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# **Towards the Total Synthesis of Apo-Neocarzinostatin**



**University College London**

**Nick Callan**

**Submitted for Mphil**

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## STATEMENT

I hereby declare that this thesis has not been submitted, either in the same or another form, to this or any other University for a degree.

Signature:

Nick Callan January 2009

## **ACKNOWLEDGEMENTS**

I would like to thank my supervisor Prof. Steve Caddick for his help and advice during my course and for obtaining the funding for my project. I would also like to thank Dr. Jon Wilden and Dr. Richard Fitzmaurice for their help and knowledge with regards to my subject and direction given.

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## ABSTRACT

Neocarzinostatin is a dienediyne chromo protein antibiotic that is made up of a chromophore and an apoprotein (apo-Neocarzinostatin) in a 1:1 ratio.

Neocarzinostatin (NCS) is an antitumor agent isolated from *Streptomyces carzinostaticus* that has shown the effective treatment against acute leukaemia and certain cancers. The aim of the project was to synthesize apo-Neocarzinostatin and there are currently no reports of it being synthesized. The C-terminal fragment was synthesized and did not require further purification. A 12-mer was synthesized that was not part of Apo-NCS but it showed that thioester fragments can be synthesized and cleaved successfully from the resin. Part of the middle fragment of Apo-NCS was synthesized but only as far as 30 residues; also only SPPS was shown to be successful.

## Abbreviations

Ac	Acetyl
AcOH	Acetic acid
Ala	Alanine
All	Allyl
Acm	Acetomidomethyl
Asp	Aspartic acid
Bzl	Benzoyl
Boc	Butoxycarbonyl
Cys	Cysteine
DBU	Diazabicyclo [5.4.0] undec-7-ene
DCC	Dicyclocarbodiimide
DEAD	Diethyl azodicarboxylate
DIC	Diisopropylcarbodiimide
DCM	Dichloromethane
DIPEA	Diisopropylethylamine
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
EDT	Ethanedithiol
Et	Ethyl
Fmoc	Fluorenylmethylloxycarbonyl
Gly	Glycine
HBr	Hydrogen Bromide
HF	Hydrogen Fluoride
HOBt	Hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
LiBr	Lithium Bromide
Me	Methyl
Me <sub>2</sub> AlCl	Dimethylaluminiumchloride
Na	Sodium
NBS	N-Bromosuccinimide
NEt <sup>3</sup>	Triethylamine
Ph	Phenyl
Phe	Phenylalanine
PPh <sub>3</sub>	Triphenylphosphine
Pyr	Pyridine
Ser	Serine
<i>Tert</i>	Tertiary
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TFMSA	Trifluoromethanesulfonic acid
TIPS	Triisopropylsilane
TMSOTf	Trimethylsilyl trifluoromethane sulfonate
Trt	Trityl
Val	Valine



## TABLE OF CONTENTS

STATEMENT

ACKNOWLEDGEMENTS

TABLE OF COTENTS

<b>1. Introduction: Methods for the Total Synthesis of Peptides and Proteins</b>	<b>8</b>
1.1 An introduction to solid phase peptide synthesis (SPPS)	8
1.1.1 SPPS and the Boc protecting group	9
1.1.2 SPPS and the Fmoc protecting group	10
1.1.3 Coupling reagents in solid phase peptide synthesis	11
1.1.4 Side reactions of solid phase peptide synthesis	13
1.2 Native chemical ligation (NCL) methods for peptide and protein synthesis	14
1.2.1 Three step ligation methods for NCL	17
1.2.2 Selenocysteine in NCL	20
1.2.3 On-resin NCL	21
1.2.4 Methionine strategy for NCL	22
1.2.5 Staudinger ligation	23
1.2.6 Cysteine Desulfurization for NCL	25
1.2.7 Non-native chemical ligation	26
1.3 Expressed Protein Ligation	26
1.4 Methods for NCL and EPL: Thioesters	26
1.4.1 Fmoc chemistry and thioesters	27
1.4.2 Thioesters from Wang resin	28
1.4.3 Backbone amide linker (BAL) and thioesters	29

1.4.4	Safety-catch linker and thioesters	30
1.4.4.1	Amino acid fluorides for generation of safety catch linkers	33
1.4.5	Chlorotrityl resins for thioester generation	36
1.4.6	Thioesters from a hydrazine-based support	38
1.4.7	Thioesters using intramolecular <i>N,S</i> -acyl shift	39
1.4.8	Diothioethanol esters for <i>in situ</i> thioester formation	41
1.4.9	Thioester synthesis with self-purification	42
1.5	Introduction to Neocarzinostatin (NCS)	44
<b>2.</b>	<b>Results and Discussion</b>	<b>48</b>
2.1	Aim of the project	48
2.1.1	Thioester model studies	55
2.1.2	2-Chlorotrityl resins	56
2.1.3	Boc chemistry	57
2.1.4	Sulfamyl resins	58
2.1.5	Loading the resin	58
2.1.6	Rink Amide Resin	59
<b>3.</b>	<b>Conclusions</b>	<b>63</b>
<b>4.</b>	<b>Experimental</b>	<b>64</b>
<b>5.</b>	<b>References</b>	<b>74</b>

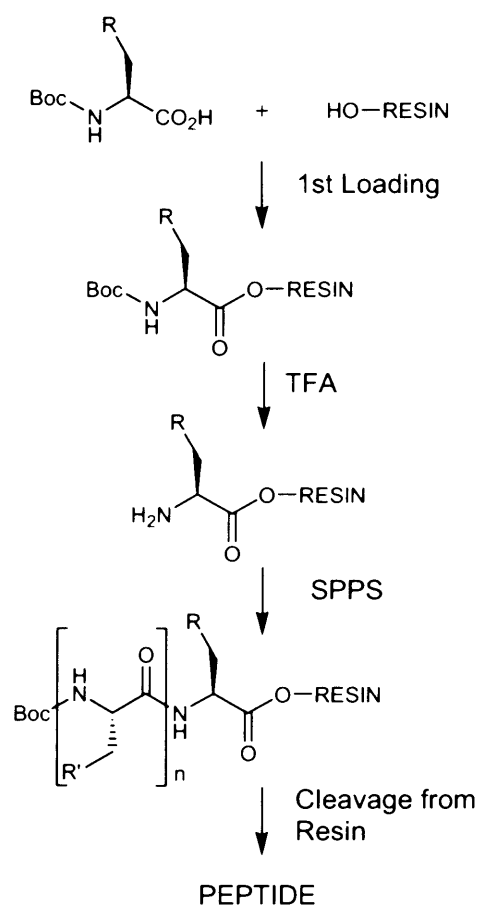
## 1. Introduction: Methods for the Total Synthesis of Peptides and Proteins

### 1.1 An introduction to solid phase peptide synthesis (SPPS)

Solid Phase Peptide Synthesis (SPPS) is a technique where  $\alpha$ -amino amino acids with relevant side-chain protecting groups are sequentially added to a polymer resin. Some amino acids have acid or amine groups in their side-chains; these are usually protected during peptide synthesis to avoid unwanted side reactions. After each addition, the N- $\alpha$ -protecting group is removed, followed by the next amino acid in the sequence. Coupling reagents or pre-activated amino acids are used in the dehydration reaction, which adds the next amino acid in the sequence. The synthesis of peptides is most commonly started from the C-terminal end. After completion of the synthesis the peptide is cleaved from the solid support, usually as a C-terminal acid or amide. The side-chain protecting groups are usually globally deprotected with the cleavage from the solid support.<sup>(1)</sup> There are two approaches to SPPS: continuous flow or batch. With continuous flow, the resin is held in a column and reagents and solvents are pumped continuously; this can be carried out manually or using a peptide synthesizer. With the batch procedure, the resin is placed into a sintered reaction vessel and reagents and solvents are added and drained from the vessel; this can also be carried out manually or using a peptide synthesizer. When using Fmoc chemistry it is possible to monitor the completion of the coupling reactions by using the Kaiser test.<sup>(2)</sup> This test checks for the presence of primary amines (hence is not useful when coupling the secondary amine proline) and indicates incomplete coupling to the resin.

### 1.1.1 Solid Phase Peptide Synthesis (SPPS) and the Boc protecting group

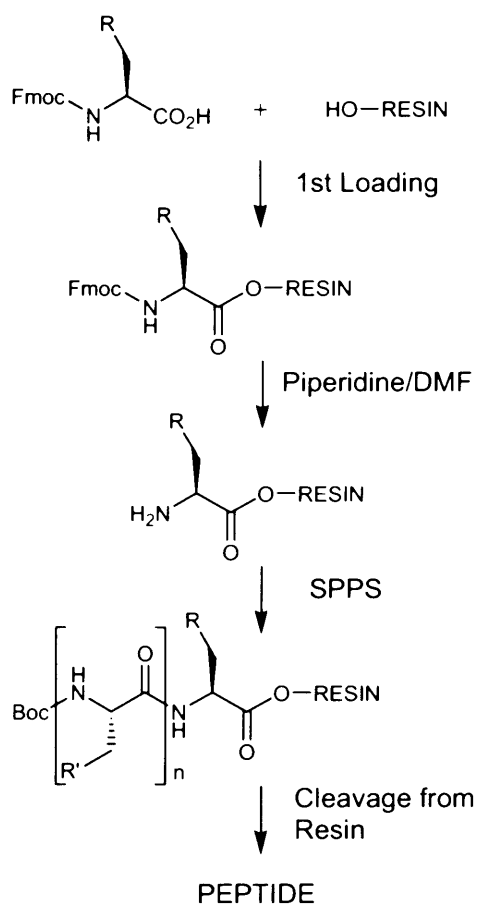
In Boc chemistry, the *N*-protecting group is the *tert*-butoxycarbonyl group, which is cleaved with TFA – one disadvantage is that the side chain protecting groups must be totally resistant to the acidic conditions of the Boc cleavage.<sup>(3)</sup> TFA is not a strong enough acid to cleave the benzyl protecting groups from the amino acid side chains. After the completion of the synthesis the peptide is cleaved from the resin using HF, so the resin must also be stable to the repeated exposure of the TFA deprotection of the *N*-Boc group. HF is very toxic and corrosive and also dissolves glass, so special apparatus must be used. Alternative acids, such as TFMSA, TMSOTf and HBr<sup>(4)</sup>, have been used to cleave peptides from the resins with limited success. A general scheme of Boc SPPS is shown (Scheme 1).



Scheme 1 - Boc SPPS Diagram

### 1.1.2 Solid phase peptide synthesis (SPPS) and the Fmoc protecting group

Fmoc SPPS (Scheme 2) uses the same principle as Boc except that the *N*-terminal is protected using the Fmoc group and the side-chain protecting groups are acid-labile (these can be removed with TFA). The Fmoc group is cleaved under basic conditions, using piperidine in DMF. The *N*-terminal amino acid is capped with Boc anhydride before cleavage from the resin. If the final Fmoc group remained attached then another step would be required to remove the group. Capping the resin with Boc anhydride allows the peptide/protein to be cleaved from the resin to liberate a fully deprotected crude product, ready to be purified. The final cleavage of the peptide from the resin is achieved under acidic conditions (approximately 95% TFA, with scavengers).<sup>(5)</sup>



Scheme 2 - Fmoc SPPS Diagram

### 1.1.3 Coupling reagents in solid phase peptide synthesis (SPPS)

Coupling reagents are used in peptide synthesis to form the amide bond in high yield. One of the first reagents used in peptide synthesis was DCC employing HOBT activation (used as a catalyst and to reduce racemization). The inclusive of HOBT was shown to increase the efficiency of the coupling reactions. DCC is very toxic <sup>(6)</sup> so is often replaced with the less toxic DIC, where the urea by-product is washed away with very polar solvents (typically DMF). Carbodiimide coupling is commonly used for reactive amino acids without bulky side chains. Apo-NCS contains a high proportion of non-hindered amino acids but also contains some that are more hindered, such as lysine and tryptophan. For this reason, carbodiimide coupling was deemed unsuitable for the peptide synthesis. BOP <sup>(8)</sup> was one of the first phosphonium coupling reagents but due to the respiratory toxicity and carcinogenicity a replacement was found. PyBOP and HBTU were the coupling reagents used in the attempt to synthesize Apo-NCS.

HOAt<sup>(10)</sup> has been shown to be an even better additive than HOBt for hindered amino acids, increasing yields and with improvements in coupling time. The completion of reaction can also be seen visually with HOBt, where there is a colour change once the reaction has finished. HOAt is much more expensive than HOBt and for the synthesis of large peptides was not practical. WSCDI is a carbodiimide coupling reagent that is especially useful when carrying out solution phase reactions as the by-product can be readily extracted with an aqueous workup.<sup>(11)</sup> Some coupling reagents are shown (Figure 1).

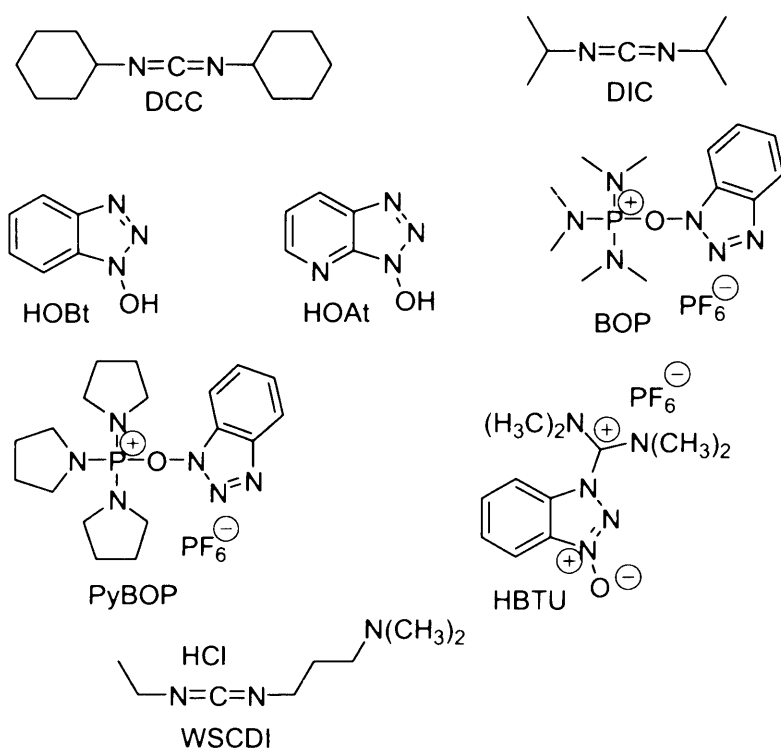
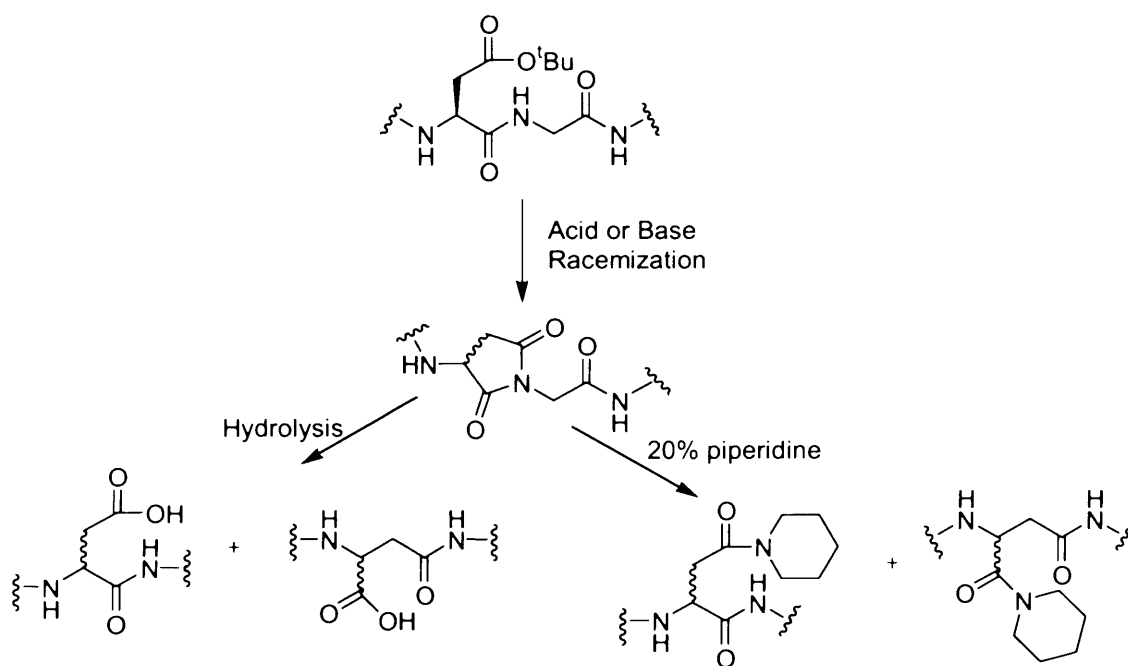


Figure 1 – Coupling Reagents and Activating Reagents

### 1.1.4 Side reactions of solid phase peptide synthesis

One of the most problematical side reactions in Fmoc SPPS is the formation of aspartimide, most commonly where there is an aspartic acid residue adjacent to a glycine residue in the sequence. The R group of glycine is a hydrogen atom, so it is the least hindered amino acid. Aspartic acid is commonly side chain protected with a bulky group (such as the *t*-butyl group) but this does not completely prevent aspartimide formation. The continued exposure to piperidine can lead to the formation of aspartimide.<sup>(12)</sup> This by-product is then prone to hydrolysis, forming the racemic enantiomers of deprotected aspartic acid and/or forming the piperidine amines (Scheme 3).



