A SOLID PHASE APPROACH TOWARDS THE SYNTHESIS OF UBIQUITIN

by

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To my parents, Bill and Muriel

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ABSTRACT

A previously developed novel linking group, 4-hydroxymethyl-β-(trimethylsilyl)hydrocinnamic acid, has been modified further for use in the solid phase synthesis of fully protected peptides to facilitate a fragment assembly approach to the synthesis of ubiquitin, a biologically very important polypeptide. An organosilyl benzyl ester is used as the peptide-resin link and this leads to a mild cleavage by fluoride ion thus providing the potential for an orthogonal protection scheme in conjunction with acid- and base-labile groups. The original linker molecule has been enlarged in different ways to enable an acceptable degree of its coupling to the solid support and of successful peptide chain construction to be obtained, and the feasibility of various strategies for achieving these aims has been investigated. This type of link has been applied to the syntheses of four peptides with good cleavage yields and acceptable purity of peptide products, culminating in the synthesis of fully protected ubiquitin (1-35). It has been utilised along with Fmoc N^{α}-protection, t-butyl-derived side-chain protection, and symmetrical anhydride/active ester activation involving a commercially available automated synthesiser.

CONTENTS

ABBREVIATIONS

aa	amino acid
Acm	acetamidomethyl
ATP	adenosine 5'-triphosphate
Bnpeoc	2,2-bis(4-nitrophenyl)ethoxycarbonyl
Boc	t-butyloxycarbonyl
Bpoc	2-(4-biphenyl)isopropyloxycarbonyl
Bzh	benzhydryl
DBN	1,5-diazabicyclo[4.3.0]non-5-ene
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCCI	1,3-dicyclohexylcarbodiimide
DCM	dichloromethane
DCU	1,3-dicyclohexylurea
Ddz	α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl
DICI	1,3-diisopropylcarbodiimide
DMA	N,N-dimethylacetamide
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DNA	2'-deoxyribonucleic acid
Dpp	diphenylphosphinyl
Fmoc	9-fluorenylmethoxycarbonyl
H-Aua-OH	11-aminoundecanoic acid, *i.e.* $H_2N(CH_2)_{10}COOH$
H-Nle-OH	norleucine, *i.e.* $H_2NCH((CH_2)_3CH_3)COOH$
HOBt	1-hydroxybenzotriazole
HO-Ⓗ-COOH	4-hydroxymethyl-β-(trimethylsilyl)-hydrocinnamic acid

HPLC	high-performance liquid chromatography
Me	methyl
NMM	N-methylmorpholine
NMR	nuclear magnetic resonance
ONSu	N-oxysuccinimide
Ph	phenyl
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
Poc	2-phenylisopropyloxycarbonyl
-Ⓡ	resin support
TBAF	tetrabutylammonium fluoride
t-Bu	tertiary-butyl
Teoc	2-(trimethylsilyl)ethoxycarbonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin-layer chromatography
TMS	trimethylsilyl
Tmz	α,2,4,5-tetramethylbenzyloxycarbonyl
Tos	*p*-toluenesulphonyl
Ub	ubiquitin
X	N^{α}-protecting group
Z	benzyloxycarbonyl

CHAPTER 1 : INTRODUCTION

1.1 UBIQUITIN

1.1.1 Introduction

Ubiquitin (see Fig. 1.1) is a 76 amino acid residue protein (m.w. 8565) and was first reported by Goldstein *et al.*[1] in 1975 during work on the isolation of thymic hormones. It was shown subsequently to occur in all living cells and its amino acid sequence is conserved to an extent unparalleled among known proteins[2,3]; the fact that there are only 3 amino acid residue variations between yeast and human ubiquitin (see Fig. 1.2) illustrates the almost total conservation of its primary structure across the whole spectrum of species.

These observations clearly suggested that ubiquitin is involved in basic cellular functions, although it took several years before a clear picture began to emerge, in no small part due to the fact that information about the biological roles of ubiquitin was furnished from seemingly unrelated areas of study, *e.g.* the isolation and characterisation of thymic hormones, the structure of chromatin, and proteolysis of cellular proteins.

1.1.2 The Ubiquitin Gene

Cloning of ubiquitin-coding DNA sequences from a variety of eukaryotes has revealed that ubiquitin-

H-Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-
Lys-Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-Ser-
Asp-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-
Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-
Gln-Arg-Leu-Ile-Phe-Ala-Gly-Lys-Gln-Leu-
Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Tyr-Asn-
Ile-Gln-Lys-Glu-Ser-Thr-Leu-His-Leu-Val-
Leu-Arg-Leu-Arg-Gly-Gly-OH

Figure 1.1 - Amino Acid Sequence of Human Ubiquitin

	Ubiquitin Source		
Residue No.	Animal[a]	Plant[b]	Yeast
19	Pro	Ser	Ser
24	Glu	Asp	Asp
28	Ala	Ala	Ser
57	Ser	Ala	Ser

a. Animal refers to ubiquitin isolated from man, cattle, mouse, chicken, toad and fly.
b. Plant refers to ubiquitin isolated from oat and barley.

Figure 1.2 - Amino Acid Sequence Variants of Ubiquitin

coding elements are typically organised into head-to-tail arrays with no internal spacers, and so it appears that ubiquitin is generated by the processing of a polyubiquitin precursor protein. Yeast polyubiquitin[4] (see Fig. 1.3) starts with the N-terminal methionine residue of the mature (monomer) ubiquitin, and consists of 6 consecutive repeats of mature ubiquitin joined via Gly-Met bonds followed by a single extra residue, asparagine. The polyubiquitin precursor thus liberates mature ubiquitin upon proteolytic cleavage of Gly-Met and Gly-Asn bonds.

Toad[5], chicken[6], human[7] and plant[8] cells also exhibit ubiquitin-coding genes with multiple consecutive repeats, and while this polyubiquitin organisation is a general feature of ubiquitin genes from yeast through to man, the number of ubiquitin-coding repeats per gene is not highly conserved, e.g. it varies from 6 in yeast, through 9 in man, to possibly more than 12 in toad, and indeed, it probably varies between cell types.

A wide variation is also found in the extra, non-ubiquitin residue, it being asparagine in yeast, tyrosine in chicken and valine in human polyubiquitin. This blocking residue may be present to prevent participation of unprocessed polyubiquitin in ubiquitin-protein conjugation, although it may not be

1 76 1 76 1 76 1 76 1 76 1 76
Met——Gly - Met——Gly - Met——Gly - Met——Gly - Met——Gly - Met——Gly - As

Figure 1.3 - Structural Organisation of Polyubiquitin Precursor Protein in Yeast Deduced from the Nucleotide Sequence of the Yeast Ubiquitin Gene

an essential part of polyubiquitin precursor protein, since the clone from the toad _Xenopus laevis_ has no extra amino acid residue.

In addition, polyubiquitin genes are unusual in the fact that they encode identical amino acid sequences even though they may differ considerably in their nucleotide sequences, thus underlining the fundamental importance of ubiquitin over the huge time-span of eukaryote evolution. Also, it is thought that polyubiquitin genes are specifically required for cell resistance to stress[9].

As exceptions to the polyubiquitin organisation characteristic, however, ubiquitin-coding genes have been found in human[10] and yeast[9,11] cells which encode "hybrid" proteins consisting of a ubiquitin unit bound to another short, basic protein. In humans, for example, a 516 base pair DNA encodes a protein consisting of ubiquitin attached to an 80 amino acid sequence that bears no obvious resemblance to ubiquitin, and indeed, there is a good deal of similarity between the basic, non-ubiquitin portions of the human and yeast proteins. The high basicity of the non-ubiquitin portion suggests that its intended destination is the cell nucleus.

1.1.3 Ubiquitin-Histone Conjugation

The structure elucidation of the chromosomal protein A24 (now called semihistone uH2A)[12] provided the first clue to the functions of ubiquitin. Goldknopf, Busch and co-workers carried out detailed sequence analyses which showed that uH2A had a branched structure[13,14] in which the ε-amino group of lysine 119 of histone 2A forms an amide bond with the C-terminal glycine of ubiquitin, and subsequent work has shown similar ubiquitin conjugates with sub-types of histone 2A and with histone 2B. Unlike other ubiquitin conjugates, however, it is not thought likely that they are intermediates in histone proteolysis[15] (see later), since for example, most H2A moieties in uH2A are not degraded as a result of ubiquitin conjugation[16]. Thus, if ubiquitin is used to mark specific chromosomal regions, then recognition of ubiquitin in its conjugates to histones and other chromosomal proteins might be involved in processes other than proteolysis.

The ubiquitin moiety in uH2A and uH2B is in a rapid equilibrium with a pool of free ubiquitin in interphase cells[17], and these semihistones represent about 10% of histone 2A[18] and 1-2% of histone 2B[19] and are heavily involved with the nucleosomes[18]. Levels of uH2A throughout the cell cycle have been studied in various cell types, for example, the slime mould Physarum polycephalum[20], a eukaryotic organism which facilitates precise observations as a result of the synchronous division of its nuclei. It was found in this case that in the early prophase uH2A.1, uH2A.z and uH2B were strongly present, only to disappear minutes later in metaphase and reappear again in anaphase. These results suggest that ubiquitin cleavage and recoupling is a very important event in the cell cycle. At the present time, however, these and other similar results and their implications for the role of ubiquitination of histones in chromatin structure are not fully understood, and certainly further study is necessary.

1.1.4 Ubiquitin in Proteolysis

This is the most active area of ubiquitin research, and Hershko, Rose and Haas have been in the forefront of the increasing understanding of ubiquitin's biological role in an ATP-dependent proteolytic system[16]. It has been established that ubiquitin becomes covalently bonded to proteins bound for ATP-requiring degradation[21] and proposed that multiple ubiquitin attachment is a prerequisite[22] for the degradation of the substrate protein by proteases. In common with the nuclear protein uH2A[14], the covalent bond involves the ε-amino groups of lysine residues[22]. The ubiquitin-dependent proteolytic pathway[23] is non-lysosomal in nature, as is the degradation of short-lived and abnormal intracellular proteins under normal metabolic conditions, and indeed the ubiquitin-dependent system has been shown to act upon abnormal proteins and is believed to be involved in the degradation of short-lived proteins[24].

When proteins destined for degradation conjugate to ubiquitin, multiple conjugates are found in which a single substrate molecule is bound to several molecules of ubiquitin[22,25], but these high molecular weight species are quickly degraded by the ubiquitin system[26]. Conjugates with a greater than expected molecular weight, however, have been observed[25,26] e.g. lysozyme contains 7 amino groups (6 lysine residues and the N-terminus) and the calculated molecular weight of Ub_7-lysozyme is 74,000, but electrophoresis showed more than 12 bands, of which the highest has an apparent molecular weight of over 100,000. Blocking of the amino groups of ubiquitin by methylation with formaldehyde and sodium cyanoborohydride[25] prevented formation of these higher molecular weight conjugates. These results indicate that ubiquitin can form a conjugate with an amino group of another ubiquitin molecule which is already bound to the protein substrate, so possibly increasing the degradation rate of the substrate. Reductive methylation of ubiquitin and subsequent conjugation to ^{125}I-labelled lysozyme supported this conclusion when only 7 prominent bands were observed on electrophoresis. Cyanogen bromide treatment of the ubiquitin-lysozyme conjugates failed to cleave any ^{125}I-labelled ubiquitin, so eliminating the possibility that multiply ubiquitinated lysozyme was obtained from head-to-tail polyubiquitin, as the gene sequence might suggest.

While the formation of conjugates between the ε-amino groups of lysine residues and the C-terminus of ubiquitin is well documented, N-terminal conjugation to ubiquitin has also been shown[27] and even appears to be essential for degradation to take place. Degradation of globin and lysozyme by the ubiquitin proteolytic system was prevented by selective modification of the α-amino groups, thus supporting this conclusion. In addition, however, α-amino-blocking of proteins prevents ubiquitin conjugation to ε-amino groups, and in proteins where the ε-amino groups are blocked but the α-amino group is free, degradation does occur but at a slower rate. This would suggest that a free α-amino group is an essential requirement for degradation and that conjugation of ubiquitin to this α-amino group initiates the formation of ε-amino conjugates and subsequent degradation. Thus, degradation of proteins in vivo may be prevented by their acetylation.

Further studies on ε-amino-blocked lysozyme[28] found that degradation in an ATP-dependent proteolytic system was stimulated by ubiquitin addition without, however, the presence of an α-amino-ubiquitinated derivative being observed. These results are in accordance with the hypothesis that several ATP-dependent proteolytic pathways exist[29], and that ubiquitination of substrates is not essential. Substrates that cannot undergo ubiquitin conjugation, i.e. proteins with blocked amino groups, can still compete with substrates which can be ubiquitinated for binding to the enzymes that promote ubiquitin conjugation of recognised potential substrates of the ubiquitin pathway[30]. The fact that these amino-blocked proteins have inhibiting properties in turn suggests that recognition of the polypeptide backbone may be a common factor in binding to ubiquitin-conjugating enzymes (see later).

To help elucidate the functional importance of N-terminal ubiquitination of proteins, a gene for encoding ubiquitin bound to the N-terminus of the bacterial protein β-galactosidase, a long-lived protein, was constructed. The resulting hybrid protein in the yeast Saccharomyces cerevisiae[31] was cloned, but as soon as the gene product was made, the cells cleaved the ubiquitin from the β-galactosidase. So, by changing a gene, a selection of hybrids with different amino acids at the N-terminus of β-galactosidase was made in an attempt to fool the enzyme responsible for cleaving the hybrid; but to no avail. As before, deubiquitination occurred, irrespective of the N-terminus of the β-galactosidase. Although this was a disappointment, a nevertheless completely unexpected result was obtained. Synthesis of β-galactosidase variants could be carried out with any N-terminal amino acid, in contrast to the standard methionine residue (which every protein must have since AUG, the genetic base code for methionine, is also the start signal for protein synthesis). In addition, they then discovered that the in vivo half-lives of the β-galactosidase proteins were dependent on the N-terminal amino acid, varying between more than 20 hours and less than 3 minutes. This enabled them to propose a simple rule for the stability of proteins, viz. the "N-end rule" (see Fig. 1.4). This rule agrees with the known half-lives of all known cellular proteins with identified N-termini, although these are almost all long-lived proteins due to the difficulty in synthesising and sequencing unstable proteins.

1.1.5 Ubiquitin and the Heat-Shock Response

One of the most interesting aspects of the heat-shock response is the communication between the environment and the genetic apparatus: somehow the cell detects a rise in ambient temperature, or some other environmental stress, and rapidly and specifically alters its pattern of gene expression, i.e. heat-shock proteins are produced. This response has the effect of accumulating denatured or damaged proteins within the cell. In normal circumstances these proteins would be quickly degraded by the ubiquitin proteolytic system, but after heat-shock, efficient degradation of abnormal

Residue Y in Ub-Y-β-Galactosidase	Half-life of Y-β-Galactosidase
Met, Ser, Ala, Thr, Val, Gly	> 20 hours
Ile, Glu	~ 30 minutes
Tyr, Gln	~ 10 minutes
Phe, Leu, Asp, Lys	~ 3 minutes
Arg	~ 2 minutes

Figure 1.4 - Stability of Proteins

proteins does not occur.

Recent studies on fruit fly, mouse and chicken cells[32,16,6], while demonstrating the involvement of the ubiquitin system, have failed to identify positively the trigger for the heat-shock response, i.e. whether it is a lack of free ubiquitin or a failure to ubiquitinate proteins. The behaviour of temperature-sensitive mouse cells (ts85) argues against the first possibility, since at the non-permissive temperature they cannot couple ubiquitin to anything, and so presumably have high levels of free ubiquitin. One theory[33], however, suggests that in normal cells a heat-shock transcription factor is suppressed by ubiquitination, an analogous situation to that of the histones being in equilibrium with a pool of free ubiquitin. Treatments of the cell which either inhibit ubiquitinating enzymes or produce a large pool of abnormal protein substrate would allow accumulation of non-ubiquitinated active factor so promoting transcription of heat-shock genes. A simplified version of this theory[34] suggests that ubiquitinated histones by themselves may be enough to prevent heat-shock gene transcription. A system such as this would be inherently self-regulating: as soon as either degradation of the abnormal proteins or increased synthesis of ubiquitin made sufficient free ubiquitin available, the synthesis of heat-shock proteins would be repressed.

More recent work[35] into chicken cells, however, does not support hypotheses invoking ubiquitin as a factor in stress protein induction. Schlesinger and co-workers found the change in the free ubiquitin pool during stress to be insignificant compared to its size, i.e. during stress there is still an ample supply of ubiquitin. This led them to postulate a model in which stress turns on signals, as yet undefined, which lead to a shutdown of nuclear activity. When the stress conditions are removed, the cell returns to its original metabolic state, with the heat-shock proteins providing for some of those functions that are needed to re-establish the former state of the cell. Since heat-shock proteins also serve to protect cells from damage imposed by the stress, this model proposes a dual function for stress proteins.

1.1.6 Ubiquitin at the Cell Surface

In contrast to all the previously mentioned functions of ubiquitin which take place in the cell nucleus or cytoplasm, recent studies have shown ubiquitin's influence to extend to the outer membrane of the cell. Weissman and his colleagues[36,37,38] have shown that ubiquitin is part of the receptor by which lymphocytes home in on and enter the lymph nodes, an activity that is necessary for normal immune responses.

They also showed the existence of a series of ubiquitinated cell surface proteins, including the glycopeptide example with a molecular weight of 90,000 involved in the lymphocyte receptor, and so it would appear that ubiquitination of cell surface proteins is fairly widespread.

Lymphocytes enter the lymphoid organs through specialised blood vessels called postcapillary high endothelial venules, and Weissman's studies suggest that the ubiquitin moiety may be critical for the lymphocyte-endothelial cell interaction that is mediated by the homing receptor. They also indicate that its conformation at the homing receptor is different from that of free ubiquitin or of ubiquitin bound to other proteins. Such conformational changes may be a result of interactions with the glycoprotein part of the homing receptor and might enable ubiquitin to contribute to specific receptor interactions. Such an explanation needs to be advanced as the presence alone of ubiquitin, whose distribution is widespread, does not seem to provide the basis for the highly selective recognition factor required of lymphocyte homing receptors.

As to how ubiquitin gets to the cell surface, Weissman suggests that it is added to the receptor protein in the cytoplasm during the protein synthesis when it is exposed to ubiquitin, before being transported to the cell membrane.

1.1.7 Ubiquitin Structure

The three-dimensional structure of human erythrocytic ubiquitin has been determined by Cook and his colleagues by X-ray analysis at 2.8Å resolution[39], with a further refinement to 1.8Å resolution[40]. Similar crystal structures[41] are found with the yeast and oat ubiquitin sequence variants.

A compact globular structure for ubiquitin, stable over a wide pH and temperature range, was indicated by NMR investigations[42,43], and the X-ray analysis supported this in revealing a markedly hydrophobic core and a tightly hydrogen-bonded secondary structure. The prominent features of this secondary structure include 3.5 turns of α-helix (residues 23 to 34), a short piece of 3_{10}-helix (residues 56 to 59), a mixed β-sheet that contains 5 strands, and 7 reverse turns, thus providing a significant contrast to previous circular dichroism studies[44].

The C-terminal region protrudes from the globular structure rather like a tail and thus is not involved with the remainder of the structure by means of either hydrophobic interactions or hydrogen bonding. This means that the active centre of the molecule can be accessed easily by the enzymes involved in the formation and cleavage of isopeptide bonds. This is consistent with amide bonding between the ubiquitin C-terminus and lysine ε-amino groups which is a common factor in the various biological functions of ubiquitin. Electron density maps of the C-terminal region are of poorer definition than the rest of the molecule since this region requires and has a considerable freedom of motion, and also the ease of proteolysis of the C-terminal GlyGly dipeptide in ubiquitin isolation[45] probably gives rise to a crystal sample which is heterogeneous.

The N-terminal methionine residue is hidden deep in the hydrophobic core of the molecule, effectively making the N-terminus inaccessible and so preventing proteolysis of ubiquitin by its own pathway. Looking at the structure of the ubiquitin gene, however, with the precursor protein having head-to-tail repeat units of ubiquitin joined C-terminus of one molecule to N-terminus of the next, considerable changes in conformation must be forced upon the liberated individual ubiquitin molecules.

A recent study[46] of mutants of ubiquitin has shown that in almost every case investigated, the mutations only affected the overall structure at the points where they occurred, and that these minor changes only affected the biological activity when they occurred at points of contact between ubiquitin and enzymes of its pathway. Mutations of the C-terminal region completely destroyed ubiquitin's biological activity. This should thus provide an interesting model for further study of protein-protein interactions during enzymatic reactions.

1.1.8 Ubiquitin Fragment Synthesis

The hexadecapeptide fragment 59-74 of ubiquitin has been chemically synthesised by two groups using different synthetic strategies. Schlesinger et al.[47] used Merrifield's solid phase strategy[48], employing Boc-protected amino acids[49] activated with DCCI[50]. Abiko and Sekino[51] on the other hand employed a solution phase strategy involving three fragment couplings. Again, they used α-amino-Boc protection, with the stepwise couplings being carried out with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide [52] and HOBt[53]. The fragment couplings were performed using the azide method of carboxyl activation.

The products of both syntheses were biologically assayed, and in both cases the fragments were found to have a significant proportion of the activity of ubiquitin. It was deduced, therefore, that the synthetic fragment incorporates the portion of ubiquitin necessary for biological activity, and that the full tertiary structure of ubiquitin is not essential for activity in the assays undertaken. These results, however, would seem to contradict the evolutionary conservation of the ubiquitin molecule which suggests that there are very strong reasons as to why the sequence is so highly conserved. Also, the lack of the C-terminal GlyGly dipeptide, which is required for activity[54,55], from the fragment casts doubts over the dependability of these results.

1.2 SOLID PHASE PEPTIDE SYNTHESIS

1.2.1 Introduction

Peptides are a class of naturally occurring compounds having important and varied roles in biology; for example, as hormones, releasing factors, growth factors, ion carriers, antibiotics, toxins and neuropeptides. This biological importance has prompted a great amount of study into peptide synthesis with a view to understanding the effect of peptide structure on biological activity.

In 1903 Fischer provided the breakthrough when he synthesised the first peptide and coined the term[56]. The basic requirements of this achievement were to block the carboxyl group of one amino acid and the amino group of the other amino acid to prevent formation of a whole family of possible products. Subsequent activation of the free carboxyl group would then facilitate the formation of the peptide bond, and then selective removal of the two protecting groups would give the free dipeptide. Fischer, however, never managed to find a suitable temporary protecting group for the amino function, but a former student of his, Bergmann, along with Zervas, successfully produced such a group[57] in 1932. Their use of the Z group helped considerably in the production of peptide chains, and as a result, this versatile urethane-type amino protection was modified extensively to suit a wide variety of conditions in the following years[58].

Further advances were made in peptide synthesis in 1951 when Wieland published results on amino acid carboxyl activation by formation of mixed anhydrides with ethyl chloroformate[59] and by formation of active esters with thiophenol[60]. The mixed anhydride approach was also described at the same time by Boissonnas[61] using ethyl chloroformate, and by Vaughan[62] using isobutyl chloroformate. These developments contributed to du Vigneaud's 1954 synthesis of oxytocin[63], the first reported synthesis of a peptide hormone.

A major leap forward, however, was made in 1963 when Merrifield conceived the idea of synthesising peptides on an insoluble polymeric support[48], a development which earned him the Nobel Prize in 1984[64]. This technique, the first example of the use of polymers in performing organic synthetic transformations, was developed to combat problems faced by solution phase methodology, such as possible poor solubility of large or protected peptides. Also, the steps of protection, coupling, deprotection, isolation and characaterisation of intermediates are time-consuming and are a discouragingly repetitive exercise.

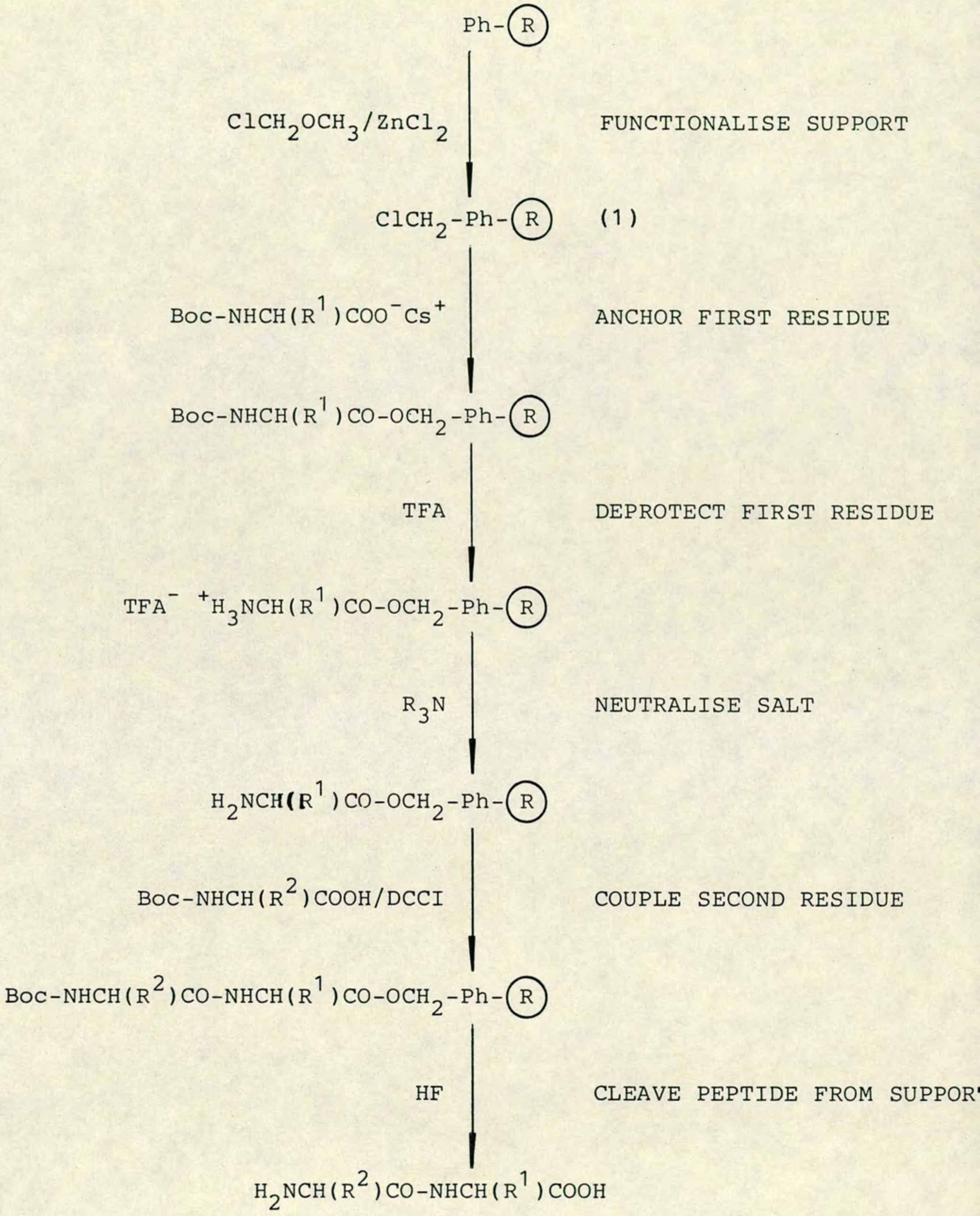
1.2.2 The Solid Phase Principle

In the solid phase system of peptide synthesis, the C-terminal amino acid of a desired peptide chain is attached to an insoluble solid support and the chain subsequently extended towards the N-terminus by stepwise coupling of activated amino acid derivatives. Filtration and washing removes any excess soluble reagents and by-products, and after the chain has been assembled it can be cleaved from the support and purified. This protocol confers several advantages: (a) intermediate purification is eliminated, (b) reactions can be driven to completion by using excess reagents, (c) vessel transfer is eliminated so cutting down mechanical losses and (d) automation is possible.

There are several requirements, however, for the polymer to meet in order to be a suitable support. It must be insoluble to enable filtration, and rigid to give good mechanical properties. A significant degree of functionalisation must be obtainable. Also, the matrix should swell in appropriate solvents, with good accessibility to solvents and reactants, whether swelled or rigid. Accessibility of the functional groups can be improved by incorporation of a handle or spacer molecule between the functional group and the polymer backbone. In addition, the functionalised support should have no side reactions, be physiochemically compatible with the bound substrate, reagents and solvents, and be able to be regenerated. Many varied supports and linkers/

spacers have been developed with the above considerations in mind and these are described later.

The standard system for solid phase peptide synthesis[64] is shown in Scheme 1. The support is a synthetic resin made from copolymerisation of styrene with 1% divinylbenzene to give crosslinking[48]. Functionalisation is achieved using chloromethylation to introduce benzylic chloride groups into the polymer. Ester bond formation between the protected amino acid salt and the chloromethyl group of the resin (1) then anchors the first residue to the polymer. Subsequent removal of the Boc group with 50% TFA in DCM followed by neutralisation of the resultant salt with a tertiary amine produces the free amino group of the resin-bound amino acid. The second amino acid in the chain is then activated with DCCI[50] and coupled to the first residue, although this coupling can also be carried out using anhydrides[65] or active esters[66]. To extend the peptide chain, the deprotection, neutralisation and coupling steps are repeated until the desired sequence has been constructed, and the completed peptide finally is deprotected and cleaved from the solid support. With the chemistry employed here, this is done using a strong anhydrous acid such as HF[67]. The free peptide product can then be purified and characterised.


Ph-(R)
ClCH2OCH3/ZnCl2
FUNCTIONALISE SUPPORT
ClCH2-Ph-(R)
(1)
Boc-NHCH(R1)COO−Cs+
ANCHOR FIRST RESIDUE
Boc-NHCH(R1)CO-OCH2-Ph-(R)
TFA
DEPROTECT FIRST RESIDUE
TFA− +H3NCH(R1)CO-OCH2-Ph-(R)
R3N
NEUTRALISE SALT
H2NCH(R1)CO-OCH2-Ph-(R)
Boc-NHCH(R2)COOH/DCCI
COUPLE SECOND RESIDUE
Boc-NHCH(R2)CO-NHCH(R1)CO-OCH2-Ph-(R)
HF
CLEAVE PEPTIDE FROM SUPPOR
H2NCH(R2)CO-NHCH(R1)COOH

Scheme 1

All of these reactions are carried out under non-aqueous conditions in organic solvents that swell the resin and give acceptable reaction rates. DCM and DMF were found to be the best. Also, due to the presence of highly reactive acylating groups in excess, all functional groups on amino acid side-chains must be protected before being liberated again at the end of the synthesis. It is very important that the steps which are repeated proceed rapidly, in high yields, and with the minimum of side reactions so as to prevent accumulation of by-products.

In nature, proteins are formed in a stepwise manner starting from the N-terminus and working towards the C-terminus, and one of the earliest attempts at solid phase peptide synthesis employed this strategy using activation of peptide intermediates in the form of mixed anhydrides and coupling to esters of single amino acids[68]. A major problem here, however, is the lack of protection against racemisation since the activated residues are not provided with a urethane-type protecting group for the amino function. In contrast, no significant racemisation occurs when constructing the chain from the C-terminus, the method of choice for solid phase peptide synthesis today.

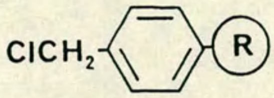
One other aspect which needs to be considered is that of reaction monitoring. The degree of completion of the deprotection and coupling reactions dictates the purity of the final peptide product, and even a departure of as little as 1% from completion can give deleted peptides and failure sequences. Thus, the deprotection and coupling steps must give at least 99% completion. Several analytical methods have been used to monitor the various steps, the most popular of which is the Kaiser ninhydrin test. This test can be used either qualitatively[69] or quantitatively[70] to monitor deprotection, coupling, or chain termination. Other methods include the fluorescamine test[71], titration with perchloric acid[72] or picric acid[73], or continuous monitoring of the ultraviolet absorbance of the reaction solution[74]. Each of these methods has its pros and cons, although over the piece, the Kaiser ninhydrin test appears to be the most practical. As an additional aid to the above techniques, insertion of an unnatural amino acid as an internal reference[75] helps to determine peptide loss from the resin by means of amino acid analysis.

