Aspects of Peptide Chemistry

by

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Only by walking the road of excess will you reach the palace of wisdom

William Blake

This thesis is submitted in part fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Edinburgh. Unless otherwise stated, the work described is original and has not been previously submitted in whole or in part, for any degree at this, or any other university.

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ABSTRACT

The design of a solid phase peptide synthesis linker, which on cleavage with trifluoroacetic acid leaves the peptide C-terminus with a hydroxy ester functionality, is described herein.

The enhanced rate of hydrolysis of hydroxy esters and carbamates under basic conditions has been investigated with a view to utilising this effect in the orthogonal protection of amino acid side chains during the syntheses of large peptides. The development of protecting groups for both acid and amine side chains has been investigated in order that the syntheses of proteins via chemical fragment coupling can proceed with a high degree of fidelity. The base hydrolysis of various esters and carbamates has been studied, as has the stability of these compounds to acidic conditions. The syntheses of a number of protecting group candidates based on tris(hydroxymethyl) derivatives has been undertaken and conditions for their removal optimised. The syntheses of various test compounds and small peptides utilising these types of protecting groups has been completed. The synthesis, purification and deprotection of a 32 residue peptide, with one lysine protected as the tris(hydroxymethyl)nitromethyl carbamate is also reported.

The synthesis of a section of the CD4 binding region of the HIV coat protein Gp120 has been investigated. Various methods of cysteine protection have been studied and the difficulties associated with solubility and protecting group removal highlighted.

ABBREVIATIONS

a.a	amino acid
A.A.A	amino acid analysis
ABI	applied biosystems
Acm	acetamidomethyl
AcOH	acetic acid
AIDS	aquired immunodeficiency syndrome
ARC	AIDS related complex
b	broad
Boc	tert-butoxycarbonyl
BOP	benzotriazolyl-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
tBuS	tertiary-butylsulphide
tBu	tertiary-butyl
d	doublet
DCC	N,N'-dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	N,N'-diisopropylcarbodiimide
DIEA	N,N-diisopropyl ethyl amine
DIU	diisopropylurea
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiolthreitol
EDT	ethane-1,2-dithiol
FAB	fast atom bombardment
Fmoc	9-fluorenylmethyloxycarbonyl
Gdm.Cl	guanidinium hydrochloride
HIV	human immunodeficiency virus
HOBt	1-hydroxybenzotriazole
HOCt	ethyl 1-hydroxy-1H-1,2,3-triazole-4-carboxylate
HPLC	high performance liquid chromatography
HR	high resolution
IR	infra red
k	rate constant
m	meta
μ	micro
m	multiplet
min	minutes
Mpt	melting point
MS	mass spectrometry
NMR	nuclear magnetic resonance

PBL	peripheral blood lymphocytes
Pfp	pentafluorophenyl
p	para
PAM	p-aminomethylated
q	quartet
PGC	porous graphitised carbon
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
PyBOP	benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
Rp	reverse phase
Rt	retention time
s	singlet
SPPS	Solid Phase Peptide Synthesis
SPS	Solid Phase Synthesis
t	tertiary
t	triplet
Tacm	trimethylacetamidomethyl
Tbfmoc	17-tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluroniumtetrafluoroborate
TEA	triethylamine
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TFMSA	trifluoromethanesulphonic acid
t.l.c	thin layer chromatography
TMSBr	trimethylbromosilane
Tme	tris(hydroxymethyl)ethane derived protecting group
Tnm	tris(hydroxymethyl)nitromethane derived protecting group
Tris	tris(hydroxymethyl)aminomethane
Trt	triphenylmethyl
UV	ultra violet
Z	benzyloxycarbonyl

AMINO ACIDS

Amino Acid	3 Letter Code	1 Letter Code
Alanine	Ala	A
Arginine	Arg	R
Aspartic Acid	Asp	D
Asparagine	Asn	N
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER 1 : INTRODUCTION

1.1 Introduction

Solid phase synthesis owes its origins to a paper published in 1963 by Merrifield[1]. In this seminal paper Merrifield described a method of synthesising a polypeptide by attaching the growing chain to a solid support. Previous to this work the synthesis of peptides had been carried out in solution and had required considerable skill and effort. The isolation and purification of intermediates at each step of the synthesis was difficult and time consuming. The Merrifield approach eliminated the need for these time consuming isolations and enabled vast excesses of reagents to be used which allowed equilibrium constants to be forced in one direction. The field of solid phase synthesis has grown imeasureable in the subsequent thirty years firstly in peptides, then DNA and now today in what is probable the "hottest" area of chemical research, combinatorial libaries.

The application of solid phase techniques to peptide synthesis has been highly refined and automated synthesisers are now capable of producing 100 residue peptides almost routinely. The purification and folding of such molecules is however less routine and can still present problems to even the most experienced peptide chemist. As time progressess and ever more ingenious strategies are developed to overcome these problems the chemical synthesis of large proteins is likely to make a an even larger impact on the fields of biological and medical research than it does today.

1.2 The Merrifield -Boc Strategy

The basic principles of the Merrifield approach are shown in figure 1.1 which shows the methodology he used to synthesis bradykinine[2]. Merrifield had originally used the benzyloxycarbonyl (Z) **1** group to protect the N^{α} but soon replaced this with the more acid labile t-butoxycarbonyl (Boc) **2** group, figure 1.2.

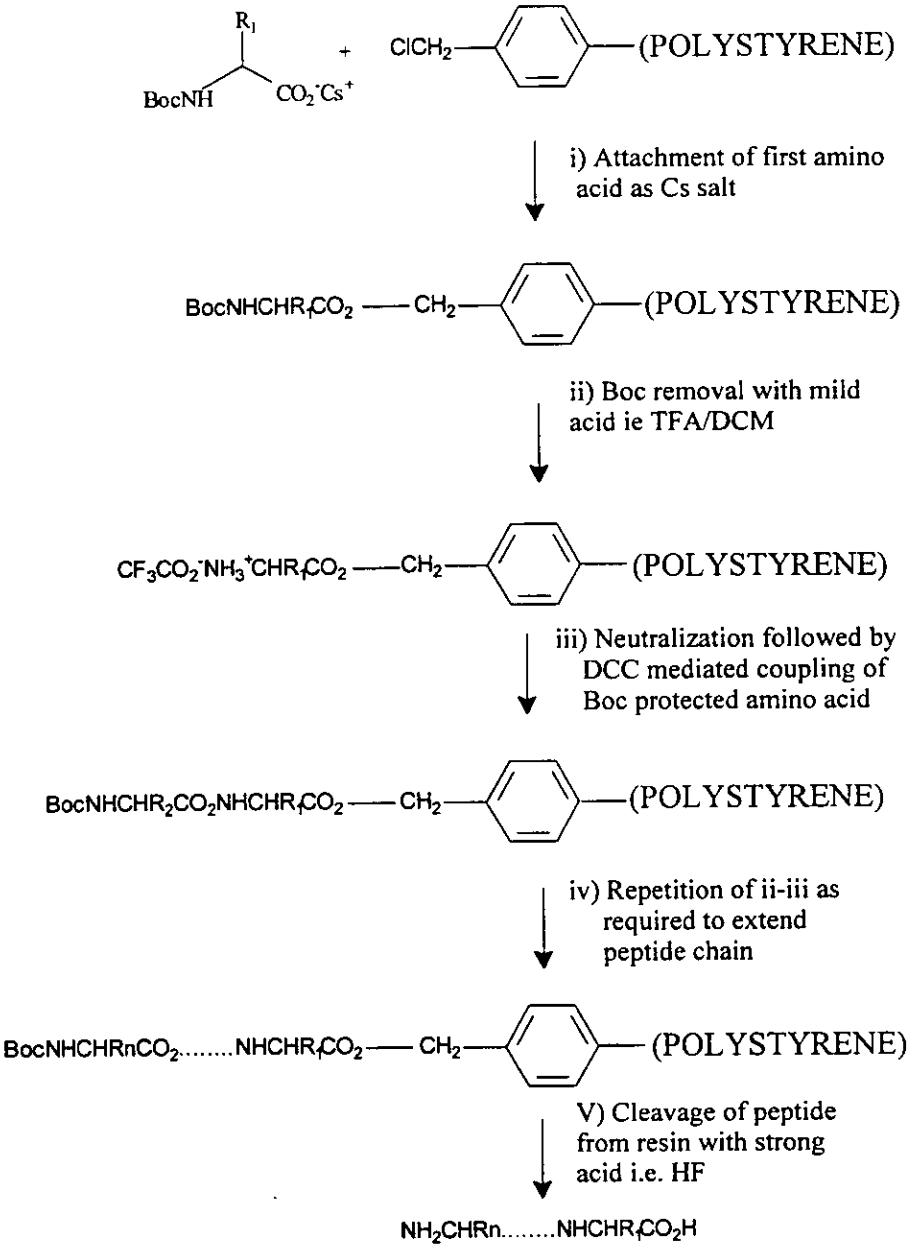
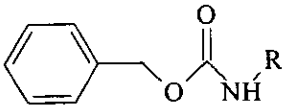
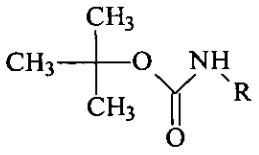

R1
BocNH
CO2-Cs+
ClCH2
(POLYSTYRENE)
i) Attachment of first amino acid as Cs salt
BocNHCHR1CO2 — CH2
(POLYSTYRENE)
ii) Boc removal with mild acid ie TFA/DCM
CF3CO2-NH3+CHR1CO2 — CH2
(POLYSTYRENE)
iii) Neutralization followed by DCC mediated coupling of Boc protected amino acid
BocNHCHR2CO2NHCHR1CO2 — CH2
(POLYSTYRENE)
iv) Repetition of ii-iii as required to extend peptide chain
BocNHCHRnCO2.....NHCHR1CO2 — CH2
(POLYSTYRENE)
V) Cleavage of peptide from resin with strong acid i.e. HF
NH2CHRn.....NHCHR1CO2H

Figure1.1



(1) Z-Group



(2) Boc-Group

Figure1.2

In this approach, polystyrene which has been crosslinked by incorporating 1-2% divinylbenzene into the polymerisation process is used as the support. The polystyrene is functionalized by chloromethylation, and the first amino acid incorporated by nucleophilic displacement. The Boc group is then removed as indicated and the TFA salt neutralised. Subsequent amino acids are incorporated using the coupleing agent N,N-dicyclohexylcarbodiimide (DCC). By repeating these steps peptides of the desired length can be manufactured before final cleavage using strong acid. During the assembly stage all the side chain functional groups are protected using semi-permanent protecting groups which are stable to the mild acid conditions which are used to cleave the N^{α} Boc protecting group. At the end of each step the insoluble polymer is extensively washed to remove excess reagents and reaction by-products.

The Merrifield approach, which has changed little over the years has been used with great success for the fully automated synthesis of peptides and there are numerous reviews[3] on its use.

The main problems encountered relate to 1) non-quantitative reactions; 2) incomplete orthogonality between the temporary and permanent protecting groups and peptide polymer link; 3) side reactions particularly at the final complete deprotection stage using the strong acid

1.3 Fmoc strategy: An overview

The development of an alternative to the Boc methodology grew out of the above mentioned problems, particularly the losses associated with cleavage of the peptide polymer link by repetitive exposure to TFA. The undesirability of working with HF also added to the demand for alternative methods.

The Fmoc group **3** developed by Carpino and Han[4] and utilized for peptide synthesis by Atherton[5,6] offers several advantages over Boc as an N^{α} protecting group. The strategy developed around the Fmoc group offers complete orthogonality between side chain and N^{α} protection and avoids the need to use strong acid during the final cleavage from the support. Figure 1.3 shows the basic Fmoc strategy

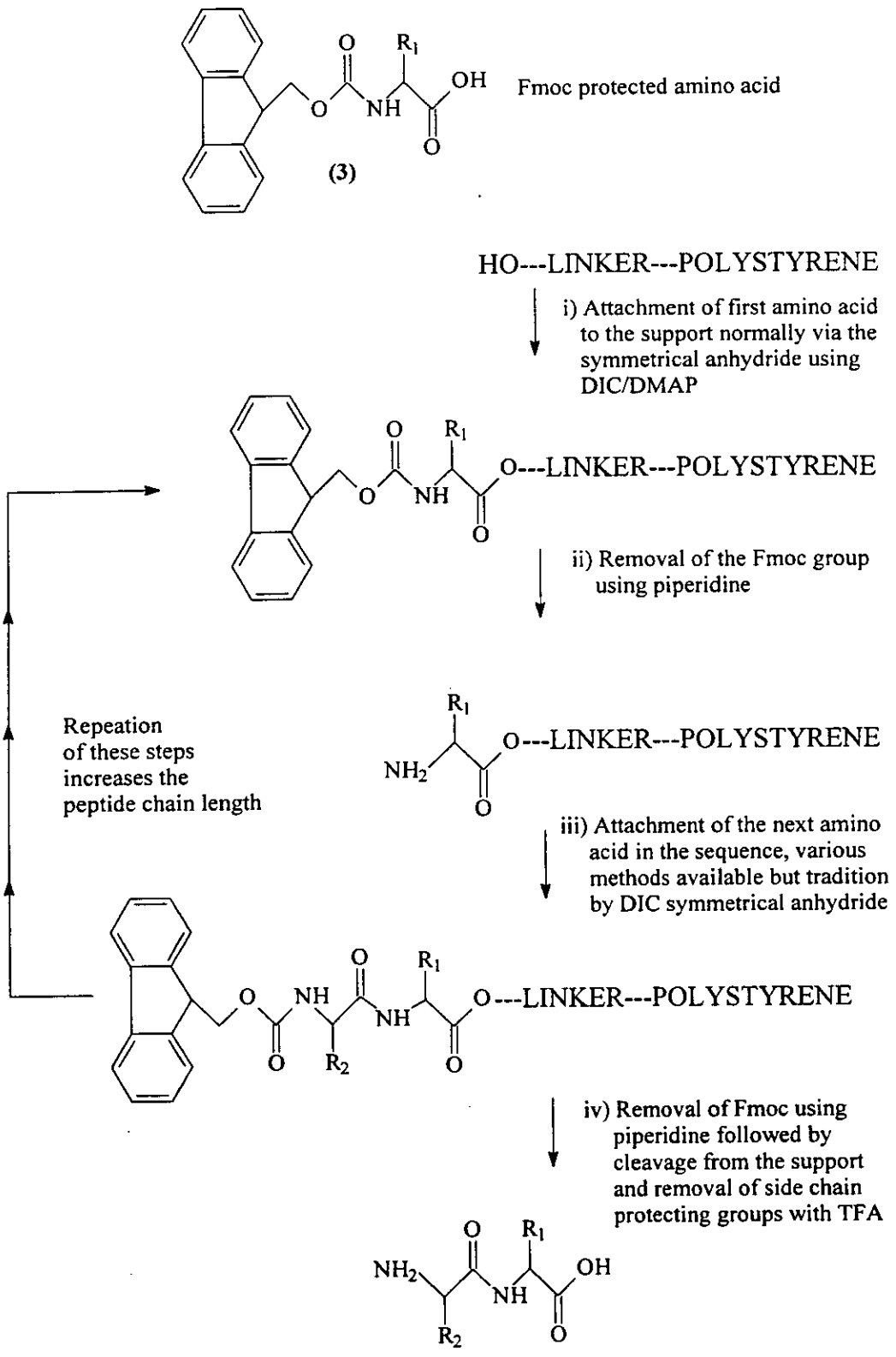
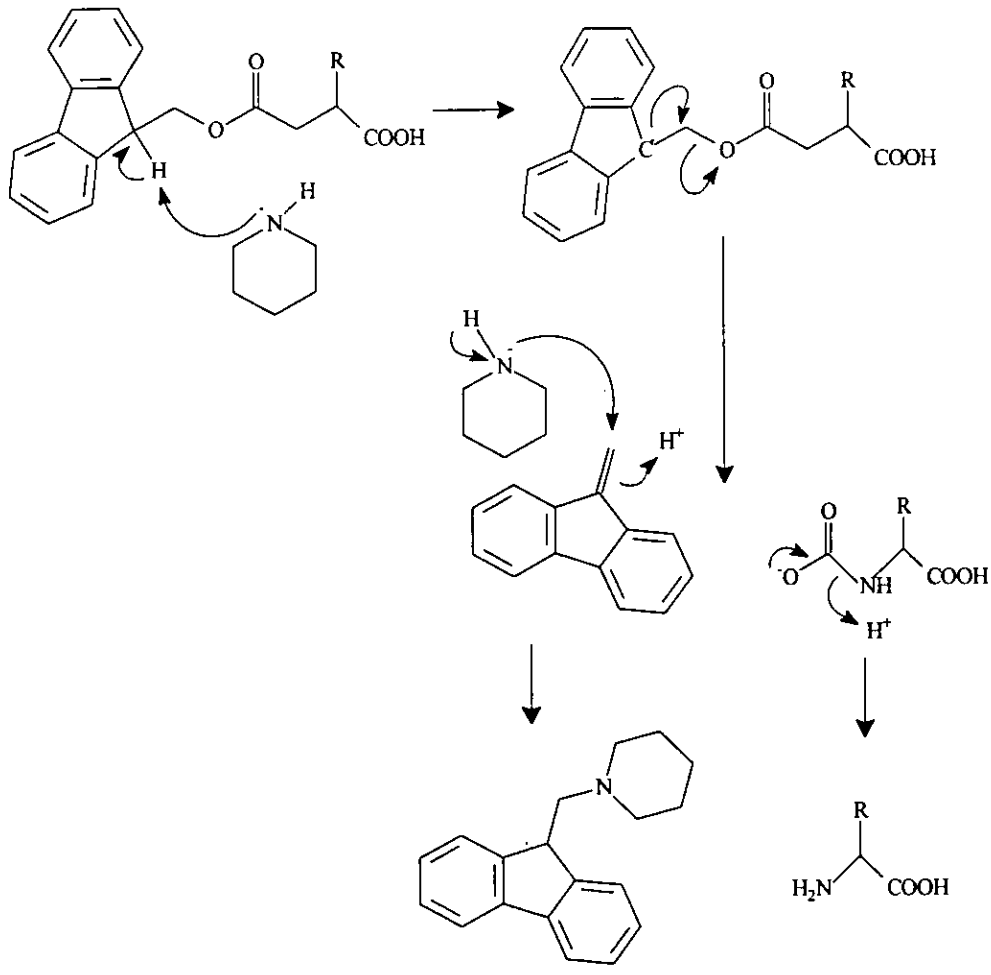

Fmoc protected amino acid
(3)
HO---LINKER---POLYSTYRENE
i) Attachment of first amino acid to the support normally via the symmetrical anhydride using DIC/DMAP
O---LINKER---POLYSTYRENE
ii) Removal of the Fmoc group using piperidine
Repeation of these steps increases the peptide chain length
O---LINKER---POLYSTYRENE
iii) Attachment of the next amino acid in the sequence, various methods available but tradition by DIC symmetrical anhydride
O---LINKER---POLYSTYRENE
iv) Removal of Fmoc using piperidine followed by cleavage from the support and removal of side chain protecting groups with TFA

Figure 1.3

The solid support used in standard Fmoc SPPS is a cross linked polystyrene which has been functionalised with an acid sensitive linker. The first amino acid is attached to this as the Fmoc protected derivative. There are various methods of attachment although the most common is through the formation of a symmetrical anhydride via reaction with DIC in situ. This is followed by displacement by the resin hydroxyl catalyse by DMAP. The N^{α} protecting Fmoc group is then removed with base, almost always piperidine, to leave a carbamate salt which breaks down to leave the free amine. The fluorescent properties of the Fmoc group can be utilise to follow the efficency of synthesis since the adduct **4** figure 1.4, formed by piperidine deprotection has a distinct absorbance at 302nm. As the resin is washed after each step in the synthetic cycle the washings from the Fmoc deprotection steps can be passed through a UV monitior to provide a quantitative assessment of the degree to which coupling has occurred[7]. This is useful in assessing when and where there are problems in a synthesis.



(4)

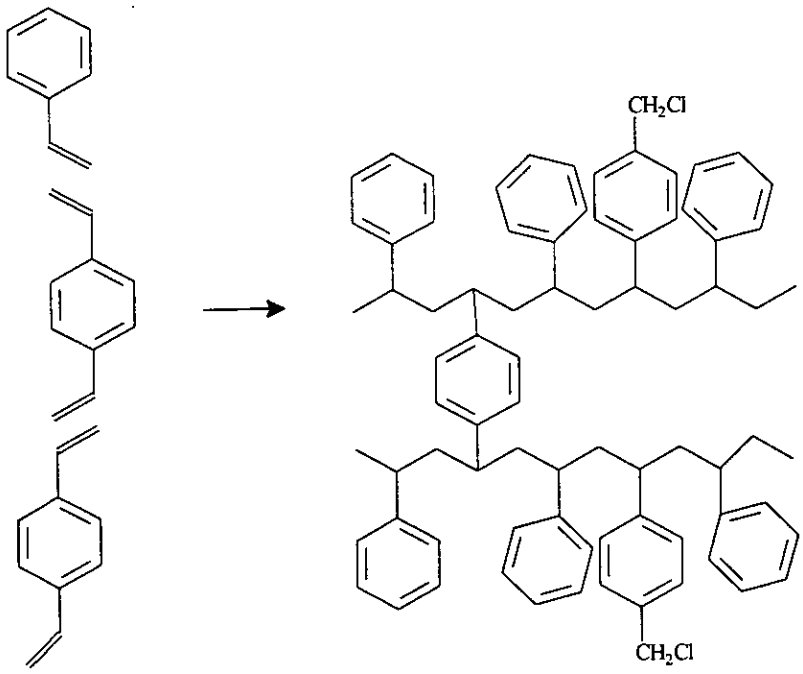
Figure 1.4

Amino acids are coupled to the growing chain as Fmoc protected derivatives, there are various methods of activating the amino acids and these will be discussed later. The cycles of deprotection and coupling are repeated until the last amino acid has been added the peptide is then cleaved from the resin and the side chain protecting groups removed in one step using TFA. The limited number of synthetic steps and the capacity to monitor the efficiency of synthesis has enable Fmoc SPPS to become highly automated.

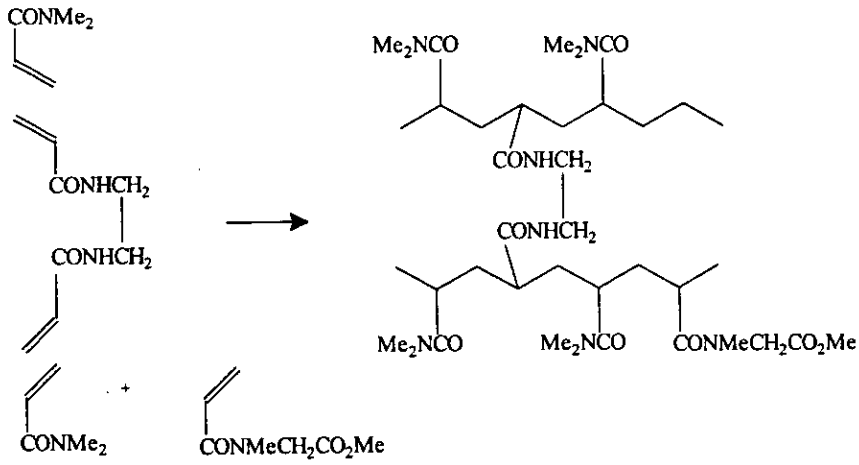
1.4 Fmoc strategy: The Details

1.4.1 Resins

For a resin to be suitable for the synthesis of the growing peptide chain it must possess several important characteristics. Firstly, it must have a functionality which allows for the attachment of a linker in order to couple amino acids. Secondly, the resin must be robust enough to withstand the chemical and mechanical transformations which it must under go in the course of a synthesis. Thirdly, the resin must swell in a suitable solvent in order for reagents to gain access to reaction sites. These properties are associated with polystyrene resins with approximately 1% crosslinking with divinylbenzene. The crosslinking provides stability but inhibits swelling, 1% has been found to be the approximate optimum in trade off between these characteristics. This resin is used worldwide in the synthesis of peptides using both the Boc and Fmoc methodologies and has proved highly successful. The main draw back of resins of this type however is that they are not greatly solvated by the solvents which swell them best. This can lead to aggregation and reduced availability of reactive groups. A more hydrophilic support such as the polyamide resins used by Atherton & Sheppard[8] should aid solvation of the growing peptide chain and in so doing aid diffusion of reagents and lead to increased coupling yields and a purer product. The disadvantages of polyamide resins are their limited capacity to withstand the continuous flow conditions of modern automated synthesis. This can however be overcome by chemically binding them to an inorganic support such as Kieselguhr which provides the necessary stability.



Merrifield resin



Sheppard's Pepsyn Resin

Figure 1.5 Resins

1.4.2 Linkers

The Fmoc strategy enables the cleavage of the peptide from the resin to be undertaken using a much milder acid than is possible for Boc synthesis. This has led to the development of convenient acid labile linkers. The Wang[9] resin **5,** which is still popular today, was first utilized for peptide synthesis by Chang and Meienhofer[10]

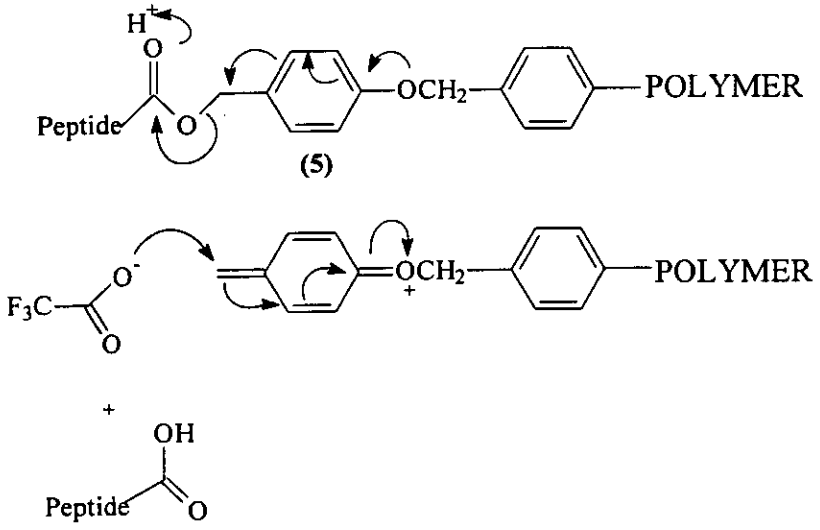
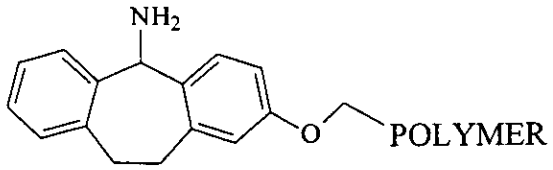


Figure 1.6 showing Wang resin linker and TFA cleavage

There has been considerable effort devoted to the development of novel linkers which, on cleavage, leave the C-terminal amino acid with a functionality other than peptide acid.

These have allowed the synthesis of among others peptide amides[11,12] and hydrazides[11,13]



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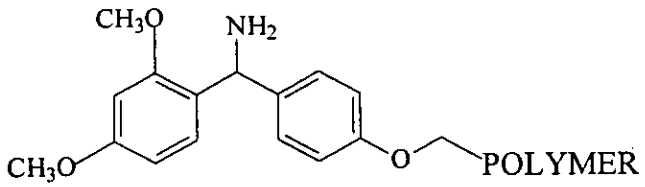
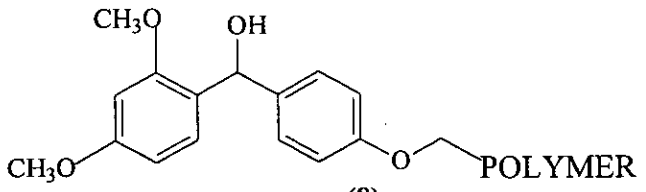


(7)

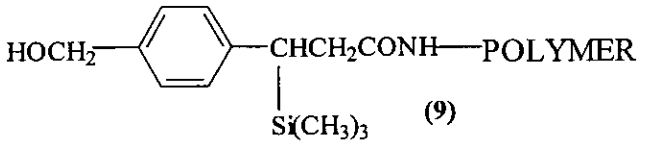
Figure 1.7 examples of peptide amide resins

The need to produce protected fragments has led to the development of very acid labile linkers such as the Rink resin[14] **8** which can be cleaved while leaving the side chain protecting groups intact. Problems can however occur with the use of these types of linker as cleavage can occur with some of the more acidic coupling agents. Other linkers do exist which can be cleaved by mild chemical means, such as those cleaved by fluoride ions[9]. And it seems likely that, as the need for protected fragments grows, ever more ingenious linkers are likely to be developed.
Linkers can also be designed which inhibit racemisation and side reactions associated with certain C-terminal amino acids. The 2-chlorotritylchloride[16] **10** linker is for instance particularly useful for peptides containing His or Cys as C-terminal residues or where Pro is present in the C-terminal dipeptide sequence.



(8)

Super acid labile Rink resin



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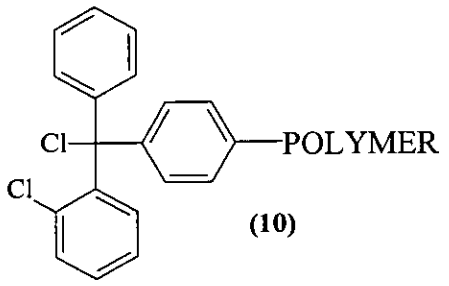
Fluoride Cleavable Linker



(10)

2-Chlorotritylchloride Linker Cleaved by acetic acid

Figure1.8

1.4.3 Amide bond formation

The solid phase synthesis of peptides involves the repetitive formation of an amide bond. As this reaction is performed over and over again without purification the yield must be as near as possible to 100%. In order to achieve this many agents have been developed which activate the carbonyl of the incoming amino acid to attack by the NH_2 of the growing peptide chain. The attachment of a leaving group to the acyl carbon of the carboxy component is necessary because ordinary carboxylic acids simple form salts with amines at ambient temperatures.

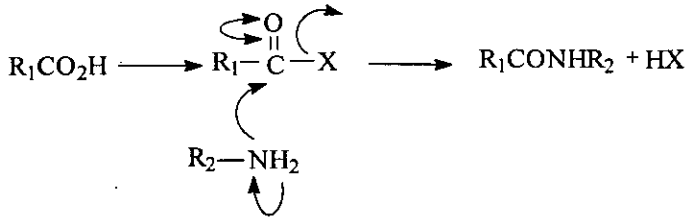


Figure 1.9 Mechanism of Amide Bond formation

In general the better the leaving group X the more efficient the coupling will be.

1.4.3.1 Carbodiimides

Carbodiimides have been the single most important reagents for activating carboxy groups ever since there use was first reported by Sheehan and Hess in 1955[16]. Carbodiimides may be used in direct coupling through an O-acylisourea **11** or, more commonly through an active ester **12** or symmetrical anhydride **13**. The latter strategy helps alleviate serious side reactions such as racemisation, carboxamide dehydration of asparagine/glutamine and the formation of N-acylureas. The two most commonly used carbodiimides are dicyclohexylcarbodiimide (DCC) **14** and diisopropylcarbodiimide (DIC) **15,** the latter finding favour in automated synthesisers due to its urea being more soluble than that of the former. Figure 1.10.

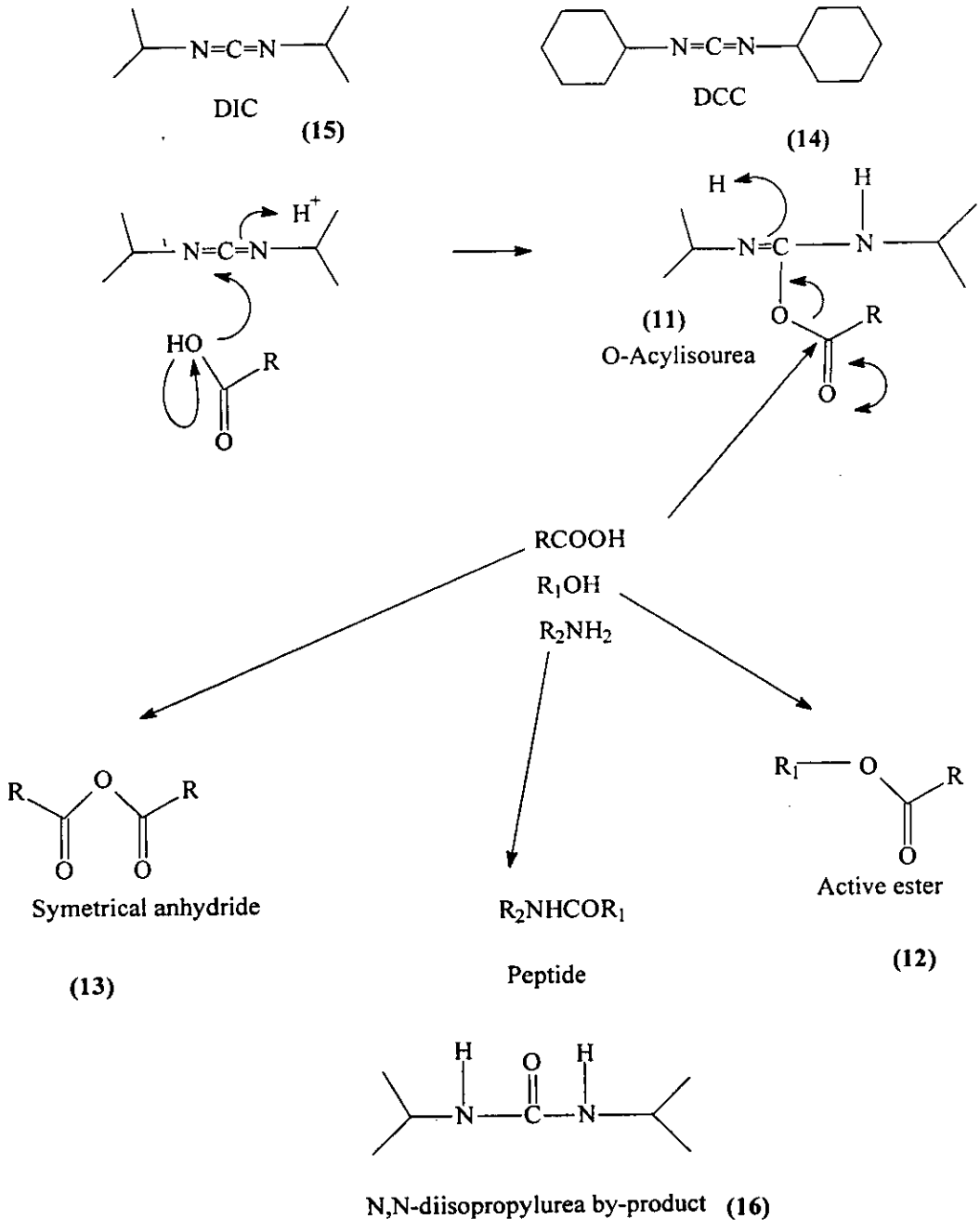

DIC
(15)
DCC
(14)
(11)
O-Acylisourea
RCOOH
R1OH
R2NH2
Symetrical anhydride
(13)
R2NHCOR1
Peptide
Active ester
(12)
N,N-diisopropylurea by-product (16)

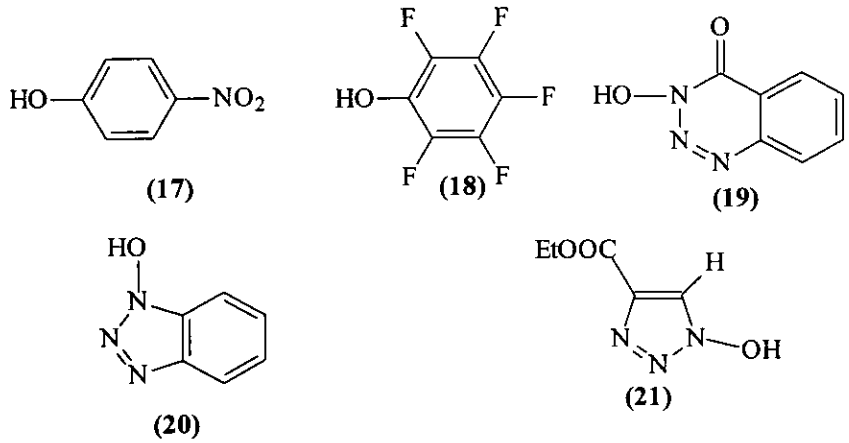
Figure 1.10

1.4.3.2 Symmetrical anhydrides

The formation of symmetrical anhydrides is relatively straight forward and these compounds can be isolated as crystalline solids, however it is more normal to form them insitu during synthesis. The use of symmetrical anhydrides has become widespread and generally speaking they provide excellent activation and unambiguous aminolysis. The drawback associated with their use is that they are wasteful in that one complete equivalent is discarded in every coupling. This is economically unsatisfactory. The chemical problems associated with them are that certain amino acids give serious side reactions. Particularly asparagine and glutamine which dehydrate with carbodiimides and histidine which can racemise

1.4.3.3 Active esters

The aforementioned problems, and the need for highly efficient couplings has led to the development of a number of active esters which again can be pre-formed or synthesised in situ using a carbodiimide reagent. As they are at a low level of activation (compared to O-acylisoureas and symmetrical anhydrides) there is less chance of side reactions including racemisation occurring. Contrary to the situation of ester hydrolysis which will be looked at in detail later, the rate determining step in aminolysis of esters is collapse of the tetrahedral adduct not its formation. The most important consideration for an activating ester therefore is the leaving ability of the ester group. Figure 1.8 shows a number of alcohol components which are all excellent leaving groups and have been utilized in peptide chemistry.



Some examples of the alcohol components of active esters

Figure 1.11

17) p nitrophenol[17]; 18) pentaflurophenol[18]; 19) 3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine[19,20]; 20) hydroxybenzotriazole[19]; 21) 1-hydroxy-4-ethoxycarbonyl 1,2,3-triazole[21].

1.4.3.4 Other coupling reagents

Until recently the carbodiimides were relatively unchallenged for peptide work and in combination with active ester and symmetrical anhydride derivatives accounted for the vast majority of peptides synthesised. With the development of phosphonium related compounds such as Castro's[22] benzotriazolyloxy-tris-(dimethylamino)phosphonium hexaflurophosphate, (BOP) **22** reagent this is begining to change. Figure 1.12 shows what is thought to be the main mechanistic route.

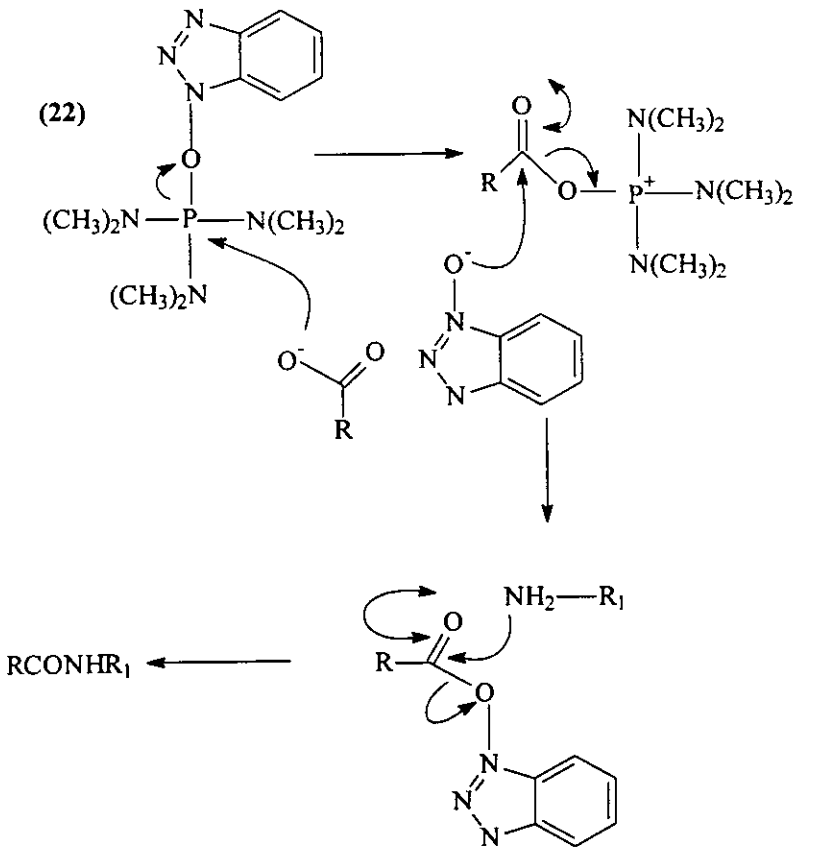
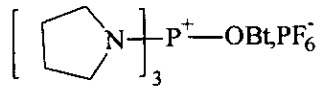


Figure 1.12 use of BOP reagent

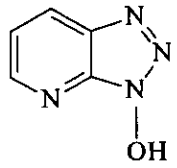
The main advantages of BOP-like compounds is that they require no preactivation the coupling reagent is simply mixed together with the resin bound amino functional group, the N^{α} protected amino acid and some tertiary base to ensure the acid is in the anionic form. This leads to shorter coupling times and increases the speed of peptide synthesis. When one considers that the limiting step in many drug development programs is not the screening of candidates but their availability anything which speeds up synthesis is looked upon favourably. The main disadvantage of the BOP reagent is that the co-product generated by its use is hexamethylphosphoramide a highly toxic substance. This has led to the development by Castro[23] of the related PyBop reagent **23** (figure 1.13)



(23)

Figure 1.13

Recent work by Carpino[24,25] suggests that by replacing the 1-hydroxybenzoltriazole portion of the BOP and Py BOP reagents with the aza analogue **24** increases in coupling effficiency and reduced racemisation can be gained.



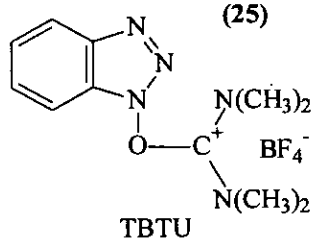
(24)

Aza analogue of 1-hydroxybenzotriazole

Figure 1.14

It is thought that neighbouring group effects may be important in increasing the reactivity of these compounds.

Another class of direct coupling agent beginning to grow in popularity are the uroniumsalts such as 2-(1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylisouroniumtetrafluoroborate (TBTU)[26] **25**. These compounds show similar reactivity to BOP and their aza analogues have been prepared.



(25)

TBTU

Figure 1.15

One problem associated with these new reagents as opposed to the carbodiimides is there relative cost, TBTU, BOP and Py-BOP being far more expensive than the carbodiimide.

1.4.4. Side chain protecting groups

In the Fmoc strategy, as aforementioned the N^{α} is protected by the base labile Fmoc group and the side chains by acid-labile groups. The following table summarises the common side chain protecting groups used.

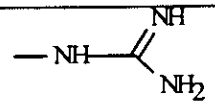
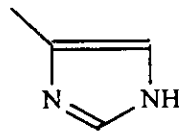
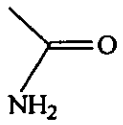
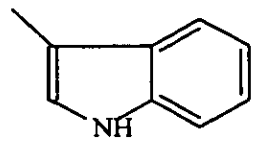
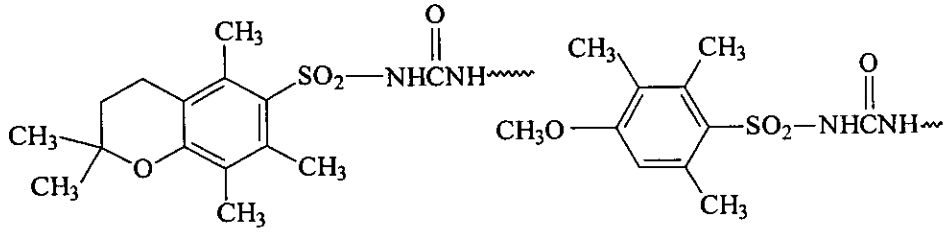
Functional Group	Amino Acids	Protection
-OH	Ser,Thr,Tyr	tBu
-SH	Cys	Acm, Trt, StBu
$-CO_2H$	Asp,Glu	OBu^t
$-NH_2$	Lys	Boc
	Arg	Mtr, Pmc
	His	Boc,Trt,Bum
	Asn,Gln	Trt or unprotected
	Trp	Boc or unprotected
$-S-CH_3$	Met	Sulphoxide or unprotected

Table 1.1

The mechanisms and principles of alcohol and acid protecting group strategy is fairly straightforward. Complications however occur with certain other residues which can't be protected in the normal manner.

1.4.4.1 Arginine

The guanidino side chain of arginine is highly basic and easily acylated during SPPS if not protected. Protection of the ω–nitrogen normally suffices and attempts have been made to protect it as the nitro, urethane, trityl and aryl sulphonyl[28]. The nitro is normally removed by H_2/Pd [29] although it is prone to a number of side reactions during acylation and cleavage[30]. Urethane protection via the Boc[31] and bis-adamantyl-oxycarbonyl $(Adoc)_2$ [32]groups has been attempted. This has proved unsuccessful as both side chain protecting groups are susceptible to acylation by Fmoc-Phe symmetrical anhydride and can subsequently be converted to ornithine by piperidine[27]. The trityl residues of arginine are not soluble in DMF/DCM[33] and therefore of little use for SPPS. The sulphonyl derivatives have hence emerged as the main arginine protecting groups. The two most commonly used groups are the 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr)[34] **26** and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc)[35,36] **27**. The Pmc group has quickly been established as the group of choice as it requires significantly less time to cleave in TFA. This greatly diminishes the likelihood of alkylation of tryptophan residues by the sulphonium ions generated during cleavage.



Pmc **(27)** Mtr **(26)**

Figure 1.16 showing Pmc and Mtr groups

1.4.4.2 Cysteine

Cysteine presents a different set of protection problems as completed peptides often have disulphide bonds between particular cysteine residues. Commonly cysteine is

simply protected as the Trt, which is removed during TFA cleavage. This can create problems when more than two cysteines are involved in a sequence and a disulphide bond is to be formed, as there is no control over disulfide formation. Disulphide bonds can hence form randomly and present serious purification and yield problems. In order to prevent this and provide a degree of control over disulphide formation a series of post-cleavage protecting groups Acm, tBuS, Tacm, tBu have been developed which withstand ordinary TFA cleavage. It has been reported that by using these groups it is possible to cleave and purify a protected peptide containing multiple cysteines and then selectively remove the cysteine protecting groups and form disulphide bonds[37]. Although all of the previously mentioned protecting groups have been shown to work in the literature none of them is particularly satisfactory. The Acm group can be difficult to remove in certain sequences and the tBuS group causes solubility problems during purification. The field of cysteine protection is still awaiting the arrival of a truly user-friendly, sequence independent, protecting group.

