

**SYNTHESIS AND PURIFICATION
OF PEPTIDES**

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To Lorna
and to my parents

ABSTRACT

Improved routes to tetrabenzobenzofluorene derivatives have been developed, allowing the synthesis of N^{α} -17-tetrabenzobenzofluorenylmethoxycarbonyl (Tbfmoc) urethane derivatives of alanine, leucine, isoleucine, methionine and valine. The chloroformate and pentafluorophenyl carbonate of 17-tetrabenzobenzofluorenylmethanol have been prepared and used to introduce the base-labile Tbfmoc group onto the N^{α} -termini of resin-bound peptides.

The high affinity of the Tbfmoc group for porous graphitised carbon (PGC) has been exploited for the purification of a range of synthetic peptides (23-85 residues). A comparison of various basic solvent systems used to elute the purified peptide from PGC is presented. The hydrophobicity of the Tbfmoc group has been used to simplify the purification of a ubiquitin analogue, UbY59F (76 residues), by the enhanced retention of the Tbfmoc peptide on RP-HPLC.

A new synthesis of 2-hydroxydibenzocycloheptadien-5-one has been devised. This compound has been used to develop acid-labile linkers for the synthesis of peptide C-terminal alkyl amides and aza-glycine peptides, compatible with the Fmoc/^tBu solid phase method. Alternative modes of attachment of the linker to polystyrene resin are compared for the synthesis of bombesin, a peptide amide.

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ABBREVIATIONS

Acm	acetamidomethyl
AcOH	acetic acid
Bnpeoc	2,2- <i>bis</i> -(4-nitrophenyl)ethoxycarbonyl
Boc	<i>t</i> -butyloxycarbonyl
BOP	benzotriazole-1-yl-oxy- <i>tris</i> -(dimethylamino)phosphonium hexafluorophosphate
br	broad
^t Bu	tertiary-butyl
Bum	<i>t</i> -butyloxymethyl
d	doublet
dd	doublet of doublets
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DEAE	diethylaminoethyl
Dhbt	3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine
DIBAL	diisobutylaluminium hydride
DIC	diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	<i>N,N</i> -dimethylformamide
DMS	dimethyl sulphide
DMSO	dimethyl sulphoxide
Ether	diethyl ether
EDT	1,2-ethanedithiol
EI	electron impact

Et	ethyl
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
Gdm.HCl	guanidinium hydrochloride
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
hGRP	human gastrin releasing peptide
HIV	human immunodeficiency virus
HMPB	4-hydroxymethyl-3-methoxyphenylbutyric acid
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
IR	infrared
LHRH	luteinising hormone releasing hormone
m	multiplet
Mbh	4,4-dimethoxybenzhydryl
Me	methyl
MS	mass spectrometry
Mtr	4-methoxy-2,3,6-trimethylbenzenesulphonyl
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser enhancement
Np	<i>p</i> -nitrophenyl
NpSSPeoc	2-[(2-nitrophenyl)dithio]-1-phenylethoxycarbonyl
Ⓟ	polymeric support
PAM	phenylacetamidomethyl
PAL	peptide amide linker
Pfp	pentafluorophenyl

PGC	porous graphitised carbon
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
PPA	polyphosphoric acid
<i>i</i> -Pr	isopropyl
PyBOP	benzotriazol-1-yl-oxy- <i>tris</i> -pyrrolidino-phosphonium hexafluorophosphate
PyBrOP	bromo- <i>tris</i> -pyrrolidino-phosphonium hexafluorophosphate
q	quartet
RP-HPLC	reverse phase high performance liquid chromatography
R _t	retention time
s	singlet
SASRIN	super acid sensitive resin
SIV	simian immunodeficiency virus
SPPS	solid phase peptide synthesis
Su	succinimide
t	triplet
Tbfmoc	17-tetrabenzo[<i>a,c,g,i</i>]fluorenylmethoxycarbonyl
TBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulphonic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Tos	<i>p</i> -toluenesulphonyl
Trt	trityl
UV	ultraviolet
Z	benzyloxycarbonyl

CHAPTER 1. INTRODUCTION

1.1 Solid Phase Peptide Synthesis (SPPS)

1.1.1 Introduction

The chemical synthesis of peptides relies upon the formation of an amide bond by the reaction of the amine function of one amino acid with the activated carboxyl function of another. In order to prevent unwanted coupling between two molecules of the same species, the amine group of the activated amino acid and the carboxyl of the other are protected (Figure 1.1).

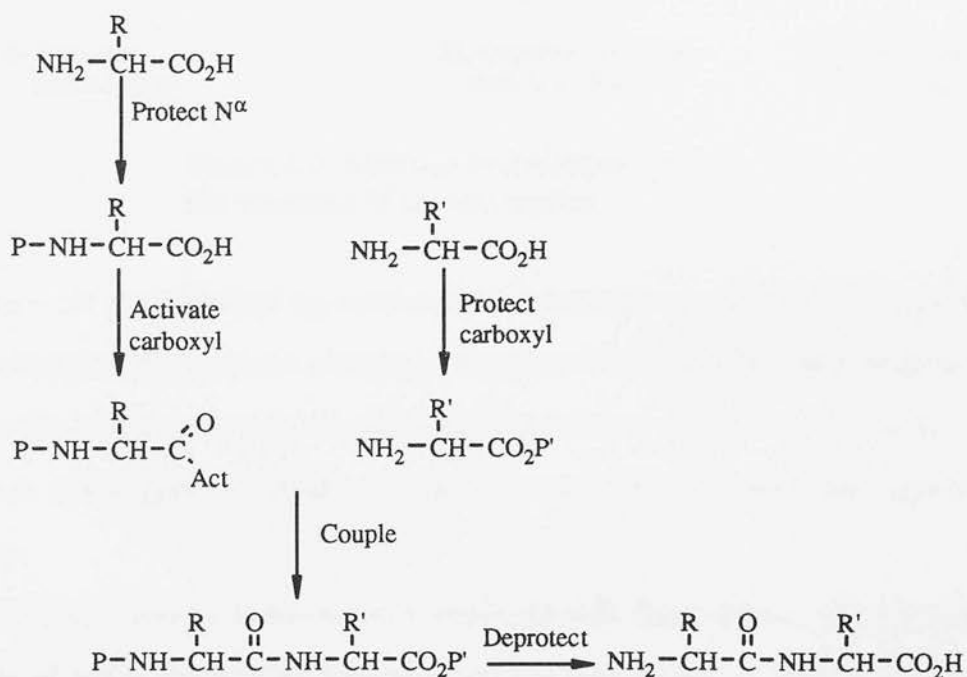


Figure 1.1: General scheme for peptide synthesis.
Synthesis of a dipeptide

For longer peptides, the question arises as to the strategy of assembly of the

peptide. Three possible routes for the synthesis of an octapeptide, A-B-C-D-E-F-G-H, where A-H represent amino acids, are outlined in Figure 1.2.

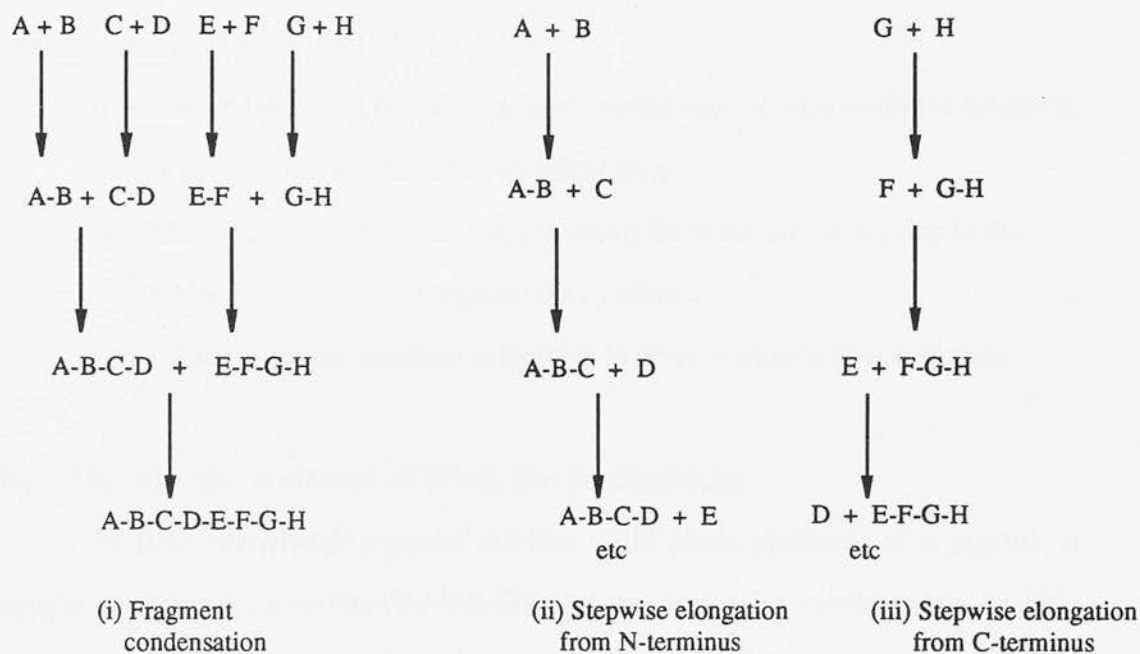


Figure 1.2: Alternative strategies for the assembly of an octapeptide

In practise (ii) is discounted by racemisation considerations, leaving (i) fragment condensation and (iii) stepwise elongation from the C-terminus. Of these, fragment condensation would seem to be the preferred route when an average yield of 90% for each stage would give an overall yield of 73%, compared with 48% for stepwise assembly.

Fragment condensation has been employed with great success, allowing the synthesis of many biologically important peptides e.g. oxytocin¹ (9 residues), the first peptide hormone to be chemically synthesised. Over the years, however, it became increasingly clear that a prodigious effort would be required to extend this method to the synthesis of longer, more complex peptides.

In 1962 Merrifield² proposed the simple but ingenious idea of carrying out stepwise peptide synthesis from the C-terminus with the first amino acid covalently bound to an insoluble solid polystyrene support. This greatly simplified the task of peptide synthesis by:

- (i) removing the need for conventional purification of intermediates (reagents and by products are removed by filtration);
- (ii) minimising physical losses by retaining the resin-bound peptide in the same reaction vessel throughout the synthesis;
- (iii) allowing excess reagents to be used to drive reactions to completion.

1.1.2 The Merrifield method of SPPS. Boc Methodology

In 1963 Merrifield³ reported the first solid phase synthesis of a peptide, a simple tetrapeptide, Leu-Ala-Gly-Val-OH, by the sequential condensation of N^α-benzyloxycarbonyl amino acids. Although several truncated peptides were formed due to poor coupling reactions and/or incomplete deprotection of the N^α-benzyloxycarbonyl protecting groups, the feasibility of the method had been clearly demonstrated.

A year later Merrifield⁴ proposed several modifications to the method, most notably the replacement of the N^α-benzyloxycarbonyl protecting group by the t-butyloxycarbonyl (Boc) group. In this method (Figure 1.3), the caesium salt of the first Boc-protected amino acid is anchored to chloromethylated polystyrene resin by the formation of a benzyl ester. The N^α-Boc group is removed with TFA and the resulting TFA salt neutralised. The liberated amino group is then coupled to an amino acid using dicyclohexycarbodiimide (DCC) as an activating agent. These steps are repeated until the desired peptide has been assembled when it can be liberated from the resin, with concomitant removal of benzyl-based side chain protection, upon treatment with anhydrous HF.

Using this method, Merrifield was able to singlehandedly synthesise a nonapeptide, bradykinin, in highly purified form, a remarkable achievement at this time and a feat that would have taken a team of workers several weeks using classical solution phase techniques.

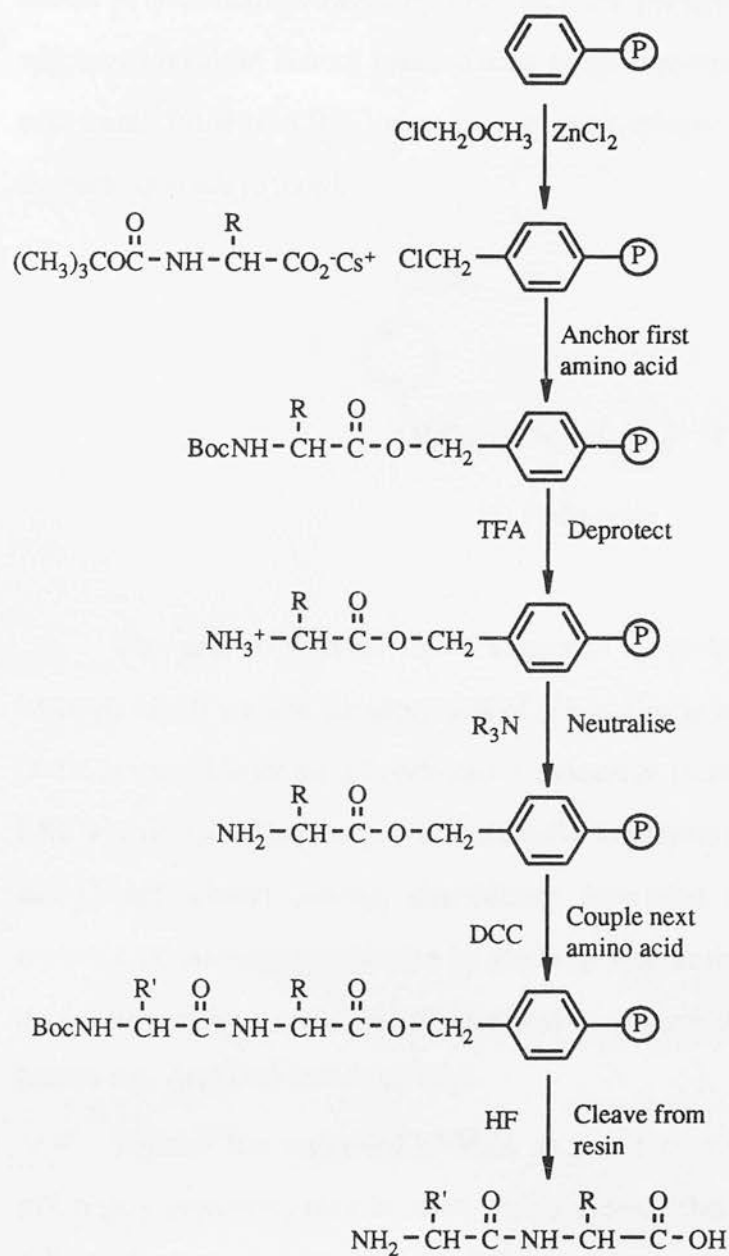
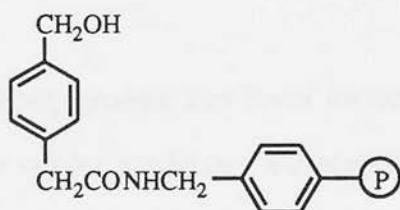


Figure 1.3: Merrifield method of SPPS

Merrifield's contribution to peptide chemistry was subsequently recognised by the award of the Nobel prize in 1984⁵.

Over the years, numerous refinements to the Boc method have been proposed. For instance, the peptide-resin benzyl ester linkage (Figure 1.3) has been shown to be partially cleaved by TFA, *ca.*1-2% per cycle, as the synthesis proceeds, which can result in serious losses during longer syntheses. For this reason the more acid-stable PAM resin (1) has come into use⁶, where bleeding of the peptide from the resin is much reduced.



(1) PAM resin

Tam and co-workers⁷ have suggested a low-high HF cleavage procedure, whereby initially a low concentration of HF in a large amount of scavenger, such as DMS, is used. Under these conditions the cleavage mechanism changes from SN1 to SN2 (Figure 1.4). This has proved successful in suppressing alkylation of tyrosine by benzyl and t-butyl cations, succinimide formation in Asp-Gly sequences and acylation of scavenger molecules by glutamyl side chains. The low HF procedure is then followed by a standard HF cleavage to remove the more resistant protecting groups e.g. Arg(Tos) and Arg(NO₂).

Yajima⁸ has suggested TFMSA as an alternative to HF. TFMSA, although still highly corrosive, may be used with standard laboratory glassware, however it will not deprotect Arg(Tos) or Arg(NO₂).

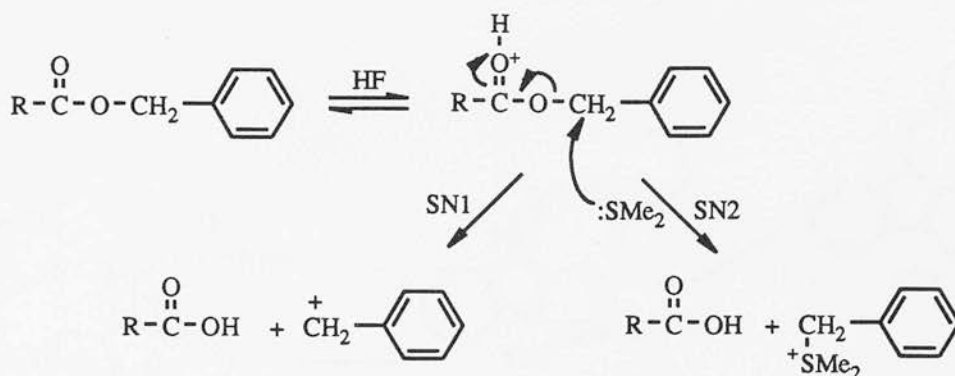


Figure 1.4: High (SN1) and low (SN2) HF cleavage mechanism

1.1.3 Base-labile N^α protecting groups. The Fmoc method of SPPS

The search for ever milder conditions for peptide synthesis prompted the introduction of the 9-fluorenylmethoxycarbonyl (Fmoc) group for N^α amino protection⁹.

The Fmoc group is base-labile by virtue of the acidic proton at the 9-position, allowing the group to be expelled by a β -elimination mechanism on treatment with base (Figure 1.5). A variety of bases have been employed but secondary amines (e.g 20% piperidine in DMF) are most commonly used.

Piperidine forms a stable adduct (15) with the liberated dibenzofulvene (14)⁹ and the formation of this adduct can be detected by its UV absorbance at 300 nm. This has formed the basis of a UV monitoring method for automated SPPS¹⁰.

The base-lability of the Fmoc group combined with acid-labile side chain protection offers a truly orthogonal approach to peptide synthesis but although the Fmoc group was introduced by Carpino in 1972, it wasn't until almost a decade later that its potential in SPPS began to be realised, when Atherton and Sheppard adopted the Fmoc group for peptide synthesis on polyamide supports¹¹⁻¹³.

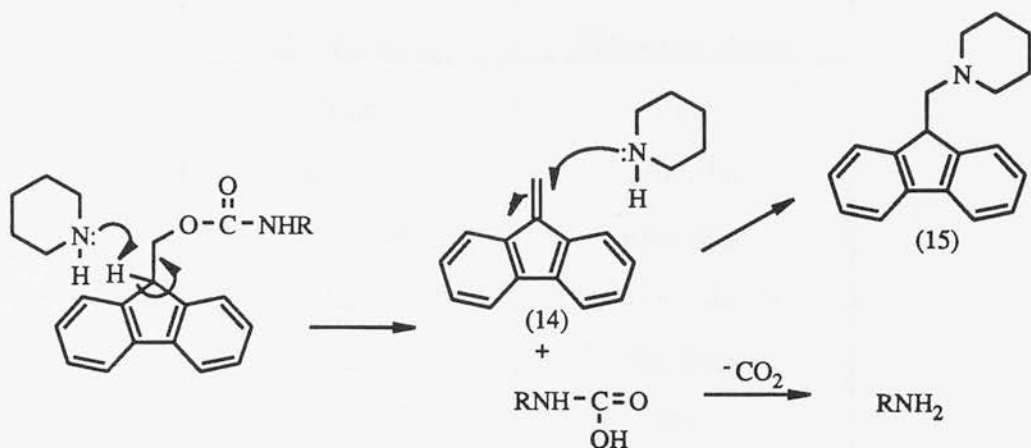
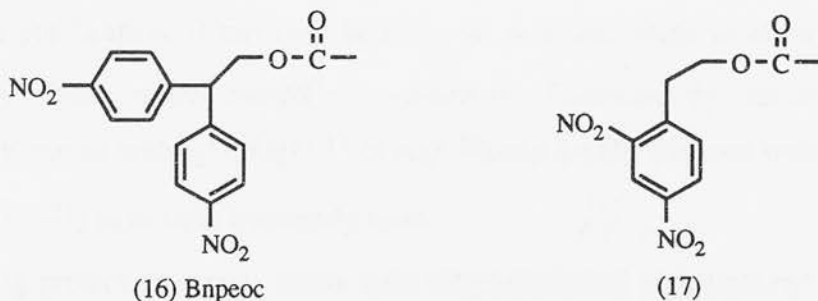


Figure 1.5: Fmoc deprotection with piperidine

Following the success of the Fmoc group, other base-labile N^α -protecting groups have since been suggested. These include the *bis*-nitrophenylethoxycarbonyl (Bnpeoc)^{14,15} (16) and dinitrophenylethoxycarbonyl¹⁶ (17) groups but none have yet come into routine use.



1.1.4 Fmoc methodology. Side chain protection

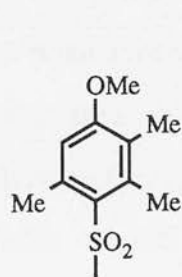
A base-labile N^α -protecting group allows more acid labile side chain protection and *t*-butyl based groups (Table 1.1), removed with TFA, have become widely used in Fmoc SPPS.

Amino acid	Protecting group
Arg	Pmc
Asn, Gln	Mbh, Trt
Asp, Glu	t-Bu ester
Cys	Acm, S-t-Bu, Trt
His	Trt, Bum
Lys	Boc
Trp	Boc
Ser, Thr, Tyr	t-Bu ether

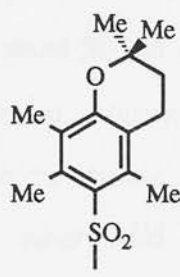
Table 1.1: Commonly used side-chain protecting groups in Fmoc methodology

Notable exceptions are Cys, His and Arg. Due to problems with unwanted disulphide formation Cys protection is often retained during cleavage from the resin and initial purification. It can then be removed at a later stage to allow disulphide formation under more controlled conditions. Consequently acetamidomethyl (Acm)¹⁷ (cleaved with I₂¹⁸, Hg²⁺¹⁷ or Ag⁺¹⁹) and S-t-Bu (cleaved under reductive conditions^{20,21}) have been frequently used.

Arg protecting groups based upon ring substituted arylsulphonyl derivatives such as the Mtr²² group (18) have proved the most effective.



(18) Mtr



(19) Pmc

The Mtr group has proved somewhat resistant to TFA cleavage^{22,24}, often requiring extended cleavage times and the more acid-labile 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc) (19) group is now preferred²⁵.

Histidine also presents special problems, as the basic imidazole ring can catalyse racemisation. N- τ -trityl protection is effective for preventing racemisation during HOBt activation but substantial racemisation occurs if DCC or DIC is used in the absence of HOBt²⁶. A superior protecting group for histidine is the N- π -t-butyloxymethyl (Bum) group²⁷ which is highly effective in suppressing racemisation. Unfortunately, this derivative has proved rather troublesome to synthesise, making it somewhat expensive and its use has remained limited.

1.1.5 The solid support

The general requirements for a suitable solid support for solid phase synthesis have been outlined by Erickson and Merrifield²⁸. It must:

- (a) have reactive sites at which the peptide can be attached, synthesised and subsequently obtained in useful yield;
- (b) allow good contact between peptide and reagents;
- (c) be easily separated from excess reagents and by-products;
- (d) be stable to the reaction conditions;
- (e) minimise interactions between bound peptide chains.

As outlined above, Merrifield selected polystyrene cross-linked with 1% divinylbenzene. These small spherical beads are about 50 μm in diameter but swell to five or six times their original volume in organic solvents such as dichloromethane²⁹, allowing the reagents access to the peptide.

Since Merrifield's original work, many other solid supports have been suggested. These include cellulose³⁰⁻³², polypropylene membranes³³ and porous glass³⁴⁻³⁶. Only the polyamide resins developed by Sheppard and co-workers^{37,38}

have come into widespread use. These resins, when supported on kieselguhr, are mechanically robust and have proved suitable for continuous flow SPPS³⁹.

1.1.5 Coupling methods

The success of the peptide bond forming reaction is obviously of crucial importance to the assembly of a peptide by either classical solution phase methods or SPPS. Accordingly, this area has attracted much attention and many coupling agents have been proposed⁴⁰.

The selection of a suitable reagent is often dictated by the need for racemisation free coupling. Only a few methods have met these stringent requirements and passed into routine use.

Racemisation of amino acids occurs upon activation of the carboxyl group with an electron-withdrawing substituent (20). The mechanism⁴¹ involves the formation and enolisation of an oxazolone (21a). The intermediate enol (21b), which is presumably stabilised by its pseudo aromatic character, can subsequently convert into either the D- or L- amino acid derivative

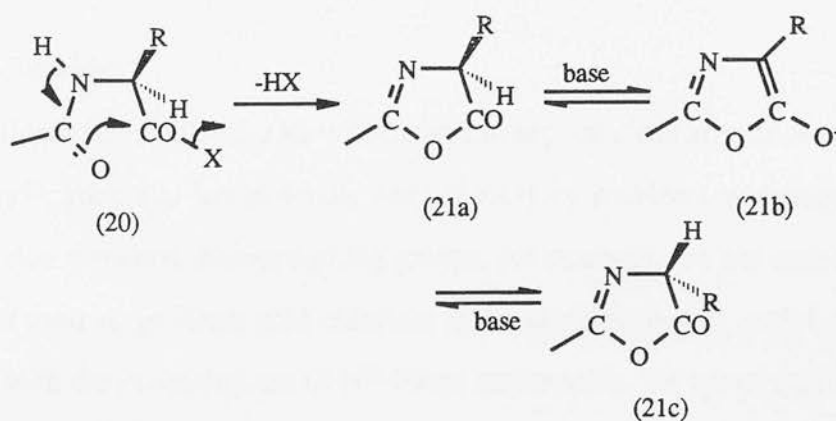
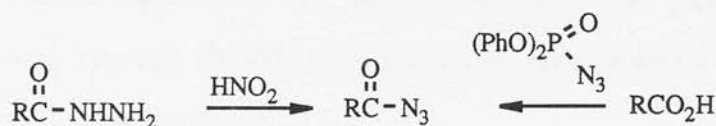


Figure 1.6: Mechanism of racemisation on activation

The various methods of activating amino acids to nucleophilic attack have been extensively reviewed⁴⁰ and only a selection of the most prominent methods are presented here.

(a) Azides

Azide activation is one of the oldest methods of activating a carboxylic acid to nucleophilic attack⁴² and is relatively racemisation-free. Acyl azides can be generated by the diazotisation of a hydrazide using nitrous acid or alkyl nitrites⁴³. Alternatively, diphenylphosphoryl azide can be used to form the acyl azide directly from the carboxylic acid⁴⁴:



However, this method has the drawback of a highly toxic by-product, hydrazoic acid, and can suffer from the formation of urea derivatives by the Curtius rearrangement.

(b) Acid Chlorides

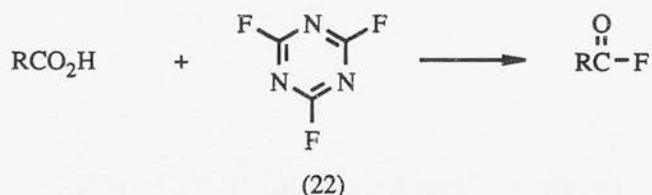
Although acid chlorides have been used in peptide chemistry since the turn of the century⁴⁵, their use has generally been limited by problems with racemisation and other side reactions. Boc-protecting groups, for example, are not entirely stable to reagents used to generate acid chlorides (thionyl chloride and oxalyl chloride). However, with the increasing use of N^α-Fmoc amino acids, the use of acid chlorides has been revived^{46,47}.

The Fmoc group is stable to thionyl chloride and Fmoc amino acid _α ^{chlorides} having aliphatic side chains can be isolated as stable compounds. These have been used successfully in solution phase synthesis but in solid phase synthesis their reactions

proved sluggish due to competing oxazolone formation. However, efficient coupling occurred on the addition of an auxiliary nucleophile e.g. HOBt. In the future, due to the widespread use of t-butyl side chain protecting groups, the use of acid chlorides is likely to remain limited.

(c) Fluorides

Recently Carpino^{48,49} and others⁵⁰ have proposed the use of amino acid fluorides as isolable activated species for peptide synthesis. Acyl fluorides show much greater stability towards hydrolysis than the corresponding acid chlorides, while retaining a high reactivity towards nitrogen nucleophiles. Many Boc and Fmoc amino acid fluoride derivatives can be prepared from the parent amino acid derivatives using cyanuric fluoride (22) and these have proved effective in both solution and solid phase synthesis.



(d) Carbodiimides

Carbodiimides such as dicyclohexylcarbodiimide (DCC)⁵¹ (23) have been used for activation of carboxylic acids to nucleophilic attack since the 1950's. DCC was used in Merrifield's first reports on SPPS³ and has remained popular.

Although a highly successful reagent, DCC has several drawbacks. It causes dehydration of Asn and Gln residues⁵²⁻⁵⁴ and activated amino acids can spontaneously rearrange to form N-acyl ureas (24)⁵⁵. Also, a DCM-insoluble by-product, dicyclohexylurea (25) is formed during coupling. Other carbodiimides⁵⁶⁻⁵⁹

which form more soluble ureas have been suggested and of these, diisopropylcarbodiimide (DIC)^{56,57} has found favour in SPPS.

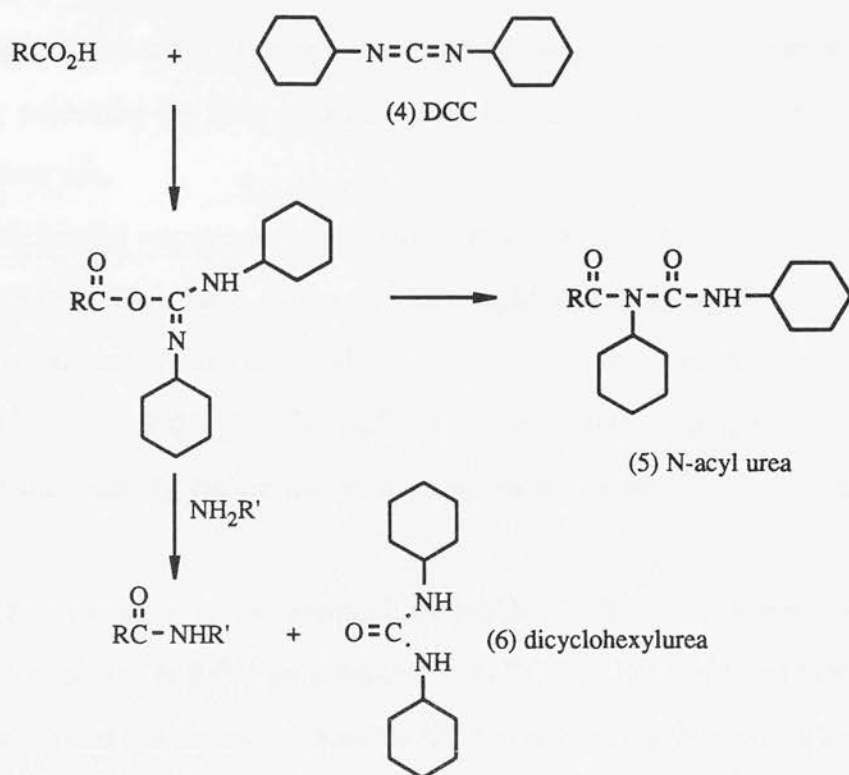
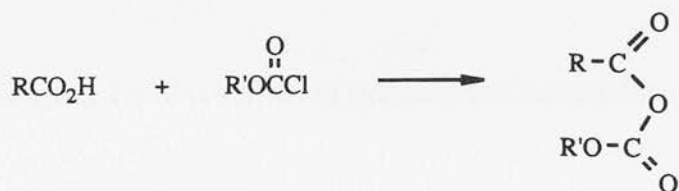


Figure 1.7: Peptide bond and N-acylurea formation using DCC

(e) Anhydrides

Mixed anhydrides have been used in peptide chemistry since the 1940s. Carboxylic-carbonic anhydrides have been generated from isobutyl^{60,61} or isopropyl chloroformate⁶²⁻⁶⁴ for use in SPPS.



Phosphinic-carboxylic anhydrides have been also been used. These can be generated with reagents such as diphenylphosphinyl chloride⁶⁵, 1-oxo-1-chlorophospholane⁶⁶, diphenylphosphinic anhydride⁶⁷ and pentafluorophenyl diphenylphosphinate⁶⁸. Although these mixed anhydrides have shown excellent coupling properties the long term instability of reagents used to generate them has limited their use.

Preformed symmetrical anhydrides (Figure 1.7) have found widespread use in SPPS. They have been shown to react rapidly in high yield and there is no ambiguity as to which carbonyl is attacked by the amine. Although some symmetrical anhydrides can be isolated as crystalline solids, they are usually prepared immediately before use with an activating carbodiimide such as DCC or DIC.

Disadvantages of symmetrical anhydrides include the already mentioned dehydration of Asn and Gln on treatment with DCC or DIC⁵²⁻⁵⁴ and high levels of racemisation that can occur with various His derivatives, e.g. Fmoc-His(Trt)²⁶. Also, symmetrical anhydrides are wasteful of amino acid derivatives since one equivalent of amino acid is not used in the coupling reaction.

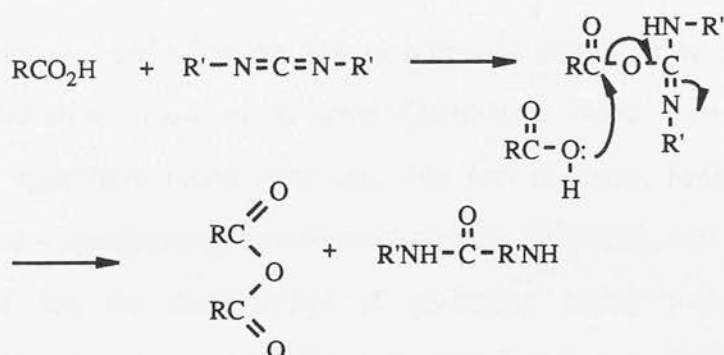
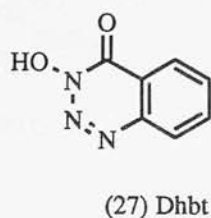
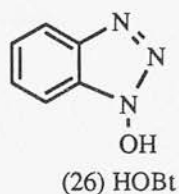


Figure 1.8: Formation of symmetrical anhydrides

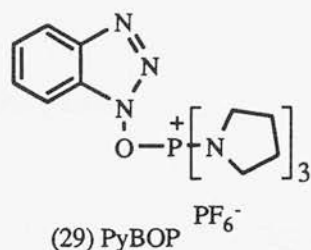
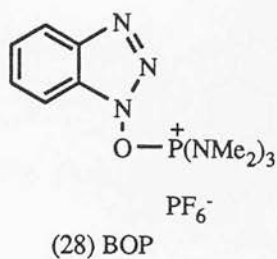
(f) Active esters

Due to some of the problems encountered in using carbodiimides and symmetrical anhydrides, activated esters have been widely studied. These include *ortho*- and *para*-nitrophenyl^{12,69}, trichlorophenyl^{70,71}, N-hydroxysuccinimidyl⁷² and recently, 1-hydroxy-4-ethoxycarbonyl-1,2,3-triazole⁷³ esters. However, the esters of N-hydroxybenzotriazole (HOBt)⁷⁴ (26), pentafluorophenol⁷⁵⁻⁷⁸ and 3,3-dihydro-4-oxobenzotriazin-3-ol (Dhbt)⁷⁹⁻⁸² (27) have attracted the most attention. These can be prepared by treating the amino acid derivative with DCC or DIC in the presence of the alcohol and used either *in situ* or isolated as relatively stable crystalline solids. These esters have been used widely and have produced efficient coupling with low levels of racemisation.

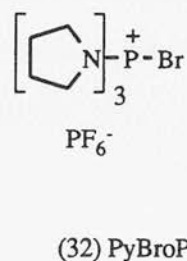
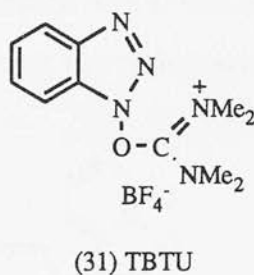
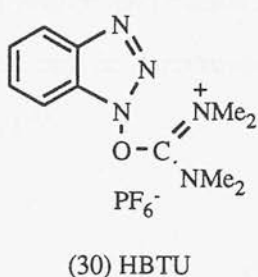


(g) Other coupling agents

The success of carbodiimides has prompted the search for other reagents for the *in situ* activation of carboxylic acids. Compounds based upon benzotriazole phosphonium salts have found wide use. The first of these, benzotriazolyl-oxyl-trisdimethylaminophosphonium hexafluorophosphate, BOP (28), was developed by Castro⁸³, but has the disadvantage of producing highly toxic hexamethyl phosphotriamide as a by-product. The pyrrolidine analogue, PyBOP (29), was introduced as a similar reagent without this drawback⁸⁴.



Knorr^{85,86} introduced benzotriazole tetramethyluronium salts, HBTU (30) and TBTU (31), as coupling agents while more recently Coste⁸⁷ has introduced PyBroP (32), which has been shown to be highly effective for even hindered couplings such as those of N-methyl amino acids.

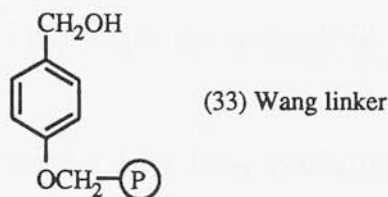


All of the reagents mentioned above can be used with an auxiliary nucleophile such as HOBT and the approximate order of reactivity is⁸⁸:



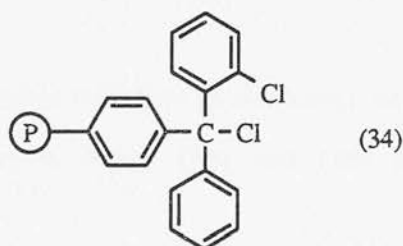
1.1.7 Peptide-resin link. Synthesis of C-terminal acids

With the advent of Fmoc methodology and the use of t-butyl side chain protecting groups came a need for more acid-labile linkers. The Wang linker⁸⁹ (33) was originally designed for the synthesis of protected fragments in combination with the highly acid-labile N^α -biphenyloxycarbonyl protecting group but it has found more general use in Fmoc SPPS.



The first amino acid is introduced by activation as a symmetrical anhydride and coupled to the resin-bound hydroxyl group in the presence of a catalytic amount of DMAP. The peptide can be cleaved from the resin in mild conditions (TFA), the incipient carbocation being stabilised by the *para*-alkoxy group.

Disadvantages of this linker are 2,5-diketopiperazine formation with C-terminal proline and partial racemisation with C-terminal Cys and His. Both of these problems can be circumvented using the highly acid labile 2-chlorotritylchloride linker (21)⁹⁰:



Diketopiperazine formation is suppressed by steric hindrance at the C-terminus, while racemisation is not an issue since the first amino acid is introduced as a diisopropylethylammonium salt. The chlorotrityl linker also offers the possibility of cleaving the side-chain protected peptide from the resin for use in fragment condensation.