The major plus, however, concerning solid phase peptide synthesis is the possibility of automation, and indeed several automated peptide synthesisers are now on the market. Even so, automation can only go as far as the chemical constraints allow, and can only fulfil its true potential when these problems are under control.

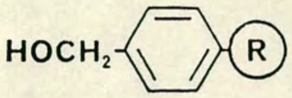
1.2.3 The Solid Support

A suitable solid support (see before) and a satisfactory method of binding the first amino acid to it are of crucial importance for successful solid phase syntheses. In the case of the Merrifield support, 1% divinylbenzene was found to afford the polymer the best mechanical properties; that with 2% was too rigid with insufficient swelling, while that with less than 1% was very soft and could not be filtered or manipulated in the usual way. A pure polystyrene polymer is soluble in the organic solvents used. The chloromethyl resin (1), however, has some problems associated with it. In the esterification procedure, a possible side reaction is the formation of quaternary ammonium salts on the resin, and these salts give the polymer the characteristics of an anion-exchange resin so possibly causing problems during the synthesis. Also, amino acids which contain easily alkylatable functional groups, e.g. histidine, cysteine and methionine, cause difficulties in the esterification reaction.

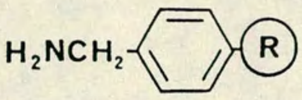
These problems can all be avoided by altering the functional group on the resin (see Fig. 1.5). The chloromethyl group can be modified with potassium acetate and hydrazine[76] to give a hydroxymethyl resin (2), or with potassium phthalimide and hydrazine[77] to give an aminomethyl resin (3) which can also be produced directly from the polystyrene-1% divinylbenzene



(1)



(2)



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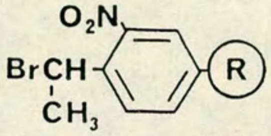

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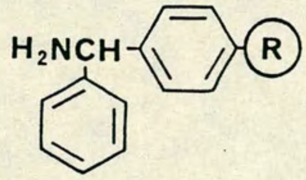
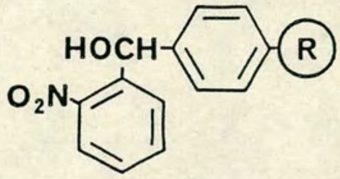


(5)



(6)

Figure 1.5 - Resins Based on Polystyrene-1% Divinylbenzene Copolymer

copolymer to avoid use of the carcinogenic chloromethyl methyl ether. Other modifications to the chloromethyl group by treatment with various alkylamines to produce a range of N-alkylamine resins have been performed and used to synthesise several peptide N-alkylamides[78], but other workers have reported problems with additional crosslinking in such cases[79] casting doubts over the widespread applicability of such resins. Also, a 4-step modification of the copolymer has been carried out recently[80] to produce a photosensitive o-nitro(α-methyl)bromobenzyl resin (4) allowing the synthesis of fully protected peptides to be performed.

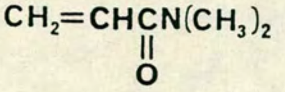
Treatment of the original copolymer with N-(α-chlorobenzyl)phthalimide and hydrazine[81] yields a benzhydrylamine resin (5) which is useful for preparing peptide amides, and again, a recent 2-step modification of the copolymer to produce a photosensitive 2'-nitrobenzhydrol resin (6) has been reported[82] resulting in a greater acid-stability than that of (5).

A new direction was taken by Sheppard in 1975[83] when he introduced polyamide resins onto the scene. In the early days of solid phase work, some sequences cropped up which defied all attempts to produce them in good yield. It was found with polystyrene resins that a significant fraction of the growing peptide chains apparently terminated and refused to couple with any subsequent activated Boc-amino acids. He suggested that these problems were due to the hydrophilic peptide and hydrophobic resin having incompatible solvation properties, <u>i.e.</u> solvents which swelled the resin collapsed the peptide chain so losing reactivity and those which opened up the peptide chain collapsed the matrix preventing reagent access. Thus, he developed polyamide resins similar in nature to peptide chains, enabling one solvent to solvate both equally, and they have proved to be useful in synthesising difficult peptide sequences.

The first resin was a copolymer of a mixture of dimethylacrylamide (7) (the basic monomer), N,N'-bis-acryloylethylenediamine (8) (the crosslinking agent) and N-t-butoxycarbonyl-β-alanyl-N'-acryloylhexamethylenediamine (9) (see Fig. 1.6). The last monomer acts as a spacer, provides an internal reference amino acid, and after the cleavage of the Boc protecting group, also provides a primary amino function for subsequent coupling to the first amino acid of a peptide chain.

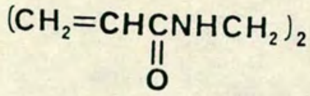
Problems were encountered, however, in the preparation of (9) and in its tendency to produce an amorphous rather than a beaded polymer, and so a new polymeric support was developed[84,85] in which N-acryloysarcosine methyl ester (10) replaced (9) (see Fig. 1.6).

The resultant polymer in this case was readily and reproducibly obtained in a beaded form, and although it



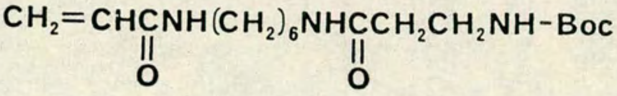
$CH_2{=}CH\underset{\overset{\|}{O}}{C}N(CH_3)_2$

(7)



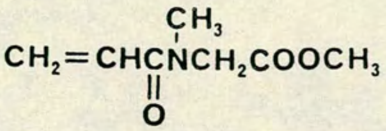
$(CH_2{=}CH\underset{\overset{\|}{O}}{C}NHCH_2)_2$

(8)



$CH_2{=}CH\underset{\overset{\|}{O}}{C}NH(CH_2)_6NH\underset{\overset{\|}{O}}{C}CH_2CH_2NH\text{-}Boc$

(9)



$CH_2{=}CH\underset{\overset{\|}{O}}{C}\overset{CH_3}{\overset{|}{N}}CH_2COOCH_3$

(10)

Figure 1.6 - Monomers Involved in Polyamide Resins

now carried methoxycarbonyl groups rather than protected primary amines, amino functions were readily obtained upon treatment with ethylene diamine. In addition, the close similarity in structure between (7) and (10) suggests a random distribution of (10) and hence functional groups throughout the matrix. Also, the swelling properties of these resins are excellent in a wide range of polar solvents including water, methanol, pyridine, DCM and DMF.

Sheppard subsequently has gone on to prove the utility of these resins in a series of peptide syntheses, as have others with polystyrene resins, although it is difficult to make a comparison between the polyamide and polystyrene resins since there has been only one example, acyl carrier protein (ACP) (65-74), of a careful comparison between them, and similar good results were obtained[85,86]. Recently, however, Sheppard has reported the development of polyamide resin supported on kieselguhr[87], opening up new possibilities for continuous-flow peptide synthesis, which he has indeed utilised.

1.2.4 The Peptide-Resin Link

The standard synthetic scheme for solid phase peptide synthesis (see Scheme 1) employs a benzyl ester linkage between the peptide and the resin. When the chloromethyl resin (1) is used, however, there are the aforementioned problems with quaternary ammonium salt formation on the resin and the alkylation of certain amino acid side-chains. Utilisation of the hydroxymethyl resin (2) prevents this side-chain alkylation and this is a significant advantage. In this case, the esterification of the Boc-amino acid is mediated by DCCI with a catalytic amount of DMAP present[88]; too much DMAP can cause racemisation of amino acids[89].

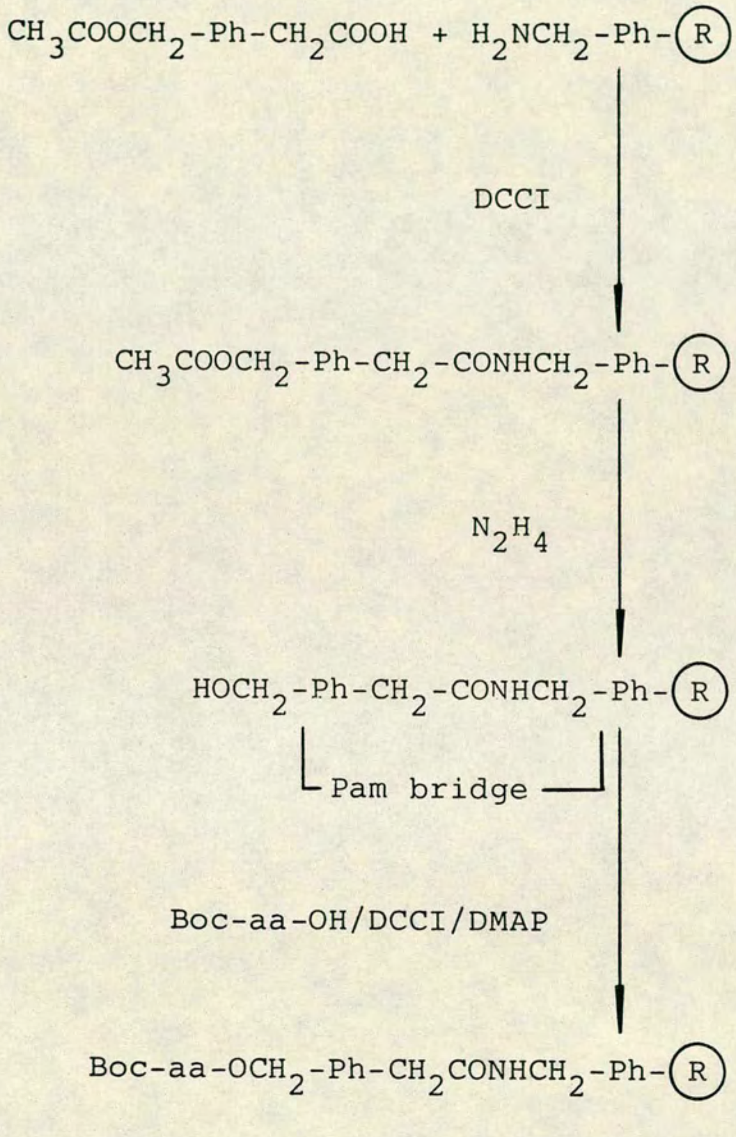
The amino protected support thus obtained, however, is not completely stable under the acidic conditions repeatedly employed for Boc cleavage[90] due to the electron-donating ability of the alkyl chain of the polystyrene resin. Using 50% TFA in DCM for deprotection results in premature cleavage of peptide of about 1.5% per cycle. This production of new hydroxymethyl functionalities can cause late initiation of peptides and formation of deletion peptides which have one or more C-terminal residues missing. Although this situation might be admissable in a short peptide synthesis, it is a major problem when large peptides are being considered.

Several solutions to this problem have been successfully employed, including the use of (a) more acid-labile protecting groups, (b) base-labile protecting groups, or (c) a more acid-stable support. The last of these was described originally by

Merrifield[48] when he nitrated or brominated the chloromethyl resin (1). This produced a significantly more acid-resistant support through the introduction of electron-withdrawing groups on the aromatic ring of the resin. Indeed, the acid-stability of the nitrated support was too great in that saponification with sodium hydroxide was required to cleave the peptide. In the case of the brominated support, it did not swell adequately in DCM and DMF so preventing sufficient access of reagents to the beads, and thus was not satisfactory for general application.

A far more flexible approach to support stability has been the development, and use in the solid phase synthesis of large peptides, of supports with some kind of linker or spacer molecule between the functional group on the resin and the peptide chain. By varying the nature of this linker molecule, varying degrees of acid- or base-lability, or indeed photolability or multidetachability, can be conferred upon the support.

The phenylacetamidomethyl (Pam) resin (12) (see Scheme 2) introduced by Merrifield[91] increases the stability of the peptide-resin link by a factor of 100 over that of the classical system (see Scheme 1); this 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. This destabilises the developing carbocation so increasing the stability to



(11) $CH_3COOCH_2\text{-Ph-}CH_2COOH + H_2NCH_2\text{-Ph-}Ⓡ$ (3)

$$\Big\downarrow \text{DCCI}$$

$$CH_3COOCH_2\text{-Ph-}CH_2\text{-CONH}CH_2\text{-Ph-}Ⓡ$$

$$\Big\downarrow N_2H_4$$

(12) $HOCH_2\text{-Ph-}CH_2\text{-CONH}CH_2\text{-Ph-}Ⓡ$

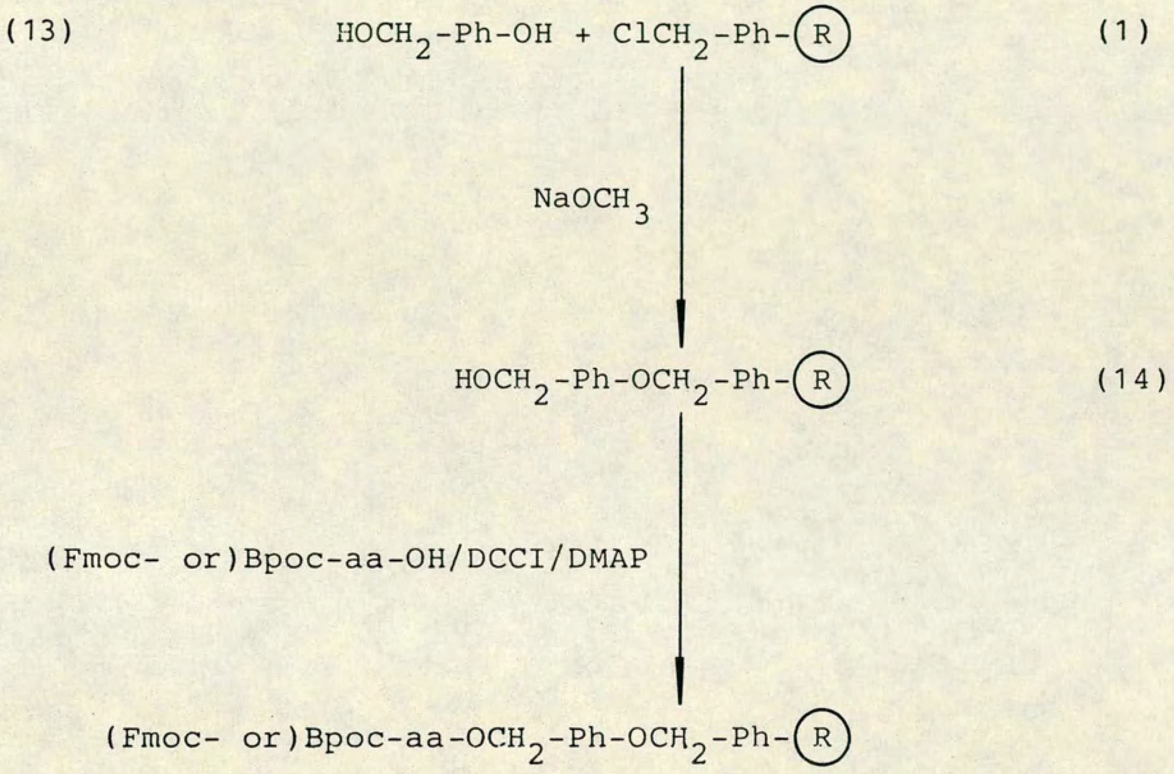
$$\Big\downarrow \text{Boc-aa-OH/DCCI/DMAP}$$

$$\text{Boc-aa-O}CH_2\text{-Ph-}CH_2\text{CONH}CH_2\text{-Ph-}Ⓡ$$

Scheme 2

TFA, while at the same time it has little effect on the S_N2 cleavage of the peptide in liquid HF acidolysis. As a result of this, peptide chain loss during synthesis is reduced to less than 0.01% per cycle and hence production of new hydroxymethyl sites is similarly almost eliminated. In addition, peptide cleavage from the Pam support is almost quantitative[92] under the mild cleavage conditions necessary for sensitive peptides[93]. The more convenient synthesis of the Pam resin (12) (see Scheme 2) involves acetylating the aminomethyl resin (3) with *p*-acetoxymethylphenylacetic acid (11) using DCCI, and removing the acetyl group using hydrazinolysis to yield the desired product. Boc-amino acids can then be esterified in a manner completely analagous to that used with the hydroxymethyl resin (2).

As peptides become larger and more complex, their stability to harsh reagents such as HF and TFA decreases, and for this reason much study has gone into the use of more labile peptide-resin links which allow final peptide cleavage from the resin by a milder reagent. To meet this criterion, the *p*-alkoxybenzylalcohol resin (14) (see Scheme 3) was developed by Wang[94]; in this case, the peptide-resin link is made labile by the electron-donating ability of the ether oxygen in the para position on the ring. Final cleavage of the peptide from the resin is achieved with TFA in DCM, and hence this precludes the use of Boc-amino

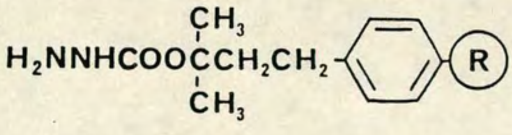

(13)
HOCH$_2$-Ph-OH + ClCH$_2$-Ph-Ⓡ
(1)
NaOCH$_3$
HOCH$_2$-Ph-OCH$_2$-Ph-Ⓡ
(14)
(Fmoc- or)Bpoc-aa-OH/DCCI/DMAP
(Fmoc- or)Bpoc-aa-OCH$_2$-Ph-OCH$_2$-Ph-Ⓡ

Scheme 3

acids. Amino protection here is performed by the Bpoc group[95], and this protocol allows the synthesis of protected peptide free acids if desired by the use of TFA-stable side-chain protection and avoiding exposure of the final peptide to strong acidic reagents such as HF. One possible drawback occurs if TFA-labile t-Bu-derived side-chain protecting groups are used. During the final peptide cleavage reaction, the peptide is exposed to a large concentration of t-Bu carbocations which in some cases may be more harmful than exposure to HF, e.g. tryptophan may be alkylated. Use of this system also incurs the added complication of working with expensive Bpoc-amino acids, which as a result of their extreme lability require to be stored as salts. In spite of these drawbacks, however, this system's ability to produce protected peptide fragments, which can also be done using base-labile Fmoc-amino protection[96] and benzyl-derived side-chain protection, is a distinct advantage. The synthesis of the p-alkoxybenzylalcohol resin (14) has since been improved by Merrifield and co-workers[97] and involves simply heating the chloromethyl resin (1) with p-hydroxybenzylalcohol (13) and sodium methoxide under a precise set of conditions to minimise competing reactions. Bpoc-(or Fmoc-) amino acids can then be esterified (see Scheme 3) as before in the case of the hydroxymethyl resin (2).

Other acid-labile peptide-resin linkages have been developed including those involving modifications to the benzhydrylamine resin (5) used in producing peptide amides. The HF-stability of the peptide-resin link with the original resin (5) was found to be too great in some cases, and hence para-substitution of electron-donating groups in the free phenyl ring was carried out to give increased acid-lability. Two such moieties introduced were the methyl group[98] and the methoxy group[99].

Protected peptide hydrazides can be made using a hydrazide resin (15), developed by Wang and Merrifield[100], with Fmoc-amino acids and benzyl-derived side-chain protection. The hydrazide yielded on final TFA cleavage can then be used in subsequent coupling by the azide procedure.

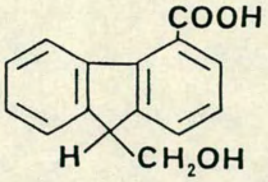


(15)

Although the peptide-resin links mentioned so far all have been acid-labile, there are some which are cleaved under basic conditions. Acid-labile links have a limitation in that they cannot be used easily for the synthesis of fully protected peptide fragments when acid-labile protection is employed. As mentioned previously, the *p*-alkoxybenzylalcohol resin (14) can be used in an orthogonal system with base-labile protecting groups, but here a limitation is the small range of base-labile protecting groups available. In contrast, however, base-labile linkers or "handles" allow use of the large range of acid-labile protecting groups available, thus maintaining the orthogonality of the system.

One such base-labile handle, 9-(hydroxymethyl)-fluorene-4-carboxylic acid (HO_2CFmOH) (16) (see Fig. 1.7), was developed recently by Mutter and Bellof[101], following on from the introduction of 9-fluorenylmethyl (Fm) esters as base-labile C-terminal protecting groups in peptide synthesis[102]. One procedure for the use of this handle involves its binding to the aminomethyl resin (3) with DCCI followed by coupling of the amino protected C-terminal residue to the resulting hydroxymethyl resin with DCCI and DMAP as before. This handle is acid-stable, is cleaved readily with 15% piperidine in DMF and has been used in both solid phase and solution phase syntheses of peptides.

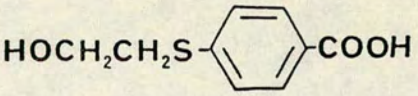
Other handles which operate on the basis of base-catalysed β-elimination have been developed by Tesser[103] (17) and Schwyzer[104] (18,19,20) (see Fig. 1.7). In these cases cleavage is obtained by oxidation of the sulphur to the sulphone, if necessary, followed by base-catalysed β-elimination with sodium hydroxide, although the oxidations required for (17), (18) and (19)



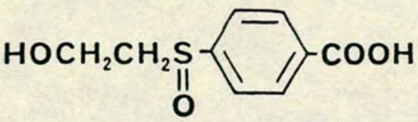
(16)

$HOCH_2CH_2SH$

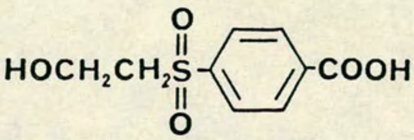
(17)



(18)



(19)

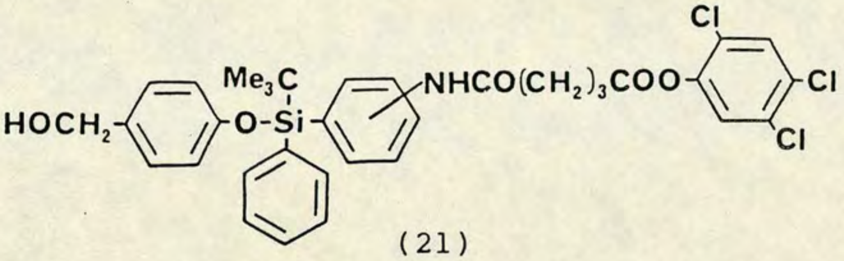


(20)

Figure 1.7 - Handles Cleaved by Base-Catalysed β-Elimination

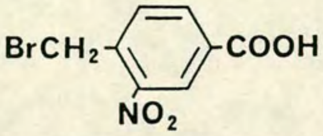
may cause problems with some amino acid side-chains.

A three-dimensional orthogonal system was introduced by Barany[105] in which he used acid-labile side-chain protection, base-labile amino protection and a fluoride-labile peptide-resin link. He incorporated a fluoride-labile silicon-oxygen bond into the 2,4,5-trichlorophenyl N-(3 or 4)-((4-(hydroxymethyl)phenoxy)-tert-butylphenylsilyl)phenyl pentanedioate monoamide (Pbs) handle (21) thus producing a system which allowed the synthesis of protected peptide fragments. The involved multistep synthesis of (21), however, remains a considerable drawback in the use of this handle.

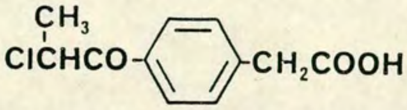


(21)

Several photolabile handles have been developed, including the 3-nitro-4-bromomethylbenzoic acid (22) of Rich[106] and the (4-(2-chloropropionyl)phenyl)acetic acid (23) of Tjoeng[107]. Again, both of these handles were employed in the synthesis of protected peptide fragments in an orthogonal system using the aminomethyl resin (3).



(22)



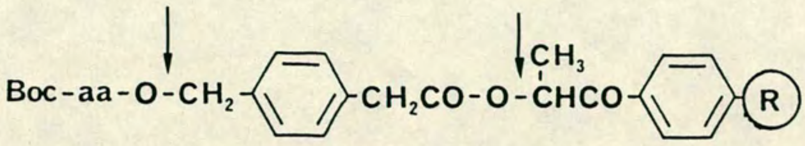
(23)

The peptide-resin links mentioned so far generally have had only one labile anchoring bond cleaved by only one or a few related methods, and as such have been applicable only to one synthetic strategy. This state of affairs has prompted the development of multidetachable systems designed to allow cleavage by a selection of reagents. Two such systems[108], (24) and (25) (see Fig. 1.8), have been devised in which two selectively cleavable (orthogonal) ester bonds can be treated with a variety of reagents to give the peptide in various stages of deprotection.

A further type of multidetachable resin (28) based on the benzhydrylamine resin (5) has been introduced by Tam[109]. It has two advantages over (5) in that it is made (see Scheme 4) from a chemically defined benzhydrylamine handle, N-Boc-(4-hydroxy)benzhydrylamine (27), which is purified and then attached to the resin support (26), and also it is a multidetachable resin which can produce protected peptide fragments. Although it is not as versatile as the POP and PON resins, this resin yields peptide amides and as such is complementary to the aforementioned resins.

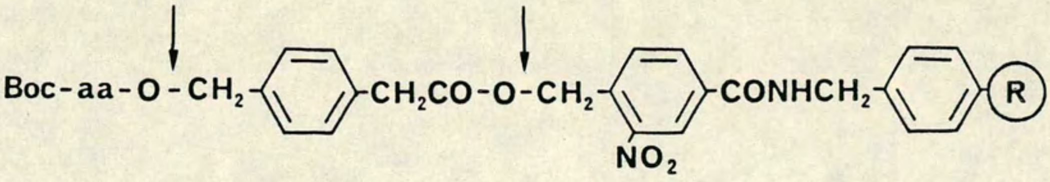
1.2.5 α-Amino Group Protection

The principle of using carboxyl protecting groups for the protection of amino groups through their conversion into urethanes (29) was the most successful



Boc-aminoacyl-2-(4-(oxymethyl)phenylacetoxy)-propionyl resin (24)

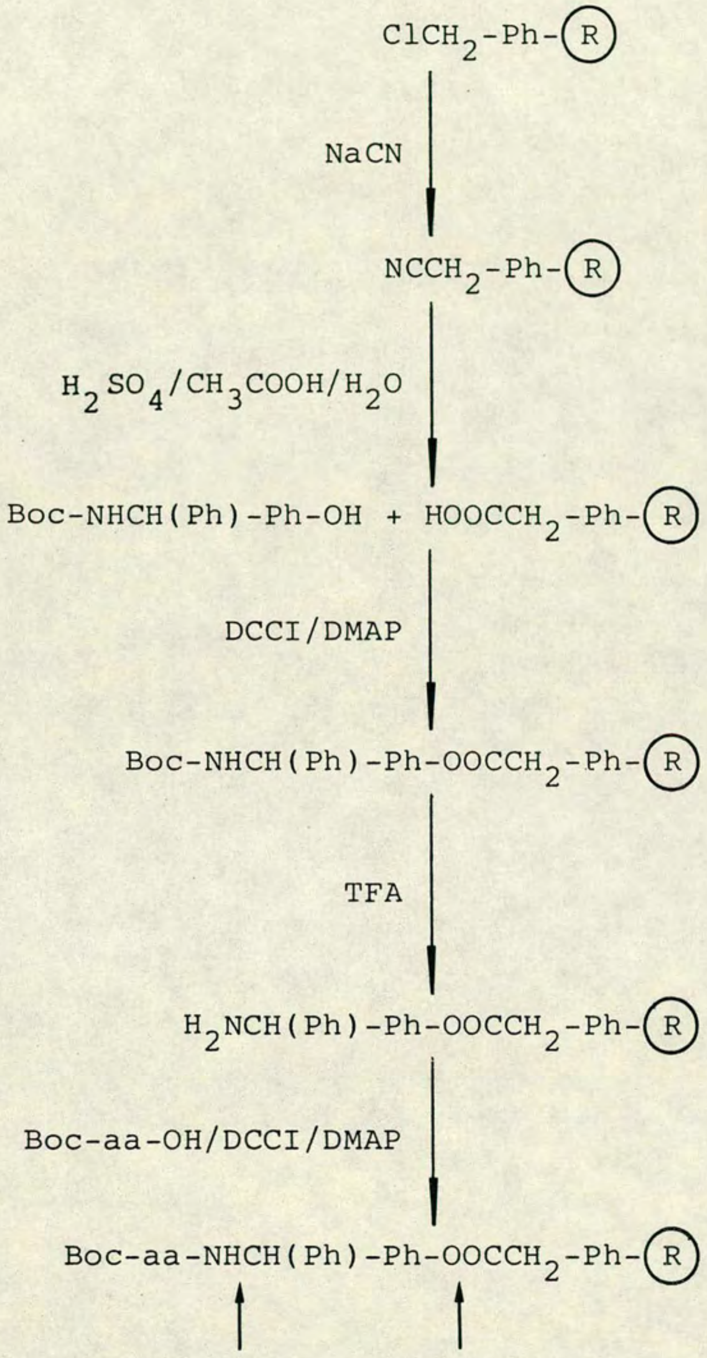
(Boc-aminoacyl-OCH_2-POP-resin)



Boc-aminoacyl-4-(4-(oxymethyl)phenylacetoxymethyl)-3-nitrobenzamidomethyl resin (25)

(Boc- aminoacyl-OCH_2-PON-resin)

Figure 1.8 - Multidetachable POP and PON Resins



(1)

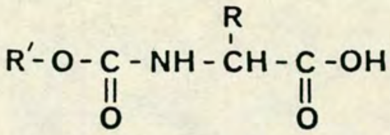
(27)

(26)

(28)

Scheme 4

innovation in peptide synthesis, and the introduction of the Z group in 1932[57] has spawned a vast array of urethane-type amino protecting groups in the intervening years making them the most widely used amino protecting



$$R'\text{-}O\text{-}\underset{\underset{O}{\|}}{C}\text{-}NH\text{-}\overset{\overset{R}{|}}{C}H\text{-}\underset{\underset{O}{\|}}{C}\text{-}OH$$

(29)

agents in peptide synthesis today. Removal of these amino protecting groups starts with the scission of the urethane ester bond to give the carbamoic acid which then spontaneously decarboxylates to regenerate the free amine, and as an added bonus, urethane amino protection confers stability towards racemisation of the residue concerned upon activation.

The most commonly used group is the Boc group[49] due to its convenient lability-stability characteristics and the easy availability of its derivatives. Also, the Z group is stable under the conditions for Boc cleavage hence allowing the use of Z side-chain protection with Boc N^{α}-protection. As mentioned before, however, there can be problems with the Boc group having a similar acid-stability to the peptide-resin link giving premature peptide cleavage, and also with the carbocations generated during cleavage giving unwanted side reactions. The latter necessitates the use of cation scavengers such as anisole or dimethylsulphide.

To circumvent the problem of premature peptide cleavage, the very acid-labile Bpoc group[110] was developed. This group is several thousand times more labile to acidolysis than the Boc group and so Boc side-chain protection can be used with Bpoc N^{α}-protection. Again, stable carbocations are formed upon cleavage thus requiring the use of scavengers. Also, the instability of the free Bpoc-amino acids is a drawback forcing them to be stored as salts and liberated immediately before use.

Other protecting groups of intermediate acid-lability to the Boc and Bpoc groups have been developed, e.g. the Ddz group of Birr[111], the Poc group of Ragnarsson[112] and the Tmz group of Matsueda and Stewart[113]. The derivatives of all of these groups, however, are either very expensive or not commercially available and thus have not been widely adopted in synthesis.

Foremost among the base-labile amino protecting groups is the Fmoc group[96]. Sheppard[114] has used this group widely in the synthesis of peptides employing an orthogonal scheme with acid-labile (e.g. Boc) side-chain protection. Fmoc is usually cleaved with 20% piperidine in DMF, but this can give premature peptide cleavage from classical Merrifield resins, hence it is used with acid-labile (and thus base-stable) Wang[94] or polyamide[83] resins.

