH₁³CO₂Na, (b) ¹³CH₃CO₂Na, and (c) ¹³CH₃¹³CO₂Na

¹³C-¹³C coupling between C-11 and C-15 in avenaciolide (27). Similar metabolic transformations give rise to ¹³C-¹³C couplings when [2,¹³C]glycine is used.

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Scheme 7

incorporated via serine into prodigiosin and [5-13C]-δ-aminolaevulinic acid is incorporated into vitamin B12 via porphobilinogen. 64

B. Doubly Labelled Precursors.—This has been without doubt one of the most important developments in biosynthetic studies in recent years. Most studies have used [1,2-13C]acetate in which both the carboxyl and methyl carbons are highly enriched. This means that with this precursor all acetate-derived atoms are

labelled and adjacent atoms derived from incorporation of an intact acetate unit will exhibit a $^{13}\text{C}-^{13}\text{C}$ coupling. Generally, no coupling will be observed between adjacent units owing to the low probability of more than one added precursor unit being incorporated into any one metabolite molecule. This coupling can be of great use in structural and spectral assignment studies in addition to providing biosynthetic information.

The first application of this technique was to dihydrolatumicidin (25). On incorporation of $[1,2-^{13}\text{C}]$acetate all 10 carbons exhibited $^{13}\text{C}-^{13}\text{C}$ couplings, confirming its origin from five acetate units. Feeding of a 50:50 mixture of $[1-^{13}\text{C}]$acetate and $[2-^{13}\text{C}]$acetate gave rise to couplings for the carbon–carbon bonds between adjacent acetate units. Although four combinations between these singly labelled acetates are possible, only one ($\text{CH}_3^{13}\text{C}=\text{CO}^{13}\text{CH}_3^{13}\text{CO}$) gives the desired coupling. This latter technique requires high incorporations and so is of limited use. The size of the $^{13}\text{C}-^{13}\text{C}$ coupling, generally 30—90 Hz, is related to the hybridization of the atoms involved, increasing with increased $'y'$ character, and so in conjunction with chemical shift data is an important source of structural information.

Tenellin (28), a metabolite of Beauvaria sp., has been studied using $[1,2,^{13}\text{C}]$acetate to provide both structural and biosynthetic information. An interesting feature of this study was growth of the organism using K$^{15}\text{NO}_3$ as the sole nitrogen source, resulting in $^{13}\text{C}-^{15}\text{N}$ couplings on C-6 and C-2. $[^{13}\text{C}]$Methionine indicated the origin of the C-10 and C-12 methyl groups and feeding of $[1-^{13}\text{C}]$-

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and [2-13C]-phenylalanine proved the origin of the remainder of the molecule and indicated that a carboxy-carbon migration must take place during biosynthesis. A major advantage of doubly labelled precursors is in the elucidation of anomalous biosynthetic pathways. If the biosynthesis involves cleavage of an original acetate unit, the 13C-13C coupling is lost and the respective carbons now appear merely as enhanced singlets. When [1,2-13C]acetate is incorporated into pyrone (23), three of the nine carbons appear as singlets; the remaining six all exhibit 13C-13C coupling (Figure 6c), suggesting biosynthesis via cleavage of a pre-formed carbocyclic ring as in Scheme 7.

The lack of couplings on C-1 and C-11 of mollisin (29) enriched with [1,2-13C]acetate was interpreted in favour of a two-chain derivation as in path (b) of Scheme 8; path (a) had been suggested on the basis of proton satellite studies.

Scheme 8


with singly labelled acetate. However, cleavage of a single polyketide chain, path (c), would give similar results and cannot be excluded.

13C-13C Couplings from incorporation of [1,2-13C]acetate aided in the placing of the substituents on the tetronic acid ring of multicolic acid (30), a metabolite of *Penicillium multicolor*.68 The labelling pattern from [1-13C]- and [2-13C] acetate was not as expected from the normal pathway of fungal tetronic acid biosynthesis and suggested formation via ring cleavage of n-pentylresorcylic acid (31). The absence of couplings on C-1, C-3, and C-11 on feeding [1,2-13C] acetate confirmed this hypothesis.

\[
\begin{align*}
\text{CH}_2\text{CO}_2\text{Na} & \rightarrow \text{HO} \quad \text{HO} \\
\text{[o]} & \quad \text{HO} \quad \text{HO} \\
\text{(31)} & \quad \text{(30)}
\end{align*}
\]

Incorporation of [1,2-13C]acetate into ascochlorin (32) in *Nectria coccinea* resulted in only five 13C-13C couplings in the triprenyl side-chain, showing that the methyl from C-6 rather than from C-2 of mevalonate migrates during biosynthesis.59

\[
\begin{align*}
\text{CH}_2\text{CO}_2\text{H} & \rightarrow \text{HO} \quad \text{HO} \\
\text{(32)} & \quad \text{HO} \quad \text{HO}
\end{align*}
\]

It has been suggested that the cyclopentenol (33), a metabolite of *Periconia macrospina*, and related fungal cyclopentenones are formed by ring contraction of a benzenoid precursor.70 The incorporation of singly and doubly labelled [13C]acetate shows that this is the case, but the labelling pattern obtained indicates a different mechanism from that proposed.71

\[
\begin{align*}
\text{CH}_2\text{CO}_2\text{Na} & \rightarrow \text{Cl} \quad \text{Cl} \\
\text{Cl} \quad \text{Cl} & \quad \text{Cl} \quad \text{Cl} \\
\text{Cl} \quad \text{Cl} & \quad \text{Cl} \quad \text{Cl}
\end{align*}
\]

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The use of a doubly labelled precursor other than acetate is illustrated by the elegant work of Battersby on the biosynthesis of uroporphyrinogen III (35).[^1] [2,11-13C]-PBG (34) was synthesized and showed a long-range coupling of ca. 4 Hz. This was incorporated into (35) using a cell-free enzyme system from avian blood. Analysis of the resultant 13C n.m.r. spectrum showed three doublets, each of 5 Hz splitting, corresponding to the α-, β-, and δ-carbons, and one doublet of 72 Hz for the γ-carbon, indicating that PBG unit d must undergo an intramolecular rearrangement with respect to itself during biosynthesis.

To date, the only other doubly labelled precursor that has been employed is [4,5-13C]mevalonate. This was synthesized by Hanson and incorporated into the fungal sesquiterpenes cyclonerodiol (36a) and cyclonerotriol (36b). All three sets of 13C-13C couplings are retained, showing that mevalonate is incorporated without rearrangement.[^2]

7 Higher Plant Metabolites
All the above 13C studies have involved micro-organisms or partially purified enzyme systems. The main problems with higher plants are (a) the low incorporations generally obtained and (b) dilution of the labelled metabolite by unlabelled endogenous material, which can be so large that the 13C content of the isolated substance does not differ significantly from natural abundance despite good incorporation. Despite these difficulties, two successful studies have been reported.

[^2]: J. R. Hanson, personal communication.
Simpson

$^{14}$C-Labelling studies indicated that the lactone (37) was an intermediate in the biosynthesis of camptothecin (38) in *Camptotheca acuminata*.[74] Owing to the absence of suitable degradations, $^{13}$C n.m.r. was used to prove specificity of incorporation. $[1-{\textsuperscript{13}}C]$Tryptamine was synthesized from K$^{13}$CN and thereby the $[5-{\textsuperscript{13}}C]$-lactone (37), 38 mg of which was wick-fed to intact plants. After two days growth, 20 mg of camptothecin was isolated which showed an enhancement of only the C-5 resonance intensity of ca. 55%.

![Chemical structure of lactone (37) and camptothecin (38)](image)

$[1-{\textsuperscript{13}}C]$Autumnaline (39) has been synthesized and injected as the hydrochloride (300 mg) into seed capsules (1 mg per capsule) of *Colchicum autumnale* (autumn crocus). After two weeks growth 1.24 g colchicine (40) was isolated. The resultant $^{13}$C n.m.r. spectrum showed ca. 2.5-fold enrichment of the C-7 signal only.[75]

![Chemical structure of autumnaline (39) and colchicine (40)](image)

However, the general extension of $^{13}$C n.m.r. biosynthetic studies to higher-plant metabolites is likely to prove difficult. This may be alleviated by the use of cell-free enzyme systems in which the problems of penetration of precursors to the active site and dilution by endogenous material are eliminated. Work with $^{14}$C-labelled material indicates that the dilution values and yields obtainable make $^{13}$C studies feasible.[76] Similar considerations apply to studies using tissue cultures.

A second possibility is the use of $^{12}$C-labelled compounds completely flushed of $^{13}$C which are becoming available as a by-product of $^{13}$C-enrichment and so are

relatively inexpensive. Their use in mechanistic\textsuperscript{77} and biosynthetic studies\textsuperscript{78} has already been advocated; elimination of a peak intensity rather than enhancement would be observed. Though their direct use in biosynthetic studies is impractical owing to the high enrichments that would be required to obtain significant results,\textsuperscript{79} they may be of use. Scott has suggested growth of a plant from seed or tissue culture in an atmosphere of $^{12}\text{CO}_2$ which should ensure a very low (0.1 to 0.2\%$^{13}\text{C}$ content in the various pools of organic intermediates.\textsuperscript{80} Thus the $^{13}\text{C}$ 'natural abundance' is lowered by an order of magnitude, making subsequent $^{12}\text{C}$-enrichment studies possible. It is perhaps noteworthy at this stage that it is only the fact that $^{13}\text{C}$ natural abundance is so relatively low that makes any biosynthetic studies possible at all, despite making spectra difficult to determine.

8 Conclusions

Besides the examples given many other metabolites have been studied. These studies include the incorporation of $[^{13}\text{C}]$acetate into ochratoxin,\textsuperscript{81} palmitoleic acid,\textsuperscript{82} malicin,\textsuperscript{83} showdomycin,\textsuperscript{84} epoxydol,\textsuperscript{85} and thermoyzomocin,\textsuperscript{86} of $[^{12},2-{^{13}\text{C}}]$acetate into penicillicacid,\textsuperscript{87} ovallicin,\textsuperscript{88} sterigmatocystin,\textsuperscript{89} and bikaverin.\textsuperscript{90} of $[2-{^{12}\text{C}}]$invalonate into gibberellic acid,\textsuperscript{81} of DL-triptophan-[3-$^{12}\text{C}$]alanine into pyrrolinitril,\textsuperscript{92} and of $[1-{^{13}\text{C}}]$glycerate into rifamycin.\textsuperscript{93} There can be no doubt that the use of $^{13}\text{C}$ methods will continue to expand rapidly, perhaps even superseding $^{14}\text{C}$ in biosynthetic studies of micro-organisms.

The use of doubly labelled precursors makes simultaneous determination of structure and biosynthesis possible for the first time and makes possible studies where classical $^{14}\text{C}$ methods could not provide unequivocal answers.

The extension of the method to higher plants and metabolic studies in man where the risk attached to the use of radio-isotopes is removed, seems imminent.
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A Review of the Literature Published
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Biosynthesis of Polyketides

BY T. J. SIMPSON

1 Introduction

Polyketides form a large class of natural products possessing structures of great
diversity related by their common formation via the acetate–polymalonate biosyn-
thetic pathway.1 Acyl units other than acetate, such as propionate, benzoate, and
farnesylcinnamate, can act as chain-initiating species, and propionate and butyrate as chain
elongation units, so that a wide variety of fatty acids, polyacetylenes, single and
multiringed phenols, macrolides, flavonoids, and other compounds can be included
in this classification.

The literature appearing during 1975 and to mid-1976 is covered in this chapter. An
outstanding feature of this period has been the continued growth in 13C n.m.r. methods,
in particular the use of doubly labelled 13C-acetate. Analysis of the resultant
13C-13C
spin–spin couplings provides a powerful method for determining the manner in which
polyketide molecules are assembled on the enzyme surface before the first stable
compounds are released, and for probing subsequent molecular rearrangements and
cleavage pathways. The scope and methodology of the technique have been discussed
in recent reviews.2,3

2 Fatty Acids, Polyacetylenes, and Prostaglandins

Lynen has studied the condensation reaction in fatty acid biosynthesis using dideu-
terio malonyl-CoA.4 No primary isotope effect was observed on the reaction velocity
of the yeast-enzyme-catalysed fatty acid synthesis, in which the rate-limiting step is
the condensation, or on the condensation itself, studied separately using the β-
ketoseryl-(acyl-carrier-protein) synthetase of Escherichia coli. When the condensation

Scheme 1

Biosynthesis was carried out in the presence of tritiated water, no tritium was incorporated into the product. These results exclude condensation mechanisms involving acylation of a malonyl carbanion and indicate a concerted mechanism, (Scheme 1).

2,4,6,8-Tetramethyldecanoic acid (1) is the major fatty acid in the uropygial gland of the goose.\(^5\) Crude cell-free extracts from the gland catalyse the carboxylation of propionyl-CoA but not of acetyl-CoA, whereas more highly purified extracts catalyse both carboxylations. This behaviour was explained by the isolation of a highly specific malonyl-CoA decarboxylase from the crude extracts. Thus acetyl-CoA and methylmalonyl-CoA are respectively the major chain-primer and elongation agents present in the gland, resulting in the production of the multi-branched fatty acid. Propionate incorporated during chain elongation has been shown to be the branching methyl group donor in biosynthesis of 3- and 13-methylpentacosane (2) and (3), the major cuticular hydrocarbons in the cockroaches _Periplaneta americana_ and _P. fuliginosa_, respectively.\(^6,7\) In plants, n-alkanes are formed by an elongation of fatty acids followed by decarboxylation, and the 2- and 3-methylalkanes originate from the appropriately branched starter acyl-CoA derived from valine and leucine,\(^8\) whereas in algae the active methyl group from methionine serves as the branching methyl group donor.\(^9\) Cell-free preparations from pea leaves, _Pisum sativum_, catalysed the decarboxylation of n-dotriacontanoic acid (4), requiring the presence of both ascorbic acid and oxygen, and giving both n-C\(_{31}\) and n-C\(_{30}\) alkanes. Thus decarboxylation and \(\alpha\)-oxidation appear to be connected processes; in confirmation, 2-hydroxydotriacontanoic acid, the intermediate in \(\alpha\)-oxidation of (4), was converted into the same two alkanes.\(^10\)

\[
\begin{align*}
(1) & \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{CO}_2\text{H} \\
(2) & \quad \text{Me(CH}_2)_2 \text{CHCH}_2\text{Me} \quad \text{Me(CH}_2)_2 \text{CH(CH}_3)_1 \text{Me} \\
(3) & \quad \text{Me(CH}_2)_3 \text{CH}_2 \text{Me} \quad \text{Me(CH}_2)_2 \text{CH(CH}_3)_1 \text{Me} \\
(4) & \quad \text{Me(CH}_2)_3 \text{CO}_2\text{H} \\
(5) & \quad \text{CO}_2\text{H} \\
(6) & \quad \text{CH}_2\text{CH}_2\text{CO}_2\text{H}
\end{align*}
\]

When [1-\(1^4\)C]aleprolic acid (5) was supplied to leaves and seeds of plants belonging to the Flacourtiaceae, and also to whole cells of _Chlorella vulgaris_, cyclopentenyl fatty acids (occurring naturally in seeds and leaves of Flacourtiaceae) were synthesized.\(^11\)

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suggesting that these fatty acids are formed by elongation of aleprolic acid rather than by cyclization of an acyclic fatty acid precursor. Both the availability of aleprolic acid and the ability to use it as a primer for fatty acid synthesis appear as specific characteristics of the Flacourtaceae and thus jointly determine the fatty acid pattern. The main fatty acids in ten strains of acidophilic, thermophilic bacteria isolated from Japanese hot springs are 6-cyclohexyl fatty acids, e.g. (6). Increasing the concentration of glucose in the culture medium increased the production of the cyclohexyl but not the acyclic acids, and from incorporation studies with 14C- and 2H-labelled glucose it was confirmed that the acids are produced by elongation of cyclohexylcarboxylate derived from shikimate, rather than by elongation of cyclohexylpropionate derived from decarboxylation of prephenate. The results are in full agreement with previous studies on cyclohexyl fatty acids from Bacillus acidocaldarius, in which cyclohexylcarboxylate competes with straight- and branched-chain precursors of similar molecular length to determine the fatty acid spectrum. These acids may be the precursors of the n-alkylcyclohexanes present in a number of sediments and crude oils, previously postulated as arising by intramolecular cyclization of unsaturated fatty acids.

Several papers have appeared on the routes to unsaturated fatty acids. Stumpf and co-workers have shown that preparations from safflower seeds and avocado mesocarp rapidly desaturate stearyl-ACP to oleic acid in the presence of oxygen. Mazliak et al. on the other hand have shown that fractions from a cauliflower homogenate synthesize radioactive oleic acid by an aerobic process from [14C]decanoate, in the presence of ATP, NADPH, Coenzyme A, and oxygen. They proposed a scheme analogous to oleic acid synthesis in anaerobic bacteria, which hardly accounts for the oxygen requirement (Scheme 2): 3-hydroxylauric acid (7) formed from decanoate undergoes β,γ-dehydration to 3-dodecenoic acid which is then elongated to oleic acid (8).

\[
\begin{align*}
\text{(7)} & \quad \text{Me}(\text{CH}_2)_m\text{CO}_2\text{H} + \text{C}_2\text{H}_5\text{O} & \quad \text{Me}(\text{CH}_2)_m\text{CHCH}_2\text{CO}_2\text{H} \\
\text{(8)} & \quad \text{Me}(\text{CH}_2)_n\text{CH} = \text{CHCH}_2\text{CO}_2\text{H} + 3\text{C}_2\text{H}_5 & \quad \text{Me}(\text{CH}_2)_n\text{CH} = \text{CH}(\text{CH}_2)_m\text{CO}_2\text{H}
\end{align*}
\]

Scheme 2

\[
\text{Me}(\text{CH}_2)_m(\text{CH} = \text{CHCH}_2\text{CH}_2)_n\text{CO}_2\text{H}
\]

(9) \( m = 12 \text{ or } 14; \ n = 4, 5, \text{ or } 6 \)

Biosynthesis

The phleic acids (9) are polyunsaturated acids produced by *Mycobacterium phlei*, with an unusual distribution of double bonds. When $^{14}$Cacetate is incubated with *M. phlei*, the saturated and unsaturated sections are unequally labelled. $^{14}$CMyristic and $^{14}$Cpalmitic acids serve as precursors for the phleic acids with $m = 12$ and 14, respectively. A chain-elongation process involving two acetate units at a time, possibly via crotonate, is postulated; $\beta$-hydroxybutyric acid, however, is not incorporated without prior degradation. The biosynthesis of cerulenin (10), an important inhibitor of fatty acid synthetase, has been studied in cultures of *Cephalosporium caerulescens*. The alternate labelling obtained with $\text{[1-}^{13}\text{C}^\text{acetate}$ rules out the possible intermediacy of succinate or glycerol and indicates that the biosynthesis is closely related to that of fatty acids.

$\alpha$-Linolenic acid (11) has been shown to be formed by desaturation of oleic and linoleic acids in several organs of higher plants and in algae. A chloroplast preparation from *Thea sinensis* leaves converts linolenic acid and 13-t-hydroxylinolenic acid into cis-3-hexenal, the precursor of 'leaf alcohol'. The biosynthesis of the $C_8$ and $C_{10}$ acetylenes, diatetryne 2 (14) and diatetryne 3 (15), respectively, via oleate, linoleate, crepenynate (12), and trans-dehydromatricariate (13) has been demonstrated in labelling experiments with the fungus *Lepista diem* (Scheme 3). Although the neces-

\[
\text{MeCH} = \text{CHCH}_2\text{CH} = \text{CHCH}_2\text{CH} = \text{CH(CH}_2)_2\text{CO}_2\text{H}
\]

(11)

Oleate $\rightarrow$ linoleate

\[
\text{Me(CH}_2)_4\text{CCCH}_2\text{CH} = \text{CHCH}_2\text{CH} = \text{CH(CH}_2)_2\text{CO}_2\text{Me}
\]

(12)

\[
\text{Me} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CH} = \text{CHCH}_2\text{CH} = \text{CHCH}_2\text{CH} = \text{CH(CH}_2)_2\text{CO}_2\text{Me}
\]

\[
\text{HOCH}_2\text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CH} = \text{CHCH}_2\text{CH} = \text{CHCH}_2\text{CH} = \text{CH(CH}_2)_2\text{CO}_2\text{Me}
\]

(13)

\[
\text{Me} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CH} = \text{CHCO}_2\text{Me}
\]

(14)

\[
\text{Me} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CH} = \text{CHCO}_2\text{Me}
\]

(15)

\[
\text{Me} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CH} = \text{CHCO}_2\text{H}
\]

(16)

\[
\text{Me} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CC} = \text{CH} = \text{CHCO(CH}_2)_2\text{CO}_2\text{Me}
\]

(17)

---

Biosynthesis of Polyketides

Sary ω-oxidation appears to be possible at both the C\textsubscript{18} and C\textsubscript{10} stages, the ready incorporation of (13) into both diatrynes and of diatryne 3 into diatryne 2 suggests that chain shortening takes place before ω-oxidation.\textsuperscript{21} Incorporation experiments with several species of Compositae confirm that crepenynate is also an intermediate in the biosynthetic conversion of oleic acid into polyacetylenes in higher plants. The more efficient incorporation of the C\textsubscript{16} compound (16; \(n = 5\)) compared to the C\textsubscript{18} compounds (16; \(n = 7\)) and (17) into \textit{cis}-dehydromatricaria ester (13) in \textit{Artemisia vulgaris} does not support the hypothesis that a direct Baeyer–Villiger type oxidation of a C\textsubscript{16} precursor is involved in the biosynthesis of (13). On incorporation of [\textsuperscript{10,\textsuperscript{14}C\textsubscript{9},\textsuperscript{10}H\textsubscript{2}]}oleate and crepenynate an unexpected loss of tritium was observed.\textsuperscript{22} This loss, which was also observed in similar experiments with \textit{Lepista diemii}, cannot yet be explained.

\[
\begin{align*}
\text{Scheme 4}
\end{align*}
\]

pathway to prostaglandin A\textsubscript{2} (PG\textsubscript{A}A\textsubscript{2}; 18) in the coral, \textit{Plexaura homomalla}, does not involve PG\textsubscript{E}A\textsubscript{2} (19), PG\textsubscript{H}A\textsubscript{2} (22), PG\textsubscript{G}A\textsubscript{2} (23), or \textit{11-epi-PGF}A\textsubscript{2} (20), and hence the epi-endoperoxide, and so differs from that of mammalian systems.\textsuperscript{24} Samuelsson's group have detected a labile intermediate of high biological activity in the conversion of arachidonic acid (21) or of the endoperoxide PG\textsubscript{G}A\textsubscript{2} (23) into the hemiacetal derivative, thromboxane B\textsubscript{2} (25), by washed human platelets.\textsuperscript{25} This intermediate, designated thromboxane A\textsubscript{2} (24), appears to be identical with rabbit aorta-contracting

\[
\begin{align*}
\text{Me} & \quad \text{[C-CH\textsubscript{2}]\_2} \quad \text{CO}_2\text{H} \\
(27)
\end{align*}
\]

substance (RCS). An enzyme system has been isolated from the microsomal fractions of disrupted horse and human platelets\textsuperscript{26} which mediates the conversion of (22) and (23) into (24), as shown in Scheme 4. Incubation of labelled arachidonic acid with a homogenate of rat stomach led to the isolation of 6-ketoprostaglandin F\textsubscript{1\alpha}, which exists predominantly in the lactol form (26).\textsuperscript{27}

### 3 \(\beta\)-Polyketomethylene Derivatives

The compounds discussed in this section may be formally considered to be formed \textit{via} polyketomethylene chains of general type (27), in which reduction of the intermediate \(\beta\)-keto-ester after each condensation step, as in fatty acid synthesis, has not taken place. These compounds are classified, as before,\textsuperscript{4} into groups according to the number of \(C\textsubscript{2}\) -units in the intermediate chain, though no distinction has been made between aromatic and non-aromatic compounds; this division is becoming increasingly artificial as interrelationships become clearer.

#### Tetraketides

Recent results reported by Lynen extend the enzyme characterization of the biosynthetic pathway to patulin (33) in \textit{Penicillium patulum}. Two separable enzyme fractions hydroxylate \(m\)-cresol (28) to \(m\)-hydroxybenzyl alcohol (31) and 2,5-dihydroxytoluene (29), respectively.\textsuperscript{28} Time studies of the appearance of activity from \([1\textsuperscript{14}C]\)acetate into metabolites of the patulin biosynthetic pathway and of the utilization of labelled intermediates show that the methyl hydroxylation of \(m\)-cresol is an important reaction on the pathway, whereas the ring hydroxylation appears to be a side-reaction. A further enzyme preparation\textsuperscript{29} ring-hydroxylates \(m\)-hydroxybenzyl alcohol to gentisyl alcohol (30), but \(m\)-hydroxybenzaldehyde (32) was not ring-hydroxylated by any preparation from \textit{P. patulum}. It was concluded that the main

\textsuperscript{28} G. Murphy, G. Vogel, G. Krippahl, and F. Lynen, \textit{European J. Biochem.}, 1974, 49, 443.
\textsuperscript{29} G. Murphy and F. Lynen, \textit{European J. Biochem.}, 1975, 58, 467.
Biosynthesis of Polyketides

\[ 4 \text{CH}_3\text{COSCoA} \rightarrow \text{(28)} \rightarrow \text{(29)} \]

\[ \text{(28)} \rightarrow \text{(30)} \rightarrow \text{(31)} \rightarrow \text{(32)} \]

\[ \text{(32)} \rightarrow \text{(33)} \]

*Scheme 5*

pathway to patulin is via \textit{m}-hydroxybenzyl alcohol, gentisyl alcohol, and gentisaldehyde (Scheme 5).

Incorporation of \textsuperscript{14}C-labelled precursors demonstrates the biosynthetic sequence, Scheme 6, leading to penicillic acid (34) in \textit{Penicillium cyclopium}.\textsuperscript{30} \textsuperscript{[1-\textsuperscript{14}C]}Methylorcinol is also incorporated but it may not be on the direct pathway. An enzyme system which carries out the final oxidative ring-opening reaction has been isolated;

*Scheme 6*

\[ \text{HO-} \text{Me} \rightarrow \text{HO-} \text{Me} \rightarrow \text{HO-} \text{Me} \rightarrow \text{HO-} \text{Me} \rightarrow \text{(34)} \]

\[ \text{(34)} \rightarrow \left[ \begin{array}{c} \text{O} \text{Me} \\ \text{O} \text{Me} \end{array} \right] \rightarrow \left[ \begin{array}{c} \text{O} \text{Me} \\ \text{O} \text{Me} \end{array} \right] \rightarrow \text{OMe} \]

\[ \text{OMe} \rightarrow \text{OMe} \rightarrow \text{OMe} \]

Biosynthesis

Co-factor requirements indicate a mono-oxygenase system. A similar mechanism for cleavage of aromatic rings via a quinonoid structure may be involved in the biosynthesis of a number of natural products, such as patulin, sulochrin, multicolic acid, and the aflatoxins.

Further analogues of mycophenolic acid (35) were produced by cultures of *P. brevicompactum*, supplemented with 4,6-dihydroxycoumaran-3-one (36), 5,7-dihydroxyindan-1-one (37), and 2,4-dihydroxyacetophenone (38). Both 3- and 5-trans, trans-farnesyl-2,4-dihydroxyacetophenone were metabolized to the corresponding analogues (39) and (40); it appears that the tendency of the side-chain double bond to be oxidized depends upon its distance from the carbonyl group in the enzyme-substrate complex. Further details have appeared on biosynthetic studies of epoxydon (41), a major antibiotic metabolite of *Phylostepia* spp. Incorporations of [13C]-acetate and [14C]gentisyl alcohol confirm the tetraketide origin of epoxydon via

\[
\text{CH}_3\text{CO}_2\text{Na} \rightarrow \text{CO}_2\text{H} \rightarrow \text{CH}_2\text{OH} \rightarrow \text{HOH}_2\text{C} \rightarrow \text{Cl} \rightarrow \text{Cl}
\]

\[
\text{HOH}_2\text{Cl} \rightarrow \text{HOH}_2\text{Cl} \rightarrow \text{HOH}_2\text{Cl}
\]

Biosynthesis of Polyketides

6-methylsalicylic acid and gentisyl alcohol, known co-metabolites. The remaining co-metabolites (42), (42a) and (42b), may be artefacts as they can be isolated after allowing epoxydon to stand in sterile culture medium (chloride concentration ca. 130 p.p.m.) for several days. The $^{13}$C n.m.r. spectra of colletodiol (43) enriched with $[^{13}$C$]$acetate in Colletotrichum capsici show enrichments consistent with a biosynthetic pathway via the union of triketide and tetraketide components.

**Pentaketides.**—Incorporation of $[^{13}$C$]$acetate and $[^{13}$C$]$methionine by cultures of Sclerotinia sclerotiorum establish that sclerin (44) is derived from five intact acetate units with the introduction of three C-methyl groups from methionine. The results are in accord with a biosynthesis from two separate polyketide chains, though this would involve an aldol condensation and methylation at the methyl group of one of the polyketide chains, both reactions for which there is almost no biosynthetic precedent. To overcome this difficulty, Staunton has proposed an interesting pathway via

---

aromatic ring-cleavage of the known co-metabolite, sclerotinin A (45) (Scheme 7). Feeding of labelled (45) or [13C]malonate might solve this intriguing problem.

Incorporations of the 14C-labelled dihydroisocoumarin (46), and of [1-13C]- and [1,2-13C2]-acetate, into the cyclopentenone terrein (47) by cultures of *Aspergillus terreus*, indicate a biosynthesis involving contraction of the aromatic ring of (46) with loss of carbon 7, carbons 8 and 9 becoming carbons 6 and 5, respectively, of terrein.35

![Diagram of biosynthesis](image)

A somewhat different mechanism leads to the chlorine-containing cyclopentenol (49) and the dihydroisocoumarin (48) in *Periconia macrospinosa*. Incorporations of singly- and doubly-labelled [13C]acetate show that (48) is derived as expected from five intact acetate units assembled as shown in Scheme 8.36 The cyclopentenol (49) is also derived from five acetates, but only three are intact, consistent with a biosynthesis by ring-contraction, involving fission of the 7—8 bond of an aromatic precursor related to (48) as shown. A previous postulate involved fission of the 4—9 bond. Incorporation of [1-13C]-, [2-13C]- and [1,2-13C2]-acetate into the pyrone (50) by cultures of *A. melleus* also gave an unusual labelling pattern. Detailed examination of the 13C n.m.r. spectrum of [1,2-13C2]acetate-enriched (50) revealed the presence of a two-bond 13C—13C coupling (6.2 Hz) between carbons 1 and 7, proving their origin.

from an originally intact acetate pair. A pathway involving a Favorovski-type rearrangement and decarboxylation of a precursor pentaketide, as indicated, was proposed.

$^{14}$C-Labelling experiments indicate a polyketide origin for flavolin (51) in A. niger and for 2,7-dimethoxynaphthazarin (53) in a Streptomyces. Labelled flavolin and mompain (52) are efficiently incorporated into (53) by the Streptomyces. As 2,7-dimethylflavolin could not be detected, a pathway in which hydroxylation of flavolin occurs exclusively before introduction of the O-methyl groups was proposed.

Melanin pigments formed by polymerization of 1,8-dihydroxynaphthalene (1,8-DHN), play an important role in the survival of fungi, being present in structures resistant to extreme conditions and protecting cell walls from the action of bacterial degradative enzymes. The biosynthetic pathway to 1,8-DHN in *Verticillium dahliae* has been explored (Scheme 9) by isolation of a series of melanin-deficient mutants. The mutant brm-1 (brown microsclerotia) accumulated (+)-scytalone (54) which could be converted into melanin by alm (albino microsclerotia) mutants. When fed to a second brown mutant, brm-2, (+)-scytalone (54) was dehydrated to 1,3,8-
Biosynthesis

5CH$_3$CO$_2$H $\xrightarrow{\text{brm-1}}$ trihydroxynaphthalene (55). On feeding (55) to brm-1 cultures, a new metabolite \((-\)-vermelone (56) accumulated.$^{41}$ \((-\)-Vermelone was dehydrated to 1,8-DHN by another mutant, alm-1. Both 1,8-DHN and (56) are rapidly converted into melanin by alm or brm-2 cultures. It appears that the brm-1 mutant lacks the enzyme activity necessary to convert either of the 3-hydroxytetralones (54) and (56) into their corresponding naphthols, which suggests that the same enzyme might catalyse both dehydratase steps in the conversion of \((+\)-scytalone (54) into 1,8-DHN. The acetate origin of scytalone has recently been demonstrated in Phialaphora lagerbergii.$^{42}$

4,7-Dimethoxy-5-methylicoumarin (58) has been shown to be polyketide in origin by $[^{14}\text{C}]$acetate incorporations in Aspergillus variecolor.$^{43}$ This compound represents an unusual cyclization since in the cyclization of a polyketide intermediate, the uncyclized residue from the methyl end of the chain is not normally shorter than the residue from the carboxyl end.$^{44}$ Siderin, a metabolite of the higher plants Sideritis canariensis and S. romana, has been shown to be identical with (58).$^{45}$ It would be interesting to see if it is of similar origin in the plants. Incorporation of $[^{14}\text{C}]$malonate by Pinus jeffreyi seedlings indicates that carbons 2 and 7 of \((-\)-pindine (59) are de-

Biosynthesis of Polyketides

rived from the 'starter' acetate unit of the precursor pentaketide chain. Feeding the 14C-labelled potential precursors 5,9-dioxo- and 3,7-dioxo-decanoic acid, nona-2,6-dione, and decanoic acid gave negligible incorporations.

Heptaketides.—Due to their co-occurrence in various fungi, it has been suggested that griseofulvin (61) and other heptaketides may be formed from a common precursor, itself derived by ring-cleavage of a preformed carbocyclic precursor, rather than by simple folding of a polyketide chain. Incorporation of [2-3H,2-14C]acetate into griseofulvin by Penicillium urticae cultures proceeds without isotopic exchange, resulting in griseofulvin with six tritium labels on the molecule, as shown in Scheme 10. Degradation studies show that three of the tritiums are located on the 6'-methyl confirming that it is part of a chain-initiating acetate unit in the parent benzophenone (60), and so cannot be formed via a ring-cleavage process as the above postulate would require. The remaining tritiums are located at 3’, 5’α, and 5. The exclusive a location of tritium at the 5’ position indicates trans-diaxial stereochemistry in the reduction of ring C at a late stage in griseofulvin biosynthesis.

[14C]Acetate is incorporated into nepodin (62), a naphthol metabolite of Rumex alpinus, with a labelling pattern consistent with a polyketide origin. Incorporation of [13C]malonate in the presence of inactive sodium acetate revealed that carbons 3 and 3’ and not carbons 2’ and 2” were derived from the chain-initiating acetate unit. The naphthol (63) co-occurs with (62), and on feeding labelled nepodin, the former became radioactive suggesting a derivation by deacetylation of (62).

Three different foldings of a single polyketide chain, as well as multichain condensations, could account for the formation of the phenalenone ring system in deoxyherqueinone (64). Analysis of the enrichments and 13C-13C couplings in 13C n.m.r.

47 Ref. 44, p. 154.
spectra of deoxyherqueinone triacetate labelled with $[1^{-13}C]$, $[2^{-13}C]$, and $[1,2^{-13}C_2]$-acetate, and $[2^{-13}C]$malonate by cultures of *Penicillium herquei* indicated formation of the phenalenone ring system from the heptaketide folding shown. Malonate enriched carbon 14 to a lesser extent than carbons 12, 10, 2, 4, 6, and 8. This is the first observation of a 'starter' effect in $^{13}C$ biosynthetic studies. In order to facilitate comparison of incorporation efficiencies into different sites, the $^{13}C$ n.m.r. spectra were determined in the presence of the relaxation agent Cr(acac)$_3$ under GATED-2 decoupling conditions, whereupon the very wide range of resonance intensities due to variable relaxation times and nuclear Overhauser factors was

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Biosynthesis of Polyketides

Scheme II

\[ \text{CH}_3\text{CO}_2\text{Na} \]

Scheme 11

Scheme 12

\[ \text{CH}_3\text{CO}_2\text{Na} \]
OH \text{Me} \text{OH} \text{Me} \text{OH}

(72) \text{R} = \text{H} \text{or} \text{CH}_2\text{CH} = \text{CMe}_2

(74) \text{R} = \text{H} \text{or} \text{CH}_2\text{CH} = \text{CMe}_2

(75) \text{R} = \text{H}, \text{R}^2 = \text{CH}_2\text{CH} = \text{CMe}_2

(76) \text{R} = \text{H}, \text{R}^2 = \text{CH}_2\text{CH} = \text{CMe}_2

(77) \text{R} = \text{Me}, \text{R}^2 = \text{H}, \text{R}^3 = \text{CH}_2\text{CH} = \text{CMe}_2

(80) \text{R}^1 = \text{H}

(81) \text{R}^1 = \text{CH}_2\text{CH} = \text{CMe}_2

(82) \text{R}^1 = \text{CH}_3\text{CH} = \text{CMe}_2
Scheme 13
removed and almost integral intensities for all resonances in the natural abundance spectrum were obtained. This technique could be usefully applied in other cases where secondary incorporation due to metabolic conversion of the labelled precursor is observed (e.g. in the macrolides, see below).

Incorporation of [1-13C]acetate and [1-13C]formate into cercosporin (65) by cultures of Cercospora kikuchii indicates its biosynthesis via oxidative coupling of two hepta-ketide units, the methoxyl and methylenedioxy carbons being derived from the C7 pool.53

Octaketides.—Anthraquinones or their related anthrones have been implicated in the biosynthesis of several natural products, particularly by ring-cleavages. Feeding of biosynthetically prepared [U-14C]emodin (66) to cultures of Aspergillus terreus yielded radioactive geodin (68) and dihydrogeodin (67), suggesting their formation by way of oxidative ring-cleavage from emodin.54 Two acetate assembly patterns are possible in islandicin (69) (Scheme 11). Incorporation of singly- and doubly-labelled [1-13C]acetate by cultures of Penicillium islandicum indicates that islandicin is assembled as in pathway (a).55 The xanthone ravenelin (71) is a metabolite of Helminthosporium ravenellii and H. gramum.56 13C Labelling studies show that acetate and malonate are incorporated equally into both benzenoid rings; no ‘starter’ effects could be detected. Incorporation of [1-1-13C]acetate confirmed the 13C results, and analysis of the 13C-13C couplings in the 13C n.m.r. spectrum of ravenelin enriched from [1,2-13C2]acetate revealed that it is biosynthesised via a benzophenone (70) which can undergo cyclo-dehydration at two positions to give an equimolar mixture of ravenelin with labelling patterns (71a) and (71b) (Scheme 12).56 Although a two-chain mechanism cannot be ruled out, the acetate assembly pattern is consistent with the derivation of ravenelin from islandicin (69), with the assembly pattern established in P. islandicum. A similar cyclization to alternative sites is possible in the biosynthesis of griseofulvin via griseophenone C (60) (Scheme 10 above) and analysis of the labelling pattern resulting from [1,2-13C2]acetate would indicate the degree of enzyme binding of intermediates of the griseofulvin biosynthetic pathway. No such randomization (via a symmetrical cleavage product) occurs in the formation of sterigmatocystin (see below).

Previously reported 13C-labelling studies of shamixanthone (86) and tajixanthone (87), metabolites of Aspergillus variecolor, were consistent with a derivation by oxidative fission of a polyketide anthrone with introduction of two prenyl residues from mevalonate.57 It was suggested that these metabolites, together with arugosin A (75),

\[
\begin{align*}
\text{HOH}_2\text{C} & \quad \text{O} \\
\text{Me} & \quad \text{OH}
\end{align*}
\]

(92)

Biosynthesis of Polyketides

B (76), and C (78), metabolites of A. rugulosus, constitute a biogenetically related group. Further studies on the metabolites of nine variant strains of A. variecolor in an attempt to throw light on the biogenetic relations of these compounds, led to the isolation of arugosin A, B, and C and ten new metabolites: variecoxanthones A, B, and C, (80)—(82), tajixanthone hydrate (88) and methanolate (89), 14-hydroxy-, and 14-methoxy-tajixanthone-25-acetate, (90) and (91), epi-isoshamixanthone (83), 25-O-methylarugosin B (77), and arugosin D (79). The biosynthesis of shamixanthone from the supposed intermediate (72) requires (i) cyclodehydration to the xanthone and (ii) cyclization of the O-prenyloxy-aldehyde unit to the substituted dihydropyran ring, but it is not clear which occurs first. Isolation of the variecoxanthones tended to suggest the prior formation of the xanthone ring and that these compounds are formed by subsequent reduction to the benzyl alcohols. However, the aldehyde (74, \( R^1 = R^2 = H \)) derived from variecoxanthone A (80) readily cyclizes in vitro to give des-C-prenyl-epi-shamixanthone (84) in which the substituents in the dihydropyran ring are cis-related, in contrast to the trans relationship in shamixanthone. If the in vitro cyclization follows the same stereochemical course, it seems unlikely that shamixanthone arises in this way, although the minor metabolite (83) may. It was suggested that the trans-relationship is more likely to arise, due to stereoelectronic requirements, by formation of the dihydropyran ring before xanthone formation. It is probable that arugosin C (78) arises from such an intermediate (73) by the alternate cyclodehydration between the C-25 and C-10 hydroxyls to the dibenzoepin system. Tajixanthone (87) and the metabolites (88)—(91) appear to be derived via epoxidation of the C-prenyl residue of shamixanthone. Arugosin D (79) would be formed from arugosin B by rearrangement of a similar epoxide. The isolation of shamixanthone and variecoxanthone B (81) from A. nidulans, and shamixanthone, (81), and epishamixanthone (85) from A. rugulosus has been reported. It is interesting chemotaxonomically that all three of the above organisms also produce sterigmatocystin and related compounds. The xanthone (92) has also been isolated from Cyathus intermedius.

\[ \text{OH} \quad \text{Me} \quad \text{COR} \]
\[ \text{OH} \quad \text{Me} \quad \text{CO}_2\text{H} \]

\[ \text{OH} \quad \text{Me} \quad \text{O} \quad \text{CO}_2\text{H} \]


The neomycins A, B, C, and D (93)-(96) have been isolated from *Streptomyces rosa*. Incorporation of \([1-\text{13C}]\)acetate established their polyketide origin and the position of the phenolic hydroxy-group. Neomycin D is enantiomeric with kalifungin, another streptomycete metabolite. In contrast to a previous report that palmitate was incorporated intact into brefeldin A (97), addition of \([16-\text{14C}]\)palmitate to cultures of *Penicillium brefeldianum* gave uniformly labelled (97), indicating incorporation via \(\beta\)-oxidation to \([2-\text{14C}]\)acetate. The labelling pattern previously reported for incorporation of \([1,2-\text{13C}]\)acetate into mollisin (98) by cultures of *Mollisia caesia* has been confirmed. Improved culture conditions gave higher incorporation rates and solubilization of mollisin by conversion into its acetate made possible the use of Cr(acac)\(_3\) and enabled all the couplings to be determined. Biosynthesis of mollisin by cleavage of a single octaketide chain (Scheme 14) would appear to be at least as likely as the two-chain pathway proposed.

---

**Scheme 14**

[Chemical structures and reactions]

---


65 R. W. Rickards, personal communication.
Biosynthesis of Polyketides

Nonaketides.—Incorporation of \( [1,2-\text{\textsuperscript{13}C}] \) acetate, in conjunction with homonuclear \( \text{\textsuperscript{13}C} \) decoupling, overcame the difficulties of low enrichments encountered with singly-labelled \( \text{\textsuperscript{13}C} \) acetate, and established the polyketide origin of bikaverin (99) in Fusarium oxysporum, via the acetate assembly pattern shown in Scheme 15.

\[
\text{CH}_3\text{CO}_2\text{Na} \quad \text{MeO} \quad \text{Me}
\]

Scheme 15

Decaketides.—The biosynthesis of the aflatoxins has recently been an area of much activity and not a little confusion. The use of \( \text{\textsuperscript{13}C} \)-labelled precursors has predominated. Reports of the incorporation of \( [1,2-\text{\textsuperscript{13}C}] \) acetate into aflatoxin B\(_1\) (102) and sterigmatocystin (101) led to conflicting conclusions: Steyn and co-workers reported an acetate assembly pattern in aflatoxin B\(_1\), produced in Aspergillus flavus, inconsistent with the original postulate of a C\(_{14}\) naphthacene precursor, and in agreement with formation of aflatoxin B\(_1\) from a C\(_{20}\) polyketide precursor, folded as in path (a) in Scheme 16, to give averufin (100), sterigmatocystin, and subsequently aflatoxin B\(_1\) by oxidative fissions. However, the previously reported assembly pattern of acetate units in sterigmatocystin indicated an alternative folding, path (b), of the precursor decaketide chain. This apparent conflict has been resolved by a reassignment by Steyn \textit{et al}. of the chemical shifts in the \( \text{\textsuperscript{13}C} \) n.m.r. spectrum of sterigmatocystin, by an analysis of the long-range \( \text{\textsuperscript{1}H}-\text{\textsuperscript{13}C} \) couplings observed in the high-resolution \( \text{\textsuperscript{13}C} \) spectrum, and lanthanide shift studies, as well as comparison with closely related model compounds. This brings the sterigmatocystin assembly pattern into agreement with that observed for aflatoxin B\(_1\). In their original study, Seto \textit{et al}. based their assignments on a comparison of the \( \text{\textsuperscript{13}C} \) chemical shifts of carbons 2–7 of sterigmatocystin and 5-methoxysterigmatocystin, and on the predicted effects of methoxy-substitution on aromatic carbon chemical shifts, and although these effects are in fact in close agreement with those observed for the corrected shifts in sterigmatocystin and those reported for 5-methoxysterigmatocystin, a completely erroneous assignment resulted. Hsieh \textit{et al}. have studied the incorporation of \( [1-\text{\textsuperscript{13}C}] \)- and \( [2-\text{\textsuperscript{13}C}] \)-acetate into aflatoxin B\(_1\) by \textit{A. parasiticus}, and they also misassigned the \( \text{\textsuperscript{13}C} \) n.m.r. spectrum on the basis of comparison with model compounds, though the biosynthetic conclusions are not affected in this case. However, it may be seen that great caution is necessary when using model compounds for the assignment of \( \text{\textsuperscript{13}C} \) resonances in complex organic molecules, while it cannot be emphasized too strongly that an unambiguous assignment of the \( \text{\textsuperscript{13}C} \) n.m.r. spectrum is an essential pre-requisite of

Biosynthesis

\[ \text{CH}_3\text{CO}_2\text{Na} \]

Scheme 16
any biosynthetic study using $^{13}$C-enriched precursors. This often requires the expenditure of considerable effort. The recent paper\textsuperscript{71} by Steyn \textit{et al.} giving full details of their studies is essential reading for anyone using $^{13}$C n.m.r. in biosynthetic studies. In the original sterigmatocystin study, most of the couplings on the quaternary carbons could not be observed due either to their low intensity, or to being obscured by the intense signals of the protonated carbons, so that the assembly pattern was deduced from the observation of only one coupled pair of carbons, \textit{i.e.} 4 and 5. The application of Cr(acac)$_3$ or of one of the known methods for selectively enhancing quaternary carbon resonances\textsuperscript{72} would have enabled more definitive coupling evidence to be elucidated.

