

**SOLID PHASE PEPTIDE SYNTHESIS**

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

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To my parents,  
Dominique and Jennifer

## ABSTRACT

A new method of purification has been developed for peptides synthesised by the Merrifield solid phase methodology.

$N^{\alpha}$ -17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl glycine (Tbfmoc Gly OH) has been prepared, and coupled via its symmetrical anhydride or its HOBT ester to the N-terminus of a peptide at the final stage of the synthesis.

The strong affinity of the Tbfmoc group for graphitised carbon (PGC) allows the retention of Tbfmoc-protected peptides on a column packed with this material, whilst incorrectly terminated sequences and other materials are washed away. Following deprotection of the Tbfmoc group under basic conditions, the free peptide can be eluted from the column.

This method has been exemplified by the purification of Ubiquitin (53-76) OH and Ubiquitin (35-76) OH.

Preliminary results indicate that the technique may be developed into a form of affinity purification suitable for the resolution of racemates using ligand-exchange chromatography (LEC).

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## ABBREVIATIONS

Bnpeoc	2,2-bis-(4-nitrophenyl)ethoxycarbonyl
Boc	tert-butyloxycarbonyl
BOP	benzotriazole-1-yl-oxy-tris-(dimethyl- amino)phosphonium hexafluorophosphate
Bpoc	2-(4-biphenyl)propyl-2-oxycarbonyl
DCC	1,3-dicyclohexylcarbodiimide
DCCC	droplet counter-current chromatography
DCM	dichloromethane
DIBAL-H	diisobutylaluminium hydride
DIC	1,3-diisopropylcarbodiimide
DMF	N,N-dimethylformamide
DMS	dimethyl sulphide
DMSO	dimethyl sulphoxide
DVB	divinylbenzene
EDTA	ethylenediaminetetraacetic acid
EEDQ	N-ethoxycarbonyl-2-ethoxy-1,2-dihydro- quinoline
EI	electron impact ionisation
FAB	fast atom bombardment
Fmoc	9-fluorenylmethoxycarbonyl
HIC	hydrophobic-interaction chromatography
HOBt	1-hydroxybenzotriazole
HONSu	N-hydroxysuccinimide
HPLC	high performance liquid chromatography
IR	infrared

Mbh	4,4'-dimethoxybenzhydryl
n.m.r.	nuclear magnetic resonance
P	polystyrene
PAM	phenylacetamidomethyl
PCC	pyridinium chlorochromate
PDC	pyridinium dichromate
PGC	porous graphitised carbon
Pmc	2,2,5,7,8-pentamethylchroman-6-sulphonyl
Pon	4-[4-(oxymethyl)phenylacetoxymethyl]-3-nitrobenzamidomethyl
Pop	2-[4-(oxymethyl)phenylacetoxyl]-propionyl
PPA	polyphosphoric acid
Ppoc	2-phenylpropyl-2-oxycarbonyl
R	resin support
SPPS	solid phase peptide synthesis
Tbfm	17-tetrabenzo(a,c,g,i)fluorenylmethyl
Tbfmoc	17-tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl
TBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
THF	tetrahydrofuran
t.l.c.	thin layer chromatography
TFA	trifluoroacetic acid
Z	benzyloxycarbonyl

## CHAPTER 1 : INTRODUCTION

### 1.1 SOLID PHASE PEPTIDE SYNTHESIS

#### 1.1.1 Introduction

Peptide chemistry was founded at the turn of the century by Emil Fischer<sup>1</sup> and Theodor Curtius and since then has been developed into an area of great sophistication. The basic requirement of peptide chemistry is to protect both the carboxyl group of one amino acid and the amino group of the other to direct synthesis towards the desired peptide product. Subsequent activation of the free carboxyl group followed by selective removal of the protecting groups after peptide bond formation, gave the first dipeptide.

A major achievement in the history of peptide chemistry was the introduction in 1963 of solid phase peptide synthesis (SPPS) by Merrifield<sup>2</sup>, for which he was awarded the Nobel Prize in 1984<sup>3</sup>. He introduced the concept of peptide synthesis on an insoluble polymeric

support in order to overcome many of the problems of peptide synthesis in solution (such as poor solubility of large or protected peptides and the need for purification and characterisation of intermediates).

### 1.1.2 The Solid Phase Principle

The principle of SPPS is simple. The growing peptide chain is elaborated while covalently attached to an insoluble polymeric support.

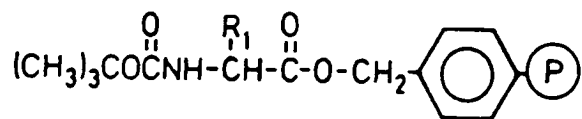
This technique has several advantages over the solution phase method:

1. Physical losses are minimised by the use of a single reaction vessel at all times.
2. The assembly of peptide chain is greatly accelerated and simplified as intermediate purification is eliminated.
3. Reactions can be driven to completion using excess reagents. This allows a high efficiency in the various chemical steps and thereby increases the overall yield of product. Excess reagents and by-products can easily be removed by filtration.
4. The repetitive steps can be automated. This is an enormous time and labour advantage over solution phase chemistry.

The standard protocol for SPPS developed by Merrifield<sup>3</sup> is illustrated in Scheme 1.

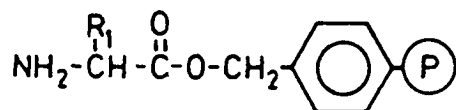
chloromethylpolystyrene-1% DVB

1 Anchoring

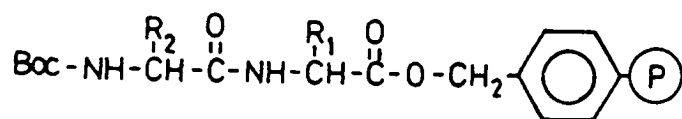


2 Deprotection (TFA 50%)

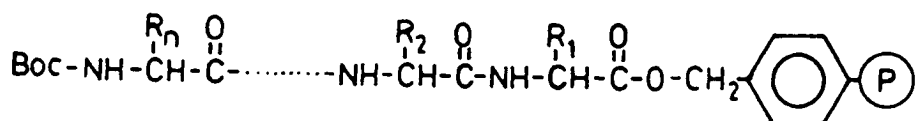
3 Basification ( $\text{R}_3\text{N}$ )



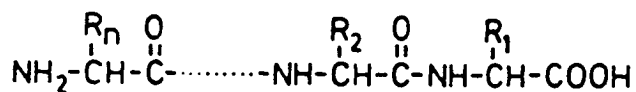
4 Coupling (DCC)



Repetition of steps  
2, 3 and 4  
n cycles



Final Deprotection and  
Cleavage from the Resin  
(HF 0°C)



Scheme 1

The polymeric support chosen was polystyrene cross-linked with 1% divinylbenzene (DVB). Functionalisation of the resin was achieved by chloromethylation ( $\text{ClCH}_2\text{OCH}_3/\text{ZnCl}_2$ ). The caesium salt of the first  $\text{n}^\alpha$ -tert-butyloxycarbonyl (Boc) protected amino acid was reacted with the chloromethylated resin. After removal of the Boc group using trifluoroacetic acid (TFA), the resulting salt was basified with a tertiary amine to regenerate the  $\alpha$ -amino function. The next  $\text{N}^\alpha$ -Boc protected amino acid was then activated with dicyclohexylcarbodiimide (DCC) and coupled to the first residue. The steps of deprotection, basification and coupling were then repeated to extend the peptide chain. Finally, the completed peptide was detached from the support using anhydrous HF at  $0^\circ\text{C}$  and subsequently purified.

### 1.1.3 The Polymer Support

The major requirements for a suitable support are outlined below:

1. The support must be capable of a high degree of functionalisation to give a useful yield of peptide.
2. It must be stable to the physical and chemical conditions of the synthesis.
3. It must allow rapid contact between the growing peptide chain and the reagents.
4. It must be readily separable at every stage.

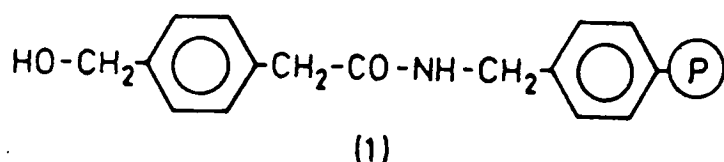
Merrifield found the most satisfactory material to be a gel prepared by copolymerisation of styrene with 1% divinylbenzene<sup>2</sup>. It provided a totally insoluble resin, mechanically robust and possessing good swelling properties in organic solvents such as dichloromethane (DCM) or N,N -dimethylformamide (DMF).

Other solid supports such as polysaccharides, silica, porous glass and polyacrylamides have also been examined and employed in solid phase synthesis from time to time. Of these, perhaps the most significant have been the polyamide resins developed by Sheppard<sup>4,6-8</sup>. They were specifically designed to overcome the problems of synthesising, in good yield, some particularly troublesome sequences of peptides (e.g. residues 65-74 of acyl carrier protein)<sup>4,5</sup>.

More recently, Sheppard has introduced the more robust polyamide resin supported on kieselguhr<sup>9</sup>, suitable for continuous-flow SPSS.

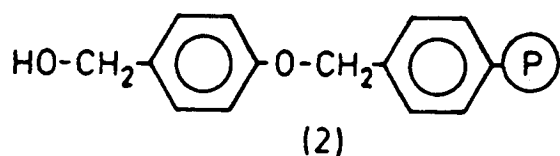
#### 1.1.4 The Peptide-Resin Link

In the original method, the use of 50% TFA in DCM for the repetitive removal of the Boc group, was found to cleave the benzyl ester linkage between the peptide and the resin by about 1% per cycle<sup>10</sup>, which is barely acceptable. A solution to this problem was the introduction by Merrifield of the phenylacetamidomethyl (PAM) resin<sup>11</sup> (1).



The electron withdrawing effect of the acetamido group was found to increase the stability of the peptide esters of (1) towards acids by a factor of 100. However, cleavage of the PAM resin and simultaneous side chain deprotection in the final step required the use of anhydrous HF which can cause damage to the peptide itself and has been found to be incompatible with some scavengers<sup>12</sup>. To reduce these side reactions to a minimum, notable success has been obtained with the introduction of the HF/DMS (low-high) methodology<sup>13</sup>.

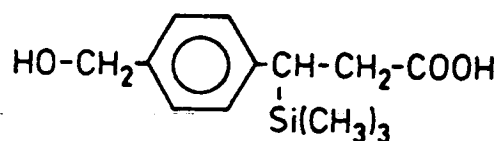
A far more flexible approach to the solid phase synthesis of large peptides has been the development of the *p*-alkoxybenzyl alcohol resin<sup>14,15</sup> (2).



In this case, the electron donation by the *para* alkoxy group stabilises the incipient benzylic carbonium ion and hence enables cleavage of the peptide from the resin under

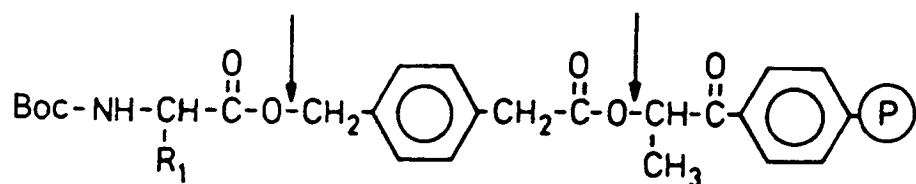
milder conditions (50% TFA in DCM). The *p*-alkoxybenzyl-alcohol resin has been extensively used in an orthogonal strategy with the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) amino protecting group<sup>16-18</sup>.

Other handles have also been reported which allow a three-dimensional orthogonal strategy. The advantage of such an approach is the opportunity to synthesise protected fragments. This in turn allows a fragment condensation approach for the synthesis of peptides to be adopted. For example a fluoride-labile linker has been reported by Barany<sup>19</sup>, and more recently, the 2-(4-hydroxymethylphenyl)-2-trimethylsilyl-propanoic acid handle (3) has been successfully used by Ramage<sup>20</sup> as an alternative fluoride-labile linker.

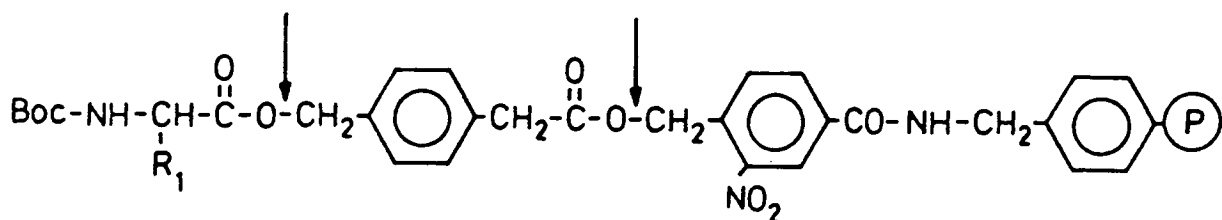


(3)

Rich<sup>21</sup> and Tjoeng<sup>22</sup> have introduced photolabile handles while Merrifield<sup>23,24</sup> has developed the multi-detachable Pop and Pon-resins (4), (5), in which two ester bonds can selectively be cleaved by different reagents, to give a peptide in various stages of deprotection.

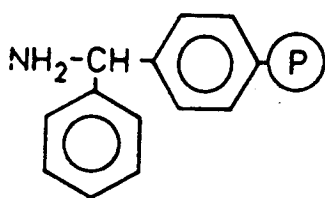


tert-butoxycarbonylaminoacyl-2-[4-(oxymethyl)phenylacetoxyl]-propionyl Pop-resin (4)

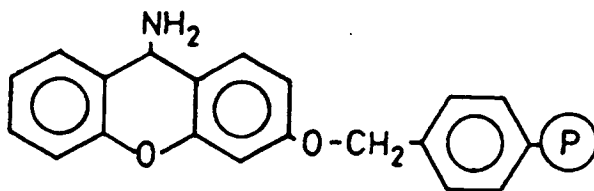


tert-butoxycarbonylaminoacyl-4-[4-(oxymethyl)phenylacetoxymethyl]-3-nitrobenzamidomethyl Pon-resin (5).

Finally, other acid-labile handles have been developed to produce peptide amides, including those derived from the benzhydrylamine-resin<sup>25,26</sup> (6) and the more recent xanthenyl-resin (7) reported by Sieber<sup>27</sup>.



(6)



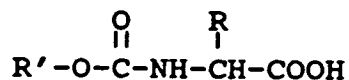
(7)

In both cases cleavage of the peptide amides can be performed using very mild acidolysis.

#### 1.1.5 N<sup>α</sup>-Protecting Groups

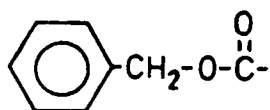
Though many protecting groups have been recommended, only a few have found general application.

Among these, the urethane-type amino protecting group (8) has been widely used for the important reason that these derivatives minimise racemisation during the activation step.



(8)

The first urethane group, the benzyloxycarbonyl (Z) group (9) was introduced by Bergmann and Zervas in 1932<sup>28</sup>.

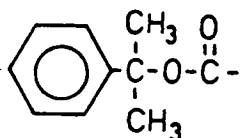


(9)

Removal of this group may be achieved by a number of methods including catalytic hydrogenolysis, acid-catalysed solvolysis in HBr/CH<sub>3</sub>COOH and reduction using sodium in liquid ammonia.

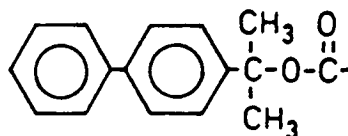
An alternative to the Z group has been the Boc<sup>29</sup> group which can be cleaved using 50% TFA in DCM.

Extensive work has been done on acid sensitive N $\alpha$ -protecting groups such as Ppoc<sup>30</sup> (10) or Bpoc<sup>31</sup> (11) and has shown that their cleavage can be performed under very mildly acidic conditions.



2-phenylpropyl-2-oxycarbonyl

Ppoc (10)



2-(4-biphenyl)propyl-2-oxycarbonyl

Bpoc (11)

However, a major problem in using acid-labile urethane protecting groups is the generation of relatively long-lived carbonium ions on acidolytic cleavage. These highly reactive species can modify the side chain functionality of tryptophan<sup>31,32</sup>, tyrosine<sup>33</sup>, methionine and cysteine<sup>34</sup>, necessitating the presence of scavengers such as anisole, thioanisole and 1,2-ethanedithiol during the deprotection step.

In comparison to acid-labile protecting groups, the base-labile protecting group Fmoc<sup>35,36</sup> offers considerable advantages:

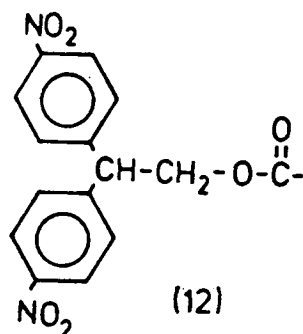
1. The mild conditions of removal (via  $\beta$ -elimination) reduce the number of side reactions inherent in

repetitive acidolysis.

2. The possibility of using this group along with acid-labile side-chain protection allows an orthogonal strategy to be adopted.

The Fmoc, *t*-butyl, alkoxybenzyl ester methodology has become a successful strategy for peptide synthesis combining mild conditions with efficiency.

Recently, the 2,2-bis-(4-nitrophenyl)ethoxycarbonyl (Bnpeoc) group<sup>37,38</sup> (12) has been developed as an alternative base-labile  $N^\alpha$ -protecting group and offers advantages over the relatively expensive Fmoc reagents.



#### 1.1.6 Side-Chain Protection

In SPPS, reaction conditions tend to be rather severe: large excess of reagents and extended reaction times are used. For these reasons, the protection of side-chain functional groups is necessary.

We have successfully used the Fmoc, *t*-butyl, alkoxybenzyl ester methodology in which the following acid-labile side-chain protecting groups were employed:

$\gamma$ -trityl for histidine, Pmc<sup>39,40</sup> for arginine, Mbh<sup>41</sup> for asparagine and glutamine, and *tert*-butyl for tyrosine, glutamic and aspartic acid, threonine, and serine.

A large number of protecting groups for amino acid side-chain functions have been reported in the literature. Although these are not described here, some excellent reviews<sup>42-44</sup> exist on this topic.

### 1.1.7 Coupling Methods

The formation of a peptide bond requires nucleophilic attack of the  $\alpha$ -amino group of one amino acid upon the activated carboxyl function of another.

Obtaining optimal conditions for the coupling reactions is of crucial importance in SPPS as mixtures of free-peptide products are often difficult to separate. For this reason much effort has been put into the search for activating procedures which effect rapid and clean acylations of amines without side reactions.

One of the major potential side reactions is racemisation which can proceed through a mechanism involving a 5(4H)-oxazolone formation<sup>45</sup>. Numerous studies on the racemisation dependence of different peptide coupling methods have revealed that only a few techniques are suitable for routine synthesis.

Some of the most prominent methods and reagents are discussed below.

### The Acid Chloride Method

$N^{\alpha}$ -protected amino acid chlorides are very reactive and were tried out in solution phase peptide synthesis around the turn of the century<sup>46</sup>; their use in SPPS has remained very limited.

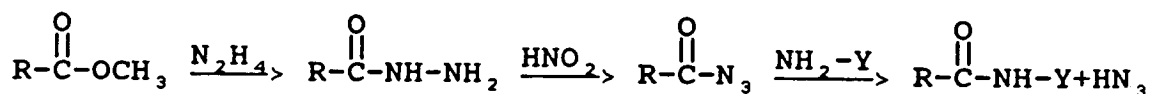
Conversion of the carboxyl group of a protected amino acid to the acid chloride generally requires the use of thionyl chloride or oxalyl chloride in DCM. Under the reaction conditions, some  $N^{\alpha}$ -protecting groups (i.e. Boc) are not completely stable and give rise to the formation of N-carboxyanhydrides<sup>47</sup>. Other problems, which limit their use in peptide chemistry are racemisation and the high level of unwanted side reactions.

Despite these considerable drawbacks, the revival of the acid chloride method has been made possible through the introduction of novel acid-stable  $N^{\alpha}$ -protecting groups. The Fmoc group was found to be stable during acid chloride preparation with thionyl chloride<sup>48</sup>. Furthermore, Fmoc amino acid chlorides having aliphatic side-chains are stable compounds<sup>48</sup> and have been shown to react rapidly in solution and in solid phase synthesis with very low levels of racemisation<sup>48,49</sup>.

### The Azide Method

The azide method<sup>50</sup> has been shown to be one of the safest methods to avoid racemisation.

Azides can be generated from alkyl esters via the formation of hydrazides (Scheme 2).



Scheme 2. Peptide synthesis by azide coupling

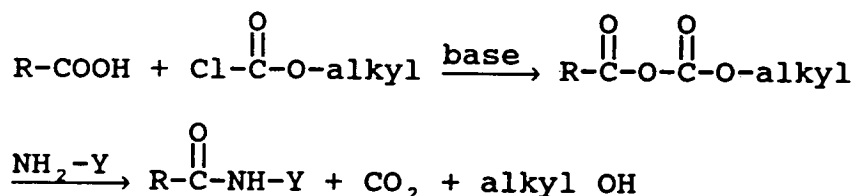
Following recent improvements of the method, direct conversion of carboxylic acids to acid azides is now possible with the use of diphenylphosphoryl azide<sup>51</sup>. Alkyl nitrites, in place of nitrous acid, may also be used for the transformation of hydrazides to azides<sup>52</sup>.

The possible drawbacks of the method are the generation of the highly toxic hydrazoic acid during acylation and the Curtius rearrangement leading to the formation of urea derivatives<sup>53</sup>.

#### The Anhydride Method

Mixed anhydrides between  $\text{N}^\alpha$ -protected amino acids and a variety of other acids were introduced in peptide chemistry in the early forties.

Among these, the mixed carboxylic-carbonic anhydrides have been the most popular. Due to their instability over long periods, they are usually prepared at low temperature immediately prior to coupling using reagents such as ethyl<sup>54,55</sup> or isobutyl chloroformate<sup>56</sup> (Scheme 3).



Scheme 3. Mixed anhydride method with alkyl chloroformate as activating agent.

The use of phosphinic-carboxylic mixed anhydrides has also been investigated. In particular diphenylphosphinyl chloride<sup>57</sup>, 1-oxo-1-chlorophospholane<sup>58</sup> and recently diphenylphosphinic anhydride<sup>59</sup> have been successfully used as reagents in both solution<sup>57</sup> and solid phase synthesis<sup>59,60</sup>.

With symmetrical anhydrides, there is no ambiguity concerning which carbonyl is attacked by the amine, and they react exceptionally cleanly and in high yield. Their disadvantage is that two equivalents of expensive amino acid derivative are required to generate one of the active species.

Symmetrical anhydrides are stable and have been stored cold for long periods, but are commonly prepared immediately prior to use with a reagent such as DCC.

#### The Active Ester Method

Schwyzler was the first to recognise that an increase in the reactivity of alkyl esters<sup>61-63</sup> was necessary for the formation of the peptide bond and introduced cyano-

methyl esters<sup>64</sup>.

In this case, the electron-withdrawing effect of the cyano group renders the carbon atom of the carbonyl group more electrophilic. They have not been generally adopted in peptide synthesis, as satisfactory rates are only obtained when the reactants are used in large excess.

Aryl esters proved to be more powerful, and enhancement of their inherent reactivity by electro-negative substituents gave rise to a whole series of acylating agents. Noteworthy examples are *p*-nitrophenyl<sup>65</sup>, 2,4,5-trichlorophenyl<sup>66</sup> and pentachloro or pentafluorophenyl esters<sup>67</sup>, although the last two have a possible problem with steric hindrance.

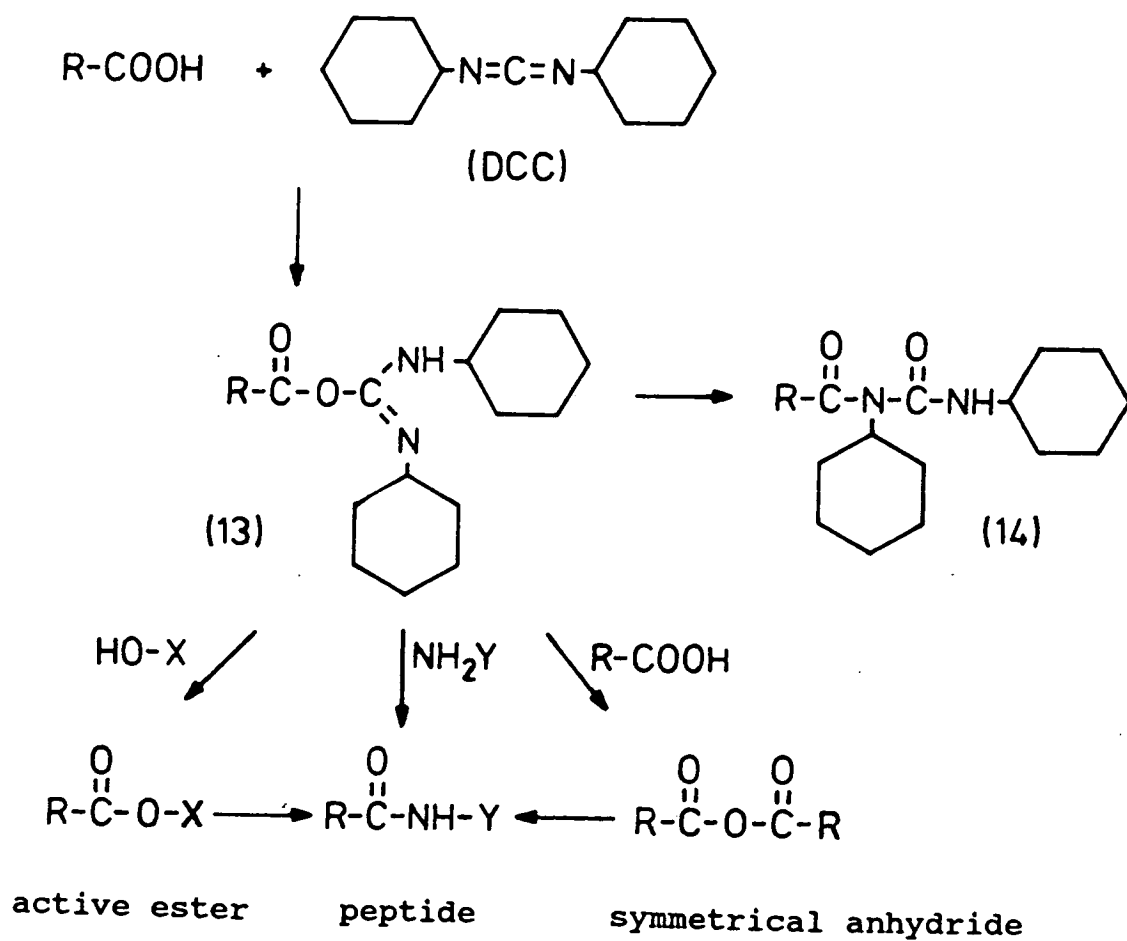
More recently, active esters of 1-hydroxybenzotriazole<sup>68</sup> (HOBt) and *N*-hydroxysuccinimide<sup>69</sup> (HONSu) have gained considerable popularity among peptide chemists. These esters may be preformed or generated *in situ*, are highly reactive and have substantially improved coupling reactions in some difficult sequences.

### Coupling Reagents

The main advantage of coupling reagents such as DCC<sup>70</sup> is that they can be added to *N*<sup>α</sup>-protected amino acids in the presence of the amino component. Thus, *in situ* activation can be achieved immediately prior to or at the time of the coupling.

DCC is highly reactive and its ease of application has

made it the most frequently used reagent in peptide synthesis (Scheme 4).



Scheme 4. Peptide Formation with DCC.

However, there are several disadvantages which limit the use of DCC. Compound (13) can spontaneously rearrange to the unreactive by-product N-acylurea (14). Furthermore, the high reactivity of intermediate (13) can lead to oxazolone formation during fragment condensation. Both these problems can be avoided by the addition of auxiliary nucleophiles such as HOBT<sup>71</sup> to give *in situ* active ester formation.

Different carbodiimides are available; DCC gives as by-product dicyclohexylurea which in some solvents can be separated almost quantitatively from soluble peptide derivatives by simple filtration. On the other hand, diisopropylcarbodiimide (DIC) has found favour in SPPS. It gives the much more soluble diisopropylurea which can be easily washed out together with excess amino acid derivatives and reagents.

The success of DCC has prompted a further search for even better coupling reagents. Some examples are carbonyldiimidazole<sup>72</sup>, N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)<sup>73</sup> and some phosphonium derivatives such as the benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) reagent<sup>74</sup> and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)<sup>75</sup> recently developed by Knorr.

This is by no means an exhaustive list of the coupling methods available to the peptide chemist. There are excellent reviews on this subject<sup>76,77</sup>; in particular

Hudson has recently compared different coupling procedures and has selected BOP in the presence of HOBT as the best coupling reagent<sup>78</sup>.

#### 1.1.8 Monitoring and Capping

Despite the use of highly efficient coupling techniques, acylation reactions cannot be assumed to go to completion. Some sequence effects such as steric hindrance or the folding of the growing peptide chain can result in some dramatic drops in the coupling efficiencies as synthesis progresses. However, for long peptide syntheses coupling and deprotection steps must be driven virtually to completion (~99.9%), as even a slight lowering in efficiency will produce deletion peptides and complicate purification.

Monitoring of both acylation and deprotection reactions can therefore help to identify these failures.

Several monitoring techniques, such as ninhydrin (Kaiser test)<sup>79,80</sup>, picric acid<sup>81</sup>, 2,4,6-trinitrobenzenesulphonic acid<sup>82</sup>, fluorescamine<sup>83</sup>, and chloranil<sup>84</sup>, are based upon the reaction of a reagent with free amino groups to form a coloured compound. Other techniques include: titration with perchloric acid<sup>85</sup>, specific absorption in the ultraviolet of the eliminated material produced during acylation (e.g. 3,4-dihydro-4-oxo-1,2,3-benzotriazine)<sup>86</sup>, and continuous spectrophotometric monitoring of the reaction when Fmoc-derivatives are

used<sup>87</sup>.

The most popular method remains, however, the qualitative colour test with ninhydrin<sup>79</sup>.

A method to circumvent the formation of deletion peptides is to covalently block (cap) with a powerful acylating reagent the residual free  $\alpha$ -amino groups where peptide coupling has failed. Eventual separation of the terminated peptides from the desired product is in theory simpler due to the differences in charge or solubility conferred by the blocking group.

The capping procedure is usually performed with acetic anhydride in the presence of a secondary or tertiary base in DCM or DMF<sup>2</sup>. Other acylating reagents such as N-acetylimidazole<sup>88</sup>, isopropenyl formate<sup>89</sup>, fluorescamine<sup>90</sup>, 3-nitrophthalic anhydride<sup>91</sup> and 3-sulphopropanoic acid anhydride<sup>92</sup>, have also been used as an alternative to acetic anhydride.

## 1.2 PEPTIDE PURIFICATION

### 1.2.1 Introduction

There are a great many separation techniques available for the isolation and purification of peptides and proteins. Small peptides can often be separated from by-products by standard techniques such as gel filtration, ion exchange and high-performance liquid chromatography, which exploit the physico-chemical properties of molecules. Purification of larger synthetic peptides or

proteins is more difficult to achieve as the differences in physical or chemical properties are relatively smaller. These difficulties are magnified with the stepwise solid phase method, where purification is possible only at the end of the synthesis. In this case, highly selective purification techniques, such as affinity chromatography, can be extremely useful as alternatives to the standard purification methods.

Optimising the separation of a complex peptide mixture should not, however, rely on a single purification method but should be achieved by a combination of these techniques.

### 1.2.2 Gel Filtration

Since its introduction<sup>93</sup>, gel filtration has occupied a key position in the purification of numerous biological molecules because of its simplicity and reliability as a separation technique.

The separation in gel filtration depends on the differential ability of the molecules to enter the stationary portion of the liquid phase through the pores in the gel. Very large molecules do not enter the stationary phase and move quickly through the chromatographic bed whereas smaller molecules, which can enter the gel pores, move more slowly through the column. Molecules are, therefore, eluted in order of decreasing molecular size.

Several bead-formed gels derived from dextran (Sephadex, Sephacryl) or agarose (Sepharose) are commercially available. They differ in their structures, in the degree of cross-linking and are available with different fractionation ranges.

Gel filtration can readily be used for removing scavengers, desalting peptides and for buffer exchanges (the peptide can be transferred to the appropriate starting buffer for subsequent purification techniques). With a careful choice of gels and operating conditions, this technique can be employed for the fractionation of peptides.

Ultrafiltration membranes have also been used as an alternative for removing low molecular weight impurities<sup>94</sup>.

### 1.2.3 Ion Exchange Chromatography

Ion exchange chromatography has proved to be a high resolution technique for the separation of biological molecules. Ion exchange is capable of separating molecules with very close isoelectric points. This characteristic combined with its simplicity of use and a good recovery of material has made ion exchange a very popular technique.

An ion exchanger consists of an insoluble matrix to which charged groups have been covalently bound. Positively charged matrices have negatively charged

counter-ions (anions) available for exchange, and therefore are termed anion exchangers (vice versa for cation exchangers).

The matrix may be based on inorganic compounds (such as hydroxyapatite), synthetic resins (e.g. AG, Bio-Rex, Chelex), or polysaccharides (e.g. Sephadex, Sepharose and Sephacel). The nature of the charged groups determines the strength of the ion exchanger whereas their total number and accessibility are responsible for the capacity.

Some of these groups are shown in the following table.

