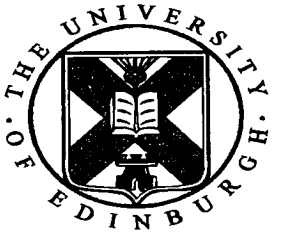
Investigations In Chemical Protein Synthesis



Carolyn Alexandra Gordon

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University of Edinburgh, 2001

To my family.

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

Abstract

The chemical synthesis of the 184aa anti-angiogenic peptide, endostatin, was undertaken. Preparation *via* the stepwise synthesis of two large fragments approximately 90aa in length was attempted but was unsuccessful. A method that would allow the efficient, sequential coupling of several small fragments was required. To this end, the segment coupling of minimally protected fragments *via* transfer active ester condensation (TAEC), a recently developed technique, was investigated.

The fragments for synthesis were first selected based on hydrophobicity and potential coupling sites. These peptide fragments were then optimised for stepwise SPPS using the Fmoc strategy. Peptides containing two types of *C*-terminal functionality, hydrazides and semi-carbazides, were prepared. An alternative strategy for the synthesis of the Wang resin-based hydrazide linker, first proposed by Wang and Merrifield in 1969, was developed to facilitate this process. Peptide fragments of up to 20aa in length were successfully coupled using TAEC.

A novel approach for the protection of arginine side chains was also investigated. This target was based on dibenzocycloheptenyl linker methodology, which was originally designed for use in the preparation of peptide amides. Recent work by Noda[1] involved the use of a derivative of this linker that has proven to be significantly more acid labile than current arginine protecting groups. Concurrent work on an improved design led to an alternative system – the dimethoxy-suberyl group - being investigated. The synthesis of the target molecule was achieved in seven steps. Key steps involved a Perkin reaction and an intramolecular Friedel-Crafts acylation. The coupling of the dimethoxysuberyl moiety to arginine was undertaken but proved unsuccessful.

[1] Noda M. and Kiffe M., *J. Peptide Res.*, 1997, **50**, 329.

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Abbreviations

aa	amino acid
AAA	amino acid analysis
Acm	acetamidomethyl
AcOH	acetic acid
Adoc	adamantyloxycarbonyl
Boc	tertiary-butoxycarbonyl
d	doublet
DBU	1,8-diazabicyclo[5.4.0]undecane
DCC	N,N-dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	N,N-diisopropylcarbodiimide
DIEA	N,N-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
EPO	erythropoietin
EDT	ethanedithiol
ES	electrospray
ESMS	electrospray mass spectroscopy
FAB	fast atom bombardment
Fmoc	9-fluorenylmethyloxycarbonyl
FPLC	fast protein liquid chromatography
HBTU	*O*-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium hexafluorophosphate
HF	hydrogen fluoride
HIV	human immunodeficiency virus
HOAt	1-hydroxy-7-azobenzotriazole
HOBt	1-hydroxybenzotriazole
HOCt	ethyl-1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate
HOSu	*N*-hydroxysuccinamide
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
IR	infra red
IPA	isopropyl alcohol (2-propanol)
LCMS	liquid chromatography-mass spectrometry
m	multiplet
MALDI	matrix assisted laser desorption ionisation
MeO-Sub	2-methoxy-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5-yl
Msc	methylsulphonylethyloxycarbonyl

MS	mass spectroscopy
Mtr	4-methoxy-2,3,6-trimethylbenzenesulphonyl
MW	molecular weight
Nsc	2-(4-Nitrophenylsulphonyl)ethoxycarbonyl
Nbs	N_G-4-Nitrobenzenesulphonyl-L-arginine
nm	nanometre
NMM	N-methyl morpholine
NMP	N-methyl pyrrolidinone
NMR	nuclear magnetic resonance
PEG	polyethyleneglycol
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl
Pic	picolyl
Pip	piperidine
Pmc	2,2,5,7,8-pentamethonylchroman-6-sulphonyl
PPA	polyphosphoric acid
RT	room temperature
Rt	retention time
s	singlet
SPFC	solid phase fragment condensation
SPPS	solid phase peptide synthesis
Sub	10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5-yl
Suben	5H-dibenzo[*a,d*]cyclohepten-5-yl
t	triplet
t	tertiary
TAEC	transfer active ester condensation
TASP	template-assembled synthetic protein
Tbfmoc	17-tetrabenzo[*a,c,g*]fluorenylmethoxycarbonyl
TEA	triethylamine
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
Tnm	3-Hydroxymethyl-3-nitro-1,5-dioxaspiro-5:5-undecane
TOF	time of flight
Trt, Trityl	triphenylmethyl
UV	ultraviolet
Z	benzyloxycarbonyl

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Appendix 1 Chemically Synthesised Peptide Fragments – *Numbers & Sequences*
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1. Introduction

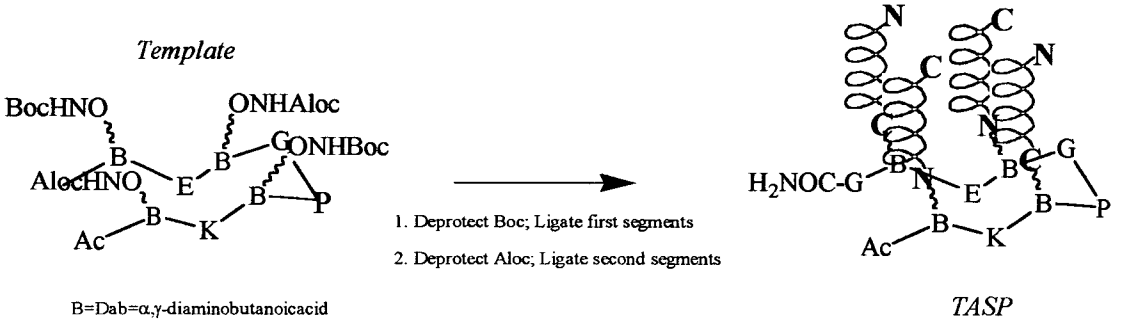
1.1 Peptide Synthesis – An Overview

Peptides and proteins are a fundamental constituent of molecular biology. With the sequencing of the human genome virtually complete, the relevance of proteins in medical research is set to balloon, since the focus must now shift from raw DNA to the proteins encoded. This area of research is known as proteomics[1], a term which refers to the complex array of proteins required to sustain each different cell. At present, about 99% of all commercially produced drugs either function *via* interaction with proteins or are proteins themselves. As new targets are identified, the ability to rapidly synthesise a wide range of peptides and proteins will become increasingly important if we are to further explore areas such as DNA replication, hormone action or enzyme function. For example, the immunoglobulins must be understood if we are ever to produce synthetic antibodies for vaccines on demand. Technology for the chemical synthesis of peptides and proteins has a potentially huge part to play, especially where unusual or mutated sequences are required.

The chemical synthesis of proteins has already been applied to molecular biology in a variety of different ways, and has proved particularly useful for structural studies. An interesting example is the use of template assembled synthetic proteins (TASPs) to investigate certain aspects of tertiary structure[2,3,4,5] (Figure 1.1). TASPs may be considered as artificially constrained systems with locked tertiary folds, and are a means to bypass the folding problem of linear polypeptides. They provide model systems of reduced structural and functional complexity, which nevertheless allow fundamental questions related to the structure, function and assembly of proteins to be addressed.

Tuchscherer[6], Kent[7] and others have applied TASP methodology to the examination of the packing topology of the 4α-helical bundle, a structural motif which is found in many natural proteins. A combination of stepwise synthesis (Section 1.2) and chemical ligation methods (Section 1.3.3) was used to construct a TASP (Figure 1.1) which was then subjected to thermodynamic studies, in order to determine whether the alpha helices were packed in a parallel or an antiparallel fashion.

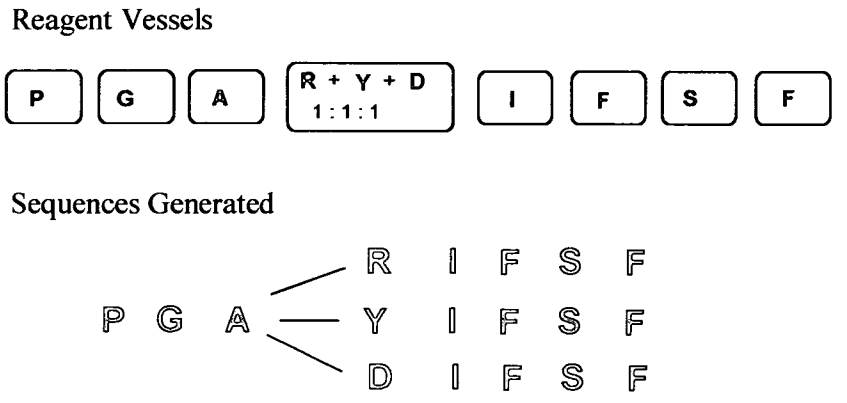
Figure 1.1 *Template Assembled Synthetic Protein – TASP*



A major strength of automated Solid Phase Peptide Synthesis (SPPS) is the ability to prepare alternative or unnatural sequences. The preparation of analogues provides the means to determine which parts of a sequence are important and helps to elucidate structure/function relationships. Protein folding mechanisms have been investigated in this way and peptide analogues have also been used to study small molecule binding, in the search for an understanding of drug/receptor interaction – an essential tool in rational drug design.

The substitution of one amino acid for another is a simple matter in automated syntheses, requiring only that the vessel containing the protected amino acid reagent be filled with the desired alternative. In biological syntheses, which are usually based on gene cloning and expression[8,9,10], the generation of such a point mutation is not such an elementary task. Although biological systems can be used to provide modified proteins[11,12,13], they are not really suitable for the production of the large numbers of systematically varied structures which are required for analogue based studies. In automated synthesis, however, peptide libraries[14] may easily be generated by filling the reagent vessel with an equally proportioned mixture of amino acids (Figure 1.2).

Figure 1.2 *The generation of peptide libraries*



Cyclic peptides have proven particularly useful as protein analogues, as work by Dutta and others has shown[15,16]. One of the problems associated with proteins as drug candidates is their bioavailablity – they are large molecules of disparate lipid/aqueous solubilities and as such are difficult to administer to the areas of the body where they are required. Cyclic peptides are much smaller and hence are more viable drug molecules. The cyclic peptide can be prepared in place of the larger molecule, and contains only the sequence of amino acids pertaining to the binding site. The cyclic nature of the molecule means that the binding region is relatively rigid and it can therefore mimic the topology it would have were it part of a larger peptide. A non-cyclic peptide has greater conformational freedom and is therefore less likely to bind in the desired way.

A further advantage of SPPS is that, once the synthesis of a particular sequence has been optimised, it may be more amenable to scale up than the, sometimes capricious, biological expression methods. Nevertheless some peptide sequences have proven extremely difficult to prepare by SPPS and biological expression may be the best, or only, means available for the preparation of these compounds[17,18,19]. Chemical synthesis also has some way to go before the synthesis of sequences greater than 200 residues in length becomes routinely possible. The most logical way forward in peptide and protein research is the use of the available methods in a complementary fashion, indeed there are already examples where large proteins have been assembled using a combination of biological expression, stepwise SPPS and fragment coupling techniques[20,21].

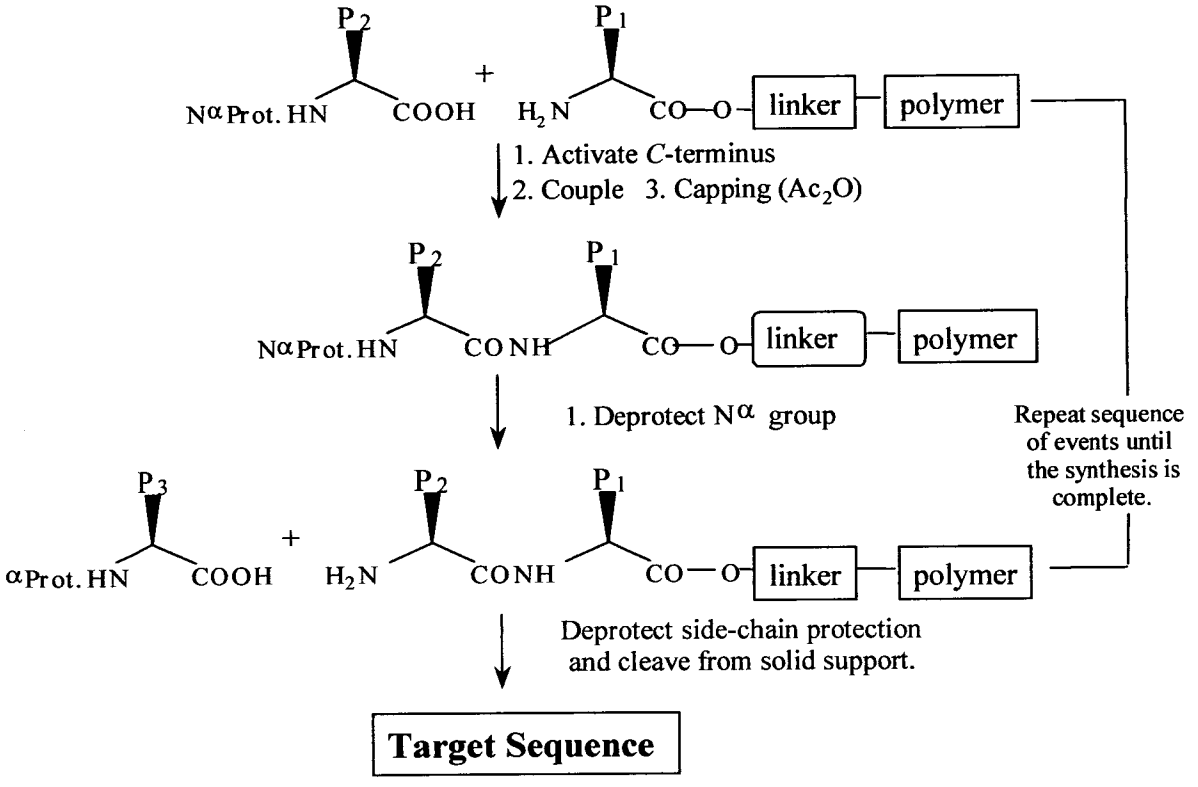
1.2 The Development and Basic Principles of SPPS

1.2.1 Background

The origins of chemical protein synthesis can be traced back to 1901, when Emil Fischer first synthesised a dipeptide in solution[22]. Other milestones in the field since then include the synthesis of insulin by Zahn[23], Katsoyannis[24], Kung[25] and Rittel[26], and the synthesis of ribonuclease A by Yajima and Fujii[27,28]. The synthesis of peptides in solution is fraught with problems, not the least of which is the cumulative loss of material, which means only relatively short peptides (< 10aa residues) may be prepared in acceptable yield. Impurities are likely to be very similar in chemical reactivity and structure to the desired product and are consequently difficult to separate. In order to ensure the formation of the correct material, any functional groups on the amino acid side chains must be blocked. Unfortunately, fully protected peptides are notoriously insoluble and are prone to aggregation and secondary structure formation, even in polar aprotic solvents such as dimethylformamide (DMF) or dimethylsulphoxide (DMSO). Epimerisation at the activated *C*-terminus must also be prevented.

The advent of Solid Phase Peptide Synthesis (SPPS)[29], originally conceived and also developed by Merrifield[30,31], has gone some way to solving these problems and peptide synthesis today is a useful and versatile technique. Peptides of 50-100 residues in length are now routinely prepared in good yield. In SPPS, the growing peptide chain is covalently anchored to a solid support. This allows an excess of reagents to be employed, thus maximising the yield of each reaction, but at the same time means that these reagents are easily removed from the peptide products by washing with appropriate solvents. The solid support also helps to combat solubility problems. The synthesis of a peptide, at its simplest level, involves repeated amide bond formation and is a relatively time consuming technique. SPPS methodology lends itself to automation, thus greatly simplifying the procedure and helping to eliminate human error. The general principles are outlined in the following diagram (Figure 1.3).

Figure 1.3 *Solid Phase Peptide Synthesis*



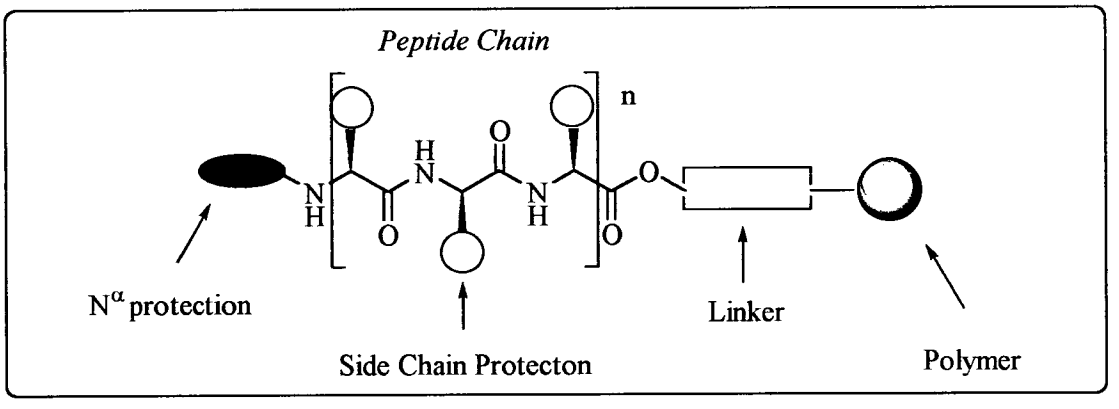
Px = Amino acid side chain + protecting group

There is a wide opportunity for reagent design in SPPS, with four main factors available for manipulation:

1 The Solid Support
2 Linker Technology
3 N^{α} & Side Chain Protection
4 Activation Methods

The following diagram (Figure 1.4) illustrates the key areas to be considered when synthesising a peptide or protein.

Figure 1.4 *Peptide Synthesis – areas for manipulation*



1.2.2 The Solid Support

Merrifield's original resin[31] was a cross-linked form of polystyrene containing reactive chloromethyl groups, onto which the carboxyl groups of the relevant amino acids were substituted. Following Merrifield, Sheppard designed a resin that would be more compatible with the growing polarity of the peptide chain, the beaded polyacrylamide resin[32]. Cross-linked polystyrene and polyacrylamide resins have been the most successful, but polyoxyethylene grafted onto polystyrene supports (Tentagel)[33,34] is becoming more important. The choice of resin depends on circumstances such as instrument design and the cost and reliability of each resin, as well as factors such as the physical stability of the peptide-loaded resin.

These resins all have certain common properties. They swell in organic solvents such as dichloromethane (DCM) and thereby allow reactants and solvents to permeate the macrostructure. This means that the necessary coupling and deprotection reactions can occur within the resin and excess reagents, together with soluble, unwanted products, can filter through the resin bound peptide. Since the desired product is "insoluble" as long as it is attached to the solid support, excess reagents may be used, making it easier for quantitative yields to be approached.

In summary, the essential features of a resin are that it should have reactive sites to attach, synthesise and remove the peptide in good yield but that these should be far enough apart that good contact between peptide/reagents may be achieved. It also must permit easy separation of the product from excess reagents and by-products, be stable to the various reaction conditions and minimise interactions between growing peptide chains.

1.2.3 Protecting Group Strategies

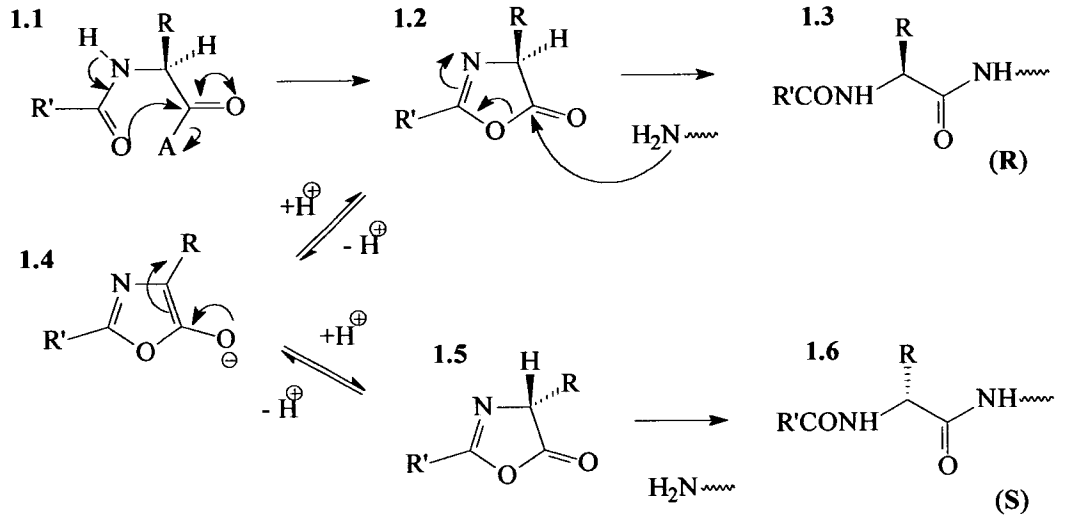
As previously mentioned (Figure 1.4), there are two main areas to consider when preparing amino acids for peptide synthesis – side chain protection and N^{α} protection.

The remit of side chain protection is that it must stay in place throughout the chain building steps to prevent undesired reactions involving side chain functionalies, and be easily removed when required, usually at the same time as the finished peptide is cleaved from the resin. Perhaps the most important consideration is that the side-chain protecting group must be stable to the conditions required for N^{α}deprotection. Side-chain protection can also be used to influence the chemistry of the peptide; this will be discussed further in later chapters.

The *N*-terminal deprotection conditions must be specific, and capable of giving quantitative release of the N^{α} amino groups. The failure of this deprotection would contaminate the crude product with residual protected peptide; this would eventually be deprotected in a subsequent step, leading to the formation of deletion peptides, *i.e.* peptides lacking certain amino acid residues. Deletion peptides may also form as a result of incomplete coupling reactions. This side reaction is avoided by the acetylation, or "capping", of unreacted amino groups after each coupling, thus preventing their participation in further reactions. The capping procedure leads to truncated sequences, which are as just as undesirable as deletion peptides but are easier to separate from the products.

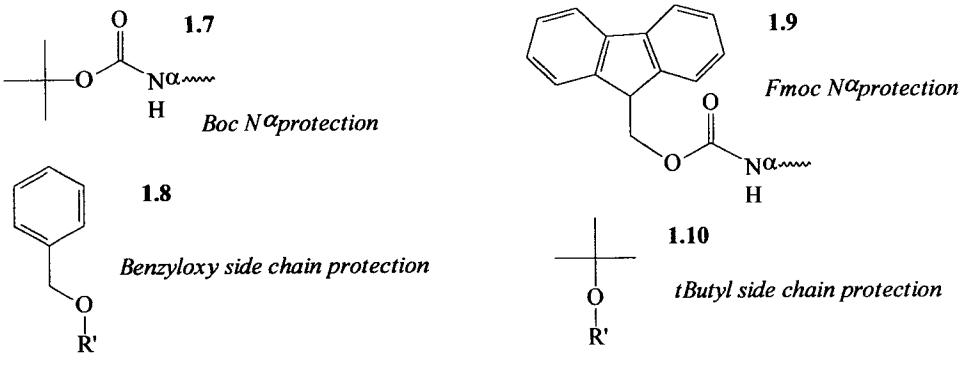
N-Terminal protecting groups fall into two broad areas – those that are acid labile, and those that are base labile. The main class of N^{α}-protecting groups used are the urethanes (oxycarbonyls), two of the most important being *t*-butoxycarbonyl (Boc) and 9-fluorenylmethyloxycarbonyl (Fmoc). These groups overcome the problems of racemisation which were inherent to the previously used acyl protecting groups, since they are slow to undergo intramolecular oxazolone formation (Figure 1.5).

Figure 1.5 *Oxazolone formation*



The Boc protecting group (Figure 1.6, **1.7**) is labile to cleavage in mild acid. The "Boc Strategy" was initially used by Merrifield[35] and utilises side chain protection that is relatively stable in mild acid, such as benzyloxycarbonyl groups, benzyl ethers and esters (**1.8**). The repeated N^{α} cleavage steps are carried out in TFA, without significant loss of side chain protection. Unfortunately, final side chain and peptide-resin cleavage requires the use of strong acid, typically HF, which can be detrimental to the peptide product. In contrast, the "Fmoc strategy", first introduced by Carpino[36,37] and applied independently by Meienhofer[38] and Sheppard[39], is a good deal more selective. The protecting group Fmoc (Figure1.6, **1.9**) is base labile. Piperidine is typically the base of choice for cleavage during synthesis, thus side chain protection which is labile under mild acidic conditions, for example the *t*Butyl ethers and esters (**1.10**), may be used. The improvements in linker technology over the recent decade have also helped improve the selectivity of this strategy.

Figure 1.6 *Boc and Fmoc*

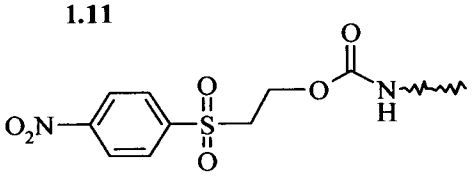


In practice, when cleaving the peptide, a scavenger cocktail is used to react with the carbocations which are produced as intermediates during the deprotection step and which would otherwise react in an undesirable manner with the reactive components of the side chains of cysteine, methionine, tryptophan and tyrosine.

There are other types of N^{α} protection in use, however these tend to be related to either Boc or Fmoc, in terms of their basic principles of deprotection and cleavage, and tend to be used only in unusual circumstances. 2-(4-Nitrophenylsulphonyl)ethoxycarbonyl (Nsc)[40,41], **1.11** (Figure 1.7), has recently been employed as an alternative to Fmoc, and is useful where increased polarity is required to improve the solubility of the protected peptide, or where racemisation of His and Cys residues has been a problem. The Nsc protecting group requires the use of 1,8-diazabicyclo[5.4.0]undecane (DBU), as well as piperidine, for cleavage. 17-tetrabenzo[*a,c,g*]fluorenylmethoxycarbonyl (Tbfmoc) has been used as *N*-terminal protection for the final amino acid of a synthesis[42,43], since its affinity for polystyrene can be exploited as an aid to purification. The Tbfmoc moiety, **1.12**, also shows fluorescent activity at 364nm and this property can be employed in experiments which involve peptide "tagging".

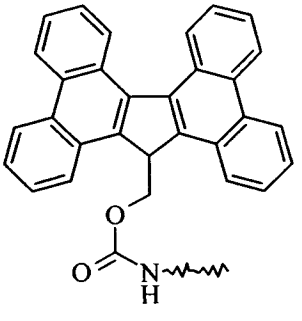
Figure 1.7 *Nsc and Tbfmoc*

1.11



Nsc N^{α} protection

1.12



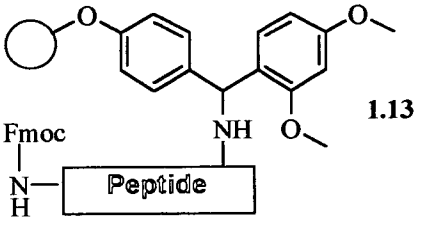
Tbfmoc N^{α} protection

1.2.4 Linker Technology

In the early days of solid phase peptide synthesis, the carboxyl terminus of the growing peptide chain was attached directly to the resin. Since then, however, groups known as linkers have been introduced, and are ever growing in complexity, since they are an extremely important variable in the synthesis.

The use of a linker permits fine-tuning of the peptide-resin bond, in that its lability may be altered to suit a particular synthetic strategy. The chemistry of a linker may also be designed to modify that of the peptide. For example, the Rink linker[44,45], **1.13** (Figure1.8), uses electron donation to stabilise the cation formed on cleavage, which reduces the risk of undesired side reactions. The linker may also be designed in order that the *C*-terminal functionality of the peptide can be adapted to specific requirements, for instance, the generation of *C*-terminal amides for use as neurotransmitters[46], or the preparation of *C*-terminal esters for use in convergent synthesis[47]. All of this chemistry takes place under relatively mild conditions which are non-destructive to the peptide chain.

Figure 1.8 *The Rink amide linker*



1.13

Acid labile, yields peptide amide

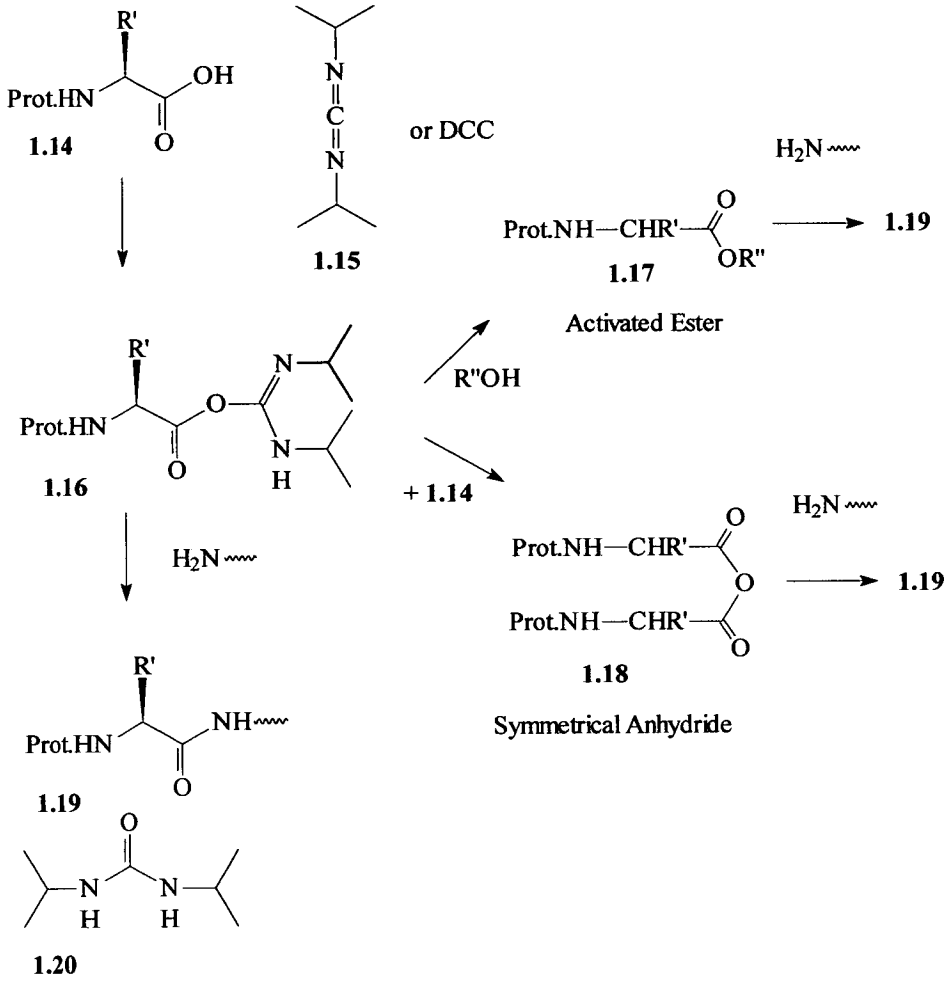
Linker technology has developed to the point where there are many different types available, for use in both Fmoc and Boc strategies. Linkers have also been designed which allow the peptide to be cleaved from the resin in different ways, such as hydrogenolysis[48] or photolysis[49].

1.2.5 Carboxyl Activation

With all the foundations in place, the peptide bond formation step must now be considered. The free carboxyl group must be activated in some way, such that it will react with the newly deprotected amino terminus of the growing chain. The selection of the activation method must take into account the solubility of the N^{α} amino acid derivative and the compatibility of the reagent with the N^{α} protected amino acid. Traditional carboxyl activation methods involve acid chlorides, but this method is not suitable for peptide synthesis due to the potential for *N*-carboxyanhydride formation

The most common carboxyl activation methods employ carbodiimides - *N,N*-dicyclohexylcarbodiimide (DCC) and *N,N*-diisopropylcarbodiimide (DIC), which has superseded DCC simply because the urea formed is more soluble in the preferred solvents. The initial carboxylic acid derivative is the *O*-acyl substituted urea, **1.16**. This active species can react directly with an amine to form an amide, **1.19**, with liberation of the urea, **1.20**, or with a variety of nucleophiles to produce more stable activated intermediates, such as active esters, **1.17,** or symmetrical anhydrides, **1.18** (Figure 1.9).

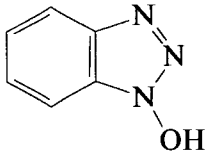
Figure 1.9 *Carboxyl Activation*



The symmetrical anhydride, **1.18** (Figure 1.9), is the most reactive of the activated N^{α}-protected amino acids and is formed from the reaction of two equivalents of the amino acid with one equivalent of DIC. This derivative undergoes rapid coupling, but the disadvantage is that only 50% of the N^{α}-protected amino acid is used. Since cycle times need to be kept as low as possible this is a favoured option, although expensive. The other advantages are that it is relatively easily prepared, and has unambiguous acylation properties. Mixed anhydrides have also been used[50], these are prepared using reagents such as isobutylchloroformate (IBCF) and diphenylphosphine (DPP). The latter derivative is preferred, since the IBCF method shows urethane formation.

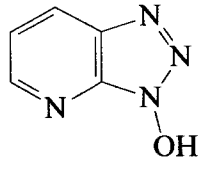
In the cases of asparagine and glutamine, which have side chain amide groups, the use of DIC alone for carboxyl activation may lead to impurities being formed as a result of side chain nitrile formation, since DIC can act as a dehydrating agent with amides. The use of *N*-hydroxybenzotriazole (HOBt)[51], **1.21** (Figure 1.10) as an additive avoids this by causing active ester formation, **1.17** (Figure 1.9). Such active esters are formed *in situ* from one equivalent of Fmoc amino acid, one of DIC and one of HOBt. Although the anhydrides give good yields, racemisation is still possible and HOBt was originally designed to minimise racemisation during SPPS. Analogues which improve upon this property have been developed - 1-hydroxy-7-azobenzotriazole (HOAt)[52], **1.22**, ethyl-1-hydroxy-1*H*-1,2,3-triazole-4-carboxylate (HOCt)[53], **1.23**. The use of these, as well as other triazole-based systems, has led to active esters been employed as highly effective coupling reagents.

Figure 1.10 *Coupling reagents*



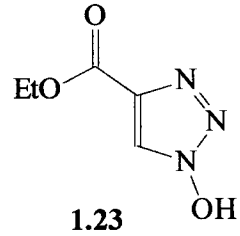
1.21

HOBt



1.22

HOAt



1.23

HOCt

1.2.6 The Solubility Problem

Solubility has been found to be a key factor in peptide synthesis. It is known that low solubility of the reacting fragment or fragments has a deleterious effect on coupling yield, and that the cause of these problems is the formation of intermolecular H-bonds, which lead to β-sheet formation. α-Helices and random coils are formed from intramolecular bonding, which acts to minimise inter-chain aggregation. In solution syntheses, low solubility leads to slow and incomplete coupling. In solid phase, reduced solvation restricts the access of the incoming activated amino acid and reduces coupling yield but, because the H-bonding effects are reversible, deletion peptides can also be formed. On the other hand, the presence of a solid support is acts to reduce the occurrence of aggregation and secondary structure formation.

When undertaking the synthesis of "difficult" sequences, some degree of predictability would be advantageous. To this end, Mutter and colleagues[54,55] studied the relationship between the primary sequences of peptides, preferred conformation and physicochemical properties such as aggregation, solubility and reactivity. A series of oligo and co-oligopeptides of between 9 and 20 residues in length were prepared by liquid phase synthesis using linear, soluble polyethylene glycol (PEG) as a support[56]. The PEG enhanced the solubility of the peptides, such that conformational studies could be carried out in a variety of solvents, but it did not alter the conformation of the bound peptide chains. The oligomers with a tendency to form helical or unordered structures showed no significant changes in solubility or coupling kinetics as the synthesis progressed. Those with the tendency to form β-sheets showed a marked decrease in solubility and reactivity when β-sheets began to form, usually about 5 residues into the synthesis. The PEG was found to increase the solubility of single strands, but could not disrupt fully aggregated structures. The insertion of a proline residue was found to increase solubility[57], since it was capable of disrupting intermolecular H-bonding by altering the dihedral angle of the peptide backbone (Figure 1.11). The β-structure was found to be

enhanced by conditions such as high concentration, high temperature and increased ionic strength.

Figure 1.11 *Dihedral Angles*

Amide link *Proline/imide link*

Further work by Narita *et al*[58] supported that of Mutter. Narita also derived a method to predict which sequences are likely to be soluble and hence suitable for solution-based condensations, based on the potential for random coil conformation, "Pc".

Methods to combat the solubility problem have, of course, been investigated. Concentration is a key factor and, in SPPS, the use of a resin with a low initial substitution is often beneficial. Microwave radiation and ultrasound have both been reported to increase the rate and yield of syntheses[59]. Solvent effects are also important, with the general finding being that polar solvents which are good proton donors or acceptors are of most use. Solvents such as DMF, DMSO, N-methylpyrrolidinone (NMP), hexafluoroisopropanol and hexamethylphosphoramide have all been used, and act to increase resin swelling as well as improving solvation of the peptide chain. The "Magic Mixture" was reported by Zhang *et al*[60] as "a powerful solvent for the solid phase synthesis of difficult sequences". It contains ethylene carbonate and non-ionic detergent, in order to disrupt secondary structure and inter-chain interactions; as well as polar solvents, which reduce viscosity. The use of inorganic salt additives during coupling has also been reported[61], as has the use of N^{α}-substitution to alter the structure of the peptide chain[62].

1.3 Convergent Peptide Synthesis – Fragment Coupling Strategies

1.3.1 Background

Stepwise SPPS has progressed to such an extent that it is now routinely possible to synthesise peptides of at least 50 residues in length with great efficiency. For peptides and proteins greater in length than 100 residues, the formation of impurities, however minor, becomes a problem. For example, the deletion of a single amino acid results in an impurity that has virtually identical physicochemical properties to the product and may never be able to be completely removed. During a lengthy synthesis, if coupling is ever less than quantitative, the final yield of peptide will be lowered, sometimes drastically. Although specific measures may be taken to circumvent these problems, a convergent approach to the synthesis of large peptides is often advantageous. In a convergent synthesis, the small intermediate peptides may be purified before coupling. Any impurities formed therefore differ by several amino acids and are considerably easier to remove from the final product.

In the early 1970's, the convergent synthesis of peptides[63] emerged as an alternative, or at least complementary, technique to stepwise SPPS. At first, convergent synthesis merely entailed the building of a peptide chain *via* the sequential coupling of fragments two to three amino acids in length. It has now progressed to the point where relatively large peptide chains, which may be up to 100 residues in length, are synthesised using stepwise SPPS or biological methods and are then coupled to produce proteins whose size renders conventional SPPS impractical. The technique has also been used to overcome "difficult" sequences - fragment coupling is used to get past the area of poor yield and the synthesis is then continued using stepwise SPPS.

The field of fragment coupling has expanded to the extent where there are several different strategies that may be considered when approaching a synthesis. The coupling reactions may be carried out either in solution or using solid phase methodology, in an analogous fashion to SPPS.

As in SPPS, both side-chain and *N*-terminal protecting groups must be considered. The protection scheme chosen is governed by the coupling method that is to be used, and this is in turn dependant on the specific properties of the peptide involved. There are several factors to be taken into account:

1 Solubility and secondary structure formation.
2 Fragment size, in terms of ease of synthesis and purification.
3 Frequency of potential coupling sites.
4 Direction of coupling desired (*C* to *N* or *N* to *C*).
5 Functionalisation of the *C*-terminal residue.

The coupling methods used in convergent synthesis fall into two broad areas, which have the following essential features:

Segment Coupling : non-regiospecific - requires full or minimal protection; organic solvents.
Chemical Ligation : regiospecific - no protection required; aqueous.

1.3.2 Segment Coupling

The term segment coupling refers to coupling reactions where the bond between peptide fragments is formed by methods which are non-regiospecific, that is the activation/coupling method is such that moieties other than the *C* or *N* termini could be affected. The protecting group strategy reflects this and the peptide fragments are either fully or partially protected. The coupling reactions are generally carried out in organic solvents. Another important feature is that racemisation can occur as an unwanted side reaction and measures must be taken to avoid this.

1.3.2.1 Fragment Condensation[64]

Fragment condensation was the first strategy to be considered for fragment coupling and was developed concurrently with SPPS. The fragments are coupled using a condensation reaction in the *N* to *C* direction, just as in conventional SPPS. The lack of selectivity in this coupling reaction requires that the peptides be fully protected. Specially designed protecting groups are not essential, the Fmoc/tBu or Boc/Bzl strategies can be employed.

The need for complete side chain protection raises two major issues. Firstly, side chain protection would normally be removed when the peptide fragments were cleaved from their respective resins - to prevent this, milder resin cleavage conditions must be employed. The second issue is that fully protected peptides are particularly insoluble. Solvent choice is restricted to DMF and DMSO, solvents which cannot be removed by lyophilisation. Additional reagents may also be required, to improve solubility. Anti aggregation agents, such as the chaotropic salts previously mentioned (Section 1.2.6), have been used for this purpose and another strategy has been to choose side chain protection that increases polarity - the picolyl protecting group[65] has been successfully applied in this way.