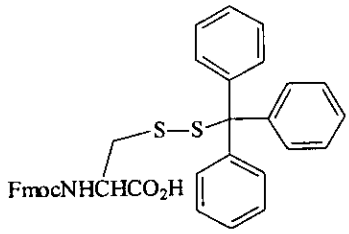
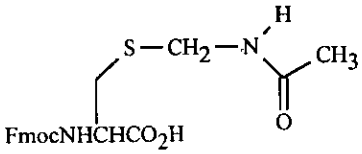
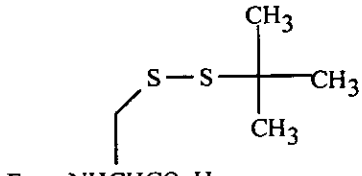
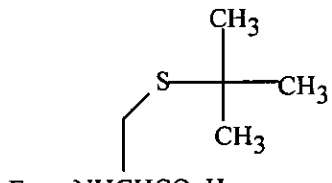
Cysteine Protecting Group	Cleaved By	Stable To
Trityl 	TFA, I_2,[38*] thallium (111) trifluroacetate[50]	Not applicable
Acm[41] 	mercury acetate[40], silvertrifluromethanesulphonate[41], ICN,[42] I_2,[38,43] $AgBF_4$/TFA[42]	TFA, TFMSA, TMSBr, HF
tBuS 	tributylphosphine,[37], β–mercaptoethanol,[44] TMSBr[45]	TFA, $AgBF_4$
tBu 	TFMSA,[46,47] mercury acetate,[46,47,48,49,]	TFA, $AgBF_4$/TFA

Table 1.2

*Care must be taken using iodine as it can attack tryptophan residues[38] this can however be mininised.[39]

**Both Met and Trp residues must be protected when using this protocol.[50]

1.4.5 Side reactions associated with SPPS

1.4.5.1 Racemisation

During the synthesis of a peptide there are a number side reactions which can occur. One of the main concerns when dealing with optically pure compounds is racemisation and a great deal of effort has been put in to keeping this to a minimum[51]. Due to the conditions used during synthesis, there is, except in the case a few special amino acids (α–phenylglycine residues, certain His and Cys derivatives), very little racemisation by direct enolisation. The main source of racemisation is the tendency of activated acylamino acids and peptides to cyclize under the influence of base to give oxazolones **28**. The oxazolones formed are themselves activated towards the incoming amino acid, but because the rate of racemisation via resonance stabilized anions is usually much faster than the rate of peptide bond formation, any peptide produced is likely to be highly racemised.

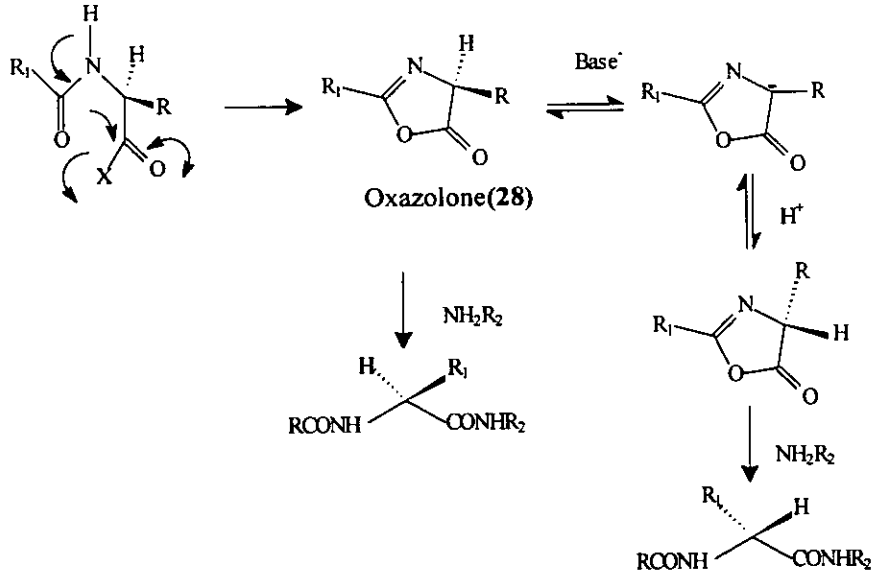


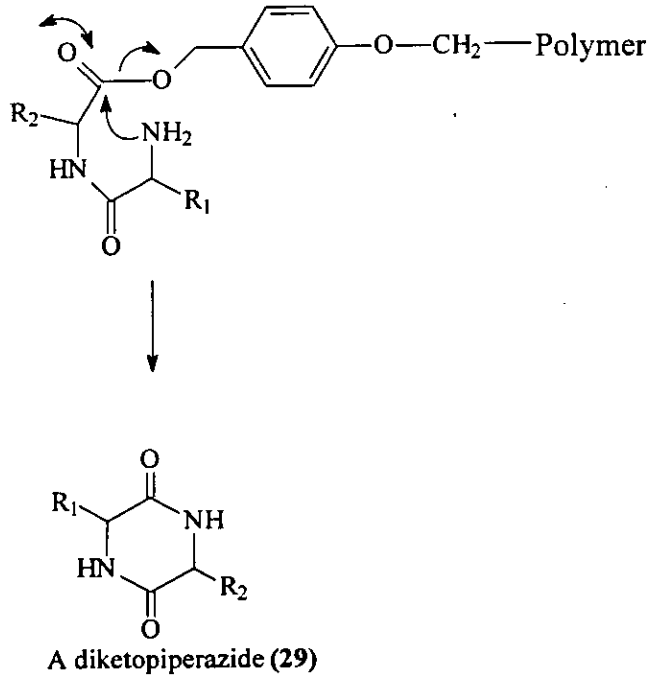
Figure 1.17 Racemisation via oxazolone

Oxazolone formation occurs with most good leaving groups X. Fortunately however this is not so easy when the acyl substitution is an alkoxycarbonyl protecting group. Further more the alkoxyoxazolones are less easily racemised and more easily

aminolysed than are the oxazolones. Hence for Z, Boc and Fmoc protected amino acids there is normally little danger of racemisation occurring.

1.4.5.2 Diketopiperazine (DKP) formation

The main area of concern with this side reaction is the deprotection of the second amino acid in a growing peptide chain. Under certain conditions cyclization can occur which causes premature cleavage of the growing peptide from the solid support.



A diketopiperazide **(29)**

Figure 1.18 Diketopipeazine formation

Most of the time losses due to DKP **29** formation are minimal. Certain residues such as glycine, proline, valine, and isoleucine enhance the tendency to cyclize, as does the incorporation of L and D amino acids as the side chains will lie on opposite sides of the general plane of the DKP ring and hence make cyclization more favourable. Inhibition of cyclization is possible by using t-Butyl protecting groups or in extreme cases by loading the second and third amino acids as a dipeptide. Protected and

activated derivatives of glycyl-proline also cyclise by a similar mechanism to yield acyldiketopiperazines[52].

1.4.5.3 Aspartimide formation.

Aspartimide **30,** formation is perhaps the most serious side reaction in peptide synthesis it can occur by both acid and base catalyzed mechanisms. It is however not normally a problem when amino acids with bulky side chains[53] are C-terminally adjacent to the aspartic acid residues. These bulky side chains inhibit attack by the lone pair of the nitrogen by steric means, thus limiting aspartimide formation. Serious problems can however occur when less hindered systems are present. This is particularly troublesome in the case of adjacent glycine[54] and unprotected serine[55] residues.

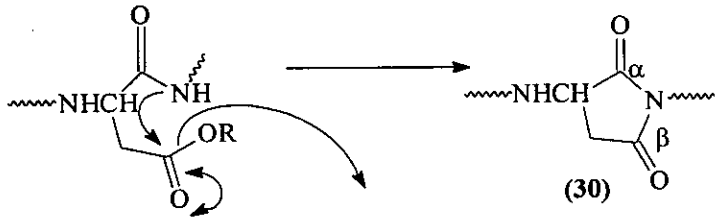


Figure 1.15 Aspartimide formation

Figure 1.15 shows the general mechanism of aspartimide formation, hydrolytic ring opening at the β-carbonyl regenerates the original peptide (less the side chain ester) attack at the α-carbonyl is however favoured and leads to the β-linked peptide. Aspartic acid is normally protected as the t-butyl ester this gives effective protection as regards external nucleophiles but is inadequate in halting aspartimide formation[33]. Several efforts have been made to synthesise a reliable β-aspartic acid protecting group which will prevent the troublesome Asp-Gly rearrangement. Little success has yet been achieved although recent work on adjacent glycine backbone protection has proved promising[56]. Other current techniques which seem to limit the rearrangement include the practice of adding an active ester such as HOBt or Pfp to the deprotection cycle in the Fmoc protocol to act as a buffer[57].

1.4.6 Cleavage and scavengers

Cleavage of the completed peptide from the resin is normally achieved in Fmoc chemistry using TFA. The side chain protecting groups are, in the main, also cleaved by the TFA. This creates a number of stable cations which can attack electron rich sites in the deprotected peptide. The side chains of tryptophan, tyrosine and methionine are particularly susceptible in this respect. In order to prevent this, additives such as water, thioanisole, anisol, phenol, and 1,2-ethanedithiol which "scavenge" these reactive cations are added to the cleavage mix. These additives act not only by trapping carbocations, but also as nucleophiles in the cleavage step by shifting the mechanism of cleavage from S_N1 to S_N2 in certain less easily cleaved protecting groups.[35] Normally, cleavages involving peptides without such sensitive amino acids and only requiring the deprotection of t-butyl groups can be achieved using 95% TFA/5% water. For other sequences however scavengers are required. In an attempt to cut cleavage time several groups have experimented with the use of stronger acids than TFA such as TFMSA[58] and TMSBr [45,59]. These are promising developments and could be particularly useful for the cleavage of large peptides.

1.5 Large peptide/protein synthesis

The technology of SPPS ultimately has limitations and it is difficult to foresee the stepwise synthesis of peptides containing over 200 residues becoming commonplace. The main problem is that structural interactions in the growing peptide chain cause the accessibility of the primary amine to be reduced. Coupling efficiency gradually drops and a point is eventually reached whereby a synthesis is no longer viable. In an effort to over come these intrinsic problems several groups have attempted to synthesis large peptides by convergent means. The basic strategy involves synthesising small fragments of a larger peptide by traditional SPPS techniques, purifying them and then coupling these purified fragments together to form the larger peptide. Other than overcoming the problem of reduced coupling efficiency the main advantage of this technique is that it is obviously easier to separate a 100 mer from a

mixture of two pure 50 mers than from a mixture containing any number of truncations.

There are several ways in which the fragments can be coupled and not all of them involve peptide bonds. Work by Kent on the synthesis of HIV-1 protease has utilized a novel thio ester linkage[60] and the Tam group has reported conjugation through a five membered ring[61]. While these are interesting asides it is obviously desirable to link fragments through peptide bonds as only then can the structure be guaranteed to resemble the natural state. The Kent group have made significant progress in this area by linking fragments through a peptide bond formed by the reaction of an N terminal cysteine and a C terminal thio ester. The coupling is carried out in guanidine solution at pH 7.6; both fragments being unprotected despite the presence of other cysteine residues in the sequence.[62]

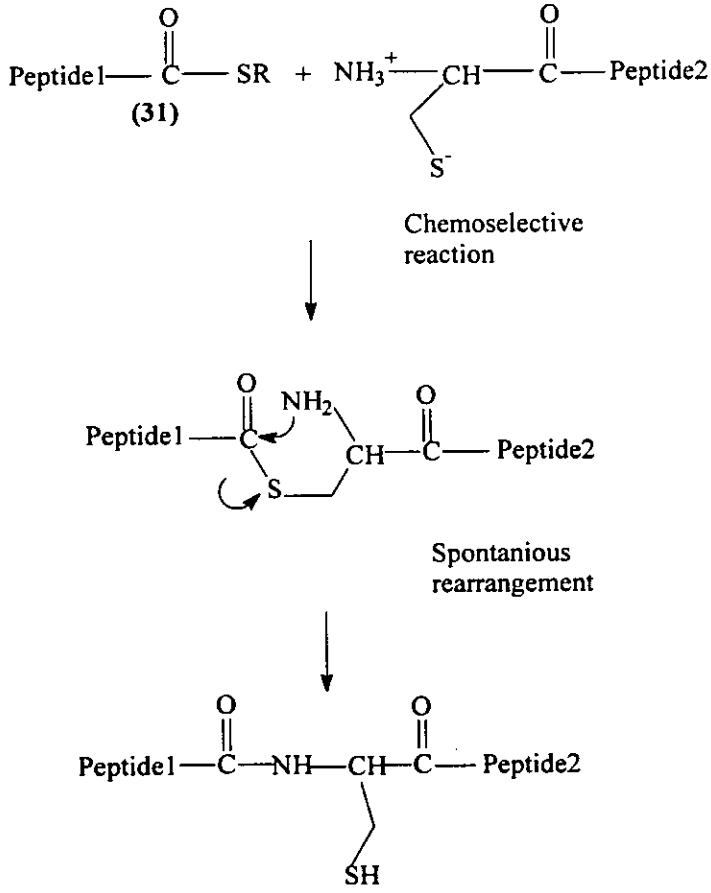


Figure1.14 after Kent

More traditional forms of coupling between fragments include coupling via enzymatic means by manipulating the equilibrium constants of certain proteolitic enzymes[63] and those involving an azide **32** mediated linkage[64,65,66] first introduced by Curtius at the turn of the century[67].

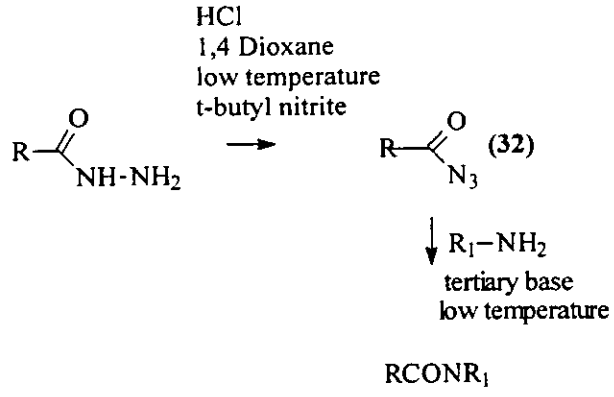


Figure 1.15 hydrazide/azide peptide method by Honzl & Rudinger method[64]

The obvious draw back of these methods is that the side chain amino groups of all the lysine units, in both fragments, have to be protected in order to prevent them interfering with the fidelity of coupling. This has presented several problems as lysine is normally protected by the Boc group, which is of course removed by TFA during cleavage from the resin and therefore unsuitable for fragment coupling work. What is required therefore, is a lysine protecting group which has a number of exacting qualities.

1) It must be able to withstand the conditions of SPPS.

2) It must be stable to TFA cleavage.

3) It must be unaffected by the conditions required for fragment coupling.

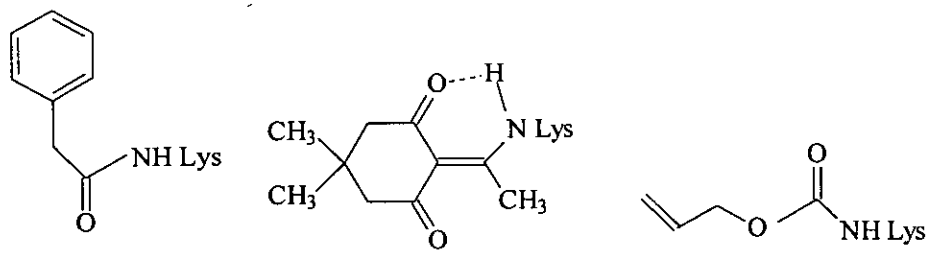
4) It must be easily removable after fragment coupling.

5) It must be relatively cheap and easy to introduce to the lysine side chain.

6) It must be as solublising as possible.

The presence of aromatic groups within post cleavage protecting groups is limited as they drastically reduce the solubility of the fragments they protect. Large peptides protected in such a manner can be insoluble thus rendering purification or further reactions exceedingly difficult. There are a number of protecting groups which have

been used to protect lysine orthogonally in conjunction with the Fmoc and Boc strategies, such as the penicillin acylase cleaved PhAc[68] group **33**, the Dde[69,70] **34**, Alloc[71] **35** groups, as well as various light sensitive molecules.



(33) **(34)** **(35)**

Figure 1.16 Orthogonal lysine protecting groups

There is as yet however no protecting group currently on the market which adequately fulfills the aforementioned requirements for the synthesis of large systems and subsequently these methods of fragment coupling have been under used. Later text will endeavour to describe the discovery, development and testing of a highly promising multipurpose lysine protecting group.

1.6 References

1) R.B. Merrifield *J. Am. Chem.Soc.* 1963, **85**, 2149

2) R.B. Merrifield, *Biochemistry,* 1964, **3**, 1385

3) J.P. Tam, *Macromolecular sequencing and synthesis*, ed D. H. Schlesinger, 1988, Alan R. Liss, New York pg 153-184

4) L.A. Carpino and G.Y. Han, J. Am. Chem. Soc. 1970, **92**, 5748

5) E. Atherton, D. Harkiss, C J Logan, R.C. Sheppard and B.J. Williams, *J Chem Soc., Chem. Commun.* 1978, 537

6) E. Atherton, D. Harkiss, C J Logan, R.C. Sheppard, J. *Chem. Soc., Chem Commun.* 1978, 539

7) Kevin Shaw, *personal communication*

8) E. Atherton and R.C. Sheppard, *Solid phase synthesis a practical approach*, Oxford Uni press, Oxford 1989.

9) S.S. Wang, *J. Am. Chem. Soc.* 1973, **95**, 1328

10) C.D. Chang & J. Meienhofer, *Int. J. Peptide Protein Res.,* 1978, **11**, 246.

11) R. Ramage, S.L. Irving, C.M. McInnes, *Tetrahedron Lett*, 1987, **28**, 3787

12) H. Rink, *Tetrhedron Lett,* 1987, **28**, 3787

13) S.S. Wang, *J. Org. Chem.* 1975, **49**, 1235

14) L.A. Carpino & G.Y. Han, *J. Org. Chem,* 1972, **37**, 3404

15) R.Ramage, C.A. Barron, S. Bielecki, D.W. Thomas, *Tetrahedron Lett*, 1987, **28**, 4105

16) J.C. Sheehan & G.P. Hess, *J. Am. Chem. Soc.* 1955, **77**, 1067

17) M. Bodanszky, M. Sheehan, *J. Inst. Chem. (India).* (1966), 1597

18) L. Kisfauldy & I Shon, *Synthesis* 1983, 325.

19) W. Konig & R. Geiger, *Chem. Ber.* 1970, **103**, 788

20) E. Atherton, J L Holder, M. Meldal, R.C. Sheppard and R.M. Valerio, *J. Chem. Soc. Perkin Trans 1.* 1988, 2887.

21) Nicola Robertson, *personal communication.*

22) B. Castro, J.R. Dormoy, B. Dourtoglon, G. Erin, C. Selve & J.C. Zieglar. *Synthesis*, 1976, 751.

23) J. Coste, D. Le Nguyen and B. Castro, *Tetrahedron Lett,* 1990, **31**, 205

24) L.A. Carpino, *J. Am. Chem. Soc.* 1993, **115**, 4397.

25) L.A. Carpino, A. El. Faham, C.A. Minor and F Albericio, *J. Chem. Soc. Chem. Commun.* 1994, 201.

26) R. Knorr, A. Trzeciak, W. Banworth and D. Gillessen, *Tetrahedron Lett,* 1989, **30**, 1927.

27) H. Rink, P. Sieber, F. Raschdorf, *Tetrahedron Lett.* 1984, **25**, 625,

28) J. Meienhofer, *Chemistry and Biology Amino acids*: ed G.C. Barnett, Chapman and Hall, London, 1985, 297

29) T. Copeland and S. Oroszlani, *Gen. Anal. Techn.* 1988, **5**, 109,

30) E. Wunsch, Houben, *Weyls, Methoden der organischem chemie*, ed E. Muller, 15, parts 1 & 2, Thieme, Stuttgart (1974).

31) M. Bodansky, J.C. Tolle, S.S. Deshmane, A. Bodansky, *Int. J. Pept. Protein Res.* 1978, 12, 57,

32) J.P Tam, M.W. Rieman, & R.B. Merrifield, *Pep, Res,* 1988, **1**, 6,

33) E. Nicolas, E. Perdoso & E. Gigalt, *Tetrahedron Lett,* 1989, **30**, 497,

34) E. Atherton, *Peptides 1984*, ed U. Ragnarson, 1985, 153

35) R. Ramage and J Green, *Tetrahedron Lett,* 1987, **28,** 2287,

36) J. Green, R.Ramage, O.M. Ocinjobi, A.S.J. Stewart, S. M^{c}urdy, R. Noble, *Tetrahedron Lett*, 1988, **29**, 4341,

37) E. Atherton, R. Sheppard, P. Ward, *J. Chem. Soc. Perkin. Trans* 1985, **1**, 2065,

38) B. Kamber, A. Hartman, K. Eisler, B. Riniker, H. Rimk, P. Sieber, W. Rittel, *Helv. Chim.* Acta. 1980, **63,** 899,

39) Y. Kiso, M. Satomi, K. Ukawa & T. Akita, *J. Chem, Soc, Chem, Commun.* 1980, 1063,.

40) D.F. Veber, J.D. Milkowski, S.L. Varga, R.G. Darkewalter, R. Hirschmann, *J. Am. Chem. Soc.* 1972, **94**, 5456.

41) H. Yajima, N. Fujii, A. Otaka, t. Watanabe, A. Okamach, H. Tamamura, Y. Inagaki, M. Nomizu, K. Asano, *J. Chem. Soc. Chem. Commun.* 1989, 283-284

42) M. Yoshida, T. Tatsumi, K. Akaji, S. Iinuma, Y. Fujiwara, T. Kimura & Y. Kiso, *Chem. Pharm. Bull.* 1990, **38**, 273,

43) J.M. Stewart & J.D. Young, *Solid phase peptide synthesis*; 2nd edition 1984, Pierce chemical company, Rockford IL.

44) R. Eritja, J. P. Ziehler-Martin, P. A. Walker, T. D. Lee, K. Legesse, F. Abericio, B. Kaplan, *Tetrahedron.* 1987, **43**, 2675-2680,

45) H. Yamjima & N. Fujii; *The peptides: analysis synthesis and biology,* 1979 ed E gross and J Meienhofer, vol 5, Academic press, New York pg 65-109.

46) S.N. McCurdy, in *ABI user Bulletin, peptide synthesisers*, Applied Biosystems, Foster City. C.A. 25, 1988

47) S. N. McCurdy, *Pept. Res.* 1989, **2**, 147,

48) A.Chimiak, *Peptides* 1962, ed G.T. Young, 1963, 53

49) H.C. Beyerman, *Peptides* 1962, ed G.T. Young, 1963, 53.

50) G. Fields & R L Noble, *Int. J. Pept.Protein Res.* 1960, **35,** 161.

51) D.S. Kemp, *The Peptides*: Analysis, Synthesis and Biology; eds E. Gross & J Meienhofer; Vol 1 academic press, New York, pg 315-382.

52) M. Goodman & K. Steuben. *J. Am. Chem. Soc.* 1962, **84**, 1279.

53) M. Bodansky & J. Kwei. *Int. J. Pept. Protein Res.* 1978, **12,** 69.

54) M. Ondetti, A. Deer, J. Sheehan, J. Pluscec, O. Kocy. *Biochemistry.* 1968, **7**, 4069.

55) S. Bernhard, A. Berger, J. Carter, E. Katachalski, M.Sela, Y. Shalitin, *J. Am. Chem. Soc.* 1962, **84**, 2421.

56) M. Quibell, D. Owen, L.C. Packmann & T. Johnson, *J. Chem. Soc., Chem. Commun.* 1994, 2343-2344.

57) J. Martinez & M. Bodansky, *Int. J. Pept. Protein Res.* 1978, **12,** 277-283.

58) H. Beilan, R, Nobel, C. Ashton, & D. Hadfield, in *Peptides 1988, Chemistry & Biology*, ed G.R. Marshall, Escom, Leiden, pg 198-201.

59) J.P. Tam & R.B. Merrifield, In the *Peptides, Analysis, Synthesis and Biology,* 1987, eds, S. Udenfriend and J. Meienhofer, vol 9, Academic press, New York pg 65-109.

60) M. Schnolzer & S.B.H. Kent, *Science*. 1992, **256,** 221-225.

61) Chuan-Fa lui & James.P. Tam. *J. Am. Chem. Soc.* 116, 4149-4153

62) P. E. Dawson, T.W. Muir, I. Clarke-Lewis, S.B.H. Kent. *Science.* 226, 776-778.

63) I. M. Chaiken, A. Komoriya, M. Ohno, F. Widmer, *Applied Biochemistry & Biotechnology*. 1982, **7,** 385-395.

64) J. Honzl & J Rudinger, *J. Collect. Czech Chem. Commun.* 1961, **26**, 2333-2344.

65) M. Ondetti, U.L. Narayanan, M. Von Salta, J.T.H. Sheehan, E.F. Sabo, M. Bodasky, *J. Am. Chem. Soc.* 1968, **90**, 4711-4716.

66) T. Shioiri, K. Ninomiya, S. Yamada. *J. Am. Chem. Soc.* 1972, **94**, 6203-6205.

67) T.H. Curtius, *Ber.dtsch. Chem Ges.* 1902, **35**, 3226.

68) H. Waldmann & D Sebastian, *Chem. Rev. 1*994, **94**, 911-937.

69) N. Hone, S.R. Chlabra, W.C. Chan, B.W. Bycroft. *In Peptides*, eds C.H. Shneider & A.N. Eberle, 1992, pg 290-291.

70) B. Bycroft, W.C. Chan, S.R. Chlabra, P.H. Teesdale-Spittle, P.M. Hardy, *J. Chem. Soc. Chem. Commun.*1993, 776-777.

71) R. Beugelmans, L. Neuville, M. Boischoussy, J. Chastanet, J Zhu. *Tetrahedron Lett,* 1995, **36**, 3129-3132.

CHAPTER 2 : POST CLEAVAGE PROTECTING GROUPS

2.1 Evolution of a post cleavage protecting group

As previously discussed, one of the main areas of current research into protein synthesis by chemistry methods is fragment coupling. The concept being that several of the limiting factors associated with SPPS can be overcome by coupling together two, or more, smaller fragments to make up a protein. As outlined in the introduction this can be facilitated by a number of techniques. These can, however be subdivided into two main categories.

1) Enzymatic fragment condensation.

2) Chemical fragment coupling.

In our initial investigations into fragment coupling we sought to devise a linker which, when cleaved from the resin, would leave the peptide with a novel C-terminal ester functionality. This novel functionality would, hopefully, give us two options for uniting peptide fragments.

1) The ester moiety would act as a substrate for enzymatic condensations.

2) The ester functionality would be designed in such a way as to give the opportunity for chemical coupling via catalysis with a Lewis acid such as boron.

With these dual aims in mind the structure **36** shown in figure 2.1 was chosen as the one most likely to possess the novel functionality required.

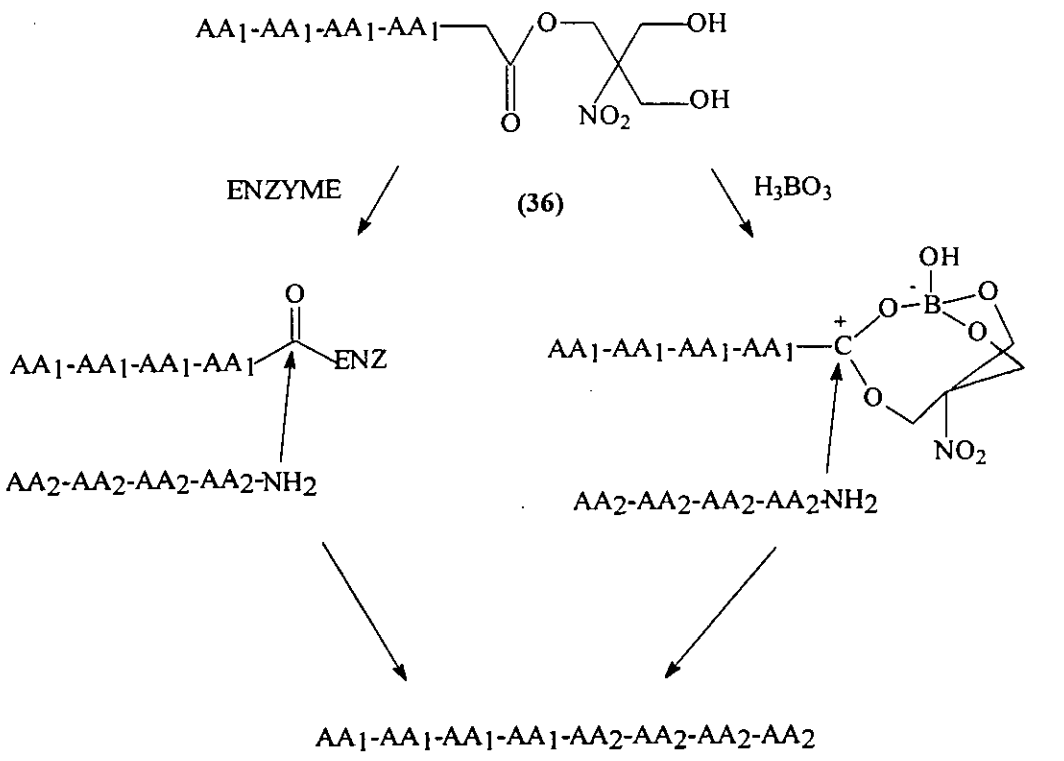
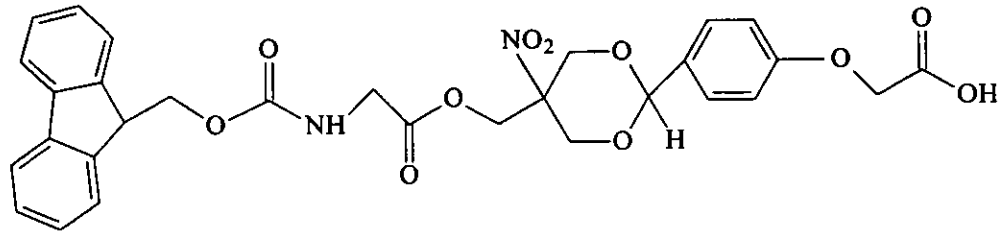


Figure 2.1

2.1.1 Linker synthesis

It was necessary then to synthesis a linker which could easily be attached to a resin, would be stable to SPPS using Fmoc methodology, and which would subsequently be cleaved under normal (TFA) cleavage conditions to give the structure **36** shown in figure 2.1.

The compound shown in figure 2.2 **37** was selected as the molecule most likely to satisfy these criteria.



(37)

Figure2.2

Several synthetic routes were attempted before a successful synthesis with practicable yields was found. The final route varies slightly, in that the target molecule is synthesised as the pentachlorophenyl ester **44** rather than the acid. See figure 2.3.

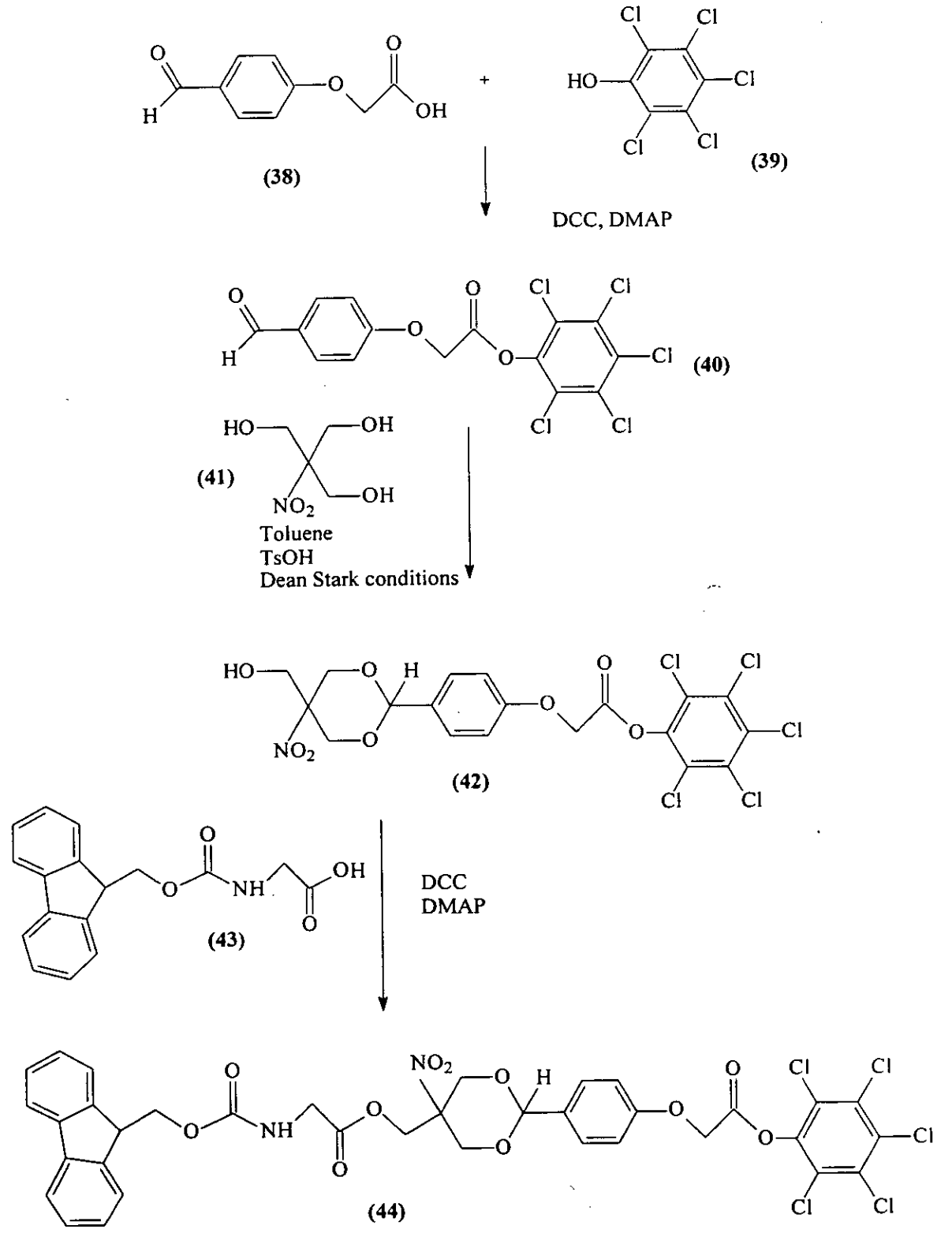


Figure 2.3 Linker Synthesis

This was because isolation of the acid proved to be difficult due to nonselective hydrolysis of the two ester functionalities present. As a result the linker was loaded on to the resin via the pentachlorophenyl active ester rather than using DIC to generate the symmetrical anhydride. The yield of this step was > 70% hence this approach did not decrease the efficiency of the route. The synthesis of a short test peptide Leu-Ile-Phe-Ala-Gly incorporating the linker was undertaken using Fmoc SPPS methodology. Unfortunately this initial synthesis failed and analysis of the deprotection profile[1] showed a major drop in dibenzofulvene-piperidine adduct **4** absorption after addition of the third residue (section 1.3). This was almost certainly due to diketopiperazine **29** formation (section 1.4.5.2). Resynthesis of the test peptide Ala-Asp-Leu-Ile-Leu-dPhe-Gly **45** in which introduction of the second and third amino acids as a dipeptide resulted in a more successful synthesis albeit with a low yield. As can be seen from the deprotection profile of this second peptide (figure 2.5) there is gradual levelling off of the profile as further amino acids are added. Whether or not this trend would continue on a long sequence is problematic. The reasons for the initial steep fall off even discounting diketopiperizine formation are not known and would require further work.

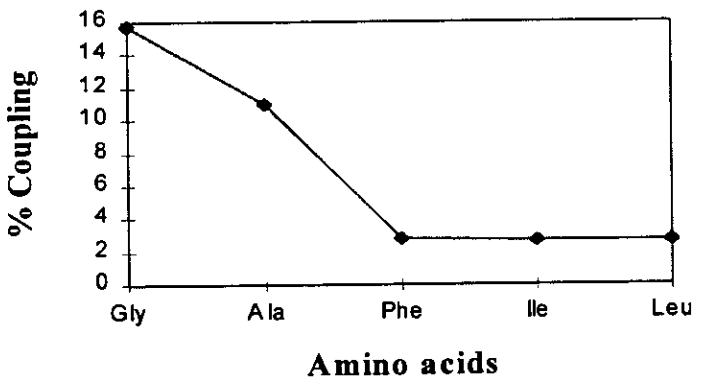


Figure 2.4 deprotection profile of failed peptide

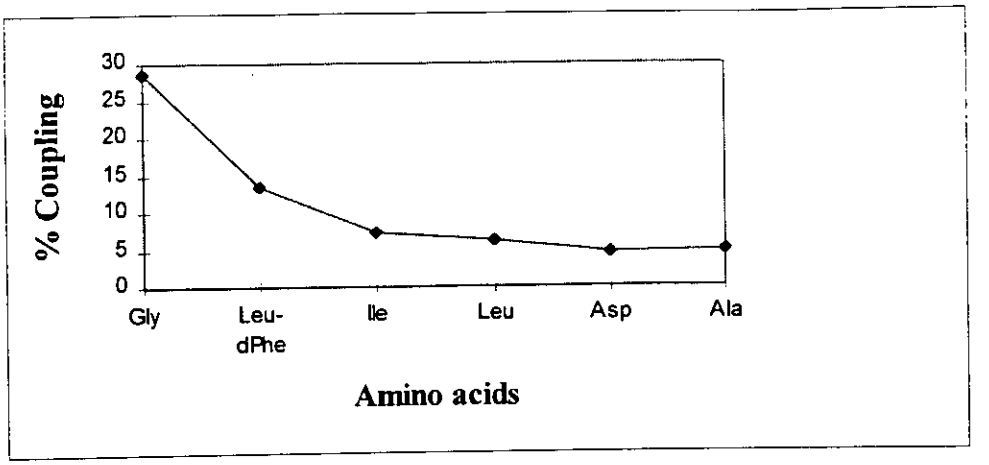
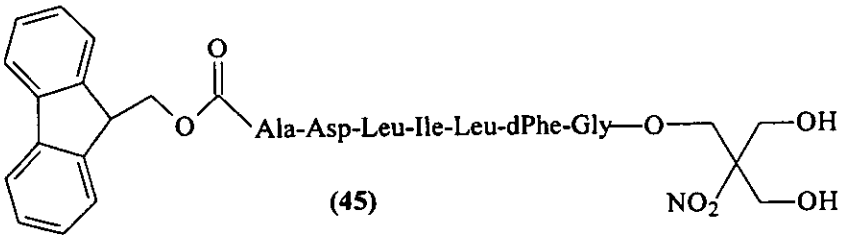


*Figure 2.5 deprotection profile peptide **45***

2.1.2 Chemical fragment coupling

After successfully synthesising the functionalised peptide a series of attempts were made at coupling it through its ester moiety to incoming amino acids. To this end a series of reactions were set up whereby the Fmoc protected peptide **45** was added to Lewis acid buffers such as H_3BO_3, $AlCl_3$[2], $SnCl_2$[3] at various pH's. A number of amino acids or dipeptides were then added in an attempt to find conditions under which a Lewis acid would catalyse fragment coupling. These elusive conditions were not found. What was discovered however was that under relatively mild basic conditions the diol ester **45** could be cleaved to yield the acid.



(45)

Figure 2.6

This discovery opened up a number of interesting possibilities. Firstly, as the diol ester appeared to be cleaved relatively easily in basic solution but its precursor, the protected acetal, was stable to Fmoc SPPS protocols, it was potentially a post-cleavage protecting group. The necessity of which has been highlighted earlier.

Secondly, as a hydroxylic compound its utility as a protecting group could be enhanced by solublising the protected peptide. The problem of protecting groups causing drastic reductions in the solubility of peptides is well known not least in this laboratory[4]. The properties of the group also mean that it would be compatible with the modified form of the Honzl & Rudinger[5] (section 1.5) azide coupling strategy currently used for the fragment condensation of peptides[6].

Azide Fragment Coupling Strategy

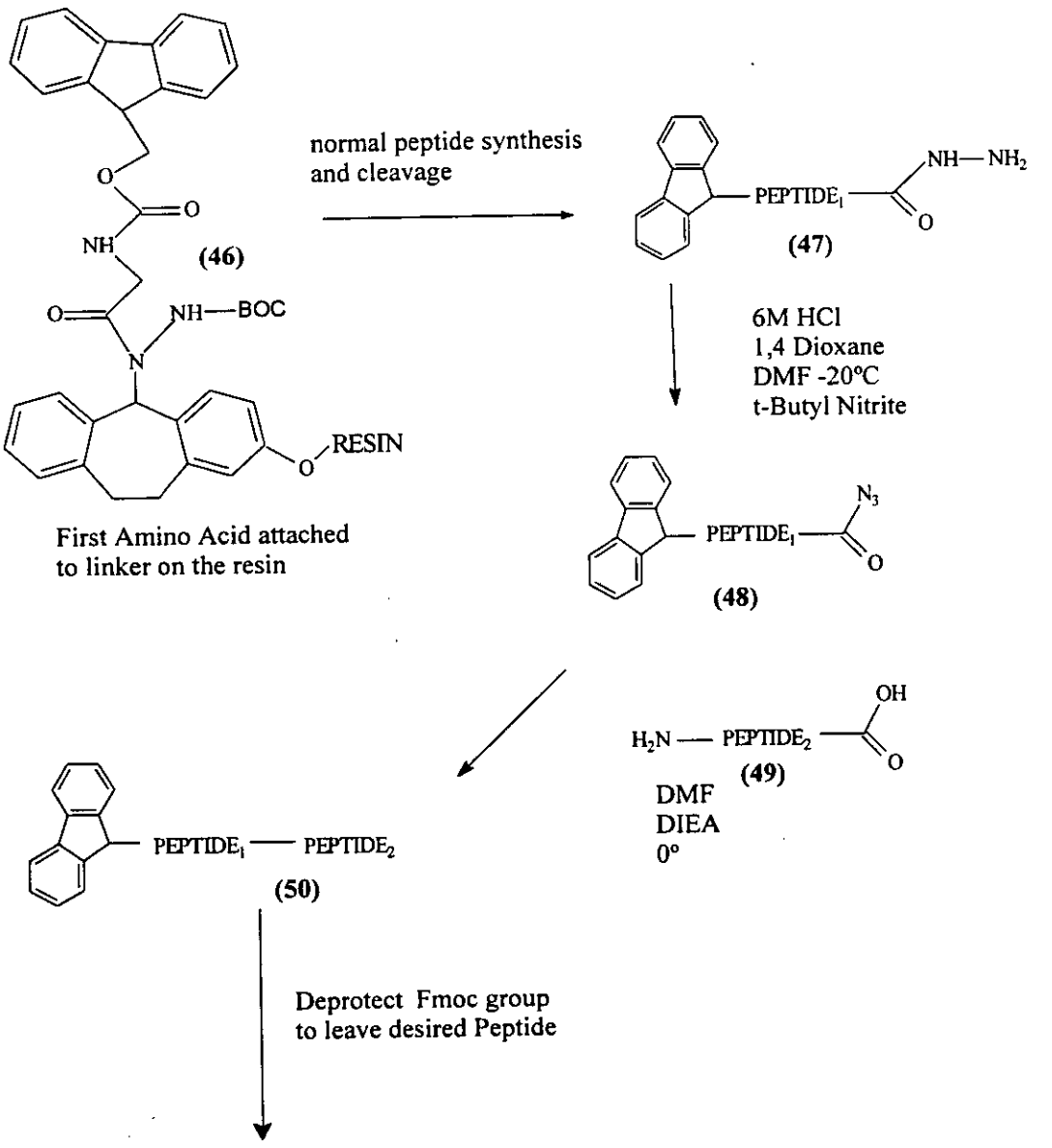
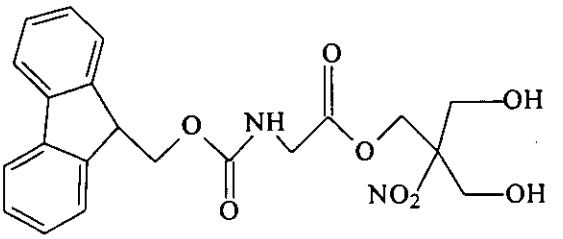


Figure 2.7 Modified Azide coupling

2.2 Ester hydrolysis

In order to examine the extent to which the diol functionalised ester could be cleaved in basic solution it was necessary to synthesis a model compound which could then be used for testing. The compound **51** was chosen as it provided a simple example of a carboxylic acid, possessed a fluorescent tag and had a suitable retention time on HPLC for monitoring the progress of the hydrolysis reactions.



(51)

Figure 2.8

The retention time was important as reactions were followed by HPLC. The synthesis of **51** was relatively straightforward and a program of research was undertaken to examine the required conditions for base-catalysed cleavage of such esters.

