"The Development of Novel Solid Phase Methodologies for the Synthesis of Atypical Peptides and Non-peptide Entities"

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This thesis is dedicated to my parents - Thank you.

"You see things and you say,

'Why ?' But I dream things that

never were; and I say 'Why not ?'

Thomas Edison

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Abbreviations

Abbreviations for amino acids and peptides follow the IUPAC-IUB nomenclature where applicable (*Eur. J. Biochem.*, **1984**, 9-37).

Ac	Acetyl
$(Ac)_2O$	Acetic anhydride
AcOH	Acetic acid
Acm	Acetamidomethyl
Alloc	Allyloxycarbonyl
BASEC	2-[(*N*-Biotinyl)-aminoethylsulphonyl]ethyl *p*-nitrophenyl-carbonate
Boc	*tert*-Butoxycarbonyl
BOP	Benzotriazol-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate
BSA	Bovine serum albumin
^{t}Bu	*tert*-Butyl
***n*-BuLi**	*n*-Butyl lithium
Bzl	Benzyl
CD	Circular dichroism
$CDCl_3$	Deuteriochloroform
CE	Capillary electrophoresis
2-ClZ	2-Chlorobenzyloxycarbonyl
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
2,6-DCB	2,6-Dichlorobenzene
DCC	*N,N'*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	*N,N'*-Dicyclohexylurea
Dde	(4,4-Dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl

DIEA	Diisopropylamine
DIPCDI	*N,N'*-Diisopropylcarbodiimide
DKP	Diketopiperazine
DMAP	4-Dimethylaminopyridine
Dmc	4,4-Dimethyl-2,6-dioxocyclohexylidenemethylene
DMMD	5,5-dimethyl-2-(diethylaminomethylene)cyclohexane-1,3-dione
DMF	Dimethylformamide
DNP	Dinitrophenyl
DSC	*N,N'*-Disuccinimidyl carbonate
DSS	3-(Trimethylsilyl)-1-propanesulphonic acid sodium salt
DVB	Divinylbenzene
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride
EDT	1,2-Ethanedithiol
EI	Electron impact (MS)
EMS	Ethyl methyl sulphide
Eq.	Molar equivalent
ES-MS	Electrospray mass spectroscopy
EtOH	Ethanol
FAB-MS	Fast atomic bombardment mass spectroscopy
Fmoc	9-Fluorenylmethoxycarbonyl
Fmoc-Cl	9-Fluorenylmethylchloroformate
For	Formyl
HATU	*O*-(7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.
HBTU	*O*-(Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate.

HF	Hydrogen fluoride
HMB	2-Hydroxy-4-methoxybenzyl
HMPA	4-Hydroxymethylphenoxyacetic acid
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
KLH	Keyhole limpet haemocyanin
LDA	Lithium diisopropylamide
MALDI-TOF MS	Matrix assisted laser desorption time of flight mass spectroscopy
MAP	Multiple antigenic peptide
4-MeBzl	4-Methylbenzyl
MeOH	Methanol
m.p.	Melting point
MS	Mass spectroscopy
NCA	*N*-Carboxyanhydride
Nde	1-(4-Nitro-1,3-dioxoindan-2-ylidene)ethyl
NOE	Nuclear Overhauser enhancement
NMR	Nuclear magnetic resonance
OcHex	Cyclohexyl ester
OdDHL	*N*-(3-Oxododecanoyl)-L-homoserine lactone
ODmab	4-{*N*-[1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester
OHHL	*N*-(3-Oxohexanoyl)-L-homoserine lactone
PAM	Phenylacetamidomethyl
Pd/C	Palladium on charcoal
PEG	Polyethyleneglycol

PGC	Porous graphitised carbon
PLC	Preparative thin layer chromatography
PS	Polystyrene
RAFT	Regioselectively addressable functionalised template
RPHPLC	Reverse phase high performance liquid chromatography
SPOC	Solid phase organic chemistry
SPPS	Solid phase peptide synthesis
Tbfmoc	Tetrabenzo[*a,c,g,i*]fluorenyl-17-methoxycarbonyl
TBTU	*O*-(Benzotriazol-1-yl)-1,1,3,3,-tetramethylurounium tetrafluoroborate
TCA	Tricarboxylic acid
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TFFH	Tetramethylfluoroforamidinium hexafluorophosphate
TFMSA	Trifluromethylsulphonic acid
THF	Tetrahydrofuran
TIPs	Triisopropylsilane
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNBS	2,4,6-Trinitrobenzenesulphonic acid
Tos	Toluenesulphonyl
UNCA	Urethane protected *N*-carboxyanhydrides
UV	Ultraviolet spectroscopy
Z	Benzyloxycarbonyl

Abstract

Solid phase peptide synthesis (SPPS) of branched/cyclic peptides, multiple antigenic peptides (MAPs), pseudopeptide toxins etc. requires amine protection orthogonal to the established Fmoc/^{t}Boc protocols. It was envisaged that progression from Dde to *N*-1-(4-Nitro-1,3-dioxoindan-2-ylidene)ethyl (Nde) amino acid protection would maintain the stipulated orthogonality, whilst improving the hydrazine mediated deprotection. A selection of N^{α}-Nde-amino acids were efficiently synthesised and their compatibility with SPPS conditions demonstrated by the synthesis of a number of peptides. The Nde group displayed a faster and more easily monitored deprotection profile and similar orthogonality when compared with the Dde group. The selective primary amine protecting characteristics of the Nde group was illustrated by the synthesis of N^{1},N^{8}-*bis* Nde-spermidine which was subsequently utilised in the solid phase synthesis of the natural product, dihydrotrypanothione.

Large peptides synthesised by SPPS often demand elaborate, expensive and cumbersome purification protocols. Dde based reversible amine protecting groups incorporating hydrophobic and affinity probes have been developed. Their ease of preparation and efficacy in the purification of synthetic peptides has been demonstrated.

Intercellular communication in various Gram-negative microorganisms is often mediated by small signalling molecules, e.g. *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL). Detection of these molecules is often extremely difficult. To address this, SPPS procedures have been employed to couple *N*-β-ketoacyl-L-homoserine lactone containing haptens to a dendritic lysine scaffolding, and the resultant macromolecule evaluated for its ability to raise anti-*N*-β-ketoacyl-L-homoserine lactone antibodies.

1-Carbapen-2-em-3-carboxylic acid is a broad spectrum antibiotic produced by the Gram-negative *Erwinia* and *Serratia* microorganisms. Some key intermediates for the putative synthetic precursors have been successfully

prepared in order to study the biosynthetic pathways by feeding blocked mutants of the above bacteria, and also to transpose the methodologies to a solid phase to construct carbapenem libraries.

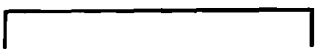
1

Introduction

Thousands of organic molecules owe their biosynthetic origins to nature; the foremost generator of molecular diversity. For decades, organic chemists have attempted to recreate many of these molecules within their laboratories; a task which has provided a continually evolving and fluxing challenge.

Peptides and proteins are two such natural product families which have created an enormous area of interest for the organic chemist. The initial attention was generated from the recognition by Hofmeister[1] and Fisher[2] that protein structure was ideally represented by chains of amino acids, linked to each other *via* amide bonds. This observation being predated by the synthesis of the first simple peptides by Curtis[3] and later by Fisher.[4] By the middle of the century, realisation that other biologically important molecules had simpler amino acid sequences escalated the stimulus and reduced the dimension of peptide synthesis to attainable levels.

A new era in both chemistry and biology was begun in the early 1950's, when du Vigneaud and his co-workers isolated,[5] determined the structure[6] and synthesised[7] the lactogenic nonapeptide amide hormone oxytocin (**1**).



H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

(**1**)

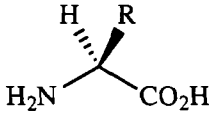
This was soon followed by Sanger's historical structural elucidation of insulin.[8] His work set new and higher aims for peptide synthesis, since it could facilitate

medicine in the study of peptide hormones and other pharmacologically relevant peptides which exhibit a function in the regulation of life processes.

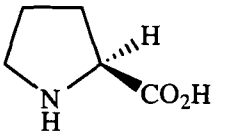
Biologically active peptides were isolated apace, many requiring novel and improved methods for their solution synthesis. However, the expectations have taken longer than expected to come to fruition, and the synthesis of complex peptides is still as much of a challenge now as in the early half of the century.

1.1. The biosynthetic origins of peptides and proteins

Naturally occurring peptides and proteins are macromolecules assembled under nucleic acid control from the nineteen L-amino acids of general structure (**2**) and the imino acid L-proline (**3**). For a full list of the proteinogenic amino acids and their coding systems (see Appendix, p.229).

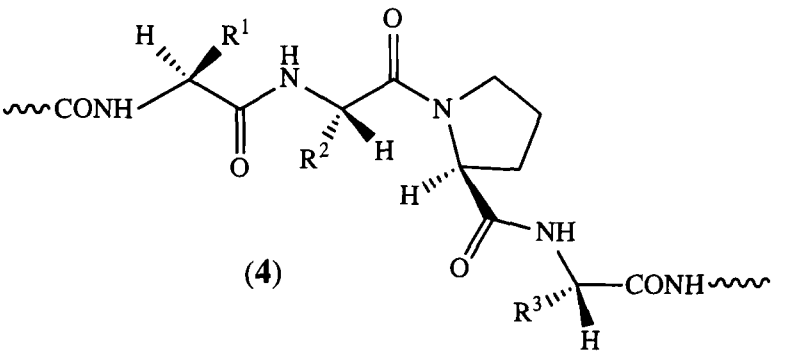


(**2**)



(**3**)

'Peptide' bonds (-CO-NH-) link the amino acids, forming macromolecules (**4**) containing 'polypeptide' backbones and diverse side chain functionalities depending upon the amino acid. Peptides, by definition, contain between two and fifty amino acid residues within their structure and, unlike proteins, naturally occurring peptides often include modified sequences.



(4)

These may be expressed as *N*- or *C*- terminal modifications, or the inclusion of D or atypical amino acids. The other common structural modification exhibited is the existence of the peptide in a cyclic or polycyclic form. These characteristics often bestow the conformational restrictions essential for biological activity and confer an increased resistance to enzymatic degradation.

1.1.1 The formation of the peptide bond

Nature synthesises peptides and proteins via stepwise assembly from the amino acid building blocks, and the organic chemist generally proceeds this way in the laboratory. However, the products formed by the uncontrolled solution synthesis of even a simple dipeptide (**5**), bearing no side chain functional groups, remains uncertain.

$$H_2NCHR^1CO_2H + H_2NCHR^2CO_2H \longrightarrow H_2NCHR^1CONHCHR^2CO_2H$$

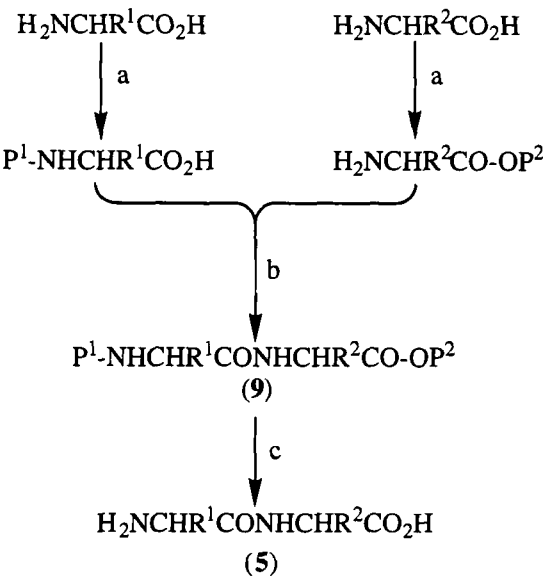
(**5**)

The target product (**5**) would be accompanied by the dipeptides (**6-8**) and any polycondensation permutations from the four possible dipeptides.

$H_2NCHR^1CONHCHR^1CO_2H$ (**6**) $H_2NCHR^2CONHCHR^1CO_2H$ (**7**)

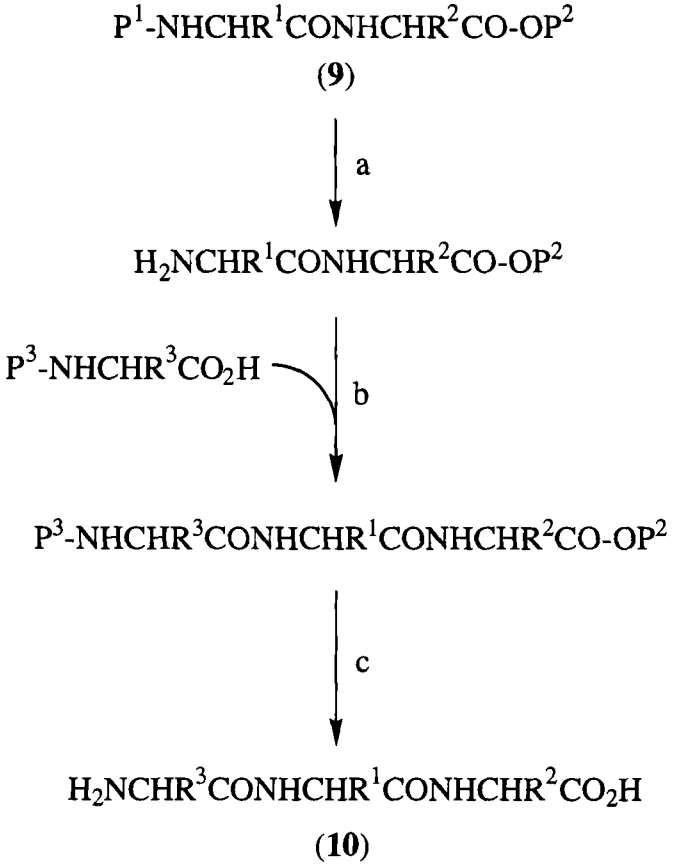
$H_2NCHR^2CONHCHR^2CO_2H$ (**8**)

A means of controlling the synthesis of (**5**) would require the blockade (or 'protection') of the amine of one amino acid, and the carboxyl group of the other. Condensation of these two components followed by removal of the blocking groups (or 'protecting groups') from the intermediate (**9**) would afford the dipeptide (**5**) as one product (Scheme 1), the whole process requiring a minimum of four reactions.



Conditions: a) protection; b) condensation; c) global deprotection
Scheme 1. Controlled synthesis of a dipeptide

Further elongation of the peptide can be accomplished by selective removal of the amine protecting group (P^1) over the carboxyl protecting group (P^2) in an orthogonal manner (Scheme 2).



Conditions : a) Selective deprotection; b) Condensation;
c) Global deprotection.
Scheme 2. Controlled synthesis of a tripeptide

An orthogonal system means one using two or more independent classes of protecting groups that are removable by differing chemical mechanisms.[9,10]

Stepwise selective deprotection, followed by condensation (or 'coupling') with the next *N*-protected amino acid can be repeated until the desired peptide is formed. The possibility of participation of side chain functional groups in the above reactions adds a further level of complexity to the procedure, which must be addressed if the synthesis is to proceed in an unambiguous manner. Consequently there is a requirement for side chain protection which can be removed only at the very end of the synthesis.

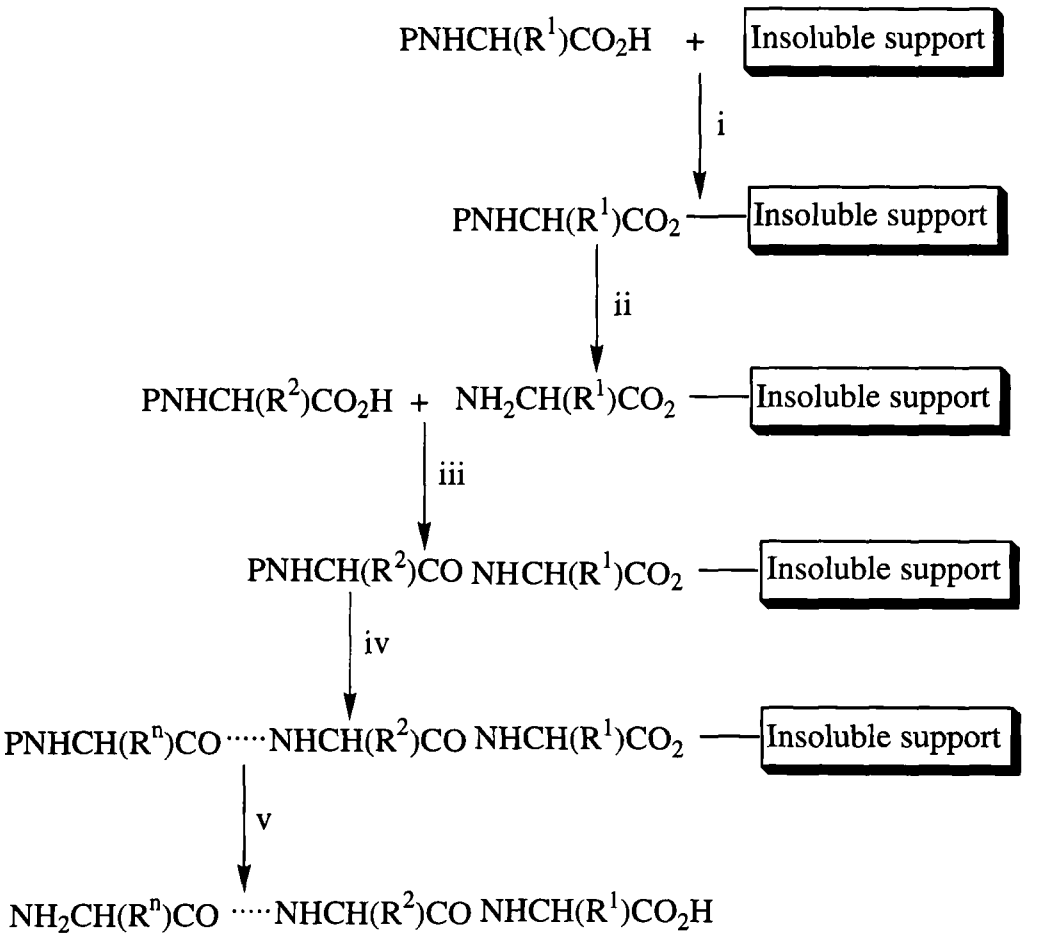
Furthermore, all of the transformations in peptide synthesis must be performed without the loss of chirality at any of the α-centres, as production of often inseparable diastereoisomers then becomes an issue. Finally, the global deprotection of the finished peptide must be executed without destruction of the peptide backbone, or deleterious side reactions.

The chemistry required to achieve these synthetic challenges has been attained and the solution phase synthesis of small peptides is now a reasonably efficient process. However, as the target peptide chain length increases, the efficiency of a solution based synthesis decreases rapidly. Solubility problems of the protected fragments leads to poor acylation rates resulting in low yields and high levels of impurities. Lengthy purification procedures are then required to isolate the target molecule, with the overall process becoming highly labour intensive and inefficient. The development of solid phase peptide synthesis (SPPS) attempted to address these problems.

1.2 Solid phase peptide synthesis

More than three decades ago the concept of solid phase peptide synthesis was introduced by Merrifield,[11] with the full experimental paper published in 1963.[12] The principle employed is outlined below (Scheme 3).

The *N*α-amine and side chain protected *C*-terminal amino acid of the target peptide was covalently attached to an insoluble resin support *via* an ester bond. The resin was thus acting in a dual faceted mode; as both a heterogeneous support and semi-permanent α-carboxy protecting group. The temporary α-amino protecting group was then removed allowing subsequent acylation of the resin bound amino acid with the second residue of the peptide.



Scheme 3. General principle of SPPS. (i) attachment of protected amino acid to insoluble polymeric support; (ii) N^{α}-deprotection; (iii) coupling of second protected amino acid; (iv) repetition of (ii) and (iii) as required; (v) cleavage of peptide-polymer link and side chain deprotection.

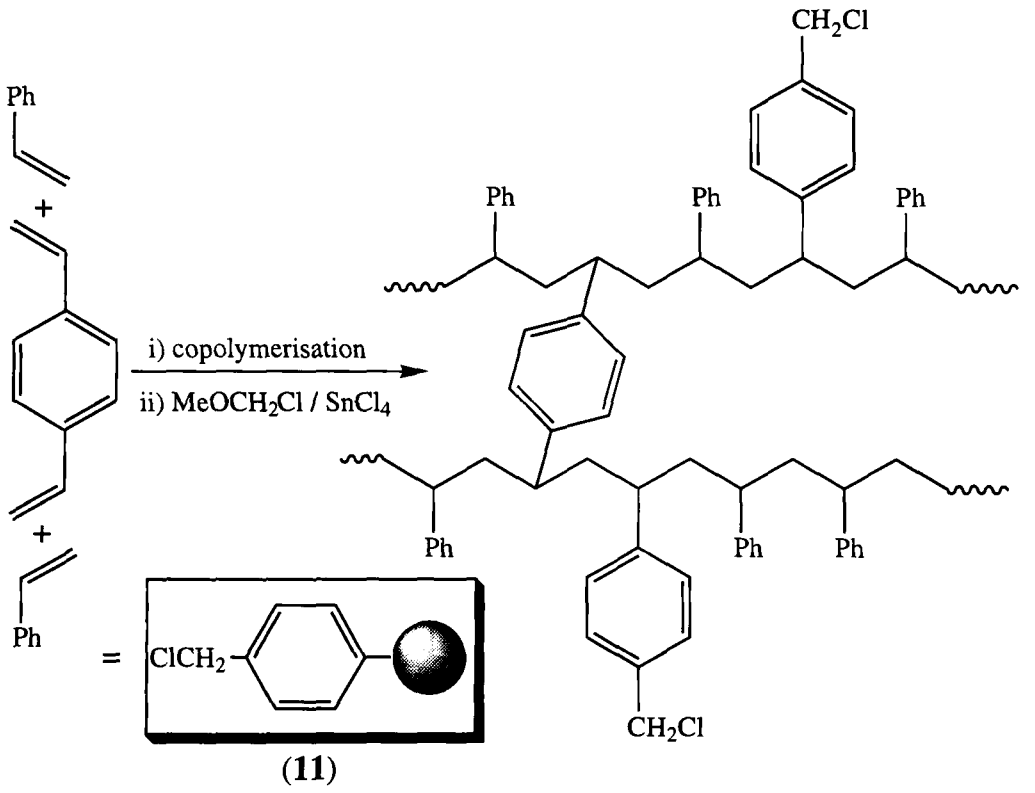
The N^{α}-amino protection utilised in the Merrifield approach was the acid labile *t*-butoxycarbonyl- (Boc) group, the chemistry of which is discussed later (see section 1.5.1, p.16).

This sequential process was continued until the desired peptide sequence was completed. The most obvious advantage of this methodology was the ability to exploit large excesses of reagents in order to drive the acylation and

deprotection reactions to completion, after which any unused materials could be removed from the resin by a simple washing procedure. Secondly, since the *C*-terminal amino acid was anchored to a heterogeneous solid support, the problems associated with purification of intermediates were effectively removed.

1.3 The solid support

The first naturally occurring peptide synthesised by Merrifield *et al*[13] *via* solid phase methodology was bradykinin. In this example the solid support consisted of polystyrene strands cross-linked together by the inclusion of a small amount of divinylbenzene during the polymerisation process (Scheme 4).



(11)

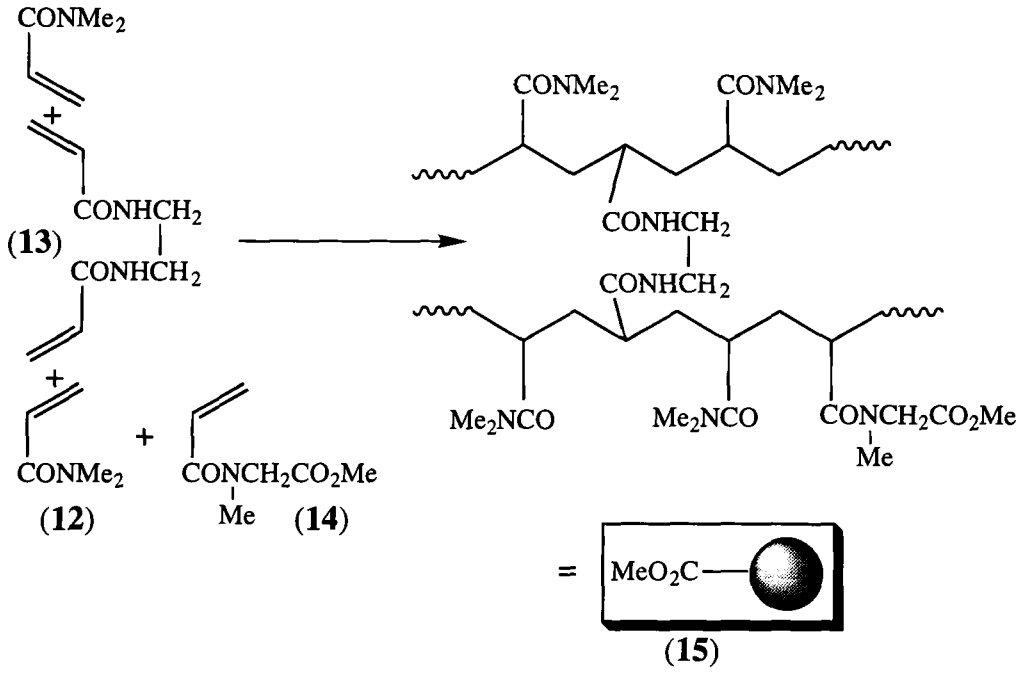
Scheme 4. Synthesis of the 'Merrifield Resin'.

Functionalisation of the inert resin via chloromethylation was essential in order to incorporate the first *N*-protected amino acid residue. This was subsequently achieved via a nucleophilic displacement with the triethylammonium salt of the *N*-protected amino acid to afford a benzyl ester type linkage. After completion of the required synthetic sequence, the peptide-resin bond was cleaved via an acidolytic mechanism employing liquid HF with concomitant removal of the acid labile side chain protecting groups.

This synthesis was indeed a landmark in peptide chemistry, taking just a few days to complete (it would probably have taken as many weeks using solution chemistry), and presented an ideal solution to the incredible demand for peptides in both pharmacological and other biological sciences. However, very little new chemistry appeared after the seminal 1964 Merrifield paper, and the technique was widely practised unchanged for a number of years.

It was not until 1971, when Sheppard *et al*[14] proposed that the growing peptide-polymer conjugate could be regarded as analogous to a graft co-polymer system consisting of various types of chains, that a major step-change in SPPS was realised. From this observation he argued that it would be advantageous for the polymer support to be chemically similar to the growing peptide chain. This would allow both the polymer and peptide to be well solvated by an appropriate polar solvent, forming an open gel system which permitted greater access of reagents to reactive sites. The Merrifield approach favoured the use of dichloromethane (DCM) as the solvent for swelling the polystyrene resin core (**11**). Unfortunately, DCM does not adequately solvate the growing peptide chain and aggregation (depending upon both the sequence and choice of side-chain protection) can often result, decreasing the availability

of reactive sites. From these considerations emerged the chemistry of the current Fmoc-polyamide system,[15,16] with the insoluble polymer matrix being synthesised from dimethylacrylamide (**12**), bisacryloylethylene diamine (**13**) and acryloylsarcosine methyl ester (**14**) (Scheme 5). Solid phase resins based on this polar polydimethylacrylamide support allowed peptide synthesis to be carried out using DMF as the solvent, thereby improving the overall solvation as originally proposed.



Scheme 5. Synthesis of polydimethylacrylamide resin.
Conditions: Suspension co-polymerisation of dimethylacrylamide (**12**) with approx. 5% molar portions of the two other monomers performed in DCM/DMF/H_2O at 50 ^{0}C, in the presence of cellulose acetate butyrate and ammonium persulphate.

The polystyrene and polyamide based polymer matrices described above still provide the insoluble core of many modern solid phase supports.

1.3.1 Base resins commonly exploited in SPPS

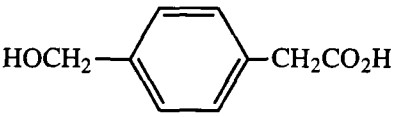
A selection of base resin supports commonly used in SPPS are illustrated below (Table 1).

Table 1. Base resins commonly exploited in SPPS.

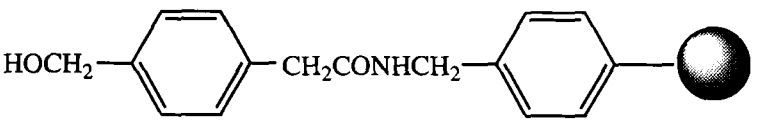
Solid support	General comments	Ref.
Kieselguhr/polyamide (Pepsyn K)	Pressure stable, yet displays low swelling properties due to the inorganic nature of the support. Fairly brittle so not very robust.	17
PS/DVB copolymer (1-5% cross-linking)	Good swelling properties (up to five times its dry volume)	18,19
Poly[*N*-{2-(4-hydroxy-phenyl)ethyl}]acrylamide (Core Q)	Base resin support displaying high loading values.	20
Poly(*N*-acryloylpyrrol-idine)resins, PAP- and SPARE-polyamide resins	Base resin support that swells well in many solvents, e.g. H_2O, DMF and CH_2Cl_2	21,22
PolyHipe, PS/polydimet-hylacrylamide copolymer	High loading base resin support with high pore volume and porosity.	23
TentaGel, PEG-PS/DVB co-polymer	Displays excellent physical stability and swells in a wide variety of solvents, e.g H_2O, MeOH, MeCN, DMF and CH_2Cl_2.	24,25 & 26

1.4 The peptide-resin linker

As stated earlier, the Merrifield approach to SPPS utilised both an acid labile *N*α-amino and side chain protecting group strategy alongside an acid labile peptide-resin bond. Despite the latter requiring strong acid for its cleavage, significant acidolysis was observed during TFA mediated *N*α -deprotection, resulting in premature loss of peptide from the insoluble support.[27] This deleterious side reaction prompted the development of resin modifications *via* electronically modulated linking reagents. The first example of this chemical tempering was demonstrated by the acylation of an aminomethylpolystyrene polymer matrix with the 4-hydroxymethylphenylacetic (**16**) acid linker to generate the so called PAM resin (**17**).[28]



(**16**)



(**17**)

Once formed, the acid stability of the benzyl ester linkage between an amino acid and the resin support is substantially enhanced due to the electron-withdrawing nature of the *p*-carboxamidomethyl substituent decreasing the potential to protonate the now electron denuded benzyl ester carbonyl.[29]

However, the general hazards of HF use initiated the search for milder conditions with which to remove the completed peptide from the resin support,

and the development of the Sheppard's polydimethylacrylamide polymer matrix, coupled with the exploitation of the base labile 9-fluorenylmethoxycarbonyl (Fmoc) (see Section 1.5.2, p.19) amino protecting group allowed such an evolution to occur.

In the Sheppard approach, the growing peptide chain is exposed to mild base treatment using piperidine during Fmoc group deprotection. Acid treatment is therefore only necessary for the final cleavage and deprotection of the target peptide from the resin. The orthogonal nature of this methodology allowed the development of a vast array of acid labile linkers displaying a broad spectrum of physico-chemical properties (Table 2).

Table 2. Commonly employed linkers in Fmoc based SPPS.

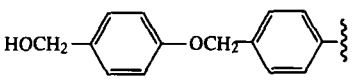
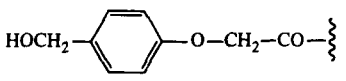
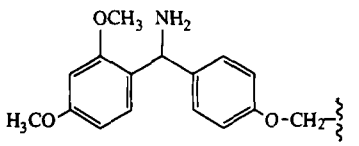
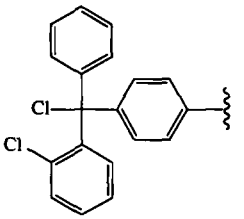
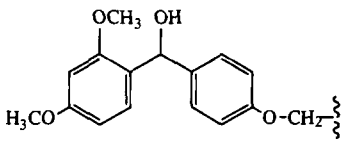
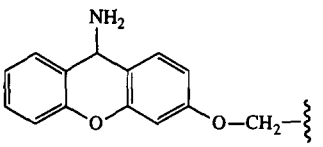
Linker	Cleavage Conditions	Cleavage Product	Ref.
Wang Linker 	95% TFA	Peptide Acid	30
4-Hydroxymethylphenoxyacetic acid (HMPA) 	95% TFA	Peptide Acid	31
Rink Amide 	95% TFA	Peptide Amide	32

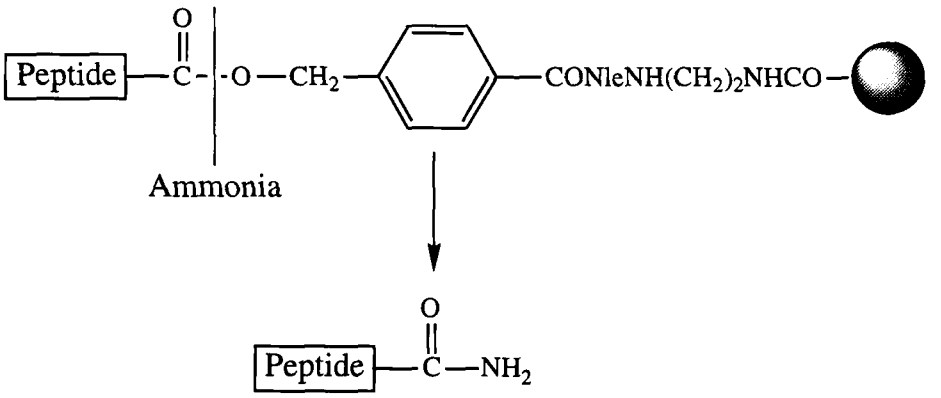
Table 2. Continued.

Linker	Cleavage Conditions	Cleavage Product	Ref.
2-Chlorotritylchloride 	**AcOH/TFE /DCM (1:1:8)**	**Protected Peptide Acid†**	**33**
Rink Acid 	**20% AcOH in DCM**	**Protected Peptide Acid†**	**32**
Sieber Amide 	**1% TFA in DCM**	**Protected Peptide Amide†**	**34**

† The extremely mild acid mediated cleavage conditions leave side-chain protecting groups intact, thereby allowing the synthesis of protected fragments.

Two of the linkers illustrated in Table 2. afford a peptide amide upon cleavage. This requirement arose from the need to synthesise biologically active peptides expressing an amide function at the *C*-terminal, eg. vasopressin.

Historically, ammonolysis of a 4-hydroxymethylbenzoic acid linker was the common practise,[35] (Scheme 6). However side reactions such as esterification were frequently reported,[36] and the newer acid-sensitive amide-producing linkers are now recommended.



Scheme 6. Peptide amide formation via ammonolysis of the 4-hydroxymethylbenzoic acid linker.

1.4.1 Continuous flow SPPS

Larger scale production of *N*α-Fmoc protected amino acids along with competitive pricing when compared with their *N*α-Boc counterparts have contributed to the much wider acceptance of the Fmoc strategy towards SPPS. This was supported by reports of the superiority of the Fmoc strategy for the synthesis of difficult sequences.[37] However, it was the ability to monitor the release of the cleaved Fmoc group at 300-320nm that truly highlighted the advantage of this strategy, and allowed the development of automated techniques where acylation, deprotection and washes were monitored by UV spectroscopic analysis of the DMF solutions passing through the resin. Continuous-flow Fmoc SPPS, as it was termed, has since proved to be an

efficient and reliable technique for the routine production of linear peptides and numerous manual and automatic synthesizers suitable for this approach are now available.[38]

A review and discussion of the current chemistry of amino acid protecting groups and protocols for peptide bond formation is essential in order to ascertain areas of SPPS which still require further development.

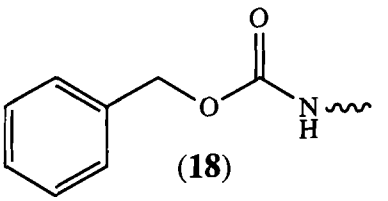
1.5 N^{α}-Amino protection

Within the field of SPPS, $N\alpha$-amino protection must suppress the reactivity of the amine group by either decreasing its nucleophilicity or creating a steric barrier. The protecting group should be easily introduced, stable to the conditions of amino acid coupling and preserve the chiral integrity of any asymmetric centres. On completion of the aforementioned condensation, it should be easily removed under conditions that will not damage the rest of the peptide or peptide resin bond. A degree of compromise has frequently been necessary to meet these exacting requirements, as illustrated below.

1.5.1 Acid-labile urethane protection

The original use of $N\alpha$-urethane protection arose from the substantial racemisation observed[39] upon carboxy activation of $N\alpha$-acyl protected amino acids such as $N\alpha$-formyl[40] and $N\alpha$-trifluoroacetyl.[41] Urethane protection can be regarded as both amide and ester in nature. As amides, they possess low nucleophilic reactivity at the nitrogen atom, and as esters, the +M effect of the ester oxygen reduces the -I effect of the amide and thereby renders the α-proton

less acidic, dramatically reducing the risk of racemisation during coupling. Urethane protection, unlike amide protection, also displays the advantage of milder acidolytic cleavage conditions, thus making deprotection less deleterious to the growing peptide chain. The first successful urethane type protecting group was the benzyloxycarbonyl (or 'carbobenzyloxy', Cbz or Z) group (**18**).[42]



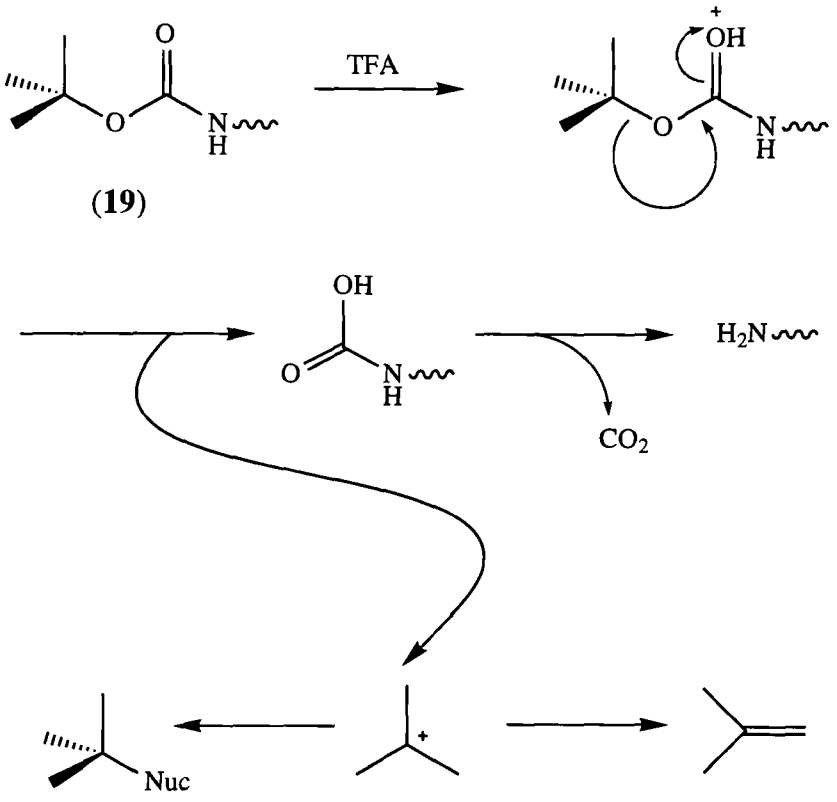
(**18**)

Unfortunately, the strong acid required for its removal (HBr/AcOH)[43] dictated its unsuitability as an *N*α-protecting group in SPPS.

The Merrifield approach to SPPS utilised a benzyl ester linkage between the *C*-terminal carboxylic acid of the peptide and the resin support, again necessitating the use of a strong acid for its final cleavage. The semi-permanent *N*α-protection therefore had to be removed under milder acidic conditions, ruling out reagents like the Z group. Consequently the *N*α-*t*-butoxycarbonyl, or Boc group (**19**)[44] was chosen, which merely required exposure to 50% TFA in DCM at room temperature for 30 min. for its removal, (Scheme 7).

The mild acid lability of the Boc group can be explained mechanistically by the ease of formation of the relatively stable *t*-butyl carbocation in an S_N1 type process, following protonation of the urethane carbonyl. The resulting unstable carbamic acid intermediate spontaneously decomposes to yield the amine as its corresponding TFA salt. In the Merrifield approach to SPPS, the trifluoroacetate salt is neutralised by exposure of the peptide-resin to 5% DIEA

in DCM, regenerating the amine for subsequent coupling to the next amino acid residue .

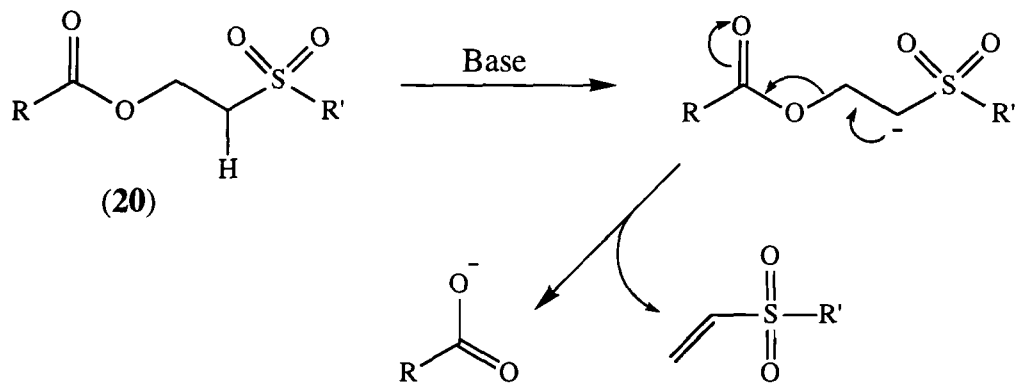


Scheme 7.TFA mediated deprotection of the *N*-Boc protecting group.

A common obstacle of most acid sensitive protecting groups is the potential alkylation of amino acid side chains by the carbocation generated during the deprotection. This is manifested by electrophilic aromatic substitution of the phenol in tyrosine or the indole in tryptophan. Alternatively, products formed with the deprotection solvent, e.g. *tert*-butyl trifluoroacetate,[45] can act as alkylating agents. The remedy to this problem lies in the addition of cation scavengers to the deprotection medium,[46] and this is discussed in more detail later (see Section 1.11.6, p.58).

1.5.2 Base-labile urethane protection

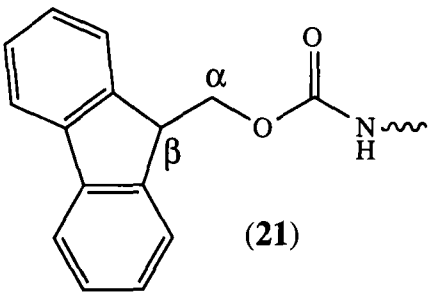
The problem of side chain alkylation and possible damage to the peptide by strong acids led to the exploration of alternative deprotection procedures to acidolysis. Carboxyl-protection using ethyl esters containing electron withdrawing substituents at their β-carbon (**20**) provided the necessary template for adaption to an amine protecting group[47, 48]. In the presence of base, proton abstraction occurs and the resulting carbanion β-eliminates to form, in the case illustrated, a vinylsulfone and the carboxylic acid (Scheme 8).



(**20**)

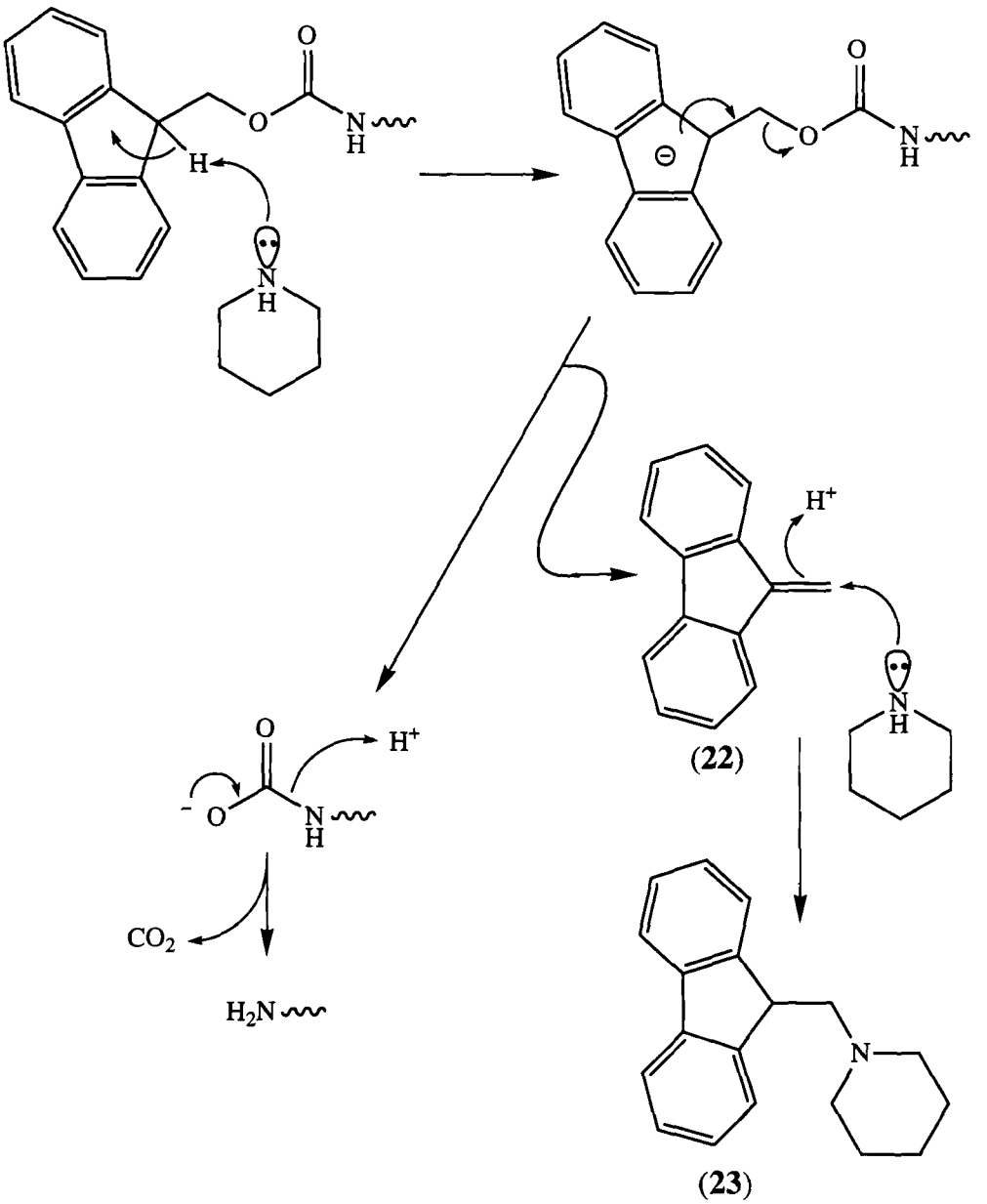
Scheme 8. Base catalysed deprotection of a β-sulphone ester.

Transposition of this mechanism to a urethane led to the development of the 9-fluorenylmethyloxycarbonyl (Fmoc) group[49, 50] (**21**), where the β-carbon is, in this example, part of the fluorenyl ring system.



(**21**)

The Fmoc group is very stable to acidic reagents, yet is readily cleaved at room temperature under basic conditions, e.g. 20% piperidine in DMF. The cleavage proceeds through an E1cb type mechanism, via the stabilised dibenzocyclopentadienide anion (Scheme 9). The dibenzofulvene (**22**) produced reacts with piperidine to form the adduct (**23**) as the co-product.

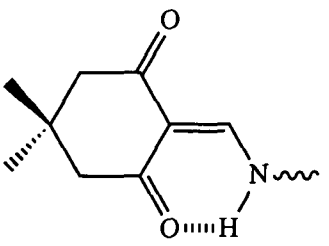


Scheme 9. Piperidine mediated deprotection of the *N*-Fmoc group.

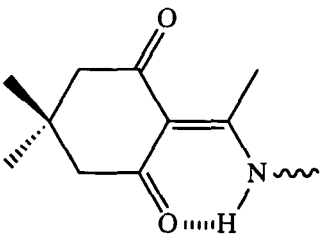
The Boc and Fmoc amino-protecting groups, both based on urethane chemistry, have between them dominated the field of SPPS for almost three decades, and will probably continue to do so. They both exhibit good stability and mild cleavage conditions, with carboxy activation and coupling normally proceeding with retention of chiral integrity. There are however, other amino protecting groups which meet these requirements and these are beginning to gain popularity in modern SPPS.

1.5.3 The *N*-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) protecting group

Vinylogous amides have found some application as amine protection in peptide synthesis using β-dicarbonyl compounds, such as pentane-2,4-dione, as the protecting group[51, 52]. However, their lability to aqueous and weakly acidic conditions limited their use. The interest in vinylogous amides was rekindled with the initial introduction of the *N*-(4,4-dimethyl-2,6-dioxocyclohexylidenemethylene (Dmc)[53] group (**24**), quickly followed by its successor, the *N*-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) group[54] (**25**).

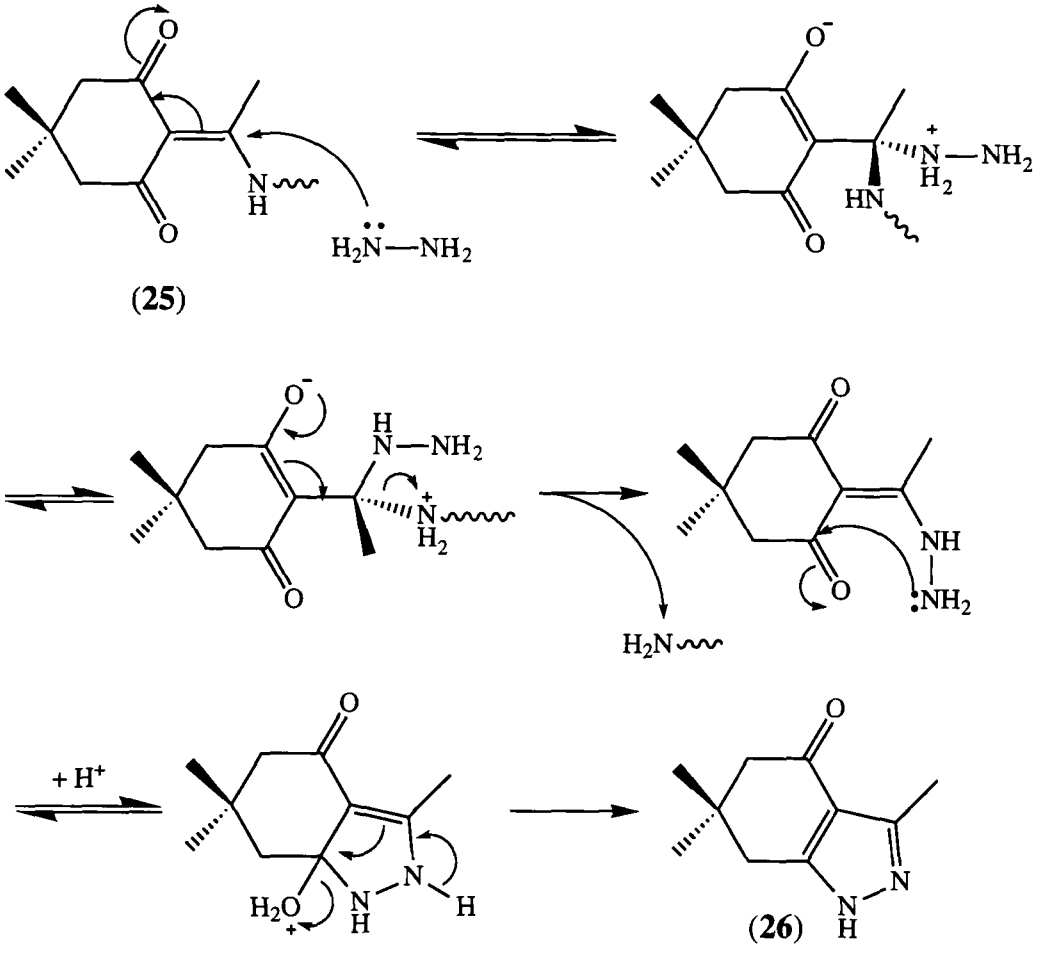


(**24**)



(**25**)

The Dde group is stable to acid and many bases, including piperidine, but is readily removed at room temperature by dinucleophiles such as hydrazine (Scheme 10). The process being aromaticity driven by the formation of the deprotection by-product 3,6,6-trimethyl-4,5,6,7-tetrahydro-1*H*-indazol-4-one (**26**).



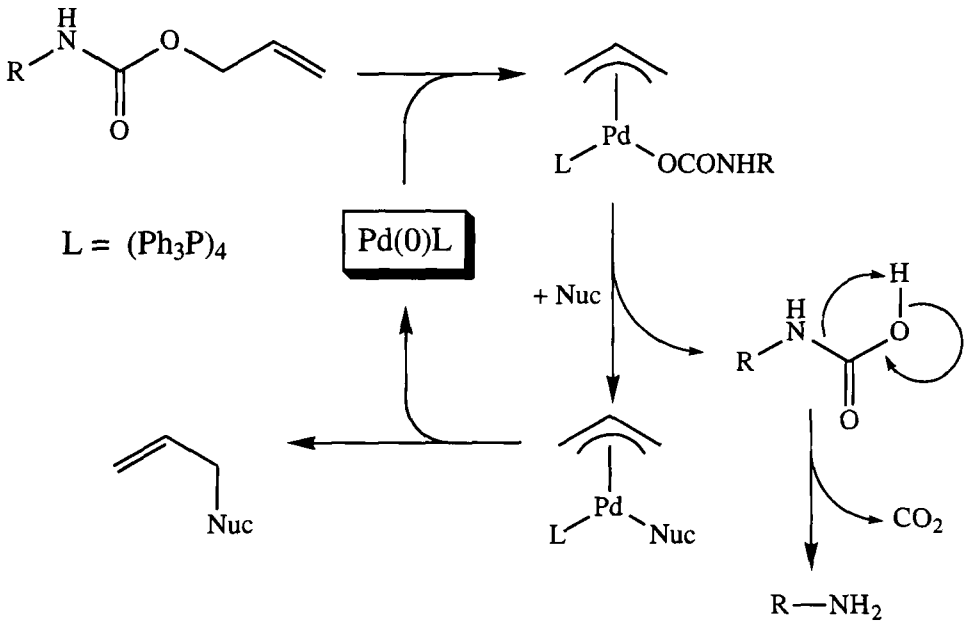
Scheme 10. Deprotection of the Dde amine protecting group using the dinucleophile hydrazine.

Dde's stability to piperidine introduces a level of orthogonality to the Fmoc protecting group, and thus can be exploited in amino acids possessing two amine functions within their structure, e.g. lysine and ornithine. Difficult

synthetic targets such as cyclic peptides,[55] multiple antigenic peptides[56] (MAPs) and spider toxins[57] have all benefitted from this orthogonality in their solid phase assembly.

1.5.4 The *N*-allyloxycarbonyl (Alloc) protecting group

The development of 'π-allyl palladium complex' chemistry has led to a powerful and increasingly exploited means of protection strategy in organic chemistry.[58] Thus, allyl carbamates,[59] or any function possessing an acidic leaving group, can be readily cleaved *via* an irreversible Pd(0)-catalysed transfer of the allyl moiety to an acceptor nucleophile, e.g. dimedone (Scheme 11).



Scheme 11. Pd(0) catalysed Alloc cleavage.