A rather different type of amino protecting group was developed by Kenner and Ramage in 1976[115] when they introduced the Dpp group. It has an advantage over the urethane-type amino protecting groups in that no reactive intermediates are produced on deprotection so preventing unwanted side reactions. Also, it does not interfere with commonly used coupling procedures, and it allows only minimal racemisation to take place due to the prevention of oxazolone formation. Cleavage is brought about by a 6-fold excess of methanolic HCl at 35-50°C - its acid-lability is a lot less than that of Bpoc and slightly greater than that of Boc. Success has been achieved in solution phase peptide synthesis with the Dpp group, although the acidic conditions required for Dpp cleavage may pose problems with some handles and side-chain protecting groups in the solid phase synthesis of large peptides.

1.2.6 Side-chain Protection

The system with more acid-stable benzyl-derived side-chain blocking groups originally used by Merrifield has continued to be the most widely used along with more acid-labile Boc-amino acids. Currently, serine and threonine are adequately protected as benzyl ethers, while tyrosine requires 2,6-dichlorobenzyl ether protection, as the unsubstituted benzyl ether can give migration to the 3-position of the aromatic ring.

Aspartic and glutamic acids are protected as benzyl esters, but at the present time there appears to be no completely ideal group available for asparagine and glutamine and their side-chains are commonly left unprotected. Arginine is usually protected with nitro or tosyl groups, the latter also being used for histidine, while a urethane is formed with the side chain of lysine using the Z or 2-chlorobenzyloxycarbonyl groups, and cysteine is protected as the Acm[116] derivative or the S-benzyl ether.

When the very acid-labile Bpoc or the base-labile Fmoc groups are used for N^{α}-protection, side-chains may be blocked with more labile moieties such as t-Bu-derived groups. A whole variety of protecting groups for the amino acids above, as well as methionine and tryptophan, have been developed including recent work in our research group on arginine[117,118] and aspartic and glutamic acids. Although these are not discussed here there are several excellent reviews on this topic[119,120].

1.2.7 Coupling Methods

Obtaining a quantitative coupling reaction presents the major technical difficulty in solid phase peptide synthesis. A coupling reaction requires to be a rapid and efficient acylation with a minimum of racemisation and other side reactions. To help achieve this end, a variety of strategies are employed, e.g. active ester, symmetrical anhydride, or mixed anhydride. Carbodiimide activation, however, remains the most widely used method.

DCCI[50] is the most popular carbodiimide and is considered to be the standard for the comparison of others. Carbodiimides give rapid acylation due to the high degree of activation afforded by the O-acyl urea, but there are problems associated with their use, however; oxazolone formation can occur in fragment condensation and also the O-acyl urea can rearrange easily to the unreactive N-acyl urea resulting in an undesirable loss of activated amino acid. The latter problem is worse in polar aprotic solvents such as DMF thus necessitating the use of a minimum amount of DMF in the coupling procedure. In common with all acylating agents, DCCI is required in excess, with 2.5 equivalents giving acylation within 30 minutes. As well as mediating the coupling reaction directly, DCCI is also used to prepare symmetrical anhydrides and active esters. Other carbodiimides are now coming to the fore, however, such as DICI which has advantages over DCCI; whereas DCCI is a waxy solid, DICI is a liquid so making for easier handling, and also the urea by-product from DICI is more soluble than that from DCCI and is thus more easily removed from a solid phase system.

Preformed symmetrical anhydrides[121] are widely utilised in peptide synthesis, although the method is wasteful in view of the excess of protected amino acid used to give quantitative acylation; 4 equivalents of protected amino acid are required to give 2 equivalents of symmetrical anhydride. Boc-anhydrides are usually prepared immediately before use due to their instability, although Fmoc-anhydrides are stable and can be made in advance and stored. They frequently give dramatically increased reaction rates in difficult coupling reactions, especially when an equivalent of tertiary amine is added to the acylation mixture, and they also prevent peptide chain termination by DCCI activation of any residual TFA or acetic acid remaining in the resin at the coupling step.

Another modification of the carbodiimide reaction involves the use of active esters. In this case, HOBt esters[53], either preformed or generated in situ, are the most popular and have improved substantially coupling reactions in some difficult sequences both in solution and solid phase syntheses. Other popular active esters are trichlorophenyl[122] and pentachloro- or pentafluorophenyl[123] esters, although the last two have a possible problem with steric hindrance. Trichlorophenyl esters are stable and so can be used in the synthesis of activated handles which have to be purified before attachment to the resin, for example, the Pbs handle of Barany[105]. A less popular active ester is the p-nitrophenyl ester[124]; this is due to low reactivity requiring the use of a large excess of activated derivative coupled with a prolonged reaction time.

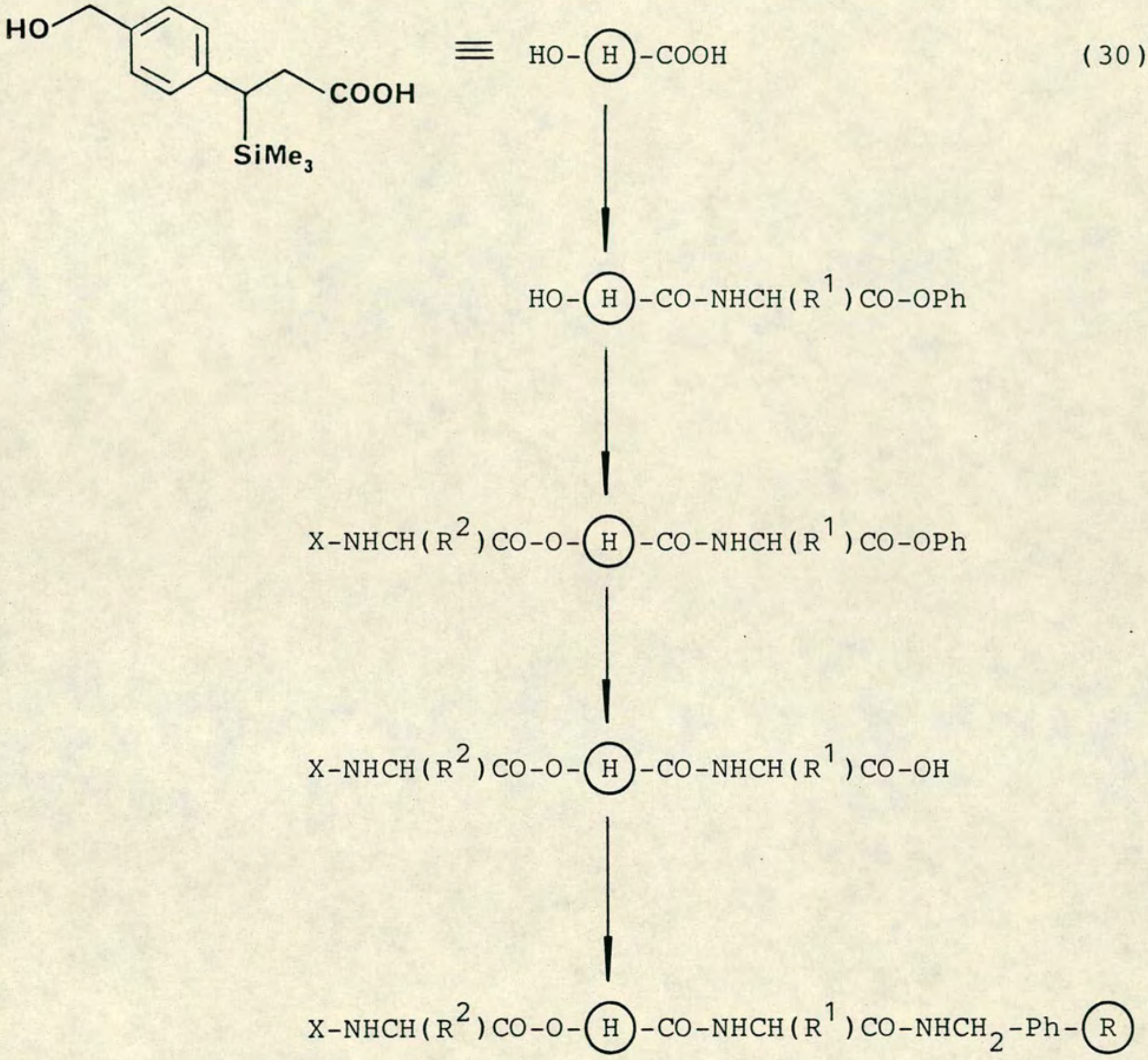
A method not involving the use of carbodiimides has recently been reported; here, phosphinic-carboxylic mixed anhydrides have been utilised in both solution[125] and solid phase[126] synthesis. Both the diphenylphosphinyl chloride (DppCl)[125] and 1-oxo-1-chlorophospholane (CptCl)[127] used for activation give a quick reaction and stability to disproportionation, an advantage over other mixed anhydrides. Also, the amine component attacks the mixed anhydride regiospecifically at the carbonyl group thus giving the desired amide with no side reactions. Unfortunately, however, there are problems associated with these two activating agents; DppCl is a hygroscopic liquid which can react with amide solvents and is difficult to handle so making it unsuitable for use in an automated system using large amounts of DMF, while the synthesis of CptCl is expensive and time-consuming.

CHAPTER 2 : DISCUSSION

2.1 INTRODUCTION

As is evident from chapter one, there has been and is much research going into the development of handles with varying modes of cleavage and stability-lability properties, with a three-dimensional orthogonal system such as that used by Barany[105] being the most versatile. In this context, we have attempted to develop a similar three-dimensional system by constructing a fluoride-cleavable handle based on the principle of fragmentation of esters of β-silylalcohol by fluoride ion. It has been reported[128] that β-trimethylsilylethyl esters of protected amino acids can be cleaved with fluoride ion and that they are stable over a wide variety of conditions encountered in peptide synthesis. Conversion of this system to a urethane-type amino protecting group was also carried out by Carpino[129] when he introduced the Teoc group.

A fluoride-cleavable handle (30) based on this fragmentation has been successfully developed in our research group[130] and applied in peptide synthesis, giving a 62% yield after purification of an N^{α}-protected hexapeptide. The strategy used to accomplish this synthesis (see Scheme 5) involved coupling the handle (30) to an amino acid protected as the phenyl ester;



HO
COOH
SiMe3
HO-(H)-COOH
HO-(H)-CO-NHCH(R1)CO-OPh
X-NHCH(R2)CO-O-(H)-CO-NHCH(R1)CO-OPh
X-NHCH(R2)CO-O-(H)-CO-NHCH(R1)CO-OH
X-NHCH(R2)CO-O-(H)-CO-NHCH(R1)CO-NHCH2-Ph-(R)

(30)

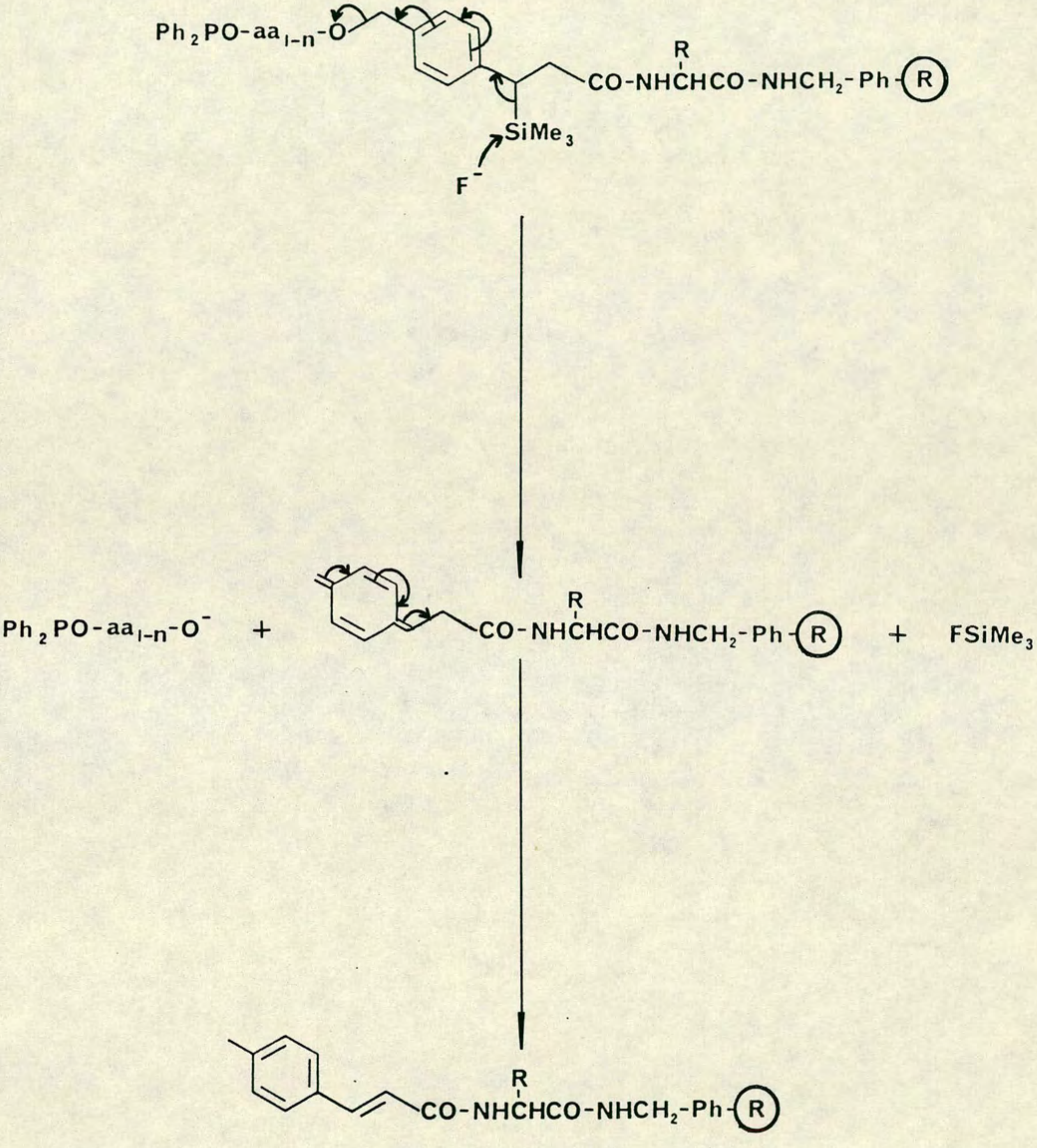
Scheme 5

this amino acid acting as an extension to the handle and an internal reference amino acid. Coupling of the first protected residue followed by deprotection of the phenyl ester gave the protected amino acid-handle-reference amino acid which was subsequently coupled to the aminomethyl resin (3) producing the required acylated support. Following the sequential synthesis of the desired peptide, treatment of the resin-bound product with dry TBAF in DMF liberated the peptide (see Scheme 6) after 6 minutes.

2.2 HANDLE STRATEGY

In using the handle strategy, coupling the handle to the resin with the first protected amino acid already attached to the handle is advantageous in that the protected amino acid-handle can be isolated, purified and characterised before being coupled to the resin, and the formation of free hydroxymethyl sites is prevented, the presence of which can lead to deletion peptides, late initiation of peptides and trifluoroacetylation or acetylation of deprotected amino groups. The aminomethyl resin (3) allows the coupling reaction to be monitored using both the Kaiser test and microanalysis, as well as giving a stronger amide rather than an ester linkage.

Initial attempts[131] to produce a protected amino acid-handle from the methyl ester of the handle floundered at the hydrolysis stage when quantitative



Ph₂PO-aa₁₋ₙ-O
R
CO-NHCHCO-NHCH₂-Ph-R
SiMe₃
F⁻
Ph₂PO-aa₁₋ₙ-O⁻
+
R
CO-NHCHCO-NHCH₂-Ph-R
+
FSiMe₃
R
CO-NHCHCO-NHCH₂-Ph-R

Scheme 6

cleavage of the benzyl ester linkage was obtained upon treatment with sodium hydroxide. It was thus decided to use the more labile phenyl ester. As a consequence of the presence of the methyl ester throughout the synthesis, however, it was considered easier to prepare the handle (30) and then couple an extra spacer, *viz.* an amino acid phenyl ester. Elongating the handle in this manner would also aid the hydrolysis if steric hindrance by the TMS group was a factor.

This phenyl ester approach was indeed successful and two examples of the final derivatised handle, Dpp-Met-O-Ⓗ-CO-Gly-OH and Dpp-Leu-O-Ⓗ-CO-Ala-OH, were produced. The former was quantitatively coupled to the aminomethyl resin (3) *via* the trichlorophenyl active ester, but subsequent peptide synthesis failed due to benzyl ester cleavage under the acidic conditions required for Dpp cleavage. Unfortunately, this clearly demonstrates that the handle (30) is not compatible with acid-labile N^{α}-protection, and subsequently[130] the base-labile Fmoc group was used.

An alternative approach[131] involving the direct coupling of a protected amino acid-handle-trichlorophenyl active ester to the aminomethyl resin (3) was also attempted. In this case a very poor coupling was achieved in contrast to the quantitative result obtained with the glycine extension example. Again, this may point to steric hindrance coming into consideration.

Extending the aminomethyl moiety on the resin by first adding phenylalanine to the resin before coupling the active ester resulted in a significant improvement, the coupling yield being 37% by amino acid analysis. These results tend to suggest that the carboxyl extension of the handle (30) is a desirable feature in attempting to maximise the coupling yield.

2.3 EXTENDED HANDLE SYNTHESIS

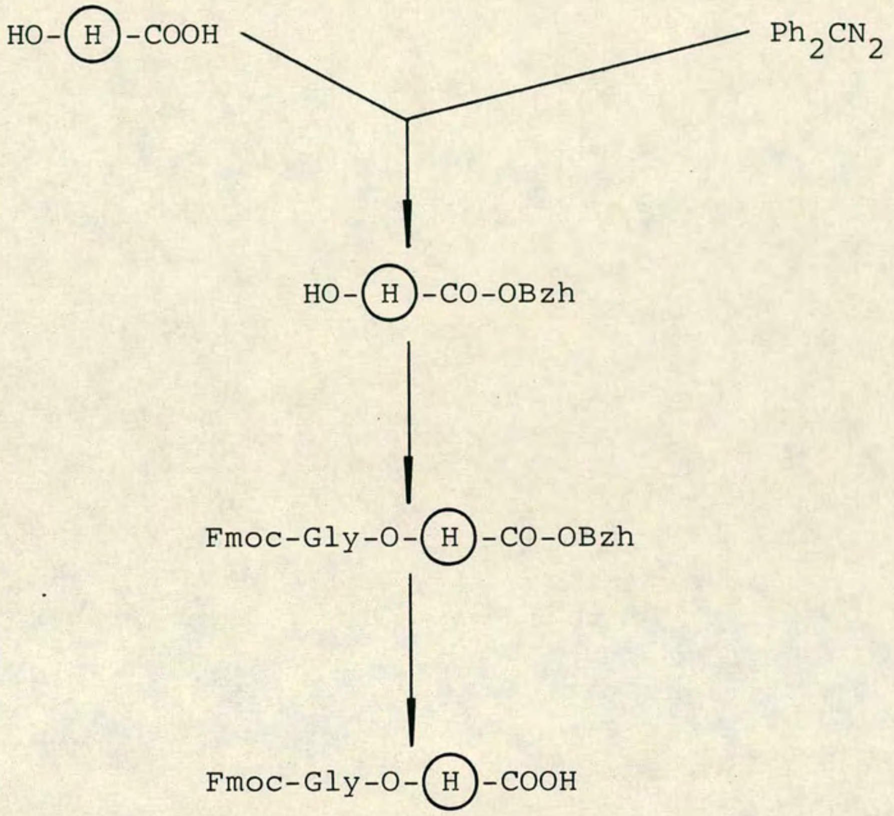
2.3.1 Benzhydryl Ester Approach

A brief first attempt (see Scheme 7) was made to protect the handle (30) as the benzhydryl ester in an effort to produce a protected amino acid-handle with no carboxyl extension. Esterification of (30) with diphenyldiazomethane (31) gave the handle-ester (32) in 88% yield. Coupling of Fmoc-glycine using DCCI with DMAP as catalyst then furnished the protected amino acid-handle-ester (33) in 98% yield. Subsequent treatment with TFA and phenol over a variety of concentrations and reaction times, however, resulted in either no reaction or a multispot TLC mixture from which the desired product could not be isolated. This line of approach was thus dropped in favour of the carboxyl extension strategy.

2.3.2 Methyl Ester Approach

In order to maximise any benefits gained from the carboxyl extension of (30), such as easier hydrolysis of

(30) (31) (32) (33)



Scheme 7

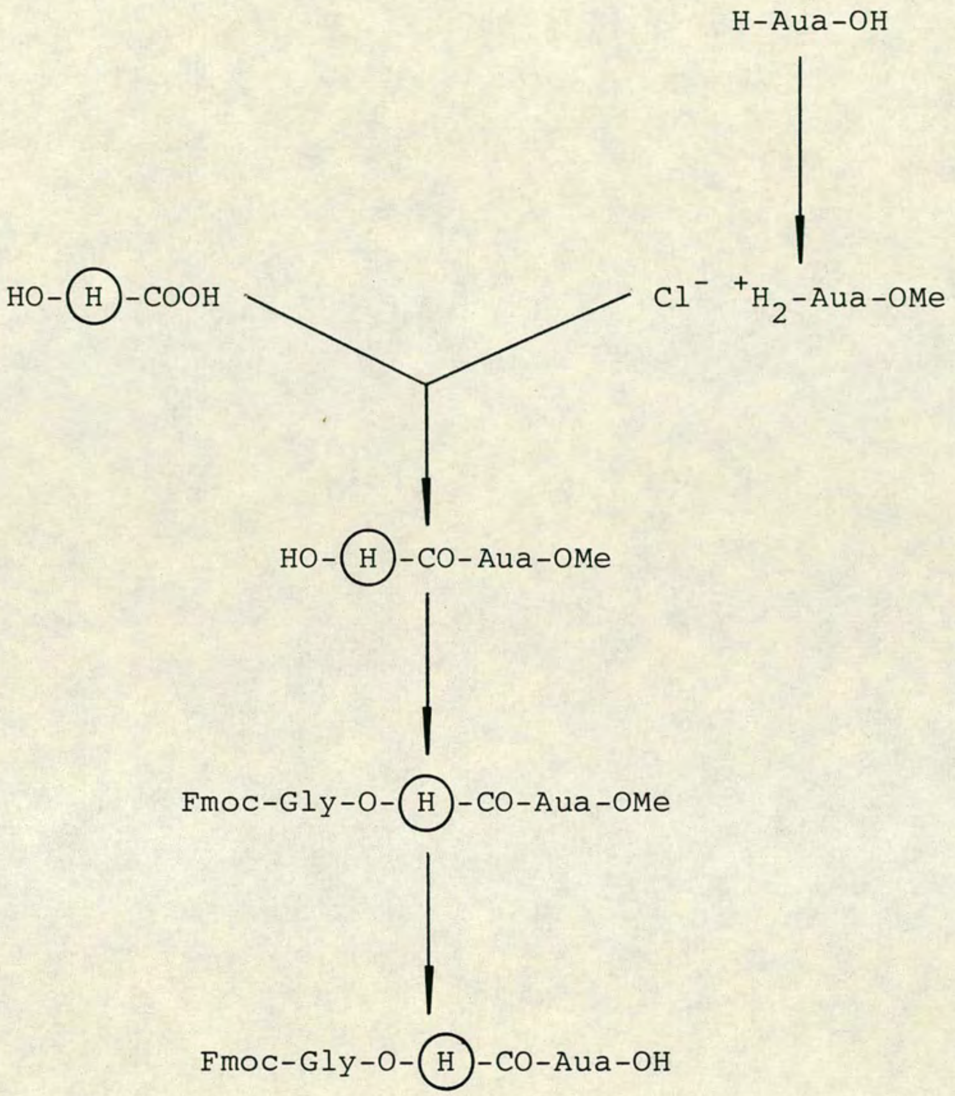
the final ester, more efficient coupling or easier reagent access after coupling, it was decided to use the long-chain, saturated 11-aminoundecanoic acid as the extension. Use of a methyl ester would also help to determine whether the earlier[131] hydrolysis failure was due to steric hindrance by the TMS group or to the methyl ester being too stable. The attempted synthesis (see Scheme 8) of the fully extended handle species commenced with the reaction of 11-aminoundecanoic acid with thionyl chloride and methanol to produce the reference amino acid-ester-salt (34) in 81% yield. A DCCI coupling of (34) to (30) with NMM gave the handle-reference amino acid-ester (35) in 40% yield, with a further DCCI coupling of Fmoc-glycine to (35) with catalytic DMAP giving the protected amino acid-handle-reference amino acid-ester (36) in 64% yield. Treatment of (36) with sodium hydroxide resulted in the liberation of dibenzofulvene, i.e. the Fmoc group was being cleaved. This was obviously unacceptable and hence the methyl ester approach was discontinued in favour of that involving the phenyl ester.

2.3.3 Phenyl Ester Approach-1

The synthetic route here (see Scheme 9) to the fully extended handle species (47) was very similar to that employed in section 2.3.2, although the preparation of the reference amino acid-ester-salt (40,42) was more

(30)

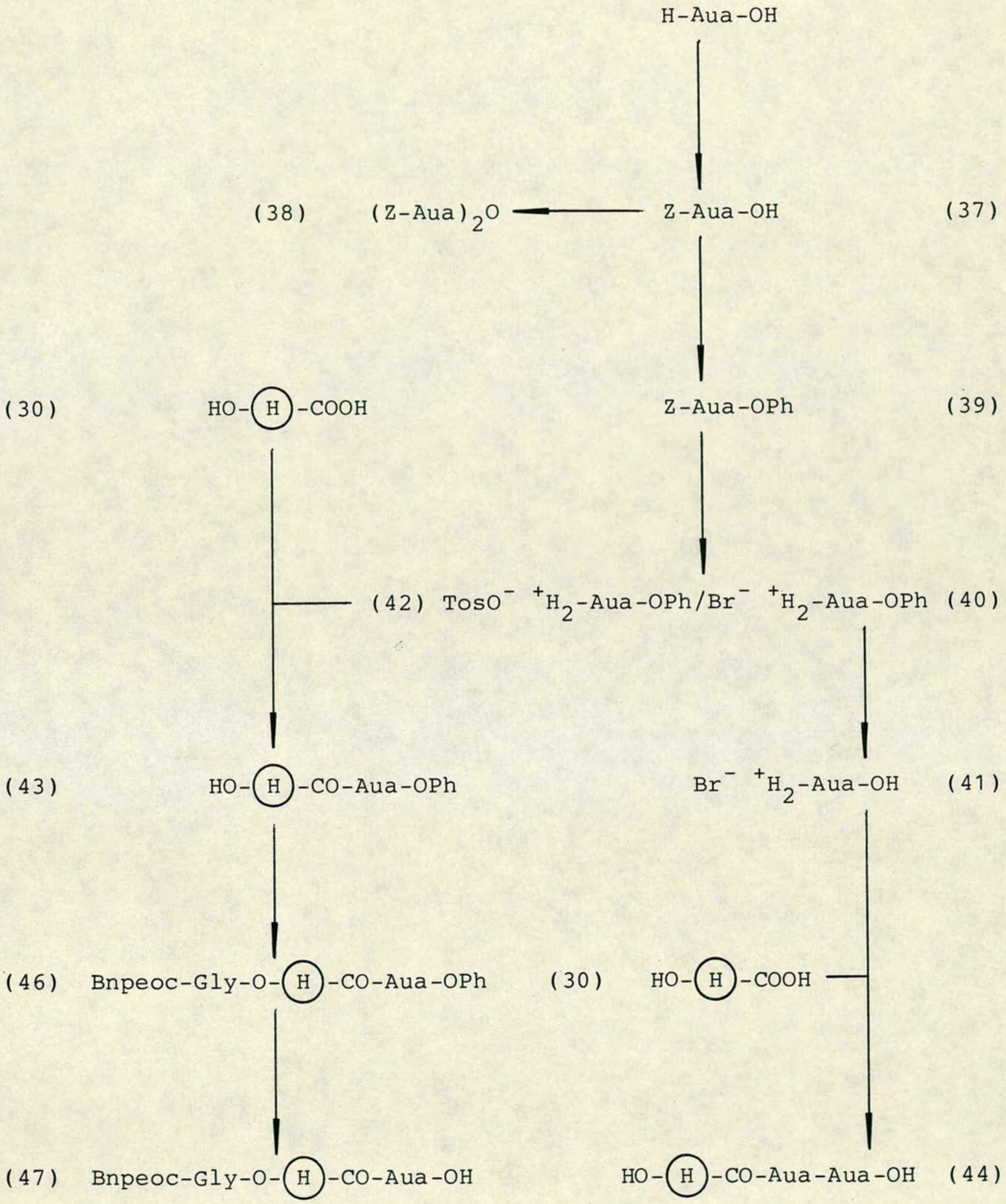
(34)



(35)

(36)

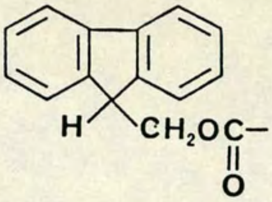
Scheme 8


H-Aua-OH
(38) (Z-Aua)₂O
Z-Aua-OH (37)
(30) HO-(H)-COOH
Z-Aua-OPh (39)
(42) TosO⁻ ⁺H₂-Aua-OPh/Br⁻ ⁺H₂-Aua-OPh (40)
(43) HO-(H)-CO-Aua-OPh
Br⁻ ⁺H₂-Aua-OH (41)
(46) Bnpeoc-Gly-O-(H)-CO-Aua-OPh
(30) HO-(H)-COOH
(47) Bnpeoc-Gly-O-(H)-CO-Aua-OH
HO-(H)-CO-Aua-Aua-OH (44)

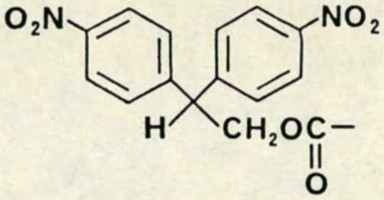
Scheme 9

involved. Firstly, 11-aminoundecanoic acid was N^{α}-Z-protected by reaction with benzyl chloroformate in sodium hydroxide and sodium bicarbonate solution to produce quantitatively the protected reference amino acid (37), although a curious solvent effect was observed here whereby use of dioxan instead of THF resulted in a yield of only 30%. Conversion of (37) to the phenyl ester (39) was achieved via a 2-step process using oxalyl chloride and catalytic DMAP to yield the acid chloride followed by phenol and NMM to give the protected reference amino acid-ester (39) in 91% yield. A first attempt to produce (39) directly from (37) using DCCI and phenol, both with and without pyridine, succeeded only in forming the symmetrical anhydride (38). Initially, the reference amino acid-ester-salt formed was the hydrobromide (40) in 76% yield with HBr in acetic acid, and indeed this was successfully coupled to (30) with DCCI and NMM to give the handle-reference amino acid-ester (43) in 50% yield; however, (40) was found to be unstable over a long time period even at refrigerator temperatures as was evidenced by its decay to the reference amino acid-salt (41) and the production of handle-(reference amino acid)$_2$ (44) in later attempts at the handle coupling reaction. This problem was solved by turning to the tosylate (42) which was obtained in 75% yield from (39) by hydrogenation on a palladium/charcoal catalyst with p-toluenesulphonic acid. Again, this was coupled to (30) as for the hydrobromide (40) to give (43) in 68% yield.

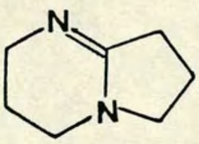
At this point, another development in our research group[132] was incorporated into the synthesis, viz. the Bnpeoc protecting group. This base-labile protecting group is similar to, and indeed was developed as a cheaper alternative to, the Fmoc group. It is cleaved by DBN or DBU as well as by piperidine which is used to cleave the Fmoc group, and it appears also to be more stable towards alkali or primary amines.