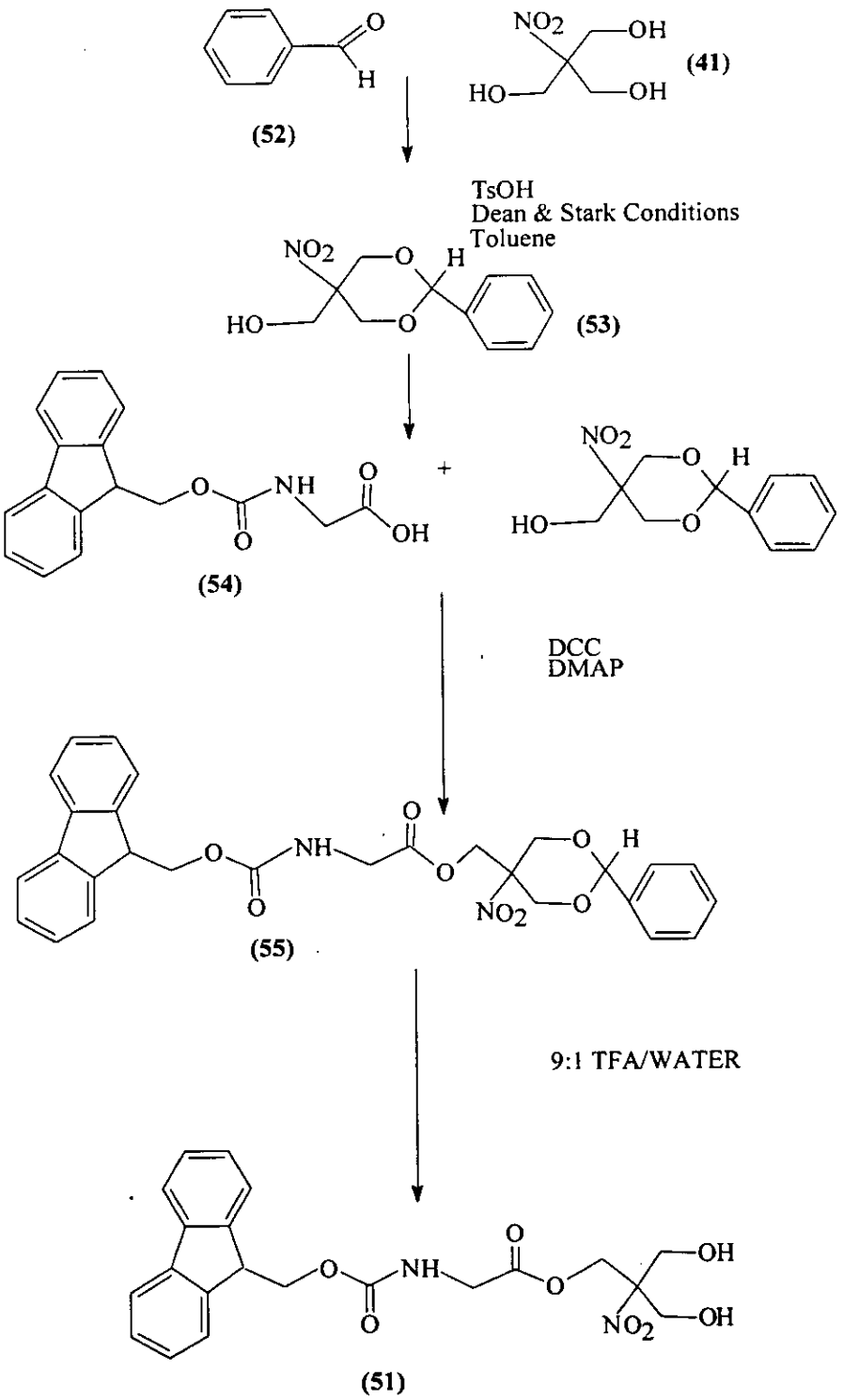


*Figure 2.9 showing synthetic route to compound **51***

2.2.1 Cleavage conditions

Initially a solution of **51** (2mg/ml) in acetonitrile was made up. This was then added in 1ml aliquots to epindorphs containing 1ml of 1% (0.162M) boric acid solution which was buffered with a mixture of HCl and Na_2CO_3 to give the required pH. The Epindorph tubes were allowed to stand and at convenient time intervals 15µl samples were withdrawn, diluted to 0.1ml with pure acetonitrile and 20µl of this solution injected then onto a analytical HPLC column. As can be seen from figure 2.10, two peaks are evident one of which corresponds to the unreacted ester the other to the product Fmoc-glycine. The relative height of each peak is dependent on the degree to which hydrolysis has occurred when the sample is injected onto the column. By assuming that at time zero the ester peak represents 100% and then calculating the proportion of this relative to the growing acid peak, over time a decay curve can be plotted. Statistical analysis of which correlated well with pseudo first order rate kinetics and therefore gave a dimensions rate constant. A series of reactions were carried out in this manner over a pH range from 2-9 the results of which are summarised in graph (2.1) and table (2.1).

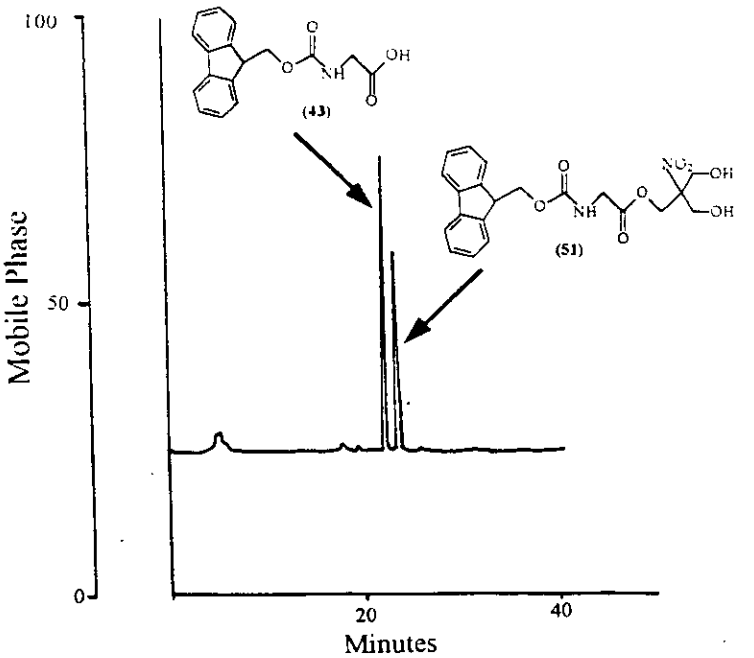
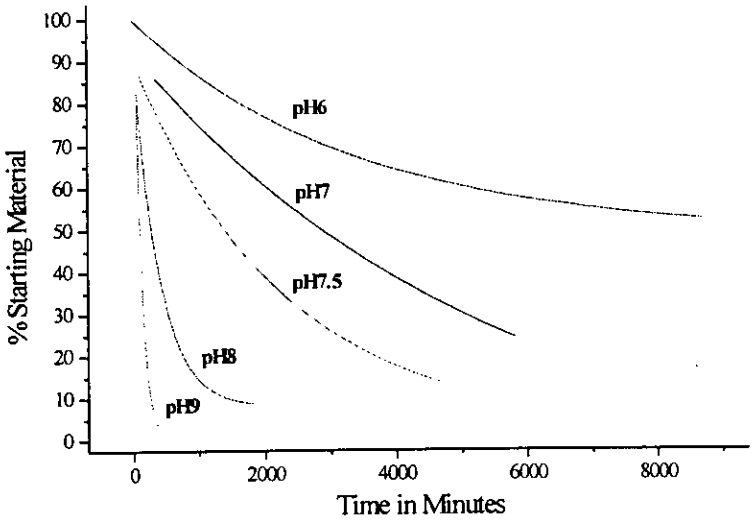


Figure2.1

Graph (2.1) showing rate of reaction at varying pH



pH	% Ester remaining	Time
9	50	1.05 Hours
8	50	2.40 Hours
7.5	50	16.00 Hours
7	50	41.20 Hours
6.5	83	53.30 Hours
2	100	15 Days

Table 2.1

The rate of reaction was initially compared against the hydrolysis of Fmoc-glycine phenyl ester **56** and compound **55**.(Table 2.2)

Compound	pH	Half life
Compound **51**	8	2.40 Hours
Fmoc phenyl ester **56**	8	23.45 Hours
Compound **55**	8	26.10 Hours

Table 2.2

There were indeed differences in the rates of hydrolysis of the three compounds. The diol compound having by far the fastest rate confirming the earlier suspicions encountered with the C-terminal peptide derivative **45**.

Examination of table (1) indicates that the rate of hydrolysis for **51** is dependent on pH, the half life being inversely proportional to the OH^- ion concentration. The subject compound also appears to be completely stable in strongly acidic conditions suggesting that hydrolysis is catalysed by OH^- ions alone.

2.2.2 Theory of ester hydrolysis

There are a number of mechanisms in the literature which explain ester hydrolysis, this is unsurprising since hydrolysis of an ester can take place under a number of different conditions and is catalysed by both acid and base. A classification of these various mechanisms was carried out by Ingold[7] and the naming system he provides proves most useful when discussing these types of reaction. The mechanisms are classified according to the following criteria: acid or base catalysis, denoted **A** or **B**; which carbon oxygen bond is cleaved, acyl **AC** or alkyl **AL**; molecularity,**1** or **2**.

The acid catalysed reactions are reversible, and mechanistically ester formation is equivalent to hydrolysis. Base catalysis however forces the equilibrium in the direction of hydrolysis by the effectively irreversible ionisation of the carboxylic acid.

These mechanistic abbreviations will be referenced in the later text.

Of the possible mechanisms for hydrolysis the one which at first glance would appear to be most likely for molecule **51** is the $B_{AC}2$ mechanism shown below.

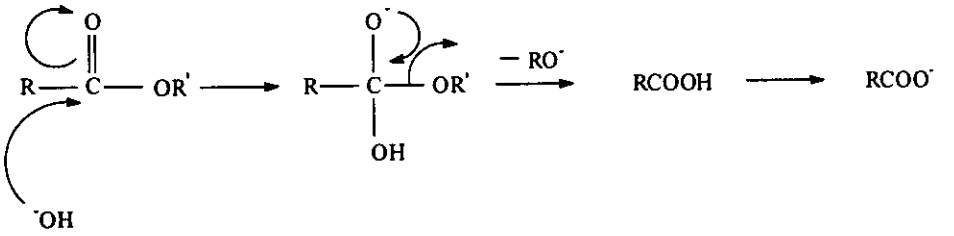
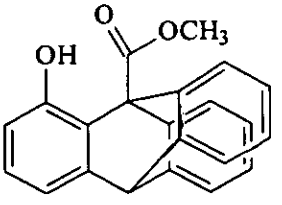
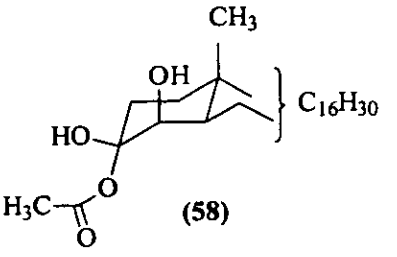


Figure 2.11

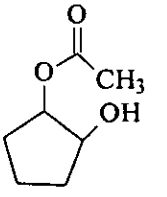
This mechanism does not of course account for the increased rate of hydrolysis over and above that of the control compounds. The increased lability of **51** must be related to its structure. This could manifest itself in a number of ways, through intra-molecular catalysis by the OH groups, through interaction with the boron from the buffer or possibly via some solvent sorting mechanism. A survey of the literature showed that the increased rate of hydrolysis of hydroxylic esters as opposed to their non-hydroxylic analogues has been investigated before, indeed there is a considerable body of work on the subject which appears to have been in particular vogue in the early 1970's. The first paper discussing the subject was that by Bartlett and Green[10] who noticed that a hydroxyl group in the 2-position of methyl triptoate increased the rate of hydrolysis. While methyl triptoate was hydrolysed by KOH in ethylene glycol on heating for one hour at 130°C , the 2-hydroxy ester **57** was saponified in 2 minutes. Further early work included that by Kupchan Slade and Young[8] who examined the rate of base hydrolysis of cholestane-3b,4b,-diol monoacetates **58** . The neighbouring hydroxyl group was reported to increase the rate of ester hydrolysis in these vicinal cyclohexane-diol monoacetates by 8 to 9 times over that noted in corresponding cyclohexane monoacetates. These observations were supported by Bruice and Fife[9] on a series of 2-hydroxycyclopentyl carboxylates **59** and support the conclusion that neighbouring hydroxyl groups increase the rate of hydrolysis.



(57)



(58)



(59)

Figure 2.12

The data in table 1 is roughly in line with these later findings so it would appear that compound **51** exhibits an example of what has become known as known as the Henbest-Kupchan[8,9,11] effect. A situation whereby the neighbouring OH group is not acting in a nucleophilic manner, in which case a lactone would be formed, rather it acts in solvating the transition state for nucleophilic attack by an external hydroxyl ion on the ester carbonyl ie. a modified form of the $B_{AC}2$ mechaism.

Later work by Capon[12] suggested that in some instances solvent sorting may also have an important role to play, however for most cases the above theory is generally accepted. In addition to anchimeric assistance by the OH group there is also literature supporting a catalytic effect[12,13,14,15,] by the borate which will be discussed later.

What appeared to have been a reasonably serindepidous discovery, i.e that diol esters are cleaved relatively quickly in basic acetonitrile/water mixtures proved to be an example of a well known effect. What does not appear to have been realized however is the potential of these compounds to act as protecting groups, similarly there appears to have been very little effort made to optimise the conditions for removal of these hydroxy esters. A program of research was therefore undertaken to try and optimise these conditions and adapt them for use in peptide chemistry.

2.3 Tris(hydroxymethyl)nitromethane (41) derivatives as protecting groups in peptide chemistry

2.3.1 Deprotection optimisation

In any attempt to increase the rate of a reaction the first variables to be altered are generally temperature and mixing. As peptides are natural products it was desirable to limit temperature rises to 37°C as this is the natural operating temperature of the body. In the case of mixing it was thought that sonication would be the best, and certainly the most reproducible method of agitation. From the initial studies it was also known that increased pH would increase the rate of hydrolysis. It is however desirable, when dealing with proteins, to minimise their exposure to high pH as this

can cause unwanted reactions with the side chains of certain amino acids and also begin hydrolysis of the amide backbone. With these limits in mind the role of the co-solvent used with the aqueous buffer in the hydrolysis reaction was investigated. In these experiments a 1mg/ml solution of **51** was made up in various solvents 0.4ml aliquots were removed and placed in epindorph tubes containing 0.2ml of borate buffer {0.2M sodium borate, with 0.2M boric acid proportionately mixed to give the required pH}. This buffer replaced the 1%(0.162M) boric acid buffer previously used purely due to ease of preparation and in did not appear to effect the rate of reaction. The reaction vessels were then floated in a sonic bath at room temperature. At convenient time intervals aliquots were removed and injected on to an analytical HPLC column whereupon it was discovered fairly quickly that by switching from acetonitrile to DMSO as the chosen co-solvent a large increase in the rate of reaction could be achieved (see table 2.3). Again a rationale for explaining this was to be found in the literature[16,17,18,19,20].

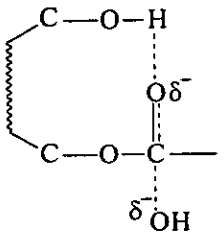
Compound	Solvent	pH	Half life	Buffer
Compound **51**	Ethanol	8.0	4.00Hours	borax
Compound **51**	Acetonitrile	8.0	2.40Hours	borax
Compound **51**	DMSO	8.0	10 minutes	borax

Table 2.3

2.3.2 Aprotic dipolar solvents

DMSO is a dipolar aprotic solvent similarly so are DMF, acetonitrile and, to a lesser extent, acetone. These solvents in spite of being highly polar lack the ability to *donate* hydrogen to form hydrogen bonds and this is important for a number of reasons. Firstly in any reaction which involves nucleophilic anions in aqueous solution there exists around each anion a solvent shell of water molecules which inhibits the reactivity of the ion. Dipolar aprotic solvents lacking the ability to form hydrogen bonds do not solvate anions and hence the effective reactivity of the anions

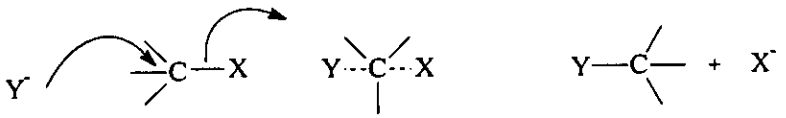
is increased. Provided there is sufficient mole fraction of dipolar aprotic solvent within an aqueous mix the anions will exist effectively as naked species, since the dipolar aprotic solvent will engage the hydrogen bond donating water molecules as dipolar aprotic solvents are strong hydrogen bond *acceptors*. This capacity to desolvate anions influences the rate increases in many nucleophilic substitution reactions. There is however a secondary effect which takes place when a neighbouring group is capable of anchimeric assistance[12]. In this scenario an internally hydrogen bonded transition state structure as in figure (2.13) is more highly favoured by aprotic as opposed to protic solvents.



Stablised transition state proposed by Bruice & Fife

Figure 2.13

This is due to the charge distribution being more similar to an S_N2 transition state as opposed to that of normal esters in which there is a localisation of negative charge on the carbonyl oxygen[9].



S_N2 Mechanism

Figure 2.14

Dipolar aprotic solvents are better able to solvate these charge dissipated transition states and therefore lower the activation energy necessary to reach them. This in turn increases the rate of reaction.

As an aside it is interesting to note that the rate of removal of the Fmoc group, compared with the Tnm group, is far faster in protic solvents than in dipolar aprotic

solvents as is illustrated in the below HPLC trace (Fig 2.15) which shows compound **51** after 2hours in ethanol/borate at pH8 (compare with figure 2.10).

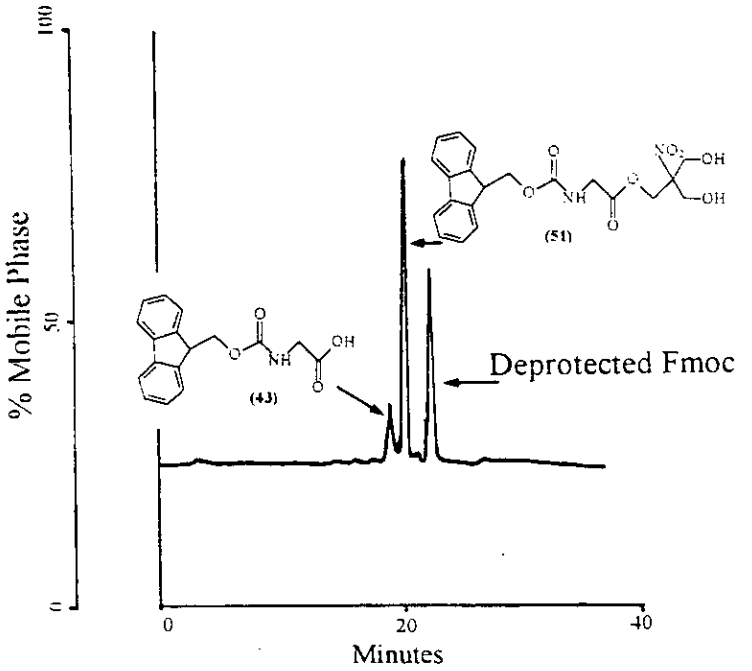


Figure 2.15

This lends extra evidence to the theory that some special form of intramolecular catalysis is taking place in the case of the tris(hydroxymethyl)nitromethane (Tnm) derivative.

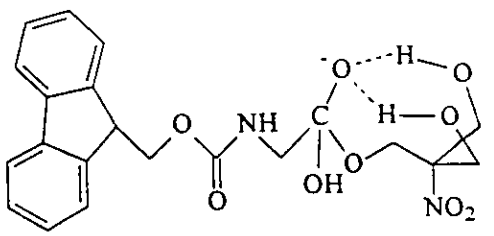


Figure 2.16 Showing how Bruice & Fife model might be applied to compound 51

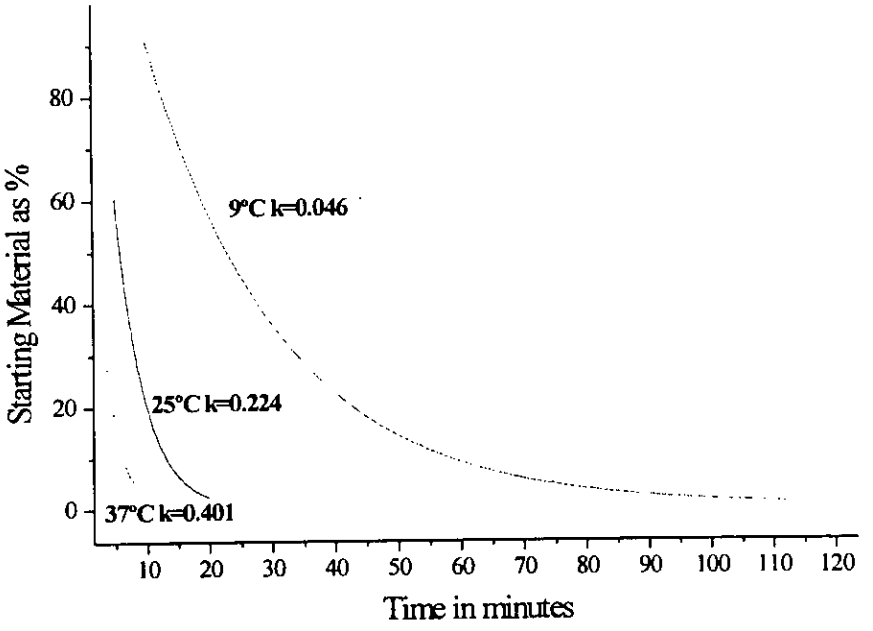
2.3.3 Kinetics

In an effort to find out how the rate of reaction in DMSO would be effected by temperature a series of reactions were carried out in which the rate of hydrolysis was monitored at three different temperatures.

2.3.3.1 Method

A stock solution of compound **51** was made up (1mg/ml in DMSO), again 0.4ml aliquots of this were taken and placed in epindorph tubes which were then placed in the cold room and allowed to freeze. This procedure was necessary because on adding buffer to DMSO there is a large enthalpy of mixing of the two solvents and this raises the temperature quite considerably in the confines of an epindorf tube. Consequently without some effort being made to negate this temperature rise any kinetic data relating to temperature would be highly inaccurate. At the beginning of each kinetic run 0.2ml of buffer (pH 8.5 borate) was added to the top of a frozen epindorph tube and the epindorph tube floated in a sonic bath. At the designated time the reaction was quenched by injection of a further 0.2ml of acetic acid. The quenching of the reaction allowed several epindorph tubes to be sonicated at the same time and therefore under the same conditions. These could then be analysed separately and the results averaged. The decay curves were calculated as previously described and are summarised in graph (2.2).

Hydrolysis of compound **51** at three different temperatures in pH 8.5 Borate buffer/DMSO



Graph 2.2

As can be seen the rate of reaction is increased by temperature as would be expected. The rate of cleavage is also exceptionally fast and hence the diol ester should prove an excellent protecting group.

2.3.4 Buffer effects.

As previously mentioned there is some evidence to suggest that under certain situations borate buffers can act in a catalytic manner. Work by Capon[7] suggests this may be through a borate stabilized intermediate as shown in figure 2.17

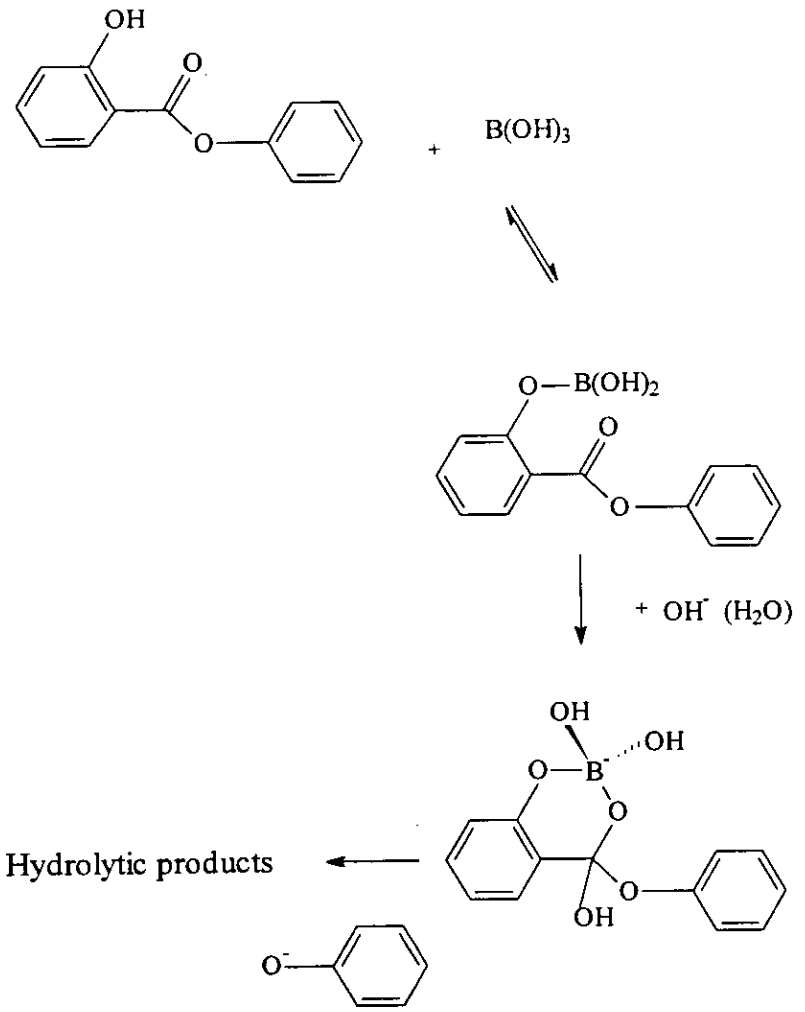


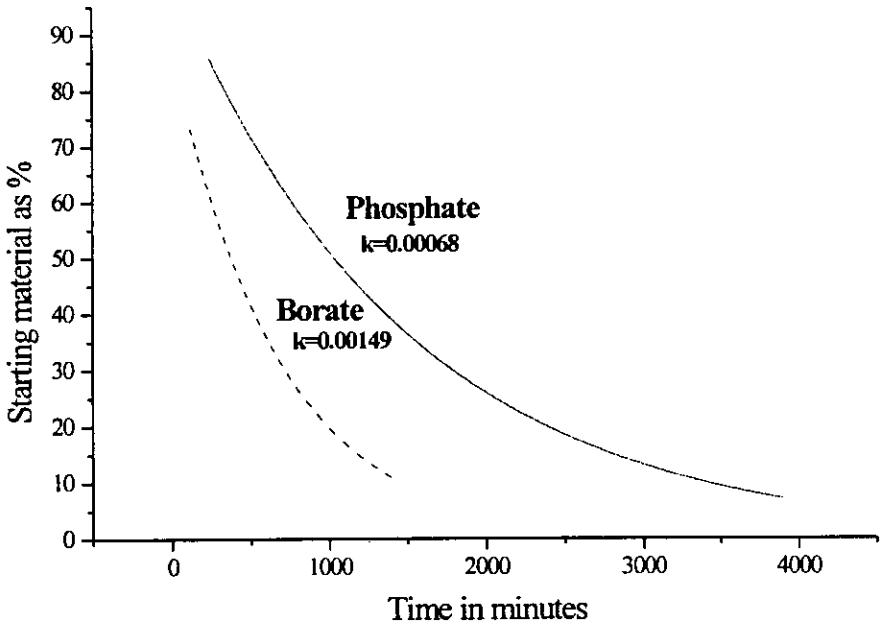
Figure 2.17 after Capon[14]

In order to examine this phenomena a series of kinetic experiments were under taken useing borate, phosphate and Tris, [tris(hydroxymethyl)aminomethane] buffers. Unfortunately the reactions involving Tris were difficult to follow due to the appearance of an unknown product, possibly the result of a reaction between the buffer and cleaved Fmoc. The rate of reaction with Tris where it could be assessed, was however much slower than for either borate or phosphate buffers cleavage taking over 20 hours at pH 8 in DMSO at room temperature.

The experiments involving phosphate and borate were carried out using exactly the same method as described previously and in the experimental section. The buffers were however at pH 8 rather than 8.5 in order to make the reactions slower and

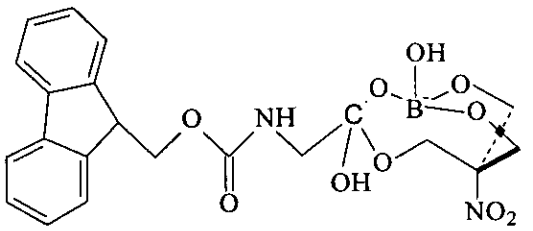
illustrate any rate differences more dramatically. As can be seen from graph (2.3) there are indeed differences in the rate of reaction in the two buffers.

Comparison of hydrolysis of compound **51** in phosphate and borate buffers at 10°C and pH8



Graph2.3

It would appear that borate does have some catalytic effect under the conditions examined and that phosphate may also to a lesser extent act in a similar way. Whether this is through a complex with the hydroxyl groups or through some interaction with the solvent is difficult say. The results do however support the evidence of the previously cited literature and could be due to existence of intermediates similar to those shown in figure 2.18



(60)

Figure 2.18 Showing how borate might stabilise the transition state

Intermediates of this type were of course implicit in the original plan (figure 2.1); although for completely different reasons.

Whichever mechanism is operating, and it is far from certain at this stage, the removal of the diol has been achieved in mildly basic solution and in good time. Hence we can be practically, if not intellectually satisfied. A survey of possible mechanisms will be discussed later.

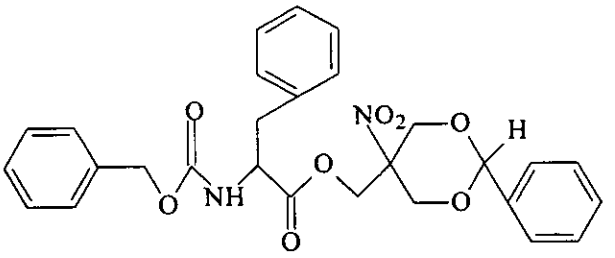
2.3.5 Other Systems

Further work was carried out to check the validity of the diol on other amino acids, whether or not it was stable to piperidine/DMF and if it could be applied to amino protection.

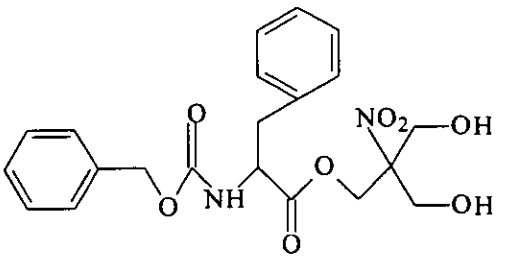
2.3.5.1 Stability

At this juncture we have a protecting group which as an acetal appears to be stable to the basic deprotection conditions of SPPS. However when the acetal is cleaved in aqueous TFA to yield a diol we have a protecting group which can easily be removed to give the acid function. Although the synthesis of peptide **44** has been achieved under SPPS conditions, there was, as reported, a low yield. In order to check that this was not due to instability of the proposed acetal protecting group to DMF/piperidine, the Z-phenylalanine derivative (**61**) was synthesised and a 1mg

sample subsequently dissolved in 1ml of DMF/piperidine (4:1). Aliquots were taken at convenient time intervals and injected on to an analytical HPLC column. After four hours there was still no change in the HPLC trace and the acetal was thus deemed to be stable to SPPS conditions.



(61)



(62)

Figure 2.19 compounds 61 & 62

It was also necessary to find out if the diol itself could be cleaved by DMF/ piperidine as it was important to know whether or not it would be stable to the conditions used to remove the Fmoc and Tbfmoc groups. In order to assess this the Z-phenylalanine derivative **62** was synthesised and tested briefly with borate/DMSO, to confirm that the diol group was removed with similar ease to that of the Fmoc-glycine derivative suggesting broad-based applicability for acid protection.
The stability of **62** was then tested against DMF/piperidine (4:1). Figure 2.20 shows an HPLC trace of **62** after 70 minutes in the DMF piperidine mix.
The half life for removal of the diol proved on average to be approximately 90 minutes which is much slower than for either Fmoc or Tbfmoc. An investigation as

to whether the piperidide or the acid was formed during the reaction was carried out to try and shed some light on the mechanism.

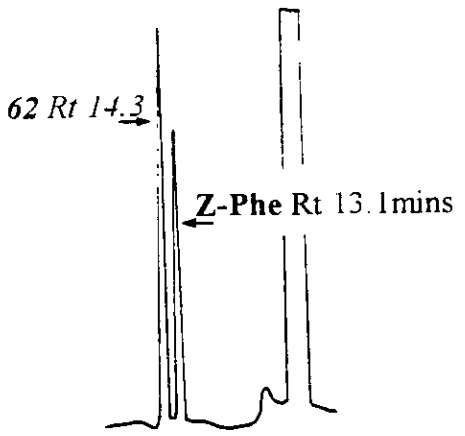


Figure 2.20

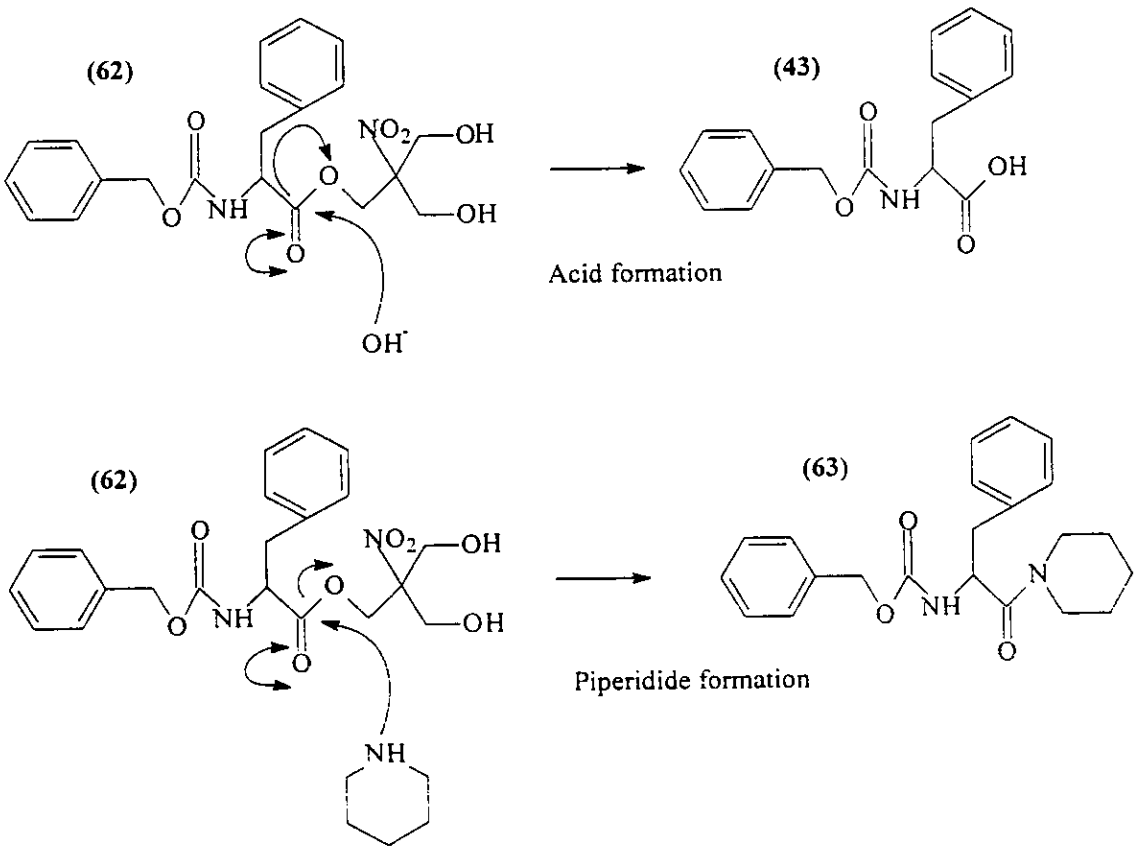


Figure 2.21 showing the two possible mechanism

The Z-phenylalanine piperidide **63** was synthesised and examined under HPLC in comparison with **62** and Z-Phenylalanine-OH.

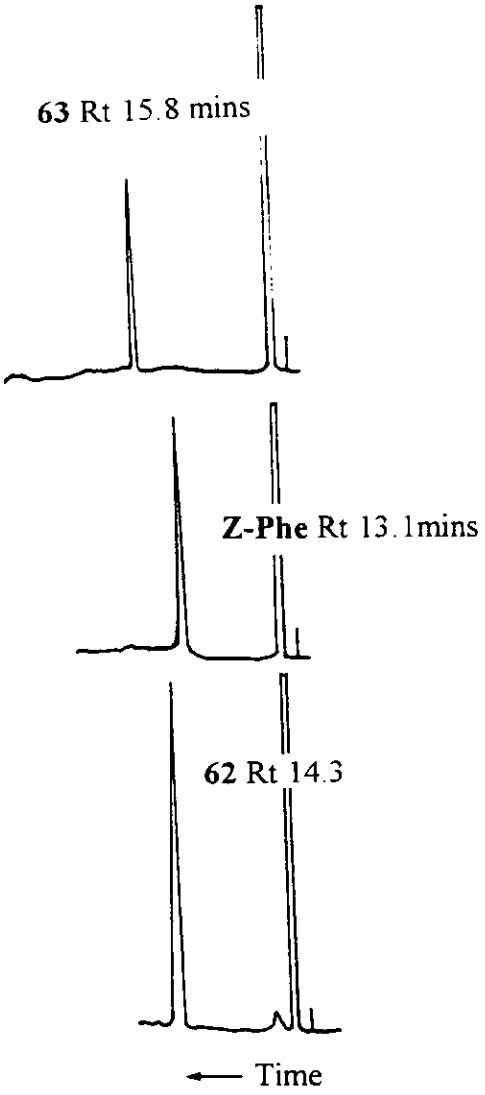


Figure 2.22

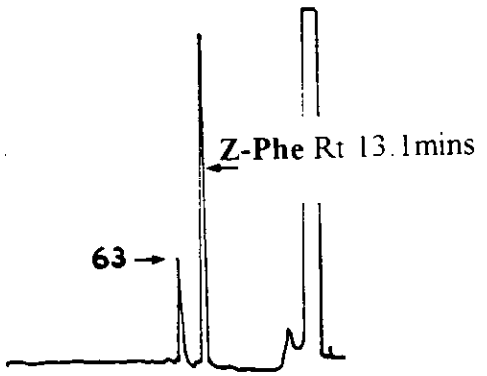


Figure 2.23

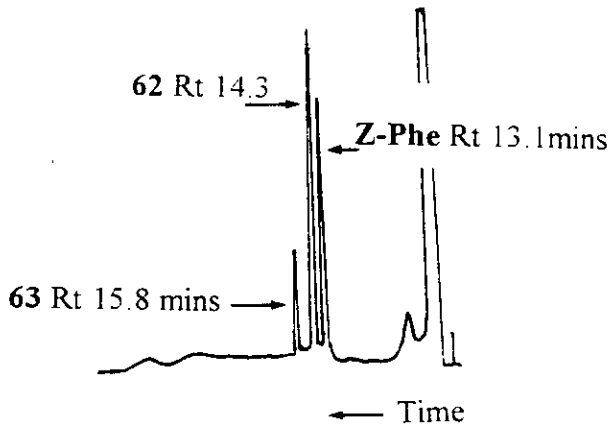


Figure 2.24

*Figure 2.22 shows the HPLC traces of the three pure compounds. Figure 2.23 shows the trace of compound **63** & Z-Phe-OH. While figure 2.24 shows the HPLC of an injection of all three.*

As can be seen from the HPLC traces, the acid, rather than the later eluting piperidide is formed. This suggests that it is OH^- ions produced by the basic piperidine reacting with water in the DMF, rather than the piperidine itself which cleaves the diol group. This leaves open the possibility of selective cleavage of

Tbfmoc in the presence of the diol ester but would require very dry solvents which may be difficult to reproduce. Whether this is feasible or not would require further research.

2.4 Other tris(hydroxymethyl)ester derivatives

So far the only compounds actually tested have been derivatives of Tnm **41** thus in order to see if other compounds of this type have similar properties the methyl derivative **64** was synthesised from the 1,1,1-tris-(hydroxymethyl)ethane (Tme) **64a** precursor. (Figure 2.25)

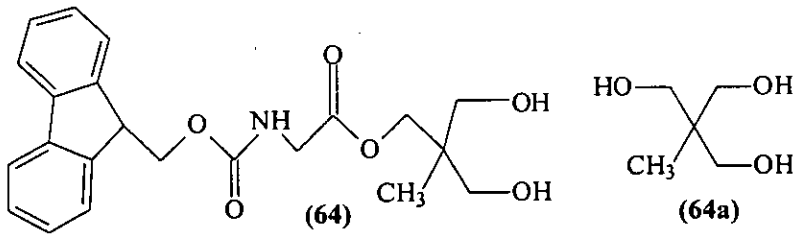
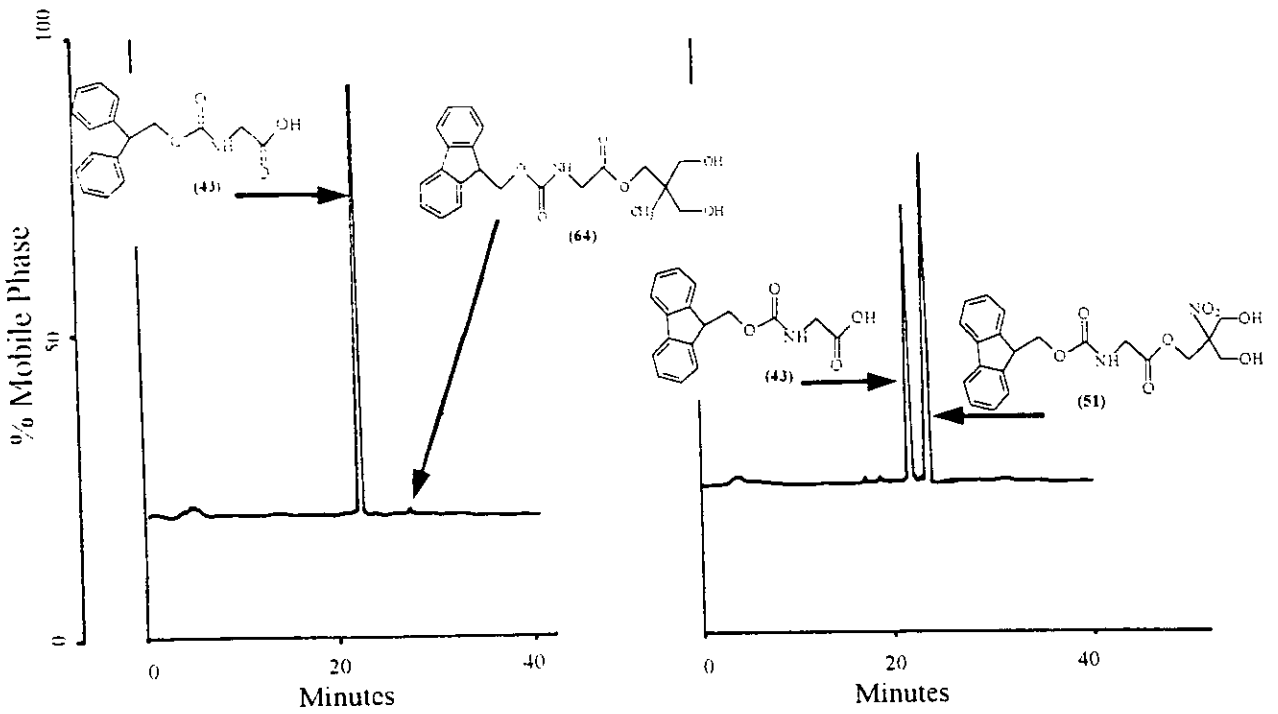


*Figure 2.25 showing compound **64***

The synthesis was analogous to that used for **51** and presented no problems. The compound **64** was tested under the same optimised conditions as for **51** and appeared to cleave faster than **51**, quantification of these results proved difficult because of the speed of reaction. As can be seen from figure 2.27, 2.28 the rate of hydrolysis of **64** is dramatically quicker than for **51**. In addition to this figure 2.29 shows that compound **64** can be cleaved by deionised water if DMSO is present.



Figures 2.27 (left **64**) and 2.28 (right **51**): Comparison of Hydrolysis of both compounds in DMSO/ Borate 2:1 pH8.5 after 5mins.

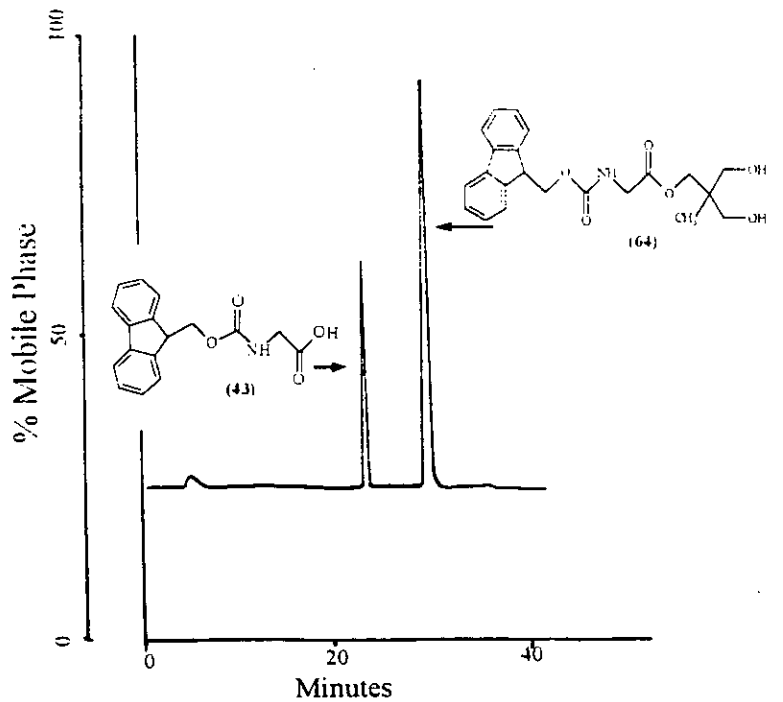
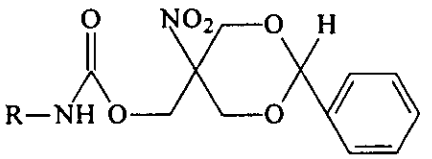


Figure 2.29 Compound **64** after 25 mins in DMSO/Deionised Water 2:1

It was later found that **64** was unstable when exposed to the atmosphere slowly breaking down to form Fmoc-glycine (half life approximately one month). Since **51** appears to be completely stable and has been stored as both as a gum and in solution for over a year this is a curious result. The capacity of both compounds to form gums on exposure to air, presumably by absorbing water, may be important. Compound **64** being less stable is perhaps hydrolysed by the absorbed water. This hypothesis is supported by the fact that compound **64** has good stability when stored in water free solvents such as DCM. It is difficult to make inferences as to the role of the nitro and methyl groups in these compounds because of the uncertainty in information gained from studies on the methyl derivative.