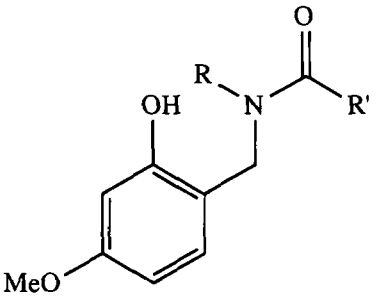
Kunz first introduced the Alloc protection for the α-amino group in solution phase peptide synthesis,[60,61] and recently a few Alloc based solid

phase syntheses have been reported.[62-64] For the solution phase peptide synthesis, standard nucleophilic reagents such as dimedone or morpholine have proved highly successful. Unfortunately, these did not prove as efficient in SPPS where side reaction free cleavage of the Alloc group was essential.[62,63] Instead use of premixed 8eq:3eq trimethylsilylazide/tetrabutylammonium fluoride ($TMSN_3/Bu_4NF[3H_20]$) and 20mol% $Pd(PPh_3)_4$ has been demonstrated to quantitatively cleave resin bound *N*-Alloc protected peptide fragments.[65]

1.6 Amide bond protection in SPPS

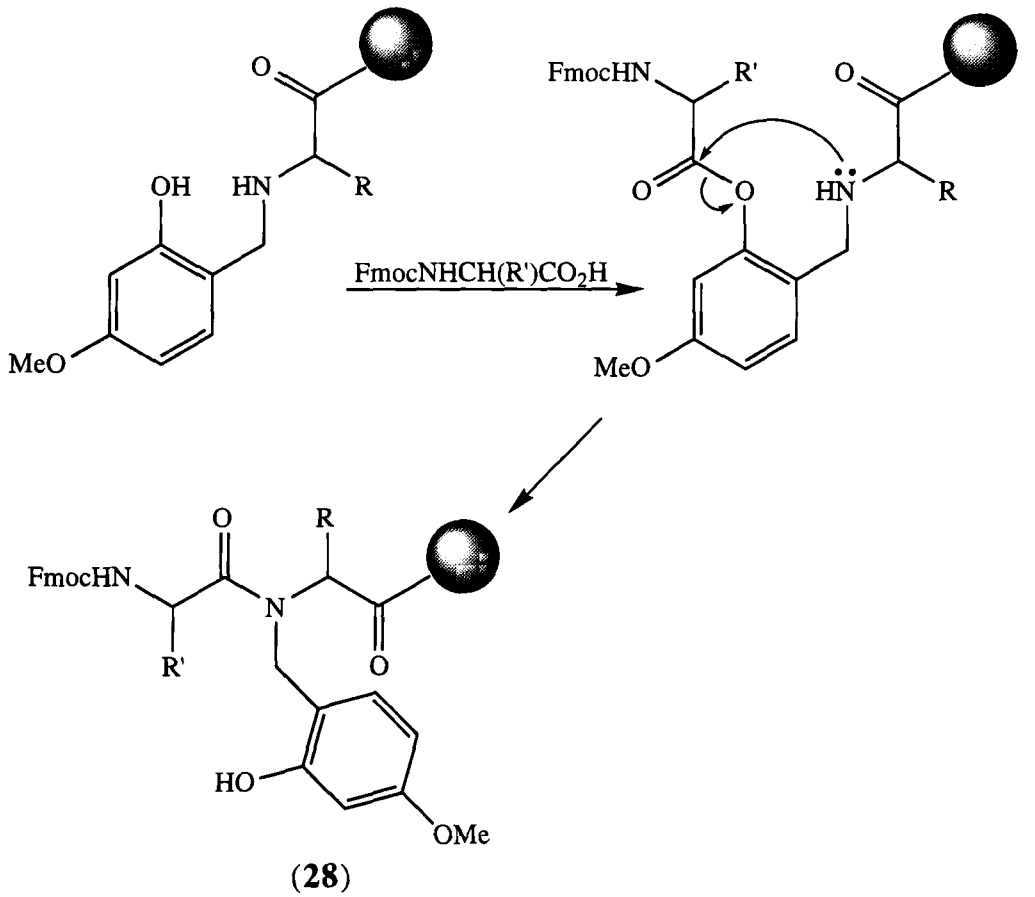
Over 20 years ago it was recognised that certain peptide sequences posed special problems associated with their solid phase synthesis.[66] These have subsequently become termed 'difficult sequences'[67] and are postulated to arise from the inter-chain association of resin-bound peptides into extended β-sheet structures.[68] The ensuing steric hindrance leads to a serious reduction in reagent penetration resulting in significant decreased reaction rates in both acylation and deprotection processes.[69,70]

Chain association had been observed when both polystyrene[71] and polydimethylacrylamide-amide[72] resins were employed for SPPS. However, the observation that replacement of secondary by tertiary amide bonds prevented this hydrogen bonded association of resin bound peptide chains,[73] led to the development of a reversible *N*-amide protecting group, namely the *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) (**27**) group.[74] The 2-hydroxy substituent is the essential feature of the reagent.



(**27**)

During coupling to a resin bound *N*-Hmb derivatised amino acid, initial acylation occurs *via* an internally base catalysed mechanism onto the phenolic-OH. This is then followed by an intramolecular *O* →*N* acyl transfer[74] (Scheme 12), to yield the fully protected dipeptide fragment (**28**).



(**28**)

Scheme 12. Two step acylation of a resin bound Hmb-derivatised amino acid.

N-Substituted amino acids tend to exhibit massive steric hindrance towards subsequent acylation.[75] Despite the intramolecular acyl transfer described, *O*-acylation tends to be slower than normal *N*-acylation. Therefore care must be exercised in the selection of amino acid residues within the target peptide that are to be protected with the Hmb group, to ensure quantitative incorporation of the next residue proceeds efficiently.

The Hmb group is removed under the normal conditions required for cleavage and side chain deprotection of peptides synthesised *via* the Fmoc/*tert*-butyl strategy, however acylation of the Hmb group with acetic anhydride in the presence of DIEA, greatly increases its acid stability, allowing backbone protected fragments also to be isolated.[76]

Hmb protection of residues sequentially preceeding aspartic acid has also been demonstrated to prevent aspartimide formation,[77] and coupled with its general applications to SPPS this group has received significant attention.[78-80]

1.7 Carboxy protection

In the context of SPPS, the *C*-terminal carboxylic acid is effectively protected during the synthetic sequence by the resin-linker support. Side chain carboxylic acids, present in aspartate and glutamate residues, are also blocked during the synthesis by protecting groups displaying a similar orthogonality towards the deprotection conditions of the semi-permanent N^{α}-protecting group employed as that of the linker (see section 1.8, p.29).

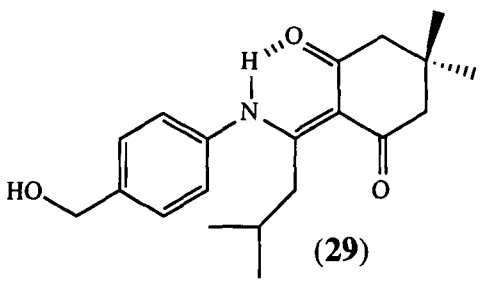
However, the ability to protect/deprotect either the α- or side chain carboxyl groups of such residues in an orthogonal manner to either Boc or Fmoc deprotection potentially allows for the synthesis of atypical peptides.

1.7.1 Allyl ester protection

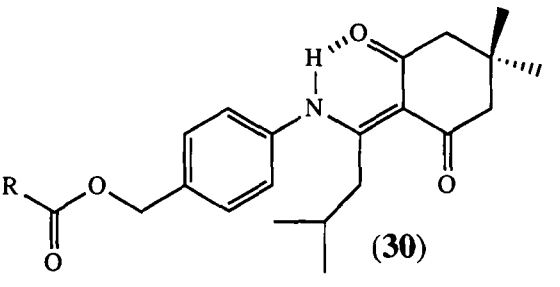
Exploitation of 'π-allyl palladium complex' chemistry with respect to carboxyl protection has proved as effective as that of the *N*-Alloc group. Displaying a similar orthogonal deprotection mechanism as that of the carbamate reagent, the allyl ester has been successfully exploited in the solid phase synthesis of many atypical peptides.[81,82]

1.7.2 The 4-{*N*-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino}benzyl ester(ODmab) protecting group.

Utilising the *N*-Dde amino protecting group, it was reasoned that the combination of its deprotection characteristics with the well documented lability of 4-aminobenzyl esters,[83] should furnish a novel carboxy-protecting group. From this hypothesis emerged the reagent, 4-{N-[1-(4,4-dimethyl-2,6-dioxocyclohexylid-ene)-3-methylbutyl]amino}benzyl alcohol (**29**) and hence the 4-{N-[1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl]amino} benzyl ester (ODmab) protecting group (**30**).[84]

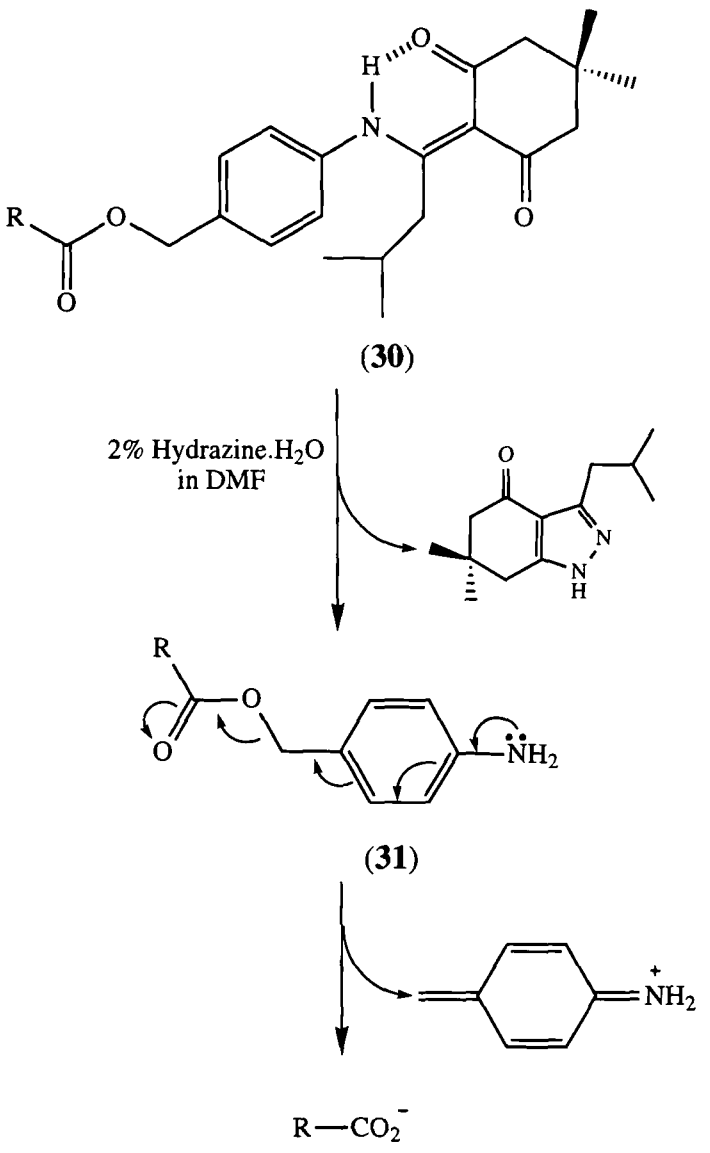


(**29**)



(**30**)

The ODmab protecting group is stable to both TFA and 20% piperidine in DMF, but is quantitatively removed with 2% hydrazine monohydrate in DMF within minutes (Scheme 13).



Scheme 13. Hydrazine mediated ODmab deprotection.

The dinucleophilic reagent initially removes the vinylogous amide in a transamination type process, followed by spontaneous collapse of the resultant 4-aminobenzyl ester (**31**) *via* a 1,6 electron pair shift to relinquish the carboxylic acid.

The ODmab group has been subsequently utilised in cyclic peptide synthesis by incorporation onto the α-carboxyl of aspartic acid of Fmoc

-Asp(O^{t}Bu)-OH *via* a carbodiimide mediated condensation. Removal of the side chain protection and subsequent coupling of the free carboxylic acid to 2-chlorotrityl chloride polystyrene[85] then allowed Fmoc deprotection and construction of the linear peptide. Final deprotection of the *N*-terminus with piperidine and the *C*-terminal with hydrazine, allowed the DCC-HOAt mediated coupling of the two unmasked groups to yield the cyclic product in good yield.[84]

1.8 Side chain protecting groups

More than half the amino acids commonly encountered in peptides and proteins contain functional side chains. These include carboxylic acids (aspartic and glutamic acid); basic residues (lysine, arginine, histidine); hydroxyl/phenolic groups (serine, threonine, tyrosine); sulphur containing amino acids (cysteine, methionine); and finally heterocyclic side chains (tryptophan and histidine). Due to the potential ability of these side chain functional groups to participate in side reactions during amino acid couplings, their semi-permanent protection is required. These protecting groups must be stable to coupling conditions and the deprotection conditions for the semi-permanent $N\alpha$-amino masking group. Upon completion of the peptide/protein synthesis, they should ideally be removed in a global manner, along with the carboxy terminal protecting group. A vast array have been reported in the literature but we will concern ourselves with those that have become favoured for solid phase peptide synthesis.

1.8.1 Side chain protection employed in Boc SPPS

Table 3. Commonly employed side chain protection in Boc SPPS.

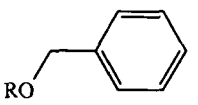
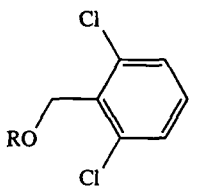
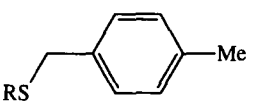
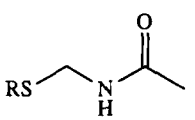
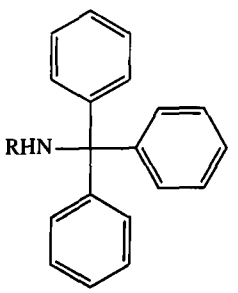
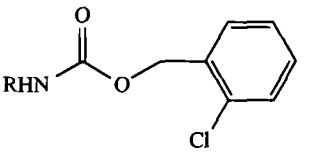
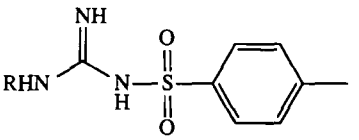
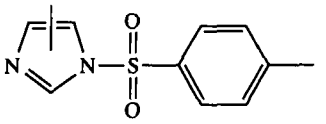
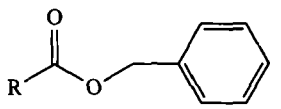
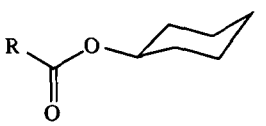
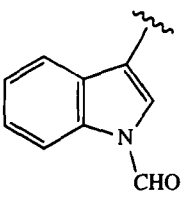
Amino Acid	Side chain protection	Abbreviation	Deprot^{n}. Condition	Ref.
Ser Thr	**Benzyl ethers** 	Bzl	$HF_{(l)}$ 0 °C	-
Tyr	**2,6-Dichlorobenzyl ether** 	2,6-Dcb	$HF_{(l)}$ 0 °C	-
Cys	***S*-4-Methyl-Benzyl** 	4-MeBzl	$HF_{(l)}$ 0 °C	-
	***S*-Acetamidomethyl** 	Acm	Hg^{2+} or I_2	86
Asn† Gln†	***N*-Trityl** 	Trt	TFA	87

Table 3. continued

Amino Acid	Side chain protection	Abbreviation	Deprotn. Condition	Ref.
Lys	2-Chlorobenzyloxycarbonyl 	2-ClZ	$HF_{(l)}$ 0 °C	88-91
Arg	*Ng*-*p*-Toluenesulphonyl 	Tos	$HF_{(l)}$ 0 °C	92
His	*Nim*-*p*-Toluenesulphonyl 	Tos	$HF_{(l)}$ 0 °C	93, 94
Asp	Benzyl ester 	OBzl	$HF_{(l)}$ 0 °C	-
Glu	Cyclohexyl ester 	OcHex	$HF_{(l)}$ 0 °C	95, 96
Trp	*Ni*-Formyl 	For	HF/Me_2S ('low' HF cleavage)	97

† Asn and Gln side chain protection is generally not necessary although its inclusion protects against dehydration during coupling. The *N*-trityl group is removed during *Nα*-Boc deprotection.

1.8.2 Side chain protection employed in Fmoc SPPS

One of the foremost advantages inherent in the Sheppard approach to SPPS is the absolute orthogonality displayed between the *N*-α-Fmoc protecting group and the side chain protecting groups; the former being removed with base treatment and the latter requiring exposure to mild acid. Hence, premature loss of the side-chain moieties during *Nα*-Fmoc deprotection is not problematic, unlike the Boc based methodology. The most commonly exploited side-chain protecting groups in Fmoc based SPPS are illustrated below (Table 4).

Table 4. Commonly employed side chain protection in Fmoc SPPS.

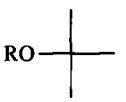
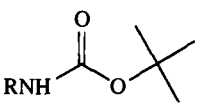
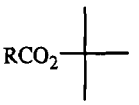
Amino Acid	Side chain protection	Abbrevi-ation	Deprotⁿ condition	Ref.
Ser Thr Tyr	***t*-Butyl ethers** 	^{t}Bu	TFA	-
Lys	***t*-Butoxycarbonyl** 	Boc	TFA	44
Asp Glu	***t*-Butyl esters** 	O-^{t}Bu	TFA	-

Table 4. Continued.

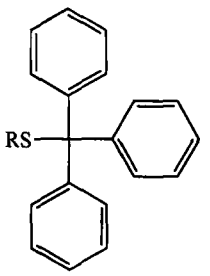
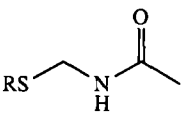
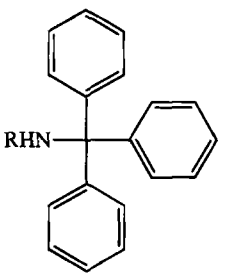
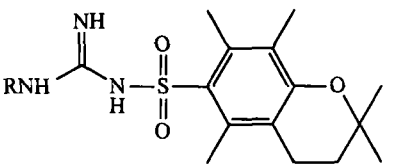
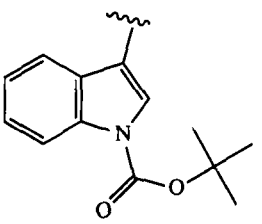
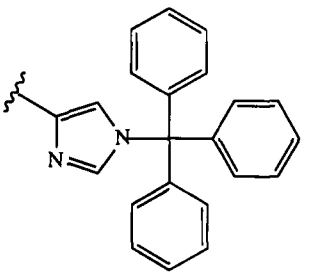
Amino Acid	Side chain protection	Abbrevi-ation	Deprot^{n} condition	Ref.
Cys	*S*-Trityl 	Trt	TFA	98
	S-Acetamidomethyl 	Acm	Hg^{2+} or I_2	86
Asn Gln	*N*-Trityl 	Trt	TFA	87
Arg	*Nω*-2,2,5,7,8-Pentamethyl-chroman-6-sulphonyl 	Pmc	TFA	99
Trp	*Ni*-*t*-Butoxycarbonyl 	Boc	TFA‡	100

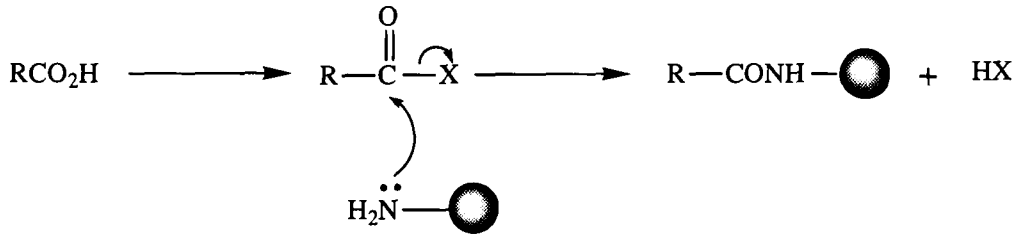
Table 4. Continued.

Amino Acid	Side chain protection	Abbrevi-ation	Deprot^{n} condition	Ref.
His	*N* $^{\tau}$-im-Trityl 	Trt	TFA	101

‡ deprotection with TFA yields a carbamic acid intermediate which protects against alkylation during peptide cleavage/deprotection. Decarboxylation ensues during aqueous work up.

1.9 Synthetic methods of peptide bond formation

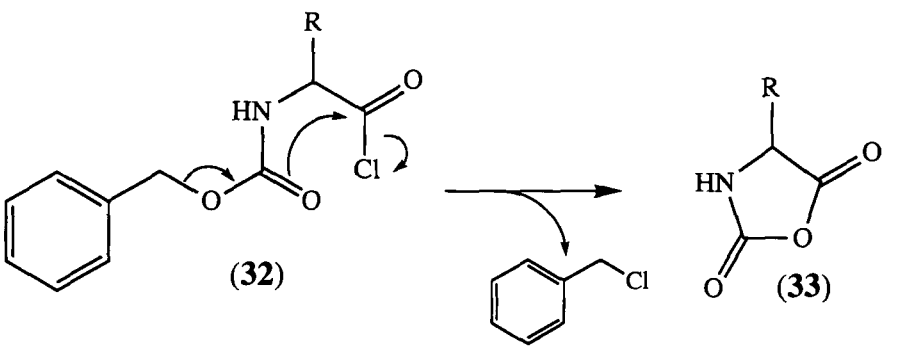
Generally, solid phase peptide bond formation involves the aminolysis of the reactive carboxy component (-COX) of one amino acid with the free amine of another resin bound amino acid (Scheme 14).



Scheme 14. Simplified representation of peptide bond formation.

Conversion of the carboxylic acid into an acylating agent requires replacement of the hydroxyl group by an electron withdrawing substituent (X). Polarisation of the carbonyl group is thus enhanced creating a more electrophilic carbon centre, thereby facilitating the nucleophilic attack of the amine group of the amino acid to be acylated.

The level of activation must be sufficient to promote fast, unambiguous coupling, yet not excessive so as to cause 'overactivation' of the carboxy component. If this occurs, then intramolecular attack by the weakly nucleophilic protected amine is a real risk. An excellent example is the formation of an *N*-carboxyanhydride (NCA) (**33**) when a Z-protected amino acid is activated to the corresponding acid chloride (**32**), Scheme 15.[102]



Scheme 15. *N*-Carboxyanhydride formation from a Z-protected amino acid chloride.

The three general methods for coupling are;

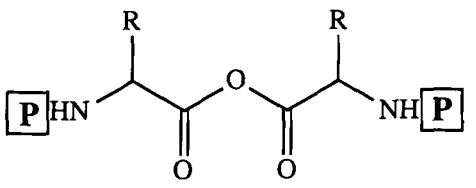
1) Formation of the reactive acylating agent from the carboxylic acid in a separate step or steps, followed by immediate treatment with the amine.

2) Formation of the reactive acylating agent separately, in a form which can be both isolated and even purified before aminolysis.
3) Formation of the reactive acylating agent in the presence of the amine, by introduction of an activating (coupling) agent to a mixture of the two compounds.

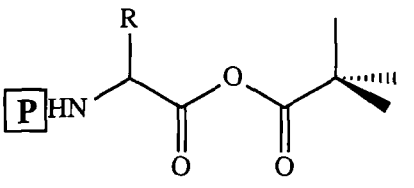
It is important to note that the distinctions between the above are blurred, with the underpinning chemistry being essentially universal.

1.9.1 *Via* anhydrides

Treatment of an amine, or any nucleophile, with an anhydride of a carboxylic acid is one of the most simple and efficient methods of acylation. Acetylation using acetic anhydride was common practise amongst synthetic chemists, yet the use of anhydrides for peptide synthesis played no part in the earliest experiments. This was presumably owing to the requirement of two moles of protected amino acid to form one mole of activated species. Certainly a wasteful process, yet now, with protected amino acids being prepared in a cheap and efficient manner, the use of *symmetrical anhydrides* (**34**) is more frequently applied.[103]



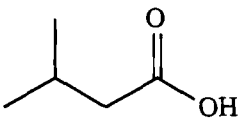
(**34**)



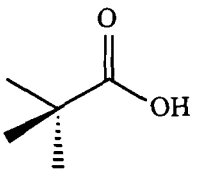
(**35**)

Formation of the peptide bond *via mixed* or *unsymmetrical anhydrides* e.g.

(**35**) gained popularity after their introduction by Chantrenne in 1947.[104] These particular intermediates are designed such that the protected amino acid is practically the sole acylating species, while the second acid of the anhydride is eliminated. The physicochemical basis behind this mode of selective aminolysis requires a decrease in the reactivity of the unwanted partner within the anhydride. Branched alkyl chain acids, such as isovaleric acid[105] (**36**) and trimethylacetic (pivalic) acid[106] (**37**) fulfilled these requirements.

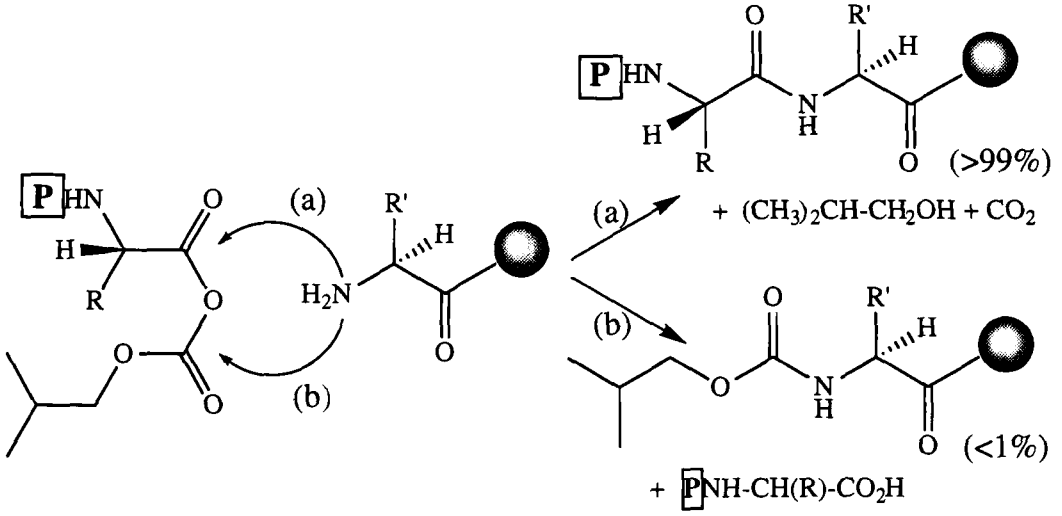


(**36**)



(**37**)

A further extension of this mode of activation was attained by the use of alkyl chloroformates to form the reactive intermediates, e.g. isobutylcarbonic acid mixed anhydrides,[107] (Scheme 16).



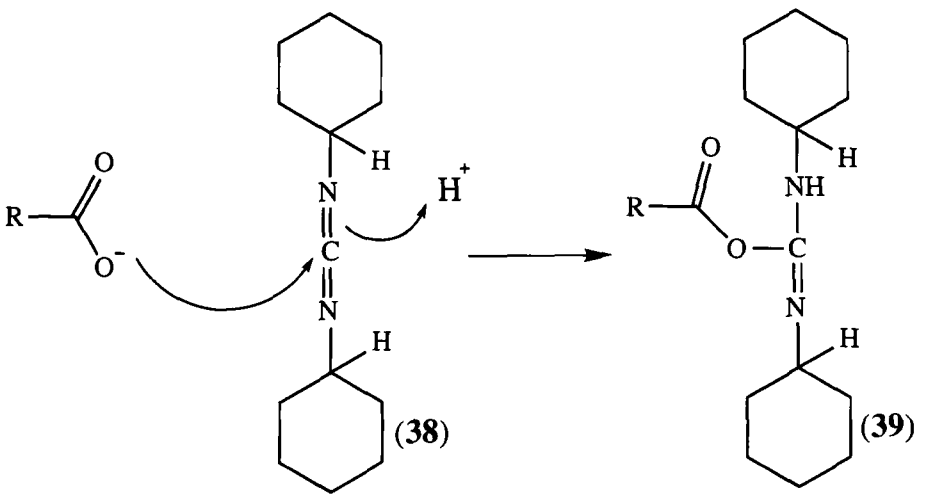
Scheme 16. Dipeptide formation via isobutylcarbonic acid mixed anhydride activation.

In this example, undesired attack of the amine at the carbonic acid carbonyl, route (b), is suppressed since its electrophilicity, and thus its reactivity, is substantially reduced by the unshared pairs of electrons on the neighbouring oxygen atom.

Mixed anhydrides formed from an *N*-protected amino acid and diphenylphosphinyl chloride[108] are extremely reactive and more regioselective than the mixed carboxylic and carbonic anhydrides. Aminolysis occurs exclusively at the amino acid carbonyl, even when steric factors might normally dictate otherwise.

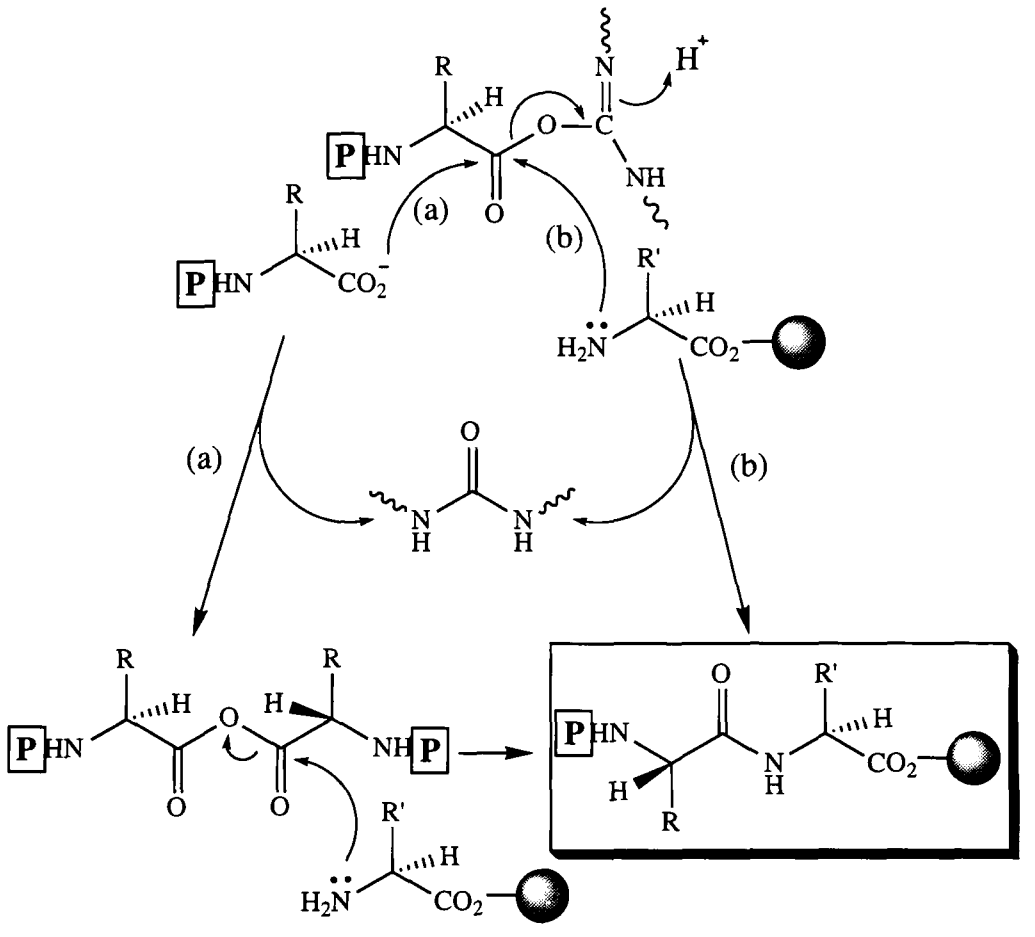
1.9.2 *Via* carbodiimide reagents

Since its inception in 1955,[109] dicyclohexylcarbodiimide, DCC, (**38**) has achieved global recognition as one of the most important agents for carboxy activation in peptide synthesis. The primary mode of activation involves addition of the carboxylate anion to the carbodiimide functionality to generate an *O*-acylisourea (**39**), which is a powerful acylating species (Scheme 17).



Scheme 17. Carbodiimide mediated activation of a carboxylic acid.

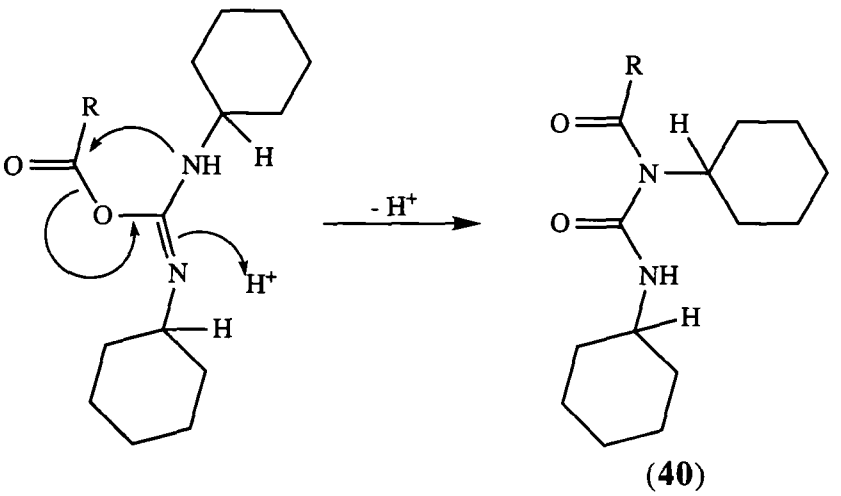
Peptide coupling simply requires mixing of an *N*-protected amino acid, resin bound amino acid and DCC in an anhydrous organic solvent at or a little below room temperature. *O*-Acylisourea generation proceeds rapidly, with peptide formation occurring *via* direct aminolysis of the above species, or *via* the symmetrical anhydride (Scheme 18).



Scheme 18. Carbodiimide mediated dipeptide formation *via* the (a) symmetrical anhydride or (b) direct aminolysis of the *O*-acylisourea intermediate.

The main drawback to the use of DCC is the formation of the dicyclohexylurea by-product, which is only sparingly soluble in most common solvents and therefore thorough washing of the resin is essential. Alternative carbodiimides which form DCM soluble ureas are now more commonly employed, e.g. diisopropylcarbodiimide (DIPCDI)[110,111]

A perturbing side reaction associated with carbodiimides is the intramolecular rearrangement of the *O*-acylisourea to the *N*-acylurea (**40**) (Scheme 19).



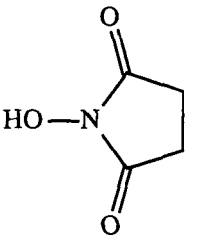
(**40**)

Scheme 19. Intramolecular *N*-acylurea formation.

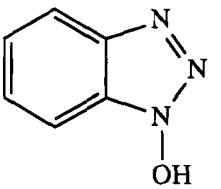
This side reaction can significantly lower the yield of the coupling unless large excess of the acylating species and activating reagent are employed, a distinct advantage of solid phase methodology. The final problem associated with carbodiimide couplings is substantial racemisation of the activated amino acid (see Section 1.10. p.51).

These side reactions can be greatly reduced by the introduction of an auxiliary nucleophile, such as *N*-hydroxysuccinimide[112] (**41**) or 1-hydroxybenzotriazole[113] (**42**) (HOBt). These reagents will rapidly react with

the *O*-acylisourea before the above side reactions occur.

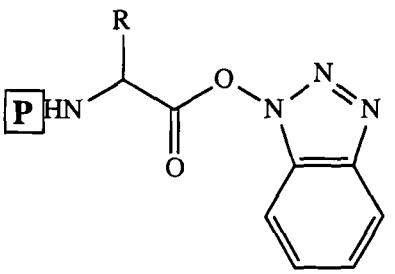


(41)



(42)

Ideally an acylating agent, such as (**43**), of decreased reactivity is formed, which is still adequately electrophilic in nature with respect to subsequent aminolysis, yet does not racemise or participate in alternative side reactions.

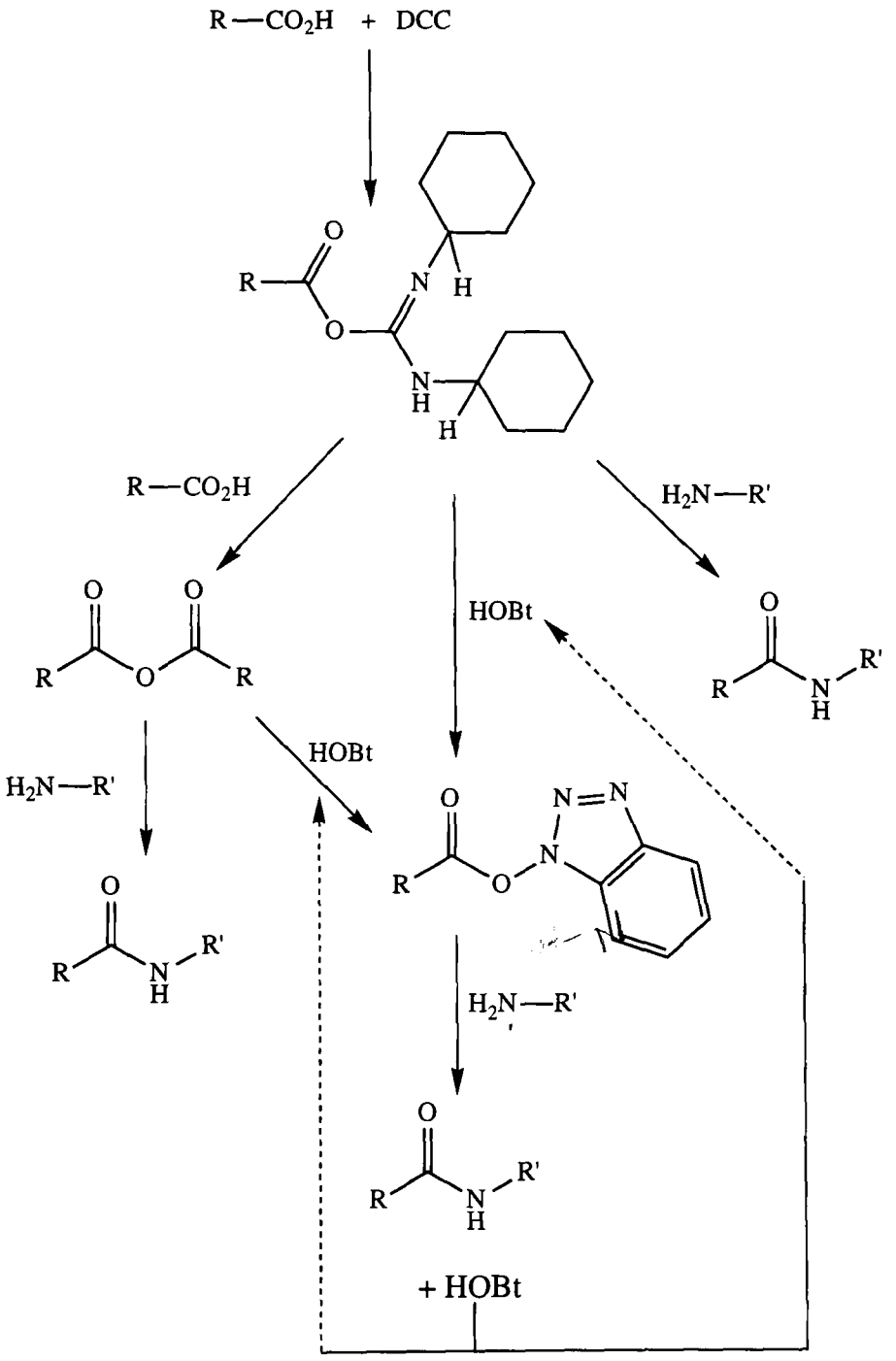


(43)

The ability of additives such as HOBt to provide alternative pathways to the required product, whilst continually being regenerated and providing the kinetic drive for completion of the coupling with minimum deleterious side reactions, makes this a powerful reagent for the peptide chemist (Scheme 20).

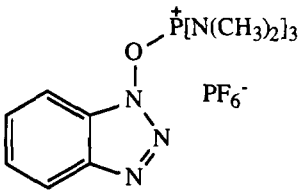
1.9.3 *Via* phosphonium and uronium reagents

The success of HOBt as an auxiliary nucleophile initiated the development of coupling reagents for the *in situ* generation of benzotriazolyl esters.

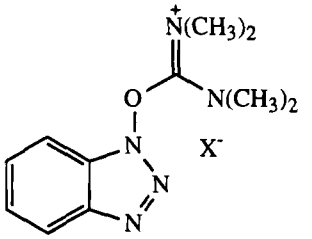


Scheme 20. The role of the auxiliary nucleophile HOBt in the DCC mediated formation of a peptide bond.

Benzotriazol-1-yloxy-tris-dimethylaminophosphonium hexafluorophosphate (BOP) (**44**),[114] and more recently *O*-(benzotriazol-1-yl-1,1,3,3-tetramethyluronium salts, HBTU (**45**) and TBTU (**46**)[115] displayed this mechanistic requirement.



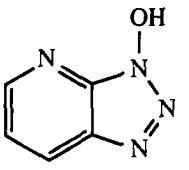
(**44**)



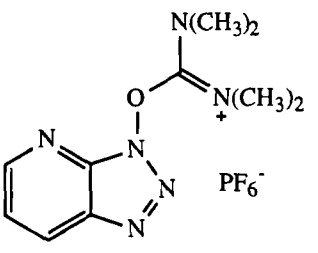
(**45**) X=PF_6
(**46**) X=BF_4

A typical coupling requires addition of an equivalent of the reagent to a 1:1 mixture of the two protected amino acids, in a suitable solvent, in the presence of a tertiary amine. The coupling proceeds *via* the intermediary benzotriazolyl ester (Scheme 21). This method of coupling affords excellent yields with few side reactions and low, but not always acceptable, levels of racemisation.

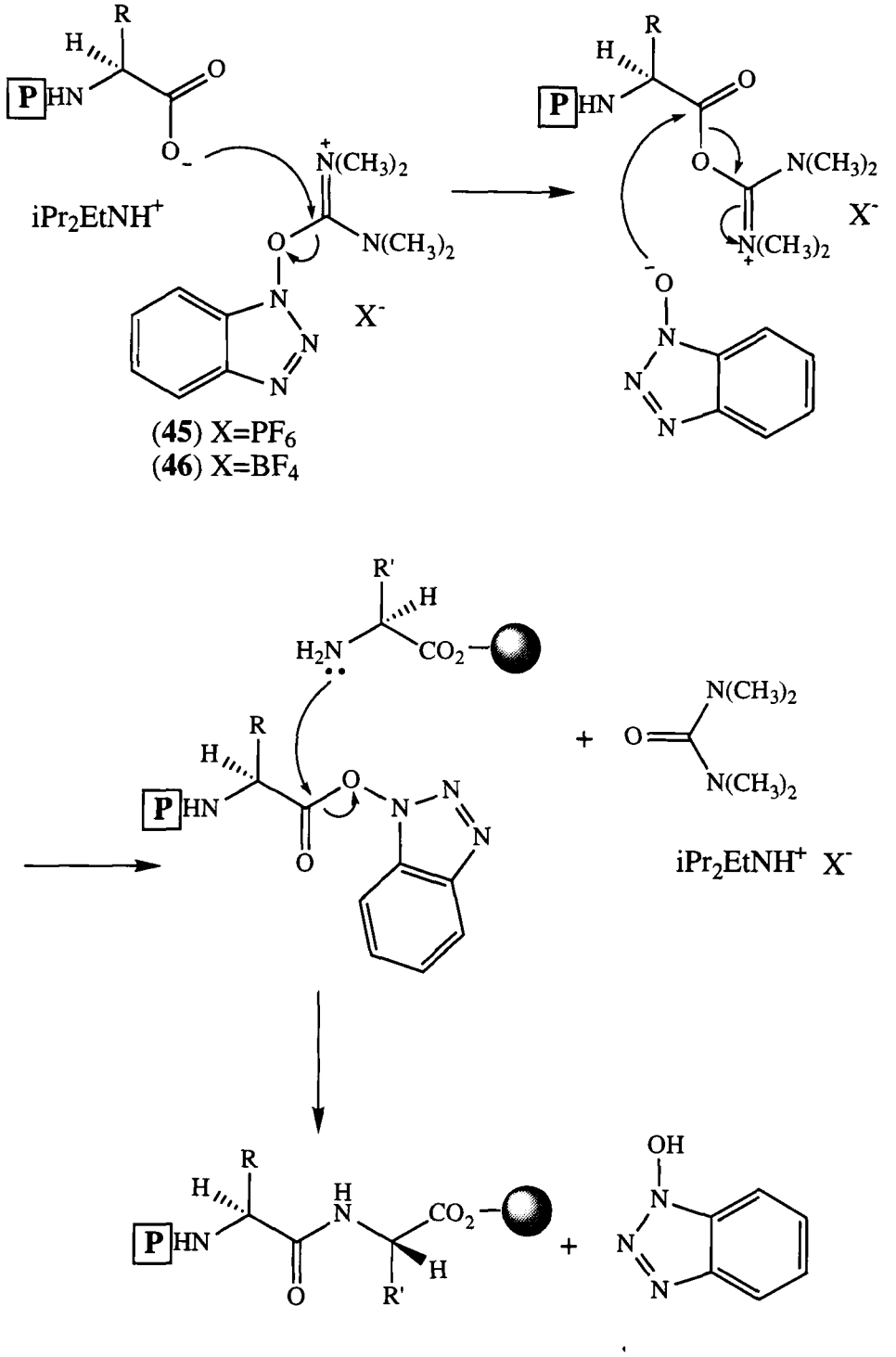
Recently, the pyridyl analogue of HOBt, namely 7-azabenzotriazole (**47**)[116] (HOAt) and its uronium salt HATU (**48**) have revealed themselves as more efficient reagents; accelerating coupling processes, reducing the loss of chiral integrity even further and providing visual evidence (yellow to colourless) of the reaction end-point.



(**47**)

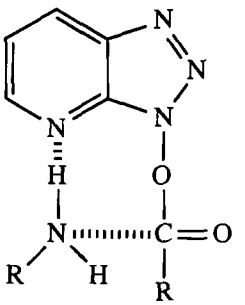


(**48**)



Scheme 21. HBTU/TBTU mediated mechanism of dipeptide formation.

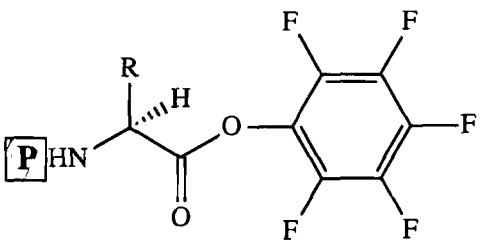
Reaction rates of HOAt esters appear to be enhanced with respect to the HOBt esters and although precise mechanistic details have not been established, it has been postulated that the neighbouring group effect, as shown in (**49**) plays a prominent role.



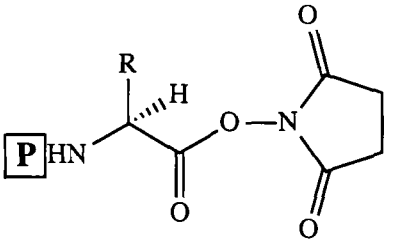
(**49**)

1.9.4 *Via* active esters

Unlike anhydrides, esters possess only one electrophilic centre available for nucleophilic attack, thus reducing the ambiguity of aminolysis. It was realized in the early 1950's that the process of aminolysis of a protected amino acid ester would be expedited if the ester had a good leaving group; e.g if they were esters of phenols or similar acidic moieties. Pentafluorophenyl (**50**)[117] or succinimidyl (**51**)[118] esters are two examples of stable crystalline compounds, which meet this requirement and are convenient to use and store.



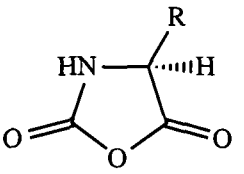
(**50**)



(**51**)

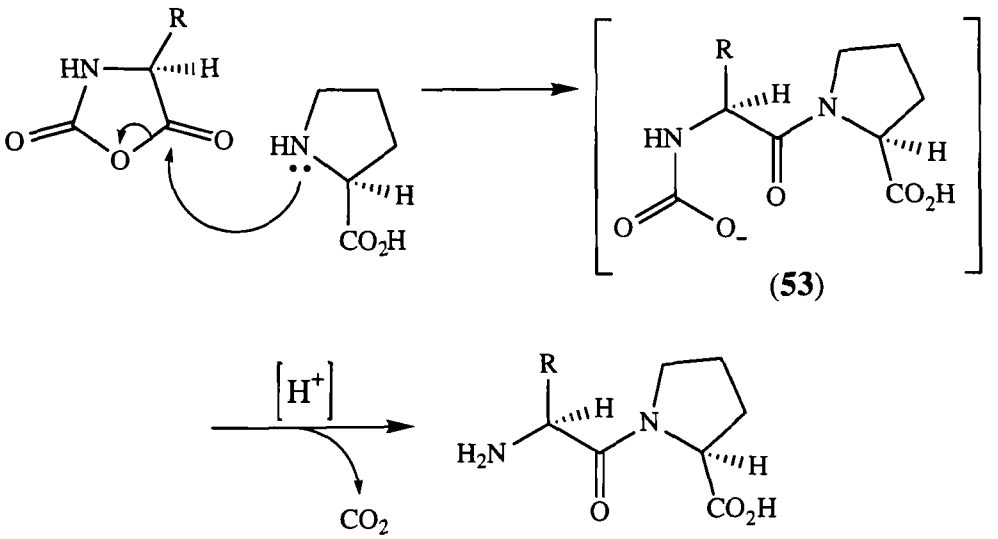
1.9.5 *Via N*-carboxyanhydrides

N-Carboxy or Leuchs' anhydrides of α-amino acids (NCAs) (**52**),[119] are readily obtained by treatment of the amino acid in question with phosgene, or as mentioned in section 1.9, decomposition of alkoxycarbonyl-α-amino acid chlorides (Scheme 15).



(**52**)

Provided conditions are carefully controlled, NCAs can be employed for peptide synthesis in aqueous media (Scheme 22). Nucleophiles attack predominately at the carbonyl of the amino acid, with acylation being rapidly followed by decarboxylation of the carbamic acid intermediate (**53**).

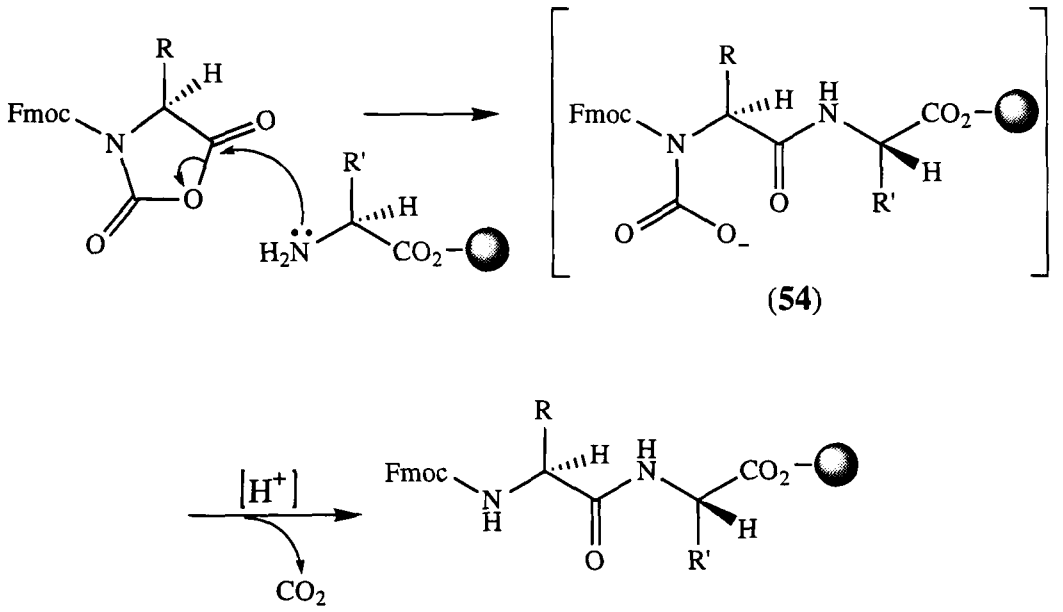


(**53**)

Scheme 22. An example dipeptide synthesis using an NCA.

The ease and simplicity of this regime attracted the interest of many investigators at the time.[120-124] However, premature decarboxylation of the unstable carbamic acid intermediate (**53**), before aminolysis is complete, can lead to double incorporation of the same amino acid residue since the free amine generated can attack any unreacted NCA still present.[122]

A recent development has attempted to circumvent this problem by utilising urethane protected NCAs.[125-127] Fmoc and Boc protected NCA derivatives are normally crystalline compounds exhibiting good stability even in DMF solution. Aminolysis and subsequent decarboxylation of the carbamic acid intermediate (**54**) results, in this case, in the formation of a urethane protected peptide which cannot participate in any further reactions (Scheme 23).



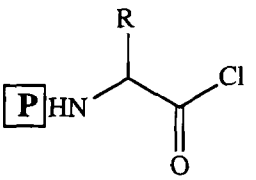
Scheme 23. Peptide coupling using an Fmoc protected NCA.

1.9.6 *Via* acyl halides

Both *N*-protected amino acid chlorides and fluorides have received considerably attention.

1.9.6.1 Amino acid chlorides

The chloride ion seemed an ideal choice for the role of the electron-withdrawing substituent. The powerful activation displayed in acid chlorides was well established when Fischer both prepared and utilised amino acid chlorides (**55**) in coupling reactions.[128]



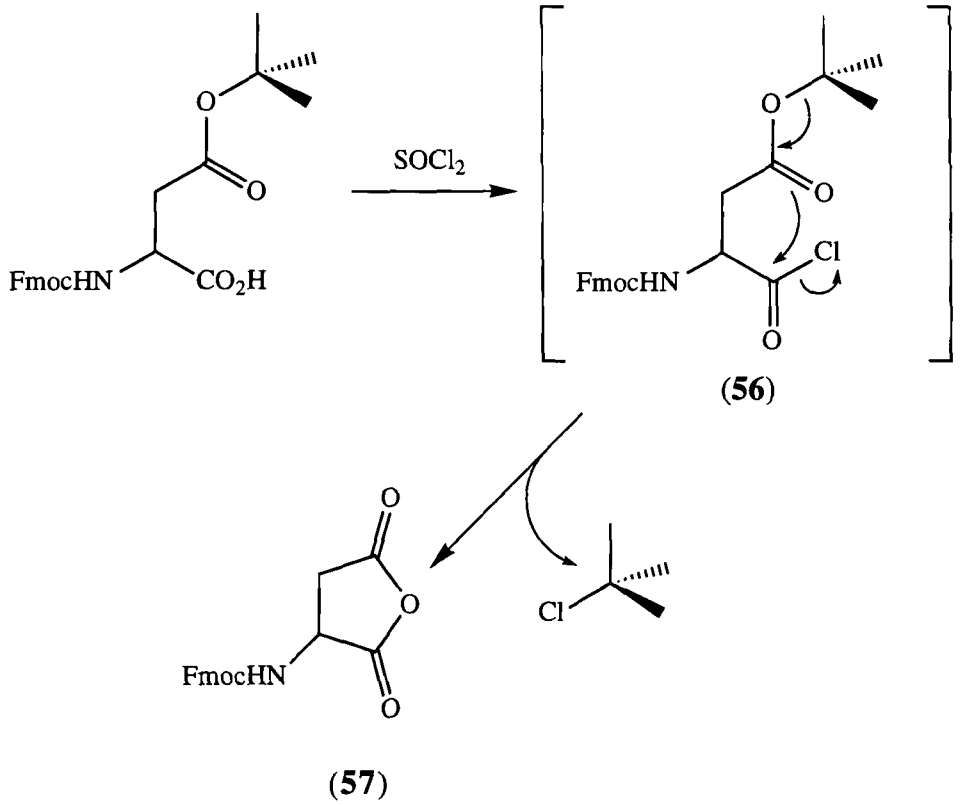
(55)

The reagents required for acid chloride synthesis (thionyl chloride, phosphorous pentachloride, etc.) were however, too vigorous, and were capable of affecting side chain functionalities, e.g. they can convert the carboxamide group in asparagine to a nitrile. Coupled with the problem of NCA formation from Z-protected acid chlorides,[102] and significant racemisation *via* oxazolone formation from simple *N*-acyl amino acid chlorides (Scheme 15), it was hardly surprising that this method of activation lost its popularity. This position has been dramatically altered by the recent discovery that *N*α-Fmoc protected amino acid chlorides are simple to prepare, and are stable to the acid chloride preparation with thionyl chloride.[129] Fmoc amino acid chlorides are

stable crystalline compounds offering rapid acylation rates (in the presence of pivalic acid/TEA or HOBt/DIEA) with low levels of racemisation reported.[130-133]

1.9.6.2 Amino acid fluorides

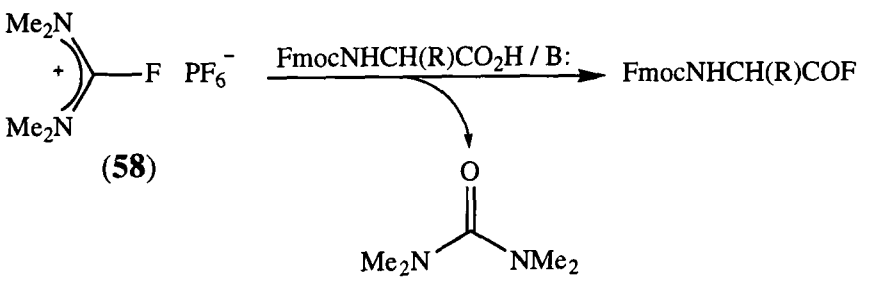
The potential offered by amino acid chlorides unfortunately did not extend to any residues bearing side-chain protection containing the *tert*-butyl moiety. For example, Fmoc-Asp(O^{t}Bu)-OH reacts with thionyl chloride to form the cyclic anhydride (**57**) presumably *via* the acid chloride intermediate (**56**) (Scheme 24).