1.1.9 Synthesis of C-terminal amides and other C-terminally functionalised peptides

Many biologically important peptides have a C-terminal amide functionality (e.g. hormones and many neurotransmitters). This, and the ability to modify peptide

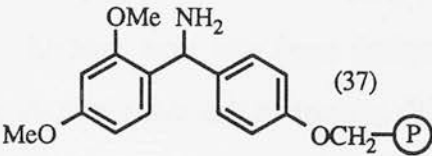
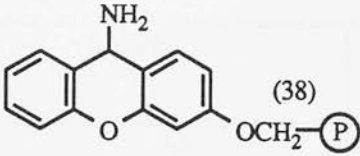
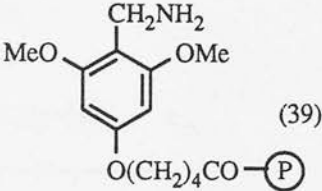
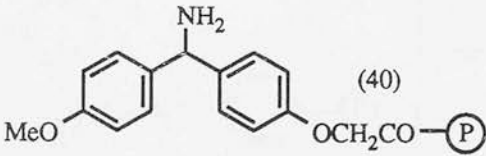
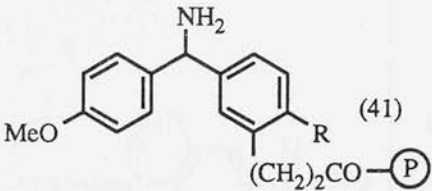
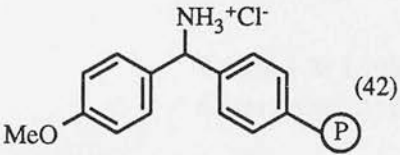
Linker	Cleavage Conditions	Ref.
 <p>(37)</p>	Dilute TFA	99
 <p>(38)</p>	Dilute TFA	100
 <p>(39)</p>	Dilute TFA	101
 <p>(40)</p>	TFA	102
 <p>(41)</p>	R=OMe Dilute TFA R=H Me ₃ SiBr/TFA	103, 104
 <p>(42)</p>	TFA	97, 105

Table 1.2: Linkers developed for the synthesis of peptide amides

trialkoxymethylamine linker (37) introduced by Rink⁹⁹ seems to have gained the most popularity.

Linkers have also been designed to offer other C-terminal functionalities. These include esters and hydrazides (Table 1.3), but this area has received relatively little attention compared with C-terminal acids and amides.

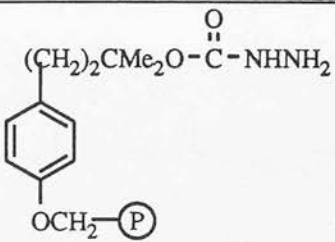
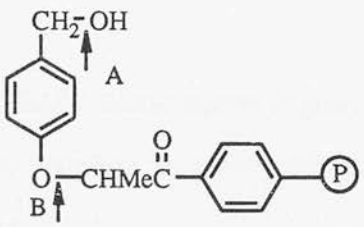
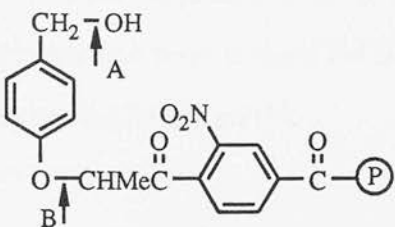
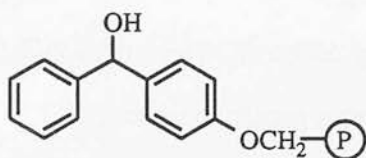
Linker	Cleavage Conditions	C-terminus	Ref.
	50% TFA/DCM	Hydrazide	106
	A= TFA B= hν 350 nm	A acid B 4-hydroxy-benzyl ester	107
	A= TFA B= hν 350 nm	A acid B 4-hydroxy-benzyl ester	108

Table 1.3: Linkers for the synthesis of C-terminal hydrazides and esters

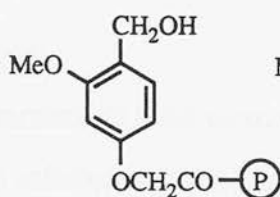
1.1.10 Synthesis of protected fragments

The mild procedures developed for Fmoc peptide chemistry have resulted in a number of linkers from which the peptide can be cleaved by mild acidolysis leaving side chain protected peptides, useful for fragment condensation reactions.

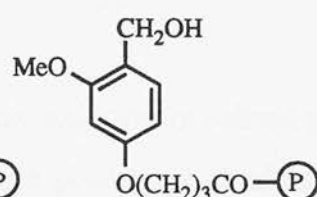
The chlorotrityl linker⁹² (34) has already been mentioned in this context but other examples include the alcohol variant of the Rink linker¹⁰⁰ (30), the SASRIN linker¹⁰⁹ (44) and the recently introduced HMPB linker¹¹⁰ (45):



(43)



(44)



(45)

The HMPB linker shows slightly more acid stability than the Rink linker and enables acidic coupling reagents, such as HOBt, to be used without cleavage of the peptide-resin linkage.

Other approaches have been employed for the synthesis of protected fragments and these include the fluoride labile linkers of Ramage¹¹¹ and Barany¹¹², and photolabile linkers¹¹³.

1.2 Peptide Purification

Although the synthesis of peptides on a solid phase support has developed into a highly sophisticated science, the product cleaved from the resin is seldom pure. This may be due to side reactions that can occur during assembly or cleavage or due to incomplete coupling steps. All of these factors combine to produce an impure product. Often the crude material may contain only a minor amount of

contaminants but nevertheless, one or more purification steps are required to obtain the peptide in pure form.

A whole range of purification techniques are available to purify synthetic peptides. While many were initially developed for the purification of biological molecules isolated from natural sources, others have been designed specifically to overcome problems associated with synthetic peptides. A few of the more common techniques are outlined below.

1.2.1 Gel Filtration Chromatography

Gel filtration utilises a polysaccharide solid matrix with closely defined pore sizes. Separation depends upon the relative size (or more precisely hydrodynamic volume) of the constituents of the mixture. Larger molecules are completely excluded from the pores of the solid support and elute with the void volume of the column. Smaller molecules have access to the pores in different degrees and are retained depending on the extent of this access.

A variety of gel filtration media are available based on dextran (sephadex, sephacryl) or agarose. The amount of cross-linking in these materials can be varied, giving rise to solid supports with different pore and particle sizes.

Gel filtration lends itself readily to the separation of peptides from lower molecular weight species such as scavengers or salts but with careful choice of gel and operating conditions it can also be used for the fractionation of peptides.

1.2.2 Ion-exchange Chromatography

Proteins and peptides carry both positive and negatively charged groups. Positive charges are carried by basic groups (N-terminus, lysine, arginine, histidine) which can be protonated, while negative charges are carried by ionised acidic groups

(C-terminus, aspartic and glutamic acids). The net charge on a peptide depends on the relative numbers of these groups and varies with pH.

In ion exchange chromatography the different charges on peptides and proteins is used as a basis for separation of the components of a mixture. Peptides can be bound to an ion-exchange column by electrostatic interactions with an immobilised inorganic counter ion. Subsequent elution is accomplished by bringing the pH closer to the isoelectric point, pI, of the peptide or by changing the ionic strength of the eluent. In practice a gradient which varies either or both of these factors can be used to effect peptide purification.

1.2.3 Reverse Phase High Performance Liquid Chromatography (RP-HPLC)

Separation by RP-HPLC relies upon the hydrophobic interaction of the components of a mixture with long chain alkyl groups, covalently bound to a solid support. Usually these are C₈ or C₁₈ alkyl chains bound to silica but other immobilised organic moieties are available (e.g. C₃, C₄, C₆ and phenyl).

The mobile phase consists of water, a miscible organic solvent and dissolved buffers and salts. Separation is achieved by a gradient elution, gradually increasing the proportion of the organic component (usually acetonitrile or isopropanol) in the aqueous eluent. Rapid separations with high resolution can easily be achieved and RP-HPLC is probably the most widely used technique for the purification of synthetic peptides.

A drawback to the method has been the instability of the silica matrix at pH values greater than 7. This can be a problem with acidic peptides which are insoluble in the buffers normally used. However, non-silica based packing materials (polyethers and polystyrene) are now available and are stable over a wide range of pH values.

1.2.4 Affinity Chromatography

Molecules such as proteins and peptides carry out their biological function by a highly specific binding to substrates. This interaction has been utilised for the purification of biomolecules in affinity chromatography, where a ligand L, immobilised on a solid support, can be used to separate a substrate S, for which it exhibits a strong binding affinity (Figure 1.9).

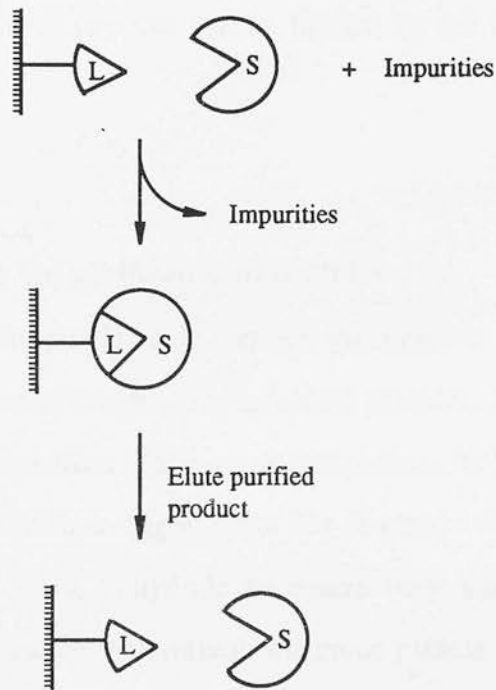


Figure 1.9: Affinity Chromatography

Immobilisation of the ligand is accomplished by activating hydroxyl groups on the solid support (typically agarose) to attack by nucleophiles on the ligand (amino, thiol). Activating species include cyanogen bromide and sulphonyl chloride.

Once the mixture has been applied to the column and the non-specifically bound components eluted then the substance of interest can be eluted. This is usually

accomplished by a change in the solvent conditions e.g. ionic strength, pH, use of denaturants, or temperature etc.

This method can be very powerful, and may be used to obtain the desired substance in high purity by a single step purification, even when present in low abundance. For example, Merrifield⁵ used immobilised polyclonal human leucocyte interferon antibodies for the purification of synthetic human leucocyte interferon α_1 and α_2 (166 and 155 residues respectively). However, the more general application of this method to synthetic peptides can be limited by the availability of suitable ligands.

1.2.5 Affinity labels for the purification of peptides

One of the main problems in peptide purification, particularly for long sequences, is the removal of accumulated truncated peptides. Truncated peptides are formed when coupling reactions fail to go to completion, for reasons such as steric hindrance, folding or inter-chain aggregation. The N-termini of these truncations are routinely capped with acetic anhydride to ensure they play no further part in synthesis, so that at the end of the synthesis the crude peptide consists largely of the desired sequence and these acetylated failure sequences, the separation of which can prove difficult using conventional chromatographic techniques.

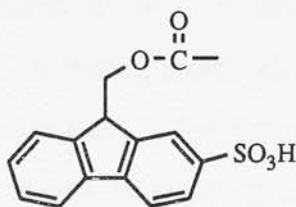
In a variation of the affinity method described above, several workers have suggested methods whereby the N-terminus of the desired peptide is derivatised with a group capable of a covalent or affinity binding to a solid support. This allows the peptide to be selectively immobilised while the acetylated impurities elute unimpeded.

(a) Polar groups

Early efforts on the the design of affinity tags concentrated on the introduction of polar groups to enhance the charge on the peptide and hence its retention on ion exchange columns.

Suzuki¹¹⁴ introduced an additional lysine onto the N-terminus of small fragments of the B chain of human insulin. After purification on a carboxymethyl-cellulose column the amino acid extension was removed by an Edmann degradation.

Using a similar strategy, Merrifield¹¹⁵ functionalised peptide N-termini with a sulphonic acid derivative of the Fmoc group (46) to facilitate purification on a DEAE-cellulose column. This group had the advantage of being readily cleaved from the purified peptide with mild base e.g. 5% triethylamine.



(46) SulFmoc

Reciprocal strategies have also been suggested, whereby polar anhydrides are used in place of acetic anhydride for capping the truncated peptides. 3-Nitrophthalic anhydride¹¹⁶, 2-sulphobenzoic acid anhydride¹¹⁷ and 3-sulphopropionic anhydride¹¹⁸ have all been used in this manner when it was the truncations that were strongly retained on ion exchange columns and the desired peptide eluted.

Although all the above cases were successful, only relatively short peptides (< 10 residues) were purified. It is doubtful whether this methodology could be applied to longer peptides where there will be intrinsically more polar groups present, diluting the effect of the added polar handle.

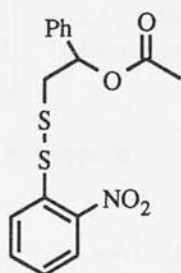
(b) N-terminal thiol groups

The reactivity of thiol groups i.e. nucleophilicity, disulphide formation and metal-ion complexation have all been exploited to selectively bind peptides to solid supports.

The first such method was suggested by Merrifield¹¹⁹ and involved extending the peptide by an additional two residues, Cys-Met-. The cysteine moiety enabled the peptide to be selectively bound to an organomercury-sepharose column, while the methionine group enabled the two residue extension to the peptide to be selectively removed with cyanogen bromide. This was used successfully for the purification of ribonuclease (111-124), but is not readily applicable to other cysteine and methionine containing peptides.

In a similar method, Funakoshi *et al*¹²⁰ derivatised the N-termini of the completed peptide with a thiol-containing group. This enabled the peptide to react selectively with an iodoacetamide resin. After elution of the acetylated truncated impurities, the free peptide could be obtained on treatment with base, the base-lability being conferred by an electron-withdrawing sulphone group in the affinity tag (Figure 1.10). This method has been successfully applied to the purification of cholecystokinin (33 residues) and human growth hormone-releasing factor (44 residues).

Lansbury¹²¹ recently suggested the use of the N-terminal (2-[(2-nitrophenyl)dithio]-1-phenylethoxy)carbonyl, NpSSPeoc (47) group.



(47) NpSSPeoc

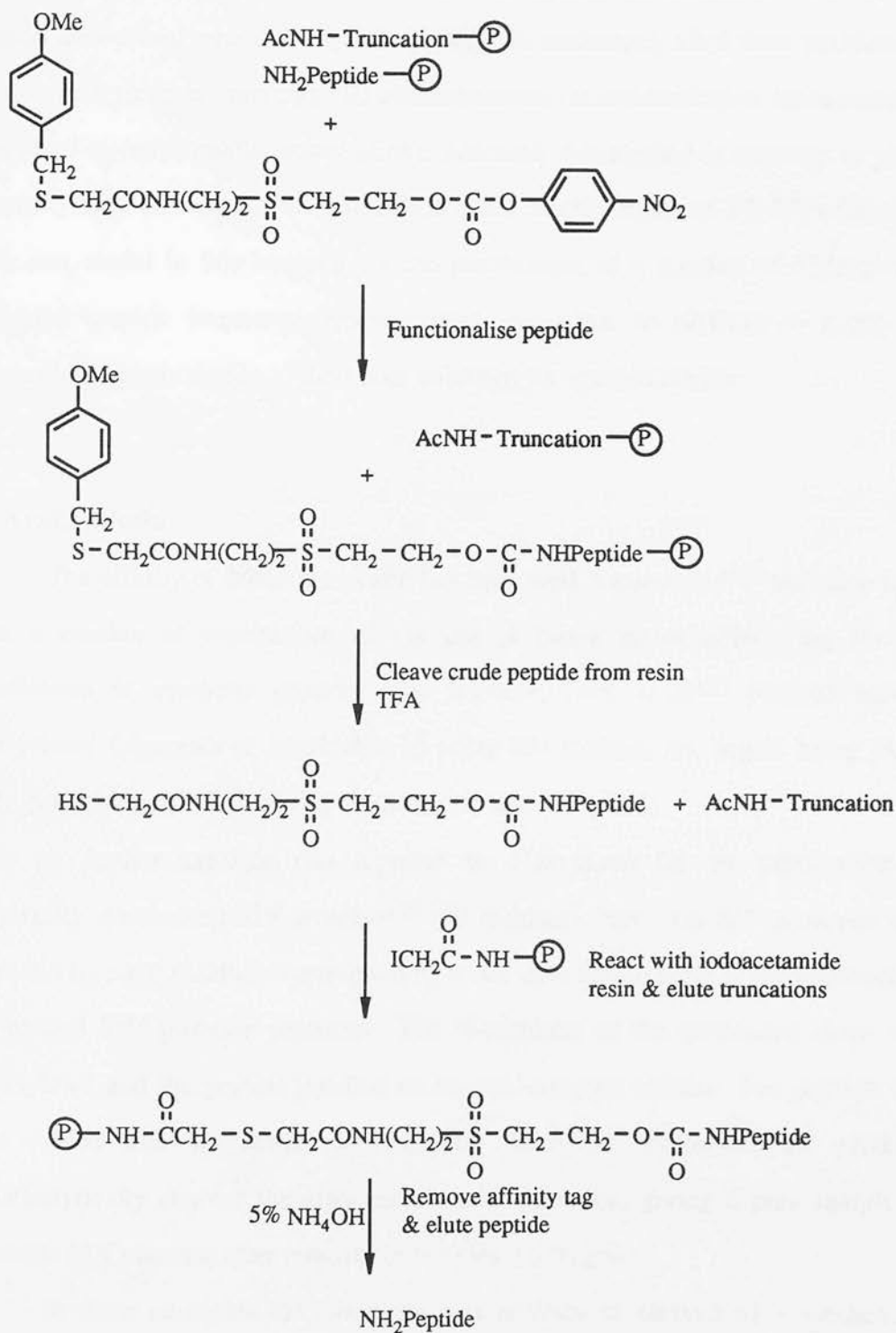


Figure 1.10. Affinity-type purification of synthetic peptides on iodoacetamide resin¹²⁰

The disulphide, or reduced thiol form, was used to bind the peptide to a polystyrene support derivatised with a disulphide (disulphide exchange), alkyl thiol (oxidation - disulphide formation), mercury (II) trifluoroacetate (complexation) or iodoacetamide functionality (nucleophilic substitution). Although this method is unlikely to prove generally applicable, since the NpSSPeoc group is only stable to 1% TFA for *ca.* 1 h, it was useful in this instance for the purification of a number of hydrophobic protected peptide fragments, species which can often be difficult to purify by conventional methods due to their poor solubility in aqueous media.

(c) Avidin-biotin

The affinity of biotin for avidin has been well documented¹²² and there has been a number of applications of the use of biotin as an affinity tag for the purification of synthetic peptides. For instance, Lobl *et al*¹²³ purified several biotinylated fragments of interleukin-1 β using this method, the largest being [Asp-205]-interleukin-1 β (117-269), present in less than 1% yield.

A further example was reported by Heinrikson for the purification of chemically synthesised SIV-protease¹²⁴ (99 residues). Here, the SIV sequence was extended by eight residues, corresponding to the next amino acids in the sequence of the natural SIV protease precursor. The N-terminus of the completed chain was biotinylated and the peptide purified on an avidin-agarose column. The peptide was then folded into its active conformation when the synthetic SIV protease autocatalytically cleaved the eight amino acid extension, giving a pure sample of synthetic SIV protease after removal of the cleaved fragment.

In these examples the biotin tag was retained or cleaved by a mechanism specific to the peptide. For more general applications, the affinity label should be readily removed after purification. In this respect Wilchek¹²⁵ has employed biotinylated methionine which can be cleaved with cyanogen bromide, although this

suffers from the same problem of other similar applications in not being useful for sequences containing other methionine residues.

A number of other affinity labels have been developed over the years. For example highly immunogenic dinitrophenyl tags have been used, which can be bound to a column derivatised with anti-dinitrophenyl antibodies¹²⁶. Mascagni¹²⁷ *et al* have developed a modified Fmoc group, incorporating an amino decanoic acid dipeptide at the 4-position, which imparts hydrophobic character to the peptide, resulting in enhanced retention times on RP-HPLC. This proved useful for the purification of synthetic HIV-1 p24 GAG (270-373) (104 residues), and when taken with the examples above, would seem to augur well for the future use of affinity tags for the purification of chemically synthesised proteins.

CHAPTER 2

AFFINITY PURIFICATION OF SYNTHETIC PEPTIDES ON POROUS GRAPHITISED CARBON

2.1 Design of an affinity tag for purification of peptides on porous graphitised carbon

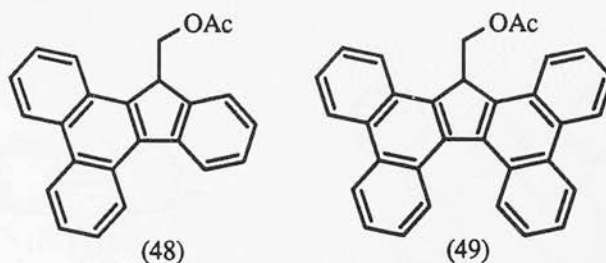
Porous graphitised carbon (PGC) is a chemically and mechanically robust two dimensional graphite with a large surface area (*ca.* 150 m²/g)¹²⁸. It is made by polymerising a phenol-hexamine mixture within the pores of silica gel and pyrolysing the resin in an atmosphere of nitrogen. The silica template is then dissolved and the residue heated to a temperature in excess of 2000°C. This material was developed by Knox¹²⁸ as a solid support for HPLC and has proved to be a strong adsorbent for hydrophobic species, in particular for large flat molecules¹²⁹.

Inspired by this work, Ramage and Raphy^{130,131} attempted to exploit the hydrophobicity of this substance for the purification of synthetic peptides. They set out to design an aromatic molecule that would adsorb strongly to PGC. If the N-terminus of a peptide could be tagged with this species then in principle the peptide could be separated from truncated sequences by selective binding to PGC. Such a molecule would:

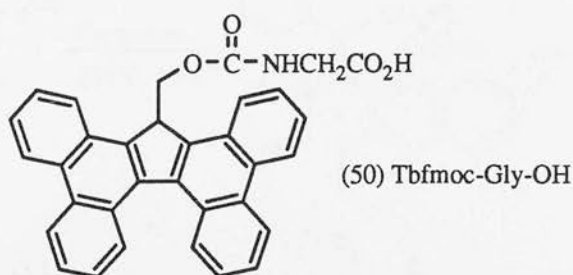
- (a) be sufficiently large and flat, so as to be strongly retained on PGC;
- (b) be stable to conditions used to cleave the peptide from the resin (TFA/scavengers);
- (c) ideally have a chromophoric group (>350 nm) to allow UV monitoring;
- (d) be readily removed from the peptide when required.

Fused benzofluorenes meet these requirements and Ramage and Raphy synthesised two such systems, dibenzofluorene and tetrabenzo[*a,c,g,i*]fluorene. Dibenzofluorene

derivative (48) failed to exhibit sufficiently strong retention on PGC, but the tetrabenzo[*a,c,g,i*]fluorene system (49) showed excellent properties.



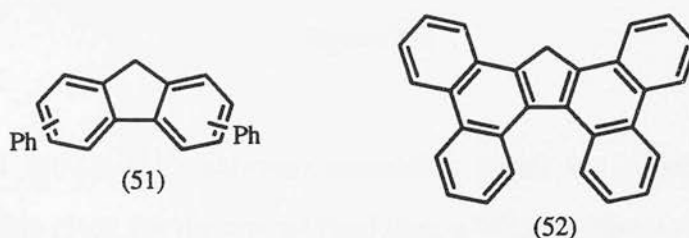
The tetrabenzo[*a,c,g,i*]fluorene group was incorporated into an amino acid, glycine, as a base-labile N^{α} -urethane protecting group, tetrabenzo[*a,c,g,i*]fluorenyl-17-methoxycarbonyl (Tbfmoc). Tbfmoc-Gly-OH (50) was used as the N-terminal amino acid in the solid phase synthesis of several peptides (6, 23 and 42 residues) and these were found to selectively bind to PGC in the presence of acetylated truncated sequences. Following separation of the truncations, the peptides could be released from the PGC by removal of the Tbfmoc group with base, clearly demonstrating the potential of this methodology for peptide purification.



Further studies on the preparation and application of Tbfmoc derivatives are now reported.

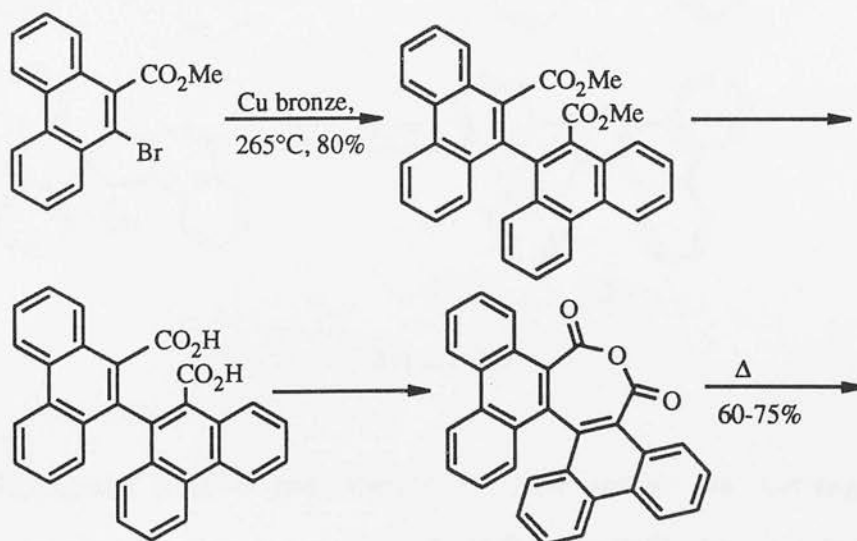
2.2 Synthesis of tetrabenzo[*a,c,g,i*]fluorene derivatives. Historical perspective

Many procedures have been described for the synthesis of fused benzofluorene derivatives (51) and these have been reviewed^{132, 133}.



Of the many fused benzofluorenes reported, derivatives of tetrabenzofluorene (52) have received relatively little attention and until recently only two syntheses had been described, both due to Martin and co-workers^{134,135}.

The first route (Figure 2.1) requires a multistep synthesis to obtain the 9,10-disubstituted phenanthrene starting material. Thereafter, tetrabenzofluorene (5) is obtained in low overall yield (<12%)¹³⁴.



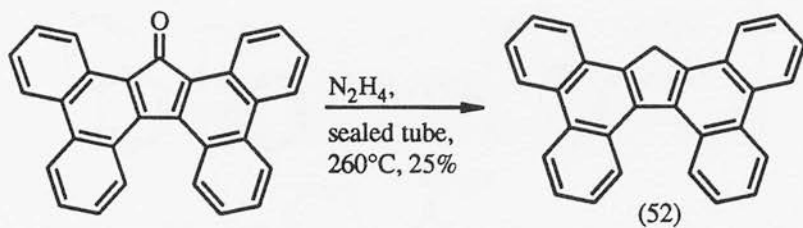


Figure 2.1

In the second synthesis¹³⁵, although reasonable yields are quoted for the earlier steps, no yield is given for the crucial final step, a Wagner-Meerwein rearrangement (Figure 2.2).

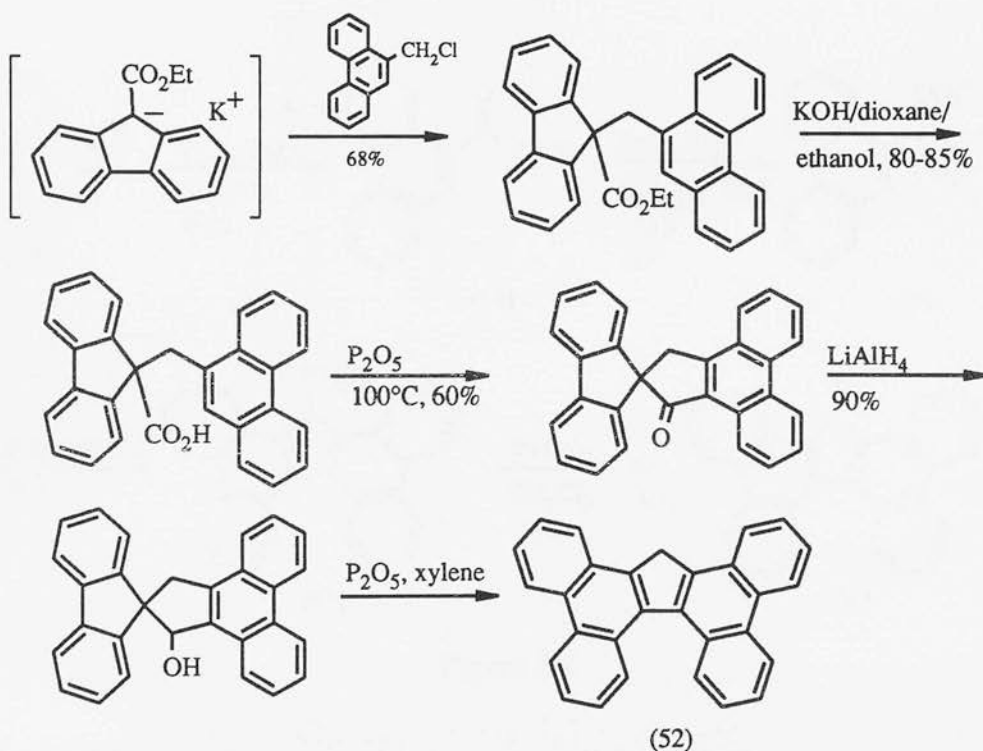


Figure 2.2

Hopkinson *et al*¹³⁶ and others^{137,138} have studied the rearrangement of electron-deficient diarylmethyl cations to form fluorene derivatives, e.g. Figure 2.3:

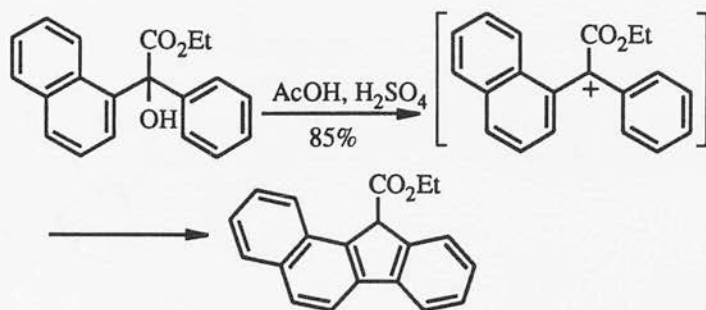


Figure 2.3: Rearrangement of electron-deficient diaryl cations¹³⁶

Recently, Ramage and Raphy^{130,131} exploited this reaction for the synthesis of 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol (56) (Figure 2.4).

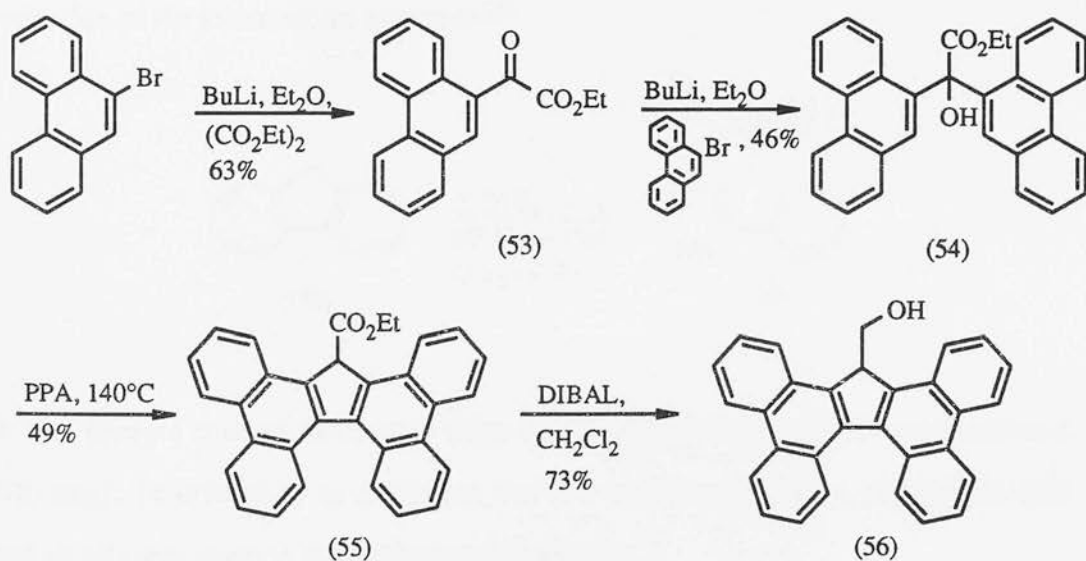
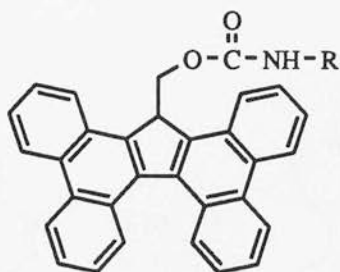


Figure 2.4

A low overall yield (10%) after several chromatographic separations made this route unattractive for obtaining multigram quantities of (56). Initial studies therefore concentrated on improving the synthesis of (56), a key intermediate in the synthesis of Tbfmoc amino acids and peptides (57).

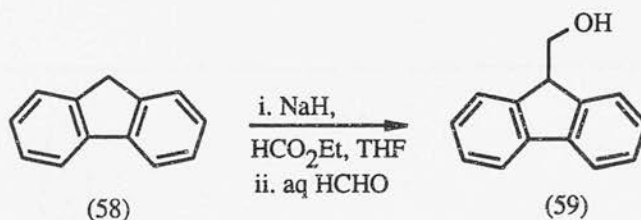


(57)

R = -NH-Peptide,
-NHCHR'COOH

2.3 Synthesis and reactions of tetrabenzo[*a,c,g,i*]fluorene (52)

Carpino has reported the synthesis of 9-fluorenylmethanol (59) by reacting of the anion of fluorene (58) with ethyl formate, followed by *in situ* cross-Cannizzaro reduction of the intermediate aldehyde¹³⁹:



It was thought that an alternative route to 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol (56) might be offered by an analogous reaction with tetrabenzo[*a,c,g,i*]fluorene (52) and an efficient route to this molecule was sought

The Grignard reagent of 9-bromophenanthrene was generated in ether and following addition of ethyl formate, alcohol (60) was isolated in *ca.* 40% yield. The yield improved to 52% in THF which proved a better solvent for both starting material and intermediates.

Attempts were then made to cyclise alcohol (60) to form tetrabenzo[*a,c,g,i*]fluorene (52). Both PPA¹³⁰ and Eaton's reagent¹⁴⁰ gave intractable brown solids, while with $\text{CH}_2\text{SO}_4/\text{AcOH}$ ¹³⁶ only traces of the fluorescent product were detected by TLC. However, a [2+2] Nazarov-type cyclisation occurred

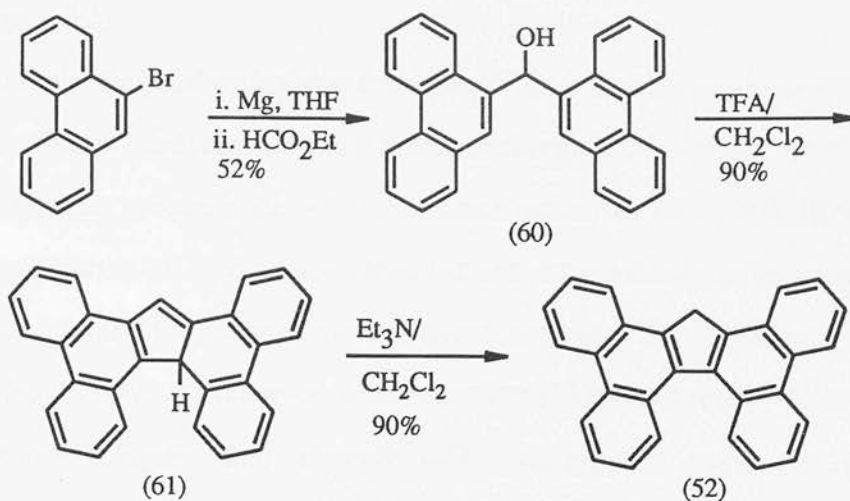


Figure 2.5: Synthesis of tetrabenzo[*a,c,g,i*]fluorene (52)

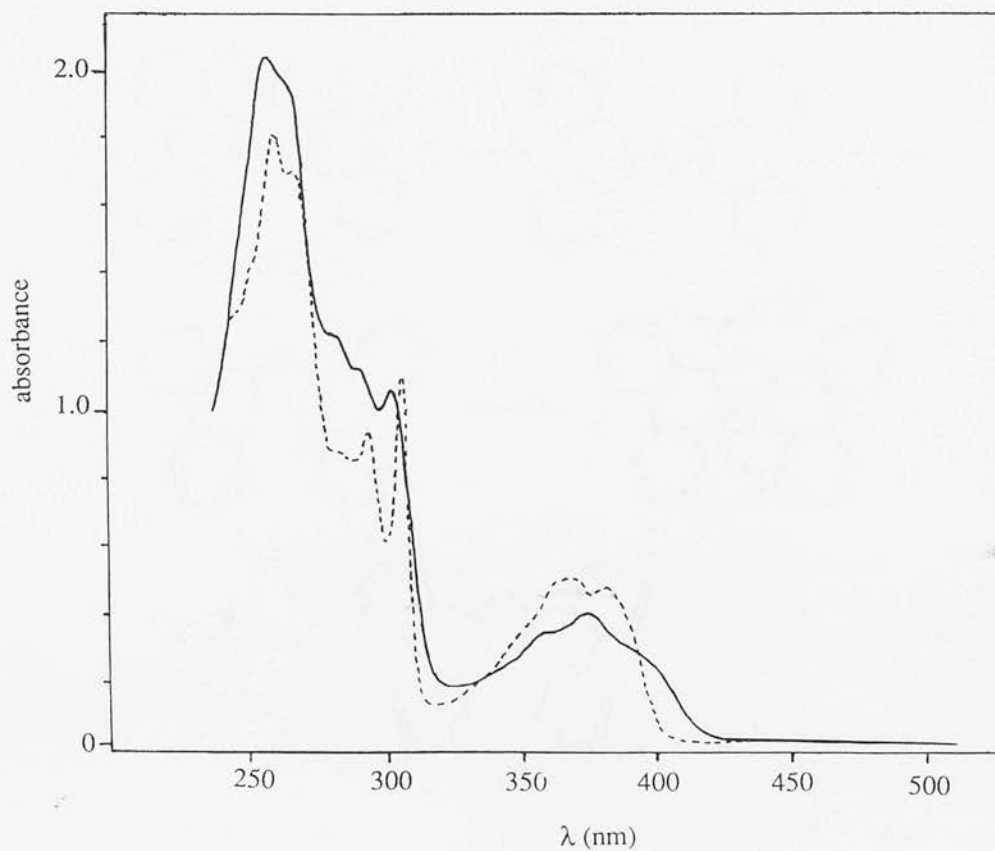


Figure 2.6: UV spectra of --- (52) ($2.73 \times 10^{-5}M$) and — (61) ($3.01 \times 10^{-5}M$)

smoothly, upon treatment of (60) with TFA in DCM and (61) was isolated in 90% yield (Figure 2.5).