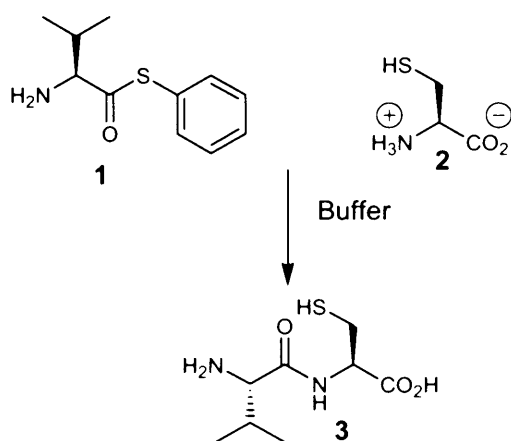
*Scheme 3 - Side Reactions During SPPS*

If stronger bases than piperidine are used, such as DBU, then aspartimide formation can be enhanced.<sup>(13)</sup> The amino acid sequence for apo-NCS contains two Asp-Gly bonds and so some aspartimide formation can occur. Steps that can be taken to reduce this side reaction are the use of more bulky trityl protecting group and to ensure the addition of HOBt to the Fmoc cleavage mixture.



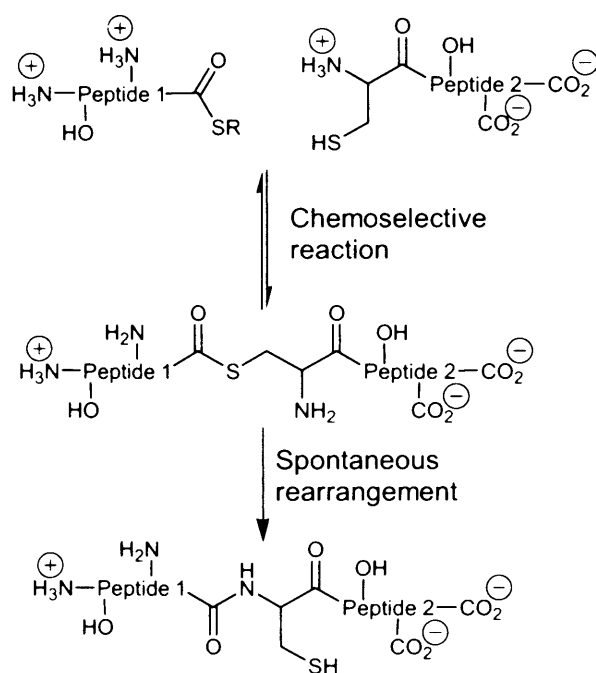
## 1.2 Native chemical ligation (NCL) methods for peptide and protein synthesis

The limitations of SPPS are that the length of the peptide chain that can be synthesized is sequence dependant and eventually there is a cut-off limit where synthesis is no longer viable, which is commonly caused by aggregation of the peptide chain. Chemical ligation is a technique where two unprotected peptide are added together in aqueous buffer leaving a peptide bond at the point of ligation. The ligation method was first discovered in 1953<sup>(14)</sup> when ValSPh **1** was reacted with cysteine **2** in aqueous buffer, resulting in the dipeptide ValCysOH **3** (Scheme 4).



*Scheme 4 - First Ligation Reaction*

Native Chemical Ligation (NCL) is an extension of this technique developed by Dawson *et al.*<sup>(15)</sup> They used two peptides that contained two mutually reactive functionalities, which are condensed *via* a two-step reaction. An unprotected peptide containing a C-terminal  $\alpha$ -thioester reacts with another unprotected peptide containing an N-terminal cysteine residue. The first step is the reversible displacement of the thioester from one peptide by the side chain of the cysteine residue from the other peptide. The second step is the spontaneous rearrangement and non-reversible S to N acyl transfer, forming an amide bond (Scheme 5). Peptides can also contain internal cysteine residues, as the first step is reversible.

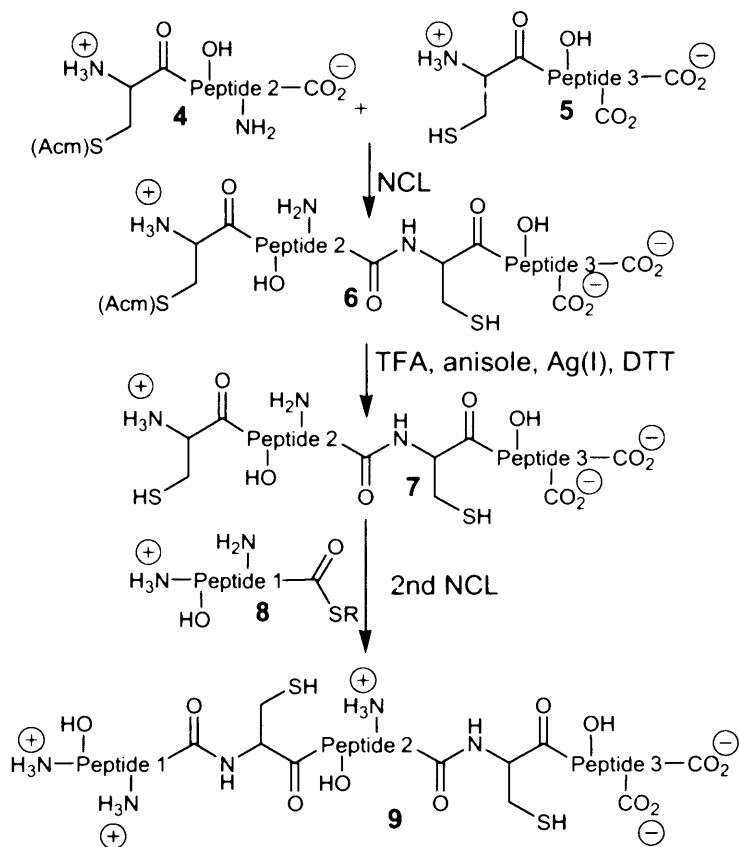


*Scheme 5 - Native Chemical Ligation*

The reaction is carried out at slightly basic pH and guanidine hydrochloride is added to aid the denaturing conditions of the peptides, allowing increased concentration of peptides, speeding up reaction times.<sup>(16)</sup> Due to the relatively unreactive nature of the aryl  $\alpha$ -thioester, more reactive thiols are usually added to the reaction in excess to encourage the in situ formation of more reactive thioesters. The excess thiols are also beneficial in preventing the oxidation of the free cysteine side chains and to reverse any trans-thioesterification of internal cysteine residues that may have occurred. There are two typical catalytic thiol systems that are added. The first being thiophenol (with or without benzyl mercaptan), both of which are sparingly soluble in buffer; and MESNa (a salt), which is soluble in the ligation conditions.

### 1.2.1 Three step ligation methods for NCL

It was proposed that our target (NCS – see section 1.6) would be synthesized in three fragments and a two-step ligation would be carried out, due to the total chain length and the inconvenient placements of cysteine residues in the sequence. The first ligation must be performed with a protecting group masking the side chain of cysteine of the peptide with the C-terminal thioester **4**. The additional protecting group is required to prevent the thioester peptide cyclising with itself and also to limit competing reactions. The two main protecting groups used are the Acm group<sup>(17)</sup> (Scheme 6) and thioproline.<sup>(18)</sup> The first ligation occurs, the product **6** is purified and isolated and then the Acm group is removed using either silver triflate or mercury (II) **7**.<sup>(19)</sup> After purification a second ligation then occurs with peptide **8** resulting in the final product.



Scheme 6 - Three Step NCL

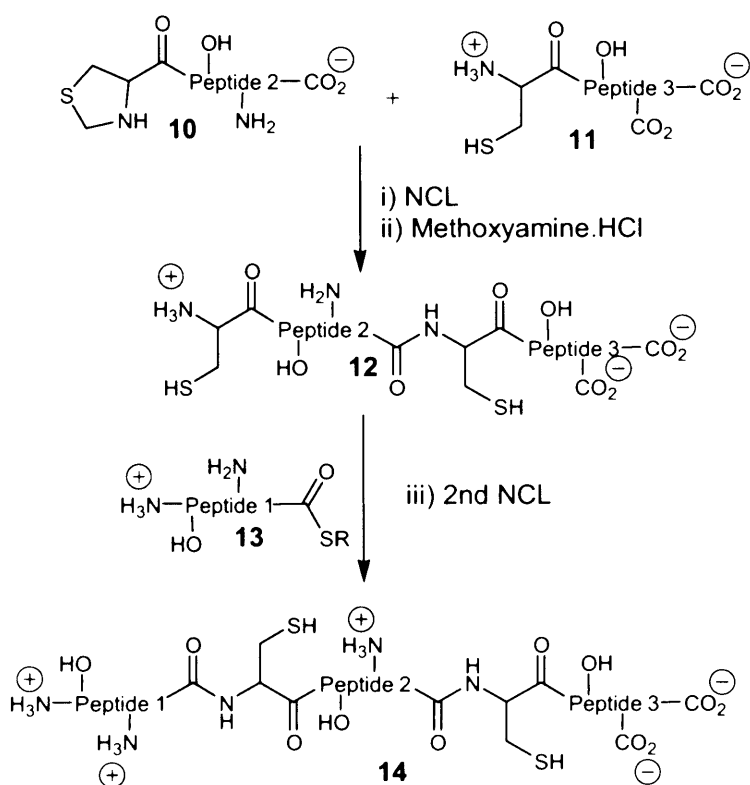
An early example of a three fragment ligation is the synthesis of Crambin <sup>(20)</sup> (Figure 2), a 46-mer by Bang and Kent *et al.* The underlined residues signify the three points of ligation.

**TTCCPSIVARSNFNACRLPGTPEALCATTTGCIIPGATCPGDYAN**

*Figure 2 – Crambin sequence*

They had previously tried to synthesize Crambin in a two-step ligation reaction but had very limited success. When they performed the three-step ligation there were difficulties removing the Ac group, despite extensive attempts at optimisation. Also after the first ligation reaction was completed there was a purification step. Then after the removal of the Ac group there was a second purification step.

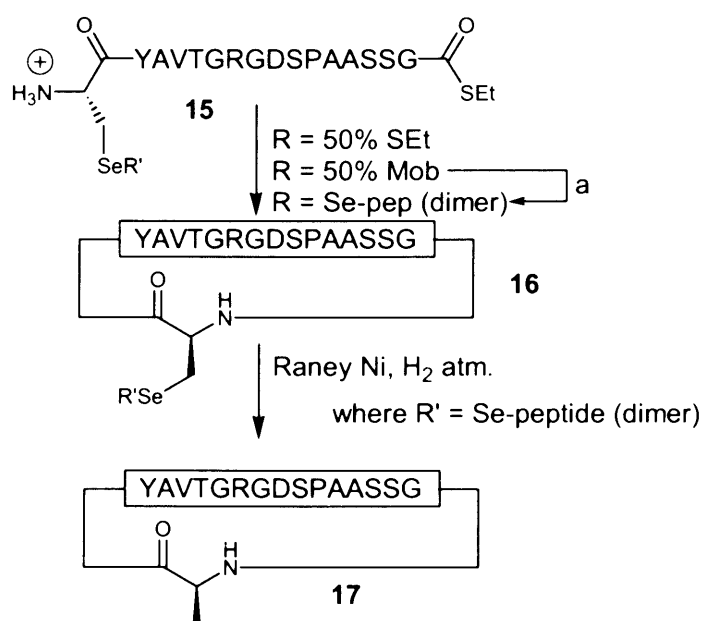
Bang and Kent *et al.* encountered problems with removing the Ac group so instead used the 1,3-thiazolidine-4-carboxy (Thz) group (thioprolinone). They were able to perform a one-pot three fragment ligation. After the first ligation reaction had gone to completion, the addition of methoxyamine hydrochloride salt to the crude reaction converted the Thz group to *N*-terminal cysteine **12**. The pH was then readjusted to pH 7 required for the ligation reaction, then the third peptide fragment **13** was added to the crude mixture and a second ligation reaction then took place. After this one pot three fragment ligation the crude was purified by HPLC and the desired fragment obtained **14** (Scheme 7).