Scheme 24. Side-reaction during acid chloride generation from Fmoc-Asp(O^{t}Bu)-OH.

However, the well documented stability of *tert*-butyl fluoroformate compared to the corresponding chloro analogue,[134,135] inspired Carpino *et al* to investigate Fmoc amino acid fluorides as an alternative.[136] Using the commercially available cyanuric fluoride,[137] Fmoc-Asp(O^{t}Bu)-OH gave the corresponding acid fluoride as a stable, crystalline solid. This was also the case for all Fmoc amino acids bearing tert-butyl side-chain protection containing the *tert*-butyl moiety.

This means of carboxy activation has subsequently been demonstrated to afford efficient, fast-acting coupling reagents for both solution and solid phase peptide synthesis, being particularly valuable in the case of hindered amino acids, or those containing α, α-dialkyl substituents.[138-141] A recent publication highlighted the methodology available to generate Fmoc amino acid fluorides *in situ* using tetramethylfluoroformamidinium hexafluorophosphate (TFFH) (**58**) (Scheme 25).[142] This overcame the problem that suitably side-chain protected His and Arg residues could not be converted to shelf-stable amino acid fluorides.



Scheme 25. TFFH mediated *in situ* generation of an Fmoc amino acid fluoride.

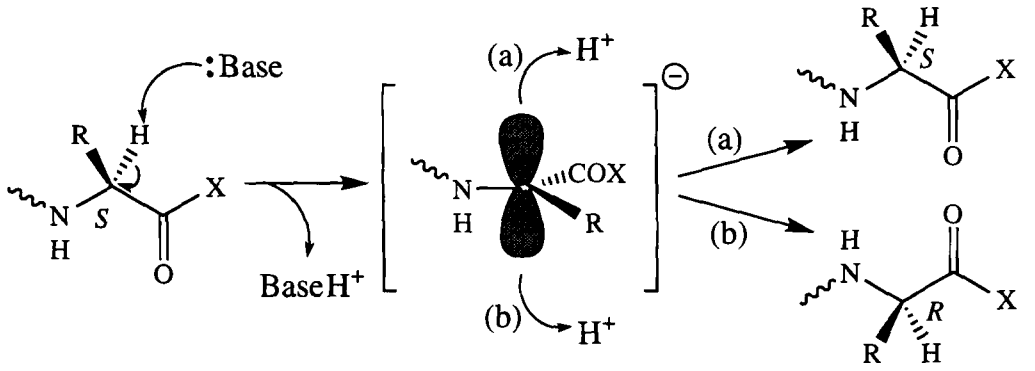
1.10 Racemisation

Within the field of peptide chemistry the term racemisation covers both the strict definition (conversion of a single enantiomer to a mixture of enantiomers) and epimerisation, i.e. loss of chiral integrity at one of two or more chiral centres within a molecule. With the exception of glycine, all the α-amino acids that are constituents of proteins are optically active at the α-carbon atom. To obtain an enantiomerically pure target peptide *via* synthetic methodology, chiral integrity must remain intact throughout the various operations during the synthesis. If this criterion is not fulfilled, a complex mixture will be produced and subsequent separation and purification of the target peptide from a mass of similar compounds becomes an onerous task.

Racemisation is predominately a base-induced process and is generally only a concern at the activation and coupling stages of synthesis. The two chief mechanisms are direct enolisation and oxazolone formation.

1.10.1 Direct enolisation

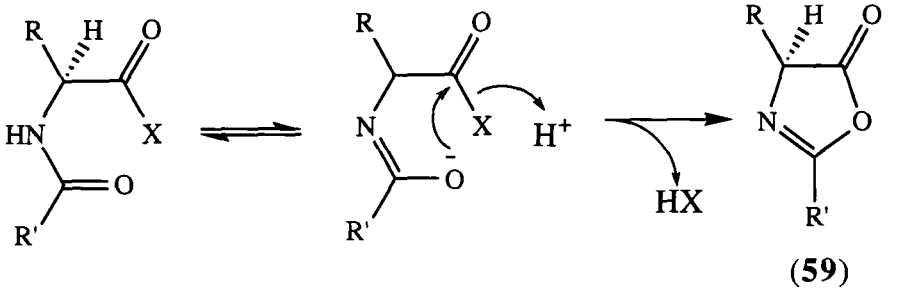
Carboxyl activated *N*-protected α-amino acids suffer from extension of the electron-withdrawing effect of the activating group (X) through to the α-carbon atom, thus facilitating α-proton abstraction in the presence of a suitable base (Scheme 26). This mode of racemisation is a unimolecular process and occurs most readily when (X) is strongly electron withdrawing, and activation/coupling is carried out in a polar aprotic solvent such as DMF. Danger of racemisation is over once coupling is complete, so apart from certain specific amino acids and couplings that are renowned for their slowness, the degree of racemisation produced by this route is very slight.



Scheme 26. Carboxy-activated amino acid racemisation *via* direct enolisation

1.10.2 Oxazolone formation

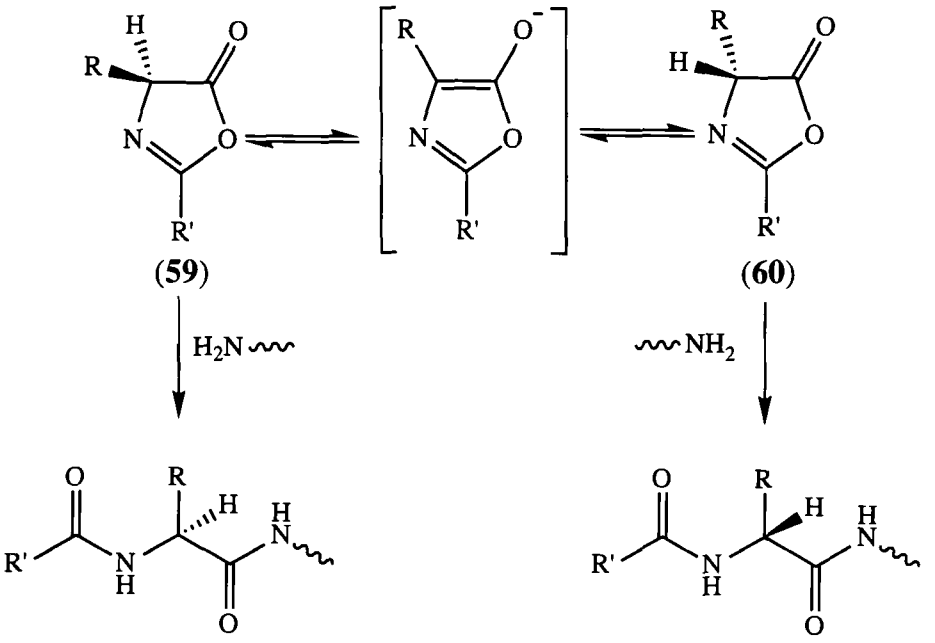
This is the most frequently invoked route of racemisation encountered in peptide synthesis, and is highly detrimental to the chiral integrity of *N*-acyl protected amino acids or the *C*-terminal of a peptide chain upon activation (Scheme 27).



(59)

Scheme 27. 5(4*H*)-Oxazolone formation under basic conditions.

Proton abstraction from the chiral centre affords a resonance stabilised anion that was initially postulated and subsequently proven by ir spectral measurements[143] (Scheme 28).



Scheme 28. Base mediated racemisation of an 5(4*H*)-oxazolone.

As shown, the oxazolones formed, (**59**) and (**60**), are themselves activated towards aminolysis, with reaction leading to peptide bond formation. However, racemisation proceeds at a faster rate when compared to the latter and any peptide produced is substantially racemised.

When the amino protecting group is urethane in nature, oxazolone formation is not such a facile process. Until recently it was assumed that these particular compounds did not produce oxazolones and were therefore resistant to racemisation during activation and coupling. Isolation of optically pure oxazolones from the reaction of Boc-L-valine with water soluble carbodiimides[144] invalidates this hypothesis, suggesting that chiral protection is afforded by the electron release provided by the urethane moiety, destabilising the anion which could form *via* proton abstraction. This evidence indicates that oxazolone formation *per se* is not a sufficient explanation of racemisation, and the stability of the cyclic intermediate towards various bases demands further

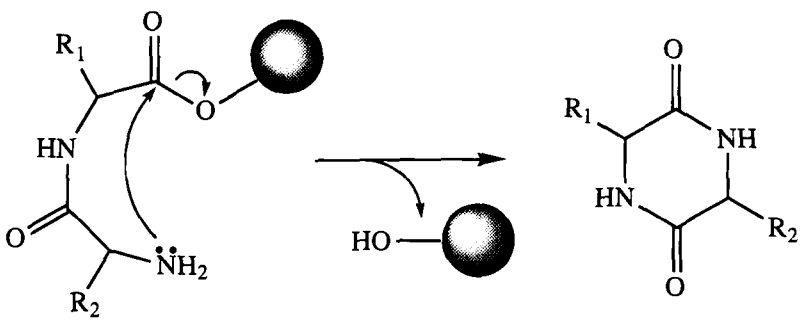
appraisal.

1.11 Common side reactions observed in SPPS

General side reactions, such as those associated with amino acid chlorides, carbodiimides and base catalysed racemisation, have already been discussed. However, there are also several amino acid specific side reactions which have been reported during acylation, deprotection and peptide resin cleavage/deprotection in SPPS. Since the work embodied within this thesis is concerned with the Fmoc/*t*-butyl strategy we will limit the discussion to this particular area.

1.11.1 Diketopiperazine formation

Upon completion of the dipeptide stage of SPPS, deprotection of the *N*α-Fmoc group can result in nucleophilic attack of the free amine at the peptide-resin ester linkage.[145] The overall result is release of the dipeptide from the resin support as its corresponding cyclic diketopiperazine (DKP) (Scheme 29).

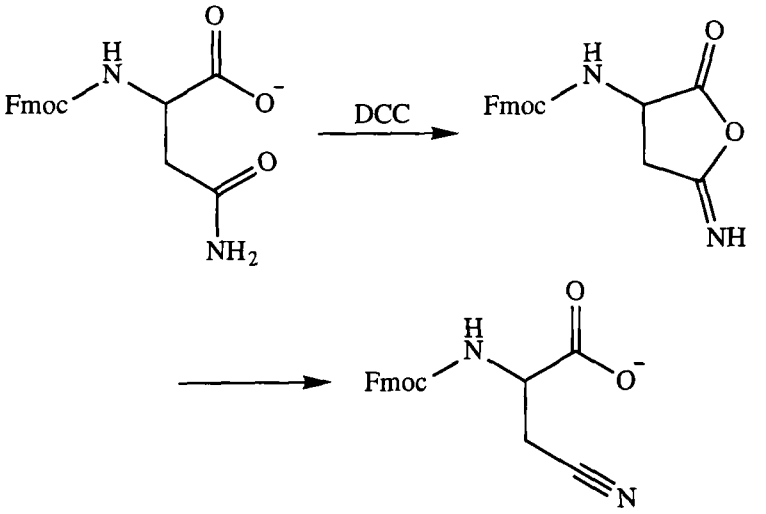


Scheme 29. Intramolecular diketopiperazine (DKP) formation.

This intramolecular aminolysis has been demonstrated to be sequence dependent, with the presence of Pro, Gly, D-amino acids and *N*-methyl amino acids accelerating the overall process.[146-149] The cyclisation is also catalysed by acids[150] and bases[151,152] commonly employed for deprotection in SPPS.

1.11.2 Asparagine and glutamine

The most commonly encountered side reaction when Asn and Gln (without side-chain protection) are coupled to a resin bound peptide is dehydration of the carboxamide to form the corresponding nitrile[153-156] (Scheme 30).

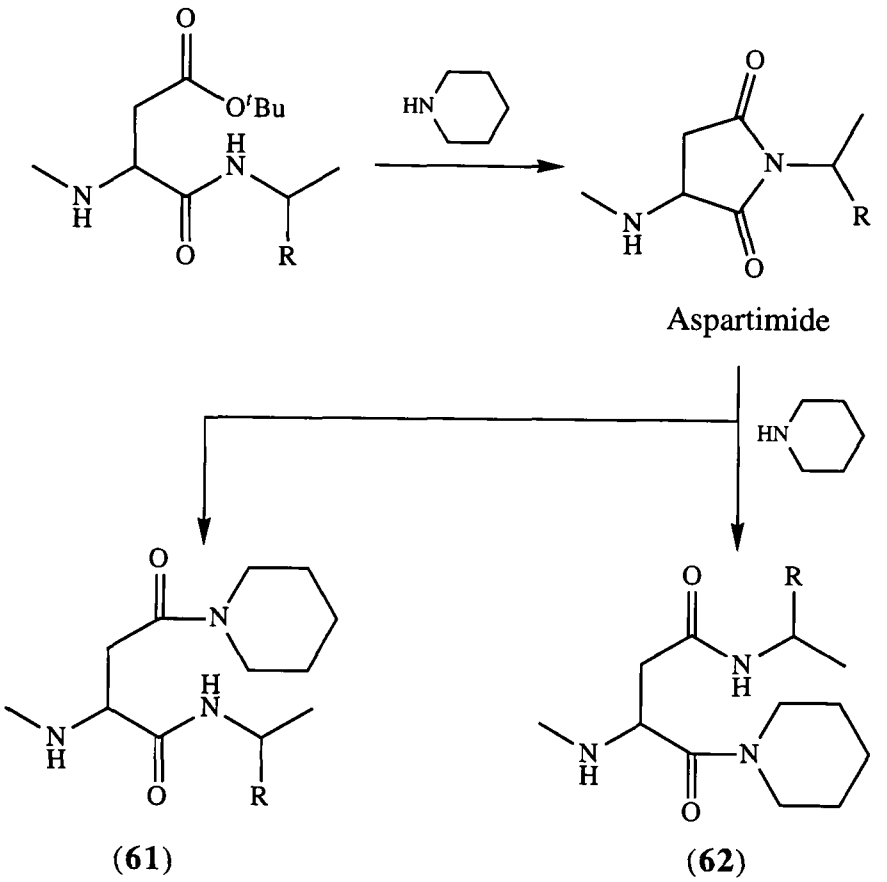


Scheme 30. Intramolecular dehydration of carboxy activated Asn.

Despite acylation with active esters demonstrating minimal dehydration,[10,157-159] side-chain protection is the only reliable method to completely eliminate nitrile formation, with trityl protection[87] now regarded as the methodology of choice.

1.11.3 Aspartic and glutamic acid

The aspartic acid residue is the source of numerous undesired side-reactions during both synthesis and purification. Cyclisation and ensuing β-Asp peptide (**62**) formation is by far the most troublesome in SPPS (Scheme 31).



Scheme 31. Piperidine mediated aspartimide formation.

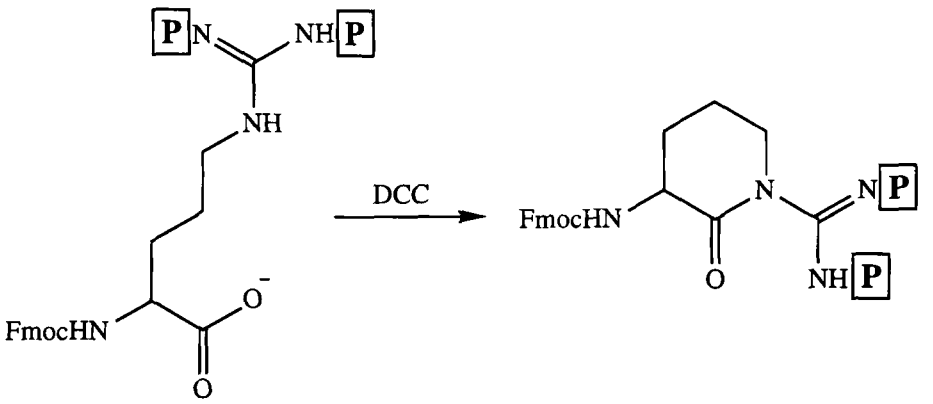
Aspartimide formation is dependent upon the nature of the catalyst, the side chain protecting group and the residue at the Asp carboxyl terminus.[160-164] The stability of the β-*tert*-butyl ester towards piperidine in DMF appeared to endow sufficient protection against cyclic imide formation.[162] However, as shown in Scheme 31, aspartimide and piperidide formation has been observed

in peptides containing the Asp(O^{t}Bu)-Asn(Trt) sequence.[165] The base catalysed abstraction of the amide NH is key to the overall progress of the side-reaction and exploitation of the previously described Hmb amide protecting group has unsurprisingly been shown to completely suppress this mechanism.[166]

The base catalysed cyclisation of glutamate residues to form γ-peptides has not yet been reported in SPPS. However, base catalysed pyroglutamate formation from Fmoc-Glu(OBzl) occurs during piperidine deprotection,[168] although this can be prevented by the use of high concentration/short deprotection times; e.g. 50% piperidine in DMF for 1 min.[167]

1.11.4 Arginine

The most deleterious side reaction of activated Fmoc-Arg is δ-lactam formation (Scheme 32).[168]



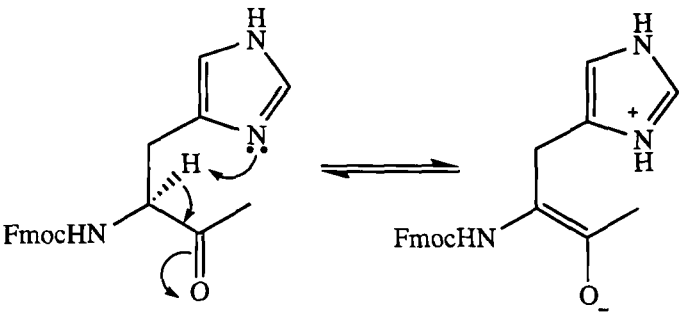
Scheme 32. Intramolecular δ-lactam formation from carboxy activated Arg.

Any Fmoc-Arg derivative without δ-protection can be assumed to undergo a degree of δ-lactam formation.[169] It should be noted that addition of HOBt to the coupling mixture inhibits δ-lactam formation in all mono-protected Fmoc-

Arg derivatives, including Fmoc-Arg(Pmc)-OH[170] and Fmoc-Arg(Mtr)[171-173] which tend to be the reagents of choice for Fmoc SPPS.

1.11.5 Histidine

Racemisation is the most serious problem during the coupling of a histidine residue.[10,174-176] It is believed to proceed *via* intramolecular α-proton abstraction by the imidazole π-nitrogen (Scheme 33).[177]



Scheme 33. Intramolecular His racemisation.

Racemisation can therefore be suppressed by blocking the π-nitrogen,[178] e.g Fmoc-His(Bum)-OH.[179] However in actuality, most His protecting groups block the τ-nitrogen, suppressing racemisation by their electron withdrawing nature, thereby decreasing the overall basicity of the π-nitrogen, e.g. Fmoc-His(Trt)-OH.[101]

1.11.6 Side reactions during peptide-resin cleavage and deprotection

During TFA mediated cleavage of the majority of peptides synthesised using the Fmoc/*t*Bu strategy, the side chain protecting groups are

concomitantly removed. TFA cleavage of ^{t}Bu and Boc groups generates the *t*-butyl carbocation,[180,181] and *t*-butyl trifluoroacetate,[45] and these reactive species can potentially *t*-butylate Tyr, Met and Trp.[182] These side reactions can be minimised by the addition of cationic scavengers to the deprotection cocktail.

1,2-Ethanedithiol (EDT) has been demonstrated to be the most efficient scavenger of *t*-butyl trifluoroacetate but as a stand alone scavenger is not enough to completely prevent Trp alkylation.[183,184] However, the introduction of Fmoc-Trp(Boc)-OH,[100] has dramatically altered the indole alkylation problems associated with this residue.[185] TFA cleavage generates the relatively stable N^{in}-carboxy intermediate, which has been demonstrated to provide adequate protection against both alkylation and sulfonation. Spontaneous decarboxylation then occurs during aqueous work up.

Acid catalysed oxidation of the Met residue to Met sulphoxide during cleavage/deprotection of the peptide resin is another problematic side reaction.[186,187] It has been shown however, that addition of ethyl methyl sulphide (EMS),[188] thioanisole[189] or EDT to the deprotection mixture can prevent such Met sulphoxide formation.

One other important consideration arises form the TFA mediated deprotection of the Cys(Trt), which if not performed in the presence of a suitable scavenger, e.g. EDT or triisopropylsilane,[190] is a reversible process. It is also of worth to note that a very recent publication reports that racemisation free coupling of Fmoc-Cys(Trt)-OH can only be achieved in a medium devoid of base.[191]

With an armoury of protecting groups, coupling reagents and automated continuous flow equipment, the peptide chemist has been able to exploit the

Merrifield and Sheppard philosophies by synthesising a plethora of complex natural and atypical peptides; and in so-doing substantiating the Fmoc based approach as probably the most popular, and generally successful, of all strategies to chemical peptide synthesis. Research continues to be highly active within this still advancing science, and the following chapters illustrate this point with investigations into the;

i) Development of new amino protecting groups.

ii) Development of reversible reagents for the chromatographic or affinity based purification of peptides synthesised *via* SPPS.

iii) Exploitation of a poly-lysine SPPS constructed scaffold for anchoring of immunogenic haptens, in an attempt to raise anti-hapten antibodies.

iv) Potential exploitation of an asymmetric solution based synthesis of biosynthetic precursors to the carbapenem antibiotics, in order to raise a library of side-chain and ring modified β-lactams.

These and alternative improvements of new and existing methodologies will transport solid phase peptide synthesis (SPPS) and solid phase organic chemistry (SPOC) into the next millennium.

2

Development of a Primary Amine Protecting Group Orthogonal to the Fmoc/ ^{t}Boc Strategy for SPPS

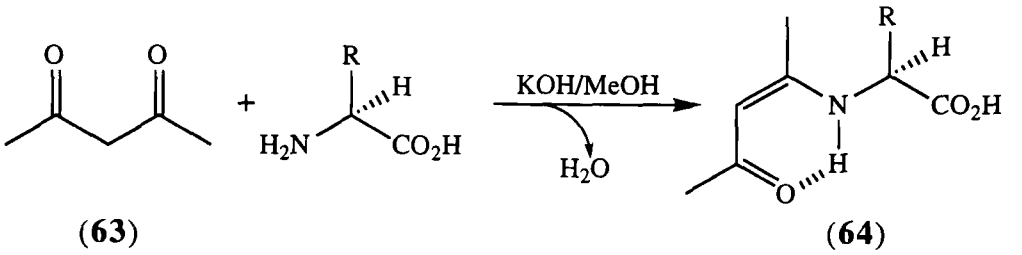
2.1 Introduction

Advances over the last 50 years associated with the chemical synthesis of peptides and proteins, as detailed within the previous chapter, have been enormous. However, the necessary requirement for side-chain modifications of a linear peptide to synthesise for example; cyclic/branched peptides,[55] multiple antigenic peptides (MAPs),[56] spider toxins[57] and regioselectively addressable functionalised templates (RAFTs)[192] via solid phase methodologies, requires a side-chain protecting group that is cheap to synthesise, displays orthogonality towards the Fmoc group and can be easily removed under, ideally, continuous flow conditions. The side-chain primary amines contained in both lysine and ornithine residues have provided an excellent functional group with which to further modify a linear peptide sequence, and work within this Department was initiated to develop a novel amine protecting group displaying the stipulated orthogonality described above.

2.1.1 Vinylogous amides as amine protection

The concept of vinylogous amides as N^{α}-amino acid protection had already received some attention.[51,52] The inability of the carboxy activated intermediate to form an oxazolone precluded racemisation *via* this route (see

section 1.10.2, p.52), and subsequently *N*-(4-oxopent-2-enyl)amino acids (**64**), derived from pentane-2,4-dione (**63**), were successfully activated *via* mixed anhydrides and cyanomethyl esters[193] and utilised in peptide synthesis (Scheme 34).



(**63**) (**64**)

Scheme 34. Vinylogous amide protection of an amino acid.

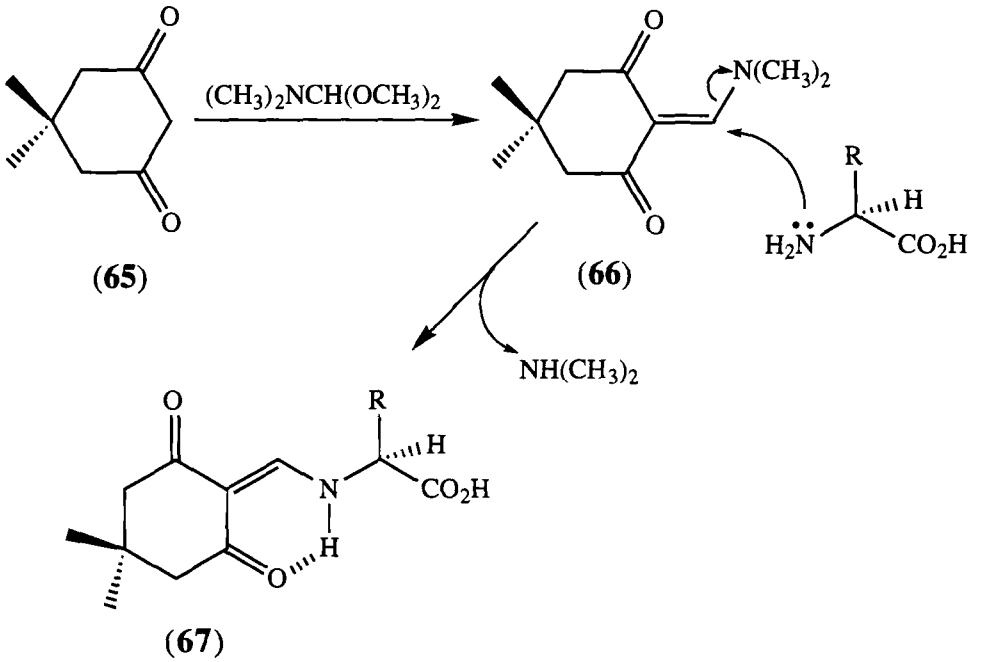
However, the lability displayed by *N*-(4-oxopent-2-enyl)amino acids towards aqueous and mildly acidic conditions limited their use as synthetic reagents.

2.1.2 Development of the *N*-1-(4,4-dimethyl-2,6-dioxocyclohexylidenemethylene) (Dmc) amino protecting group

Within our laboratories the cyclic 1,3-diketone, 5,5-dimethylcyclohexane-1,3-dione (dimedone) (**65**) was employed as the template starting material. The reaction between (**65**) and dimethylformamide dimethylacetal[194] afforded 5,5-dimethyl-2-(dimethylaminomethylene)cyclohexane-1,3-dione (DMMD) (**66**), which was demonstrated further to react with amino acids at room temperature to yield stable, crystalline *N*α-Dmc-derivatives (**67**)[53] (Scheme 35).

Intramolecular hydrogen bonding provides the driving force for the formation and chemical stability of (**64**) and (**67**). This hydrogen-bond formation is not possible with secondary amines and although *N*-Dmc-proline

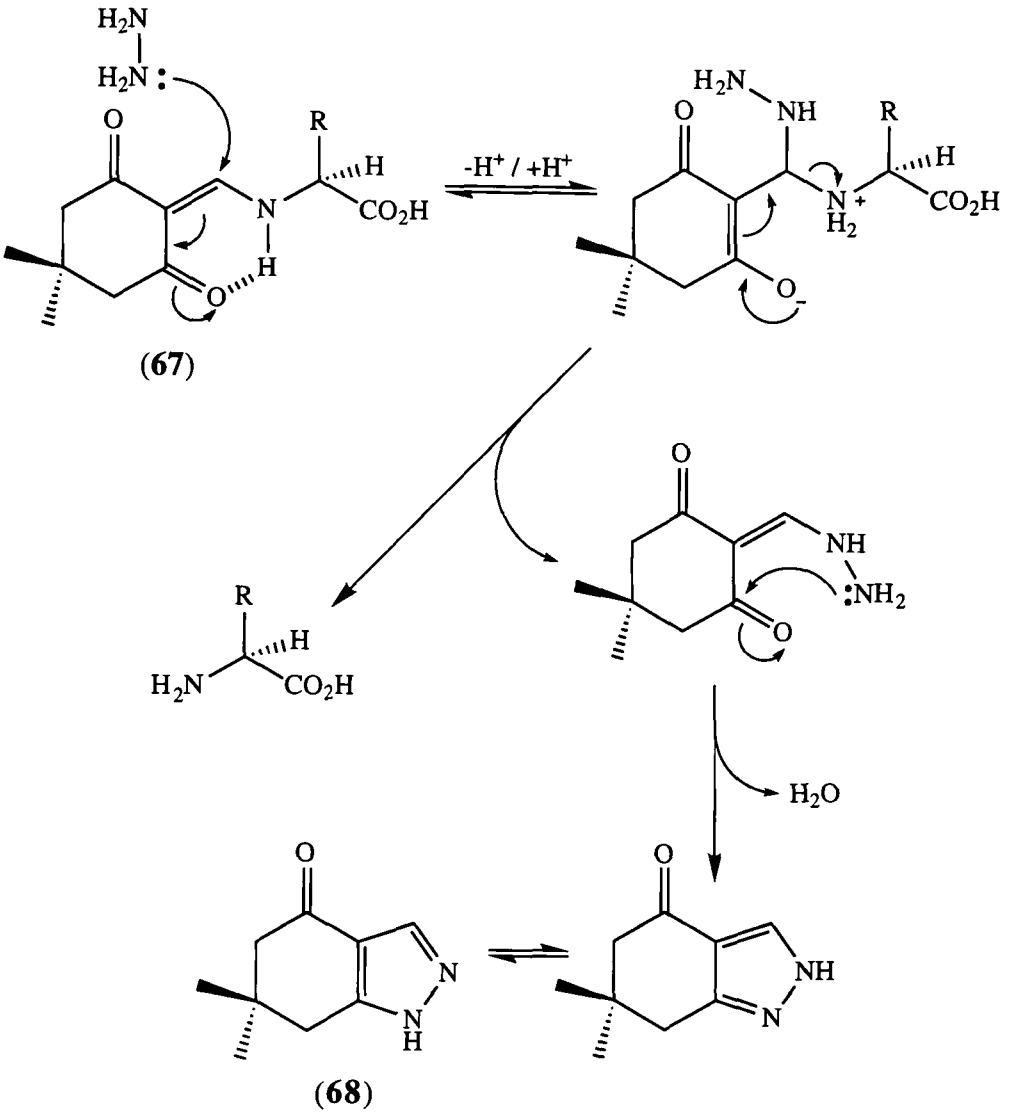
and other *N*-methyl-*N*-Dmc amino acids can be synthesised, they lack adequate stability for use in SPPS.



Scheme 35. Protection of an amino acid as its *N*-(4,4-dimethyl-2,6-dioxocyclohexylidenemethylene) (Dmc) derivative (**67**).

Cyclic 1,3-diketone derivatised amino acids were found to be more robust than their open chain counterparts and showed remarkable stability towards acidic conditions. However, their deprotection could be rapidly effected by a dilute hydrazine solution in a polar solvent at room temperature, with the overall process being driven by the formation of the 1,2-diazole[195,196] (**68**) (Scheme 36).

Carbodiimide mediated coupling of *N*α-Dmc-amino acids under SPPS conditions were found to be racemisation free, as demonstrated by the synthesis of Leu-enkephalin, where deprotection of the *N*α-Dmc group was achieved, following each coupling, using continuous flow 2% hydrazine in DMF.



Scheme 36. Hydrazine mediated deprotection of a *N*-Dmc amino acid.

However, the use of uronium salt reagents for carboxyl activation (see section 1.9.3, p.41), which requires the employment of strong non-nucleophilic bases such as DIEA, led to red coloration of the activation mixture and a subsequent complex mixture of products.[197] This result coupled with the observation that the Dmc group was removed in 20% piperidine in DMF under continuous flow conditions meant that the protecting group was both lacking orthogonality with Fmoc and a realistic application to SPPS.

2.1.3 Development of the *N*-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) amino protecting group

The instability of The Dmc group to piperidine was postulated to be a result of nucleophilic attack at the enamine double bond, with ensuing displacement of the amino acid. It was proposed that introduction of steric hinderance at the enamine position would discourage this attack and thereby render the protecting group more stable to piperidine. This reasoning led to the introduction of a methyl group, resulting in the *N*-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) amino protecting group.[54]

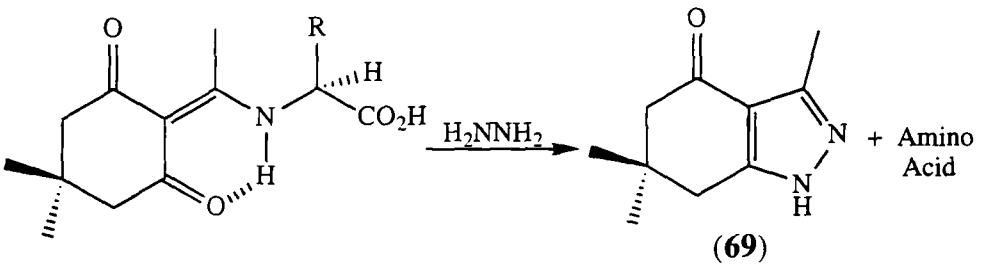
The Dde group has been demonstrated as displaying a quasi-orthogonality to Fmoc (6.5% loss after 4 h treatment with 20% piperidine in DMF under continuous flow conditions), allowing the synthesis of medium sized peptide chains without serious loss of the Dde group. Unlike *N*α-Dmc-amino acids the *N*α-Dde equivalents (except glycine)[198] are compatible with uronium salt coupling reagents. The *N*α-Dde group has received considerable attention and its application within SPPS has already been dealt with in more detail in the previous chapter (see Section 1.5.3, p.21).

2.2 Development of the *N*-1-(4-nitro-1,3-dioxoindan-2-ylidene)ethyl (Nde) amino protecting group

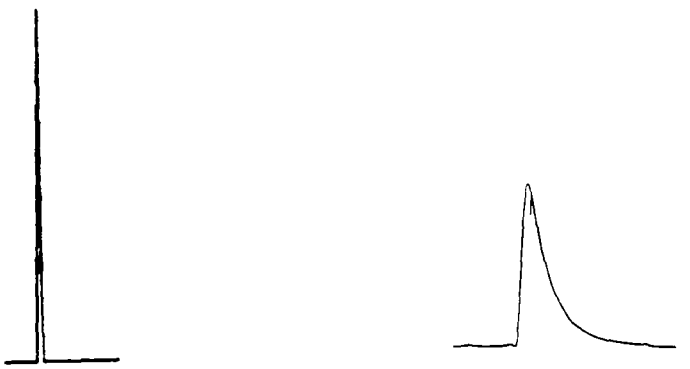
The *N*α-Dde group has provided an excellent template for amine protection displaying quasi-orthogonality to Fmoc. However, hydrazine does cause significant removal of the Fmoc group, dictating that in a *bis*-protected residue (e.g Fmoc-Lys(Dde)-peptide) the Fmoc group must be removed first

and that chain extended before the peptide is exposed to the 2% hydrazine in DMF for Dde deprotection.

Another problem that has been identified with the Dde group is associated with its removal. It has on several occasions displayed a slow deprotection profile, and also in all instances requires monitoring at 300-310nm as the deprotection adduct, 3,6,6-trimethyl-4,5,6,7-tetrahydro-1*H*-indazol-4-one (**69**) has a less powerful chromophore than the Fmoc deprotection adduct (Scheme 37). This requires re-calibration of automated equipment designed to monitor the Fmoc deprotection adduct at 290nm.



(**69**)

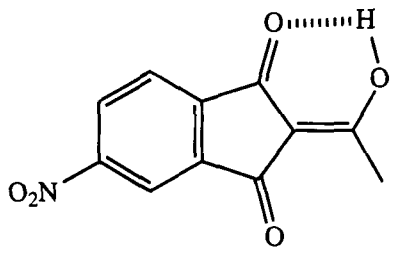


(a) (b)

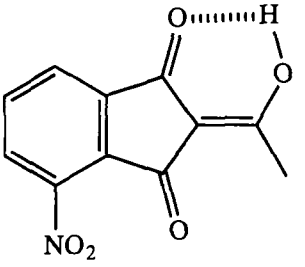
Scheme 37. Comparison between (a) a typical Fmoc deprotection profile monitored at 290nm and (b) a poor Dde deprotection profile monitored at 310nm.

It was envisaged that incorporation of an extended chromophore onto the already well established ring encased diketone nucleus, would generate a structure that could be more efficiently monitored during the deprotection cycle, preferably at the Fmoc wavelength of 290nm. It was anticipated that an extended chromophore with a fused aromatic ring would meet this requirement. Furthermore, if the ring had an electron withdrawing substituent, the increased electrophilicity of the exocyclic enol may generate a more reactive entity. Thus the potential for a reagent capable of coupling to amino acids quicker than 2-acetyldimedone, and subsequently its deprotection easier than *N*α-Dde, could produce an ideal amine protecting group that exhibits total orthogonality to Fmoc; since it may potentially be removed using extremely weak nucleophiles such as thiourea that, unlike hydrazine, would not affect the integrity of Fmoc protection.

These considerations led us to formulate 2-acetyl-5-nitroindane-1,3-dione (**70**) as our target, which possessed both the extended chromophore and electron withdrawing substituent on the fused aromatic ring. Presence of the methyl substituent on the exocyclic enol, should once again deter the attack of piperidine.



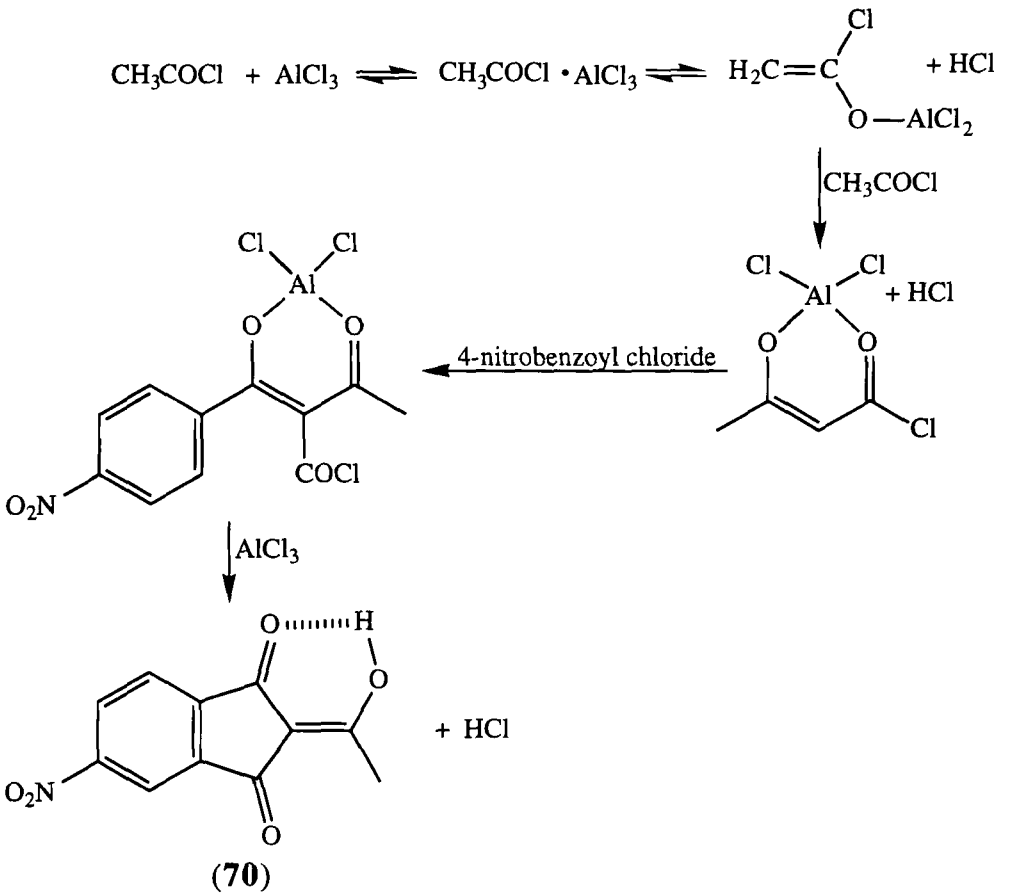
(**70**)



(**71**)

Fortunately, the literature search revealed that the desired compound (**70**) could be synthesised by a regioselective one-pot reaction, involving a

sequential cross-condensation-cycloacylation of 4-nitrobenzoyl chloride with acetyl chloride in the presence of aluminium chloride (Scheme 38).[199]



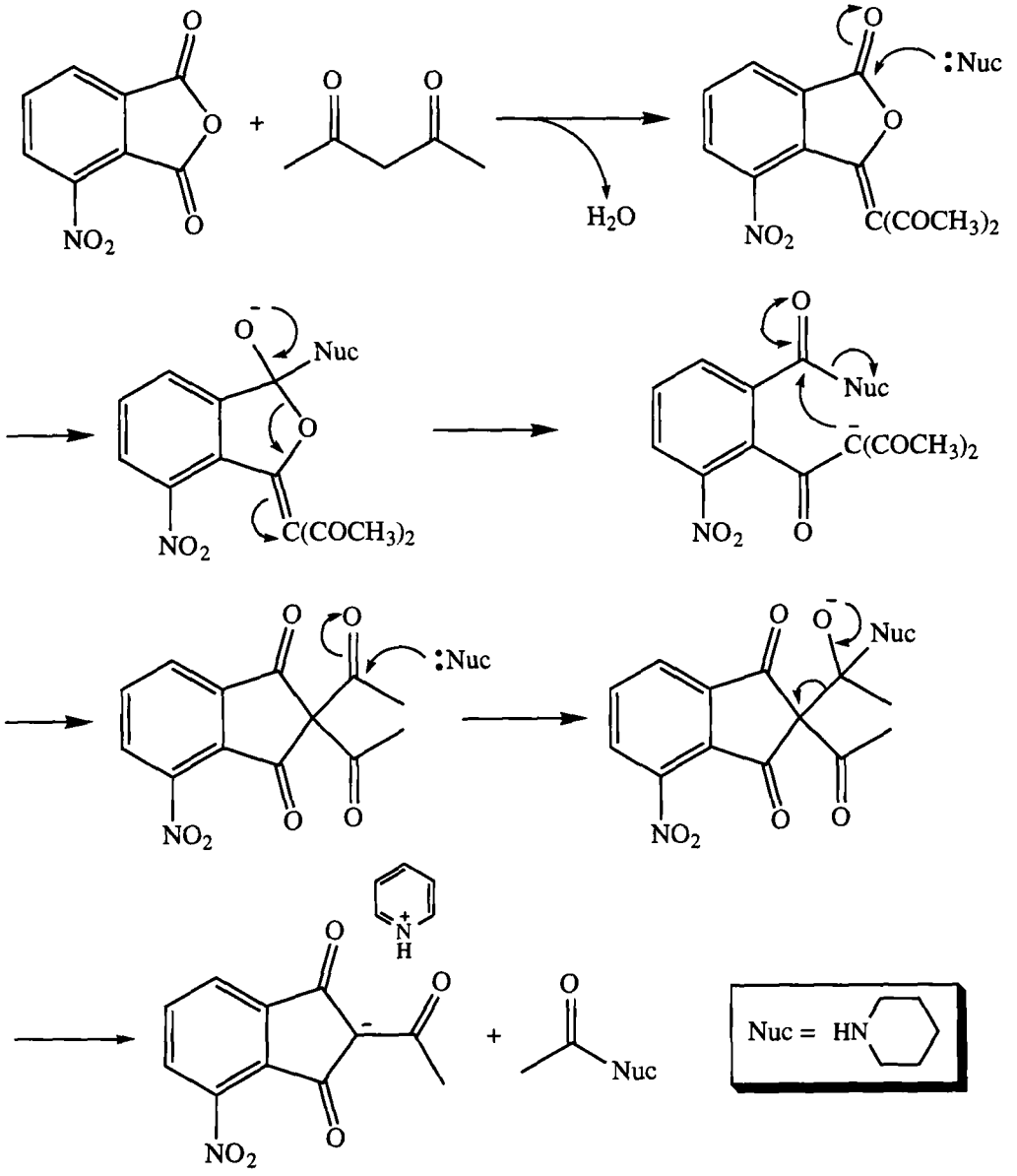
(**70**)

Scheme 38. Synthesis of 2-acetyl-5-nitroindane-1,3-dione (**70**)

Unfortunately, even after several attempts, the originally reported poor 35% yield could not be improved. This led us to return to the literature to locate an efficient and facile synthesis of either (**70**) or (**71**).

A paper published by Mosher and Meier in 1970 described the synthesis of both the 2-acetyl-4-nitroindane-1,3-dione (**71**) and 2-acetyl-5-nitroindane-1,3-dione (**70**) molecules, with the former being attainable in higher yield. The synthesis involved the reaction between a nitrophthalic anhydride and 2,4-pentanedione using pyridine as a solvent.[200] A putative mechanistic

interpretation suggests an initial condensation reaction followed by two nucleophile (piperidine) assisted rearrangements to furnish the target molecule as its pyridinium salt (Scheme 39). In our hands, synthesis of 2-acetyl-4-nitroindane-1,3-dione (**71**) was achieved with a similar overall yield of 63%, affording fine yellow crystals after neutralisation of the pyridinium salt.

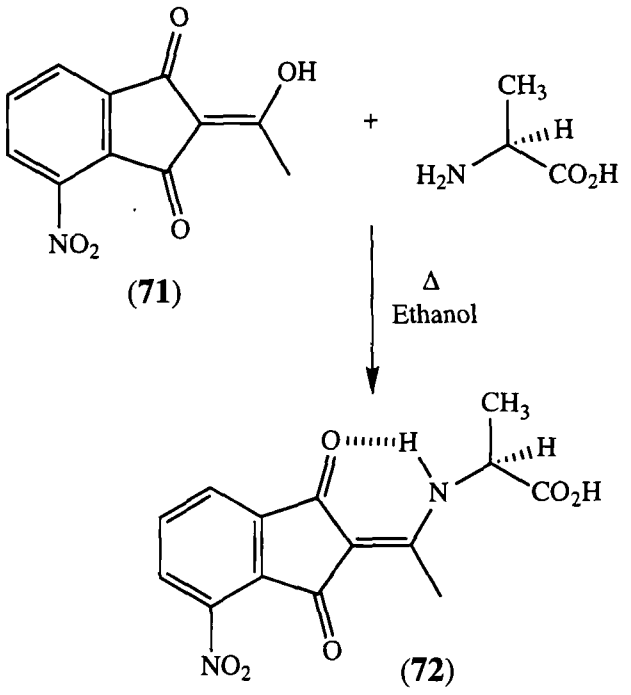


Scheme 39. Synthesis of 2-acetyl-4-nitroindane-1,3-dione.pyridinium salt.

It is noteworthy that the ^1H NMR ($CDCl_3$) spectrum of both (**70**) and (**71**) show the enol proton as an exchangeable singlet at δ11.5, providing evidence for the proposed hydrogen bonded structure.

2.2.1 Synthesis of *N*α-Nde-L-amino acids

In order to generate a cost-effective strategy, the higher yielding 2-acetyl-4-nitroindane-1,3-dione (**71**) was employed as the reagent of choice for *N*α-amino acid protection. Initially, L-alanine and 2-acetyl-4-nitroindane-1,3-dione (2:1) were refluxed together in anhydrous ethanol, with the reaction closely monitored by TLC, indicating a complete loss of (**71**) after 3 h. Following work-up *N*-Nde-L-alanine (**72**) was recovered as a yellow amorphous powder in 65% yield (Scheme 40).



Scheme 40. Synthesis of *N*-Nde-L-alanine.

This reaction time was significantly quicker when compared with the preparation of *N*-Dde-amino acids, which typically required refluxing overnight to obtain acceptable yields. It is also noteworthy that the increased reactivity displayed by 2-acetyl-4-nitroindane-1,3-dione means only a 1.5-2.0 mole equivalent of the amino acid is required in order to drive the reaction to completion. In contrast, synthesis of *N*-Dde-amino acids dictated the use of a 4 molar excess of 2-acetyldimedone in order to obtain comparable yields.

With the condensation reaction conditions optimised, a series of representative amino acids were *N*α-protected with 2-acetyl-4-nitroindane-1,3-dione (**71**) to afford in all cases a yellow amorphous powder of good to excellent yield and purity (Table 5).

Table 5. A summary of *N*α-Nde-amino acids synthesised.

Nde Amino acid	Yield / %	$[\alpha]_D$ (c=1, MeOH)
Phe	69	-135.1$^{0\#}$
Lys†	68	-26.2^0
Val	77	+18.5^0
Tyr†	78	-136.5^0
Ile	70	+10.2$^{0\#}$
Leu	83	-31.5^0
His††	40	- 86.9^0
Gly	81	n/a
Ala	65	+26.4^0

† Side chain protection : ^{t}Boc
†† Side chain protection : Trt
# (c=1, EtOH)

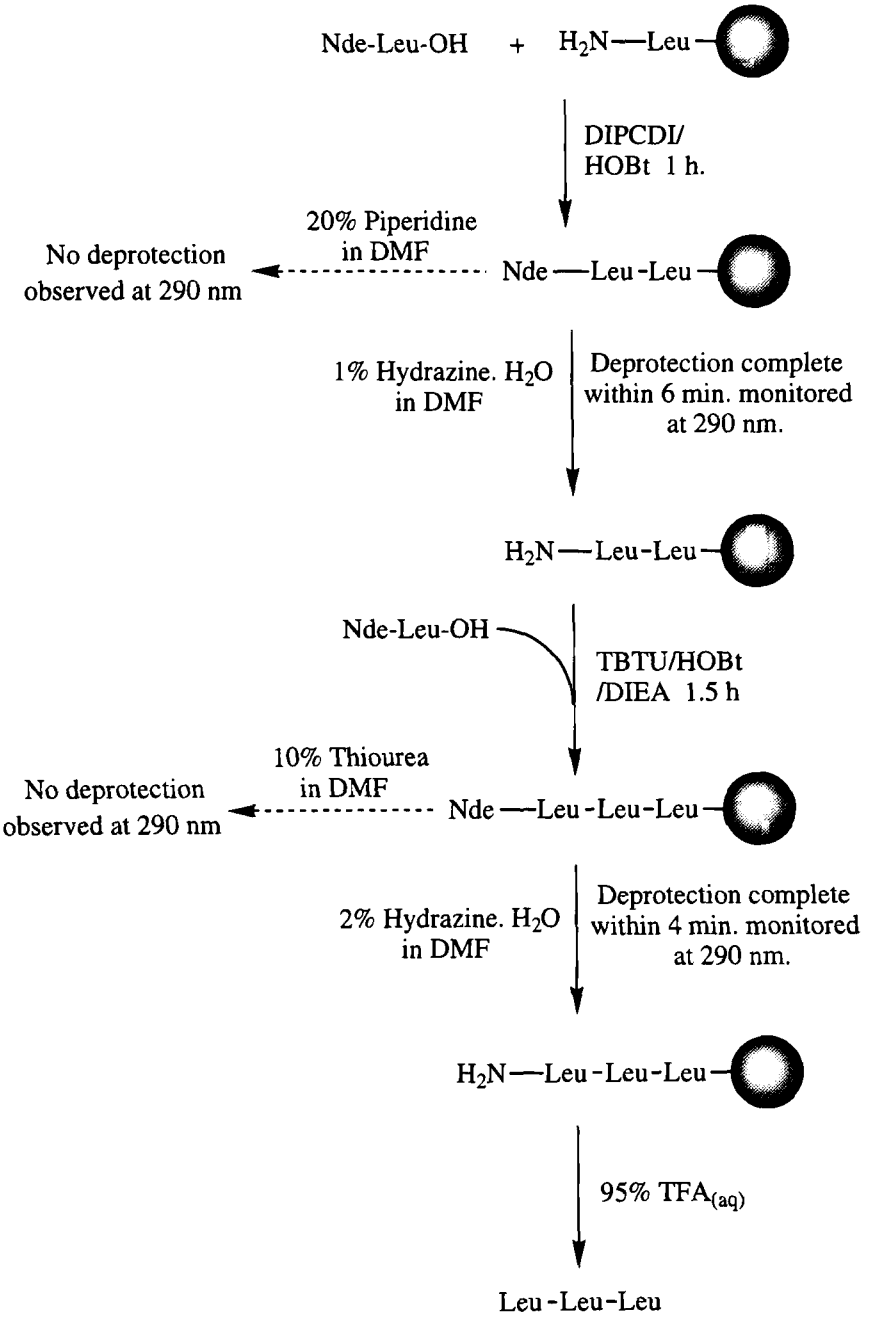
All Nde amino acids were obtained as yellow amorphous powders *via* hexane precipitation from a concentrated ethyl acetate solution. These compounds were extremely difficult to recrystallise. The ^{1}H NMR displayed a small hexane impurity that could not be removed by vacuum desiccation over wax and this small degree of solvent occlusion prevented meaningful elemental analysis. Release of occluded solvent from the amorphous powder during melting point determination caused a glass formation prior to liquification/decomposition. Therefore, to ascertain the purity of individual N^{α}-Nde-amino acids, HRMS and RPHPLC analyses were undertaken for each compound, and found to display single peak traces of the correct mass in all cases.

Interestingly, the ^{1}H NMR of all N^{α}-Nde amino acids displayed split signals (of *ca* ratio 2:1) for the characteristic vinylogous amide NH (δ 11.0-11.2) and vinyl methyl protons (δ 2.30-2.60). This phenomenon was postulated to be a consequence of *cis/trans* isomerism around the vinyl bond, with sample heating resulting in collapse of the signals to the expected doublet and singlet for the NH and CH_3 protons respectively.