Anion Exchangers	Cation Exchangers
Aminoethyl (AE) -OCH <sub>2</sub> CH <sub>2</sub> NH <sub>3</sub> <sup>⊕</sup>	Carboxymethyl (CM) -OCH <sub>2</sub> COO <sup>⊖</sup>
Diethylaminoethyl (DEAE) -OCH <sub>2</sub> CH <sub>2</sub> NH <sup>⊕</sup> (CH <sub>2</sub> CH <sub>3</sub> ) <sub>2</sub>	Phospho -PO <sub>4</sub> H <sup>⊖</sup>
Quaternary aminoethyl (QAE) -O(CH <sub>2</sub> ) <sub>2</sub> N <sup>⊕</sup> (C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub> CH <sub>2</sub> CH(OH)CH <sub>3</sub>	Sulphopropyl (SP) -CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> SO <sub>3</sub> <sup>⊖</sup>

Table 1. Functional groups used for ion exchangers.

This technique has been widely employed in SPSS for

separating the desired free amino peptide product from terminated sequences. Where capping reagents such as acetic anhydride, N-acetylimidazole or isopropenyl formate have been used, both anion and cation exchange chromatography have proved to be useful for isolating the peptide of interest. In the case of 3-nitrophthalic anhydride, the resulting 2-carboxy-3-nitrobenzoyl derivatives are strong acids and can be readily separated on an anion exchanger<sup>91</sup>. 3-Sulphopropanoic acid anhydride<sup>92</sup> as well as the structurally closely related 2-sulphobenzoic acid anhydride<sup>95</sup> have also been employed to facilitate product purification.

In a reciprocal strategy described by Krieger *et al.*<sup>96</sup> (Section 1.2.7), in which a tag (affinity reagent) is attached selectively to the desired peptide, ion exchange chromatography has also been useful. Suzuki *et al.*<sup>97</sup> used lysine as a highly polar handle (removable by Edman degradation) for simplifying the purification of the B chain of human insulin. Other examples include the use of *m* or *p*-sulphophenylisothiocyanate<sup>98,99</sup> and the 9-(2-sulpho)fluorenylmethyloxycarbonyl (Sulfmoc) group<sup>100</sup>. In the latter case, the strongly acidic Sulfmoc-peptide is easily separated from terminated sequences by anion exchange chromatography. Removal of the Sulfmoc group can be carried out using mild bases to give the desired free amino peptide.

#### 1.2.4 High Performance Liquid Chromatography

Reversed-phase HPLC has been by far the most widely used mode of HPLC.

Octadecyl ( $C_{18}$ ) and octyl ( $C_8$ ) stationary phases, chemically bonded on a microparticulate silica gel support, have been commonly used as column packing materials. Supports containing  $C_3$  and  $C_4$  functionalities have occasionally been used for hydrophobic peptides and proteins. In this technique, molecules are separated on the basis of differences in their hydrophobicities.

Gradients with an increasing percentage of the organic solvent in an aqueous mobile phase are commonly used for elution. The ability to separate peptides with closely related structures, has made this technique, an extremely powerful tool for both analytical and preparative purposes. Parameters such as pH, mobile phase, counterion, etc. can be varied for optimum resolution. The flexibility as well as the rapidity of this technique has made it the obvious choice to gauge the complexity of a peptide mixture.

Displacement chromatography is a recently developed mode of HPLC<sup>101</sup> which also takes advantage of differences in the hydrophobicity of molecules. Hydrophobic impurities are isolated on a short precolumn trap while hydrophilic impurities are displaced from the column with an aqueous mobile phase (0.05% aqueous TFA), leaving the main column filled with homogeneous product. A gradient

elution is then applied to remove the peptide product from the main column.

This method has been particularly useful for the isolation of a synthetic polypeptide which is a promising malaria vaccine<sup>102</sup>.

#### 1.2.5 Droplet Counter-Current Chromatography

Complementary to conventional chromatographic methods, droplet counter-current chromatography (DCCC)<sup>103</sup> has been widely applied to the isolation of natural products.

DCCC is a liquid-liquid separation technique relying on the partition of a solute between two immiscible solvents.

The main advantages of this technique are outlined below:

1. As no solid support is present, irreversible adsorption is avoided; thus, all material is recoverable.
2. Quantities ranging from mg to g can easily be handled and the solvent consumption is small.
3. DCCC is a simple, economical method and is therefore particularly suited for preparative large scale purification.

Some applications of DCCC for the purification of peptides have been reviewed by Hostettmann<sup>104</sup>. Recently, the transferability of this technique on an industrial

setting has been clearly demonstrated in the case of somatocrinin<sup>105</sup>.

However, the efficiency of the method depends entirely upon droplet formation which is a serious limitation. Furthermore, separations remain slow (up to several days) and resolution is poorer than, for example, HPLC.

#### 1.2.6 Hydrophobic-Interaction Chromatography

In hydrophobic-interaction chromatography (HIC), substances are separated on the basis of their varying hydrophobic interactions with an uncharged bed material containing hydrophobic groups (e.g. octyl and phenyl Sepharose).

This technique has been widely applied for the purification of proteins. Its application in peptide synthesis remains very limited. Examples include the purification of  $\alpha$ -factor<sup>106</sup> and the separation of a particular hydrophobic peptide from a complex mixture<sup>107</sup>.

Recently, HI media have been examined as an alternative to reversed-phase media for the resolution of peptide mixtures by HPLC. Significant differences in selectivity were noted with peptides large enough to possess secondary or tertiary structures<sup>108,109</sup>.

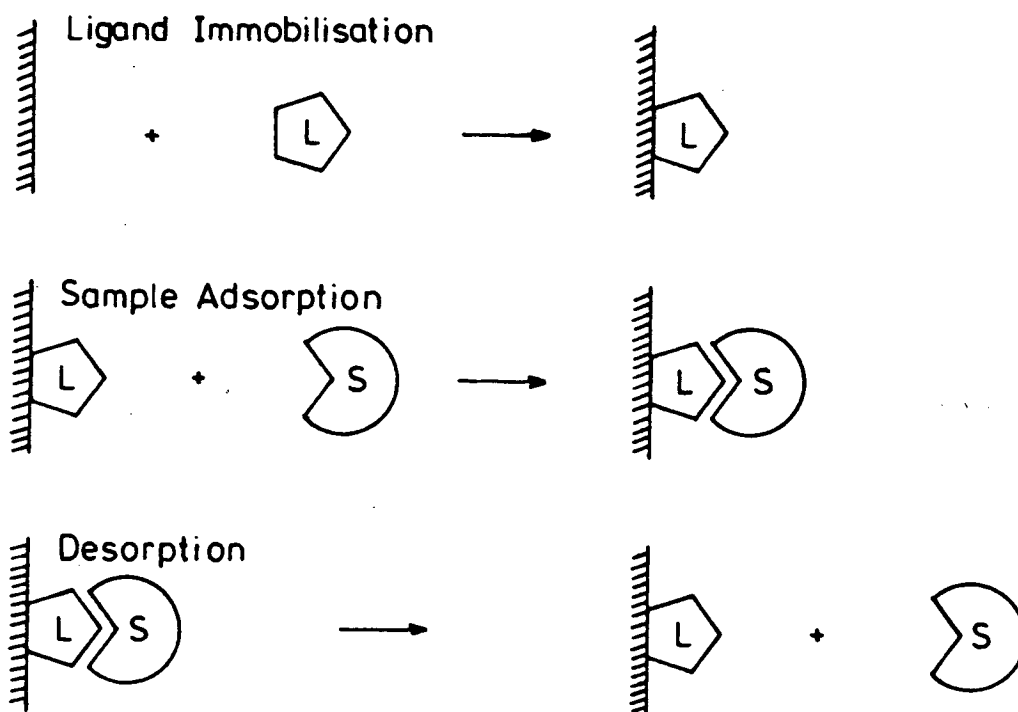
#### 1.2.7 Affinity Chromatography

Affinity chromatography is a highly selective method which allows the purification of molecules on the basis of

their biological functions or individual chemical structures. This technique makes it possible to remove large amounts of undesired materials from the solutions of interest. The method has found wide acceptance in biological research since its introduction<sup>110</sup>.

In principle, the method is based on the interactions between a specific ligand (covalently bonded to a support) and the desired substance. For example, an enzyme bonded as the ligand can be used for the purification of a substrate analogue, whereas an immobilised antibody may be suitable for the purification of an antigen.

The method involves three stages (Scheme 7).



Scheme 7. Principle of affinity chromatography.

1. A specific ligand is covalently bound to a chromatographic bed material, such as an agarose gel (Sepharose 4B, Sepharose CL), previously activated (e.g. by cyanogen bromide).
2. The gel with the immobilised ligand is packed into a column of suitable dimensions. After sample application, substances capable of specific interactions with the ligand are adsorbed on the column, while the non-adsorbed impurities are washed away.
3. The adsorbed substances are eluted from the column. This is usually achieved by either a stepwise or a continuous change in properties of the eluent. Non-selective elution involves pH or ionic strength gradients whereas selective elution uses an eluting agent (e.g. NaCl, urea) that competes for binding to the adsorbed substance or the ligand.

Affinity chromatography has been employed from time to time in SPPS and the general strategy developed by Krieger *et al.*<sup>96</sup> has been particularly useful for the purification of numerous synthetic peptides. The method involves functional selection of the N-terminal amino acid of the peptide by chemical reaction with an affinity reagent A-B, bearing an affinity group A and a binding group B. The affinity-labelled peptide can be separated from terminated sequences by selective binding to an affinity receptor.

The affinity-labelled peptide is then dissociated from the receptor, and the affinity reagent is finally removed to give the peptide of interest.

#### Covalent Chromatography

One suitable affinity reagent has been the dipeptide cysteinyl-methionine, which was employed for the purification of ribonuclease (111-124)<sup>96</sup>. The desired peptide is distinguished from terminated peptide chains by attachment of the affinity reagent (cysteinyl-methionine) before removal from the support. The carboxyl group of methionine is attached to the N-terminus of the desired peptide whereas the thiol group of cysteine can be bound to an organomercurial-agarose column (through a covalent mercury-sulphur bond). This allows separation of the derivatised peptide from terminated sequences lacking the affinity group. Following displacement from the column by elution with an organic thiol (e.g. cysteine), the peptide of interest can be obtained after removing the Cys-Met dipeptide by cyanogen bromide cleavage. This technique has also enabled the purification of a synthetic hexadecapeptide present in the sequence of allergen M from cod<sup>111</sup>.

#### "Functional" Purification

Since the interactions that form and stabilise the structure of proteins are highly characteristic of each

molecule, another approach has involved the capacity of biologically active peptides for complex formation.

This principle has been applied to the purification of synthetic ribonuclease S-peptide derivatives<sup>112</sup>. The latter were prepared by the solid phase method and purified by specific complex formation with RNase S-protein, which had been immobilised by attachment to cyanogen bromide activated agarose (Sepharose 4B). Hofmann, Smithers and Finn<sup>113</sup> have used a similar approach for the purification of synthetic S-peptide (1-20).

In this instance, advantage was taken of the complementary fit of the synthetic peptide onto natural S-protein.

#### Avidin-Biotin Purification

The high affinity of avidin for biotin<sup>114</sup> provides an example of an affinity purification procedure for large peptides, chemically synthesised by solid phase methodology.

The peptide, terminally deprotected and neutralised on the resin, is biotinylated with N-hydroxysuccinimido-biotin. Following cleavage, the crude material is applied to an avidin agarose column which is then washed with a phosphate buffer, until all unbound material has been eluted. The biotinylated peptide is finally eluted with 0.1M glycine HCl, pH 2.0.

This method has allowed the purification of several

synthetic peptide fragments including [Asp<sup>205</sup>]-interleukin-1  $\beta$  (117-269), present in less than 1% yield in the crude HF cleavage mixture<sup>115</sup>.

#### Metal Chelate Affinity Chromatography

In this technique, the separation is achieved by differential binding of substances to metal ions immobilised by chelation<sup>116</sup>. Zn, Cu, Cd, Hg, Co and Ni can be immobilised on a Sepharose or Sephadex column previously derivatised with a metal chelating ligand (e.g. iminodiacetic acid). Some amino acids (e.g. histidine, cysteine) form complexes with these metal ions, allowing the adsorbed peptide or protein to be washed free of contaminants in the sample. Elution is usually carried out by either lowering the pH of the mobile phase or introducing a displacing ligand in the buffer such as ethylenediaminetetraacetic acid (EDTA).

The potential of this technique has been illustrated by the resolution of angiotensins I and II and their seven synthetic analogues by HPLC<sup>117</sup>.

#### Antibody Binding Chromatography

Specific antibodies can be induced against almost all small molecules, and can be covalently bound to a solid support to serve as highly specific sorbents for the desired peptides that contain these small molecules.

The potential of this method has been investigated

with the use of anti-dinitrophenyl antibodies for the isolation of peptides containing at their N-terminus a dinitrophenyl moiety<sup>118</sup>.

#### Graphite Based Chromatography

In a recent article by Knox et al.<sup>119</sup>, the use of porous graphitised carbon (PGC) was examined as a potential carbon material for HPLC. This material was described as a porous 2-dimensional graphite consisting of 3-10  $\mu\text{m}$  spherical particles having a specific surface area of about 150  $\text{m}^2/\text{g}$ . PGC was found to give good peak shapes for elution of a wide range of molecules. It acted as a very strong hydrophobic adsorbent and could be deactivated by adsorption of high-molecular-weight additives.

These interesting results suggested to us that the strong affinity of PGC for hydrophobic molecules could serve as a basis for a new method of purification in peptide chemistry.

Isolation of the desired peptide from terminated sequences would be achieved if at the end of the synthesis the free amino terminal residue was blocked with a group X having the following properties:

1. It must be sufficiently large and flat to be retained on a PGC column.
2. It must be stable to the conditions of final cleavage of the peptide from the support (i.e. 95% TFA).
3. It must contain a chromophoric group ( $\lambda_{\text{max}} > 350\text{nm}$ ) in

order to enable monitoring.

The desired peptide temporarily blocked with the affinity reagent X should be retained to a greater degree than the terminated sequences when passed through a PGC column.

Fluorene derivatives meet the above criteria and were therefore chosen for further investigation.

## CHAPTER 2 : DISCUSSION

### 2.1 SYNTHESIS OF DIBENZOFLUORENES AND DERIVATIVES

Fused diarylfluorenes were selected as the target compounds.

However, a number of possible structures can be envisaged depending on the position of the additional benzenoid fused rings (Fig. 1).

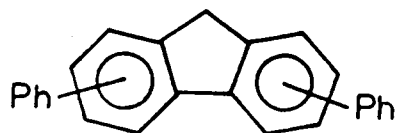


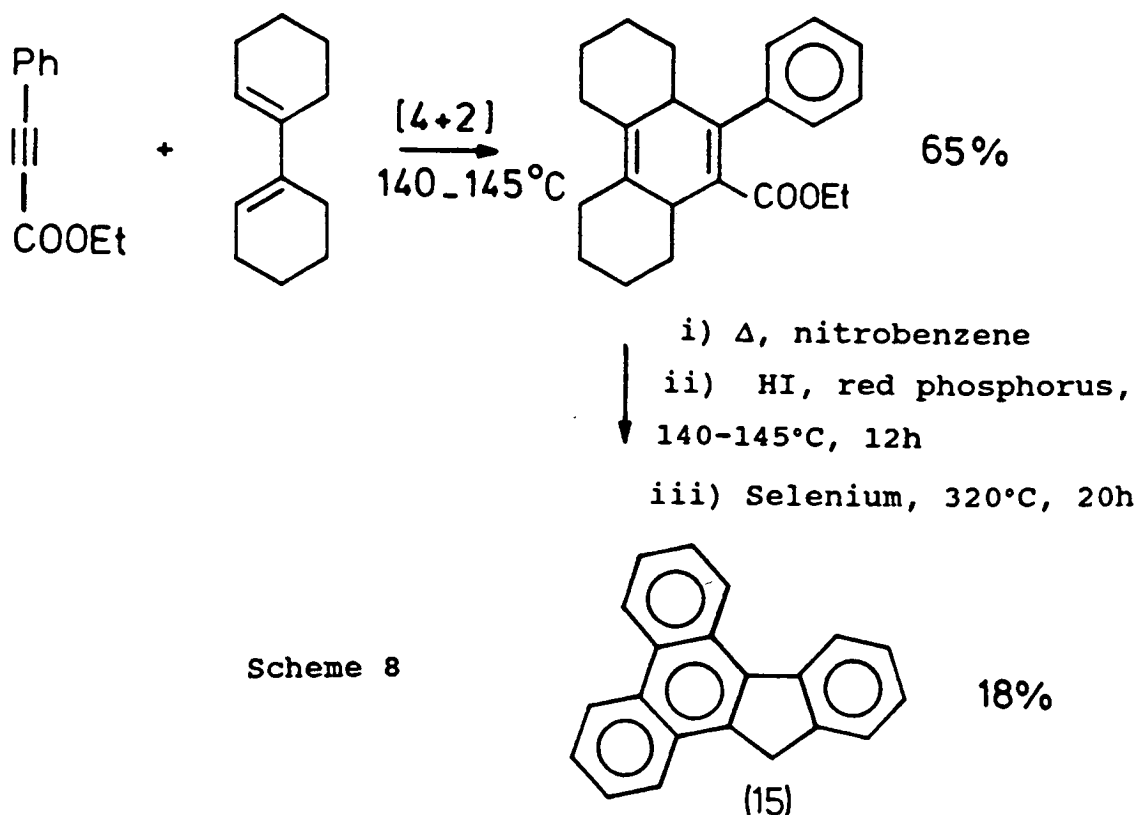
Fig.1

A review of the literature was undertaken with a view to ascertaining which ring system would be most suitable for our purpose. We required a compound which would be planar, could be prepared in reasonable quantities, and could be obtained relatively easily.

### 2.1.1 Historical Background

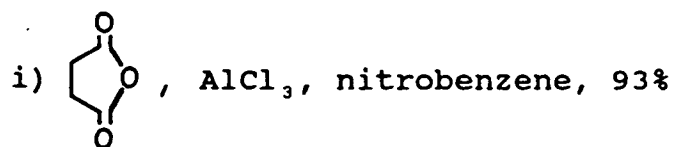
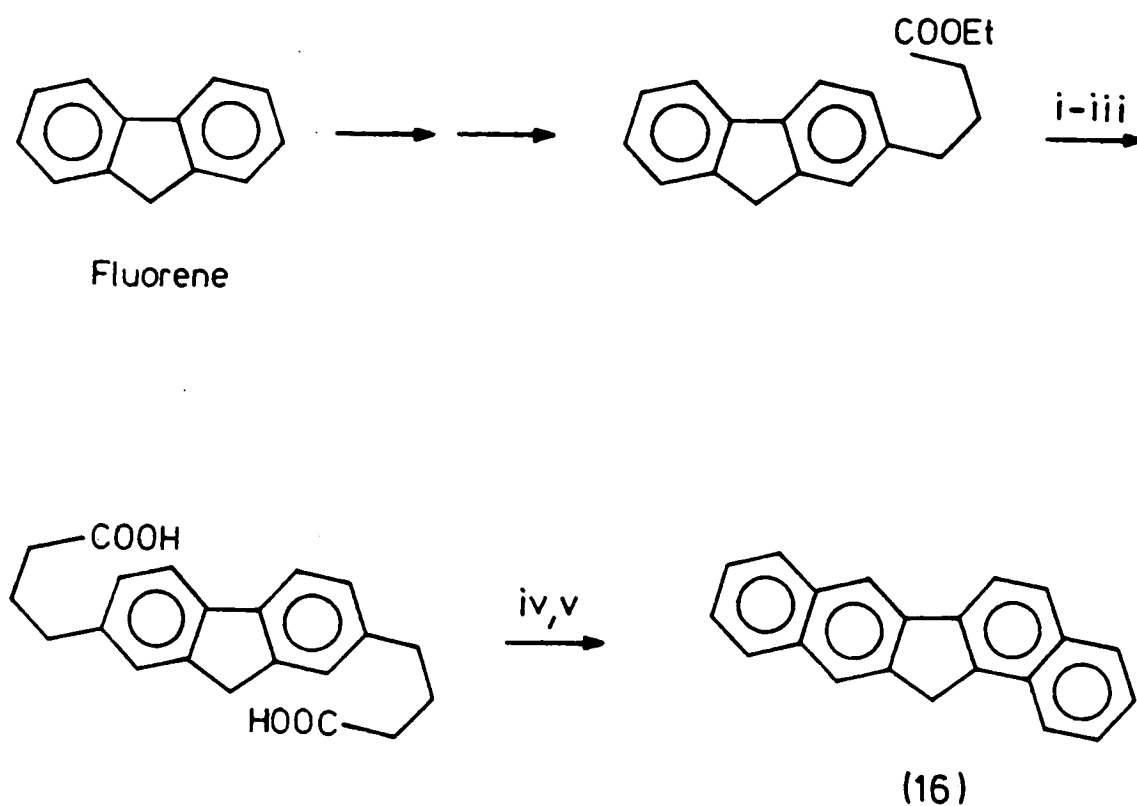
Few practicable syntheses have been described. Several steps as well as forcing conditions (e.g. heating at 300°C for several hours) are generally required, and poor yields are usually obtained. A number of the more relevant methods are outlined below.

(i) In the method of Bandyopadhyay *et al.* a Diels-Alder reaction was used to synthesise a useful intermediate which on subsequent aromatisation and cyclisation afforded 13H-dibenzo[*a,c*]fluorene<sup>120</sup> (15) (Scheme 8).



The yields for this route were moderate and the conditions difficult, making it somewhat impractical for a large scale production (notably selenium at 320°C).

(ii) Rahman and Tombesi<sup>121</sup> used a double succinoylation of fluorene followed by double cyclisation, reduction and aromatisation for their synthesis of 1,2:6,7 dibenzofluorene (16) (Scheme 9).



ii) KOH/MeOH, 87%

iii) Huang-Minlon reduction, 80%

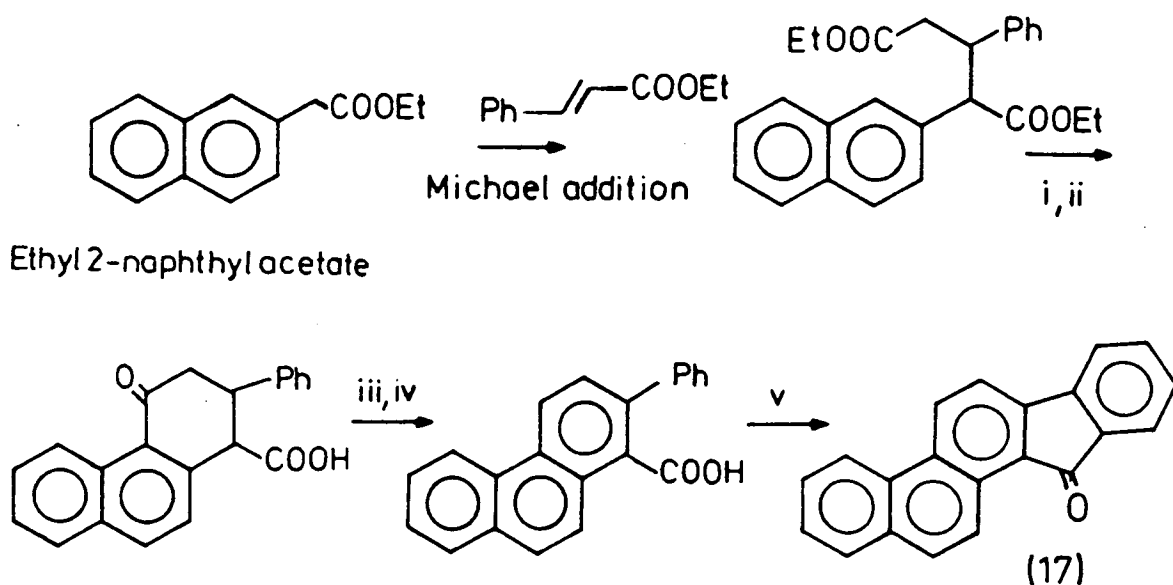
iv) PCl<sub>5</sub>, benzene

v) PtO/280-300°C, 40 min., 82%

Scheme 9

The major drawbacks of this synthesis are the cyclisation reactions which lead to the formation of other isomers (no yields are given) and the aromatisation step which requires heating to 280-300°C in the presence of PtO.

(iii) The synthesis of a polycyclic aromatic ring system was also achieved through the use of a Michael addition<sup>122</sup>. Ethyl 2-naphthylacetate was condensed with ethyl cinnamate to give ethyl  $\alpha$ -(2-naphthyl)- $\beta$ -phenylglutarate. Hydrolysis of the latter followed by cyclisation gave 4-oxo-2-phenyl-1,2,3,4-tetrahydrophenanthrene-1-carboxylic acid. After reduction, dehydrogenation and cyclisation of this intermediate, 2',1'-naphtho[a] fluorenone (17) was prepared (Scheme 10).

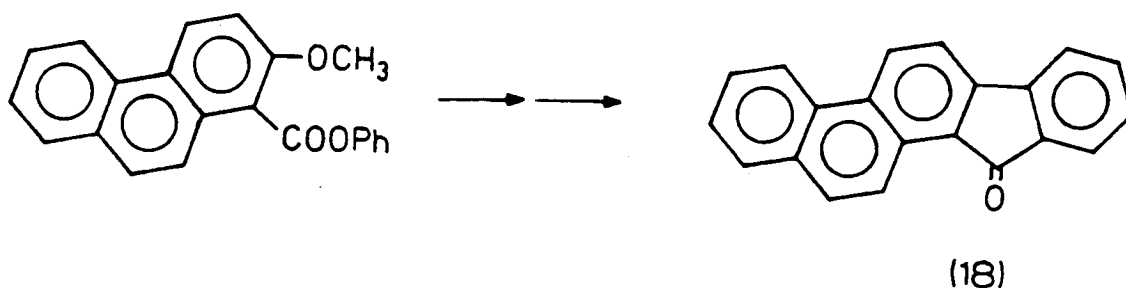


i) Hydrolysis ii) Anhydrous SnCl<sub>2</sub> iii) Huang-Minlon reduction  
iv) Pd/C 10%, boiling p-cymene v) H<sub>2</sub>SO<sub>4</sub>

Scheme 10

The main disadvantage of this approach lies in the last cyclisation step which proceeds with low yield.

(iv) In a further route, phenyl 2-methoxy-1-phenanthrenecarboxylate was utilised as a key intermediate<sup>123</sup>. Treatment of this compound with phenylmagnesium bromide furnished phenyl 2-phenyl-1-phenanthrenecarboxylate in 80% yield. Subsequent cyclisation in the presence of conc.  $H_2SO_4$  gave 11H-indeno[2,1-a]phenanthren-11-one (18) in good yield (Scheme 11).

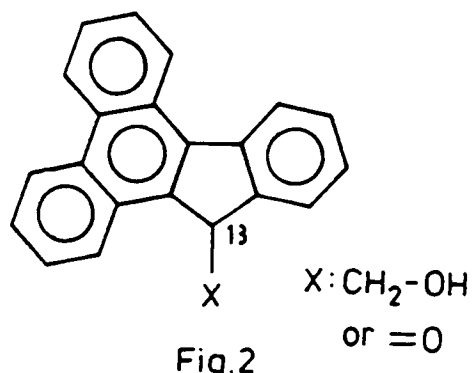


Scheme 11

(v) A photochemical method proved successful for the synthesis of 13H-dibenzo[a,c]fluorene (15) from 2,3-diphenylindene<sup>124</sup>.

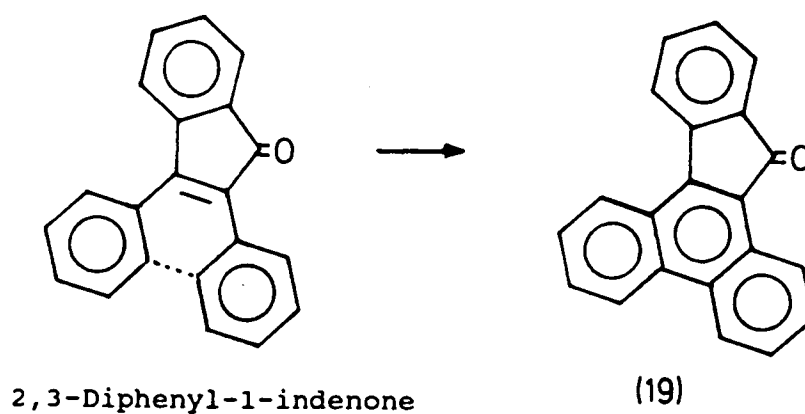
In the last two approaches, however, the major limitations are the syntheses of the key intermediates which involve difficult steps and proceed in low yields.

After considering the literature we decided to aim mainly for dibenzo(a,c)fluorenes functionalised at the C(13) position (fig. 2) and four possible routes to the synthesis of these derivatives were then investigated.



### 2.1.2 Synthesis of Dibenzo(a,c)fluorenes

The possible cyclisation of 2,3-diphenyl-1-indenone to dibenzo[a,c]fluorene-11-one (19) was studied first (Scheme 12).



Scheme 12

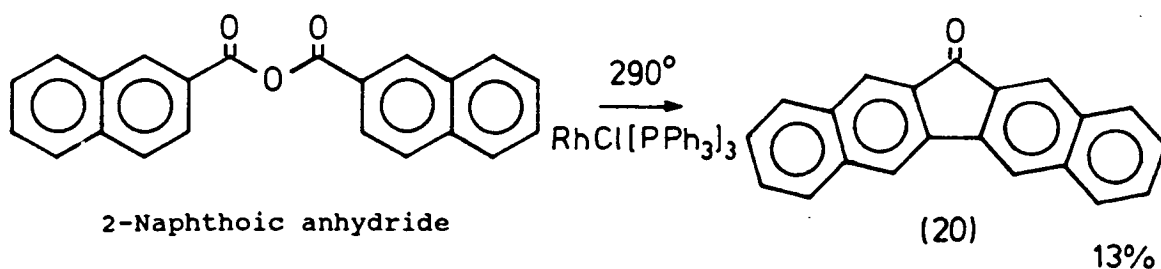
The cyclisation was attempted by several methods:

- $\text{AlCl}_3$ ,  $\text{NaCl}$ ,  $200^\circ\text{C}$  (Scholl reaction).
- $\text{AlCl}_3$ ,  $\text{CH}_2\text{Cl}_2$ , reflux (Friedel-Crafts reaction).
- $h\nu$ ,  $\text{I}_2$  (photochemistry)<sup>125</sup>.
- $h\nu$ ,  $\text{I}_2$ , trifluoromethanesulphonic acid (photochemistry)<sup>125</sup>.

Unfortunately, none of these methods resulted in formation of the desired product as judged by t.l.c. or  $^1\text{H}$  n.m.r. of the crude material. Either no reaction occurred or a black tar was formed.

This approach was abandoned.

Although the initial target compounds were dibenzo-(a,c)fluorenes, we were not confined to this ring system. Blum et al. reported the synthesis of dibenzo[b,h]-fluorene-12-one (20)<sup>126</sup>. If we could prepare this material and also derivatives functionalised at position 13, this method would provide a short synthesis of compounds suited to our purposes (Scheme 13).



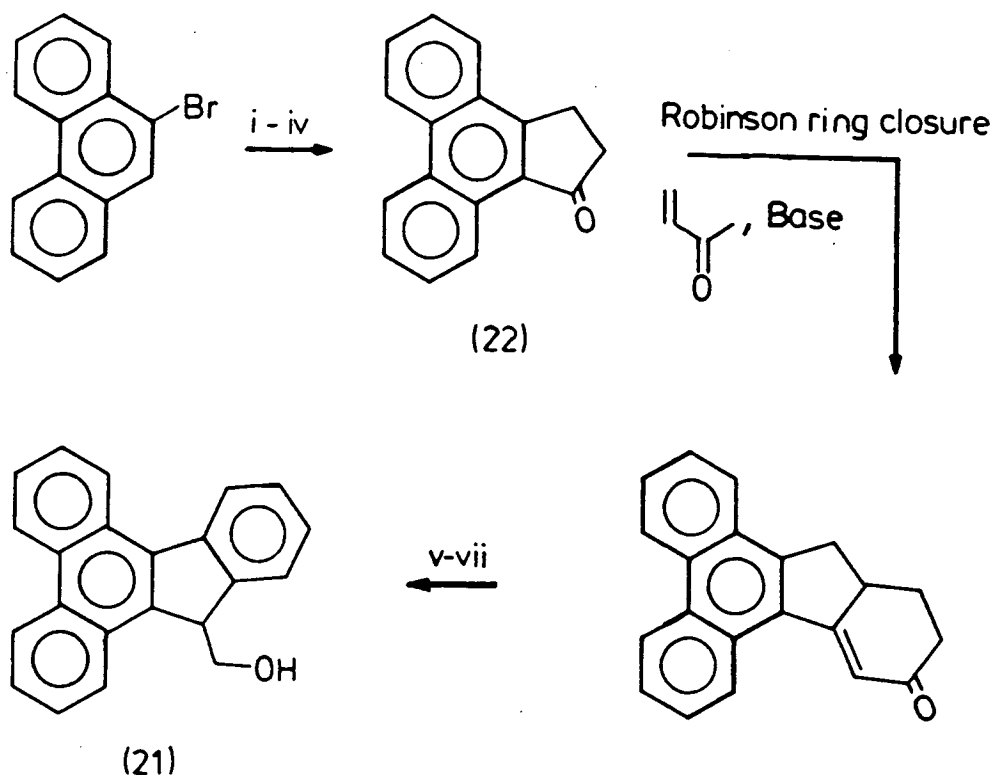
Scheme 13

2-Naphthoic anhydride was heated in the presence of  $\text{RhCl}[\text{P}(\text{C}_6\text{H}_5)_3]_3$  catalyst at  $290^\circ\text{C}$ , according to the literature method. Unfortunately, the desired product could not be isolated.

