RECENT DEVELOPMENTS IN THE SYNTHESIS
OF PEPTIDES AND GLYCOPEPTIDES

by

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To my parents, Thomas and Diane,

and to Mandy – with many thanks.
Further development of the 2,2-\textit{bis} (4'-nitrophenyl)ethoxycarbonyl (Bnpeoc) group, recently introduced as an N$^\alpha$-protecting group in peptide synthesis, is presented. Comparison between this moiety and the analogous Fmoc protecting group has led to a greater understanding of its protection and deprotection requirements.

The use of this group is exemplified by the preparation of a tripeptide, N$^\alpha$-[2,2-\textit{bis} (4'-nitrophenyl)ethoxycarbonyl]-alanylphenylalanylglycine, by two syntheses in solution. A solid phase synthesis was also carried out manually, at the same time re-introducing acid-chloride coupling methodology to the field.

The preparation of N$^\alpha$-(benzyloxycarbonyl)alanylalanylserine (O-2,3,4,6-tetra-O-benzyl-\alpha/\beta-glucopyranosyl)benzyl ester has shown that Bnpeoc may also have a useful role in the new area of glycopeptide synthesis.

A novel coupling reagent, diphenylphosphinic anhydride, useful in the mediation of both amide and ester bond formation is introduced. Several attempts at the preparation of the reagent are discussed. Phosphorus n.m.r. is shown to be an excellent method for monitoring its long-term stability and its reactivity towards nucleophiles.

These new protection and activation/coupling methodologies have been fitted into the automated solid phase synthesis protocol, alongside more conventional methods (\textit{e.g.} carbodiimide/HOBt), enabling several test peptides to be prepared.
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<table>
<thead>
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<th>Full Form</th>
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<tr>
<td>Ac</td>
<td>acetyl</td>
</tr>
<tr>
<td>AcOH</td>
<td>acetic acid</td>
</tr>
<tr>
<td>AFGP</td>
<td>antifreeze glycoproteins</td>
</tr>
<tr>
<td>AFP</td>
<td>antifreeze proteins</td>
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<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>Bnpeoc</td>
<td>2,2-bis(4'-nitrophenyl)ethoxycarbonyl</td>
</tr>
<tr>
<td>Boc</td>
<td>tertbutyloxy carbonyl</td>
</tr>
<tr>
<td>BzI</td>
<td>benzyl</td>
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<tr>
<td>CHA</td>
<td>cyclohexylamine</td>
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<td>DBN</td>
<td>1,5-diazabicyclo[4.3.0]non-3-ene</td>
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<td>DBU</td>
<td>1,8-diazabicyclo[5.4.0]undec-7-ene</td>
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<tr>
<td>DCCI</td>
<td>1,3-dicyclohexyl carbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
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<tr>
<td>DICI</td>
<td>1,3-diisopropyl carbodiimide</td>
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<tr>
<td>DMA</td>
<td>N,N-dimethylacetamide</td>
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<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<td>Dpp</td>
<td>diphenylphosphinyl</td>
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<td>Fmoc</td>
<td>9-fluorenlymethoxycarbonyl</td>
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<tr>
<td>Gal</td>
<td>galactose</td>
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<tr>
<td>Gluc</td>
<td>glucose</td>
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<tr>
<td>HOObt</td>
<td>N-hydroxybenzotriazole</td>
</tr>
<tr>
<td>Hplc</td>
<td>high-performance liquid chromatography</td>
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<tr>
<td>Mbb</td>
<td>N-bis(4'-methoxyphenyl)methyl</td>
</tr>
<tr>
<td>NMM</td>
<td>N-methyloxymorphone</td>
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<tr>
<td>NMP</td>
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<tr>
<td>Pmc</td>
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<td>SPPS</td>
<td>solid phase peptide synthesis</td>
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<td>tetraethylammonium fluoride</td>
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<td>trifluoroacetic acid</td>
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<tr>
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<tr>
<td>TosO</td>
<td>p-toluenesulphonyl</td>
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<td>Trt</td>
<td>trityl</td>
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CHAPTER 1
INTRODUCTION

1.1. Ubiquitin

1.1.1. Introduction

Ubiquitin, a 76 α-amino acid polyfunctional protein, was first reported as being present in bovine thymus by Goldstein et al.\textsuperscript{1} in 1975, during studies on the isolation of the thymic polypeptide hormone, thymopoietin.

Its presence in animal cells, yeast, bacteria and higher plants led the author to postulate that the protein was in fact a universal constituent of living cells.

Subsequent radioimmunoassay studies have shown ubiquitin to be present, either free or covalently bound, in the nucleus, cytoplasm and at the cell surface of all eukaryotic cells.

The primary amino acid sequence of ubiquitin (Figure 1.1) is the most evolutionarily conserved of all known proteins\textsuperscript{2,3}, with variations confined to four residues over all species studied to date, namely residues 19, 24, 28 and 51 (Figure 1.2).

\begin{verbatim}
H-Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-Lys-Thr-Ile-Thr-Leu-
  20     25     30
-Glu-Val-Glu-Pro-Ser-Asp-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-
  35     40     45
-Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-Ile-Phe-
  50     55     60
-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Tyr-Asn-
  65     70     75
-Ile-Gln-Lys-Glu-Ser-Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-Gly-
-Gly-OH
\end{verbatim}

Figure 1.1 Primary amino acid sequence of human erythrocytic ubiquitin.
Ubiquitin source

<table>
<thead>
<tr>
<th>Residue</th>
<th>Animal&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Plant&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Yeast</th>
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<tr>
<td>19</td>
<td>Pro</td>
<td>Ser</td>
<td>Ser</td>
</tr>
<tr>
<td>24</td>
<td>Glu</td>
<td>Asp</td>
<td>Asp</td>
</tr>
<tr>
<td>28</td>
<td>Ala</td>
<td>Ala</td>
<td>Ser</td>
</tr>
<tr>
<td>57</td>
<td>Ser</td>
<td>Ala</td>
<td>Ser</td>
</tr>
</tbody>
</table>

a. animal refers to that isolated from man, cattle, mouse, chicken, toad and fly.
b. plant refers to that isolated from oat and barley.

Figure 1.2 Primary sequence variations of ubiquitin.

These observations clearly suggest that ubiquitin has a fundamental role in basic cellular chemistry, and recent investigations into the polyfunctional nature of ubiquitin, together with the elucidation of its crystal structure (see below), have led to a greater understanding of its physiological significance.

1.1.2. Biological significance of ubiquitin

Ubiquitin-coding DNA sequences have been cloned from a number of eukaryotes<sup>4,5,6,7</sup>. These investigations have shown ubiquitin-coding genes to be organised into head-to-tail repeat units with no internal spacers. Thus suggesting mature ubiquitin is produced by selective proteolytic processing of the precursor protein, polyubiquitin.

In the case of yeast ubiquitin<sup>4</sup>, six consecutive ubiquitin monomers are bound directly via Gly-Met peptide bonds of the first and last residues of adjacent ubiquitin molecules, with a final non-ubiquitin amino acid, asparagine, also present.

A study of the crystal structure of ubiquitin (see below) shows the amino terminal methionine residue to be buried within the molecule. Thus considerable conformational change must take place, after cleavage of the polyubiquitin, in order to produce the mature ubiquitin monomer.

However the number of consecutive repeats per gene is not conserved over all species, for example, human ubiquitin has been shown to have nine. Another
feature which is not species conserved is the identity of the final, non-ubiquitin amino acid; it being asparagine in yeast, valine in human and tyrosine in chicken ubiquitin. However this is not thought essential to the precursor protein, due to the discovery that the clone isolated from the toad *Xenopus laevis* had no additional amino acid present.

Ubiquitin demonstrates a fundamental and perhaps diverse role in the life of the cell, and has been identified as present in the nucleus, in the cytoplasm and on the cell-surface membrane of eukaryotic cells (for review).

Its major function is thought to be the targetting of ubiquitin-protein conjugates for degradation by non-lysosomal ATP-dependent proteolysis systems i.e. ubiquitin may act as a signal for the attack of proteinases specific to ubiquitin-protein conjugates. Protein breakdown by this pathway requires the formation of a covalent bond between the carboxyl terminus of ubiquitin and the target protein. Two distinct classes of ubiquitin-protein conjugate have been identified to date: the first displays an isopeptide bond formed with the ε-NH$_2$ group of a protein lysine residue, the other formed by ubiquitination of the terminal α-NH$_2$ group of the acceptor protein.

Selective *in vitro* modification of the terminal α-NH$_2$ groups of proteins prevents degradation, thus suggesting degradation requires the NH$_2$-terminal ubiquitination of proteolytic substrates. Investigations have shown that a reduced rate of degradation by the ubiquitin pathway exists for proteins with blocked ε-NH$_2$ groups, but free α-NH$_2$ groups.

There appear to be a number of highly specific enzymes which mediate the concerted reactions that result in the formation of ubiquitinated proteins (Figure 1.3). The resulting conjugates, which may contain more than one ubiquitin molecule, are either degraded or simply deubiquitinated.

The existence of at least two distinct enzymes, with different specificities for the non-ubiquitin moiety, which catalyse the deubiquitination process, has also been shown.

The four stages in the formation of ubiquitin-protein conjugates (and the enzymes of the ubiquitin ligase system) are:

1. ubiquitin carboxy-terminal adenylation (ubiquitin activating enzyme, E1)
2. thiolester bond formation (enzyme E1)

3. transesterification (enzymes E1 and E2)

4. conjugation of ubiquitin to the target protein (enzymes E2 and E3).

Figure 1.3 Pathways of the ubiquitin system.

The first such ubiquitin–protein conjugate reported displayed the formation of an isopeptide bond, between the ε-NH₂ group of lysine-119 of histone H2A, and the carboxyl terminus of ubiquitin. The function of this conjugate is as yet unproved, but it is believed that this 'branched' ubiquitin-H2A (uH2A) semi-histone may result in specific degradation at transcriptionally active chromosome sites, and that ubiquitination may indirectly influence the cell-cycle by regulating the lifetime of 'cyclins'. 
The first example of a protein at the cell-surface being covalently ubiquitinated was reported by Weissman et al.\textsuperscript{19,20}, who discovered a 90 kDa glycoprotein lymphocyte homing receptor bound to ubiquitin.

Biochemical investigations of the lymphocyte cell-surface receptor involved in this homing were made possible by the development of a monoclonal antibody (MEL-14) which detects the receptor on only the lymphocytes that home in on the peripheral lymph node high endothelial venules (HEVs). It is known that a large number of circulating lymphocytes enter the bloodstream through this specialized endothelium. Subsequent screening of λgt 11 expression libraries with MEL-14, revealed the ubiquitin sequence in several reactive clones\textsuperscript{21}. Further studies confirmed the obtained glycoprotein to be ubiquitinated at the $\varepsilon$-$\text{NH}_2$ group of a lysine residue analogous to the ubiquitination of the histone H2A.

The discovery of ubiquitin at the cell surface merits further investigation to determine, for example, the mode of transport of ubiquitin to that location and what difference, if any, in function, there is between cell-surface ubiquitin and its intracellular counterpart.

1.1.3. Structure of ubiquitin

Apart from the physiological significance and the unparalleled sequence conservation of ubiquitin, its structure has been of some interest due to the remarkable stability of this relatively small polypeptide.

It is noteworthy that the molecule is extremely resistant to trypsic digestion despite the presence of seven lysine and four arginine residues, as well as being stable to a wide range of pH and temperature. Nuclear magnetic resonance studies have demonstrated no apparent denaturation over a pH range of 1.18–8.48 and a temperature range of 23–80°C, whilst predicting a compact, globular structure\textsuperscript{22}.

More recently the three dimensional structure by X-ray analysis at 2.8Å resolution of human erythrocytic ubiquitin\textsuperscript{23}, together with a further refinement to 1.8Å\textsuperscript{24}, have been reported.

These studies have shown ubiquitin to be extremely compact, with a pronounced hydrophobic core (of the 21 valine, leucine, isoleucine and methionine residues, 16 are buried within the interior of the molecule), and tightly hydrogen-bonded – with approximately 87% of the polypeptide chain involved in hydrogen-bonded secondary structure.
Noteworthy secondary structure features include three and one-half turns of a \( \alpha \)-helix, a short piece of \( 3_{10} \)-helix, a mixed \( \beta \)-sheet that contains five strands, and seven reverse turns. The molecule also displays a number of unusual secondary structure features, namely a parallel G1 \( \beta \)-bulge, two reverse Asx turns, and a symmetrical hydrogen-bonding region that involves the two helices and two of the reverse turns.

A comparison of the three dimensional crystal structures of yeast and oat ubiquitin\(^{25} \) has shown both to have a similar structure to that of human ubiquitin, with all amino acid variations contained in two small sections on one surface of the molecule. It is therefore concluded that this surface is not involved in conjugation with proteins destined for ATP-dependent ubiquitin proteolysis.

As previously stated, the conjugates formed between ubiquitin and other proteins involves the formation of isopeptide bonds between the carboxyl terminus of ubiquitin and the \( \epsilon-NH_2 \) groups of lysine residues of other proteins. In the only fully characterised ubiquitin-protein complex to date, ubiquitin-histone H2A, it has been reported that there are probably no allosteric, noncovalent interactions between ubiquitin and histone H2A\(^{26} \). This is consistent with the crystal structure\(^{24} \), in that the carboxyl terminus protrudes from, and therefore does not interact with, the rest of the molecule by way of hydrogen bonding or hydrophobic interactions. Thus, this portion of the molecule has considerable freedom of motion (confirmed by the poorer definition of the electron density maps for this region\(^{24} \) and should be accessible to enzymes involved with the formation or cleavage of the isopeptide bond.

Conversely the amino-terminal methionine is buried in the interior of the molecule, with the sulphur atom hydrogen bonded to the backbone nitrogen atom of lysine-63. This inaccessibility shields the terminus from solvent and prevents the proteolysis of the mature ubiquitin by its own pathway.

1.1.4. Synthesis and fragment synthesis of ubiquitin

In 1978, Schlesinger \textit{et al.}\(^{27} \) synthesised and purified a hexadecapeptide, corresponding to positions 59–74 of ubiquitin, by the solid phase strategy of Merrifield\(^{28} \) using Boc N\( \alpha \)-protection\(^{29} \) and DCCI\(^{30} \) activation. The peptide was shown to possess approximately 40\% of the activity of the native ubiquitin in each of three \textit{in vitro} biological assays. More recently, Abiko and Sekino\(^{31} \) chose solution phase methodology to synthesise the same fragment, employing a
combination of activation strategies which included the azide method$^{32}$, DCCI-HOBT$^{33}$ and active ester methodology$^{34}$. Again the fragment was shown to have considerable biological activity.

It was at about this time that ubiquitin, which had been thought of as a 74 residue protein, was confirmed as actually containing 76 residues — with a C-terminus of Gly-Gly-OH$^{35}$. Subsequent structural and activity studies of ubiquitin have suggested that the C-terminus, which protrudes from the bulk of the molecule, is fundamental to its activity. This somewhat contradicts the discovery that small fragments of ubiquitin have significant activity $i.e.$ suggesting that there is no need for (1) the complete tertiary structure of the molecule or (2) the C-terminal Gly-Gly region. These more recent investigations, together with the knowledge of ubiquitin's unique structure conservation, must therefore cast some doubt on the representativeness of the earlier fragment activity experiments.

Recent work in this research group has led to the synthesis and purification of a number of fragments of ubiquitin (for example, residues 1-35$^{36}$, 36-53$^{37}$, 60-76$^{38}$) as well as the total synthesis of human ubiquitin itself$^{39}$. This was undertaken using the Merrifield solid phase strategy$^{40}$, based on the Fmoc group designed by Carpino$^{41}$, and developed by Sheppard$^{42}$. 
1.2. Solid Phase Peptide Synthesis

1.2.1. Introduction

The importance of peptides and proteins, substances upon which the primary functions of the living cell depend, is now well established and is the subject of much research.

The area of peptide and protein chemistry is a vast one, with the many types of molecule exhibiting a large and varied range of functions *e.g.* as enzymes, oxygen carriers, electron-transfer agents, hormones, hormone precursors, antibiotics and growth factors.

There is no distinct division between the terms peptide and protein, however the former term is generally reserved for species of more than 100 amino acids, whereas 'peptide' is reserved for those substances of a lesser number. Hence, whereas protein chemistry has largely been thought of as a subdiscipline of biochemistry, peptide chemistry has been of more interest to organic chemists.

Fischer\(^{43}\) synthesised, and coined the name of, the first peptide at the beginning of this century. His results were impressive but not practical, due largely to his failure to discover a suitable reversible protecting group for the amine function. However one of his former students, Bergmann, with Zervas, was successful and in 1932 the benzyloxycarbonyl or Z- group was introduced\(^{44}\). The design of this urethane-type protecting group led to a new era in peptide chemistry.

The methodology of peptide synthesis has progressed quickly since that introduction, with improvements and modifications in all the relevant areas of synthesis frequently reported. However the most conspicuous milestone was the move away from solution phase synthesis and the introduction of Solid Phase Peptide Synthesis (SPPS) by Merrifield\(^{28}\) in 1963, for which he was later awarded the Nobel Prize\(^{40}\).

His plan to synthesise peptides in a stepwise manner while one end was attached to an insoluble support revolutionized the entire peptide field, and its influence has since spread to other areas of chemical synthesis *e.g.* oligonucleotide synthesis\(^{45}\), with equally dramatic results.
1.2.2. 'Classical' Solid Phase Peptide Synthesis

Standard solution phase synthesis of peptides containing more than just a few amino acids is a demanding task.

The introduction of protecting groups, coupling and deprotection reactions involves a large number of physical operations, such as neutralising the reaction mixture after coupling, precipitation or crystallisation of intermediates, collection of solid products by filtration etc. These manipulations lead to a great deal of mechanical loss of product during a synthesis. Add to these the problems of purification and increasing lack of solubility of the growing peptide chain, and it becomes apparent why the solution phase synthesis of a 20 amino acid peptide is indeed a heroic effort.

Merrifield, and others, were aware of the repetitiveness of the procedures involved in peptide synthesis when he introduced the method whereby the first amino acid was attached to an insoluble support, and the peptide synthesised by a stepwise procedure of repetitive activation, coupling and deprotection cycles (Scheme 1.1).

The insoluble support is a synthetic polymer (a 'resin') which bears reactive groups (X). A Nα-protected derivative of the first amino acid which will form the C-terminal residue of the peptide is activated by the appropriate chemistry (A) and coupled to the reactive polymer.

The nature of this labile Nα-protection (L) must be such that it can be removed by the addition of a reagent which must not either harm the link formed between the polymer and the C-terminal residue or remove any stable (semi-permanent) protecting groups (S) which are employed in masking the side-chain functionality of the initial, or subsequent, residues.

The orthogonal nature of the protecting groups and reagents involved permits this deprotection-coupling cycle to continue until all the amino acids have been incorporated into the chain.

Finally the fully side-chain-protected peptide is cleaved from the resin and taken into solution using a reagent of a different class. The semi-permanent protecting groups must also be removed, and are usually chosen such that they are removed simultaneously with cleavage of the peptide from the resin.
Scheme 1.1 ‘Classical’ solid phase peptide synthesis.
Merrifield demonstrated that once the amino acid or peptide was attached to the insoluble matrix, it too would be insoluble and hence in a suitable physical form to permit rapid filtration and washing.

The advantages of such a system are numerous:

1. The multi-step synthesis is greatly simplified by the use of a single reaction vessel and hence the mechanical losses associated with the repeated transfer of material is avoided.

2. It avoids the large losses which are encountered during the isolation and purification of intermediates.

3. Excess starting materials and reagents can be used to force individual reactions to completion and hence increase overall yield of the product.

4. Excess starting materials and reagents, as well as deprotection products and byproducts from the reactions, can be removed by simply washing the peptidyl polymer with appropriate solvents such as DCM and DMF.

5. The repetitive nature of the cycles lends SPPS to automation.

6. Increased speed of synthesis; modern automated synthesisers assemble peptides at a rate of one residue every four hours or so.

1.2.3. The Polymer Support

The fundamental requirements for a suitable insoluble support for SPPS are as follows:

1. The polymer must consist of particles of a physical size and shape to permit ready manipulation and rapid filtration from liquids.

2. The polymer must undergo sufficient chemical modification to permit attachment of the first amino acid, and yet be inert to all subsequent reagents and solvents used in the synthesis.
3. The polymer must swell in the appropriate solvents - the greater the swelling, the more efficiently the reactants will penetrate the polymer matrix by diffusion.

The support (2) developed initially by Merrifield was prepared by the chloromethylation of a copolymer (1) obtained from 2% divinylbenzene in styrene. The first Nα-protected amino acid (the C-terminal residue of the peptide) was then attached by the reaction of its triethylammonium salt with the chloromethylated resin (2) (Scheme 1.2).

\[
\text{CH}_3\text{-O-CH}_2\text{-Cl} \quad + \quad \text{POLYMER} \quad \text{(1)}
\]

\[
\text{CH}_3\text{OH} \quad + \quad \text{Cl-CH}_2 \quad \text{POLYMER} \quad \text{(2)}
\]

\[
R' S \quad \text{L-NH-CH-CO}_2 \quad \text{Et}_3\text{HN} \quad + \quad \text{POLYMER}
\]

\[
\text{deprotection etc}
\]

Scheme 1.2 Development of the chloromethyl resin (2).

If benzyloxycarbonyl Nα-protected amino acids are used, a solution of HBr in acetic acid is required for the subsequent deprotection.

The next step is the removal of the temporary Nα-protecting group. While it is possible to do this without cleaving to a significant extent the benzyl ester-like
linkage between the support and the C-terminal residue, this selectivity is simply not sufficient for practical purposes. With each deprotection cycle a small, but not negligible amount, of peptide is cleaved from the support. While this could be tolerated in the synthesis of short peptides, it is unacceptable in larger ones.

Nitration and bromination of the aromatic nuclei of the polymer were considered as two possible methods of increasing the electron-withdrawing effect on the benzylic position and thus improving this selectivity. However while both products exhibited an increased stability towards acidolysis, the nitrated derivative yielded a peptide-resin link that was resistant to acidolysis with HBr and HF, and required the somewhat unsatisfactory method of saponification for removal, the brominated resin, although used successfully in some instances, suffers from a reduction in swelling properties in both DCM and DMF, and hence has not been adopted extensively.

Merrifield therefore sought an improvement by introducing the tert-butyloxycarbonyl (Boc) N\(^\alpha\)-protecting group to replace the Z- group in his revised strategy in 1964.

The milder deprotection conditions for this more acid-labile group, namely 1M HCl in acetic acid or trifluoroacetic acid in DCM, were shown to degrade the peptide-resin bond to a lesser extent during the course of the synthesis.

An alternative development has been the modification of the Merrifield copolymer itself.

A reduction in the divinylbenzene content from 2% to 1% has resulted in a resin with greater swelling characteristics in both DCM and DMF. This improvement permits more efficient delivery of the reagents to the reactive sites by increasing the penetration of the acylating agents around the amino sites, but also the 1% cross-linked polymer will have fewer sites hidden in areas inaccessible to hindered amino acid derivatives e.g. those of valine and isoleucine. The cost of this increased 'reactivity' is a reduction in the mechanical stability of the polymer.

If less than 1% divinylbenzene is used, Birr has shown that although the resulting resin can be used, it is very soft and filtration can no longer be used as a means of separation. On the other hand, Merrifield also reported that 8 and 16% cross-linked supports resulted in a matrix that was too rigid to allow easy
penetration of reagents, and therefore caused a reduction in both reaction rate and yield\textsuperscript{28}.

In addition to the physical developments, the chemistry of the chloromethylated resin (2) has also been improved.

\[ \text{Cl-CH}_2-\text{POLYMER} \]

\[ \text{CH}_3\text{CO}_2^- \text{K}^+ \]

\[ \text{CH}_3-\text{CO}-\text{O-CH}_2-\text{POLYMER} \]

\[ \text{a KOH/EtOH} \]

\[ \text{b NH}_3/\text{MeOH} \]

\[ \text{c N}_2\text{H}_4 \]

\[ \text{HO-CH}_2-\text{POLYMER} \]

Scheme 1.3 Synthesis of the hydroxymethyl resin (3).

The heating of the resin in a solution of potassium acetate in a high boiling alcohol results in the displacement of the chloride by the acetate ion. Subsequent saponification\textsuperscript{50}, ammonolysis in methanol\textsuperscript{50} or treatment with hydrazine\textsuperscript{51} yields the hydroxymethyl resin (3) (Scheme 1.3).

One of the problems associated with the original resin (2) is that the chloromethyl group may alkylate nucleophilic side chain amino acid functions \textit{e.g.} the imidazole nucleus of histidine and the thioether sulphur atom of methionine, during incorporation of the C-terminal amino acid. The hydroxymethyl resin (3) avoids this problem, due to the possibility of employing a number of milder coupling reagents for the first (and subsequent) amino acids – the most commonly used being \textit{DCCI}\textsuperscript{30}.  

In the early years after the introduction of SPPS a number of peptide sequences were encountered that proved essentially impossible to synthesise in good yield e.g. the decapeptide corresponding to residues 65-74 of acyl carrier protein (ACP)\textsuperscript{52}.

Sheppard\textsuperscript{53} suggested that this was a result of the incompatibility between the nature of the polymer support and the peptide chain, that is the hydrophobic support is in marked contrast to the essentially hydrophilic chain. Therefore a solvent such as DCM, while it sufficiently swells the resin, causes the collapse of the peptide chain and renders it unreactive. The use of DMF, which solvates both the peptide chain and the support (but to a lesser extent), partially alleviates the problem. However DMF, while usefully promoting nucleophilic reactions, does give rise to other problems in SPPS (see Discussion).

Sheppard therefore sought to develop cross-linked polyamide resins\textsuperscript{53,54,55}, which are fundamentally very similar in nature to the chain itself.

While these resins are being used increasingly in synthesis, as yet they have been shown to exhibit no general fundamental advantage over the traditional polystyrene support – for although Sheppard\textsuperscript{53} has successfully synthesised the ACP 65-74 peptide using polyamide resins, Merrifield\textsuperscript{56} has also more recently adapted the polystyrene strategy to obtain this same product.

Sheppard has however introduced polyamide resin supported on Kieselguhr\textsuperscript{57}, and has been using this resin as the basis for his successful continuous flow peptide synthesis strategy.

1.2.4. The Peptide-Resin Link

Unfortunately the introduction of the Boc group as the transient $N^\alpha$-protection in SPPS did not end the problems of peptide-resin bond cleavage during deprotection.

It has been reported that the standard SPPS conditions employed for the repetitive removal of Boc, namely 50% TFA in DCM, results in the loss of approximately 1% of the peptide per deprotection cycle. As was discussed in the previous section, increasing the stability of the peptide-resin link by the introduction of electron-withdrawing groups to the aromatic nuclei of the resin was shown to be unsatisfactory for general application.
The phenylacetamidomethyl (Pam) resin (6) of Merrifield et al.\textsuperscript{58} increased the stability of this link by a factor of 100 over the classical system through the inclusion of a 'handle' between the peptide and the resin. Merrifield's original synthesis of the Pam resin\textsuperscript{58} was a time-consuming process, and stimulated the introduction of a much improved synthesis\textsuperscript{59}. This involves the acylation of the aminomethyl resin (5) (generated by the treatment of the chloromethyl support (2) with potassium phthalimide then hydrazine\textsuperscript{60}) with p-acetoxymethylphenylacetic acid (4) using DCCI\textsuperscript{30}. The acetyl group is subsequently removed by hydrazinolysis to yield the hydroxymethyl Pam resin (6) (Scheme 1.4).

\textbf{Scheme 1.4 Synthesis of the hydroxymethyl Pam resin (6).}

The increased stability of the peptide–resin linkage in the Pam resin is due to the electron-withdrawing effect of the acetamido group in the para position of the phenyl ring to which the peptide is attached.

A further advantage gained by the inclusion of a 'handle' attached to the resin is that a 'spacer' is also introduced \textit{i.e.} the distance between the peptide chain and the solid support is increased – hence there is a reduction in crowding of the polymer-bound amine sites and a greater accessibility for reagents.
However there is a problem with this type of resin. As peptides become larger and more complex, they become less stable towards the reagents used for the final cleavage of the peptide from the support, namely HF. Therefore although the Pam resin has been used to synthesise large peptides, researchers have considered the possibility of more labile peptide-resin linkages being used in SPPS.

The most successful such resin to date is the p-alkoxybenzyl alcohol resin (8), introduced by Wang\textsuperscript{61} which was developed primarily for the synthesis of protected peptide fragments.

The synthesis of (8) involves the treatment of the chloromethyl resin (2) with 4-hydroxy-benzyl alcohol (7) and sodium methoxide (Scheme 1.5). Merrifield later improved this synthesis by more rigorously controlling the reaction conditions\textsuperscript{62}.

![Scheme 1.5 Synthesis of the Wang resin (8).](image)

The p-alkoxy substituent of the benzyl alcohol moiety of this resin enhances the acid lability of the ester linkage by electron-donation – hence a solution of TFA in DCM can be used for the final cleavage of the peptide from the resin. Consequently the N\textsuperscript{α}-protecting group chosen for a synthesis involving the Wang resin must be either more acid-labile (\textit{e.g.} the Bpoc group\textsuperscript{63}), or alternatively, base-labile (\textit{e.g.} the Fmoc group\textsuperscript{41}) (see section 1.2.5).

An alternative approach to the development of a suitable handle was reported by Barany\textsuperscript{64}, when he introduced a fluoride-labile linker (9). The advantages of a SPPS strategy involving such a handle include the possibility of the final
peptide-resin cleavage being performed at neutral pH (i.e. using TBAF in DMF) and also it would enable a fully protected peptide to be cleaved and purified - hence a fragment condensation approach for peptide and protein synthesis may be adopted.

\[(9)\]

Investigations in this research group have led to the introduction of the 2-(4-hydroxymethylphenyl)-2-trimethylsilyl-propanoic acid group\(^65\) (10) as an alternative fluoride-labile handle, with more recent work yielding a fully protected peptide corresponding to residues 1–35 of ubiquitin\(^36\).

\[(10)\]

Other handles and strategies have also been reported, for example, while both Rich\(^66\) and Tjoeng\(^67\) have produced photolabile handles, Tam\(^68\) has developed a multi-detachable linker for peptide synthesis, where careful selection of reagents has resulted in the possibility of cleavage at more than one site of the resin during the synthesis.
1.2.5. Nα-Protection

The most commonly used amine protecting groups in peptide synthesis are of the urethane-type (11) due to their relatively mild deprotection requirements and the suppression of racemization during the activation and coupling of the amino acid derivatives.\(^{69}\)

\[
\begin{align*}
\text{O} & \quad \text{R} \\
\| & \quad | \\
\text{R'}-\text{O-}\text{C-}\text{NH-CH-CO}_2\text{H}
\end{align*}
\]

(11)

**Z protecting group**

The first such urethane group, the benzylloxycarbonyl or Z-protecting group\(^{44}\) (12), was introduced in 1932 and still has a key role in peptide synthesis. The Z-derivatives of amino acids are stable and readily prepared.

\[
\begin{align*}
\text{O} & \quad | \\
\| & \quad \text{CH}_2-\text{O-}\text{C-}
\end{align*}
\]

(12)

Removal of the group may be achieved by a number of methods; hydrogenolysis and acid-catalysed solvolysis using HBr being by far the most widely used procedures. However the use of this group has significant limitations, for example, catalytic hydrogenolysis of sulphur-containing amino acids usually fails due to catalytic poisoning, difficulties may result in the hydrogenation of aromatic amino acids (particularly tryptophan), and premature peptide-resin cleavage may occur during repetitive Nα-deprotection by acidolysis in SPPS.

The principle alternatives to the Z- group are:
The tertbutyloxycarbonyl (Boc) group\(^{29,47}\) (13) is the most commonly used acid-labile N\(^\alpha\)-protecting group in SPPS - this is due to the ready availability of its derivatives and its satisfactory lability-stability characteristics (this is only the case in SPPS if used in conjunction with a suitable resin *e.g.* the Pam resin). Cleavage is usually performed by 50% TFA in DCM.

A disadvantage of the Boc group is the generation of reactive intermediate carbonium ions (Scheme 1.6) during deprotection which may participate in side reactions with sulphur containing amino acids (methionine and cysteine) or the aromatic ring systems of tyrosine and tryptophan which readily undergo side reactions.

Scheme 1.6 Cleavage of the Boc protecting group.
electrophilic substitution. In the case of small peptides this can be overcome by the use of cation scavengers *e.g.* anisole or dimethyl sulphide\(^{70}\), however, these may reduce the desired selectivity of cleavage and thus lower the yield of the product.

**Bpoc protecting group**

The 2-(4-biphenyl)isopropyloxycarbonyl (Bpoc) group (14) was introduced by Sieber and Iselin\(^{71}\) in 1968 and displays a significantly greater acid-lability than that of the the Boc group.

![Bpoc urethane structure](14)

Bpoc urethanes are 2000–9000 times more labile to acidolysis that Boc urethanes – therefore \(N^\alpha\)-deprotection conditions of 0.2–0.5% TFA in DCM can be employed. As with Boc derivatives, deprotection yields stable carbonium ions and hence scavengers are required.

Although Bpoc-amino acids exhibit near ideal lability characteristics, they are not in general use due to their instability as free acids *i.e.* they must be stored as their acid salts and liberated before use.

Other urethane-type protecting groups of intermediate acid-lability include the Ddz group of Birr\(^{72}\), the Tmz group of Matsueda and Stewart\(^{73}\) and the Poc group of Ragnarsson\(^{74}\). These require 3% TFA in DCM for adequate deprotection, but due to the expensive nature of their synthesis, have not been generally adopted in peptide synthesis.

**Fmoc protecting group**

The principle base-labile \(N^\alpha\)-protecting group, the 9-fluorenlymethoxycarbonyl (Fmoc) group (15) was introduced by Carpino\(^{41}\), and applied to peptide synthesis by Sheppard\(^{42,75}\).
The use of a base-labile protecting group enables an orthogonal protection strategy to be adopted for peptide synthesis, in that acid-labile side chain protection and peptide-resin linkage may be used. Hence this group has been employed a great deal in SPPS\textsuperscript{76,77}.

Fmoc displays essentially complete acid stability and a quite remarkable lability to secondary amines. Therefore deprotection, a $\beta$-elimination process, is generally effected by 20\% piperidine in DMF and as no intermediate carbonium ions are produced with Fmoc, no scavengers are required.

**Bnpeoc protecting group**

More recently an alternative base-labile $N^\text{a}$-protecting group, the 2,2-\textit{bis} (4'-nitrophenyl)ethoxycarbonyl (Bnpeoc) group (16) has been introduced by Ramage and Florence\textsuperscript{78}, and Valentine\textsuperscript{79}.

The synthesis of the protecting group is based on the observation of Kharasch and Clapp\textsuperscript{80} that the opening of styrene oxide with phenylmagnesium bromide to give either the expected secondary alcohol (17) (after attack at the least hindered centre) or the primary alcohol (18), is dependent on the order of addition of reactants \textit{i.e.} the primary alcohol can be obtained in 80\% yield if the oxide is added to the Grignard reagent (Scheme 1.7).
The subsequent para nitration of the phenyl rings gave $2,2$-bis (4'-nitrophenyl)-ethanol (19), from which both the chloroformate (20) and the succinimidy active ester (21) derivative can be readily synthesised (Scheme 1.8). The active ester derivative (21) has been used to protect the $N^\alpha$-functionality of a number of amino acids by the method of Lapatsanis \cite{81}, or more recently by the method of Rich \cite{82}.

The deprotection of the Bnpeoc group, as with the Fmoc group, was found to proceed via a $\beta$-elimination mechanism, and is performed by the addition of either DBN or DBU. These are strong bases, but importantly, weak nucleophiles, hence the possibility of unwanted side reactions is considerably reduced. Also extremes of pH can be avoided by the use of a system buffered with acetic acid.

The authors have reported Bnpeoc to be stable to the following reagents commonly used in peptide synthesis: TFA, HCl-methanol, acetic acid, NaOH, pyridine, triethylamine, $N$-methylmorpholine and piperidine.
Scheme 1.8 Bnpeoc protecting group synthesis.
1.2.6. Side Chain Protection

The use of the acid-labile Boc $N^\alpha$-protection in SPPS necessitates a side chain protection strategy based on increased acid stability. The one in general use is that which involves benzyl derived protecting groups.

In the case of serine, threonine and tyrosine, benzyl ethers may be employed. However as the tyrosine derivative is not completely stable to the Boc cleavage conditions, the 2,6-dichlorobenzyl ether group is more commonly used (an unwanted side reaction involves the migration of the unsubstituted benzyl ether to the 3-position of the aromatic ring).

The acidic side chains of aspartic and glutamic acids are protected using benzyl esters, arginine protection employs the nitro or tosyl group, histidine the tosyl, and lysine uses a urethane with the Z- or 2-chlorobenzyloxy carbonyl group. As for cystine, the Acm group is being used increasingly in synthesis. Asparagine and glutamine are generally left unprotected, as there is no ideal method available for their protection.

If the very acid-labile Bpoc or the base-labile Fmoc protecting groups are used, a side chain protection strategy based on tert-butyl is more commonly employed e.g. serine and threonine are adequately protected as their tert-butyl ethers.

As for the remaining amino acid residues, a large number of alternative side chain protecting groups have been reported (see reviews), including a novel acid-labile arginine protecting group, the Pmc group, developed recently in this research group.

1.2.7. Coupling Methods

The standard procedure for peptide bond formation must involve the activation of the carboxylic acid component of the incoming $N^\alpha$-protected amino acid to nucleophilic attack by the polymer bound amine component i.e. the hydroxyl group must be replaced by an electron-withdrawing group (X) (Scheme 1.9).
Ensuring complete coupling of the acid and amine components represents the greatest technical difficulty in SPPS at the present time. Hence many alternative procedures have been investigated, with the result that there are now many different coupling methods and reagents available to the peptide chemist.

The ideal coupling reagent must exhibit all of the following characteristics:

1. It must be easy to handle and use (of particular importance if automated synthesisers are to be used).
2. It must result in racemization-free couplings.
3. It must give rapid and efficient coupling (the degree of racemization is increased if the coupling is slow and prolonged).

Some of the most widely used methods and reagents are discussed below:

**The Acid Chloride method**

Perhaps the most obvious choice of an electron-withdrawing group (X) is the chlorine atom. The high reactivity of protected amino acid chlorides had been well documented before the earliest days of peptide synthesis. However their use in peptide chemistry has been limited due to the problem of racemization (via
azlactone formation) occurring during the activation procedure, the high incidence of unwanted side reactions and the generally difficult deprotection of protecting groups which are stable enough to survive the conversion of a carboxylic acid to an acid chloride.

Notwithstanding these drawbacks, the introduction of novel acid-stable Nα-protecting groups has led to a re-evaluation of the acid chloride methodology.

Bechtolsheimer and Kunz have employed acid chlorides (generated using oxalyl chloride in DCM at 0°C) for the preparation of sterically hindered peptide bonds using the extremely acid-stable 2-(triphenylphosphonio)-ethoxycarbonyl (Peoc) Nα-protecting group.

More recently, Carpino et al. have used thionyl chloride in DCM to generate the acid chlorides of several Fmoc-amino acids. Again these have been successfully employed in synthesis, and can be isolated and stored indefinitely in a dry atmosphere. On the question of racemization, Carpino et al. have shown that the acid chloride couplings of Fmoc-amino acids result in a loss of chirality of less than 0.1%.

The Azide method

This technique was first introduced in 1902 by Curtius and to this day it remains a powerful and practical approach for the synthesis of peptides of high optical purity.

The original method involves the treatment of alkyl esters (often methyl) with hydrazine, and the resulting hydrazides (22) are smoothly converted to the corresponding acid azides (23) by the addition of nitrous acid (Scheme 1.10).

More recent improvements have resulted in the use of diphenylphosphonyl
azide for the conversion of carboxylic acids to acid azides, and the use of alkyl nitrites, instead of nitrous acid, for the transformation of hydrazides to azides.

These modifications however, have had little effect on the inherent shortcoming of the azide method, that is the side-reaction involving the conversion of acid azides to isocyanates by the Curtius rearrangement. Therefore if the azide coupling method is to be successful, a number of precautions must be taken: the reaction must be performed at low temperature, with a high concentration of reactants, and the coupling step must immediately follow the preparation of the azide.

The Carbodiimide Method

The usefulness of this procedure for peptide bond formation was first recognised by Sheehan and Hess. Merrifield employed the N,N'-dicyclohexylcarbodiimide (DCCI) coupling reagent when he first introduced SPPS, and it remains the most widely used in peptide synthesis.

The mechanism of DCCI-mediated coupling reactions was first investigated by Khorana, and more recently discussed by De Tar et al. These studies have shown that the reactions proceed rapidly due to the high activation afforded by the O-acyl urea (25) which is formed (Scheme 1.11).

However this isourea spontaneously rearranges to the unreactive N-acyl urea (26), and leads to the loss of Nα-protected amino acid from the coupling reaction. The rate of this undesirable rearrangement can be troublesome in polar aprotic solvents such as DMF, and hence DCCI-mediated couplings require the use of the minimum volume of such solvents.

The high degree of activation afforded by the O-acyl urea can also lead to the formation of azlactones. This is not considered a serious problem if the species being activated is a single amino acid with urethane-type Nα-protection (hence the suitability of DCCI to SPPS), but can result in racemization if used in a fragment condensation procedure.
More recently, diisopropylcarbodiimide (DICI)\textsuperscript{99} has become commercially available, and has gained increasing popularity with peptide chemists. This carbodiimide is a liquid and hence much easier to handle than DCCI which is a waxy solid. Also the byproducts, diisopropylurea and the N-acyl ureas, are more soluble than their DCCI counterparts, and therefore can be removed more readily and efficiently from the reaction system (of particular importance in SPPS).

One further point to note is that DCCI (and DICI) cannot be used to couple asparagine and glutamine that have no side chain protection, as this results in the dehydration of the side chain amide to the corresponding nitrile\textsuperscript{100}. 

Scheme 1.11 DCCI-mediated coupling mechanism.
The Preformed Symmetrical Anhydride Method

Preformed Boc-amino acid symmetrical anhydrides (27)\textsuperscript{95} have been reported as resulting in the greatest coupling yields in many difficult sequences.

Their preparation can be performed using phosgene, but is more usually carried out using condensing agents such as carbodiimides (e.g. DCCI) (Scheme 1.12).