2.2.2 Compatibility of N^{α}-Nde-amino acids in SPPS

Indications of improved reactivity in the condensation reaction between amino acids and 2-acetyl-4-nitroindane-1,3-dione (**71**), suggested that the deprotection may also proceed faster than that already established for N^{α}-Dde-amino acids. This hypothesis was investigated by coupling N^{α}-Nde-L-Leu to a leucine derivatised resin *via* carbodiimide (DIPCDI)/HOBt mediated carboxy

activation (Scheme 41).



Scheme 41. Compatibility of Nα-Nde-amino acids in SPPS.

Acylation was monitored by the trinitrobenzenesulphonic acid (TNBS) amine test[201] and was complete after 1 hour. The resin was initially exposed to 20%

piperidine in DMF under continuous flow conditions, with the post column eluent monitoring at 290 nm. No deprotection peak was observed during the 10 min cycle. After washing with DMF, the resin was subjected to a continuous flow of 1% hydrazine monohydrate in DMF. Post column monitoring of the eluent, at the Fmoc deprotection adduct wavelength of 290 nm, displayed a sharp deprotection peak indicating complete removal of the Nde group within approximately 5-6 min (Figure. 1). It is noteworthy that the deprotection is also visual, with the yellow resin (resulting from the Nde protected residue) changing to red and finally restored to the original resin colour upon complete removal of the Nde group.

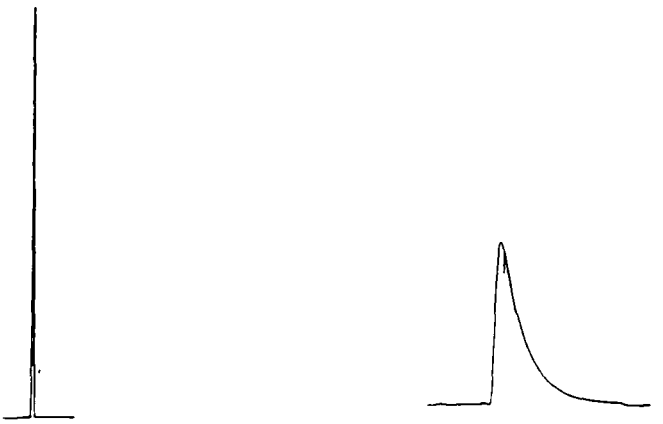


Fig. 1. Comparison between (a) a typical Nde deprotection profile monitored at 290nm and (b) a poor Dde deprotection profile monitored at 310nm.

This experiment indicated that the Nde group displayed stability to piperidine, yet could be efficiently removed with 1% hydrazine, with post column monitoring possible at the same wavelength as that established for the Fmoc group.

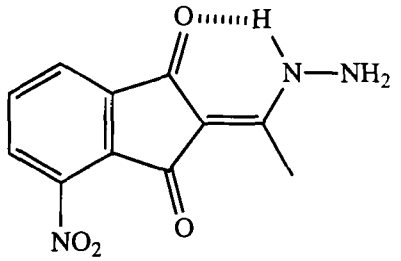
To test the compatibility of N^{α}-Nde-amino acids with uronium salt reagents, the Leu-Leu-derivatised resin was acylated with a second N^{α}-Nde-L-Leu residue via TBTU/HOBt/DIEA activation (Scheme 41). Coloration of the reaction mixture was no different than that observed with the carbodiimide reagent, and acylation was halted after 1.5 h. The TNBS test indicated a small but detectable amount of free amine was still present on the resin, and it was apparent that the Leu-Leu-Leu sequence was presenting coupling problems at the third residue. However, the strong yellow coloration of the resin indicated that the third coupling had proceeded sufficiently well to test the lability of Nde towards thiourea, which if successful, would provide an additional level of orthogonality. Unfortunately, exposure of the resin to 10% thiourea in DMF under continuous flow conditions showed no detectable signs of deprotection.

The terminal Nde group removal was therefore effected using 2% hydrazine in DMF under continuous flow conditions with complete deprotection after approximately 3-4 min. The tripeptide was cleaved from the resin, and shown to be two major peaks by RPHPLC, the largest of which was characterised as $(Leu)_3$ by MALDI-TOF MS, with the main impurity being confirmed as $(Leu)_2$.

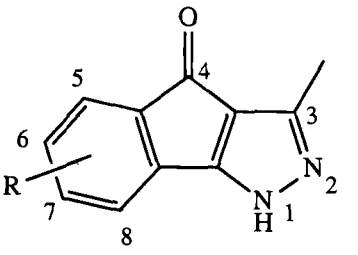
2.2.3 Racemisation of N^{α}-Nde-amino acids during synthesis

With the previous experiment indicating the promising application of N^{α}-Nde-amino acids in SPPS it was important to ascertain whether chiral integrity of the amino acid was not jeopardised during the condensation reaction with 2-acetyl-4-nitroindane-1,3-dione (**71**). A sample of N^{α}-Nde-L-

phenylalanine was deprotected with 1% hydrazine monohydrate in super-dry ethanol. TLC monitoring indicated that deprotection was complete after 3 h, during which an orange/red precipitate had formed. The solid was collected and dried and was surprisingly elucidated as the Nde-hydrazine deprotection product (**73**), by ^{1}H NMR and mass spectrometry.



(**73**)



R = 5 or 8 NO_2

(**74**)

It was anticipated that hydrazine mediated Nde deprotection would yield the 3-methyl-5/8-nitroindeno[1,2-*c*]pyrazol-4(1*H*)-one (**74**) byproduct in a similar mechanistic fashion to that previously displayed with *N*-Dmc and *N*-Dde amino acids. However it would appear that the electron withdrawing nature of the fused nitro-aromatic ring sufficiently activates the reactive vinyl bond to allow a straightforward transamination type deprotection.

A UV spectrum of the deprotection byproduct was obtained (Figure. 2) and indeed showed a λ_{max} at 294 nm, which made this an ideal reagent for monitoring at the identical deprotection wavelength associated with the Fmoc group.

Evaporation of the deprotection media, resulted in an oily residue which was partitioned between chloroform and water. Evaporation of the aqueous layer returned L-phenylalanine with an optical rotation identical to that reported

in the literature, and an identical R_f to an authentic L-Phe sample when run together on a chiral TLC plate. This confirmed that no racemisation occurred during the synthesis of N^{α}-Nde-L-Phe.

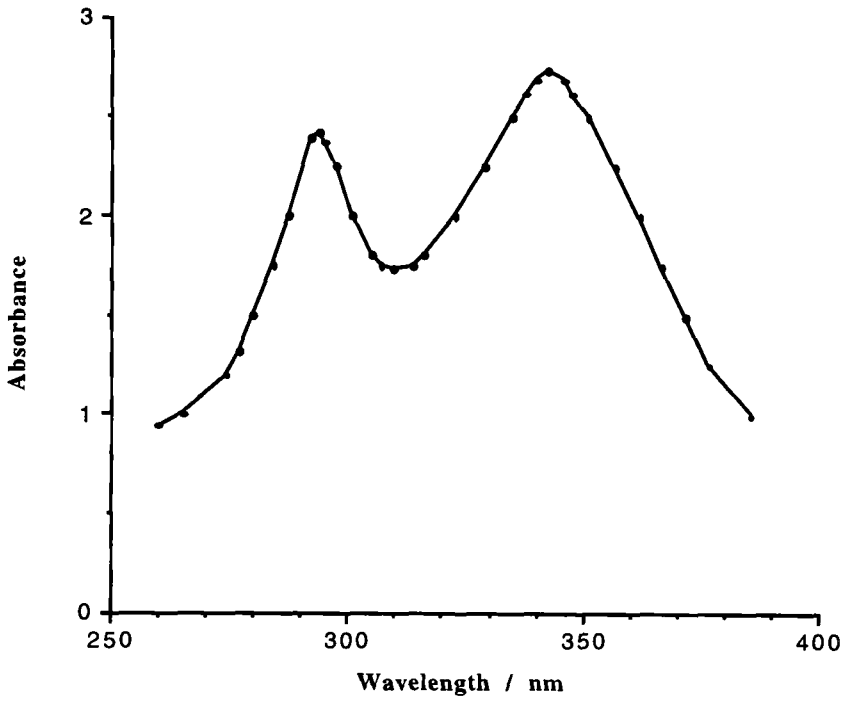
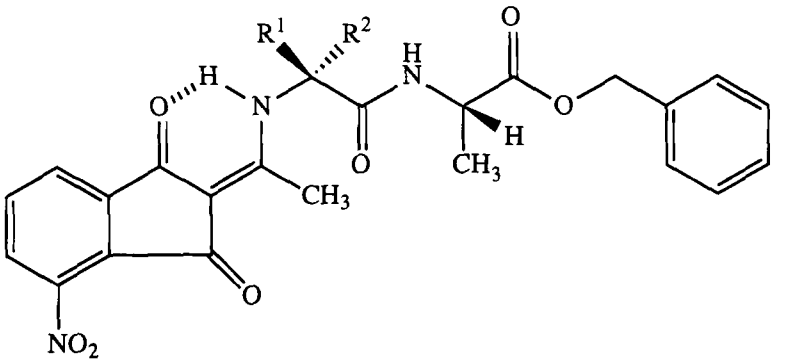


Fig.2. UV Absorption spectrum of the Nde deprotection by-product, Nde-hydrazine in DMF

2.2.4 Racemisation of N^{α}-Nde-amino acids during coupling

An estimate of racemisation levels encountered when coupling N^{α}-Nde-amino acids was required, since despite oxazolone formation being effectively impossible, the enhanced electron-withdrawing nature of the Nde substituent may predispose the α-proton to base (e.g. DIEA) abstraction following carboxy

activation. L-Phenylalanine was once again chosen as the model amino acid as it is one of the most easily racemised and thus ideal for this experiment.[202] Accordingly *N*α-Nde-L-Phe and *N*α-Nde-D-Phe were coupled to L-Ala-OBzl using both DCC/HOBt and TBTU/HOBt/TEA protocols. The two diastereoisomers produced, *N*α-Nde-L-Phe-L-Ala-OBzl (**75**) and *N*α-Nde-D-Phe-L-Ala-OBzl (**76**) were readily separable by RPHPLC when aqueous methanol was used as the mobile phase for gradient elution.



(**75**) R^1 = CH_2-Ph, R^2 = H
(**76**) R^1 = H, R^2 = CH_2-Ph

No detectable signs of epimerisation were evident when either DCC or TBTU were employed as the coupling reagent. These results demonstrated that *N*α-Nde-amino acids were compatible for SPPS with their chiral integrity remaining unscathed during both their synthesis and subsequent coupling.

2.3 SPPS synthesis of Neuromendin N amide using *N*α-Nde-amino acids

The potential use of *N*α-Nde-amino acids in SPPS was examined by the synthesis of three peptides. The first of which, Neuromendin N (**77**), was

synthesised as its corresponding amide, using the N^{α}-Nde-amino acids, Lys(Boc)OH, Ile, Tyr(O^{t}Bu) and Leu prepared as described earlier.

H-Lys-Ile-Pro-Tyr-Ile-Leu-OH

(77)

The inability to synthesise a stable *N*-Nde-Pro derivative was circumvented by using *N*-Fmoc-Pro-OH for the incorporation of the proline residue. A peptide amide forming resin (Novasyn® KR 100) carrying the modified Rink linker,[32] was chosen because of the ease of initial acylation of the first residue onto an amine rather than an alcohol.

Synthesis was accomplished on an LKB 4175 Biolynx manual peptide synthesizer, using a DIPCDI/HOBt coupling strategy under continuous flow conditions. Four equivalents of acylating reagents were employed for each residue, and every coupling was allowed to continue until the TNBS amine test was negative.

Coupling of the first three residues progressed with relative ease, displaying excellent deprotection profiles using 2% hydrazine, both visually and with post column UV monitoring at 290 nm. As mentioned earlier, *N*-Fmoc-Pro was then incorporated into the synthetic strategy, requiring double coupling to obtain a negative TNBS test. Following 20% piperidine mediated Fmoc deprotection, the final two N^{α}-Nde-residues were incorporated smoothly, and the crude cleaved peptide was shown to be one major peak by RPHPLC, with the purified peptide subsequently characterised by +ve-ES-MS.

2.4 SPPS synthesis of the angiotensin II receptor binding protein fragment as its corresponding amide using N^{α}-Nde-amino acids

The second model peptide, angiotensin II receptor binding protein fragment (**78**), was once again synthesised as its corresponding amide using the N^{α}-Nde-amino acids, Lys(Boc)OH, Val, Tyr(O^{t}Bu), Ile, His(Trt), Ala and Leu. The reported problems encountered with N^{α}-Dde-Gly[198] dictated the replacement of the corresponding N^{α}-Nde residue with *N*-Fmoc-Gly-OH.

H-Lys-Gly-Val-Tyr-Ile-His-Ala-Leu-OH

(78)

Under analogous conditions as those described for the synthesis of neuromendin N amide, the first two residues of the angiotensin II binding protein fragment were incorporated readily, exhibiting excellent deprotection profiles. However, introduction of the histidine residue, even after double coupling, produced a small but significant positive TNBS test. At this juncture, there was insufficient N^{α}-Nde-His(Trt) with which to perform another coupling, however it was decided to proceed with the total synthesis to assess how poor the His incorporation had been, and to investigate whether any of the following residues posed problems in coupling. Fortunately, the remaining synthesis, including incorporation of the N^{α}-Fmoc-Gly residue, proceeded without further complications.

The RPHPLC trace of the crude cleaved peptide indicated two major peaks as anticipated, with +ve-ES-MS of the purified peaks confirming them as the required peptide and the histidine deletion product, Fig. 3.

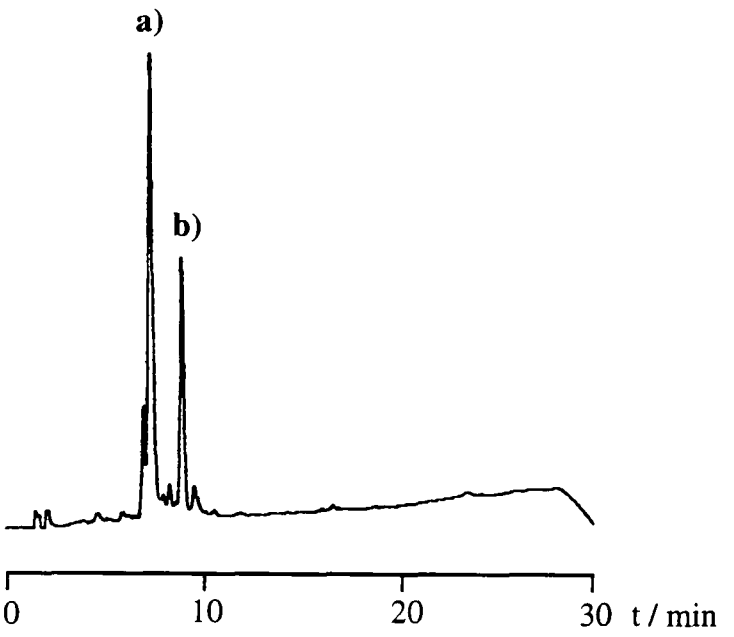


Fig.3. RPHPLC trace of the crude cleaved angiotensin II receptor binding protein (as its corresponding amide) indicating (a) the required peptide, and (b) the histidine deletion product.

The *N*α-Nde-His(Trt) residue was re-synthesised, and a second attempt at the above peptide sequence was undertaken. Once again, the histidine residue proved difficult to couple to the Ala-Leu substituted resin, and required the sequential exposure to four individual acylating mixtures in order to obtain a negative TNBS test. As in the initial experiment, all remaining residues proved non-problematic in their incorporation and the crude cleaved peptide was on this occasion shown to be one major peak by RPHPLC, with the purified peptide exhibiting the correct mass by +ve-ES-MS. It is interesting, but not surprising, that the forced coupling conditions of the histidine residue led to a small but significant degree of epimerisation (approximately 5%), which is not uncommon with this particular residue.[10, 174-176]

2.5 SPPS synthesis of Leu-enkephalinamide using *N*-α-Nde-amino acids

To further demonstrate the applicability of Nde as an *N*α-protecting group in SPPS, a final peptide, Leu-enkephalinamide (**79**), was chosen as a synthetic target via TBTU/HOBt/DIEA coupling procedures using the *N*α-Nde-amino acids Tyr(O^{t}Bu)OH, Phe and Leu. Once again *N*-Nde-Gly was replaced by *N*-Fmoc-Gly for the introduction of these particular residues within the sequence.

H-Tyr-Gly-Gly-Phe-Leu-NH_2

(79)

The SPPS was once again performed using an LKB 4175 Biolynx manual peptide synthesizer and Novasyn® KR 100 resin. The total synthesis proceeded satisfactorily, with the crude cleaved peptide shown to be one major peak by RPHPLC, which on subsequent purification was characterised by +ve-ES-MS.

The three peptides synthesised above demonstrate that *N*α-Nde-amino acids are suitable for use in SPPS producing peptides of good to excellent purity with little or no epimerisation. The deprotection is rapid and visual, or can be monitored by UV at the same wavelength required for Fmoc deprotection analysis. In order to establish whether Nde displayed similar or better orthogonality to Fmoc than Dde, stability studies against TFA and piperidine were performed.

2.6 Stability of Nde to TFA

To confirm the stability of Nde to TFA, a sample of *N*α-Nde-L-Phe was dissolved in freshly distilled anhydrous TFA, and monitored by RPHPLC over

a 24 h period. During this time no additional peak was observed, and after 24 h the sample was evaporated to dryness and shown to be identical to the starting material by ^{1}H NMR and FAB-MS.

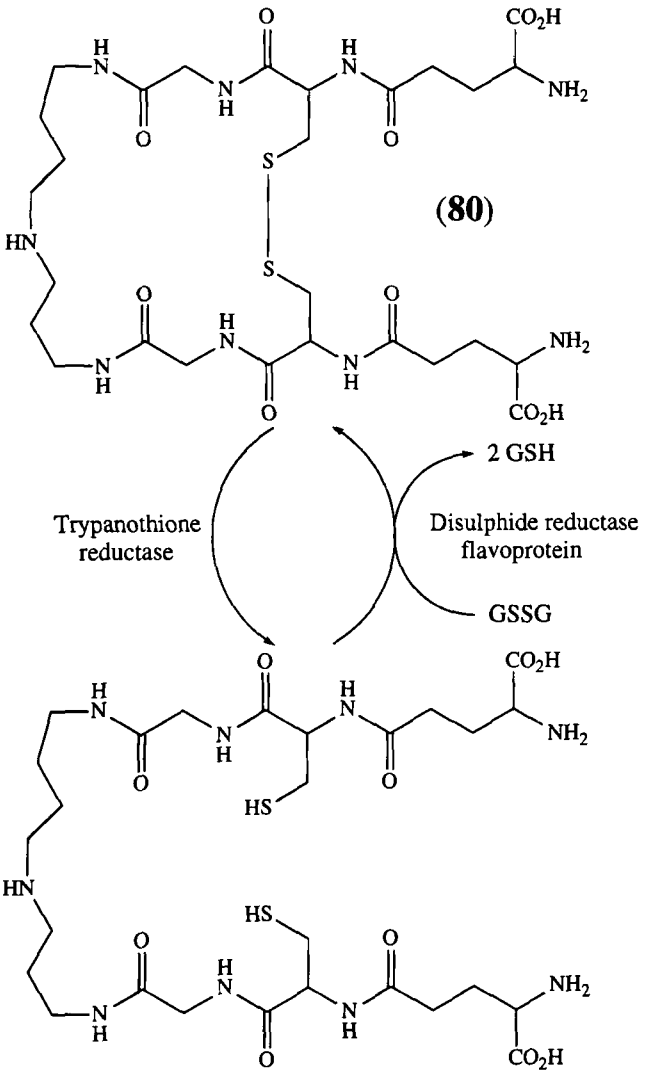
2.7 Stability of Nde to piperidine

A test peptide was synthesised in order to ascertain the stability of Nde towards piperidine. Leu-enkephalinamide was synthesised on Novasyn® KR 100 resin *via* standard Fmoc chemistry. At this juncture, *N*$^{\alpha}$-Nde-Leu-OH was employed as the terminal amino acid and was coupled to the peptide using the DIPCDI/HOBt procedure. The Nde group was left intact and the resin bound protected fragment was utilised in a stability test against a continuous flow of 20% v/v piperidine in DMF for 24 h. Periodic cleavage of a portion of the resin and RPHPLC analysis of the product indicated the emergence of small quantities of the unprotected peptide. From the visual examination of the relative peak heights, it could be concluded that the % loss of Nde is *ca.* 6% and 13% after 3 and 6 h respectively. A contact time of 3 h with 20% piperidine represents 25 x 7min Fmoc deprotection cycles, thus allowing the synthesis of medium sized branched peptide chains, via Fmoc chemistry, without significant loss of the Nde group. This result is comparable to that obtained for the Dde group, but with improved deprotection characteristics.

2.8 Solid phase synthesis of trypanothione disulphide utilising Nde as a primary amine protecting group

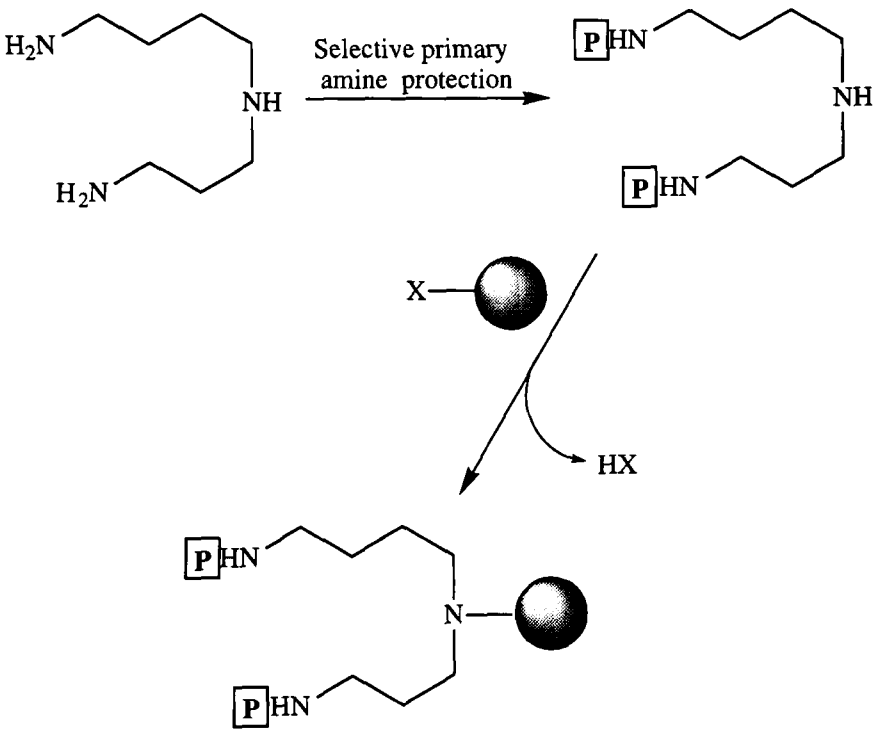
The pathogenic *Trypanosomidae* species responsible for such diseases as African sleeping sickness and South American Chagas' do not use the

classical glutathione (GSH) based redox system present within mammalian cells to guard against cellular damage.[203] They instead use the GSH-spermidine conjugate, *N1*, *N8*-bis(glutathionyldisulphide)spermidine, commonly referred to as trypanothione disulphide (**80**).[204] This metabolite plays a pivotal role in the maintenance of cellular glutathione in its reduced state (Scheme 42) and is significantly detached from the mammalian system to make it an attractive target for drug development against these parasitic diseases.



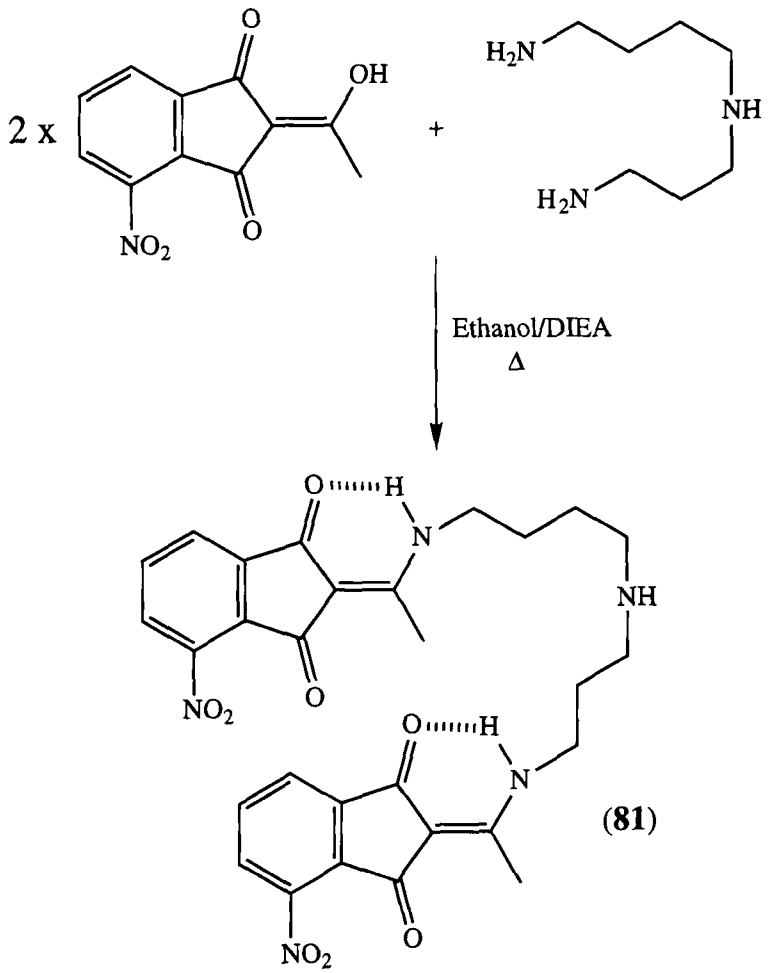
Scheme 42. Trypanothione disulphide mediated glutathione regulation within trypanosomatids.

Until now only one solution phase,[205] and one solid phase[206] synthesis of trypanothione disulphide have been reported, both with poor to moderate overall yields. It was envisaged that the inability of 2-acetyl-4-nitroindane-1,3-dione to form stable protected secondary amine derivatives meant that this reagent could be utilised as a selective primary amine protecting group. Compounds such as spermidine, displaying both primary and secondary amines within its structure, provided an ideal target to investigate this hypothesis. If selective primary amine protection was possible, the remaining secondary amine functionality could be employed to attach the molecule onto a solid support, (Scheme 43).



Scheme 43. Attachment of *N1,N8-bis* protected spermidine to a solid support.

Initial attempts to synthesise N^1,N^8-*bis*-Nde-spermidine (**81**), *via* the methodology developed for N^{α}-Nde amino acid synthesis, proved unsuccessful, with the Nde salt crashing out as a bright yellow precipitate almost instantaneously. However, modification of the above protocol to include an equivalent of base (DIEA) in the reaction mixture, afforded N^1,N^8-*bis*-Nde-spermidine (**81**) in almost quantitative yield (90%) (Scheme 44). The mono-protected impurity was easily removed by treatment of the precipitate with 1% AcOH in ethanol. The amorphous product, like the N^{α}-Nde amino acids could not be further recrystallised.



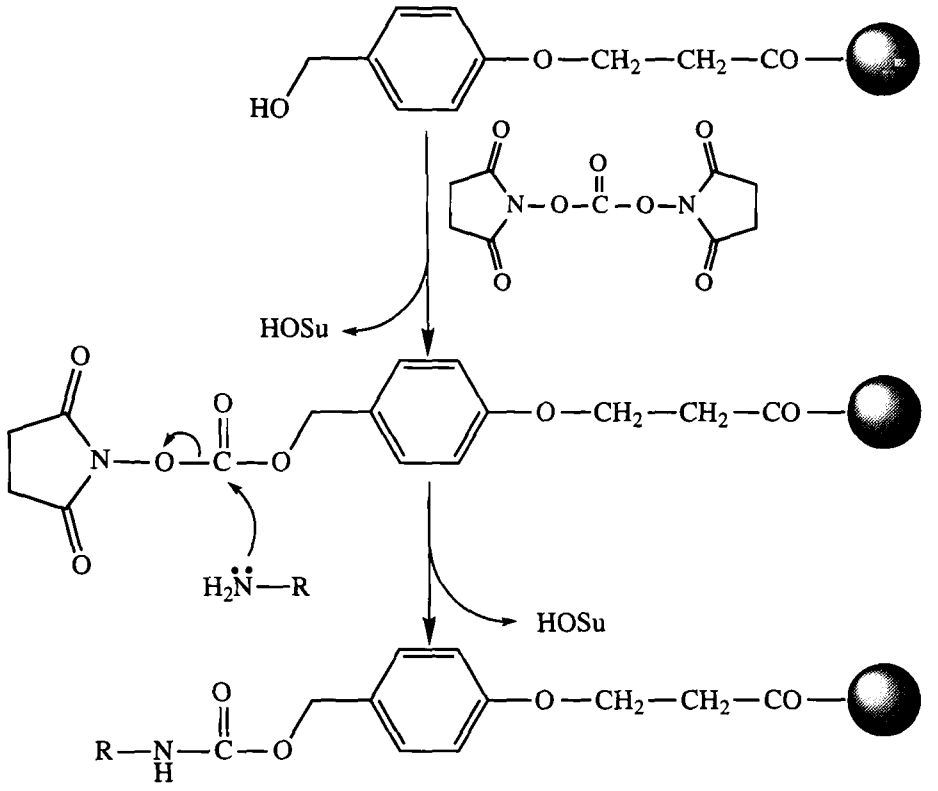
Scheme 44. Synthesis of N^1,N^8-bis-Nde-spermidine (**81**)

Solid phase peptide chain elongation is traditionally carried out from the $C \rightarrow N$ terminals in order to reduce racemisation, hence few reports were available in the literature where the amine group of an amino acid was linked to a resin.

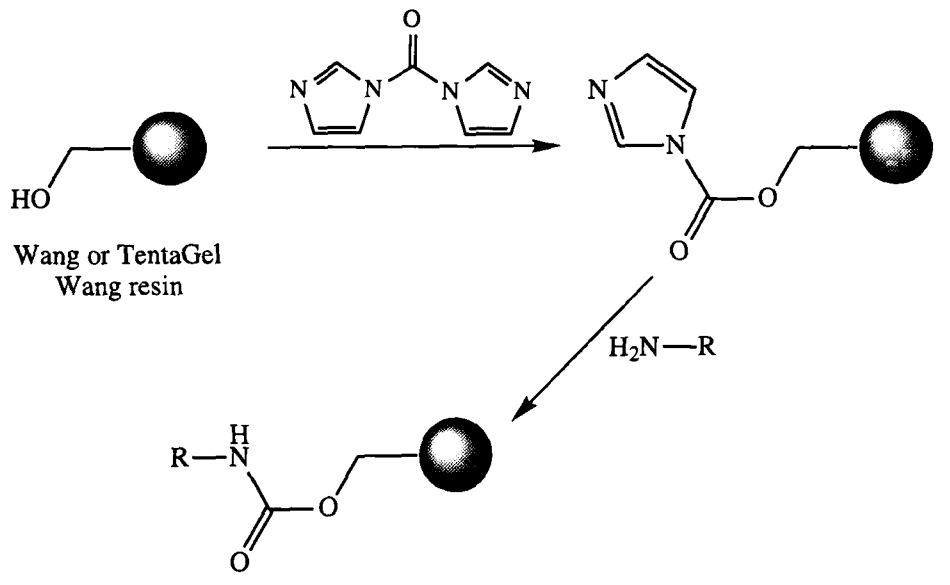
The earliest example was a carbamate linker described by Letsinger,[207,208] in which a resin bound chloroformate was generated from hydroxymethyl polystyrene and phosgene. This method was exploited by Felix and Merrifield for the azide mediated elongation of a linear peptide in the $C \rightarrow N$ direction.[209] This particular method suffered from three drawbacks;

i) The use of phosgene is dangerous and should be avoided if possible.
ii) The high loading Merrifield resins are prone to a decrease in functionalisation, possibly due to internal carbonate formation.
iii) Phosgene in not compatible with the TFA labile *p*-alkoxybenzyl alcohol resins used for Fmoc synthesis.

A recent publication reported the application of *N,N'*-disuccinimidyl carbonate (DSC),[210] as a means of activating an hydroxymethylphenoxypropionic acid (HMP) based linker in order to anchor the amine side chain of a lysine residue for subsequent cyclic peptide synthesis (Scheme 45). More recently, a method for the synthesis of tripeptides has been described which exploits a polymer bound acylimidazole as a means of generating a carbamate linker from the Wang resin (Scheme 46).[211]



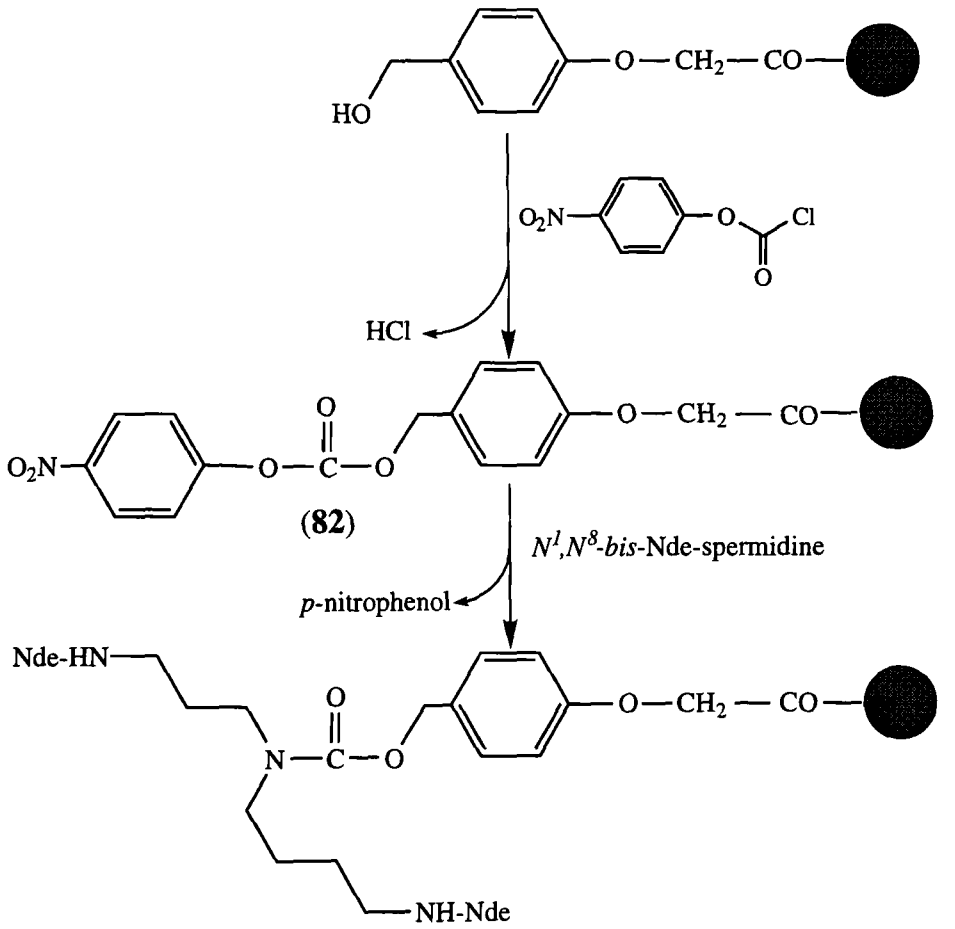
Scheme 45. DSC activation of an HMP functionalised resin, where H_2N-R = *N*α-Fmoc-Lys(NH_2).OH.



Scheme 46. Carbonyldiimidazole mediated activation of Wang Resin.

However, our choice of activation was based on the seminal papers published by Leznoff,[212,213] in which he describes the use of *p*-nitrophenyl chloroformate to prefunctionalise a polymer bound benzyl alcohol for the synthesis of unsymmetrical diamines. This method has also received recent attention for the solid phase synthesis of a hydantoin library.[214]

NovaSyn® TGA resin, derivatised with the TFA labile 4-hydroxymethylphenoxyacetic acid (HMPA) linker,[31] was readily converted to the mixed carbonate (**82**) by overnight exposure to a ten fold excess of *p*-nitrophenyl chloroformate and DIEA in DCM (Scheme 47).



Scheme 47. Attachment of N^1,N^8-bis-Nde-spermidine to a *p*-nitrophenyl chloroformate pre-activated HMPA resin.

The extent of activation was demonstrated by overnight exposure of (**82**) to a four fold excess of N^1,N^8-bis-Nde-spermidine (**81**) in DMF. Nde deprotection was subsequently executed using 2% hydrazine monohydrate in DMF under continuous flow conditions. After thorough washing of the resin and acetic anhydride capping, a four fold excess of Fmoc-Cl /DIEA was introduced and the resin regularly monitored until a negative TNBS test was obtained. The resin was sequentially washed with DMF, DCM and finally methanol, and dried *in vacuo* overnight over KOH. An Fmoc loading test on the dried resin indicated a nearly quantitative incorporation of spermidine (>95%) onto the derivatised solid support.

With the preliminary investigation into the synthetic validity proving successful, the full synthesis of trypanothione was undertaken by the sequential TBTU/HOBt/DIEA mediated acylation of Fmoc-Gly-OH, Cys(Trt) and Glu(OH)OtBu onto the hydrazine deprotected resin bound spermidine. Each acylation was monitored using the TNBS amine test, and N^{α}-deprotection was accomplished using 20% piperidine in DMF.

Peptide resin cleavage with concomitant side-chain deprotection was effected using TFA/TIPS/EDT/H_2O (9.25 : 0.25 : 0.25 : 0.25), and a crude yield of *ca* 90% was obtained. RPHPLC indicated one major peak (*ca.* 80% purity) which was characterised by +ve-ES-MS to be dihydrotrypanothione. This was aerially oxidised at a 0.1 mg ml^{-1} concentration in ammonium $acetate_{(aq)}$ 0.04M (adjusted to pH 8.5 with concentrated ammonia) under atmospheric oxygen for 72 h. Lyophilisation and RPHPLC analysis of the resultant white amorphous powder displayed one major peak of reduced retention time when compared to the dihydrotrypanothione precursor, which

was confirmed by accurate FAB-MS to be trypanothione disulphide (**80**).

2.9 Conclusions

We have described the development, synthesis and exploitation of the *N*-Nde group as a primary amine protecting group displaying good stability towards the deprotection conditions required for *N*α-Fmoc removal and hence offering quasi-orthogonality to the well established Fmoc/^{t}Boc combination commonly used in SPPS. The piperidine stability of the *N*-Nde protecting group is similar to that of *N*-Dde, however it displays an improved deprotection profile that can be monitored both visually, or by UV detection at the same wavelength as that required for the Fmoc group.

We have also illustrated *N*-Nde as a selective primary amine protecting group, which can be utilised to temporarily block the primary amines present in polyamine structures, such as spermidine, whilst leaving the secondary amine free to derivatise a resin support, and thereby allow the solid phase synthesis of complex structures such as trypanothione disulphide (**80**).

3

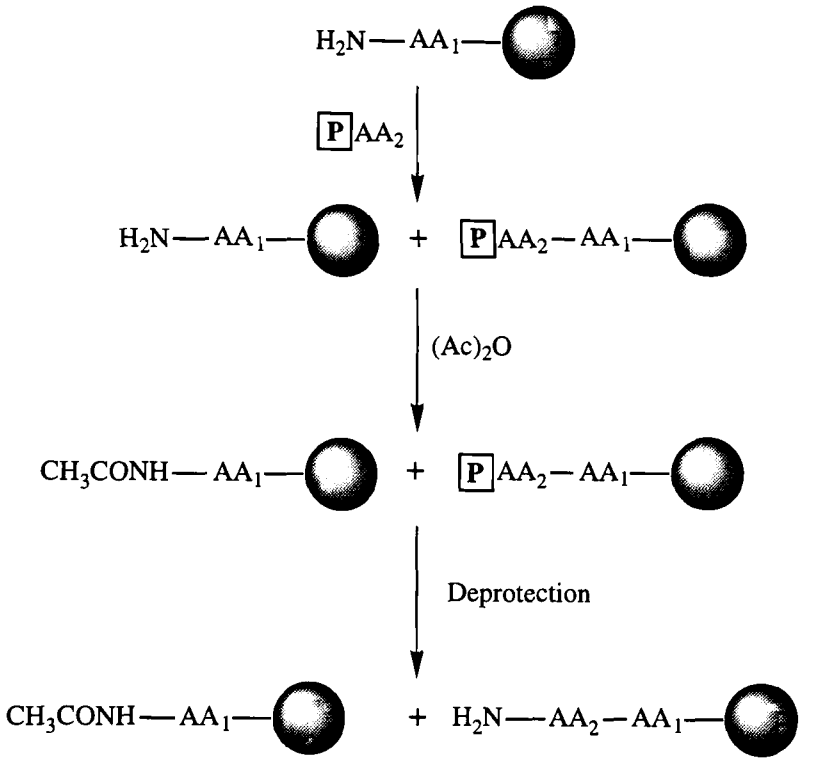
The Development of Reversible Chromatographic Probes for the Purification of Synthetic Peptides

3.1 Introduction

Despite enormous advances in the protecting groups, coupling reagents and resin supports utilised in SPPS, the process suffers intrinsically from the accumulation of complex mixtures of deletion peptides. The onset of either secondary structure,[215] 'hydrophobic collapse',[216] interchain clustering or phase transitional change,[217] in resin bound peptides frequently hinders acylation of the free amino groups, which can react with the next or ensuing residues.[218] The individual entities of the crude product often differ by only gradual deletions, endowing similar chromatographic properties upon them. Techniques such as HPLC or CE are often insensitive to these subtle differences, with individual components manifesting as one peak and therefore making purification laborious and costly.

To minimize the formation of deletion sequences, acetic anhydride is often employed as a capping reagent at the final juncture of an acylation cycle, (Scheme 48)[39] resulting in the formation of truncated sequences. However, these also frequently display similar chemical and chromatographic properties as the desired peptide, as indicated above. Understandably, the problem of impurities manifests itself further when attempting the linear synthesis of proteins, hence very few, eg. HIV-1 protease[219] (99 residues) and ubiquitin[220]

(76 residues), have surrendered to chemical synthesis and crystallisation.



Scheme 48. Acetic anhydride capping of unacylated residues during linear SPPS.

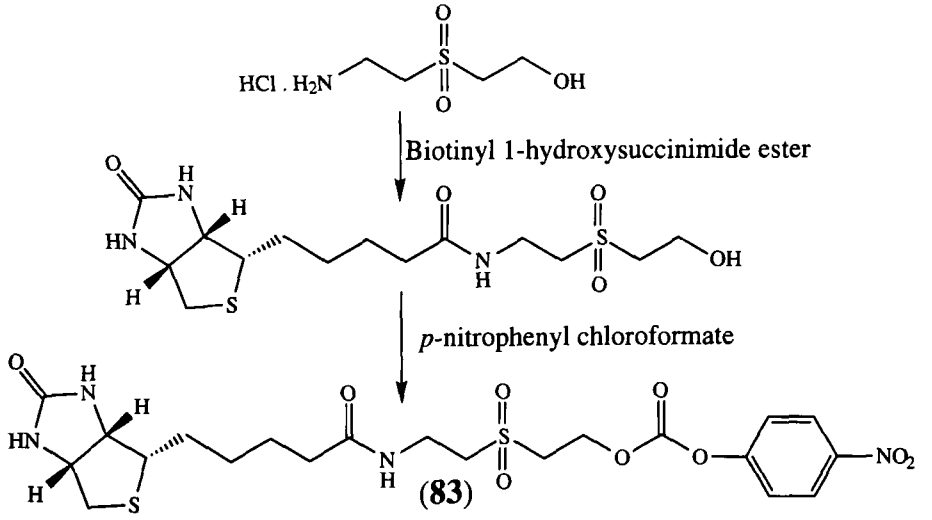
Initially several methods were suggested for separating the target molecule from the truncated impurities by derivatising the *N*-terminus of the finished peptide with a reagent capable of an affinity-type binding to a solid support. Examples of which included the binding of *N*α-dinitrophenyl (DNP) peptides to immobilised DNP antibodies,[221] *N*α-biotinylated peptides to immobilised avidin[222] and the reaction of an *N*α-thiol with an iodoacetamide resin.[223] The two former reagents had the drawback of being covalently linked to the peptide in a non-reversible manner and were therefore not suitable if the target peptide or protein was required in its physiological form. With respect to

the latter example; the chemical affinity of SH-iodoacetamide was found to be weaker than the biological affinity at low concentrations and therefore the method proved ineffectual at the purification of long peptides or proteins.

Consequently, investigations were initiated by various groups to address the requirement for a reversible chromatographic probe that could be introduced onto the *N*-terminus of the completed protected fragment, was stable to the cleavage/side-chain deprotection conditions, yet could be easily removed, following purification, to relinquish the target peptide/protein.

3.1.1 Previously reported reversible affinity or hydrophobic chromatographic probes

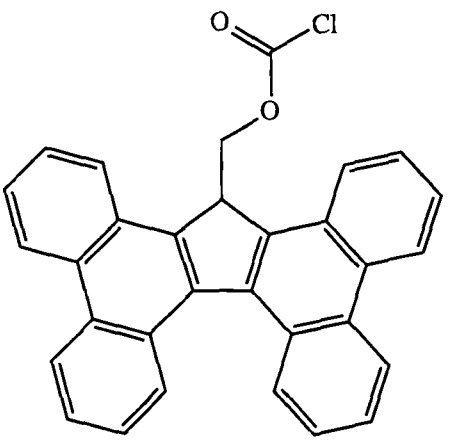
The first example relied once more upon the specific interaction between biotin and avidin as an efficient method of chromatographic purification.[224,225] The biotinylating reagent, 2-[(*N*-biotinyl)-aminoethylsulphonyl]ethyl *p*-nitrophenylcarbonate (BASEC) (**83**) was readily synthesised from 2-(aminoethylsulphonyl)ethanol,[226] (Scheme 49) in a 48% overall yield.



Scheme 49. 2-[(*N*-biotinyl)-aminoethylsulphonyl]ethyl *p*-nitro-phenyl-carbonate (BASEC) (**83**) synthesis.

N-Terminal modification of the finished peptide chain was achieved through the base labile sulphonylethoxycarbonyl linkage, and after cleavage of the biotinylated peptide from the resin, purification was effected on an avidin-agarose column. Finally, base removal (5% ammonia solution) of the BASEC group afforded the pure peptide; in this instance magainin 2 (23 residues), hGRF (44 residues) and the transducin γ-subunit 1-67 were used as examples.

Ramage and co-workers focused their attention on the tetrabenzo substituted Fmoc reagent, tetrabenzo[*a,c,g,i*]fluorenyl-17-methoxycarbonyl chloride (Tbfmoc-Cl) (**84**).[227]

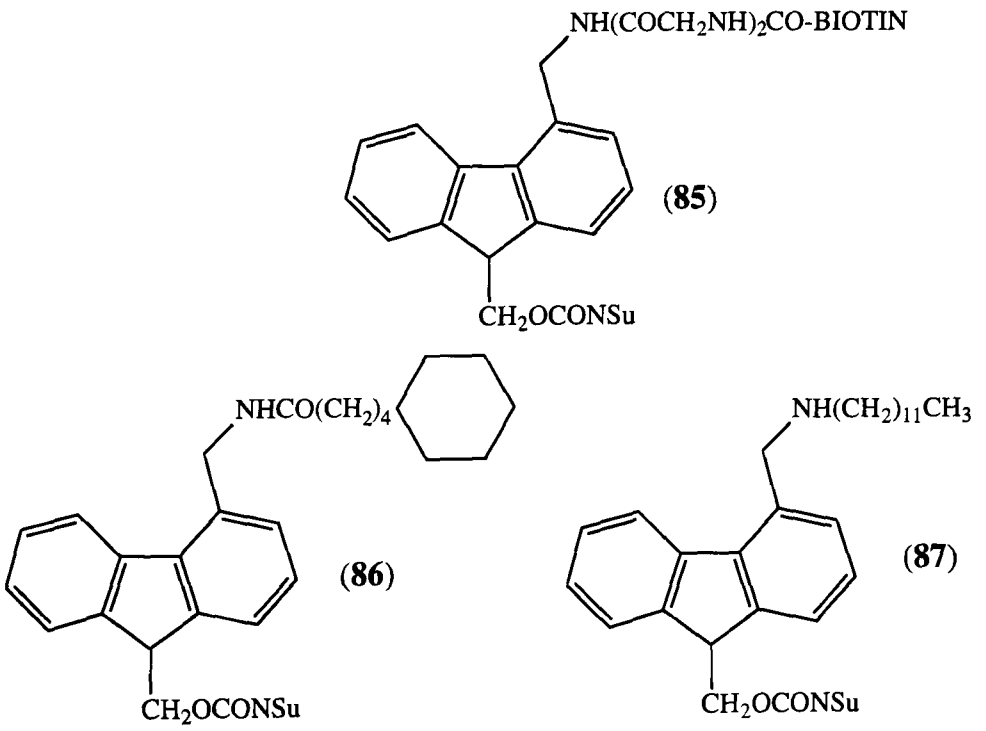


(**84**)

The aromatic polycyclic reagent, synthesised in five steps, demonstrates a high affinity towards porous graphitised carbon (PGC). After synthesis of Hepatitis B surface antigen (23 residues) via Fmoc/^{t}Bu methodology with acetic anhydride capping at each residue, Tbfmoc was attached to the resin bound peptide via the piperidine labile urethane linker. TFA mediated cleavage of the peptide-resin bond and application of the crude product to a short column of PGC in 70% $MeCN_{(aq)}$ resulted in complete retention of the Tbfmoc peptide whilst the truncated impurities eluted off. The pure peptide was released from

the Tbfmoc group by treatment of the PGC column with 10% (v/v) piperidine in 70% $MeCN_{(aq)}$. Later experiments with larger peptides, e.g. bacteriophage λ Ral $(Acm)_4$ (66 residues) and MeCP2 methylated DNA binding domain (78-162) (85 residues) indicated that it was crucial to maintain deprotection conditions in which the peptide is soluble; the most effective being 10% (v/v) piperidine in 1:1 6M guanidine HCl/isopropanol. Despite this reagent demonstrating promising early results, the polyaromatic nature of the molecule raises doubts about its potential carcinogenicity and the possible solubility problems of the affinity probe-peptide conjugate.

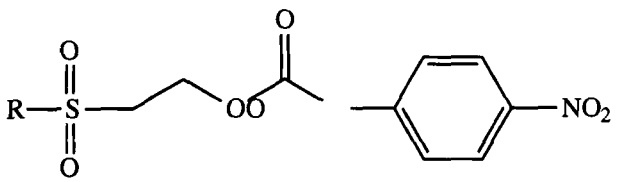
An alternative modification of the Fmoc nucleus by Mascagni *et al* generated both an affinity based reagent; {4-[(biotinylglycylglycyl)aminomethyl] fluoren-9-yl}methyl succinimidyl carbonate (**85**) and two hydrophobic probes; {4-[(4-cyclohexylbutyryl)aminomethyl]fluoren-9-yl}methyl (**86**) and [4-(dodecylaminocarbonyl)fluoren-9-yl}methyl (**87**) succinimidyl carbonates.[228]



(**85**) (**86**) (**87**)

Synthesised from the 9-(hydroxymethyl)fluorene-4-carboxylic acid precursor, these reagents were reversibly attached to the *N*-terminus of the resin-bound protected peptide, in a similar manner to Tbfmoc, through a base labile urethane linkage. The efficiency of the probe reagents was demonstrated by the purification of two polypeptides of 46 and 101 residues in length. The biotinylated peptides were purified using an immobilised avidin column, whereas the hydrophobic probes allowed simple RPHPLC purification by imparting a significant shift in retention time for the modified peptides, thereby removing them from the more hydrophillic truncated impurities. In both cases probe removal was effected using 5% $TEA_{(aq)}$.

The latter method of purification was also exploited recently; using the sulphonylethoxycarbonyl template described earlier, only replacing the biotin moiety with C_{10} (**88**) or C_{16} (**89**) *n*-alkyl chains.[229]

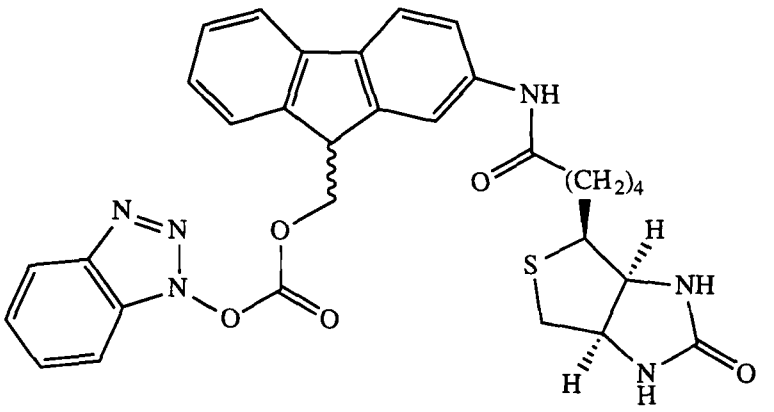


R=*n*-$C_{10}H_{21}$ (**88**)
R=*n*-$C_{16}H_{33}$ (**89**)

Subsequent synthesis and purification of magainin 1 using a RPHPLC technique afforded the pure peptide after deprotection of the probe using a 10% solution of NH_4OH in 2,2,2-trifluroethanol. The reaction was monitored by analytical RPHPLC, and indicated that complete conversion to the free peptide took 18 h at room temperature.

