BIOSYNTHESIS OF FUNGAL MELANIN

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Doctor of Philosophy
University of Edinburgh
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"Well," said Owl, "the customary procedure in such cases is as follows."
"What does Crustimoney Proceedcake mean?" said Pooh. "For I am a Bear of Very Little Brain, and long words Bother me."

A.A. Milne,
Winnie-the-Pooh, Ch. 4.
ABSTRACT

Rice blast disease, caused by the fungus *Pyricularia oryzae*, presents major problems in temperate climates. Chapter 1 is a review of the methods used to control it. The most effective group of anti-fungal agents currently in use are antipenetrants such as tricyclazole. These have been shown to interfere with melanin biosynthesis in the fungus. The biosynthesis of melanin in various organisms is discussed, and the evidence for the DHN melanin pathway in *P. oryzae* presented. The role of melanin inhibition in blast control is discussed. Finally there is a brief account of the process by which invasion of the host plant by the pathogen occurs.

Chapter 2 describes the synthesis of intermediates on the melanin biosynthetic pathway. 1,3-Dihydroxy-6,8-dimethoxynaphthalene, a protected form of 1,3,6,8-THN was synthesised, and the assignment of both $^1$H and $^{13}$C n.m.r. spectra performed using spin-spin decoupling and n.O.e. difference spectroscopy. 1,3,8-THN was prepared from natural scytalone, and biologically converted to vermelone. The $^1$H and $^{13}$C n.m.r. spectra of both of these were assigned. The synthesis of 1-acetoxy-3,6,8-trimethoxynaphthalene was carried out, and x-ray crystallography used to prove its structure definitively, and the regiochemistry of the photo-Fries rearrangement to 2-acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene.

(iii)
In Chapter 3, biosynthetic studies on scytalone in *P. lagerbergii* and *V. dahliae brm-1* are discussed. These utilised deuterium n.m.r., and isotope-induced shifts in $^{13}$C n.m.r. spectra to follow the incorporation of $[^2H_3]$acetate and acetate doubly-labelled with $^{13}$C and $^2$H or $^{18}$O. A relatively new technique of $^1$H, $^2$H-decoupled $^1$H,$^{13}$C correlation n.m.r. spectroscopy was also employed. Derivatives of scytalone were prepared in advance of x-ray crystallographic studies with derivatives from chiral esters, to determine the absolute configuration of scytalone. The optical purity of scytalone was investigated via the monomethyl ether, using chiral shift reagents in proton n.m.r. studies. Vermelone was also investigated using this method.

In Chapter 4, cell-free enzyme methods were used to study melanin biosynthesis. H.P.L.C. techniques were developed to analyse melanin intermediates. The intermediates were fed to cell-free preparations of *P. oryzae* and *V. dahliae brm-1*, and H.P.L.C. used to monitor enzymic conversions in the presence or absence of the melanin inhibitors tricyclazole and PP-389.
DECLARATION

I declare that this thesis is my own composition, that the work of which it is a record has been carried out by myself, and that it has not been submitted in any previous application for a higher degree.

The thesis describes the results of research carried out in the Department of Chemistry, University of Edinburgh, and latterly in the Department of Chemistry, University of Leicester, both under the supervision of Professor T.J. Simpson. The work described in Chapter 4 was carried out at I.C.I. Plant Protection Division, Jealott’s Hill, Bracknell, Berkshire, under the supervision of Drs. B. Baldwin and P. Worthington.
ACKNOWLEDGEMENTS

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My thanks to Suzan Kasperek for tuition in Microbiology, which formed an important part of the work.

Dr. Brian Baldwin and Dr. Paul Worthington of I.C.I. provided invaluable support and advice throughout the project, especially during the period spent at Jealott's Hill Research Station.

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Particular thanks also to Dr. Sandy Blake, for the collection of crystal data sets, and for many hours spent in patient instruction on how to solve the data sets and produce crystal structures from them.

(vi)
POSTGRADUATE LECTURE COURSES

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Medicinal Chemistry (3 lectures), Prof. P.Sammes, S.K.& F.
Sugar Chemistry (5 lectures), Prof. R.Ramage.
Multipulse n.m.r. Spectroscopy of Liquid Samples (5 lectures),
Dr. I.Sadler.
Ortho-xylylenes (1 lecture), Dr. I.Gosney.
Shikimic Acid Pathway (1 lecture), Dr. T.J.Simpson.
Glycopeptides (1 lecture), Prof. R.Ramage.
Captodative Radicals (1 lecture), Dr. H.McNab.

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CHAPTER 1
Table 1.2 Damage of rice plants by four kinds of pests in Japan (unit = 1000 ton)

<table>
<thead>
<tr>
<th>Year</th>
<th>Blast</th>
<th>Sheath blight</th>
<th>Stem borer</th>
<th>Virus (leaf hopper)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1960</td>
<td>270</td>
<td>143</td>
<td>137</td>
<td>73</td>
</tr>
<tr>
<td>1961</td>
<td>263</td>
<td>132</td>
<td>185</td>
<td>37</td>
</tr>
<tr>
<td>1962</td>
<td>299</td>
<td>93</td>
<td>167</td>
<td>28</td>
</tr>
<tr>
<td>1963</td>
<td>649</td>
<td>93</td>
<td>96</td>
<td>18</td>
</tr>
<tr>
<td>1964</td>
<td>324</td>
<td>162</td>
<td>68</td>
<td>39</td>
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<tr>
<td>1965</td>
<td>305</td>
<td>72</td>
<td>77</td>
<td>28</td>
</tr>
<tr>
<td>1966</td>
<td>243</td>
<td>103</td>
<td>63</td>
<td>349</td>
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<tr>
<td>1967</td>
<td>175</td>
<td>111</td>
<td>68</td>
<td>72</td>
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<td>1968</td>
<td>290</td>
<td>98</td>
<td>89</td>
<td>34</td>
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<td>1969</td>
<td>213</td>
<td>97</td>
<td>75</td>
<td>177</td>
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<td>1970</td>
<td>223</td>
<td>103</td>
<td>72</td>
<td>68</td>
</tr>
<tr>
<td>1971</td>
<td>302</td>
<td>110</td>
<td>54</td>
<td>33</td>
</tr>
<tr>
<td>1972</td>
<td>182</td>
<td>90</td>
<td>36</td>
<td>33</td>
</tr>
</tbody>
</table>
## Table 1.1 Rice diseases and the causal organisms in Japan

<table>
<thead>
<tr>
<th>Disease</th>
<th>Causal organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal diseases:</td>
<td></td>
</tr>
<tr>
<td>Blast</td>
<td>Pyricularia oryzae</td>
</tr>
<tr>
<td>Sheath blight</td>
<td>Pellicularia sasakii (=Corticium sasakii)</td>
</tr>
<tr>
<td>Bacterial leaf blight</td>
<td>Xanthomonas oryzae</td>
</tr>
<tr>
<td>Virus diseases</td>
<td></td>
</tr>
<tr>
<td>Dwarf Yellow dwarf, Stripe disease, Black-streaked dwarf</td>
<td>Virus that is transmitted by leaf-hoppers</td>
</tr>
<tr>
<td>Stem rot, culm rot</td>
<td>Helminthosporium sigmoidum or H.sigmoidum var.irregulare</td>
</tr>
<tr>
<td>Helminthosporium leaf spot</td>
<td>Cochliobolus miyabeanus.</td>
</tr>
<tr>
<td>Minor diseases:</td>
<td></td>
</tr>
<tr>
<td>White tip</td>
<td>Aphelenchoides besseyi</td>
</tr>
<tr>
<td>Downy mildew</td>
<td>Phytophthora macrospora</td>
</tr>
<tr>
<td>False smut</td>
<td>Ustilaginoidea virens</td>
</tr>
<tr>
<td>&quot;Bakanae&quot; disease</td>
<td>Gibberella fujikuroi</td>
</tr>
<tr>
<td>Cercospora leaf spot</td>
<td>Sphaerulina oryzina</td>
</tr>
<tr>
<td>Seed and seedling rot</td>
<td>Spp. of Pythium, Achlya, Pythiomorpha and Dictyuchus</td>
</tr>
<tr>
<td>Brown leaf blight</td>
<td>Rhynchosporium oryzae</td>
</tr>
<tr>
<td>Leaf spot</td>
<td>Entyloma oryza</td>
</tr>
<tr>
<td>Brown Sclerotium disease</td>
<td>Sclerotium oryzae-sativae</td>
</tr>
<tr>
<td>Sheath rot</td>
<td>Acrocylindrium oryzae</td>
</tr>
<tr>
<td>Sheath net-blotch</td>
<td>Cylindrocladium scoparium</td>
</tr>
<tr>
<td>Kernel smut</td>
<td>Tilletia horrida</td>
</tr>
</tbody>
</table>
1.1 RICE DISEASES

1.1.1 Introduction

Diseases take a heavy toll from rice production every year. In countries such as Japan whose agricultural economy depends on it, the problem is a serious one. Most of the research on rice disease has centred in Japan for this reason. Diseases affect 70-80% of the total acreage planted with rice, and typically cause 4-5% loss in yields.

The main diseases affecting rice are blast, sheath blight, bacterial leaf blight, virus diseases, Helminthosporium leaf spot and stem rot (Table 1.1).

1.1.2 Rice-Blast

Rice-blast disease caused by the fungus Pyricularia oryzae is the most serious and damaging of all rice diseases due to its wide geographical distribution and the severity of the damage it causes. It occurs in almost every rice-growing country, and under favourable conditions results in heavy and sometimes total yield losses. The extent of damage varies from year to year depending on climatic conditions (Table 1.2). The problem is most serious in temperate climates such as Japan. In tropical climates disease outbreaks are more weather-dependent.

Disease development is favoured by high humidity, extensive rainfall and cool nights. Airborne conidia of the fungus infect leaves nodes and panicles, and other above-ground parts of the plant at all stages of its growth. Nursery beds where planting is dense and the temperature and humidity are high, are particularly
Lesions on the leaves of mature rice plants, caused by the blast fungus *P. oryzae.*
susceptible; but all stages of growth are at risk.\textsuperscript{8,9}

The disease appears as yellowing, striping and stunting of seedlings, and as lesions on the leaves of mature plants. Lesions are typically elliptical with pointed ends, and greyish or whitish in colour (Fig.1.1). The disease often infects nodes, appearing as "neck-rot".\textsuperscript{4}

Intensive cultivation practices used to improve the yields of rice accentuate the problem. The density of planting involved in such methods and the heavy use of nitrogen fertilisers, has been proven to increase the severity of disease outbreaks, providing ideal conditions for the fungus as well as the rice plant.\textsuperscript{7}

1.2 DISEASE CONTROL\textsuperscript{6}

The most economical way to control rice-blast is by the development of resistant cultivars of the rice plant. Many resistant varieties with high yields and good quality characteristics have been introduced in the field, but these soon became susceptible to the disease. This problem arises because \textit{P.oryzae} forms physiological races which differ in pathogenicity. The fungus adapts readily to produce new strains that are able to attack so-called "highly-resistant" varieties of rice.\textsuperscript{1} It is also difficult to produce varieties which combine high disease-resistance with good yields.\textsuperscript{4}

Other cultivation practices are used to improve blast-control, including the disinfection of fields prior to planting, the disinfection of seeds and the restricted use and split
<table>
<thead>
<tr>
<th>Copper fungicides</th>
<th>Basic copper chloride or basic copper sulphate (16, 10 or 2% copper)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper mercury fungicides</td>
<td>Basic copper chloride or basic copper sulphate (2 - 3% copper) with phenylmercuric acetate, ethyl mercuric phosphate (0.15 - 0.08% mercury)</td>
</tr>
</tbody>
</table>

(1) \( \text{AcO-Mg-Ph} \)
Table 1.3 Consumption of pesticides for the treatment of diseases and pests of rice in 1985 in Japan.

<table>
<thead>
<tr>
<th>Disease / pest</th>
<th>Total area treated ( / 1000 ha.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blast</td>
<td>5391</td>
</tr>
<tr>
<td>Sheath blight</td>
<td>2176</td>
</tr>
<tr>
<td>White-backed planthopper</td>
<td>1697</td>
</tr>
<tr>
<td>Brown planthopper</td>
<td>1653</td>
</tr>
<tr>
<td>Smaller brown planthopper</td>
<td>1642</td>
</tr>
<tr>
<td>Green leaf-hopper</td>
<td>1637</td>
</tr>
<tr>
<td>Stem borer</td>
<td>1556</td>
</tr>
<tr>
<td>Stink bug</td>
<td>1452</td>
</tr>
<tr>
<td>Leaf beetle</td>
<td>836</td>
</tr>
<tr>
<td>Grass leaf roller</td>
<td>463</td>
</tr>
<tr>
<td>Seedling blight</td>
<td>348</td>
</tr>
</tbody>
</table>
application of nitrogen fertilisers.  

Effectiveness of disease-control by these methods is limited by climatic conditions, and control by chemical methods is thus economically worthwhile. From the beginning of the twentieth century fungicides have been used in practical application, and many excellent anti-blast agents have since been discovered. In 1985 the pesticide market in Japan totalled $1,657 million, of which rice accounted for 46.7% usage, and rice-blast 28.6% of this (Table 1.3).

1.3 ANTI-BLAST AGENTS

1.3.1 Copper Fungicides

The first group of fungicides to be used were copper compounds. (Table 1.4) In 1914 the effectiveness of Bordeaux mixture (widely used in vineyards for disease control) was demonstrated, and this was used extensively from 1923 onwards, especially in the cooler areas of Japan where blast damage was serious. Copper fungicides however are phytotoxic and often reduce yields rather than increase them, particularly when blast attack is not severe. They are also ineffective under epidemic conditions.

1.3.2 Organomercury Fungicides

It was then discovered that when used in conjunction with phenylmercuric acetate (PMA, 1) these copper compounds were more effective and less phytotoxic in disease control. In 1950 it was found that a simple mixture of PMA and slaked lime was very
effective, cheap, and non-phytotoxic. It could be readily and easily applied as foliar dust sprays with remarkable results. Research with phenylmercuric compounds produced a wide range of fungicides $R-Hg-X^3$ which showed lasting effectiveness in the field and also a degree of systemic activity.

The fungicides are absorbed onto the leaves, and restrict fungal growth not only on the treated areas but on untreated parts and also subsequent new growth. Fungicidal activity lasts for several weeks and is lost as the compounds react with glutathione and sulphhydryl groups on enzymes. The fungicides are thought to be strong inhibitors of the respiratory enzymes of pathogenic fungi.$^4$

In the late 1950s mercuric poisoning was reported in several areas of Japan, and although these outbreaks were later shown to be caused by industrial mercuric waste and not agricultural residues, the compounds are nevertheless highly toxic and cause environmental pollution. This led to the legal prohibition of the use of mercury compounds as agricultural chemicals in 1968 in Japan.$^6$

1.3.3 Antibiotics$^5,6,7$

The urgent need for blast control agents in Japan especially led to extensive research, and many non-mercuric compounds were developed including antibiotics. Various promising antibiotics such as antiblastin, antimycin A (2) and Blasticidin $A^3$ were found but not developed for practical use either because they were chemically unstable or toxic to fish.
Blasticidin S (3) was first isolated from a soil organism *Streptomyces griseochromogenes*. It is effective against rice-blast at concentrations of only 10 - 20 p.p.m., although at higher concentrations does cause damage to other crops such as tobacco, eggplant, tomato and beans. A formulation of the benzylaminobenzene sulphonate salt was found to be the least phytotoxic without reducing efficacy.

At 5 p.p.m. blasticidin S inhibited the spore germination of the fungus completely and was thus better than PMA (which achieves this at 20 p.p.m.) although it decomposes more rapidly. Blasticidin S was found to inhibit protein synthesis in *P. oryzae*.

Another antibiotic kasugamycin (4) was isolated from *Streptomyces kasugaensis* in 1965. At 20 - 30 p.p.m. it is very effective against rice-blast, although it does not inhibit sporulation or appressorial formation, and therefore penetration of the host by the fungus still occurs. It is systemic, although lacking in residual activity, and prevents mycelial growth within the leaf. As with blasticidin S it inhibits protein biosynthesis in *P. oryzae*, although at a different stage.

### 1.3.4 Organochlorine Agents

Organochlorine compounds such as PCBA (pentachlorobenzyl alcohol, blastin, 5) and tetrachlorophthalide (fthalide, rabcide, 6) were also developed from 1960.\(^5\) PCBA showed excellent preventive action in field tests, inhibiting penetration of blast spores.\(^1\) It was found in practical use that it is oxidised in
soil to pentachlorobenzoic acid, which causes a virus-disease-like abnormality of vegetables such as tomato and cucumber at very low concentrations, and its use was banned.

Tetrachlorophthalide was then developed. This inhibits sporulation on lesions and penetration of the rice plant epidermis and has very low mammalian toxicity. The major metabolites are slightly toxic to vegetables, but this problem is minimised by restricted use.

Both PCBA and tetrachlorophthalide inhibit the germinating spore of *P. oryzae* from growing through the plant epidermis. The spores germinate laterally and do not form the appressoria from which infection pegs develop. This was observed on inert nitrocellulose membranes as well as living tissue, implying that the mode of action is not host-mediated. The observation that these two inhibit melanisation in the fungus led to the proposal that along with other non-fungitoxic anti-blast agents, this effect was the criterion for disease-control ability. Albino (unmelanised) mutants of *P. oryzae* were also unable to penetrate either living epidermis or inert membranes. However most evidence suggests that this is a false interpretation of the results, and that they act by preventing the maturation process of appressoria before the formation of infection pegs. Higher concentrations of the chemicals are required for melanin inhibition than to prevent penetration, and the time of application of the chemicals is different than for other melanin inhibitors (see below).
Tetrachlorophthalide is widely used in Japan owing to its potency, and its ability to persist after rainfall, hence having the greatest residual effect of these anti-blast agents.

1.3.5 Organophosphorous Agents

The discovery of anti-fungal activity in kitazin (EPP) led to the organophosphorous thiolates (PTL compounds), already in use for insect control, becoming the major group of anti-blast chemicals in practical use. The PTL compounds IBP (kitazin P, \(^7\))\(^{18,19}\) and EDDP (hinosan, ediphenphos, \(^8\))\(^{20}\) are effective in foliar application at 400-500ppm. They inhibit spore germination, appressorial formation, penetration into the leaf epidermis and sporulation on lesions. IBP is systemic, but EDDP has the greater residual activity despite being only weakly systemic.

IBP was originally thought to interfere with chitin biosynthesis. Studies with *Pythium debaryanum*, a chitin-free fungus, along with evidence that penetration into nitrocellulose membranes is also prevented contradicted this. Both these agents have now been shown to inhibit the enzyme phospholipid N-methyltransferase, which is responsible for the transmethylation reaction of S-adenosyl-L-methionine in phosphatidylcholine biosynthesis.\(^5,19\)

Isoprothiolane (fuji-one, \(^9\)) although containing no phosphorous seems to have a similar but not identical mode of action to the PTL compounds, and fungal cultures show cross-resistance to both (see below). Isoprothiolane strongly inhibits penetration of the epidermis and mycelial growth, and is
systemic. It seems to inhibit either the synthesis of the cell membrane or the permeability of the membrane, rather than phosphatidylcholine biosynthesis. ⁵

1.4 RESISTANCE PROBLEMS

Many microorganisms develop resistant strains to anti-fungal agents, and as mentioned earlier P. oryzae is particularly prone to this. ⁴ This is a major problem especially when chemicals having a specific mode of action are involved such as the PTL compounds. Resistance to many anti-blast chemicals occurred in the field due to the confined use of one type of chemical. ⁶ Cyclical use, or the use of several compounds in combination often provides better disease control.

In 1958 resistance to PMA was seen when P. oryzae was repeatedly grown on medium supplemented with it. ²¹ Resistance to copper sulphate, boric acid and mercuric chloride was also found. Prolonged use of kasugamycin resulted in a significant decrease in effectiveness due to the appearance of resistant strains of the fungus. ⁷

Resistance to IBP, EDDP and isoprothiolane was recognised in 1976 and considered to be due to natural selection processes and spontaneous mutagenesis in the fungus. Resistance often declines in the field after the use of a particular agent is discontinued, hence rotation of different agents provides control. Strains resistant to IBP, EDDP and isoprothiolane are not generally resistant to tetrachlorophthalide and kasugamycin. Hence a
combination of the two groups is often used to overcome the problem.

Legislation governing the use of agrochemicals has become much stricter. An increasing amount of research has been devoted to more effective and less toxic methods of disease control. Much of this has concentrated on studies of the modes of action of the fungicides, with the eventual aim of designing new agents specific for blast control.

1.5 **ANTIPENETRANTS**

The disease control abilities of copper, mercury and PTLC fungicides are more or less related to their fungitoxicity in vitro. In 1974 a new anti-blast chemical probenazole (oryzexnate, 10) was registered which showed striking disease control in vivo at concentrations well below those required for fungitoxicity. Since then, other compounds have emerged which show similar characteristics. Among these notably tricyclazole (11), pyroquilon (12) and PP-389 (13). They are mostly systemic agents which provide long-term control and do not have the problems of induced host-resistance associated with earlier fungicides. Application is protectant, that is, the chemicals are not effective against plants already infected, as they are not primarily fungitoxic.

In view of their lack of toxicity to the vegetative growth of the fungus, the effect of these compounds in disease control must
be sought elsewhere. Possible modes of action are:

1) Metabolism of chemicals in the plant to fungitoxins,
2) Enhancement of the plant resistance mechanism,
3) Suppression of fungal pathogenicity.\textsuperscript{5,29}

Control of plant disease by chemicals that enhance host-resistance or suppress pathogenicity of the parasite is a very attractive and potentially promising approach towards new anti-fungal agents. Compounds of this type would only be toxic towards the parasitic phase of the pathogen.\textsuperscript{22,30} Much research has therefore gone into elucidating the mechanisms by which these chemicals provide disease control.

Probenazole has been shown to act both by inhibition of stages of fungal development and by enhancement of the host defence mechanism.\textsuperscript{24,25} When applied to infected rice plants, these produced anti-microbial substances such as $\alpha$-linolenic acid and momilactones A and B. The activity of enzymes such as peroxidase, phenylalanine ammonia lyase and catechol $o$-methyltransferase was accentuated, promoting the formation of lignified walls within the leaf, which prevent the spread of the mycelium into cells adjacent to the invasion site.\textsuperscript{6}

The other anti-fungal agents in this group, despite their diversity of structure all appear to share a common mode of action. They were all demonstrated to inhibit melanin biosynthesis in \textit{P.oryzae}, but a direct relationship between the loss of pigmentation and the loss of pathogenicity had yet to be firmly established. Evidence for this was subsequently provided.
1.6 MELANINS\textsuperscript{31,32}

1.6.1 Introduction

Melanins are dark brown to black pigments of high molecular weight, many of which are formed by the oxidative polymerisation of phenolic compounds. They occur in humans, both warm- and cold-blooded vertebrates, invertebrates including insects, higher plants, fungi and bacteria. They are not essential for the growth and development of these organisms, but contribute to their survival ability and competitiveness. Animal melanins have been widely studied due to their association with skin disorders and malignant melanomas. They have a protective role against sunlight, and are used as camouflage by squid and octopus, whose "ink" consists of a fine suspension of melanin granules.

In fungi melanins have long been recognised as an important factor in the survival of spores and other structures designed to withstand stress and extreme environmental conditions. They have a protective role against heat, uv-irradiation, desiccation and degradation by enzymes such as endo-\(\beta\)-1,3-glucanase and chitinase. The structure of melanins, involving combinations of quinones and hydroquinones, enables them to exist as stable free radicals that are formed on exposure to uv-irradiation, \(\gamma\)-rays or high temperatures, or by chemical reduction. It is this radical stability that gives them their protective ability under harsh environmental conditions.
Fig. 1.2 Biosynthesis of DOPA melanin from tyrosine by the action of tyrosinase (t). DOPA melanin is a polymer formed from a number of quinone intermediates.
In animals, melanin synthesis occurs mainly in specialised structures such as melanocytes or melanophores. Melanins are found almost entirely in melanosomes or as granules in specialised cells. This is in distinct contrast to fungi, where the melanin is located either in the cell walls, or as extracellular polymers formed in the medium around the cells.

Melanins are generally difficult to degrade and study, due to their polymeric nature. They are inert, and usually poorly soluble in water, aqueous acids, organic solvents and alkali. Purification and characterisation require drastic procedures, and hence they are usually classified in terms of their biosynthetic origin.