Boc-amino acid symmetrical anhydrides are not stable and therefore they must be prepared immediately before use. While this is of little inconvenience in solution phase peptide synthesis and manual SPPS, it causes significant problems with storage, transfer \textit{etc.} in automated synthesisers. In contrast Fmoc-amino acid symmetrical anhydrides have been reported as being stable and hence can be prepared in advance and stored until they are required.

\begin{center}
\begin{tikzpicture}
\node (A) at (0,0) {\text{\textit{Boc-NH-CH-C-OH}}};
\node (B) at (2,0) {\text{\textit{\textit{Boc-NH-CH-C-O-C}}}};
\node (C) at (-2,0) {\text{\textit{\textit{Boc-NH-CH-C-OH}}}};
\draw[->] (A) -- (B);
\draw[->] (A) -- (C);
\end{tikzpicture}
\end{center}

\textbf{Scheme 1.12 Preparation of symmetrical anhydrides.}

Disadvantages associated with the use of such anhydrides include the slow and incomplete couplings experienced when anhydrides of hindered amino acids (such as valine, isoleucine and threonine) are reacted with sterically hindered polymer-bound amine sites, and also the wastefulness of the symmetrical
anhydride procedure in that it requires 2 moles of (expensive!) N$^{\alpha}$-protected amino acid to generate 1 mole of the symmetrical anhydride.

**The Active Ester method**

At the very beginning of synthetic peptide chemistry, peptide bonds were prepared using amino acid methyl and ethyl esters as acylating agents. Both Fischer$^{101}$ and Curtius$^{102}$ prepared peptides by this approach.

However Schwyzer et al.$^{103}$ recognised that by increasing the electron-withdrawing effect of the alcohol component of the ester, the electrophilic character of the carbon atom is increased and therefore becomes more susceptible to attack by nucleophiles e.g. the amino group of the amine component. Thus he introduced the cyanomethyl esters (28) for use in peptide synthesis. However although used with limited success, they have not been generally adopted as satisfactory reaction rates are only obtained if the reactants are applied in high concentration.

Aryl esters proved to be more promising, with the most popular derivative being that reported by Bodanszky$^{104}$, the p-nitrophenyl active ester (29).

![Chemical structure of cyanomethyl ester (28) and p-nitrophenyl ester (29).](image)

These esters (29) can be prepared by treatment of the N$^{\alpha}$-protected amino acid with either p-nitrophenyl sulphite or phosphite$^{105}$, or by condensation with p-nitrophenol using DCCI$^{106}$. They are easily purified, crystalline solids and couple under mild conditions (although the reaction rate is dependent on the nature of the solvent and some racemization difficulties have been reported$^{107}$).

However these esters were largely superceeded by the introduction of the active esters of 1-hydroxybenzotriazole (HOBt) (30) of Konig and Geiger$^{108}$, and N-hydroxysuccinimide (HONSu) (31) of Anderson et al.$^{109}$.
Following the dramatic improvement in the rate of solution phase coupling reactions involving HOBT active esters, whether in catalytic (with p-nitrophenyl active esters) or equimolar quantities, this procedure was successfully applied to SPPS. These esters may be preformed or generated in situ, and in the example of coupling asparagine and glutamine, HOBT esters have been commonly adopted without the need for protection on the side chain amide.

N-hydroxysuccinimide esters have attained increasing practical importance and successful application in both solution and solid phase peptide synthesis. Their high reactivity results in the preparation of the desired peptides with the expected ease — however their application is not without problems; the succinimide carbonyls are not entirely inert toward the amine component. An attractive feature of the use of this method, however, is that the byproduct formed, N-hydroxysuccinimide, is water soluble and readily removed from the reaction system.

This is by no means an exhaustive list of the active esters available for use in peptide synthesis — for example, the trichlorophenyl110 and the pentachloro(fluoro)phenyl111 esters have also been employed extensively.
The Mixed Anhydride method

The use of mixed or unsymmetrical anhydrides (32) for peptide bond formation has been an area of interest for many years.

![Chemical structure of mixed anhydride](image)

(32)

One of the first examples investigated involved the use of benzoyl chlorides (R' = Ph)\textsuperscript{112,113}.

![Chemical reaction](image)

(33)

Scheme 1.13 An example of the use of mixed anhydrides in peptide synthesis.

However these were found to be unsatisfactory due to the electron-withdrawing effect of the phenyl group. This effect renders the adjacent carbonyl carbon sufficiently electrophilic to compete in the acylation reaction.

More satisfactory results were obtained when electron-donating R' groups were used, e.g. mixed anhydrides formed from isovaleric\textsuperscript{114} and trimethylacetic (pivalic)\textsuperscript{115} acid chlorides (33) were shown to couple in excellent yield (Scheme 1.13).

This is not only due to the electron-donating effect of the R' groups, but also the increased steric bulk of these groups promote the attack at the carbonyl derived from the carboxy component.
Mixed carbonic anhydrides (34) have also been introduced. Examples of these now commonly employed include the ethylcarbonic$^{116,117}$ and the improved isobutylcarbonic$^{118}$ acid mixed anhydrides, and they remain a valuable asset to the peptide chemist.

The problems with mixed anhydrides are their tendency towards disproportionation to symmetrical anhydrides$^{117}$, and the possibility of racemization. The former can largely be avoided by employing low temperatures for the anhydride preparation and the latter by the use of short activation times$^{118}$ (less than 10 minutes) with the minimum quantity of tertiary amine present during their generation.

Mixed anhydrides with organophosphorus acids have more recently received a great deal of attention and will be discussed in Section 1.3.
1.3. Organophosphorus coupling reagents in peptide synthesis

1.3.1. Introduction

Mixed carboxylic anhydrides of $N^0$-protected amino acids (34), as discussed in the previous section, have been employed extensively in both solution and solid phase peptide synthesis.\textsuperscript{119}

The choice of group (X), based on a consideration of steric and electronic factors, is an important one. It must promote attack by the amine component ($R''NH_2$) at position a therefore yielding the desired peptide bond, rather than at position b, which would result in reduced yields and unwanted byproducts.

The advantages of this methodology, not least of which is the relatively fast nature of the couplings involved (important if the amine component has a limited lifetime \textit{e.g.} if it is a dipeptide ester favourably disposed toward cyclisation to a piperazine-2,5-dione), have resulted in the repetitive excess mixed anhydride (REMA) method becoming increasingly popular with peptide chemists.

Problems with this method include the facile thermal disproportionation of the mixed anhydrides and the occurrence of racemization. However a further limitation concerns the size of the groups R' and R", for if they are bulky, the unwanted reaction at position b may become competitive.\textsuperscript{120}

Researchers have therefore sought a class of anhydrides that would not only be more stable to disproportionation but which would react with nucleophiles only at the desired carbonyl. It was also noted that stability and crystallinity of the anhydrides would be an added advantage.

One approach that has been studied extensively in recent years is the use of anhydrides derived from organophosphorus acids. The role of such reagents in
carbonyl activation has been reviewed\textsuperscript{121} and continuing developments in this area are periodically collated\textsuperscript{122}.

### 1.3.2. Anhydrides with Phosphoric acid derivatives

Research in this area was stimulated by the early hypothesis by Lipmann\textsuperscript{123} that the formation of the peptide bond in living cells may be ascribed to energy-rich acyl phosphates derived from amino acids. This led to Chantrene\textsuperscript{124} and, later, Sheehan\textsuperscript{125} studying the potentialities of the mixed anhydrides (35) and (36) respectively, in peptide synthesis.

\[ \text{R} \quad \text{O} \]
\[ \text{Z-NH-CH-CO-O-P-OPh} \]

(35)

\[ \text{O} \quad \text{R} \]
\[ \text{\text{N-CH-CO-0-P-OBzl}} \]

(36)

The reaction of phthaloylglycyl chloride and dibenzylphosphate afforded (36) in good yield, and peptide derivatives were prepared using this acyl phosphate at pH 7.4 and 37°C i.e. approaching physiological conditions.

Cramer and Gartner\textsuperscript{126} found that the reaction of acylamino acids with the enol phosphate (37) provided the O,O-diethylphosphoric mixed anhydride (38) and that this could be used in peptide synthesis (Scheme 1.14). Interestingly, no disproportionation to the symmetrical anhydrides was observed despite the initial reaction being carried out at 70°C.

An alternative strategy was introduced by Zervas \textit{et al}\textsuperscript{127} when he employed diphenyl phosphorochloridate to form the mixed anhydride (39) which gave racemization-free coupling in the synthesis of the dipeptides H-Met-Met-OH and H-Phe-Gly-OH.
Scheme 1.14 Use of O,O-diethylphosphoric mixed anhydride (38).

Diphenylphosphorazidate (40)\textsuperscript{128} (prepared by the reaction of diphenylphosphoric chloride and sodium azide in acetone) and diethyl-phosphorocyanidate (41)\textsuperscript{129} (from an Arbusov reaction between triethyl phosphite and cyanogen bromide) were introduced as coupling reagents by Shiori and Yamada in 1972 and 1973 respectively.
These reagents were shown to be effective in peptide bond formation in both solution and solid phase synthesis. However using (40) in the Young test\textsuperscript{130}, that is the formation of Bz-Leu-Gly-OEt in which the oxazalone might be expected to be an intermediate, the optical purity of the product was found to be only 89%. The Izumiya test\textsuperscript{131}, involving the condensation of Boc-Gly-Ala-OH with H-Leu-(resin), was employed to determine the viability of this reagent for fragment couplings in solid phase work. After deprotection to give H-Gly-Ala-Leu-OH, it was shown that the extent of racemization was 2.5% at 20°C and 2.0% at 0°C. No difficulties were encountered with functional side chains such as serine, threonine, asparagine, glutamine, histidine, methionine, tyrosine and tryptophan.

With reagent (41) even less racemization was observed in the Young test (96% optically pure) and in the Izumiya test (1.0% racemization at 20°C and 0.5% at 0°C).

However, whilst the anhydrides derived from O,O-dialkyl and O,O-diaryl phosphoric acids have previously been employed in peptide synthesis, they have failed to gain widespread acceptance.

1.3.3. Anhydrides with Phosphinic acid derivatives

More recently researchers have turned their attentions toward the analogous phosphinic-carboxylic mixed anhydrides. The following considerations suggested that these anhydrides may be useful intermediates in peptide synthesis:

1. The cyclic system (42) (Scheme 1.15) undergoes aminolysis at the carbonyl group $a$ but alcoholsysis occurs by attack at phosphorus $b$\textsuperscript{132}. This change in regiospecificity depending on the nature of the nucleophile is entirely compatible with the requirements of peptide synthesis.
2. Dimethylphosphoryl chloride (43) has been shown to be less reactive toward oxygen nucleophiles than dimethylphosphinyl chloride (44). The rate of solvolysis of (44) in absolute alcohol is 300 times as fast as that of (43), which would suggest that phosphinyl chlorides should react with carboxylate anions to form mixed anhydrides very much faster than phosphoryl chlorides.

3. Published evidence suggested that phosphinic-carboxylic mixed anhydrides are more stable to disproportionation than mixed carboxylic anhydrides.

It was with this limited knowledge that Ramage et al. introduced the readily available diphenylphosphinyl chloride (Dpp-Cl) (45) as a means of preparing phosphinic-carboxylic mixed anhydrides (46).

In an attempt to determine the relative reactivity of mixed carboxylic anhydrides and phosphinic-carboxylic mixed anhydrides, the Izumiya test sequence was applied using both pivalic and Dpp mixed anhydrides. Since
this test is a measure of the racemization derived from an oxazolone intermediate, it gives a measure of the electron density on the terminal peptide carbonyl group. The extent of racemization found for Dpp (5.7%) and pivalic (2.6%) mixed anhydrides indicated a greater activation in the phosphinic example.

The standard conditions for the use of Dpp-Cl (45) in stepwise peptide synthesis involves the initial formation of the mixed anhydride at -20°C in DCM (or EtOAc) using NMM as base. After 20 minutes the amino component is added and the reaction mixture is allowed to warm to room temperature (Scheme 1.16).

\[
\begin{align*}
R' \text{NH-CH-CO}_2\text{H} + (\text{Ph})_2\text{P-Cl} & \rightarrow R' \text{NH-CH-CO}_2\text{P(Ph)}_2 \\
\text{NMM} & \rightarrow \\
\text{CH}_2\text{Cl}_2 & \\
(45) & \\
\end{align*}
\]

Scheme 1.16 Diphenylphosphinic anhydrides in peptide synthesis.

The yields of crystalline products and the ease of purification were shown to be generally superior to those involving mixed carboxylic anhydrides.

Subsequent $^{31}\text{P n.m.r.}$ studies found that the rates of disproportionation of a series of phosphinic-carboxylic mixed anhydrides were insignificant from a preparative point of view in comparison with aminolysis at 0°C.

Also these studies, as well as proving that mixed anhydride formation with Dpp-Cl was almost instantaneous, confirmed that the resulting anhydrides react rapidly with a nucleophilic amine to form the desired peptide bond \textit{i.e.} because the amino group has a greater affinity for the carbonyl group rather than the phosphorus centre, attack occurs regiospecifically at the carbonyl centre.

The initial investigations with diphenylphosphinic mixed anhydrides had shown that the extent of racemization that had occurred during the coupling of
Z-Gly-Ala-OH to H-Leu-OBzl (i.e. the Izumiya test sequence) was 5.7%.

Galpin et al.\textsuperscript{139} chose to study further this racemization process by considering the activation and coupling of N-methylated amino acids. Some research has found these to be particularly susceptible to racemization during coupling with DCCI\textsuperscript{140,141} (it should be noted that Benoitin has reported exceptions to this\textsuperscript{142}), however with HOBT and HONSu as additives, problematic oxazolone formation has been minimised. The researchers found that within the limits of the proton n.m.r. method\textsuperscript{141,143} they employed, no racemization was observed when urethane protection was used for the N-methylated amino acids. However if the benzoyl group was employed for protection, this led to extensive racemization.

The use of diphenylphosphinic mixed anhydrides in manual SPPS was initially reported by Galpin et al.\textsuperscript{144,145}.

The researchers confirmed that there were no problems with 'wrong-way' opening (in SPPS, attack by the amine component at P=O instead of C=O would result in chain termination, whereby the extending amino terminus would become blocked as the corresponding diphenylphosphinamide) or thermal disproportionation. In fact, \textsuperscript{31}P and \textsuperscript{13}C n.m.r. studies had shown that the Z-Gly-ODpp mixed anhydride was stable for at least 48 hours at room temperature in CD\textsubscript{3}CN, and after that time, the mixed anhydride had retained its potential for promoting acylation (e.g. it reacted immediately with benzylamine). This is in marked contrast to the related pivalic anhydride of the same amino acid derivative, which displayed some disproportionation after 30 minutes at 0°C.

Galpin et al. subsequently employed diphenylphosphinic mixed anhydrides in the synthesis of the protected and unprotected derivatives of the Bombesin-like peptide sequence (47), using a phenolic resin\textsuperscript{146,147} and Bpoc N\textsubscript{\alpha}-protection\textsuperscript{71}.

\begin{equation}
H-Ala-Pro-Val-Ser-Val-Gly-Tyr-OH
\end{equation}

(47)

Results in this research group on the application of diphenylphosphinic mixed anhydrides to SPPS\textsuperscript{148} have suggested that the byproduct from the coupling reaction, diphenylphosphinic acid, forms a salt with the liberated amino component, thus removing the latter from the reaction and reducing the efficiency
of the coupling. To counteract this, one further equivalent of base was added to
the reaction mixture after the activated amino acid and the NMM had been added
to the amine component. The base used was 2,6-lutidine (2,6-dimethylpyridine)
which was thought sufficiently weak so as not to abstract an α-proton from the
amino acid residues, but strong enough to neutralise any diphenylphosphinic acid.

Although Dpp-Cl has been successfully employed in both solution and in
manual SPPS, the search continued for a phosphinic chloride with all the
aforementioned advantages of Dpp-Cl, but whose corresponding acid – formed as
a byproduct of the acylation reaction – would be water soluble, thereby permitting
its ready removal from the final reaction mixture. Diphenylphosphinic acid
(Dpp-OH) has occasionally proved troublesome in this respect.

One possible alternative, 1-oxo-1-chlorophospholane (Cpt-Cl) (48), was
introduced by Ramage et al\textsuperscript{149} in 1984.

\begin{center}
\begin{tikzpicture}[thick]
\draw (0,0) circle (1cm);
\draw (0,0) -- (90:1cm);
\draw (0,0) -- (180:1cm);
\draw (0,0) -- (270:1cm);
\draw (0,0) -- (45:1cm);
\draw (0,0) -- (135:1cm);
\draw (0,0) -- (315:1cm);
\draw (0,0) -- (225:1cm);
\draw (0,0) -- (67.5:1cm);
\draw (0,0) -- (112.5:1cm);
\draw (0,0) -- (202.5:1cm);
\draw (0,0) -- (247.5:1cm);
\draw (0,0) -- (327.5:1cm);
\end{tikzpicture}
\end{center}

\text{(48)}

\begin{center}
\begin{tikzpicture}[thick]
\draw (0,0) circle (1cm);
\draw (0,0) -- (90:1cm);
\draw (0,0) -- (180:1cm);
\draw (0,0) -- (270:1cm);
\draw (0,0) -- (45:1cm);
\draw (0,0) -- (135:1cm);
\draw (0,0) -- (315:1cm);
\draw (0,0) -- (225:1cm);
\draw (0,0) -- (67.5:1cm);
\draw (0,0) -- (112.5:1cm);
\draw (0,0) -- (202.5:1cm);
\draw (0,0) -- (247.5:1cm);
\draw (0,0) -- (327.5:1cm);
\end{tikzpicture}
\end{center}

\text{(49)}

Cpt-Cl was shown to be an efficient promotor of peptide bond formation
through phosphinic-carboxylic mixed anhydrides, with the byproduct, Cpt-OH,
readily removed from the reaction system to leave peptides of high quality and
requiring the minimum of purification. Regiospecific attack by amine on the mixed
anhydride and stability to disproportionation at 0°C were also confirmed.

Later work had demonstrated that the mixed anhydrides derived from Cpt-Cl
have greater stability than those of Dpp-Cl and to the suitability of this reagent to
fragment coupling has been postulated. However this reagent is expensive and
time-consuming to prepare. It is hoped therefore, that the recently introduced
1-oxo-1-chloro-3,4-dimethyl-3-phospholene (49)\textsuperscript{38} which is more readily prepared,
will be of some use in the mediation of amide bond formation. The preliminary
results\textsuperscript{38} appear promising.
1.3.4. Anhydrides with Phosphorous acid derivatives

A great deal of work on this class of anhydride has been reported. Examples include diethyl chlorophosphite (50) and tetraethyl pyrophosphite (51) introduced by Anderson et al.\textsuperscript{150}, both of which have been employed in peptide synthesis, but there are many others. Reagent (51) is preferred to (50) due to its simpler purification requirements and its use in peptide bond formation without a solvent.

\[
\begin{align*}
\text{Cl-P(OEt)}_2 & \quad \text{(50)} \\
\text{(EtO)}_2\text{P-O-P(OEt)}_2 & \quad \text{(51)}
\end{align*}
\]

For other examples - see aforementioned reviews\textsuperscript{121,122}.

1.3.5. Other phosphorus reagents useful in peptide synthesis

\[
\begin{align*}
\text{(Me}_2\text{N)}_3\text{P-O-P(NMe}_2\text{)}_3 & \quad \text{(52)} \\
\text{(Me}_2\text{N)}_3\text{P-O-N} & \text{PF}_6 & \quad \text{(53)}
\end{align*}
\]

Again, many possible coupling reagents have been reported - see reviews\textsuperscript{121,122}. Noteworthy examples include the Bates' reagent (52) of Ramage et al.\textsuperscript{151}, and the BOP reagent (53) of Castro\textsuperscript{152,153}.

An alternative approach was reported by Ueki et al.\textsuperscript{154} when he introduced dimethylphosphinothioyl chloride (54) (Mpt-Cl) as a reagent for the preparation of dimethylphosphinothioic mixed anhydrides (55).

It was found that the preparation of the mixed anhydrides could be performed in polar, protic solvents \textit{e.g.} MeOH. MeOH cannot be used in traditional peptide bond formation reactions due to its high reactivity toward the activated amino acid derivatives. The applicability of this reagent has been demonstrated by the synthesis of sparingly soluble C-terminal segments of vasoactive intestinal peptide
(VIP) up to the heptapeptide stage.

\[
\begin{align*}
S & \quad (\text{Me})_2\text{P-Cl} \\
R & \quad R'-\text{NH-CH-C-O-P(\text{Me})}_2 \\
\end{align*}
\]

(54) 

(55)
1.4. Glycopeptides and glycoproteins

1.4.1. Introduction

Chemical and biochemical researchers had long considered proteins and carbohydrates to be two distinct classes of natural products, and due to the belief that biological specificity could be attributed to proteins, with carbohydrates largely serving only a secondary role as structural and protective moieties, or as energy sources, protein research had enjoyed by far the greater attention.

It is only since the 1950's that the existence, and the diversity, of a novel class of ubiquitous natural products, the glycoproteins, has been proved. These substances, in which proteins are bound covalently to carbohydrate units, have been found to occur in cells both in soluble and membrane-bound forms, as well as in the intercellular matrix and the extracellular fluids.

In fact, early researchers working on the isolation and determination of protein and carbohydrate structures often went to some length to remove what they thought were sugar 'impurities' from proteins and protein 'impurities' from polysaccharides. Now it seems many proteins (and many polysaccharides) exist in vivo as glycoproteins, and far from being biologically insignificant, there is evidence to suggest that the carbohydrate moieties perform important biological functions, for example, in the stabilisation of protein conformation - hence stabilisation of activity, the specification of blood types, the control of the lifetimes of glycoproteins in the circulatory system of higher animals, and the control of glycoprotein uptake by cells.

While research has shown there is a vast number of natural glycoproteins, there is seemingly little variation in the type of linkage employed between the protein and carbohydrate units. The principle linkages concern the formation of a covalent bond between the anomeric centre of a carbohydrate residue, and the amide of the side chain of an asparagine residue, or the side chain hydroxyl functions of serine and threonine residues. The former are collectively known as N-glycoproteins, the latter as O-glycoproteins.

There are at least 200 monosaccharides known to occur in nature, however, only a limited number of these (about 20) have been shown to be present in glycoproteins. These are mostly hexoses or their simple derivatives, such as N-acetyliminosamines and uronic acids. In addition to these, some animal
glycoproteins have been shown to contain the more complex sugars, the sialic acids. More than 20 different sialic acids (derived from the nine-carbon straight-chain sugar, neuraminic acid\textsuperscript{156}) have been identified, including N-acetylneuraminic (the most common) and N-glycolylneuraminic acid.

The carbohydrate moiety need not just be a monosaccharide unit, oligosaccharides and polysaccharides, in which the carbohydrate moieties are linked through O-glycosidic bonds, are also found in abundance. In glycoproteins such as serum glycoproteins, the carbohydrate unit is an oligosaccharide consisting of up to 20 monosaccharides, generally arranged in branched sequences with no repeating structures. The proteoglycans, such as chondroitin sulphate and dermatan sulphate, contain linear units of 50 to 100 carbohydrate residues made up of repeating disaccharide structures.

One point to note is that carbohydrate moieties which cross-link protein chains have yet to be encountered.

\[
\text{---NH-CH-CO---}
\]

\[
\begin{array}{c}
\text{CH}_2 \\
\text{OH} \\
\text{HO} \\
\text{AcNH}
\end{array}
\]

\begin{center}
(56)
\end{center}

The most common N-glycoprotein involves asparagine bound through a $\beta$-N-glycosidic linkage to N-acetylglucosamine (56). Examples in nature of this linkage include immunoglobulins, hormones (e.g. chorionic gonadotropin), hormone precursors (e.g. thyroglobulin) and membrane glycoproteins (e.g. glycosyl transferases).

The biosynthesis of these N-glycoproteins (56) has been investigated at some length\textsuperscript{157}, and it has been found that they are further characterised by a common sequence in the connecting region: N-glycosylated asparagine appears to be always followed by a serine or threonine residue at the next but one C-terminal amino acid. It is thought that the hydroxyl function of this residue catalyses the glycosyl transfer\textsuperscript{158}. 
Recent studies have confirmed the existence of alternatives to the type (56) N-glycosidic linkage. Investigations of cell surface glycoproteins of halobacteria have shown that they contain N-glucosyl-\textsuperscript{159} and N-galactosaminylasparagine\textsuperscript{160}, and also the involvement of glutamine as a binding partner has been postulated\textsuperscript{161}.

Proteoglycans of the extracellular matrix and connective tissue display a characteristic $\beta$-O-glycosidic protein to carbohydrate linkage between xylose and serine (57). On the other hand, the linkage between N-acetylgalactosamine and serine (and threonine) (58), is commonly an $\alpha$-O-glycosidic bond. Although this connecting element was first reported as present in mucous glycoproteins (mucins)\textsuperscript{155}, it is characteristic of many other membrane and serum glycoproteins, including the blood group glycoproteins\textsuperscript{162}, human glycophorin\textsuperscript{163}, and the glycoproteins of the tumor-associated $T_N$ and $T$ antigens\textsuperscript{164}.

\begin{center}
\begin{align*}
\text{(57)} & & \text{(58)}
\end{align*}
\end{center}

In glycoproteins which contain more than one N-glycosidic unit, several amino acids separate the points of attachment of the carbohydrate moieties. In contrast, O-glycosidic chains may be attached at adjacent amino acid residues. In glycophorin\textsuperscript{163}, for example, residues 2 to 4 and 10 to 15 are all hydroxyamino acids with attached carbohydrate units, and residues threonine-25 and asparagine-26 are O- and N-glycosylated respectively.

The highest concentration of serine and threonine residues with attached carbohydrate units is found in two classes of substance. One is the class of glycoproteins known as antifreeze glycoproteins (AFGPs)\textsuperscript{165,166} (see below). These are polymers of the monomer unit, Ala-Ala-Thr, with all the threonine residues substituted with the galactosyl-$\beta$1$\rightarrow$3-N-acetylgalactosamine disaccharide. The other is ovine submaxillary mucin, in which one in every three residues is either
serine or threonine with an attached $N$-acetylneuraminyl-$\alpha$2\(\rightarrow\)6-$N$-acetyl-
galactosamine unit.

The large variation in both size and number of oligosaccharide chains has
resulted in glycoproteins with very different proportions of carbohydrate present
\textit{i.e.} from 1\% (\textit{e.g.} collagen) to 85\% (soluble blood group substances) carbohydrate.

1.4.2. Glycoprotein biosynthesis

The polypeptide chains of glycoproteins are produced in the same manner as
non-glycosylated proteins. Their structure is therefore determined by information
contained in the genetic code and as a result all the molecules of a given protein
are chemically identical. Oligo- and polysaccharides, on the other hand, whether
free or covalently bound to polypeptide chains in glycoproteins, are not primary
gene products.

The attachment of carbohydrate moieties to polypeptide chains is a
co-translational event, catalysed by enzymes that permit the transfer of saccharide
units from nucleotides, known as glycosyltransferases. These glycosyltransferases,
for example, sialyltransferases and galactosyltransferases, act in a stepwise
manner, with the product of one enzymically controlled process acting as a
substrate for the following one. The identity of the carbohydrate sequence is
therefore controlled indirectly by the properties of the glycosyltransferases, which
are themselves primary gene products. However, this method of synthesis results
in microheterogeneity within the carbohydrate unit\(^{167}\), and thus renders the
isolation and characterisation of natural glycoproteins difficult.

One further point to note is that examples of carbohydrate units being added
nonenzymically to proteins \textit{in vivo} are known\(^{168}\).

Until the 1970's it was thought that all the carbohydrate units of glycoproteins
were produced by this stepwise transfer of monosaccharides from nucleotides to
the growing oligosaccharide structures. While this appears to be the case in the
synthesis of O-glycosidic units, researchers\(^{168}\) have demonstrated an alternative
mechanism for the process of N-glycosylation. The existence of a lipid-linked
oligosaccharide or "G-oligosaccharide" precursor, central to the \textit{in vivo} process,
has been discovered.

The formation of this G-oligosaccharide, which consists of two residues of
$N$-acetylglucosamine, nine residues of mannose, and two or three residues of
glucose, is controlled by the dolichol phosphate cycle. The function of these lipid-linked saccharides appears to involve the transfer of the hydrophilic carbohydrates into or through the hydrophobic membrane to a position where the formation of glycoproteins occurs.

The G-oligosaccharide is attached to the side chain of an asparagine residue of the growing polypeptide chain. The subsequent removal of some of the carbohydrate units of the oligosaccharide, by α-glycosidases and α-mannosidases, and incorporation of additional saccharide residues, by glycosyltransferases, results in the formation of the required glycoproteins.

This is by no means a lengthy consideration of the extensive process of glycoprotein biosynthesis. Reviews can furnish the reader with much greater detail.

1.4.3. Biological significance of glycoproteins

Cellular biological and biochemical investigations have clearly demonstrated the fundamental biological functions that glycoproteins perform.

Although glycoproteins are widely distributed, both as soluble and membrane-bound forms, perhaps their most important roles are in post-translational biological selectivity, particularly in biological recognition, and in cell differentiation and the regulation of cell growth.

Studies of human erythrocytes and lymphocytes have demonstrated the amphiphatic nature of cell-membrane glycoproteins, that is, in addition to the hydrophilic sections commonly present in soluble proteins, they contain a hydrophobic region that anchors them to the lipid bilayer. Biological selection processes, which occur predominately on the intracellular and outer-cell membranes, are responsible for the transportation of a recently synthesised protein to its required location. In eukaryotic cells at least, glycoproteins perform an important function in this post-translational biological selectivity which is clearly based on a receptor-effector interaction.

Researchers have investigated at some length the functions of the carbohydrate moieties of both soluble and membrane-bound glycoproteins - for some examples, see above.

It is important to be aware that the glycosylation of proteins is an expensive
procedure for an organism to perform. It requires a considerable amount of genetic information, the synthesis of enzymes, cofactors and intermediates, and the expenditure of much energy. Hence the inclusion of a carbohydrate unit must be of considerable value to the cell.

Carbohydrates in soluble glycoproteins appear to perform a variety of functions. In particular, they may alter the physicochemical properties of the protein by changing its hydrophobicity, electrical charge, mass and size. However, although the sugar rarely affects the biological activities of the protein\textsuperscript{172}, it may act as a recognition marker on soluble glycoproteins. This has been demonstrated by the control of the lifetime of serum glycoproteins in the circulatory system and their uptake into liver cells.

Glycoproteins, especially if they are carbohydrate rich, are more resistant to proteolysis \textit{in vitro} than are non-glycoslated proteins\textsuperscript{172}. Also if the sugar content is very high (particularly if considerable amounts of sialic acid are present), the conformation of the protein may be altered.

Cell-surface glycoprotein carbohydrate units have been shown to perform roles in intercellular adhesion, which is an important stage in fundamental biological processes such as fertilization and cellular differentiation\textsuperscript{167}, as well as serving as initial attachment sites for viruses\textsuperscript{173}.

1.4.4. Antifreeze glycoproteins

A unique class of serum proteins, the antifreeze glycoproteins (AFGPs)\textsuperscript{165,166}, are found in some Antarctic and Arctic fish species and overwintering insects. These glycoproteins depress the freezing point of the aqueous body fluids (and of water) by means of a non-colligative mechanism\textsuperscript{174,175}, without significantly lowering the melting temperature\textsuperscript{176}. One third of the freezing temperature depression in the blood sera of the Antarctic fish species, \textit{Trematomus borchgrevinki} is attributed to the presence of these antifreeze glycoproteins\textsuperscript{177}.

To date eight closely related glycoproteins and glycopeptides have been identified, numbered 1 to 8 (AFGP 1 to 8) in order of decreasing size according to their relative migrations on gel electrophoresis. AFGP 1 to 5 (m. wt. 10500 to 32000) have similar antifreeze capabilities and are polymers of the monomer, Ala-Ala-Thr, with each threonine residue substituted with a galactosyl-\textbeta\textsubscript{1}→3-N-acetylgalactosamine disaccharide unit. AFGP 6 to 8 have basically the
same repeating monomer, except that proline residues are often present after the threonines. They have smaller molecular weights (2600 to 10000), are found in larger quantities than AFGP 1 to 5 and by themselves exhibit lower antifreeze activities\textsuperscript{178}.

Research has shown, however, that the largest freezing point depression values are obtained with mixtures of AFGP 1 to 5 and 6 to 8 \textit{i.e.} there appears to be a cooperative function between the two different components\textsuperscript{179}.

It should be noted that a class of nonglycoprotein polypeptides (AFPs)\textsuperscript{180,181} exist, which, although containing no carbohydrate, also depress the freezing temperature of water non-colligatively. They display a unique compositional similarity to AFGP, in that approximately two thirds of their amino acid content is alanine.

1.4.5. Glycopeptide synthesis

Introduction

Perhaps the most attractive strategy for the synthesis of glycopeptides would involve the coupling of the peptide and carbohydrate moieties following their separate preparation by established methods. However, this method has been hampered by the lack of reliable methods for the stereoselective introduction of the glycosidic linkage and by the limited solubility of peptides in solvents which in turn determine the outcome of the glycosylation reaction. Therefore the most practical approach to date involves the preparation of adequately protected N- and O-glycosyl amino acid derivatives, followed by extension from either or both of the C- and N-terminals. One advantage of this method is that the final glycopeptide may contain both glycosyl and non-glycosylated serine, threonine and asparagine residues.

Glycopeptide synthesis requires protecting groups in order to direct the site of required reaction, that is, in the case of the carbohydrate moiety, the anomic centre must be selectively exposed and functionalised, and with the amino acids, protecting groups must be chosen in order to selectively protect and deprotect both the amine and carboxyl terminals. In short, the problems of peptide and carbohydrate chemistry merge in the synthesis of such polyfunctional molecules.

The requirements of glycopeptide synthesis can therefore be summarised as follows:
1. Deprotection of peptide residues to permit coupling at the C- and N-terminals must be both selective and compatible with the traditional carbohydrate hydroxyl protecting groups (i.e. benzyl ethers and acetates).

2. Protecting group and coupling methodology must permit the glycosidic linkage to be formed stereoselectively and to be retained unchanged during the course of the synthesis.

3. Amino acid protecting groups must be acid stable due to the acid-catalysed nature of the glycosidation reaction.

The stability of the glycosidic linkage itself must also be considered. The N-glycosidic linkage is relatively stable to mild acid, but is hydrolysed under harsh conditions. The various O-glycosidic linkages are also acid-labile, and differ markedly in their sensitivity to base\(^{182}\) (they are cleaved by base by a reaction known as β-elimination). Recent investigations have shown that the lability of the O-glycosidic linkage appears to be dependent on the structure of the glycopeptide itself (see review\(^{183}\)).

The synthesis of oligosaccharides represents a separate area of chemistry and has been the subject of many recent reviews\(^{184,185}\), however, it will not be considered further here. The synthesis of N- and O-glycopeptides has been comprehensively reviewed in 1985\(^{186}\) and 1983\(^{187}\) respectively, and again in 1987\(^{183}\). The work of this project does not involve N-glycopeptide synthesis, and therefore only the more significant methods and recent advances in O-glycopeptide synthesis will be discussed.
The β-glucosyl-serine/threonine linkage

The β-glycosidic linkage between carbohydrate residues and serine (and threonine) side chain hydroxyl functions is found extensively in the glycoproteins of fungi, animals and plants. Xylosylserine derivatives of the type (57), for example, are especially characteristic of the animal proteoglycans, and have been prepared by Lindberg et al under Koenigs-Knorr glycosylation reaction conditions. However, in plants and fungi, other carbohydrates commonly occur as connecting partners.

The use of Koenigs-Knorr, or the modified Helferich, conditions has been extensive in glycopeptide synthesis, and a number of syntheses are discussed in the aforementioned reviews. One notable example concerns the synthesis of the glycotripeptide alanyl-(O-β-D-galactopyranosylseryl)-alanine, by van Boom et al. The glycosylation of the (9,10-anthraquinon-2-yl)methyl (Maq) ester (see below) of Fmoc-serine and threonine (59) with 2,3,4,6-tetra-0-acetyl-β-D-galactopyranosyl bromide (Ac₄αGal-Br) (60) was initially attempted using the Helferich modification of the classical Koenigs-Knorr method. However, low yields of the desired product (61) were obtained even when the mercuric cyanide was activated by the addition of mercuric bromide. It was only when pure mercuric bromide and two equivalents of the monosaccharide were used that acceptable results were obtained (Scheme 1.17).

Modern variants of the Koenigs-Knorr reaction have also been used in the synthesis of O-glycosylserine (and threonine) linkages. Kunz et al for example, have successfully employed the silver trifluoromethanesulphonate (silver triflate) / tetramethyl urea method to couple the 2-bromoethyl ester of Z-serine and the benzyl ester of Fmoc-serine (62) to α-D-galactopyranosyl bromides (63) to give protected glucosylserine derivatives (64) (Scheme 1.18).

The trifluoromethanesulphonic (triflic) anhydride method has also been employed. Both Gobbo et al and Lacombe and Pavia, have synthesised glycosylthreonine derivatives in good yield. However, in both cases the authors have reported that a mixture of the α and β anomeric products are obtained, and these have to be separated. It is interesting to note that Lacombe and Pavia chose threonine, rather than serine, for their glycosylation reactions. This is because of the presence of a methyl group on the former, which enables the stereochemistry of the anomeric linkage and the racemization at either or both the asymmetric amino acid centres to be monitored by ¹³C n.m.r. spectroscopy.
Scheme 1.17.

Scheme 1.18.
Scheme 1.19.
Alternatives to the Koenigs-Knorr method have also been reported. The xylopyranose (65), for example, has been treated with the allylic ester of Z-serine (66) by means of the trichloroacetimidate method\textsuperscript{185,201} to give (67) in good yield\textsuperscript{202} (Scheme 1.19). This product (67) has also been obtained by the reaction of the xylopyranosyl fluoride derivative (68) with the serine ester (66) in the presence of boron trifluoride etherate and triethylamine\textsuperscript{203} (Scheme 1.19). However, as with the triflic anhydride method, both \(\alpha\) and \(\beta\) anomers are formed and must be separated by chromatography.

The \(\alpha\)-glycosidic serine/threonine-N-acetylgalactosamine linkage

For many years the preparation of the \(\alpha\)-glycosidic linkage between N-acetylgalactosamine and serine and threonine residues, which is characteristic of many serum and membrane-bound glycoproteins, presented considerable problems. Attempts to synthesis these derivatives in the corresponding glucose series using Koenigs-Knorr or Helferich methodology had resulted in almost exclusively the \(\beta\)-anomer.

It was only when Paulsen \textit{et al.}\textsuperscript{204} introduced glycosyl chlorides that contained the 2-azido function as the reactive carbohydrate moiety that an answer was found. By adapting this methodology and applying it with the triflic anhydride coupling procedure, Ferrari and Pavia\textsuperscript{205} glycosylated Fmoc-serine and threonine active esters (69) (Scheme 1.20) in order to synthesise two antigenic \(T_N\) triglycosylated pentapeptides.

Advantages of this method include the ready availability of the 2-azido-glycosyl halides \textit{via} the azidonitration route\textsuperscript{206}, and the possible conversion of the azido group to the acetamido group by treatment with dimethyl(phenyl)phosphine in glacial acetic acid\textsuperscript{207} (Scheme 1.21).

More recently \(\alpha\)-glycosides have been reported as being prepared by two alternative routes. While one involves the coupling of 2-azido-2-deoxygalactopyranose with serine derivatives using the trichloroacetimidate method\textsuperscript{208}, the other employs triflic anhydride as the coupling reagent\textsuperscript{209}.
\[
\text{Scheme 1.20.}
\]

\[
\text{Scheme 1.21.}
\]
Advances in glycosyl amino acid protecting groups

\( N^\alpha \) -protecting groups

Chemists have traditionally made use of the Fmoc \( N^\alpha \)-protecting group\(^{41} \) in the synthesis of glycopeptides. Its removal, under basic conditions that are sufficiently weak, permits the base-labile 0-glycosylserine linkage to remain intact during deprotection. While some syntheses have employed the commonly applied piperidine in DMF deprotection conditions\(^{198} \), more recent researchers have turned to the weaker base, morpholine, for the selective cleavage of the Fmoc moiety\(^{192,196} \).

An alternative protecting group, the triphenylphosphonioethoxycarbonyl (\( \text{Peoc} \)) group\(^{70} \), was introduced in 1976 by Kunz\(^{90,91} \). The author employed this group in the synthesis of \( N \)-glycopeptides in conjunction with the C-terminal benzyl and \( \text{tert.} \)butyl ester groups\(^{183} \). It was shown to exhibit high acid stability, while being cleaved in very mild basic conditions – typically, a solution of morpholine in DCM – and hence base-labile 0-glycosylserine linkages remain completely intact during deprotection. There are, however, limitations: the bulky nature of the group hinders the condensation reaction, the group is so base-labile that the reaction conditions must be very carefully controlled, and as the Peoc group is a cationic moiety, its halogen counter ion (\( i.e. \) chloride) may interfere with glycosylation reactions involving mercury or silver reagents. Hence the \( Z \)-group had to be used in the glycosylation step (and the Peoc group for subsequent chain extension), and this in turn prohibited the use of the benzyl ester carboxyl protecting group.

Kunz et al have also reported the use of the 2-(2-pyridyl)ethoxycarbonyl (2-Pyoc)\(^{210} \) (71) and the 2-(4-pyridyl)ethoxycarbonyl (4-Pyoc)\(^{183} \) (72) \( N^\alpha \)-protecting groups, as well as more recently, the \( p \)-nitrocinnamylxocarbonyl (Noc)\(^{211} \) (73) protecting group.

The 2-Pyoc and the 4-Pyoc groups are themselves extremely stable toward acid and base, but, following a simple modification, may be cleaved from the amino function under very mild conditions. This involves the quaternization of the pyridine nitrogen with methyl iodide, which renders the \( \alpha \)-methylene group very acidic, and subsequent treatment with a mild base, such as morpholine, in DCM.

The Noc protecting group can be removed under conditions that are practically neutral, that is, by palladium(0)-catalysed allylic transfer, in which
N,N'-dimethylbarbituric acid (DMBS) is employed as a suitable allylic acceptor\textsuperscript{212}. One further advantage of this group is its stability to the rhodium(1)-catalysed isomerisation and hydrolysis conditions that are used to cleave the allylic ester carboxyl protecting group\textsuperscript{202}.

**Carboxyl protecting groups**

The benzyl and tert-butyl ester carboxyl protecting groups have both been employed in glycopeptide synthesis\textsuperscript{183}. The problems associated with these groups, for example, the variable stability of Fmoc derivatives during the hydrogenolysis of benzyl esters, and the problematic tert-butyl carbocation side reactions during deprotection of tert-butyl esters, are well documented\textsuperscript{183}, and hence alternative protecting groups have been sought.

One alternative, first described by Ugi \textit{et al}\textsuperscript{194}, and applied to glycopeptide synthesis by Kunz and Buchholz\textsuperscript{195}, is the 2-bromoethyl ester group (74). The authors\textsuperscript{195} have shown that both the 2-chloro- and 2-bromoethyl esters of amino acids and peptides, after conversion to the iodoethyl ester, can be removed selectively with zinc in DMF. These neutral conditions permit the deprotection of a carboxyl function in the presence of the Z-protecting group, whilst leaving the glycosylserine linkage intact.