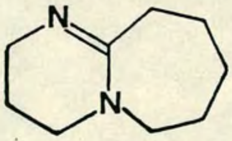
Fmoc



Bnpeoc



DBN



DBU

Thus, Bnpeoc-glycine (45) was coupled to (43) with DCCI and catalytic DMAP, giving the protected amino acid-handle-reference amino acid-ester (46) in 91% yield. In the final hydrolysis with sodium hydroxide, it turned out to be a very painstaking exercise to optimise the conditions for the reaction; a whole host of variations of alkali concentration and amount, presence or absence and amount of peroxide, solvent and reaction time were attempted. The best conditions ultimately proved to be 1 equivalent of 0.1M sodium hydroxide and 2 equivalents of 100 volumes hydrogen peroxide in 20% aqueous acetone for 15 minutes at room temperature, which produced the required protected amino acid-handle-reference amino acid (47) in 65% yield. Undoubtedly, the reason for the great sensitivity of this reaction towards the conditions is the presence in a species such as (46) of several possible sites for hydrolysis, <u>viz.</u> the N^{α}-protecting group, benzyl ester and phenyl ester.

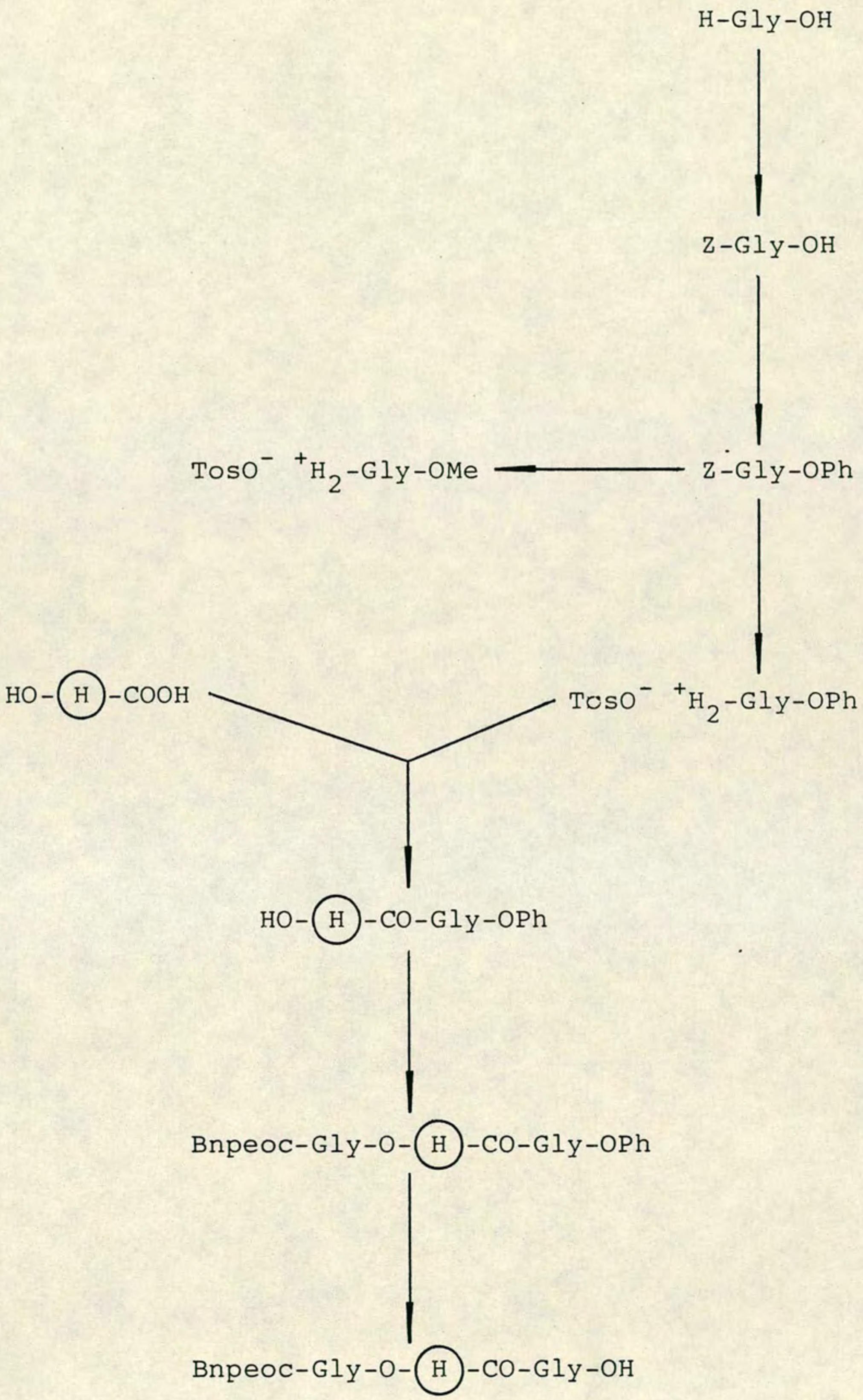
The Fmoc derivative corresponding to (46) was also made, the method being identical and giving Fmoc-Gly-O-Ⓗ-CO-Aua-OPh in 84% yield. In common with (46) the hydrolysis proved to be troublesome, and again after much experimentation the optimum conditions[133] were found to be 1 equivalent of 1M sodium hydroxide and 1 equivalent of 100 volumes hydrogen peroxide in 20% aqueous dioxan for 15 minutes at room temperature: this produced Fmoc-Gly-O-Ⓗ-CO-Aua-OH in 60% yield, although this compound was put aside in favour of the Bnpeoc analogue. The difference in optimum conditions for the hydrolysis of the two analogues perhaps reflects the difference in the acidity of the two β-hydrogens or the different steric constraints of the two N^{α}-protecting groups or a combination of both.

The desired product (47) having been successfully obtained in acceptable yield and purity, it was then coupled to a modified aminomethyl resin (3) and utilised in a solid phase peptide synthesis (see later); however, the result of this synthesis was considered to be disappointing at the time and hence it was decided to look into the use of glycine as the carboxyl extension of the handle (30). It could be that the saturated chain in (47) was too long allowing the molecule too high a degree of freedom of movement once it was bound to the polymeric support so making access to the α-amino function more difficult for incoming activated amino acids.

Another researcher[134], however, investigating a related extension strategy obtained a rather different result. He modified the Pam resin (12) by inserting two 11-aminoundecanoic acid units into the amide link of the Pam bridge to produce a very large spacer chain between the polymer matrix and the functional group of the resin. When this modified Pam resin was applied to peptide synthesis a significant improvement in crude product yield was obtained together with a vast improvement in peptide product homogeneity resulting in a three-fold improvement in yield of the final purified peptide. One reason for these apparently contradictory results could be that the use of two 11-aminoundecanoic acid units gives enough clearance to the peptide chain to minimise peptide-polymer interactions while the use of one such unit, as in the case of (47), still allows significant peptide-polymer interactions to occur.

2.3.4 Phenyl Ester Approach-2

In this case, the synthesis (see Scheme 10) of the fully extended handle species (54) was almost identical to that for (47) except for a few minor differences. The protected reference amino acid (48) was prepared in 89% yield using benzyl chloroformate and sodium hydroxide solution[135], with the subsequent esterification being successfully carried out by DCCI, phenol and pyridine[136] to produce the protected reference amino acid-ester (49) in 78% yield. In view of the long-term instability of the previous hydrobromide (40), the reference amino acid-ester-salt made was the tosylate (51) - in exactly the same manner as (42) - in 74% yield. An attempt to form (51) using methanol as solvent in place of the usual DMF resulted in transesterification to give the methyl ester (50), in 62% yield, instead of the phenyl ester (51). Again, the following steps to produce (52)[131] and (53) were carried out exactly as for (43)


H-Gly-OH
Z-Gly-OH
(48)
(50)
TosO$^-$ $^+H_2$-Gly-OMe
Z-Gly-OPh
(49)
(30)
HO-(H)-COOH
TosO$^-$ $^+H_2$-Gly-OPh
(51)
HO-(H)-CO-Gly-OPh
(52)
Bnpeoc-Gly-O-(H)-CO-Gly-OPh
(53)
Bnpeoc-Gly-O-(H)-CO-Gly-OH
(54)

Scheme 10

and (46) with the respective yields being 65% and 87%. Similar problems were encountered once more in the final hydrolysis with the same lengthy experimentation required to find the optimum conditions; surprisingly, however, in spite of the considerable difference in size of the two carboxyl extensions, these turned out to be identical to those for (47), and the final product (54) was obtained in 81% yield.

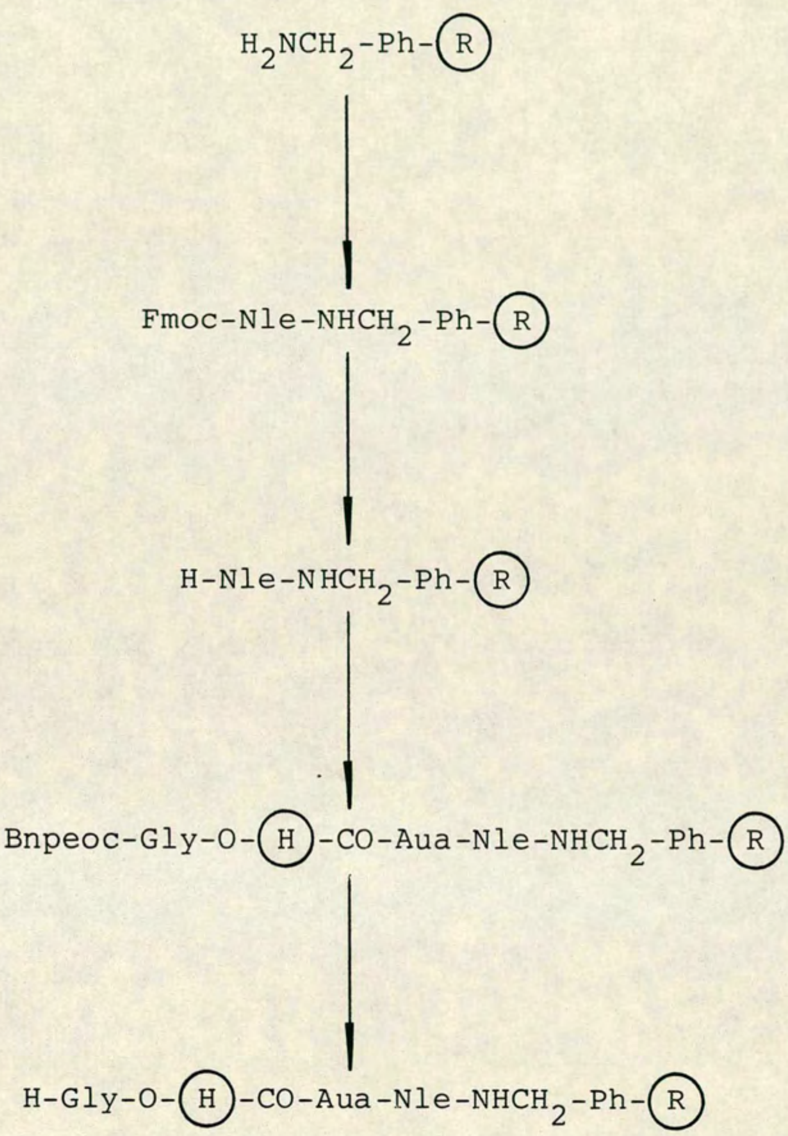
An alternative transesterification approach[137] to the hydrolysis of (53) was attempted and involved reacting (53) with 50% v/v dimethylaminoethanol in DMA at room temperature for 24 hours to replace the phenyl ester with the dimethylaminoethyl ester, and then hydrolysing this product with 15% v/v water in DMA and catalytic quantity of imidazole at room temperature for 24 hours to produce hopefully the desired acid (54). It was found, unfortunately, that almost quantitative production of the Bnpeoc-derived olefin had taken place, so indicating incompatibility of the Bnpeoc moiety with this system. Later studies[138] on Bnpeoc solvent stability, however, showed that the quality of a solvent such as DMA was crucial to the long-term stability of the Bnpeoc moiety in its presence, thus it was possible that the failure of the transesterification system was in some part attributable to the quality of the DMA, although due to the success in producing (54) by other means, this possibility was not investigated.

The purification of the fully extended handle species (47) and (54) also proved to be troublesome; wet flash silica chromatography (twice) followed by gel filtration was sufficient to purify (47), as the fatty nature of the carboxyl end ensured an adequate mobility on silica. On the other hand, the purification of (54) was an altogether different proposition; alumina quantitatively produced the olefin elimination product of the Bnpeoc group, while exposure to silica for any significant length of time caused the compound to stick quite badly to the column requiring unacceptable amounts of polar solvent to elute it, undoubtedly having an adverse effect on its stability. Attempts to purify (54) by differential solubility, treatment with an acidic buffer, or conversion to an amine salt all yielded no improvement, and treatment with charcoal and Celite suffered from the same problem as did the silica chromatography. Eventually, (54) was obtained in acceptable purity by gel filtration followed by rapid dry flash silica chromatography on a short 1" column.

In the synthesis of (47) and (54) glycine was used as the first residue in the peptide chain for two reasons: firstly, glycine is the smallest and only achiral natural amino acid and so when it is used as the C-terminal residue in fragment condensation it gives better coupling with no problem over racemisation; and secondly, because glycine is the least sterically hindered amino acid the relevant compounds in Schemes 9 and 10 are the most vulnerable to unwanted side reactions, and hence success with glycine should mean that corresponding derivatives with other amino acids if desired should present no greater problem.

2.4 RESIN LOADING OF EXTENDED HANDLE (47)

Unfortunately, 11-aminoundecanoic acid was not detectable under amino acid analysis, hence another reference amino acid was required. To avoid confusion with the natural amino acids in any peptide being constructed, the unnatural amino acid norleucine was chosen. Fmoc-norleucine (see Scheme 11) was coupled (X4) to the aminomethyl resin (3) using DCCI and catalytic DMAP to give a negative Kaiser result for (55), i.e. quantitative coupling was achieved. Subsequent piperidine deprotection of the Fmoc group to give (56) and coupling (X2) of the extended handle (47) with DCCI and catalytic DMAP was then carried out. Kaiser testing indicated that functionalisation of the resin was incomplete and thus capping to a negative Kaiser test with acetic anhydride and triethylamine of any unreacted free amino groups on the resin was performed. Amino acid analysis of the resin-bound product (57) indicated a yield of 87%, while



(3)

(55)

(56)

(57)

(58)

Scheme 11

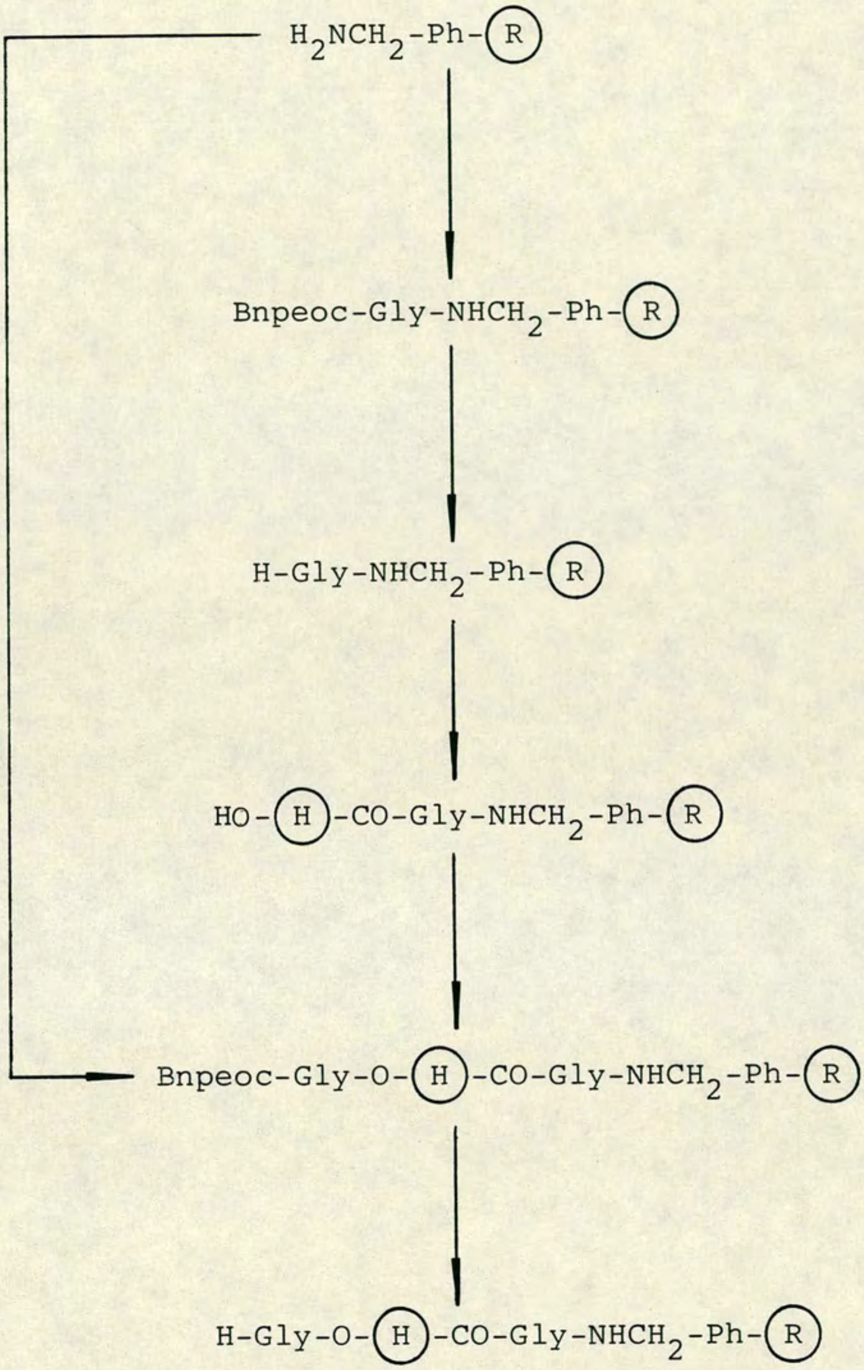
microanalysis of (57) gave a yield of 84%, i.e. consistent results. The resin-bound extended handle (57) was then prepared for peptide synthesis by deprotection of the Bnpeoc group with DBN and acetic acid to give the extended amino resin (58). All of these operations were performed on a continually-inverting manual peptide synthesiser of our own design, and in common with all subsequent use of the aminomethyl resin (3), its initial functionality was determined by repeated microanalysis.

2.5 STEPWISE RESIN LOADING OF EXTENDED HANDLE (54)

2.5.1 Complete Fragmentation

While the lengthy experimentation into the synthesis and purification of (54) was being carried out, an investigation was also undertaken into the possibility of loading (54) onto the resin in three stages (see Scheme 12) as a means of circumventing the problems involved in the production of (54) in acceptable purity.

Acid chloride methodology was employed in the first attempt to couple Bnpeoc-glycine (45) to the aminomethyl resin (3). Refluxing (45) with thionyl chloride furnished the acid chloride (59), as confirmed by infrared spectroscopy, which was then coupled to (3) using NMM and catalytic DMAP to give the resin-bound product: microanalysis indicated a coupling yield of


H2NCH2-Ph-(R) (3)
Bnpeoc-Gly-NHCH2-Ph-(R) (60)
H-Gly-NHCH2-Ph-(R) (61)
HO-(H)-CO-Gly-NHCH2-Ph-(R) (62)
Bnpeoc-Gly-O-(H)-CO-Gly-NHCH2-Ph-(R) (63)
H-Gly-O-(H)-CO-Gly-NHCH2-Ph-(R) (64)

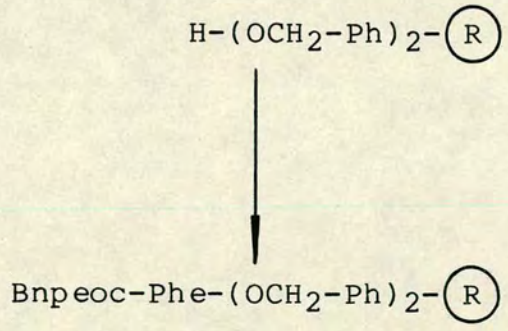
Scheme 12

83%. Replacement of NMM by pyridine in this procedure resulted in very little coupling. A similar result was obtained when (45) was coupled (X2) to (3) with DCCI. Kaiser testing indicated incomplete reaction so acetic anhydride/triethylamine capping was carried out to give the resin-bound product (60) in 90% yield from microanalysis.

The Bnpeoc group was removed with DBU and acetic acid to give (61) which was then treated with (30) and DCCI. A negative Kaiser test was obtained on the product of this coupling which microanalysis indicated had gone to completion. This was convenient since capping could not be performed in this case due to the presence, in theory, of free hydroxyl groups on the resin. Subsequent coupling of (45) with DCCI (X2) and with DCCI, HOBt and catalytic DMAP produced no reaction - the infrared spectrum of the product was identical to that of the starting material. A similar outcome was achieved using the acid chloride approach as for production of (60); both the pyridine and NMM/catalytic DMAP approaches resulted in no incorporation of (45) onto the modified resin.

This last result is in conflict with standard Wang resin methodology and with a resin loading carried out in the research group involving Bnpeoc- and Fmoc-amino acid chlorides and Wang's p-alkoxybenzylalcohol resin (14). Recently there has been a revival of interest in the use of acid chlorides[139] as coupling agents in peptide synthesis, especially with hindered substrates[140], and this prompted our solid phase study which successfully culminated in an apparently racemisation-free synthesis of a tripeptide[138].

As part of this work, the acid chloride (65) of Bnpeoc-phenylalanine was prepared by refluxing with thionyl chloride, formation being confirmed by its infrared spectrum. As in the coupling of Bnpeoc-glycine (45) to the aminomethyl resin (3), success in obtaining the resin-bound product (66) was achieved with NMM and catalytic DMAP while replacement of NMM with pyridine resulted in coupling failure : microanalysis of (66) showed a yield of 30% after two couplings. This is consistent with other results from the group study[132] which showed a similar degree of ester formation for amino acids with sterically bulky side-chains : Bnpeoc-glycine gave a considerably higher coupling yield under identical conditions.



(14)

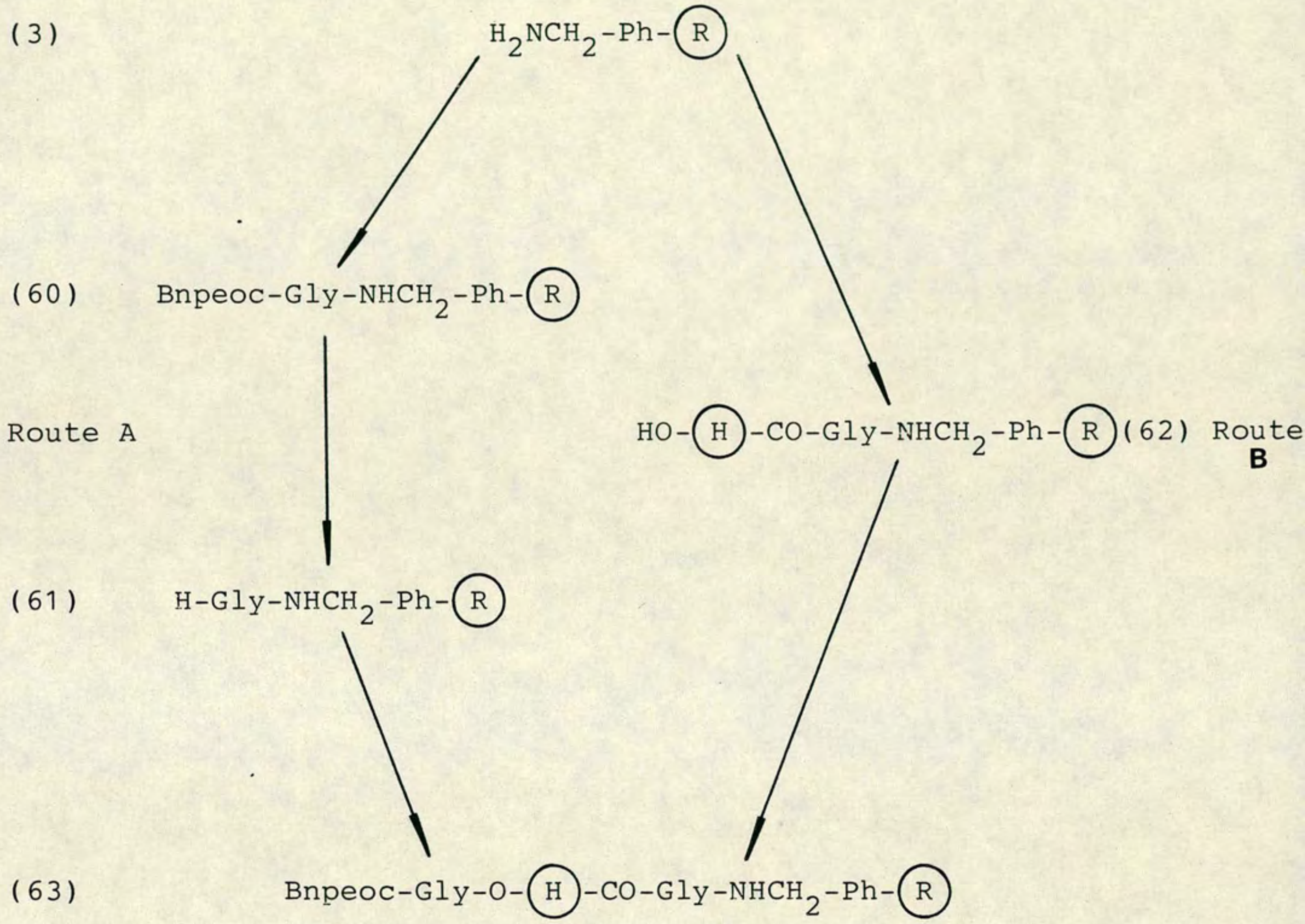
(66)

A further consequence of this study was a differentiation between the methods of agitation employed in the resin loading procedure[132]. Mechanical stirring is the least desirable as this can possibly cause damage to the resin beads, and rotary agitation has problems in that there is a conflict between the requirements of low viscosity and optimum immersion of the reaction mixture for continual vessel inversion, and high concentration of reagents for optimum coupling. The former requires the reaction vessel to be about half-full which obviously contradicts the latter. There is also the problem of the "dead-volume" below the sinter which can effectively remove a small quantity of acylating agent from the reaction mixture. Sonication provides the least disruptive method of agitation and allows the use of the minimum amount of solvent giving high reagent concentration, although it has a disadvantage with respect to rotary inversion in that removal of the resin product from the vessel is necessary for washing, drying and repeat couplings if needed. Results showed, however that sonication was superior to rotary inversion, and in view of these findings, all of the resin modification steps in the attempted stepwise loading of the extended handle (54) were carried out using sonication for the first treatment and rotary inversion for any subsequent treatments so minimising mechanical losses of material. Also, while microanalysis was used as a more accurate measure of reaction yield, KBr disk infrared spectroscopy provided a qualitative guide to the proceedings, with particular attention being paid to the carbonyl region and the characteristic absorptions of the nitro groups of the Bnpeoc moiety.

Returning to the stepwise resin loading of (54), standard Wang resin methodology and the research group acid chloride studies suggest by analogy that the final coupling in the sequence to produce (63) should have been successful, and the fact that it was not indicates that the product obtained from the coupling of (30) to (61) was not the desired product (62). The nature of this product is unclear, but as little could be done to remedy the situation, this line of approach was taken no further.

2.5.2 Partial Fragmentation

Other strategies for producing resin-bound (54) involving a two- rather than a three-fragment approach are outlined in Scheme 13. As can be seen from Schemes 7 and 12, although the resin-bound carboxyl extension amino acid (61) can be obtained successfully, route A falls down on the inability to produce, without further lengthy experimentation, a protected amino acid-handle species with no carboxyl extension.


(3) H_2NCH_2-Ph-(R)
(60) Bnpeoc-Gly-$NHCH_2$-Ph-(R)
Route A
HO-(H)-CO-Gly-$NHCH_2$-Ph-(R) (62)
Route B
(61) H-Gly-$NHCH_2$-Ph-(R)
(63) Bnpeoc-Gly-O-(H)-CO-Gly-$NHCH_2$-Ph-(R)

Scheme 13

As for route B, brief attempts to hydrolyse the phenyl ester (52) (and also (43)) using a similar protocol to that for (53) (and (46)) resulted in a similar outcome to that for (53) (and (46)), *viz.* a complex product mixture requiring further investigation to maximise the desired product yield. In the case of (43), NMR evidence indicated that the desired product, HO-Ⓗ-CO-Aua-OH, had been obtained thus suggesting that improvements in yield and purity could be obtained from further experimentation. While these initial brief attempts at the hydrolysis of (52) (and (43)) were being carried out, however, success was achieved in the direct coupling of (54) (and (47)) to the aminomethyl resin (3) and hence these stepwise approaches were abandoned.

2.6 MONITORING OF RESIN LOADING

Unlike the previous example with the resin loading of the extended handle (47) where amino acid analysis could be used as a simple and fairly accurate measure of the coupling yield, other methods must be employed with the extended handle (54) due to the fact that (54) contains only glycine and that no other different amino acid was coupled to the resin (3) prior to the loading of (54).

When embarking on a solid phase synthesis utilising a handle strategy, it is vital to have at least an approximate idea of the extent to which the handle species has coupled to the resin in order to ensure that some coupling has indeed taken place and, having established that, to avoid unnecessary wastage of materials by adjusting the scale of subsequent reactions accordingly.

In the case of the extended handle (54) being coupled to the aminomethyl resin (3), qualitative monitoring of the reaction was performed by Kaiser testing and KBr disk infrared spectroscopy of the product resin, with the appearance of the relevant carbonyl absorptions and characteristic Bnpeoc-nitro group absorptions at 1350 and 1520 cm^{-1} being particularly significant. Quantitatively, the coupling was monitored in three ways; microanalysis, amine determination by Kaiser testing, and olefin determination by deprotection and ultraviolet absorbance. The last of these methods, devised in the research group, involved deprotecting a sample of the product resin to give the Bnpeoc-derived olefin, measuring the ultraviolet absorbance of the resulting solution at a chosen wavelength and comparing this absorbance with a previously measured standard to ascertain the amount of olefin liberated from the sample. To save on repeated arithmetical effort, BASIC computer programs for use on the Apple II microcomputer were written (see Appendix) to process the raw data from these quantitative tests and display the results in the form of a printout.

The procedures for the quantitative tests were as follows:

Amine Determination using the Kaiser Test[70]

(I) Weigh the resin sample (ca. 2-3 mg) into a tared test-tube.

(II) Add the test solutions to an empty tube as a blank, and the sample, as follows:

Solution 1 : 76% w/w phenol in ethanol (75 μl)
Solution 2 : 0.002M potassium cyanide in pyridine (100 μl)
Solution 3 : 0.28M ninhydrin in ethanol (75 μl).

(III) Incubate the tubes in a heating block at 100°C for 7 minutes.

(IV) Remove the tubes from the block and immediately add 4.75 ml of 60% ethanol in water to give a final volume of 5.00 ml.

(V) Vortex the tubes to mix the solutions thoroughly and let the resin settle to the bottom of the tube.

(VI) Zero an ultraviolet spectrophotometer at 570 nm with the 60% ethanol in water. Note the absorbance of the blank and the sample at 570 nm.

(VII) For manual calculation, the expression

$$\frac{(\text{absorbance sample}-\text{absorbance blank}) \times \text{dilution (ml)} \times 10^6}{\text{extinction coefficient} \times \text{mass of sample (mg)}}$$

where dilution = 5 ml & extinction coefficient = 15000 M^{-1} cm^{-1}

gives the functionality of the resin sample with respect to free amino groups in micromoles per gram.

(VIII) The program RESFUNC-KAISER then uses this expression to calculate the percentage coupling yield for the resin loading step and the functionality of the product resin in terms of the species being loaded. Sampling can be carried out either before the capping step or after the deprotection step.