2.5 Lysine protection

As previously discussed (section 1.5) in order to perform reliable fragment coupling, be it by chemical or enzymatic means, it is necessary to protect amine as well as acid groups. To this end, and after the success of the Tnm group in protection of acid functions, attempts were made to assess its potential as an amine protector. The protection being in the form of the carbamate derivative of Tnm.

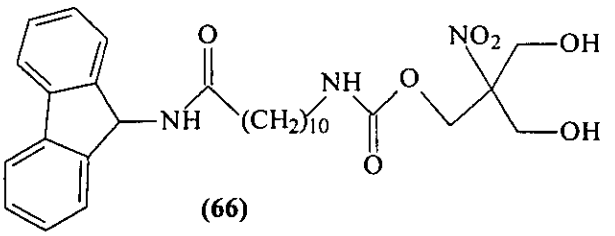


(65)

Figure 2.30 Protection of an Amine as a Carbamate

It was necessary therefore to synthesis a test compound which could be used in a similar manner to **51** for optimising the conditions necessary for deprotection.
It was perceived that removal of an amine protecting group of the above type would be more difficult than in the protected acid situation. This is because the lone pair of electrons on the nitrogen are capable of entering into resonance with the carbonyl double bond and therefore stabilizing the compound to hydrolysis. Due to the increased stability of urethanes towards hydrolysis it was decided to synthesise a

model compound without the Fmoc moiety as this could be deprotected if the conditions ultimately used had to involve a high pH. As it was still desirable to have a fluorescent tag and suitable retention time on HPLC the compound **66** was chosen as the target test molecule.



(66)

*Figure 2.31 Compound **66***

The synthesis of this compound is relatively straight forward the only problem occurring in the activation of the carbamate for attack by the amine. This was initially attempted by trying to form the chloroformate of compound **53** (figure 2.9, section 2.2) but this proved a difficult compound to isolate due to its instability. It was hence decided to convert the unstable chloroformate into an activated carbonate and thus avoid the need to isolate the unstable intermediate. Various methods of activating the alcohol were tried and some success was achieved with the p-nitrophenol mixed carbonate which was formed by reaction of **53** with triphosgene and p-nitrophenol.

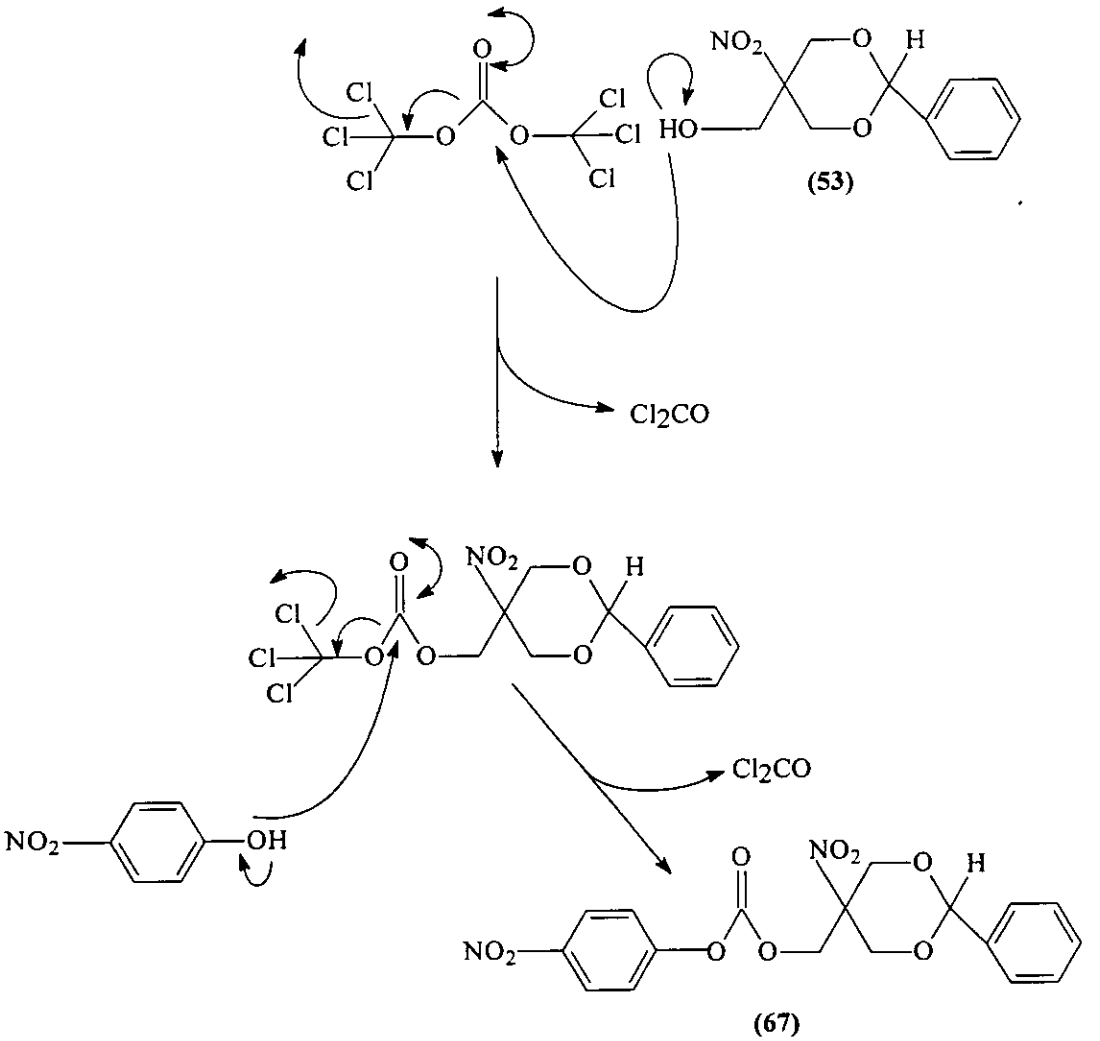


Figure 2.32 Synthesis of Compound 67

The best over all yields were however achieved with use of the pentaflurophenyl derivative **69** which had the added advantage of being easy to crystallise. It was again synthesised using triphosgene.

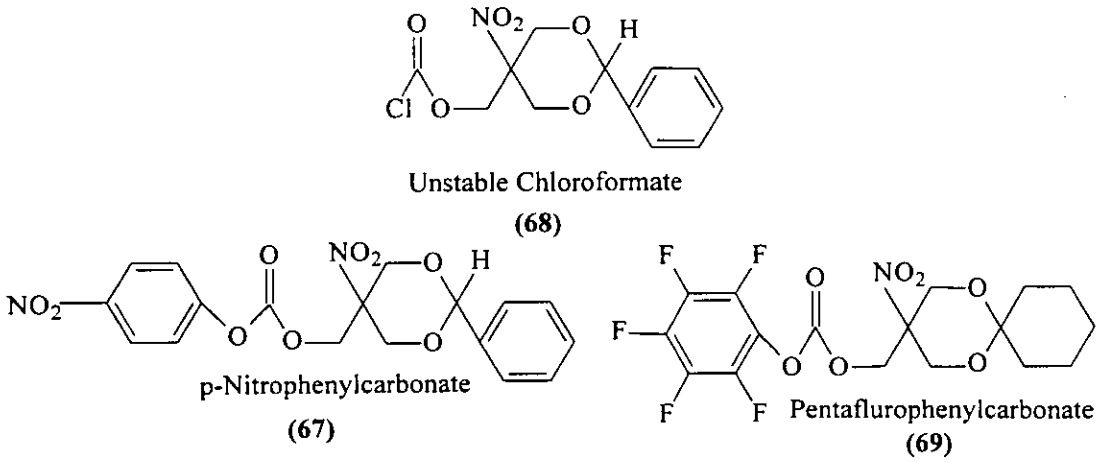


Figure 2.33

Once **66** had been synthesised a number of reactions were carried out to assess its stability and the optimum conditions for hydrolysis.

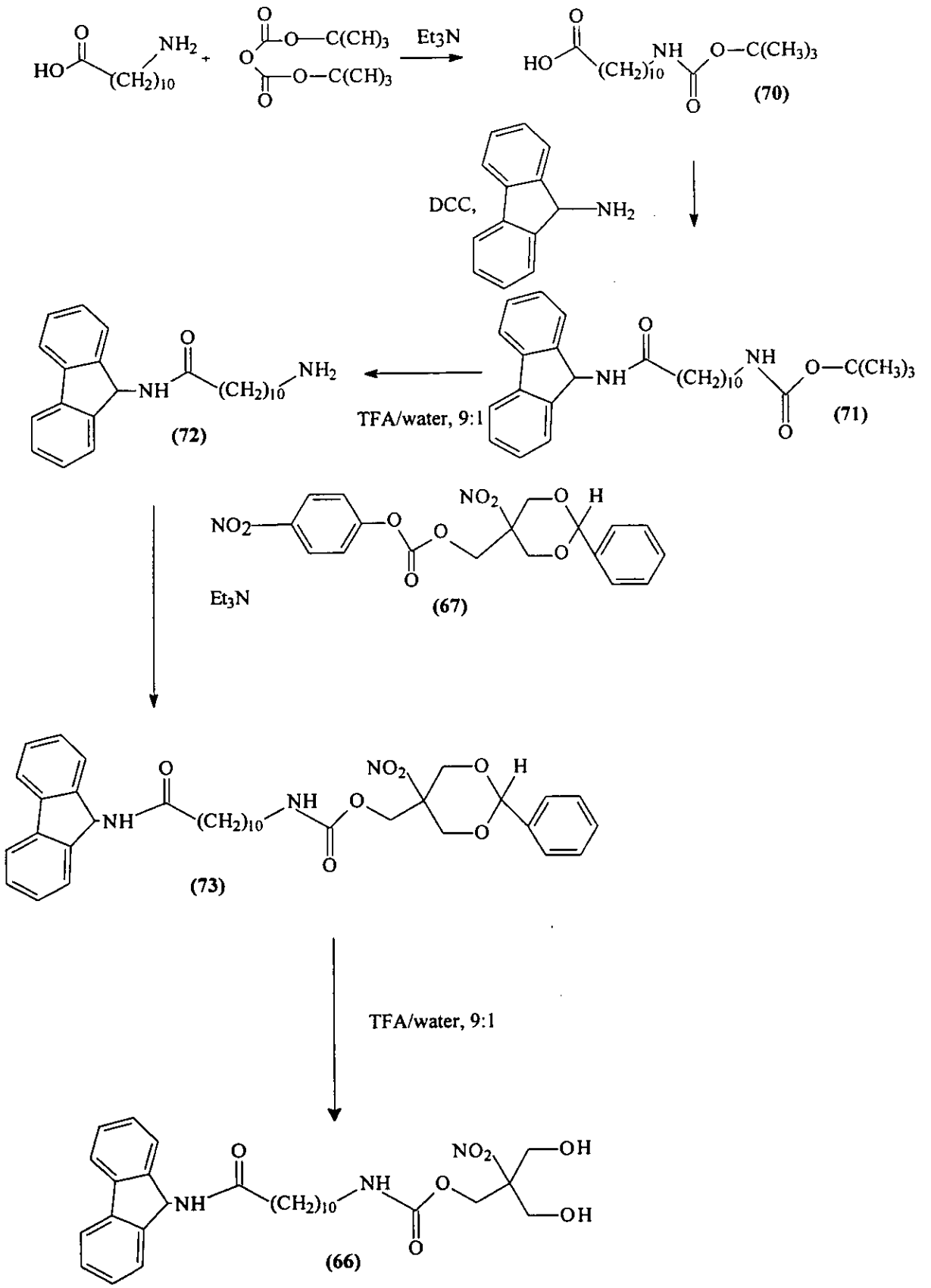


Figure 2.34 Synthesis of compound 66

Before any investigation into the conditions for hydrolysis of **66** was undertaken the synthetic precursor of **66**, namely **73** was tested for its stability to piperidine/DMF and therefore the ability of the protected carbamate to withstand conditions on the synthesiser. There was no noticeable change in HPLC profile after 4 hours in 20% piperidine/DMF and hence the overall structure was thought to be compatible with SPPS. A series of hydrolysis reactions were then carried out on **66** in a number of solvents to assess their effect (Table 2.3).

Solvent	pH	% Hydrolysis	Buffer	Time
DMSO	8	100%	Borate	30 mins
DMF	8	100%	Borate	50 mins
ACETONITRILE	8	100%	Borate	5 hours
ETHANOL	8	50%	Borate	3.30 hours

Table 2.3

Broadly speaking the results were very similar to those for **51** the rate of deprotection being maximised in DMSO/borate. There are however some important differences. If the below HPLC traces are examined it can be seen that the hydrolysis of **66** is less "clean" than for that of **51** and that the choice of solvent will affect the cleanliness of reaction. The more dipolar aprotic in nature, the cleaner the reaction, also the higher the pH the cleaner the reaction.

Hydrolysis of Compound **66** in DMSO/Borate at pH8

Hydrolysis of Compound **66** in Acetonitrile /Borate at pH8

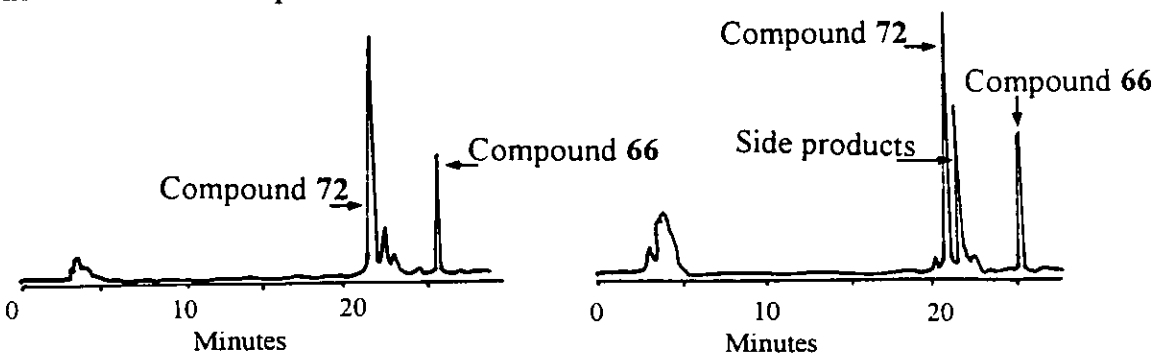


Figure 2.35(A) showing how solvent affects cleanliness of reaction

These additional peaks are obviously intermediates or side products in the hydrolysis of **51**; exactly what they might be and how they can be removed will be discussed later. It is worth noting however that although the rate of reaction is slower using Tris buffer it is much cleaner than with borate.

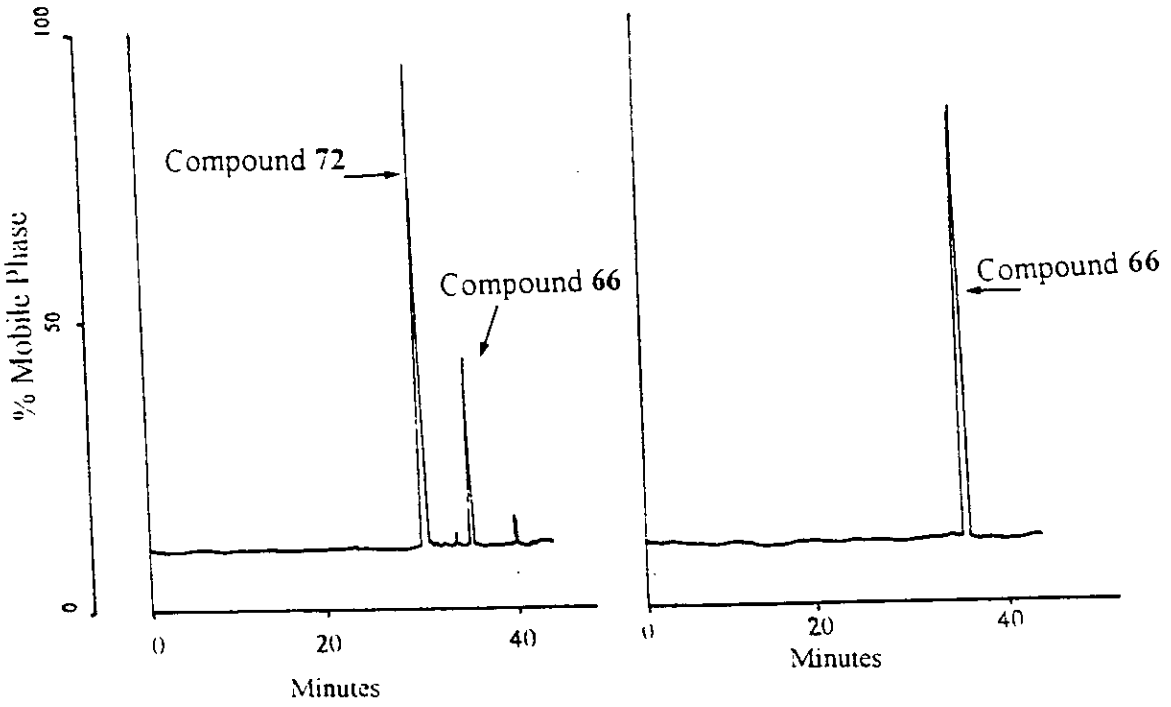
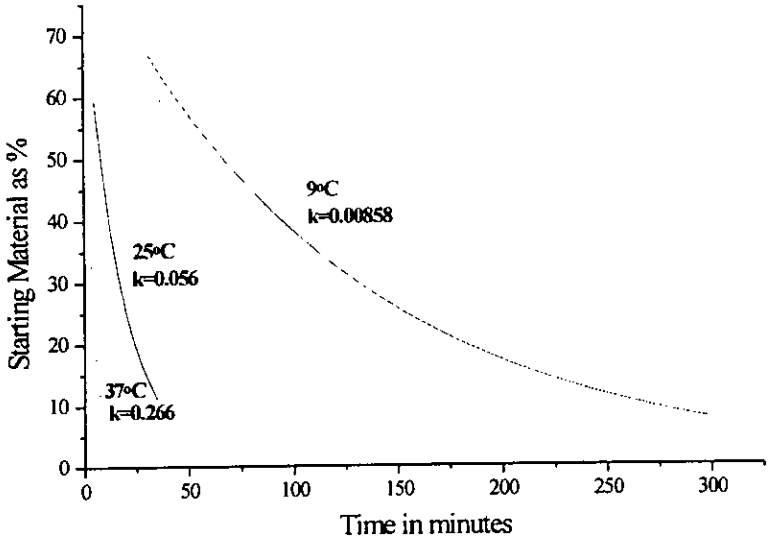


Figure 2.35(B) showing how buffer affects cleanliness of reaction: right trace pure 66; Left trace same sample after 7 hours in pH8 DMSO/Tris.

2.5.1 Kinetics

As with compound **51** and using the same method a series of reactions were subsequently performed at three different temperatures to provide kinetic data. As can be seen from the graph 2.3 the rate of reaction is temperature dependent also the reaction becomes cleaner at higher temperature with fewer side products being observed.

Hydrolysis of compound **66** at three different temperatures in pH 8.5 Borate buffer/DMSO



Graph 2.3

2.5.2 Protection of lysine in a peptide

The ability of the carbamate acetal structure to withstand mock SPPS conditions has been ascertained, as has the capacity to remove the diol compound formed after hydrolysis. All that remained was to test the strategy on a real peptide. In order to achieve the broadest possible specification it was decided to attempt to protect lysine as the Tme **64a** derivative. As it was thought that this could be more easily incorporated into the synthesis of protected glutamic/aspartic acid residues as well as lysine. The reason for this is, that although it is by no means the only route, the most convenient synthesis of these protected Glu/Asp residues involves hydrogenation (figure 2.36) and it was thought that the Tnm nitro group may be converted to an amine during this process. As this would obviously compromise the use of the Tnm derived molecule as a protecting group the Tme derivative was used for this initial synthesis.

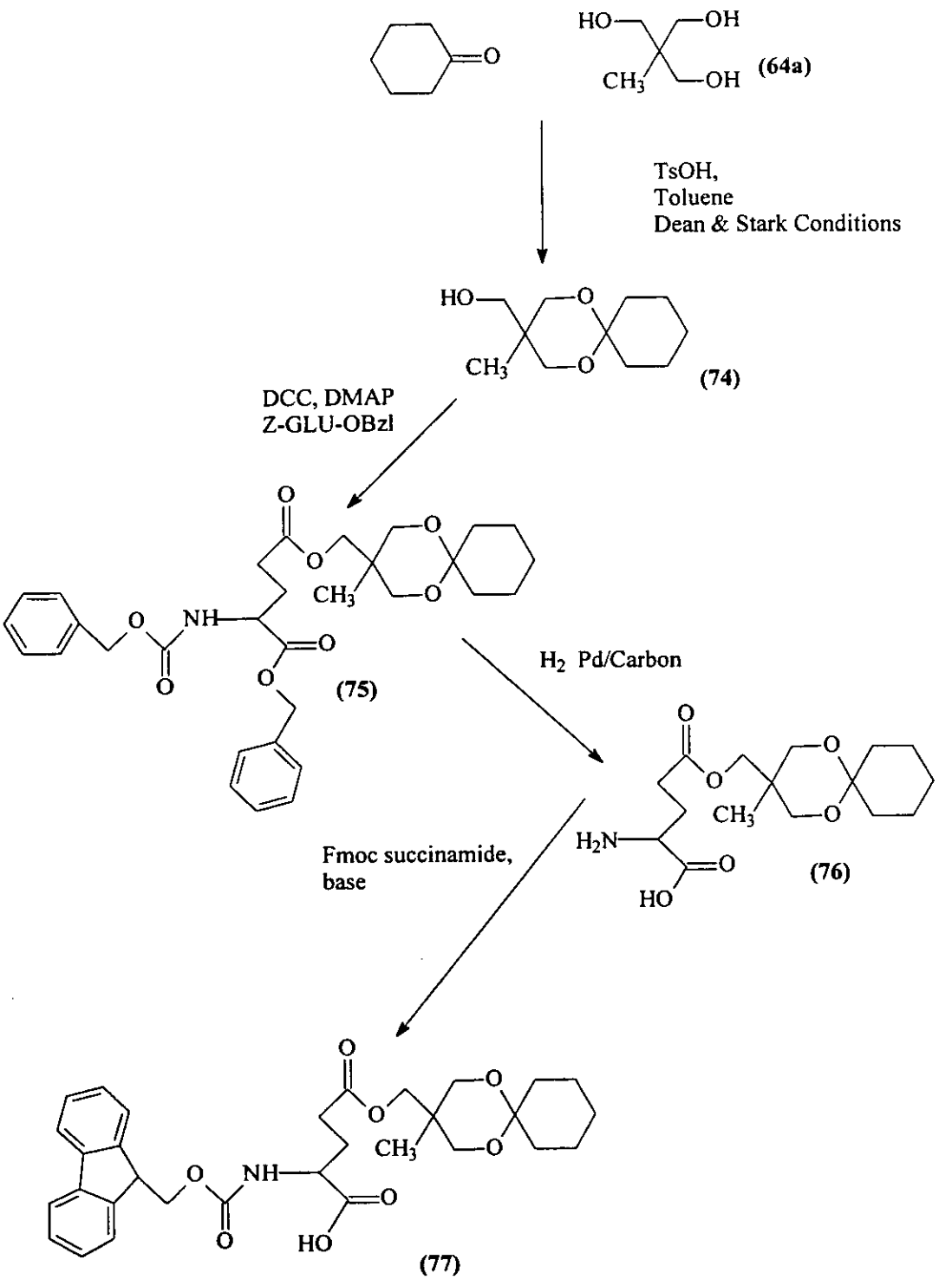


Figure 2.36 Synthesis of the Tme derivative of Glutamic acid

Having decided to protect lysine using the Tme derivative, and having attached it to Fmoc-lysine via the pentaflurophenyl carbonate **78,** to give compound **79,** all that remained to do was to synthesise a test peptide.

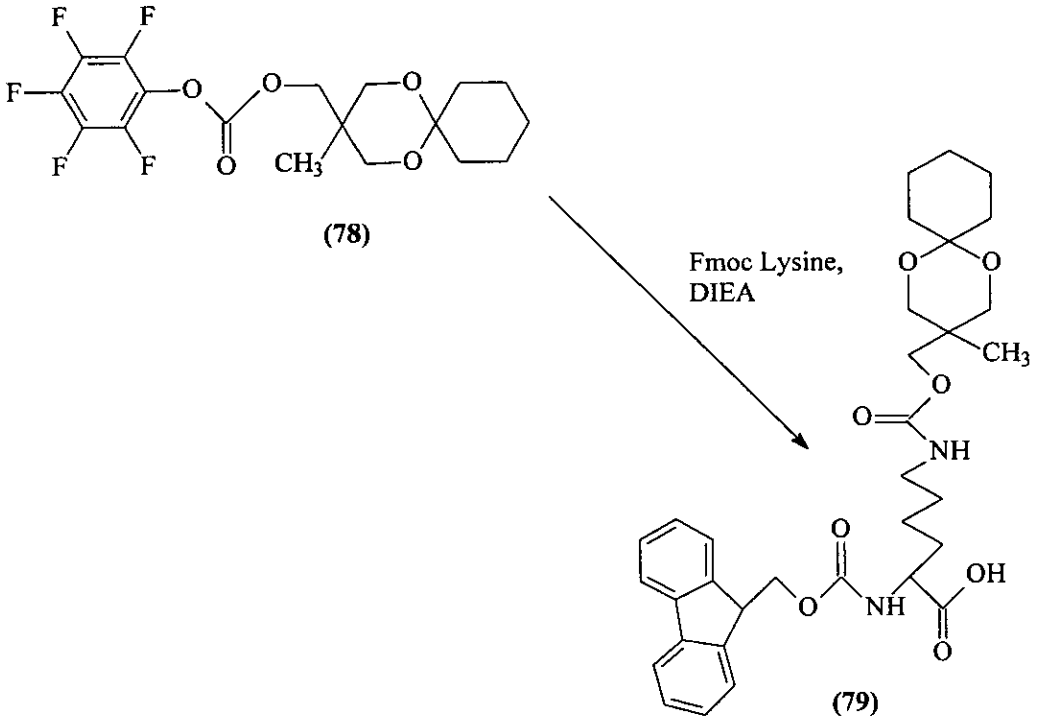


Figure 2.37 Synthesis of Compound 32

The short peptide Phe-Gly-Lys(Tme)-Ala-Gly-Gly (**80**) was synthesised on an Applied Biosystems 430A synthesiser using the method outlined in the experimental section.

After standard purification, via semi-preparative HPLC, removal of the protecting group was attempted with borate buffer at pH8.5 in DMSO; this was however unsuccessful. The pH was increased to 10 without effecting deprotection. This was a curious result considering the rate of deprotection for the methyl group was faster than that of the nitro derivative in the esters previously tested. This was somewhat disappointing as the protecting group had failed in its first real test. In order to assess whether there was an intrinsic problem associated with removal from peptides, as had been experienced with other protecting groups, or whether the methyl group was

stable to hydrolysis in the carbamate form yet subject to hydrolysis in the ester form, compound (**82**) was synthesised.

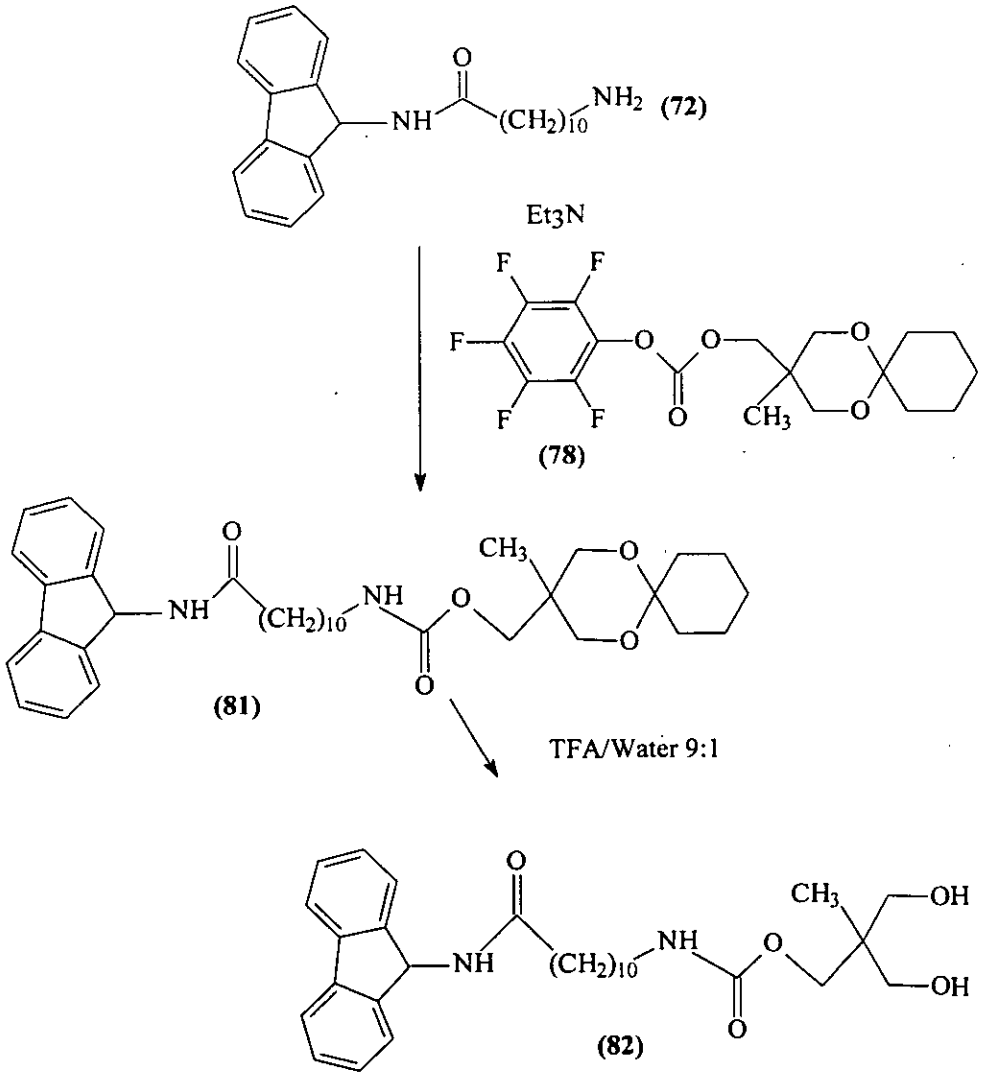


Figure 2.38 Synthesis of Compound 82

This compound is analogous to **66** and as such should reveal the required information on the effects of the methyl group on hydrolysis. The compound was tested with borate/DMSO pH 8.75 in the same manner as previously discussed, there was no hydrolysis, even when the pH was raised to 10 and the reaction epindorph tube allowed to sonicate for 24 hours. This was a very interesting result and indicated that for hydrolysis of carbamates the nitro group is important. In order to confirm this the

hydrolyses of the acetals **73** and **81** were investigated. Figure 2.39 shows example HPLC profiles of both **73** and **81** these indicate that there is very little difference between the rate of hydrolysis of the methyl and nitro acetal analogues and it is only when the diol is formed that differences in the rate can be observed. It also adds to the evidence suggesting that in the case of the carbamate both the nitro and hydroxyl function are needed to catalyse hydrolysis.

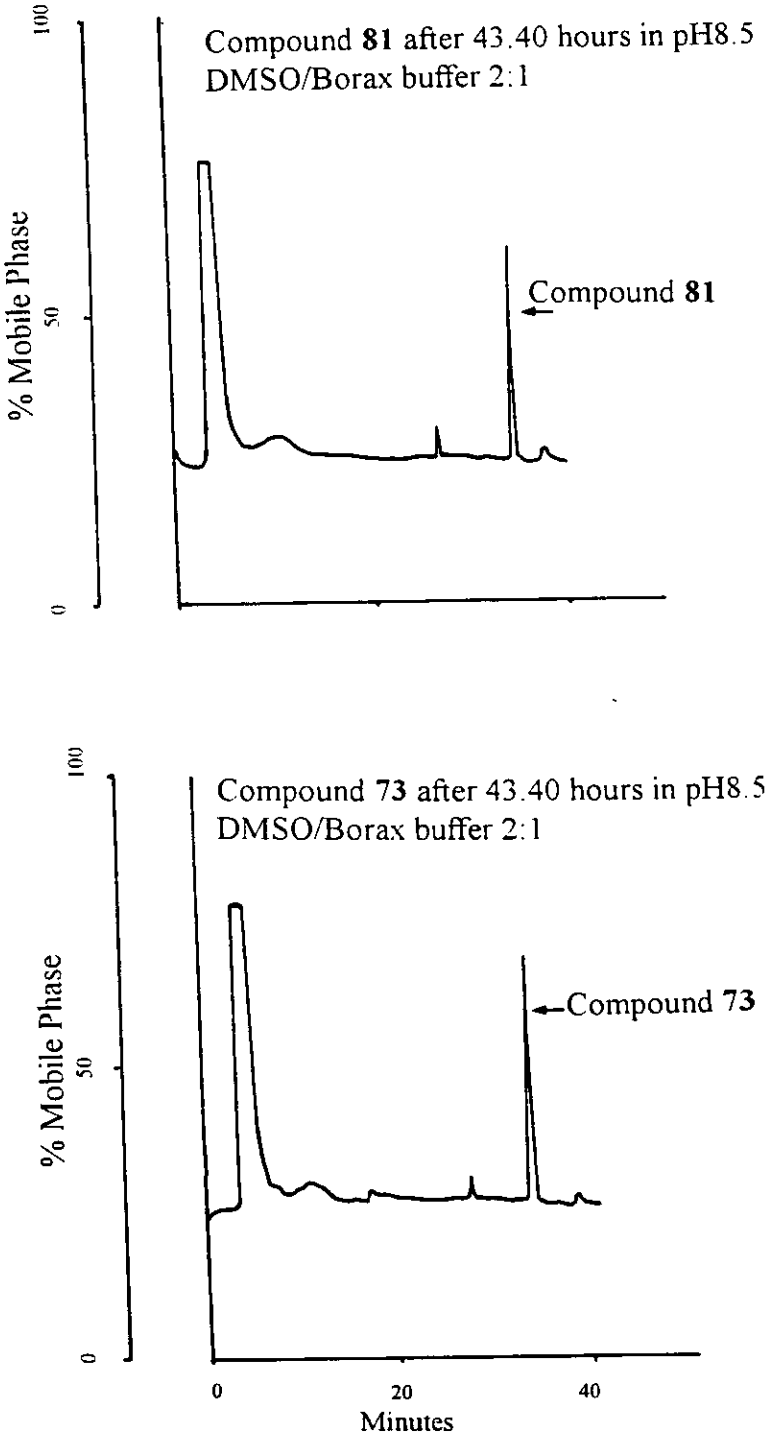


Figure 2.39

2.5.3 Use of the Tnm group in peptides

As the methyl derivative Tme was ineffective as a protecting group the Tnm group was reinvestigated. The lysine derivative **83** was synthesized in good yield by reaction of the pentafluorophenyl carbonate **69** with Fmoc-lysine.

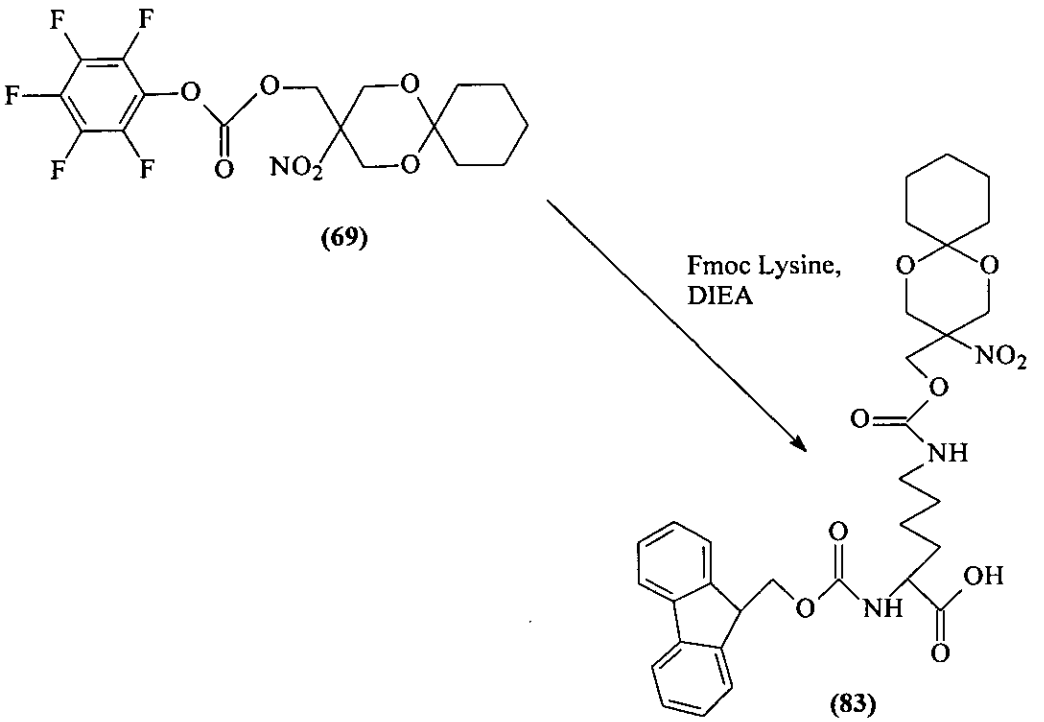


Figure 2.40 Synthesis of compound 83

The test peptide Lys-Phe-Gly-Lys(Tnm)-Ala-Gly-Gly **84** was then synthesized as outlined in the experimental section. The peptide was subsequently acetylated with phenylacetic acid to give it an extended retention time on HPLC. This was particularly useful as a number of reactions would be performed in DMSO. As DMSO has a large solvent front it was helpful to have as large a retention time as possible so that the solvent front did not mask the compounds under study. After cleavage from the resin and purification, via semi-preparative HPLC, the peptide was tested for the lability of the lysine protecting group.

At pH 8.75 in DMSO/borax buffer (2:1) the protecting group was removed with sonication in less than 30 minutes. This was an excellent result and proved that the protecting group strategy could be used in a peptide situation.

The removal of the protecting group was subsequently examined in a number solvents.

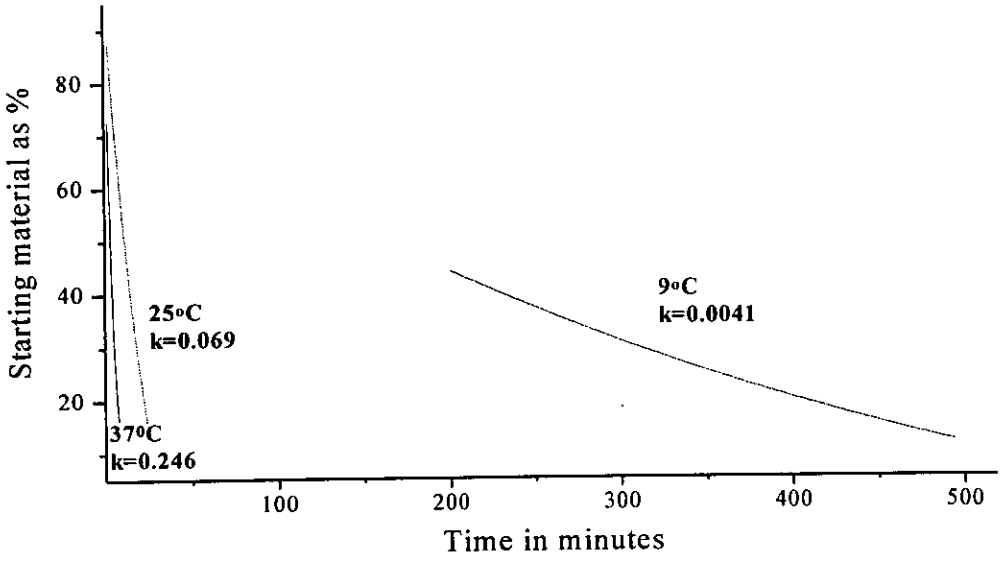
Solvent	pH	Buffer	Time
DMSO	8	Borate	40 mins
DMF	8	Borate	60 mins
ACETONITRILE	8	Borate	5 hours
METHANOL	8	Borate	>24 hours
DMF	8.7	Tris	>24 hours
DMF	8	Phosphate	>24 hours
DMF	10.5	Carbonate	<50 mins
DMF	8.75	Borate	<20 mins
DMSO	8.75	Borate	<20 mins
DMSO	8.7	Tris	8 hours

Table 2.4

2.5.4 Kinetics of hydrolysis of peptide 84

As with **51** and **66** a series of kinetic experiments were performed at three different temperatures, the results of which are shown in graph 2.4.

Graph of Hydrolysis of Peptide **84** at three different temperatures in Borate Buffer pH 8.5/ DMSO.



Graph 2.4

The protected peptide appears to cleave at a similar rate to the test molecule **66** at 37°C and 25°C but appears to be slower at 9°C. The reasons for this are uncertain although reduced solubility of the phenylacetylated peptide **84** at the lower temperature would seem to be the most likely cause. The comparability of the model compound and the peptide at the higher temperatures indicates there is no fundamental difference between the model and the real situation.

2.6 Side products

The problem of side products in the break down of the diol structure remains. While these can be kept to a minimum by using a high pH this is obviously not the desired answer. In an effort to find out what was taking place during cleavage, an experiment was designed using acetonitrile at pH 8 with borate buffer. These

conditions gave the highest proportion of side product in the shortest time. Once this unknown product had been produced it was isolated via semi-preparative HPLC. Having isolated the side product, attempts were made to break it down to give the free amine. The most promising results were achieved by stirring in Tris at pH 8.5. Figure 2.41 shows that the side product can be converted quite quickly to the desired amine, at least to a reasonable degree. Figure 2.42 which shows the side product after being stored for a week in a loosely stoppered bottle, indicates that the compound is only semi-stable and will in fact break down of its own accord with time. It is possible that the lack of cleanliness of the reaction may create problems when more than one lysine is protected, but this should not prove to be an insurmountable problem since the intermediate is intrinsically unstable.

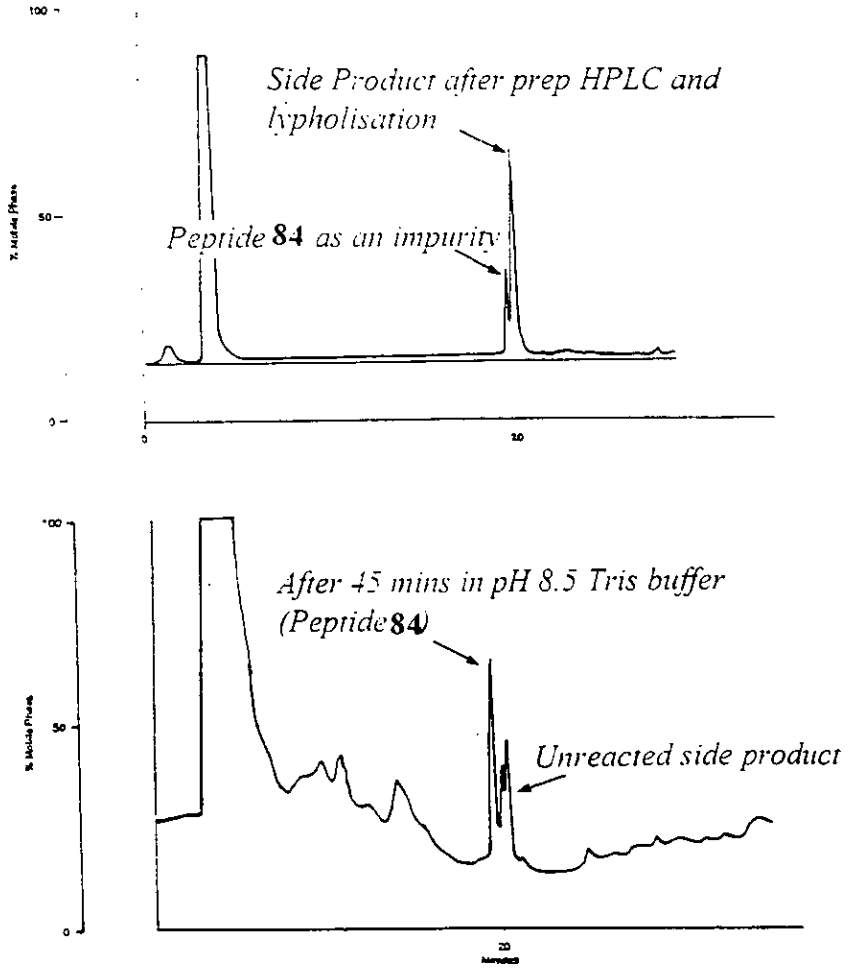


Figure 2.41

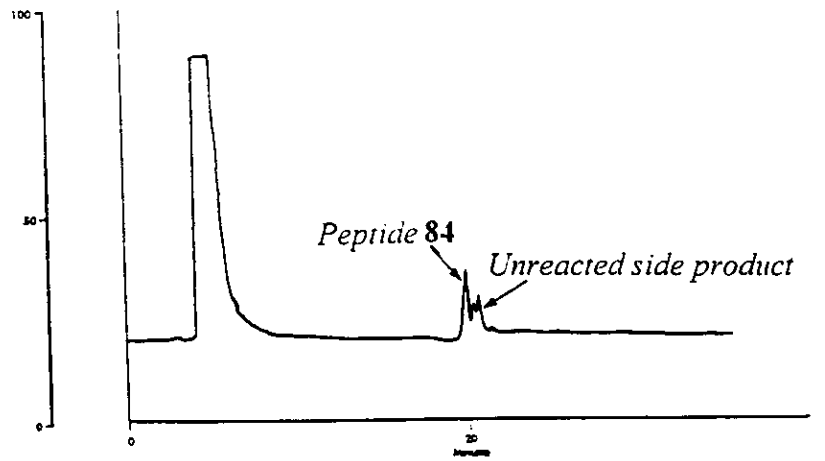


Figure 2.42

2.7 Hydrolysis hypothesis.

The experiments undertaken so far have elucidated a number of structural factors vital for the enhanced rate of base hydrolysis of the protecting groups studied. Table 2.5 gives a summary of this information.