At about the same time, Kemp and Reczek\textsuperscript{193} introduced the (9,10-anthraquinon-2-yl)methyl (Maq) ester (75) as a possible carboxyl protecting group. Van Boom \textit{et al}\textsuperscript{192} successfully employed this ester, which can be cleaved under reductive conditions milder than catalytic hydrogenation (\textit{e.g.} by reaction with sodium dithionite in aqueous-organic solution), in O-glycopeptide synthesis.

More recently, Kunz and Waldmann have made use of allylic esters (76) in the preparation of both N-glycopeptides\textsuperscript{213} and O-glycopeptides\textsuperscript{202}. In the case of O-glycopeptides, quantitative deprotection of the allylic ester is effected by treatment with a solution of tetrakis(triphenylphosphane)palladium(0) in THF, with morpholine employed as the acceptor nucleophile. For further syntheses involving this group – see review\textsuperscript{183}. 
Recent advances in glycopeptide synthesis

A few examples of the chemical glycosylation of suitably protected peptides and proteins have now been reported\(^{214,215}\). Kessler \textit{et al.}\(^{215}\), for example, have adapted the N-iodosuccinimide (NIS) method, introduced by Thiem \textit{et al.}\(^{216}\) for the synthesis of 2-deoxyoligosaccharides, for the stereoselective glycosylation of peptide fragments. The authors have shown that peptides up to the pentapeptide size, react directly to yield products of almost complete \(\alpha\)-diastereoselectivity.

However, the most noteworthy advance in the preparation of glycopeptides has been the introduction of solid phase synthesis, where preliminary reports have begun to appear.

Kunz and Dombo\(^{217}\) have employed polymeric supports with allylic spacer groups to synthesise, for example, glycotripeptides. The base-labile Fmoc N\(^\alpha\)-protecting group\(^{41}\) has been used (as the deprotection conditions for acid-labile groups would result in the loss of the glycosidic linkage – see above), and the final cleavage of the glycopeptide from the resin was effected by the standard conditions employed by Kunz and Waldmann\(^{202}\) for the cleavage of the allylic ester carboxyl protecting group, namely a solution of tetrakis-(triphenylphosphane)palladium(0) and morpholine in THF.

Paulsen \textit{et al.}\(^{218}\) have synthesised, using solid phase methodology, two hexapeptides, corresponding to the partial structures of ovine submaxillary mucin and porcine glycophorin, by employing a strategy that includes the Wang resin (8) and Fmoc N\(^\alpha\)-protection. Interestingly, the final cleavage of the glycopeptide from the resin, performed in aqueous TFA (95%), did not result in cleavage of the \(\alpha\)-glycosidic threonine-N-acetylgalactosamine linkage.

Solid phase glycopeptide synthesis is very much in its infancy, but in forthcoming years it will surely become the most active area of glycopeptide preparation.
2.1. The 2,2- \textit{bis}(4'-Nitrophenyl)ethoxycarbonyl (Bnpeoc) N\textsuperscript{\alpha}-protecting group

2.1.1. Introduction

The 9-fluorenylmethoxycarbonyl (Fmoc) group (15) has enjoyed considerable employment as the principle base-labile N\textsuperscript{\alpha}-protecting group for solution and solid phase peptide synthesis since its introduction by Carpino and Han\textsuperscript{41} in 1972. The advantage of this transient protecting group is that it allows the use of acid-labile semi-permanent protecting groups based on the \textit{tert}butyl moiety to be employed in the protection of most side-chain functionality. Together with a suitable peptide-resin linkage (see Introduction), it introduced a truly orthogonal approach to solid phase peptide synthesis, and eliminated several of the problems associated with the initial Merrifield resin-Boc protection strategy.

The removal of the Fmoc group, effected by secondary amines - typically 20\% (v/v) piperidine in DMF (Scheme 2.1); has been shown to proceed via a $\beta$-elimination process.

The byproducts of this cleavage reaction, dibenzofulvene (77) and the tertiary amine (78) formed from the addition of piperidine to the double bond of (77) (Scheme 2.2) are soluble in DMF and are readily removed.

The principle drawback to the Fmoc methodology is its cost - Fmoc amino acid derivatives are 2-5 times more expensive than their Boc counterparts. It was with this in mind that Ramage and Florence\textsuperscript{78} and Valentine\textsuperscript{79}, sought to design and develop a cheaper alternative to the Fmoc moiety. The stringent criteria which must be achieved for the successful introduction of such a protecting group are as follows:

1. the protecting group must be easily prepared and introduced
2. the protecting group must not introduce an extra chiral centre
3. the protected amino acid derivatives must have good shelf lives
4. the deprotection reaction must be selective, high yielding and
Scheme 2.1

Scheme 2.2
Scheme 2.9

Scheme 2.10
must proceed under mild conditions

5. separation of the deprotected derivative from the byproducts etc. must be readily performed

6. all reagents, byproducts etc. must be non-toxic.

The researchers\textsuperscript{78,79} believed that the 2,2-\textit{bis} (4'-nitrophenyl)ethoxycarbonyl (Bnpeoc) group (16) meets these criteria.

2.1.2. Preparation of 2,2-\textit{bis} (4'-nitrophenyl)ethanol

The method employed at present in this research group for the synthesis of 2,2-\textit{bis} (4'-nitrophenyl)ethanol (Bnpe-OH) (19) has been outlined above (see Introduction). While the yields for each of the four steps involved are generally acceptable (58-100\%), the overall yield for such a synthesis, based on styrene oxide and phenylmagnesium bromide, is 40\%. Hence it was hoped a reduction in the number of steps of the process would decrease the duration of the synthesis, and result in an increase in yield (and therefore a reduction in cost).

Jarczewski and Leffek\textsuperscript{219} have shown that it is possible to nitrate the 4-position of both phenyl rings in diphenylmethane (79) by simply adding the solid (79) to a vigorously stirred solution of conc. nitric acid and conc. sulphuric acid at reduced temperature. Although the desired product, di-(4-nitrophenyl)methane (80), could be separated from any byproducts (\textit{i.e.} mono-nitrated, 2,4'-dinitro derivatives etc.) by recrystallisation from benzene, no yield for the preparation was quoted by the authors.

By employing (80) and adopting the approach outlined by Hamrick and Hauser\textsuperscript{220} for the preparation of 2,2-diphenylethanol either directly by the formylation of potassium diphenylmethide or by the reduction of 2,2-diphenylacetic acid (which is produced by the quenching of the potassium diphenylmethide with carbon dioxide), a 2-step synthesis of (19) could be envisaged (Scheme 2.3).

Attempts to repeat the process of Jarczewski and Leffek were hindered by their poorly defined work up procedure and the aggregation of large quantities of solid diphenylmethane on addition to the acid mixture. In order to improve this, the diphenylmethane (79) was dissolved in nitromethane and added very slowly to the very vigorously stirred reaction mixture at -15 to -10°C, and stirred for 45 minutes at this temperature. An analytical hplc trace of the resulting solid did indeed reveal
the existence of more than one product, however, as suggested by Jarczewski and Leffek, recrystallisation from benzene (twice) afforded pure di-(4-nitrophenyl)-methane (80) in 37% yield.

\[
\begin{align*}
&\text{conc. HNO}_3 \\
&\text{conc. H}_2\text{SO}_4
\end{align*}
\]

Scheme 2.3

In an attempt to increase the yield of (80), a number of alternative reaction temperatures/reaction times were studied (Figure 2.1).

The conclusion that was drawn from these studies was that by increasing the duration of the reaction (to 180 minutes), a significant increase in the reaction yield is obtained (Scheme 2.4). As for lowering the reaction temperature, it is unclear from the above experiments whether this in itself results in any increase in yield.
<table>
<thead>
<tr>
<th>reaction temperature</th>
<th>reaction duration</th>
<th>yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 -15 to -10°C</td>
<td>30 minutes</td>
<td>37%</td>
</tr>
<tr>
<td>2 -15 to -10°C</td>
<td>45 minutes</td>
<td>37%</td>
</tr>
<tr>
<td>3 -15 to -10°C</td>
<td>90 minutes</td>
<td>45%</td>
</tr>
<tr>
<td>4 -25 to -20°C</td>
<td>30 minutes</td>
<td>34%</td>
</tr>
<tr>
<td>5 -25 to -20°C</td>
<td>90 minutes</td>
<td>47%</td>
</tr>
<tr>
<td>6 -25 to -20°C</td>
<td>180 minutes</td>
<td>53%</td>
</tr>
<tr>
<td>7 -25 to -20°C</td>
<td>180 minutes</td>
<td>52%</td>
</tr>
</tbody>
</table>

**Figure 2.1**

![Scheme 2.4](image)

**Scheme 2.4**

Attempts were then made to react di-(4-nitrophenyl)methane (80) with a suitable base to form the carbanion intermediate (81) and to formylate it. Formation of the carbanion using butyl lithium proceeded smoothly (as evidenced by the instant generation of the characteristic blue colouration). However formylation, using dry formaldehyde afforded only starting material and no (19). Repeating this reaction, generating the carbanion both with butyl lithium at -78°C, and with piperidine at room temperature, again resulted in none of the desired product.

Carpino\textsuperscript{221} more recently introduced a more convenient and higher yielding synthesis for the preparation of 9-fluorenylethanol (84) (Scheme 2.5). In this,
fluorene (82) was reacted with sodium hydride and ethyl formate to afford the aldehyde intermediate (83), which was subsequently reduced to the corresponding alcohol (84) by treatment with sodium borohydride. It was hoped that sodium borohydride could be used in the presence of nitro groups without resulting in their conversion to the corresponding amines.

\[
\text{fluorene (82)} \xrightarrow{\text{NaH, HCO}_2\text{CH}_2\text{CH}_3} \text{aldehyde intermediate (83)} \xrightarrow{\text{NaBH}_4} \text{alcohol (84)}
\]

Scheme 2.5

The above scheme was therefore repeated using di-(4-nitrophenyl)methane (80). However, while the formation of the carbanion intermediate proceeded as above, none of the desired aldehyde product could be isolated. Repeating the reaction, using sodium ethoxide instead of sodium hydride as the base, had no beneficial effect.

The data in Figure 2.2 suggests that the carbanion intermediate (81) of di-(4-nitrophenyl)methane (80)\textsuperscript{219} should form rapidly and easily — and by the generation of the characteristic blue colour in each of the above experiments, this appears to be the case. Figure 2.2 summarises the importance of the effect of electron withdrawing groups on $\beta$-carbon acidity — the electron withdrawing power of the $\beta$-substituent results in the stabilisation of the ionised carbon through delocalisation into the electronegative centre. However, this considerable delocalisation most certainly results in a substantial reduction in the nucleophilicity of the $\beta$-carbon, and hence is probably the reason why to date it has been impossible to achieve reaction at that carbanion centre.
2.1.3. Bnpeoc $\text{N}^{\alpha}$-protection of amino acids

The first derivative of $2,2'$-bis (4'-nitrophenyl)ethanol (19) to be considered for the protection of the $\text{N}^{\alpha}$-functionality of amino acids was the corresponding chloroformate (20). Florence\textsuperscript{78} had shown that this derivative could be prepared in good yield by the reaction of (19) with a solution of phosgene in toluene and N-methylmorpholine in DCM. However, while (20) has been successfully employed in the protection of the amine functionality of penicillins and cephalosporins\textsuperscript{222}, and the 5' sugar hydroxyl of DNA deoxyribose units\textsuperscript{223}, in amino acids it led to the formation of significant quantities of the undesired Bnpeoc dipeptide. Researchers have been aware of this problem with the corresponding Fmoc-Cl\textsuperscript{224}, with which up to 20% oligopeptide contaminant may be formed during the protection reaction. This can be rationalised by the reaction of the carboxyl function of the amino acid with the chloroformate, resulting in the formation of a mixed anhydride intermediate, which goes on to react with the free amine.

A number of mixed carbonate derivatives\textsuperscript{225} were then considered, with the
active ester (21) formed from the reaction of (20) with N-hydroxysuccinimide\textsuperscript{109} being chosen due to its ease of preparation, ease of use and the resulting high acylating yields.

Florence\textsuperscript{78} has shown (21) to be useful in the preparation of many Bnpeoc N\textsuperscript{O}-protected amino acids in good yield. His method of choice for the protection reaction was an adaptation of the method introduced by Lapatsanis\textsuperscript{81}, which involves dissolving the amino acid in 2 eq. of a cooled, aqueous solution of 10% Na\textsubscript{2}CO\textsubscript{3}, followed by the slow addition of the active ester in DMF.

![Scheme 2.6](image)

Applying this method, a number of Bnpeoc amino acids have been prepared (e.g. Bnpeoc-Ala-OH (85), Bnpeoc-Phe-OH (86) and Bnpeoc-Gly-OH (87)) in good yield (65-75%). However, when the reaction was repeated with serine and threonine, lower reaction yields were obtained (50-65%). Florence has shown that in order to ensure an excess of base (without significantly altering the pH), an additional 1 eq. of an aqueous solution of 5% NaHCO\textsubscript{3} could be added and it was shown that in the case of tyrosine, this resulted in a slight increase in yield. By applying this to serine and threonine the reaction yields were increased by 10-15% (Scheme 2.6).

An alternative method of protection with ONSu active esters was introduced by Rich\textsuperscript{82} in 1987, for use with his Teoc N\textsuperscript{O}-protecting group. This requires the suspension of the amino acid in water, before the addition of 1.5 eq. of triethylamine in dioxan and the solid ONSu active ester. His work also displayed the possibility of purifying the protected amino acid derivative by the formation,
and the liberation of the cyclohexylamine (CHA) salt. This optional purification step would not be possible with Fmoc amino acids, as the protecting group would be cleaved by treatment with CHA.

Bnpeoc-Ser-OH (88), Bnpeoc-Thr-OH (89) and Bnpeoc-Phe-OH (86) have been prepared in good yield (70–80%) by the Rich method, both with and without the CHA salt purification step (Scheme 2.7).

\[
\begin{align*}
H_2N-CH-CO_2H & \quad \text{Et}_3N \\
\text{Bnpeoc-ONSu} & \quad \text{Bnpeoc-OH} \\
(21) & \quad \text{H}_2\text{O/dioxan}
\end{align*}
\]

Scheme 2.7

2.1.4. Deprotection of Bnpeoc N\(^\alpha\)-protected amino acid derivatives

On the introduction of the Bnpeoc group, Florence\(^{78}\) had demonstrated the possibility of employing bases such as 1,5-diazabicyclo[4.3.0]non-5-ene (DBN) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in stoichiometric quantities for its removal. These are strong bases, but importantly weak nucleophiles, and hence the possible occurrence of unwanted side reactions is reduced.

Florence has also shown that the deprotection reaction proceeded at a much increased rate when DMF was used as the solvent instead of DCM. Also, DBU reduced the deprotection time when used in preference to DBN, and increasing the number of equivalents of DBU from 1 to 2 effectively resulted in a 50% reduction in the elimination time. However, the system now used routinely for the deprotection involves the addition of an equimolar quantity of glacial acetic acid to the DBU solution. This suppresses the free amine that is generated, together with the side reactions that it may cause, but reduces only slightly the effective concentration of the DBU and the rate of the \(\beta\)-elimination reaction.

In order to determine the possible conditions for the removal of the Bnpeoc moiety, a study of Bnpeoc/Fmoc deprotections with both DBU/AcOH in DMF and
20% piperidine in DMF was undertaken.

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{H} \quad \text{CH}_2\text{OCONH} - \text{CH}_2 - \text{CO}_2\text{CH}_2 \\
\text{NO}_2 & 
\end{align*}
\]

(90)

\[
\text{DBU/AcOH} \quad \text{DMF}
\]

\[
\begin{align*}
\text{O}_2\text{N} & \quad \text{H}_\text{2} \quad \text{CH}_2 \\
\text{NO}_2 & 
\end{align*}
\]

(91)

\[
\text{DBU} + \text{CO}_2 + \text{H}_2\text{N} - \text{CH}_2 - \text{CO}_2\text{CH}_2 
\]

Scheme 2.8

Bnpeoc-Gly-OBzl (90) was deprotected with a solution of DBU (1 eq.) and AcOH (1 eq.) in DMF (Scheme 2.8). At 5 minute intervals, aliquots were removed from the reaction mixture, quenched by the addition of excess AcOH and monitored by analytical hplc. This revealed that the deprotection reaction was complete within 30 minutes. When this elimination was repeated with the corresponding Fmoc-Gly-OBzl, the deprotection was again shown to be complete within 30 minutes i.e. the conditions outlined by Florence for the removal of Bnpeoc are not specific to Bnpeoc.

The standard SPPS conditions for the removal of Fmoc i.e. 20% piperidine in DMF, were shown to effect the Fmoc elimination reaction within 10 minutes.
However the deprotection solution is known to contain not only the liberated dibenzofulvene (77), but also the tertiary amine (78) formed from the reaction between the base and the olefin (77).

Florence has reported the apparent lack of reactivity of the $1,1\text{-bis}(4'\text{-nitrophenyl})\text{ethene}$ moiety (91), in so much that it was thought to be particularly stable towards addition. Certainly in the case of DBU/AcOH mediated deprotections, analytical hplc has been used to show that no adduct is formed between the base and the produced olefin. However if 20% piperidine in DMF is employed, the deprotection solution immediately turns dark blue, and analytical hplc reveals no peak corresponding to the standard olefin (91). This suggested therefore that (91) does indeed form an adduct (92) similar to that which is produced with dibenzofulvene (Scheme 2.9).

In order to confirm the existence of this adduct, an authentic sample of $1,1\text{-bis}(4'\text{-nitrophenyl})\text{ethene}$ (91) was prepared, and reacted with an excess of piperidine in DMF.

After attempts to dehydrate the alcohol (19) with acid (p-toluenesulphonic acid) had failed, $2,2\text{-bis}(4'\text{-nitrophenyl})\text{ethanol}$ (19) was acetylated with an excess of acetic anhydride and pyridine to afford $2,2\text{-bis}(4'\text{-nitrophenyl})\text{ethyl acetate}$ (Bnpe-Ac) (93) as an off-white solid in 78% yield. The elimination of 1 mole of acetic acid from (93) was accomplished by the addition of 2 eq. of DBN to give the required $1,1\text{-bis}(4'\text{-nitrophenyl})\text{ethene}$ (91) as a yellow solid (Scheme 2.10). A sample of this olefin was dissolved in DMF, and to this was added piperidine (to give a 25% piperidine in DMF solution). The solution immediately turned dark blue, however, over 20 minutes this colour faded to a pale yellow. The removal of the solvents gave an orange oil which was identified as the proposed tertiary amine (92). i.e. $1,1\text{-bis}(4'\text{-nitrophenyl})\text{ethene}$ (91) reacts in a similar fashion to dibenzofulvene (77) with piperidine.

A point of particular interest is that the $\lambda_{\text{max}}$ for (91) in DMF occurs at 304 nm, whereas for the tertiary amine adduct (92) it occurs at 275 nm (see below).

Ramage and Florence, and Valentine, have not been the only researchers interested in the Bnpeoc protecting group. In 1985, Konig et al. published work they had performed using this group. While the synthesis of the protecting group and its ONSu active ester derivative proceeded smoothly, the researchers were only able to prepare the crystalline phenylalanine derivative, and experienced
significant problems with the subsequent removal of the protecting group. Using 10 equivalents of diethylamine in DMF for 45 minutes to deprotect Bnpeoc-Phe-OH (86) resulted in the isolation of only 67% of the phenylalanine. N.m.r. data revealed the following combination of products: 1,1-\textit{bis} (4'-nitrophenyl)ethene (91) (65%), N-[2,2-\textit{bis} (4'-nitrophenyl)ethyl]-diethylamine (15%) and N-[2,2-\textit{bis} (4'-nitrophenyl)ethyl]-phenylalanine (20%) \textit{i.e.} somewhat surprisingly the researchers found the olefin (91) adds more readily to phenylalanine than to a 10 fold excess of diethylamine. When the deprotection was repeated using n-propylamine, only 6% of the olefin was detected together with 94% of the N-[2,2-\textit{bis} (4'-nitrophenyl)ethyl]-n-propylamine.

Workers in this research group have yet to detect any significant addition of the olefin (91) to amine-free amino acid derivatives using the DBU/AcOH deprotection conditions, and in the case of 20% piperidine in DMF, only large quantities of the tertiary amine adduct (92) have been shown to be present.
2.2. Preparation of a glycotripeptide by solution phase methodology

2.2.1. Introduction

Antifreeze glycopeptides/proteins (AFGP) are polymers of a tripeptide monomer unit (with some exceptions), with covalently attached carbohydrate moieties. The tripeptide unit consists of two residues of alanine and one of threonine, and the carbohydrate, of a disaccharide unit.

It was the objective of this section of work to synthesise a fully protected analogous glycotripeptide unit, \(N^\alpha-(\text{benzyl} \text{oxycarbonyl})\text{alan}y\text{alan}y\text{serine-}(O-2,3,4,6-\text{tetra-}O\text{-benzyl-}\alpha/\beta-D\text{-glucopyranosyl}) \text{benzyl ester (Z-Ala-Ala-Ser-(}[\alpha/\beta-D\text{-Gluc-(BzI)}_4]-\text{OBzl})}\) (94), in order to investigate known glycosylation reactions and to determine the suitability of the Bnpeoc group for the \(N^\alpha\)-protection of amino acid residues of glycopeptides. To date, Fmoc has been used as the principle \(N^\alpha\)-protecting group in glycopeptide synthesis - with the milder base, \(N\)-methylmorpholine, used for its deprotection in preference to piperidine, due to base sensitivity of the glycosidic linkage. It was therefore hoped that Bnpeoc, which can be removed by stoichiometric quantities of DBU/AcOH, would provide even milder deprotection conditions.

2.2.2. Preparation of Bnpeoc-Ser-OBzl

It was anticipated that the synthesis of \(N^\alpha-[2,2\text{-bis (4'-nitrophenyl)-ethoxycarbonyl}]\text{-serine benzyl ester (Bnpeoc-Ser-OBzl)}\) (95) would proceed as the analogous preparation of \(N^\alpha-(9\text{-fluorenylmethoxycarbonyl})\text{serine benzyl ester (Fmoc-Ser-OBzl)}\) (96). However, whilst the synthesis of (96) was shown to proceed in good yield by an adaptation of the method of Wang \textit{et al.} \cite{227} (and Chang \textit{et al.} \cite{228}), that is, \textit{via} the formation of the caesium salt of Fmoc-Ser-OH, followed by reaction of this salt with a slight excess of benzyl bromide in DMF for 48 hours, attempts to repeat this synthesis with Bnpeoc-Ser-OH (88) resulted in the almost quantitative isolation of the olefin (91) after 24 hours.

A study was therefore performed in which Bnpe-OAc (93) was simply stirred in DMF, and the analogous solvents, \(N,N\text{-dimethylacetamide (DMA) and N-methylpyrrolidinone (NMP)}}\), for 48 hours. The results for the production of the olefin (91), as determined by analytical hplc (monitoring at 254 nm), are summarised in Figure 2.3.
<table>
<thead>
<tr>
<th>solvent</th>
<th>% olefin formed after 24 hours</th>
<th>% olefin formed after 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bnpe-OAc</td>
<td>DMF</td>
<td>29</td>
</tr>
<tr>
<td>Bnpe-OAc</td>
<td>DMA</td>
<td>7</td>
</tr>
<tr>
<td>Bnpe-OAc</td>
<td>NMP</td>
<td>100</td>
</tr>
</tbody>
</table>

concentration of Bnpe-OAc = \(1.25 \times 10^{-5}\) mol/ml

Figure 2.3

These data clearly suggested that DMA, in this form, contains the smallest quantity of basic impurities.

The synthesis of Bnpeoc-Ser-OBzI (95) was therefore carried out in DMA via the caesium salt of the acid as above (Scheme 2.11). In order to increase the rate of the reaction, and hence have the amino acid derivative in the solvent for the least amount of time, a larger excess of benzyl bromide (4-5 eq.) was used. Tlc confirmed the completion of the reaction within 90 minutes, and Bnpeoc-Ser-OBzI (95) was isolated in 89% yield.

![Scheme 2.11](image)

When the above studies were repeated on a sample of Bnpeoc-Thr-OH (89), no olefin (91) was detected after 48 hours in any of the three solvent systems. This can presumably be attributed to the free acid group neutralising any amines
present in the solvents.

<table>
<thead>
<tr>
<th>solvent</th>
<th>% olefin formed after 24 hours</th>
<th>% olefin formed after 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bnpeoc-Ser-OBzl</td>
<td>DMF 14</td>
<td>26</td>
</tr>
<tr>
<td>Bnpeoc-Ser-OBzl</td>
<td>DMA 0</td>
<td>10</td>
</tr>
<tr>
<td>Bnpeoc-Ser-OBzl</td>
<td>NMP 44</td>
<td>61</td>
</tr>
<tr>
<td>Fmoc-Ser-OBzl</td>
<td>DMF 0</td>
<td>7</td>
</tr>
<tr>
<td>Fmoc-Ser-OBzl</td>
<td>DMA 0</td>
<td>5</td>
</tr>
<tr>
<td>Fmoc-Ser-OBzl</td>
<td>NMP 5</td>
<td>9</td>
</tr>
</tbody>
</table>

concentration = $2.96 \times 10^{-5}$ mol/ml

Figure 2.4

A more pertinent study was performed with both Bnpeoc-Ser-OBzl (95) and Fmoc-Ser-OBzl (96) in DMF, DMA and NMP. A consideration of the results for the two amino acid derivatives (Figure 2.4) clearly reveals support for the above conclusion that the DMA, at least as supplied by Aldrich, contains the fewest basic impurities.

While these data suggest that this problem is more acute with Bnpeoc amino acid derivatives than Fmoc (this is supported by the $pK_a$ values listed in Figure 2.2), no actual quantitative comparison can be drawn. This is due to variations in $\varepsilon$ between the $N^\alpha$-protected amino acid derivatives and their respective olefins, which have not been considered in compiling the above data, and which would have to be taken into account.

2.2.3. Preparation of Bnpeoc-Ser[\(\alpha/\beta-\text{D-(Bzl)}\)\_4Gluc]-OBzl

As was discussed previously (see above), the most practical approach to date for the synthesis of glycopeptides has involved the preparation of adequately protected N- and O-glycosyl amino acid derivatives, followed by extension from either or both of the C- and N-terminals. Therefore in an attempt to incorporate the Bnpeoc $N^\alpha$-protecting group into glycopeptide synthesis, Bnpeoc-Ser-OBzl
(95), was glycosylated with a suitably protected carbohydrate moiety.

The selectively protected carbohydrate that was chosen was 2,3,4,6-tetra-O-benzyl-α-D-glucopyranose [(Bzl)$_4$αGluc-OH] (98). This was prepared from methyl α-D-glucopyranose (97) by the method of Koto et al.\textsuperscript{229} (Scheme 2.12), and involved the protection of the non-anomeric hydroxyls as benzyl ethers by the reaction of (97) with sodium hydride and benzyl chloride at elevated temperature. The selective hydrolysis of the methyl ether, and therefore the exposure of the anomeric hydroxyl, was performed by a mixture of 6M HCl and acetic acid, and afforded (98) in 33% yield (based on (97)).

![Scheme 2.12](image)

The glycosylation of Bnpeoc-Ser-OBzl (95) with (Bzl)$_4$αGluc-OH (98) was initially attempted by both the classical Koenigs-Knorr glycosylation conditions\textsuperscript{190} (i.e. employing mercuric bromide as the catalyst) and the modified Helferich conditions\textsuperscript{191}, which involves using mercuric cyanide as the catalyst and a mixture of toluene and acetonitrile as solvent. However, the reaction mixtures of both these preparations were shown (by tlc and hplc) to contain a great many products, and this made purification impractical.

More recently researchers\textsuperscript{197,198} have turned to the trifluoromethanesulphonic
(triflic) anhydride method for use in glycosylation reactions. While this has afforded glyco-amino acid derivatives in good yields, it has been noted\textsuperscript{197,198} that both the α and β anomic glycosidic products are obtained, and therefore at some point they must be separated.

The reaction of Bnpeoc-Ser-OBzl (95) (2 eq.) and (Bzl)\textsubscript{4}αGluc-OH (98) (1 eq.), mediated by triflic anhydride, and performed in a solvent mixture of acetonitrile and DCM, afforded $N^α$-[2,2-\textit{bis} (4'-nitrophenyl)ethoxycarbonyl]-serine-(O-2,3,4,6-tetra-O-benzyl-α/β-D-glucopyranosyl) benzyl ester (Bnpeoc-Ser-[α/β-D-(Bzl)\textsubscript{4}-Gluc]-OBzl) (99) in 34\% yield in the ratio of 1:1 for α and β products, but only after a great deal of work on the purification method (Scheme 2.13).

A tlc of the final reaction mixture revealed the existence of many byproducts of the glycosylation reaction, and hence still presented an overwhelming purification problem. Attempts to isolate the desired material (99) by gel filtration, in a variety of different solvents and gel media, failed completely. Eventually the final method
of purification that was applied involved a small flash chromatography column, followed by preparative hplc. The preparative hplc obviously presents problems of its own - for example, employing the semi-preparative reverse phase columns that are routinely used for peptide purification permits only a small quantity of material (5-10 mg) to be loaded on to the column per injection. Separation is not helped by the aromatic nature of both the product (99) and the starting materials/byproducts, that is, they each have a intense chromophore (cf. peptides) and this in turn reduces the quantity of material that the detector can handle per run. Hence this method of purification is both time consuming and expensive, and while it was possible with this preparation to purify sufficient material in order to proceed with the synthesis, steps have to be taken in the future in order to reduce the problems and time required to purify such intermediates. One possible solution could be the use of much larger bore preparative hplc columns, and this research group has recently acquired such a column, that can reputedly handle up to 100 mg of peptide per injection.

What the hplc purification step failed to do in this synthesis, however, was to separate the two anomic products. It was therefore decided to proceed with the synthesis of the glycotripeptide in the hope that the \( \alpha/\beta \) separation could be performed at a later stage.

### 2.2.4. Preparation of H-Ser[\( \alpha/\beta \)-D-(BzI)\(_4\)] GlucJ-OBzI

The next stage of the synthesis was the selective removal of the Bnpeoc N\(^{\alpha} \)-protecting group. Florence has shown that while the deprotection of the Bnpeoc group proceeds rapidly in a solution of DBU/AcOH in DMF, in DCM it takes 6-10 times as long. This can presumably be attributed to the greater solvation of the charged intermediates of the reaction in the solvent of higher dielectric constant.

This observation was repeated with small-scale deprotections of Bnpeoc-Ser[\( \alpha/\beta \)-D-(BzI)\(_4\)] GlucJ-OBzI (99) with DBU (1.2 eq.)/AcOH (1.2 eq.) in both DCM and DMF. For while the deprotection was shown (by tlc and hplc) to be complete in less than 20 minutes in DMF, in DCM it took more than 3 hours. Mass spectroscopy confirmed both the absence of the starting material (99), and the absence of any products that may have been present as a result of the loss of the glycosidic linkage, in the DMF reaction mixture.

The deprotection of (99) was therefore repeated on a larger scale with DBU (1.2
eq.)/AcOH (1.2 eq.) in DMF, and afforded serine(O-2,3,4,6-tetra-O-benzyl-\(^{\alpha/\beta}\)-D-glucopyranosyl) benzyl ester (H-Ser[\(\alpha/\beta\)-D-(Bzl)\(_4\)Gluc]-OBzl) (100) in almost quantitative yield (Scheme 2.14).

\[
\begin{align*}
\text{Bnpeoc-CH-CO}_{2}\text{CH}_2 & \quad \text{DBU} \\
\text{OBzl} & \quad \text{AcOH} \\
\end{align*}
\]

(99)

(100)

Scheme 2.14

2.2.5. Preparation of Z-Ala-Ala-OH

At this point, the synthesis of Z-Ala-Ala-Ser[\(\alpha/\beta\)-D-(Bzl)\(_4\)Gluc]-OBzl (94) could have been performed by two alternative routes from (100). One would have firstly involved the coupling of Bnpeoc-Ala-OH (85) to (100), followed by the removal the Bnpeoc group, and finally by coupling N\(^{\alpha}\)-(benzyloxycarbonyl)alanine (Z-Ala-OH) (101) to the N\(^{\alpha}\)-deprotected glycodipeptide. The other, by preparing N\(^{\alpha}\)-(benzyloxycarbonyl)alanylalanine (Z-Ala-Ala-OH) (103), and coupling it to (100) to give (94). The second method was chosen as difficulties were anticipated in purifying the glycopeptide intermediates that would result from following the first.

Z-Ala-OH (101) was prepared in good yield by established literature methods, and N\(^{\alpha}\)-(benzyloxycarbonyl)alanine N-succinimidyl ester (Z-Ala-ONSu) (102), the ONSu active ester of (101), was synthesised by the method of Anderson et al.\(^{109}\) As discussed below, the purification of these active esters can be greatly simplified by quickly washing the material (dissolved in ethyl acetate) with water.

The subsequent coupling of Z-Ala-ONSu (102) and alanine was accomplished by the aforementioned method of Rich\(^{81}\), and provided Z-Ala-Ala-OH (103) in 65% yield (Scheme 2.15).
2.2.6. Preparation of Z-Ala-Ala-Ser(α/β-D-(Bzl)$_4$Gluc]-OBzl

The preparation of Z-Ala-Ala-Ser(α/β-D-(Bzl)$_4$Gluc]-OBzl (94) from Z-Ala-Ala-OH (103) and H-Ser(α/β-D-(Bzl)$_4$Gluc]-OBzl (100) was accomplished using the standard DCCI/HOBt activation and coupling procedure, and using triethylamine to adjust the pH of the reaction mixture to 9.0.

The Bnpeoc-Ser(α/β-D-(Bzl)$_4$Gluc]-OBzl (99) was deprotected as above with a solution of DBU (1.2 eq.)/AcOH (1.2 eq.) in DMF, followed by the partition of the reaction mixture between ethyl acetate and water. After washing the ethyl acetate layer with further water, the solvent was removed to afford a yellow oil. It was decided that due to the small scale of the reaction (i.e. 100 mg of (99)), no further purification of (100) would be possible. Therefore in an attempt to maximise the isolated yield of the final glycotripeptide product (94) with respect to (100), two equivalents of the dipeptide (103) and the coupling reagents were subsequently employed (Scheme 2.16).

The purification of (94) was also very problematic and was finally performed by preparative hplc to afford (94) as a white solid in only 15% yield. While some of the reason for this poor yield can be attributed to the problems with the purification, it was felt that when considering a repeat of the synthesis, it would have to be done on such a scale as to permit the purification of (100). However,
there was also some evidence to suggest that the glycotripeptide product was not completely stable to the weakly acidic nature of the standard preparative hplc solvents (i.e. water/acetonitrile (+0.05% TFA)), and therefore it must be suggested that further hplc work in this area be carried out in neutral solvent systems only.

Scheme 2.16

The conclusion that can be drawn from this work is that the Bnpeoc group is indeed applicable to solution phase glycopeptide synthesis, as the conditions for its removal are suitably mild and selective so as to leave intact the glycosidic linkage. However, the nature of the coupling of amino acid and peptide derivatives to glyco-amino acid units and their subsequent purification requirements must be further investigated if such syntheses are to be understood and are to become a matter of routine.
2.3. Preparation of Bnpeoc-Ala-Phe-Gly-OH

In an attempt to further determine the suitability and applicability of the Bnpeoc N\(^\alpha\)-protecting group to both solution and solid phase peptide synthesis, a test peptide, N\(^\alpha\)-[2,2-\(bis\) (4'-nitrophenyl)ethoxycarbonyl]-alanylphenylalanylglucose (Bnpeoc-Ala-Phe-Gly-OH) (106) was prepared by three alternative routes:

2.3.1. Saponification of Bnpeoc-Ala-Phe-Gly-OMe

Florence\(^78\) has shown that N\(^\alpha\)-[2,2-\(bis\) (4'-nitrophenyl)ethoxycarbonyl]-alanylphenylalanylglucose methyl ester (Bnpeoc-Ala-Phe-Gly-OMe) (105) can be prepared by the coupling of Bnpeoc-Ala-OH (85) and the trifluoroacetate salt of H-Phe-Gly-OMe (104) (Scheme 2.17). The coupling agent that was chosen was that introduced by Ramage \textit{et al.}\(^{136,137}\) in 1976, diphenylphosphinyl chloride (45). Repeating the preparation afforded the desired protected tripeptide (105) in 60% yield.

\[
\begin{align*}
\text{Bnpeoc-Ala-OH} &\quad + \quad \text{CF}_3\text{CO}^- \quad \text{H}_2^+ - \text{Phe-Gly-OMe} \\
(85) &\quad (104) \\
\text{Ph}_2\text{POCl} &\quad \text{NMM} \\
(45) &\quad 2,6\text{-lutidine}
\end{align*}
\]

Scheme 2.17

Florence has also discussed the possibility of removing methyl esters with a dilute sodium hydroxide solution, without the loss of the N\(^\alpha\)-protecting group. However, Holden\(^36\) had experienced great difficulties in removing the analogous phenyl esters by saponification from Bnpeoc-Gly-OH derivatives. He found that significant quantities of the Bnpeoc group were cleaved under a number of alternative reaction conditions - confirmed by the presence of ninhydrin active spots on tlc's of reaction mixtures and by the isolation and characterisation of byproducts. After much experimentation, Holden eventually found conditions \textit{i.e.} 0.1M NaOH (1 eq.) and a solution of H\(_2\)O\(_2\) (2 eq.) in water, in 20% aqueous acetone
for 15 minutes at room temperature) that produced the protected amino acid derivative in 65% yield.

Florence has shown that the methyl ester of Bnpeoc-Ala-Phe-Gly-OMe (105) can be cleaved, using 0.025M NaOH (1 eq.), without resulting in the significant loss of the Bnpeoc group, in 60% yield (with the remainder isolated as starting material (35%) and olefin (91) (5%)). Attempts to repeat that reaction, under a variety of reaction conditions, all resulted in the formation of significant quantities of ninhydrin active material, and the reaction mixtures were confirmed as containing the olefin (91) and free-amine amino acid derivatives by analytical hplc.

The optimum, but not wholly satisfactory, conditions found for the reaction were those introduced by Holden, namely 0.1M NaOH (1 eq.) and a solution of H₂O₂ (2 eq.) (as an 'α'-effect nucleophile) in water, in 20% aqueous acetone at room temperature. This resulted in the isolation of the desired product (106) as a white solid (Scheme 2.18), but in only a 55% yield.

\[
\begin{align*}
\text{Bnpeoc-Ala-Phe-Gly-OMe} & \quad \text{Bnpeoc-Ala-Phe-Gly-OH} \\
\text{(105)} & \quad \text{(106)} \\
\text{H₂O₂} & \quad \text{acetone/H₂O}
\end{align*}
\]

Scheme 2.18

The conclusion that must be drawn from these studies is that experimental conditions may be tailored in order to increase the viability of a route involving the removal of methyl esters by saponification in the presence of Bnpeoc, however, the insufficiently qualified claim that Bnpeoc is stable to NaOH is one which merits further investigation.
2.3.2. Utilising ONSu active ester methodology

A second solution phase synthesis of Bnpeoc-Ala-Phe-Gly-OH (106) was performed, in which the final step involved the incorporation of the Bnpeoc-Ala-OH residue as its ONSu active ester (Scheme 2.19).

Glycine benzyl ester p-toluenesulphonate (TosO\(^{-}\)H\(_2\)\(^+\)-Gly-OBzl) (107) was prepared from glycine, p-toluenesulphonic acid and benzyl alcohol in benzene by the method of Zervas et al\(^{230}\). The resulting salt (107) (prepared in 92% yield) was then coupled to N\(^{\alpha}\)-(benzylxoycarbonyl)phenylalanine (Z-Phe-OH) (108) (prepared by the established literature procedure) by the DCCI/HOBt activation and coupling procedure to give N\(^{\alpha}\)-(benzylxoycarbonyl)-phenylalanylglycine benzyl ester (Z-Phe-Gly-OBzl) (109) (88%).

The removal of both the Z- and benzyl ester protecting groups was performed (simultaneously) by hydrogenation in the presence of 10% palladium-charcoal catalyst and p-toluenesulphonic acid (1 eq.) (to prevent the unwanted side reaction of cyclisation\(^{231}\)), at room temperature and pressure, and afforded phenylalanylglycine p-toluenesulphonate (TosO\(^{-}\)H\(_2\)\(^+\)-Phe-Gly-OH) (110) as a green oil. All attempts at crystallisation failed, and hence the product (110) was employed without further manipulation in the next stage of the synthesis.

The preparation of the N-succinimidyl ester (Bnpeoc-Ala-ONSu) (111) of Bnpeoc-Ala-OH (85) by the method of Anderson et al\(^{109}\) proceeded satisfactorily. Often a problem associated with such a preparation is the removal of the excess/unreacted N-hydroxysuccinimide from the final reaction mixture. However, as earlier work has demonstrated, this can be accomplished by redissolving the reaction mixture in ethyl acetate and quickly washing (once) with water (even though these active esters are water sensitive). In the case of the synthesis of (111) this was deemed unnecessary and the product was obtained in 88% yield.

The subsequent coupling of (110) and (111) was performed under the conditions outlined by Rich\(^{82}\), and applied to Bnpeoc N\(^{\alpha}\)-protection (see above), to afford Bnpeoc-Ala-Phe-Gly-OH (106) as a white solid (66% - based on Z-Phe-Gly-OBzl (109)).

This synthesis of (106) has demonstrated the ready preparation of Bnpeoc amino acid ONSu active esters, the possibility of successfully incorporating them into peptide synthesis, and has confirmed the required stability of Bnpeoc to triethylamine (under peptide synthesis conditions).
Scheme 2.19
2.3.3. Utilising solid phase/acid chloride methodology

As was discussed in the introduction, acid chloride coupling methodology for peptide synthesis has been enjoying a recent revival of interest\(^{(89,92)}\). The use of acid-stable urethane-type \(N^\alpha\)-protecting groups \(e.g.\) Fmoc has permitted peptide synthesis to be achieved with acid chlorides with very little loss of optical purity \(< 0.1\%)\(^{(93)}\).

In an attempt to determine the feasibility of the preparation and reaction of acid chlorides of Bnpeoc amino acids, the tripeptide, Bnpeoc-Ala-Phe-Gly-OH (106) was prepared by manual solid phase synthesis on the \(p\)-alkoxybenzyl alcohol (Wang) resin (8), and employing a manual shaker (Scheme 2.20).