The acetate origin of averufin (100) has been confirmed.\textsuperscript{73} A mutant of \textit{A. parasiticus} defective in aflatoxin production accumulates averufin and was used by Steyn \textit{et al.} for detailed $^{13}$C-acetate studies which established pathway (a) of Scheme 16 for this case also.\textsuperscript{74} Incorporation of labelling from (100), labelled biosynthetically from $[^{13}$C]acetate, into aflatoxin B, has been reported, in agreement with previous $^{13}$C studies.\textsuperscript{75} [Senior Reporter’s note: these fall short of proving that averufin is a true precursor of the aflatoxins, since degradation of the side-chain of (100) to acetate should proceed without randomization of acetate-derived labelling, and indeed the oxygenation pattern of (100) is not wholly appropriate for the formation of sterigmatocystins (or aflatoxins), where a 5-deoxy-analogue of (100) would be more appropriate. In the postulated anthraquinone $\rightarrow$ xanthone conversion it is also noteworthy that the $^{13}$C data establish that the non-equivalence of potentially equivalent carbon atoms is retained; contrast the case of ravenelin (above, Scheme 12)].

\begin{center}
\begin{tabular}{c}
\includegraphics[width=\textwidth]{Scheme17.png}
\end{tabular}
\end{center}

The mechanism of conversion of the C₆ side-chain into the bisfuran moiety of the aflatoxins also requires some clarification. Studies on the later stages of aflatoxin biosynthesis in *A. flavus* using biosynthetically ¹⁴C-enriched compounds indicate that aflatoxin B₁ is converted into most of the other aflatoxins,¹⁵ including B₂, B₂₄, G₁, G₂, and G₂₄, but not into aflatoxin M₁ (15-hydroxyaflatoxin B₁). The authors suggest the order of formation of the aflatoxins to be M₁ → B₁ → Gr₁ (103; R = H), though it appears more likely that M₁ is a precursor of GrM₁ (103; R = OH) only and is itself formed via 15-hydroxysterigmatocystin rather than by the main pathway via sterigmatocystin.

**Undecaketides.**—¹³C N.m.r. analysis of chartreusin aglycone (104) biosynthesized from singly- and doubly-labelled [¹³C]acetate in *Streptomyces chartreusis* has shown that the metabolite is formed by condensation and subsequent scission of a polyketide chain of 11 acetate units,¹⁶ rather than from a decaketide as previously postulated. This study provides a compelling demonstration of the power of the ¹³C double-labelling technique in indicating the intermediates involved at the enzyme level; it would have been very difficult to predict the involvement of a benzopyrene molecule as a possible intermediate.

### 4 Other Acetate-derived Compounds

The compounds discussed in the previous section were, other than introduced C₁ and C₅ units, entirely derived from condensation of acetyl-CoA with varying numbers of malonyl-CoA molecules. In the biosynthesis of the following compounds additional chain initiation or elongation species, particularly propionate, are involved, or the compounds are only partially polyketide.

---

Macrolides.—Omura and co-workers have made extensive use of $^{13}$C precursors in studying the origin of the aglycone carbons of several macrolide antibiotics. Incorporation of $[1^{-13}C], [2^{-13}C], and [1,2^{-13}C]_2$ acetate, $[1^{-13}C]$ propionate, and $[1,2^{-13}C]$ butyrate by cultures of *Streptomyces kitasatoensis* into leucomycin (105) indicated formation of the aglycone ring from five intact acetates, one propionate, and one butyrate,$^{77}$ but carbons 3 and 4 were not labelled by any of the added precursors and their origin remains obscure. In the related tylosin (106), carbons 3 and 4 are derived from carbons 1 and 2 of propionate. Incorporation of $[1^{-13}C]$-acetate, -propionate, and -butyrate, and $[4^{-13}C]$-ethylmalonate by *Streptomyces fradiae* demonstrated formation of the aglycone from two acetates, five propionates, and one butyrate.$^{78}$ Metabolism of $[1^{-13}C]$-acetate resulted in secondary enrichment of the butyrate-derived carbons 5 and 19, and possibly of those carbons derived from C-1 of propionate, though the rather large variation in apparent enrichments is confusing. More reliable information on these secondary incorporation pathways could be obtained by application of the resonance equalization methods discussed above. Both the added butyrate and ethylmalonate are extensively metabolized to propionate, but by different pathways since they respectively enrich carbons originating from C-1 and C-2 of propionate. No enrichment of acetate-derived carbons was observed so neither was metabolized by $\beta$-oxidation.$^{13}$C-Studies in *S. flavochromogenes* indicate the derivation of picromycin (107) from six propionates and one acetate.$^{79}$

$$\text{(108)}$$

$$\text{(109)}$$

$$\begin{align*}
A_1: & \quad R = \text{CH}_3\text{CHMe}_2 \\
A_2: & \quad R = \text{Et} \\
A_3: & \quad R = \text{Me}
\end{align*}$$

The platenomycins (109) are closely related to leucomycins. In a detailed study of their biosynthesis, a variety of intermediate molecules have been isolated from mutant strains of *S. platensis*.$^{80}$ The interrelationships among these compounds and the detailed steps leading from the aglycone (108) to the platenomycins have been delineated by metabolism studies using growing cultures or washing mycelia of blocked mutants. The erythromycin biosynthetic pathway has been extended by the isolation of erythromycin E (111) to which erythromycin A (110) is slowly metabolized by certain strains of *S. erythreus*.$^{81}$

Ansamycins.—Studies on the origins of the ansa chains and aromatic nuclei of rifamycins, streptovaricins, and geldanamycin were discussed in a previous Report. Current work has centred on the biogenetic interrelationships among the streptovaricins and the rifamycins. Rinehart’s group has isolated several new compounds, damaravicin D (DmD; 112), damavarkin C (DmC; 112a), and protostreptovaricins (PSv) I-V, (113)—(117) from the antibiotic complex produced by *Streptomyces spectabilis*. 14C-Labelled streptovaricin D (SvD, 118) is converted into SvC (119) by growing cultures of *S. spectabilis*, whereas (119) is converted by a cell-free system into SvA and SvB. Scheme 18 summarizes the present view of the biogenetic relationship of these compounds. Parallel pathways are required to accommodate the full range of

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metabolites [e.g. both (112) and (114), (116) and (112a)] and the known interconversions.

Rifamycin W (120), closely related to DmD, has a continuous ansa chain, not interrupted by oxygen, and as it was converted into rifamycin S (121) by the mycelium of Nocardia mediterranei, it may be considered as a precursor to the remaining rifamycins. An interesting new compound, rifamycin Gf (122), has been isolated from normal rifamycin fermentations. The pyrone ring presumably arises by oxidative removal of the C-1 carbonyl of (121), which is indeed converted in good yield into both rifamycin B and (122) by washed mycelium of N. mediterranei.

Flavonoids.—Further work on the enzymology of the early steps in flavonoid biosynthesis in Petroselinum hortense (parsley) has been reported. Kreuzaler and Hahlbrock have isolated and purified flavanone synthase from cell suspension cultures. This enzyme catalysed the formation of the flavanone naringenin (125) from labelled p-coumaryl-CoA (123) and three moles of malonyl-CoA. Trapping experiments with an enzyme preparation free of chalcone isomerase activity revealed that the flavanone, and not the isomeric chalcone as previously postulated, was the immediate product.

of the synthase. On treatment of the enzyme system with mercaptoethanol or dithio-
erythritol, naringenin formation was inhibited and the styrylpyrone, bis-noryangonin
(124) was formed. A possible explanation (Scheme 19) is that the thiols act as acceptors
for the intermediate enzyme-bound di-β-keto-acid. The resultant thiol ester would
most likely be highly unstable and immediately be converted into (124).** Cell-free
extracts from very young leaves of parsley plants catalyse the oxidation of naringenin
(125) to the flavone, apigenin (126) and of 7,4′-dihydroxyflavanone (127) to the corre-
ponding flavone (128).** The isomeric chalcone (129; R = H) cannot serve as sub-
strate, and only after cyclization to the flavanone with chalcone–flavanone isomerase
is oxidation to the flavone (128) observed.

Cytochalasins.—Tamm's group have reported further work on cytochalasin bio-
synthesis. The utilization of 13C- and 14C-labelled sodium acetate, propionate, and

![Scheme 19](image-url)

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Biosynthesis of Polyketides

The labelled precursors were all incorporated primarily by the acetate–malonate pathway to generate (130) from nine intact acetate units, eight of which are linked head-to-tail to form the C_{16} polyketide moiety as shown in Scheme 20. While most of the [1-{\textsuperscript{13}C}]propionate is oxidatively decarboxylated to give unlabelled acetate, some is transformed into phosphoenol pyruvate which condenses with shikimic acid to produce C-1 labelled phenylalanine, resulting in a low enrichment of C-4 of cyto-
chalin D. Rapid equilibration of D- and L-phenylalanines with phenylpyruvic acid was shown to account for their equally efficient incorporation into cytochalasin D. Incorporation proceeds with complete loss of tritium at the α-position and extensive loss at the β-position. Considerable suppression of the incorporation of D-phenylalanine was observed on addition of phenylpyruvic acid, indicating that the L-isomer is the primary precursor of cytochalasin D. Labelled deoxaphomin (131) was well incorporated into cytochalasin B (132) by cultures of Phoma sp. (S298), demonstrating that deoxaphomin is an immediate precursor of cytochalasin B, probably via a Baeyer–Villiger type oxidation. A further oxidation of this type would account for the formation of the carbonic ester moiety found in cytochalasin E.

Miscellaneous Compounds. — The metabolite responsible for the antifungal activity of cultures of Streptomyces sp. (E/877) was shown to be the 9-methyl derivative (133) of the known strepimidone. Incorporations of [14C]methionine and [14C]acetate indicate that the 'extra' 9-methyl group is derived from the C1-pool rather than by retention of one of the carboxyls of the terminal malonate unit, both of which are lost.

Scheme 21

---

Biosynthesis of Polyketides

The $^{13}$C n.m.r. spectrum of glauconic acid (134) enriched with [2,3-$^{13}$C$_2$]succinate in Penicillium purpurogenum shows only two pairs of $^{13}$C-$^{13}$C couplings, indicating incorporation into the oxalacetate-derived residue as shown in Scheme 21. In contrast to the previous $^{14}$C studies, no randomization of the label from [2,3-$^{13}$C$_2$]succinate was observed. This inhibition of randomization of label when feeding the greater amounts of precursor required in $^{13}$C studies has been observed previously.

Four of the C-methyl groups in mavioquinone (135), a new quinone isolated from lipid extracts of Mycobacterium avium, were shown to be labelled by [3-$^{14}$C]-propionate. $^{14}$C-Methionine labels the methoxyl carbon but not the ring C-methyl, which is presumably formed by reduction of the carboxyl of acetate. A number of assembly patterns are possible for the biosynthesis of the antibiotic daunomycin (136) via an acetate-malonate or propionate-malonate route. Incorporation of [1-$^{13}$C], [2-$^{13}$C], and [1,2-$^{13}$C$_2$]-acetate by Streptomyces peucetius was poor, but indicated a derivation from a propionate starter unit and nine malonates, as shown in Scheme 22. The biosynthesis of piericidin A, a metabolite of S. mobaraensis, has previously been studied.

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been studied by $^{14}$C- and $^1$H-satellite methods. The $^{13}$C n.m.r. spectra of piericidin A enriched with $[1-^{13}$C$]$-, and $[2-^{13}$C$]$-acetate and $[1-^{13}$C$]$propionate confirmed its origin from four acetates and five propionates, but necessitated a revision of the structure to (137). The 5—6 double bond was previously located between carbons 4 and 5. Extensive transformation of acetate into all positions of propionate was also observed. $[2-^{13}$C$]$Acetate was randomized to give a significant quantity of $[1,2-^{13}$C$]_2$acetate with resulting $^{13}$C—$^{13}$C couplings being observed. This behaviour can be explained by frequent cycling of the highly labelled acetyl-CoA in the Krebs cycle, and supports the proposed conversion mechanism of acetate into propionyl-CoA via succinyl-CoA and methylmalonyl-CoA.

Incorporation of $[1^{14}$C$]$acetate into verrucarin A (138) in *Myrothecium roridum* demonstrated that the cis,trans-muconic acid moiety (139) was derived from acetate. Cleavage of an aromatic precursor was proposed to account for its formation and for the analogous C$_8$ moiety in roridin A. Full details have appeared of the incorpora-

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Tracer studies with $^{13}$C and $^{14}$C indicate that vulgamycin (142), an antibiotic metabolite of *Streptomyces hygroscopicus*, is derived from seven acetates with benzoate as a starter unit. Analysis of the $^{13}$C-$^{13}$C couplings in $^{13}$C-$^{13}$C acetate-enriched vulgamycin, and the detection of a two-bond coupling of 3 Hz between the C-1 and C-3 resonances, indicate that vulgamycin biosynthesis involves a similar rearrangement to that previously observed for pyrone (50).

Chapter 6 of this volume notes work on *Lobelia* alkaloids (Table) and discusses the role of an irregular 'tetra-acetate' precursor in the biosynthesis of dioscorine from nicotinic acid (p. 148).

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A Specialist Periodical Report

Biosynthesis

Volume 6

A Review of the Literature Published during 1977 and 1978

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Biosynthesis of Polyketides

BY T. J. SIMPSON

1 Introduction

This chapter follows the format of the previous report with the inclusion of an extra section on compounds of mixed polyketide-terpenoid origin, and covers the literature from late 1976 until the end of 1978. ¹³C-Labelling studies now greatly outnumber those using ¹⁴C, although ¹⁴C-methods are still almost exclusively limited to studies with micro-organisms. An interesting and potentially very useful development has been in the use of ²H-labelling, the label being detected either directly by ²H n.m.r., or indirectly through its coupling to ¹³C in the ¹³C n.m.r. spectra of metabolites enriched from doubly labelled [²H, ¹³C] precursors. These methods may be of even more use in the study of terpenoid biosynthesis. ²H-Labelling in conjunction with ³H n.m.r. should also be of use, but it has been applied in only one major study to date. There has been an encouraging number of papers describing the incorporation of larger molecules to obtain details of the later stages of polyketide biosynthesis; and close examination of the fate of ¹⁴C-label through secondary incorporation routes has shed light on the overall metabolic pathways operating in many organisms. An excellent short review of the fundamentals of polyketide biosynthesis has appeared.

2 Fatty Acids, Polyacetylenes, and Prostaglandins

In a series of related papers, from Cornforth and co-workers, the precise stereochemical course of individual reactions in the biosynthesis of fatty acids was investigated. Incubation of chiral acetates with either chicken-liver or bakers’ yeast fatty-acid synthetase showed that in palmitic acid a higher proportion of tritium was retained from (S)-1²⁻¹⁴C, ³H-acetyl-CoA than from the (R) isomer, indicating an overall stereospecificity in the formation of fatty acids from chiral acetate. The discrimination between the (R) and (S) isomers was small because the small hydrogen isotope effect, operative in the acetyl-CoA carboxylase reaction, led to nearly equal proportions of (R)- and (S)-tritiated malonyl-CoA species. A partial

and non-specific exchange of hydrogen catalysed by the synthetase was also observed, and this further reduced the net retention of tritium. Malonyl thiol esters stereospecifically labelled with tritium at C-2 were prepared, and incubation of these chiral malonates with the purified yeast synthetase showed that (2S)-malonate retained 51% of the original tritium whereas the (2R)-isomer retained only 23%. Comparison of these results with those from chiral acetates indicates that carboxylation of acetyl-CoA occurs with retention of configuration. It is known that reduction of the 3-ketoacyl product (1) from condensation of acetyl-CoA with malonyl-CoA is stereospecific, giving rise to the (3R)-hydroxyacyl intermediate (2) which in turn dehydrates to give exclusively the trans-2-enoyl derivative (3).

\[
\begin{align*}
\text{H}_2\text{C} & \text{C} = \text{C} - \text{O} - \text{C} - \text{CO}_2\text{H} \\
\text{H}_3\text{C} & \text{C} \text{C} - \text{O} - \text{C} - \text{CO}_2\text{H} \\
\text{H}_2\text{C} & \text{C} \text{C} \text{C} - \text{O} - \text{C} - \text{CO}_2\text{H}
\end{align*}
\]

Dehydration of (2R,3R)-3-hydroxy[2-\text{H}_1]butyryl thioester by yeast fatty acid synthetase proceeded with retention of tritium to give the trans-2-enoyl derivative, by means of a syn elimination of the elements of water. Assignment of the syn stereochemistry for the dehydration, together with the findings that tritium is retained preferentially from (2S)-[2-\text{H}_1]malonate, means that the condensation reaction in fatty acid biosynthesis must proceed with inversion of configuration at C-2 of malonate. Thus the overall stereochemistry of the process is as shown in Scheme 1.

Lynen has examined the selective inhibition of fatty acid synthetase to obtain information on the condensation step in fatty acid biosynthesis. The synthetase

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Biosynthesis of Polyketides

reacts with three moles of iodoacetamide, resulting in inhibition of condensation activity, but greatly increasing the malonyl-CoA decarboxylase activity which is normally low. To account for this, a mechanism is proposed (Scheme 2) in which binding of iodoacetamide to the peripheral thiol groups induces the same change in enzyme conformation as does acetyl-CoA, permitting binding of malonate to the active site as usual. Now, however, instead of concerted loss of CO₂ and transfer of malonate to the acyl residue [path (a)], simple decarboxylation results as shown in

Further reaction of the inhibited synthetase with three moles of N-ethylmaleimide results in inhibition of the decarboxylase activity also.

The biosynthesis of cyclopentenyl fatty acids from 2-cyclopentenyl carboxylic (aleprolic) acid (4) was tested in seeds and leaves of the Flacourtiaeae *viz.* Caloncoba echinata and Hydnocarpus anthelmintica, and in various preparations of other higher plants. Only tissues of the Flacourtiaeae, where the cyclopentenyl fatty acids occur naturally, were able to accept aleprolic acid as a starter for fatty acid synthesis. The labelling pattern of both straight chain and cyclic fatty acids synthesized after incubation of Flacourtiaeae seeds with [1-¹⁴C]acetate indicated initial *de novo* synthesis of C₁₀ fatty acids in each case, followed by chain elongation to higher homologues.⁸ Cyclopentenylglycine, which is found in the tissues of Flacourtiaeae, where it is believed to be formed from acetate and glutamate, serves as a precursor for the cyclic fatty acids, transamination and decarboxylation converting it to aleprolic acid.⁹

Multibranched fatty acids, *e.g.* 2,4,6,8-tetramethyldecanoic acid (5), are the major fatty acids produced by goose uropygal gland. The enzyme systems from

Biosynthesis

This gland and from goose liver have both been shown to be equally capable of producing both branched and non-branched fatty acids, so that the production of methyl substituted fatty acids is not an inherent property of the enzyme system but simply reflects the availability of methylmalonyl-CoA in the uropygial gland.\(^{10}\) Radiclonic acid (6) is a multi-branched fatty acid produced by a *Pencillium* sp. Incorporation of \(^{13}C\) acetate and methionine confirmed its biosynthesis by the usual fungal pathway \textit{via} methylolation of an intermediate polyketide chain and not from propionate.\(^{11}\)

\[
\text{CO}_2\text{H} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{CO}_2\text{H}
\]

(4) (5) (6)

Incorporation of octanoic acids tritiated at C-5, C-6, C-7, and C-8 into lipoic acid (7) by cultures of *E. coli* indicated complete tritium retention at C-5, C-7, and C-8 thus excluding unsaturated intermediates.\(^{12}\) Fifty per cent of the tritium at C-6, however, was lost, and a further experiment\(^{13}\) with \((6R)-\text{ and } (6S)-[6-\text{H}_1]\) octanoate indicated that sulphur was introduced at C-6 with loss of the 6-pro-R hydrogen and inversion of configuration (Scheme 3).

\[
\text{CO}_2\text{H}
\]

(7)

Scheme 3

Polyacetylenes with eight carbon atoms are produced exclusively in fungal cultures. Feedings of \([18-\text{H}^1]\text{crepenynate (8), [18-\text{H}^1]hydroxyester (10), and [10-}
\text{\textsuperscript{1}}\text{H}]\text{-10-hydroxydehydromatricaria ester (11) established the origin of the metabolites (12)—(15) from C-18 to C-11 of crepenynate in cultures of *Agrocybe dura, Psilocybe merdaria*, and *Daedalea juniperina* as shown in Scheme 4. Junipal (15) is one of only three fungal thiophen-acetylenes. They are mostly associated with the Compositae and their derivation from polyacetylenes by the addition of the elements of hydrogen sulphide is probable.\(^{14}\)


Biosynthetic studies on mycomycin (16), one of the few natural allenes, have been thwarted by its instability and the consequent difficulty of purifying it. However, it has been found possible to isolate the alkali isomerization product (17) by h.p.l.c., and feedings of [9-14C]- and [18-14C]-labelled crepenynate (8) and its triacetylenic derivative (9) to cultures of Resinicium bicolor indicate that mycomycin is derived from C-5 to C-17 of crepenynate as shown in Scheme 5. Drosophilin C (19) is formed along with its allenic isomer, drosophilin D (20), and both are converted by alkali into the stable readily purified methyl ester (21). The triacetylene (9) was not incorporated into drosophilin C (19) but the [18-14C]diacetylene (18) was (Scheme 6). Thus it appears that the terminal ethynyl group in (19) does not always arise, as it must do...
in mycomycin and other acetylenes with odd numbers of carbon atoms, by elimination of C-18 of crepenynate by decarboxylation or an equivalent process.\textsuperscript{13}

A large number of C\textsubscript{10} polyacetylenes has been isolated from \textit{Polyporus anthracophilus}. When a mixture of E,E- and 2E,8Z-[1-\textsuperscript{14}C]matricaria esters (22) were fed to cultures of \textit{P. anthracophilus}, one or both of these esters were specifically incorporated into E,E- and Z,Z-matricananol (23), and dimethyl E,E-deca-2,8-diene-4,6 diyne-1,10-dioate (24), to provide evidence that in this organism matricaria esters are intermediates on the route to the many other polyacetylenic metabolites.\textsuperscript{16}

A major development in the prostaglandin field has been the discovery of prostacyclin (PGX or PGI\textsubscript{2}; 27).\textsuperscript{17} Prostacyclin, in contrast to the thromb oxanes which cause blood clot formation, prevents clot formation and causes preformed clots to disperse. Prostacyclin is formed from PGG\textsubscript{2} (25); polarization of the endoperoxide in the opposite sense to that required for thromboxane formation could lead to participation of the 5,6-double bond leading to the carbonium ion (26) which by loss of the proton from C-6 gives prostacyclin (Scheme 7).\textsuperscript{18} Reviews of

\textsuperscript{12} D. G. Davies, P. Hodge, P. Yates, and M. J. Wright, \textit{J.C.S. Perkin I}, 1978, 1602.
Biosynthesis of Polyketides

Scheme 6

18

19

20

21

Me—==—==—==—CO₂R

22

Me—==—==—==—CO₂H

23

Me—==—==—==—CH₂OH

24

MeO₂C—==—==—==—CO₂Me
the formation, synthesis, and biological properties of prostaglandins and other metabolites of arachidonic acid have appeared in the recent chemical literature.\textsuperscript{18,19}

\section*{3 Tetraketides}

Previous studies have demonstrated the polyketide character of barnol (32) and the origin of the alkyl groups: one of the ring methyls is derived from the C\textsubscript{1}-pool, and the other from reduction of the terminal carboxy-group; and the ethyl group is formed by methylation of an original acetate methyl group. Further details of the biosynthetic pathway have been elucidated by feeding phenolic substances to \textit{Penicillium baarnense}.\textsuperscript{20} Neither orsellenic acid nor 5-methylorsellenic acid were metabolized. However, administration of \textsuperscript{14}C-labelled 5-methylorcylaldehyde (28) led to the isolation of two radioactive phenols, identified as 4,5,6-trimethylresorcinol (29) and 4,5,6-trimethylpyrogallol (30). Similarly \textsuperscript{14}C orcylaldehyde was converted to 4,5-dimethylresorcinol, and a cell-free homogenate from \textit{P. baarnense} converted (29) to (30). Finally, 2,4-dihydroxy-6-ethyl-5-methylbenz-
aldehyde (31) was incorporated to the extent of 24% into barnol. These results indicate the biosynthetic sequence shown in Scheme 8 and reveal that both ring and side-chain methyls must be introduced before cyclization of the polyketide, and that reduction of the terminal carboxyl precedes ring hydroxylation. Methylation of a polyketide methyl, rather than a methylene, is very unusual. Mosbach's suggestion
Biosynthesis

that methylation occurred via a quinone-methide intermediate is ruled out by the above results. Another possibility is that methylation occurs on a pentaketide precursor followed by the loss of the 'starter' unit. Stellatin (33), a recently isolated metabolite of *Aspergillus variicolor*, has an apparently similar bis-C-methylated tetraketide skeleton to barnol, and pentaketide metabolites have been isolated from *A. variicolor*. Experiments to establish the presence of a 'starter' group in these molecules would be of interest.

![Chemical structures](image)

$^3$H n.m.r. has been used to determine directly the regio- and stereoselectivity of labelling in penicillic acid (35) biosynthesis in *Penicillium cyclopium* from $[^3]$Hacetate, $[^3]$Hmalonate, and $[3,5-^3$H]orsellinic acid (34). This reveals specific labelling of the 5-methylene with the $^3$H mainly trans to the C-methyl; in the case of the acetate feed there is a partial loss of label from C-5 relative to C-3, and from C-3 relative to the C-methyl. In addition the C-methyl is not labelled by malonate, revealing a clear acetate 'starter' effect, nor is it labelled by the orsellinic acid (so

![Chemical structures](image)


that label from the orsellinic acid is not being incorporated through prior degradation to acetate). The previously postulated intermediate 2,5-dihydroxy-3-methoxytoluene (36) has been isolated from penicillic acid producing cultures of Penicillium baarnense.24

Incorporations of [13C]acetate and methionine into the fungal α-pyrones rosellisin (37), a metabolite of Hypomyces rosellus,25 and coarcatin (38), a metabolite of Chaetomium coarcatum26 reveal that both are tetraketide in origin, with the extra carbons being derived from the C5-pool. The structurally similar nectriapyrone (39) was isolated from Gyrostroma missouriense and on the basis of incorporation of activity from [2-14C]mevalonic acid it was said to be a monoterpenoid.27 This assumption has since been repeated several times in the literature. No degradations to establish the specificity of labelling were reported, however, and nectriapyrone is almost certainly a tetraketide with the ‘extra’ methyls deriving from the C5-pool.

4 Pentaketides

The detection of 2H through its coupling to 13C in the 13C n.m.r. spectra of metabolites derived from doubly labelled [1H,13C] precursors offers the possibility of establishing the integrity of C—H bonds during the course of a biosynthetic pathway. This has been demonstrated by Staunton who proposes a new method for the detection of chain starter units by this means. On incorporation of [1-2H,2-13C]acetate into terrein (40) by cultures of Aspergillus terreus the presence of 2H on C-1, C-3, and C-8 could be inferred by their lowered intensity compared to C-5 (also enriched by 13C acetate but not carrying 2H). In addition the C-3 signal showed 2H-13C coupling satellites.28 On redetermining the 13C n.m.r. spectrum with deuterium noise-decoupling a singlet at 17.95 p.p.m. (0.81 p.p.m. upfield of the normal chemical shift value for C-1) was assigned to molecules trisubstituted with 2H at this position (the normal chemical shift difference for isotopic substitution is ca. 0.3 p.p.m. for each deuterium) thus confirming that C-1 is a chain starter unit.29 There was also a doublet (6Hz, 123 Hz) centred at 18.22 p.p.m. (0.55 p.p.m. upfield from normal) corresponding to molecules labelled with CHD2, and the presence of an enriched CH3 singlet (in the 1H noise decoupled spectrum) shows that there is considerable exchange of hydrogen from the methyl group during

\[
\text{CD}_{3}\text{CO}_2\text{Na} \rightarrow \text{HO} \quad 2H \quad \text{HO}
\]

\(\text{(40)}\)

23 M. S. R. Nair, Phytochemistry, 1976, 15, 1090.
biosynthesis. The $^2$H noise decoupled spectrum also showed singlets at 125.3 and 124.8 for C-3 and C-8 each carrying one $^2$H only.

Previous studies suggested that sclerin (43) a metabolite of Sclerotinia sclerotiorum was formed by condensation of two preformed polyketide chains. The principal difficulty with this proposal was that it necessitated introduction of a methyl from the C$_1$-pool on to the methyl, rather than the usual methylene, of a polyketide chain (see barnol, above). To overcome this Staunton suggested that sclerin was formed via a novel structural reorganization of the known co-metabolite, scleritonin A (41), Scheme 9. This has been confirmed by incorporation of both (41) and (42), which are probably readily interconverted in vitro. The

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incorporations were low, 0.01 and 0.45% respectively, presumably due to poor cell permeability, but degradation confirmed their specific incorporation. Interestingly, label from the isocoumarin (44) was incorporated with much greater efficiency, but (44) was clearly shown to be incorporated only through prior degradation to [2-14C]acetate, a salutary reminder of the need for rigorous proof of specific incorporation. Incorporation of [14C]acetate and [13C]malonate into sclerin was reported independently earlier. As required by the above mechanism, incorporation of [13C]malonate in the presence of unlabelled acetate showed a clear acetate starter effect for C-12 only. However the authors then interpreted this as evidence for a two-chain pathway, with loss of the starter acetate unit from one of the polyketides.31

The tetralone scytalone (45) has also been the subject of further study. Scytalone and related naphthols and naphthaquinones typify a minority of polyketides which lack a clear acetate 'starter' unit, so that several cyclization modes for the precursor...
pentaketide are feasible. Two separate studies\textsuperscript{22-23} have shown that \textsuperscript{13}C\textsubscript{2}acetate is incorporated into scytalone by cultures of \textit{Phialaphora lagerbergii} with both of the two different dispositions shown in Scheme 10, implying that scytalone is formed via a reduction of a symmetrical intermediate such as 1,3,6,8-tetrahydroxynaphthalene (46). The co-metabolite flavolin (47) was thought to be formed from scytalone via (46), but this work demonstrates that (46) is in fact more likely a common precursor of both scytalone and flavolin. Incorporation of \textsuperscript{2-13}H\textsubscript{2,2-13}C\textsubscript{2}acetate into scytalone is also reported,\textsuperscript{23} and under proton noise decoupling the \textsuperscript{13}C n.m.r. spectrum showed a triplet due to \textsuperscript{3}H-\textsuperscript{13}C coupling for C-4. No other \textsuperscript{13}C-\textsuperscript{3}H coupled signals could be detected, but the presence of \textsuperscript{3}H on C-5, but not on C-2 or C-7, was suggested by the decrease in signal intensity. This was confirmed on redetermining the spectrum under \textsuperscript{2}H noise decoupling,\textsuperscript{24} when the C-4 signal appeared as a doublet (\(J_{\text{13}C-\text{3}H} = 127\) Hz) and that of C-5 as a singlet. The presence of \textsuperscript{3}H on C-4 and C-5 but not on C-2 and C-7 could be accounted for if C-4/C-5 were derived from the acetate starter unit; then G-2/C-7 being derived entirely from malonate might lose \textsuperscript{3}H label more readily. Incorporation of \textsuperscript{13}C\textsubscript{1-2}acetate ought to confirm this, and further define the exact cyclization mode of the pentaketide chain.

\[
\text{MeO} \quad \text{Me} \quad -\text{C} = \text{O} \quad \text{Na} \quad \rightarrow \quad \text{MeO} \quad \text{Me} \quad \text{O} \quad \text{H} \\
\text{MeO} \quad \text{Me} \quad \text{OH} \quad \text{OH} \quad \text{Me} \quad \text{O} \quad \text{Me} \quad \text{OH} \quad \text{OH} \\
(48) \quad (49)
\]

\textbf{Scheme 11}

Fungal infection, or treatment with ethrel, induces formation of the phytoalexins (stress compounds) 6-methoxymellein (48) and eugenin (49) in carrot roots. Incorporation of \textsuperscript{13}C\textsubscript{2}acetate by ethrel treated carrot roots reveals a pentaketide origin for both metabolites.\textsuperscript{23} The two modes of acetate incorporation into the benzenoid ring of eugenin (Scheme 11) indicates that the heterocyclic ring is formed by ring closure onto the two equivalent hydroxyls of a symmetrical intermediate which is not firmly enzyme bound. In both cases it is the non-chelated hydroxyl which is methylated, suggesting that O-methylation occurs after cyclization.

Culture filtrates of *Streptomyces mobaraensis* produce reticulol (50) and several related isocoumarins, which are inhibitors of cyclic nucleotide phosphodiesterases. Incorporations of $^{13}$C-lactates and $^{14}$C-methionine indicate a pentaketide origin with the methoxyl being derived from the C$_1$-pool.\textsuperscript{34}

### 5 Hexaketides

Feeding experiments with the $[^1H, ^{14}C]$-labelled pyrone (51) and dioxopentanoic acid (52) indicate that the former, and not the latter, is a biosynthetic precursor of aloenin (53) in aloe plants (Scheme 12), so that methoxylation precedes glucosylation.\textsuperscript{33}


6 Heptaketides

Incorporation of $[^{13}C]$$^3$acetates by cultures of Aspergillus melleus have confirmed the heptaketide origin of xanthomegnin and viomellein. The labelling patterns suggested by this study are readily reconciled with the much more satisfactory revised structure proposed for xanthomegnin (54) from consideration of long range $^1$H--$^{13}$C couplings in the fully $^1$H coupled $^{13}$C n.m.r. spectrum. This also necessitates revision of the structures of the co-metabolites viomellein (55), rubrosulphin (56), and viopurpurin (57) which can now be seen to form a biogenetically homogeneous grouping, along with vioxanthin (58) and floccosin (59), derived by dimerization and further modification of a single naphtol precursor (60) (Scheme 13).

\[ (54) \]

\[ (55) \]

\[ (56) \]

\[ (57) \]

\[ (58) \]

\[ (59) \]

---

Incorporations of singly and doubly labelled $^{13}$C acetates are consistent with previous $^{13}$C results that citromycetin (61) is formed by condensation of two preformed polyketide chains. 43.

The biosynthesis of the antifungal antibiotic griscofulvin (68) continues to attract much attention. Incorporation studies with singly and doubly labelled $^{13}$C acetates using a high producing strain of *Penicillium patulum* showed a clear preferential incorporation of label into the acetate starter unit (i.e. C-6' and 6'-methyl) confirming that grisofulvin is formed from a single heptaketide chain to refute previous proposals of a two-chain pathway. 44. The spectrum from incorporation of $^{13}$C$_{2}$ acetate was difficult to interpret, as despite very low overall enrichment the specific incorporation of $^{13}$C-label into those molecules which were labelled was

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very high, so that extensive $^{13}$C-$^{13}$C coupling was observed between acetate units. This effect is presumably due to inhibition of endogenous acetate production by the high levels of exogenous acetate added, resulting in the metabolite being formed first almost entirely from the added acetate; when the acetate levels fall below a
(necessarily very low) threshold level endogenous acetate production is resumed, to give unlabelled metabolite. Hence the very low overall enrichment level observed. Similar results have been observed in the terpenoid field and the biosynthetic significance of $^{13}$C-$^{13}$C couplings observed under these conditions requires careful interpretation.45 Extensive metabolism of acetate in these experiments also resulted in high enrichment of the C1-pool (and thus of the methoxyl carbons) from C-2 but not C-1 of acetate. Also, randomization of label from [2-$^{13}$C]acetate (via the Krebs cycle) resulted in a much lower apparent enrichment in the $^{13}$C n.m.r. of [2-$^{13}$C]acetate enriched griseofulvin compared to [1-$^{13}$C]acetate, though the isotope peaks in the mass spectra revealed comparable levels of incorporation. Similar studies with *Penicillium urticae* were not subject to the same complications and in this case analysis of the couplings in [13C]acetate-derived griseofulvin46 clearly showed the scrambling of label in the A-ring, consistent with the intermediacy of symmetrical benzophenone intermediates not tightly bound to the enzyme surface. Harris, using isotope dilution and feeding studies with suitably labelled potential intermediates, has delineated the pathway shown in Scheme 14.47 Contrary to some previous suggestions, he has demonstrated that the initial product from cyclization of the heptaketide chain must be the benzophenone (62) which is methylated to give (63) and (64) successively, followed by chlorination to (65). Oxidative cyclization of (65) to the grisan (66) is followed by a final methylation to give dehydrogriseofulvin (67), from which griseofulvin (68) is formed by reduction. Separate studies by Sato et al. showed that the stereochemistry of this final reduction was trans overall, by incorporation of [5'-$^{2}$H]-griseofulvin B (66) and [5'-$^{2}$H]-4-demethyl dehydrogriseofulvin (66). $^{13}$C N.m.r. of the resultant deuterated griseofulvins demonstrated that the $^{2}$H was at the 5'$\alpha$ position, trans to the 6'$\alpha$-methyl.48 In an earlier paper the same workers had reported the incorporation of $^{2}$H from [2-$^{2}$H]acetate, and the first use of $^{2}$H n.m.r. in a polyketide study.49 In agreement with the above results with *P. patulum*, $^{2}$H from the [2-$^{2}$H]acetate was also incorporated into the methoxy-groups.

### 7 Octaketides

Analysis of the $^{13}$C-$^{13}$C coupling pattern of secalonic acid A (69), produced by cultures of *Pyrenochaeta terrestris* supplemented with [$^{13}$C]acetate, is consistent with formation of the tetrahydroxanthone moiety via ring cleavage of an antherquinonoid precursor to give a benzophenone intermediate. The randomization of $^{13}$C-$^{13}$C couplings in the A ring of secalonic acid A indicates that xanthone ring formation must occur with equal facility to either of the hydroxyls in a symmetrically substituted ring so that ring cleavage and xanthone ring formation must precede dimerization (Scheme 15).50 This is an excellent illustration of the

---

\[ \text{CH}_2\text{CO}_2\text{Na} \]

\[ \text{O} \quad \text{O} \quad \text{O} \quad \text{COSX} \quad \text{Me} \]

\[ \text{OH} \quad \text{O} \quad \text{OH} \quad \text{Me} \]

\[ \text{OH} \quad \text{O} \quad \text{OH} \quad \text{Me} \]

\[ \text{OH} \quad \text{O} \quad \text{OH} \quad \text{Me} \]

\( (A) \)

\( (B) \)

\[ \text{A} + \text{A} \]

\[ \text{A} + \text{B} \]

\[ \text{B} + \text{B} \]

\[ \text{OH} \quad \text{O} \quad \text{OH} \quad \text{Me} \]

\[ \text{MeO}_2\text{C} \quad \text{OH} \]

\[ \text{2} \]

\[ \text{(69)} \]

Scheme 15
information that incorporation of doubly $^{13}$C-labelled early precursors can provide on much later stages of a biosynthetic pathway.

The biosynthetic relationships among tetrahydroanthracene and anthraquinone metabolites of *Aloe saponaria* has been demonstrated. $[^{13}$C$]$Acetate was incorporated into aloe saponol (70), aloe saponarin (71), and laccic acid D methyl ester (72). Labelled (70) was incorporated into (71), but no conversion of (70) into (72) or vice versa was observed, showing that (71) and (72) must be biosynthesized in parallel and not in sequence (Scheme 16). The incorporation pattern of $^{2-2}$H$_2$-$^{13}$C acetate into rugulosin (73) in cultures of *Penicillium brunneum*, determined in both $^1$H and $^2$H noise decoupled $^{13}$C n.m.r. spectra is consistent with dimerization of two octaketide-derived anthraquinonoid moieties. Deuterium is detected at C-1, C-3, C-8, and the 7-methyl indicating retention of acetate hydrogen throughout biosynthesis, so that the absence of deuterium at C-6 indicates that the necessary decarboxylation of the octaketide precursor occurs after formation of the aromatic rings. The 7-methyl group shows a singlet (due to CD$_3$) and a doublet (due to CHD$_2$) in the $^2$H noise decoupled $^{13}$C n.m.r. spectrum indicating some loss of hydrogen, possibly via reversible reaction between acetyl-CoA and malonyl-CoA.

The biosynthesis of the fungal metabolite brefeldin A (74) is of great interest due to its obvious structural similarities to the prostaglandins. When *Penicillium brefeldianum* was grown in the presence of $[^{18}$O$_2$]acetate, $^{14}$O$_2$, and $^{16}$O$_2$, mass spectral analysis of the resultant brefeldin A clearly showed that the oxygens on

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**Scheme 16**

$[^{13}$C$]$acetate into rugulosin (73) in cultures of *Penicillium brunneum*, determined in both $^1$H and $^2$H noise decoupled $^{13}$C n.m.r. spectra is consistent with dimerization of two octaketide-derived anthraquinonoid moieties. Deuterium is detected at C-1, C-3, C-8, and the 7-methyl indicating retention of acetate hydrogen throughout biosynthesis, so that the absence of deuterium at C-6 indicates that the necessary decarboxylation of the octaketide precursor occurs after formation of the aromatic rings. The 7-methyl group shows a singlet (due to CD$_3$) and a doublet (due to CHD$_2$) in the $^2$H noise decoupled $^{13}$C n.m.r. spectrum indicating some loss of hydrogen, possibly via reversible reaction between acetyl-CoA and malonyl-CoA.

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C-1 and C-15 were derived from acetate whereas the oxygens on C-4 and C-7 were derived from two different oxygen molecules. Thus the biosynthetic mechanism by which the cyclopentane ring is formed does not closely parallel prostaglandin biosynthesis and the fact that the C-15 oxygen atom does not come from atmospheric oxygen further supports a non-fatty acid, polyketide origin for (74) since C-15 oxygenation of a saturated or unsaturated fatty acid would be expected to occur via a mixed function mono-oxygenase and so would involve molecular oxygen.

In an interesting but unsuccessful experiment, Cross has tried to convert °F-labelled fatty acids into analogues of avenociolide (75) in cultures of Aspergillus avenaceus. However this could yet prove to be a useful alternative method of labelling precursors.

8 Decaketides

The aflatoxins and related metabolites have continued to be the subject of much study. Aflatoxin B₁ (80) production by Aspergillus flavus and Aspergillus parasiticus is greatly reduced in the presence of the insecticide dichlorvos, with the concomitant appearance of an orange pigment, versiconal acetate. Two groups have studied the incorporation of [¹³C]acetate into versicon acetate, and have revised its structure to (77). The further conversion of [¹³C]acetate-enriched (77) into aflatoxin B₁ is reported, but the resultant [¹³C]-enrichment pattern

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Biosynthesis of Polyketides

\[ \text{Me—CO}_2\text{Na} \]

\[
\begin{align*}
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{Me} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{COSX} \\
\end{align*}
\]

\[ \text{HO—O—Me—O—H} \quad \text{(76)} \]

\[
\begin{align*}
\text{HO} & \quad \text{O} & \quad \text{O} & \quad \text{OH} & \quad \text{O—Me} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} & \quad \text{HO—S—O} & \quad \text{Me—OH} & \quad \text{OMe} \\
\end{align*}
\]

\[ \text{HO—O—S—O—Me—OH} \quad \text{(77)} \]

\[ \text{HO—5—O—OH} \quad \text{(78)} \]

\[ \text{HO—O—OH} \quad \text{(79)} \]

\[ \text{O—O—Me} \quad \text{(80)} \]

Scheme 17

- Metabolic block
~ Metabolic inhibitor
observed in aflatoxin B₁ could also be accounted for by degradation of (77) to ¹⁴C acetate followed by reincorporation. Unfortunately much of the work in this area has been characterized by this lack of rigorous proof of incorporation of intact metabolites. Incorporations of singly and doubly labelled ¹³C acetates into averufin (76) and versicolorin A (78) reveal the acetate assembly patterns shown in Scheme 17, and these are fully consistent with the assembly pattern observed in aflatoxin B₁. Steyn has proposed a further mechanism involving an epoxide rearrangement for the conversion of the C₆ side-chain of averufin to the bis-furan of versicolorin A and the aflatoxins. At least three other mechanisms for this conversion have been proposed recently, and it would be of interest to see some detailed studies to test these various proposals and possibly solve this intriguing problem. Two mutants of A. parasiticus, both deficient in aflatoxin production, one of which accumulates averufin (76) and one which accumulates versicolorin A (78), have been used to clarify the role of proposed intermediates on the biosynthetic pathway to aflatoxins. The averufin-producing mutant efficiently converted biosynthetically ¹⁴C-labelled versiconal acetate (77), versicolorin A (78), and sterigmatocystin (79) into aflatoxin B₁, indicating that averufin preceded these compounds on the biosynthetic pathway. In the presence of dichlorvos, the conversion of (78) and (79) was unaffected, but the conversion of versiconal acetate was markedly inhibited. None of the precursors apart from [¹⁴C]acetate were incorporated into averufin, indicating that the conversions are not reversible, and ruling out incorporation via degradation to acetate. The versicolorin A-accumulating mutant incorporated ¹⁴C-labelled acetate, averufin, and versiconal acetate into versicolorin A. In the presence of dichlorvos, however, the major conversion product was versiconal acetate, indicating that dichlorvos inhibited the step of conversion of (77) to (78). This mutant resumed production of aflatoxin B₁ if sterigmatocystin was added to resting cell cultures, indicating that the mutant was blocked at the step converting versicolorin A to the sterigmatocystin. This work would appear to provide the most compelling evidence to date for the biosynthetic pathway summarized in Scheme 17. Similar results have been reported for the conversion of the above anthraquinones into sterigmatocystin by both cultures and by a cell free system from Aspergillus versicolor. The important problems now requiring study would appear to be: how and when the loss of phenolic oxygen occurs on going from the anthraquinone to xanthone; what is the role, if any, of 6-deoxyversicolorin A and 5-hydroxysterigmatocystin in aflatoxin biosynthesis; and of course, the mechanisms of the side-chain rearrangement, and of the oxidative degradations required for the anthraquinone to xanomarin conversions.

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The anthraquinone dothistromin (81) is very similar to the versicolorins. It is isolated from Dothistroma pini, the fungus responsible for blight of pine needles. The incorporation of $^{13}$Clacetates indicates an entirely polyketide origin analogous to versicolorin A.

Incorporations of $[^{13}\text{C}]$acetates by cultures of *Streptomyces nogalater* and *S. elegretus* respectively indicate a decaketide origin for the anthracyclonone antibiotics nogalamycin (82) and steffamycin B (83). The remaining carbons are derived from glucose and the C$_7$-pool.

Scheme 18

Biosynthesis of Polyketides

9 Meroterpenoids

A number of metabolites of mixed polyketide-terpenoid biosynthesis have been studied and it is convenient to discuss these compounds as a group.