Olefin Determination

(I) Weigh the resin sample (*ca.* 2-3 mg) into a tared 10 ml volumetric flask.

(II) Make up the volume to 10 ml with 20% v/v piperidine in DMF or DMA and sonicate for 30 minutes before allowing the resin to settle to the bottom of the flask. Piperidine is used in preference to DBU/DBN as there are problems with the latter remaining in the resin matrix after washing.

(III) For Bnpeoc products, remove 1.00 ml of the solution, place in another 10 ml volumetric flask, and again make up the volume to 10 ml with the piperidine solution. This dilution is necessary in view of the Bnpeoc chromophore being an order of magnitude stronger than that of Fmoc and dilution of the sample is preferable to reduction of the sample size. For Fmoc products, omit this dilution step.

(IV) Record the ultraviolet spectrum of a blank and the sample between 350 and 280 nm. Note the absorbance of the sample relative to the blank at 300 nm.

(V) The program RESFUNC-UV then compares this absorbance value with the previously measured standard to determine the amount of olefin released from the sample and hence calculate the percentage coupling yield for the resin loading step and the functionality of the product resin in terms of the species being loaded. Sampling can be carried out either before or after the capping step but before the deprotection step.

Microanalysis

The program RESFUNC-CHN uses the nitrogen percentages in the starting material and product resins to give the coupling yield and the product resin functionality. As before, sampling can be carried out either before or after the capping step but before the deprotection step.

In all of the above quantitative tests, it is imperative to have the product sample absolutely dry as the presence of solvent will give an inaccurate microanalysis and also, since the Kaiser and olefin tests depend for their results on a very small mass of sample, any error in this mass gives a grossly inaccurate final result for the coupling yield. The best way to ensure sample dryness is to give any sample a thorough washing with DCM before drying in either a vacuum desiccator, a vacuum oven, or a drying pistol for at least 24 hours. This ensures the removal of any traces of less volatile solvents, such as DMF, which otherwise can linger throughout the drying process.

The tests are also complementary in the sense that both the microanalysis and olefin determination measure the amount of successfully coupled extended handle while, on the other hand, the Kaiser test - if performed before the capping step - measures the amount of unreacted amine sites remaining on the product resin. As for the accuracy of the tests, microanalysis suffers from the fact that the operational error of ± 0.3% is significant compared to the nitrogen percentages being dealt with, although repetition of the analysis helps to combat this. The olefin and Kaiser tests, as mentioned before, are very sensitive to the sample mass and so again repetition of the test allows a more confident result to be obtained.

2.7 DIRECT RESIN LOADING OF EXTENDED HANDLE (54)

In an attempt to utilise the parent phenyl ester (53) for the resin loading as opposed to the acid (54), the phenyl ester (53) was applied directly to the aminomethyl resin (3) with sonication and reflux in both DCM and DMA. In both cases, however, microanalysis of the product resin showed that no coupling had taken place indicating that the phenyl ester (53) was too inactive for successful coupling in this manner.

To cut down the number of steps involved in producing the functionalised resin (63) and for the possible steric reasons discussed at the end of section 2.3.3; no additional reference amino acid was coupled to the resin (3) prior to its functionalisation with the acid (54) although, as mentioned before, this rules out amino acid analysis as a means of monitoring the coupling reaction. Successful coupling of the extended handle (54) to the aminomethyl resin (3) to give the desired resin-bound product (63) (see Scheme 12) was achieved with the active ester approach using DCCI and HOBt. Small scale studies showed that triple coupling was required and so this was implemented in the larger scale preparation of (63): sonication was used in all three couplings to maximise the coupling yield. Kaiser testing after the third coupling indicated incomplete reaction, thus capping to a negative Kaiser result with acetic anhydride and triethylamine was carried out (see

Fig. 2.1 for quantitative test results).

% Coupling from	Coupling No. 1	2	3	Capping
Microanalysis	17	39	54	37
Olefin Determination	18	35	42	53
Kaiser Testing	26	60	72	45

Figure 2.1 - Coupling Yields Observed in Synthesis of Functionalised Resin (63)

While these results are not in complete accord, it is evident that, especially after capping, there is reasonably good agreement between all three tests; it is also clear that there is good agreement throughout between microanalysis and olefin determination, whereas Kaiser testing gives significantly higher couplng yields, except after capping. It is interesting to note that Kaiser testing gives far better agreement after capping, since for the couplings it detects the unreacted amino groups whereas after capping it detects the amino groups which had previously been coupled to by the desired product (54) and subsequently had the Bnpeoc group removed. Another interesting observation was that an attempt to use the coupling reaction as a purification step for the acid (54) by applying the crude product of a preparation of (54) directly to the resin without any chromatographic purification resulted in none of the desired product (54) being coupled to the resin. A possible explanation for these last two observations is that after purification of (54) there remained a small amount of an unidentified impurity which occupied some amino sites on the resin during the coupling reactions - this would give the high Kaiser test results. Assuming that this impurity was small in relation to (54) and had an accessible, unprotected functional group, then capping would ensure that it took no further part in the proceedings and its small size would render olefin determination and microanalysis relatively insensitive to its presence. Thus the results from these two testing methods were considered more reliable.

For the purposes of later peptide synthesis, the results from the microanalysis and olefin determination of the third coupling and the capping were averaged to give a yield of 47% for the resin loading step, and as can be seen from Fig. 2.1, this was in good agreement with the Kaiser test of the capped and deprotected resin-bound product (64) which gave a 45% yield. As the automated peptide synthesiser used in the subsequent solid phase work begins a synthesis with a deprotection step (see Experimental section), the resin-bound extended handle was left in the N^{α}-protected form (63).

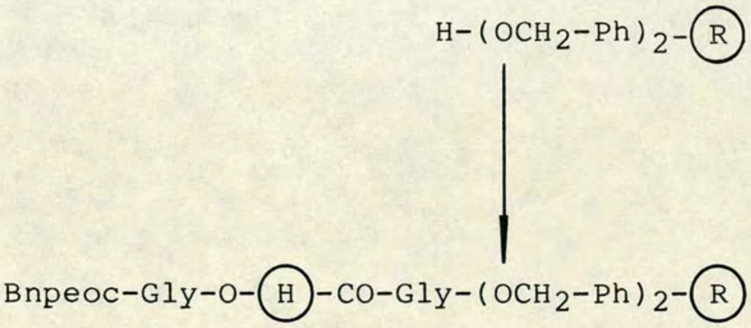
A comparison between the coupling yields for the extended handles (54) and (47), viz. 47% and 86%

respectively, shows a large difference in favour of (47). This suggests that while the long saturated chain of (47) may possibly be a disadvantage when it comes to peptide chain construction (see later), it may also cut down the steric hindrance in the coupling step by distancing the acid function from the bulky remainder of the molecule. Another factor which must also be considered in this instance, however, is the norleucine extension to the aminomethyl resin (3) in the case of (47). It was seen earlier[131] (see section 2.2) that, keeping all other factors constant, extending the functional group on the resin by precoupling a spacer amino acid resulted in a significant improvement in the coupling yield of an extended handle, and this may be the dominant effect in creating the difference in coupling yields. Obviously, a peptide synthesis utilising (54) and the norleucine resin extension is required to offer a direct comparison between the two extended handles, and although this has not yet been attempted, this system may provide the best balance between a high coupling yield and a good peptide synthesis with the added bonus of amino acid analysis as another monitor of the situation.

A second interesting comparison can also be made between the coupling of (54) and previous work[131] on a similar system. Using the active ester protocol with DCCI and HOBt, the extended handle Fmoc-Leu-O-Ⓗ-

CO-Gly-OH was coupled to the aminomethyl resin (3) quantitatively. Since the only major difference here is the N^{α}-protecting group, this result suggests that use of the Bnpeoc group for N^{α}-protection of the extended handle is detrimental and that a better coupling yield might be obtained through use of the Fmoc group.

Coupling of the extended handle (54) to the Wang resin (14) was also attempted using the standard active ester approach which was successful with the aminomethyl resin (3). On this occasion, however, even after two couplings, microanalysis of the intended resin-bound product (67) demonstrated that negligible functionalisation of the Wang resin (14) had been achieved.



$\text{H-(OCH}_2\text{-Ph)}_2\text{-}\textcircled{R}$ (14)

$\text{Bnpeoc-Gly-O-}\textcircled{H}\text{-CO-Gly-(OCH}_2\text{-Ph)}_2\text{-}\textcircled{R}$ (67)

This may be due to steric factors as in this case the functionality of the Wang resin (14) is 2.5 times that of the aminomethyl resin (3), hence the functional groups in (14) are more tightly packed so possibly preventing access of such a large molecule as (54). One way to establish if this was the case would be to couple an extra spacer amino acid to the resin (14) prior to coupling (54) as was demonstrated successfully in previous work[131] in our research group (see section 2.2). Another possibility would be to use an alternative active ester such as the trichlorophenyl ester or the more active pentafluorophenyl ester to see if varying the activity of the coupling ester made any difference to the outcome.

As a footnote to all of the above resin loading studies, a recent publication[141] has justified the use of the traditional Merrifield-type polystyrene-1% divinylbenzene copolymer as the solid support in peptide synthesis. In a study to evaluate the influence of the chemical and physical constitution of the support on the coupling efficiency of protected peptides, the aforementioned polymer gave the best result when compared with four other polymers; a more porous one, a non-crosslinked one, a more polar one and one with a rigid matrix. Since the extended handles (47) and (54) can be likened sterically to protected peptide fragments, this result justifies the use of a resin such as (3) in the work described here and in the fragment condensation approach to the synthesis of ubiquitin in general, to which goal it is hoped the work described here will eventually lead.

2.8 SOLID PHASE SYNTHESIS OF PEPTIDES

All of the peptides discussed here (see Experimental section for details) were assembled on the Applied Biosystems model 430A automated peptide synthesiser using, unless otherwise stated, Fmoc-amino acids and double coupling; the first with symmetrical anhydride (DICI) and the second with active ester (DICI/HOBt). Final cleavage of peptide from the resin was achieved using TBAF which had to be fully dried as the presence of any water forms HF which would attack any acid-labile protecting groups on the peptide. It has recently been reported[142], nevertheless, that N^{α}-protection can be retained intact after peptide cleavage with the commercially available TBAF trihydrate but this practice was not adopted here and the TBAF continued to be thoroughly dried before use. For small peptides, three TBAF treatments were found to be sufficient to remove the vast majority of peptide from the resin, with the third treatment producing only a fraction of that released by the first two. DMF was used as the solvent for these TBAF treatments as a polar solvent was required to ensure adequate solvation of peptide product while at the same time giving acceptable resin swelling, and previous studies in our group[131] showed DMF to be superior to others in this respect.

Dpp-Leu-Ile-Phe-Ala-Gly-OH (68)

Having successfully obtained the resin-bound handle (57), it was utilised in its deprotected form (58) in the synthesis of a pentapeptide. Although the aim of the extended handle strategy was to produce fully protected peptide fragments, it was decided not to complicate matters too soon but to synthesise a short peptide with no side-chain functionality solely in order to test the amenability of the system to peptide synthesis. In keeping with the ubiquitin goal, the peptide chosen for synthesis was ubiquitin (43-47), with the N-terminal amino acid, leucine, having Dpp N^{α}-protection. The completed coupling cycle of each amino acid was monitored quantitatively using the Kaiser test which showed reasonable, but not outstanding, coupling efficiencies (see Fig. 2.2). Capping was not performed during this synthesis as the machine cycles had not been altered to incorporate it at this stage.

Amino Acid	% Coupling from Kaiser Test
Ala [46]	98.8
Phe [45]	98.7
Ile [44]	98.0
Leu [43]	99.5

Figure 2.2 - Coupling Yields Observed in Synthesis of Ubiquitin (43-47) (68) using (58)

The amino acid analysis of the resin-bound peptide: Nle_1 1.00, Gly_1 0.91, Ala_1 0.69, Phe_1 0.62, Ile_1 0.57, Leu_1 0.61 reflected the possible non-quantitative nature of the glycine-alanine coupling, however, the high glycine ratio perhaps alternatively indicated the presence of some capped glycine from the coupling of (47). Excepting this, the analysis also pointed to a reasonable homogeneity of peptide product, although the high leucine ratio with respect to isoleucine suggested some deletion peptide formation. After TBAF cleavage of the peptide, the amino acid analysis of the residue resin: Nle_1 1.00, Gly_1 0.16, Ala_1 0.23, Phe_1 0.26, Ile_1 0.15, Leu_1 0.18 indicated very little peptide remaining on the resin. Purification of the crude cleavage product by gel filtration removed a trace amount of impurity to give the desired peptide (68) in 40% yield. Even though on reflection this yield is not too bad, it was considered to be disappointing at the time and this prompted the investigation into the use of the extended handle (54) in place of (47).

H-Leu-Ile-Phe-Ala-Gly-OH (69)

This peptide was made using the resin-bound extended handle (63) in order to obtain some measure of comparison of the utility of the extended handles (47) and (54) in peptide synthesis. The only differences between here and the synthesis of (68) were that capping after each amino acid was carried out so ensuring no deletion peptide formation and that the N-terminal leucine was deprotected before TBAF peptide cleavage. No amino acid analysis of the resin-bound peptide was carried out but that of the residue resin: Gly_2 2.00, Ala_1 0.43, Phe_1 0.58, Ile_1 0.23, Leu_1 0.29 suggested at first glance that there was more peptide remaining on the resin in this case than with (68), but little could be said in this instance about capped glycine since the carboxyl end of the extended handle (54) is also glycine. Purification of the crude cleavage product by gel filtration, however, gave the desired peptide (69) in 65% yield. It would thus appear that use of the extended handle (54) gives a better yield that that of (47), but as mentioned earlier (see section 2.7), (47) may have some points in its favour.

H-Val-Lys-Gly-Arg-Gly-OH (70)

In order to confirm the suitability of the system employing (54) towards peptide synthesis, it was considered necessary to prepare a second short peptide before tackling a fragment of ubiquitin; the one chosen here was the C-terminal fragment of glucagon-like peptide I. This time, however, amino acids with side-chain functionality were used necessitating the use of side-chain protecting groups so producing adducts with a much greater steric bulk than those used for

(69). For this example, the side-chain of lysine was protected with the Boc group while that of arginine was protected by the Pmc group, an acid-labile moiety developed recently in our research group[117,118]; the N-terminal valine was Boc-protected and the second active ester coupling was omitted for glycine to prevent any possible formation of a glycine-glycine sequence. Also, both couplings for arginine used the active ester and acetyl imidazole was used as the capping agent. Again, no amino acid analysis was performed on the resin-bound peptide and that of the residue resin : Gly_3 3.00, Arg_1 0.49, Lys_1 0.44, Val_1 0.48 showed that most of the peptide had been removed from the resin and, in addition, that excellent homogeneity of product had been obtained. The crude cleavage product was purified by gel filtration after which deprotection was carried out using aqueous TFA. Further purification by gel filtration and then by preparative reverse phase HPLC resulted in a 60% yield of the desired peptide (70).

Fully Protected Ubiquitin (1-35) (71)

Now that it had been clearly demonstrated that the extended handle (54) was suitable for solid phase peptide synthesis, an attempt could now be made to synthesise a fully protected fragment of ubiquitin.

Earlier work in our group[118,143] with the Applied Biosystems machine and the Wang resin (14) had involved the successful synthesis of unprotected ubiquitin (48-76) and indeed of the complete ubiquitin molecule itself, although the purification of the latter proved to be extremely difficult due to, amongst other things, several bad couplings on route; thus it was decided to tackle ubiquitin (1-35). As before, all couplings employed Fmoc-amino acids except for the last one where Boc-methionine was used; TBAF is known to cleave the Fmoc group[144] thus necessitating the use of an alternative protecting group for the N-terminus. The side-chains of glutamic and aspartic acids, threonine and serine were protected with the t-Bu group while that of lysine was Boc-protected. As for (70), glycine was single-coupled using the symmetrical anhydride, while for asparagine and glutamine both couplings employed the active ester with extended reaction times. The ultraviolet deprotection trace (see Experimental section) from the complete ubiquitin synthesis[143] helped to pinpoint the potentially problematic couplings in ubiquitin (1-35) which required checking and these were monitored using the previously described olefin determination test (see Fig. 2.3).

Coupling	% Coupling by Olefin Determination after Coupling Cycle No. 1	2	3
Ile[30]/Gln[31]	102	-	-
Ile[23]/Glu[24]	68	74*	90
Ile[13]/Thr[14]	72	72	-
Gly[10]/Lys[11]	61	-	-

*active ester coupling only repeated

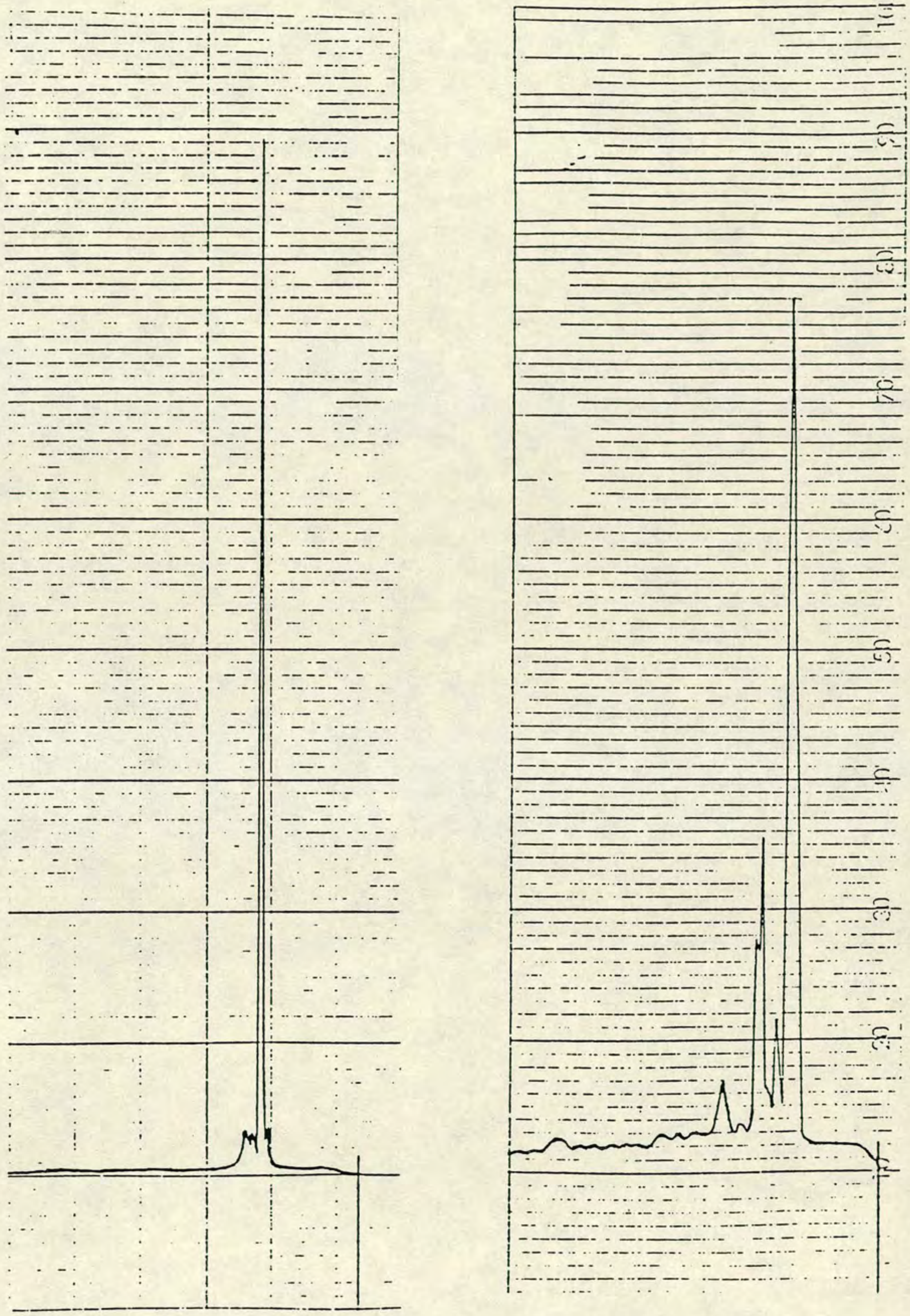
Figure 2.3 - Coupling Yields Observed in Synthesis of Ubiquitin (1-35) (71) using (63)

When TBAF cleavage of the peptide from the resin was attempted, it was found that the crude mass obtained each time increased steadily over the first three treatments, hence the TBAF treatments were continued and seven were performed in all. The crude mass liberated reached a maximum with the fifth treatment after which it began to decline again. Since it was not immediately obvious how many treatments would be required to release all the available peptide, it was decided to stop after the seventh treatment as it was felt in any event at this stage that the quality of the product would deteriorate with repeated TBAF applications liberating an increasing proportion of impurity. This slow leeching of the peptide from the resin points to steric effects coming into play which is not entirely unexpected due to the highly significant increase in steric bulk from the presence of the side-chain protecting groups coupled with the considerable size of TBAF itself. Amino acid analysis of the residue resin was carried out after the fourth, fifth, sixth and seventh TBAF treatments (see Fig. 2.4). Glycine was used to standardise the figures and it can be seen clearly that the ratio of each amino acid to glycine decreased steadily with continuing TBAF treatment showing a steady loss of peptide from the resin. It can also be seen that a significant amount of peptide remained on the resin after the seventh treatment.

As a consequence of the extremely fatty nature of the fully protected peptide, reverse phase HPLC of the product could not be performed on account of its very low solubility in acetonitrile and/or water; indeed its solubility in DMF was just sufficient enough to allow gel filtration to be carried out. Fortunately, the product was soluble enough in DCM to facilitate normal phase HPLC, and this indicated that the product from TBAF treatments one to four was of greater purity than that from the later treatments. These two batches of product were thus combined and purified separately by gel filtration: the first four TBAF treatments produced peptide of excellent quality while that from the later TBAF treatments contained small amounts of impurity (see Fig. 2.5) . A combined yield of 32% was achieved, but

Amino Acid	Frequency in Ubiquitin (1-35)	Amino Acid Analysis of Residue Resin after TBAF Treatment No. 4	5	6	7
Asp/Asn	3	9.19	7.70	6.10	4.46
Thr	5	13.63	11.41	9.29	6.74
Ser	1	3.63	2.78	2.39	1.74
Glu/Gln	6	19.15	15.99	12.48	9.08
Pro	1	4.27	2.71	-	-
Gly	2	10.00	10.00	10.00	10.00
Ala	1	3.38	2.87	2.34	1.89
Val	3	7.82	6.85	5.70	4.59
Met	1	1.39	1.17	0.97	0.50
Ile	4	10.09	8.92	7.47	5.61
Leu	2	5.21	4.69	3.91	2.89
Phe	1	1.92	1.51	1.38	1.09
Lys	5	13.76	12.05	9.92	7.14

Figure 2.4 - Amino Acid Analyses of Resin-bound Ubiquitin (1-35) (71)



TBAF treatments (I)-(IV) TBAF treatments (V)-(VII)

Figure 2.5 - HPLC Traces of Gel Filtered Ubiquitin (1-35) (71)

as a third of the resin-bound product after the coupling of glutamine[2] was removed for other purposes, this amounted to a projected yield of 48% for the desired peptide (71). The amino acid analysis of the product: Asx_3 3.06, Thr_5 4.71, Ser_1 0.93, Glx_6 6.40, Pro_1 1.13, Gly_2 1.97, Ala_1 1.05, Val_3 2.95, Met_1 0.59, Ile_4 3.84, Leu_2 1.95, Phe_1 0.80, Lys_5 5.03 gave a fair result and the presence of the required molecular ion was confirmed by high-resolution mass spectrometry. Proton NMR evidence also supported the existence of the desired product showing strong signals in the correct region for the Boc and t-Bu groups, and so it would appear that the synthesis of fully protected ubiquitin (1-35) (71) was successful which hopefully bodes well for any future attempts at a fragment assembly approach to the synthesis of ubiquitin.

CHAPTER 3 : EXPERIMENTAL

3.1 NOTES

All amino acids used were purchased from the SAS group of companies, except for 11-aminoundecanoic acid which was purchased from Fluka, and used as supplied. Z-amino acid derivatives were prepared by literature methods. Melting points were taken in open capillaries on an electrically heated Buchi 510 melting point apparatus, or on microscope slides on an electrically heated Reichert 7905 melting point apparatus, and are uncorrected. Thin-layer chromatography (TLC) was carried out on plastic sheets coated with silica gel 60GF-254 (Merck 5735) in the following systems:

(A) 50/50, ether/petrol ether (40-60)
(B) 100% ether
(C) 50/50, ethyl acetate/petrol ether (40-60)
(D) 100% ethyl acetate
(E) 80/20, ethyl acetate/methanol
(F) 60/40, ethyl acetate/methanol
(G) 85/15, chloroform/isopropanol
(H) 85/14/1, chloroform/methanol/acetic acid
(I) 60/20/20, n-butanol/acetic acid/water

Visualisation of the compounds was achieved by a suitable combination of the following methods: iodine vapour, ultraviolet absorption at 254 nm, neutral potassium permanganate and bromophenol blue sprays, Mary's reagent (4,4'-bis-(dimethylamino)-phenylcarbinol) for acid functions, and ninhydrin for peptides with free amino groups. High-performance liquid chromatography (HPLC) was carried out 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, i.e. 2 x 1406A solvent delivery systems, a 1480A injector/mixer, and a 1783A detector/controller. Analytical separations were carried out on the following columns:

(I) ODS3 5µ Partisil (4.6 x 250 mm) (reverse phase)
(II) ODS2 5µ Spherisorb (4.6 x 250 mm) (reverse phase)
(III) µ Porasil (3.9 x 300 mm) (normal phase)

using with column (I) a gradient, as specified in parentheses, between solvent A (0.05% TFA in water) and solvent B (0.05% TFA in acetonitrile). 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. Amino acid analyses were carried out on an LKB 4150 alpha amino acid analyser following sealed tube hydrolysis in constant boiling hydrochloric acid at 110°C for 18 hours. Infrared spectra were recorded on a Perkin Elmer 781 spectrophotometer in the solvent indicated, or by the nujol mull technique, using polystyrene as the standard (1603 cm^{-1}). Resin-bound samples were recorded using the KBr disk technique.

Ultraviolet spectra were recorded in HPLC grade acetonitrile on a Varian Cary 210 spectrophotometer. Mass spectra were measured on a Kratos MS 50TC machine. Proton nuclear magnetic resonance (NMR) spectra were recorded on either Bruker WP80 (80 MHz), WP200 (200 MHz), or WH360 (360 MHz) machines in the solvent indicated, using tetramethylsilane as the standard ($\delta = 0.00$). Carbon-13 NMR spectra were recorded on a Bruker WP200 (50 MHz) machine in the solvent indicated, again using tetramethylsilane as the standard ($\delta = 0.00$). Elemental analyses were carried out on a Carlo Erba model 1106 elemental analyser. Halogen analyses were carried out using the oxygen flask combustion technique followed by mercurimetric titration. All solvents were distilled before use, and the following were dried using the reagents given in parentheses when required: acetonitrile (calcium hydride), chloroform (phosphorus pentoxide), dichloromethane (calcium hydride), diethylether (sodium wire), N,N-dimethylformamide/N,N-dimethylacetamide (calcium hydride or 4A molecular sieves), ethyl acetate (anhydrous potassium carbonate), isopropanol (calcium hydride), methanol (magnesium-iodine), tetrahydrofuran (benzophenone-sodium metal), benzene (sodium wire). Petrol ether (40-60) refers to that fraction which boils between 40°C and 60°C.

3.2 EXPERIMENTAL

4-Hydroxymethyl-β-(trimethylsilyl)hydrocinnamic acid (30) HO-Ⓗ-COOH[130]

HO-Ⓗ-COOH (30) was prepared from *p*-tolualdehyde following the experimental procedure given by Ramage *et al.*[130]. The crude product thus obtained was recrystallised from aqueous ethanol to yield pure (30) as a white powder (overall yield 6%), m.p. 120-122°C (lit.[130] 123-124.5°C); δ_H (80 MHz, $(CD_3)_2CO$) 7.24 (2H, d, J 8.0 Hz, aromatics), 7.04 (2H, d, J 8.0 Hz, aromatics), 4.56 (2H, s, benzyl alcohol CH_2), 2.76-2.64 (3H, m, cinnamate CH, CH_2), -0.01 (9H, s, $Si(CH_3)_3$); m/z (FAB) 251, 235, 162, 147, 134; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 5.5 min; TLC (D) R_f 0.40.

Diphenyldiazomethane (31) Ph_2CN_2[145]

Ph_2CN_2 (31) was prepared from benzophenone hydrazone (15.00 g, 76.5 mmol) following the experimental procedure given by Miller[145]. The crude product thus obtained was crystallised from an oil by cooling in a refrigerator to yield (31) as a red crystalline solid (7.80 g, 53%); ν_{max} (CH_2Cl_2) 2040 1590 cm^{-1} $(C=\overset{-}{N}=\overset{+}{N})$.

Benzhydryl 4-(hydroxymethyl)-β-(trimethylsilyl)hydrocinnamate (32) HO-Ⓗ-CO-OBzh

To a stirred solution of HO-Ⓗ-COOH (30)[130] (10.1 g, 40.0 mmol) in chloroform (200 ml) and ethyl acetate

(100 ml) at room temperature was added a solution of diphenyldiazomethane (31)[145] (7.8 g, 40.0 mmol) in chloroform (100 ml) dropwise. The reaction mixture was stirred at room temperature for a further 4 hours, with the nitrogen gas evolved detected using a bubbler. During reaction, the colour of the solution changed from deep purple to pale pink after 4 hours. The reaction mixture was then kept at room temperature in the dark for a further 24 hours, by which time the solution was a pale yellow/green colour. After removal of the reaction solvent under reduced pressure, remaining starting materials were removed by dry flash silica chromatography using a petrol ether (40-60) to ether gradient, and the product was subsequently recrystallised (X3) from 50/50, petrol ether (40-60)/ ether to yield pure (32) (10.00 g, 88%), m.p. 90-91°C; (Found: C, 74.9; H, 7.31; N, 0%; $C_{26}H_{30}O_3Si$ requires C, 74.6; H, 7.22; N, 0%); ν_{max} (CH_2Cl_2) 3600 (O-H), 2960 2930 2870 (aliphatic C-H), 1730 (C=O), 865 845 cm^{-1} (aromatic C-H); δ_H (200 MHz, $CDCl_3$) 7.35-7.10 (10 H, m, aromatics), 6.87 (1H, s, benzhydryl CH), 4.63 (2H, s, benzyl alcohol CH_2), 3.29 (1H, broad s, OH), 3.14-2.78 (3H, m, cinnamate CH, CH_2), 0.08 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 172.3 (C=O), 141.4 (cinnamate C-1), 140.1 (benzhydryl C-1 (X2)), 137.7 (cinnamate C-4), 128.3-127.0 (aromatic CH (X14)), 77.1 (benzhydryl CH), 64.7 (benzyl alcohol CH_2), 35.2 (cinnamate CH_2), 32.5

(cinnamate CH), -0.1 ($Si(CH_3)_3$); m/z (FAB) 419, 401, 357, 226, 167, 117, 74; HRMS 419.2042, $C_{26}H_{31}O_3Si$ (MH^+) requires 419.2042; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 21.4 min; TLC (A) R_f 0.39.