1.6.2 DOPA Melanin

DOPA melanin is formed by the oxidation of tyrosine by tyrosinase, via 3,4-dihydroxyphenylalanine (DOPA) (Fig.1.2). The black pigments of animals are formed by this route, and related reactions involving cysteine give rise to yellow, brown and red pigments.33

Tyrosine occurs in all living organisms, and tyrosinases have been isolated from several fungal species. It appears that extracellular melanins in fungi may arise from this route; but there is no proof that the wall-bound melanins have the same origin. Oxidation of DOPA in cell walls does occur, but the enzyme involved appears to be laccase.
Fig. 1.4 Biosynthesis of catechol melanin from catechol.
Fig. 1.3 Biosynthesis of γ-glutaminyl-3,4-dihydrobenzene (GDHB) melanin from γ-glutaminyl-4-hydroxybenzene (GHB) by the action of peroxidase / phenolase (pe / ph) and γ-glutamyltransferase (γ-g).
1.6.3 GDHB Melanin

Cell walls of *Agaricus brunnescens* contain nitrogenous melanins which appear to come from \( \gamma \)-glutaminyl-4-hydroxybenzene (GHB) (Fig.1.3).\(^\text{37}\) GHB is a product of the shikimic acid pathway, and is oxidised to melanin via \( \gamma \)-glutaminyl-3,4-dihydroxybenzene (GDHB) and \( \gamma \)-glutaminyl-3,4-benzoquinone (GBQ).\(^\text{38}\) Synthetic melanin produced by the action of tyrosinase prepared from *A.brunnescens* resembles the natural melanin chemically and ultrastructurally.\(^\text{37}\) Hence GDHB seems to be the natural precursor to melanin in this species, and may also be in other species of *Basidiomycotina* which produce tyrosinases and have also been shown to contain GHB and GDHB.

1.6.4 Catechol Melanin

Melanin in teliospores of *Ustilago maydis* apparently originates from catechol. Elemental analysis is identical to that of synthetic melanin produced by the action of mushroom tyrosinase on catechol.\(^\text{39,40}\) Biosynthesis probably occurs via free radicals or quinone-catechol adducts (Fig.1.4); as these dimeric species are found as products of enzymic oxidation of the melanin.

Catechol was suggested as precursor to melanins in some other species, including *V.albo-atrum* and *V.dahliae*.\(^\text{40,41}\) These have since been shown to produce DHN melanin\(^\text{35,36}\) (see below). However, *U.maydis* and other fungi may also produce catechol melanin.
Table 1.5 Results of tricyclazole and homogenate studies to show 1,8-DUN melanin

Ascomycotina
Cochliobolus carbonum, TI,EA,PP
C.miyabeanus, TI,EA,PP
Pleospora infectoria, TI,EA,PP
Sclerotinia minor, EA,PP
S.trifoliorum, TI,EA,PP
Wetzelinia sclerotiorum, TI,EA,PP

Fungi Imperfecti
Alternaria alternata, TI,PP,HP
A.brassicicola, TI,EA,PP
A.solani, TI,PP
Aspergillus niger, -,HP
Aureobasidium pullulans, TI
Bipolaris sorokinaria, TI,EA,PP
Botrytis cinerea, TI,PP
Cladosporium carrionii, TI,HP
Colletotrichum gossypii, TI,PP
C.lagenarium, TI,PP
C.lindemuthianum, TI,PP
Curvularia protuberata, TI,EA,PP
Diplodia gossypina, TI,EA,PP
D.natalensis, TI,EA,PP
Exophiala jeaneselmei, TI,HP
Fonsecaea compacta, TI,HP
F.pedrosoi, TI,HP
Hendersonula toruloidea, TI,EA,PP,HP
Macrophomina phaseoli, TI,PP
Microdochium bolleyi, TI,PP
Monilinia fructicola, TI,PP
Phaeoannelloryces werneckii, TI,HP
Phaeococcomyces sp., TI
Phialaphora richardiae, TI,HP
P.verrucosa, TI,HP
Pyricularia oryzae, TI,EA,PP
Rhizoctonia leguminicola, TI,EA,PP
Sclerotium cepivorum, EA,PP
Thielaviopsis basicola, TI,PP
Verticillium albo-atrum, TI,EA,PP
V.dahliae, TI,EA,PP
V.nigrescens, TI,PP
V.tricorpus, TI,EA,PP
Wangiella dermatitidis, TI,EA,HP
Xylohypha bantiana, TI,HP

Basidiomycotina
Thanatephorus cucumeris, -,PP
Sphacelotheca reiliana, -,PP
Sclerotium rolfsii, -,PP
Typhula idahoensis, -,PP
T.ishikariensis, -,PP
Ustilago maydis, -,PP

* TI = DUN melanin demonstrated by tricyclazole inhibition; EA = DUN melanin demonstrated by enzyme assay; - = no effect with tricyclazole inhibition or enzyme assay; PP = plant pathogen; HP = human pathogen.
1.6.5 Extracellular Melanins

Melanins that are synthesised completely apart from the fungal cell walls are generally referred to as extra-cellular or heterogeneous melanins. This does not include melanins which have broken off from the fibrillar matrix surrounding the cell wall.

They may be formed by the action of phenol oxidases such as tyrosinase, which have been released from the cell, on phenolic compounds in the medium. Many wood-rot fungi, and fungi which decompose plant tissue use such extra-cellular enzymes.

Saprophytic fungi often accumulate brown pigments in the culture medium, which may be likened to humic acids in soil and are thus sometimes referred to as "fungal humic acids". These are formed by the secretion of phenols from the cells, which are autoxidised, or may be oxidised by phenol oxidases later released. Aspergillus glaucus and other fungi produce considerable amounts of these extra-cellular melanins. They are also produced by many Actinomycetes.

1.6.6 DHN Melanin

A number of Ascomycetes and fungi imperfecti produce brown to black melanins from 1,8-dihydroxynaphthalene (1,8-DHN, 18, Table 1.5). These include human pathogens such as Wangiella dermatitidis and other dematiaceous fungi found in soil, and plant pathogens as mentioned such as Thielaviopsis basicola, Colletotrichum lagenarium, Colletotrichum lindemuthianum, and Leptosphaeria maculans, as well as P. oryzae.
Table 1.6 Characteristics of melanin-deficient mutations in *Verticillium dahliae*

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Enzyme deficiency</th>
<th>Major product accumulated</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. dahliae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alm-1</td>
<td>unknown</td>
<td>none</td>
</tr>
<tr>
<td>alm-2</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>alm-3</td>
<td>dehydratase</td>
<td>scytalone, flaviolin, 4-HS</td>
</tr>
<tr>
<td>brm-1</td>
<td>reductase</td>
<td>2-HJ, 3, 4, 8-DHT, 4, 8-DHT, flaviolin</td>
</tr>
<tr>
<td>brm-3</td>
<td></td>
<td>DHN, 2, 2'-dimer of DHN</td>
</tr>
<tr>
<td>brm-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gym-1</td>
<td>oxidase</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1.6 Pentaketide assembly of 1,3,6,8-THN (NB. Several folding patterns of the pentaketide intermediate are possible, of which one is shown).
Fig. 1.5 Polyketide (DHN) pathway of melanin biosynthesis, including the related flaviolin and 2-HJ branch pathways. Postulated intermediates are shown in brackets. Blockage sites for tricyclazole are indicated (t).
1,8-DHN is formed via the polyketide pathway (Fig.1.5). A pentaketide intermediate derived from acetate assembles and cyclises on the polyketide synthase surface, to form the symmetrical intermediate 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN, 14, Fig.1.6). This is reduced to scytalone (15) and dehydrated to 1,3,8-tri hydroxynaphthalene (1,3,8-THN, 16). A second reduction/dehydration sequence gives first verinelone (17) and then 1,8-DHN (18). This is presumably polymerised oxidatively to melanin.

The biosynthesis of melanin via 1,8-DHN was characterised using genetic mutants of Verticillium dahliae. The mutations and their genetic characteristics have been ascertained (Table 1.6). Mutant cultures accumulate both direct intermediates on the pathway, and products of shunt pathways resulting from oxidation of unstable intermediates on the main pathway.

Hence the intermediates were isolated and characterised, and cross-feeding experiments between mutants demonstrated that "normal" melanin was made from these (see below).

The brm-1 mutant lacks the dehydratase which converts scytalone to 1,3,8-THN and vermelone to 1,8-DHN. Cultures accumulate scytalone as a result. Flaviolin (19) and cis-4-hydroxyscytalone (4-HS, 20) which are shunt products of 1,3,6,8-THN, also accumulate. Feeding 1,3,8-THN to brm-1 produces vermelone, because the reduction occurs downstream from the genetic block. The dehydration of vermelone to 1,8-DHN is also blocked in brm-1, presumably because the same enzyme is involved.
The brm-2 mutant of V. dahliae lacks the reductase enzyme and cannot convert 1,3,8-THN to vermelone. Thus 1,3,8-THN and the shunt product 2-hydroxyjuglone (2-HJ, 21) accumulate. Other metabolites 3,4,8-trihydroxytetralone (3,4,8-THT, 22), juglone (23), 3-hydroxy-juglone (3-HJ, 24) and 4,8-dihydroxytetralone (4,8-DHT, 25) also build up. The reduction of 1,3,6,8-THN is also inhibited, but only partially under culture conditions.

The albino mutations alm-1, alm-2 and alm-3 block an early stage in the biosynthesis and do not produce 1,3,6,8-THN. However when treated with scytalone, vermelone or 1,8-DHTN they are able to synthesise "normal" melanin.

The brm-3 and brm-1 mutants accumulate the same metabolites but complement each other to produce melanin. Brm-4 complements brm-2, but is blocked at the reduction of 1,3,6,8-THN more effectively than brm-2, and thus accumulates flaviolin. The selectivity of brm-2 for the second reduction step suggests that if the same reductase is involved in both steps, the active site has a higher binding affinity for 1,3,6,8-THN than 1,3,8-THN.

1.7 MELANIN BIOSYNTHESIS INHIBITORS

The first of these, tricyclazole (11) is a highly potent systemic agent with long residual activity. Because of its systemic nature it may be applied in a number of ways in order to provide the most effective disease control. While allowing spore germination, appressorial formation and mycelial growth, it specifically prevents the in vivo invasion of P. oryzae into the
Fig. 1.7 Effect of increasing concentrations of tricyclazole on pigmentation in P. oryzae.
host plant tissue. In vivo, its efficacy is some thirty times greater than in vitro.

Early research noted a correlation between the loss of pathogenicity of the culture and the loss of pigmentation. At concentrations of tricyclazole application well below those which affect vegetative growth of the fungus, the mycelium loses its normal grey-black colour and becomes buff-coloured (Fig.1.7). The fungus is also unable to invade the host. The mode of action was thus proposed to be 2) or 3) above (Section 1.5). The inhibitory effect was seen to be quite selective to P. oryzae.26,29 In field trials, tricyclazole did not appear to reduce the incidence of the rice diseases caused by Cochliobolus miyabeanus, Corticium sasakii or Xanthomonas oryzae, even at levels of application of 1.0 p.p.m., at which concentration it was completely effective against P. oryzae. These findings indicated that either this anti-blast agent had an effect within the host which was not expressed in vitro, or that a host-resistance mechanism was induced which was specific for P. oryzae.

The fact that the chemicals were preventative but not curative supported the idea that they were primarily non-fungitoxic.

The effect of tricyclazole on the production of pyriculol (26) was considered as a reason for its effectiveness.29 Pyriculol is a polyketide-derived phytotoxin produced by P. oryzae, which was thought to be a factor in its phyto-toxicity.56,57
Table 1.7 Effects of various concentrations of tricyclazole, pyroquilon and PP-389 on melanisation and pigment excretion in cultures of *P. oryzae* grown at 27°C for 7 days on sucrose-nitrate medium.

<table>
<thead>
<tr>
<th>Compound</th>
<th>0.0001</th>
<th>0.001</th>
<th>0.01</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
<th>50.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricyclazole</td>
<td>+++ N</td>
<td>++ N</td>
<td>+ Y</td>
<td>O Y</td>
<td>O YR</td>
<td>O R</td>
<td>O R</td>
</tr>
<tr>
<td>Pyroquilon</td>
<td>+++ N</td>
<td>+++ N</td>
<td>++ Y</td>
<td>+ Y</td>
<td>O Y</td>
<td>O YR</td>
<td>O R</td>
</tr>
<tr>
<td>PP-389</td>
<td>+++ N</td>
<td>+++ N</td>
<td>+++ N</td>
<td>++ Y</td>
<td>+ Y</td>
<td>O YR</td>
<td>O R</td>
</tr>
</tbody>
</table>

(i) Effect on melanisation:
- +++ no effect
- ++ slight inhibition
- + moderate inhibition
- O complete inhibition

Excreted pigment:
- N none
- Y yellow
- YR yellow-orange
- R red
Studies on cultures treated with tricyclazole revealed that, if anything, pyriculol production was enhanced rather than suppressed compared to untreated cultures. Pyriculol was also produced in non-pathogenic albino and pink mutants of *P. oryzae*. Hence it seemed to be essentially irrelevant to the pathogenicity of the fungus.

Pyroquilon (12) and PP-389 (13) would appear to share the mode of action of tricyclazole. Treatment of the fungus with either of these results in a similar change of mycelial colour from the normal grey-black to buff. With increasing levels of application, other changes in mycelial colour are seen (Table 1.7) as a result of the excretion of other metabolites which are shunt products of the main melanin biosynthetic pathway (see below). Melanisation is extremely sensitive to tricyclazole and the other anti-blast agents in this group.

Melanin-deficient (buff or albino) mutants of *P. oryzae*, produced by uv-irradiation of colonies, or arising from spontaneous mutations, were shown to phenotypically resemble the tricyclazole-treated wild-type cultures. Both were non-pathogenic to the rice-plant. The fact that the buff mutants were non-pathogenic supported the idea of melanisation being necessary for the invasion process.

The possibility of a host-mediated defence mechanism was ruled out by several experiments. Epidermal strips from *Bryophyllum pinnatum* leaves were heat-treated to kill the cells, treated with tricyclazole and infected with *P. oryzae*. As with
living epidermis, melanisation of *P. oryzae* was inhibited, and penetration of the membrane prevented. Another method of evaluating the effects of anti-fungal agents on penetration by *P. oryzae* was developed which used inert cellophane film or nitrocellulose membranes as a model for the cell wall.\(^\text{12}\)

Again, penetration of these was prevented by application of tricyclazole or the other melanin inhibitors.\(^\text{13}\) The application of tricyclazole after appressorial formation still prevented membrane penetration, but the effectiveness of the treatment was closely linked to the degree of melanisation of the appressoria at the time of application. Cultures with heavily melanised appressoria were still able to penetrate the membranes, thus providing further evidence for the importance of melanin in the invasion process.

Tricyclazole was seen to protect unwounded but not wounded rice plants from infection by *P. oryzae* (see also Section 1.8). Buff mutant strains were also able to infect wounded plants although non-pathogenic towards healthy ones.\(^\text{30}\) Tricyclazole at concentrations of 0.1 - 10 p.p.m. had no effect on spore germination or the formation of appressoria, but did prevent melanisation of the appressoria, and subsequent penetration of the leaf cell wall.

At dose levels as low as 0.1 p.p.m., the biosynthesis of vermelone (17) in the melanin pathway (Fig.1.5) was completely inhibited.\(^\text{42}\) Early intermediates such as scytalone (15) and shunt
(27)

(28)
products of 1,3,8-THN (16) such as 2-HJ (21) accumulated, causing a yellowish discolouration.

At higher concentrations of the chemicals (1 - 10 p.p.m.) flaviolin (19) accumulated, resulting in a reddish colouration. Flaviolin is an oxidation product of 1,3,6,8-THN (14), indicating a secondary site of inhibition in the pathway. This supported the idea suggested by the studies on the genetic mutants V.dahliae brm-2 and brm-4. The same reductase may be involved in the reduction of both 1,3,6,8-THN and 1,3,8-THN, but the enzyme has a higher binding affinity for 1,3,6,8-THN.

It was suggested that the accumulation of the cytotoxic metabolite 2-HJ was responsible for the anti-fungal activity of tricyclazole in P.oryzae. This may contribute to the effect but is unlikely to be solely responsible, as buff mutants of the fungus are non-pathogenic, yet do not produce 2-HJ.

It was also shown that addition of vermelone or 1,8-DHN to treated cultures restored the ability to penetrate nitrocellulose film along with melanisation. Melanin thus formed resembled wild-type melanin chemically and ultra-structurally. Restoration of penetrative ability was incomplete, probably due to the cytotoxicity of the 2-HJ formed.

Evidence that 2-HJ accumulation does have a role in reducing the pathogenicity of P.oryzae came from studies with cerulenin (27). Cerulenin inhibits condensation of acetyl CoA and malonyl CoA, and hence blocks polyketide biosynthesis at an early stage. The addition of cerulenin along with tricyclazole or
tetrachlorophthalide (6) prevented the accumulation of 2-HJ. When vermeline or 1,8-DHN was added, restoration of penetrative ability was markedly improved.

In albino mutants, melanisation and penetrative ability were induced by the addition of scytalone or 1,8-DHN. If treated with tricyclazole, only 1,8-DHN was able to do this, supporting the idea of tricyclazole blockage occurring to inhibit the reduction of 1,3,8-THN to vermeline.

Further work with both tricyclazole-treated wild-type, and the non-pathogenic buff mutants showed that the appressoria of both became melanised when melanin precursors such as DOPA and 1,8-DHN were added, and concurrently recovered penetration ability, although the melanin thus formed was chemically different from the natural product. This was observed both in vivo and on nitrocellulose membranes.

Chlobenthiazone (4-chloro-3-methyl-2(3H)-benzothiazolone, 28), a more recent systemic anti-blast agent, was shown to have the same features in disease control. The melanisation process was very sensitive to the chemical, again at non-fungitoxic concentrations of as little as 0.1 p.p.m. Detailed studies on the interference of the chemical in the process of infection of the rice-plant by P.oryzae showed a very close correlation between the melanisation of appressoria and the formation of infection pegs to invade the host. If the chemical was applied too late to inhibit melanisation, or if the host plant was deliberately wounded, then no disease control was
observed. In the case of wounded plants, infection can take place from hyphae, and the formation of infection pegs is unnecessary.

The effects of chlobenthiazole and the other melanin inhibitors were compared with tetrachlorophthalide and PCBA. The results reinforced the idea that although the latter do inhibit melanisation, this occurs at higher concentrations than are required for disease control. Also, melanin inhibitors were effective when applied until just before the start of appressorial melanisation, whereas tetrachlorophthalide and PCBA were only effective if applied early on during appressorial formation. 17

Similar observations to these studies on the action of tricyclazole and other melanin biosynthesis inhibitors on P. oryzae have been made for Colletotrichum species. 16,46 The melanin inhibitors prevented the infection of Phaseolus vulgaris (bean plants) by C. lindemuthianum at low concentrations which inhibited melanisation of appressoria. Infection of wounded plants was not prevented. The treated cultures germinated laterally. The untreated appressoria contained an electron-dense layer which increased in thickness around the infection pore. 46,48 In C. lagenarium (anthracnose of cucumber), unmelanised albino mutants 45 and tricyclazole-treated cultures 46 both failed to penetrate nitrocellulose membranes, and the colourless appressoria germinated laterally. Treatment of the cultures with DOPA resulted in pigmentation and restoration of penetrative ability in albino mutants. 64 In tricyclazole-treated cultures,
accumulation of 4,8-dihydroxytetralone (4,8-DHT, 25) was seen, indicating blockage of the melanin biosynthetic pathway again between 1,3,8-THN and vermelone. Colourless appressoria were formed which germinated laterally and failed to penetrate nitrocellulose membranes. Vermelone addition restored penetrative ability as for albino mutants, along with pigmentation.

The evidence demonstrates clearly that tricyclazole protects the rice plants by specifically interfering with penetration of the epidermal cell wall, and that it acts on the pathogen, not via the host defence mechanism. The relationship between melanisation and pathogenicity in P. oryzae is also fairly conclusive.

A brief consideration of the biological processes by which fungal invasion of the rice plant occurs, follows.

1.8 BIOLOGY OF LEAF INVASION 65,66,67

To infect a plant and thus gain access to the nutrients contained within it, a parasitic fungus must first invade the plant. Three routes of entry are via wounds, natural openings such as stomata, or through the intact plant surface. Different fungi are specialised to use different routes. Some, such as Penicillium digitatum which infects oranges, may only be able to infect wounded fruit. Rusts and downy mildews utilise structural apertures to gain entry. Yet others, such as powdery mildews, are able to penetrate the cuticle directly. These fungi may also take
Fig. 1.8 Leaf invasion by parasitic fungi; direct penetration process.
advantage of wounds or natural openings if necessary or available.

To aid in the direct penetration process, fungi may secrete enzymes or other chemicals which help to soften the cuticle. However the basis of the process in many cases is the mechanical force exerted on the cell walls of the plant by the pathogen.

When the fungus and the plant come into contact, intermolecular forces arise which keep them together. The hypha or radicle then enlarges to form a flattened, bulbous structure termed an "appressorium", which secures the pathogen in place (Fig.1.8). From this a very fine hyphal tip, the "infection peg" develops, which forces its way through the cuticle. Once inside the leaf, the hypha expands to its normal size.

Inside the leaf, mycelial growth is accomplished largely by chemical and enzymic processes, to dissolve cell wall barriers and neutralise structures and chemicals produced by the plant in response to the fungal invasion.

Penetration therefore depends on the balance of forces between the host plant, and the hyphal tip of the infection peg and the appressorium from which it emerges.

Ultrastructural studies in *P. oryzae* indicate that melanin provides the necessary strength and rigidity for the appressorium, as the basis for infection peg development. Melanin biosynthesis would therefore seem to be an ideal target for the control of rice blast disease, as evidenced by the remarkable effect of antipenetrant anti-fungal agents such as tricyclazole.
1.9 AIMS

The melanin inhibitors appear to act primarily at the reduction of 1,3,8-THN to vermelone in the main biosynthetic pathway, and also at higher concentrations, on the reduction of 1,3,6,8-THN to scytalone. The melanin pathway could be blocked at other sites.

The research proposed to confirm the general pathway as outlined, and elucidate further the mechanism of the reduction and dehydration steps to aid further investigation of the mechanism of inhibition of the anti-blast agents.

The exact polyketide origins of the first enzyme-free intermediate 1,3,6,8-THN were still in question, due to conflicting results from earlier precursor feeding experiments.

The absolute stereochemistry of the intermediates scytalone and vermelone has not been established, and indeed there is some conflict in the literature over their optical characteristics and purity from different sources. It was proposed to investigate these further.

Techniques to study melanin biosynthetic reactions in cell-free homogenates of *P. oryzae* and *V. dahliae* have been developed. These techniques are much more sensitive and versatile than traditional whole-cell studies. Conditions may be varied using different mutants, and in the presence or absence of NADPH and enzyme inhibitors, to study different steps on the pathway. HPLC techniques greatly facilitate evaluation of the results of such experiments.
The mechanism of deoxygenation of natural products is an interesting problem which has recently received attention\textsuperscript{71} and merits further investigation in pathways such as melanin biosynthesis.
1.10 REFERENCES

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CHAPTER 2
2.1 INTRODUCTION

To study the incorporation of substrates in any biochemical pathway, these substrates must first be synthesised. To follow biochemical interconversions, isotopically-labelled substrates are required.

Early biosynthetic studies made extensive use of $^{14}\text{C}$ and $^3\text{H}$ radioactive isotopes. These were used to monitor uptake of precursors such as acetate and malonate during biosynthesis, and also to determine the ultimate fate of the precursors in metabolites. Radioactive measurements involved using a scintillant "cocktail" containing phosphors, able to convert the $\beta$-radiation emitted by the radioactive compound into light, measured in a photomultiplier. The reliability of this method was dependent on the purity of the sample to be counted, and obtaining a strong enough sample count comparative to an inactive blank sample.

The fate of radioactive precursors was determined by degradation of the radioactive metabolite to isolate the site of label, and hence establish the specificity of incorporation. The path of degradation had to be known precisely, and the process was often complex. The method also destroyed the sample.

Advances in n.m.r. spectroscopy provided a non-destructive analytical tool of great sensitivity and versatility, which utilises stable isotopes.¹ In biosynthesis the most useful of these are $^{13}\text{C}$, $^2\text{H}$, $^{15}\text{N}$ and $^{18}\text{O}$. These are present in natural abundance in all molecules, but this is a very low level. By
feeding precursors or intermediates enriched with these isotopes to an organism, its metabolites may be obtained in labelled form. The location and relative quantity of label incorporated in biosynthesis, or retained during a biological transformation, may be determined using a variety of n.m.r. techniques.

$^{14}$C radioactive labelling studies are still used, but mainly to establish the most efficient feeding regimen for organisms prior to the use of stable isotopes (see also Chapter 3.2.2). The high sensitivity of the method means that $^{14}$C labels may be fed in trace amounts with minimal disruption of normal metabolism, to determine the overall efficiency of incorporation when feeding at different stages of growth. Thus the optimum time to feed may be established.