The proposed biosynthetic pathway for mycophenolic acid (90) is based on the incorporation of labelled compounds in vivo, with 5,7-dihydroxy-4-methylphthalide (85), 6-farnesyl-5,7-dihydroxy-4-methylphthalide (86), and demethylmycophenolic acid (89) as sequential intermediates (Scheme 18). In support of this an enzyme fraction has been isolated from *Penicillum brevicompactum* that catalyses the synthesis of (86) from 5,7-dihydroxy-4-methylphthalide and either (3RS)-mevalonic acid or farnesyl pyrophosphate (84). The enzymatic synthesis was shown to proceed with inversion of configuration at C-5 of mevalonic acid (i.e. C-1 of farnesyl pyrophosphate) by incorporation of (5R)- and (5S)-[5-¹⁴C, 5-¹³H]-mevalonic acids, isolation of C-1' of (86) as propionic acid, and enzymic conversion to (S)-methylmalonyl-CoA (87). The mechanism leading from 6-farnesyl-5,7-dihydroxy-4-methylphthalate (86) to mycophenolic acid (90) has also been investigated. A total enzyme extract has been isolated from *P. brevicompactum* which in the presence of ATP converts 5,7-dihydroxy-4-methylphthalide (85) and farnesyl pyrophosphate to (86) and mycophenolic acid. However in the absence of ATP, the enzyme system caused the oxidation of (86) to the hydroxyketone (88),

\[ \text{Me-CO}_2\text{Na} \quad \longrightarrow \quad \text{HCO}_2\text{Na} \]

*Scheme 19*

\[ \text{(91) } R = R' = \text{H} \]

\[ \text{(92) } R = \text{H}, R' = \text{CHO} \]

\[ \text{(93) } R = \text{CHO}, R' = \text{H} \]

whose structure was confirmed by comparison with a synthetic sample. The hydroxyketone was further metabolized by the enzyme system to (89) and (90). Administration of $^{13}$C-labelled (88) to the in vivo culture resulted in a 45% conversion to mycophenolic acid.

Incorporations of singly and doubly labelled $[^{13}]$Cacetates, $[^{13}]$Cformate, and $[^{5-13}]$Cmevalonate by cultures of Colletotrichum nicotianae labelled colletotrichin (91) as shown in Scheme 19, indicating its formation via alkylation of a mono-C-methylated polyketide by a novel geranylgeranyl pyrophosphate derived, bicyclic, nor-diterpenoid. The incorporation pattern suggests that the diterpenoid carbon is lost via an epoxide rearrangement followed by Baeyer–Villiger type oxidation as shown in Scheme 20. The formyl esters (92) and (93) are co-metabolites of (91), and the formyl carbon in each is enriched from [2-$^{13}$C]acetate, as required by the scheme, and not by $[^{13}]$Cformate. The authors suggest a triketide origin for the pyrone, but analogy with known polyprenylphenols strongly suggest alkylation of 5-methylorsellinic acid followed by oxidative degradation to the pyrone. In this light it should be noted that prenylated orsellinic acid derivatives, e.g. (94), have been isolated from C. nicotianae. The desacetyl derivative of sesquicillin (95), a metabolite of Sesquicillium globulosporum, would appear to be likely near precursor to colletotrichin.

Incorporations of singly and doubly labelled $[^{13}]$Cacetates and $[^{13}]$Cmethionine by cultures of Aspergillus variecolor indicate that andibenin (97), for which a

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Biosynthesis of Polyketides

A possible route involving the alkylation of the precursor phenol by farnesylylpyrophosphate is shown in Scheme 21, an interesting feature being the proposed formal 4 + 2 cycloaddition which generates the correct carbon skeleton. A sesterterpenoid origin has also been proposed for austin (98), a mycotoxin produced by Aspergillus usus; however, a pathway to austin from the common intermediate (96) can be formulated.

Cochlioquinones, e.g. (99), metabolites of Cochliobolus miyabeanus, are formed via prenylation of the bis-C-methylated hexaketide by a farnesyl pyrophosphate derived moiety. Oxygen-18 labelling together with mass spectrometric studies indicate that the two oxygen atoms of the unusual 2-(2-hydroxypropyl)-tetrahydropyran system are derived from two different oxygen atoms at separate steps on the biosynthetic pathway. A regular heptaketide origin is indicated for flavoglaucin (100) by incorporation of [13C2]-acetate in cultures of Aspergillus amstelodami. The labelling pattern of the dimethylallyl group indicates that there is no change in stereochemistry around the double bond during aromatic isoprenylation.

References:
Scheme 21

Biosynthesis

Me—CO₂Na

C₁O

C₁O

COSCoA

HO

CO₂H

(96)

(97)
Mikrolin (102), a metabolite of *Gilmaniella humicola*, has been shown by incorporation of singly and doubly labelled \(^{13}C\)acetates to be a highly modified prenylated pentaketide.\(^{33}\) On the basis of the labelling pattern as revealed by \(^{13}C\) n.m.r., the biosynthetic pathway to mikrolin and its co-metabolite 6-hydroxymellein (101) shown in Scheme 22 is suggested. The absence of randomization of \(^{13}C\)--\(^{13}C\) couplings in the quinonoid ring indicates oxidative decarboxylation to give directly a 1,2,4-trihydroxyphenol, rather than decarboxylation to a symmetrical 1,4-dihydroxyphenol followed by hydroxylation.

10 Macrolides and Other Compounds

The ansamycin group of antibiotics are usually formed by elongation of an aromatic ‘\(C_2N\)’ starter unit, itself derived from glucose by a shikimate type pathway, by acetate and propionate. On this basis, inspection of the ansa chain of geldanamycin (103) suggested an origin from four propionates and three acetates. However, incorporation of \(2-^{13}C\)malonate by cultures of *Streptomyces hygroscopicus* resulted in enrichment of C-4 only and not C-6 and C-12 as anticipated.\(^{34}\) Both C-6 and C-12 are oxygenated, and on feeding of either \(1-^{13}C\)glycerate or \(1-^{13}C\)glycollate these two C\(_2\) units were equally labelled, as indicated in Scheme.


Biosynthesis

\[
\text{Me-CO}_2\text{Na} \rightarrow \text{Me-OH}
\]

\[
\begin{align*}
\text{OH} & \quad \text{O} \\
\text{HO} & \quad \text{O}
\end{align*}
\]

(101)

\[
\begin{align*}
\text{OH} & \quad \text{O} \\
\text{HO} & \quad \text{O}
\end{align*}
\]

Scheme 22

\[
\begin{align*}
\text{HOCH}_2\text{CO}_2\text{H} & \quad \text{MeCO}_2\text{H} \\
\text{HOCH}_2\text{CHCO}_2\text{H} & \quad \text{MeCH}_2\text{CO}_2\text{H}
\end{align*}
\]

(102)

Scheme 23
Biosynthesis of Polyketides

23. This is a novel variation of the usual acetate–propionate pathway. Incorporation of [6-13C]glucose resulted in enrichment of 21 of the 29 carbons. Apart from the expected enrichment of C-17 and C-21 of the C2N unit, secondary incorporation of label occurred via conversion of glucose into propionate, acetate, and methione by established metabolic pathways.

Further studies and fuller details of previous work on the origin of the skeletal carbons of the lactone ring of the 16-membered macrolide antibiotics has appeared. 13C-Enriched leucomycins and tylosins have been obtained from cultures of *Streptomyces kiiusatoensis* and *Streptomyces fradiae* respectively, fed with the appropriate precursors, and 13C n.m.r. analysis reveals that the aglycone of leucomycin A3 (104) is derived from five acetates, one propionate, and one butyrate, and an unknown precursor corresponding to two carbons. 13C-Glycine, malonate, succinate, and oxalate all failed to label this C2 unit. However, from its structural similarity to the glycollate-derived C2 units of geldanamycin, a similar origin must be highly likely. Tylosin (105) originates entirely from two acetates, five propionates, and one butyrate. In addition to the expected enrichments from primary incorporation, and in contrast to leucomycin, tylosin presented evidence for extensive secondary incorporation of precursors, especially of butyrate and 2-ethylmalonate: [1-13C]butyrate also enriched those carbons derived from the carboxyl of propionate, and [1-13C]ethyl malonate enriched those carbons derived from C-2 of propionate. To account for this, a pathway involving ω-oxidation of butyrate to succinate, and hence via the vitamin-B12-dependent methylmalonyl mutase was proposed (Scheme 24). In support of this, feeding of [1,4-13C]succinate resulted in enrichment of C-11, C-13, and C-15 as predicted. Incorporation of [1,3,1-13C]2-ethylmalonate resulted in incorporation of [1,2-13C]propionate into tylosin and clear 13C–13C couplings were observed between carbons 11 and 12, 13 and 14, and 15 and 16. Somewhat surprisingly, no enrichment of carbons 3, 4, 7 and 8 was observed, suggesting some difference in the mode of biosynthesis of these and other propionate derived carbons.

Incorporation of singly and doubly labelled [13C]acetates, [13C]methionine, and [1,13C]propionate into pseudomonic acid (106) an antibiotic metabolite of Pseudomonas fluorescens indicated the labelling pattern shown. To account for this a biosynthetic route via C12, C9, and C5 units as shown in Scheme 25 was proposed. The origin of the C11 unit from a pentaketide with introduction of two carbons from methionine is unexceptional. This C11 unit would then condense with a branched C5 unit derived from hydroxymethylglutaryl-CoA (107) as indicated; no incorporation of mevalonate was observed. The very odd labelling pattern of the C9 unit, in which C-7' is enriched by both [1,13C]acetate and [1,13C]propionate, is accounted for by both HMG-CoA and homo-HMG-CoA acting as chain starters, which then condense with two malonates with reduction, loss of the methyl or ethyl then giving the straight chain 9-hydroxynonanoate.

---

Scheme 24

---

Further investigation of this moiety is warranted, and in particular incorporations of \[^{13}C\text{malonate}\] may be revealing. The remaining observation of interest is the efficient incorporation of label from \[^{3,13}C\text{propionate}\] into those carbons derived from C-2 of acetate.

The incorporation of \[^{13}C\text{-labelled precursors}\] into the ionophore antibiotics lysocellin (108) and narasin (109) has been reported. These indicate that narasin is derived from five acetates, seven propionates, and three butyrates; and lysocellin from one acetate, eight propionates, and two butyrates, as indicated. Some interconversion of butyrate and propionate is noted in the case of narasin, and conversion of butyrate into propionate in the case of lysocellin.

The antibiotic pactamycin (110), from *Streptomyces pactum* contains two aromatic rings. Incorporation of \[^{13}C\text{acetate}\] reveals a normal tetraketide origin for

\[
\text{(108)}
\]

\[
\text{(109)}
\]

\[
\text{(110)}
\]

---

\[^{13}N.\text{Otake}, H.\text{Seto}, \text{and M. Koenuma, Agric. Biol. Chem. (Japan), 1978, 42, 1879.}\]

the 6-methylsalicylic acid moiety. In the m-amino-acetophenone moiety, however, only the methyl carbon is derived from C-2 of acetate. The labelling pattern from [6-13C]glucose suggests that the same ‘C,N’ moiety which acts as a starter in the ansamycins condenses with acetate or malonate followed by decarboxylation to produce the acetophenone. The urea carbon is enriched by both C-1 or C-2 of acetate, suggesting its origin from CO₂ produced in the Krebs cycle. The cyclopentane ring and 9-methylene are derived from glucose, but the remaining branching carbons C-6, C-7, and C-8 are derived from methionine. The labelling of both carbons of the ethyl group by methionine is of particular interest, as this feature has previously only been observed in stigmasterol and other 24-ethylsterols.⁷⁹

Further details of the biosynthesis of tenellin (111) have appeared.⁸⁰ [13N]-Phenylalanine is incorporated intact, which confirms that it is the amino-acid and

not the corresponding keto-acid that participates. The proposed pathway is summarized in Scheme 26. Condensation of polyketide and phenylalanine is suggested, to give a tetramic acid intermediate. Subsequent hydroxylation of the aromatic ring might give a quinomethane (112) which could induce rearrangement to the pyridone ring.

Full papers have appeared describing the details of previously reported work on chartreusin, daunomycin and islandicin, the prodigiosins, and piericidin A.

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V. International IUPAC Symposium on MYCOTOXINS and PHYCOTOXINS
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2H-LABELLING STUDIES ON AFLATOXIN B₁ AND ITS PRECURSORS

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The aflatoxins are highly modified decaketide metabolites. Several compounds which appear to be intermediates on the biosynthetic pathway to aflatoxin B₁ have been isolated from both aflatoxin-producing and non-aflatoxin producing fungi. The outlines of the proposed pathway are shown in Scheme 1 and although widely accepted, await rigorous proof. Many studies describing interconversions of various of these proposed intermediates have been reported but virtually all this work has used metabolites which have themselves been labelled biosynthetically from acetate (14C or 13C). This method is notoriously prone to producing misleading results. Moreover, very little is known of the detailed mechanisms involved in the interconversion of the key intermediates eg conversion of the C6 side chain of averufin into the bis-furanoid moiety; conversion of anthraquinone to xanthone; and xanthone to coumarin metabolites. We describe here preliminary results from a programme designed to study all of these points.

![Scheme 1](attachment:Scheme_1.png)

[4'-2H₂]-averufin (1), prepared by acid catalysed exchange, was fed in acetone solution to shaken cultures of Aspergillus flavus and the
Aflatoxin B₁ (2) produced was analysed by 55 MHz ²H nmr. The resultant spectrum showed a strong signal at 6H 6.45 corresponding to the anticipated signal for H-16. On conversion of the labelled aflatoxin B₁ to tetrahydrodeoxyaflatoxin B₁ (3) on catalytic hydrogenation, the ²H nmr spectrum now showed two signals at 6H 3.59 and 4.07 due to the 16-pro-S and 16-pro-R hydrogens respectively, the greater intensity of the latter signal indicating that reduction from the less hindered 15 re, 16 re face predominates. Thus averufin is incorporated intact into aflatoxin B₁ and is rigorously established as an intermediate on the aflatoxin biosynthetic pathway.

In order to obtain preliminary information on the mechanisms of the biochemical transformations of the intermediates on the aflatoxin biosynthetic pathway, we have studied the mode of incorporation of acetate-derived hydrogen into averufin, sterigmatocystin and aflatoxin B₁. Both direct (²H nmr) and indirect (¹³C nmr) methods of monitoring ²H incorporation have been used. The 90.6 MHz nmr spectrum resulting from incorporation of [1-¹³C,²H₃]acetate into averufin by static cultures of A. toxicarius (ATCC 24551) shows, figure 1, ²H-isotope shifts on carbons 6, 8, 1', 3' and 5' consistent with the incorporation of one ²H on carbons 5, 7, 2' and 4'; and 1-3 ²H atoms on C-6', confirming its origin from the starter acetate group of the precursor decaketide. Significantly no ²H was incorporated at C-4 and much less at C-7 than C-5. These observations were confirmed by direct ²H nmr studies of [²H₃]acetate-enriched averufin. The loss of ²H label from these positions may simply reflect random exchange processes occurring in the very early stages of
biosynthesis. However both sterigmatocystin and aflatoxin B1 (see below) do show similar loss of the acetate-derived hydrogen at C-11 and C-9 respectively (ie their equivalent positions to C-4 of averufin). However they do show markedly different behaviour at their equivalent of C-7 in averufin (ie C-4 in sterigmatocystin and C-4 in aflatoxin B1). These differences are the subject of further investigations in progress.

The complete $^{13}\text{C}_{13}\text{C}$ coupling pattern in sterigmatocystin enriched from [1,2-$^{13}\text{C}_2$]acetate by cultures of A. versicolor (NRRL 5219) has been determined and confirms that no randomisation of labelling occurs. Thus a symmetrical intermediate as recently proposed appears to be unlikely. $^2\text{H}$-labelling studies (both direct and indirect) indicate that acetate-derived hydrogen is incorporated on carbons 17, 15 and 6, but not 4 or 11. The observed retention of acetate derived hydrogen at C-6 is of crucial importance as it rules out all mechanisms for xanthone formation necessitating introduction of phenolic hydroxyl at this carbon during the biosynthesis of sterigmatocystin. On the basis of existing information, three mechanisms appear to be feasible. These, outlined in Scheme 2, are Michael addition followed by oxidative decarboxylation, path (a); oxidative coupling to give a spiro-intermediate, cf erdin', followed by rearrangement and decarboxylation, path (b); or addition to an epoxide, path (c).

This latter mechanism is the one we prefer as it has the merit of leading to a previously proposed intermediate which by concerted decarboxylation and elimination would give sterigmatocystin. The timing and mechanism of the required loss of phenolic hydroxyl from C-6 of versicolor A remains to be determined and is the subject of work in progress.

Similar $^2\text{H}$ labelling studies on aflatoxin B1 itself show that acetate derived hydrogen is incorporated on carbons 16, 14, 5 and 4. The $^2\text{H}$ nmr spectrum of the [2-$$^2\text{H}_3$]acetate-enriched aflatoxin B1 is shown in figure 2a, along with the spectrum of universally labelled aflatoxin B1 for comparison. This sample is produced by the simple expedient of growing
A. flavus on a medium supplemented with 10% D_2O. Thus it would appear that the first essential step in the conversion of sterigmatocystin to aflatoxin B_1 is hydroxylation at C-6 with migration of the 4'H at C-6 of sterigmatocystin to C-5 of 6-hydroxysterigmatocystin by a NIH shift mechanism. Cleavage of the aromatic ring and formation of the cyclopentanone ring can then occur by preceded procedures as shown in Scheme 3.

Scheme 3

A Specialist Periodical Report

Biosynthesis
Volume 7

A Review of the Literature Published during 1979, 1980, and 1981

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The Biosynthesis of Polyketides

BY T.J. SIMPSON

1 Introduction

This chapter covers the literature appearing between January 1979 and December 1981 and follows the format of the previous report. It has been a particularly active period with a welcome increasing trend towards studies aimed at elucidating the mechanisms of the intermediate steps in polyketide biosynthesis. The potential of $^2$H-labelling mentioned previously has been realised and extended in the review period with several studies using both direct methods, i.e. $^2$H n.m.r., and indirect methods, i.e. $^2$H α-isotope shifts and $^2$H-$^1$C couplings in $^{13}$C n.m.r. using doubly labelled [${^2}$H,${^{13}$C]} precursors. These methods have been reviewed. A potentially more useful technique than the α-isotope shift method has been described. In this, $^2$H is placed β to the reporter $^{13}$C nucleus in a doubly labelled precursor: an isotope shift is still observable but the unfavourable relaxation and noe effects associated with $^2$H directly attached to $^{13}$C are avoided. This has been applied to only one study in the review period, but will clearly find much use. A related and also extremely useful technique makes use of $^{18}$O-induced isotope shifts in $^{13}$C n.m.r. to detect the biosynthetic origins of oxygen by incorporating doubly labelled [$^{16}$O,${^{13}$C]} precursors or by growing organisms in an atmosphere containing $^{18}$O and subsequent $^{13}$C n.m.r. analysis of the labelled metabolites. The number of studies using advanced intermediates continues to increase and $^2$H-labelling has great potential in this area. A number of books which cover aspects of polyketide biosynthesis have appeared, with Steyn’s book on the biosynthesis of mycotoxins being particularly valuable.

2 Fatty Acids

The stereochemical mechanism of enoyl reductase, the enzyme catalysing the final reduction in the cycle of condensation-reduction-dehydration-reduction that lengthens the fatty acid
chain by one \(-\text{CH}_2\text{CH}_2\)- unit at each turn of the cycle on fatty acid synthetase, appears to be species specific. As shown in Scheme 1, the enoyl reductase from yeast converts the enoyl thioester (1) to the acyl thioester (2) by an anti-addition of hydride from NADPH to the \(\text{si}\) face of the 8-carbon with protonation of the \(\alpha\)-carbon from the \(\text{si}\) face. However with the reductase from both \(E.\ coli\) and \(D.\ ammoniagenes\), a syn addition of hydrogen via a 2-re, 3-si attack occurs, whereas the reductase from rat liver also carries out a syn-addition, but this time via 2-si, 3-re attack. The stereochemistry of hydride donation from NADPH is related to the stereochemistry of addition, with the pro-4S hydrogen being used for 3-si addition and the pro-4R hydrogen being used for 3-re addition.

Incorporation studies with \([2\text{-}^{13}\text{C},\text{H}_3\text{]}\text{acetate}\) and analysis of the \(\text{H}\) isotope shifts in the simultaneously \(\text{H}\) and \(\text{H}\) decoupled \(^{13}\text{C}\) n.m.r. spectrum has shown that palmitic acid (3) is biosynthesised in the alga \(A.\ nidulans\) with a gradation of \(\text{H}\) retention along the acyl chain as shown in Scheme 2. The

\[\begin{align*}
\text{CD}_3 & = \ (79) \\
\text{CD}_2\text{H} & = \ (14)
\end{align*}\]

Relative amounts of \(\text{H}\) at each \(^{13}\text{C}\)-labelled position, as a percentage of that theoretically expected
The results are interpreted as being consistent with a 'post-malonate' exchange process, presumably associated with reversible transfer of the growing acyl chain from the acyl carrier protein to a cysteine residue of \( \delta \)-keto acyl ACP synthetase. Similar results have been obtained using \( [{}^{2}H_{3}] \) acetate in \( E. \text{coli} \).\(^{13}\)

The incorporation of \( {}^{2}H \) from \( [{}^{2}H_{3}] \) acetate into lipoic acid (4) is consistent with its formation from octanoic acid with the loss of one \( {}^{2}H \) label from C-8. The \( {}^{2}H \) incorporated at C-6 of octanoic acid is retained, and since this \( {}^{2}H \) is incorporated with the L-configuration during fatty acid biosynthesis but is known to have the D-configuration in lipoic acid, an inversion of configuration must occur at C-6 during sulphur insertion.\(^{14}\) This suggested the involvement of hydroxylated octanoic acids as intermediates. However, feeding studies\(^{15}\) with \( {}^{2}H \)-labelled 6-hydroxy-, 8-hydroxy-, and 6,8-dihydroxyoctanoic acids gave negligible incorporations and so direct introduction of sulphur at the saturated carbons of octanoic acid seems likely.

An authoritative review of the structure of fatty acid synthetase has appeared.\(^{16}\)

3 Tetraketides

Both \( {}^{2}H \) n.m.r. spectroscopy and \( \delta \)-isotope shifts in \( ^{13}C \) n.m.r. have been used to measure the incorporation of \( {}^{2}H \) from \( [{}^{2}H_{3}] \)- and \( [{}^{1}{}^{13}C, {}^{2}H_{3}] \) acetates into 6-methyl-salicylic acid (5) by \( \text{Penicillium griseofulvum} \).\(^{3}\) Both methods show that there is a preferential incorporation into the methyl of the acetyl-CO\( \text{A} \)-
derived starter unit and significantly more $^2\text{H}$ is retained at C-3 than at C-5. It is suggested that the non-uniform incorporation could arise from differing degrees of random exchange during the chain assembly process or, more interestingly, it could reflect the actual mechanisms of cyclisation and aromatisation of the precursor polyketide. More examples will be needed to test the validity of this observation.

Addition of 5-chloroorsellinic acid to growing cultures of Penicillium cyclopium inhibits the biosynthesis of penicillic acid (8) and results in the accumulation of the previously indicated intermediates orsellinic acid (6) and 3-methoxyluguinol (7) and its corresponding quinone.17

The post-gentisaldehyde part of the biosynthetic pathway to patulin (13) has been extensively investigated, using mutant strains of Penicillium urticae.18 A patulin-minus mutant, J1, accumulates phyllostine (10) and isoeoxydon (11). Another patulin-minus mutant, J2, which is blocked immediately after gentisaldehyde (9),
The Biosynthesis of Polyketides

converts both (10) and (11) to patulin in yields of 90% and 60% respectively. They are interconverted by a specific dehydrogenase and cell-free systems have been isolated from the mutants which carry out their interconversion and further transformation. A further mutant, S15, accumulates isopatulin (12) and immobilised cells of the wild-type strain convert phyllostine to isopatulin in good yield. Finally, a cell suspension of mutant P3, blocked between phyllostine and isopatulin, converts isopatulin to patulin. These results are summarised in Scheme 3. The changes in oxidation levels occurring during these transformations are somewhat puzzling and labelling studies would be useful. This and earlier works are summarised in a review which also compares patulin biosynthesis with the pathways leading to a number of other fungal lactones.

Elasnin (14), a novel inhibitor of human granulocyte elastase, has been isolated from Streptomyces noboritoensis. Incorporation of [$^{13}$C$_2$]acetate has shown it to be derived from twelve acetates but it would appear to be best regarded as a tetraketide, as the most plausible route is extension of a hexanoate starter by three 2-butylmalonate units as indicated in Scheme 4.
Acetate is incorporated into zinniol (15) by cultures of
Alternaria solani as shown.\textsuperscript{22}

Pentaketides

Incorporation studies\textsuperscript{23,24} with singly and doubly labelled
$^{13}$C-acetates have confirmed that the dihydroisocoumarin moiety of

the important mycotoxin ochratoxin A (16) has a regular poly-
ketide origin and so previous proposals of a phenylpropanoid
precursor are no longer tenable. Similar results have been
reported for mellein (17) in Aspergillus melleus.\textsuperscript{25}

Austdiol (19) is a toxin produced by Aspergillus ustus. In-

\begin{equation}
\begin{aligned}
\text{Me-} &\text{COONa} \\
\rightarrow &\text{C}_{1}\text{H}_{2}\text{OBBR}
\end{aligned}
\end{equation}

\begin{enumerate}
\item[(16)]
\begin{equation}
\begin{aligned}
\text{Me-} &\text{COONa} \\
\rightarrow &\text{C}_{1}\text{H}_{2}\text{OBBR}
\end{aligned}
\end{equation}

\end{enumerate}

\begin{enumerate}
\item[(17)]
\begin{equation}
\begin{aligned}
\text{Me-} &\text{COONa} \\
\rightarrow &\text{C}_{1}\text{H}_{2}\text{OBBR}
\end{aligned}
\end{equation}

\end{enumerate}

\begin{enumerate}
\item[(18)]
\begin{equation}
\begin{aligned}
\text{Me-} &\text{COONa} \\
\rightarrow &\text{C}_{1}\text{H}_{2}\text{OBBR}
\end{aligned}
\end{equation}

\end{enumerate}

Scheme 5
The Biosynthesis of Polyketides

corporation of $^{13}$C$_2$acetate resulted in two $^{13}$C-$^{13}$C couplings being observed for carbons 5, 6, 7, 8, and 9 while carbons 11, 3, 4, 10, 12, and 1 showed only one coupling. On feeding $^{13}$Cmethionine, C-13 was enriched approximately twice as much as C-1 and C-12. This labelling pattern is consistent with a biosynthetic pathway, shown in Scheme 5, where a methionine-derived methyl is oxidised and the polyketide carboxyl is reduced to give the symmetrical dialdehyde (18) as an intermediate. 26

Full details of $^{13}$C-labelling studies on aspyrone (20) have appeared. 25 Asperlactone (21) is a co-metabolite of aspyrone in A. melleus and has the same carbon skeleton. Computer-aided resolution enhancement of the $^{13}$C n.m.r. spectrum of $^{13}$C$_2$acetate-enriched asperlactone shows a 2-bond $^{13}$C-$^{13}$C coupling between C-2 and C-8. Pathways involving rearrangement of a linear polyketide (22), or rearrangement and cleavage of an aromatic precursor (23), were proposed. 27 However, on incorporation of [2-$^{13}$C, $^2$H$_3$]acetate and determination of the simultaneously $^1$H and $^2$H noise-decoupled $^{13}$C n.m.r. spectrum, the resonance for the C-7 methyl showed two isotopically shifted signals, indicating that two acetate-derived hydrogens are retained on C-7, and so intermediates in which this carbon forms part of an aromatic ring are excluded. 28 On the basis of stereochemical differences between aspyrone and asper-
lactone, the epoxide (24) is proposed as a common intermediate; alternative modes of attack by the carboxylate on the epoxide would lead to (20) and (21) as shown in Scheme 6. Oxygen-18 labelling studies should yield further information on this point.

The biosynthesis of diplosporin (25), a toxic metabolite of Diplodia macrospora, has been studied, using $^{13}$C-labelled acetates and methionine.\textsuperscript{29} The results indicate its derivation from a pentaketide chain, folded as shown in Scheme 7, with C-5 and C-2 derived from the C\textsubscript{1}-pool. The presence of a methionine-derived carbon atom in a carbocyclic ring is highly unusual; cf biogenesis of tropolones via rearrangement of 3-methylorsellinic acid. The mechanism may proceed via methylation of the pentaketide at either C-4 or C-8 followed by oxidative activation of the newly formed C-methyl to facilitate ring closure. The introduction of a methionine-derived carbon into a heterocyclic ring is also uncommon and the possibility that it is introduced via O-methylation, cf rotenone, cannot be excluded.
Full details of $^{13}$C, $^2$H, and advanced precursor studies on sclerin biosynthesis in _Sclerotinia sclerotiorum_ have appeared. These are consistent with formation of sclerin (27) via ring cleavage and reorganisation of the carbon skeleton of sclerotinin A (26). A full paper has appeared on $^{13}$C- and $^2$H-labelling studies on scytalone biosynthesis in _Phialaphora lagerbergii_. To account for the lack of $^2$H incorporation from acetate on C-2 and C-7 it is suggested that scytalone (28) may be formed via deacylation of a hexaketide-derived naphthol, (29). Such compounds are known – see _9-methylasparvenone_ (46) below. Attempts to incorporate $[^{13}$C]malonate to check for a ‘starter’ effect were unsuccessful in this study. However, [2-$^{13}$C]malonate has been incorporated with high efficiency into scytalone and no ‘starter’ effect was observed. 

The antifungal metabolite citrinin (34), produced by _Penicillium citrinum_, has been the subject of intensive study by several research groups and notable use has been made of advanced precursors. The isocoumarin (35), labelled with $^{14}$C at C-9, was specifically incorporated into citrinin whereas label from (36) was only incorporated after prior degradation to acetate, indicating that methylation of the polyketide precursor occurs before aromatisation. However, in a study using a novel technique where _P. citrinum_ was cultured in D$_2$O, incorporation of [1,2-$^{13}$C$_2$, $^2$H$_3$]-acetate and subsequent $^{13}$C n.m.r. analysis allowed the origin of the hydrogens to be elucidated. This indicated that the hydrogen on C-4 of citrinin was acetate-derived and so, although (35) is specifically incorporated, it cannot be an obligatory intermediate on the pathway. (This was confirmed by a $^2$H-labelling study using [$^2$H$_3$]acetate and $^2$H n.m.r. (35). This study also revealed a marked difference in the protium content at C-1 and C-3, suggesting that the necessary reductions at these two sites are carried out at markedly different stages in the biosynthesis. Taken with the non-intermediacy of (35), this indicated that either the lactone (37) (reduction at C-3 but not C-1) or the aldehyde (30) (reduction at C-1 but not C-3) must be the first enzyme-free intermediate on the pathway. Both these compounds were synthesised with a single $^2$H label on the C-11 methyl and fed to cultures of _P. citrinum_. $^2$H n.m.r. analysis showed that only (30) was incorporated into citrinin. The incorporation efficiency was 6.5%, with a dilution value of ca 62.5. In a further interesting experiment _P. citrinum_ was grown in the presence of ethionine, which is known to inhibit methylation,
resulting in suppression of citrinin production.\textsuperscript{38} On incorporation of (30) labelled with \textsuperscript{2}H at C-1 in the presence of ethionine in a replacement medium, a small amount of citrinin was isolated. Now, it was so highly enriched that the specific incorporation of (30) could be demonstrated by \textsuperscript{1}H n.m.r. The incorporation rate was now 9.5\% but the dilution was only 1.25.

Parallel work by Scolastico and co-workers, using specifically \textsuperscript{14}C-labelled precursors, has shown that both (30) and (32) are specifically incorporated,\textsuperscript{39, 40} and so the pathway shown in Scheme
8 is indicated for citrinin biosynthesis. Sankawa and co-workers have reported incorporations of \([2-^{13}C,^{2}H_{3}]\), \([1-^{13}C,^{18}O_{2}]\), and \([1-^{13}C,^{17}O]\) acetates into citrinin. The results are consistent with Scheme 8, and in the \(^{13}C\) n.m.r. spectrum of \([1-^{13}C,^{18}O_{2}]\) acetate-enriched citrinin, isotopically shifted signals are observed for the resonances due to C-3, C-6, and C-8, indicating origin of the attached oxygens from acetate, so that the quinone-methide structure must be formed by elimination of the hemi-acetal hydroxyl from (32).

**Hexaketides**

Incorporations of \(^{13}C\)-labelled acetates and methionine and of \(^{14}C\)-labelled advanced precursors into ascochitine (41), a
metabolite of the phytotoxic fungus *Ascocytta fabae*, have been reported. These show its derivation from a single hexaketide chain with introduction of three C\(_1\) units from methionine to give a quinone-methide structure related to citrinin. The aldehyde (39) and quinone-methide (40) are specifically incorporated. The specific incorporation of the methyl ester (42) shows that the organism can convert it directly to the enzyme-bound thioester (38). The enol lactone (43) is also specifically incorporated, but 'enzyme trap' experiments show that it is not on the direct pathway, indicating that aldehyde (39) is formed by direct reduction of the thioester. Thus, the pathway shown in Scheme 9 can be proposed.

Incorporations of singly and doubly \(^{13}\)C-labelled acetates into \(0\)-methylasparvenone (46), a dihydronaphthalene metabolite of *Aspergillus parvulus*, indicated a hexaketide origin with the novel acetate-assembly pattern shown in Scheme 10. Incorporation of \(^{2}\text{H}_3\)acetate and analysis of the resultant \(^2\text{H}\) n.m.r. spectrum showed labelling of the 10-methyl, 5-, 2-axial, 3-axial hydrogens and significantly no labelling at C-4. The loss of label from C-4 and its appearance on C-3 can only be explained by an N.I.H. shift, which implies that hydroxylation of a 1,6,8-trihydroxy-naphthalene (44) to the corresponding 1,4,6,8-tetrahydroxy-naphthalene (45) is a necessary step in the biosynthesis of (46). The 10-methyl is labelled to less than twice the level of H-5. This is significant as, in a number of \(^2\text{H}\)-labelling studies, preferential labelling of the acetyl-CoA-derived 'starter' position relative to positions derived from malonyl-CoA is
observed. This therefore suggests that the necessary loss of ketide oxygen from C-9 occurs after aromatisation, allowing loss of label from C-10 relative to C-5 by exchange from an acetyl side-chain and via reduction to and dehydration of the resultant 1'-hydroxyethyl group during conversion to the ethyl side-chain. Support from this comes from a related study on the biosynthesis of the naphthoquinone (47), a metabolite of *Hendersonula toruloidea.*

![Scheme II](image)

Incorporation of [13C]acetate gave the assembly pattern shown and incorporation of [2-13C,2H3]acetate indicated retention of only two acetate-derived hydrogens on C-10, consistent with its derivation from the acetate ‘starter’ and via the sequence \( \text{CH}_3\text{CO}^- + \text{CH}_3\text{CHOH} + \text{CH}_2\text{CH}^- + \text{CH}_3\text{CH}_2^- \).

Further details have appeared of biosynthetic studies on multicolic, multicolic, and multicolosic acids (51; \( R = \text{Me}, \text{CH}_2\text{OH}, \) and \( \text{CO}_2\text{H} \) respectively) in *Penicillium multicolor.* Specific incorporation of 6-pentyl[2-14C]resorcylic acid (48), fed as the ethyl ester, confirmed the conclusions from [13C]acetate incorporations that these compounds are formed by oxidative cleavage of the
aromatic ring of (48). The absence of randomisation of \(^{13}\text{C}-^{13}\text{C}\) couplings in the \(^{13}\text{C}_2\)acetate-enriched metabolites excludes symmetrical intermediates, eg 5-pentylresorcinol, and, as shown in Scheme 11, the suggested pathway proceeds via the arene oxide (49) and the trihydroxyphenol (50). It seems likely that \(\omega\)-oxidation of the pentyl side-chain occurs at a late stage in the biosynthesis.

6 Heptaketides

A long-standing biogenetic postulate is the derivation of a structurally diverse group of fungal metabolites by modification of a common heptaketide-derived precursor.47 Although the structure of fulvic acid (53) strongly suggests its formation by oxidative

![Scheme 12](image)

ring cleavage of an intermediate, eg (52), derived from a single heptaketide precursor, there is some evidence that the closely related citromycetin is formed by condensation of two separate polyketide chains.48 Incorporation of \(^{13}\text{C}_2\)acetate into fulvic acid in Penicillium brevisporum results in high enrichment and in addition to the anticipated \textit{intra}-acetate \(^{13}\text{C}-^{13}\text{C}\) couplings, extra satellites were observed due to \textit{inter}-acetate unit coupling arising from multiple enrichment of individual molecules.49 The \(^{13}\text{C}\)-enrichment of the individual acetate-derived units was calculated to be ca 45% at the time of polyketide chain assembly in
the presence of labelled acetate (subsequent dilution due to biosynthesis from endogenous acetate gives an overall enrichment of ca 2.6%). As the same level of inter-acetate couplings is observed between all the acetate units it is therefore suggested that a single chain origin is more likely than one in which separately formed chains come together. However, while the single-chain hypothesis is most attractive, in view of our poor understanding of the fundamentals of polyketide assembly processes at the enzyme level, these observations cannot be taken in any way as conclusive evidence. $^{13}$C-Malonate or possibly $^{18}O$-labelling studies would be more definitive, and ultimately advanced precursor studies are essential here. A potential precursor would be fusarubin, and the acetate assembly pattern of dihydrofusarubin (54) has been determined by feeding $^{13}$C$_2$-acetate to Fusarium solani cultures. This shows that (54) is biosynthesised via a heptaketide chain, folded as shown in Scheme 12. Deuterium from (2-$^{13}$C, $^2$H$_3$)-acetate is incorporated only into the 11-methyl and the observed isotopically shifted signals show that up to three $^2$H atoms are retained, thus proving its origin from the acetate 'starter' unit. Interestingly, parallel $^2$H n.m.r. analysis shows the presence of some molecules containing $^2$H but not $^{13}$C. This suggests that during conversion of the enriched acetyl-CoA to malonyl-CoA, some $^2$H is transferred to BCCP, where it does not exchange rapidly with the medium and is available for conversion of endogenous malonyl-CoA to $^2$H-enriched acetyl-CoA.

Similar results are reported for incorporation of $^2$H- and $^{13}$C-labelled acetates into elsinichromes C and D (55) by Pyrenochaeta terrestris. Both the C-14 and C-16 methyls incorporate up to three $^2$H atoms. Thus both are derived from acetate 'starter'
units, excluding possible one-chain pathways, and so (55) is derived by dimerisation of two heptaketide-derived moieties, presumably by oxidative coupling after aromatisation. Note that in relation to the fulvic acid results above, extensive inter-acetate coupling was observed in \(^{13}C_{2}\)acetate-enriched (55) and there were no differences in the levels between and within the separate heptaketide-derived moieties.

Full details have appeared of biosynthetic studies with \(^{13}C\)-labelled acetates and malonate on the phenalenone antibiotics deoxyherqueinone (59) and herqueichrysin (57) in *Penicillium hernuei.*\(^{52}\) Herqueichrysin is the only member of the group to have

![Scheme 13](image)

the alternative orientation of the fused dihydrofuran ring. The co-occurrence of (57) and (58) suggests their formation from a common precursor (56) in which the 1,1-dimethylallyl moiety can cyclise to either of the adjacent phenolic hydroxyls, Scheme 13. The absolute configuration of herqueinone has been determined and a biosynthetic mechanism is proposed to account for the stereochemistry by initial introduction of the prenyl substituent at a chiral bridgehead carbon.\(^{53}\)

Further studies on incorporation of \(^2H\) label from both acetate and the medium into griseofulvin by cultures of *P. urticae* are reported.\(^{54}\)
The results of $^{13}$C and $^2$H n.m.r. analyses of $[^3H_3]$- and $[^{13}C_2]$-acetate-enriched tajixanthone (60), prepared by feeding experiments with Aspergillus variicolor, are reported. These indicate derivation of the xanthone via ring cleavage of a precursor derived from an octaketide, folded as shown in Scheme 14.
Scrambling of label from \(^{13}\text{C}_2\)acetate in ring C indicates that ring cleavage must precede C-prenylation and the stereospecificity of \(^{13}\text{C}\)-labelling in the dihydropyran ring moiety is consistent with cyclisation of the C-prenylaldehyde moiety in (59) by a concerted 'ene' reaction, suggesting that dihydropyran ring formation precedes formation of the xanthone ring system. The lack of \(^{2}\text{H}\) label on C-25 implies cleavage of an anthraquinone rather than an anthrone as previously suggested; and the observation of \(^{2}\text{H}\) on C-2 but not C-5 indicates that decarboxylation of the octaketide precursor occurs after cyclisation and aromatisation.

This latter result contrasts somewhat with studies on anthraquinone biosynthesis in *Penicillium islandicum*. The synthetically \(^{14}\text{C}\)-labelled diketonaphthol (61) was fed to surface cultures to yield radioactive islandicin (62) and skyrin (63) with significant incorporation rates, 0.61 and 0.46\% respectively. This would appear to be the first detection of bicyclic intermediates in anthraquinone biosynthesis in microorganisms.

However, two problems posed by these results are the required aldol condensation onto a mono-activated methyl rather than the usual doubly activated methylene; and the incorporation into skyrin implies loss of ketide oxygen and subsequent reoxidation of the same, an unactivated position, which seems highly inefficient biosynthetically. Oxygen-18 studies to confirm the origins of the oxygens would be worthwhile.

Incorporation of \(^{13}\text{C}_2\)acetate by *Alternaria solani* into
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altersolanol A (64) reveals the expected assembly pattern for an octaketide-derived anthraquinone. On refeeding the $^{13}$C-enriched altersolanol A to a mutant blocked for altersolanol A production, macrosporin A (67) was isolated and shown to be highly $^{13}$C-enriched. Small amounts of altersolanol B (65) and (66) were also isolated. Thus, it is claimed that altersolanol A is metabolised by A. solani to altersolanol B and macrosporin A. However, the mechanisms are somewhat difficult to visualise.

Concurrent incorporation of [3-$^{14}$C]emodin (68) and [11-$^{14}$C]emodinanthrone (69) into secalonic acid D (70) by cultures of Penicillium oxalicum, and proving specificity of incorporation by Kuhn-Roth oxidation followed by Schmidt degradation of the resultant acetic acid, showed that the anthrone was incorporated 4.5 times better than the anthraquinone. These results again necessitate loss of phenolic hydroxyl from the precursors.

The benzisochromanequinone system is found in a large class of microbial metabolites. Several studies on the biosynthesis of this system have been reported. [1-$^{13}$C] and [2-$^{13}$C]acetates are incorporated into granticin (72) by Streptomyces violaceoruber as indicated in Scheme 15. Similar results are reported using Streptomyces olivaceus. The remaining carbons are derived from...
glucose, which is converted into a 2,6-dideoxyhexose and attached to the aromatic moiety by C-C linkages at C-1 and C-4. Conversion of glucose proceeds with retention of H-1, H-2, H-4, and the hydrogens at C-6 and loss of H-3 and H-5. Feeding (6R)- and (6S)-D-[4,5,6,7,13C]glucose, followed by Kuhn–Roth oxidation, and determination of the chirality of the methyl of the resultant acetic acid showed that the hydroxyl group at C-6 of glucose is replaced with inversion of configuration by intramolecular transfer of a hydrogen from C-4. The hydroxyl group at C-2 is replaced by hydrogen with retention of configuration. The last step in the biosynthesis of granticin seems to be formation of the lactone ring, as a cell-free extract of *S. violaceoruber* was shown to catalyse
formation of (72) from dihydrogranticin (71) without incorporation
of $^{18}$O from $^{18}$O$_2$ to rule out a hydroxylation-lactonisation
mechanism, and direct cyclisation of the carboxyl onto a quinone-
methide intermediate is proposed. Contrasting results have
been found in biocconversion studies of the nanomycins in cultures of
Streptomyces rosa treated with cerulenin, which is known to inhibit
the early stages of polyketide biosynthesis. These suggest that
nanomycin D (73) is formed first and is converted to nanomycin A
(74), then to E (75), and finally to B (76).

A further benzoisochromane-quinone metabolite, actinorhodin
(77), is elaborated by Streptomyces coelicolor. Its structure
was known, apart from the point of dimerisation. Incorporation
of $[^{13}C_2]$acetate established the acetate-assembly pattern shown
in Scheme 16, and in addition the point of dimerisation was shown
to be a carbon enriched from $[2,^{13}C]$acetate, $^{13}$C-10.\(^61\)

8. Nonaketides

Incorporations of singly and doubly $^{13}$C-labelled acetates and
[$2-{^{14}}C$]- and [$2,^{13}$C]malonates by cultures of Pyrenochaeta
terrestriis indicate that the aza-anthraquinone phomazarin (78) is biosynthesised by condensation followed by oxidative ring fission of a nonaketide precursor, folded as indicated in Scheme 17.62 Although a large number of metabolites appear to be formed by oxidative metabolism of anthraquinones, the cleavage of a benzenoid ring as in phomazarin biosynthesis, rather than the quinonoid ring, is highly unusual.

Incorporations of [l-13C] and [13C2]acetates into the novel 9,9'-bianthryl antibiotic setomycin (79) by cultures of Streptomyces pseudovenezuelae show that (79) is formed by oxidative coupling of two nonaketide-derived moieties, assembled as shown in Scheme 18 with loss of the terminal carboxyls.63

Two parallel pathways have been shown to operate simultaneously in the biosynthesis of aurovertins B (80) and D (81) in Calcarisporium arbuscula.64 Incorporations of 13C-labelled acetates, methionine, and propionate indicate that C-1, C-2, and C-3 can be derived either from propionate or from an acetate unit plus a methyl from methionine. In the first pathway therefore a propionate 'starter' is extended by eight malonates, with the
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methyls attached to carbons 4, 6, and 16 derived from methionine. Incorporations of [2-\(^{13}\)C]malonate and [2-\(^{13}\)C,\(^2\)H\(_3\)]acetate reveal the absence of an acetate 'starter' unit and so the second pathway involves methylation of a C\(_{20}\) polyketide precursor at C-18, followed by loss of the chain-initiating acetate unit, C-19—C-20, with the other methyls again derived from methionine. This simultaneous operation of two independent pathways is unique amongst fungal metabolites.

9 Decaketides

The biosynthesis of the important mycotoxin aflatoxin B\(_1\) (86) continues to attract considerable attention. The generally accepted pathway is summarised in Scheme 19, but there are still major gaps and many problems to be solved in this complex pathway. Further evidence that averufin (82) is an obligatory intermediate on the pathway is provided by experiments\(^65\) in which averufin enriched biosynthetically from either \([1-^{14}\text{C}]\) or \([^{13}\text{C}_2]\)-acetates is incorporated into aflatoxin B\(_1\) with 18.5% efficiency (dilution value 5.1) by cultures of Aspergillus parasiticus ATCC 15517. Despite these excellent incorporation rates, the definitive study using a specifically labelled sample of averufin must be carried out.

The incorporation pattern resulting from incorporation of \(^{13}\text{C}\)-labelled acetates into versiconal acetate (83) by dichlorvos-treated cultures of A. parasiticus is consistent with its origin from a single C\(_{20}\) polyketide and its proposed intermediacy in aflatoxin biosynthesis.\(^66\) Two reports\(^67\),\(^68\) have appeared on the isolation of cell-free enzyme systems capable of converting versiconal acetate to versicolorin A (84). Plausible mechanisms are proposed for this interesting conversion but their rigorous establishment awaits further study.