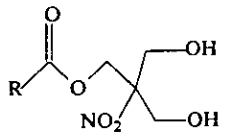
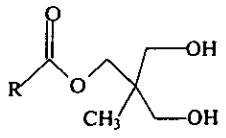
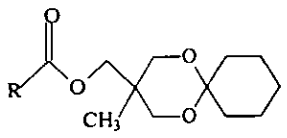
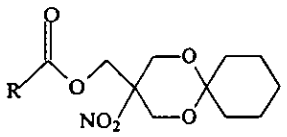
Protecting Group	R Group Ester	R Group Carbamate
	Fast hydrolysis	Fast hydrolysis
	Fast hydrolysis	No hydrolysis
	Very slow hydrolysis	No hydrolysis
	Very slow hydrolysis	No hydrolysis

Table 2.5

The enhanced rate of hydrolysis is only apparent when the OH and NO_2 groups are present in the carbamate situation. Although for esters the presence of the NO_2 group does not seem to be a necessity as cleavage can be achieved at an enhanced rate when the CH_3 group is present. It seems likely, that for esters **51** and **64**, the enhanced rate of hydrolysis can be accounted for by the Henbest-Kupan effect. The overall mechanism for hydrolysis being as shown in figure 2.43.

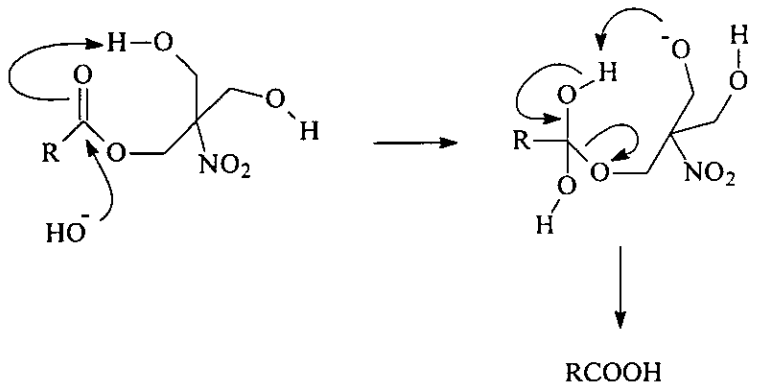
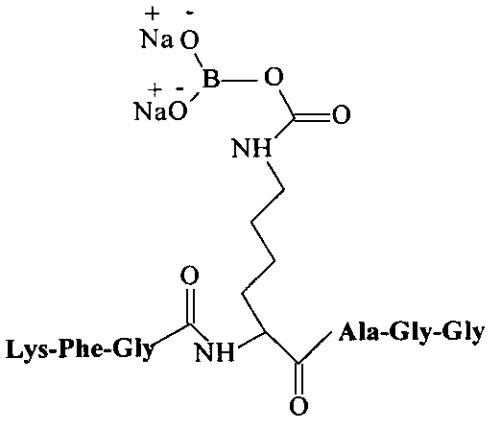


Figure 2.43

Hydrolysis of the carbamates **66** and **82** however, can not be accounted for solely by the Henbest-Kupan effect, since this does not take into account the difference in the CH_3 and NO_2 groups. In addition, it should be noted that whereas the ester compounds give a clean hydrolysis in all the basic buffers tested, the carbamate compounds produce side products in a number of solvent/buffer mixtures. These side products are likely to be formed by the reaction of intermediates, formed during the breakdown of the carbamate, and the buffer.

Any hypothesis suggested for the mechanism of cleavage of the Tnm protecting group must be able to account for all these pieces of information. In an effort to elucidate what was taking place during cleavage of the carbamate **84,** the side product isolated from the hydrolysis of **84,** (section 2.6) was sent for mass spectral analysis. The result of which gave a mass of 900.37798 as opposed to 767.40426 for the completely deprotected compound. It is possible to draw a number of structures which will allow for the extra 133 mass units. The structure **(86)** shown in figure 2.44 is particularly appealing as its accurate mass is so similar to that found.



(86) Mass 900.37777

Figure 2.44

As aforementioned, such side products are not formed in the deprotection of esters (section 2.2) nor are they formed when Tris is used as the buffer (section 2.5). We can infer, therefore, that the side product is not formed via a reaction of a carbamic acid break-down product, since the acid functions are essentially the same in both the product amino acids and any carbamic acid which could be formed.(Figure 2.45)

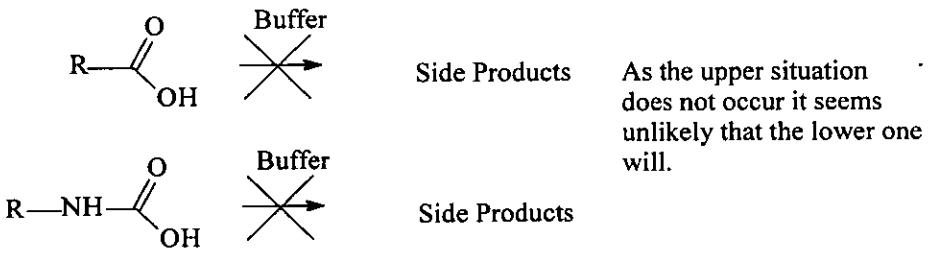


Figure 2.45

Additionally, since the stable intermediate is not formed when Tris is used as the buffer, it is likely that it is the product of a reaction of the borate and some reactive intermediate. There are several possible mechanisms which could account for this.

2.7.1 Hypothesis (A)

One explanation is that the hydrolysis proceeds via an elimination mechanism not dissimilar to that of aromatic carbamates with strong leaving groups[21,22,23,24,25]. See figures 2.46, 2.47

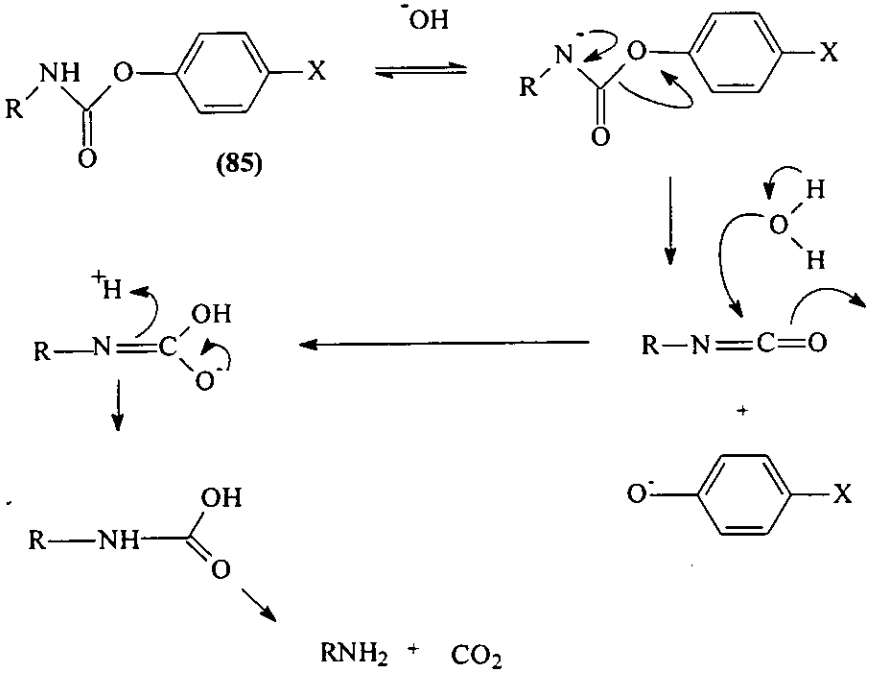


Figure 2.46 $E1_cB$ mechanism for hydrolysis of carbamates

If we apply this hypothesis to current situation it can be seen that the isocyanate initially formed could well act as a competitive substrate for the borate and OH^- present (see figure 2.47).

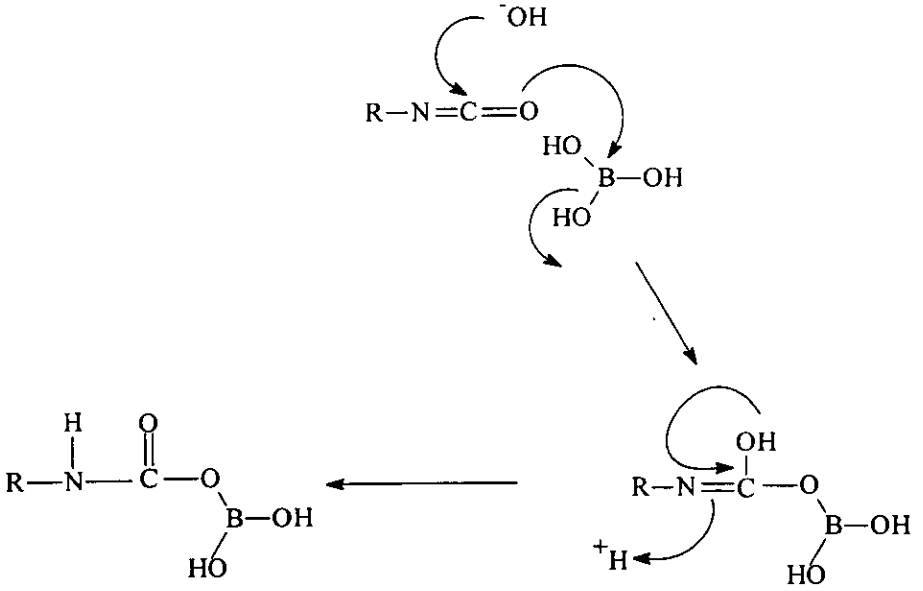


Figure 2.47

If this occurred with peptide **84** it could account for a mass spectrum fragment of mass 900 **(86)**.

This hypothesis is however flawed since the isocyanate formed would likely react with any amine present or with Tris buffer to form a urea for which there is no evidence. Also the hypothesis does not explain the necessity for both OH and NO_2 groups, since if the mechanism depended solely on the electron withdrawing effect of the NO_2 group the acetal compound **73** should be hydrolysed at an increased rate and it is not.

2.7.2 Hypothesis (B)

In order to reconcile the Henbest-Kupan theory, which almost certainly operates in the case of the ester derivatives, with the situation present in the nitro carbamates, the following mechanistic path was devised. (See figure 2.48)

In this theory the nitro group attacks the hydroxy imine, formed after initial proton abstraction, to yield a six member ring. This ring breaks up to yield a highly efficient leaving group. This group may leave by one of two different mechanistic routes.

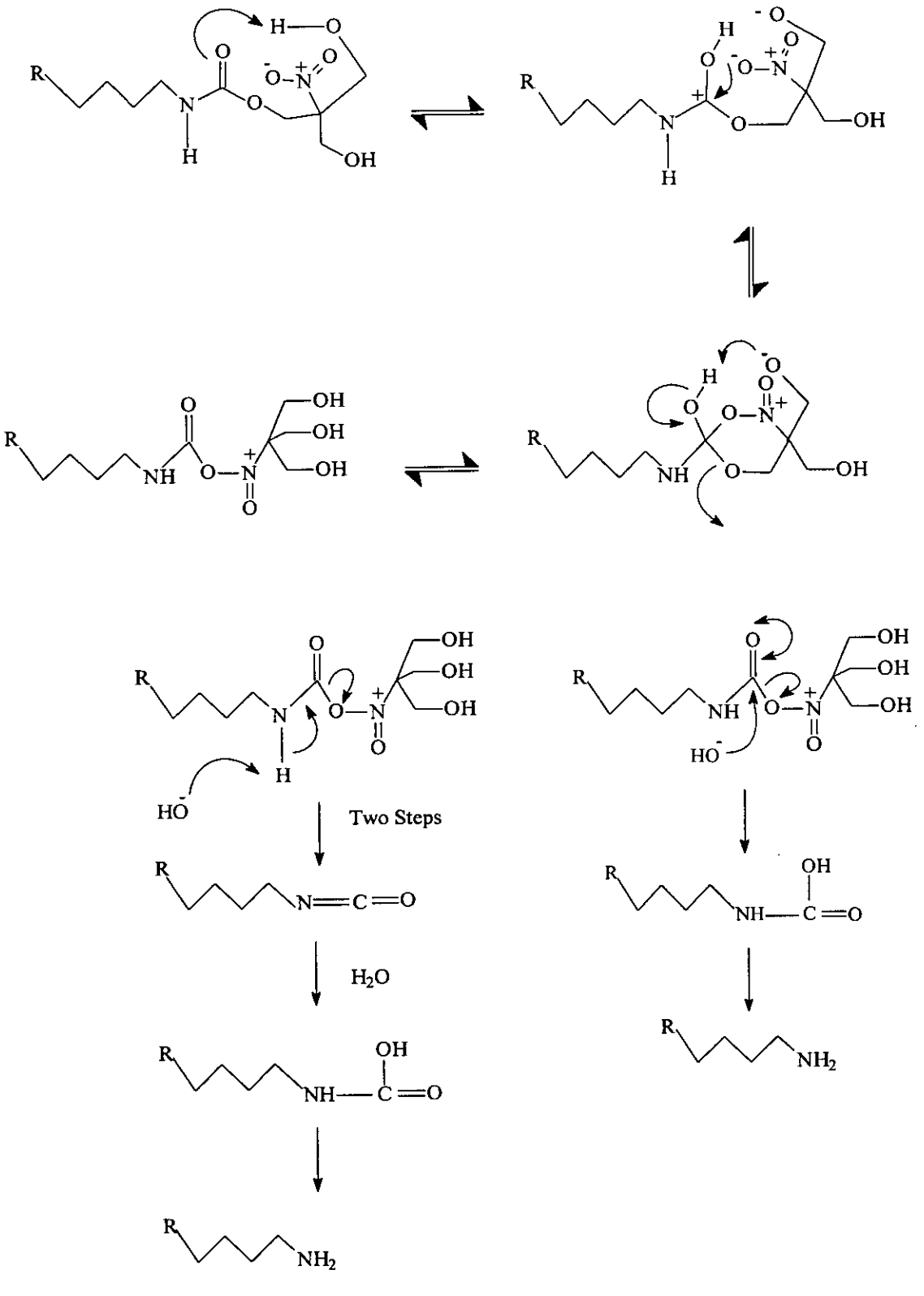
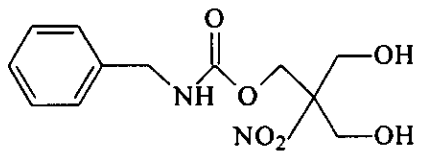


Figure 2.48

The route to the left involves an E1cB elimination mechanism whereas that to the right involves the $B_{AC}2$ hydrolysis. As aforementioned, the lack of urea formation when Tris buffer is used tends to suggest that the mechanism does not involve an isocyanate intermediate. This coupled with the fact that borate could easily replace the OH in the right hand route of figure 2.48 to give the semi-stable intermediates **86**, which have been found to be formed during hydrolysis of the carbamates, suggests that the right hand route may be the more likely. Hypothesis B also offers an explanation of why both OH and NO_2 groups are needed for hydrolysis of the carbamate to take place.

The hypotheses A and B are attempts to logically explain a set of observed phenomenon, hypothesis B perhaps offers the most convenient answer but it is by no means certain that the mechanism follows this route, indeed it would be foolish to state that one mechanism is correct since there is insufficient evidence to settle the matter. NMR experiments on the test compound **89** were undertaken in an attempt to shed further light on the mechanism of hydrolysis. These however proved inconclusive primarily due to a large water peak from the buffer obscuring the area in the spectrum of most interest, making it difficult to follow the reaction.



(89)

Figure 2.49

2.8 Thermodynamic considerations

In an effort to shed further light on the mechanism of reaction, the kinetic data from the hydrolysis of **51** and **66** was used to calculate the value of a number of thermodynamic variables. Before this is discussed however, it must be pointed out that as only three temperatures were used the results from any such calculation must be fraught with error. Several problems occurred in the measurement of data,

notably the difficulty in maintaining constant temperature under the conditions used i.e. in a sonic bath. This made it impractical to take readings at anything less than ten degree intervals. The physical properties of DMSO, its viscosity and freezing point, also made readings difficult, as did the speed of reaction. As previously mentioned however, the objective of the exercise was to look at rates under optimised experimental conditions. Taking these points into consideration, it is still possible to arrive at approximate estimations of the entropy and enthalpy of activation for hydrolysis.

The Arrhenius equation $k=A.e^{-Ea/RT}$ shows the relationship between activation energy, rate and temperature. It is possible however to derive an expression which relates temperature and rate to the entropy and enthalpy of the activated state of reaction this expression is termed the Eyring equation[26].

$k=k_BT/h.e^{\Delta S^*/R}.e^{-\Delta H^*/RT}$.

k=Rate constant, k_B=Boltzmann constant, h=Planks constant, R=universal gas constant, T=Temperature.

This expression can be expressed logarithmically as.

$\ln(k\,h/k_B\,T)=\Delta S^*/R - \Delta H^*/RT$

This is a linear equation of the form of Y=A+BX.

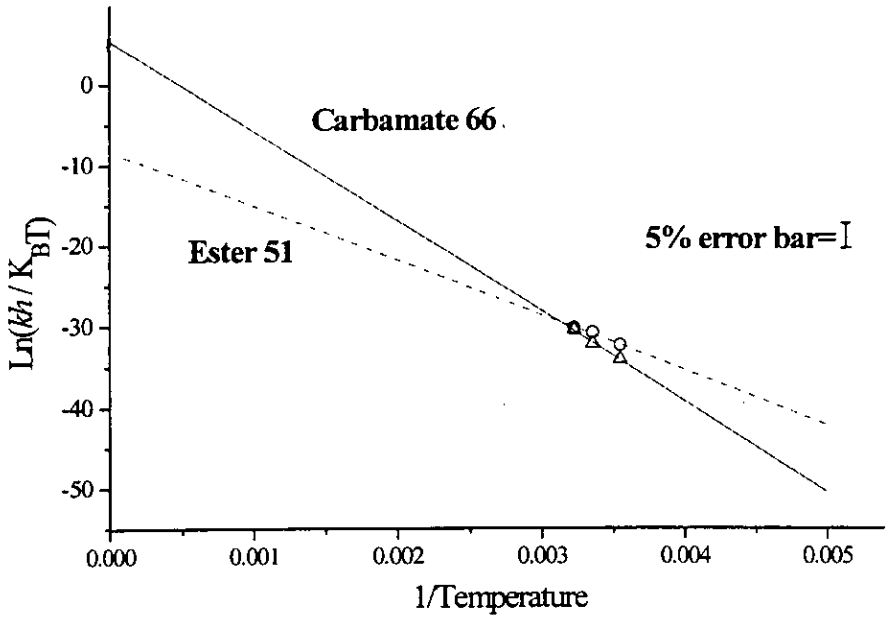
where $Y= \ln(k\,h/k_B\,T)$

$A= \Delta S^*/R$

$B=-\Delta H^*/R$

$X=1/T$

Hence a plot of X v's Y should give a gradient equal to - $\Delta H^*/R$ and an intercept of $\Delta S^*/R$ graph 2.5 shows the Eyring plot for **51** and **66** respectively. Giving a ΔS^* value of +44.4 J K^{-1} for the carbamate and -68.89 J K^{-1} for the ester.



Graph 2.5

Although as previously mentioned it is dubious to quote results derived from such a limited pool of information, as the error bar included on the graph indicates. It is however interesting to note that the entropy of activation ΔS^* value for the carbamate is positive while that for the ester is negative. Work by Christenson[25] on the hydrolysis of carbamates suggests that hydrolysis by the $E1_CB$ mechanism has a positive entropy value. Whereas the hydrolysis of most carbamates and esters via the $B_{AC}2$ mechanism involves a negative entropy, as is shown in table 2.6 and found by Bruice and Fife[9] when investigating the Henbest-Kupan effect[8,9,11]. This perhaps adds further weight to the suggestion that there are different mechanisms of hydrolysis operating in the ester and carbamate situations.

Thermodynamic data for the alkaline hydrolysis of selected esters and carbamates is shown in table 2.6.

Formula	$\Delta H^{\ddagger}$ (Kcal/ mol)	$\Delta S^{\ddagger}$ (E.U)
$C_6H_5NHCOOC_2H_5$	15.9	-25.5
$C_6H_5NHCOOC_6H_4N(CH_3)_3$	16.6	+5.0
$C_6H_5NHCOOC_6H_5$	18.3	+11.4
$CH_3COOCH(CH_3)$	10.5	-30.6
CH_3COOCH_2Cl	10.6	-20
$CH_3COOCHCH_2$	12.8	-12.7
$CH_3COOC_2H_5$	10.8	-26.8

Table 2.6 from Patai[27] & Christenson[25]

2.9 Mechanistic comments

2.9.1 Esters

It seem likely that in both the Tnm and Tme derivatives the rate enhancement is due to the Henbest-Kupan effect. The Tme derivative having a faster rate of hydrolysis than the Tnm due to partial ionisation of the Tnm or some other process whereby interaction of OH and NO_2 groups hinders the anchimeric assistance. The situation as regards borate has already been discussed, and it may well have a catalytic effect. The hydrolysis of the diol compounds does however proceed at an accelerated rate even without its presence so any effect is likely to be an enhancement of effects already present rather than a special effect

2.9.2 Carbamates

As has been illustrated it is possible to write a number mechanisms which will account for the hydrolysis of the Tnm derivative at enhanced rate. The thermodynamic data while interesting, is dubious in nature, so it is difficult to propose an $E1_CB$ over a $B_{AC}2$ hydrolysis mechanism on this evidence alone. The lack of urea formation suggests that an isocyanate is not formed during cleavage which would suggest a $B_{AC}2$ mechanism. It is impossible with the evidence available to be certain one way or the other, what is certain however is that the NO_2 and OH groups interact in some way to enhance the rate of hydrolysis.

2.10 Protection of a large peptide Ub 36-76 (90)

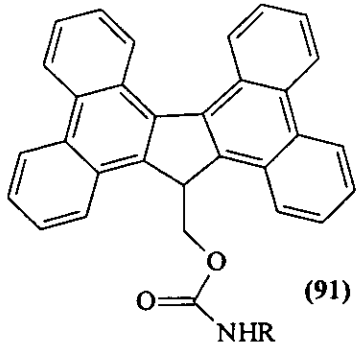
As a crucial test, lysine (63) of the Ubiquitin fragment 36-76 **(90)** was protected with Tnm. This would prove to be a particularly useful target since the fragment had already been synthesised with the penicillin acylase cleavable 4-hydroxyphenylacetyl protecting group[28]. Removal of this protecting group by enzymatic means had not been achieved probably because tertiary structural interactions cause the lysine residue to be sterically hindered and therefore unable to fit into the active site of the enzyme. Attempts in the group were made to denature the target peptide and hence better present the lysine to the enzyme. This was however largely unsuccessful and presents a flawed strategy since conditions which denature the target peptide are also likely to denature the enzyme.

If the Tnm protecting group could succeed on a relatively large peptide, where enzyme-cleavable groups had failed, its utility would be proved and its use in the fragment coupling approach could be considered.

The peptide was synthesised as outlined in the experimental section without any significant problems and with no loss of coupling on introduction of the Tnm protected lysine.

2.10.1 Purification

It was decided to purify the peptide *via* the Tbfmoc[29] **91** affinity purification group since this was likely to be the N^{α} protecting group used in any subsequent fragment couplings.



(91)

Figure 2.50

The Tbfmoc group was utilised in both of the relevant purification protocols developed in this laboratory. Firstly as a hydrophobic group to extend the retention time of the peptide on HPLC and thus make the separation of truncations much easier via semi-preparative HPLC. And secondly by affinity binding to porous graphitised (PGC). This is a novel protocol whereby the impure Tbfmoc-peptide is vortexed in solution with some PGC, the carbon adsorbs the large flat Tbfmoc group causing the labelled peptide to become stuck to it. The truncations formed during synthesis lack Tbfmoc and can easily be removed by washing. Once the truncations have been removed the peptide is liberated from the carbon by cleavage with piperidine/DMF.

In accordance with the first of these methods the peptide N-terminus was protected with Tbfmoc and subsequently purified by semi-preparative HPLC. The Tnm and Tbfmoc protecting groups removed by borate buffer pH 8.75 in DMSO (1:2) in under 30 minutes. The DMSO was subsequently removed *in vacuo*, the peptide gel filtered and freeze dried to give the desired product.

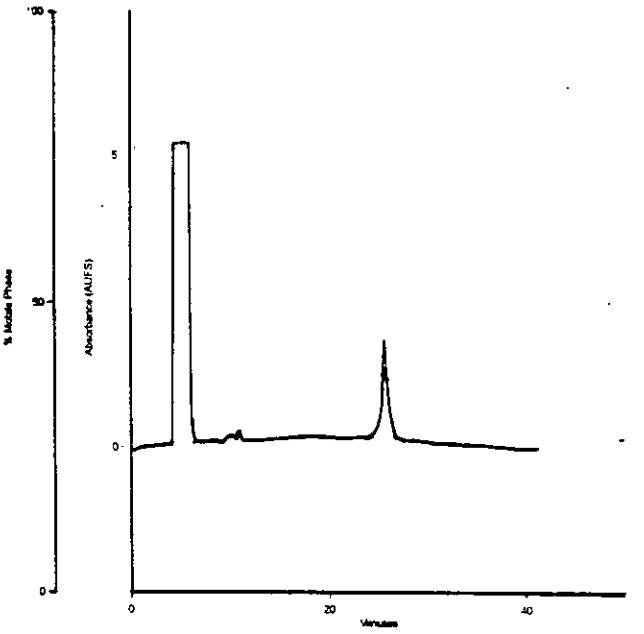


Figure 2.51 TbFmoc -Peptide

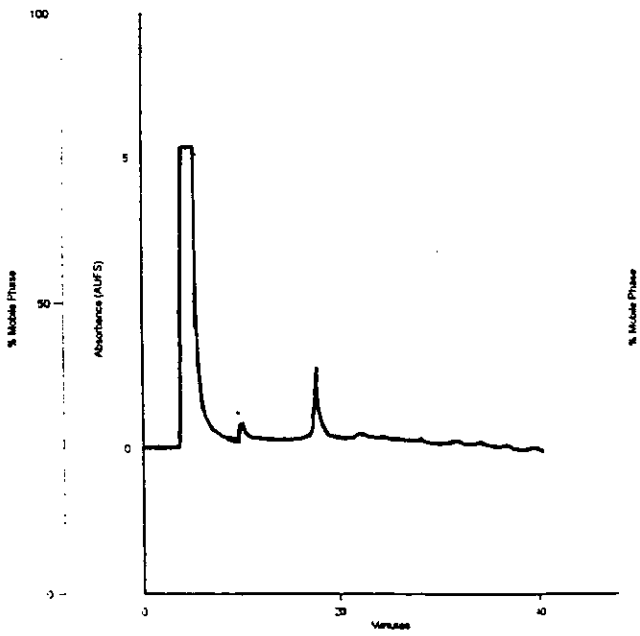


Figure 2.52 Removal of Tnm & TbFmoc peptidprotecting groups by sonicating for 30 mins in pH 8.75 borate / DMSO

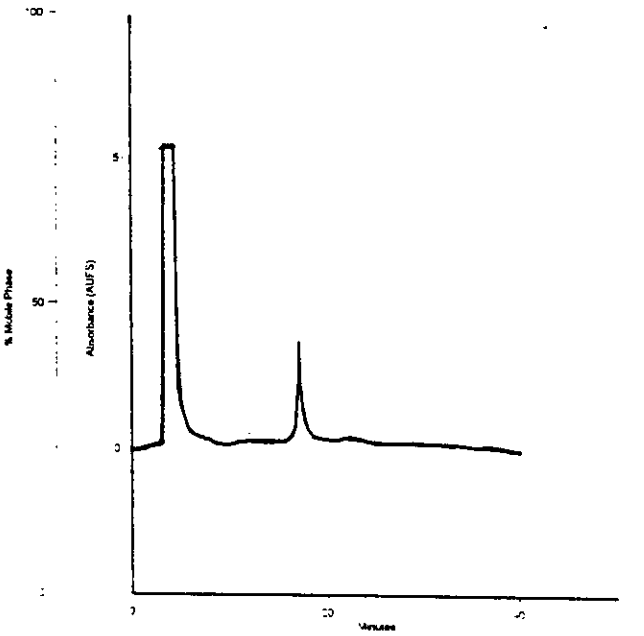


*Figure 2.53 Dual injection of peptide **90** after deprotection & a known sample of Ub36-76*

The second purification strategy worked equally well and is in some ways superior the Tbfmoc and Tnm protecting groups being removed by vortexing the carbon with 10% piperidine in 1:1 6M Guanidine.HCl/propan-2-ol. This solution is then neutralised, concentrated *in vacuo* and gel filtered to give pure Ub 36-76. It is worth noting however that although the vortexing only takes approximately 5mins the pH at this time may be as high as 13 or 14.

It can be seen then, that the Tnm protecting group satisfies the criteria earlier set out for a post cleavage protecting group (section 1.5) and should be compatible with the hydrazide fragment coupling strategy. Recent work[31] in this laboratory indicates that the this is indeed so. The successful coupling of two small peptides using the azide method (section 2.1.2) one of which contained a lysine protected with Tnm has been achieved.

2.11 References

1) Kevin Shaw, *personal communication.*

2) Portu, *Gazz. Chem. Ital.* 1988, **118**, 475-477.

3) S. Wang, *Tetrahedron Lett*, 1993, **34**, 7217-7220.

4) Nicola Robertson & Linda Draffon, *personal comunication.*

5) J. Honzl & J Rudinger, *J. Collect. Czech Chem. Commun.* 1961, **26**, 2333-2344.

6) Nicola Robertson, *personal communication.*

7) C.K. Ingold, In: *Structure and Mechanism in Organic Chemistry,* 2nd Edn,1969, Bell, London, pg, 104,141,175,265.

8) S.M. Kupan, P. Slade and R.J. Young, *Tetrahedron Lett.* 1960, **24**, 22

9) T.C. Bruice & T.H. Fife, *J. Am. Chem. Soc.* 1962, **84**, 1973.

10) P.D. Bartlett & F. D. Greene, *J. Am. Chem. Soc.* 1954, **76**, 1088.

11) H.B. Henbest & B.J. Lovell, *J. Chem.Soc.* 1957, 1965.

12) B. Capon & M.Page, *J. Chem. Soc. (B).* 1971, 741-744.

13) M. Balakrishnan, G. Venkoba Rao, N. Venkatasabramanian. *J. Sci. Ind. Res.* 1974,

14) B. Capon, Bidham Ch. Gosh, *J. Chem. Soc.* (B). 1966, 472-478.

15) D. W. Tanner & T.C. Bruice., *J. Am. Chem. Soc.* 1967, **89**, 6954.

16) A.J. Parker, *Chem. Rev.* 1969, **69**, 1-31.

17) E. Buncel & H. Wilson, *Adv. Phy. Org. Chem*, 1977, **14**, 203-352.

18) R. Fuchs, C. P. Hagan, R. F. Rodewald, *J. Physical. Chem.* 1974, **78**, 1509-1511

19)M. Balakrishnan, N. Venkatasubramanian, G. Venkoba Rao, *Int. J. Chem.kinet.* 1974, **6**, 103-110.

20) N. Venkatasubramanian, G. Venkoba Rao, *Tetrahedron Lett.* 1967, **52**, 5275-5280.

21) J. March, in *Advanced Organic Chemistry*, 382, Wiley-Interscience 1992.

22) A. Williams & K.T. Douglas, *Chem. Rev.* 1975, **75**, 627-649.

23) M.L. Bender & R. B. Homer, *J. Org. Chem.* 1965, **30**, 3975

24) A. Williams, *J. Chem. Soc., Perkin Trans. 2.* 1972, 808, & 1973, 1244.

25)I Christenson, *Acta Chem. Scand.*, 1964, **18**, 904.

26) S. Gladstone, K.J. Laidlier, H. Eyring, In: *The Theory of Rate Processes.* McGraw Hill 1941.

27) E. Euranto, In: *The Chemistry of Carboxylic Acids & Esters*, ed S. Patai, Chap. XI (Interscience Publishers Inc, New York) 1969.

28) Linda Draffon, *personal communication.*

29) R. Ramage & G. Raphy, *Tetrahedron Lett.*, 1992, **33**, 385

30) Nicoal Robertson, *personal communication.*

CHAPTER 3 : SYNTHETIC PEPTIDES AS VACCINES AGAINST HIV INFECTION

3.1 Immunology: A brief survey

The immune systems which operate in living organisms are some of the most complicated and least understood in all biology. Having said this they can broadly speaking be separated into two distinct branches, the humoral response and the cell-mediated response.

3.1.1 Humoral response

The humoral response is centered around the production of neutralising antibodies. In this process an antigen is first recognised as foreign by a macrophage which engulfs it and packages its coat proteins so that they are expressed on the surface of the macrophage. These packaged antigens are then recognised by both B-cell and T_H-cell receptors. The B-cells are stimulated by the T_H-cells to enlarge and divide forming a population of antibody secreting plasma cells and a smaller number of memory cells. These secreted antibodies then recognise and neutralise the invading antigens directly. Memory cells are long lived cells which lie dormant, on a second exposure to the antigen however they are quickly transformed into plasma cells and begin secreting antibodies.

3.1.2 Cell mediated response

The cell mediated response refers to actions of the immune system whereby cells of the immune system are actively involved but antibody production is of minor importance. The cell mediated response involves two major classes of lymphocytes. Cytotoxic T_C-cells recognise antigens on the surface of infected cells and lyse those

cells. Natural killer cells on the other hand, are able to kill foreign cells without the necessity of a packaged antigen for recognition.

T-lymphocytes, macrophages, other leukocytes, and natural killer cells can also act to eliminate invading micro organisms and other foreign material by releasing lymphokines. These proteins act in stimulating and regulating the immune responses, they also act to induce inflammation and as toxic proteins for tumor cells. Figure 3.1 shows how the various facets of the immune system are linked.

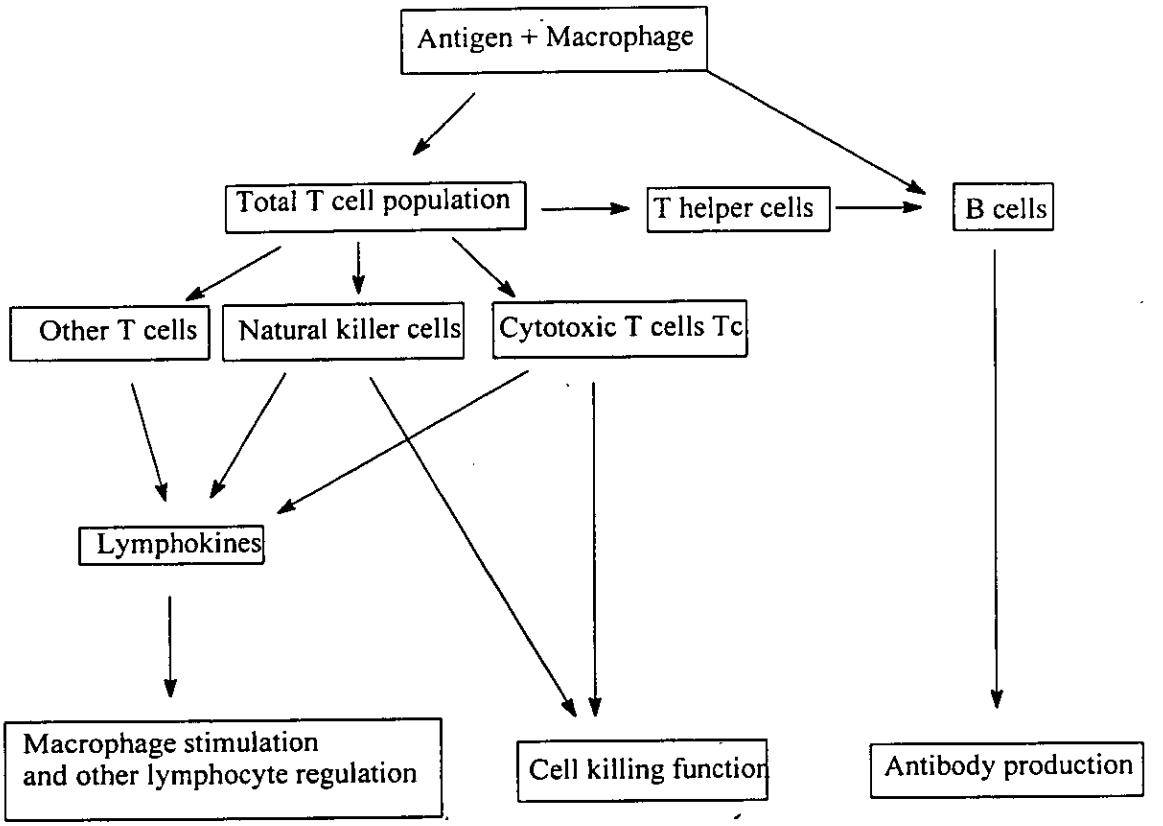


Figure 3.1

3.2 Vaccines

Antigens within the body are in the main recognised by their surface characteristics. As the surfaces of many infectious agents are predominantly proteinous in nature, it is these surface proteins, which, by inducing recognition by B and T cells elicit the

body's defences. Since it is often only particular areas of these proteins that induce this effect it is often possible to synthesise relatively short peptide sequences which posses the important moiety of the larger protein molecule. It is this principle that underlies the use of synthetic peptides as vaccines.

To date, most vaccines are whole inactive or live attenuated vaccines, the whole infectious organism being used as the vaccine particle. The use of a live virus guarantees a natural presentation of immunogens and has a historical background stretching back over almost two hundred years. Edward Jenner first used the live cowpox virus as a vaccine against the far more dangerous, yet related, smallpox in 1798. There is however not always a conveniently related live virus and the use of attenuated viruses has inherent risks. In any rapidly mutating virus and in particular with retro viruses such as HIV, the risk that an attenuated virus acting as a vaccine could revert to a viable form is a constant worry. Retroviral replication involves chromosomal integration in the vaccine recipient and may establish life long persistence. Transmission to others cannot be ruled out and transient episodes of immunosuppression have to be anticipated. The use therefore of a subunit or synthetic vaccine is particularly attractive for combating HIV.

As synthetic molecules, peptide vaccines have a low inherent risk, it is also possible to exclude immunosuppresive and infection enhancing epitopes which may be present on the native virus. One of the difficulties however may be the inclusion of adequate B and T-cell epitopes. This however may be overcome by immunisation with a cocktail of peptides each representing a number of epitopes. So far peptides have only been of limited use for immunisation[1,2]. However they represent a relatively new field and it is likely that with time will become a more useful form of vaccination.

3.3 Human Immuno deficiency virus (HIV)

3.3.1 introduction

Human Immune Deficiency Virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS)[3,4]. AIDS is a disease of the immune system characterised by depletion of CD4+ve T-lymphocytes in the body[5].

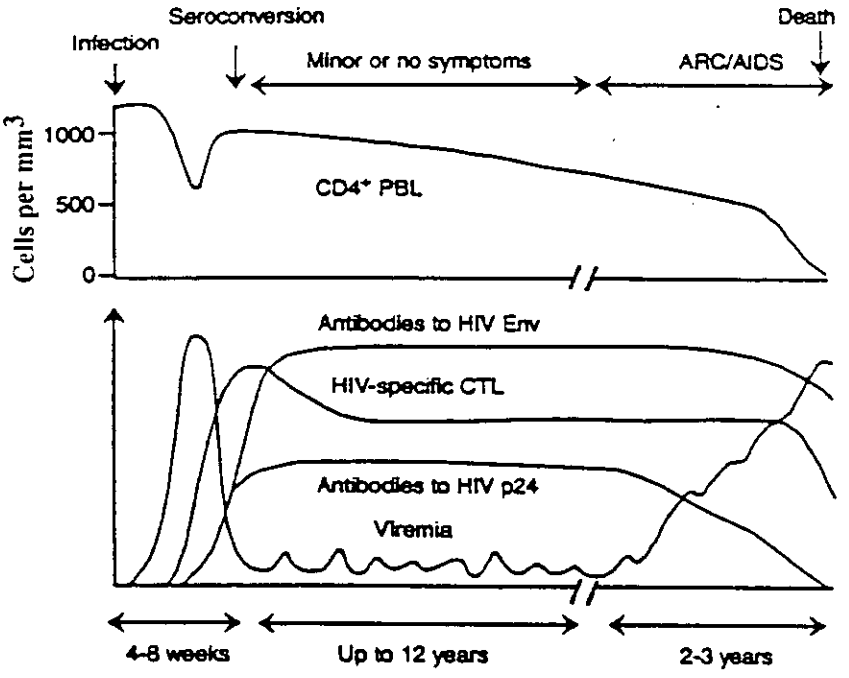


Figure 3.2

Although the main point of entry for infection is through CD4+ve lymphocytes (T_H helper cells)[6] the virus also affects other CD4 bearing cells such as macrophages[7]. There is also evidence to suggest that the virus can enter cells which do not possess the CD4 receptor,[8] although this is generally thought to be of secondary importance. Current theories[9] on the progress from HIV positive status to the onset of full blown

AIDS suggest that there is a rapid turn over of virus and infected cells.[10,11,12] the immune system eventually being overcome resulting in death from opportunistic infections. This is in contrast to the previously held view which suggested that HIV infection was a relatively indolent process during the extended period of clinical latency[13].

There are two main subtypes of HIV, termed HIV-1 and HIV-2, these two types are prevalent in two different geographical areas HIV-1 being predominantly associated with the USA and Western Europe[13], whereas HIV-2 is mainly a disease of the African continent[13]. These two strains have approximately 40% homology[14] between their envelope coat proteins. There are also animal versions of HIV such as the simian version (SIV), and feline version (FIV), both have proved useful models for researchers[15,16].

3.3.2 Structure

HIV (figure 3.3) is a retrovirus, its genetic make up consisting of viral RNA which codes for two structural genes gag (internal) and env (envelope) glyco-proteins, and the regulatory genes pol, vif, vpr, rev, tat, and ref. The outer layer of the virus consists of envelope comprising a host derived lipid bilayer, interspersed with a glycoprotein. This protein, is translated as a 88Kd precursor that is subsequently modified by glucosidase 1 to give a 160Kd glucosylated molecule. This, so called Gp160, is cleaved by a cellular protease during virus maturation to the external envelope protein Gp120, and the transmembrane protein Gp41. Gp41 anchors Gp120 to the virus through non covalent interactions. The Gp120 molecule, is the recognition site to which CD4 receptors on T_H lymphocytes[17] bind during viral entry into the host cell.

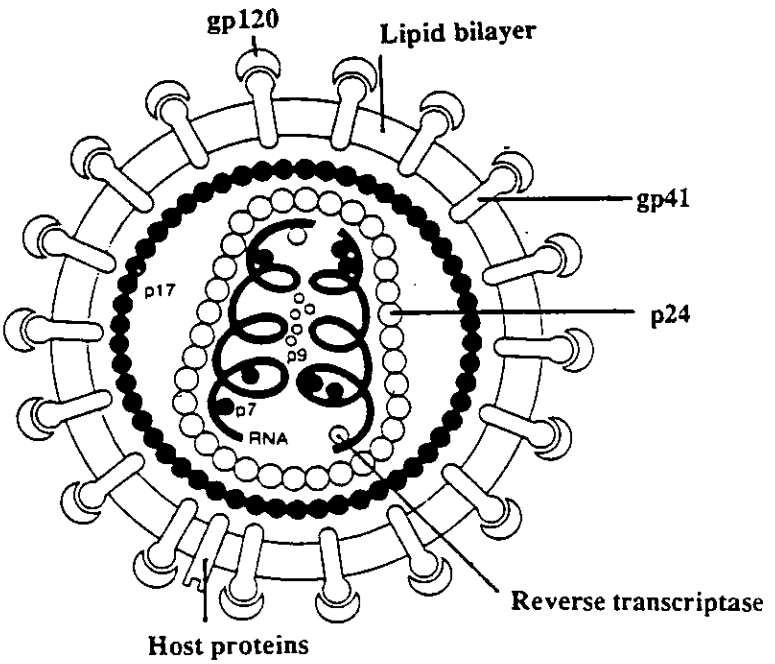
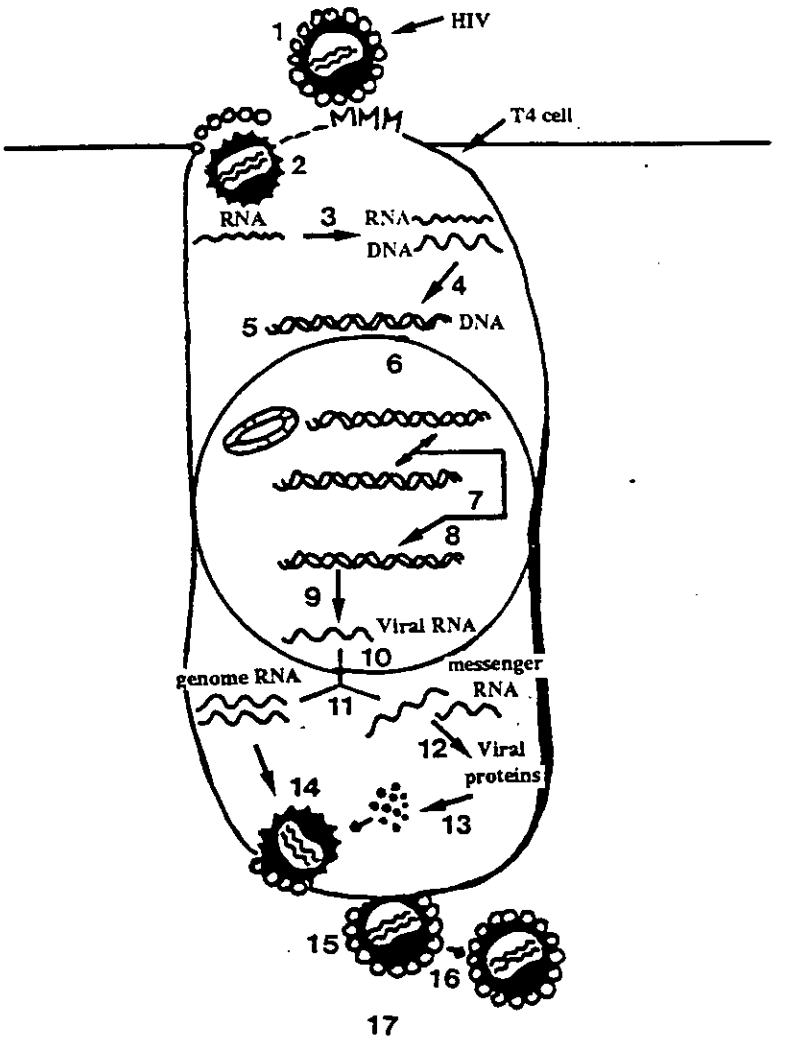


Figure 3.3 HIV Virus

3.3.3 Viral replication

The virus becomes attached to T_H cells via the affinity of CD4 to Gp120.[18] The method by which the viral and host membranes then fuse to allow viral RNA into the host cell is not fully understood[19-25]. Once the viral RNA has entered the host cell however, it is quickly transcribed into DNA by reverse transcriptase and incorporated into the hosts genome. This DNA is then read by the normal transcribing apparatus of the host cell and produces viral proteins and RNA. These proteins and RNA, are then assembled and bud off from the surface of the host, acquiring host lipid bilayer as they do so. (Figure 3.4)



1) Attachment, 2) Uncoating, 3) Reverse transcription, 4) RNASEH Degredation, 5) DNA synthesis of second strand, 6) Migration to nucleus, 7) Integration, 8) Latency, 9) Viral transcription, 10) RNA nuclear transport, 11) Protein synthesis, 12) RNA stability, 13) Protein glycosylation, 14) RNA packaging and virion assembly, 15) Release of the virus, 16) Maturation, 17) Other. From ref 7

Figure 3.4

3.3.4 Gp 120

In order to develop a successful vaccine it seems likely that the interaction between GP120 and CD4 must be blocked, as this interaction is fundamental to disease development[25]. Ideally a successful vaccine would elicit an antibody response which

would block the binding of Gp120 to CD4. In addition, a vaccine should also affect a cytotoxic T-cell response which will neutralize viral particles.