One possible explanation of this result is that the crude 2-naphthoic anhydride used may have contained impurities which could have poisoned the catalyst. The crude anhydride was prepared from 2-naphthoic acid using a large excess of acetic anhydride and a catalytic amount of sulphuric acid.

Considering the difficulty of obtaining 2-naphthoic anhydride sufficiently pure, and the poor literature yield (13%) for the synthesis of (20), this approach was not pursued further.

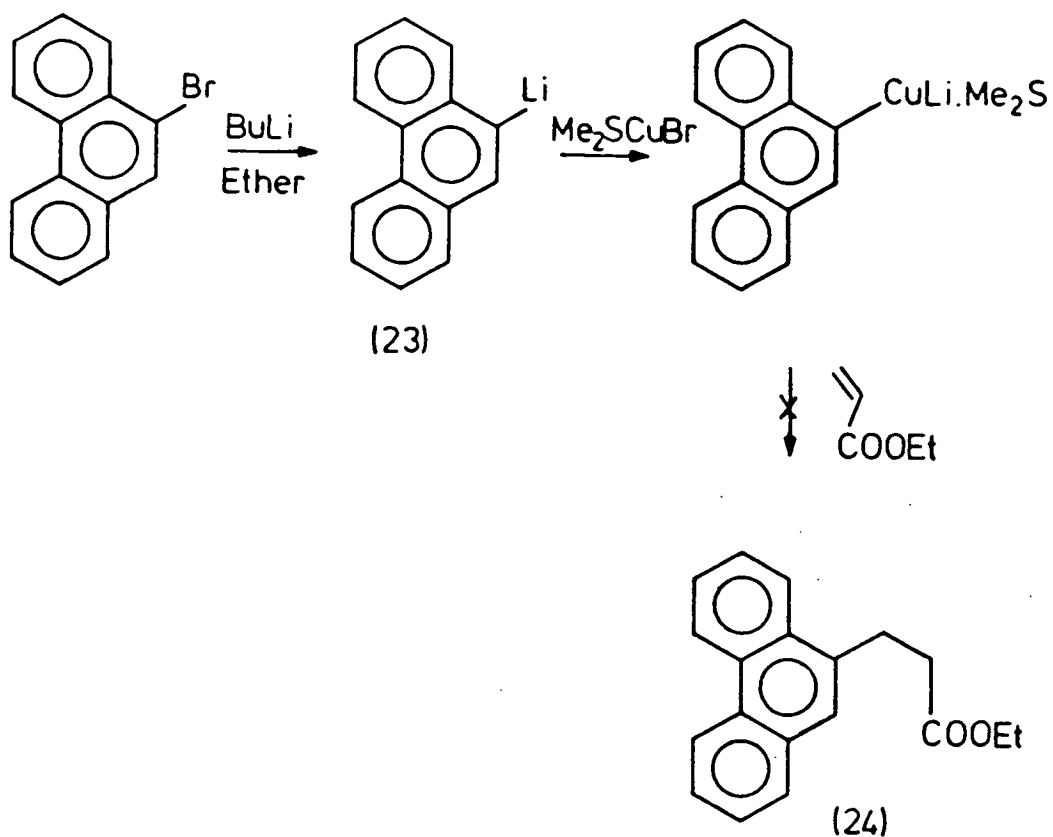
The possibility of using a Robinson ring closure as the key step for the preparation of 13-hydroxymethyl dibenzo(a,c)fluorene (21) was also investigated. The approach we adopted is outlined in Scheme 14. An important intermediate is ketone (22) but the synthesis of this compound proved difficult.



i) BuLi, ether ii)  $\text{Me}_2\text{S} \cdot \text{CuBr}$ , cuprate iii)  $\text{CH}_2=\text{CHCOOEt}$ , Michael addition iv) Cyclisation v) Reduction vi) Aromatisation vii) Base, HCHO

#### Scheme 14

9-Bromophenanthrene was first converted to 9-phenanthrenyl lithium (23) by treatment with BuLi in  $\text{Et}_2\text{O}$ <sup>127</sup>. The corresponding lithium organocuprate reagent was then generated according to the methodology described by House et al.<sup>128</sup>. A Michael addition with ethyl acrylate was then attempted at several temperatures between  $-65^\circ\text{C}$  and room temperature but unfortunately the required product (24) could not be obtained (Scheme 15). In each case, phenanthrene was isolated as the major product.

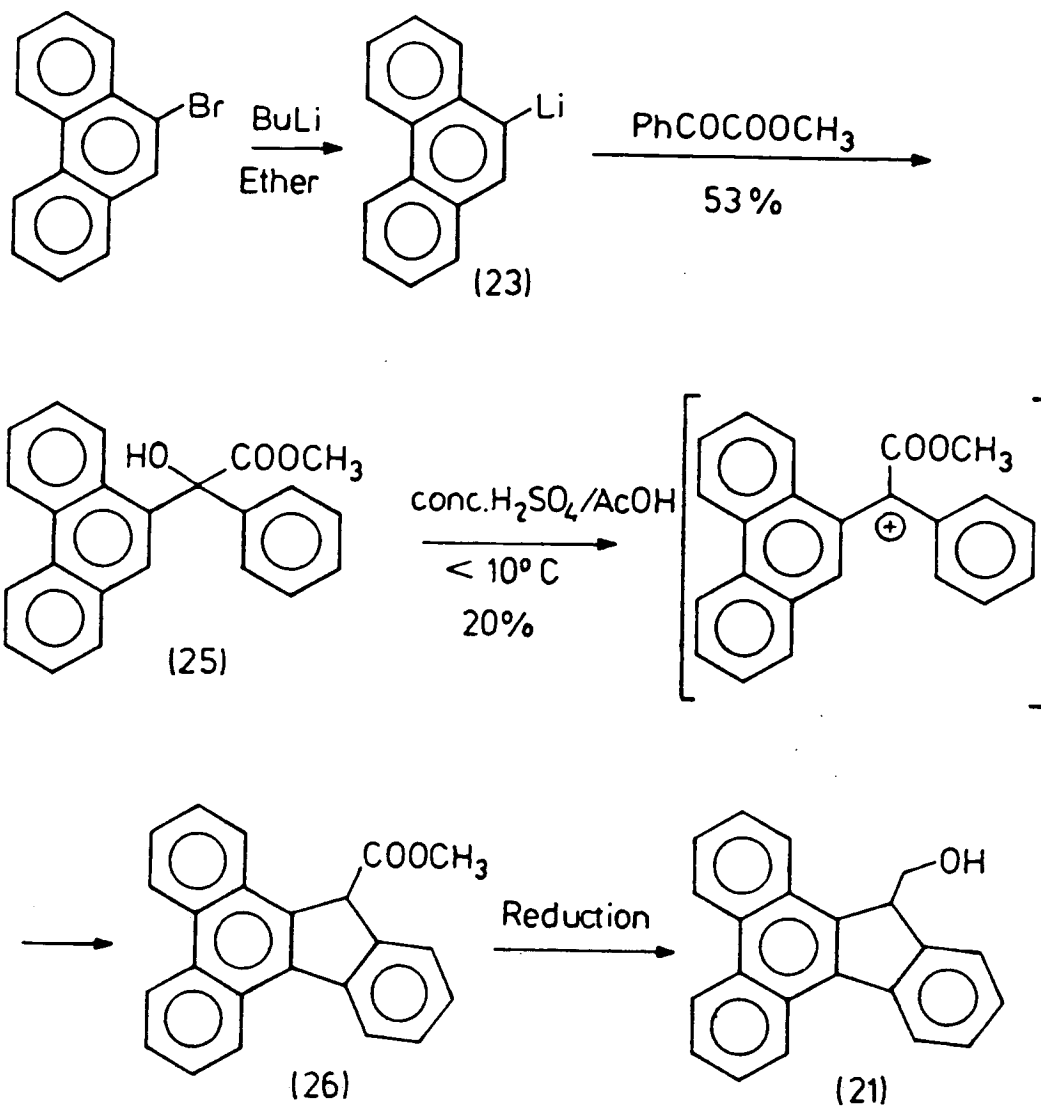


Scheme 15

It is possible that the phenanthrenyl cuprate reagent was not generated *in situ*, because of steric hindrance.

This approach was also abandoned.

Finally, the procedure by Hopkinson *et al.*<sup>129</sup> for the synthesis of benzofluorenes via the rearrangements of  $\alpha$ -alkoxycarbonyldiarylmethyl cations was examined (Scheme 16). This method enables 13-methoxycarbonyldibenzo(a,c)fluorene (26) to be readily obtained in three steps.

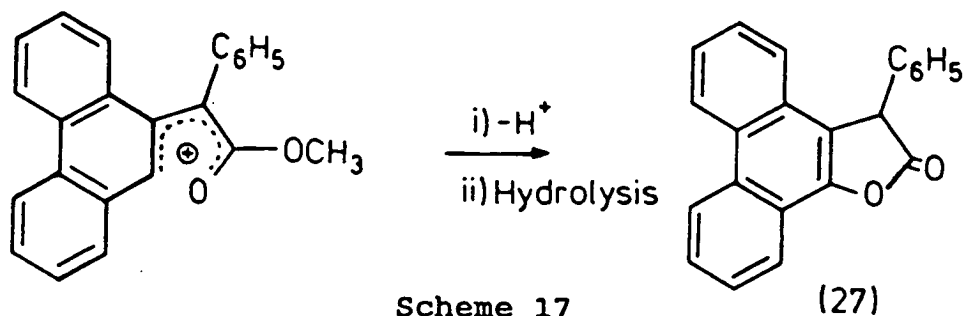


Scheme 16

If this compound (26) could be reduced, this approach would provide a facile synthesis of the required alcohol (21).

The synthesis of methyl (phenanthren-9-yl, phenyl) glycolate (25) was carried out in ether by reaction of 9-phenanthrenyl lithium (23) with methyl benzoyl-

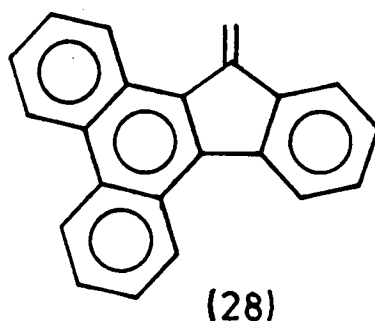
formate. Treatment of (25) under acidic conditions (conc.  $\text{H}_2\text{SO}_4$ ,  $\text{CH}_3\text{COOH}$  at  $10^\circ\text{C}$ ) led to the formation of (26) in poor yield. The major product was the lactone (27) (46%) (the formation of which may be rationalised by  $4\pi$ -electrocyclisation of the carbonium ion shown in Scheme 17).



These results are in agreement with those described in the literature.

In an attempt to improve the yield of the reaction, polyphosphoric acid (PPA) was tried as an alternative catalyst. Surprisingly, treatment of (25) with PPA at  $110^\circ\text{C}$ , led to the formation of the desired ester (26) in 42% yield. The lactone (27), in this case, was a minor product (11%).

The reduction of ester (26) proved difficult. The use of excess (3 equiv.)  $\text{LiAlH}_4$  at  $0^\circ\text{C}$  did not give the expected alcohol (21), whereas  $\text{LiBH}_4$  gave a trace of the desired product, which could not be isolated.  $^1\text{H}$  n.m.r. showed the presence of 13H-dibenzo(a,c)fluorene (15) (singlet at 3.9 ppm corresponding to the  $\text{CH}_2$ ) and also the olefin (28) (singlet at 5.3 ppm corresponding to the  $=\text{CH}_2$ ).

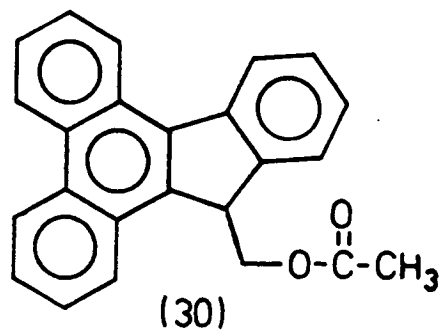
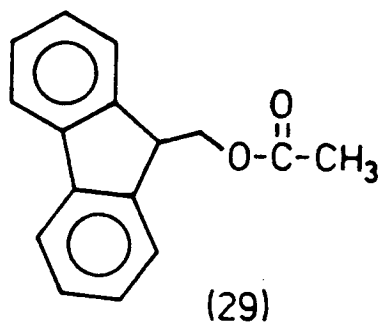


Two further attempts using  $\text{NaBH}_4$  in a large excess (5 to 16 equiv.) in a methanol-dichloromethane mixture were unsuccessful. The reduction was finally achieved in 48% yield using three equivalents of diisobutylaluminium hydride (DIBAL-H) in dichloromethane at  $-50^\circ\text{C}$ .

This last route provided a synthesis of 13-hydroxymethyldibenzo(a,c)fluorene (21) in four steps with an overall yield of 10%.

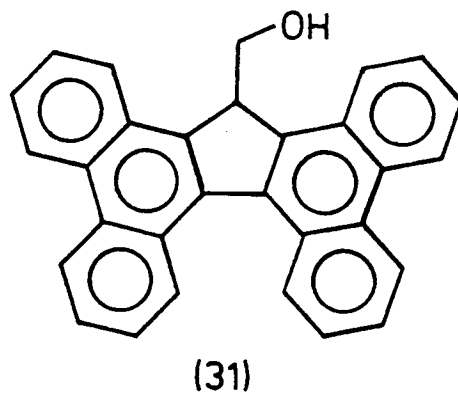
The acetate of both the alcohol (21) and 9-fluorene-methanol were then prepared in order to compare their behaviour on an HPLC column packed with PGC.

9-Fluorenylmethyl acetate (29) gave a retention time of 3.3 min (eluted with  $\text{CHCl}_3$ ), whereas 13-acetoxymethyl dibenzo(a,c)fluorene (30) under the same conditions, was not completely retained on the column but was slowly eluted.



These results were encouraging. However, in an ideal situation the compound would be totally retained. Hence modification of alcohol (21) was required.

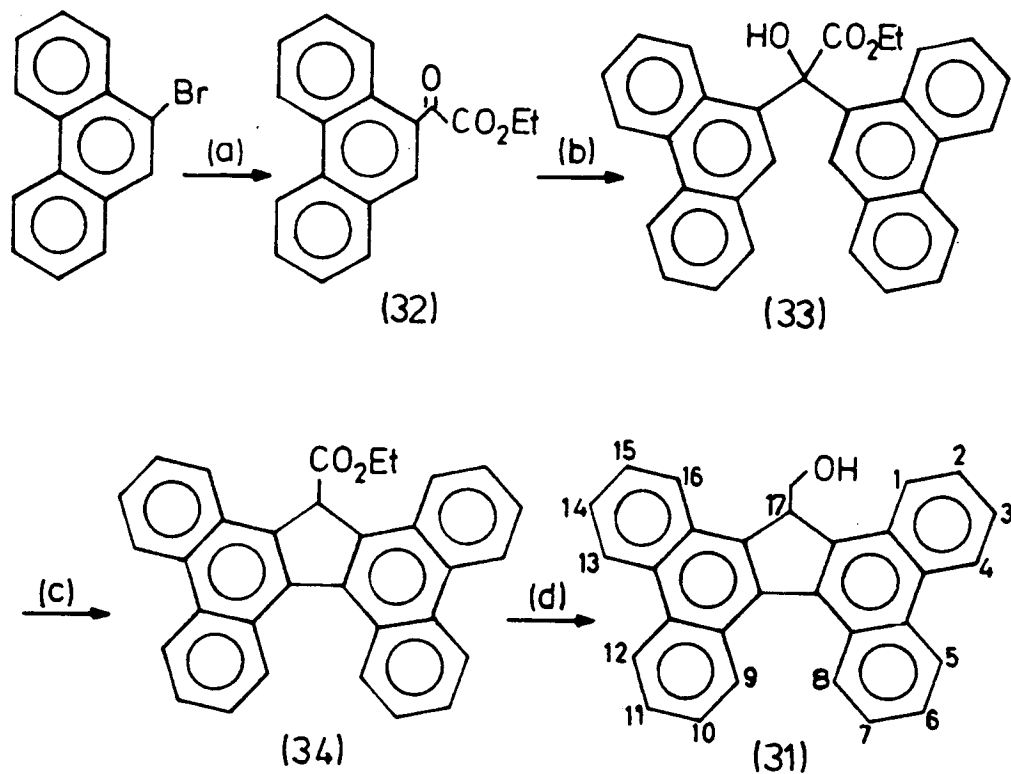
A bigger and symmetrical compound (31) was designed by adding two more aromatic rings on (21), to both improve the retention on the column and avoid the problem of a chiral centre at C(13), which could complicate purification of intermediates.



## 2.2 SYNTHESIS OF TETRABENZOFLUORENES AND DERIVATIVES

### 2.2.1 Synthesis of 17-Hydroxymethyltetrabenzo(a,c,g,i)fluorene

The tetrabenzofluorenyl alcohol (31) was prepared in a way analogous to that described for the dibenzo derivative (21) (Scheme 18).



(a) BuLi, Et<sub>2</sub>O, r.t., 30 min; EtOCCOOEt, Et<sub>2</sub>O, 0°C-5°C, 2h, 63%; (b) 9-Bromophenanthrene, BuLi, Et<sub>2</sub>O, r.t., 30 min; (32), Et<sub>2</sub>O, 0°C-5°C, 2h, 46%; (c) PPA, 140°C, 4h, 49%; (d) DIBAL-H (3 equiv.), CH<sub>2</sub>Cl<sub>2</sub>, -65°C, 1h, 73%.

Scheme 18

Initially the direct synthesis of compound (33) from 9-phenanthrenyl lithium (23) (obtained upon treatment of 9-bromophenanthrene with BuLi in ether<sup>127</sup>) was attempted. Reaction of 2 equivalents of the lithium salt with 1 equivalent of diethyl oxalate, gave a mixture of products from which the expected tertiary alcohol (33) could not be isolated.

Fortunately, synthesis and isolation of the  $\alpha$ -keto ester (32) was possible. This was achieved by adding 9-phenanthrenyl lithium to a solution of diethyl oxalate (20% excess) in ether at 0°C. The reaction mixture was subsequently heated under reflux for 2 hours. Following purification by flash chromatography, the product was isolated in 23% yield.

In an attempt to improve the yield of this reaction, tetrahydrofuran (THF) was tried in place of ether as the solvent.

In THF a clear orange solution of 9-phenanthrenyl lithium was obtained and the subsequent reaction in THF with diethyl oxalate gave a complex mixture, in which the major product was found to be 9(1'-butyl)-phenanthrene (10%).

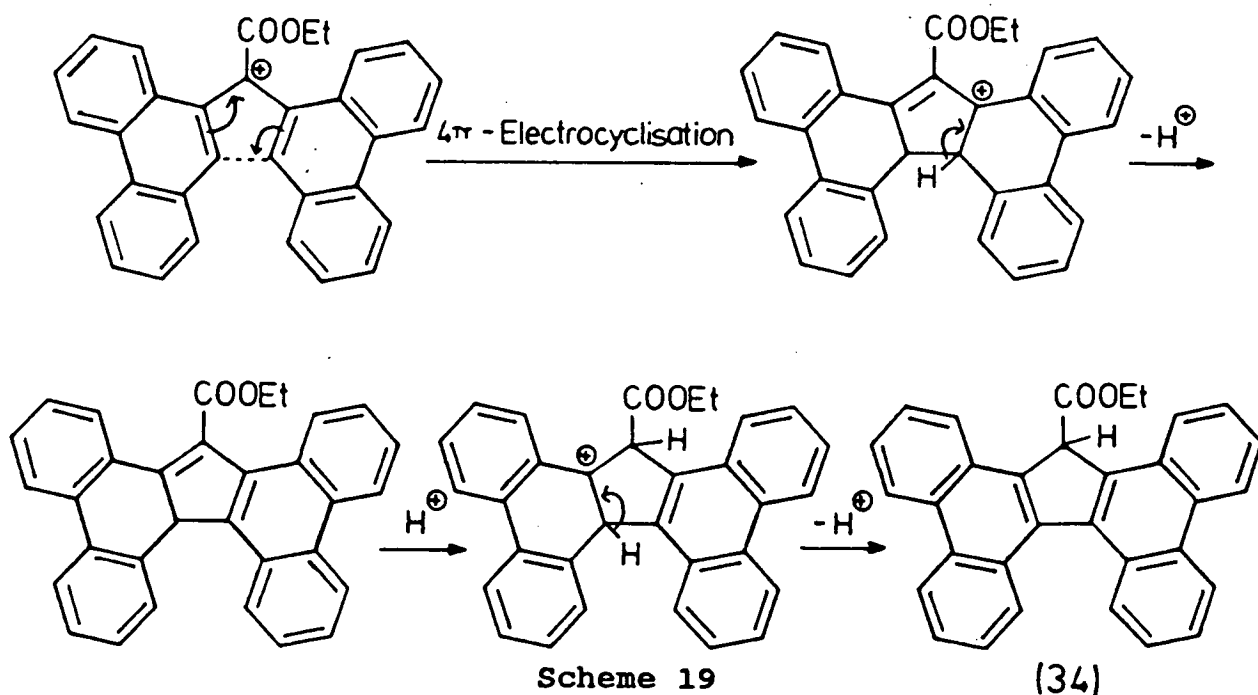
An explanation of these results might be that 9-phenanthrenyl lithium (23) forms a precipitate when the reaction is carried out in ether, and this allows the by-product, n-butyl bromide, to be almost entirely removed by

syringe<sup>127</sup>. This precaution reduces the competing reaction of 9-phenanthrenyl lithium with n-butyl bromide, which results in the formation of 9(1'-butyl)-phenanthrene.

The best synthesis of (32) was finally achieved in ether, by carefully adding 9-phenanthrenyl lithium to a solution of diethyl oxalate (20% excess), between 0°C and 5°C, and subsequently stirring the reaction at room temperature (63% yield).

The tertiary alcohol (33) was then synthesised in adequate yield (46%) under similar conditions using (32) as the keto ester component.

Treatment of (33) with polyphosphoric acid at 140°C led to the formation of the cyclic ester (34) in 49% yield via 4 $\pi$ -electrocyclisation of the  $\alpha$ -ethoxycarbonyl diaryl cation (Scheme 19).



Purification of (34) proved difficult, due to the formation of side products whose structures have not been identified. Recrystallisation of the crude material following flash chromatography, was not sufficient to give completely satisfactory analytical results (cf: elemental analysis and infrared spectroscopy). A small peak at  $1810\text{ cm}^{-1}$  compatible with a  $\alpha,\beta$ -unsaturated lactone could be detected in the IR spectrum.

The modified procedure developed by Eaton *et al.*<sup>130</sup>, for general cyclisation of this type, involved the use of a 1:10 phosphorus pentoxide - methanesulphonic acid solution in place of polyphosphoric acid. However, under these conditions, the cyclisation of the tertiary alcohol (33) was unsuccessful; at room temperature no reaction occurred, and upon heating at  $90\text{-}100^\circ\text{C}$  for 1h, a black polymer was formed yet again.

Reduction of ester (34) to the corresponding alcohol (31) proceeded in a straightforward manner (73% yield). Interestingly, a trace of the aldehyde could be detected. This was confirmed by mass spectrometry:  $m/z$  (EI)  $394(\text{M}^+, 100)$ ,  $365(\text{M}^+-\text{CHO}, 30)$ ,  $178(48)$ . The reaction was carried out in DCM at  $-65^\circ\text{C}$  using 3 equivalents of DIBAL-H.

Following flash chromatography and recrystallisation from DCM, crystals of (31) were obtained and proved suitable for X-ray analysis.



The crystal structure of alcohol (31) reveals several interesting features (Fig. 3a and Fig. 3b). It indicates that there are two molecules held together by hydrogen bonding between the two hydroxyl groups. The exocyclic hydroxymethyl group has two different conformations, probably as this is required for the formation of the hydrogen-bonded dimer. The interaction between H<sub>8</sub> and H<sub>9</sub> (d = 2.03 Å, Van der Waal's radius for hydrogen = 1.2 Å) is responsible for the degree of non-planarity in the molecule.

This molecule is chiral if it adopts a fixed orientation, as in the crystal structure. Therefore it is possible that in solution at low temperature, the molecule may also adopt a fixed orientation and be chiral. <sup>1</sup>H n.m.r. at room temperature in CDCl<sub>3</sub> showed a triplet (δ = 5.05 ppm) as well as a doublet (δ = 4.5 ppm), corresponding to H(17) and the two protons of the methylene group respectively. A probable explanation is that the phenanthrene rings can oscillate rapidly during the acquisition period and consequently the protons of the CH<sub>2</sub> group are magnetically equivalent (as are the matched pairs of aromatic protons, e.g. H(2) and H(15)).

Nuclear Overhauser experiments carried out on this compound showed the close through-space interactions between the methylene group and the two aromatic protons H(1) and H(16).

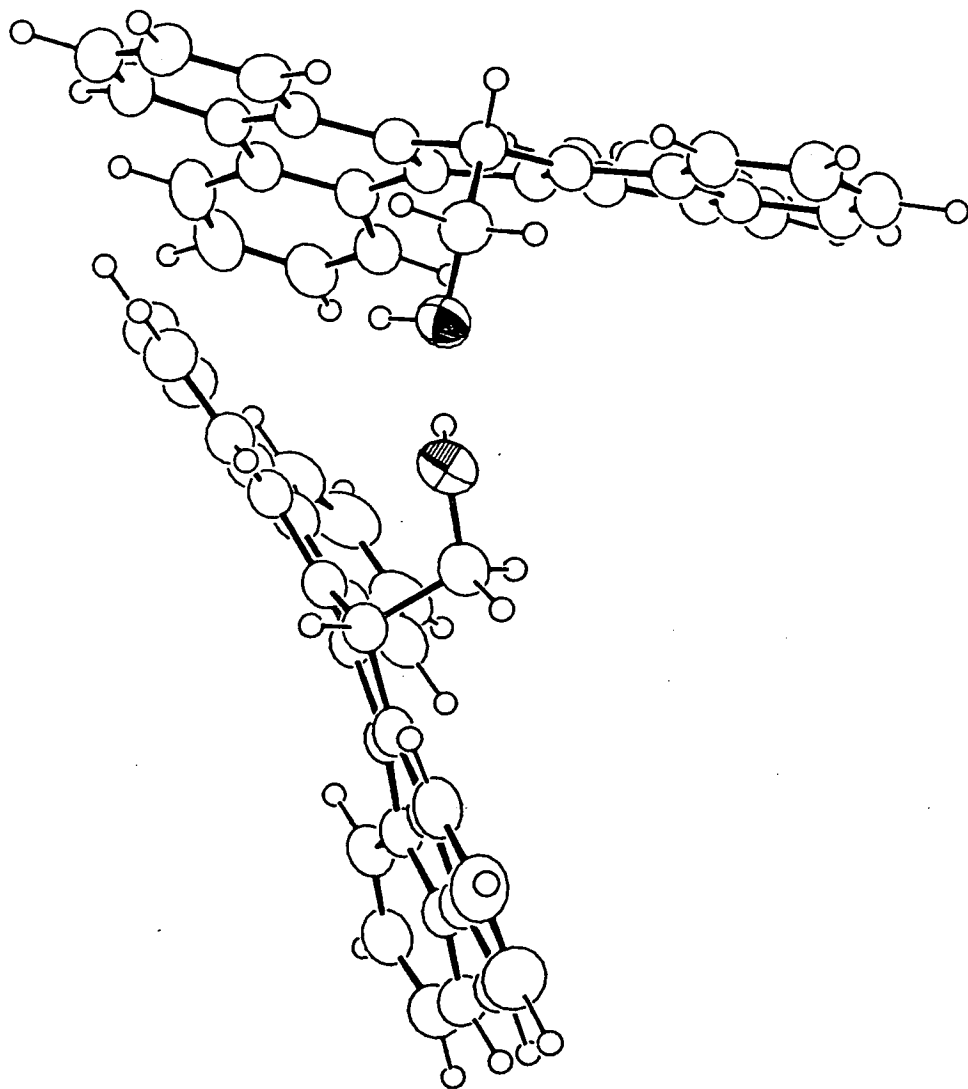


Fig. 3. X-ray crystal structure, (a) 'side' view of (31) showing the unit cell.

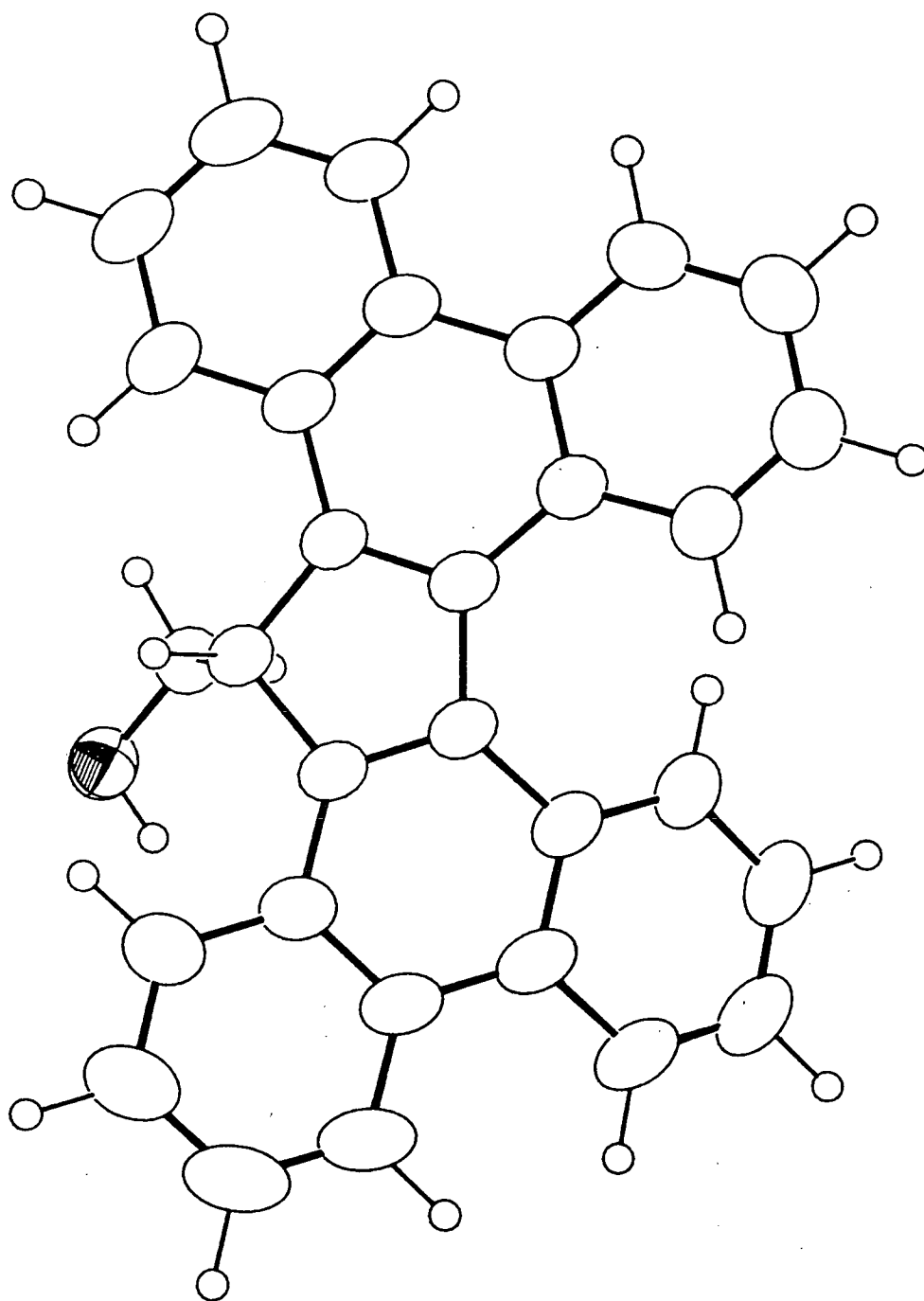
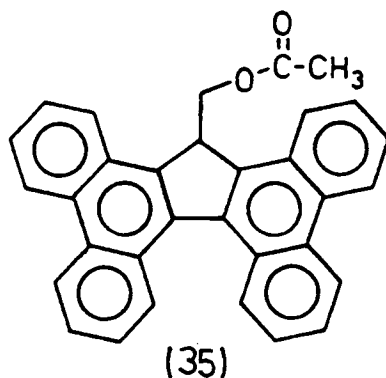


Fig. 3. X-ray crystal structure, (b) 'overhead' view of (31).

Finally, 17-acetoxymethyltetra benzo(a,c,g,i)fluorene (35) was easily prepared in 80% yield from alcohol (31) using a large excess of acetic anhydride and a catalytic amount of sulphuric acid.



As expected, the acetate (35) was totally retained when passed through an HPLC column packed with PGC (eluent  $\text{CHCl}_3$ )<sup>131</sup> whilst under the same conditions 13-acetoxymethyldibenzo(a,c)fluorene (30) was slowly eluted.