A kinetic pulse-labelling technique for the detection of transitory intermediates on the aflatoxin biosynthetic pathway has been described. It has been applied to both sterigmatocystin (85) formation in Aspergillus versicolor\(^69\) and aflatoxin formation in A. parasiticus.\(^70\) The results are largely in accord with the accepted sequence of intermediates. However, one ambiguity was the observation that radioactivity from \([1-^{14}\text{C}]\)acetate appeared in the aflatoxins before it appeared in sterigmatocystin. This suggests that sterigmatocystin may not
actually be on the direct aflatoxin pathway and might explain, *inter alia*, why *A. versicolor* does not produce aflatoxins.

In one of the highlights of the review period, the first reported biosynthetic application of $^{18}$O isotope shifts in $^{13}$C n.m.r. has elucidated the origins of all the oxygen atoms in averufin. $^{71}$ [l-$^{13}$C,$^{18}$O$_2$]Acetate was added to cultures of *A. parasiticus* and the enriched averufin was analysed by $^{13}$C n.m.r. As expected, carbons 1, 9, 8, 6, 11, 14, 3, 1', 3', and 5' were highly enriched. However, on expansion of the signals for carbons
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directly attached to oxygen, all except C-5' and C-10 showed isotopically shifted resonances (\(\Delta C 0.01-0.03\) p.p.m.), indicating incorporation of \(^{18}O\), at C-1, C-3, C-6, C-8, C-9, and C-1'. Comparison of the integral ratios of the shifted and non-shifted signals suggested that about half the oxygen label was lost. In a second experiment, A. parasiticus was grown in an atmosphere highly enriched with \(^{18}O_2\). This resulted in an isotopically shifted signal being observed for C-10 only.

10 Macrolides and Ionophores

The macrolide and ionophore group of antibiotics consist of long, usually polyoxygenated, carbon chains derived by combination of acetate, propionate, and butyrate units. Several groups of workers have reported studies using mainly \(^{13}C\)-, \(^2H\)-, and \(^{18}O\)-labelled precursors, aimed at establishing the mechanistic details involved in the biosynthesis of these compounds, in particular with a view to establishing the extent to which the obvious similarities to classical fatty acid biosynthesis are in fact applicable to these functionally and stereochemically far more complex compounds.

One of the simpler systems to be studied has been brefeldin A
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(87), a metabolite of *Penicillium brefeldianum*. Oxygen-labelling studies have established that the C-1 and C-15 oxygens are labelled equally by $[^{18}O]$acetate and that the C-4 and C-7 oxygens are labelled by two different molecules of oxygen. Further studies using $[^{14}C, {^3}H]$, $[^{2-13}C, ^2H_3]$, and $[^2H_3]$acetates have established that acetate-derived hydrogen is incorporated into brefeldin A with the regiospecificity and stereospecificity shown in Scheme 20. The results are fully discussed in terms of their similarities and differences to fatty acid biosynthesis. Noteworthy features are the unexplained lack of acetate-derived hydrogen on C-12 and the retention of both acetate-derived hydrogens on C-14, suggesting that the initially formed acetoacetyl-enzyme is rapidly reduced to $\beta$-hydroxybutyryl-enzyme before significant loss by exchange can occur. A proposed mechanism for brefeldin A formation via an epoxide-initiated cyclisation is outlined in Scheme 20; cf monensin below.

Three possible mechanisms can be postulated for formation of the oxygen-bearing centres in the macrolides and ionophores: (a) retention or direct reduction of the keto group in the growing $\beta$-ketoacyl chain; (b) reduction followed by dehydration and stereospecific rehydration of the resulting enone; or (c) multistep reduction to a fully saturated deoxygenated chain followed by aerobic oxidation. A number of studies have shown path (a) to be the predominant one.

Incorporation of $[1-^{13}C, ^{18}O_2]$propionate and $[1-^{13}C, ^{18}O_2]$butyrate into lasalocid A (88) by cultures of *Streptomyces lasaliensis* establish the presence of intact $^{13}C-^{18}O$ units at C-3/O-3, C-11/O-11, C-13/O-13, and C-15/O-15. The incorporation of $^2H$ from acetate, propionate, and butyrate has also been studied and the somewhat complex results are again analysed in relation to fatty acid biosynthetic processes. All three $^2H$ labels from $[1-^{13}C, ^2H_3]$acetate are incorporated into the propionate- and butyrate-derived methyls but oddly the apparent
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acetate 'starter'-derived methyl, C-24, of (88) retains no $^2$H labels.

Incorporation of $^{13}$C-labelled precursors established the biosynthetic origins of all the carbons of monensin (91), an important antibiotic produced by *Streptomyces cinnamoniensis*. When [1-$^{13}$C, $^{18}$O]propionate was fed, the oxygens attached to C-1, C-3, and C-5 were enriched, and [1-$^{13}$C, $^{18}$O]acetate enriched the oxygens attached to C-7, C-9, and C-25. It is likely that the remaining oxygens are derived from molecular oxygen. These results are therefore consistent with the derivation of monensin by formation of the triene (89), which can be converted to monensin via cyclisation of the triepoxide (90) as shown in Scheme 21.

Similarly, incorporation of [1-$^{13}$C, $^{18}$O$_2$]propionate by
Streptomyces erythreus established that in the biosynthesis of erythromycin the oxygens attached to C-1, C-3, C-5, C-9, C-11, and C-13 of the aglycone (92) are derived from propionate.

The biosynthesis of boromycin (93) has been studied by feeding experiments with $^{13}$C-labelled malonate and methionine and $^{2}$H$_2$-valine in *Streptomyces* sp. MA 4423 followed by $^{13}$C n.m.r. and mass spectral analysis. The results show that the carbon skeleton is derived from 14 acetate/malonate units, providing carbons 1-14 and 1'-14'; methionine gives rise to the methyis at C-4 and C-4' and the gem-dimethyl groups at C-8 and C-8'. D-Valine, rather than the L-isomer, is the immediate precursor of
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The D-valyl moiety of (93). Parallel studies\(^{80}\) using \([1,3-^{13}C_2]glycerol\) with the closely related aliclomycin (94), produced by *Streptomyces griseus*, have shown that glycerol is the intact precursor of the 3-carbon 'starter' unit of the polyketide chain, C-15, C-16, and C-17. It is proposed that glycerol is converted via methylglyoxal to give lactate as the chain-initiating moiety.

![Diagram of glycerol conversion](image)

The ansamycin group of antibiotics are formed by elongation of a 'C\(_8\)N' starter unit by acetate and propionate. The exact nature of the 'C\(_8\)N' unit was unknown but two independent studies have identified it as 3-amino-5-hydroxybenzoic acid (95). [Carboxy-\(^{14}C\)]-(95) was specifically incorporated by *Streptomyces* sp. E/784 into the novel ansamycin antibiotic actamycin (96).\(^{81}\) Subsequent work showed that it was also incorporated into mitomycin (97) and h.p.l.c. analysis showed the presence of the free acid in culture filtrates of *Streptomyces verticillatus*; and its presence was also demonstrated by an isotopic dilution...
experiment using $^{13}$C-labelled (95).\textsuperscript{82} In an independent study using mutants of the rifamycin (99) producer Nocardia mediterranei it was found that a mutant P14 produced compound (98), which contains the 'C$_7$N' starter and the first three acetate/propionate chain-extending units of the ansa chain.\textsuperscript{83} On cofermentation of P14 with a second mutant A8, which produced no rifamycin and instead accumulated shikimate, or on addition of (95) to A8, the normal rifamycin-producing capability of the parent strain was restored.\textsuperscript{84}

\[ \text{MeCOONa} \quad \begin{array}{c} \text{HCOH} \ \text{CH(NH$_2$)COOH} \\ \text{Me} \quad \text{MeCOO} \end{array} \]

\[ \text{Scheme 22} \]

13C-Labelled acetates, methionine, and [3-$^{13}$C]-serine are incorporated into virginiamycin M (100) by Streptomyces virginiae as indicated in Scheme 22.\textsuperscript{85} Noteworthy is the origin of the 10a-methyl group from an external acetate. Serine enriches C-17a consistent with the formation of the oxazole ring from an acylserine precursor. Isobutyryl CoA derived from valine is the likely 'starter' unit.

14C- And $^{13}$C-labelling studies have shown that the polyketide chain methyl groups in streptolydigin (101), a metabolite of Streptomyces lydicus, are derived from propionate and not from methionine.\textsuperscript{86} The origins of the remaining carbons are yet to be
established but β-methylaspartic acid probably provides the remainder of the acyltetramic acid moiety.

\[ \text{MeCH}_2\text{COONa} \rightarrow \]

\[ \text{(101)} \]

$^{13}$C-Labelling studies have shown that methionine, acetate, propionate, and butyrate provide most of the skeleton of aurodox (102), a metabolite of *Streptomyces goldiniiensis*, but the origins of the pyridone moiety are obscure. Whereas C-2, C-4, and C-6 are enriched by C-1 of acetate, C-2 of acetate failed to enrich C-3 and C-5 of (102).
Two separate studies of mycophenolic acid biosynthesis have shown that the prenylogue (104) is converted to mycophenolic acid (107) by whole cells of *Penicillium brevicompactum*. Cell-free extracts have been shown to convert the phthalide (103) into the acyloin (105), which in turn is converted to mycophenolic acid by whole cells; Scheme 23. However, combined radioisotope chromatography-mass spectrometric analysis of *P. brevicompactum* cultures indicate that both (103) and (104) but not the acyloin (105) are active in mycophenolic acid biosynthesis.
Scheme 24
Previous $^{13}$C-labelling studies had indicated that andibenin B (110) was formed by a novel pathway in which a bis-C-methylated tetraketide-derived phenolic precursor is alkylated by farnesyl pyrophosphate to give (109) followed by cyclisation, intramolecular cycloaddition, and oxidative modification. Further evidence for this pathway has been obtained by the specific incorporation of $^{14}$C- and $^2$H-labelled 3,5-dimethylorsellinate (108) into andibenin B by cultures of *Aspergillus variicolor*. 

Orsellinic acid is not incorporated, providing clear evidence that biological C-methylation precedes aromatisation, in contrast to the post-aromatic introduction of the farnesyl moiety. Incorporation studies with $^{13}$C-labelled acetates and methionine have shown that anditomin (112), austin (111), and terretonin (113), metabolites of *Aspergillus variicolor*, *Aspergillus ustus*, and *Aspergillus terreus* respectively, despite their varied structures, can also be derived by this novel pathway. Whereas in andibenin B, the carbocyclic skeleton of (108) is retained intact, the remaining metabolites exhibit (Scheme 24) increasing degrees of cleavage and rearrangement of the original aromatic precursor. Pathways for these processes have been proposed. Interestingly, a mutant of the andibenin-producing culture produces the sesquiterpenoids, astellolides A (114) and B. 

Closely related compounds, pebrolides, have also been isolated from cultures of *P. brevicompactum* impaired in mycophenolic acid production.

### 12 Flavonoids

Though these compounds are discussed more fully in chapter 2, some features of relevance to polyketide biosynthesis are included here.

$[^{13}]$C$_2$acetate was incorporated into the flavone apigenin (116) and the flavonol kaempferol (117) by cell suspension cultures of parsley, *Petroselinum hortense*, with randomisation of $^{13}$C-$^{13}$C couplings in ring A, showing that a symmetrical intermediate, presumably the chalcone (115), is an intermediate in their biosynthesis. In contrast, $[^{13}]$C$_2$acetate is incorporated into ring A of the phytoalexin pisatin (118) in *Pisum sativum* without randomisation, showing that deoxygenation of the polyketide precursor occurs before cyclisation and aromatisation.

Incorporation of $^{14}$C- and $^{13}$C-labelled precursors into chloroflavonin (119) by cultures of *Aspergillus candidus* revealed
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Scheme 25
a distinctly different route to the flavonoid skeleton compared to plants. Phenylalanine is converted to benzoic acid, which then acts as a chain initiator, combining with four malonates to give a pentaketide. Randomisation of $^{13}\text{C}$-$^{13}\text{C}$ couplings in ring A of $[^{13}\text{C}]$acetate-enriched (119) indicates that a symmetrical intermediate must be involved, as indicated in Scheme 25.

13 Miscellaneous Metabolites

The stereochemical course of the incorporation of an intact malonate unit into the glutarimide ring of cycloheximide (120) was investigated by feeding [1,2,3-$^{13}\text{C}$]malonate to Streptomyces naraensis. The $^{13}\text{C}$ n.m.r. of the enriched cycloheximide and its derivatives indicated that the incorporation of an intact malonate unit was completely stereospecific, with the pro-$S$ acetate unit and methine carbon (C-4, C-5, and C-6) being labelled from malonate. A separate study of incorporations of $^{13}\text{C}$-labelled acetates and bicarbonate into cycloheximide, using Streptomyces griseus, also indicated stereospecific labelling of the glutarimide carbons but reached the opposite conclusion that C-2, C-3, and C-4 were derived from an intact malonate. These different conclusions appear to arise from differing assignments of the crucial C-2, C-3, C-5, and C-6 resonances.

$^{13}\text{C}$-Labelled acetates, malonate, and succinate were incorporated into rubratoxin B (122) by cultures of Penicillium rubrum as shown in Scheme 26. The results are in agreement with the assumption that the C$_{10}$-chain is formed by the fatty acid pathway and the C$_{3}$-unit via the Krebs cycle. However, when the likely intermediate (121), specifically labelled at the C-3 methyl, was fed, randomisation of label was observed, so its
status as a biosynthetic intermediate is not established.

Incorporation studies using $^2$H- and $^{13}$C-labelled acetates and methionine and $^2$H-, $^{14}$C-, and $^{15}$N-labelled tryptophan indicate the pathway shown in Scheme 27 for the biosynthesis of chaetoglobosin A (126) and 19-O-acetylchaetoglobosin A (127) in Chaetomium globosum. It is suggested that a C$_{18}$ polyketide combines with L-tryptophan, probably forming an amide linkage first, and subsequently closing the lactam ring to form the tetramic acid (123). This is then transformed by reduction and dehydration to (124), which can undergo an internal Diels-Alder cyclisation to form
chaetoglobosin J (125) before final acetylation to give (126).

A tetramic acid intermediate (128) has also been proposed in the biosynthesis of pseurotin A (129), a metabolite of *Pseudorotium ovalis*; Scheme 28. Feeding studies with $^{13}$C-labelled propionate, acetate, methionine, and phenylalanine confirm the origin of all
the carbons. Incorporation of $[2^{-13}C,^{15}N]$ phenylalanine shows that $^{15}N$ is retained, and so nitrogen-free intermediates can be excluded. 104

Incorporation of $[^{13}C_2]$ acetate into verrucarin E (130) by cultures of Myrothecium verrucaria indicates its formation by condensation of two molecules of acetacetate as shown in Scheme 29. 107
$^{13}$C and $^2$H n.m.r. analysis of cytochalasin B (131) and cytochalasin D, enriched by feeding [2-13C,2H]acetate to cultures of Phoma exigua and Zygosporium masonii respectively, indicate most of the $^2$H label was lost except at the C-11 methyl, which is derived from the acetyl-CoA-derived chain-initiating unit. Incorporation of [1-13C,18O$_2$]acetate into cytochalasin B shows that only the doubly bonded oxygens of the lactone and lactam moieties are enriched, suggesting that the hydroxyl functions are introduced in the later stages of biosynthesis.

References
14 R.H. White, Biochemistry, 1980, 19, 15.
The Biosynthesis of Polyketides

20 G.M. Gaucher, J. Food Protect., 1979, 42, 810.
32 E. Bardshiri and T.J. Simpson, unpublished results.
Biosynthesis

52 T.J. Simpson, J.C.S. Perkin 1, 1979, 1233.
The Biosynthesis of Polyketides


Introduction

This report follows the format of reports appearing previously in the series of Specialist Periodical Reports on 'Biosynthesis'.

The period of the review has been characterized by the large number of studies using precursors that are labelled with $^{13}$C and/or $^2$H and $^{18}$O. These allow the detection of labelled hydrogen and oxygen atoms, using the changes in chemical shift (isotope shifts) that are induced in $^{13}$C n.m.r. spectra by or $^{18}$O. These techniques enable more detailed biosynthetic mechanisms to be proposed and tested, and limit the possible oxidation states of intermediates which can be involved in the biosynthesis of metabolites. This is illustrated by an interesting study in which the pattern of incorporation of deuterium from $[^{2}$H$_3]$acetate into the fungal metabolites 6-methylsalicylic acid (1), mellein (2), rubrofusarin (3), and alternariol (4) has been studied and the results have been interpreted in terms of a proposed mode of action for polyketide synthetases. In the formation of aromatic products, the appropriate sites on the polyketide chain must be brought together for cyclization to occur, and the enzyme can achieve this by forming a cis-double-bond, so that the three contiguous C—C bonds that are marked by heavy lines in (5) are held in a syn arrangement. This key double-bond can be formed as shown in Scheme I either (i) by formation of an enol (5; R = OH), or (ii) by reduction and dehydration to form an unfunctionalized double-bond, i.e. (5; R = H). If the former occurs it will be a reversible process, and so one might expect greater loss of $^2$H label at site (a) relative to site (b) than in the latter case, where formation of a double-bond would be essentially irreversible, and so extra loss of label at site (a) would not be expected. The metabolites (1)—(4) provide two examples of path (i) and two of path (ii). The relative amounts of $^2$H retained at the positions corresponding to sites (a) and (b) are indicated in Scheme I and the higher retention observed at (a) for path (ii) and the lower retention observed at (a) for path (i) provide support for the ideas put forward in this paper. The patterns of retention of deuterium at the positions corresponding to sites (a) and (b) in a number of
other highly elaborated polyketides, all corresponding to path (i), e.g. averufen (6), alterolsol A (7), and scytalone (8) are also consistent with the above ideas. It is through studies of this type and the detailed "H- and "O-labelling studies which are appearing on other polyketides, in particular the macrolides and polyethers, that a picture of what is occurring at the enzymatic level in the early stages of polyketide biosynthesis can be obtained. There continues to be an encouraging number of studies using advanced intermediates, again with stable isotopes often being used as the label of choice. Reviews have appeared on applications of "O-labelling, on the 13C n.m.r. spectra of ionophore antibiotics, and on biosynthetic studies on macrolides and ionophores. The results contained in these will be discussed below, where appropriate. A new textbook on biosynthesis has a chapter on polyketides. A feature of this book is its mechanistic approach.

2 Fatty Acids

The stereochemistry of the dehydration of ß-hydroxydecanoyl thioester (9) during the biosynthesis of oleic acid has been studied, using fatty acid synthetase from Brevibacterium ammoniagenes. Deuterium-labelled oleic acid (12) was synthesized from [2-2H]malonyl-CoA by the synthetase. Mass-spectral analysis revealed that there was no deuterium on C-10. As the deuterium atom that is derived from [2-2H]malonyl-CoA is known to be located on the pro-4S position of (9), the pro-4S deuterium of (10) must have been abstracted during the prototropic rearrangement to (11) as indicated in Scheme 2. Further analysis showed that the deuterium at C-8 of oleic acid (12) occupies the pro-2R position of (11) so that a proton was added to the 2-si face during the rearrangement.

Scheme 2

\[ \text{CH}_3\text{CO}_2\text{Na} \rightarrow \text{CH}_3\text{CO}_2\text{H} \]

\[ \rightarrow \text{HO}_2\text{C} \text{CH}_3 \]

\[ \rightarrow \text{HO}_2\text{C} \text{CH}_3 \]

\[ \rightarrow \text{HO}_2\text{C} \text{CH}_3 \]

3 Tetraketides

Astepyrone (16), a novel metabolite of Aspegillus terreus, showed an incorporation pattern from [1-13C]acetate content with its being formed by oxidative cleavage of an orsellinic acid derivative. Carbonyl-13C]Orsellinic acid (13; R = CO) and orsellinic aldehyde (14) were both incorporated. Slightly higher incorporation of the aldehyde (22%), versus 13C suggests that it is the first enzyme-free intermediate, being formed by reduction of the enzyme-bound thioester (R = SEnz) (cf. citrinin and ascochitine), the incorporation of the acid being accounted for by a 'transferesterification' to thioester. Orsellinic acid 2-methyl ether was not incorporated so that O-methylation is probably a late process, occurring after a ring cleavage. Oxygen-18-labelling studies would provide interesting information on the mechanism of the ring-clos processes from the acyclic intermediate (15).

Cell-free preparations of patulin-minus mutants of Peni- lium urticae convert isopatulin (17) into both (E)- and (Z)-isomers (18, 19). However, only the (E)-isomer is further converted into patulin (19). The (Z)-isomer was shown to be produced by a non-enzymatic isomerization, catalysed by sulphhydril compounds. The biosynthetic pathway to patin in P. urticae can now be summarized as shown in Scheme 3.

4 Pentaketides

The biosynthesis of chlorine-containing metabolites of P. conia macrospinosa has been studied, using dihydro-3,14 isocoumarins. Compounds (20), (21), (22), and (23) were efficiently incorporated into the cyclopentenol cryptosporidol (25), confirming that a ring-contraction step is involved in the biosynthetic pathway. The corresponding 6-O-methyl esters were also incorporated, but at a much lower level presumably via prior biological O-demethylation. Compounds (20) and (22) were incorporated with high efficiency into co-metabolite 5-chloro-3,4-dihydro-6-methoxy methylisocoumarin (24). As with astepyrone (see above), O-methylation occurs as a late biosynthetic step. Growth P. macrospinosa on a chloride-depleted medium resulted in the production of the dihydroxydihydroisocoumarin (20) and its O-methyl analogue but no ring-contracted metabolite was detected, suggesting that chlorination is an early, essential step in the pathway. Isotope-trapping experiments provided evidence for the presence of (21) and (23) in cultures of macrospinosa, allowing the relationships shown in Scheme 3 to be proposed.
6-methylsalicylic acid \rightleftharpoons \text{acetyl-CoA + 3-malonyl-CoA}

\[
\begin{align*}
\text{Scheme 4} \\
\text{MeCO}_2\text{H} & \quad \text{HO} \\
\text{HO} & \quad \text{Me} \\
\end{align*}
\]

\[
\begin{align*}
\text{Scheme 5} \\
\text{CD}_3\text{CO}_2\text{Na} & \quad \text{OH} \\
\text{OH} & \quad \text{O} \\
\text{(26)} & \quad \text{(27)} \\
\end{align*}
\]

\[
\begin{align*}
\text{5 Hexaketides} \\
\text{Previous studies, using [}^{2}\text{H}_3\text{]acetate and }^{2}\text{H n.m.r. to study the biosynthesis of O-methylasparvenone (27) in }\text{Aspergillus parvulus, had suggested that one hydrogen was lost from the methyl of the starter acetate group, implying in turn that the necessary loss of ketide oxygen from C-9 occurred after condensation and aromatization of the linear polyketide precursor. This has been confirmed by incorporation of [}^{1-13}\text{C,}^{2}\text{H}_3\text{]acetate and analysis of the }^{2}\text{H-induced }\beta\text{-isotope shifts in the resultant }^{13}\text{C n.m.r. spectrum of the enriched metabolite.}^{14}\text{ C-9 showed two isotopically shifted resonances, indicating that a maximum of two }^{2}\text{H atoms are incorporated at C-10. In contrast to }^{2}\text{H-induced }\alpha\text{-shifts, the }\beta\text{-shifts show a marked dependence on the functionality of the reporter }^{13}\text{C nucleus, and for carbonyl carbons the shifts may even be downfield or zero (see rubrofusarin below). In addition, this study indicated that }\beta\text{-shifts can also show a marked dependence on the stereospecificity of incorporation of }^{2}\text{H. It will be interesting to see if this potentially useful observation is also made in further studies.}
\end{align*}
\]
derived 6-pentylresorcylicate (28) and establish that lactonization to form the tetronic acid ring occurs by displacement from the carboxy-group at C-1 by the enolic oxygen at C-4 of (29) as indicated in Scheme 6, and not by formation of a lactol. In this and a number of other studies the isotopically shifted resonances were obscured by signals due to long-range carbon—carbon couplings resulting from multiple incorporation of $^{13}$C labels within the same molecule. These were eliminated, using a spin-echo technique which eliminates signals due to coupled $^{13}$C nuclei. It should be noted that problems arising from multiple incorporation of precursor units into individual molecules of the final metabolite can also be overcome by the expedient of diluting the labelled substrate with unlabelled material prior to feeding.

6 Heptaketides

Griseofulvin (34) is possibly the most studied polyketide metabolite of all time! However, new information on its biosynthesis is still forthcoming. In the generally accepted route to griseofulvin, griseophenone A (31) undergoes oxidative coupling to produce, after O-methylation, the co-occurring dehydrogriseofulvin (32 $R = Me$). A possible alternative mechanism, shown in Scheme 7, would involve aerobic oxidation of (31) to the hydroxyquinone intermediate (33), which could cyclize to (32) by an addition—elimination mechanism. In this case the bridging oxygen atom would be derived from atmospheric oxygen. However, incorporation of $[1-13C, 18O]$acetate by cultures of Penicillium griseofulvum, and analysis of the $^{13}$C n.m.r. spectrum, showed that all oxygen atoms in griseofulvin are acetate-derived, so that the latter mechanism can be discounted.

Incorporation of $[13C]acetate into rubrofusarin (35) by cultures of Fusarium culmorum and analysis of the resultant $^{13}$C—$^{13}$C couplings indicates that the mode of folding of precursor heptaketide chain is as shown in Scheme 8, contrary to previous assumptions, but consistent with its post-intermediacy in the biosynthesis of fulvic acid. Incorporation of $[1-13C, 17O]$acetate resulted in deuterium $\beta$-isotope shifts being observed for carbons 8, 6, and 14, thus indicating incorporation of deuterium at carbons 7, 9, and 10. A broad unresolved envelope of $\beta$-shifted peaks was observed for which was stated as showing incorporation of acetate units retaining one, two, or three deuterium atoms at C-15. Anticipated incorporation of deuterium at C-3 could not be demonstrated by the $\beta$-shift technique because the shift on C-4 carbonyl was too small. However, its presence has been proved by direct $^2$H n.m.r. It should be noted that observation of $\beta$-shifted signals provides an alternative labelling technique for demonstrating the origin of acetate units. Contrasting results have been reported for closely related fonsecin (36). Incorporation of $[13C]acetate into (36) by cultures of Aspergillus carbonarius apparently gives an alternative folding pattern for the precursor polyketide. However, the differences in these two studies may be due to misassignment of the $^{13}$C n.m.r. spectra and we shall await details of the spectral assignments.

Cultures of Fusarium solani produce exclusively the diastereoisomeric 4a,10a-dihydropusarinins (37) and javanicin (38) if maintained at pH ~3, by providing a medium rich in maltose or by the addition of acetic acid. However, the pH is not maintained in this way, (37) and (38) are formed during the first two days when the pH is still low, but thereafter the pH increases rapidly and the amount of (37) decreases rapidly, to be replaced by an increasing portion of fusaricin (40) and norjavanicin (39). Addition of NaOH to cultures were harvested after 3 days had the same result. Thus (39) and (40) are produced non-enzymatically in cultures of Fusar culmorum. A new compound (41), which would be
intermediate in the conversion of (37) into (39), has been obtained by alkaline oxidation of (37).

Incorporation experiments with acetate that is singly and doubly labelled with $^{13}$C show that phacidin (42) is formed from a heptaketide precursor in the fungus *Pozebniamyces alsamicola*. Results with $[^{13}$C$^2$]formate show that the methoxyl and aldehyde carbons are derived from the C$_1$ pool.

Brefeldin C (43) is a deoxygenated analogue of brefeldin A (44), another much-studied compound. Feeding of [4-$^2$H$^2$]-brefeldin C to brefeldin-A-producing cultures of *Eupenicillium refeldianum* resulted in a high conversion of (43) into (44), as demonstrated by $^2$H n.m.r. and also by the observation of a triplet ($J = 19.1$ Hz) due to $^{13}$C-$^2$H coupling in the $^{13}$C n.m.r. spectrum of the enriched brefeldin A. Repeating the experiment in the presence of caerulein, which is a known inhibitor of the early steps of biosynthesis of polyketides and of fatty acids, resulted in higher specific enrichment. Thus the oxygen at C-7 of brefeldin A is not involved in the formation of the cyclopentane ring, but is introduced in the last step of biosynthesis of brefeldin A. Previous studies on the labelling of brefeldin A with $^{13}$C, $^{2}$H, and $^{18}$O and their relationship to the biosynthesis of fatty acids have been reviewed.4

Octaketides

The biosynthetic relationships in the naphthocyclinone series of isochromanonequinone antibiotics have been studied.22

Compounds (45)–(51) were produced by feeding [1-$^{14}$C$^2$]acetate to cultures of *Streptomyces arenae*. The labelled compounds were then individually re-administered to the cultures and the distribution of radioactivity among the individual metabolites was determined. This established the biosynthetic sequence γ-naphthocyclinone (45) → β-naphthocyclinone (46) → epoxide (47) → α-naphthocyclinone (48) → α-naphthocyclinone acid (49). Some conversion of the monomeric unit (50), but not of the dimer (51), was also observed, but further work is required to define the exact role of (50) in the formation of the dimeric naphthocyclinones. The conversion sequence (45) → (46) → (47) parallels the sequence of steps that has been established in the naphthomycinone series, where the lactone nanaomycin D (52) is reduced to nanaomycin A (53), which is then epoxidized to nanaomycin E. This contrasts with the situation which appears to exist in the granaticin series, where the appearance of the lactone granaticin (56) is preceded by the open-chain dihydrogranaticin (55), and the enzymatic conversion of (55) into (56) has been demonstrated. This seems a much more reasonable sequence of events. Incorporation of [1-$^{13}$C, $^{18}$O$_2$]acetate into granaticin in *Streptomyces violaceoruber* indicates that the oxygens at C-1, C-3, C-11, and probably C-13 are acetate-derived. Observations of an isotope shift at C-3 but not at C-15 demonstrate that the C-3 carbon–oxygen bond is preserved during the formation of the dihydropyran ring.5 Nanaomycin reductase, which catalyses the conversion of (52) into (53), has been isolated and studies with the enzyme have led to a proposal that (53) is formed from (52) via a hydroquinone intermediate (54), as shown in Scheme 9.23 This is essentially the same as (but in reverse to) that earlier proposed for the formation of granaticin (56) from dihydrogranaticin (55).
Previous studies with $^{13}$C- and $^{14}$C-labelled acetates had demonstrated that a benzophenone, e.g. (59), itself derived by oxidative ring-cleavage of chrysophanol (57) or islandicin (58), was a likely intermediate in the biosynthesis of the xanthone ravenelolin (60). Incorporation of $[^1-13]$C,$^{18}$O$_2$-acetate into ravenelolin in Drechslera ravenelii results in $^{13}$O isotope shifts at C-1, C-8, C-9, and C-10a but not at C-4a. These results indicate that ring-closure proceeds by nucleophilic attack of the hydroxy-group on ring A of either rotamer (59a) or (59b) on the ortho position of ring B, as shown in Scheme 10, to give a mixture of (60b) and (60c), and not (60a). The B ortho-substituent on ring A which is eliminated need not be a hydroxy-group, but (59) is a chemically reasonable hypothetical intermediate. Although an addition-elimination mechanism is proposed for ring-closure, an oxidative coupling mechanism would also be possible. The potential precursors chrysophanol (57) and islandicin (58) have not yet been proven to be on the direct biogenetic pathway, but both have been shown to occur with ravenelolin.\textsuperscript{24}\textsuperscript{25}

Full details of previously reported preliminary studies on the biosynthesis of altersolanol A (61) and related metabolites of Alternaria solani have appeared.\textsuperscript{27} Incorporation of $[^1-13]$C,$^2$H$_3$-acetate into (61) and the related macrosporin A (62) results in incorporation of $^2$H being observed at C-1, C-6, and C-9 and the methyl that is derived from the acetate starter unit. The lack of $^2$H at C-3 is consistent with derivation from an octaketide precursor, with decarboxylation occurring after cyclization and aromatization. The presence of $^2$H at C-1 of (61) indicates that hydroxylation at this position must occur after reduction to the tetrahydroaromatic level. Altersolanol A (61) is reported to be incorporated intact into (62) but the mechanism is not at all easy to visualize.

Incorporation of singly and doubly labelled $[^1]$Cacetates into achaetolide (63), which is a metabolite of Achaetomium crystalliferum, has established that it is formed from an octaketide.\textsuperscript{28} This metabolite represents a highly reduced and deoxygenated polyketide, and $^2$H- and $^{18}$O-labelling studies might reveal some interesting information on possible relationships with the biosynthesis of fatty acids and the mechanisms of removal of oxygen.

8 Nonaketides

Mevinolin (64) is representative of a group of closely related substances which have provoked considerable interest in recent years due to their ability to block the biosynthesis of cholesterol. The incorporation of $[^1-13]$C,$[^2-13]$C,$[^1,2-13]$C,$[^1-' 13]$C, $[^1-' 13]$C, and $[^1-' 13]$C, $[^2-13]$C acetates and of Me$_3$C-acetates and of Me$_3$C-methionine into mevinolin in Aspergillus terreus has been studied.\textsuperscript{29} Analysis of the enriched metabolites by $^{13}$C n.m.r. and $^2$H n.m.r. spectroscopy gave the labelling patterns summarized in Scheme 11. Oxygen-18 could be detected only at the double bonded oxygen that is attached to C-1. The lack of oxygen-18 at the expected labelling sites, i.e. C-11, C-13, and C-15, presumably due to solvent exchange during biosynthesis, though the difference between the nonaketide-derived skeleton and the a-methylbutyryl side-chain may be significant. Deuterium was incorporated at all the expected sites except C-11, C-13, and C-15, and the observation of signals due to molecules with three deuterium atoms at C-4′ and at the methyl group at C-6 confirms that these carbons are starter units of the polyketid
hams. These results are consistent with biogenesis of (64) by intramolecular Diels-Alder cyclization of a C₁₈ polyunsaturated acid or by intramolecular anionic condensations of a partially reduced polyketide. Further analysis of these results would give valuable information on the stereochemistry of the secondary modification processes, such as the formation of the double-bond between C-3 and C-4, C-methylation at C-6 and C-2, and oxidation at C-8. A notable feature of this study is the application of a two-dimensional INADEQUATE experiment to demvinolin, after heavy incorporation of [¹³C₂]acetate, to obtain a complete ¹³C n.m.r. assignment on only 20 mg of sample, because of the large increase in the relative intensity of signals for coupled carbons (ca 200-fold). Incorporation of [¹-¹³C]-, [2-¹³C]-, and [1,2-¹³C₂]-acetate into oxytetracycline (65) by Streptomyces rimosus has established the polyketide origin of the tetracyclic nucleus and the direction of folding of the hypothetical linear intermediate as that shown. Incorporation of [1,2,3-¹³C₃]malonate confirms the derivation of C-1, C-4, and the carboxamide substituent from an intact malonate, although analysis was complicated by extensive degradation of the malonate to [1,2-¹³C₂]acetate. Labelling studies have also been reported on the mycotoxin viriducatumtoxin (66), which, although a fungal metabolite, is structurally closely related to the tetracyclines. Incorporation of singly and doubly ¹³C-labelled acetates into (66) in Penicillium expansum indicated a different mode of polyketide folding to that observed for the tetracyclines. The observed incorporation of ¹⁸O from [1-¹³C, ¹⁸O₂]acetate at C-4a (also incorporated at C-1, C-8, C-12, and C-13) precludes a pathway involving a fully aromatic intermediate analogous to pretetramid, and the derivation of C-2 and of the carboxamide carbon of (66) from an intact acetate unit, together with the non-acetate origin of C-3, further distinguishes it biosynthetically from the Streptomyocyte tetracycline antibiotics. The exact nature of the starter unit of the polyketide chain is uncertain. If it is malonyl-CoA then either C-3, C-4, C-4a or C-5, C-2, and C-3 could originate from such an intact unit. Incorporation studies with [1,2,3-¹⁴C₃]-malonate, [¹⁴C]bicarbonate, or [1-¹⁴C]pyruvate failed to clarify the situation. The side-chain is presumably derived from geranyl pyrophosphate but exactly how is not clear, and should provide an interesting problem.

Incorporation studies with ¹³C- and ²H-labelled glycine suggest that, in the biosynthesis of lankicidin C (67) in a species of the genus Streptomyces, the seventeen-membered carbocyclic ring is formed by ring-contraction of an eighteen-membered ring by a Favorskii-type rearrangement, as shown in Scheme 12. The polyketide precursor is formed from glycine as a starter unit and eight intact acetate units.

The biosynthesis of citreoviridin (70), which is a mycotoxin produced by Penicillium pullulorum, has been studied, using ¹³C-labelled precursors. It is formed from a nonaketide chain, five methyl groups being derived from methionine. Incorporation of ¹³C-labelled precursors into citreomontanin (68) in Penicillium pedemontanum gives a similar labelling pattern, consistent with (68) being a possible precursor to (70). The bis-epoxide (69) would be a likely intermediate, as indicated in Scheme 13. Oxygen-18-labelling studies would test this mechanism.

### 9 Decaketides

The biosynthetic pathway to the important mycotoxin aflatoxin B₁ (84), as always, has attracted considerable attention. The doubts concerning the intermediacy of averufin (71) have been removed by two studies with specifically labelled...
Averufin was prepared by an acid-catalysed exchange reaction and incorporated into (84) by Aspergillus flavus. An analysis of the enriched aflatoxin B₁ (84) by ²H n.m.r. spectroscopy showed specific incorporation of deuterium into H-16. In this study, universally ²H-labelled aflatoxin B₁, required for comparison of its ²H n.m.r. spectrum with that of biosynthetically enriched (84), was prepared by the simple expedient of growing A. flavus in a medium that was supplemented with 10% of ²H₂O. This is a useful general technique for the preparation of universally deuteriated metabolites for determination of their complete ²H n.m.r. spectra. In a separate study, Townsend has used his recently developed synthetic route to averufin to prepare both [⁴⁻¹³C]- and [¹⁻¹³C,¹⁻²H]averufins. These were incorporated into aflatoxin B₁ in A. flavus and their intact incorporation was demonstrated by ¹³C n.m.r. spectroscopy. In particular, the spectrum of a sample of aflatoxin B₁ that had been derived from [¹⁻¹³C,¹⁻²H]averufin had a triplet (28.5 Hz) due to ¹³C-²H coupling at 113.0 p.p.m., showing that the bond between C-1⁻¹³C and H₁⁻²H coupling at 113.0 p.p.m. remained intact throughout the pathway from (71) to (84), so ruling out Favoristski-type processes in the rearrangement of the side-chain. A possible mechanism for the rearrangement of the side-chain of averufin is shown in Scheme 14. Hydroxylation of (71) to the known co-metabolite nidurufin (72), followed by pinacol-type rearrangement via (73), which (on hydrolysis) would yield the aldehyde-ketone (75), followed by Baeyer–Villiger-type oxidation of (75) would produce (76). Some evidence that the rearrangement precedes oxidation comes from the isolation of versicolorone (74) from Aspergillus versicolor. This could be a shunt metabolite from the intermediate (75).

The incorporation of acetate-derived hydrogen into averufin (71), sterigmatocystin (82), and aflatoxin B₁ (84) has been studied by incorporation of [²H₃]- and [¹⁻¹³C,¹⁻²H]acetate and analysis by either ²H n.m.r. or ¹³C n.m.r. spectroscopy (isotope shifts). The results are summarized in Scheme 14, along with results for the incorporation of [¹⁻¹³C]acetate in sterigmatocystin (82) and [¹⁻¹³C,¹⁻²H]acetate into versicolorone A (77) and sterigmatocystin (82). Three deuterium atoms were incorporated at C-6 of averufin, which confirms its origin from an acetate starter unit. Only one deuterium atom was incorporated at C-2' and another at C-4', consistent with the loss of oxygen from the side-chain by processes analogous to those in the biosynthesis of fatty acids; in the aromatic positions, it was found that significantly more deuterium was retained at C-5 compared to C-7, and that none was retained at C-4. However, in a separate study, using [⁴⁻¹³C,¹⁻²H]acetate the incorporation of [²H] at C-4 was reported, but this was done by looking at relative decreases in ¹³C resonances, which is a less reliable method.

Studies with Aspergillus versicolor showed that acetate-derived deuterium was retained at C-6, C-15, C-17, at possibly C-4 and C-11 of sterigmatocystin (82). Incorporation with [¹⁻¹³C]acetate confirms that the carbon-label pattern in ring A is not randomized, so that no intermediates are involved that are potentially symmetrical with regard to ring and oxygen-18-labelling studies show that the oxygen atom that is attached to C-1, C-3, C-8, and C-10 are acetate derived. The retention of acetate-derived hydrogen at C-6 (82) rules out previous proposals for the conversion of (77) in (82) involving the introduction of a phenolic hydroxy-group to this carbon either at the anthraquinone or the benzophenone stage. The results with oxygen-18 indicate that the hydroxyl group at C-1 of versicolorin A (77) becomes the oxygen atom of the xanthone ring of sterigmatocystin. Thus it appears that versicolorin A (77) (or a derivative of it) is oxidatively cleaved to give a benzophenone-carboxylic acid intermediate, e.g. (7 \* R = H) or (78; R = OH). Scheme 14 indicates two possible mechanisms for the closure by which the xanthone ring formed, each of which is consistent with the observed labelling. Path (a) involves oxidative coupling to give the intermediate (79; R = H) followed by rearrangement and decarboxylation. An outstanding problem is the necessary loss of the hydroxy-group from C of versicolorin A (77) on conversion into sterigmatocystin (82) and it has been suggested that this could be lost in the rearrangement of (79; R = OH) to (82). In path (b), (78) converted into an arene oxide (80), addition of the hydroxyl group of ring B followed by concerted decarboxylation as elimination then giving (82).

Incorporation of [³⁻¹³C]acetate into aflatoxin B₁ (84) showed that acetate-derived hydrogen is retained at C-4, C-5, C-14, at C-16, and possibly at C-9. The crucial observation is the retention of deuterium at C-5, which is a position that is derived originally from carboxyl of acetate, and so it must assumed that the label has migrated to C-5 from the adjacent carbon atom during the conversion of (82) into (84). This most likely to occur as a result of an NIH shift, indicating the hydroxylation at C-6 of (82) is a key step in the conversion (82) into (84). Thus 5-hydroxysterigmatocystin (83) is a likely intermediate. Scheme 14 summarizes the likely pathway from averufin to aflatoxin B₁, based on current results, but, clearly, much further work is required before the details can be known for certain. In contrast to work with whole cells, a cell-free system from Aspergillus flavus failed to convert versicolorin (77) into aflatoxin B₁ (84), but did convert versicolorin hemiacetal (85; R = H) and the corresponding acetate (8 R = Ac) into (84), so it is suggested that (77) is not on the main pathway but is simply a shunt product.
A real product report, 1984

Scheme 14
Resistomycin (86) has been shown, by incorporation of \(^{13}\text{C}\)-labelled acetates in \textit{Streptomyces griseoflavus}, to be formed from a decaketide, folded as shown in Scheme 15. The gem-dimethyls on C-1 are presumably derived from the C-1 pool. \(^{47}\) Vineomycin A (87) and vineomycin B (88) are antibacterial and antitumour metabolites of \textit{Streptomyces matensis}. Results of \(^{13}\text{C}\)-labelling experiments show that the benz[a]-anthraquinone moiety of (87) is derived as shown in Scheme 16, and that (88) is formed via cleavage of a C—C bond of (87) as indicated. \(^{48}\) Rabelomycin (89) was found to be a co-metabolite, suggesting that the necessary loss of acetate-derived oxygen from C-6 in (87) and (88) may occur (somewhat unusually) at a post-aromatic stage.

### 10 Ansamycins

A number of ansamycins bear different substituents, e.g. hydroxyl, thiomethyl, chlorine, or methyl, in position 3 of the aromatic nucleus. 4-Substituted 3-amino-5-hydroxybenzoic acids were tested in mutasynthesis experiments as potential starter units for the biosynthesis of 3-substituted rifamycins (91) in \textit{Nocardi a mediterranei}. No mutasynthesis of rifamycins was observed with compounds of the type (90), and so it is concluded from these and previous results that 3-substituents in rifamycin (and, by implication, in other ansamycin chromophores) must be introduced in a late biosynthetic step. \(^{49}\) Transformation experiments with intact mycelium of \textit{N. mediterranei} and feeding studies with \(^{13}\text{C}\)-labelled precursors show that rifamycin B (94) and rifamycin L (95) are formed from rifamycin S (92) via rifamycin SV (93) by different pathways (Scheme 17), using different C\(_3\) precursors: glycerol predominantly labels the glycolic acid moiety of rifamycin B whereas pyruvate labels the glycolic acid moiety of rifamycin L (95). Inhibition experiments indicated that thiamine-dependent enzyme, \textit{i.e.} a decarboxylase, is involved in the transformations. \(^{50}\)

Ansamitocin P-3 (97) is a maytansinoid antitumour antibiotic that is produced by a species of the genus \textit{Nocardia}. Amino-5-hydroxy[\textit{carboxy-}\(^{13}\text{C}\)]benzoic acid (96) was incorporated with high efficiency into (97) and so is the primary precursor of the aromatic nucleus. \(^{51}\) A sample of \(^{13}\text{C}\)-glucose that was diluted with \(^{13}\text{C}\)-depleted glucose was fed \textit{Streptomyces hygroscopicus} and the \(^{13}\text{C}—^{13}\text{C}\) couplings in the enriched geldanamycin (98) were analysed, using homonuclear \(^{13}\text{C}\) decoupling. \(^{52}\) This showed that the benzoquinone ring was formed from glucose-derived C\(_3\) and C\(_4\) units, as would be expected if 3-amino-5-hydroxybenzoic acid is the starter unit for the ansamycin chain. Mycotrienin (99) and macbecin (100) are unique among ansamycin antibiotics in having a 2-membered macrocyclic lactam ring. Incorporation of \(^{13}\text{C}\)-labelled precursors into (99) and (100) by cultures of \textit{Streptomyces rishiriensis} and by a species of the genus \textit{Nocardia} gave the labelling patterns summarized in Scheme 18. The cyclohexylcarboxylic acid moiety of (99) is probably derived from shikimate. The origin of the C\(_2\) unit corresponding to C-17 and C-18 of (100) remains obscure, but it is possibly derived as leucomycin (see below) from glycollate via glycerol.

### 11 Polyether Ionophores

Full details have appeared of preliminary studies of the incorporation of \(^{13}\text{C}\)- and \(^{18}\text{O}\)-labelled acetates and propionates into the polyanions.
into monensin. In addition, analysis of the monensin (103) that was produced by *Streptomyces cinnamonensis* that grew in \(^{18}O_2\) atmosphere indicated that the oxygen atoms bridging bonds 13 and 16, 17 and 20, and 21 and 25 are derived from atmosphere. A separate study confirmed these results and showed that the primary hydroxyl function at C-26 was also produced by an oxidative process. All of the results are consistent with the formation of monensin via the tri-epoxide (93), itself derived from the triene (101). Incorporation of \([\text{I-}^{13}\text{C}]\)butyrate and \([\text{I-}^{13}\text{C}]\)isobutyrate resulted in high enrichment of C-15 of monensin A and also of those carbons derived from the carboxyl carbon of propionate. These results show that isobutyrate can be isomerized to n-butyrate and n-butyrate be degraded to propionate in *S. cinnamonensis*.