The synthesis of the fragments is normally carried out in a stepwise manner, using SPPS. The peptide is then detached from the resin in such a way as to leave both the N^{α} and side chain protection intact. The *C*-terminal of the segment is either the free carboxylic acid, or a derivative of this which is suitable for coupling. The *C*-terminal of the coupling site is usually chosen so that it contains Pro or Gly, in order to avoid epimerisation during coupling.

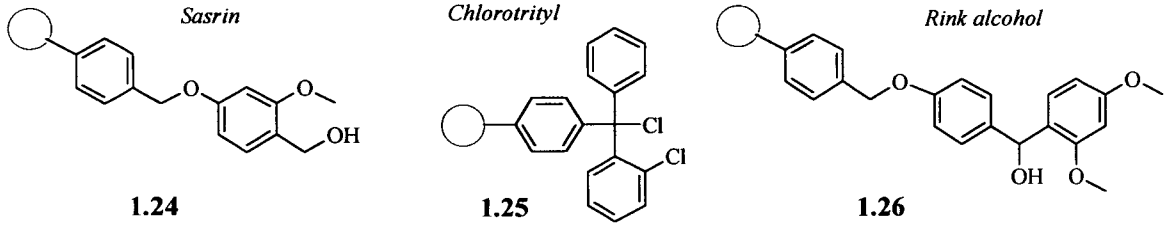
Although the condensation of peptide fragments may be carried out in solution, the procedure is subject to many of the difficulties encountered in solution-phase, stepwise peptide syntheses. It has therefore been transferred to solid phase chemistry for the same reasons – increased solubility, ease of purification and the opportunity to use excess reagents. One disadvantage of solid phase fragment condensation (SPFC)[64,65] is that the first peptide segment remains attached to the resin, together with any impurities formed. Fortunately, SPPS is efficient enough to mean that is not always a problem. However, if the matter should arise, multidetachable resins have been developed which permit the reattachment of a protected peptide to a resin[66,67]. Such a procedure enables the inclusion of a purification step, although the reattachment does not always proceed in good yield.

Linker Design

The main variable in this strategy is the linker, which must be carefully chosen to furnish a peptide with the correct side-chain protection or functional groups. The most widely used resin cleavage method is acidolysis. The Sasrin linker[68], **1.24**, was first proposed by Mergler. Cleavage is carried out using only 1%TFA in DCM, conditions which are compatible with Fmoc SPPS since they are sufficiently mild to permit retention of the acid-labile side chain protecting groups. This resin is also suitable for use in conjunction with Boc N^{α}-protection.

The chlorotrityl linker[69], **1.25**, developed by Barlos, is highly acid labile and is thus compatible with Fmoc/tBu protection; cleavage is carried out in a mixture of AcOH/TFE/DCM for 1-2hrs. The resin below, **1.26** (Figure 1.12), was used by Rink[44] and is cleaved using 0.2%TFA in DCM for 3-5min. However, this resin proved to be so acid labile that premature cleavage was found to occur if HOBt was used during coupling.

Figure 1.12 *Linkers*



1.24 **1.25** **1.26**

Cleavage conditions need not be acidic. Base cleavable linkers are available, but these are only compatible with the Boc/Bzl strategy. Phenacyl resins are cleaved by photolysis[70], while hydrogenolysis has been used to detach protected segments from the o-nitrobenzoyl PEG support[49]. Ammonolysis[71] and hydrazinolysis[72] have also been used; these methods give rise to peptide amides and peptide hydrazides, respectively, as opposed to the free peptide acids.

The Coupling Reaction

The coupling reaction itself is carried out *via* the activated *C*-terminal ester, using the same type of chemistry as stepwise SPPS, and reagents have evolved concurrently. Early procedures involved oxidation-reduction and the azide method (Section 1.3.2.2). These were superseded by DCC/DIC; or DCC/DIC in combination with HOBt, HOCt or *N*-hydroxysuccinamide (HOSu), as discussed in the previous section.

1.3.2.2 The Azide Method[73,74]

Azide coupling was first used for the formation of peptide bonds by Curtius, in the 1900's[75,76]. It was later employed by Merrifield[31] and others as a coupling method in SPPS, using the amino acid Boc-hydrazides developed by Honzl and Rudinger[77]. The significance of this method was that the azide was contained on the solid support and the amino component was in solution, such that the peptide was assembled in an *N* to *C* fashion, in the opposite direction to conventional SPPS. This approach eliminated the possibility of racemisation. However, the coupling reaction could not be carried out at room temperature and the overall yields were to low for the method to be a replacement for DCC-based fragment condensation in SPPS.

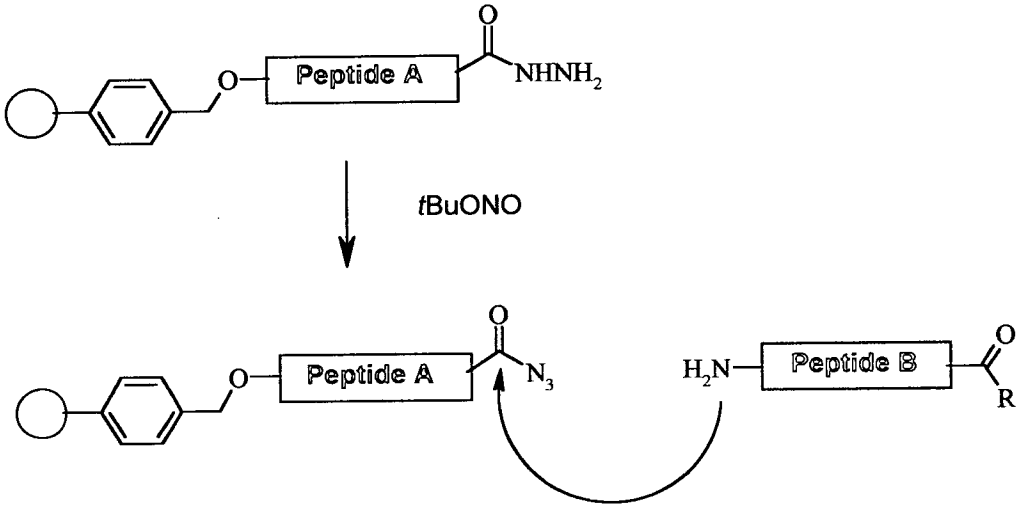
The azide coupling reaction has since been applied to other areas of peptide synthesis, notably to the preparation of cyclic peptides[78,79,80] and to polymerisation reactions[81,82] as well as its use within the area of peptide segment coupling.

As previously discussed, fully protected peptides are notoriously insoluble. Minimal side chain protection may be used when the coupling reaction exhibits some selectivity, and this considerably lessens fragment solubility problems. This is the case in the azide method, where the activation step is selective. However, the nucleophilic side chains of Cys and Lys, as well as the *N*-terminus of the first fragment, must be protected in order to prevent cross-linking or, in the latter case, cyclisation. A major strength of the azide method is that it minimises racemisation. In addition, the required hydrazide starting materials are relatively easy to prepare. However, in practice there are drawbacks. These are mainly due to the instability of the acyl azide itself, which compromises peptide solubility and restricts solvent choice. The coupling must be carried out at a low temperature, it is difficult to monitor and is also relatively slow and low yielding.

The Coupling Reaction

In azide coupling (Figure 1.13), the azide is not introduced directly, but instead is generated *in situ*, usually from the peptide hydrazide, which then undergoes nucleophilic attack by the free amino terminus of the second fragment. Azide formation takes place in acidic conditions, generally using acetic acid, TFA or hydrochloric acid, in DMF, IPA or THF. Sodium nitrite, or an organic nitrite such as *t*-butyl nitrite, butyl nitrite or isoamyl nitrite, is added to generate the azide, which forms rapidly (1-4hrs).

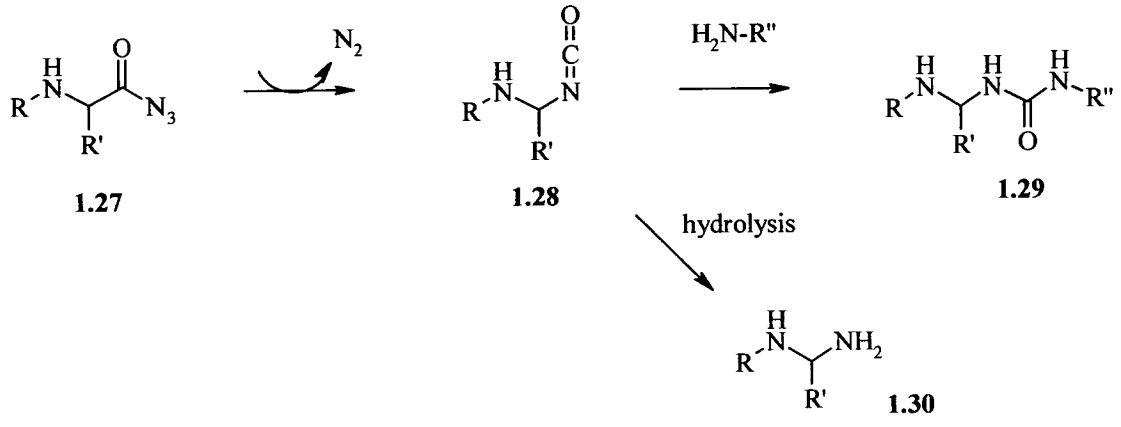
Figure 1.13 *Azide coupling*



Azide Coupling -key step

Recognised techniques for azide formation include the Honzl-Rudinger[77] and the Medzihradsky[83] methods. Potential side reactions include isocyanate formation, **1.28**, which occurs *via* the Curtius rearrangement (Figure1.14). This rearrangement is disfavoured at low temperature and, ideally, the azide should be generated at –30°C, while the coupling should be carried out between –10 and 4°C. Reactions generally take 1-4 days to complete - the rate-determining step is the coupling reaction.

Figure 1.14 *The Curtius rearrangement*

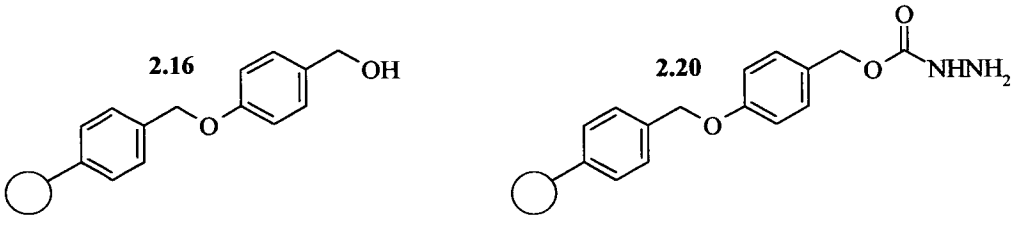


The issue of racemisation is of fundamental importance in peptide synthesis, since optically impure peptides show reduced biological activity. The azide method was initially thought to be racemisation free, but was later proven to show some degree of racemisation[84], albeit to a very small extent under conventional conditions (<1%)[85] and certainly less than that exhibited in most other coupling methods. Techniques are available to suppress the racemisation *via* oxazolone formation which is encountered when using traditional coupling methods, but the mechanism of azide racemisation is not fully understood.

Linker Design - The Preparation of Hydrazides

The peptide hydrazides required for azide coupling were generally prepared by the hydrazinolysis of the peptide-resin bond, as demonstrated by Bodansky and Sheehan[86]. Both benzyl ester and alkyl ester linkages have been successfully hydrazinolysed. Wang and Merrifield[87,88] later introduced resins designed to yield the peptide hydrazide directly, following acidolytic cleavage. *t*-Alkoxycarbonyl hydrazide and *p*-alkoxybenzyloxycarbonylhydrazide resins (Figure 1.15) were prepared. The latter resin was applied as part of this project and is further discussed in Section 2.4.8. A modified form of the tricyclic amide linker, developed by Ramage and Irving[89], was previously employed by the Ramage group for the preparation of peptide hydrazides[90].

Figure 1.15 *Hydrazide resins*



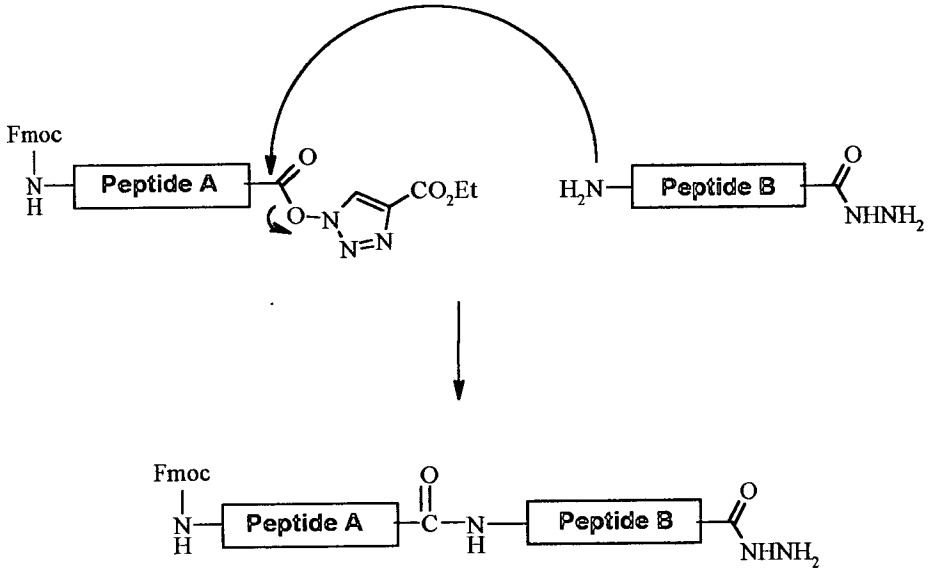
p-alkoxybenzyl alcohol (Wang) resin

p-alkoxybenzyloxycarbonylhydrazide resin

1.3.2.3 Transfer Active Ester Condensation

Transfer Active Ester Condensation (TAEC) is based on the same principles as the azide method, however the azide is trapped as its HOCt active ester prior to coupling (Figure 1.16). The TAEC technique was developed by the Ramage group[90]. It has been investigated and applied as part of this project and will therefore be discussed in detail in later chapters.

Figure 1.16 *Transfer Active Ester Condensation – key step*



1.3.3 Chemical Ligation

The term "chemical ligation" refers to the coupling of two peptide fragments *via* two mutually reactive functional groups, in a chemoselective manner. This technique represents a significant departure from the other coupling strategies discussed. The coupling reaction is specific, only the *N*- and *C*- termini can participate and so unprotected peptides may be used, either in solution or attached to a solid support. Solution phase reactions are rendered less problematic due to the increased aqueous solubility of the unprotected peptides, indeed the coupling reactions are generally carried out in water. Where a solid support is employed, the peptide must be reattached to the resin after cleavage and purification – linkers have been developed which facilitate such procedures[66,67].

Since side chain protection is not required, the peptide fragments may be obtained using biological as well as chemical syntheses. So-called "semi-synthetic" peptides have been obtained using a combination of both techniques, as illustrated by the work of Muir and Evans in the area of expressed protein ligation[20,21]. In terms of SPPS, the strategy chosen largely depends on the methods chosen for the preparation of the relevant *C*-terminal esters.

Significantly, unprotected peptides are unsuitable for sequential ligation, since they are open to the possibility of cyclisation. Muir has developed the methylsulphonylethyloxycarbonyl (Msc)[91] protecting group for the temporary protection of *N*-terminal cysteine, while Kent has used the acetamidomethyl (Acm)[92] group for this purpose. Photolabile or reduction sensitive protecting groups are also suitable, since they are compatible with the Boc strategy and can be removed under mild, aqueous conditions.

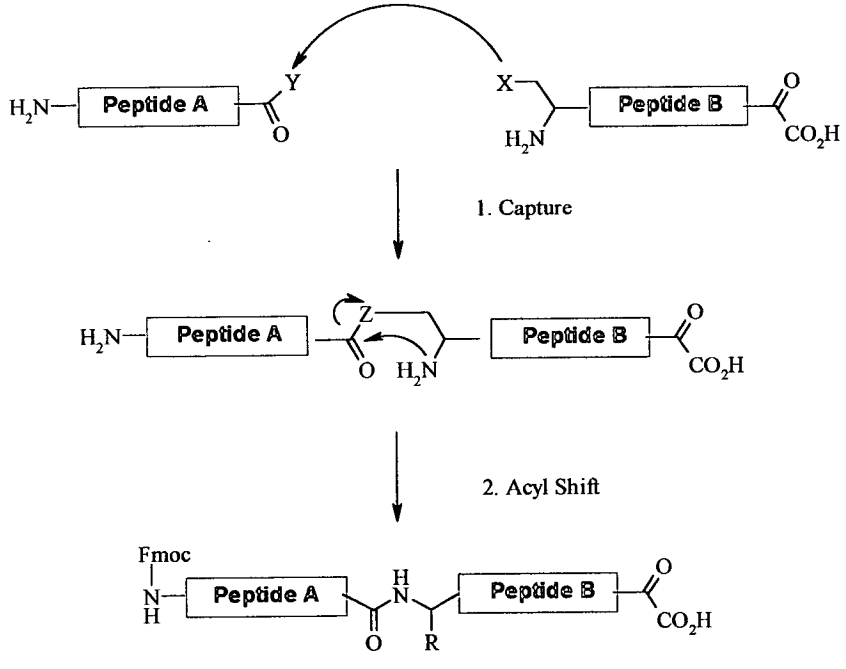
In some cases ligation results in a native amide bond, in others a non-amide link, such as a thioester[93], is formed. The native amide link would, intuitively, seem the most favourable outcome, since the use of alternative backbone linkages in key locations may drastically alter the folding properties of the peptide. Some peptides prepared with alternative backbones have shown normal biological activity, however, and such analogues are valuable tools in the investigation of peptide structure or ligand-binding activity. As well as the thioester, peptides including thioether[94,95], hydrazone[96,97] and oxime[98] linkages have been successfully synthesised. Nevertheless, as a general peptide ligation strategy, this is far from ideal and only ligation methods which result in a native amide link will be further discussed. These ligations work by the common principle of an initial, rate determining, step which brings the reacting termini into close proximity, followed by a second reaction where the amide bond is formed in an intramolecular fashion.

1.3.3.1 Orthogonal Ligation

The term orthogonal ligation, coined by Tam[99], refers to ligation methods which are chemoselective and which lead to a native amide bond *via* the two-step process of intermolecular chemoselective capture to form a covalent bond, followed by an intra molecular acyl shift (Figure 1.17). This procedure has also been referred to as chemoselective ligation, or native chemical ligation, although the latter term is more specifically associated with the work of Kent[100] in the area of Cys-thioester ligation.

Generally speaking, four functional groups are clustered in the reaction centre – amine, ester, nucleophile and electrophile. One nucleophile or electrophile, located at the amino acid side chain, is placed close in space to the reacting *N*-terminal amine of one segment, and the complementary member of the reacting pair is placed close to the acylating *C*-terminal ester of another segment.

Figure 1.17 *Chemical ligation – general*



Native Amide Link

X, Y = nucleophile/electrophile pair; Z = bond of capture intermediate; R = amino acid side chain.

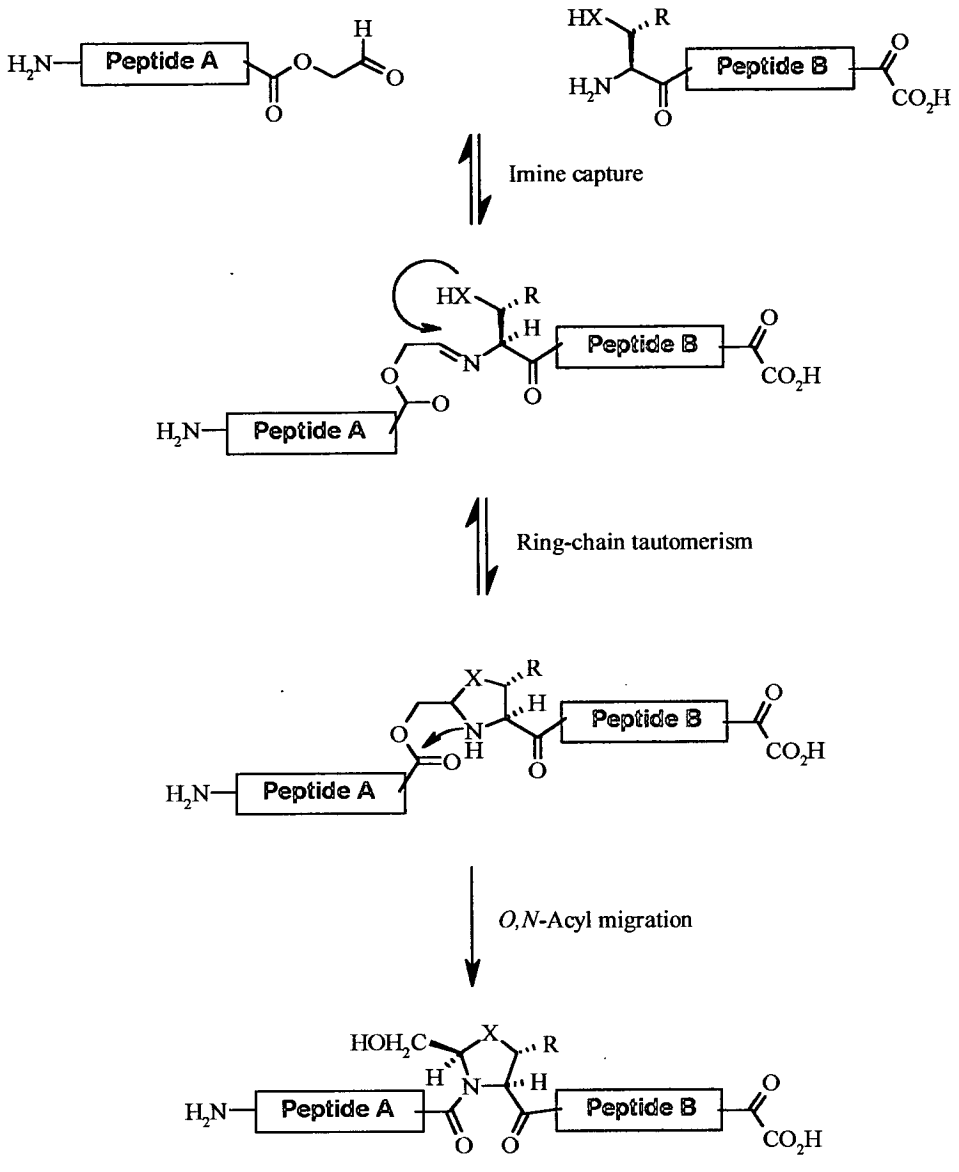
In segment coupling the main source of chemical complexity is the protecting group strategy. This is not an issue in orthogonal ligation. Instead, the main variable to be considered is the choice of coupling site, since only particular amino acids may be activated to form moieties suitable for coupling. Fragments are generally chosen based on the location of these amino acids, especially as the size of the fragments is less of a factor due to the increased solubility of unprotected peptides. The most successful strategies use *N*-terminal cysteine, but this is somewhat restrictive since not all peptides have these groups conveniently spaced, or indeed contain any cysteine residues. In consequence, the method has been extended to allow a wider range of amino acids to participate. This may mean varying the coupling chemistry for successive fragments, but should permit fragment choice to reflect the ease with which the coupling segments may be synthesised. In general, all ligations share the central concept of acyl transfer, but the method used in the initial capture step is subject to variation.

Imine Ligation

In imine ligation the initial capture step proceeds *via* an imine intermediate, which results from the reaction of an acyl aldehyde with an *N*-terminal amine. The imine then undergoes rapid ring/chain tautomerism. The resulting heterocycle facilitates the acyl transfer of the ester intermediate, leading to a proline-like imidic bond (Figure 1.18).

N-terminal Cys, Ser and Thr containing peptides have all been used in this procedure. Cys forms a thiaproline[101] intermediate, while Ser and Thr both proceed *via* the oxaproline[102]. The thiazolidine formation step is particularly rapid (5-15 min); such high reactivity means the reaction is highly regiospecific. Only weakly basic nucleophiles are used. Imine ligation requires aqueous acidic conditions and as a result the strongly basic Lys and Arg side chains are protonated – they are therefore excluded as nucleophiles and do not require side-chain protection. The acyl capture reaction initially forms a Schiff base, which would normally be unstable and reversible under aqueous conditions. The reaction with the second nucleophile effectively traps the Schiff base to form the more stable, heterocyclic system.

Figure 1.18 *Imine ligation*



Thiaproline ligation: X=S, R=H; Oxaproline ligation: X=O, R=H or R=CH_3

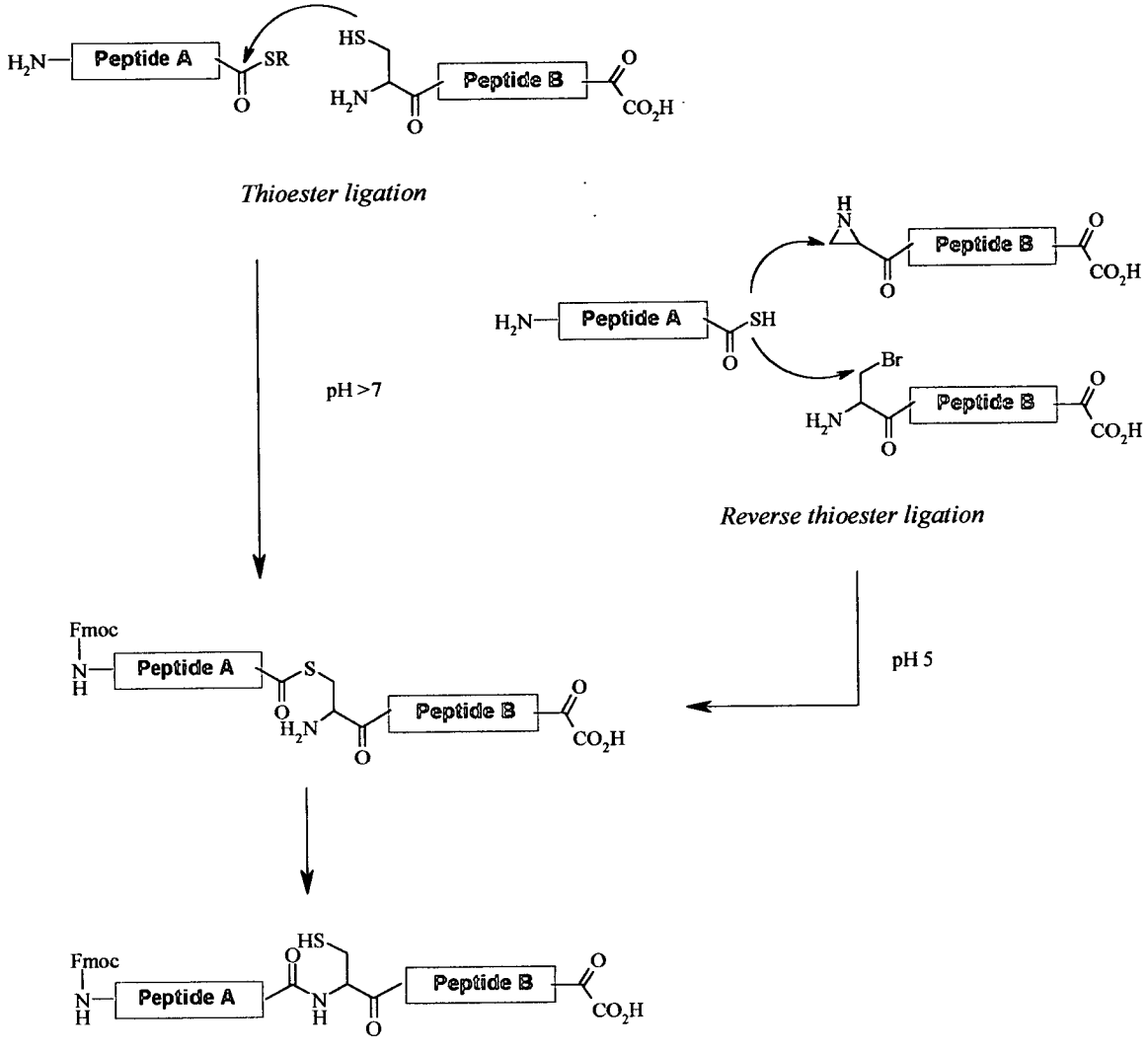
Thioester Ligation

The capture step in thioester ligation is centred around the reaction of a thiol with a *C*-terminal thioester, leading to a second thioester *via* transesterification. When a suitable *N*-terminal side chain nucleophile is present, the thioester linkage forming a covalent intermediate spontaneously undergoes an *S* to *N* acyl migration to form a native amide bond, through a five membered ring intermediate (Figure 1.18). Reverse thioester ligation, where the initial capture step proceeds in the opposite direction, is also possible[103] (Figure 1.19).

The thioester reaction involving cysteine was first reported by Wieland in 1953[104], but only began to be applied to segment coupling in the early 1990s. Since then, a great deal of work has been carried out in this area, notably by Tam[99], Kent[100], Muir[20] and Evans[21]. More recently, the methodology has been extended to other amino acids - His[105], Met[106]and even Gly[107] have been used. All are weak base nucleophiles and are separated from the coupling site by at least two carbons.

The Cys-thioester ligation reaction is the most well known and also the most effective for coupling. Its success lies in the fact that it is a ready-made nucleophile/electrophile pair, with a favourable spatial arrangement, which leads to a stable 5-membered intermediate. The reaction is usually carried out in aqueous conditions, buffered at pH 7-8; at this pH the thiol is more reactive than the amino side chains. Acidic conditions are used for the reverse thioester coupling and this process is therefore useful where base sensitive sequences are involved - for example, analogues of HIV-protease were prepared using the reverse thioester method[108]. Generally speaking, non-denaturing conditions work best, as do unhindered *C*-terminal thioesters. Most importantly, a reducing environment must be maintained in order to avoid the formation of disulphides or bis-acylated products. Hydrolysis of the thioester is also possible but, at basic pH, this is usually too slow to compete with the more rapid ligation reaction.

Figure 1.19 *Thioester ligation*



The methionine ligation[106] technique uses the unmasked homocysteine as the *N*-terminal nucleophile. The reaction proceeds in an analogous manner to Cys ligation, the only difference being that a methylation step is required to regenerate the Met side chain, after ligation has taken place. Canne *et al*[107] have used Gly as the *N*-terminal nucleophile, to form an *N*-oxyalkyl product, which is reduced after purification using zinc dust in acid. The capture step is still rapid and regiospecific, but the rearrangement proceeds much more slowly, via a less favoured 6-membered ring.

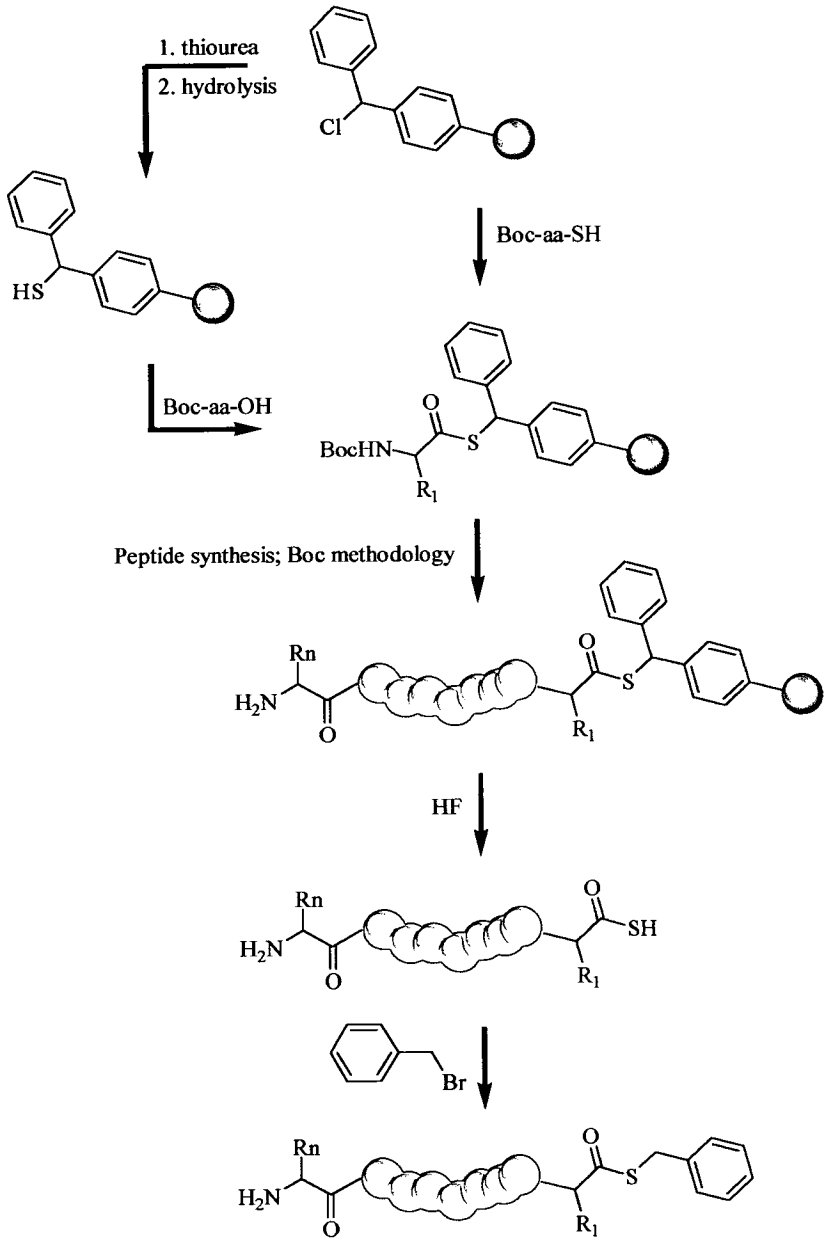
Histidine ligation[105] is an attempt to transfer thioester ligation to non-cysteinyl *N*-terminal amino acids. The imidazole is exploited as a weak base in order to catalyse the acyl transfer, but the *C*-terminal ester requires activation before capture will occur. An acyl segment containing a *C*-terminal thioacid is used and this is activated using a thiophilic promoter, such as Ellmans reagent (dinitrothiobenzoic acid (DNTB))[109].

Linker Design

The preparation of the *C*-terminal electrophile segments is an important area in chemical ligation. If SPPS is to be used, linkers must be designed to provide peptides with the correct *C*-terminal functionality. The versatility of linker design means there are several approaches available for the preparation of peptide esters, both direct and indirect.

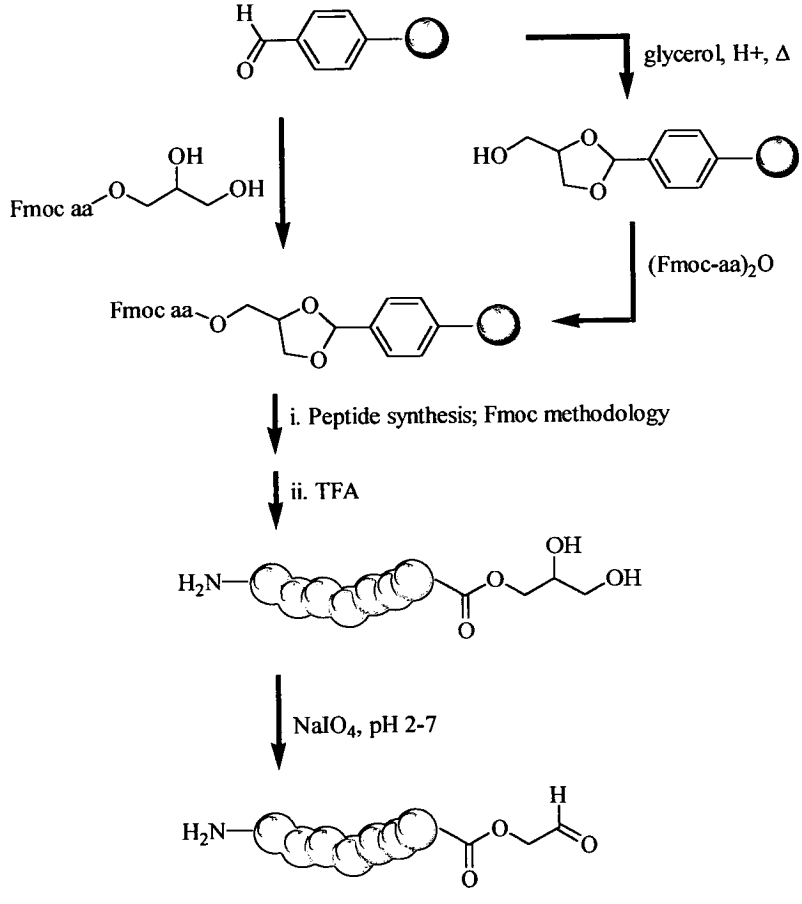
An indirect method, reported by Dawson *et al*[110] (Figure 1.20), uses the Boc strategy for SPPS in conjunction with mercaptobenzhydryl resin[111,112]. HF cleavage liberates the *C*-terminal thioacids, which are then esterified in solution *via* reaction with alkyl halides or symmetrical disulfides. For direct preparation, the alkyl thioester resin, designed by Hojo and Aimoto[47] may be used. This resin is also compatible with the Boc strategy, and uses HF cleavage. The Fmoc strategy is generally avoided for thioester preparation, since the linkers designd to yield thioesters are adversely affected by the repeated exposure to piperidine during N^{α} deprotection steps. Direct preparation of thioesters is advantageous since thioacids are relatively vulnerable to HF and are susceptible to hydrolysis and side-product formation during purification. However, such indirect preparation does allow *C*-terminal reactivity to be varied without the need for the repetition of the initial synthesis.

Figure 1.20 *Dawson protocol for the synthesis of peptide thioesters*



Peptide glycoaldehydes for imine ligation may be prepared in solution from their corresponding peptide esters[113,114] or thioesters[115], or prepared directly using functionalised resins[116] (Figure 1.21). The glyceric ester resin[117] leads to a diol precursor which is oxidised prior to coupling. In semi-synthesis enzymes which produce peptide esters are used[118]. *C*-terminal esters are intermediates in protein splicing and mutant enzymes can be used to trap these products.

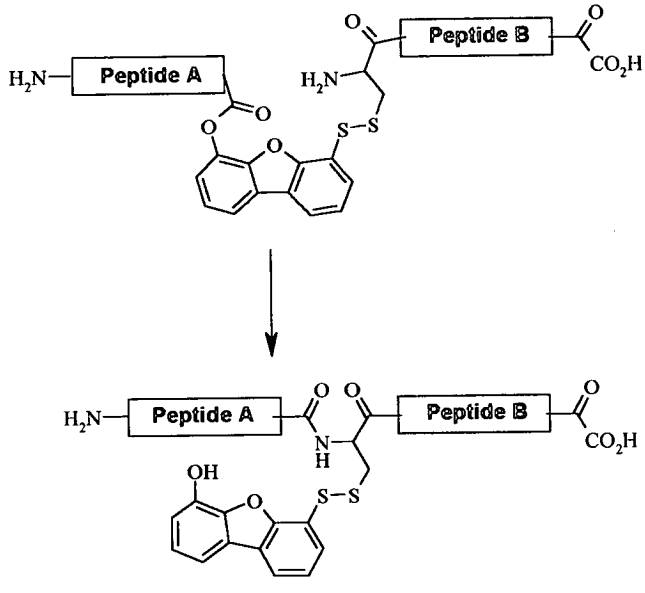
Figure 1.21 *The synthesis of peptide glycoaldehydes*



1.3.3.2 Entropic Activation

The principle of entropic activation has been applied in bioorganic strategies[119], where fragment complementarity has allowed the formation of a stable non-covalent intermediate. A proximity driven acylation, mediated either chemically or enzymatically then takes place. Such sequence complementarity is, however, hard to predict and organic templates have been used to provide more control. Kemp and co workers carried out a version of this method, known as "prior thiol capture"[120,121] (Figure 1.22), which used a tricyclic template to capture the *C*- and *N*-termini *via* disulfide bond formation. A intramolecular acyl migration then ensues, followed by reduction to regenerate the thiol.

Figure 1.22 *"Prior thiol capture"*



Prior Thiol Capture -key intermdiates

1.3.3.3 Enzymatic Coupling

Enzymes have been used to couple unprotected synthetic peptide fragments and this strategy is also compatible with minimal side chain protection. Successful couplings have been achieved using synthetic ligases, based on mutated subtilisins[122] and antibodies designed to catalyse amide bond formation have also been investigated[123].

1.4 Conclusions

Since its inception, SPPS has progressed far beyond what could have been imagined and the threshold of size is ever increasing. By comparison, the field of convergent peptide synthesis is in its infancy, however there is no reason why, one day, the two strategies might not be effortlessly combined, or even automated. This could lead to bulk preparation of large proteins, or even peptide libraries based on segments rather than single amino acids. The huge advances in molecular biology, as well as the increasing interest in proteomics means that demand for such reliable techniques is growing. With such discoveries taking place, the difficulties involved in the synthesis of large proteins on demand may soon pale in comparison to the new challenges that are about to be encountered.