The most recent example of a chromatographic probe once more returned to biotin-avidin affinity to effect purification. The transient biotinylating reagent

(**90**) was again based upon the Fmoc nucleus; its synthesis involving seven individual steps with an overall yield not quoted. Its efficiency was demonstrated using 10 and 17 residue peptides, with final deprotection proceeding with piperidine treatment.[230]

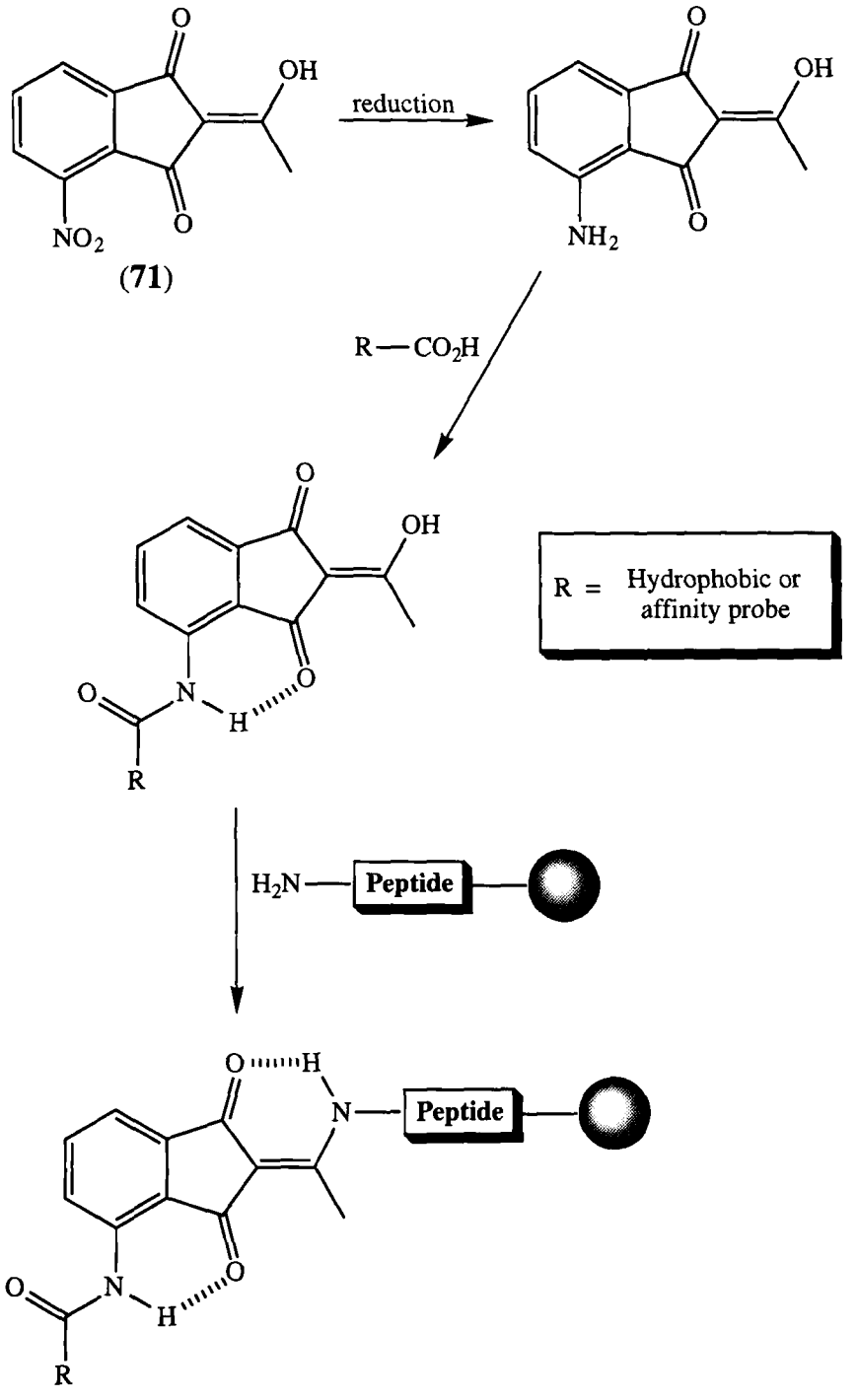


(**90**)

3.2 2-Acetyl-4-nitroindane-1,3-dione (71) as a potential precursor for a chromatographic probe

The above review of the literature highlights that in general, all of the reported chromatographic probes suffered from lengthy syntheses, on occasions resulting in poor overall yields, with the requirement for prior activation adding yet another step. However, acylation of a resin bound amine with 2-acetyldimedone to afford the *N*-Dde derivative has been demonstrated to proceed without prior activation, and has recently been exploited in the synthesis of polyamine spider and wasp toxins.[231] It was therefore envisaged that reduction of the aromatic nitro group of 2-acetyl-4-nitroindane-1,3-dione (**71**) (see Chapter 2) and its subsequent acylation with either an affinity or hydrophobic probe would afford a potential reagent for peptide purification (Scheme 50). The absence of activation reduced the number of synthetic steps

to three, the first of which was already well established as high yielding (63%).



Scheme 50. A potential chromatographic probe from 2-acetyl-4-nitroindane-1,3-dione (**71**).

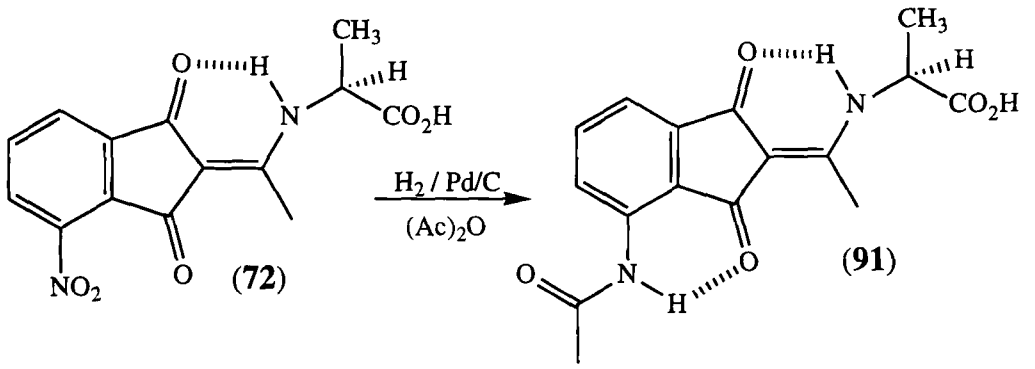
3.2.1 Acylation of a resin-bound amine using 2-acetyl-4-nitroindane-1,3-dione

To test the validity of our hypothesis, resin-bound Leu-enkephalinamide (on Novasyn® KR 100) was incubated overnight with a four fold excess of 2-acetyl-4-nitroindane-1,3-dione in DMF. A TNBS test performed on the resin was strongly positive indicating that unacylated amine was still present. A repeat of the experiment with larger equivalents of 2-acetyl-4-nitroindane-1,3-dione were attempted, but all failed to quantitatively acylate the resin. A measurement of the pK_a of 2-acetyl-4-nitroindane-1,3-dione indicated a surprisingly low value of 2.5,[232] and it was therefore apparent that potential salt formation with the resin bound amine was inhibiting the acylation process.

3.2.2 Reduction and concomitant acetylation of *N*α-Nde-Ala *via* catalytic hydrogenation in the presence of acetic anhydride

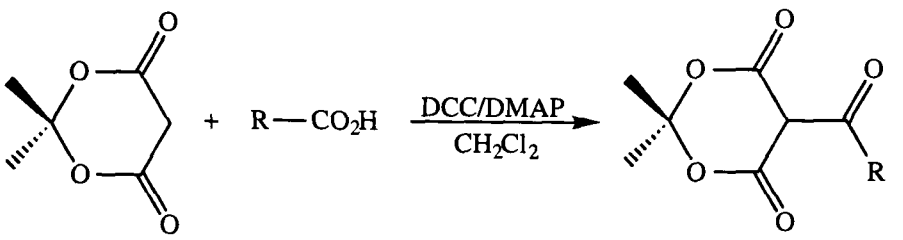
With the disappointing result obtained with the attempted on resin amino acylation with 2-acetyl-4-nitroindane-1,3-dione, it was felt that even prior reduction and ensuing acylation of the generated amine would not significantly alter the pK_a of the resultant molecule, and therefore the affinity probe would probably need to be introduced at the final residue stage, coupled to the relevant amino acid. In order to investigate whether the nitro group could be selectively reduced in this situation, *N*α-Nde-Ala was dissolved in methanol and hydrogenated overnight in the presence of a 50 fold excess of acetic anhydride (Scheme 51). In the original paper,[200] catalytic hydrogenation proved to be the most efficient means of reducing 2-acetyl-4-nitroindane-1,3-dione (**71**) to 2-

acetyl-4-aminoindane-1,3-dione, yet in our hands the modified process at best afforded the *N*-acetylated product (**91**)as a pale yellow amorphous powder in a very poor 20% overall yield. Various alternative catalytic transfer hydrogenation reductions were attempted; all returning the starting material.



Scheme 51. Catalytic hydrogenation and acetylation of *N*α-Nde-Ala.OH

The unrewarding results so far obtained suggested that our initial ambitions to endow a multi-faceted nature to the *N*-Nde protecting group were not going to be realised. However, the ease with which *C*-acylated Meldrum's acids could be prepared[233] (see Chapter 4) led us to readdress the problem by exploiting the cyclic 1,3-diketone, 5,5-dimethylcyclohexane-1,3-dione (dimedone) (**65**), as our template for further modification towards a chromatographic probe. Generation of the *C*-acylated Meldrum's acid exploited the acidity of the carbonyl flanked methylene protons. This intrinsic nucleophilicity allowed the 4-dimethylaminopyridine (DMAP) catalysed condensation between Meldrum's acid and a carbodiimide activated carboxylic acid (Scheme 52).



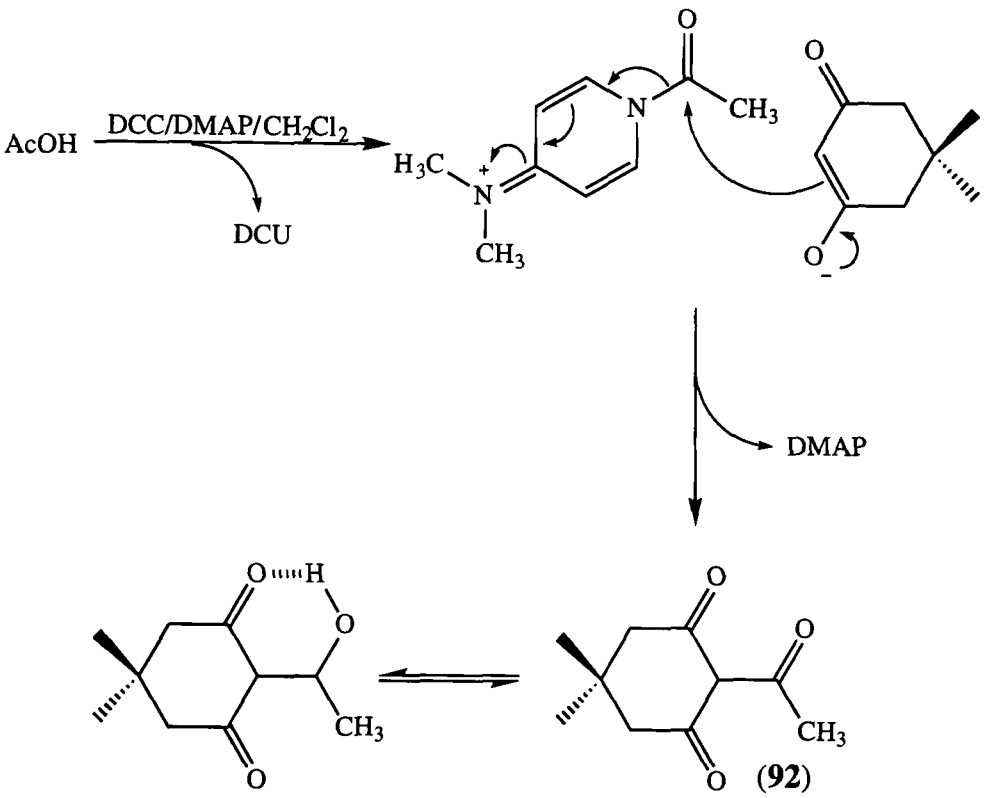
Scheme 52. DCC/DMAP mediated acylation of Meldrum's acid.

Until now, the synthesis of 2-acetyldimedone, required 5,5-dimethylcyclohexane-1,3-dione to be refluxed in acetic anhydride in the presence of anhydrous sodium acetate, with the mechanism proceeding through initial *O*-acetylation followed by an intramolecular Fries-type rearrangement to afford the required product as a pale yellow crystalline solid in a 64% yield. This particular approach required both vacuum distillation and flash column chromatography to purify the product, which itself was limited to the acetyl derivative. However the structural similarity displayed between Meldrum's acid and dimedone prompted us to readdress the synthetic strategy.

3.3 A new synthesis of 2-acetyldimedone

In accordance with the conditions used for *C*-acylation of Meldrum's acid, dimedone, DCC and DMAP (1:1.2:1) were allowed to stir at room temperature in dichloromethane (DCM). A precipitate of DCU was soon formed, and after overnight reaction work-up afforded 2-acetyldimedone (**92**) as a pale yellow crystalline solid in an 80% yield. During the reaction, TLC analysis revealed that no *O*-acetyl dimedone formation occurred. This observation led us to postulate that mechanistically, the presence of the DMAP auxiliary nucleophile promoted *C*-acylation *via* attack of the soft 'Lewis base'

carbon centre of dimedone at the activated acid (Scheme 53).



(92)

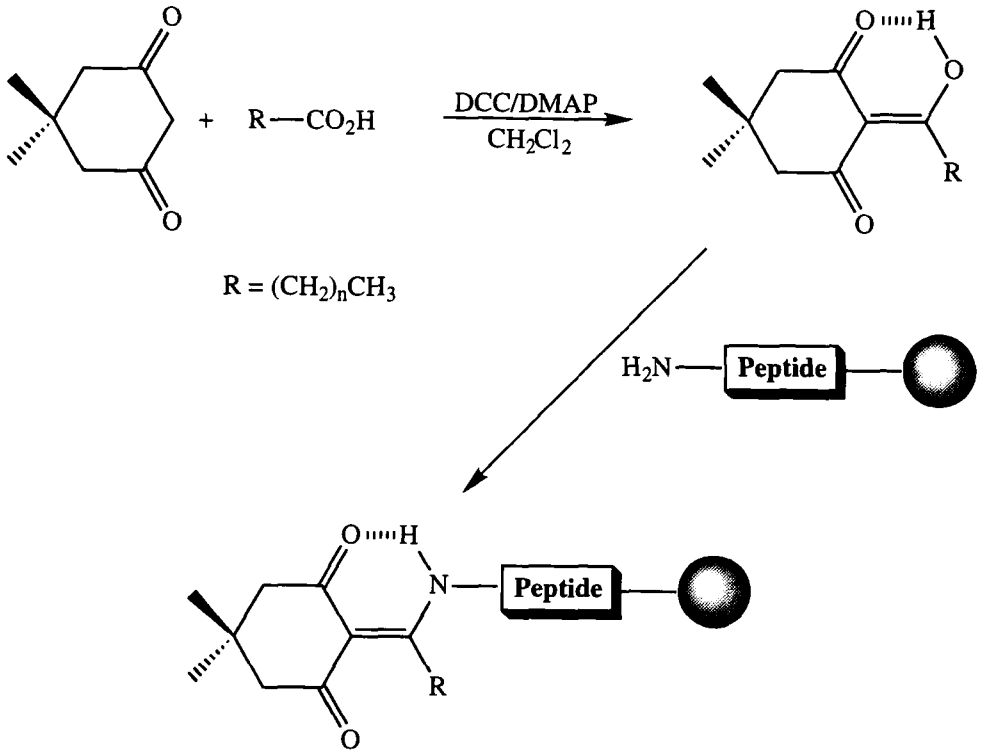
Scheme 53. DCC/DMAP mediated acetylation of dimedone

The significant amount of *O*-acylation observed with the original procedure, or alternatively treatment of dimedone with acetyl chloride,[234] is presumably a result of the hard enolic oxygen anion participating as the predominant nucleophile towards, in these examples, the hard carbonyl centre of the acid anhydride or chloride.

3.4 Development of a hydrophobic chromatographic probe

Encouraged by this result, it was felt that replacement of acetic acid in

the above scheme by a long chain fatty acid, would generate a hydrophobic chromatographic probe that could be coupled to the *N*-terminus of a resin bound peptide chain without the requirement for pre-activation (Scheme 54).



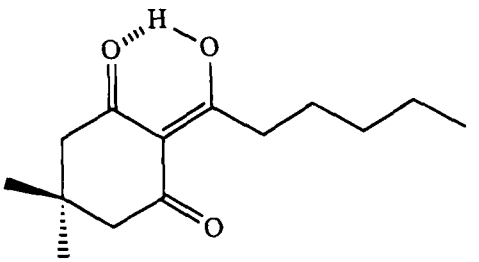
Scheme 54. Synthesis of a hydrophobic chromatographic probe and subsequent *N*-acylation of a resin-bound peptide.

Hexanoic acid was chosen, as it was envisaged that a five carbon chain would endow a substantial increase in the capped peptides hydrophobicity, without compromising its solubility.

3.4.1 Synthesis of 2-hexanoyldimedone

Using analogous conditions used to synthesise 2-acetyldimedone;

dimedone, hexanoic acid, DCC and DMAP were allowed to stir overnight in dry DCM at room temperature. Filtration of the *N,N'*-dicyclohexylurea, and acid/base work-up afforded 2-hexanoyldimedone (**93**) as a yellow oil, single spot by TLC with an overall yield of 84%. Mass spectral and ^{1}H NMR analysis confirmed the structure, with the latter showing an extremely low field singlet at δ18.14 expected for the strongly hydrogen-bonded enol proton.



(**93**)

3.4.2 On resin *N*-acylation of the angiotensin receptor binding protein fragment using 2-hexanoyldimedone

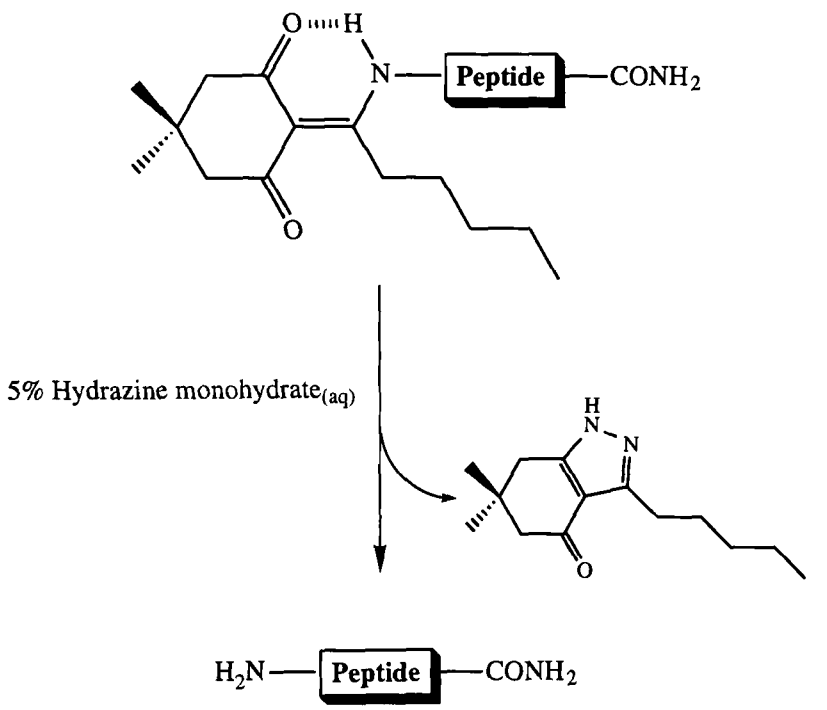
The eight residue angiotensin receptor binding protein fragment was chosen as the test sequence to validate the ability of 2-hexanoyldimedone to acylate the *N*-terminus of a resin-bound peptide. The peptide was synthesised by standard Fmoc SPPS, with RPHPLC analysis of the crude product showing it to be one major peak (*ca.* 98% purity), with the gradient conditions employed eluting the peptide after 7.5 min. The purified peptide was characterised by +ve ES-MS.

A portion of the resin, with the peptide still attached, was incubated overnight with a four fold excess of 2-hexanoyldimedone in DMF, showing a negative TNBS test the following morning. Consequently, a portion of the

resin was cleaved and side-chain deprotected to afford a white amorphous powder which was subsequently demonstrated by RPHPLC, identical to the gradient used for the free peptide, to be one major peak eluting at 16.8 min. The purified peak was characterised by +ve ES-MS as the *N*-acylated peptide. It is felt that the significant (9.3 min in this example) shift in retention time is sufficient to separate any required peptide from the more hydrophillic truncated impurities obtained from the synthesis of a difficult sequence.

3.4.3 Deprotection and recovery of the target peptide

Removal of the hydrophobic probe under mild conditions was required to completely substantiate this reagent. Therefore, the *N*-acylated peptide was allowed to stir in 5% hydrazine monohydrate$_{(aq)}$ and the deprotection monitored by RPHPLC (Scheme 55).



Scheme 55. Hydrazine mediated deprotection of the hydrophobic probe.

After 30 min the complete removal of the hydrophobic probe was evident. TFA was then added dropwise to the cooled solution until pH 2 was attained. The solution was desalted and evaporated to dryness, with ether trituration affording the product as a white amorphous powder in almost quantitative yield. The ether soluble heterocyclic deprotection byproduct being removed during trituration. The RPHPLC of the crude deprotection product indicated one major peak, of the original retention time 7.5 min., with the purified peak being characterised by +ve ES-MS as the angiotensin receptor binding protein fragment. The three RPHPLC traces of the original peptide, *N*-modified and post deprotection are displayed overleaf (Fig.4).

In summary, we have described the one-pot synthesis of a reversible hydrophobic chromatographic probe, which can quantitatively acylate the *N*-terminus of a resin bound peptide without the need for any form of pre-activation. Post-purification removal of the probe with 5% hydrazine monohydrate$_{(aq)}$ provides a mild, inexpensive and convenient procedure for recovery of the required peptide in almost quantitative yields.

3.5 Development of an affinity chromatographic probe

The facile synthesis of the hydrophobic probe from the dimedone and hexanoic acid precursors suggested that any reagent bearing a carboxylic acid could also be coupled onto the cyclic 1,3-diketone template to generate a raft of potential chromatographic probes. The well established non-covalent interactions displayed between biotin and avidin,[223,224] has been previously exploited in affinity probes; as indicated in the introduction to this chapter.

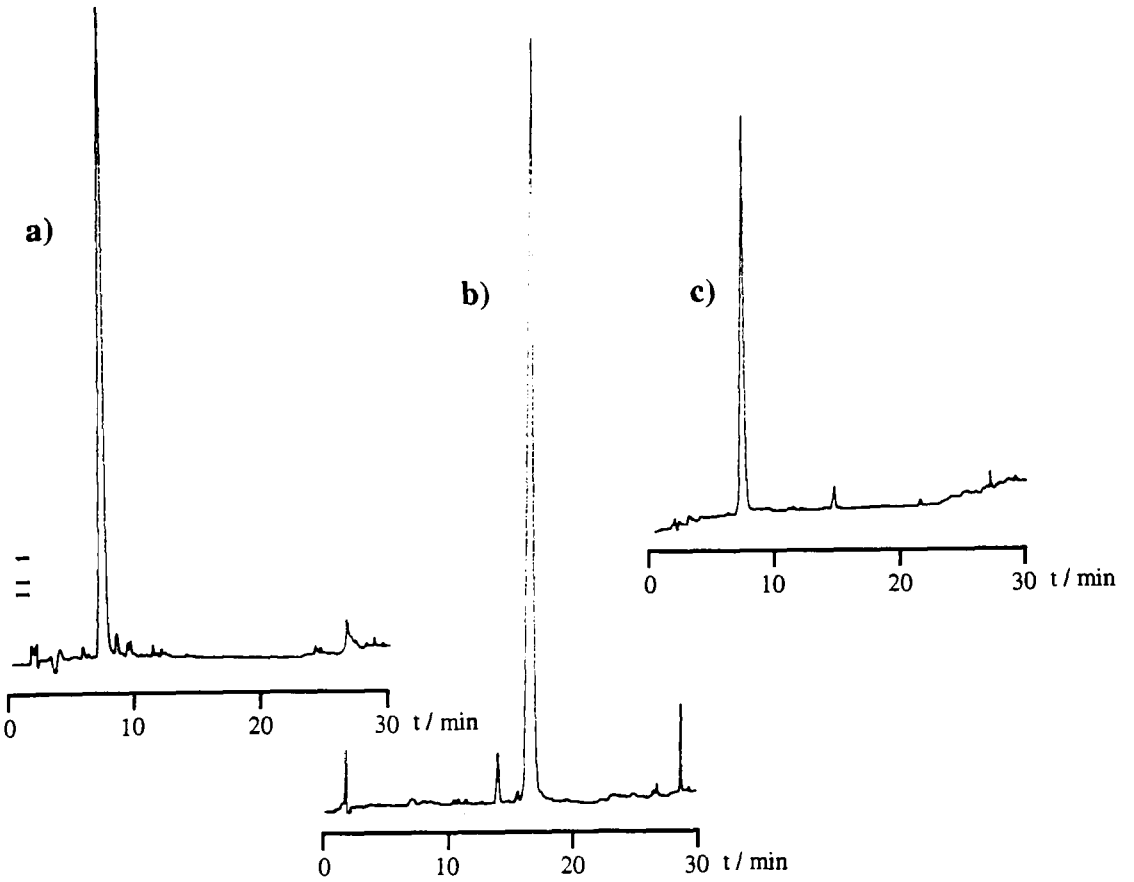
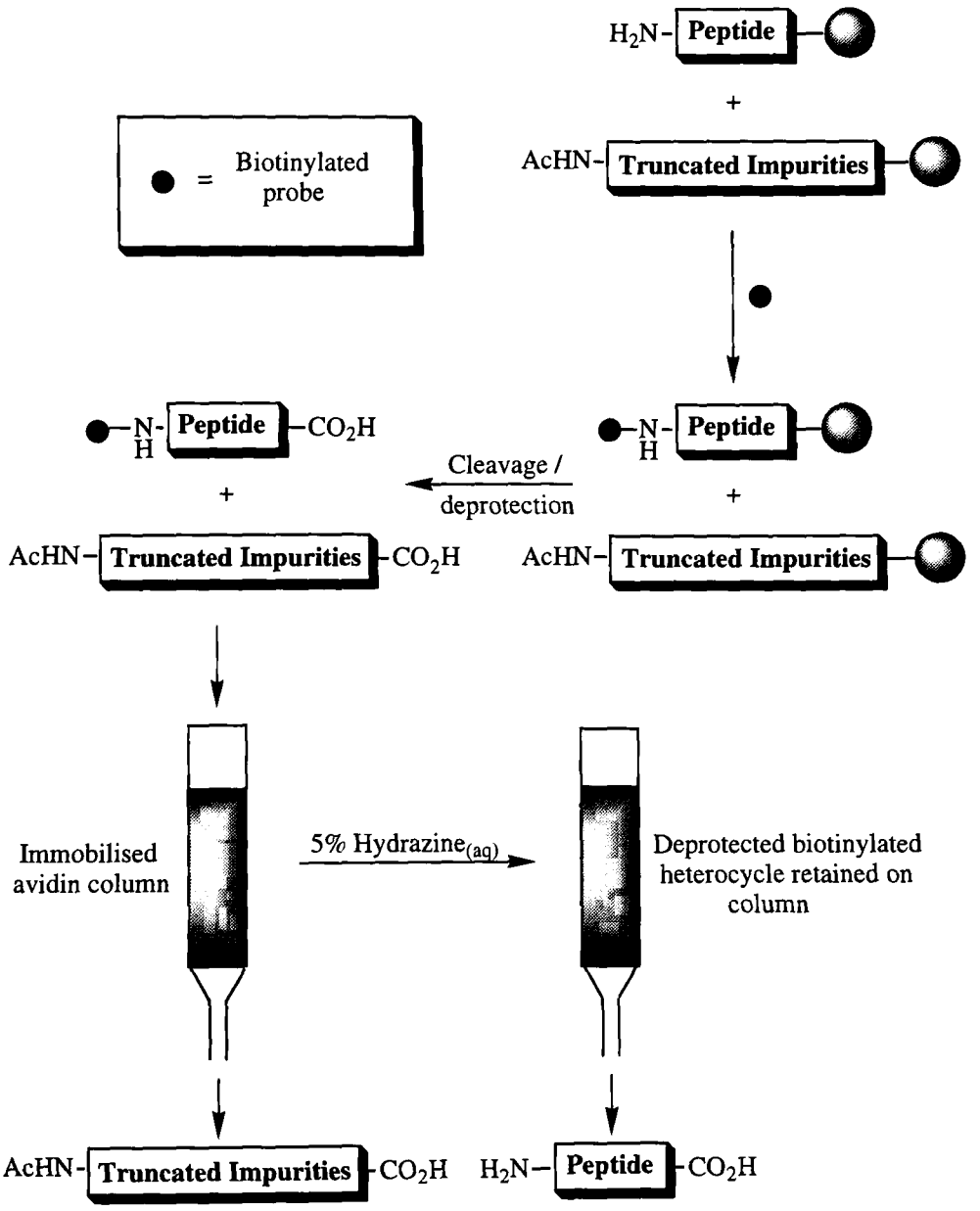


Figure 4. RPHPLC traces of (a) angiotensin receptor binding protein fragment; (b) *N*-terminal modified angiotensin receptor binding protein fragment; (c) crude hydrazine deprotection product.

However, the synthesis of these reagents is cumbersome and often protracted, with only moderate overall yields. The methodology we have developed allows the synthesis to be complete in one step.

We envisaged that acylation of the *N*-terminus of a resin bound peptide, containing truncated capped impurities, with biotinylated dimedone should be possible without prior activation. Application of the crude cleavage product to an immobilised avidin column should result in retention of the *N*-modified peptide, whilst the truncated impurities elute off. The pure peptide could then

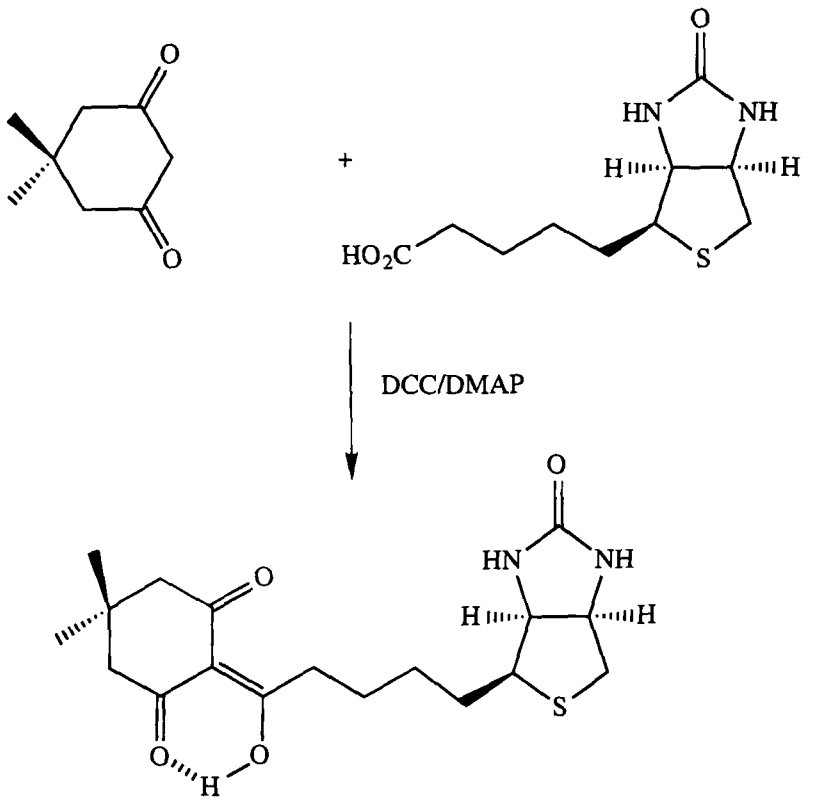
be recovered by treating the avidin column with a 5% hydrazine$_{(aq)}$ solution, thereby leaving the biotinylated heterocyclic byproduct attached to the column (Scheme 56).



Scheme 56. Immobilised avidin affinity column purification of a reversibly biotinylated peptide.

3.5.1 Synthesis of 2-biotinyldimedone (94)

It was envisaged that the DCC/DMAP mediated acylation of dimedone with biotin should afford 2-biotinyldimedone (**94**) in high yield (Scheme 57). However, due to the poor solubility displayed by biotin in DCM, the reaction was extremely slow. Replacement of DCM with DMF overcame the solubility problem and after allowing the reaction to proceed for 48 h., work-up afforded 2-biotinyldimedone as a white crystalline solid in 69% yield.



(**94**)

Scheme 57. Synthesis of 2-biotinyldimedone

The compound was fully characterised by ^{1}H NMR, ^{13}C NMR and accurate FAB-MS and microanalysis. RPHPLC confirmed the compound to be >99% pure.

3.5.2 On resin *N*-acylation of the angiotensin receptor binding protein fragment using 2-biotinyl-dimedone (94)

Once again, the eight residue angiotensin receptor binding protein fragment was chosen as the peptide with which to test the validity of the biotinylating reagent. Attached to NovaSyn®KR 125 resin, the side-chain protected fragment was incubated overnight with a four-fold excess of 2-biotinyldimedone, producing a negative TNBS test. A portion of the resin was cleaved and the side-chain deprotected peptide was analysed by RPHPLC. The trace indicated the product to be one major peak, confirming quantitative incorporation of the probe onto the resin bound peptide. +ve ES-MS analysis of the purified peak exhibited the correct mass for the *N*-biotinylated peptide.

3.5.3 Immobilisation and deprotection of the biotinylated angiotensin binding protein fragment on an immobilised avidin column

An immobilised avidin column (Pierce) was equilibrated with phosphate buffered saline (pH 7.5). The crude biotinylated peptide was dissolved in the same buffer solution and run onto the column. The column was allowed to incubate for 1 h, and was then washed with ten column volumes of binding buffer. RPHPLC analysis of the washings indicated complete loss of the biotinylated peptide peak (Figure 5).

The column was treated with 5% hydrazine monohydrate$_{(aq)}$ (5 column volumes, each allowed to incubate for 5 min.) and the eluent collected into a 10% acetic acid solution. Subsequent de-salting of the eluent and RPHPLC

analysis exhibited one major peak of identical retention time to the required peptide, Fig. 5. The purified product was characterised by +ve ES-MS and confirmed as the angiotensin receptor binding protein fragment.

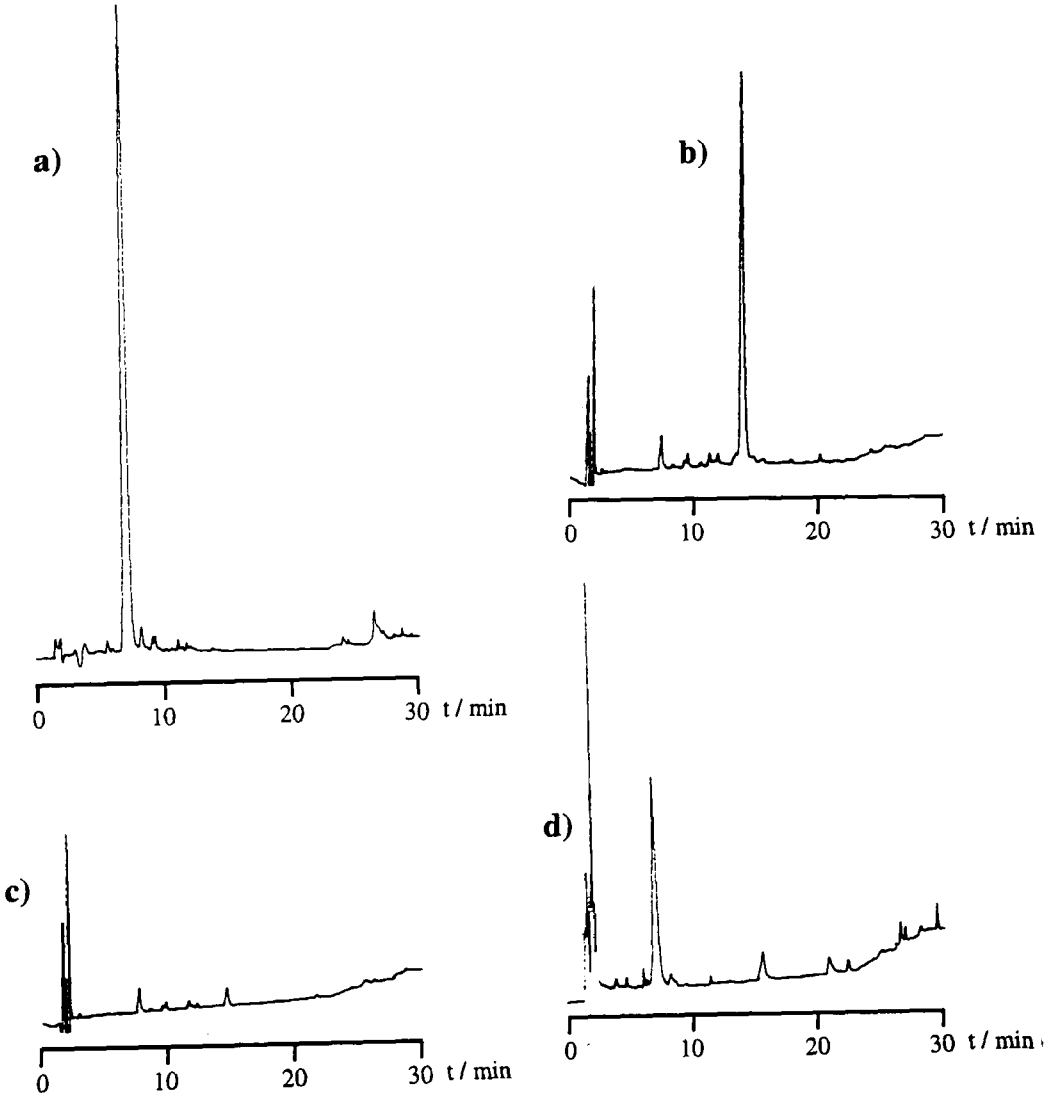
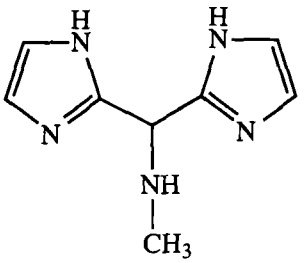


Figure 5. RPHPLC traces of (a) angiotensin receptor binding protein fragment; (b) *N*-biotinylated angiotensin receptor binding protein fragment in binding buffer; (c) post incubation column washings; (d) crude hydrazine deprotection product.

In summary, we have described a facile and high yielding one-pot synthesis of 2-biotinyldimedone (**94**), a reversible biotinylating reagent which can be quantitatively incorporated onto the *N*-terminus of a resin bound protected-peptide fragment without the requirement of pre-activation. Following immobilised avidin affinity chromatography, 5% hydrazine monohydrate$_{(aq)}$ mediated deprotection provides a mild and efficient means of peptide recovery.

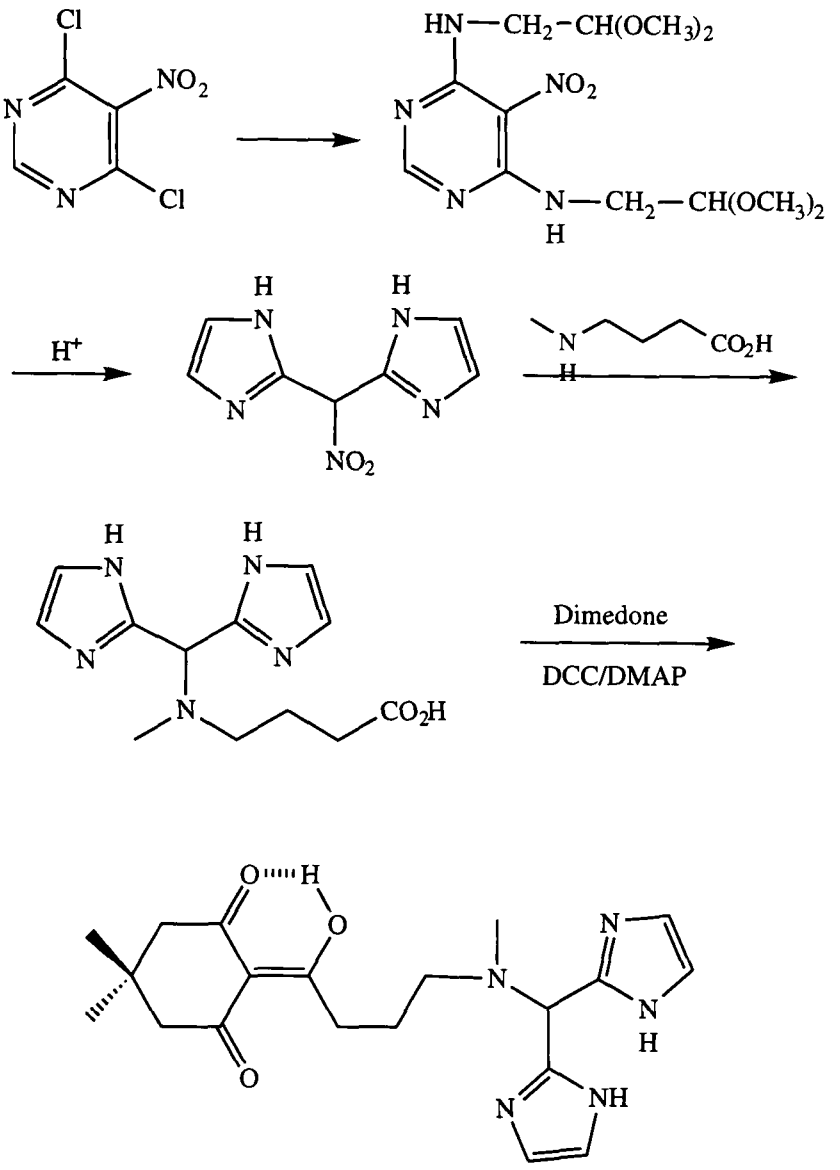
3.6 Future work

The facile nature of both the synthesis and use of these dimedone derived reagents offers wide exploitation. Recently, a *bis*-imidazole reagent, *bis*-(imidazo-2-yl)methylaminomethane,[235] (bimam) (**95**) was anchored to solid support and subsequently demonstrated to display an excellent affinity for Cu^{2+} ions.



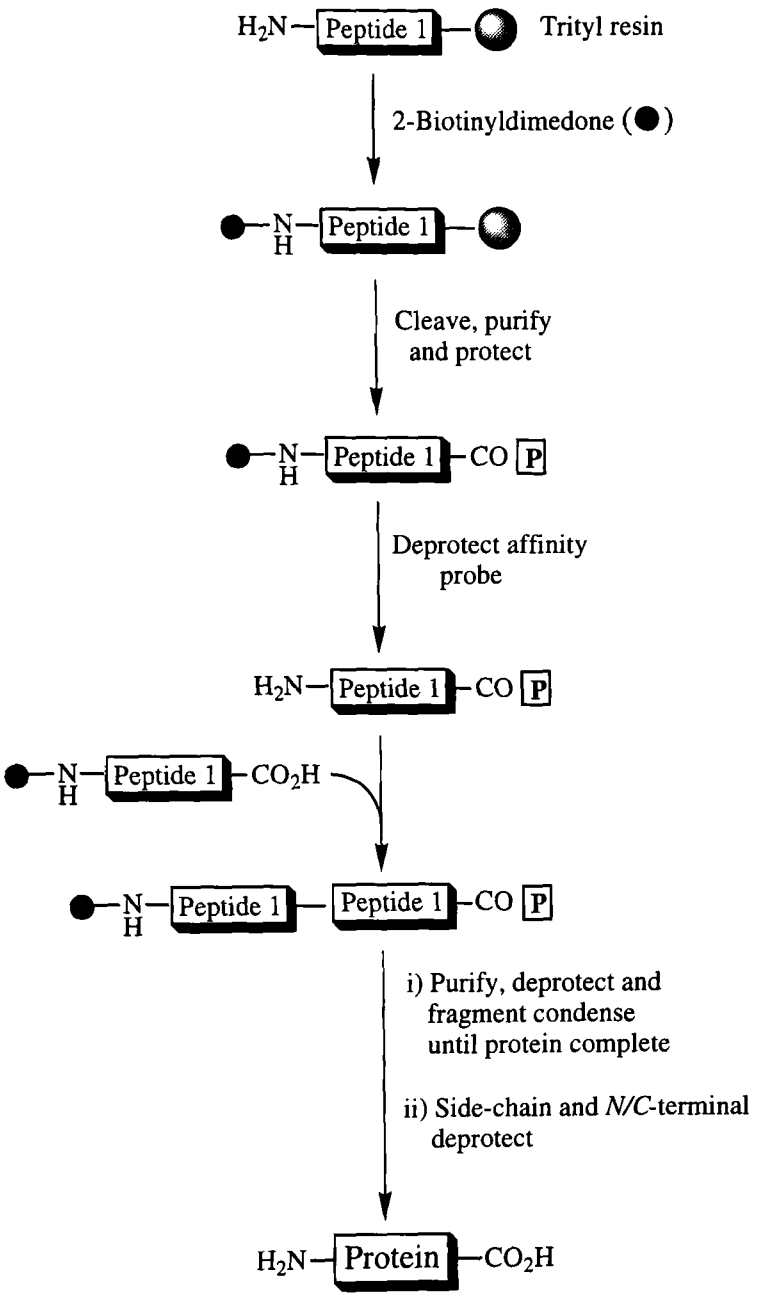
(**95**)

It is envisaged that slight modification of the molecule, in order to incorporate a carboxylic acid containing linker, would furnish a molecule that once coupled to dimedone, could be utilised as an affinity reagent exploiting the pH reversible binding it would display to an immobilised Cu^{2+} ion column (Scheme 58).



Scheme 58. Potential exploitation of the bimam Cu^{2+} affinity reagent as a reversible peptide purification probe.

Another exciting application of the reversible affinity chromatographic probes would be the ability to rapidly purify protected peptide fragments for subsequent segment condensation towards protein synthesis (Scheme 59).



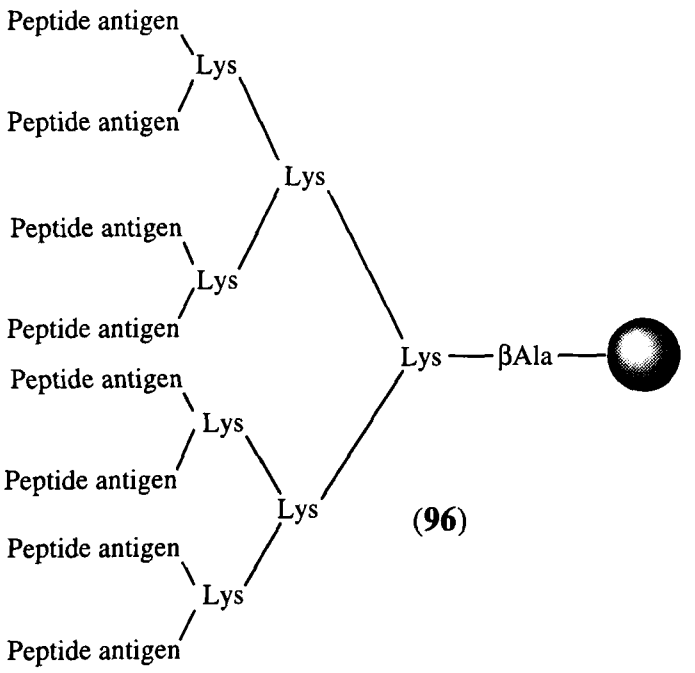
Scheme 59. Hypothetical route for protein synthesis *via* rapid purification of protected peptide fragments using the reversible biotinylating reagent 2-biotinyl-dimedone (**94**).

4

Synthesis of a Multiple Antigenic Vector, Expressing a Non-Peptidic Hapten, *via* Solid Phase Methodology

4.1 Introduction

Work pioneered by Tam *et al* during the late eighties introduced the concept of multiple antigenic peptides (MAPs) as a means of eliciting the generation of anti-peptide antibodies.[236] The ability to express multiple copies of the required hapten on a poly-lysine framework (**96**) via SPPS methodology, complemented the previous protocols of coupling such peptidic/non-peptidic immunogens onto carrier proteins such as keyhole limpet haemocyanin (KLH) or bovine serum albumin (BSA).[237]



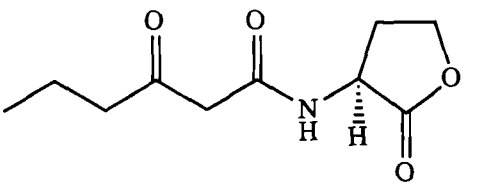
(**96**)

The MAP system has been demonstrated as extremely valuable in experimental vaccine development,[238-242] with the inherent advantage of enhanced reproducibility, not possible with the protein bound peptide antigens.

These encouraging reports prompted our research group to explore this methodology in an attempt to raise antibodies to the *N*-β-ketoacyl-L-homoserine lactone structure of bacterial autoinducers such as *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL) (**97**).

4.2 OHHL; structure and function

Research within this Department, focussed towards the elucidation of the biosynthetic pathway responsible for carbapenem antibiotic production (see Chapter 5) in the Gram-negative plant pathogen *Erwinia carotovora* , uncovered a small molecular weight, diffusible substance that regulated carbapenem synthesis in a cell-density dependent fashion.[243,244] Spectroscopic analysis of the molecule confirmed that it was identical to the pheromone responsible for bioluminescence control in the unrelated marine bacteria *Vibrio fisheri*; namely OHHL (**97**).



(**97**)

4.2.1 Autoinduction of bioluminescence in *Vibrio fisheri*

Before the discovery of OHHL in *Erwinia carotovora*, the only reports of its

existence were as a pheromone controlling bioluminescence in the marine bacteria *Vibrio fisheri*.[245,246] The light emission is catalysed by a collection of enzymes known as luciferases, whose only common feature is their requirement for molecular oxygen.[247,248] The process involves intracellular oxidation of a long-chain aliphatic aldehyde (e.g. tetradecanal) and the reduced form of flavin mononucleotide ($FMNH_2$) by luciferase, with the free energy liberated as blue-green light.

$$RCHO + FMNH_2 + O_2 \xrightarrow{\text{Luciferase}} RCO_2H + FMN + H_2O + h\nu$$

A 9 kilobase-pair gene was isolated from *V. fisheri* and demonstrated to encode all the functions required for light production and regulation (Figure 6).

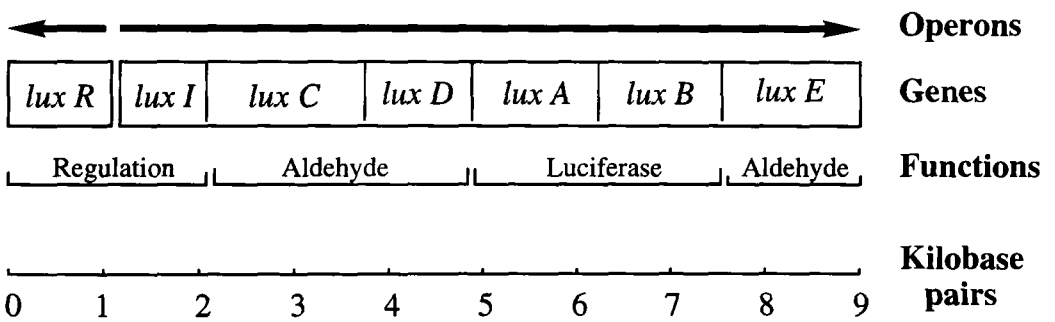


Figure 6. Organisation and function of *lux* genes cloned from *V. fisheri.* (adapted from[249])

Lux A and *lux B* code for the luciferase enzyme, *lux C, D* and *E* code for the sub-units of a fatty acid reductase, whereas *lux R* and *I* are involved in regulation of *lux* expression. Bioluminescence in *V. fisheri* only occurs in dense bacterial cultures, as then, the pheromone or autoinducer can attain the

threshold concentration required to initiate transcription of the *lux* gene. The pheromone was established as OHHL (**97**) by Eberhard *et al*,[250] and has subsequently been shown to be generated from the *lux I* gene product.[247,248] OHHL then binds to and activates the LuxR protein, with the OHHL-Lux R adduct stimulating transcription of the operon indicated above (Figure 6). Since the first gene located within the sequence produces the Lux I protein, transcription results in increased levels of OHHL, ensuring a positive feedback circuit that generates a substantial and rapid response to a small initial stimulus (Figure 7).

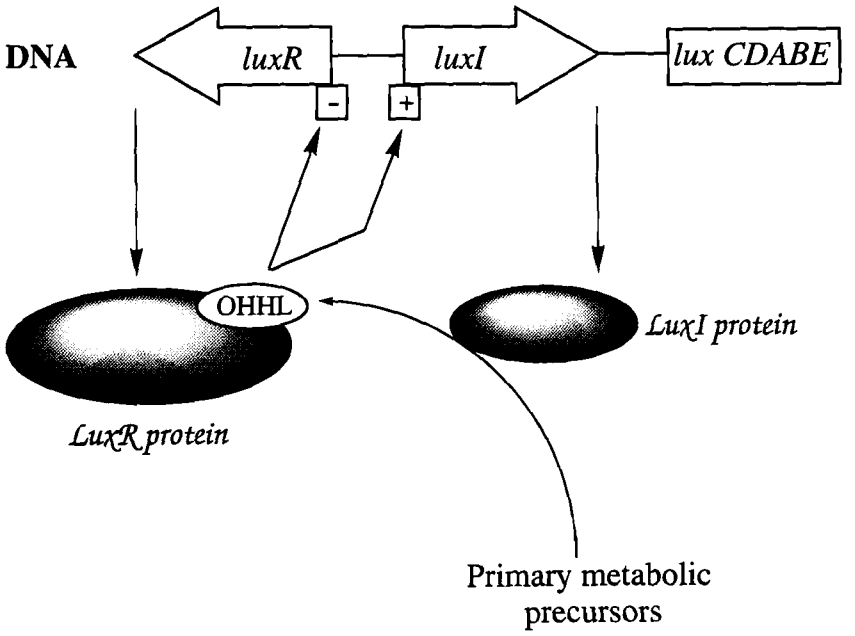


Figure 7. Regulation of the *lux* operon in *V. fisheri* by the OHHL autoinducer.

4.2.2 Other bacteria possessing autoinducer molecules

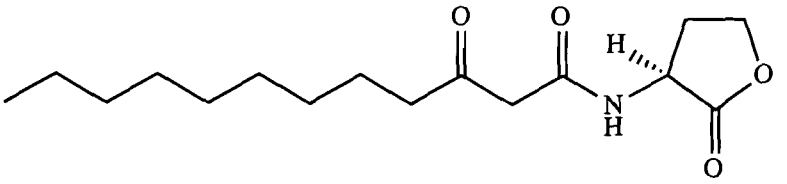
The unexpected discovery that carbapenem biosynthesis within a terrestrial bacterium was controlled in a similar fashion, and by exactly the same

autoinducer, as the bioluminescent pathway of a few marine bacteria, implied that other bacteria employed this mode of intracellular communication. Using recombinant technology, an *E. coli* vector was constructed to bioluminesce only in the presence of exogenous OHHL and a long chain fatty aldehyde.[244,251] Studies using this vector demonstrated that over 20 different Gram-negative bacterial cultures could induce bioluminescence. For *Pseudomonas aeruginosa, Serratia marcescens, Erwinia herbicola, Erwinia carotovora, Enterobacter agglomerans and Yersinia enterocolitica,* the inducer molecule was characterised by IR, MS and ^{1}H NMR to be OHHL.[251]

An indication that OHHL plays a pivotal role in the pathogenesis of human infection has come from studies associated with the opportunistic pathogen *Pseudomonas aeruginosa*. This bacterium can infect almost any body site given the correct predisposing conditions, and now displays multiple resistance to most commonly employed antibiotics. Secretion of extracelluar toxic factors, such as an exotoxin which inhibits protein synthesis in mammalian cells and various tissue damaging enzymes, e.g. an alkaline protease and an elastase, is key to its pathogenicity. The latter enzyme has been demonstrated to be the product of a gene termed *lasR*, and shares significant amino acid sequence homology with LuxR.[252] The well established observation of the growth phase dependency of elastase production, coupled with the discovery in our Department that *P. aeruginosa* produces OHHL, strongly suggested the involvement of OHHL, or structurally similar compounds, in the control of this virulence determinant.

It has recently been shown that *P. aeruginosa* produces another *N*-acyl homoserine lactone, *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL)

(**98**), also involved in autoinduction pathways.[253]



(**98**)

The similarity of the OHHL and OdDHL structures, coupled with work carried out in our Department,[254] led us to postulate that the *N*-β-ketoacyl-L-homoserine lactone was the key structural feature responsible for the molecules activity, with the alkyl chain providing the major feature of family/species diversity. Interference with the synthesis or transmission of these molecular messengers therefore presents an ideal target for the treatment or prevention of infection. However, a means of rapidly screening bacteria that produce such molecules was not yet available.