A point to note is that there have been lengthy discussions as to the best method of agitation to be employed in the loading of the first amino acid residue to the resin\(^{(36,78)}\). Mechanical stirring can essentially be dismissed due to the possibility of damage occurring to the resin beads, and while this is not a problem with rotary agitation (in a manual shaker) and ultrasonication, there are disadvantages with both of these methods. With rotary inversion there is a conflict concerning the volume of solvent in the vessel during the coupling reaction - a large volume is required to permit optimum immersion of the resin in the reaction mixture during the inversion, however, the higher the concentration of reagents, the higher the generally obtained coupling yields. There is also the problem of a small quantity of the reaction mixture 'leaking' through the sinter, and thus effectively removing it from the reaction vessel. In the case of ultrasonication, the minimum volume of solvent may be used to ensure a high concentration of reagents. The problem with this method, however, is the resulting mechanical loss of product during its removal, washing, drying and repeated couplings \(etc.\)

It was therefore decided that while all manual solid phase peptide couplings would be performed by rotary inversion (hence minimum loss of the peptide during the synthesis), the coupling of the first amino acid derivative would, in general, be performed by sonication (a higher coupling yield is more desirable than ensuring no loss of resin, if a subsequent wastage of material is to be avoided). However, due to the nature of the reagents involved, and the expectation of high coupling yields \(via\) acid chlorides, the loading of the first amino acid derivative in this synthesis was performed by rotary inversion.

The acid chloride (112) of Bnpeoc-Gly-OH (87) was prepared by heating under
Scheme 2.20

Bnpeoc—Gly—OH

\[ \text{SOCl}_2 \]

Bnpeoc—Gly—Cl

(112)

pyridine \[ \rightarrow \text{Wang resin (8)} \]

\[ \text{DBU} \rightarrow \text{AcOH} \]

H—Gly—OCH\(_2\)

Bnpeoc—Phe—Cl \[ \rightarrow \text{NMM (2X)} \]

DBU \[ \rightarrow \text{AcOH} \]

Bnpeoc—Ala—Cl \[ \rightarrow \text{NMM (1X)} \]

\[ \text{TFA} \]

Bnpeoc—Ala—Phe—Gly—OH

(106)
reflux in excess thionyl chloride - its formation being confirmed by infrared spectroscopy. The coupling of (112) with the p-alkoxybenzyl alcohol resin (8) was performed in DCM, using an excess of pyridine as base, to afford Bnpeoc-Gly-(O-CH₂C₆H₄-OR) (113). Microanalysis of (113) revealed a yield of 62% after one coupling, and hence a second coupling, undertaken exactly as the first, was required to achieve a satisfactory 87% (by microanalysis) coupling yield.

Bnpeoc-Gly-(O-CH₂C₆H₄-OR) (113) was deprotected with a solution of DBU (2 eq.)/AcOH (2 eq.) in DMF for 1 hour at room temperature. To this was coupled Bnpeoc-Phe-Cl, prepared as above, in DCM and using N-methylmorpholine as base. Attempts to mediate this coupling in pyridine resulted in little of the desired product. Two couplings of Bnpeoc-Phe-Cl, each of a duration of 2 hours, were required before a sample of the functionalised resin gave a negative Kaiser test result. As a precaution (perhaps unnecessary considering the result of the Kaiser test), any unreacted amine sites were capped with an excess of acetic anhydride and pyridine to ensure no subsequent build-up of a deletion peptide.

Deprotection of the phenylalanine residue was again effected by a solution of DBU/AcOH in DMF, before the final coupling of Bnpeoc-Ala-Cl was performed. A single (2 hour) coupling was shown to be sufficient to elicit a negative result from the Kaiser test.

The removal of the tripeptide from the resin was performed using TFA in DCM and gave a yellow solid, which on purification by gel filtration afforded Bnpeoc-Ala-Phe-Gly-OH (106) as a white solid (overall yield = 38%).

The above synthesis clearly demonstrated the effectiveness of acid chloride coupling methodology for use in peptide synthesis. The preparation of acid chlorides of Bnpeoc amino acids was shown to be a simple, high yielding procedure and also, it is noteworthy that during the course of the synthesis, these acid chloride derivatives were shown to be stable overnight if stored in an anhydrous atmosphere (at -15°C).
2.4. Preparation of Bnpeoc-Ser(tBu)-OH and Bnpeoc-Thr(tBu)-OH

While the syntheses of Bnpeoc-Ser-OH (88) and Bnpeoc-Thr-OH (89) have been reported above, these derivatives are of little use in solution and solid phase peptide synthesis due to the presence of the free hydroxyl functionality on the side chains of the amino acid residues. Clearly these hydroxyls have to protected, and as discussed above, the use of a base-labile Nα-protecting group (such as Bnpeoc) for peptide synthesis permits acid-labile tert.butyl ethers to be employed for the protection of the side-chain functionality. This in turn allows for the simultaneous removal of the protecting group with the acid mediated cleavage of the peptide from the resin.

The syntheses of Nα-[2,2-bis (4'-nitrophenyl)ethoxycarbonyl]-serine (O-tert-butyl) (Bnpeoc-Ser(tBu)-OH) (119) and Nα-[2,2-bis (4'-nitrophenyl)ethoxycarbonyl]-threonine(O-tert-butyl) (Bnpeoc-Thr(tBu)-OH) (124) were therefore undertaken.

2.4.1. Bnpeoc-Ser(tBu)-OH

The synthesis of Bnpeoc-Ser(tBu)-OH (119) essentially consists of the preparation of (O-tert-butyl)serine (H-Ser(tBu)-OH) (118) by the now established methods of amino acid derivatisation, followed by the incorporation of the Bnpeoc Nα-protecting group (Scheme 2.21).

Serine methyl ester hydrochloride (ClH₂⁺-Ser-OMe) (114) was prepared by the method of Guttmann and Boissonnas²³³, which consists of the addition of thionyl chloride and serine to methanol at reduced temperature. The resulting salt (114) (prepared in 90% yield) was subsequently reacted with benzyloxy carbonyl chloride (Z-Cl) in chloroform, while employing a mixture of triethylamine and pyridine as base. This afforded Nα-(benzyloxy carbonyl)serine methyl ester (Z-Ser-OMe) (115) as a white solid in 87% yield.

Nα-(Benzyloxy carbonyl)serine(O-tert-butyl) methyl ester (Z-Ser(tBu)-OMe) (116) was prepared by firstly dissolving Z-Ser-OMe (115) in DCM and cooling in a CO₂/acetone bath, and then adding condensed isobutylene together with conc. sulphuric acid (as a catalyst) and copper (I) chloride (to prevent the polymerisation of the isobutylene). The reaction mixture was securely stoppered and placed in a screened area for 7 days, before being worked up to afford Z-Ser(tBu)-OMe (116) (47%).

The subsequent removal of the amine and carboxyl protecting groups was
H-Ser-OH $\xrightarrow{\text{SOCl}_2}$ MeOH $\rightarrow$ Cl$^-$H$_2^+$-Ser-OMe

$\text{Et}_3\text{N, pyridine}$

Z-Ser-OMe $\xrightarrow{\text{isobutylene}}$ Z-Ser(tBu)-OMe

$\text{H}^+$/CuCl $\rightarrow$

H$_2$/Pd-C $\rightarrow$ H-Ser(tBu)-OH

Bnpeoc-ONSu $\text{Et}_3\text{N}$

$\text{H}_2\text{OCONH-CH-CO}_2\text{H}$

Scheme 2.21
performed in two stages.

The methyl ester was removed by the method of Shields and Renner\textsuperscript{234}, which involves the reaction of (116) with 1.0 M NaOH (1.1 eq.) in aqueous acetone at room temperature, to give \(\text{N}^\alpha\text{-(benzylloxycarbonyl)serine} (\text{O-} \text{tertbutyl}) \) (117). The cleavage of the Z- group required hydrogenation in the presence of 10\% palladium-charcoal catalyst and in DMF at room temperature and pressure. This gave \(\text{O-} \text{tertbutyl} \text{serine} (\text{H-Ser}^{1}\text{Bu}) \text{-OH} \) (118) in 82\% yield.

The incorporation of the Bnpeoc N\(\alpha\)-protecting group was performed as above, employing Bnpeoc-ONSu (21) and the method of Rich\textsuperscript{82} (with the optional CHA purification), to afford Bnpeoc-Ser\(^{1}\text{Bu})\text{-OH} \) (119) (93\%).

2.4.2. Bnpeoc-Thr\(^{1}\text{Bu})\text{-OH}

The preparation of Bnpeoc-Thr\(^{1}\text{Bu})\text{-OH} \) (124) followed a similar route to the analogous serine derivative (see above), that is, \(\text{O-} \text{tertbutyl} \text{threonine} \) (123) was synthesised by established methods, followed by the incorporation of the Bnpeoc N\(\alpha\)-protecting group (Scheme 2.22).

However in an attempt to reduce the number of steps in the synthesis, and hence increase the overall yield of (124), a benzyl ester was used for the carboxyl protection rather than the methyl ester. This permitted the simultaneous removal of both the amine and the carboxyl protecting groups by hydrogenation.

\(\text{N}^\alpha\text{-}\text{Benzyloxycarbonyl} \text{threonine} (\text{Z-Thr-OH}) \) (120) was prepared by an established literature method in good yield. The reaction of (120) with triethylamine and an excess of benzyl bromide gave \(\text{N}^\alpha\text{-}\text{Benzyloxycarbonyl} \text{threonine benzyl ester} (\text{Z-Thr-OBzl}) \) (121) as a white solid (55\%).

The incorporation of the tertbutyl ether protecting group was performed as above \textit{i.e.} by the addition of condensed isobutylene, conc. sulphuric acid and copper (I) chloride to a cooled solution of Z-Thr-OBzl (121) in DCM. This afforded \(\text{N}^\alpha\text{-}\text{Benzyloxycarbonyl} \text{threonine}(\text{O-} \text{tertbutyl}) \text{benzyl ester} (\text{Z-Thr}^{1}\text{Bu)-OBzl}) \) (122) as a white solid in 76\% yield.

The removal of both the amine and carboxyl groups was accomplished by hydrogenation at room temperature and pressure in the presence of 10\% palladium-charcoal catalyst and in DMF, as outlined by Wunsch and Jentsch\textsuperscript{235}. This gave \(\text{O-} \text{tertbutyl} \text{threonine} (\text{H-Thr}^{1}\text{Bu}-\text{OH}) \) (123) in a somewhat
Scheme 2.22
disappointing yield of 39%.

The reaction of (123) with Bnpeoc-ONSu (21) by the method of Rich\textsuperscript{82} gave Bnpeoc-Thr(\textsuperscript{1}Bu)-OH (124), however, unlike the analogous serine derivative (119), Bnpeoc-Thr(\textsuperscript{1}Bu)-OH required purification by flash chromatography as well as by the formation, and the liberation, of the CHA salt.
2.5. The design and development of a new coupling reagent for peptide synthesis

2.5.1. Introduction

Diphenylphosphinyl chloride (Ph$_2$POCl) (45), the coupling reagent introduced and developed by Ramage et al.\textsuperscript{136,137} has enjoyed extensive usage in solution and manual solid phase peptide synthesis. However, due to the hygroscopic nature of the acid chloride (45), it is not suitable for application to automated SPPS. The hydrolysis of (45) to the corresponding acid (Ph$_2$PO$_2$H) (125), which is relatively insoluble (even in DMF), would result in the possibility of costly blockages in the solvent transfer lines and the valve block of an automated synthesiser.

A reagent that would result in the formation of the same phosphinic-carboxylic mixed anhydride (46) as is generated with (45), but yet would be more stable to hydrolysis (i.e. less air sensitive), was therefore sought.

The initial studies in this area were centred on the attempts to prepare the HOBt ester derivative (126) of Ph$_2$POCl. These were largely a failure however, due to the lack of a suitable work up procedure for the isolation of (126).

![Diagram](126)

![Diagram](127)

Attention was therefore directed to diphenylphosphinic anhydride (Ph$_2$PO$_2$POPh$_2$) (127). This was a known literature compound, and its preparation was shown to be possible by a number of alternative methods.

The synthesis of Ph$_2$PO$_2$POPh$_2$ (127), its stability and reactivity, and the possibility of employing it to mediate peptide couplings were then investigated.
2.5.2. Preparation of Ph$_2$PO$_2$POPh$_2$

A number of preparations of phosphinic anhydrides are present in the literature.

In general, they may be prepared by the heating of the appropriate phosphinyl chloride with the sodium phosphinate in an inert solvent$^{236}$. An alternative method, however, involves the treatment of the phosphinyl chloride with triethylamine and a restricted quantity of water$^{237}$.

Perhaps the most versatile route to both aliphatic$^{238}$ and aromatic$^{239}$ phosphinic anhydrides is the reaction of phosphinyl chlorides with ethyl phosphinates at elevated temperature (150-200°C) without a solvent. Alternatively Moedritzer$^{240}$ has demonstrated the slightly unusual method of preparing symmetrical anhydrides from phosphinyl chlorides and paraformaldehyde.

The first attempts at the synthesis of Ph$_2$PO$_2$POPh$_2$ (127) involved the treatment of Ph$_2$POCl (45) with water (0.5 eq.) in a mixture of DCM and DMF (Scheme 2.23). While there was evidence ($^{31}$P n.m.r.) to suggest that some of the desired product had been formed, the reaction was by no means near completion, and hence presented an impractical purification problem.

$$\text{Ph}_2\text{P}=\text{O} + \text{H}_2\text{O} \rightarrow \text{Ph}_2\text{P}O\text{Ph}_2$$

(Scheme 2.23)

Attention was therefore directed to the method of Crofts et al$^{239}$, in which the anhydride can be prepared by the heating of (45) and ethyl diphenylphosphinite (Ph$_2$P(O)OEt) (128) without a solvent and in an inert atmosphere. Ph$_2$P(O)OEt (128) was prepared by the method of Kosalapoff and Watson$^{238}$, which involves the addition of sodium ethoxide to a cooled solution of (45) in benzene. After 24 hours the sodium chloride formed was filtered off and the solvent removed to give (128) as a clear oil. The purification of (128) was achieved by distillation and afforded the ester as a white solid in 75% yield.
Ph$_2$P(O)OEt (128) and Ph$_2$POCl (45) were then heated without a solvent to 200°C for 2 hours under an argon atmosphere (Scheme 2.24). It was at this point that problems with this synthesis were encountered. Crofts et al.\textsuperscript{239} had demonstrated that on cooling, the reaction mixture solidified and that the desired anhydride (127) can be obtained pure simply by the recrystallisation of this solid from toluene. Efforts to repeat this work up procedure resulted in the contamination of the product (127) with a significant quantity (up to 50% by $^{31}$P n.m.r.) of diphenylphosphinic acid (125). Adopting a more rigorous approach to the drying of the toluene yielded no beneficial effect.

In our modification the reaction was allowed to cool to 60°C, a sufficient volume of toluene was added to dissolve the product, and the vessel was placed in an ice/salt bath. Vigorous scratching encouraged the formation of a white precipitate which, after storing in a refrigerator for 30 minutes, was filtered off and dried. This gave (127) in 59% yield as a white solid and its purity was confirmed by $^{31}$P n.m.r.

While the above two-step synthesis of (127) gave a tolerable overall yield of
44% (based on initial $\text{Ph}_2\text{POCl}$), it was felt that improvements could be made. The distillation of the intermediate ester (128) was both time consuming and predictably wasteful of material, and yet $^{31}\text{P}$ n.m.r. had shown the crude (128) to be only one product. It was therefore decided to omit the distillation procedure and, on removing the benzene from $\text{Ph}_2\text{P(O)OEt}$ (128), to simply add the acid chloride (45) and heat as before. This simple modification increased the obtained yield of (127) from 44% to 60% with no loss of purity.

The route introduced by Moedritzer $^{240}$ for the synthesis of phosphinic (and phosphonic) anhydrides was also investigated. In this, phosphinyl chlorides are heated to 150°C in the presence of paraformaldehyde (1 eq.) (Scheme 2.25).

However, attempts to repeat the author's preparation of (127) from (45) proved to be somewhat problematic. In the majority of the occasions that this reaction was performed (using paraformaldehyde (1 eq.)), the final product (127) was shown to be contaminated with the acid (125). After much experimentation, it was decided that, due to the unreliable nature of this reaction, the resulting low yield (at best, 45%) and the failure to separate the anhydride from the anhydride/acid mixtures, this method would not be pursued.

It should also be noted that efforts to perform this synthesis using 0.5 and 2 eq. of paraformaldehyde afforded no reliable increase in yield or guarantee of purity.

Hence the method of choice for the synthesis of $\text{Ph}_2\text{PO}_2\text{POPh}_2$ (127) was that which involved the preparation, and the use (without distillation), of the intermediate $\text{Ph}_2\text{P(O)OEt}$ (128).
Before any experimentation on the stability and relative reactivity etc. of Ph$_2$PO$_2$POPh$_2$ (127) was performed, an attempt had to be made to discover if (127) did indeed mediate the formation of amide bonds. This was accomplished by the successful preparation of two simple dipeptides, N$_\alpha$-(benzyloxy carbonyl) phenylalanylglycine benzyl ester (Z-Phe-Gly-OBzl) (109) and N$_\alpha$-(benzyloxy carbonyl) phenyl-alanylalanine benzyl ester (Z-Phe-Ala-OBzl) (132), using (127) as the coupling reagent.

Z-Phe-OH (108) (1 eq.) was activated by the addition of (127) (1 eq.) and
N-methylmorpholine (1 eq.) at -5°C in DCM. After 10 minutes at -5°C
TosO⁻H₂⁺-Gly-OBzl·(107) (1 eq.) and N-methylmorpholine (2 eq.) were added, and
the resulting mixture stirred at room temperature for 2.5 hours. (Scheme 2.26).
Work up afforded the crude dipeptide, which was purified by recrystallisation to
give Z-Phe-Gly-OBzl (109) as a white solid in 60% yield.

In solid phase peptide synthesis, the incoming activated amino acid derivative
(e.g. (129)) is applied in excess (typically 2-4 times excess) in order to push the
coupling reaction to completion. In an attempt therefore to mimic SPPS and to
increase the yield of (109) the above reaction scheme was repeated using
Z-Phe-OH (108) (2 eq.), N-methylmorpholine (2 eq.) and Ph₂PO₂POPh₂ (127) (2 eq.)
for the activation step, followed by the addition of TosO⁻H₂⁺-Gly-OBzl (107) (1 eq.)
and N-methylmorpholine (2 eq.). This increased the yield of (109), as predicted to
76%.

After the preparation of TosO⁻H₂⁺-Ala-OBzl (131), in 91% yield, by the general
method of Zervas et al. 230, as outlined above for the synthesis of the
Corresponding glycine derivative (107), the above procedure was subsequently
applied to the coupling of Z-Phe-OH (108) (2 eq.) to (131) (1 eq.), employing
Ph₂PO₂POPh₂ (127). This afforded Z-Phe-Ala-OBzl (132) as a white solid in 70%
yield.

The preparation of Z-Phe-Gly-OBzl (109) and Z-Phe-Ala-OBzl (132) in good
yield clearly demonstrated the possibility of employing Ph₂PO₂POPh₂ (127) for the
mediation of peptide bond formation, and hence, further stability and reactivity
studies were performed in order to gain a better understanding of the nature of
the reagent.

2.5.4. ³¹P n.m.r. monitored stability studies of Ph₂PO₂POPh₂

The heat of hydrolysis of the P-O-P linkage of phosphinic anhydrides is known
to be more than that for the corresponding pyrophosphate reaction. 241 There is
evidence confirming the rapid hydrolysis of phosphinic anhydrides with water, 242
and their ready cleavage by alcoholysis 243, 244 and aminolysis . 240

As regards the use of Ph₂PO₂POPh₂ (127) as a peptide coupling reagent, the
questions that must be considered are, how air sensitive is (127) (at both reduced
and room temperature), and what is the relative rate of reaction of (127) with
carboxylic acids (to form phosphinic-carboxylic mixed anhydrides) as against
alcoholysis and, more importantly, aminolysis?

**Air sensitivity of Ph$_2$PO$_2$POPh$_2$**

After the preparation of Ph$_2$PO$_2$POPh$_2$ (127), the compound was stored at -15°C, and it has been subsequently shown to be completely stable for more than four months at that temperature (by $^{31}$P n.m.r.). This permits the synthesis of large quantities of the anhydride for storage prior to being required.

At room temperature, however, the anhydride is not so stable. While its stability may be guaranteed for 24 hours in a sealed vessel, longer periods of time result in the hydrolysis of the anhydride to yield significant quantities of the analogous acid, Ph$_2$PO$_2$H (125) - up to 60% after 30 days. The storing of some of the solid (127) in a sealed Applied Biosystems SPPS capsule (see below), flushed with nitrogen, confirmed this observation. The anhydride was shown to be completely stable at -15°C for 30 days, and at room temperature for 48 hours, however, after 30 days at approximately 40% of the Ph$_2$PO$_2$H (125) was found to be present.

The conclusions that may be drawn from these studies are:

1. on preparation of the anhydride (127), it must be stored in a freezer (-15°C) prior to use

2. the anhydride has sufficient stability to be handled (for weighing purposes etc) at room temperature and in air

3. at room temperature, the stability of the anhydride can only be guaranteed for 24 hours in a sealed vessel (48 hours if sealed under nitrogen), after that a slow but steady build up of Ph$_2$PO$_2$H (125) takes place.

**Stability of Ph$_2$PO$_2$POPh$_2$ to N-methylmorpholine**

Before the stability of Ph$_2$PO$_2$POPh$_2$ (127) to N-methylmorpholine was examined, samples of the anhydride were dissolved in DMF, DMA and NMP. The removal of aliquots of these solutions at 15 minute intervals for 60 minutes, and monitoring by $^{31}$P n.m.r., confirmed no significant loss of the starting anhydride over the duration of the study.
Subsequently (127) was dissolved in a mixture of CD$_2$Cl$_2$/DMA at room temperature, and N-methylmorpholine (3 eq.) was added. $^{31}$P n.m.r. was again used to confirm that no loss of the starting anhydride (due to the base) was observed over the 60 minute period.

**Stability of Ph$_2$PO$_2$POPh$_2$ to free-amine amino acid derivatives**

As discussed above, the ready aminolysis of phosphinic anhydrides is known to take place. It was interesting therefore to compare the relative rates of reaction of (127) with TosO$^-$H$_2$-$^+$-Gly-OBzl (107) (1 eq.) in the presence of N-methylmorpholine, with that of the formation of phosphinic-carboxylic mixed anhydrides (see below). $^{31}$P n.m.r. revealed the existence (after 15 minutes) of the diphenylphosphinate anion (Ph$_2$PO$_2^-$) (130) signal, and its steady but slow increase in size.

**Stability of Ph$_2$PO$_2$POPh$_2$ to alcohols**

Again, the known reaction of alcoholysis of phosphinic anhydrides has been discussed.

Dissolving the anhydride (127) in a solution of CD$_2$Cl$_2$/methanol, and a solution of CD$_2$Cl$_2$/benzyl alcohol, resulted in no significant loss of the anhydride signal over a period of 30 minutes (by $^{31}$P n.m.r.) in either case. On the addition of N-methylmorpholine (2 eq.) to the methanol solution, the anhydride signal diminished completely within 30 minutes to afford two signals, one corresponding to the phosphinyl ester and the other to the phosphinate anion (130). The benzyl alcohol solution on the other hand, revealed a much reduced loss of the anhydride signal, and the introduction of a small phosphinate anion signal.

These studies clearly demonstrate the possibility of performing both the aminolysis and the alcoholysis of Ph$_2$PO$_2$POPh$_2$ (127), but at a relatively slow rate compared to that of phosphinic-anhydride mixed anhydride formation (see below).

2.5.5. $^{31}$P n.m.r. monitored peptide couplings with Ph$_2$PO$_2$POPh$_2$

In an attempt to further understand the Ph$_2$PO$_2$POPh$_2$ (127) mediated coupling reaction of Z-Phe-OH (108) and TosO$^-$H$_2$-$^+$-Gly-OBzl (107), the reaction was repeated on a scale such that it could be followed by $^{31}$P n.m.r.

The activation reaction (Scheme 2.26) was shown to be complete within 15 minutes, that is , $^{31}$P n.m.r. revealed the presence of only two signals – one
corresponding to the mixed anhydride (129), the other to the phosphinate anion (130) – after such time (see Figure 2.5).

The subsequent coupling to TosO⁻H₂⁺-Gly-OBzl (107), in the presence of N-methylmorpholine, was shown to be complete within 20 minutes – only a large phosphinate anion signal remained after this time.

A similar coupling of Fmoc-Arg(Pmc)-OH (133) and (107) was performed using Ph₂PO₂POPh₂ and followed by ³¹P n.m.r. A somewhat different activation conclusion was reached, in which a significant quantity of the starting anhydride (127) remained as well as the formation of the mixed anhydride (46) and the liberation of the phosphinate anion (130). However, on the addition to the reaction of the TosO⁻H₂⁺-Gly-OBzl (107) and further N-methylmorpholine, the reaction was driven to completion and the coupling performed within 20 minutes.

A number of alternative N⁰-protected amino acid derivatives with protected side-chain functionality (e.g. Fmoc-Cys(Acm)-OH and Fmoc-His(Trt)-OH) were activated using (127) and coupled to (107), and monitored by ³¹P n.m.r. In all cases the activation step was shown to be complete, whether actually to the complete reaction of (127), or to an 'equilibrium' position concerning the starting anhydride (127), the mixed anhydride (46) and the phosphinate anion (130) as above, within 15 minutes, and the coupling complete within 30 minutes.

The studies above concerning the alcoholysis of (127) were also considered further. In a simple attempt to study the relative rates of mixed anhydride formation as against alcoholysis, Bnpeoc-Ser-OH (88) was activated with (127) and coupled to (107), and monitored by ³¹P n.m.r. A position was reached almost immediately with the activation reaction, whereby the starting anhydride (127), the mixed anhydride and the phosphinate anion were all present. The relative sizes of the peak heights of these signals changed little over a 20 minute period. No signal for the phosphinyl ester that may have been formed by the reaction of the anhydride (127) with the free hydroxyl function of the side-chain of the serine residue was observed.

The subsequent coupling was shown to be complete within 20 minutes of the addition of the TosO⁻H₂⁺-Gly-OBzl (107) and N-methylmorpholine.

Fmoc-Asn-OH (134), that is, Nα-protected asparagine with free side-chain amide functionality, is routinely coupled as the HOBt ester derivative (prepared
Figure 2.5 Activation of Z-Phe-OH and coupling to TosO⁻H₂⁻Gly-OBzl.

(A) Z-Phe-OH / Ph₂PO₂POPh₂ / NMM 5 minutes after mixing

(B) Z-Phe-OH / Ph₂PO₂POPh₂ / NMM 15 minutes after mixing
(C) $\text{Z-Phe-ODpp / TosO}^-\text{H}_2\text{^+}-\text{Gly-OBzl / NMM}$

15 minutes after mixing

(D) $\text{Z-Phe-ODpp / TosO}^-\text{H}_2\text{^+}-\text{Gly-OBzl / NMM}$

30 minutes after mixing

Figure 2.5
using a carbodiimide) in solid phase synthesis. This is due to the fact that mixed anhydrides of, for example, Fmoc-Asn-OH, undergo an internal cyclisation which results in the dehydration of the side-chain functionality (Scheme 2.27).

![Scheme 2.27](image)

It was predicted therefore that attempts to activate Fmoc-Asn-OH (134) with Ph₂PO₂POPh₂ would result in the aforementioned dehydration. Indeed when this activation was performed on a ³¹P n.m.r. scale, it revealed the complete decay of the starting anhydride (127) signal to afford only the phosphinate anion signal within 20 minutes. An infrared spectrum of the reaction mixture confirmed the presence of the characteristic nitrile peak (2260 cm⁻¹).

At this point it was decided to investigate the suitability of the Mbh side-chain protecting group for asparagine and glutamine. Fmoc-Gln(Mbh)-OH (135) was activated as above with (127). ³¹P n.m.r. revealed the presence of only two signals after 15 minutes, one corresponding to the activated mixed anhydride, the other to the phosphinate anion (130). The ratio of the peak heights of these two signals changed little over a 60 minute period. The coupling was shown to be complete within 15 minutes of the addition of TosO⁻H₂⁺-Gly-OBzl (107) and further N-methylmorpholine.
The stability of the formed phosphinic-carboxylic mixed anhydride (129) was also investigated. Z-Phe-OH (108) was activated with (127) and N-methylmorpholine by the method outlined above. After 10 minutes, a position had been reached whereby three signals were present by $^{31}$P n.m.r., the mixed anhydride (129), the starting anhydride (127) and the phosphinate anion (130). As the ratio of the peak heights of these signals remained unchanged over 4 hours at room temperature, it was assumed that the mixed anhydride (129) had the required stability and was not prone to unwanted side reactions (e.g. disproportionation).

2.5.6. Further solution phase peptide couplings with $\text{Ph}_2\text{PO}_2\text{POPh}_2$.

The $^{31}$P n.m.r. coupling experiment with Bnpeoc-Ser-OH (88) and TosO%H$_2$Gly-OBzl (107) suggested that it may be possible to activate, and couple, a $N^\alpha$-protected amino acid derivative with free hydroxyl side-chain functionality (Scheme 2.28). The above experiment was therefore repeated on a preparative scale. A tlc of the final reaction mixture revealed a number of reaction products, however, separation was possible by flash chromatography and $N^{\alpha}$-[2,2-\textit{bis} (4'-nitrophenyl)ethoxycarbonyl]-serylglycine benzyl ester (Bnpeoc-Ser-Gly-OBzl) (136) was obtained as a yellow oil in 61% yield. Apart from the limited preparative applications of this reaction, it confirms the observation of selective phosphinic-carboxylic mixed anhydride formation over phosphinyl ester generation.

The coupling of Fmoc-Gln(Mbh)-OH (135) to (107) was also repeated on a preparative scale to afford $N^{\alpha}$-(9-fluorenylethoxycarbonyl)glutamyl [N-\textit{bis} (4'-methoxyphenyl)methyl]-glycine benzyl ester (Fmoc-Gln(Mbh)-Gly-OBzl) (137) as a white solid in 77% yield. This confirmed the suitability of the Mbh group for use in side chain amide protection while employing a phosphinic-carboxylic mixed anhydride activation and coupling methodology.
2.5.7. Racemization studies

Investigations concerning the possible occurrence of racemization of the activated amino acid derivatives that are generated with Ph$_2$POCl (45) have been carried out$^{139,144}$, and have identified no such problems providing urethane-type N-protecting groups are used. As the phosphinic-carboxylic mixed anhydride (46) that is produced with Ph$_2$PO$_2$POPh$_2$ (127) is the same as is generated with (45), no problems with the loss of optical purity are anticipated.

However, by way of a check, (127) was employed in the activation and coupling of Fmoc-L-Phe-OH and Fmoc-D-Phe-OH to TosO$^+$-H$_2$-Gly-OBzl (131) to afford N$_{9}$-(9-fluorenylmethoxycarbonyl)-L-phenylalanyl-L-alanine benzyl ester (Fmoc-L-Phe-L-Ala-OBzl) (138) and N$_{9}$-(9-fluorenylmethoxycarbonyl)-D-phenylalanyl-L-alanine benzyl ester (Fmoc-D-Phe-L-Ala-OBzl) (139) respectively (in 58 and 62% yields). Any racemization would lead to the partial inversion of the phenylalanine centre and therefore lead to the contamination of both of the diastereomers with the other.

Analytical reverse phase hplc traces of the two products revealed a slight difference in retention times, but not one sufficient to give base-line resolution of the two peaks when a hplc trace was run of a mixture of the two products.
Therefore, while the studies revealed that certainly no major degree of racemization (i.e. 20%) had taken place during the activation and coupling, the limits were not such that lower quantities could be identified.

\(\text{Ph}_2\text{PO}_2\text{POPh}_2\) (127) has also been employed in the loading of the first amino acid derivative (i.e. the C-terminal amino acid residue of a peptide chain) on to the \(\text{p-alkoxybenzyl alcohol resin (8)}\) (see below). That is, it has been used in the mediation of ester, as well as amide, bond formation.

The possibility of a loss of optical purity of an \(\text{N}^\alpha\)-protected amino acid derivative when activated with (127) and coupled to an alcohol, to form an ester bond, was also similarly investigated. Fmoc-\(\text{L-Ala-OH}\) and Fmoc-\(\text{D-Ala-OH}\) were coupled to \((-\)-menthol to afford \(\text{N}^\alpha\)-(9-fluorenlymethoxycarbonyl)-L-alanine (-)-menthyl ester (Fmoc-\(\text{L-Ala-O[(-)Men]}\)) (140) and \(\text{N}^\alpha\)-(9-fluorenlymethoxycarbonyl)-D-alanine (-)-menthyl ester (Fmoc-\(\text{D-Ala-O[(-)Men]}\)) (141) respectively (in 43 and 48% yields). Again, while a separation of the 2 diastereomers was possible by reverse phase hplc, it was not such that base-line resolution could be obtained. Hence no small quantities of racemization, if they had occcured, could be identified.

These studies on the possibility of racemization with \(\text{Ph}_2\text{PO}_2\text{POPh}_2\) (127) are by no means complete, and are continuing at present within this research group.

2.5.8. Peptide couplings with \(\text{Ph}_2\text{PO}_2\text{POPh}_2\) and \(\text{ZnI}_2\)

Jacquier has recently discussed (but not yet published) work on peptide couplings with \(\text{Ph}_2\text{POCl}\) (45) when used in conjunction with \(\text{ZnI}_2\). His research has suggested that the addition of a quantity of \(\text{ZnI}_2\) (approximately 0.1 eq.) to his couplings of dipeptides to amino acid derivatives has resulted in a significant reduction in the observed racemization (due to azlactone formation) of the dipeptide moiety.

It was therefore decided to investigate the effect of adding \(\text{ZnI}_2\) to \(\text{Ph}_2\text{PO}_2\text{POPh}_2\) (127), and to the couplings mediated with this reagent.

Stability of \(\text{Ph}_2\text{PO}_2\text{POPh}_2\) to \(\text{ZnI}_2\)

The addition of \(\text{ZnI}_2\) (0.1 eq.) and N-methylmorpholine (1 eq.) to a solution of (127) in \(\text{CD}_2\text{Cl}_2/\text{DMF}\) resulted in no change in the \(31\text{P}\) n.m.r. signal for the anhydride (127) over a 75 minute period. However, if \(\text{ZnI}_2\) (1 eq.) was added, \(31\text{P}\)
n.m.r. revealed the instantaneous and complete generation of an alternative phosphorus species. The addition of N-methylmorpholine caused a further shift in the location of the phosphorus signal. Clearly some form of complex is being generated with the Zn\(_2\). However the identity of this 'complex' has yet to be ascertained.

\[ ^{31}\text{P n.m.r. monitored peptide couplings with Ph}_2\text{PO}_2\text{PPh}_2 \text{ and Zn}_2 \]

A repeat of the reaction of Z-Phe-OH (108) with \(\text{Ph}_2\text{PO}_2\text{PPh}_2\) (127) and N-methylmorpholine, gave complete activation within 15 minutes as above. The addition of Zn\(_2\) (0.1 eq.) to this activation solution resulted in a change in the \(^{31}\text{P n.m.r. spectrum}\) - what had been a sharp peak for the phosphinate anion (130), had shifted in position and become a broad hump. No relative change in the integrals of these signals occurred over a 10 minute period, and therefore TosO\(^+\text{H}_2\text{Gly-OBzl}\) (107) and further N-methylmorpholine were added. The coupling was shown to be complete within 10 minutes.

Performing the above reaction again, but with the Zn\(_2\) (0.1 eq.) present at the beginning of the activation, rather than adding it at the end, gave, immediately on mixing, three signals: one corresponding to the activated mixed anhydride (129), one to the starting anhydride (127) and one to the phosphinate anion (130) (a broad signal). The ratio of the peak heights of these signals remained unchanged over a 10 minute period. The subsequent coupling of (129) and (107) was shown to be complete within 10 minutes of the addition of (107) and further N-methylmorpholine.

As mentioned above Jacquier has been using Zn\(_2\) to suppress the racemisation taking place in dipeptide centres during activation and coupling. It was therefore decided to investigate this observation by coupling Fmoc-Met-Gly-OH to TosO\(^+\text{H}_2\text{-Gly-OBzl}\) (107) using \(\text{Ph}_2\text{PO}_2\text{PPh}_2\) (127). As above, the reaction was performed twice - firstly with the Zn\(_2\) added after the activation, and secondly, with it present at the start of the activation.

Fmoc-Met-Gly-OH was therefore activated with (127) in the presence of N-methylmorpholine as outlined above. Immediately on mixing \(^{31}\text{P n.m.r. revealed three signals: the activated anhydride, the starting anhydride (127) and the phosphinate anion (130). Over the following 20 minute period, the signals for the mixed anhydride and the starting anhydride decreased, while that of the phosphinate anion increased in relative size. This can be attributed to the
formation of an azlactone intermediate, and hence is responsible for the racemization which is observed in couplings of this type. However, on the addition of ZnI₂ (0.1 eq.), an unexpected observation was made. The previously discussed broad hump for the phosphinate anion was revealed, but a large, sharp signal was also immediately present. It was not possible, however, to ascertain the identity of this signal i.e. whether or not it was the starting anhydride or the mixed anhydride. Over a 10 minute period the ratio of the relative peak heights of these signals remained unchanged. TosO⁻H₂⁺-Gly-OBzl (107) and further N-methylmorpholine were added, and ³¹P n.m.r. revealed the completion of the coupling within 20 minutes. Mass spectroscopy confirmed the presence of a peak at 576 (i.e. corresponding to Fmoc-Met-Gly-Gly-OBzl) in the spectrum of the reaction mixture.

The reaction was repeated with the ZnI₂ present at the beginning of the activation. 5 minutes after the activation components were mixed, ³¹P n.m.r. revealed three signals: the mixed anhydride, the starting anhydride (127) and the phosphinate anion (130) (broad signal). The relative ratio of the peak heights of these signals did not change over the following 15 minute period. The subsequent coupling with (107) in the presence of further N-methylmorpholine was shown to be complete within 20 minutes (mass spectroscopy again confirming the presence of a peak at 576).

In an attempt to study further the effect of the presence of ZnI₂, the coupling of Z-Phe-OH (108) and TosO⁻H₂⁺-Ala-OBzl (131) was repeated as above on a preparative scale, but with ZnI₂ (0.1 eq.) present. Work up afforded the crude dipeptide, which was purified by flash chromatography to give Z-Phe-Ala-OBzl (132) in 81% yield. This compares favourably with the aforementioned yield of 70% for the coupling without the addition of the ZnI₂.

The conclusions that can be drawn from the above experiments are, that in the example of the coupling of Z-Phe-OH (108) and TosO⁻H₂⁺-Gly-OBzl (107), the addition of the ZnI₂ appears to increase the rate of both the activation and coupling reactions, as well as the yield. In the coupling of Fmoc-Met-Gly-OH to (107), the ZnI₂ (if present at the start of the activation) appears to tie up the activated dipeptide in such a fashion that azlactone formation does not take place, and this in turn prevents racemization. Beyond this, the role of ZnI₂ in these reactions remains somewhat of a mystery, and one which merits further investigation and understanding.
2.5.9. Regeneration of Ph$_2$POCl

One advantage of the use of the coupling reagents Ph$_2$POCl (45) and Ph$_2$PO$_2$POPh$_2$ (127) is that they may be regenerated from re-isolated Ph$_2$PO$_2$H (125). This has been of particular importance in this work bearing in mind the considerable number of attempted preparations of (127) which have resulted in inseparable mixtures of (127) and (125).

The Ph$_2$PO$_2$POPh$_2$/Ph$_2$PO$_2$H mixtures from these failed preparations were converted completely into Ph$_2$PO$_2$H by simply partitioning them between ethyl acetate and 1M NaOH. The separated aqueous layer was acidified to pH 2.0 by the addition of 1M HCl, and the resulting white precipitate worked up to afford Ph$_2$PO$_2$H (125). The conversion of (125) to Ph$_2$POCl (45) was performed by the method of Kreutzkamp and Schlindler$^{236}$, which involves simply heating the acid in thionyl chloride, followed by distillation, to give (45) as a yellow oil in 86% yield.
2.6. Solid Phase Peptide Synthesis

The objectives of this section of work were to prepare a number of peptides using current solid phase methodology, while introducing the Bnpeoc Nα-protecting group (16) and the diphenylphosphinic anhydride (127) activation and coupling reagent, on an Applied Biosystems 430A automated solid phase peptide synthesiser.

2.6.1. Loading the first residue onto the p-alkoxybenzyl alcohol resin

As discussed above, the coupling of the first amino acid residue (i.e. the C-terminal residue of a peptide chain) to the p-alkoxybenzyl alcohol resin (8) was performed outside the automated synthesiser. This permitted the coupling to be accomplished by ultrasonication, and hence provide the optimum coupling yield. The standard method employed in this research group for this coupling involves the formation of the activated symmetrical anhydride of the Nα-protected amino acid derivative using a carbodiimide (DICI). However, this method often requires the use of a second coupling in order to obtain an acceptable yield. Hence a considerable excess of Nα-protected amino acid must be used and is therefore wasted. Figure 2.6 provides some examples of the yields that have been obtained using this DICI mediated symmetrical anhydride coupling method.

<table>
<thead>
<tr>
<th>amino acid derivative</th>
<th>quantity</th>
<th>coupling yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Gly-OH</td>
<td>1 x 4 eq.</td>
<td>54%</td>
</tr>
<tr>
<td>Bnpeoc-Gly-OH</td>
<td>1 x 4 eq.</td>
<td>52%</td>
</tr>
<tr>
<td>Bnpeoc-Ala-OH</td>
<td>2 x 4 eq.</td>
<td>78%</td>
</tr>
</tbody>
</table>

Figure 2.6

Figure 2.6 shows that a considerable quantity of Nα-protected amino acid must be used in order to give an acceptable coupling of the first residue. While this can be tolerated with the cheaper amino acid derivatives (e.g. Fmoc-Gly-OH), it becomes unacceptable with the costly ones (e.g. Fmoc-Arg(Pmc)-OH).

Attempts were therefore made to mediate this ester bond formation using the Ph₂PO₂POPPh₂ (127) coupling reagent (Scheme 2.29). A number of Nα-protected
amino acids were activated by the addition of (127) and N-methylmorpholine at room temperature in DMA (or DMF). After stirring for 15 minutes, the activated mixture was transferred to a vessel containing the p-alkoxybenzyl alcohol resin (8) previously swollen in DMA (or DMF), and further N-methylmorpholine, together with a catalytic quantity of DMAP, were added. Agitation by the preferred method of ultrasonication was performed for 3 hours at room temperature before the resin was filtered, washed and dried.

Figure 2.7 summarises the yields that were obtained when the coupling of the first amino acid residue was performed using Ph₂PO₂POPh₂ (127).
<table>
<thead>
<tr>
<th>amino acid derivative</th>
<th>quantity</th>
<th>coupling yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fmoc-Gly-OH</td>
<td>1 x 4 eq.</td>
<td>80%</td>
</tr>
<tr>
<td>Fmoc-Ala-OH</td>
<td>1 x 4 eq.</td>
<td>78%</td>
</tr>
<tr>
<td>Fmoc-Ala-OH</td>
<td>2 x 2 eq.</td>
<td>79%</td>
</tr>
</tbody>
</table>

Figure 2.7

The data in Figure 2.7 clearly suggested that when a similar quantity of $N^\alpha$-protected amino acid derivative was employed in the $\text{Ph}_2\text{PO}_2\text{POPh}_2$ mediated couplings as to the DICl mediated ones, the obtained yields were significantly higher. This therefore permits a smaller number of equivalents of the incoming first amino acid to be used in order to obtain similar coupling yields with the $\text{Ph}_2\text{PO}_2\text{POPh}_2$ reagent as to the that which are provided with DICl.