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[1] Lawn, R.M., Lasky, L.A., *Curr.Opin Biotechnol.*, 2000, **11**, 579.
[2] Mutter, M., Tuchscherer, G., *Makromol.Chem. Rapid Commun.* 1988, **9**, 437.
[3] Mutter M., *Trends Biochem Sci.* 1988, **13**, 261.
[4] Dumy, P., Eggleston I.M., Esposito G., Nicula S., Mutter M., *Biopolymers*, 1996, **39**, 297.
[5] Dumy, P., Eggleston, I.M., Cervigni, S., Sila, U., Sun, X., Mutter, M., *Tetrahedron Lett.*, 1995, **36**, 1255.
[6] Tuchscherer, G., Grell, D., Mathieu, M., Mutter M., *J. Peptide Res.*, 1999, **54**, 185.
[7] Dawson, P.E., Kent, S.B.H., *J. Am. Chem. Soc.*, 1993, **115**, 7263.
[8] Smith, M., *Ann. Rev. Genet.*, 1985, **19**, 423.
[9] Carter, P., *Biochem. J.*, 1986, **237**, 1.
[10] Wells, J.A., Vasser, M., Powers D.B., *Gene*, 1985, **34**, 315.
[11] Noren, C.J., Anthony-Cahill S.J., Griffith M.C., Schultz P.G., *Science*, 1989, **244**, 182.
[12] Bain, J.D., Diala, E.S., Glabe, C.G., Wacker, D.A., Lyttle, M.H., Dix, T.A., Chamberlain A.R., *Biochemistry*, 1991, **30**, 5411.

[13] Mendel, D., Ellman, J.A., Chang, Z., Veenstra, D.L., Kollman, P.A., Schultz P.G., *Science*, 1992, **256**, 1798.
[14] Ramage, R., Swenson, H.R., Shaw, K.T., *Tetrahedron Lett.*, 1998, **39**, 8715.
[15] Dutta, A.S., *Adv. Drug Res.*, 1991, **21**, 145.
[16] Dutta, A.S., *Proc.10th RSC-SCI Med.Chem.Symp.*, 1999.
[17] Merrifield, R.B., in *Recent Progress in Hormone Research,* Pincus, G. (ed.), Academic Press New York, **23**, 451.
[18] Hancock, W.S., Prescott, D.J., Vagelos, P.R., Marshall, G.R., *J.Org.Chem.*, 1973, **38**, 774.
[19] Deber, C.M., Lutek, M.K., Heimer, E.P., Felix, A.M., *J.Pept.Res.*, 1989, **2**, 184.
[20] Ayers, B., Blaschke, U.K., Cameraro, J.A., Cotton, G.J., Holford, M., Muir, T.W., *Biopolymers*, 1999, **51**, 343.
[21] Evans, T.C. jnr., Benner, J., Xu M.-Q., *Protein Sci.*, 1998, **7**, 2256.
[22] Fischer, E., Fourneau, E., *Ber. Dtsch. Chem. Ges.*, 1901, **34**, 2868.
[23] Meienhofer, J., Schnabel, E., Bremer, H., Brinkoff, O., Zabel, R., Sroka, W., Klostermeyer, H, Brandenburg, D., Okuda, T., Zahn, H., *Z. Naturforsch*, 1963, **18b**, 1120.
[24] Katsoyannis, P. G., Fukuda, K., Tometsko, A., Suzuki, K., Tilak, M., *J. Am.Chem. Soc,* 1964, **86**, 930.
[25] Kung, Y.-T., Du, Y.-C., Huang W.-T., Chen, C.-C., Ke, L.-T., Hu, S.-C., Jiang R.-Q., Chu S.-Q., Niu, C.-I., Hsu, J.-Z., Chang, W.-C., Cheng, L.-L., Li, H.-S., Wang, Y., Loh, T.-P., Chi, A.-H., Li, C.-H., Shi, P.-T., Yieh, Y.-H., Tang, K.-L., Hsing, C.-Y., *Scientia Sinica*, 1965, **14**, 1710.
[26] Sieber, P., Kamber, B., Hartmann, A., Johl, A., Riiker, B., Rittel, W., *Helv. Chim. Acta.*, 1974, **57**, 2617.
[27] Yajima, H., Fujii, N., *Biopolymers*, 1981, **20**, 1859.
[28] Yajima, H., Fujii, N., *J. Chem. Soc. Chem. Commun.*, 1980, 115.
[29] Merrifield, R.B., in *Peptides-Synthesis, Structure and Applications*, Gutte, B. (ed.), Academic Press Inc., 1995, 93.
[30] Merrifield R.B., *J. Am. Chem. Soc.*, 1963, **85**, 2149.
[31] Merrifield R.B., *Science*, 1986, **232**, 341.
[32] Arshady R., Atherton E., Clive D.L.J. and Sheppard R.C., *J.C.S. Perkin Trans. I*, 1981, 529.
[33] Bayer, E., Dengler, M., Hemmasi, B., *Int.J.Pept.Prot.Res.*, 1985, **25**, 178.
[34] Rapp, W., Zhang L., Haebich, R., Bayer, E., *Proc.20th Eur.Pept.Symp.*, 1989, 199.
[35] Merrifield R.B., *Biochemistry*, 1964, **3**, 1385.
[36] Carpino E.A., Han G.Y., *J. Am. Chem. Soc.*, 1970, **92**, 5748.
[37] Carpino E.A., Han G.Y., *J. Org. Chem.*, 1972, **37**, 3404.
[38] Chang, C.D., Meienhofer, J., *Int.J.Pept.Prot.Res.*, 1978, **11**, 246.
[39] Atherton E., Harkiss D. and Sheppard R.C., *Bioorganic Chem.*, 1979, **8**, 351.
[40] Verhart, C.G.I., Tesser, G.I., *Recl. Trav. Chim. Pav. B.*, 1988, **107**, 621.
[41] Samukov, V.V., Sabirov, A.N., Pozdnyakov, P.I., *Tetrahedron Lett.*, 1994, **35**, 7821.
[42] Ramage R., Raphy G., *Tetrahedron Lett.*, 1992, **33**, 385.
[43] Brown A.R., Irving S.L., Ramage R., *Tetrahedron Lett.*, 1993, **34**, 7129.
[44] Rink, H., *Tetrahedron Lett.*, 1987, **38**, 3787.
[45] Bernatowicz, M.S., Daniels, S.B., Koster H., *Tetrahedron Lett.*, 1989, **30**, 4645.
[46] Noda, K., Gazis, D., Gross, E., *Int.J.Pept.Prot.Res.*, 1983, **19**, 413.
[47] Hojo, H., Aimoto, S., *Bull. Chem. Soc. Jpn.*, 1991, **64**, 111.
[48] Tjoeng, F.S., Hodges, R.S., *Tetrahedron Lett.*, 1979, 1273.
[49] Rich, D.H., Gurwara, S.K., *J.Am. Chem. Soc.*, 1975, **97**, 1575.
[50] Sole, N., Torres, J.L., Garcia Anton, J.M., Valencia, G., Reig, F., *Tetrahedron*, 1986, **42**, 193.
[51] Windridge, G., Jorgensen, E.C., *J.Am.Chem.Soc.*, 1971, **93**, 6318.
[52] Carpino, L., *J.Am.Chem.Soc.*, 1993, **115**, 4397.
[53] Jiang L, Davison, A., Tennant, G., Ramage, R., *Tetrahedron*, 1998, **54**, 14233.
[54] El Rahman, S.A., Anzinger, H., Mutter, M., *Biopolymers*, 1980, **19**, 173.
[55] Pilai, V.N.R., Mutter, M., *Acc. Chem. Res.*, 1981, **14**, 122.

[56] Toniolo, C., Bonora, G.M., Mutter, M., *J.Am.Chem.Soc.*, 1979, **101**, 811.
[57] Narita, M., Fuknaga, T., Wakabayashi, A., Ishikawa, K., Nakana, H., *Int. J. Pept. Prot. Res* , 1984, **23**, 306.
[58] Narita, M., Ishikawa, K., Chen, J-Y., Kim, Y., *Int. J. Pept. Prot. Res.*, 1984, **24**, 580.
[59] Blaha, I, Nemec, J., Toszer, J., Oroszlan, S., *Int.J.Pep.Prot.Res.*, 1991, **38**, 453.
[60] Zhang, L., Goldammer, C., Henkel, B., Zuhl, F., Panhaus, G., Jung, G., Bayer, E., in *Innovation and Perspectives in Solid Phase Peptide Synthesis,* Epton, R. (ed.), Mayflower, Birmingham, 1994, 711.
[61] Seebach, D., Thaler, A., Beck, A.K., *Helv.Chim.Acta.*, 1989, **72**, 857.
[62] Eckert, H., Seidel, C., *Angew.Chem.Intl.Ed.Engl.*, 1986, **25**, 159.
[63] Lloyd-Williams, P., *Tetrahedron*, 1993, **49**, 11065.
[64] Benz, H., *Synthesis,* 1994, 337.
[65] Macrae, R., Young, G.T., *J.Chem.Soc.Perkin Trans.I*, 1975, 1185.
[66] Tam, J.P., Tjoeng, F.S., Merrifield, R.B., *J.Am.Chem.Soc.* 1980, **102**, 6117.
[67] Tam, J.P., DiMarchi, R.D., Merrifield, R.B., *Int. J. Pep. Prot.Res.*, 1980, **16**, 412.
[68] Fivush, A.M., Willson, T.M., *Tetrhedron Lett.*, 1997, **38**, 7151.
[69] Barlos K., Gatos D., Kallitsis J., Papaphotiu G., Sotiriu P., Wenquing Y. and Schafer W., *Tetrahedron Lett.*, 1989, **30**, 3943.
[70] Wang, S.S., *J.Org.Chem*, 1976, **41**, 3258.
[71] Bodansky, M., Sheehan, J.T., *Chem.Ind.(London),* 1964, 1423.
[72] Ohno, M., Anfinsen, C.B., *J.Am.Chem.Soc,* 1967, **49**, 1330.
[73] Meienhofer J., in *The Peptides: Analysis, Synthesis, Biology*, Gross E. and Meienhofer J. (Eds), Academic Press Inc., New York U.S.A., 1979, **1**, 197.
[74] Bodansky, M., Klausner, S.Y., *Synthesis,* 1974, 549.
[75] Curtius, T., *Ber. Dtsch. Chem. Ges.*, 1902, **35**, 3226.
[76] Curtius, T., *J.Prakt.Chem.*, 1904, **70**, 230.
[77] Honzl,, J., Rudinger, J., *Coll. Czech. Chem. Commun.*, 1961, **26**, 2333.
[78] Sheehan, J.C., Goodman, M., Richardson, W.L., *J.Am.Chem.Soc.*, 1955, **77**, 6391.
[79] Schwyzer, R., Aung T.K., *Helv. Chem. Acta.*, 1962, **45**, 859.
[80] Zahn, H., Piper, W., *Chem. Ber.*, 1962, **95**, 1069.
[81] Magee, M.Z., Hofmann, K., *J.Am.Chem. Soc.*, 1949, **71**, 1515.
[82] Rydon, H.N., Smith, P.W.G., *J.Chem.Soc.*, 1955, 2542.
[83] Medzihradsky, K., *Acta.Chim.Acad.Sci.Hung.* 1962, **30**, 102.
[84] Weygand, F., Prox, A., Konig, W., *Chem.Ber.*, 1963, **7**, 335.
[85] Kemp, D.S., Wang, W., Busby, G., Hugel, G., *J.Am.Chem.Soc.,* 1970, **92**, 4756.
[86] Bodansky, M., Sheehan, J.T., *Chem. & Ind.(London),* 1964, 1423.
[87] Wang, S.S., Merrifield, R.B., *J.Am.Chem.Soc.*, 1969, **91**, 6488.
[88] Wang, S.S., Merrifield, R.B., *Int. J. Pep. Prot.Res.*, 1972, **4**, 309.
[89] Irving S.L., McInnes C., Ramage R., *Tetrahedron Lett.*, 1993, **34**, 6599.
[90] Wang P., Layfield R., Landon M., Mayer R.J., Ramage R., *Tetrahedron Lett.*, 1998, **47**, 8711.
[91] Muir, T., Dawson, P.E., Kent, S.B.H., *Methods Enzymol*, 1997, **289**, 266.
[92] Canne, L.E., Botti, P., Simon, R.J., Chen, Y., Dennis, E.A., Kent, S.B.H., *J.Am.Chem.Soc.,* 1999, **121**, 8720.
[93] Schnolzer M., Kent S.B.H., *Science*, 1992, **256**, 221.
[94] Muir T.W., Williams M.J., Ginsberg M.H., Kent S.B.H., *Biochemistry*, 1994, **33**, 7701.
[95] Englebretsen D.R., Garnham B.G., Bergman D.A., Alewood P.F., *Tetrahedron Lett.*, 1995, **36**, 8871.
[96] Pietrolovski, S. *Protein Sci.,* 1998, **7**, 64.
[97] Perler, F.B., Olsen, G.J, Adam, E., *Nucleic Acids Res.,* 1997, **25**, 1087.
[98] Rose K., *J. Am. Chem. Soc.*, 1994, **116**, 30.
[99] Tam, J.P., Yu, Q., Miao, Z., *Biopolymers*, 1999, **51**, 311.
[100] Clarke-Lewis I., Abersold R., Ziltener H., Schrader J.W., Hood L.E., Kent S.B.H., *Science*, 1986, **231**, 134.
[101] Liu, C.F..,Tam, J.P., *J.Am.Chem.Soc.,* 1994, **116**, 4149.

[102] Tam, J.P., Rao, C., Shao, J., Liu, C.F., *Int. J. Pept. Prot. Res.*, 1994, **45**, 209.
[103] Tam, J.P., Lu, Y.-A., Liu C.-F., Shao J.J., *Proc Natl. Acad. Sci. USA*, 1995, **92**, 12485.
[104] Wieland, T., Schneider, G., *Liebigs. Ann. Chem.*, 1953, **583**, 159.
[105] Zhang, L., Tam, J.P., *Tetrahedron Lett.*, 1997, **38**, 3.
[106] Tam, J.P., Yu, Q., *Biopolymers*, 1998, **46**, 319.
[107] Canne, L.E., Bark, S.J., Kent, S.B.H., *J. Am. Chem. Soc.*, 1996, **118**, 5891.
[108] Schnolzer, M., Kent, S.B.H., *Science*, 1992, **256**, 221.
[109] Ellman, G.L., *Arch.Bichem.Biophys.*, 1959, **82**, 70.
[110] Dawson, P.E., Muir, T.W., Clark-Lewis, I., Kent, S.B.H., *Science*, 1994, **266**, 776.
[111] Blake, J., *Int. J. Pept. Prot. Res.*, 1986, **27**, 191.
[112] Yamashiro, D., Li, C.H., *Int. J. Pept. Prot. Res.*, 1988, **31**, 322.
[113] Liu, C.F., Tam, J.P., *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 6584.
[114] Barbas, C.F., III, Matos J.R., West, J.B., Wong, C.H., *J.Am. Chem. Soc.*, 1988, **110**, 5162.
[115] Liu, C.F., Rao, C.,Tam, J.P., *J.Am.Chem.Soc.*, 1996, **118**, 307.
[116] Botti, P., Pallin, T.D., Tam, J.P., *J.Am.Chem.Soc.*, 1996, **118**, 10018.
[117] Frechet, M.J., Scheurch, C., *J.Am.Chem.Soc.*, 1971, **93**, 492.
[118] Evans, T.C., Xu, M.-Q., *Biopolymers*, 1999, **51**, 333.
[119] Brenner, M., Zimmermann, J. P., Wehrmuller, J., Quitt, P., Hartmann, A., Schneider, W., Beglinger, U., *Helv. Chem. Acta.*, 1957, **40**, 1497.
[120] Kemp, D.S., *Biopolymers*, 1981, **20**, 1793.
[121] Kemp, D.S., Carey, R.I., *J.Org.Chem.*, 1993, **58**, 2216.
[122] Sears, P., Schuster, M., Wang, P., Witte, K., Wong, C-H, *J.Am.Chem.Soc.*, 1994, **116**, 6521.
[123] Smithrud, D.B., Benkovic, P.A, Benkovic, S.J., Roberts, V., Liu, J., Neagu, I., Iwama, S., Phillips, B.W., Smith, A.B., Hirschmann, R., *Proc.Natl.Acad.Sci.USA*, 2000, **97**, 1953.

2. Studies Towards The Chemical Synthesis of Endostatin

2.1 Introduction

2.1.1 Angiogenesis Inhibition

The main challenge in cancer therapy today is to overcome the problem of acquired drug resistance. The very nature of tumour cells means that they show a high rate of mutation, that they are genetically unstable, and tend to be heterogeneous. Conventional drug therapy is seldom able to completely eradicate a tumour. For this reason, angiogenesis inhibitors[1,2] have come to the fore. These compounds represent a new strategy in cancer therapy and the hope is that they will avoid the problem of drug resistance by targeting tumour cells indirectly.

Angiogenesis is defined as the sprouting of capillaries from pre-existing blood vessels. It is a fundamental process during embryonic development but it also takes place under certain circumstances during adulthood. Examples include wound healing processes, diabetic retinopathy and, significantly, the growth of solid tumours[3,4]. Solid tumours need to furnish themselves with a blood supply, in order to obtain the oxygen and nutrients necessary to fuel their growth. To do this they recruit host endothelial cells, which are the building blocks of new blood vessels. The chemical signals that direct the endothelial cells are produced by the tumour. They induce chemotaxis - movement along a chemical gradient; and mitogenesis - the cells replicate and divide to form new cells.

These chemical signals have been investigated[5,6], and it seems that angiogenesisis is controlled by sets of positive and negative regulators, and that the negative regulators - angiogenesis inhibitors - are the breakdown products of the positive regulators. Depending on the net balance between the two, angiogenesis is either suppressed or induced. It follows that, if the proportion of negative regulators is increased and maintained at a certain level, angiogenesis might be completely suppressed. This in

turn should result in a slowing of tumour cell growth and, eventually, tumour cell death, especially in hypoxic areas of the tumour. Recent experiments with compounds such as endostatin and angiostatin have shown this to be true[7,8,9]. A further advantage of this approach to tumour therapy is that the cells under attack, since they are not descended from the tumour, are not subject to high rates of mutation and are therefore less likely to become resistant[10]. Therapy is normally carried out by direct injection at the tumour site. One hypothesis is that, serendipitously, this approach works by keeping the concentration of the drug high enough at the tumour site to inhibit angiogenesis, but low enough in the rest of the bloodstream that the desirable processes of wound healing are not affected[11]. More recently, other therapeutic avenues have been explored, such as antiangiogenic gene therapy, using both viruses[12] and cationic lipids[13] as delivery systems for the systemic administration of DNA which codes for antiangiogenic proteins. The viral therapy has had particularly promising results, but neither technique is as yet sufficiently advanced to have widespread clinical utility.

2.1.2 Angiostatin & Endostatin

The recent interest in Endostatin was preceded by the discovery of another angiogensis inhibitor, Angiostatin. Angiostatin is a 38 kDa specific inhibitor of endothelial cell proliferation and was isolated in 1994 from a subclone of Lewis lung carcinoma[14]. It was generated by the primary tumour in order to inhibit the growth of its metastases and has subsequently been shown to be the product of the proteolytic cleavage of plasminogen by a serine protease[15]. Angiostatin therapy has been successful in inhibiting the growth of at least three different types of murine primary tumours and, at the doses so far tested, neither resistance nor toxicity due to the therapy have been observed[16]. Recombinant fragments have shown inhibitory activity in vitro[17].

Endostatin was first isolated in 1996, from a tumour found in mice[18,19]. It is a 184 aa, 20kDa peptide fragment from a *C*-terminal non-collagenous domain of collagen XVIII and has been found to be a specific inhibitor of murine endothelial proliferation. Systemic therapy causes a near complete suppression of tumour-induced angiogenesis, resulting in strong anti tumour activity. The *E.coli*-derived endostatin was administered

as a non-refolded suspension and primary tumours were regressed to dormant, microscopic lesions[20]. Combined therapy using both endostain and angiostatin is also purported to work well[21], and shows a more than additive effect on tumour cell growth inhibition. Endostatin has already entered phase IV clinical trials and is due for launch in 2004[22].

2.1.3 Endostatin – structure[23]

The three dimensional structure of Endostatin is composed primarily of β-sheets and loops. The protein is described as being prone to aggregation[24], a property which is partly due to this extensive β-type structure. The most prominent structural feature is a highly twisted mixed β-sheet composed of seven strands. There are two alpha helices and two disulphide bridges. The overall structure is described as an irregular β-barrel, propped open at one side by an α–helix (α2).

Endostatin is rich in basic residues, especially arginine. These residues are highly conserved between different subtypes and are located on the surface of the protein, suggesting a location for the proposed heparin binding site. A water molecule is hydrogen bonded to three residues in the hydrophobic core. Endostatin also chelates zinc, however the coordination is variable and the zinc is thought to play a structural role rather than a functional one[25].

Figure 2.1 *The crystal structure of endostatin*

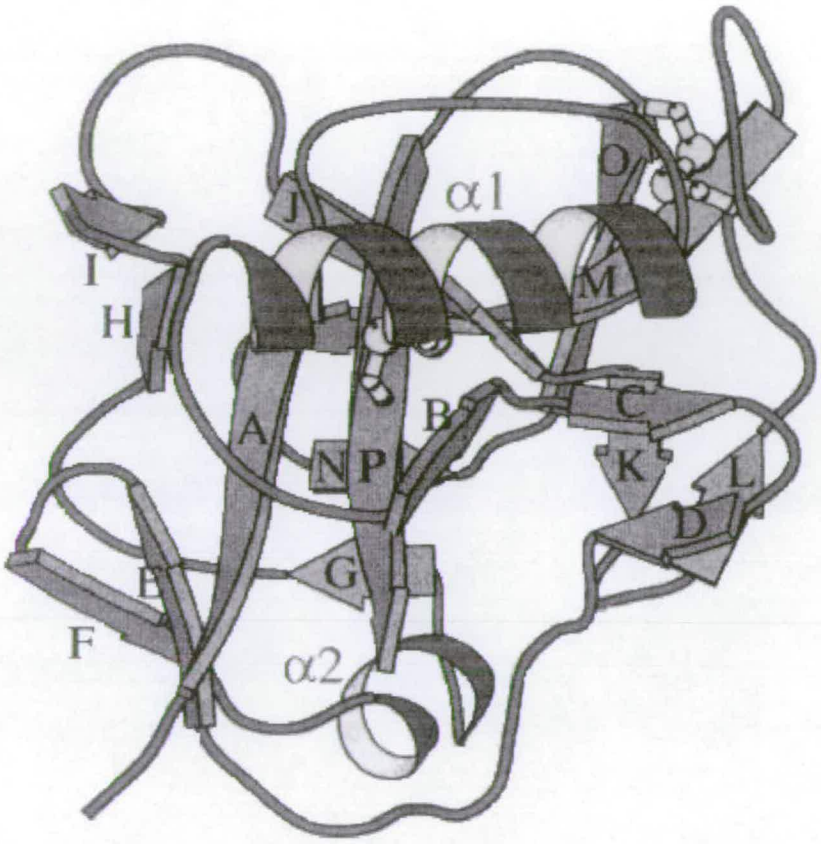
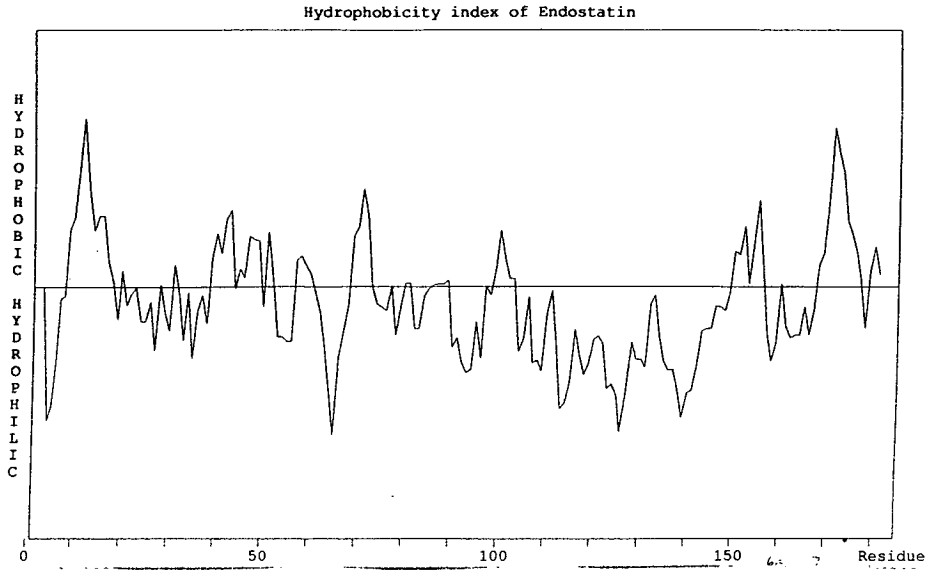


Figure 2.2 *Endostatin – sequence, numbered relative to the NC1 domain of collagen XVIII*

H_2N-H[132]THQDFQPVL	HLVALNTPLS	GGMRGIRGAD	FQCFQQARAV
GLSGTFRAFL	SSRLQDLYSI	VRRADRGSVP	IVNLKDEVLS
PSWDSLFSGS	QGQLQPGARI	FSFDGRDVLR	HPAWPQKSVW
HGSDPSGRRL	MESYCETWRT	ETTGATGQAS	SLLSGRLLEQ
KAASCHNSYI	VLCIENSFMT	SFSK[315]-CO_2H	

Figure 2.3 *Endostatin - hydrophobicity plot*

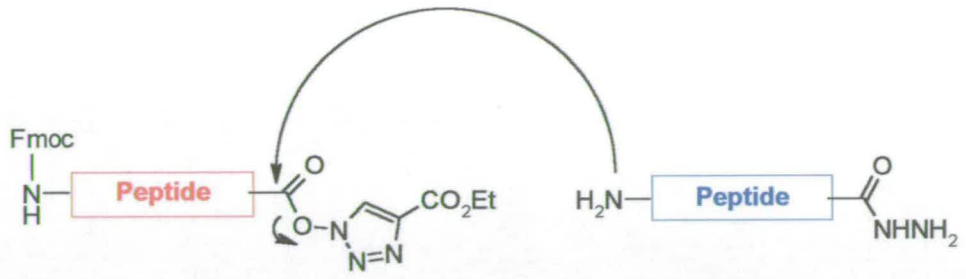


2.2 Research Overview

If endostatin is to prove useful as a cancer treatment there are important hurdles to be overcome. Bulk production must be made possible, especially due to the need for repeated administration[26]. Extended structural studies to determine the site of action are necessary since, although the crystal structure of endostatin has been published, the precise receptors and mechanisms underlying angiogenesis remain undefined. For these reasons, the synthesis of Endostatin by SPPS was undertaken. Once perfected, the synthesis could be carried out on a reasonably large scale, and, more importantly, specific peptide fragments can be produced, which could facilitate the determination of the active portion of the protein.

Endostatin is a relatively large peptide and, initially, the condensation of two large fragments, approximately 90aa in length was considered. However, the stepwise SPPS of the first of these large fragments was unsuccessful. A method that would allow the efficient, sequential coupling of several small fragments was required. The segment coupling of minimally protected fragments *via* transfer active ester condensation (TAEC)[27], a technique recently developed at Edinburgh, was therefore investigated. The key step is illustrated below (Figure 2.4).

Figure 2.4 *Transfer Active Ester Condensation - key step*



The fragments for synthesis were provisionally selected based on hydrophobicity and potential coupling sites. These peptide fragments were then optimised for stepwise SPPS using the Fmoc strategy. Peptides containing two types of *C*-terminal functionality - hydrazides and semi-carbazides - were investigated. An alternative strategy for the synthesis of the Wang resin-based hydrazide linker, first proposed by Wang and Merrifield in 1969[28], was developed to facilitate this process.

2.3 Initial Strategy

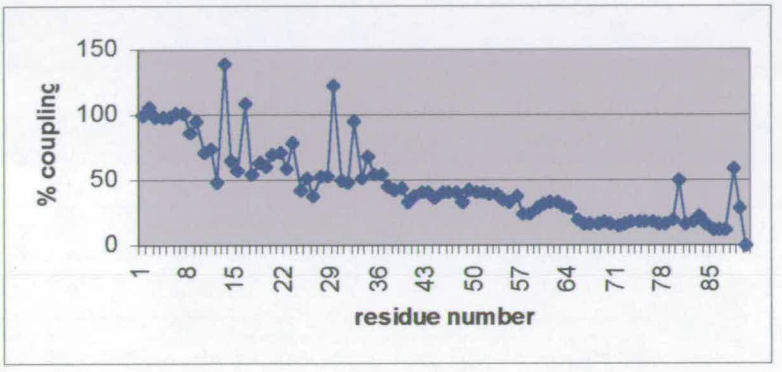
Since Endostatin is a relatively large peptide (184aa), the original strategy was to synthesise it in two fragments, which would then be coupled by solution phase fragment condensation. The sequence was considered and a convenient dividing point was decided upon (Figure 2.5). Two fragments of approximately equal length were selected. A Gly residue was chosen as the coupling *C*-terminus, in order to eliminate the danger of racemisation.

Figure 2.5 *Endostatin - Ligation sites (i)*

H[1]THQDFQPVL	HLVALNTPLS	GGMRGIRGAD	FQCFQQARAV
GLSGTFRAFL	SSRLQDLYSI	VRRADRGSVP	IVNLKDEVLS
PSWDSLFSGS	QG[92]	**Q[93]LQPGARI**	**FSFDGRDVLR**
HPAWPQKSVW	**HGSDPSGRRL**	**MESYCETWRT**	**ETTGATGQAS**
SLLSGRLLEQ	**KAASCHNSYI**	**VLCIENSFMT**	**SFSK[184]**

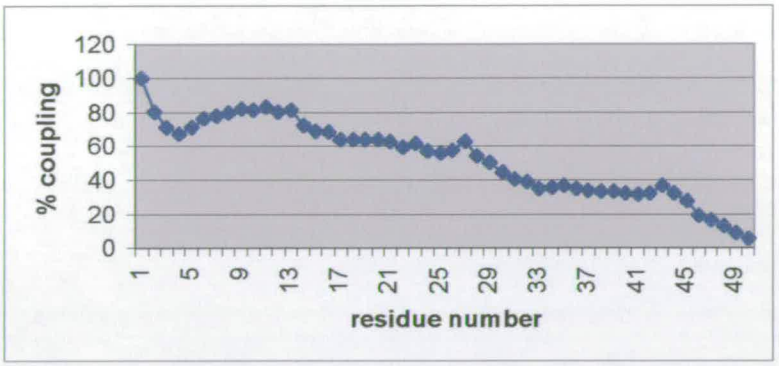
Synthesis commenced from the carboxy terminus using Wang resin with a relatively low loading. This synthesis was carried out to completion, yielding peptide **1**, however Fmoc loading test measurements indicated that the end yield was very low. Examination of the deprotection profile (Figure 2.6) revealed that, although the coupling yield had been dropping consistently throughout, the main drop was at the beginning of the synthesis. A second attempt was made, using PEG resin with decreased loading, but this also failed.

Figure 2.6 *Peptide* **1** *- Deprotection Profile*



The hydrophobicity plot (Figure 2.3) showed an extensive hydrophobic region at the beginning of the synthesis, where the largest drop occurred, and it therefore seemed likely that the peptide was folding in on itself, thus restricting the access of active carboxyl to the free amide. For this reason, the side chain protection was altered, with trityl protection being employed on Ser and Thr amino acid residues, in the hope that these sterically demanding functionalities would prevent the aggregation of the growing peptide. PEG resin with a loading of 0.18 mmol/g was used. This new strategy appeared to work well, and showed a significant improvement at the beginning of the synthesis (Figure 2.7), before yields again began to drop (peptide **2**). The synthesis was stopped approximately half way through.

Figure 2.7 *Peptide* **2** *– Deprotection profile*



It was next decided that the protein, should be broken down into even smaller fragments for ligation. The use of orthogonal ligation was considered, so the first division was made at a cysteine residue (Cys^{165}). The proposed ligation sites are shown below (Figure 2.8).

Figure 2.8 *Endostatin – ligation sites (ii)*

H^{1}THQDFQPVL HLVALNTPLS GGMRGIRGAD FQ^{32}

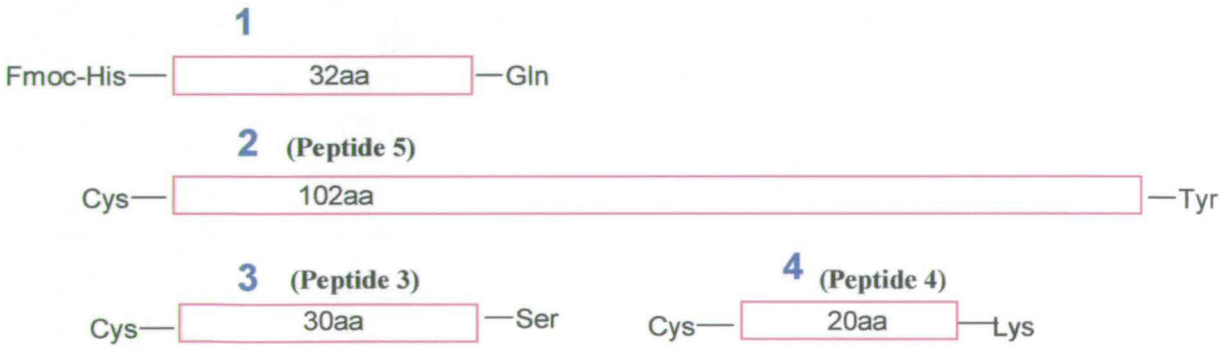
C^{33}FQQARAV GLSGTFRAFL SSRLQDLYSI VRRADRGSVP

IVNLKDEVLS PSWDSLFSG S QGQLQPGARI FSFDGRDVLR

HPAWPQKSVW HGSDPSGRRL MESY^{134} C^{135}ETWRT

ETTGATGQAS SLLSGRLLEQ KAAS^{164} **C^{165}HNSYI**

VLCIENSFMT SFSK^{184}



At this point, a trial synthesis of peptide **3** (fragment 3, Figure 2.8), was attempted, the reasoning being that the success or failure of this fragment's synthesis would give the best indication with regard to the viability of the strategy. The synthesis went reasonably well, with analysis indicating that a 32% yield had been achieved. The fragment was cleaved from the resin, purified by preparative HPLC and retained for analytical purposes. This sample itself would be unsuitable for ligation since it did not have the necessary *C*-terminal ester functionality.

The preparation of peptide **4** was next undertaken, using PEG resin. This was achieved successfully, with a coupling yield of 70%. This sample remains resin-bound.

The central fragment, peptide **5** was next attempted and at this point it became obvious that this peptide would also prove too "difficult" for stepwise synthesis. The spacing of the Cys residues is such that further division into conveniently sized fragments was not possible and it was therefore decided to employ the newly developed Transfer Active Ester Condensation (TAEC)[27] strategy for segment coupling, which would allow fragment size to be optimised according to ease of synthesis. TAEC is compatible with minimal side chain protection, a feature that was especially desirable since the insolubility of the early peptide sequence was likely to be a contributing factor in the problems thus far encountered.

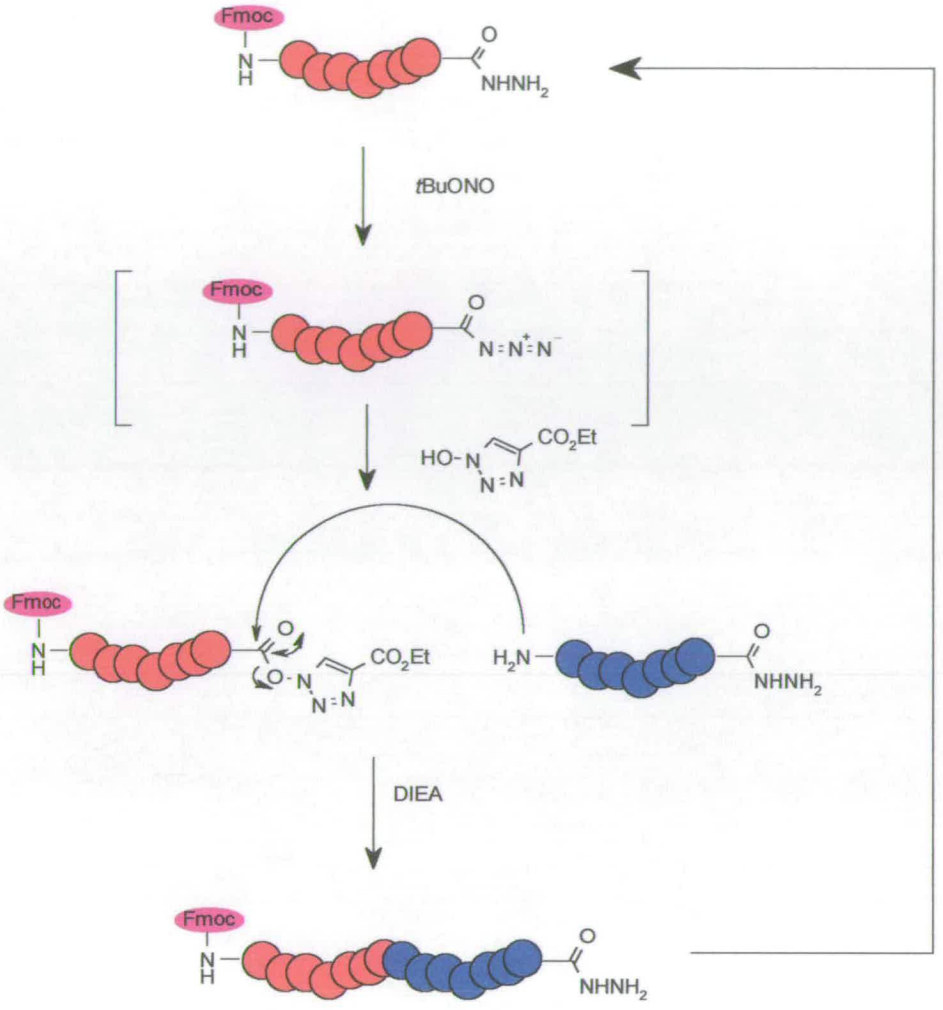
2.4 Segment Coupling *via* Transfer Active Ester Condensation

2.4.1 The Coupling Reaction

Transfer active ester condensation has previously been used to couple peptide segments *via* the incorporation of peptide bonds and *via* the incorporation of isopeptide bonds. It has also been applied to the selective coupling of peptides containing *C*-terminal cysteine residues and for the synthesis of modified ubiquitin derivatives[27]. During this project, the method was employed in the formation of native amide bonds between fragments of varying size, which contained non-sterically hindered *C*-terminal amino acids.

In TAEC, the coupling reaction itself is based on that of the more widely known azide method[29,30], discussed in Section 1.3.2.2, where the azide is formed from the *C*-terminal hydrazide following the introduction of either $NaNO_2$ or *t*BuONO, together with a strong acid, such as HCl. TAEC differs by employing a coupling reagent, usually HOCt or HOAt, to form an active ester from the azide which has been generated *in situ*, resulting in a more stable, long-lived intermediate which is still active enough to undergo coupling, as illustrated in Figure 2.9. *t*BuONO is still used but the acidity of the coupling reagent means HCl is not required. The trapping of the active species means that the major side reaction in azide coupling – isocyanate formation – is reduced and the procedure can therefore be carried out at room temperature. The most significant side reaction is the hydrolysis of the active ester, a reaction which is not rapid enough to directly compete with amide bond formation. In model studies, this method has resulted in higher yields and faster reaction rates.

Figure 2.9 *Transfer Active Ester Condensation*



2.4.2 The Coupling Reagent

The coupling reagent of choice in TAEC is HOCt, although HOAt is also viable. HOCt was devoloped as part of our own research[31] as a novel, racemisation free reagent for the activation of the carboxyl activation and is now used routinely in automated SPPS, in conjunction with DIC (see Section 1.2.5).

The use of HOCt in excess provides the mildly acidic conditions necessary for the activation step to occur and also acts to solubilise the peptide. Following activation, the HOCt then reacts with the peptide azide, thus forming the "Active Ester", as shown in Figure 2.9.

2.4.3 Protecting Groups

TAEC requires that the nucleophilic side chains of Cys and Lys, as well as the N-terminus of the peptide azide, be protected, in order to prevent cross linking of the former and cyclisation of the latter species. The side chain protection must be stable to resin cleavage conditions, but must be easily removed following ligation.

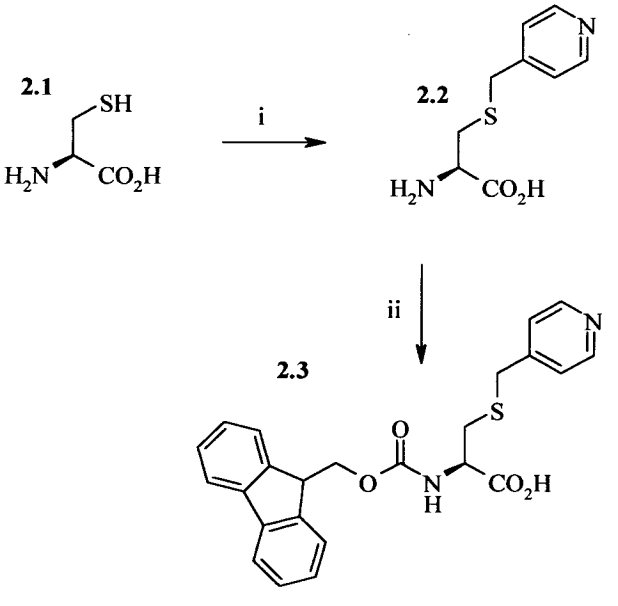
2.4.3.1 Nα Protection

The peptide fragments were synthesised using Fmoc/tBu protection. The Fmoc group was retained after completion of the synthesis

2.4.3.2 Cysteine Protection

Cysteine was protected using the picolyl group[32, 33]. This group is stable to TFA and is thus retained following cleavage of the peptide-resin. The picolyl group may be removed after the coupling reaction using zinc dust in acetic acid. The picolyl functionality also increases the polarity of the peptide, acting to improve solubility.