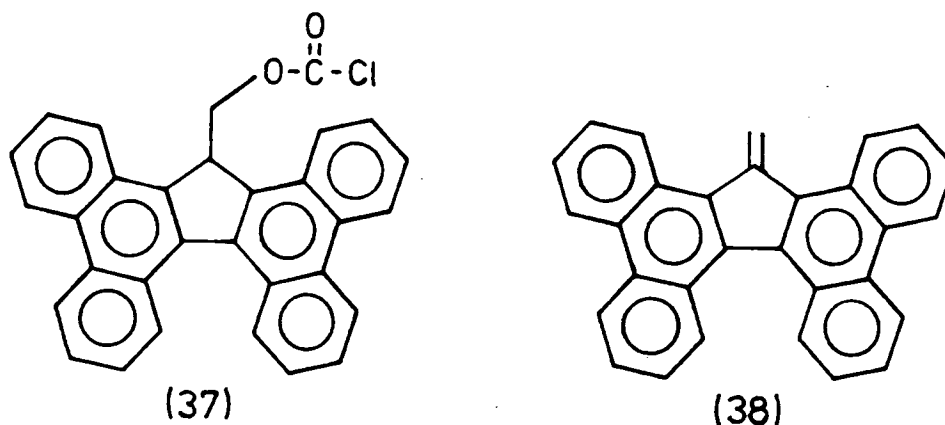
These results were considered to be satisfactory, and hence the possibility of linking alcohol (31) to an amino acid via the chloroformate or the N-succinimidyl carbonate was investigated.

### 2.2.2 Synthesis of Tbfmoc Gly OH

Preparation of  $\text{N}^\alpha$ -17-tetra benzo(a,c,g,i)fluorenyl-methoxycarbonylglycine (Tbfmoc Gly OH) (36) was first examined via the aryl chloroformate (37).

Unfortunately, reaction of the tetrabenzofluorene alcohol (31) with phosgene in the presence of a strong

base such as triethylamine or N-methylmorpholine led to the formation of the olefin (38) as the major product (indicated by a singlet at 6.9 ppm by  $^1\text{H}$  n.m.r. corresponding to the methylene protons).



The possibility of repeating the reaction using, this time, a weaker base such as *N,N'*-dimethylaniline ( $\text{pK}_a = 5.15$ )<sup>132</sup> was then investigated. Under these conditions, the desired product could be obtained *in situ* (reaction followed by IR) together with the hydrochloride salt of the base and a trace of the olefin.

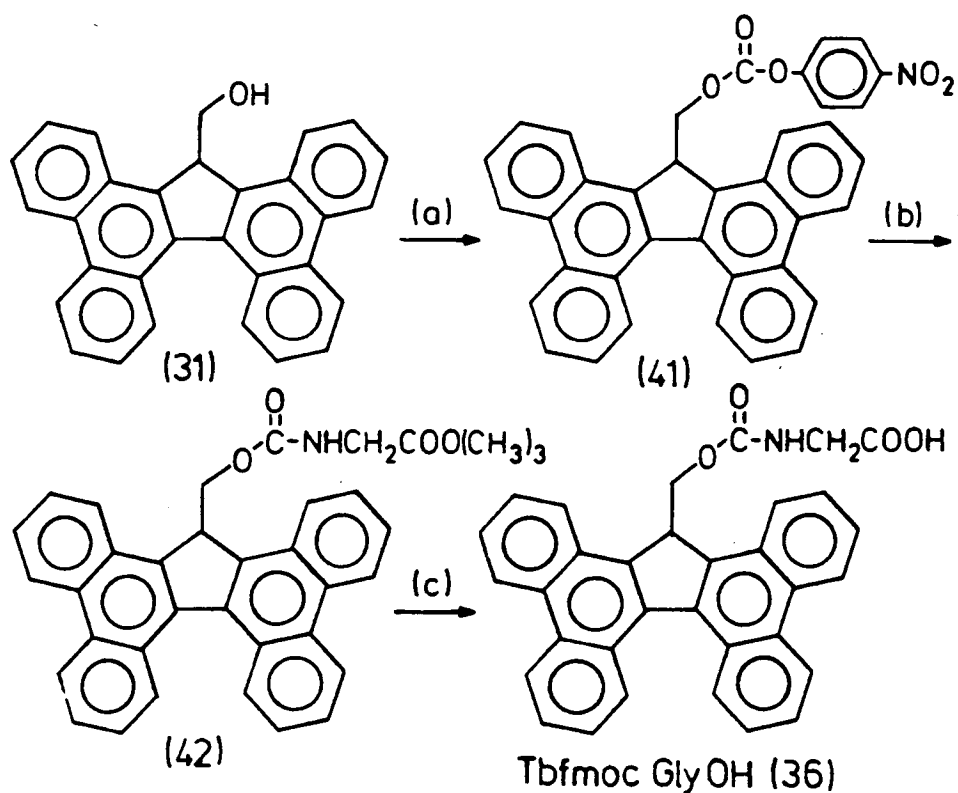
However, purification by chromatography or aqueous work-up to remove the hydrochloride salt remained unsuccessful, due to the instability of this compound in solution with respect to water and base.

The chloroformate (37) was finally synthesised by the following sequence of reactions: treatment of alcohol (31) with *N,N'*-bis-trimethylsilyl urea (2.5 equiv) to give the corresponding trimethylsilyl ether (39), followed by



However, it was not possible to separate the carbonate (present in 37% yield) from unreacted *N*-hydroxy-succinimide.

Tbfmoc Gly OH was finally synthesised in three steps from alcohol (31) via the *p*-nitrophenyl carbonate (41) as depicted in Scheme 21.



(a) *para*-Nitrophenyl chloroformate (2 equiv), *N,N'*-dimethylaniline (1 equiv), CH<sub>2</sub>Cl<sub>2</sub>, r.t., 72h, 80%;  
 (b) CH<sub>3</sub>COOH · NH<sub>2</sub>CH<sub>2</sub>COOC(Me)<sub>3</sub>, *N,N'*-dimethylaniline (2 equiv), CH<sub>2</sub>Cl<sub>2</sub>, r.t., 24h, 79%; (c) *para*-toluenesulfonic acid (0.3 equiv), CH<sub>2</sub>Cl<sub>2</sub>, reflux, 5h, 90%.

Scheme 21

Reaction of commercially available *para*-nitrophenyl chloroformate (2 equiv) with alcohol (31) and *N,N'*-dimethylaniline gave the mixed carbonate (41) in 80% yield. This material was in turn reacted with the acetate salt of glycine *tert*-butyl ester in the presence of two equivalents of *N,N'*-dimethylaniline to afford the protected glycine *tert*-butyl ester (42) (79%). Simple hydrolysis of the latter using *p*-toluenesulphonic acid in refluxing DCM gave the desired acid (36) in 90% yield. This provided an easy synthesis of Tbfmoc Gly OH using mildly basic conditions in a 57% overall yield based on alcohol (31).

### 2.3 APPLICATION TO THE SYNTHESIS AND PURIFICATION OF PEPTIDES

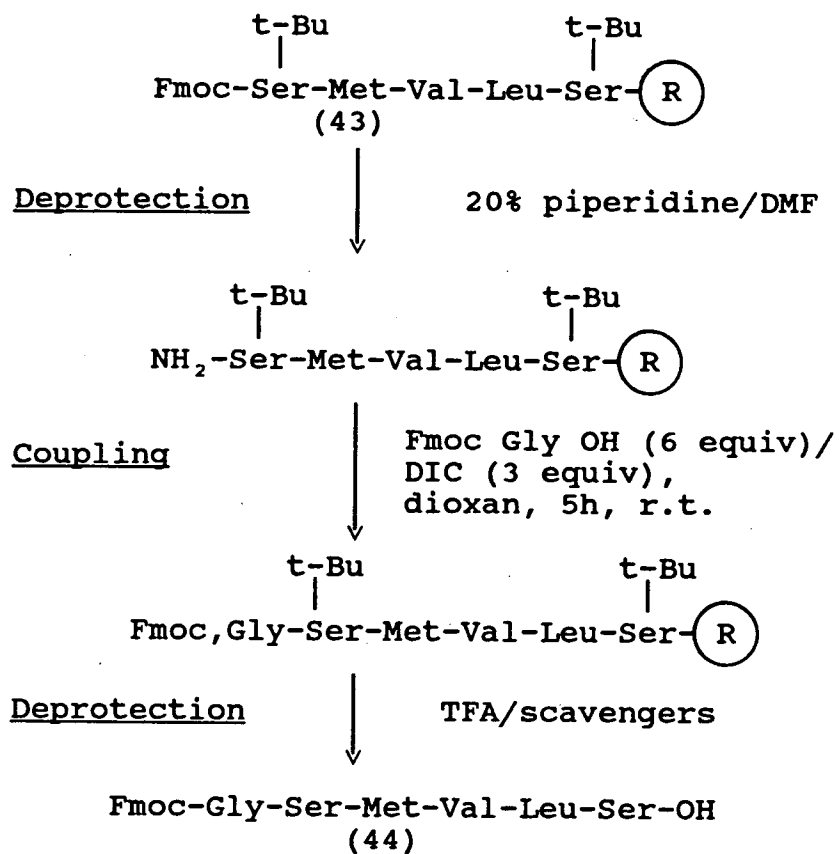
#### 2.3.1 Synthesis of Fmoc Gly Ser Met Val Leu Ser OH

This peptide was readily available in our laboratory and was selected as the first test peptide.

The protected resin-bound peptide Fmoc Ser ( $O^t\text{Bu}$ ) Met Val Leu Ser ( $O^t\text{Bu}$ ) (43) was prepared on the peptide synthesiser using an orthogonal strategy<sup>133</sup>. Fmoc was used for the temporary protection of the amine function of each amino acid and was removed before each coupling with a 20% piperidine in DMF solution. The side-chain function of serine was protected with the *t*-butyl group. The coupling procedures involved a symmetrical anhydride

followed by an HOBt ester coupling. Acetic anhydride and pyridine were used to acetylate any unreacted amino functions. The Merrifield resin was employed with the *p*-alkoxybenzylalcohol group as the linker.

Fmoc Gly Ser Met Val Leu Ser OH (44) was synthesised from the resin-bound peptide (43) via the series of reactions outlined in Scheme 22.



Scheme 22

After deprotection of the Fmoc group under basic conditions the amino-terminus of the peptide was coupled in dioxan to Fmoc Gly OH via its symmetrical anhydride (3 equiv) with a coupling efficiency of 88%. Cleavage of the hexapeptide from the resin and removal of the t-butyl groups was carried out using TFA, and in the presence of ethyl/methyl/sulphide and anisole as cation scavengers. After removal of the solvent *in vacuo* the peptide was finally precipitated with ether, filtered off and dried. X

Purification of the peptide was difficult. Removal of the scavengers by gel filtration (Sephadex G10, 30% acetic acid) was not possible as it was insoluble in aqueous media. Purification using HPLC was then considered, and for that purpose the best solvent was found to be dioxan in which the peptide was soluble at approximately 1.5 mg/ml. Preparative HPLC gave the peptide (44) together with a trace of the sulfoxide derivative, as indicated by mass spectrometry ( $MH+16^+$ ). The identity of the peptide was confirmed by mass spectrometry and amino acid analysis.

### 2.3.2 Synthesis of Tbfmoc Gly Ser Met Val Leu Ser OH

Tbfmoc Gly Ser Met Val Leu Ser OH (45) was then prepared from the resin-bound peptide (43) via a similar method.

Tbfmoc Gly OH was coupled in dioxan with an 80% coupling efficiency. Removal of the peptide from the

resin and *t*-butyl cleavage were carried out with TFA in the presence of the same scavengers. The peptide was finally precipitated with ether, filtered off and dried (69% yield).

As before, purification proved difficult. Also, a fresh solution of the crude peptide in dioxan gave one peak on HPLC, but on standing (30 min) the same solution gave two peaks corresponding to the desired peptide and the sulfoxide derivative. After isolation of these two peptides by preparative HPLC, these assignments were confirmed by mass spectrometry ( $MH^+$  and  $MH+16^+$ ) and amino acid analysis.

The facile oxidation of methionine to the sulfoxide might be due to the presence of peroxides in the dioxan used to dissolve both the Fmoc and Tbfmoc peptides. However, this does not explain why the corresponding Fmoc peptide was not oxidised at the same rate.

### 2.3.3 Behaviour of Tbfmoc and Fmoc Derivatives on PGC

The retention of both Fmoc Gly OH and Tbfmoc Gly OH was compared on a column packed with graphitised carbon.

A solution of Fmoc Gly OH (10.2 mg, 34.3  $\mu$ mole) in dioxan was loaded on a 6 mm diameter column packed with PGC (1.5 g). Only 15 ml of dioxan was required to elute the compound completely (as monitored by t.l.c.). In contrast, when Tbfmoc Gly OH (10.2 mg, 20.5  $\mu$ mole) was

loaded under the same conditions, none of this material was eluted in the first 35 ml. Indeed the total volume of dioxan needed to elute the compound was 300 ml.

The behaviour on a graphitised carbon column of Fmoc Gly Ser Met Val Leu Ser OH (44) was then compared with the Tbfmoc derivative (45). To increase the overall retention time, a mixture of dioxan/water (2:1) was used to elute both peptides. After loading a solution of the Fmoc hexapeptide (9.2 mg, 11.3  $\mu$ mole) in a mixture of dioxan/water (2:1) onto the column, the peptide was eluted completely with 75 ml of the solvent mixture. In contrast, the Tbfmoc derivative (10.5 mg, 10.3  $\mu$ mole) was not eluted at all in the first 80 ml of solvent and was subsequently only very slowly eluted from the column.

As these results were promising, the deprotection of the Tbfmoc group from the peptide, while still retained on the graphitised carbon column was investigated.

The best results were obtained when the deprotection was carried out using 20% piperidine in a mixture of dioxan/water (2:1). Under these conditions the Tbfmoc hexapeptide was totally deprotected. The free peptide (HGly Ser Met Val Leu Ser OH (46)) was eluted and was monitored by t.l.c. using ninhydrin to reveal the amino function. The subsequent isolation of the product was simple; the piperidine and solvents were removed *in vacuo* and the peptide was precipitated with ether, filtered and

dried (85%). Mass spectrometry showed both the presence of the peptide ( $MH^+$ ) and the sulfoxide derivative ( $MH + 16^+$ ). Amino acid analysis confirmed the composition of the peptide.

Finally, the tetrabenzofluorene olefin (38) or its piperidine adduct, the by-products from the deprotection step, were removed from the column by eluting with hot dioxan.

Considering the good degree of retention shown by the small Tbfmoc hexapeptide (45), this purification method was ready for testing on larger peptides.

#### 2.3.4 Synthesis of Fmoc Ubiquitin (54-76) OH

Ubiquitin is a protein of 76 amino acids and its synthesis is currently being undertaken in our laboratory. Consequently fragments of ubiquitin were selected as more challenging peptides.

The resin-bound peptide Fmoc ubiquitin (54-76) (47) was prepared on the peptide synthesiser<sup>134</sup> under the conditions described in section 2.3.1 (for the protection of side-chain functional groups (see Section 1.1.6)). Most coupling procedures involved a symmetrical anhydride followed by an HOBt ester coupling in a mixture of DMF/dioxan (1:1) as the solvent.



### 2.3.6 Behaviour of N<sup>α</sup>-Acetyl-Ubiquitin (54-76) OH and Tbfmoc Ubiquitin (53-76) OH on PGC

N<sup>α</sup>-Acetyl-ubiquitin (54-76) OH (49) was prepared from the resin-bound peptide (47) by the following reactions.

Deprotection of the Fmoc group from the peptide using 20% piperidine in DMF gave the peptide with a free N-terminal amino group, which was then acetylated with a mixture of acetic anhydride/pyridine (1:1) (100 equiv). Removal of the peptide from the resin and cleavage of the side-chain protecting groups were carried out using TFA in the presence of anisole and thioanisole as scavengers. Subsequent precipitation with ether afforded crude N<sup>α</sup>-acetyl-ubiquitin (54-76) OH (49).

The behaviour of both the N<sup>α</sup>-acetyl and Tbfmoc ubiquitin peptides on a PGC column was then examined.

In a typical experiment a solution containing 5 mg of each peptide was loaded onto the column which was then eluted using different solvent mixtures. After collecting the fractions, the solvent was removed *in vacuo* and the residue obtained was redissolved in fresh solvent before analysis by HPLC.

Initially, a 10% aqueous solution of acetic acid was used as the eluent, but in this system, neither peptide was sufficiently soluble and both were slowly eluted. No separation was achieved. The same results were observed

when a mixture of MeOH/H<sub>2</sub>O (1:1) was tried. The best results were obtained when a mixture of CH<sub>3</sub>CN/H<sub>2</sub>O (1:1) was used. Under these conditions, the N<sup>α</sup>-acetyl-peptide was eluted completely by 30 ml of the mixture, whereas the Tbfmoc peptide was retained.

The total chromatographic separation of these closely related peptides clearly demonstrated the efficiency of the method and its potential for the purification of peptides synthesised by solid phase methodology, provided careful attention is paid to the choice of solvents.

As these results were promising, the technique was used in the purification of ubiquitin (53-76) OH (50), and subsequently extended to a second N-terminal glycine peptide, ubiquitin (35-76) OH (51).

#### 2.3.7 Purification of Ubiquitin (53-76) OH

A simple chromatographic elution on a column packed with graphitised carbon was used as the first purification step (as described in Section 2.3.6). Crude Tbfmoc ubiquitin (53-76) OH (48) dissolved in a mixture of CH<sub>3</sub>CN/H<sub>2</sub>O (1:1; 0.5% TFA) was loaded onto a column packed with PGC (50 x mass of peptide). 50 ml of a mixture of the same solvent was required to completely elute the main impurities (probably N<sup>α</sup>-acetyl ubiquitin (55<sup>1</sup>-76) and unreacted ubiquitin (54-76)). None of the Tbfmoc peptide

was eluted even after flushing the column with a further 50 ml of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1). Deprotection of the Tbfmoc group was carried out using 20% piperidine in a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1) and only the desired peptide, ubiquitin (53-76) OH, was eluted. After removal of the solvent *in vacuo*, the peptide was finally precipitated with ether. Subsequent purification by preparative HPLC gave pure ubiquitin (53-76) OH (50) in 15% overall yield (see Fig. 4).

The identities of both Tbfmoc ubiquitin (53-76) OH and ubiquitin (53-76) OH peptides were established by high resolution mass spectrometry and amino acid analysis.

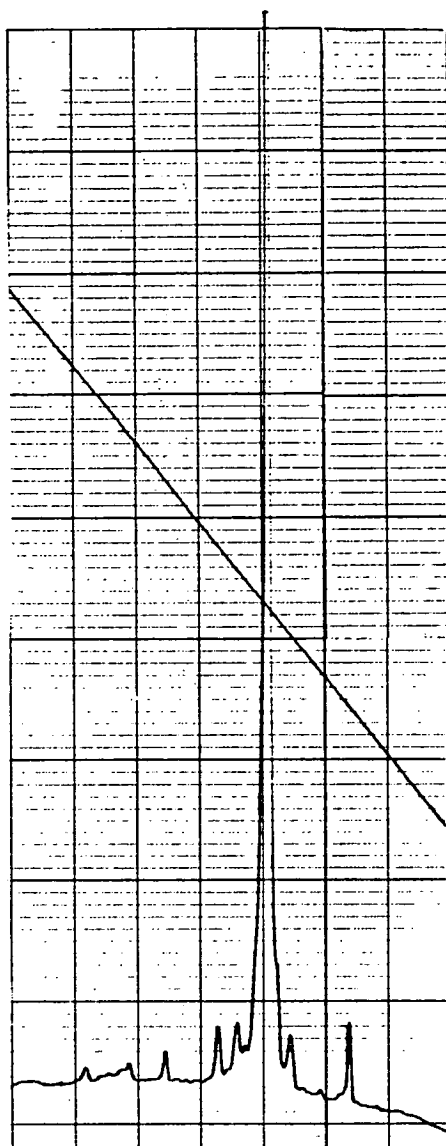
#### 2.3.8 Synthesis and Purification of Ubiquitin (35-76)

The purification of this peptide did not pose any problems and was carried out as with the smaller ubiquitin fragment (50); Tbfmoc ubiquitin (35-76) OH (52) was prepared by coupling *in situ* Tbfmoc Gly OBT to the resin-bound peptide ubiquitin (36-76)<sup>135</sup> (75% yield). Following chromatography on a PGC column and preparative HPLC, the desired peptide (51) could be obtained (see Fig. 5).

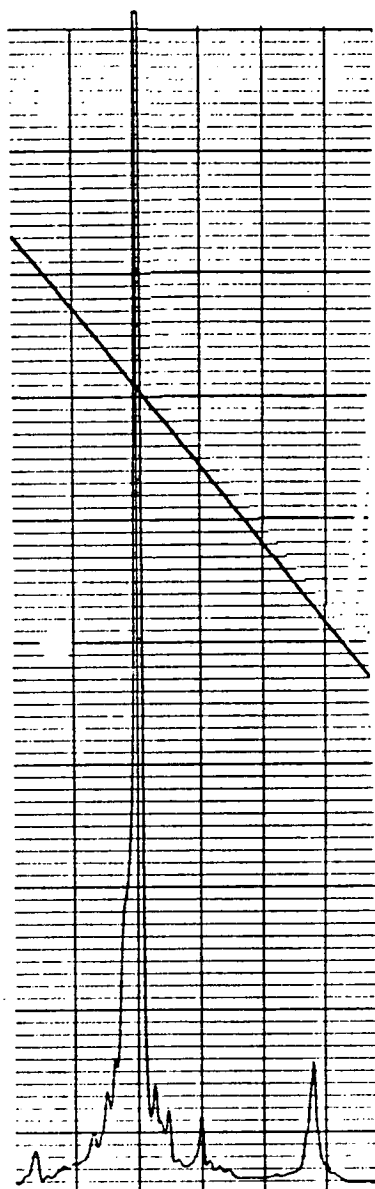
The authenticity of (51) was also confirmed by mass spectrometry and amino acid analysis, and its purity by analytical HPLC.

#### 2.4 PREPARATION OF OTHER TBFMOC AMINO ACIDS

Initially, the potentiality of directly linking the

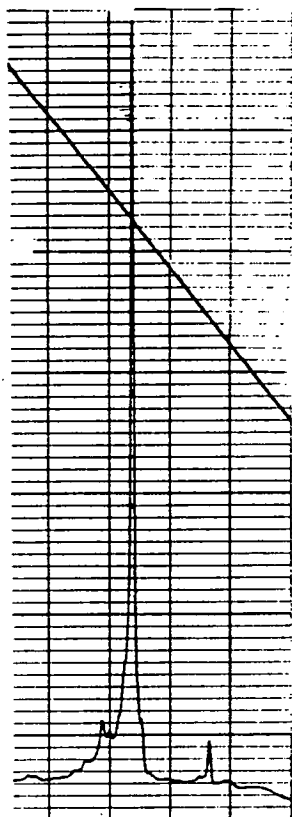


Crude Fmoc Ubiquitin  
(54-76) OH (47)



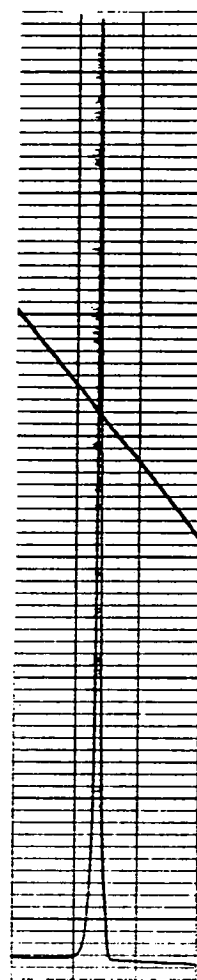
Crude Tbfmoc Ubiquitin  
(53-76) OH (48)

Fig. 4. Analytical HPLC: column ABI, aquapore ODS-300, C-18,  
300 Å pore size, 7 μm spherical silica (220 x 4.6 mm)  
solvents: A(H<sub>2</sub>O/TFA (0.1%)), B(CH<sub>3</sub>CN/TFA (0.1%))  
conditions: A(90%)  $\xrightarrow{25 \text{ min}}$  A(10%); λ=214 nm; AUFS=1  
flow rate: 1 ml/min.



Crude Ubiquitin  
(53-76) OH (50)

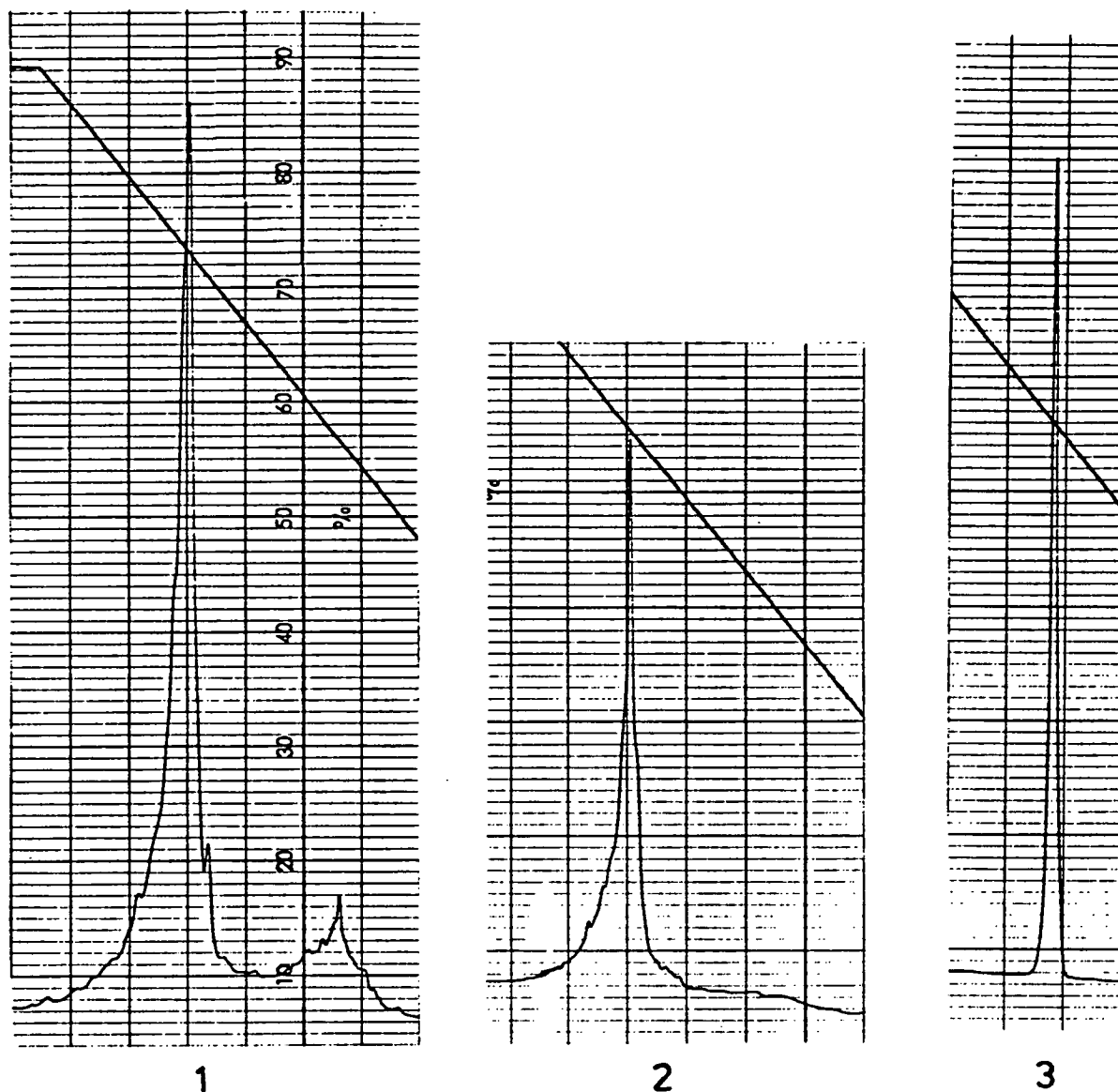
(after PGC column chromatography)



Purified Ubiquitin  
(53-76) OH (50)

(after preparative HPLC)

Fig. 4. Analytical HPLC: column ABI, aquaporeODS-300, C-18,  
300 Å pore size, 7 μm spherical silica (220 x 4.6 mm)  
solvents: A(H<sub>2</sub>O/TFA (0.1%)), B(CH<sub>3</sub>CN/TFA (0.1%))  
conditions: A(90%)  $\xrightarrow{25 \text{ min}}$  A(10%); λ=214 nm; AUFS=1  
flow rate: 1 ml/min.



1. Crude Tbfmoc Ubiquitin (35-76) OH (52)
2. Crude Ubiquitin(35-76)OH(51)after PGC column chromatography
3. Purified Ubiquitin (35-76) OH (51) (after preparative HPLC)

Fig. 5. Analytical HPLC: column ABI, aquaporeODS-300, C-18, 300 Å pore size, 7 μm spherical silica (220 x 4.6 mm)  
 solvents: A(H<sub>2</sub>O/TFA (0.1%)), B(CH<sub>3</sub>CN/TFA (0.1%))  
 conditions: A(90%)  $\xrightarrow{25 \text{ min}}$  A(10%); λ=214 nm; AUFS=1  
 flow rate: 1 ml/min.

Tbfmoc group to a resin-bound peptide via the *p*-nitrophenyl carbonate or the chloroformate was investigated.

Coupling of the mixed carbonate (41) (three to five equivalents either in dioxan or DCM in the presence of one equivalent of *N,N'*-dimethylaniline) to the resin-bound peptide ubiquitin (36-76), did not afford the corresponding Tbfmoc peptide. The major products were found to be the olefin (38) and *p*-nitrophenol, as judged by t.l.c. of the filtrate.

In the case of the chloroformate (37), generated *in situ* as described in Section 2.2.2 and coupled in large excess (10 equiv), the reaction with either ubiquitin (54-76) or ubiquitin (36-76) resin-bound peptides, resulted in low coupling efficiencies (17% and 25% respectively).

As described in Section 2.2.2, and illustrated with Tbfmoc Gly OH, the alternative approach is synthesis of the peptide less its *N*-terminal amino acid, and a final coupling of this amino acid previously protected with the Tbfmoc group.

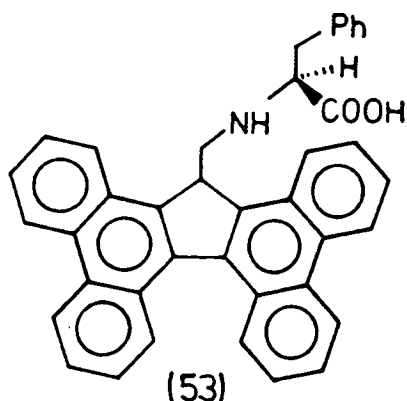
This necessitates the preparation of a range of Tbfmoc amino acids.

#### 2.4.1 Synthesis of Tbfmoc-L-Phe OH

Preparation of this compound proved difficult.

The free amine function of commercially available *L*-phenylalanine *tert*-butyl ester hydrochloride was initially

liberated with triethylamine. The amine in 20% excess was then reacted with the mixed carbonate (41) in the presence of *N,N'*-dimethylaniline. However, after removal of the *t*-butyl group with TFA, compound (53) was obtained.



The formation of (53) may be rationalised by  $\beta$ -elimination of the mixed carbonate (41), 1,2 addition of the amino ester onto the resulting olefin (38) and finally cleavage of the *t*-butyl ester.

As the acetate of glycine was successful in the synthesis of Tbfmoc Gly OH, this method may also be useful in this case; thus the acetate salt of L-phenylalanine *tert*-butyl ester was prepared.

The hydrochloride salt of the amino acid ester was neutralised with triethylamine and the precipitated  $\text{Et}_3\text{N}\cdot\text{HCl}$  generated was filtered off. Following removal of the solvent, the residue obtained was redissolved in acetic acid before lyophilisation. Subsequent reaction with the mixed carbonate (41) in the presence of two equivalents of *N,N'*-dimethylaniline afforded Tbfmoc-L-Phe

O<sup>t</sup>Bu (54) in 46% yield after purification by flash chromatography and recrystallisation. Final cleavage of the *t*-butyl ester using a mixture of TFA/H<sub>2</sub>O (95:5) gave Tbfmoc-L-Phe OH (55) in excellent yield.

A possible drawback of the method is the first step.

Due to the insolubility of L-phenylalanine *t*-butyl ester hydrochloride in ethyl acetate, neutralisation with triethylamine fails to reach completion, even after a long period. Thus, the precipitate which is largely triethylamine hydrochloride also includes some unreacted L-phenylalanine *t*-butyl ester hydrochloride (as judged by t.l.c.). This explains the significant losses of material encountered after lyophilisation. This method has the advantage, however, of eliminating any traces of triethylamine present in the filtrate.

#### 2.4.2 Attempted Syntheses of Tbfmoc-L-Met OH

Methionine was the next target as this amino acid often forms the N-terminal residue of numerous biologically active peptides and proteins.

Kricheldorf's method<sup>136</sup> for the preparation of  $\beta$ -amino acids, based on silicon chemistry, was examined first. L-methionine was treated in DCM with two equivalents of both chlorotrimethylsilane and triethylamine to give N $\alpha$ -trimethylsilylmethionine trimethylsilyl ester. Subsequent reaction of the latter with chloroformate (37) followed by

aqueous work-up led, however, to the formation of several products. <sup>1</sup>H n.m.r. and mass spectrometry did not show the presence of the expected compound.