Scheme 7 – One Pot Three Fragment Ligation

### 1.2.2 Selenocysteine in NCL

Selenocysteine has been referred to as the “21<sup>st</sup> amino acid” and because of the similarities between selenium and sulphur it was proposed that a peptide with an *N*-terminal selenocysteine (instead of cysteine) would undergo NCL.<sup>(21)</sup> Okely *et al.* incorporated Fmoc-Sec(Ph)-OH into a peptide sequence and, after global deprotection, the cyclic peptide was selectively oxidised to dehydroalanine using sodium periodate.<sup>(22)</sup> It is not practical to have internal selenocysteine residues when using Fmoc chemistry as piperidine can cause the protected selenium to deselenate, forming piperidine adducts via dehydroalanine (Dha). Hilvert *et al.*<sup>(23)</sup> utilised selenocysteine in their synthesis of a cyclic peptide and showed that with a *C*-terminal thioester and an *N*-terminal selenocysteine present in a peptide, it can be cyclized when exposed to thiophenol in buffer to form a selenol dimer. They also used Raney nickel to reduce the cyclic diselenide peptide to a single cyclic peptide with Ala in place of the selenocysteine (Scheme 8).



a: 1M TMSBr in TFA/thioanisole/*m*-cresol

Scheme 8 – Selenocysteine in NCL

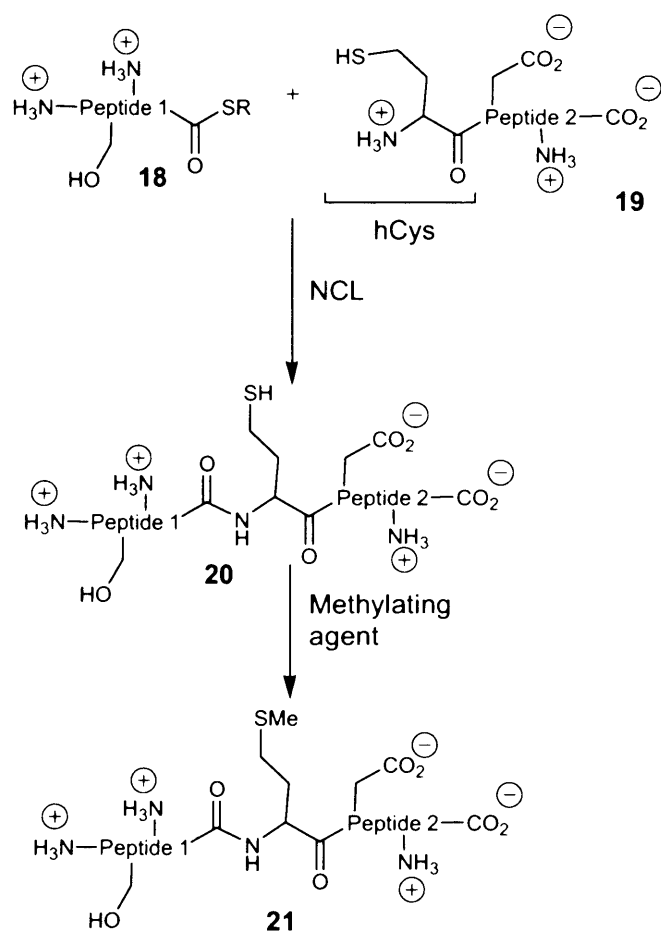
### 1.2.3 On-resin NCL

Baraney *et al.* <sup>(24)</sup> reported the synthesis of a cyclic peptide that incorporated an on-resin NCL reaction. Fmoc-Asp-OAl was anchored to the solid phase via its  $\beta$ -carboxyl group. After subsequent removal of the Fmoc group SPPS was carried out with Trt-Cys(Xan)-OH as the *N*-terminal amino acid. Removal of the allyl group with palladium and coupling with an amino acid resulted in a peptide thioester being attached to the resin. Mild acidic cleavage of the *N*-terminal acid-labile trityl group, followed by ligation conditions resulted in the on-resin ligation cyclization. High concentrations of TFA then cleaved the cyclic peptide from the resin with global deprotection.



### 1.2.4 Methionine strategy for NCL

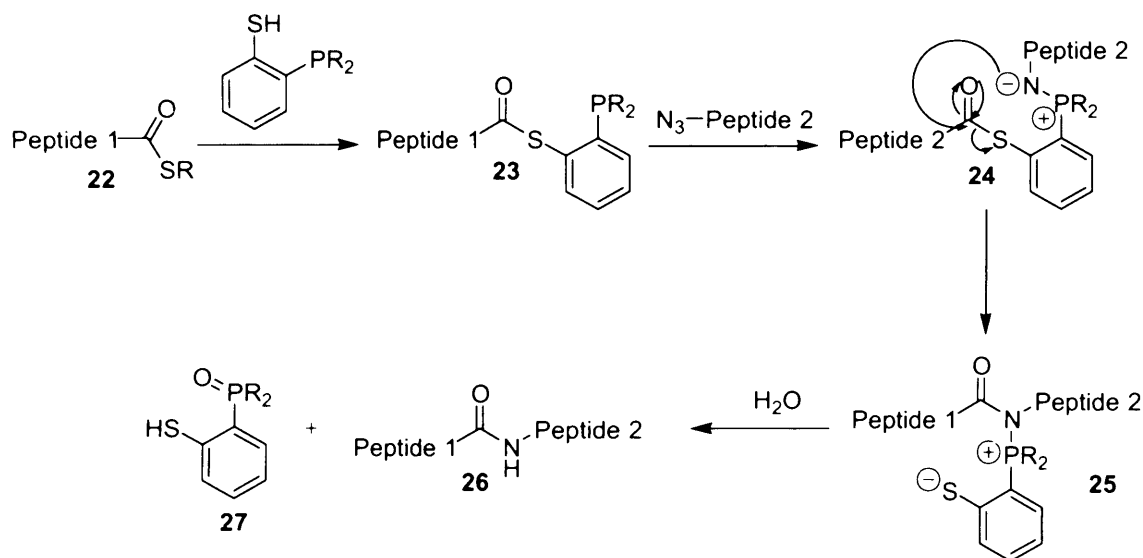
Yu *et al.*<sup>(25)</sup> were interested in performing ligation reactions on peptide segments that do not contain any cysteine residues. Some of their target peptides contained methionine so they used homocysteine (a homologue of cysteine) as the *N*-terminal amino acid. The side chain of homocysteine methylated is methionine. They synthesized their peptides using Boc chemistry, performed the ligation reaction with the homocysteine present and then they selectively methylated the homocysteine side chain in the presence of lysine, aspartic acid, arginine, histidine and asparagines (Scheme 9). They used a large methyl *p*-nitrobenzenesulfonate as the methylating agent in large excess. This strategy is severely limited because the peptide needs to contain a methionine residue at a convenient ligation position.



Scheme 9 - Methionine Ligation Strategy

### 1.2.5 Staudinger ligation

Nilsson and Raines *et al.*<sup>(26)</sup> developed a technique that utilises the Staudinger ligation to add small peptide fragments together in the absence of an *N*-terminal cysteine residue. They noted that cysteine comprises only 1.7% of residues<sup>(27)</sup> in proteins and that because cysteine is one of the most reactive amino acids, simply inserting an extra cysteine into a chain is not practical. They prepared a small thioester **22**, which was then trans-thioesterified with phosphinothioester. Reacting this intermediate **23** formed with a peptide containing an *N*-terminal azide **24** produces an iminophosphorane **25**, as the nucleophilic nitrogen atom displaces the thioester. Subsequent hydrolysis of this intermediate produces the amide bond **26** (Scheme 10).



Scheme 10 – Staudinger Ligation

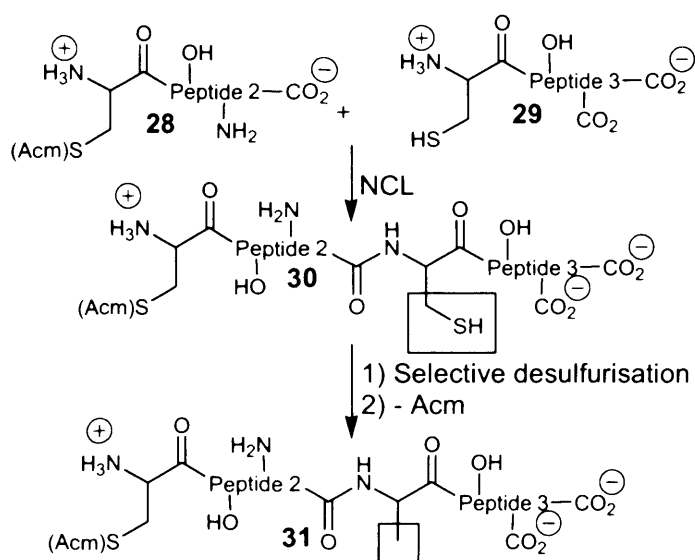
This reaction is traceless, in that no atoms from the phosphinothioester are found in the product.<sup>(28)</sup> After their initial model studies work they have gone on to use their approach with larger peptides. It should be noted that this technique has only been used on small-scale peptides (Figure 3), where the group synthesized a 12-mer using Fmoc chemistry. It should also be noted that the peptide contained two proline residues, which help to inhibit chain aggregation. Aggregation can be a problem with long chain peptides, especially when there are consecutive non-polar residues.



*Figure 3 - Peptide Azide Synthesized and Employed in Staudinger Ligation*

### 1.2.6 Cysteine Desulfurization for NCL

Kent *et al.* <sup>(29)</sup> extended their work in NCL where a peptide with an *N*-terminal cysteine and also an internal cysteine protected with AcM was added to a peptide thioester that also contained an internal Cys(AcM). It was previously thought that selective desulfurization on a peptide that contained a protected cysteine was not practical. They proposed that a thiol could be reduced in the presence of a thioether (the AcM group). Using Boc chemistry, they synthesized a small peptide with Cys(AcM) and an *N*-terminal cysteine **28** and were able to selectively convert the Cys to Ala using Raney nickel after NCL with **29** giving **30**. They synthesized a 37-mer using NCL and they were then able to selectively reduce the cysteine residue that participated in the ligation reaction to an alanine residue **31** (Scheme 11).



Scheme 11 – Cysteine Desulfurisation

### 1.2.7 Non-native chemical ligation

Work has been carried out by Caddick *et al.*<sup>(30)</sup> has shown that non-native reactions can take place. Cross-methathesis using Grubbs' second generation catalyst was used to incorporate a linker which was the equivalent length to two amino acids. Simple coupling reactions have been carried out at the present, with the intention of synthesizing larger peptides in the future. It is proposed that the modification of proteins will help gain insight into the folding of proteins and also their internal interactions.

### 1.3 Expressed Protein Ligation

Expressed Protein Ligation (EPL) or Intein-mediated Protein Ligation is a semi-synthesis method<sup>(31)</sup>, where a synthesized peptide (or a recombinant peptide/protein) with an *N*-terminal cysteine residue is chemoselectively ligated to a recombinant protein with a *C*-terminal  $\alpha$ -thioester. This technique was first used because it has advantages over NCL, in that large amounts of peptide can be made in high purity.

### 1.4. Methods for NCL and EPL: Thioester synthesis

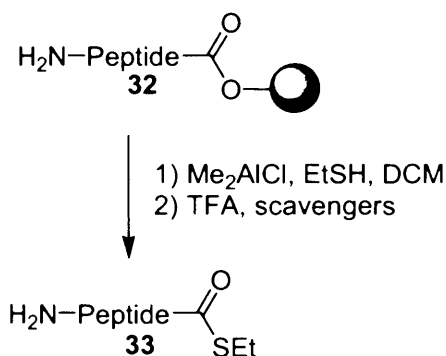
Methods are shown below for producing thioesters directly and indirectly from resins. To synthesize thioesters from resins is a non-trivial procedure and there are a relatively small number of reliable methods in the literature for achieving this. In Fmoc chemistry the resin bound peptide is repeatedly treated with base to cleave the Fmoc group. These basic conditions result in the cleavage of thioesters from the resin via aminolysis and also the *C*-terminal chiral carbon is prone to racemization, so to simply load a thioester to a Wang type resin is not appropriate.

#### 1.4.1 Fmoc chemistry and thioesters

Aimoto *et al.*<sup>(32)</sup> prepared a previously synthesized *N*-terminal Fmoc thioester and they subjected it to alternative amines to find the optimum conditions for the Fmoc removal, without displacement of the thioester. Piperidine, cyclohexylamine, 4-aminomethylpiperidine and morpholine were all shown to cleave the Fmoc group but also completely displaced the thioester. Their results showed that a solution of 1-methylpyrrolidine (25%), hexamethyleneimine (25%), and HOBt (2%) in NMP/DMSO was successful in removing the Fmoc group, without displacing the thioester. However this work was reported in 1998<sup>(33)</sup> and yet this method has not been widely used by other groups. Guo *et al.*<sup>(34)</sup> tried to use this procedure but found that the deblocking reagent proved insufficiently capable of removing the Fmoc group of the initial amino acids.

### 1.4.2 Thioesters from Wang resin

Hilvert *et al.* <sup>(35)</sup> developed a procedure from work carried out by Corey *et al.*, where peptides are cleaved directly from Wang (or similar) resin to produce thioesters using Fmoc chemistry (Scheme 12). This method had a very limited scope as the reaction proceeds via intermediates that lead to racemization of the  $\alpha$ -carbon. Side reactions that also occurred were the formation of aspartimide and aspartic acid side chain thioesters. They proposed that the racemization of the  $\alpha$ -carbon was due to the large excess of the  $\text{Me}_2\text{AlCl}$ .

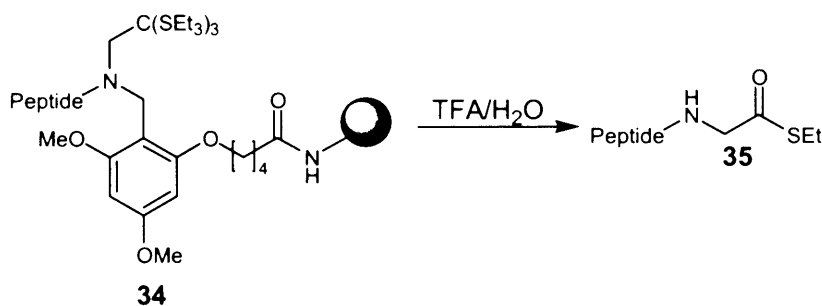


Scheme 12 – Thioesters from Wang Resin

Following on from their work, Hilvert *et al.* <sup>(36)</sup> then used the less acidic  $\text{Me}_3\text{Al}$  in place of  $\text{Me}_2\text{AlCl}$  and were able to greatly reduce the formation of the bis-thioesters of the aspartimide and aspartic acid side chains. By using this alternative reagent they were also able to partly suppress the level of racemization of the  $\alpha$ -carbon, although they were not able to eliminate racemization altogether. This method is therefore only relevant where the amino acid at the C-terminal position is glycine.

### 1.4.3 Backbone amide linker (BAL) and thioesters

A procedure was developed by Brask *et al.*<sup>(37)</sup> utilised a novel backbone amide linker (BAL) allowing the preparation of C-terminal thioesters to be synthesized on the resin using Fmoc chemistry. The thioester is then liberated when the peptide attached to the resin is treated with TFA/water (Scheme 13). This method is limited to peptides that have glycine at the C-terminus. This method is less prone to the formation of side reactions when compared to the procedure cleaving directly from Wang.

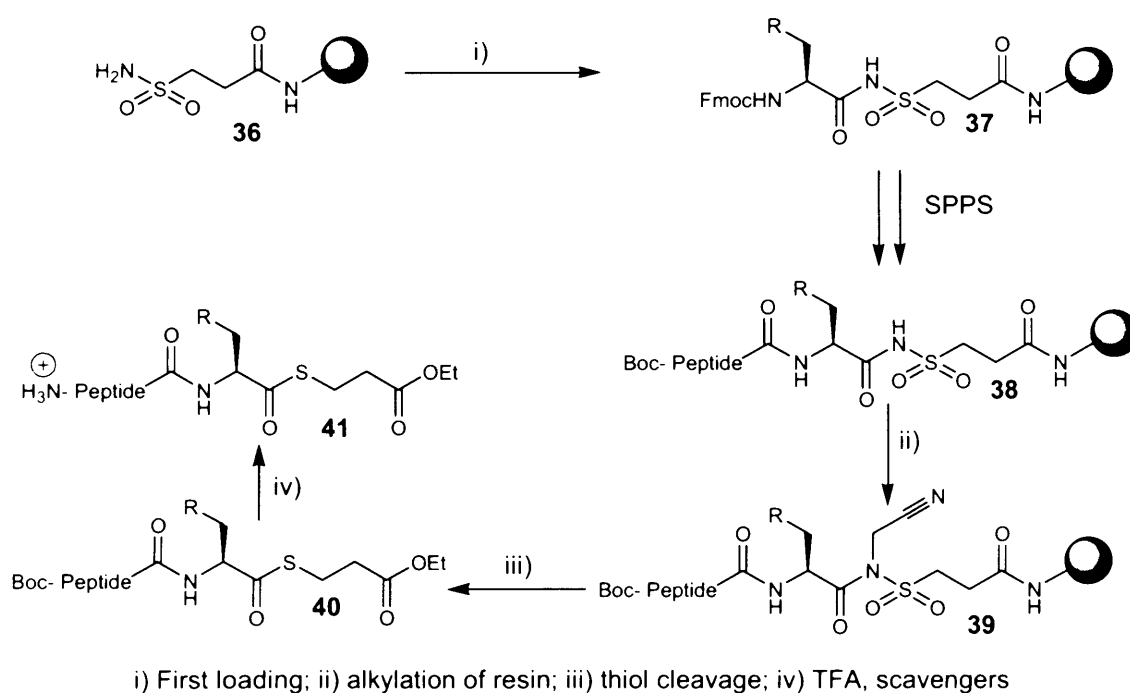


Scheme 13 – BAL



#### 1.4.4 Safety-catch linker and thioesters

This method is based on Kenner's safety-catch linker and was modified more recently by Backes *et al.*<sup>(38)</sup>, where carboxyl groups of amino acids are loaded onto either sulfamylbutyryl or 3-carboxypropane sulfonamide resin.<sup>(39)</sup> The resin linker is stable to basic and nucleophilic conditions, so will not be cleaved from the resin by repeated exposure to piperidine and coupling cycles. The first amino acid is usually loaded using PyBOP (or to a lesser extent DIC/Melm) and then the peptide is synthesized using standard Fmoc chemistry. The final amino acid in the sequence is protected with a Boc group (acid labile) and the resin is then activated with an alkylating agent.