Initially the product isolated from the TFA cyclisation reaction was thought to be (52). The isolated material had a similar melting point to the literature value for (52) and the mass spectrum showed the expected molecular ion at 366. However, the ^1H NMR spectrum showed a more complex aromatic splitting pattern than would have been expected for a symmetrical structure such as (52) and only a one proton signal at δ 5.42 ppm was observed where structure (52) would require two. Also, the UV spectrum (Figure 2.6) showed subtle differences from the published spectrum¹³⁴. Based upon these observations and taking into account the possible intermediates that would be formed via the mechanism shown in figure 2.7, structure (61) was assigned, and this was supported by later work (see section 2.4).

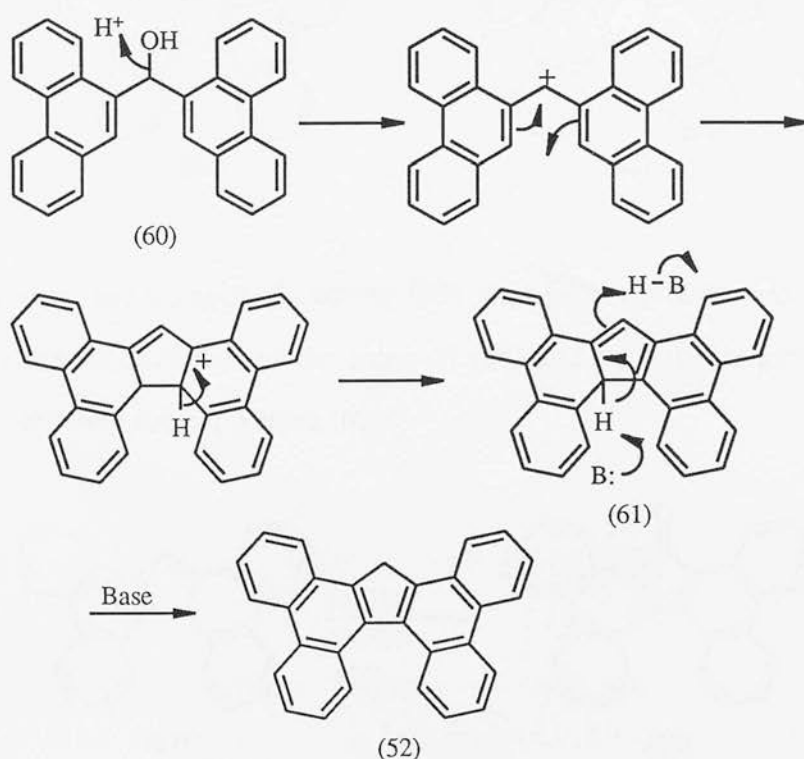
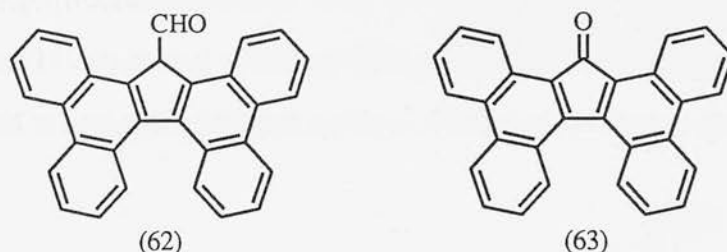


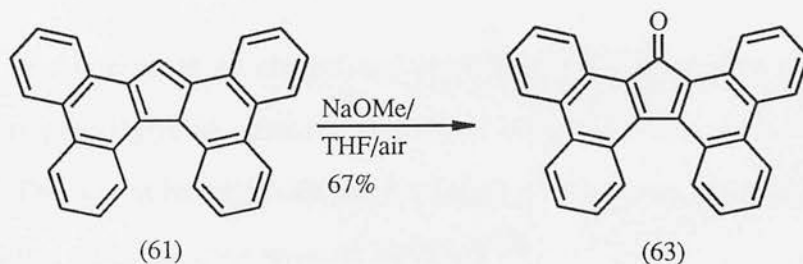
Figure 2.7: Cyclisation of *bis*-phenanthrylmethanol (60) in acid and base-catalysed rearrangement to tetrabenzo[*a,c,g,i*]fluorene system

Previous reports on analogous cyclisations¹³⁶⁻¹³⁸, have referred only to the isolation of the expected fluorene system, invoking a hydride shift¹³⁷ as a mechanistic explanation for its formation. This would not appear to be the case, at least in this instance, where the formation of the fluorene system requires a deprotonation and reprotonation, catalysed by base (Figure 2.7).

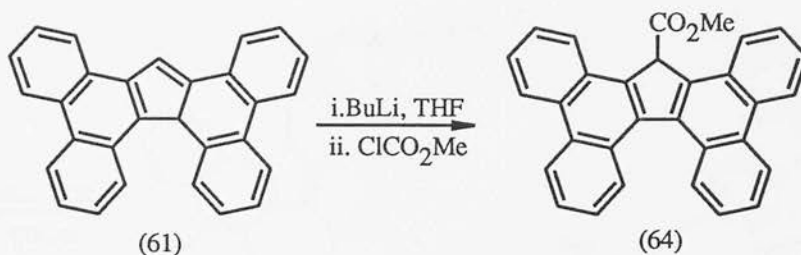
The next stage of the synthesis required the reaction of the anion of (61) with a suitable electrophile. Treatment of (61) with ethyl formate in the presence of sodium hydride followed by an aqueous formaldehyde work-up¹³⁹ failed to produce any of the desired alcohol (56). Quenching the reaction with water in place of formaldehyde showed that none of the expected aldehyde intermediate (62) had been formed.



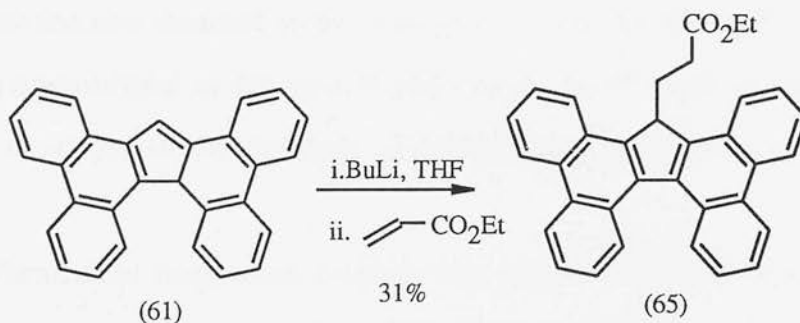
Instead, a dark red compound, ketone (63) was isolated. This was presumably formed by the air oxidation of the anion of (61) and in fact this proved to be a reasonably efficient route to ketone (63):



Attempts to react the anion of (61) with another suitable electrophile, methyl chloroformate also proved fruitless and ester (64) could only be isolated in poor yield (20%).



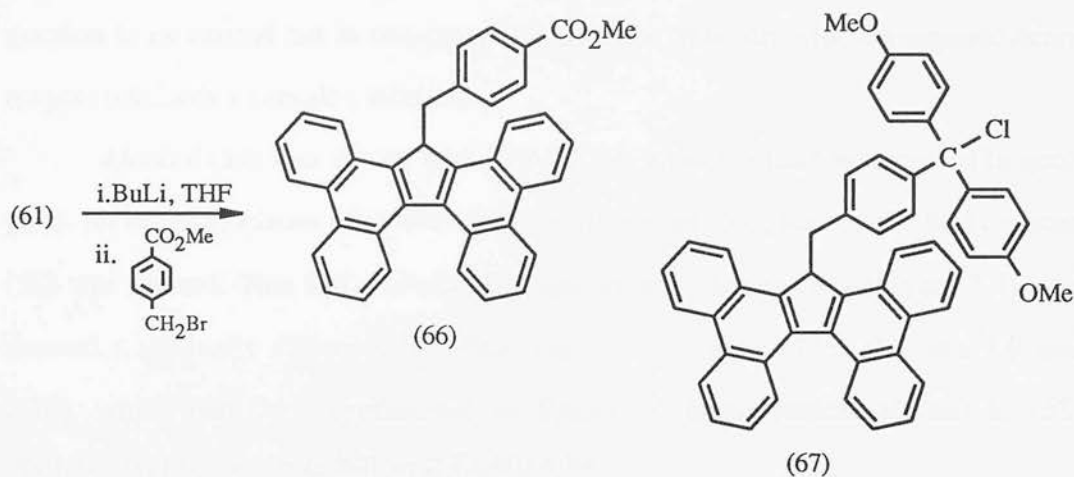
In light of the poor reactivity of the anion of (61), with suitable electrophiles, this strategy was abandoned for the synthesis of 17-tetrabenzofluorenylmethanol (56). However, it may provide a convenient route to other 17-substituted tetrabenzofluorene derivatives. For instance, (61) was found to undergo a Michael addition with ethyl acrylate to give ester (65):



Reduction of this ester to an alcohol and esterification of the alcohol with a chiral acid such as phenylglycine would give a chiral compound that could be adsorbed onto PGC. This might be useful for the resolution of racemates, in a similar fashion to the modified silica columns reported by Pirkle¹⁴¹.

In addition, Wahl has recently reported that the reaction of (61) with allyl bromide or benzylic bromides takes place in reasonable yield¹⁴². For example (66)

was synthesised as a key intermediate in the synthesis of chlorotrityl derivative (67) which was used as a hydrophobic 5'-OH protecting group for the purification of oligonucleotides by HPLC¹⁴².

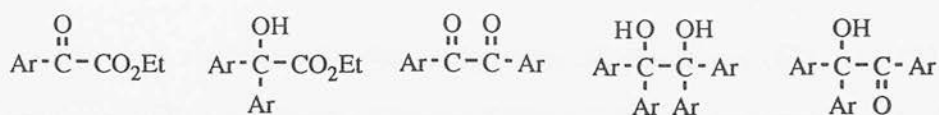


2.4 Synthesis of 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol (56)

Attention now switched to the strategy developed by Raphy¹³⁰ (Figure 2.4) to see if improvements to the overall yield could be achieved by applying the chemistry developed in the synthesis of tetrabenzo[*a,c,g,i*]fluorene (52), (section 2.2).

9-Phenanthryl magnesium bromide was treated with ethyl oxalate, giving alcohol (54) in a reasonable yield of 57% (Figure 2.8). This contrasts with the reaction of 9-phenanthryllithium reported by Raphy¹³⁰, where a one pot synthesis of (54) failed, the reaction having to be carried out in two separate reactions with a combined yield of 29%.

This reflects the relative reactivities of the two aryl-metallic reagents. Ethyl oxalate has two ester groups and could conceivably react with an organometallic reagent to form five possible products:



Presumably, the Grignard reagent is sufficiently selective to allow the two step reaction to be carried out in one-pot while a similar reaction with the organolithium reagent produces a complex mixture.

Alcohol (54) was treated with TFA/DCM, when cyclisation occurred in good yield. As in the synthesis of tetrabenzo[*a,c,g,i*]fluorene (52), mainly the 8*b*-H isomer (68) was formed. This had a similar UV spectrum to that of (61) (Figure 2.6), and showed a distinctly different ¹H NMR spectrum to isomer (55) (Figures 2.9 and 2.10), which had been synthesised by Raphy¹³⁰. Isomerisation of (68) to (55) occurred rapidly on treatment with Et₃N/DCM.

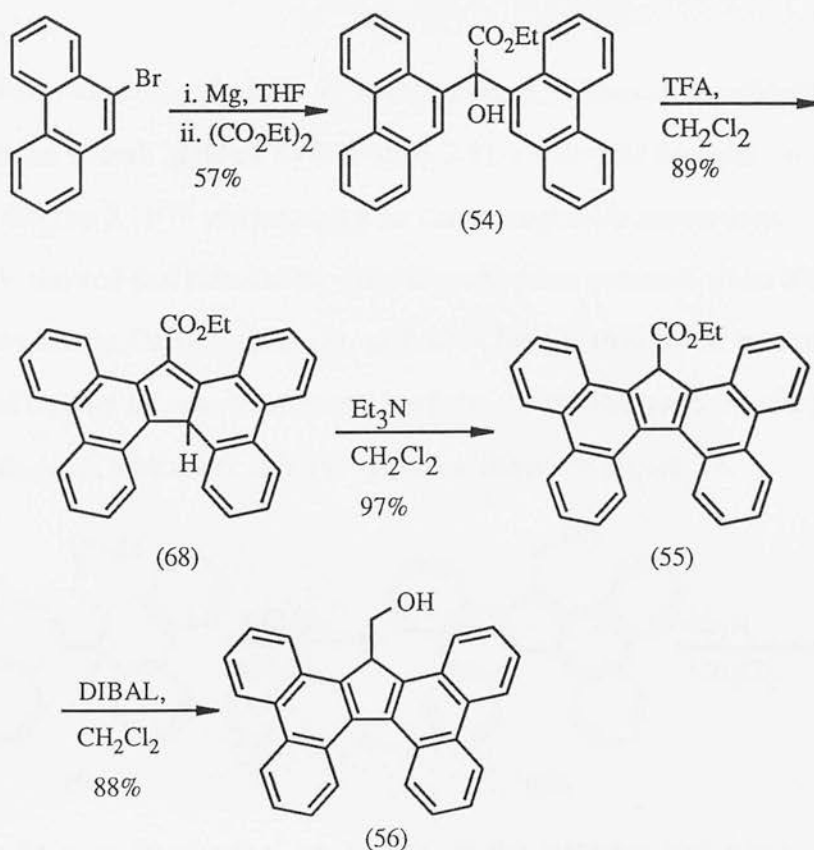


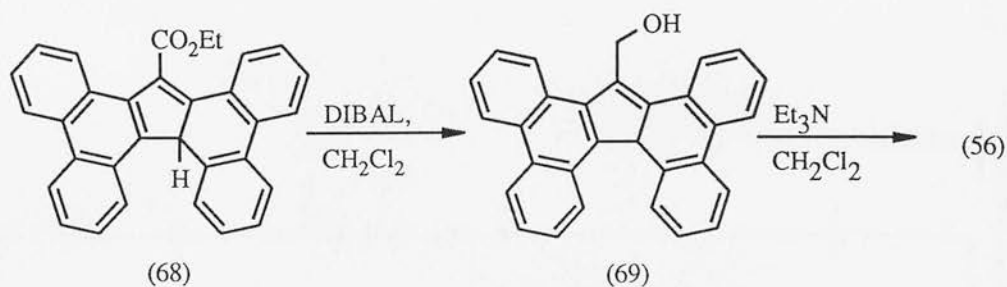
Figure 2.8: Synthesis of 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol

Additional evidence for the assignment of structures (68) and (55) was obtained from NOE experiments (Figures 2.9 and 2.10). In each case, irradiation of the ethyl ester CH₂ and the 5-membered ring CH were carried out and the NMR difference spectrum plotted. In isomer (68), irradiation of the CH₂ produced positive NOE's for the two one proton resonances centred at 8.11 ppm and 7.73 ppm (H-1 and H-16), while irradiation of the 8*b*-H gave a positive NOE at a different one proton aromatic resonance centred at 8.01 ppm (H-8). This was consistent with structure (68), the ethyl CH₂ and 8*b*-H being located close to different aromatic protons.

When a similar experiment was performed on isomer (55), the same aromatic resonance was affected in each case (equivalent protons H-1 and H-16), in line with the much closer proximity of the ester and 5-membered ring CH required by structure (55).

After reduction of (55), 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol (56) was obtained in an overall yield of 41% (Figure 2.8), a four-fold increase on the original synthesis (Figure 2.4)¹³⁰ and requiring no chromatographic separations.

UV showed that remarkably little isomerisation occurred when (68) was used as substrate for the DIBAL reduction and 8*b*-H isomer (69) could be converted into the desired alcohol on base treatment. However, the overall yield on carrying out the steps in this order was lower than the sequence shown in Figure 2.8.



No problems were encountered on scaling up the reactions and access to 30-40 g batches of alcohol (56) was readily achieved.

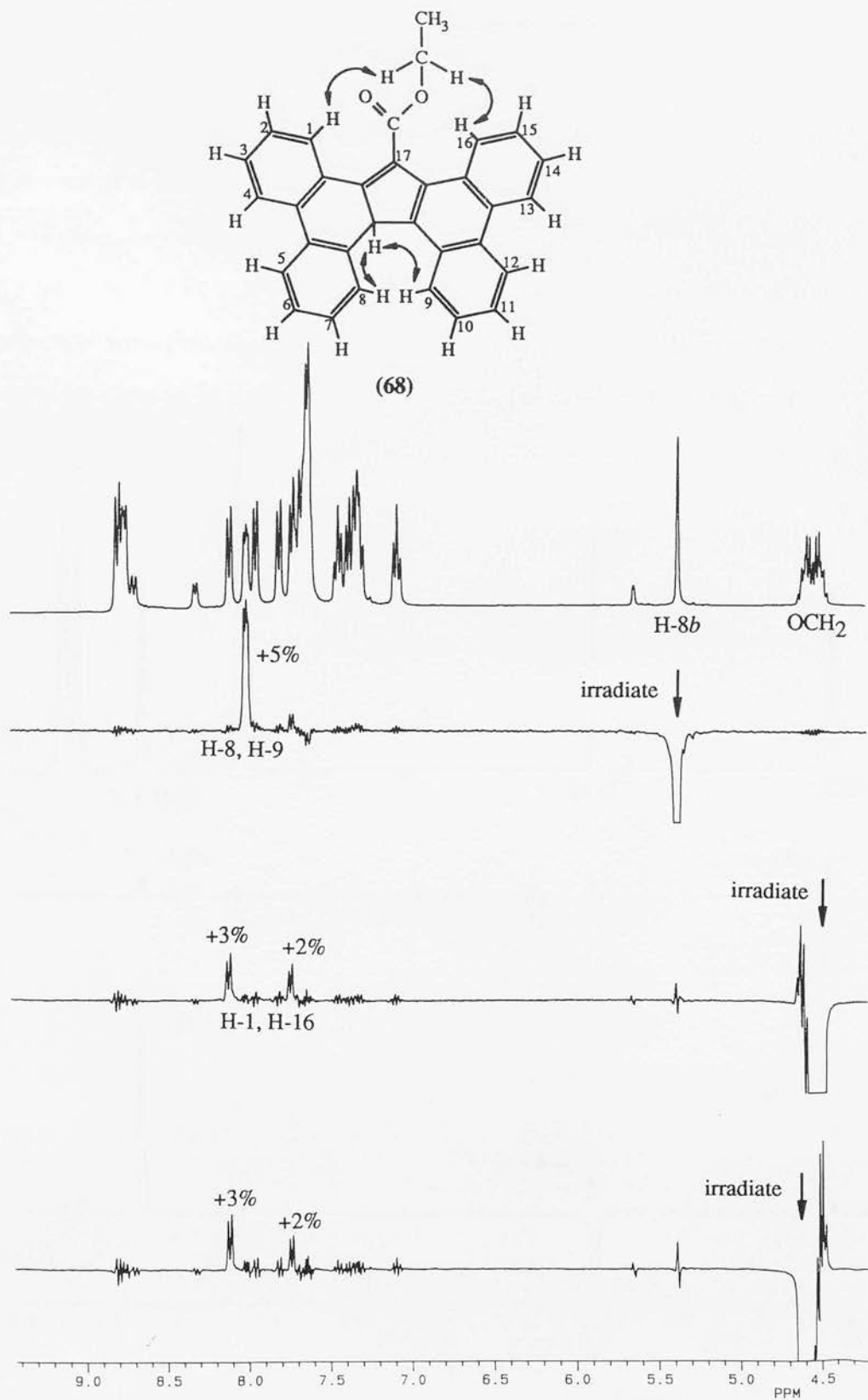


Figure 2.9: ^1H NMR spectrum (360 MHz) and NOE experiment on isomer (68)

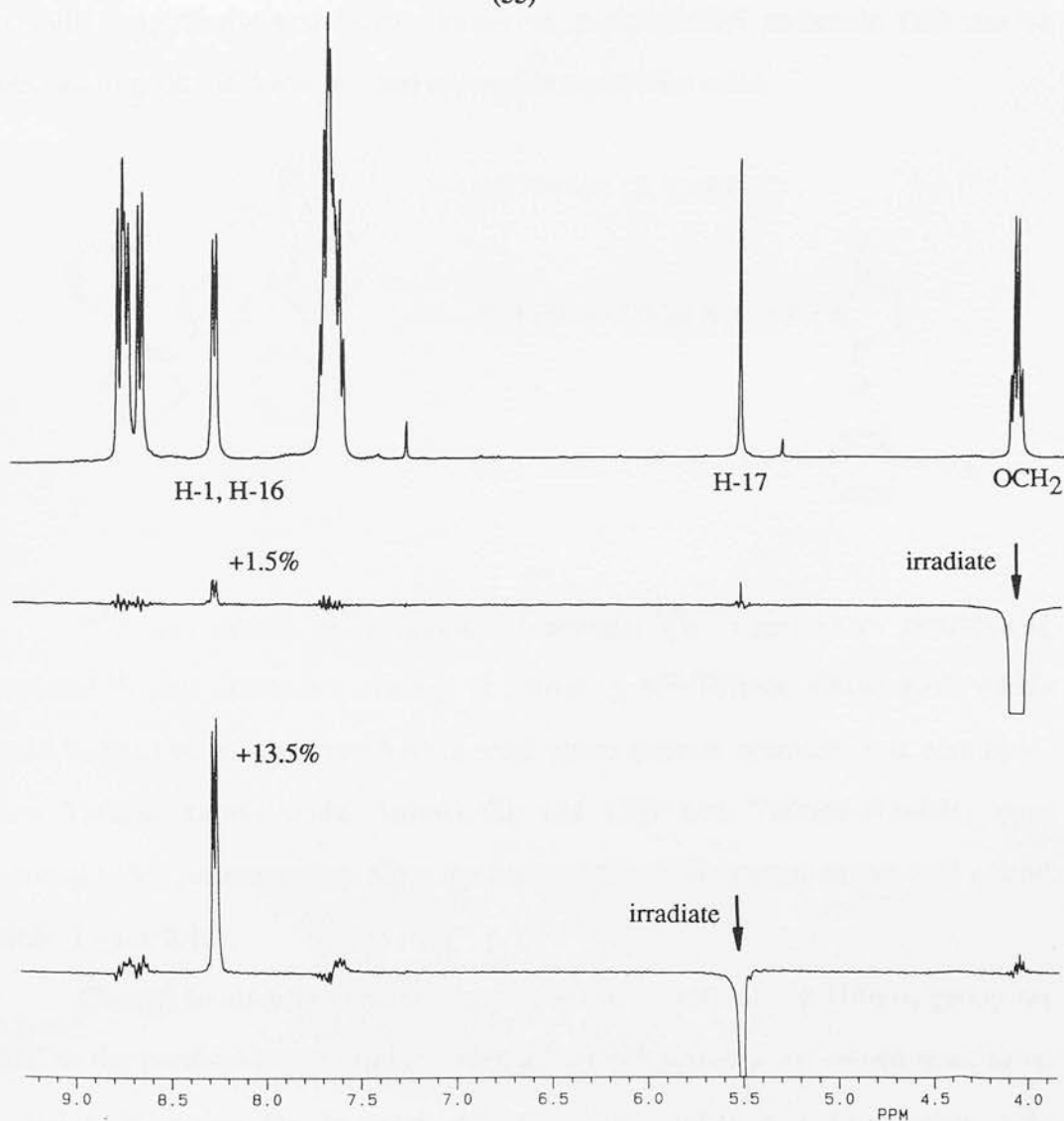
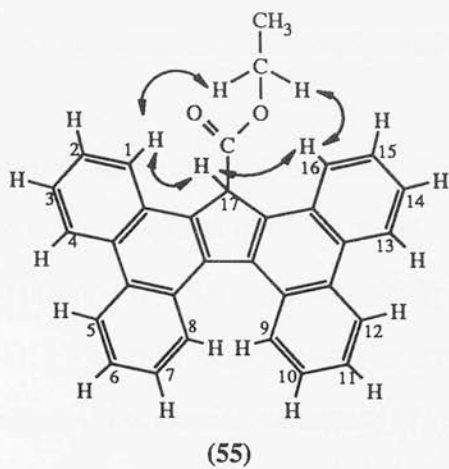
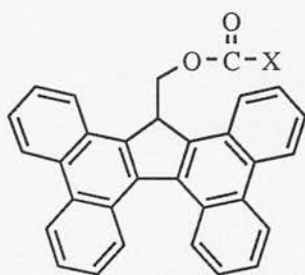


Figure 2.10: ¹H NMR spectrum (360 MHz) and NOE experiment on isomer (55)

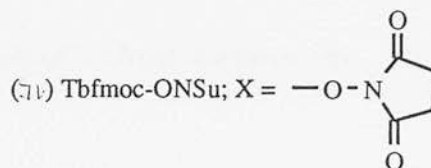
2.5 Synthesis of N^α-17-tetrabenz[*a,c,g,i*]fluorenylmethoxycarbonyl (Tbfmoc) amino acid derivatives

Raphy¹³⁰ used alcohol (56) to synthesise several reagents for the introduction of the Tbfmoc group onto the N^α position of amino acids.

Chloroformate, Tbfmoc-Cl, (70) was reportedly difficult to synthesise directly from alcohol (56), although it was obtained in low yield by the reaction of its TMS ether with phosgene. Succinimide carbonate (71) was also reported to be difficult to synthesise and isolate. However, *p*-nitrophenyl carbonate (72) can be obtained in good yield and isolated as a stable crystalline solid.



(70) Tbfmoc-Cl; X = Cl



(72) Tbfmoc-ONp; X =

(72) was shown to be unreactive towards the N-termini of resin-bound peptides¹³⁰. The alternative strategy of preparing N^α-Tbfmoc amino acids which could be used as the final residues in solid phase peptide synthesis was employed. Two Tbfmoc amino acids, Tbfmoc-Gly-OH (50) and Tbfmoc-Phe-OH, were reported and these were made from the acetate salts of the parent amino acid t-butyl esters (Figure 2.11).

Clearly, to allow general application of the affinity of the Tbfmoc group for PGC to the purification of peptides, then all 20 N^α-amino acids would need to be available. With this goal in mind, synthesis of a number of Tbfmoc amino acid derivatives was undertaken using the nitrophenyl carbonate (72).

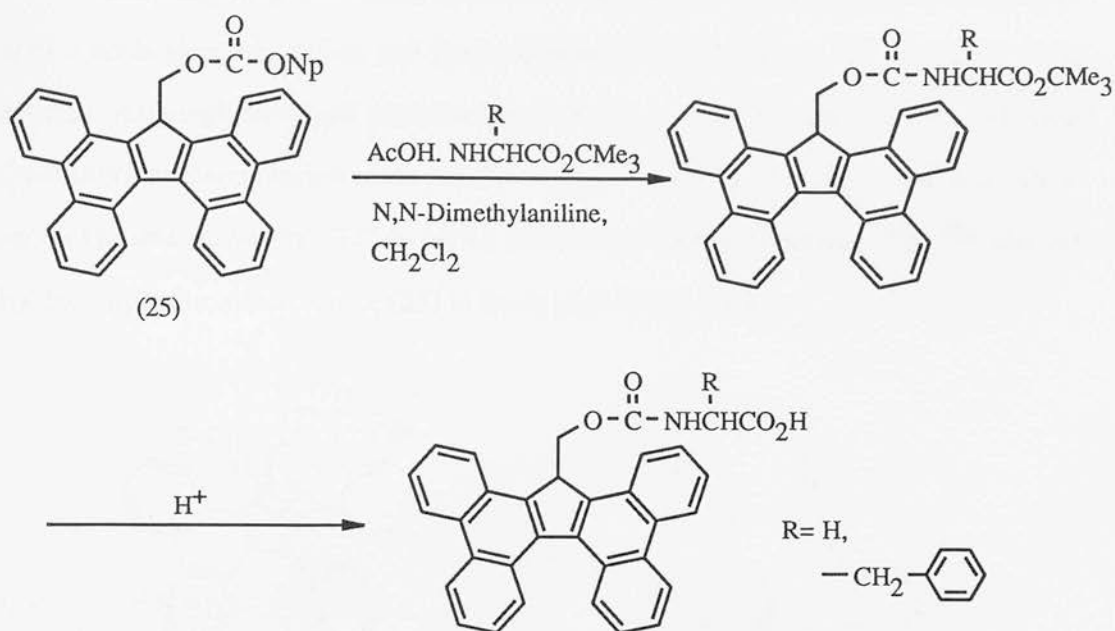


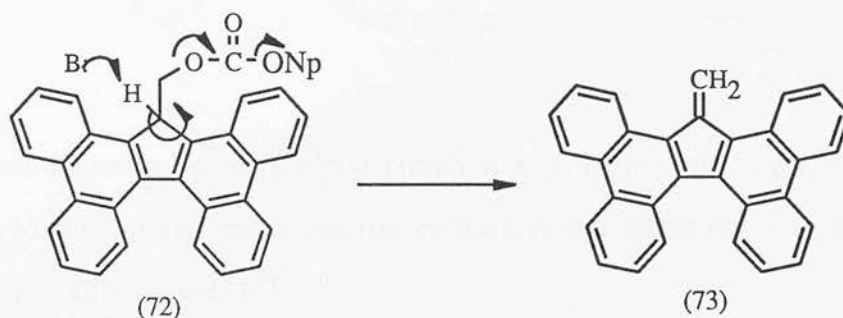
Figure 2.11: Synthesis of Tbfmoc amino acids

2.5.1 Alternative routes to Tbfmoc amino acids

Attempts to simplify the synthesis of Tbfmoc amino acids were made by employing Schotten-Baumann conditions. It was hoped this would obviate the need for expensive amino acid t-butyl esters and would also allow one step syntheses of many of the simpler Tbfmoc amino acids.

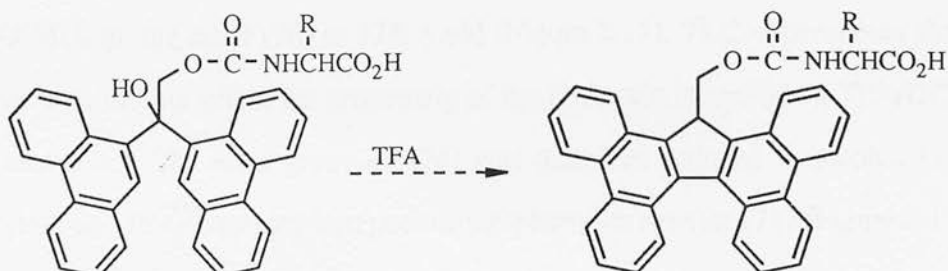
As a trial, glycine was taken in dioxane/aqueous Na_2CO_3 and added to a solution of Tbfmoc-ONp (72) in dioxane. Although keeping the hydrophobic carbonate (72) and the hydrophilic amino acid mutually soluble proved troublesome, Tbfmoc-Gly-OH was obtained in a moderate yield of 45%. This compares unfavourably with Raphy's two step synthesis from GlyO^tBu (Yield 79%), but nevertheless offers a much quicker and more economical route to Tbfmoc-Gly-OH (50).

Encouraged by this result, attempts were made to extend this reaction to other amino acids (leucine, valine and phenylalanine). Unfortunately, this met with little success. Although traces of products were detected by TLC, reactions of (72) with these more hindered amino acids proceeded much more slowly. The main product in each case was shown by TLC to be the red-orange coloured fulvene (73)¹³⁰, formed by decomposition of carbonate (25) in basic reaction conditions:



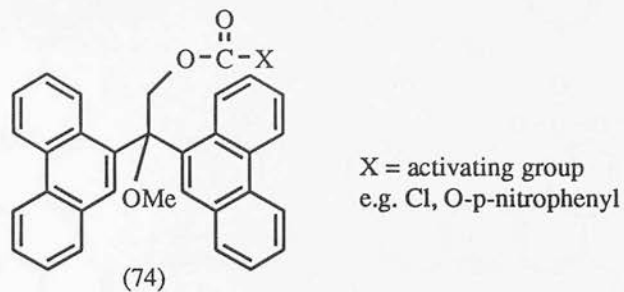
This method was not pursued further.

One of the main problems in synthesising Tbfmoc amino acids seemed to be the high base-lability of derivatives such as (72) that are required. If the base-lability of the Tbfmoc group could somehow be masked, or introduced at a later stage, then this should lead to higher yielding reactions with amino acid derivatives. This would be achievable if the cyclisation of the *bis*-phenanthryl system (54) to the tetrabenzofluorene system in TFA could be brought about after coupling to an amino acid:

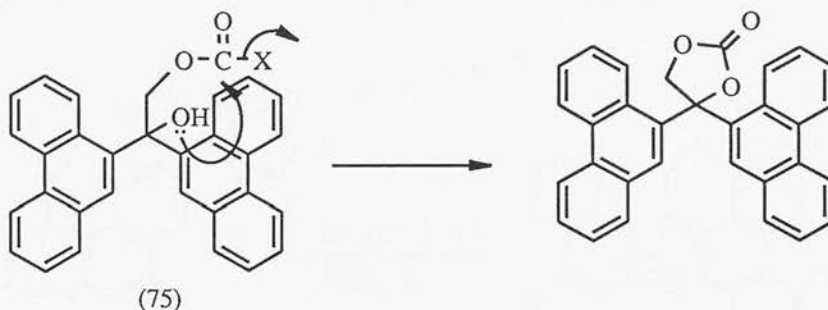


This would necessitate the preparation of an activated carbonate of the form

(74):



(74) shows the tertiary alcohol group protected as a methyl ether, which would be desirable to prevent any intramolecular cyclisation that might occur if the alcohol had remained unprotected (75):



Compound (54) was taken as starting material, available in large quantities via the reaction shown in Figure 2.8. Initial attempts to protect the tertiary alcohol with MeI using either NaH or KO^tBu as base failed, with only starting material being isolated. However, the methyl group was finally introduced with KOH-DMSO-MeI, giving ether (76) in 57% yield (Figure 2.12). TLC experiments showed that the ether did not affect the propensity of the molecule to cyclise in TFA/DCM to form ester (68). The ester group of (76) was therefore reduced to alcohol (77) in good yield and this^{was} converted into pentafluorophenyl carbonate (78) (Figure 2.12).

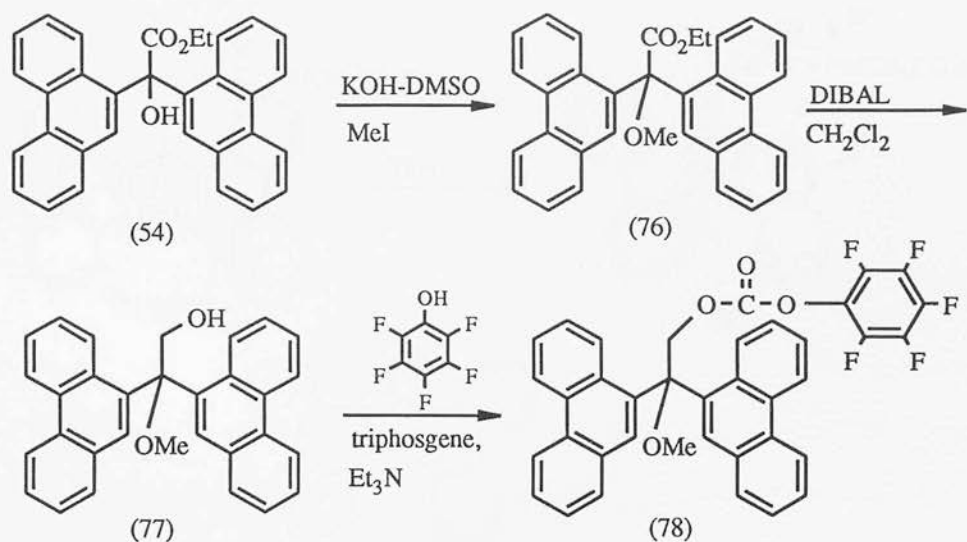
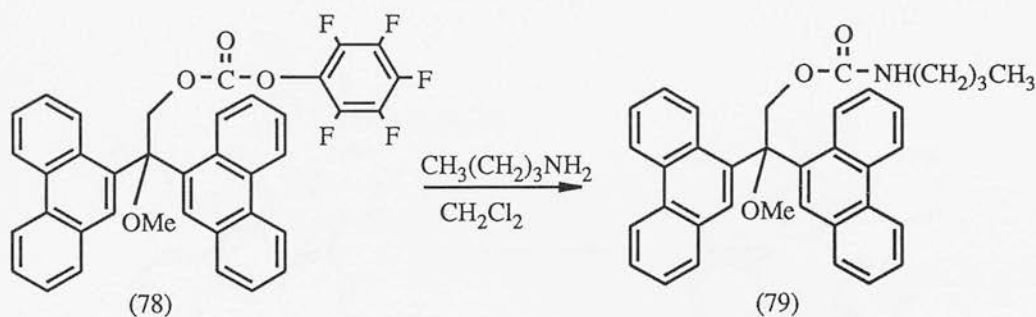
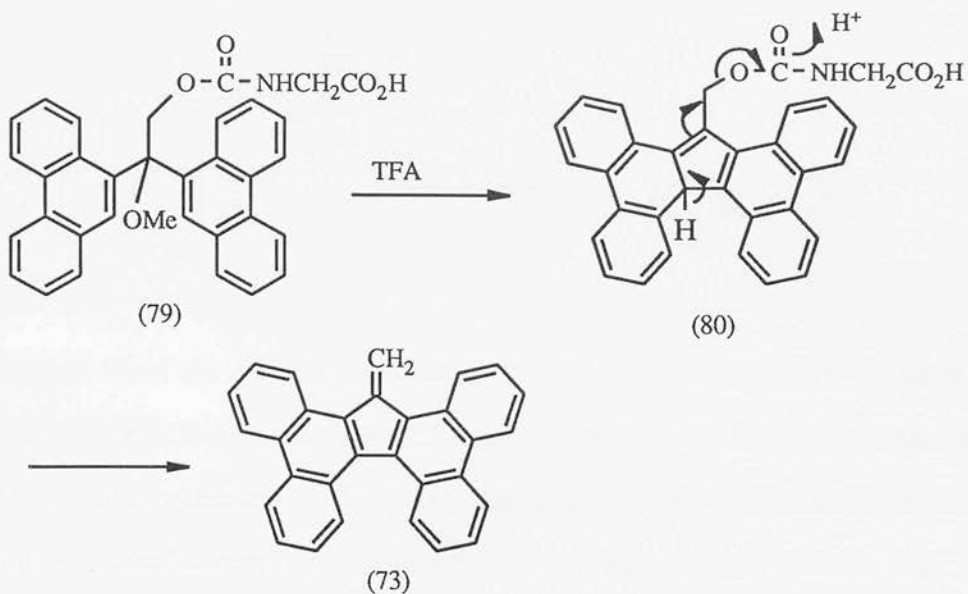


Figure 2.12

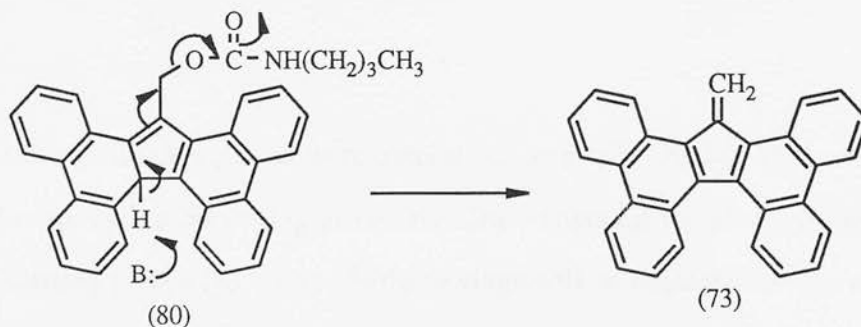
(78) was used to prepare the urethane derivative of n-butylamine (79) as a model compound:



Trials were carried out on the cyclisation of this compound to the tetrabenzofluorene system in TFA/DCM. Although TLC showed the initial appearance of the fluorescent product, all attempts to isolate it failed, the deep red-orange fulvene (73) being among several products present. This would appear to be due to the formation of the *8b*-H isomer (80). Unlike the desired Tbfmoc-derivative, the *8b*H-isomer (80) can readily undergo an acid catalysed decomposition:

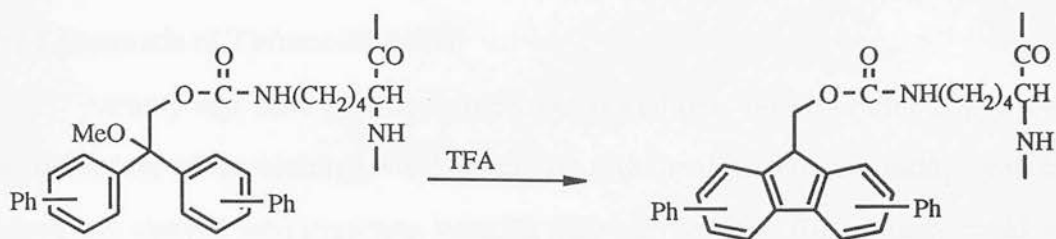


Unfortunately, attempting to isomerise (80) to the Tbfmoc-derivative by treatment with mild base, *N,N*-dimethylaniline, failed. Presumably, in this case, base-catalysed decomposition occurs in preference to isomerisation:

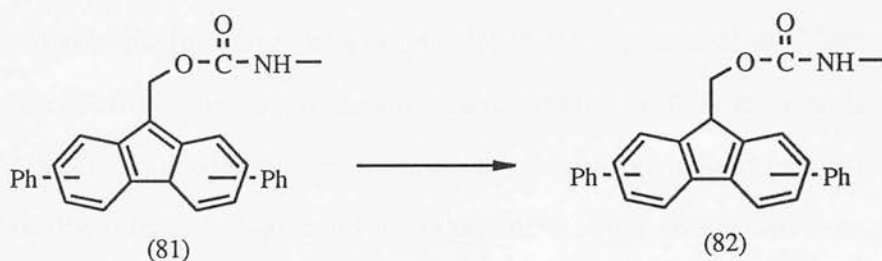


At this stage this approach to the synthesis of Tbfmoc amino acids was abandoned. However, in the future a similar approach might prove useful with other fused benzofluorene systems. This would provide a novel approach to lysine side-chain protection, when treatment with TFA (e.g. during cleavage of other side-chain protecting groups) would effect cyclisation of the N^{ϵ} -protecting group:

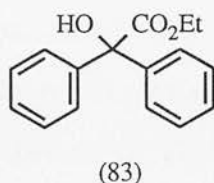




This would leave the peptide protected on the N^E-amino function of lysine with a base-labile benzofluorene urethane. This would find use in fragment condensation where selective protection of the lysine side-chain is needed. However, this would require a benzofluorene system where the initially formed isomer, e.g. (81), spontaneously rearranges to the required fluorene system (82):



Some preliminary trials were carried out on ethyl benzilate (83), with a view to developing such a protecting group, but this compound failed to cyclise in TFA, and presumably one or more fused benzene rings will be required for this to become a favoured process.