Development of synthetic methodology for the synthesis of labelled compounds must take account of several considerations apart from being chemically feasible. The position of the label in the molecule is often important, and in any case must be unambiguous. It is also desirable to incorporate the label at a late stage in synthesis, and in a step which is clean and high yielding, to minimise loss of activity. In the use of radioactive isotopes this is especially desirable for reasons of safety.
Scheme 2.1
Fig. 2.1 Major intermediates on the pentaketide (DHN) melanin biosynthetic pathway.
2.2 SYNTHESIS OF INTERMEDIATES

The main metabolites in the melanin biosynthetic pathway in \textit{P. oryzae}, \textit{V. dahliae} and other \textit{fungi imperfecti} are shown in Fig. 2.1. These are: 1,3,6,8-THN (14), scytalone (15), 1,3,8-THN (16), vermelone (17) and 1,8-DHN (18).

Scytalone may be isolated in good yields (200 - 300 mg/l) from cultures of the fungi \textit{P. lagerbergii}\textsuperscript{2} or the \textit{brm-1} mutant of \textit{V. dahliae}\textsuperscript{3}. Chemical dehydration of scytalone with either acid or base yields 1,3,8-THN.\textsuperscript{2} Vermelone may then be obtained by feeding the chemically-prepared 1,3,8-THN to \textit{V. dahliae} \textit{brm-1}.\textsuperscript{4}

The major synthetic task was the preparation of the symmetrical intermediate 1,3,6,8-THN; the presumed first enzyme-free intermediate on the melanin biosynthetic pathway (Chapter 1.6.6). This was first synthesised according to the route described (Scheme 2.1),\textsuperscript{5} modified from a previous synthesis.\textsuperscript{6,7}

Citric acid, a cheap and readily-available starting material, was converted to acetone dicarboxylic acid by addition to fuming nitric acid.\textsuperscript{8} The crude product was esterified using HCl-saturated ethanol to give ethyl acetone dicarboxylate (29).\textsuperscript{9} Treatment of this with sodium metal at 140°C gave the cyclised aromatic product ethyl 2,6-diethoxy-carbonyl-3,5-dihydroxy-phenylacetate (30),\textsuperscript{10} followed by hydrolysis and decarboxylation to 3,5-dihydroxyphenylacetic acid (31). Before proceeding further, this was protected by esterification of the side-chain carboxyl with HCl-saturated ethanol (32), and methylation of the
Scheme 2.2
ring hydroxyls with methyl iodide under basic conditions provided by potassium carbonate.

The resulting ethyl 3,5-dimethoxyphenylacetate (33) was treated with acetic anhydride and boron trifluoride etherate in a Friedel-Crafts acylation step to form ethyl 2-acetyl-3,5-dimethoxyphenylacetate (34). This was set up for cyclisation of the 2-acetyl function onto the ethyl ester, which was achieved with refluxing sodium methoxide in methanol, generated in situ. This gave 1,3-dihydroxy-6,8-dimethoxynaphthalene (35), which was purified by flash column chromatography.

The first part of this route, leading to ethyl 2-acetyl-3,5-dimethoxyphenylacetate (34) was cumbersome and low-yielding. An improved synthesis was devised to produce methyl 3,5-dimethoxyphenyl acetate (41), which was used similarly to the ethyl ester (33) to form the dimethoxynaphthalene (35).

The new method (Scheme 2.2) started from 3,5-dihydroxybenzoic acid. This was converted to the methyl ester (36) with HCl-saturated methanol, and the hydroxyl functions protected by methylation using methyl iodide and potassium carbonate, to give methyl 3,5-dimethoxybenzoate (37). The ester function was reduced using lithium aluminium hydride, giving 3,5-dimethoxybenzyl alcohol (38). Bromination with phosphorous tribromide gave 3,5-dimethoxybenzyl bromide (39), and an extra carbon atom introduced into the side-chain via sodium cyanide, to give 3,5-dimethoxybenzyl cyanide (40).
Hydrolysis of the cyanide with HCl-saturated methanol and water gave the methyl 3,5-dimethoxyphenylacetate (41). Acylation with acetic anhydride and boron trifluoride etherate as before to give methyl 2-acetyl-3,5-dimethoxyphenylacetate (42) followed by sodium methoxide ring closure, gave 1,3-dihydroxy-6,8-dimethoxy-naphthalene (35).

The final step in the preparation was then the removal of the protecting methoxyl functions. This was achieved by refluxing a solution of (35) in glacial acetic acid with hydrobromic acid. The product 1,3,6,8-THN (14) was purified by flash column chromatography.

There were several advantages to the new synthetic route used. Yields in the first 6 steps were all in excess of 75%, resulting in an overall yield of methyl 3,5-dimethoxyphenylacetate (41) of 50% from 3,5-dihydroxybenzoic acid. This compared very favourably with the original route (Scheme 2.1), where the first two steps in particular gave poor yields, resulting in only 45% of ethyl acetone dicarboxylate (29) from citric acid.

The synthetic routes were designed to allow efficient use of labelled isotopes, to enable preparation of labelled substrates for use in future feeding experiments to the fungal cultures. The Friedel-Crafts acylation step (Schemes 2.1 and 2.2) provided an ideal site for introduction of label, as it was both clean and high-yielding.

In addition, in Scheme 2.2, the cyanide reaction was also clean and high-yielding, providing an alternative site for
Scheme 2.3

"pentaketide"

"hexaketide"

(14)

(15)

(43)
introduction of label. The 1,3,6,8-THN could thus be synthesised incorporating $^{13}$C label at C-1, C-2 or C-3.

2.3 X-RAY CRYSTALLOGRAPHIC STUDIES

Previous work on the incorporation of $[^2$H$_3$]acetate into scytalone in *Phialaphora lagerbergii* had shown deuterium incorporation at H-4 equatorial (H-4$\text{eq}$.) and H-5. A series of reverse-exchange experiments showed that of the coincident H-4 axial (H-4$\text{ax}$.) and H-2 equatorial (H-2$\text{eq}$.) positions, only H-4$\text{ax}$ was labelled. No incorporation at H-2 or H-7 was seen.$^{14}$

Although $[2-^{13}$C$]$acetate incorporation studies suggested a pentaketide origin for scytalone,$^8$ and studies with $[1,2-^{13}$C$]$acetate$^9,10$ showed a randomisation of $^{13}$C-$^{13}$C couplings consistent with the involvement of the symmetrical intermediate 1,3,6,8-THN (14) in biosynthesis, this lack of incorporation of deuterium from acetate at H-2 and H-7 was interpreted to suggest the possibility of scytalone being derived from a hexaketide rather than a pentaketide.$^6$

The symmetrical intermediate 1,3,6,8-THN would be formed by loss of the acetyl moiety from 2-acetyl-1,3,6,8-THN (43), which would be formed by the cyclisation of the hexaketide (Scheme 2.3). There is precedent for naphthalene derivatives being formed via hexaketides in several fungi, including the *Scytalidium* species from which scytalone was first isolated and identified. Results from $[1,2-^{13}$C$]$acetate labelling studies have recently shown a number of substituted naphthoquinones to be of hexaketide
Scheme 2.5
(46) 

(47) 
   a R = H 
   b R = Me 
   c R = Et 

(48) 
   a R = H 
   b R = OAc 

(49) → (50)
Scheme 2.4
origin (Scheme 2.4, c.f. Scheme 2.3). The fungus Aspergillus parvulus produces 6-ethyl-7-methoxyjuglone (44), O-methyl-asparvenone (45), asparvenone (46), parvulenone (47a), 1'-O-methylparvulenone (47b) and 1'-O-ethylparvulenone (47c). The species Hendersonula toruloidea (also known as Scytalidium hyalinum) produces 2,7-dimethoxy-6-ethyljuglone (48a) and 2,7-dimethoxy-5-hydroxy-6(1-acetoxyethyl)-1,4-naphthoquinone (48b).

In addition, labelling studies in the plant Rumex alpinus showed that the metabolite 1,8-dihydroxy-3-methylnaphthalene (50) was formed by the loss of the acetyl moiety from nepodin (2-acetyl-1,8-dihydroxy-3-methylnaphthalene, 49), which was of hexaketide origin.

Studies on the incorporation of [2-\(^{13}\)C]malonate into scytalone failed to reveal a "starter effect" which would have resolved the matter.

The proposed hexaketide intermediate 2-acetyl-1,3,6,8-tetrahydroxynaphthalene (43) was synthesised for use in incorporation studies (Scheme 2.5). 1,3-dihydroxy-6,8-dimethoxynaphthalene (35) was treated with ethereal diazomethane at 0°C to afford 1-hydroxy-3,6,8-trimethoxynaphthalene (51). Acylation with acetic anhydride in pyridine gave the O-acylated 1-acetoxy-3,6,8-trimethoxynaphthalene (52). A photo-Fries rearrangement of this, using a 400W medium pressure mercury lamp, gave the C-acylated 2-acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (53). 2-Acetyl-1,3,6,8-tetrahydroxynaphthalene (43) would be obtained from this
Scheme 2.6

(61)
by removal of the protecting methoxyl functions using hydrobromic and glacial acetic acids.

During the synthesis, doubt arose as to the disposition of the photo-Fries rearrangement, as it seemed that the product could be 4-acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (54) and not the desired 2-acetyl isomer (53).

The photo-Fries rearrangement of naphthyl esters of the type (55) has been studied for use in the synthesis of the natural product daunomycinone (56) and analogues. 7 Irradiation of 1-naphthyl acetate (57) with uv light gave 41% of 2-acetyl-1-hydroxynaphthalene (58), 27% 4-acetyl-1-hydroxynaphthalene (59) and 18% 1-hydroxynaphthalene (60).22

The mechanism of this reaction appeared to involve homolytic cleavage of the acyl oxygen to give a pair of radicals within a solvent cage. 23,24 The radicals could recombine to form either starting material or rearranged products. Diffusion of radicals from the cage allowed abstraction of hydrogen from the solvent by the phenol radical and thus the formation of phenolic products (Scheme 2.6).

In naphthyl esters of this type, the 4-position was more reactive than the 2-position, on the basis of Molecular Orbital calculations, and the higher yield of rearranged ortho-product was explained by the proximity of the positions favouring recombination here.22

It was found also that the presence of a substituent in the 5-position on the naphthyl skeleton promoted rearrangement to the
2-position by increasing the steric hindrance at the 4-position. Where \( R_1 = \text{COCH}_3 \) and \( R_2 = \text{OCH}_3 \), no para-isomer was isolated at all. Yield of the ortho-product varied considerably with solvent. This was dependent on the ability of the solvent to donate hydrogen atoms. Ethyl acetate, t-butyl alcohol and methanol were thus good reaction solvents, being poor hydrogen donors. Polarity and viscosity of the solvent did not appear to be important.

In the photo-Fries rearrangement of 1-acetoxy-3,6,8-tri-methoxynaphthalene (52), methanol was chosen as the reaction solvent, and the product was expected to be exclusively 2-acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (53), on the basis of the relative ease of recombination at the 2-position.

If a 4-acetyl isomer was produced, a reason could be that methylation of the dimethoxynaphthalene (35) had occurred at the 1-hydroxyl, not the 3-hydroxyl position. If so, then 0-acylation would occur on the unprotected 3-hydroxyl, and rearrangement would result in 3-hydroxy-4-acetyl-1,6,8-trimethoxynaphthalene (61). If this were the case, then an alternative route to the desired isomer would have to be designed.

The \(^1\text{H n.m.r.} \) spectra of these compounds did not resolve the positions of methylation, acetylation and rearrangement. The spectrum of the trimethoxynaphthalene showed three singlets due to methoxyl protons at 3.84, 3.86 and 3.92; four meta-coupled doublets at 6.29, 6.4, 6.57 and 6.61 due to the aromatic protons, and a singlet due to the hydroxyl proton at 9.14.
The O-acetylated trimethoxynaphthalene showed again, four doublets due to aromatic protons, three singlets due to the methoxyl protons, and at δ2.31, a signal due to the acetoxy proton.

The spectrum of the rearranged product clearly showed a hydroxyl signal at δ15.5. The three methoxyls appeared as in the other compounds. The aromatic region now showed two doublets and one singlet, due to the uncoupled proton, and the acetyl group now on the naphthalene ring appeared at δ2.9.

The appearance of the hydroxyls in both the trimethoxy-naphthalene and the rearranged acetyl-hydroxy-trimethoxy-naphthalene at downfield positions suggested that these were hydrogen-bonded and therefore attached to C-1. However, hydrogen bonding would also be observed if the rearrangement product was 4-acetyl-3-hydroxy-1,6,8-trimethoxynaphthalene (61).

Mass spectroscopy would obviously be unable to distinguish between these two. The ideal method was seen to be x-ray crystallography. Determination of the absolute structures of both starting material and product of the photo-Fries reaction would prove where rearrangement occurred.

The compound believed to be 1-acetoxy-3,6,8-trimethoxy-naphthalene (52) was prepared as described, and a sample purified by preparative t.l.c., followed by recrystallisation from ether to furnish crystals suitable for x-ray structural determination. The remainder was dissolved in methanol and irradiated as described.
Fig. 2.3 Numbering schemes for crystal structures of 1-acetoxy-3,6,8-trimethoxynaphthalene (52) and 2-acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (53).
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<th>Torsion Angle (°)</th>
<th>Standard Deviation (°)</th>
</tr>
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<td>---------</td>
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<tr>
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### Table 2.2 Angles (degrees) with standard deviations for (52).

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Table 2.1 Bond lengths (Å) with standard deviations for (52).

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C(2C) - C(3C) 1.403(6)
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C(5C) - C(4aC) 1.425(5)
C(5C) - C(7C) 1.394(6)
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C(7C) - C(8C) 1.361(6)
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C(3LC) - C(31C) 1.420(6)
C(1D) - C(2D) 1.347(7)
C(1D) - C(8aD) 1.409(7)
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C(2D) - C(3D) 1.391(6)
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O(3D) - C(31D) 1.425(6)
O(6D) - C(61D) 1.427(6)
O(8D) - C(81D) 1.405(6)
Fig. 2.2 Crystal structure of 1-acetoxy-3,6,8-trimethoxynaphthalene (52) showing the ordered molecules A, B and C in the asymmetric unit.
The product of this reaction was purified by preparative t.l.c. followed by recrystallisation from ether to provide good crystals, also for x-ray structural determination.

Crystals of each compound were mounted on a goniometer head and x-ray diffraction data collected on a four-circle diffractometer. The structures were solved using computer direct methods, refined, and plotted using computer graphics.

Refinement of the structure of the acetylated trimethoxy-naphthalene was difficult as the asymmetric unit was made up of four independent molecules, in one of which the acetyl group was disordered. Molecular modelling to allow fractional occupancy of the sites, enabled refinement to be achieved. The refined structure was plotted, and the structure shown to be the expected 1-acetoxy-3,6,8-trimethoxynaphthalene (52). This crystal structure is shown in Fig.2.2. The disordered molecule D is omitted, as the computer plot only shows a partial structure.

Included are tables of bond lengths (Table 2.1), bond angles (Table 2.2) and selected torsion angles, omitting endocyclic torsions (Table 2.3). The numbering scheme for the molecules is shown in Fig.2.3. The four molecules in the asymmetric unit are designated A, B, C, and D (D being the disordered molecule as mentioned).

The structure of the rearranged product was refined with less difficulty. The asymmetric unit comprises only one molecule, which is ordered. This was plotted, and the structure shown
Table 2.6  Torsion angles (degrees) with standard deviations for (53).

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Table 2.4 Bond lengths (Å) with standard deviations for (53).

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Table 2.5 Angles (degrees) with standard deviations for (53).

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Fig. 2.4 Crystal structure of 2-acetyl-1-hydroxy-3,6,8-trimethoxy-naphthalene (53).
definitively to be the 2-acetyl-1-hydroxy-3,6,8-trimethoxy-
naphthalene (53). The crystal structure is shown in Fig.2.4.
Tables of bond lengths (Table 2.4), bond angles (Table 2.5) and
torsion angles (Table 2.6) are included.

Thus the photo-Fries rearrangement in this case did proceed
as predicted.

The lack of incorporation of deuterium from acetate at H-2
and H-7 was later explained by the observation that the 2- and
7-protons in 1,3,6,8-THN readily exchange with protons in the
surrounding medium. Thus it seemed that 1,3,6,8-THN is indeed
derived from a pentaketide precursor as originally supposed. The
synthesis of these "hexaketide precursors" was therefore not
followed up with incorporation experiments.

2.4 N.M.R. STUDIES

The effectiveness of n.m.r. techniques in biosynthetic
studies depends on the unambiguous assignment of spectra obtained
first for unlabelled metabolites. Once this has been achieved,
the positions of label may be ascertained or confirmed, and their
retention or loss during biological transformations followed.

Current high-field n.m.r. allows good resolution of signals in
the $^1$H and $^{13}$C spectra of compounds. Techniques such as the
nuclear Overhauser effect (n.O.e., usually using difference
spectroscopy), decoupling experiments and correlation experiments
may be used to assign signals unambiguously to certain positions
in the molecule examined.

45
Table 2.10 $^{13}\text{C}$ chemical shifts for vermelone (17) in CDCl$_3$.

<table>
<thead>
<tr>
<th>$\delta_C$ (p.p.m.)</th>
<th>Assignment</th>
</tr>
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<tbody>
<tr>
<td>202.74</td>
<td>1</td>
</tr>
<tr>
<td>162.49</td>
<td>8</td>
</tr>
<tr>
<td>141.08</td>
<td>4a</td>
</tr>
<tr>
<td>116.61</td>
<td>8a</td>
</tr>
<tr>
<td>136.70</td>
<td>6</td>
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<tr>
<td>119.67</td>
<td>5</td>
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<tr>
<td>115.96</td>
<td>7</td>
</tr>
<tr>
<td>66.14</td>
<td>3</td>
</tr>
<tr>
<td>47.02</td>
<td>2</td>
</tr>
<tr>
<td>38.16</td>
<td>4</td>
</tr>
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</table>
Fig. 2.8  90.55 MHz $^{13}$C n.m.r. spectrum of vermelone (17) in CDCl$_3$. 
Table 2.9 \(^1\)H chemical shifts for vermelone (17) in CDCl\(_3\).

<table>
<thead>
<tr>
<th>(\delta_H) (p.p.m.)</th>
<th>Multiplicity</th>
<th>Assignment</th>
<th>J / Hz</th>
</tr>
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<tr>
<td>7.40</td>
<td>t</td>
<td>H-6</td>
<td>7.65</td>
</tr>
<tr>
<td>6.82</td>
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<tr>
<td>6.74</td>
<td>dd</td>
<td>H-7</td>
<td>7.41, 0.91</td>
</tr>
<tr>
<td>4.43</td>
<td>tt</td>
<td>H-3</td>
<td>3.9, 7.8</td>
</tr>
<tr>
<td>3.22</td>
<td>dd</td>
<td>H-4e</td>
<td>16.0, 3.9</td>
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<tr>
<td>3.00</td>
<td>dd</td>
<td>H-4a</td>
<td>16.0, 7.8</td>
</tr>
<tr>
<td>2.97</td>
<td>dd</td>
<td>H-2e</td>
<td>16.0, 3.9</td>
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<tr>
<td>2.77</td>
<td>dd</td>
<td>H-2a</td>
<td>16.0, 7.8</td>
</tr>
</tbody>
</table>
Fig. 2.7 300 MHz $^1$H n.m.r. spectrum of vermelone (17) in CDCl$_3$. 
Table 2.7 $^1$H chemical shifts for scytalone (15) in (CD$_3$)$_2$CO.

<table>
<thead>
<tr>
<th>$\delta_H$ (p.p.m.)</th>
<th>Multiplicity</th>
<th>Assignment</th>
<th>$J$ / Hz</th>
</tr>
</thead>
<tbody>
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<td>9.5</td>
<td>s</td>
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<tr>
<td>6.28</td>
<td>dt</td>
<td>H-5</td>
<td>2.2, 1.1</td>
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<tr>
<td>6.15</td>
<td>dd</td>
<td>H-7</td>
<td>2.2, 0.6</td>
</tr>
<tr>
<td>4.32</td>
<td>tt</td>
<td>H-3</td>
<td>7.8, 3.9</td>
</tr>
<tr>
<td>3.15</td>
<td>dddd</td>
<td>H-4eq.</td>
<td>16.1, 3.9, 1.1, 1.1</td>
</tr>
<tr>
<td>2.88</td>
<td>dddd</td>
<td>H-4ax.</td>
<td>16.1, 7.8, 1.1, 1.0, 0.6</td>
</tr>
<tr>
<td>2.84</td>
<td>ddd</td>
<td>H-2eq.</td>
<td>17.1, 3.9, 1.0</td>
</tr>
<tr>
<td>2.62</td>
<td>ddd</td>
<td>H-2ax.</td>
<td>17.1, 7.8, 1.1</td>
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Table 2.8 $^{13}$C chemical shifts for scytalone (15) in (CD$_3$)$_2$CO.

<table>
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<tr>
<th>$\delta_C$ (p.p.m.)</th>
<th>Assignment</th>
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<td>201.1</td>
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<tr>
<td>166.0</td>
<td>C-8</td>
</tr>
<tr>
<td>165.4</td>
<td>C-6</td>
</tr>
<tr>
<td>145.8</td>
<td>C-4a</td>
</tr>
<tr>
<td>111.5</td>
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<td>108.9</td>
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<td>101.3</td>
<td>C-7</td>
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<tr>
<td>66.4</td>
<td>C-3</td>
</tr>
<tr>
<td>47.1</td>
<td>C-2</td>
</tr>
<tr>
<td>38.9</td>
<td>C-4</td>
</tr>
</tbody>
</table>
Fig. 2.6 75 MHz $^{13}$C n.m.r. spectrum of scytalone (15) enriched with [1-$^{13}$C]acetate, in (CD$_3$)$_2$CO.
Fig. 2.5 269MHz $^1$H n.m.r. spectrum of scytalone (15) in (CD$_3$)$_2$CO.
Of the major intermediates in the melanin biosynthetic pathway (Scheme 2.1), the $^1$H $^5$ and $^{13}$C $^{25,26}$ spectra of scytalone (15) had been rigorously assigned previously (Fig. 2.5 and 2.6, Tables 2.7 and 2.8. \textit{NB} Fig. 2.6 shows the carbon-13 n.m.r. spectrum of scytalone enriched with [1-\textsuperscript{13}C]acetate).

2.4.1 Vermelone (17):-

Vermelone (17) was produced by feeding 1,3,8-THN to cultures of \textit{V. dahliae} \textit{brm-1} (Ch. 3.2.3).\textsuperscript{17} The $^1$H n.m.r. spectrum (Fig. 2.7) was very similar to that of scytalone, and was assigned with reference to it.\textsuperscript{1}

The hydrogen-bonded phenolic group appeared at δ12.25. The aromatic protons H-5 and H-7 were shifted downfield from their positions in scytalone, due to the absence of the shielding effect of the 6-hydroxyl group. They appeared as two doublets at δ6.82 and 6.74. H-6 was seen as a triplet at δ7.40. The methine proton H-3 was seen as a septet at δ4.43, and the four methylene protons as complex multiplets centred at δ3.10 and 2.86. These results are summarised in Table 2.9.

The $^{13}$C n.m.r. spectrum of vermelone (Fig. 2.8) was also very similar to that of scytalone (Fig. 2.6 and Table 2.8) and was again assigned with reference to it. The signals due to C-1 (δ202.74), C-8 (δ162.49), C-4a (δ141.08), C-3 (δ66.14), C-2 (δ47.02) and C-4 (δ38.16) are within 5 p.p.m. of the positions they appear at in scytalone. The hydroxyl group at C-6 in scytalone has a strong deshielding effect on C-6. In vermelone, C-6 lacks this substituent, and resonates 28.7 p.p.m. upfield of
Table 2.11 $^1$H chemical shifts for 1,3,8-THN in (CD$_3$)$_2$CO.