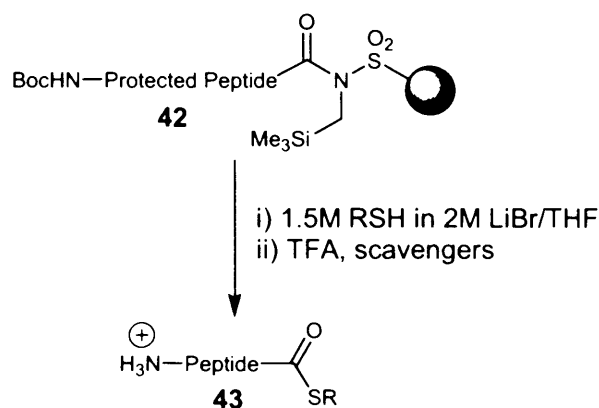


Scheme 14 - Safety-catch linker

Activation of the safety-catch linker is with iodoacetonitrile and TMS-diazomethane.<sup>(40)</sup> The most likely reason for the alkylation is that the subsequent nucleophilic alkylation is more reactive under basic conditions and the amide proton is highly acidic, so could be deprotonated. This would then be less susceptible to nucleophilic attack. The alkylated resin is then exposed to a nucleophilic thiol, which liberates the fully protected thioester from the resin. The fully protected thioester is then globally deprotected with TFA in the presence of thiol scavengers (Scheme 14).

Using this procedure, Ingenito *et al.*<sup>(41)</sup> added catalytic NaSPh to the thiol cleavage mixture, suggesting that the peptide would first cleave as the more reactive but less stable aromatic thioester. This intermediate was not isolated but was displaced with an alkyl thiol that is used in large excess (commonly ethyl-3-mercaptopropionate or benzyl mercaptan). The likely explanation for not using NaSPh in excess is that the peptides would be prone to racemization. The group prepared several different tripeptides as well as a 20-mer to show that the addition of the catalyst enhanced the cleavage rate.

Quaderer *et al.*<sup>(42)</sup> tried to synthesize a 22-mer thioester using this procedure towards the total synthesis of bovine pancreatic trypsin inhibitor (BPTI) but encountered problems with the cleavage. LiBr in anhydrous THF was used as the solvent system for the thiol cleavage, where DMF is commonly used, greatly improving the yield of the thioester (Scheme 15). The group also tested their modified procedure on a difficult to prepare 14-mer from cytochrome C using their LiBr/THF solvent system for the cleavage, producing a four times higher yield.



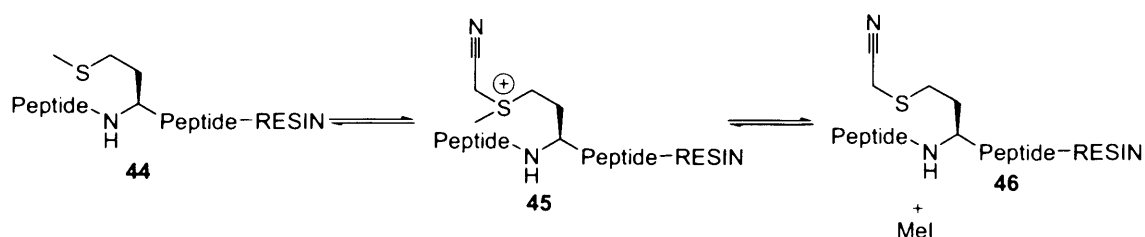
*Scheme 15 - LiBr in thioester cleavage cocktail*

Work by Flavell *et al.* <sup>(43)</sup> was carried out using the safety-catch method to synthesize a tetraphosphorylated peptide that was cleaved from the resin to produce a thioester that contained phospho-groups (Figure 4).

### TTLKDLIYDMpTTpSGpSGpSGLPL-C(O)SBzl

*Figure 4 - Sequence of tetraphosphorylated peptide thioester.*

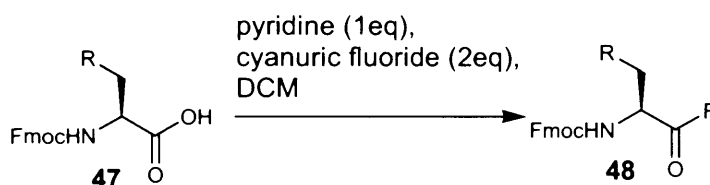
This thioester was used in a ligation reaction, joining two fragments together without degradation or elimination of the phosphorylated groups. Problems were encountered with yield and side reactions because the resin was alkylated with iodoacetonitrile, where the side chain of methionine reacted (Scheme 16). The alkylation of the resin could not be carried out using TMS-diazomethane because this reagent also alkylated the phosphorylated groups. Instead norleucine was substituted in place of methionine.



*Scheme 16 - Methionine alkylation*

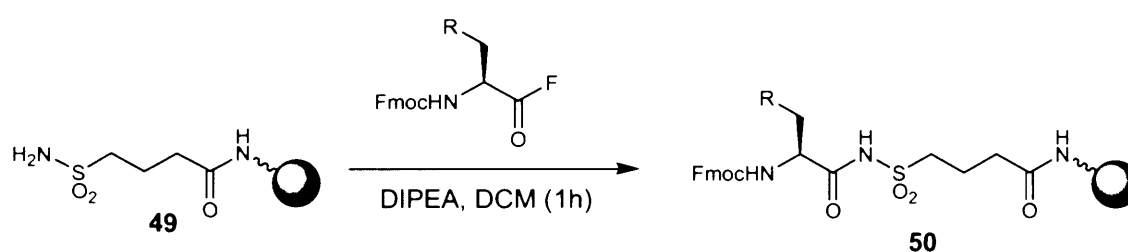
#### 1.4.4.1 Amino acid fluorides for generation of safety catch linkers

Due to the relatively low reactivity of the sulfonamide the first loading can be problematic, with long reaction times required and also repeated couplings. This led Ingenito *et al.* <sup>(44)</sup> to develop a procedure increasing the reactivity of the first loading. They investigated the synthesis of amino acid fluorides and optimised the conditions so that racemization of the C-terminal chiral carbon was low (0.5%) (Scheme 17). The coupling reactions of amino acid fluorides occur in a weak organic base that is not strong enough to cleave the Fmoc group, so loss of configuration of the  $\alpha$ -stereocentre is minimised.



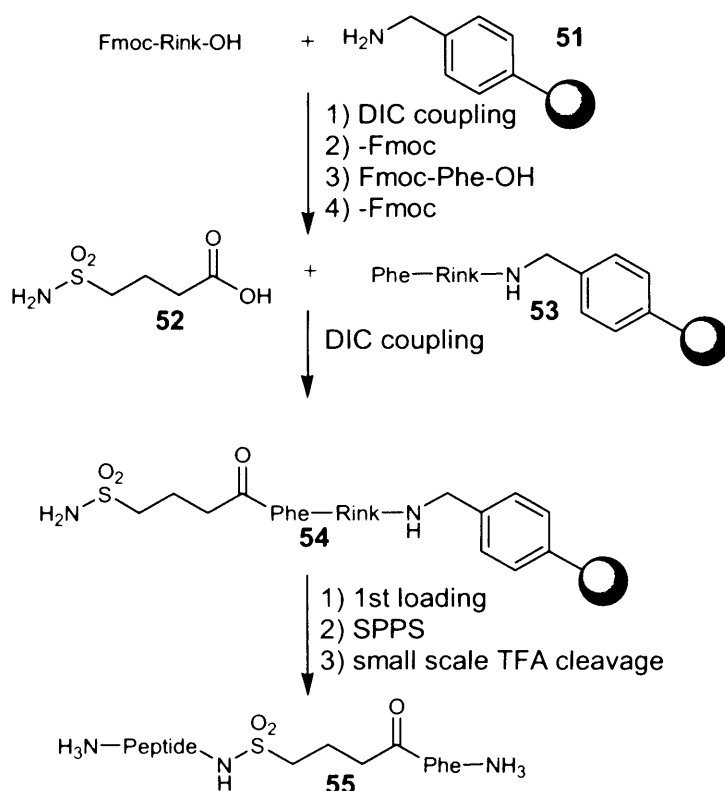
*Scheme 17 - Amino Acid Fluoride Synthesis*

The group tested a broad spectrum of amino acid fluorides, loading them onto three different types of sulfamylbutyryl resins, with high loadings in most cases and reduced loading times 8 hours with PyBOP or DIC/Melm to 1 hour with the amino acid fluorides (Scheme 18). This procedure is compatible with hindered amino acids, where bulky side chain protecting groups such as trityl and *tert*-butyl are required. Problems were encountered with two amino acids: Fmoc-His(Trt)-F, where it was found to have only short-term stability, and Fmoc-Arg(Pbf)-F, where the product cyclized to the corresponding lactam.



*Scheme 18 - Loading Fmoc Amino Acids onto Sulfamylbutyryl resins*

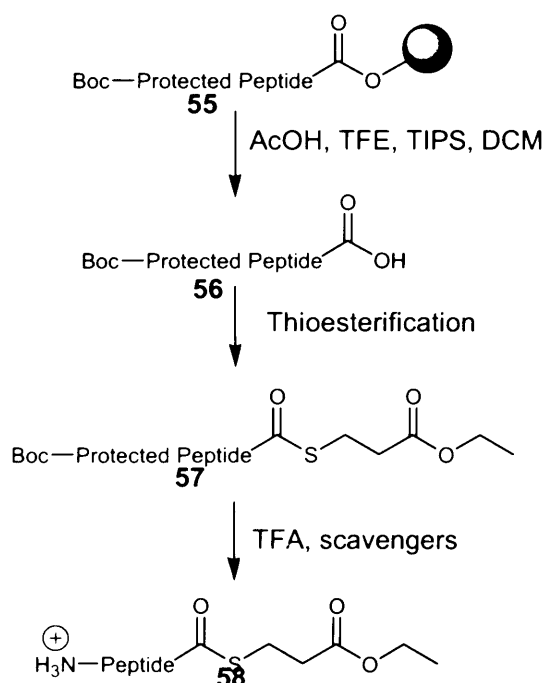
One of the major disadvantages of the safety-catch procedure is that because the resin needs to be alkylated before being subjected to nucleophilic attack, followed by cleavage of the protecting groups, the monitoring of the synthesis can be difficult. When using other resins, small portions can be removed and cleaved to check the purity and success of the synthesis in a relatively short time. This is not practical with the safety-catch linker, so Mezzato *et al.* <sup>(45)</sup> utilised a double linker procedure. Rink linker was loaded onto a typical resin, phenylalanine was added as a spacer and then 3-carboxypropane sulfonamide linker was attached (Scheme 19). The advantage is that small portions of the resin can be removed and subjected to TFA cleavage to check the progress of the synthesis.



Scheme 19 – Rink Linker SPPS

### 1.4.5 Chlorotriyl resins for thioester generation

Futaki *et al.* <sup>(46)</sup> developed a procedure where SPPS was carried out using a 2-chlorotriyl chloride resin and Fmoc chemistry, where a fully side chain protected peptide acid **56** was cleaved from the resin under mild acidic conditions, without cleaving any of the acid labile protecting groups. The crude product was then purified before a solution-phase coupling reaction was carried out to produce the thioester **57**, before cleaving all protecting groups giving **58**. The drawback of this method in comparison to the safety-catch method is that there is an extra step to synthesize the thioester. Also, the handling and purification of the fully protected peptide must be taken into account before thioesterification (column chromatography is often needed). The group produced a 23-mer thioester using this method (Scheme 20), although it should be noted that the C-terminal amino acids in the fragments they produced were glycine.



*Scheme 20 – Thioesters from chlorotriyl chloride resins*

Eggelkraut-Gottanks *et al.* <sup>(47)</sup> eliminated the requirement for the extra purification step by cleaving the peptide acid from the resin, concentrating and then synthesizing the thioester directly from the crude, using a reduced excess of activating reagents and thiol. Side chain cleavage and subsequent purification were then carried out as in the safety-catch method. A 40-mer was synthesized using this procedure (Figure 5), although it contains four conveniently paced proline residues. They also synthesized a 21-mer but this also contained two proline residues. This suggests that when using this procedure they have so far synthesized peptides with conveniently placed residues. Slight levels of racemisation of the  $\alpha$ -carbon of the thioester were observed.

**YPSKPDNPGEDAPAEDLARYYSALRHYINLITRQRYGKRS-C(O)SR'**

*Figure 5 - Peptide sequence of chlorotrityl resin thioester*

In 2004 Kitagawa *et al.* <sup>(48)</sup> used this procedure to produce a peptide hormone, Cholecystokinin, which can be found in the brain and gastrointestinal tract. There are different isoforms of this peptide but all have an *O*-sulfated tyrosine at position 7 from the *C*-terminus. Instead of using AcOH in the mild cleavage of the peptide acid, they used hexafluoro-2-propane so that they could directly synthesize the thioester using the crude peptide. Unusually the *N*-terminal Fmoc group was still attached while the thioester was subjected to a ligation reaction and was subsequently removed. The 24-mer that they synthesized (Figure 6) contained two proline residues, helping reduce chain aggregation.