Figure 2.11 *The synthesis of Fmoc-4-S-picolyl-L-Cysteine*

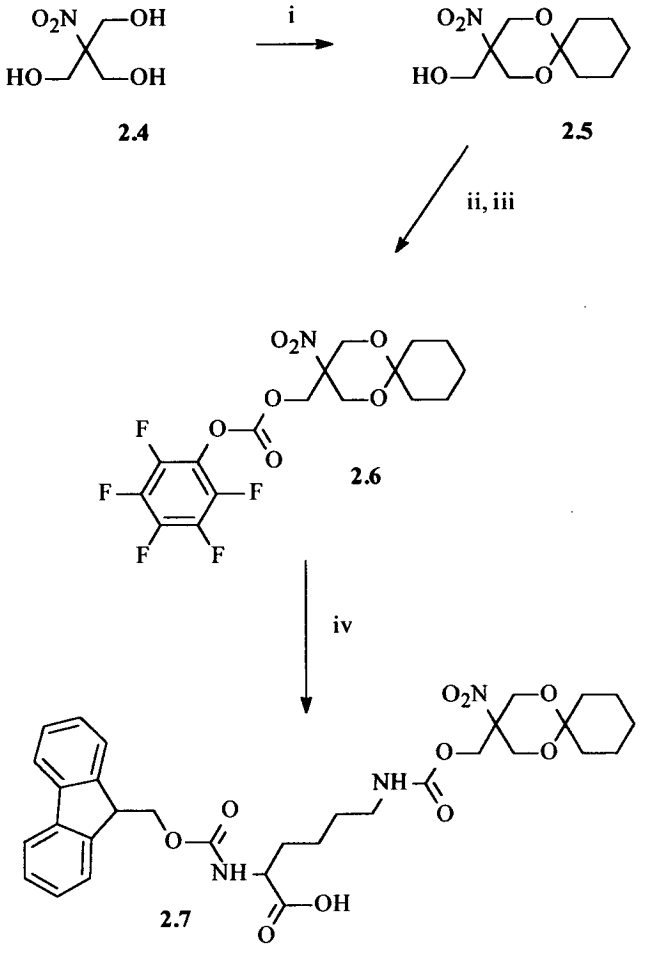


(i) 4-picolyl chloride.HCl, NaOH/EtOH (ii) Fmoc-*N*-hydroxy succinamide, 10%Na_2CO_3/1,4-dioxane

2.4.3.3 Lysine Protection

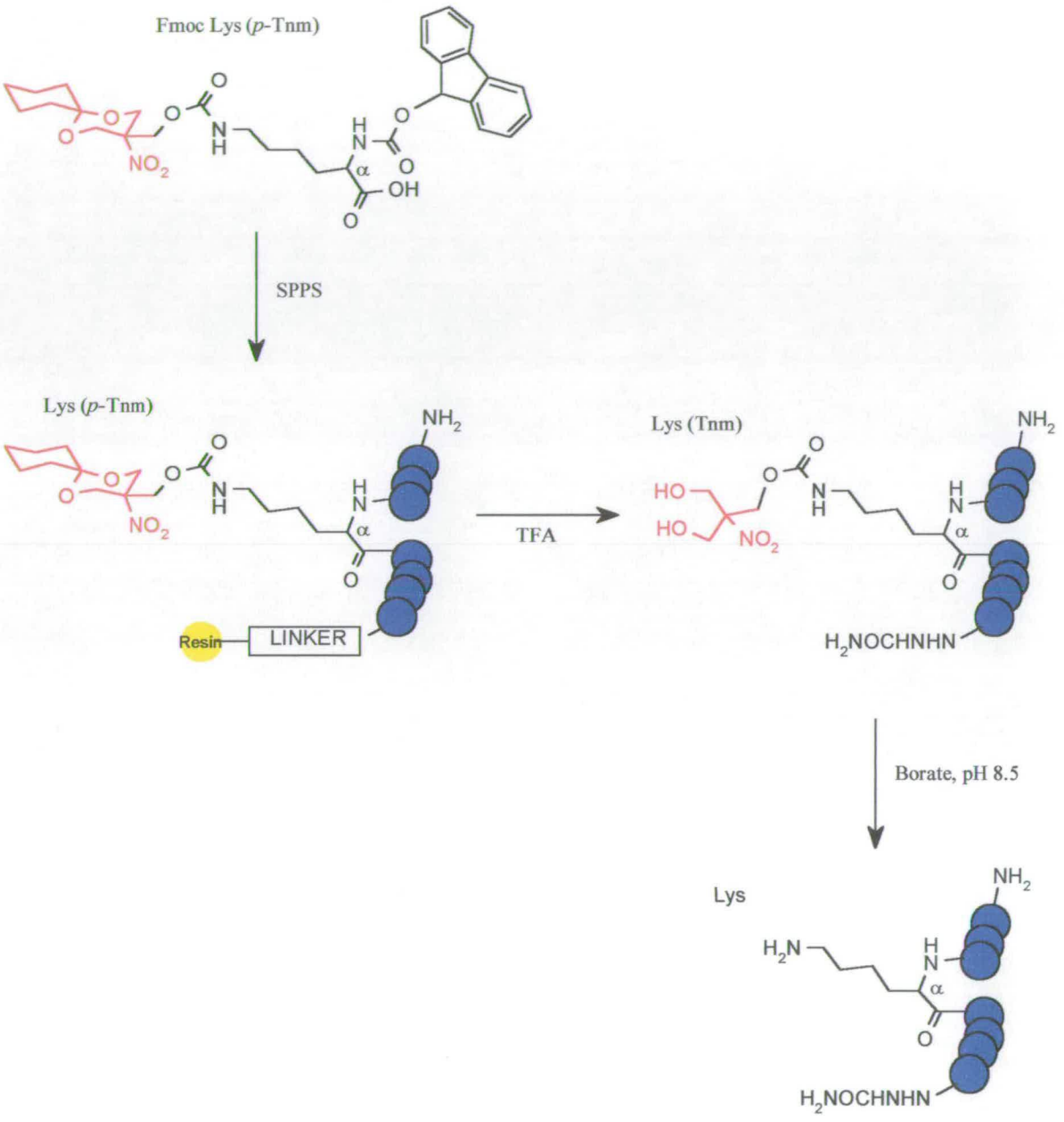
Lysine was protected using the Tnm group[34] (Figure 2.12). Tnm is cleaved *via* a two step mechanism as shown (Figure 2.13). Initial exposure to TFA leads to diol formation, then final cleavage is carried out in aqueous base (borate buffer, pH 8.5).

Figure 2.12 *The synthesis of Fmoc-1,5-dioxaspiro-5:5-undecane-3-nitro-3-methoxycarbonyl-Lysine*



(i) Cyclohexanone, TsOH, Dean&Stark conditions/ benzene; (ii) Triphosgene, DIEA/ toluene; (iii) Pentafluorophenol, DIEA/ toluene; (iv) Fmoc-Lys-OH, TEA/ 1,4 dioxane/water (2:1).

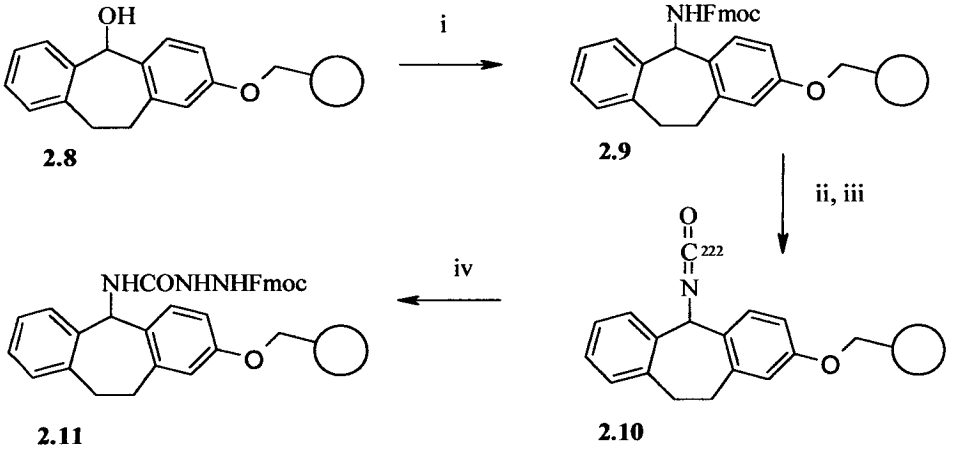
Figure 2.13 *Cleavage of Fmoc-Lys(Tnm)-OH*



2.4.4 Semicarbazide Linker

The initial linker used to prepare fragments for TAEC was chosen to provide the peptide-semicarbazide. The dibenzosuberyl linker used to yield this C-terminal functionality was first investigated by Ramage and Irving[35] and has been used by Patterson in the synthesis of peptide aldehydes[36]. It is readily prepared from tricyclic amide resin, **2.9**, which was originally designed for use in the preparation of peptide amides. The amide resin is first transformed into its isocyanate, this is then trapped using freshly prepared Fmoc-hydrazine[37]. An initial capping step is carried out, on the automated synthesiser, to block any unreacted amino groups. The Fmoc is then removed in the normal way and the first aa is coupled directly to the resin. Following synthesis, the peptide is cleaved from the resin using 90% TFA.

Figure 2.14 *The synthesis of the semicarbazide linker*



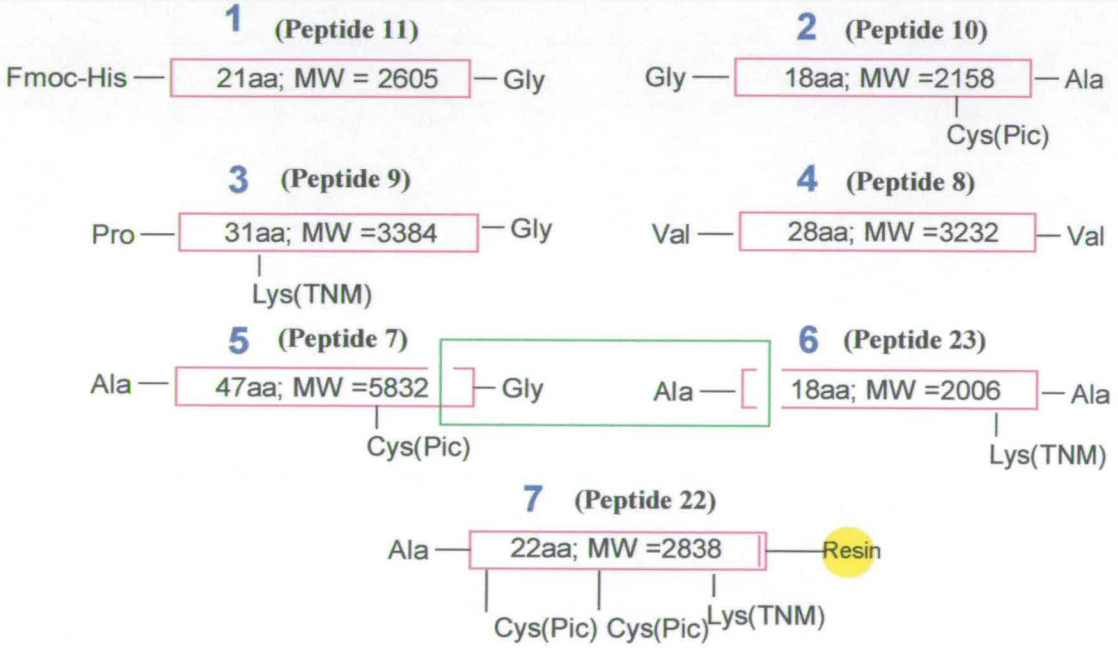
(i) $FmocNH_2$, $PhSO_3H$/DMF (ii) 20% piperidine/DMF (iii) DIEA, Triphosgene/DCM (iv) $FmocNHNH_2$/DCM

It was hoped that this species would prove a viable alternative to the peptide hydrazide, since it is potentially more stable and the peptide products are easier to differentiate from peptide acids or peptide amides by mass spectrometry. Also, the use of this linker would allow access to alternative functionalities[27].

2.4.5 Fragment optimisation

As discussed in Section 2.2, the fragments to be synthesised for use in TAEC coupling were provisionally selected, based on hydrophobicity and potential coupling sites. Following trial syntheses and optimisation, the protein was divided into the seven fragments shown in the following diagram (Figure 2.14). The optimal size of each fragment is largely sequence dependant - for example, fragments 6 and 7 (peptides **23** & **22**) were originally a single fragment 40aa in length (peptide **6**), which was subsequently divided into two 20mers, whereas fragment 5 (peptide **7**) contained 47aa and was synthesised successfully. The fragments were purified using Preparative HPLC and analysed by electrospray mass spectrometry and amino acid analysis.

Figure 2.14 *Endostatin – final fragments*



2.4.5.1 Peptide Acid

The *C*-terminal fragment, was prepared using Wang linker loaded with Fmoc-Lys-(Tnm)-OH, in order that the final coupled product would be the peptide acid.

Resin loading using conventional methods was unsuccessful, probably due to the sterically demanding Tnm functionality, and loading studies were therefore carried out. The results are summarised in table 2.1. The final loading result of 0.16 mmol/g was consistent and reproducible. Due to the long reaction time required, a Merrifield bubbler was used to agitate the mixture, as this method is least likely to degrade the resin.

Table 2.1 *Fmoc-Lys(TNM)OH - resin loading study*

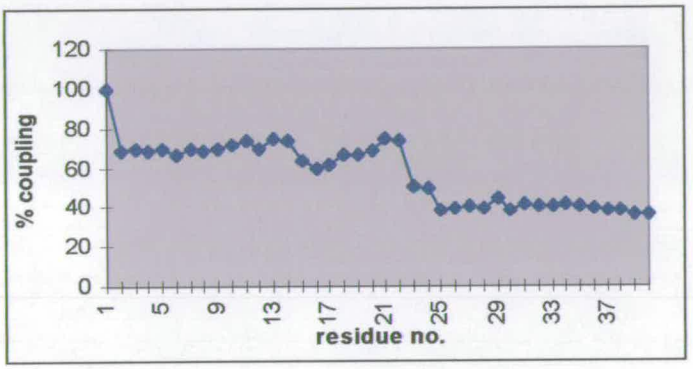
Resin type	**Activation method**	**No. of eq. of activated aa**	**Solvent**	**Reaction Time**	**Fmoc loading**
Wang	Symmetrical anhydride	1.5	DCM	2.5hrs (sonicated)	0.025 mmol/g
Wang	Symmetrical anhydride	1.5	DMF/dioxane 1:1	3hrs (Merrifield bubbler)	0.022 mmol/g
Wang	Active ester	3	DMF/dioxane 1:1	3hrs (sonicated)	0.06 mmol/g
Chlorotrityl*	Symmetrical anhydride	2	DMF/dioxane 1:1	0.5hrs (sonicated)	0.01 mmol/g
Wang	**Active ester**	**3**	**DMF/dioxane 1:1**	**20hrs (Merrifield bubbler)**	**0.16 mmol/g**

* Chlorotrityl resin is particularly active and maximum loading is expected after 30 mins.

Since the preparation of peptides **3** and **4** had gone well (Section 2.3), it was reasoned that the *C*-terminal fragment could be extended to contain 40aa. However, the product of this synthesis, peptide **6**, proved to be extremely insoluble in water/acetonitrile. Such insolubility led to difficulty with electrospray mass spectrometry and a product of the correct mass could not be conclusively detected. FAB mass spectrometry was also

attempted, without success. The deprotection profile (Figure 2.15) indicated that the synthesis had gone relatively well and truncated products were not detected. It is possible that the side chain protecting groups were not completely removed during the acidolytic cleavage from the resin. Although the synthesis could not be said to have failed, purification and deprotection would be simplified with smaller peptides and it was therefore decided to reduce the size of the *C*-terminal fragment.

Figure 2.15 ***Peptide*** 2 – ***Deprotection profile***



Fragment 7 (peptide **22**) was 22aa in length and was prepared in good yield. From earlier syntheses, areas of potentially poor coupling had been identified and double coupling procedures were therefore carried out at the residues most affected. The trityl protection previously employed in the synthesis of this fragment (Section 2.3) was not employed in the final synthesis of fragment 7, and no decrease in yield was encountered. It is thought that the *C*-terminal Tnm side chain provides a similar amount of steric bulk as the trityl group, and therefore also acts to prevent the active *N*-terminus being buried by the growing peptide.

2.4.5.2 Peptide Semicarbazides

Fragments 1-5, as shown in Figure 2.14 (peptides **7-11**), were prepared and optimised in terms of size and ease of synthesis using the semicarbazide linker, with side chain protection as previously described (Section 2.4.3). Within the remit of TAEC, racemisation-free coupling sites are desirable, although non-essential, and the presence of bulky side chains at these sites should be avoided. Fragment solubility is also a factor, although the use of minimal side chain protection goes some way to circumventing this problem. The segment coupling reaction is carried out in an *N* to *C* direction, and the original strategy was to build up the peptide segments in this direction using solution based TAEC coupling, thus avoiding the need for repeated deprotection at the N-terminus (Figure 2.9). However, conventional SPPS is carried out in the *C* to *N* direction, and for the coupling fragments to be optimised it was most efficient to prepare the *C*-terminal fragments first and work backwards.

2.4.6 Peptide Semicarbazides - Trial Coupling

Initial ligation studies were carried out on two smaller peptides (**12** & **13**), the sequence of which was taken from the five amino acids either side of the fifth coupling site, as shown in Figure 2.16 and as highlighted in Figure 2.14.

Figure 2.16 *Peptides for trial coupling*

Peptide 12

Fmoc-HN — T E T T G — $NHNHCONH_2$

(Fmoc-R-NHNHCONH2)
MW = 788.5

Peptide 13

H_2N — A T G Q A — CO_2H

(R^1- NH2)
MW = 446.4

All reagents were dried and the reaction was carried out under nitrogen. The reaction was monitored by HPLC, see Figure 2.17. The reagents and conditions used are listed in table 2.2).

Figure 2.17 *Trial Ligation (I) -monitored by HPLC*

HPLC (1) HPLC (2) HPLC (3)

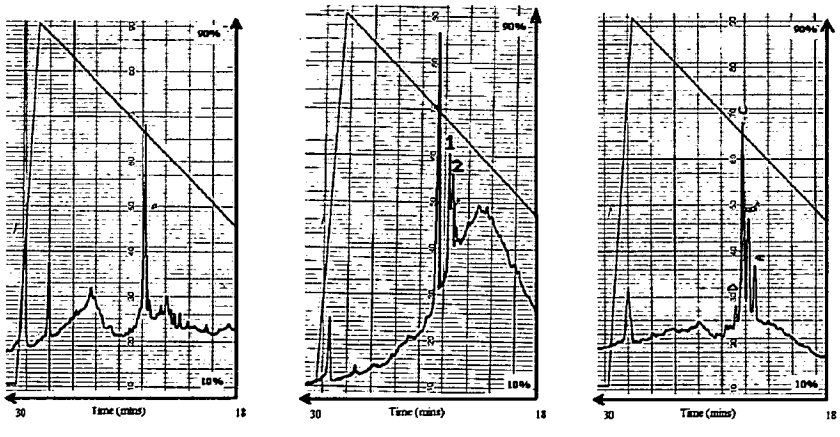


Table 2.2 ***Trial Ligation (I) – reagents & conditions***

	Reagents added / conditions	HPLC Profile
HPLC (1)	Fmoc-R-NHNHCONH2 (5mg), HOCt (1.5mmol) / RT, stirred 1h	**Major Peak – Peptide 12 (Rt 20.8 min, 66% AcCN).**
HPLC (2);	tBuONO (2eq) / 0°C, stirred 2.5h	**Major Peak - Active Ester (Rt 22.0 min, 70% AcCN);** Minor Peak 1 - Peptide Acid (Rt 20.0 min, 67%AcCN); Minor Peak 2 – Peptide **12** (Rt 20.8 min, 66%AcCN).
HPLC (3);	Peptide **13** (1eq), DIEA (1.5mmol) / RT, stirred 16h	Peak B – Peptide **13** (Rt 21.2 min, 65%AcCN); **Peak C – ligated product, Peptide 24 (Rt 20.4 min, 66% AcCN);** Peak D - Peptide Acid (Rt 20.0 min, 67%AcCN).

From the mass spectrum, it could be seen that the coupled product (peptide **24**) had been formed successfully. The only significant side product observed was the peptide acid, the result of the hydrolysis of the active ester. This side reaction may have occurred *in situ*, however it may also be the result of exposure to the aqueous solvent system of the reverse phase HPLC. After 16h at room temperature, the active ester peak had disappeared and the reaction was judged to be complete.

2.4.7 Peptide Semicarbazides – Large Fragment Coupling

Following the successful coupling of the small peptide **12** to peptide **13**, the coupling of fragments 1 and 2 (Figure 2.14, peptides **11** & **10**) of Endostatin was attempted. This reaction was also monitored by HPLC. Fragment 1 (**11**) was exposed to the conditions required for active ester formation, however no new peak was observed. To rule out co-elution, fragment 2 (**10**) was introduced and monitoring was continued for several hours, however no product formation was evident.

From the initial studies carried out, it seemed that the problem lay with the formation if the initial active ester, since no evidence of its presence could be detected by HPLC or mass spectrometry. It was later observed that the peptide acid and semi-carbazide peaks of fragment 1 share the same retention time under these conditions of elution. The mass

spectra indicated that a peak with a molecular weight identical to that of the peptide acid was present after at least 12h under the reaction conditions. The reaction was repeated under scrupulously dry conditions, with similar results. Concurrent work on semi-carbazide based coupling, with erythropoietin (EPO) fragments, was also unsuccessful[38].

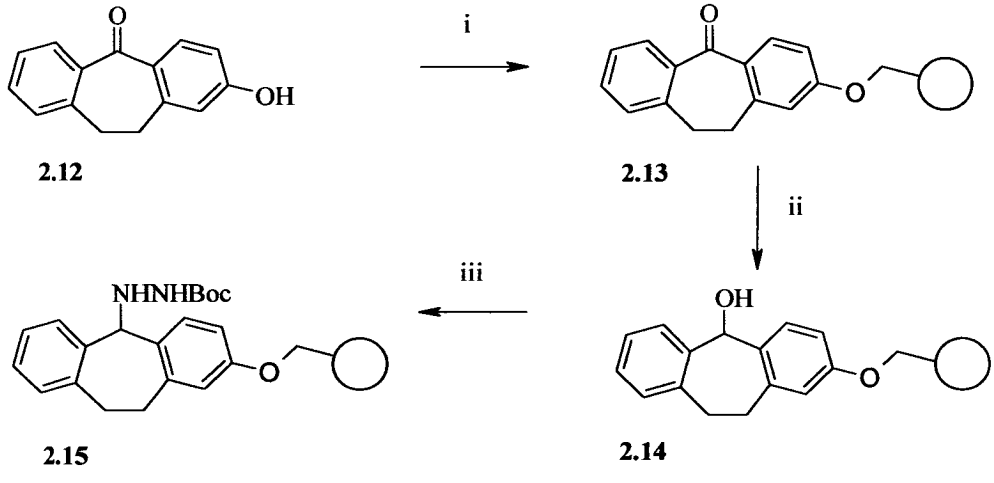
Since none of these problems were encountered with the small fragments, it seems likely that the size of the peptide is the primary factor. Some secondary structure formation is likely to have occurred, thus restricting the access of reagents to the *N*-terminus. It is unclear whether the product thought to be the peptide acid results from the direct breakdown of the semi-carbazide functionality, or whether the active ester formed successfully but was hydrolysed before it was able to undergo coupling. The peptide amide has a similar mass (+/- 1) to the peptide acid and could have formed as a breakdown product during azide formation[39], although TAEC was designed to avoid such side reactions. Fragmentation may also have occurred during mass spectrometry, however no such pattern was observed when the pure starting material was analysed using a similar cone voltage.

2.4.8 The Hydrazide Linker

2.4.8.1 Overview

Since the semi-carbazide linker had proved unsuitable for the coupling of larger fragments, it was decided to repeat the TAEC strategy using peptide hydrazides. This presented a problem, however, since the preparation of the corresponding tricyclic hydrazide linker[34] would require a more complex series of reactions, as can be seen from the following diagram (Figure 2.18). Methods for the synthesis of the starting tricyclic system, **2.12,** are further discussed in Section 3.2 and typically involve at least four steps. In an attempt to simplify the process, an alternative linker for the preparation of peptide hydrazides was investigated.

Figure 2.18 *The synthesis of the tricyclic hydrazide linker*

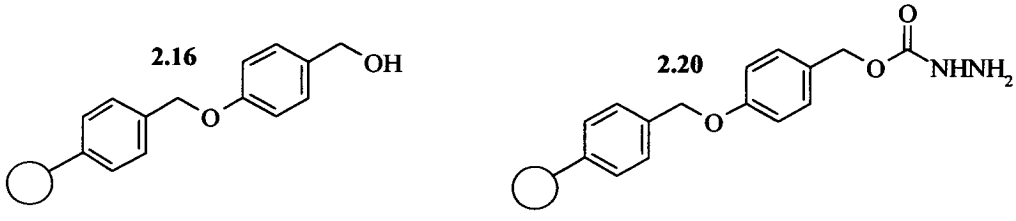


(i) CsOH, Merrifield Resin/DMF (ii) $LiBH_4$/THF (iii) $BocNHNH_2$, DIEA/DCM

The *p*-alkoxybenzylalcohol (Wang) linker is very versatile and has been used as the basis of a wide range of *C*-terminal functionality, both directly and indirectly[40]. A modified version of the Wang linker, shown below (Figure 2.19), was proposed. Wang and Merrifield originally applied this linker to the synthesis of peptide hydrazides in 1969[28] (Figure 2.20). The desired hydrazide, **2.20**, was obtained through the hydrazinolysis of the *p*-alkoxybenzylformate species, **2.19**, a procedure which involved

the treatment of the resin with anhydrous hydrazine for a period of 6h. The initial ester was prepared by the reaction of the *p*-alkoxybenzylalcohol resin, **2.16**, with phenyl chloroformate and pyridine at 0°C overnight.

Figure 2.19 *Wang resins*

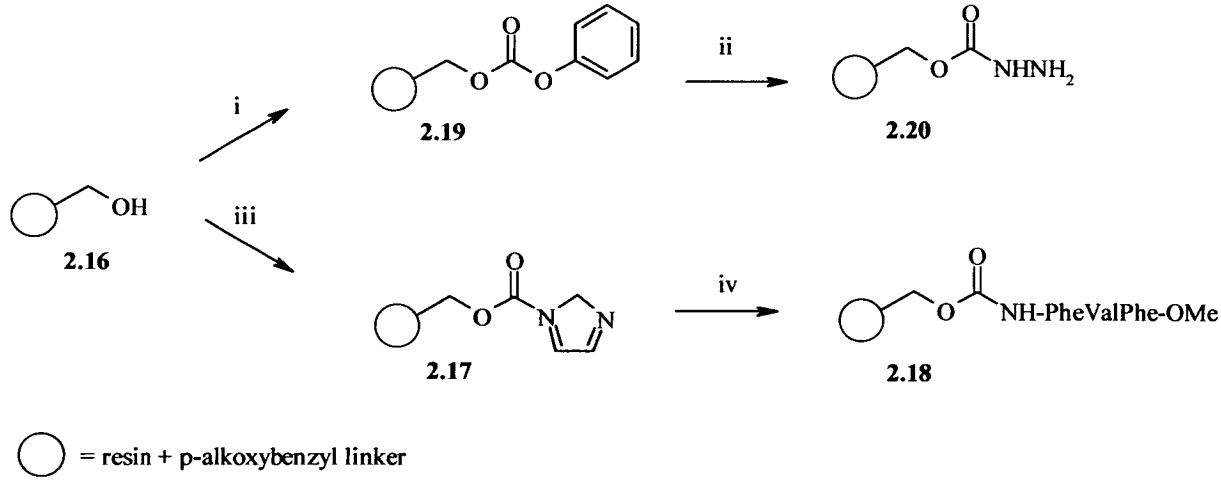


p-alkoxybenzyl alcohol (Wang) resin

p-alkoxybenzyloxycarbonylhydrazide resin

In 1995, Hauske and Dorff[41] used commercially available Wang resin for the preparation of the *p*-alkoxybenzyl-imidazolide carbamate species, **2.17**, as a substitute for the corresponding chloroformate, which could not be readily prepared. This was intended for use as a TFA-cleavable linker in the synthesis of non-peptide libraries. Protected amino acids were coupled directly to this linker and it was therefore reasoned that Fmoc-hydrazine could be reacted with the *p*-alkoxybenzyloxyimidazole carbamate, to give the Fmoc protected *p*-alkoxybenzylalcohol-hydrazide, **2.18** (Figure 2.20).

Figure 2.20 *Modification of Wang resin*



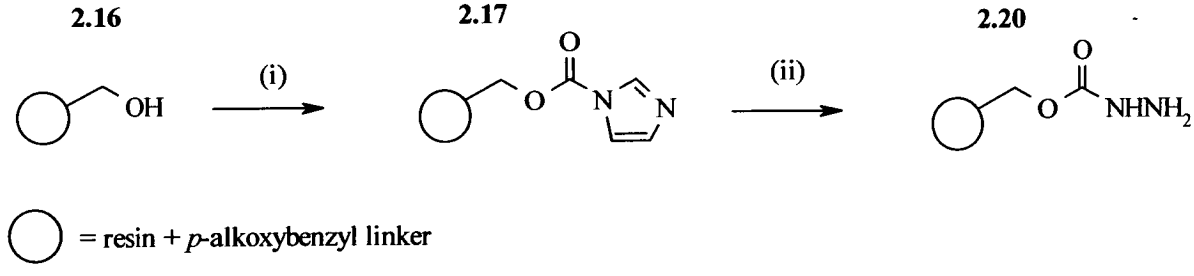
(i) phenyl chloroformate, pyridine/DCM; (ii) anhydrous $NHNH_2$ / DMF; (iii) carbonyl diimidazole/THF; (iv) PheValPhe-OMe.HCl, *N*-methylpyrrolidinone, *N*-methylmorpholine/THF, Δ.

2.4.8.2 Linker Synthesis

The *p*-alkoxybenzyl-imidazolide carbamate resin was prepared, according to the procedure described in the literature[40]. The presence of the desired product was confirmed by the detection of the imidazolide stretch in the IR spectrum (1755cm^{-1}).

This resin was then treated with Fmoc hydrazine. Upon carrying out an Fmoc loading test, no Fmoc was detected, although the imidazolide peak was no longer visible by IR spectroscopy. Since the resin had been heated to 60°C, it was possible that the Fmoc protection had been removed *in situ*, and a ninhydrin test[42] was therefore carried out, in order to determine whether a free amine was present. A sample of the Wang-imidazole resin was also tested to ensure that a false positive would not be obtained. The ninhydrin test indicated that some form of free amine was present, so it was probable that the hydrazine species had been formed. In order to confirm this, a test peptide was prepared and subjected to analysis (see Section 2.4.8.4). Mass spectrometry confirmed the presence of the desired peptide hydrazide (peptide **14**).

Figure 2.21 *Wang-hydrazine linker, final prep.*



(i) 1,1 carbonyldiimidazole/THF, N_2 (ii) $FmocNHNH_2$,DIEA/THF,N-Methylpyrrolidine, Δ.

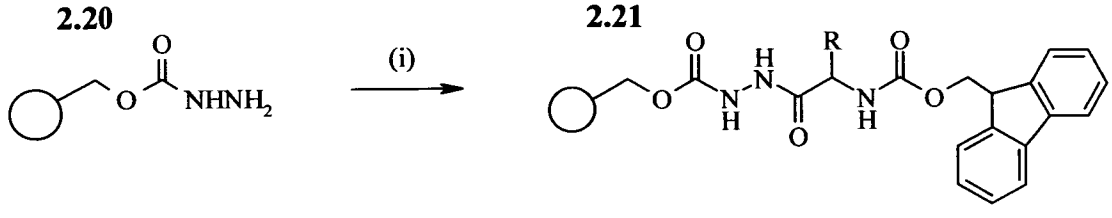
Having successfully prepared the unprotected peptide hydrazide, milder reaction conditions were investigated in an attempt to retain the *N*-Fmoc protection. None of these attempts proved successful. The preparation of a more active alternative to the imidazole species, the Wang-chloroformate, was attempted and some reaction with Fmoc-hydrazine was detected, however the yield of the initial reaction was poor and resins of suitably high functionality could not be prepared. It was therefore decided to

continue with the original method. Although it may seem wasteful, Fmoc-hydrazine is a more convenient reagent than anhydrous hydrazine, since it can be freshly prepared in high purity and is dried easily.

2.4.8.3 Resin Loading

Since the unprotected hydrazine was the final species desired for peptide synthesis, the *in situ* removal of the Fmoc protection was not regarded as an immediate problem. However, the presence of Fmoc group would have provided a convenient means to quantify the initial resin functionality. Since this test was no longer possible, an alternative method had to be employed. The quantitative ninhydrin test[43] could be used, and it is also possible to detect the presence of resin bound hydroxyl groups[44]. The most consistent results were obtained by the manual loading of the resin with the first Fmoc amino acid of the relevant sequence (Figure 2.22), followed by an Fmoc loading test. The maximum loading achieved compared favourably with the initial Wang-OH functionality and a qualitative ninhydrin test of this resin proved negative, indicating that no further free hydrazine groups were accessible. The capping procedure carried out on the automated synthesiser prior to the deprotection step acts as a further safeguard against the formation of deletion peptides.

Figure 2.22 *Wang-hydrazine linker, resin loading.*



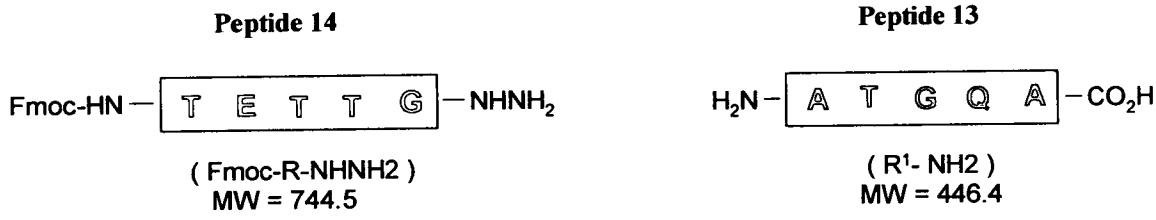
◯ = resin + *p*-alkoxybenzyl linker; (i) Fmoc-amino acid, HOCt, DIC/DMF; R = amino acid side chain

To avoid the participation of any unreacted Wang-OH groups, the method used for loading the resin was free of the 4-dimethylaminopyridine (DMAP) which would normally be employed as a catalyst. The HOCt ester was formed directly and coupled to the free hydrazine. The initial resin functionality was 0.64 mmol/g. The maximum loading achieved was 0.4 mmol/g, both Gly and Ala were loaded successfully.

2.4.8.4 Test Peptides

A peptide containing five amino acids (peptide **14**) was synthesised using the Wang-hydrazine resin. The sequence was identical to the 5mer previously prepared using the semicarbazide linker (peptide **12**), in order that TAEC coupling profiles could be compared. Cleavage was carried out in 90% TFA and was complete in two hours. Mass spectrometry implied that the peptide hydrazide had been successfully prepared and a small scale coupling reaction was therefore attempted.

Figure 2.23 *Peptide for trial coupling (II)*



The reaction was monitored by HPLC and electrospray mass spectrometry, as before. The peptide was stirred in DMF with HOCt until it had dissolved. Following the addition of *t*BuONO, a new peak was observed after 30mins. The second peptide (peptide **13**) was added after 45 mins, and the ligated product peak (peptide **25**) was observed after 1hour. The peptide acid was also observed and, as expected, the peptide hydrazide was considerably more reactive than the peptide semi-carbazide. It was clear that the Wang based hydrazide linker had provided a peptide of the correct functionality which was capable of undergoing coupling.

Table 2.3 *Coupling methods compared*

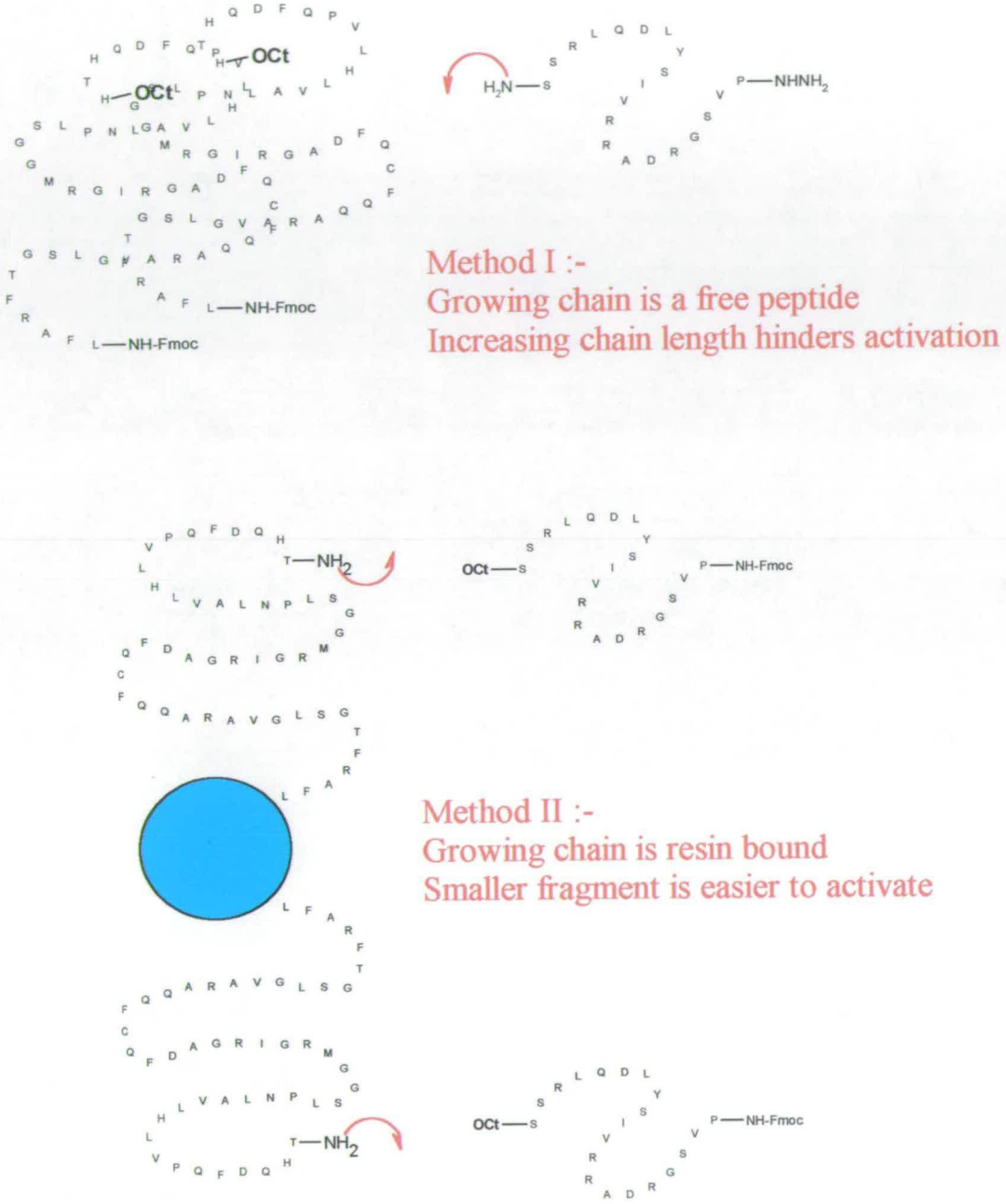
	Azide Method	Semicarbazide	Hydrazide
Active Ester observed	1 – 4h	2.5h, Rt: SM+1.5min.	0.5h, Rt: SM+1min.
Product observed	1-4 days	16h	1.0h

2.4.9 Sequential Coupling – Resin Based

2.4.9.1 Overview

Since the eventual aim of this project was to couple several large peptide fragments in a sequential manner, it was decided to carry out trial coupling reactions involving more than one fragment. The difficulties encountered with the large semi-carbazide fragments were considered. It was concluded that both fragment size and the accessibility of the activated *C*-terminus were important factors. Although the peptide hydrazide is significantly more active, the activated *C*-terminus would probably become more and more hindered as chain length increases, thus drastically lowering yields. An alternative coupling strategy was therefore proposed – that of resin based segment coupling, with successive fragments being added in the *C* to *N* direction. This would proceed in an analogous manner to SPPS, with capping and *N*-deprotection steps being carried out following each coupling reaction. The use of such a strategy would mean that the species to be activated in solution would be a relatively short, soluble single fragment. The coupled product would be resin bound, thus aiding purification and also helping to anchor/solubilise the growing chain, as illustrated in the following diagram (Figure 2.24).