<table>
<thead>
<tr>
<th>$\delta_H$ (p.p.m.)</th>
<th>Multiplicity</th>
<th>Assignment</th>
<th>J / Hz</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.14</td>
<td>dd</td>
<td>H-6</td>
<td>8.49, 7.21</td>
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<tr>
<td>7.08</td>
<td>dd</td>
<td>H-5</td>
<td>8.49, 1.42</td>
</tr>
<tr>
<td>6.68</td>
<td>d</td>
<td>H-4</td>
<td>2.25</td>
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<td>6.57</td>
<td>dd</td>
<td>H-7</td>
<td>7.21, 1.42</td>
</tr>
<tr>
<td>6.47</td>
<td>d</td>
<td>H-2</td>
<td>2.25</td>
</tr>
</tbody>
</table>
Fig. 2.11 300 MHz $^1$H n.O.e. difference spectra of 1,3,8-TUN (16) arising from (a) Irradiation at $\delta$6.45 (b) Irradiation at $\delta$6.5 (c) Irradiation at $\delta$6.6.
Fig. 2.10 Homonuclear spin-decoupling experiments in the 300 MHz $^1$H n.m.r. spectrum of (16) (a) No decoupling (b) Irradiation at $\delta$6.47 (c) Irradiation at $\delta$6.57 (d) Irradiation at $\delta$6.68.
Fig.2.9 300 MHz $^1$H n.m.r. spectrum of 1,3,8-THN (16) in (CD$_3$)$_2$CO.
the C-6 resonance in scytalone.

In scytalone, the hydroxyl group at C-6 has a small shielding effect on C-5 and C-7, which is absent in verinelone. C-5 and C-7 in verinelone give rise to signals at δ119.67 and 115.96; 10 - 15 p.p.m. downfield of the resonances due to the same carbon positions in scytalone.

These results are summarised in Table 2.10.

2.4.2 1,3,8-Trihydroxynaphthalene (16, 1,3,8-TEN):-

The proton spectrum of 1,3,8-THN (16) was assigned using the techniques mentioned above (Fig.2.9). A preliminary assignment of the signals was made, based on expectation of the shielding effects of the hydroxyl groups on the five aromatic protons. Hydroxyl groups attached to aromatic rings have a strong shielding effect on ortho-substituents, and a slightly less strong shielding effect on para-substituents. H-2, situated ortho to two hydroxyls, was expected to appear at the highest field as a doublet, meta-coupled to H-4 (Fig.2.10a).

Irradiation of the signal at highest field (δ6.47, 1H, d, J 2.27Hz) caused the coupling at δ6.68 to collapse, suggesting that this was due to H-4, meta-coupled to H-2 (J 2.22Hz, Fig.2.10b). Irradiation of the signal at δ6.68 due to H-4 similarly resulted in loss of coupling of the signal due to H-2 (Fig.2.10d).

The doublet of doublets δ6.57 was predicted to be due to H-7, showing a large ortho-coupling (J 7.19Hz) to H-6 and a small meta-coupling (J 1.43Hz) to H-5. Irradiation in this region
Table 2.12 $^{13}$C chemical shifts for 1,3,8-THN in $(CD_3)_2CO$.

<table>
<thead>
<tr>
<th>$\delta_C$ (p.p.m.)</th>
<th>Assignment</th>
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<tbody>
<tr>
<td>157.06</td>
<td>C-1*</td>
</tr>
<tr>
<td>156.43</td>
<td>C-3*</td>
</tr>
<tr>
<td>154.94</td>
<td>C-8</td>
</tr>
<tr>
<td>139.12</td>
<td>C-4a</td>
</tr>
<tr>
<td>127.85</td>
<td>C-6</td>
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<td>118.88</td>
<td>C-5</td>
</tr>
<tr>
<td>110.73</td>
<td>C-8a</td>
</tr>
<tr>
<td>106.67</td>
<td>C-7</td>
</tr>
<tr>
<td>102.20</td>
<td>C-4'</td>
</tr>
<tr>
<td>101.73</td>
<td>C-2</td>
</tr>
</tbody>
</table>

* may be interchanged
Fig. 2.13 Two-dimensional $^1H,^{13}C$ correlation spectrum for 1,3,8-THN (16) in (CD$_3$)$_2$CO.
Fig. 2.12 75 MHz $^1$H n.m.r. spectrum of 1,3,8-THN (16) in (CD$_3$)$_2$CO.
caused the two doublets of doublets at δ7.08 and 7.14 to collapse into doublets, i.e. removing the couplings of H-7 to H-5 and H-6 (Fig.2.10c).

n.O.e.s are used to show spatial proximity of two protons in a molecule. Irradiation of the sample at the resonance frequency of one proton causes an enhancement (which may be positive or negative) of the signal due to a proton close to it in space. Such effects are usually observed by plotting n.O.e. difference spectra such as those shown here.

Irradiation of the signal at δ6.45 due to H-2 was not expected to show an n.O.e. enhancement, as it is not close to any other protons (Fig.2.11a).

Irradiation of the doublet at δ6.5 due to H-7 was expected to result in an n.O.e. on the signal due to H-6. When this experiment was run, an n.O.e. enhancement was observed on the doublet of doublets at δ7.14; hence this signal was due to H-6 (Fig.2.11b).

H-4 and H-5 are also close in space. Irradiation at δ6.6 (due to H-4) confirmed the signal at δ7.05 to be due to H-5, as an n.O.e. enhancement was observed here (Fig.2.11c).

These results are summarised in Table 2.11.

The 13C n.m.r. spectrum of 1,3,8-THN (Fig.2.12) was assigned with the help of a two-dimensional (2D) 1H,13C correlation experiment (Fig.2.13). In the 2D contour plot, contours show where protons are directly bonded to carbon atoms. If the protons have been assigned, the corresponding carbon signals may thus be
Table 2.13 $^1\text{H}$ chemical shifts for 1,3-dihydroxy-6,8-dimethoxynaphthalene (35) in CDCl$_3$.

<table>
<thead>
<tr>
<th>$\delta$$_H$ (p.p.m.)</th>
<th>Multiplicity</th>
<th>Assignment</th>
<th>J / Hz</th>
</tr>
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<tbody>
<tr>
<td>9.22</td>
<td>s</td>
<td>C-1 OH</td>
<td></td>
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<tr>
<td>6.56</td>
<td>d</td>
<td>H-4</td>
<td>2.32</td>
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<tr>
<td>6.50</td>
<td>d</td>
<td>H-5</td>
<td>2.18</td>
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<tr>
<td>6.49 (-7.0)</td>
<td>s</td>
<td>C-8 OH</td>
<td></td>
</tr>
<tr>
<td>6.38</td>
<td>d</td>
<td>H-2</td>
<td>2.31</td>
</tr>
<tr>
<td>6.25</td>
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<td>3.95</td>
<td>s</td>
<td>C-8 OCH$_3$</td>
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</tr>
<tr>
<td>3.82</td>
<td>s</td>
<td>C-6 OCH$_3$</td>
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</table>
Fig. 2.16
Fig. 2.15 300 MHz $^1$H n.O.e. difference spectra of (35) arising from (a) Irradiation at 83.95 (b) Irradiation at 83.82.
Fig. 2.14 300 MHz $^1$H n.m.r. spectrum of 1,3-dihydroxy-6,8-dimethoxynaphthalene (35) in CDCl$_3$. 
assigned. Using the assignment of the proton signals as described, the signals due to carbon atoms with bonded hydrogens were assigned.

The signals at δ139.12 and 110.73 were typical signals due to quaternary carbons, and were assigned to C-4a and C-8a. C-8a is subject to the shielding effect of two ortho-hydroxyl groups, and appeared at higher field.

The remaining three carbon signals are due to the hydroxyl-bearing carbons, and were not further distinguished.

These results are summarised in Table 2.12.

2.4.3 1,3-Dihydroxy-6,8-dimethoxynaphthalene (35):

The $^1$H and $^{13}$C n.m.r. spectra of 1,3-dihydroxy-6,8-dimethoxynaphthalene (35) were also assigned. In the proton spectrum (Fig.2.14) the aromatic protons expected to appear at highest field were H-2 and H-7. Both are situated ortho to two electron-donating substituents and are strongly shielded.

The two methoxyl signals appear at δ3.95 and 3.82, and were expected to be due to C-8 methoxyl and C-6 methoxyl respectively. Irradiation of the signal at δ3.95 should produce an n.O.e. enhancement of the signal due to H-7. This experiment was performed (Fig.2.15a), and an enhancement of the signal at δ6.25 seen, which confirmed the assignment of both methoxyls and H-7.

Irradiation at δ3.82, due to the C-6 methoxyl, should result in an n.O.e. enhancement of the signal due to H-5. When this experiment was run, the proton signal at δ6.50 showed a large enhancement (Fig.2.15b). A small enhancement of the H-7 signal
Table 2.14 $^{13}$C chemical shifts for 1,3-dihydroxy-6,8-dimethoxynaphthalene (35) in CDCl$_3$.

<table>
<thead>
<tr>
<th>$\delta_C$ (p.p.m.)$^a$</th>
<th>$\delta_C$ (p.p.m)$^b$</th>
<th>Assignment</th>
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<td>156.6</td>
<td>C-8 COCH$_3$</td>
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<tr>
<td>156.11</td>
<td>154.4</td>
<td>C-3 COH</td>
</tr>
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<td>155.66</td>
<td>151.8</td>
<td>C-1 COH</td>
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<td>138.3</td>
<td>C-4$^a$</td>
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<td>105.70</td>
<td>109.3</td>
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<td>95.10</td>
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<td>C-7</td>
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<td>56.03</td>
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<tr>
<td>55.86</td>
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<td>6-OCH$_3$</td>
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</tbody>
</table>

$^a$ observed value
$^b$ calculated value

$^c$ cou
Fig. 2.18 Two-dimensional $^1\text{H}, ^{13}\text{C}$ correlation spectrum for (35).
Fig. 2.17 75 MHz $^{13}$C n.m.r. spectrum of (35) in CDCl$_3$. 
was also seen. This presumably occurs because the molecule exists as an equilibrium between several conformations, such as those shown in Fig.2.16. The methoxy groups are constrained to be coplanar with the π-system, to allow maximum overlap of the π-system with the oxygen lone-pairs. The preferred conformation has the methoxy groups arrayed anti-clockwise around the naphthalene backbone (Fig.2.16 A). In this conformation, the C-6 methoxy is close in space to H-5. In a small proportion of molecules the substituent groups are arranged otherwise, with the C-6 methoxy close to H-7 (e.g. Fig.2.16 B and C).

Using the same criteria of shielding effects, the signals at δ6.38 and 6.56 were assigned to H-2 and H-4 respectively. H-2 like H-7 is ortho to two electron-donating substituents, and is hence strongly shielded and the signal is expected to appear at higher field than that due to H-4. The hydroxyl attached to C-1 appeared at δ6.49, and the C-3 hydroxyl appeared as a broad singlet between δ6.50 and 7.00. These results are summarised in Table 2.13.

The 13C n.m.r. spectrum of 1,3-dihydroxy-6,8-dimethoxy-naphthalene (Fig.2.17) was assigned with the help of 2D 1H,13C correlation experiments (Fig.2.18). This enabled assignment of the proton-bearing carbons C-2, C-4, C-5 and C-7 to the signals at δ99.57, 101.09, 98.3 and 95.10 respectively, by correlation with the corresponding proton chemical shifts. The signals due to the methoxy carbons attached to C-6 and C-8 were also assigned
from this spectrum. The signal due to the methoxyl carbon attached to C-6 appears at δ 55.86, and that due to the methoxyl attached to C-8 at δ 56.03.

The expected $^{13}$C chemical shifts for this compound were calculated from previous work on $^{13}$C chemical shifts in substituted naphthalenes. The calculated values are shown in Table 2.14, with the observed chemical shifts. The predicted values enabled assignment of the four signals at lowest field, due to the hydroxyl- and methoxyl-bearing carbons. The signals at δ 158.06 and 157.17 were expected to be due to the methoxyl-bearing carbons C-6 and C-8, as they showed greater splitting and broader peaks. The calculated values confirmed this, and assigned the signal at δ 158.06 as due to C-6, and that at δ 157.17 as due to C-8. The signals at δ 156.11 and 155.66 were assigned to C-3 and C-1 respectively. Predicted values appeared to agree well with those observed.

The signal due to C-4a was expected to appear at lower field than that due to C-8a, as it is subject to the shielding effects of two ortho-substituents, and two para-substituents. C-8a is meta to these groups, and hence does not experience such large effects.

The predicted $^{13}$C chemical shifts confirmed the assignments also of the signals due to C-5 and C-7. All the predicted values agreed well with the observed $^{13}$C chemical shifts. The values for C-1, C-8a and C-2 were calculated on the basis of a methoxyl substituent at C-1 instead of the hydroxyl, as the available data
on substituent effects was incomplete.

These results are summarised in Table 2.14 as mentioned.

2.5 GENERAL EXPERIMENTAL PROCEDURES

Melting points were determined on a Reichert hot-stage microscope and are uncorrected. Microanalyses were performed on a Perkin-Elmer 204 Elemental Analyser. Optical rotations were measured on a Perkin-Elmer 141 Polarimeter. Mass spectra were obtained on a single-focussing Micromass 16B machine (V.G. Micromass Ltd.).

Routine proton n.m.r. spectra were obtained on Varian EM390 90 MHz and Jeol JNM PMX60 60 MHz continuous-wave machines. High-field proton and carbon-13 spectra were run on Bruker WP200SY, AM300Y and WH360 Fourier-transform machines. Deuterium spectra were run on the Bruker WH360 Fourier-transform machine. In all cases, quoted chemical shifts are relative to tetramethylsilane $\delta_H$ and $\delta_C = 0.0$ p.p.m.

Radioactivity was measured on a Beckmann LS7000 liquid scintillation counter. The scintillant was butyl-PBD (10 g/l) in methanol-toluene (50:50). Samples for radiocounting were purified for constant activity by recrystallisation.

Unless otherwise specified, thin layer chromatography (t.l.c.) was carried out using either analytical (5 x 20cm) or preparative (20 x 20cm) glass plates coated with a 0.5 - 0.7mm layer of silica gel (Merck Art. 7730 Kieselgel G$_{F254}^+$). Chromatograms were visualised under ultra-violet light at 254nm.
Flash chromatography was performed using glass columns varying between 10 - 35mm diameter as appropriate,\textsuperscript{12} an air pump generating 5 - 10 p.s.i., and 6" unpacked column length of Sorbasil C60 silica gel (Crosfield Chemicals).

Solvents were purified using standard procedures. Culture media solutions and equipment were sterilised by autoclaving for 15 minutes at 15 p.s.i.

2.6 \textbf{EXPERIMENTAL}

2.6.1 Synthesis of intermediates:-

\textbf{1,3,8-Trihydroxynaphthalene (16)}\textsuperscript{2}

Scytalone (15, 232mg) was dissolved under nitrogen in trifluoroacetic acid (10ml) and heated to 60°C under reflux and nitrogen for 2 hours. The solvent was evaporated over a warm water bath. Purification by preparative t.l.c. (acetone-chloroform, 15:85) gave 1,3,8-trihydroxynaphthalene (1,3,8-THN, 16, 87%) as a pale green-brown solid m.p. 192-194°C (Lit. m.p. 197-199°C\textsuperscript{8}). For both n.m.r. studies and culture work, a second purification by preparative t.l.c. was similarly performed.

\textbf{Ethyl 2,6-diethoxycarbonyl-3,5-dihydroxyphenylacetate (30)}\textsuperscript{10}

Fine-cut pieces of sodium (0.1g) were dissolved in ethyl acetone dicarboxylate (29, 10ml) and heated on an oil bath at 140°C for 2 hours. The hot orange solution was poured into a conical flask where it crystallised to a deep orange solid. This was purified by suspension in 50% aqueous ethanol (20ml), filtration and further washing with 50% aqueous ethanol (2 x
20ml) to give pale yellow crystalline ethyl 2,6-diethoxy-carbonyl-3,5-dihydroxyphenylacetate (30) m.p. 94-96°C (Lit. m.p. 98°C, Aldrich).

3,5-Dihydroxyphenylacetic acid (31)

The product (30, 4.4g) was refluxed for 2 hours with 28% sodium hydroxide solution (40ml), cooled and acidified (6M H₂SO₄). Water was removed by evaporation until sodium sulphate precipitated and ethyl acetate (50ml) was added. The solution was dried over MgSO₄ and evaporated to give 3,5-dihydroxyphenylacetic acid (31) as an orange-brown oil. This was seen by t.l.c. to be one major product, Rf 0.15 (ethyl acetate-light petroleum 40-60°C-formic acid, 40:60:2) and was used without further purification.

Ethyl 3,5-dihydroxyphenylacetic acid (32)

The acid (31, 2.5g) was dissolved in HCl-saturated ethanol (150ml) and stirred for 24 hours. The solvent was removed and ethyl acetate (30ml) added. This was washed with water (2 x 10ml), dried over MgSO₄ and evaporated to give ethyl 3,5-dihydroxyphenylacetic acid (32) as a yellow oil. δH 1.3 (3H, t, CH₂CH₃); 2.1 (2H, s, OH); 3.5 (2H, s, CH₂CO₂Et); 4.2 (2H, q, CH₂CH₃); 6.2-6.5 (3H, m, Ar-H).

ethyl 3,5-dimethoxyphenylacetic acid (33)

Ethyl 3,5-dihydroxyphenylacetic acid (32, 2.17g) was dissolved in acetone (100ml) and excess potassium carbonate (3g) and methyl iodide (3.5g) added. This was refluxed 12 hours, allowed to cool to room temperature and filtered. The solvent was
evaporated and the resulting yellow oil dissolved in ethyl acetate (100ml). This was washed with water (2 x 20ml), dried over MgSO₄ and evaporated to give ethyl 3,5-dimethoxyphenylacetetic acid (33) as a yellow oil which was seen by t.l.c to be one major product and used without further purification. δH 0.35 (3H, t, CH₂CH₃); 2.65 (2H, s, CH₂); 2.9 (6H, s, OCH₃); 3.3 (2H, q, CH₂CH₃); 5.6 (3h, s, Ar-H).

**Ethyl 2-acetyl-3,5-dimethoxyphenylacetic acid (34)**

Boron trifluoride etherate (7.14ml) was added to ethyl 3,5-dimethoxyphenylacetic acid (33, 1g) in acetic anhydride (0.86ml) and heated to 60°C under reflux with stirring for 2 hours. The flask was allowed to cool to room temperature and left to stir for a further 12 hours.

Excess solvent was removed by rotary evaporation and ethyl acetate (30ml) added. The solution was washed with water (3 x 20ml), dried over MgSO₄ and evaporated to give ethyl 2-acetyl-3,5-dimethoxyphenylacetic acid (34, 1.18g, 98%) as a yellow oil. δH 1.3 (3H, t, CH₂CH₃); 2.6 (2H, s, COCH₃); 3.75 (6H, s, OCH₃); 3.88 (2H, s, CH₂); 4.2 (2H, q, CH₂CH₃); 6.5 (2H, s, Ar-H). This was used without further purification.

**1,3-Dihydroxy-6,8-dimethoxynaphthalene (35)**

Ethyl 2-acetyl-3,5-dimethoxyphenylacetic acid (34, 1.029g) in dry methanol (10ml) was added dropwise to a refluxing solution of sodium metal (200mg) in dry methanol (5ml) under a nitrogen atmosphere. A gradual colour change from brown to dark purple was observed. Reflux was continued for 2 hours.
The solution was cooled to room temperature, acidified with 2M H₂SO₄ and poured onto water (150ml). This was extracted into ether (4 x 100ml), dried over MgSO₄ and evaporated to give 1,3-dihydroxy-6,8-dimethoxynaphthalene (35, 0.947g, 84%) as an orange-brown crystalline oil which was unstable and kept refrigerated under nitrogen. [m/z 220 (M⁺). C₁₂H₁₂O₄ requires 220]; δH 3.4 (6H, m, OCH₃); 5.6–6.3 (4H, m, Ar-H); 6.7 (1H, s, OH); 9.0 (1H, s, OH).

**Methyl 3,5-dihydroxybenzoate (36)**

3,5-Dihydroxybenzoic acid (50g) was dissolved in HCl-saturated methanol (300ml) for 12 hours. The solvent was evaporated and ethyl acetate (200ml) added. This was washed with water (4 x 50ml), dried over MgSO₄ and evaporated to give methyl 3,5-dihydroxybenzoate (36, 90%) as a brown crystalline solid. M.p. 162–164°C (Lit. m.p. 164–166°C). C₁₂H₁₀O₃ requires 220]; δH 3.85 (6H, s, Ar-OCH₃); 3.88 (3H, s, CO₂CH₃); 6.63 (1H, t, p-Ar-H); 7.12 (2H, d, o-Ar-H).

**Methyl 3,5-dimethoxybenzoate (37)**

Methyl 3,5-dihydroxybenzoate (36, 54g) was dissolved in acetone (600ml) and methyl iodide (3eq., 138.3g) and potassium carbonate (3eq., 134.4g) added. The mixture was stirred mechanically for 24 hours, and then filtered. The filtrate was evaporated, and ethyl acetate (200ml) added to the residue. This was washed with water (2 x 50ml), dried over MgSO₄ and evaporated to give methyl 3,5-dimethoxybenzoate (37, 98%) as a yellow crystalline solid m.p. 40–43°C (Lit. m.p. 42–44°C). C₁₂H₁₀O₃ requires 220]; δH 3.85 (6H, s, Ar-OCH₃); 3.88 (3H, s, CO₂CH₃); 6.63 (1H, t, p-Ar-H); 7.12 (2H, d, o-Ar-H).
3,5-Dimethoxybenzyl alcohol (38)

Methyl 3,5-dimethoxybenzoate (37, 63g) in dry ether (200ml) was added dropwise with stirring to a suspension of lithium aluminium hydride (2/3 eq., 8.23g) in dry ether (300ml) in a flask fitted with a reflux condenser and flushed with nitrogen. Once addition was complete, the reaction was allowed to subside and 10% aqueous methanol (100ml) added cautiously, followed by water (100ml). Ethyl acetate (10ml) was added to help disperse the aluminium complex formed, and the reaction left to stir for 15 minutes.

A white precipitate formed, which was then removed by Büchner filtration, and the filtrate separated. The aqueous layer was extracted into ethyl acetate (2 x 100ml) and these extracts and the ether layer were combined. The solution was then washed with water (2 x 50ml), dried over MgSO₄ and evaporated to give 3,5-dimethoxybenzyl alcohol (38, 83%) as a yellow oil. This was seen by t.l.c. to be one major product, Rf 0.69 (ethyl acetate-light petroleum 60-80°, 50:50) and was used without further purification. δH 3.35 (1H, m, OH); 3.75 (6H, s, Ar-OCH₃); 4.53 (2H, broad s, ArCH₂OH); 6.50 (3H, m, Ar-H).

3,5-Dimethoxybenzyl bromide (39)

Phosphorous tribromide (¼ eq, 44g) was added dropwise with stirring to a solution of 3,5-dimethoxybenzyl alcohol (38, 40g) in dry ether (300ml) in a flask from which light was excluded. The reaction was left stirring for 12 hours.

The solution was poured onto ice-water (200ml), the ether
layer separated and the flask rinsed thoroughly with ethyl acetate as the product is not very soluble in ether. The aqueous layer was extracted into ethyl acetate (2 x 200ml), and the combined organic solutions dried over MgSO₄ and evaporated to give 3,5-dimethoxybenzyl bromide (39) as a yellow crystalline solid. Recrystallisation from ether gave white needles, m.p. 45-48°C (75%). By t.l.c. these showed as one spot, Rf 0.364 (ethyl acetate-light petroleum 60-80°, 50:50).

3,5-Dimethoxybenzyl cyanide (40)

Sodium cyanide (1.5eq., 20.4g) in water (30ml) was added in one go to a suspension of 3,5-dimethoxybenzyl bromide (39, 40g) in methanol (200ml), and the reaction refluxed for 4-5 hours.

The reaction was cooled, the solvent evaporated over a tepid water bath and ethyl acetate (200ml) added. This was washed repeatedly with water to remove cyanide, dried over MgSO₄ and evaporated to give 3,5-dimethoxybenzyl cyanide (40, 97%) as a brown crystalline solid. Recrystallisation from light petroleum 30-40° gave pure pale brown crystals, m.p. 48-53°C [Found: C, 67.6; H, 6.21; N, 7.84%, C₁₀H₁₄O₂N requires C, 67.79; H, 6.21; N, 7.91%]; δH 3.55 (2H, s, CH₂); 3.65 (6H, s, OCH₃); 6.3 (3H, s, Ar-H).

Methyl 3,5-dimethoxyphenyl acetate (41)

Hydrogen chloride gas was bubbled briskly through a solution of 3,5-dimethoxybenzyl cyanide (40, 30g) in methanol (150ml) and water (10ml) for 1½ hours. The reaction mixture was refluxed for 1 hour to remove excess gas, and the solvent removed by evap-
oration. Ethyl acetate (200ml) was added and the solution was washed with water (100ml), dried over MgSO₄ and evaporated to give methyl 3,5-dimethoxyphenyl acetate (41, 95%) as a yellow-orange oil which crystallised slowly on standing. T.l.c. showed one major spot, Rf 0.268 (ethyl acetate-light petroleum 60-80°, 20:80). This was used without further purification.