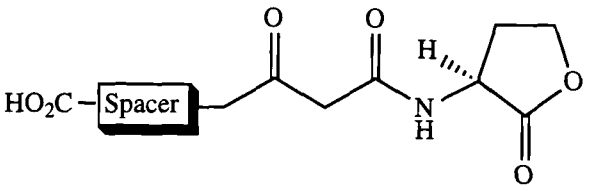
It was envisaged that generation of a monoclonal antibody designed to recognise the *N*-β-ketoacyl-L-homoserine lactone molecule, should therefore furnish a biological probe that could be used to screen any bacterial colony for the presence of compounds containing this particular chemical entity. The poly-lysine structure described earlier in this chapter was felt to be the ideal vector system with which to attempt this antibody generation, with the overall approach presenting two important advantages;

i) The reproducibility of the immunogenic reagent.

ii) The potential to use the resin bound MAP type structure as an immobilised affinity column for both isolation and purification of the required antibody from a polyclonal source.

4.3 Synthesis of a *N*-β-ketoacyl-L-homoserine lactone containing hapten

Construction of the desired MAP type structure, incorporating the 3-oxoalkanoyl homoserine lactone molecule, required the synthesis of the latter carrying a spacer and a carboxyl functionality (**99**) to acylate the amino residues of the poly-lysine MAP framework. The spacer was required to separate the hapten molecule from the steric bulk of the MAP macromolecule.

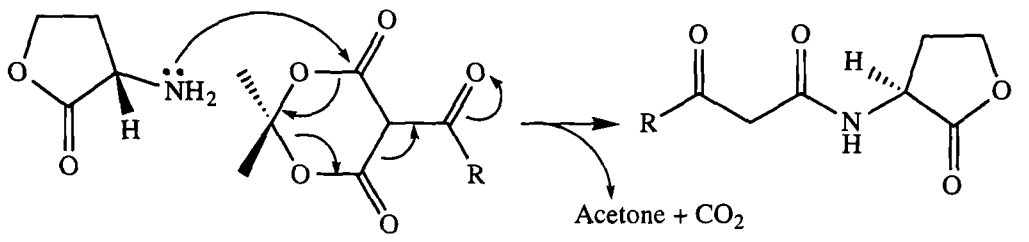


(**99**)

The 3-oxoacylated homoserine lactone has been reported,[254] and involves the cumbersome preparation of a 3-oxoalkanoic acid *via* acylating malonic ester, 3-oxo ketalisation, amine acylation and deprotection. Obviously, this method would have been even lengthier in our case, hence we sought to prepare (**99**) by a different strategy.

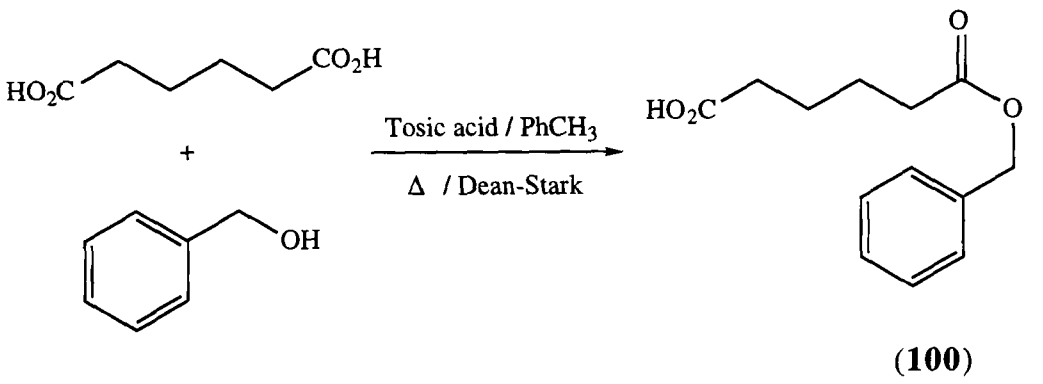
The synthesis of β-ketoesters via alcoholysis and spontaneous decarboxylation of *C*-acylated Meldrum's acid is well documented.[233] It was envisaged that replacement of the alcohol with the amine of L-homoserine lactone would instead afford the β-ketoamide structural unit characterising molecules such as OHHL (Scheme 60).

The DMAP catalysed *C*-acylation of Meldrum's acid using a carbodiimide activated carboxylic acid has been reported to proceed with excellent yields.[255]



Scheme 60. β-Ketoamide synthesis.

Acylation of Meldrum's acid with mono-protected adipic acid and aminolysis of the resultant molecule with L-homoserine lactone would furnish the required molecule with adequate spacer length. Adipic acid was therefore monoprotected as its benzyl ester (**100**),[256] in a 60% yield (Scheme 61).

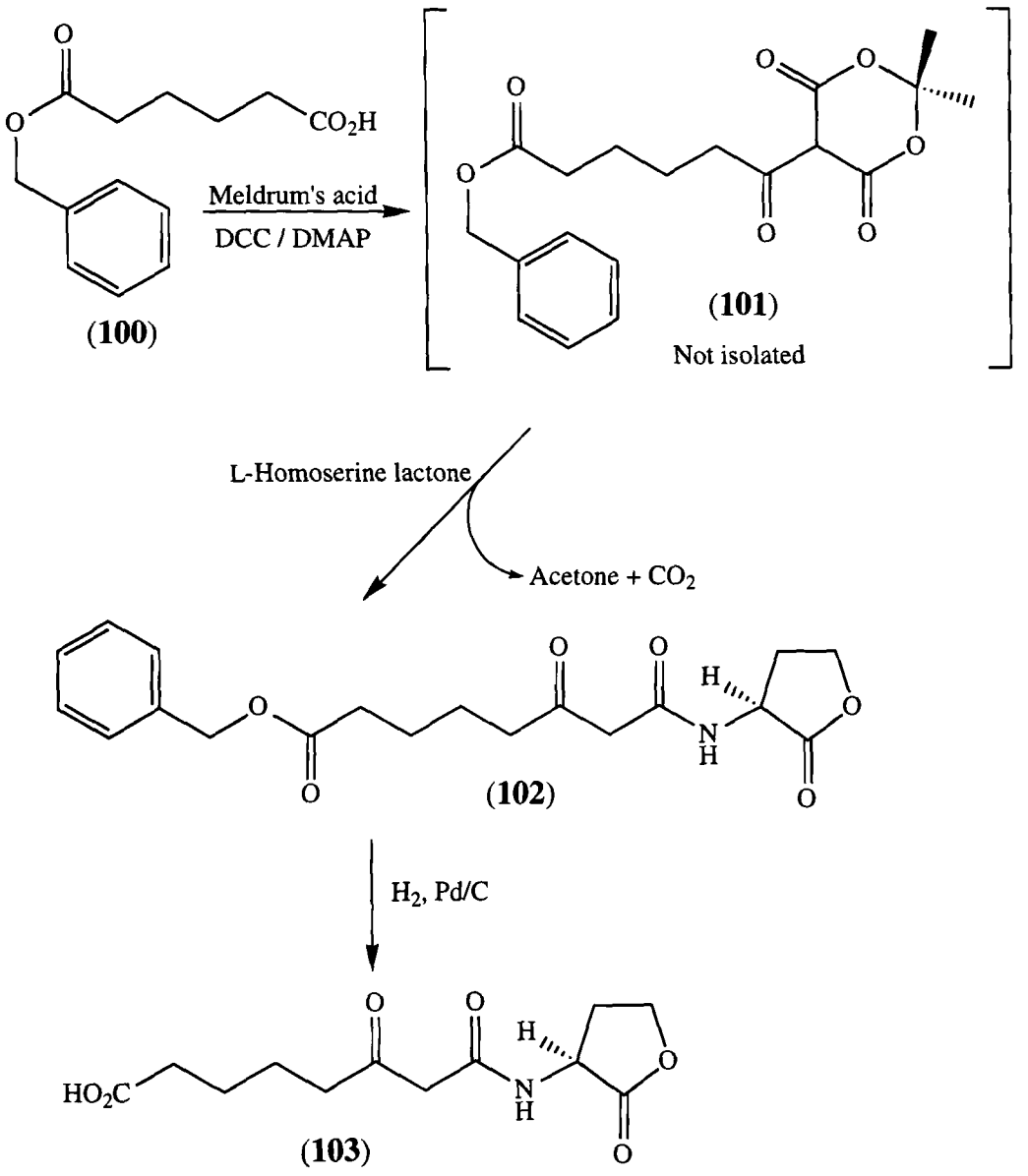


(**100**)

Scheme 61. Mono-benzyl adipate synthesis.

Benzyl ester protection was chosen as catalytic hydrogenolysis provided a convenient and mild means of deprotection, which should not effect any deleterious side reactions on the rest of the completed molecule. *C*-acylation of Meldrum's acid was achieved via DCC activation of the mono-benzyl adipate (**100**) with DMAP catalysis. The intermediate (**101**), without isolation, was treated with L-homoserine lactone in anhydrous acetonitrile, which after work-

up, afforded *N*-(7-benzyloxycarbonyl-3-oxoheptanoyl)-L-homoserine lactone (**102**) as a white crystalline solid with an overall yield of 42% obtained for the two steps (Scheme 62).



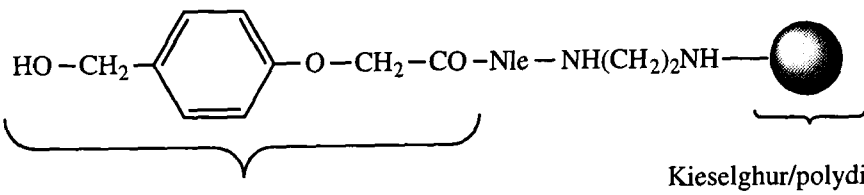
Scheme 62. Synthesis of *N*-(7-carboxy-3-oxoheptanoyl)-L-homoserine lactone.

The ^1H NMR of (**102**) displayed the characteristic singlet at δ3.45, expected for the carbonyl flanked methylene protons, with peak integration indicating a predominantly keto, rather than enol structure. FAB-MS and microanalysis of the product confirmed its overall purity. Catalytic hydrogenation removed the benzyl ester protection in almost quantitative yield, to afford the free acid (**103**) as a waxy solid. RPHPLC analysis revealed the product to be of single peak purity, ready for use in SPPS.

4.4 Initial attempt at the MAP synthesis

It was envisaged that a MAP core similar to that shown earlier (**96**), with four outermost lysine residues, should provide a vector capable of eliciting an immune response when injected with a suitable adjuvant. Two β-alanine residues, initially coupled to the resin, would also provide an adequate spacer between the poly-lysine core and the solid support to allow unhindered synthesis of the peptide skeleton.

A kieselguhr/polydimethylacrylamide composite support functionalised with the TFA labile HMPA linker, was used as the solid phase resin (**104**).[31]



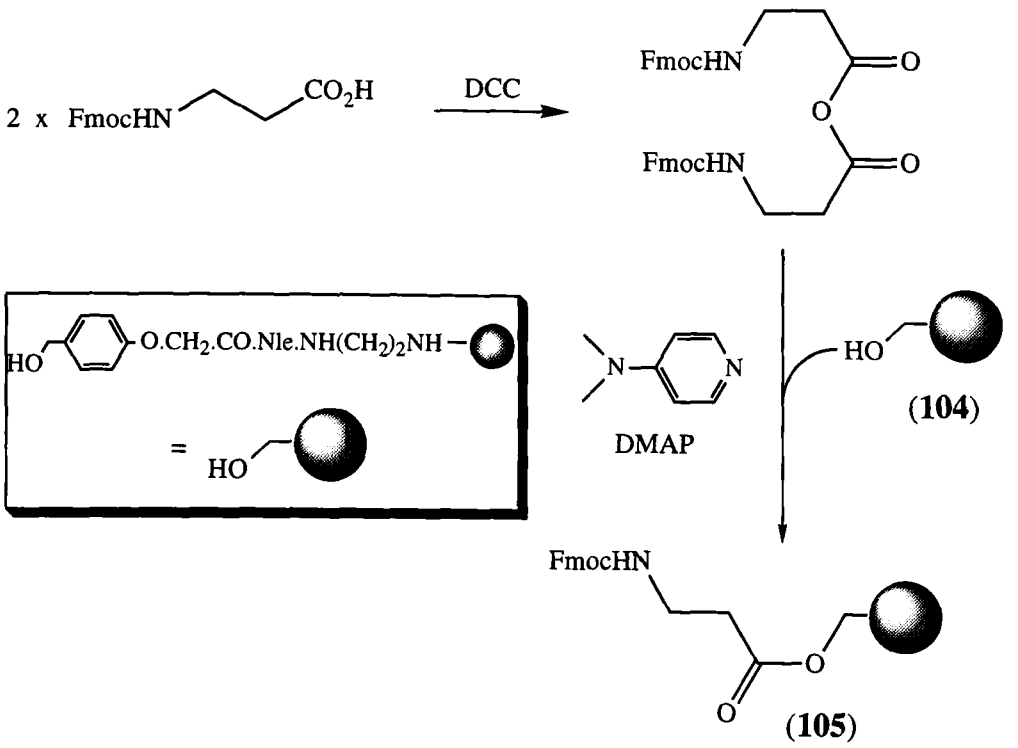
4-HMPA linker

Kieselghur/polydimethyl-acyrlamide resin support

(**104**)

Esterification of an *N*-protected amino acid onto a hydroxymethylated resin is readily achieved *via* the preformed symmetrical anhydride with DMAP catalysis.[257,258] Hence *N*-Fmoc-β-alanine was coupled to the resin with an

overall efficiency estimated to be 90% using the calibration plot provided,[259] (Scheme 63).



Scheme 63. First residue (*N*-Fmoc-β-alanine) attachment to an HMPA modified resin.

Using an LKB 4175 Biolynx manual peptide synthesizer, the Fmoc group was deprotected under continuous flow conditions using 20% piperidine in DMF. Acylation of the generated free amino group with a second *N*-Fmoc-β-alanine residue was effected *via* TBTU/DIEA/HOBt activation. A negative TNBS amine test indicated coupling was complete after 30 min. Piperidine mediated *N*-Fmoc deprotection was followed by incorporation of the Fmoc-Lys(Fmoc)-OH residue, which after Fmoc removal exposed the N^{α}-and N^{ε}-amino groups for subsequent acylation. Sequential addition and deprotection of

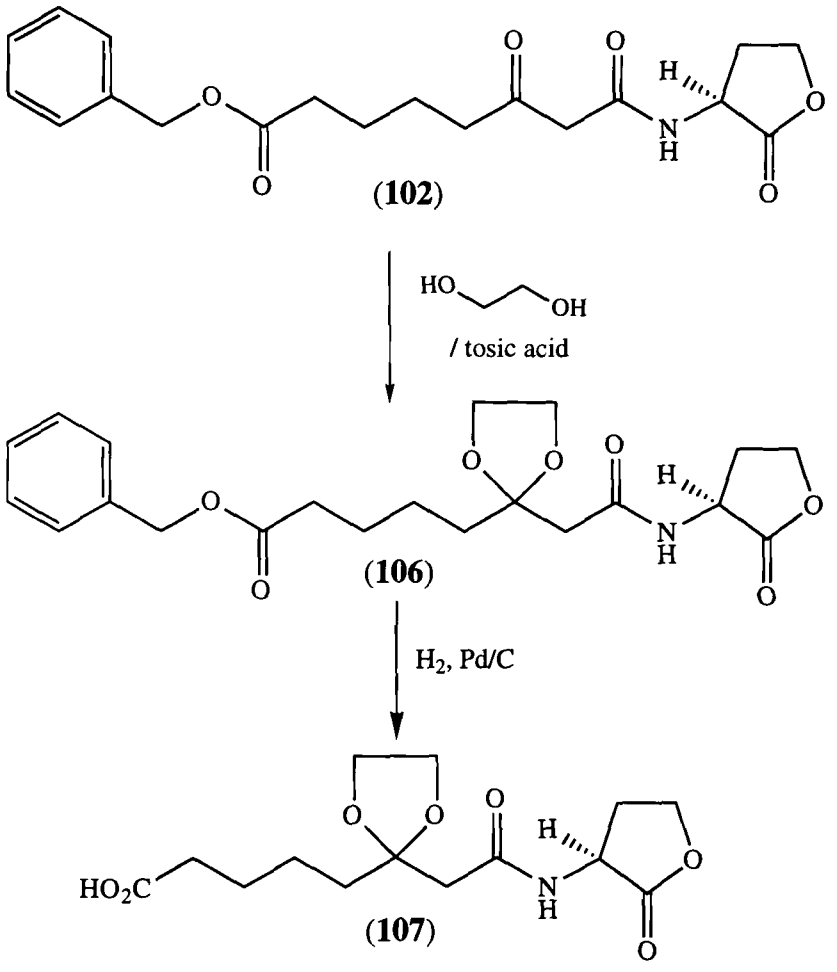
Fmoc-Lys(Fmoc)-OH residues to the growing peptide can therefore supply a poly-lysine core of any required dimension. As stated earlier, our synthesis was halted at the four lysine residue stage, allowing the incorporation of eight hapten molecules. Introduction of the *N*-β-ketoacyl-L-homoserine lactone containing hapten (**103**) was attempted using DIPCDI/HOBt activation. The TNBS amine test remained strongly positive even after extended double coupling procedures were attempted. This disappointing result dictated the requirement for further solution based experiments in an attempt to ascertain the reason for coupling difficulties.

4.4.1 Attempted coupling of hapten (103) to L-valine methyl ester

A solution based coupling of *N*-(7-carboxy-3-oxoheptanoyl)-L-homoserine lactone (**103**) to L-valine methyl ester was attempted using a DCC/HOBT activation procedure. After allowing the reaction to proceed overnight, TLC indicated mainly the presence of starting materials. Since the acylating species was an alkyl carboxylic acid, there was no mechanistic reason why the coupling should not proceed almost quantitatively. It was therefore postulated that the potential keto-enol tautomerism inherent within the molecule may be generating a conformationally restricted structure that is either deactivated or sufficiently hindered sterically to suppress activation and subsequent aminolysis. Temporary blockade of this enolisation *via* ketal formation, was hoped to improve the acylation efficiency.

The benzyl ester (**102**) was converted to the corresponding ethylene ketal (**106**) by the acid catalysed ketalisation with ethylene glycol in refluxing

benzene, with azeotropic removal of water achieved using Dean-Stark distillation apparatus (Scheme 64).



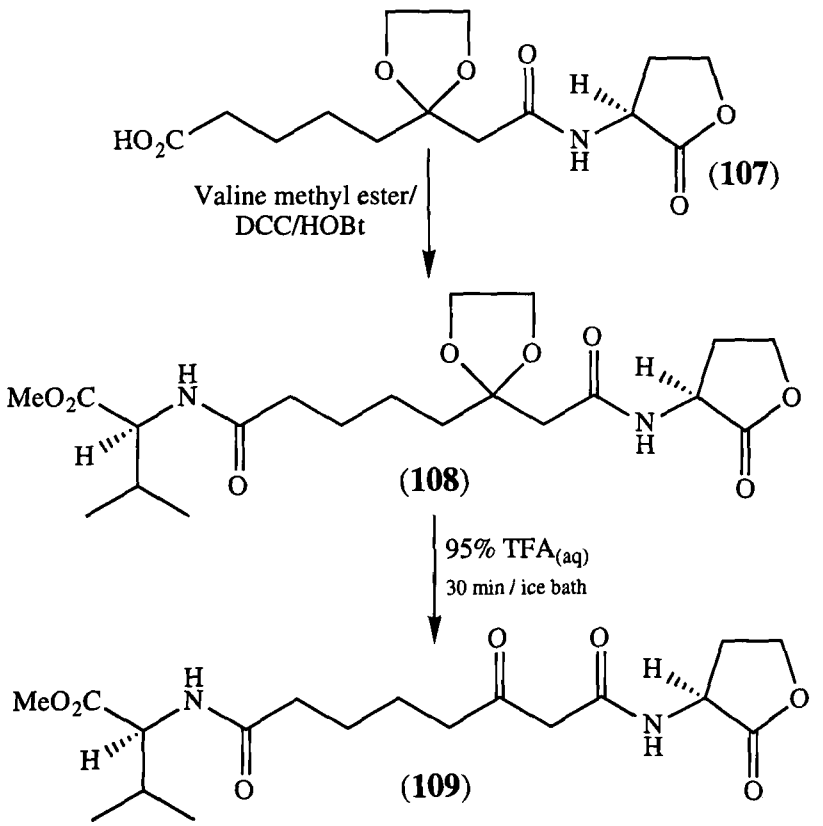
Scheme 64. Acid catalysed ketalisation of (**102**) and subsequent benzyl ester deprotection.

Work-up and purification by preparative thin layer chromatography (PLC) afforded the required compound as a colourless oil, with the ^1H NMR confirming the ketal incorporation by the characteristic singlet at δ 4.0 for the ethylene protons and the expected upfield shift of the neighbouring methylene

protons. Catalytic hydrogenation of (**106**) afforded the ketal protected free acid (**107**).

4.4.2 Attempted coupling of the ketal protected free acid (107) to L-valine methyl ester

The ketal protected free acid (**107**) was coupled to L-valine methyl ester *via* DCC/HOBt carboxy activation. In this instance, a DCU precipitate was rapidly formed, and monitoring of the reaction by TLC indicated its completion within four hours. Work-up afforded the desired *N*-acylated valine residue (**108**) as a colourless oil in a much improved 60% yield (Scheme 65).



Scheme 65. Coupling of the ketal free acid (**107**) to valine methyl ester and subsequent ketal deprotection.

If coupling to the MAP core could now be achieved, it was hoped that the resin cleavage conditions, 95% TFA, would concomitantly remove the ketal protection to yield the required hapten. To test this hypothesis, (**108**) was treated with 95% $TFA_{(aq)}$, and was subsequently shown by TLC monitoring to be deprotected within 30 min. (Scheme 65). Removal of TFA *in vacuo* followed by a simple aqueous work-up afforded (**109**) in a quantitative yield, with the ^1H NMR indicating complete loss of the ketal protons and a shift downfield of the now di-carbonyl flanked methylene protons.

4.5 Second attempt at the MAP synthesis

Using synthetic methodology analogous to that described in section 4.4, the MAP core was once again constructed on an HMPA modified kieselguhr/polydimethylacrylamide resin (**104**). Acylation proceeded more favourably with the ketal protection in place, yet TNBS monitoring and RPHPLC analysis of the poorly soluble crude cleaved product demonstrated that coupling was still not quantitative. Double coupling of the hapten failed to improve the situation and it was subsequently felt that the 60% coupling efficiency displayed in solution combined with potential steric factors inherent with the MAP core made this particular target unattainable.

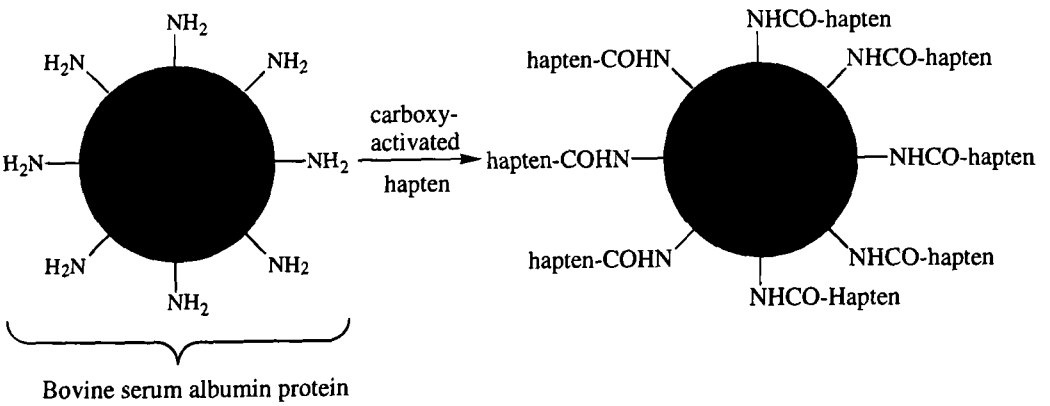
4.5.1 Synthesis of a 4-branch MAP core

In order to minimise the steric factors that may have been suppressing the overall coupling efficiency, it was envisaged that reduction of the MAP core to four available acylation sites should produce an immunogen of higher purity.

In this instance the synthesis of the title MAP core proceeded extremely smoothly, with the RPHPLC indicating predominantly one product, which was characterised by MALDI-TOF MS to be of the correct mass. However, it was generally regarded that the decrease in both expression of the non-peptidic hapten on the poly-lysine framework, and the overall reduction in molecular weight of the immunogenic vector would seriously compromise the ability of the compound to elicit antibody production. It was therefore decided to attempt to couple the MAP vector onto a carrier protein.

4.6 Attempted coupling of the MAP type vector to a carrier protein

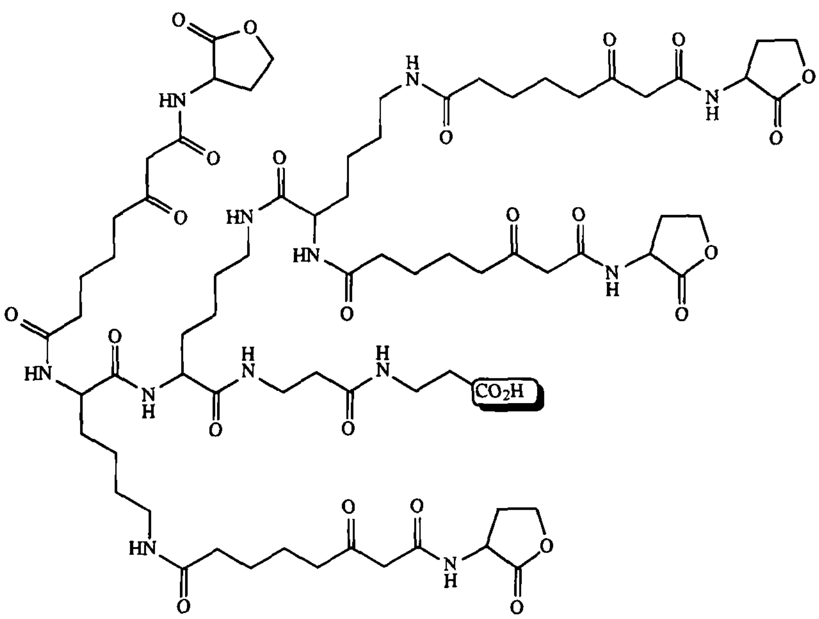
Bovine serum albumin (BSA) is one of the most frequently used carrier proteins with which to elicit antibody production towards small organic molecules. Lysine residues, located on the outer surface of the globular protein, provide an ideal acylation sites with which to couple a carboxy-bearing hapten (Scheme 66).



Scheme 66. Conjugation of a low molecular weight hapten to the lysine residues of BSA.

Various methods for hapten-protein conjugation are available,[260] however the base lability of the lactone ring dictated coupling in a mildly acidic pH. Water soluble carbodiimide carboxy activation was therefore employed, with the reaction performed in phosphate buffered saline with the pH held at 6.4.

Following overnight reaction, lyophilisation and subsequent MALDI-TOF MS analysis of the crude product failed to indicate any incorporation of the *N*-β-ketoacyl-L-homoserine lactone containing MAP vector. Once more, steric factors were potentially the greatest hinderance, with the carboxylic acid being almost completely obscured by the dendritic arms of the MAP framework (**110**).



(110)

It is likely that carboxy activation will occur, but approach of the *o*-acylisourea intermediate towards the globular protein surface will be disfavoured because of the potential inter-chain hydrophobic interactions that would occur between the MAP vector and the BSA amino acid side chains which extend into the solvent.

4.7 Exploitation of a solubilisable resin suitable for injection into experimental animals

With the synthesis of an 8-branch MAP system producing large impurities, and the inability to acylate a 4-branch MAP onto a suitable carrier protein, another option was inspired by a paper published by Sheppard *et al.*[261] A polymer support is described which contains cleavable cross-links, thereby making it suitable for SPPS but can subsequently be solubilised for immunisation. Novabiochem produced a similar resin, NovaSyn® KD 100, which consists of a polydimethylacrylamide/kieselguhr support functionalised with ethylene diamine (**111**).[262,263]

(**111**)

The peptide antigen is synthesised on the support and is permanently attached *via* an amide bond. In our case TFA mediated ketal deprotection would be followed by homogenisation of the resin and injection into a suitable experimental animal.

4.7.1 Synthesis of the MAP vector on NovaSyn® KD100 resin

Two β-alanine spacers were initially attached to the resin support *via* a TBTU/DIEA/HOBt coupling regime. Synthesis of the 4-branch poly-lysine core proceeded smoothly, as did the final addition of the ketal protected *N*-β-

ketoacyl-L-homoserine lactone hapten, with all couplings monitored until a negative TNBS amine test was obtained. Since the MAP vector was permanently attached to the resin support, it was impossible to characterise the final product. However, since our previous synthesis of this particular vector on the HMPA modified resin produced a relatively pure compound, it was felt that proper monitoring of each acylation step should furnish the expected resin bound immunogen.

On completion of the synthesis, incubation of the resin in 95% $TFA_{(aq)}$ for 2 h was regarded to be significantly long enough to remove the ketal protection. The resin was subsequently washed and dried overnight *in vacuo.*

4.7.2 Preparation and injection of the homogenised resin into experimental rabbits.

High power sonication of 20mg of peptide resin in 1 ml of water for injections, resulted in the formation of an milky white paste. Introduction of 1 ml of Freund's complete adjuvant and subsequent emulsification, provided enough injectable material for two experimental rabbits, both requiring six individual injection sites. Secondary boost injections with the same amount of resin were carried out week and one month after the initial injection. Bleeds were taken from the animals prior to the initial and subsequent booster injections.

4.7.3 Analysis of plasma samples for anti-*N*-β-ketoacyl-L-homoserine lactone antibodies

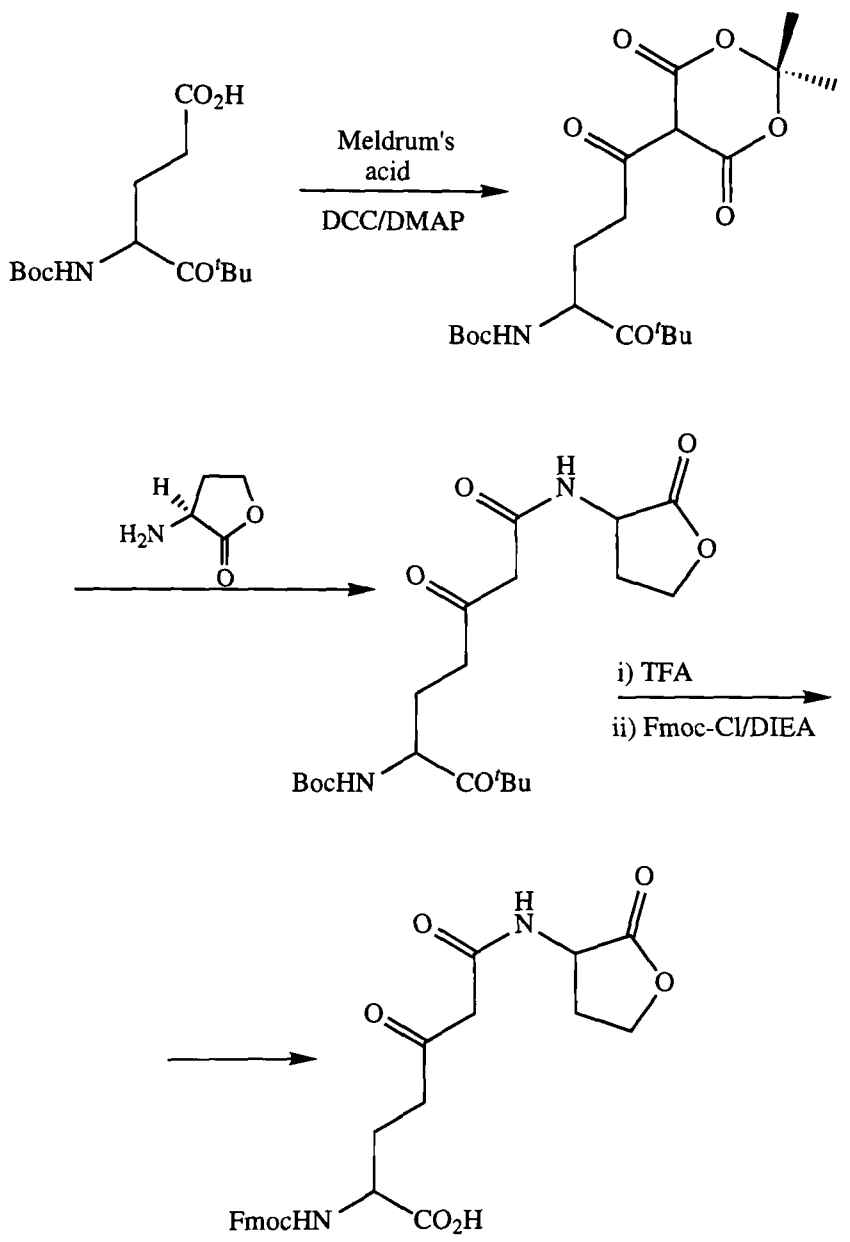
A nitrocellulose dot blot assay was performed on plasma samples from all three bleeds, but failed to indicate the presence of any anti-*N*-β-ketoacyl-L-

homoserine lactone antibodies. This result confirmed our fears that the copy number and hence the molecular weight of the immunogen was perhaps too low to illicit a measurable immune response.

4.8 Conclusions and future work

In summary we have described a facile and efficient novel synthesis of the key *N*-β-ketoacyl-L-homoserine lactone portion of many bacterial autoinducers. By including a carboxyl group at the terminus of the alkyl chain we successfully constructed a reagent that, once attached to the correct immunogenic vector, had the potential to raise antibodies to the above moiety. Attempts to generate an immunogenic vector *via* solid phase peptide synthetic techniques unfortunately proved unsuccessful, due probably to the overall low molecular weight of the completed construct.

To attain the requirement of antibody generation, future work should address glutamic acid as the reagent to initially acylate Meldrum's acid. Aminolysis of the resultant molecule using L-homoserine lactone and correct re-protection of the amino acid should afford a reagent which could employed to generate multiple copies of the required hapten on an appropriate immunogenic scaffold (Scheme 67).



Scheme 67. A Potential reagent for the SPPS of a MAP type structure incorporating multiple copies of the required *N*-β-ketoacyl-L-homoserine hapten.

5

Preliminary Solution Phase Synthesis of Methyl (3*S*, 5*R*) Carbapenam-3-carboxylate for Potential Transposition onto Solid Phase.

5.1 Introduction

Within the last decade, the extension of SPPS techniques to solid phase organic synthesis (SPOS) has advanced at an unprecedented rate.[264-269] The ability to perform a vast array of chemical transformations on a resin-bound synthon has allowed the generation of vast chemical libraries, for lead molecule identification within both the pharmaceutical and agrochemical industries.

Within our Department, the requirement for metabolic precursors of the biosynthetic pathway involved in production of the β-lactam carbapenem antibiotics, prompted us to address an asymmetric synthetic strategy that could:

i) provide the required biological target molecules.

ii) generate a library of side-chain and ring modified carbapenems upon transposition of the solution based synthesis onto solid phase.

5.2 The development of β-lactam antibiotics

Louis Pasteur's initial observation of antibiosis, i.e. the inhibition of one microorganism by products diffusing from another, heralded the birth of antimicrobial chemotherapy in 1877. The next significant advance was made in

1940 when Florey and Chain solved the problem of isolating the labile antibacterial substance from *Penicillium notatum* , named penicillin by Alexander Fleming in 1929. The efficacy of penicillin against a wide range of bacteria was demonstrated *in vitro* and was subsequently used to treat experimental infections in mice, with quite outstanding results. The problem of mass production was solved in the United States and purified penicillin was used to save many lives during the latter stages of the Second World War.

During the last fifty years the antibiotic field has continued to advance in a wide variety of chemotherapeutic areas, and throughout its development the ß-lactams have continued to contribute a major part. However, the battle between man and bacteria has never remained a one-sided battle. Many ß-lactam containing antimicrobials can be rendered inactive by ß-lactamase enzymes produced by various bacteria. Now, even many semi-synthetic derivatives have been rendered ineffective, encouraging the search for new compounds, e.g. the cephalosporins and most recently, the carbapenems.

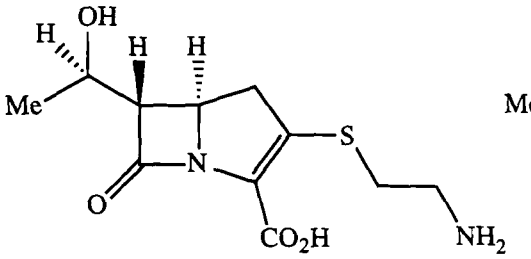
5.2.1 The carbapenems

The carbapenems form a diverse group of ß-lactam antibiotics and since 1970 over 40 different examples have been isolated from the actinomycetes, with *Streptomyces* spp. acting as major producers.[270,271] The carbapenems possess the broadest antibacterial spectra of all the β-lactam containing antibiotics. It is however, their high intrinsic resistance to β-lactamases that once again makes them attractive targets to develop as clinical antimicrobial agents.

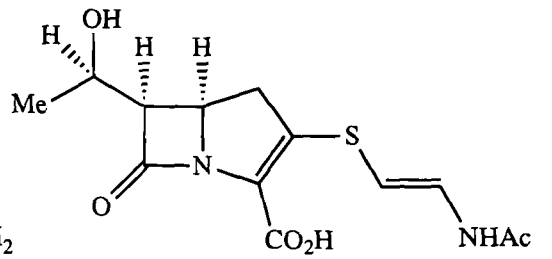
In general, the carbapenems belong to two main groups:

i) the thienamycins, e.g. (**112**)

ii) the olivanic acids, e.g. (**113**)



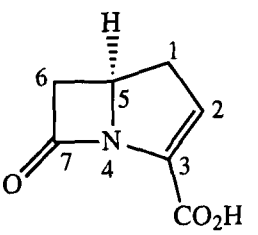
(**112**)



(**113**)

Despite their promise, the development of carbapenems as chemotherapeutic agents has been troublesome, with low titre concentrations and stability problems associated with attempts to produce them *via* fermentation processes. Consequently, the only two carbapenems licensed for use, imipenem and meropenem, are produced *via* expensive total chemical synthetic methodologies.

However, isolation of the structurally very simple parent compound, (5*R*)-carbapen-2-em-3-carboxylic acid (**114**), as its *p*-nitrobenzyl ester,[272] demonstrated it to possess the same absolute configuration at the C-5 position as the products isolated from *Streptomyces*,[273] and was shown to display excellent antimicrobial activity.



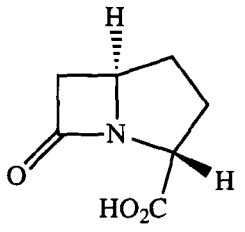
(**114**)

This molecule or its biosynthetic precursor, in an analogous fashion to 6-aminopenicillanic acid, could potentially provide lead structures for semi-synthetic manipulations, to generate a raft of possible antimicrobial agents. Attempts in this Department were therefore embarked upon to elucidate the biosynthetic pathways that produced (**114**) and other secondary metabolites contained within the growth medium.[274]

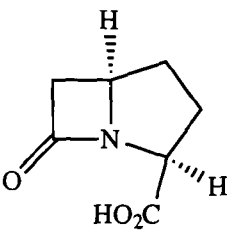
5.2.2 Biosynthetic studies

An initial investigation, using labelled L-[U-^{14}C]-glutamate as a primary metabolic precursor in the bacterial growth medium, confirmed that L-glutamic acid was involved in the synthesis of pyrroline ring of the bicyclic β-lactam (**114**). However, during the experimental study it became increasingly evident that two other labelled compounds, with a similar RPHPLC retention time as (**114**), were also present within the culture medium. Extraction and isolation of the unknown compounds indicated they were present in a ratio of *ca.* 9:1 and both displayed masses two units greater than the carbapenem (**114**). The IR spectra of the two molecules demonstrated strong stretching frequencies centred at 1760 and 1745 cm^{-1} respectively, indicative of a β-lactam carbonyl. They were also shown to possess a high intrinsic resistance to β-lactamases. Mass spectral comparisons of the two unknown compounds with (**114**) combined with their ^{1}H NMR identified the major isomer as the *trans*-carbapenam (**115**) and the minor compound as the *cis*-isomer (**116**).[275]

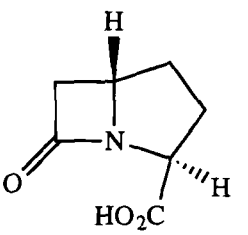
In a parallel experiment, it was discovered that catalytic hydrogenation of (**114**) resulted in the production of two isomers, again in the ratio of 9:1.



(115)



(116)



(117)

Understandably, in this instance the larger proportion was of the *cis*-isomer. The CD spectrum of the *cis*-isomer obtained via hydrogenation was shown to be identical to that of (**116**) from the previous labelling experiment, thereby establishing the absolute configuration around the two asymmetric centres. Due to the low yield of the *trans*-isomer, it was impossible to compare it to the natural product, so a tentative assignment of (3*R*, 5*R*) was made on the basis of comparative CD data. This also correlated well with the observation that the ring junction stereochemistry of all naturally occurring carbapenems was also 5*R*.[276]

It was not until the *trans*-isomer (**115**) was unambiguously synthesised,[277] and its CD spectrum compared with that of the natural *trans*-carbapenam, that discrepancies were apparent. The CD spectrum of the synthetic carbapenam exhibited a differential absorbance at 230 nm that was equal and opposite to that of the natural product. It followed therefore, that the major isomer obtained from the original radioactive tracer experiment possessed the enantiomeric (3*S*,5*S*) configuration (**117**).

This discovery suggested that L-glutamic acid was the biological precursor to the pyrroline moiety of the carbapenems. To confirm this, a further study was undertaken, which demonstrated the incorporation of [1,2-

$^{13}C_2$] acetate into both the pyrroline and lactam rings. Incorporation of the radiolabelled acetate into glutamic acid *via* the TCA cycle accounts for the appearance of the $^{13}C_2$ unit in the five membered ring (Figure 8).

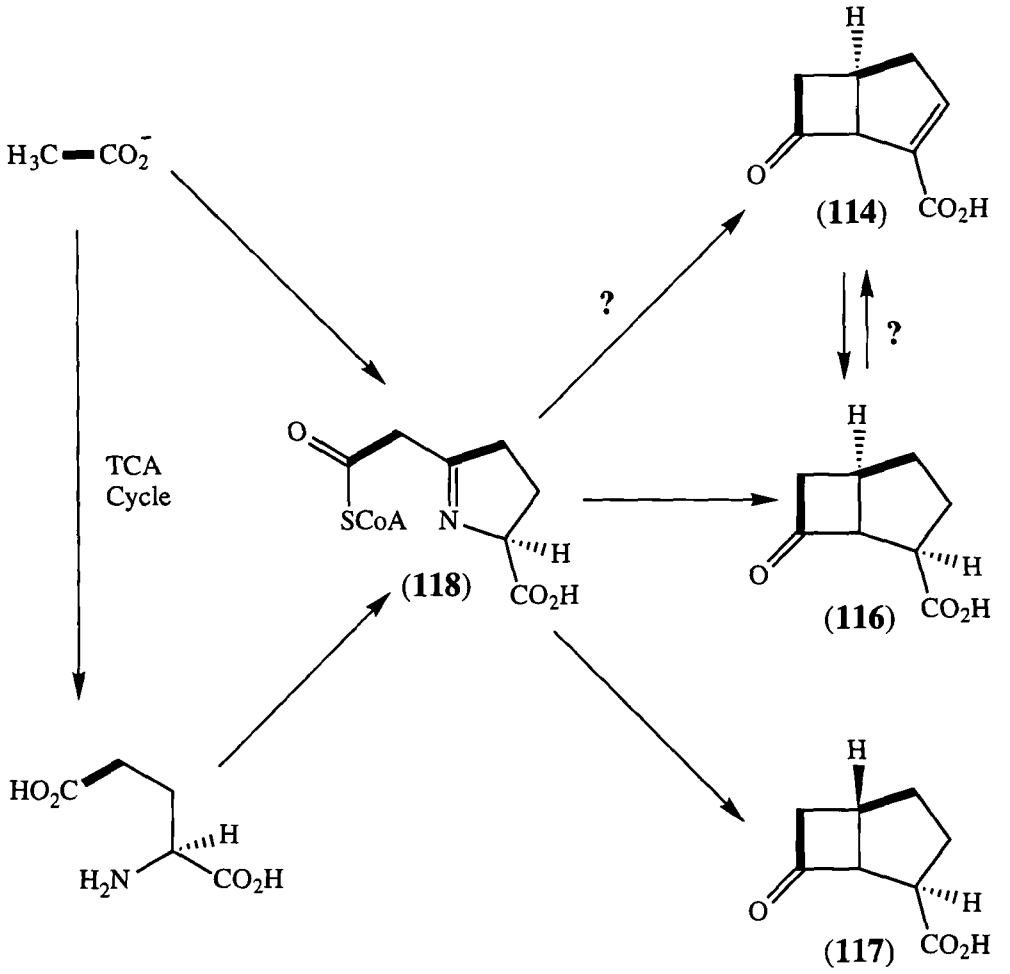


Figure 8. Putative biosynthetic pathway accounting for the incorporation of [1,2-$^{13}C_2$] acetate into (**114**), (**116**) and (**117**).

The hypothetical monocyclic intermediate (**118**) would probably be generated from the condensation of acetyl-coenzyme A (acetyl-S-CoA), and γ-glutamyl pyrophosphate.

Conversion of the carbapenam (**116**) to the active carbapenem (**114**) was still unproven. It was therefore envisaged that an unambiguous synthesis of the *cis*-isomer would allow further investigations into this area by allowing this potential biosynthetic precursor to be fed to a mutant *Erwinia* strain, blocked in the early stages of the biosynthetic gene responsible for carbapenem production. If the carbapenem (**114**) was subsequently isolated, it could only have been synthesised from the fed compound. If so proven, then a potential biosynthetic route, capable of exploitation for the semi-synthetic preparation of various modified carbapenems, would have been demonstrated.

5.3 Solution phase synthesis of *cis*-(3*S*,5*R*)-carbapenem-3-carboxylic acid biosynthetic precursors with further potential for the generation of a carbapenem library

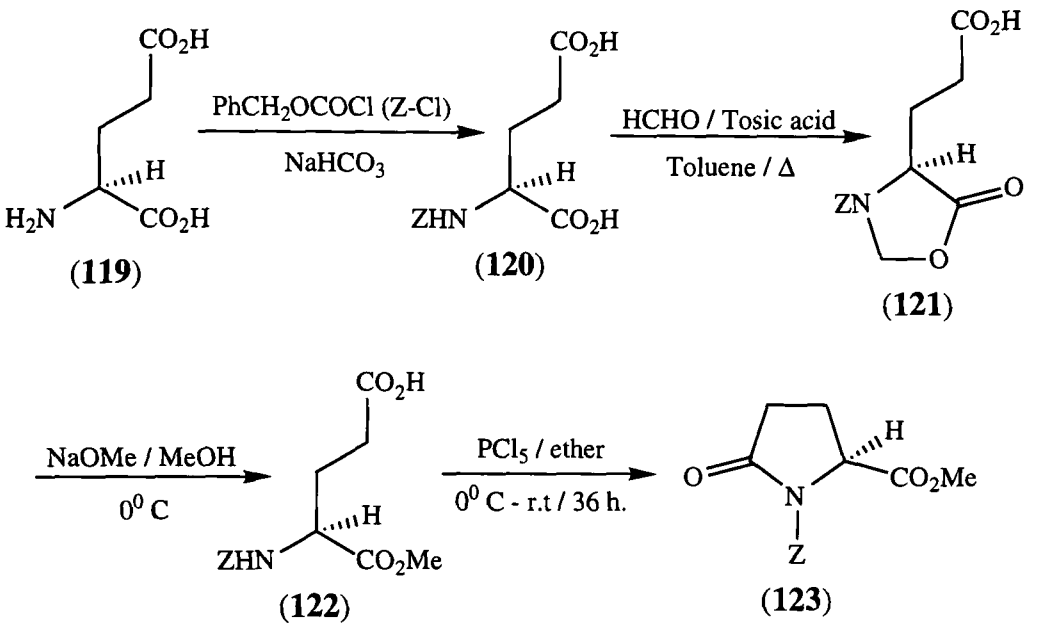
Our initial goal was the asymmetric synthesis of the *cis*-carbapenam (**116**), with the ambition of transposing the solution based strategy onto a solid phase support, with the ultimate target being the generation of a carbapenem library exhibiting subtle side-chain and ring modifications.

To this end, L-pyroglutamic acid was chosen from the 'chiral pool' of potential synthons as our starting material. The 2*S*-stereochemistry inherent within the molecule, would therefore be maintained during the synthesis to provide the required 3*S*-configuration of the carbapenam pyrrolidine ring. It was also envisaged that the L-pyroglutamic acid synthon could be chemically manipulated to afford potential biosynthetic precursors, e.g the Co-enzyme A thioester analogue (**136**), which could then be utilised as primary metabolic

feeder molecules to *Erwinwia* and *Serratia* strains, to ascertain whether they can be converted to the carbapenem-3-carboxylate.

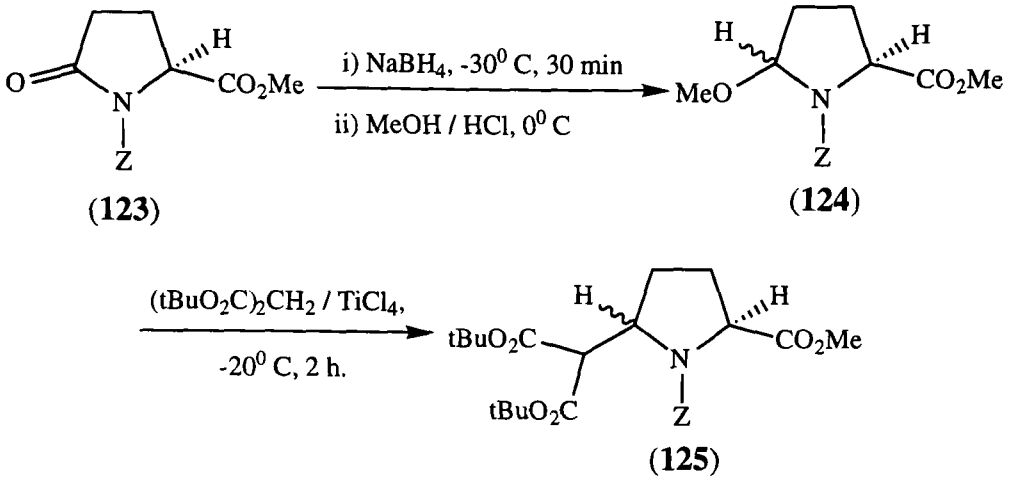
5.3.1 Attempted Synthesis of methyl (3*S*, 5*R*) - carbapenam-3-carboxylate

The *N*-Z-pyroglutamic acid methyl ester was synthesised from L-glutamic acid (**119**) in four steps (Scheme 68), by the literature methods.[278,279]



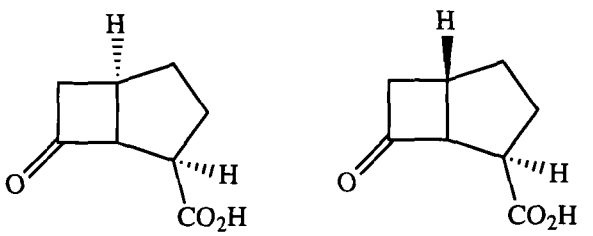
Scheme 68. Synthesis of *N*-Z-pyroglutamic acid methyl ester.

The reduction of the lactam (**123**) to the hemi-aminal (**124**) and subsequent condensation with di-tbutylmalonate, has been reported to proceed smoothly to furnish (**125**) in good yield (Scheme 69).[280]



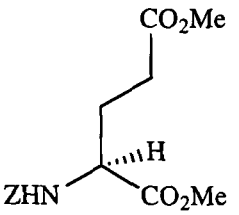
Scheme 69. Synthesis of the hemi-aminal (**124**) from Z-L-pyroglutamic acid and subsequent Lewis acid catalysed nucleophilic displacement of the methoxy group.

Deprotection of the *tert*-butyl esters of (**125**) should result in mono-decarboxylation. Removal of the Z-protecting group, with subsequent lactamisation and saponification of the methyl ester was then expected to yield the carbapenam as a mixture of diastereoisomers (**116**) and (**117**), which could be readily resolved using RPHPLC.



(**116**) (**117**)

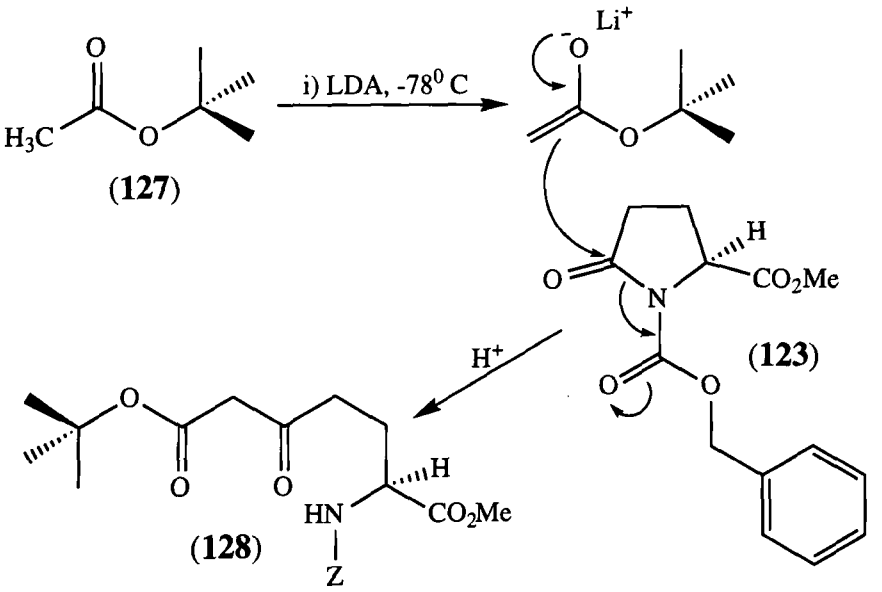
However, the initial reduction of (**123**) with sodium borohydride in methanol[281] did not afford the hemi-aminal as hoped. Instead, alcoholysis of the lactam ring resulted, generating the dimethyl ester (**126**).



(126)

Other reducing agents such as sodium cyanoborohydride and lithium aluminium *tert* -butoxide were also unsuccessfully explored.

An alternative approach was therefore undertaken, still using methyl Z-L-pyroglutamate as the synthetic starting material. Ohta *et al*[282] had described an efficient addition of lithium enolates to *N*-acylpyroglutamates with concomitant ring opening. Using *tert*-butyl acetate (**127**) as the enolisable ester, we attempted the corresponding lithium enolate addition to (**123**) with outstanding success, with the product recovered as a pale yellow oil in an 77% yield (Scheme 70).