Another attempt involved the use of resin-bound methionine. 9-Fluorenylmethoxycarbonyl-L-methionine was coupled to the *p*-alkoxybenzylalcohol resin via its symmetrical anhydride in the presence of a catalytic amount of 4-dimethylaminopyridine. Following deprotection of the Fmoc group, the free amino function was reacted with chloroformate (37) under the conditions described above. Unfortunately, the reaction gave a very low coupling efficiency (8%).

Both of these approaches were abandoned.

In an approach similar to that described in section 2.4.1, methionine methyl ester was treated with the mixed carbonate (41) in the presence of base, and the desired compound, Tbfmoc-L-Met OCH<sub>3</sub> (56) was obtained in 53% yield. Complete hydrolysis of the methyl ester required the use of HBr in aqueous acetic acid at 60°C for 5h. Under these rather severe conditions, only a poor yield of Tbfmoc-L-Met OH could be obtained.

The difficulties encountered in the hydrolysis of the methyl ester indicated that this route was not viable

either, and was therefore abandoned.

Synthesis of Tbfmoc-L-Met OH should be possible and is worth exploration in the future through the use of L-methionine tert-butyl ester. Unfortunately, it is not commercially available. Preparation of this compound has been described in the literature from N $\alpha$ -phthalyl-L-methionine, 2-methylpropene and a catalytic amount of sulphuric acid<sup>137</sup>.

## 2.5 CONCLUDING REMARKS

The efficiency of this new method of purification has been clearly demonstrated for the purification of N-terminal glycine peptides prepared by solid phase methodology.

Although the coupling of other Tbfmoc amino acids (such as Tbfmoc-L-Phe OH) to a resin-bound peptide have not been tried, this should be possible via their symmetrical anhydrides or HOBt esters.

The properties of the Tbfmoc group could also be used advantageously in different areas of peptide and protein chemistry.

It could be employed as a fluorescent label (see Appendix I : excitation wavelength : 383 nm; emission wavelength : 424 nm), particularly in immunology, where detection of compounds present in very low concentrations

requires highly sensitive techniques.

The strong affinity of the Tbfmoc group for graphitised carbon could also be applied in other fields of organic chemistry.

One such example is the resolution of racemates.

## 2.6 APPLICATION TO THE RESOLUTION OF RACEMATES

### 2.6.1 Introduction

Ligand-exchange chromatography (LEC) was developed by Davankov and Rogozhin in 1971<sup>138</sup>. It involves the reversible formation of complexes between a central metal ion (usually  $\text{Cu}^{2+}$  and  $\text{Ni}^{2+}$ ) and two chelating chiral bifunctional molecules (typically  $\alpha$ -amino acids)<sup>139</sup>.

When the chelators are  $\alpha$ -amino acids, both the amino and the carboxylate groups of the two chelators participate in the formation of the complexes. If one of the chiral chelators is immobilised on an inert support to provide a chiral stationary phase, a mixture of enantiomers added in an analyte will bind to give a pair of diastereoisomeric complexes, each with two bidentate ligands.

The difference between the relative stabilities of the diastereoisomeric adsorbates is responsible for the chromatographic separation of the enantiomers.

The strong affinity of the 17-tetrabenzo(a,c,g,i)-

fluorenylmethyl (Tbfm) group for graphitised carbon suggested to us a possible use in that particular field.

Indeed, an optically active amino acid could be linked to the Tbfm group to provide a chiral chelator, and a PGC column could easily be used to provide a support for it.

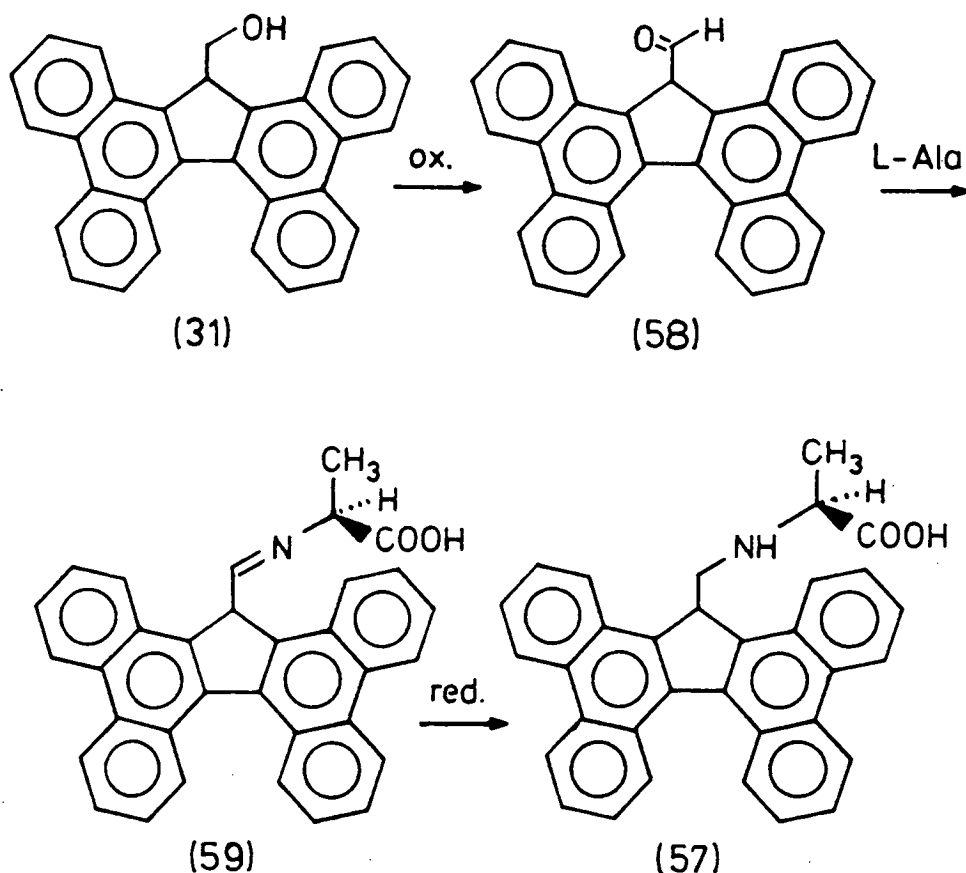
Alanine was initially chosen as the chiral chelator because of its simplicity.

#### 2.6.2 Synthesis of Tbfm-L-Ala OH

This work was done in parallel with the development of the purification method (see section 2.3). Although Tbfm-L-Phe OH (53) was obtained as the major product during the synthesis of Tbfmoc-L-Phe OH (55), the structure of this compound had not been elucidated when this work on the resolution of racemates was started.

A number of routes to Tbfm-L-Ala OH (57) were explored.

Initially the synthesis of this compound via the addition of the amino acid onto the tetrabenzofluorene aldehyde (58) was investigated as outlined in Scheme 24.



Scheme 24

Careful oxidation of alcohol (31) should give aldehyde (58), which in turn, could be reacted with L-alanine to give the intermediate imine (59). Reduction of the latter using  $\text{H}_2/\text{Pd}$  on charcoal or  $\text{NaBH}_3\text{CN}$  could provide Tbfm-L-Ala OH.

Unfortunately, synthesis of aldehyde (58) was not straightforward. The following reagents were used in an attempt to oxidise alcohol (31) to the corresponding aldehyde:

- pyridinium dichromate (PDC)<sup>140</sup> (1.5 equiv/CH<sub>2</sub>Cl<sub>2</sub>/r.t.)
- pyridinium chlorochromate (PCC)<sup>141</sup> (1.5 equiv/CH<sub>2</sub>Cl<sub>2</sub>/r.t.)
- Swern oxidation<sup>142</sup> (DMSO/oxalyl chloride/Et<sub>3</sub>N/CH<sub>2</sub>Cl<sub>2</sub>/-50°C)
- tetrapropylammonium perruthenate<sup>143</sup>/4-methylmorpholine-N-oxide/CH<sub>2</sub>Cl<sub>2</sub>/r.t.

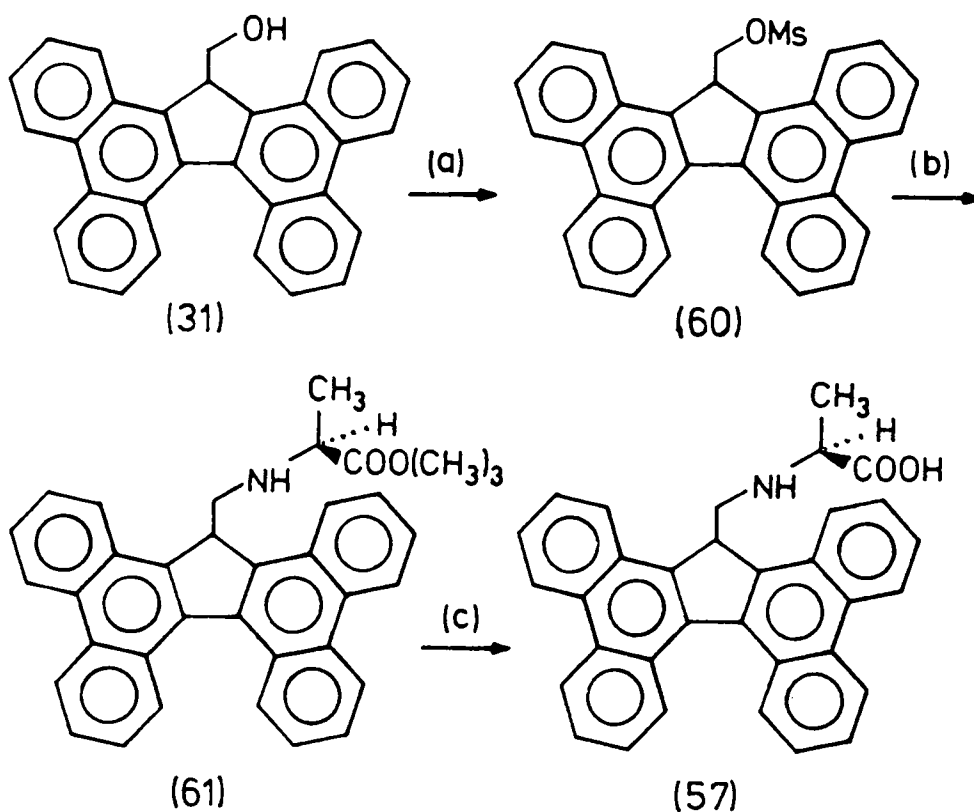
In all cases a complex mixture of products was obtained.

Only under the conditions of the Swern oxidation could the aldehyde be detected. This was indicated by n.m.r. (singlet at 10.5 ppm) and infrared spectroscopy peak at 1670 cm<sup>-1</sup>).

A second approach involved reduction of 17-ethoxycarbonyltetrabenzo(a,c,g,i)fluorene (34) using one equivalent of DIBAL-H at -100°C in DCM or toluene.

Under these conditions, however, alcohol (31) was obtained as the major product. In view of the problems at the aldehyde stage this route was abandoned.

Finally, in a third approach, the amino acid was successfully reacted with the mesylate derivative (60) and this provided an alternative route to the synthesis of Tbfm-L-Ala OH (Scheme 25).



(a) Methanesulfonic anhydride (10.5 equiv), *N,N'*-dimethylaniline (2.5 equiv), CH<sub>2</sub>Cl<sub>2</sub>, r.t., overnight, 76%; (b) L-Ala *t*-butyl ester. HCl (2 equiv), Et<sub>3</sub>N (2 equiv), EtOAc, r.t., 3h; (60) (1 equiv), *N,N'*-dimethylaniline (1 equiv), EtOAc, r.t., overnight, 76%; (c) TFA/H<sub>2</sub>O (95:5, r.t., 3.5h, 69%.

#### Scheme 25

Preparation of mesylate (60) from alcohol (31) was initially carried out using methanesulfonyl chloride. Due to the low reactivity of alcohol (31) it was necessary to heat the reaction at 60°C for 4h in methanesulphonyl

chloride (solvent) and *N,N'*-dimethylaniline (4 equiv).

Even under these conditions, the mesylate could only be obtained in 32% yield. The major side products were found to be the olefin (38) and methanesulfonic acid. In an attempt to improve the yield of the reaction, an alternative reagent, methanesulfonic anhydride, was tried. Using a large excess of methanesulfonic anhydride (10.5 equiv) and *N,N'*-dimethylaniline (2.5 equiv) in dichloromethane, the mesylate (60) was finally obtained in 76% yield.

The remaining reactions proceeded in a straightforward manner. L-alanine *tert*-butyl ester hydrochloride was liberated as the free amine with one equivalent of triethylamine in ethyl acetate, and reacted with mesylate (60) in the presence of *N,N'*-dimethylaniline (1 equiv), to give the protected L-alanine *tert*-butyl ester (61) (76% yield). Simple hydrolysis of the latter using a mixture of TFA/H<sub>2</sub>O (95:5) afforded Tbfm-L-AlaOH (57) in 69% yield.

### 2.6.3 Ligand-Exchange Chromatography

This work was carried out by a colleague (Quianhong Wan)<sup>131</sup>.

A solution of Tbfm-L-Ala OH in acetic acid (3 mg/ml) was loaded onto an analytical HPLC column packed with graphitised carbon (50 x 4.6 mm). The percolation of the material through the column was monitored by UV at 320 nm. When the column was fully saturated with the amino acid

derivative, the now coated column was then eluted with an aqueous copper acetate solution ( $C = 1 \text{ mM}$ ; monitored by UV at 254 nm).

In a typical experiment 1 to 3  $\mu\text{g}$  of racemates dissolved in water were used per injection. Detection of the amino acids was by UV at 254 nm. For each enantiomeric mixture resolved, the absolute configuration was assigned by loading pure samples of each of the enantiomers on the column in separate experiments.

The results obtained are described below (Table 2).

racemates	$k'$ (min)	$\alpha_{DL} = k'_L/k'_D$
± Pro	D 7.15 L 12	1.6
± Thr	D 6.0 L 14.6	2.33
± Ser	D 7.75 L 13.0	1.68
± Val	D 8.25 L 13.25	1.61

$k'$  refers to the capacity factor (retention time).  $\alpha_{DL}$  is the ratio of the capacity factors for the D and L enantiomers of the analyte and gives an indication of the selectivity.

Table 2

In all the cases complete resolution was achieved with

a good selectivity ( $> 1.5$ ).

In view of the good results obtained, for Tbfm-L-Ala OH, other Tbfm amino acid derivatives were prepared and tested as chiral chelators.

Both Tbfm-L-Phe OH (53) and Tbfm-L-Pro OH (62) were synthesised from mesylate (60) and obtained in good yields, following the procedure used for Tbfm-L-Ala OH (57) (section 2.6.2).

Unfortunately, the results obtained by ligand-exchange chromatography on a PGC column proved disappointing.

In the case of Tbfm-L-Phe OH, the initial resolution of the racemate ( $\pm$  Pro) was successful and complete separation of the enantiomers was obtained. However, upon regenerating the column with the copper acetate solution, the resolution was lost. Perhaps this was because the coating of this compound onto the PGC column was carried out differently. In this case, the adsorption was obtained by directly immersing the packing material in the solution instead of pumping the solution through the column.

Using Tbfm-L-Pro OH, no resolution of racemates could be achieved. This is possibly due to the fact that this compound is not stable in solution (e.g.  $\text{CH}_3\text{COOH}$  or  $\text{CHCl}_3$ ) and decomposes to give the corresponding olefin and

proline (as judged by t.l.c.). Under the slightly acidic conditions of the experiment, the tertiary amine may be protonated allowing a possible  $\beta$ -elimination to take place.

To improve reliability, the method needs further development.

In particular, the use of homologous derivatives with an additional methylene group between the Tbfm group and the amino acid would be advantageous. This modification would prevent  $\beta$ -elimination and act as a spacer.

## 2.7 CONCLUDING REMARKS

The strong affinity of the Tbfm group for graphitised carbon (PGC) has found application for both the purification of peptides and the resolution of racemates by ligand-exchange chromatography.

Although PGC was used for both these studies, a recent survey of other support materials has shown that equivalent binding of the Tbfm group can be obtained with activated charcoal. This could provide a cheaper support material than the rather expensive PGC, and thus its use should be considered.

## CHAPTER 3 : EXPERIMENTAL

## 3.1 NOTES

All amino acid derivatives were purchased from either Fluka, Aldrich or Sigma. Melting points were taken in open capillaries on an electrically heated Büchi 510 melting point apparatus, or on microscope slides on an electrically heated Reichert 7905 hot stage and are uncorrected. Thin-layer chromatography (t.l.c.) was carried out using plastic sheets coated with silica gel 60 GF-254 (Merck 5735) in the following systems:

- (A) ethyl acetate/petrol (b.p. 40-60°C) (1:4)
- (B) methanol/chloroform (1:9)
- (C) toluene
- (D) chloroform
- (E) ethyl acetate
- (F) benzene
- (G) dichloromethane
- (H) methanol/chloroform/acetic acid (1:9:0.5)
- (I) n-butanol/acetic acid/water (3:1:1)

Visualisation of the compounds was achieved by a suitable combination of the following methods: iodine vapour, ultraviolet absorption at 254 nm or 352 nm, neutral potassium permanganate and bromophenol blue sprays, Mary's reagent (4,4'-bis (dimethylamino) diphenyl carbinol) for acid functions and ninhydrin for compounds with free amino groups. Optical rotations were measured

on an AA 1000 polarimeter using a 1 dm cell in the solvents quoted in the text. High performance liquid chromatography (HPLC) was performed using either a Waters system *i.e.* 2 x 600 A pumps, a U6K injector, a 680 automatic gradient controller and a model 441 ultraviolet detector; or an Applied Biosystems system, *i.e.* 2 x 1406 A solvent delivery system, a 1480 A injector/mixer and a 1783 A detector/controller. Analytical separations were carried out on a Whatman Partisil-5 ODS-3 (4.6 mm ID x 250 mm) and on a ABI (RP18) aquapore OD-300, 300 Å pore size, 7 µm spherical silica (4.6 mm ID x 220 mm) column under the conditions indicated in the text. Flash chromatography was performed using silica gel 60 (230-400 mesh (Fluka); 60-100 g of silica per gram of crude material; active length 15-20 cm). Infrared spectra were recorded on a Perkin Elmer 781 spectrophotometer in the solvent indicated or by the KBr disc technique, using polystyrene as the standard (1603 cm<sup>-1</sup>). Ultraviolet spectra were recorded on a Cary 210 spectrophotometer and fluorescence spectra on a Perkin Elmer LS-5 luminescence spectrophotometer. Mass spectra were measured on a Kratos MS 50 TC machine. Proton nuclear magnetic resonance (NMR) spectra were recorded on either Brüker WP 80 (80 MHz), WP 200 (200 MHz) or WH 360 (360 MHz) machines in the solvent indicated, using external tetramethylsilane as the standard ( $\delta = 0.00$ ). Carbon-13 NMR spectra were recorded on a Brüker WP 200 (50 MHz) or WH 360 (90 MHz) machines in

the solvent indicated, using tetramethylsilane as the standard. Elemental analyses were carried out on a Carlo Erba model 1106 elemental analyser. Single crystal X-ray structure determination was performed on a Stoë Stadi-4 four-circle diffractometer, graphite-monochromated (CU-K $\alpha$  radiation;  $\lambda = 1.54184 \text{ \AA}$ ). All solvents were distilled before use and the following were dried using the reagents given in parentheses when required: benzene (sodium wire), chloroform (phosphorus pentoxide), dichloromethane (calcium hydride), diethyl ether (sodium wire), dioxan (neutral alumina and sodium wire), methanol (magnesium-iodine), toluene (sodium wire). Petrol (b.p. 40-60°C) refers to that fraction which boils between 40°C and 60°C.

### 3.2 EXPERIMENTAL

#### Methyl(phenanthren-9-yl,phenyl)glycolate (25)<sup>129</sup>

To a cold (0°C) solution of 9-bromophenanthrene (2.57 g, 10 mmol) in dry ether (10 ml) under nitrogen, was added a solution of n-butyllithium in hexane<sup>127</sup> (8 ml, 11 mmol; 1.1 equiv; 1.38M titrated<sup>144</sup>) dropwise over 10 min. The reaction mixture was stirred for one hour at room temperature. The resultant suspension was allowed to settle and then the supernatant removed by syringe. The residue was resuspended in ether (5 ml). This solution was added, under nitrogen, to a cold (0°C) solution of methyl benzoylformate (1.64 g, 1.4 ml, 10 mmol) in 50 ml

of diethylether. The resulting mixture was heated under reflux for 2h and allowed to stir overnight. After addition of dilute (10% v/v) HCl (50 ml; pH=1), the organic layer was separated, combined with ether washings (2 x 50 ml) of the aqueous layer, washed with water (2 x 50 ml) and dried with MgSO<sub>4</sub>. The solvent was removed under vacuum to give a yellow oil. After purification by flash chromatography (eluent: (A)), a yellow oil was obtained which was triturated with petrol. A white solid was obtained which was filtered and dried in a vacuum desiccator to give the required compound (1.75 g, 51%); m.p. 143-145°C (lit.<sup>129</sup> 138.5-139.5°C); (Found: C, 79.7; H, 5.24 C<sub>23</sub>H<sub>18</sub>O<sub>3</sub> requires: C, 80.7; H, 5.26%); t.l.c. R<sub>f</sub>(A) 0.32; R<sub>f</sub>(B) 0.76; ν<sub>max</sub> CH<sub>2</sub>Cl<sub>2</sub> 3690 (free OH), 3510 (H bonded OH), 3025 (CH stretching, aryl), 2960, 2880, 2860 (CH stretching, alkyl), 1730 (CO, ester), 1600, 1500 (aromatic rings), 1225, 1185, 1170, 1110, 1100, 1070 (CO stretching) cm<sup>-1</sup>; λ<sub>max</sub> 296 (9832), 284 (9200), 276 (11981), 254 (48450), 248 (37620) nm; δ<sub>H</sub> (CDCl<sub>3</sub>, 200 MHz) 8.72 (1H, d, <sup>3</sup>J = 8 Hz, aromatic), 8.66 (1H, d, <sup>3</sup>J = 8 Hz, aromatic), 8.16 (1H, d, <sup>3</sup>J = 8 Hz, aromatic), 7.75-7.25 (11H, m, aromatic), 4.32 (1H, s, OH), 3.85 (3H, s, CH<sub>3</sub>); δ<sub>C</sub> (CDCl<sub>3</sub>, 50 MHz) 175.7 (CO, ester), 141.1, 135.6, 131.3, 130.6, 130.3, 129.8 (quaternary aromatic C's), 129.1, 128.2, 128.1, 127.3, 127.0, 126.9, 126.6, 126.2, 126.1, 122.9, 122.2 (aromatic CH's), 82.1 (C<sub>1</sub>, quaternary), 53.4 (CH<sub>3</sub>); m/z (EI) 342, 283, 105.

13-Methoxycarbonyldibenzo(a,c)fluorene (26)<sup>129</sup>

Polyphosphoric acid<sup>145</sup> (60 g) was heated to 110°C and stirred with a mechanical stirring paddle. To this was added methyl(phenanthren-9-yl,phenyl)glycolate (5 g, 14.6 mmol). The mixture was stirred for 1 hour at 110°C. The reaction was then cooled to room temperature, diluted with water (150 ml) and extracted with ethyl acetate (4 x 250 ml). The organic layers were combined, washed with NaHCO<sub>3</sub> (10% v/v; 200 ml), water (100 ml) and dried over MgSO<sub>4</sub>. The solvent was removed *in vacuo* to give a yellow residue. This crude material was purified by flash chromatography using toluene as the eluent. After chromatography the *required compound* (2.82 g, 60%) could be isolated. This solid was recrystallised from CH<sub>2</sub>Cl<sub>2</sub>/ petrol (b.p. 40-60°C) to afford a white powder, (1.96 g, 42%); m.p. 190-191°C (lit.<sup>129</sup> 195-197°C); (Found: C, 85.2; H, 4.93 C<sub>23</sub>H<sub>16</sub>O<sub>2</sub> requires: C, 85.2; H, 4.94%); t.l.c. R<sub>f</sub>(C) 0.50; R<sub>f</sub>(D) 0.56;  $\nu_{\max}$  (CH<sub>2</sub>Cl<sub>2</sub>), 3040, 3020 (CH stretching, aryl), 2970 (CH stretching, alkyl), 1730 (CO, ester), 1610, 1600, 1580 (aromatic rings)cm<sup>-1</sup>;  $\lambda_{\max}$  364 (540), 346 (16200), 322 (18360), 268 (64800) nm;  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 200 MHz), 8.84-8.67 (3H, m, aromatic), 8.36 (1H, d, <sup>3</sup>J = 7.8 Hz, aromatic), 7.98-7.90 (1H, m, aromatic), 7.80-7.35 (7H, m, aromatic), 5.16 (1H, s, CH), 3.63 (3H, s, CH<sub>3</sub>);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 50 MHz), 172.0 (CO, ester), 142.7, 141.9, 137.4, 135.6, 131.1, 130.0, 128.7, 128.4 (quaternary aromatic C's),

128.1, 127.0, 126.7, 126.3, 126.2, 124.3, 124.1, 123.8, 123.3, 123.2, 122.9 (aromatic CH's), 53.4 (CH<sub>3</sub>), 52.4 (CH); m/z (EI) 324, 265, 262, 132.

13-Hydroxymethyldibenzo(a,c)fluorene (21)

Diisobutylaluminium hydride (4.6 ml, 4.62 mmol; 1 M in CH<sub>2</sub>Cl<sub>2</sub>) was added at -65°C to a solution of 13-methoxycarbonyldibenzo(a,c)fluorene (0.5 g, 1.54 mmol) in dichloromethane (10 ml). The reaction mixture was stirred for 3h and the temperature maintained between -50°C and -40°C. A white precipitate was obtained. The reaction mixture was treated with a mixture of acetic acid and water (1:1; 30 ml) and extracted with dichloromethane (3 x 60 ml). The organic layer was washed with saturated bicarbonate (50 ml), water (30 ml) and dried over MgSO<sub>4</sub>. The solvent was removed *in vacuo* to afford a crude solid. This crude solid was purified by flash chromatography using chloroform as the eluent. The *title compound* was obtained as a white solid which was washed with petrol (0.217 g, 48%); m.p. 167-168°C; (Found: C, 89.0; H, 5.39; C<sub>22</sub>H<sub>16</sub>O requires: C, 89.2; H, 5.41%); t.l.c. R<sub>f</sub>(D) 0.26; R<sub>f</sub>(E) 0.64; ν<sub>max</sub> (CH<sub>2</sub>Cl<sub>2</sub>) 3610 (free OH), 3490 (H bonded OH), 3060 (CH stretching), 2940, 2890 (CH stretching, alkyl), 1610, 1600, 1580 (aromatic rings) cm<sup>-1</sup>; λ<sub>max</sub> 366 (888), 338 (12728), 322 (14504), 266 (27232), 246 (31968) nm; δ<sub>H</sub> (CDCl<sub>3</sub>, 200 MHz), 8.88-8.68 (3H, m, aromatic), 8.38 (1H, d, <sup>3</sup>J = 7.9 Hz, aromatic), 8.14-8.08 (1H, m,

aromatic), 7.81-7.33 (7H, m, aromatic), 4.45 (2H, m, H<sub>a</sub> (CH<sub>2</sub>) and H<sub>c</sub>), 3.85 (1H, m, H<sub>b</sub>, CH<sub>2</sub>), 1.64 (1H, s, OH),  $\delta_C$  (CDCl<sub>3</sub>, 50 MHz), 146.7, 142.5, 139.4, 134.9, 130.9, 130.1, 128.7 (quaternary aromatic C's), 127.5, 126.8, 126.1, 125.9, 125.8, 124.7, 124.2, 124.1, 123.8, 123.4, 122.9 (aromatic CH's), 65.7 (CH<sub>2</sub>), 50.0 (CH); m/z (EI) 296, 265.

13-Acetoxymethyldibenzo(a,c)fluorene (30)

13-Hydroxymethyldibenzo(a,c)fluorene (215.2 mg, 0.727 mmol) was dissolved in acetic anhydride<sup>146</sup> (5 ml). To this solution was added one drop of sulphuric acid (2M). The reaction mixture was stirred for 30 min. A white precipitate was obtained which was filtered and washed with water (150 ml). The solid was finally dried to constant weight over P<sub>2</sub>O<sub>5</sub> in a vacuum desiccator to afford 13-acetoxymethyldibenzo(a,c)fluorene as a white solid, (206.3 mg, 84%); m.p. 135-136°C; (Found: C, 84.9; H, 5.33 C<sub>24</sub>H<sub>18</sub>O<sub>2</sub> requires: C, 85.2; H, 5.33%; t.l.c. R<sub>f</sub>(D) 0.57; R<sub>f</sub>(E) 0.68;  $\nu_{\max}$  (CH<sub>2</sub>Cl<sub>2</sub>) 3030 (CH stretching, aryl) 2980, 2920 (CH stretching, alkyl) 1735 (CO, ester), 1610, 1600, 1570 (aromatic rings) cm<sup>-1</sup>;  $\lambda_{\max}$  366 (1184), 338 (25160), 322 (27528), 265 (52688), 246 (53020) nm;  $\delta_H$  (CDCl<sub>3</sub>, 200 MHz), 8.86-8.69 (3H, m, aromatic), 8.38 (1H, d, <sup>3</sup>J = 7.9 Hz, aromatic), 8.30-8.25 (1H, m, aromatic), 7.78-7.35 (7H, m, aromatic), 5.16 (1H, dxd, <sup>3</sup>J<sub>a,c</sub> = 4.1 Hz, <sup>2</sup>J<sub>a,b</sub> = 10.8 Hz, H<sub>a</sub>, CH<sub>2</sub>), 4.56 (1H, dxd, <sup>3</sup>J<sub>c,a</sub> = 4.1 Hz, <sup>3</sup>J<sub>c,b</sub> = 9.3 Hz, H<sub>c</sub>), 3.76 (1H, dxd, <sup>2</sup>J<sub>b,a</sub> = 10.8 Hz,

$^3J_{b,c} = 9.3$  Hz,  $H_b$ ,  $CH_2$ ), 2.17 (3H, s,  $CH_3$ );  $\delta_C$  ( $CDCl_3$ , 50 MHz) 171.0 (CO, ester), 146.7, 142.1, 138.7, 134.8 131.1, 130.1, 128.7, 128.6 (quaternary aromatic C's), 127.6, 127.0, 126.8, 126.2, 126.1, 125.7, 124.9, 124.8, 124.3, 123.4, 123.3, 122.9 (aromatic CH's), 67.3 ( $CH_2$ ), 46.5 (CH), 20.9 ( $CH_3$ ); m/z (EI) 338, 277, 265, 139, 43.

HPLC: column (ODS3-PL5-393, solvents: A( $H_2O$ ), B( $CH_3CN$ ), conditions: B(50%) 2 min.

B(50%)  $\xrightarrow{30 \text{ min.}}$  B (95%)

$\lambda = 254$  nm, flow rate: 1 ml/min., injection: 5  $\mu$ l, C = 1.42 mg/ml, AUFS = 2, retention time : 21.6 min.

### 9-Fluorenylmethyl acetate (29)

9-Fluorenylmethanol (0.215 g, 1.1 mmol) was dissolved in acetic anhydride<sup>146</sup> (5 ml). To this solution was added 2 drops of sulphuric acid (2M). This was stirred for 30 min. The clear solution was poured onto cold water (20 ml). The precipitate obtained was collected on a Büchner filter and dried to constant weight over  $P_2O_5$  in a vacuum desiccator to afford 9-fluorenylmethyl acetate as a white solid, (0.175g, 67%); m.p. 83-84°C (lit.<sup>148</sup> 87°C); (Found: C, 80.5; H, 5.98  $C_{16}H_{14}O_2$  requires: C, 80.7; H, 5.88%); t.l.c.  $R_f$  (D) 0.50;  $R_f$  (E) 0.68;  $\nu_{max}$  ( $CH_2Cl_2$ ) 3030 (CH stretching), 2960, 2910 (CH stretching, alkyl), 1735 (CO, ester), 1610 (aromatic rings)  $cm^{-1}$ ;  $\lambda_{max}$  300 (2476), 290 (2063), 268 (7634) nm;  $\delta_H$  ( $CDCl_3$ , 80 MHz), 7.83-7.29 (8H, m, aromatic), 4.46-4.28 (3H, m, CH,  $CH_2$ ),

2.14 (3H, s, CH<sub>3</sub>);  $\delta_C$  (CDCl<sub>3</sub>, 50 MHz), 170.8 (CO, ester), 143.6, 141.1 (quaternary aromatic C's), 127.6, 126.9, 124.9, 119.9 (aromatic CH's), 66.2 (CH<sub>2</sub>), 46.5 (CH), 20.8 (CH<sub>3</sub>); m/z (EI) 238, 178, 165, 149, 60, 43.

HPLC: column (ODS3-PL5-393), solvents: A(H<sub>2</sub>O), B(CH<sub>3</sub>CN)

Conditions: B(50%) 2 min.

B(50%)  $\xrightarrow{30 \text{ min.}}$  B (95%)

$\lambda$  = 254 nm, flow rate: 1 ml/min.,

injection: 5  $\mu$ l, C = 1.46 mg/ml, AUFS = 2, retention time : 8.4 min.