**Methyl 2-acetyl-3,5-dimethoxyphenyl acetate (42)**

Methyl 3,5-dimethoxyphenyl acetate (41, 1g) was treated with acetic anhydride (0.86ml) and boron trifluoride etherate (7.14ml) as for ethyl 3,5-dimethoxyphenyl acetate (33). It was worked-up identically to give methyl 2-acetyl-3,5-dimethoxyphenyl acetate (42) as a dark brown oil, Rf 0.37 (ethyl acetate-light petroleum 60-80°, 30:70). δH 2.5 (2H, s, CH₂); 3.7 (3H, s, CO₂CH₃); 3.9 (6H, s, OCH₃); 6.4 (2H, m, Ar-H).

**1,3-Dihydroxy-6,8-dimethoxynaphthalene (35)**

Methyl 2-acetyl-3,5-dimethoxyphenylacetic acid (42, 1.01g) was added to a refluxing solution of sodium metal (200mg) in methanol as for ethyl 2-acetyl-3,5-dimethoxyphenylacetic acid (34). It was worked up identically to give 1,3-dihydroxy-6,8-dimethoxynaphthalene (35) which was kept refrigerated under nitrogen.

Analytical t.l.c. showed one major spot, Rf 0.1 (ethyl acetate-benzene, 1.5:98.5) which reddened on standing in air. Purification was performed by flash column chromatography using a 35mm diameter column and the same solvent system for elution. The crude product was applied to the column dissolved in 4ml
eluent. Fractions of 200ml were collected and tested. The product came off as fractions 4, 5, 6 and 7, which were combined and evaporated to give an orange crystalline solid (75%) m.p. 116-121°C (Lit. m.p. 126-127°C). 

Further purification for high-field n.m.r. studies was performed by preparative t.l.c. under nitrogen, using the same eluent.

**1,3,6,8-Tetrahydroxynaphthalene (14)**

1,3-Dihydroxy-6,8-dimethoxynaphthalene (35, 800mg) was dissolved under nitrogen in glacial acetic acid (20ml) and hydrobromic acid (6ml) added. This was heated under reflux and nitrogen for 3 hours, cooled, and the solvent mixture evaporated over a warm water bath. Water (20ml) was added to the residue, and extracted into ethyl acetate (2 x 50ml). The extracts were washed with water (30ml), dried over MgSO₄ and evaporated to leave a brown solid. This showed as one major spot by t.l.c., Rf 0.52 (acetone-n-hexane, 60:40).

Purification was by performed by flash column chromatography using a 35mm diameter column and the same eluent. The crude product was applied to the column dissolved in 2ml eluent, and fractions of 30ml collected and tested. The product was contained in fractions 3, 4, 5, 6 and 7, which were combined and evaporated to give 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN, 14, 81%) as a pale brown solid. This was unstable towards air and hence kept refrigerated under nitrogen. δH 6.25 (2H, d, H-2 and H-7); 6.47 (2H, d, H-4 and H-5).
Hydroxy-3,6,8-trimethoxynaphthalene (51)

1,3-dihydroxy-6,8-dimethoxynaphthalene (35, 376mg) in dry ether (10ml) was cooled to 0°C and treated with ethereal diazomethane (0.32g, 4.5eq.) This was left to stir for 3 hours, after which an equal amount of ethereal diazomethane was added. This was then left to stir for 12 hours, filtered through celite, and evaporated to leave a gummy orange solid. T.l.c. showed two spots, one corresponding to the starting material, Rf 0.188 (ether-light petroleum 40-60°, 40:60). The other, Rf 0.46, was isolated by preparative t.l.c. to give 1-hydroxy-3,6,8-trimethoxynaphthalene (51, 40%) as orange crystals M.P. 88-90°C. [Found C, 66.54; H, 5.8%. \( \text{C}_{13} \text{H}_{14} \text{O}_4 \) requires C, 66.67; H, 5.98%].

\[ \delta_H 3.84 (3H, s, \text{OCH}_3); 3.86 (3H, s, \text{OCH}_3); 3.98 (3H, s, \text{OCH}_3); 6.29, 6.40, 6.57, 6.61 (\text{all 1H, d, Ar-H}); 9.14 (1H, s, \text{OH}). \]

1-Acetoxy-3,6,8-trimethoxynaphthalene (52)

1-Hydroxy-3,6,8-trimethoxynaphthalene (51, 439mg) was dissolved in pyridine (4ml) and acetic anhydride (6ml) added. The reaction was stirred at room temperature for 12 hours, and the solvent mixture evaporated under high vacuum to leave a dark orange oil. Initial purification by preparative t.l.c. (ether-light petroleum 30-40°, 40:60), followed by recrystallisation from ether-light petroleum 30-40° gave 1-acetoxy-3,6,8-trimethoxynaphthalene (52, 64%) as pale yellow crystals, m.p. 108-110°C. \( \delta_H 2.3 (3H, s, \text{COCH}_3); 3.8 (9H, s, \text{OCH}_3); 6.3, 6.6, 6.9 (\text{all 1H, d, Ar-H}). \)
2-Acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (53)  

1-Acetoxy-3,6,8-trimethoxynaphthalene (52, 300mg) was dissolved in nitrogen-purged methanol (100ml) in a test-tube fitted with a condenser. The tube was irradiated for 24 hours using a 400W medium-pressure mercury photochemical reactor with water cooling. The solution was evaporated, and the residue purified by preparative t.l.c. (benzene-ether-formic acid, 95:4:1). After 2 elutions, the major band separated out and was isolated as very pale yellow crystals of 2-acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (53, 28%).

This was recrystallised from ether-light petroleum 30-40° to give near-colourless crystals, m.p. 137-139°C [Found C, 66.8; H, 6.46%; C_{15}H_{16}O_{5} requires C, 65.2; H, 5.7%]; δ_H 2.90 (3H, s, COCH_3); 3.87 (6H, s, OCH_3); 3.97 (3H, s, OCH_3); 6.29, 6.48 (both 1H, d, Ar-H); 6.97 (1H, s, Ar-H); 15.5 (1H, s, exchangeable OH).

2.6.2 X-ray studies:–

1-Acetoxy-3,6,8-trimethoxynaphthalene (52)

Crystal data:–

C_{15}H_{16}O_{5}, M = 276.29, monoclinic, space group P2_1/c (No. 14), a = 15.156(4), b = 18.604(7), c = 20.132(7)Å, β = 92.02(3)°, V = 5763Å³ [from 2θ values of 13 centred reflections with 2θ = 36-40°, λ = 1.54184Å], Z = 16, D_{calc} = 1.294 g cm⁻³, T = 298K, colourless lath, 0.96 x 0.54 x 0.19 mm, μ = 0.77 mm⁻¹, F(000) = 2336.

Data Collection and Processing:–

Stoë STADI-4 four-circle diffractometer, graphite-monochromated Cu-Kα X-radiation, T = 298K, ω-2θ scans using the
learnt-profile method, 7958 reflections measured (2θ\text{max} 120°, 
h -16 → 16, k 0 → 20, l 0 → 22), 7172 unique (R_{int} = 0.046), 
semi-empirical absorption correction applied, giving 5761 with 
F ≥ 6σ(F) for use in all calculations. No significant crystal 
decay or movement was observed.

Structure Solution and Refinement:–

Automatic direct methods located all ordered non-H atoms 
which were then refined (by least-squares on $F^{2}$) with 
anisotropic thermal parameters and with H-atoms included at 
fixed, calculated positions. The asymmetric unit comprises four 
independent molecules, one of which contains a disordered acetyl 
group which required modelling: this was achieved by allowing 
fractional occupation of alternative sites. At final convergence 
$R, R_w = 0.709, 0.0985$ respectively, $S = 1.237$ for 715 parameters 
refined in two blocks; the final ΔF synthesis showed no peak 
above 0.57 eÅ⁻³; the weighting scheme $w^{-1} = σ^2(F) + 0.000183F^2$ 
gave satisfactory agreement analyses and in the final cycle 
($Δ/σ)_{\text{max}}$ was 0.28.

Atomic scattering factors were inlaid, molecular geometry 
calculations utilised CALC and the figure was produced by 
ORTEPII.

2-Acetyl-1-hydroxy-3,6,8-trimethoxynaphthalene (53)

Crystal data:–

C_{15}H_{16}O_{5}, \ M = 276.29, monoclinic, space group P2₁/c, 
a = 7.2659(4), b = 11.3101(7), c = 15.7807(10)Å, β = 93.564(3)°, 
V = 1294.32Å³ [from 2θ values of 40 reflections measured at
\[ \pm \omega(2\theta = 30 - 35^\circ, \lambda = 1.54184\text{Å}), Z = 4, D_{\text{calc}} = 1.418\text{g cm}^{-3}, \]

\[ T = 298\text{K}, \] pale yellow tablet, \( 0.23 \times 0.23 \times 0.077\text{mm}, \mu = 0.85\text{mm}^{-1}, F(000) = 584. \]

**Data Collection and Processing:**

Stoë STADI-4 four-circle diffractometer, graphite-monochromated Cu-K\( \alpha \) X-radiation, \( T = 298\text{K}, \) \( \omega-2\theta \) scans with \( \omega \) scan width \( (1.05 + 0.347\tan\theta)^\circ, \) 1916 unique data \( (2\theta_{\text{max}} = 120^\circ, h = 8 \rightarrow 7, k 0 \rightarrow 12, l 0 \rightarrow 17) \) of which 1078 with \( F > 6\sigma(F) \) were used in all calculations. No significant crystal decay or movement was observed.

**Structure Solution and Refinement:**

Solution was by direct methods. 32 Non-H atoms were then refined (by least squares on \( F^2 \)) with anisotropic thermal parameters, with H atoms included at fixed, calculated positions. At final convergence, \( R, R_{\text{w}} = 0.0440, 0.0700 \) respectively, \( S = 0.828 \) for 187 parameters; the final \( \Delta F \) synthesis showed no peaks above 0.50 eÅ\(^{-3}\); the weighting scheme \( w^{-1} = \sigma^2(F) + 0.00656F^2 \) and in the final cycle \( (\Delta/\sigma)_{\text{max}} \) was 0.15.

Atomic scattering factors were inlaid, 33 molecular geometry calculations utilised CALC \(^{34}\) and the figure was produced by ORTEPII.\(^{35}\)
2.7 REFERENCES

1. For general texts on n.m.r. spectroscopy:–


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33. SHELX76, program for crystal structure refinement, G.M.Sheldrick, University of Cambridge, England, 1976.
CHAPTER 3
Fig. 3.2 Incorporation of $[1^{-13}\text{C}]$acetate (•) into scytalone.
Fig. 3.1 Biosynthesis of scythalone (15) and flavilin (19) via a pentaketide precursor.
3.1 INTRODUCTION

3.1.1 Previous studies on scytalone and vermelone

Scytalone (15, Fig. 1.5) was first isolated from a Scyalidium species\textsuperscript{1} and from its oxygenation pattern anticipated to be closely related to flaviolin (19).\textsuperscript{2} Both could be derived from a pentaketide precursor (Fig. 3.1, c.f. Fig. 1.6 and Scheme 2.3). Scytalone was also isolated from Phialaphora lagerbergii,\textsuperscript{3} and incorporation of $[\text{1-}^{13}\text{C}]$acetate confirmed the pentaketide origin. In the $^{13}\text{C}$ n.m.r. spectrum, signals due to C-1, C-3, C-4a, C-6 and C-8 are enhanced to twice natural intensity (Fig. 3.2).

The use of genetic mutants of Verticilliium daedliae (Table 1.6) enabled intermediates to be isolated, and cross-feeding experiments confirmed that "normal" fungal melanin was made from these.\textsuperscript{4} The brown mutant brm-1 produced a metabolite which restored pigmentation in the albino mutant alm-1. This was isolated and shown to be scytalone. Unlike scytalone from Scyalidium and Phialaphora, this was reported to have an optical rotation $[\alpha]_D^{25} = +32^\circ$, and was designated (+)-scytalone.\textsuperscript{5} Scytalone from Scyalidium (m.p. 160-168\textdegree C\textsuperscript{1}) and P. lagerbergii (m.p. 172-174\textdegree C\textsuperscript{3}) was reported to have $[\alpha]_D^{25} = 0$, but on the basis of a weak negative Cotton effect at 220 nm was believed to be optically active.\textsuperscript{2} This could be due to there being a very small enantiomeric excess of the (-)-isomer in scytalone from this source.

A biomimetic synthesis of scytalone from methyl curvulinate
was later described. The racemic synthetic material had identical $^1$H and $^{13}$C n.m.r. spectra to the natural metabolite. On comparison with the material from *Scytaledium* and *P. lagerbergii*, noticeable differences were observed in the i.r. spectra (KBr) and the melting points. These differences were seen to be consistent with those which could be expected from a racemate and one of its enantiomers. Neither the differences or the absolute value for the melting point of the synthetic scytalone were quoted in the literature.

The *V. dahiae* brown mutant *brm-2*, when fed (+)-scytalone enzymatically dehydrated this to 1,3,8-THN (16). When this was fed to the *brm-1* mutant, no melanin was produced, but a new metabolite vermelone (17) was isolated. This was found to have an optical rotation $[\alpha]_{D}^{25} = -18^\circ$, and was designated (-)-vermelone. The (-)-vermelone was converted to "normal" melanin by *brm-2* or *alm* mutants, thus establishing it as a melanin intermediate. Dehydration of vermelone under alkaline conditions gave 1,8-DHN (18), which is the final monomeric intermediate in melanin biosynthesis. It appears that the same enzyme catalyses both the reduction of 1,3,6,8-THN (14) and 1,3,8-THN, and similarly one enzyme catalyses the dehydration of both scytalone and vermelone.

The *brm-1* mutant accumulated flaviolin (19) and cis-4-hydroxyscytalone (20) as shunt products of the pathway, presumably formed by oxidation of 1,3,6,8-THN. This had been proposed as an intermediate of flaviolin biosynthesis previously.
Fig. 3.3 Incorporation of [1,2-\textsuperscript{13}C]acetate (\textsuperscript{13}C\textsuperscript{*}) into scytalone in \textit{P. lagerbergii}.
and the biomimetic synthesis of scytalone from methyl curvulinate had involved chemical reduction of 1,3,6,8-THN. Experiments feeding [1-\textsuperscript{13}C\textsubscript{1}]acetate and [1,2-\textsuperscript{13}C\textsubscript{2}]acetate provided strong evidence for the involvement of the symmetrical 1,3,6,8-THN in the natural pathway to melanin. In the 13C n.m.r. spectrum each signal showed two kinds of 13C-13C couplings, indicating two different arrangements of acetate units (Fig.3.3). This was explained by the equal probability of reduction occurring in either aromatic ring in 1,3,6,8-THN. Further work confirmed this, and that 1,3,6,8-THN was an intermediate in the biosynthesis of scytalone and very probably flaviolin also. Previously it had been thought that flaviolin was formed from scytalone.

The reductase and dehydratase enzymes of DHN melanin biosynthesis are unique to this pathway. Tricyclazole and other antifungal agents displaying similar disease-control characteristics were shown to specifically block the reductase, mimicking the brm-2 and brm-4 mutations in V. dahliae (Chapter 1). Thus the demonstration of inhibition of melanin biosynthesis by tricyclazole is evidence for the operation of the DHN melanin biosynthetic pathway in a species of fungus, and can be used as a criterion for it (see Table 1.5).

At 1.0 p.p.m. of tricyclazole the reduction of 1,3,8-THN to vermelone was blocked, and at higher concentrations that of 1,3,6,8-THN to scytalone also. This seemed to indicate that although the same reductase was operating in both cases, it had a higher binding affinity for 1,3,6,8-THN than for 1,3,8-THN. Other
studies confirmed these results.\textsuperscript{13,14,15}

The synthesis of scytalone from a pentaketide intermediate via 1,3,6,8-THN was demonstrated using [1-\textsuperscript{13}C]acetate, [2-\textsuperscript{13}C]acetate, [\textsuperscript{2}H\textsubscript{3},2-\textsuperscript{13}C]acetate and [1,2-\textsuperscript{13}C]acetate.\textsuperscript{10,11} The observation that deuterium was incorporated at both C-4 positions and C-5, but not at C-2 or C-7, led to the suggestion of a hexaketide rather than a pentaketide precursor in biosynthesis (see Chapter 2.3).\textsuperscript{16} This was later discounted when it was discovered that the 2- and 7-protons of 1,3,6,8-THN readily exchange with protons in the surrounding medium.

The biosynthetic pathway to melanin in \textit{fungi imperfecti} was thus well established, but there remained important questions about the stereochemistry of the reduction and elimination processes involved. The question of the optical activity and/or optical purity of scytalone and vermelone produced by the different fungal species had not been fully resolved, and literature reports conflicted.

It was thus proposed to study the biosynthesis of scytalone and vermelone in \textit{V.dahliae} and \textit{P.lagerbergii} in more detail, using recent advances in n.m.r. techniques developed for use in biosynthetic studies.

\textbf{3.1.2 N.m.r. techniques in biosynthesis}

With the advent of sensitive Fourier-transform n.m.r. techniques it became feasible to use \textsuperscript{13}C n.m.r. spectroscopy as a routine method for biosynthetic studies.\textsuperscript{17} Classical biosynthetic studies using radioisotope methods and degradation of the
labelled metabolites, were becoming increasingly complex as the natural products under investigation became more complex, especially as the quantities of these available were often limited.

Not only did $^{13}\text{C}$ n.m.r. facilitate the determination of labelling patterns, it also enabled the mode of incorporation of intact precursor units to be followed. For example, if doubly labelled [$1,2-^{13}\text{C}]$acetate was fed as a precursor, the observation of $^{13}\text{C}$-$^{13}\text{C}$ couplings indicated the incorporation of intact acetate units, whereas a loss of coupling indicated that bond cleavage or rearrangement processes had occurred during biosynthesis.

The availability of precursors enriched with a high level of $^{13}\text{C}$ was also an important factor in the development of $^{13}\text{C}$ n.m.r. for use in biosynthesis. The natural abundance of carbon-13 is 1.1%, and to observe enrichment in the $^{13}\text{C}$ n.m.r. spectrum of the natural products, dilution of the label of less than 100 is necessary. Radioactive carbon-14 is now used mainly as a tracer to determine whether incorporation of label from the precursor is sufficient to make $^{13}\text{C}$ labelling studies worthwhile (see below).

The use of precursors doubly-labelled with $^2\text{H}$, $^{18}\text{O}$ or $^{15}\text{N}$ along with $^{13}\text{C}$, enables the biosynthetic origins of hydrogen, oxygen and nitrogen to be established, by the use of isotope-induced shifts in the $^{13}\text{C}$ n.m.r. spectrum (see below) or by direct observation of $^2\text{H}$ or $^{15}\text{N}$.
Fig. 3.4 Simulated p.n.d. $^{13}$C n.m.r. spectra of a polyketide-derived moiety enriched with (a) $[^{2}$H$_3,2^{-13}$C]acetate, (b) $[^{2}$H$_3,1^{-13}$C]acetate, (c) $[1^{-13}$C, $^{18}$O$_2$]acetate.
Deuterium incorporation into metabolites may be detected by changes in the $^1$H n.m.r. With the increased sensitivity of Fourier-transform techniques, deuterium n.m.r. became possible, which meant that deuterium could be directly observed. Deuterium is a quadropole nucleus of spin 1. It has a lower magnetogyrnic ratio than hydrogen, and consequently resonates at lower field. The chemical shift values are very similar to those of hydrogen, although due to the smaller frequency range the spectrum is often poorly resolved. Relaxation of deuterium is governed by the quadropole, and is efficient, so that sample saturation is not a problem. It does give rise to broadening of the signals, but with the lack of n.O.e. enhancements, integration of the spectrum can give accurate information as to the extent of deuterium incorporation.

Due to the very low natural abundance of deuterium (0.016%), isotopic enrichment is easily observed. In contrast with $^{13}$C, dilutions of 6000-fold are tolerable in $^2$H n.m.r. Deuterium n.m.r. is particularly useful in determining the stereo-specificity of deuterium incorporation into metabolites.

Another n.m.r. technique in increasing use for biosynthetic work utilises isotope-induced shifts in the $^{13}$C n.m.r. spectrum as mentioned above. This effect is due to the presence of isotopes of different mass bonded to carbon, which cause an upfield shift in the signal due to that carbon (Fig.3.4). In a sample containing a mixture of labelled and unlabelled molecules, the shifted signal is seen as a low intensity peak upfield of the
main carbon resonance. The magnitude of the effect is greatest when deuterium is present instead of hydrogen, as the relative difference in mass of the isotopes is greatest. Isotopically shifted signals are also observable for other nuclei such as $^{18}\text{O}$, but the magnitude of the effect is smaller.$^{19}$

So-called "α-shifts" arise when the isotopic substitution occurs on the carbon being observed (i.e. α to this carbon).$^{20}$ This splits and causes (usually) an upfield shift in the carbon resonance (Fig.3.4a and c). These shifts are additive, and are typically 0.3 p.p.m. for each deuterium, but are often difficult to observe due to coupling effects which split the carbon signals, complicating the spectrum and resulting in a decrease of intensity of individual signals. The $^{13}\text{C}-^2\text{H}$ relaxation is less efficient than $^{13}\text{C}-^1\text{H}$ relaxation, and the loss of n.O.e. enhancement also results in a decrease of signal intensities. Deuterium-induced α-shifts in the carbon-13 n.m.r. had been used to detect deuterium incorporation from $[^2\text{H}_3,2-{^{13}\text{C}}]$acetate in scytalone and other polyketides, but the results were complicated due to couplings of the $^{13}\text{C}$ to hydrogen.$^{21}$ Ideally, simultaneous $^1\text{H}$ and $^2\text{H}$ noise-decoupling are required for satisfactory results, but the instrumentation required for this is not widely available.

Of more widely practical use are "β-shifts".$^{22,23}$ These are upfield shifts due to isotopic substitution two bonds away from the carbon atom being observed (i.e. β to this carbon). β-shifts are much smaller, of the order of 0.05 p.p.m. for deuterium, but
Fig. 3.5 Simulated two-dimensional $^1$H-$^2$H-decoupled $^1$H-$^{13}$C correlation spectrum of a polyketide derived moiety enriched with [${^2}$H$_3$, 2- $^{13}$C]acetate.
are not complicated by coupling, as the $^{2}{H}^{13}{C}$ coupling over two bonds is negligible (Fig.3.4b). β-shifts are useful to quantify the extent of deuterium incorporation during biosynthesis.

A very recent technique developed for the stereochemical analysis of deuterium into natural products utilises $^{1}{H},^{2}{H}$-decoupled two-dimensional (2D) $^{1}{H},^{13}{C}$ correlation n.m.r. spectroscopy. This enables differentiation of diastereotopic hydrogens on a methylene group, if one is deuterium labelled.

The technique makes use of the high chemical shift dispersion available in $^{13}{C}$ n.m.r. to separate signals of interest. It is ideal for molecules having extensive overlap of proton and carbon resonances, as these are often separated in the 2D spectrum. Identification of diastereotopic hydrogens can be done if the proton resonances are separated by 0.1 p.p.m.

The method relies upon having both hydrogen and deuterium on the same carbon atom. Thus it cannot be applied to fully deuterated carbons. A simulation of such an experiment is shown in Fig.3.5. In the one-dimensional $^{13}{C}$ n.m.r. spectrum, signals due to carbons bearing deuterium atoms show α-shifted resonances. C-1 shows α-shifted signals due to carbons bearing both one and two deuteriums. There is a correlation between both proton signals and the α-shifted signals due to carbon bearing one deuterium. Thus deuterium incorporation here is non-stereospecific.

C-2 shows an α-shifted signal due to the presence of one deuterium atom. The α-shifted signal only correlates to the
Fig. 3.6 Production of scytalone in P. lagerbergii.
Table 3.1 Production of scytalone in *P. lagerbergii*

<table>
<thead>
<tr>
<th>Day</th>
<th>Scytalone (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2½</td>
<td>4.7</td>
</tr>
<tr>
<td>3½</td>
<td>9.1</td>
</tr>
<tr>
<td>4½</td>
<td>19.6</td>
</tr>
<tr>
<td>5½</td>
<td>25.3</td>
</tr>
<tr>
<td>6½</td>
<td>23.6</td>
</tr>
</tbody>
</table>

\(^a\) Inoculated on day 0
downfield proton resonance, therefore deuterium incorporation is stereospecific, and at the proton position resonating upfield. If the proton resonances can be assigned, experiments such as this can yield valuable stereochemical detail.