2.5.2 Synthesis of Tbfmoc-Met-OH

Affinity tags have previously been introduced onto the N-termini of peptides as derivatives of methionine^{115,125}, since the additional methionine residue can be selectively cleaved with cyanogen bromide after purification. This strategy could be extended to the synthesis of Tbfmoc peptides, whereby the peptide N-terminus could be capped with Tbfmoc-Met-OH.

In addition, methionine forms the N-terminus of ubiquitin, a small protein (76 residues) which was the subject of on-going synthetic and structural studies in this laboratory. Of all the amino acids, Tbfmoc-Met-OH was considered the most important at this stage and its synthesis was undertaken.

Raphy¹³⁰ had previously prepared Tbfmoc-Met-OMe, however it was not possible to selectively cleave the methyl ester in the presence of the Tbfmoc group. In contrast, Tbfmoc-Met-O^tBu should cleave readily in TFA to liberate Tbfmoc-Met-OH. Methionine t-butyl ester is not commercially available but its synthesis has been described from N^α-phthalyl methionine¹⁴³. This compound was therefore prepared by the literature procedure from methionine and phthalic anhydride¹⁴⁴. The t-butyl ester was then made by the standard method (isobutylene/ CH_2SO_4) and the phthalyl group removed with hydrazine to give Met-O^tBu as a colourless liquid, after distillation.

Met-O^tBu was then taken in DCM with N,N-dimethylaniline and nitrophenyl carbonate (72). After several days, an intensely red-orange coloured solution had formed, indicating extensive decomposition of the carbonate (72) to fulvene (73). None of the required product was detected by TLC.

In the synthesis of Tbfmoc-Gly-OH, Raphy¹³⁰ had employed the acetate salt of Gly-O^tBu. The reaction was therefore repeated in the presence of one equivalent of acetic acid. After several hours TLC showed a new fluorescent spot and after ^{three} days the product was isolated and shown to be the desired compound, Tbfmoc-Met-O^tBu.

The successful synthesis of Tbfmoc-Met-O^tBu, in the presence of one equivalent of acetic acid, served to outline the importance of adequately buffering the reaction to suppress the basic nature of the amine group of the amino acid derivative. In the absence of the acetic acid, the basic nature of the amine simply serves to cleave nitrophenyl carbonate (72) to give fulvene (73). The acetate salt of Met-O^tBu was therefore prepared and used in further syntheses of Tbfmoc-Met-O^tBu, giving the product in moderate yields of 36-39%.

Cleavage of the t-Bu ester was then attempted in 95% TFA/5% H₂O. TLC after 3 h showed the reaction to be complete. After removal of the TFA *in vacuo* a solid was obtained on trituration with ether. TLC showed several components and further work-up of the crude product exacerbated the problem. Methionine has previously been reported to be susceptible to photooxidation in the presence of sensitising agents such as benzophenone¹⁴⁵, and it may be that the Tbfmoc group was acting as a photosensitising agent, promoting side reactions such as oxidation.

In an effort to suppress any methionine oxidation, the reaction was repeated in the presence of a mixture of EDT and sulphides (ethyl methyl sulphide and thioanisole). In this case, the product, Tbfmoc-Met-OH was isolated in 85% yield when TLC and HPLC showed only traces of by-products.

2.5.3 Synthesis of Tbfmoc-Ala-O^tBu, Tbfmoc-Ile-O^tBu, Tbfmoc-Leu-O^tBu and Tbfmoc-Val-O^tBu

Ala-O^tBu, Ile-O^tBu, Leu-O^tBu and Val-O^tBu are all commercially available compounds. This and the fact that no side-chain protecting groups are required made the Tbfmoc derivatives of these amino acids obvious synthetic targets.

The acetate salts of each amino acid t-butyl ester were prepared and these reacted with carbonate (72) in the presence of N,N-dimethylaniline to give the N^α-Tbfmoc derivatives in moderate yields (Table 2.1)

	Yield %
Tbfmoc-Ala-O ^t Bu	42
Tbfmoc-Gly-O ^t Bu ¹³⁰	79
Tbfmoc-Ile-O ^t Bu	46
Tbfmoc-Leu-O ^t Bu	34
Tbfmoc-Met-O ^t Bu	39
Tbfmoc-Phe-O ^t Bu ¹³⁰	46
Tbfmoc-Val-O ^t Bu	56

Table 2.1: Yields of Tbfmoc amino acid t-butyl esters

2.6 Synthesis and purification of Tbfmoc peptides

2.6.1 Synthesis of Tbfmoc Human Gastrin Releasing Peptide

Having synthesised a range of Tbfmoc amino acid derivatives, a suitable peptide target was sought to allow study of both the coupling of the Tbfmoc-amino acids and behaviour of the resulting Tbfmoc-peptides on PGC.

Human gastrin releasing peptide¹⁴⁶ (hGRP) (84) was selected since it is of reasonable size (27-residues) and has an N-terminal valine, a Tbfmoc derivative of which was now available.

The peptide was synthesised minus its N-terminal valine using amide resin (108). An Fmoc loading test¹⁴⁷ after coupling of the final amino acid (residue 2, proline) indicated that assembly of the peptide on the resin had been achieved in 77% yield.

Tbfmoc-Val-OH was obtained in 90% yield by treatment of Tbfmoc-Val-O^tBu with 95% TFA/5% H₂O. This was then coupled by sonicating 5 equivalents of its HOBt ester in dioxane with the resin bound hGRP (2-27). A small sample of the resin-bound Tbfmoc-hGRP was then taken and the incorporation of the Tbfmoc group estimated by the UV method developed by Raphy¹³⁰. This indicated a disappointingly low coupling efficiency of 49%. Tbfmoc-Val-OH was therefore recoupled with a further 5 equivalents of HOBt ester but only a slight increase (53%) in Tbfmoc incorporation was observed.

Removal of the side-chain protecting groups and cleavage of the peptide from the resin was accomplished using TFA in the presence of water, EDT, ethyl methyl sulphide, thioanisole and phenol. After concentration of the cleavage mixture, the crude Tbfmoc peptide was precipitated with ether. Encouragingly, an analytical HPLC of the crude material indicated considerably better incorporation of Tbfmoc-Val-OH than had been originally estimated. Only one main peak was observed (Figure 2.13) when a large truncated peptide peak (hGRP 2-27) would have been expected had a coupling efficiency of only 53% been achieved. This suggested serious shortcomings in the UV monitoring method for estimating Tbfmoc coupling efficiency developed by Raphy¹³⁰ (see also section 2.6.3).

2.6.2 Behaviour of Tbfmoc hGRP on PGC

A solution of the crude Tbfmoc peptide was taken in 50% aqueous CH₃CN/0.5% TFA and applied to a PGC column (particle size 50-100 μm). The column was subjected to the washing protocol outlined below and the eluent monitored by HPLC :

1. 50% aqueous CH₃CN/0.5% TFA (50 ml)
2. 50% aqueous CH₃CN (2 x 50 ml)
3. Tbfmoc deprotection: 50% aqueous CH₃CN/

- 1% piperidine(50 ml), 30 min
4. 50% aqueous CH₃CN (20 ml)
5. 50% aqueous CH₃CN/0.5% TFA (50 ml)
6. 60% aqueous CH₃CN/0.5% TFA (50 ml)
7. 70% aqueous CH₃CN/0.5% TFA (3 x 50 ml)

Analysis of the eluent showed that the Tbfmoc peptide bound strongly to the column, while non-Tbfmoc material was eluted in the filtrate and washes 1,2. The Tbfmoc group was removed with piperidine (wash 3), and the deprotected peptide eluted in washes 6,7. The combined fractions were lyophilised to give an oil, which formed a white solid on trituration with ether. Although the quality of the eluted peptide was reasonably good (Fig 2.13), the recovery of material was lower than might have been expected. The two small satellite peaks present were separated by HPLC and shown by FAB-MS to be methionine sulphoxide peptides, formed by aerial oxidation of the two methionine residues.

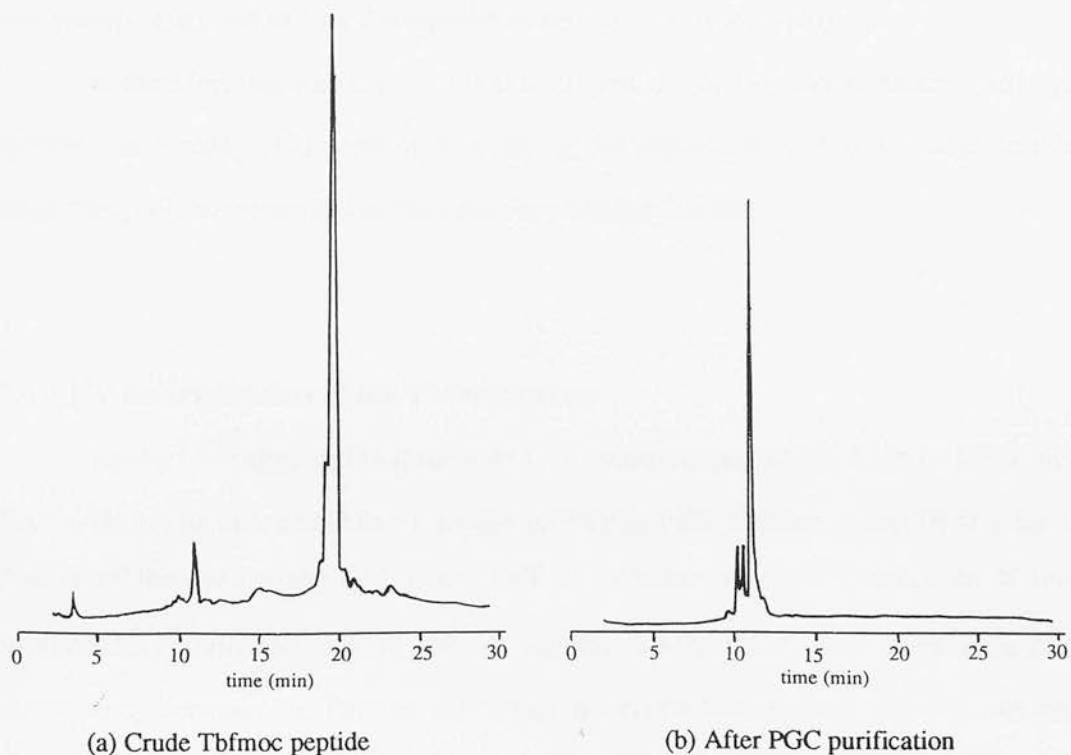


Figure 2.13: HPLC profiles of hGRP

Other similar experiments were carried out. These included application of the crude TFA cleavage mixture directly to the PGC column, when the peptide could be successfully bound while the scavengers and other impurities eluted. In all these trials, reasonably good quality material was obtained, but with variable recoveries (typically, 10 mg of crude peptide gave 1.5-2 mg of product). This was possibly due to two factors:

- (a) poor solubility of hGRP in the piperidine solution used to remove the Tbfmoc group, and subsequently only partial dissolution of the peptide on aqueous CH₃CN/0.5% TFA washes;
- (b) incomplete removal of the Tbfmoc group.

The latter seemed unlikely as no further material was obtained after additional piperidine washes. However, the former was consistent with the relatively large volume required to elute the Tbfmoc-deprotected peptide from the PGC (*ca.* 200 ml on a 3 g column) and with solution studies, when a faint precipitate was noticed (previously assumed to be a the piperidine adduct of fulvene (73)).

It therefore seemed crucial for the success of the method to select a solvent system that would avoid precipitation during the deprotection step, although at this stage this problem remained unresolved (see section 2.6.10).

2.6.3 UV determination of the Tbfmoc group

Raphy¹³⁰ suggested that the extent of incorporation of the Tbfmoc label into a resin-bound peptide could be estimated by cleaving the Tbfmoc group from a small portion of the resin using 20% Et₃N/DMF and examining the UV spectrum of this solution. As indicated for hGRP in section 2.6.2, there was a considerable discrepancy between the Tbfmoc content estimated by this method and that inferred by analytical HPLC of the crude material. Closer examination of the UV spectra of

such solutions showed considerable fluctuations in profile over periods of up to 2.5 h, when the spectrum stabilised.

20% piperidine in DMF has been used in a similar context, for estimating the Fmoc group¹⁴⁷. This was tried as an alternative, but showed poorer UV characteristics than the Et₃N solutions. However, the UV profile in 20% piperidine/dioxane remained constant over a period of 24 h. Various concentrations of Tbfmoc-Leu-OtBu were then prepared in this solvent and their UV spectra recorded. The absorption at 366 nm was used as the basis for a revised Tbfmoc loading test (Figure 2.14).

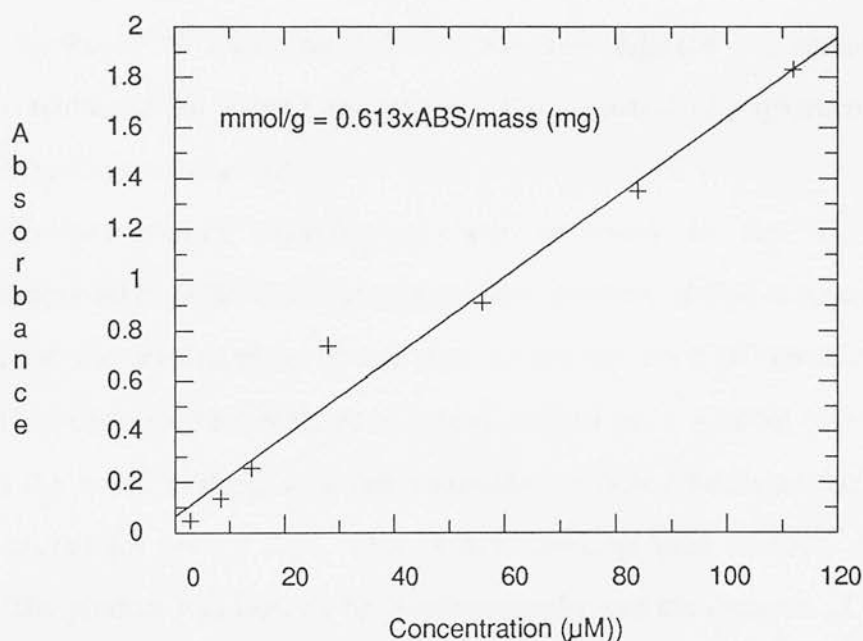


Figure 2.14: UV absorbance at 366 nm of various concentrations of Tbfmoc-Leu-O^tBu in 20% piperidine/dioxane

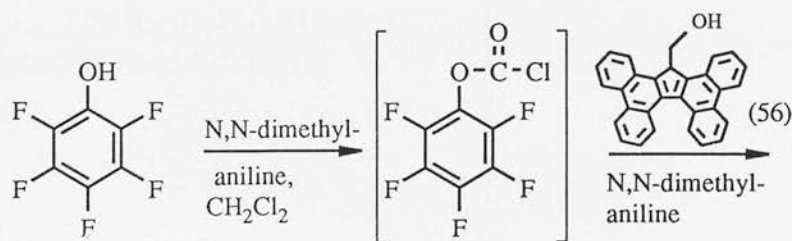
2.6.4 Direct incorporation of the Tbfmoc group into resin bound peptides

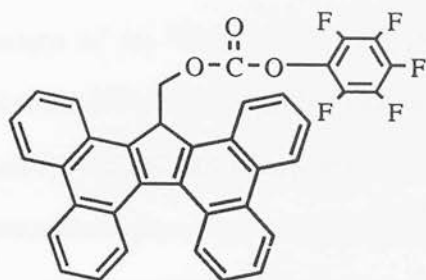
Although a number of Tbfmoc amino acid derivatives had been synthesised from amino acid t-butyl esters, it became clear that this generally poor yielding

reaction would render the synthesis of all suitably protected 20 amino acids a difficult and laborious task. As outlined earlier, it would be more efficient and convenient if an activated Tbfmoc derivative could be used to introduce the group directly onto the N $^{\alpha}$ -terminus of the resin-bound peptide. This possibility was now investigated in some detail using a simple pentapeptide, Leu.Ile.Phe.Ala.Gly-Resin, as a model system. At this time a reliable UV monitoring method had not been established and the incorporation of the Tbfmoc label was monitored by HPLC of the crude cleaved product (95% TFA/water).

Initial trials with *p*-nitrophenyl carbonate (72), using several equivalents in various solvents (DCM, dioxane, DMF) were unsuccessful, confirming earlier work reported by Raphy¹³⁰. Carbonate (72) was therefore rejected and attempts were made to synthesise an alternative reagent, 17-tetrabenzo[*a,c,g,i*]fluorenylmethyl pentafluorophenyl carbonate (85).

Pentafluorophenyl chloroformate was generated *in situ* by treating pentafluorophenol in DCM with triphosgene in the presence of N,N-dimethylaniline. The reaction was monitored by IR and after 30 minutes the triphosgene carbonate peak at 1820 cm⁻¹ had been replaced by a peak at 1810 cm⁻¹. Alcohol (56) was then added to the solution along with one equivalent of N,N-dimethylaniline and the mixture stirred for several days, when a new carbonyl peak at 1775 cm⁻¹ was evident. The product was isolated by chromatography and the presence of Tbfmoc-OPfp (85) confirmed by accurate mass FAB-MS.

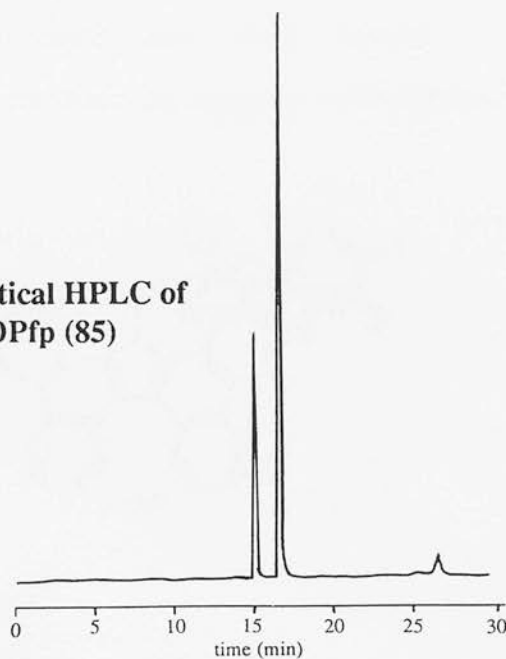




(85) Tbfmoc-OPfp

However, neither the ^1H nor ^{13}C NMR spectra were consistent with the expected Tbfmoc-OPfp structure (85). ^{13}C NMR showed two carbonyl signals at 150.8 and 150.4 ppm and two CH_2 signals at 75.4 and 73.2 ppm. A high resolution ^1H NMR spectrum resolved a three proton multiplet around 5 ppm into two distinct triplets at 5.12 and 5.08 ppm (integral ratio 2:1) and two distinct doublets at 4.82 and 4.74 ppm (integral ratio 2:1). This suggested that the compound was in fact a 2:1 mixture of two similar substances. Although TLC in several systems showed only one spot, the presence of two components was confirmed by HPLC (5 μm silica, A=n-hexane, B=DCM, linear gradient of 0-50% B over 30 min., monitoring at 254 nm), when two peaks were observed (Figure 2.15).

Figure 2.15 Analytical HPLC of isolated Tbfmoc-OPfp (85)



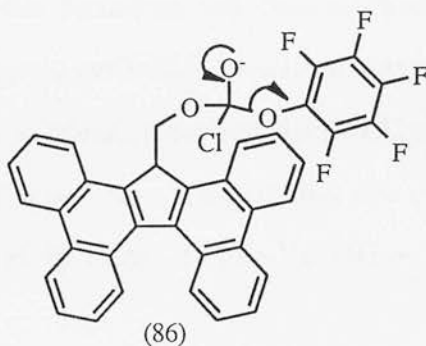
A comparison of the ^1H and ^{13}C NMR shifts with an authentic spectrum of Tbfmoc chloroformate¹³⁰ (70) suggested that this was ^{the} minor component present. This was confirmed by C, H and Cl microanalyses which were all consistent with a 2:1 mixture of pentafluorophenyl carbonate (85) and chloroformate (70) (Table 2.2).

This was a rather surprising result as the chloroformate had previously been reported to be unstable. It also raised the question of how the chloroformate had been formed, since prior to addition of alcohol (56) to the reaction mixture no unreacted triphosgene had been detected by IR.

	<u>Found</u> %	<u>Calculated for</u> <u>(37), %</u>	<u>Calculated for</u> <u>(23), %</u>	<u>Calculated for</u> <u>2:1 mixture of</u> <u>(37) & (23),</u> <u>%</u>
C	74.9	73.3	81.1	75.4
H	3.58	3.14	4.14	3.41
Cl	1.82	-	7.74	2.12

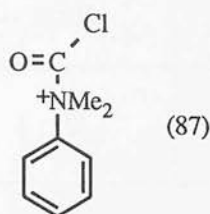
Table 2.2: Elemental analysis results for isolated Tbfmoc-OPfp (85)

The chloroformate could have been formed if elimination of pentafluorophenol from intermediate (86) competed with the expected elimination of HCl:



This seems unlikely on the basis of leaving group abilities but might become more favoured if the intermediate is sterically crowded.

However, the most likely explanation would seem to be from residual species such as (87), which could conceivably be formed during the reaction¹⁴⁸.



At this stage, since both components of the mixture could effect the desired reaction (incorporation of the Tbfmoc group) it was expedient to use this crude product for coupling trials with resin-bound pentapeptide, Leu-Ile-Phe-Ala-Gly. Encouragingly, *ca.* 50% incorporation of the Tbfmoc group was accomplished by sonicating with a 3-fold excess of reagent in either DCM or dioxane. DMF was also tried with moderate success but proved unsuitable due to the limited stability of Tbfmoc derivatives in this solvent.

Improved results were obtained by carrying out the reaction in the presence of a mild tertiary base (N,N-dimethylaniline) but virtually quantitative reaction was achieved using DCM as solvent in the presence of diisopropylethylamine as tertiary base. The generality of this procedure was demonstrated with a number of other resin-bound peptides that were available in this laboratory¹⁴⁹⁻¹⁵² (Table 2.3).

In the case of gastrin releasing peptide, the HPLC profile and FAB-MS of the material cleaved from the resin was compared with that obtained when the Tbfmoc group had been introduced by using Tbfmoc-Val-OH as the final amino acid. No significant differences were noted.

The other peptides shown in the table were used for further study (see sections 2.6.7-2.6.11). Tbfmoc peptide (88) proved to be unsuitable since the free

cysteine thiol group, liberated by removal of the trityl group during the TFA cleavage, showed a tendency to dimerise when the peptide was treated with base to remove of the Tbfmoc group.

<u>Peptide</u>	<u>No. of amino acids</u>	<u>N-terminus</u>	<u>Estimated Tbfmoc incorporation</u>
$a(88)^{149}$	19	Cys(Trt)	<i>ca. 100%^b</i>
Hepatitis B, surface antigen, PreS1 (1-23) <u>ayw</u> (89) ¹⁵⁰	23	Met	<i>ca. 100%^b</i>
Gastrin releasing peptide (84)	27	Val	<i>ca. 100%^b</i>
Ubiquitin Y59F (90) ¹⁵¹	76	Met	85% ^c
MeCP2 methylated DNA binding domain (91) ¹⁵²	85	Ser(^t Bu)	62% ^c

^aSequence: Cys-Arg-Lys-Phe-Glu-Glu-Tyr-Pro-Lys-Phe-Arg-Lys-Ile-Ile-Ile-Pro-Phe-Leu-Phe-OH; ^bHPLC; ^cmodified UV method (section 2.7.3)

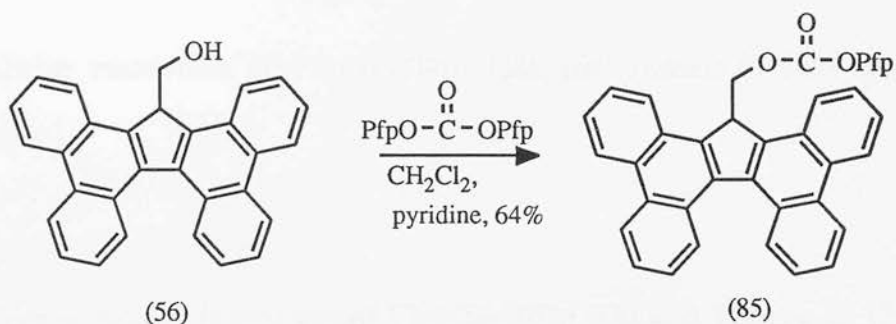
Table 2.3: Tbfmoc peptides synthesised

2.6.5 Synthesis of Tbfmoc-OPfp (85) and Tbfmoc-Cl (70)

Although a general method had now been developed for the introduction of the Tbfmoc group onto the N-termini of resin-bound peptides, the reagent so far used to accomplish this was a 2:1 mixture of chloroformate (70) and pentafluorophenyl carbonate (85). As indicated above, TLC in various systems showed no separation of the two components of the mixture. Recrystallisation also gave no separation.

Although both components could in principle accomplish the same task, presumably one would react with peptide N-termini more efficiently than the other. To investigate this, pure samples of each compound were obtained by alternative routes.

Tbfmoc-OPfp (85) was prepared from dipentafluorophenyl carbonate¹⁵³ in a reasonable yield:



Low stability to base had previously been attributed to Tbfmoc-Cl¹³⁰ (70) and an earlier synthesis of this molecule proceeded via the TMS ether of 17-tetrabenzofluorenylmethanol (56), giving a poor overall yield (18%). The alternative and more direct route to (70) would be to simply react alcohol (56) with phosgene or a phosgene substitute.

Equimolar amounts of alcohol (56) and *N,N*-dimethylaniline were therefore taken in DCM with one third of an equivalent of triphosgene. Although with longer reaction times (> 2-3 h), TLC showed significant decomposition of the product to fulvene (73), if the reaction was quenched with water and acidified with 2M HCl after 2 h then the chloroformate could be obtained in a moderate yield of 49%.

Even better results were obtained if an excess of triphosgene was used (2/3 equivalents) when the product precipitated out of the reaction mixture and could be isolated in good yield:

Clearly, both reagents give the desired reaction with the resin-bound peptide, but the chloroformate (70) reacts much more quickly than the pentafluorophenyl carbonate (85). In fact, further studies showed the reaction with the chloroformate (85) to be complete in less than 30 min. This contrasts markedly with earlier reports¹³⁰ which suggested that the chloroformate (70) gave only low coupling efficiencies with resin-bound peptides. It was also found that the tertiary base was only necessary for Tbfmoc-Cl (70), to neutralise the liberated HCl. Similar results could be obtained for Tbfmoc-OPfp (85) without the addition of any base.

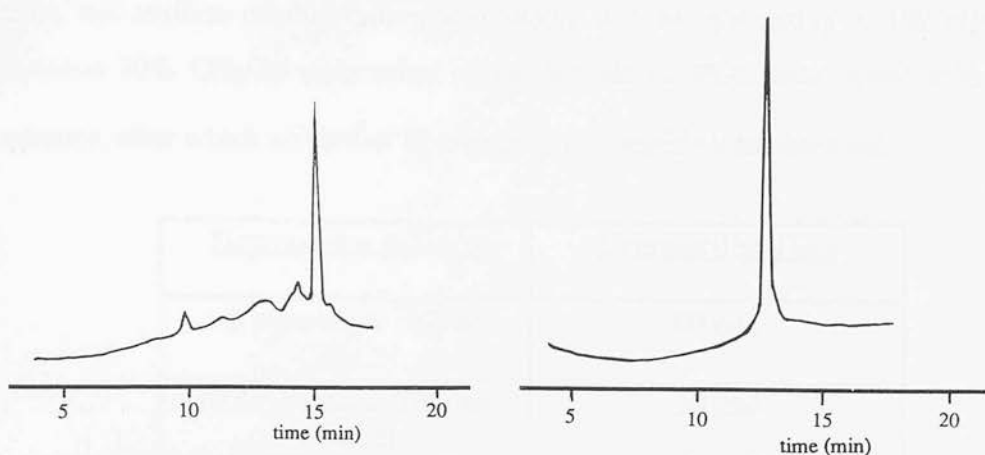
As a further comparison, the relative reactivities of the two species with a hindered peptide N-terminus (peptide (88), N-terminus Cys(Trt)), were investigated. Again quantitative incorporation was obtained with Tbfmoc-Cl (70), but only poor yields were obtained using Tbfmoc-OPfp (85) (14%) although this could be increased to 36% in the presence of diisopropylethylamine.

2.6.7 Hepatitis B surface antigen PreS1 (1-23) ayw:

Met-Gly-Gln-Asn-Leu-Ser-Thr-Ser-Asn-Pro-Leu-Gly-Phe-Phe-Pro-Asp-His-Gln-Leu-Asp-Pro-Ala-Phe-OH¹⁵⁰ (89)

In contrast to hGRP, this acidic peptide was shown to be freely soluble in 70% aqueous CH₃CN/1% piperidine and therefore the peptide was expected to show much better elution properties on PGC after removal of the Tbfmoc group.

The Tbfmoc-peptide was cleaved from the resin with TFA/scavengers. A sample of the crude peptide was taken in 70 % aqueous CH₃CN and applied to a PGC column (particle size 50-100 μm). The Tbfmoc group was removed and the peptide eluted with 70% aqueous CH₃CN/1% piperidine. The combined fractions were neutralised with acetic acid, diluted, desalted and lyophilised to give a good crop of pure peptide (Fig 2.16).



(a) Crude Tbfmoc peptide

(b) After PGC purification

Figure 2.16: HPLC profiles of Pre S1 (1-23) ayw

2.6.8 Tbfmoc deprotection studies on PGC

Encouraged by this result, Tbfmoc-peptide (89) seemed an ideal system with which to investigate and optimise the deprotection of the Tbfmoc group on PGC. In a typical experiment, 1.5 mg of Tbfmoc-peptide was taken in 5 ml 70% aqueous CH_3CN and 120 mg of PGC (particle size 50-100 μm) added. The adsorption of the Tbfmoc peptide from the solution was monitored by the UV absorbance at 350-400 nm. The supernatant liquor was then pipetted off and 5 ml of the deprotection solution added. Samples of the basic solution were taken periodically, and after neutralising with acetic acid the release of the peptide from the PGC was monitored by HPLC. The results are summarised in Table 2.5.

These figures should be compared with Tbfmoc deprotection in solution which is usually complete in 1 minute using 1% piperidine solutions. Clearly, the

deprotection is greatly retarded when the peptide has been adsorbed onto PGC. The possibility that this was merely reflecting slow diffusion of the deprotected peptide from the surface of the carbon was discounted by quenching a 1% piperidine/aqueous 70% CH₃CN suspension of the peptide on PGC with acetic acid after 5 minutes, after which no further liberation of the peptide was observed.

<u>Deprotection solution</u>	<u>Deprotection Time</u>
1% piperidine/70% aq. CH ₃ CN	60 min
10% piperidine/70% aq. CH ₃ CN	10 min
1% piperidine/70% aq. CH ₃ CN/2.6 M urea	30 min
1% piperidine/50% aq. CH ₃ CN/1.5M Gdm.HCl	60 min
5% piperidine/50% aq. CH ₃ CN/1.5M Gdm.HCl	20 min
10% pyrrolidine/70% aq. CH ₃ CN	10 min
1% DBU/70% aq. CH ₃ CN	1 min ^a
10% triethylamine/70% aq. CH ₃ CN	>3 h

^aextensive peptide degradation noted after 10 min.

Table 2.5: Deprotection of Tbfmoc group from peptide (89) adsorbed onto PGC

This lower deprotection rate on PGC is probably due to the acidic hydrogen (H-17) being shielded by the carbon surface from basic attack. In any case, this study showed that a piperidine concentration of 10% is necessary for reasonably fast deprotection of the Tbfmoc group on PGC.

2.6.9 Comparison of different grades of PGC for adsorption of peptides

Up until this time, large particulate PGC (particle size 50-100 μm) had been used. A comparison was made between this material and HPLC grade PGC (particle size 7 μm). PGC was added to solutions of Tbfmoc-peptide (89) (1.5 mg in 5 ml of 70% aqueous CH_3CN) and the adsorption of the peptide onto the surface of the carbon monitored by the UV absorbance of the supernatant liquor at 350-400 nm:

<u>PGC particle size</u>	<u>Mass required to adsorb peptide</u>
50-100 μm	120 mg
7 μm	12 mg

The smaller mass of HPLC grade PGC required at least partially reflects the larger surface area of this material (175-200 m^2/g , compared with 68 m^2/g), but other factors may be involved. For instance, the larger particulate material had been used in previous experiments and not all of the surface activity may have been restored on washing with hot dioxane¹³⁰.

In light of this finding, however, further peptide purifications were carried out using HPLC grade material, with the carbon being added to the peptide solution and the supernatant liquor removed after centrifugation.

2.6.10 MeCP2 Methylated DNA Binding Domain

Ser-Ala-Ser-Pro-Lys-Gln-Arg-Arg-Ser-Ile-Ile-Arg-Asp-Arg-Gly-Pro-Met-Tyr-Asp-Asp-Pro-Thr-Leu-Pro-Glu-Gly-Trp-Thr-Arg-Lys-Leu-Lys-Gln-Arg-Lys-Ser-Gly-Arg-Ser-Ala-Gly-Lys-Tyr-Asp-Val-Tyr-Leu-Ile-Asn-Pro-Gln-Gly-Lys-Ala-Phe-Arg-Ser-Lys-Val-Glu-Leu-Ile-Ala-Tyr-Phe-Glu-Lys-Val-Gly-Asp-Thr-Ser-Leu-Asp-Pro-Asn-Asp-Phe-Asp-Phe-Thr-Val-Thr-Gly-Arg-OH (91)

This peptide is an 85 amino acid section of a larger protein which binds to methylated DNA¹⁵⁴. It has recently been synthesised in this laboratory¹⁵² and was thought to provide a good test of the Tbfmoc/PGC methodology, especially as it, like hGRP, proved to be insoluble in aqueous CH₃CN/piperidine solutions.

The Tbfmoc-peptide was cleaved from the resin with TFA/scavengers. As the Tbfmoc peptide represented a relatively small amount of the crude material (Fig 2.17) any losses due to precipitation on the PGC would not be tolerated. A solvent system that would prevent precipitation of the peptide at alkaline pH was therefore sought.

It was thought that addition of chaotropic agents, such as guanidinium hydrochloride (Gdm.HCl) or urea might prove useful, since these are frequently used as solubilising agents for peptides and proteins. To facilitate this study, a small amount of the Tbfmoc peptide was isolated in solution by preparative HPLC. HPLC grade PGC was then added to samples of the eluent to adsorb the Tbfmoc peptide. The supernatant liquor was removed, a deprotection solution added and any release of the peptide from the PGC monitored by HPLC. 10% piperidine in 50% aqueous CH₃CN/1.5M Gdm.HCl failed to elute the peptide, but increasing the Gdm.HCl concentration to 3M eluted some product. 10% Piperidine in 50% aqueous CH₃CN/4M urea also proved reasonably successful. However, best results were obtained using 10% piperidine in 50% aqueous isopropanol, a solvent system which has the advantage of being completely volatile.

Having found a suitable solvent system a sample of the crude Tbfmoc peptide was taken in 2.5 % acetic acid/50% aqueous isopropanol. HPLC grade PGC was added to adsorb the Tbfmoc peptide then the solution centrifuged and the supernatant pipetted off. The carbon was then subjected to the following washing protocol:

1. 2.5% acetic acid/50% aqueous isopropanol (5 ml)
2. 50% aqueous isopropanol (5 ml)

3. Tbfmoc deprotection: 10% piperidine/50% aqueous isopropanol (2 ml), wait 10 min
4. 50% aqueous isopropanol (2 ml)
5. 10% piperidine/50% aqueous isopropanol (2 ml)
6. 50% aqueous isopropanol (5 ml)

Washes 3, 4, 5, and 6 were combined and the solvent removed *in vacuo*. The peptide obtained was of reasonable purity (Fig 2.17), but was subjected to a final purification by HPLC to give single peak material.