Figure 2.24 *Coupling Strategies*



2.4.9.2 Resin Based Coupling – Initial Trial

The 5aa fragment (peptide **14**) prepared in Section 2.4.8.4 was coupled to a second, resin-bound fragment (peptide **15**). The conditions used were identical to those of solution based coupling. The reaction was monitored by HPLC (Figure 2.25) and the formation of the peptide-active ester could be detected as before. Since the desired product was resin bound, the reaction was stopped following the disappearance of this active ester peak. Some hydrolysis had occurred to give the peptide acid, but this was to be expected since the peptide active ester was present in excess. The resin bound product was detected by testing for the reappearance of the Fmoc group. From this it was clear that the reaction had proceeded in reasonable yield, and as confirmation, the product (peptide **26**) was cleaved from the resin and analysed.

Figure 2.25 *Trial Coupling (III) – monitored by HPLC*

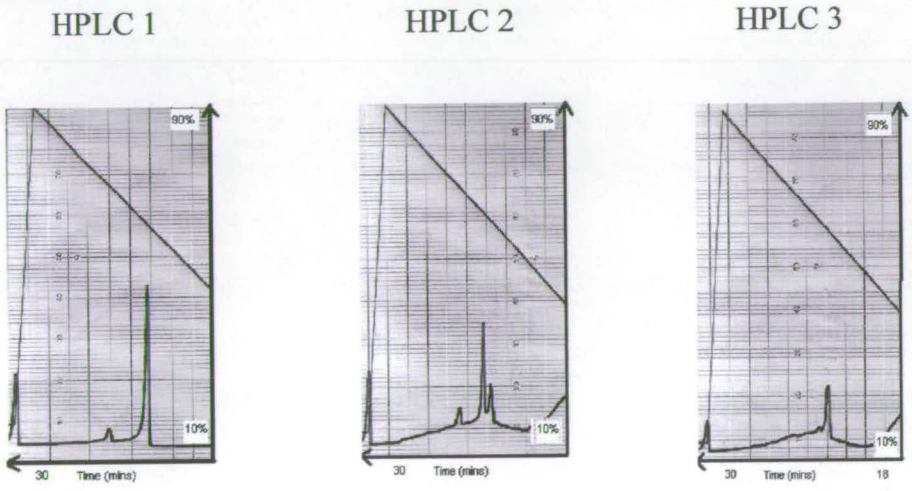


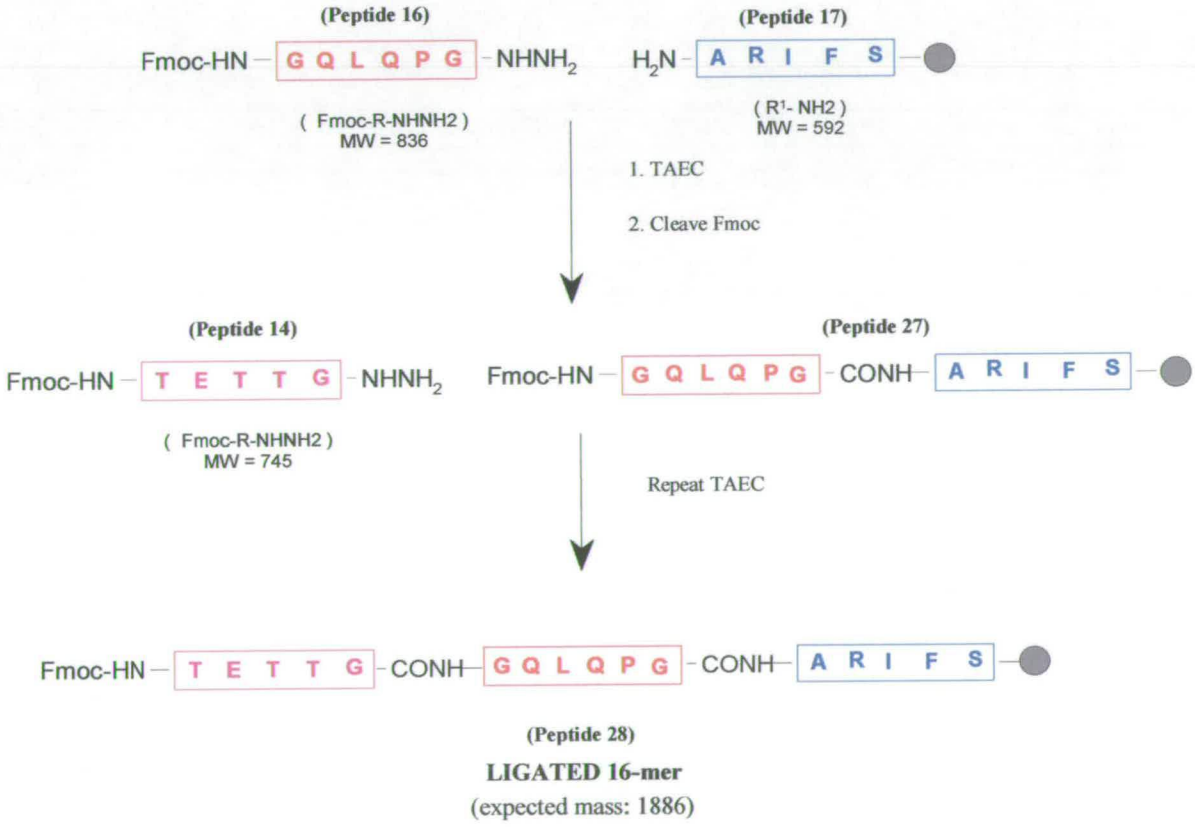
Table 2.4 *Trial coupling (III) – reagents & conditions*

	Reagents added / conditions	HPLC Profile
HPLC (1)	Peptide **14**; HOCt / DMF, RT, stirred 2h.	**Major Peak – Peptide 14 (Rt 14.4 min, 47% AcCN)**
HPLC (2)	tBuONO / 0°C, stirred 0.5h.	**Major Peak - Active Ester (Rt 15.4 min, 50% AcCN);** Minor peak - Peptide Acid (Rt 14.6 min, 48% AcCN)
HPLC (3)	Peptide **15-*Resin***, DIEA / RT, stirred overnight.	Major Peak - Peptide Acid (Rt 14.6 min, 48% AcCN)

2.4.9.3 The Synthesis of a 16aa Peptide Fragment *via* Resin Based Sequential Segment Coupling

Three peptide fragments were selected for use in the segment coupling reactions, two of which were derived from the second Endostatin coupling site. The *C*-terminal fragment (peptide **17**) was synthesised by SPPS using unmodified Wang resin. A trial cleavage was carried out to ensure the synthesis had been successful. The remainder of the peptide was retained on the resin, with its side chain protection intact. The remaining two fragments (peptides **14** & **16**) were synthesised using the Wang-hydrazine resin. The fragments are shown in Figure 2.26.

Figure 2.26 *Resin based sequential segment coupling*



Having successfully prepared the desired peptide hydrazides, the initial TAEC coupling to the resin bound fragment (peptide **17**) was attempted. Following the first coupling, the yield based on the Fmoc loading was 35%. The Fmoc protection was removed and the resin was washed and resubmitted for a second coupling reaction, which was carried out using the same procedure. The active ester peak had disappeared after 0.5hrs and the reaction was stopped after 1hr. A final Fmoc loading test indicated that an overall yield of 22% had been achieved. The peptide products were cleaved from the resin and isolated using standard conditions. The resulting peptide was analysed and proved to be a mixture of products. Mass spectrometry and amino acid analysis detected both the desired 16mer (peptide **28**) as well as the 11aa deletion peptide, indicating that an intermediate capping step might be necessary in future reactions. A third component, the unreacted, deprotected peptide **17** was also detected. The products were sufficiently different in sequence as to be separable by HPLC.

2.4.9.4 Optimisation of Coupling Conditions

Having proven that resin based coupling was possible with small fragments, a second set of reactions was carried out using peptides of approximately 10aa in length (peptides **18 – 21**). The purpose of this investigation was two-fold. The primary purpose was the optimisation of the coupling conditions in an attempt to improve yields and, in addition, the use of larger fragments would provide further information with regard to the fragment size threshold.

The various reaction conditions are tabulated below (table 2.5). It should be noted that the stop time denotes the time after which each reaction was worked up. In general, the active ester peak had appeared after 30min and had decreased to a constant height after 1.5-2hrs, as was observed for the smaller fragments. The times in brackets indicate when the final aliquot was removed for analysis.

Table 2.5 *Optimisation of coupling conditions*

Reaction number / peptide numbers	Solvent	HOCt concentration.	Resin concentration.	Peptide Hydrazide excess (approx.)	DIEA addition (time after resin)	Reaction time – by HPLC.	Stop time. (relative to active ester formation)	%Yield (based on final Fmoc)
1. / 29	DMF	1mmol/ml	12.5mg/ml	3eq	0mins	2.0h	2.5h, stirred, RT	56%
2. / 30	DCM	1mmol/ml	12.5mg/ml	3eq	0mins	1.5h	2.0h, stirred, RT	59%
3. / 31	DMF	2mmol/ml	50mg/ml	2eq	30mins	1.5h, (3.0h)	3.5h, stirred, RT	100%
4. / 33	DMF	2mmol/ml	35mg/ml	3eq	5mins	2.0h	2.5h, sonicated	40%
5. / 32 (alternative sequence)	DMF	2mmol/ml	50mg/ml	2eq	15mins	1.5h, (3.0h)	3.5h, sonicated	80%
6. HOAt	DMF	2mmol/ml	50mg/ml	2eq	0mins	2.0h	2.5h, stirred,RT	No Rxn.

From the conditions tested, DMF proved the best solvent, since the peptide hydrazide/HOCt mixture took almost twice as long to dissolve in DCM, although the active ester formation itself was unaffected. It was found that larger fragments couple better at higher concentration, although resin swelling must be considered. The resin was added dry in order that the HOCt concentration would remain constant.

As can be seen from table 2.5, reactions 3 and 5 show increased yields. Although reactions were continued for an increased length of time, no further change was observed by HPLC after 1.5h.

In addition to the increased concentration, it is possible that delaying the addition of DIEA for at least 15mins following the addition of the resin has helped to improve the yield of the reaction. Resin swelling may effect the distribution of reagents, such that the DIEA is best added after swelling has had time to occur, especially when such high resin concentrations are necessary. Preswollen resin could avoid this problem, but this would decrease the reagent concentration. The addition of DIEA also considerably increases the amount of liquid present. Since the reactions were carried out on a relatively small scale, slight changes in procedure can have a large impact. However, even if such conditions are directly aiding the coupling reaction, this procedure can only

be adopted when the coupling is non-sequential, or is carried out in the *C* to *N* direction. This is due to the fact that *t*BuONO was present in a slight excess, such that the free peptide hydrazide could itself undergo active ester formation were the HOCt not neutralised immediately.

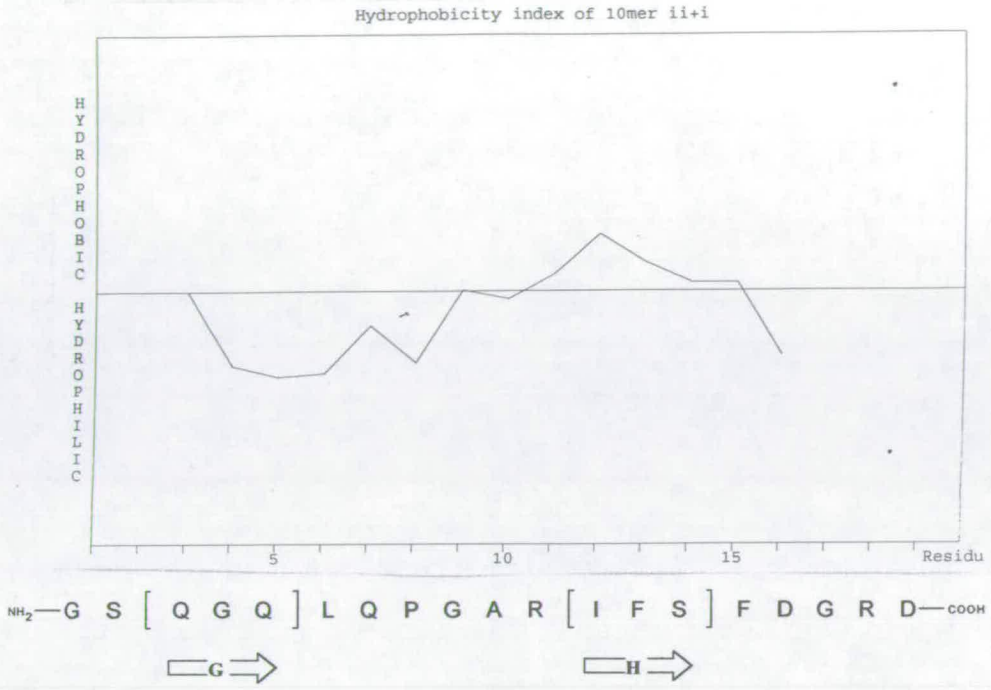
Sonication became the method of choice for agitation when the peptide hydrazide solution prepared in Reaction 4 became gel-like. Sonication is also considered less likely to break down the resin beads. The use of HOAt as the coupling agent was also investigated but was unsuccessful due to its poor solubility in DMF, the solvent of choice for the peptide fragments.

The synthesis of a 29aa peptide (peptide **34**), assembled from three 9-10aa fragments (peptides **18+19+21**), was also carried out on a small scale. The coupling conditions were based on 2mmol/ml of HOCt in DMF. The resin bound 19mer (peptide **33**) was treated with 20% piperidine in DMF prior to coupling, however, due to limited material, no capping procedure was carried out. The yield based on the final Fmoc loading was 20%, with individual yields being 40% and 50% respectively. Some 29mer product was detected by mass spectrometry, although it could not be isolated by HPLC. As expected, the deletion peptide - peptide **18** coupled to peptide **21** - was also detected.

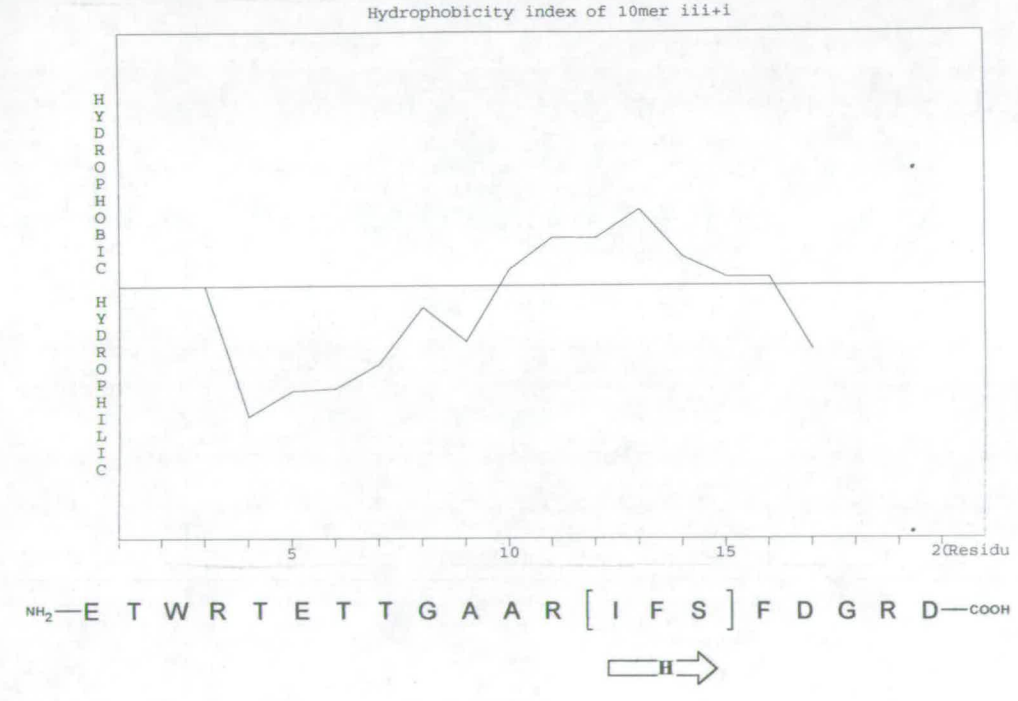
It is possible that the gel-like nature observed for peptide **19** was due to secondary structure formation. On comparison with the crystal structure (Figure 2.1), peptides **19** – **21** all contain regions that are part of β–sheets (Figure 2.27). Peptide **18** does not contain a β–sheet forming region, so it should be less likely to aggregate. This could explain why the deletion peptide formed in appreciable yield during the synthesis of peptide **34**. The 5aa peptides **16** and **17** share these β–sheet forming regions, containing the Gln-Gly-Gln and the Ile-Phe-Ser patterns respectively.

Figure 2.27 *Coupled 20mers - comparison of hydrophobicity and β-sheet regions (square brackets).*

(i) True Sequence – **19 + 21**



(ii) Deletion Peptide – **18 + 21**



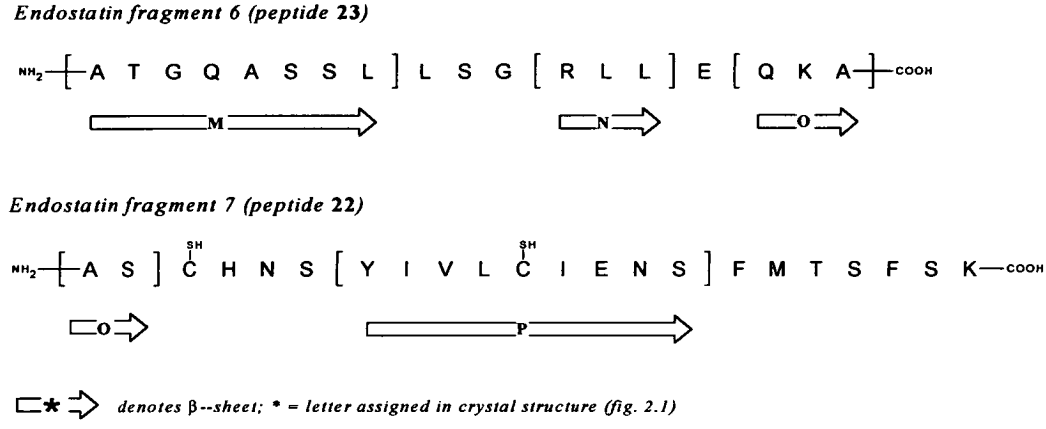
*denotes β--sheet; * = letter assigned in crystal structure (fig. 2.1)*

2.4.9.5 Resin Based Coupling - Large Fragments

With the coupling of 10mer fragments having proven successful, the Endostatin fragments 6 and 7 (peptides **23** & **22**; Figure 2.14) were prepared. Fragment 6, peptide **23**, was synthesised using the hydrazide linker. Synthesis proceeded smoothly and in good yield. The synthesis of the resin-bound peptide acid, **22**, is discussed in Section 2.4.5.1.

Referring to the crystal structure (Figure 2.1), it can be seen that fragments contain two of the longest β-sheet regions (Figure 2.28). Secondary structure formation was therefore anticipated.

Figure 2.28 *Fragments 6 & 7 - β-sheet regions (square brackets)*



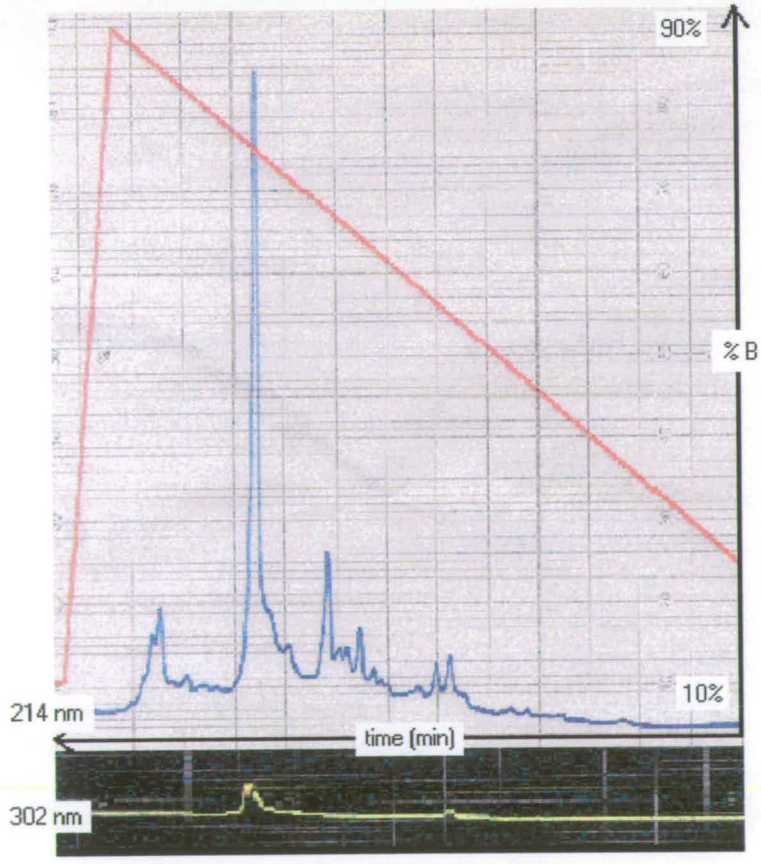
The coupling of the 20aa Endostatin fragments, peptides **23** and **22**, was next attempted. The results are summarised in table 2.6. In contrast to the semi-carbazide fragment, where the active ester could not be detected (Section 2.4.7), active ester formation was observed by HPLC after 30 minutes. Concerns regarding the solubility and potential for secondary structure formation meant initial attempts used increased quantities of solvent. However, it became clear that, for coupling to be successful, a more concentrated solution was required, the increased yield of reaction 3 reflects the efficacy of this measure. It should also be noted that, having gained an idea of approximate reaction times, reaction 3 was not monitored as closely as previous attempts and was therefore not disturbed to the same degree.

Table 2.6 *20mer coupling – conditions & results*

Reaction number	Solvent	HOCt concentration.	Resin concentration.	Peptide Hydrazide excess (approx.)	Stop time. (relative to active ester formation)	%Yield (based on final Fmoc)
1.	DMF	0.7mmol/ml	50mg/ml	2eq	2.5h, sonicated	10%
2.	DMF	1mmol/ml	80mg/ml	2eq	16h, sonicated	10%
3.	DMF	2mmol/ml	160mg/ml	2eq	3.5h, sonicated	64%

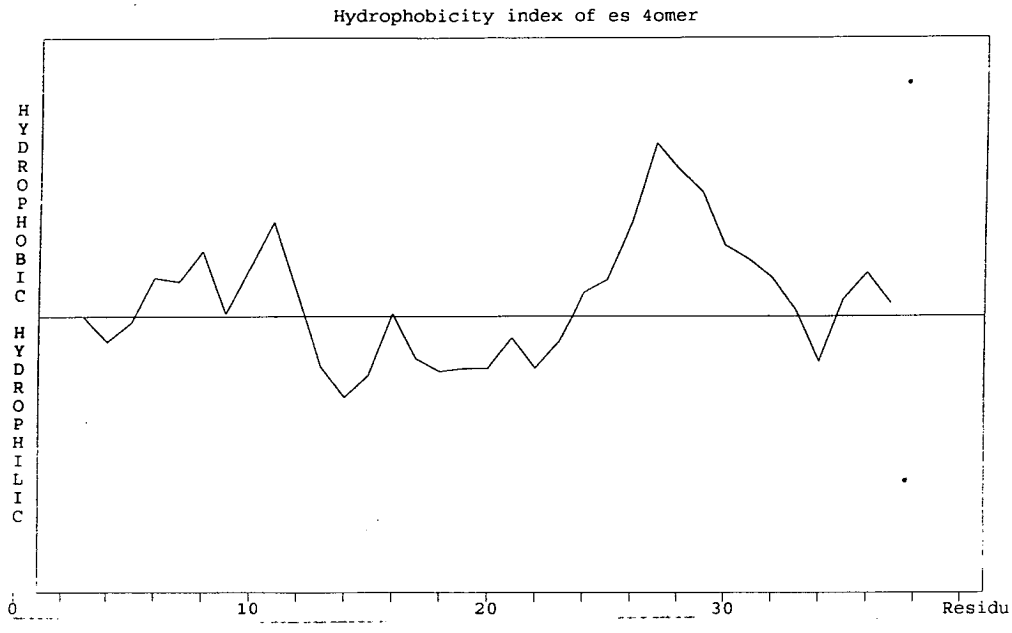
The product of reaction 3 (peptide **35**) was cleaved (using standard conditions for 4hrs) and analysed. Analytical HPLC showed a large new peak at $\lambda = 302$, indicating the presence of a product containing Fmoc (Figure 2.29). A product with molecular weight corresponding to that of the coupled 40mer was detected by mass spectrometry, using both manual and computer based deconvolution.

Figure 2.29 *Reaction 3 – Analytical HPLC; RP18 C_{18}; A=H_2O, 0.1%TFA, B=CH_3CN; 2ml loop, 1ml/min; 0-2min 10%B, 2-32min 10-90%B; λ=302nm*



The peptide was found to be extremely insoluble in AcCN/H_2O, as corroborated by the hydrophobicity plot (Figure 2.30), and purification difficulties similar to those encountered when the peptide was prepared by stepwise methods (Section 2.3) were experienced.

Figure 2.30 *40-mer; peptide* **35** *- hydrophobicity*



The initial HPLC studies had shown that the material was reasonably pure, however further purification by HPLC was unsuccessful due to equipment failure. In addition, some form of contamination seemed to have occurred during the process and insufficient material was available for alternative purification methods to be explored. Nevertheless this reaction has shown that the coupling of fragments of at least 20aa in length is possible using peptide hydrazides, together with the TAEC method. Were the entire peptide to be prepared, purification at this 40-mer stage would not be necessary. Indeed, the latter part of the sequence is considerably less hydrophobic (Section 2.1.3) and, relatively speaking, the peptide as a whole is unlikely to be as insoluble. In addition, its increased size would permit the use of purification methods other than HPLC, for example size exclusion chromatography, ion exchange chromatography, FPLC or TbFmoc/polystyrene techniques.

2.5 Conclusions and Future Work

The experiments to date have shown that the *p*-alkoxybenzylyoxycarbonylhydrazide linker works well and provides peptide hydrazides suitable for use in TAEC. In addition, the TAEC method has been shown to work for fragments of up to 20aa in length. TAEC has been found to be a useful coupling method in its own right which successfully complements existing methods for stepwise peptide synthesis.

In future studies, the sequential coupling of more than two large fragments remains to be carried out, followed by the cleavage of the remaining side chain protection. It would also be useful to investigate the reattachment of the *C*-terminal fragment to a new resin[45], following cleavage and purification, as opposed to carrying out coupling onto the uncleaved species immediately following synthesis. As well as improving purity, this technique would allow milder conditions to be employed for the final cleavage of the coupled product. In addition, the final cleavage of Tnm could be carried out on this resin bound product, since all the fragments would now have been subjected to TFA treatment and the cleavable diol moiety would be in place throughout the resin bound product.

It would also be interesting to apply the resin/hydrazide-based TAEC technique to the synthesis of a peptide which, unlike Endostatin, has previously been successfully prepared by stepwise SPPS; in order that respective yields might be compared. The synthesis of Erythropoietin (EPO) is possible using SPPS, and work has already been carried out towards its preparation using solution phase, semi-carbazide based TAEC[36]. This approach was unsuccessful, however, and a repeated attempt using the hydrazide methodology is likely to have more success.

In the longer term, the application of this work within a clinical area would be desirable. The ultimate goal would of course be a reliable procedure for the preparation of the entire protein by SPPS. In previous trials, endostatin was administered as a non-refolded suspension, suggesting that the primary or secondary, rather than the tertiary, structure is responsible for its activity. Should this be the case, the fragments that have been prepared by stepwise SPPS could prove useful in the identification of the active site of the protein.

[1] Boehm T., Folkman J., Browder T. and O'Reilly M.S., *Nature*, 1997, **390**, 404.
[2] O'Reilly M.S., Boehm T., Shing Y., Fukai N., Vasios G., Lane W.S., Flynn E. and Birkhead J.R., *Cell*, 1997, **88**, 277.
[3] Millauer B., Shawver L.K., Plate K.H., Risau W. and Ullrich A., *Nature*, 1994, **367**, 576.
[4] Kim K.J., Li B., Winer J., Armanini M., Gillett N., Phillips H.S. and Ferrara N., *Nature*, 1993, **362**, 841.
[5] Parangi S., O'Reilly M., Christofori G., Holmgren L., Grosfeld J., Folkman J. and Hanahan D., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 2002.
[6] O'Reilly M.S., Holmgren L., Shing Y., Chen C., Rosenthal R.A., Moses M., Lane W.S., Cao Y., Sage E.H. and Folkman J., *Cell*, 1994, **70**, 315.
[7] Harris A.L., *Lancet*, 1998, **351**, 1598.
[8] O'Reilly M.S., Boehm T., Shing Y., Fukai N., Vasios G., Lane W.S., Flynn E. and Birkhead J.R., *Cell*, 1997, **88**, 277.
[9] O'Reilly M.S., Holmgren L., Chen C.C. and Folkman J., *Nature Med.*, 1996, **2**, 689.
[10] Boehm T., Folkman J., Browder T., and O'Reilly M.S., *Nature*, 1997, **390**, 404.
[11] Black W.R. and Agner R.C., *Nature*, 1988, **391**, 450.
[12] Zhang, L., Chen, Q.R,, Mixson A.J., *Curr. Genomics*, 2000, **1(2)**, 17.
[13] Blezinger, P., Yin, G., Xie, L., Wang, J., Mater, M., Bishop, J.S., Min, W., *Angiogenesis*, 2000, **3**, 205.
[14] O'Reilly M.S., Holmgren L., Shing Y., Chen C., Rosenthal R.A., Moses M., Lane W.S., Cao Y., Sage E.H. and Folkman J., *Cell*, 1994, **70**, 315.
[15] Gately S., Twardowski P., Stack M.S., Patrick M., Boggio L., Cundiff D.L., Schnaper H.W., Madison L., Volpert O., Bouck N., Enghild J., Kwaan H.C. anhd Soff G.A., *Cancer Res.*, 1996, **56**, 4887.
[16] O'Reilly M.S., Holmgren L., Chen C.C. and Folkman J., *Nature Med.*, 1996, **2**, 689.
[17] Cao Y., Ji R.W., Davidson D., Schaller J., Marti D., Sohndel S., Mc Cance S.G., O'Reilly M.S., Llinas M. and Folkman J., *J. Biol. Chem.*, 1996, **271**, 29461.
[18] Rehn M., Pihlajaniemi T., *J. Biol. Chem.*, 1995, **270**, 4705.
[19] Rehn M., Pihlajaniemi T., *Proc. Natl. Acad. Sci. USA*, 1994, **91**, 4234.
[20] O'Reilly M.S., Boehm T., Shing Y., Fukai N., Vasios G., Lane W.S., Flynn E. and Birkhead J.R., *Cell*, 1997, **88**, 277.
[21] Yokoyama, Y., Dhanabal, M., Griffioen, A.W., Sukhatme, V.P., Ramakrishnan, S., *Cancer Res.*, 2000, **60(8)**, 2190.
[22] Grosios, K., *I. Drugs*, 2000, **3**, 799.
[23] Hohenester E., Sasaki T., Olsen B.R. and Timpl R., *EMBO J.*, 1998, **17**, 1656.
[24] Nishihara, K., Kanemori, M., Yanagi, H., Yura, T., *Appl. Environ. Microbiol.*, 2000, **66**, 884.
[25] Hohenester, E., Sasaki, T., Mann, K., Timpl, R., *J.Mol.Biol.*, 2000, **297**, 1.
[26] Sim B.K.L., O'Reilly M.S., Liang H. *et al*, *Cancer Res.*, 1997, **57**, 1329.
[27] Wang P., Layfield R., Landon M., Mayer R.J. and Ramage R., *Tetrahedron Lett.* 1998, **47**, 8711.
[28] Wang S.S. and Merrifield R.B., *J.Am.Chem. Soc.*, 1969, **91**, 6488-6493; Wang, S.S., *J. Am. Chem. Soc.*, 1973, **95**, 1328.
[29] Gross E. and Meienhofer J. (Eds)., *The Peptides: Analysis, Synthesis, Biology.*, Academic Press Inc., New York U.S.A., 1979, **1**, 197.
[30] Bodansky M., Klausner S.Y., *Synthesis*, 1974, 549.

[31] Robertson N., *Ph.D. Thesis*, University of Edinburgh, 1996.
[32] Gosden A., Macrae R. and Young G.T., *J. Chem. Soc. Perkin Trans. I*, 1977, **8**, 317.
[33] Bland L., *Ph.D. Thesis*, University of Edinburgh, 1999.
[34] Ramage, R., Biggin G.W., Brown A.R., Comer A.J., Davidson A., Draffan L.C., Jiang L., Morton G.H., Robertson N., Shaw K.T., Tennant, G., Urqhart, K. and Wilken J., *Proc. 4th Int. Symp. In Solid Phase Synth. and Combinatorial Chemical Libraries*, Edinburgh, 1995.
[35] Irving S.L., McInnes C. and Ramage R., *Tetrahedron Lett.*, 1993, **34**, 6599.
[36] Patterson, J.A. and Ramage, R., *Tetrahedron Lett.*, 1999, **40**, 6121.
[37] Zhang, R.E., Cao, Y.-L. and Hearn, M.W., *Anal. Biochem.*, 1991, **195**, 160.
[38] Bland L., *Ph.D. Thesis*, University of Edinburgh, 1999.
[39] Bodansky M., Klausner S.Y., *Synthesis*, 1974, 549.
[40] James, I.W., *Tetrahedron*, 1999, **55**, 4855.
[41] Hauske J.R., Dorff P., *Tetrahedron Letters*, 1995, **36**, 1589.
[42] Kaiser, E., Colescott R.L., Bossinger C.D., Cook, P.I., *Anal. Biochem.*, 1970, **34**, 595.
[43] Sarin V.K., Kent S.B.H., Tam J.P. and Merrifield R.B., *Anal. Biochem.*, 1981, **117**, 147.
[44] Kuisle, O., Lolo, M., Quinoa, E., Riguera, R., *Tetrahedron*, 1999, **55**, 14807.
[45] Tam, J.P., Tjoeng, F.S., Merrifield, R.B., *J.Am.Chem.Soc.* 1980, **102**, 6117.

3. The Investigation of a Novel Strategy for the Protection of Arginine in Solid Phase Peptide Synthesis

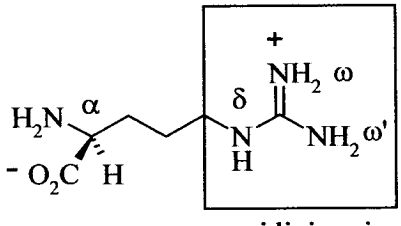
3.1 Introduction

3.1.1 Overview

As previously detailed in Section 1.2.3, the Fmoc strategy for SPPS was designed to allow both the amino acid side-chain protection and the peptide-resin bond to be cleaved simultaneously, under mild conditions using TFA. In doing so, this method attempts to minimise acid treatment of the peptide products, thus preventing their deterioration[1,2,3]. Most of the side-chain protecting groups used in the Fmoc strategy are easily cleaved by TFA. However, when the same acid conditions are used for the deprotection of the guanidino group of arginine, significant problems may be encountered.

These difficulties are due to the basicity of the arginine side chain, as shown below (Figure 3.1). The trifunctional guanidino group is strongly nucleophilic and is particularly prone to acylation. The pKa of the guanidinium proton is 12.

Figure 3.1 *Arginine*



guanidinium ion

Intramolecular δ-lactam formation (Figure 3.2) is also a possibility - this reaction competes directly with peptide bond formation. Nevertheless, the protection of all three nitrogens is not used in practice. Instead, electron-withdrawing groups are employed to block one or both of the ω–nitrogens, in an attempt to alter the electronics enough to decrease the nucleophilicity.

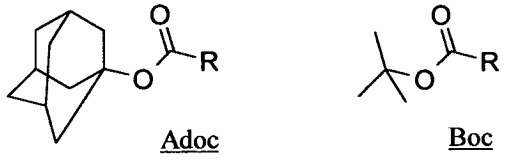
Figure 3.2 *δ-lactam formation*

R = protecting group; R' = active ester eg. -OCt, -OBt

3.1.2 Strategies for Arginine Side-Chain Protection

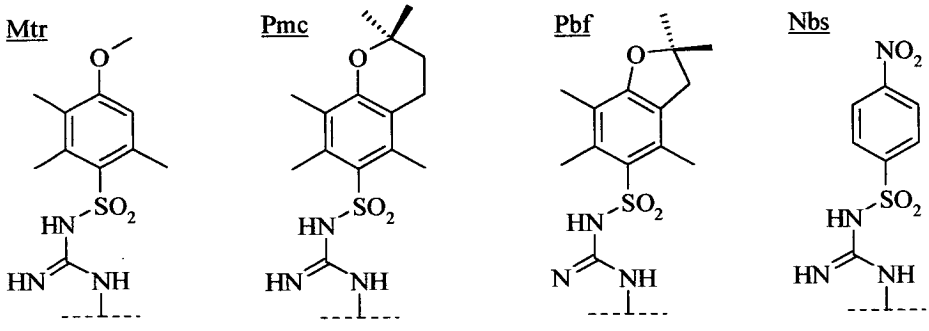
Previous methods of arginine protection have used nitration and urethane formation[4]. The nitro protection is difficult to remove, however, and was found to be prone to side reactions such as δ-lactam formation[4] (Figure 3.2). It has also been reported, by Merrifield, that the lactam is capable of transferring the protected amidino moiety onto amines, resulting in premature termination of the peptide chain[5]. Examples of urethane protection include adamantyloxycarbonyl (Adoc)[6] and Boc[7] (Figure 3.3). The urethane strategy was successful in some cases[8] but was largely abandoned when studies proved that the acylation of guanidine was not being prevented during peptide synthesis and that conversion to ornithine was taking place under the basic conditions required for cleavage of the Nα protection[9].

Figure 3.3 *Urethane protection*



Protecting groups in current use are based on the arylsulphonyl group, for example 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr)[10] and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc)[11,12,13] (fig.3.1.2.1). The Pmc group can be removed under relatively mild conditions, but this involves long cleavage times - up to 2h - which are often deleterious to peptide products. Subsequent developments in this area include the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl (Pbf) [14] residue, which has been reported to be slightly easier to cleave than Pmc. Nevertheless, the acidolytic cleavage of arginine still proceeds at a much slower rate than that of the groups employed to protect other amino acid side chains in the Fmoc strategy. This presents a problem, especially where peptides containing multiple Arg residues are involved. It seems evident that an entirely new strategy is needed.

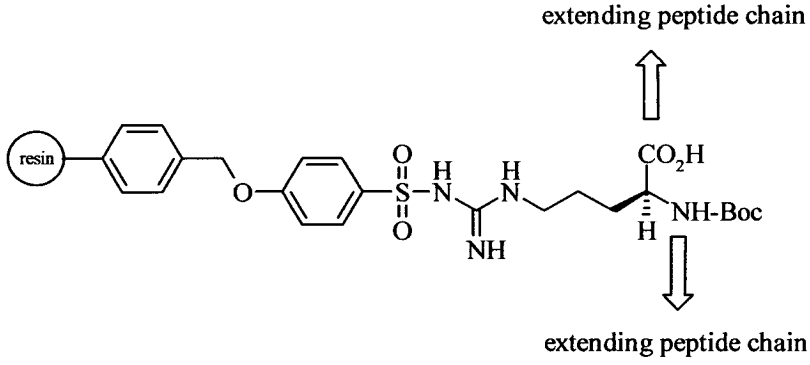
Figure 3.4 *Sulphonyl protection.*



In a tangential approach, the N_G-4-nitrobenzenesulphonyl-L-arginine (Nbs)[15] group (fig. 3.4) was developed to exploit an alternative cleavage mechanism for sulphonyl protection. Instead of attempting to improve TFA lability, the Nbs group is designed to be stable to TFA, for up to 24hrs. Following synthesis, the *tert*-butyl and other acid labile side chain protection is removed using TFA. Final cleavage of the Nbs-arginine residues is then carried out by treatment with a thiolate reagent, usually 2-mercaptoethanol, together with either DIEA or 30% piperidine in DMF. Nbs is stable to organic bases in DMF and is therefore suitable for use with Fmoc, or Nsc Nα protection. Upon addition of thiol to such a mixture, however, cleavage is rapid and is complete in less than 30 minutes. The product of Nbs cleavage has been identified as 2-(4-nitrophenylthio)ethanol. It has also been shown that the selective cleavage of Nbs in the presence of Nsc is possible, where DIEA is the organic base employed.