Gp120 is a 120Kd glycosylated protein (figure 3.5), it consists of a number of distinct regions which have been studied and mapped extensively[26-28]. The regions of Gp120 can be divided into two distinct sub sets, the conserved regions, and the variable regions. The variable regions V1,V2,V3,V4,V5, are regions of the protein

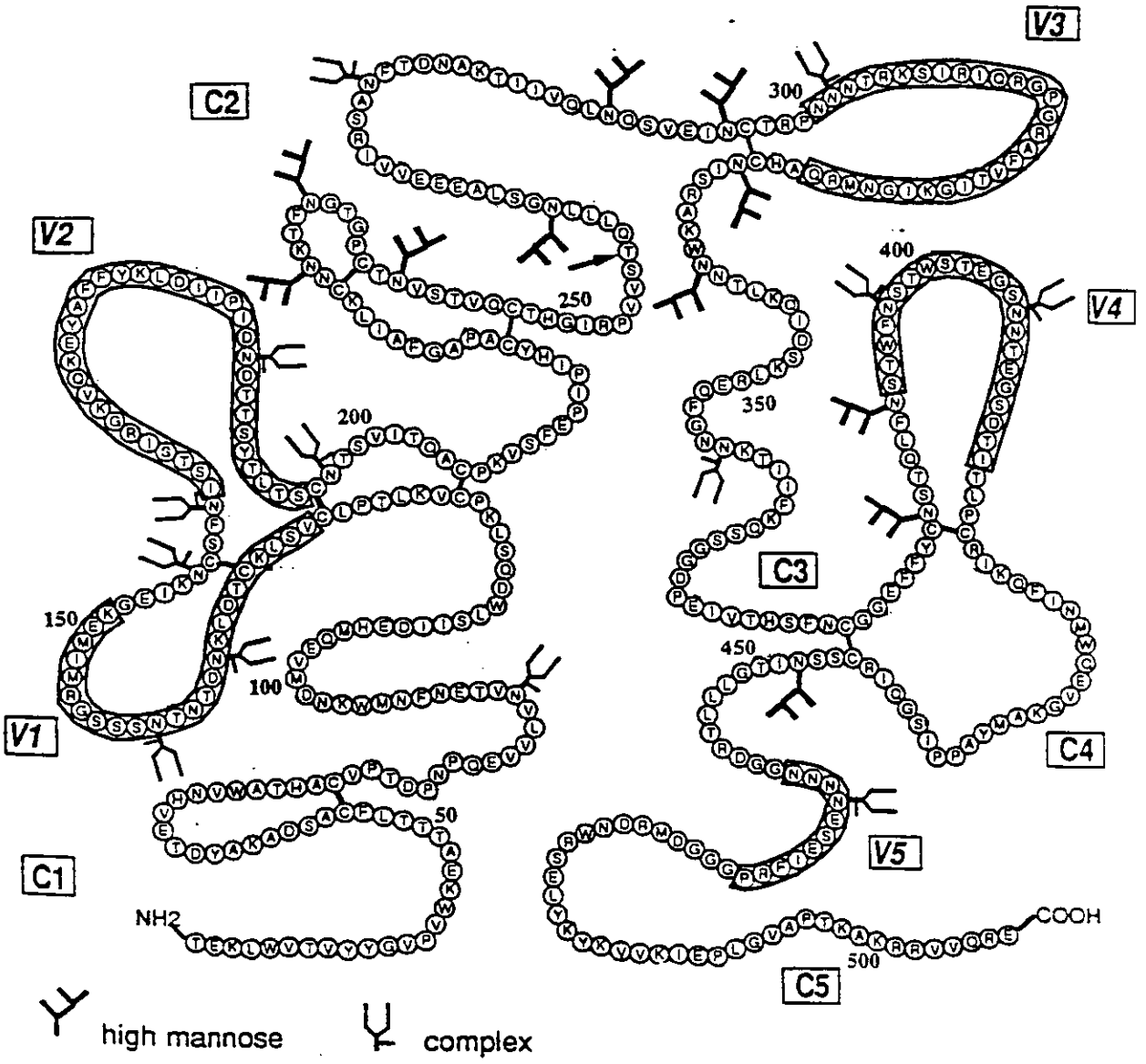


Figure 3.5 Gp120

where the amino acid sequence is highly variable between strains. The V3 loop, one of the variable regions, is the immuno dominant epitope of the GP120 molecule. This means to say, if antibodies are raised against Gp120 they will in the main be composed of antibodies which recognise the V3 loop. Although immunisation with synthetic peptides mimicking the V3 loop has had some success in chimpanzees,[29,30] it seems unlikely that a vaccine based on such a variable region would have sufficient cross reactivity to offer any degree of protection in the real world. Interestingly the tip of the V3 loop G,P,G,R is in fact fairly well conserved[31] and has been implicated in secondary binding processess[30,32]. On the whole however the V3 loop is thought to be a poor target for vaccine studies due to its excessive variability. The conserved regions of the Gp120 molecule would obviously make better targets since they show less strain variance. One conserved region of the GP120 molecule which has been shown to elicit neutralising antibodies is the CD4 binding region[33]. The highly conserved nature of this region and the fact that it is involved in binding to CD4 make it an ideal vaccine target. By removing the CD4 binding region from the whole of Gp120 the problem of the immunodominance of the V3 loop should be circumvented.

3.3.5 GP120-CD4 binding

The C4 region of Gp120 is important in binding of CD4, the binding site is however a discontinuous site involving linearly separated amino acids which are spatially proximal in the three dimensional structure. This binding site for CD4 involves a number of highly conserved amino acids which have been identified by point mutations as being vital for binding. The residues Thr-257, Asp-368, Glu-370, Asp-457 and Trp-427 are believed to be amongst the most important. It was originally thought however, that the C4 region was in itself the binding site, as a deletion mutation lacking this area did not bind to CD4[17]. The C4 region has also been shown to be exposed on the surface of the native protein[34] strengthening the evidence behind it being involved in binding. The residue Trp-427, was initially implicated in binding[35,36] but it is now thought that it may not be involved in actual contact.

Rather that its presence is crucial to a flip from β-sheet to α-helix, which occurs on moving from a polar to a non-polar environment. It is thought that this flip is necessary for CD4 binding to take place[37].

3.4 Discontinuous epitopes

The obvious problem in trying to synthesis a molecule which mimics a discontinuous epitope is the problem of trying to bring together the amino acids involved in binding in such a way that they mimic the structure of the natural binding site. Without a crystal structure (which currently does not exist for Gp120) the shape of the binding site can only be guessed through the empirical evidence supplied by point mutation studies. Earlier work in this laboratory[38] in the area of discontinues epitopes has sought to utilise a Cys-Val-Cys construct to bring together linearly separated sequences, which, through a disulfide bond are proximal to one another in the natural structure. Some success has been achieved through the synthesis of the GC1 peptide (figure 3.5), a highly immunogenic peptide involving amino acids important for CD4 binding.

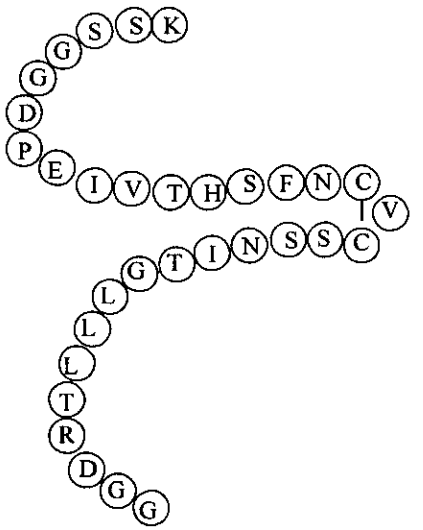


Figure 3.5 GC1

The initial aim of this project, was to use the Cys-Val-Cys technique to synthesis a number of other peptides corresponding to discontinuous epitopes. Antibodies would then be raised to these synthetic peptides, and binding studies with CD4 and Gp120 carried out.

3.5 Discussion

3.5.1 Introduction

The first peptide to be synthesised in this program was a 31 amino acid sequence comprising the C4 region (figure 3.6). This peptide **92**, contains the WQEVGKAMYA sequence, which has been identified as being exposed on the surface of natural Gp120[34]. The presence of the Trp-427 residue, which is important for the switch from β–sheet to α-helix that is involved in the binding of CD4 to Gp120 is also important. It was hoped that after a successful synthesis of this peptide, a larger structure involving the GC 1 amino acids might be constructed (figure 3.7). This would enable the immunogenically important amino acids of both structures to be present in the same molecule and hence increase the chances for antibody recognition of a discontinuous epitope mimicking the CD4 binding site.

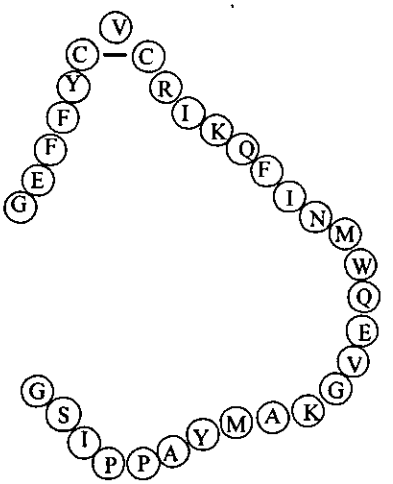


Figure 3.6

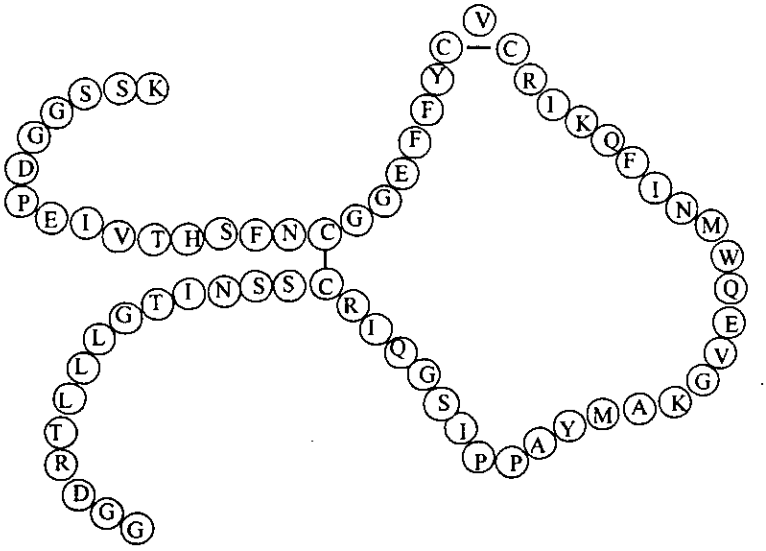


Figure 3.7

3.5.2 Synthesis of C4 peptide (Acm protected) (92)

The 31 amino acid peptide GGEFFYCVCRIKQFINMWQEVGKAMYAPPISG **92** was synthesised as outlined in the experimental section using Acm protection for the cysteine residues. The Acm protected peptide was purified using reverse phase semi-preparative HPLC. There were some problems during purification due to the insolubility of the peptide. It was however, possible to produce purified, protected peptide, in reasonable yield (200mg resin yields 40mg of Acm peptide).

In order for the peptide target to have the correct structure the disulfide bond between the two cysteines must be formed. For this to happen, the Acm protecting groups must be removed and the sulfur bond formed under oxidising conditions. The removal of the Acm group was first attempted using Mercury (II) Acetate[39] as outlined in the literature. This initial procedure proved ineffective so a second attempt was made using a vast excess of the reagents (see experimental). This second attempt, after work up, revealed a change in the HPLC profile. Mass spectroscopy of the major peak suggested a situation whereby only one Acm had been removed (figure 3.8).

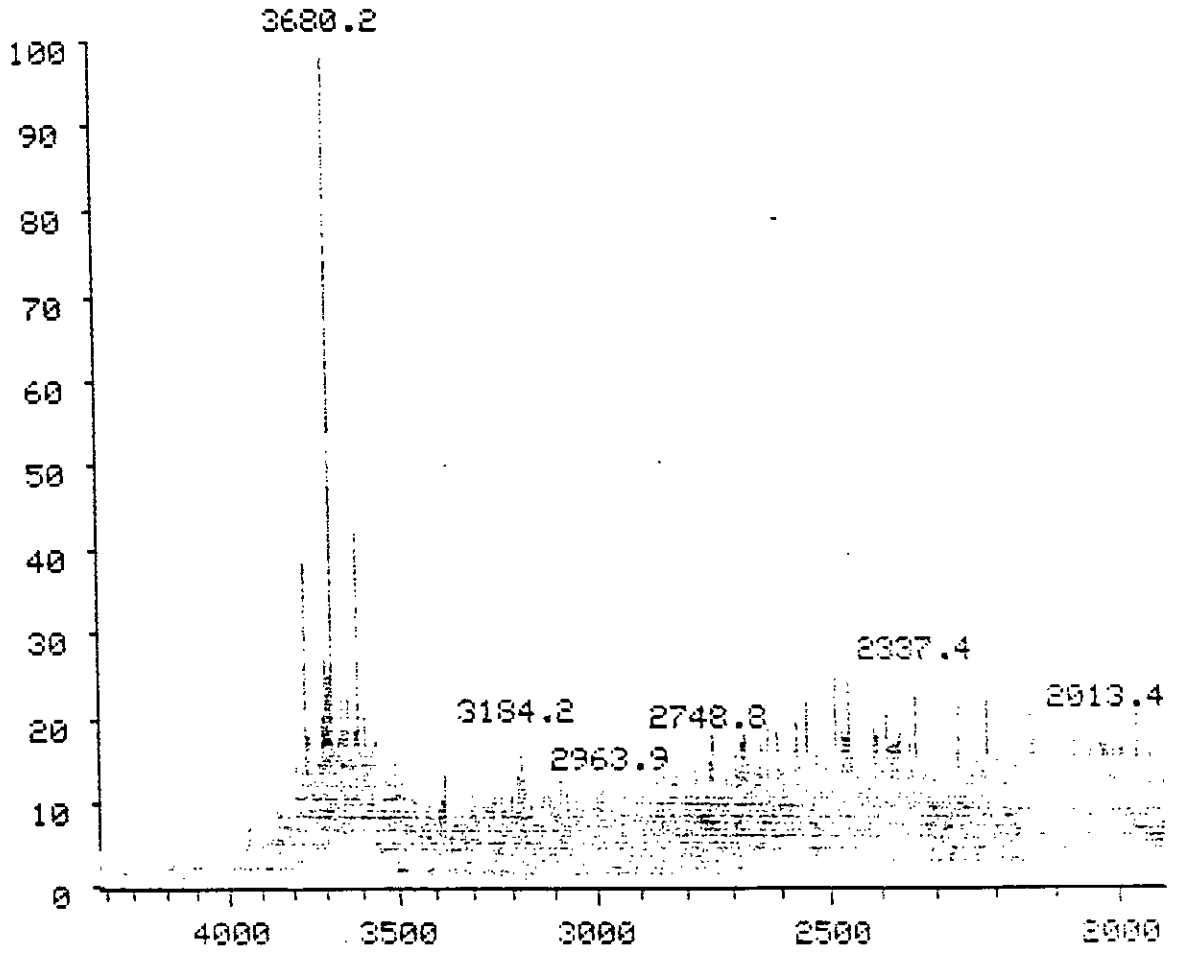


Figure 3.8

The single deprotected Acm peptide was purified and the Mercury (II) acetate deprotection protocol attempted a second time. HPLC analysis of the resultant product indicated no change in retention time, mass spectra confirmed the single deprotected species as being the major component. This was a somewhat curious result as Acm deprotection via Mercury (II) acetate has been reported in the literature[39] and used in this laboratory for a number of years.

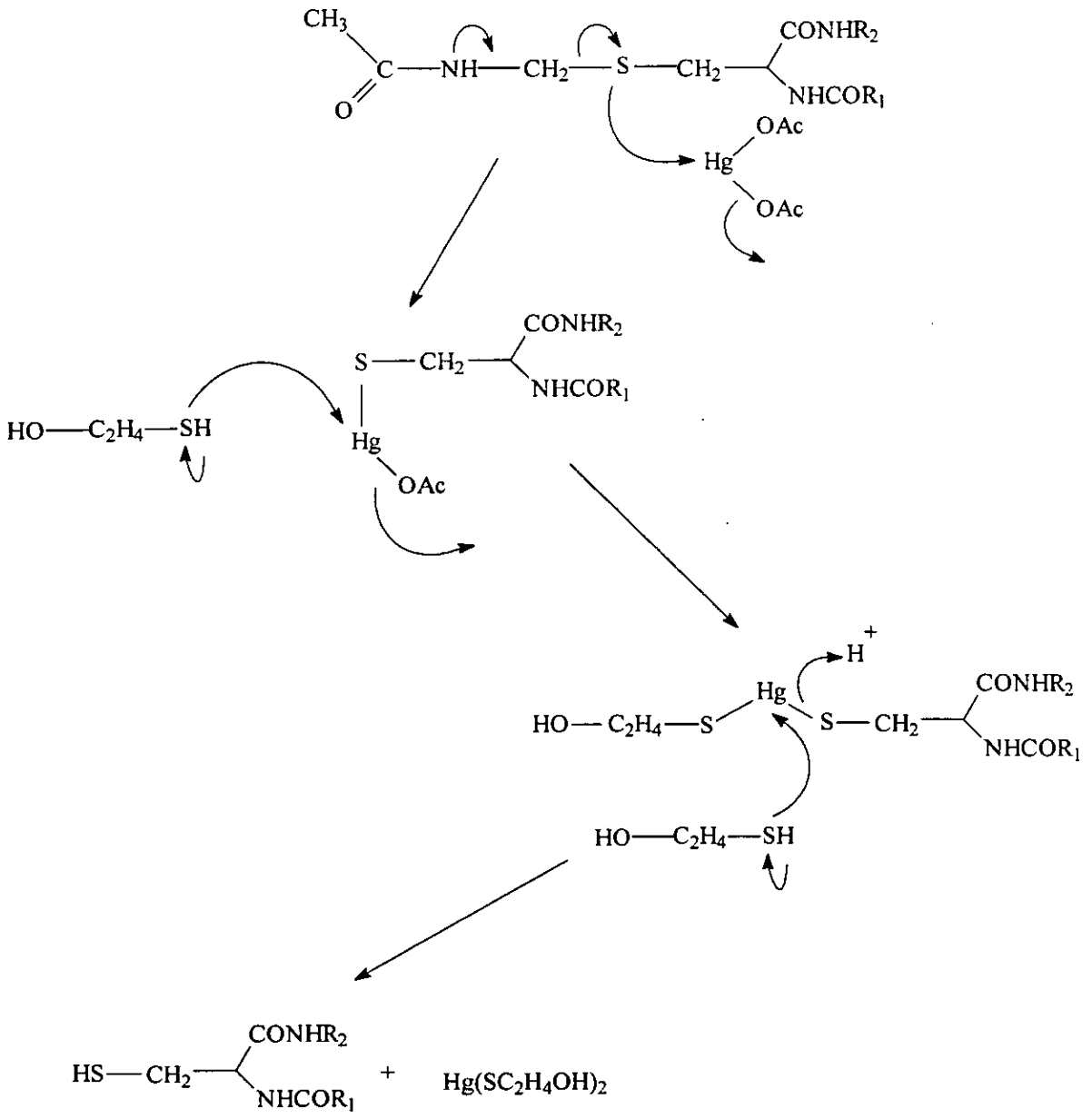


Figure 3.9 suggested mechanism for Mercury(II) Acetate/Mercaptoethanol deprotection

It was thought that the deprotection reaction was failing due to some steric effect hindering the access of the $Hg(OAc)_2$ to the Acm group. In an attempt to overcome this problem the reaction mix was place in sonic bath at 40°C overnight. On inspection of the resultant mixture by HPLC two distinct peaks could be seen. The mass spectrum of these two peaks indicated that the peptide had been broken up by

the overnight sonication in to two smaller fragments. Interestingly, the mass spectrum of one of these fragments showed a distinctive pattern which could be interpreted as involving removal of one, none, and both Acm groups. (Figure 3.10).

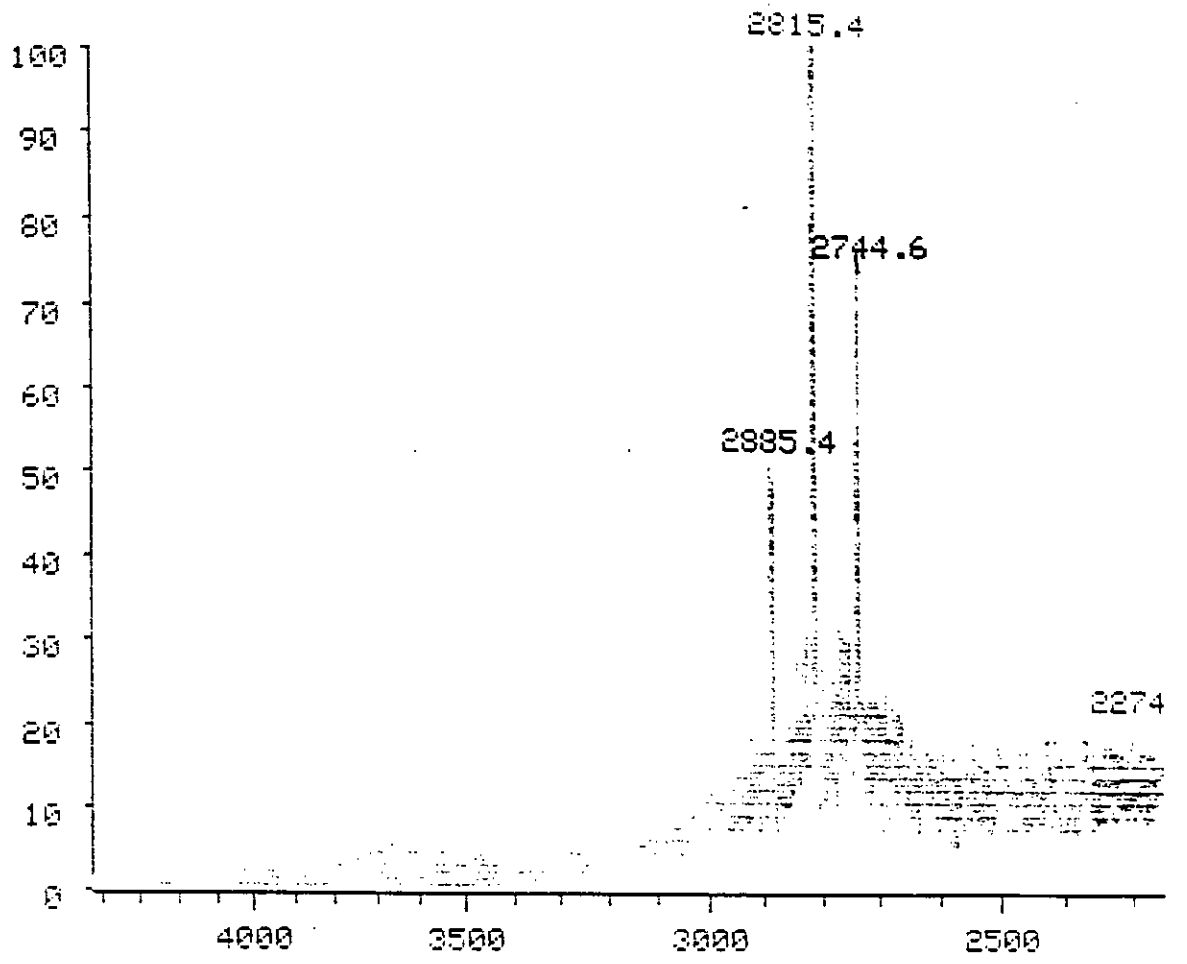


Figure 3.10

After this failure the mercury (II) acetate protocol was abandoned and replaced with the silver trifluromethanesulphonate[40] reagent (see experimental section). Removal of the Acm groups using this reagent also proved unsuccessful. Literature precedents exist for the removal of the Acm group using iodine[41] and Ag/BF_4[42]. Iodine was used in an attempt to remove the Acm groups, but the reaction resulted in iodination of the tryptophan ring so this method was abandoned.

Clearly there are considerable difficulties present in the efficient removal of the Acm group from this problematic sequence. It was hence decided to re-make peptide **92** using tBuS as the protecting groups.

3 5.3 C4 peptide (tBuS protected) (93)

The tBuS protected peptide **93** was synthesised as out lined in the experimental section, purification via HPLC proved extremely difficult due to solubility problems. It was therefore decided to remove the tBuS protecting groups prior to purification, in order to aid solubility. This was achieved using the tributylphosphine/trifluoroethanol/water procedure[43].

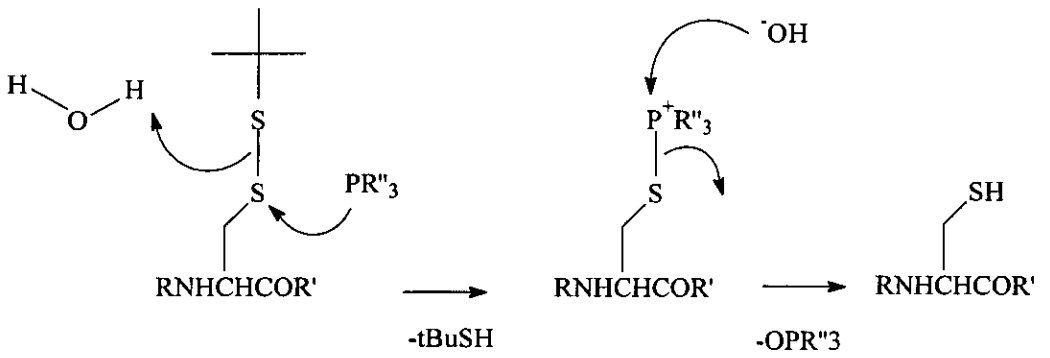


Figure 3.11.Removal of tBuS group using trialkylphosphine/water

This method did remove the tBuS groups, although the resultant peptide was still very insoluble, and exceptionally difficult to work with. On average 90-95% of peptide was lost when purifying the crude tBuS free peptide.
Various attempts were made to oxidise the sulfur bond in the deprotected peptide, both in pure form, and crude form, none of these was however successful. Failure being confirmed by unchanged HPLC profile, positive Ellman's[44] test and mass spectroscopy.

3.5.4 C4 peptide [Pro (D)Val] (94)

In order to aid oxidative formation of the disulfide bond the Cys-Val-Cys bridge present in peptide **93** was replaced with the β-turn mimic Cys-Pro-(D)Val-Cys in peptide **94**. This structural replacement has been found to encourage the oxidation of disulfides[45].

The peptide was synthesised as outlined in the experimental section, the change in sequence having little effect on the overall synthesis. Removal of the tBuS groups was achieved using the tributylphosphine technique as with peptide **93**. Purification of the deprotected peptide proved exceptionally difficult, solubility again being a major problem.

Oxidation was again attempted with very limited success. It was possible to oxidise the peptide using DMSO at pH4, however this also resulted in oxidation of the methionine residues present in the sequence. In addition, the dramatic losses of peptide during HPLC purification due to very poor solubility, made it impossible to attempt to reduce the methionines as there was simply too little product by this stage of the purification process. Due to the extensive practical problems and uncertainty in immunogenicity of the product, this work was abandoned with a view to returning at a later date.

3.6 Conclusions and summary

The insolubility of the chosen sequence dramatically hindered the purification of the desired peptide. A somewhat unusual situation prevailed, whereby the removal of the cysteine protecting groups did not actually aid solubility. This made it practically impossible to arrive at purified oxidised peptide. There is always a possibility when one synthesises unnatural peptides that the desired sequence will be problematical. The use of a β-turn mimic such as cyclopentyl was mooted as a way around the difficulty of forming sulfur bridges, although these compounds were latter found to be non-immunogenic and therefore effectively pointless for our studies. The problem in purification is caused by the dramatic losses at each HPLC stage, hence it

would be necessary to reduce the number of HPLC steps to a minimum in order to produce the desired product. Although the development of a reliable, solublising, cysteine protecting group would certainly aid the synthesis of this troublesome peptide, it is the intrinsic insolubility of the molecule which causes problems.

3.7 References

1) J.P Langeueld, J, Ignacio Casal, R.D.M.E, Osterhaus, E. Cortes, R. De Swart, C. Vela, C. Dalsgaard, W.C. Puijk, W. Schaaper, Rob. Meloen, *J. Virol,* 1994, 4506-4513.

2)M. V. Valero, L.R. Amador, C. Galindo, J. Figueroa, M.S. Bello, L.A. Murillo, A.L. Mora, G. Patarroyo, C.L. Rocha, M. Rojas, J.J. Aponte, L.E. Sarmiento, D.M. Lozada, C.G. Coronell, N.M. Ortega, J.E. Rosas, P.L.Alonso, M.E. Patarroyo, *Lancet*, 1993, **341**,705-710

3) L. Montagnier, F. Barre-Sinoussi, J. Cherman, F. Rey, M. Nugeyre, S. Charmet, J. Greust, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, *Science.* 1983, **220**, 868-871.

4) R. Gallo, S.Z. Salhuddin, M, Popovic, g. M. Shearer, M. Kaplan, B.F. Haynes, T.J. Parker, R. Redfield, J. Oleske, B. Safai, G. White, P. Foster, P.O. Markham. *Science.* 1984, **224**, 500-503.

5) M.C. Lane, J.L. Depper, W.C. Greene, G. Whalen, T. Waldmann, & A.S.N Fauci. *Eng. J. Med.* 1985, **313,** 79.

6) Jon Cohen, *Science, 1*993, **260**, 1254-1261.

7) J. Levy, *J. Microbiol. Rev.* 1993, **57**, 183.

8) J. Homsy, M. Meyer, M. Taterno, S. Clarkson, J.A. Levy, *Science* 1989, **244**, 1357-1460

9) J. Coffin, *Science*, 1995, **267**, 483-488

10) D. Ho, D. Avidan, U. Neumann, A. Sperelson, Wen Chen, J. Leonard, M. Markowitz, *Science*, 1995, **273**, 123-126

11) J. Coffin *Nature,* 1995, **375**, 534-535

12) S. Wain-Hobson, *Nature*, 1995, **373**, 102,

13) R.M. Brown, *in Chemical and Engineering News,* 24/8/1992

14) F. Brun-Vezinet, C. Katlama, D. Roulot, L. Lenoble, M. Alizon, J.J. Madjar, M.A. Rey, P.M. Girard, P. Yeni, F. Clavel, S. Gabelle, & M. Hazric. *Lancet I,* 1987, 128.

15) M.D. Daniel, Y. Li, Y.M. Naidu, J.P. Durda, D.K. Schimdt, C.D. Troup, D.P. Silva, J.J. Mackey, H.W. Kestler, P.K. Sehgal, N.W. King, Y. Ohta, M. Hayami, & R.C. Desrosiers, *J. Virol.* 1988, **62**, 4123.

16) E.E. Sparger, P.A. Luciw, J.H. Elder, J.K. Yamamoto, L.J. Lowenstine, & N.C. Pederson, *AIDS,* 1989, **3**, S43.

17) L.A. Larsky, G.M. Nakamura, D.H. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, & D.J. Capon. *Cell*, 1987, **50**, 975.

18) F.D. Veronese, A.L. DeVico, T.D. Copeland, S. Oroszlan, R.C. Gallo, & M.G. Sarngadharan. *Science,* 1985, **137**, 1402.

19) Q.J. Sattentau, & J.P. Moore, *J. Exp. Med.* 1991, **174**, 407.

20) F. Celada, C. Cambiaggi, J. Maccari, S. Burasero, T. Gregory, E. Patzer, J. Porter, C. McDanal, & T.J.Mattews, *J. Exp Med.* 1990, **172**, 1143..

21) G.J. Clements, M.J. Price-Jones, P.E. Stephens, C. Sutton, T.F. Schulz, P.R. Clapman, J.A. McKeating, M.O. McClure, S. Thompson, M. Marsh, J. Kay, R.A. Wiess & J.P. Moore, *AIDS Res. Hum. Retroviruses,* 1991, **7**, 3.

22) R.D. Harrington, A.P. Gabelle, *J. Virol.* 1993, **67**, 5939.

23) W.R. Gallaher, *Cell,* 1987, **50**, 327.

24) L.E. Eiden, J.D. Lifson, *Immunol. Today.* 1992, **13**, 201.

25) D. Ho, J. McKeating, Xi Ling Li, T. Moudgil, E. Daar, Nai-Chau Sun, J.E. Robinson, *J. Virol,* 1991, **65,** 489-493.

26) C.K. Leonard, W.M. Spellman, L. Riddel, R.J. Harris, N.J. Thomas, & T.J. Gregory, *J. Biol. Chem.* 1990, **265**, 10373.

27) M. Guyander, M. Emerman, P. Songio, F. Clavel, L. Montagnier, M. Alizon, *Nature,* 1987, 326, 662.

28) M. Muesing, D. Smith, C. Cabradilla, C. Benton, L. Lasky, & D. Capon, *Nature* 1985, **313,** 450.

29) Marc Girard, M.P. Kieny, A. Pinter, F. Barre-Sinoussi, P. Nara, H. Kolbe, K. Kusumi, A. Chaput, T. Reinhart, E. Muchmore, J. Ronco, M. Kaczorek, E. Gomard, J.C. Gluckman, & P. Fultz, *Proc. Natl. Acad. Sci. USA*. 1991, **88**, 542-546.

30) S.A. Tilley, *AIDS Res. Hum. Retroviruses*, 1992, **8**, 451-459.

31) G.J Larosa, J. Davide, K. Weinhold, J. Waterbury, A. Profy, J. Lewis, A. Langlois, G. Dreesman, R. Boswell, P. Shadduck, L. Holley, M. Karplus, D. Bolognesi, T. Mattews, E. Emini, S. Putney, *Science*, 1990, **249**, 932-935

32) Scott Putney, *Tibs,* 1992, **17**, 191-197.

33) Markus Thall, Graig Furman, David Ho, James Robinson, Shermaine Tilley, Abraham Pinter, Joseph Sodroski, *J. Virol,* 1992, 5635-5641.

34) Jane Mckeating, John Moore, Morag ferguson, Howard Marsden, Susan Graham, Jeffrey Almond, David Evans & Robin Weiss, *AIDS Res. Hum. Retroviruses*, 1992, **8,** 451-459.

35) U. Olshevsky, E. Helseth, F. Furman, W. Haseltine, J. Sodroski. *J. Virol.* 1990, **64**, 5701.

36) A. Cordonnier, Y. Riviere, L. Montagnier, M. Emerman. *J. Virol.* 1989, **63**, 4464.

37) J. Reed, V. Kinzell, *Proc. Natl. Acad. Sci. USA*. 1993, **90**, 6761.

38) R. Ramage & G. Cotton, *personal communication.*

39) D.F. Veber, J.D. Milkowski, S.L. Varga, R.G. Darkewalter, R. Hirschmann, *J. Am. Chem. Soc.* 1972, **15**, 5456.

40) H. Yajima, N. Fujii, A. Otaka, t. Watanabe, A. Okamach, H. Tamamura, Y. Inagaki, M. Nomizu, K. Asano, *J. Chem. Soc. Chem. Commun.* 1989, 283-284

41) B. Kamber, A. Hartman, K. Eisler, B. Riniker, H. Rimk, P. Sieber, W. Rittel, *Helv. Chim.* Acta. 1980, **63,** 899,

42) M. Yoshida, T. Tatsumi, K. Akaji, S. Iinuma, Y. Fujiwara, T. Kimura & Y. Kiso, *Chem. Pharm. Bull.* 1990, **38**, 273,

43) E. Atherton, R. Sheppard, P. Ward, *J. Chem. Soc. Perkin. Trans* 1, 2065, 1985

44) G. L. Ellman, *Arch, Biochem, Biophys,* 1959, **82,** 70.

45) K. Urquart & R. Ramage, *personal communication.*

CHAPTER 4: EXPERIMENTAL

4.1 Notes

All amino acids were purchased from either Bachem, Novabiochem, or alternatively were synthesised as outlined in the text. They were all of the L configuration unless otherwise stated. Melting points were determined using a Koffler hot stage melting point apparatus and are uncorrected. Optical rotations were measured on an AA1000 polarimeter (Optical activity limited) using a 10.0cm cell in the solvent indicated in the text. Analytical thin layer chromatography (t.l.c) was carried out using a 0.3mm layer of silica (Merck, kieselgel 609) containing 0.5% Woelm fluorescent indicator, on foil plates in the solvents indicated in the text. The components were observed under ultra-violet light, by reaction with iodine vapour and by charring of the plate after spraying with 20% sulphuric acid in methanol. Infrared spectra were recorded on a Bio-RAD SPC 3200 instrument. Ultraviolet spectra were recorded on a Varian Cary 210 spectrophotometer in the solvents indicated in the text. High and low resolution fast atom bombardment (FAB) mass spectra were measured on a Kratos MS50TC instrument, using either thioglycerol, 3-nitrobenzyl alcohol or glycerol as matrix. Nuclear magnetic resonance (NMR) spectra were recorded on either a Jeol FX-60 (60MHz), a Brucker WH-80 (80MHz), a Brucker WP-200 (200MHz), a Brucker AC-250 (250MHz), a BruckerWH-360 (360MHz) instrument in the solvents stated. Elemental analyses were performed on a Perkin-Elmer 2400CHN analyser. Amino acid analysis was performed on a LKB 4150 alpha amino acid analyser on the hydrolyse obtained after heating samples at 110°C for 24 hours in a sealed Carious tube, followed by evaporation to dryness.

All solvents were distilled before use and the following were dried using the reagents given in parenthesis when required: dichloromethane (calcium hydride), diethyl ether (sodium wire). Peptide synthesis grade dimethylformamide (DMF), 1,4-dioxan and piperidine were obtained from Rathburn Chemicals, Walkerburn Scotland. Peptide synthesis grade trifluroacetic acid was obtained from Applied

Biosystems (ABI) . High performance liquid chromatography (HPLC) was carried out using either an ABI system, comprising 2 x 1406A solvent delivery systems, an 1480A injector/mixer and an 1783A detector/controller, or a Gilson system, comprising 2 x 306 solvent delivery systems, an 811C dynamic mixer, an 805 manometeric module, a 119 UV/VIS detector and Gilson 715 or Unipoint software driven gradient controller. Components were eluted from various columns, as described in the text, by a linear gradient of acetonitrile (far UV grade, Rathburn Chemicals) in Milli-Q grade water, where both solvents contained 0.1% v/v of HPLC grade trifluoroacetic acid (Fisons).

4.2 Solid phase peptide synthesis.

The polypeptides described were synthesised on an Applied Biosystems 430A automated peptide synthesiser fitted with a UV monitoring system as described previously[1]. All peptides were synthesised using the 9-fluorenylmethoxycarbonyl (Fmoc) strategy of N^{α} protection. This involves the complementary use of orthogonal acid labile side chain protection and an acid labile peptide-resin linker. The side chain protecting groups used were as follows : t-butyl (Bu^{t}) ethers for serine, threonine and tyrosine; t-Bu esters for aspartic and glutamic acid; t-butoxycarbonyl (Boc), 1,5 -dioxaspiro-5:5undecane-3-nitro-3-methoxy carbonyl (Tnm) for lysine; τ-triphenylmethyl (trityl) for histidine; (Pmc) for arginine. The carboxamide side chains of arginine and glutamine were incorporated as either the 4,4'-dimethoxybenzhydryl (MBH) or trityl derivatives, or without side chain protection as indicated in the text. Cysteine was incorporated as indicated in the text.

4.2.1 Coupling of the C-terminal amino acid onto 4-alkoxybenzylalcohol (Wang) resin.

A solution of Fmoc amino acid (1mM) and N,N'-diisopropylcarbodiimide (DIC) (0.5mM) in DMF (20ml) was stirred for 15 minutes at room temperature , then 4-benzyloxybenzylalcohol functionalised polystyrene resin (Wang) (1.0g, 0.8mM) was

added, together with a catalytic amount of 4-(N,N'-dimethylamino)- pyridine and the mixture was then sonicated for 1-2 hours. The functionalised resin was then removed by filtration and sequentially washed with DMF, 1,4-dioxan and dichloromethane, before being dried under a vacuum. The loading of the functionalised resin was then determined by treating a known weight of resin with 20% piperidine/DMF in a 10ml volumetric flask, for 20 minutes in a sonic bath. The UV absorbance of the supernatant was then measured at 302nm and the loading calculated using the Beer-Lambert law (ε_{302}= 15400 for fulvene-piperidine adduct). This procedure generally gives a resin with a functionality in the region of 0.1mM/g. To obtain higher resin loadings (0.5-0.6mM/g), the amounts of Fmoc amino acid and DIC were increased to 5.0mM and 2.5mM respectively.

4.2.2 Automated SPPS

Synthetic procedures were pre-programmed into the ABI 430A synthesiser prior to the commencement of synthesis. Routinely most residues were incorporated using double couple cycles, in which the first coupling cycle utilised a preformed symmetrical anhydride, followed by the second coupling using a preformed 1-hydroxybenzotriazole (HOBt) ester. The exceptions to this were the amino acids asparagine, glutamine and histidine, which were coupled twice via their HOBt esters and glycine, which was coupled singly as a symmetric anhydride. An alternative coupling strategy was used for the peptides **84, 90, 99** whereby the amino acids were all single coupled using the HOCt[2] activating agent.

1. Capping - The resin was vortexed with a solution of 20% piperidine/DMF for 3 minutes before being drained . An aliquot of the deprotection solution was then sent to a UV detector in order to quantify the amount of fulvene-piperidine adduct present and hence subsequently gave an indication of the percentage incorporation of each residue. Deprotection was then repeated for a second time for one minute in order to

establish if the N^{α} protecting group had been completely removed. Finally the resin was washed with six portions of DMF/1,4-dioxan (1:1).

3. Coupling-(I) The resin was vortexed with a solution of 0.5mM Fmoc amino acid preformed symmeterical anhydride (formed from 1mM Fmoc AA and 0.5mM DIC in the activator vessel). The first coupling cycle was allowed to continue for 30 minutes before the solution was drained from the reaction vessel and the resin washed with two portions of DMF. The resin was then vortexed for a second 30 minute period with 0.5mm of Fmoc amino acid HOBt active ester (preformed from 0.5mM Fmoc amino acid, 0.5mM HOBt and 0.5mM DIC) before being drained and washed with four portions of DMF. For certain difficult couplings the coupling cycle time was extended.

(II) The peptides synthesised using the HOCt activating agent used a slightly different coupling cycle. Each cartridge contained 1mM of Fmoc amino acid and 1mM of HOCt, the mixture was dissolved up in 4mls of DMF and passed to the activation vessel. The activation vessel contained 1mM of DIC dissolved in 4mls of 1,4-dioxan, both mixtures were vortexed together for 20 minutes to form the activated ester. The mixture was then passed to the reaction vessel and vortexed for 30 minutes before being drained and washed.

4.3 Ellman's assay for free thiol groups

Qualitative Ellman's assay[3] enables the number of free thiols per molecule to be calculated. Accurately weighed peptide (0.02-0.05 μmol) is dissolved in 0.1M sodium phosphate buffer pH 7.3 (2.5 ml), containing 0.01M EDTA and 6M GdmCl. A fresh solution of 3mM 5,5'-dithiobis-(2-nitrobenzoic acid), (DTNB), in 0.1 M sodium phosphate buffer pH 7.3 is prepared. Denaturing buffer (2.5 ml) is placed in the reference cell and denaturing buffer plus peptide placed in the sample cell of a UV spectrometer. DTNB solution (100μl) is added to each and mixed by inversion, the absorbance at 412nm is then recorded after a few minutes. The concentration of

free thiols can then be calculated knowing ε_{412} of nitrothiobenzoate (NTB) in 6M GdmCl is 13700 $M^{-1}cm^{-1}$.

4.4 Kinetics

The mechanical processes involved in the taking of kinetic data are described in chapter 2, as is the primary mathematical manipulation. Each kinetic run was performed at least twice and the data points averaged to reduce errors. The graphs presented in the text were produced using Micro Soft Origin software, the curves being modelled to the $A_o \exp(-A_1 t)$ function.