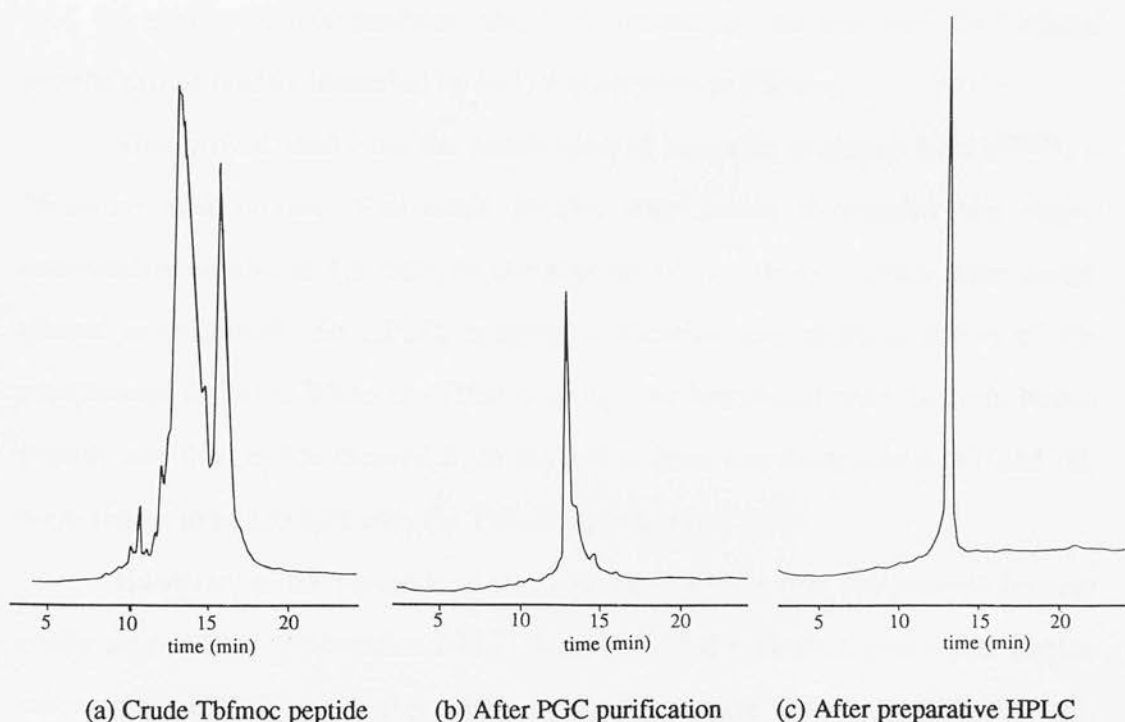


Figure 2.17: HPLC profiles of MeCP2 Methylated DNA Binding Domain (91)

Recent experience suggests that the solvent system used here should be applicable to a wide range of peptides. For instance, much better recoveries of hGRP (section 2.6.2) were obtained when the acetonitrile based solvent system was replaced with an aqueous isopropanol system.

2.6.11. Tbfmoc as a hydrophobic group for purification of peptides by RP-HPLC: Ubiquitin analogue, UbY59F

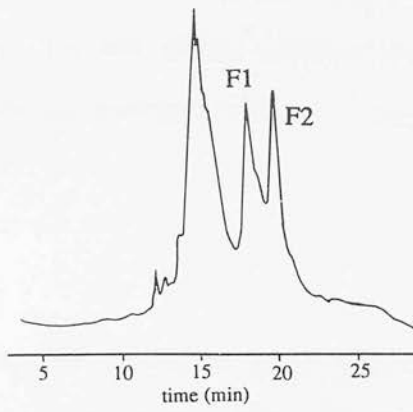
Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-Lys-Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-Ser-Asp-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-Ile-Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Phe-Asn-Ile-Gln-Lys-Glu-Ser-Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-OH¹⁵¹ (90)

The hydrophobic nature of the Tbfmoc group markedly increases the RP-HPLC retention times of peptides to which it is attached. This property can also be used for purification of peptides, and is facilitated by the fact that the Tbfmoc peptide can be readily identified by its UV absorption at 364 nm.

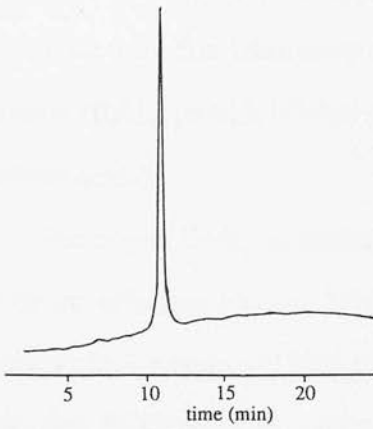
This proved useful for the purification of ubiquitin analogue UbY59F¹⁵¹, a 76-amino acid protein. The crude peptide was shown to contain two major components, in almost 1:1 ratio. In the absence of the Tbfmoc group these eluted almost coincidentally on HPLC, making purification and characterisation of the components difficult. When the Tbfmoc group was introduced onto the resin-bound peptide and the peptide cleaved from the resin, these two components, F1 and F2, were shown to be labelled with the Tbfmoc group (Fig 2.18a).

However, in the presence of the Tbfmoc-label the two components became easily separable by preparative HPLC. Removal of the Tbfmoc group and further preparative HPLC gave the two peptides in pure form (Fig 2.18b, c). Characterisation by amino acid analysis and laser-desorption mass spectrometry (Figure 2.19) showed the later-eluting peptide, F2 to be the required sequence, whilst the earlier-eluting peptide, F1 was the desired peptide with an oxidised methionine. This was further confirmed by oxidation-reduction studies when the two species could be interconverted¹⁵⁵.

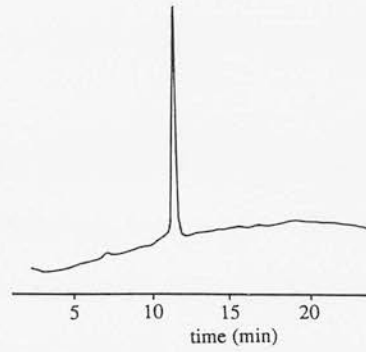
This seems to indicate that the Tbfmoc-group may have some potential for enhancing the HPLC separation of even very similar sequences (e.g. where a residue



(a) Crude Tbfmoc peptide



(b) Purified F1



(c) Purified F2

Figure 2.18: HPLC profiles of Ubiquitin analogue, UbY59F

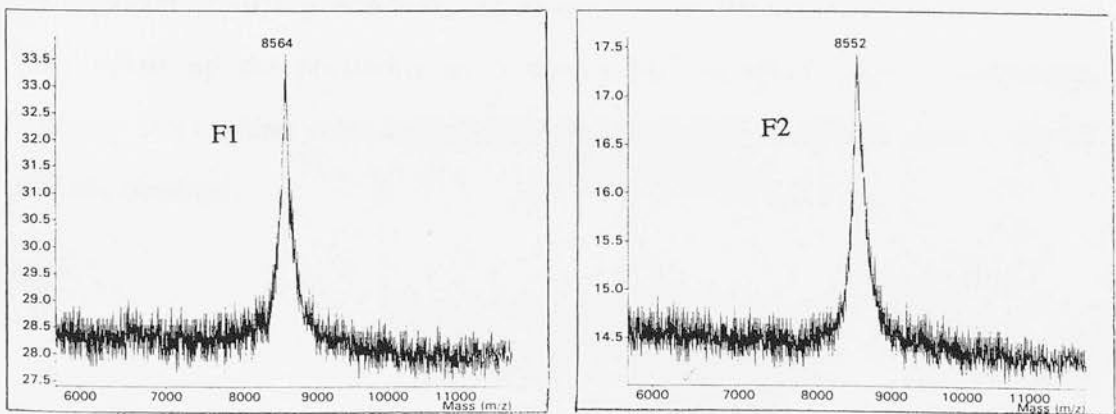


Figure 2.19: Laser desorption mass spectra of peptides F1 and F2 (instrument calibrated with bovine ubiquitin, $\sigma \pm 5.2$ Da)

has been modified by a side reaction during cleavage), although the large separation encountered in the above case was almost certainly due to the proximity of the Tbfmoc group to the N-terminal methionine that had been oxidised.

2.7 Concluding remarks

The Tbfmoc group has proved to be an effective affinity label for the purification of peptides up to 85 amino acids in length on PGC or by RP-HPLC. This method has shown wide applicability and in addition to the peptides discussed has been used in this laboratory for the purification of bacteriophage λ restriction alleviation (RAL) peptide¹⁵⁶ (66 residues) and for a range of ubiquitin analogues¹⁵⁷ (76 amino acids).

The upper limit, in terms of peptide size, at which this methodology will cease to be effective has not been reached during these studies. Preliminary trials with synthetic lysozyme (129 residues) have shown that the affinity of the Tbfmoc-peptide for PGC remains strong and that it will be useful to effect at least a preliminary purification.

In the future this methodology should allow other large synthetic peptides to become more readily accessible while the stability of PGC to TFA/scavengers offers the potential of directly adsorbing the crude Tbfmoc cleavage mixture onto carbon. This opens up the possibility of a completely automated peptide synthesiser, whereby from amino acids loaded onto the synthesiser, a purified peptide can be directly obtained.

CHAPTER 3

SYNTHESIS OF C-TERMINAL PEPTIDE DERIVATIVES

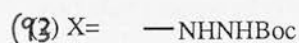
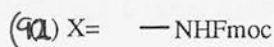
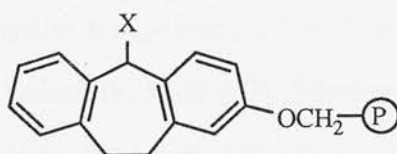
3.1 Design of a linker for SPPS

A suitable linker for SPPS must form a relatively stable carbocation to facilitate cleavage of the peptide by an SN1 mechanism on acid treatment:



Pless¹⁵⁸ has studied the relative stabilities of various benzhydryl carbocations (Table 3.1). Based upon these findings and the estimated carbocation-stabilising effect of an alkoxy substituent¹⁵⁹, McInnes¹⁶⁰ reasoned that an alkoxydibenzocycloheptadiene system would form the basis of a linker, suitable for SPPS, from which the peptide could be released on mild acidolysis.

Linkers (92) and (93) were subsequently developed for the synthesis of peptide C-terminal amides and hydrazides, when the peptide could be liberated from the resin on treatment with dilute TFA.



R ⁺	pK _{R⁺}
	-3.7
	-6.6
	-8.0
	-13.3
	-14.0



Table 3.1: Relative carbocation stabilities¹⁵⁸

3.2 Synthesis of 2-hydroxydibenzocycloheptadien-5-one (100)

In the synthesis of linkers (92) and (93), 2-hydroxydibenzocycloheptadien-5-one (100) is a key intermediate. The route to this molecule developed by McInnes¹⁶⁰ is shown in figure 3.1 and, although a good overall yield of 38% was achieved, two of the intermediates were oils and three steps involved chromatographic separations. To facilitate large scale synthesis of the linker some modifications were necessary.

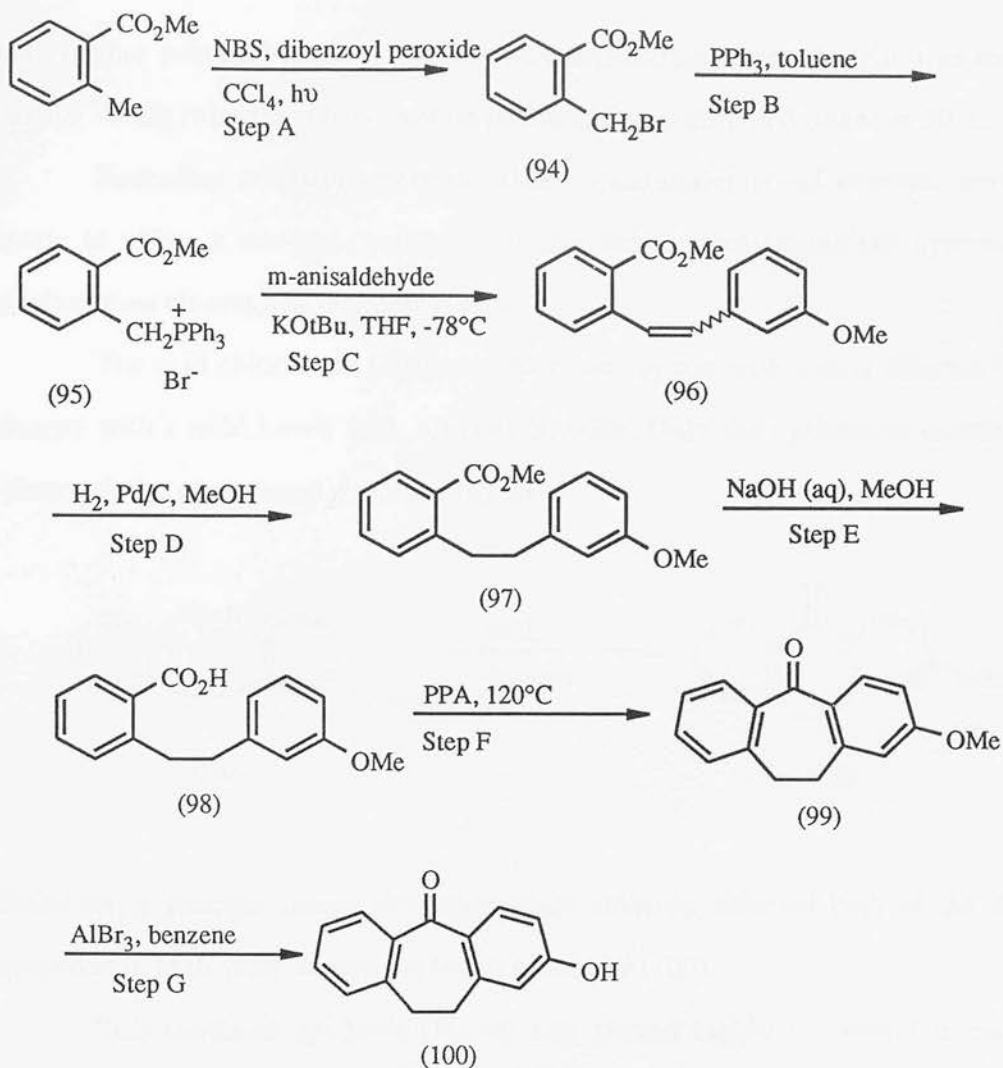


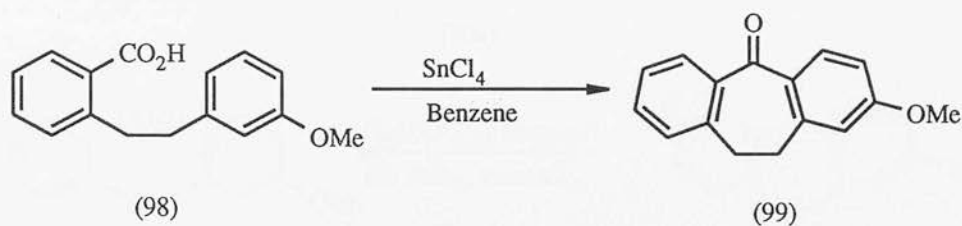
Figure 3.1: M^CInnes synthesis of 2-hydroxydibenzocycloheptadien-5-one (100)¹⁶⁰

In the Wittig reaction (Fig 3.1, step C) removal of the triphenylphosphine oxide by-product had proved troublesome¹⁶⁰. Initial attempts to circumvent this problem using the Horner-Emmons procedure, where water-soluble by-products are formed, were unsuccessful. Instead, by immediately hydrolysing the crude stilbene ester product (96) then the triphenylphosphine oxide by-product could be easily removed on aqueous work up of the resulting acid (101) (Figure 3.2). The procedure

was further refined by using 1,8-diazabicyclo[5.4.0]undecene-5 (DBU) as the base for the Wittig reaction, when reaction proceeded smoothly in dioxane at 50°C.

Reduction of (101) proceeded almost quantitatively and attempts were then made to effect a one-pot cyclisation to the dibenzocycloheptadiene system, with concomitant cleavage of the methyl ether.

The acid chloride of (101) was generated *in situ* with oxalyl chloride¹⁶¹ and treated with a mild Lewis acid, tin (IV) chloride. Only the cyclisation reaction was observed, the ether group remained intact.



However, a stronger Lewis acid, aluminium chloride, effected both of the desired reactions in high yield to give the linker precursor (100).

This modified synthesis (Figure 3.2) proved highly successful in enabling large quantities of the linker precursor to be obtained in an increased overall yield of 52% and without recourse to chromatographic purification. No problems were encountered on scaling up the reactions and each step has been carried out on at least 100 g scale, except the final step where the largest scale tried was 30 g.

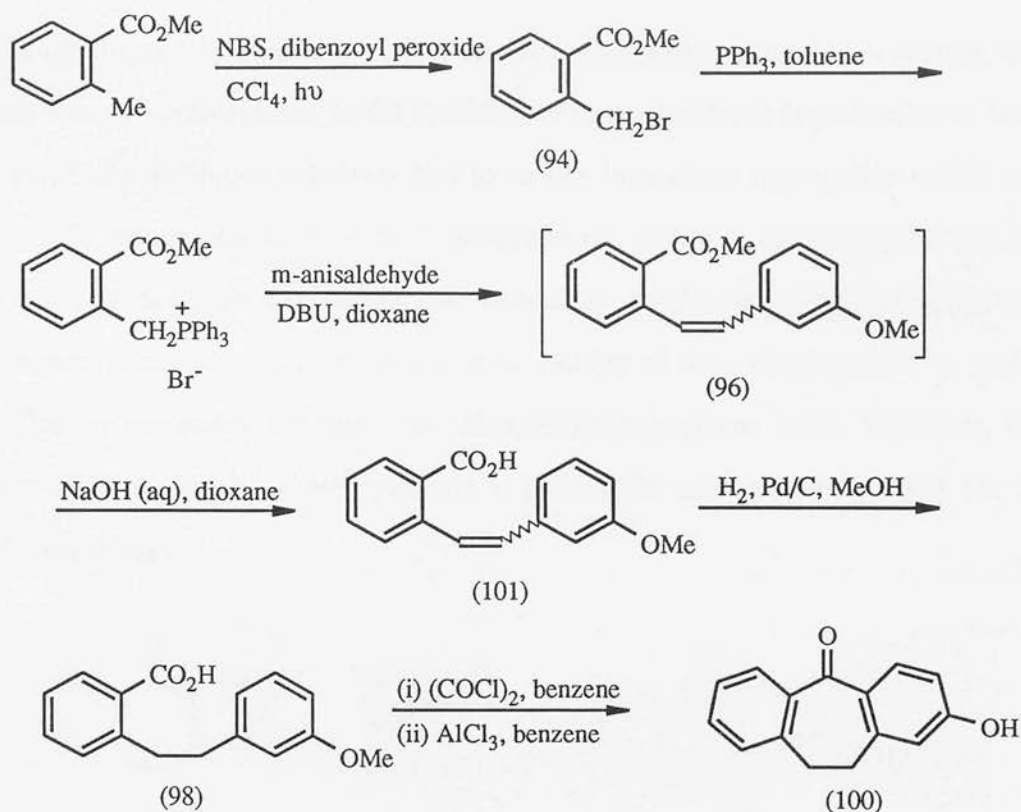
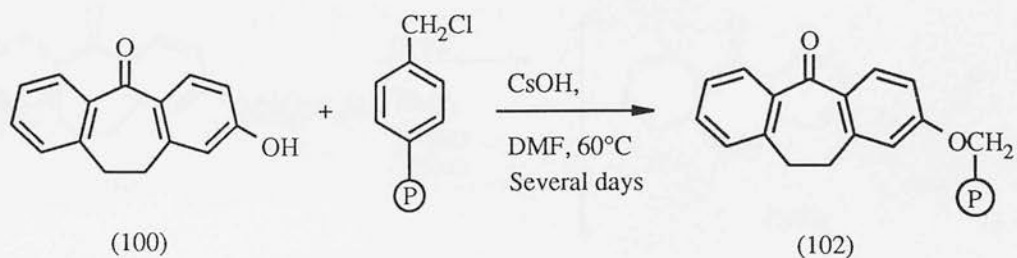


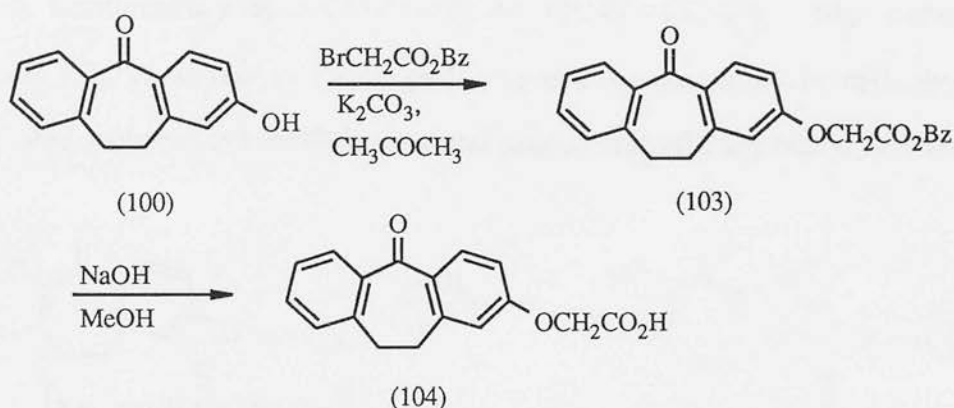
Figure 3.2: Modified synthesis of 2-hydroxydibenzocycloheptadien-5-one

3.3 Attachment of the linker to polystyrene resin

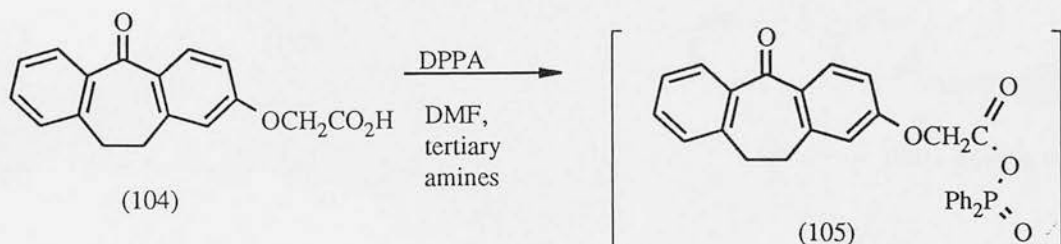
In the initial report¹⁶⁰, the linker was loaded onto polystyrene resin by a Williamson reaction with chloromethylpolystyrene (Merrifield) resin forming an ether linkage:

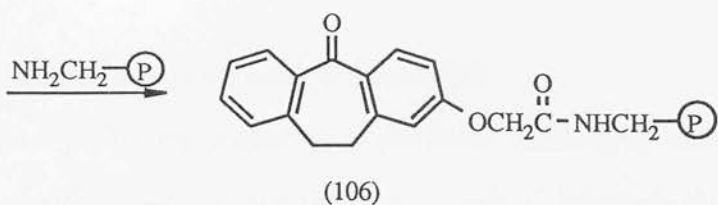


Although this reaction resulted in quantitative incorporation of the linker moiety, this is not always advantageous. In the synthesis of long peptides it is preferable to keep the level of substitution relatively low to reduce inter-chain aggregation which can drastically reduce coupling yields. Furthermore the ability to cap unreacted sites on the resin is desirable to prevent any unwanted attachment of amino acids and subsequent chain assembly during synthesis. Neither of these requirements is easily fulfilled by anchoring the linker to chloromethylpolystyrene resin. Therefore, the linker was modified by incorporating a carboxylic acid extension onto the 2-hydroxyl group:



Acid (104) was then activated as a diphenylphosphinic mixed anhydride⁶⁷ (105) and reacted with an aminomethylpolystyrene resin. This reaction proceeded smoothly in a few hours and a quantitative Kaiser test showed the reaction to be complete.





This quick and efficient reaction should enable the loading of the linker to be readily controlled by the amount of mixed anhydride used. Unreacted aminomethyl groups can then be capped with acetic anhydride using standard cycles on an automated peptide synthesiser.

The ketone group of (106) was reduced with lithium borohydride and the alcohol (107) so obtained used to derivatise the resin with protected hydrazine and amine functionalities by reaction with BocNHNH₂ and FmocNH₂, respectively (Figure 3.3). These reactions were analogous to those carried out by McInnes¹⁶⁰ on the linker anchored to Merrifield resin and employed similar reaction conditions.

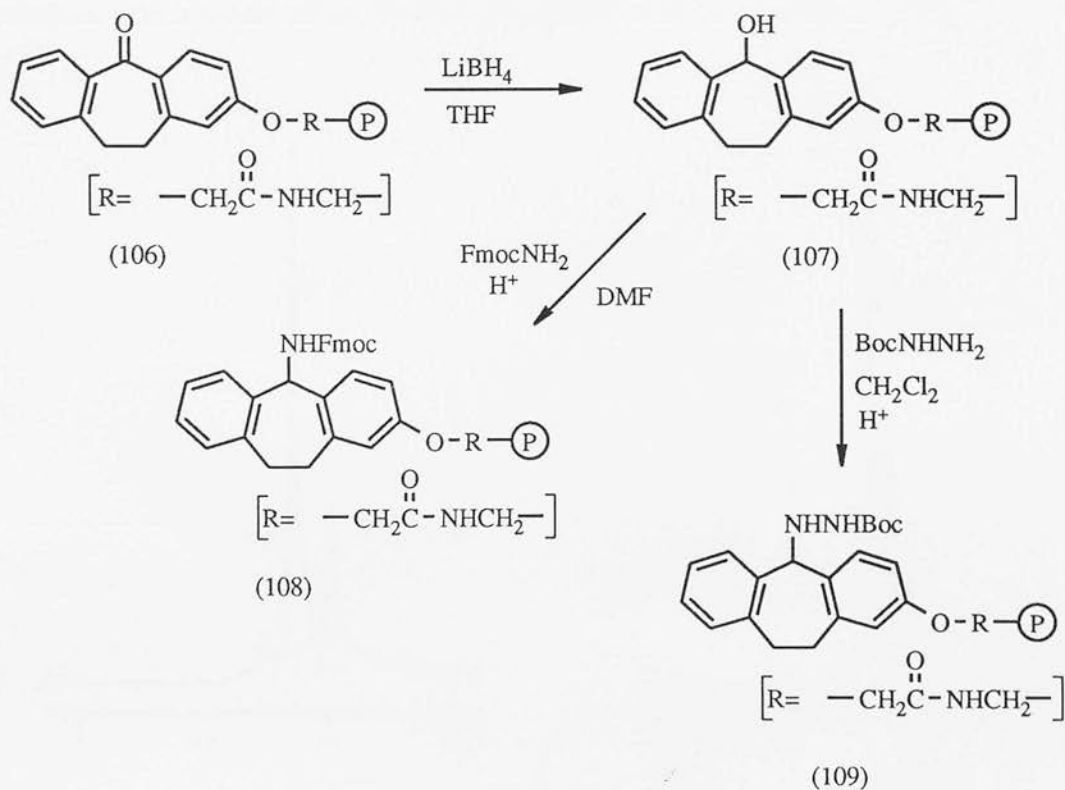
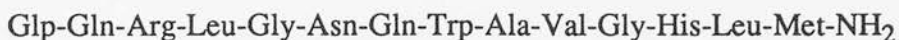


Figure 3.3: Synthesis of amide and hydrazide linkers

3.4 Synthesis of a peptide amide: Bombesin

To compare the properties of the linker attached to both Merrifield resin and to aminomethyl resin, bombesin¹⁶², a peptide C-terminal amide, was synthesised using resins (92) and (108).

Bombesin:



The yields of crude peptide cleaved from the resin were 74% and 97% respectively. This seems to indicate the superiority of resin (108), with the linker anchored to aminomethyl polystyrene resin. However, these figures should be treated with caution since the peptides could vary in the quantity of residual scavengers associated with them and in the extent of removal of TFA from the crude cleavage mixture, which would affect the ether precipitation of the peptide.

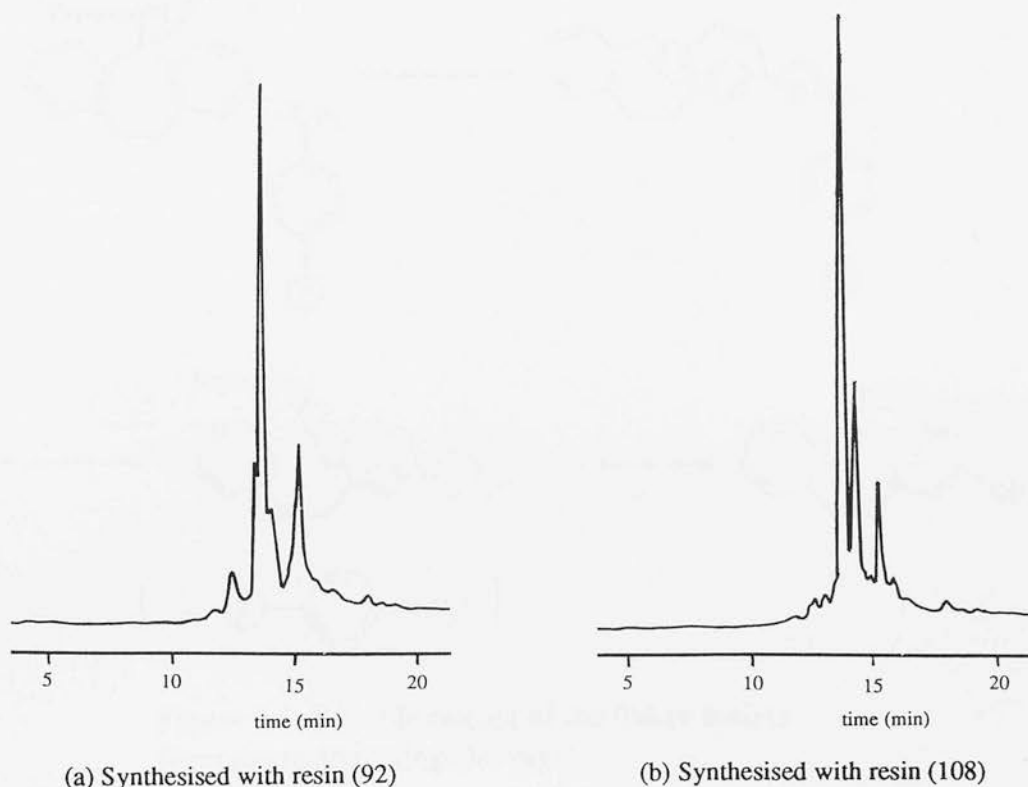


Figure 3.4: HPLC profiles of crude bombesin

HPLC traces of the crude peptides are shown in Figure 3.4. Although there are slight differences, in each case the major peak corresponded to the required peptide and from this limited comparison it would seem that both resins can be used effectively for the synthesis of peptide amides. However, resin (108) seems to be preferable on the basis of the amount of crude product obtained. Furthermore, this resin has other intrinsic features that make it more attractive than resin (92). For instance, the linker molecule could conceivably be released from the resin during cleavage, with undesirable consequences. This could occur by the mechanism shown in Figure 3.6, when expulsion of the linker moiety would form a relatively stable benzyl cation on the resin. The reactive dienone released could then react with the peptide if not efficiently scavenged. Any tendency for this to occur would be suppressed using resin (108) where the amide spacer prevents the formation of a benzyl cation.

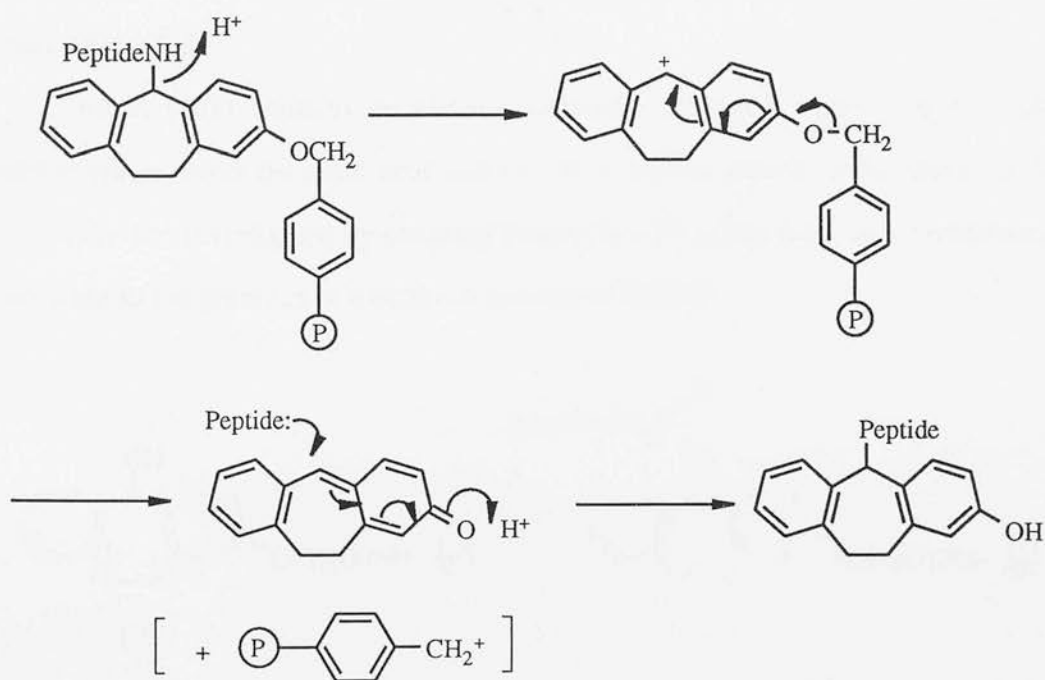
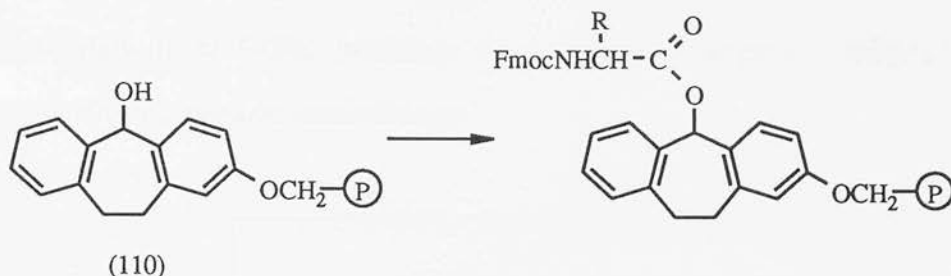


Figure 3.5: Possible release of the linker moiety from the resin during cleavage

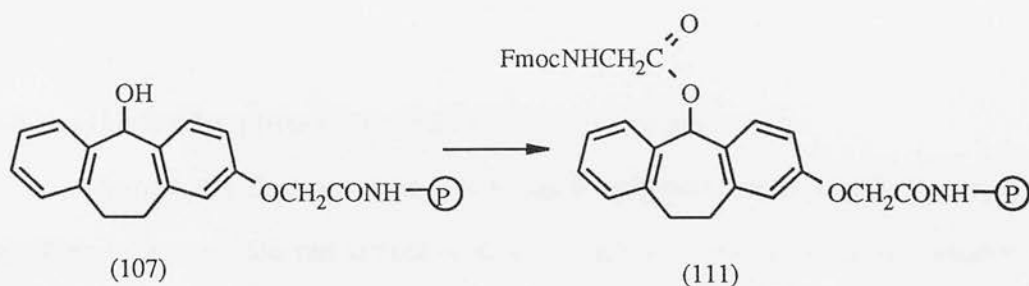
3.5 Synthesis of peptide C-terminal acids

McInnes¹⁶⁰ attempted to synthesise peptide acids using resin (110), by anchoring the first amino acid by the formation of an ester with the secondary alcohol:



However, when the synthesis of a hexapeptide was carried out using standard double couple cycles (symmetrical anhydride then HOBt ester couplings), only half of the expected peptide remained on the resin at the end of the synthesis. This was attributed to the use of the acidic HOBt coupling agent, which cleaved the peptide-linker ester bond.

Resin (107) contains an electron-withdrawing amide group, which might exert a minor effect on the acid stability of a peptide bound to the linker as an ester. This was investigated by coupling Fmoc-Gly-OH to the resin as a symmetrical anhydride in the presence of a catalytic amount of DMAP.



Resin (111) was sonicated in a solution of HOBt/DMF. Samples of the resin were taken periodically and the quantity of amino acid on the resin estimated by the Fmoc

UV loading test¹⁴⁷. As a control, the same experiment was carried out on the Wang linker loaded with Fmoc-Gly-OH, when the amino acid-resin linkage proved stable over a period of 24 h.

The results are shown in Figure 3.6, and clearly the amino acid-resin bond has low stability to HOBt, rendering the synthesis of peptide C-terminal acids impractical using standard methodology.

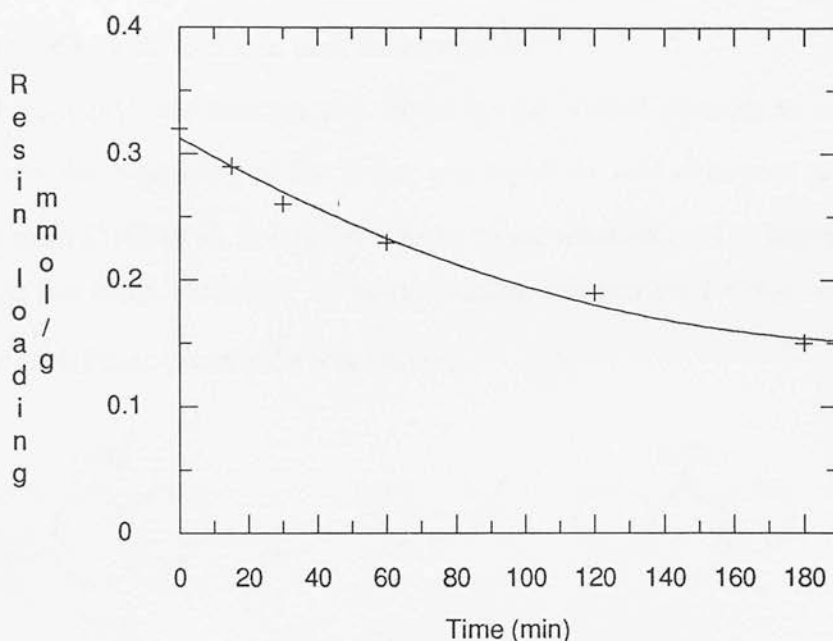


Figure 3.6: Stability of resin (111) to HOBt (0.5 M/DMF)

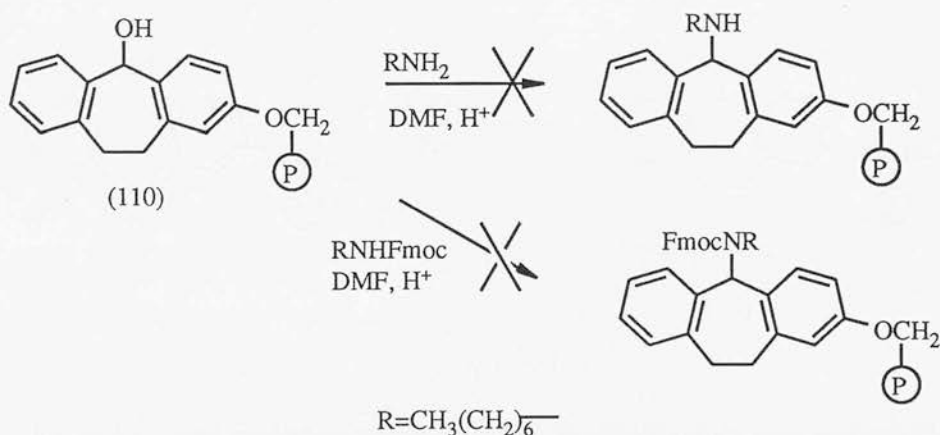
3.6 Synthesis of peptide C-terminal secondary amides

Despite the fact that many biological receptors are activated by peptides, peptides in general are not attractive drug candidates. One of the main reasons for this is the widespread occurrence of proteolytic enzymes in the body, leading to short lifetimes of administered peptides. Furthermore peptides are highly polar and poorly soluble in organic solvents hence will not pass through lipid membranes unless actively transported. It might therefore be desirable to incorporate a feature into a

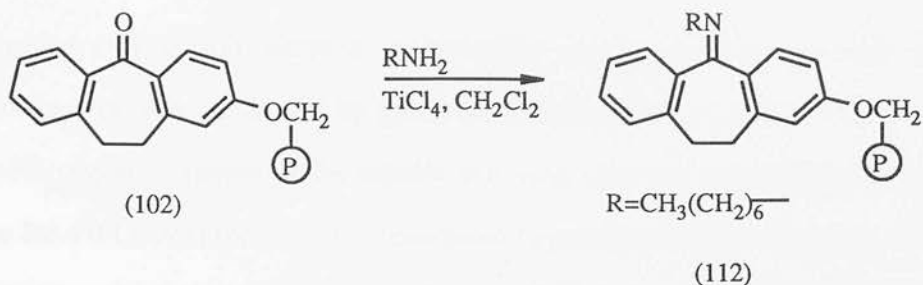
peptide molecule that will enhance lipid solubility. A number of recent studies^{163,164} have focused on lipophilic modifications to enhance transport of peptidic molecules into the central nervous system, e.g. cholesteryl ester derivatives of Leu-enkephalin¹⁶⁴.