2.6.2. Solid Phase Peptide Synthesis

A number of peptides were prepared introducing $\text{Ph}_2\text{PO}_2\text{POPh}_2$ (127) and Bnpeoc (16) to solid phase methodology.

The first peptide synthesised by employing the (127) coupling reagent was tyrosylisoleucylphenylalanylalanylglycine (H-Tyr-Ile-Phe-Ala-Gly-OH) (144). The synthesis was performed on a 0.5 mmol scale and was accomplished using Fmoc amino acid derivatives, the functionalised resin Fmoc-Gly-(O-$\text{CH}_2\text{C}_6\text{H}_4$-OR) (142) and tertbutyl ether protection for the tyrosine side chain hydroxyl.

A preprogrammable 'double couple' cycle was prepared and employed which involved, firstly, the activation of the incoming Fmoc amino acid (2 eq.) with $\text{Ph}_2\text{PO}_2\text{POPh}_2$ (127) (2 eq.) in the presence of N-methylmorpholine (2 eq.) at room temperature (what is known as) the activation vessel on the synthesiser. The resulting phosphinic-carboxylic mixed anhydride was then transferred to the reaction vessel and coupled to the free-amine resin bound product, with the addition of further N-methylmorpholine (1 eq.) and 2,6-lutidine (4 eq. – see before). After 40 minutes coupling time the reaction mixture was removed and the resin and washed with further solvent. The activation and coupling cycle was then repeated (and hence the term 'double couple') in order to force the coupling to
completion, before the functionalised resin was washed well and capped (i.e. any unreacted amine sites were blocked by acetylation in a solution of acetic anhydride and pyridine). Deprotection was performed by the now-standard conditions for the cleavage of Fmoc, that is, 20% piperidine in DMF (or DMA) (Scheme 2.30).

Throughout the above synthesis the $\text{Ph}_2\text{PO}_2\text{POPh}_2$ (127) was stored in a solution of DMA and simply transferred to the activation vessel when required.
The cleavage of the peptide from the resin, performed with a solution of TFA in DCM, together with the simultaneous removal of the tert-butyl ether protecting group from the side-chain hydroxyl function of the tyrosine residue, afforded the crude pentapeptide as a yellow solid. Analytical reverse phase hplc revealed this solid to contain a number of reaction products, however, purification by preparative reverse phase hplc proceeded smoothly and the desired pentapeptide (144) was isolated as a white solid in an overall yield of 41%.

At this point checks were made as to the long term stability of the anhydride (127) in the DMF and DMA solutions, bearing in mind its susceptibility to hydrolysis at room temperature as the solid. $^{31}$P n.m.r. revealed that a significant quantity of the anhydride was hydrolysed to the corresponding acid (125) if stored in solution for a number of days. This observation therefore rules out the possibility of preparing large volumes of the anhydride in a solution of DMF (or DMA) for prolonged use on the automated synthesiser. Clearly a method had to be found whereby the anhydride was only taken into solution immediately prior to being required. The method employed for the synthesis of the remaining Ph$_2$PO$_2$POPh$_2$ prepared peptides in this section, was to have the anhydride stored on the synthesiser in capsules similar to those ones which are used to contain the N$^{\beta}$-protected amino acid derivatives. In the case of the amino acid derivatives, solvent is added to the capsule immediately prior to the derivative being required, it is taken into solution and then transferred to the activation vessel. By simply alternating the amino acid capsules with the anhydride ones, the anhydride could be taken into solution and transferred to the activation vessel when required.

The pentapeptide, leucylisoleucylphenylalanylalanylglycine (H-Leu-Ile-Phe-Ala-Gly-OH (145), corresponding to residues 43-47 of ubiquitin, was subsequently prepared using the aforementioned Fmoc/Ph$_2$PO$_2$POPh$_2$/p-alkoxybenzyl alcohol resin 'double couple' (2 x 2eq.) cycle but with the anhydride stored on the synthesiser as a solid, rather than in solution. Analytical reverse phase hplc of the crude peptide on cleavage from the resin and work up revealed only one product. Nevertheless the peptide was passed down a gel filtration column and lyophilised to afford (145) as a white solid in excellent yield (78%).

By way of comparison the same peptide, H-Leu-Ile-Phe-Ala-Gly-OH (145), was prepared using Fmoc amino acids and Fmoc-Gly-(O-CH$_3$C$_6$H$_4$-OR) (142), but employing the DICl/HOBt activation and coupling methodology which is routinely
used on this research group’s 430A synthesiser (for cycle – see Experimental). This synthesis also afforded the crude pentapeptide as one peak by analytical reverse phase hplc, and subsequently resulted in the isolation of (145) as a white solid in 73% yield after gel filtration.

What is interesting to note is that the same peptide was prepared in almost the same yield (if anything, a yield consideration would favour the Ph₂PO₂Poph₂ prepared peptide) with the Fmoc/Ph₂PO₂Poph₂ (127) methodology as with the Fmoc/DICl/HOBt methodology. However, in the case of the Ph₂PO₂Poph₂ prepared peptide, only a total of four equivalents of the Fmoc amino acid derivative was required per residue, compared with the six required when DICl/HOBt methodology is employed. Another beneficial aspect of using Ph₂PO₂Poph₂ methodology is that the residue time (i.e. the length of time it takes to incorporate one residue into the peptide chain) is reduced from 4–5 hours (with DICl/HOBt) to 2–3 hours. This is more significant than might be first imagined as often the limiting factor in solid phase synthesis is the lack of available machine time, rather than the subsequent purification and analysis.

The pentapeptide H-Leu-Ile-Phe-Ala-Gly-OH (145) was synthesised on two subsequent occasions – again using both Ph₂PO₂Poph₂ and DICl/HOBt methodology, but employing Bnpeoc Nα-protected amino acid derivatives and Bnpeoc-Gly(O-CH₂C₆H₄-OR) (113) for both preparations.

In the case of the Bnpeoc/DICl/HOBt prepared peptide, analytical reverse phase hplc of the crude peptide after cleavage from the resin and work up, revealed two products: a major peak (corresponding to the desired pentapeptide), and a minor peak. Amino acid analysis of the crude product (Gly₁1.08, Ala₁0.77, Ile₁1.03, Leu₁1.03, Phe₁1.06) suggested the impurity to be the tetrapeptide H-Leu-Ile-Phe-4′-OH. On purification of the material by preparative reverse phase hplc, the major product was indeed identified as the desired pentapeptide (145) (in 61% yield), and the minor as the suggested tetrapeptide. Clearly the initial deprotection of the glycine residue had not gone to completion. However, on the subsequent alanine residue deprotection, it appears that the remainder of the glycine was also deprotected. While steric constraints could be regarded as one possible explanation for the incomplete removal of the Bnpeoc protecting group from the glycine residue (Bnpeoc is significantly larger than Fmoc), it is more difficult to explain why the remaining Bnpeoc-Gly(O-CH₂C₆H₄-OR) (113) should be deprotected during the second set of deprotections, where if anything, the
problems of steric bulk around the surface of the resin must surely be greater.

When the peptide (145) was prepared by Bnpeoc/Ph$_2$PO$_2$POPh$_2$ methodology, a shoulder was also found to be present on the major product peak (which corresponded to the desired pentapeptide) in the analytical reverse phase hplc trace of the crude product on cleavage from the resin and work up. Purification was again accomplished by preparative reverse phase hplc, and afforded the pentapeptide (145) on the removal of the hplc solvents by lyophilisation as a white solid in 58% yield.

What these studies appear to have shown is that the deprotection conditions/timings etc. for the Fmoc protecting group can not be automatically applied to the Bnpeoc deprotection. Considerable further experimentation must be performed if the Bnpeoc is to be successfully incorporated into existing solid phase methodology.

In an attempt to gain further solid phase experience with Ph$_2$PO$_2$POPh$_2$ (127), a further two peptides were prepared using the aforementioned Fmoc/Ph$_2$PO$_2$POPh$_2$ 'double couple' (2 x 2 eq.) cycle.

Firstly, prolylglycylprolyl-D-phenylalanylalanine (H-Pro-Gly-Pro-D-Phe-Ala-OH) (146) was prepared using the above cycle for the incorporation of all the residues with the exception of glycine - this was incorporated in a single (1 x 4 eq.) coupling rather than the standard (2 x 2 eq.) double cycle. Also in an attempt to reduce the volume of solvent present in the reaction vessel during the coupling (a result of reducing the volume was that the effective concentration of reagents was increased, and it was hoped that this in turn would lead to an increase in coupling efficiency), a solution of N-methylmorpholine (2 eq.) in DMF (or DMA) was used to dissolve the anhydride (127) in the capsule - rather than dissolving the anhydride in DMF (or DMA) and then adding the base solution.

Analytical reverse phase hplc of the crude product on cleavage from the support revealed essentially one product. However, all attempts at this stage to obtain the peptide as a solid failed and therefore preparative reverse phase hplc was employed to purify the obtained oil. This gave the desired pentapeptide as a white solid in excellent yield (79%). Clearly the modifications to the employed cycle had no detrimental effect on the quality and quantity of the prepared peptide.

The synthesis of a larger fragment of ubiquitin,
leucylvalylleucylarginyleucylarginylglycylglycine (H-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-OH) (ubiquitin 69-76) (147), was also performed. Again the standard Fmoc/Ph$_2$PO$_2$POPh$_2$ ‘double couple’ cycle was used for all residues with the exception of glycine. Studies on the swelling characteristics of functionalised resins in DCM, DMF, DMA, and mixtures of DCM/DMF and DCM/DMA have revealed that while the resins swell more in DCM than in DMF or DMA, in mixtures of DCM/DMF and DCM/DMA the swelling was actually less than that found with DMF and DMA on their own. It was therefore decided to synthesise (147) in DMA only rather than in the mixtures of DCM/DMF and DCM/DMA that had been used previously.

During initial attempts at the preparation of (147) both the coupling of the first arginine residue (with side-chain Pmc protection) and the valine residue did not go to completion. This can presumably be attributed to the steric problems involved with having such bulky side chains. Therefore when the synthesis was repeated, the second coupling of each of these two residues was left overnight rather than for the standard 40 minutes. This enabled the peptide (147) to be successfully synthesised, removed from the resin (with a solution of TFA in DCM, which also removed the side-chain Pmc protecting groups) and purified by preparative reverse phase hplc. The removal of the hplc solvents by lyophilisation afforded the octapeptide (147) as a white solid in 44% yield. The relatively low yield reflects the difficulty of the sequence from a preparative point of view due the bulky nature of the residues involved.

At this point investigations into the actual cycles employed in conjunction with Ph$_2$PO$_2$POPh$_2$ (127) were performed. All the aforementioned peptide synthesis with (127) had made use of the standard (2 x 2 eq.) ‘double couple’ cycle. It was therefore decided to prepare a peptide by this standard cycle (as a reference), but also by two other cycles: a ‘triple couple’ cycle - involving one coupling of 2 equivalents of the N$^\alpha$-protected amino acid derivative and (127), and two couplings of one equivalent of each (i.e. 1 x 2 eq. + 2 x 1 eq.), and a ‘quadruple couple’ cycle - involving four couplings of one equivalent of each (4 x 1 eq.). The peptide chosen for this study was threonylleucylserylisoleucylglycine (H-Thr-Leu-Ser-Ile-Gly-OH) (148), due to the bulkiness of each of the side chains (with the exception of glycine) i.e. it was hoped that any deficiencies in the activation and coupling procedure would be amplified by the steric constraints placed on the peptide by the nature of the residues involved.
The three syntheses of (148) were performed\(^{37,246}\), and in each case the crude peptide was removed from the resin, examined by analytical reverse phase hplc and purified by preparative reverse phase hplc. Somewhat disappointingly the results revealed no appreciable difference in both the quality of the crude peptide and the quantity obtained by preparative hplc from each of the syntheses. The standard cycle (2 x 2 eq.) has therefore been retained, due to the simplicity of the cycles involved, and the fact that the residue time for the standard cycle is considerably less than that for the other two (this is due to increased washing time etc.).

The final peptide prepared in this section of work returned to the use of the Bnpeoc N\(^\alpha\)-protecting group. It was a repeat of the synthesis of H-Thr-Leu-Ser-Ile-Gly-OH (148), but employing Bnpeoc N\(^\alpha\)-protection, tert-butyl ether side-chain hydroxyl protection (see above for the preparation of Bnpeoc-Ser(tBu)-OH (119) and Bnpeoc-Thr(tBu)-OH (124)), the functionalised resin, Bnpeoc-Gly(O-CH\(_2\)C\(_6\)H\(_4\)-OR) (113), and DICl/HOBt activation and coupling.

The synthesis appeared to proceed smoothly (followed by UV deprotection trace - see Experimental), and on cleavage of the peptide from the resin, a yellow solid was obtained. Analytical reverse phase hplc of this solid revealed essentially one product, however, on purification by preparative reverse phase hplc, the pentapeptide was isolated in a disappointing yield of only 33%. At this point in time, simply no explanation of why such a low yield of the peptide was obtained can be suggested, but obviously further experimentation must be performed if conditions/timings etc. are to be found that are compatible with the Bnpeoc group.

In summary, the solid phase peptide work discussed in this section has proved the worth of the Ph\(_2\)PO\(_2\)POPPh\(_2\) (127) coupling reagent for the mediation of both amide and ester bond formation. It has been shown to afford peptides of a similar quality and quantity as might be expected if the DICl/HOBt activation and coupling procedure had been adopted, while using considerably less amounts of N\(^\alpha\)-protected amino acids. This work, while clearly indicating the potential of the Bnpeoc protecting group, suggests that further experimentation will have to be performed in order to optimise the conditions for its use in solid phase peptide synthesis.
CHAPTER 3
EXPERIMENTAL

All amino acids and protected amino acid derivatives were purchased from the SAS group of companies and from Novabiochem respectively, and unless otherwise stated were of the L-configuration. All Z- amino acids were prepared by literature methods. All Bnpeoc- amino acids were prepared by one of the two methods discussed below for the synthesis of Bnpeoc-Ser-OH (88), with the obtained characterisation data in agreement with that provided by Florence. Melting points were recorded in open capillary tubes on an electrically heated Buchi 510 melting point apparatus and are uncorrected. Optical rotations were measured on an AA1000 polarimeter using a 10 cm cell in the solvents quoted in the text. Thin layer chromatography (tlc) was carried out on commercially available plastic sheets precoated with silica gel 60 Gf-254 (Merck) in the following systems:

(A1) 33/67 ethyl acetate/chloroform
(A2) 40/60 methanol/chloroform
(A3) 25/75 ethyl acetate/chloroform
(A4) 20/80 methanol/chloroform
(A5) 50/50 ethyl acetate/chloroform
(A6) 60/20/20 n-butanol/acetic acid/water
(A7) ethyl acetate
(A8) 25/75 methanol/chloroform
(A9) 15/85 ethyl acetate/chloroform
(A10) 50/50 methanol/chloroform

Visualisation of the compounds was achieved by a suitable combination of the following methods: UV absorption at 254 nm, potassium permanganate, bromophenol blue, 4,4'-bis (dimethylamino)phenyl carbinol (Mary's reagent) and ninhydrin sprays. High-performance liquid chromatography (hplc) was performed using either a Waters system i.e. 2 x 600A pumps, a U6K injector, a 680 automatic gradient controller, a model 441 ultraviolet detector, and a 308 computing
integrator; or an Applied Biosystems system \textit{i.e.} 2 x 1406A solvent delivery systems, a 1480A injector/mixer, and a 1783A detector/controller. Analytical separations were performed on the following columns and gradients of water/acetonitrile (+0.05\% TFA):

(B1) Spherisorb C$_{18}'$, 50/50 to 0/100 (28 minutes), 254 nm

(B2) Spherisorb C$_{8}'$, 80/20 to 0/100 (28 minutes), 214 nm

(B3) Spherisorb C$_{18}'$, 50/50 to 0/100 (25 minutes), 229 nm

(B4) Spherisorb C$_{18}'$, 80/20 to 0/100 (25 minutes), 229 nm

(B5) Aquapore C$_{18}'$, 70/30 to 0/100 (28 minutes), 214 and 254 nm

(B6) Aquapore C$_{18}'$, 90/10 (5 minutes), 90/10 to 0/100 (20 minutes), 229 nm

(B7) Spherisorb C$_{18}'$, 50/50 to 30/70 (40 minutes), 229 nm

(B8) Spherisorb C$_{18}'$, 50/50 to 0/100 (50 minutes), 229 nm

(B9) Aquapore C$_{18}'$, 90/10 to 40/60 (25 minutes), 229 nm

(B10) Aquapore C$_{8}'$, 90/10 to 0/100 (28 minutes), 229 nm

(B11) Aquapore C$_{18}'$, 100/0 (10 minutes), 100/0 to 60/40 (20 minutes), 229 nm

(B12) Aquapore C$_{18}'$, 95/5 (10 minutes), 95/5 to 20/80 (20 minutes), 229 nm

(B13) Aquapore C$_{18}'$, 80/20 (5 minutes), 80/20 to 0/100 (30 minutes), 214 nm

The flow rate was 1 ml/min., and elution of the samples was monitored by ultraviolet absorption at 214, 229 or 254 nm as indicated (see above). Amino acid analysis were carried out on a LKB 4150 amino acid analyser following sealed tube hydrolysis with constant boiling hydrochloric acid at 110\°C for 18 hours. Infra-red spectra were recorded on a Perkin Elmer 781 double beam spectrophotometer in the solvent indicated or by the KBr disc technique, with polystyrene (1603 cm$^{-1}$) employed as the standard. Ultraviolet spectra were recorded in SPPS-grade DMF (unless otherwise stated) on a Pye-Unicam SP8-400 spectrophotometer. High and low resolution fast atom bombardment (FAB) spectra were measured on a Kratos MS 50TC machine. Proton n.m.r. were recorded on a Jeol FX-60 (60 MHz), a Bruker WP80 (80 MHz), a WP200 (200 MHz) or a WH360 (360 MHz) machine in the solvent
indicated, using tetramethylsilane (TMS) as the external standard (δ = 0.000). Carbon-13 n.m.r. spectra were recorded on a Bruker WP200 machine operating at 50.3 MHz. Samples were dissolved in the solvent indicated and chemical shifts were measured relative to TMS assigned to zero. Phosphorus-31 n.m.r. were recorded on a Jeol FX-90 (36.3 MHz) or a Bruker WP200 (81.1 MHz) machine, and the chemical shifts measured relative to an external 85% aqueous phosphoric acid standard assigned as zero. Elemental analysis were carried out on a Carlo Erba elemental analyser (model 1106). All solvents were distilled before use and the following were dried using the reagents given in parentheses when required: benzene (sodium wire), chloroform (phosphorus pentoxide), dichloromethane (calcium hydride), diethyl ether (sodium wire), ethanol (magnesium – iodine) and toluene (sodium wire). N,N-Dimethylformamide and N,N-dimethylacetamide were commercially supplied by Applied Biosystems, Rathbone and Aldrich, were stored over 4Å molecular sieves and were used without distillation.
Di-(4-nitrophenyl)methane (80)

The compound was prepared by the adaptation of the general method of Jarczewski and Leffek.\(^{219}\)

Diphenylmethane (79) (10.0 g, 59.5 mmol) was dissolved in nitromethane (33 ml) and added slowly to a vigorously stirred solution of conc. HNO\(_3\) (52 ml) and conc. H\(_2\)SO\(_4\) (63 ml) maintained at -15 to -25°C. Stirring was continued for 3 hours keeping the temperature below -20°C, after which the solution was poured into an excess of ice-water. The resulting precipitate was filtered off and washed with water (300 ml), cold methanol (15 ml), and diethyl ether (50 ml). Recrystallisation from benzene (twice) afforded the title compound (8.10 g, 53%) as yellow needles: tlc 
R\(_f\) (A1) 0.70; m.p. 182-183°C (lit.\(^{219}\) 183°C); \(\nu_{\text{max}}\) (bromoform mull) 1520, 1350 (NO\(_2\)), 860 (p-disubstit. benzene) cm\(^{-1}\); \(\delta_H\) (200 MHz, d\(_7\)-DMF) 8.16 (4H, d, J\(_{AB}\) 8.8 Hz, Bnpe aromatic CH's), 7.32 (4H, d, J\(_{AB}\) 8.8 Hz, Bnpe aromatic CH's), 4.17 (2H, s, CH\(_2\)); \(m/z\) (FAB), 259 (MH\(^+\)), 243, 195; hplc 
R\(_t\) (B1) 13.8 minutes.

N\(^\alpha\)-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]-serine (88)

Bnpeoc-Ser-OH

Method A

The compound was prepared by the general method of Lapatsanis\(^{81}\), and applied to Bnpeoc \(N\alpha\)-protection by Florence.\(^{78}\)

Serine (2.415 g, 23.0 mmol) was dissolved in an aqueous solution of 10% Na\(_2\)CO\(_3\) (52.0 ml, 46.0 mmol) and 5% NaHCO\(_3\) (38.6 ml, 23.0 mmol), and cooled to 0°C. Bnpeoc-ONSu (21) (7.990 g, 18.6 mmol) in DMF (55 ml) was added slowly, and the resulting white suspension was stirred at 0°C for 20 minutes and at room temperature for 2 hours. Water (600 ml) was added and the aqueous mixture extracted with diethyl ether (2 x 150 ml) and ethyl acetate (3 x 150 ml). The aqueous phase was cooled, acidified with conc. HCl to pH1.5 and extracted with ethyl acetate (4 x 200 ml). The combined organic phases were washed with water and brine, and dried over Na\(_2\)SO\(_4\). The solvent was removed \textit{in vacuo} to afford a green oil which crystallised from diethyl ether/chloroform. The material was recrystallised from acetone/light petroleum (b.p. 40-60°C) to give the title compound (5.570 g, 71%) as a white solid: tlc 
R\(_f\) (A2) 0.27; m.p. 171-173°C (lit.\(^{78}\)
169-173°C); [α]D27 +2.9° (c = 1, DMF) (lit.78 +3.1° (c = 1, DMF)); νmax (bromof orm
mull) 1730, 1670 (C=O), 1520, 1350 (NO2), 1155 cm⁻¹; δH (200 MHz, CD3COCD3) 8.21
(4H, d, JAB 8.8 Hz, Bnpeoc aromatic CH's), 7.69 (4H, d, JAB 8.8 Hz, Bnpeoc aromatic
CH's), 6.43 (1H, bd, NH), 4.77 (3H, m, Bnpeoc CH,CH2), 4.28 (1H, m, Ser αCH), 3.88
(2H, m, Ser βCH2); δC (50 MHz, CD3COCD3) 170.3 (acid C=O), 154.9 (urethane C=O),
147.1, 146.3 (Bnpeoc quaternary aromatic C's), 128.8, 122.8 (Bnpeoc aromatic CH's),
64.9 (Bnpeoc CH2), 61.0 (Ser βCH2), 55.3 (Ser αCH), 48.8 (Bnpeoc CH); m/z (FAB),
420 (MH+), 325, 307; hrms, found 420.10427, C18H17N3O9 requires 420.10429 (< 1
ppm).

Method B

The compound was prepared by the general method of Rich82.

To a stirred suspension of serine (4.893 g, 46.6 mmol) in water (46 ml) was
added a solution of triethylamine (9.72 ml, 69.9 mmol) in dioxan (46 ml). To the
resultant solution was added Bnpeoc-ONSu (21) (20.001 g, 46.6 mmol) and the
mixture stirred at room temperature for 24 hours. Water (300 ml) was added and
the aqueous mixture extracted with ethyl acetate (3 x 300 ml). The aqueous phase
was acidified to pH2 with a saturated aqueous solution of KHSO4 and extracted
with ethyl acetate (3 x 300 ml). The combined organic phases were dried over
Na2SO4 and the solvent removed in vacuo to yield a white foam. The material was
triturated with diethyl ether/chloroform and was recrystallised from acetone/light
petroleum (b.p. 40–60°C) to afford the title compound (15.238 g, 78%) as a white
solid. Characterisation was in agreement with the above, plus the following
additional data: Found: C, 51.2; H, 4.03; N, 9.87 Calc. for C18H17N3O9 C, 51.6; H, 4.05;
N, 10.0%.

Deprotection of Bnpeoc–Gly–OBzl (90) with DBU/AcOH

Bnpeoc–Gly–OBzl (90) (0.064 g, 0.13 mmol) was dissolved in DMF (5 ml) at
room temperature and given 5 minutes to equilibrate. To this was added DBU (20.0
µl, 0.13 mmol) and AcOH (7.7 µl, 0.13 mmol), and the resulting mixture was stirred
for 30 minutes. At 5 minute intervals, aliquots (0.5 ml) of the reaction solution
were removed and quenched by adding to 1M AcOH (1.0 ml) and hplc grade
acetonitrile (2.0 ml). After running the standard Bnpeoc–Gly–OBzl (90) and 1,1–bis
(4′-nitrophenyl)ethene (91), a sample of each aliquot was monitored by analytical
hplc. The deprotection was shown to be complete in less than 30 minutes.
Deprotection of Fmoc-Gly-OBzl with DBU/AcOH

Fmoc-Gly-OBzl (0.052 g, 0.13 mmol) was dissolved in DMF (5 ml) at room temperature and given 5 minutes to equilibrate. To this was added DBU (20.0 µl, 0.13 mmol) and AcOH (7.7 µl, 0.13 mmol), and the resulting mixture was stirred for 30 minutes. At 5 minute intervals, aliquots (0.5 ml) were removed and prepared as above for monitoring by analytical hplc. The deprotection reaction was shown to be complete within 30 minutes.

2,2-bis (4'-Nitrophenyl)ethanol (19) (3.000 g, 10.4 mmol) was dissolved in pyridine (20 ml) and acetic anhydride (1.169 g, 11.5 mmol), and stirred at room temperature for 24 hours. The reaction mixture was concentrated in vacuo, co-evaporating (three times) with toluene, to yield a yellow solid. The product was recrystallised from chloroform/diethyl ether (twice) to afford the title compound (2.680 g, 78%) as an off-white solid: tlc R_f (A3) 0.65; m.p. 113-114°C (lit. 78 107-108°C); Found: C, 58.2; H, 4.36; N, 8.40 Calc. for C_{16}H_{14}N_{2}O_{6} C, 58.2; H, 4.24; N, 8.48%; \nu_{max} (CH_2Cl_2) 1740 (C=O), 1520, 1350 (NO_2), 1220, 1040, 860 cm^{-1}; \delta_H (80 MHz, CDCl_3) 8.16 (4H, d, J_{AB} 8.9 Hz, Bnpeoc aromatic CH's), 7.38 (4H, d, J_{AB} 8.9 Hz, Bnpeoc aromatic CH's), 4.63 (3H, m, Bnpeoc CH(CH_2)_2), 1.96 (3H, s, CH_3); \delta_C (50 MHz, CDCl_3) 170.4 (C=O), 147.2, 146.8 (quaternary aromatic C's), 124.0, 123.5 (aromatic CH's), 65.2 (Bnpeoc CH_2), 49.4 (Bnpeoc CH), 20.5 (CH_3).

1,1-bis (4'-Nitrophenyl)ethene (91)

The compound was prepared by the method of Florence. 78

2,2-bis (4'-Nitrophenyl)ethyl acetate (93) (1.824 g, 5.53 mmol) was dissolved in chloroform (18 ml) and to this was added DBN (1.37 ml, 11.1 mmol). On addition of the base, the initially pale yellow solution turned dark blue and a white precipitate formed. After 15 minutes the reaction mixture was diluted with further chloroform (12 ml) and washed with 2M HCl (2 x 30 ml), water (1 x 30 ml), 1M NaOH (2 x 30
ml) and brine (2 x 30 ml). After drying over Na$_2$SO$_4$, the solvent was removed in vacuo to yield a yellow solid. The product was recrystallised from chloroform/light petroleum (b.p. 40-60°C) to afford the title compound (1.031 g, 69%) as a yellow solid: tlc $R_f$ (A3) 0.75; m.p. 174-175°C (lit. 78 175-176°C); Found: C, 61.8; H, 3.73; N, 10.3 Calc. for C$_{14}$H$_{10}$N$_2$O$_4$ C, 62.2; H, 3.70; N, 10.4%; $\nu_{max}$ (CH$_2$Cl$_2$) 1605, 1520, 1350 (NO$_2$), 870 cm$^{-1}$; $\lambda_{max}$ 304 nm; $\delta_H$ (200 MHz, CDCl$_3$) 8.22 (4H, d, J$_{AB}$ 9.0 Hz, Bnpe aromatic CH's), 7.46 (4H, d, J$_{AB}$ 9.0 Hz, Bnpe aromatic CH's), 5.77 (2H, s, CH$_2$); $\delta_C$ (50 MHz, CDCl$_3$) 147.6, 146.5, 146.4 (3 x quaternary C's), 128.8, 123.7 (aromatic CH's), 120.0 (CH$_2$); m/z (FAB), 271 (MH$^+$), 255, 240, 176; hplc $R_t$ (B1) 16.8 minutes.

N-[2,2-bis (4'-Nitrophenyl)ethyl]-piperidine (92)

1,1-bis (4'-Nitrophenylethene (91) (0.105 g, 0.39 mmol) was dissolved in DMF (3.0 ml) at room temperature. On addition of piperidine (1.0 ml), the initially pale yellow solution turned dark blue. After 20 minutes the solution had returned to a pale yellow colour. The removal of the solvent in vacuo co-evaporating with DCM (twice), afforded an orange oily residue; $\lambda_{max}$ 275 nm; $\delta_H$ (200 MHz, CDCl$_3$) 8.12 (4H, d, J$_{AB}$ 8.9 Hz, Bnpe aromatic CH's), 7.37 (4H, d, J$_{AB}$ 8.9 Hz, Bnpe aromatic CH's), 4.41 (1H, t, J 7.7 Hz, Bnpe CH), 2.89 (2H, d, J 7.7 Hz, Bnpe CH$_2$), 2.40 (4H, m, 2 x CH$_2$), 1.41 (6H, m, 3 x CH$_2$); $\delta_C$ (50 MHz, CDCl$_3$) 149.8, 146.5 (quaternary aromatic C's), 129.0, 123.5 (aromatic CH's), 63.3 (Bnpe CH$_2$), 54.5 (CH$_2$), 48.3 (Bnpe CH), 25.7, 24.0 (CH$_2$).

N$^\alpha$-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]-serine benzyl ester (95)

Bnpeoc-Ser-OBzl

N$^\alpha$-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]-serine (88) (3.018 g, 7.20 mmol) was dissolved in dioxan/water (1:1) (80 ml) and cooled to 0°C. The solution was neutralised by the addition of an aqueous solution of 20% Cs$_2$CO$_3$ and lyophilised overnight. The resulting salt was dissolved in DMA (40 ml), and to this was added benzyl bromide (6.156 g, 36.0 mmol). The reaction mixture was stirred at room temperature for 90 minutes, before being filtered (to remove CsBr) and concentrated in vacuo. The residue was redissolved in ethyl acetate (100 ml), washed with water (2 x 100 ml), a saturated aqueous solution of NaHCO$_3$ (2 x 100 ml) and brine (2 x 100 ml), and dried over Na$_2$SO$_4$. The removal of the solvent in
vacuo afforded a yellow oil which was purified by dry flash chromatography on silica (employing a gradient of light petroleum (b.p. 40–60°C)/ethyl acetate) to give the title compound (3.278 g, 89%) as a yellow foam: tlc Rf (A3) 0.20; [α]27 -11.6° (c = 1, DMF); vmax. (CH2Cl2) 3420 (NH), 1725 (C=O), 1520, 1350 (NO2), 1190 cm⁻¹; δH (200 MHz, CDCl3) 8.13 (4H, d, JAB 8.7 Hz, Bnpeoc aromatic CH’s), 7.34 (9H, m, Bnpeoc aromatic CH’s, benzyl CH’s), 5.74 (1H, d, J 8.2 Hz, NH), 5.15 (2H, s, benzyl CH2), 4.62 (3H, m, Bnpeoc CH,CH2), 4.38 (1H, m, Ser αCH), 3.75 (2H, m, Ser βCH2), 2.58 (1H, bs, OH); δC (50 MHz, CDCl3) 170.0 (ester C=O), 155.5 (urethane C=O), 147.1, 146.2 (Bnpeoc quaternary aromatic C’s), 134.9 (benzyl quaternary C), 129.0 (Bnpeoc aromatic CH), 128.5, 127.9 (benzyl CH’s), 123.9 (Bnpeoc aromatic CH), 67.3 (benzyl CH2), 66.0 (Bnpeoc CH2), 62.3 (Ser βCH2), 56.0 (Ser αCH), 49.5 (Bnpeoc CH); m/z (FAB), 510 (MH+), 345, 181, 109; hrms. found 510.15125, C25H24N3O9 requires 510.15124 (< 1 ppm); hplc Rf (B1) 11.8 minutes.

Nα-(9-Fluorenylmethoxycarbonyl)serine benzyl ester (96)

Fmoc-Ser-OBzl

The compound was prepared from Nα-(9-fluorenylmethoxycarbonyl)serine by the general method described for compound (95) as discussed above. The compound was described previously less the following additional data: δH (200 MHz, CDCl3) 7.78–7.25 (13H, m, Fmoc aromatic CH’s, benzyl aromatic CH’s), 5.89 (1H, d, J 7.8 Hz, NH), 5.21 (2H, s, benzyl CH2), 4.44 (3H, m, Fmoc CH,CH2), 4.20 (1H, t, J 6.9 Hz, Ser αCH), 3.96 (2H, m, Ser βCH2), 2.57 (1H, bs, OH); δC (50 MHz, CDCl3) 170.3 (ester C=O), 156.1 (urethane C=O), 143.7, 143.5, 141.2 (Fmoc quaternary aromatic C’s), 135.0 (benzyl quaternary aromatic C), 129.4–119.8 (Fmoc aromatic CH’s, benzyl CH’s), 67.4, 67.1 (Fmoc CH2, benzyl CH2), 63.0 (Ser βCH2), 56.1 (Ser αCH), 47.0 (Fmoc CH); m/z (FAB), 418 (MH+), 403, 268, 239, 215, 203.
2,3,4,6-Tetra-O-benzyl-α-D-glucopyranose (98)

(Bzl)\textsubscript{4}αGluc-OH

The compound was prepared by the method of Koto et al.\textsuperscript{229}.

The material was purified by recrystallisation from ethyl acetate/hexane (yield = 33\%): tlc \textit{R}_f (A4) 0.79; m.p. 149-150°C (lit.\textsuperscript{229} 149-149.5°C); [\alpha]_D\textsuperscript{27} +46.1° (c = 1, dioxan) (lit.\textsuperscript{229} +48° (c = 1, dioxan)); $\nu_{\text{max}}$ (CH$_\text{2}$Cl$_2$) 1500, 1365, 1070 cm$^{-1}$; Found C, 75.5; H, 6.85 Calc. for C$_{34}$H$_{36}$O$_6$ C, 75.6; H, 6.67%; $\delta_H$ (200 MHz, CDCl$_3$) 7.33 (20H, m, aromatic CH’s), 5.26 (d, J 3.6 Hz, equatorial CH [C$_{1}$]), 5.01-4.47 (91-1, m, axial CH [C$_{1}$], 4 x benzyl CH$_{2}$), 4.07-3.55 (6H, m, 4 x glucose CH, glucose CH$_2$); $\delta_C$ (50 MHz, CDCl$_3$) 138.5-137.6 (quaternary aromatic C’s), 128.2, 127.8, 127.5 (aromatic CH’s), 97.4 (equatorial CH [C$_{1}$]), 91.1 (axial CH [C$_{1}$]), 84.5, 83.1, 81.7, 80.0, 77.8 (CH’s), 75.5, 74.9, 73.4, 73.1 (benzyl CH$_2$’s), 70.2 (CH), 69.0, 68.7 (glucose CH$_2$); hplc \textit{R}_f (B2) 23.0 minutes.

\textit{N}^{\alpha}-(2,2-bis (4’-Nitrophenyl)ethoxycarbonyl]-serine-(O-2,3,4,6-tetra-O-benzyl-α/β-D-glucopyranosyl) benzyl ester (99)

Bnpeoc-Ser(α/β-D-((Bzl)$_4$Gluc)-OBzl

Trifluoromethanesulphonic anhydride (0.175 ml, 1.05 mmol) was added to a solution of Bnpeoc-Ser-OBzl (95) (1.070 g, 2.10 mmol) in acetonitrile/DCM (1:1) (10 ml) at -15°C. 2,3,4,6-Tetra-O-benzyl-α-D-glucopyranose (98) (0.653 g, 1.20 mmol) in DCM (12 ml) was added, maintaining the temperature at -15°C. The reaction mixture was then allowed to warm to room temperature and stirred for a further 90 minutes. Water (30 ml) was added and the aqueous solution extracted with DCM (3 x 30 ml). The combined organic phases were washed with a saturated aqueous solution of NaHCO$_3$ (2 x 50 ml), water (1 x 50 ml) and dried over Na$_2$SO$_4$. The removal of the solvent \textit{in vacuo} gave a yellow oil, an aliquot of which was purified by, firstly, flash chromatography on silica (employing a gradient of light petroleum (b.p. 40-60°C)/diethyl ether), and, secondly, preparative hplc, using an Aquapore C$_8$ reverse phase prep. column with acetonitrile/water (+0.05% TFA) as the eluent and monitoring at 254 nm. Removal of the solvents by lyophilisation afforded the title compound (0.145 g, calculated yield = 34\%) as a yellow solid: tlc \textit{R}_f (A3) 0.78, 0.80;
$\delta_H$ (360 MHz, CDCl$_3$) 8.12 (4H, m, Bnpeoc aromatic CH's), 7.21 (29H, m, Bnpeoc aromatic CH's, benzyl CH's), 5.94 + 5.67 (1H, d + d, J 8.6, 8.0 Hz, NH), 5.15 (2H, m, benzyl ester CH$_2$), 4.91-4.27 (13H, m, anomic CH, 4 x benzyl ether CH$_2$, Ser $\alpha$CH, Bnpeoc CH,CH$_2$), 4.12-3.29 (8H, m, Ser $\beta$CH$_2$, 4 x glucose CH, glucose CH$_2$); m/z (FAB), 1032 (MH$^+$), 1002, 558, 523, 325; hrms, found 1032.39184, C$_{59}$H$_{58}$N$_3$O$_{14}$ requires 1032.39185 (< 0.1 ppm); hplc $R_t$ (B5) 21.8, 22.6 minutes.

Serine-(O-2,3,4,6-tetra-O-benzyl-α/β-D-glucopyranosyl) benzyl ester (100)

H-Ser(α/β-D-(Bzl)$_4$Gluc)-OBzl

Bnpeoc-Ser(α/β-D-(Bzl)$_4$Gluc)-OBzl (99) (0.194 g, 0.19 mmol) was dissolved in DMF (8 ml), and to this was added DBU (1.57 ml of a solution of 0.219 g in DMF (10 ml), 0.22 mmol) and glacial acetic acid (0.72 ml of a solution of 0.187 g in DMF (10 ml), 0.22 mmol). The reaction was stirred for 45 minutes, monitoring by tlc. Ethyl acetate (150 ml) and water (150 ml) were added, and the separated organic layer washed with water (1 x 100 ml), an aqueous solution of 5% citric acid (1 x 100 ml), water (1 x 100 ml) and brine (1 x 100 ml), and dried over Na$_2$SO$_4$. The removal of the solvent in vacuo afforded a yellow oil which was purified by flash chromatography on silica (employing a gradient of light petroleum (b.p. 40-60°C)/ethyl acetate/methanol). The removal of the solvent in vacuo gave the title compound (0.126 g, 93%) as a yellow oil: tlc $R_t$ (A7) 0.54; $\delta_H$ (360 MHz, CDCl$_3$) 7.28 (25H, m, benzyl CH's), 5.15 (2H, m, benzyl ester CH$_2$), 4.94-4.27 (10H, m, Ser $\alpha$CH, anomic CH, 4 x benzyl ether CH$_2$), 4.14-3.34 (8H, m, Ser $\beta$CH$_2$, glucose CH$_2$, 4 x glucose CH); m/z (FAB), 718 (MH$^+$), 628; hrms, found 718.3381, C$_{44}$H$_{48}$NO$_8$ requires 718.33797 (< 1 ppm).

N$^\alpha$-(Benzyloxy-carbonyl)alanine N-succinimidyl ester (102)

Z-Ala-ONSu

The compound was prepared by the general method of Anderson et al.$^{109}$

1,3-Dicyclohexylcarbodiimide (1.860 g, 9.01 mmol) was added slowly to a solution of Z-Ala-OH (101) and N-hydroxysuccinimide (1.036 g, 9.01 mmol) in dioxan (22 ml), keeping the temperature at 14-16°C during the addition. The
reaction mixture was transferred to a refrigerator and stored for 24 hours. The formed dicyclohexylurea was filtered off and washed with dioxan. The combined filtrates were concentrated in vacuo to yield a white oil which soon crystallised. The solid was redissolved in ethyl acetate (100 ml), washed quickly with water (1 x 100 ml), and dried over Na$_2$SO$_4$. The removal of the solvent in vacuo afforded a white solid which was recrystallised from ethyl acetate/light petroleum (b.p. 40-60°C) to give the title compound (2.546 g, 88%) as a white solid: tlc $R_f$ (A5) 0.37; m.p. 122-123°C (lit.$^{109}$ 123-123.5°C); $[\alpha]_{D}^{27}$ -39.9° (c = 2, dioxan) (lit.$^{109}$ -37.2° (c = 2, dioxan)); Found C, 56.2; H, 5.24; N, 8.91 Calc. for C$_{15}$H$_{16}$N$_2$O$_6$ C, 56.2; H, 5.03; N, 8.75%; $\nu_{\text{max}}$ (CH$_2$Cl$_2$) 3435 (NH), 1820, 1790, 1750 (C=O), 1510, 1205, 1065 cm$^{-1}$; $\delta$H (200 MHz, CDCl$_3$) 7.35 (5H, s, aromatic CH's), 5.31 (1H, d, J 8.2 Hz, NH), 5.12 (2H, s, Z- CH$_2$), 4.78 (1H, m, Ala CH), 2.83 (4H, s, 2 x succinimidyl CH$_2$), 1.59 (3H, d, J 7.2 Hz, CH$_3$); $\delta$C (50 MHz, CDCl$_3$) 168.6 (ester, 2 x succinimidyl C=O), 155.2 (urethane C=O), 135.8 (aromatic quaternary C), 128.4, 128.0 (aromatic CH's), 67.1 (Z- CH$_2$), 47.9 (Ala CH), 25.4 (succinimidyl CH$_2$'s), 18.3 (CH$_3$); m/z (FAB), 321 (MH$^+$), 277, 181.