The method has been applied to the study of a number of natural products. Camphor (62)\textsuperscript{24a,b} was originally used as a test case, and it was seen that labelling of the diastereotopic hydrogens at C-3 could be distinguished. The method was then extended to other problems in natural product biosynthesis. It has been used in comparisons of the stereochemistry of fatty acid and polyketide biosynthesis in a number of fungi.\textsuperscript{24c,d,e,f} For example, the biosynthesis of the polyketide cladosporin (63) and fats derived from oleic acid (64) and stearic acid (65) in Cladosporium cladosporioides\textsuperscript{24c} was investigated, and shown to be different in several respects. Similarly, stereochemical features of enoyl thiol ester reductase in the biosynthesis of averufin (66) and oleic acid were studied in Aspergillus parasiticus.

3.2 BIOSYNTHETIC STUDIES ON SCYTALONE AND VERMELONE

3.2.1 Isolation of scytalone from \textit{P.lagerbergii}

Preliminary experiments were carried out to determine the production of scytalone in \textit{P.lagerbergii}. Production was seen to commence on day 1, and reached a maximum 5½ days after inoculation. These results are summarised in Table 3.1 and Fig.3.6.

This established conditions for the growth of \textit{P.lagerbergii}
Fig. 3.7 55 MHz $^2$H n.m.r. spectrum of scytalone from *P. lagerbergii* enriched with $[^3\text{H}]$acetate.
in order to isolate scytalone in quantity, and also indicated suitable timing for feeding experiments. Yields of scytalone from the culture when isolated at 5-6 days were typically 200 - 300 mg/l.

The optical rotation of scytalone from this source was measured, and seen to be zero, as expected.

A feeding experiment was performed using $[^2H_3]$acetate in this culture. *P.lagerbergii* was grown in liquid medium and fed on day 3 with $[^2H_3]$acetate. The scytalone was isolated after 6 days and purified by preparative t.l.c. The 55.28 MHz $^2$H n.m.r. spectrum of this was obtained (Fig.3.7). This showed signals at $\delta$ 6.27, 3.05 and 2.79 p.p.m., due to H-5, H-4 equatorial and H-4 axial respectively, as had been previously determined.\textsuperscript{11,25} The presence of deuterium incorporation from acetate at both H-4 positions suggests that the enzymic reduction of 1,3,6,8-THN to scytalone is non-stereospecific here, as previous work had shown that only one deuterium from acetate could be incorporated here.\textsuperscript{10,21} There does seem to be some degree of stereo-selectivity, as the level of incorporation is consistently greater at the 4-equatorial than at the 4-axial position.

No incorporation of deuterium from acetate was seen at either H-2 or H-7, despite the expectation that this would occur. This was also consistent with earlier observations as mentioned.

3.2.2 Isolation of scytalone from *V.dahliae brm-1*

Scytalone from *V.dahliae brm-1* was reported to be different from that produced by *P.lagerbergii* in its optical activity.
Fig. 3.9 Production of scyetalone in V. dahliae brm-1.
Fig. 3.8 H.P.L.C. standard curve for scytalone (eluent methanol-water, 40:60).
Table 3.2 Production of scytalone in *V. dahliae* brm-1

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Peak height (mm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Yield (µg/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>72</td>
<td>1.5</td>
<td>2</td>
</tr>
<tr>
<td>96</td>
<td>2.0</td>
<td>4</td>
</tr>
<tr>
<td>104</td>
<td>2.5</td>
<td>4</td>
</tr>
<tr>
<td>120</td>
<td>6.5</td>
<td>13</td>
</tr>
<tr>
<td>128</td>
<td>3.75</td>
<td>6</td>
</tr>
<tr>
<td>144</td>
<td>6.5</td>
<td>13</td>
</tr>
<tr>
<td>168</td>
<td>12.5</td>
<td>29</td>
</tr>
<tr>
<td>176</td>
<td>17.0</td>
<td>39</td>
</tr>
<tr>
<td>240</td>
<td>35.5</td>
<td>108</td>
</tr>
<tr>
<td>248</td>
<td>27.0</td>
<td>67</td>
</tr>
<tr>
<td>264</td>
<td>46.5</td>
<td>172</td>
</tr>
<tr>
<td>288</td>
<td>47.0</td>
<td>174</td>
</tr>
<tr>
<td>296</td>
<td>54.0</td>
<td>220</td>
</tr>
<tr>
<td>313</td>
<td>58.0</td>
<td>248</td>
</tr>
<tr>
<td>320</td>
<td>65.0</td>
<td>282</td>
</tr>
<tr>
<td>336</td>
<td>73.0</td>
<td>308</td>
</tr>
<tr>
<td>344</td>
<td>77.0</td>
<td>322</td>
</tr>
<tr>
<td>408</td>
<td>127.0 [135.0]&lt;sup&gt;c&lt;/sup&gt;</td>
<td>447 [460]&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reading from H.P.L.C. trace
<sup>b</sup> Read from H.P.L.C. standard curve
<sup>c</sup> Alternative sample from control flasks
Hence a number of feeding studies were carried out with this culture, using acetate labelled variously with $^2$H, $^{13}$C and $^{18}$O, and the scytalone produced was examined using the n.m.r. techniques mentioned. Again, it was necessary to determine the production of scytalone in the culture, in order to establish a feeding regimen for labelled precursors.

Ten flasks of liquid culture were grown under static conditions in darkness at 24°C. From five of these flasks, aliquots of 0.2 ml culture medium each were withdrawn, combined and filtered to remove mycelium, at approximately 12 hour intervals over a period of 17 days. At the last sampling time, a similar set of 0.2 ml aliquots was taken, combined and filtered, from the remaining 5 undisturbed culture flasks. This was used as a control to ensure that sampling did not disrupt normal metabolism in the culture.

Analysis of the samples was performed by H.P.L.C., using an elution system of methanol-water, 40:60. Scytalone had a retention time of 5½ minutes in this system, and gave sharp, well-defined peaks. A standard curve was plotted for known concentrations of scytalone against measured peak height (Fig.3.8) The concentrations of the unknown samples were then read off against the corresponding peak height obtained (Table 3.2).

A growth-production curve was plotted for scytalone in V. dahliae brm-1 (Fig.3.9). Production commenced at about 72 hours, and by the end of the sampling period of 17 days, did not
appear to have reached a maximum. For feeding studies however the first phases of growth are the important ones for establishing a feeding regimen. It is desirable to feed precursors when production of the metabolite starts to accelerate, to obtain maximum incorporation. Incorporation may be very sensitive to the time of addition of precursors.

The predicted yield of scytalone at 17 days' growth was 460 mg/l by the H.P.L.C. analysis. When, at 21 days, the flasks were worked up by standard procedures, the mass of scytalone isolated corresponded to a yield of 220 mg/l. This is normal for H.P.L.C. yield predictions which are often higher than those obtained in practice.

Using the results of the growth-production study, preliminary incorporation studies were performed using $[^{14}\text{C}]$acetate to monitor incorporation of the precursor into scytalone. In order to make $^{13}\text{C}$ feeding studies worthwhile, for the incorporation of 90% enriched precursors, dilution values of less than 100 are required, so that the observed enhancement of signals in the $^{13}\text{C}$ n.m.r. spectrum is unequivocal. For $^{14}\text{C}$, dilution per labelled site is given by:

$$\frac{\text{specific activity of precursor} \times \text{no. of labelled sites}}{\text{specific activity of product}}$$

The labelled acetate was fed at four different times; 72, 144, 168 and 192 hours, and one set of flasks kept as a control to check that normal production of scytalone was not interfered
Fig.3.10 55 MHz $^2$H n.m.r. spectrum of scytalone from V.dahliae brm-1 enriched with [$^3$H$_3$]acetate.
Table 3.3 Incorporation of \([^{14}\text{C}]\)acetate into scytalone in *V. dahliae* brm-1

<table>
<thead>
<tr>
<th>Time of feed (hours)</th>
<th>Specific activity of precursor (dpm/mmol)</th>
<th>Scytalone isolated (mg)</th>
<th>Specific activity of product (dpm/mmol)</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>42.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>72</td>
<td>(9.02 \times 10^6)</td>
<td>30.8</td>
<td>(3.17 \times 10^6)</td>
<td>14.2</td>
</tr>
<tr>
<td>( &quot;</td>
<td>&quot;</td>
<td>23.1</td>
<td>(2.78 \times 10^6)</td>
<td>16.2 )a</td>
</tr>
<tr>
<td>144</td>
<td>&quot;</td>
<td>32.0</td>
<td>(4.12 \times 10^6)</td>
<td>10.9</td>
</tr>
<tr>
<td>168</td>
<td>&quot;</td>
<td>33.0</td>
<td>(4.45 \times 10^6)</td>
<td>10.1</td>
</tr>
<tr>
<td>192</td>
<td>&quot;</td>
<td>42.2</td>
<td>(3.92 \times 10^6)</td>
<td>11.5</td>
</tr>
<tr>
<td>( &quot;</td>
<td>&quot;</td>
<td>32.9</td>
<td>(3.99 \times 10^6)</td>
<td>11.3 )a</td>
</tr>
</tbody>
</table>

\(a\) after repurification
with. At 14 days the five sets of cultures were worked-up separately and the scytalone isolated. The samples were purified by preparative t.l.c. and the radioactivity measured. The samples of scytalone from the cultures fed at 72 and 192 hours were re-purified by preparative t.l.c. and the radioactivity again measured. Dilution values were calculated on the basis of the level of radioactivity in each feed, and these were seen to be excellent (Table 3.3). The optimum feeding time appeared to be between 144 and 168 hours' growth.

A number of feeding experiments were then carried out using acetates labelled with stable isotopes. The sodium acetate was fed to the cultures between 144 and 168 hours' growth, and scytalone isolated after 13-14 days. The samples of scytalone were purified by preparative t.l.c. for n.m.r. analysis.

Deuterium n.m.r. at 55.28 MHz of scytalone from cultures fed with \( \text{[}^{2}H_{3}\text{]} \) acetate (Fig.3.10) shows a significant difference to that of the material obtained by feeding \( \text{[}^{2}H_{3}\text{]} \) acetate to P.lagerbergii. The relative intensities of the signals indicate more equal incorporation of the label at the proton positions resonating at \( 63.0 \) and \( 2.8 \). The protons at H-4ax. and H-2eq. in scytalone both resonate at the same frequency. In scytalone from P.lagerbergii, the signal at \( 62.8 \) was shown to be entirely due to deuterium incorporated at H-4ax. Initially, it was assumed that this was also the case for scytalone from V.dahliae brm-1 (see below). There is also clearly incorporation of deuterium at H-7. This had not been observed in any previous labelling studies, and
Table 3.4 Isotope-induced $\alpha$-shifts due to $^{18}\text{O}$ in the $^{13}\text{C}$ n.m.r. spectrum of scyatalone from cultures of \textit{V.dahliae} brm-1 enriched with [1-$^{13}\text{C}$, $^{18}\text{O}_2$]acetate.

<table>
<thead>
<tr>
<th>$\delta_\text{C}$ (p.p.m.)</th>
<th>Carbon</th>
<th>Shift (p.p.m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>201.76</td>
<td>1</td>
<td>0.037</td>
</tr>
<tr>
<td>165.60</td>
<td>8</td>
<td>0.011</td>
</tr>
<tr>
<td>164.94</td>
<td>6</td>
<td>0.011</td>
</tr>
<tr>
<td>65.79</td>
<td>3</td>
<td>0.0196</td>
</tr>
</tbody>
</table>

Fig. 3.12 Incorporation of [1-$^{13}\text{C}$, $^{18}\text{O}_2$]acetate into scyatalone in \textit{V.dahliae} brm-1.
Fig. 3.11 90.55 MHz $^{13}$C n.m.r. spectrum of scyhalone from V. dahliae brm-1 enriched with [1-$^{13}$C, $^{18}$O$_2$]acetate.
it had been assumed that all the deuterium at this position was lost by exchange with protons in the surrounding medium from 1,3,6,8-THN, reported to be a facile process. The stereo-selectivity of the enzymic reduction process appears to differ between these two species, which produce scytalone of different optical activity.

Feeding *V. dahliae* brm-1 liquid culture with [1-\textsuperscript{13}C,\textsuperscript{18}O\textsubscript{2}]-acetate resulted in doubly \textsuperscript{18}O,\textsuperscript{13}C-labelled scytalone. The \textsuperscript{13}C n.m.r. spectrum of this at 90.56 MHz was obtained (Fig.3.11). This shows \(\alpha\)-isotope-induced shifts on C-1, C-3, C-6 and C-8, indicating the incorporation of intact C-O units from the labelled acetate. This confirmed the acetate origin of the oxygens in scytalone (Fig.3.12). The magnitude of the shifts are summarised in Table 3.4. The largest shift was seen on the carbonyl carbon, of 0.037 p.p.m. A large shift of 0.019 p.p.m. was also observed on the signal due to C-3, a secondary alcohol. Signals due to the phenolic carbons exhibited smaller shifts of 0.011 p.p.m. These results agree with the expected magnitudes of such \textsuperscript{18}O-induced \(\alpha\)-shifts in the \textsuperscript{13}C n.m.r., based on earlier work.\textsuperscript{19}

The culture was also fed with doubly-labelled \([\textsuperscript{2}H\textsubscript{3},1-\textsuperscript{13}C]\)-acetate, and the resulting scytalone examined by n.m.r. The deuterium n.m.r. again shows incorporation at H-5, both H-4eq. and the H-4ax./H-2eq. positions, and slight incorporation at H-7. The \textsuperscript{13}C spectrum was then examined to look for \(\beta\)-shifts on the carbon signals due to deuterium 2 bonds away (Fig.3.13).
Table 3.5 Isotope-induced δ-shifts due to deuterium in the $^{13}\text{C}$ n.m.r. spectrum of scytonal from cultures of $V.dahliae$ brm-1 enriched with [2-$^2\text{H}_3,1-{^{13}\text{C}}$]-acetate.

<table>
<thead>
<tr>
<th>$\delta_C$ (p.p.m.)</th>
<th>Carbon</th>
<th>Shift (p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>201.8</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>165.6</td>
<td>8</td>
<td>0.024</td>
</tr>
<tr>
<td>164.9</td>
<td>6</td>
<td>0.037</td>
</tr>
<tr>
<td>145.4</td>
<td>4a</td>
<td>0.085</td>
</tr>
<tr>
<td>65.79</td>
<td>3</td>
<td>0.046</td>
</tr>
</tbody>
</table>
Fig. 3.13 90.55 MHz $^{13}$C n.m.r. spectrum of scyatalone from V. dahliae brm-1 enriched with [\(^{1}$H$_{3}$, 1-$^{13}$C]acetate.
C-1 shows no shifted signal, as expected since there was no deuterium incorporated at C-2. C-3 appears to show two shifted signals, 0.0455 and 0.0949 p.p.m. upfield of the main signal. This would appear to suggest that in some molecules C-4 carries two deuterium atoms from acetate. As earlier studies had shown that this was not possible, the weak signal 0.0949 p.p.m. upfield must be due to long-range $^{13}\text{C}-^{13}\text{C}$ coupling of the shifted signal (where two labelled acetate units have been incorporated in one molecule), which would result in a triplet as shown. The other signal in this triplet is hidden below the main carbon resonance.

C-4a shows a β-shifted signal 0.085 p.p.m. upfield, due to incorporation of deuterium on C-5 or C-4, depending on the arrangement of acetate units in scytalone (c.f. Fig.3.3). The signals are not very "clean", which could be due to long-range couplings as for C-3, or because the shifts due to deuterium on C-4 and C-5 are of slightly different magnitudes.

The signal due to C-6 shows a shifted resonance 0.037 p.p.m. upfield, as expected due to the incorporation of deuterium at C-5, and partly due to C-7. There also appears to be a signal shifted downfield by 0.028 p.p.m. This would be unusual, and as its intensity was low, this signal was also attributed to long-range $^{13}\text{C}-^{13}\text{C}$ coupling.

The remaining carbon resonance to show a β-shifted signal was that of C-8. The shifted signal here was small, and although this would be expected from the low level of incorporation seen in the
Fig. 3.15 Two-dimensional $^1\text{H}, ^2\text{H}$-decoupled $^1\text{H}, ^{13}\text{C}$ correlation n.m.r. spectrum of scytalone from V.dahliae brm-1 enriched with $[^2\text{H}_3, ^{13}\text{C}]$acetate.
Table 3.6 Isotope-induced α-shifts due to deuterium in the $^{13}$C n.m.r. spectrum of scytalone from cultures of *V. dahlia* *brm-*1 enriched with $[2-^{2}H_{3}, 2-^{13}C]$acetate.

<table>
<thead>
<tr>
<th>δ$_C$ (p.p.m.)</th>
<th>Carbon</th>
<th>Shift (p.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47.49</td>
<td>2</td>
<td>0.299</td>
</tr>
<tr>
<td>39.21</td>
<td>4</td>
<td>0.321</td>
</tr>
</tbody>
</table>

Fig. 3.16 Incorporation of $[2-^{2}H_{3}, 2-^{13}C]$acetate into scytalone in *V. dahlia* *brm-*1.
Fig. 3.14 100.62 MHz $^{13}$C n.m.r. spectrum of scytalone from *V. dahliae bhm-l* enriched with [$^{2}$H$_{3}$,2-$^{13}$C]acetate.
deuterium n.m.r. spectrum, the signal could also be due to coupling effects again. These results are summarised in Table 3.5.

The results of this experiment were therefore not entirely clear-cut, and to provide more information on the incorporation of deuterium in the saturated ring particularly, the complementary feeding experiment using \([^{2}\text{H}_3,^{2}\text{H}_2}\text{C}^{12}\text{C}^{13}\text{C}]\)-labelled acetate was performed.

The carbon-13 deuterium-decoupled n.m.r. spectrum of this was obtained at 100.62 MHz, with expansions for the signals due to the saturated carbons C-2 and C-4. The signal at 839.21 due to C-4 clearly shows an isotopically-shifted signal 0.32 p.p.m. upfield of the main carbon resonance (Fig.3.14) due to \(\alpha\)-deuterium. Only one shifted signal appears, as C-4 may only carry one deuterium derived from acetate. The slight doubling of the shifted signal may be due to a slight difference in the magnitude of the \(\alpha\)-shift caused by deuterium in the axial and equatorial positions.

The spectrum also shows an \(\alpha\)-shifted signal on the resonance at 847.49, due to C-2 (Fig.3.14). The shifted signal is of very low intensity, but is of the order of magnitude expected for deuterium-induced \(\alpha\)-shifts (Table 3.6).

The two-dimensional \(^1\text{H},^2\text{H}\)-decoupled \(^1\text{H},^{13}\text{C}\) correlation spectrum for this sample was obtained (Fig.3.15). This shows a correlation between the shifted \(^{13}\text{C}\) signal at 838.89 (due to C-4) and the upfield hydrogen (H-4ax). This means that the deuterium
must reside at the downfield position, which has been assigned to H-4equatorial. The correlation of C-4 with H-4eq. appears to show no shifted signal, indicating a lack of deuterium at H-4axial. However, the correlation of the fully-protonated C-4 resonance with H-4eq. is rather broad, and may be obscuring a correlation between the shifted CHD signal and H-4eq.

The signal at 647.5 due to C-2 attached to protium in this spectrum also shows a very weak shifted signal on the upfield proton resonance, due to H-2axial. This signifies that there could be a very low level of deuterium incorporation at H-2equatorial (Fig.3.16). This result agrees with the deuterium n.m.r. results, where a small amount of deuterium incorporation was seen at H-7 for scytalone isolated from *V. dahliae* *brm-1* (Fig.3.10). Since the melanin intermediate 1,3,6,8-THN is symmetrical, it would be expected that deuterium incorporation would be seen at neither or both H-2 and H-7.

The apparent lack, or at any rate very low level of incorporation of deuterium at H-4 axial as shown by these α-shift experiments, contradicts the initial assignment of the ²H n.m.r. spectrum for scytalone from *V. dahliae* *brm-1* enriched with [²H₃]acetate. In the ²H n.m.r. spectrum of scytalone, the H-4ax. and H-2eq. signals are coincident at 62.8. For scytalone isolated from *P. lagerbergii*, the deuterium was shown to be incorporated at H-4ax. and not H-2eq., by a series of reverse-exchange experiments. The recent results suggest that in the deuterium n.m.r. of scytalone obtained from *V. dahliae* *brm-1*, the signal at
62.8 is due mainly (at least) to deuterium at H-2eq. The deuterium is situated at H-5, H-7, H-2eq. and H-4eq., and perhaps at H-4ax. To resolve the question as to whether deuterium from acetate is incorporated at H-4ax., it would be necessary to carry out a series of reverse-exchange experiments such as were done for scytalone derived from \textit{P.lagerbergii} enriched with $[^2\text{H}_3]\text{acetate}$. It would also be worthwhile to re-examine the incorporation of deuterium into scytalone in \textit{P.lagerbergii}, using $[^2\text{H}_3,2^{13}\text{C}]$-acetate as a precursor, and obtaining similar $^{13}\text{C}$ n.m.r. and 2D $^1\text{H},^2\text{H}$-decoupled $^1\text{H},^{13}\text{C}$ correlation spectra.

3.2.3 Production of (−)-vermelone in \textit{V.dahliae brom-1}

The mutant \textit{V.dahliae brom-1} lacks the dehydratase enzyme necessary to convert scytalone to 1,3,8-THN. The same enzyme appears to operate on vermelone to convert this to 1,8-DHN. Thus if 1,3,8-THN is fed to the culture, vermelone (17) is produced.

The experimental procedure used was based on that of previous studies with some modifications. The culture had been grown on Petri dishes containing Potato Dextrose Agar (PDA), and inoculated with a spore suspension. The spore suspension was carefully prepared by washing conidia and spores from the outer 1 cm of fungal colonies into distilled water, and diluting to $10^6$ conidia/ml. This procedure was adapted in our experiments. The inoculum was prepared by gentle scraping of the conidia and spores from a PDA stock slope of the fungus into distilled water (5 ml) and adding this to one flask of modified Brandt’s liquid.
medium. This was incubated overnight on shaken culture in darkness at 24°C to provide an actively growing inoculum.

The culture was grown on PDA plates. These were inoculated with 0.2 ml of the suspension, which was spread over the entire surface of the plate. The plates were incubated at 24°C in the dark.

The previous studies had added the substrate 1,3,8-THN to the culture as a saturated solution in potassium phosphate buffer solution containing sucrose. 1,3,8-THN is not very water-soluble, and it was difficult to quantify the amount of substrate added to the culture. A more efficient procedure was found to be to dissolve the compound in a minimum quantity of ethanol prior to adding it to the buffer solution. This did not appear to disrupt the system at all. Quantification of the amount of substrate fed was therefore more precise.

The timing of addition of the substrate in any biological system is important, and depends very much on the activity of the particular batch of a culture. Of the previous studies on this system, feeding times had varied between 52-56 hours, and 144 hours. Due to the nature of the procedure for isolation of vermelone by this method, it was impractical to attempt a growth-production study.

Instead, a good visual guide to the state of growth of the culture was seen to be its colour. The beginning of activity of the enzymes involved in melanin biosynthesis was marked by the observation of a pinkish-brown colouration of the plates, due to
the production of flaviolin and other highly-coloured shunt products of the pathway. Feeding when this colouration was observed, gave good results. In these experiments, this varied between 78 and 144 hours' growth of the fungus.

Incubation of the plates after addition of the substrate was normally 12-18 hours, although it has been shown that most vermelone is produced within 1-2 hours of feeding.14

The original work-up procedure had isolated vermelone from the aqueous liquid decanted from the plates. Yields from this were low, and it was found that extraction of the agar gave nearly three times the yield, as the metabolites are excreted into the agar as well as the supernatant liquid. Thereafter, the complete contents of the plates were extracted. Yields from these biological conversions were typically 18-25%, and sometimes as high as 35%, which are good yields for such experiments.

The metabolite from V.dahliae brm-1 was reported to be (-)-vermelone, showing an optical rotation $[\alpha]_D^{25} = -18^\circ$. Samples of vermelone isolated from different growths of the fungus in these studies varied in optical activity. Optical rotations of 17° and 9° were obtained for two samples isolated, but several culture batches yielded vermelone which showed no optical activity. The question of the optical nature of vermelone from V.dahliae brm-1 as well as the scytalone from this source would also benefit from further investigation.
(67)

(68)

(69)

(70)  \( R = \text{Me} \)

(71)  \( R = \text{CH}_2\text{OH} \)

(72)  \( R = \text{COOH} \)
3.3 SCYTALONE DERIVATIVES

It was originally assumed that natural scytalone would be optically pure, as, in nature, fungi usually contain enzymes which specifically produce one optical isomer.