Benzhydryl 4-(N^{α}-(9-fluorenylmethoxycarbonyl)glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamate (33) Fmoc-Gly-O-Ⓗ-CO-OBzh

To a stirred solution of HO-Ⓗ-CO-OBzh (32) (2.50 g, 6.0 mmol) in DCM (10 ml) cooled to 0°C was added a solution of Fmoc-Gly-OH (1.95 g, 6.6 mmol) in DCM (10 ml) and DMF (4 ml) cooled to 0°C. DCCI (1.36 g, 6.6 mmol) in DCM (5 ml) cooled to 0°C was then added followed by a catalytic amount of solid DMAP. The reaction mixture was left stirring to come to room temperature overnight, after which time the precipitated DCU was filtered off and the reaction solvent removed under reduced pressure. Dissolving the product in ethyl acetate afforded a further precipitate of DCU which was removed by filtration, and the filtrate was washed with 5% citric acid solution (X2), saturated sodium bicarbonate solution (X2), water (X2), brine (X2), and dried over anhydrous magnesium sulphate. Removal of the drying agent and the solvent gave the product as a pale yellow oil. Purification was carried out using dry flash silica chromatography with a petrol ether (40-60) to ether gradient, followed by recolumning with chloroform as the eluent to afford the product as a clear, colourless oil, which on lyophilisation yielded pure (33) as a fluffy white powder (4.09 g, 98%), m.p. 47-49°C; (Found: C, 74.1; H, 6.66; N, 1.95%; $C_{43}H_{43}NO_6Si$ requires C, 74.0; H, 6.21; N, 2.01%); ν_{max} (CH_2Cl_2) 3460 (N-H), 3040 2980 2920 2880 (aliphatic C-H), 1740 (C=O), 1520 (urethane N-H), 875 855 cm^{-1}(aromatic C-H); λ_{max} (CH_3CN) 299 nm (ε=6993), 289 (5944), 265 (21678), 227 (23427), 220 (32867); δ_H (200 MHz, $CDCl_3$) 7.82-7.08 (22H, m, aromatics), 6.80 (1H, s, benzhydryl CH), 5.57 (1H, t, J 5.6 Hz, Fmoc-Gly NH), 5.19 (2H, s, benzyl CH_2), 4.46 (2H, d, J 7.1 Hz, Fmoc CH_2), 4.28 (1H, t, J 6.9 Hz, Fmoc CH), 4.04 (2H, d, J 5.7 Hz, Fmoc-Gly CH_2), 3.08-2.71 (3H, m, cinnamate CH, CH_2), 0.01 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 172.0 (benzhydryl ester C=O), 169.8 (Fmoc-Gly C=O), 156.1 (Fmoc C=O), 143.6 141.0 (Fmoc quaternary aromatic (2X2)), 139.8 (benzhydryl quaternary aromatic (X2)), 142.7 (cinnamate C-1), 131.3 (cinnamate C-4), 128.3-119.8 (aromatic CH (X22)), 76.9 (benzhydryl CH), 66.9 (Fmoc CH_2, benzyl CH_2), 46.9 (Fmoc CH), 42.6 (Fmoc-Gly CH_2), 34.7 (cinnamate CH_2), 32.4 (cinnamate CH), -3.4 ($Si(CH_3)_3$); m/z (FAB) 698, 532, 476, 400, 167; HRMS 698.2938, $C_{43}H_{44}NO_6Si$ (MH^+) requires 698.2938; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 26.4 min; TLC (A) R_f 0.26.

Methyl 11-aminoundecanoate hydrochloride (34) $Cl^{-}H_2^{+}$-Aua-OMe

Thionyl chloride (11.91 g, 100.1 mmol) was dissolved in methanol (50 ml) and cooled to below -40°C in a dry ice bath with stirring. 11-aminoundecanoic acid (10.00 g, 49.8 mmol) was added as a solid in small portions over a period of 40 minutes so as to maintain the low temperature. The reaction mixture was left overnight to rise to room temperature, giving a clear, colourless solution and a white precipitate. This was filtered off, and the solvent removed under reduced pressure from the filtrate to give the product as a white powder. Recrystallisation (X2) of the precipitate from methanol afforded further crops of product from the filtrate. All crops of the product were combined and recrystallised from DCM to yield pure (34) (10.16 g, 81%), m.p. 158-160°C; (Found: C, 56.9; H, 10.44; N, 5.57; Cl, 14.0%; $C_{12}H_{26}NO_2Cl$ requires C, 57.2; H, 10.41; N, 5.56; Cl, 14.1%); ν_{max} ($CHCl_3$) 2980 2930 2860 (NH_3^+, aliphatic C-H), 1730 cm^{-1} (C=O); δ_H (80 MHz, $CDCl_3$) 3.56 (3H, s, methyl ester CH_3), 2.73 (2H, m, Aua CH_2), 2.26 (2H, m, Aua CH_2), 1.75-1.23 (16H, m, Aua CH_2 (X8)); δ_C (50 MHz, $CDCl_3$) 173.3 (C=O), 51.0 (methyl ester CH_3), 38.6-24.3 (Aua CH_2 (X10)); m/z (FAB) 216; HRMS 216.1963, $C_{12}H_{26}NO_2$ requires 216.1963; TLC (F) R_f 0.50.

Methyl N^{α}-(4-(hydroxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoate (35) HO-Ⓗ-CO-Aua-OMe

A solution of $Cl^-H_2^+$-Aua-OMe (34) (5.81 g, 23.1 mmol) in DCM (70 ml) and DMF (70 ml) cooled to 0°C was added to a solution of HO-Ⓗ-COOH (30)[130] (5.29 g, 21.0 mmol) in DCM (40 ml) and DMF (10 ml) cooled to 0°C. DCCI (4.76 g, 23.1 mmol) in DCM (20 ml) cooled to 0°C was then added after a period of 5 minutes, followed by neat NMM (2.54 ml, 23.1 mmol) after a further period of 5 minutes. The reaction mixture was left stirring to come to room temperature overnight, after which time the work-up was as for (33). The crude product was obtained as a yellow oil which was purified by dry flash silica chromatography (X2) using a petrol ether (40-60) to ether gradient to yield pure (35) after lyophilisation as a pale yellow oil (3.77 g, 40%); (Found: C, 67.0; H, 9.83; N, 3.17%; $C_{25}H_{43}NO_4Si$ requires C, 66.8; H, 9.64; N, 3.11%); ν_{max} (CH_2Cl_2) 3600 (O-H), 3440 (N-H), 3040 2930 2860 (aliphatic C-H), 1730 (ester C=O), 1665 1510 (amide C=O, N-H), 865 845 cm^{-1} (aromatic C-H); δ_H (80 MHz, $CDCl_3$) 7.16 (2H, d, J 8.3 Hz, aromatics), 6.93 (2H, d, J 8.4 Hz, aromatics), 5.63 (1H, broad s, handle-Aua NH), 4.52 (2H, s, benzyl alcohol CH_2), 3.57 (3H, s, methyl ester CH_3), 2.95 (2H, m, Aua CH_2), 2.70 (1H, broad s, OH), 2.51 (3H, m, cinnamate CH, CH_2), 2.22 (2H, m, Aua CH_2), 1.75-1.00 (16H, m, Aua CH_2 (X8)),

-0.14 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 174.0 (amide C=O), 172.3 (ester C=O), 141.1 (cinnamate C-1), 137.5 (cinnamate C-4), 126.9 (cinnamate C-3, C-5), 126.7 (cinnamate C-2,C-6), 64.2 (benzyl alcohol CH_2), 51.0 (methyl ester CH_3), 36.3 (cinnamate CH_2), 32.4 (cinnamate CH), 39.0-24.5 (Aua CH_2 (X10)), -3.4 ($Si(CH_3)_3$); m/z (FAB) 450, 432, 216, 145; HRMS 450.3039, $C_{25}H_{44}NO_4Si$ (MH^+) requires 450.3039; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 18.6 min; TLC (B) R_f 0.56.

Methyl N^{α}-(4-(N^{α}-(9-fluorenylmethoxycarbonyl)glycyloxymethyl)- β -(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoate (36) Fmoc-Gly-O-Ⓗ-CO-Aua-OMe

A solution of Fmoc-Gly-OH (1.29 g, 4.4 mmol) in DCM (20 ml) and DMF (5 ml) cooled to 0°C was added to a solution of HO-Ⓗ-CO-Aua-OMe (35) (1.77 g, 4.0 mmol) in DCM (30 ml) cooled to 0°C. DCCI (0.90 g, 4.4 mmol) in DCM (10 ml) cooled to 0°C was then added after a period of 5 minutes, followed by a catalytic amount of solid DMAP. The reaction mixture was left stirring to come to room temperature overnight, after which time the work-up was as for (33). The crude product was obtained as a pale yellow oil which was purified by dry flash silica chromatography using a petrol ether (40-60) to ether gradient, followed by a chromatotron using ether as eluent, to yield pure (36) after lyophilisation as a colourless, tacky oil (1.84 g, 64%); (Found: C, 68.8; H, 7.95; N, 3.98%; $C_{42}H_{56}N_2O_7Si$ requires C, 69.2; H, 7.74; N, 3.84%); ν_{max} (CH_2Cl_2) 3460 (N-H), 3040 2950 2870 (aliphatic C-H), 1740 (urethane, benzyl ester, methyl ester C=O), 1670 (amide C=O), 1520 (urethane, amide N-H), 870 850 cm^{-1} (aromatic C-H); λ_{max} (CH_3CN) 300 nm (ε=6461), 289 (5337), 266 (20225), 228 (18258), 221 (21910); δ_H (200 MHz, $CDCl_3$) 7.72-6.98 (12H, m, aromatics), 5.62 (2H, broad m, Fmoc-Gly NH, handle-Aua NH), 5.08 (2H, s, benzyl CH_2), 4.35 (2H, d, J 6.8 Hz, Fmoc CH_2), 4.20 (1H, d, J 6.9 Hz, Fmoc CH), 3.97 (2H, s, Fmoc-Gly CH_2), 3.60 (3H, s, methyl ester CH_3), 3.03 (2H, m, Aua CH_2), 2.56 (3H, m, cinnamate CH, CH_2), 2.25 (2H, m, Aua CH_2), 1.75-1.00 (16H, m, Aua CH_2 (X8)), -0.08 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 174.0 (amide C=O), 171.8 (methyl ester C=O), 169.6 (Fmoc-Gly C=O), 156.1 (Fmoc C=O), 143.5 140.9 (Fmoc quaternary aromatic (2X2)), 142.9 (cinnamate C-1), 131.4 (cinnamate C-4), 128.2-119.6 (aromatic CH (X12)), 66.9 66.6 (Fmoc CH_2, benzyl CH_2), 51.1 (methyl ester CH_3), 46.8 (Fmoc CH), 42.5 (Fmoc-Gly CH_2), 36.4 (cinnamate CH_2), 32.7 (cinnamate CH), 39.1-24.6 (Aua CH_2 (X10)), -3.3 ($Si(CH_3)_3$); m/z (FAB) 729, 713, 506, 432, 179; HRMS 729.3935, $C_{42}H_{57}N_2O_7Si$ (MH^+) requires 729.3935; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 23.2 min; TLC (B) R_f 0.37.

N^{α}-Benzyloxycarbonyl-11-aminoundecanoic acid (37)
Z-Aua-OH

A 4M solution of sodium hydroxide (100 ml, 400 mmol) and a 1M solution of sodium bicarbonate (190 ml, 190 mmol) were added to 11-aminoundecanoic acid (25.01 g, 124.4 mmol). THF (400 ml) was also added. The resulting suspension was cooled to 0°C with mechanical stirring being applied. Benzyl chloroformate (23.32 g, 136.8 mmol) was added dropwise over a period of 1 hour and the reaction mixture was left stirring to come to room temperature overnight. The reaction mixture was filtered, and the filtrate was brought to pH2 with concentrated HCl then stirred at room temperature for 1 hour. Aqueous and organic phases were separated and the aqueous phase was extracted with ethyl acetate. The combined organic phase was dried over anhydrous magnesium sulphate and removal of the solvent gave the product as a white powder. Recrystallisation from ethyl acetate yielded pure (37) as a white powder (41.68 g, 100%), m.p. 96-98°C; (Found: C, 68.2; H, 8.82; N, 4.12%; $C_{19}H_{29}NO_4$ requires C, 68.1; H, 8.66; N, 4.18%); ν_{max} (mull) 3330 (N-H), 3100-2500 (O-H, aliphatic C-H, masked by nujol), 1685 (urethane, acid C=O), 1530 (urethane N-H), 750 695 cm^{-1} (aromatic C-H); δ_H (200 MHz, $(CD_3)_2SO$) 7.36-7.28 (5H, m, aromatics), 7.17 (1H, broad s, NH), 5.03 (2H, s, benzyl CH_2), 3.01

(2H, m, Aua CH_2), 2.20 (2H, t, J 7.3 Hz, Aua CH_2), 1.60-1.20 (16H, m, Aua CH_2 (X8)); δ_c (50 MHz, $(CD_3)_2SO$) 174.4 (acid C=O), 156.0 (urethane C=O), 137.2 (quaternary aromatic), 128.1-126.7 (aromatic CH (X5)), 65.0 (benzyl CH_2), 40.1-24.4 (Aua CH_2 (X10)); m/z (FAB) 336, 292, 202, 181, 158; HRMS 336.2175, $C_{19}H_{30}NO_4$ (MH^+) requires 336.2175; TLC (F) R_f 0.72.

N^{α}-Benzyloxycarbonyl-11-aminoundecanoic anhydride (38)
(Z-Aua)$_2$O

To a stirred solution of Z-Aua-OH (37) (5.00 g, 14.9 mmol) and phenol (1.41 g, 14.9 mmol) in ethyl acetate (600 ml) at 0°C were added pyridine (1.21 g, 15.2 mmol) (optional) at 0°C and DCCI (3.35 g, 16.2 mmol) in ethyl acetate (100 ml) at 0°C. The reaction mixture was allowed to come to room temperature overnight, after which time glacial acetic acid (1 ml) was added and the reaction mixture stirred for 1 hour. DCU was then filtered off, and the work-up was as for (33). Removal of the drying agent and the solvent gave the product as a yellow/white powder. Treatment of this powder with hot ethyl acetate enabled residual DCU to be filtered off. Solvent removal from the filtrate afforded (38) as a white powder (2.91 g, 60%), m.p. 89-91°C; ν_{max} (mull) 3330 (N-H), 3100-2500 (aliphatic C-H, masked by nujol), 1800 1740 (anhydride C=O), 1685

1530 (urethane C=O, N-H), 750 695 cm^{-1} (aromatic C-H); δ_H (80 MHz, $(CD_3)_2SO$) as for Z-Aua-OH (37); m/z (FAB) 670, 541, 430, 336, 292, 281, 225, 202, 181, 158.

Phenyl N$^{\alpha}$-benzyloxycarbonyl-11-aminoundecanoate (39)
Z-Aua-OPh

Z-Aua-OH (37) (3.11 g, 9.3 mmol) was covered in benzene (100 ml), and oxalyl chloride (1.42 g, 11.2 mmol) and DMAP (catalytic amount) were added. The reaction mixture was stirred at room temperature for 2 hours, then the solvent was removed in vacuo. Benzene was reapplied and removed several times to ensure no oxalyl chloride remained. Phenol (0.88 g, 9.3 mmol) and NMM (0.94 g, 9.3 mmol) in DCM (100 ml) was then added to the product, and the reaction mixture was stirred at room temperature for 24 hours, after which time the work-up was as for (33). Removal of the drying agent and the solvent furnished the product as a white powder which was recrystallised from ether/DCM and washed with petrol ether (40-60) to yield pure (39) as a white powder (3.48 g, 91%), m.p. 63-65°C; (Found: C, 73.0; H, 8.15; N, 3.37%; $C_{25}H_{33}NO_4$ requires C, 73.0; H, 8.03; N, 3.41%); ν_{max} (CH_2Cl_2) 3440 (N-H), 2930 2850 (aliphatic C-H), 1755 (ester C=O), 1720 1510 cm^{-1} (urethane C=O, N-H); δ_H (200 MHz, $CDCl_3$) 7.39-7.06 (10H, m, aromatics), 5.21 (1H, broad s, NH), 5.10 (2H, s, benzyl CH_2), 3.14

(2H, m, Aua CH_2), 2.53 (2H, t, J 7.4 Hz, Aua CH_2), 1.78-1.30 (16H, m, Aua CH_2 (X8)); δ_C (50 MHz, $CDCl_3$) 171.9 (ester C=O), 156.4 (urethane C=O), 150.8 (phenyl ester quaternary aromatic), 136.8 (Z quaternary aromatic), 129.1 125.4 121.4 (phenyl ester aromatic CH (X5)), 128.3 127.8 (Z aromatic CH (X5)), 66.2 (Z CH_2), 41.0-24.8 (Aua CH_2 (X10)); m/z (FAB) 412, 332, 278, 210, 184; HRMS 412.2488, $C_{25}H_{34}NO_4$ (MH^+) requires 412.2488; TLC (C) R_f 0.57.

<u>Phenyl 11-aminoundecanoate hydrobromide</u> (40) $Br^-H_2{}^+$-Aua-OPh

Z-Aua-OPh (39) (25.00 g, 60.8 mmol) was dissolved in glacial acetic acid (650 ml) and 45% HBr in glacial acetic acid (54.8 g, 304.9 mmol) was added. The reaction mixture was stirred at room temperature for 24 hours, after which time the solvent was removed to give a yellow/brown oil. Trituration of this oil with ether produced an off-white solid which was filtered off and recrystallised (X3) from acetonitrile and washed with ether to yield pure (40) as a white powder (16.60 g, 76%), m.p. 115-119°C; ν_{max} (mull) 3300-2500 ($NH_3{}^+$, aliphatic C-H, masked by nujol), 1725 (C=O), 1605 1585 1500 cm^{-1} ($NH_3{}^+$); δ_H (200 MHz, $(CD_3)_2CO$) 8.30 (3H, broad s, $NH_3{}^+$), 7.45-7.08 (5H, m, aromatics), 2.57 (2H, t, J 7.4 Hz, Aua CH_2), 2.26 (2H, t, J 7.3 Hz, Aua CH_2), 1.85-1.28 (16H, m, Aua CH_2 (X8)); m/z (EI) 279, 184, 94; full characterisation not possible due to long-term instability of the product.

<u>11-Aminoundecanoic acid hydrobromide</u> (41) $Br^-H_2^+$-Aua-OH

$Br^-H_2^+$-Aua-OH (41) was formed quantitatively in the breakdown of $Br^-H_2^+$-Aua-OPh (40). Recrystallisation from acetonitrile and washing with ether yielded pure (41) as a white powder, m.p. 130-131°C; (Found: C, 46.0; H, 8.56; N, 4.98; Br, 28.3%; $C_{11}H_{24}NO_2Br$ requires C, 46.8; H, 8.57; N, 4.96; Br, 28.3%); ν_{max} (mull) 3410 (O-H), 3200-2500 (NH_3^+, aliphatic C-H, masked by nujol), 1720 (C=O), 1610 1590 1505 cm^{-1} (NH_3^+); δ_H (80 MHz, $(CD_3)_2SO$) 7.87 (3H, broad s, NH_3^+), 2.69 (2H, m, Aua CH_2), 2.12 (2H, m, Aua CH_2), 1.75-1.00 (16H, m, Aua CH_2 (X8)); δ_C (50 MHz, $(CD_3)_2SO$) 174.3 (C=O), 38.6-24.3 (Aua CH_2 (X10)); m/z (FAB) 202 184 158 142; HRMS 202.1807, $C_{11}H_{24}NO_2$ requires 202.1807; TLC (F) R_f 0.50.

<u>Phenyl 11-aminoundecanoate p-toluenesulphonate</u> (42) $TosO^-H_2^+$-Aua-OPh

Z-Aua-OPh (39) (3.88 g, 9.4 mmol) and 4-toluenesulphonic acid (1.88 g, 9.9 mmol) were dissolved in DMF (25 ml) under nitrogen. The reaction mixture was hydrogenated at room temperature for 72 hours in the presence of 10% palladium on charcoal (0.39 g). Filtration through Celite was carried out and the solvent removed in vacuo to give a pale green oil which was taken up in isopropanol. Addition of ether to this solution precipitated out the desired product which was filtered and washed with petrol ether (40-60) to yield pure (42) as a white powder (3.18 g, 75%), m.p. 95-98°C; (Found: C, 64.0; H, 8.12; N, 3.21%; $C_{24}H_{35}NO_5S$ requires C, 64.1; H, 7.85; N, 3.12%); ν_{max} (CH_2Cl_2) 3300-3000 (NH_3^+), 2930 2860 (aliphatic C-H), 1755 (C=O), 1595 1495 (NH_3^+), 815 cm^{-1} (aromatic C-H); δ_H (200 MHz, $CDCl_3$) 7.60 (3H, broad s, NH_3^+), 7.76-7.04 (9H, m, aromatics), 2.75 (2H, m, Aua CH_2), 2.54 (2H, t, J 7.3 Hz, Aua CH_2), 2.35 (3H, s, tosyl CH_3), 1.80-1.10 (16H, m, Aua CH_2 (X8)); δ_C (50 MHz, $CDCl_3$) 171.6 (C=O), 150.3 (phenyl ester quaternary aromatic), 141.2 139.9 (tosyl quaternary aromatic), 128.8-121.0 (aromatic CH (X9)), 39.5-24.4 (Aua CH_2 (X10)), 20.8 (tosyl CH_3); m/z (FAB) 278, 202, 184, 158; HRMS 278.2120, $C_{17}H_{28}NO_2$ requires 278.2120; TLC (G) R_f 0.62.

Phenyl N^{α}-(4-(hydroxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoate (43) HO-Ⓗ-CO-Aua-OPh

A solution of HO-Ⓗ-COOH (30)[130] (2.61 g, 10.3 mmol) in DCM (100 ml) and DMF (5 ml) cooled to 0°C was added to a solution of $TosO^-H_2^+$-Aua-OPh (42) (4.73 g, 10.5 mmol) in DCM (180 ml) and DMF (10 ml) cooled to 0°C. DCCI (2.24 g, 10.9 mmol) in DCM (20 ml) cooled to 0°C was then added after a period of 5 minutes followed by neat NMM (1.20 ml, 10.9 mmol) after a further period of 5 minutes. The reaction mixture was left stirring to come to room temperature overnight, after which time the work-up was as for (33). The crude product was obtained as a pale yellow oil which was purified by wet flash silica chromatography using a petrol ether (40-60) to ether gradient, followed by a chromatotron using 50/50, ether/ethyl acetate as eluent. The chromatotron product was then further purified by gel filtration on Sephadex LH20 eluting with methanol to yield pure (43) after lyophilisation as a colourless oil (5.84 g, 68%); satisfactory microanalysis could not be obtained; ν_{max} ($CHCl_3$) 3600 (O-H), 3450-3200 (O-H, N-H), 3000 2930 2860 (aliphatic C-H), 1750 (ester C=O), 1650 1510 (amide C=O, N-H), 865 845 cm^{-1} (aromatic C-H); δ_H (200 MHz, $CDCl_3$) 7.31-6.91 (9H, m, aromatics), 6.01 (1H, broad s, handle-Aua NH), 4.48 (2H, s, benzyl alcohol CH_2), 2.93 (2H, m, Aua CH_2), 2.52-2.43 (5H, m, cinnamate CH, CH_2; Aua CH_2), 1.70-1.00 (16H, m, Aua CH_2 (X8)), -0.12 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 172.4 172.1 (ester, amide C=O), 150.6 (phenyl ester quaternary aromatic), 141.3 (cinnamate C-1), 137.7 (cinnamate C-4), 129.1 125.5 121.3 (phenyl ester aromatic CH (X5)), 127.1 (cinnamate C-3, C-5), 126.8 (cinnamate C-2, C-6), 64.3 (benzyl alcohol CH_2), 36.6 (cinnamate CH_2), 32.6 (cinnamate CH), 39.2-24.7 (Aua CH_2 (X10)), 0.01 ($Si(CH_3)_3$); m/z (FAB) 512, 265, 132; HRMS 512.3196,

$C_{30}H_{46}NO_4Si$ (MH^+) requires 512.3196; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 18.4 min; TLC (D) R_f 0.56.

N^{α}-(N^{α}-(4-(Hydroxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoyl)-11-aminoundecanoic acid

(44) HO-Ⓗ-CO-Aua-Aua-OH

HO-Ⓗ-CO-Aua-Aua-OH (44) was obtained as a by-product in the formation of HO-Ⓗ-CO-Aua-OPh (43) when $Br^-H_2^+$-Aua-OPh (40) was used in place of $TosO^-H_2^+$-Aua-OPh (42). Recrystallisation from DCM and washing with ether yielded pure (44) as a white powder (1.02 g, 16%), m.p. 111-113°C; (Found: c, 68.4; H, 10.58; N, 4.99%; $C_{35}H_{62}N_2O_5Si$ requires C, 67.9; H, 10.10; N, 4.53%); ν_{max} (mull) 3380-3200 (O-H, N-H), 3100-2500 (aliphatic C-H, masked by nujol), 1705 (acid C=O), 1660 1640 (amide C=O), 1545 (amide N-H, masked by nujol), 870 840 cm^{-1} (aromatic C-H); δ_H (200 MHz, $(CD_3)_2SO$) 7.76 (2H, broad s, handle-Aua-Aua NH (X2)), 7.14 (2H, d, J 8.0 Hz, aromatics), 6.94 (2H, d, J 8.0 Hz, aromatics), 4.41 (2H, s, benzyl alcohol CH_2), 2.94 (4H, m, Aua CH_2 (X2)), 2.60 (3H, m, cinnamate CH, CH_2), 2.10 (4H, m, Aua CH_2 (X2)), 1.50-1.20 (32H, m, Aua CH_2 (X16)), -0.11 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $(CD_3)_2SO$) 174.5 (acid C=O), 172.1 171.3 (amide C=O (X2)), 141.3 (cinnamate C-1), 138.4 (cinnamate C-4), 127.0 (cinnamate C-3, C-5), 126.2 (cinnamate C-2, C-6), 63.0 (benzyl alcohol CH_2), 35.5

(cinnamate CH_2), 31.7 (cinnamate CH), 38.3-24.6 (Aua CH_2 (X20)), -3.0 ($Si(CH_3)_3$); m/z (FAB) 619, 601, 385, 225, 216, 185, 145; HRMS 619.4506, $C_{35}H_{63}N_2O_5Si$ (MH^+) requires 619.4506; TLC (D) R_f 0.25.

N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)-glycine (45)

Bnpeoc-Gly-OH[132]

Method 1

Glycine (5.53 g, 73.7 mmol) was dissolved in 20% sodium carbonate solution (78 ml, 147.2 mmol) and cooled to 0°C. To this solution was added Bnpeoc-ONSu[132] (26.3 g, 61.4 mmol) in DMF (100 ml) cooled to 0°C. The resulting suspension was stirred at room temperature for 15 minutes before the addition of a quantity of water sufficient to obtain a clear solution. This solution was then extracted (X5) with ethyl acetate to remove any unreacted Bnpeoc-ONSu, and the aqueous phase was cooled to 0°C and acidified to pH 1-2 with concentrated HCl. A white suspension was immediately formed which was extracted (X5) with ethyl acetate, and the organic fraction was washed with water (X2), brine (X2), and dried over anhydrous sodium sulphate. Removal of the solvent *in vacuo* gave a green oil which was recrystallised from acetone/petrol ether (40-60) to yield pure (45) as a white powder (20.92 g, 88%), m.p. 155-157°C (lit.[132] 156-158°C).

Method 2

Bnpeoc-Gly-OH (45) was prepared from glycine (3.97 g, 53.0 mmol) following the experimental procedure given by Rich and Shute[146] for the synthesis of Teoc-amino acids using, however, Bnpeoc-ONSu in place of Teoc-ONSu. The crude product thus obtained was recrystallised from acetone/petrol ether (40-60) to yield pure (45) as a white powder (17.32 g, 84%), m.p. 155-157°C (lit.[132] 156-158°C); ν_{max} (CH_2Cl_2) 3460 (N-H), 1730 (urethane, acid C=O), 1610 (aromatic C-C), 1520 (urethane N-H; NO_2), 1350 (NO_2), 860 cm^{-1} (aromatic C-H); δ_H (200 MHz, $(CD_3)_2SO$) 8.19 (4H, d, J 8.6 Hz, Bnpeoc aromatics), 7.69 (4H, d, J 8.6 Hz, Bnpeoc aromatics), 7.49 (1H, t, J 6.0 Hz, NH), 4.82-4.67 (3H, m, Bnpeoc CH, CH_2), 3.63 (2H, d, J 6.0 Hz, Gly CH_2); δ_C (50 MHz, $(CD_3)_2SO$) 171.3 (acid C=O), 156.2 (urethane C=O), 148.1 146.6 (Bnpeoc quaternary aromatic (2X2)), 129.6 123.7 (Bnpeoc aromatic CH (2X4)), 65.2 (Bnpeoc CH_2), 49.0 (Bnpeoc CH), 42.1 (Gly CH_2).

Phenyl N^{α}-(4-(N^{α}-(2,2-bis(4-nitrophenyl)ethoxycarbonyl)-glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoate (46) Bnpeoc-Gly-O-Ⓗ-CO-Aua-OPh

A solution of Bnpeoc-Gly-OH (45)[132] (4.45 g, 11.5 mmol) in DCM (60 ml) and DMF (15 ml) cooled to 0°C was added to a solution of HO-Ⓗ-CO-Aua-OPh (43) (5.57 g, 10.9 mmol) in DCM (100 ml) cooled to 0°C. DCCI

(2.47 g, 12.0 mmol) in DCM (25 ml) cooled to 0°C was then added after a period of 5 minutes, followed by a catalytic amount of solid DMAP. The reaction mixture was left stirring to come to room temperature overnight, after which time the work-up was as for (33). The crude product was obtained as a green oil which was purified by wet flash silica chromatography using a petrol ether (40-60) to ethyl acetate gradient, followed by a chromatotron using 50/50, ether/ethyl acetate as eluent. Further purification was carried out using gel filtration on Sephadex LH20 eluting with methanol to yield pure (46) after lyophilisation as a yellow powder (8.73 g, 91%), m.p. 47-50°C; (Found: C, 64.4; H, 6.50; N, 6.45%; $C_{47}H_{58}N_4O_{11}Si$ requires C, 64.0; H, 6.58; N, 6.35%); ν_{max} (CH_2Cl_2) 3460 (N-H), 2950 2870 (aliphatic C-H), 1735 (urethane, benzyl ester, phenyl ester C=O), 1670 (amide C=O), 1525 (urethane, amide N-H; NO_2), 1355 (NO_2), 865 845 cm^{-1} (aromatic C-H); λ_{max} (CH_3CN) 274 nm (ε = 33529), 229 (23529), 220, (22941); δ_H (200 MHz, $CDCl_3$) 8.21-7.01 (17H, m, aromatics), 5.33-5.10 (4H, m, Bnpeoc-Gly NH, handle-Aua NH, benzyl CH_2), 4.68-4.56 (3H, m, Bnpeoc CH, CH_2), 3.94 (2H, d, J 5.5 Hz, Bnpeoc-Gly CH_2), 3.05 (2H, m, Aua CH_2), 2.59-2.50 (5H, m, Aua CH_2, cinnamate CH, CH_2), 1.73-1.05 (16H, m, Aua CH_2 (X8)), -0.05 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 171.9 171.8 (phenyl ester, amide C=O), 169.3 (Bnpeoc-Gly C=O), 155.5 (Bnpeoc C=O), 150.4 (phenyl ester quaternary aromatic), 146.8 (Bnpeoc quaternary aromatic (X4)), 143.0 (cinnamate C-1), 131.2 (cinnamate C-4), 128.9-121.2 (aromatic CH (X17)), 66.7 (benzyl CH_2), 65.7 (Bnpeoc CH_2), 49.4 (Bnpeoc CH), 42.4 (Bnpeoc-Gly CH_2), 36.3 (cinnamate CH_2), 32.6 (cinnamate CH), 39.0-24.5 (Aua CH_2 (X10)), -3.4 ($Si(CH_3)_3$); m/z (FAB) 883, 867, 851, 789, 579, 568, 510, 494, 145; HRMS 883.3949, $C_{47}H_{59}N_4O_{11}Si$ (MH^+) requires 883.3949; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 23.8 min; TLC (C) R_f 0.48.