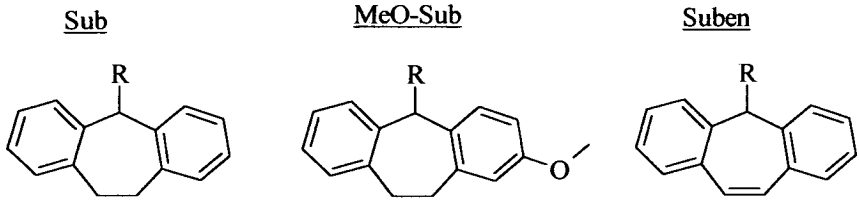
Another interesting strategy is the use of the sulphonyl moiety as a linker rather than as side chain protection[16]. The species was intended for use in the solid phase synthesis of small organic molecules, but was also investigated for use as a solid support in the synthesis of peptides containing arginine (Figure 3.5). The arginine was attached to the resin *via* its guanidine side chain and the rest of the peptide was extended in both directions, by coupling at both the *C* and *N* terminii. The resin is cleaved using HF and is compatible with both Boc and Fmoc methodology.

Figure 3.5 *Sulphonyl linker*



Recent work by Noda and Kiffe[17] has focused on the 10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5-yl [5-dibenzosuberyl(Sub)], 2-methoxy-10,11-dihydro-5H-dibenzo[*a,d*]cyclohepten-5-yl [2-methoxy-5-dibenzosuberyl(MeO-Sub)], and 5H-dibenzo[*a,d*]cyclohepten-5-yl [5-dibenzosuberenyl (Suben)] groups, previously used as linkers (Figure 3.6).

Figure 3.6 *Suberyl and suberenyl protection*



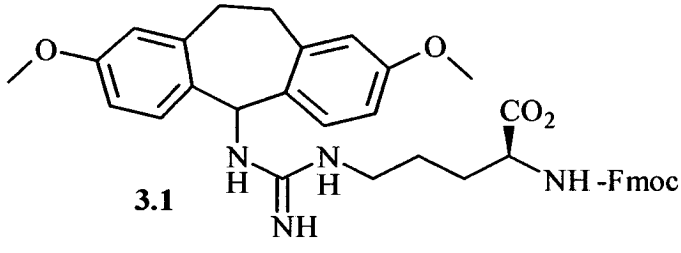
R = Arginine

Noda and Kiffe reasoned that, since Sub and Suben are readily cleaved under mild acid conditions to give the peptide amide, these groups would perhaps be more suited to Arg protection than the arylsulphonyls. In addition, they are not removed by either bases or nucleophiles and the carbonium cations, which originate under acid conditions, are stable and efficiently trapped by a thiol scavenger, meaning freed side chains are not at risk. When these groups were employed as Arg side chain protection, they did indeed prove to be significantly more acid labile than those previously employed, showing relatively short cleavage times (20 mins) at low TFA concentrations (5%).

3.1.3 Project aims

The aim of this project was to improve upon current arginine protection strategies by synthesising a further modified Sub/Suben style protecting group. The dimethoxy compound shown, **3.1**, (fig 3.7) would be subject to an increased inductive effect. It was therefore expected to show a higher degree of resonance stabilisation at the tricyclic carbocation, thus increasing the overall acid lability and ease of removal, whilst taking advantage of the associated stability to bases and nucleophiles.

Figure 3.7 *Dimethoxysuberyl-protected Arginine*



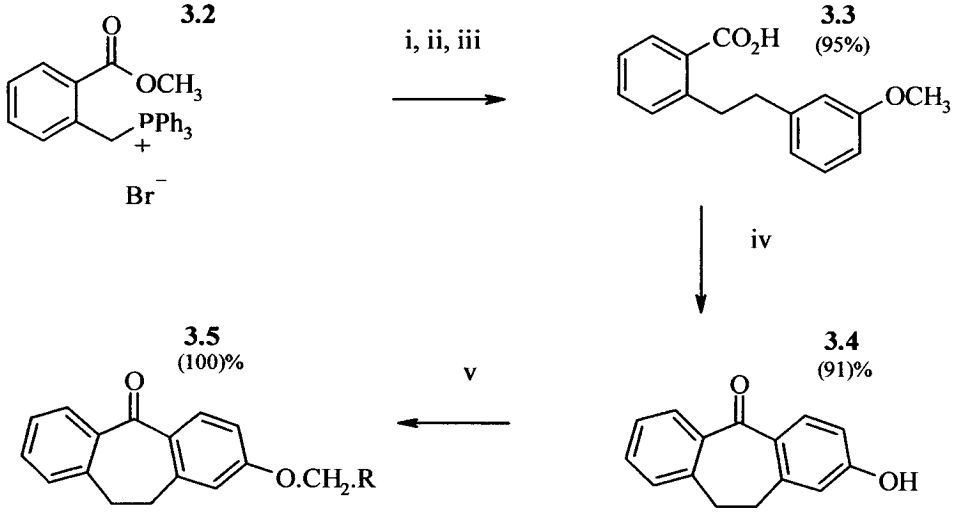
3.1

3.2 Synthesis Design

3.2.1 Previous Routes

When designing the synthesis of the dimethoxy species, **3.1**, (Figure 3.7), the synthetic routes to the suberyl linker/protecting group, which contains a single methoxy group, were first considered. The tricyclic amide linker below, **3.5**, (Figure 3.8) was synthesised by Ramage, Irving and McInnes[18], using Wittig chemistry as the key step.

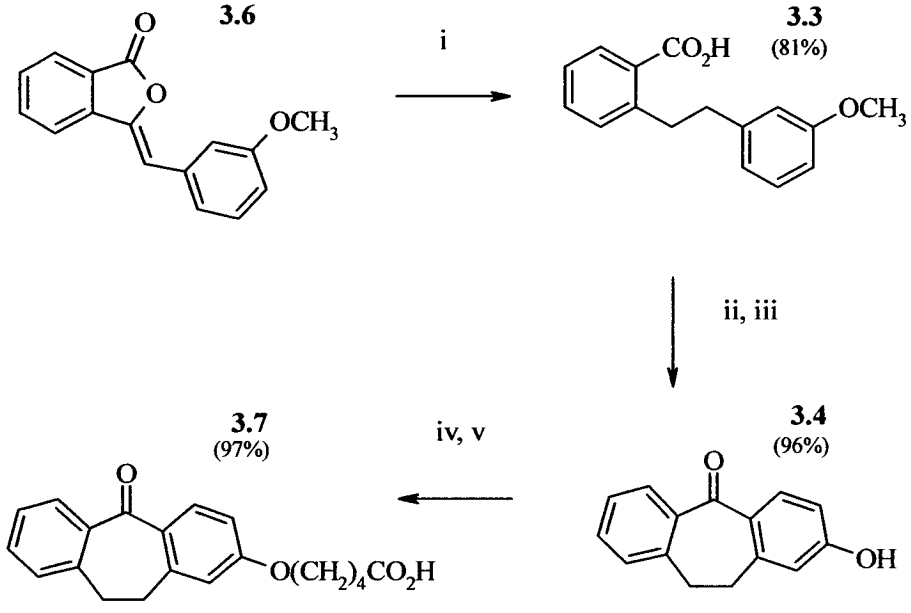
Figure 3.8 *Wittig-based Route*



(i) m-anisaldehyde, DBU/ dioxan, 50°C; (ii) NaOH(aq)/dioxan, reflux 45 min; (iii) H_2, Pd/C / MeOH; (iv) (a) $(COCl)_2$ / benzene, 3.5h. (b)excess $AlCl_3$, reflux 30min; (v) CsOH, chloromethylpolystyrene / DMF, 60°C, 4days; R=polystyrene.

Noda also prepared this species[19] for use as a linker, before applying it to the protection of arginine (Section 3.1.2). This alternative route used a Perkin reaction to prepare **3.6** (Figure 3.9) from the readily available starting materials of phthalic anhydride and *m*-methoxyphenylacetic acid.

Figure 3.9 *Noda Route*

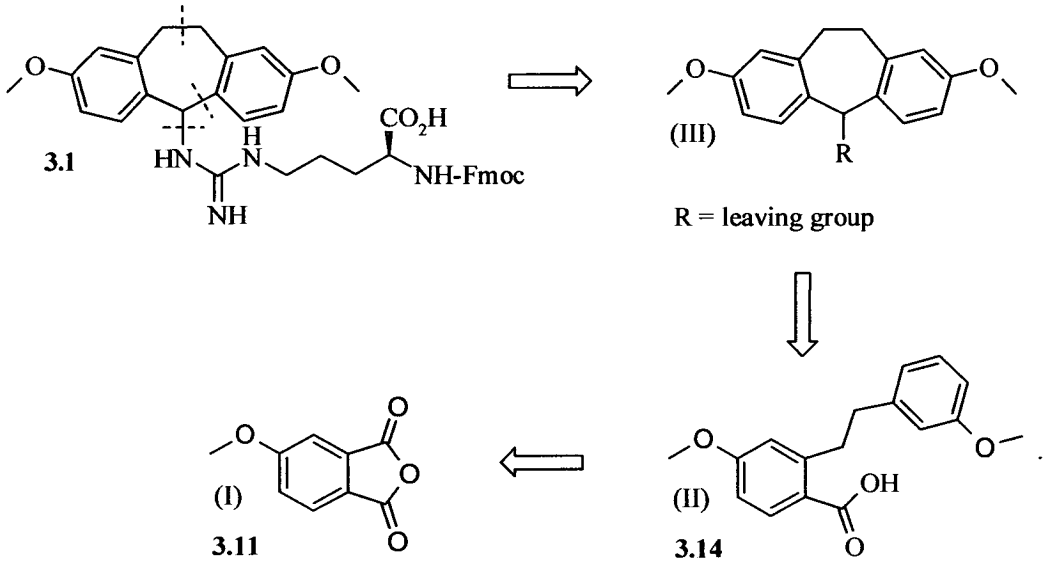


(i) Raney Ni, H_2 / THF; (ii) PPA; (iii) $AlCl_3$ / benzene; (iv) tert-BuOK, $Br(CH_2)_4COOC_2H_5$/DMF; (v) NaOH(aq)/dioxan

3.2.2 Proposed Route

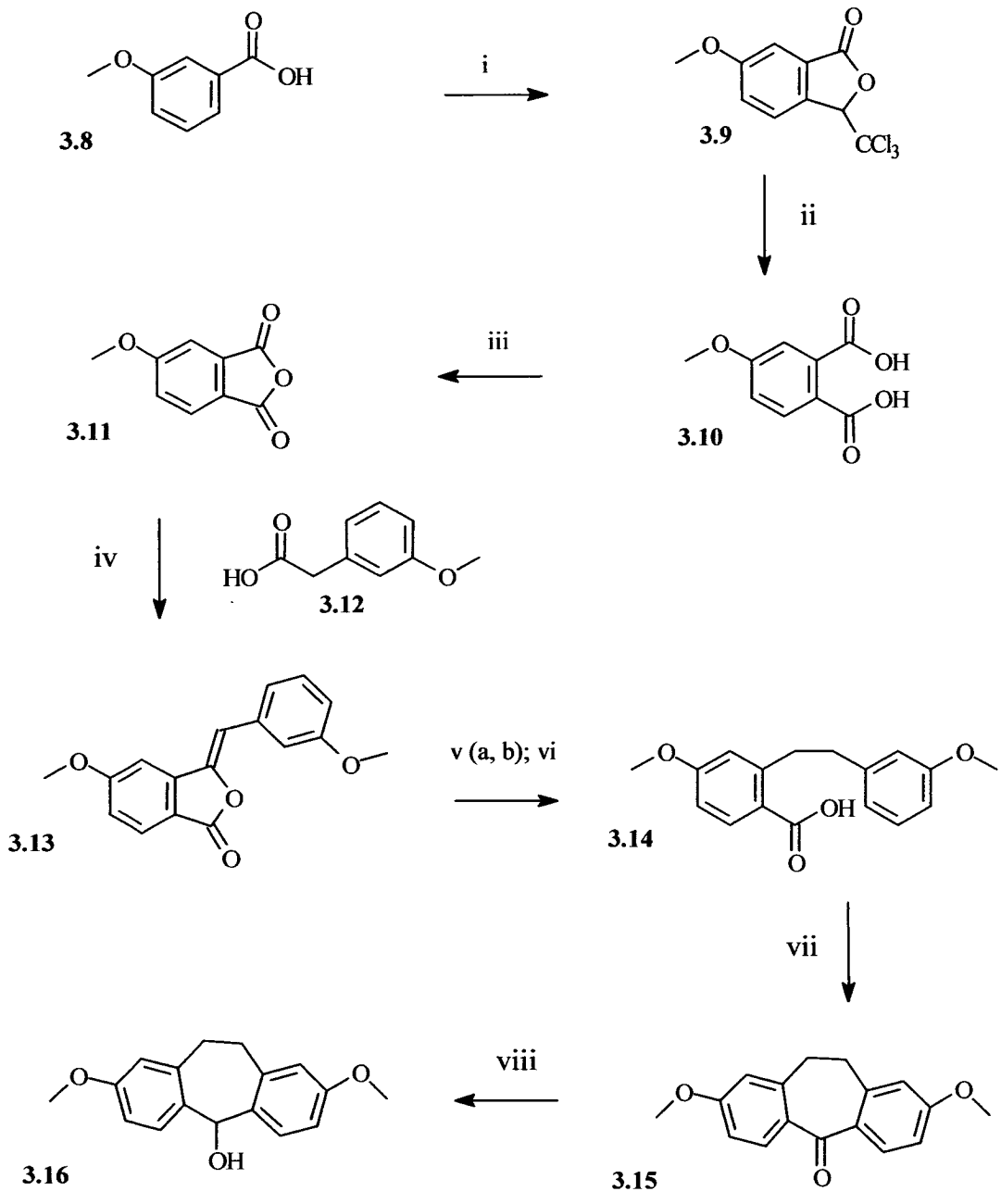
The key intermediates (I – III) of the proposed route to **3.1** are shown in the following diagram (Figure 3.10).

Figure 3.10 *Key intermediates*



The main factor for consideration in the synthesis of **3.1** is the incorporation of the additional methoxy group, which significantly affects the early stages of the route. The electron donating properties of the methoxy group result in strong *ortho, para* direction, giving an activated aromatic system with considerably altered properties. This renders the Wittig-based route unsuitable, since a system containing the ylid in the required position, *meta* to the methoxy group, would be disfavoured. The route employed by Noda is viable, however an analogous starting material cannot be purchased directly. The preparation of an aromatic system with suitable functionality was therefore investigated. Starting from *m*-anisic acid (**3.7**, Figure 3.11) a suitable molecule can be prepared in four steps. The route converges with that of Noda's linker synthesis at the Perkin reaction, which yields **3.13**, and follows a protocol originally outlined by Humber[20] for the preparation of **3.15** from **3.11**. The synthetic route, as far as key intermediate (III), is detailed in Figure 3.11.

Figure 3.11 *Reaction Scheme*



(i) chloral hydrate; conc. H_2SO_4 (ii) $KMnO_4$; aqueous NaOH; 0°C (iii) Ac_2O; Δ (iv) NaOAc; 265°C
(v) a. aqueous NaOH; Δ b. 3%HCl - pH 8.5 (vi) H_2; Pd/C (vii) PPA, Δ (viii) $NaBH_4$ / IPA.

3.3 Results & Discussion

3.3.1 The Preparation of 4-methoxyphthalic anhydride

The initial step in the synthesis concerned the introduction of a new carbon atom *via* electrophilic aromatic substitution, followed by an intramolecular cyclisation. This step proceeded smoothly and was amenable to scale up. The resulting system, **3.9**, contained the electron rich carbon-trichloride group, which was expected to permit a facile ring opening upon treatment with base, in order that the cyclic system might eventually be oxidised.

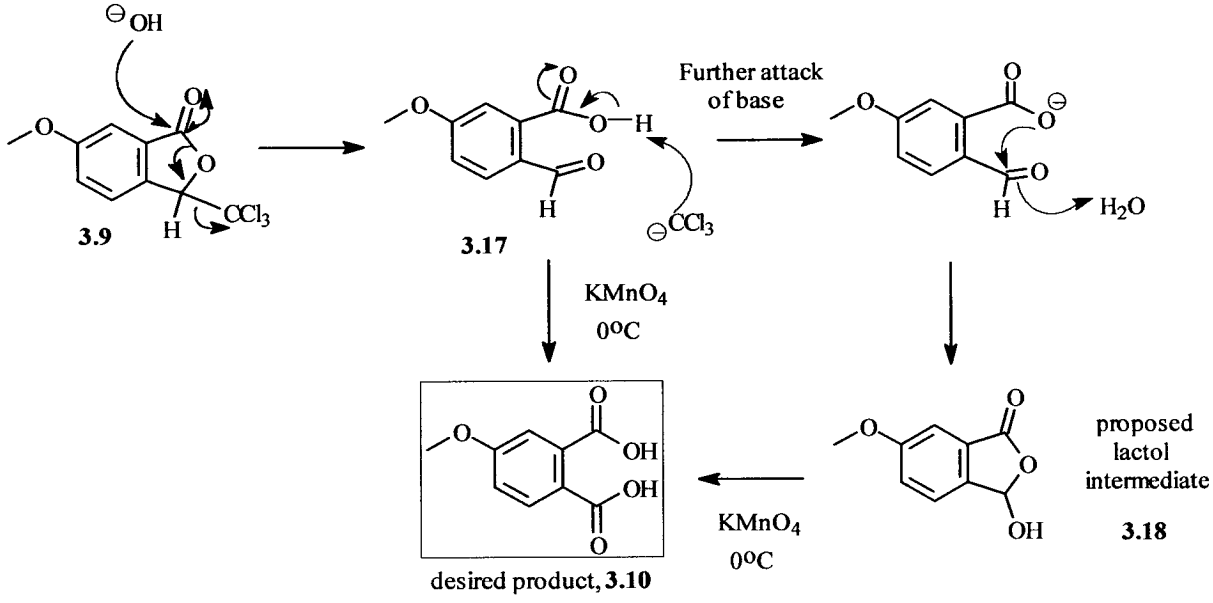
The following oxidation step[21,22] proved problematic. At first, yields were low and inconsistent. Various conditions were investigated and it was discovered that product formation occurred if the permanganate addition was halted at the halfway stage, that is after 0.6 equivalents had been added dropwise over a period of 5 hours. The reaction was then allowed to stir overnight before the remaining oxidant was added. Following acidification and extraction of the crystalline product, the filtrate was reoxidised, producing yields in excess of 70%.

It was postulated that the reagents were becoming trapped at an intermediate stage, since starting material was not recovered in significant quantities. A possible mechanism is as shown below (Figure 3.12). It seemed likely that the lactol intermediate, **3.18**, was being formed and was resistant to oxidation, thus making reoxidation necessary. This hypothesis was corroborated by the fact that an impurity with the corresponding molecular ion was observed by mass spectrometry.

Considering the conditions employed, it appeared that the rate determining step was the destruction of the carbon-trichloride anhydride, **3.9**, by base. Without this going to completion, oxidation could not take place. If the permanganate was added too fast, it was simply destroyed by the base, and *vice versa*. The addition of more base half way through the permanganate addition is thought to produce more of the aldehyde intermediate, **3.17**, which should be easily oxidised, but this does not happen fast

enough to prevent some of this aldehyde being trapped as the lactol, **3.18**. Allowing the reaction to warm up should speed up the oxidation, resulting in the formation of the desired product, **3.10**. Allowing the reaction mixture to warm to room temperature half way through the permanganate addition has perhaps facilitated the oxidation of a portion of the lactol, thus improving the yield of **3.10**.

Figure 3.12 *Proposed oxidation Mechanism*

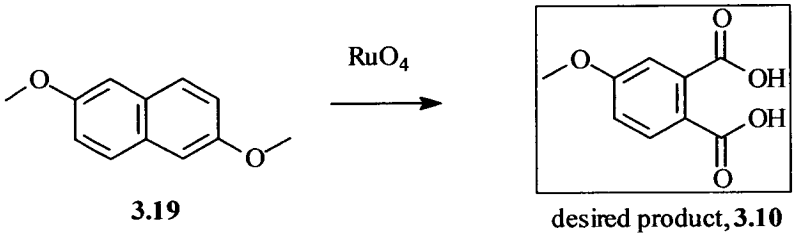


Due to the problems encountered with this reaction, coupled with the length of time required to carry it out to its maximum yield, alternative routes to 4-methoxyphthalic acid, **3.10**, were investigated.

Alternative oxidation methods were first considered, for example the use of Jones reagent[23], and selenium oxidation[24], but these proved unsuccessful. In the latter case, only starting material, **3.9**, was recovered. The former also proved unsuitable, since the product is not soluble in organic solvents and can only be extracted by acidification. Since the Jones reagent is highly acidic, it was assumed that no reaction had occurred since no precipitate was produced and no product was recovered upon chilling and concentration.

The use of an aromatic ring as a synthetic equivalent for the carboxyl group (Figure 3.13) has been reported by Martin and Nuñez[25] and was attempted here. Successful oxidation of the symmetrical species **3.19** should produce the desired product, **3.10**. Once again, however, no precipitate was obtained on acidification and product formation could not be detected.

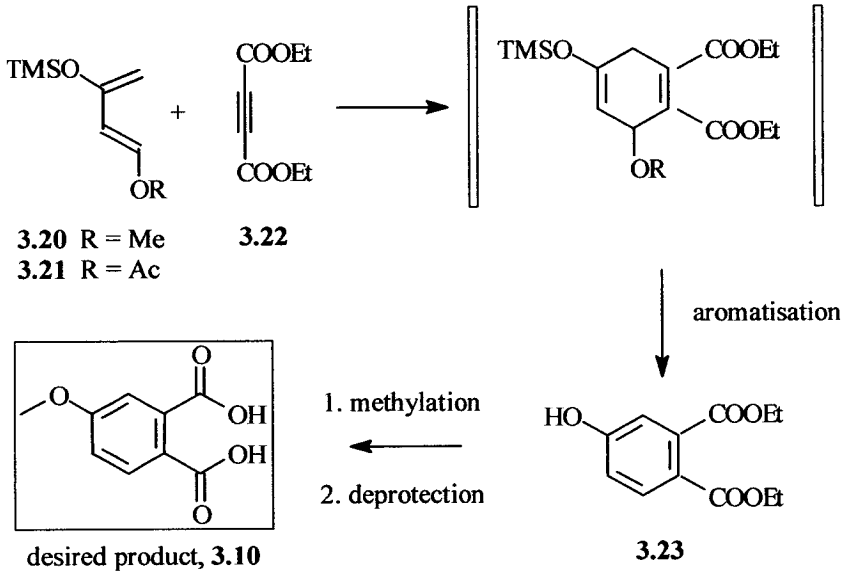
Figure 3.13 *Route (2)*



3.19 desired product, **3.10**

The third approach examined used Diels Alder chemistry. Two potential dienes were considered (Figure 3.14), both of which would be expected to react with the protected acetylene, **3.22**. Attempts to use Danishefsky's Diene[26], **3.20**, were unsuccessful.

Figure 3.14 *Route (3)*



3.20 R = Me
3.21 R = Ac

3.22

3.23

desired product, **3.10**

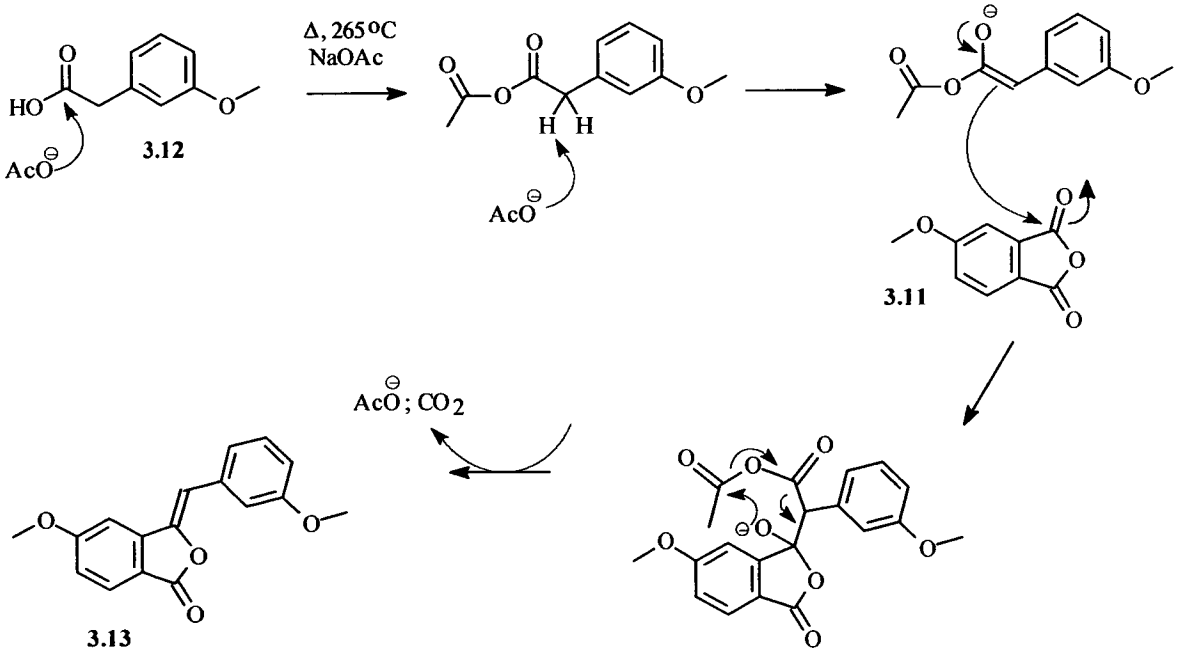
The alternative diene, **3.21**, is not commercially available or readily synthesised and, since no literature precedent is available for the Diels Alder step, this route was abandoned when the initial scheme (Figure 3.11, step (ii)) began to be productive.

Step (iii) (Figure 3.11), anhydride formation, was carried out using acetic anhydride as the dehydrating agent, at reflux. The acetic anhydride was removed under high vacuum. Due to differing solubility properties, the desired cyclic anhydride (Figure 3.11, **3.11**) was relatively easy to separate from any unreacted starting material, **3.10**, and was further purified by recrystallisation.

3.3.2 The Perkin Reaction

The synthesis of **3.13**, *via* a Perkin reaction[27, 28], was next carried out. Reactions of this type involve the condensation of an aromatic aldehyde with an anhydride, in the presence of base, which is usually the salt of the acid corresponding to the anhydride. In this instance, sodium acetate was employed. This is not a typical Perkin reaction however. The cyclic anhydride is participating in the manner of an aromatic aldehyde. The required anhydride moiety is formed *in situ* from the self-promoted esterification of the aromatic acid with sodium acetate. The Perkin reaction itself, followed by dehydration, then takes place. The literature precedence[28] indicates that cyclisation is expected to occur, to give **3.13** (Figure 3.15).

Figure 3.15 *Perkin reaction – proposed mechanism*

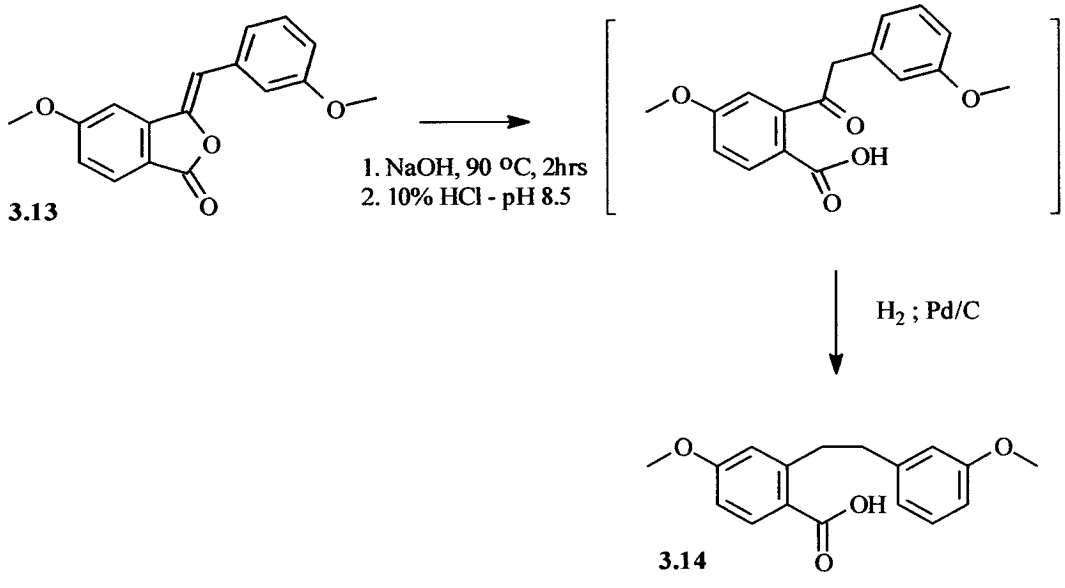


The Perkin reaction was pioneered as an industrial process, and works best on at least a gram scale. Thus, in order to prevent wastage, model studies were performed. The reaction of **3.12** with phthalic anhydride proved unsuccessful, however some product, was obtained on test reaction with **3.9**. The reaction is carried out as a melt and the most troublesome part was safely maintaining the required temperature of 280-300°C. To this end, a graphite bath was employed, as was a Liebeg condenser. Nitrogen was blown across the reagents in an attempt to aid the removal of water. Yields were generally low, however, and it is thought that some product may have been lost by sublimation. The use of high purity starting materials is also essential, in order to prevent the formation of polyaromatic impurities.

3.3.3 Hydrogenation

Having formed the structural backbone, the next step in the strategy is to adjust the functionality, as illustrated below (fig. 3.16), in preparation for the final cyclisation to form the seven membered ring. Ring opening with aqueous base is first carried out. The pH is adjusted to 8.5 with a strong acid and hydrogenation is carried out using a noble metal catalyst - in this case palladium on charcoal was employed. The reaction is complete when two moles of hydrogen have been absorbed by the reaction mixture.

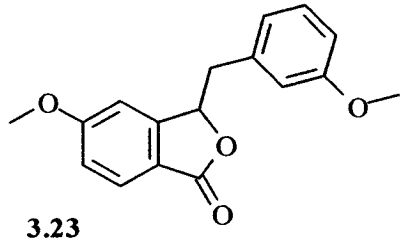
Figure 3.16 *Hydrogenation*



The literature[20] indicates that hydrogenation should be carried out at 95°C with a pressure of 65psi. The apparatus used could not support such conditions, and the reaction was therefore attempted using 60psi at 80°C, and the reaction time was extended accordingly. These conditions proved successful. The yield was decreased at larger scales, however, this may have been improved were increased pressures able to be used. Raney nickel has been used for the direct hydrogenation of a species analogous to **3.13**[19] and this reagent might prove a useful alternative, although it has the drawback of toxicity and associated disposal problems.

The desired product was successfully obtained, however a structurally related impurity, **3.23** (Figure 3.17), was also detected. This was thought to be the hydrogenated product of an incomplete ring opening step, that is a lactone as opposed to the expected carboxylic acid. Alterations to the conditions were made in an attempt to optimise this step, however it was found that the presence of some **3.23** could be tolerated in the subsequent cyclisation procedure.

Figure 3.17 *Observed Impurity*

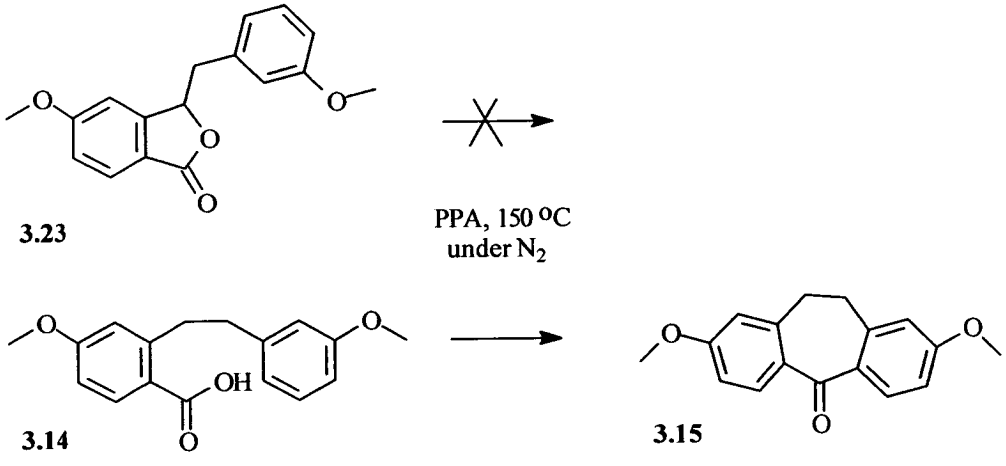


3.23

3.3.4 Cyclisation

The formation of the seven membered ring shown below (**3.15**, Figure 3.18) is carried out *via* an intramolecular Friedel Crafts acylation[19], using polyphosphoric acid as the catalyst. The reaction was monitored by TLC. The formation of a new product was first observed after 0.75h, and a single spot was obtained after 1.5h. Both the starting material and the lactol impurity appeared to have been consumed, with no evidence of side product formation, although yields were not quantitative. The lactone, **3.23**, was initially thought to have reacted in the same manner as **3.14,** to give **3.15**, however a plausible mechanism for this reaction could not be postulated. It therefore seems likely that **3.23**, or its breakdown products, were removed during the basic workup which was carried out on a small scale for each TLC sample, as well as upon completion of the reaction. The product, **3.15**, was purified by recrystallisation and showed UV absorption at the characteristic wavelength as well as the expected symmetry by ^{1}H and ^{13}C NMR spectroscopy.

Figure 3.18 *Friedel Crafts ring-closure*

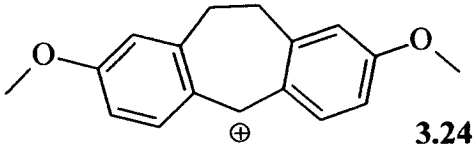


The tricyclic species, **3.15**, has three active moieties available for functionalisation and can be modified in a number of ways. For example, treatment with a Lewis acid in benzene, in an analogous fashion to the methods employed by Noda[17] and Ramage[18] (Section 3.2.1), should yield a system suitable for use as a linker in SPPS. In order to prepare the protected arginine derivative, selective reduction of the ketone to the alcohol was necessary. Sodium borohydride[19,29] proved to be a suitably mild reagent and the reaction was complete after 2hrs.

3.3.5 The Protection of Arginine

Having successfully prepared the desired dimethoxy-suberyl species, **3.16**, coupling to arginine was attempted. The desired nucleophile in this reaction was the guanidino group of arginine. Considering the approximate pKa values of each reagent, it is clear that the alcohol is not a suitable leaving group, since it would be deprotonated under the basic conditions required. The exchange of this hydroxyl for a suitable leaving group was therefore attempted. In the first instance, the preparation of the chloride was undertaken. This was expected to be unstable and was therefore reacted directly with arginine, following quenching of the chloride reagent. Thionyl chloride and HCl[29] were both employed. An excess of the prospective protecting group was used and the reaction was monitored by HPLC. The arginine concentration did not decrease and no product formation was observed. Analysis using both MS+ and MS- techniques was attempted in order to confirm that the chlorinated species had been successfully formed. However, the only species detected was the carbonium ion shown (Figure 3.19), a product which may be derived from either the hydroxyl or the chloro starting material.

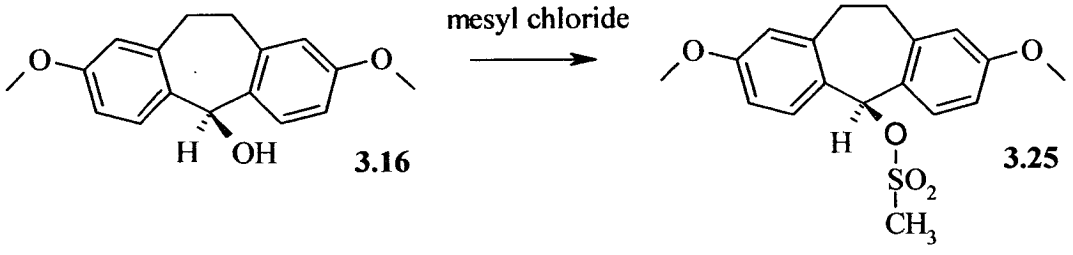
Figure 3.19 *Carbonium ion*



3.24

The replacement of the alcohol with a mesyl group was next attempted. Since this product (Figure 3.20) contains an additional carbon atom it, could be differentiated from the alcohol by ^{1}H NMR spectroscopy, and should also show a different profile by mass spectrometry, although the carbocation would be expected at high cone voltages. Several sets of conditions were attempted, however the desired mesylate could not be prepared.

Figure 3.20 *Alcohol and mesylate*



3.4 Conclusions and Future Work

Having considered the data obtained by mass spectroscopy, it is evident that the above carbocation (Figure 3.19) is particularly stable. Although this was considered a desirable property for the protecting group, it is probable that the intrinsic stability of this carbocation is responsible for the coupling difficulties encountered, especially as the analogous mono-methoxy species was successfully coupled under similar conditions[17]. Reverse phase HPLC monitoring may have interfered with the coupling reaction, however a protecting group which is highly sensitive to such conditions would not be suitable for application in peptide synthesis.

A direct comparison using the mono-methoxy species would give a final indication as to the usefulness of the dimethoxysuberyl protecting group. Should application in the protection of arginine protection prove unfeasible, the tricyclic ketone (Figure 3.11, **3.15**) may be functionalised in alternative ways, as mentioned previously, and it may yet prove useful as a linker in SPPS.

[1] Tam J.P., *Proc. Natl. Acad. Sci. USA*, 1988, **85**, 5409.
[2] Morton G.H., *Ph.D. Thesis*, University of Edinburgh, 1997.
[3] Carpino E.A. and Han G.Y., *J. Am. Chem. Soc.*, 1970, **92**, 5748.
[4] Rzeszortarska B., Masiukiewicz E., *Org. Prep. Proc. Int.*, 1988, **20**, 427.
[5] Barany G, Merrifield, R.B., in *The Peptides: Analysis, Synthesis, Biology*, Gross E. and Meienhofer J. (Eds), Academic Press Inc., New York U.S.A., 1980, **2**.
[6] Jaeger G., Geiger R., *Chem. Ber.*, 1970, **103**, 1727.
[7] Arold H., Riessmann S., *Z.Chem.*, 1968, **8**, 107.
[8] Neri P., Antoni G., Barbarulli G., Casagli C., Mariani M., Nencioti L., Presentini R. and Tagliabu A., *Mol. Immunol.*, 1984, **21**, 151.
[9] Rink H., Sieber P. and Raschdorf F., *Tetrahedrn Lett.*, 1984, **25**, 621.
[10] Fujino M., Mitsuhiro W. and Kitada C., *Chem. Pharm. Bull.*, 1981, **29**, 2825.
[11] Ramage R. and Green J., *Tetrahedron Lett.*, 1987, **28**, 2287.
[12] Green J., Ogunobio O., Ramage R. and Stewart A.S.J., *Tetrahedron Lett.*, 1988, **29**, 4341.
[13] Ramage R., Green J. and Blake A.J., *Tetrahedron*, 1991, **47**, 6353.
[14] Carpino L.A., Schroff H., Triolo S.A., Mansour El.M.E., Wenschuh H. and Albericio F., *Tetrahedron Lett.*, 1993, **34**, 7829.
[15] Sabirov A., Samukov V., *Proc. 25th Eur. Pept. Symp.* 1998, 112.
[16] Zhong H.M., Greco M.N., Maryanoff B.E., 1997, **62**, 9326.
[17] Noda M. and Kiffe M., *J. Peptide Res.*, 1997, **50**, 329.
[18] Irving S.L., McInnes C. and Ramage R., *Tetrahedron Lett.*, 1993, **34**, 6599.
[19] Noda .M., Yamaguchi M., Ando E., Takeda K. and Nokihara K., *J. Org. Chem.*, 1994, **59**, 7968.
[20] Humber L.G., *United States Patent no. 3697581*, 1972.
[21] Chakravarti S.N., Perkin W.H., *J.Chem. Soc.*, 1929, 197.
[22] McAlees, *J.Chem Soc.Perkin I*, 1977, 2030.
[23] Jackman L.M. and Thompson D.T., *J. Chem. Soc.*, 1961, 4794.
[24] Umbreit M.A. and Sharpless K.B., *J. Am. Chem. Soc.*, 1977, 5526.
[25] Nuñez M.T. and Martin V.S., *J. Org. Chem.*, 1990, **55**, 1928.
[26] Danishefsky S. and Kitahara T., *J. Am. Chem. Soc.*, 1974, 7807.
[27] Edwards, Perkin W.H. and Stoyle, *J.Chem. Soc.*, 1925, **127**, 197.
[28] Johnson, *Org. React.*, 1942, **1**, 210.
[29] Dhainaut A., Regnier G., Atassi G., Pierre A., Leonce S., *J. Med. Chem.*, 1992, 2481.

4. Experimental Data

4.1 Notes

All Fmoc amino acids were purchased from either Bachem or Novabiochem and were of L-configuration. The *p*-alkoxybenzylalcohol (Wang) resin and the tricyclic amide resin were supplied by Bachem and the polyethyleneglycol (PEG) resin by Perseptive Biosystems. Peptide synthesis grade dimethylformamide (DMF), 1,4 dioxane and piperidine were supplied by Rathburn Chemicals. *N,N*-diisopropylethylamine (DIEA) and trifluoroacetic acid (TFA) were also of peptide synthesis grade and were purchased from Applied Biosystems (ABI). Dichloromethane (DCM) was dried by distillation over potassium carbonate. Tetrahydrofuran (THF) was dried by distillation over sodium wire. 1-Hydroxy-4-ethoxycarbonyl-1,2,3,-triazole (HOCt), and tetrabenzo[*a,c,g,i*]fluorenyl-17-methoxy-carbonyl (Tbfmoc) were synthesised by members of the research group.