Where racemic metabolites are produced, this is usually an indication of the operation of a spontaneous (i.e. non-enzymic) process in the generation of a chiral centre. Such is the case in Alternaria species, which produce racemic (+)-dehydroaltnenusin (68) and (+)-alternuene (69). These are formed from alternariol (67) by non-enzymic oxidative processes.26

Only rarely do fungi produce both optical isomers of a metabolite. For example, (+)-mitorubrin (70), (+)-mitorubrinol (71) and (+)-mitorubrinic acid (72) were isolated from fruiting bodies of the Ascomycete Hypoxylon fragiforme. These compounds were isolated in the (-)-form from species of Penicillium.27

The absolute stereochemistry of scytalone had not been established, from any of the natural sources. This could be achieved by reaction of the chiral centre at C-3 in scytalone with a chiral ester, followed by x-ray structure determination. The configuration of the chiral centre could be assigned with reference to the known configuration of the chiral auxiliary.

Protection of the aromatic hydroxyls was first necessary. Methylation with ethereal diazomethane produced the monomethyl ether (73).1 The hydroxyl group on C-8 is intramolecularly hydrogen-bonded to the ketone function, and is thus unreactive. Methylation of scytalone using other reagents and conditions:
methyl iodide/potassium carbonate or methyl iodide/silver(I) oxide, also produced only the monomethyl ether. Further protection appeared to be unnecessary as a result.

The acetate and benzoate derivatives were then prepared to test the reactivity of scytalone monomethyl ether, and thus the potential for preparation of chiral derivatives.

A previous attempt to prepare the acetate of scytalone monomethyl ether had resulted in a crude product which seemed to be this derivative, but which was not characterised as it decomposed on attempts at purification. Here, methyl scytalone acetate was successfully prepared and purified for analysis.

Scytalone monomethyl ether in pyridine was cooled in ice and treated with acetic anhydride, and allowed to stir for 2 hours in the ice-bath. Work-up by pouring the reaction mixture onto crushed ice, extraction into chloroform and drying, was performed carefully and rapidly. The solution was evaporated over a tepid water bath to give the crude product. This was purified first by preparative t.l.c., followed by recrystallisation from ether to give a pale brown crystalline product, m.p. 115-118°C in 62% yield. This was confirmed by n.m.r., elemental analysis and mass spectroscopy to be methyl scytalone acetate (74). The mass spectrum showed a weak parent ion at m/z 250, and a base peak at 190, which corresponds to the facile loss of acetic acid (60 mass units).

The benzoate derivative was prepared in a similar fashion, by treatment of a cooled solution of scytalone monomethyl ether in
pyridine with benzoyl chloride. Careful work-up and purification by preparative t.l.c. and recrystallisation from ether-light petroleum 40-60° gave a pale brown crystalline product, m.p. 104-108°C. Examination of the proton n.m.r. spectrum of this suggested that the product was the dibenzoate derivative (75, 67% yield). The hydroxyl signal at \( \delta 12 \) had disappeared, and the aromatic region due to the phenyl groups showed a pattern indicating two monosubstituted phenyl rings in slightly different chemical environments. This was confirmed by elemental analysis and mass spectroscopy. The mass spectrum showed a weak parent ion at \( m/z \) 416, and a base peak at 294, corresponding to loss of benzoic acid (122 mass units).

Conditions for the derivatisation of scytalone thus need to be mild in order to prevent decomposition of the products, as seen by the earlier failure to purify methyl scytalone acetate, and facile loss of the substituent groups. The reagents need to be mild also, as was seen by the attempt to produce the benzoate derivative. Despite failure to methylate the C-8 hydroxyl under various reaction conditions, it did react with the acid chloride.

Doubt over the enantiomeric purity of natural scytalone isolated from the different sources, put the usefulness of such a structural determination into question, and this was not pursued, pending a solution to the other problem.
3.4 $^1$H N.M.R CHIRAL SHIFT STUDIES

Further to the study of melanin biosynthesis in fungi imperfecti, the DHN melanin pathway was shown to operate in Wangiella dermatitidis. Tricyclazole inhibition in this fungus caused the accumulation of scytalone and other metabolites. The scytalone was apparently racemic. Optical measurements on recrystallised scytalone from this source showed $[\alpha]_D^{25} = -1.37^\circ$. The optically active PMR shift reagent Eu(hfbc)$_3$ was used to examine scytalone from both W.dermatitidis and V.dahliae brm-1.

Proton Magnetic Resonance shift reagents are chiral complexing reagents which can be used to determine the enantiomeric purity of chiral compounds. The reagents are pure enantiomers which complex reversibly with pairs of enantiomers, to form pairs of diastereomers, and cause changes in the observed chemical shifts. In the diastereomers, protons which were chemically equivalent in the enantiomers, now become non-equivalent, and hence experience slightly different shifts. Hence if two enantiomers are present in a sample, a single signal in the proton n.m.r. spectrum may split into two on the addition of a shift reagent. The effect is most easily observable in the aromatic region as the spectrum here is least complicated by coupling. Eu(hfbc)$_3$ normally causes a downfield shift in the proton resonances. The method has been used to determine the enantiomeric excess in samples of chiral compounds. One disadvantage to these reagents is that they cause broadening of
Fig. 3.17 Aromatic region of the $^1$H n.m.r. spectrum of scytalone from *P. lagerbergii* in CDCl$_3$; (a) No Eu(hfbc)$_3$, (b) With Eu(hfbc)$_3$. 
the proton resonances, especially at high field strengths. However, only minute quantities of the reagent are usually necessary, which minimises this problem.

In the studies with *W. dermatitidis* and *V. dahliae*, proton n.m.r. spectra of scytalone were obtained in CDCl$_3$, and the aromatic protons were reported to appear as signals at $\delta$7.86 and 6.7. Increments of Eu(hfbc)$_3$ were added, and the spectra re-run. In the case of scytalone from *W. dermatitidis*, each of these aromatic peaks divided into two, of which the inner ones converged as more shift reagent was added. When the same experiment was run using scytalone isolated from *V. dahliae brm-1*, these peaks remained single and constant. These results were taken to confirm the optical purity of scytalone from *V. dahliae brm-1*, and the racemic nature of that from *W. dermatitidis*.

The results of this study appear to be fundamentally questionable, for the basic reason that scytalone is very sparingly soluble in deuterochloroform.

A sample of scytalone from *P. lagerbergii* was dissolved in CDCl$_3$, and the proton n.m.r. spectrum at 300 MHz obtained (Fig.3.17). The scytalone signals are very weak compared to the solvent peak, but the signals due to the aromatic protons H-5 and H-7 are visible at approximately $\delta$6.25. The signals observed at $\delta$7.6 and 6.9 in this spectrum, comparable to the signals attributed to the aromatic protons in the previous studies, are clearly satellite peaks of CDCl$_3$. Thus the validity of those studies is extremely doubtful.
Fig. 3.19 Aromatic region of the $^1$H n.m.r. spectrum of the monomethyl ether of scytalone from *V. dahliae* brm-1 in CDCl$_3$ as increments of Eu(hfbc)$_3$ are added.
Fig. 3.18 Aromatic region of the $^1$H n.m.r. spectrum of the monomethyl ether of scytalone from *P. lagerbergii* in CDCl$_3$ as increments of Eu(hfbc)$_3$ are added.
Eu(hfbc)$_3$ was added to the solution of scytalone in CDCl$_3$, and the aromatic proton signals broadened and shifted considerably, showing that complexation had occurred. No splitting of the resonances was seen.

The effect of the chiral shift reagent on scytalone from *P. lagerbergii* was re-examined, especially since samples of scytalone isolated from *V. dahliae* brm-1 in the present studies had shown no optical rotation. To overcome the solubility problem, the monomethyl ether (73) of samples of scytalone from both fungi was made (see Ch.3.3), and the n.m.r. experiments carried out using these. Scytalone monomethyl ether is soluble in CDCl$_3$, and the aromatic signals due to H-5 and H-7 appear at the same positions as in scytalone, at 6.28 and 6.15 respectively, and distant from the solvent peak.

The $^1$H n.m.r. spectrum was obtained for the monomethyl ether of scytalone isolated from *P. lagerbergii*, and re-run as increments of Eu(hfbc)$_3$ were added (Fig.3.18). The aromatic signals experienced a downfield shift, and broadened considerably as more shift reagent was added. No splitting of the signals was observed.

The same experiment was then performed on the monomethyl ether of scytalone from *V. dahliae* brm-1 (Fig.3.19). Again, a downfield shift and broadening of the signals was seen, but no splitting occurred.
Fig. 3.20 Aromatic region of the $^1$H n.m.r spectrum of vermelone from V. dahliae brm-1 in CDCl$_3$ as increments of Eu(hfbc)$_3$ are added.
Samples of scytalone from both fungi were also applied to a Percol chiral chromatography column at I.C.I. Plant Protection Division. In each case, scytalone was eluted as one narrow band.

Vermelone produced by the biological conversion of 1,3,8-THN in V.dahliae brm-1 was also examined using the PNR chiral shift reagent technique. This vermelone had shown a negative optical rotation, and was thus not racemic. It was not known however whether it was optically pure, or a mixture of enantiomers with one isomer predominant.

A sample of vermelone was dissolved in CDCl₃, and ¹H n.m.r. spectra obtained as increments of the shift reagent were added (Fig.3.20). No splitting of the signals due to the aromatic protons was observed, but there was also no broadening or shifting of the signals. It could be that the shift reagent does not complex to vermelone.

The question of the optical activity and purity of scytalone from different fungal species was therefore not resolved, and appears to present difficulties. The synthesis of racemic scytalone and its examination by ¹H n.m.r. using chiral shift reagents, and comparison with the natural metabolites, would be the next step. The x-ray crystallographic methods already mentioned for determination of the absolute configuration of chiral compounds could also be used to determine whether pure enantiomers are present, but would be a more drastic way of approaching the problem.
3.5 EXPERIMENTAL

For general experimental conditions, see Chapter 2.5.

3.5.1 Phialaphora lagerbergii

Culture conditions:–

Phialaphora lagerbergii (CMI 96745) was maintained on stock slopes of Malt Extract Agar (MEA, Oxoid). It was grown on shaken culture at 26°C in 500 ml conical flasks each containing 200 ml of Czapek-Dox medium (Oxoid) supplemented with 0.1% yeast extract and 5% sucrose. Distilled water (10 ml) was added to one stock slope and the spores and conidia loosened using a sterilised wire loop. This was used to inoculate one flask containing the liquid medium, which was grown as described for two days to produce an active suspension of the culture. This was then used in 10 ml aliquots to inoculate further flasks of the liquid medium.

Isolation of scytalone:–

Liquid cultures were filtered to remove mycelium and the filtrate adjusted to pH 3 using 2M H₃PO₄. Extraction with ethyl acetate, drying over MgSO₄ and evaporation yielded a gummy brown oil.

This was seen by t.l.c. (acetone–light petroleum 60–80°, 50:50) to be a mixture of products, of which scytalone was the major spot, Rf 0.55. Purification was by preparative t.l.c. in the same solvent system, or by "dry-flash" column chromatography using a 70 mm diameter sinter funnel as the column and ether as eluent. Fractions of 30 ml were collected,
and tested for the product. This gave pure scytalione as
ear-colourless crystals m.p. 160-164°C, $[\alpha]_D^{25} = 0^\circ$ (c; 0.25;
95% EtOH). $\delta_H$ 2.62 - 3.20 (4H, m, 2 x CH$_2$); 4.31 (1H, septet,
CHOH); 6.15 (1H, dd, H-7); 6.28 (1H, dt, H-5); 9.5 (1H, broad s,
OH); 12.82 (1H, s, OH).

**Growth-production study:**

Ten flasks were inoculated and grown as described above. Pairs of flasks were taken off and worked up as described, at
24-hour intervals. Scytalione was isolated and purified by
preparative t.l.c. in acetone-light petroleum 60-80°C, 50:50. Production was seen to commence at 2 days' growth and reach a
maximum after 5½ days.

The yield of scytalione at 5½ days corresponded to 250 mg /
litre.

(a) Incorporation of sodium [2H$_3$]acetate:--

Sodium [2H$_3$]acetate (226 mg) was dissolved in sterilised
distilled water (6 ml) and divided between two flasks of liquid
culture medium inoculated with *P.lagerbergii*, after 3 days' growth. After a further 4 days' growth the contents were filtered
to remove mycelium. The filtrate (400 ml) was acidified and
extracted with ethyl acetate (3 x 200 ml). The extracts were
dried over MgSO$_4$ and evaporated to give a gummy brown oil. This
was purified by preparative t.l.c. in the solvent system
described, to give pure scytalione (69 mg).
3.5.2 *Verticillium dahliae* brm-1

**Culture conditions:**

*Verticillium dahliae* Kleb. brm-1 (ATCC 44571) was maintained on stock slopes of Potato Dextrose Agar (PDA, Oxoid). It was grown in static liquid culture at 24°C in darkness, in 125 ml conical flasks each containing 40 ml of Brandt's liquid sucrose-nitrate medium modified to contain $3 \times 10^{-5}$ M catechol. Sterile distilled water (10 ml) was added to one stock slope and the conidia and spores loosened using a sterilised wire loop. This suspension was used to inoculate liquid culture medium in 2 ml aliquots.

*V. dahliae* brm-1 was also grown on PDA in 150 x 10 mm Petri dishes (Sterilin) at 24°C in the dark, for the production of vermelone (see below).

**Isolation of scytalone:**

The liquid cultures were filtered to remove mycelium, adjusted to pH 5 using 2M $\text{H}_3\text{PO}_4$ and saturated with salt. The solution was extracted into ethyl acetate, dried over MgSO$_4$ and evaporated to give a gummy brown oil. The crude product was purified by preparative t.l.c. (acetone-light petroleum 60-80°, 50:50) to give pure scytalone m.p. 160-163°C, $[\alpha]_D^{25} = 0^\circ$ (c; 0.25; 95% EtOH).
Growth-production study:

Ten flasks were inoculated and grown as described. Samples of 0.2ml were taken from each of five flasks and combined, at intervals of approximately 12 hours over a period of 17 days. When the final set of samples were taken, a duplicate set was also taken, to provide a control ensuring that no disruption of normal metabolism had occurred as a result of sampling. When the samples were taken, each 1 ml was filtered through a disposable filter (GWSP0130, pore size 0.22μm, Millipore) to remove mycelium and halt growth.

The samples were analysed by H.P.L.C., using a 4.1 x 250mm Versapak C18 10μm reverse-phase column (Gilson). The eluent was methanol-water 40:60 v/v; mobile phase flow rate 1.0 ml/min; injection size 20μl. Detection was at 254 nm; scytalone retention time \( R_t = 5\frac{1}{2} \) minutes. A standard curve was plotted for known concentrations of scytalone against measured peak height (Fig.3.8). This was used to quantify the concentrations of the samples taken from the cultures of V.dahliae brm-1. At 17 days' growth, the estimated yield of scytalone was 440 mg/litre.

Work-up of the remaining culture medium at 21 days' growth gave, after purification, a yield of scytalone corresponding to 220 mg/litre.
(a) Incorporation of sodium $^{14}$C acetate:

Twenty-five flasks of liquid culture of V.dahliae brm-l were inoculated and grown as described. Sodium acetate (800 mg) was dissolved in sterile distilled water (40 ml) and spiked with $^{14}$C acetate (40 µCi). This was divided into four equal portions, and each portion fed equally between five flasks of the culture, at times of 72, 144, 168 and 192 hours. The five remaining flasks were kept as a control of scytalone production.

At 14 days' growth each set of flasks was separately worked up and the scytalone isolated as described. Crude scytalone was purified by preparative t.l.c. (acetone-light petroleum 68-80°, 50:50) and the radioactivity measured. Two samples were re-purified by the same method, and radioactivity measurements repeated. Dilution values for the samples were calculated on the basis of the radioactivity fed (Table 3.3). On the basis of these, the optimum feeding time appeared to be between 144 and 168 hours.

(b) Incorporation of sodium $^{2}$H$_{3}$ acetate:

Sodium $^{2}$H$_{3}$ acetate (600 mg) was dissolved in sterile distilled water (10 ml) and divided between 10 flasks of V.dahliae brm-l liquid culture at 144 hours' growth.

The flasks were worked-up after a total of 13 days' growth, and the scytalone isolated was purified twice by preparative t.l.c. (1. acetone-light petroleum 60-80°, 50:50, 2. methanol-chloroform, 10:90) to give pure scytalone (38 mg).
(c) Incorporation of sodium \([1-^{13}C,^{18}O_2]\)acetate:—

Sodium \([1-^{13}C,^{18}O_2]\)acetate (180 mg) was dissolved in sterile distilled water (5 ml) and divided between 5 flasks of \(V.\text{dahliae brm-1}\) liquid culture at 168 hours' growth.

After 14 days total growth the cultures were worked-up and scytalone isolated and purified twice as described to give pure scytalone (31 mg).

(d) Incorporation of sodium \([^{2}H_3,1-^{13}C]\)acetate:—

Sodium \([^{2}H_3,1-^{13}C]\)acetate (500 mg) was dissolved in sterile distilled water (5 ml) and divided between 5 flasks of \(V.\text{dahliae brm-1}\) liquid culture at 168 hours' growth. At 14 days these were worked-up and the scytalone isolated and purified twice as described to give pure scytalone (35.2 mg).

(e) Incorporation of sodium \([^{2}H_3,2-^{13}C]\)acetate:—

Sodium \([^{2}H_3,2-^{13}C]\)acetate (214 mg) was dissolved in sterile distilled water (6 ml) and divided between 6 flasks of \(V.\text{dahliae brm-1}\) liquid culture at 156 hours' growth. At 14 days these were worked up and the scytalone isolated and purified twice as described to give the pure compound (20 mg).

N.m.r. studies

Carbon-13 n.m.r. spectra for \(\delta\)-shift studies were obtained on a Bruker WH360 instrument at the University of Edinburgh, Scotland. Carbon-13 spectra for \(\alpha\)-shift studies and 2D-\(\text{H},^{13}\text{C}\) correlation spectroscopy were run on a Bruker WH400 instrument at the University of Alberta, Canada.
Conversion of 1,3,8-THN to (-)-vermelone (17)

V. dahliae brm-1 was grown in 25 Petri dishes containing 20 ml each of PDA, and inoculated with an active suspension of the fungus. The inoculum was prepared by adding sterile distilled water (5 ml) to one stock slope of the culture, loosening the spores and conidia using a sterilised wire loop and adding the contents to one flask of modified Brandt's medium. The flask was incubated overnight on shaken culture in darkness and used in 0.2 ml aliquots to inoculate the plates.

The inoculum was smeared over the whole surface of the plate using a bent glass rod sterilised in ethanol. The plates were incubated at 24°C in darkness.

Scytalone (388 mg) was chemically dehydrated to 1,3,8-THN (16, 288 mg) as described in Chapter 2.6.

The 1,3,8-THN (288 mg) was dissolved in ethanol (1 ml) and added to a solution of 0.01 M potassium phosphate buffer (250 ml, pH 6.0) containing 1% sucrose, which had been purged with nitrogen for 20 minutes.

At 78 hours' growth the solution of 1,3,8-THN was divided between the plates in 10 ml portions, and incubated a further 18 hours. The supernatant liquid was decanted, and the agar cultures chopped and added. Acetone (1 l) was added and the mixture homogenised using a tissue homogeniser (Silverson), and left to stir for 10 hours. The mixture was then filtered through Büchner apparatus, and the filtrate evaporated to leave an aqueous residue. This was acidified to pH 5 with H₃PO₄, saturated with
salt and extracted into ethyl acetate (3 x 100 ml). The extracts were dried over MgSO$_4$ and evaporated to give a crude product. This was purified by preparative t.l.c., Rf 0.62 (chloroform-acetone, 9:1) to give pure vermelone (17, 109 mg, 37.5%). Recrystallisation from cyclohexane gave pale yellow crystals m.p. 88-93°C (Lit. m.p. 91-94°C$^{13}$). [Found C, 67.0; H, 5.81%. C$_{10}$H$_{10}$O$_3$ requires C, 67.4; H, 5.6%].

3.5.3 Scyaltalone derivatives

Scyaltalone monomethyl ether (73)$^6$

Scyaltalone (15, 154 mg) was dissolved in dry acetone (15 ml), cooled to 0°C and treated with ethereal diazomethane (0.32g). This was left to stir for 12 hours, after which time t.l.c. (acetone-light petroleum 60-80°C, 50:50) showed complete reaction. Evaporation of the solvents and purification by preparative t.l.c. gave pale brown crystals of pure scyaltalone monomethyl ether (73, 150 mg, 91%) m.p. 75-79°C (Lit. m.p. 76-82°C$^6$).

Methyl scyaltalone acetate (74)

Scyaltalone monomethyl ether (73, 162 mg) was dissolved in pyridine (1ml), cooled to 0°C and acetic anhydride (0.5 ml) added with stirring. After 2 hours the reaction mixture was poured onto ice-water (30 ml) and extracted into chloroform (2 x 50 ml). The chloroform solution was quickly washed with water (3 x 20 ml), dried over MgSO$_4$ and evaporated over a tepid water bath. Purification by preparative t.l.c., Rf 0.65 (ethyl acetate-
chloroform, 5:95) followed by recrystallisation from ether gave pale brown crystals of methyl scytalone acetate (74, 120 mg, 62%) m.p. 115-118°C. [Found C, 61.8; H, 5.61%; m/z 250 (M$^+$), 190 (M$^+$ - CH$_3$CO$_2$H). C$_{13}$H$_{14}$O$_5$ requires C, 62.4; H, 5.6%; M$^+$, 250]. $\delta_H$ 2.01 (3H, s, COCH$_3$); 2.85 (2H on AB of ABX, m, H-2); 3.09 (2H on AB of ABX, m, H-4); 3.81 (3H, s, OCH$_3$); 5.36 (1H, septet, H-3); 6.26 (2H, m, Ar-H); 12.6 (1H, s, OH).

**Methyl scytalone dibenzoate (75)**

Scytalone monomethyl ether (73, 71 mg) was dissolved in pyridine (1ml), cooled to 0°C and benzoyl chloride (0.25 ml) added with stirring. After 2 hours this was poured onto ice-water (30 ml) and extracted into chloroform (3 x 30 ml). The chloroform solution was quickly washed with 2M HCl (30 ml) and water (3 x 50 ml), dried over MgSO$_4$ and evaporated. Purification by preparative t.l.c. (acetone-light petroleum 60-80°, 50:50) followed by recrystallisation from ether-light petroleum 40-60° gave off-white crystals of pure methyl scytalone dibenzoate (75, 96 mg, 67%) m.p. 104-108°. [Found C, 70.82; H, 4.87%; m/z 416 (M$^+$), 294 (M$^+$ - PhCO$_2$H. C$_{25}$H$_{20}$O$_6$ requires C, 72.12; H, 4.81%; M$^+$ 416]. $\delta_H$ 2.91 (2H on AB of ABX, m, H-2); 3.35 (2H on AB of ABX, m, H-4); 3.81 (3H, s, OCH$_3$); 5.62 (1H, septet, J 3.44Hz, H-3); 6.65 (1H, d, J 2.49Hz, H-7); 6.69 (1H, d, J 2.45Hz, H-5); 7.35-8.23 (10H, m, OCOPh).
3.6 REFERENCES


4.1 INTRODUCTION

4.1.1 Whole-cell vs. cell-free systems

The study of secondary metabolism was until relatively recently mainly confined to whole-cell systems. There can be problems with these studies such as the lack of incorporation of substrates, not because they are not biosynthetic intermediates but because they may be unable to reach the site of metabolism in the cell, eg. they are unable to permeate the cell wall. This can be especially true of the more advanced and complex intermediates in biosynthesis.

The use of whole-cell systems often involves large volumes of culture media, which once fungal growth has reached the desired stage, must be killed, the mycelium removed, and the metabolites extracted into organic solvents. Yields of metabolites are frequently less than one hundred milligrams per litre of culture medium.

The application of cell-free systems such as those used in biochemistry for the study of primary metabolism, is an attractive prospect for the study of secondary metabolic processes. Cell-free systems provide a more simple and direct way of studying individual reactions. In cell-free systems it is easier to achieve the anaerobic conditions required to prevent unwanted oxidative reactions. The reactions and extraction procedures may be done on a smaller scale and hence with a greater chance of achieving good recovery of metabolites or undigested substrates.
These methods have met with some but limited success. This may be due to the enzymes involved existing in the cell as aggregates or complexes which are dependent on the structure of the complex as a whole for their activity. These aggregates may be dispersed during disruption of the cell causing loss of enzyme activity. The enzymes may also be intimately connected to structural features of the cell (e.g. cell membranes) which are disrupted during the process of preparation of the cell-free extracts.

Procedures designed to give active cell-free preparations for the study of secondary biosynthetic processes were often difficult in the past and ultimately unreliable. As biochemical techniques have improved, these methods have become more accessible and more widely applicable.