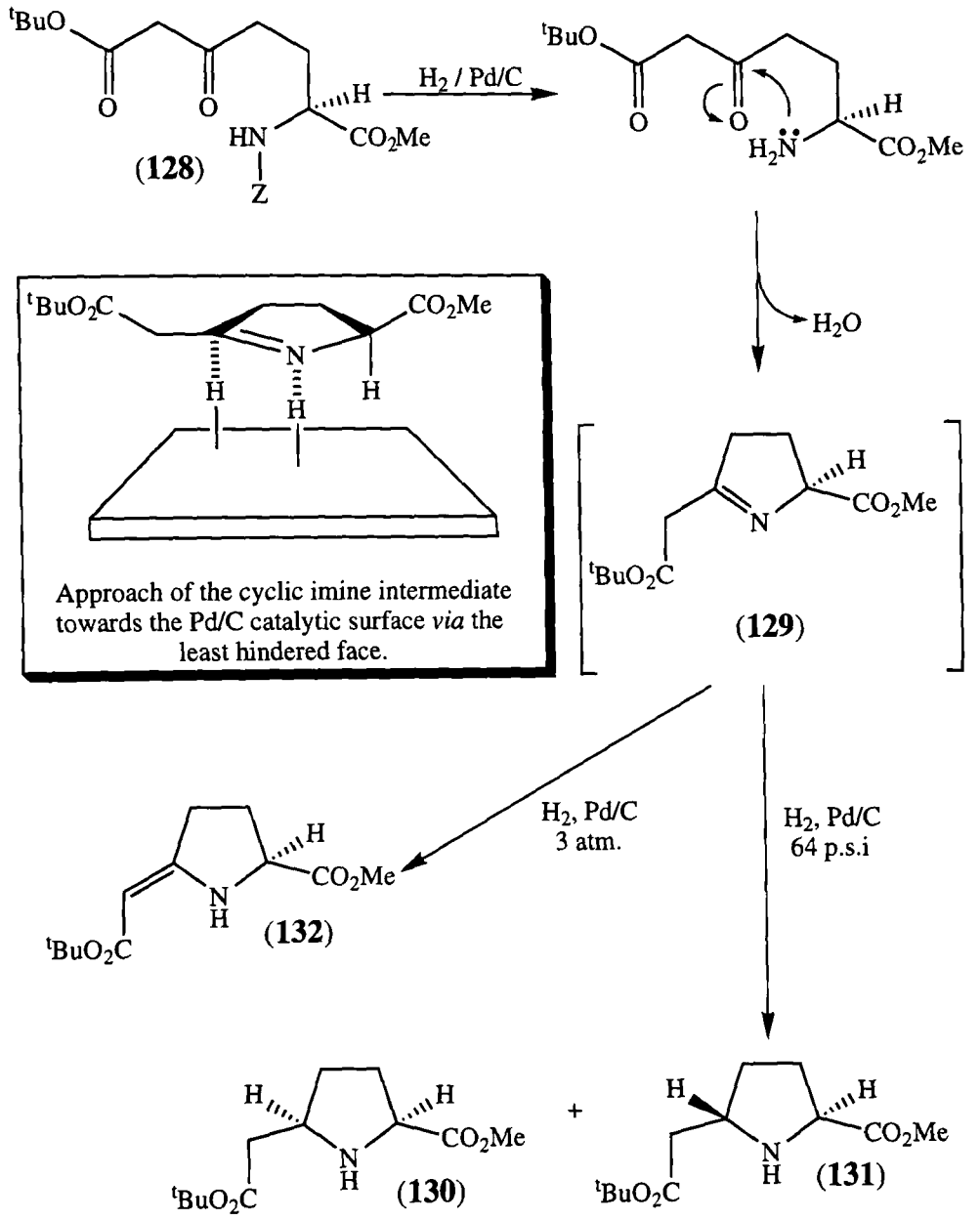
Scheme 70. Lithium enolate mediated addition of *tert*-butyl acetate to (**123**).

The identity of (**128**) was confirmed by ^{1}H NMR which displayed signals for the *tert*-butyl ester and the urethane NH.

5.3.1.1 Synthesis of *tert*-butyl (2*R*,5*S*)-5-methoxycarbonyl-2-pyrrolidineacetate *via* chirally induced catalytic hydrogenation

The precursor (**128**) could now be readily converted to the required pyrrolidine ring by catalytic hydrogenation. The reaction should theoretically proceed in two steps; initial hydrogenolytic deprotection of the Z group would release the primary amine, which spontaneously undergoes an intramolecular condensation to form an endocyclic imine intermediate (**129**). Ensuing reduction of this species should proceed under chiral induction from the asymmetric centre of L-glutamic acid moiety. Approach of (**129**) towards the Pd/C catalytic surface should proceed *via* the least hindered face, resulting in delivery of hydrogen to the lower face of the pyrroline ring and ultimately yielding a product of predominately *cis*-configuration (Scheme 71). From the results obtained from the catalytic hydrogenation of carbapen-2-em-3-carboxylic acid (**113**), (see 5.1.4), it was anticipated that a ratio of *ca.* 9:1 (*cis*:*trans*) would be obtained for (**130**) and (**131**) respectively.

When hydrogenation was initially performed at 3 atm., the resultant yellow oil still possessed a powerful chromophore, despite the ^{1}H NMR confirmation of the Z-group deprotection. EI-MS displayed a *m/z* =241 (mass required 243) suggesting that the Schiff's base had not been reduced. The NH signal in the ^{1}H NMR intimated that the endocyclic imine structure (**129**) had isomerised to the thermodynamically more stable exocyclic enamine (**132**).



Scheme 71. One-pot catalytic hydrogenolysis of the Z-protecting group and subsequent hydrogenation of the resulting endocyclic Schiff base intermediate.

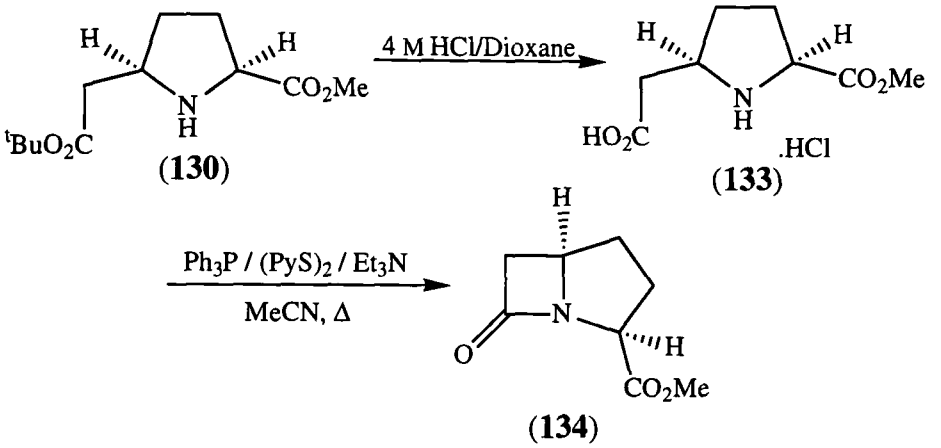
With the double bond situated in the exocyclic position, the molecule (**132**) is a vinylogous urethane, possessing extended conjugation through to the *tert*-butyl ester, and thereby increasing its resistant to reduction. By performing the hydrogenation at a very high pressure, namely 64 p.s.i., and allowing the reaction to proceed overnight, the resulting product was this time obtained as a pale yellow oil (97% yield) with the correct FAB-MS = 244 [M + H+]. The ^{1}H NMR confirmed complete loss of the Z-protection, with 1H multiplets of the C-2 and C-5 protons resonating either side of the methyl ester singlet at δ3.70.

To ascertain that the C-2 and C-5 protons were predominately held in the *cis*-configuration, a ^{1}H 400 MHz NOE difference experiment was performed. The results indicated that both protons appeared to be split into two discrete multiplets, in a ratio of 4:1 by integration, resonating either side of the methyl ester at δ3.70. The uneven value of the integrations seemed to discount NH coupling. This was confirmed by re-recording the NMR after a D_2O shake; both sets of signals remained unaltered.

An NOE difference experiment was therefore performed upon the two largest representative peaks for the C-2 and C-5 protons. Irradiation at the C-2 proton frequency produced a positive NOE difference at the C-5 position and also at the pyrrolidine ring proton frequencies. Irradiation of the C-5 proton confirmed the predominant *cis*-configuration by producing a positive NOE difference at the C-2 proton frequency and again on the ring protons. It is important to note that no NOE difference was observed between the two smaller C-2 and C-5 peaks indicating a possible 20% *trans*-configuration impurity. Since purification of the epimers at this stage was impossible, it was decided to lactamise the chirally impure pyrrolidine rings to their respective carbapenams

(**116**) and (**117**), and purify the β-lactams by RPHPLC.

Deprotection of the *tert*-butyl ester in 4 M HCl in dioxane proceeded in a quantitative yield, and the attempted lactamisation of the resultant amino acid was initially tried using triphenylphosphine and pyridyl disulphide (Scheme 72).[283]

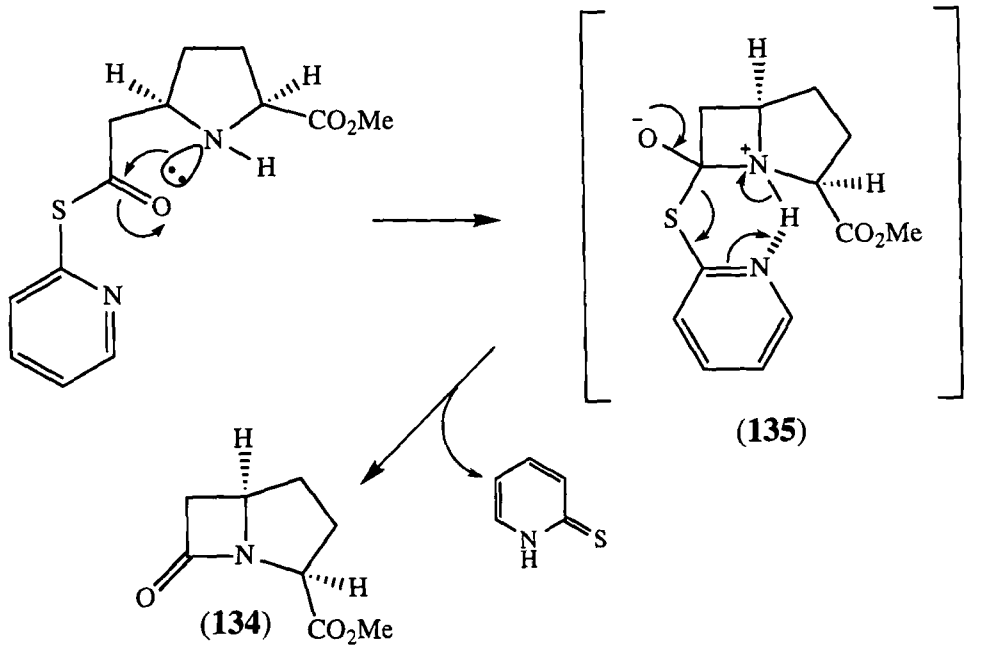


Scheme 72. Deprotection and lactamisation of the *cis*-pyrrolidine intermediate.

It is noteworthy that initial attempts at *tert*-butyl ester deprotection using 2 M HCl in ether, originally exploited in the synthesis of the *trans*-carbapenam,[277] only resulted in precipitation of the hydrochloride salt. Despite replacement of ether with dioxane to increase the products solubility, deprotection of the *cis*-pyrrolidine *tert*-butyl ester took 48 h to complete, indicating a significant difference in the energetics of the two epimers. The anomaly of the *cis*-product was born out further in the attempted lactamisation.

The triphenylphosphine, pyridyl disulphide mediated lactamisation proceeded with reasonable yields when performed on the *trans*-precursor.[277] The mechanism proceeds *via* a quaternary intermediate (**135**), with the generation of a stabilised hydrogen bonded six membered ring intermediate

directing elimination of the pyridylthione and β-lactam formation (Scheme 73).



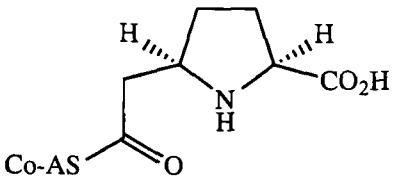
Scheme 73. Mechanism of pyridyl disulphide mediated lactamisation.

However, with the *cis*-epimer, work-up afforded a thin oil of 3-4% overall yield, with the ^{1}H NMR proving highly unsatisfactory. Various different coupling procedures were therefore explored, including DCC/DMAP, diphenylphosphinic chloride,[284] and *N,N-bis*[2-oxo-3-oxazolidinyl]phosphoro-diamidic chloride,[285] but none produced any tangible results.

5.3.2 Synthesis of a Co-enzyme A analogue of the *cis*-pyrrolidine intermediate

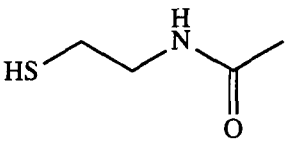
It was felt that if the *cis*-carbapenam was thermodynamically unfavoured and its synthesis beyond our means, then conversion of the free acid to a Co-enzyme A thioester analogue (**136**), would yet furnish a potential biosynthetic precursor very similar to the putative cyclic imine intermediate (**118**), which

could be used as a biological feeder molecule for various mutant strains of *Erwinia* or *Serratia.*



(136)

Methodology has been described in linking Co-enzyme A to fatty acids,[286] and 2-arylpropionate anti-inflammatory drugs.[287] However it was felt that the efficiency of thioester formation should be established before an expensive reagent such as purified Co-enzyme A was committed. The model thiol cysteamine protected as its *N*-acetyl derivative (**137**) was therefore initially used.



(137)

A DCC/DMAP coupling procedure in dry DCM was performed at room temperature, and following overnight reaction, the thioester was recovered as an oil in a 44% overall yield. The ^{1}H NMR confirmed the presence the CH_3CONH- peak, with the FAB-MS indicating the required mass for the compound. An improved synthesis should be obtained by protection of the pyrrolidine NH, thereby preventing intermolecular amide bond formation.

It was envisaged that this model thioester could itself be used as a metabolic precursor, and is now awaiting the correct mutant bacterial construct.

6

Experimental

All melting points were determined on a Kofler hot-stage apparatus and are uncorrected. Ultraviolet spectra were recorded on a Cecil 1020S scanning spectrophotomter and infrared spectra were recorded using a Perkin Elmer 257 or Philips PU9716 spectrophotometer. Routine ^{1}H NMR spectra were recorded on a Varian EM390 at 90MHz. High resolution ^{1}H NMR were measured using either a Bruker AM 250 or a Bruker AM 400 operating at 250 and 400MHz respectively. The spectra were recorded in deuteriochloroform unless otherwise stated, and the chemical shifts stated are relative to an internal tetramethylsilane standard. The multiplicity of a signal is designated one of the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; br, broad; m, multiplet. All coupling constants, *J* values, are reported in Hertz. ^{13}C NMR were recorded in deuteriochloroform unless otherwise stated on a Bruker AM 250 at 62.9 MHz. The chemical shifts are reported relative to an internal deuteriochloroform standard on a broad band decoupled mode, and the assignments were obtained using a DEPT pulse sequence.

Electron impact (EI) mass spectra were recorded on a AE1 MS-902. Fast atomic bombardment (FAB) mass spectra were obtained using a VG micromass 70 E. Positive ion electrospray MS (+ve ES-MS) were recorded on a Micromass VG platform instrument and MALDI-TOF spectra were recorded using a Kratos MALDI II instrument. Microanalytical data were obtained on a Perkin-Elmer 240B elemental analyser. Optical rotations were measured on a Bendix NPL automatic polarimeter type 143 C with a digital output.

Analytical thin layer chromatography (TLC) was performed using Merck silicagel 60 F_{254} precoated (0.2 mm) aluminium sheets. Preparative thin layer chromatography (PLC) was performed using Merck silicagel 60 GF_{254} coated (1.0 mm) glass plates (20 cm x 20 cm). Flash chromatography was performed using Merck Kieselgel 60 (230-400 mesh) silica. Chiral TLC were performed using Chiraplate®, manufactured by CAMLAB, Germany.

Analytical reverse phase high performance liquid chromatography (RPHPLC) was performed using a Hypersil Pep C_{18} column (4.6 x 150mm) at a constant flow rate of 1.2 ml min^{-1}, and semi-preparative RPHPLC performed with a Hypersil Pep C_{18} column (8 x 150mm) at a constant flow rate of 2.2 ml min^{-1}, unless otherwise stated. Mobile phases were; Eluent A - 0.06% $TFA_{(aq)}$, degassed with helium for 30 min, and Eluent B - 0.06% TFA in 90% $MeCN_{(aq)}$, degassed *via* sonication also for 30 min unless otherwise stated. RPHPLC was performed using either an LKB 2150 twin pump system, 2152 controller and 2151 variable wavelength monitor or a Waters 510 twin pump system and 484 tunable absorbance detector. Post column eluent was monitored by UV absorbance at 220 nm, and sample lyophilisation was performed using an Edwards Modulyo FD freeze dryer.

Diethyl ether, hexane and toluene were dried over sodium wire, whereas all other solvents were dried by distillation from the following heterogenous drying reagents; THF (lithium aluminium hydride), dichloromethane (phosphorus pentoxide), acetonitrile (calcium hydride), pyridine (sodium hydroxide pellets), methanol (magnesium turnings) and ethanol (magnesium turnings). Dried organic solvents were stored under nitrogen.

Protected amino acids, coupling reagents and resins were purchased from Novabiochem (UK) Ltd.

Chapter 2.

2-Acetyl-4-nitroindan-1,3-dione (**66**).

A mixture of 3-nitrophthalic anhydride (12 g, 60 mmol), anhydrous pyridine (25 ml), piperidine (0.2 ml) and 2,4-pentanedione (6.25 g, 60 mmol) were stirred together at 35-40 ^{0}C under anhydrous conditions. A dark solution was rapidly formed and a bright yellow precipitate soon appeared. The solution was allowed to stir for a further 6 h, after which the reaction mass was cooled and the thick crystalline mass was collected at the pump, washed with ether and dried to give the yellow pyridinium salt (15.3 g, 79%). The salt was treated with 6 M HCl (100 ml) to furnish a yellow crystalline precipitate, which was collected at the pump, washed with water and dried.

The product was recrystallised from *tert*-butanol to afford 2-acetyl-4-nitroindane-1,3-dione as fine yellow needles (8.74 g, 63%), m.p. 145-148 ^{0}C (lit[200], 148-150 ^{0}C).

ν_{max} (KBr) 3080, 1710, 1650, 1600, 1540, 1360, 1340, 1210 cm^{-1}

^{1}H NMR δ 2.60 (3 H, d, *J* 3.0, CH_3), 7.95 (3 H, m, ArH's) 11.3 (1 H, br, s, enol -OH)

m/z (FAB) 234 (M + H)

N$^{\alpha}$-Nde-amino acids

N-1-(4-nitro-1,3-dioxoindan-2-ylidene)ethyl-L-alanine (Nde-L-Ala) (**72**).

A suspension of finely powdered L-alanine (0.178 g, 2.0 mmol) and 2-acetyl-4-nitroindane-1,3-dione (0.233 g, 1 mmol) were refluxed in anhydrous ethanol (20 ml) for 6-8 h. Ethanol was then removed *in vacuo* and the residue redissolved in ethyl acetate. The organic solution was washed with 1M $KHSO_{4(aq)}$ and then extracted with sat. $NaHCO_{3(aq)}$ (2 x 20 ml). Extracts were combined, carefully acidified using 6 M HCl, and the resultant yellow oil extracted using ethyl acetate. The organic extract was washed with brine, dried ($MgSO_4$) and evaporated to dryness. The yellow residual oil was dissolved in a minimal amount of ethyl acetate, the product being precipitated by flooding with hexane. The mother liquor was decanted off and the product dried to afford a yellow amorphous powder (0.197 g, 65%) which could not be further recrystallised. m.p. 250-253 ^{0}C, Decomp.

ν_{max} (KBr) 3180, 1750, 1690, 1640, 1600, 1540, 1500, 1415, 1360cm^{-1}

^{1}H NMR ($(CD_3)_2SO$, 250 MHz) δ 1.53 (3 H, d, *J* 7.5, β-CH_3), 2.60 and 2.65 (3H, 2 x s, *E/Z* C=CCH_3), 4.74 (1 H, m, α-CH), 7.87 (2 H, m, Ar C6 and C7 H's), 7.99 (1 H, m, Ar C5-H), 11.02 and 11.10 (1 H, 2 x d, *J* 8.0, *E/Z* NH), 13.65 (1H, br, s, CO_2H)

m/z (FAB) 305 (M + H), 259 (M- CO_2H)

$[\alpha]_D^{25}$ +26.4^{0} (*c* 1.0, MeOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 4.0 min.

Nde-L and D-phenylalanine

The use of L-phenylalanine (0.330 g, 2 mmol) and D-phenylalanine (0.330 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-L-Phe (0.262 g, 69%) and Nde-D-Phe (0.243 g, 64%) respectively as yellow amorphous powders, m.p. 166-168 ^{0}C respectively

Nde-L-Phe.

ν_{max} (KBr) 3100, 1750, 1600, 1560, 1520, 1430, 1375, 1210 cm^{-1}

^{1}H NMR ($(CD_3)_2SO$, 250 MHz) δ 2.37 and 2.39 (3 H, 2 x s, *E/Z* C=CCH_3), 3.15 and 3.30 (2 H, 2 x dd, *J* 12.5, 5.0, β-CH_2), 5.00 (1 H, m, α-CH), 7.26 (5 H, m, Ph), 7.84 (2 H, m, Ar C6 and C7-H's), 8.0 (1 H, m, Ar C5-H), 10.92 and 11.0 (1H, 2 x d, *J* 8.0, *E/Z* NH), 13.78 (1H, br, s, CO_2H)

m/z (FAB) 381 (M + H), 335 (M - CO_2H)

HRMS (FAB) Found: *m/z* 381.102180. Calcd for $C_{20}H_{17}N_2O_6$: (M+H), 381.108662.

$[\alpha]_D^{25}$ -135.1^0 (*c* 1.0, EtOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 14.0 min.

Nde-L-Lysine(Boc)-OH

The use of L-lysine(*N*ε-Boc)-OH (0.493 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-L-Lys(Boc)-OH (0.313 g, 68%) as a yellow amorphous powder.

ν_{max} (KBr) 3400, 2960, 1700, 1650, 1600, 1545, 1360 cm^{-1}

^1H NMR (250 MHz) δ 1.41 (9 H, s, $C(CH_3)_3$), 1.46 (2 H, m, γ-CH_2), 1.54 (2 H, m, δ-CH_2), 2.01 (2 H, m, β-CH_2), 2.62 and 2.64 (3 H, 2 x s, *E/Z* C=CCH_3), 3.14 (2 H, m, ε-CH_2), 4.45 (1 H, m, α-CH), 6.45 (1 H, m, ε-NH), 7.83 (3 H, m, ArH's), 8.27 (1 H, br, s, CO_2H), 11.18 and 11.23 (1 H, 2 x d, *J* 8.0, *E/Z* NH)

m/z (FAB) 462 (M + H), 388 (M - $(CH_3)_3CO$)

HRMS (FAB) Found: *m/z* 462.184750. Calcd for $C_{22}H_{28}N_3O_8$: (M+H), 462.187640.

$[\alpha]_D^{25}$ -26.2^0 (*c* 1.0, MeOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 13.0 min.

Nde-L-Valine

The use of L-valine (0.234 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-L-Val (0.256 g, 68%) as a yellow amorphous powder.

ν_{max} (KBr) 3120, 2970, 1730, 1670, 1620, 1555, 1380, 1350, 1230 cm^{-1}

^1H NMR (250 MHz) δ 1.11 (3 H, d, *J* 3.0, γ-CH_3), 1.14 (3 H, d, *J* 3.0, γ-CH_3), 2.44 (1 H, m, β-CH), 2.63 and 2.66 (3 H, 2 x s, *E/Z* C=CCH_3), 4.32 (1H, m, α-CH), 7.80 (3 H, m, ArH's) 7.91 (1 H, br, s, CO_2H), 11.22 and 11.28 (1H, 2 x d, *J* 10.0 *E/Z* NH)

m/z (FAB) 333 (M+H), 287 (M - CO_2H).

HRMS (FAB) Found: *m/z* 333.104073. Calcd for $C_{16}H_{17}N_2O_6$: (M+H), 333.108662.

$[\alpha]_D^{25}$ +18.5^0 (*c* 1.0, MeOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 12.0 min.

Nde-L-Tyrosine (O^{t}Bu)-OH

The use of L-tyrosine(O^{t}Bu)-OH (0.475 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-L-Tyr(O^{t}Bu)-OH (0.353 g, 78%) as a yellow amorphous powder.

ν_{max} (KBr) 3080, 1700, 1600, 1550, 1520, 1370 cm^{-1}

^{1}H NMR (250 MHz) δ 1.32 (9 H, s, $C(CH_3)_3$), 2.20 and 2.23 (3 H, 2 x s, *E/Z* $C=CCH_3$), 3.10 and 3.40 (2 H, 2 x dd, *J* 15.0, 4.0, β-CH_2), 4.63 (1 H, m, α-CH), 7.05 (4 H, ABq, *J* 8.0, Ph), 7.67 (1H, br, s, CO_2H), 7.71 (2 H, m, Ar C6 and C7-H's), 7.87 (1 H, m, Ar C5-H), 11.19 and 11.26 (1H, 2 x d, *J* 8.0, *E/Z* NH)

m/z (FAB) 453 (M + H), 351 (M - CO_2H - $C(CH_3)_3$ + H)

HRMS (FAB) Found: *m/z* 453.165775. Calcd for $C_{24}H_{25}N_2O_7$: (M+H), 453.166176.

$[\alpha]_D^{25}$ -135.5^0 (*c* 1.0, EtOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 16.5 min.

Nde-L-Isoleucine

The use of L-isoleucine (0.262 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-L-Ile (0.242 g, 70%) as a yellow amorphous powder.

ν_{max} (KBr) 3000, 1750, 1700, 1655, 1600, 1550, 1500, 1415, 1360 cm^{-1}

^1H NMR (250 MHz) δ 1.01 (3 H, t, *J* 5.0, δ-CH_3), 1.08 (3 H, m, γ-CH_3), 1.54 (2 H, 2 x m, γ-CH_2), 2.17 (1 H, m, β-CH), 2.59 and 2.65 (3 H, 2 x s, *E/Z* C=CCH_3), 4.39 (1H, m, α-CH), 7.85 (3 H, m, ArH's) 8.10 (1 H, br, s, CO_2H), 11.21 and 11.28 (1H, 2 x d, *J* 10.0, *E/Z* NH)

m/z (FAB) 347 (M+H), 301 (M - CO_2H).

HRMS (FAB) Found: *m/z* 347.126755. Calcd for $C_{17}H_{19}N_2O_6$: (M+H), 347.124312.

$[\alpha]_D^{25}$ $+10.2^0$ (*c* 1.0, EtOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 14.0 min.

Nde-L-Leucine

The use of L-leucine (0.262 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-L-Leu (0.287 g, 83%) as a yellow amorphous

powder.

ν_{max} (KBr) 3000, 1750, 1700, 1660, 1600, 1550, 1510, 1420, 1360 cm^{-1}

^1H NMR (250 MHz) δ 1.01 (6 H, m, $CH(CH_3)_2$), 1.90 (3 H, m, $CH\text{-}CH_2$), 2.64 and 2.67 (3 H, 2 x s, *E/Z* $C{=}CCH_3$), 4.45 (1H, m, α-CH), 7.85 (3 H, m, ArH's), 7.95 (1 H, br, s, CO_2H), 11.02 and 11.09 (1H, 2 x d, *J* 10.0, *E/Z* NH)

m/z (FAB) 347 (M+H), 302 (M - CO_2H).

HRMS (FAB) Found: *m/z* 347.124918. Calcd for $C_{17}H_{19}N_2O_6$: (M+H), 347.124312.

$[\alpha]_D^{25}$ -31.5^0 (*c* 1.0, MeOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 14.7 min.

Nde-L-Histidine(N^{im}Trt)-OH

The use of L-histidine(*N^{im}*-Trt)-OH (0.795 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-L-His(Trt)-OH (0.244 g, 40%) as a yellow amorphous powder.

ν_{max} (KBr) 3100, 3020, 1750, 1715, 1665, 1610, 1560, 1515, 1470 cm^{-1}

^1H NMR δ 2.45 and 2.51 (3 H, 2 x s, *E/Z* $C{=}CCH_3$), 3.40 (2 H, m, β-CH_2), 4.85 (1 H, m, α-CH), 7.05 and 7.26 (15 H, 2 x m, CPh_3), 7.65 (2 H, m, Ar C6 and C7-H's), 7.80 (1 H, m, Ar C5-H), 8.15 (1H, br, s, CO_2H), 9.85 (2 H, m, imidazole 2 x CH), 11.05 and 11.15 (1H, 2 x d, *J* 8.0, *E/Z* NH)

m/z (FAB) 613 (M+H), 568 (M - CO_2H + H).

HRMS (FAB) Found: *m/z* 613.207948. Calcd for $C_{36}H_{29}N_4O_6$: M, 613.208710.

$[\alpha]_D^{25}$ -86.9° (*c* 1.0, MeOH)

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 14.1 min.

Nde-Glycine

The use of glycine (0.150 g, 2 mmol) under the conditions for the synthesis of (**72**) afforded Nde-Gly (0.235 g, 81%) as a yellow amorphous powder.

ν_{max} (KBr) 3100, 1730, 1650,1600, 1560, 1500 cm^{-1}

^1H NMR ($(CD_3)_2SO$, 250 MHz) δ 2.61 and 2.64 (3 H, 2 x s, *E/Z* $C=CCH_3$), 4.23 (2H, d, *J* 5.0, α-CH_2), 6.85 (1 H, br, s, CO_2H), 7.85 (3 H, m, ArH's), 11.03 and 11.07 (1H, 2 x d, *J* 10.0, *E/Z* NH)

m/z (FAB) 290.8 (M+H).

HRMS (FAB) Found: *m/z* 291.062411. Calcd for $C_{13}H_{11}N_2O_6$: (M+H), 291.061711.

RPHPLC analysis

Gradient - 40% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 7.8 min.

L-Leucyl-L-Leucyl-L-Leucine

UltraSyn-USA resin, functionalised with Fmoc-L-leucine at 0.1 mmol g^{-1} (0.50 g) was swelled in DMF for 60 min. Using an LKB 4175 Biolynx manual peptide synthesiser, the following operations were performed on the resin under continuous flow conditions. A constant flow rate of 3.0 ml min^{-1} was maintained, with post-column monitoring performed at 290nm.

	Operation	*Time*
1.	DMF Wash.	10 min.
2.	20% Piperidine in DMF Wash.	7 min.
3.	DMF Wash.	5 min.
4.	Nde-Leu-OH (70 mg, 0.20 mmol) HOBt.H_2O (31 mg, 0.20 mmol), and DIPCDI (31µl, 0.20 mmol) load.	
5.	Recycle.	60 min.
6.	DMF Wash.	2 min.
7.	Load Wash.	
8.	DMF Wash.	10 min.
9.	Picrylsulphonic acid amine test.	
10.	20% Piperidine in DMF Wash. *(no deprotection observed at 290 nm)*	10 min.
11.	DMF Wash.	5 min.
12.	1% hydrazine monohydrate in DMF wash. *(deprotection complete after 6 min., monitoring at 290 nm)*	10 min.
13.	Nde-Leu-OH (70 mg, 0.20 mmol), HOBt.H_2O (15 mg, 0.10 mmol), TBTU (64 mg, 0.20 mmol)	

and DIEA (70µl, 0.40 mmol) load.

Repeat steps 5 to 9.

14. 10% thiourea in DMF wash 10 min.

(no deprotection observed at 290 nm)

15. 2% hydrazine monohydrate in DMF wash. 10 min.

(deprotection complete after 4 min. monitoring at 290 nm)

16. DMF wash. 10 min.

The resin was transferred to a sintered glass funnel and sequentially washed with DMF (25 ml), *t*-amyl alcohol (10 ml), acetic acid (5 ml), *t*-amyl alcohol (5 ml) and ether (20 ml). The resin was then dried *in vacuo* over KOH overnight. A portion of the resin (50 mg) was suspended in TFA-H_2O (95:5, 10 ml) for 2 h with occasional agitation. The mixture was then filtered through a glass sinter and the filtrate evaporated. The solid residue was triturated several times with HPLC grade ether, dissolved in distilled water and lyophilised to yield the product as a white amorphous powder.

RPHPLC Analysis

Gradient - 20% to 70% Eluent B in 20 min. linearly.

Observed - 2 major peaks at 7.4 and 12.4 min respectively.

m/z (MALDI-TOF) Peak 1; 359.1 (M + 1 for Leu-Leu-Leu). Peak 2; 245.4 (M + 1 for Leu-Leu).

Racemisation studies with N^{α}-Nde amino acids.

Racemisation during the synthesis of N-Nde-L-Phe.

A solution of *N*-Nde-L-Phe (0.190 g, 0.5 mmol) was dissolved in 1%

hydrazine monohydrate in anhydrous ethanol (5 ml) and allowed to stand at room temperature. After 3 h a yellow/orange precipitate had formed which was collected at the pump, washed several times with ethanol and dried to yield Nde-hydrazine (**73**) as an amorphous powder (0.120 g, 97%), m.p. 194-198 ^{0}C (dec).

The hydrazine/ethanol solution was evaporated to dryness and the residue partitioned between chloroform and water. Removal and evaporation of the aqueous layer afforded L-phenylalanine (0.048 g, 58%).

Nde hydrazine

ν_{max} (KBr) 3330, 3260, 3100, 1650, 1570, 1490, 1350 cm^{-1}

^{1}H NMR ($(CD_3)_2SO$-$CDCl_3$) δ 2.53 (3 H, s, C=CCH_3), 5.50 (2 H, br, s, NH_2), 7.75 (3 H, m, ArH's) 11.4 (1 H, d, NH)

m/z (+ve ES-MS) 248.2 (M + 1).

L-phenylalanine

^{1}H NMR (D_2O, DSS external standard) δ 3.10 and 3.25 (2 H, 2 x dd, *J* 8.0, 5.5, β-CH_2), 3.95 (1 H, dd, *J* 8.1, 5.5, α-CH), 7.35 (5 H, m, Ph)

$[\alpha]_D^{28}$ -31.5^{0} (*c* 1.0, H_2O) {lit.,[288] $[\alpha]_D^{20}$ -33.7^{0} to -35.2^{0} (*c* 2, H_2O).

Chiral TLC analysis

R_f = 0.625, (L-Phe; R_f = 0.625, D-Phe; R_f = 0.50).

Racemisation during coupling of N-Nde-L-Phe.

*N-Nde-L-Phenylalanyl-L-alanine benzyl ester (**75**) and N-Nde-D-Phenylalanyl-L-alanine benzyl ester (**76**) via DCC mediated coupling.*

To stirred individual solutions of *N*-Nde-L-phenylalanine and *N*-Nde-D-phenylalanine (0.057 g, 0.15 mmol) in dry DCM (30 ml) both containing L-alanine benzyl ester tosylate salt (0.053 g, 0.15 mmol), HOBt (0.023 g, 0.15 mmol) and triethylamine (0.021 ml, 0.15 mmol), was added DCC (0.034 g, 0.165 mmol) at 0 ^{0}C. Stirring was continued at room temperature for 3 h, followed by evaporation of volatiles *in vacuo*. The individual residues were redissolved in cold ethyl acetate and the DCU precipitate removed *via* filtration. The organic solutions were then washed with 1M $KHSO_{4(aq)}$, sat. $NaHCO_{3(aq)}$, water and brine. The organic layers were then dried ($MgSO_4$) and evaporated to dryness to yield *N*-Nde-L-Phe-L-Ala-OBzl (**75**) (0.062 g, 77%) and *N*-Nde-D-Phe-L-Ala-OBzl (**76**) (0.057 g, 70%) as yellow oils.

N-Nde-L-Phenylalanyl-L-alanine benzyl ester (75) and N-Nde-D-Phenylalanyl-L-alanyl benzyl ester (76) via TBTU mediated coupling.

To stirred individual solutions of *N*-Nde-L-phenylalanine and *N*-Nde-D-phenylalanine (0.057 g, 0.15 mmol) in dry DCM (30 ml) both containing L-alanine benzyl ester tosylate salt (0.053 g, 0.15 mmol), HOBt (0.023 g, 0.15 mmol) and triethylamine (0.021 ml, 0.15 mmol), was added TBTU (0.048 g, 0.15 mmol) at 0 ^{0}C. Stirring was continued at room temperature for 2.5 h, followed by evaporation of volatiles *in vacuo*. The individual residues were redissolved in cold ethyl acetate and washed with 1M $KHSO_{4(aq)}$, sat. $NaHCO_{3(aq)}$, water and brine. The organic layers were then dried ($MgSO_4$) and evaporated to dryness to yield *N*-Nde-L-Phe-L-Ala-OBzl (**75**) (0.060 g, 74%) and *N*-Nde-D-Phe-L-Ala-OBzl (**76**) (0.065 g, 80%) as yellow oils.

N-Nde-L-Phe-L-Ala-OBzl (**75**)

ν_{max} (thin film) 3300, 1750, 1650, 1580, 1535, 1495, 1350 cm^{-1}

^1H NMR (250 MHz) δ 1.53 (3 H, d, *J* 7.5, Ala β-CH_3), 2.40 and 2.46 (3 H, 2 x s, *E/Z* C=CCH_3), 3.25 and 3.48 (2 H, 2 x dd, *J* 12.5, 5.0, Phe β-CH_2), 4.55 (1 H, m, Phe α-CH), 4.76 (1 H, m, Ala α-CH), 5.26 (2 H, s, PhCH_2), 6.70 and 6.75 (1 H, 2 x d, *J* 7.5, *E/Z* CONH), 7.37 (5 H, m, Ph), 7.45 (5 H, s, Ph), 7.85 (3 H, m, ArH's), 11.20 and 11.23 (1H, 2 x d, *J* 7.5, *E/Z* C=CNH)

m/z (FAB) 542 (M + H), 335 (M - CO-Ala-OBzl)

N-Nde-D-Phe-L-Ala-OBzl (**76**)

^1H NMR (250 MHz) δ 1.44 (3 H, d, *J* 7.5, Ala β-CH_3), 2.41 and 2.42 (3 H, 2 x s, *E/Z* C=CCH_3), 3.19 and 3.41 (2 H, 2 x dd, *J* 8.5, 6.25, Phe β-CH_2), 4.52 (1 H, m, Phe α-CH), 4.71 (1 H, m, Ala α-CH), 5.23 (2 H, s, PhCH_2), 6.52 and 6.75 (1 H, 2 x d, *J* 7.5 *E/Z* CONH), 7.35 (5 H, m, Ph), 7.41 (5 H, s, Ph), 7.85 (3 H, m, ArH's), 11.24 and 11.32 (1H, 2 x d, *J* 7.5, *E/Z* C=CNH)

m/z (FAB) 542 (M + H), 335 (M - CO-Ala-OBzl)

RPHPLC Analysis; (Eluent A: H_2O, Eluent B: 90% $MeOH_{(aq)}$.)

Gradient 70% -100% Eluent B in 20 min. linearly.

Observed - For (**75**) 1 peak only at 18.4 min.

For (**76**) 1 peak only at 16.5 min.

Neuromendin N amide (Lys-Ile-Pro-Tyr-Ile-Leu-NH_2).

NovaSyn® KR 100 resin functionalised at 0.12 $mmolg^{-1}$ with an Fmoc protected modified Rink amide linker (0.10 g, 12μmol functionality) was

swelled in DMF for 60 min. Using an LKB 4175 Biolynx manual peptide synthesiser, the following operations were performed on the resin under continuous flow conditions. A constant flow rate of 3.0 ml min^{-1} was maintained, with post-column monitoring performed at 290nm.

Operation	*Time*
1. DMF Wash.	10 min.
2. 20% Piperidine in DMF Wash.	20 min.
3. DMF Wash.	5 min.
4. Nde-Leu-OH (17 mg, 0.05 mmol) HOBt.H_2O (4 mg, 0.025 mmol), and DIPCDI (7.6 µl, 0.05 mmol) in DMF (1 ml) load.	
5. Recycle.	60 min.
6. DMF Wash.	2 min.
7. Load Wash.	
8. DMF Wash.	10 min.
9. Picrylsulphonic acid amine test.	
10. 2% Hydrazine monohydrate in DMF wash	10 min.
11. DMF Wash.	5 min.

4. to 11. repeated using Nde-Ile (17 mg, 0.05 mmol)
then Nde-Tyr(^{t}Bu)-OH (22 mg, 0.05 mmol)
then Fmoc-Pro (16 mg, 0.05 mmol)*
then Nde-Ile (17 mg, 0.05 mmol)
then Nde-Lys(Boc)-OH (22 mg, 0.05 mmol).

* Substitute step 10. with 20% piperidine in DMF wash (10 min.).

The resin was transferred to a sintered glass funnel, and sequentially

washed with DMF (25 ml), *t*-amyl alcohol (10 ml), acetic acid (5 ml), *t*-amyl alcohol (5 ml) and ether (20 ml). The resin was then dried *in vacuo* over KOH overnight. A portion of the resin (50 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5, 10 ml) for 2 h with occasional agitation. The mixture was then filtered through a glass sinter and the filtrate evaporated. The solid residue was triturated several times with HPLC grade ether, dissolved in distilled water and lyophilised to yield the product as a white amorphous powder.

RPHPLC analysis

Gradient - 20% to 50% Eluent B in 20 min. linearly.

Observed - 1 peak at 11.2 min.

m/z (+ve ES-MS) 746.2 (M + H), 617.1 (M - Leu-NH_2).

Angiotensin II receptor binding protein fragment synthesised as its corresponding amide (Lys-Gly-Tyr-Ile-His-Ala-Leu-NH_2).

NovaSyn® KR 100 resin functionalised at 0.12 mmolg^{-1} with an Fmoc protected modified Rink amide linker (0.20 g, 24μmol functionality) was swelled in DMF for 60 min. Using an LKB 4175 Biolynx manual peptide synthesiser, the following operations were performed on the resin under continuous flow conditions. A constant flow rate of 3.0 ml min^{-1} was maintained, with post-column monitoring performed at 290nm.

Operation	*Time*
1. DMF Wash.	10 min.
2. 20% Piperidine in DMF Wash.	20 min.
3. DMF Wash.	5 min.

4. Nde-Leu-OH (35 mg, 0.10 mmol)
   HOBt.H_2O (7 mg, 0.05 mmol), and
   DIPCDI (15.0 µl, 0.10 mmol) in DMF (1 ml) load.
5. Recycle. 60 min.
6. DMF Wash. 2 min.
7. Load Wash.
8. DMF Wash. 10 min.
9. Picrylsulphonic acid amine test.
10. 2% Hydrazine monohydrate in DMF wash 10 min.
11. DMF Wash. 5 min.

4. to 11. repeated using Nde-Ala (30 mg, 0.10 mmol)
then Nde-His(Trt)-OH (61 mg, 0.10 mmol)*
then Nde-Ile (35 mg, 0.10 mmol)
then Nde-Tyr(*t*Bu)-OH (45 mg, 0.10 mmol)
then Nde-Val (33 mg, 0.10 mmol)
then Fmoc-Gly (30 mg, 0.10 mmol)‡
then Nde-Lys(Boc)-OH (46 mg, 0.10 mmol).

* Required resin incubation with four individual coupling solutions to obtain a negative TNBS test

‡ Substitute step 10. with 20% piperidine in DMF wash (10 min.).

the resin was transferred to a sintered glass funnel, and sequentially washed with DMF (25 ml), *t*-amyl alcohol (10 ml), acetic acid (5 ml), *t*-amyl alcohol (5 ml) and ether (20 ml). The resin was then dried *in vacuo* over KOH overnight. A portion of the resin (50 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5,

10 ml) for 2 h with occasional agitation. The mixture was then filtered through a glass sinter and the filtrate evaporated. The solid residue was triturated several times with HPLC grade ether, dissolved in distilled water and lyophilised to yield the product as a white amorphous powder.

RPHPLC analysis

Gradient - 20% to 50% Eluent B in 20 min. linearly.

Observed - 1 major peak at 7.0 min.

The major peak was purified by semi-preparative RPHPLC under analogous conditions.

m/z (+ve ES-MS) 900.2 (M + H), 769.5 (M - Leu-NH_2), 561.8 (M - His-Ala-Leu-NH_2), 450.1 (M - Ile-His-Ala-Leu-NH_2 + H).

*Leucine enkephalinamide (Tyr-Gly-Gly-Phe-Leu-NH_2) (**79**).*

NovaSyn® KR 100 resin functionalised at 0.12 mmolg^{-1} with an Fmoc protected modified Rink amide linker (0.20 g, 24μmol functionality) was swelled in DMF for 60 min. Using an LKB 4175 Biolynx manual peptide synthesiser, the following operations were performed on the resin under continuous flow conditions. A constant flow rate of 3.0 ml min^{-1} was maintained, with post-column monitoring performed at 290nm.

Operation	*Time*
1. DMF Wash.	10 min.
2. 20% Piperidine in DMF Wash.	20 min.
3. DMF Wash.	5 min.
4. Nde-Leu-OH (35 mg, 0.10 mmol)	

HOBt.H_2O (7 mg, 0.05 mmol), TBTU
(32 mg, 0.10 mmol) and DIEA (34 µl, 0.20 mmol)
in DMF (1 ml) load.

5. Recycle. 60 min.
6. DMF Wash. 2 min.
7. Load Wash.
8. DMF Wash. 10 min.
9. Picrylsulphonic acid amine test.
10. 2% Hydrazine monohydrate in DMF wash 10 min.
11. DMF Wash. 5 min.

4. to 11. repeated using Nde-Phe (38 mg, 0.10 mmol)
then Fmoc-Gly (30 mg, 0.10 mmol)*
then Fmoc-Gly (30 mg, 0.10 mmol)*
then Nde-Tyr(*t*Bu)-OH (45 mg, 0.10 mmol)

* Substitute step 10. with 20% piperidine in DMF wash (10 min.).

The resin was transferred to a sintered glass funnel, and sequentially washed with DMF (25 ml), *t*-amyl alcohol (10 ml), acetic acid (5 ml), *t*-amyl alcohol (5 ml) and ether (20 ml). The resin was then dried *in vacuo* over KOH overnight. A portion of the resin (50 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5, 10 ml) for 2 h with occasional agitation. The mixture was then filtered through a glass sinter and the filtrate evaporated. The solid residue was triturated several times with HPLC grade ether, dissolved in distilled water and lyophilised to yield the product as a white amorphous powder.

RPHPLC analysis

Gradient - 20% to 60% Eluent B in 20 min. linearly.

Observed - 1 major peak at 11.5 min and 1 impurity peak at 13.0 min.

The major peak was purified by semi-preparative RPHPLC under analogous conditions.

m/z (+ve ES-MS) 555.1 (M + H), 538.8 (M - NH_2 + H), 510.1 (M - $CONH_2$), 425.0 (M - Leu-NH_2), 397.7 (M - CO-Leu-NH_2).

Assessment of Nde stability towards TFA.

N-Nde-L-Phe (0.190 g, 0.5 mmol) was dissolved in neat TFA (50 ml) and allowed to stand at room temperature for 24 hours. At 2, 4, 6 and 24 h intervals a 2 ml sample was removed and evaporated to dryness, with 2 ml fresh TFA added to the flask to maintain the original volume. The residue was redissolved in 0.06% TFA in 90% $MeCN_{(aq)}$ (mobile phase B) and a 50µl sample was analysed by analytical RPHPLC. After 24 h, the solution was evaporated to dryness and the residue analysed by ^1H NMR and FAB-MS.

RPHPLC analysis

Gradient - 20% to 80% Eluent B in 25 min. linearly.

Observed at: t = 0 h, 1 peak at 14.0 min.

t = 2 h, 1 peak at 14.0 min.

t = 4 h, 1 peak at 14.0 min.

t = 6 h, 1 peak at 14.0 min.

t = 24h, 1 peak at 14.0 min.

An authentic sample of L-Phe displays a retention time of 3.5 min. under analogous conditions.

^{1}H NMR ($(CD_3)_2SO$, 250 MHz) δ 2.37 and 2.39 (3 H, 2 x s, *E/Z* C=CCH_3), 3.15 and 3.30 (2 H, 2 x dd, *J* 12.5, 5.0, β-CH_2), 5.00 (1 H, m, α-CH), 7.26 (5 H, m, Ph), 7.84 (2 H, m, Ar C6 and C7-H's), 8.0 (1 H, m, Ar C5-H), 10.92 and 11.0 (1H, 2 x d, *J* 8.0, *E/Z* NH), 13.78 (1H, br, s, CO_2H)

m/z (FAB) 381.1 (M + H), 335.1 (M - CO_2H).

Assessment of Nde stability towards 20% piperidine under continuous flow conditions.

Nde-Leu-Tyr-Gly-Gly-Phe-Leu-NovaSyn® KR 100 resin.

NovaSyn® KR 100 resin functionalised at 0.12 mmolg^{-1} with an Fmoc protected modified Rink amide linker (0.20 g, 24μmol functionality) was swelled in DMF for 60 min. The title peptide was synthesised using Fmoc-Leu-OH (35 mg, 0.10 mmol), Fmoc-Phe (39 mg, 0.10 mmol), 2 x Fmoc-Gly (30 mg, 0.10 mmol), Fmoc-Tyr(^{t}Bu)-OH (46 mg, 0.10 mmol) and Nde-Leu-OH (35 mg, 0.10 mmol) *via* TBTU (32 mg, 0.10 mmol)/HOBt.H_2O (7 mg, 0.05 mmol)/DIEA (35 μl, 0.20 mmol) mediated coupling. The synthesis was accomplished using an LKB 4175 Biolynx manual peptide synthesiser, following the synthetic strategy outlined for (**79**), with Fmoc deprotection achieved with 20% piperidine in DMF.

The resin was then washed and dried as for (**79**). A portion of the resin (10 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5, 5 ml) for 2 h with occasional agitation. The *N*-protected peptide product was isolated as a yellow amorphous powder as for (**79**).

RPHPLC analysis

Gradient - 25% to 55% Eluent B in 20 min. linearly.

Observed - 1 major peak at 19.2 min.

The major peak was purified by semi-preparative RPHPLC under analogous conditions.

m/z (+ve ES-MS) 883.2 (M + H), 866.1 (M - NH_2), 754.7 (M - Leu-NH_2), 606.0 (M - Phe-Leu-NH_2), 549.1 (M - Gly-Phe-Leu-NH_2), 492.2 (M - Gly-Gly-Phe-Leu-NH_2).

Leu-Tyr-Gly-Gly-Phe-Leu-NovaSyn® KR 100 resin.

A sample of the Nde-Leu-Tyr-Gly-Gly-Phe-Leu-NovaSyn® KR 100 resin (20 mg) was Nde deprotected using 2% hydrazine monohydrate in DMF under continuous flow conditions. The resin was then washed and dried as for (**79**). A portion of the resin (10 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5, 5 ml) for 2 h with occasional agitation. The *N*-protected peptide product was isolated as a yellow amorphous powder as for (**79**).

RPHPLC analysis

Gradient - 25% to 55% Eluent B in 20 min. linearly.

Observed - 1 peak at 8.0 min.

m/z (+ve ES-MS) 668.2 (M + H), 651.6 (M - NH_2), 538.1 (M - Leu-NH_2), 390.2 (M - Phe-Leu-NH_2), 277.5 (M - Gly-Gly-Phe-Leu-NH_2).

Exposure of Nde-Leu-Tyr-Gly-Gly-Phe-Leu-NovaSyn® KR 100 resin to piperidine under continuous flow conditions.

The resin-bound peptide (0.10 g) was swelled in DMF for 1 h, and using an LKB 4175 Biolynx manual peptide synthesiser, was exposed to a continuous flow of 20% piperidine in DMF at a constant flow rate of 0.5 ml min^{-1} for 24 h. Samples of resin (20 mg) were removed at 3, 6 and 24 h intervals and washed and dried as described for (**79**). The resin samples were each ^{t}Bu deprotected and cleaved using TFA-H_2O-TIPS (95:2.5:2.5, 2 ml) at room temperature for 2 h. The individual peptide products were then recovered as described for (**79**) as yellow amorphous powders.

RPHPLC analysis

Gradient - 25% to 55% Eluent B in 20 min. linearly.

at t = 0 h.

Observed - 1 peak at 19.2 min

at t = 3 h.

Observed - 1 major peak at 19.2 min. and a second peak at 8.0 min.

Relative peak heights (94:6)

at t = 6 h.

Observed - 1 major peak at 19.2 min. and a second peak at 8.0 min.

Relative peak heights (87:13)

at t = 24 h.

Observed - 1 peak at 19.2 min. and a second peak at 8.0 min.

Relative peak heights (54:46)

Solid phase synthesis of dihydrotrypanothione

N^1, N^8-bis-Nde-Spermidine (81).

To a solution of spermidine (79 μl, 0.50 mmol) in anhydrous ethanol was added 2-acetyl-4-nitroindane-1,3-dione (0.350 g, 1.50 mmol) and DIEA (261 μl, 1.50 mmol) and the resultant solution was refluxed for 6-8 h. A bright yellow precipitate had formed which was collected at the pump and washed with ethanol and dried. TLC indicated a ninhydrin positive base-line spot which was subsequently removed by digestion of the yellow powder with 1% AcOH in ethanol. The solid was once again collected at the pump, washed several times with ethanol, and dried to afford N^1, N^8-bis-Nde-Spermidine (**81**), as a yellow amorphous powder (0.253 g, 88%), m.p. 165-169 ^{0}C.

ν_{max} (KBr) 3440, 3100, 2940, 1688, 1645, 1600, 1535, 1495, 1350 cm^{-1}

^{1}H NMR (CF_3CO_2H) δ 2.10 (4 H, m, NH-CH_2-CH_2-CH_2-CH_2), 2.49 (2 H, m, NH-CH_2-CH_2-CH_2-NH_2), 2.83 (6 H, s, 2 x =CCH_3), 3.54 (4 H, m, CH_2-NH-CH_2), 3.85 (4 H, m, 2 x =CNH-CH_2), 7.39 (2 H, br, s, CH_2-N+H_2-CH_2), 8.00 (6 H, m, ArH's), 11.54 (2 H, d, br, 2 x =CNH)

m/z (+ve ES-MS) 576.1 (M + H), 344.4 (M - Nde-NH), 114.3 (M - {2 x Nde-NH}).

Activation of NovaSyn® TGA resin with para-nitrophenylchloroformate.

NovaSyn® TGA resin functionalised at 0.28 mmol g^{-1} with an HMPA linker (0.20 g, 0.056 mmol of free alcohol), was swelled in dry DCM for 30 mins. To this solution was added 4-nitrophenylchloroformate (0.113 g, 0.56 mmol) and DIEA (98.0 μl, 0.56 mmol) and the suspension was allowed to stir

at room temperature overnight.

The resin was then washed 5 times with dry DCM and then washed 5 times with, and finally suspended in, peptide synthesis grade DMF. To this was added *Bis*-Nde-spermidine (0.161 g, 0.28 mmol) and DIEA (49 µl, 0.28 mmol) and the suspension was again allowed to stir overnight.

The resin was then washed with DMF and subsequently treated with acetic anhydride/DIEA to cap any unreacted resin bound alcohol. Deprotection of the Nde protecting group was then achieved using 2% hydrazine v/v in DMF under continuous flow conditions for 7 min., at a flow rate of 3.0 ml min^{-1}. A portion of the resin (20 mg) was then removed and treated with Fmoc-Cl (0.012 g, 0.045 mmol) and DIEA (8.0 µl, 0.045 mmol) until a negative TNBS test was obtained, the resin was then washed and dried as for (**79**).

The remainder of the Nde deprotected resin was used for the synthesis of dihydrotrypanothione (see p. 179).

Determination of Fmoc loading

The Fmoc-protected spermidine-resin (1 mg) was placed in a 1 cm path length quartz cuvette and treated with 3 ml 20% piperidine in DMF. After allowing the resin to stand, with occasional agitation, for 4 minutes the UV absorbtion at 290 nm was recorded against a blank of 20% piperidine in DMF.