Prior to each peptide coupling reaction, all reagents were dried under vacuum, over $POCl_5$, for at least 24h before use. The reactions were carried out under a positive pressure of dried N_2, using a balloon rather than a continuous gas flow. DMF and DIEA were of peptide synthesis grade.

The chemicals for solution phase organic synthesis were purchased from Acros Organics or Aldrich. Sulphur dioxide was generated by the addition of concentrated hydrochloric acid to sodium metabisulphite. Before use, 4-methoxyphthalic anhydride (**3.11**), 3-methoxyphenyl acetic acid (**3.12**) and sodium acetate were dried overnight at 40°C, under vacuum.

Melting points were recorded in open capillaries using a Buchi 510 oil immersion melting point apparatus. Analytical thin layer chromatography (TLC) was performed using plastic sheets precoated with silica gel, 0.25mm, UV_{254} (Macherey-Nagel) using the solvent systems described within the text. Infrared (IR) spectra were recorded on a Bio-Rad SPC 3200 instrument. Ultraviolet (UV) spectra were recorded on a Perkin Elmer single beam spectrophotometer and were carried out in the solvents indicated. Unless otherwise stated, nuclear magnetic resonance (NMR) spectra were recorded on an AC-250 instrument, with proton NMR being recorded at 250 MHz and carbon NMR at 63 MHz.

High and low resolution fast atom bombardment mass spectra (FAB MS) were measured on a Kratos MS50TC instrument. Matrix assisted laser desorption ionisation mass spectra (MALDI) were measured on a PerSeptive Biosystems Voyager™ Workstation. Electrospray ionisation and LC-MS were performed on a Micromass Platform Mass Spectrometer using MassLynx v2.3 software and were carried out by direct infusion or *via* a Waters Alliance 2690 LC system. Amino acid analyses were performed using a Pharmacia Biochrom 20 LKB 4150 alpha acid analyser on the hydrosylates obtained after heating samples in 6M HCl at 110°C in sealed Carius tubes for the time durations indicated.

The buffers described were prepared using Milli-Q grade water, and urea (Fluka). High performance liquid chromatography (HPLC) was carried out using either an ABI system comprising 2x1406A solvent delivery systems, a 1480 injector/mixer and a 1783 detector/controller, or a Gilson system comprising 2x306 solvent delivery systems, a 811c dynamic mixer, an 805 manometric module, a 119 UV/Vis detector and a Gilson 715 software-driven gradient controller. The dialysis tubing used was purchased from Spectrum® and was a Spectra/Por® cellulose ester (CE) membrane with molecular weight cut-offs as indicated. Samples were centrifuged using MSE Mistral 2000R.

4.2 Studies Towards The Chemical Synthesis of Endostatin

4.2.1 General Procedures for Solid Phase Peptide Synthesis

4.2.1.1 Side chain protecting groups

Ala, Gly, Ile, Leu,	
Met, Phe, Pro, Val	- No protection
Arg	- 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulphonyl (Pbf)
Asn, Gln, His	- τ-triphenylmethyl (Trt)
Cys	- Picolyl (Pic), or Trt where indicated.
Asp, Glu	- *t*-butyl esters (OBu^t)
Trp	- *t*-butoxycarbonyl (Boc)
Lys	-1,5-dioxaspiro-5:5-undecane-3-nitro-3-methoxycarbonyl (Tnm), or Boc where indicated.
Ser, Thr, Tyr	- *t*-butyl ethers (Bu^t), or Trt where indicated.

4.2.1.2 The Fmoc Loading Test

The loading of the functionalised resin was determined by treating an accurately weighed quantity of resin (typically 2 x ~5mg) with 20% piperidine/DMF/1,4-dioxan (10ml). This suspension was sonicated for 10 minutes before the UV absorbance of the supernatant was recorded at 302nm. The coupling efficiency and resulting resin functionality (mmol/g) was calculated by applying the Beer-Lambert law (ε = 15,400 for the Fmoc-piperidine adduct).

$$\text{Resin functionality (Fmoc-peptide - mmol/g)} = \frac{10 \times Abs_{302nm}}{9 \times \text{mass of resin (mg)}}$$

4.2.1.3 The Ninhydrin (Kaiser) Test[1]

A few beads of washed resin were placed in an Epindorf tube and two drops of each of the following reagents were added using a Pasteur pipette.

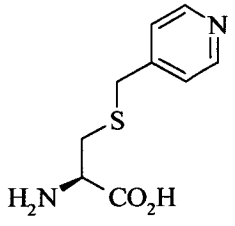
Monitor 1 - Phenol (80g)/EtOH (20ml); Monitor 2 - KCN (2ml, 0.001M) /Pyridine (98ml); Monitor 3 - Ninhydrin (5g)/EtOH (100ml).

The mixture was vortexed and the tube was incubated at 120°C in a heating block for 4-6 minutes. The presence of a free amino group is indicated by a blue reaction mixture.

4.2.2 Special Reagents for SPPS

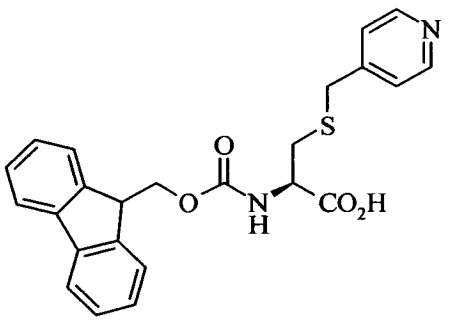
4.2.2.1 FmocCys(Pic)-OH [2, 3]

S-4-Picolyl-L-cysteine (2.2)



L-Cysteine (**2.1**) (500mg, 4.1mmol) was added to a mixture of 2N NaOH (4.1ml) and EtOH (4.9ml) stirred until it had dissolved. 4-Picolylchloride (HCl salt) was added (0.75g, 4.1mmol, 1eq) and the solution was stirred for 2hrs at RT, after which time a precipitate had formed. The reaction was then chilled in an ice bath for 1hr. The precipitate was collected by filtration, washed with EtOH and ether and dried under vacuum. **Yield** = 0.661g; 75%. **Mp** 207-209°C (lit 209.5-211°C[2]); **CHN** found, C 51.30%, H 5.70%, N 13.37% ($C_9H_{12}N_2O_2S$ requires C 50.92%, H 5.70%, N 13.20%); **MS** (FAB) m/z = 213 (MH^+); **HRMS** (FAB) 213.0694 (MH^+ requires 213.0698); **^{1}H NMR (200 MHz, D_2O δH/ppm)** 2.78 (1H, d, J = 4.8 **βH**), 2.78 (1H, d, J = 6.6, **β'H**), 3.64 (2H, s, Ar-**CH_2**), 3.72 (1H, dd, J = 4.8, 6.6, **αH**), 7.24 (2H, d, J = 6.3, Ar-**H**), 8.28 (2H, d, J = 6.3, Ar-**H**); **^{13}C{^{1}H} NMR (63 MHz, D_2O δC/ppm)** 31.41 (t), 34.02 (t), 53.31 (d), 124.61 (d), 148.67 (d), 148.65 (d), 172.68 (s); **FTIR ν_{max}/cm^{-1} (bromoform)** 1140 (C-O), 1527 (aromatic), 1602 (C=O), 2123, 2577 (NH_2), 2920 (CH), 3017 (aromatic CH), 3421 (OH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 260 (6057).

N-9-Fluorenylmethoxycarbonyl-S-4-Picolyl-L-cysteine (2.3)

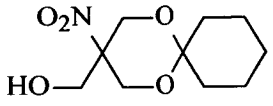


To **2.2** (300mg, 1.4mmol, 1eq) was added a solution of 10% Na_2CO_3 in water (7ml) and 1,4 dioxane (7ml). The reagents were allowed to stir until **2.2** had dissolved. 9-Fluorenylmethoxycarbonyl-*N*-hydroxysuccinamide (470mg, 1.4mmol, 1eq) was next added and the solution was allowed to stir at RT overnight. The resulting precipitate was then removed by filtration and the filtrate was acidified. This solution was extracted with EtOAc (3x20ml). The combined organic extracts were dried over $NaSO_4$ and concentrated to yield a yellow oil. This was purified by wet flash chromatography ($CHCl_3$/MeOH/AcOH; 85:10:5). The combined product fractions were concentrated *in vacuo*. Water was added to swell the resulting glassy solid. This solution was reconcentrated to yield a yellow gelatinous product which was dissolved in EtOAc (~20ml) and washed with water (2x20ml), conc. NH_4Cl (1x20ml) and brine (1x20ml). The organic extract was then dried ($MgSO_4$), filtered and concentrated to yield a pale yellow powder.

Yield = 0.468g, 77%; **Tlc** Rf = 0.39 ($CHCl_3$/MeOH/AcOH, 85:10:5); **Mp** 142-145°C (lit 142-145°C[2]); **MS** (FAB) m/z = 435 (MH^+); **HRMS** (FAB) 435.1373 (MH^+ requires 435.1379); **^{1}H NMR (200 MHz, D_2O δH/ppm)** 2.72 (1H, dd, J = 9.3, 13.7, β**H**), 2.86 (1H, dd, J = 4.6, 13.7, β'**H**), 3.77 (2H, s, Ar-**CH_2**), 4.14 - 4.34 (4H, m, fluorenyl **CH**, **CH_2**, α**H**), 7.28 - 7.43 (6H, m, fluorenyl Ar- **H**), 7.87 (2H, d, J = 7.4, picolyl Ar-**H**), 8.49 (2H, d, J = 5.5, picolyl Ar-**H**); **^{13}C{^{1}H} NMR (63 MHz, D_2O δC/ppm)** 32.54 (t), 34.27 (t), 46.78 (d), 53.85 (d), 65.93 (t), 120.27 (d), 125.03 (d), 125.44 (d), 127.25 (d), 127.83 (d), 148.15 (d), 140.89 (s) 143.94 (s), 150.05 (s), 156.21 (s), 172.28 (s); **FTIR ν_{max}/cm^{-1} (bromoform)** 1139 (C-O), 1530 (aromatic), 1611 (C=O), 1686 (urethane), 2890, 2970 (CH), 3017 (aromatic CH), 3350 (NH), 3466 (OH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 300 (4991), 288 (5359), 265 (17143); $[\alpha]_D^{24}$ – 34.0° (c = 0.1, DMF); **HPLC** (System 1, Aquapore RP18 C_{18}, λ = 214nm) 50% MeCN, Rt 15 min.

4.2.2.2 Fmoc-Lys(Tnm)-OH[4]

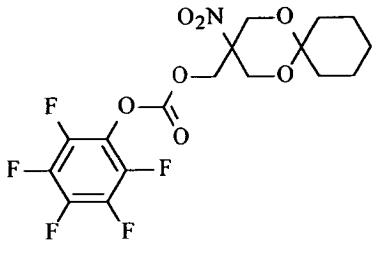
3-Hydroxymethyl-3-nitro-1,5-dioxaspiro-5:5-undecane (2.5)



Cyclohexanone (10.1ml, 9.55g, 97mmol, 1eq) and tris(hydroxymethyl)nitromethane (**2.4**) (14.84g, 98mmol; recrystallised from 1:1 EtOAc/Hexane), together with a catalytic amount of *p*-TSA, were refluxed in sodium dried benzene (150ml) under Dean-Stark conditions, until the calculated amount of water was collected in the trap (2.5 hrs). The solution was then cooled to room temperature and washed with water (2x25ml). The aqueous layers were back extracted with Toluene (2x25ml), then the combined organic layers were dried over sodium sulphate. Concentration *in vacuo* resulted in a cream coloured solid which was crystallised from hexane/diethylether 1:1. This yielded a white solid, which was recrystallised from ether.

Yield = 11.2g, 50%; **Mp** 95-97°C (lit. 97-98°C[4]); **MS** FAB m/z = 232 (MH^+); **HRMS** 232.1179 (MH^+ requires 232.1185); **CHN** found C 52.03%, H 7.41%, N 5.97% ($C_{10}H_{17}NO_5$ requires C 51.94%, H 7.41%, N 6.06%); **^{1}H NMR (250MHz, $CDCl_3$ δH/ppm)** 1.37-1.75 (10H, m, aliphatic ring, 5 x **CH_2**), 2.57 (1H, br, **OH**), 4.02 (2H, s, **CH_2**OH), 4.03 (2H, d, J = 12.7, dioxane ring 2 x **CH** *axial*), 4.38 (2H, d, J = 12.7, dioxane ring, 2 x **CH** *equatorial*); **^{13}C {^{1}H} NMR (63 MHz, $CDCl_3$ δC/ppm)** 22.25 (t), 22.37 (t), 25.19 (t), 31.66 (t), 32,19 (t), 60.51 (t), 63.38 (t), 86.65 (s), 99.52 (s); **FTIR ν_{max}/cm^{-1} (bromoform)** 1112 (C-O), 1346, 1443 (NO_2), 1544 (C-NO_2), 2856, 2936 (CH), 3147 (OH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 271 (770).

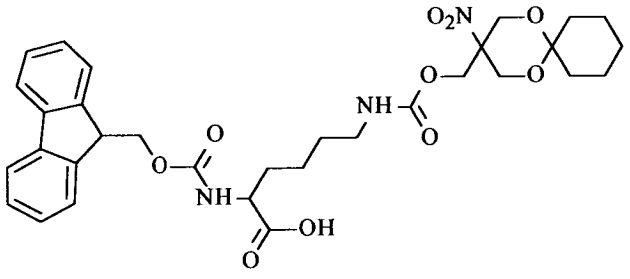
3-Hydroxymethyl-3-nitro-1,5-dioxaspiro-5:5-undecane-pentafluorophenolcarbonate (2.6)



To (**2.5**) (10g, 0.044mol) was added triphosgene (5g, 0.166mol, 4eq) and DIEA (9ml, 0.05mol, 1eq). The reagents were stirred in sodium dried toluene (100ml, 10ml/g, based on (**2.5**)) under N_2. After 20mins, a white precipitate of DIEA.HCl formed and the first step of the reaction was deemed to be complete. The newly formed chloroformate was then reacted *in situ* by the addition of a mixture of pentafluorophenol carbonate (10.1g, 0.055mol) and DIEA (9ml, 0.05 mol) dissolved in the minimum amount of toluene (~20ml). The reagents were stirred for a further 15mins, then rapidly washed with iced water (1x20ml), followed by brine (1x20ml), and dried ($NaSO_4$) before the organic extracts were concentrated *in vacuo*. **2.6** was obtained as a white solid after recrystallisation from hexane/ethyl acetate (1:1).

Yield = 11.576g, 50%; **Mp** 118-121°C (lit 121-123°C[4]); **MS** FAB m/z = 442 (MH^+); **HRMS** 442.0926 (MH^+ requires 442.0925); **CHN** found C 46.39, H 3.76%, N 3.19% ($C_{17}H_{16}F_5NO_7$ requires C 46.27%, H 3.65%, N 3.17%); **^{1}H NMR (250MHz, $CDCl_3$ δH/ppm)** 1.24-1.84 (10H, m, aliphatic ring, 5 x **CH_2**), 4.09 (2H, d, J = 12.7, dioxane ring, 2 x **CH** *axial*), 4.43 (2H, d, J = 12.7, dioxane ring, 2 x **CH** *equatorial*), 4.49 (2H, s, **CH_2O**); **^{13}C {^{1}H} NMR (63 MHz, $CDCl_3$ δC/ppm)** 22.23 (t), 22.35 (t), 25.14 (t), 31.86 (t), 60.41 (t), 68.26 (t), 83.22 (s), 99.99 (s), 126.17 – 128.34 (aromatic CF), 129.32 (s), 150.45 (s); **FTIR ν_{max}/cm^{-1} (bromoform)** 1140 (C-O), 1392, 1448 (NO_2), 1521 (aromatic), 1547 (C-NO_2), 1786 (C=O), 2873, 2940, 2957 (CH), 3379 (OH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 263 (1208), 243 (735).

N^{α}–9-Fluorenylmethoxycarbonyl-N^{ε}-1,5-dioxaspiro-5:5-undecane-3-nitro-3-methoxycarbonyllysine (2.7)

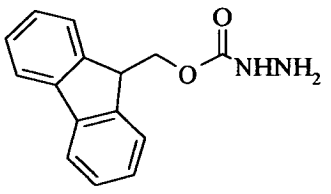


Fmoc-Lys-OH (1.6g, 4.3mmol) was suspended in 1,4 dioxane/water (2:1, 70 ml) and TEA was added (1.3ml, 8.9 mmol). The solution was then cooled to 0°C and **2.6** (2g, 4.5mmol) was added. The reaction mixture was allowed to warm to room temperature and was stirred overnight. The resulting solution was concentrated and the residue was dissolved in ethyl acetate(~50ml), washed with brine (~50ml) and dried ($NaSO_4$), before being re-concentrated to yield a white foam. This was dissolved in a minimal amount of DCM and purified by wet flash chromatography (DCM/MeOH 90:10). The concentrated sample collected from the column remained sticky/foamy, so the product was triturated with hexane to yield a white powdery solid.

Yield = 1.637g, 72%; **Tlc** Rf = 0.73 (DCM/MeOH 90:10); **Mp** 174-176°C (lit. 172°C[4]); **MS** $ESMS^+$ 627; FAB m/z = 626 (MH^+); **HRMS** 626.2717 (MH^+ requires 626.2714); **^{1}H NMR (200MHz, $CDCl_3$ δH/ppm)** 1.16-1.98 (18H, m, aliphatic ring **CH_2**, lysine side chain **CH_2**), 4.1 – 4.4 (10H, m, dioxane ring **CH_2**, **αH**, Fmoc **CH**, Fmoc **CH_2**, **CH_2O**), 7.2 – 7.4 (4H, m, Ar-**H**), 7.7 (2H, d, J = 7.3, Ar-**H**), 7.9 (2H, d, J = 7.3, Ar-**H**); **^{13}C {^{1}H} NMR (63 MHz, $CDCl_3$ δC/ppm)** 22.2 (t), 22.6 (t), 22.8 (t), 25.0 (t), 28.3 (t), 29.3 (t), 31.1 (t), 31.9 (t), 46.9 (d), 55.2 (d), 60.5 (t), 63.2 (t), 65.4 (t), 85.6 (s), 98.7 (s), 120.2 (d), 125.4 (d), 127.2 (d), 12.7 (d), 140.6 (s), 144.1 (s), 155.0 (s), 155. (s), 176.1 (s); **FTIR ν_{max}/cm^{-1} (bromoform)** 1140 (C-O), 1341, 1446 (NO_2), 1515 (aromatic), 1549 (C-NO_2), 1709 (C=O), 2859, 2937 (CH), 3020 (aromatic CH), 3331 (NH), 3416 (OH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 300 (7422), 288 (10156), 265 (23438); **$[\alpha]_D^{24}$** –6.1° (c 1.0, DMF); **HPLC** (System 1, Aquapore RP18 C_{18}, λ = 214nm) 66% MeCN, R_t 21.2min.

4.2.2.3 Fmoc-hydrazine[5]

9-fluorenylmethoxycarbonyl hydrazine (2.11)



Hydrazine monohydrate (6ml, 0.12mol) was chilled to 0°C in the dark, with stirring. A solution of Fmoc-chloride (1g, 3.8mmol) in acetonitrile (130ml) was added to this, dropwise. Stirring was then continued for 30mins, after which time a precipitate had formed. The solution was then concentrated to approx ¼ volume and filtered. The resulting white fluffy solid was washed with EtOH (3x10ml) and dried *in vacuo*.

Yield = 0.93g, 95%. 172-174°C (lit. 172-174°C[5]); **CHN**: found, C 70.76%, H 5.38%, N 11.08% ($C_{15}H_{14}N_2O_2$ requires C 70.85%, H 5.55%, N 11.02%); **MS** (FAB) m/z = 255 (MH^+); **HRMS** (FAB) 255.1132 (MH^+ requires 255.1134); **^{1}H NMR (250 MHz, d_6DMSO δH/ppm)** 4.08 (2H, br, NH_2), 4.25 (3H, m, fluorenyl CH, CH_2), 7.35 (4H, m, Ar-H), 7.67 (2H, d, J = 7.6, Ar-H), 7.87 (2H, d, J = 7.6, Ar-H), 8.35 (1H, br, NH); **^{13}C{^{1}H} NMR (63 MHz, d_6DMSO, δC/ppm)** 46.77 (d), 65.73 (t), 120.12 (d), 120.20 (d), 121.47 (d), 125.33 (d), 127.17 (d), 127.38 (d), 127.73 (d), 129.01 (d), 137.52 (s), 139.51 (s), 140.79 (s), 143.91 (s), 158.27 (s); **FTIR ν_{max}/cm^{-1} (bromoform)** 1141 (C-O), 1512 (aromatic), 1694 (C=O), 2899, 2956 (CH), 3018 (aromatic CH), 3207, 3315 (NH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 300 (5186), 289 (5186), 265 (16510)

4.2.2.4 Semi-carbazide resin[6]

Modification of the resin - removal of Fmoc (2.14)

Amine resin (**2.13**) (0.90g, 0.59mmolg^{-1}, 0.531 mmol) was sonicated in 20% v/v piperidine/DMF for 45mins. The resin was filtered, washed with DMF, DCM and ether (2x10ml) and dried under vacuum. A Kaiser test (section 4.2.2.3) confirmed the presence of a free amino group.

Kaiser test – positive.

Generation of the isocyanate intermediate (2.15)

The resin (**2.14**) (0.84g) was swollen in the minimum amount of DCM and DIEA (0.1ml, 0.6mmol) was added. The suspension was sonicated for 10mins. A solution of triphosgene (0.49g, 1.65mmol) in DCM (4ml) was added and the mixture was sonicated for a further 1hr. The resin was collected by filtration, washed with DCM and ether (2x10ml) and dried. IR confirmed the presence of the isocyanate grouping.

FTIR ν_{max}/cm^{-1} (KBr) 2250-2260 (N=C=O).

Trapping of the isocyanate with Fmoc hydrazine (2.16)

The isocyanate resin (**2.15**) was swollen in the minimum amount of DCM. A solution of FmocNHNH$_2$ (**2.11**) (0.35g, 1.37mmol) in DCM (5ml) was added and the mixture was sonicated for 2hrs. The resin was collected by filtration, washed with DMF, DCM and ether (2x10ml) and dried under vacuum. Analysis by FTIR spectroscopy indicated that the isocyanate functionality was no longer present. Before the resin was used in peptide synthesis, capping with acetic anhydride (section 4.2.4.1) was carried out, in order to prevent the participation of any unreacted amino groups in subsequent coupling steps.

Yield = 0.9g of resin; **Fmoc loading** 0.20 mmolg^{-1}.

4.2.2.5 Wang-hydrazide (*p*-alkoxybenzyloxycarbonylhydrazide) resin

Generation of Wang-imidazole species (2.21)

Wang (*p*-alkoxybenzyl) resin (**2.20**) (940mg, substitution 0.64 $mmolg^{-1}$) was swollen in dry THF (15 ml) under N_2. Carbonyl diimidazole (700mg, mmol, 6eq.) was added and the reaction was stirred at room temperature. The reaction was monitored by FTIR spectroscopy, and was deemed to be complete when a strong imidazole peak was observed. After 2h, workup was carried out. The resin was washed with THF, DCM and ether (2 x 10ml, twice) and dried under vacuum.

Yield = 810mg of resin; **FTIR** ν_{max}/cm^{-1} (KBr) 1756 (imidazole); **Kaiser test** - negative.

Trapping of the imidazole with hydrazine (2.24)

The resin (**2.21**) (500mg, 0.35mmol) was swollen in 1:1 THF (dry) / N-Methylpyrrolidinone (HPLC grade); (20ml). $FmocNHNH_2$ was added (445mg, 1.75mmol, 5eq), followed by DIEA (0.5ml, mmol,eq). The reagents were heated at 60°C, under an N_2 atmosphere, for 4hrs. The resin was then washed and dried as before. Analysis by FTIR spectroscopy indicated that the imidazole functionality was no longer present. A positive Kaiser test (section 4.2.2.3) confirmed the presence of an unprotected amino group.

Yield = 462mg of resin; **FTIR** ν_{max}/cm^{-1} (KBr) 1719 ($CONHNH_2$), 3339, 3412 ($NHNH_2$); **Fmoc loading** – blank; **Kaiser test** - positive.

4.2.3 Loading of the C-terminal acid onto the solid support

4.2.3.1 Loading of 4-alkoxybenzylalcohol (Wang) resin - general procedures

(i) Required loading = 0.1- 0.2 $mmolg^{-1}$

The *C*-terminal Fmoc-amino acid (2 eq, based on resin functonality) was dissolved in DMF (10ml) before DIC (1 eq) was added and the solution sonicated for 15 minutes. The solution was added to Wang resin (**2.20**) (1g, preswollen in a minimum quantity of DMF), mixed with a catalytic amount of DMAP (approx. 10mg). The mixture was sonicated briefly to mix the reagents, then left to stand for 20-30 minutes at room temperature. The resin was separated by filtration and sequentially washed with copious amounts of DMF, 1,4-dioxane, DCM and ether before being dried under vacuum.

(ii) Required loading 0.2-0.5 $mmolg^{-1}$

When high loadings were required, or when sterically hindered amino acids were involved, the resin and the symmetrical anhydride were sonicated for 2-3hrs.

4.2.3.2 Loading of Wang resin with Fmoc-Lys(Tnm)-OH

Fmoc-Lys(Tnm)-OH (**2.5**) (533mg, 0.84mmol, 3eq, based on resin functionality) was dissolved in 1:1 DMF/1,4 dioxane (10ml). DIC (132µL, 0.84mmol, 3eq) and HOCt (132mg, 0.84mmol, 3eq) were added and the mixture was sonicated for 15 minutes. The solution was added to Wang resin (**2.20**) (500mg, 0.28mmol, initial functionality 0.56$mmolg^{-1}$, preswollen in DMF), in the presence of a catalytic amount of DMAP. The mixture was agitated, using a Merrifield bubbler, for 20hrs.

Fmoc loading = 0.16$mmolg^{-1}$.

4.2.3.3 Loading of *p*-alkoxybenzyloxycarbonylhydrazide (Wang-hydrazide) resin

To the desired amino acid (2eq, based on initial resin functionality), dissolved in 20ml DMF, HOCt (2eq) and DIC (2eq) were added. The resulting solution was sonicated at RT for 15mins. The newly formed HOCt ester was added to the Wang-hydrazide resin (**2.24**), which had been preswollen in the minimum amount of DMF. The mixture was sonicated for 2hrs. The resin was collected by filtration, washed with DMF, DCM and ether (2x10ml) and dried. An Fmoc loading test (section 4.2.1.2) was then carried out.

Fmoc loading = $0.36 mmol g^{-1}$

4.2.4 Automated SPPS

All peptides were synthesised on the ABI 430A automated peptide synthesiser with on-line UV monitoring using an ABI 758A detector. The Fmoc strategy for N^{α} protection was employed.

Every coupling cycle, resulting in the coupling of a single amino acid involved the following sequence of events. After every step, the resin was washed repeatedly with copious amounts of solvent to ensure the complete removal of all unreacted material.

4.2.4.1 Capping - *to irreversibly block any unreacted amino groups*

The resin was vortexed with a solution of acetic anhydride (0.5M), DIEA (0.125M) and HOBt (0.2% w/v) in DMF/1,4 dioxan (1:1, 10ml) for 10 minutes, then drained and washed with 6 portions of DMF/1,4 dioxan (1:1).

4.2.4.2 Deprotection - *removal of the Fmoc N^{α} protection releasing the amino functionality for coupling*

The resin was vortexed with a solution of 20% piperidine DMF/1,4 dioxan (1:1, 10ml) for 4 minutes before being drained. After washing with DMF/1,4 dioxan (1:1, 4 times), the resin was treated with a further portion of the 20% piperidine solution (10ml) and vortexed for a further 1.5 minutes. Finally, the resin was washed with 6 portions of DMF/1,4 dioxan (1:1). In order to monitor the synthesis, following each deprotection an aliquot of filtrate was passed through the UV detector with on-line integration. The absorbance at 302nm is directly related to the concentration of the Fmoc-piperidine adduct ($\varepsilon = 15{,}400$) and thus makes it possible to estimate the coupling efficiency of every amino acid, using the same principle as the Fmoc loading test previously described (section 4.2.1.2).

4.2.4.3 Peptide Bond formation

Activation - *formation of the HOCt ester*

Fmoc amino acid (1mmol) was treated with HOCt (1mmol) and DIC (1mmol) in DMF/1,4 dioxan (1:1, 8ml). After 15 minutes, the activated ester was transferred to the reaction vessel.

In the case of His, HOBt (2mmol) was added directly to the cartridge containing the amino acid. The HOBt ester of His is formed in place of its HOCt ester since the latter derivative has been found to undergo racemisation[7].

Coupling - *reaction with growing peptide on the solid support*

On addition of the activated amino acid to the resin, the mixture was vortexed for 30 minutes before the solution was drained and the resin washed with DMF/1,4 dioxan (1:1, 6x 10ml).

Notes

The peptide bond formation step may be repeated if desired in order to maximise coupling yield. This procedure is known as "double coupling". When FmocCys(Pic) was the activated amino acid, extended solvation and coupling times (30 minutes) were employed.

4.2.4.4 Final Fmoc Loading

Following completion, the final Fmoc-amino acid loading was calculated using the Fmoc loading test. Based on the absorbance at 302 nm the overall resin functionality was determined (section 4.2.1.2) and this compared with the theoretical final loading given 100% yield in every step of the synthesis (information provided using computer software). This in turn gave an indication as to the overall success of the synthesis.

4.2.4.5 Acidolytic treatment of resin-bound peptide

Scavenger Cocktail 1 - (peptide contains Arg, Cys or Met) EDT (0.3ml), thioanisole (0.25ml), TIS (0.25ml), phenol (750mg) and H_2O (1ml).
Scavenger Cocktail 2 - H_2O (0.25ml) and TIS (0.25ml).

The N^{α}–deprotected peptide-resin (up to 1g) was swollen and stirred in the prescribed scavenger cocktail. Where the peptide contained Arg, Cys or Met, scavenger cocktail 1 was used. In all other cases scavenger cocktail 2 was employed. TFA (10ml) was added and the mixture stirred under nitrogen at room temperature for a period of time determined by the specific peptide sequence, as indicated in the text. When less than 100mg of resin was involved, the cleavage mixture was scaled down and based on 5ml of TFA. The resin was removed by filtration and the filtrate was added to ice cold diethyl-ether. The resulting white precipitate was centrifuged to a pellet, washed in ether (3 x 10ml), dissolved in water/acetonitrile (1:1), or water/acetonitrile/AcOH as necessary, and lyophilised.

4.2.5 Tbfmoc Peptides

4.2.5.1 Preparation of Tbfmoc peptides

Loading of Tbfmoc onto peptide-resin

The peptide-resin was swollen in a minimum volume of capping reagent (Ac_2O 940μL, DIEA 440μL, HOBt 40mg, made up to 20ml with DCM) and sonicated for 30 minutes. It was then filtered, washed and dried. The resin was then swollen in 20% piperidine in DMF, followed by sonication (30 mins), filtering, washing (DMF, DCM, ether, 3x10ml) and drying at the pump.

The resin was swollen in distilled DCM and Tbfmoc chloroformate (3 equivalents to peptide-resin), was introduced, followed by DIEA (1 equivalent). The mixture was sonicated in the dark for 2-3 hours before the resin was filtered and washed with copious amounts of DCM and ether (~40ml/1g v/w). The resin was then stored in a vacuum dessicator in the dark until required.

The Tbfmoc loading test

The loading of the functionalised resin was determined by treating an accurately measured quantity of resin (typically ~ 5mg) with 20% piperidine/1,4 dioxan (10ml). After sonication for 10 minutes, the UV absorbance of the supernatant was recorded between 320 and 400 nm. The Tbfmoc-peptide-resin functionality and resulting coupling efficiency was calculated from the following equations:

$$\text{Resin functionality (Tbfmoc-peptide - mmol/g)} = \frac{0.613 \times \text{Abs}_{364\text{nm}}}{\text{weight of resin (mg)}}$$

$$\text{Coupling efficiency of Tbfmoc (\%)} = \frac{\text{resin funct. mmol/g (Tbfmoc-peptide)} \times 100}{\text{resin funct. mmol/g (peptide)}}$$

The Tbfmoc peptide is then subjected to the standard acidolytic cleavage procedure (4.2.3.5).

4.2.5.2 Tbfmoc purification

Adsorption

Tbfmoc peptide, dissolved in 7M aqueous urea/*i*-propanol (1:1, ~ 10mg/ml), was vortexed with washed activated charcoal until HPLC indicated that all the Tbfmoc-peptide had adsorbed onto charcoal (negligible absorbance at 365nm). The mixture was then centrifuged and the supernatant removed.

Wash

The charcoal was washed using 7M urea/*i*-propanol (1:1, 3 times) in order to remove any impurities trapped in the charcoal pellet. In each case, the mixture was vortexed, centrifuged and the supernatant discarded.

Tbfmoc cleavage

The charcoal was vortexed in 10% piperidine/7M urea/*i*-propanol (1:1) for 15 minutes, to release the peptide from the Tbfmoc moiety. Following centrifugation, *i*-propanol was removed from the supernatent *in vacuo*. The product solution was dialysed against distilled water in order to remove the urea and the piperidine salts. The resulting aqueous solution was lyophilised.

4.2.6 HPLC Purification and Analysis of Peptides

1. Analytical scale: 15cm, 4.6mm i.d. column; A=H_2O, 0.1%TFA, B=CH_3CN; 2ml loop, 1ml/min; 0-2min 10%B, 2-32min 10-90%B; λ=214nm
2. Semi prep scale: 15cm, 10.0mm i.d. column; A=H_2O, 0.1%TFA, B=CH_3CN; 5ml loop, 9 ml/min; 0-2min 10%B, 2-32min 10-90%B; λ=214nm

4.2.7 Endostatin - Fragments Prepared by SPPS

Tbfmoc-Gln^{93}-Leu-Gln-Pro-Gly-Ala-Arg-Ile-Phe-Ser-Phe-Asp-Gly-Arg-Asp-Val-Leu-Arg-His-Pro-Ala-Trp-Pro-Gln-Lys-Ser-Val-Trp-His-Gly-Ser-Asp-Pro-Ser-Gly-Arg-Arg-Leu-Met-Glu-Ser-Tyr-Cys-Glu-Thr-Trp-Arg-Thr-Glu-Thr-Thr-Gly-Ala-Thr-Gly-Gln-Ala-Ser-Ser-Leu-Leu-Ser-Gly-Arg-Leu-Leu-Gln-Glu-Lys-Ala-Ala-Ser-Cys-His-Asn-Ser-Tyr-Ile-Val-Leu-Cys-Ile-Glu-Asn-Ser-Phe-Met-Thr-Ser-Phe-Ser-Lys^{184}-Resin (Peptide 1)

Wang resin loaded with FmocLys(Boc) (0.35g, 0.32mmolg^{-1}, 0.1mmol scale) was used to prepare a peptide containing 93aa using automated SPPS. Trityl-protected Cysteine was used. After loading with Tbfmoc, the peptide was cleaved from the resin (cleavage time 6.0h). Purification of the peptide using the Tbfmoc protocol was carried out, followed by dialysis (1L, 20%AcOH in H_2O, MW cutoff = 5000) and lyophilisation.

Yield = 20mg, 7%; **HPLC** (Aquapore C_8) Rt = 21.0min, 66%B; **AAA** (36h) – Asx_5 5.3, Thr_6 5.7, Ser_{13} 12.5, Glx_9 9.4, Gly_7 6.7, Ala_6 6.4, Cys_3 1.0, Val_3 4.7, Ile_3 6.7, Leu_8 12.5, Tyr_2 1.3, Phe_4 4.5, His_3 2.7, Lys_3 3.7, Arg_7 6.4, Pro_4 4.9.

Fmoc-Cys^{135}-Glu-Thr-Trp-Arg-Thr-Glu-Thr-Thr-Gly-Ala-Thr-Gly-Gln-Ala-Ser-Ser-Leu-Leu-Ser-Gly-Arg-Leu-Leu-Gln-Glu-Lys-Ala-Ala-Ser^{164}-CO_2H (Peptide 3)

Wang resin loaded with FmocSer(^{t}Bu) (0.50g, 0.56mmolg^{-1}, 0.15mmol scale) was used to prepare a 29aa peptide fragment by automated SPPS. Trityl-protected Cysteine was used. The peptide was then cleaved from the resin (cleavage time 6.0h) and analysed. The fragment was purified by semi-preparative HPLC.

Yield = 200mg, 32%; **MS** (MALDI-TOF) mass = 3273.21 (MW (MI) = 3273); **HPLC** (Aquapore C_8) Rt = 17.0 min, 56%B; **AAA** (24h) Thr_5 3.6, Ser_4 3.1, Glx_5 4.1, Gly_3 3.0, Ala_4 4.2, Cys_1 0.5, Leu_4 3.7, Lys_1 1.3, Arg_2 1.7.

Fmoc-Cys^{165}-His-Asn-Ser-Tyr-Ile-Val-Leu-Cys-Ile-Glu-Asn-Ser-Phe-Met-Thr-Ser-Phe-Ser-Lys^{184}-Resin (Peptide 4)

PEG resin loaded with FmocLys(Boc) (0.60g, 0.18 mmolg^{-1}, 0.1mmol scale) was used to prepare a 20aa fragment by automated SPPS. Trityl-protected Ser and Thr amino acids were used in place of their *t*Butyl ether-protected counterparts. Val^{159} was double-coupled. A trial cleavage (112mg of resin, cleavage time 6.0h) was carried out to yield 13.8mg of crude peptide for analysis. The fragment remains bound to the resin.

Coupling efficiency 70%; **MS** (ESMS+) mass = 2549 (MW (MI) = 2548.8); **HPLC** (RP18 C_{18}) Rt = 19.6 min, 62%B; **AAA** (24h) – Asx_2 1.9, Thr_1 1.0, Ser_4 3.1, Glx_1 1.1, Cys_2 0.4, Val_1 0.7, Met_1 0.9, Ile_2 1.5, Leu_1 0.9, Tyr_1 0.9, Phe_2 2.1, His_1 1.0, Lys_1 1.1.

H_2N-Ala^{145}-Thr-Gly-Gln-Ala-Ser-Ser-Leu-Leu-Ser-Gly-Arg-Leu-Leu-Gln-Glu-Lys-Ala-Ala-Ser-Cys-His-Asn-Ser-Tyr-Ile-Val-Leu-Cys-Ile-Glu-Asn-Ser-Phe-Met-Thr-Ser-Phe-Ser-Lys^{184}-OH (Peptide 6)

Wang resin, loaded with FmocLys(Tnm)OH (0.55g, 0.16mmolg^{-1}, 0.1mmol scale) was used to prepare a 40aa fragment. Ser and Thr amino acids were used in place of their *t*Butyl ether-protected counterparts. Cys^{173}, Leu^{172} and Val^{171} were double coupled. The peptide was then cleaved from the resin (cleavage time 6.0h) and analysed. The fragment was purified by semi-preparative HPLC.

Yield = 48mg 10%; **MS** (ESMS+) mass = 5099, 4832 (MW (MI) = 4828); **HPLC** (Vydac C_{18}) Rt = 21 min, 66%B; **AAA** (24h) Asx_2 2.8, Thr_2 2.1, Ser_8 8.2, Glx_4 3.8, Gly_2 1.8, Ala_4 4.4, Cys_2 0.9, Val_1 1.0, Met_1 1.3, Ile_1 0.7, Leu_5 4.3, Tyr_1 0.8, Phe_2 2.9, His_1 1.4, Lys_2 2.5, Arg_1 0.9.

H_2N-Ala^{98}-Arg-Ile-Phe-Ser-Phe-Asp-Gly-Arg-Asp-Val-Leu-Arg-His-Pro-Ala-Trp-Pro-Gln-Lys-Ser-Val-Trp-His-Gly-Ser-Asp-Pro-Ser-Gly-Arg-Arg-Leu-Met-Glu-Ser-Tyr-Cys-Glu-Thr-Trp-Arg-Thr-Glu-Thr-Thr-Gly^{144}-$NHNHCONH_2$ (Peptide 7)

Semi-carbazide resin (0.45g, 0.25mmolg^{-1}, 0.1mmol scale) was used to prepare a peptide containing 47aa using automated SPPS. The last seven residues, Asp^{104} to Ala^{98}, were double coupled. The peptide was then cleaved from the resin (cleavage time 5.0h) and analysed. The fragment was purified by semi-preparative HPLC.

Yield = 80mg, 14%; **MS** (ESMS+) mass = 5831.8 (MW (MI) = 5831.7); **HPLC** (Vydac C_8) Rt = 16.4 min, 54%B. **AAA** (36h) - Asx_3 2.6, Thr_4 5.2, Ser_5 4.1, Glx_4 5.6, Gly_4 4.7, Ala_2 1.7, Cys_1 0.2, Val_2 1.9, Ile_1 0.7, Leu_2 2.1, Tyr_1 1.6, Phe_2 1.6, His_2 2.1, Lys_1 1.3, Arg_6 6.0, Pro_3 3.1.