4.5 Synthesis

5-Hydroxymethyl-5-nitro-2-phenyl-1:3dioxane[4] **(53)**

Benzaldehyde (106.12g, 1M) was added to a solution of tris(hydroxymethyl)nitromethane (151.12g 1M) in a solvent mix consisting of water (100ml), concentrated hydrochloric acid (80ml) and methylcellusolve (100ml), after the addition the mixture is made up to 500ml with more water. The reaction mixture is then shaken at room temperature for 40 hours. The white solid obtained is washed neutral with water. Recrystallisation from aqueous ethanol gave the title compound as a white solid (172g, quantitative); m.p. 125-126°C (lit.,[4]125-126°C); (found: C,54.99;H,5.71;N,5.62, $C_{11}H_{13}O_5N$ requires: C,55.23;H,5.44;N,5.85%); t.l.c. (ethyl acetate/hexane, 3:7) Rf 0.47; υ_{max}(nujol) 3406 (OH), 1544 (NO_2); δH($CDCl_3$, 200MHz) 7.36 (5H, s, aromatic); 5.65 (1H, s, benzylidene CH); 5.59 (1H, t ,OH); 4.74 (2H, d, equatorial ring pair 2CH); 4.24 (2H, d, axial ring pair 2CH); 3.72 (2H, d, CH_2OH); δC($CDCl_3$ 200MHz) 137.77 (aromatic quaternary); 129.43, 128.49, 126.41, (aromatic CH);100.88 (benzylidene CH); 88.46 (aliphatic quaternary CH); 68.56 (2 ring CH_2's); 62.67 (CH_2OH); m/z (FAB)240 (MH+), HRMS found 240.08637, $C_{11}H_{13}O_5N$ requires 240.08720

5-Hydroxymethyl-5-methyl-2-phenyl-1;3-dioxane[5] **(95)**

Benzaldehyde (53.06g, 0.5M) was added to a solution of tris(hydroxymethyl)ethane (61g, 0.5M) in a solvent mix consisting of water (50ml), concentrated hydrochloric acid (40ml) and methycellusolve (50ml), after addition the mixture was made up to 250ml with more water. The white solid obtained was washed neutral with water. Recrystallisation from aqueous ethanol gave the title compound as a white solid (103g, quantitative); m.p 87-89°C (lit.,[5] 87-89°C); (found: C,69.07;H,7.90, $C_{12}H_{16}O_3$ requires: C,69.23;H,7.90%); t.l.c. (ethyl acetate/hexane, 3:7); Rf 0.56, υ_{max}(nujol) 3490 (b, OH); δH($CDCl_3$, 250MHz) 7.50-7.46 (5H, m, aromatic); 5.41 (1H, s, benzylidene CH); 4.03 (2H, d, J 11.81, equatorial ring pair 2CH); 3.81 (2H, d, CH_2OH); 3.62 (2H,d, J 11.81, axial ring pair); 0.74 (3H, s, CH_3); δC($CDCl_3$ 200MHz) 137.90 (aromatic quaternary); 128.73, 128.03, 125.83, (aromatic CH); 101.46 (benzylidene CH); 72.94 (ring CH_2's); 64.84 (CH_2OH); 34.61(aliphatic quaternary CH); 16.62 (CH_3); m/z (FAB)209 (MH^+); HRMS found 209.11754, $C_{12}H_{16}O_3$ requires 209.11777

3-Hydroxymethyl-3-methyl-1;5-dioxaspiro-5:5undecane[5] **(74)**

Cyclohexanone (40ml, 0.38M) and tris(hydroxymethyl)ethane (45.6g, 0.38M), plus a catalytic amount of p-toluenesulphonic acid (TsOH) were refluxed in toluene, overnight, using a Dean Stark apparatus. After 24 hours, the solution was cooled to room temperature, washed with water (2 x 100ml), and the organic layer dried over $MgSO_4$. Concentration *in vacuo* gave a white solid, recrystalization from hexane/ether gave the *title compound.*(66g, 86%); m.p. 60°c (lit[5]., 60-61°C); (found:C,66.33; H,10.25%, $C_{11}H_{20}O_3$ requires:C,65.98; H,10%); t.l.c.(ethyl acetate/hexane, 3:7) Rf 0.48; υ_{max}(nujol) 3417 (OH); δH($CDCl_3$, 200MHz) 3.60 (2H, s, CH_2OH); 3.57-3.59 (4H, d, J 2.63 Hz, dioxane ring CH_2); 2.85 (1H, s, OH); 1.79-1.34 (10H, m, aliphatic ring CH_2); 0.75 (3H, s, CH_3); δC($CDCl_3$ 50MHz) 97.80 (ring junction aliphatic quaternary); 65.46 (CH_2OH); 65.19 (2 x dioxane ring CH_2); 35.91

(aliphatic ring CH_2); 34.65 (aliphatic quaternary); 28.56-22.18 (4 x aliphatic ring CH_2); 17.47 (CH_3); m/z (FAB)201 (MH^+).

3-Hydroxymethyl-3-nitro-1:5-dioxaspiro-5:5-undecane[6] **(98)**

Cyclohexanone (44.50ml, 0.43M) and tris(hydroxymethyl)nitromethane (64.93g, 0.43M), plus a catalytic amount of TsOH were refluxed in toluene, overnight, using a Dean Stark apparatus. After 24 hours, the solution was cooled to room temperature, washed with water (2 x 100ml), and the organic layer dried over $MgSO_4$. Concentration *in vacuo* gave a cream coloured solid, recrystallization from hexane/ether gave the *title compound.* (42g, 42%); m.p. 99°C (lit.,[6] 97-98);(found: C,52.19; H,7.54;N,5.65; $C_{10}H_{17}0_5N$ requires: C,51.96; H,7.36; N, 6.06%); t.l.c. (ethyl acetate/hexane, 3:7) Rf 0.57; υ_{max}(nujol) 3415 (OH); 1545 (NO_2); δH($CDCl_3$, 200MHz) 4.36 (2H, d, J 12.56 Hz, equatorial dioxane ring CH_2); 4.09 (2H, d, J 6.33 Hz, CH_2OH) 4.07 (2H, d, J 12.57 Hz, axial dioxane ring CH_2); 2.09 (1H, t, J 6.38 Hz, OH); 1.79-1.40 (10H, m, aliphatic ring CH_2); δC($CDCl_3$, 50MHz) 99.19 (ring junction quaternary C); 86.28 (quaternary C); 63.10 (CH_2OH); 60.19 (2 x dioxane ring CH_2); 32.44-21.93 (5 x aliphatic ring CH_2); m/z (FAB) 232 (MH^+).

The 5-hydroxymethyl-5-nitro-2-phenyl-1:3dioxane ester of 9-fluorenylmethyloxycarbonylglycine **(55)**

Fmoc-glycine (1.24g, 4.17mM) was dissolved in DCM (50ml), to this was added DCC (0.86g, 4.17mM), and the mixture sonicated for 15mins. A solution of compound **(53)** (1g 4.18mM) in DCM was then added together with a tenth of an equivalent of DMAP. The resultant mixture was stirred at 5°C for twelve hours. The white solid formed (urea) was filtered off and the filtrate washed with Na_2CO_3 (2 x 50ml) and water (2 x 50ml). The solution was dried ($MgSO_4$) then evaporated *in vacuo* to yield the *title compound* as a white solid (1.83g, 85%): Upon recrystallisation from ethyl acetate/hexane, m.p 136-138°C (found: C,64.94;H,5.16;N,5.28, $C_{28}H_{26}O_8N_2$ requires: C,64.86;H,5.02;N,5.40%); t.l.c. (ethyl

acetate/hexane, 7:3) Rf 0.85, υ_{max}(nujol) 3448 (NH); 1751 (ester CO); 1724 (urethane CO);1555 (NO_2);λ_{max}(MeOH) 265nm (ε 24000 dm^3 mol^{-1} cm^{-1}), 290 (6100), 300 (7500); δ_H(360 MHz, $CDCl_3$) 7.25-7.76 (13H, m, aromatic); 5.50 (1H, s,benzylidene CH); 5.33 (1H, t, amine NH); 4.95 (2H, d, J 12.08 Hz, equatorial ring pair 2CH); 4.43 (2H, d, J 6.83 Hz, β CH_2); 4.39 (2H, s, ester CH_2); 4.22 (1H, t, J 6.85 α CH); 4.07 (2H, d, J 12.46 Hz, axial ring pair 2CH); 3.98 (2H, d, J 5.28 Hz, glycine CH_2); δ_C(50 MHz, DMSO) 169.75 (ester CO); 156.73 (Fmoc CO); 143.95, 140.93 (Fmoc quaternary); 137.33 (benzyl quaternary); 129.34-120.32 (aromatic CH); 100.61 (benzyl CH); 85.63 (aliphatic ring quaternary); 67.86 (aliphatic ring CH_2); 66.04 (Fmoc CH_2); 63.33 (ester CH_2); 46.76 (Fmoc CH); 42.15 (glycine CH_2); m/z FAB) 520 (MH^+); HRMS found 519.176575, $C_{28}H_{26}O_8N_2$ requires 519.17674.

<u>The tris(hydroxymethyl)nitromethane ester of 9-fluorenylmethoxycarbonylglycine</u>
(51)

The 3-methyl-3-nitro-1:5-diol ester of 9-fluorenylmethoxycarbonylglycine (2g, 3.86 mM) was added to TFA/H_2O 95/5 (25ml) the mixture was stirred under nitrogen for 3 hours. The solvent was then removed *in vacuo*, H_2O added, and the white solid product re-extracted into ethyl acetate (2 x 100ml). The ethyl acetate was dried with $MgSO_4$ and the product absorbed from this solvent onto silica. Wet flash chromatography, eluting with ethyl acetate/hexane gave the *title compound* as a white solid, which rapidly becomes a gum on exposure to air. (1.5g, 90%), (Found: C,58.77; H,54.90; N,6.61. $C_{21}H_{22}O_8N_2$ requires C,58.46; H,5.10; N,6.49%); t.l.c. (ethyl acetate/hexane, 7:3)Rf 0.4, υ_{max} 3411 (OH), 1750 (ester CO), 1703 (urethane CO), 1547 (NO_2); λ_{max} 265 (ε 28000 dm^3 mol^{-1} cm^{-1}), 290 (7100), 300 (9000);δH($CDCl_3$, 360MHz) 7.75-7.25 (8H, m, aromatic); 5.46 (1H, t, NH); 4.65 (2H, s, ester CH_2); 4.39 (2H, d, J 6.89 b CH_2); 4.19 (1H, t, J 6.89 a CH); 4.02 (4H, s, 2CH_2OH); 3.93 (2H, d, J 5.9 glycine CH_2); δC(DMSO, 50MHz) 156.77 (ester CO); 169.85 (Fmoc CO); 144.01, 140.96 (Fmoc quaternary); 127.91-120.37 (Fmoc aromatic CH); 93.48 (aliphatic quaternary); 66.05 (Fmoc CH_2); 60.11 (ester CH_2);

59.83 (2 x CH_2OH); 46.76 (Fmoc CH); 42.22 (glycine CH_2); m/z (FAB) 431 (MH^+), HRMS found 431.14561, $C_{21}H_{22}N_2O_8$ requires 431.14544.

The 5-hydroxymethyl-5-methyl-2-phenyl-1,5-dioxane ester of 9-fluorenylmethoxycarbonylglycine **(96)**

N-9-fluorenylmethoxycarbonylglycine (1.42g, 4.78mM) was dissolved in DCM (50ml) to this was added DCC (0.98g, 4.78mM) the mixture was then sonicated for 15mins. A solution of compound **95** (1g, 4.81mM) in DCM was then added together with a tenth of an equivalent of DMAP. The resultant mixture was stirred at 5°C for twelve hours. The white solid formed (urea) was filtered off and the filtrate washed with Na_2CO_3 (2 x 50ml) and water (2 x 50ml). The solution was dried ($MgSO_4$) then evaporated *in vacuo* to yield an oily gum. Wet flash chromatography using an ethyl acetate/hexane gradient gave the *title compound* as a white solid (1.50g, 64%); m.p 114-117°c (found: C,71.00;H,5.89;N,2.95, $C_{29}H_{29}O_6N$ requires C,71.40;H,5.99;N,2.87%); t.l.c.(ethyl acetate/hexane, 4:6)Rf 0.60, υ_{max}(nujol) 3417(NH), 1721 (ester CO), 1706 (urethane CO); λ_{max}(MeOH) 265nm (ε 27000$dm^3mol^{-1}cm^{-1}$), 290nm (6800), 300nm (8400);δH($CDCl_3$, 360MHz) 7.8-7.2 (13H, m, aromatics); 5.54(1H, t, NH); 5.41(1H, s, benzylidene CH); 4.47(2H, s, ester CH_2); 4.40(2H, d, J 7.13 Fmoc CH_2); 4.22(1H, t, J 6.87 Fmoc CH); 4.04(2H, d, J 11.90 equatorial ring pair 2CH); 3.98(2H, d, J 5.73Hz glycine CH_2); 3.65(2H, d, J 11.89 axial ring pair CH_2); 0.78(3H, s, CH_3); δC(DMSO, 50MHz) 170.35 (ester CO); 156.71 (Fmoc CO); 143.89 (Fmoc quaternary); 140.85 (benzyl quaternary); 128.88-120.26 (aromatic CH); 100.92 (benzilidene CH); 72.36 (dioxane ring CH_2); 66.27 (Fmoc CH_2); 65.93 (ester CH_2) 55.03 (aliphatic quaternary); 46.69(Fmoc CH); 42.37 (glycine CH_2); 17.02(CH_3); m/z(FAB) 488(MH^+); HRMS found 488.20854 $C_{29}H_{29}O_6N$ requires 488.20731

The tris(hydroxymethyl)ethane ester of 9-fluorenylmethoxycarbonylglycine **(64)**

The 5-hydroxymethyl-5-methyl-2-phenyl-1:5-dioxane ester of 9-fluorenylmethoxycarbonylglycine, compound **96** (2g 4.09mM), was added to TFA/H_20 (25ml) and the mixture stirred under nitrogen for three hours. The solvent was then removed *in vacuo*, H_2O added and the white solid product re-extracted into ethyl acetate (2 x 100ml). The ethyl acetate was dried with $MgSO_4$ and the product absorbed from this solvent onto silica and then purified by wet flash chromatography. Eluting with ethyl acetate/hexane gave the *title compound* as an unstable hydroscopic gum which breaks down to the ordinary glycine compound over time. (1.32g, 81%); t.l.c.(ethyl acetate/hexane, 6:4) Rf 0.44; υ_{max}(gum) 3424(OH); 2971(CH); 1785(ester CO); 1707(urethane CO); λ_{max}(MeOH) 265nm(ε 26000$dm^3mol^{-1}cm^{-1}$); 290nm(6600); 300nm(8100); δH($CDCl_3$, 360MHz); 7.76-7.25(8H, m, aromatic); 5.25(1H, t, J 5.56Hz, NH); 4.39(2H, s, β CH_2); 4.24-4.21(3H, s, ester CH_2, and obscured t, αCH); 5.98(2H, d, J 5.62Hz, glycine CH_2); 3.38(4H, s, CH_2OH); 0.87(3H, s, CH_3); δC(DMSO, 90MHz); 170.52 (ester CO); 156.78(Fmoc CO); 144.05, 140.98 (Fmoc quaternary); 127.84-120.30 (Fmoc aromatic CH); 66.79 (Fmoc CH_2); 66.03 (ester CH_2); 63.67 (2 x CH_2OH); 46.86 (Fmoc CH); 42.38 (glycine CH_2); 40.88 (aliphatic quaternary); 21.17(CH_3);m/z (FAB) 399 (MH^+), HRMS found 399.16553, $C_{22}H_{25}NO_6$ requires:399.16819

The pentafluorophenyl carbonate of 3-hydroxymethyl-3-nitro-1,5-dioxaspiro-5:5-undecane **(69)**

To a stirred flask of sodium dried toluene under an inert atmosphere of argon, compound **98** (1g, 5mM), triphosgene (0.5g, 1.66mM), and DIEA (0.9ml, 5mM) are added. After fifteen minutes a white precipitate was formed (DIEA.HCl), the first step in the reaction, formation of the chloroformate, can be adjudged to have been completed (confirmed by sharp IR peak C=O 1779cm^{-1} in toluene).

The chloroformate was reacted immediately in situ by the addition of a mixture of pentafluorophenol (1.1g, 5.5mM), and DIEA (0.9ml, 5mM), dissolved in

the minimum amount of toluene. The reaction was then stirred for a further fifteen minutes, (comparative IR indicates loss of sharp peak at 1779cm^{-1}, appearance of less well defined peak at 1787cm^{-1}). The reaction mixture was washed twice with iced water, followed by brine solution and dried over $MgSO_4$. The resultant solvent was then evaporated *in vacuo* to yield a white solid, which on recrystallization from hexane/ethyl acetate gave the *title compound* as large clear crystals. (2.09g, 95%); m.p. 121-123°C (found: C,46.26;H,3.62;N3.17, $C_{17}H_{16}NO_7$ requires:C,46.56;H,3.72;N,3.33%); t.l.c. (ethyl acetate/hexane, 3:7) Rf 0.65; υ_{max}(nujol) 1787 (CO) 1529 (NO_2); δH($CDCl_3$ 250MHz) 4.85 (2H, s, CH_2O-); 4.45 (2H, d, J 12.63Hz equatorial ring pair 2 x CH); 4.12 (2H, d, J 12.66Hz axial ring pair 2 x CH); 1.85-1.42 (10H, m, aliphatic ring CH_2); δC($CDCl_3$, 50MHz) 150.45 (CO); 100,01 (ring junction quaternary C); 83.15 (aliphatic quaternary C); 68.24 (CH_2O-); 60.42 (dioxane ring 2 x CH_2); 33.96-22.24 (aliphatic ring 5 x CH_2); m/z (FAB) 442 (MH^+), HRMS found: 442.09070; $C_{17}H_{16}NO_7$, requires: 442.09252.

The pentafluorophenyl carbonate of 3-hydroxymethyl-3-methyl-1,5-dioxaspiro-5;5-undecane **(78)**

To a stirred flask of sodium dried toluene under an inert atmosphere of argon, compound **74** (2.31g, 10mM), triphosgene (0.98g, 3mM), and DIEA (1.7ml, 10mM) are added. After fifteen minutes a white precipitate was formed (DIEA.HCl), and the first step in the reaction, formation of the chloroformate can be adjudged to have been completed (confirmed by sharp IR peak C=O 1779cm^{-1} in toluene).

The chloroformate was reacted immediately *in situ* by the addition of a mixture of pentaflurophenol (1.84g, 10mM), and DIEA (1.7ml, 10mM), dissolved in the minimum amount of toluene. The reaction was stirred for a further fifteen minutes before washing twice with iced water, followed by brine solution and dried over $MgSO_4$. The resultant solvent was then evaporated *in vacuo* to yield a white solid, which on recrystallization from hexane/ethyl acetate gave the *title compound* as large clear crystals. (3.86g, 95%); m.p. 101-102°C (found: C,52.68;H,4.63,

$C_{18}H_{19}O_5F_5$ requires: C,52.48;H,4.77%); t.l.c. (ethyl acetate/hexane, 1:1) Rf 0.76; υ_{max}(nujol) 1777 (CO); δH($CDCl_3$ 250MHz) 4.45 (2H, s, CH_2O-); 3.67 (4H, s, 2 x dioxane ring CH_2); 1.88-1.24 (10H, m, aliphatic ring CH_2); 0.89 (3H, s, CH_3); δC($CDCl_3$, 50MHz) 151.28 (CO); 98.26 (ring junction quaternary C); 73.05 (CH_2O-); 64.96 (2 x dioxane ring CH_2); 36.45-22.30 (5 x aliphatic ring CH_2); 35.01 (aliphatic quaternary) 17.19 (CH_3); m/z (FAB) 411 (MH^+), HRMS found: 411.12395; $C_{18}H_{19}O_5F_5$, requires: 411.12309.

The p-nitrophenyl carbonate of 5-hydroxymethyl-5-nitro-2-phenyl-1:3-dioxane **(67)**

To a stirred flask of DCM (100ml) under an inert atmosphere of argon, 5-hydroxymethyl-5-nitro-2-phenyl-1:3-dioxane (2g, 8.36mM) was added together with p-nitrophenylchloroformate (1.885g, 9.35mM) and pyridine (1.7ml, 21 mM). The mixture was stirred for 4 days and then washed with water (2 x 50ml) and dried over $MgSO_4$. The resultant solvent was evaporated *in vacuo* to give a white solid, recrystallization from ethyl acetate/hexane gave the *title compound* as a crystalline white solid. (1.98g, 59%);m.p.156-160°C (found:C,53.60;H,4.40;N,6.84; $C_{18}H_{16}N_2O_9$ requires: C,53.46;H,3.96;N,6.93%); t.l.c. (ethyl acetate/hexane, 1:1) Rf 0.50; υ_{max}(nujol) 1781 (CO) 1571 (NO_2); δH($CDCl_3$, 250MHz) 8.34 (2H, d, J 9.17Hz, 2 x NO_2 proximity aromatic CH); 7.60 (2H, d, J 9.15Hz, 2 x O proximity aromatic CH); 7,37 (5H, s, aromatic CH); 5.71 (1H, s, benzylidene CH); 4.89 (2H, d, J 12.77Hz, 2 x equatorial dioxane ring CH); 4.66 (2H, s, ester CH_2O-) 4.44 (2H, d, J 12.92Hz, 2 x axial dioxane ring CH); δC($CDCl_3$, 50MHz) 164.07 (CO); 155.02, 145.46, 137.19 (aromatic quaternary); 129.25-122.59 (5 aromatic CH); 100.62 (benzylidene CH); 85.22 (aliphatic quaternary); 67.72 (2 x dioxane ring CH_2); 67.32 (ester CH_2); m/z (FAB) 405 (MH^+), HRMS found:405.09463; $C_{18}H_{16}N_2O_9$, requires:405.09341.

The N-benzylamine carbamate of 5-hydroxymethyl-5-nitro-2-phenyl-1;3dioxane (97)

To a stirred flask of DCM (100ml) compound **67** (1g, 2.47mM) was added together with benzylamine (0.26g, 2.47mM) and DIEA (0.32g, 2.47mM). A strong yellow colouration was observed as p-nitrophenol is liberated. The reaction mixture was left overnight and then washed with citric acid (50ml), water (2 x 50ml) and dried over $MgSO_4$. The solvent was then removed *in vacuo* to leave an oil, wet flash chromatography eluting with ethyl acetate/hexane gave the *title product* as a white crystalline solid.(0.919g, 45%) m.p.140-142°C (found: C,61.40;H,5.59;N,7.40; $C_{19}H_{20}O_6N_2$ requires: C,61.26;H,5.37;N,7.52%); t.l.c. (ethyl acetate/hexane, 7:3) Rf 0.72; υ_{max} (KBr) 3276 (NH) 1717(CO) 1553 (NO_2); δH(DMSO, 360MHz) 8.01 (1H, t, J 6.07Hz, NH); 7.38-7.22 (10H, m, aromatic CH); 5.67 (1H, s, benzylidene CH); 4.79 (2H, d, J 12.87Hz, 2 x equatorial ring CH); 4.37 (2H, s, ester CH_2); 4.35 (2H, d, J 12.37Hz, 2 x axial ring CH); 4.18 (2H, d, J 6.12Hz, CH_2NH); δC(DMSO, 90MHz) 155.3 (urethane CO); 139.37, 137.29 (aromatic quaternary); 129.23-126.15 (aromatic CH); 100.545 (benzylidiene CH); 86.06 (aliphatic quaternary); 67.95 (2 x dioxane ring CH_2); 63.12 (ester CH_2); 43.96 (CH_2NH); m/z (FAB) 373 (MH^+), HRMS found:373.14240; $C_{19}H_{20}O_6N_2$, requires:373.13996.

The N-benzylamine carbamate of tris(hydroxymethyl)nitromethane (89)

Compound **97** (0.50g, 1.34mM) was added to a mixture of 9:1 TFA/H20 (25ml) and the mixture stirred under an inert atmosphere of nitrogen for 3 hours. The TFA was then removed *in vacuo*, water added, and the white solid produced re-extracted with ethyl acetate (2 x 50ml), The ethyl acetate was dried with $MgSO_4$ and then absorbed on to silica. Wet flash chromatography eluting with ethyl acetate/hexane gave the *title compound* as a white solid. (0.31g, 82%); (found: C,50.63;H,6.04;N,9.58, $C_{12}H_{16}N_2O_6$, requires: C,50.68;H,5.67;N,9.58%); t.l.c. (ethyl acetate/hexane, 7:3) Rf 0.45; υ_{max}(KBr) 3310 (OH) 1698 (CO) 1562 (NO_2); δH(DMSO, 250MHz) 7.35-7.20 (5H, m, aromatic CH); 5.54 (1H, t, J 5.843 NH); 4.57 (2H, s, ester CH_2O-); 4.31 (2H, d, J 5.96 CH_2NH-); 3.96 (4H, s, 2 x CH_2OH); δC(DMSO, 90 MHz) 155.78 (CO);

139.579 (aromatic quaternary); 128.401, 127.218, 126.977 (aromatic CH); 93.44 (aliphatic quaternary); 60.36 (ester CH_2O-); 59.47 (CH_2OH); 43.94 (CH_2NH-); m/z (FAB) 285 (MH+), HRMS found: 285.10863; $C_{12}H_{16}N_2O_6$, requires: 285.10866.

Pentachlorophenyl,4-formylphenoxyacetate **(40)**

4-Formylphenoxyacetic acid (4.80g, 26.6mM) was dissolved in DCM (200ml) and to this was added DCC (5.479g 26.6mM) the mixture was sonicated for 15 minutes. A solution of pentachlorophenol (7.25g, 27.2mM) in DCM was then added together with DMAP (1mM, catalytic amount). The resultant mixture was stirred at 5°C for twelve hours. The white solid formed (urea) was filtered off and the resultant solvent washed with water, dried with $MgSO_4$, and absorbed onto silica. Dry flash chromatography eluting with ether/hexane gave the *title compound* as a white crystalline solid. (8.5g, 75%) m.p. 112-115°C (found: C,42.13;H,1.76; $C_{15}H_7O_4Cl_5$ requires: C,42.01;H,1.65%); t.l.c. (ethyl acetate/hexane, 3:7) Rf 0.71, υ_{max}(nujol) 1783 (ester CO); 1677 (aldehyde CO); δH($CDCl_3$, 200MHz) 9.91 (1H, s, aldehyde CH); 7.90 (2H, d, J 8.90, aromatic CH); 9.37 (2H, d, J 8.82, aromatic CH); 5.10 (2H, s, CH_2); δC(DMSO, 50MHz) 191.44 (aldehyde CH); 170.19 (CO); 162.77, 158.32, (aromatic chloro quaternary); 150.46 (aromatic quaternary); 127.62, 114.15 (aromatic CH); 130.10, 121.46 (aromatic quaternary); 66.36 (CH_2); m/z (FAB) 428 (MH+), HRMS found: 428.88519; $C_{15}H_{17}O_4C_{15}$, requires: 428.88357.

5-Hydroxymethyl-5-nitro-2-(4-pentachlorophenoxyacetate) phenyl-1:3-dioxane ester **(42)**

To a stirred 500ml flask of sodium dried toluene (250ml), compound **40** was added (8g, 18.69mM) together with tris(hydroxy)nitromethane (4.25g, 28mM) and a catalytic amount of TsOH (100mg). The resultant mixture was then refluxed under Dean and Stark conditions for 24hrs. The toluene was removed *in vacuo* and the resultant gum re-dissolved in ethyl acetate. This solution was then washed with brine (2 x 100ml), water (2 x 100ml) and dried with $MgSO_4$. The ethyl acetate was

removed *in vacuo* to give a yellow solid, recrystallisation of this solid from ethyl acetate/hexane gave the title compound as white crystals. (9g, 85%); m.p. 156-162°C (found:C,40.69;H,2.27;N,2.40; $C_{19}H_{14}NO_8Cl_5$ requires: C,40.64;H,2.49;N,2.49%); t.l.c. (ethyl acetate/hexane, 7:3) Rf 0.43; υ_{max}(nujol) 3552 (OH); 1801 (CO); 1544 (NO_2); δH($CDCl_3$, 250MHz) 7.26 (2H, d, J 8.81, 2 x aromatic CH); 6.89 (2H, d, J 8.85, 2 x aromatic CH); 5.57 (1H, s, benzylidene CH); 4.76 (2H, d, J 12.75, dioxane ring equatorial CH's); 4.66 (2H, s, ester CH_2O); 4.20 (2H, d, J 12.82, dioxane ring axial CH's); 3.69 (2H, s, CH_2OH); δC($CDCl_3$, 60MHz) 170.18 (CO); 158.23, 150.36, 130.44, 121.48, (aromatic quaternary); 127.47, 114.04 (aromatic CH); 100.41 (benzylidene CH); 88.13 (aliphatic quaternary); 68.22 (aliphatic ring CH_2); 64.50 (ester CH_2O-); 62.40 (CH_2OH); m/z (FAB) 562 (MH+), HRMS found 561.92111, $C_{19}H_{14}NO_8Cl_5$ requires 561.92107

The 5-hydroxymethyl-5-nitro-2-(4-pentachlorophenoxyacetate)phenyl-1,3-dioxane ester of 9-fluorenylmethoxycabonylglycine **(44)**

To a stirred flask of DCM (100ml) Fmoc-glycine (0.5g, 1.68mM) was added together with DCC (0.34g, 1.68mM) the mixture was sonicated for 15 mins and then cooled in an acetone dry ice bath for 30mins. Compound **42** (1g, 1.78mM) was then added and allowed to dissolve. To the resultant solution a catalytic amount of DMAP (10mg) was added (care should be taken as the addition of too much DMAP results in the loss of the PCP group and an impure product) and the mixture transferred from the acetone bath to the cold room. The mixture was stirred for a further 3 hours and then worked up. The white solid was filtered off and the solvent washed with water (2 x 50ml), and dried with $MgSO_4$. Removal of the solvent *in vacuo* yields a gum, trituration of this gum with hexane provides a white solid which was pure enough for attachment to a resin. Improved purity was obtained by wet flash chromatography: Chromatography of the above solid eluting with ethyl acetate/hexane gave the title compound (1.28g, 90%); m.p.178-182°C (found: C,51.69;H,3.52;N,3.23, $C_{36}H_{27}O_{11}N_2Cl_5$ requires: C,51.42;H,3.21;N,3.23%); t.l.c. (ethyl acetate/hexane, 6:4) Rf 0.48; υ_{max}(KBr)3324 (NH); 2928 (CH); 1762 broad (CO, glycine & Pfp ester);

1726 (CO, urethane); 1553 (NO_2); δH(DMSO, 360MHZ) 7.88-6.87 (11H, m, aromatic CH); 5.59 (1H, s, benzylidene CH); 4.77 (2H, d, J 12.41 dioxane ring equatorial CH's); 4.66 (2H, s, -O*CH*$_2$CO); 4.46 (2H, s, ester CH_2O-); 4.34-4.27 (5H, m, dioxane ring axial CH's, α CH, β CH_2); 3.80 (2H, d, J 5.77Hz glycine CH_2); δC(DMSO, 90MHz) 169.92 (CO, PCP); 169.38 (CO, ester); 156.41 (CO, urethane); 158.09, 156.41 (aromatic chloro quaternary); 143.63, 140.61 (Fmoc quaternary); 130.01, 129.92, 121.29 (benzyl quaternary); 127.53, 127.26, 126.98, 125.02 (aromatic Fmoc CH); 119.99, 113.86 (benzyl CH); 100.19 (benzylidene CH); 85.25 (aliphatic quaternary); 67.53 (dioxane ring CH_2); 65.75 (Fmoc CH_2); 64.30, 63.03 (ester CH_2); 46.46 (Fmoc CH); 41.85 (glycine CH_2); m/z (FAB) 841 (MH^+); HRMS found: 841.01064; $C_{36}H_{27}O_{11}N_2Cl_5$, requires: 841.01061.

The tris(hydroxymethyl)nitromethane α ester of Ala-Asp-Leu-Ile-Leu-(D)Phe-Gly **(45)**

Boc-Glu(OBzl)OCH_2-PAM resin (O.5mM) was sonicated in 10ml of 9:1, TFA/H_2O for 20 mins. The resin was then washed with DMF, DCM, and diethyl ether. A Kaiser test was performed to check the removal of the Boc protecting group. The above resin (500mg, 0.64mM/g substitution) was then sonicated in DMF with compound **44** (1g, 1.18mM) and TEA (100μl, 0.72mM) for 12 hours. The resin was then filtered and washed with DMF, DCM and ether (loading 78%, weigh 625mg). The above peptide was then synthesised using this functionalized linker. All side chains were protected as described previously and all residues were coupled using standard double coupling cycles. The first two amino acids were introduced as a dipeptide to avoid the problem of diketopiperazine formation. At the completion of the assembly stage, the resin bound peptide was sequentially washed with DMF, DCM, diethyl ether and dried to give 564mg of product. The peptide was then cleaved from a 176mg batch of resin by stirring with 9:1, TFA/H_2O for 2 hours at room temperature. The resin was then removed by filtration and washed with TFA. Evaporation of the filtrate and trituration with diethyl ether yielded the crude peptide which was isolated by filtration, before being dissolved in aqueous acetic acid (10%)

and lyophilised to yield 25mg of white solid. The peptide was purified using semi-preparative HPLC (Aquapore C18, 250 x 10mm, 10μM, A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10% B for 2 mins then 10-90% B over 30 mins. λ=214nm). The main peak eluting at 51% B was isolated and lyophilised to give 10mg of the title compound. Analytical HPLC (C18 Aquapore Rp300 220 x 4.6mm ,7μm, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min . 10-90%B over 30 min. λ=214nm) Rt=14.2mins, 38%B; amino acid analysis: Asp_1 1.18, Gly_1 1.17, Ala_1 1.12, Ile_1 1.00, Leu_2 1.85, Phe_1 1.08; m/z (FAB) 881 (MH^+), HRMS found 881.46049, $C_{40}H_{65}N_8O_{14}$ requires 881.46202.

The 5-hydroxymethyl-5-nitro-2-phenyl-1;3-dioxane ester of N-benzyloxycarbonylphenylalanine **(61)**

N-benzyloxycarbonylphenylalanine (D/L) (1g, 3.34mM) was dissolved in DCM (50ml) to this was added DCC (0.688g, 3.34mM) the mixture was sonicated for 15mins. A solution of compound **53** (1g, 4.18mM) in DCM was then added together with a catalytic amount of DMAP (0.036g, 0.3mM). The resultant mixture was stirred at 5°C for 1 hour and then at room temperature for a further 2 hours. The white solid formed (urea) was filtered off and the filtrate washed with Na_2CO_3 (2 x 50ml) and water (2 x 50ml). The solution was dried over $MgSO_4$ then evaporated *in vacuo* to leave a gum. Purification by wet flash chromatography eluting with ethyl acetate/hexane gave the title compound as oily gum which on drying became a foam. (1.47g, 85%). (Found: C,64.38;H,5.44;N,5.18; $C_{28}H_{28}N_2O_8$ requires: C,64.55;H,5.37;N,5.37%); t.l.c. (ethyl acetate/hexane, 7:3) Rf 0.6; υ_{max}(gum) 3332 (NH); 3032 (aromatic CH); 2968 (aliphatic CH); 1752 (ester CO); 1712 (urethane CO); 1554cm^{-1} (NO_2); δH(DMSO, 360MHz) 7.92 (1H, d, J 7.85Hz NH); 7.38-7.23 (15H, m, aromatic CH); 5.66 (1H, s, benzylidene CH); 5.00 (2H, d, J 3.77 benzyl CH_2); 4.45 (2H, s, ester CH_2); 4.82-4.70 (2H, d of d, J 12.80 diasteriomeric dioxane ring axial CH_2's); 4.45 (2H, s, ester CH_2) 4.33-4.25 (3H, m, diasteriomeric dioxane ring equatorial CH_2's and α CH); 3.03-2.85 (2H, m, β CH_2); δC(DMSO, 90MHz) 171.158 (ester CO); 156.17 (urethane CO); 137.32, 137.28, 136.89 (aromatic

quaternary); 129.31-126.82 (aromatic CH); 100.58 (benzylidene CH); 85.56 (aliphatic quaternary); 67.83, 67.74 (dioxane ring CH_2); 65.77 (benzylmethoxy CH_2); 63.47 (ester CH_2); 55.58 (α CH); 36.25 (β CH_2); m/z (FAB) 519 (MH^+), HRMS found 521.19205, $C_{28}H_{28}N_2O_8$ requires 521.19239.

The tris(hydroxymethyl)nitromethane ester of N-benzymethloxycarbonylphenylalanine **(62)**

Compound **61** (0.5g, 0.96mM) was added to TFA/water 95:5 (5ml) the mixture was stirred under nitrogen for 3 hours. The solvent was then removed *in vacuo*, water added, and the white solid product re-extracted into ethyl acetate (2 x 50m). The ethyl acetate was dried with $MgSO_4$ and the product absorbed from this solvent onto silica and then purified by wet flash chromatography. Eluting with ethyl acetate/hexane gave the *title compound* as a yellow oil. (0.35g, 84%); $[\alpha]_D$=O.O°, c=1.0 in MeOH; (found: C,57.73;H,6.10;N,6.40, $C_{21}H_{25}N_2O_8$ requires: C,58.17;H,5.81;N,6.46%); t.l.c. (ethyl acetate/hexane, 7:3) Rf 0.5, υ_{max}(oil) 3409 (OH); 3035 (aromatic CH); 2940 (aliphatic CH); 1745 (ester CO); 1694 (urethane CO); 1551 (NO_2); δH(DMSO, 360MHz) 7.82 (1H, d, J 8.43 NH); 7.37-7.16 (10H, m, aromatic CH); 5.47 (2H, t, J 5.45 OH); 4.96 (2H, s, benzyl CH_2); 4.55-4.43 (2H, d of d, J 11.62Hz, diasteriotopic ester CH_2); 4.33 (1H, m, α CH); 3.84-3.69 (4H, m,CH_2OH); 3.16-3.03 (2H, m, βCH_2); δC($CDCl_3$,) 171.10 (ester CO); 156.22 (urethane CO); 137.50, 136.92 (aromatic quaternary); 129.162-126.140 (aromatic CH); 93.39 (aliphatic CH); 65.60 (benzyl CH_2); 60.28 (ester CH_2) 59.87, 59.81 (CH_2OH); 55.49 (αCH); 36.36 (βCH_2); (FAB) 453 (MNa^+) HRMS found: 455.14297; $C_{21}H_{25}N_2O_8Na$, requires: 455.14307.

N-benzyloxycarbonylphenylalanine piperidide **(63)**

Benzylmethoxycarbonylphenylalanine (D/L) (0.5g, 1.67mM) is dissolved in DCM, to this DCC (0.344g, 1.67mM) is added and the mixture sonicated for 15mins. Piperidine (0.165ml, 1.67mM) was then added and the mixture stirred for a further 2 hours. The mixture was then washed with brine (2 x 25ml) and water (2 x 25ml) and dried over $MgSO_4$. The DCM was removed *in vacuo* and a white solid triturated out with hexane, recrystallisation from ethyl acetate/hexane gave the *title compound* as clear crystals. m.p.225-229°C; (Found: C,72.13;H,7.88;N,7.86, $C_{28}H_{26}N_20_3$ requires:C,72.13;H,7.70;N,7.6%); t.l.c. (ethyl acetate/hexane 7:3) Rf 0.64, υ_{max}(KBr) 3242 (NH); 3030 (aromatic CH); 2927 (aliphatic CH); 1706 (urethane CO); 1633 (amide CO); δH(DMSO, 360MHz) 7.64 (1H, d, J 8.39Hz, NH); 7.35-7.19 (10H, m, aromatic CH); 4.97 (2H, d, J 3.63Hz, benzyl CH_2); 4.67 (1H, d of d, J 8.146, 8.078 αCH); 2.93-2.49 (2H, m, βCH_2); δC(DMSO, 60MHz); 169.19 (amide CO); 155.72 (urethane CO); 137.72,137.13 (aromatic quaternary); 129.42-126.43 (aromatic CH); 65.34 (benzyl CH_2); 51.83 (αCH); 45.98,42.56 (aliphatic ring NCH_2-); 37.68 (βCH_2); 25.83,25.29,24.02 (aliphatic ring CH_2); m/z (FAB) 367 (MH+); HRMS found 367.19994, $C_{28}H_{26}N_20_3$ requires: 367.20217

t-Butoxycarbonylaminoundecanoic acid **(70)**

Aminoundecanoic acid (2g, 9.9mM) was dissolved in DMF/TEA, 10:1 (50ml) to this mixture di-t-butyl dicarbonate (4.365g, 20mM) was added. The mixture was heated to 80°C and stirred until all the white slurry has dissolved and the solution become clear. The DMF was partially removed *in vacuo* and the mixture poured into ice cold water acidified to pH 2 with citric acid. The solid was then re-extracted using ethyl acetate and the resultant solution dried over MgSO4. The solvent was removed *in vacuo* to give a white solid, recrystallisation from ether/hexane gave the *title compound* as white crystals. (2.5g, 83.5%) m.p.63-65°C

(found:C,63.55;H,10.45;N,4.55, $C_{16}H_{31}NO_4$ requires: C,63.78;H,10.29;N,4.64%); t.l.c. (ethyl acetate/hexane, 1:1)Rf 0.37; υ_{max} (nujol) 3365 (NH); 3000-2500 (broad acid OH); 1684 (urethane & acid CO); δH($CDCl_3$, 250MHz); 2.89 δC(DMSO, 60MHz); 174.51 (acid CO); 155.64 (urethane CO); 77.31 (aliphatic quaternary); 33.74, 29.55, 29.04, 28.92, 28.81, 28.64 (CH_2); 28.32 (CH_3); 26.34, 24.57 (CH_2); m/z (FAB) 302 (MH+), HRMS found: 302.23384; $C_{16}H_{31}NO_4$ requires: 302.23313.

9-Fluorenylamido,t-butoxycarbonylamidoundecane **(71)**

Compound **70**, (0.692g, 2.29mM) was added to DCM (50ml) together with DCC (0.473g, 2.29mM) and the mixture was sonicated for 15 mins. A solution of 9-aminofluorene.HCl (0.5g, 2.29mM) in DCM was then added together with TEA (O.35ml, 2.51mM) and the mixture stirred for 8 hours. The white solid (urea) was filtered off and the filtrate washed with 5% aqueous citric acid (2 x 25ml), water (2 x 25ml), and dried over $MgSO_4$. The solvent was removed *in vacuo* and a white solid formed on tituration with ethyl acetate/hexane, recrystallization from ethyl acetate gave the *title compound* as fine white crystals. (0.7g, 77%); m.p. 136°C (found: C,75.04;H8.96;N,6.37; $C_{29}H_{40}N_2O_3$ requires: C,74.96;H,8.96;N,6.03%); t.l.c.(ethyl acetate/hexane, 1:1) Rf 0.54; υ_{max}(nujol) 3354 (urethane NH); 3311 (amide NH); 1675 (urethane CO); 1640 (amide CO); λ_{max}(MeOH); 269 (ε 73000 dm^3 mol^{-1} cm^{-1}); δH(CDCl3, 200MHz); 7.65-7.21 (8H, m, aromatic CH); 6.21 (1H, d, J 8.91Hz fluorene NH); 5.93 (1H, d, J 8.75Hz fluorene CH); 6.12 (2H, q, 6.52Hz, -NH*CH_2*-); 2.24 (1H, t, 7.2Hz, -*NH*CH_2-); 1.39 (8H, s, CH_2); 1.24 (9H, s, CH_3); δC($CDCl_3$, 50MHz); 173.79 (amide CO); 155.82 (urethane CO); 144.33, 140.38 (aromatic quaternary); 128.43, 127.54, 124.90, 119.77(aromatic CH); 78.88 (aliphatic quaternary); 54.43 (fluorene CH); 40.43, 36.67, 29.88, 29.25, 29.20, 29.07 (CH_2); 28.25 (CH_3); m/z (FAB) 465 (MH^+), HRMS found: 465.31677; $C_{29}H_{40}N_2O_3$ requires: 465.31172

9-Fluorenylamidoaminoundecane (**72**)

Compound **71**, (0.5g, 1.07mM) was added to 20ml of TFA/H_20, 9:1 and stirred for four hours under nitrogen. The TFA was then partially removed *in vacuo* and the concentrated mix pored into 5% sodium bicarbonate. The product was re-extracted with ethyl acetate, washed with water, and dried over $MgSO_4$. Removal of the solvent *in vacuo* gave the product as a very sticky gum/oil. HPLC (C18 Vydac 250 x 4.6 x 5μm), A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10% B for 2 mins then 10-90% B over 30 mins; λ=214nm); Rt=22.4 mins, 65%B); υ_{max}(nujol) 3271 (NH_2); 1675 (amide CO); λ_{max}(MeOH); 269 (ε 81000 dm^3mol^{-1} cm^{-1}); δH(DMSO, 250MHz); 8.40 (1H, d, J 8.40Hz, NH); 7.85-7.28 (aromatic CH); 6.04 (1H, d, J 8.35Hz, fluorene CH); 2.78 (2H, m, CH_2NH_2); 2.19 (2H, t, J 7.2, CH_2NH_2); δC(DMSO, 60MHz); 173.01 (CO); 145.06, 140.11 (aromatic quaternary); 128.39, 127.64, 124.79, 120.19, (aromatic CH); 54.07 (flourene CH); 38.91, 35.36, 28.84, 28.62, 27.06, 25.86, 25,51 (CH_2); m/z (FAB) 365 (MH+); HRMS found: 365.25822, $C_{24}H_{33}N_2O$ requires: 365.25929

The 5-hydroxymethyl-5-nitro-2-phenyl-1:3-dioxane carbamate of 9-fluorenylamidoundecane (**73**)

To a stirred flask of DMF (50ml) compound **67**, (0.655g, 1.4mM) is added together with compound **72**, (0.555g, 1.37mM) and TEA, (0.155g,1.54mM) the reaction mixture is stirred for a further twelve hours (note strong yellow colour indicates displacement of p-nitrophenol). The solvent was then removed *in vacuo* to give a sticky gum, this gum was re-dissolved in ethyl acetate, and then washed with 5% citric acid (50ml), water (2 x 50ml) and dried over $MgSO_4$ before being absorbed on to wet flash silica. Elution with ethyl acetate/hexane gave the *title compound.* (0.41g, 48%). m.p.100-104°C (found: C,68.47;H,6.83;N,6.79; $C_{36}H_{43}N_3O_7$ requires: C,68.64;H,6.83;N,6.67; t.l.c. (ethyl acetate/hexane 1:1) Rf 0.51; υ_{max}(nujol) 3325 (b,

NH); 1712 (CO, urethane); 1630 (CO, amide); 1559 (NO_2); λ_{max}(MeOH); 269 (ε 74000 $dm^3mol^{-1}cm^{-1}$); δC(CDCl3, 360MHz) 7.69-7.25 (13H, aromatic CH); 6.23 (1H, d, J 8.89Hz, fluorenyl NH); 5.77 (1H, d, J 8.77Hz, fluorenyl CH); 5.49 (1H, s, benzylidene CH); 5.01 (2H, d, J 12.95Hz, 2 x equatorial ring CH_2); 4.23 (2H, s, carbamate CH_2); 4.08 (2H, d, J 12.85Hz, 2 x axial ring CH_2); 3.12 (2H, q, J 6.42Hz, -CH_2*CH_2*NH-); 2.25 (2H, t, J 7.63Hz, -CO*CH_2*CH_2-); 1.27 (8H, bs, CH_2); δC($CDCl_3$, 90MHz); 173.92 (amide CO); 154.69 (urethane CO); 144.28, 140.45, (fluorenyl quaternary); 136.65 (aromatic quaternary); 101.82 (benzylidene CH); 86.74 (aliphatic quaternary); 68.6 (dioxane ring CH_2); 63.56 (carbamate CH_2); 54.53 (fluorenyl CH); 41.13-25.67 (CH_2); m/z (FAB) 631 (MH^+), HRMS found: 630.31636; $C_{36}H_{43}N_3O_7$; requires: 630.31793.