**N$^\alpha$-(Benzylxycarbonyl)alanylalanine (103)**

Z-Ala-Ala-Oh

To a stirred suspension of alanine (0.833 g, 9.36 mmol) in water (9 ml) was added a solution of triethylamine (1.95 ml, 14.0 mmol) in dioxan (9 ml). To this was added Z-Ala-ONSu (102) (2.997 g, 9.36 mmol) and the mixture stirred at room temperature overnight. Water (20 ml) was added, the solution acidified to pH1.5 using 2M KHSO$_4$ (aq) and extracted with ethyl acetate (3 x 50 ml). The combined organic phases were washed with water (2 x 40 ml) and dried over Na$_2$SO$_4$. The removal of the solvent in vacuo gave a white solid, which was purified by flash chromatography on silica (eluting with ethyl acetate). The resulting material was recrystallised from ethyl acetate/light petroleum (b.p. 40-60°C) to give the title compound (1.791 g, 65%) as a white solid: tlc $R_f$ (A6) 0.29; m.p. 149-150°C; $[\alpha]_{D}^{27}$ -36.4° (c = 1, MeOH); Found C, 57.5; H, 6.38; N, 9.61 Calc. for C$_{14}$H$_{18}$N$_2$O$_5$ C, 57.1; H, 6.16; N, 9.52%; $\nu_{\text{max}}$ (CH$_2$Cl$_2$) 3420 (NH), 1725, 1680 (C=O), 1510 cm$^{-1}$; $\delta$H (200 MHz, CD$_3$COCD$_3$) 7.57 (1H, bd, J 7.5 Hz, amide NH), 7.36 (5H, m, aromatic CH's), 6.54 (1H, bd, J 7.3 Hz, urethane NH), 5.08 (2H, s, Z- CH$_2$), 4.47, 4.29 (2H, m, 2 x Ala CH), 1.35 (6H, m, 2 x CH$_3$); $\delta$C (50 MHz, CD$_3$COCD$_3$) 172.3, 171.4 (amide, acid C=O), 155.0 (urethane C=O), 136.5 (quaternary aromatic C), 127.5, 126.9 (aromatic CH's), 65.2 (Z-
Nα-(Benzyloxycarbonyl)alanylalanylserine-(O-2,3,4,6-tetra-O-benzyl-α/β-D-glucopyranosyl) benzyl ester (94)

Z-Ala-Ala-Ser[α/β-D-(Bzl)₄Gluc]-OBzl

Bnpeoc-Ser[α/β-D-(Bzl)₄Gluc]-OBzl (99) (0.100 g, 0.097 mmol) was dissolved in DMF (4.0 ml), and to this was added DBU (2.78 ml of a solution of 0.062 g in DMF (10 ml), 0.11 mmol) and glacial acetic acid (0.60 ml of a solution of 0.096 g in DMF (10 ml), 0.11 mmol). The reaction mixture was stirred for 40 minutes, monitoring by tlc. Ethyl acetate (50 ml) and water (50 ml) were added, and the separated organic layer was washed with water (2 x 50 ml) and dried over Na₂SO₄. The removal of the solvent in vacuo afforded a yellow oil which was used without further manipulation.

Z-Ala-Ala-OH (103) (0.057 g, 0.19 mmol) was dissolved in DMF (2.0 ml) and to this was added HOBt (0.026 g, 0.19 mmol) in DMF (0.5 ml). After stirring for 2 minutes 1,3-dicyclohexylcarbodiimide (0.040 g, 0.19 mmol) was added and the reaction mixture stirred for a further 2 minutes. The deprotected glyco-amino acid from above (100) was dissolved in OMF (1.0 ml), added to the reaction mixture and the pH of the resulting solution adjusted to 9.0 by the addition of triethylamine. The reaction mixture was stirred for 18 hours at room temperature, before filtering (to remove the formed urea) and isolating the desired product by preparative hplc, employing an Aquapore C₁₈ reverse phase prep. column, with water/acetonitrile (+0.05% TFA) as eluent and monitoring at 229 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.015 g, 15%) as a white solid: tlc R₁ (A7) 0.71; δH (360 MHz, D₂O/CD₃COCD₃) 7.27 (30H, m, Z-, benzyl ether, benzyl ester aromatic CH's), 5.16-4.40 (14H, m, 4 x benzyl ether CH₂, benzyl ester CH₂, anomeric CH, Ser αCH, 2 x Ala αCH), 4.10-3.68 (6H, m, 4 x glucose CH, glucose CH₂), 3.55 (2H, m, Ser βCH₂), 1.25 (6H, m, 2 x Ala CH₃); m/z (FAB) 994 (M⁺), 610, 562, 472, 454, 367; hirms, found 994.44904, C₅₈H₆₅N₃O₁₂ requires 994.44896 (< 1 ppm); hplc R₁ (B13) 28.2, 28.4 minutes.
Nα-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]-alanylphenylalanylglucose methyl ester (105)

Bnpeoc-Ala-Phe-Gly-OMe

This compound was prepared by the method of Florence.78

The material was purified by recrystallisation from ethyl acetate/light petroleum (b.p. 40-60°C) (yield = 60%): tlc Rf (A4) 0.70; m.p. 159-161°C (lit. 78 159-162°C); $[\alpha]_D^{27} = -16.1^\circ$ (c = 1, DMF) (lit. 78 $-3.9^\circ$ (c = 1, DMF)); Found C, 57.7; H, 4.92; N, 11.2

Calc. for C$_{30}$H$_{31}$N$_5$O$_{10}$ C, 58.0; H, 5.03; N, 11.3%; $\nu_{\text{max}}$ (CH$_2$Cl$_2$) 3420 (NH), 1750, 1730, 1680 (C=O), 1525, 1350 (NO$_2$) cm$^{-1}$; $\delta_H$ (200 MHz, CD$_3$COCD$_3$) 8.22 (4H, d, J$_{AB}$ 8.8 Hz, Bnpeoc aromatic CH's), 7.69 (4H, d, J$_{AB}$ 8.8 Hz, Bnpeoc aromatic CH's), 7.25 (5H, m, Phe aromatic CH's), 6.68 (1H, bd, urethane NH), 4.74 (4H, m, Phe CH$_2$CH, Bnpeoc CH,CH$_2$), 4.10 (1H, t, Bnpeoc aromatic CH's), 3.95 (2H, m, Gly CH$_2$), 3.65 (3H, s, OCH$_3$), 3.10 (2H, m, Phe CH$_2$), 1.22 (3H, d, J 7.7 Hz, Ala CH$_3$); $\delta_C$ (50 MHz, CD$_3$COCD$_3$) 171.2, 170.4, 169.1 (2 x amide C=O, ester C=O), 154.8 (urethane C=O), 147.3, 146.5 (Bnpeoc quaternary aromatic C's), 136.7 (Phe quaternary aromatic C), 129.3-122.9 (aromatic CH's), 65.0 (Bnpeoc CH$_2$), 53.1, 50.5, 50.2, 48.9 (Bnpeoc CH, OCH$_3$ 2 x CH), 39.5, 37.0 (Phe CH$_2$, Gly CH$_2$), 16.6 (Ala CH$_3$); m/z (FAB), 622 (M$^+$), 254, 237, 167; amino acid analysis, Gly$_1$ 1.03, Ala$_1$ 0.99, Phe$_1$ 0.98; hplc R$_f$ (B3) 17.0 minutes.

Nα-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]-alanylphenylalanylglucose (106)

Bnpeoc-Ala-Phe-Gly-OH

Bnpeoc-Ala-Phe-Gly-OMe (105) (0.117 g, 0.19 mmol) was dissolved in acetone/water (8:2) (8 ml), and to this was added 0.10M NaOH$_{aq}$ (1.92 ml, 0.19 mmol) and H$_2$O$_2$ (3% solution in water; 216 µl, 0.19 mmol). The reaction mixture was stirred for 40 minutes at room temperature. The pH of the solution was adjusted to 1.5 by the addition of conc. HCl, extracted with ethyl acetate (3 x 40 ml), and dried over Na$_2$SO$_4$. Concentration of the reaction mixture in vacuo afforded a yellow oil, which was purified by preparative hplc, employing an Aquapore C$_{18}$ reverse phase prep. column with water/acetonitrile (+0.05% TFA) as the eluent and monitoring at 254 nm. The removal of the solvents by lyophilisation gave the title compound (0.063 g, 55%) as a white solid: tlc R$_f$ (A4) 0.19, R$_f$ (A6) 0.80; m.p. 110-113°C; $\delta_H$ (200 MHz, d$_6$-DMSO) 8.32 (1H, bt, amide NH). 8.19 (4H, d,
J\textsubscript{AB} 8.7 Hz, Bnpeoc aromatic CH's), 7.87 (1H, bd, NH), 7.68 (4H, d, J\textsubscript{AB} 8.7 Hz, Bnpeoc aromatic CH's), 7.33 (1H, d, NH), 7.21 (5H, s, Phe aromatic CH's), 4.64 (4H, m, Phe \alpha CH, Bnpeoc CH, CH\textsubscript{2}), 3.95 (1H, m, Ala \alpha CH), 3.77 (2H, d, Gly CH\textsubscript{2}), 2.97 (2H, m, Phe \beta CH\textsubscript{2}), 1.07 (3H, d, J 7.0 Hz, Ala CH\textsubscript{3}); \textit{m/z} (FAB), 608 (MH\textsuperscript{+}), 585, 329, 176; \textit{hrms} found 608.19921, C\textsubscript{29}H\textsubscript{30}N\textsubscript{5}O\textsubscript{10} requires 608.19925 (< 1 ppm); amino acid analysis, Gly\textsubscript{1} 1.02, Ala\textsubscript{1} 0.98, Phe\textsubscript{1} 1.00; hplc \textit{R}\textsubscript{1} (B4) 22.8 minutes.

**Glycine benzyl ester p-toluenesulphonate (107)**

\textit{TosO}\textsubscript{2}H\textsuperscript{+}-Gly-OBzl

The compound was prepared by the method of Zervas et al\textsuperscript{230}.

Glycine (9.466 g, 0.126 mol), p-toluenesulphonic acid monohydrate (24.543 g, 0.129 mol) and benzyl alcohol (50.0 ml, 0.483 mol) were added to benzene (50 ml) and heated under reflux, collecting the water produced in a Dean and Stark receiving apparatus, for 5 hours. The mixture was left to cool to room temperature overnight. Benzene (125 ml) and diethyl ether (200 ml) were added, and the flask stored at 5°C for 2 hours. The crystalline material was filtered, washed with diethyl ether and recrystallised from methanol/diethyl ether to give the title compound (39.053 g, 92%) as a white, crystalline solid: m.p. 132–133°C (lit.\textsuperscript{230} 132–133°C); Found C, 56.6; H, 5.62; N, 4.23 Calc. for C\textsubscript{16}H\textsubscript{19}NO\textsubscript{5}S C, 57.0; H, 5.68; N, 4.15%; \textit{v}\textsubscript{max} (bromoform mull) 1750 (C=O), 1550, 1425, 905, 810 cm\textsuperscript{-1}; \textit{δ} (200 MHz, d\textsubscript{6}-DMSO) 8.30 (3H, bs, NH\textsubscript{3}\textsuperscript{+}), 7.54 (2H, d, J\textsubscript{AB} 8.1 Hz, tosyl aromatic CH's), 7.41 (5H, m, benzyl aromatic CH's), 7.14 (2H, d, J\textsubscript{AB} 8.1 Hz, tosyl aromatic CH's), 5.23 (2H, s, benzyl CH\textsubscript{2}), 3.91 (2H, s, Gly CH\textsubscript{2}), 2.30 (3H, s, CH\textsubscript{3}); \textit{m/z} (FAB), 166 (H\textsubscript{3}N\textsuperscript{+}-CH\textsubscript{2}CO\textsubscript{2}CH\textsubscript{2}Ph), 150.

\textit{N\textsuperscript{α}-(Benzylloxy carbonyl)phenylalanylglycine benzyl ester (109)}

Z-Phe-Gly-OBzl

Z-Phe-OH (108) (10.004 g, 33.4 mmol) and N-hydroxybenzotriazole (4.986 g, 36.9 mmol) were dissolved in DMF (50 ml) and cooled to 0°C. To this solution was slowly added 1,3-dicyclohexylcarbodiimide (6.909 g, 33.4 mmol) in DMF (25 ml), maintaining the temperature at 0°C. After stirring for 5 minutes, TosO\textsuperscript{−}H\textsubscript{2}\textsuperscript{+}-Gly-OBzl (107) (11.273 g, 33.4 mmol) in DMF (25 ml) was added and the
pH adjusted to 9.0 by the addition of triethylamine. The reaction was then stirred for 17 hours at 5°C. The formed dicyclohexylurea was filtered off and the filtrate concentrated in vacuo to yield an off-white solid. This was redissolved in ethyl acetate (250 ml) and placed in a refrigerator for 2 hours, after which time the solution was again filtered. The filtrate was washed with a saturated aqueous solution of NaHCO₃ (2 x 100 ml), water (2 x 100 ml), an aqueous solution of 5% citric acid (2 x 100 ml) and brine (1 x 100 ml), and dried over Na₂SO₄. The removal of the solvent in vacuo afforded a white solid, which was recrystallised from ethyl acetate/light petroleum (b.p. 40-60°C) to yield the title compound (13.097 g, 88%) as a white solid: tlc R₇ (A₄) 0.75; m.p. 134-135°C (lit.²⁴⁷ 135°C); [α]D ²⁷ -1.82° (c = 1, acetone) (lit.²⁴⁷ -3.75° (c = 1, acetone)); Found C, 70.0; H, 5.96; N, 6.42 Calc. for C₂₆H₂₆N₂O₅ C, 69.9; H, 5.87; N, 6.27%: νmax. (CH₃Cl₂) 3425 (NH), 1745, 1685 (C=O), 1600, 1500, 1195 cm⁻¹; δH (200 MHz, CD₃COCD₃) 7.78 (1H, bs, amide NH), 7.30 (15H, m, Z- aromatic CH's, benzyl aromatic CH's, Phe aromatic CH's), 6.58 (1H, bd, urethane NH), 5.18 (2H, s, benzyl CH₂), 5.00 (2H, m, Z- CH₂), 4.57 (1H, m, Phe αCH), 4.02 (2H, m, Gly CH₂), 3.13 (2H, m, Phe βCH₂); δC (50 MHz, CD₃COCD₃) 170.9, 168.6 (amide, ester C=O), 155.1 (urethane C=O), 137.0, 136.5, 135.5 (quaternary aromatic C's), 128.6-125.6 (aromatic CH's), 65.5, 65.2 (Z- CH₂, benzyl CH₂), 55.6 (Phe αCH), 40.3 (Gly CH₂), 37.4 (Phe βCH₂); m/z (FAB), 447 (MH⁺), 403, 372, 313, 225: hrms, found 447.19199, C₂₆H₂₇N₂O₅ requires 447.19198 (< 1 ppm); hplc R₇ (B₂) 18.5 minutes.

Phenylalanylglycine p-toluenesulphonate (110)

TosO⁻H₂⁺-Phe-Gly-OH

Z-Phe-Gly-OBzl (109) (3.005 g, 6.73 mmol) was dissolved in DMF (25 ml) together with p-toluenesulphonic acid monohydrate (1.282 g, 6.73 mmol) and was hydrogenated for 7 days at room temperature and pressure in the presence of 10% palladium-charcoal catalyst (0.905 g, 30% by weight of protected dipeptide). Filtration through 'celite', followed by concentration of the reaction mixture in vacuo gave the title compound as a green oil. All attempts at recrystallisation failed, and the product was used without further manipulation: tlc R₇ (A₆) 0.25; m/z (FAB), 223 (H₃N⁺-CH(CH₂Ph)CONHCH₂CO₂H).
N^\beta-(2,2-bis (4'-Nitrophenyl)ethoxycarbonyl)-alanine N-succinimidy ester (111)

Bnpeoc-Ala-ONSu

1,3-Dicyclohexylcarbodiimide (1.537 g, 7.45 mmol) was added slowly to a solution of Bnpeoc-Ala-OH (85) (3.006 g, 7.45 mmol) and N-hydroxsuccinimide (0.859 g, 7.45 mmol) in dioxan (25 ml), maintaining the temperature between 10-15°C throughout the addition. After stirring for 1 hour at 10-15°C, the mixture was transferred to a refrigerator for 24 hours. The formed dicyclohexylurea was filtered off and washed with dioxan (70 ml). The combined filtrates were concentrated in vacuo to give a colourless oil, which was redissolved in ethyl acetate and stored at 5°C for 2 hours. Again the precipitated urea was filtered off, and the solvent was removed in vacuo to yield the title compound (3.187 g, 86%) as a white foam: m.p. 73-76°C; [a]_D^27 -24.2° (c = 1, dioxan); ν_max. (CHCl_3) 1835, 1805, 1755 (C=O), 1530, 1355 (NO_2), 1215 cm^{-1}; δ (200 MHz, CD_3COCD_3) 8.21 (4H, d, J_AB 8.7 Hz, Bnpeoc aromatic CH's), 7.69 (4H, d, J_AB 8.7 Hz, Bnpeoc aromatic CH's), 7.12 (1H, d, J 7.9 Hz, NH), 4.77 (3H, m, Bnpeoc CH,CH_2), 4.62 (1H, m, Ala αCH), 2.90 (4H, s, 2 x succinimidyl CH_2), 1.52 (3H, m, CH_3); δ (50 MHz, CD_3COCD_3) 168.5, 168.0 (2 x succinimidyl C=O, ester C=O), 155.0 (urethane C=O), 147.2, 146.5 (Bnpeoc quaternary aromatic C's), 128.9, 122.9 (Bnpeoc aromatic CH's), 65.1 (Bnpeoc CH_2), 49.0 (Ala αCH), 47.4 (Bnpeoc CH), 24.7 (succinimidyl CH_2), 16.1 (CH_3); m/z (FAB), 501 (MH^+), 485, 404, 307, 225; hrrms, found 501.12573, C_{22}H_{21}N_4O_{10} requires 501.12575 (< 1 ppm).

N^\beta-(2,2-bis (4'-Nitrophenyl)ethoxycarbonyl)-alaninophenylalaniglycine (106)

Bnpeoc-Ala-Phe-Gly-OH

TosO^+H_2^+Phe-Gly-OH (110) (2.375 g, 6.03 mmol) was dissolved in water/dioxan (1:1) (20 ml), and to this was added triethylamine (1.72 g, 17.2 mmol) in dioxan (2 ml). Bnpeoc-Ala-ONSu (111) (4.524 g, 9.05 mmol) in dioxan (20 ml) was added slowly, and the reaction mixture stirred overnight at room temperature. Water (500 ml) was added, the pH adjusted to 1.5 by the addition of conc. HCl, and the aqueous solution extracted with ethyl acetate (3 x 300 ml). The combined organic phases were dried over Na_2SO_4 and the solvent removed in vacuo to yield an oily solid. This material was purified by flash chromatography on silica (employing a
gradient of ethyl acetate/methanol) to give a white solid. Lyophilisation of this solid from dioxan/water (2:1) (60 ml) afforded the title compound (2.399 g, 66%) as a white solid: tlc Rf (A4) 0.20, Rf (A6) 0.80; m.p. 111-113°C; δH (200 MHz,d6-DMSO) 8.30 (1H, t, amide NH), 8.17 (4H, d, JAB 8.7 Hz, Bnpeoc aromatic CH's), 7.84 (1H, d, NH), 7.65 (4H, d, JAB 8.7 Hz, Bnpeoc aromatic CH's), 7.31 (1H, d, NH), 7.19 (5H, s, Phe aromatic CH's), 4.63 (4H, Phe αCH, Bnpeoc CH2), 3.95 (1H, m, Ala αCH), 3.78 (2H, d, Gly CH2), 2.95 (2H, m, Phe βCH2). 1.04 (3H, d, J 7.0 Hz, Ala CH2); m/z (FAB), 608 (MH+), 584, 329, 271; hrms, found 608.19921, C29H30N5O10 requires 608.19925 (< 1 ppm); amino acid analysis, Gly 1.01, Ala 0.98, Phe 1.01; hplc Rf (B4) 22.8 minutes.

Nα-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]-glycyl chloride (112)

Bnpeoc-Gly-Cl

Thionyl chloride (1.61 ml, 22.0 mmol) was added to Bnpeoc-Gly-OH (87) (0.857 g, 2.20 mmol) in DCM (100 ml) and heated under reflux for 90 minutes under nitrogen. The solvent and excess thionyl chloride were removed in vacuo, re-evaporating three times with DCM to ensure complete removal of the thionyl chloride. The resulting foam was used without further manipulation: vmax (CH2Cl2) 3425 (NH), 1805 (acid chloride C=O), 1740 (urethane C=O), 1525, 1350 (NO2) cm⁻¹.

Nα-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]glycyl p-alkoxybenzyl alcohol resin (113)

Bnpeoc-Gly-(O-CH2C6H4-OR)

p-Alkoxybenzyl alcohol resin (8) (Bachem, 1.01 mmol/g) (1.982 g, 2.00 mmol) was swollen in DCM (35 ml) and to this was added pyridine (4.85 ml, 60.0 mmol) and the preformed Bnpeoc-Gly-Cl (112) (2.20 mmol) in DCM (15 ml). The reaction mixture was agitated for 3 hours on a manual shaker at room temperature under nitrogen. The resin was filtered, washed with DCM (3 x 1 minute), DMF (3 x 1 minute) and DCM (3 x 1 minute), and dried; Found N, 1.90%; Bnpeoc-Gly-(O-CH2C6H4-OR) requires N, 3.08%; hence coupling yield = 62%. A second coupling reaction, performed exactly as the first, was therefore necessary: Found N, 2.68%; hence coupling yield (after second coupling) = 87%; vmax (KBr disc) 1720, 1665 (C=O), 1515, 1350 (NO2) cm⁻¹.
N^α-[2,2-\text{bis}(4'\text{-Nitrophenyl})ethoxycarbonyl]-alanylphenylalanylglucose (106)

Bnpeoc-Ala-Phe-Gly-OH

The synthesis of (106) was accomplished using the functionalised resin, Bnpeoc-Gly-(O-CH\text{2}C\text{6}H\text{4}-OR) (113), and employing a repetitive solid-phase cycle on a manual shaker which involved:

1. washing the functionalised resin - DCM (2 x 1 minute),
2. capping of any unreacted sites - acetic anhydride (2.08 ml, 22.0 mmol) and pyridine (1.78 ml, 22.0 mmol) in DCM (35 ml) (1 hour),
3. washing - DCM (2 x 1 minute), DMF (2 x 1 minute),
4. removal of N^α-protecting group - DBU (600 μl, 4.02 mmol) and acetic acid (230 μl, 4.02 mmol) in DMF (35 ml) (1 hour),
5. washing - DMF (2 x 1 minute), DCM (2 x 1 minute),
6a. coupling of Bnpeoc-Phe-Cl (2.40 mmol) in N-methylmorpholine (6.60 ml, 60.0 mmol) and DCM (35 ml) (2 hours) (double couple),
6b. coupling of Bnpeoc-Ala-Cl (2.40 mmol) in N-methylmorpholine (6.60 ml, 60.0 mmol) and DCM (35 ml) (2 hours) (single couple),
7. washing - DCM (2 x 1 minute), DMF (2 x 1 minute), DCM (2 x 1 minute).

Samples of the resin were removed after steps (6a) and (6b), and subjected to the Kaiser test\textsuperscript{232} to determine the extent of coupling.

The cleavage of the peptide from the support was performed by reaction with trifluoroacetic acid (25 ml) in DCM (25 ml). The reaction mixture was shaken for 1 hour under nitrogen at room temperature, before the resin was filtered off and washed with DMF (3 x 1 minute) and DCM (3 x 1 minute). The combined filtrates were concentrated \textit{in vacuo} to give a yellow oil, which was triturated from ethyl acetate/light petroleum (b.p. 40–60°C) to give a yellow solid. This material was purified by gel filtration on Sephadex LH20, eluting with methanol, to afford the title compound (0.458 g, 38%) as a white solid: tlc R\textsubscript{f} (A4) 0.20, R\textsubscript{f} (A6) 0.80; m.p. 113–115°C; ν\textsubscript{max} (CH\text{2}Cl\text{2}) 1725, 1670 (C=O), 1525, 1350 (NO\textsubscript{2}) cm\textsuperscript{-1}; δ\textsubscript{H} (200 MHz, CD\textsubscript{3}COCD\textsubscript{3}) 8.20 (4H, d, J\textsubscript{AB} 8.8 Hz, Bnpeoc aromatic CH's), 7.68 (4H, d, J\textsubscript{AB} 8.8 Hz, Bnpeoc aromatic CH's), 7.23 (5H, m, Phe aromatic CH's), 4.75 (4H, d, J\textsubscript{AB} 8.8 Hz, Bnpeoc aromatic CH's), 7.23 (5H, m, Phe aromatic CH's), 4.75 (4H, m, Phe αCH, Bnpeoc CH,CH\textsubscript{2}), 4.06 (1H, m, Ala αCH), 3.99 (2H, d, J 3.0 Hz, Gly CH\textsubscript{2}), 3.07 (2H, m, Phe βCH\textsubscript{2}), 1.21 (3H, d, J 7.2 Hz, Ala CH\textsubscript{3}); δ\textsubscript{C} (50 MHz, CD\textsubscript{3}COCD\textsubscript{3}) 171.4, 170.4, 169.7 (2 x amide C=O, acid C=O), 154.8 (urethane C=O), 147.3, 146.6 (Bnpeoc quaternary aromatic C's), 136.8 (Phe quaternary aromatic C), 129.0–122.9 (aromatic CH's), 65.2 (Bnpeoc CH\textsubscript{2}), 53.2, 50.2, 49.1 (Bnpeoc CH, 2 x αCH), 40.0, 37.0 (Phe
**BrCH₂·Gly CH₂), 16.7 (Ala CH₃); m/z (FAB), 608 (M⁺), 592, 323, 307, 271; hrms, found 608.19921, C₂₉H₃₀N₅O₁₀ requires 608.19925 (< 1 ppm); amino acid analysis, Gly₁0.99, Ala₁1.02, Phe₁0.99; hplc Rₜ (B4) 22.8 minutes.**

Serine methyl ester hydrochloride (114)

Cl⁺H₂⁺-Ser-OMe

The compound was prepared by the method of Guttmann and Boissonnas²³³.

Methanol (500 ml) was cooled in a CO₂/acetone bath, and thionyl chloride (130 ml) was added dropwise at such a rate as to maintain the temperature below -20°C. The bath was removed and serine (52.5 g, 0.50 mol) was added in portions over 30 minutes. The reaction mixture was allowed to warm to room temperature and stirred for 24 hours. The formed precipitate was filtered and recrystallised from methanol/diethyl ether to give the title compound (70.10 g, 90%) as a white solid: tlc Rₜ (A8) 0.22; m.p. 164-165°C (lit.²³³ 168°C); [α]D27 +5.0° (c = 2, methanol) (lit.²³³ [α]D23 +5.5° (c = 1.8, methanol)); Found: C, 30.8; H, 6.84; N, 9.00%; νmax (bromoform mull) 1750 (C=O), 1510, 1300, 1140 cm⁻¹; δH (200 MHz, CD₃OD) 5.00 (3H, bs, NH₃⁺), 4.25 (1H, m, Ser CH₂), 4.08 (2H, m, Ser CH₂), 3.95 (3H, s, CH₃); δC (50 MHz, CD₃OD) 167.5 (C=O), 58.8 (Ser CH₂), 54.2 (Ser αCH), 51.8 (CH₃); m/z (FAB), 120 (H₃N⁺-CH(CH₂OH)CO₂CH₃), 85, 32.

Nα-(Benzyloxy carbonyl)serine methyl ester (115)

Z-Ser-OMe

Cl⁺H₂⁺-Ser-OMe (114) (61.0 g, 0.392 mol) was dissolved in triethylamine (56.0 ml, 0.402 mol) and chloroform (800 ml) and cooled to 0°C. After stirring for 10 minutes, benzyloxy carbonyl chloride (32.0 ml, 0.224 mol), triethylamine (56.0 ml, 0.402 mol), benzyloxy carbonyl chloride (32.0 ml, 0.224 mol) and pyridine (72.0 ml, 0.890 mol) were added at 5 minute intervals respectively. The reaction mixture was allowed to warm to room temperature and stirred for 90 minutes. Water (500 ml) was added, and the organic and aqueous layers were separated. The organic phase was washed with water (1 x 500 ml), 2M HCl (2 x 500 ml), water (1 x 500 ml) and dried over Na₂SO₄. The removal of the solvent in vacuo gave an oil, which, after
washing well with light petroleum (b.p. 40–60°C), was purified by dry flash chromatography on silica (employing a gradient of chloroform/ethyl acetate) to yield the **title compound** (86.8 g, 87%) as a colourless oil (which solidified on standing in a refrigerator): tlc Rf (A9) 0.19; m.p. 33–34°C (lit.247 33–35°C); [α]D 27° -11.2° (c = 1, methanol) (lit.247 [α]D 20° -13.2° (c = 10, methanol)); νmax. (CH2Cl2) 3430 (NH), 1750, 1720 (C=O), 1510, 1210 cm⁻¹; δH (200 MHz, CDCl3) 7.30 (5H, s, Z- aromatic CH's), 6.01 (1H, bd, J 8.0 Hz, NH), 5.08 (2H, s, Z- CH2), 4.39 (1H, m, Ser αCH), 3.88 (2H, m, Ser βCH2), 3.70 (3H, s, CH3), 3.25 (1H, bs, OH); m/z (FAB), 254 (MH+), 210, 150.

Nα-(β-\text{benzyloxy}carbonyl)\text{serine(O-\text{tert}butyl} \text{ methyl ester (116)

Z-Ser(tBu)-OMe

Z-Ser-OMe (115) (27.25 g, 108 mmol) was dissolved in DCM (120 ml) and cooled in a CO2/acetone bath. To this was added condensed isobutylene (approximately 120 ml), conc. H2SO4 (1.2 ml) and CuCl (0.5 g). The vessel was securely stoppered and placed in a screened area for 7 days at room temperature. The reaction mixture was again cooled in CO2/acetone, before triethylamine (7.1 ml) was added. The excess isobutylene was removed by very gentle warming and the reaction mixture concentrated in vacuo to give an oil. This material was redissolved in ethyl acetate (150 ml) and washed with a saturated aqueous solution of NaHCO3 (3 x 150 ml), water (1 x 150 ml) and brine (1 x 150 ml), and dried over Na2SO4. The removal of the solvent in vacuo gave a brown oil which was triturated under light petroleum (b.p. 60–80°C) with rapid cooling to yield an off-white solid. This was recrystallised (twice) from light petroleum (b.p. 60–80°C) with rapid cooling to give the **title compound** (31.04 g, 47%) as a white solid: tlc Rf (A9) 0.64; m.p. 40°C (lit.248 40–41°C); [α]D 27° +2.5° (c = 2, ethanol) (lit.248 [α]D 25° +6° (c = 1.996, ethanol)); Found: C, 62.1; H, 7.68; N, 4.45 Calc. for C16H23NO5 C, 62.1; H, 7.49; N, 4.53%; νmax. (CH2Cl2) 3435 (NH), 1750, 1725 (C=O), 1070 cm⁻¹; δH (200 MHz, CDCl3) 7.30 (5H, m, Z- aromatic CH's), 5.63 (1H, bd, J 8.6 Hz, NH), 5.12 (2H, s, Z- CH2), 4.45 (1H, m, Ser αCH), 3.70 (2H, m, Ser βCH2), 1.11 (9H, s, 3 x CH3); δC (50 MHz, CDCl3) 171.0 (ester C=O), 156.0 (urethane C=O), 136.2 (Z- quaternary aromatic C), 128.8, 128.4, 128.0, 127.4 (Z- aromatic CH's), 73.3 (tBu quaternary C), 66.8 (Z- CH2), 61.9 (Ser βCH2), 54.6 (Ser αCH), 52.1 (ester CH3), 27.1 (tBu CH3); m/z (FAB), 310 (MH+), 254, 210, 146.
The compound was prepared by the general method of Shields and Renner.\(^{234}\)

Z-Ser(tBu)-OMe (116) (29.84 g, 96.6 mmol) was dissolved in acetone (200 ml), and to this was added 1.0M NaOH (107 ml, 107 mmol) over a period of 30 minutes. The reaction mixture was left stirring for a further 3.5 hours. The acetone was removed \textit{in vacuo} and the aqueous phase washed with diethyl ether (1 x 100 ml) before being acidified to pH 3.0 with an aqueous solution of 5% citric acid and extracted with ethyl acetate (3 x 100 ml). The combined organic phase was washed with water (2 x 100 ml), brine (1 x 100 ml) and dried over \(\text{Na}_2\text{SO}_4\). The solvent was removed \textit{in vacuo} to yield an oil, which solidified on standing. This material was recrystallised from ethyl acetate/light petroleum (b.p. 40–60\(^\circ\)C) to afford the title compound (21.78 g, 76%) as a white solid: tlc \(R_f\) (A9) 0.15; m.p. 86–87\(^\circ\)C (lit.\(^{234}\) 85–87\(^\circ\)C); \([\alpha]_D^{27} +18.2^\circ\) (c = 1, ethanol) (lit.\(^{234}\) \([\alpha]_D^{21} +21.2^\circ\) (c = 2.82, 95% ethanol)); Found: C, 60.9; H, 7.42; N, 4.70 Calc. for \(\text{C}_{15}\text{H}_{21}\text{N}_0\text{O}_5\) C, 61.0; H, 7.17; N, 4.74%; \(\nu_{\text{max}}\) (CH\(_2\)Cl\(_2\)) 3430 (NH), 1760, 1715 (C=O), 1500, 1420 cm\(^{-1}\); \(\delta_H\) (360 MHz, CD\(_3\)COCD\(_3\)) 7.35 (5H, m, Z- aromatic CH's), 6.24 (1H, bd, J 8.2 Hz, NH), 5.10 (2H, s, Z- CH\(_2\)), 4.37 (1H, m, Ser aCH), 3.76 (2H, m, Ser BCH\(_2\)), 1.15 (9H, s, 3 x CH\(_3\)); \(m/z\) (FAB), 296 (MH\(^+\)), 240, 196, 162.

H-Ser(tBu)-OH

Z-Ser(tBu)-OH (117) (9.95 g, 33.7 mmol) was dissolved in DMF (100 ml) and to this was added 10% palladium-charcoal catalyst (1.00 g, 10% by weight of the amino acid derivative), slowly under nitrogen. The reaction mixture was hydrogenated for 22 hours at room temperature and pressure. Filtration through 'celite', washing with DMF and water, followed by concentration of the aqueous washings \textit{in vacuo} gave the title compound (4.45 g, 82%) as an off-white solid: tlc \(R_f\) (A6) 0.32; m.p. > 220\(^\circ\)C (lit.\(^{248}\) 203–204\(^\circ\)C), \([\alpha]_D^{27} -15.2^\circ\) (c = 0.91, water) (lit.\(^{248}\) \([\alpha]_D^{25} -13.2^\circ\) (c = 0.91, water)); \(\nu_{\text{max}}\) (bromoform mull) 1625 (C=O), 1345, 1200, 900 cm\(^{-1}\); \(\delta_H\) (200 MHz, D\(_2\)O) 3.81 (3H, m, Ser aCH, BCH\(_2\)), 1.20 (9H, s, 3 x CH\(_3\)); \(\delta_C\) (50 MHz, D\(_2\)O) 172.2 (C=O), 74.9 (tBu quaternary C), 60.2 (Ser BCH\(_2\)), 55.2 (Ser aCH), 26.4 (CH\(_3\)):
m/z (FAB), 162 (MH⁺), 146; hrms, found 162.11300, C₇H₁₆NO₃ requires 162.11301 (< 1 ppm).

Nα-[2,2-bis (4'-Nitrophenyl)ethoxycarbonyl]-serine(O-tertbutyl) (119)

Bnpeoc-Ser(tBu)-OH

To a stirred suspension of H-Ser(tBu)-OH (118) (3.270 g, 20.3 mmol) in water (30 ml) was added a solution of triethylamine (4.21 ml, 30.5 mmol) in dioxan (20 ml). Bnpeoc-ONSu (21) (8.710 g, 20.3 mmol) was added to the resultant solution and the reaction mixture was stirred at room temperature for 24 hours. Water (250 ml) was added and the pH adjusted to 2.0 by the addition of an aqueous solution of KHSO₄. The aqueous solution was extracted with ethyl acetate (3 x 150 ml), and the combined organic phases were dried over Na₂SO₄ and concentrated in vacuo to yield an oily residue. This residue was redissolved in diethyl ether (100 ml) and cyclohexylamine (2.56 ml, 22.4 mmol) was added. After stirring for 90 minutes, the formed precipitate was filtered, washed with diethyl ether and suspended in a mixture of ethyl acetate and 20% citric acid (200 ml). The mixture was stirred for 45 minutes, before separating the organic layer and drying it over Na₂SO₄. The removal of the solvent in vacuo co-evaporating (twice) with diethyl ether, afforded the title compound (9.002 g, 93%) as a yellow foam: tlc Rf (A6) 0.77, Rf (A10) 0.70; [α]D²⁷ +13.7° (c = 1, methanol); νmax. (CH₂Cl₂) 3430 (NH), 1770, 1730 (C=O), 1605, 1595, 1525, 1350 (NO₂), 860 cm⁻¹; λmax 274 nm (ε = 19811); δH (200 MHz, CD₃COCD₃) 8.25 (4H, d, J₈.₉ Hz, Bnpeoc aromatic CH's), 7.73 (4H, d, J₈.₉ Hz, Bnpeoc aromatic CH's), 4.80 (3H, m, Bnpeoc CH₂), 4.32 (1H, m, Ser CH), 3.71 (2H, m, Ser CH₂), 1.10 (9H, s, 3 x CH₃); δC (50 MHz, CD₃COCD₃) 170.1 (acid C=O), 154.8 (urethane C=O), 147.2, 146.3 (Bnpeoc quaternary aromatic C's), 128.8, 122.8 (Bnpeoc aromatic CH's), 72.0 (tBu quaternary C), 64.8 (Bnpeoc CH₂), 60.8 (Ser CH), 53.6 (Ser CH), 48.8 (Bnpeoc CH), 25.8 (CH₃); m/z (FAB), 476 (MH⁺), 420, 254, 225; hplc Rf (B2) 17.8 minutes.

Nα-(Benzyloxycarbonyl)threonine benzyl ester (121)

Z-Thr-OBzl

Z-Thr-OH (120) (95.59 g, 0.378 mol) was dissolved in DMF (155 ml) and the stirred
solution cooled in an ice/salt bath. To this was added triethylamine (52.7 ml, 0.378 mol) and benzyl bromide (49.5 ml, 0.416 mol) over 30 minutes. The reaction mixture was allowed to warm to room temperature and stirred for 24 hours. Water (1250 ml) was added and the aqueous solution extracted with ethyl acetate (3 x 200 ml). The combined organic phases were washed with water (1 x 200 ml), brine (1 x 200 ml) and dried over Na$_2$SO$_4$. The removal of the solvent \textit{in vacuo} afforded an orange oil, which solidified on standing. This material was filtered, washed well with light petroleum (b.p. 40–60°C) and redissolved in ethyl acetate (400 ml), before being washed with a saturated aqueous solution of NaHCO$_3$ (4 x 200 ml) and dried over Na$_2$SO$_4$. The reaction mixture was concentrated \textit{in vacuo} to yield an oily residue which was recrystallised from acetone/light petroleum (b.p. 40–60°C) to afford the \textit{title compound} (71.23 g, 55%) as a white solid: \textit{t}lc $R_f$ (A3) 0.54; m.p. 78–80°C (lit.$^{249}$ 80–80.5°C); [\(\alpha\)]$_D^{27}$ -22.1° (c = 1, chloroform) (lit.$^{249}$ -13.8° (c = 2.5, chloroform)); Found: C, 66.2; H, 6.29; N, 4.12 Calc. for C$_{19}$H$_{21}$N0$_5$ C, 66.5; H, 6.16; N, 4.08%; \(\nu_{max}$ (CH$_2$Cl$_2$) 3440 (NH), 1725 (C=O), 1510, 1215 cm$^{-1}$; \(\delta_{H}$ (200 MHz, CDCl$_3$) 7.33 (10H, s. Z- and benzyl aromatic CH's), 5.79 (1H, d, J 9.2 Hz, NH), 5.18 (2H, s, benzyl CH$_2$), 5.11 (2H, s, Z- CH$_2$), 4.34 (2H, m, Thr /BCH), 2.45 (1H, bs, OH), 1.21 (3H, d, J 6.4 Hz, CH$_3$); \(\delta_{C}$ (50 MHz, CDCl$_3$) 170.9 (ester C=O), 156.6 (urethane C=O), 136.0, 135.1 (quaternary aromatic C's), 128.5, 128.4, 128.0, 127.9 (aromatic CH's), 67.8 (Thr BCH), 67.2, 67.0 (Z- and benzyl CH$_2$), 59.2 (Thr aCH), 19.7 (CH$_3$); \(m/z$ (FAB), 344 (MH$^+$), 340, 210, 164.

\(\text{N}^{\alpha-}(\text{Benzylxycarbonyl})\text{threonine(O-} t\text{ertbutyl) benzyl ester (122)}\)

\(\text{Z-Thr}^{(t} \text{Bu})-\text{OBzl}\)

\(\text{Z-Thr-OBzl (121) (35.045 g, 0.102 mol) was dissolved in DCM (120 ml) and cooled in a CO}_2/\text{acetone bath. To this was added condensed isobutylene (approximately 120 ml), conc. H}_2\text{SO}_4 (1.2 ml) and CuCl (0.5 g). The vessel was securely stoppered and placed in a screened area for 7 days at room temperature. The reaction mixture was again cooled in CO}_2/\text{acetone, before triethylamine (7.1 ml) was added and the excess isobutylene removed by allowing the bath to warm to room temperature overnight. The removal of the solvent \textit{in vacuo} gave a green oil which, on redissolution in ethyl acetate (150 ml), was washed with water (1 x 150 ml), a saturated aqueous solution of NaHCO$_3$ (3 x 150 ml), water (1 x 150 ml) and brine (1 x 150 ml). After drying over Na$_2$SO$_4$, the solvent was removed \textit{in vacuo} to give a green oil which was purified by flash chromatography on silica (eluting with}
chloroform) to afford the title compound (65.563 g, 76%) as a white solid: tlc R_f (A3) 0.86; m.p. 53-55°C; ν_max (CH_2Cl_2) 3440 (NH), 1720 (C=O), 1500, 1200, 1070 cm⁻¹; δ_H (60 MHz, CDCl_3) 7.40 (10H, m, Z- and benzyl aromatic CH's), 5.20 (4H, s, Z- and benzyl aromatic CH_2), 4.35 (2H, m, Thr α/βCH), 1.25 (3H, d, Thr CH_3), 1.10 (9H, s, 3 x 'Bu CH_3); m/z (FAB), 400 (MH⁺), 344, 300, 210.

(O-tertButyl)threonine (123)

The compound was prepared by an adaptation of the method of Wunsch and Jentsch²³⁵.