Attempts were made to modify the dibenzocycloheptadiene linker to allow the routine synthesis of peptide C-terminal secondary amides, (peptide-NH-R, where R is a long chain alkyl group), without recourse to nucleophilic cleavage, which has previously been used to obtain such molecules⁹³.

R=n-heptyl was selected as a model system. Initial attempts to introduce the amine onto the 5-position of the linker employed an acid catalysed reaction with hydroxy resin (110) in an analogous fashion to the reaction used to introduce Fmoc-NH₂ onto the resin. However, no incorporation was achieved either with the free amine or with Fmoc-protected heptylamine:

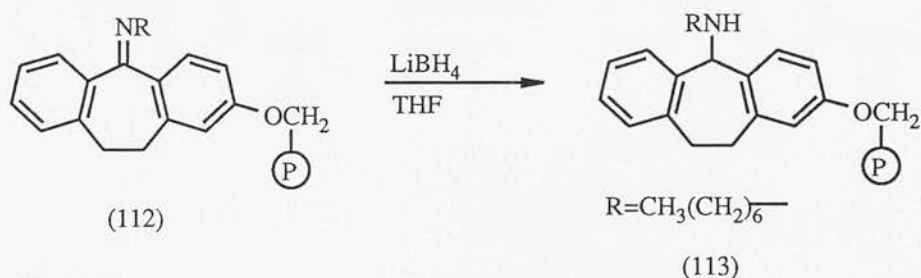


McInnes¹⁶⁰ had previously shown the ketone of alkoxy ketones such as (102) to be of rather low reactivity, failing to undergo either a Leuckart reaction or oxime formation. This is presumably due to the vinylogous ester nature of the molecule. However, the ketone function of resin (102) was found to react with heptylamine in the presence of TiCl₄ in DCM¹⁶⁵:



The reaction was monitored by IR, which showed the gradual disappearance of the ketone band at 1640 cm^{-1} over a period of 2.5 h. Virtually quantitative incorporation of the heptylamine was confirmed by nitrogen analysis. It was not possible to confirm the imine structure of the resin (112) and this structure was assigned on the basis of analogous reactions in the literature¹⁶⁵.

Imine (112) was reduced to the secondary amine (113) using lithium borohydride and this reaction was judged to be successful by the appearance of a broad signal around 3400 cm^{-1} in the IR spectrum.

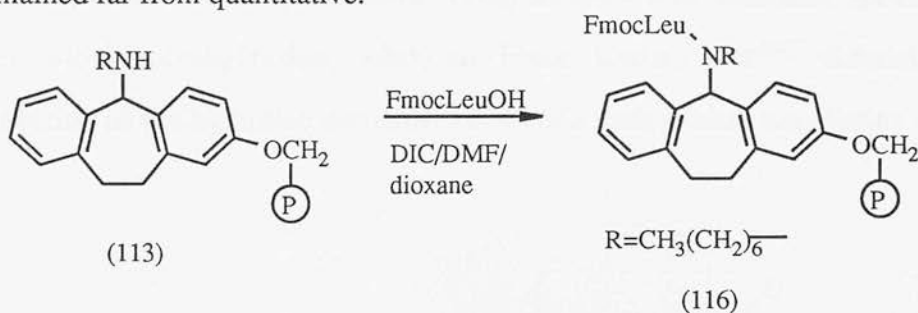


An amino acid was then coupled to the resin. Glycine was activated as its acid chloride and reacted with the resin-bound amine. After 2 h an Fmoc loading test¹⁴⁷ indicated a coupling efficiency of 40%. Higher loadings could be achieved with more extended reaction times, however some discolouration of the resin was noted.

This resin (114) was used to synthesise a pentapeptide N-heptyl amide, Leu-Ile-Phe-Ala-Gly-NH-(CH₂)CH₃ (115). The peptide was liberated from the resin in

TFA, using anisole and water as carbocation scavengers. The presence of the required peptide was confirmed by amino acid analysis and accurate FAB-MS. The enhanced lypophilic nature of the peptide was apparent from the enhanced retention time on RP-HPLC and the peptide's reluctance to precipitate from the crude cleavage mixture after stripping the mixture of TFA and adding ether. The peptide was finally obtained in solid form by partitioning between pet. ether (40-60) and water.

Attempts were then made to introduce amino acids other than glycine. Leucine symmetrical anhydride was generated using DIC and reacted with resin (113) in 1:1 DMF/dioxane. The reaction was monitored by Fmoc loading tests¹⁴⁷ on small aliquots of resin. The reaction proceeded rather sluggishly, but after sonicating the mixture overnight then adding fresh symmetrical anhydride and sonicating for a further period, sufficient incorporation of the the amino acid was achieved, although this remained far from quantitative.



The N-heptylamide derivative of Leu-enkephalin (117) was then synthesised using resin (117), giving the peptide in an overall yield of 55% after cleavage from the resin and purification by semi-preparative HPLC.

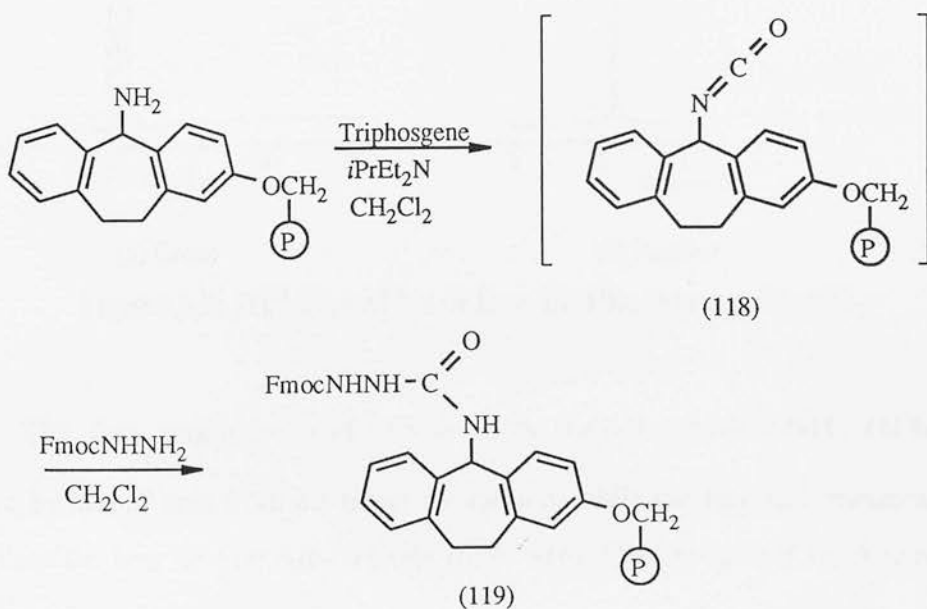
Although the utility of this resin has been demonstrated in principle, for future use quicker and more efficient coupling of the first amino acid would be desirable. With such long extended symmetrical anhydride couplings racemisation could be a risk. In this respect, the PyBroP coupling agent has been recently reported⁸⁷ to give efficient coupling of N-methyl amino acids and might prove useful for coupling the first amino acid to this secondary amine resin.

3.7 Synthesis of C-terminal aza-glycine peptides

Aza-amino acids have previously been incorporated into peptides as non-natural amino acid analogues to enhance stability to proteolytic enzymes¹⁶⁶. This has led to the development of numerous peptides of actual or potential therapeutic use. For instance, potent LHRH antagonists¹⁶⁷ and agonists¹⁶⁸ containing a C-terminal aza-glycine amide have been synthesised, the latter of which has proved valuable as a drug (Zoladex) for the treatment of prostate cancer.

Although C-terminal aza-amino acids would seem useful moieties to be routinely incorporated into peptides for biological studies, only one linker suitable for the solid phase synthesis of such species has been proposed¹⁶⁷.

The amine resin (108) was therefore modified for this use by removal of the Fmoc group with 20% piperidine in DMF, then treating the liberated amine with triphosgene, to generate an isocyanate (118) *in situ*. This activated species was trapped with Fmoc-hydrazine, when an Fmoc loading test¹⁴⁷ showed good incorporation of the hydrazine derivative, to form a resin-bound aza-glycine moiety (119).



A small peptide Leu-Ile-Phe-Ala-azaGlyNH₂ (120) was synthesised as a test sequence using resin (119). The peptide was cleaved from the resin with 95% TFA/water and analytical HPLC (Figure 3.6) showed one main product with two smaller but significant side products. These were separated by semi-preparative HPLC.

Amino acid analysis on each fraction showed the presence of the four required amino acids in equimolar ratios. Accurate mass FAB-MS showed the main component to be the required peptide, while the nominal FAB-MS of the early and late-eluting contaminants showed peaks at 477 and 462 respectively.

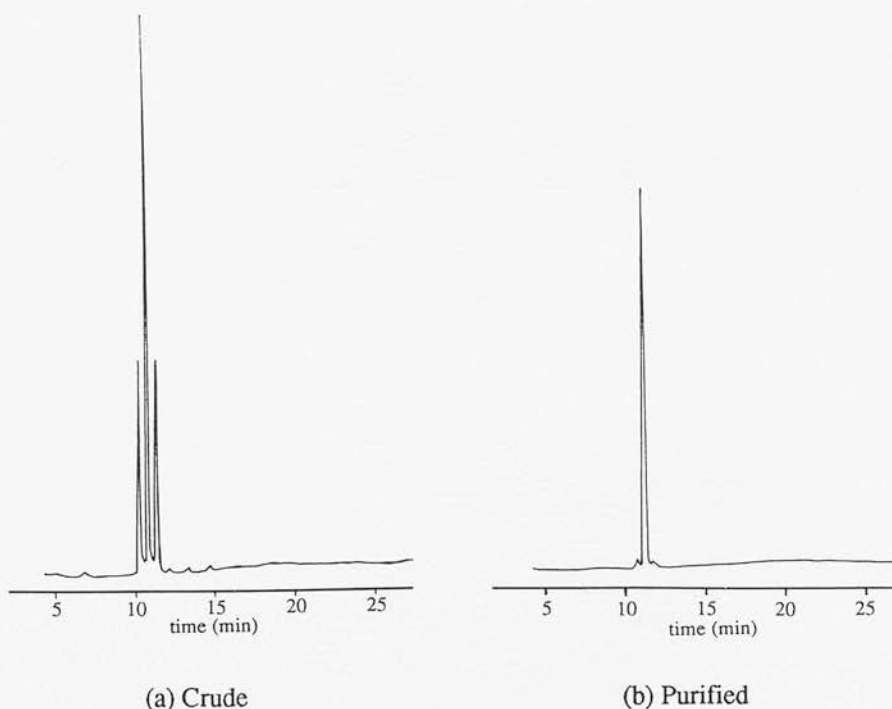


Figure 3.7: HPLC profiles of Leu-Ile-Phe-Ala-azaGlyNH₂

The first product would appear to be Leu-Ile-Phe-Ala-NH₂ (MW 461), formed by loss of urea from the target compound, while the second component could be hydrazide, Leu-Ile-Phe-Ala-NHNH₂ (MW 476). Clearly further work is required to establish the nature and extent of the side reactions that occur with this resin during cleavage. Nevertheless, the desired peptide was isolated in an overall yield of

52% and this resin would appear to be of some use for the routine synthesis of C-terminal aza-glycine peptides.

CHAPTER 4. EXPERIMENTAL

4.1 Notes

Amino acid derivatives were purchased from Aldrich, Sigma or Novabiochem and were used as supplied. Melting points were determined in open capillaries using a Büchi 510 melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed with silica gel 60GF-254 (Merck 5735) on plastic or aluminium sheets in the following solvent systems:

- A 30% ether/n-hexane
- B chloroform
- C 10% methanol/chloroform
- D 1:1 dichloromethane/n-hexane
- E dichloromethane
- F 9:1:0.5 chloroform/methanol/acetic acid
- G 30% dichloromethane/hexane
- H toluene

Compounds were visualised using suitable combinations of the following methods: iodine vapour, ultra-violet absorption at 254 and 352 nm, methanolic sulphuric acid, Mary's reagent (4,4'-bis(dimethylamino)diphenylcarbinol and ninhydrin. Optical rotations were measured on an AA 1000 polarimeter using a 1 dm cell in the solvents quoted. Flash chromatography was performed using silica gel 60 230-400 mesh (wet flash) or silica gel 60H 5-40 μm (dry flash). HPLC was performed using Applied Biosystems equipment (2 x 1406A or 2x400 solvent delivery systems, a 1480A or 491 injector mixer and a 783A programmer detector). The following columns and solvent gradients were used and in all cases A=0.1% aqueous TFA, B=CH₃CN/0.1% TFA, monitoring the eluent at 214 nm, unless stated.

<u>Column</u>	<u>Specifications</u>
A	Aquapore C ₁₈ , 220x4.6 mm, 7 μm
B	Aquapore C ₁₈ , 100x4.6 mm, 7 μm
C	Aquapore C ₈ , 100x4.6 mm, 7 μm
D	Aquapore C ₈ , 250x10 mm, 7 μm
E	Aquapore C ₁₈ , 100x10 mm, 7 μm
F	Aquapore C ₁₈ , 250x10 mm, 7 μm
G	Vydac C ₁₈ , 280x20mm, 7 μm

<u>Gradient No.</u>	<u>Initial,</u> <u>B%</u>	<u>Final,</u> <u>B%</u>	<u>Flow rate</u> <u>(ml/min)</u>	<u>Time</u> <u>(min)</u>
1	10	50	1.00	30
2	10	90	10.00	45
3	10	40	10.00	45
4	10	90	1.00	30
5	20	75	1.00	30
6	10	90	10.00	30
7	10	90	5.00	45
8	10	90	1.00	20
9	10	60	5.00	45
10	10	90	10.00	45

Infrared spectra were recorded on either a Perkin-Elmer 781 or Bio-Rad FTS-7 spectrophotometer in dichloromethane solution, as KBr discs or nujol mulls. Ultraviolet spectra were recorded on a Cary 210 spectrophotometer. Mass spectra were obtained using Kratos MS50TC, AEI MS-902, Kratos Kompact Maldi-TOF, and Finnegan Laser-Mat machines. Proton NMR spectra were recorded on either

Brüker WP 80 (80 MHz), WP 200 (200 MHz) or WH (360 MHz) machines in the solvent indicated relative to a tetramethylsilane external standard. Carbon-13 NMR spectra were recorded on a Brüker WP 200 (50 MHz) or WH 360 (90 MHz) machines in the solvents indicated relative to tetramethylsilane as external standard. Fluorine-19 NMR spectra were recorded on a Brüker WP 80 (75 MHz). Elemental analyses were carried out on Carlo Erba 1106 or Perkin-Elmer 2400 instruments. Amino acid analysis was carried out on an LKB 4151 alpha plus amino acid analyser subsequent to Carius tube hydrolysis with constant boiling hydrochloric acid at 110°C. N,N-dimethylformamide and dioxane were peptide synthesis grade. Other solvents were of analytical grade or were distilled before use. The following solvents were dried when required using the reagents indicated: benzene (sodium wire), dichloromethane (calcium hydride), diethyl ether (sodium wire), THF (sodium/benzophenone indicator), toluene (sodium wire).

4.1.1 Elemental analyses on tetrabenzo[*a,c,g,i*]fluorene derivatives

In this and related studies^{130,142} microanalysis results on tetrabenzo[*a,c,g,i*]fluorene derivatives have proved generally unsatisfactory, giving lower carbon content than that calculated for the expected molecular formulae. This has been attributed to the large number of quaternary carbons present leading to poor combustion. Increased oxygen concentrations and the addition of vanadium pentoxide have given improved results (see Appendix for an example¹⁶⁹) although these have still not proved entirely satisfactory and this is the subject of on-going investigations in this laboratory.

4.2 Experimental

(Bis-phenanthren-9-yl)methanol (60)

9-Bromophenanthrene (20 g, 77.8 mmol) was taken in THF (40 ml) and slowly added to Mg turnings (2.0 g, 8.2 mmol) together with a crystal of iodine, in an atmosphere of dry nitrogen. The mixture was stirred for 1 h then ethyl formate (2.88 g, 3.89 mmol) slowly added. After 1.5 h, ice (10 g) then aqueous NH_4Cl (20 g in 40 ml H_2O) was added and a white solid precipitated on stirring. A further 40 ml of H_2O was added, the precipitate filtered off and washed with water. The solid was taken up in DCM and combined with a DCM extract of the aqueous filtrate. The solution was dried (MgSO_4) and solvent removed *in vacuo*. The residue was triturated with ether to give a white solid (7.7 g, 52%).

m.p. 234-237°C; Found: C, 90.2; H 5.43. $\text{C}_{29}\text{H}_{20}\text{O}$ requires C, 90.6; H, 5.20%; TLC: R_f (A) 0.23; δ_{H} (200 MHz, CDCl_3): 9.10 (2H, d, $J=7.6$ Hz, aromatic), 9.03 (2H, d $J=8.3$ Hz, aromatic), 8.46 (2H, d $J=7.7$ Hz, aromatic) 8.08-8.05 (4H, m, aromatic), 7.93-7.76 (8H, m, aromatic), 7.53 (1H, d $J=6.0$ Hz, CH), 5.08 (1H, d $J=6.0$ Hz, OH); ν_{max} (Nujol): 3400 (OH), 1600 (aromatic C=C) cm^{-1} ; λ_{max} (DCM): 298 (ϵ 19700 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 286 (18900), 277 (24900), 255 nm (104000); m/z (EI): 384 (M^+), 366, 205, 178; HRMS: 384.1519, $\text{C}_{29}\text{H}_{20}\text{O}$ requires 384.15141.

8bH-Tetrabenzo[*a,c,g,i*]fluorene (61)

(Bis-phenanthren-9-yl)methanol (5.2 g, 13.5 mmol) was taken in DCM (100 ml) and TFA (15 ml) added. The mixture was swirled for 2 min then the solvent removed *in vacuo* and the residue triturated with ether to give the product as a yellow solid (4.47 g, 90%).

m.p. 277-280°C; Found: C, 94.2; H, 5.07. $\text{C}_{29}\text{H}_{18}$ requires C, 95.1; H, 4.92%; TLC: R_f (A) 0.34; δ_{H} (80 MHz, CDCl_3): 8.82-8.75 (2H, m, aromatic), 8.30-7.08 (15H, m,

aromatic & CH), 5.42 (1H, s, CH); ν_{\max} (Nujol): 1620 (aromatic C=C) cm^{-1} ; λ_{\max} (DCM): 376 (ϵ 12600 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 360 (10600), 302 (34200), 290 (36200), 280 (39500), 256 nm (66800); m/z (EI): 366 (M^+), 365, 364, 363, 182; HRMS: 366.1419, $\text{C}_{32}\text{H}_{24}\text{O}_3$ requires 366.14084.

17-(Methoxycarbonyl)tetrabenzo[*a,c,g,i*]fluorene (64)

8*b*H-Tetrabenzo[*a,c,g,i*]fluorene (0.5 g, 1.37 mmol) was taken in THF (25 ml) under an atmosphere of dry nitrogen and cooled to -78°C . A solution of *n*-butyllithium in cyclohexane (1.6 M titrated, 0.93 ml, 1.48 mmol) was added and the deep orange-coloured mixture stirred at -78°C for 15 min. Methyl chloroformate (0.2 ml, 2.59 mmol) was added and the mixture stirred at -78°C for 1 h then at room temperature for 30 min, when the orange colouration had faded significantly. The reaction was quenched with water and the organic layer extracted with DCM, dried (MgSO_4) and the solvent removed *in vacuo*. DCM (20 ml) was added to the residue, the insoluble solid filtered off and the solvent removed from the filtrate *in vacuo*. This was repeated, then the residue triturated with ether to give the *title compound* as a yellow solid (0.10 g, 16%)

m.p. $238\text{--}240^\circ\text{C}$; Found: C, 88.5; H, 4.89. $\text{C}_{31}\text{H}_{20}\text{O}_2$ requires C, 87.7; H, 4.72%; TLC: R_f (A) 0.23, R_f (H) 0.45; δ_{H} (200 MHz, CDCl_3): 8.80-8.64 (6H, m, aromatic), 8.28-8.24 (2H, m, aromatic), 7.75-7.25 (8H, m, aromatic), 5.60 (1H, s, CH), 3.50 (3H, s, OMe); ν_{\max} (DCM): 3080 (aromatic CH), 2960 (aliphatic CH), 1730 (ester C=O), 1610 (aromatic C=C), 1500, 1210, 1090 cm^{-1} ; λ_{\max} (DCM): 370 (ϵ 17000 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 302 (42700), 290 (34700), 253 nm (66200); m/z (EI): 424 (M^+), 364, 181; HRMS: 424.1474, $\text{C}_{31}\text{H}_{20}\text{O}_2$ requires 424.14632.

Ethyl 3-(17-tetrabenzo[*a,c,g,i*]fluorenyl)propanoate (65)

8*b*H-Tetrabenzo[*a,c,g,i*]fluorene (0.2 g, 0.546 mmol) was taken in THF (10 ml) under an atmosphere of dry nitrogen and cooled to -84°C. A solution of *n*-butyllithium in cyclohexane (1.61 M titrated, 0.40 ml, 0.640 mmol) was added and the deep orange-coloured mixture stirred at -84°C for 25 min. Ethyl acrylate (0.10 ml, 0.924 mmol) was added and the mixture stirred at -84°C for 30 min then at room temperature for 1 h. The solvent was removed *in vacuo* and the residue purified by dry flash chromatography using DCM as eluent. Appropriate fractions were combined and the solvent removed *in vacuo*. The residue was taken up in ether and the ether allowed to slowly evaporate to leave the *title compound* as a pale yellow solid (0.079 g, 31%).

m.p. 181-182°C; Found: C, 87.2; H 5.84. C₃₄H₂₆O₂ requires C, 87.6; H, 5.84%; TLC: R_f (A) 0.24; δ_H(200 MHz, CDCl₃): 8.83-8.65 (6H, m, aromatic), 8.28-8.23 (2H, m, aromatic), 7.75-7.58 (8H, m, aromatic), 5.09 (1H, t J=4.4 Hz, CH), 3.58 (2H, q J=7.1 Hz, OCH₂), 3.04-2.94 (2H, m, CH₂), 1.39-1.31 (2H, m, CH₂), 0.84 (3H, t J= 7.1 Hz); ν_{max}(DCM): 3080 (aromatic CH), 2995 (aliphatic CH), 1730 (ester C=O), 1610, 1500, 1380, 1215, 1175 cm⁻¹; λ_{max}(DCM): 380 (ε 12000 dm³mol⁻¹cm⁻¹), 365 (12800), 302 (28300), 289 (23600), 254 nm (48300); m/z (EI): 466 (M⁺), 424, 364, 363, 182; HRMS: 466.1926, C₃₄H₂₆O₂ requires 466.19327.

Tetrabenzo[*a,c,g,i*]fluorene-17-one (63)

8*b*H-Tetrabenzo[*a,c,g,i*]fluorene (0.3 g, 0.819 mmol) was taken in THF (20 ml) and sodium methoxide (0.22 g, 4.07 mmol) added. The mixture was stirred for 15 min then poured into water (100 ml), acidified to pH1 with concentrated hydrochloric acid and extracted with DCM (3 x 50 ml). The extract was dried (MgSO₄) and the solvent removed *in vacuo* to give the *title compound* as dark needles (0.21 g, 67%).

m.p. 302-305°C (lit¹³³ 295°C); Found: C, 89.9; H 4.22. C₂₉H₁₆O requires C, 91.6; H, 4.21%; δ_{H} (200 MHz, CDCl₃): 9.23-9.18 (2H, m, aromatic), 8.78-8.63 (4H, m, aromatic), 8.28 (2H, dd J=8.2 Hz, 1.7 Hz, aromatic), 7.83-7.60 (8H, m, aromatic); TLC: R_f (A) 0.34; ν_{max} (DCM): 1695 (C=O), 1605 (aromatic C=C), 1505 cm⁻¹; λ_{max} (DCM): 425 (ϵ 2000 dm³mol⁻¹cm⁻¹), 336 (37100), 320 (31400), 253 nm (78100); m/z (FAB): 381 (MH⁺), 380 (M⁺); HRMS: 380.12015, C₂₉H₁₆O requires 380.12011.

Tetrabenzo[*a,c,g,i*]fluorene (52)

8*b*H-Tetrabenzo[*a,c,g,i*]fluorene (0.5 g) was taken in THF (40 ml) and 3 drops of triethylamine added. The solution was mixed for 1 min, then the solvent removed *in vacuo*. Ether was added to the residue to give the *title compound* as a yellow solid (0.45 g, 90%).

m.p. 281-283°C (lit¹³³ 275-276°C); Found: C, 95.6; H 4.94. C₂₉H₁₈ requires C, 95.1; H, 4.92%; TLC: R_f (A) 0.48; δ_{H} (200 MHz, CDCl₃): 8.83-8.68 (6H, m, aromatic), 8.23-8.18 (2H, m, aromatic), 7.74-7.58 (8H, m, aromatic), 4.62 (2H, s, CH₂); ν_{max} (Nujol): 1610 (aromatic C=C), 1500 cm⁻¹; λ_{max} (DCM): 378 (ϵ 16500 dm³mol⁻¹cm⁻¹), 364 (17600), 301 (39600), 288 (33300), 261 (61500), 254 nm (65600); m/z (FAB): 366 (M⁺), 324; HRMS: 366.14987, C₂₉H₁₈ requires 366.14084.

Ethyl (bis-phenanthren-9-yl)glycolate (54)

9-Bromophenanthrene (100 g, 0.389 mol) was dissolved in dry THF (200 ml) and slowly added to Mg turnings (10 g, 0.41 mol), together with a crystal of iodine under an atmosphere of dry nitrogen. After initiation of the reaction, the mixture was cooled on an ice-bath to control the initial vigour of the reaction, then stirred at room temperature for 45 min. The mixture was cooled on an ice/salt bath and diethyl

oxalate (27.3 g, 0.187 mol) added dropwise over a period of 10 min. The mixture was then stirred at room temperature for 1.5 h, after which time ice, then aqueous 3.7M NH₄Cl (250 ml) was added. The mixture was stirred for 10 min and extracted with DCM (2 x 500 ml). A precipitate formed in the organic extract and this was filtered off and discarded. The filtrate was dried (MgSO₄) and the solvent removed *in vacuo* to leave a yellow oil which was triturated with ether and allowed to stand overnight. The precipitated solid was filtered off and recrystallised from DCM/n-hexane to give the *title compound* as a white solid (48.8 g, 57%).

m.p. 189-191°C (lit¹³⁰ 188-189°C); Found: C, 84.4; H 5.36. C₃₂H₂₄O₃ requires C, 84.2; H, 5.26%; TLC: R_f (B) 0.5; δ_H(360 MHz, CDCl₃): 8.79 (2H, d, J=8.3 Hz, aromatic), 8.72 (2H, d, J=8.5 Hz, aromatic), 8.53 (2H, d, J=8.0 Hz, aromatic), 7.70-7.47 (12H, m, aromatic), 4.51 (1H, s, OH), 4.42 (2H, q, J=7.2Hz, CH₂), 1.18 (3H, t, J=7.2 Hz, CH₃); ν_{max}(DCM): 3500 (OH), 3060 (aromatic CH), 1730 (ester C=O), 1600 cm⁻¹; λ_{max}(DCM): 300 (ε 20200 dm³mol⁻¹cm⁻¹), 289 (18900), 278 (25900), 257 nm (103000); m/z (EI): 456 (M⁺), 364, 363; HRMS: 456.1739, C₃₂H₂₄O₃ requires 456.17253.

17-(Ethoxycarbonyl)-8bH-tetrabenzo[*a,c,g,i*]fluorene (68)

Ethyl (*bis*-phenanthren-9-yl)glycolate (27 g, 59.2 mmol) was taken in DCM (270 ml) and TFA (81 ml) slowly added with stirring. After 20 min, the solvent was removed *in vacuo* and the residue triturated with ether to give the product as a yellow solid (23.1 g, 89%).

m.p. 161-163°C; Found: C, 86.5; H, 5.12. C₃₂ H₂₂O₂ requires C, 87.7; H, 5.02%; TLC: R_f (B) 0.69; δ_H(360 MHz, CDCl₃): 8.81-8.74 (2H, m, aromatic), 8.12-7.93 (3H, m, aromatic), 7.82-7.61 (6H, m aromatic), 7.47-7.29 (4H, m, aromatic), 7.11-7.06 (1H, m, aromatic), 5.38 (1H, s, CH), 4.62-4.49 (2H, m, CH₂), 1.41 (3H, t, J=7.2 Hz, CH₃); δ_C(50 MHz, CDCl₃): 168.5 (ester C=O), 146.9 (quaternary aromatic C),

138.3-123.2 (aromatic C's), 61.7 (CH₂), 52.5 (CH), 13.9 (CH₃); ν_{\max} (DCM): 2960-2900 (CH), 1730 (ester C=O), 1610 cm⁻¹ (aromatic C=C); λ_{\max} (DCM): 372 (ϵ 10300 dm³mol⁻¹cm⁻¹), 301 (32200), 290 (31200), 254 nm (66500); m/z (EI): 438 (M⁺), 364, 363; HRMS: 438.1610, C₃₂H₂₂O₂ requires 438.16197.

17-(8*b*H-Tetrabenzo[*a,c,g,i*]fluorenyl)methanol (69)

17-(Ethoxycarbonyl)-8*b*H-tetrabenzo[*a,c,g,i*]fluorene (6.0 g, 13.7 mmol) was taken in DCM (300 ml) in an atmosphere of dry nitrogen and cooled to -65°C. A 1M solution of diisobutylaluminium hydride (41.4 ml, 41.4 mmol) in DCM was added dropwise, such that the temperature remained below -60°C. The mixture was then stirred at -70°C for 1 h and for a further 1.5 h at room temperature. The mixture was cooled to -30°C and 10% aqueous acetic acid (180 ml) carefully added. A solid precipitated and was filtered off, washed with water and taken up in DCM. The solution was dried (MgSO₄) and solvent removed *in vacuo* to give a yellow solid which was recrystallised from DCM/*n*-hexane to give the *title compound* (3.78 g, 70%).

m.p. 185-187°C; Found: C, 89.9; H, 5.13. C₃₀ H₂₀O requires C, 90.9; H, 5.05%; TLC: R_f (F) 0.75, (A) 0.07, (H) 0.08; ν_{\max} (DCM): 3600 (OH), 3080 (aromatic CH), 2920 (aliphatic CH), 1610 cm⁻¹ (aromatic C=C); λ_{\max} (DCM): 372 (ϵ 9450 dm³mol⁻¹cm⁻¹), 356 (7910), 299 (28600), 287 (29000), 253 nm (59800); m/z (FAB): 396 (M⁺), 379, 366, 364; HRMS: 396.15142. C₃₀H₂₀O requires 396.15141.

17-(Ethoxycarbonyl)tetrabenzo[*a,c,g,i*]fluorene (55)

17-(Ethoxycarbonyl)-8*b*H-tetrabenzo[*a,c,g,i*]fluorene (15 g, 43.2 mmol) was taken in DCM (100 ml) and triethylamine (0.2 ml, 1.44 mmol) added with swirling. The solvent was then removed *in vacuo* and the residue triturated with ether to give the *title compound* as a yellow solid (14.5 g, 97%).

m.p. 168-169°C (lit¹³⁰ 165-166°C); Found: C, 87.1; H, 5.02. C₃₂H₂₂O₂ requires C, 87.7; H, 5.02%; TLC: R_f (B) 0.67; δ_H(360 MHz, CDCl₃): 8.76-8.63 (6H, m, aromatic), 8.27-8.24 (2H, m, aromatic), 7.70-7.57 (8H, m aromatic), 5.50 (1H, s, CH), 4.04 (2H, q, CH₂), 0.96 (3H, t, CH₃); δ_C(50 MHz, CHCl₃): 172.6 (ester C=O), 138.8, 138.7, 132.2, 131.0, 129.2, 128.3 (quaternary aromatic C's), 128.2, 127.8, 126.8, 126.7, 125.6, 124.5, 123.8 (aromatic CH's), 62.1 (CH₂), 55.4 (CH), 14.5 (CH₃); ν_{max}(DCM): 3080 (aromatic CH), 2930 (aliphatic CH), 1725 (ester C=O), 1610 cm⁻¹ (aromatic C=C); λ_{max}(DCM): 386 (ε 15200 dm³mol⁻¹cm⁻¹), 370 (17500), 302 (47500), 290 (37800), 279 (32300), 262 (71400), 253 nm (76500); m/z (EI): 438 (M⁺), 364, 363; HRMS: 438.1633, C₃₂H₂₂O₂ requires 438.16197

17-Tetrabenzo[*a,c,g,i*]fluorenylmethanol (56)

(a) 17-(Ethoxycarbonyl)tetrabenzo[*a,c,g,i*]fluorene (6.0 g, 13.7 mmol) was taken in DCM (300 ml) in an atmosphere of dry nitrogen and cooled to -65°C. A solution of 1M diisobutylaluminium hydride (41.4 ml, 41.4 mmol) in DCM was added dropwise, such that the temperature remained below -60°C. The mixture was then stirred at -70°C for 1 h and for a further 2 h at room temperature. The mixture was cooled to -30°C and 20% aqueous acetic acid (90 ml) carefully added. The mixture was diluted with water (100 ml) and extracted with DCM (300 ml + 2 x 200 ml), the organic extract dried (MgSO₄) and solvent removed *in vacuo* to give a yellow solid which was washed with ether to give the *title compound* (4.8 g, 88%).

(b) 17-(8*b*H-Tetrabenzo[*a,c,g,i*]fluorenyl)methanol (10.0 g, 25.3 mmol) was taken in DCM (200 ml) and triethylamine (0.2 ml) added. The mixture was swirled until all the solid had dissolved then the solvent was removed *in vacuo* and the residue triturated with ether to give the *title compound* (8.99 g, 90%).

m.p. 202-204°C (lit¹³⁰ 202-203°C); Found: C, 90.2; H, 5.17. C₃₀H₂₀O requires C, 90.9; H, 5.05%; TLC: R_f (C) 0.74; δ_H(200 MHz, CDCl₃): 8.82-8.64 (6H, m.

aromatic), 8.33-8.25 (2H, m, aromatic), 7.75-7.57 (8H, m aromatic), 5.15 (1H, t, $J=4.3$ Hz, CH), 4.43 (2H, d, $J=4.3$ Hz, CH_2); $\nu_{\text{max}}(\text{DCM})$: 3580 (OH), 3010 (aromatic CH), 2910, 2890 (aliphatic CH), 1605 (aromatic C=C), 1495, 1035 cm^{-1} ; $\lambda_{\text{max}}(\text{DCM})$: 380 (ϵ 16500 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 366 (17600), 301 (40300), 289 (33000), 261 (61500), 253 nm (66700); m/z (FAB): 396 (M^+), 379, 366; HRMS: 396.15142, $\text{C}_{30}\text{H}_{20}\text{O}$ requires 396.15141.

(17-tetrabenzo[*a,c,g,i*]fluorenyl)methyl-*p*-nitrophenyl carbonate (72)

(17-tetrabenzo[*a,c,g,i*]fluorenyl)methanol (6.5 g, 16.4 mmol) was taken in DCM (260 ml) along with *p*-nitrophenyl chloroformate (3.64 g, 18.1 mmol) and *N,N*-dimethylaniline (2.63 ml, 20.8 mmol). The mixture was stirred for 4 days then the solvent removed *in vacuo* and the residue re-dissolved in DCM and hexane added to precipitate the crude product. The yellow solid was recrystallised from dioxane/hexane and a further product was obtained from the mother liquor on wet flash chromatography using toluene as eluent. Yield 5.92 g (64%).

$m.p.$ 210-213°C (lit¹³⁰ 139-140°C); TLC: R_f (H) 0.33; $\delta_{\text{H}}(360$ MHz, dioxane- d_8): 9.18-9.14 (4H, m, aromatic), 8.92 (2H, dd $J=8.3, 0.82$ Hz), 8.64-8.60 (2H, m, aromatic), 8.23 (2H, d $J=9.1$ Hz, nitrophenyl *m*-CH), 8.03-7.79 (8H, m aromatic), 7.07 (2H, d $J=9.1$ Hz, nitrophenyl *o*-CH), 5.74 (1H, t, $J=4.6$ Hz, CH), 5.30 (2H, d, $J=4.6$ Hz, CH_2); $\nu_{\text{max}}(\text{DCM})$: 3060 (aromatic CH), 1770 (C=O), 1620 (aromatic C=C), 1530, 1490, 1350, 1210 cm^{-1} ; $\lambda_{\text{max}}(\text{DCM})$: 366 (ϵ 13400 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 301 (33700), 289 (30200), 261 (53100), 254 nm (56100); m/z (FAB): 561 (M^+), 379, 234; HRMS: 561.15768, $\text{C}_{37}\text{H}_{23}\text{O}_5\text{N}$ requires 561.15761.