Ethyl 2-oxo-2-(phenanthren-9'-yl)acetate (32)

To a stirred solution of 9-bromophenanthrene (5.14 g, 20 mmol) in dry ether (20 ml), was added at 0°C under nitrogen, a solution of n-butyllithium in hexane<sup>127</sup> (16 ml, 22 mmol; 1.1 equiv; 1.38M titrated)<sup>144</sup> dropwise over 10 min. The reaction was stirred for 1h at room temperature. The resultant suspension was allowed to settle and the supernatant was removed by syringe. The residue was resuspended in ether (10 ml). This suspension was added, under nitrogen, to a cold (0°C) solution of diethyl oxalate (3.4 ml, 25 mmol) in ether (100 ml). The temperature was maintained between 0°C and 5°C during the addition. The reaction mixture was then stirred for 2h between 0°C and 5°C and finally at room temperature for 2h. After addition of dilute HCl (100 ml; 10% v/v), the organic layer was separated, combined with ethyl acetate

washings (3 x 100 ml) of the aqueous layer, neutralised with NaHCO<sub>3</sub> solution (100 ml; 1M), washed with water (50 ml) and dried over MgSO<sub>4</sub>. The solvent was removed *in vacuo* to give an orange oil which was triturated with petrol (b.p. 40°C-60°C). The title compound was obtained as a yellow solid which was filtered and dried, (3.53 g, 63%); m.p. 67-68°C; (Found: C, 77.5; H, 5.02 C<sub>18</sub>H<sub>14</sub>O<sub>3</sub> requires: C, 77.7; H, 5.04%); t.l.c. R<sub>f</sub> (A) 0.56, R<sub>f</sub> (D) 0.52; ν<sub>max</sub> (CH<sub>2</sub>Cl<sub>2</sub>) 3070 (CH stretching, aromatic), 2990, 2940, 2910, 2880 (CH stretching, alkyl), 1735 (CO, ester), 1680 (CO, ketone), 1620, 1575 (aromatic rings) cm<sup>-1</sup>; λ<sub>max</sub> 328 (9822), 286 (7784), 252 (34842) nm; δ<sub>H</sub> (CDCl<sub>3</sub>, 200 MHz) 9.04 (1H, m, aromatic), 8.62 (2H, m, aromatic), 8.23 (1H, s, H<sub>10</sub> aromatic), 7.92 (1H, d, <sup>3</sup>J = 7.6 Hz, aromatic), 7.78-7.59 (4H, m, aromatic), 4.51 (2H, q, <sup>3</sup>J = 7.1 Hz, CH<sub>2</sub>), 1.48 (3H, t, <sup>3</sup>J = 7.1 Hz, CH<sub>3</sub>), δ<sub>C</sub> (CDCl<sub>3</sub>, 50 MHz) 188.5 (CO, ketone), 164.4 (CO, ester), 137.2 (aromatic CH), 132.8 (quaternary aromatic), 130.5, 130.2 (aromatic CH's), 129.2, 128.1 (quaternary aromatic C's), 128.0, 127.4, 127.1, 126.3, 122.7, 122.6 (aromatic CH's), 62.3 (CH<sub>2</sub>), 14.0 (CH<sub>3</sub>); m/z (EI) 278, 205, 177, 176.

Ethyl (bis-phenanthren-9-yl)glycolate (33)

To a stirred solution of 9-bromophenanthrene (2.57 g, 10 mmol) in dry ether (10 ml) was added, at 0°C under nitrogen, a solution of n-butyllithium in hexane<sup>127</sup> (8 ml, 11 mmol; 1.1 equiv.; 1.38 M titrated)<sup>144</sup> dropwise over

10 min. The reaction mixture was stirred for 1h at room temperature. This solution was added, under nitrogen, to a cold (0°C) solution of ethyl,2-oxo-2-(phenanthren-9'-yl)acetate (2.78 g, 10 mmol) in ether (50 ml). The temperature was maintained between 0°C and 5°C during the addition. The reaction mixture was finally stirred at room temperature for 2h. After addition of dilute HCl (50 ml; 10% v/v) the organic layer was separated, combined with ethyl acetate washings (3 x 250 ml) of the aqueous layer, neutralised with a solution of NaHCO<sub>3</sub> (1M; 200 ml), washed with water (200 ml) and finally dried over MgSO<sub>4</sub>. The solvent was removed *in vacuo* to give a residue. After purification by flash chromatography using chloroform/petrol (b.p. 40°C-60°C) (4:1), the expected product was obtained from the fractions containing material of R<sub>f</sub> = 0.23. After recrystallisation from dichloromethane/petrol (b.p. 40°C-60°C), a white solid was obtained which was filtered and dried to give the *title compound* (2.11 g, 46%); m.p. 188-189°C; (Found: C, 83.1; H, 5.23 C<sub>32</sub>H<sub>24</sub>O<sub>3</sub> requires: C, 84.2; H, 5.26%); t.l.c. R<sub>f</sub> (E) 0.71; R<sub>f</sub> (D) 0.48; ν<sub>max</sub> (CH<sub>2</sub>Cl<sub>2</sub>) 3680 (free OH), 3510 (H bonded OH), 3060 (CH stretching, aromatic), 2990, 2940 (CH stretching, alkyl), 1735 (CO, ester), 1600 (aromatic rings) cm<sup>-1</sup>; λ<sub>max</sub> 332 (629), 300 (25080), 288 (23560), 256 (123120) nm; δ<sub>H</sub> (CDCl<sub>3</sub>, 200 MHz), 8.80-8.69 (4H, m, aromatic), 8.51 (1H, s, H<sub>10</sub>, aromatic), 8.47 (1H, s, H<sub>10</sub>, aromatic), 7.72-7.41 (12H, m, aromatic), 4.46 (1H, s, OH), 4.41 (2H, q, <sup>3</sup>J =

7.1 Hz, CH<sub>2</sub>), 1.17 (3H, t, <sup>3</sup>J = 7.1 Hz, CH<sub>3</sub>), δ<sub>C</sub> (CDCl<sub>3</sub>, 50 MHz) 175.7 (CO, ester), 135.0, 131.4, 130.6, 130.5, 130.1 (quaternary aromatic C's), 129.2, 128.2, 127.3, 126.6, 126.2, 126.1, 122.9, 122.3 (aromatic CH's), 84.3 (C<sub>1</sub>, quaternary), 62.9 (CH<sub>2</sub>), 13.8 (CH<sub>3</sub>); m/z (EI) 383, 206, 177, 176.

17-Ethoxycarbonyltetrabenzo(a,c,g,i)fluorene (34)

Polyphosphoric acid<sup>145</sup> (20 g) was placed in a three necked 100 ml round bottom flask equipped with a mechanical stirring paddle. Ethyl (bis-phenanthren-9-yl) glycolate (0.402 mg, 0.881 mmol) was then added and the reaction was stirred at 140°C for 4h and subsequently at room temperature overnight. The reaction mixture was diluted with water (50 ml) and extracted with ethyl acetate (4 x 50 ml). The organic layer was neutralised with NaHCO<sub>3</sub> (1M; 2 x 30 ml), washed with water (30 ml) and dried over MgSO<sub>4</sub>. After removal of the solvent in vacuo a residue was obtained which was purified by flash chromatography (eluent : benzene). The fractions containing material of R<sub>f</sub> = 0.68 were evaporated to give the *title compound* as a yellow solid which was washed with petrol, filtered and dried (189 mg, 49%); m.p. 165-166°C; (Found: C, 84.4; H, 4.66 C<sub>32</sub>H<sub>22</sub>O<sub>2</sub> requires: C, 87.7; H, 5.02%); t.l.c. R<sub>f</sub> (F) 0.68, R<sub>f</sub> (D) 0.62; ν<sub>max</sub> (CH<sub>2</sub>Cl<sub>2</sub>) 3060 (CH stretching, aromatic), 2990, 2960, 2920, 2860 (CH stretching, alkyl), 1810 (CO, lactone), 1735 (CO, ester),

1610, 1500 (aromatic rings)  $\text{cm}^{-1}$ ;  $\lambda_{\text{max}}$  386 (6371), 370 (7964), 302 (22697), 290 (10894), 254 (42208) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz) 8.78-8.61 (6H, m, aromatic), 8.28-8.21 (2H, m, aromatic), 7.75-7.55 (8H, m, aromatic), 5.39 (1H, s, CH), 4.04 (2H, q,  $^3\text{J} = 7.1$  Hz,  $\text{CH}_2$ ), 0.97 (3H, t,  $^3\text{J} = 7.1$  Hz,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 67 MHz) 171.9 (CO, ester), 138.3, 138.2, 131.6, 130.5, 128.6, 127.7 (quaternary aromatic C's), 127.6, 127.1, 126.2, 126.1, 125.0, 123.9, 123.4, 123.2 (aromatic CH's), 61.4 ( $\text{CH}_2$ ), 54.9 (CH), 13.8 ( $\text{CH}_3$ ); m/z (EI) 438 ( $\text{M}^+$ ).

17-Hydroxymethyltetrabenzo(a,c,g,i)fluorene (31)

Diisobutylaluminium hydride (12.3 ml, 12.3 mmol; 3 equiv.; 1M solution in  $\text{CH}_2\text{Cl}_2$ ) was added dropwise at  $-65^\circ\text{C}$  to a solution of 17-ethoxycarbonyltetrabenzo(a,c,g,i)fluorene (1.795 g, 4.098 mmol) in dry distilled dichloromethane (25 ml). The temperature was maintained between  $-65^\circ\text{C}$  and  $-60^\circ\text{C}$  during the addition. The reaction mixture was stirred for 1h at  $-65^\circ\text{C}$  and at room temperature for a further hour. The reaction mixture was cooled to  $-30^\circ\text{C}$ . An aqueous solution of acetic acid (50 ml; 10% v/v) was then added dropwise. After separation of the two layers, the aqueous phase was extracted with dichloromethane (3 x 100 ml). The combined organic phases were washed with water (70 ml) and neutralised with  $\text{NaHCO}_3$ . Finally the organic phase was dried over  $\text{MgSO}_4$  and evaporated to give a crude oil (1.7 g). After

purification by flash chromatography using benzene as the eluent, the fractions containing material of  $R_f = 0.14$  were evaporated to give a yellow solid. This was recrystallised from  $\text{CH}_2\text{Cl}_2$ /petrol (b.p.  $40^\circ\text{C}$ - $60^\circ\text{C}$ ) to afford the *title compound* as a yellow solid (1.18 g, 73%); m.p.  $202$ - $203^\circ\text{C}$ ; (Found: C, 91.2; H, 4.96  $\text{C}_{30}\text{H}_{20}\text{O}$  requires: C, 90.9; H, 5.05%); t.l.c.  $R_f$  (F) 0.15,  $R_f$  (A) 0.18,  $R_f$  (B) 0.75;  $\nu_{\text{max}}$  ( $\text{CH}_2\text{Cl}_2$ ) 3600 (free OH), 3060 (CH stretching, aromatic), 2940, 2900 (CH stretching, alkyl), 1610, 1500 (aromatic rings), 1045 (CO stretching)  $\text{cm}^{-1}$ ;  $\lambda_{\text{max}}$  380 (10692), 368 (11484), 302 (27324), 290 (21780), 254 (43560) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz), 8.80-8.63 (6H, m, aromatic), 8.27-8.22 (2H, m, aromatic), 7.73-7.56 (8H, m, aromatic), 5.05 (1H, t,  $^3J = 4.3$  Hz, CH), 4.49 (2H, d,  $^3J = 4.3$  Hz,  $\text{CH}_2$ ), 1.31 (1H, s, OH);  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 50 MHz), 141.6, 137.3, 131.4, 130.4, 128.5, 127.9 (quaternary aromatic C's), 127.4, 126.9, 126.1, 125.9, 125.0, 124.5, 123.4 (aromatic CH's), 66.5 ( $\text{CH}_2$ ), 50.8 (CH); m/z (EI) 396 ( $\text{M}^+$ ), 366.

17-Acetoxymethyltetrabenzo(a,c,g,i)fluorene (35)

17-Hydroxymethyltetrabenzo(a,c,g,i)fluorene (0.207 g, 0.522 mmol) was dissolved in acetic anhydride<sup>146</sup> (4 ml). To this solution was added 2 drops of  $\text{H}_2\text{SO}_4$  (2M). The reaction mixture was stirred for 1h. The *title compound* was obtained as a yellow solid which was washed with water (80 ml) and dried to constant weight over  $\text{P}_2\text{O}_5$  in a vacuum

desiccator (183.8 mg, 80%); m.p. 209-210°C, (Found: C, 87.0; H, 5.08  $C_{32}H_{22}O_2$  requires: C, 87.7; H, 5.02%); t.l.c.  $R_f$  (A) 0.32,  $R_f$  (F) 0.22;  $\nu_{max}$  ( $CH_2Cl_2$ ) 3060 (CH stretching, aromatic), 1740 (CO, ester), 1610, 1500 (aromatic rings), 1230, 1045 (CO, stretching)  $cm^{-1}$ ;  $\lambda_{max}$  380 (17885), 368 (19345), 302 (45260), 290 (36865), 254 (72271) nm;  $\delta_H$  ( $CDCl_3$ , 80 MHz), 8.83-8.57 (6H, m, aromatic), 8.29-8.17 (2H, m, aromatic), 7.78-7.54 (8H, m, aromatic), 5.12 (1H, t,  $^3J = 5.5$  Hz, CH), 4.60 (2H, d,  $^3J = 5.5$  Hz,  $CH_2$ ), 1.84 (3H, s,  $CH_3$ );  $\delta_C$  ( $CDCl_3$ , 50 MHz) 170.7 (CO, ester), 141.8, 137.0, 131.5, 130.4, 128.6, 128.0 (quaternary aromatic C's), 127.4, 126.8, 126.1, 126.0, 125.0, 124.9, 123.5, 123.3 (aromatic CH's), 67.9 ( $CH_2$ ), 47.1 (CH), 20.7 ( $CH_3$ ); m/z (EI) 438 ( $M^+$ ), 378, 364.

#### 17-Methylenetetra benzo(a,c,g,i)fluorene (38)

A solution of 17-hydroxymethyltetra benzo(a,c,g,i)fluorene (50 mg, 0.126 mmol) and triethylamine (1 ml, 7.17 mmol; 57 equiv.) in dichloromethane (2 ml) was stirred overnight at room temperature. T.l.c. (eluent (C)) showed only the presence of the starting material. Phosgene (1 ml, 1.93 mole; 15 equiv; 1.93 M in toluene) was then added. A red colouration of the reaction mixture was immediately observed and a gas was released. The reaction mixture was stirred for 5 min, then diluted with water (50 ml) and acidified with  $H_2SO_4$  (2M; pH=1). After extraction with dichloromethane (3 x 30 ml), the combined

organic phases were washed with water (2 x 50 ml) to neutrality and dried over  $\text{MgSO}_4$ . The solvent was removed *in vacuo* to give an orange residue. Purification by flash chromatography (eluent (C)) gave the title compound as a red crystalline solid (7.3 mg, 15%); m.p. 169-170°C; (Found: C, 94.9; H, 4.90  $\text{C}_{30}\text{H}_{18}$  requires: C, 95.2; H, 4.76%); t.l.c. :  $R_f$  (C) 0.79,  $R_f$  (F) 0.87;  $\lambda_{\text{max}}$  454 (2700), 328 (44280), 314 (50220), 260 (87480) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 80 MHz) 8.82-8.41 (8H, m, aromatic), 7.77-7.51 (8H, m, aromatic), 6.92 (2H, s,  $\text{CH}_2$ );  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 50 MHz) 147.1, 136.8, 133.4, 131.6, 131.1, 128.5, 127.4 (quaternary aromatic C's and quaternary C (alkene)), 127.8, 127.3, 126.3, 125.6, 125.2, 124.7, 123.7, 123.5 (aromatic CH's), 122.8 ( $\text{CH}_2$ ); m/z (FAB) 378 ( $\text{M}^+$ ). HRMS 378.14082,  $\text{C}_{30}\text{H}_{18}$  requires : 378.14084 ( $\therefore$ ) < 1 ppm.

17-(Trimethylsilyl)oxymethyltetra benzo(a,c,g,i)fluorene

(39)

A solution of 17-hydroxymethyltetra benzo(a,c,g,i) fluorene (0.05 g, 0.126 mmol) and *N,N'*-bis-trimethylsilyl urea<sup>147</sup> (32.3 mg, 0.158 mmol; 2.6 equiv.) in dichloromethane (2 ml) was heated under reflux for 3h. The precipitated urea was filtered off and washed with dichloromethane (2 x 1 ml). The solvent was removed *in vacuo* to give a residue. Purification by wet flash chromatography (benzene/petrol (b.p. 40-60°C) (75:25)) gave the title compound (47.8 mg, 81%); m.p. 130-131°C;

(Found: C, 84.2; H, 6.06 C<sub>33</sub>H<sub>28</sub>O Si requires: C, 84.6; H, 5.98%); t.l.c. R<sub>f</sub> (F) 0.58, R<sub>f</sub> (A) 0.51, R<sub>f</sub> (G) 0.76; δ<sub>H</sub> (CDCl<sub>3</sub>, 80 MHz) 8.86-8.62 (6H, m, aromatic), 8.49-8.37 (2H, m, aromatic), 7.79-7.51 (8H, m, aromatic), 5.11 (1H, t, <sup>3</sup>J = 5.3 Hz, CH), 4.13 (2H, d, <sup>3</sup>J = 5.3 Hz, CH<sub>2</sub>), 0.4 (9H, s, 3xCH<sub>3</sub>); δ<sub>C</sub> (CDCl<sub>3</sub>, 67 MHz) 143.4, 136.5, 131.4, 130.3, 129.0, 128.1 (quaternary aromatic C's), 127.4, 126.5, 125.9, 125.8, 125.6, 124.9, 123.5, 123.0 (aromatic CH's), 67.2 (CH<sub>2</sub>), 51.4 (CH), -1.0 (3xCH<sub>3</sub>); m/z (EI) 468 (M<sup>+</sup>), 378, 364.

17-Tetrabenzo(a,c,g,i)fluorenylmethyl chloroformate (37)

To a solution of 17-(trimethylsilyl)oxymethyl tetrabenzo(a,c,g,i)fluorene (0.177 g, 0.38 mmol) in dichloromethane (5 ml) was added phosgene (1.5 ml, 2.9 mmol; 7.5 equiv; 1.93M in toluene). The reaction mixture was heated under reflux for 2h and then stirred at room temperature for 48h, under nitrogen. The solvent was removed *in vacuo* to give the *title compound* as a yellow solid which was recrystallised from dichloromethane/n-hexane and dried (36.9 mg, 21%); m.p. 188-189°C; (Found: C, 80.9; H, 4.29; N, 0.45 C<sub>31</sub>H<sub>19</sub>O<sub>2</sub>Cl requires: C, 81.1; H, 4.14%); ν<sub>max</sub> (CH<sub>2</sub>Cl<sub>2</sub>) 3060 (CH stretching, aromatic), 1775 (CO, chloroformate), 1610, 1500 (aromatic rings), 1160, 1140 (CO stretching) cm<sup>-1</sup>; δ<sub>H</sub> (CDCl<sub>3</sub>, 200 MHz) 8.79-8.60 (6H, m, aromatic), 8.20-8.15 (2H, m, aromatic), 7.76-7.57 (8H, m, aromatic), 5.17 (1H, t, <sup>3</sup>J = 5.7 Hz, CH), 4.77

(2H, d,  $^3J = 5.7$  Hz, CH<sub>2</sub>);  $\delta_C$  (CDCl<sub>3</sub>, 50 MHz) 150.5 (CO, chloroformate), 140.3, 137.2, 131.6, 130.5, 128.2, 127.7 (quaternary aromatic C's), 127.5, 127.0, 126.3, 126.2, 125.1, 124.5, 123.5, 123.4 (aromatic CH's), 74.6(CH<sub>2</sub>), 46.5(CH).

17-Tetrabenzo(a,c,g,i)fluorenylmethyl N-succinimidyl carbonate (40)

To a solution of 17-tetrabenzo(a,c,g,i)fluorenylmethyl chloroformate (26.8 mg, 0.0585 mmol) and N-hydroxysuccinimide (6.7 mg, 0.0585 mmol) in dichloromethane (2 ml) was added N,N'-dimethylaniline (7.4  $\mu$ l, 0.0585 mmol). The reaction mixture was stirred overnight under nitrogen. The solvent was removed *in vacuo* and the residue obtained was recrystallised from dichloromethane/petrol (b.p. 40-60°C) to give the title compound as a yellow solid (11.6 mg, 37%); m.p. 212°C; (Found: C, 77.0; H, 4.25; N, 2.16 C<sub>35</sub>H<sub>23</sub>NO<sub>5</sub> requires: C, 78.2; H, 4.28; N, 2.61%);  $\nu_{\max}$  (CH<sub>2</sub>Cl<sub>2</sub>) 3060 (CH stretching, aromatic), 2990 (CH stretching, alkyl), 1815, 1790, 1750 (imide carbonyl and carbonate), 1610, 1500 (aromatic rings) cm<sup>-1</sup>;  $\lambda_{\max}$  368 (39738), 302 (99882), 292 (83772), 255 (128880) nm;  $\delta_H$  (CDCl<sub>3</sub>, 80 MHz) 8.86-8.60 (6H, m, aromatic), 8.34-8.22 (2H, m, aromatic), 7.86-7.58 (8H, m, aromatic), 5.30 (1H, t,  $^3J = 5.9$  Hz, CH), 4.76 (2H, d,  $^3J = 5.9$  Hz, CH<sub>2</sub>), 2.76 (4H, s, 2xCH<sub>2</sub>, succinimide);  $\delta_C$  (CDCl<sub>3</sub>, 50 MHz) 168.2 (CO, succinimide), 151.8 (CO, carbonate), 140.7, 137.2, 131.8,

130.5, 128.4, 127.8 (quaternary aromatic C's), 127.5, 127.4, 126.4, 126.2, 125.1, 124.9, 123.6, 123.3 (aromatic CH's), 74.8 (CH<sub>2</sub>), 46.6 (CH) 25.3 (2xCH<sub>2</sub>, succinimide); m/z (FAB) HRMS (MH<sup>+</sup>) found: 538.16544; C<sub>35</sub>H<sub>24</sub>NO<sub>5</sub> requires: 538.16543 (∴) < 1 ppm.

17-Tetrabenzo(a,c,g,i)fluorenylmethyl-p-nitrophenyl carbonate (41)

To a solution of 17-hydroxymethyltetrabenzo(a,c,g,i)fluorene (0.05 g, 0.126 mmol) and p-nitrophenyl chloroformate (35.5 mg, 0.175 mmol; 1.4 equiv.) in dichloromethane (2 ml) was added N,N'-dimethylaniline (16 µl, 0.126 mmol). The reaction was stirred at room temperature, under nitrogen, for 24h. The reaction was driven to completion by adding more p-nitrophenyl chloroformate (45 mg, 0.223 mmol; 1.7 equiv.) and by stirring at room temperature for a further 48h. After purification by flash chromatography using toluene as the eluent, the fractions containing material of R<sub>f</sub>=0.29 were evaporated to give a yellow oil. Trituration in petrol (b.p. 40-60°C) gave the title compound as a yellow solid (56.5 mg, 80%); m.p. 139-140°C; (Found: C, 77.7; H, 3.94; N, 2.29 C<sub>37</sub>H<sub>23</sub>NO<sub>5</sub> requires: C, 79.1; H, 4.10; N, 2.49%); t.l.c. R<sub>f</sub> (C) 0.29, R<sub>f</sub> (A) 0.16; ν<sub>max</sub> (CH<sub>2</sub>Cl<sub>2</sub>) 3060 (CH stretching, aromatic), 2960, 2930, 2880 (CH stretching, alkyl), 1770 (CO, carbonate), 1620, 1600, 1495 (aromatic rings), 1530, 1350 (conjugated nitro-NO<sub>2</sub>), 1215 (CO

stretching), 860 (CH bending, *p*-disubstituted)  $\text{cm}^{-1}$ ;  $\lambda_{\text{max}}$  366 (20035) 301 (52092), 289 (48085), 260 (85751), 252 (88957) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz) 8.79-8.76 (4H, m, aromatic), 8.61-8.57 (2H, m, aromatic), 8.33-8.16 (2H, m, aromatic), 7.91 (2H, d,  $J_{\text{AB}} = 8.9$  Hz, *p*-nitrophenyl), 7.76-7.56 (8H, m, aromatic), 6.64 (2H, d,  $J_{\text{AB}} = 8.9$  Hz, *p*-nitrophenyl), 5.14 (1H, t,  $^3J = 4.7$  Hz, CH), 4.96 (2H, d,  $^3J = 4.7$  Hz,  $\text{CH}_2$ );  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 50 MHz) 154.9 (CO, carbonate), 151.6 (quaternary aromatic  $\text{C}_{1'}$ ), 144.9 (quaternary aromatic  $\text{C}_{4'}$ ), 140.3, 137.4, 131.5, 130.4, 128.3, 127.7 (quaternary aromatic c's), 127.4, 127.0, 126.2, 125.1, 124.7, 124.1, 123.5, 121.2 (aromatic CH's), 125.3 (aromatic  $\text{CH}_{2',6'}$ ), 121.4 (aromatic  $\text{CH}_{3',5'}$ ), 71.2 ( $\text{CH}_2$ ), 46.8 (CH);  $m/z$  (EI) 378, 139, 44; (FAB) 561 ( $\text{M}^+$ ), 379. HRMS 561.15759,  $\text{C}_{37}\text{H}_{23}\text{NO}_5$  requires: 561.15761 ( $\Delta$ ) < 1 ppm.

$N_{\alpha}$ -17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl

glycine tert-butyl ester (42)

To a solution of 17-tetrabenzo(a,c,g,i)fluorenylmethyl-*p*-nitrophenyl carbonate (39.2 mg, 0.07 mmol) and glycine tert-butyl ester acetate salt (14.7 mg, 0.077 mmol; 1.1 equiv.) in dichloromethane (1.5 ml) was added *N,N'*-dimethylaniline (18  $\mu\text{l}$ , 0.14 mmol; 2 equiv). The reaction mixture was stirred at room temperature, under nitrogen, for 72h. After addition of water (10 ml) and acidification with  $\text{KHSO}_4$  (2M; pH=1), the reaction

mixture was extracted with dichloromethane (3 x 15 ml), washed with water (3 x 20 ml) and dried over  $\text{MgSO}_4$ . The solvent was removed *in vacuo* to give an orange oil which was triturated in ether. A yellow solid was obtained which was filtered, washed with petrol (b.p. 40-60°C) and dried to give the title compound (30.6 mg, 79%); m.p. 170-171°C; (Found: C, 79.7; H, 5.51; N, 2.33  $\text{C}_{37}\text{H}_{31}\text{NO}_4$  requires: C, 80.3; H, 5.60; N, 2.53%); t.l.c.  $R_f$  (H) 0.79,  $R_f$  (I) 0.95;  $\nu_{\text{max}}$  ( $\text{CH}_2\text{Cl}_2$ ) 3440 (secondary amide NH), 3060 (CH stretching, aromatic), 2940 (CH stretching, alkyl), 1725 (CO, urethane, ester and amide I), 1600, 1500 (aromatic rings), 1520 (amide II), 1340 (OH bonding)  $\text{cm}^{-1}$ , 1220, 1160, 1110 (CO stretching), 865, 850 (out of plane CH bending)  $\text{cm}^{-1}$ ;  $\lambda_{\text{max}}$  384 (16323), 367 (17656), 302 (42641), 290 (34313), 262 (62296) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 80 MHz) 8.85-8.60 (6H, m, aromatic), 8.39-8.28 (2H, m, aromatic), 7.81-7.56 (8H, m, aromatic), 5.27 (1H, t,  $^3J = 6$  Hz, CH), 4.98 (1H, s broad, NH), 4.61 (2H, d,  $^3J = 6$  Hz,  $\text{CH}_2$ ), 3.74 (2H, d,  $^3J = 6$  Hz,  $\text{CH}_2$  glycine), 1.44 (9H, s,  $\text{CH}_3 \times 3$ );  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 50 MHz) 168.7 (CO, ester), 156.1 (CO, urethane), 141.9, 136.7, 131.5, 130.3, 128.7, 127.9 (quaternary aromatic C's), 127.4, 126.8, 126.0, 125.8, 125.3, 124.9, 123.5, 123.1 (aromatic CH's), 82.0 (quaternary C,  $\text{CMe}_3$ ), 69.0 ( $\text{CH}_2$ ), 47.5 (CH), 43.2 ( $\text{CH}_2$ , glycine), 27.9 ( $\text{CH}_3 \times 3$ ); m/z (FAB) 553, 379. HRMS 553.22527,  $\text{C}_{37}\text{H}_{31}\text{NO}_4$  requires 553.22529 ( $\Delta$ ) < 1 ppm.

$N_{\alpha}$ -17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl

glycine, Tbfmoc GLYOH (36)

A solution of  $N_{\alpha}$ -17-tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl glycine tert-butyl ester (0.392 g, 0.708 mmol) and *p*-toluenesulphonic acid (39.2 mg, 0.206 mmol) in dichloromethane (15 ml) was heated under reflux for 4.5h, during which time a precipitate was formed. This precipitate was filtered, washed with dichloromethane (2 x 15 ml) and dried to afford the title compound (319 mg, 90%); m.p. 240-241°C; (Found: C, 77.7; H, 4.5; N, 2.0  $C_{33}H_{23}NO_4$  requires: C, 79.7; H, 4.63; N, 2.82%); t.l.c. R<sub>f</sub> (H) 0.56, R<sub>f</sub> (I) 0.80;  $\nu_{\max}$  (KBr disc) 3320 (NH stretching), 3060 (CH stretching, aromatic), 1735 (CO, acid), 1710 (CO, urethane), 1680 (amide I), 1540 (amide II), 1610, 1500 (aromatic rings), 1435 (CH deformations, alkyl), 1310 (OH bending), 1260, 1240, 1175, 1160, 1040 (CO stretching), 1000, 745, 720 (out-of-plane CH bending)  $cm^{-1}$ ;  $\lambda_{\max}$  382 (21744), 368 (23297), 303 (55913), 291 (45041), 264 (82005), 255 (88839) nm;  $\delta_H$  (d-dioxan, 200 MHz), 9.05-8.97 (4H, 2xd, aromatic), 8.83 (2H, d,  $^3J = 7.8$  Hz, aromatic), 8.64 (2H, d,  $^3J = 7.8$  Hz, aromatic), 7.95-7.74 (8H, m, aromatic), 6.53 (1H, t,  $^3J = 5.8$  Hz, NH), 5.61 (1H, t,  $^3J = 5.1$  Hz, CH), 4.78 (2H, d,  $^3J = 5.1$  Hz, CH<sub>2</sub>), 3.96 (2H, d,  $^3J = 5.8$  Hz, CH<sub>2</sub> glycine);  $\delta_C$  (d-dioxan, 50 MHz) 170.6 (CO, acid), 156.2 (CO, urethane), 142.5, 136.2, 131.3, 130.0, 128.7, 127.7 (quaternary aromatic C's), 127.0, 126.5, 125.6, 125.4,

124.5, 123.3, 122.8 (aromatic CH's), 68.3 (CH<sub>2</sub>), 47.6 (CH), 41.5 (CH<sub>2</sub>, glycine); m/z (FAB) 497, 397. HRMS 498.17053, C<sub>33</sub>H<sub>24</sub>NO<sub>4</sub> requires 498.17052 (∴) < 1 ppm.

HPLC: column (RP 18), solvents: A (H<sub>2</sub>O/TFA(0.1%)), B(CH<sub>3</sub>CN/TFA(0.1%)); conditions: B (75%), A (25%); λ=229 nm,AUFS=2 or λ=368 nm,AUFS=1; flow rate: 1 ml/min; injection: 25 μl, C=1.3 mg/ml (dioxan/water (1:1)), retention time: 5.4 min.

(17-Tetrabenzo(a,c,g,i)fluorenyl)methyl methane-sulfonate (60)

To a suspension of 17-hydroxymethyltetrabenzo (a,c,g,i)fluorene (0.20 g, 0.505 mmol) and methane-sulphonic anhydride (0.92 g, 5.32 mmol, 10.5 equiv.) in dichloromethane (8 ml) was added N,N'-dimethylaniline (160 μl, 1.26 mmol; 2.5 equiv.). The reaction mixture was stirred overnight at room temperature under nitrogen. The solvent was removed *in vacuo* to give a residue. After purification by flash chromatography (eluent (F)), the fractions containing material of R<sub>f</sub> = 0.26 (F) were evaporated to give an orange oil. Trituration in diethylether gave the *title compound* as a yellow solid which was filtered and dried (181.7 mg, 76%); m.p. 178-181°C; (Found: C, 78.2; H, 4.59 C<sub>31</sub>H<sub>22</sub>O<sub>3</sub>S requires: C, 78.5; H, 4.64%); t.l.c. R<sub>f</sub> (C) 0.13, R<sub>f</sub> (F) 0.26; ν<sub>max</sub> (CH<sub>2</sub>Cl<sub>2</sub>) 3060 (CH stretching, aromatic), 2920 (CH

stretching, alkyl), 1610, 1500 (aromatic rings), 1360, 1175 (SO<sub>3</sub>), 1230, 1040 (CO stretching), 990, 970, 940 (out-of-plane CH deformation) cm<sup>-1</sup>;  $\delta_{\text{H}}$  (CDCl<sub>3</sub>, 200 MHz) 8.74 (4H, 2xd, aromatic), 8.58 (2H, d, <sup>3</sup>J = 7.9 Hz, aromatic), 8.16 (1H, d, <sup>3</sup>J = 6.6 Hz, aromatic), 8.15 (1H, d, <sup>3</sup>J = 6.2 Hz, aromatic), 7.75-7.54 (8H, m, aromatic), 5.01 (1H, t, <sup>3</sup>J = 5.0 Hz, CH), 4.76 (2H, d, <sup>3</sup>J = 5.0 Hz, CH<sub>2</sub>), 2.19 (3H, s, CH<sub>3</sub>);  $\delta_{\text{C}}$  (CDCl<sub>3</sub>, 50 MHz) 140.1, 137.3, 131.5, 130.4, 128.1, 127.6 (quaternary aromatic C's), 127.4, 127.1, 126.3, 126.2, 125.1, 124.4, 123.5, 123.4 (aromatic CH's), 72.4 (CH<sub>2</sub>), 47.1 (CH), 36.6 (CH<sub>3</sub>); m/z (FAB) 474 (M<sup>+</sup>), 379. HRMS 474.12898, C<sub>31</sub>H<sub>22</sub>O<sub>3</sub>S requires: 474.12896 (Δ) < 1 ppm.