Z-Thr('Bu)-OBzl (122) (4.627 g, 11.6 mmol) was dissolved in DMF (50 ml) and to this was added 10% palladium-charcoal catalyst (0.50 g, 10% by weight of the amino acid derivative), slowly under nitrogen. The reaction mixture was hydrogenated for 22 hours at room temperature and pressure. Filtration through 'celite', washing with DMF and water, followed by the concentration of the aqueous washings _in vacuo_ afforded the title compound (0.791 g, 39%) as a white solid: tlc R_f (A3) 0.39; m.p. >220°C (lit.²³⁵ 259-260°C); [α]_D¹⁰ -40.3 (c = 2, methanol) (lit.²³⁵ [α]_D¹⁰ -42.1 (c = 2, methanol)); ν_max (bromoform mull) 1650 (C=O), 1390, 1240, 1040 cm⁻¹; δ_H (360 MHz, D_2O) 4.30 (1H, m, Thr βCH), 3.57 (1H, d, J 3.4 Hz, Thr αCH), 1.33 (3H, d, J 6.6 Hz, Thr CH_3), 1.22 (9H, s, 3 x 'Bu CH_3); δ_C (50 MHz, D_2O) 173.0 (C=O), 75.6 ('Bu quaternary C), 66.1, 60.3 (Thr α/βCH), 27.5 ('Bu CH_3), 20.8 (Thr CH_3); m/z (FAB), 176 (MH⁺), 147, 132.

N⁵-[2,2-bis(4'-Nitrophenylethoxycarbonyl)-threonine(O-tertbutyl) (124)

Bnpeoc-Thr('Bu)-OH

To a stirred suspension of H-Thr('Bu)-OH (123) (0.729 g, 4.17 mmol) in water (6 ml) was added a solution of triethylamine (0.87 ml, 6.26 mmol) in dioxan (6 ml). Bnpeoc-ONSu (21) (1.789 g, 4.17 mmol) was added to the resultant solution and the reaction mixture stirred at room temperature for 24 hours. Water (150 ml) was added and the pH adjusted to 1.5 by the addition of a saturated aqueous solution of KHSO_4. The aqueous solution was extracted with ethyl acetate (3 x 100 ml), and
the combined organic phases were dried over Na$_2$SO$_4$ and the solvent was removed in vacuo to yield a yellow oil. This material was redissolved in ethyl acetate/diethyl ether (1:1) (50 ml) and cyclohexylamine (0.53 ml, 4.59 mmol) was added. After stirring for 4 hours, the resulting precipitate was filtered, washed with diethyl ether and suspended in a mixture of ethyl acetate and 20% citric acid (aq) (1:1) (100 ml). The reaction mixture was stirred for 90 minutes, before separating the organic layer and drying it over Na$_2$SO$_4$. The removal of the solvent in vacuo gave a yellow oil which was purified by dry flash chromatography on silica (eluting with chloroform) to yield the title compound (1.583 g, 78%) as a yellow foam: tlc R$_f$ (A4) 0.48; [α]$_D^{27}$ +9.7° (c = 1, MeOH); $\nu$$_{max}$ (CH$_2$Cl$_2$) 3420 (NH), 1770, 1730 (C=O), 1525, 1350 (NO$_2$); 860 cm$^{-1}$; δ$_H$ (200 MHz, CD$_3$COCD$_3$) 8.22 (4H, d, J$_{AB}$ 8.8 Hz, Bnpeoc aromatic CH's), 7.71 (4H, d, J$_{AB}$ 8.8 Hz, Bnpeoc aromatic CH's), 4.80 (3H, m, Bnpeoc CH,CH$_2$), 4.22, 4.12 (2H, m, Thr α/β), 1.12 (12H, m, Thr CH$_3$ 3 x tBu CH$_3$); δ$_C$ (50 MHz, CD$_3$COCD$_3$) 170.5 (acid C=O), 155.2 (urethane C=O), 147.3, 147.2, 146.3 (Bnpeoc quaternary aromatic C's), 128.8, 122.8 (Bnpeoc aromatic CH's), 72.8 (tBu quaternary C), 64.8 (Bnpeoc CH$_2$), 66.3, 58.6 (Thr α/BCH), 48.9 (Bnpeoc CH), 26.9 (tBu CH$_3$), 19.1 (Thr CH$_3$); hplc R$_t$ (B2) 21.0 minutes.

Ethyl diphenylphosphinate (128)

Ph$_2$P(O)OEt

The compound was prepared by the method of Kosolapoff and Watson$^{238}$.

Diphenylphosphinyl chloride (45) (54.30 g, 0.231 mol) was dissolved in benzene (400 ml), and the resulting solution cooled to 0°C. A solution of sodium (5.31 g, 0.231 mol) in ethanol (dried from magnesium and iodine) (100 ml) was added over 30 minutes, and the reaction mixture allowed to warm to room temperature and stirred for 24 hours. Filtration through 'celite', followed by the removal of the solvent in vacuo afforded a clear oil, which on distillation, gave the title compound (42.84 g, 75%) as a white solid: m.p. 37-38°C; b.p. 135-136°C at 0.03-0.04 mmHg (lit.$^{238}$ 140-145°C at 0.05 mmHg); Found: C, 68.4; H, 6.33 Calc: for C$_{14}$H$_{15}$O$_2$P C, 68.3; H, 6.10%; $\nu$$_{max}$ (CH$_2$Cl$_2$) 1445, 1230 (P=O), 1135, 1040, 960 cm$^{-1}$; δ$_H$ (200 MHz, CDCl$_3$) 7.76 (4H, m, aromatic CH's), 7.39 (6H, m, aromatic CH's), 4.03 (2H, m, CH$_2$), 1.29 (3H, t, J 7.1 Hz, CH$_3$); δ$_C$ (50 MHz, CDCl$_3$) 132.9 (aromatic quaternary C), 131.9, 131.5, 131.3 (aromatic CH's), 130.2 (quaternary aromatic C), 128.5, 128.2 (aromatic CH's), 60.9 (CH$_2$), 16.3 (CH$_3$); δ$_P$ (36 MHz, CDCl$_3$) 31.50; m/z (FAB) 246 (M$^+$), 218,
Diphenylphosphinyl chloride (45) (36.52 g, 0.155 mol) and ethyl diphenylphosphinate (128) (38.80 g, 0.158 mol) were heated at 200°C for 2 hours under argon. The reaction mixture was allowed to cool to 55°C, toluene (20 ml) was added, and the resulting solution cooled in an ice/salt bath. Vigorous scratching encouraged the rapid formation of a white precipitate. After storing in a refrigerator for 30 minutes, the precipitate was filtered, washed with a little toluene and dried to afford the title compound (37.95 g, 59%) as a white solid: m.p. 140-142°C (lit. 143.5-144.5°C); Found: C, 68.8; H, 4.81 Calc. for C_{24}H_{20}O_{3}P_{2} C, 68.9; H, 4.80%; ν_{max} (CH_{2}Cl_{2}) 1440, 1235 (P=O), 1130, 940 (P-O-P) cm^{-1}; δ_{H} (200 MHz, CDCl_{3}) 7.85-7.65 (8H, m, aromatic CH's), 7.53-7.25 (12H, m, aromatic CH's); δ_{C} (50 MHz, CDCl_{3}) 134.0-127.9 (aromatic CH's, quaternary aromatic C's); δ_{P} (36 MHz, CH_{2}Cl_{2}) 27.64; δ_{P} (36 MHz, CDCl_{3}) 28.60; m/z (FAB), 418 (M^+), 341, 277, 218, 202, 152; hrms, found 419.09657, C_{24}H_{21}O_{3}P_{2} requires 419.09659 (< 1 ppm).

Method B

The compound was prepared as method A, without the distillation of the ethyl diphenylphosphinate (128) intermediate.

Diphenylphosphinyl chloride (45) (13.45 g, 56.9 mmol) was dissolved in benzene (100 ml), and the resulting solution cooled to 0°C. A solution of sodium (1.31 g, 56.9 mmol) in ethanol (27 ml) was added over 90 minutes, and the reaction mixture allowed to warm to room temperature and stirred for 24 hours. Filtration through 'celite', followed by the removal of the solvent in vacuo afforded ethyl diphenylphosphonate (128) (11.89 g, 85%) (confirmed by 31P n.m.r. - δ_{P} (36 MHz, CDCl_{3}) 31.57).

To (128) (11.33 g, 46.1 mmol) was added diphenylphosphinyl chloride (45) (10.90
g, 46.1 mmol), and the resulting mixture was heated to 200°C for 2 hours under argon. The reaction mixture was allowed to cool to 55°C, toluene (20 ml) was added, and the resulting solution cooled in an ice/salt bath. Vigorous scratching encouraged the formation of a white precipitate, which was filtered and washed with a little cold toluene to afford the title compound (13.31 g, 70%) as a white solid: m.p. 138-140°C; \( \delta_H \) (60 MHz, \( \text{CDCl}_3 \)) 7.90-7.10 (m, aromatic CH's); \( \delta_P \) (36 MHz, \( \text{CD}_2\text{Cl}_2 \)) 27.37.

Method C

The compound was prepared by the general method of Moedritzer\textsuperscript{240}

Dry paraformaldehyde (based on \( \text{CH}_2\text{O} \) - 3.164 g, 0.105 mol) was added to diphenylphosphinyl chloride (45) (24.95 g, 0.105 mol), and the resulting slurry heated under argon at 150°C for 50 minutes (i.e. until the solution had become clear). After the reaction mixture had been allowed to cool to room temperature, the flask was placed under vacuum for 10 minutes. The resulting oil was dissolved in toluene (20 ml) and cooled in an ice/salt bath. Vigorous scratching resulted in the formation of a white precipitate. After storing in a refrigerator for 60 minutes, the precipitate was filtered off, washed with a little cold toluene and dried to afford the title compound (9.95 g, 45%) as a white solid: m.p. 139-142°C; \( \nu_{\text{max}} \) (\( \text{CH}_2\text{Cl}_2 \)) 1440, 1235 (P=O), 1135, 940 (P-O-P) cm\(^{-1}\); \( \delta_H \) (80 MHz, \( \text{CDCl}_3 \)) 7.92-7.52 (8H, m, aromatic CH's), 7.49-7.16 (12H, m, aromatic CH’s); \( \delta_P \) (81 MHz, \( \text{CD}_2\text{Cl}_2 \)) 27.89.

\textit{N}^\text{\(\alpha\)}-(Benzyloxy carbonyl)phenylalanlylglycine benzyl ester (109)

\textbf{Z-Phe-Gly-OBzl}

(A) \textit{Z-Phe-OH} (108) (0.501 g, 1.67 mmol) was dissolved in DCM (5 ml) and cooled to -5°C. To this was added N-methylmorpholine (0.185 ml, 1.67 mmol) and diphenylphosphinic anhydride (127) (0.716 g, 1.67 mmol), and the reaction mixture stirred at -5°C for 10 minutes. \( \text{TosO}^-\text{H}_2^-\text{Gly-OBzl} \) (107) (0.563 g, 1.67 mmol) in DMA/DCM (1:5) (6 ml) and N-methylmorpholine (0.370 ml, 3.34 mmol) were added, and the reaction mixture allowed to warm to room temperature and stirred for 2.5 hours. Water (250 ml) and ethyl acetate (250 ml) were added, and the organic and aqueous layers were separated. The organic phase was washed with a saturated
aqueous solution of NaHCO$_3$ (2 x 100 ml), water (1 x 100 ml), an aqueous solution of 5% citric acid (2 x 100 ml), water (1 x 100 ml) and brine (1 x 100 ml), and dried over Na$_2$SO$_4$. The removal of the solvent in vacuo afforded a white solid, which was recrystallised from ethyl acetate/light petroleum (b.p. 40–60°C) to give the title compound (0.450 g, 60%) as a white solid: tlc $R_f$ (A4) 0.76; m.p. 133–134°C; [$\alpha$]$^D_{27}$ -1.94° ($c = 1$, acetone) (lit.$^{247}$ -3.75° ($c = 1$, acetone)); $\nu_{\text{max}}$ (CH$_2$Cl$_2$) 3425 (NH), 1750, 1725, 1685 (C=O), 1500, 1200 cm$^{-1}$; $\delta_H$ (200 MHz, CD$_3$COCD$_3$) 7.30 (15H, m, aromatic CH's), 6.70 (1H, bd, NH), 5.18 (2H, s, benzyl CH$_2$), 5.00 (2H, m, Z-CH$_2$), 4.55 (1H, m, Phe $\alpha$CH), 4.06 (2H, d, J 5.9 Hz, Gly CH$_2$), 3.10 (2H, m, Phe $\beta$CH$_2$); $m/z$ (FAB), 447 (MH$^+$), 403, 372, 313, 225; hplc $R_f$ (B2) 18.5 minutes.

(B) Z-Phe-OH (108) (1.003 g, 3.34 mmol) was dissolved in DCM (10 ml) and cooled to -5°C. To this was added N-methylmorpholine (0.370 ml, 3.34 mmol) and diphenylphosphinic anhydride (127) (1.433 g, 3.34 mmol), and the reaction mixture stirred at -5°C for 10 minutes. TosOH$_2^+$-Gly-OBzl (107) (0.564 g, 1.67 mmol) in DMA/DCM (1:5) (6 ml) and N-methylmorpholine (0.370 ml, 3.34 mmol) were added, and the reaction mixture allowed to warm to room temperature and stirred for 2.5 hours. The reaction mixture was worked up as above to afford the title compound (0.564 g, 76%) as a white solid. Characterisation was in agreement with above.

Alanine benzyl ester p-toluenesulphonate (131)

TosOH$_2^+$-Ala-OBzl

The compound was prepared by the general method of Zervas et al.$^{230}$ as outlined above for the preparation of TosOH$_2^+$-Gly-OBzl (107).

The material was purified by recrystallisation from benzene/diethyl ether (yield = 91%): m.p. 106–108°C; [$\alpha$]$^D_{27}$ -3.8° ($c = 1$, MeOH); Found: C, 58.2; H, 6.07; N, 4.11 Calc. for C$_{17}$H$_{21}$NOS C, 58.1; H, 6.02; N, 3.99%; $\nu_{\text{max}}$ (bromoform mull) 1750 (C=O), 1600, 1520 cm$^{-1}$; $\delta_H$ (200 MHz, d$_6$-DMSO) 8.40 (3H, bs, NH$_3^+$), 7.55 (2H, d, J$_{AB}$ 7.9 Hz, tosyl aromatic CH's), 7.39 (5H, m, benzyl aromatic CH's), 7.14 (2H, d, J$_{AB}$ 7.9 Hz, tosyl aromatic CH's), 5.23 (2H, s, benzyl CH$_2$), 4.18 (1H, q, Ala $\alpha$CH), 2.29 (3H, s, tosyl CH$_3$), 1.43 (3H, d, J 7.2 Hz, Ala CH$_3$); $m/z$ (FAB), 180 (H$_3$N$^+$-CH(CH$_3$)CO$_2$CH$_2$Ph).
Nα-(Benzyloxycarbonyl)phenylalanylalanine benzyl ester (132)

Z-Phe-Ala-OBzl

Z-Phe-OH (108) (1.000 g, 3.34 mmol) was dissolved in DCM (10 ml) and cooled to -5°C. To this was added N-methylmorpholine (0.370 ml, 3.34 mmol) and diphenylphosphinic anhydride (127) (1.432 g, 3.34 mmol), and the reaction mixture was stirred for 10 minutes at -5°C. TosO-H2-Ala-OBzl (131) (0.587 g, 1.67 mmol) in DMA/DCM (1:5) (6 ml) and N-methylmorpholine (0.560 ml, 4.95 mmol) were added, and the reaction mixture was allowed to warm to room temperature and stirred for 2.5 hours. The reaction mixture was worked up as above for Z-Phe-Gly-OBzl (109), to give a white solid, which, on recrystallisation from acetone/light petroleum (b.p. 40-60°C), afforded the title compound (0.540 g, 70%) as a white solid; tlc Rf (A4) 0.67; m.p. 122-124°C; [α]D27 -20.9° (c = 1, acetone); Found: C, 70.4; H, 6.24; N, 6.03 Calc. for C27H29N2O5 C, 70.4; H, 6.13; N, 6.08%; νmax. (CH2Cl2) 3420 (NH), 1740, 1680 (C=O), 1500, 1200, 1135 cm⁻¹; δH (200 MHz, CD3COCD3) 7.78 (1H, m, amide NH), 7.33 (15H, m, aromatic CH's), 6.54 (1H, bd, urethane NH), 5.18, 5.00 (benzyl CH2, Z- CH2), 4.62 (2H, m, Phe CH, Ala CH), 3.10 (2H, m, Phe CH2), 1.39 (3H, d, J 7.3 Hz, Ala CH3); δC (50 MHz, CD3COCD3) 171.3, 170.2 (amide C=O, ester C=O), 155.0 (urethane C=O), 136.9, 136.4, 135.4 (quaternary aromatic C's), 130.4-125.5 (aromatic CH's), 65.4, 64.9 (benzyl CH2, Z- CH2), 55.1 (Phe CH), 47.1 (Ala CH), 37.1 (Phe CH2), 16.0 (Ala CH3); m/z (FAB) 461 (MH+), 242, 219, 91; hrrms. found 461.20762, C27H29N2O5 requires 461.20763 (< 1 ppm); hplc Rf (B2) 19.0 minutes.

31P n.m.r. monitored stability studies of diphenylphosphinic anhydride (127)

(1) Stability of diphenylphosphinic anhydride (127) to N-methylmorpholine

Diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol) was dissolved in CD2Cl2/DMA (2:3) (0.5 ml) at room temperature, and to this was added N-methylmorpholine (0.027 ml, 0.25 mmol). The resulting solution was monitored by 31P n.m.r. (36 MHz), acquiring spectra at given time intervals. No significant loss of the starting anhydride signal (27.09 ppm) was observed over 60 minutes.
(2) Stability of diphenylphosphinic anhydride (127) to TosO⁻H₂⁺-Gly-OBzl (107)

Diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol) was dissolved in CD₂Cl₂/DMA (2:3) (0.5 ml) at room temperature, and to this was added TosO⁻H₂⁺-Gly-OBzl (107) (0.028 g, 0.084 mmol) and N-methylmorpholine (0.020 ml, 0.168 mmol). The resulting solution was monitored by ³¹P n.m.r. (36 MHz), acquiring a spectrum immediately on the addition of the amine component and the base, and subsequently at 15 minute intervals for 60 minutes. After 15 minutes a small phosphinate anion signal (16.02 ppm) was shown to be present, which increased in size at a steady but slow rate.

(3) Stability of diphenylphosphinic anhydride (127) to methanol

Diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol) was dissolved in CD₂Cl₂/MeOH (2:3) (0.5 ml) at room temperature. The solution was monitored by ³¹P n.m.r. (36 MHz), acquiring spectra at 10 minute intervals for 30 minutes. No change in the starting anhydride signal (31.10 ppm) was observed over the 30 minutes.

To the above solution was subsequently added N-methylmorpholine (0.020 ml, 0.167 mmol) and spectra again recorded at 10 minute intervals for 30 minutes. After the 30 minutes, the starting anhydride signal had completely diminished to afford 2 other signals, one corresponding to the phosphinyl ester (35.27 ppm), and the other to the phosphate anion (20.87 ppm).

(4) Stability of diphenylphosphinic anhydride (127) to benzyl alcohol

Diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol) was dissolved in CD₂Cl₂/benzyl alcohol (2:3) (0.5 ml) at room temperature. The solution was monitored by ³¹P n.m.r. (36 MHz), acquiring spectra at 10 minute intervals for 30 minutes. No change in the starting anhydride signal (30.89 ppm) was observed over the 30 minutes.

To the above solution was added N-methylmorpholine (0.020 ml, 0.167 mmol) and spectra again acquired at 10 minute intervals for 30 minutes. Over the 30 minutes the starting anhydride signal (30.22 ppm) was reduced slightly and a small phosphinate anion signal (21.13 ppm) appeared.
$^{31}$P n.m.r. monitored couplings with diphenylphosphinic anhydride (127)

(1) Coupling of Z-Phe-OH (108) and TosO$^{-}$H$_2$*-Gly-OBzl (107)

(A) Z-Phe-OH (108) (0.050g, 0.167 mmol) was dissolved in CD$_2$Cl$_2$/DMA (2:3) (0.5 ml) at room temperature, and to this was added N-methylmorpholine (0.019 ml, 0.167 mmol) and diphenylphosphinic anhydride (127) (0.071 g, 0.167 mmol). The solution was monitored by $^{31}$P n.m.r. (36 MHz), acquiring spectra at given time intervals for 15 minutes. After the 15 minutes, the activation was shown to be complete.

To the above solution were added TosO$^{-}$H$_2$*-Gly-OBzl (107) (0.056 g, 0.167 mmol) and N-methylmorpholine (0.038 ml, 0.334 mmol), and again spectra were acquired at given time intervals. The coupling was shown to be complete after 30 minutes (i.e only the phosphinate anion signal (16.99 ppm) was present after this time).

(B) The above coupling of Z-Phe-OH (108) and TosO$^{-}$H$_2$*-Gly-OBzl (107) was repeated as above, except that d$_7$-DMF (0.5 ml) was used as the solvent instead of a mixture of CD$_2$Cl$_2$/DMA. The monitoring of the reaction solution by $^{31}$P n.m.r. (36 MHz) revealed that the activation was complete within 5 minutes, and the coupling reaction within 10.

(2) Coupling of Fmoc-Arg(Pmc)-OH (133) and TosO$^{-}$H$_2$*-Gly-OBzl (107)

Fmoc-Arg(Pmc)-OH (133) (0.055 g, 0.084 mmol) was dissolved in CD$_2$Cl$_2$/DMA (1:4) (0.5 ml) at room temperature, and to this was added N-methylmorpholine (0.010ml, 0.084 mmol) and diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol). The solution was monitored by $^{31}$P n.m.r. (36 MHz). After 5 minutes a position appeared to have been reached whereby the starting anhydride (127) (27.25 ppm), the activated mixed anhydride (26.77 ppm) and the phosphinate anion (16.37 ppm) were all present.

To this solution were added TosO$^{-}$H$_2$*-Gly-OBzl (107) (0.028 g, 0.084 mmol) and N-methylmorpholine (0.019 ml, 0.168 mmol). The reaction solution was again monitored by $^{31}$P n.m.r., acquiring spectra at given time intervals for 20 minutes. The coupling was shown to be complete within 20 minutes.
(3) Coupling of Bnpeoc-Ser-OH (88) and TosO⁻H₂⁺-Gly-OBzl (107)

Bnpeoc-Ser-OH (88) (0.035 g, 0.084 mmol) was dissolved in CD₂Cl₂/DMA (1:4) (0.5 ml) at room temperature, and to this was added N-methylmorpholine (0.010 ml, 0.084 mmol) and diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol). The solution was monitored by ³¹P n.m.r. (36 MHz), acquiring spectra at given time intervals for 20 minutes. After 5 minutes a position appeared to have been reached whereby the starting anhydride (127) (27.39 ppm), the activated mixed anhydride (26.96 ppm) and the phosphinate anion (16.56 ppm) were all present. The ratio of the peak heights of these signals remained largely unchanged over the 20 minute period.

TosO⁻H₂⁺-Gly-OBzl (107) (0.028 g, 0.084 mmol) and N-methylmorpholine (0.019 ml, 0.168 mmol) were added to the above solution, and again spectra were collected for 20 minutes. The coupling was shown to be complete within the 20 minutes.

(4) Attempted coupling of Fmoc-Asn-OH (134) and TosO⁻H₂⁺-Gly-OBzl (107)

Fmoc-Asn-OH (134) (0.030 g, 0.084 mmol) was dissolved in CD₂Cl₂/DMA (1:4) (0.5 ml) at room temperature, and to this was added N-methylmorpholine (0.010 ml, 0.084 mmol) and diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol). The monitoring of the reaction solution by ³¹P n.m.r. (36 MHz) revealed the complete decay of the starting anhydride signal (27.06 ppm) within 20 minutes, to afford only a phosphinate anion signal (17.30 ppm). The characteristic nitrile peak (2260 cm⁻¹) was shown to present in an IR spectrum of the reaction mixture.

(5) Coupling of Fmoc-Gln(Mbh)-OH (135) and TosO⁻H₂⁺-Gly-OBzl (107)

Fmoc-Gln(Mbh)-OH (135) (0.050 g, 0.084 mmol) was dissolved in CD₂Cl₂ (1:4) (0.5 ml) at room temperature, and to this was added N-methylmorpholine (0.010 ml, 0.084 mmol) and diphenylphosphinic anhydride (127) (0.035 g, 0.084 mmol). The reaction solution was monitored by ³¹P n.m.r. After 15 minutes, 2 signals were shown to be present, one corresponding to the activated mixed anhydride (27.80 ppm), the other to the phosphinic anion (16.56 ppm). The ratio of the peak heights of these 2 signals changed little over a 60 minute period.

After the 60 minutes, TosO⁻H₂⁺-Gly-OBzl (107) (0.028 g, 0.084 mmol) and
N-methylmorpholine (0.019 ml, 0.168 mmol) were added. $^{31}$P n.m.r. revealed that the coupling was complete within 15 minutes.

$^{31}$P n.m.r. monitored stability of (benzyloxy carbonyl)phenylalanyl phosphinic mixed anhydride (129)

Z-Phe-OH (108) (0.050 g, 0.167 mmol) was dissolved in CD$_2$Cl$_2$/DMA (1:4) (0.5 ml) at room temperature, and to this was added N-methylmorpholine (0.019 ml, 0.168 mmol) and diphenyl phosphinic anhydride (127) (0.071 g, 0.167 mmol). The solution was monitored by $^{31}$P n.m.r. (81 MHz), acquiring spectra at given time intervals: for 4 hours. After 10 minutes, a position had been reached with the mixed anhydride (129) (27.91 ppm), the starting anhydride (127) (27.81 ppm) and the phosphinate anion (16.64 ppm) all present. The ratio of the peak heights of these signals remained unchanged over the 4 hours.

$\text{N}^-\text{[2-O/s (4'-Nit benzyl ester (1 36) Bnpeoc-Ser-Gly-OBzl}$

Bnpeoc-Ser-Gly-OBzl

Bnpeoc-Ser-OH (88) (1.399 g, 3.34 mmol) was dissolved in DMA (5 ml) and cooled to 0°C. To this was added N-methylmorpholine (0.370 ml, 3.34 mmol) and diphenyl phosphinic anhydride (127) (1.433 g, 3.34 mmol) and the reaction mixture was stirred for 10 minutes. TosO$^-\text{H}$$_2$$^+\text{Gly-OBzl}$ (107) (0.564 g, 1.67 mmol) in DMA/DCM (1:5) (8 ml) and N-methylmorpholine (0.370 ml, 3.34 mmol) were added, and the reaction mixture was allowed to warm to room temperature and stirred for 2.5 hours. Water (200 ml) and ethyl acetate (200 ml) were added, and the organic and aqueous layers were separated. The organic phase was washed with water (1 x 100 ml), an aqueous solution of 5% citric acid (2 x 100 ml), water (1 x 100 ml), a saturated aqueous solution of NaHCO$_3$ (2 x 100 ml) and water (1 x 100 ml), and dried over Na$_2$SO$_4$. The removal of the solvent in vacuo afforded an off-white solid, which was purified by flash chromatography on silica (employing a gradient of diethyl ether/chloroform) to yield the title compound (0.580 g, 61%) as a yellow oil: tlc $R_f$ (A4) 0.70; ν$_{\text{max}}$ (CH$_2$Cl$_2$) 3430 (NH), 1735, 1685 (C=O), 1605, 1595, 1525, 1350 (NO$_2$), 1210 cm$^{-1}$; δ$_{\text{H}}$ (200 MHz, CD$_3$COCD$_3$) 8.21 (4H, d, J$_{AB}$ 8.9 Hz, Bnpeoc aromatic CH's), 7.68 (4H, d, J$_{AB}$ 8.9 Hz, Bnpeoc aromatic CH's), 6.50 (1H, bd, NH), 5.17 (2H, s, benzyl CH$_2$), 4.77 (3H, m, Bnpeoc CH,CH$_2$), 4.23 (1H, m, Ser αCH), 4.05 (2H, d, J 5.7 Hz, Gly CH$_2$), 3.78 (2H, m, Ser βCH$_2$); δ$_{\text{C}}$ (50 MHz, CD$_3$COCD$_3$) 169.9,
N\textsuperscript{\textalpha}-(9-Fluorenlymethoxycarbonyl)glutamyl[N-\textalpha i s. (4'-methoxy-phenyl)methyl]-glycine benzyl ester (137)

Fmoc-Gln(Mbh)-Gly-OBzl

Fmoc-Gln(Mbh)-OH (135) (0.300 g, 0.51 mmol) was dissolved in DMF (15 ml) and cooled to 0°C. To this was added N-methylmorpholine (0.057 ml, 0.51 mmol) and diphenylphosphinic anhydride (127) (0.211 g, 0.51 mmol), and the reaction mixture stirred for 10 minutes. TosO\textsuperscript{-}H\textsubscript{2}+\textsuperscript{-}Gly-OBzl (107) (0.168 g, 0.51 mmol) in DMF (5 ml) and N-methylmorpholine (0.114 ml, 1.01 mmol) were added, and the reaction mixture allowed to warm to room temperature and stirred for 2.5 hours. Ethyl acetate (150 ml) was added, and the resulting solution was washed with water (2 x 100 ml), a saturated aqueous solution of NaHCO\textsubscript{3} (4 x 100 ml), water (1 x 100 ml), an aqueous solution of 10% citric acid (2 x 100 ml) and brine (1 x 100 ml). After drying the organic phase over Na\textsubscript{2}SO\textsubscript{4}, the solvent was removed \textit{in vacuo} to yield a white solid. This material was recrystallised from ethylacetate/light petroleum (b.p. 40-60°C) to afford the \textit{title compound} (0.290 g, 77%) as a white solid: tlc R\textsubscript{f} (A7) 0.70; m.p. 194-196°C; [\alpha]\textsubscript{D} \textsuperscript{27} -3.9° (c = 1, DMF); \nu_{\text{max}} (bromofrom mull) 1730, 1690, 1640 (C=O), 1540, 1510, 1255 cm\textsuperscript{-1}; \delta\textsubscript{H} (200 MHz, d\textsubscript{6}-DMSO) 8.48 (1H, d, J 8.5 Hz, amide NH), 8.42 (1H, bt, amide NH), 7.91-7.21 (14H, m, Fmoc aromatic CH\textsubscript{3}s, benzyl CH\textsubscript{3}s, Mbh CH\textsubscript{3}s), 7.19 (4H, d, J\textsubscript{AB} 8.3 Hz, Mbh aromatic CH\textsubscript{3}s), 6.87 (4H, d, J\textsubscript{AB} 8.3 Hz, Mbh aromatic CH\textsubscript{3}s), 6.07 (1H, d, J 8.5 Hz, urethane NH), 5.13 (2H, s, benzyl CH\textsubscript{2}), 4.26 (4H, m, Gln aCH, Fmoc CH\textsubscript{2}CH\textsubscript{2}), 3.95 (2H, m, Gly CH\textsubscript{2}), 3.72, 3.71 (6H, 2 x s, 2 x OMe), 2.34, 2.08 (4H, m, Gln \beta/\gamma CH\textsubscript{2}s); \delta\textsubscript{C} (50 MHz, d\textsubscript{6}-DMSO) 172.4, 170.8, 169.7 (2 x amide C=O, ester C=O), 158.2 (Mbh quaternary aromatic C), 156.1 (urethane C=O), 143.9, 140.8 (Fmoc quaternary aromatic C\textsubscript{3}s), 135.9, 135.1 (Mbh quaternary aromatic C, benzyl quaternary aromatic C), 128.4-113.7 (aromatic CH\textsubscript{3}s, Mbh CH), 65.9 (Fmoc CH\textsubscript{2} benzyl CH\textsubscript{2}), 55.1, 54.6 (2 x OMe, Gln aCH), 46.8 (Fmoc CH), 43.2 (Gly CH\textsubscript{2}), 32.0, 28.3 (Gln \beta/\gamma CH\textsubscript{2}s); \textit{m/z} (FAB), 742 (M\textsuperscript{+}), 617, 419, 325, 307; \textit{hrms}, found 742.31280, C\textsubscript{44}H\textsubscript{44}N\textsubscript{3}O\textsubscript{6} requires 742.31282 (< 1 ppm); hplc R\textsubscript{f} (B3) 14.4 minutes.
**N**<sub>ε</sub>-**(9-Fluorenylethoxycarbonyl)-L-phenylalanine-L-alanine benzyl ester**

**(138)**

**Fmoc-L-Phe-L-Ala-OBzl**

Fmoc-L-Phe-OH (0.458 g, 1.18 mmol) was dissolved in DMF (15 ml) at room temperature, and to this was added N-methylmorpholine (0.135 ml, 1.18 mmol) and diphenylphosphinic anhydride (127) (0.506 g, 1.18 mmol). After stirring for 5 minutes, TosO<sub>2</sub>-H<sub>2</sub>-Ala-OBzl (131) (0.414 g, 1.18 mmol) and N-methylmorpholine (0.270 ml, 2.36 mmol) were added, and the reaction mixture was stirred for 3 hours. Ethyl acetate (150 ml) was added, and the resulting solution was washed with water (1 x 150 ml), a saturated aqueous solution of NaHCO<sub>3</sub> (2 x 150 ml), water (1 x 150 ml), an aqueous solution of 10% citric acid (2 x 150 ml), water (1 x 150 ml), a saturated aqueous solution of NaHCO<sub>3</sub> (2 x 150 ml) and brine (1 x 150 ml). After drying the organic solution over Na<sub>2</sub>SO<sub>4</sub>, the solvent was removed in vacuo to afford a white solid. This material was purified by flash chromatography on silica (eluting with ethyl acetate) to give a white solid, which was recrystallised from ethyl acetate/light petroleum (b.p. 40-60°C) to afford the **title compound** (0.374 g, 58%) as a white solid: tlc R<sub>f</sub> (A7) 0.71; m.p. 183-185°C; [α]<sub>D</sub><sup>27</sup> -19.5° (c = 1, DMF); Found: C, 74.3; H, 5.96; N, 5.38 Calc. for C<sub>34</sub>H<sub>33</sub>N<sub>2</sub>O<sub>5</sub> C, 74.4; H, 5.88; N, 5.11%; ν<sub>max</sub> (bromof orm mull) 1745, 1695, 1650 (C=O), 1540, 1260 cm<sup>-1</sup>; δ<sub>H</sub> (200 MHz, d<sub>6</sub>-DMSO) 8.57 (1H, d, J 7.3 Hz, amide NH), 7.89-7.13 (18H, m, aromatic CH's), 5.15 (2H, s, benzyl CH<sub>2</sub>), 4.25 (5H, Phe αCH, Ala αCH, Fmoc CHCH<sub>2</sub>), 3.95 (2H, m, Phe βCH<sub>2</sub>), 1.36 (3H, d, J 7.1 Hz, Ala CH<sub>3</sub>); δ<sub>C</sub> (50 MHz, d<sub>6</sub>-DMSO) 172.4, 171.8 (amide C=O, ester C=O), 155.9 (urethane C=O), 143.8, 140.7, 138.2, 136.0 (Fmoc quaternary aromatic C's, benzyl quaternary C, Phe quaternary aromatic C), 129.2-120.1 (aromatic CH's), 66.0, 65.7 (benzyl CH<sub>2</sub>, Fmoc CH<sub>2</sub>), 55.8 (Phe αCH), 47.8 (Ala αCH), 46.6 (Fmoc CH), 37.5 (Phe βCH<sub>2</sub>), 16.9 (Ala CH<sub>3</sub>); m/z (FAB), 549 (MH<sup>+</sup>), 237, 179; hrms, found 549.23897, C<sub>34</sub>H<sub>33</sub>N<sub>2</sub>O<sub>5</sub> requires 549.23893 (< 1 ppm); hplc R<sub>t</sub> (B7) 33.9 minutes.
Fmoc-D-Phe-L-Ala-OBzI

The compound (139) was prepared by repeating the above procedure for the synthesis of Fmoc-L-Phe-L-Ala-OBzI (138), but using Fmoc-D-Phe-OH instead of Fmoc-L-Phe-OH.

The product was recrystallised from ethyl acetate/light petroleum (b.p. 40-60°C) to afford the title compound (0.400 g, 62%) as a white solid: tlc Rf (A7) 0.71; m.p. 163-164°C; [α]D +13.3° (c = 1, DMF); Found: C, 74.4; H, 5.96; N, 5.25 Calc. for C34H32N2O5 C, 74.4; H, 5.88; N, 5.11%; νmax (bromoform mull) 1745, 1695, 1655 (C=O), 1540, 1260 cm⁻¹; δH (200 MHz, d6-DMSO) 8.55 (1H, d, J 7.1 Hz, amide NH), 7.90-7.15 (18H, m, aromatic CH's), 5.14 (2H, s, benzyl CH2), 4.25 (5H, m, Phe αCH, Ala αCH, Fmoc CH, CH2), 3.94 (2H, m, Phe βCH2), 1.29 (3H, d, J 7 Hz, Ala CH3); δC (50 MHz, d6-DMSO) 172.4, 171.6 (amide C=O, ester C=O), 155.8 (urethane C=O), 143.9, 140.8, 138.1, 136.0 (Fmoc quaternary aromatic C's, benzyl quaternary C, Phe quaternary aromatic C), 129.4-120.2 (aromatic CH's), 66.0, 65.8 (benzyl CH2, Fmoc CH2), 56.0 (Phe αCH), 47.9 (Ala αCH), 46.7 (Fmoc CH), 38.1 (Phe βCH2), 17.2 (Ala CH3); m/z (FAB), 549 (MH⁺), 237, 179; hrms, found 549.23889, C34H33N2O5 requires 549.23893 (< 1 ppm); hplc Rf (B7) 34.3 minutes.

Nα-(9-Fluorenylethoxycarbonyl)-L-alanine (-)-menthyl ester (140)

Fmoc-L-Ala-O[(-)Men]

Fmoc-L-Ala-OH (0.520 g, 1.67 mmol) was dissolved in DMF (10 ml) at room temperature, and to this was added N-methylmorpholine (0.190 ml, 1.67 mmol) and diphenylphosphinic anhydride (127) (0.716 g, 1.67 mmol). After stirring for 10 minutes, 2-dimethylaminopyridine (0.010 g, 0.084 mmol), N-methylmorpholine (0.190 ml, 1.67 mmol) and (−)-menthol (0.261 g, 1.67 mmol) were added, and the reaction mixture was stirred for 3 hours. Ethyl acetate (100 ml) was added, and the resulting solution was washed with water (1 x 100 ml), a saturated aqueous solution of NaHCO3 (2 x 100 ml), water (1 x 100 ml), an aqueous solution of 10% citric acid (2 x 100 ml), water (1 x 100 ml), 0.1M NaOH (aq) (2 x 100 ml) and brine (1 x 100 ml). After drying the organic phase over Na2SO4, the solvent was removed in
vacuo to afford a white solid. This material was purified by flash chromatography on silica (eluting with ethyl acetate) to give a solid, which was recrystallised from ethyl acetate/light petroleum (b.p. 40–60°C) to afford the title compound (0.323 g, 43%) as a white solid: tlc Rf (A7) 0.72; m.p. 120–121°C; [α]D27° -26.3° (c = 1, DMF); Found: C, 74.7; H, 7.89; N, 3.17 Calcd. for C28H35NO4 C, 74.8; H, 7.80; N, 3.12%; νmax.
(CH2Cl2) 3420 (NH), 1720 (C=O), 1510, 1210 cm⁻¹; δH (200 MHz, CDCl3) 7.78–7.27 (8H, m, Fmoc aromatic CH's), 5.44 (1H, bd, J 6.6 Hz, NH), 4.75 (1H, m, Men CH), 4.35 (3H, m, Fmoc CH2CH2), 4.25 (1H, m, Ala αCH), 2.00–1.25 (9H, m, Men CH3, Men signals), 1.20–0.83 (9H, m, Men signals), 0.77 (3H, d, J 6.9 Hz, Men CH3); δC (50 MHz, CDCl3) 172.5 (ester C=O), 155.4 (urethane C=O), 143.8, 143.7, 141.2 (Fmoc quaternary aromatic C's), 127.5, 126.9, 124.9, 119.8 (Fmoc aromatic CH's), 75.4 (Men CH), 66.8 (Fmoc CH2), 49.6 (Men CH), 47.1, 46.8 (Fmoc CH, Ala αCH), 40.5 (Men CH2), 34.0 (Men CH2), 31.2 (Men CH), 26.2 (Men CH3), 23.3 (Men CH), 21.8, 20.5, 18.8 (2 x Men CH, Ala CH3), 16.2 (Men CH3); m/z (FAB), 450 (MH⁺), 312, 179, 91; hplc, found 450.26444, C28H36NO4 requires 450.26442 (< 1 ppm); hplc Rf (B8) 39.3 minutes.

N°-(9-Fluorenylmethoxycarbonyl)-D-alanine (-)-menthyl ester (141)

Fmoc-D-Ala-O[(-)Men]

The compound (141) was prepared by repeating the above procedure for the synthesis of Fmoc-L-Ala-O[(-)Men] (140) but using Fmoc-D-Ala-OH instead of Fmoc-L-Ala-OH.

The product was purified by flash chromatography on silica (eluting with ethyl acetate) to afford the title compound (0.361 g, 48%) as a clear oil: tlc Rf (A7) 0.72; [α]D27° -20.7° (c = 1, DMF); νmax.
(CH2Cl2) 3440 (NH), 1720 (C=O), 1510, 1215 cm⁻¹; δH (200 MHz, CDCl3) 7.78–7.25 (8H, m, Fmoc aromatic CH's), 5.39 (1H, bs, NH), 4.75 (1H, m, Men CH), 4.38 (3H, m, Fmoc CH2CH2), 4.23 (1H, m, Ala αCH), 2.05–1.34 (9H, m, Ala CH3, Men signals), 1.11–0.83 (9H, m, Men signals), 0.78 (3H, d, J 6.9 Hz, Men CH3); m/z (FAB), 450 (MH⁺), 312, 215, 202, 179; hplc, found 450.26444, C28H36NO4 requires 450.26442 (< 1 ppm); hplc Rf (B8) 39.1 minutes.
$^{31}$P n.m.r. monitored stability studies with diphenylphosphinic anhydride (127) and ZnI$_2$

(1) Diphenylphosphinic anhydride (127) and ZnI$_2$ (0.1 eq.)

Diphenylphosphinic anhydride (127) (0.075 g, 0.18 mmol) was dissolved in CD$_2$Cl$_2$/DMF (1:4) (0.5 ml) at room temperature, and to this was added ZnI$_2$ (0.006 g, 0.018 mmol). The resulting solution was monitored by $^{31}$P n.m.r. (36 MHz), acquiring a spectrum immediately on the addition of the ZnI$_2$, and subsequently at 15 minute intervals for 30 minutes. A small signal (25.41 ppm) was shown to be present after the 30 minute period as well as the starting anhydride (127) (27.76 ppm).