N^α -(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-glycine (50)

Glycine (0.05 g, 0.667 mmol) was dissolved in 5% aqueous Na_2CO_3 (2ml) and diluted with water (5 ml) and dioxane (3 ml) then added to a solution of (17-

tetrabenzo[*a,c,g,i*]fluorenyl)methyl-*p*-nitrophenyl carbonate (0.10 g, 0.178 mmol) in dioxane (10 ml). The mixture was stirred for 1.5 h then poured into water (30 ml) and washed with ether (2 x 50 ml). The aqueous mixture was acidified to pH1 with 2M KHSO₄ and extracted with DCM (2 x 30 ml). The organic extract was washed with water and dried (MgSO₄). The solvent was removed *in vacuo* and the residue triturated with ether to give the *title compound* (0.04 g, 45%).

m.p. 224-225°C (lit¹³⁰ 240-241°C); m/z (FAB): 497 (M⁺), 379.

Ethyl 2-methoxy-2-(bis-phenanthren-9-yl)acetate (76)

Ethyl (*bis*-phenanthren-9-yl)glycolate (20 g, 43.9 mmol) was taken in DMSO (40 ml) and powdered KOH (12 g, 0.214 mol) added. the mixture was stirred for 5 min then methyl iodide (5.5 ml) added and the mixture stirred for 15 min. Ice/water (300 ml) was added and the mixture extracted with DCM (3 x 100 ml), the extract washed with water, dried (MgSO₄) and the solvent removed *in vacuo*. The residue was triturated with ether to give a white solid which was recrystallised from DCM/n-hexane to give a white solid (11.9 g, 57%).

m.p. 234-237°C; Found: C, 83.7; H, 5.44. C₃₃H₂₆O₃ requires C, 84.3; H, 5.53%; TLC: R_f (B) 0.55, R_f (G) 0.13; δ_H(80 MHz, CDCl₃): 8.81-8.53 (6H, M, aromatic), 7.78-7.32 (12H, m, aromatic), 4.31 (2H, q, J=7.1 Hz, CH₂), 3.46 (3H, s, OCH₃), 1.11 (3H, t, J=7.1 Hz, CH₃); ν_{max}(DCM): 3068 (aromatic CH), 2991, 2942 (aliphatic CH), 1733 (ester C=O), 1600 (aromatic C=C), 1447, 1239, 1097 cm⁻¹; λ_{max}(DCM): 301 (19700 dm³mol⁻¹cm⁻¹), 290 (17900), 256 nm (103000); m/z (FAB): 470 (M⁺), 439, 397, 365, 205.

2-Methoxy-2-(bis-phenanthren-9-yl)ethanol (77)

Ethyl 2-methoxy-2-(*bis*-phenanthren-9-yl)acetate (11.7 g, 24.9 mmol) was taken in DCM (100 ml) and cooled to 0°C under an atmosphere of dry nitrogen. A 1M

solution of diisobutylaluminium hydride in DCM (84 ml, 84.0 mmol) was added dropwise and then the solution allowed to stir at room temperature for 30 min. The reaction was quenched by the careful addition of 10% aqueous acetic acid and the mixture extracted with DCM (3 x 100 ml). The extract was washed with water (2 x 100 ml) then dried (MgSO_4) and the solvent removed *in vacuo*. The residue was triturated with ether to give a white solid (7.9 g, 74 %).

m.p. 222-224°C; Found: C, 86.4; H, 5.44. $\text{C}_{31}\text{H}_{24}\text{O}_2$ requires C, 86.9; H, 5.60%; TLC: R_f (B) 0.18; δ_{H} (80 MHz, CDCl_3): 8.75-7.09 (18H, m, aromatic), 4.83 (2H, apparent br d, $J=6.4$ Hz, CH_2), 3.24 (3H, s, CH_3), 1.66-1.61 (1H, br m, exchanges with D_2O , OH); ν_{max} (DCM): 3576 (OH), 3080 (aromatic CH), 2982, 2897 (aliphatic CH), 1598 (aromatic C=C), 1495, 1446, 1118, 1055 cm^{-1} ; λ_{max} (DCM): 301 (24400 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 289 (21300), 255 nm (120000); m/z (FAB): 398 (M^+-OMe), 274, 257, 232.

2-Methoxy-2-(bis-phenanthren-9-yl)ethyl pentafluorophenyl carbonate (78)

2-Methoxy-2-(bis-phenanthren-9-yl)ethanol (5.0 g, 11.7 mmol) was taken with triphosgene (1.18 g, 3.97 mmol) and triethylamine (1.65 ml, 11.8 mmol) and the mixture stirred in an atmosphere of dry nitrogen. After 30 min pentafluorophenol (2.5 g, 13.6 mmol) was added along with triethylamine (1.65 ml, 11.8 mmol) and the solution stirred overnight. the solvent was removed *in vacuo* and the residue purified by wet flash chromatography using 50% DCM/hexane as eluent. Fractions of R_f 0.47 were combined and the solvent removed *in vacuo* to leave a foam, which gave a white solid (3.91 g, 52%) on trituration with ether.

m.p. 167-169°C (decomp); Found: C, 70.8; H, 4.00. $\text{C}_{38}\text{H}_{23}\text{O}_4\text{F}_5$ requires C, 71.5; H, 3.61%; TLC: R_f (E) 0.87, R_f (D) 0.47, R_f (B) 0.66; δ_{H} (200 MHz, CDCl_3): 8.95 (6H, br s, aromatic), 8.68-7.17 (12H, br m, aromatic), 5.75 (2H, br s, CH_2), 3.40 (3H, s, CH_3); δ_{C} (50 MHz, CDCl_3): 150.9 (C=O), 143.5, 140.0, 138.5, 134.5, 131.1,

130.5, 129.7 (quaternary aromatic C's), 129.1, 128.0, 127.3, 126.6, 126.2, 122.9, 122.2 (aromatic CH's), 69.9 (br, CH₂), 51.2 (br, CH₃); ν_{\max} (DCM): 2957 (CH), 1789 (C=O), 1525, 1239, 986 cm⁻¹; λ_{\max} (DCM): 301 (24200 dm³mol⁻¹cm⁻¹), 289 (21700), 255 nm (116000); m/z (FAB): 639 (M⁺), 608.

N-(2-Methoxy-2-(bis-phenanthren-9-yl)ethoxycarbonyl)butylamine (79)

2-Methoxy-2-(bis-phenanthren-9-yl)ethyl pentafluorophenyl carbonate (0.50 g, 0.785 mmol) was taken in DCM (5 ml) and n-butylamine (0.233 mmol) added. The mixture was stirred for 5 min then the organic layer extracted with water (3 x 10 ml), washed with 2m KHSO₄ and dried (MgSO₄). The solvent was removed *in vacuo* and the residue triturated with ether to give a white solid (0.31 g, 75%).

m.p. 112-118°C ; Found: C, 80.8; H, 6.52; N, 2.50. C₃₆H₃₃NO₃ requires C, 82.0; H, 6.26; N, 2.66%; TLC: R_f (E) 0.29; δ_{H} (200 MHz, CDCl₃): 8.66 (6H, d J=8.7 Hz, aromatic), 7.96-7.22 (12H, m, aromatic), 5.36 (2H, br s, CH₂), 4.39 (1H, br t, NH), 3.25 (3H, s, OCH₃), 3.04-2.95 (2H, m, α -CH), 1.29-0.92 (7H, m, 2xCH₂, CH₃); ν_{\max} (DCM): 3449 (NH), 1724 (urethane C=O), 1520, 1221, 1128 cm⁻¹; λ_{\max} (DCM): 302 (24400 dm³mol⁻¹cm⁻¹), 257 nm (114000); m/z (FAB): 496 (M-OMe), 411, 397, 379.

N^α-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-methionine t-butyl ester, Tbfmoc-L-Met-O^tBu

L-Methionine t-butyl ester¹⁴³ (2.0 g, 9.85 mmol) was dissolved in glacial acetic acid (4.0 ml). The solution was lyophilised and the residue washed with a little ether to give the acetate salt as a white solid (2.08 g, 80%).

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl-*p*-nitrophenyl carbonate (0.5 g, 0.891 mmol) and L-methionine t-butyl ester acetate (0.28 g, 1.06 mmol) were dissolved in dichloromethane (20 ml). N,N-Dimethylaniline (0.22 g, 1.74 mmol) was added and

the mixture stirred at room temperature in an atmosphere of dry nitrogen for 4 days. Water (30 ml) was then added and the mixture acidified to pH1 with 2M KHSO₄. The mixture was extracted with dichloromethane (3 x 20 ml), the combined extracts washed with water (30 ml), dried (MgSO₄) and the solvent removed *in vacuo* to leave an orange oil. The oil was taken up in ether and the product precipitated by the slow addition of n-hexane. Recrystallisation from ether/n-hexane gave the *title compound* as an off-white solid (0.219 g, 39%).

m.p. 148.5-150°C; Found: C, 75.9; H, 5.90; N, 2.27. C₄₀H₃₇NO₄S requires C, 76.6; H, 5.90; N, 2.23%; TLC: R_f (B) 0.12; [α]_D²⁵ -13.8° (c=1, DCM); δ_H(200 MHz, CDCl₃): 8.83-8.64 (6H, m, aromatic), 8.33 (2H, d, J=7.8 Hz, aromatic), 7.75-7.57 (8H, m aromatic), 5.31-5.18 (2H, m, CH and NH), 4.60 (2H, m, CH₂), 4.32 (1H, m, CH), 2.40 (2H, m, CH₂), 2.03 (3H, s, SMe), 1.85 (2H, m, β CH₂), 1.45 (9H, s, t-Bu); δ_C(50 MHz, CDCl₃): 170.7, (CO, ester), 155.5 (CO, urethane), 142.1, 141.4, 136.6, 131.3, 128.5, 127.7 (aromatic quaternary C's), 127.2, 126.7, 125.9, 125.7, 125.3, 125.0, 124.8, 123.3, 123.0 (aromatic CH's), 82.1 (C-Me₃), 68.7 (CH₂), 53.4 (CH), 47.3 (CH), 32.0 (CH₂), 29.5 (CH₂), 27.8 (CH₃ x 3), 15.2 (S-CH₃); ν_{max}(DCM): 3410 (urethane NH), 3040 (aromatic CH), 2960, 2920 (aliphatic CH), 1720 (C=O), 1610 (aromatic C=C), 1500, 1215, 1155, 1050 cm⁻¹; λ_{max}(DCM): 380 (18800 dm³mol⁻¹cm⁻¹), 375 (20500), 300 (24200), 288 (38800), 260 (74100), 252 (79800); m/z (FAB): 627 (M⁺), 379; HRMS: 627.24428, C₄₀H₃₇NO₄S requires 627.24431.

N^α-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-methionine,

Tbfmoc-L-Met-OH

N^α-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-methionine t-butyl ester, Tbfmoc-L-Met-O^tBu (0.2 g, 0.319 mmol) was dissolved in trifluoroacetic acid (10 ml), together with ethyl methyl sulphide (0.2 ml), ethanedithiol (0.2 ml) and

thioanisole (0.2 ml). The mixture was stirred under nitrogen for 2 h then the solvent removed *in vacuo* and the residue dissolved in ethyl acetate. n-Hexane was added slowly and, after chilling overnight, the *title compound* precipitated as an off-white solid (0.155 g, 85%), m.p. 167-171°C (decomp); TLC: R_f (F) 0.51; HPLC: column A, gradient 4 R_t 27.5 min; v_{max}(DCM): 3410 (urethane NH), 3080 (aromatic CH), 2920 (aliphatic CH), 1720 (C=O), 1610 (aromatic C=C), 1510, 1050 cm⁻¹; λ_{max}(DCM): 382 (14800 dm³mol⁻¹cm⁻¹), 376 (16200), 302 (40500), 290 (34800), 254 (66700); m/z (FAB): 571 (M⁺), 379; HRMS: 571.18173, C₃₆H₂₉NO₄S requires 571.18172

N^α-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-leucine t-butyl ester, Tbfmoc-L-Leu-O^tBu

L-Leucine t-butyl ester (0.2 g, 0.894 mmol) hydrochloride was taken in ethyl acetate (5 ml) and triethylamine (0.125 ml; 0.894 mmol) added. The mixture was stirred under an atmosphere of dry nitrogen overnight and the precipitated triethylamine hydrochloride filtered off and washed with ethyl acetate (5 ml). The solvent was removed from the combined filtrate *in vacuo* and the residue dissolved in glacial acetic acid (2 ml) then lyophilised. The residue was triturated with ether to give leucine t-butyl ester acetate as a white solid (0.1 g).

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl-*p*-nitrophenyl carbonate (0.2 g, 0.357 mmol) and L-leucine t-butyl ester acetate (0.097 g, 0.393 mmol) were dissolved in dichloromethane (8 ml). N,N-Dimethylaniline (0.086 ml, 0.71 mmol) was added and the mixture stirred at room temperature in an atmosphere of dry nitrogen for 7 days. Water (10 ml) was then added and the mixture acidified to pH1 with 2M KHSO₄. The mixture was extracted with dichloromethane (3 x 10 ml), the combined extracts washed with water (10 ml), dried (MgSO₄) and the solvent removed *in vacuo* to leave an orange oil. The product was purified by dry flash chromatography

(DCM/MeOH). Fractions of R_f 0.10 (E) were combined and the solvent removed *in vacuo*. The residue was triturated with n-hexane to give the *title compound* as an off-white solid (0.075 g, 34%)

m.p. 151-154°C (decomp); Found: C, 79.7; H, 6.30; N, 2.30. $C_{41}H_{39}NO_4$ requires C, 80.8; H, 6.40; N, 2.30%; TLC: R_f (F) 0.81, R_f (E) 0.10; $[\alpha]_D^{25}$ -36.4° (c=0.25, DCM); δ_H (360 MHz, $CDCl_3$): 8.81-8.76 (4H, m, aromatic), 8.66 (2H, d, J=8.2 Hz, aromatic), 8.35 (2H, t, J=8.6 Hz, aromatic), 7.70-7.59 (8H, m, aromatic), 5.28 (1H, t, J=5.9 Hz, ring CH), 4.95 (1H, d, J=8.8 Hz, NH), 4.59 (2H, br s, CH_2), 4.27-4.23 (1H, m, α -CH), 1.62-1.50 (3H, m, γ -CH & β - CH_2), 1.45 (9H, s, 3 x CH_3), 0.92-0.89 (6H, m, 2 x CH_3); ν_{max} (DCM): 3415 (urethane NH), 3030 (aromatic CH), 2980-2875 (aliphatic CH), 1720 (C=O), 1610 (aromatic C=C), 1510 cm^{-1} ; λ_{max} (DCM): 382 (17600 $dm^3mol^{-1}cm^{-1}$), 367 (18900), 302 (45300), 290 (35500), 254 (70400); m/z (FAB): 609 (M^+), 378; HRMS: 609.28784, $C_{41}H_{39}NO_4$ requires 609.28789.

N^α -(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-isoleucine t-butyl ester, Tbfmoc-L-Ile-O^tBu

L-Isoleucine t-butyl ester (0.5 g, 2.24 mmol) hydrochloride was taken in ethyl acetate (15 ml) and triethylamine (0.313 ml; 2.24 mmol) added. The mixture was stirred under an atmosphere of dry nitrogen for 1 h and the precipitated triethylamine hydrochloride filtered off and washed with ethyl acetate (5 ml). The solvent was removed from the combined filtrate *in vacuo* and the residue dissolved in glacial acetic acid then lyophilised. The residue was triturated with n-hexane to give isoleucine t-butyl ester acetate as a white solid (0.405 g, 73%).

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl-*p*-nitrophenyl carbonate (0.5 g, 0.891 mmol) and L-isoleucine t-butyl ester acetate (0.24 g, 0.984 mmol) were dissolved in dichloromethane (20 ml). N,N-Dimethylaniline (0.217 ml, 1.78 mmol) was added

and the mixture stirred at room temperature in an atmosphere of dry nitrogen for 4 days. Water (20 ml) was then added and the mixture acidified to pH1 with 2M KHSO_4 and the mixture extracted with dichloromethane (3 x 20 ml). The combined extracts were washed with water (20 ml), dried (MgSO_4) and the solvent removed *in vacuo* to leave an orange oil. The residue was taken up in ether and the product precipitated by the addition of n-hexane to give the *title compound* as a pale orange solid (0.25 g, 46%).

m.p. 159-161°C (decomp); Found: C, 79.7; H, 6.16; N, 2.30. $\text{C}_{41}\text{H}_{39}\text{NO}_4$ requires C, 80.8; H, 6.40; N, 2.30%; TLC: R_f (F) 0.81, R_f (E) 0.10; $[\alpha]_D^{25} +3.0^\circ$ (c=0.5, DCM); δ_{H} (360 MHz, CDCl_3): 8.79 (4H, t J=8.7 Hz, aromatic), 8.67 (2H, d, J=8.2 Hz, aromatic), 8.39-8.33 (2H, m, aromatic), 7.78-7.59 (8H, m, aromatic), 5.32 (1H, t, J=6.2 Hz, ring CH), 5.15 (1H, d, J=9.1 Hz, NH), 4.58 (2H, t J=5.7 Hz, CH_2), 4.24-4.20 (1H, m, α -CH), 1.46 (9H, s, 3 x CH_3), 1.43-1.38 (2H, m, CH_2), 0.93-0.85 (6H, m, 2 x CH_3); δ_{C} (50 MHz, CDCl_3): 170.7, (CO, ester), 155.8 (CO, urethane), 142.1, 141.8, 136.6, 131.4, 130.2, 128.6, 127.8 (aromatic quaternary C's), 127.3, 126.8, 126.6, 125.7, 125.5, 124.8, 123.4, 123.0 (aromatic CH's), 81.7 (C-Me₃), 68.9 (CH_2), 58.4 (CH), 47.5 (CH), 27.9 (CH_3 x 3), 24.8 (CH_2), 15.2 (CH_3), 11.6 (CH_3); ν_{max} (DCM): 3415 (urethane NH), 3030 (aromatic CH), 2980-2875 (aliphatic CH), 1720 (C=O), 1610 cm^{-1} (aromatic C=C); λ_{max} (DCM): 382 (16700 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 367 (18300), 302 (44200), 290 (35100), 262 (67000), 254 (70000); m/z (FAB): 609 (M^+), 379; HRMS: 609.28784, $\text{C}_{41}\text{H}_{39}\text{NO}_4$ requires 609.28789.

**N^α -(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-alanine t-butyl ester,
Tbfmoc-L-Ala-O^tBu**

L-Alanine t-butyl ester (0.44 g, 2.42 mmol) hydrochloride was taken in ethyl acetate (13 ml) and triethylamine (0.338 ml, 2.42 mmol) added. The mixture was stirred for 3 h and the precipitated triethylamine hydrochloride filtered off and washed with

ethyl acetate (5 ml). The solvent was removed from the combined filtrate *in vacuo* to leave L-alanine t-butyl ester.

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl-*p*-nitrophenyl carbonate (0.5 g, 0.891 mmol) and L-alanine t-butyl ester (0.142 g, 0.98 mmol) were dissolved in dichloromethane (20 ml) along with acetic acid (0.056 ml, 0.98 mmol). *N,N*-Dimethylaniline (0.217 ml, 1.78 mmol) was added and the mixture stirred at room temperature in an atmosphere of dry nitrogen for 7 days. The solvent was then removed *in vacuo* to leave an orange oil and the product purified by dry flash chromatography using DCM as eluent. Fractions of R_f 0.12 (E) were combined and the solvent removed *in vacuo*. The residue was triturated with ether to give the *title compound* as a white solid (0.210 g, 42%)

m.p. 148-150°C (decomp); Found: C, 80.1; H, 5.82; N, 2.50. $C_{38}H_{33}NO_4$ requires C, 80.4; H, 5.82; N, 2.47%; TLC: R_f (E) 0.12; $[\alpha]_D^{25} +2.0^\circ$ (c=0.5, DCM); δ_H (360 MHz, $CDCl_3$): 8.78 (4H, t J=8.5 Hz, aromatic), 8.66 (2H, d J=8.2 Hz, aromatic), 8.35-8.31 (2H, m, aromatic), 7.77-7.59 (8H, m, aromatic), 5.28 (1H, m, ring CH), 5.15 (1H, d J=7.7 Hz, NH), 4.62-4.54 (2H, m, CH_2), 4.25-4.21 (1H, m, α -CH), 1.46 (9H,s, t-Bu), 1.31 (3H, d J=7.1 Hz, CH_3); δ_C (50 MHz, $CDCl_3$): 171.8 (CO, ester), 155.3 (CO, urethane), 142.1, 141.6, 136.6, 131.3, 130.1, 128.6, 127.8 (aromatic quaternary C's), 127.2, 126.7, 125.9, 125.4, 125.1, 124.8, 123.3, 123.0 (aromatic CH's), 81.7 (C-Me₃), 68.7 (CH_2), 49.8 (CH), 47.3 (CH), 27.7 (CH_3 x 3), 18.7 (CH_3); ν_{max} (DCM): 3415 (urethane NH), 3030 (aromatic CH), 2980-2885 (aliphatic CH), 1720 (C=O), 1610 (aromatic C=C), 1500 cm^{-1} ; λ_{max} (DCM): 380 (17900 $dm^3mol^{-1}cm^{-1}$), 365 (19200), 300 (45900), 288 (36200), 253 (74240); m/z (FAB): 567 (M⁺), 379; HRMS: 567.24094, $C_{38}H_{33}NO_4$ requires 567.24095.

**N^α-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-valine t-butyl ester,
Tbfmoc-L-Val-O^tBu**

L-Valine t-butyl ester hydrochloride (0.5 g, 2.38 mmol) was taken in ethyl acetate (15 ml) and triethylamine (0.333 ml; 2.38 mmol) added. The mixture was stirred under an atmosphere of dry nitrogen for 3 h and the precipitated triethylamine hydrochloride filtered off and washed with ethyl acetate (5 ml). The solvent was removed from the combined filtrate *in vacuo* and the residue dissolved in glacial acetic acid (2 ml) then lyophilised. The residue was triturated with n-hexane to give valine t-butyl ester acetate as a white solid (0.424 g, 81%).

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl-*p*-nitrophenyl carbonate (0.5 g, 0.891 mmol) and L-valine t-butyl ester acetate (0.23 g, 0.98 mmol) were dissolved in dichloromethane (20 ml). N,N-Dimethylaniline (0.217 ml, 1.78 mmol) was added and the mixture stirred at room temperature in an atmosphere of dry nitrogen for 7 days. Water (20 ml) was added, the mixture acidified to pH1 with 2M KHSO₄ and extracted with DCM (3 x 20 ml). The combined extracts were dried (MgSO₄) and the solvent removed *in vacuo* to leave an orange oil. The product was purified by dry flash chromatography using DCM as eluent. Fractions of R_f 0.14 (E) were combined and the solvent removed *in vacuo*. The residue was triturated with ether and the precipitated solid recrystallised from DCM/n-hexane to give the *title compound* as a white solid (0.297 g, 56%).

m.p. 159-163°C (decomp); Found: C, 81.0; H, 6.44; N, 2.37. C₄₀H₃₇NO₄ requires C, 80.7; H, 6.22; N, 2.35%; TLC: R_f (E) 0.14; [α]_D²⁵ -3.7° (c=1, DCM); δ_H(360 MHz, CDCl₃): 8.79-8.74 (6H, m, aromatic), 8.66-8.63 (2H, m, aromatic), 7.79-7.58 (8H, m, aromatic), 5.23-5.17 (2H, m, ring CH & NH), 4.61-4.49 (2H, m, CH₂), 4.24-4.20 (1H, m, α-CH), 2.17-2.11 (1H, m, CH), 1.49 (9H, s, t-Bu), 0.93 (3H, d J=6.9 Hz, CH₃), 0.81 (3H, d J=6.9 Hz, CH₃); δ_C(50 MHz, CDCl₃): 170.8 (CO, ester), 156.0 (CO, urethane), 142.0, 141.8, 136.5, 131.3, 130.1, 128.6, 127.8

(aromatic quaternary C's), 127.3, 126.7, 126.6, 125.9, 125.7, 125.5, 125.2, 124.8, 123.3, 123.0 (aromatic CH's), 81.7 (C-Me₃), 69.0 (CH₂), 59.0 (CH), 47.5 (CH), 31.2 (CH), 27.9 (CH₃ x 3), 18.7 (CH₃), 17.1 (CH₃); ν_{\max} (DCM): 3410 (urethane NH), 3060 (aromatic CH), 2960 (aliphatic CH), 1720 (C=O), 1610 (aromatic C=C), 1510 cm⁻¹; λ_{\max} (DCM): 380 (18800 dm³mol⁻¹cm⁻¹), 365 (20200), 300 (48600), 288 (38500), 253 (78000); m/z (FAB): 596 (MH⁺), 595 (M⁺), 379; HRMS: 595.27223, C₄₀H₃₇NO₄ requires 595.27224.

N^α-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-valine,

Tbfmoc-L-Val-OH

N^α-(17-Tetrabenzo[*a,c,g,i*]fluorenylmethoxycarbonyl)-L-valine t-butyl ester, Tbfmoc-L-Val-O^tBu (0.5 g, 0.84 mmol) was taken in TFA (9.5 ml)/H₂O (0.5 ml) and stirred for 1 h. The solvent was then removed *in vacuo* and the residue triturated with ether to give the *title compound* as an off-white solid (0.408 g, 90%).

m.p. 186-190°C (decomp); Found: C, 79.8; H, 5.65; N, 2.55. C₃₆H₂₉NO₄ requires C, 80.2; H, 5.38; N, 2.60%; TLC: R_f (F) 0.58; [α]_D²⁵ +40.5° (c=1, dioxane); δ_H(360 MHz, dioxane d-8): 9.06-8.99 (4H, m, aromatic), 8.83 (2H, d J=7.8 Hz, aromatic), 8.66-8.62 (2H, m, aromatic), 7.97-7.75 (8H, m, aromatic), 6.56 (1H, d J=9.2 Hz, NH), 5.66-5.60 (1H, t J=5.8 Hz, ring CH), 4.91-4.82 (2H, m, CH₂), 4.74-4.65 (1H, m, α-CH), 2.24-2.14 (1H, m, β-CH), 1.01-0.93 (6H, m, 2 x CH₃); ν_{\max} (DCM): 3504 (OH), 3455 (NH), 3068 (aromatic CH), 2984 (aliphatic CH), 1728 (C=O), 1609 (aromatic C=C), 1518 cm⁻¹; λ_{\max} (DCM): 382 (17300 dm³mol⁻¹cm⁻¹), 367 (18700), 302 (45100), 289 (35900), 260 (64700), 252 (70100); m/z (FAB): 540 (MH⁺), 379; HRMS: 539.20968, C₃₆H₂₉NO₄ requires 539.20965.

Dipentafluorophenyl carbonate

Triphosgene (1.35 g, 4.55 mmol) was taken in ether (100 ml) with pentafluorophenol (4.0 g, 21.7 mmol) and cooled on an ice-salt bath. Triethylamine (4.08 ml, 29.3 mmol) was added and the mixture allowed to warm to room temperature over 30 min. The precipitated solid was filtered off and discarded. The solvent was removed *in vacuo* and the residue recrystallised from n-hexane to give the *title compound* as white crystals (3.04 g, 71%).

m.p. 48-50°C (lit¹⁵³ 48-50°C); ν_{\max} (DCM): 1810 (carbonate C=O), 1531, 1220, 1011 cm^{-1}

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl pentafluorophenyl carbonate (85)

Dipentafluorophenyl carbonate (1.0 g, 2.54 mmol) was taken in DCM (20 ml) with 17-tetrabenzo[*a,c,g,i*]fluorenylmethanol (1.06 g, 2.54 mmol) in an atmosphere of dry nitrogen and cooled to 0°C. Pyridine (0.204 ml, 2.54 mmol) was added and the solution stirred for 2.5 h. Water (20 ml) was added and the mixture acidified to pH 1 with 2M KHSO₄. The organic layer was separated and washed with water (2 x 10 ml), dried (MgSO₄), the solvent removed *in vacuo* and the residue purified by dry flash chromatography using DCM/n-hexane as eluent. Fractions containing material of R_f 0.46 (D) were combined, the solvent removed *in vacuo* and the residue triturated with ether to give the *title compound* as a pale yellow solid (0.97 g, 60%).

m.p. 186-189°C; Found: C, 73.4; H, 3.37. C₃₇H₁₉O₃F₅ requires C, 73.3 H, 3.14%; TLC: R_f (D) 0.46, R_f (E) 0.75; δ_{H} (200 MHz, CDCl₃): 8.80-8.76 (4H, m, aromatic), 8.62 (2H, d J=7.8 Hz, aromatic), 8.23-8.18 (2H, m, aromatic), 7.77-7.58 (8H, m aromatic), 5.22 (1H, t J=5.5 Hz, CH), 4.86 (2H, d J=5.5 Hz, CH₂); δ_{C} (50 MHz, CDCl₃): 150.8, (CO), 143.5, 143.2, 140.2, 140.1, 139.7, 138.4, 138.2, 136.9, 134.9, 131.4, 130.3, 128.0 (aromatic quaternary C's), 127.5-123.3 (aromatic CH's), 74.5 (CH₂), 45.2 (CH); δ_{F} (75 MHz, CDCl₃): -152.4- -153.2 (m), -156.8 - -157.3 (m),

-161.2-162.0 (m); ν_{\max} (DCM): 3080 (aromatic CH), 2935, 2920 (aliphatic CH), 1780 (carbonate C=O) 1610 (aromatic C=C), 1527 cm^{-1} ; λ_{\max} (DCM): 366 (ϵ 19200 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 301 (45500), 289 (36900), 261 (67700), 253 nm (72200); m/z (FAB): 606 (M^+), 379, 378, 363; HRMS: 606.12541, $\text{C}_{37}\text{H}_{19}\text{O}_3\text{F}_5$ requires 606.12542.

(17-Tetrabenzo[*a,c,g,i*]fluorenyl)methyl chloroformate (70)

17-Tetrabenzo[*a,c,g,i*]fluorenylmethanol (1.0 g, 2.52 mmol) was taken in DCM (20 ml) along with triphosgene (0.5 g, 1.68 mmol) and *N,N*-dimethylaniline (0.64 ml, 5.04 mmol) added. The mixture was stirred for 45 min and the precipitated solid filtered off and recrystallised from DCM/hexane to give the *title compound* as a pale yellow solid (0.577 g). A further crop of product was obtained from the DCM filtrate after adding water, acidifying to pH1 with 2M HCl, drying the organic layer (MgSO_4), removing the solvent and recrystallising the residue from DCM/*n*-hexane. Total yield (0.819 g, 71%).

m.p. 194-195°C (lit¹³⁰ 188-189°C); Found: C, 81.3; H, 4.22; Cl, 7.66. $\text{C}_{31}\text{H}_{19}\text{O}_2\text{Cl}$ requires C, 81.1; H, 4.14; Cl 7.74%; TLC: R_f (E) 0.75; δ_{H} (200 MHz, CDCl_3): 8.80-8.75 (6H, m, aromatic), 8.63 (2H, d $J=8.0$ Hz, aromatic), 8.20-7.57 (8H, m, aromatic), 5.20 (1H, t $J=5.8$ Hz, CH), 4.78 (2H, d $J=5.8$ Hz, CH_2); δ_{C} (90 MHz, dioxane *d*-8): 149.5, (CO), 140.9, 136.7, 131.4, 130.3, 128.2, 127.6 (aromatic quaternary C's), 127.1, 126.6, 125.9, 125.8, 124.8, 124.5, 123.4 (aromatic CH's), 74.3 (CH_2), 46.3 (CH); ν_{\max} (DCM): 3069 (aromatic CH), 2980 (aliphatic CH), 1776 (carbonate C=O) 1611 (aromatic C=C), 1504, 1142 cm^{-1} ; λ_{\max} (DCM): 368 (ϵ 17600 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 302 (43000), 290 (33800), 254 nm (66200); m/z (FAB): 460, 458 (M^+); HRMS: 458.10737, $\text{C}_{31}\text{H}_{19}\text{O}_2\text{Cl}$ requires 458.10735.

Methyl 2-bromobenzoate¹⁶⁰ (94)

N-Bromosuccinimide (130.0 g, 0.73 mol) and a catalytic amount of dibenzoyl peroxide were added to a solution of methyl 2-methylbenzoate (100.0 g, 0.66 mol) in carbon tetrachloride (1.0 l). The mixture was heated under reflux and irradiated with a 500 W Halogen lamp for 2 h, switching the lamp off periodically to control the vigour of the reaction. The cooled mixture was then filtered and the filtrate concentrated *in vacuo* to give the *title compound* as a yellow oil (191.9 g, overweight), which was used without further purification.

2-(Methoxycarbonyl)benzyltriphenylphosphonium bromide¹⁶⁰ (95)

Methyl 2-bromobenzoate (191.9 g) was dissolved in toluene (1.0 l) together with triphenylphosphine (173.2 g, 0.66 mol) and the mixture stirred overnight. The resulting precipitate was filtered off, washed with toluene and recrystallised from DCM/toluene to give the *title compound* as a white solid (253.1 g, 78%), m.p. 234-238 °C (lit¹⁶⁰ 230-235°C).

2-(3'-Methoxyphenylethenyl)benzoic acid (101)

2-(Methoxycarbonyl)benzyltriphenylphosphonium bromide (200.0 g, 0.41 mol) and *m*-anisaldehyde (58.4 g, 0.43 mol) were stirred in dioxane (700 ml) under an atmosphere of dry nitrogen. A solution of DBU (64.4 g, 0.42 mol) in dioxane (100 ml) was added and the mixture stirred at 50°C for 3 h. After cooling, the precipitated DBU hydrobromide was filtered off and washed with dioxane (400 ml). Water (320 ml) then aqueous 2M NaOH (400 ml) were added to the filtrate and the mixture was heated under reflux for 45 min. The solution was then concentrated *in vacuo*, water (1.8 l) added and the precipitated solid filtered off and washed with water (200 ml). The combined filtrate was washed with ethyl acetate (2 x 500 ml) and ice added to the aqueous layer, which was then acidified to pH1 with 2M aqueous HCl. A white

solid precipitated which was filtered off, taken up in ether, dried (MgSO_4) and the solvent removed *in vacuo* to give the *title compound* as a white solid (39.7 g). Further product was obtained by extraction of the aqueous filtrate with ether (3 x 300 ml). The ethereal extract was dried (MgSO_4), and the solvent removed *in vacuo* to give an orange oil, which, on trituration with n-hexane gave the acid as a white solid (40.1 g), total yield (79.8 g, 77%). Found: C, 75.5; H, 5.63; $\text{C}_{16}\text{H}_{14}\text{O}_3$ requires C, 75.6; H, 5.51; R_f (C) 0.17; δ_{H} (200 MHz, d_6 -DMSO): 13.06 (1H, br s, COOH), 8.11-6.84 (10H, m, aromatic & alkene CH's), 3.77 & 3.52 (3H, s, cis & trans OCH_3); δ_{C} (50 MHz, d_6 -DMSO): 169.1 & 168.4 (COOH, cis & trans), 159.9 & 159.2 (quaternary aromatic C-OMe, cis & trans), 139.0-112.5 (aromatic C's & alkene C's), 55.2 & 54.8 (CH_3 , cis & trans); ν_{max} (DCM): 3504 (OH), 3060-2436 (OH), 1728 (C=O), 1688 (C=O), 1609 (C=C), 1518 cm^{-1} ; λ_{max} (MeOH): 302 (ϵ 19100 $\text{dm}^3\text{mol}^{-1}\text{cm}^{-1}$), 215 nm (19600); m/z(EI): 254, 194, 165.

2-(3'-Methoxyphenylethyl)benzoic acid (98)

2-(3'-Methoxyphenylethenyl)benzoic acid (100.0 g, 0.39 mol) was dissolved in methanol (1.0 l). 10% Pd/C (5.0 g) was added whilst the solution was cooled on an ice bath and stirred under an atmosphere of dry nitrogen. The mixture was then gently warmed to redissolve precipitated acid and, after allowing to cool to room temperature, was hydrogenated for 8 h. Ethyl acetate was then added to dissolve the precipitated product and the catalyst was filtered off on celite. The celite was washed with ethyl acetate to recover any undissolved product then the solvent was removed *in vacuo* and the residue recrystallised from methanol to give the *title compound* as a white solid (96.0 g, 95%).

m.p. 119-121°C (lit¹⁶⁰ 120-120.5°C); Found: C, 75.0; H, 6.33. $\text{C}_{16}\text{H}_{16}\text{O}_3$ requires C, 75.0; H, 6.30%; R_f (C) 0.26; δ_{H} (80 MHz, CDCl_3): 11.70 (1H, s, exchanges with D_2O , -COOH); 8.18-6.70 (8H, m, aromatic); 3.79 (3H, s, - OCH_3); 3.50-2.86 (4H, m,

-CH₂- x 2); δ_{C} (50 MHz, d₆-DMSO): 169.2 (COOH), 159.6 (quaternary aromatic, C-OMe), 143.7, 143.1 (quaternary aromatic C's) 131.9, 131.2 (aromatic CH's), 130.8 (quaternary aromatic), 130.6, 129.5, 126.4, 120.9, 114.2, 111.5 (quaternary aromatic CH's), 55.0 (CH₃), 37.9 (CH₂), 36.4 (CH₂); ν_{max} (CH₂Cl₂): 3500 & 3400-2400 (OH), 1749 & 1695 (C=O), 1600 cm⁻¹ (C=C); λ_{max} (MeOH): 280 (ϵ 5180 dm³mol⁻¹cm⁻¹), 273 (4970), 209 nm (35200); m/z(EI): 256, 238, 121.

2-Methoxydibenzocycloheptadien-5-one (99)

2-(3'-Methoxyphenylethyl)benzoic acid (1.5 g, 5.9 mmol) was taken in dry benzene (3 ml) and oxalyl chloride (1.5 ml) added while the mixture was cooled on an ice/salt bath. The mixture was stirred at room temperature under an atmosphere of dry nitrogen for 2 h, after which time the acid had dissolved and gas evolution had ceased. Excess oxalyl chloride and benzene were removed *in vacuo* and the residue taken up in dry benzene (1.5 ml). The benzene solution was then added to a stirred, cooled solution of tin (IV) chloride (3.2 g) in benzene (1.5 ml) over a period of 15 min. Ice, then concentrated HCl (3 ml) were added and the mixture heated until the precipitated solid had dissolved. After cooling, the organic layer was separated, taken up in ether, washed with water then brine and dried (MgSO₄). The solvent was removed *in vacuo* to leave a brown oil which was triturated with n-hexane to give a pale brown solid. Recrystallisation from ether/n-hexane gave the *title compound* (1.05 g, 76%).

m.p. 75-76°C (lit¹⁶⁰ 75.5-76.0°C); Found: C, 80.3; H, 5.93. C₁₆H₁₄O₂ requires C, 80.6; H, 5.9%; R_f (E) 0.46, R_f (A) 0.28); δ_{H} (360 MHz, CDCl₃): 8.16 (1H, d, J=8.9 Hz, aromatic); 8.01 (1H, dd, J=7.7, 1.5 Hz, aromatic); 7.41-7.25 (2H, m, aromatic); 7.18 (1H, dd, J=7.5, 1.1 Hz, aromatic); 6.84 (1H, dd, J=8.9, 2.6 Hz, aromatic); 6.68 (1H, d, J=2 Hz); 3.83 (3H, s, OMe); 3.14 (4H, s, -CH₂ x 2); δ_{C} (50 MHz, CDCl₃): 192.8 (CO), 162.5 (quaternary aromatic, C-OMe), 144.9, 141.3, 138.7 (quaternary

aromatic C's), 133.5, 131.8, 130.3, 128.4, 126.3, 113.9, 111.9 (aromatic CH's), 55.0 (CH₃), 35.5 (CH₂), 34.4 (CH₂); $\nu_{\max}(\text{CH}_2\text{Cl}_2)$: 1640 (C=O), 1600 cm⁻¹ (C=C); $\lambda_{\max}(\text{MeOH})$: 302 (ϵ 13300 dm³mol⁻¹cm⁻¹), 238 (8300), 209 nm (14900); m/z (EI): 238, 210, 195, 165.