N<sup>α</sup>-17-Tetrabenzo(a,c,g,i)fluorenylmethyl-L-alanine

tert-butyl ester (61)

To a suspension of alanine tert-butyl ester hydrochloride (80.7 mg, 0.444 mmol; 2 equiv.) in ethyl acetate (4 ml) was added triethylamine (62 μl, 0.444 mmol; 2 equiv.). The reaction mixture was stirred for 3h at room temperature. The precipitated triethylamine hydrochloride was filtered off, washed with ethyl acetate (2 x 0.5 ml) and dried (53.9 mg, 88%). To the filtrate containing the free amino acid ester was added (17-tetrabenzo(a,c,g,i)fluorenyl)methyl methanesulfonate (105.2 mg, 0.222 mmol) and N,N'-dimethylaniline (28.1 μl, 0.222 mmol). The reaction mixture was stirred overnight

at room temperature. After addition of water (20 ml) and acidification with  $\text{KHSO}_4$  (2M; pH=1), the reaction was extracted with dichloromethane (3 x 40 ml). The combined organic phases were washed with water (2 x 35 ml; pH 6-7) and dried over  $\text{MgSO}_4$ . After filtration the solvent was removed *in vacuo* to give an orange oil. Following purification by flash chromatography using chloroform and then a mixture of chloroform/methanol (9:1) as the eluent, the fractions containing material of  $R_f = 0.79$  (B) were evaporated to give a yellow oil. Diethyl ether was added and subsequently removed *in vacuo* to give the title compound as a yellow foam (88.5 mg, 76%); m.p. 65-73°C; (Found: C, 84.0; H, 6.39; N, 2.80  $\text{C}_{37}\text{H}_{33}\text{NO}_2$  requires: C, 84.9; H, 6.31; N, 2.68%); t.l.c.  $R_f$  (B) 0.79,  $R_f$ (I) 0.86;  $\nu_{\text{max}}$  ( $\text{CH}_2\text{Cl}_2$ ) 3060 (CH stretching, aromatic), 2960, 2940, 2870 (CH stretching, alkyl), 1725 (CO, ester), 1610, 1500 (aromatic rings), 1370 ( $\text{CH}_3$  symmetrical deformation), 1220, 1150, 1045 (Co stretching), 850 (out-of-plane CH bending)  $\text{cm}^{-1}$ ;  $\lambda_{\text{max}}$  383 (18014), 368 (18886), 302 (43874), 290 (36610), 254 (74382) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz), 8.82-8.64 (6H, m, aromatic), 8.39-8.25 (2H, m, aromatic), 7.75-7.57 (8H, m, aromatic), 4.96 (1H, dxd,  $^3J_{\text{a,c}} = 4.7$  Hz,  $^3J_{\text{b,c}} = 4.4$  Hz, CH), 3.56 (1H, dxd,  $^2J_{\text{a,b}} = 12.2$  Hz,  $^3J_{\text{a,c}} = 4.7$  Hz,  $\text{H}_\text{a}(\text{CH}_2)$ ), 3.45 (1H, dxd,  $^2J_{\text{a,b}} = 12.2$  Hz,  $^3J_{\text{b,c}} = 4.4$  Hz,  $\text{H}_\text{b}(\text{CH}_2)$ ), 2.77 (1H, q,  $^3J = 7.0$  Hz,  $\alpha$  CH), 1.15 (9H, s,  $\text{CH}_3 \times 3$ ), 0.87 (3H, d,  $^3J = 7.0$  Hz,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 50 MHz) 174.3 (CO, ester), 143.7, 143.1, 136.7,

136.5, 131.2, 130.2, 128.6, 128.5, 127.9, 127.8 (quaternary aromatic C's), 127.3, 126.6, 125.7, 125.5, 124.8, 123.3, 123.2 (aromatic CH's), 80.1 (quaternary C, C(Me)<sub>3</sub>), 57.4 (α CH), 52.0 (CH<sub>2</sub>), 49.0 (CH), 27.5 (CH<sub>3</sub>x3), 18.2 (CH<sub>3</sub>); m/z (FAB) 524 (MH<sup>+</sup>), 468. HRMS 524.25895, C<sub>37</sub>H<sub>34</sub>NO<sub>2</sub> requires: 524.25894 (..) < 1 ppm.

N<sup>α</sup>-17-Tetrabenzo(a,c,g,i)fluorenylmethyl-L-alanine,

Tbfm-L-Ala OH (57)

A solution of N<sup>α</sup>-17-tetrabenzo(a,c,g,i)fluorenylmethyl-L-alanine tert-butyl ester (52.3 mg, 0.1 mmol) in trifluoroacetic acid (0.475 ml) and water (0.025 ml) was sonicated for 3.5h. The solvent was evaporated under reduced pressure to give a residue. Trituration in ether gave the trifluoroacetate salt of the *title compound* as a yellow solid, which was filtered off, washed with ether and dried (40.4 mg, 69%). A small aliquot was dissolved in a mixture of CH<sub>3</sub>COOH and H<sub>2</sub>O and lyophilised to give an analytically pure sample of the acetate; m.p. 139-142°C; (Found: C, 79.7; H, 5.25; N, 2.75 C<sub>35</sub>H<sub>29</sub>NO<sub>4</sub> requires: C, 79.7; H, 5.50; N, 2.66%); [α]<sub>D</sub><sup>25</sup> (TFA salt) + 163.4° (C = 1 in CH<sub>3</sub>COOH); t.l.c. R<sub>f</sub> 0.50 (I), R<sub>f</sub> 0.20 (H); ν<sub>max</sub> (KBr) 3400(NH), 3070 (CH stretching aromatic), 2920, 2850(CH stretching, alkyl), 1700 (CO, acid), 1620(COO<sup>-</sup>), 1500 (aromatic rings), 1450, 1435 (CH deformation, alkyl), 1240, 1200, 1040 (CO stretching), 750, 730 (out-of-plane CH bending) cm<sup>-1</sup>; λ<sub>max</sub> (TFA salt) 382 (22917), 365

(27759), 300 (69720), 288 (56809), 252 (122660) nm;  $\delta_{\text{H}}$  ( $d_4$ -acetic acid, 200 MHz) 8.81-8.72 (4H, m, aromatic), 8.57 (2H, d,  $^3J=8.5$  Hz, aromatic), 8.21-8.14 (2H, m, aromatic), 7.77-7.54 (8H, m, aromatic), 5.11 (1H, t (broad), CH), 4.53 (1H, dxd (broad),  $H_{\text{a}}(\text{CH}_2)$ ), 4.37 (1H, dxd (broad),  $H_{\text{b}}(\text{CH}_2)$ ), 3.69 (1H, q,  $^3J = 7.2$  Hz,  $\alpha$  CH), 0.90 (3H, d,  $^3J = 7.2$  Hz,  $\text{CH}_3$ );  $\delta_{\text{C}}$  ( $d_4$ -acetic acid, 50 MHz) 172.5 (CO, acid), 138.2, 137.7, 137.4, 137.3, 131.52, 131.48, 130.1, 130.0, 127.58, 127.54, 126.9, 126.8 (quaternary aromatic C's), 127.2, 126.7, 126.2, 126.0, 125.8, 124.3, 123.7, 122.8 (aromatic CH's), 57.8 ( $\alpha$  CH), 49.2 ( $\text{CH}_2$ ), 44.0 (CH), 13.4 ( $\text{CH}_3$ ); m/z (FAB) 468 ( $\text{MH}^+$ ), 379. HRMS 468.19637,  $\text{C}_{33}\text{H}_{26}\text{NO}_2$  requires: 468.19634 (.) < 1 ppm.

$N^{\alpha}$ -17-Tetrabenzo(a,c,g,i)fluorenylmethyl-L-phenylalanine  
tert-butyl ester (63)

To a suspension of L-phenylalanine t-butyl ester hydrochloride (0.594 g, 2.30 mmol; 2 equiv) in ethyl acetate (22 ml) was added triethylamine (319.8  $\mu\text{l}$ , 2.30 mmol; 2 equiv). The reaction mixture was stirred for 2.5 h at room temperature. The precipitated triethylamine hydrochloride was filtered off, washed with EtOAc (2 x 1 ml) and dried (0.314 g, 99%). To the filtrate containing the free amino acid ester was added (17-tetrabenzo(a,c,g,i)fluorenyl)methyl methanesulfonate (0.546 g, 1.152 mmol),  $N,N'$ -dimethylaniline (146  $\mu\text{l}$ , 1.152 mmol) and

dichloromethane (5 ml). The reaction mixture was stirred overnight at room temperature under nitrogen. After addition of water (100 ml) and acidification to pH = 1 with  $\text{KHSO}_4$  (2 M), the reaction was extracted with dichloromethane (3 x 200 ml). The combined organic phases were washed with water to pH 6-7 (2 x 200 ml) and dried over  $\text{MgSO}_4$ . After filtration, the solvent was evaporated under reduced pressure to give an orange oil. After purification by flash chromatography using benzene followed by a mixture of benzene and methanol (97:3) as the eluent, the fractions containing material of  $R_F = 0.09$  (benzene) were evaporated to give a yellow oil. Diethyl ether was added and subsequently removed *in vacuo* to give the title compound as a yellow foam (0.537 g, 78%); m.p. 65-74°C; (Found: C, 85.9; H, 6.24; N, 2.38  $\text{C}_{43}\text{H}_9\text{NO}_2$  requires: C, 86.1; H, 6.18; N, 2.34%);  $[\alpha]_D^{20} + 41.6^\circ$  (C = 1,  $\text{CH}_2\text{Cl}_2$ ); t.l.c.  $R_F$  (F) 0.09,  $R_F$  (H) 0.80;  $\nu_{\text{max}}$  (KBr) 3060, 3030 (CH stretching, aryl), 2980, 2920, 2850 (CH stretching, alkyl), 1720 (CO, ester), 1610, 1500 (aromatic rings), 1450, 1435 (CH deformations, alkyl), 1390, 1370 ( $\text{CH}_3$ , symmetrical deformations), 1240, 1150, 1045 (CO stretching), 850, 750, 725, 700, 680 (CH, out-of-plane deformation)  $\text{cm}^{-1}$ ;  $\lambda_{\text{max}}$  382 (22742), 368 (23742), 303 (54982), 290 (46235), 254 (93469) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz) 8.84-8.64 (6H, m, aromatic), 8.31-8.20 (2H, m, aromatic), 7.76-7.58 (8H, m, aromatic), 6.97-6.82 (3H, m, aromatic (meta and para), phenylalanine), 6.64-6.59 (2H, m,

aromatic (*ortho*), phenylalanine), 4.90 (1H, apparent t,  $^3J_{C,a} = 4.2$  Hz,  $^3J_{C,b} = 4.5$  Hz,  $H_C$  (CH)), 3.71 (1H, d x d,  $^2J_{a,b} = 12.2$  Hz,  $^3J_{b,c} = 4.5$  Hz,  $H_b$  (CH<sub>2</sub>)), 3.45 (1H, d x d,  $^2J_{a,b} = 12.2$  Hz,  $^3J_{a,c} = 4.2$  Hz,  $H_a$  (CH<sub>2</sub>)), 2.84 (1H, t,  $^3J = 6.5$  Hz,  $\alpha$  CH), 2.41 (2H, d,  $^3J = 6.5$  Hz,  $\beta$  CH<sub>2</sub>), 2.03 (1H, s (broad), NH), 1.05 (9H, s, CH<sub>3</sub> x 3);  $\delta_C$  (CDCl<sub>3</sub>, 50 MHz) 173.1 (CO, ester), 143.5, 142.8, 137.1, 136.7, 131.3, 130.29, 130.26, 128.5, 128.0, 127.9 (quaternary aromatic C's), 128.7, 128.2, 127.6, 127.3, 126.7, 125.8, 125.6, 124.8, 124.5, 123.3 (aromatic CH's), 80.4 (quaternary C, C(CH<sub>3</sub>)<sub>3</sub>), 63.4 ( $\alpha$  CH), 51.8 (CH<sub>2</sub>), 49.1 (CH), 39.0 ( $\beta$  CH<sub>2</sub>), 27.5 (CH<sub>3</sub> x 3); m/z (FAB) 600 (MH<sup>+</sup>), 544, 379. HRMS 600.29021, C<sub>43</sub>H<sub>38</sub>NO<sub>2</sub> requires 600.29024 ( $\Delta$ ) < 1 ppm.

N $\alpha$ -17-Tetrabenzo(a,c,g,i)fluorenylmethyl-L-phenylalanine,  
Tbfm-L-Phe OH (53)

A solution of N $\alpha$ -17-tetrabenzo(a,c,g,i)fluorenylmethyl-L-phenylalanine tert-butyl ester (421.1 mg, 0.702 mmol) in trifluoroacetic acid (3.6 ml) and water (0.2 ml), was sonicated for 3.5h at room temperature. The solvent was removed *in vacuo* to give a brown residue. Trituration in diethylether gave the title compound as a yellow solid which was chilled overnight, filtered, washed with ether, and finally dried (352.7 mg, 92%); m.p. 178-180°C; (Found: C, 85.2; H, 5.47; N, 2.57 C<sub>39</sub>H<sub>29</sub>NO<sub>2</sub> requires: C, 86.2; H, 5.37; N, 2.59%);  $[\alpha]_D^{20} -40.4^\circ$  (C = 1, TFA); t.l.c. R<sub>f</sub> (H)

0.36,  $R_f$  (I) 0.62;  $\nu_{\max}$  (KBr) 3090, 3060, 3030 (CH stretching, aryl), 1620 (COO<sup>-</sup>), 1500 (aromatic rings), 1240, 1190, 1040 (CO stretching), 750, 725, 700 (CH out-of-plane deformation)  $\text{cm}^{-1}$ ;  $\lambda_{\max}$  384 (18868), 369 (21267), 304 (52447), 292 (41894), 264 (78991), 255 (86346) nm;  $\delta_C$  (TFA, 50 MHz) 169.5 (CO, acid), 137.9, 136.9, 136.1, 134.2, 131.8, 131.4, 130.5, 129.8, 126.3, 126.1, 125.8 (quaternary aromatic C's) 128.7, 127.9, 127.2, 127.0, 126.8, 124.8, 123.44, 123.34, 123.29, 122.2 (aromatic CH's), 62.2 ( $\alpha$  CH), 50.9 (CH<sub>2</sub>), 43.2 (CH), 34.2 ( $\beta$ CH<sub>2</sub>);  $m/z$  (FAB) 544 (MH<sup>+</sup>), 379. HRMS 544.22762, C<sub>39</sub>H<sub>30</sub>NO<sub>2</sub> requires: 544.22764 (Δ) < 1 ppm.

N $\alpha$ -17-Tetrabenzo(a,c,g,i)fluorenylmethyl-L-proline  
tert-butyl ester (64)

To a suspension of L-proline tert-butyl ester hydrochloride (411.5 mg, 1.981 mmol; 2 equiv) in ethyl acetate (20 ml) was added triethylamine (275  $\mu$ l; 1.981 mmol; 2 equiv). The reaction mixture was stirred for 2.5h at room temperature. The precipitated triethylamine hydrochloride was filtered off, washed with EtOAc (2 x 0.5 ml) and dried (255.4 mg, 94%). To the filtrate containing the free amino acid ester was added (17-tetrabenzo(a,c,g,i)fluorenyl)methyl methanesulfonate (469.5 mg, 0.99 mmol) and dichloromethane (5 ml). The reaction mixture was stirred overnight at room temperature

under nitrogen. A clear orange solution was obtained. After addition of water (100 ml) and acidification with  $\text{KHSO}_4$  (2M) to  $\text{pH} = 1$ , the reaction mixture was extracted with dichloromethane (3 x 200 ml). The combined organic phases were washed with water (2 x 200 ml) to  $\text{pH} 6-7$  and dried over  $\text{MgSO}_4$ . After filtration the solvent was removed *in vacuo* to give an orange oil. After purification by flash chromatography using benzene as the eluent, the fractions containing material of  $R_f = 0.23$  were evaporated to give a yellow oil. Diethyl ether was added and subsequently removed *in vacuo* to afford the *title compound* as a yellow foam which was finally dried in a vacuum desiccator (435.8 mg, 80%); m.p.  $78-87^\circ\text{C}$ ; (Found: C, 84.8; H, 6.43; N, 2.43  $\text{C}_{39}\text{H}_{35}\text{NO}_2$  requires: C, 85.2; H, 6.42; N, 2.55%);  $[\alpha]_D^{20} -56.6^\circ$  (C = 1,  $\text{CH}_2\text{Cl}_2$ ); t.l.c.  $R_f$  (H) 0.59,  $R_f$  (F) 0.23;  $\nu_{\text{max}}$  ( $\text{CH}_2\text{Cl}_2$ ) 3060 (CH stretching, aryl), 2960, 2940, 2870 (CH stretching, alkyl), 1725 (CO, ester), 1610, 1500 (aromatic rings), 1395, 1370 ( $\text{CH}_3$ , symmetrical deformations), 1220, 1150, 1040 (CO stretching)  $\text{cm}^{-1}$ ;  $\lambda_{\text{max}}$  384 (23210), 368 (23820), 302 (43060), 254 (92838) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz) 8.84-8.65 (7H, m, aromatic), 8.32 (1H, m, aromatic), 7.74-7.57 (8H, m, aromatic), 5.14 (1H, d x d,  $^3J_{\text{C},\text{a}} = 4.19$  Hz,  $^3J_{\text{C},\text{b}} = 4.21$  Hz,  $\text{H}_{\text{C}}$  (CH)), 3.77 (1H, d x d,  $^2J_{\text{a},\text{b}} = 13.9$  Hz,  $^3J_{\text{a},\text{c}} = 4.2$  Hz,  $\text{H}_{\text{a}}$  ( $\text{CH}_2$ )), 3.06 (2H, m,  $\text{H}_{\text{b}}$  ( $\text{CH}_2$ ) and  $\alpha$  CH), 2.74 (1H, m, Pro), 2.36 (1H, m, Pro), 1.87-1.57 (4H, m, Pro), 1.32 (9H, s,  $\text{CH}_3$  x 3);  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 90 MHz) 174.0 (CO,

ester), 145.9, 143.6, 136.2, 135.9, 131.3, 131.2, 130.3, 130.1, 129.2, 128.7, 128.2, 128.1 (quaternary aromatic C's), 127.3, 126.7, 126.5, 126.2, 125.6, 125.5, 125.4, 125.3, 124.8, 124.7, 123.4, 123.1, 122.7 (aromatic CH's), 80.2 (quaternary C, C (CH<sub>3</sub>)<sub>3</sub>), 66.8 (α CH), 59.4 (δ CH<sub>2</sub>), 53.6 (CH<sub>2</sub>), 49.5 (CH), 29.6 (β CH<sub>2</sub>), 27.8 (CH<sub>3</sub> x 3), 23.6 (γ CH<sub>2</sub>); m/z (FAB) 550 (MH<sup>+</sup>), 494, 379. HRMS 550.27461, C<sub>39</sub>H<sub>36</sub>NO<sub>2</sub> requires: 550.27459 (..) < 1 ppm.

N<sup>α</sup>-17-Tetrabenzo(a,c,g,i)fluorenylmethyl-L-proline

Tbfm-L-Pro OH (62)

A solution of N<sup>α</sup>-17-tetrabenzo(a,c,g,i)fluorenylmethyl-L-proline tert-butyl ester (316.6 mg, 0.576 mmol) in trifluoroacetic acid (3 ml) and water (158 μl) was sonicated for 3.5h at room temperature. The solvent was removed *in vacuo* to give a brown residue. Diethyl ether was added and the *title compound* was obtained as a yellow precipitate, which was chilled overnight, filtered and dried (295.3 mg, 84%); m.p. 121-124°C; (Found: C, 76.3; H, 4.89; N, 2.39 C<sub>37</sub>F<sub>3</sub>H<sub>28</sub>NO<sub>4</sub> requires: C, 73.1; H, 4.61; N, 2.31%); [α]<sub>D</sub><sup>20</sup> = + 28.6° (C = 1, CH<sub>2</sub>Cl<sub>2</sub>); t.l.c. R<sub>f</sub> (H) 0.28, R<sub>f</sub> (I) 0.36; ν<sub>max</sub> (KBr) 3400 (NH), 3070 (CH stretching, aryl), 2960 (CH stretching, alkyl), 1720, 1690 (CO, acid), 1500 (aromatic rings), 1450, 1435 (CH deformations, alkyl), 1240, 1180, 1130, 1045 (CO stretching), 755, 730 (out-of-plane, CH deformation) cm<sup>-1</sup>; λ<sub>max</sub> 384 (12564), 368 (14359), 302 (36796), 290 (29392),

262 (54745), 254 (61027) nm;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz) 8.74-8.48 (6H, m, aromatic), 8.04-7.96 (2H, m, aromatic), 7.75-7.47 (8H, m, aromatic), 4.99 (1H, apparent s,  $\text{H}_{\text{C}}$ , CH), 4.50 (1H, d x d,  $^2\text{J}_{\text{a,b}} = 13.5$  Hz,  $^3\text{J}_{\text{a,c}} = 4.8$  Hz,  $\text{H}_{\text{a}}$  ( $\text{CH}_2$ )), 4.10 (1H, apparent d,  $^2\text{J}_{\text{b,a}} = 13.5$  Hz,  $\text{H}_{\text{b}}$  ( $\text{CH}_2$ )), 3.49 (1H, d x d,  $^3\text{J} = 5.3$  Hz,  $^3\text{J} = 5.2$  Hz,  $\alpha$  CH, Pro), 2.28 (1H, m, Pro), 1.83-1.07 (5H, m, Pro);  $\delta_{\text{C}}$  ( $\text{CDCl}_3$ , 90 MHz) 172.3 (CO, acid), 141.6, 139.9, 137.7, 137.4, 131.7, 131.4, 130.5, 130.4, 128.1, 127.9, 127.7 (quaternary aromatic C's), 127.3, 127.25, 126.5, 126.3, 126.2, 125.3, 125.1, 124.0, 123.8, 123.6, 123.5 (aromatic CH's), 69.7 ( $\alpha$  CH), 60.7 ( $\delta$   $\text{CH}_2$ ), 55.3 ( $\text{CH}_2$ ), 47.1 (CH), 28.5 ( $\beta$   $\text{CH}_2$ ), 24.3 ( $\gamma$   $\text{CH}_2$ ); m/z (FAB) 494 ( $\text{MH}^+$ ), 379. HRMS 494.21201,  $\text{C}_{35}\text{H}_{28}\text{NO}_2$  requires: 494.21199 ( $\Delta$ ) < 1 ppm.

$\text{N}^{\alpha}$ -17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl-L-phenylalanine tert-butyl ester, Tbfmoc-L-Phe  $\text{O}^t\text{Bu}$  (54)

To a suspension of L-phenylalanine tert-butyl ester hydrochloride (126.7 mg, 0.491 mmol) in ethyl acetate (6 ml) was added triethylamine (68.5  $\mu\text{l}$ , 0.491 mmol). The reaction mixture was stirred for 2.5h at room temperature. The precipitated triethylamine hydrochloride was filtered off, washed with ethyl acetate (2 x 0.5 ml) and dried (65.3 mg, 97%). The filtrate was evaporated to give a residue which was redissolved in acetic acid. Following lyophilisation the acetate salt was obtained as a white solid (83.9 mg, 61%).

To a solution of 17-tetrabenzo(a,c,g,i)fluorenyl-methyl-para-nitrophenyl carbonate (139.4 mg, 0.248 mmol) and L-phenylalanine tert-butyl ester acetate (83.9 mg, 0.298 mmol; 1.2 equiv) in dichloromethane (5 ml) was added N,N'-dimethylaniline (63  $\mu$ l, 0.497 mmol; 2 equiv). The reaction mixture was stirred at room temperature under nitrogen for 120h. After addition of water (10 ml) and acidification with KHSO<sub>4</sub> (2M) to pH=1, the reaction mixture was extracted with dichloromethane (3 x 20 ml). The combined organic phases were washed with water (2 x 15 ml) to pH 6-7 and dried over MgSO<sub>4</sub>. After filtration the solvent was evaporated under reduced pressure to give an orange oil. After purification by flash chromatography using toluene as the eluent, the fractions containing material of R<sub>f</sub>=0.04 were evaporated to give a yellow solid. Recrystallisation from ether/petrol (b.p. 40-60°C) gave the *title compound* as a pale yellow solid which was filtered, washed with petrol and finally dried (73.9 mg, 46%); m.p. 158-162°C (dec); t.l.c. R<sub>f</sub>(C) 0.04, R<sub>f</sub>(H) 0.84;  $\nu_{\text{max}}$  (KBr) 3280 (NH stretching), 3060, 3030 (CH stretching, aryl), 2980, 2930 (CH stretching, alkyl), 1735 (CO, ester), 1715 (CO, urethane), 1680 (amide I), 1610 (aromatic rings), 1545 (amide II), 1500 (aromatic rings) 1440 (CH deformations, alkyl), 1390, 1370 (CH<sub>3</sub>, symmetrical deformations), 1290, 1255, 1220, 1155, 1050 (CO stretching), 850, 750, 720, 700 (out-of-plane CH deformation) cm<sup>-1</sup>; m/z (FAB) 643 (M<sup>+</sup>), 379. HRMS

643.27225,  $C_{44}H_{37}NO_4$  requires : 643.27224 ( $\therefore$ ) < 1 ppm.

$N^{\alpha}$ -17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl-L-phenylalanine, Tbfmoc-L-Phe OH (55)

A solution of  $N^{\alpha}$ -17-tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl-L-phenylalanine tert-butyl ester (68.2 mg, 0.106 mmol) in trifluoroacetic acid (950  $\mu$ l) and water (50  $\mu$ l) was sonicated for 2.5h. The solvent was removed in *vacuo* to give a purple residue. A mixture of diethyl ether and petrol b.p. 40-60°C (1:1) was added. The precipitate obtained was chilled overnight, filtered, washed with petrol and finally dried in a vacuum desiccator over  $P_2O_5$  to give the *title compound* (57.6 mg, 93%); m.p. 137-140°C;  $[\alpha]_D^{20}$  -50.8 (C=0.25,  $CH_2Cl_2$ ); t.l.c. :  $R_f(H)$  0.43,  $R_f(I)$  0.89;  $\nu_{max}$  (KBr) 3410 (NH stretching), 3060, 3030 (CH stretching, aryl), 2960, 2860 (CH stretching, alkyl), 1715 (CO, acid and urethane), 1610, 1500 (aromatic rings), 1435, 1420 (CH deformations, alkyl), 1340 (OH bending), 1215, 1050 (CO stretching), 750, 730, 700 (out-of-plane CH deformation)  $cm^{-1}$ ;  $\lambda_{max}$  384 (15794), 368 (16896), 302 (39670), 290 (32324), 262 (59505), 254 (64647) nm;  $\delta_H$  ( $CDCl_3$ , 200 MHz) 8.80-8.58 (6H, m, aromatic), 8.30-8.19 (2H, m, aromatic), 7.65-7.54 (8H, m, aromatic), 7.19-7.07 (5H, m, aromatic (Phe)), 5.13 (1H, apparent t, CH), 4.98 (1H, d,  $^3J=8.4$  Hz, NH), 4.79-4.64 (2H, m,  $H_a$  ( $CH_2$ ) and  $\alpha$ CH (Phe)), 4.30-4.24 (1H, m,  $H_b$  ( $CH_2$ )), 3.08 (2H, m,  $\beta$   $CH_2$  (Phe));  $\delta_C$  ( $CDCl_3$ , 90

MHz), 175.8 (CO, acid), 155.8 (CO, urethane), 142.6, 141.0, 136.8, 136.5, 135.3, 131.5, 130.3, 130.1 (quaternary aromatic C's), 129.0, 128.6, 127.4, 127.3, 127.1, 126.8, 126.6, 125.9, 125.7, 125.5, 125.1, 124.9, 124.8, 123.4, 123.1, 123.0 (aromatic CH's), 69.2 (CH<sub>2</sub>), 54.4 (α CH), 47.4 (CH), 37.4 (β CH<sub>2</sub>); m/z (FAB) 587 (M<sup>+</sup>), 379. HRMS 588.21744, C<sub>40</sub>H<sub>30</sub>NO<sub>4</sub> requires : 588.21747 (∴) < 1 ppm.

N<sup>α</sup>-17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl-L-methionine methyl ester, Tbfmoc-L-Met OCH<sub>3</sub> (56)

To a suspension of L-methionine methyl ester hydrochloride (0.300 g, 1.50 mmol) in ethyl acetate (6 ml) was added triethylamine (209.4 μl, 1.50 mmol). The reaction mixture was stirred for 40h at room temperature under nitrogen. The precipitated triethylamine hydrochloride was filtered off, washed with ethyl acetate (2 x 0.5 ml) and dried (222.3 mg; overweight). Analysis of this salt by t.l.c. (CHCl<sub>3</sub>/MeOH/CH<sub>3</sub>COOH (9:1:0.5)) revealed after ninhydrin spray the presence of some L-methionine methyl ester hydrochloride. The filtrate was evaporated under reduced pressure and the residue obtained was redissolved in glacial acetic acid. After lyophilisation the acetate salt of L-methionine methyl ester was obtained as a white solid (220.4 mg, 66%). To a solution of 17-tetrabenzo(a,c,g,i)fluorenylmethyl-para-nitrophenyl carbonate (0.238 g, 0.424 mmol) and

L-methionine methyl ester acetate (113.7 mg, 0.509 mmol; 1.2 equiv) in dichloromethane (5 ml) was added N,N'-dimethylaniline (107.6  $\mu$ l, 0.848 mmol; 2 equiv). The reaction mixture was stirred at room temperature, under nitrogen and in the dark, for 96h. After addition of water (20 ml) and acidification with  $\text{KHSO}_4$  (2M) to pH=1, the reaction mixture was extracted with dichloromethane (3 x 40 ml). The combined organic phases were washed with  $\text{H}_2\text{O}$  (2 x 40 ml) to pH 6-7 and dried over  $\text{MgSO}_4$ . After filtration the solvent was removed *in vacuo* to give an orange oil. Diethyl~~ether~~ was added and the *title compound* was obtained as a yellow precipitate which was chilled for 40h, filtered, washed with ether and dried (130.9 mg, 53%); m.p. 157-158°C; t.l.c.  $R_f$  (D) 0.20,  $R_f$  (B) 0.78;  $\nu_{\text{max}}$  (KBr) 3290 (NH stretching), 3070 (CH stretching, aryl), 2950, 2920 (CH stretching, alkyl), 1750 (CO, ester), 1715 (CO, urethane), 1680 (amide I), 1610 (aromatic rings), 1540 (amide II), 1500 (aromatic rings), 1430 (CH deformations, alkyl), 1280, 1260, 1220, 1170, 1050 (CO stretching), 750, 720 (out-of-plane CH deformation)  $\text{cm}^{-1}$ ;  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ , 200 MHz) 8.80-8.63 (6H, m, aromatic), 8.30 (2H, d,  $^3J=7.6$  Hz, aromatic), 7.73-7.57 (8H, m, aromatic), 5.22 (2H, m, CH and NH), 4.69 (1H, m,  $\text{H}_a$  ( $\text{CH}_2$ )), 4.51 (2H, m,  $\text{H}_b$  ( $\text{CH}_2$ ) and  $\alpha$  CH (Met)), 3.73 (3H, s,  $\text{CH}_3$  (ester)), 2.39 (2H, t,  $^3J=8.1$  Hz,  $\gamma$   $\text{CH}_2$  (Met)), 2.02 (3H, s,  $\epsilon$   $\text{CH}_3$  (Met)), 1.86 (2H, m,  $\beta$   $\text{CH}_2$  (Met)); m/z (FAB) 586 ( $\text{MH}^+$ ), 379. HRMS 586.20517,

$C_{37}H_{32}NO_4S$  requires : 586.20519 (..) < 1 ppm.