Two more general papers have appeared. The first discusses the \(^{13}\text{C}\) n.m.r. spectra of polyether antibiotics and gives some empirical rules for structural studies. These include the use of \(^{13}\text{C}\)-labelled precursors for structural and spectral assignment studies. The second presents a unified stereochemical model which attempts to correlate the structures and stereochemistries of more than thirty different polyether antibiotics and to show how they can be biosynthesized via polyepoxide precursors. In a further review article, Hutchinson discusses recent work on the biosynthesis of polyether and macrolide antibiotics and fragment. On the basis of incorporation of \([\text{I-}^{13}\text{C}]\), \([\text{I-}^{13}\text{C}]\), and \([\text{U-}^{13}\text{C}]\)-glucose, a shikimate origin for the C-7N-2 unit is proposed. Addition of tryptophan or anthranilic acid to the cultures inhibits the formation of A23187 and results in the formation of its demethylamo-analogue, cezomycin. Simultaneous addition of anthranilic and 3-hydroxyanthranilic acids left the production of A23187 unaffected, leading to the proposal that 3-hydroxyanthranilic acid is hydroxylated, then aminated at C-6, giving 6-methylamino-3-hydroxyanthranilic acid as the C-7N-2 unit. Two more general papers have appeared. The first discusses the \(^{13}\text{C}\) n.m.r. spectra of polyether antibiotics and gives some empirical rules for structural studies. These include the use of \(^{13}\text{C}\)-labelled precursors for structural and spectral assignment studies. The second presents a unified stereochemical model which attempts to correlate the structures and stereochemistries of more than thirty different polyether antibiotics and to show how they can be biosynthesized via polyepoxide precursors. In a further review article, Hutchinson discusses recent work on the biosynthesis of polyether and macrolide antibiotics and
carbon atoms in the tetronic acid moiety, however, remain unresolved. Similar results have been obtained for the related chlorothricin (107) in *Streptomyces antibioticus*. 

Features are the presumed cleavage of the carbocyclic system by a Baeyer-Villiger oxidation (with the insertion of oxygen at C-13). The derivation of the oxygen atom at C-13 from propionate rules out the possibility that the avermectins are biosynthesized via a late-stage oxidation of a milbemycin metabolite. The isobutyryl starter unit is not labelled by acetate or propionate, which is consistent with the previously established role of L-isoleucine as the precursor of the starter unit in this series of avermectins. Tetrocarcin A (106), which is the major antibiotic produced by *Micromonospora chalcea*, consists of a thirteen-membered ring containing a tetronic acid moiety. Carbon-13-labelled acetate and propionate gave the labelling pattern shown. The origin of three carbon atoms in the tetronic acid moiety, however, remains unresolved. Similar results have been obtained for the closely related chlorothricin (107) in *Streptomyces antibioticus*. Features are the presumed cleavage of the carbocyclic system by a Baeyer-Villiger oxidation (with the insertion of oxygen at C-13).

12 Macrolides
Under this general heading, a number of diverse compounds, all containing at least one macrocyclic lactonic ring, are discussed. The biosynthetic origins of the carbon skeletons and the oxygen atoms of the avermectins, e.g. (105), have been studied by incorporation of $^{13}$C- and $^{18}$O-labelled acetates and propionates by *Streptomyces avermitilis*; these origins are summarized in (105). The derivation of the oxygen atom at C-13 from propionate rules out the possibility that the avermectins are biosynthesized via a late-stage oxidation of a milbemycin metabolite. The isobutyryl starter unit is not labelled by acetate or propionate, which is consistent with the previously established role of L-isoleucine as the precursor of the starter unit in this series of avermectins. Tetrocarcin A (106), which is the major antibiotic produced by *Micromonospora chalcea*, consists of a thirteen-membered ring containing a tetronic acid moiety. Carbon-13-labelled acetate and propionate gave the labelling pattern shown. The origin of three carbon atoms in the tetronic acid moiety, however, remains unresolved. Similar results have been obtained for the closely related chlorothricin (107) in *Streptomyces antibioticus*. Features are the presumed cleavage of the carbocyclic system by a Baeyer-Villiger oxidation (with the insertion of oxygen at C-13).
between C-1 and C-25) and the tetraketide-derived 5-chloro-2-thoxy-6-methyisalicylic acid moiety that is present as an er residue on the terminal sugar residue.\(^\text{66}\) Incorporation of \(\text{CJ-actates and -propionates showed that azalomycin F},\) produced by \textit{Streptomyces hygroscopicus}, contains fourteen molecules of acetate and seven of propionate, and the pattern of labelling was deduced to be as shown; carbons 41–44 are probably derived from arginine, but this has not been established by feeding experiments.\(^\text{67}\) Incorporations of singly and doubly \(\text{\(^{13}\)C-labelled acetates and propionates were used in the structural elucidation of irumamycin (109), which is a metabolite of \textit{Streptomyces subflavus}. The aglycone is derived from eight propionates and five acetates.}\(^\text{68}\)

Previous biosynthetic studies had shown that the aglycone of leucomycin (110) is derived from five acetate, one propionate, and one butyrate units, leaving a bis-oxygenated C\(_2\) unit (corresponding to carbons 3 and 4) which was of unknown biosynthetic origin. Incorporations of [U-\(^{13}\)C]glucose and of [2-\(^{13}\)C]glycerol indicate that C-3 and C-4 are derived from glycerol via glycolate.\(^\text{69}\) In a separate study, the incorporation of [\(\text{U-}\)\(^{13}\)C]glucose and of [2-\(^{13}\)C]glycerol into leucomycin (110) and protylonolide (111) by cultures of \textit{Streptomyces subflavus} and \textit{Streptomyces hygroscopicus} has been examined.\(^\text{70}\) The results are summarized in Scheme 19 and indicate that both organisms can convert valine, via isobutyrate, into n-butyrate and propionate. \textit{Streptomyces hygroscopicus} further converts the [2,4-\(^{13}\)C\(_2\)]n-butyrate that is derived from the isobutyrate into [2-\(^{13}\)C]acetate and [2-\(^{13}\)C]propionate via the glyoxylate cycle. These transformations are summarized in Scheme 20. The further conversion of protylonolide (tylactone) into tylasin has been studied by examining the bioconversions of 23 potential intermediates with a mutant strain of \textit{S. fradiae} that is blocked only in tylactone biosynthesis. In this way, a preferred sequence of reactions consisting of eight steps was established.\(^\text{71}\)
13 Meroterpenoids

Full details of incorporation studies with advanced precursors and of isotopic trapping experiments on the biosynthesis of mycophenolic acid (114) in *Penicillium brevicompactum* have appeared.\(^\text{72}\) The results show that 6-farnesyl-5,7-dihydroxy-4-methylphthalide (112) is converted into (114) by at least two pathways; these are a direct oxidation of the central double-bond and a two-stage removal of the terminal and central groups. The oxidation mechanism appears to be epoxidation, rearrangement of an epoxide to a ketone, hydroxylation to an \(\alpha\)-hydroxy-ketone, and cleavage of a C—C bond to form the a (113), as shown in Scheme 21.

Incorporation of \([1^{13}C]\)- and \([1,2^{13}C_2]\)-acetates and \([2^{13}C]\)mevalonolactone into austalide D (117), which is metabolite of *Aspergillus ustus*, and the isolation of the metabolites austalide J (116), austalide K (115; \(R = H\)), and austalide L (115; \(R = OH\)) indicate its formation via a multifunctional polyketide-terpenoid pathway, as outlined in Scheme 22, with the same key intermediate (112) as occurs in the biosynthesis of mycophenolic acid.\(^\text{73}\)
Carbon-14- and deuterium-labelling experiments, together with 2H n.m.r. spectroscopy, show that 3,5-dimethylorsellinic acid (118) is a specific precursor of both austin (119) and reteronin (120) in *Aspergillus ustus* and *Aspergillus terreus* respectively, and so substantiate the mixed polyketide–furanoid origin that was proposed for these metabolites from the incorporation of 13C-labelled simple precursors, as shown in Scheme 23.74 It is noteworthy that all of the compounds discussed in this section have the alkylation of an orsellinic acid analogue by farnesy1 pyrophosphate as a key step in their osynthesis.

4 Miscellaneous Metabolites

Corporation studies with 13C-labelled acetate, propionate, and succinate in *Streptomyces griseus* indicate that nonactic acid (121), which is the monomeric unit that is present in nonactin (122), is biosynthesized as shown in Scheme 24. Extensive interconversions of label from acetate into succinate and from succinate into propionate are ascribed to the operation of the Krebs cycle and of an active methylmalonyl-CoA mutase, respectively.75

Incorporation of 13C-labelled acetate shows that, in the biosynthesis of vancomycin (123) by *Streptomyces orientalis*, the 3,5-dihydroxyphenylglycine moiety (124), forming ring D, is acetate-derived, as shown in Scheme 25, whereas the p-hydroxyphenylglycine and m-chloro-3-hydroxytyrosine residues are derived from tyrosine.76 Similar results were obtained for the biosynthesis of ristocetin (126) by *Nocardia lurida*. Rings D and F are acetate-derived, as shown. It is suggested that the biosynthesis of the aromatic rings may proceed via an intermediate (125) with an additional chain-starting acyl group.
which is lost after the ring has been formed. This would account for the otherwise improbable ring-closure onto a terminal methyl group.\textsuperscript{77} Feeding experiments with \([1-{ }^{13}\text{C}], [2-{ }^{13}\text{C}],\) and \([1,2-{ }^{13}\text{C}_2] \)-acetates, using cell suspension cultures of \textit{Ruta graveolens}, indicate that rutacridone (129) is biosynthesized \textit{via} chain-elongation of anthranilic acid (127) with three acetate units to give the aminobenzophenone (128). Randomization of \({ }^{13}\text{C} - { }^{13}\text{C} \) couplings in ring C indicates that the formation of the acridone ring occurs before prenylation,\textsuperscript{78} as shown in Scheme 26.

A feeding experiment with \([1,2-{ }^{13}\text{C}_2] \)-acetate confirms the polyketide origin of ring B of echinatin (134) and of ring A of formononetin (133) in cultured cells of \textit{Glycyrrhiza echinata}.\textsuperscript{79} Further studies with \({ }^{14}\text{C} \)-labelled advanced precursors revealed that licodione (131; \(R = \text{H} \)) is an intermediate in the conversion of the chalcone isoliquiritigenin (130) into the retrochalcone echinatir (134). The isotopic labelling of licodione was also incorporated into 7,4'-dihydroxyflavone (132), although (130) labelled (132) more efficiently. Incorporation (131) was also low compared to that of (130) into the isoflavononoid (133). These results are summarized in Scheme 27.

Experiments on the biosynthesis of flexirubin (138), from gliding bacterium \textit{Flexibacter elegans}, using \({ }^{14}\text{C} \)-and \({ }^{13}\text{C} \)-labelled acetate and blocked mutants, showed that all of the carbon atoms of ring B are acetate-derived and that, as shown in Scheme 28, orsellinic acid (135) is converted into 3-dodecorsellinic acid (136), which is decarboxylated to (137) before the ester linkage with the 2-phenylpolyenoatecarboxylic acid is formed.\textsuperscript{80}

Volume 4 of the ‘Antibiotics’ series\textsuperscript{81} is devoted to biosynthesis and contains a number of chapters on polyketide-derived molecules, \textit{viz.} tetracyclines,\textsuperscript{81e} ansamycins, polyether antibiotics,\textsuperscript{81c} erythromycins,\textsuperscript{81d} sixteen-member macrodides,\textsuperscript{81f} other macrodides,\textsuperscript{81f} and isochromanequinone antibiotics.\textsuperscript{81g}
The Chemistry of Natural Products

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3 Aromatic compounds

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Aromatic compounds can be isolated from all possible natural sources—micro-organisms, plants, insects, mammals and marine organisms. In any discussion of them a major, and in many ways unresolvable, problem arises in trying to subdivide the huge variety of compounds and structural types which occur, and in this chapter the choice of compounds is necessarily both highly selective and highly subjective. As many reviews and books have appeared on plant phenolic substances in recent years, their coverage here bears no relation to the size and importance of the field but is limited simply to allow coverage of areas which have been less adequately treated. In general this chapter will discuss compounds in order of increasing number of aromatic rings and increasing condensation of rings.

Aromatic compounds are formed by several biosynthetic routes, in particular the polyketide and shikimate pathways, but also by the terpenoid pathway, and by combinations of some or all of these. These have been thoroughly reviewed in a number of publications¹ and so biosynthetic aspects will not be covered here in any detail, but it is important to bear in mind that many of the structural studies described have been carried out along with biosynthetic studies.

Major advances in natural product chemistry in recent years have undoubtedly been the development of \(^{13}\text{C}\) n.m.r. spectroscopy,² advances in multipulse sequences in both \(^{1}\text{H}\) and \(^{13}\text{C}\) n.m.r. for structural studies,³ and associated isotopic labelling methodology for studying both the structures and biosynthesis of metabolites.⁴ Thus \(^{13}\text{C}\) n.m.r. studies will be mentioned where appropriate.

3.1 Benzenoids

A large number of compounds containing a benzene ring continue to be isolated from many sources. The number, size and disposition of substituents
on the aromatic ring varies widely and some illustrative examples are given below.

Marine organisms are a source of characteristic bromine-containing compounds. The dibromo-amide (1) has been isolated from a sponge, Verongia aurea, whereas the red alga Rhodomela subfuscus produces 2,3-dibromo-4,5-dihydroxybenzyl methyl ether (2). A number of phenolic sesquiterpenoids, e.g. laurinterol (3) are responsible for the antibiotic activity of Laurencia (red algal) species. α-Bromocuparene (4) and α-isobromocuparene (5) have been isolated from Laurencia glandulifera and L. nipponica and it is suggested that these are the precursors of the other aromatic sesquiterpenes from Laurencia species.

Fungi are a particularly prolific source of benzenoid compounds, many of
polyketide origin, and a comprehensive listing of compounds isolated up to 1982 has recently appeared. The isomeric phthalides (6) and (7) have been isolated from Alectoria nigricans and Aspergillus flavus, respectively. 5,7-Dihydroxy-4-methylphthalide (6) is also a key intermediate in the biosynthesis of the antibiotic mycophenolic acid in Penicillium brev-compactum where it is first converted to the 6-farnesyl compound (8). An interesting synthesis of (6) proceeds (Scheme 1) via the isoxazole (9). Reaction of (9) with diketene gives the acetoacetyl ester (10); reduction and hydrolysis unmasks the latent polyketide-type precursor (11) which is converted first to the butenolide (12) and is then cyclized to the phthalide (6).
Fungal metabolites with increasing length of side chain are represented by 2,4-dihydroxy-6-propylbenzoic acid (13) from *Penicillium brevicompactum*; the sorbophenone (14) from a *Scytalidium* species; and 2-(3,4-dihydroxyhepta-1,5-dienyl)-6-hydroxybenzyl alcohol (15) from *Pyricularia oryzae*. The corresponding methyl ether (16) has also been isolated from *Aspergillus variecolor*.

Long-chain phenolic substances have been isolated from many sources. The polyunsaturated acylphloroglucinol (17) has been obtained from the brown alga *Zonaria tournefortii* along with the related unsaturated and saturated chromones (18) and (19). Fruiting bodies of the basidiomycete *Byssomerulius corium* contain large quantities of the byssomeruloids, e.g. (20) and (21). Lasolicid A (22) is a benzoic acid with a long polyoxygenated chain containing both tetrahydrofuran and tetrahydropyran rings isolated from *Streptomyces lasaliensis*. It was one of the first of the polyether antibiotics, which act as ionophores, to be isolated. A recent comprehensive review discusses the isolation, structures, synthesis and properties of the long-chain phenolic substances of plant origin.

Many of the more interesting benzene-containing compounds have substituents of isoprenoid origin. The isoprenoid moieties can themselves be highly modified. *Aspergillus duricantis* produces several hydroxyphthalides, e.g. (23) and (24). The disubstituted dihydropyran (chroman) ring of (24) is presumably formed by an intramolecular `ene` reaction between the dimethylallyloxy and formyl substituents on (23). A similar system is found in *tajixanthone* (123). Pergillin (25) is a plant-growth retarding substance isolated from *Aspergillus ustus* which contains a 2-isopropyliden-benzofuran-3-one system. *Stereum frutulosin* produces an antibiotic frutulosin (26). Its structure has been confirmed by synthesis. An alternative synthesis is outlined in Scheme 2. 2,5-Dimethoxybenzyl alcohol protected as the alkyl ether (27) was metallated with butyl lithium and iodinated to give (28). Deprotection and oxidation to the aldehyde (29) was followed by coupling with the copper acetylde derived from 3-methylbut-3-en-1-yn.

The plant pathogenic fungus *Colletotrichum nicotianae* produces a series of prenylated benzaldehydes including colletochlorin D (30) and the geranyl
AROMATIC COMPOUNDS

Scheme 2 Reagents: i. BuLi/C₆H₆, 0°C; ii. ICH₂CH₂Cl; iii. H⁺; iv. PCC; v. BBr₃; vi. Cu—C≡C−C(Me)=CH₂ DMF.

homologue, colletochlorin B. These are closely related to ascochlorin (31), a metabolite of *Ascochyta viciae*, and similar substances which have a variety of biological activities, in particular antiviral, which has stimulated much recent interest in their synthesis. A recent synthesis of colletochlorin B is summarized in Scheme 3. Birch reduction of O-dimethylocinosol gave the cyclohexadiene (32) which was lithiated and alkylated with geranyl bromide. Chlorination and aromatization gave the phenol (33) which was formylated to give collectotrichin B (34). Other synthetic approaches to ascochlorin and colletochlorins have appeared.

K-76 (35), an inhibitor of the complement system, has been isolated from *Stachybotrys complementi*. It has a bicyclofarnesyl moiety attached to a bis-aldehyde to give what is formally a dihydrobenzofuran. *Aspergillus ustus* produces a group of mycotoxins with novel complex structures, the austalides, e.g. austalide A (36). It is likely that the farnesyl-substituted phthalide (8) is a key intermediate in the biogenesis of the austalides. However the farnesyl
Scheme 3  i, Li/NH$_2$/**BuOH/THF;  ii, **BuLi/HMPA/geranyl bromide;  iii, NCS;  iv, DBU;  v, hexamethylenetetramine/AcOH.
moiety has been highly cyclized and oxidized. Note that this compound is formally a chroman.

Sesquiterpenoid-substituted benzoquinones and quinols have been isolated from both sponges and brown algae. Avarol (37) has been isolated from the sponge *Disidea avaria* and zonarol (38) from the alga *Dictyopteris undulata*.

The key feature of a synthesis of zonarol is the conjugate addition of 2,5-dimethoxyphenylmagnesium bromide (39) to the enone (40) as shown in Scheme 4. Disidein (41) is unique in having a sesquiterpenoid-derived moiety fused to a quinol. It is a metabolite of *Disidea pallescens*.

### 3.2 Coumarins

A substantial monograph on coumarins is available. The $^{13}$C n.m.r. spectra of coumarin and substituted coumarins have been analysed. Siderin (42) is a polyketide-derived compound which has been isolated both from plant (*Sideritis romana* and *Cedrela toona*) and fungal (*Aspergillus variecolor*) sources. Its structure has been confirmed by synthesis. Kotanin (43), a dimer of siderin, has been isolated from a toxigenic strain of *Aspergillus glaucus* and the structure confirmed by synthesis. The most widely studied group of coumarins are the aflatoxins, e.g. aflatoxin B$_1$ (44), a group of mycotoxins produced by the common moulds *Aspergillus parasiticus* and *A. flavus*. Their biosynthesis, analysis and toxicology have been extensively studied. Much work has been done on chemical methods of detoxification of aflatoxin-infected foodstuffs. Aflatoxin M$_1$ (45), a metabolite of aflatoxin B$_1$, is commonly detected in milk, and its synthesis has been reported.

### 3.3 Isocoumarins

A number of biologically active isocoumarins have been isolated from microorganisms. The bacterium *Bacillus pumulus* produces a gastroprotective
substance Al-77-B (46). Ochratoxin A (47), a metabolite of Aspergillus ochraceus, is an important mycotoxin responsible for kidney damage in pigs. Its synthesis has recently been reported. A facile synthesis of mellein (48), a dihydroisocoumarin which is a common fungal metabolite, is summarized in Scheme 5. 5-Allyloxy-2-hydroxybenzoates are smoothly converted, presumably via (49), to 3,4-dihydro-5,8-dihydroxy-3-methylisocoumarin (50). Selective mesylation and reductive cleavage of the sulphonate-carbon bond gave mellein. 3,4-Dihydroisocoumarins have been identified as intermediates in the biosynthesis of the fungal cyclopentenones terrein and cryptosporiopsinol. They have been synthesized in isotopi-
AROMATIC COMPOUNDS

R₁ R₂ = H or CI
R₃ = H or Me

Scheme 6  i. Ac₂O; ii. NaOH; iii. NaBH₄.

cally labelled form by conversion of the homophthalates (51) with acetic anhydride to give the 3-methyl-4-carboxyisocoumarins (52), which are then decarboxylated to the 3-methylisocoumarins (53) and finally reduced to the 3-methyl-3,4-dihydroisocoumarins (Scheme 6).⁵⁷,⁵⁸

Fomajorin S (54) and fomajorin D (55) are isocoumarins of terpenoid origin produced by the common wood-rotting fungus *Fomes annosus*.⁵⁹ Stellatin (56), produced by *Aspergillus stellatus*, is unusual among fungal dihydroisocoumarins in lacking a substituent on either C-2 or C-3. Its structure was defined by a complete analysis of long-range (i.e. greater than one bond) $^1$H–$^1$C couplings in its fully $^1$H-coupled $^1$C n.m.r. spectrum.⁶⁰ The 2- and 3-bond couplings detected in a series of selective low-power decoupling experiments are indicated on structure (56). This is a very powerful technique for assigning the substitution pattern of highly substituted benzenoid compounds (see also naphthoquinones below). Monocerin (57), a metabolite of *Helminthosporium monoceras* and other fungi,⁶¹ has an unusual fused dihydrofurubenzopyrrole ring system. It has been isolated along with the closely related fusarentins as an insecticidal metabolite of *Aspergillus parvulus*.⁶²

Sclerin (60) is a highly substituted homophthalic anhydride metabolite of *Sclerotinia sclerotiorum*⁶² and *Aspergillus carneus*⁶³ which stimulates plant root formation. It has been synthesized (Scheme 7) by condensation of the bis-trimethyl-silyl ether (58) of methyl 3-oxopentanoate with methyl orthoacetate in the presence of titanium tetrachloride. The resulting homophthalate (59) is then methylated and hydrolysed to produce sclerin.⁶⁴ The elaboration of (59) can be considered as a controlled condensation of diketide and triketide moieties and so constitutes a biomimetic synthesis.
3.4 Chromanones and chromones

LL-D253α (62), an antibiotic metabolite of *Phoma pigmentivora*, *P. violacea*, and *Sclerotinia fructigena*, was originally assigned structure (61), 5-hydroxy-6-(2'-hydroxyethyl)-7-methoxy-2-methylchromanone. However its structure has been revised to (62) by analysis of the fully $^1$H-coupled
AROMATIC COMPOUNDS

\[
\begin{align*}
\text{(62)} & & \text{(63)} R = H, OH \\
\text{(64)} R = O \\
\text{(65)} & & \text{(61)}
\end{align*}
\]

\[\text{OMe} \quad \text{OH} \quad \text{Me} \quad \text{Me} \quad \text{Me} \quad \text{OH} \quad \text{Me} \quad \text{Me} \quad \text{OH} \quad \text{CO}_2\text{Me} \quad \text{CO}_2\text{Me} \]

\[\text{^13C n.m.r. spectrum and by unambiguous synthesis of both structures (61) and (62).} \]

Mycochromanol (63) and mycochromanone (64) are co-metabolites in *Myrothecum roridum*. Mycochromone (65) has been isolated from the phytotoxic fungus *Mycosphaerella rosigena* which is responsible for leaf spot of greenhouse roses. It co-occurs with mycoxanthone (126) which suggests it is formed from (126) by oxidative degradation of the methoxylated benzenoid ring. Chromenes, chromones, and chromanones have been reviewed in a recent text.

3.5 Cannabinoids

Cannabinoids can be regarded as chroman derivatives. Their chemistry, analysis and synthesis have been extensively reviewed. Two of the more interesting recent syntheses are described in Schemes 8 and 9. The key step in a synthesis of cannabinol (70) makes use of the remarkably smooth displacement of methoxyl groups in O-methoxy-aryloxazolines described by Meyers. Thus reaction of oxazoline (66) with the aryl Grignard reagent (67) gives the sterically hindered biphenyl intermediate (68) which on hydrolysis and deprotection is converted to lactone (69). Methylolation with methylmagnesium iodide completes the synthesis of cannabinol. In a second biogenetically patterned approach, condensation of 1,3-bis-trimethylsilyloxy-1-methoxybutadiene (71) with the acid chloride (72) gave methyl olivetolate (73) which on condensation under carefully controlled conditions with (+)-\text{*p*}-mentha-2,8-dien-1-ol gave methyl \(\Delta^2\)-tetrahydrocannabinolate (74). Hydrolysis and decarboxylation then gave \(\Delta^2\)-tetrahydrocannabinol (75).
Scheme 8  i, HI/Ac₂O; ii, MeMgI.

Scheme 9  i, TiCl₄/CH₂Cl₂; ii, MgSO₄; iii, BF₃·Et₂O; iv, NaHCO₃; v, NaOH.
3.6 Macrocyclic lactones

Zearalenone (80), a mycotoxin isolated from *Fusarium* fungi, is notable because of its oestrogenic and anabolic activity in animals.75 A large number of related compounds has been isolated from fungal sources76 and all feature a macrocyclic lactone attached to a benzene ring. Zearalenone has been synthesized as shown in Scheme 10 via a palladium-catalysed carbonylation of the iodothioether (76) in the presence of the iodoalcohol (77) to give the ester (78).

\[
\begin{align*}
\text{(76)} & \quad + \quad \text{(77)} \quad \rightarrow \quad \text{(78)} \\
\end{align*}
\]

Scheme 10  i, PdCl₂/K₂CO₃/C₆H₆/120°C; ii, KN(SiMe₃)₂; iii, NaIO₄; iv, TsOH.

(78). This was cyclized using potassium hexamethyldisilazide as base to give, after oxidation and elimination, zearalenone dimethyl ether (79).77 A similar approach has been used to synthesize curvularin78 and lasiodiplodin.79 Lasiodiplodin (81), first obtained from the fungus *Lasiodiplodia theobromae*,80 has recently also been isolated from a plant source, *Euphorbia splendens*,81 to join the list of metabolites produced by both lower and higher organisms.

3.7 Pyrones and butenolides

A number of biologically interesting compounds contain a benzenoid ring linked to a pyrone. The nitrobenzene moiety is found in luteoreticulin (82) and aureothin (83), toxic metabolites of *Streptomyces luteoreticuli*82 and *S. thioluteus*83 respectively; and in spectinabilin (84), an antibiotic metabolite of *S. spectabilis*.84 A new kawain derivative (85) has been isolated from the roots of *Piper sanctum* and its structure confirmed by synthesis.85 Piperolide (87) is a closely related metabolite,86 containing the 4-hydroxybutenolide (tetronic
acid) moiety. It has been synthesized (Scheme 11) by reaction of the anion of 4-
methoxytetronate (86) with 3-phenylpropanal. Methylation followed by
allylic bromination and elimination as shown gave piperolide; presumably
the co-occurring pyrone and butenolide metabolites have a common biogen-
etic precursor, probably formed from cinnamic acid and two acetate units
(Ar-C7). Fadyenolide (90), isolated from *Piper fadyenii*, has two carbons (one
acetate) less than piperolide. It has been synthesized (Scheme 12) by
conversion of 4-methoxytetronate to 2-trimethylsilyloxy-4-methoxyfuran
(88) followed by condensation with methyl orthobenzoate to give (89)
followed by base-catalysed elimination of methanol to give fadyenolide.
AROMATIC COMPOUNDS

\[ (86) \]

\[ (87) \]

Scheme 11: i. LDA/HMPA/THF; ii. ArCH\(_2\)CH\(_2\)CHO; iii. BuLi/HMPA/\(-78^\circ\); iv. MeI; v. NBS/CH\(_2\)Cl; vi. DBU/CH\(_2\)Cl.

\[ (88) \]

\[ (89) \]

\[ (90) \]

Scheme 12: i. BuLi/Me\(_3\)SiCl; ii. PhC(OMe)\(_3\)/ZnBr\(_2\); iii. Bu'Li.

A similar Ar-C\(_2\) derived pyrone moiety is present in the territrems, e.g. territrem A (91), tremorgenic mycotoxins isolated from Aspergillus terreus.\(^9\) These have a bicyclofarnesyl-derived moiety substituted on to the pyrone ring to form a chroman ring.

\[ (92) \]
Scheme 13

i. Bu'Li/THF/ -78°C/ZnCl₂;
ii. Ni(acac)₃/Br₂/THF/P₅Br₅/ -20°C;
iii. Bu'Li/(S-(CH₂)₃S=CH₂)Me₂/THF/ -20°C;
iv. HgCl₂/HCl/MeOH;
v. H₂/Pd/C;
vii. MeO₂CCO₂Me;
viii. pyrrolidine;
vii. PCl₃;
ix, KOH/HCHO;
xii, resolution;
xiii, xylene/reflux.
3.8 Lignans

Steganacin (92), isolated from the Ethiopian plant *Steganotaenia araliacea*, belongs to a novel class of dibenzocyclooctadiene lignan lactones which display significant antileukaemic properties. The first synthesis was reported in 1976. More recently a synthesis of enantiomerically pure (-)steganone (98) has been described. This proceeds (Scheme 13) via the biphenyl (93) formed by coupling of 1-bromo-3,4-methylenedioxybenzene with the cyclohexylimine of 2-iodo-3,4,5-trimethoxybenzaldehyde. Conversion of (93) via the dithian gave the aryl acetate (94), which was ring closed leading to the enamine (95). Cycloaddition and ring expansion gave the cyclooctatetraene (96) which was converted by hydrolysis and reduction to the ester (97) which was resolved via the (S)-2-amino-3-phenylpropan-1-olamide derivative of the acid. Further elaboration of the keto-acid gave (+)-isosteganone which on heating isomerized to (-)-steganone (98).

![Scheme 14](https://example.com/scheme14)

Scheme 14 i, BuLi/THF/ -78°C; ii, Ra—Ni/EtOH; iii, BBr₃; iv, Hg(O₂CCF₃)₂/O₂.

In the first reported isolation of lignans from humans, the *trans*-dibenzylbutyrolactone (99) was found to be excreted by females during the luteal phase of the menstrual cycle and during early pregnancy. The structure was proved by synthesis. A further interesting synthesis (Scheme 14) used a tandem conjugate addition involving Michael addition of the anion of an aryldiphenylthiomethane to butenolide at -78°C, followed by trapping of the intermediate with a benzyl bromide. Raney nickel treatment of the in-
termediate (100) gave the desired butyrolactone (99) after deprotection. When treated with mercuric trifluoroacetate, compound (100) gave the arylnapthalene lignan (101). It had been hoped to stop this reaction at the dihydronaphthalene oxidation level which would have provided a good route to another class of lignans with significant antitumour activity, e.g. isopicro-podophyllone (102) which has been isolated from the roots and rhizomes of *Podophyllum pleianthum*. Synthetic routes to a variety of lignans have been reviewed.

3.9 Benzofurans

Two interesting approaches to the related benzofurans (104) and (105), isolated from *Sophora tomentosa*, have been described. In the first (Scheme 15) the heterocycle was constructed via an intramolecular Wittig reaction of the phenolic ester (103). The second (Scheme 16) proceeds by condensation of an ortho-iodophenolic acetate with a copper acetylide itself formed from the corresponding methyl ketone via the hydrazone and vinyl iodide. These

![Scheme 15](image)

Scheme 15 i. (CCl₃)₂/C₅H₅N; ii. NaBH₄; iii. Ph₃P·HBr/MeCN; iv. PhMe/Et₃N; v. Pd/C—H₂.
AROMATIC COMPOUNDS

Scheme 16

i, Et$_3$N/H$_2$NNH$_2$; ii, I$_2$/Et$_3$N/THF; iii, NaH/THF; iv, CuSO$_4$·HONH$_2$·HCl aq. NH$_3$; v, C$_5$H$_5$N/heat

compounds are of increasing importance as several are antifungal phytoalexins, e.g. vignafuran (106) which is the phytoalexin of cow pea (Vigna unguicalata) leaves infected with Colletotrichum lindemuthianum.

3.10 Terphenyls

Many diphenylbutenolides and related systems are formed via diphenylbenzoquinones and the related quinols. The large variety of structures and structural types have been reviewed so a few representative examples only are given here. All are fungal or lichen metabolites. In corticin A (107), a metabolite of Corticium caeruleum, the three phenolic rings are linked by ether bridges, whereas pigment C$_3$ (108), isolated from Suillus grevillei, has only one ether linkage. The central ring can be subjected to a variety of oxidative changes and rearrangement processes to give metabolites such as atromentic acid (109), a tetronic acid isolated from several fungal sources, and aspulvinone I (110), one of a series of prenylated butenolides produced by Aspergillus terreus. The structures and syntheses of these butenolides have
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(107)

(108)

(109) $R^1 = \text{CO}_2\text{H} \cdot R^2 = \text{H}$

(110) $R^1 = \text{H}, R^2 = \text{prenyl}$

(111)

(112)

(113)

(114)

(115)
been the subject of a good comprehensive review.\textsuperscript{106} They can be regarded as being derived formally by a cleavage of the bond between C-2 and C-3 of the central ring of a terphenyl precursor. Grevillin A has the pyrone structure (111)\textsuperscript{107} which can be formed via a formal fission of the bond between C-1 and C-2 of a terphenyl, whereas pigment A (112), also from \textit{S. grevillei},\textsuperscript{108} represents an alternative ring closure of the same intermediate to a furan-3-one. In gynosporin (113), a metabolite of \textit{(inter alia) Chamonixia caespitosa},\textsuperscript{109} the central terphenyl ring has been contracted to form a furan-1,2,4-trione. Finally, in hydnuferrugin (114), produced by \textit{Hydnellum ferrugineum}, one of the outer benzenoid rings has been cleaved to form a spiro-fused dihydropyranlybutenolide moiety.\textsuperscript{110}

3.11 Flavanoids

An authoritative book covering many aspects of flavanoid isolation, structure and synthesis has appeared.\textsuperscript{111} Two more recent books\textsuperscript{112,113} cover plant phenolics, the shikimate pathway, phenolic acids, phenylpropanoids, lignin, flavanoids and tannins. In addition very comprehensive reviews have appeared on isoflavonoids, including rotenoids and aryl benzofurans,\textsuperscript{114} homoisoflavones,\textsuperscript{115} neo lignans,\textsuperscript{116} tannins,\textsuperscript{117} and gallic acid metabolites and ellagitannins.\textsuperscript{118}

Of the many unusual structures to appear in this area, two are worthy of special mention. The isolation and synthesis of melanervin (115) from \textit{Maleuca quincinerva}\textsuperscript{119} has been reported. This is the first naturally occurring compound with a triphenylmethane structure. The synthesis of scillescillin (119), the only naturally-occurring benzocyclobutene, isolated from the bulbs of \textit{Scilla scilloides},\textsuperscript{120} has been reported (Scheme 17).\textsuperscript{121} The benzocyclobutene nitrile (116) was converted to the phenylketone (117). Introduction of

\textsuperscript{1} HCl/Et\textsubscript{2}O/ZnCl\textsubscript{2}/0\textdegree C; ii, Bu\textsuperscript{t}Me\textsubscript{2}SiCl/DMF/imidazole; iii, Bu\textsuperscript{t}OK/HCHO; iv, HF/MeCN; v, TsOH/C\textsubscript{6}H\textsubscript{6}.
the hydroxymethyl substituent necessitated prior protection of the phenolic hydroxyls as their silyl ethers. Surprisingly, the product of the reaction with formaldehyde was the silyl ether (118) in which migration of a silyl group had occurred. Deprotection and cyclization produced scillescillin (119).

3.12 Xanthones, benzophenones and grisans

Major reviews of xanthones from plant sources have appeared. A systematic $^{13}$C n.m.r. study of substituted xanthones allows the effect of substituents on chemical shifts to be deduced. Although many compounds

(120) $R^1 = R^2 = H$
(121) $R^1 = \text{OCH}_2\text{CH} = \text{CMe}_3$; $R^2 = H$
(122) $R^1 = \text{OCH}_2\text{CH} = \text{CMe}_3$; $R^2 = \text{CH}_2\text{CH} = \text{CMe}_3$

(124)

(125)

(126)

(129)
have been isolated from plant sources, fungi have also proved a source of several interesting systems in recent years. A relatively simple compound (120) has been isolated from the bird’s nest fungus *Cyathus intermedius*. A group of biogenetically related xanthones and benzophenones have been isolated from *Aspergillus variecolor*, *A. rugulosus*, and *A. nidulans*. These include the xanthones varieioxanthone A (121) and varieixanthone B (122), tajixanthone (123), and the benzophenone arugosin A (124) which contains a hemi-acetal linkage between the two aromatic rings. A closely related benzhydrol, silvaticamide (125) has been isolated from *Aspergillus silvaticus*. This has an unusual amide linkage between the rings. All these compounds are apparently formed via anthraquinonoid precursors. Mycoxanthone (126) has been isolated along with mycochromone (65).

The ergochromes are dimeric tetrahydroxanthones. Further compounds related to the ergochromes have been isolated. These include the secalonic acids, e.g. (127), isolated from several fungi; the eumetrins, e.g. (128), isolated from the lichen *Usnea baylei*; and the monomeric diversonol (129), a metabolite of *Penicillium diversum*. The eumetrins are of particular biosynthetic interest because the monomeric units present represent alternate modes of cleavage of the quinonoid ring of the presumed anthraquinonoid precursor. Some biomimetic synthetic studies on the ergochromes have been reviewed.

Bikaverin (132), a red pigment with specific antiprotozoal activity, has been isolated from several fungi including *Fusarium oxysporum*. It has been synthesized as shown in Scheme 18. The intermediate xanthone (130) was oxidized to the quinone (131) which was then selectively demethylated with
Scheme 18 i, ZnCl₂/HCl; ii, (EtO₂C)₂/NaOEt; iii, TsOH; iv, Me₂SO₄; v, KOH/EtOH; vi, SOCl₂; vii, BF₃; viii, K₂Cr₂O₇/AcOH; ix, LiI/MeCOClMe₃.
iodide ion. Leprocybin, a glucoside responsible for the yellow fluorescence of the fruiting bodies under u.v. light, has been isolated from Cortinarius cotoneus and related Leprocybes. It has the unusual pyranoxanthone structure (133). A number of xanthones containing a bis-furano side chain and modified prenyl substituents have been isolated from a toxigenic strain of Aspergillus ustus. These are the austocystins, e.g. austocystin E (134); their structures have been assigned by extensive $^{13}$C n.m.r. studies.

A new total synthesis of griseofulvin (140) has been described (Scheme 19). The key step is a cycloaddition between 1,1-dimethoxy-3-trimethylsilyloxy-
1,3-butadiene (138) and the sulphoxide (137) to afford dehydrogriseofulvin (139) which was converted to (140) by hydrogenation. The benzofuranone (136) was prepared by base-catalysed rearrangement and cyclization of the phenolic acetate (135). Thelepin (141) is a grisan derivative isolated from the marine worm Thelepus setosus, where it co-occurs with a number of brominated phenols and diphenylmethanes. Depsides and depsidones are predominantly lichen products. Comprehensive lists of those isolated have been prepared.

Scheme 20  i, K₂Fe(CN)₆/H₂O/K₂CO₃.

It is generally accepted that depsidones are derived from depsides. However, the facile conversion of benzophenones to depsidones via grisandiones (Scheme 20) reported by Sargent raises the interesting possibility that this sequence may be involved in depsidone biosynthesis.

3.13 Naphthalenes and naphthoquinones

The isochroman quinones are a class of antibiotic substances isolated mainly from various streptomycetes. Ventilagone and the eleuthrins are members of the class which had previously been found in higher plants. The simplest class are the nanomycins, e.g. nanomycin D (142) isolated from S. rosa. The griseusins, e.g. (143), a metabolite of S. griseus, have a more complex highly oxygenated side chain which forms a tri-substituted spiro-tetrahydropyran ring. A synthetic approach to the griseusins and their absolute configuration has been reported. The granaticins, e.g. (144), metabolites of S. olivaceus, have a six-carbon substituent derived from glucose fused to the quinone moiety unusually through two carbon-carbon bonds. Dimeric compounds also occur. The actinorhodins and phenocyclinone are produced by S. coelicolor. The most complex structures however are found in
the naphthocyclinones, e.g. 2-naphthocyclinone (145), metabolites of *S. avenae*.\(^{150}\) In these compounds the dimerization results in the modification of one of the isochroman quinone units to an aryl ketone and a two-carbon unit has been lost from the other in (145).

The unusual polychlorinated dimeric naphthoquinone (146) has been isolated from an unidentified soil fungus.\(^{151}\) The naphthoquinone moiety in viomellein (147) and related metabolites was originally assigned an angular structure, but detailed \(^{13}C\) n.m.r. studies and in particular the observation of a 3-bond \(^1H-^{13}C\) coupling between the aromatic hydrogen and a quinonoid carbonyl indicated that the linear structure was correct.\(^{152}\) Viomellein and related naphthalenoid dimers have been isolated from several fungi, *Aspergillus sulphureus*,\(^{153}\) *A. melleus*, *Penicillium citreo-viride*,\(^{154}\) *P. viridicaturn*,\(^{155}\) and *Microsporum cookei*.\(^{156}\) The monomeric unit semi-vioxanthin (148) has been isolated from *P. citreo-viride*.\(^{154}\) Xanthoviridicatum D (149), also from *P. viridicaturn*,\(^{157}\) has a semi-vioxanthin moiety linked to 2-methoxy-5-hydroxy-1,4-naphthoquinone.

Another synthesis of the spinochrome (152) from methoxynaphthazarin (150) has been described.\(^{158}\) This uses (Scheme 21) a photo-Fries rearrangement of the diacetate (151) to introduce the acetyl moiety. 7-Methyljuglones bearing 4-hydroxy-5-methylcoumarin-3-yl units at C-2 and both C-2 and C-3, e.g. (153), have been isolated from *Diospyros ismailii*, and 2-methyljuglone with the coumarinyl unit at C-3 has been isolated from the bark of *Diospyros*
canaliculata. The structures have been confirmed by synthesis.\textsuperscript{159} The tryptopholones, e.g. (154), are a group of 1,2-naphthoquinones isolated from the fungal mycosymbiont of the tropical lichen \textit{Trypethelium eleuteria}.\textsuperscript{160} Their structures suggest that they may be formed by degradation of the phenalenone, deoxyherqueinone.\textsuperscript{210} Trichione (155, $n = 2$) is the main red pigment found in the fruiting bodies of the slime mould \textit{Trichia floriformis}, whereas homotrichione (155, $n = 4$) is found in \textit{Metatrichia vespertinum}.\textsuperscript{161}

Isohemigossypol (157) is a phytoalexin isolated from a cotton plant infected with \textit{Verticillium dahliae}.\textsuperscript{162} The asparvenones, e.g. (158) and (159), are tetralone metabolites of \textit{Aspergillus parvulus}.\textsuperscript{163} Systematic $^{13}$C n.m.r. studies of substituted naphthalenes\textsuperscript{164} and substituted naphthoquinones\textsuperscript{165} have been reported. The effect of substituents on the chemical shifts of the quinonoid carbonyls and observation of long-range $^1$H-$^{13}$C couplings provide valuable methods for obtaining the relative dispositions of substituents in the benzenoid and quinonoid rings.
3.14 Anthraquinones and anthracenes

A number of prenylated anthracenes, e.g. ferruginin A (160), have been isolated from the berries of Vismia species. $^{13}$C n.m.r. was used to establish their structures and to study the keto-enol tautomerism of ring C. $^{156}$ The mycelium of Aspergillus cristatus produces the pigments viocristin (161) and isoviocristin (162). These are the first 1,4-anthraquinones to be found in nature. $^{167}$ Phomazarin is an aza-anthraquinone present in Phoma terrestris, the fungus responsible for 'pink root' disease of onions. Its structure has undergone many mutations over the years. Detailed $^{13}$C n.m.r. studies finally established its structure as (163).$^{168}$ A feature of this study was the biosynthetic incorporation of $^{15}$N and (1,2-$^{13}$C$_2$)acetate to enable $^{13}$C-$^{15}$N, $^{13}$C-$^{13}$C, in addition to $^{13}$C-$^{1}$H couplings to be found, which greatly facilitated the structure determination. Note that the molecule exists in the pyridol rather than pyridone tautomeric system.
The treatment of aflatoxin-producing cultures of *Aspergillus parasiticus* with the insecticide dichlorvos inhibits the production of aflatoxin B₁ and causes accumulation of the anthraquinone versicol acetate (164). Nidurufin (165), which is also believed to be on the biosynthetic pathway to aflatoxin B₁, has been isolated from *Aspergillus nidulans*; Dothistroma pini, and as the 6,8-di-O-methylether from *Aspergillus versicolor*, though none of these fungi are themselves aflatoxin producers. Averufin (168) is another anthraquinoid precursor of the aflatoxins. Townsend has developed a synthesis of...
Aromatic Compounds

Averufin (Scheme 22) in which the key steps are (i) the regiospecific coupling of the anion of phthalide (166) and the benzyne derived from the aryl bromide (167) to give the desired anthraquinone, and (ii) the use of methoxymethyl (phenol) protecting groups for regiospecific aryl metallation and the introduction of electrophiles to selectively elaborate simple oxygenated benzenoid precursors. This synthesis has been used to prepare a variety of specifically ($^{13}$C and $^{2}$H) labelled averufins which have been used for biosynthetic studies.

Setomimycin (169) is a novel 9,9'-bianthryl antibiotic isolated from Streptomyces pseudovenezuelae. Its structure was deduced largely by $^{13}$C n.m.r. and established by X-ray analysis. An interesting feature is the non-aromaticity of the cyclohexadienone ring, which presumably prefers to have C-1 sp$^3$ hybridized for steric reasons.