2-Hydroxydibenzocycloheptadien-5-one (100)

2-(3'-Methoxyphenylethyl)benzoic acid (30 g, 0.12 mol) was taken in dry benzene (60 ml) together with oxalyl chloride (30 g, 0.24 mol) and the mixture stirred in an atmosphere of dry nitrogen for 3.5 h, after which time the acid had dissolved and gas evolution had ceased. The benzene and excess oxalyl chloride were removed *in vacuo*, the residue taken up in dry benzene (125 ml) and a suspension of anhydrous aluminium chloride (45 g, 0.34 mol) in dry benzene (125 ml) slowly added whilst cooling and stirring on an ice bath. The mixture was heated under reflux for 30 min then cooled on an ice/salt bath and 2M HCl (300 ml) slowly added with mixing. The precipitated solid was filtered off, taken up in ether and the ethereal solution filtered to remove the dark insoluble solid. The filtrate was washed with water (100 ml) then dried (MgSO₄) and the solvent removed *in vacuo*. The residue was recrystallised from ether/n-hexane to yield pale needles (23.2 g). A further 0.7 g of product was obtained from the benzene layer of the acidified reaction mixture filtrate by extracting with 2M NaOH (2 x 100 ml), acidifying the extract to pH1 with 2M HCl and extracting with ether. The additional product was obtained from the ethereal solution similarly to the main fraction of product. Total yield 23.9 g (91 %).

m.p. 141-142°C (lit¹⁶⁰ 141-141.5°C); Found: C, 80.4; H, 5.38. C₁₅H₁₂O₂ requires C, 80.4; H, 5.4%; R_f (A) 0.06, R_f (C) 0.31; δ_{H} (80 MHz, CDCl₃): 8.14-6.82 (7H, m, aromatic); 6.65 (1H, s, exchanges with D₂O, OH); 3.12 (4H, s, -CH₂ x 2); δ_{C} (50 MHz, CDCl₃): 192.2 (CO), 162.0 (quaternary aromatic, C-OH), 145.8, 141.9, 139.2 (quaternary aromatic C's), 134.0, 132.2, 130.5, 129.0, 126.7, 116.1, 114.4 (aromatic

CH's), 35.6 (CH₂), 34.4 (CH₂); ν_{\max} (CH₂Cl₂): 3570 (OH), 1640 (C=O), 1605 cm⁻¹ (C=C); λ_{\max} (MeOH): 302 (ϵ 16700 dm³mol⁻¹cm⁻¹), 240 (12300), 208 nm (28800); m/z (EI): 224, 196, 181, 165.

2-Copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadiene-5-one¹⁶⁰ (102)

2-Hydroxydibenzocycloheptadien-5-one (5.0 g, 22.3 mmol) was dissolved in t-butanol/water (1:1, 50 ml) along with caesium hydroxide (3.75 g, 25 mmol). The solution was stirred for 10 min then the t-butanol removed *in vacuo*. The caesium salt was dried by azeotropic distillation with pyridine (2 x 100 ml) and DMF (3 x 100 ml), then the salt was dissolved in DMF (50 ml) and added to chloromethylpolystyrene resin (10 g, 0.7 mmol Cl/g), preswollen in DMF (50 ml). The mixture was stirred for 4 days at 60°C, after which time the resin was filtered off and washed with copious DMF, isopropanol and finally DCM. The resin was dried *in vacuo* to yield 10.8 g of resin (94%).

ν_{\max} (KBr disc): 3100-3000 (Ar CH), 2920-2840 (CH), 1640 (C=O), 1600 cm⁻¹ (C=C); Found: Cl <0.7%; expected for 100% loading 0%.

2-Copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadien-5-ol¹⁶⁰ (110)

THF (100 ml) was added to the above resin (0.7 mmol/g: 5.0 g, 3.5 mmol) along with lithium borohydride (0.6 g, 27.3 mmol) and the mixture heated under reflux for 1 h under an atmosphere of dry nitrogen. The mixture was then cooled on an ice/salt bath and methanol (10 ml), followed by acetone (10 ml) slowly added with stirring. The resin was filtered off and washed with copious methanol, 0.001M HCl then methanol. The resin was dried *in vacuo* to give 4.91 g of product.

ν_{\max} (KBr disc): 3560 & 3420 (OH), 3100-3000 (Ar CH), 2940-2860 (CH), 1600 cm^{-1} (C=C).

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(9-fluorenylmethoxy-carbonyl)aminodibenzocycloheptadiene¹⁶⁰ (92)

2-Copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadiene-5-ol (0.7 mmol/g: 2.5 g, 1.75 mmol) was taken in DMF (70 ml) along with 9-fluorenylmethylcarbamate (1.7 g, 7.1 mmol). Benzenesulphonic acid (0.1 g, 0.6 mmol) was added and the mixture stirred overnight under an atmosphere of nitrogen. The resin was then filtered off, washed with copious DMF then DCM and dried *in vacuo* to leave the *title compound* as an off-white solid (2.67 g, functionality 0.57 mmol/g by uv monitoring¹⁴⁷).

ν_{\max} (KBr disc): 3420 (NH), 3100-2840 (CH), 1720-1660 (C=O), 1600 cm^{-1} (C=C);

N-t-Butyloxycarbonyl-N'-2-copoly(styrene-1%-divinylbenzene)methoxy-dibenzocycloheptadien-5-ylhydrazine¹⁶⁰ (93)

2-Copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadiene-5-ol (0.7 mmol/g: 1.5 g, 1.05 mmol) was taken in DCM (100 ml), along with t-butyloxycarbonylhydrazine (0.97 g, 7.3 mmol) and benzenesulphonic acid (0.08 g, 0.51 mmol) added. The mixture was heated under reflux overnight and the resin filtered off, washed with copious DMF, DCM and ether to leave the *title compound* as a white solid (1.55 g).

Found: N, 1.55; expected for 100% loading 1.62%; ν_{\max} (KBr disc): 3410 & 3300 (NH), 3100-2820 (CH), 1710 (C=O), 1600 cm^{-1} (C=C).

2-(Benzyloxycarbonyloxy)dibenzocycloheptadien-5-one¹⁶⁰ (103)

2-Hydroxydibenzocycloheptadiene-5-one (3.0 g, 13.4 mmol) was taken in acetone (100 ml) along with anhydrous potassium carbonate (18.5 g, 134 mmol) and benzyl-2-bromoacetate (3.09 g, 13.5 mmol) and the mixture stirred at room temperature overnight. The excess potassium carbonate was then filtered off and the filtrate concentrated *in vacuo*. The residue was taken up in ethyl acetate (50 ml) and washed with saturated sodium carbonate solution (3 x 25 ml), then water (25 ml). The organic layer was dried (MgSO₄) and the solvent removed *in vacuo* to yield a white solid which was washed with a little ether. Yield 4.45 g (89%).

m.p. 80-82°C (lit¹⁶⁰ 81.5-82°C).

2-(Oxyacetic acid)dibenzocycloheptadien-5-one¹⁶⁰ (104)

2-(Benzyloxycarbonyloxy)dibenzocycloheptadien-5-one (4.0 g, 10.8 mmol) was dissolved in methanol (50 ml) along with 2M sodium hydroxide (11 ml) and the mixture heated under reflux for 1 h. The methanol was then removed *in vacuo* and water (50 ml) added to the residue. Ice was added and the mixture was acidified to pH1 with 2M HCl. The aqueous layer was extracted with ethyl acetate (2 x 100 ml) and the organic layer dried (MgSO₄) and the solvent removed *in vacuo* to leave the title compound as a white solid (2.8 g, 93 %).

m.p. 164-166°C (lit¹⁶⁰ 164-165°C).

**2-Copoly(styrene-1%-divinylbenzene)methylaminocarbonyloxy-
dibenzocycloheptadien-5-one (106)**

2-(Oxyacetic acid)dibenzocycloheptadiene-5-one (2.97 g, 10.5 mmol) was taken in DMF (15 ml) together with diphenylphosphinic anhydride (4.39 g, 10.5 mmol) and N-methylmorpholine (1.11 g, 11.0 mmol) and the mixture stirred at room

temperature for 15 min. The mixture was then added to aminopolystyrene resin (4.5 g; 0.89 mmol NH₂/g, 4.0 mmol), preswollen in DMF, together with N-methylmorpholine (1.11 g, 11.0 mmol) and 2,6-lutidine (2.22 g, 20.7 mmol) and the mixture stirred at room temperature for 3 h, after which time the resin showed only pale blue to Kaiser test. The resin was filtered off, washed with copious DMF the DCM and dried *in vacuo* to yield a white solid (5.4 g). Quantitative Kaiser test showed only 0.003 mmol/g of unreacted NH₂.

ν_{\max} (KBr disc): 3410-3300 (NH), 3080-2840 (CH), 1680-1630 (C=O x 2), 1600 (C=C), 1530 cm⁻¹ (amide II).

2-Copoly(styrene-1%-divinylbenzene)methylaminocarboxymethoxy-dibenzocycloheptadien-5-ol (107)

THF (75 ml) was added to the above resin (3.0 g) along with lithium borohydride (0.4 g, 18.3 mmol) and the mixture heated under reflux for 1 h under an atmosphere of dry nitrogen. The mixture was then cooled on an ice/salt bath and methanol (10 ml), followed by acetone (10 ml) slowly added with stirring. The resin was filtered off and washed with copious methanol, 0.001M HCl then methanol. The resin was dried *in vacuo* to give 2.81 g of product.

ν_{\max} (KBr disc): 3530 (OH), 3420 (NH), 3080-2840 (CH), 1660 (C=O amide I), 1600 (C=C), 1530 (amide II) cm⁻¹.

2-Copoly(styrene-1%-divinylbenzene)methylaminocarboxymethoxy-5-(9'-fluorenylmethoxycarbonyl)aminodibenzocycloheptadiene (108)

2-Copoly(styrene-1%-divinylbenzene)methylaminocarboxymethoxydibenzocycloheptadiene-5-ol (2.0 g) was taken in DMF (70 ml) along with 9-fluorenylmethylcarbamate (1.7 g, 7.1 mmol). Benzenesulphonic acid (0.1 g, 0.6 mmol) was added and the mixture stirred overnight under an atmosphere of nitrogen.

The resin was then filtered off, washed with copious DMF then DCM and dried *in vacuo* to leave the *title compound* as an off-white solid (2.10 g, functionality 0.85 mmol/g by uv monitoring¹⁴⁷).

ν_{\max} (KBr disc): 3440 (NH), 3080-2820 (CH), 1690 (C=O), 1630 cm^{-1} (C=O), 1600 cm^{-1} (C=C).

N-t-Butyloxycarbonyl-N'-2-copoly(styrene-1%-divinylbenzene)methylaminocarboxymethoxydibenzocycloheptadien-5-ylhydrazine (109)

2-Copoly(styrene-1%-divinylbenzene)methylaminocarboxymethoxydibenzocycloheptadiene-5-ol (0.5 g) was taken in DCM (30 ml) along with t-butyloxycarbonylhydrazine (0.5 g, 3.8 mmol) and benzenesulphonic acid (0.03 g, 0.19 mmol) added. The mixture was heated under reflux overnight and the resin filtered off, washed with copious DMF, DCM and ether to leave the *title compound* as a white solid (0.5 g).

Found: N, 2.30; expected for 100% loading 2.52%; ν_{\max} (KBr disc): 3410 & 3310 (NH), 3080-2840 (CH), 1710-1670 (2 x C=O), 1600 cm^{-1} (C=C).

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-heptyliminodibenzocycloheptadiene (112)

2-Copoly(styrene-1%-divinylbenzene)methoxydibenzocycloheptadiene-5-one (2.0 g) was taken in DCM (40 ml) along with heptylamine (2.4 g, 20.9 mmol), cooled on an ice/salt bath and stirred under an atmosphere of dry nitrogen. A solution of 1.0M titanium (IV) chloride (3.5 ml, 3.5 mmol) was added and the mixture heated under reflux for 2.5 h. The resin was then filtered off, washed copiously with DCM and dried *in vacuo* to yield 2.19 g of pale yellow resin.

Found: N, 0.79%; expected for 100% loading, 0.81%; ν_{\max} (KBr disc): 3080-2840 (CH), 1600 cm^{-1} (C=C).

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-heptylamino-dibenzocycloheptadiene (113)

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-heptylimino-dibenzocycloheptadiene (1.5 g) was taken with lithium borohydride (0.2 g, 9.1 mmol) in THF (40 ml) and heated under reflux for 1.5 h in an atmosphere of dry nitrogen. The mixture was then cooled in an ice/salt bath and methanol (5 ml) then acetone (5 ml) carefully added. The resin was then filtered off, washed with copious methanol, water then methanol again to give 1.4 g of white solid.

ν_{\max} (KBr disc): 3420 (NH), 3080-2840 (CH), 1600 cm^{-1} (C=C).

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(9'-fluorenyl-methoxy-carbonyl-glycylaminoheptyl)-dibenzocycloheptadiene (114)

To Fmoc-Gly-OH (1.7 g, 5.8 mmol) in DCM (5 ml) was added thionyl chloride (7 ml, 68 mmol). The mixture was heated under reflux for 2 h, then cooled and concentrated *in vacuo*. DCM was added (3 x 15 ml) and the mixture concentrated a further three times, then the residue taken up in DCM (15 ml) and the solution added to 2-copoly(styrene-1%-divinylbenzene)methoxy-5-heptylamino-dibenzocycloheptadiene resin (1.2 g), preswollen in DCM (10 ml) and pyridine (2 ml). The mixture was stirred at room temperature under an atmosphere of dry nitrogen for 7 h, then the resin filtered off and washed with copious DMF and DCM to yield 1.25 g of pale yellow solid (functionality by uv monitoring¹⁴⁷, 0.15 mmol/g)

ν_{\max} (KBr disc): 3410 (NH), 3080-2840 (CH), 1730 (C=O), 1640 (C=O), 1600 cm^{-1} (C=C).

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(9'-fluorenyl-methoxy-carbonylleucylaminoheptyl)dibenzocycloheptadiene (116)

Resin (113) (0.20 g) was added to Fmoc-Leu-OH (0.20 g, 0.566 mmol) in 0.5M solution of DIC in 1:1 dioxane/DMF (0.56 ml). 1 ml of dioxane/DMF (1:1) was added and the mixture sonicated overnight. the resin was filtered off and washed copiously with DMF, DCM then ether. A UV loading test¹⁴⁷ showed 0.11 mmol/g incorporation of the amino acid. The resin was sonicated for a further 15 h with a fresh solution of Fmoc-Leu-OH/DIC then the resin filtered and washed with DMF, DCM then ether. An Fmoc UV loading test¹⁴⁷ showed an Fmoc functionality of 0.16 mmol/g.

2-Copoly(styrene-1%-divinylbenzene)methoxy-5-(9'-fluorenyl-methoxy-carbonyl-azaglycylamino)dibenzocycloheptadiene (119)

2-Copoly(styrene-1%-divinylbenzene)methylaminocarbomethoxy-5-(9'-fluorenyl-methoxycarbonyl)aminodibenzocycloheptadiene resin (1.0 g, 0.29 mmol/g; 0.29 mmol) was sonicated in 20% piperidine/DMF for 15 min then the resin was filtered off and washed copiously with DMF then DCM. A solution of triphosgene (0.086 g, 0.29 mmol) and diisopropylethylamine (0.10 ml) in DCM (10 ml) was added to the resin and nitrogen bubbled through the solution for 30 min. The resin was filtered off and washed with DCM (2 x 10 ml). 9-Fluorenylmethoxycarbonylhydrazine¹⁶⁰ (0.22 g, 0.87 mmol) in DCM (20 ml) was added and nitrogen bubbled through the solution for 1.5 h. The resin was filtered off and washed copiously with DMF then DCM. Resin functionality by uv monitoring¹⁴⁷, 0.29 mmol/g.

4.3 Peptide Synthesis

Solid phase peptide synthesis was carried out on polystyrene-1%-divinylbenzene resin, functionalised with the appropriate linker, using programmed cycles on an Applied Biosystems 430A synthesiser. The programme details are summarised below.

(1) Capping using protocol A: acetic anhydride (0.5 M in DMF, 1 ml) + pyridine (0.5 M in DMF, 1 ml) diluted to 8 ml with DMF, 2.5 and 3.7 min, with 1:1 DMF dioxane wash between; or protocol B: 8 ml 0.5 M acetic anhydride/0.125 M diisopropylethylamine/0.2% HOBt, 10 min.

(2) Removal of N^α-protection: 20% piperidine /DMF (2 x 9 ml; 3+1 min).

(3) Washing: 1:1 DMF/dioxane (6x).

(4) Activation: First coupling, symmetrical anhydride formation (2 equiv. unless stated) using DIC, 15 min*

Second coupling, HOBt active ester formation (2 equiv. unless stated) 30 min.

(5) Coupling: activated species transferred to reaction vessel, 30 min.

(6) Washing, 1:1 DMF/dioxane (5x).

*Symmetrical anhydride activation was not used for histidine, asparagine or glutamine, HOBt activation being substituted. Glycine was single coupled using 4 equiv. of symmetrical anhydride.

Unless stated, the following side-chain protecting groups were used: Arg (Pmc); Asp, Glu (t-Bu); Glp (Z); His (Bum); Lys (Boc); Ser, Thr Tyr (t-Bu).

The progress of the synthesis was monitored by passing the Fmoc deprotection effluent through a UV flow cell (302 nm), allowing a semi-quantitative analysis of the deprotection and acylation cycles

Fmoc-Gastrin-Releasing Peptide (2-27), Fmoc-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH₂

The peptide was synthesised using resin (108) (0.51 g, 0.49 mmol/g, 0.25 mmol), capping protocol A. The coupling time was extended for residues 2, 3, 9, 10 and 11: symmetrical anhydride (45 min), HOBt ester (1.5 h). Histidine was coupled as its trityl derivative (HOBt activation). After completion of the synthesis the resin was washed with DMF then DCM to give 1.66g of resin-bound Fmoc-peptide (Fmoc functionality, 0.133 mmol/g).

Tbfmoc-Gastrin-Releasing Peptide, Tbfmoc-Val-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met-NH₂

A sample of resin-bound Fmoc-Gastrin-releasing peptide (2-27), (0.133 mmol/g, 0.20 g) was taken in 20% piperidine/DMF (25 ml) and sonicated for 30 min. The resin was filtered off and washed with DMF then DCM and swollen in dioxane (1 ml). Tbfmoc-Val-OH (77 mg, 0.133 mmol, 5 equiv) was dissolved in dioxane (3 ml) along with DIC (0.021 ml, 0.133 mmol) and HOBt (18 mg, 0.133 mmol) and after sonicating for 5 minutes was added to the resin. The mixture was sonicated overnight then for a further 2.5 h with a further 5 equiv of Tbfmoc-Val-OH HOBt ester.

A sample of the resin-bound Tbfmoc-peptide (50 mg) was taken in TFA (9.5 ml)/water (0.5 ml)/EDT (0.2 ml)/EMS (0.2 ml)/thioanisole (0.2 ml)/phenol (0.5 g)

and stirred in an atmosphere of dry nitrogen for 3 h, after which time the resin was filtered off and washed with TFA (2 ml). The filtrate was concentrated *in vacuo* and the crude peptide (26 mg) precipitated with ether and filtered off.

m/z (FAB): 3281.9 (MH⁺);

HPLC: (column B, gradient 4) R_t 19.8 min.

Purification of Gastrin-Releasing peptide on PGC

Crude Tbfmoc-gastrin-releasing peptide (50.6 mg) was dissolved in 50% aqueous CH₃CN/1% TFA (30 ml) and applied to a glass column packed with PGC (50-100 μm, 68 m²/g, 3 g, 1 cm diameter), monitoring the eluent by analytical HPLC (column B, gradient 4). The column was then subjected to the following wash protocol:

1. 50% aqueous CH₃CN/0.5% TFA, (50 ml);
2. 50% aqueous CH₃CN, (2 x 50 ml);
3. 50% aqueous CH₃CN/1% piperidine, (50 ml);
4. 50% aqueous CH₃CN, (20 ml);
5. 50% aqueous CH₃CN/0.5% TFA, (50 ml);
6. 60% aqueous CH₃CN/0.5% TFA, (50 ml);
7. 70% aqueous CH₃CN/0.5% TFA (3 x 50 ml);

The product eluted in washes 5-7 and these were combined and lyophilised to give an oily solid which on trituration with ether gave a white solid (20 mg).

Amino acid analysis: Thr 2.20, Pro 3.17, Gly 5.32, Ala 2.36, Val 3.06, Met 1.95, Leu 3.07, Tyr 0.99, His 2.12, Lys 1.00, Arg 1.06;

m/z (FAB): 2863.9;

HRMS: 2859.51071. C₁₃₀H₂₀₅N₃₈O₃₁S₂ requires 2859.51068.

HPLC: (column B, gradient 4) R_t 11.6 min.

Leu-Ile-Phe-Ala-Gly-Resin

Wang resin (0.80 mmol/g, 1.0 g), pre-swollen in DMF (4 ml), was sonicated with 2 equiv of glycine symmetrical anhydride (formed using DIC) in DMF (5 ml) for 3.5 h in the presence of DMAP (10 mg). The resin was filtered and washed with DMF, DCM then ether (Fmoc loading test indicated a functionality of 0.61 mmol/g). A sample of this resin (0.42 g, 0.25 mmol) was taken and the peptide assembled using standard cycles, capping protocol B. The resin-bound peptide was washed with DMF DCM then ether to give 0.51 g of white solid.

General procedure for incorporation of Tbfmoc group onto the N-termini of resin-bound peptides

Typically 50 mg of resin-bound peptide is sonicated for 3 h with 3 equivalents of either Tbfmoc-Cl or a 2:1 mixture of Tbfmoc-OPfp/Tbfmoc-Cl in DCM (2 ml) in the presence of 3 equivalents of diisopropylethylamine. The resin is then filtered off and washed copiously with DCM then ether. The Tbfmoc functionality is estimated by measuring the UV spectrum of an accurately weighed portion of the resin (1-2 mg) in 20% piperidine/dioxane:

$$\text{Tbfmoc functionality} = \frac{0.613 \times \text{ABS}_{366 \text{ nm}}}{m}, m = \text{mass of resin (mg)}$$

Tbfmoc-Hepatitis B surface antigen, PreS1 (1-23) ayw, Tbfmoc-Met-Gly-Gln-Asn-Leu-Ser-Thr-Ser-Asn-Pro-Leu-Gly-Phe-Phe-Pro-Asp-His-Gln-Leu-Asp-Pro-Ala-Phe-OH

A sample of the resin-bound Tbfmoc-peptide (0.3 g, Tbfmoc functionality 0.122 mmol/g) was stirred in TFA (10 ml)/EDT (0.25 ml), water (0.5 ml)/phenol (0.75 g)

for 2 h in an atmosphere of dry nitrogen. The resin was filtered off and the filtrate concentrated *in vacuo*, then the crude peptide (0.198 g) precipitated with ether.

HPLC: (column B, gradient 8) R_t 15.9 min.

Purification of Hepatitis B surface antigen, PreS1 (1-23) ayw, Met-Gly-Gln-Asn-Leu-Ser-Thr-Ser-Asn-Pro-Leu-Gly-Phe-Phe-Pro-Asp-His-Gln-Leu-Asp-Pro-Ala-Phe-OH on PGC

A solution of the crude Tbfmoc-peptide (9.3 mg) in 70% aqueous CH_3CN (10 ml) was applied to a glass column packed with PGC (50-100 μm , 68 m^2/g , 0.5 g, column length 5.2 cm), monitoring the eluent by analytical HPLC (column B, gradient 8). The column was then eluted with 4 x 10 ml of 70% aqueous $\text{CH}_3\text{CN}/1\%$ piperidine, the fractions combined and acidified with acetic acid. The solution was diluted with water (200 ml) and desalted on a HPLC column, eluting the salt with 100% solvent A then the peptide with 90% B. The eluent was lyophilised to give the peptide as a white fluffy solid (5.9 mg).

Amino acid analysis: Asx 4.10, Thr 1.03, Ser 1.76, Glx 2.19, Pro 3.12, Gly 2.16, Ala 1.05, Met 0.91, Leu 2.88, Phe 3.16, His 0.59;

m/z (FAB): 2538.2;

HRMS: 2534.18554. $\text{C}_{114}\text{H}_{166}\text{N}_{29}\text{O}_{35}\text{S}$ requires 2534.18545;

HPLC: (column B, gradient 8) R_t 10.4 min.

Tbfmoc deprotection studies on Tbfmoc-Hepatitis B surface antigen, PreS1 (1-23) ayw, Tbfmoc-Met-Gly-Gln-Asn-Leu-Ser-Thr-Ser-Asn-Pro-Leu-Gly-Phe-Phe-Pro-Asp-His-Gln-Leu-Asp-Pro-Ala-Phe-OH, adsorbed on PGC

A sample of the crude Tbfmoc-peptide (1-1.5 mg) was taken in THF (0.10 ml) and diluted to 5 ml with 70% aqueous CH_3CN . PGC (50-100 μm , 68 m^2/g , 0.12 g) was

added to the solution and the quantitative adsorption of the Tbfmoc-peptide confirmed by the UV spectrum of the supernatant between 350-400 nm. The supernatant was discarded and the PGC suspended in 5 ml of the deprotection mixture. 0.2 ml aliquots of the supernatant were taken at 1, 2, 5, 10, 15, 30, 60, 120 and 180 min intervals as appropriate, the samples neutralised with acetic acid and the release of the Tbfmoc-deprotected peptide monitored by analytical HPLC (column B, gradient 8). Tbfmoc deprotection was judged to be complete when no further increase in the peptide peak height (R_t 10.4 min) was observed.

Tbfmoc-MeCP2 Methylated DNA Binding Domain, Tbfmoc-Ser-Ala-Ser-Pro-Lys-Gln-Arg-Arg-Ser-Ile-Ile-Arg-Asp-Arg-Gly-Pro-Met-Tyr-Asp-Asp-Pro-Thr-Leu-Pro-Glu-Gly-Trp-Thr-Arg-Lys-Leu-Lys-Gln-Arg-Lys-Ser-Gly-Arg-Ser-Ala-Gly-Lys-Tyr-Asp-Val-Tyr-Leu-Ile-Asn-Pro-Gln-Gly-Lys-Ala-Phe-Arg-Ser-Lys-Val-Glu-Leu-Ile-Ala-Tyr-Phe-Glu-Lys-Val-Gly-Asp-Thr-Ser-Leu-Asp-Pro-Asn-Asp-Phe-Asp-Phe-Thr-Val-Thr-Gly-Arg-OH

Resin bound Tbfmoc-peptide (0.10 g, Tbfmoc functionality 0.014 mmol/g, 62% incorporation) was stirred in TFA (5ml)/EDT (0.38 ml)/Phenol (0.38 g)/thioanisole (0.25 ml)/water (0.25 ml) for 3 h in an atmosphere of dry nitrogen. The resin was filtered off, washed with a little TFA and the filtrate concentrated *in vacuo* and the crude Tbfmoc-peptide (0.052 g) precipitated with ether.

Purification of MeCP2 Methylated DNA Binding Domain on PGC

Crude Tbfmoc-peptide (21.2 mg) was dissolved in 50% acetic acid and diluted to 5 ml with 50% aqueous isopropanol. The solution was filtered and PGC (HPLC grade, 5 μ m, 60 mg) added. Adsorption of the Tbfmoc-peptide was monitored by analytical

HPLC (column B, gradient 4). After centrifugation, the solvent was decanted off and the PGC subjected to the following wash protocol:

1. 2.5% acetic acid/50% aqueous isopropanol (5 ml);
2. 50% aqueous isopropanol (5 ml);
3. 50% aqueous isopropanol/10% piperidine (2 ml), 10 min;
4. 50% aqueous isopropanol (2 ml);
5. 50% aqueous isopropanol/10% piperidine (2 ml);
6. 50% aqueous isopropanol (2 ml);

Washes 3-6 were combined and the solvent removed *in vacuo*. The residue was dissolved in aqueous acetic acid, filtered to remove fine carbon particulate and a final purification effected by semi-preparative HPLC (column G, gradient 10). The eluent was lyophilised to give the product as a white fluffy solid (1.0 mg)

Amino acid analysis: Asx 10.47, Thr 5.06, Ser 5.67, Glx 6.39, Pro 5.15, Gly 6.53, Ala 4.74, Val 4.51, Met 0.80, Ile 3.38, Tyr 3.41, Phe 3.61, Lys 6.97, Arg 7.2;

m/z (Laser desorption): 9726 (required 9627).

HPLC: (column B, gradient 4) R_t 12.4 min.

Tbfmoc-Ubiquitin Y59F, Tbfmoc-Met-Gln-Ile-Phe-Val-Lys-Thr-Leu-Thr-Gly-Lys-Thr-Ile-Thr-Leu-Glu-Val-Glu-Pro-Ser-Asp-Thr-Ile-Glu-Asn-Val-Lys-Ala-Lys-Ile-Gln-Asp-Lys-Glu-Gly-Ile-Pro-Pro-Asp-Gln-Gln-Arg-Leu-Ile-Phe-Ala-Gly-Lys-Gln-Leu-Glu-Asp-Gly-Arg-Thr-Leu-Ser-Asp-Phe-Asn-Ile-Gln-Lys-Glu-Ser-Thr-Leu-His-Leu-Val-Leu-Arg-Leu-Arg-Gly-Gly-OH

The resin-bound Tbfmoc peptide (Tbfmoc-functionality, 0.026 mmol/g, 85% incorporation, 88 mg) was taken in TFA (4 ml)/EMS (0.25 ml)/thioanisole (0.25 ml)/anisole (0.25 ml)/water (0.25 ml) and stirred for 4h in an atmosphere of dry nitrogen. The resin was filtered off, washed with a little TFA and concentrated *in*

vacuo and the crude Tbfmoc-peptide precipitated with ether. The solid was dissolved in aqueous acetic acid and lyophilised to give a white solid (42.2 mg).

Purification of Ubiquitin Y59F

Crude Tbfmoc-Ubiquitin Y59F (30 mg) was dissolved in 30% acetic acid and passed down a sephadex G-50 column (dimensions 100x2.5 cm), combining the fluorescent fractions. After lyophilisation this material was subjected to semi-preparative HPLC (wavelength 364 nm, column D, gradient 7), when two main fractions were isolated, Tbfmoc-F1 (3.9 mg), R_t 14.8 min (column C, gradient 4), and Tbfmoc-F2 (3.8 mg), R_t 16.6 (column C, gradient 4), after lyophilisation. The fractions were dissolved in 10% aqueous CH_3CN (2 ml) and piperidine (0.020 ml) added. After 5 min the solutions were acidified with acetic acid (0.20 ml) and purified by semi-preparative HPLC (column D, gradient 9), giving F1 (0.8 mg) and F2 (0.5 mg).

Amino acid analysis F1: Asx 8.47, Thr 7.49, Ser 3.09, Glx 16.3, Pro, 2.80, Gly 6.11, Ala 2.03, Val 3.86, Met 0.69, Ile 6.65, Leu 9.36, Phe 3.26, His 1.16, Lys 8.03, Arg 4.32;

m/z (laser desorption): 8564;

HPLC: (column C, gradient 4) R_t 11.0 min

Amino acid analysis: Asx 8.21, Thr 7.35, Ser 3.15, Glx 16.08, Pro 2.98, Gly 6.18, Ala 2.19, Val 3.91, Met 0.62, Ile 6.53, Leu 9.19, Phe 3.31, His 1.05, Lys 7.86, Arg 4.14;

m/z (laser desorption): 8552;

HPLC: (column C, gradient 4) R_t 11.8 min.

Bombesin, GIp-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH₂

Bombesin was prepared twice using (a) resin (92) (0.88 g, 0.57 mmol, 0.5 mmol) and (b) resin (108) (0.51 g, 0.49 mmol/g, 0.25 mmol), capping protocol A.

(a) A portion of the resin-bound peptide (0.5 g of total 1.47 g) was stirred with TFA (24 ml)/EDT (0.5 ml)/EMS (0.5 ml)/water (1.25 ml)/anisole (0.5 ml)/thioanisole (0.5 ml)/phenol (0.5 g) for 3 h in an atmosphere of dry nitrogen. The resin was filtered off and the filtrate concentrated *in vacuo*. The crude peptide was precipitated with ether to yield a white solid (0.204 g). A sample of this material (0.05 g) was purified by semi-preparative HPLC on column G, gradient 2 to give the product as a white fluffy solid after lyophilisation (0.02 g, 29%).

Amino acid analysis: Asp 1.00, Glu 3.27, Gly 2.00, Ala 0.99, Val 0.95, Met 0.97, Leu 1.89, His 0.98, Arg 0.93, Trp 1.02;

m/z (FAB): 1619.9 (MH⁺), 965.7, 809.9;

HRMS: 1619.82282. C₇₁H₁₁₁N₂₄O₁₈S requires 1619.82283;

HPLC: (Column A, gradient 1) R_t 21.5 min.

(b) A sample of the dried resin-bound peptide (0.5 g of 1.02 g) was treated as in (a) above to give 0.193 g of crude peptide, a portion of which (29.6 mg) was purified by semi-preparative HPLC (column G, gradient 3) to give 6.5 mg of the *title compound*.

Amino acid analysis: Asp 0.81, Glu 3.29, Gly 2.17, Ala 1.04, Val 1.10, Met 0.87, Leu 1.84, His 0.96, Arg 0.90, Trp 1.29;

m/z (FAB): 1619.1 (MH⁺);

HRMS: 1619.82282. C₇₁H₁₁₁N₂₄O₁₈S requires 1619.82283;

HPLC: (Column B, gradient 4) R_t 8.5 min.

Leu-Ile-Phe-Ala-Gly-NH-(CH₂)₆CH₃

The peptide was prepared using resin (114) (1.0 g, 0.15 mmol/g, 0.15 mmol) using single symmetrical anhydride coupling (6.7 equiv), capping protocol A.

A sample of the resin-bound peptide (0.5 g of total 0.95 g) was stirred in TFA (25 ml)/water (1.25 ml)/anisole (1.25 ml) for 1 h under an atmosphere of dry nitrogen. The resin was filtered off and the filtrate concentrated *in vacuo*. The product was precipitated with water/petroleum ether (40-60) to give the peptide as an off-white solid (0.035 g).

Amino acid analysis; Gly 1.07, Ala 1.02, Ile 0.94, Leu 0.98, Phe, 0.99;

m/z (FAB): 617 (MH⁺), 428, 322;

HRMS: 617.43906. C₃₃H₅₇N₆O₅ requires 617.43902.

HPLC: (column A, gradient 5) R_t 23.6 min.

Leu-enkephalin, N-heptylamide, Tyr-Gly-Gly-Phe-Leu-(CH₂)₆CH₃

This peptide was prepared using resin (116) (0.15 mmol/g, 1.0 g, 0.15 mmol), using double couple cycles with 3.33 equiv of activated species, capping protocol A.

The resin-bound peptide was washed with DMF then DCM and dried *in vacuo* to give 1.03 g. A sample of the resin (0.5 g) was taken in TFA (10 ml)/water (0.5 ml)/anisole (0.5 ml) and the mixture stirred in an atmosphere of dry nitrogen for 1 h. The resin was filtered off and the peptide precipitated with ether, filtered off, dissolved in aqueous acetic acid and lyophilised to give 31.6 mg of crude peptide. A small portion of this (6.0 mg) was dissolved in 50% acetic acid and purified by semi-preparative HPLC (column G, gradient 6) to give 5.0 mg (55%) of pure peptide after lyophilisation.

Amino acid analysis: Gly 1.99, Leu 1.02, Tyr 0.93, Phe 1.06;

m/z (FAB: 654 (MH⁺);

HRMS: 653.40261. C₃₅H₅₃N₆O₆ requires 653.40263.

HPLC: (column B, gradient 4) R_t 15.1 min.

Leu-Ile-Phe-Ala-azaGly-NH₂

This peptide was prepared using resin (119) (0.29 mmol/g, 0.86g, 0.25 mmol) using capping protocol B.

The resin-bound peptide was awashed with DMF then DCM and dried *in vacuo*. A portion of the resin (0.5 g) was taken in TFA (9.5 ml)/water (0.5 ml) and sonicated for 1 h. The resin was filtered off and washed with a little TFA, then the filtrate was concentrated *in vacuo* and the peptide (85 mg) precipitated with ether. A sample of the crude peptide (46.8 mg) was dissolved in 5% acetic acid and purified by semi-preparative HPLC in three portions (column F, gradient 7) to give the *title compound* as a white fluffy solid (16.5 mg, 52%) after lyophilisation.

Amino acid analysis: Ala 1.04, Ile 0.93, Leu 0.99, Phe 1.03;

m/z (FAB): 520 (MH⁺);

HRMS: 520.32474. C₂₅H₄₂N₇O₅ requires 520.32474..

HPLC: (column A, gradient 4) R_t 11.4 min.

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APPENDIX

Microanalysis Results for 8*b*H-Tetrabenz[*a,c,g,i*]fluorene (61)¹⁶⁹

Instrument: Perkin-Elmer 2400 elemental analyser

<u>Mass (mg)</u>	<u>Operating Conditions</u>	<u>% C</u>	<u>% H</u>
1.323	Normal running conditions ^a	93.3	4.91
1.474	Extended combustion, 40 sec.	92.8	4.50
1.376	Extended combustion, 60 sec.	92.4	4.14
1.377	Oxygen fill +2 sec. Oxygen boost +1 sec.	93.4	4.86
1.343	Oxygen fill +2 sec. Oxygen boost +1 sec., +V ₂ O ₅	93.7	4.96
1.801	Oxygen fill +3 sec. Oxygen boost +2 sec., +V ₂ O ₅	94.2	5.07
1.642	Oxygen fill +5 sec. Oxygen boost +2 sec., +V ₂ O ₅	93.5	5.12
1.374	Oxygen fill +3 sec. Oxygen boost +2 sec., +V ₂ O ₅	94.0	5.08

^aAs recommended by the manufacturer

(Molecular formula C₂₉H₁₈ requires C, 95.1%; H, 4.92%)

Courses attended

Organic research seminars (various speakers)

Topics in Organic Chemistry (various speakers, University of Edinburgh)

NMR Spectroscopy (Dr I.H. Sadler and Dr J. Parkinson)

Medicinal Chemistry (Professor R. Baker and colleagues, Merck Sharp and Dohme)

Mass Spectrometry (various speakers, ICI)

Chemical Development in the Pharmaceutical Industry (various speakers, SmithKline Beecham)

Discovery and Development of Zoladex (ICI, various speakers)

Hirst Memorial Lecture, University of St Andrews (Professor S.J. Benkovic, Pennsylvania State University)

Symposium on Protein Engineering, Royal Society of Edinburgh (various speakers)