### 3.3 SOLID PHASE SYNTHESIS

All protected amino acid derivatives were purchased from Novabiochem and have L-stereochemistry. Solid phase peptide synthesis was carried out on an Applied Biosystems 430A peptide synthesiser. The DMF used was supplied by Rathburn Chemicals Ltd. (peptide synthesis grade). The first residue of each sequence (*i.e.* the C-terminal residue) was coupled to the *p*-alkoxybenzyl alcohol resin outside the synthesiser. The extent of coupling was determined by deprotecting a small sample of the loaded resin and quantitatively checking the olefin produced by UV at 300 nm in the case of Fmoc-peptides and at 364 nm in the case of Tbfmoc-peptides (see Appendix II). Each residue was coupled twice (2 equiv.), firstly by the symmetrical anhydride method and secondly by the HOBt active ester method ('double coupling') (except Arg, Asn, Gln double coupled *via* HOBt ester and Gly coupled once *via* symmetrical anhydride (4 equiv.)). Preformed symmetrical anhydrides were prepared from Fmoc-amino acids (1 mmol) and DICI (0.5 mmol) with an activation time of 15 minutes, and HOBt esters from Fmoc-amino acids (0.5 mmol), DICI (0.5 mmol) and HOBt (0.5 mmol) with an activation time of 30 minutes. Coupling reactions were carried out over a period of 30-60 minutes. Capping of unreacted amino functions was performed for 6 minutes using acetic

anhydride and pyridine in DMF. Deprotection of the Fmoc-peptide-resin was achieved over a 12 minute period (5+3+3+1 minutes) using a solution of 20% piperidine in DMF. The resin was washed thoroughly with DMF at the end of each cycle. As a rough monitor of the couplings, the product solution from the deprotection steps was fed through an ultraviolet detector (313 nm) and its absorption recorded to give a series of peaks. Removal of peptides from the resin and simultaneous cleavage of side chain protecting groups were performed using a mixture of trifluoroacetic acid/water/scavenger (95:5:5) for 2-3 hours. Chromatography of  $N^{\alpha}$ -protected peptides was carried out using graphitised carbon (PGC 220-224; 150-180  $\mu\text{m}$ , 100  $\text{m}^2/\text{g}$ ) in a glass column. HPLC was carried out on a Waters system using an ABI aquapore Prep 10 C-18 300 Å pore size 20  $\mu\text{m}$  spherical silica (10 mm ID x 250 mm) column for preparative separations and an ABI RP 18 aquapore OD 300 7  $\mu\text{m}$  spherical silica (4.6 mm ID x 220 mm) column for analytical separations. A gradient was used, as specified in parentheses, between solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The flow rate was 1 ml/min for analytical HPLC and 5 ml/min for preparative HPLC. Elution of the samples was monitored by ultraviolet absorption at 214 nm. Amino acid analyses were carried out on a LKB 4150 alpha amino acid analyser following sealed tube hydrolysis in constant-boiling hydrochloric acid at 110°C for 18-36 hours.

N<sup>α</sup>-9-Fluorenylmethoxycarbonyl glycylyserylmethionyl-  
valylleucylserine, Fmoc Gly Ser Met Val Leu Ser OH (44)

The resin-bound peptide Fmoc Ser (O<sup>t</sup>Bu) Met Val Leu Ser (O<sup>t</sup>Bu) (130.5 mg, 0.05 mmol) was sonicated in a solution of 20% piperidine in N,N-dimethylformamide (5 ml) for 20 min, then filtered and finally washed well with N,N-dimethylformamide, dichloromethane and dioxan. Fmoc Gly OH (89.2 mg, 0.3 mmol; 6 equiv.) was dissolved in dioxan (2 ml); 1,3-diisopropylcarbodiimide (47 μl, 0.3 mmol; 6 equiv.) was then added and the reaction mixture was sonicated for 5 min. This solution was then added to the resin-bound peptide previously swollen in dioxan (1 ml). The reaction mixture was sonicated for 4.5h. The resin-bound peptide was filtered, washed with dichloromethane, ether, and dried (129.8 mg); product resin functionality in mmol/g: 0.334, resin loading/coupling percentage: 88; A<sub>300 nm</sub> = 0.76 (resin-bound peptide: 2.52 mg). To a mixture of resin-bound peptide (126.1 mg, 0.042 mmol) anisole (0.25 ml), ethyl/methyl/sulphide (0.25 ml) and water (0.25 ml) was added trifluoroacetic acid (10 ml). The reaction mixture was sonicated for 2h. The resin was filtered, washed with trifluoroacetic acid (2 x 1 ml) and chloroform (4 ml). The solvent was removed *in vacuo* to give a residue. The peptide was precipitated on addition of ether, filtered, washed with ether and dried (42.7 mg); (Found: Ser<sub>2</sub>, 1.90, Gly, 1.05, Val, 0.99, Met, 1.02, Leu, 1.01); m/z (FAB) 853, 837, 815 (MH<sup>+</sup>). HRMS 815.36488,

$C_{39}H_{55}N_6O_{11}S_1$ , requires: 815.36492 (..) < 1 ppm.

HPLC: column RP 18, solvents: A ( $H_2O/TFA$  (0.1%)), B ( $CH_3CN/TFA$  (0.1%)); conditions: A (90%)  $\xrightarrow{25 \text{ min.}}$  A (10%);  $\lambda = 229 \text{ nm}$ ; AUFS = 0.2; flow rate: 1 ml/min.; injection: 25  $\mu$ l (C = 0.4 mg/0.5 ml (dioxan)); retention time: 14.2 min.

$N_\alpha$ -17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl-glycylserylmethionylvalylleucylserine, Tbfmoc Gly Ser Met Val Leu Ser OH (45)

The resin-bound peptide Fmoc Ser ( $O^tBu$ ) Met Val Leu Ser ( $OBu^t$ ) (65.3 mg, 0.025 mmol) was sonicated in a solution of 20% piperidine in DMF (5 ml) for 20 min. The resin-bound peptide was then filtered and washed well with DMF, dichloromethane and dioxan. Tbfmoc Gly OH (74.5 mg, 0.15 mmol; 6 equiv.) was sonicated for 30 min. in dioxan (2 ml). 1,3-Diisopropylcarbodiimide (23.5  $\mu$ l, 0.15 mmol; 6 equiv.) was then added and the reaction mixture was sonicated for 5 min. This solution was then added to the resin-bound peptide previously swollen in dioxan (1 ml). The reaction mixture was sonicated for 5h. The resin was filtered, washed well with dichloromethane, dioxan and ether, and then dried (58.3 mg); product resin functionality in mmol/g: 0.28; resin loading/coupling percentage: 80;  $A_{364 \text{ nm}} = 0.43$  (resin-bound peptide: 0.85 mg). To a mixture of resin-bound peptide (57.3 mg, 0.016 mmol), anisole (0.25 ml), ethylmethylsulphide (0.25 ml)

and water (0.25 ml), was added trifluoroacetic acid (5 ml). The reaction mixture was sonicated for 2h. The resin was filtered and washed with trifluoroacetic acid (1 ml) and chloroform (4 ml) and the solvent was removed *in vacuo*. Trituration of the residue in ether gave the desired peptide as a yellow solid which was filtered, washed well with ether and dried (17.2 mg, 69%); (Found: Ser<sub>2</sub> 1.8, Gly<sub>1</sub> 0.08, Val<sub>1</sub> 1.00, Met<sub>1</sub> 0.96, Leu<sub>1</sub> 1.00; m/z (FAB) 1053, 1037, 1015 (MH<sup>+</sup>). HRMS 1015.42761, C<sub>55</sub>H<sub>63</sub>N<sub>6</sub>O<sub>11</sub>S, requires: 1015.42752 (Δ) < 1 ppm.

HPLC: column RP18, solvents: A (H<sub>2</sub>O/TFA (0.1%)); B (CH<sub>3</sub>CN/TFA (0.1%)); conditions: A (75%) 22 min. A (5%); λ = 229 nm; AUFS = 1; flow rate: 1 ml/min.; injection: 25 μl (C = 0.2 mg/0.2 ml (dioxan)); retention time: 16.1 min.

Glycylserylmethionylvalylleucylserine, H Gly Ser Met Val  
Leu Ser OH (46)

A solution of Tbfmoc Gly Ser Met Val Leu Ser OH (29.2 mg, 28.8 μmol) in a mixture of dioxan and water (2:1; 15 ml) was loaded on a 9 mm diameter column packed with graphitised carbon (4 g). The column was then eluted with a mixture of dioxan and water (2:1; 60 ml). The deprotection was carried out by eluting the column with 20% piperidine in a mixture of dioxan and water (2:1; 20 ml). The fractions containing the peptide (monitored by t.l.c., R<sub>f</sub> = 0 (MeOH/CHCl<sub>3</sub>/CH<sub>3</sub>COOH (1:9:0.5) (using ninhydrin) were combined and evaporated to give a residue.

The peptide was precipitated with ether, filtered and dried (14.5 mg, 85%); (Found: Ser<sub>2</sub>, 1.92, Gly, 0.98, Val, 1.03, Met, 0.87, Leu, 1.02; m/z (FAB) 609, 593 (MH<sup>+</sup>). HRMS 593.29689, C<sub>24</sub>H<sub>45</sub>O<sub>9</sub>N<sub>8</sub>S requires: 593.29685 (∴) < 1 ppm.

N<sup>α</sup>-(17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl)glycylarginylthreonylleucylseryllaspartyltyrosylasparagyl-isoleucylglutaminyllsylvglutamylserylthreonylleucylhistidylleucylvalylleucylarginylleucylarginylglycylglycine.  
Tbfmoc-ubiquitin (53-76) OH (48)

To the Fmoc-ubiquitin (54-76) resin-bound peptide (150 mg, 22.65 μmol) in DMF (1 ml) was added acetic anhydride (21.4 μl, 226 μmol; 10 equiv.) and pyridine (18.2 μl, 226 μmol; 10 equiv.). The reaction mixture was sonicated for 30 min. After filtration and washing with DMF and ether, the resin-bound peptide was sonicated for 15 min in a solution of 20% piperidine in DMF (25 ml). The resin bound peptide was then filtered and washed well with DMF and ether. Tbfmoc Gly OH (45 mg, 0.09 mmol; 4 equiv.) was sonicated for 30 min. in dioxan (1.5 ml) until complete dissolution. To this solution was added 1,3-diisopropylcarbodiimide (14 μl, 0.09 mmol; 4 equiv.) and 1-hydroxybenzotriazole (12.2 mg, 0.09 mmol; 4 equiv.). The reaction mixture was sonicated for 2 min. This solution was then added to the resin-bound peptide previously swollen for 30 min. in dioxan (1.5 ml). After

sonication for 3.5h, the resin-bound peptide was filtered, washed with dioxan and ether and dried overnight in a desiccator under vacuum (148.1 mg).

Substitution efficiency: The Tbfmoc resin-bound peptide (2.1 mg) was sonicated for 30 min. in a solution of 20% triethylamine in DMF (10 ml). The specific absorption at 364 nm was recorded and the following results were derived from it: product resin functionality in mmol/g: 0.133; resin loading/coupling percentage: 88;  $A_{364 \text{ nm}} = 0.505$  (resin-bound peptide: 2.1 mg).

To the resin-bound Tbfmoc-peptide (146 mg) was added water (75  $\mu$ l), thioanisole (75  $\mu$ l), anisole (75  $\mu$ l) and trifluoroacetic acid (3 ml). The reaction mixture was sonicated for 2.5h. The resin was filtered, washed with TFA (2 x 0.5 ml) and chloroform (2 x 1 ml), and dried (24 mg). The filtrate was evaporated under reduced pressure to give a residue. Trituration in ether gave the crude peptide as a yellow solid which was chilled overnight, filtered and dried (68.5 mg). A suspension of crude Tbfmoc-ubiquitin (53-76) (12 mg) in  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 0.1% TFA; 12 ml) was then sonicated for 2h until complete dissolution had occurred. Purification of this product was finally achieved by preparative HPLC using a reverse phase C-18 column (10 x 250 mm) with a gradient of A:B, 20 to 80% B over 25 min. and ultraviolet detection at 214 nm, to yield pure Tbfmoc ubiquitin (53-76) OH after lyophilisation as a white powder (4.5 mg); amino acid analysis:

Asx<sub>2</sub> 2.14, Thr<sub>2</sub> 1.94, Ser<sub>2</sub> 1.87, Glx<sub>2</sub> 2.38, Gly<sub>3</sub> 2.73,  
 Val<sub>1</sub> 1.14, Ile<sub>1</sub> 0.94, Leu<sub>5</sub> 5.02, Tyr<sub>1</sub> 0.94, His<sub>1</sub> 1.01,  
 Lys<sub>1</sub> 0.94, Arg<sub>3</sub> 2.94; m/z (FAB) 3147.4 (MH<sup>+</sup>),  
 HRMS 3149.64063, C<sub>149</sub>H<sub>219</sub>N<sub>38</sub>O<sub>38</sub> requires: 3149.64048 (Δ) <  
 1 ppm; HPLC (A:B, A (90%)  $\xrightarrow{25 \text{ min.}}$  A (10%); 214 nm; C =  
 0.2 mg/0.2 ml (A), injection: 25 μl), R<sub>t</sub> = 18.2 min.

N<sup>α</sup>-(Acetyl)arginylthreonylleucylserylaspartyltyrosyl-  
 asparagylisoleucylglutaminyllysylglutamylserylthreonyl-  
 histidylleucylvalylleucylarginylleucylarginylglycylglycine.

N<sup>α</sup>-acetyl-ubiquitin (54-76) OH (49)

The resin-bound peptide Fmoc-ubiquitin (54-76) (100 mg, 0.0151 mmol) was sonicated for 30 min. in a solution of 20% piperidine in DMF (25 ml), then filtered, and finally washed well with DMF and ether. To the resin bound peptide previously swollen for 30 min. in DMF (1 ml) was added acetic anhydride (143 μl, 1.51 mmol; 100 equiv.) and pyridine (122 μl, 1.51 mmol; 100 equiv.). The reaction mixture was sonicated for 1h. The resin-bound peptide was filtered and washed with DMF and ether. To the resin-bound N<sup>α</sup>-acetyl-peptide was added water (50 μl), thioanisole (50 μl), anisole (50 μl) and trifluoroacetic acid (2 ml). The reaction mixture was sonicated for 2.5h. The resin was filtered, washed with TFA (2 x 0.5 ml) and chloroform (2 x 0.5 ml). The solvent was removed *in vacuo*. Trituration in ether gave the product as a white precipitate which was chilled overnight, filtered and

dried (43.6 mg). Purification of this peptide (23.4 mg) was by preparative HPLC using the same reverse phase C-18 column with a gradient of A:B, 10 to 60% B over 16 min. and UV detection at 214 nm, to give pure N<sup>α</sup>-acetyl-ubiquitin (54-76) OH as a white solid after lyophilisation (9.7 mg); amino acid analysis: Asx<sub>2</sub> 1.98, Thr<sub>2</sub> 1.82, Ser<sub>2</sub> 1.69, Glx<sub>2</sub> 2.33, Gly<sub>2</sub> 2.36, Val<sub>1</sub> 1.14, Ile<sub>1</sub> 0.91, Leu<sub>5</sub> 5.06, Tyr<sub>1</sub> 0.85, His<sub>1</sub> 1.01, Lys<sub>1</sub> 0.98, Arg<sub>3</sub> 2.87; m/z (FAB) 2712.1 (MH<sup>+</sup>), HRMS 2712.49903, C<sub>118</sub>H<sub>200</sub>N<sub>37</sub>O<sub>36</sub> requires 2712.49891 (Δ) < 1 ppm; HPLC (A:B, A (90%) 25 min., A (10%); 214 nm; C = 0.2 mg/0.2 ml (A), injection: 25 μl), R<sub>t</sub> = 13.2 min.

Behaviour of N<sup>α</sup>-acetyl-ubiquitin (54-76) OH and Tbfmoc ubiquitin (53-76) OH on PGC

A solution of Tbfmoc ubiquitin (53-76) OH (48) (5 mg) and N<sup>α</sup>-acetyl-ubiquitin (54-76) OH (49) (5 mg) was sonicated for one hour in a mixture of CH<sub>3</sub>CN/H<sub>2</sub>O/TFA (5 ml; 6:4:0.5) and loaded onto a 5 mm diameter glass column packed with graphitised carbon (1.4 g; PGC 220-224; 150-180 μm; 100 m<sup>2</sup>/g; length after packing (140 mm)). The column was then eluted with a 50% aqueous solution of CH<sub>3</sub>CN and monitored by HPLC. For each fraction the solvent was removed *in vacuo*, lyophilised and examined by analytical HPLC.

The N<sup>α</sup>-acetyl-peptide (5.1 mg recovered) was totally eluted by 30 ml of the mixture (R<sub>t</sub>=13.2 min). Nothing was

eluted by the next 325 ml of the mixture. The column was then washed with pure dioxan (600 ml). HPLC showed that the Tbfmoc peptide ( $R_t=18.2$  min) was slowly eluted from the column along with some impurities.

The deprotection was carried out using a 20% piperidine solution in a mixture of dioxan and water (1:1; 10 ml) followed by elution of the column with dioxan (50 ml). The solvent was evaporated under reduced pressure to give a residue, trituration of which in ether gave a white solid (3.3 mg) corresponding to ubiquitin (53-76) OH (50) ( $R_t = 12.6$  min).

Glycylarginylthreonylleucylserylaspartyltyrosylasparagyl-  
isoleucylglutaminyllysylglutamylserylthreonylleucylhistidyl-  
leucylvalylleucylarginylleucylarginylglycylglycine,  
ubiquitin (53-76) OH (50)

A solution of Tbfmoc-ubiquitin (53-76) OH (30 mg; crude peptide) in a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 0.5% TFA; 30 ml) was sonicated for 30 min until complete dissolution and loaded on a 5 mm diameter glass column packed with graphitised carbon (1.4g; PGC 220-224; 150-180  $\mu\text{m}$ ; 100  $\text{m}^2/\text{g}$ ; length after packing: 140 mm). The column was first eluted with a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 0.5% TFA; 50 ml). After lyophilisation a residue (6.1 mg) was obtained which gave a major peak on HPLC ( $R_t = 13$  min.,  $\text{N}^\alpha$ -acetyl-ubiquitin (55-76) or ubiquitin (54-76)). The column was then eluted with a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1;

50 ml). A residue (0.4 mg) was obtained after lyophilisation which was found by HPLC to contain impurities. The deprotection was carried out using a 20% piperidine solution in a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 50 ml). The solvent was removed *in vacuo* to give a residue which was triturated in ether, filtered and dried (11 mg). This crude peptide (11 mg) was finally purified by preparative HPLC (reverse phase C-18 column (10 x 250 mm); gradient (A:B), 10 to 60% B over 25 min; detection at 214 nm) to give ubiquitin (53-76) OH as a white solid after lyophilisation (4.5 mg, 15%); amino acid analysis: Asx<sub>2</sub>, 2.04, Thr<sub>2</sub>, 1.88, Ser<sub>2</sub>, 1.67, Glx<sub>2</sub>, 2.27, Gly<sub>3</sub>, 3.37, Val<sub>1</sub>, 1.01, Ile<sub>1</sub>, 0.90, Leu<sub>5</sub>, 4.99, Tyr<sub>1</sub>, 0.93, His<sub>1</sub>, 1.01, Lys<sub>1</sub>, 0.96, Arg<sub>3</sub>, 2.95; m/z (FAB) 2727.8 (MH<sup>+</sup>), HRMS 2727.50986, C<sub>118</sub>H<sub>201</sub>N<sub>38</sub>O<sub>36</sub> requires: 2727.50981 (..) < 1 ppm; HPLC (A:B, A (90%)  $\xrightarrow{25 \text{ min}}$  A (10%); 214 nm; C = 0.4 mg/0.4 ml (A), injection: 25  $\mu\text{l}$ ), R<sub>t</sub> = 12.6 min.

N<sup>α</sup>-(17-Tetrabenzo(a,c,g,i)fluorenylmethoxycarbonyl)  
glycylisoleucylprolylprolylaspartylglutaminyglutaminyl-  
arginylleucylisoleucylphenylalanylalanylglycyllysylglutaminyl-  
leucylglutamylaspartylglycylarginylthreonylleucylserylaspartyl-  
tyrosylasparagylisoleucylglutaminyllsylglutamylserylthreonyl-  
leucylhistidylleucylvalylleucylarginylleucylarginylglycyl-  
glycine, Tbfmoc ubiquitin (35-76) OH (52)

To the Fmoc ubiquitin (36-76) resin-bound peptide (109.3 mg, 7.88  $\mu\text{mol}$ ) in DMF (1 ml) was added acetic

anhydride (14.8  $\mu\text{l}$ , 157.6  $\mu\text{mol}$ ; 20 equiv) and pyridine (12.8  $\mu\text{l}$ , 157.6  $\mu\text{mol}$ ; 20 equiv). The reaction mixture was sonicated for 1h. After filtration and washing with DMF and ether, the resin-bound peptide was sonicated for 15 min in a solution of 20% piperidine in DMF (25 ml). The resin-bound peptide was then filtered and washed well with DMF and ether. Tbfmoc GlyOH (19.6 mg, 39.4  $\mu\text{mol}$ ; 5 equiv) was sonicated for 30 min in dioxan (1 ml). To this solution was added 1,3-diisopropylcarbodiimide (6.2  $\mu\text{l}$ , 39.4  $\mu\text{mol}$ ; 5 equiv) and 1-hydroxybenzotriazole (5.3 mg, 39.4  $\mu\text{mol}$ ; 5 equiv). The reaction mixture was sonicated for 5 min. This solution was then added to the resin-bound peptide previously swollen in dioxan (1 ml) for 1h. The reaction mixture was sonicated for 19h. The resin-bound peptide was filtered, washed with dioxan, dichloromethane and ether, and finally dried for 48h in a vacuum desiccator (102 mg).

Substitution efficiency: The Tbfmoc resin-bound peptide (2.9 mg) was sonicated for 30 min. in a solution of 20% triethylamine in DMF (10 ml). The specific absorption at 364 nm was recorded and the following results were derived from it:  $A = 0.265$ ; product resin functionality in  $\mu\text{mol/g}$ : 50.51; resin loading/coupling percentage: 70. The coupling was then repeated under the same conditions (4 equivalents of each Tbfmoc GlyOH, 1,3-diisopropylcarbodiimide and 1-hydroxybenzotriazole; reaction time: 3.5h). The resin-bound peptide was filtered, washed with

dioxan, dichloromethane and ether, and finally dried overnight in a vacuum desiccator (99.5 mg).

Substitution efficiency: A = 0.265 (resin-bound peptide : 2.7 mg); product resin functionality in  $\mu\text{mol/g}$ : 54.26; resin loading/coupling percentage: 75.

To the resin-bound Tbfmoc peptide (96.8 mg) was added water (75  $\mu\text{l}$ ), thioanisole (50  $\mu\text{l}$ ) and TFA (2.1 ml). The reaction mixture was sonicated for 2.5h. The resin was filtered, washed with TFA (2 x 0.5 ml) and  $\text{CHCl}_3$  (2 x 1 ml), and dried (19.1 mg). The solvent was removed *in vacuo* to give a residue. Trituration in diethyl ether gave the crude peptide as a yellow solid which was chilled overnight, washed well with ether and finally dried (49.2 mg). HPLC (A:B; A (90%) 25 min., A (10%; 214 nm; C = 0.5 mg/0.5 ml (A/B (1:1)); injection 30  $\mu\text{l}$ )  $R_t$  = 19.2 min.

Glycylisoleucylprolylprolylaspartylglutaminyglutaminy-  
arginylleucylisoleucylphenylalanylalanylglycyllysylglutaminy-  
leucylglutamylaspartylglycylarginylthreonylleucylserylaspartyl-  
tyrosylasparagylisoleucylglutaminyllsylglutamylserylthreonyl-  
leucylhistidylleucylvalylleucylarginylleucylarginylglycyl-  
glycine, ubiquitin (35-76) OH (51)

A suspension of crude Tbfmoc-ubiquitin (35-76) OH (30 mg) in a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 0.5% TFA, 25 ml) was sonicated for 20 min (until complete dissolution) and

loaded on a 5 mm diameter glass column packed with graphitised carbon (1.5 g; PGC 220-224; 150-180  $\mu\text{m}$ ; 100  $\text{m}^2/\text{g}$ ; active length: 15 cm). The column was first eluted with a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 0.5% TFA; 50 ml). After lyophilisation of the eluent a residue (2 mg) was obtained which gave a broad peak on HPLC ( $R_t = 13.8$  min (impurities)). The column was then eluted with a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 50 ml). A white solid (4.5 mg) was obtained after lyophilisation which was found by HPLC also to contain impurities (broad peak,  $R_t = 14.1$  min). The column was again eluted with 50 ml of a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1) and a residue (0.3 mg) was obtained after lyophilisation ( $R_t = 14.2$  min). The deprotection was carried out using a 20% piperidine solution in a mixture of  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 50 ml) and was followed by an elution of the column with  $\text{CH}_3\text{CN}/\text{H}_2\text{O}$  (1:1; 50 ml). The solvent was removed *in vacuo* to give a brown residue. Diethyl ether was added and the precipitate obtained was chilled overnight, filtered and washed well with ether, and finally dried (11.7 mg). The crude peptide (24.8 mg) was finally purified by preparative HPLC (reverse phase C-18 column (10 x 250 nm); gradient (A:B), 20 to 60% B over 22 min; detection at 214 nm) to give pure ubiquitin (35-76) OH as a white solid after lyophilisation (4.4 mg); amino acid analysis:  $\text{Asx}_4$  3.98,  $\text{Thr}_2$  1.83,  $\text{Ser}_2$  1.75,  $\text{Glx}_6$  6.67,  $\text{Pro}_2$  1.91,  $\text{Gly}_5$  5.10,  $\text{Ala}_1$  0.96,  $\text{Val}_1$  1.01,  $\text{Ile}_3$  2.99,  $\text{Leu}_7$  7.00,  $\text{Tyr}_1$  0.94,  $\text{Phe}_1$  1.04,  $\text{His}_1$  1.11,  $\text{Lys}_2$  1.91,

Arg<sub>4</sub> 3.81; m/z (FAB) 4734.2 (MH<sup>+</sup>). HRMS 4734.57186,  
C<sub>208</sub>H<sub>344</sub>N<sub>63</sub>O<sub>63</sub> requires: 4734.57161 (∴) < 1 ppm; HPLC  
(A:B, A (90%) 25 min. > A (10%); λ = 214 nm; C = 0.1 mg/  
0.1 ml (A); injection: 12 μl), R<sub>t</sub> = 13.8 min.

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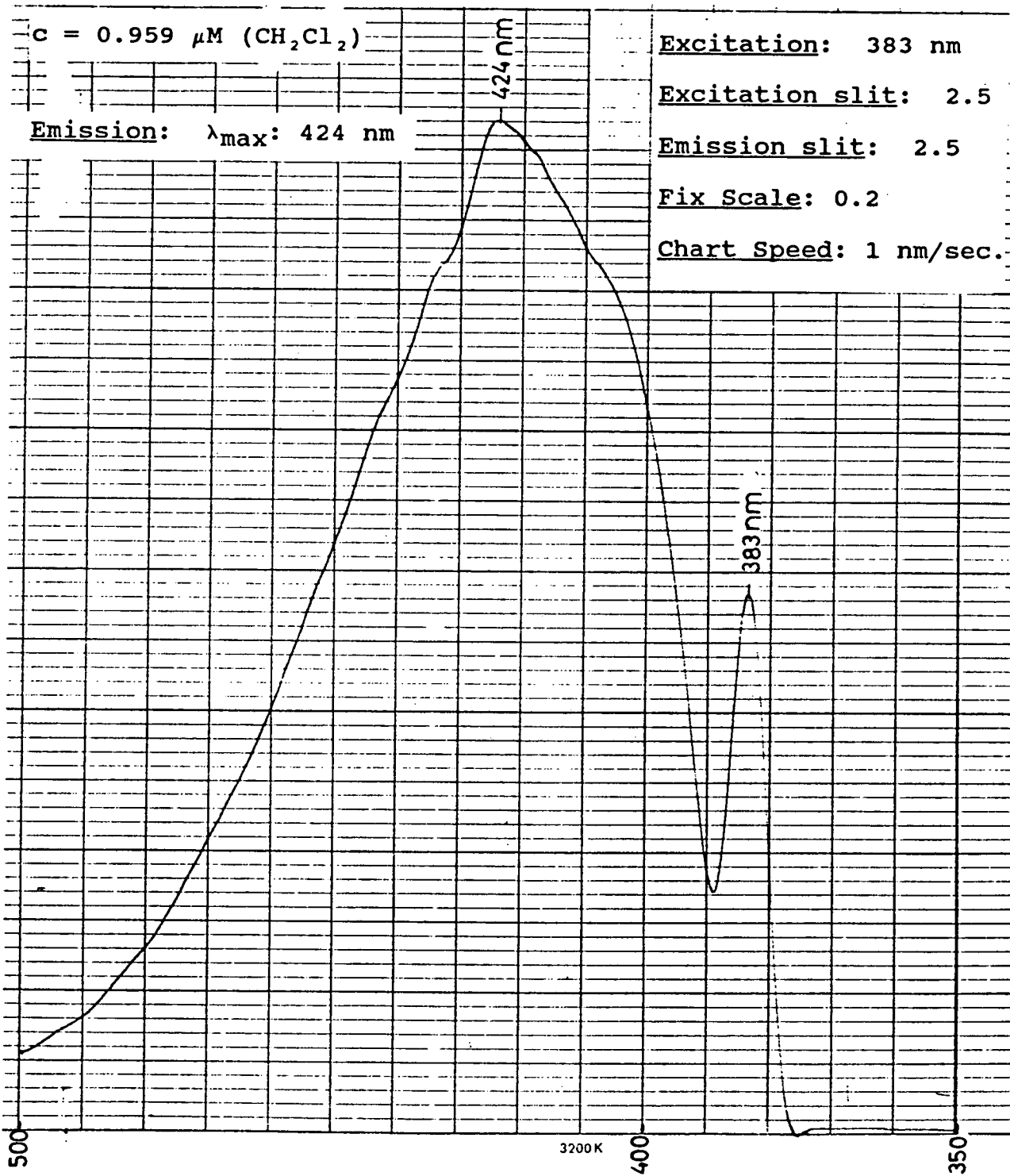
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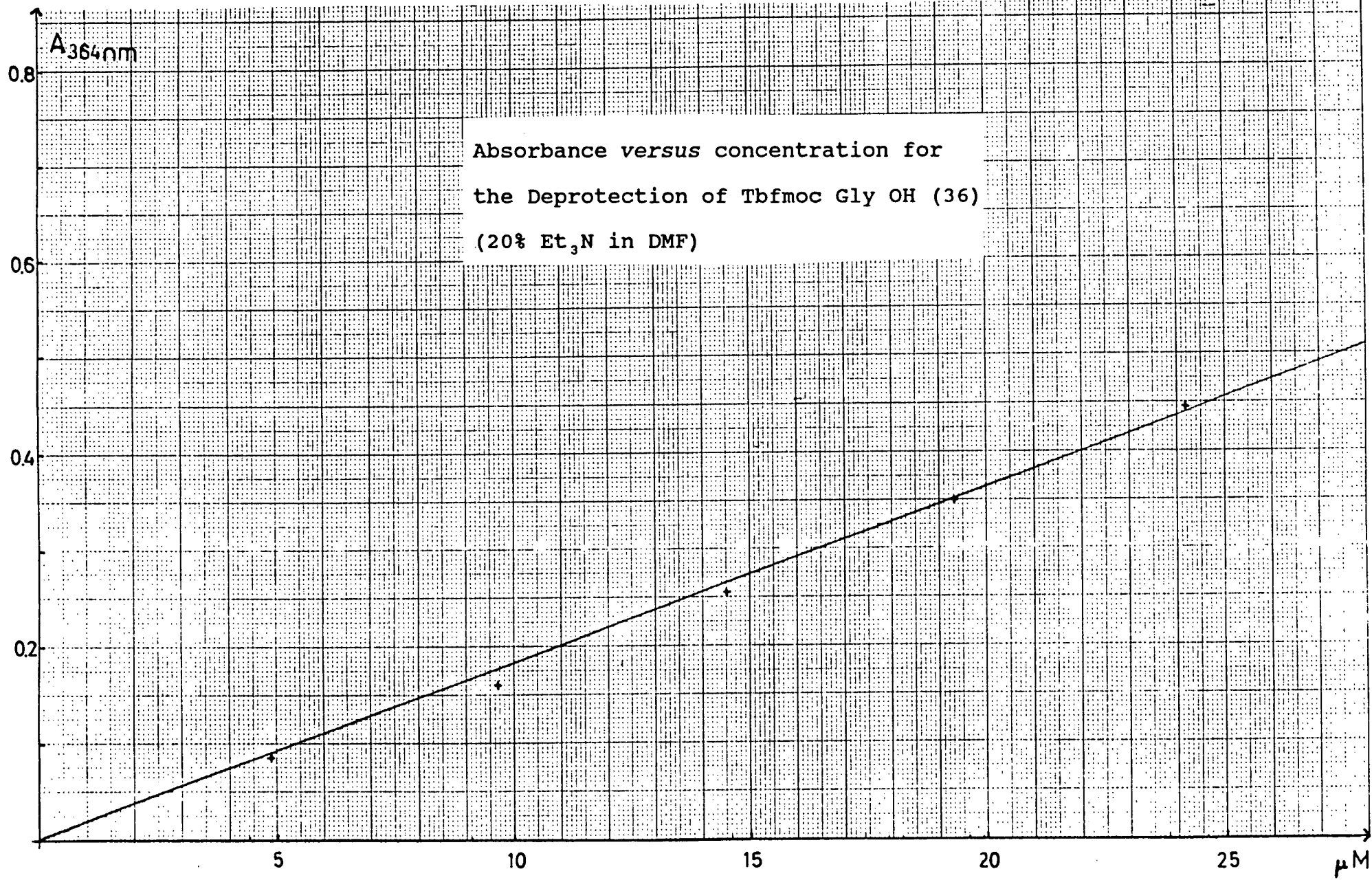
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## COURSES ATTENDED

Organic Research Seminars (various speakers).

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

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

Solid Phase Peptide Synthesis, Symposium (various speakers, University of Oxford).

Peptide and Protein Group of the Biochemical Society and Royal Society of Chemistry (various speakers, Gregynog).

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

Scottish Protein Group (various speakers, University of Edinburgh).