**YIQQARKAPSGRMSIVKNLQNLDP-C(O)S(CH<sub>2</sub>)<sub>2</sub>CO<sub>2</sub>Et**

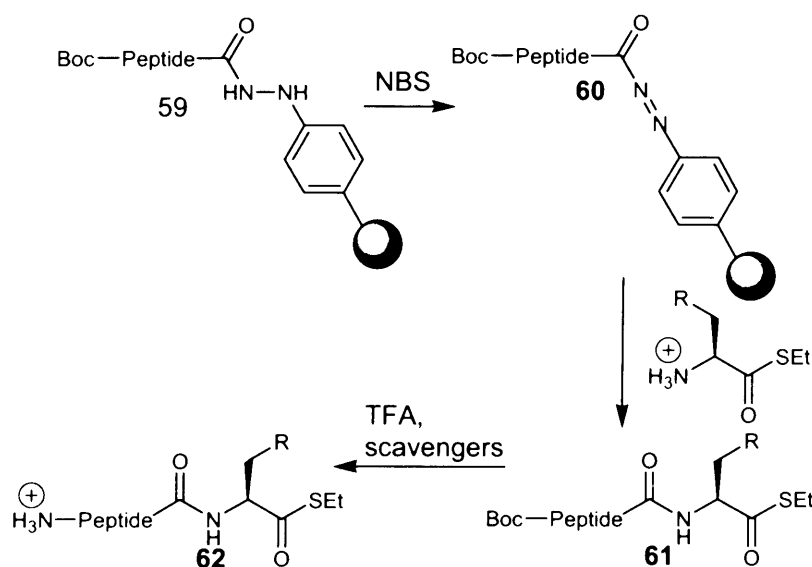
*Figure 6 - 24-mer thioester from 2-chlorotrityl chloride resin*



G

#### 1.4.6 Thioesters from a hydrazine-based support

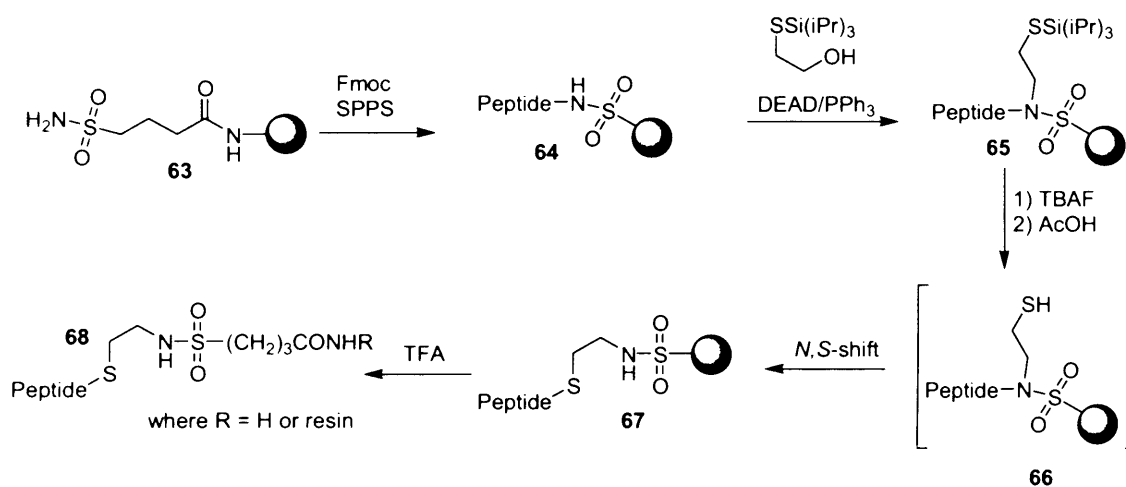
Camarero *et al.*<sup>(49)</sup> developed a procedure where a hydrazine safety-catch linker was attached to, typically, Rink Amide resin. They were then able to carry out Fmoc SPPS with the linker being completely stable to the conditions of the synthesis. At the end of the synthesis they treated the resin-linker (with attached protected peptide) with a mild oxidant to form a reactive acyl diazene intermediate. This oxidation reaction did not affect the peptide still attached to the linker. The protected peptide is then liberated from the resin-linker by an  $\alpha$ -amino acid alkyl thioester, after which the protecting groups were removed with TFA and scavengers. SPPS was carried out from the *C*-terminal end, starting with the second amino acid. After the peptide had been synthesized the linker was oxidised, then reacted with the alkyl thioester of the first amino acid in the sequence. This liberated the protected peptide thioester, after which the side chain protecting groups were cleaved to give the product that was then used in NCL after purification (Scheme 21). It should be noted that almost all peptides synthesized using this procedure had a *C*-terminal glycine residue.



Scheme 21 – Thioester using Hydrazine Support

### 1.4.7 Thioesters using intramolecular *N,S*-acyl shift

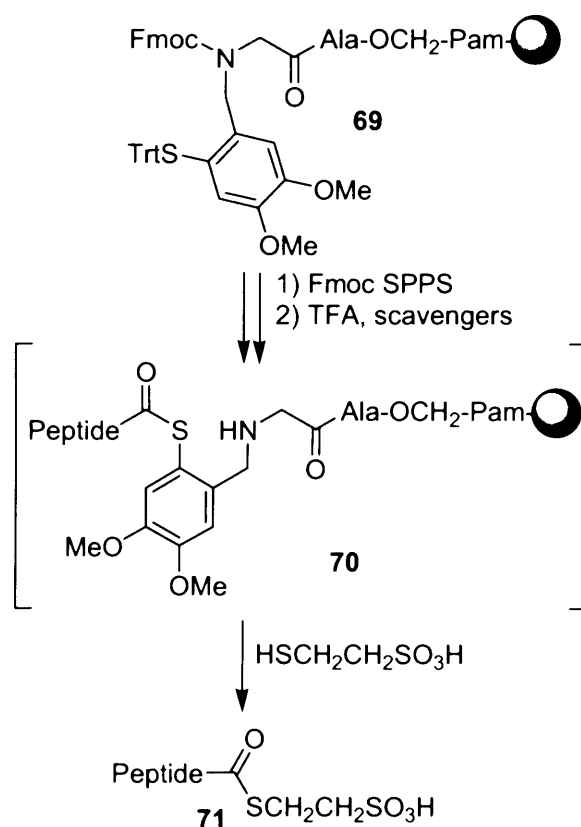
Ollivier *et al.* <sup>(50)</sup> extended Kenner's safety-catch linker procedure in another way. By using Mitsunobu alkylation <sup>(51)</sup> of the acidic sulfonamide proton after SPPS, they removed the protecting group whilst on the resin and a spontaneous rearrangement occurred. The resulting thioester was then be cleaved from the resin using TFA and used in a ligation reaction (Scheme 22). The benefit of this procedure is that SPPS can be carried out using Fmoc chemistry without the undesired thioester cleavage by piperidine or racemization of the  $\alpha$ -carbon because no thioester bond is present until after chain assembly. This procedure was very limited in that only small peptides (8mers) were synthesized and all their small fragments contain an internal proline residue.



Scheme 22 – Intramolecular *N,S*-shift

After SPPS has been carried out **64** the Mitsunobu reaction results in the alkylated sulfonamide **65**. Mild acidic cleavage of the silicon-containing protecting group liberates the thiol **66**, which then undergoes rearrangement to produce the thioester that is cleaved from the resin.

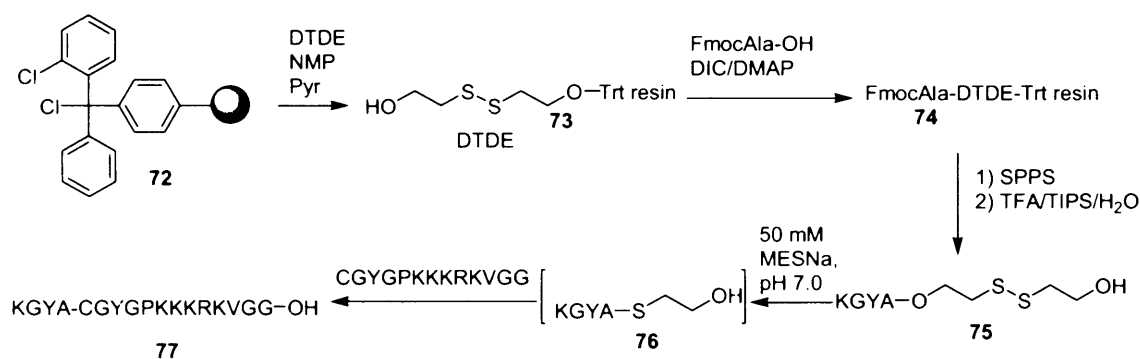
Aimoto *et al.* <sup>(52)</sup> also investigated the use of an *N,S* acyl shift. They developed the *N*-4, 5-dimethoxy-2-mercaptobenzyl group (Dmmb), which was loaded onto Pam resin with an Gly-Ala linker and the mercaptan of the Dmmb was protected with the trityl group. Standard Fmoc SPPS was carried out, the side chain protecting groups and the trityl of the mercapto group was cleaved and then the *N-S* acyl shift occurred to form an intermediate. After the addition of excess thiol, transthioesterification took place, liberating the thioester from the resin **71** (Scheme 23). They reported racemisation at the  $\alpha$ -carbon at the *C*-terminal end so this is another procedure limited to the synthesis of fragments with a *C*-terminal glycine residue.



*Scheme 23 – Dmmb with Pam Linker*

### 1.4.8 Dithiodiethanol esters for *in situ* thioester formation

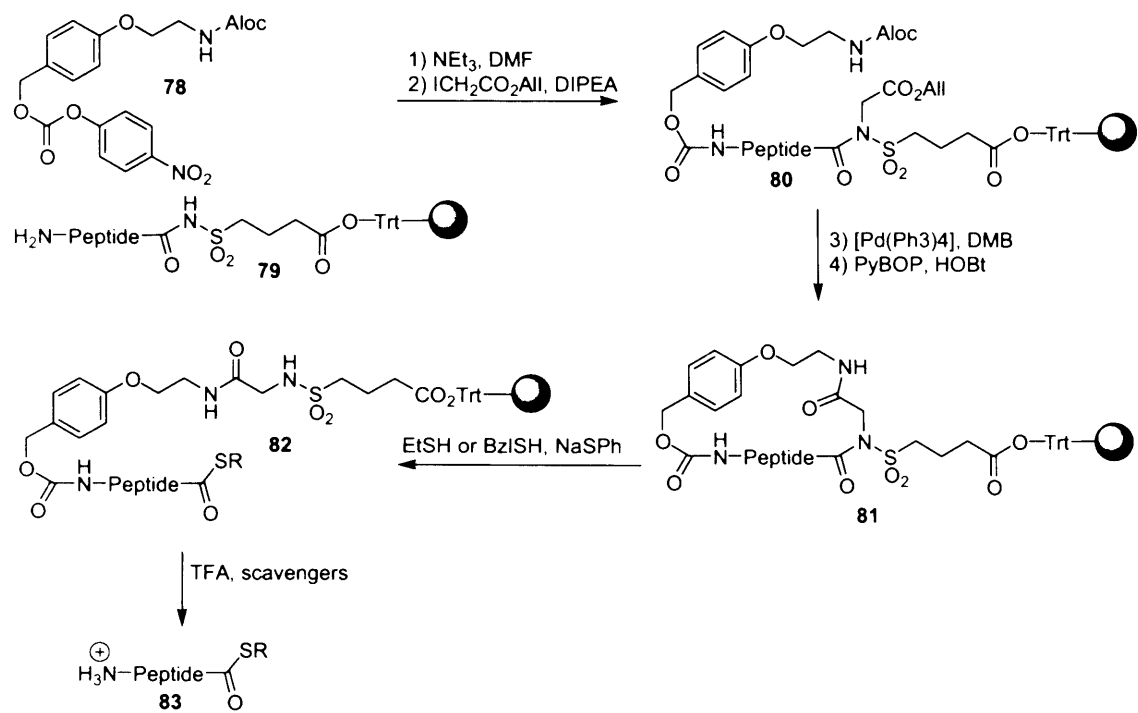
Tofteng *et al.*<sup>(53)</sup> developed further the *in situ* *O* to *S* acyl shift procedure. 2-Chlorotrityl chloride resin **72** was treated with DTDE **73** and then used both Fmoc-Phe-OH and Fmoc-Ala-OH were each used as the initial residues to couple to the linker. After the loading of the first amino acid, SPPS was carried out and then the peptide was cleaved from the resin using TFA/TIPS **75**. The peptide with a *C*-terminal ester bond with a disulfide linker incorporated was then exposed to ligation conditions and underwent rearrangement to produce a thioester **76**. Another peptide was added that contained an *N*-terminal cysteine residue and they were able to isolate the desired product **77** (Scheme 24).



Scheme 24 – Dithioethanol esters

#### 1.4.9 Thioester synthesis with self-purification

Seiz *et al.*<sup>(54)</sup> noted that because of incomplete coupling reactions during a synthesis, truncated products are also formed. When synthesizing thioesters this problem adds to the difficulty of purification. They developed a two-linker approach to try to eliminate the need for final HPLC purification. They attached 3-carboxypropane linker to trityl resin then performed standard Fmoc SPPS **79**, cleaving the final *N*-terminal Fmoc group. They then attached a linker with an *N*-terminal Alloc group **78**, followed by activation of the acidic sulfonamide group using ICH<sub>2</sub>CO<sub>2</sub>All **80**. Subsequent deallylation with palladium followed by macrolactamization with PyBOP produced an on-resin cyclized product with the sulfonamide group alkylated **81**, in preparation for nucleophilic cleavage with a thiol. By acetylating after each coupling reaction these truncated products were not incorporated into the macrolactam. Thiolysis at this stage opens the macrocycle to produce an on-resin fully protected thioester of high purity **82**. As the *N*-acetylated truncated products do not react with the linker that contains the *N*-terminal Alloc group, they therefore do not undergo macrolactamization. These by-products are liberated into solution at this stage. The fully deprotected thioester was then liberated from the resin with TFA and scavengers **83** (Scheme 25). As the first linker was attached to acid-labile trityl resin, the group were able to cleave the *C*-terminal acid from the resin using mild acidolysis (followed by side chain removal) at various stages. They showed that the purity of a small peptide at the macrolactamization step was excellent. This is a very recent technique and has only been tried on a few peptides.



*Scheme 25 – Thioester Synthesis with Self-Purification*

## 1.5 Introduction to Neocarzinostatin (NCS)

NCS is from the family of dinediyne chromoprotein antibiotics and is the most studied of the family;<sup>(55)</sup> it consists of a chromophore **1** (Figure 7) and an apoprotein (Figure 8) in a 1:1 ratio, which was reported in 1979. NCS is an antitumour agent first isolated in 1965 from *Streptomyces carzinostaticus* and clinical studies in Japan have shown to be an effective treatment against acute leukaemia and certain cancers.<sup>(56)</sup> NCS has a high toxicity and a very short half-life (approximately two minutes) so has not been used clinically worldwide.<sup>(57)</sup>

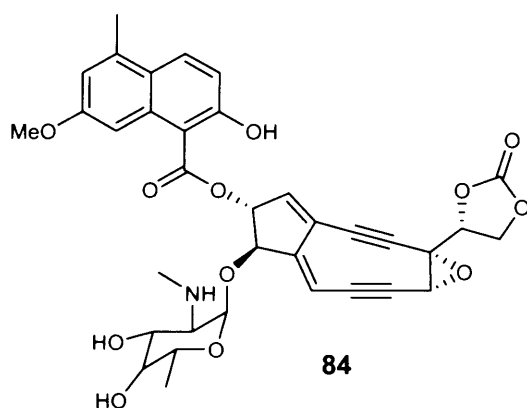


Figure 7 – NCS chromophore



Figure 8 – NCS apoprotein

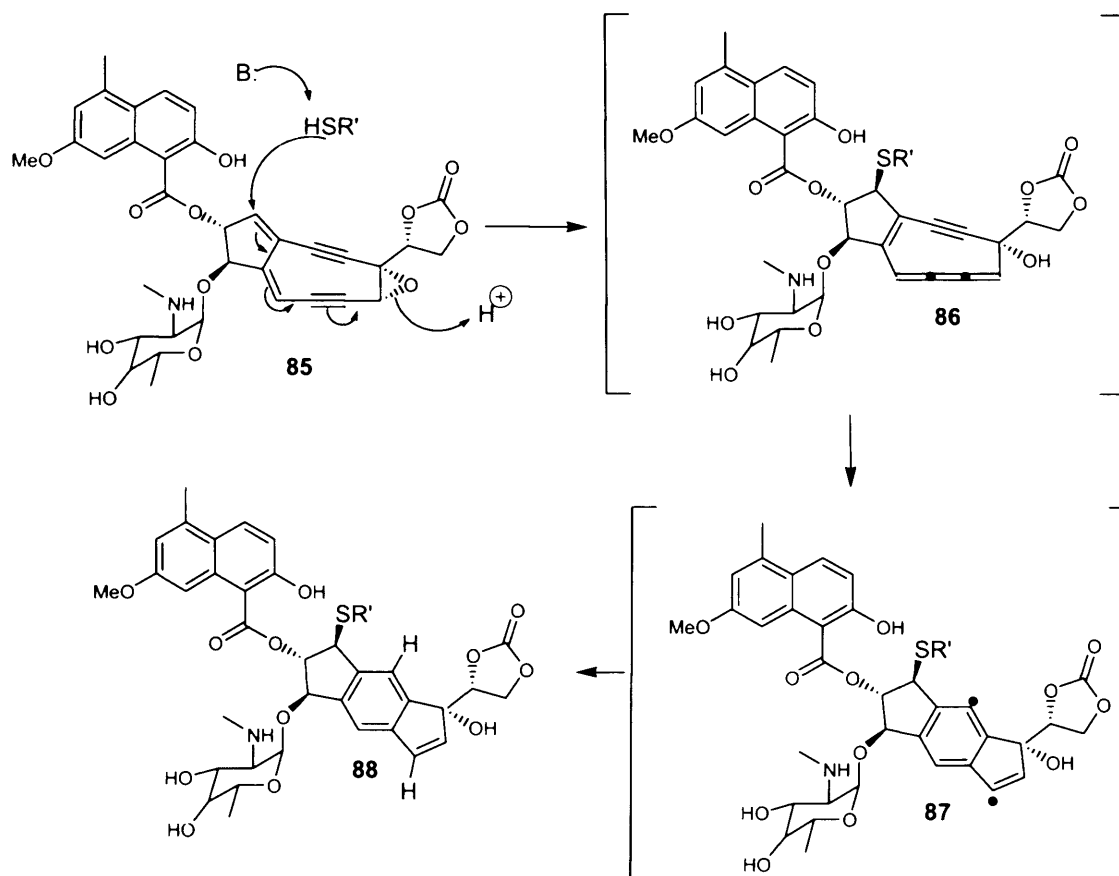


In 1972 Meinhofer *et al.* were the first to provide information on the amino acid sequence of the apoprotein, which was later updated by Biemann *et al.*, then Gibson *et al.*, before the final sequence was reported by Hirayama *et al.* Apo-NCS is an 11 kDa protein made up of 113 amino acids, with a relatively high proportion of alanine, glycine, serine, and threonine residues.<sup>(4)</sup> It also contains a relatively low concentration of amino acid residues that have the potential for modification, such as tyrosine, tryptophan, lysine and arginine. The protein sequence (Figure 9) is available from the Protein Data Bank.<sup>(58)</sup>