Much effort has gone into developing new routes to anthraquinones, stimulated in part by their close structural relationship to the anthracyclines (see below). Reaction of 1,1-dimethoxyethene with stypandrone (170) (Scheme 23) gave the 1:2 addition product which on deprotection gave the insect pigment acetylmodin (171). Similar reaction with juglone gave a

\[
\begin{align*}
\text{(170)} & \quad + \quad \text{OMe} & \quad \text{Me} & \quad \text{OH} & \quad \text{Me} & \quad \text{OH} \\
\text{Me} & \quad \text{OH} & \quad \text{Me} & \quad \text{OH} & \quad \text{(171)} \\
\text{Me} & \quad \text{Me} & \quad \text{Br} & \quad \text{Me} & \quad \text{OMe} & \quad \text{OMe} & \quad \text{(172)} & \quad \text{Me} & \quad \text{OH} & \quad \text{Me} & \quad \text{OH} & \quad \text{(173)} \\
\text{Me} & \quad \text{Me} & \quad \text{OH} & \quad \text{Me} & \quad \text{OMe} & \quad \text{OMe} & \quad \text{(174)} & \quad \text{Me} & \quad \text{OH} & \quad \text{Me} & \quad \text{OH} & \quad \text{(175)} \\
\end{align*}
\]

Scheme 23: i, DMSO; ii, AlCl$_3$/NaCl; iii, DMF; iv, PhMe/heat; v, Ag$_2$O/MgSO$_4$; vi, HBr/HOAc.
good yield of emodin.\textsuperscript{178} It appears that the presence of a 5-hydroxyl in these naphthoquinones has a strong directing effect so that the required 1,3,8-trihydroxyanthraquinones are the main products. In the absence of a 5-hydroxyl, a halogen substituent is required in the quinonoid ring to direct the regiospecificity of addition. Thus synthesis of deoxyerythrolaccin (173) required the use of the bromo-quinone (172).\textsuperscript{179} Jung has developed a convenient synthesis of chrysophanol (175) by reaction of the readily available 6-methoxy-3-methylpyrone (174) with juglone.\textsuperscript{180} A further cycloaddition approach to the synthesis of anthraquinones uses 1,1-dimethoxybutadienes, e.g. (176), and naphthoquinones. Thus the permethylated crinoid pigment (177) has been synthesized by the route shown in Scheme 24.\textsuperscript{181}

%chem
c15h10o3 + c15h10o5 \rightarrow c38h24o15

\textbf{Scheme 24} i, C\textsubscript{6}H\textsubscript{6}/heat; ii, NaOMe/Cul/MeOH/DMF; iii, Me\textsubscript{3}SO\textsubscript{4}/K\textsubscript{2}CO\textsubscript{3}; iv, O\textsubscript{2}/EtOH/hv
The tetrahydroanthracenones (179) prepared by the addition of the anions of the sulphones (178) to substituted cyclohexenones (Scheme 25) can be aromatized and oxidized to give a simple route to 1-hydroxyanthraquinones. Harris has reviewed the use of masked polyketide synthons in the biomimetic synthesis of a wide range of aromatic compounds. The approach is exemplified by the synthesis of chrysophanol (175) from the glutarate derivative (180) as shown in Scheme 26.

3.15 Anthracyclones

One of the main areas of activity in natural products chemistry in the past decade has been the study of the anthracycline group of antitumour antibiotics. They are all glycosides of tetracyclic anthraquinones and are produced by a variety of actinomycetes. In addition to isolation, structure determination and biosynthetic studies, there has been an enormous amount of work done on the synthesis of these compounds, particularly to find analogues which retain the desired antitumour activity but have lower toxicity to non-cancerous mammalian cells. Four main aglycones are known—daunomycinone (181), its 14-hydroxy derivative adriamycinone (182), aklavinone (183), and steffimycinone (184). The recently isolated 11-deoxydaunomycin and aclacinomycin have particularly low toxicity and this has prompted several recent syntheses of the corresponding aglycones, 11-deoxydaunomycinone and aklavinone. Other earlier work on the synthesis of the anthracyclones has been reviewed, and some more recent approaches are described below.

In Hauser's approach (Scheme 27) to 7,9-dideoxy daunomycinone (190), the anion of sulphone (185) is condensed with 2-ethoxybutenolide leading to (186)
which is converted to the sulphone (187). The anion of (187) is then condensed with the cyclohexanone (188) to give the heterocyclic intermediate (189) which is converted using established chemistry to (190).¹⁹³

Most other approaches utilize cycloaddition reactions in the key steps. A flexible route which gives daunomycinone, adriamycinone, and their 6-deoxy analogues, uses the diethoxycyclobutene (191) prepared as shown in Scheme 28. On heating (191) with 5-methoxy-1,4-naphthoquinone, the tetracyclic ketone (192) is formed and this again is converted to the anthracycinones using standard methodology.¹⁹⁴ In a related approach to the synthesis of 11-deoxydaunomycinone the diene (193) was prepared as shown in Scheme 29.
AROMATIC COMPOUNDS

Scheme 28
i, Ac$_2$O; ii, hv; iii, LiOEt; iv, heat; v, NaOEt/O$_2$.

Scheme 29
i, Me(Li)CS(CH$_2$)$_3$S/THF/HMPA; ii, CO(O)Me$_2$/NaOH/C$_2$H$_5$; iii, NaH/THF,(EtO)$_3$POCl/Me$_2$CuLi; iv, LDA/Me$_3$SiCl; v, THF/20°C; vi, HgCl$_2$/CaCO$_3$/MeCN/H$_2$O.
and subsequent cycloaddition with 3-bromo-5-methoxy-1,4-naphthoquinone gave, after acidic work-up and oxidative cleavage of the dithian, the known ketone (194).\(^{195}\)

In an extension of previous work, Kishi has prepared optically pure (+)aklavinone. The key intermediate was the aldehyde (199) prepared as shown in Scheme 30. Cycloaddition between 3-bromo-5-methoxy-1,4-naphthoquinone and the triene (195) led to the anthraquinone (196) which was converted to the allyl derivative (197). Palladium-catalysed cyclization

Scheme 30 i. Et\(_2\)O/CH\(_2\)Cl\(_2\)/−40°C; ii. CH\(_3\)=CHCH\(_2\)Br/Ag\(_2\)O; iii. o-C\(_6\)H\(_5\)Cl/refsu; iv. PdCl\(_2\)(C\(_6\)H\(_5\)CN)/C\(_6\)H\(_6\)/reflux; v. BO\(_3\); vi. O\(_3\)/CH\(_2\)Cl\(_2\)/−78°C; vii. D(−)-2,3-butandiol/TsOH; viii. SnCl\(_2\)/MeCN/−20°C; ix. K\(_2\)CO\(_3\)/MeOH; x. CF\(_3\)CO\(_2\)H/−78°C.
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3.16 Ansamycins

Another major area involving aromatic compounds has been the study of the ansamycin antibiotics which display important antibacterial, antiviral, and antitumour activities. They are characterized by having either a benzenoid or a naphthalenoid chromophore spanned by a long (polyketide-derived) aliphatic bridge which usually contains an amide linkage. The major groups are the rifamycins, e.g. (202) produced by *Nocardia mediterranei*, the streptovaricins, e.g. (203) produced by *Streptomyces spectabilis*, and naphthomycin, produced by *Streptomyces collinus*. These all contain naphthalenoid chromophores. However, geldanamycin, produced by *Streptomyces hygroscopicus*, and the recently isolated mycotrienins (*Streptomyces richiniensis*), macebins (a *Nocardia* species), and ansatrienins (*Streptomyces collinus*) all contain a benzoquinone or benzenoid chromophore. Perhaps the most interesting of all are the maytansinoids, e.g. (204). These were first isolated from the higher plants *Maytenus ovatus*, *M. buchanii,*
and *Colubrina texensis*, but have recently been isolated from a microorganism, a *Nocardia* species, which suggests that these compounds may not be true plant metabolites but are produced by symbiotic micro-organisms. *Tridentoquinone (205)*, produced by the fungus *Suillus tridentinus*, has an ansa-type chain of terpenoid origin.
A five-step synthesis of the naphthalene nucleus (208) of streptovaricin D uses phenylthiomethyl azide for the introduction of the amino group (Scheme 31). The naphthalene system is built up using a cycloaddition between the benzoquinone (206) and 1,3-bis(trimethylsilyloxy)-methoxy-2-methylbutadiene (207). A similar regiospecific cycloaddition-based synthesis of the naphthofuranone chromophore of the rifamycins has been described (Scheme 32). In this the trimethylsilyl ether (209) is reacted with 2-bromo-6-acetamido-1,4-benzoquinone. Palladium-catalysed conversion of (210), derived from the adduct, to the methyl ketone (211) and selenium dioxide gave (212) which on hydrolysis produced (213). A total synthesis of rifamycin has been developed but this used a naphthalene precursor derived from the natural product, and a total synthesis of maytansine (204) has been described.

\[ \text{Herqueichrysin (214), a} \]

3.17 Some other polycyclic antibiotics

Phenalenones can be isolated from many fungal and plant sources and a comprehensive review of the area has appeared. Herqueichrysin (214), a
metabolite of *Penicillium herquei*, has the dihydrofuran ring in the opposite orientation to the other members of the group. Its structure has been established by synthesis and detailed $^{13}$C n.m.r. studies which provided information on the tautomerism occurring in these systems.

Ravidomycin (215), recently isolated from *Streptomyces ravidus*, represents the newest member of the chrysomycin family of antitumour antibiotics. These compounds have now been found in a number of organisms. They are apparently formed by cleavage of a benzphenanthrene precursor. This skeleton is found intact in vineomycin A (216) produced by *Streptomyces matensis*. Its co-metabolite vineomycin B (217) is presumably formed by an alternative cleavage pathway.

Scheme 33

- i, cyclohexylamine; ii, BuLi/TMCDA/THF; iii, ICH$_2$CO$_2$H/C$_5$H$_5$N/C$_6$H$_6$/heat; iv, C$_5$H$_5$N.HCl/heat.
Viridicatum toxin (218) is a mycotoxin produced by the fungus *Penicillium viridicatum*.\(^{215}\) It has a structure closely related to that of the tetracycline antibiotics. Resistomycin (221) is a polycyclic antibiotic produced by *Streptomyces griseoflavus*. It has been synthesized for the first time by an intramolecular cycloaddition of the isobenzofuran (219) which was itself synthesized in masked form as shown in Scheme 33. The Diels-Alder adduct (220) was converted to (221) in high yield in a remarkable one-pot desilylation, aromatization, demethylation and cyclization sequence using pyridinium hydrochloride.\(^{216}\)

Ristocetin is a glycopeptide elaborated by *Nocardia lurida*. It is a member of the vancomycin class of antibiotics which inhibit bacterial cell wall synthesis.\(^{217}\) The structure of the aglycone has been established as (222), mainly by detailed n.m.r.\(^{218}\) and degradative studies.\(^{219}\) Containing as it does a biphenyl, a diphenyl ether and a phenoxydiphenyl ether it is perhaps one of the most complex 'aromatic' natural products to be isolated so far!

References

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66. G.C. Crawley, and C.J. Strawson, unpublished results cited in ref. 11, p. 98.
150 THE CHEMISTRY OF NATURAL PRODUCTS

100. K. Chamberlain, and R.A. Skipp, 1975, Phytochemistry, 14, 1843.
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The Biosynthesis of Polyketides

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Reviewing the literature published between July 1983 and June 1984

(Continuing the coverage of the literature in Natural Product Reports, 1984, Vol. 1, p. 281)

1 Introduction

This report follows the format of previous reports appearing in the 'Biosynthesis' series of Specialist Periodical Reports and in Natural Product Reports and it covers the literature appearing in the period between July 1983 and June 1984. During the review period the application of oxygen-18 and deuterium labelling, using isotope-induced shifts in 13C n.m.r. spectra to detect the isotope label, has continued to develop and provides increasingly useful and often very subtle information on the intermediate processes in the biosynthesis of polyketides. In this context, the recent symposium-in-print in 'N.m.r. Spectroscopic Techniques for Studying Metabolic Processes', as well as containing several articles that are discussed below and which are of immediate relevance to the biosynthesis of polyketides, also makes essential reading for anyone involved in applying n.m.r. techniques to biosynthetic studies. In general, polyketide biosynthesis continues to be an active and healthy area. Several of the studies that are discussed are worthy of special mention. These include Schwab's elegant stereochemical studies on the β-hydroxydecanoylthioester-dehydrase-catalysed allylic rearrangement of (E)-dec-

2 Fatty Acids

β-Hydroxydecanoyl thiohydrase, which is the pivotal enzyme in the biosynthesis of unsaturated fatty acids in anaerobic micro-organisms, mediates the interconversion of acyl-carrier-protein thioesters of (R)-3-hydroxydecanoic acid (1), (E)-dec-2-enoic acid (2), and (Z)-dec-3-enoic acid (3) as indicated in Scheme 1. The key allylic rearrangement which interconverts (2) and (3) has been studied, using the dehydrase that is produced by Escherichia coli DM51 A (a cloned, over-producing, mutant strain). Scheme 2 shows the route by which (4R)- and (4S)-(E)-4.5,5-2H1 dec-2-enoic acids were synthesized. These, as their N-acetylcysteinamine thioesters, were converted by the dehydrase into the corresponding (Z)-dec-3-enoic acid that was isolated after reduction with sodium borohydride and conversion of the resulting alcohols into the corresponding p-phenylphenzoates (4). Analysis of these by 1H n.m.r. showed that the 4(pro-4S)-hydrogen of (2) was retained and that the 4(pro-4R)-hydrogen was removed in the course of the rearrangement to (3). The stereochemical course at C-2 was then studied by preparation of the corresponding thioester of (E)-[2-2H]dec-2-enoic acid and studying its conver-
sion into the ester of (Z)-dec-3-enolic acid by the dehydrase. The configuration of the deuterium label at C-2 of this ester was determined by $^1$H n.m.r., using the method of Parker. The configuration of the deuterium label at C-2 of this ester was now resolved. A complementary experiment was carried out in which (E)-dec-2-enolic acid was used with dehydrase in H$_2$O. These experiments clearly show that the enzyme-catalysed protonation occurs at the $s_i$ face at C-2, so that the hydrogen at C-2 in (2) occupies the 2(pro-2S)-position.

The stereochemistry of hybridalactone (18) (an isosanoic acid that has been isolated from the marine alga Laurencia hhydrargyra) has been established by X-ray crystallography. Prior to the stereochemistry was deduced by a combined analysis molecular-mechanics calculations and $^1$H n.m.r. data, at from biogenetic considerations. Based on the absolute stereochemistry that was deduced, a procedure synthesis was initiated. The biogenetic pathway that is outlined in Scheme 6 was proposed. The first step is lipoxygenase-mediated oxidation, the biosynthesis appears to take place via an elongation-decarboxylation mechanism (Scheme 3) analogous to that which operates in higher plants.

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Scheme 4

Scheme 5

Scheme 6
nucleophilic attack by the carboxylate group generates hybridolactone (18) with the correct absolute configuration.

3 Triketides

Triketides are very uncommon,4,5 the only established representatives being triacetic acid lactone (19)12 and colletodiol (20) and related metabolites.1,3 Several pyrones (see radicinin, citreopyrone, and alternaric acid below) possibly contain a triketide moiety, though this has not yet been satisfactorily demonstrated. However, incorporation studies with \(^1\text{[\text{[1-\(^{13}\text{C}\)]}]\text{propionate and with singly and doubly \(^{13}\text{C}\)-labelled acetates indicate that the antibiotic furanomycin (22), which is a metabolite of \textit{Streptomyces threomyceticus,} is derived (Scheme 7) from the triketide (21)\(^1\text{4}\) This contrasts with the structurally similar agaric toxin muscarine (23), which is biosynthesized...
from pyruvate and glutamate.\textsuperscript{15} [U-\textsuperscript{14}C]Lactate was not incorporated into (22), so that the necessary hydroxylation at C-2 of the propionate moiety occurs after, rather than before, assembly of the carbon chain.\textsuperscript{[1-\textsuperscript{14}C]-\textsuperscript{2},2\textsuperscript{3}H\textsubscript{2}]Propionate was incorporated with 45\% retention of \textsuperscript{3}H, so the possible formation of a keto-function at C-2 of the propionate-derived carbon chain during biosynthesis can also be ruled out.

4 Tetraketides

Two interesting studies involving novel tetraketides have been reported. Botryodiplodin (24), first isolated from \textit{Botryodiplodia theobromae},\textsuperscript{16} has recently been isolated from a toxigenic strain of \textit{Penicillium roqueforti}.	extsuperscript{17} Incorporation studies with \textsuperscript{13}C-labelled acetates gave a labelling pattern in (24) that is consistent with its formation via ring-cleavage of orsellinic acid (25) as indicated in Scheme 8. This was confirmed by synthesizing \textsuperscript{[2,\textsuperscript{13}C,\textsuperscript{2},\textsuperscript{2}H\textsubscript{2}] and \textsuperscript{[3,4-\textsuperscript{13}C\textsubscript{0},\textsuperscript{2}H\textsubscript{2}] orsellinic acids and studying their incorporation. As required by Scheme 8, the labelled botryodiplodin, respectively, at both C-4 and C-6 and at C-7 only.\textsuperscript{18} The biosynthesis of verrucarin E (27)\textsuperscript{19} can also be explained via ring-cleavage of orsellinic acid.\textsuperscript{20} Botryodiplodin (and possibly verrucarin E) therefore joins the increasing group of compounds (which includes penicillic acid (28), patulin (29),\textsuperscript{5} and the recently isolated astepyrone (30)) which are all formed by oxidative ring-cleavage of orsellinic acid (25) or 6-methylsalicylic acid (26), as indicated in Scheme 9. It is interesting to note that a strain of \textit{P. roqueforti} is reported to produce either penicillic acid or patulin, depending on the culture conditions.\textsuperscript{21}

Asticolorins A (31), B (32), and C (33) are novel mycotoxins, isolated from toxic extracts of cultures of \textit{Aspergillus multicolor} on whole maize.\textsuperscript{22} Incorporation studies\textsuperscript{23} on asticolorin C (33), using a variety of \textsuperscript{13}C- and \textsuperscript{2}H-labelled precursors, indicate a biosynthetic pathway involving one molecule of mevalonate and four molecules of orcinol (34). The labelling patterns from \textsuperscript{[1,2-\textsuperscript{13}C\textsubscript{0},\textsuperscript{2}H\textsubscript{2}]acetates are summarized in Scheme 10. These are consistent with a biosynthesis of asticolorin C as shown in Scheme 11. Oxidative coupling of orcinol, which is formed by decarboxylation of orsellinic acid, yields pannarol (35),\textsuperscript{24} which is then prenylated to (36) and hydroxylated to (37). Further oxidative coupling between (36) and (37), followed by formation of the C-15—C-31 bond as indicated, allows formation of the D and E rings of the asticolorins as shown. However, the observed stereochemistry and labelling patterns would also be consistent with an intermolecular cycloaddition between the diene (38) and the orthoquinone (39), formed from (36) and (37) respectively.

5 Pentaketides

The biosynthesis of diplosporin (40), which is a mycotoxin that is elaborated by the maize contaminant \textit{Diplodia macropora}, has previously been shown to involve the incorporation of methionine-derived carbon atoms into both the carbocyclic and the heterocyclic ring, as shown in Scheme 12. In an effort to obtain information on the mechanisms of formation of the rings, the origins and fates of the oxygen and hydrogen atoms in diplosporin have been investigated, using \textsuperscript{3}H- and \textsuperscript{18}O-labelled precursors.\textsuperscript{25} In the proton-noise-decoupled (p.n.d.)\textsuperscript{13}C n.m.r. spectrum of diplosporin that had been derived from \textsuperscript{[1,\textsuperscript{13}C\textsubscript{0},\textsuperscript{2}H\textsubscript{2}]acetate, the resonance due to C-9 shows \textsuperscript{3}H isotope-induced shifts, indicating that 1 to 3 \textsuperscript{2}H atoms are incorporated at C-10 and so confirming its derivation from the acetate 'starter' unit of the polyketide chain. Interestingly, no acetate-derived \textsuperscript{2}H was incorporated elsewhere in the molecule. On incorporation of \textsuperscript{[1-\textsuperscript{13}C\textsubscript{0},\textsuperscript{18}O\textsubscript{2}]acetate, only the C-3 resonance showed an \textsuperscript{18}O isotope-induced shift in the \textsuperscript{13}C n.m.r.
The lack of $^{18}$O at C-1 is surprising. On incorporation of [methyl-$^2$H]methionine, $^2$H label was observed (by $^2$H n.m.r.) at C-11 and C-12, showing that oxidation of these two carbons does not go beyond the aldehyde level during the biosynthesis. On the basis of these results it is suggested that C-11 arises by $C$-methylation at C-2 rather than by $O$-methylation at C-5. No overall pathway is proposed but a reasonable mechanism for the biosynthesis, consistent with the above observations, is shown in Scheme 13. It is noteworthy that no randomization of labelling between C-1 and C-11 is observed, so that the involvement of intermediates with the same oxidation level at these centres is ruled out.

Citreothiolactone (41) is an unusual sulphur-containing metabolite which has been isolated from the mycelium of *Penicillium citreo-viride* Bourge* and with citreopyrone (42) and pyrenocine B (43) (a phytotoxin that had previously been isolated from *Pyrenochaeta terrestris*). The labelling pattern resulting from incorporation of [1,2-$^{13}$C]acetate into these metabolites has been determined by obtaining the $^{13}$C n.m.r. spectrum by using an inadequate pulse sequence. On the basis of these results, a two-chain pathway (shown in Scheme 14) has been proposed. Condensation of a triketide moiety with a diketide as shown in path (a) would give citreothiolactone whereas an alternative condensation between these two moieties (path (b)) would give citreopyrone and pyrenocine B. It is suggested that the sulphur atom originates from the enzyme-bound thioacetyl. This Scheme embodies a number of features, each of which is unusual: (i) there is a lack of firmly established precedent for involvement of diketide or triketide moieties in biosynthesis; (ii) multi-chain pathways are rare; and (iii) the existence of alternative modes of condensation of two pre-formed chains in one organism is unprecedented. An alternative pathway which would account for the formation of these compounds from a common intermediate is shown in Scheme 15. This involves alternative ring-cleavages of a pentaketide-derived coumarin (44). The $O$-methyl ether of (44) is a known fungal metabolite. Further studies to test which of these pathways operates would be very worthwhile.

Previously reported incorporation experiments with $^{13}$C labelled acetates and methionine gave a labelling pattern that is consistent with the intermediacy of the symmetrical dialdehyde (47) in the biosynthesis of austriol (49), which is the main toxic metabolite of a strain of *Aspergillus usatus*. By analogy with previous results for citrinin and ascochitin, a reasonable pathway to (47) would involve reduction of an enzyme-bound thioester (45) to give (46) followed by oxidation of the methyl group that had been derived from methionine (Scheme 16). This has been tested by synthesizing a sample of the ketoaldehyde (46) that was specifically labelled with $^2$H at the
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nethyl group C-13. This was fed to A. usus, and $^2$H n.m.r. showed that label from (46) was specifically incorporated into the methyl group C-13 of austdiol with ca 10% efficiency. Further confirmation that (46) was an obligatory intermediate was provided by an "isotopic trap" experiment, using [Me-$^{14}$C]-methionine and unlabelled (46). In a further study, the incorporation of $^{18}$O resulted in an isotopic-induced shift being observed in the $^{13}$C n.m.r. for C-7 only. This suggests an oxidation mechanism involving a mono-oxygenase-mediated addition of an oxygen atom to C-7 of either the dialdehyde (47) or the quinone methide (48), as shown in Scheme 17(a) for the quinone methide. The involvement of an intermediate [e.g. (50)] that is derived through the action of a dioxygenase (Scheme 17(b)) would result in austdiol that contains $^{18}$O atoms on C-7 and C-8 that are derived from the same molecule of $^{18}$O$_2$. Surprisingly, no $^{18}$O isotope-induced shift on the resonance for the aldehyde group C-12 was observed in the $^{13}$C n.m.r. spectrum. The authors explain this as being due to facile exchange of the oxygen in carbonyl groups, as has been noted in previous biosynthetic experiments involving $^{18}$O-labelled precursors. If this is so then $^{18}$O label might equally well be lost from the oxo function at C-8 in the intermediate (51) on the dioxygenase pathway.

Full details have appeared of the incorporation of $^2$H, $^{18}$O, and $^{17}$O from [1-$^{13}$C,$^{18}$O$_2$], [2-$^{13}$C$_2$H$_2$], and [1-$^{13}$C,$^{17}$O]-acetates into citrinin (52) by cultures of Penicillium citrinum. Deuterium and oxygen-18 were detected by observation of $^{13}$C-$^5$H couplings and isotope-induced shifts in the $^{13}$C n.m.r.
Aspyrone (53) and asperlactone (54) are closely related metabolites of *Aspergillus melleus*. Their biosynthesis has been extensively studied. A full account of previously reported results and details of further studies have appeared. Three possible pathways were postulated as a basis for a series of detailed studies, using $^{13}$C-, $^{14}$C-, $^2$H-, and $^3$H-labelled simple precursors and potential advanced intermediates. These pathways are summarized in Scheme 19. Pathways (a) and (b) are based on the observation that mellein (55), 4-hydroxymellein (56), and penicillic acid (57) are all co-metabolites aspyrone and asperlactone. Incorporation of $^{14}$C-labelled acetate and malonate into aspyrone, followed by comprehen...
rative degradations, established that C-9 and C-10 were derived from an acetate 'starter' unit. On incorporation of [3H]acetate, extensive retention of 3H was observed (particularly at C-7 of [53]), which immediately ruled out pathway (a) in Scheme 19. Compounds (58)-(61) were each synthesized with a 14C label in the methyl group, but none was significantly incorporated into [53], making pathway (b) unlikely. This was subsequently confirmed by incorporating [2-13C]acetate into aspyrone, followed by analysis of the 13C n.m.r. spectrum of the product, which showed that two acetate-derived hydrogen atoms were incorporated at C-7, therefore intermediates in which C-7 is part of an aromatic ring are excluded. This experiment also showed that up to three acetate-derived hydrogen atoms are incorporated at C-10, confirming its origin from the 'starter' acetate of the polyketide. Thus pathway (c), involving the rearrangement of a linear pentaketide, remained the only possibility (if the possibility that there are two chain mechanisms is excluded). Stereochemical studies on aspyrone and asperlactone established the absolute stereochemistries shown in (53) and (54) and indicated that the δ- and γ-lactone rings could be formed by alternative ring-openings of an epoxide intermediate (62) by carboxylate, as shown in Scheme 20.34 On incorporation of [1,2-3H]acetate into asperlactone, a two-bond 13C-13C coupling between C-2 and C-8 was detected, indicating their derivation from a rearrangement involving an originally intact acetate unit.35 This confirms previous observations for aspyrone.36 Finally, 1H n.m.r. analysis of [3H]acetate-enriched asperlactone confirmed the retention of acetatederived hydrogens on C-5, C-7, C-8, and C-10. A biosynthetic pathway (Scheme 20) has been proposed to accommodate all of the above results and to account for the formation of all of the pentaketide-derived metabolites of A. melleus. The steps that are proposed in this pathway bear comparison with those in the postulated biosynthesis of monensin and other polyether antibiotics, where ring-closure onto epoxide intermediates is suggested for the formation of five- and six-membered oxygen-containing rings.32 Clearly, 1H-labeling studies would be of value in providing further evidence for this pathway.

Rice blast disease, which is caused by the fungus Pycnopus oryzae, is the most serious disease of rice. Several anti-blast chemicals, e.g. tricyclazole (63), are very effective for control of blast disease in vitro but are not toxic to P. oryzae.37 They appear to act by inhibition of the biosynthetic pathway to melanin, melanization of the cell wall being essential for pathogenicity.38 Studies with P. oryzae have resulted in the biosynthetic pathway shown in Scheme 21 being proposed for the formation of melanin. In this, 1,3,6,8-tetrahydroxynaphthalene (64) (derived from a pentaketide precursor) is reduced to scytalone (65); elimination of water to give 1,3,8-trihydroxynaphthalene (66) and a second sequence of reduction and elimination converts (66), iso vermelone (67), into 1,8-dihydroxynaphthalene (68), which is then polymerized to melanin. Treatment of cultures with tricyclazole inhibits the reduction step, resulting in the accumulation of shunt products, inter alia flavinol (69), iso sclerone (70), and 2-hydroxyjuglone (71). Recent work has shown that this pathway is common to a large number of ascomycetes and fungi imperfecti.39 The regio- and stereospecificity of incorporation of label from [3H]acetate into scytalone (65) in Phialophora lagerbergii has been studied. Carbon label was incorporated at C-4 and C-5 only, with no detectable label at C-2 or C-7 (Scheme 22). The 3H was incorporated into both axial and equatorial positions at C-4, so reduction of the established intermediate (64) to the dihydroxy-naphthalene level is not stereospecific [2-13C]Malonate was incorporated with equal efficiency into all of the positions that are indicated in Scheme 22. No 'starter' effect was observed. On the basis of this result, and the non-incorporation of 3H at C-2 or C-7, it is suggested that scytalone may not be a pentaketide-derived metabolite but may be formed via deacetylation of a hexaketide-derived naphthol (72), as indicated in Scheme 22. The co-occurrence of the naphthol (73) and

\[
\text{MeCO}_2\text{Na} \rightarrow \text{MeCO}_2\text{H} \rightarrow \text{HOOCCH}_2\text{OH} \rightarrow \text{HOOCCH}_2\text{OH} \rightarrow \text{HOOCCH}_2\text{OH} \rightarrow \text{HOOCCH}_2\text{OH}
\]

\[
\text{R} = \text{OH} \rightarrow \text{R} = \text{H} \rightarrow \text{R} = \text{OH} \rightarrow \text{R} = \text{H}
\]

\[
\begin{align*}
\text{Scheme 19} \\
\text{Scheme 20} \\
\text{Scheme 21} \\
\end{align*}
\]

\[
\begin{align*}
\text{(53)} \\
\text{(54)} \\
\text{(55)} \\
\text{(56)} \\
\text{(58)} \rightarrow \text{(60)} \rightarrow \text{(61)} \rightarrow \text{(59)} \\
\end{align*}
\]
nepodin (74) in *Rumex alpinus* provides some precedent for this proposal.\textsuperscript{41}

Alterperylenol (75) and dihydroalterperylenol (76) are metabolites of an unidentified species of the genus *Alternaria* which show activity against the plant-pathogenic fungus *Valsa ceratosperma*.\textsuperscript{42} Incorporation of [\textsuperscript{2-13}C]acetate indicates a pentaketide origin for (75) and (76) via dimerization of 1,3,8-trihydroxynaphthalene, as shown in Scheme 23.

**Scheme 21**

**Scheme 22**

**Scheme 23**

**6 Hexaketides**

3-epi-Deoxyradicinol (77) has been isolated, along with deoxyradicinin (78); these are new phytotoxic metabolites of *Alternaria helianthi*, which is the causative agent of seedling blight and leaf spot (these are major diseases of sunflower).\textsuperscript{43}
Radicinol (80) has also been isolated, along with the known compound radicinin (79), from the closely related Alternaria brachyanthemi. Radicinin is of interest as it was the first compound to have its biosynthesis studied by direct $^{13}$C n.m.r. methods. The incorporation of $[^1-13C]$- and of $[^2-13C]$-acetate into deoxyradicinin has been reported. A two-chain pathway has been generally accepted but a ring-cleavage pathway, as shown in Scheme 24, could account for the formation of these metabolites. Studies on the inter-relationships among this group of metabolites would be of interest, as would $^2H$- and $^18O$-labelling studies to ascertain the oxidation levels of the enzyme-bound intermediates.

LL-13253a, which is a chromanone that was first isolated from Phoma pigmenhivora and subsequently from several other organisms, was originally assigned the structure (81). This has been revised to (82), on the basis of a complete analysis of the fully proton-coupled $^{13}$C n.m.r. spectrum of LL-D253a acetate (83), and confirmed by synthesis. The biosynthesis of the chromanone has been studied by incorporation of $^{13}$C-, $^2H$, and $^{18}O$-labelled acetates; the resulting labelling patterns are summarized in Scheme 25. A particularly interesting feature was the partial randomization of label from singly $^{13}$C-labelled acetates between C-10 and C-11 in the hydroxethyl side-chain. On incorporation of $[1-13C,2H]$acetate, two $^2H$ atoms were incorporated at both C-10 and C-11 and only one at C-3. With the $^{18}O$ labelling (Scheme 25), this indicated that the chromanone ring was formed by conjugate addition of a phenolic hydroxyl group to the corresponding 2p-ununsaturated ketone (Scheme 26). Because LL-D253a is optically active, the ring-closure is stereospecific with respect to C-2, but analysis of the $^2H$ n.m.r. spectrum showed that the two hydrogen atoms at C-3 were labelled to an equal extent, so that protonation of the intermediate enolate must occur with equal facility from both faces, as indicated in Scheme 26. This contrasts with the corresponding ring-closure by which a chalcone is converted into a flavanone, which is known to be stereospecific with respect to both positions. LL-D253a must be biosynthesized into two pre-formed polyketide chains. One possibility is shown in Scheme 27. The observed randomization of labelling in $80\%$ of the molecules is accounted for by the formation of a symmetrical cyclopropyl intermediate (84), as shown. This intermediate can undergo hydrolytic ring-opening at either the $\alpha$- or the $\beta$-carbon. According to this Scheme, the $20\%$ of the molecules that are not undergoing randomization should have the $11$-hydroxyl group derived from the atmosphere; in accord with this, fermentation in an atmosphere of $^{18}O_2$ resulted in an $^{18}O$ isotope shift being observed on the resonance due to C-11 in the $^{13}$C n.m.r. spectrum, the intensity of the shifted peak being ca $20\%$ that of the unshifted peak. It is not clear whether the randomization is a process that occurs in vivo or in vitro.
7 Heptaketides

The incorporation of \([2,13\text{C}]\) and \([1,2,13\text{C}_2]\) acetates into marticine (85), which is a phytotoxic metabolite of *Fusarium murrinii*, gave the labelling pattern that is shown in Scheme 27. This is consistent with the addition of a C₃ unit (derived from an intermediate in the Krebs cycle) to a heptaketide which probably related to the intermediate leading to fusarubin (84) and related compounds. No mechanism is proposed, but a possible sequence is shown in Scheme 28. It should be possible by appropriate labelling studies, to establish which of the dicarboxylic acids is involved; oxaloacetate or malate would appear to be the most likely compounds.

Incorporation of \([13\text{C}]-, {^2}\text{H}-, \text{and} \,[1,2,15\text{O}]\) labelled acetates into monocerin (87) by cultures of *Drechslera ravenelii* and analysis by \(13\text{C}\) and \(2\text{H}\) n.m.r. spectroscopy gave the labelling pattern that is shown in Scheme 29. A particularly interesting feature is the retention of two acetate-derived hydrogen atoms at C-10, which suggests that reduction of the \(\beta\)-ketoacids intermediate to the corresponding \(\beta\)-hydroxyacids intermediate takes place during assembly of the carbon chain. Only one of the diasterotopic hydrogen atoms at C-12 is labelled, but the absolute stereochemistry remains to be established. The trihydroxylated moiety (88) is proposed as the likely enzymatic precursor (Scheme 30). The \(15\text{O}\)-labelling pattern (see Scheme 29) means that the benzopyrone ring must be formed by nucleophilic attack on the terminal carboxyl moiety by the hydroxyl group at C-9. It is likely that the cyclization takes place on the thioester (89) to give (90) as the first enzyme-bound intermediate. The retention of the acetate carbon-oxygen bond at C-11 indicates that the tetrahydrofuran ring is formed by a nucleophilic attack at the terminal carboxyl moiety by the hydroxyl group at C-9. A mechanism for this would be nucleophilic addition onto the quinone methide intermediate (92) that is formed by oxidation of (91), which is the hydroxylated derivative of (90). This is supported by the co-occurrence of monocerin and the fusarubin methyl ether (93) in *Fusarium laveranii*.

Brefeldin A (94) is a metabolite of *Penicillium brefeldianum*. Its biosynthesis has been much studied as a model for that of the macrolide antibiotics, also due to its structural similarity to the prostaglandins, and to determine the relationships (if any) between the biosynthesis of fatty acids and of polyketides.
In this regard, the stereochemistry of labelling from [2-\textsuperscript{13}C,\textsuperscript{2}H\textsubscript{3}]acetate in the fatty acids of \textit{P. brefeldianum} has been determined and compared with the labelling of the corresponding positions in brefeldin A. This study\textsuperscript{52} was based on the hypothesis that labelling in different positions in the brefeldin A molecule might correspond to the labelling in the different intermediates of the biosynthesis of fatty acids. Thus, as shown in Scheme 31, positions 2, 3, and 11 of (94) may correspond to the enoylthioester intermediate (97); positions 4, 6, 8, and 12 to the \textalpha-carbon of the saturated thioester intermediate (98); positions 5, 7, 9, and 13 to the \textbeta-carbon of (98); position 14 to the \textalpha-carbon of the \textbeta-ketothioester intermediate (95); and position 15 to the \textbeta-hydroxy-thioester intermediate (96). The labelling patterns in stearic and oleic acids of \textit{P. brefeldianum} were determined. The labelling for palmitic acid (99) was assumed to be the same, and is compared with that of brefeldin A in Scheme 31. The stereochemistry at C-6 and C-8 in brefeldin A differs from that of the fatty acid, suggesting that the configuration at these centres is determined by some
The epoxy-lactones (100) and (101) have been proposed as the immediate biosynthetic precursors of brefeldin A. Both of these, and their alkene precursors (102) and (103), have been synthesized, presumably as a preliminary to testing them as biosynthetic precursors. These results will be awaited with great interest.

8 Octaketides

Betaenones A (104), B (105), and C (106) are phytotoxins that have recently been isolated from Phoma betae, which is the fungus that is responsible for leaf spot disease of beetroot. They are closely related to the phytotoxins stemphyloxin 1 (107) and diplodiatoxin (108). Although the highly branched structures are suggestive of a propionate origin, the incorporation of $^{13}$C-labelled acetates and methionine into betaenone B indicates an origin via an acetate-derived polyketide, with five methyl groups being derived from the C-1 pool, as indicated in Scheme 32. It would appear that betaenone A is formed via an intramolecular aldol condensation between C-1 and C-17 of betaenone C (106), which would also be the precursor (by reduction at C-18 and oxidation at the methyl group C-1 respectively) of betaenone B (105) and stemphyloxin 1 (107). A large number of polyketides containing a highly deoxygenated decalin ring system have been isolated recently. These include compactin, mevinolin, nargenicin A, chlorothricin, kijanimicin, the tetrocarcins, and ilicicolin H. A variety of mechanisms, such as intramolecular aldol condensation, intramolecular cycloadditions, and electrophilic cyclization of polyolefins, have been proposed for the formation of these bicyclic systems. Studies to elucidate these mechanisms are in progress (see below) and should produce interesting results.

Previous studies on the biosynthesis of streptolydigin (109), which is an acyltetramic acid antibiotic that is produced by Streptomyces lydicus, had shown that the methyl groups C-1, C-16, and C-17 as well as C-18 were derived from propionate. However, establishing which parts of the structure we derived from acetate proved difficult because the incorporation of singly labelled $^{13}$C-acetate was too low to demonstrate.
enrichment of individual carbon atoms and because there is no degradation scheme that would be suitable for $^{14}$C studies. However, by feeding $[1,2-^{13}$C$]_2$acetate and detecting low-level $^{12}$C-$^{13}$C coupling satellites in the $^{13}$C n.m.r. spectrum, the origin of carbons 2' and 3', 1 and 2, 9 and 10, and 13 and 14 from intact acetate units was established. The results to date are summarized in Scheme 33. The remainder of the tetramic acid moiety is probably derived from $\beta$-methylaspartic acid as glutamate, which is known to be a precursor to $\beta$-methylaspartic acid and seems to be incorporated exclusively into this part of the molecule.$^{64}$

Germichrysone (110) (an octaketide-derived hydroanthracene) is produced in high yields by callus cultures of Cassia torosa. It is also the characteristic pigment of the seedlings, whereas the main pigment in the seeds is an anthraquinone glycoside. However, the main pigment in six-week-old callus cultures was the xanthone pinselin (111) and the germichrysone content was markedly decreased, suggesting that pinselin is derived from germichrysone.$^{65}$

Ilicicolin H (114) is a metabolite of Cylindrocladium ilicicola, containing a 5-(4-hydroxyphenyl)-$\alpha$-pyridone chromophore. Feeding experiments with $^{13}$C-labelled acetates and with $^{15}$N- and $^{14}$C-labelled phenylalanine indicate a biosynthetic pathway (Scheme 34) in which the decalin system is derived by cyclization of a bis-C-methylated octaketide precursor, which then condenses with phenylalanine to give an acyltetramic acid intermediate (112). Rearrangement of the tetramic acid via an intermediate quinonmethide (113) would then generate the $\alpha$-pyridone.$^{63}$

The incorporation of $^2$H from $[^2$H$_2]$acetate into the alkyl side-chains of 2-n-hexyl-5-n-propylresorcinol, which is a polyketide metabolite of Pseudomonas sp. B-9004, has been studied.$^{66}$ Different levels of incorporation were found, as indicated in (115). The $^2$H content was established by $^2$H n.m.r. studies on the deuteriated n-hexyl(phenyl)carbinol (116) and n-propyl(phenyl)carbinol (117) which were obtained from the enriched metabolite as shown in Scheme 35. The (1S) enantiomers were resolved by preparative g.l.c. of their (–)-menthoxycarbonyl esters and recovered by cleavage of the esters with LiAlH$_4$. Deuteron n.m.r. studies in the presence of Eu(fod)$_3$, revealed that deuterium was incorporated specifically into the (2(pro-2S))-position of (117) and the (3(pro-3S))-position of (116), using a method that is described in an accompanying paper$^{67}$ and which is generally applicable to determining enantiomeric composition and absolute configuration of 2- and/or 3-deuteriated 3-alkyl-propan-1-ols.

9 Nonaketides

The incorporation of $[1,2-^{13}$C$]_2$acetate into alternaric acid (118) by cultures of Alternaria solani gave the labelling pattern that is shown in Scheme 36.$^{68}$ Alternaric acid is derived from nine intact acetate units, with the remaining carbon atoms being derived from the $C_1$ pool. The levels of incorporation into $C$-13 and $C$-14 and into $C$-3' and $C$-4' were slightly higher than into the remaining carbon atoms, indicating that there are small acetate 'starter' effects, consistent with a two-chain pathway for the biosynthesis of alternaric acid. To try to distinguish between the two possible pathways (a) and (b) in Scheme 36, $[1,2-^{13}$C$]_2$acetate was fed to the fungus along with unlabelled triacetic acid lactone (119), its dihydro-derivative (120), sodium acetoacetate, and sodium 3-hydroxybutyrate (as possible specific precursors for $C_0$ or $C_4$ side-chains). However, in each case the enrichment was essentially the same as for acetate alone. Similarly, feedings of $^2$H-labelled (119) or 3-hydroxybutyrate gave alternaric acid which was unlabelled (according to $^2$H n.m.r.). It seems that recourse to cell-free enzyme systems will be required to resolve this problem, which is common to the biosynthesis of a number of polyketides for which a two-chain pathway has been invoked.

The antibiotics nodusmicin and its pyrrolecarboxylate ester nargenicin A$_1$ (122) represent a novel group of macrolide

![Image](https://example.com/image.png)
antibiotics that has recently been isolated from *Saccharopolyspora hirsuta* and *Nocardioid argentienss*, respectively. The derivation of the carbon skeletons of these antibiotics from acetate and propionate has been established by two separate studies, using 

\[ { }_{13}C^- \text{and } { }_{12}C^- \text{-labelled precursors, to be as}

shown in Scheme 37. The observed labelling of the pyrrolecarboxylate moiety is consistent with the conversion of propionate into succinate and thence into dehydroproline via α-ketoglutarate. Incorporation of [1-13C, 18O] acetate and -propionate indicated that the oxygen atoms that are attached to C-1 and C-11 were derived from acetate and those attached to C-9 and C-17 were derived from propionate. The remaining oxygen atoms are presumably derived from molecular oxygen. The observed derivation of the ether and carbonyl oxygens of the lactone from separate acetate and propionate units is consistent with previous observations for macrolides. The fact that neither the oxygen at C-9 nor that at C-11 is derived from molecular oxygen rules out various ring-forming mechanisms.
based on cyclizations of epoxy-olefins and epoxy-alcohols, and the absence of propionate-derived oxygen on C-13 suggests that the C-4-C-13 bond has not been formed by an aldol reaction, as has been suggested for the formation of the cyclohexane ring in the biosynthesis of avermectins. Therefore an intramolecular Diels-Alder reaction of an intermediate (121) is proposed. This would account for the observed labelling, stereochemistry, and functionality.

 Cultures of *Fusarium roseum* were treated with $[^{14}C]$-zearealenone (123), $[^{14}C]$-a-zearealenol (124), and $[^{14}C]$-b-zearealenol (125) to determine whether precursor-product relationships existed amongst them. Compounds (124) and (125) were converted into zearealenone (123) within 7 and 14 days, respectively, but (123) was not converted into other compounds.

Recent $^{13}$C n.m.r. studies have established the biosynthetic origins of the carbon skeleton of oxytetracycline (128) in *Streptomyces rimosus*. Further results on the incorporation of $^2$H from $[^1-^{13}C, ^2H]$acetate have been reported. The observation of $^2$H isotope-induced shifts for C-6a and C-8 indicates the acetate origin of the hydrogen atoms that are attached to C-7 and to C-9. The lack of $^3$H at C-4 is consistent with the introduction of the amine functionality onto an aromatic precursor such as 6-methylpretetramid (126). The biosynthesis of oxytetracycline is generally thought to deviate from that of tetracycline (129) after dehydroractyclcycline (127) has been formed (see Scheme 38). The conversion of (127) into (128) involves an oxidative step leading to the introduction of a 5a-hydroxyl group. Stereospecific hydroxylation with either retention or inversion of configuration would necessitate the elimination of either the a- or the b-hydrogen atom at this prochiral centre in (127), only one of which is likely to be derived from the 5-H of 6-methylpretetramid (126) and hence acetate. Assuming that $^2$H from acetate is incorporated at C-5 of (126) with comparable efficiency to the incorporation that is observed at C-7 and C-9 of (128), it follows that only one of the diastereotopic hydrogen atoms at C-5 of (127) is derived from acetate and that this is stereospecifically eliminated when (127) is hydroxylated to form oxytetracycline (128). Thus the

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**Scheme 37**

**Scheme 38**
determination of the stereospecificity of incorporation of $^3$H from acetate at C-5 of tetracycline (129) or 7-chlorotetracycline would allow the stereochemistry of the hydroxylation step in the biosynthesis of oxytetracycline to be elucidated.

10 Decaketides

Cetocycline (130) is a broad-spectrum antibiotic that is closely related to the tetracyclines. The incorporation of [1-13C]acetate into cetocycline in cultures of Nocardia sulphurens indicated its origin from a decaketide precursor with acetyl-CoA as the 'starter' unit, as shown in Scheme 39. The 'starter' unit for the polyketide chain in the tetracyclines is malonamyl-CoA, which suggests that acetacetyl-CoA (rather than acetyl-CoA) may in fact be the actual chain-initiating unit in cetocycline, though this might be fairly difficult to establish. No incorporation of label from [1-13C]propionate was observed, so the methyl groups that are attached to C-6 and C-9 are probably derived from the C3 pool.

Aklanonic acid (131) has been isolated from cultures of a species of the genus Streptomyces. Its status as a possible intermediate in the biosynthesis of anthracyclines has been tested by studying its transformation by daunorubicin-negative mutants of daunorubicin-producing strains of Streptomyces griseus. Strain O$_7$P$_7$ converted aklanonic acid and its methyl ester into ε-rhodomycinone (132), 7-deoxy-ε-rhodomycinone (133), and glycosides of daunomycinone (134), as shown in Scheme 40. A second mutant strain, 1P$_5$, converted aklanonic acid into the fully aromatic substance 1P/II (135). Oxygen-18- and deuterium-labelling studies might provide evidence for or against the intermediacy of fully aromatic tetracyclic compounds in the biosynthesis of anthracyclines.