UV_{290} absorbance = 0.792

Now $A = \varepsilon.c.l$ where $A = 0.792$, $\varepsilon = 5253$ and $l = 1$

$$\text{therefore } c = \frac{0.792}{5253}$$

$= 1.51 \times 10^{-4}$ mol L^{-1} of piperidine adduct

Therefore in 3 ml of deprotection media there is 4.52×10^{-7} mol of piperidine adduct. Taking into account the mass difference associated with incorporation of *bis*-Fmoc-spermidine onto the resin this value is equivalent to a molar amine functionality found on 0.849 mg of resin. Therefore, the loading on 1 g would be 0.53 mmol. If 100% incorporation had occurred, then the theoretical value would be 0.56 mmol g^{-1}.

Hence, % incorporation of N^1, N^8-bis-Nde-Spermidine (**81**) onto the urethane modified linker = 95%.

Dihydrotrypanothione.

Following Nde deprotection with 2% hydrazine in DMF, peptide synthesis was then carried out on the spermidine-NovaSyn® TGA resin using Fmoc-Gly-OH (133 mg, 0.448 mmol), Fmoc-Cys(Trt)-OH (262 mg, 0.448 mmol) and Fmoc-Glu-OtBu (191 mg, 0.448 mmol) *via* TBTU (143 mg, 0.448 mmol)/HOBt.H_2O (34 mg, 0.224 mmol)/DIEA(87 µl, 0.896 mmol) mediated coupling. The synthesis was accomplished using an LKB 4175 Biolynx manual peptide synthesiser, following the synthetic strategy outlined for (**79**), with Fmoc deprotection achieved with 20% piperidine in DMF.

The resin was then washed and dried as for (**79**). A portion of the resin (10 mg) was suspended in TFA-EDT-H_2O-TIPS (92.5 : 2.5 : 2.5 : 2.5, 10 ml) for 2 h with occasional agitation. The peptide product was isolated as a white amorphous powder (7 mg) as for (**79**).

RPHPLC Analysis

Gradient - 5% Eluent B for 5 min. then

5% to 50% Eluent B in 20 min. linearly.

Observed - 1 major peak at 3.0 min.

The major peak was purified by semi-preparative RPHPLC under analogous conditions.

m/z (+ve ES-MS) 724.1 (M + 1)

*Trypanothione disulphide (**80**).*

Dihydrotrypanothione (7 mg) was dissolved in ammonium acetate 0.04 M (70 ml, adjusted to pH 8.5 with conc. ammonia) under atmospheric oxygen at room temperature and allowed to stir for 72 h. The solution was then carefully concentrated *in vacuo* and finally lyophilised to afford a white amorphous solid.

RPHPLC Analysis

Gradient - 5% Eluent B for 5 min. then

5% to 50% Eluent B in 20 min. linearly.

Observed - 1 major peak at 2.2 min.

The major peak was purified by semi-preparative RPHPLC under analogous conditions.

m/z (+ve ES-MS) 722.2 (M + 1)

HRMS (FAB) Found: *m/z* 722.293257 Calcd for $C_{27}H_{48}N_9O_{10}S_2$: (M+H), 722.296558.

Chapter 3.

N-1-(4-acetamido-1,3-dioxoindan-2-ylidene)ethyl-L-alanine.

In a high pressure hydrogenation vessel, Nde-L-alanine (0.20 g, 0.66 mmol) was dissolved in methanol (50 ml). The solution was purged with N_2 and 10% Pd/C catalyst (40 mg) and acetic anhydride (5 ml) were introduced. The vessel was evacuated and filled with hydrogen to obtain a pressure of 50 psi. The suspension was shaken at room temperature for 18 h, after which, the vessel was evacuated and purged with N_2. The suspension was filtered through a damp kieselguhr pad and evaporated to dryness to yield a yellow amorphous powder (42 mg, 20%)

ν_{max} (KBr) 3660, 1745, 1680, 1635, 1590, 1530, 1300 cm^{-1}

^1H NMR ($(CD_3)_2SO$, 250 MHz) δ 1.50 and 1.51 (3 H, 2 x d, *J* 7.5, *E/Z* β-CH_3),2.18 and 2.19 (3 H, 2 x s, *E/Z* $COCH_3$), 2.59 and 2.60 (3H, 2 x s, *E/Z* $C=CCH_3$), 4.72 and 4.83 (1 H, 2 x m, *E/Z* α-CH), 7.27 (1 H, m, Ar C5-H), 7.58 (1 H, m, Ar C6-H), 8.47 (1 H, m, Ar C7-H) 10.12 and 10.42 (1 H, 2 x br, s, *E/Z* CONH), 10.68 and 13.89 (1H, 2 x d, *J* 7.5, *E/Z* =CNH)

m/z (FAB) 317.5 (M + H)

2-Acetyldimedone (23).

To a stirred solution of acetic acid (0.096 g, 3.0 mmol) in dry dichloromethane (10 ml) was added dimedone (0.280 g, 2.0 mmol), DCC (0.412 g, 2.0 mmol) and DMAP (0.244 g, 2.0 mmol). The resulting solution was allowed to stir at room temperature for 18 h.

The solution was cooled and filtered to remove the precipitated DCU.

The dichloromethane was removed *in vacuo* and the residue redissolved in ethyl acetate (20 ml) and washed with 1M $KHSO_{4(aq)}$. The organic extract was washed with brine (2 x 10 ml), dried ($MgSO_4$) and rotary evaporated to yield 2-acetyldimedone (**23**) as a pale yellow crystalline solid (0.211 g, 80%), m.p. 34 ^{0}C (lit.,[234] 34-36 ^{0}C).

ν_{max} (Thin film) 2960, 1667, 1560, 1445 cm^{-1}

^{1}H NMR δ 1.10 (6 H, s, $C(CH_3)_2$), 2.33 (2 H, s, CH_2), 2.51 (2 H, s, CH_2), 2.20 (3 H, s, $=CCH_3$), 17.92 (1 H, s, OH) ppm.

m/z (+ve ES-MS) 183.2 (M+H).

*2-Hexanoyldimedone (**92**).*

To a stirred solution of hexanoic acid (0.116 g, 1.0 mmol) in dry dichloromethane (5 ml) was added dimedone (0.140 g, 1.0 mmol), DCC (0.226 g, 1.1 mmol) and DMAP (0.183 g, 1.5 mmol). The resulting solution was allowed to stir at room temperature for 18 h.

The solution was cooled and filtered to remove the precipitated *N,N'*-dicyclohexylurea. The dichloromethane was removed *in vacuo* and the residue redissolved in ethyl acetate and washed with 1M $KHSO_{4(aq)}$. The organic extract was washed with brine, dried ($MgSO_4$) and rotary evaporated to yield 2-hexanoyldimedone (**92**) as a yellow oil (0.20 g, 84%).

ν_{max} (Thin film) 2995, 2900, 1680, 1560, 1450 cm^{-1}

^{1}H NMR δ 0.85 (3 H, t, 6 Hz CH_2CH_3), 1.05 (6 H, s, $C(CH_3)_2$), 1.40 (6 H, m, $((CH_2)_3CH_3)$), 2.30 (2 H, s, $(COCH_2)$), 2.47 (2 H, s, $(COCH_2)$), 2.93 (2 H, t, 7 Hz, $(=C(OH)CH_2)$), 18.14 (1 H, s, enol -OH)

m/z (+ve ES-MS) 239.1 (M+H)

Angiotensin II receptor binding protein fragment synthesised as its corresponding amide (Lys-Gly-Tyr-Ile-His-Ala-Leu-NH_2) using Fmoc amino acids.

NovaSyn®KR 125 resin functionalised at 0.12 mmol g^{-1} with an Fmoc protected modified Rink amide linker (0.2 g, 24 μmol functionality) was swelled in DMF for 60 min. The title peptide was then synthesised using Fmoc-Leu-OH (34 mg, 0.096 mmol), Fmoc-Ala-OH (30 mg, 0.096 mmol), Fmoc-His(Trt)-OH (60 mg, 0.096 mmol), Fmoc-Ile-OH (34 mg, 0.096 mmol), Fmoc-Tyr(^{t}Bu)-OH (44 mg, 0.096 mmol), Fmoc-Val-OH (33 mg, 0.096 mmol), Fmoc-Gly-OH (29 mg, 0.096 mmol) and Fmoc-Lys(Boc)-OH (45 mg, 0.096 mmol) *via* TBTU (31 mg, 0.096 mmol)/HOBt.H_2O (7 mg, 0.048 mmol)/DIEA (33 μl, 0.192 mmol) mediated coupling. The synthesis was accomplished using an LKB 4175 Biolynx manual peptide synthesiser, following the synthetic strategy outlined for (**79**), with Fmoc deprotection achieved with 20% piperidine in DMF.

The resin was then washed and dried as for (**79**). A portion of the resin (10 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5, 10 ml) for 2 h with occasional agitation. The peptide product was isolated as a white amorphous powder as for (**79**).

RPHPLC Analysis

Gradient - 10% to 50% Eluent B in 20 min. linearly.

Observed - 1 peak at 7.5 min.

m/z (+ve ES-MS) 900.2 (M + H), 769.5 (M - Leu-NH_2), 561.8 (M - His-Ala-Leu-NH_2), 450.1 (M - Ile-His-Ala-Leu-NH_2 + H).

*On resin acylation of the angiotensin receptor binding protein fragment using 2-hexanoyldimedone (**92**).*

Angiotensin receptor binding protein fragment attached to NovaSyn®KR 125 resin (50 mg, 0.12 mmol g^{-1}), with a theoretical total amine functionality of 6 μmol, was swelled in DMF for 60 min. 2-Hexanoyldimedone (**92**) (6 mg, 0.025 mmol) was dissolved in DMF (1 ml) and subsequently added to the resin bound peptide and was allowed to stand at room temperature overnight. The picrylsulphonic acid amine test was negative, so the resin was transferred to a sintered glass funnel and sequentially washed and dried as for (**79**). A portion of the resin (10 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5, 10 ml) for 2 h with occasional agitation. The peptide product was isolated as a white amorphous powder as for (**79**).

RPHPLC Analysis

Gradient - 10% to 50% Eluent B in 20 min. linearly.

Observed - 1 peak at 16.8 min. (a 9.3 min. shift in retention time)

m/z (+ve ES-MS) 1120.2 (M + 1), 669.3 (M - Ile-His-Ala-Leu-NH_2).

Deprotection and recovery of the angiotensin receptor binding protein fragment.

The hexanoylated peptide amide fragment (3 mg) was dissolved in 5% $hydrazine_{(aq)}$ (3 ml) and allowed to stir at room temperature for 30 min. Trifluoroacetic acid was added dropwise to the cooled solution until pH 2 was

attained. The solution was then de-salted using a Millipore C_{18} Sep-Pak cartridge. The de-salted solution was evaporated to dryness using a high vacuum rotary evaporator, and the residue triturated with HPLC grade ether. The white amorphous powder was dissolved in distilled water and lyophilised to yield the angiotensin receptor binding protein fragment as a white amorphous powder (2 mg).

RPHPLC Analysis

Gradient - 10% to 50% Eluent B in 20 min. linearly.

Observed - 1 major peak at 7.5 min.

m/z (+ve ES-MS) 900.2 (M + H), 561.8 (M - His-Ala-Leu-NH_2), 450.1 (M - Ile-His-Ala-Leu-NH_2 + H).

*2-Biotinyldimedone (**93**).*

To a stirred solution of biotin (0.244 g, 1.0 mmol) in peptide synthesis grade DMF, (10 ml) was added dimedone (0.154 g, 1.1 mmol), DCC (0.206 g, 1.0 mmol) and DMAP (0.122 g, 1.0 mmol). The resulting solution was allowed to stir at room temperature for 48 h.

The solution was cooled and filtered to remove the precipitated *N,N'*-dicyclohexylurea. The DMF was removed *in vacuo* using a high vacuum rotary evaporator and the residue redissolved in ethyl acetate and washed with 1M $KHSO_{4(aq)}$. The organic layer was then extracted with saturated $NaHCO_{3(aq)}$, the extracts combined, cooled and acidified to pH 2 using 6M HCl and the oily product extracted with dichloromethane. The organic extract was washed with brine (2 x 10 ml), dried ($MgSO_4$) and evaporated to dryness to yield a white/yellow amorphous powder. Recrystallisation from methanol-water (1:1)

afforded 2-biotinyldimedone (**93**) as a white crystalline solid (252 mg, 69%), m.p. 142-144 ^{0}C

ν_{max} (KBr) 3300, 2595, 1750, 1700, 1575, 1475 cm^{-1}

^{1}H NMR (250 MHz) δ 1.07 (6 H, s, $C(CH_3)_2$), 1.54 (6 H, m, $(CH_2)_3$), 2.35 (2 H, s, $COCH_2$), 2.54 (2 H, s, $COCH_2$), 2.73 (1 H, d, *J* 13 Hz, $CHSCH_{\beta}$), 2.91 (1 H, dd, *J* 13, 5 Hz, $CHSCH_{\alpha}$), 3.04 (2 H, t, *J* 7 Hz, $=C(OH)CH_2$), 3.16 (1 H, m, $CHSCH_2$), 4.31 (1H, m, ring junction H), 4.56 (1H, m, ring junction H), 5.87 (1H, s, NH), 6.15 (1H, s, NH), 14.85 (1H, s, OH)

^{13}C NMR δ 24.26 ($C(OH)CH_2\mathbf{C}H_2$), 28.02 ($C(\mathbf{C}H_3)_2$), 28.19 ($C(OH)CH_2$-$CH_2CH_2\mathbf{C}H_2$), 28.45 ($C(OH)CH_2CH_2\mathbf{C}H_2$), 30.50 ($\mathbf{C}(CH3)_2$), 39.87 ($C(OH)\mathbf{C}H_2$), 40.44 ($SCH_2$), 46.60 ($\mathbf{C}H_2CO$), 52.44 ($\mathbf{C}H_2CO$), 55.47 ($S\mathbf{C}HCH_2$), 60.00 ($SCH_2\mathbf{C}H$), 61.86 ($SCHCH$), 111.80 ($\mathbf{C}=C(OH)CH_2$), 164.01 ($NHCONH$), 195.01 ($CH_2\mathbf{C}O$), 197.55 ($CH_2\mathbf{C}O$), 205.191 ($=\mathbf{C}(OH)CH_2$)

Found: C, 59.15; H, 7.07; N, 7.39. $C_{18}H_{27}N_2O_4S$ requires C, 59.00; H, 7.15; N, 7.65%.

m/z (FAB) 367.2 (M+H).

HRMS (FAB) Found: *m/z* 367.162687. Calcd for $C_{18}H_{27}N_2O_4S$: (M+H), 367.169154.

RPHPLC Analysis

Gradient - 20% to 80% Eluent B in 20 min. linearly.

Observed - 1 peak at 10.5 min.

On resin acylation of the angiotensin receptor binding protein fragment using 2-biotinyldimedone (93).

Angiotensin receptor binding protein fragment attached to NovaSyn®KR 125 resin (50 mg, 0.12 mmol g^{-1}) with a total functionality of 6 μmol was swelled in DMF for 60 min. 2-Biotinyl dimedone (9 mg, 0.025 mmol) was dissolved in DMF (1 ml) and subsequently added to the resin bound peptide and allowed to stand at room temperature overnight. The picrylsulphonic acid amine test was negative, so the resin was transferred to a sintered glass funnel and sequentially washed and dried as for (**79**). A portion of the resin (10 mg) was suspended in TFA-H_2O-TIPS (95:2.5:2.5, 10 ml) for 2 h with occasional agitation. The peptide product was isolated as a white amorphous powder as for (**79**).

RPHPLC Analysis

Gradient - 10% to 50% Eluent B in 20 min. linearly.

Observed - 1 peak at 14.0 min.

m/z (+ve ES-MS) 1248.0 (M + 1), 1047.0 (M - Ala-Leu-NH_2), 911.1 (M - His-Ala-Leu-NH_2), 796.5 (M - Ile-His-Ala-Leu-NH_2).

Immobilisation of the biotinylated angiotensin receptor binding protein fragment on an immobilised avidin column.

A pre-packed immobilised avidin column (Pierce) was equilibrated using five column volumes of 20 mM phosphate buffered saline (pH 7.5, 500 mM NaCl). Biotinylated angiotensin receptor binding protein (1 mg) was dissolved in the buffer solution (1 ml) and was run into the column. The flow was halted, and the column allowed to stand at room temperature for 1 h. The

column was washed with binding buffer and the eluent monitored by RPHPLC. No biotinylated peptide was detected in the washings.

The column was then treated with 5% hydrazine$_{(aq)}$ (5 x 1ml), allowing each deprotection volume to incubate for 5 min. The deprotection medium was eluted into 10% acetic acid$_{(aq)}$ and the resulting solution was de-salted using a millipore C_{18} Sep-Pac Cartridge. The de-salted solution was lyophilised to yield a white amorphous solid.

RPHPLC Analysis

Gradient - 10% to 50% Eluent B in 20 min. linearly.

Observed - 1 major peak at 7.5 min.

The major peak was purified by semi-preparative RPHPLC under analogous conditions.

m/z (+ve ES-MS) 900.0 (M + 1), 561.7 (M - His-Ala-Leu-NH_2), 450.7 (M - Ile-His-Ala-Leu-NH_2).

Chapter 4.

Mono-benzyl adipate (100).

Adipic acid (11.0 g, 0.075 mol), benzyl alcohol (12.0 g, 0.111 mol) and tosic acid (0.190 g, 1.0 mmol) were mixed together in anhydrous toluene and heated under reflux for 2 h, using Dean-Stark apparatus for the azeotropic removal of water. The reaction mixture was cooled, water (100 ml) added and the solution carefully adjusted to pH 8 using 6 M NaOH. The aqueous layer was separated and washed with ether (2 x 25 ml). Fresh ether (50 ml) was added and the solution adjusted to pH 2 using 6 M HCl. The ether layer was

separated, dried ($MgSO_4$), and evaporated to dryness to yield mono-benzyl adipate (**91**) as a colourless oil (10.97 g, 62%).

^1H NMR δ 1.55 (4 H, m, $CH_2CH_2CH_2CH_2$), 2.26 (4 H, m, 2 x $COCH_2$), 5.02 (2 H, m, OCH_2), 7.29 (5 H, s, Ph), 11.31 (1 H, s, CO_2H).

*5-(5-benzyloxycarbonylpentanoyl)Meldrum's acid (**101**).*

To a stirred solution of mono-benzyl adipate (**100**) (2.36 g, 0.01 mol), Meldrum's acid (1.50 g, 0.0105 mol) in dry DCM (40 ml) was added DCC (2.36 g, 0.115 mol), and DMAP (1.83 g, 0.015 mol) and the mixture was allowed to stir for 18 h. The solvent was removed and the residue taken-up into cold ethyl acetate and filtered. The organic filtrate was then washed with 2M HCl (2 x 20 ml), brine (2 x 20 ml) and dried ($MgSO_4$). Volatiles were removed *in vacuo* to afford a yellow solid, (1.74 g, 48% crude yield) which was used in the following reaction, without purification.

^1H NMR δ 1.70 (6 H, s, $C(CH_3)_2$), 1.75 (4 H, m, $CH_2CH_2CH_2CH_2$), 2.38 (2 H, m, $BzlOCOCH_2$), 3.10 (2 H, m, CH_2CO-Meldrum's acid), 5.12 (2 H, m, OCH_2Ph), 7.34 (5 H, s, Ph)

*N-(7-Benzyloxycarbonyl-3-oxoheptanoyl)-L-homoserine lactone (**102**).*

To a stirred solution of 5-(5-benzyloxycarbonylpentanoyl)Meldrum's acid (**101**) (1.74 g, 4.8 mmol) in anhydrous MeCN (90 ml) was added L-homoserine lactone hydrochloride (0.66 g, 5.3 mmol) and TEA (0.74 ml, 5.0 mmol) and the resulting mixture was allowed to stir at room temperature for 18 h and then heated under reflux for 3 h. The volatiles were removed *in vacuo* and the residue partitioned between ethyl acetate and water. The organic layer

was separated, washed with 1M $KHSO_{4(aq)}$ and brine, dried ($MgSO_4$) and evaporated to dryness to yield a colourless oil. Trituration of the oil with diethyl ether afforded *N-(7-Benzyloxycarbonyl-3-oxoheptanoyl)-L-homoserine lactone* (**102**) as a white solid (1.52 g, 89%), m.p. 77-78 ^{0}C, after recrystallisation from ethyl acetate.

ν_{max} (KBr) 3400, 2950, 1780, 1730, 1660, 1530 cm^{-1}

^{1}H NMR (250 MHz) δ 1.62 (4 H, m, $CH_2CH_2CH_2CH_2$), 2.24 (1 H, m, NHCHC*H*), 2.37 (2 H, t, *J* 7.5 $OCOCH_2$), 2.55 (2H, t, *J* 7.5, $CH_2CH_2COCH_2$), 2.73 (1 H, m, NHCHC*H*), 3.45 (2 H, s, $COCH_2CO$), 4.25 (1H, m, α-CH), 4.53 (2 H, m, $CO_2CH_2CH_2$) 5.10 (2 H, s, OCH_2Ph), 7.35 (5 H, s, Ph), 7.64 (1 H, d, *J* 7.5, NH)

Found: C, 63.42; H, 6.52; N, 3.89. $C_{21}H_{27}NO_7$ requires C, 63.14; H, 6.42; N, 3.88%.

m/z (FAB) 362 (M+H), 154 (M - $PhCH_2O$ - homoserine lactone).

*N-(7-Carboxy-3-oxoheptanoyl)-L-homoserine lactone (**103**).*

In a high pressure hydrogenation vessel, *N*-(7-Benzyloxycarbonyl-3-oxoheptanoyl)-L-homoserine lactone (**102**) (1.387 g, 3.8 mmol) was dissolved in methanol (50 ml). The solution was purged with N_2 and 10% Pd/C catalyst (140 mg) was added. The vessel was evacuated and filled with hydrogen to obtain a pressure of 3 psig. The suspension was shaken at room temperature for 2 h, after which, the vessel was evacuated and purged with N_2. The suspension was filtered through a damp kieselguhr pad and evaporated to dryness to yield N-(7-Carboxy-3-oxoheptanoyl)-L-homoserine lactone (**103**) as

a waxy solid (944 mg, 92%).

ν_{max} (thin film) 3350, 2950, 1775, 1720, 1660, 1540 cm^{-1}

^1H NMR ($(CD_3)_2SO$-$CDCl_3$) δ 1.60 (4 H, m, $CH_2CH_2CH_2CH_2$), 2.25 (3 H, m, CH_2CO_2H and NHCHC*H*), 2.59 (3 H, m, $CH_2CH_2COCH_2$ and NHCHC*H*), 3.41 (2 H, s, $COCH_2CO$), 4.40 (3H, m, $CO_2CH_2CH_2$ and α-CH), 7.40 (1 H, s, br, CO_2H), 8.34 (1 H, d, *J* 7.5, NH)

m/z (FAB) 272 (M+H), 254 (M - OH), 226 (M - CO_2H), 171 (M - homoserine lactone).

RPHPLC Analysis

Gradient - 5% to 30% Eluent B in 25 min. linearly.

Observed - 1 peak at 8.5 min.

*Attempted coupling of N-(7-Carboxy-3-oxoheptanoyl)-L-homoserine lactone (**103**) to L-valine methyl ester.*

To a stirred solution of *N*-(7-carboxy-3-oxoheptanoyl)-L-homoserine lactone (**103**) (0.03 g, 0.11 mmol), L-valine methyl ester tosylate (0.03 g, 0.1 mmol) and TEA (14 µl, 0.1 mmol) in dry DCM (5 ml) was added DCC (0.023 g, 0.11 mmol) and HOBt (0.017 g, 0.11 mmol) and the solution was allowed to stir for 18 h. TLC indicated mainly the presence of starting materials, however the presence of a small DCU precipitate warranted overall work-up. Volatiles were removed *in vacuo*, and the residue dissolved in cold ethyl acetate and filtered. The organic solution was washed with 1M $KHSO_{4(aq)}$, sat. $NaHCO_{3(aq)}$ and brine. The solution was then dried ($MgSO_4$) and evaporated to dryness to yield a colourless oil (8mg, 21% crude yield). The ^1H NMR

indicated mainly the presence of DCU.

*N-(7-Benzyloxycarbonyl-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**106**).*

N-(7-Benzyloxycarbonyl-3-oxoheptanoyl)-L-homoserine lactone (**102**) (0.10 g, 0.28 mmol), ethanediol (16 µl, 0.3 mmol) and tosic acid (1 mg) in dry benzene (50 ml) were heated together under reflux for 18 h. A dean-Stark apparatus was attached for azeotropic removal of water.

The reaction mixture was cooled and evaporated to dryness. The residue was redissolved in ethyl acetate, washed with water and brine, dried ($MgSO_4$) and rotary evaporated to yield an oil which was subsequently purified by PLC (EtOAc-Hexane-MeOH, 4.5:4.5:1) to afford *N*-(7-Benzyloxycarbonyl-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**106**) as a pale yellow oil (96 mg, 86%).

ν_{max} (thin film) 3460, 2950, 1780, 1730, 1660, 1530 cm^{-1}

^1H NMR δ 1.60 (6 H, m, $HO_2CCH_2CH_2CH_2CH_2$), 2.15 (1 H, m, NHCHC*H*), 2.35 (2 H, t, *J* 7.5, CH_2CO_2H), 2.59 (2 H, s, $C(O_2(CH_2)_2)CH_2$ CO), 2.72 (1 H, m, NHCHC*H*), 3.96 (4 H, m, OCH_2CH_2O), 4.45 (3H, m, $CO_2CH_2CH_2$ and α-CH), 5.12 (2 H, s, OCH_2Ph), 6.98 (1 H, d, *J* 7.5, NH), 7.38 (5 H, s, Ph)

m/z (FAB) 406.1 (M+H), 154 (M - $PhCH_2O$ - homoserine lactone).

*N-(7-Carboxy-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**107**)*

In a high pressure hydrogenation vessel, (**106**) (0.203 g, 0.50

mmol) was dissolved in methanol (20 ml), The vessel was purged with N_2 and 10% Pd/C catalyst (21 mg) was added. The vessel was evacuated and filled with hydrogen to obtain a pressure of 3 psig. The suspension was shaken at room temperature for 2 h, after which, the vessel was evacuated and purged with N_2. The suspension was filtered through a damp kieselguhr pad and evaporated to dryness to yield *N*-(7-Carboxy-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**107**) a colourless oil (150 mg, 95%).

ν_{max} (thin film) 3330, 2950, 1780, 1730, 1660, 1545 cm^{-1}

^1H NMR δ 1.42 (6 H, m, $HO_2C.CH_2CH_2CH_2CH_2$), 2.18 (3 H, m, CH_2CO_2H and NHCHC*H*), 2.48 (2 H, s, $C(O_2(CH_2)_2)CH_2$ CO) 2.62 (1 H, m, NHCHC*H*), 3.83 (4 H, m, OCH_2CH_2O), 4.30 (3 H, m, $CO_2CH_2CH_2$ and α-CH), 7.12 (1 H, d, *J* 7.5, NH), 9.60 (1 H, s, br, CO_2H)

m/z (FAB) 316.2 (M+H), 297.2 (M - OH), 255.9 (M - OCH_2CH_2O + H)

*Coupling of N-(7-Carboxy-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**107**) to valine methyl ester.*

To a stirred solution of *N*-(7-Carboxy-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**107**) (0.10 g, 0.32 mmol), L-valine methyl ester hydrochloride (0.06 g, 0.36 mmol) and TEA (50 µl, 0.36 mmol) in dry DCM (20 ml) at 0 ^0C was added DCC (0.074 g, 0.36 mmol) and HOBt (0.055 g, 0.36 mmol) and the resulting solution allowed to stir for 18 h. DCM was removed *in vacuo* and the residue was redissolved in cold ethyl acetate and filtered. The filtrate was then successively washed with 1M $KHSO_{4(aq)}$, sat. $NaHCO_{3(aq)}$, water and brine. The organic layer was dried ($MgSO_4$) and

volatiles evaporated to yield an oil which was subsequently purified by PLC (EtOAc-Hexane, 1:1) to afford the expected *N*-acylated valine methyl ester (**108**) as a colourless oil (82 mg, 60%).

ν_{max} (thin film) 3350, 2940, 2860, 1780, 1740, 1670, 1540 cm^{-1}

^1H NMR δ 1.11 (6 H, d, *J* 8.0, 2 x Val-γ-CH_3), 1.55 (6 H, m, $NHCOCH_2$-$CH_2CH_2CH_2$), 2.20 (3 H, m, CH_2CO_2H and NHCHC*H*), 2.35 (1 H, m, Val-β-CH), 2.60 (2 H, s, $C(O_2(CH_2)_2)CH_2$ CO) 2.72 (1 H, m, NHCHC*H*), 3.68 (3 H, s, OCH_3), 3.92 (4 H, m, OCH_2CH_2O), 4.30 (4 H, m, $CO_2CH_2CH_2$, Val-α-CH and homoserine lactone-α-CH), 6.58 (1 H, d, *J* 7.5, Val-NH), 7.18 (1 H, d, *J* 7.5, homoserine lactone-NH)

m/z (FAB) 429.6 (M+H), 397.7 (M - OMe), 328.6 (M - homoserine lactone).

Deketalisation of (108).

The compound (**107**) (0.050g, 0.12 mmol) was dissolved in 95% $TFA_{(aq)}$ and allowed to stand for 2 h. The aqueous TFA was removed on a high vacuum rotary evaporator and the residue partitioned between ethyl acetate and water. The organic layer was washed with water (3 x 20 ml), brine and dried ($MgSO_4$). Removal of ethyl acetate *in vacuo* afforded the desired deketalised product as a colourless oil (46 mg, 99%).

ν_{max} (thin film) 3300, 2930, 1770, 1730, 1660, 1565, 1200 cm^{-1}

^1H NMR δ 1.12 (6 H, d, *J* 7.0, 2 x Val-γ-CH_3), 1.65 (4 H, m, CH_2CH_2-CH_2CH_2), 2.35 (4 H, m, CH_2CO_2H, NHCHC*H* and Val-β-CH), 2.60 (3 H, m, CH_2CH_2 $COCH_2$ and NHCHC*H*), 3.60 (2 H, s, $COCH_2CO$), 3.74 (3 H, s, OCH_3), 3.95 (1 H, m, Val-α-CH), 4.55 (3 H, m, $CO_2CH_2CH_2$ and

homoserine lactone-α-CH), 7.00 (1 H, d, *J* 7.5, Val-NH),
8.08 (1 H, d, *J* 7.5, homoserine lactone-NH)
m/z (FAB) 385.2 (M+H)

*Acylation of H-Lys$_2$β-Ala$_2$-OUSA with N-(7-Carboxy-3,3-ethylenedioxy-heptanoyl)-L-homoserine lactone (**107**)*

Ultrasyn A resin, functionalised with the HMPA linker at 0.09 mmol g^{-1} was employed. To a stirred solution of Fmoc-β-alanine (0.062 g, 0.2 mmol) in dry DCM (5 ml) was added DCC (0.022 g, 0.1 mmol) at 0 ^{0}C. Stirring was continued for 10 min at 0 ^{0}C and then 15 min at room temperature. The mixture as then filtered to remove DCU and evaporated to dryness at ambient temperature to afford Fmoc-β-alanine anhydride as oily residue.

The residue was dissolved in DMF (0.5 ml) and added to the pre-swollen resin (0.2 g, 18 μmol -OH functionality), followed by a solution of DMAP (0.0024g, 0.02 mmol) in DMF (0.5 ml). The mixture was allowed to stand for 2 h at room temperature with occasional agitation. The resin was then washed and dried as for (**79**).

Estimation of the amount of Fmoc-β-alanine attached to the resin.

A portion of resin (10 mg) was suspended in 20% piperidine in DMF (3 ml) for 5 min with occasional agitation. The UV absorbance of the supernatant was measured at 290 nm against a blank solution of 20% piperidine in DMF. The observed absorbance of 1.501 corresponded to a resin substitution level of 90% (0.081 mmol g^{-1}) using the calibration plot,[259]

Using the remainder of the resin, Fmoc-β-Ala-OH (20 mg, 0.064

mmol), Fmoc-Lys(Fmoc)-OH (38 mg, 0.064 mmol) and Fmoc-Lys(Fmoc)-OH (77 mg, 0.13 mmol), the title peptide was synthesised using HBTU/HOBt/ DIEA *in situ* activation. The synthesis was accomplished using an LKB 4175 Biolynx manual peptide synthesiser following the synthetic strategy outlined for (**79**), with Fmoc deprotection achieved with 20% piperidine in DMF.

After the final Fmoc deprotection cycle, *N*-(7-Carboxy-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**107**) (80 mg, 0.26 mmol), HOBt.H_2O (40 mg, 0.26 mmol) and DIPCDI (40 µl, 0.26 mmol) were employed to acylate the resin-bound MAP core. The resin was transferred to a sintered glass funnel and sequentially washed and dried as for (**79**). A portion of the resin (10 mg) was suspended in TFA-H_2O (95:5, 10 ml) for 2 h with occasional agitation. The peptide product was isolated as a white amorphous powder as for (**79**).

RPHPLC analysis

Gradient - 10% to 50% Eluent B in 20 min. linearly.

Observed - 1 major peak at 10.5 min and 1 impurity peak at 6.5 min.

The major peak was purified by semi-preparative RPHPLC under analogous conditions.

m/z (MALDI-TOF) 1557.6 (M + H).

Attempted coupling of Nα, Nα, Nε, Nε-tetra[7-{N-((S)-2-oxotetrahydro-3-furyl)carbamoyl-6,6-ethylenedioxyheptanoyl-Lys$_2$-β-Ala$_2$-OH to bovine serum albumin

Nα, Nα, Nε, Nε-tetra[7-{*N*-((*S*)-2-oxotetrahydro-3-furyl)carbamoyl-6,6

-ethylenedioxyheptanoyl-Lys_2-β-Ala_2-OH (10 mg, 6.43 μmol) was dissolved in PBS buffer 12.5 mM at pH 5.0-5.5 (1 ml). To this was added EDC (1.0 mg) and the Ph of the solution maintained by the careful addition of 0.01M HCl for 1-2 min before the addition of BSA (5 mg ml^{-1}, 34 μl) with rapid adjustment to pH 6.4. The pH was maintained at 6.4 for 90 min and then the solution was left to stir in the dark for 18 h. The solution was the lyophilised to afforded a white amorphous powder.

MALDI-TOF MS displayed no signs of incorporation of the MAP vector.

Nα, Nα, Nε, Nε-tetra[7-{N-((S)-2-oxotetrahydro-3-furyl)carbamoyl-6,6-oxo-heptanoyl-Lys_2-β-Ala_2-O-NovaSyn® KD.

NovaSyn® KD resin, functionalised ethylene diamine at 0.07 mmol g^{-1} (0.50 g) was swelled in DMF for 60 min. Using 2 x Fmoc-β-Ala-OH (44 mg, 0.14 mmol), Fmoc-Lys(Fmoc)-OH (83 mg, 0.14 mmol) and Fmoc-Lys(Fmoc)-OH (166 mg, 0.28 mmol) and TBTU/HOBt/DIEA *in situ* activation, the MAP core synthesis was accomplished using an LKB 4175 Biolynx manual peptide synthesiser following the synthetic strategy outlined for (**79**), with Fmoc deprotection achieved with 20% piperidine in DMF.

After the final Fmoc deprotection cycle, *N*-(7-Carboxy-3,3-ethylenedioxyheptanoyl)-L-homoserine lactone (**107**) (166 mg, 0.28 mmol), HOBt.H_2O (97 mg, 0.64 mmol) and DIPCDI (99 μl, 0.64 mmol) were employed to acylate the resin-bound MAP core. The resin was transferred to a sintered glass funnel and sequentially washed and dried as for (**79**).

The resin was then suspended in TFA-H_2O (95:5, 10 ml) for 2 h with occasional agitation, transferred to a sintered glass funnel and once more

sequentially washed and dried as for (**79**).

Preparation of N^{α}, N^{α}, N^{ε}, N^{ε}-tetra[7-{N-((S)-2-oxotetrahydro-3-furyl)carbamoyl-6,6-oxoheptanoyl-Lys_2-β-Ala_2-O-NovaSyn® KD for injection into experimental rabbits.

N^{α}, N^{α}, N^{ε}, N^{ε}-*tetra*[7-{*N*-((*S*)-2-oxotetrahydro-3-furyl)carbamoyl-6,6-oxoheptanoyl-Lys_2-β-Ala_2-O-NovaSyn® KD (20 mg) was sonicated in water for injections (1 ml) until a milky white paste was obtained. Introduction of Freund's Complete Adjuvant and subsequent emulsification provided enough injectable material for two experimental rabbits (6 injection sites per animal). A bleed was taken prior to the first injection. Two secondary boosters were given at one week and one month intervals after the initial injection, with bleeds being taken on each occasion.

*Analysis of plasma samples for the presence of anti-N-(7-carboxy-3-oxoheptanoyl)-L-homoserine lactone (**103**) antibodies.*

N^{α}, N^{α}, N^{ε}, N^{ε}-*tetra*[7-{*N*-((*S*)-2-oxotetrahydro-3-furyl)carbamoyl-6,6-oxoheptanoyl-Lys_2β-Ala_2-OH (5 mg) was dissolved in *tris* buffered saline and blotted onto nitrocellulose paper and allowed to dry. The strip was washed in Tween for 1 h to block all unbound sites, washed twice in *tris* buffered saline and cut into six squares, (Figure 9).

Each square was placed into individual sample tubes and covered with *tris* buffered saline (15 ml) and the relevant plasma sample (20 μl), see overleaf;

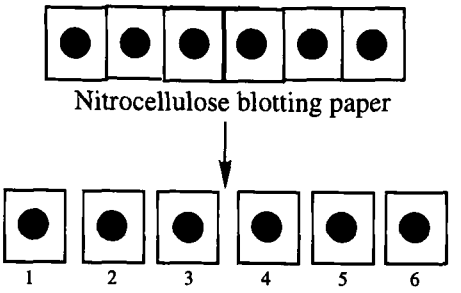


Figure 9. Preparation of nitrocellulose dot blot strips.

1. Rabbit No 40. Pre-immunisation bleed
2. Rabbit No 40. Post immunisation bleed (1 week)
3. Rabbit No 40. Post immunisation bleed (1 month)
4. Rabbit No 69. Pre-immunisation bleed
5. Rabbit No 69. Post immunisation bleed (1 week)
6. Rabbit No 69. Post immunisation bleed (1 month)

After 1.5 h incubation the strips were all washed twice with *tris* buffered saline. A solution of Protein A Peroxidase (PAP) (15 ml) was added to each sample tube and were left to incubate for 1 h. Meanwhile a visualisation solution was then made-up, consisting of chloronapthol (60 mg in MeOH, 3 ml) and 30% H_2O_2 (50 µl) in 100 mM *tris* buffered saline (pH 8). After washing each strip twice with *tris* buffered saline, 2-3 drops of the visualising solution were applied to each strip, and allowed to develop at ambient temperature.

None of the blot sites darkened , indicating the absence of anti-(*N*-(3-oxoheptanoyl)-L-homoserine lactone)$_4$Lys$_2$β-Ala$_2$-OH antibodies.

Chapter 5.

*N-Benzyloxycarbonyl-L-glutamic acid (**120**);*

The compound was prepared using the method described in Greenstein[278].

To a suspension of $NaHCO_3$ (31.5 g, 0.375 mol) in water (200 ml) was added L-glutamic acid (14.7 g, 0.1 mol) and the mixture stirred vigorously. To the mixture was added benzyl chloroformate (15.7 ml, 0.11 mol) dropwise over 30 min. The stirring was continued for 1 h longer. The reaction mixture was then washed with ether (50 ml) and the aqueous layer was removed and acidified using conc. HCl with cooling and stirring. The oily product was extracted with ethyl acetate (2 x 100 ml), dried ($MgSO_4$) and evaporated *in vacuo* to obtain a syrup/solid mixture. Addition of a small amount of ether and subsequent trituration resulted in the production of white crystals, which were collected at the pump and air dried (16.9 g, 60%), mp. 118-119^0 (lit.,[278] mp. 120-121^0)

ν_{max} (KBr) 1712, 1553, 1420, 1044 cm^{-1}.

^1H NMR δ 2.00 (4 H, m, CH_2CH_2), 4.11 (1 H, m, α-CH), 4.85 (2 H, s, PhCH_2), 5.68 (1 H, d, br, *J* 7.5Hz, NH), 7.24 (5 H, s, Ph), 8.98 (2 H, s, br, 2 x CO_2H)

*(S)-3-Benzyloxycarbonyl-5-oxo-4-oxazolidine propionic acid (**121**);*

The compound was prepared by the method described by Itoh *et al*[279]

N-Benzyloxycarbonyl-L-glutamic acid (7.0 g, 25 mmol), paraformaldehyde (1.25 g, 14 mmol) and toluene-*p*-sulphonic acid (0.25 g) were mixed together in dry toluene (175 ml) and refluxed for 1 h. The water

liberated during the reaction was removed azeotropically by means of a Dean-Stark distilling apparatus. The toluene solution was decanted from the brown residue and washed with water and then extracted with 5% $NaHCO_{3\ (aq)}$ solution (100 ml). The aqueous layer was removed and carefully acidified using 6N HCl, with ice cooling. The oily product was extracted with ethyl acetate (2 x 50 ml) and dried ($MgSO_4$). The solvent was evaporated to afford a colourless syrup (6.13 g, 84%).

ν_{max} (thin film) 3020, 1810, 1720, 1425 cm^{-1}.

^1H NMR δ 2.38 (4 H, m, CH_2CH_2), 4.38 (1 H, m, α-CH), 5.19 (2 H, s, PhCH_2), 5.22 (1H, d, br, *J* 4.5Hz, 1 x NHC*H*-O), 5.52 (1 H, d, br, *J* 4.5Hz, 1 x NHC*H*-O), 7.39 (5 H, s, Ph), 10.41 (1 H, br, s, CO_2H)

m/z (FAB) 294 (M + H).

m/z (EI) 275 (M - H_2O), 158 (M - $PhCH_2OCO$), 91 (Ph-CH_2).

*N-Benzyloxycarbonyl-L-glutamic acid α-methyl ester (**122**);*

(*S*)-3-Benzyloxycarbonyl-5-oxo-4-oxazolidine propionic acid (4.3 g, 15 mmol) was mixed with 0.1M sodium methoxide (Na, 0.68 g) in methanol (300 ml) at 0^0. The solution was stirred for 2 h and then neutralised by the careful addition of 2 M HCl. Methanol was removed *in vacuo* and the residue dissolved in ethyl acetate (50 ml). The organic solvent was washed with water (2 x 20 ml) and finally with brine (20 ml). Drying ($MgSO_4$) and evaporation of solvent gave a colourless syrup (3.6 g, 85%).

ν_{max} (thin film) 3338, 2960, 1740, 1720, 1530, 1470 cm^{-1}.

^1H NMR δ 2.28 (4 H, m, CH_2CH_2), 3.67 (3 H, s, OCH_3), 4.37 (1 H, m, α-

CH), 5.10 (2 H, s, PhCH_2), 5.86 (1 H, d, *J* 4.5, NH), 7.32 (5 H, s, Ph), 10.23 (1 H,br, s, CO_2H)

m/z (FAB) 296 (M + H)

m/z (EI) 250 (M - CO_2H), 91 ($PhCH_2$).

*N-Benzyloxycarbonyl-L-pyroglutamic acid methyl ester (**123**)*

Phosphorus pentachloride (3.0 g, 14 mmol) was added in one portion to a stirred solution of *N*-benzyloxycarbonyl-L-glutamic acid methyl ester (3.5 g, 10 mmol) in dry ether (150 ml) at 0^0. The solution was allowed to warm to room temperature and stirred for a further 36 h. Ether was removed *in vacuo*, followed by the complete removal of $POCl_3$ using a high vacuum pump for 1.5 h. The residue was dissolved in ethyl acetate, washed with $NaHCO_{3(aq)}$ and dried (Na_2SO_4). Evaporation gave the title product as an orange oil (2.68 g, 97%) which was sufficiently pure for the following step.

^{1}H NMR δ 2.30 (4 H, m, CH_2CH_2), 3.63 (3 H, s, OCH_3), 4.62 (1 H, m, α-CH), 5.23 (2 H, d, *J* 3Hz, PhCH_2), 7.35 (5 H, s, Ph)

m/z (FAB) 278 (M + H).

*Attempted synthesis of hemi-aminal (**124**).*

To a solution of *N*-benzyloxycarbonyl-L-pyroglutamic acid methyl ester (0.277 g, 1.0 mmol) in dry methanol (10 ml) at -30 ^{0}C, was added $NaCNBH_4$ (0.250 g, 4 mmol) over a period of 4 h, and allowed to stir for 2 h. 2 M Methanolic HCl was then added (10 ml) and the reaction mixture left to stir at ambient temperature for a further 18 h.

Volatiles were removed *in vacuo* and the residue partitioned between ethyl acetate and sat. $NaHCO_{3(aq)}$. The organic layer was washed with brine, dried ($MgSO_4$) and evaporated to dryness to yield a colourless oil which was identified as being the *N*-benzyloxycarbonyl-L-pyroglutamic acid dimethyl ester (**126**).

^1H NMR δ 2.28 (4 H, m, CH_2CH_2), 3.52 (3 H, s, γ-OCH_3), 3.67 (3 H, s, α-OCH_3), 4.37 (1 H, m, α-CH), 5.10 (2 H, s, PhCH_2), 5.86 (1 H, d, br, *J* 4.5Hz, NH), 7.32 (5 H, s, Ph)

m/z (EI) 250 (M - CO_2CH_3), 91 (Ph-CH_2).

*1-Methyl 7-tert-butyl (S) 2-benzyloxycarbonylamino-5-oxoheptanedioate(**128**),* The procedure by Ohta *et al*[282] was followed.

To a 2-neck flask, equipped with a suba-seal and a magnetic stirrer bar, was added dry THF (40 ml) and dry diisopropylamine (1.6 ml, 11.0 mmol). An atmosphere of nitrogen was maintained and the flask cooled to -25 ^{0}C followed by the careful addition of *n*-butyllithium (1.6 M in hexanes, 6.7 ml, 11.0 mmol) over a period of five minutes. The temperature was allowed to rise to -5 ^{0}C and maintained at that temperature for 40 min. This was then lowered to -78 ^{0}C and *t*-butyl acetate (1.25 ml, 9.0 mmol) was added and the mixture stirred at that temperature for 30 min. A solution of *N*-Benzyloxycarbonyl-L-pyroglutamic acid methyl ester (2.5 g, 9.0 mmol) in dry THF (20 ml) was added to the reaction mixture and stirred for 30 min. at -78 ^{0}C and then quenched with acetic acid:methanol (1:1, 4 ml). The solvent was removed *in vacuo* and the residue partitioned between ethyl acetate and water. The organic

layer was dried ($MgSO_4$) and evaporated to a pale yellow oil which was purified by flash silica chromatography in ethyl acetate-hexane, 1:3 to furnish the title product as an pale yellow oil (2.7 g, 77%).

ν_{max} (thin film) 3200-3500, 2990, 1700-1750, 1530, 1460 cm^{-1}.

^1H NMR δ 1.47 (9 H, s, $C(CH_3)_3$), 2.10 (2 H, m $COCH_2CH_2$), 2.60 (2 H, t, *J* 6Hz, $COCH_2CH_2$), 3.30 (2 H, s, $COCH_2CO$), 3.69 (3 H, s, OCH_3), 4.30 (1 H, m, α-CH), 5.12 (2 H, s, $PhCH_2$), 5.78 (1 H, d, br, *J* 7.5Hz, NH), 7.33 (5 H, s, Ph)

m/z (FAB) 376 (M + H - H_2O)

m/z (EI) 319 (M - *t*Bu-OH), 185 (M - $PhCH_2OCO$ -*t*BuO).

*tert-Butyl (2R,5S) 5-methoxycarbonyl-2-pyrrolidineacetate (**130**).*

In a high pressure hydrogenation vessel 1-methyl 7-*t*-butyl 2-benzyloxycarbonylamino-5-oxoheptanedioate (**128**) (2.0 g, 5 mmol) was dissolved in methanol (200 ml) and glacial acetic acid (1 ml). The solution was purged with N_2 and 10% Pd/C catalyst (0.4 g) was added. The vessel was evacuated and filled with hydrogen to obtain a pressure of 64 p.s.i. The suspension was shaken at room temperature for 18 h, after which, the vessel was evacuated and purged with N_2. The suspension was filtered through a damp kieselguhr pad and evaporated to dryness to yield the acetate salt. This was dissolved in 5% $NaHCO_{3(aq)}$ and extracted into ethyl acetate. The organic extract was dried ($MgSO_4$) and evaporated to afford *t*-butyl 5-methoxycarbonyl-2-pyrrolidine acetate (**130**) as a pale yellow oil (1.20 g, 97%). The product was pure by TLC ($CHCl_3$:MeOH, 9:1), R_f = 0.40.

ν_{max} 3360, 2910, 1730, 1440, 1370 cm^{-1}.

^1H NMR δ 1.50 (9 H, s, $C(CH_3)_3$), 2.05 (4 H, m, CH_2CH_2), 2.45 (2 H, dd, *J* 7.0, $COCH_2$), 2.58 (1 H, s, br, NH), 3.45 (1 H, m, C2-H), 3.73 (3 H, s, OCH_3), 3.90 (1 H, m, C5-H)

m/z (FAB) 244 (M + H)

m/z (EI) 186 (M - $C(CH_3)_3$).

*(2R,5S) 5-Methoxycarbonyl-2-pyrrolidineacetic acid hydrochloride (**133**).*

t-Butyl (2*R*, 5*S*) 5-methoxycarbonyl-2-pyrrolidineacetate (**130**) (1.277g, 5 mmol) was added to 4 M dioxane/HCl and left to stir for 48 h. The solution was evaporated to dryness , redissolved in dioxane and evaporated to dryness again. This process was repeated two to three times to remove all traces of HCl, finally affording the title compound as a brown glass (1.17 g, 100%).

^1H NMR (D_2O, DSS external standard) δ 2.10 (4 H, m, CH_2CH_2), 2.88 (2 H, t, *J* 7Hz, HO_2CCH_2), 3.65 (1 H, m, C2-H), 3.76 (3 H, s, OCH_3), 3.99 (1 H, m, C5-H)

m/z (FAB) 188 (M + H (-HCl))

*Attempted synthesis of methyl (3S,5R)-carbapenam-3-carboxylate (**134**).*

To a solution of (2*R*,5*S*) 5-methoxycarbonyl-2-pyrrolidineacetic acid hydrochloride (**133**) (0.073 g, 0.33 mmol) in dry acetonitrile (80 ml) was added TEA (75 μl, 0.54 mmol) with stirring. After 10 min. triphenylphosphine (0.120 mg, 0.46 mmol) and 2,2'-dipyridyl disulphide (0.102 g, 0.46 mmol)

and the resulting solution heated under reflux for 7 h. Mainly starting materials were returned.

S-(2-Acetamidoethyl)(2R,5S)-5-methoxycarbonyl-2-pyrrolidinethioacetate (136).

(2*R*,5*S*) 5-Methoxycarbonyl-2-pyrrolidineacetic acid hydrochloride (**133**) (0.025 g, 0.11 mmol) was dissolved in dry DCM (5 ml) with TEA (16 µl, 0.11 mmol). To this was added DCC (0.024 g, 0.121 mmol) and *N*-acetylcysteamine(0.026 g, 0.22 mmol) at 0 ^{0}C and the mixture allowed to warm to room temperature and stirred for 18 h. The solution was evaporated to dryness, redissolved in cold ethyl acetate and filtered. The organic solution was evaporated to dryness and the crude residue purified by PLC ($CHCl_3$:MeOH, 9:1) and the title compound recovered as an oil (14 mg, 44%).

ν_{max} 3460, 2910, 1740, 1680, 1510, 1365 cm^{-1}.

^{1}H NMR δ 1.95 (3 H, s, $COCH_3$), 2.10 (4 H, m, CH_2CH_2), 2.29 (1 H, br, s NH), 2.62 (2 H, d, *J* 7.0, $SCOCH_2$), 3.05 (2 H, t, *J* 6.0, SCH_2), 3.43 (2 H, dt, *J* 6.0 and 6.0, SCH_2CH_2), 3.65 (1 H, m, C2-H), 3.74 (3 H, s, OCH_3), 3.85 (1 H, m, C5-H), 6.10 (1 H, s, br, NHCO)

m/z (FAB) 289 (M + H)

m/z (EI) 229 (M - CO_2CH_3)

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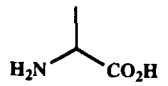
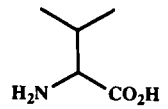
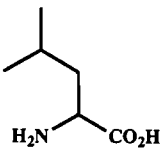
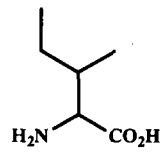
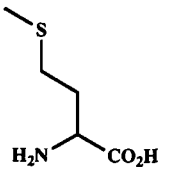
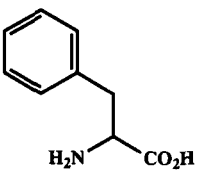
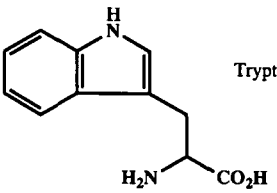
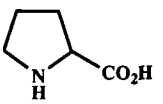
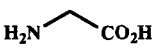
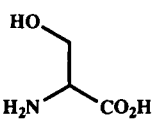
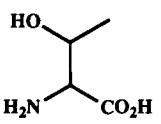
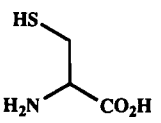
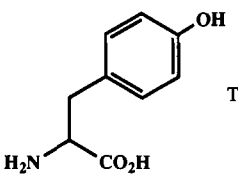
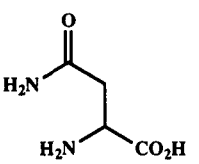
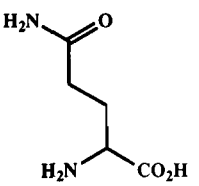
Appendix. The genetically coded amino acids

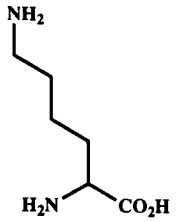
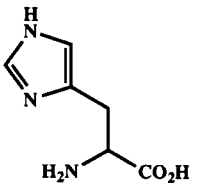
(a) Non-polar

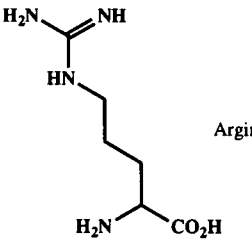
	Name	Abbreviation 3 Letter	Abbreviation 1 Letter
	Alanine	Ala	A
	Valine	Val	V
	Leucine	Leu	L
	Isoleucine	Ile	I
	Methionine	Met	M
	Phenylalanine	Phe	F
	Tryptophan	Trp	W
	Proline	Pro	P

(b) Polar uncharged

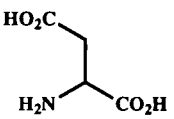
	Name	Abbreviation 3 Letter	Abbreviation 1 Letter
	Glycine	Gly	G
	Serine	Ser	S
	Threonine	Thr	T
	Cysteine	Cys	C
	Tyrosine	Tyr	Y
	Asparagine	Asn	N
	Glutamine	Gln	Q

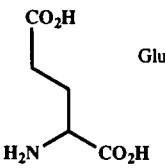
(c) Basic amino acids

	Name	Abbreviation 3 Letter	Abbreviation 1 Letter
	Lysine	Lys	K
	Histidine	His	H

	Name	Abbreviation 3 Letter	Abbreviation 1 Letter
	Arginine	Arg	R

(d) Acidic amino acids

	Name	Abbreviation 3 Letter	Abbreviation 1 Letter
	Aspartic acid	Asp	D

	Name	Abbreviation 3 Letter	Abbreviation 1 Letter
	Glutamic acid	Glu	E