AAPTATVTPSSGLSDGTVVKVAGAGLQAGTAYDVGQCAWVDTGVLACNPA  
DFSSVTADANGSASTSLTVRRSFEGFLFDGTRWGTVSCTTAACQVGLSDAAG  
NGPEGVAISFN

*Figure 9 – NCS Peptide Sequence*

NCS is made up of a strained nine-membered dienediyne chromophore, which is non-covalently but tightly bound to the apo-protein. The chromophore, specifically, is involved in inhibiting DNA replication, and it does this by the Bergman style cycloaromatization of the strained nine-membered ring.<sup>(59)</sup> A diradical species is formed which is able to abstract hydrogen, cleaving DNA. The apoprotein is not thought to be cytotoxic on its own but acts as a drug delivery system, and also offers stability to the highly labile chromophore, shielding it from harmful heat, UV light and from nucleophiles. The highly strained nine-membered ring is thought to be the reason that the chromophore is very unstable and there have only been two total syntheses to date.<sup>(60)</sup>



*Scheme 26 – Bergman Cyclization*

Apo-NCS has also been shown to bind to other molecules, so there could be a possibility of it being used to deliver other molecules *in vivo*. Work has been carried out by Caddick *et al.* where nitrogen mustard derivatives were synthesized and conjugated to the naphthoate moiety of NCS. They showed that small molecules such as chlorambucil and melphalan can be attached to this naphthoate moiety and can maintain, and in the case of melphalan, their cytotoxic activity. This was the first example of small non-natural molecules binding to apo-NCS and it was proposed that they were also shielded from heat, light and nucleophiles as with the natural chromophore. This suggested that the apo-protein could perhaps be utilised as a drug delivery system.

NCS was the first in the family of enediyne natural products to be discovered. Other chromoproteins include macromomycin, kedarcidin, C-1027 and actinoxanthin and are also bound to proteins of approximately 11kDa. Other members of the family include calicheamicin, dynemicin and esperamicin; they are all ten-membered ring enediynes as opposed to the nine-membered ring NCS and so have less ring strain. Another difference is that they are not bound to an apo-protein.

## **Chapter 2. Results and Discussion**

### 2.1 Aim of the project

The aim of the work was to develop a synthetic approach to the protein chain of apo-NCS (Figure 10) which is 113 amino acids in length.

**AAPTATVTPSSGLSDGTVVKVAGAGLQAGTAYDVGQCAWVDTGVLACN  
PADFSSVTADANGSASTSLTVRRSFEGFLFDGTRWGTVDCTTAACQVGLS  
DAAGNGPEGVAISFN**

*Figure 10 – Protein sequence of apo-NCS*

It appeared that it would not be feasible to develop a synthesis based on a linear sequence and thus it was proposed that the protein be synthesised via a 3 fragments ligation strategy employing Fmoc chemistry. The 3 proposed fragments (Figure 11) are below.

#### Fragment 1

**AAPTATVTPSSGLSDGTVVKVAGAGLQAGTAYDVGQCAWVDTGVLA**

#### Fragment 2

**CNPADFSSVTADANGSASTSLTVRRSFEGFLFDGTRWGTVS**

#### Fragment 3

**CTTAACQVGLSDAAGNGPEGVAISFN**

*Figure 11 – Proposed fragments of apo-NCS*

Fragment 3 is a 26-mer with a C-terminal arginine carboxylic acid and an N-terminal cysteine residue. Fragment 2 was proposed as a 41-mer with a C-terminal serine  $\alpha$ -thioester and an N-terminal cysteine residue. Fragment 3 was proposed as a 46-mer with a C-terminal alanine  $\alpha$ -thioester and an N-terminal alanine. The intention was to synthesize the three fragments using SPPS and then couple fragments 2 and 3 using NCL, and thence fragment 1 to the coupled 2-3 fragment in a second NCL reaction. The cysteine residue of fragment 2 would have to contain a protecting group that could be removed after the NCL reaction to stop the fragment cyclising with itself.

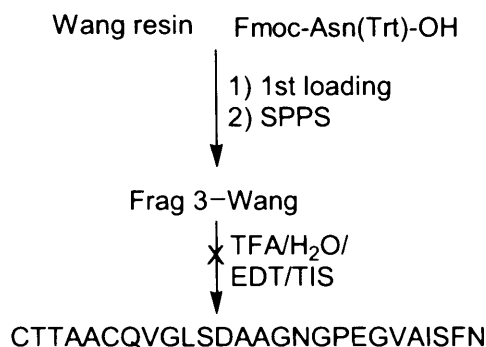
### Fragment 3

The third fragment (Figure 12) was conceivably the easiest to synthesize of the three because it was the shortest length (a 26-mer) but more importantly was the C-terminal end of the 113 amino acid sequence of apo-NCS. The C-terminal end is a carboxyl group, unlike fragments 2 and 3 which need to contain a thioester motif to enable NCL to take place. It should also be noted that the ninth residue is proline, which could help reduce chain aggregation.



*Figure 12 – Fragment 3 of apo-NCS*

The synthesis was first attempted using Fmoc chemistry with PyBOP/HOBt (4eq) as the coupling reagents on Wang resin. The initial attempt (Scheme 27) was not successful. When the peptide was eventually synthesized the fragment had the ninhydrin test carried out after every coupling reaction. Numerous residues required two or three coupling cycles and every residue had the resin capped with acetic anhydride after coupling and before cleavage of the Fmoc group.



*Scheme 27 – Attempted synthesis of fragment 3*

The synthesis was then re-attempted using a 2-chlorotrityl resin instead of Wang but this was also unsuccessful.

Instead of attempting the synthesis again without any insight into the reasons for the lack of successful synthesis it was proposed that a model study be carried out using a 3-mer made containing the first 3 amino acids in the chain (Figure 13).

**SFN**

*Figure 13 – Proposed 3-mer*

Several attempts to synthesize this 3-mer with Wang resin and 2-chlorotrityl resin led to failure but it should be observed that 3-mer's are often difficult to cleave from the resin and isolate.

It was then decided that the synthesis would be reattempted on Wang resin and after successful coupling of each residue (confirmed by ninhydrin test) the resin would be capped with acetic anhydride. The chain was successfully synthesized (Figure 14) in very low yield (4%) according to the first loading, where isolation issues and the capping of the resin after subsequent couplings played their part.

**CTTAACQVGLSDAAGNGPEGVAISFN**

*Figure 14 – Fragment 3*

The residues that are underlined (at positions 5, 8, 10 and 17 in the sequence) required two coupling reactions each to go to completion according to the ninhydrin test. The residue in italics (at position 11 in the sequence) required three coupling reactions to go to completion according to the ninhydrin test.

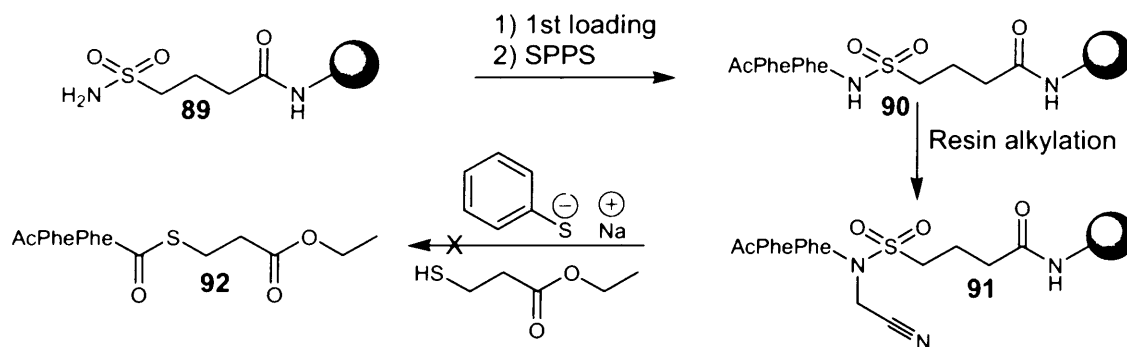
Fragment 2

Before attempting the synthesis of the middle fragment (Figure 15) it was thought beneficial to establish which type of resin would be appropriate for the synthesis of the thioesters.

**CNPADFSSVTADANGSASTSLTVRRSFEGFLFDGTRWGTVS**

*Figure 15 – Fragment 2 of apo-NCS*

It was decided that model studies should be carried out on the sulfamyl-style resins. A dipeptide Phe-Phe was proposed because it contained no reactive side-chain groups and the side-chains are aromatic therefore non-polar. It was intended that product be capped with acetic anhydride so that the cleaved product could be purified by flash column chromatography (Scheme 28).



*Scheme 28 – Model studies of sulfamyl resins*

The first attempt at synthesising the dimer used the procedure described by Ingenito *et al.* No desired product was obtained and only thiol was recovered. A larger batch was prepared and divided into small portions, and coupling attempts were carried out using different cleavage protocols were used at both ambient and elevated temperatures.

After getting no successful cleavage of products from these experiments using this resin it was decided to try a similar style resin (Figure 16).

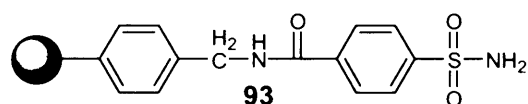
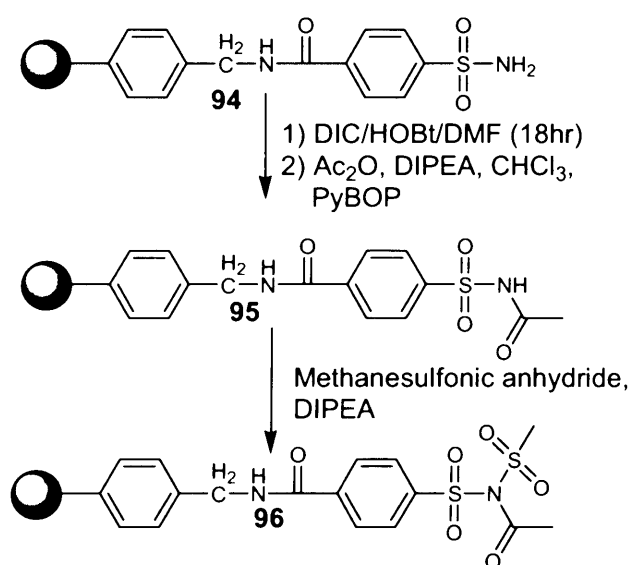


Figure 16 – Sulfamylbenzoyl resin

As there are two steps to the cleavage it was not known if the alkylation step was the limiting factor or the cleavage step (or both). A different activating reagent (Scheme 29) was used and then subjected to various cleavage conditions (Table 1).



Scheme 29 – Activation of sulfamylbenzoyl resin with methane sulfonic anhydride

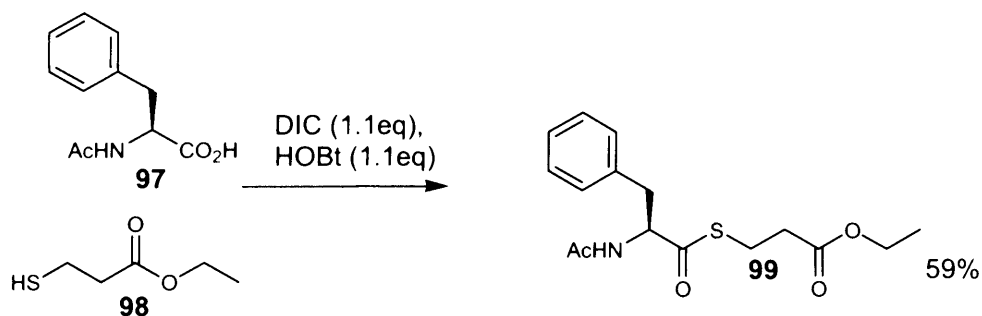


Starting material	Conditions	Temperature °C
<b>96</b>	Ethyl-3-mercaptopropionate, NaSPh, DMF	ambient
<b>96</b>	2-Methoxybenzyl mercaptan, NaSPh, DMF	ambient
<b>96</b>	Ethyl-3-mercaptopropionate, NaSPh, THF	60
<b>96</b>	Thiophenol, NaSPh, THF	60

*Table 1 – Conditions tried to cleave from resin*

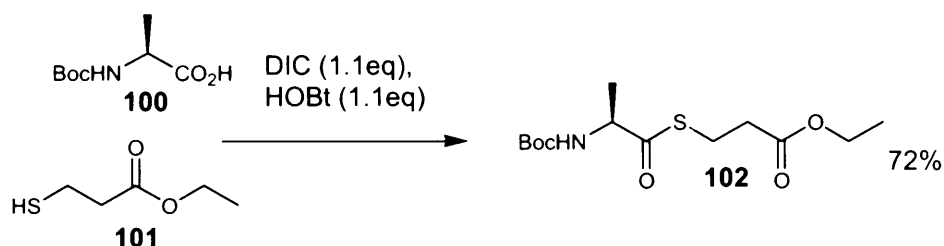
So far no product had been cleaved from the resin using the conditions in Table 2.

It was then decided that two amino acids would be loaded onto sulfamyl resins and the corresponding thioesters that should be cleaved would be synthesized so that the authentic samples could be used for comparison. The first thioester synthesized was using Ac-Phe-OH (Scheme 30).



*Scheme 30 – Thioester synthesis*

The second thioester synthesized was using Boc-Ala-OH (Scheme 31).



*Scheme 31 – Thioester synthesis*

With the Rf value of these thioesters **99** and **102** known their corresponding carboxylic acids **97** and **100** were loaded onto the sulfamyl resins, activated using both ICH<sub>2</sub>CN and TMS-diazomethane and subjected to cleavage with ethyl-3-mercaptopropionate. Again no products were cleaved.