Carbon-13-labelled acetates and propionate were incorporated into the antitumour antibiotic ravidomycin (136) by a Streptomyces species. The pairs of coupled carbon atoms in the 13C n.m.r. spectrum of [1,2-13C]acetate-enriched ravidomycin were identified by a two-dimensional INADEQUATE pulse sequence. [1,2-13C]Propionate enriched C-4', indicating that the vinyl group is derived from a propionate 'starter' unit. On the basis of the observed labelling pattern, the biosynthetic pathway that is shown in Scheme 41 has been proposed. Condensation of a decaketide chain, with loss of oxygen from C-7 and introduction of oxygen at C-6, gives the tetracyclic intermediate (139). Oxidative cleavage of (139), followed by
decarboxylation, gives (140); bond rotation and lactonization of this gives (141), which is converted into ravidomycin by \( \beta \)-methylation, dehydrogenation of the ethyl group, and \( \beta \)-glycosylation. Similar results have been reported for the closely related compounds gilvocarcin V (137) and gilvocarcin M (138), which are antitumour substances that have been isolated from *Streptomyces gthotanareus*.\(^6\) Carbon-13-labelled acetates and propionates were incorporated into (137) and (138), which were not separated but were analysed as a ca 7:1 mixture. It is clear that the \( \beta \)-methyl group in (138) and the \( \beta \)-vinyl substituent in (137) are derived from acetate and propionate, respectively. It was found that \([2-^{13}\text{C}]\) and \([3-^{13}\text{C}]\)-propionate specifically enriched the \( \alpha \) and \( \beta \)-carbons, respectively, of the vinyl group in (137). A small enrichment of the \( \beta \)-methyl in (138) from \([2-^{13}\text{C}]\)propionate was observed. \([2-^{13}\text{C}]\)Acetate enriched both carbon atoms of the vinyl group, but this can be accounted for by the recycling of acetate through the Krebs cycle and production of propionate from succinate via methylmalonate, as shown in Scheme 42. To explain the labelling, a pathway that is shown in Scheme 43 was proposed. In this, a common tetracyclic intermediate is alkylated by either acetate or propionate to generate either gilvocarcin M or gilvocarcin V, respectively. However, the results for ravidomycin (see above) rule out this pathway. The fault in this study is that the \( ^{13}\text{C} \) n.m.r. analysis was carried out on a mixture. This has obscured the derivation of C-8 in (138) from C-1 of propionate rather than acetate, as would be required by the pathway that is shown in Scheme 43.
As usual, there have been several papers on the biosynthetic pathway leading to the most important mycotoxin aflatoxin B$_1$ (153). By far the most significant of these is the reported observation of a hexanoate 'starter' effect in the biosynthesis of averufin (145). Feeding of [1-13C]hexanoate to Aspergillus parasiticus (ATCC 24551) resulted in high specific incorporation of label at C-1' of averufin. Some incorporation, but at a much lower level, was also observed at positions that would be derived from C-1 of acetate if this were produced as the result of some breakdown of hexanoate to acetate. Two different feeding protocols were used: (a) addition of labelled hexanoate to 48-hour-old mycelial pellets that had been resuspended in a replacement medium that contained a low level of sugar and which were shaken for a further 48 hours, and (b) pulse addition to standing cultures after 48, 72, and 96 hours, followed by work-up after a further 24 hours. This is a most significant result, as it provides the first firm evidence for the previously postulated involvement of linear 'starter' units of C$_4$ or longer carbon chains in the biosynthesis of polyketides. Previous attempts to incorporate these compounds, using 14C-labelled precursors, have mostly resulted in rapid catabolism of the precursors by $\beta$-oxidation, resulting in the secondary incorporation of label via acetate.

In addition, the observation rationalizes some hitherto curious observations on the biosynthesis of aflatoxins, such as the first isolable intermediate on the pathway being norsolorinic acid (143), followed by averantin (144) and then averufin (145), as shown in Scheme 44. Thus norsolorinic acid can be
formally regarded as being formed by the addition of seven malonate units to a separately formed hexanoyl 'starter' to give an octaketide intermediate (142). However, an alternative explanation which cannot be totally excluded at present is that a single polyketide synthetase is involved; this produces the initial C₆ segment, which is able to exchange with free hexanoyl-CoA. Interestingly, no specific incorporation could be obtained when the experiment was repeated with [1-¹³C]butyrate, [1-¹³C]-5-oxohexanate, or [1-¹³C]-3-oxo-octanoate; these all resulted in low, uniform enrichments due to their prior degradation to [1-¹³C]-acetate. These results also help to explain the long-standing observation that the carbon atoms in the bis-furanoid moiety of sterigmatocystin (152) are labelled to a lower extent (ca 10%) than those of the xanthone nucleus (ca 11%) if [1-¹³C]acetate is fed to Aspergillus versicolor.

The incorporation of [4'-¹³C]- and of [1'-¹³C,1'-²H]-averufin into versicolorin A (151) by Aspergillus parasiticus (ATCC 36537) has also been studied. The former enriched C₄ of versicolorin A, in the p.n.d. ¹²C n.m.r. spectrum of versicolorin A that had been enriched from the latter, the resonance for C-1 showed a triplet, due to ¹³C-H coupling. This confirms that the H label at C-1' of averufin is retained during the rearrangement steps in which the linear C₆ side-chain of averufin is converted into the branched C₆ side-chain of versicolorin A and of aflatoxin B, itself. It is proposed that the next intermediate after averufin (145) is nidurufin (146). The relative stereochemistry of the 2'-hydroxyl group of nidurufin has been revised from endo to exo by synthesis of both epimers. This enables the sequence that is shown in Scheme 44 to be proposed for the key rearrangement step. In the former structure for nidurufin the bond between C-2' and oxygen was orthogonal to the migrating bond between C-2 and C-1', whereas in the revised structure these bonds are essentially antiperiplanar, and so ideally disposed stereolectronically for rearrangement to yield the oxonium ion (147), which would be hydrolysed to the aldehyde (148) and would cyclize to the hemiacetal (149). Baeyer-Villiger oxidation of (149) would generate versiconal acetate (ISO), which on hydrolysis and oxidation would yield versicolorin A (151).

Further evidence for the mode of incorporation of averufin into aflatoxin B₁ has been provided by the synthesis of [5,6-¹³C]- and [8,11-¹³C,¹²C]-averufin and by their incorporation into aflatoxin B₁ by cultures of A. parasiticus (ATCC 15517). As expected, analysis of the ¹³C n.m.r. spectra shows that the C-8-C-11 bond of averufin (145) becomes the C-2-C-3 bond of aflatoxin B₁ (153) whereas C-6 of averufin is transformed into C-5 of aflatoxin B, with loss of C-5 of averufin in the process.

A large number of metabolites that are related to the aflatoxin pathway have been isolated from Bipolaris sorokiniana. Averufanin (154), versicolorin C (155), and bipolarin (156) were isolated, along with sterigmatocystin (152), from Bipolaris sorokiniana that had been cultured on maize. When cultured on a liquid medium, several other metabolites were also produced. These are averufin (145), versicolin (157), versicolin acetate (158), versicolinol acetate (150), and a novel xanthone (159).

No bipolarin was isolated, and a re-examination of the data suggests that the previously isolated compound that was formulated as bipolarin (156) was in fact versicolin (157). Versicolin acetate and versicolinol acetate were previously only obtained by treatment of aflatoxin-producing cultures of Aspergillus flavus and A. parasiticus with the enzyme inhibitor dichlorvos. This report of their production under natural conditions strongly supports their intermediacy in the biosynthesis of aflatoxins and of sterigmatocystin.

The austocystins, e.g. austocystin D (160), are toxic metabolites of Aspergillus ustus. Interestingly, these xanthones show a linear fusion of the xanthone and bis-dihydrofuran moieties, in contrast to the angular fusion in sterigmatocystin (152). The incorporation of [1,2,3-¹³C]-acetate into austocystin D results in the labelling pattern that is shown in Scheme 45, consistent with a biosynthesis via ring-cleavage of an anthraquinone, e.g. versicolorin A (151). In support of this, averufin, versicolorin C, and 8-deoxy-6-O-methylversicolorin A are coremetabolites of austocystin D in A. ustus.

### 11 Macrolides
Tylosin (161) is a sixteen-membered-ring macrolide antibiotic whose carbon skeleton is derived from acetate, propionate, and butyrate. The origin of the oxygen atoms in tylactone (162), which is a biosynthetic precursor of tylosin, has been
determined by feeding \([^{13}C,^{18}O}\text{-acetate}, \text{-propionate}, \text{-butyrate}\) to a blocked mutant of *Streptomyces fradiae*. The labelling patterns that are shown in Scheme 46 were obtained.\[^{13}C,^{18}O\]Butyrate, as well as labelling the oxygen that is attached to C-5, also enriched (to a lower extent) the propionate-derived oxygen atoms that are attached to C-3 and to C-15. The results are consistent with macrolide ring-closure by direct displacement of thiol from a C-1 thioester by a hydroxyl group at C-15.

Full details of studies that were designed to elucidate the origins of the carbon skeleton and the oxygen atoms of the erythromycins have been reported.\[^{13}C\]Feeding of \([^{13}C]\text{-and } [^{2-}C] \text{-propionates to cultures of Streptomyces erythreus gave erythromycin A (163) and erythromycin B (164) with the labelling pattern that is shown in Scheme 47. A slightly greater enhancement of the signals due to C-13 in the \[^{13}C\]n.m.r. spectrum of each sample indicated a propionate 'starter' effect. In agreement with these observations, Kuhn–Roth oxidation of a labelled erythromycin that was obtained from feeding \([^{13}C]\text{-propionate to the bacterium gave proponic acid whose p-phenylphenacyl ester had 21.3\% of the specific activity of the intact macrolide. A number of assignments in the \[^{13}C\]n.m.r. spectra were ambiguous. These were resolved by feeding experiments with \([2,3-^{13}C]\text{-succinate. This acts, in } \text{vivo, as a precursor of } [2,2,-1^{13}C] \text{methylmalonyl-CoA, which is generated by the action of methylmalonyl-CoA mutase, and it can therefore be considered as an equivalent, in } \text{vivo, of } [2,3-^{13}C] \text{-propionate. The resultant } [^{13}C,^{13}C \text{-couplings in the } [^{13}C}\n.m.r. spectrum enabled most of the dubious assignments to be resolved. Incorporation of \([^{13}C,^{18}O] \text{-propionate resulted in the incorporation of } [^{18}O] \text{into the oxygen atoms that are attached to C-1, C-3, C-5, C-9, C-11, and C-13. Variable (but small) amounts of oxygen exchange were observed to occur at most sites, resulting in a partial loss of } [^{18}O \text{ label relative to } [^{13}C]. The ketone carbonyl oxygen at C-9 showed more exchange, and this was attributed to the basic conditions (pH 9) that are required during isolation. From these results it is clear that each of the six oxygen atoms that are present in the initially formed aglycon (165) is derived from propionate. The ring-closure must occur by addition of a hydroxyl group at C-13 onto a carboxyl function (C-1). The four secondary alcohol or ether functions on the macrolide ring were all labelled, irrespective of their configuration, i.e. d (C-13) or l (C-3, C-5, and C-11). These results suggest that the oxidation level that is observed in the aglycon (165) is established during elongation of the carbon chain, thereby excluding alternative oxidation or dehydration-re-hydration mechanisms which have been previously proposed.

3-Amino-5-hydroxybenzoic acid (166) has been identified as the key amino acid 'starter' for the biosynthesis of a number of ansamycin antibiotics. Yields of actamycin (171) were increased 4.6-fold when actamycin-producing cultures of *Streptomyces* sp. E/784 were supplemented with (166). However, addition of the 4-chloro-, 6-chloro-, N-methyl, and O-methyl...
analogues (167)-(170) reduced the yield of actamycin but did not cause any structurally modified actamycins to be produced.\textsuperscript{85} These results suggest that the corresponding chlorine, N-methyl, and O-methyl substituents that are present in the nuclei of various ansamycins are introduced at biosynthetic stages beyond the level of the amino acid (166).

The biosynthetic origin of the milbemycins, which are important insecticidal and anthelmintic substances that are produced by \textit{Streptomyces hygroscopicus}, has been studied.\textsuperscript{86} Twenty milbemycins have been isolated but strain Au-3, which was chosen for the biosynthetic study, produces milbemycin \textit{a} (172), milbemycin \textit{a}1 (173), and milbemycin D (174) as its major metabolites. [1-\textsuperscript{13}C\textsubscript{2}]isobutyrate, \textit{dL}(2-\textsuperscript{13}C\textsubscript{2})valine, [1-\textsuperscript{13}C\textsubscript{2}]acetate, \textit{[\textsuperscript{13}C\textsubscript{3}]\textsuperscript{13}C\textsubscript{3}}propionate, and \textit{L}-[\textsuperscript{13}C\textsubscript{3}]methionine were all fed. This revealed that, except for C-25, the carbon skeletons of milbemycins \textit{a}, \textit{a}1, and D are derived from seven acetate units and five propionates, as indicated in Scheme 48. The methyl, ethyl, and isopropyl
groups at C-25 are derived from acetate, propionate, and isobutyrate (itself derived from valine), respectively, and the methoxyl group at C-5 of milbemycins \( \alpha_2 \) and \( \alpha_4 \) is derived from methionine.

12 Polyether Ionophores

Cationomycin (175) is a polyether antibiotic that is produced by a rare actinomycete, *Actinomadura azurea*. It is structurally unique in having an aromatic acyl substituent. Carbon-13-labelled acetate, propionate, and methionine were incorporated to give the labelling pattern that is shown in Scheme 49. Label from \( [2-\text{13}C] \) acetate was extensively randomized, giving rise to multiply labelled propionate, so that extensive \( ^{13}C \)--\( ^{13}C \) coupling was observed among the propionate-derived carbon atoms. This may be rationalized by multiple passages of acetate through the Krebs cycle followed by the formation of methylmalonate via succinate, as shown in Scheme 42.

Somewhat surprisingly, neither labelled orsellinate nor 4-methoxy-6-methylsalicylate was incorporated.

The macrotetrolide ionophore antibiotics (176)-(180) are macrocyclic tetraesters that are built up from both enantiomers of nonactic acid (181) and homononactic acid (182), which in turn are derived from acetate, propionate, and succinate as indicated in Scheme 50. \( [1-\text{13}C,\text{18}O] \) Acetate and -propionate have been incorporated into monactin (176) and monactin (177) by cultures of *Streptomyces griseus* ETHA 7796. The incorporation of \( \text{18}O \) was determined by the reduction of (176) and (177) to the diols (183) and (184), respectively, which were converted into their \( (-\alpha-)\alpha\text{-methoxy-}\alpha\text{-trifluoromethylphenylacetyl esters} \) (185)-(188) for separation by h.p.l.c. and subsequent \( ^{13}C \) n.m.r. analysis. Incorporation of \( [1-\text{13}C,\text{18}O] \) acetate led only to enrichment of the oxygen that is attached to C-8 in (185) and (186) whereas \( [1-\text{13}C,\text{18}O] \) propionate enriched the oxygen atoms that are attached to C-1, C-6, and C-8 in (187) and (188) and to C-1 and C-6 in (185) and (186). These results are consistent with the conversion of propionate into succinate (see Scheme 42) and with the formation of the thioester (191) by reduction of an intermediate such as (189), which is converted [via reduction to the diol (190) and intramolecular Michael reaction] into (191) as shown in Scheme 51. Since the observed retention of \( ^{18}O \) relative to \( ^{13}C \) at C-1 in samples of (187) and (188) that had been derived from \( [1-\text{13}C,\text{18}O] \) propionate is >50%, it follows that homononactic acid (182) [and therefore nonactic acid (181)] is not an obligatory intermediate on the biosynthetic pathway to the macrotetrolides, but that a thioester intermediate such as (191) can react to generate an ester bond by direct displacement of the thiol activating group by the hydroxyl group that is attached to C-8 of a second building block. Thus the biosynthesis probably proceeds from acetate, propionate, and succinate without the intervention of free unactivated intermediates, despite the fact that they can be isolated from cultures and incorporated into the macrotetrolides.

13 Miscellaneous Metabolites

Full details of an extensive study, using stable isotopes, to investigate the biosynthesis of the antibiotic virginiamycin M1 (192) in *Streptomyces virginiiae* have appeared. The skeleton is derived from valine, seven acetate units, glycine, serine, and proline, as summarized in Scheme 52. The methyl group at C-32 is derived from methionine but the methyl at C-33 is derived by a novel pathway involving decarboxylation of an acetate unit, as shown in Scheme 53. That the methyl group is derived from an acetate unit that is added to a pre-formed polyketide

![Scheme 50](image)

![Scheme 51](image)
chain, rather than by one of the alternative mechanisms that have been proposed, was demonstrated by a rather subtle experiment in which \(^{[3,13]C}\)serine was used as a delayed source of \(^{[2,13]C}\)acetate. \(^{[2,13]C}\)Acetate enriched carbons 4, 6, 11, 13, 15, 17, and 33 to the same extent. However, while \(^{[3,13]C}\)serine enriched carbons 4, 6, 11, 13, 15, and 17 to the extent of 0.8%, the enrichment at C-33 was 1.9%. This experiment depends on the conversion of serine into acetyl-CoA via dehydration and deamination to pyruvate. It would have been interesting to see if feeding \(^{[2,13]C}\)malonate also gave differential labelling. The oxazole ring is formed from serine, presumably by a pathway such as shown in Scheme 54.

A similar derivation of a methyl group from C-2 of an acetate unit, which is added to a pre-formed polyketide, has been demonstrated during the biosynthesis of two metabolites that have been isolated from gliding bacteria. The first of these is myxopyronin A (193), which is an antibiotic that has been isolated from Myxococcus fulvus.\(^{90}\) Incorporation studies with


13C-labelled acetate, glycine, and methionine indicated that the carbon skeleton is derived from two polylketide chains (Scheme 55). On incorporation of [1-13C,1-14N]glycine, C-12 showed both 13C-13C and 13C-15N couplings, so that glycine is incorporated intact as a ‘starter’ unit in one of the chains. The methyl groups C-8 and C-17 are derived from methionine whereas C-21 is enriched from [2-13C]acetate.

A similar result was observed for C-33 in the antibiotic myxoviricin A₁ (194), which is a metabolite of Myxococcus virescens. Carbon-13-labelled acetates and methionine are incorporated as shown in Scheme 56, C-33 being derived from C-2 of a cleaved acetate unit. Even more curiously, both carbon atoms of the ethyl group that is attached to C-13 are derived from C-2 of acetate. Although no pathway is suggested for this, derivation via an intermediate in the Krebs cycle appears to be likely. Glycine functions as the ‘starter’ unit of a polylketide chain, which is then alkylated at acetate-derived methylenes (C-2 and C-4) and carbonyls (C-13 and C-17). The remaining moiety appears to be a C₂ hydroxy-acid that has been derived from acetate and methionine, but at what stage it is incorporated [and which bond (ester or amide) is formed] is not yet known. Further results on this intriguing compound will be awaited with eager anticipation.

14 References

21 J. S. E. Holker, personal communication to T. J. Simpson.
13C-NMR in Metabolic Studies

T. J. SIMPSON

1 Introduction

The study of natural products and the development of organic chemistry have always been closely related, and the rapid growth of organic chemistry in the 19th century was due largely to the efforts made in isolating and characterising some of the more biologically active plant metabolites. A lasting result of this is the predominance of the methodology of organic chemistry in the study of the biosynthesis and metabolism of secondary plant products, in contrast to the study of primary metabolism in which biochemical methods have held sway.

In this chapter the general principles involved in applying stable isotope labelling methods in conjunction with 13C NMR spectroscopy to the study of secondary metabolism will be described. For reasons which will become obvious below, these techniques will be illustrated in many cases by reference to studies in microorganisms. Whether micro-organisms, in particular the lower fungi, can be regarded as plants is debatable, but as many of the techniques have been developed and applied in studies of fungal metabolism, their use for illustrative purposes is appropriate. The latter parts of the sections will survey the metabolic pathways in higher plants which have been studied using 13C NMR. It is hoped to convey the basic requirements for carrying out 13C-labelling studies to both potential users and those interested in the results, not only to give some idea of the strengths and advantages of these techniques but also their limitations and the difficulties and disadvantages associated with their use, and general considerations in interpreting the results.

As a necessary preamble, a brief discussion follows of some basic principles and considerations in biosynthetic studies and of radioisotope tracer methods.

2 The Design and Interpretation of Labelling Experiments

Some of the principles involved in carrying out biosynthetic experiments have been usefully discussed in a number of texts (Bu’lock 1965; Manitto 1981; Herbert 1981; Brown 1972). The most fundamental method for establishing the intermediates involved in a biosynthetic pathway is to label likely compounds with an isotope and then use this isotopic label to assess the extent to which the compounds are incorporated into the products of the pathway. A number of problems are associated with this.
Low or negligible incorporation, even of true precursors, is often observed. Compounds of general metabolic importance may show low incorporation into the metabolites of interest due to the diversity of other routes open to them. In studies using whole organisms, particularly in the higher plants, structurally complex or highly polar intermediates may not be able to cross cell membranes, or may not be transported to the site of biosynthesis in highly differentiated organisms, or they may be degraded or otherwise metabolised before they can be utilised. Therefore a negative result is always inconclusive and must be regarded with caution.

Careful interpretation is also required in the case of apparently positive results, particularly when using the isolation and incorporation of other metabolites to obtain information on the later stages of a pathway. Scheme 1 illustrates some

![Scheme 1](image)

of the problems. Thus, a compound (L) may be isolated, which when labelled appears to be incorporated into the final metabolite. However, unless it can be shown that the label is incorporated specifically (i.e. from a known position in the precursor into a corresponding position in the metabolite of interest), the possibility remains that degradation to a common early precursor followed by incorporation of this degradation product has taken place. A striking example of this was provided by the reported intact incorporation of \([14-\text{¹⁴C}]\) palmitic acid (1) into brefeldin A (2) by cultures of *Penicillium brefeldianum*. However, subsequent work (Cross and Hendley 1975) showed that the observed incorporation was due to degradation of palmitic acid to produce \([2-\text{¹⁴C}]\)-acyl CoA (3) which was then incorporated to produce brefeldin A labelled as shown in Scheme 2.

![Scheme 2](image)

Even if specific incorporation can be demonstrated, the supposed intermediate may not lie on the main pathway, but may be formed as shown in scheme 1 reversibly as a “shunt” metabolite (E) from a compound (D) which is on the pathway. A further complicating factor is the often observed lack of specificity of the
enzymes of secondary metabolism giving rise to a metabolic grid, as illustrated by compounds (D), (F), (G), (H), (I), and (J) in Scheme 1. As indicated there are a priori three possible routes from (D) to (J), so that (F), (G), (H), and (I) are all possible but not obligatory intermediates. An interesting example of this and of its solution has been described by Campbell in a study of mycophenolic acid (4) biosynthesis (Doerfier et al. 1980).

3 Radioisotope Labelling

Until the advent of $^{13}$C NMR techniques, labelling with the radioactive isotopes $^{14}$C and $^3$H was the most powerful method of biosynthetic investigation. The major advantage of these radionuclides is their negligible natural abundance, which, when coupled with the low limits of detection of modern liquid scintillation counters (Faires and Boswell 1981) allows very low levels of precursor incorporation to be accurately measured. Only very small quantities of labelled precursors are required, providing that the specific activity is sufficiently high, thus the usual assumption that the addition of tracer does not itself affect the metabolic pathway under study is likely to be valid.

The main disadvantages are the need to obtain radiochemically pure compounds and the problem of establishing specificity of incorporation of label. This usually requires experimentally demanding and often impractical degradative chemistry, especially where the metabolite has been labelled from a simple precursor such as acetate or mevalonate and is labelled in many sites. More complex intermediates may be labelled in two sites with both $^3$H and $^{14}$C in a known ratio and administered to an organism. The isolation of a product with the same $^3$H:$^{14}$C ratio as the precursor provides good evidence for intact incorporation of the precursor. This remains a good method for obtaining information on the stereochemical fate of particular hydrogen atoms in a molecule, although $^2$H, $^{13}$C double labelling (see Sects. 8.1 and 8.2) and $^2$H labelling in conjunction with $^2$H NMR analysis (Garson and Staunton 1979) are replacing it.

Nonetheless, if accurate quantitative data on the incorporation of labelled atoms into a metabolite is needed, but precise location of the label is not important, the use of radioisotopes is still indicated. Also it is the method of choice for establishing the most efficient precursor feeding regime prior to $^{13}$C labelling studies (see Sect. 5.1).
4 $^{13}$C NMR Spectroscopy

The most important stable isotopes in biosynthetic studies are $^{13}$C, $^2$H, $^{18}$O and $^{15}$N. The advent of high field Fourier-transform spectrometers has allowed the development of $^{13}$C NMR spectroscopy to provide a non-destructive detection system, capable of determining the location and relative concentration of each chemically non-equivalent carbon in a molecule, as will be shown. It also enables the presence of $^2$H, $^{18}$O, and $^{15}$N to be demonstrated by using isotope-induced shifts in $^{13}$C NMR resonance frequencies or by spin-spin coupling to $^{13}$C.

A large number of texts (Levy et al. 1980; Abraham and Loftus 1978; Breitmaier and Voelter 1978; Stothers 1972) are available which describe the basic principles of $^{13}$C NMR spectroscopy in greater or lesser detail, so only the essential details are summarised here. Since the $^{13}$C nucleus has a nuclear spin ($I = \frac{1}{2}$), nuclear magnetic resonance signals may be observed, and with the development of sensitive instruments with the means to accumulate (and thus enhance) weak signals, the amount of data available on $^{13}$C chemical shifts and spin-spin coupling constants (Marshall 1983) to $^{13}$C, $^1$H, and $^{15}$N is increasing rapidly. The undecoupled $^{13}$C NMR spectrum shows coupling to both directly bonded hydrogens and longer-range couplings to non-bonded hydrogens. Analysis of these couplings is often of great utility in both structural elucidation and spectral assignments (see Sect. 4.1.3) but the spectra are usually proton noise-decoupled so that a single line is obtained for each carbon in the molecule. Thus, although the natural abundance of $^{13}$C is only 1.1%, it is now relatively easy to obtain $^{13}$C NMR spectra for any compound given a minimal amount of material (e.g., 50 mg of a compound of molecular weight 500). One great advantage of $^{13}$C NMR in the considerable spread of chemical shifts commonly observed: about 200 ppm compared with 10 ppm for $^1$H shifts. These shifts are of the same relative order as $^1$H shifts, i.e. alkyl carbons have shifts at higher field (smaller $\delta$ value) than aromatic or olefinic carbons, and carbons carrying oxygen or other electronegative substituents have larger $\delta$ values than those bearing only carbon or hydrogen substituents. Carbonyl carbons usually provide the lowest field signals. Thus even complex molecules generally give proton noise-decoupled (PND) $^{13}$C NMR spectra in which a single line is observed for each carbon in the molecule and coincident lines are relatively rare.

4.1 Assignment of $^{13}$C NMR Spectra

4.1.1 Known Chemical Shifts and Substituent Chemical Shift Effects

The chemical shifts of different functional types fall into well defined ranges; carbonyl carbons resonate at low field (ca. 200 ppm), aromatic and olefinic carbons at 160–100 ppm, aliphatic carbons with electronegative substituents at 90–50 ppm, and simple aliphatics at highest field 50–10 ppm. Substituent effects are generally found to be additive, and rules for predicting chemical shifts in hydrocarbons and aromatics are available. Useful compilations of chemical shifts are available (Breitmaier et al. 1979).
4.1.2 Off-Resonance and Specific Proton Decoupling

In the PND spectrum all $^1$H-$^{13}$C coupling information is lost. In the off-resonance decoupled spectrum, the $^1$H irradiation is kept at high power levels but the decoupling frequency is moved ca. 500 Hz away from the protons being irradiated, so that one bond $^1$H-$^{13}$C coupling patterns partially return and so quaternary, methine, methylene and methyl carbons appear as singlets, doublets, triplets and quartets respectively. If the $^1$H NMR spectrum has been fully or partially assigned, the $^1$H and $^{13}$C resonances can be interrelated by single-frequency decoupling with the decoupler set at the exact $^1$H frequency. The attached carbon appears as a singlet in the $^{13}$C spectrum, whereas the remaining carbons show off-resonance patterns. This process becomes time-consuming if many resonances require decoupling, but can be overcome by plotting the line frequencies in the $^{13}$C NMR spectrum as the $^1$H irradiating frequency is stepped through the $^1$H NMR spectrum. Where the lines cross gives the exact $^1$H and $^{13}$C frequencies (Birdsall 1972). The four oxygen-bearing carbons in aspyrone (5) have been assigned in this way, as shown in Fig. 1 (Holker and Simpson 1981).

4.1.3 Analysis of Long-Range $^1$H-$^{13}$C Couplings

A particularly useful method for both spectral assignment and structural elucidation is to analyse the origins of long-range, i.e., greater than one-bond $^1$H-$^{13}$C couplings using specific, low-power decoupling to selectively remove long-range couplings to $^{13}$C only from the hydrogen which is being irradiated. This has been used to great effect in the structural reassignment and spectral assignment of the antibiotic LL-D253s (6), a chromanone metabolite of *Phoma pigmenticora*. 
(McIntyre and Simpson 1984). Figure 2 shows the downfield region of the fully $^1$H coupled $^{13}$C NMR spectrum of (6) and the results of a series of specific decoupling experiments. Thus it may be seen that C-7 which appears as a quartet in Fig. 2a simplifies to triplet on irradiation of H-6 (Fig. 2e) and to a doublet on irradiation of the benzylic methylene hydrogens (Fig. 2d) due to the removal of

![Chemical structure](image)

**Fig. 2a-f.** The low-field region of the fully $^1$H-coupled 50 MHz $^{13}$C NMR spectrum of LL-D253α diacetate and the results of selective $^1$H-decoupling experiments
2- and 3-bond couplings respectively. Irradiation of H-6 simplifies the pentet due to C-5 in Fig. 2a to a quartet (Fig. 2e). This quartet splitting is due to coupling to the methoxyl hydrogens as an irradiation of these the signal due to C-5 now appears as a doublet (Fig. 2f). Similarly, it may be seen that C-8 which appears as a multiplet in Fig. 2a shows 2-bond coupling to the C-10 methylene hydrogens and 3-bond couplings to H-6 and to the C-11 methylene hydrogens. The long-range couplings which have been identified in this way are summarised in Fig. 3. These permit only one structure and simultaneously assign the $^{13}$C NMR spectrum prior to biosynthetic studies.

4.1.4 Polarisation Transfer and 2D NMR Methods (Benn and Gunther 1983)

The methods described in Sections 4.1.2 and 4.1.3 have been partly superceded, e.g. by polarisation transfer methods such as DEPT and INEPT which provide a more sensitive method of identifying and distinguishing methyl, methylene, methine and quaternary carbons. These methods utilise pulse sequences which enable only those carbons with a given number of hydrogens attached to be observed selectively. Thus they can be used to simplify congested spectral regions so that only carbons bearing one, two or three, or indeed no hydrogens, are observed. This is illustrated in Fig. 4 which shows the DEPT spectra of austin (7), a mycotoxin isolated from Aspergillus usus. Correlation of $^1$H and $^{13}$C chemical shifts can be achieved by two-dimensional heteroscalar correlation NMR spectra. Figure 5 shows how the $^1$H and $^{13}$C resonances for the methyl groups in terretonin (8), a metabolite of Aspergillus terreus, can be matched. Other pulse sequences such as 2D INADEQUATE permit carbon connectivity patterns to be established. These methods have been very well reviewed (Benn and Gunther 1983) but
Fig. 4. a Normal PND $^{13}$C NMR spectrum of austin (7) and DEPT spectra showing
b methines only, c methylenes only, d methyls only and e quaternary carbons only.

Fig. 5. Two-dimensional heteroscalar correlation NMR spectrum of terretonin (8) matching the $^1$H and $^{13}$C resonances of each methyl group.
it should be noted that they require not only sophisticated spectrometers with very good data-handling facilities, but also highly skilled operators. They are far from being the routine methods that the literature often implies.

4.1.5 Lanthanide-Induced Shift Studies
These are not as useful in $^{13}$C studies as in $^1$H NMR because, as for solvent and anisotropy effects, the actual shifts are of the same absolute value in both and so are relatively small in $^{13}$C NMR. However, they can be useful for separating overlapping resonances. In complicatic acid (9) the resonances due to C-3 and C-10 both occur at 46 ppm. However addition of Eu(fod)$_3$ separated these resonances and showed that C-3 but not C-10 was enriched from [1-$^{13}$C]acetate in cultures of Stereum complicatum (Feline et al. 1974).

Another important use of shift reagents is to resolve the $^1$H NMR spectrum prior to specific proton decoupling studies on the $^{13}$C NMR spectrum.

4.1.6 Model and Derivative Studies
Model compounds whose chemical are known can be helpful in assigning the spectrum of a new compound, though they must be used with care. Quaternary carbons always present the greatest difficulties in assignment, and studying the variation in chemical shifts in a series of closely related compounds may be the only method of reaching an assignment. The $^{13}$C NMR spectrum of tajixanthone (10), a prenylated xanthone, was fully assigned by a study of eleven derivatives prior to biosynthetic studies (Holker et al. 1974).

4.1.7 Synthesis of Isotopically Labelled Compounds
Synthesis of a compound enriched at a known site with $^{13}$C or $^2$H may be used for assignment. This has the effect either of enhancing the signal of the labelled carbon or deleting the signal from the spectrum in the case of $^2$H substitution.
4.1.8 Incorporation Studies

$^{15}$N is an isotope of spin $\frac{1}{2}$ and so is NMR active and will couple with $^{13}$C nuclei. Thus a metabolite grown in the presence of, say, K$^{15}$NO$_3$ may incorporate $^{15}$N and will exhibit $^{15}$N-$^{13}$C couplings for any carbons bonded to nitrogen. Both one- and two-bond couplings may be observed. This technique was used in both structural elucidation and spectral assignment studies (Birch and Simpson 1979) of phomazarin (11). The couplings observed are indicated in Fig. 6. Obviously the $^{15}$N may also be incorporated by chemical synthesis.

Similarly any compound with adjacent $^{13}$C nuclei will exhibit $^{13}$C-$^{13}$C couplings (see Sect. 6.2.2) between these nuclei. Again, the $^{13}$C may be incorporated metabolically or by synthesis.

5 Biosynthetic Methodology

The availability of compounds enriched with $^{13}$C and other stable isotopes has increased rapidly and a wide range is now available at enrichments of up to 99%. In addition, many others may be readily synthesised by standard methods (Ott 1980) from simple precursors such as $^{13}$CO$_2$, $^{13}$CH$_3$I, K$^{15}$CN, $^{15}$NH$_4$Cl, and H$_2$$^{18}$O.

Quite apart from the convenience of using a non-radioactive isotope, the main advantage of $^{13}$C NMR studies over $^{14}$C radio-labelling is the absence of the need for chemical degradations to establish the sites of labelling. This is particularly important as degradative chemistry is now little used in structure determination, whereas $^{13}$C NMR is routinely used for this purpose.

A disadvantage of $^{13}$C NMR, one not shared by radio-labelling, is its insensitivity. This is due mostly to the low energy of the transitions measured in NMR generally, but the problem is more acute in the case of $^{13}$C than for protons because of the smaller magnetogyric ratio ($\gamma$) of $^{13}$C (only 0.252 times that of proton). Sensitivity, atom for atom, is proportional to $\gamma^3$. In addition, the natural abundance of $^{13}$C is only 1.1%. This is a mixed blessing. It means that natural abundance spectra are reasonably easy to obtain without the complications of $^{13}$C-$^{13}$C coupling; however, it also requires that very efficient incorporation of precursors into products is achieved. For this reason, the method has found more limited application in plant studies, as high incorporations are difficult to achieve and whatever labelled precursor is incorporated tends to become diluted by large pools of endogenous material.
5.1 Precursor Incorporation (Simpson 1975)

Before carrying out experiments with stable isotopes, it is necessary to carry out extensive experiments to optimise conditions for feeding of labelled substrates. It is routine procedure to perform these preliminary experiments with $^{14}$C-labelled precursors. Precursor efficiency may be assessed in several ways in biosynthetic experiments (Brown 1972) but for $^{13}$C studies the important criterion is dilution of added label. For $^{14}$C, dilution per labelled site is given by:

\[
\text{specific activity of precursor} \times \text{no. of labelled sites} \div \text{specific activity of product}
\]

To obtain unequivocal results in $^{13}$C studies using typically 90% enriched precursors, dilutions per labelled site of ca. 100 or less are therefore required (see Sect. 6.1.1).

Three main parameters require studying: time of precursor addition, period of growth after addition, and amount of precursor required. Maximum incorporation usually occurs when precursors are added at the start of maximum metabolite production, e.g. the start of the idiophase in the case of microbial fermentations. This necessitates determination of cell growth and metabolite production curves. The period of growth after addition of precursor may be critical. If the turnover of exogenous precursor is rapid, then prolonged growth results merely in increased dilution of labelled metabolite by further metabolite production from unlabelled endogenous substrate. Thus it is important to note that neither maximum yield of metabolite or maximum total incorporation of label is the important factor, the prime consideration being to obtain the minimum dilution of label given a sufficient yield of metabolite for the $^{13}$C NMR spectrum to be determined. In general, dilution decreases with increasing amounts of precursor. Thus mass versus incorporation studies will determine the minimum amount of labelled precursor that must be added to obtain a satisfactory enrichment.

The amount of labelled precursor that can be used is limited both by the actual expense of the isotopic label and also due to the adverse effects that high levels of added precursor may have on the metabolism of the organism and may in fact change the biosynthetic pathway under study. Toxicity effects may be overcome by pulsed addition of precursor or by continuous slow addition over a prolonged period by use of e.g. peristaltic pumps.

6 $^{13}$C Enrichment Studies

The labelling pattern resulting from incorporation of $^{13}$C-enriched precursors is determined by obtaining the PND $^{13}$C NMR spectrum of the labelled metabolite and comparing it with the spectrum of the unlabelled metabolite. A large number of different types of experiments are now available.
6.1 Single $^{13}$C-Labelling

This was the first method to be developed and remains the simplest. It is best understood by examining a formal model system.

If we consider any four contiguous carbons in a polyketide derived molecule, normally these carbons will be derived from endogenous acetyl CoA produced by

(a) $\text{MeCOSCoA} \rightarrow -\text{C}_1\text{-C}_2\text{-C}_3\text{-C}_4$

(b) $\text{MeC}\text{O}_2\text{Na} \rightarrow -\text{C}_1\text{-C}_2\text{-C}_3\text{-C}_4$

(c) $^{13}\text{CH}_3\text{C}_2\text{O}_2\text{Na} \rightarrow -\text{C}_1\text{-C}_2\text{-C}_3\text{-C}_4$

Fig. 7a–c. Simulated PND $^{13}$C NMR spectra of a polyketide-derived moiety a at natural abundance, b enriched from [1-$^{13}$C]acetate, and c enriched from [2-$^{13}$C]acetate

Fig. 8a–c. PND $^{13}$C NMR spectra of the diacetate of deoxyherqueirone: a at natural abundance, b enriched from [1-$^{13}$C]acetate, and c enriched from [2-$^{13}$C]acetate
the cell’s normal metabolism and will contain only natural abundance $^{13}$C (1.1%) and so in the PND $^{13}$C NMR spectrum each carbon will give rise to one sharp line of more or less equal intensity, Fig. 7a. Now, if sodium acetate, in which the carboxyl carbon is highly enriched (ca. 95%) with $^{13}$C, ([l-$^{13}$C]acetate) is added, then this exogenous acetate will be diluted to a greater or lesser extent by the endogenous acetate pool, but some of it will be incorporated into the metabolite and so those carbons, say C-2 and C-4, which were originally derived from the carboxyl carbon of acetate, will contain extra $^{13}$C and this will manifest itself as an increase in the appropriate signal intensities in the $^{13}$C NMR spectrum of the enriched metabolite, Fig. 7b. If acetate in which the methyl carbon is enriched ([2-$^{13}$C]acetate) is now added, then enhancement of the alternate signals is obtained, Fig. 7c. Thus, simply by feeding $^{13}$C-labelled precursor and determining the $^{13}$C NMR spectrum, the signals which show enhanced intensities indicate the sites of enrichment provided the $^{13}$C NMR spectrum has been assigned unambiguously. It is important to note that this does not give us any further information than (in theory at least) could have been obtained by classical $^{14}$C techniques, though in practice it is easier and usually more comprehensive.

An example of single labelling studies with acetate is the incorporation of [l-$^{13}$C]- and [2-$^{13}$C]acetates into deoxyherqueinone (12), a metabolite of Penicillium herquei (Simpson 1979). The resultant spectra are shown in Fig. 8. If high enrichments are obtained, as in this case, the labelled sites are readily apparent from visual inspection of the spectrum. However, in general, enrichments are lower and identification of enriched sites with certainty can be more difficult, as discussed below.

### 6.1.1 Quantitative $^{13}$C Measurements

In principle at least, quantitative values for the degree of enrichment by labelled precursors and intermediates can be determined by comparing the integrated intensities of different carbon resonances in the PND $^{13}$C NMR spectrum. The percentage of $^{13}$C isotope above natural abundance has been defined as:

\[
\text{%age enrichment} = 1.1 \times \frac{\text{(integrated intensity at labelled centre)}}{\text{(integrated intensity at unlabelled centre)}} - 1.1.
\]
In practice, however, comparing signal heights or integrals is problematic and the complications have been widely discussed (Abraham and Loftus 1978; Simpson 1975). Due to the wide range of spin-lattice relaxation times ($T_1$'s) encountered in $^{13}$C NMR spectroscopy and the variable effects of the nuclear Overhauser enhancement (NOE) induced by proton decoupling, the line intensities in a $^{13}$C spectrum usually show wide variation even in a natural abundance spectrum. Fourier transform NMR spectrometers exacerbate this problem using normal pulse sequences and add another one: the digitization of data often results in a poorly defined peak shape. The first problem can be circumvented to an extent by comparing unenriched and enriched resonances of carbons in a similar environment, but a better solution is to obtain natural abundance and enriched spectra under identical conditions, normalise both spectra to a standard which is known to be unlabelled, and compare the intensities of the lines in the two spectra directly. An alternative approach is to add a paramagnetic relaxation agent such as chromium Tris-acetoacetonate which suppresses the NOE and shortens the $T_1$'s. It may also be necessary to use "inverse gated" proton decoupling in which the NOE is reduced by only decoupling during acquisition. Further alleviation of the effects of long $T_1$'s may be achieved by the use of a long delay between pulses.

The second problem, that of data handling, manifests itself in random variations in intensities in the lines of spectra obtained from the same sample under the same experimental parameters. These variations can be reduced by using more data points to define the spectrum or by acquiring the spectrum several times and averaging the results. As can be seen, the price to be paid in instrument time, in return for reliable enrichment data, is considerable, and some compromise is generally required.

### 6.1.2 Single $^{13}$C-Labelling Studies in Plants

Due to the reasons discussed above, $^{13}$C-labelling studies are much more difficult to carry out on higher plant metabolites than on microbial metabolites. Some of the problems associated with precursor incorporation and transport, and dilution by endogenous pools of precursors and metabolites can be overcome by use of cell-free enzyme systems and in particular by use of tissue cultures. Obviously both of these require much effort to develop workable systems. However, there have been a number of successful applications, particularly in the alkaloid field and in the important area of phytoalexin metabolites.

$^{14}$C-Labelling studies indicated that the lactam (14) was an intermediate in the biosynthesis of camptothecin (15) in *Camptotheca acuminata*. Owing to the ab-

\[ \text{[13]} \rightarrow \text{[14]} \rightarrow \text{[15]} \]
ence of suitable degradations, $^{13}$C NMR was used to prove specificity of incorporation. Thus, [1-$^{13}$C]tryptamine (13) was synthesised from K$^{13}$CN and converted to the [5-$^{13}$C]lactam (14), 38 mg of which was wick-fed to intact plants. After 2 days growth, 20 mg of comptothecin was isolated which showed a ca. 55\% enhancement of the C-5 resonance only (Hutchinson et al. 1974).

[1-$^{13}$C]Autamnaline (16) was synthesised and injected as the hydrochloride (300 mg) into seed capsules (1 mg per capsule) of Colchicum autumnale. After 2 weeks’ growth 1.24 g colchicine (17) was isolated. The resultant $^{13}$C NMR spectrum showed a 2.5-fold enhancement of the C-7 signal only (Battersby et al. 1974).

By feeding [2’-$^{13}$C,$^{14}$C]anatabine (18) to various Nicotania species it was established that this alkaloid is a precursor of the $\alpha\beta$-bipyridyl (19), the $^{13}$C label being detected by the enrichment observed for C-2 (10\%) by $^{13}$C NMR. The precursor was fed to plants in hydroponics. However the transformation was only observed in plants which had been allowed to dry in the sun for several days and no conversion could be detected in freshly harvested plants so it is not clear whether it is an enzymic process or simply due to aerial oxidation (Leete et al. 1979).

Feeding [N-Methyl-$^{13}$C]stylopine $\alpha$-methochloride (20) to Chelidonium majus resulted in a 10\% enrichment of the N-methyl signal of protopine (21) and a 29\%
enrichment of the N-methyl signal of chelidonine (22). Similarly [N-methyl-\(^{13}\)C]protopine enriched the N-methyl signals of chelidonine (22) and sanguinarene (23) by ca. 40% in each case. Other transformations are reported in this paper but the level of enrichments observed are probably too low to be regarded as certain (Takao et al. 1976).

The isolation of a range of naphthoquinones and anthraquinones from plant cell cultures has allowed their biosynthesis to be studied by \(^{13}\)C NMR. Thus, o-succinylbenzoic acid (24), labelled as shown in was incorporated into dunnione (30), \(\alpha\)-dunnione (29) and 8-hydroxydunnione (31), and the anthraquinones (32) and (33) as shown. The location of the introduced label led to the conclusion that the naphthoquinones were biosynthesised via (25) and lawsone (26) which is O-prenylated to give (27) (Scheme 3). A Claisen-type rearrangement then leads to (28) which can then be converted into (29) or into (30) and (31) (Inoue et al. 1982). The anthraquinones appear to be produced by prenylation at C-2 of (25) as shown. However, feeding \(^{13}\)C-labelled o-succinylbenzoic acid to cell cultures of Gallium mollugo gave lucidin primeveroside (34) labelled as shown demonstrating that prenylation occurs at C-3 in this case (Inoue et al. 1979).

3,10-Dihydro-1,4-dimethylazulene (35) is an unusual product of cultured cells of the liverwort Calyprogeia granulata (Takeda and Katoh 1983). Biosynthetic studies using \([2-{^{13}}\text{C}]\text{acetate}\) have established a terpenoid origin for (35) and its co-metabolite (36).

### 6.2 \(^{13}\)C—\(^{13}\)C Spin-Spin Coupling

In natural abundance \(^{13}\)C NMR spectra \(^{13}\)C—\(^{13}\)C spin-spin coupling is not normally observed as the probability of \(^{13}\)C nuclei being adjacent is equal to the square of the natural abundance, giving satellites of 0.55% of the intensity of the main signal. They can be observed by resorting to the double quantum resonance technique, INADEQUATE, which allow observation only of coupled signals (Benn and Gunther 1983), but this requires very large samples and sophisticated spectrometers. However, in enriched metabolites, the probability of having adjacent \(^{13}\)C nuclei is much higher and the detection of a \(^{13}\)C—\(^{13}\)C coupling can provide conclusive evidence that two labels have been incorporated into adjacent positions in a molecule. This is normally done by using doubly labelled precursors, but \(^{12}\)C—\(^{13}\)C couplings can also arise from administration of singly labelled precursors.