The tris(hydroxymethyl)nitromethane carbamate of 9-fluorenylamidoundecane **(66)**

To a flask of TFA/H_2O, (9:1), (10ml), under an inert atmosphere of nitrogen compound **73** (1g, 1.58mM) was added. The mixture was stirred for three hours, the TFA was then removed *in vacuo* and water added, the cloudy white product was then extracted into ethyl acetate (2 x 50ml). The ethyl acetate was dried with $MgSO_4$ and the product absorbed from this solvent onto silica and then purified via wet flash chromatography. Elution with ethyl acetate/hexane gave the *title compound* as a gum. (0.78g, 92%); HPLC (C18 Vydac 250 x 4.6 x 5μm), A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 10% B for 2 mins then 10-90% B over 30 mins; λ=300nm; Rt=25.34 mins, 72.24%B); υ_{max}(KBr) 3280 b,(OH); 2924 (CH); 1688 (urethane); 1637 (amide); 1548 (NO_2); λ_{max}(MeOH); 269 (ε 76000 dm^3mol^{-1} cm^{-1}); δH(DMSO, 360MHz) 8.38 (1H, d, J 8.53Hz, fluorenyl NH); 7.85-7.21 (8H, m, aromatic CH); 6.01 (1H, d, J 8.63Hz, fluorenyl CH); 4.32 (2H, s, ester CH_2); 3.78 (4H, s, CH_2OH); 2.95 (2H, q, J 6.32Hz, -CH_2*CH_2*NH-); 2.19 (2H, t, J 7.1Hz, -$COCH_2$-); 1.27-1.24 (8H, bs, undecane CH_2's); δC(DMSO, 90MHz); 173.42 (amide CO); 155.52 (urethane CO); 145.09,140.25 (aromatic quaternary); 128.45,127.72,124.85,120.25 (aromatic CH); 93.43 (aliphatic CH); 60.16 (ester CH); 59.40 (CH_2OH); 40.42 (-

CH_2CH_2NH-); 35.40 (-$COCH_2CH_2$-); 29.42-25.57 (-CH_2-); m/z (FAB) 542 (MH^+); HRMS found: 542.28508, $C_{29}H_{39}N_3O_7$ requires:542.28663.

N^{α}–9–fluorenylmethoxycarbonyl-N^{ε}-1:5-dioxaspiro-5:5undecane-3-nitro-3-methoxycarbonyl-lysine.**(83)**

To a stirred solution of dioxane/H_2O (1:1, 50ml), at $O^{\circ}C$ Fmoc lysine (0.5g, 1.357mM) was added together with compound **69** (0.9, 1.587mM) and TEA (0.379ml, 2.714mM). The reaction mixture was then allowed to warm to room temperature and stirred over night. The reaction mixture is then concentrated *in vacuo* and the residue dissolved in ethyl acetate. The ethyl acetate is washed with brine (2 x 50ml) and water (2 x 50ml) before being dried over $MgSO_4$. The product was then absorbed from the dry solvent onto wet flash silica, elution using DCM/MeOH (9:1) gave the *title compound* as a white solid. (0.68g, 81%); $[\alpha]_D$= $+12^{\circ}$, c=1.0 in MeOH; m.p.172°C; (found: C,61.10H,6.63N,6.41 $C_{32}H_{39}O_{10}N_3$; requires: C61.40,H6.30,N6.71); t.l.c.(DCM/MeOH, 9:1) Rf 0.30; υ_{max}(KBr) 3408 (b, NH); 2937 (CH); 1708 (CO); 1552 (NO_2); λ_{max}(MeOH) 265nm (ε 19000 $dm^3\ mol^{-1}\ cm^{-1}$), 290 (4800), 300 (5700); δH(DMSO, 250MHz); 7.90-7.30 (8H, m, aromatic CH); 4.39-4.33 (6H, bm, 2 x equatorial dioxane ring CH, carbamate CH_2, Fmoc CH_2); 4.23-4.12 (4H, bm, 2 x axial dioxane ring CH_2, α CH, Fmoc CH); 2.91 (2H, bs, -$NHCH_2$-); 1.84-1.23 (16H, bm, CH_2); δC(DMSO, 60MHz); 173.37, (CO, acid); 155.79, 155.01 (CO, urethane); 143.96,140.84 (Fmoc quaternary); 129.06,127.42,121.52,120.17 (Fmoc CH); 98.65 (aliphatic ring junction quaternary); 85.64 (aliphatic quaternary); 65.45 (Fmoc CH_2); 63.24 (ester CH_2); 60.50 (dioxane ring CH_2); 46.89 (Fmoc CH); 38.60, 28.23, 25.03, 22.20 (βCH_2 + ring CH_2); 30.82 (αCH); m/z (FAB) 665 (MH^+), HRMS found 664.22861 $C_{32}H_{39}N_3O_{10}K$ requires:664.22725.

N^{α}-9-fluorenylmethoxycarbonyl-N^{ε}-1,5-dioxaspiro-5:5undecane-3-methyl-3-methoxycarbonyl-lysine. **(79)**

To a stirred solution of dioxane/H_2O (1:1, 50ml) at O°C Fmoc lysine (1g, 2.714mM) was added together with compound **78** (1.3g, 3.174mM) and TEA (0.758ml, 5.428mM). The reaction mixture was allowed to warm to room temperature and stirred overnight. The reaction mixture was then concentrated *in vacuo* and the residue dissolved in ethyl acetate. The ethyl acetate was washed with brine (2 x 50ml) and water (2 x 50ml) before being dried over $MgSO_4$. The product was then absorbed from the dry solvent on to wet flash silica, elution using DCM/MeOH (9:1) gave the *title compound* as a white foam. (1.12g, 70%); $[\alpha]_D$=24°, c=1.0 in MeOH; (found: C,66.53;H,7.07;N,4.76; $C_{33}H_{43}N_2O_8$ requires: C,66.60;H,7.07;N,4.76); t.l.c.(dichloromethane/methanol 9:1) Rf 0.32; υ_{max}(nujol) 3313 (NH); 1701 (b, CO); λ_{max}(MeOH) 265nm (ε 22000 $dm^3\,mol^{-1}\,cm^{-1}$), 290 (6000), 300 (7300); δH(DMSO, 360MHz); 7.88-7.30 (8H, m, aromatic CH); 4.28-4.19 (3H, m, Fmoc CH_2 & α CH); 3.93 (2H, s, ester CH_2); 3.53 (4H, d of d, J 7.57Hz, equatorial & axial dioxane ring CH); 2.96 (2H, q, J 6.04Hz, -NH*CH_2*-); 1.73-1.24 (16H, m, CH_2); 0.77 (3H, s, CH_3); δH(DMSO, 360MHz); 174.27 (acid CO); 156.57, 156.28 (urethane CO); 144.02, 143.95 (aromatic quaternary); 127.78, 127.21, 125.43, 120.22 (aromatic CH); 97.36 (aliphatic quaternary, ring bridge carbon); 66.51 (2 x dioxane ring CH_2); 64.65 (Fmoc CH_2); 59.91 (carbamate CH_2); 53.37 (αCH); 46.83 (Fmoc CH); 41.70-22.28 (CH_2); 33.61 (aliphatic quaternary C); 17.57 (CH_3); m/z (FAB) 595 (MH^+); HRMS found 595.30170; $C_{33}H_{43}N_2O_8$ requires:595.30194

The 3-hydroxymethyl-3-methyl-1:5-dioxaspiro-5:5-undecane carbamate of 9-fluorenylamidoundecane.**(81)**

To a stirred flask of DMF (50ml) compound **72**, (0.55g, 1.51mM) was added together with compound **78**, (0.62g, 1.51mM) and TEA, (0.30, 3.00mM) the reaction mixture was stirred for a further twelve hours. The solvent was then removed *in vacuo* to give a sticky gum, this was re-dissolved in ethyl acetate, the ethyl acetate

was then washed with 5% citric acid (50ml), water (2 x 50ml), and dried over $MgSO_4$ before being absorbed on to wet flash silica. Elution with ethyl acetate/hexane gave the title compound as a white solid. (0.70g, 78%) m.p. 90-94°C; (found: C,72.93;H,8.64;N,4.48; $C_{36}H_{50}N_2O_5$ requires: C,73.20;H,8.47;N,4.75); t.l.c.(ethyl acetate/hexane)Rf 0.53; υ_{max}(nujol) 3276 (NH); 1695 (CO urethane); 1639 (CO amide); λ_{max}(MeOH); 269 (ε 79000 $dm^3mol^{-1}cm^{-1}$); δH (DMSO, 360MHz) 8.4 (1H, d, J 8.42Hz NH); 7.85-7.29 (8H, m, aromatic CH); 6.04 (1H, d, J 8.33Hz CH); 3.93 (2H, s, ester CH_2); 3.58 (2H, d, J 11.43Hz, 2 x equatorial dioxane ring CH); 3.52 (2H, d, J 11.56Hz, 2 x axial dioxane ring CH); 2.96 (2H, q, 6.29Hz ,CH_2CH_2NH-); 2.21 (2H, t, 7.26Hz, -COCH_2CH_2-); 1.75- 1.03 (18H, m, CH_2); (3H, s, CH_3) δC(DMSO, 90MHz); 173.31 (amide CO); 156.47 (urethane CO); 145.09, 140.121, (fluorenyl quaternary); 128.38, 127.65, 124.82, 124.82, 120.19 (fluorenyl CH); 97.28 (aliphatic quaternary, ring junction C); 66.08 (carbamate CH_2); 64.58 (dioxane ring CH_2); 54.04 (fluorenyl CH); 40.29-22.17 (CH_2); 33.57 (aliphatic quaternary C);17.54 (CH_3); m/z (FAB) 591 (MH^+); HRMS found: 591.38071; $C_{36}H_{50}N_2O_5$ requires: 591.37980.

The tris(hydroxymethyl)ethane carbamate of 9-fluorenylamidoundecane **(82)**

To a flask of TFA/H_2O, (9:1), (10ml), under an inert atmosphere of nitrogen compound **81**, (0.5g, 0.85mM) is added. The mixture was stirred for three hours the TFA removed *in vacuo*, water added and the cloudy white product re-extracted into ethyl acetate (2 x 50ml). The ethyl acetate was dried with $MgSO_4$ and the product absorbed from this solvent onto silica and then purified via wet flash chromatography. Elution with ethyl acetate/hexane gave the *title compound* as an oil. (0.38g, 89%); HPLC (Vydac C18, 250 x 4.6mm, 5μM, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 10% B for 2 mins then 10-100% B over 35 mins; λ=300nm); Rt=31.28 mins, 85.59%B; t.l.c. (ethyl acetate/hexane 1:1) Rf 0.46; υ_{max}(film); 3301(NH); 1690 (CO urethane); 1637 (CO amide); λ_{max}(MeOH); 269 (ε 80000 $dm^3mol^{-1}cm^{-1}$); δH(DMSO, 360MHz) 8.40 (1H, d, J 8.39Hz, fluorenyl NH); 7.85-7.29 (8H, m, aromatic CH); 6.03 (1H, d, J 8.36Hz, fluorenyl CH); 4.39 (1H, t, J

5.32Hz, NH); 3.78 (2H, s, ester CH_2); 3.24 (4H, m, CH_2OH); 2.95 (2H, q, J 6.55Hz, -CH_2CH_2NH-); 2.19 (2H, t, J 7.25Hz, -$COCH_2$-); 1.27-1.24 (8H, bs, undecane CH_2's); 0.76 (3H, s, CH_3); δC(DMSO, 90MHz); 173.40 (amide CO); 156.79 (urethane CO); 145.06, 140.13 (aromatic quaternary); 128.44,127.70,124.84,120.24 (aromatic CH); 65.98 (ester CH); 63.648 (CH_2OH); 54.065 (florenyl CH); 40.97 (-CH_2CH_2NH-); 35.38 (-$COCH_2CH_2$-); 29.42-25.57 (-CH_2-); 16.54 (CH_3); m/z (FAB) 511 (MH^+); HRMS 511.32198 $C_{30}H_{42}N_2O_5$ requires: 511.3172

Phe-Gly-Lys*(Tme)*-Ala-Gly-Gly **(80)**

The synthesis was carried out on 0.25 mM scale using Fmoc-glycine functionalized 4-alkoxybenzylalcohol resin. All side chains were protected as previously described and all residues were coupled using standard double coupling cycles. At the completion of the assembly phase, the resin bound peptide was sequentially washed with DMF, DCM, ether and dried to give 0.720g of yellow solid. The peptide was then cleaved from the resin by treatment with TFA/water (9:1) for one hour at room temperature. The resin was then removed by filtration and washed with TFA. Evaporation of the filtrate and trituration with diethyl ether yielded the crude product which was isolated by filtration before being dissolved in acetic acid (10%) and lyophilised to yield 111mg of white solid. The peptide was purified by semi-preparative HPLC (ABI
Aquapore C18,250 x 9.2mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 5% B for 15 mins then 5-60% B over 40 mins; λ 214nm). The main peak, eluting at 17%B was isolated and lyophilised to give 62mg (21%) of the title compound. Analytical HPLC (Vydac C18, 250 x 4.6mm, 5μM, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 5% B for 15 mins then 5-60% B over 40 mins; λ 214nm); Rt 27.5 mins; 22.5% B; amino acid analysis: Gly_3 2.91, Ala_1 1.09, Phe_1 1.08, Lys_1 1.078; m/z (FAB) 6812, (MH+) HRMS found 682.33732; $C_{12}H_{47}N_7O_{11}$ requires: 682.34118.

Phenylacetyl-Lys-Phe-Gly-Lys*(Tnm)*-Ala-Gly-Gly **(84)**

The synthesis was carried out on 0.25mM scale using the Fmoc-Gly functionalised 4-alkoxybenzylalcohol resin. All side chains were protected as described previously, except for Lysine which was protected with the experimental Tnm group. All couplings were carried out using the HOCt coupling agent[8] and single coupling cycles, an extended period of time was given for the lysine compound to dissolve. After assembly the resin bound peptide was sequentially washed with DMF, DCM, and diethyl ether before being dried to give 672mg of white solid. The Fmoc group was removed by sonication of the resin in DMF/piperidine, 4:1 for 20mins. The resin was filtered and washed with DFM, DCM, and ether. The resin was then added to a round bottomed flask with 4mls of DMF/dioxane(1:1), phenylacetic acid (0.136g, 1mM), HOBt, and sonicated for 2 hours. The resin was washed as above and added to a cleavage mixture consisting of 25ml TFA/H_2O (95:5); and stirred under nitrogen for 2 hours. The resin was then removed by filtration and washed with TFA. Evaporation of the filtrate and trituration with diethyl ether yielded the crude peptide which was isolated by filtration, before being dissolved in aqueous acetic acid (15%) and lyophilised to yield 242mg of white solid. The peptide was purified using semi-preparative HPLC (Aquapore C18, 250 x 10mm, 10μM, A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10% B for 2 mins then 10-90% B over 30 mins. Λ=214nm); Rt 15.22mins; 47%B. The appropriate resultant fractions were combined and lyophilised to yield *the title compound* 102mg. Analytical HPLC (Vydac C18, 250 x 4.6mm, 5μM, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 10% B for 2 mins then 10-90% B over 30 mins; λ 214nm); Rt=24mins; amino acid analysis Gly_3 2.95, Ala_1 1.047, Leu_1 0.81, Phe_1 0.938, Lys_1 1.021, m/z (FAB) 944 (MH^+); HRMS 944.43527; $C_{43}H_{62}N_9O_{15}$ requires:944.43654.

Phenylacetyl-Lys-Phe-Gly-Lys-Ala-Gly-Gly **(99)**

The synthesis was carried out on 0.25mM scale using the Fmoc-Gly functionalised 4-alkoxybenzylalcohol resin. All side chains were protected as described previously, lysine with the ordinary Boc group. All couplings were carried out using the HOCt coupling agent and single coupling cycles. After assembly the resin bound peptide was sequentially washed with DMF, DCM, diethyl ether and dried to give 752mg of solid. The Fmoc group was removed by sonication of the resin in DMF/piperidine, 4:1 for 20mins. The resin was filtered and washed with DFM, DCM, and diethyl ether. The resin is the added to a round bottomed flask with 4mls of DMF/dioxane (1:1), phenylacetic acid (0.136g, 1mM), HOBt, and sonicated for 2 hours. The resin was washed as above and added to a cleavage mixture consisting of 25ml TFA/H_2O (95:5); and stirred under nitrogen for 2 hours. The resin was then removed by filtration and washed with TFA . Evaporation of the filtrate and trituration with diethyl ether yielded the crude peptide which was isolated by filtration, before being dissolved in aqueous acetic acid (15%) and lyophilised to yield 286mg of white solid. The peptide was purified using semi-preparative HPLC (Aquapore C18, 250 x 10mm, 10μM, A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10% B for 2 mins then 10-90% B over 30 mins. λ=214nm); the appropriate resultant fractions were combined and lyophilised to yield *the title compound* 110mg. Analytical HPLC (Vydac C18, 250 x 4.6mm, 5μM, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 10% B for 2 mins then 10-90% B over 30 mins; λ 214nm); Rt=24mins; amino acid analysis Gly_3 2.97, Ala_1 1.12, Leu_1 0.86, Phe_1 0.92, Lys_1 1.04, m/z (FAB) 768 (MH+); HRMS 767.41470; $C_{38}H_{55}N_8O_9$ requires: 767.40920

Ubiquitin segment peptide (36-76) **(90)**

Ile-Pro-Pro-Asp-Gln-Arg-Leu-Ile-Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Tyr-Asn-Ile-Gln-Lys(*Tnm*)-Glu-Ser-Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-OH

The synthesis was carried out on a 0.25mM scale using the Fmoc-Gly functionalised 4-alkoxybenzylalcohol resin. All side chains were protected as previously described except Lys63 which was protected using the Tnm group. Most amino acids were coupled using standard coupling cycles for the HOCt coupling agent. The exceptions include, Asn, Gln and Arg, where the initial time period was extended to allow for reduced solubility and Gln41 to Ile36 where extended coupling times were used. His was also treble coupled as the HOBt ester.

After the assembly stage the resin was sequentially washed with DMF, DCM, diethyl ether and dried to give 1.521g of yellow solid.

Tbfmoc/Carbon[7] purification.

The resin (200mg) was taken and sonicated in piperidine/DMF 1:4 for twenty minutes and subsequently washed with DMF, DCM and diethyl ether. The resin bound peptide was then added to a suspension of TbfmocCl (15mg, 0.03mM) and DIEA (6µl, 0.03mM) in DCM (5ml), covered in aluminium foil and sonicated at room temperature for 3 hours. The Tbfmoc-peptide-resin was separated by filtration and washed thoroughly with DCM and ether. The dried Tbfmoc-peptide-resin was cleaved for 4 hours using TFA (10ml), H_2O (0.5ml), phenol (0.5g), EDT (1.5ml) and thioanisol (0.5ml) under nitrogen and shielded from light. The resin was then removed by filtration and washed with TFA. Evaporation of the filtrate and trituration with diethyl ether yielded the crude peptide, which was isolated by filtration, before being dissolved in aqueous acetic acid (15%) and lyophilised to yield 131mg of crude peptide. A 75mg portion of this Tbfmoc-Ub36-76(*Tnm*)-peptide was taken and dissolved in 20ml of 1:1 6M guanidine.HCl / isopropanol, to this was added 267mg of PGC and the suspension vortexed and then centrifuged. Adsorption of the Tbfmoc-protein was monitored by analytical HPLC of the

supernatant at 364nm. The PGC was then subjected to the following washing protocol with alternate vortexing and centrifugation:

1) 1:1 6M Gu.HCl / propan-2-ol (40ml)

2) 1:1 6M Gu.HCl / propan-2-ol (40ml)

3) 1:1 6M Gu.HCl / propan-2-ol (40ml)

4) 10% piperidine in 1:1 6M Gu.HCl / propan-2-ol (20ml)

5) 10% piperidine in 1:1 6M Gu.HCl / propan-2-ol (20ml)

The supernatant from washes 4&5 were combined and neutralised to pH4.5 with AcOH and concentrated *in vacuo* prior to being loaded onto a Sephadex G25 (fine) column (10 x 800mm) pre-equilibrated with 30% AcOH. The column was then eluted with 30% AcOH for 48hours (monitoring at 226 and 277nm) and collecting 20 minute fractions. The relevant fractions were combined, diluted with water and lyophilised, yielding 30mg of white solid. Further purification by semi-preparative HPLC (Aquapore C18, 250 x 10mm, 10μM, A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10% B for 2 mins then 10-90% B over 30 mins. λ=214nm) and lyophilisation gave Ub36-76 (19mg). Analytical HPLC(Vydac C18, 250 x 4.6mm, 5μM, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 10% B for 2 mins then 10-90% B over 30 mins; λ 214nm); Rt 18.20mins; 52%B amino acid analysis (40hrs): Asn_4 4.13, Thr_2 1.95, Ser_2 1.95, Glu_6 6.09, Pro_2 1.58, Gly_4 4.65, Ala_1 1.18, Val_1 1.22, Ile_3 2.64, Leu_7 7.00, Tyr_1 0.93, Phe_1 1.07, His_1 0.727, Lys_2 2.025, Arg_4 4.00; m/z (FAB) 4674; HRMS 4676.55031; $C_{206}H_{341}N_{62}O_{62}$ requires: 4676.54252

Tbfmoc HPLC purification

Tbfmoc-Ub36-76(Tnm)-peptide (71mg) was purified by semi-preparative HPLC (Aquapore C18, 250 x 10mm, 10μM, A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10% B for 5 mins then 10-40% B over 10 mins then 40-90%B over 30mins; λ=214nm; Rt 24.5; 56%B) lyophilisation gave Ub(Tmn)36-76 (18.5mg). Analytical HPLC (Vydac C18, 250 x 4.6mm, 5μM, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 10% B for 2 mins then 10-90% B over 30 mins; λ 214nm); Rt 25.8mins; 72%B; amino acid analysis (40hrs): Asn_4 3.84, Thr_2 1.67, Ser_2 1.67, Glu_6 5.82, Pro_2 1.65., Gly_4 3.98, Ala_1 1.01, Val_1 1.05, Ile_3 2.76, Leu_7 6.51, Tyr_1 0.80, Phe_1 0.92, His_1 0.63, Lys_2 1.74,

Arg_4 3.43; m/z (FAB) 5277; HRMS 5277.70047; $C_{242}H_{367}N_{63}O_{70}$ requires: 5277.71619

Tbfmoc & Tnm removal

Tbfmoc-peptide 13mg was added to 8ml of 3:1 DMSO/0.2M borate buffer (pH 8.5) and shaken vigorously before being sonicated for 30 minutes. Acetic acid was then added to adjust the pH to neutral and the solvent concentrated *in vacuo*. The solvent was loaded onto a Sephadex G25 (fine) column (10 x 800mm) pre-equilibrated with 30% AcOH. The column was eluted with 30% AcOH for 48hours (monitoring at 226 and 277nm) and 20minute fractions collected. The relevant fractions were combined, diluted with water and lyophilised to give Ub36-79 (9mg). Analytical HPLC (Vydac C18, 250 x 4.6mm, 5μM, A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min. 10% B for 2 mins then 10-90% B over 30 mins; λ 214nm); Rt 18.2mins; 52%B; amino acid analysis (40hrs): Asn_4 4.08, Thr_2 1.90, Ser_2 1.85, Glu_6 5.85, Pro_2 1.54., Gly_4 4.44, Ala_1 1.09, Val_1 1.10, Ile_3 2.50, Leu_7 7.00, Tyr_1 0.92, Phe_1 1.05, His_1 0.69, Lys_2 1.97, Arg_4 3.82; m/z (FAB) 4676 HRMS 4676.53916; $C_{206}H_{341}N_{62}O_{62}$ requires:4676.54252

N-benzyloxycarbonylglutamic acid-α-benzyl-β-1,5-dioxaspiro-5:5-undecane-3-methyl-3-methyl ester **(75)**

Z-Glu-OBzl (1g, 2.695mMol) was dissolved in dioxane/DMF 80:20 (50ml) to this was added DIC (0.34g, 2.695mMol), 3-hydroxymethyl-3-methyl-1,5-dioxaspiro-5:5-undecane (0.5929g, 2.965mMol) and a catalytic amount of DMAP. The mixture was stirred at 5°C for 1hour, and then left to stir at room temperature overnight. The solvent was then removed *in vacuo* to leave an oil. The oil was subsequently dissolved ethyl acetate and washed with Na_2CO_3 (2 x 50ml) and water (2 x 50ml). The resultant mixture was dried with $MgSO_4$ and the solvent removed *in vacuo* to yield the *title compound* as an oil. Further purification was carried out by wet flash chromatography. (0.91g, 57%); $[\alpha]_D$=11, c=1.0 in MeOH; (found: C,66.84;H,7.52; N,2.40, $C_{31}H_{39}N_1O_8$ requires: C,66.70;H,6.90;N,2.5%);t.l.c. (ethyl acetate/hexane,

1:1); Rf 0.74, υ_{max}(oil) 3344 (NH), 2921 (CH), 1734 (b, CO); δH(DMSO, 250MHz) 7.32 (10H, s, aromatic); 5.15, 5.08 (4H, s, 2 x benzyl CH_2); 4.38-4.48 (1H, m, αCH);1.37-1.81 (14H, bm, 2 x side chain CH_2s', & cyclohexane ring CH_2s') 0.77 (3H, s, CH_3); 4.12 (2H, s, ester CH_2) 3.57 (4H, s, 2 x dioxane ring CH_2); δC(DMSO, 50 MHz) 171.16 (CO, 2 x ester); 155.76 (CO, urethane); 134.87, 135.92 9 (aromatic quaternary C); 128.26-127.63 (aromatic CH); 97.29 (aliphatic quaternary); 66.34-63.58 (β, CH_2, ester CH_2, dioxane ring CH_2);52.58 (α, CH);30.19-22.22 (cyclohexane ring CH_2); 16.23 (CH_3);m/z (FAB) 554 (MH^+), HRMS found 544.10175 $C_{31}H_{39}N_1O_8$ requires 544.10201.

N-9-fluorenylmethoxycarbonylglutamic acid-α-benzyl-β-1,5-dioxaspiro-5:5-undecane-3-methyl-3-methyl ester.**(77)**

Compound **75** (1g, 1.80mM) was added to a round bottomed flask containing ethanol (50ml). The flask was cooled in iced water and Pd/C catalyst (300mg) added. The mixture was then hydrogenated overnight. The Pd/C catalyst was filtered off using a celite packed sinter funnel (the residue being washed extensively with ethanol; 200ml). The ethanol is then removed *in vacuo* to leave a white powder, this was the free amine free acid compound, and was used without further purification. To this white powder was added Fmoc-succinamide (0.546g, 1.62mM) and K_2CO_3 (0.49g, 3.6mM) the mixture was then dissolved in a 1:1 mixture of dioxane/H_2O and stirred overnight. The dioxane was removed *in vacuo* and the solution washed with diethyl ether. The aqueous component is then acidified with potassium hydrogen sulphate and the product retrieved by extraction with ethyl acetate. Wet flash chromatography was used as a final purification and yields the title product as a white solid. (0.51g, 52%); Mpt 174-178°C; $[\alpha_D]$=+11 c=1.0 in MeOH; (found: C,66.81;H,7.20;N,2.38; requires: C,67.39;H,6.88;N,2.50; t.l.c.(ethyl acetate /hexane 7:1) Rf = 0.40; υ_{max}(nujol) 3342 (b, NH/COOH) 1733 (b, CO);λ_{Max} (MeOH) 265nm (ε 23000 dm^3 mol^{-1} cm^{-1}), 290 (5700) 300 (7000); δH(DMSO, 360MHz) 7.92-7.29 (aromatic CH); 4.33-4.18 (2H, m, Fmoc CH_2); 4.04-3.92 (1H, m, Fmoc CH); 3.93 (2H, s, ester CH_2); 3.26 (4H, s, dioxane ring CH_2); 2.13-1.29 (14H, bm, β CH_2's & ring CH_2's); 0.78

(CH_3);δC(DMSO, 90MHz) 173.44, 172.22 (CO, acid, ester); 156.16 (CO, urethane); 143.89,140.77 (aromatic quaternary); 127.69-120.16 (aromatic CH); 65.67 (Fmoc CH_2); 59.93 (2 x CH_2OH & ester CH_2); 52.99 (α CH); 46.71 (Fmoc CH); 30.19, 26.13 (β CH_2's); 14.13 (CH_3); m/z (FAB) 552 (MH^+), HRMS found 552.25961; $C_{31}H_{37}N0_8$ requires 552.25974.

Gp-120 Peptide (92)

Gly-Glu-Phe-Phe-Tyr-Cys*(Acm)*-Val-Cys*(Acm)*-Arg-Ile-Lys-Gln-Phe-Ile-Asn-Met-Trp-Gln-Glu-Val-Gly-Lys-Ala-Met-Tyr-Ala-Pro-Pro-Ile-Ser-Gly

The synthesis was carried out on a 0.268 mM scale using Fmoc-glycine functionalised 4-alkoxybenzylalcohol resin, all amino acid side chains were protected as previously described, cysteine being protected as the Acm derivative. Amino acids were double coupled using standard coupling cycles, except for the last 10 residues which were allowed to couple for an extended period of time. The N-terminal Fmoc group was left on at the completion of the assembly phase. The resin bound product was washed sequentially with DMF, 1,4-dioxan, DCM and diethyl ether to give 1.428g of resin bound product.

Fmoc protected resin bound product (105g) was treated with 20% piperidine/DMF (10mls) in a sonic bath for 15 mins, after which the resin bound product was filtered, washed as previously, and dried to give 95mg of resin bound product. The peptide was then cleaved from the resin using a cleavage mixture consisting of 250μl EDT, 250μl EMS, 250μl H_2O, 250mg Phenol and 9mls TFA. The mixture was stirred with the resin bound peptide under an atmosphere of nitrogen for 3 Hours. Subsequently the resin was removed by filtration and the filtrate concentrated *in vacuo* to give an oil. This oil yielded solid peptide on trituration with diethyl ether. The solid peptide was then filtered off, washed with diethyl ether, and dissolved in aqueous acetic acid (15%). The aqueous solvent was then removed by lyophilisation to leave 42mg of crude product. This impure peptide was then re-dissolved in 30% acetic acid and purified by semi-preparative Rp HPLC (ABI, Aquapore Rp300; C18, 250 x 9.2 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min, 10%B for 2 mins then 10-90%B over

30 min. λ=214nm). The appropriate resultant fractions were combined and lyophilised to give a white solid. (20mg); Analytical HPLC (ABI, Aquapore Rp300; C18, 220 x 4.6 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min; 10%B for 2 mins then 10-90%B over 30 min, λ=214nm) Rt=22mins 64.5%B; amino acid analysis Asp_1 1.28, Ser_1 1.20, Glu_4 4.76, Pro_2 2.21, Gly_3 3.38, Ala_2 2.36, Cys_2 0.3(Acm), Val_2 2.08, Met_2 0.8, Ile_3 3.29, Tyr_2 1.13, Phe_3 3.37, Lys_2 2.25, Arg_1 0.97; m/z (FAB) 3754 (MH+), HRMS found 3754.81128; $C_{173}H_{257}N_{42}O_{44}S_4$ requires 3754.80472.

Removal of the Acm cysteine protecting group

1) Mercury (II) Acetate (HgAc) method.

The peptide **92** (15mg, 3.99 x 10^{-4}mM) was dissolved in the minimum volume of 30% aqueous acetic acid, to this was added 100mg of Hg(Ac) and the mixture stirred for one hour at room temperature. 0.8mls of β–mercaptoethanol was then added and the mixture stirred for an additional hour. The precipitated mercury salt was removed by filtering through celite and the filtrate examined under Analytical HPLC (ABI, Aquapore Rp300; C18, 250 x 4.6 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min; 10%B for 2 mins then 10-90%B over 30 min. λ=214nm). Two peaks were present, one corresponding to the unreacted protected peptide (Rt=22mins), (Mass 3754), and the other (Rt=21.5mins) to a mono Acm protected derivative (Mass 3680). The mono protected peptide (3mg, 8.152 x 10^{-4}mM), after isolation via semi-preparative HPLC, was stirred with Hg(Ac) (100mg, 0.31mM) for 18hrs: β-mercaptoethanol (0.8mls, 11.40mM) was then added, the residue removed by filtration and the filtrate examined under analytical HPLC. There was no change in retention time, the peak was isolated by semi-preparative HPLC and lyophilised (1mg). Quantitative Ellman's[3] test suggested only one Acm had been removed i.e. no change; m/z (FAB) 3680, HRMS found 3683.76431; $C_{170}H_{252}N_{41}O_{43}$ requires 3683.76773.

In a final effort to remove the Acm protecting groups using the Hg(Ac) method, peptide **92** (20mg, 5.3 x 10^{-3}mM) was dissolved in 10mls of 1:1 acetic acid, Hg(Ac) (110mg, 0.35 mM) added, and the mixture placed in a sonic bath at 40°C overnight.

β–mercaptoethanol (1ml, 14mM) was added and the mixture stirred for two hours before being filtered through celite. The filtrate was diluted and freeze dried. Analytical HPLC showed a number of peaks, the major constituents were separated as best as possible by semi-preparative HPLC and sent for mass spectroscopy. This yielded poor results and indicated that the peptide had broken up during sonication. The pattern of peaks around a fragment weighing 2812 and giving a qualitatively positive Ellman's test suggests that one of the Acm groups remains attached even though the peptide has broken up. This suggests removal of both Acm's using this method is unlikely to be successful.

Removal of Acm using silver trifilate.

Peptide **92** (8mg, $2x10^{-3}$mM) was added to a round bottomed flask containing 5mls of TFA and stirred with 20 equivalents of silver trifluromethanesulphonate (11mg 0.0426mM) at 0°C in the dark for 2 hours. The TFA was removed *in vacuo* and the residue triturated with cold ether to precipitate the silver salt of the peptide. This was taken up in 50% AcOH (4mls) and treated with 100 equivalents of DTT (31mg, 0.2mM) overnight in the dark at room temperature. After centrifugation (4500 revs for 30mins) the supernatant was decanted from the brown pellet and diluted with water to 10ml volume. The residual brown pellet was washed with 50% AcOH (2ml) and the supernatant again collected after centrifugation of the solution. This was diluted with water to 5mls volume and combined with the 10ml solution. The peptide was isolated by semi-preparative HPLC (ABI, Aquapore Rp300; C18, 250 x 9.2 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min, 10%B for 2 mins then 10-90%B over 30 min, λ=214nm). The appropriate fractions were lyophilised to yield 3mg of product. Ellman's test proved negative; m/z 3754; therefore Acm groups have not been removed, hence procedure abandoned.

Gp-120 Peptide (93)

Gly-Glu-Phe-Phe-Tyr-Cys*(tBuS)*-Val-Cys*(tBuS)*-Arg-Ile-Lys-Gln-Phe-Ile-Asn-Met-Trp-Gln-Glu-Val-Gly-Lys-Ala-Met-Tyr-Ala-Pro-Pro-Ile-Ser-Gly

The synthesis was carried out on a 0.25 mM scale using Fmoc-Glycine functionalised 4-alkoxybenzylalcohol resin, all amino acid side chains were protected as previously described, cysteine being protected as the tBuS derivative. Amino acids were double coupled using standard coupling cycles, except for the last 10 residues which were allowed to couple for an extended period of time. The synthesis was interrupted after the arginine residue and two thirds of the resin removed and stored. The synthesis was continued on the remaining third incorporating tBuS protected cysteines. The N-terminal Fmoc group was left on at the completion of the assembly phase, after which the resin bound product was washed sequentially with DMF, 1,4-dioxin, DCM and diethyl ether to give 0.750g of resin bound product. Fmoc protected resin bound product (100mg), was treated with 20% piperidine/DMF (10mls) in a sonic bath for 15 mins, after which the resin bound product was filtered off, washed as previously, and dried to give 91mg of resin bound product. The peptide was cleaved from the resin using a cleavage mixture consisting of 250µl EDT, 250µl EMS, 250µl H_2O, 250mg Phenol and 9mls TFA. The mixture was stirred with the resin bound peptide under an atmosphere of nitrogen for 3 hours. The resin was removed by filtration and the filtrate concentrated *in vacuo* to give an oil. This oil yielded solid peptide on trituration with diethyl ether. The solid peptide was then filtered off, washed with diethyl ether, and dissolved in aqueous acetic acid (15%). The aqueous solvent was then removed by lyophilisation to leave 54mg of crude product. The crude product was added to a vial containing 45mls of a mixture composed of 95% trifluroethanol and 5% H_20. This mixture was placed in a sonic bath for 40mins, 180µl of tributylphosphine was then added and sonication continued for a further hour. The solvent was removed *in vacuo* and the peptide triturated out of solution by the addition of an excess of diethyl ether, filtered and re-dissolved in 15% acetic acid before being lyophilised. The peptide was then re-dissolved in 50% acetic acid and purified by semi-preparative Rp HPLC (this proved very difficult due

to constant crashing out of peptide on the guard column) (ABI, Aquapore Rp300; C18, 250 x 9.2 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10-90%B over 30 min, λ=214nm). The appropriate resultant fractions were lyophilised to give a white solid. (2mg); positive Ellman's test; analytical HPLC (ABI, Aquapore Rp300; C18, 110 x 4.6 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min; 10%B for 2mins then 10-90%B over 30 min, λ=214nm) Rt=20mins, 62%B; amino acid analysis Asp_1 1.31, Ser_1 1.18, Glu_4 4.66, Pro_2 2.24, Gly_3 3.38, Ala_2 2.37, Cys_2 1.57, Val_2 2.06, Met_2 1.84, Ile_3 3.19, Tyr_2 1.63, Phe_3 3.36, Lys_2 2.31, Arg_1 1.07; m/z (FAB) 3612 (MH+), HRMS found 3612.72640, $C_{167}H_{246}N_{40}O_{42}S_4$ requires 3612.72584.

Oxidation of peptide **(93)**

Crude peptide **93** (tBuS removed), (20mg, 5.6 x 10^{-3}mM), was added to 25mls of 5% acetic acid, the pH adjusted to 3.5 with NH_4CO_3, 20% by volume of DMSO added and the mixture left to stir overnight. The volume was doubled by adding 50% acetic acid and the product purified by semi-preparative RP-HPLC (ABI, Aquapore Rp300; C18, 250 x 9.2 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min; 10%B for 2mins then 10-90%B over 30 min, λ=214nm). (This proved very difficult due to constant crashing out of the product on the guard column). Appropriate fractions were combined and lyophilised to give a white smear (0.2mg); positive Ellman's test: oxidation not successful.

Gp-120 Peptide **(94)**

Gly-Glu-Phe-Phe-Tyr-Cys*(tBuS)-Pro-(D)*Val-Cys*(tBuS)*-Arg-Ile-Lys-Gln-Phe-Ile-Asn-Met-Trp-Gln-Glu-Val-Gly-Lys-Ala-Met-Tyr-Ala-Pro-Pro-Ile-Ser-Gly

The synthesis was carried out on the 2/3rds of resin which had been removed during the assembly of peptide **93**. The synthesis was carried out in exactly the same manner as for peptide **93** only the Proline and (D)Valine residues were introduced. The N-terminal Fmoc group was left on at the completion of the assembly phase, after which the resin bound product was washed sequentially with DMF, 1,4-dioxin, DCM and ether to give 1.112g of resin bound product.

100mg of Fmoc protected, resin bound product was treated with 20% piperidine/DMF (10mls) in a sonic bath for 15 mins, before the resin bound product was filtered off, washed as previously, and dried to give 93mg of resin bound product. The peptide was cleaved from the resin using a cleavage mixture consisting of 250µl EDT, 250µl EMS, 250µl H_2O, 250mg Phenol and 9mls TFA. The mixture was stirred with the resin bound peptide under an atmosphere of nitrogen for 3 hours. The resin was removed by filtration and the filtrate concentrated *in vacuo* to give an oil. This oil yielded solid peptide on trituration with diethyl ether. The solid peptide was then filtered off, washed with diethyl ether, and dissolved in aqueous acetic acid (15%). The aqueous solution was then removed by lyophilisation to leave 57mg of crude product. The crude product was added to a vial containing 50mls of a mixture composed of 95% trifluroethanol and 5% H_20. This mixture was placed in a sonic bath for 40mins, 180µl of tributylphosphine was then added and sonication continued for a further hour. The solvent was then removed *in vacuo* and the peptide triturated out of solution by the addition of an excess of diethyl ether, filtered, and re-dissolved in 15% acetic acid before being lyophilised. The peptide was then re-dissolved in 50% acetic acid and purified by semi-preparative Rp HPLC (this proved very difficult due to constant crashing out of peptide on the guard column) (ABI, Aquapore Rp300; C18, 250 x 9.2 mm, 7µm A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min; 10% B for 2mins then 10-90%B over 30 min, λ=214nm). The appropriate resultant fractions were combined and lyophilised to give a white solid. (3mg); positive Ellman's test; Analytical HPLC (ABI, Aquapore Rp300; C18, 110 x 4.6 mm, 7µm A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min, 10% B for 2mins then 10-90%B over 30 min, λ=214nm) Rt=17mins, 55% B; amino acid analysis Asp_1 1.11, Ser_1 0.88, Glu_4 4.52, Pro_2 3.80, Gly_3 3.23, Ala_2 2.24, Cys_2 1.32, Val_2 2.06, Met_2 1.81, Ile_3 3.30, Tyr_2 1.65, Phe_3 3.13, Lys_2 2.28, Arg_1 1.18; m/z (FAB) 3711 (MH+), HRMS found 3710.78721, $C_{172}H_{254}N_{41}O_{43}S_4$ requires 3710.78657.

Oxidation of peptide **(94)**

Peptide **94** crude (tBuS removed) (13mg, 3.5 x 10^{-4}mM) was added to 20ml of 5% aqueous acetic acid and the pH adjusted to 4 with ammonium acetate. DMSO 5ml

was then added and the mixture left to stir overnight. The mixture was then diluted to double its volume with 50% aqueous acetic acid and the product purified via semi-preparative RP-HPLC (ABI, Aquapore Rp300; C18, 250 x 9.2 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 5ml/min. 10-90%B over 30 min. λ=214nm). (This proved very difficult due to constant crashing out of the product on the guard column.) Appropriate fractions were combined and lyophilised to give a white powder. (1mg); negative Ellman's test Analytical HPLC (ABI, Aquapore Rp300; C18, 250 x 4.6 mm, 7μm A=H_2O, B=CH_3CN, 0.1% TFA; 1ml/min; 10-30%B over 10 min, 30-50% over 35 mins λ=214nm) Rt=19mins, 43%B; m/z (FAB) 3740.1 (MH+), HRMS found 3740.77116, $C_{172}H_{252}N_{41}O_{45}S_4$ requires 3740.77078. (Mass spec suggests that unfortunately the methionine residues have been oxidised as well as the disulfide. Several attempts were made to selectively oxidise the disulphide without the methionine but these proved unsuccessful).

4.6 References

1) Kevin Shaw, *personal communication.*

2) Nicola Robertson, *personal communication.*

3) G.L. Ellman, *Arch. Bioche. Biophys.* 1959, 82, 70.

4) R. Ramage, *personal communication.*

5) W.E. Conrad, B.D. Gesner, L.A. Levasseur, R.F. Murphy & H. Conrad, *J. Org. Chem.* 1961, 26, 3571-3574.

6) F.F. Blicke & E.L. Schumann, *J.Am.Chem. Soc.* 1954, 76, 3153-3156.

7) A.R. Brown, S.L. Irving & R. Ramage, *Tetrahedron Lett.* 1993, 34, 7129-7132.

Courses and Conferences Attended

Organic Research Seminars,University of Edinburgh, various speakers, 1992-1995.

NMR Spectroscopy, Drs I. Sadler & J. Parkinson, University of Edinburgh, 1993.

Royal Society of Chemistry Perkin Division, Regional Meetings, various speakers, Edinburgh 1992, Aberdeen 1993.

3rd International Symposium on Solid Phase Synthesis, various speakers, Oxford 1993.

Medicinal Chemistry, Prof R. Baker and colleagues, Merck Sharp & Dohme, Terlings Park, UK. 1993-1995.

Chemical Development in the Pharmaceutical Industry, various speakers, SmithKline Beecham, UK. 1993-1994.

4th International Symposium on Solid Phase Synthesis & Combinatorial Chemical Libraries, various speakers, Edinburgh 1995.