A previously prepared 3-mer (with the sequence KPA) that had been synthesized by Ingenito *et al.* was attempted but no product was obtained. Previously methanesulfonic anhydride was used as an alternative alkylating reagent; now the first loadings were tried in the microwave, with normal alkylating conditions. The attempted cleavage with the thiol was also carried out in the microwave in the hope that any product could be cleaved from the resin under these more vigorous conditions. Again no product was cleaved from the resin. The next step was to carry out a double coupling along with a third coupling using DIC (48hr). In a lot of cases the first loading required six repeated couplings to get a high yield (approximately 70%) with respect to the stated loading. Interestingly, performing a single coupling reaction in the microwave for thirty minutes gave a respectable yield (35%). On occasions there would be no loading after three coupling reactions, so the procedure

was not consistent. Once the first loadings were established the synthesis of fragment 2 was tried many times on a peptide synthesizer with different coupling reagents (DIC/HOBt, HBTU) with varying equivalents, different first loading (from 0.1 mmol/g upto 0.95 mmol/g with ranges in between). Also different scales were tried (ranging from 0.1 mmol/g to 2 mmol/g).

### 2.1.1 Thioester model studies

Due to the lack of success in synthesising thioesters it was considered beneficial to try to produce a smaller thioester using resin chemistry so that the conditions could be used for fragment 2 (and 1) of apo-NCS. A 12-mer (Figure 17) was chosen with a C-terminal serine  $\alpha$ -thioester and an N-terminal cysteine residue that could be protected with an AcM group.

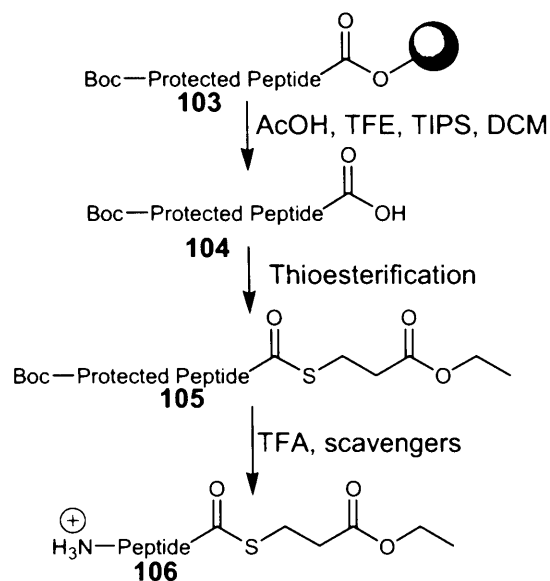


*Figure 17 – Small Thioester*

Three approaches were attempted simultaneously (sulfamyl resins, 2-chlorotrityl resins and using Boc chemistry). Even the synthesis of this small thioester proved to be very difficult; several initial attempts proved to be futile. The only success occurred when checking the coupling had gone to completion (some residues required multiple coupling cycles) using the ninhydrin test. The resin was capped with acetic anhydride each time before cleavage of the Fmoc group.

### 2.1.2 2-Chlorotrityl chloride resin

The first approach involved trying to manually synthesize the peptide sequence on 2-chlorotrityl resin and then cleave off from the resin with mild acid conditions (AcOH, TFE, DCM) so that a coupling reaction with a thioester could then occur with a thioester. The protected groups could then be removed to liberate the product.

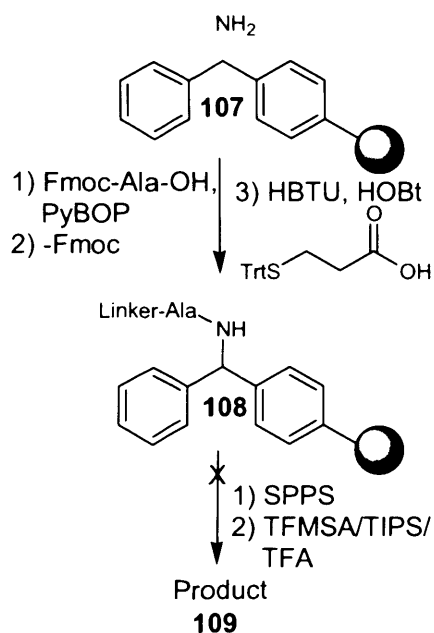


*Scheme 32 – 2-chlorotrityl chloride SPPS*

This procedure was tried many times with different coupling reagents and loading levels on the resin. The synthesis was also tried in the microwave on numerous occasions but all attempts proved to be unsuccessful.

### 2.1.3 Boc Chemistry

In this procedure (Scheme 32) Fmoc-Ala-OH is added to MBHA resin **102** and then once the first loading had been checked the Fmoc group was removed. A linker is then added that has a protected thiol group. Subsequent removal of the trityl protecting group gives **103** and then SPPS was carried out using Boc chemistry. Final cleavage attempt was using TFMSA in place of HF. Several attempts were made to synthesize the 12-mer using this chemistry but none were successful.



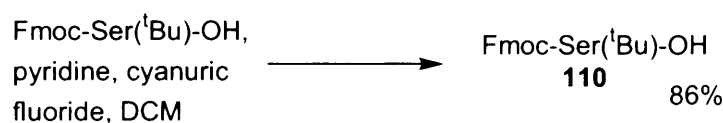
*Scheme 33 – Boc SPPS*

#### 2.1.4 Sulfamyl resin

The 12-mer was synthesized in 37% yield from the first loading using sulfamylbutyryl resin and didn't require further purification. This showed that the chemistry worked but only on a 12-mer so far.

#### 2.1.5 Loading the Resin

After the success of synthesizing the 12-mer using sulfamyl resins there were still difficulties with the loading of amino acids. Previously loading sulfamyl resins with Fmoc-Ser(<sup>t</sup>Bu)-OH had taken 6 couplings to achieve 0.54mmol/g loading (theoretical = 1.1mmol/g). Below is a scheme of the synthesis of amino acid fluorides (Scheme 34).



*Scheme 34 – Amino acid fluoride synthesis*

Below (Figure 18) shows the loadings and methods using approximately 50mg of resin.

Fmoc-Ser(<sup>t</sup>Bu)-OH, using DIC/Melm (18hrs): No loading

Fmoc-Ser(<sup>t</sup>Bu)-OH, using PyBOP (8hrs): Loading = 0.40mmol/g (2<sup>nd</sup> = 0.57mmol/g)

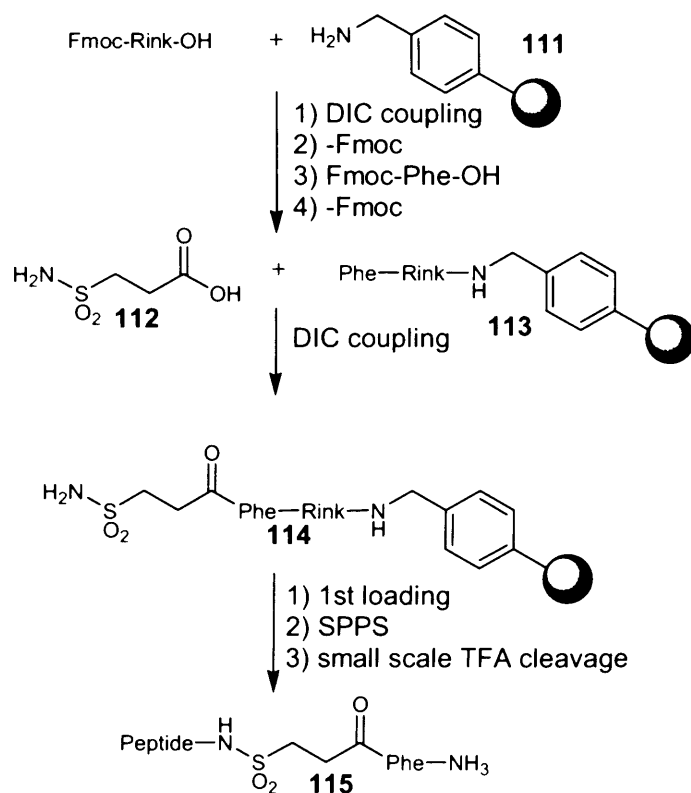
Fmoc-Ser(<sup>t</sup>Bu)-F, DIPEA (1hr): Loading = 0.35mmol/g (2<sup>nd</sup> = 0.24mmol/g)

Fmoc-Ser(<sup>t</sup>Bu)-F, in situ and direct loading: No loading

*Figure 18 – Amino acid fluoride loadings vs PyBOP*

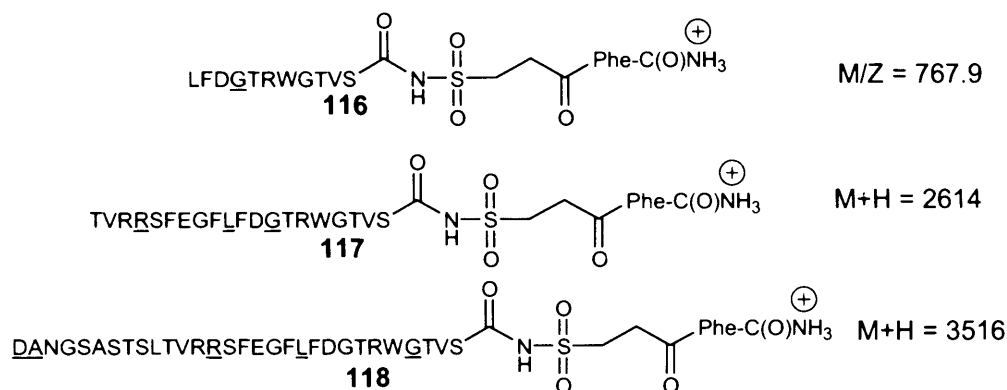
### 2.1.6 Rink Amide Resin

An extension to the sulfamyl type resins is a double-linker strategy. Here the acid-labile rink linker was attached to polystyrene resin **106**, the protecting group was removed then an amino acid spacer followed by the attachment of a second linker **107**. 3-Carboxypropane linker was then added and SPPS was then carried out. The couplings were checked by ninhydrin test and after every ten residues a small portion of resin was cleaved to give the peptide amide **110**. The advantage was that the product liberated could be monitored by HPLC and MS to check the sequence was being synthesized correctly (Scheme 35).



Scheme 35 – Rink SPPS

The first 10 amino acid section of fragment 2 was synthesized on the resin and cleaved off successfully in this way. Two more sections were synthesized on the resin also (Figure 19). No other residues were added successfully to the chain after the 30<sup>th</sup> amino acid residue. The underlined residues required multi-couplings.



*Figure 19 – Sections of fragment 2 synthesized*

This showed that the synthesis was successful on the resin but only as far as residue 30. No more amino acids could be added to this sequence and a repeat attempt using the same conditions was unsuccessful. The crude was impure and also the HPLC trace was messy, suggesting that the resin had been loaded to its maximum potential. Attempts to re-synthesize the chain on the resin were not successful (even though the first loadings were the same). Attempts with lower loading of the first amino acid also resulted in an incomplete synthesis of the peptide chain.

The sequence was also attempted using a microwave peptide synthesizer with varying first loading levels but with no success. The synthesis was also carried out on the first ten amino acids and subjected to cleavage of the peptide amide (as in Figure 16) but with no success. The first five amino acids were added to the resin and the cleavage of the peptide amide was attempted but also with no success.



### Fragment 3

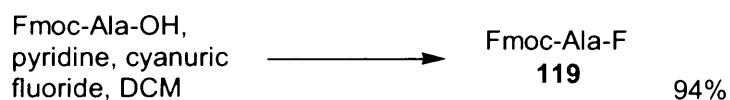
The first attempt at synthesizing fragment 3 (Figure 20) was tried manually using sulfamylbutyryl resin. The fragment was a 46-mer, the largest chain of the three.

This attempt was unsuccessful.

**AAPTATVTPSSGLSDGTVVKVAGAGLQAGTAYDVGQCAWVDTGVLA**

*Figure 20 – Fragment 1 of apo-NCS*

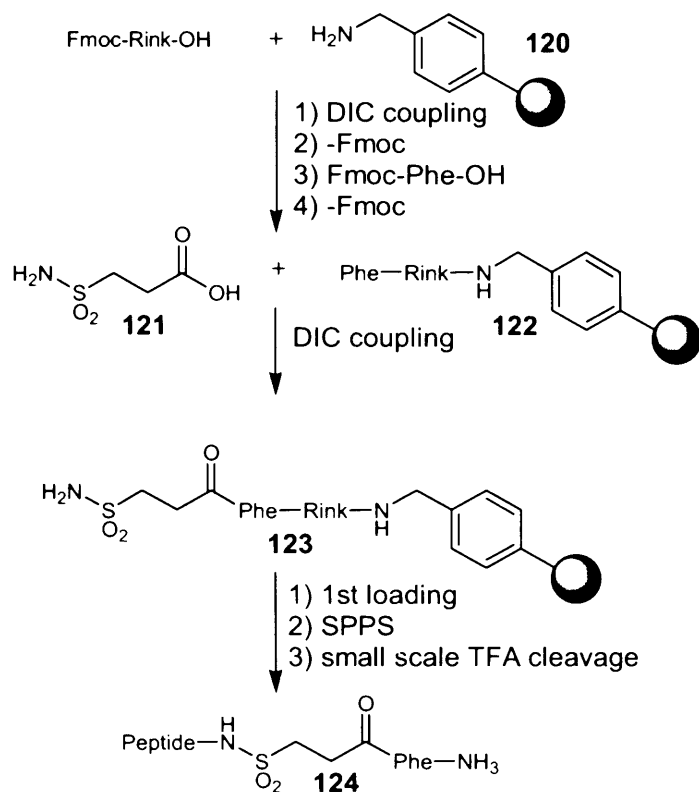
The attempted synthesis was repeated using a peptide synthesizer on a small scale. No product was cleaved from the resin. The synthesis was then re-tried using a peptide synthesizer on a larger scale; there was a varying range of first loadings tried with no success. The alkylation of the sulfamyl resin was tried with both TMS-diazomethane and iodoacetonitrile. Problems were encountered with the loadings so the amino acid fluoride synthesis was investigated (Scheme 36).



*Scheme 36 – Amino acid fluoride synthesis*

Loading the Fmoc-Ala-F onto sulfamyl resins proved more effective than using the literature procedure.

Once the loading was established the next step was to try the two-linker approach (Scheme 37).



*Scheme 37 – Rink SPPS*

We envisaged monitoring the efficiency of the coupling after every ten residues when the peptide amide was cleaved from the resin. No success was gained from trying to synthesize the first 10-mer when using varying levels of loading. After many attempts the first five were attempted but no successful product was cleaved.

### **3. Conclusions**

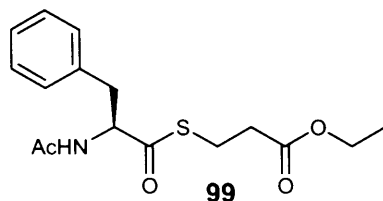
Fragment 3 was synthesized from Wang resin in low yield but was very pure. A 12-mer thioester was synthesized using sulfamylbutyryl resin in good yield and purity so it was shown that the thioester chemistry worked but only on smaller fragments. The middle fragment 2 was not fully synthesized; the sequence was synthesized on the resin successfully only up to the residue 30 (there were 41 residues in the proposed fragment). The first loading of amino acids onto the sulfamyl style resins was optimised and successful using amino acid fluoride derivatives. No success was gained with fragment 1. It is proposed that for further work Boc chemistry be used to attempt to synthesize the fragments; if they were able to be synthesized then at least ligation reactions could be tried.