N^{α}-(4-(N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoic acid (47) Bnpeoc-Gly-O-Ⓗ-CO-Aua-OH

A solution of 100 volumes hydrogen peroxide (508.2 μl, 4.56 mmol) followed by a 0.1M solution of sodium hydroxide (22.83 ml, 2.28 mmol) was added to a solution of Bnpeoc-Gly-O-Ⓗ-CO-Aua-OPh (46) (2.010 g, 2.28 mmol) in 80/20, acetone/water (125 ml), and the reaction mixture was stirred at room temperature for 15 minutes. Quenching of the reaction mixture to pH 2-3 with a saturated aqueous solution of citric acid was then carried out, and the reaction mixture was partitioned between ethyl acetate and water. The organic fraction was washed with water (X2), brine (X2), and dried over anhydrous magnesium sulphate. Purification of the product was by wet flash silica chromatography (X2) using a petrol ether (40-60) to ethyl acetate gradient, followed by gel filtration on Sephadex LH20 eluting with methanol to yield pure (47) after lyophilisation as an off-white powder (1.194 g, 65%), m.p. 56-69°C; (Found: C, 61.0; H, 6.80; N, 7.09%; $C_{41}H_{54}N_4O_{11}Si$ requires C, 61.0; H, 6.74; N, 6.94%); ν_{max} (CH_2Cl_2) 3470 (N-H), 3350-2500 (O-H, aliphatic C-H), 1740 (urethane, ester, acid C=O), 1675 (amide C=O), 1530 (urethane, amide N-H; NO_2), 1360 (NO_2), 870 855 cm^{-1} (aromatic C-H); λ_{max} (CH_3CN) 274 nm (ε = 40173), 230 (24277), 220 (22832); δ_H (200 MHz, $CDCl_3$) 8.14 (4H, d, J 8.8 Hz, Bnpeoc aromatics), 7.37 (4H, d, J 8.8 Hz, Bnpeoc aromatics), 7.16 (2H, d, J 8.2 Hz, cinnamate aromatics), 7.00 (2H, d, J 8.2 Hz, cinnamate aromatics), 5.56 5.43 (1H, broad s (X2), Bnpeoc-Gly NH, handle-Aua NH), 5.06 (2H, s, benzyl CH_2), 4.68-4.58 (3H, m, Bnpeoc CH, CH_2), 3.90 (2H, d, J 5.7 Hz, Bnpeoc-Gly CH_2), 3.00 (2H, m, Aua CH_2), 2.57 (3H, m, cinnamate CH, CH_2), 2.27 (2H, t, J 7.4 Hz, Aua CH_2), 1.60-1.00 (16H, m, Aua CH_2 (X8)), -0.09 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 177.7 (acid C=O), 172.4 (amide C=O), 169.6 (Bnpeoc-Gly C=O), 155.6 (Bnpeoc C=O), 147.0 146.8 (Bnpeoc quaternary aromatic (2X2)), 143.0 (cinnamate C-1), 131.3 (cinnamate C-4), 129.1 123.9 (Bnpeoc aromatic CH (2X4)), 128.4 (cinnamate C-3, C-5), 127.4 (cinnamate C-2, C-6), 66.9 (benzyl CH_2), 66.0 (Bnpeoc CH_2), 49.6 (Bnpeoc CH), 42.5 (Bnpeoc-Gly CH_2), 36.5 (cinnamate CH_2), 32.8 (cinnamate

CH), 39.2-24.6 (Aua CH_2 (X10)), -3.3 ($Si(CH_3)_3$); m/z (FAB) 807, 792, 506, 490, 476, 462, 450, 418, 404, 145; HRMS 807.3636, $C_{41}H_{55}N_4O_{11}Si$ (MH^+) requires 807.3636; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 17.6 min; TLC (D) R_f 0.45.

N^{α}-Benzyloycarbonyl-glycine (48) Z-Gly-OH[135]

Z-Gly-OH (48) was prepared from glycine (113.21 g, 1.5 mol) following the experimental procedure given by Greenstein and Winitz[135]. The crude product thus obtained was recrystallised from ethyl acetate with petrol ether (40-60) to yield pure (48) as a white crystalline solid (280.50 g, 89%), m.p. 119-120°C (lit.[135] 120-121°C); ν_{max} (CH_3CN) 3480 (N-H), 3240 (O-H), 3000-2800 (aliphatic C-H), 1770 (acid C=O), 1725 1525 (urethane C=O, N-H), 740 705 cm^{-1} (aromatic C-H); δ_H (80 MHz, $(CD_3)_2CO$) 7.34 (5H, s, aromatics), 5.09 (2H, s, benzyl CH_2), 3.91 (2H, t, J 3.1 Hz, Gly CH_2); m/z (EI) 209, 108, 91; TLC (H) R_f 0.50.

Phenyl N^{α}-benzyloxycarbonyl-glycinate (49) Z-Gly-OPh[136]

Z-Gly-OPh (49) was prepared from Z-Gly-OH (48)[135] (61.60 g, 0.3 mol) following the experimental procedure given by Ramage et al.[136]. The crude product thus obtained was taken up in ethyl acetate and precipitated out by the addition of petrol ether (40-60) to yield pure (49) as a white powder (65.30 g, 78%), m.p. 66-67°C (lit.[136] 67-68°C); ν_{max} (CH_2Cl_2) 3460 (N-H), 3040 2960 (aliphatic C-H), 1775 (ester C=O), 1730 1515 cm^{-1} (urethane C=O, N-H); δ_H (80 MHz, $CDCl_3$) 7.46-7.01 (10H, m, aromatics), 5.36 (1H, broad s, NH), 5.15 (2H, s, benzyl CH_2), 4.20 (2H, d, J 5.7 Hz, Gly CH_2); m/z (FAB) 286, 242, 185, 181, 152; TLC (D) R_f 0.60.

Phenyl glycinate p-toluenesulphonate (51) $TosO^-H_2^+$-Gly-OPh

Z-Gly-OPh (49)[136] (11.39 g, 39.9 mmol) and 4-toluenesulphonic acid (8.35 g, 43.9 mmol) were dissolved in DMF (100 ml) under nitrogen. The reaction mixture was hydrogenated at room temperature for 72 hours in the presence of 10% palladium on charcoal (1.14 g). Filtration through Celite was carried out and the solvent removed in vacuo to give a pale green oil which was recrystallised from isopropanol with petrol ether (40-60) to yield pure (51) as a white powder (9.54 g, 74%), m.p. 154-156°C; (Found: C, 56.0; H, 5.26; N, 4.40%; $C_{15}H_{17}NO_5S$ requires C, 55.7; H, 5.30; N, 4.33%); ν_{max} (mull) 3300-3000 (NH_3^+), 3000-2500 (aliphatic C-H, masked by nujol), 1780 (C=O), 1600 (NH_3^+), 815 755 685 cm^{-1} (aromatic C-H); δ_H (80 MHz, $(CD_3)_2SO$) 8.38 (3H, broad s, NH_3^+), 7.60-7.05 (9H, m, aromatics), 4.14 (2H, s, Gly CH_2), 2.30 (3H, s, tosyl CH_3); δ_C (50 MHz, $(CD_3)_2SO$) 166.2 (C=O), 149.6 (phenyl ester quaternary aromatic), 143.7 138.9 (tosyl quaternary aromatic), 129.5-121.3 (aromatic CH (X9)), 39.9 (Gly CH_2), 20.6 (tosyl CH_3); m/z (FAB) 324, 152, 31; HRMS 324.0905, $C_{15}H_{18}NO_5S$ (MH^+) requires 324.0906; 152.0712, $C_8H_{10}NO_2$ requires 152.0712; TLC (G) R_f 0.24.

Methyl glycinate p-toluenesulphonate (50) $TosO^-H_2^+$-Gly-OMe

The procedure for the formation of $TosO^-H_2^+$-Gly-OMe (50) from Z-Gly-OPh (49)[136] (52.40 g, 183.9 mmol) was exactly the same as that for $TosO^-H_2^+$-Gly-OPh (51) except that methanol (250 ml) was used as the solvent rather than DMF. Purification of the crude golden brown oil again was achieved by recrystallisation from isopropanol with petrol ether (40-60) to yield pure (50) as a white powder (36.80 g, 62%), m.p. 109-112°C; (Found: C, 46.3; H, 5.69; N, 5.41%; $C_{10}H_{15}NO_5S$ requires C, 46.0; H, 5.79; N, 5.36%); ν_{max} (mull) 3200-3000 (NH_3^+), 3000-2500 (aliphatic C-H, masked by nujol), 1760 (C=O), 1620 1530 (NH_3^+), 820 cm^{-1} (aromatic C-H); δ_H (200 MHz, $(CD_3)_2SO$) 8.25 (3H, broad s, NH_3^+), 7.50 (2H, d, J 8.0 Hz, aromatics), 7.12 (2H, d, J 8.0 Hz, aromatics), 3.83 (2H, s, Gly CH_2), 3.74 (3H, s, methyl ester CH_3), 2.30 (3H, s, tosyl CH_3); δ_C (50 MHz, $(CD_3)_2SO$) 167.9 (C=O), 145.3 137.8 (tosyl quaternary aromatic), 128.0 125.5 (aromatic CH (2X2)), 52.4 (methyl ester CH_3), 39.7 (Gly CH_2), 20.7 (tosyl CH_3); m/z (FAB) 262, 185, 152, 92, 31; HRMS 262.0749, $C_{10}H_{16}NO_5S$ requires 262.0749; TLC (E) R_f 0.24.

Phenyl N^{α}-(4-(hydroxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-glycinate (52) HO-Ⓗ-CO-Gly-OPh[131]

A solution of HO-Ⓗ-COOH (30)[30] (10.16 g, 40.3 mmol) in DCM (48 ml) and DMF (12 ml) cooled to 0°C was added to a solution of $\text{TosO}^-\text{H}_2^+$-Gly-OPh (51) (13.28 g, 41.1 mmol) in DCM (160 ml) and DMF (80 ml) cooled to 0°C. DCCI (8.72 g, 42.3 mmol) in DCM (40 ml) cooled to 0°C was then added after a period of 5 minutes followed by neat NMM (4.65 ml, 42.4 mmol) after a further period of 5 minutes. The reaction mixture was left stirring to come to room temperature overnight, after which time the work-up was as for (33). The crude product was obtained as a yellow oil which was purified by dry flash silica chromatography (X2) using a hexane to ether gradient, followed by a chromatotron using ether as eluent, to yield pure (52) after lyophilisation as a white powder (10.09 g, 65%), m.p. 79-81°C (lit.[131] 80-82°C); ν_{max} (CH_2Cl_2) 3610 (O-H), 3440 (N-H), 2960 2900 2880 (aliphatic C-H), 1765 (ester C=O), 1675 1510 (amide C=O, N-H), 865 845 cm^{-1} (aromatic C-H); δ_H (80 MHz, $CDCl_3$) 7.36-6.91 (9H, m, aromatics), 6.04 (1H, broad s, handle-Gly NH), 4.54 (2H, s, benzyl alcohol CH_2), 4.08 (2H, d, J 5.3 Hz, Gly CH_2), 2.67 (4H, m, OH, cinnamate CH, CH_2), -0.05 (9H, s, $Si(CH_3)_3$); m/z (EI) 385, 368, 292, 248, 205, 117, 73; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 8.2 min; TLC (D) R_f 0.50.

Phenyl N^{α}-(4-(N^{α}-(2,2-bis(4-nitrophenyl)ethoxycarbonyl)-glycyloxymethyl)- β -(trimethylsilyl)hydrocinnamyl)-glycinate (53) Bnpeoc-Gly-O-Ⓗ-CO-Gly-OPh

A solution of Bnpeoc-Gly-OH (45)[132] (9.67 g, 24.9 mmol) in DCM (80 ml) and DMF (20 ml) cooled to 0°C was added to a solution of HO-Ⓗ-CO-Gly-OPh (52)[131] (8.69 g, 22.6 mmol) in DCM (20 ml) cooled to 0°C. DCCI (5.00 g, 24.9 mmol) in DCM (20 ml) cooled to 0°C was then added after a period of 5 minutes, followed by a catalytic amount of solid DMAP. The reaction mixture was left stirring to come to room temperature overnight, after which time the work-up was as for (33). The crude product was obtained as a yellow oil which was purified by dry flash silica chromatography (X2) using a hexane through ether to ethyl acetate gradient, followed by a chromatotron using 50/50, ether/ethyl acetate as eluent, to yield pure (53) after lyophilisation as a white powder (14.92 g, 87%), m.p. 68-71°C; (Found: C, 59.9; H, 5.54; N, 7.11%; $C_{38}H_{40}N_{4}O_{11}Si$ requires C, 60.3; H, 5.33; N, 7.40%); ν_{max} (CH_2Cl_2) 3450 (N-H), 2970 2860 (aliphatic C-H), 1735 (urethane, benzyl ester, phenyl ester C=O), 1680 (amide C=O), 1525 (urethane, amide N-H; NO_2), 1350 (NO_2), 865 845 cm^{-1} (aromatic C-H); λ_{max} (CH_3CN) 274 nm (ε= 34483), 230 (19655), 220 (19655); δ_H (200 MHz, $CDCl_3$) 8.11-6.91 (17H, m, aromatics), 6.50 (1H, broad s, handle-Gly NH), 5.57 (1H, broad s, Bnpeoc-Gly NH), 4.99 (2H, s, benzyl CH_2), 4.64-4.56 (3H, m, Bnpeoc CH, CH_2), 4.07-4.02 (2H, m, handle-Gly CH_2), 3.83 (2H, d, J 5.3 Hz, Bnpeoc-Gly CH_2), 2.68-2.60 (3H, m, cinnamate CH, CH_2), -0.09 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 172.7 (amide C=O), 169.5 (Bnpeoc-Gly C=O), 168.4 (phenyl ester C=O), 155.9 (Bnpeoc C=O), 150.2 (phenyl ester quaternary aromatic), 147.0 (Bnpeoc quaternary aromatic (X4)), 143.0 (cinnamate C-1), 131.5 (cinnamate C-4), 129.1-121.1 (aromatic CH (X17)), 66.8 (benzyl CH_2), 66.0 (Bnpeoc CH_2), 49.6 (Bnpeoc CH), 42.6 (Bnpeoc-Gly CH_2), 41.4 (handle-Gly CH_2), 36.0 (cinnamate CH_2), 32.4 (cinnamate CH), -3.2 ($Si(CH_3)_3$); m/z (FAB) 757, 742, 664, 603, 442, 307, 226, 117; HRMS 752.2541, $C_{38}H_{41}N_4O_{11}Si$ (MH^+) requires 757.2541; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 16.9 min; TLC (D) R_f 0.69.

N^{α}-(4-(N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-glycine

(54) Bnpeoc-Gly-O-Ⓗ-CO-Gly-OH

A solution of 100 volumes hydrogen peroxide (740.0 µl, 6.64 mmol) followed by a 0.1M solution of sodium hydroxide (32.90 ml, 3.31 mmol) was added to a solution of Bnpeoc-Gly-O-Ⓗ-CO-Gly-OPh (53) (2.500 g, 3.31 mmol) in 80/20, acetone/water (250 ml), and the reaction mixture was stirred at room temperature for 15 minutes. The reaction mixture was then diluted with water (150 ml) and the acetone was removed in vacuo. Phenol formed as a by-product was removed with ether extraction, and the remaining aqueous reaction mixture was acidified to pH 2-3 with a saturated solution of citric acid in water. This quenching of the reaction took place after 45 minutes. The product mixture was then extracted with ethyl acetate, and the organic fraction was washed with water (X2), brine (X2), and dried over anhydrous magnesium sulphate. Purification of the product was by gel filtration on Sephadex LH20 eluting with methanol, followed by rapid dry flash silica chromatography using an ethyl acetate to methanol gradient with approximately a one-inch bed of silica, to yield (54) after lyophilisation as an off-white powder (1.825 g, 81%), m.p. 105-107°C; (Found: C, 56.2; H, 5.44; N, 7.86%; $C_{32}H_{36}N_4O_{11}Si$ requires C, 56.5; H, 5.33; N, 8.23%); $\nu_{max}(CH_2Cl_2)$ 3450 (N-H), 3400-2500 (O-H, aliphatic C-H), 1730 (urethane, ester, acid C=O), 1660 (amide C=O), 1525 (urethane, amide N-H; NO_2), 1350 (NO_2), 860 845 cm^{-1} (aromatic C-H); λ_{max} (CH_3CN) 274 nm (ε = 33333), 230 (18027), 220 (16667); δ_H (200 MHz, $CDCl_3$) 8.14 (4H, d, J 8.7 Hz, Bnpeoc aromatics), 7.37 (4H, d, J 8.7 Hz, Bnpeoc aromatics), 7.17 (2H, d, J 7.9 Hz cinnamate aromatics), 7.00 (2H, d, J 7.9 Hz, cinnamate aromatics), 5.90 (1H, broad s, handle-Gly NH), 5.35 (1H, broad s, Bnpeoc-Gly NH), 5.05 (2H, s, benzyl CH_2), 4.66-4.57 (3H, m, Bnpeoc CH, CH_2), 3.87 (2H, d, J 5.6 Hz, handle-Gly CH_2), 3.76 (2H, d, J 4.6 Hz, Bnpeoc-Gly CH_2), 2.66-2.57 (3H, m, cinnamate CH, CH_2), -0.07 (9H, s, $Si(CH_3)_3$); δ_C (50 MHz, $CDCl_3$) 173.3 (acid C=O), 171.2 (amide C=O), 169.5 (Bnpeoc-Gly C=O), 155.8 (Bnpeoc C=O), 146.7 146.6 (Bnpeoc quaternary aromatic (2X2)), 142.4 (cinnamate C-1), 131.2 (cinnamate C-4), 128.9 123.5 (Bnpeoc aromatic CH (2X4)), 128.1 (cinnamate C-3, C-5), 127.1 (cinnamate C-2, C-6), 66.5 (benzyl CH_2), 65.8 (Bnpeoc CH_2), 49.2 (Bnpeoc CH), 42.3 (Bnpeoc-Gly CH_2), 40.8 (handle-Gly CH_2), 35.6 (cinnamate CH_2), 32.3 (cinnamate CH), -3.6 ($Si(CH_3)_3$); m/z (FAB) 703, 681, 664, 527, 364, 308, 292, 278, 117; HRMS 681.2228, $C_{32}H_{37}N_4O_{11}Si$ (MH^+) requires 681.2228; HPLC (I) (A:B, 50:50-B, 100%; 28 min; 254 nm) RT = 11.6 min; TLC (F) R_f 0.32.

N^{α}-(9-Fluorenylmethoxycarbonyl)norleucyl aminomethyl resin (55) Fmoc-Nle-NHCH_2-Ph-Ⓡ

Aminomethyl resin (3)[77] (Peninsula, 0.278 mmol/g) (2.52 g, 0.70 mmol) was swollen in DCM and then treated with a solution of Fmoc-Nle-OH (0.62 g, 1.77 mmol) in DCM/DMF, DCCI (0.36 g, 1.77 mmol) in DCM, and a catalytic amount of DMAP. The reaction mixture was shaken for 12 hours in a manual peptide synthesiser of our own design. Agitation of the resin was by repeated inversion of the reaction vessel through 180°. After 12 hours reaction time, the resin was washed with DMF (X3) and DCM (X3). The above process was repeated a further three times, each coupling being monitored using the Kaiser test to detect unreacted primary amine sites on the resin. These tests indicated that complete functionalisation of the resin (3) occurred after the fourth coupling, after which the product resin (55) was thoroughly dried; Found: N, 0.71% (fourth coupling); Fmoc-Nle-NHCH$_2$-Ph-Ⓡ requires N, 0.71%; thus, after fourth coupling, coupling yield = 100%; product resin (55) functionality = 0.256 mmol/g.

Norleucyl aminomethyl resin (56) H-Nle-NHCH$_2$-Ph-Ⓡ

Fmoc-Nle-NHCH$_2$-Ph-Ⓡ (55) (0.256 mmol/g) (2.65 g, 0.68 mmol) was swollen in DMF and then treated with a solution of 20% piperidine in DMF. The reaction mixture was shaken for 15 minutes in the manual peptide synthesiser, washed with DMF (X3), DCM (X3), and dried. A Kaiser test of the product gave a strongly positive result; product resin (56) functionality = 0.271 mmol/g.

N^{α}-(4-(N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoylnorleucyl aminomethyl resin (57) Bnpeoc-Gly-O-Ⓗ-CO-Aua-Nle-NHCH$_2$-Ph-Ⓡ

H-Nle-NHCH$_2$-Ph-Ⓡ (56) (0.271 mmol/g) (2.46 g, 0.67 mmol) was swollen in DCM and then treated with a solution of Bnpeoc-Gly-O-Ⓗ-CO-Aua-OH (47) (1.08 g, 1.34 mmol) in DCM, DCCI (0.28 g, 1.34 mmol) in DCM, and a catalytic amount of DMAP. The reaction mixture was shaken for 12 hours in the manual peptide synthesiser, after which time the product resin was washed thoroughly with DMF (X3), DCM (X3), and dried. A second coupling was carried out exactly as above, each coupling being monitored using the Kaiser test. These tests indicated that incomplete functionalisation of the resin (56) had occurred, thus the product resin was swollen in DMF and then treated with a solution of acetic anhydride (0.63 ml, 6.7 mmol) and triethylamine (0.93 ml, 6.7 mmol) in DMF. The reaction mixture was shaken for 4 hours in the manual peptide synthesiser, after which time Kaiser testing indicated that capping was complete. Thus, the product resin (57) was washed thoroughly with DMF (X3), DCM (X3), and dried; Found: N, 1.72% (capped product); Bnpeoc-Gly-O-Ⓗ-CO-Aua-Nle-NHCH$_2$-Ph-Ⓡ requires N, 1.88%; thus, after capping, coupling yield = 84%; amino acid analysis : Nle$_1$ 1.00, Gly$_1$ 0.87; thus, after capping, coupling yield = 87%. For the purposes of subsequent solid phase peptide synthesis, these two yields were averaged to give 86%; product resin (57) functionality = 0.196 mmol/g.

N$^{\alpha}$-(4-(Glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-11-aminoundecanoylnorleucyl aminomethyl resin (58)

H-Gly-O-Ⓗ-CO-Aua-Nle-NHCH$_2$-Ph-Ⓡ

Bnpeoc-Gly-O-Ⓗ-CO-Aua-Nle-NHCH$_2$-Ph-Ⓡ (57)

(0.196 mmol/g) (2.81 g, 0.55 mmol) was swollen in DMF and then treated with a solution of DBN (136 µl, 1.10 mmol) and acetic acid (63 µl, 1.10 mmol) in DMF. The reaction mixture was shaken for 15 minutes in the manual peptide synthesiser, washed with DMF (X3), DCM (X3), and dried. A Kaiser test of the product gave a strongly positive result; product resin (58) functionality = 0.209 mmol/g.

N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)glycyl chloride (59) Bnpeoc-Gly-Cl

Bnpeoc-Gly-OH (45)[132] (63 mg, 0.16 mmol) was covered in DCM (25 ml) along with thionyl chloride (0.12 ml, 1.63 mmol). The mixture was refluxed for 2 hours, after which time complete solution was achieved; the solvent and excess thionyl chloride were removed under reduced pressure and the residue taken up in DCM and re-evaporated three times to ensure complete removal of thionyl chloride. The crude material was used without further purification; ν_{max} (CH_2Cl_2) 3450 (N-H), 1805 (acid chloride C=O), 1740 (urethane C=O), 1525 (urethane N-H; NO_2), 1350 (NO_2), 830 cm^{-1} (aromatic C-H).

N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)glycyl aminomethyl resin (60) Bnpeoc-Gly-NHCH$_2$-Ph-Ⓡ

Method 1

Aminomethyl resin (3)[77] (Peninsula, 0.278 mmol/g)

(0.53 g, 0.15 mmol) was swollen in DCM (10 ml) and then treated with a solution of Bnpeoc-Gly-Cl (59) (0.16 mmol) in DCM (15 ml), NMM (0.49 ml, 4.44 mmol), and DMAP (1.0 mg, 0.01 mmol). The reaction mixture was sonicated for 2 hours, filtered, washed thoroughly with DMF then DCM, and dried; Found: N, 1.26% (uncapped product); Bnpeoc-Gly-NHCH$_2$-Ph-Ⓡ requires N, 1.42%; thus, after first coupling, coupling yield = 83%; ν_{max} (KBr disk) 3410 (N-H), 1730 (urethane C=O), 1680 (amide C=O), 1520 (urethane, amide N-H; NO$_2$), 1345 (NO$_2$), 830 cm^{-1} (aromatic C-H). An attempt to carry out this coupling with pyridine (0.36 ml, 4.46 mmol) in place of NMM and DMAP gave very little functionalisation of (3).

Method 2

Aminomethyl resin (3)[77] (Peninsula, 0.278 mmol/g) (5.06 g, 1.41 mmol) was swollen in DCM (40 ml) and then treated with a solution of Bnpeoc-Gly-OH (45)[132] (1.10 g, 2.83 mmol) in DMF (3 ml) and DCCI (0.58 g, 2.83 mmol) in DCM (10 ml). The reaction mixture was sonicated for 4 hours, filtered, washed thoroughly with DMF then DCM, and dried. A second coupling was carried out as above except that the manual peptide synthesiser was used instead of sonication. Kaiser testing indicated incomplete functionalisation of the resin (3), thus the product resin was swollen in DCM (40 ml) and treated with a solution of acetic anhydride (1.40 ml, 14.8 mmol) and triethylamine (2.00 ml, 14.4 mmol) in DCM (10 ml). This reaction mixture was sonicated for 1 hour, after which time Kaiser testing indicated that capping was complete. Thus, the product resin (60) was washed thoroughly with DMF then DCM, and dried; Found: N, 1.32% (capped product); Bnpeoc-Gly-NHCH$_2$-Ph-Ⓡ requires N, 1.42%; thus, after capping, coupling yield = 90%; product resin (60) functionality = 0.230 mmol/g; ν_{max} (KBr disk) 3410 (N-H), 1735 (urethane C=O), 1680 (amide C=O), 1520 (urethane, amide N-H; NO$_2$), 1345 (NO$_2$), 830 cm^{-1} (aromatic C-H).

Glycyl aminomethyl resin (61) H-Gly-NHCH$_2$-Ph-Ⓡ

Bnpeoc-Gly-NHCH$_2$-Ph-Ⓡ (60) (0.230 mmol/g) (4.78 g, 1.10 mmol) was swollen in DMF and then treated with a solution of DBU (328 µl, 2.20 mmol) and acetic acid (126 µl, 2.20 mmol) in DMF. The reaction mixture was shaken for 15 minutes in the manual peptide synthesiser, washed with DMF (X3), DCM (X3), and dried. A Kaiser test of the product gave a strongly positive result; product resin (61) functionality = 0.248 mmol/g; ν_{max} (KBr disk) 3340 (N-H), 1680 (amide C=O), 1540 cm^{-1} (amide N-H).

N$^{\alpha}$-(4-(Hydroxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-glycyl aminomethyl resin (62) HO-Ⓗ-CO-Gly-NHCH$_2$-Ph-Ⓡ

H-Gly-NHCH$_2$-Ph-Ⓡ (61) (0.248 mmol/g) (6.01 g, 1.49 mmol) was swollen in DCM (55 ml) and then treated with a solution of HO-Ⓗ-COOH (30)[130] (0.75 g, 2.98 mmol) in DMF (2 ml) and DCCI (0.61 g, 2.98 mmol) in DCM (5 ml). The reaction mixture was sonicated for 5 hours, filtered, washed thoroughly with DMF then DCM, and dried. A Kaiser test indicated that complete functionalisation of the resin (61) had occurred; Found: N, 0.69% (uncapped product); HO-Ⓗ-CO-Gly-$NHCH_2$-Ph-Ⓡ requires N, 0.69%; thus, after first coupling, coupling yield = 100%; product resin (62) functionality = 0.235 mmol/g; ν_{max} (KBr disk) 3410 3320 (N-H), 1675 (amide C=O), 1545 (amide N-H), 850 cm^{-1} (aromatic C-H).

N^{α}-(4-(N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)glycyl aminomethyl resin (63) Bnpeoc-Gly-O-Ⓗ-CO-Gly-$NHCH_2$-Ph-Ⓡ

Method 1

HO-Ⓗ-CO-Gly-$NHCH_2$-Ph-Ⓡ (62) (0.235 mmol/g) (0.51 g, 0.12 mmol) was swollen in DCM (5 ml) and then treated with a solution of Bnpeoc-Gly-OH (45)[132] (93 mg, 0.24 mmol) in DCM (5 ml)/DMF (1 ml) and DCCI (49 mg, 0.24 mmol) in DCM (5 ml). The reaction mixture was sonicated for 4 hours, filtered, washed thoroughly with DMF then DCM, and dried. A second coupling was carried out as above except that the manual peptide synthesiser was used instead of sonication. A third coupling was carried out as for the second coupling except that HOBt (32 mg, 0.24 mmol) in DMF (1 ml) and a catalytic amount of DMAP were used, the procedure being one of those successfully employed for coupling protected amino acids to the Wang resin (14); ν_{max} (KBr disk) - identical to that for (62) i.e. no product absorptions. Attempts to carry out this coupling using acid chloride methodology both with pyridine and with NMM and DMAP gave a similar result to above, i.e. no functionalisation of (62).

Method 2

To a solution of Bnpeoc-Gly-O-Ⓗ-CO-Gly-OH (54) (1.22 g, 1.80 mmol) in DCM (10 ml) was added a solution of HOBt (0.24 g, 1.80 mmol) in DMF (2 ml). This mixture was shaken for 2 minutes, after which time a solution of DCCI (0.37 g, 1.80 mmol) in DCM (5 ml) was added, and the resulting mixture shaken for 2 minutes. This solution was then added to aminomethyl resin (3)[77] (Peninsula, 0.400 mmol/g) (3.00 g, 1.20 mmol) swollen in DCM (40 ml), and the reaction mixture was sonicated for 5 hours, filtered, washed thoroughly with DMF then DCM, and dried. Second and third couplings were carried out exactly as for the first. Each coupling was monitored quantitatively in the three ways described in section 2.6. Kaiser testing after the third coupling showed that complete functionalisation of the resin (3) had not been achieved hence capping was performed by adding a solution of acetic anhydride (1.13 ml, 12.0 mmol) and triethylamine (1.67 ml, 12.0 mmol) in DCM (10 ml) to the product resin swollen in DCM (40 ml) and sonicating the mixture for 1 hour. After this time, Kaiser testing showed that capping was complete, thus the product resin (63) was washed thoroughly with DMF then DCM, and dried. The capped product (63) was also tested quantitatively in the manner described in section 2.6 (see Fig. 2.1); ν_{max} (KBr disk) 3410 3330 (N-H), 1735 (urethane, ester C=O), 1670 (amide C=O), 1520 (urethane, amide N-H; NO_2), 1345 (NO_2), 850 cm^{-1} (aromatic C-H). For the purposes of subsequent solid phase peptide synthesis and as explained in section 2.7, a yield of 47% was determined for this coupling step; product resin (63) functionality = 0.167 mmol/g.

N^{α}-(4-(Glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)-glycyl aminomethyl resin (64) H-Gly-O-Ⓗ-CO-Gly-NHCH_2-Ph-Ⓡ

Bnpeoc-Gly-O-Ⓗ-CO-Gly-NHCH_2-Ph-Ⓡ (63) (unknown functionality) (3.73 g) was swollen in DMF (40 ml) and then treated with a solution of DBU (267 µl, 1.78 mmol) and acetic acid (102 µl, 1.78 mmol) in DMF (10 ml). The reaction mixture was sonicated for 2 hours, filtered, washed thoroughly with DMF then DCM, and dried.

Quantitative Kaiser testing gave product resin (64) functionality = 0.150 mmol/g; ν_{max} (KBr disk) 3410 3310 (N-H), 1745 (ester C=O), 1665 (amide C=O), 1515 (amide N-H), 850 cm^{-1} (aromatic C-H).