4.1.2 Cell-free techniques for melanin biosynthesis

In the study of the melanin biosynthetic pathway in fungi imperfecti, cell-free methods have been demonstrated to work in several species, and various preparation procedures used.

In V. dahliae, homogenates were prepared from 6-day-old fungal tissues. These were placed in a potassium phosphate buffer, blended to a suspension and mixed with glass beads. A tissue homogeniser was used to ballistically disrupt the cells, and the glass beads, cellular debris and remaining unbroken cells removed by centrifuging the mixture and decanting the supernatant liquid. This formed the extract, and was further purified by filtration to remove lipids and suspended debris.
Cell-free homogenates were used to study the metabolism of 1,3,6,8-THN, scytalone, 1,3,8-THN and vermelone in homogenates of both wild-type *V. dahliae* and also albino (*alm*-1) and brown (*brm*-1 and *brm*-2) mutants. The reductase reactions required NADPH, and both reduction and dehydration reactions were inactivated by heating homogenates to 70°C. The dehydratase enzyme appeared to be more stable to heat and ageing than the reductase. Inhibition of the reductase reactions was demonstrated with tricyclazole, pyroquilon and PP-389. T.l.c. was used to evaluate the results of these studies. Although the techniques were successful, the activity of individual homogenates varied considerably. All homogenates were used within several hours of preparation.

The technique of homogenate preparation was applied successfully also to *P. oryzae*¹ and *Wangiella dermatitidis*³, and interconversion of 1,3,6,8-THN, scytalone, 1,3,8-THN and vermelone demonstrated as for *V. dahliae*.

Analysis of the results of such experiments was greatly improved by the development of High Performance Liquid Chromatography (H.P.L.C.) techniques for the purification and separation of melanin intermediates and related shunt products of the biosynthetic pathway.⁴ The extraction of the compounds from acidified aqueous brine was accomplished using reverse-phase Sep-Pak cartridges, and they were separated on a reverse-phase H.P.L.C. column. Twelve of the fourteen compounds were separated using a linear gradient of 12-14% acetonitrile-water with 2% acetic acid, and the remaining two using an isocratic system of
5% aqueous acetonitrile with 2% acetic acid.

The sensitivity of H.P.L.C. for the detection of these compounds was obviously an advantage, as much lower levels of substrates and metabolites could be detected and quantified. Quantification previously was based on visual estimation of the intensities of spots on t.l.c. plates when viewed under uv light, and was therefore not very precise. H.P.L.C. offered a much more accurate and reproducible determination of the quantities involved.

Subsequently the method of preparation of cell-free homogenates of *P*. *oryzae* was improved. 5 7-Day-old cultures of the fungus were frozen at -70°C and then freeze-dried to disrupt the cells. The freeze-dried culture was pulverised, and the resulting powder stored at -70°C. To prepare extracts, the powder was first washed with acetone to remove any endogenous melanin intermediates, filtered, and the powder dried. This was suspended in phosphate buffer and stirred for 20 minutes. After centrifugation, the supernatant liquid was decanted, and finally filtered to remove remaining lipids and cell debris. The whole process was carried out at about 6°C.

This procedure gave two advantages over the original one. Firstly it was much simpler and quicker. Secondly, quantities of the fungus could be stored as the freeze-dried powder for use when required, thereby reducing the amount of preliminary preparation needed for each experiment.

Thus with improved techniques for both preparation and
analysis of cell-free systems active in melanin biosynthesis, more detailed study of this pathway should be possible.

It was planned to try and reproduce some of these results to test their validity, and to modify and extend the use of the H.P.L.C. system.

4.2 MODIFICATION OF H.P.L.C. ANALYSIS

Under anaerobic conditions in which unwanted oxidative reactions of melanin intermediates are eliminated, the two branch pathways resulting from oxidation of 1,3,6,8-THN and 1,3,8-THN will not be active. Thus the intermediates present in reaction mixtures should only include 1,3,6,8-THN, scytalone, 1,3,8-THN, vermelone and 1,8-DHN. Melanin would not be expected as this is formed by oxidation of 1,8-DHN.

The H.P.L.C. system for the analysis of homogenate reactions in P. oryzae used a linear gradient system for elution, as mentioned, increasing the concentration of acetonitrile in water and acetic acid. The system produced good separation of intermediates, but the total run time was 40 minutes. For an experiment involving several samples and controls, the time required for analysis was considerable. It was desirable to be able to prepare homogenates, run experiments and analyse them all within one day to obtain accuracy of results. Thus it was proposed to try and find an isocratic elution system, which would adequately separate the compounds and have a shorter run time. Using a gradient system also means that if a run is stopped in
Fig. 4.2 (c) vermelone, (d) 1,3,8-THN, (e) mixture.
Fig. 4.2 Wavelength profiles of intermediates including peak shape at 254nm (a) scytalone, (b) 1,3,6,8-THN.
Fig. 4.1  HPLC separations in acetonitrile-water-acetic acid, 26:72:2; scytalone (15), 1,3,6,8-THN (14), vermelone (17) and 1,3,8-THN (16).
the middle, the column still has to be re-equilibrated before the
next run. With an isocratic system this is obviously unnecessary.

Based on the information available on isocratic systems, the
first one tried was a 26:72:2 v/v mixture of acetonitrile–water–
acetic acid. Standard solutions were made up of 1,3,6,8-THN, scytalone, 1,3,8-THN and vermelone in the eluent at known
concentrations (approx. 1 mg/ml), and chromatographed individ-
ually. Sharp peaks were obtained, and the retention times ($R_t$) differed sufficiently to expect good resolution of a mixture.
Scytalone had the shortest $R_t$, then 1,3,6,8-THN, vermelone and
1,3,8-THN in that order. The longest $R_t$ (for 1,3,8-THN) was less
than 8 minutes. A mixture of the standards was made up and run.
In the mixture, separation of 1,3,8-THN from vermelone, and
vermelone from 1,3,6,8-THN was good. Resolution of scytalone and
1,3,6,8-THN was only partial, the scytalone appearing as a
shoulder on the larger peak (Fig.4.1). It seemed that in the
mixture, the $R_t$ of the compounds were significantly affected by
each other.

An alternative to altering the solvent system was thought to
be the use of different wavelengths to detect the compounds. A
Diode-Array Detector was used to obtain wavelength profiles of
each compound and the mixture (Fig.4.2a–e). These were used to
predict wavelengths at which the intensity of absorption of
scytalone would increase relative to 1,3,6,8-THN, and thus enable
better resolution of these peaks.

Wavelengths of between 254 and 300nm looked promising, and
Fig. 4.5 HPLC separations of scytalone (15) and 1,3,6,8-THN (14) in mixtures of varying proportions (for % of each component, see Table 4.1).
Fig. 4.4 HPLC separations in acetonitrile–water–acetic acid, 20:78:2; scytalone (15), 1,3,6,8-THN (14), vermelone (17) and 1,3,8-THN (16).
Fig. 4.3 HPLC detection of intermediates at different wavelengths
(a) 254 nm, (b) 270 nm, (c) 280 nm.
the standard mixture was chromatographed at 254, 270 and 280nm (Fig.4.3). At 254nm no resolution of 1,3,6,8-THN and scytalone was seen, due to a slightly higher column operating temperature than before. This also shortened the total run time.

At 270 and 280nm, the ratios of the different peaks were considerably altered, but there was still no resolution of the two coincident peaks. This idea was thus abandoned, as the quantification of the compounds would also be complicated by altering the detection wavelength.

To resolve the scytalone and 1,3,6,8-THN peaks more efficiently the eluent system was then changed to decrease the acetonitrile component and hence increase the $R_t$ of each compound (also the total run time). The composition used was then 20:78:2 v/v acetonitrile-water-acetic acid. Runs of individual compounds again gave sharp, well-separated peaks. In the mixture, although the scytalone and 1,3,6,8-THN peaks again converged, resolution was good enough to be able to distinguish them (Fig.4.4).

To test the scope for quantifying the two compounds in mixtures of scytalone and 1,3,6,8-THN, mixtures of the two standard solutions were made up in varying proportions and chromatographed. As the scytalone peak is less intense at 254nm, at the same substrate concentration, it was feared that in mixtures containing trace amounts of this relative to 1,3,6,8-THN, the scytalone peak would not be distinguished. Ten different mixtures were tested and satisfactory results were obtained (Fig.4.5).
Fig. 4.6 HPLC standard curves in mixtures of scytalone and 1,3,6,8-THN (a) scytalone, (b) 1,3,6,8-THN.
Table 4.1 HPLC quantification in mixtures of varying proportions of (a) scytalone (15) and (b) 1,3,6,8-THN (14).

(a)  | Sample | % (15) | (15)/μg | Peak area |
-----|--------|--------|---------|-----------|
  a  | 100    | 33.2   | 3,890,257 |
  b  | 90     | 29.9   | 4,244,971 |
  c  | 80     | 26.6   | 3,133,984 |
  d  | 70     | 23.2   | 2,660,150 |
  e  | 50     | 16.6   | 1,833,983 |
  f  | 30     | 9.7    | 1,098,383 |
  g  | 25     | 8.3    | 896,217   |
  h  | 20     | 6.6    | 702,372   |
  i  | 15     | 4.9    | 520,643   |
  j  | 10     | 3.3    | 347,682   |

(b)  | Sample | % (14) | (14)/μg | Peak area |
-----|--------|--------|---------|-----------|
  b  | 10     | 2.2    | -       |
  c  | 20     | 4.4    | 1,759,610 |
  d  | 30     | 6.5    | 2,804,543 |
  e  | 50     | 10.9   | 4,609,684 |
  f  | 70     | 15.3   | 6,676,396 |
  g  | 75     | 16.4   | 5,093,255 |
  h  | 80     | 17.4   | 5,417,301 |
  i  | 85     | 18.5   | 5,696,423 |
  j  | 90     | 19.6   | 6,154,293 |
  k  | 100    | 21.8   | 8,937,088 |
Integration of peak area was compared to the absolute amount of substrate present for each sample (Table 4.1a & b). Sensitivity was good enough to detect accurately as little as 3μg scytalone in a 100μl sample injection (Fig.4.6a), and increase of peak area was linear with increase in substrate concentration. Quantification of 1,3,6,8-THN was also good (Fig.4.6b).

The isocratic system consisting of 20:78:2 v/v acetonitrile-water-acetic acid was settled on as the optimum one, and used for analysis of the cell-free homogenate experiments. Retention times of the compounds were apt to vary with the operating conditions of the H.P.L.C., but in practice the substrates were easily identifiable.

4.3 EXPERIMENTS WITH CELL-FREE HOMOGENATES

4.3.1 P. oryzae

Cell-free homogenates of P. oryzae were prepared from freeze-dried powders of the fungus stored at -80°C as described. All steps of the preparation were carried out at 4-6°C and the homogenates kept cold until used. The homogenates were then used to reproduce the in vivo melanin reactions in vitro, and to assess the effects of the potent systemic anti-fungal agents tricyclazole and PP-389 on the metabolic pathway. The results were analysed by H.P.L.C. using the system discussed (4.2).

Initial experiments had little success. As a guide to whether the extraction procedure was responsible, a determination of the protein concentration of the extract was performed using the
Fig. 4.8 Non-conversion of 1,3,6,8-THN (14) in P. oryzae (a) Control containing 1,3,6,8-THN but no enzyme, (b) Enzyme reaction.
Fig. 4.7 Conversion of scytalone (15) to 1,3,8-THN (16) in *P. oryzae* (a) Control containing scytalone but no enzyme, (b) Enzyme reaction.
Bradford method. Protein extracted was calculated to be 6.6mg per gram fungal powder, i.e. 0.6% w/w. This was compared to reports of protein extraction from the fungus Botrytis cinerea, and appeared to be in the expected range. The extraction procedure thus seemed adequate, and further experiments with the homogenates were tried.

Scytalone was fed to fresh homogenates, and incubated anaerobically overnight. Analysis of the reaction mixtures showed good activity of the dehydratase enzyme, as the peak due to scytalone had largely disappeared in enzyme-containing mixtures, and a peak corresponding to 1,3,8-THN had appeared (Fig.4.7). Further metabolism of the 1,3,8-THN was not expected, as the reductase requires NADPH as co-factor. The activity of the cell-free homogenates varied, so that scytalone was sometimes completely metabolised and sometimes only partially.

1,3,6,8-THN was fed to extracts, with the addition of NADPH as co-factor, and incubated overnight. H.P.L.C. analysis showed that no reduction had occurred, and the same was observed in repeat experiments (Fig.4.8).

The preliminary acetone wash in homogenate preparation was suggested as a possible reason for the lack of reductase activity. Omitting this step made no difference and the lack of reductase activity remained unexplained.

The dehydratase conversions were performed with the addition of tricyclazole to reaction mixtures. Tricyclazole inhibits the reductive conversions in P.oryzae, and no effect was anticipated,
Fig. 4.10 Conversion of 1,3,6,8-THN (14) to scytalone (15) in V. dahliae brm-1 (a) Control containing 1,3,6,8-THN but no enzyme, (b) Enzyme reaction, (c) Control containing enzyme extract but no 1,3,6,8-THN.
Fig. 4.9 Conversion of scytalone (15) to 1,3,8-THN (16) in *P. oryzae* in the presence of inhibitors (a) No enzyme, (b) Enzyme and PP-389, (c) Enzyme and tricyclazole.
or seen. The same result was expected and observed for the anti-fungal agent PP-389 (Fig.4.9).

4.3.2 V.dahliae

The brown mutant brm-1 of V.dahliae lacks the dehydratase enzyme involved in melanin biosynthesis, and thus accumulates scyitalone. If treated with 1,3,8-THN, vermelone is produced. It was decided to apply the cell-free homogenate preparation procedure used for P.oryzae on this brm-1 mutant of V.dahliae to investigate whether reductase activity could be observed here.

A similar preparation procedure was followed, with slight modifications such as the volume of buffer used to prepare extracts from known weight of fungal mycelium. The concentration of buffer was as for previous experiments with V.dahliae.2

1,3,6,8-mN was added to the extracts with NADPH and incubated anaerobically for 3 hours. Analysis of this showed an apparent decrease of the level of substrate, and the appearance of a peak corresponding to scyitalone (Fig.4.10). Control reactions containing extract but without substrate also showed trace amounts of scyitalone (Fig.4.10c). Thus it was difficult to interpret the results of these experiments with any certainty. A broad peak at Rf 3.8 - 4.0 also appeared in all traces, but was hence disregarded.

When 1,3,8-THN was added to extracts along with NADPH, because the retention times of substrate and metabolite were much
Fig. 4.13 Conversion of 1,3,8-THN (16) to vermelone (17) in V. dahliae brm-1 in the presence of tricyclazole (a) No inhibitor, (b) 12 p.p.m., (c) 24 p.p.m., (d) 36 p.p.m.
Fig. 4.12 Disappearance of 1,3,8-THN (16) and coincident appearance of vermione (17) in enzyme extract of V. dahliae brm-1.
Fig. 4.11 Conversion of 1,3,8-THN (16) to vermelone (17) in V. dahliae brm-1 (a) Control containing 1,3,8-THN but no enzyme, (b) Enzyme reaction.
greater than that of endogenous scytalone, the result was clear. The extract had metabolised 1,3,8-THN and produced vermelone (Fig.4.11). After 3 hours' reaction time there was 80% conversion of the substrate.

A time-course experiment was then undertaken with this cell-free system, removing samples from the reaction mixture at time intervals for analysis. The results showed an initially fast rate of disappearance of 1,3,8-THN, which decreased with time. A correspondingly fast initial rate of appearance of vermelone also decreased with time (Fig.4.12). At 3 hours there was 60% conversion of the substrate, thus a slightly different level of enzyme activity from the previous enzyme extract.

A time-course experiment was attempted for the metabolism of 1,3,6,8-THN, but the results were inconclusive for the reasons mentioned above.

The reductase inhibitor tricyclazole was tested in this system using 1,3,8-THN as substrate and NADPH as co-factor. One reaction was left untreated, and three treated with tricyclazole at dose levels of 12, 24 and 36 p.p.m.

The untreated reaction showed almost complete conversion of 1,3,8-THN after overnight incubation (Fig.4.13a). Treated reactions showed significant inhibition of the reductase. Inhibition even at 12 p.p.m. was good, and only a small increase was seen by raising the dose to 24 or 36 p.p.m. (Fig.4.13b, c, and d).
Thus the melanin biosynthetic reactions of *P. oryzae* and *V. dahliae* *brm-1* were conveniently and directly observed *in vitro* by the use of cell-free preparations of freeze-dried powders of the fungi, and analysed by H.P.L.C. The lack of reductase activity from extracts of *P. oryzae* was strange, especially as the same preparation procedure succeeded for *V. dahliae* *brm-1*.

The inhibition of reductase activity *in vitro* by tricyclazole supports the evidence that it is melanin biosynthesis inhibition which is responsible for the anti-fungal activity of this and other anti-penetrant anti-blast agents *in vivo*.

**4.3.3 Future work**

The next major task in the study of melanin biosynthesis in *fungi imperfecti* would be to isolate, purify and characterise the enzymes involved in the reduction and dehydration steps. One problem with the cell-free homogenate methods at present is the variability of extract activity as mentioned. If purification and characterisation could be achieved, more precise quantification of substrate metabolism would be feasible and profitable. The affinity of the enzymes for substrates could also be investigated, as each enzyme appears to accept several substrates (see Chapter 1).

Characterisation of the type of enzyme inhibition and the affinity of the inhibitors, using classical enzyme kinetics would be worthwhile. This could possibly be achieved by spectrophotometric methods to monitor the rate of loss of NADPH during the reductase reaction, in preference to H.P.L.C.
4.4 EXPERIMENTAL

Chemicals:-

The melanin metabolites scytalione and vermelone were isolated from cultures of *P. lagerbergii* and *V. dahliae brm-1* as described (Chapter 3). 1,3,6,8-THN was synthesised from 3,5-dihydroxybenzoic acid, and 1,3,8-THN prepared from scytalione (Chapter 2). Tricyclazole and PP-389 were supplied by ICI.

Isolates:-

Wild-type *P. oryzae* (strains K1017C and K1017D) was supplied by I.C.I. *V. dahliae Klebahn brm-1* (ATCC 44571) was supplied by the U.S.A. National Cotton Pathology Research Laboratory, Texas.

Culture conditions:-

All cultures were maintained on Potato Dextrose Agar (PDA, Oxoid) and grown on PDA in 100x15mm Petri dishes (Sterilin) to prepare freeze-dried powders for extraction. PDA was covered with a single sheet of cellophane (Type 1850-221, Pharmacia) which had been sterilised at 15 p.s.i. for 15 minutes and inoculated with 0.5ml of a fungal suspension containing conidia and spores.

Inoculum for *P. oryzae* was obtained by loosening conidia and spores from 14- to 21-day-old plate cultures into 15ml sterile distilled water using a sterilised wire loop. Inoculum for *V. dahliae brm-1* was obtained by loosening conidia and spores from 10- to 14-day-old slope cultures into 10ml sterile distilled water using a sterilised wire loop.

PDA plate cultures were incubated at 24°C in the dark for 7 days (*P. oryzae*) or 6 days (*V. dahliae brm-1*).
Preparation of extracts:-

P. oryzae or V. dahliae brm-1 was scraped from PDA cultures and frozen at -80°C. The fungus was freeze-dried, ground to a powder in a pestle and mortar and stored at -80°C. Extracts were prepared from the dry powders, all steps being carried out at about 6°C.

**P. oryzae:**

The dried powder (1.0g) was suspended in acetone (100ml) with stirring for 15 minutes. The suspension was filtered through Whatman no.1 filter paper and the dried powder (0.84g) resuspended in 0.2mM potassium phosphate buffer (20ml, pH 7) containing 2.0mM EDTA and 2.0mM DTT. After stirring for 20 minutes the solution was centrifuged at 30,700g (16,000 rpm, Sorvall centrifuge with no.5 rotor) for 20 minutes at 4°C. The supernatant liquid was decanted and formed the extract. This was used without further purification.

**V. dahliae brm-1:**

The dried powder (1.0g) was suspended in acetone (100ml) and stirred 15 minutes. The suspension was filtered through Whatman no.1 paper and the dried powder resuspended in 100mM potassium phosphate buffer (10ml, pH 6.8) containing 1.0mM EDTA and 1.0mM DTT. After stirring for 20 minutes the solution was centrifuged at 30,700g (16,000 rpm) for 20 minutes at 4°C. The supernatant
liquid was decanted to form the extract and used without further purification.

All extracts were kept at 4°C and used within 4 hours of preparation.

**Enzymatic conversions:**

**P. oryzae:**

To measure the enzymatic conversion of 1,3,6,8-THN, reaction mixtures contained 1.8 ml extract, 2.6 ml 20 mM potassium phosphate buffer (pH 7) and 6.0 μmol exogenous NADPH.

To measure the conversion of scytalone to 1,3,8-THN, reaction mixtures contained 0.55 ml extract and 3.85 ml buffer without NADPH.

To measure the conversion of scytalone to 1,8-DHN, reaction mixtures contained 0.55 ml extract, 3.85 ml buffer and 6 μmol exogenous NADPH.

**V. dahliae brm-1:**

To measure the enzymatic conversions of 1,3,6,8-THN to scytalone or 1,3,8-THN to vermelone, reaction mixtures contained 2.4 ml extract, 2.5 ml 100 mM potassium phosphate buffer and 6 μmol exogenous NADPH.

Mixtures were pre-incubated in 165 x 16 mm Vacutainer tubes (Becton-Dickinson) at 25°C for 15 minutes prior to addition of substrate. Nitrogen was bubbled through the solutions for 5 minutes before and after addition, via a luered double-needle gas-purge unit (18 gauge, Aldrich) to ensure anaerobic conditions. Substrates (5 μmol) were dissolved in ethanol (0.1 ml),
mixed with buffer (0.4ml) and added to the mixtures to start reaction.

Where inhibitors were used, these were added in ethanol (0.1ml) at the start of pre-incubation. For both P. oryzae and V. dahiae brm-l reactions, controls containing extract and buffer but no substrate had 0.2ml ethanol added. Other controls contained only buffer and NADPH if required, to which substrates (5μmol) in ethanol (0.1ml) and buffer (0.4ml) were added. Controls were similarly made anaerobic.

Reactions were incubated 3 hours or overnight. The reactions were stopped by the addition of glacial acetic acid (0.4ml) and salt (1g).

**Analysis by H.P.L.C.:-**

The acidified and salted reaction mixtures were applied to C18 reverse-phase Sep-Pak cartridges (Waters Associates) prepared according to manufacturers instructions. The cartridges were washed with water (10ml) and eluted with a volume of acetonitrile-water-acetic acid (55:43:2 v/v) sufficient to collect 5ml. Aliquots were diluted with 2 volumes water and chromatographed.

A modular H.P.L.C. system was used, consisting of LC-6A pump, SCL-6A system-controller, SPD-6AV uv-visible spectrophotometric detector (Shimadzu) and TRIO computing integrator (Trivector). For variable wavelength scans a diode-array detector (Philips PU6003) and computer (Philips) were used.

The column was a pre-packed 4.0 x 250mm Spherisorb ODS2 5μm
Fig. 4.14 Standard curve of net absorbance at 595nm against known concentration of Bovine Serum Albumin (BSA) for the Bradford protein assay.
reverse-phase protected with a 4.0 x 10mm ODS2 5μm guard column (Pharmacia LKB). The mobile phase flow rate was 0.7 ml/minute.

Detection of 1,3,6,8-THN, scytalone, 1,3,8-THN and vermelone was at 254 nm unless otherwise stated. Quantitative determination of substrates and metabolites was obtained using an isocratic elution system of acetonitrile-water-acetic acid (20:78:2 v/v). The isocratic system took 10 - 30 minutes to run, depending on substrates used.

Bradford protein assay\(^6\):-

The Bradford protein assay is based on the absorbance shift in a Coomassie Brilliant blue G-250 solution. A series of known protein concentrations was prepared from a stock solution of Bovine Serum Albumin (BSA). Protein Assay Reagent (Pierce, 0.5ml) was added to each dilution (0.1ml) and mixed. The absorbance of each mixture was measured at 595nm in a uv-spectrophotometer (Perkin-Elmer Lambda 3B uv-vis) and a standard curve of absorbance against known protein concentration plotted (Fig.4.14).

The sample of cell-free homogenate of \(P.\)oryzae (0.1ml) was similarly mixed with Protein Assay Reagent (0.5ml) and its absorbance at 595nm measured. The corresponding protein concentration was then read from the standard curve.

From this, the weight of protein extracted from a known weight was calculated to be 6.6mg per gram fungal powder, i.e. 0.6% w/w.
4.5 **EXPERIMENTAL**