## 4. Experimental

### General

$^1\text{H}$  and  $^{13}\text{C}$  nmr spectra were recorded on a 300MHz Bruker AMX300 machine using deuterium as in internal standard. Chemical shifts are recorded in parts per million, standard abbreviations are used (s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet). Mass spec samples were run on a Varian MALDI or electrospray. Column chromatography was performed on silica gel (32-70 micron). TLC's were run on Merk silica gel 60 F<sub>254</sub>, using UV and/or permanganate dip. Melting points were obtained using a Gallenkamp heater. IR's were obtained from a SHIMADZU FT-IR 8700. Optical rotations were run on a PolaAr 2000 polarimeter. Piperidine and DIPEA were distilled from calcium hydride; THF was distilled from sodiumbenzophenone ketyl. General reagents were commercially available.

### 3-(2-Acetylamino-3-phenyl-propionylsulfanyl)-propionic acid ethyl ester



N-Ac-Phe-OH (3.73g, 18.0 mmol), ethyl-3-mercaptopropionate (2.3 mL, 18.2 mmol), DIC (2.38g, 18.9 mmol) were added to a round bottom flask and then dissolved in dry DCM (30 mL). The reaction was stirred for 3 hours at ambient temperature until the reaction had gone to completion, which was checked by TLC. The solution was then diluted with DCM (30 mL) and washed with 2M HCl (2 x 50 mL), saturated NaHCO<sub>3</sub> (2 x 50 mL) and H<sub>2</sub>O (2 x 50 mL). The organic phase was separated, dried over magnesium sulphate, filtered and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (5:1 petroleum ether 40-60°C: ethyl acetate) to give the title compound as a white solid (3.42g, 59%).

**Rf** 0.29 (2:1 petroleum ether: ethyl acetate)

**<sup>1</sup>H (CDCl<sub>3</sub>)** 7.32-7.21 (m, 5H, PhH), 5.84 (d, 1H, *J* = 8.2, -NH), 4.93 (dd, 1H, NH-CH-C=O), 4.11 (q, 2H, -CH<sub>2</sub>CH<sub>3</sub>), 3.04-3.18 (m, 4H, PhCH<sub>2</sub> & -S-CH<sub>2</sub>), 2.59 (s, 3H, CH<sub>3</sub>C=O), 1.38 (t, 3H, *J* = 6.2, -CH<sub>2</sub>-CH<sub>3</sub>)

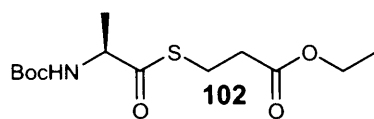
**<sup>13</sup>C (CDCl<sub>3</sub>)** 199.8 (CH<sub>3</sub>C=O), 171.5 (C=O ester), 170.0 (S-C=O), 135.6, 129.3, 128.7, 127.2, (PhC), 60.9 (CH<sub>2</sub>CH<sub>3</sub>) 38.1, (S-CH<sub>2</sub>), 34.1 (CH<sub>2</sub>CH<sub>2</sub>), 24.0 (PhCH<sub>2</sub>), 23.1 (CH<sub>3</sub>C=O), 14.2 (CH<sub>2</sub>CH<sub>3</sub>)

**LRMS (ES+)** 346 (M<sup>+</sup> + Na)

**HRMS (ES+)** Theoretical mass 346.1084, measured mass 346.1092

**ν<sub>max</sub> (cm<sup>-1</sup>)** 3282, 3100, 1734, 1655, 1541

### 3-(2-*tert*-Butoxycarbonylamino-propionylsulfanyl)-propionic acid ethyl ester



Boc-Ala-OH (0.965 g, 5.10 mmol), ethyl-3-mercaptopropionate (3.5 mL, 27.7 mmol), DIC (0.725 g, 5.75 mmol) were added to a round bottom flask and then dissolved in dry DCM (25 mL). The reaction was stirred for 3 hours at ambient temperature until the reaction had gone to completion. The solution was then diluted with DCM (30 mL) and washed with 2M HCl (2 x 50 mL), saturated NaHCO<sub>3</sub> (2 x 50 mL) and H<sub>2</sub>O (2 x 50 mL). The organic phase was separated, dried over magnesium sulphate, filtered and concentrated *in vacuo*. The crude residue was purified by flash column chromatography (50:1 petroleum ether 40-60°C: ethyl acetate) to give the title compound as a white solid (1.13 g, 72%).

**Rf** 0.5 (4:1 petroleum ether: ethyl acetate)

**<sup>1</sup>H (CDCl<sub>3</sub>)** 4.95 (d, 1H, *J* = 7.4, -NH), 4.64 (m, 1H, -CHCH<sub>3</sub>), 4.17 (q, 2H, CH<sub>2</sub>CH<sub>3</sub>), 3.12 (t, 3H, *J* = 7.0, -SCH<sub>2</sub>CH<sub>2</sub>-), 2.82 (t, 3H, *J* = 3.2, -CH<sub>2</sub>CH<sub>2</sub>-), 1.44 (s, 9H CH<sub>3</sub>-C), 1.37 (d, 3H, *J* = 7.2, CHCH<sub>3</sub>), 1.27 (t, 3H, *J* = 7.1, CH<sub>2</sub>CH<sub>3</sub>)

**<sup>13</sup>C (CDCl<sub>3</sub>)** 201.9 (C=O thioester), 171.6 (C=O ester), 154.9 (C=O amide), 80.2 (C *tert*-butoxy), 60.72 (CH<sub>2</sub>CH<sub>3</sub>), 56.3 (CHCH<sub>3</sub>), 34.2 (CH<sub>2</sub>C=O), 28.3 (CH<sub>3</sub>C), 23.7 (-SCH<sub>2</sub>), 18.5 (CH<sub>3</sub>CH), 14.1 (CH<sub>3</sub>CH<sub>2</sub>)

**LRMS (ES<sup>+</sup>)** 328 (M<sup>+</sup> + Na)

**HRMS (ES<sup>+</sup>)** Theoretical mass 328.1189, measured mass 328.1190

**ν<sub>max</sub> (cm<sup>-1</sup>)** 3363, 2984, 2936, 1732, 1686, 1504

## 12-mer thioester



Commercially available 4-sulfamylbutyryl resin (1.00 g) was swollen in chloroform for 1 hour in a sintered flask. The resin was then drained. Fmoc-Ser(tBu)-OH (4 eq) was added to PyBOP (4 eq) and DIPEA (8 eq) and pre-activated for ten minutes in residual DCM. The reagent was added to the resin and shaken for eight hours. The resin was then drained and washed with DMF (5 x 3 mL), DCM (5 x 3 mL), and then DMF (5 x 3 mL). This coupling procedure was repeated five times. The first loading of the resin was 0.96 mmol/g. The synthesis was then completed with PyBOP (4 eq) and DIPEA (8eq) as the coupling reagent for 2 hours and drained and washed as before. The ninhydrin test was used to check the success of each coupling reaction. After a negative ninhydrin the resin was capped with acetic anhydride (20%) in DMF before the removal of the Fmoc group. Fmoc groups were removed in piperidine (20%) in DMF; the resin was shaken with the cleavage solution for one minute, drained, washed and then shaken with fresh cleavage solution. The residues underlined above required double coupling reaction to give a negative ninhydrin. The resin was alkylated with iodoacetonitrile (3.5 mL, 48.4 mmol) that had been previously filtered through basic alumina and DIPEA (2.5 mL, 14.4 mmol) in DMF for 24 hours. After 24 hours the resin was rinsed and washed (as previously). Ethyl-3-mercaptopropionate (6.8 mL, 53.7 mmol), and sodium thiophenolate (67 mg, 0.5 mmol) were dissolved in DMF (5 mL) and added to the resin and shaken for 24 hours. The resin was filtered and washed with DCM (20 x 10 mL) and methanol (20 x 10 mL) and the combined solvent was concentrated *in vacuo*. The crude was subjected to column chromatography to remove the excess thiol and to remove residual DMF. After the thiol had eluted from the column the product was flushed with methanol:

ethyl acetate (50: 50) and concentrated *in vacuo*. TFA/H<sub>2</sub>O/TIPS/phenol (88/5/2/5, 10 mL) was added to the crude and stirred at ambient temperature for 4 hours. The reaction was then concentrated *in vacuo*, and allowed to precipitate in a 20 fold excess of cold ether. The crude peptide was centrifuged and then the solvent was decanted; the crude was washed with ether (20 mL), centrifuged and then the solvent decanted. The crude was then dissolved in 10 %AcOH (10 mL), dissolved and then freeze dried to give a white solid (0.59 g, 0.35 mmol). The product was characterised by analytical HPLC and ES<sup>+</sup>.

**ES+** found 1675.7, actual = 1675.1

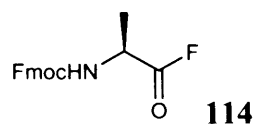


### Fragment 3 of apo-NCS

CTTAACQVGLSDAAGNGPEGVAISFN

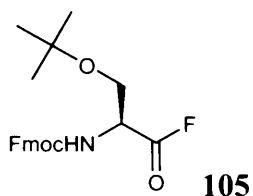
Wang resin was swollen in DMF for 30 minutes. Fmoc-Asn(Trt)-OH (1.39811 g, 2.34 mmol) was dissolved in dry DCM (10 mL) and residual DMF. DIC (0.18 ml, 1.16 mmol) was dissolved in dry DCM (1 mL) and added to the amino acid solution. The reaction was stirred at 0°C for 20 minutes and then added to the resin. DMAP (approximately 3 mg) was then added and the reaction was stirred for 1 hour. The resin was then filtered, washed with DMF (10 x 5 mL), DCM (10 x 5 mL), and shrunken in ether. The resin was dried and then the first loading was measured (0.65 mmol/g). SPPS was then carried out using amino acid (3 eq), PyBOP (4 eq), DIPEA (8 eq). The coupling was checked by ninhydrin test. Residues that required extra coupling step are underlined in the sequence above. The resin was capped with 20% acetic anhydride/DMF after each successful coupling. The final amino acid was capped with Boc anhydride (10 eq) in DMF. The peptide was dried after synthesis and then TFA/H<sub>2</sub>O/EDT/TIPS (94.5/2.0/2.5/1.0, 4 mL) was added. The resin was shaken for 3 hours and then filtered and washed with TFA (30 x 3 mL). The combined cleavage cocktail and washings were combined and concentrated *in vacuo*. The crude was then allowed to precipitate in a 20 fold excess of cold ether to give a white solid (14 mg, 4%). The product was characterised by analytical HPLC and ES<sup>+</sup>. ES<sup>+</sup> found 2454.9, actual = 2453.7.

**(1-Flouocrbonyl-ethyl)-carbamic acid 9H-fluoren-9-ylmethyl ester**



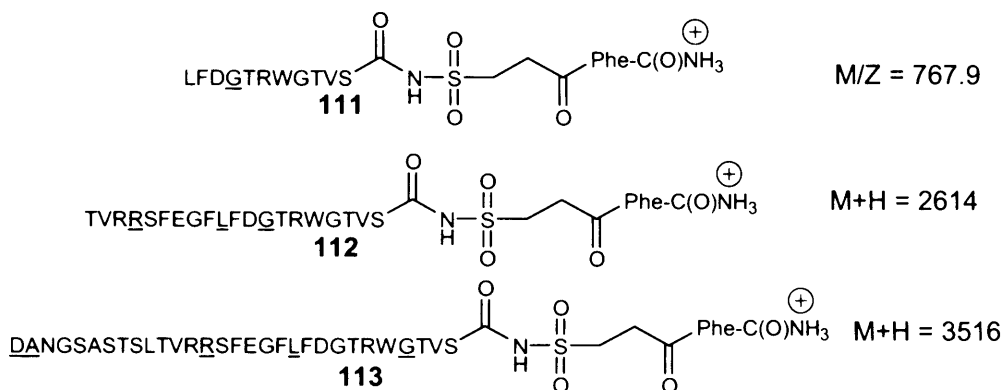
Fmoc-Ala-OH (2.01 g, 6.47 mmol) was dissolved in dry DCM (30 mL) and then distilled pyridine (0.52 ml, 6.46 mmol) and cyanuric fluoride (1.1 mL, 12.9 mmol) were added under a nitrogen atmosphere. The cyanuric fluoride was added dropwise and then the mixture was stirred for 2 hours. The reaction was dissolved in DCM (150 mL) and then washed with cold distilled water (2 x 100 mL), dried over magnesium sulphate, filtered and concentrated *in vacuo*. The product was then recrystallised from petrol. The product was then filtered and dried to give a white solid (1.90 g, 6.07 mmol, 94%). The product was used crude as used in the literature.

**(2-*tert*-Butoxy-1-fluorocarbonyl-ethyl)-carbamic acid 9*H*-fluoren-9-ylmethyl ester**



Fmoc-Ser(*t*-Bu)-OH (0.593 g, 1.55 mmol) was dissolved in dry DCM (5 mL) and then distilled pyridine (0.12 ml, 1.49 mmol) and cyanuric fluoride (1.1 mL, 3.1 mmol) were added under a nitrogen atmosphere. The cyanuric fluoride was added dropwise and then the mixture was stirred for 2 hours. The reaction was dissolved in DCM (10 mL) and then washed with cold distilled water (2 x 25 mL), dried over magnesium sulphate, filtered and concentrated *in vacuo*. The product was then recrystallised from petrol. The product was then filtered and dried to give a white solid (0.518 g, 1.3 mmol, 86%). The product was used crude as used in the literature.

## Fragment 2 of apo-NCS



Aminomethylpolystyrene-Rink-Fmoc resin (2.026 g) was swollen in DMF for 1 hour. The Fmoc group was cleaved with 20% piperidine/DMF (as previously) then drained and washed in DMF (10 x10 mL), DCM (10 x10 mL) and DMF (10 x10 mL). Fmoc-Phe-OH (1.72 g, 4.44 mmol), HBTU (1.688g, 4.45 mmol), HOBt (0.615 g, 4.56 mmol) and DIPEA (2.1 mL, 12.1 mmol) were dissolved in DMF (10 mL) and allowed to pre-activate for 10 minutes. The mixture was then added to the resin and shaken for 2 hours and then drained and washed as previously. After a negative ninhydrin test the resin was capped with 20% acetic anhydride/DMF and then the Fmoc was removed. 3-Carboxypropylsulfonamide linker (1.27 g, 7.51 mmol), DIC (1.15 mL, 7.43 mmol) and HOBt (1.011 g, 7.48 mmol) were dissolved in DMF (10 mL) and allowed to pre-activate for 10 minutes. Then the solution was added to the resin and shaken for 24 hours then drained and washed as above. The resin was then dried. A portion of this resin (1.77 g) was then swollen in DMF for 1 hour. Fmoc-Ser(*t*-Bu)-OH (1.92 g, 5.01 mmol), PyBOP (2.96 g, 5.69 mmol) and DIPEA (1.8 mL, 10.6 mmol) were dissolved in dry DCM (5 mL) and allowed to pre-activate for 10 minutes. The solution was then added to the resin and shaken for 8 hours before being drained and then washed as previously. The resin was dried and then the first loading was measured (0.65 mmol/g). SPPS was then carried out using amino acid (3 eq), PyBOP (4 eq), DIPEA (8 eq). The coupling was checked by ninhydrin test. Residues that

required extra coupling step are underlined in the sequence above.

TFA/H<sub>2</sub>O/TIPS/phenol (88/5/2/5, 1 mL) was added to the some dried resin beads and shaken at ambient temperature for 4 hours. The reaction was then concentrated *in vacuo*, and allowed to precipitate in a 20 fold excess of cold ether. The crude peptide was centrifuged and then the solvent was decanted; the crude was washed with ether (5 mL), centrifuged and then the solvent decanted. The crude was then dissolved in 10 %AcOH (10 mL), dissolved and then freeze dried. The product was characterised by analytical HPLC and either ES<sup>+</sup> or MALDI.

**111 ES<sup>+</sup> m/z found 767.9, actual = 767.9**

**112 MALDI found 2613, actual 2612**

**113 MALDI found 3517, actual 3516**

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