#### 6.2.1 Singly \(^{13}\)C-Labelled Precursors

Molecular rearrangement of a biosynthetic intermediate may give rise to a \(^{13}\)C—\(^{13}\)C coupling. Aspyrone (5), a metabolite of Aspergillus melleus (Holker and Simpson 1981), when enriched from \([2-{^{13}}\text{C}]\text{acetate}\) shows a coupling of 61 Hz between C-2 and C-7 (Fig. 9b). This coupling arises due to an intramolecular rearrangement of the precursor pentaketide chain as shown in Scheme 4.
Incorporation of $[1^{-13}C]$acetate into the sesquiterpene dihydrobotrydial (38) in Botrytis cinerea resulted in a coupling being observed between C-7 and C-8 (Bradshaw et al. 1977). Conversion of $[2^{-13}C]$acetate into succinate in the Krebs cycle results in a $^{13}C-^{13}C$ coupling between C-11 and C-15 in avenaciolide (39) (Tanabe et al. 1973). Similar metabolic transformations give rise to $^{13}C-^{13}C$ cou-
6.2.2 Double $^{13}$C-Labelled Precursors

This has been one of the major developments in biosynthetic methodology and permits information to be obtained which would have been impossible or at best extremely difficult to obtain by classical radio-isotope labelling techniques. Again, the basic concept can be illustrated by consideration of a model polyketide system (Fig. 10).

If we consider a molecule of acetate in which both carbons are entirely $^{13}$C, ([1,2-$^{13}$C$_2$]acetate), it contains two adjacent nuclei of spin $\frac{1}{2}$ and so they will couple to each other. If this acetate molecule is incorporated intact into a metabolite, then in any individual molecule, those pairs of carbons derived from an originally intact acetate unit must necessarily both be enriched simultaneously and so will show a mutual $^{13}$C-$^{13}$C coupling. Thus if C-1 is enriched, then C-2 must also be enriched. In the resultant $^{13}$C NMR spectrum, the natural abundance signal is flanked by $^{13}$C-$^{13}$C coupling satellites (Fig. 10b). By analysing the coupling...
patterns, information is obtained on the way in which the precursor molecules are assembled on the enzyme surface, and on the way the, in this case, polyketide, chain folds up prior to condensation and cyclisation. If at any stage in the biosynthesis the bond between two carbons originally derived from an intact acetate unit is broken, then the $^{13}\text{C}^{13}\text{C}$ coupling is lost and these carbons then appear simply as enhanced singlets, as shown for C-3 and C-4 in Fig. 10c. In this way bond cleavage and rearrangement processes occurring during biosynthesis can be detected.

$^{13}\text{C}^{13}\text{C}$ Couplings are generally between 30 and 80 Hz and increase in proportion to the amount of “s” character of the atoms in the bond. Hence sp$^3$—sp$^3$ bonds are typically 35 Hz; sp$^2$—sp$^3$ are 45 Hz, and sp$^2$—sp$^2$ are 60 Hz (Marshall 1983). Substitution of one or both atoms by oxygen increases the size of the coupling. Usually the couplings will be sufficiently different in magnitude to enable the pairs of coupled carbons to be matched up. In addition, if the spectrum has been unambiguously assigned it will be apparent which carbons are mutually coupled. Couplings may also be confirmed by selective homonuclear $^{13}\text{C}^{13}\text{C}$ decoupling or by a 2D INADEQUATE spectrum (Benn and Gunther 1983).

The contiguous double labelling technique has been used extensively. This is partly because it extends the permissable dilution factor to at least 2000, as small $^{13}\text{C}^{13}\text{C}$ coupling satellites can be observed with more certainty than the corresponding enrichment from a singly labelled precursor. However, its success is largely because of the extra information obtainable in respect of bond cleavages, rearrangements and symmetrical elements of intermediates.

The $^{13}\text{C}$ NMR spectrum resulting from incorporation of [1,2-$^{13}\text{C}_2$]acetate into aspyrone (5) is shown in Fig. 9c. $^{13}\text{C}^{13}\text{C}$ couplings of 68, 41 and 44 Hz are observed between C-2 and C-3, C-4 and C-5, and C-8 and C-9 respectively, indicating their derivation from originally intact acetate units. C-1, C-6 and C-7 how-

---

Fig. 10a–c. Simulated PND $^{13}\text{C}$ NMR spectra of a polyketide derived moiety: a at natural abundance, b enriched from [1,2-$^{13}\text{C}_2$]acetate and c after cleavage or rearrangement of an originally intact acetate unit.
ever appear as enriched singlets indicating their derivation from cleaved acetate units as indicated above in Scheme 4.

Due to its extra sensitivity the technique has been applied to many higher plant metabolites. Some of these studies are discussed briefly below to indicate the type of information these studies can provide.

$[1,2-^{13}C_2]$Acetate was incorporated into the flavone apigenin (45) and the flavonol (46) by cell suspension cultures of parsley, *Petroselinum hortense*, with randomisation of $^{13}$C–$^{13}$C couplings in ring A (Light and Hahlbrock 1980). Each carbon shows two sets of coupling satellites due to coupling to both adjacent carbons. This means that a symmetrical intermediate, presumably the chalcone (44) in which ring A is free to rotate, is an intermediate in the biosynthesis of (45) and (46) as shown in Scheme 5. Detection of symmetrical intermediates by this type of randomisation of coupling is one of the more important applications of double $^{13}$C-labelling experiments. In contrast, $[1,2-^{13}C_2]$acetate is incorporated into ring A of the phytoalexin pisatin (47) in *Pisum sativum* without randomisation, showing that deoxygenation of the polyketide precursor must occur before cyclisation and aromatisation (Scheme 6) (Stoessl and Stothers 1979). In a similar experiment, the labelling pattern in a sample of the retrochalcone echinatin (49) produced when cell cultures of *Glycyrrhiza echinata* were fed $[1,2-^{13}C_2]$acetate was...
analysed. Ring B rather than ring A was labelled and the coupling satellites in the
$^{13}$C NMR spectrum demonstrated a specific folding of the polyketide chain and
reductive loss of the oxygen function during the formation of the normal chalcone
intermediate (48) as shown in Scheme 7 (Ayabe and Furuya 1982).

Patterns of $^{13}$C labelling in the isoflavonoid phytoalexins phaseolin (50) and
kevitone (51) produced by cotyledons of wounded French beans (*Phaseolus vulgaris*)
in the presence of [1,2-$^{13}$C$_2$]acetate has been determined (Dewick et al.
1982). These show that specific folding of the polyketide chain and reductive loss
of an oxygen atom occur prior to ring closure in phaseolin (50) so that only one
acetate-enrichment pattern is observed. In contrast, free rotation of a symmetrical chalcone intermediate results in randomisation of label and two labelling patterns in kevitone (51). The biosynthetic routes to these 5-hydroxy- and 5-deoxyisoflavonoids thus diverge prior to the formation of the chalcones as shown in Scheme 8.

Incorporation of [2,3-\(^{13}\)C\(_2\)]phenylalanine into psilotin (52) and psilotin epoxide (53) by excised shoots of *Psilotum nudum* and analysis of their \(^{13}\)C NMR spectra showed that incorporation of the intact \(^{13}\)C\(_2\) unit had occurred consistent with their biosynthesis via p-coumaric acid, chain extension with malonate, cyclisation and glucosylation as shown in Scheme 9 (Leete et al. 1982).

Incorporation of [1,2-\(^{13}\)C\(_2\)]acetate by carrot roots treated with ethrel into the phytoalexins 6-methoxymellein (54) and eugenin (55) confirmed a pentaketide origin for both metabolites. The two modes of acetate incorporation (Scheme 10)

![Scheme 9](image)

![Scheme 10](image)

into the benzenoid ring of eugenin indicate that the heterocyclic ring is formed by ring closure onto the two equivalent hydroxyls of a symmetrical intermediate which is not enzyme-bound (Stoessl and Stothers 1973).

A number of studies have been reported with terpenoid metabolites. The labelling pattern of paniculide (56) derived from [1,2-\(^{13}\)C\(_2\)]acetate in callus tissue cultures *Andrographis paniculata* demonstrates that the manner of folding of the farnesyl pyrophosphate in the biosynthesis of (56) must be as shown in Scheme 11 (Overton and Picken 1976). The sesquiterpenoid stress compounds of the solanaceae have been extensively studied. The labelling patterns observed on incorpo-
ration of \( [1,2-^{13}C_2] \)acetate into 2,3-germacrenediol (57), lubimin (58) and hydroxylubimin (59), the major stress metabolites of \textit{Datura stramonium} indicate the pathway shown in Scheme 12 (Stoessl et al. 1976). The biosynthesis of the eudesmane sesquiterpenoid, capsidiol (60) which is formed by the sweet pepper \textit{Capsicum annum} when it is inoculated with spores of \textit{Monilia fruiticola} has also been studied with \( [1,2-^{13}C_2] \)acetate. The mode of incorporation of the acetate units suggests a biosynthetic pathway in which the angular methyl group has undergone a 1,2-shift as shown in Scheme 13 (Baker and Brooks 1976).

Both singly and doubly labelled precursors have been used to study triterpenoid biosynthesis. Incorporation of \( [4-^{13}C] \)mevalonic acid into oleanolic acid (61), ursolic acid (62) and several other metabolites was achieved using tissue cultures of \textit{Isodon japonicus} (Seo et al. 1975 a). The \( ^{13}C \) NMR spectra showed enrichments of carbons 13, 18, and 19 in (61) confirming operation of the sequence (63)–(66) in formation of the oleanes (Scheme 14). The rearrangements involved in ursolic acid biosynthesis could not be identified unambiguously. However, incorporation of \( [1,2-^{13}C_2] \)acetate gave oleanic acid (67) and ursolic acid (68) containing a large number of non-adjacent \( ^{13}C \) atoms (Seo et al. 1975 b). Although some of these singlets derive from C-2 of mevalonate (69), the absence of coupling between C-20/21 and C-19/29 in (68) establishes the pathway to ursane-type triter-
penoids (Scheme 15) and eliminates alternative skeletal rearrangements such as conversion of (70) to (68). Similar studies on a number of triterpenes produced in tissue cultures of a number of plants have been described (Seo et al. 1983).

The technique has also been applied to several alkaloids. [5,6-\textsuperscript{13}C\textsubscript{2}]Nicotinic acid (71) was wick-fed to intact *Nicotiana tabacum* plants. A very low incorporation (0.07\%) into nicotine (72) was obtained, but even this very low specific incorporation could be detected in the \textsuperscript{13}C NMR spectrum by the satellites due to the contiguous \textsuperscript{13}C atoms (Leete 1977).

[4,5-\textsuperscript{13}C\textsubscript{2}]Lysine was incorporated into anabasine (73) by *Nicotiana glauca*. The \textsuperscript{13}C labels were incorporated into C-4' and C-5' only (Leete 1982). In contrast, [2,3-\textsuperscript{13}C\textsubscript{2}]ornithine was incorporated into both C-2'/3' and C-4'/5' of nicotine (72) to indicate the involvement of a symmetrical intermediate, presumably putrescine (74) (Leete and Yu 1980). Incorporation of [2,3-\textsuperscript{13}C\textsubscript{2}]putrescine into retronecine (75) by seedlings of *Senecio isatideus* and observation of coupling satellites on C-1/2 and C-6/7 established the symmetrical labelling of the two halves of the pyrrolizidine alkaloids (Khan and Robins 1981).

Incorporation of [4,5-\textsuperscript{13}C\textsubscript{2}]lysine into vertine (76) by *Heima salicifolia* and analysis of the resultant \textsuperscript{13}C NMR spectrum showed that C-8 was coupled to
both C-7 and C-9. It follows that (76) is biosynthesised from lysine via a symmetrical intermediate, i.e. cadaverine (77) as shown in Scheme 16 (Hedges et al. 1983).

Incorporation of \([1,2-^{13}C_2]\)acetate into the acridone alkaloid, rutacridone (79) using a cell suspension culture of \textit{Ruta graveolens} shows that ring C is derived from three intact acetate units (Scheme 17). The scrambling of labelling however, shows that a symmetrical intermediate e.g. (78) must be involved in the pathway (Zschunke 1982).
6.2.3 Non-Contiguous Double $^{13}$C-Labeling

All the above examples in Section 6.2.2 have involved precursors in which there is a one-bond coupling between $^{13}$C labels in both precursors and final metabolites. Other studies yielding valuable biosynthetic information have involved precursors with contiguous labels which become non-contiguous in the metabolite, non-contiguous labels which become contiguous, or even non-contiguous labels which remain non-contiguous but detectable during biosynthesis.

6.2.3.1 Two-Bond $^{13}$C-$^{13}$C Couplings from Contiguous $^{13}$C$_2$-Labelled Precursors

When two $^{13}$C-labelled atoms are in a two-bonded relationship to each other, they may or may not display a mutual $^{13}$C-$^{13}$C coupling. These couplings, when observed, are much smaller than one-bond couplings and typically range from 1 to 15 Hz (Marshall 1983). However, with careful spectral determination they can be observed.

In the study of aspyrone (5) discussed above, the rearrangement proposed in Scheme 4 involves cleavage of an intact acetate unit during an intramolecular rearrangement. Thus C-1 and C-7 which are in a 2-bonded relationship in aspyrone arise from the same acetate unit. This was confirmed by redetermination of the spectrum of [1,2-$^{13}$C$_2$]acetate-enriched aspyrone under conditions of higher resolution, whereupon a mutual $^{13}$C-$^{13}$C coupling of 6.2 Hz was observed between C-1 and C-7, Fig. 9c. Similar 2-bond couplings arising from intramolecular rearrangements involving pairs of carbons derived from the same acetate unit have also been observed for vulgamycin (80) and the tetrahydrofuran (81) (Seto et al. 1975; Seto et al. 1979).

6.2.3.2 One-Bond $^{13}$C-$^{13}$C Couplings from Non-Contiguous $^{13}$C$_2$-Labelled Precursors

This is an especially valuable method for elucidating the direction of intramolecular rearrangements and also for establishing the intact incorporation of precursors. It is in essence the reversal of the process described in Section 6.2.3.1 and is best illustrated by examples.

Tropic acid (82) as found in scopolamine (83) is formed from phenylalanine with the side-chain rearrangement involving an intramolecular 1,2-shift of the
carboxy-group. This was established by synthesis of [1,3-^{13}C_2]phenylalanine and its incorporation into (82) and (83) in *Datura inoxia* (Leete 1975). In the $^{13}$C NMR of the resultant alkaloids, a $^{13}$C-$^{13}$C coupling of 38 Hz was observed between C-2' and C-3' to prove that the rearrangement is an intramolecular and not intermolecular process (Scheme 18). Similar studies have been carried out on tenellin (84), an insecticidal metabolite of *Beauveria bassiana*. On incorporation of [1,3-^{13}C_2]phenylalanine, the labelled tenellin (84) showed a $^{13}$C-$^{13}$C coupling of 45 Hz between C-5 and C-6, indicating that these two carbons have migrated together from C-1 and C-3 of phenylalanine during biosynthesis (Leete et al. 1975).

In a study of cholesterol (87) biosynthesis [4,6-$^{13}$C_2]mevalonic acid was fed to a cell-free enzyme preparation from rat liver, C-13 and C-18 showed a $^{13}$C-$^{13}$C coupling of 35 Hz, proving that they were derived from the same mevalonate unit of squalene (85) and confirming that C-18 becomes bonded to C-13 by a 1,2-methyl migration from C-14 of the initial cyclisation product (86) rather than by a 1,3 migration from C-8, as indicated in Scheme 19 (Popjak et al. 1977).

One of the first and most elegant examples of this approach was in Battersby's study of uroporphyrinogen III (89) biosynthesis. [2,11-$^{13}$C_2]porphobilinogen (88) was synthesised and showed a long-range coupling of 4 Hz. This was incor-
porated into (89) using a cell-free enzyme system from avian blood. Analysis of the resultant $^{13}$C NMR spectrum showed three doublets each of 5 Hz splitting corresponding to the $\alpha$, $\beta$ and $\delta$ carbons, and one doublet of 72 Hz for the $\gamma$ carbon, indicating that PBG unit D must undergo an intramolecular rearrangement with respect to itself during biosynthesis (Battersby et al. 1973).

6.2.3.3 Two-Bond Couplings from Non-Contiguous $^{13}$C$_2$-Labelled Precursors

The value of this method lies in its ability to display intact incorporation of complex biosynthetic intermediates. A powerful illustration of the method is the demonstrated intact incorporation of homospermidine into the alkaloid retronecine. [1,9-$^{13}$C$_2$]Homospermidine (90) was synthesised and fed to Senecio isatoides. The $^{13}$C NMR spectrum of the derived retronecine (91) showed doublets for C-8 and C-9 that were superimposed on the natural abundance singlets (Rana and Robins 1983).

The long-range couplings observed for uroporphyrinogen III (see Sect. 6.2.3.2), of course, also demonstrates the intact incorporation of PBG units A, B and C.

7 $^{13}$C, $^{15}$N Doubly Labelled Precursors

As discussed above, $^{15}$N has spin $\frac{1}{2}$ and so will couple to $^{13}$C. Thus, observation of a $^{13}$C-$^{15}$N coupling on incorporation of a precursor doubly labelled with $^{13}$C-$^{15}$N can be used to establish intact incorporation of precursor or to probe whether the integrity of a particular carbon-nitrogen bond is maintained during a biosynthetic sequence. $^{13}$C-$^{15}$N Couplings are in general smaller and less predictable than $^{13}$C-$^{13}$C couplings, and in certain cases they may not be observable at all, so results must be interpreted with caution (Levy and Lichter 1979).

As with doubly $^{13}$C-labelled precursors we can use precursors in which the $^{13}$C and $^{15}$N are already in a one-bonded relationship or we can use precursors labelled so that the $^{13}$C and $^{15}$N become bonded during biosynthesis. An elegant series of experiments encompassing both approaches was carried out during studies on the biosynthesis of the antibiotic streptothricin F (92). As shown in Scheme 20 a series of arginines specifically labelled with $^{13}$C and $^{15}$N were synthesised and fed to Streptomyces L-1689-23. The streptothricin F isolated in each case was analysed by $^{13}$C NMR and the $^{13}$C-$^{15}$N couplings indicated by heavy
lines in Scheme 20 were observed (Martinkus et al. 1983). A particularly elegant application to penicillin biosynthesis has been reported (Baxter et al. 1985). δ-(α-L-Aminoadipoyl)-L-[3-13C]cysteinyl-L-[15N]valine (93), the Arnstein tripeptide, was efficiently incorporated into isopenicillin N (94) by a cell-free enzyme system prepared from cells of *Cephalosporium acremonium*. The 13C NMR spectrum showed a 13C–15N coupling of 4.4 Hz for the enriched C-5 resonance of (94).

The technique has also been applied in a number of higher plant alkaloids. [1-13C,methylamino-15N]N-methyl putrescine has been incorporated into scopalamine (95) and nicotine (96). In both cases C-5' show 13C–15N coupling satellites due to the intact incorporation of the contiguous 13C and 15N atoms as shown (Leete and McDonnell 1981). [1-13C,1-15N]Putrescine has been incorporated into retronecine (98). The resultant 13C NMR spectrum had enhanced 13C signals due to C-3, C-5, C-8 and C-9. In addition both C-3 and C-5 showed 13C–15N coupling satellites (4.1 and 3.8 Hz respectively), indicating the intermediacy of a symmet-
Rational intermediate (97) in the biosynthesis of retronecine (Scheme 21) (Khan and Robins 1981).

Similar results have been reported for the quinolizidine alkaloids. [4,5-\textsuperscript{13}C\textsubscript{2}]Lysine was incorporated into lupinine (100) in Lupinus luteus plants with the labelling pattern shown in Scheme 22, indicating that a symmetrical intermediate, i.e. cadaverine (99) is involved in the biosynthesis of lupinine (Rana and Robins 1984). In further studies [1-\textsuperscript{15}N,1-\textsuperscript{13}C]cadaverine was incorporated into both lupinine (100) and sparteine (102). In the spectrum of the enriched lupinine, a \textsuperscript{13}C-

\[ \text{Scheme 21} \]

\[ \text{Scheme 22} \]

\[ ^{15}\text{N} \text{ coupling was observed between C-6 and N-5, but not between C-4 and N-5, so that a symmetrical intermediate, e.g. (101), such as is found in the biosynthesis of the pyrrolizidine alkaloids, can be excluded (Golebiewski and Spenser 1983; Rana and Robins 1984). Examination of the }^{13}\text{C NMR spectrum of sparteine (102) enriched from [1-}^{13}\text{C,1-}^{15}\text{N]cadaverine gave results which were similar to those obtained for lupinine. For sparteine, three separate molecules of cadaverine are used and intact incorporation of }^{13}\text{C-}^{15}\text{N was observed for C-2– N-1, and C-15 – N-16 only. Carbons, 6, 10, 11 and 17 appeared as enriched singlets in the }^{13}\text{C NMR spectrum (Rana and Robins 1984; Rana and Robins 1983).} \]

\textbf{8 Isotope-Induced Shifts in }^{13}\text{C NMR}

In studying the nature of the intermediates on a biosynthetic pathway and in particular elucidating the detailed mechanisms of their interconversions, it is essential to determine the biosynthetic origins and fate of the hydrogen and oxygen atoms.
Hydrogen and oxygen can be monitored directly by NMR by using the NMR active isotopes $^2$H, $^3$H or $^{17}$O (Garson and Staunton 1979; Sankawa et al. 1983). However all of these have distinct disadvantages and a great advance in biosynthetic studies has been made by the use of $^{13}$C as a “reporter nucleus” in labelling studies. The presence of $^2$H alpha or beta to a $^{13}$C atom can be deduced from the appearance in the PND $^{13}$C NMR spectrum of $^{13}$C-$^2$H coupling and/or an isotope-induced shift. Similarly, the presence of $^{18}$O alpha to a $^{13}$C atom can be detected by an isotope-induced shift in the $^{13}$C NMR spectrum. Before discussing specific applications of these methods, some general points on the effect should be noted (Batiz-Hernandez and Benheim 1967).

a) Substitution by a heavy isotope shortens the average length of the bond holding the isotope, and, less so, the remaining bonds of the molecule. This almost always shifts the NMR signal of neighbouring nuclei to high field, although the magnitude of the shift is inversely dependent on the remoteness of the substitution.

b) The shift is largest where the fractional change in mass is largest and is also roughly proportional to the number of atoms substituted.

c) The magnitude of the shift decreases with an increase in “s” character of the bond holding the isotope and depends also on the resonant nucleus, correlating with the range of chemical shifts observed for that nucleus.

8.1 The Deuterium Alpha-Shift Technique (Garson and Staunton 1979)

In this technique, the deuterium label is directly attached to the $^{13}$C nucleus in the precursor molecule. The PND $^{13}$C NMR spectrum of the enriched metabolite shows, for carbon atoms which have retained deuterium label, a series of resonances upfield of the normal singlet. The presence of each deuterium shifts the centre of the carbon resonance by 0.3–0.6 ppm to low frequency and spin-spin

Fig. 11. Expected appearance of the PND $^{13}$C NMR spectra of carbons bearing zero, one, two or three deuterium atoms (alpha-shifted)
coupling ($^{1}J_{CD}$) gives rise to a characteristic multiplet pattern; hence CD appears as a triplet centred 0.3–0.6 ppm upfield of the normal singlet, CD$_{2}$ gives a quintet centred 0.6–1.2 ppm upfield and CD$_{3}$ gives a septet (Fig. 11). Shifted signals arising from carbons which bear no protium suffer reduced signal-to-noise ratio caused by poor relaxation and lack of NOE enhancement, a disadvantage of the method which is compounded by the multiplicities due to coupling. Deuterium decoupling can assist in this by removing the $^{13}$C–$^{2}$H coupling. However, information not obtainable by direct $^{2}$H NMR spectroscopy, such as the distribution of label as CH$_{2}$D, CHD$_{2}$ and CD$_{3}$ and the integrity of carbon-hydrogen bonds during biosynthesis, may be gained.

One of the first applications of this technique was to terrein (103), a cyclopentenone metabolite of Aspergillus terreus. In the PND decoupled $^{13}$C NMR of terrein (103) enriched from [2-$^{13}$C, $^{2}$H$_{3}$]acetate, the signals for isotopically shifted resonance were not clearly observed. However, on redetermining the spectrum with deuterium decoupling, a signal at 17.95 ppm, 0.8 ppm upfield of the normal C-1 signal was observed indicating the presence of molecules triply labelled with deuterium at C-1. In addition a weak doublet signal ($J = 133$ Hz) centred at 18.2 ppm indicated the presence of molecules labelled as CHD$_{2}$. The detection of molecules labelled with three deuteriums at C-1 confirmed that the methyl is derived intact from the methyl of the acetate “starter” unit of the precursor polyketide chain (Garson et al. 1977).

The method has been used most successfully when the $^{13}$C NMR spectrum of the enriched metabolite can be determined with simultaneous proton and deuterium decoupling (Hutchinson et al. 1981). Thus on incorporation of [2-$^{13}$C, $^{2}$H$_{3}$]acetate into brefeldin A (104) by cultures of Penicillium brefeldianum, the resultant proton and deuterium decoupled $^{13}$C NMR spectrum showed isotopically shifted signals indicating incorporation of up to three deuteriums on C-16, two on C-14 and one on carbons 2, 4, 6, 8, and 10 (Fig. 12).

More recently, a pulse sequence has been described which allows the selective observation of deuterated $^{13}$C signals by selective suppression of signals from protonated carbons (Doddrell et al. 1983). This makes the technique more sensitive, but like the simultaneous proton and deuterium decoupling method requires instrumentation and expertise which are not widely available.

The $\alpha$-shift technique has also found use in terpenoid biosynthesis where it enables hydride shifts to be detected. [3-$^{13}$C, $^{4}$H$_{2}$]Mevalonate was incorporated
Fig. 12. $^1$H, $^2$H-noise decoupled $^{13}$C NMR spectrum of brefeldin A enriched from [2-$^{13}$C, $^{2}$H$_3$]acetate

into the fungal triprenylphenol ascochlorin (105). In the PND $^{13}$C NMR spectrum the signal for C-9 was enhanced relative to natural abundance and showed two lines, one for molecules with $^{13}$C at C-9 and $^2$H at C-10 (a $\beta$-isotope shift, see Sect. 8.2) and the other, more intense peak for molecules with $^1$H at C-10. Neither C-1 nor C-5, which are also derived from C-3 of mevalonate, appeared to be significantly enriched relative to natural abundance, consistent with suppression of these signals by attached deuterium. However, two lines of a triplet ($J = 24$ Hz) centred at 40.65 ppm, were detected upfield of the normal signal for C-5 but no deuterated signal was visible for C-1. Thus the biosynthetic sequence shown in Scheme 22 is indicated (Tanabe andSusuki 1974).
8.2 The Deuterium Beta-Shift Technique (Abell and Staunton 1981)

Many of the problems associated with directly attached deuterium are avoided by placing the deuterium label two bonds away from the $^{13}$C reporter nucleus. The isotope shift, although reduced, is still observable, and as $\beta$-hydrogens only contribute markedly to the relaxation of non-protonated $^{13}$C nuclei, the shifted signals otherwise retain any NOE effect also experienced by the unshifted signals on proton decoupling. As geminal carbon-proton coupling constants are generally small anyway (Marshall 1983), and carbon-deuteron couplings are over six times smaller again, the shifted signals are effectively singlets (Fig. 13), even without deuterium decoupling, and this gives a further increase in the signal to noise ratio compared with the corresponding $\alpha$-shift experiment.

The method was first applied to biosynthesis in a study of 6-methylsalicylic acid (106). [1-$^{13}$C,2$^2$H$_3$]acetate was fed to cultures of Penicillium griseofulvum. The PND $^{13}$C NMR spectrum of the methyl ester of the resulting 6-MSA showed shifted signals for C-2, C-4, and C-6, corresponding to the presence of deuterium label at positions 3, 5, and 7 respectively. Thus the integrity of an acetate unit (heavy lines) can be established in certain cases without recourse to a double $^{13}$C-labelled experiment (Abell and Staunton 1981). In a similar study on the aflatoxin intermediate averufin (107), the regiospecificity of incorporation of $^3$H from [1-$^{13}$C,2$^2$H$_3$]acetate into (107) by cultures of Aspergillus toxicarius was determined using the $\beta$-$^2$H isotope shifts observed in the PND $^{13}$C NMR spectrum which is shown in Fig. 14. This shows isotopically shifted signals for C-5′ (the reporter nucleus) indicative of the incorporation of 1–3 deuteriums on C-6′, and for C-1′, C-3′, C-6, and C-8 consistent with the incorporation of one deuterium on C-2′, C-4′, C-5′, and C-7 respectively (Simpson et al. 1982).

Unlike the $\alpha$-shifts, the $\beta$-shifts show a marked dependence on both stereochemistry of the deuterium label and the functionality of the $^{13}$C reporter nucleus. Carbonyl resonances may show irresolvable or even downfield shifts. However $\beta$-shifts do appear to be additive (Simpson and Stenzel 1982).
In the only application to date to a higher plant metabolite, the postulated migration of hydrogen from C-5 to C-4 in the biosynthesis of the potato phytoalexins lubimin (108), 3-hydroxylubimin (109), and rishitin (110) has been confirmed by the incorporation of [2-13C, 2H3]acetate and direct observation of the expected β-shift of the 13C NMR signal of C-5 due to deuterium label at C-4 (Stoessl and Stothers 1983).

$$\text{DCO}_2\text{Na} \rightarrow \text{DODC}_2\text{H}_5\text{CO}_2\text{H}$$

$$\text{CD}_3\text{CO}_2\text{Na} \rightarrow \text{DODCD}_{3}$$

$$\text{CD}_3\text{CO}_2\text{Na} \rightarrow \text{DODCD}_{3}$$

Fig. 14. 90.6 MHz PND 13C NMR spectrum of averufin (107) enriched from [1-13C, 2H3]acetate
8.3 $^{18}$O Isotope Induced Shifts in $^{13}$C NMR

Until recently, the biosynthetic origin or fate of oxygen was determined almost exclusively by mass spectrometry, and locating the position of a label, present perhaps in only low concentrations, was difficult. It was known that the heaviest oxygen isotope, $^{18}$O, (natural abundance – 0.204%) was capable of inducing resolvable isotope shifts in the NMR spectra of certain other elements and this had been exploited in biological studies, e.g. the mechanisms and kinetics of enzymatic phosphoryl group transfer had been investigated via the $^{18}$O isotope shift in the $^{31}$P NMR spectrum (Lowe and Sproat 1978). The detection (Risley and Van Etten 1979) of such an isotope shift in the $^{13}$C spectrum provided a more general technique for biosynthetic research, first employed in a study of averufin (111). *Aspergillus parasiticus*, when grown under an atmosphere containing $^{18}$O$_2$ gas, produced averufin, the $^{13}$C NMR spectrum of which showed an isotopically shifted resonance for C-10 only. In a second experiment feeding with [1-$^{13}$C,$^{18}$O$_2$]acetate, carbons 1, 3, 6, 8, 9, and 10 showed prominent shifted signals (Vederas and Nakashima 1980). Thus, at the incorporation levels typically achieved with early precursors, carbon-oxygen bonds which have been preserved intact throughout the course of biosynthesis can be distinguished from those which have arisen between precursor units.

Application of the $^{18}$O shift technique has been rapid and the method and its applications have been the subject of a review (Vederas 1982). The shifts show a marked dependence on structure, which could be of use in $^{13}$C spectral assignments, but they are not generally much larger than 0.05 ppm. These are very small effects and are the same general magnitude as $^{1}$H isotope shifts. They are only readily observed with high field spectrometers which can determine $^{13}$C NMR spectra at ca. 100 MHz.

Although no applications to higher plant metabolites have been reported as yet, the method will certainly find use. One of the more interesting studies reports the incorporation of [1-$^{13}$C,1,4-$^{18}$O$_3$]-$\delta$-amino-laevulinic acid (112) into bac-
Fig. 15a, b. Partial PND $^{13}$C NMR spectra of: a bacteriochlorophyll a (114) and b δ-aminolaevulinic acid (112) illustrating the isotopic distribution at C-17 and C-1 respectively.

teriochlorophyll a (114) by cultures of Rhodopseudomonas spheroides. In the resultant $^{13}$C NMR spectrum the signal due to C-17 shows isotopically shifted signals as indicated in Fig. 15, with shifts of 0.014, 0.037 and mainly 0.051 ppm to prove that both the carbonyl and ether oxygens of the phytol ester linkage are derived from (112). This indicates that phytlation occurs by nucleophilic attack of the C-17 carboxy group of bacteriochlorophyllide a (113) on the phytol pyrophosphate to yield (114) with retention of both C-17 oxygen atoms (Emery and Akhtar 1985).

9 Conclusion

The development of $^{13}$C NMR and associated stable isotope labelling techniques has provided a major stimulus to biosynthetic and metabolic studies. Not only does $^{13}$C labelling provide information on the origins of the carbon skeletons of metabolites but in association with $^2$H, $^{15}$N and $^{18}$O it also indicates the origins of hydrogen, nitrogen and oxygen atoms and so provides an invaluable insight into the nature of the intermediates on biosynthetic pathways and the detailed mechanisms of their interconversions. It can be safely anticipated that the number of applications of these techniques will grow rapidly in the next few years.
References


Baker FC, Brooks CIW (1976) Biosynthesis of the sesquiterpenoid capsidiol in sweet pepper fruits inoculated with fungal spores. Phytochemistry 15:689–894


Breitmaier E, Voelter L (1978) 13C NMR spectroscopy. Verlag Chemie, Weinheim


Leete E, Kowanko N, Newmark RA (1975a) Use of carbon-13 nuclear magnetic resonance to show that the biosynthesis of tropic acid involves an intramolecular rearrangement of phenylalanine. J Amer Chem Soc 97:682–683
Leete E, Kowanko N, Newmark RA, Vining LC, McInnes AG, Wright JLC (1975b) The use of carbon-13 nuclear magnetic resonance to establish that the biosynthesis of tenellin involves an intramolecular rearrangement of phenylalanine. Tetrahedron Letters 4103–4106
13C-NMR in Metabolic Studies


Manitto P (1981) Biosynthesis of natural products. Ellis Horwood, Chichester


Stoessl A, Stothers JB (1979) The incorporation of \([1,2,\text{C}^{13}]\)acetate into pisatin to establish the biosynthesis of its polyketide moiety. Z Naturforsch 34C:87–89
STUDIES OF POLYKETIDE CHAIN-ASSEMBLY PROCESSES

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ABSTRACT

$^{13}$C, $^2$H and $^{18}$O-labelling studies on the polyketide-derived fungal metabolites, colletodiol, aspyrone and averufin are described. From these results conclusions are drawn regarding the nature of the intermediates involved in polyketide chain assembly processes.

INTRODUCTION

Mycotoxins are products of fungal secondary metabolism and as the polyketide pathway is probably the major pathway of secondary metabolism in fungi, it is involved in the biosynthesis of many mycotoxins. Polyketide metabolites can be divided into aromatic and non-aromatic metabolites. Patulin, ochratoxin A, aflatoxins and zearalenone being representatives of the former class; and diploporin and citreoviridin of the latter. Note that patulin, though non-aromatic itself should be classified as such due to its biosynthesis via 6-methylsalicylic acid. Despite much effort over the last 30 years since the recognition of the pathway, little is known of the exact nature of the intermediates involved in the early stages of polyketide chain assembly. At its simplest, it is thought that poly-$\delta$-ketide intermediates (I) are built up by a cyclic process (scheme 1) analogous to fatty acid biosynthesis but omitting the reduction-elimination-reduction sequence responsible for the loss of acetate oxygen. However these intermediates have not been observed in the free state and they remain enzyme-bound while modifications such as cyclisation, alklyation and reduction occur and metabolites are released from the enzyme surface. Further, sometimes drastic, modifications may occur after release from the chain-assembly enzymes and many of these secondary modification processes have been well studied, e.g. in the aflatoxins where many of the intermediates have been characterised and an increasing amount is being elucidated on their mechanisms of interconversion.

However what I would like to describe in this talk are studies aimed at trying to obtain some information on the nature of the intermediates involved in the chain assembly process and the events occurring before the initial
release of metabolites from the enzyme surface. The inherently unstable polyketide intermediates, where in fact they exist, must be stabilised on the enzyme surface and it has been suggested that this could be achieved by hydration of the carbonyl groups; formation of polyenolic systems which could chelate to metal ions, or to specific amino acids on the enzyme. However most metabolites show varying degrees of reduction and deoxygenation and an increasing body of evidence suggests that this occurs by processes analogous to fatty acid biosynthesis before the initial release from the chain-assembly enzymes. Thus path (a) in Scheme I would simply produce poly-β-ketide but by invoking pathways (b), (c) and (d) intermediates with varying degrees of reduction may be formed.

There has been little progress in enzymatic or other direct methods of observing these early intermediates. However, with recent developments in n.m.r. based methods\(^4\); \(^2\)H and \(^18\)O isotope induced shifts in \(^{13}\)C n.m.r., and \(^2\)H n.m.r., which facilitate determination of the biosynthetic origins of hydrogen and oxygen, we are now in a position to be able to obtain significant indirect evidence for the nature of the intermediates involved in the early stages of polyketide chain assembly processes. Recent work has shown that both malonate derived hydrogens are retained from the first malonate unit to be added to the acetyl CoA starter unit during the biosynthesis of melacin (2)\(^5\) and brefeldin A (3)\(^6\), and we have shown that both hydrogens are retained from the second malonate unit added during the biosynthesis of monocerin (4)\(^7\). These results imply that polyketide chains are being reduced and deoxygenated as chain assembly proceeds and not after it is complete. From the results of
$^2$H and $^{18}$O labelling studies we can deduce that in monocerin biosynthesis the precursor is probably assembled in a stepwise manner as shown in scheme 2, so that the actual enzyme-bound species is (5) and not the more classically accepted heptaketide (1, n=5).

![Scheme 2. Proposed assembly of Monocerin biosynthetic precursors](image)

We are now trying to expand on these observations by, for example, studying metabolites whose structures bear a close resemblance to likely enzyme bound precursors. The fundamental questions we seek to answer are:

1) Is the polyketide chain assembled in its entirety on the enzyme surface and then modified? OR
2) Do the modifications occur during chain assembly? OR
3) Do they occur both during and after assembly?
4) What is the stereochemistry of the reduction and deoxygenation processes?
5) What is the timing and mechanism of other modifications such as introduction of oxygen functionality?

**COLLETOODIOL/GRABAMICIN A**

Colletodiol (6) and colletoketol (7) are macrocyclic dilactonic metabolites originally isolated from the plant pathogen, Colletotrichum capsici. More recently grabamycin A was isolated as a broad spectrum antibiotic from a Cytospora sp. and was subsequently shown to be identical to colletoketol.

![Colletodiol (6)](image) ![Colletoketol (7)](image)
Incorporation studies with singly $^{13}$C-labelled acetates have confirmed the acetate-origin of colletodiol in _C. capsici._\textsuperscript{10} These metabolites can be seen to be derived by combination of C$_6$ (triketide) and C$_8$ (tetraketide) moieties. A priori, one can postulate a number of triketide-derived moieties with differing oxidation levels as the actual enzyme-bound precursor for the C$_6$ moiety in colletodiol. Some of these are shown in scheme 3 along with a number of possible tetraketide-derived C$_8$ precursors. Depending on the nature of the actual intermediates, a number of mechanisms can be proposed for the formation of the lactone functions. These are summarised in scheme 4 which also indicates the predicted origins of the associated oxygen and hydrogen atoms. Similarly, a number of different mechanisms can be proposed for the formation of the 1,2-diol and α-ketol systems found in colletodiol and colletoketol respectively. These are shown in scheme 5 and again they may be differentiated by appropriate $^{18}$O and $^2$H labelling experiments.
Scheme 5 Possible mechanisms for 1,2-diol formation

Fig 1 2D $^1$H-$^1$C Correlation Spectrum of Colletodiol
Before carrying out labelling studies it was necessary to rigorously assign the $^{13}$C n.m.r. spectrum of colletodiol. The $^1$H n.m.r. spectrum has been fully assigned by decoupling and difference NOE experiments. All the signals except those assigned to the 3 and 3' olefinic hydrogens and the methyl hydrogens are well resolved. From this, a two dimensional carbon-proton correlation experiment (Figure 1) enabled carbons 2, 4, 5, 6, 7, 2', 4' and 5' to be assigned. The remaining carbons were assigned by analysis of long-range couplings in the fully $^1$H-coupled $^{13}$C spectrum. For example, irradiation of H-5' removed a three-bond coupling through oxygen to C-1 (Figure 2). Interestingly C-3' showed couplings to H-2, H-4o, H-48 and H-5'. The C-8 methyl could be assigned by its coupling inter alia to H-7.

In our hands Cytospora sp. has produced colletodiol as the major metabolite and only smaller amounts of grahamycin A. Fermentations were carried out in the presence of [1-$^{13}$C, $^2$H$_3$]- and [1-$^{13}$C, $^{18}$O$_2$]-acetates and under an atmosphere of $^{18}$O$_2$. The $^2$H and $^{18}$O isotope shifts observed in the p.n.d. $^{13}$C n.m.r. spectra of the colletodiol isolated in each case are shown in Figures 3 and 4. No $^2$H isotope shifts could be observed at C-1 or C-1' in the $^{13}$C spectrum of the [1-$^{13}$C, $^2$H$_3$] acetate-enriched colletodiol, but the presence of $^2$H label at C-2 and C-2' was shown by $^2$H n.m.r. analysis as indicated in Figure 5, which also shows the spectrum of universally $^2$H-enriched colletodiol for comparison. The labelling pattern is summarised in Scheme 6.

The retention of acetate derived oxygens on both the carbonyl and ether carbons of the lactone functions indicate that the ring closure must proceed by path (a) in Scheme 4 and so the enzyme bound intermediates must retain the oxygen of the acetate starter units as hydroxyl functions with the R absolute stereochemistry.

![Diagram](image-url)
Fig. 5. 55 MHz 1H nmr spectra of (a) $\text{H}_2\text{O}$- and (b) $\text{H}_3$-acetate enriched Colletodiol.
If we now consider the formation of the diol system, the results indicate that the 5-hydroxyl function is derived from the atmosphere i.e. via an oxidative process, whereas the 4-hydroxyl must be derived from the medium. The absolute configuration at both these centres is $R_{11}$. A mechanism consistent with the observed labelling and stereochemistry is shown in Scheme 7. Epoxidation of a $\delta$-olefin from the $\beta$-face is followed by hydrolytic ring opening by attack of water from the $\alpha$-face at C-5.

On the basis of these results we can therefore propose the thioesters (8) and (9) as the likely enzyme-bound precursors for colletodiol. These could be built up by the sequence shown in Scheme 8 where the diol (10) is proposed as a common intermediate, trans-elimination of water giving rise to the C$_6$ precursor directly, whereas cis elimination followed by addition of further C$_2$ unit produces the C$_8$ precursor. The relative timing of the diol formation step is not known but it may occur after lactonisation and release from the enzyme surface.
This provides a further example of how ²H and ¹⁸O labelling studies provide rather compelling evidence on the nature of the enzyme-bound intermediates in polyketide biosynthesis. Aspyrone (11) and asperlactone (12) are closely related metabolites of Aspergillus melleus. Their biosynthesis has been the subject of study by a number of groups and mainly on the basis of incorporation of doubly-labelled acetate a pathway was proposed via decarboxylation and Favorksi-type rearrangement of a linear pentaketide intermediate as shown in Scheme 9. In support of this a 2-bond ¹³C-¹³C coupling was observed between C-2 and C-8 in aspyrone. The relative stereochemistry at C-5 and C-6 suggested that the lactone rings could be formed via alternative openings of an epoxide ring by the carboxyl group generated as a result of the Favorksi rearrangement. To obtain evidence for this, [¹³C,¹⁸O]acetate and ¹⁸O₂ were incorporated into aspyrone. To our surprise there was no incorporation of ¹⁸O label in the acetate experiment, despite high ¹³C incorporation being achieved. This suggested involvement of intermediates in which all acetate-derived oxygen had been lost. On incorporation of ¹⁸O₂ the isotope shifts shown in Figure 6 were observed. C-2 shows two isotopically shifted signals and C-6 shows one. Within experimental error, the intensities of these signals are essentially equal to one another, but half those observed at C-5, C-8 and C-9. The most reasonable interpretation is that one ¹⁸O atom has been incorporated equally into both the carbonyl and ether oxygens of the lactone moiety. Thus three of the oxygen atoms in aspyrone appear to be derived from the atmosphere and so the remaining oxygen on C-2 must be derived from the medium.
To account for these results the pathway shown in Scheme 10 is proposed. The enzyme-bound precursor produced by the polyketide synthetase would be the triene (13). Decarboxylation, followed by epoxidation and rearrangement would generate the aldehyde (14). This could then be converted to the key epoxy-carboxylic acid intermediate (15), required for ring closure to aspyrone and asper lactone, by further epoxidation and NAD⁺ mediated oxidation of the aldehyde as indicated.

Scheme 10 Proposed biosynthetic route to Aspyrone (11) and Asper lactone (12)

AVERUFIN

The studies described above provide strong indirect evidence for the nature of the enzyme-bound intermediates being produced by the polyketide synthesising enzymes in different organisms. They also enable us to predict with a reasonable degree of accuracy the sequence of events in their formation. Obviously we now wish to test this by synthesising these intermediates and studying their incorporation to obtain direct evidence for the assembly processes. To date there has been virtually no success in experiments of this type. This is probably related to the fact that people have been feeding the wrong precursors and in general to whole cells. I suspect that we
shall have to use cell-free enzyme preparations to achieve real success in this area. However some recent results suggest that some success may be achieved with whole cells. In a very significant result, Todd and reported the intact and specific incorporation of $^{13}$C-labelled hexanoic acid into averufin and on the basis of this suggested that averufin and therefore the aflatoxins were not decaketides as previously accepted but rather were really octaketide-derived metabolites being formed from a hexanoate starter unit and 7 malonates as shown in Scheme 11. To test this further we have examined the incorporation of $^{13}$C-labelled malonate to look for a hexanoate starter effect. The results are summarised in Scheme 12. What we observe is a clear acetate starter effect.

![Scheme 11](image)

**Scheme 11** Octaketide and decaketide routes to Averufin

![Scheme 12](image)

**Scheme 12** Incorporation of $[^{13}$C]malonate into Averufin
Thus it appears that averufin is a decaketid. Presumably acetate is chain extended by two malonates and the full reduction-elimination-reduction sequence is used to produce enzyme-bound hexanoate. Assembly then proceeds by addition of a further 7 malonate unit with no further reduction to give the requisite precursor for direct cyclisation and aromatization to produce norsolorinic acid and subsequently averufin. The interesting and significant observation therefore is that exogenous hexanoate can equilibrate with the endogenous enzyme-bound intermediate and so be incorporated. This holds out the hope that if one can feed intermediates with the correct oxidation level under the right conditions then success can be achieved in the direct study of these polyketide chain assembly processes.

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REFERENCES