To the above solution was added N-methylmorpholine (0.020 ml, 0.18 mmol) and again spectra were recorded at given time intervals for 75 minutes. Immediately on addition of the base, only one signal (27.80 ppm) (i.e. the starting anhydride (127)) was shown to be present, and this remained unchanged for the duration of the experiment.

(2) Diphenylphosphinic anhydride (127) and ZnI$_2$ (1.0 eq.)

The above experiment was repeated with ZnI$_2$ (1.0 eq.) (0.057 g, 0.18 mmol). 30 minutes after the addition of the ZnI$_2$ only one signal was shown to be present (26.90 ppm) - i.e. not the starting anhydride (127).

The addition of the base resulted in a shift of this signal to 25.11 ppm.

$^{31}$P n.m.r. monitored couplings with diphenylphosphinic anhydride (127) and ZnI$_2$

(1) Coupling of Z-Phe-OH (108) and TosO$^-$/H$_2$Gly-OBzl (107)

(A) Z-Phe-OH (108) (0.200 g, 0.67 mmol) was dissolved in CD$_2$Cl$_2$/DMF (1:4) (3.0 ml) at room temperature, and to this was added N-methylmorpholine (0.076 ml, 0.67 mmol) and diphenylphosphinic anhydride (127) (0.284 g, 0.67 mmol). The solution was monitored by $^{31}$P n.m.r. (36 MHz), acquiring spectra at given time intervals for 15 minutes. After the 15 minutes the activation was shown to be complete.
To the above solution was added ZnI\(_2\) (0.021 g, 0.067 mmol). \(^{31}\)P n.m.r. revealed a change in the phosphinate anion signal \(i.e.\) what had been a sharp peak at 16.42 ppm had become a broad hump at 17.6 ppm.

After 10 minutes TosO\(^-\)H\(_2\)^+Gly-OBzl (107) (0.224 g, 0.67 mmol) and N-methylmorpholine (0.152 ml, 1.34 mmol) were added, and again spectra were acquired for 10 minutes. The coupling was shown to be complete within 10 minutes and mass spec. (FAB\(^+\)) confirmed the presence of a peak at 447 (MH\(^+\)).

(B) Z-Phe-OH (108) (0.100 g, 0.33 mmol) and ZnI\(_2\) (0.010 g, 0.033 mmol) were dissolved in CD\(_2\)Cl\(_2\)/DMF (1:4) (1.5 ml), and to this was added N-methylmorpholine (0.038 ml, 0.33 mmol) and diphenylphosphinic anhydride (127) (0.142 g, 0.33 mmol). Immediately on mixing, \(^{31}\)P n.m.r. (36 MHz) revealed 3 signals: one corresponding to the activated mixed anhydride (27.53 ppm), one to the starting anhydride (127) (27.43 ppm) and a broad signal for the phosphinate anion (17.50 ppm). The ratio of the peak heights of these signals remained unchanged over 10 minutes.

After the 10 minutes TosO\(^-\)H\(_2\)^+Gly-OBzl (107) (0.112 g, 0.33 mmol) and N-methylmorpholine (0.076 ml, 0.67 mmol) were added. \(^{31}\)P n.m.r. revealed that the coupling was complete within 20 minutes and mass spec. (FAB\(^+\)) confirmed the presence of a peak at 447 (MH\(^+\)).

(2) Coupling of Fmoc-Met-Gly-OH and TosO\(^-\)H\(_2\)^+Gly-OBzl (107)

(A) Fmoc-Met-Gly-OH (0.143 g, 0.33 mmol) was dissolved in CD\(_2\)Cl\(_2\)/DMF (1:4) (2.0 ml) at room temperature, and to this was added N-methylmorpholine (0.038 ml, 0.33 mmol) and diphenylphosphinic anhydride (127) (0.142 g, 0.33 mmol). Immediately on mixing \(^{31}\)P n.m.r. (36 MHz) revealed 3 signals: the activated mixed anhydride, the starting anhydride (27.50, 27.41 ppm) and the phosphinate anion (17.26 ppm). Over a 20 minute period the signals at 27.50 and 27.41 ppm reduced substantially in height, while that of the anion signal increased.

ZnI\(_2\) (0.010 g, 0.033 mmol) was added to the above solution. \(^{31}\)P n.m.r. revealed that immediately on mixing a large signal (27.43 ppm) and a broad signal (17.26 ppm) were present. Over a 10 minute period the ratio of the peak heights of the signals remained unchanged.

TosO\(^-\)H\(_2\)^+Gly-OBzl (107) (0.112 g, 0.33 mmol) and N-methylmorpholine (0.076 ml, 0.67 mmol) were added, and again spectra were acquired at given time
intervals. The coupling was shown to be complete within 20 minutes and mass spec. (FAB+) confirmed the presence of a peak at 576 (MH+).

(B) Fmoc-Met-Gly-OH (0.143 g, 0.33 mmol) and ZnI₂ (0.010 g, 0.033 mmol) were dissolved in CD₂Cl₂/DMF (1:4) (2.0 ml) at room temperature, and to this was added N-methylmorpholine (0.038 ml, 0.33 mmol) and diphenylphosphinic anhydride (127) (0.142 g, 0.33 mmol). The solution was monitored by ³¹P n.m.r. (36 MHz), acquiring spectra at 5 minute intervals for 15 minutes. After 5 minutes three signals were shown to be present: the activated mixed anhydride (27.56 ppm), the starting anhydride (27.23 ppm), and the phosphinate anion (broad signal). The ratio of the peak heights of these signals remained unchanged over the 15 minute period.

To the above solution were added TosOH₂⁺-Gly-OBzl (107) (0.112 g, 0.33 mmol) and N-methylmorpholine (0.076 g, 0.67 mmol), and spectra were again acquired at given time intervals. The coupling was shown to be complete within 20 minutes and mass spec. (FAB+) confirmed the presence of a peak at 576 (MH+).

Nα-(Benzoyloxycarbonyl)phenylalanylalanylalanine benzyl ester (132)

Z-Phe-Ala-OBzl

Z-Phe-OH (108) (0.501 g, 1.67 mmol) and ZnI₂ (0.053 g, 0.167 mmol) were dissolved in DMF (15 ml) at room temperature, and to this were added N-methylmorpholine (0.185 ml, 1.67 mmol) and diphenylphosphinic anhydride (127) (0.716 g, 1.67 mmol). After stirring the resulting solution for 5 minutes, TosOH₂⁺-Ala-OBzl (131) (0.586 g, 1.67 mmol) and N-methylmorpholine (0.370 ml, 3.34 mmol) were added, and the reaction mixture was stirred for 3 hours. Ethyl acetate (150 ml) was added, and the organic solution was washed with water (1 x 150 ml), a saturated aqueous solution of NaHCO₃ (3 x 150 ml), water (1 x 150 ml), an aqueous solution of 10% citric acid (3 x 150 ml) and brine (1 x 150 ml), and dried over Na₂SO₄. The removal of the solvent in vacuo gave a white solid which was purified by flash chromatography on silica (eluting with ethyl acetate). The resulting material was recrystallised from acetone/light petroleum (b.p. 40-60°C) to afford the title compound (0.619 g, 81%) as a white solid: tlc RF (A4) 0.69; m.p. 124-126°C; [α]D 27° -23.4° (c = 1, acetone); Found: C, 70.5; H, 6.20; N, 6.20 Calc. for C₂₇H₂₈N₂O₅ C, 70.4; H, 6.13; N, 6.08%; v max. (CH₂Cl₂) 3420 (NH), 1740, 1670 (C=O), 1500, 1200 cm⁻¹; δH (200 MHz, CD₃COCD₃) 8.75 (1H, bd, amide NH), 7.30 (15H, m, aromatic CH's), 6.49 (1H, d, J 8.8 Hz, urethane...
NH), 5.18, 5.00 (Z-CH<sub>2</sub>, benzyl CH<sub>2</sub>), 4.55 (2H, m, Phe αCH, Ala αCH), 3.08 (2H, m, Phe βCH<sub>2</sub>), 1.38 (3H, d, J 7.2 Hz, Ala CH<sub>3</sub>); δ<sub>C</sub> (50 MHz, CD<sub>3</sub>COCD<sub>3</sub>) 171.3, 170.2 (amide C=O, ester C=O), 155.1 (urethane C=O), 136.9, 136.4, 135.4 (quaternary aromatic C's), 128.5-125.5 (aromatic CH's), 65.4, 64.9 (Z-CH<sub>2</sub>, benzyl CH<sub>2</sub>), 55.1 (Phe αCH), 47.2 (Ala αCH), 37.2 (Phe βCH<sub>2</sub>), 16.0 (Ala CH<sub>3</sub>); m/z (FAB), 461 (MH<sup>+</sup>), 417, 327, 210; hplc R<sub>t</sub> (B2) 20.5 minutes.

Regeneration of diphenylphosphinic acid (125)

Ph<sub>2</sub>PO<sub>2</sub>H

A mixture of diphenylphosphinic anhydride (127)/diphenylphosphinic acid (125) (50.0 g) was suspended in a mixture of 1M NaOH (200 ml) and ethyl acetate (100 ml), and was stirred for 60 minutes. The organic and aqueous layers were separated, and the aqueous layer was washed with further ethyl acetate (2 x 100 ml), before the pH was adjusted to 2.0 by the addition of 1M HCl. The resulting white precipitate was extracted with ethyl acetate (3 x 100 ml), washed with water (1 x 100 ml), and dried over Na<sub>2</sub>SO<sub>4</sub>. The removal of the solvent in vacuo afforded the title compound (40.8 g) as a white solid: m.p. 193-195 °C (lit. 193-195 °C); Found: C, 65.7; H, 5.12 Calc. for C<sub>12</sub>H<sub>11</sub>O<sub>2</sub>P C, 65.8; H, 5.02%; δ<sub>δ</sub> (36 MHz, CDCl<sub>3</sub>/DMF (1:1)) 24.90; m/z (FAB), 219 (MH<sup>+</sup>), 141.

Regeneration of diphenylphosphinyl chloride (45)

Ph<sub>2</sub>POCl

The compound was prepared by the method Kreutzkamp and Schindler<sup>236</sup>. Diphenylphosphinic acid (125) (20.00 g, 0.092 mol) was suspended in thionyl chloride (40.0 ml, 0.548 mol) and warmed to 60°C for 30 minutes. The reaction mixture was then heated to 90°C at atmospheric pressure for 10 minutes, and at water pump pressure for 3 hours, before being distilled to afford the title compound (18.58 g, 86%) as a yellow oil: b.p. 144°C at 0.5-0.7 mmHg (lit. 135-140°C at 0.01 mmHg); δ<sub>H</sub> (60 MHz, CDCl<sub>3</sub>) 8.70-6.95 (m, aromatic CH's); δ<sub>δ</sub> (36 MHz, CDCl<sub>3</sub>) 44.69; m/z (FAB), 237 (M<sup>+</sup>), 219, 201, 141.
Loading first residue to p-alkoxybenzyl alcohol resin (8)

(1) \(N^\alpha-(9\text{-Fluorenlymethoxycarbonyl})\text{glycyl } p\text{-alkoxybenzyl alcohol resin (142)}\)

Fmoc-Gly-(O-CH\(_2\)C\(_6\)H\(_4\)-OR)

(A) Fmoc-Gly-OH (2.497 g, 8.40 mmol) was dissolved in DMA (15 ml) at room temperature, and to this was added diisopropylcarbodiimide (0.65 ml, 4.20 mmol). After stirring for 15 minutes, this was added to p-alkoxybenzyl alcohol resin (8) (Bachem, 1.05 mmol/g) (2.00 g, 2.10 mmol) which had been swollen in DMA (25 ml) in the presence of DMAP (0.013 g, 0.11 mmol). The reaction mixture was agitated for 3 hours in a ultrasonic bath at room temperature. The resin was filtered, washed with DCM (3 x 30 ml), DMA (3 x 30 ml) and DCM (3 x 30 ml), and dried. The coupling yield was found to be 54% (0.489 mmol/g) (by UV deprotection study\(^{36}\)).

(B) Fmoc-Gly-OH (3.942 g, 13.3 mmol) was dissolved in DMF (30 ml) at room temperature, and to this was added N-methylmorpholine (1.47 ml, 13.3 mmol) and diphenylphosphinic anhydride (127) (5.548 g, 13.3 mmol). After stirring for 5 minutes, this was added to p-alkoxybenzyl alcohol resin (8) (Bachem, 0.79 mmol/g) (4.20 g, 3.32 mmol) which had been swollen in DMF (30 ml) in the presence of DMAP (0.020 g, 0.17 mmol). The reaction mixture was agitated for 3 hours in a ultrasonic bath at room temperature, followed by washing and drying as above. The coupling yield was found to be 80% (0.537 mmol/g) (by UV deprotection study\(^{36}\)).

\(N^\alpha-[2,2\text{-bis (4\text{'-Nitrophenyl)ethoxycarbonyl})\text{glycyl } p\text{-alkoxybenzyl alcohol resin (113)}\)

Bnpeoc-Gly-(O-CH\(_2\)C\(_6\)H\(_4\)-OR)

Bnpeoc-Gly-OH (3.268 g, 8.40 mmol) was dissolved in DMA (15 ml) at room temperature, and to this was added diisopropylcarbodiimide (0.65 ml, 4.20 mmol). After stirring for 15 minutes, this was added to p-alkoxybenzyl alcohol resin (8) (Bachem, 1.05 mmol/g) (2.00 g, 2.10 mmol) which had been swollen in DMA (25 ml) in the presence of DMAP (0.013 g, 0.11 mmol). The reaction mixture was agitated for 3 hours in a ultrasonic bath at room temperature, followed by washing and
drying as above. The coupling yield was found to be 52% (0.454 mmol/g) (by UV deprotection study\textsuperscript{36}).

\[ \text{N}^\alpha-\text{(9-Fluorenlymethoxy carbonyl)alanyl } p\text{-alkoxybenzyl alcohol resin (143)} \]

\[ \text{Fmoc-Ala-(O-CH}_2\text{C}_6\text{H}_4\text{-OR)} \]

(A) \text{Fmoc-Ala-OH (1.961 g, 6.30 mmol) was dissolved in DMF (23 ml) at room temperature, and to this was added diisopropylcarbodiimide (0.50 ml, 3.15 mmol). After stirring for 15 minutes, this was added to } p\text{-alkoxybenzyl alcohol resin (8) (Bachem, 1.05 mmol/g) (1.50 g, 1.58 mmol) which had been swollen in DMF (23 ml) in the presence of DMAP (0.010 g, 0.08 mmol). The reaction mixture was agitated and washed as above. The coupling yield was found to be 36\% (0.340 mmol/g) (by UV deprotection study\textsuperscript{36}). A second coupling, performed exactly as the first, was therefore necessary. The coupling yield was then found to be 78\% (0.660 mmol/g).}

(B) \text{Fmoc-Ala-OH (0.981 g, 3.15 mmol) was dissolved in DMF (15 ml) at room temperature, and to this was added N-methylmorpholine (0.35 ml, 3.15 mmol) and diphenylphosphinic anhydride (127) (1.317 g, 3.15 mmol). After stirring for 5 minutes, this was added to } p\text{-alkoxybenzyl alcohol resin (8) (Bachem, 1.05 mmol/g) (1.50 g, 1.58 mmol) which had been swollen in DMF (15 ml) in the presence of DMAP (0.010 g, 0.08 mmol). The reaction mixture was agitated and washed as above. The coupling yield was found to be 56\% (0.492 mmol/g) (by UV deprotection study\textsuperscript{36}). A second coupling, performed exactly as the first, was therefore necessary and resulted in an overall coupling yield of 79\% (0.666 mmol/g).}

(C) \text{Fmoc-Ala-OH (0.981 g, 3.15 mmol) was dissolved in DMF (15 ml) at room temperature, and to this was added N-methylmorpholine (0.35 ml, 3.15 mmol) and diphenylphosphinic anhydride (127) (1.317 g, 3.15 mmol). After stirring for 5 minutes, this was added to } p\text{-alkoxybenzyl alcohol resin (8) (Bachem, 0.79 mmol/g) (1.00 g, 0.79 mmol) which had been swollen in DMF (15 ml) in the presence of DMAP (0.010 g, 0.08 mmol). The reaction mixture was agitated and washed as above. The coupling yield was found to be 78\% (0.522 mmol/g) (by UV deprotection study\textsuperscript{36}).}
Automated Solid Phase Peptide Synthesis

General notes

All peptides prepared in this section were done so using an Applied Biosystems 430A automated solid phase peptide synthesiser. All solvents used were either freshly distilled (in the case of DCM) or used as commercially supplied by Applied Biosystems, Rathbone and Aldrich (DMF and DMA).

The first residue of each sequence (i.e. the C-terminal residue) was coupled to the p-alkoxybenzyl alcohol resin (8) outside the synthesiser. The extent of the coupling was determined by deprotecting a small sample of the loaded resin and quantitatively checking the olefin produced by UV36.

A number of programmed cycles were employed. The standard DIC (HOBt) activation and coupling cycles, used routinely on the 430A synthesiser, are summarised below. The diphenylphosphinic anhydride (127) cycles are also introduced, with the relative changes and developments in the cycles discussed with the relevant peptide.

All washings were transferred to a waste outlet, with the exception of the deprotection solutions. These were first passed through a UV cell (monitoring at 313 nm) before being put to waste. This enabled the relative efficiency of the coupling/deprotection cycles to be determined.

Tyrosylisoleucylphenylalanylanlynlglycine (144)

H-Tyr-Ile-Phe-Ala-Gly-OH

Fmoc/diphenylphosphinic anhydride (127) methodology

The synthesis of (144) was accomplished using the functionalised resin, Fmoc-Gly-(O-CH\textsubscript{2}C\textsubscript{6}H\textsubscript{4}-OR) (142), on a 0.5 mmol scale, employing Fmoc N\textsuperscript{a}-protected amino acid derivatives and diphenylphosphinic anhydride (127) activation. The side-chain hydroxyl function of the tyrosine residue was protected as a 'Bu ether, and a 'double couple' cycle was employed which involved:

(1) washing the functionalised resin - DCM, DMA (5 x, total time = 5 minutes),
(2) removal of the Nα-protecting group - 20% piperidine in DMA (10.8 ml) (4 x, 3 + 3 + 3 + 1 minutes), (3) washing - DCM, DMA (10 x, total time = 14 minutes), (4) activation - Fmoc-AA-OH (1.0 mmol) + diphenylphosphinic anhydride (127) (1.0 mmol in DMA (4.0 ml)) + N-methylmorpholine (1.0 mmol in DMA (2.0 ml)) (2 minutes), (5) coupling - activated mixed anhydride transferred to the reaction vessel + N-methylmorpholine (0.5 mmol)/2,6-lutidine (2.0 mmol) (in DMA (3.0 ml)) (40 minutes), (6) washing - DCM, DMA (2 x, total time = 3 minutes), (7) repeat the activation and coupling steps, (8) washing - DCM, DMA (5 x, total time = 5 minutes), (9) capping - acetic anhydride (1.0 ml of a 0.5M solution in DMA) + pyridine (1.0 ml of a 0.5M solution in DMA) (2 x, 2.5 + 3.5 minutes).

The cycle was terminated with the removal of the Nα-protecting group from the tyrosine residue and the subsequent washings.

The cleavage of the peptide from the support (and the simultaneous removal of the tBu protecting group) was performed by trifluoroacetic acid/water/thioanisole (95:5:5) (33 ml). The reaction mixture was agitated in an ultrasonic bath for 90 minutes at room temperature, before the resin was filtered off and washed with DMA (6 x 10 ml). The combined filtrates were concentrated in vacuo to give a yellow solid, which was washed with diethyl ether and filtered. This material was purified by preparative hplc, employing a Spherisorb C18 reverse phase prep. column, with water/acetonitrile (+0.05% TFA) as eluent and monitoring at 254 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.117 g, 41%) as a white solid: δH (360 MHz, D2O/CD3COCD3) 7.24-7.10 (5H, m, Phe aromatic CH's), 6.97 (2H, d, J 8.5 Hz, Tyr aromatic CH's), 8.70 (2H, d, J 8.5 Hz, Tyr aromatic CH's), 4.60 (1H, q, Ala αCH), 4.30, 4.23 (2H, m, Phe αCH, Tyr αCH), 4.10 (1H, d, J 8.0 Hz, Ile αCH), 3.82 (2H, m, Gly CH2), 3.05 (4H, m, Phe βCH2, Tyr βCH2), 1.62 (1H, m, Ile βCH), 1.27 (3H, d, J 7.2 Hz, Ala CH3), 1.12 (1H, m, Ile γCH), 0.95 (1H, m, Ile γCH), 0.69 (6H, m, 2 x Ile CH3); m/z (FAB), 570 (MH+), 323, 136; hrrms, found 570.29272, C29H40N5O7 requires 570.29275 (< 1 ppm); amino acid analysis, Gly1.06, Ala1.01, Ile1.03, Tyr1.088, Phe1.03; hplc Rf (B2) 10.5 minutes.
Leucylisoleucylphenylalanylalanylglycine (145)

H-Leu-Ile-Phe-Ala-Gly-OH

(A) Fmoc/DIC (HOBT) methodology

The synthesis of (145) was accomplished using the functionalised resin, Fmoc-Gly-(O-CH$_2$C$_6$H$_4$-OR) (142) on a 0.5 mmol scale, employing Fmoc N$^\alpha$-protected amino acid derivatives and a combination of DIC and HOBT activation. A 'double couple' cycle was employed which involved:

1. washing the functionalised resin - DCM, DMF (5 x, total time = 5 minutes),
2. removal of the N$^\alpha$-protecting group - 20% piperidine in DMF (10.8 ml) (4 x, 3 + 3 + 3 + 1 minutes),
3. washing DCM, DMF (10 x, total time = 14 minutes),
4. activation - Fmoc-AA-OH (2.0 mmol) + DIC (1.0 mmol in DMF (2.0 ml)) (15 minutes),
5. coupling - activated symmetrical anhydride transferred to the reaction vessel (90 minutes),
6. washing - DCM, DMF (2 x, total time = 3 minutes),
7. activation - Fmoc-AA-OH (1.0 mmol) + HOBT (1.0 mmol in DMF (2.0 ml)) + DIC (1.0 mmol in DMF (2.0 ml)) (30 minutes),
8. coupling - HOBT active ester transferred to the reaction vessel (90 minutes),
9. washing - DCM, DMF (5 x, total time = 5 minutes),
10. capping - acetic anhydride (1.0 ml of a 0.5M solution in DMF) + pyridine (1.0 ml of a 0.5M solution in DMF) (2 x, 2.5 + 3.5 minutes).

The cycle was terminated with the removal of the N$^\alpha$-protecting group from the leucine residue and the subsequent washings.

The cleavage of the peptide from the support was performed by trifluoroacetic acid/water (95:5) (30 ml). Agitation of the reaction mixture in an ultrasonic bath at room temperature for 90 minutes, followed by filtration of the resin and washing with DMF (6 x 10 ml), gave a white solid on removal of the solvents in vacuo. This material was purified by Sephadex LH20 gel filtration (eluting with DMF) to afford the title compound (0.189 g, 73%) as a white solid: $\delta_H$ (200 MHz, D$_2$O/TFA) 6.15, m, Phe aromatic CH's), 3.52 (1H, t, J 7.4 Hz, Leu $\alpha$CH), 3.23 (1H, m, Ala $\alpha$CH), 2.95 (2H, m, Ile $\alpha$CH, Phe $\alpha$CH), 2.76 (2H, d, J 3.8 Hz, Gly CH$_2$), 1.89 (2H, m, Phe $\beta$CH$_2$), 0.47 (4H, m, Ile $\beta$CH, Leu $\beta$CH$_2$/γCH), 0.17 (5H, m, Ala CH$_3$', Ile γCH$_2$), -0.31 (12H, m, 2 x Leu CH$_3$, 2 x Ile CH$_3$); $m/z$ (FAB), 520 (MH$^+$), 294, 227, 199, 147; hrms, found 520.31350, C$_{26}$H$_{42}$N$_5$O$_6$ requires 520.31349 (< 1 ppm); amino acid analysis, Gly$_1$1.00, Ala$_1$1.00, Ile$_1$0.97, Leu$_1$0.99, Phe$_1$1.04; hplc R$_t$ (B9) 17.4 minutes.
The synthesis of (145) was performed using the functionalised resin, Bnpeoc-Gly-(O-CH$_2$C$_6$H$_4$-OR) (113) on a 0.5 mmol scale, employing Bnpeoc N$^\alpha$-protected amino acid derivatives and a combination of DIC and HOBt activation. A 'double couple' cycle was used as in method (A).

The unprotected peptide was removed from the support and manipulated as above to yield a yellow solid. An analytical hplc (139) trace of this solid revealed two products: a major peak, $R_t$ 17.5 minutes (the desired pentapeptide), and a minor peak, $R_t$ 18.2 minutes (impurity). Amino acid analysis of the crude material (Gly$_1$,1.08, Ala$_1$0.77, Ile$_1$1.05, Leu$_1$1.03, Phe$_1$1.06) suggested the impurity to be the tetrapeptide, H-Leu-Ile-Phe-Gly-OH.

An aliquot of this material was purified by preparative hplc, employing a Spherisorb C$_{18}$ reverse phase prep. column, eluting with water/acetonitrile (+0.05% TFA) and monitoring at 229 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.076 g, calculated yield = 61%) as a white solid: $\delta_H$ (360 MHz, D$_2$O/TFA) 7.47–7.35 (5H, m, Phe aromatic CH's), 4.85 (1H, t, J 7.5 Hz, Leu aCH), 4.55 (1H, q, Ala aCH), 4.33 (1H, d, J 7.9 Hz, Ile aCH), 4.24 (1H, m, Phe aCH), 4.08 (2H, m, Gly CH$_2$), 3.17 (2H, m, Phe CH$_2$), 1.90–1.71 (4H, m, Ile CH, Leu CH$_2$/γCH), 1.56 (1H, m, Ile γCH), 1.50 (3H, d, J 7.1 Hz, Ala CH$_3$), 1.21 (1H, m, Ile γCH), 1.09–0.92 (12H, m, 2 x Leu CH$_3$, 2 x Ile CH$_3$); $m/z$ (FAB), 520 (MH$^+$), 294, 228, 200, 147; hrms, found 520.31350, C$_{26}$H$_{42}$N$_5$O$_6$ requires 520.31349 (< 1 ppm); amino acid analysis, Gly$_1$1.08, Ala$_1$0.99, Ile$_1$0.98, Leu$_1$0.99, Phe$_1$0.96; hplc $R_t$ (B9) 17.6 minutes.

The removal of the hplc solvents of the fractions containing the 'impurity' by lyophilisation afforded a white solid (0.006g). Mass spec. (FAB$^+$) confirmed the presence of a peak at 449 (H-Leu-Ile-Phe-Gly-OH m.wt. 448).

(C) Fmoc/diphenylphosphinic anhydride (127) methodology

The synthesis of (145) was performed using the functionalised resin, Fmoc-Gly-(O-CH$_2$C$_6$H$_4$-OR) (142) on a 0.5 mmol scale, employing Fmoc N$^\alpha$-protected amino acid derivatives and diphenylphosphinic anhydride (127) activation. A 'double couple' cycle was employed which involved:
(1) washing the functionalised resin - DCM, DMF (5 x, total time = 5 minutes),
(2) removal of the Nα-protecting group - 20% piperidine in DMF (10.8 ml) (4 x, 3 + 3 + 3 + 1 minutes), (3) washing - DCM, DMF (10 x, total time = 14 minutes), (4) activation - Fmoc-AA-OH (1.0 mmol) + diphenylphosphinic anhydride (127) (1.0 mmol) + N-methylmorpholine (1.0 mmol in DMF (2.0 ml)), (5) coupling - activated mixed anhydride transferred to the reaction vessel + N-methylmorpholine (0.5 mmol)/2,6-lutidine (2.0 mmol) (in DMF (3.0 ml)), (6) washing - DCM, DMF (2 x, total time = 3 minutes), (7) repeat activation and coupling steps, (8) washing - DCM, DMF (5 x, total time = 5 minutes), (9) capping - acetic anhydride (1.0 ml of a 0.5M solution in DMF) + pyridine (1.0 ml of a 0.5M solution in DMF) (2 x, 2.5 + 3.5 minutes).

The cycle was terminated with the removal of Nα-protecting group from the leucine residue and the subsequent washings.

The cleavage of the unprotected peptide from the support was performed as method (A), to give on filtration, a white solid. This material was purified by Sephadex LH20 gel filtration (eluting with DMF) to afford the title compound (0.204 g, 78%) as a white solid: δH (200 MHz, D2O/TFA) 6.12 (5H, m, Phe aromatic CH's), 3.54 (1H, t, Leu αCH), 3.25 (1H, m, Ala αCH), 3.00 (2H, m, Ile αCH, Phe αCH), 2.77 (2H, m, Gly CH2), 1.91 (2H, m, Phe βCH2), 0.49 (4H, m, Ile βCH, Leu βCH2/γCH), 0.20 (5H, m, Ala CH3, Ile γCH2), −0.33 (12H, m, 2 x Leu CH3, 2 x Ile CH3); m/z (FAB), 520 (MH+), 294, 227, 199, 147; hrms, found 520.31350, C26H42N5O6 requires 520.31349 (< 1 ppm); amino acid analysis, Gly 1.03, Ala 1.01, Ile 1.00, Leu 0.99, Phe 1.00; hplc Rf (B9) 17.4 minutes.

(D) Bnpeoc/diphenylphosphinic anhydride (127) methodology

The synthesis of (145) was performed using the functionalised resin, Bnpeoc-Gly-(O-CH2C6H4-OR) (113) on a 0.5 mmol scale, employing Bnpeoc Nα-protected amino acid derivatives and diphenylphosphinic anhydride (127) activation. A 'double couple' cycle was used as in method (C).

The unprotected peptide was removed from the support and manipulated as above to give a yellow solid. An analytical hplc (B9) trace of this material revealed a major peak (Rf 17.4 minutes) with an accompanying small shoulder. Amino acid analysis of this crude material (Gly 1.00, Ala 1.00, Ile 0.98, Leu 0.94, Phe 1.08) gave no obvious clue as to the identity of the impurity.
The solid was purified by preparative hplc, employing an Aquapore C_{18} reverse phase prep. column, eluting with water/acetonitrile (+0.05% TFA) and monitoring at 229 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.151 g, 58%) as a white solid: δ_H (360 MHz, D_2O/TFA) 7.47-7.34 (5H, m, Phe aromatic CH's), 4.86 (1H, t, J 7.5 Hz, Leu αCH), 4.57 (1H, q, Ala αCH), 4.34 (1H, d, J 7.8 Hz, Ile αCH), 4.26 (1H, m, Phe αCH), 4.09 (2H, d, Gly CH_2), 3.23 (2H, m, Phe BCH_2), 1.89-1.74 (4H, m, Ile BCH, Leu BCH_2/γCH), 1.54 (1H, m, Ile γCH), 1.51 (3H, d, J 7.1 Hz, Ala CH_3), 1.23 (1H, m, Ile γCH), 1.10-0.94 (12H, m, 2 x Leu CH_3, 2 x Ile CH_3); m/z (FAB), 520 (MH^+), 294, 227, 199, 147; hrms, found 520.31350, C_{26}H_{42}N_6O_6 requires 520.31349 (< 1 ppm); amino acid analysis,. Gly 1 0.98, Ala 1 1.00, Ile 1 1.00, Leu 1 1.03, Phe 0.99; hplc R_t (B9) 17.5 minutes.

**Prolylglyclylprolyl-D-phenylalanylalanine (146)**

H-Pro-Gly-Pro-D-Phe-Ala-OH

**Fmoc/diphenylphosphinic anhydride (127) methodology**

The synthesis of (146) was accomplished using the functionalised resin, Fmoc-Ala-(O-CH_2C_6H_4-OR) (143) on a 0.5 mmol scale, employing Fmoc N_0-protected amino acid derivatives and diphenylphosphinic anhydride (145) activation. A 'double couple' cycle (2 x 2 eq.), outlined above for the synthesis of H-Leu-Ile-Phe-Ala-Gly-OH (145) (method (C)), was used, with one exception - the glycine residue was incorporated in a single (1 x 4 eq.) coupling rather than the standard (2 x 2 eq.) double cycle.

The unprotected peptide was removed from the support and manipulated as above to give a yellow oil. This material was purified by preparative hplc, employing an Aquapore C_{8} reverse phase prep. column, eluting with water/acetonitrile (+0.05% TFA) and monitoring at 229 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.192 g, 79%) as a white solid: δ_H (360 MHz, D_2O) 7.36 (5H, m, Phe aromatic CH's), 4.77 (1H, m, Phe αCH), 4.46 (1H, m, Ala αCH), 4.35 (2H, m, 2 x Pro αCH), 4.15 (2H, m, Pro CH_2), 3.83 (2H, m, Gly CH_2), 3.45 (2H, m, Pro CH_2), 3.10 (2H, m, Phe BCH_2), 2.45 (1H, m, Pro CH), 2.08 (4H, m, 2 x Pro CH, Pro CH_2), 1.90 (2H, m, 2 x Pro CH), 1.50 (1H, m, Pro CH), 1.36 (3H, d, J 7.3 Hz, Ala CH_3); m/z (FAB), 488 (MH^+), 334, 155, 139; hrms, found 488.25091, C_{24}H_{34}N_5O_6 requires 488.25089; amino acid analysis, Pro 1.94, Gly 0.98,
Ala1,1.03, Phe1,0.99; hplc R₁ (B10) 14.7 minutes.

\[ \text{Leucylvalyleucylarginyleucylarginylglycylglycine (147)} \]

\[ \text{H-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-OH} \]

**Fmoc/diphenylphosphinic anhydride (127) methodology**

The synthesis of (147) was accomplished using the functionalised resin, Fmoc-Gly-(O-CH₂C₆H₄-OR) (142) on a 0.5 mmol scale, employing Fmoc Nα-protected amino acid derivatives and diphenylphosphinic anhydride (127) activation. A 'double couple' cycle (2 x 2 eq.), outlined above for the synthesis of H-Leu-Ile-Phe-Ala-Gly-OH (145) (method (C)), was used for each of the residues, with the exception of glycine, which was incorporated in a single (1 x 4 eq.) coupling. Also, unlike the above cycles, which employed a mixture of DCM and DMA/DMF as the solvent, only DMA was used. The second couplings of both the first arginine and the valine residues were left overnight, rather than for the standard 40 minutes. The arginine side-chain function was protected with the Pmc group.

The cleavage of the peptide from the support (and the simultaneous removal of the Pmc protecting group) was performed by trifluoroacetic acid/water/thioanisole (95:5:5) (33 ml). The reaction mixture was agitated in an ultrasonic bath for 90 minutes at room temperature, before the resin was filtered off and washed with DMA (6 x 10 ml). The combined filtrates were concentrated in vacuo to give an off-white solid, which was washed with diethyl ether and filtered. An aliquot of this material was purified by preparative hplc, employing an Aquapore C₁₈ reverse phase prep. column with water/acetonitrile (+0.05% TFA) as eluent and monitoring at 229 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.030 g, calculated yield = 44%) as a white solid: δH (360 MHz, D₂O) 4.38 (4H, m, 2 x Arg αCH, 2 x Leu αCH), 4.17 (1H, d, J 8.7 Hz, Val αCH), 4.09 (1H, m, Leu αCH), 3.99 (4H, m, 2 x Gly CH₂), 3.24 (4H, m, 2 x Arg βCH₂), 2.05 (1H, m, Val βCH), 1.91–1.55 (17H, m, 3 x Leu βCH₂/γCH, 2 x Arg γCH₂/δCH₂), 1.00–0.87 (24H, m, 6 x Leu CH₃, 2 x Val CH₃); m/z (FAB), 883 (MH⁺), 870, 213, 149; h rms, found 883.584113, C₃₉H₇₅N₁₄O₉ requires 883.58411 (< 1 ppm); amino acid analysis, Gly₂2.13, Val₁0.92, Leu₃3.17, Arg₂2.09; hplc, R₁ (B12) 20.0 minutes.
Threonylleucylserylisoleucylglycine (148)

H-Thr-Leu-Ser-Ile-Gly-OH

(A) Fmoc/diphenylphosphinic anhydride (127) methodology

The synthesis of (148) was accomplished using the functionalised resin, Fmoc-Gly-(O-CH$_2$C$_6$H$_4$-OR) (142) on a 0.5 mmol scale, employing Fmoc N$^\alpha$-protected amino acid derivatives and diphenylphosphinic anhydride (127) activation. The side-chain hydroxyl functions of the serine and threonine residues were protected as 'Bu ethers. A 'quadruple couple' cycle was employed which involved:

1. washing the functionalised resin - DMF (5 x, total time = 5 minutes),
2. removal of the N$^\alpha$-protecting group - 20% piperidine in DMF (10.8 ml) (4 x, 5 + 3 + 3 + 1 minutes),
3. washing - DMF (10 x, total time = 14 minutes),
4. activation - Fmoc-AA-OH (0.5 mmol) + diphenylphosphinic anhydride (127) (0.5 mmol) + N-methylmorpholine (0.5 mmol in DMF (1.0 ml)) (2 minutes),
5. coupling - activated mixed anhydride transferred to the reaction vessel + N-methylmorpholine (0.5 mmol)/2,6-lutidine (2.0 mmol) (in DMF (3.0 ml)) (20 minutes),
6. washing - DMF (2 x, total time = 3 minutes),
7. repeat the activation and coupling steps three times,
8. washing - DMF (5 x, total time = 5 minutes),
9. capping - acetic anhydride (1.0 ml of a 0.5M solution in DMF) + pyridine (1.0 ml of a 0.5M solution in DMF) (2 x, 2.5 + 3.5 minutes).

The cycle was terminated with the removal of the N$^\alpha$-protecting group from the threonine residue and the subsequent washings.

The cleavage of the peptide from the support (and the simultaneous removal of the 'Bu side-chain protecting groups) was performed with trifluoroacetic acid/water/thioanisole (95:5:5) (33 ml). The reaction mixture was agitated in an ultrasonic bath for 90 minutes at room temperature, before the resin was filtered off and washed with DMF (6 x 10 ml). The combined filtrates were concentrated in vacuo to give a yellow solid, which was washed with diethyl ether and filtered. Amino acid analysis of the crude material gave the following: Thr$_1$0.89, Ser$_1$0.89, Gly$_1$1.15, Ile$_1$1.01, Leu$_1$1.05. An aliquot of this material was purified by preparative hplc, employing an Aquapore C$_{18}$ reverse phase prep. column, with
water/acetonitrile (+0.05% TFA) as eluent and monitoring at 229 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.057 g, calculated yield = 60%) as a white solid: $\delta_H$ (360 MHz, $D_2O$) 4.50 (2H, m, Leu $\alpha$CH, Ser $\alpha$CH), 4.29 (1H, d, J 7.3 Hz, Ile $\alpha$CH), 4.17 (1H, m, Thr $\beta$CH), 4.00 (2H, s, Gly CH$_2$), 3.91 (1H, d, J 6.4 Hz, Thr $\alpha$CH), 3.85 (2H, m, Ser $\beta$CH$_2$), 1.94 (1H, m, Ile $\beta$CH), 1.67 (3H, m, Leu $\beta$CH$_2$/yCH), 1.50 (1H, m, Ile yCH), 1.34 (3H, d, J 6.5 Hz, Thr CH$_3$), 1.24 (1H, m, Ile yCH), 0.98–0.88 (12H, m, 2 x Leu CH$_3$, 2 x Ile CH$_3$); m/z (FAB), 490 (MH$^+$). 474, 446, 201, 173; $hrms$, found 490.28769, C$_{21}$H$_{40}$N$_5$O$_8$ requires 490.28767 (< 1 ppm); amino acid analysis, Thr$_1$ 0.99, Ser$_1$ 0.94, Gly$_1$ 1.07, Ile$_1$ 0.99, Leu$_1$ 1.01; hplc $R_t$ (B11) 23.5 minutes.

(B) Bnpeoc/DIC (HOBt) methodology

The synthesis of (148) was performed using the functionalised resin, Bnpeoc–Gly–(O–CH$_2$C$_6$H$_4$–OR) (113) on a 0.25 mmol scale, employing Bnpeoc N$\alpha$-protected amino acid derivatives and a combination of DIC and HOBt activation. The side-chain hydroxyl functions of the serine and threonine residues were protected as $^t$Bu ethers (see above for the preparation of Bnpeoc–Ser($^t$Bu–OH (119) and Bnpeoc–Thr($^t$Bu–OH (124)). A 'double couple' cycle was used as outlined above.

The peptide was deprotected, removed from the support, and manipulated as above to give a yellow solid. This material was purified by preparative hplc, employing an Aquapore C$_{18}$ reverse phase prep. column, with water/acetonitrile (+0.05% TFA) as eluent and monitoring at 229 nm. The removal of the hplc solvents by lyophilisation afforded the title compound (0.041 g, 33%) as a white solid: $\delta_H$ (360 MHz, $D_2O$) 4.54 (2H, m, Leu $\alpha$CH, Ser $\alpha$CH), 4.30 (1H, d, J 7.3 Hz, Ile $\alpha$CH), 4.18 (1H, m, Thr $\beta$CH), 3.99 (2H, s, Gly CH$_2$), 3.91 (1H, d, J 6.4 Hz, Thr $\alpha$CH), 3.88 (2H, m, Ser $\beta$CH$_2$), 1.95 (1H, m, Ile $\beta$CH), 1.67 (3H, m, Leu $\beta$CH$_2$/yCH), 1.51 (1H, m, Ile yCH), 1.35 (3H, d, J 6.5 Hz, Thr CH$_3$), 1.24 (1H, m, Ile yCH), 0.99–0.86 (12H, m, 2 x Leu CH$_3$, 2 x Ile CH$_3$); m/z (FAB), 490 (MH$^+$), 257, 234, 199; $hrms$, found 490.28770, C$_{21}$H$_{40}$N$_5$O$_8$ requires 490.28767 (< 1 ppm); amino acid analysis, Thr$_1$ 0.99, Ser$_1$ 0.92, Gly$_1$ 1.06, Ile$_1$ 1.02, Leu$_1$ 1.01; hplc $R_t$ (B11) 23.6 minutes.
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245. K. Shaw, personal communication.

246. J. Henry, personal communication.


Courses Attended

Organic research seminars - various speakers, University of Edinburgh.

Current topics in organic chemistry - various speakers, University of Edinburgh.

X-ray crystallography - Dr. R. O. Gould and Dr. A. J. Blake, University of Edinburgh.

N.m.r. spectroscopy - Dr. I. H. Sadler, University of Edinburgh.

Mass spectroscopy - Prof. K. R. Jennings, Warwick University.

Medicinal chemistry - various speakers, ICI and Beecham.

Medicinal chemistry - Prof. P. G. Sammes, SK & F.

Cell biology - Dr. J. Phillips, Biochemistry Department, University of Edinburgh.