N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)phenylalanyl chloride (65) Bnpeoc-Phe-Cl

Bnpeoc-Phe-OH[132] (0.25 g, 0.52 mmol) was dissolved in DCM (40 ml) along with thionyl chloride (0.38 ml, 5.22 mmol). The solution was refluxed for 2 hours, after which time the solvent and excess thionyl chloride were removed under reduced pressure and the residue taken up in DCM and re-evaporated three times to ensure complete removal of thionyl chloride. The crude material was used without further purification; ν_{max} (CH_2Cl_2) 3430 (N-H), 1795 (acid chloride C=O), 1735 (urethane C=O), 1525 (urethane N-H; NO_2), 1350 (NO_2), 835 cm^{-1} (aromatic C-H).

N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)phenylalanyl p-alkoxybenzyl alcohol resin (66) Bnpeoc-Phe-$(OCH_2$-$Ph)_2$-Ⓡ

p-Alkoxybenzyl alcohol resin (14)[97] (Bachem, 1.01 mmol/g) (0.47 g, 0.48 mmol) was swollen in DCM (10 ml) and then treated with a solution of Bnpeoc-Phe-Cl (65) (0.52 mmol) in DCM (5 ml), NMM (1.60 ml, 14.57 mmol), and DMAP (2.9 mg, 0.02 mmol). The reaction mixture was sonicated for 2 hours, filtered, washed thoroughly with DMF then DCM, and dried. A second coupling was carried out exactly as for the first; Found: N, 0.68% (first coupling), 1.11% (second coupling); Bnpeoc-Phe-$(OCH_2$-$Ph)_2$-Ⓡ requires N, 2.89%; thus, after first coupling, coupling yield = 17%; after second coupling, coupling yield = 30%; ν_{max} (KBr disk) 3560 (resin O-H), 3430 (N-H), 1725 (urethane, ester C=O), 1520 (urethane N-H; NO_2), 1345 (NO_2), 830 cm^{-1} (aromatic C-H). Attempts to carry out this coupling with pyridine (1.15 ml, 14.24 mmol) in place of NMM and DMAP failed completely.

N^{α}-(4-(N^{α}-(2,2-Bis(4-nitrophenyl)ethoxycarbonyl)glycyloxymethyl)-β-(trimethylsilyl)hydrocinnamyl)glycyl p-alkoxybenzyl alcohol resin (67) Bnpeoc-Gly-O-Ⓗ-CO-Gly-$(OCH_2$-$Ph)_2$-Ⓡ

To a solution of Bnpeoc-Gly-O-Ⓗ-CO-Gly-OH (54) (0.14 g, 0.20 mmol) in DCM (10 ml) was added a solution of HOBt (0.03 g, 0.21 mmol) in DMA (2 ml). This mixture was shaken for 2 minutes, after which time a solution of DCCI (0.04 g, 0.20 mmol) in DCM (2 ml) was added, and the resulting mixture shaken for 2 minutes. This solution was then added to p-alkoxybenzyl alcohol resin (14)[97] (Bachem, 1.01 mmol/g) (0.10 g, 0.10 mmol) swollen in DCM (10 ml), and the reaction mixture was sonicated for 4 hours, filtered, washed thoroughly with DMA then DCM, and dried. A second coupling was carried out exactly as for the first; Found: N, 0% (first coupling), 0.32% (second coupling); Bnpeoc-Gly-O-Ⓗ-CO-Gly-(OCH_2-Ph)$_2$-Ⓡ requires N, 3.39%; thus, after first coupling, coupling yield = 0%; after second coupling, coupling yield = 6%.

3.3 SOLID PHASE SYNTHESIS

The automated peptide synthesiser used in the following syntheses was the Applied Biosystems model 430A. The DMF used was supplied by Rathburn Chemicals Ltd. (peptide synthesis grade).

Unless otherwise stated, each residue was double coupled; firstly by the symmetrical anhydride method and secondly by the HOBt active ester method. Preformed symmetrical anhydrides were prepared from Fmoc-amino acids (1.0 mmol) and DICI (0.5 mmol) in DMF with an activation time of 15 minutes, whilst simultaneous deprotection of the Fmoc-peptide-resin was achieved over a 10 minute period using 20% piperidine in DMF. The symmetrical anhydride was then reacted with the free amino resin for 1 hour. Subsequent activation of each Fmoc-amino acid (0.5 mmol) with DICI (0.5 mmol) and HOBt (0.5 mmol) was carried out over a period of 30 minutes before addition to the resin and allowing reaction to proceed for a further 2 hours. Capping of unreacted amino functions was then performed over a period of 6 minutes using acetic anhydride and pyridine in DMF. The resin was washed thoroughly with DMF at the end of each cycle, and with DMF then DCM at the end of the synthesis. Each synthesis was commenced with a deprotection step. As a rough monitor of the couplings, the product solution from the deprotection steps was fed through an ultraviolet detector (276 nm) and recorder to give a series of peaks for the olefin liberated from each residue which should in theory have all had the same total peak area. Removal of peptide from the resin was accomplished using three separate 5 minute treatments with dry TBAF (0.25 mmol) in DMF. These cleavages were performed on the manual peptide synthesiser of our own design, after which the residue resin was washed thoroughly with DMF then DCM and dried. In the purification of the peptides by gel filtration, product containing fractions were identified by ultraviolet absorbance (276 nm) and optical rotation (589 nm).

N^{α}-Diphenylphosphinylleucylisoleucylphenylalanylalanylglycine (68) Dpp-Leu-Ile-Phe-Ala-Gly-OH

H-Gly-O-Ⓗ-CO-Aua-Nle-$NHCH_2$-Ph-Ⓡ (58) (0.209 mmol/g) (2.38 g, 0.50 mmol) was placed in the reaction vessel of the automated peptide synthesiser and thus, in this case, the amounts used of all reactants were doubled. All the N^{α}-protected amino acids used were Fmoc-protected except for leucine which was Dpp-protected. Capping was not carried out. After the completed coupling cycle for each amino acid, a small sample of resin-bound product was automatically removed for a quantitative Kaiser test (see Fig. 2.2).

Resin-bound peptide amino acid analysis: Gly_1 0.91, Ala_1 0.69, Ile_1 0.57, Leu_1 0.61, Nle_1 1.00, Phe_1 0.62.

All three TBAF treatments were worked-up as follows: removal of solvent in vacuo to give a yellow oil, addition of water (20 ml) to give a white precipitate, and addition of one equivalent (50 ml) of 0.01M HCl. The resulting white solids were filtered off and dried in vacuo over phosphorus pentoxide.

Residue resin amino acid analysis: Gly_1 0.16, Ala_1 0.23, Ile_1 0.15, Leu_1 0.18, Nle_1 1.00, Phe_1 0.26.

Amino acid analysis, mass spectrometry, and HPLC of the three product fractions indicated that the major component in each case was the desired product, thus all three fractions were combined and purified by gel filtration on Sephadex LH20 eluting with DMF to yield pure (68) after lyophilisation as an off-white powder (143 mg, 40%); amino acid analysis: Gly_1 1.00, Ala_1 0.95, Ile_1 0.84, Leu_1 0.88, Phe_1 0.95; m/z (FAB) 742, 720, 645, 574, 427, 400, 286, 201; HRMS 720.3526, $C_{38}H_{51}N_5O_7P$ (MH^+) requires 720.3526; HPLC (II) (A:B, 50:50-B, 100%; 28 min, 214 nm) RT = 7.2 min; TLC (I) R_f 0.76 (ninhydrin negative).

Leucylisoleucylphenylalanylalanylglycine (69) H-Leu-Ile-Phe-Ala-Gly-OH

Bnpeoc-Gly-O-$\textcircled{H}$-CO-Gly-NHCH$_2$-Ph-$\textcircled{R}$ (63) (0.167 mmol/g) (1.50 g, 0.25 mmol) was placed in the reaction vessel of the automated peptide synthesiser. Leucine was deprotected before the TBAF treatments.

Residue resin amino acid analysis: Gly$_2$ 2.00, Ala$_1$ 0.43, Ile$_1$ 0.23, Leu$_1$ 0.29, Phe$_1$ 0.58.

HPLC of the three product fractions indicated that the first TBAF treatment was the most pure of the three, and that the second and third treatments were identical; hence the last two were combined. The first and second/third TBAF treatments were purified separately by gel filtration on Sephadex G15 eluting with 30% acetic acid in water, to yield pure (69) as a white powder (85 mg, 65%); amino acid analysis; Gly$_1$ 1.00, Ala$_1$ 0.99, Ile$_1$ 0.93, Leu$_1$ 0.94, Phe$_1$ 0.94; m/z (FAB) 520, 242, 184, 142; HRMS 520.3135, $C_{26}H_{42}N_5O_6$ (MH$^+$) requires 520.3135; HPLC (II) (A:B, 90:10-A:B, 40:60; 25 min; A:B, 40:60-B, 100%; 2 min; 214 nm) RT = 18.6 min; TLC (I) R$_f$ 0.31 (ninhydrin positive).

Valyllysylglycylarginylglycine (70) H-Val-Lys-Gly-Arg-Gly-OH

H-Gly-O-$\textcircled{H}$-CO-Gly-NHCH$_2$-Ph-$\textcircled{R}$ (64) (0.150 mmol/g) (1.67 g, 0.25 mmol) was placed in the reaction vessel of the automated peptide synthesiser. All the N$^\alpha$-protected amino acids used were Fmoc-protected except for valine which was Boc-protected; side-chain protection was as follows - Arg (Pmc), Lys (Boc). Single coupling only was employed for Fmoc-Gly-OH, the second HOBt active ester coupling being omitted. Both couplings for Fmoc-Arg(Pmc)-OH were performed using the HOBt active ester protocol, and acetyl imidazole was used as the capping agent.

Residue resin amino acid analysis: Gly_3 3.00, Val_1 0.48, Lys_1 0.44, Arg_1 0.49.

All three TBAF treatments were combined and purified by gel filtration on Sephadex LH20 eluting with methanol to yield a white powder (295 mg) which on TLC in system (I) showed two major ultraviolet-active spots, neither of which was ninhydrin positive. Side-chain protection was then removed by treating the product with TFA/DCM/water, 50/45/5 (75 ml) and stirring the reaction mixture at room temperature for 1 hour, after which time the solvent was removed and the residue triturated with ether to give a white powder. This was dried *in vacuo* over phosphorus pentoxide to give a yield of 162 mg, and then purified by gel filtration on Sephadex G25 eluting with 30% acetic acid in water to yield a white powder (90 mg). Purification of this product was by preparative HPLC using a reverse phase Spherisorb ODS2 5μ column (10 x 250 mm) with a gradient of A:B, 90:10-A:B, 10:90 over 28 minutes and ultraviolet detection at 254 nm, to yield pure (70) after lyophilisation as a white powder (78 mg, 60%); amino acid analysis: Gly_2 2.00, Val_1 1.02, Lys_1 1.04, Arg_1 1.05; m/z (FAB) 517, 473, 242, 171, 147; HRMS 516.3258, $C_{21}H_{42}N_9O_6$ (MH^+) requires 516.3258; HPLC (I) (A:B, 90:10-A:B, 10:90; 28 min; 214 nm) RT = 4.7 min.

Fully protected ubiquitin (1-35) (71)

Bnpeoc-Gly-O-Ⓗ-CO-Gly-$NHCH_2$-Ph-Ⓡ (63) (0.167 mmol/g) (1.50 g, 0.25 mmol) was placed in the reaction vessel of the automated peptide synthesiser. All the N^{α}-protected amino acids used were Fmoc-protected except for the N-terminal methionine which was Boc-protected; side-chain protection was as follows - Lys (Boc); Glu, Asp, Thr, Ser (t-Bu). Single coupling only was employed for Fmoc-Gly-OH, the second HOBt active ester coupling being omitted. For Fmoc-Asn-OH and Fmoc-Gln-OH, both couplings employed the HOBt active ester protocol with the first coupling lasting for 1 hour and the second coupling lasting for 90 minutes. Cleavage of the fully protected peptide from the resin was done using seven TBAF treatments of 5 minutes duration - four with one equivalent followed by three with two equivalents of TBAF. After several selected couplings, small samples of resin-bound product were removed for quantitative olefin determination by ultraviolet absorbance (see Fig. 2.3). Amino acid analyses of the residue resin were carried out after the fourth, fifth, sixth, and seventh TBAF treatments (see Fig. 2.4). The product obtained from TBAF treatments (I)-(V) was a tacky white solid, whereas that from (VI) and (VII) was a yellow oil, the crude yields being as follows: (I) 67 mg (5%), (II) 91 mg (7%), (III) 107 mg (8%), (IV) 135 mg (10%), (V) 300 mg (23%), (VI) 197 mg (15%), (VII) 159 mg (12%). TBAF treatments (I)-(IV) and (V)-(VII) were combined and purified separately by gel filtration on Sephadex LH20 eluting with DMF to yield (71) after trituration as an off-white powder (416 mg, 32%). This yield, however, was recovered from only two-thirds of the resin-bound peptide obtained after the coupling of Gln [2], and thus projected yield = 624 mg, 48%; amino acid analysis: Asx_3 3.06, Thr_5 4.71, Ser_1 0.93, Glx_6 6.40, Pro_1 1.13, Gly_2 1.97, Ala_1 1.05, Val_3 2.95, Met_1 0.59, Ile_4 3.84, Leu_2 1.95, Phe_1 0.80, Lys_5 5.03; δ_H (360 MHz, $(CD_3)_2SO$) 1.50-0.82 (indeterminate H, m, t-Bu and Boc); m/z (FAB) HRMS 5179.1716, $C_{250}H_{436}N_{43}O_{69}S$ (MH^+) requires 5179.1716; HPLC (III) (isocratic-0.1% isopropanol in DCM, 254 nm) RT = 2.75 min (see Fig. 2.5).

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COMPOUND INDEX

APPENDIX

I

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10  HOME : INVERSE
20  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "********************"
30  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "RESIN FUNCTIONALITY"
40  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "********************"
50 Y$ = "O":W$ = "O"
60 A = B = C = D = E = F = G = H = I = X = Y = Z = 0:H = 42
70  PRINT : PRINT : PRINT "INPUT FUNCTIONALITY OF STARTING RESIN INMMOL/G": PRINT : INPUT A: PRINT
80  PRINT "INPUT MOLECULAR WEIGHT OF PROTECTED": PRINT "AMINOACID SPECIES BEING LOADED": PRINT : INP
UT C: PRINT
90 D = C - 18
100  PRINT "INPUT AMINO PROTECTING GROUP USED": PRINT : INPUT Y$: PRINT
110  IF Y$ = "FMOC" THEN G = 900: GOTO 140
120  IF Y$ = "BNPEOC" THEN G = 163.75: GOTO 140
130  GOTO 100
140  PRINT "INPUT MASS OF RESIN-BOUND PRODUCT IN MG": PRINT : INPUT E: PRINT
150  PRINT "INPUT OBSERVED UV ABSORBANCE AT 300NM": PRINT : INPUT F: PRINT
160  PRINT "INPUT IF SAMPLE IS CAPPED OR UNCAPPED": PRINT "(C OR U)": PRINT : INPUT W$: PRINT
170  IF W$ = "U" THEN  GOTO 210
180  IF W$ = "C" THEN  GOTO 200
190  GOTO 160
200 B = ((100 * F * (1000 + (A * H))) / (A * ((E * G) + (H * F) - (D * F)))) + 0.5:B =  INT (B):I =
100 - B: GOTO 220
210 B = ((100000 * F) / (A * ((E * G) - (D * F)))) + 0.5:B =  INT (B):I = 100 - B
220 X = (A * B * 1000) / ((A * B * D) + (A * H * I) + 100000):X = (X * 1000) + 0.5:X =  INT (X):X =
X / 1000
230 Y = X
240  IF W$ = "C" THEN  GOTO 260
250 Z = (1000 * A * B) / (100000 + (A * B * D)):Z = (Z * 1000) + 0.5:Z =  INT (Z):Z = Z / 1000
260 D$ =  CHR$ (4): PRINT D$"PR#1"
270  PRINT "**************************************": PRINT
280  PRINT "  RESIN LOADING/COUPLING PERCENTAGE  :"; TAB( 22)" ";B: PRINT
290  PRINT "**************************************": PRINT : PRINT
300  PRINT "**************************************": PRINT
310  PRINT "PRODUCT RESIN FUNCTIONALITY IN MMOL/G:"; TAB( 22)" ";
320  IF Y < 1 THEN  GOTO 350
330  PRINT Y;"(CAPPED)": IF W$ = "C" THEN  GOTO 400
340  GOTO 360
350  PRINT "0";Y;"(CAPPED)": IF W$ = "C" THEN  GOTO 400
360  IF Z < 1 THEN  GOTO 390
370  PRINT  TAB( 60)" ";Z;"(UNCAPPED)"
380  GOTO 410
390  PRINT  TAB( 60)" ";"0";Z;"(UNCAPPED)": GOTO 410
400  PRINT
410  PRINT "**************************************": PRINT : PRINT : PRINT
420  PRINT "YOUR DATA WERE AS FOLLOWS:": PRINT : PRINT
430  PRINT "FUNCTIONALITY OF STARTING RESIN IN MMOL/G:"; TAB( 18)" ";
440 D = A: GOSUB 540
450  PRINT "MOLECULAR WEIGHT OF PROTECTED AMINO ACID:"; TAB( 19)" ";C: PRINT
460  PRINT "AMINO PROTECTING GROUP:"; TAB( 37)" ";Y$: PRINT
470  PRINT "MASS OF RESIN-BOUND PRODUCT IN MG:"; TAB( 26)" ";
480 D = E: GOSUB 540
490  PRINT "OBSERVED UV ABSORBANCE AT 300NM:"; TAB( 28)" ";
500 D = F: GOSUB 540
510  PRINT "CAPPED OR UNCAPPED (C OR U):"; TAB( 32)" ";W$: PRINT
520  PRINT D$"PR#0": NORMAL : HOME
530  END
540  IF D < 1 THEN  GOTO 560
550  GOTO 570
560  IF D > 0 THEN  GOTO 580
570  PRINT D: PRINT : GOTO 590
580  PRINT "0";D: PRINT
590  RETURN
```

```
****************************************

  RESIN LOADING/COUPLING PERCENTAGE  :                   18

****************************************


****************************************

PRODUCT RESIN FUNCTIONALITY IN MMOL/G:                  0.068(CAPPED)
                                                        0.069(UNCAPPED)
****************************************


YOUR DATA WERE AS FOLLOWS:

FUNCTIONALITY OF STARTING RESIN IN MMOL/G:              0.4

MOLECULAR WEIGHT OF PROTECTED AMINO ACID:               680

AMINO PROTECTING GROUP:                                 BNPEOC

MASS OF RESIN-BOUND PRODUCT IN MG:                      3

OBSERVED UV ABSORBANCE AT 300NM:                        0.034

CAPPED OR UNCAPPED (C OR U):                            U




****************************************

  RESIN LOADING/COUPLING PERCENTAGE  :                   100

****************************************


****************************************

PRODUCT RESIN FUNCTIONALITY IN MMOL/G:                  0.788(CAPPED)

****************************************


YOUR DATA WERE AS FOLLOWS:

FUNCTIONALITY OF STARTING RESIN IN MMOL/G:              1.01

MOLECULAR WEIGHT OF PROTECTED AMINO ACID:               297

AMINO PROTECTING GROUP:                                 FMOC

MASS OF RESIN-BOUND PRODUCT IN MG:                      1.1

OBSERVED UV ABSORBANCE AT 300NM:                        0.777

CAPPED OR UNCAPPED (C OR U):                            C
```

```
10  HOME : INVERSE
20  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "********************"
30  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "RESIN FUNCTIONALITY"
40  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "********************"
50 W$ = "0"
60 A = B = C = D = E = F = I = K = X = Y = Z = 0:H = 42:G = 314
70  PRINT : PRINT : PRINT "INPUT FUNCTIONALITY OF STARTING RESIN INMMOL/G": PRINT : INPUT A: PRINT
80  PRINT "INPUT MOLECULAR WEIGHT OF PROTECTED": PRINT "AMINOACID SPECIES BEING LOADED": PRINT : INP
UT C: PRINT
90 D = C - 18
100  PRINT "INPUT MASS OF RESIN-BOUND PRODUCT IN MG": PRINT : INPUT E: PRINT
110  PRINT "INPUT UV ABSORBANCE OF BLANK AT 570NM": PRINT : INPUT F: PRINT
120  PRINT "INPUT UV ABSORBANCE OF SAMPLE AT 570NM": PRINT : INPUT K: PRINT
130  PRINT "INPUT IF SAMPLE IS CAPPED/DEPROTECTED ORUNCAPPED/PROTECTED (C OR U)": PRINT : INPUT W$:
PRINT
140 X = (K - F) / (E * 3):X = (X * 1000) + 0.5:X =  INT (X):X = X / 1000
150  IF W$ = "U" THEN  GOTO 180
160  IF W$ = "C" THEN  GOTO 190
170  GOTO 130
180 B = ((100000 * (A - X)) / ((1000 * A) + (A * D * X))) + 0.5:B =  INT (B):I = 100 - B: GOTO 200
190 B = ((100 * X * (1000 + (A * H))) / (A * (1000 + (X * (H + G - D))))) + 0.5:B =  INT (B):I = 100
 - B
200 Y = (A * B * 1000) / ((A * B * D) + (A * H * I) + 100000):Y = (Y * 1000) + 0.5:Y =  INT (Y):Y =
Y / 1000
210 Z = (A * B * 1000) / ((A * B * D) + 100000):Z = (Z * 1000) + 0.5:Z =  INT (Z):Z = Z / 1000
220 D$ =  CHR$ (4): PRINT D$"PR#1"
230  PRINT "****************************************": PRINT
240  PRINT "  RESIN LOADING/COUPLING PERCENTAGE  :"; TAB( 22)" ";B: PRINT
250  PRINT "****************************************": PRINT : PRINT
260  PRINT "****************************************": PRINT
270  PRINT "PRODUCT RESIN FUNCTIONALITY IN MMOL/G:"; TAB( 22)" ";
280  IF Y < 1 THEN  GOTO 310
290  PRINT Y;"(CAPPED)": IF W$ = "C" THEN  GOTO 360
300  GOTO 320
310  PRINT "0";Y;"(CAPPED)": IF W$ = "C" THEN  GOTO 360
320  IF Z < 1 THEN  GOTO 350
330  PRINT  TAB( 60)" ";Z;"(UNCAPPED)"
340  GOTO 370
350  PRINT  TAB( 60)" ";"0";Z;"(UNCAPPED)": GOTO 370
360  PRINT
370  PRINT "****************************************": PRINT : PRINT : PRINT
380  PRINT "YOUR DATA WERE AS FOLLOWS:": PRINT : PRINT
390  PRINT "FUNCTIONALITY OF STARTING RESIN IN MMOL/G:"; TAB( 18)" ";
400 D = A: GOSUB 510
410  PRINT "MOLECULAR WEIGHT OF PROTECTED AMINO ACID:"; TAB( 19)" ";C: PRINT
420  PRINT "MASS OF RESIN-BOUND PRODUCT IN MG:"; TAB( 26)" ";
430 D = E: GOSUB 510
440  PRINT "UV ABSORBANCE OF BLANK AT 570NM:"; TAB( 28)" ";
450 D = F: GOSUB 510
460  PRINT "UV ABSORBANCE OF SAMPLE AT 570NM:"; TAB( 27)" ";
470 D = K: GOSUB 510
480  PRINT "CAPPED/DEPROTECTED OR UNCAPPED/PROTECTED (C OR U):"; TAB( 10)" ";W$: PRINT
490  PRINT D$"PR#0": NORMAL : HOME
500  END
510  IF D < 1 THEN  GOTO 530
520  GOTO 540
530  IF D > 0 THEN  GOTO 550
540  PRINT D: PRINT : GOTO 560
550  PRINT "0";D: PRINT
560  RETURN
```

```
**************************************
  RESIN LOADING/COUPLING PERCENTAGE  :                      32
**************************************

**************************************
PRODUCT RESIN FUNCTIONALITY IN MMOL/G:                      0.117(CAPPED)
                                                            0.118(UNCAPPED)
**************************************


YOUR DATA WERE AS FOLLOWS:

FUNCTIONALITY OF STARTING RESIN IN MMOL/G:                  0.4

MOLECULAR WEIGHT OF PROTECTED AMINO ACID:                   680

MASS OF RESIN-BOUND PRODUCT IN MG:                          3.3

UV ABSORBANCE OF BLANK AT 570NM:                            0.02

UV ABSORBANCE OF SAMPLE AT 570NM:                           2.49

CAPPED/DEPROTECTED OR UNCAPPED/PROTECTED (C OR U):          U




**************************************
  RESIN LOADING/COUPLING PERCENTAGE  :                      58
**************************************

**************************************
PRODUCT RESIN FUNCTIONALITY IN MMOL/G:                      0.146(CAPPED)
**************************************


YOUR DATA WERE AS FOLLOWS:

FUNCTIONALITY OF STARTING RESIN IN MMOL/G:                  0.28

MOLECULAR WEIGHT OF PROTECTED AMINO ACID:                   680

MASS OF RESIN-BOUND PRODUCT IN MG:                          1.7

UV ABSORBANCE OF BLANK AT 570NM:                            0.01

UV ABSORBANCE OF SAMPLE AT 570NM:                           0.79

CAPPED/DEPROTECTED OR UNCAPPED/PROTECTED (C OR U):          C
```

```
10  HOME : INVERSE
20  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "*******************"
30  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "RESIN FUNCTIONALITY"
40  NORMAL : PRINT  TAB( 10)" ";: INVERSE : PRINT "*******************"
50 W$ = "0"
60 A = B = C = D = E = F = I = K = Y = Z = 0:H = 42:G = 14
70  PRINT : PRINT : PRINT "INPUT FUNCTIONALITY OF STARTING RESIN INMMOL/G": PRINT : INPUT A: PRINT
80  PRINT "INPUT MOLECULAR WEIGHT OF PROTECTED": PRINT "AMINOACID SPECIES BEING LOADED": PRINT : INP
UT C: PRINT
90 D = C - 18
100  PRINT "INPUT NUMBER OF NITROGEN ATOMS IN": PRINT "SPECIES BEING LOADED": PRINT : INPUT E: PRINT

110  PRINT "INPUT % NITROGEN IN STARTING RESIN": PRINT : INPUT F: PRINT
120  PRINT "INPUT % NITROGEN IN PRODUCT RESIN": PRINT : INPUT K: PRINT
130  PRINT "INPUT IF SAMPLE IS CAPPED OR UNCAPPED": PRINT "(C OR U)": PRINT : INPUT W$: PRINT
150  IF W$ = "U" THEN  GOTO 180
160  IF W$ = "C" THEN  GOTO 190
170  GOTO 130
180 B = ((100000 * (K - F)) / (A * ((100 * E * G) - (D * K)))) + 0.5:B =  INT (B):I = 100 - B: GOTO
200
190 B = ((100 * ((1000 * K) + (A * H * K) - (1000 * F))) / (A * ((100 * E * G) + (H * K) - (D * K)))
) + 0.5:B =  INT (B):I = 100 - B
200 Y = (A * B * 1000) / ((A * B * D) + (A * H * I) + 100000):Y = (Y * 1000) + 0.5:Y =  INT (Y):Y =
Y / 1000
210 Z = (A * B * 1000) / ((A * B * D) + 100000):Z = (Z * 1000) + 0.5:Z =  INT (Z):Z = Z / 1000
220 D$ =  CHR$ (4): PRINT D$"PR#1"
230  PRINT "***************************************": PRINT
240  PRINT "  RESIN LOADING/COUPLING PERCENTAGE  :"; TAB( 22)" ";B: PRINT
250  PRINT "***************************************": PRINT : PRINT
260  PRINT "***************************************": PRINT
270  PRINT "PRODUCT RESIN FUNCTIONALITY IN MMOL/G:"; TAB( 22)" ";
280  IF Y < 1 THEN  GOTO 310
290  PRINT Y;"(CAPPED)": IF W$ = "C" THEN  GOTO 360
300  GOTO 320
310  PRINT "0";Y;"(CAPPED)": IF W$ = "C" THEN  GOTO 360
320  IF Z < 1 THEN  GOTO 350
330  PRINT  TAB( 60)" ";Z;"(UNCAPPED)"
340  GOTO 370
350  PRINT  TAB( 60)" ";"0";Z;"(UNCAPPED)": GOTO 370
360  PRINT
370  PRINT "***************************************": PRINT : PRINT : PRINT
380  PRINT "YOUR DATA WERE AS FOLLOWS:": PRINT : PRINT
390  PRINT "FUNCTIONALITY OF STARTING RESIN IN MMOL/G:"; TAB( 18)" ";
400 D = A: GOSUB 510
410  PRINT "MOLECULAR WEIGHT OF PROTECTED AMINO ACID:"; TAB( 19)" ";C: PRINT
420  PRINT "NUMBER OF NITROGEN ATOMS IN SPECIES BEING LOADED:"; TAB( 11)" ";
430 D = E: GOSUB 510
440  PRINT "% NITROGEN IN STARTING RESIN:"; TAB( 31)" ";
450 D = F: GOSUB 510
460  PRINT "% NITROGEN IN PRODUCT RESIN:"; TAB( 32)" ";
470 D = K: GOSUB 510
480  PRINT "CAPPED OR UNCAPPED (C OR U):"; TAB( 32)" ";W$: PRINT
490  PRINT D$"PR#0": NORMAL : HOME
500  END
510  IF D < 1 THEN  GOTO 530
520  GOTO 540
530  IF D > 0 THEN  GOTO 550
540  PRINT D: PRINT : GOTO 560
550  PRINT "0";D: PRINT
560  RETURN
```

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***************************************
  RESIN LOADING/COUPLING PERCENTAGE  :                       30
***************************************

***************************************
PRODUCT RESIN FUNCTIONALITY IN MMOL/G:                       0.259(CAPPED)
                                                             0.266(UNCAPPED)
***************************************


YOUR DATA WERE AS FOLLOWS:

FUNCTIONALITY OF STARTING RESIN IN MMOL/G:                   1.01

MOLECULAR WEIGHT OF PROTECTED AMINO ACID:                    479

NUMBER OF NITROGEN ATOMS IN SPECIES BEING LOADED:            3

% NITROGEN IN STARTING RESIN:                                0

% NITROGEN IN PRODUCT RESIN:                                 1.12

CAPPED OR UNCAPPED (C OR U):                                 U




***************************************
  RESIN LOADING/COUPLING PERCENTAGE  :                       84
***************************************

***************************************
PRODUCT RESIN FUNCTIONALITY IN MMOL/G:                       0.192(CAPPED)
***************************************


YOUR DATA WERE AS FOLLOWS:

FUNCTIONALITY OF STARTING RESIN IN MMOL/G:                   0.27

MOLECULAR WEIGHT OF PROTECTED AMINO ACID:                    806

NUMBER OF NITROGEN ATOMS IN SPECIES BEING LOADED:            4

% NITROGEN IN STARTING RESIN:                                0.76

% NITROGEN IN PRODUCT RESIN:                                 1.72

CAPPED OR UNCAPPED (C OR U):                                 C
```

COURSES ATTENDED

Organic Research Seminars (Various Speakers)

Current Topics in Organic Chemistry (Various Speakers, University of Edinburgh)

X-Ray Crystallography/Cambridge Structural Database (Dr. O. Kennard et al., University of Cambridge)

X-Ray Crystallography (Dr. R.O. Gould & Dr. A.J. Blake, University of Edinburgh)

NMR Spectroscopy (Dr. I.H. Sadler, University of Edinburgh)

Mass Spectroscopy (Dr. G. Elliot & Dr. A. Ashcroft, Kratos)

Mass Spectroscopy (Prof. K.R. Jennings, University of Warwick)

Medicinal Chemistry (Various Speakers, ICI & Beecham Pharmaceuticals)

Medicinal Chemistry (Prof. P.G. Sammes, SK & F)

Cell Biology (Dr. J. Philips, University of Edinburgh, Department of Biochemistry)