H_2N-Pro^{70}-Ile-Val-Asn-Leu-Lys-Asp-Glu-Val-Leu-Ser-Pro-Ser-Trp-Asp-Ser-Leu-Phe-Ser-Gly-Ser-Gln-Gly-Gln-Leu-Gln-Pro-Gly^{97}-$NHNHCONH_2$ (Peptide 8)

Semi-carbazide resin (0.44g, 0.25mmolg^{-1}, 0.1mmol scale) was used to prepare a peptide containing 28aa using automated SPPS. Leu^{87}, Phe^{86}, Leu^{94} were double coupled. The coupling time of Lys^{75} was extended. The peptide was then cleaved from the resin (cleavage time 6.0h) and analysed. The fragment was purified by semi-preparative HPLC.

Yield = 167mg, 52%; **MS** (ESMS+) mass = 3235.25 (MW (MI)= 3232.3); **HPLC** (RP18 C_{18}) Rt = 18.0 min, 57%B.

H_2N-Val^{40}-Gly-Leu-Ser-Gly-Thr-Phe-Arg-Ala-Phe-Leu-Ser-Ser-Arg-Leu-Gln-Asp-Leu-Tyr-Ser-Ile-Val-Arg-Arg-Ala-Asp-Arg-Gly-Ser-Val^{69}-$NHNHCONH_2$ (Peptide 9)

Semi-carbazide resin (0.35g, 0.25mmolg^{-1}, 0.1 mmol scale) was used to prepare a peptide containing 30aa using automated SPPS. Ser^{59} Tyr^{58}, Leu^{54} Arg^{53} Phe^{52} and Arg^{47} Phe^{46} were double coupled. The synthesis was interrupted at Val^{69} and half the resin was removed. The retained peptide was cleaved from the resin (cleavage time 5.5h) and analysed. The fragment was purified by semi-preparative HPLC. An attempt was made to couple a further 10aa's to the resin-bound Peptide 9, but this was unsuccessful.

Yield 130mg 38%; **MS** (LCMS; ES+) mass = 3382.6, *3425.6* (Peptide 9 - MW (MI)= 3383.77, *acetylated (capped) Peptide 9 - MW (MI) = 3425.77*); **HPLC** (Vydac C_8) Rt = 22.2 min, 63%B; **AAA** (24h) – Asx_2 2.2, Thr_1 0.6, Ser_5 3.5, Glx_1 1.9, Gly_3 2.5, Ala_2 3.1, Val_3 2.6, Ile_1 0.9, Leu_4 3.0, Tyr_1 0.4, Phe_2 2.3, Arg_5 5.1.

H_2N-Gly^{22}-Met-Arg-Gly-Ile-Arg-Gly-Ala-Asp-Phe-Gln-Cys-Phe-Gln-Gln-Ala-Arg-Ala^{39}-$NHNHCONH_2$ (Peptide 10)

Semi-carbazide resin (0.41g, 0.25mmolg^{-1}, 0.1mmol scale) was used to prepare a peptide containing 18aa using automated SPPS. The peptide was then cleaved from the resin (cleavage time 5.0h) and analysed. The fragment was purified by semi-preparative HPLC.

Yield = 132mg, 61%; **MS** (ESMS+) mass = 2160.38 (MW (MI)= 2158.01); **HPLC** (RP18 C_{18}) Rt = 12.2 min, 43%B. **AAA** (24h) – Asx_1 1.0, Glx_3 3.1, Gly_3 2.1, Ala_3 3.1, Cys_1 0.2, Met_1 0.3, Ile_1 0.7, Phe_2 2.0, Arg_3 2.1.

Fmoc-His^{1}-Thr-His-Gln-Asp-Phe-Gln-Pro-Val-Leu-His-Leu-Val-Ala-Leu-Asn-Thr-Pro-Leu-Ser-Gly^{21}-NHNHCONH$_2$ (Peptide 11)

Semi-carbazide resin (0.32g, 0.31mmolg^{-1}, 0.1 mmol scale) was used to prepare a peptide containing 21aa using automated SPPS. The peptide was then cleaved from the resin (cleavage time 5.0h) and analysed. The fragment was purified by semi-preparative HPLC.

Yield = 162mg, 62%; **MS** (ESMS+) mass = 2603.00 (MW (MI) = 2602.24); **HPLC** (RP18 C_{18}) Rt = 16.0 min, 53%B. **AAA** (24h) – Asx_2 1.8, Thr_2 1.5, Ser_1 1.0, Glx_2 1.5, Gly_1 1.1, Ala_1 1.1, Val_2 2.0, Leu_4 3.6, Phe_1 0.9, His_3 2.2, Pro_2 2.2.

Fmoc-Thr-Glu-Thr-Thr-Gly-NHNHCONH$_2$ (Peptide 12)

Semi-carbazide resin (605mg, 0.25mmol/g, 0.15mmol scale) was used to synthesise a 5aa fragment. The peptide was cleaved from the resin and analysed (cleavage time 2.5h). Analytical HPLC showed a single peak and no further purification was carried out.

Yield = 90mg, 76%; **MS** (ESMS+) m/z = 787.31 (MW (MI) = 786.5); **HPLC** (Vydac C_{18}) Rt = 18 min, 58%B; RP18 C_{18}, Rt = 20.8 min, 66%B; **AAA** (24h) - Thr_3 1.7, Glu_1 1.0, Gly_1 1.0.

Ala-Thr-Gly-Gln-Ala-OH (Peptide 13)

Wang resin loaded with Fmoc-Ala (635g, 0.18mmolg^{-1}, 0.1mmol scale) was used to prepare a 5aa peptide acid fragment. The peptide was then cleaved from the resin (cleavage time 3.5h) and analysed. No further purification was carried out.

Yield = 42mg, 82%. **MS** (ESMS+) m/z 447.10 (MW (MI) = 446); **HPLC** (Vydac C_{18}) Rt = 15 min, 51%B; RP18 C_{18}, Rt = 20.0 min, 64%B; **AAA** (24h) – Thr_1 0.9, Glu_1 1.0, Gly_1 1.1, Ala_2 1.8.

Fmoc-Thr-Glu-Thr-Thr-Gly-$NHNH_2$ (Peptide 14)

Wang-hydrazine resin loaded with Gly (460mg, 0.35mmolg^{-1}, 0.15mmol scale) was used to prepare a 5aa fragment. The peptide was cleaved from the resin and analysed (cleavage time 2 hrs). Purification was carried out by semi-prep HPLC.

Yield = 85mg, 71%; **MS** (ESMS+) m/z = 744.36 (MW (MI) = 744); **HPLC** (RP18 C_{18}) Rt = 17.2 min, 51%B; **AAA** (18h) - Thr_3 2.2, Glu_1 1.0, Gly_1 1.0.

Ala-Thr-Gly-Gln-Ala-(resin) (Peptide 15)

Wang resin loaded with Fmoc-Ala (900mg, 0.13mmolg^{-1}, 0.1mmol scale) was used to prepare a 5aa peptide acid fragment. Following the completion of the automated synthesis, Fmoc was manually cleaved from the resin (10% piperidine/DMF, 20ml; sonicated 30mins; filtered, washed (DCM, Et_2O, 2x10ml), dried under vacuum). A trial cleavage was carried out, stirring was continued for 3.5hrs.

Yield (resin + peptide) = 920mg; **Final Fmoc loading** = 0.08mmolg^{-1}; **MS** (ESMS+) m/z = 447 (MW (MI) = 446); **HPLC** (RP18 C_{18}) Rt = 20.0min, 64%B.

Fmoc-Gly-Gln-Leu-Gln-Pro-Gly-$NHNH_2$ (Peptide 16)

Wang-hydrazine resin loaded with Fmoc-Gly (450mg, 0.28mmolg^{-1}, 0.1mmol scale) was used to prepare a 6aa fragment. The peptide was cleaved from the resin and analysed (cleavage time 2 hrs).

Yield = 95mg, 90%; **MS** (ESMS+) m/z = 835.37 (MW (MI) = 835); **HPLC** (RP18 C_{18}) Rt = 18.8 min, 56%B.

Ala-Arg-Ile-Phe-Ser-Resin (Peptide 17)

Wang resin loaded with Fmoc-Ser (1.19g, 0.15mmolg^{-1}, 0.2mmol scale) was used to prepare a 5aa fragment. Following the completion of the automated synthesis, Fmoc was manually cleaved from the resin (10% piperidine/DMF, 20ml; sonicated 30mins; filtered, washed (DCM, Et_2O, 2x10ml), dried under vacuum). A trial cleavage was carried out using standard conditions; stirring was continued for 5h.

Yield (resin + peptide) = 1.18g; **Final Fmoc loading** = 0.11mmolg^{-1}; **MS** (ESMS+) m/z 593.27 (MW (MI) = 592); **HPLC** (RP18 C_{18}) Rt = 19.4 min, 63%B; **AAA** (18h) – Ser_1 0.7, Ala_1 1.1, Ile_1 1.0, Phe_1 1.0, Arg_1 1.0.

Fmoc-Glu-Thr-Trp-Arg-Thr-Glu-Thr-Thr-Gly-Ala-$NHNH_2$ (Peptide 18)

Wang-hydrazine resin loaded with Fmoc-Ala (400mg, 0.38mmolg^{-1}, 0.15mmol scale) was used to prepare a 10aa fragment. The peptide was cleaved from the resin using the standard conditions; stirring was continued for 5.5hrs. Purification was carried out using semi-prep HPLC.

Yield = 125mg, 60%; **MS** (ESMS+) mass = 1387.4 (MW (MI) =1388.5); **HPLC** (RP18 C_{18}) Rt = 17.4 min, 57%B; **AAA** (24h) - Thr_4 3.6, Glx_2 2.0, Gly_1 1.0, Ala_1 1.0, Arg_1 1.0, Trp_1 0.

Fmoc-Gly-Ser-Gln-Gly-Gln-Leu-Gln-Pro-Gly-$NHNH_2$ (Peptide 19)

Wang-hydrazine functionalised resin loaded with Fmoc-Gly (500mg, 0.35mmolg^{-1}, 0.15mmol scale) was used to prepare a 9aa fragment. The peptide was cleaved from the resin using the standard conditions; stirring was continued for 3hrs. Purification was carried out using semi-preparative HPLC.

Yield = 78mg, 40%; **MS** (ESMS+) mass = 1107.25 (MW (MI) = 1108.4); **HPLC** (RP18 C_{18}) Rt = 15.6 min, 54%B; **AAA** (24h) - Asx_1 0.9, Glx_3 3.0, Gly_3 3.0, Leu_1 1.1, Pro_1 0.9.

Fmoc-Gly-Ser-Gln-Gly-Gln-Leu-Gln-Pro-$NHNH_2$ (Peptide 20)

Wang-hydrazine functionalised resin loaded with Fmoc-Ala (400mg, 0.43mmolg^{-1}, 0.15mmol scale) was used to prepare an 8aa fragment. The peptide was cleaved from the resin using the standard conditions; stirring was continued for 3.5hrs. Purification was carried out using semi-preparative HPLC.

Yield = 55mg, 31%; **MS** (ESMS+) m/z = 1050.28 (MW (MI) = 1049.4); **HPLC** (RP18 C_{18}) Rt = 12.2 min, 45%B; **AAA** (24h) - Asx_1 0.9, Glx_3 3.0, Gly_2 2.3, Leu_1 1.1, Pro_1 1.1.

Ala-Arg-Ile-Phe-Ser-Phe-Asp-Gly-Arg-Asp-(resin) (Peptide 21)

Wang resin loaded with Fmoc-Asp (560mg, 0.35mmolg^{-1}, 0.2mmol scale) was used to prepare a 10aa fragment. A final Fmoc loading test was carried out and Fmoc was then removed manually (10% piperidine/DMF, 20ml; sonicated 30mins; filtered, washed (DCM, Et_2O, 2x10ml), dried under vacuum). A trial cleavage was carried out using standard conditions; stirring was continued for 5.5hrs.

Yield (resin+peptide) = 600mg; **Final Fmoc loading** = 0.16mmolg^{-1}; **MS** (ESMS+) m/z = 1405.50 (MW (MI) = 1404.57); **HPLC** (RP18 C_{18}) Rt = 19.8 min, 63%B; **AAA** (24h) - Asx_2 1.9, Ser_1 0.8, Gly_1 1.0, Ala_1 0.8, Ile_1 1.1, Phe_2 1.9, Arg_2 1.8.

Ala^{163}-Ser-Cys-His-Asn-Ser-Tyr-Ile-Val-Leu-Cys-Ile-Glu-Asn-Ser-Phe-Met-Thr-Ser-Phe-Ser-Lys^{184}- (resin) (Peptide 22)

Wang resin loaded with Fmoc-Lys(Tnm) (530mg, 0.21mmolg^{-1}, 0.1mmol scale) was used to prepare a 22aa fragment. The coupling times of the two Fmoc-Cys(Pic) residues were extended and double coupling procedures were employed at Ser^{183}, Phe^{182}, and at Leu^{172}, Val^{171}. A trial cleavage was carried out using standard conditions; stirring was continued for 6hrs. Fmoc was manually cleaved from the remaining resin (10% piperidine/DMF, 20ml; sonicated 30mins; filtered, washed (DCM, Et_2O, 2x10ml), dried under vacuum).

Yield (resin+peptide) = 715mg; **Final Fmoc loading** = 0.07mmolg^{-1}; **MS** (ESMS+) mass = 3062.80±0.44 (MW (MI+Fmoc) = 3060.9); **HPLC** (RP18 C_{18}) Rt = 15.8 min, 51%B; **AAA** (36h) – Asx_2 1.9, Thr_1 1.0, Ser_5 3.4, Glx_1 1.2, Ala_1 0.4, Cys_2 0.1, Val_1 0.8, Met_1 1.0, Ile_2 1.7, Leu_1 1.0, Tyr_1 0.3, Phe_2 2.4, His_1 0.7, Lys_1 1.3.

Fmoc-Ala^{145}-Thr-Gly-Gln-Ala-Ser-Ser-Leu-Leu-Ser-Gly-Arg-Leu-Leu-Gln-Glu-Lys-Ala^{162}-NHNH$_2$ (Peptide 23)

Wang-hydrazine resin was loaded with Fmoc-Ala (500mg, 0.35mmolg^{-1}, 0.2mmol scale) and an18aa fragment was prepared. The solvation time of Fmoc-Lys(Tnm)-OH was extended and double couple cycles were used at Glu^{160}, Gln^{159}. The peptide was cleaved from the resin using the standard conditions and stirring was continued for 5.5hrs. Purification was carried out using semi-prep HPLC.

Yield = 58mg, 13%; **MS** (ESMS+) mass = 2243.12±0.18 (MW (MI)= 2241.05); **HPLC** (RP18 C_{18}) Rt = 21.0 min, 66%B; **AAA** (24h) – Thr_1 0.9, Ser_3 2.5, Glx_3 3.2, Gly_2 2.0, Ala_3 3.0, Leu_4 4.2, Lys_1 1.0, Arg_1 1.1.

4.2.8 The Coupling of Endostatin Fragments *via* Transfer Active Ester Condensation

Fmoc-Thr-Glu-Thr-Thr-Gly-Ala-Thr-Gly-Gln-Ala-OH (Peptide 24)

Peptide **12** (5.3mg, 6.7μmol) together with HOCt (235mg, 1.5mmol) was dissolved in DMF (1.5ml). The reagents were stirred for 1h, then chilled to 0°C. Following the addition of *t*BuONO (2μL, 14μmol, 2eq) as a single portion the reagents were allowed to warm to room temperature. Stirring was continued for 2.5h when HPLC monitoring indicated that formation of the HOCt ester was complete. Peptide **13** (3.2mg, 7.1μmol, 1eq,) was added as to the mixture as solid, followed immediately by DIEA (1.5mmol, 194mg, 0.26ml) in one portion. After 18h, a large new peak was observed by HPLC and stirring was discontinued. Desalting was carried out by analytical scale HPLC and the collected fractions were characterized by ESMS+.

MS (ESMS+) m/z 1158.7 (MW (MI) = 1157.9); **HPLC** (RP18 C_{18}) Rt = 20.4 min, 66%B.

Fmoc-Thr-Glu-Thr-Thr-Gly-Ala-Thr-Gly-Gln-Ala-OH (Peptide 25)

Peptide **14** (5.4mg, 7.3μmol) together with HOCt (235mg, 1.5mmol) was dissolved in DMF (1.5ml). The reagents were stirred for 2h and the solution was then chilled to 0°C. Following the addition of *t*BuONO (1.4mg, 2μL, 14μmol, 2eq) in one portion the reagents were allowed to warm to room temperature. Stirring was continued for 45min when HPLC monitoring indicated complete formation of the active ester intermediate. Peptide **13** (3.2mg, 7.1μmol, 1eq) was added as a solid, followed immediately by DIEA (1.5mmol, 194mg, 0.26ml) in one portion. After 1h, HPLC monitoring showed the reaction to be complete. The reaction was sampled after 1h. Product formation was evident, as was the disappearance of the active ester moiety. Desalting was carried out by semi-prep HPLC and the collected fractions were characterized by ESMS+. The major peak proved to be *title compound.*

MS (ESMS+) m/z 1155.9 (MW (MI) = 1157.9); **HPLC** (Hichrom C_{18}) Rt = 17.8 min, 57%B.

Fmoc-Thr-Glu-Thr-Thr-Gly-Ala-Thr-Gly-Gln-Ala-OH (Peptide 26)

Peptide **14** (50mg, 0.067mmol) together with HOCt (1.57g, 10mmol) was dissolved in DMF (10ml). The reagents were stirred for 2h, then chilled to 0°C. Following the addition of *t*BuONO (10.4mg, 12μL, 0.1mmol, 1.5eq) in one portion the reagents were allowed to warm to room temperature. Stirring was continued for 30min, before HPLC monitoring indicated that formation of the HOCt ester intermediate was complete. The resin, to which was attached Peptide **15** (0.425g, 0.08mmolg^{-1}, 0.034mmol, 0.5eq), was added to the reaction mixture, followed immediately by DIEA (10mmol, 1.29g, 1.74ml) in one portion. Stirring was continued overnight until HPLC monitoring indicated that no further reaction was taking place. An Fmoc loading test on the filtered resin confirmed that coupling to the resin-bound peptide had occurred. The product was cleaved from the resin under standard conditions, for 4h. No precipitation in ice cold ether occurred, and the ether layers were therefore washed with H_2O and concentrated *in vacuo*. The mixture was purified by semi-prep HPLC at 302nm; the collected fractions were lyophilised and characterized by ESMS+. **Final Fmoc loading** = 0.02mmolg^{-1}, 25%; **MS** (ESMS+) m/z 1158.2 (MW (MI) = 1157.9); **HPLC** (RP18 C_{18}) Rt = 20.2 min, 61%B; **AAA** (24h) – Thr_4 3.9, Glx_2 2.0, Gly_2 2.0, Ala_2 2.0.

Fmoc-Thr-Glu-Thr-Thr-Gly-Gly-Gln-Leu-Gln-Pro-Gly-Ala-Arg-Ile-Phe-Ser-NHNH$_2$ (Peptide 28)

Peptide **16** (37mg, 0.044mmol) together with HOCt (1.57g, 10mmol) was dissolved in DMF (10ml). The reagents were stirred gently for 1.5h, then chilled to 0°C. Following the addition of *t*BuONO (2eq, 0.1mmol, 10.4mg, 12μL) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 30min, before HPLC monitoring indicated that formation of the HOCt active ester was now complete. The dry resin, to which was attached Peptide **17** (0.169g, 0.13mmolg^{-1}, 0.022mmol, 0.5eq) was added to the mixture, followed immediately by DIEA (10mmol, 1.29g, 1.74ml) in one portion. HPLC monitoring indicated that the reaction was complete after 30mins. An Fmoc

loading test on the filtered resin confirmed that coupling had occurred. Fmoc was manually cleaved from the resin (10% piperidine/DMF, 20ml; sonicated 30mins; filtered, washed (DCM, Et_2O, 2x10ml), dried under vacuum), to yield H-GQLQPGARIFS-® (Peptide **27**). Peptide **14** (33mg, 0.044mmol) together with HOCt (1.57g, 10mmol) was dissolved in DMF (10ml) and stirred until it had dissolved (1h). The solution was chilled to 0°C and *t*BuONO (2eq, 0.1mmol, 10.4mg, 12μL) was added. As before, the reaction was monitored by HPLC and upon detection of the active ester (30min), Peptide **27** was added, followed immediately by DIEA (10mmol, 1.29g, 1.74ml) in one portion. HPLC monitoring indicated that the reaction was complete after 1h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. A final Fmoc loading test was carried out to confirm coupling had occurred, before the peptide was cleaved from the resin under standard conditions for 4h. Analysis by HPLC indicated that three components were present; these were separated by analytical scale HPLC and characterised.

Yield = 9mg; **Final Fmoc loading** = 0.07mmolg^{-1}, 22%; ***Coupling 1 – Fmoc loading*** = *0.038mmolg^{-1}, 35%*; **MS** (ESMS+) mass 1884.24±0.19 (MW (MI) = 1883.8); **HPLC** (RP18 C_{18}) Rt = 22.0 min, 66%B; **AAA** (18h) – Thr_3 2.6, Ser_1 0.7, Glu_1+Gln_2 2.8, Gly_3 2.7, Ala_1 1.1, Ile_1 1.2, Leu_1 1.1, Phe_1 1.1, Arg_1 1.0, Pro_1 0.8. ***Main impurity***: 10aa, deletion (TETTGARIFS); **MS** (ESMS+) mass = 1303.5 (MW (MI) = 1303); **HPLC** (RP18 C_{18}) Rt = 23.0 min, 68%B; **AAA** (18h) – Thr_3 2.5, Ser_1 0.6, Glu_1+Gln_0 1.2, Gly_1 1.1, Ala_1 1.0, Ile_1 1.3, Phe_1 1.0, Arg_1 0.9. ***Minor impurity***: 5aa, starting material (Peptide **17**); **MS** (ESMS+) m/z 593 (MW (MI) = 592); **HPLC** (RP18 C_{18}) Rt = 16.0 min, 51%B.

Fmoc-Gly-Ser-Gln-Gly-Gln-Leu-Gln-Pro-Gly-Ala-Arg-Ile-Phe-Ser-Phe-Asp-Gly-Arg-Asp-OH

(Peptide 29)

Peptide **19** (40mg, 0.04mmol, 2eq) together with HOCt (628mg, 4mmol) was dissolved in DMF (4ml). The reagents were stirred gently for 1.5h, then chilled to 0°C. Following the addition of *t*BuONO (4.8μL, 0.03mmol, 1.1eq) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 1h, when HPLC monitoring indicated that formation of the HOCt active ester was now complete. The resin-bound Peptide **21** (50mg, 0.16mmolg^{-1}, 0.4eq) was added, followed immediately by DIEA (0.7ml, 4mmol). The reagents were stirred gently at room temperature and the reaction was stopped after 2.5h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. An Fmoc loading test confirmed that coupling to the resin-bound peptide had occurred. The product was then cleaved from the resin under standard conditions, for 5h.

Final Fmoc loading = 0.092 mmolg^{-1}, 56%; **MS** (ESMS+) mass = 2255.63 (MW (MI) = 2256); **HPLC** (RP18 C_{18}) Rt = 19.8 min, 64%B. *Main impurity:* 10aa, starting material, (Peptide **22**); **MS** (LCMS, ES+) Rt = 16.2 min, λ=280nm, mass = 1182.49 (MW (MI) = 1182.57); **HPLC** (RP18 C_{18}) Rt = 15.0 min, 52%B.

(Peptide 30)

Peptide **19** (40mg, 0.04mmol, 2eq) together with HOCt (628mg, 4mmol) was dissolved in dry DCM (4ml). The reagents were stirred gently until all the solid material had dissolved (1h), then chilled to 0°C. Following the addition of *t*BuONO (4.8μL, 0.03mmol, 1.1eq) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 1h, when HPLC monitoring indicated that formation of the HOCt active ester was now complete. The resin-bound Peptide **21** (50mg, 0.16mmolg^{-1}, 0.4eq) was added, followed immediately by DIEA (0.7ml, 4mmol). The reagents were stirred gently at room

temperature and the reaction was stopped after 2h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. An Fmoc loading test confirmed that coupling to the resin-bound peptide had occurred. The product was then cleaved from the resin under standard conditions, for 5h.

Final Fmoc loading = 0.095 $mmolg^{-1}$, 59%; **MS** (ESMS+) m/z 2255.59 (MW (MI) = 2256); **HPLC** (RP18 C_{18}) Rt = 19.8 min, 64%B. *Main impurity*: 10aa, starting material, (Peptide **22**); **MS** (LCMS, ES+) Rt = 16.2 min, λ=280nm, mass = 1182.49 (MW (MI) = 1182.57); **HPLC** (RP18 C_{18}) Rt = 15.0 min, 52%B.

(Peptide 31)

Peptide **19** (60mg, 0.04mmol, 2eq) together with HOCt (628mg, 4mmol) was dissolved in DMF (2ml). The reagents were stirred gently for 0.5h, then chilled to 0°C. Following the addition of *t*BuONO (4.8μL, 0.03mmol, 1.1eq) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 1.5h, when HPLC monitoring indicated that formation of the HOCt active ester was now complete. The resin-bound Peptide **21** (100mg, 0.16$mmolg^{-1}$, 0.8eq) was added, followed after 30min by DIEA (0.7ml, 4mmol). The reagents were stirred gently at room temperature and the reaction was stopped after 3.5h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. An Fmoc loading test confirmed that coupling to the resin-bound peptide had occurred. The product was then cleaved from the resin under standard conditions, for 5h.

Final Fmoc loading = 0.19 $mmolg^{-1}$, 100%; **MS** (ESMS+) m/z = 2257.63 (MW (MI) = 2256); **HPLC** (RP18 C_{18}) Rt = 16.4min, 56%B; **AAA** (crude, 24h) – Asx_2 3.0, Ser_2 2.0, Glx_3 2.7, Gly_4 3.9, Ala_1 2.0, Ile_1 1.6, Leu_1 1.2, Phe_2 3.5, Arg_2 3.0, Pro_1 0.9.

Fmoc-Gly-Ser-Gln-Gly-Gln-Leu-Gln-Pro-Ala-Arg-Ile-Phe-Ser-Phe-Asp-Gly-Arg-Asp-OH (Peptide 32)

Peptide **20** (20mg, 0.02mmol, 2eq) together with HOCt (314g, 2mmol) was dissolved in DMF (1ml). The reagents were stirred gently for 0.5h, then chilled to 0°C. Following the addition of *t*BuONO (2.4μL, 0.015mmol, 1.1eq) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 1h, when HPLC monitoring indicated that formation of the HOCt active ester was now complete. The resin-bound Peptide **21** (50mg, 0.16mmolg^{-1}, 0.8eq) was added, followed after 15min by DIEA (0.35ml, 2mmol). The reagents were sonicated and the reaction was stopped after 3.5h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. An Fmoc loading test confirmed that coupling to the resin-bound peptide had occurred. The product was then cleaved from the resin under standard conditions, for 5.5h.

Final Fmoc loading = 0.13mmolg^{-1}, 80%; **MS** (ESMS+) mass 2200.41 (MW (MI) = 2201); **HPLC** (RP18 C_{18}) Rt = 16.2min, 55%B; **AAA** (24h) – Asx_2 1.9, Ser_2 1.6, Glx_3 2.4, Gly_3 2.5, Ala_1 0.9, Ile_1 0.9, Leu_1 1.7, Phe_2 2.2, Arg_2 1.8, Pro_1 0.8.

Fmoc-Glu-Thr-Trp-Arg-Thr-Glu-Thr-Thr-Gly-Ala-Gly-Ser-Gln-Gly-Gln-Leu-Gln-Pro-Gly-Ala-Arg-Ile-Phe-Ser-Phe-Asp-Gly-Arg-Asp-OH (Peptide 34)

Peptide **19** (20mg, 0.02mmol, 2eq) together with HOCt (314mg, 2mmol) was dissolved in DMF (2ml). The reagents were stirred gently for 0.5h, then chilled to 0°C. Following the addition of *t*BuONO (2.4μL, 0.015mmol, 1.1eq) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 1.5h, when HPLC monitoring indicated that formation of the HOCt active ester was now complete. The resin-bound Peptide **21** (70mg, 0.16mmolg^{-1}, 1.1eq) was added, followed after 5min by DIEA (0.35ml, 2mmol). The reagents were sonicated and the reaction was stopped after 2.5h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. An Fmoc loading test confirmed that coupling to the resin-bound peptide had occurred. Fmoc was manually cleaved from the resin and left swollen (10% piperidine/DMF, 20ml;

sonicated 30mins; filtered, washed (DMF, 2x10ml)), yielding H-GSQGQLQPGARIFSFDGRD-® (Peptide **33**). Due to lack of material, no capping procedure was carried out. The coupling of a third 10aa fragment to this 20mer was then attempted. Peptide **18** (20mg, 0.02mmol, 2eq) together with HOCt (314mg, 2mmol) was dissolved in DMF (1ml). The reagents were stirred gently for 1h, then chilled to 0°C. Following the addition of *t*BuONO (2.4μL, 0.015mmol, 1.1eq) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 1h, when HPLC monitoring indicated that formation of the HOCt active ester was now complete. Peptide **33** (27mg) was added, followed after 15min by DIEA (0.35ml, 2mmol). The reagents were sonicated and the reaction was stopped after 2.5h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. An Fmoc loading test confirmed that coupling to the resin-bound peptide had occurred. The product was then cleaved from the resin under standard conditions, for 5.25h. Although the final yield was low, the desired product was detected by ESMS.

Final Fmoc loading = 0.031 $mmolg^{-1}$, 48% (20% overall); *Coupling 1 – Fmoc loading = 0.064mmolg^{-1}, 40%*; **MS** (ESMS+) mass 3371.00 (MW (MI) = 3371.5); **HPLC** (RP18 C_{18}) Rt = 19.0 min, 62%B.

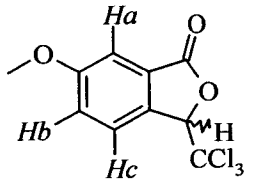
Fmoc-Ala^{145}-Thr-Gly-Gln-Ala-Ser-Ser-Leu-Leu-Ser-Gly-Arg-Leu-Leu-Gln-Glu-Lys-Ala-Ala-Ser-Cys-His-Asn-Ser-Tyr-Ile-Val-Leu-Cys-Ile-Glu-Asn-Ser-Phe-Met-Thr-Ser-Phe-Ser-Lys^{184}-OH (Peptide 35)

Peptide **23** (20mg, 0.01mmol, 2eq) together with HOCt (157mg, 1mmol) was dissolved in DMF (0.5ml). The reagents were stirred gently for 0.5h, then chilled to 0°C. Following the addition of *t*BuONO (1.7μL, 0.015mmol, 1.1eq) in one portion, the reagents were allowed to warm to room temperature. Stirring was continued for 1.1h, when HPLC monitoring indicated that formation of the HOCt active ester was now complete. The dry, resin-bound Peptide **22** (80mg, 0.07mmolg^{-1}, 0.6eq) was added and allowed to swell. DIEA (0.18ml, 1mmol) was next added, after 15min and the reagents were sonicated. The reaction was stopped after 3.5h. The resin was collected by filtration, washed (DMF, DCM, Et_2O, 2x10ml), and dried at the pump. An Fmoc loading test confirmed that coupling to the resin-bound peptide had occurred. The peptide was subjected to the standard cleavage conditions, based on 1ml TFA, for a period of 4hrs. The peptide product was precipitated into ice-cold ether, washed by centrifugation and lyophilised. Analysis of the crude material was carried out before purification was attempted. Initial HPLC studies showed that the material was relatively pure, however the peptide was extremely insoluble and further purification by HPLC was unsuccessful due to equipment failure.

Yield = 8.3mg; **Final Fmoc loading** = 0.05 mmolg^{-1}, 64%; **MS** (ESMS+) mass 5051.53±3.95, 5061.24±1.60 (MW (MI)= 5049.85); **HPLC** (RP18 C_{18}, λ = 302) Rt = 24.2 min, 75%B; **AAA** (crude, 24h) – Asx_2 3.6, Thr_2 2.5, Ser_8 9.5, Glx_4 3.5, Gly_2 1.9, Ala_4 2.2, Cys_2 0.4, Val_1 1.5, Met_1 2.4, Ile_1 3.1, Leu_5 3.8, Tyr_1 1.2, Phe_2 5.0, His_1 1.4, Lys_2 3.2, Arg_1 0.5. *Side product:* 18aa, (Peptide **23** - peptide acid); **HPLC** (RP18 C_{18}) Rt = 16.4 min, 55%B; **AAA** (24h) – Thr_1 0.9, Ser_3 2.4, Glu_1+ Gln_2 3.1, Gly_2 2.1, Ala_3 2.8, Leu_4 3.6, Lys_1 1.1, Arg_1 1.1.

4.3 The Investigation of a Novel Strategy for the Protection of Arginine in Solid Phase Peptide Synthesis

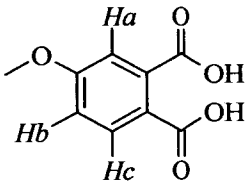
6-Methoxy-3-trichloromethyl phthalide (3.9)



m-Anisic acid (**3.8**) (20g, 0.13 moles) was mixed with chloral hydrate (24g, 0.14 moles, 1.1eq). To this was added conc. H_2SO_4 (200ml, 10 vols.) and the reagents were stirred until no solid remained. The mixture was poured into a large excess of ice/water. The resulting precipitate was collected by filtration and recrystallised from ethanol, giving white, needle-like crystals.

Yield = 20.05g, 54%; **Mp** 129-132°C; **MS** FAB m/z = 280 (MH^+); **HRMS** 280.9539 (MH^+ requires 280.9540); **CHN** found C 42.06% H 2.51% ($C_{10}H_6O_3Cl_3$ requires C 42.66%, H 2.54%); **^{1}H NMR (250 MHz, D_2O δH/ppm)** 3.90 (3H, s, **CH_3O**), 5.86 (1H, s, **CH**-CCl_3), 7.27 (1H, dd, J = 2.6, 8.4 Ar-**H_b**), 7.38 (1H, d, J = 2.6, Ar-**H_a**), 7.80 (1H, d, J = 8.4, Ar-**H_c**); **^{13}C {^{1}H} NMR (63 MHz, $CDCl_3$ δC/ppm)** 55.82 (q), 86.72 (d), 97.69 (s), 108.03 (d), 122.82 (d), 125.65 (d), 129.03 (s), 135.04 (s), 162.03 (s), 168.09 (s). **FTIR ν_{max}/cm^{-1} (nujol)** 1112 (methoxy C-O), 1690, 1776 (lactone C=O), 2856, 2936 (methoxy CH); **λ_{max}/nm (MeOH)** 299.

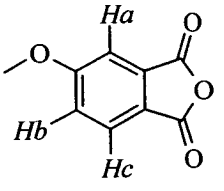
4-Methoxy phthalic acid (3.10)[8]



To **3.9** (5g, 0.018mmoles) was added a solution of NaOH (3M, 40ml, 7eq). This suspension was heated at 100°C for approximately 1 hour, until a brown solution was formed. The solution was then chilled in an ice bath and $KMnO_4$ solution (0.15M, 50mls, 0.6eq) was added, dropwise. After approximately 5h, a solution of NaOH was added in one portion (16.5ml, 1M, 1eq). The solution was then left to stir overnight before a further portion of $KMnO_4$ (0.15M, 50mls, 0.6eq) was added, dropwise. The solution was left to stir for a further 48h at room temperature, until it became dark brown in colour. The solution was next acidified with conc. HCl and saturated with SO_2. The resulting clear solution was concentrated *in vacuo* and chilled to yield a precipitate of white needles, which was isolated by filtration. The filtrate was reacidified and saturated with SO_2 until no further product was produced. The remaining filtrate was evaporated to a solid and reoxidised using the same conditions as before. The product was purified by recrystallisation from water.

Yield = 2.569g, 74%; **Mp** 166-169°C (lit 168-170°C[9]); **MS** FAB m/z = 197 (MH^+); **HRMS** 197.0453 (MH^+ requires 197.0451); **^{1}H NMR (250MHz, D_2O δH/ppm)** 3.69 (3H, s, **CH_3O**), 7.10 (1H, dd, J = 2.6, 8.3, Ar-**H_b**), 7.14 (1H, d, J = 2.6, Ar-**H_a**), 7.86 (1H, s, J = 8.4, Ar-**H_c**); **^{13}C {^{1}H} NMR (63 MHz, $CDCl_3$ δC/ppm)** 55.71 (q), 113.42 (d), 115.78 (d), 120.92 (s), 132.13 (d), 136.26 (s), 162.21 (s), 170.12 (s), 173.24 (s). **FTIR ν_{max}/cm^{-1} (nujol)** 1502, 1578, 1603 (aromatic ring), 1677, 1730 (C=O), 2846 (methoxy CH), 2983 (OH); **λ_{max}/nm (MeOH))** 254.

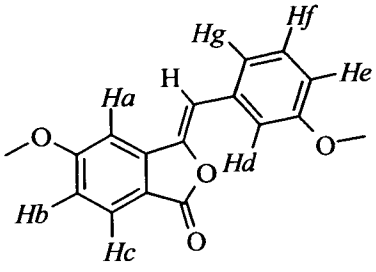
4-Methoxy phthalic anhydride (3.11)



To **3.10** (1.12g, 5.7mmols) was added Ac_2O (56ml, peptide synthesis grade). The mixture was then refluxed, at 160°C, for 2h. Ac_2O was removed *in vacuo*. The resulting beige solid was dissolved in DCM and filtered, to remove any unreacted starting material. The filtrate was concentrated to yield **3.11**, which was recrystallised from ethanol to yield white, needle-like crystals.

Yield = 0.930g, 91%; **Rf** 0.3 (2:1 DCM/Hexane); **Mp** 165-167°C; **MS** EI m/z = 178 (M), FAB m/z = 179 (MH^+); **HRMS** 179.0339 (MH^+ requires 179.0345); **CHN** found C 60.02%, H 3.47% ($C_9H_6O_4$ requires C 60.58%, H 3.39%); **^{1}H NMR (250MHz, D^{6}DMSO, δH/ppm)** 3.76 (3H, s, **CH_3O**), 7.06 (1H, d, J = 2.7, Ar-**H_a**), 7.11 (1H, dd, J = 2.7, 8.6, Ar-**H_b**), 7.80 (1H, d, J = 8.6, Ar-**H_c**); **^{13}C {^{1}H} NMR (63 MHz, D^{6}DMSO, δC/ppm)** 52.72 (q), 113.26 (d), 115.76 (d), 122.33 (s), 131.93 (d), 136.45 (s), 161.97 (s), 167.09 (s), 168.84 (s); **FTIR ν_{max}/cm^{-1} (KBr)** 1770, 1847 (cyclic anhydride C=O), 2846 (CH); **λ_{max}/nm (MeOH)** 252.

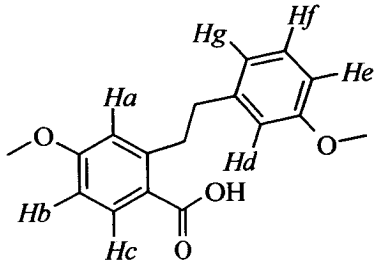
5-Methoxy-3-(3-methoxybenzyl) phthalide (3.13)[10]



To **3.11** (3.04g, 17.1mmol) was added 3-methoxy phenyl acetic acid (**3.12**) (3.12g, 18.8mmol, 1.1eq) together with a catalytic amount of sodium acetate (100 mg). The reagents were then mixed intimately (mortar and pestle), and heated to 260°C under N_2 for 1.5h. The product solidified on cooling. It was recrystallised twice from ethanol and once from acetonitrile, to yield **3.13** as an off-white, amorphous solid.

Yield = 0.52g, 11%; **Mp** 132-134°C (lit 141-142°C[10]); **MS** FAB m/z = 283 (MH^+); **HRMS** 283.0971 (MH^+ requires 283.0971); **^{1}H NMR (250MHz, D^{6}DMSO, δH/ppm)** 3.86 (3H, s, **CH_3O**), 3.95 (3H, s, **CH_3O**), 6.34 (1H, s, olefinic **CH**), 6.85 (1H, ddd, J = 1.2, 2.6, 8.1, Ar-**H_e**), 7.06 (1H, dd, J = 2.0, 8.5, Ar-**H_b**), 7.13 (1H, d, J = 2.0, Ar-**H_a**), 7.31 (1H, t, J = 8.1, Ar-**H_f**), 7.42 (2H, m, Ar-**H_d**, Ar-**H_g**), 7.82 (1H, d, J = 8.5, Ar-**H_c**); **^{13}C {^{1}H} NMR (63 MHz, D^{6}DMSO, δC/ppm)** 55.22 (q), 55.85 (q), 102.57 (d), 106.58 (d), 114.35 (d), 114.85 (d), 116.01 (q), 118.18 (d), 122.68 (d), 126.99 (d), 129.57 (d), 134.26 (s), 143.06 (s), 144.58 (s), 159.65 (s), 164.97 (s), 166.54 (s); **FTIR ν_{max}/cm^{-1} (KBr)** - 1661 (conjugated C=C), 1750 (conjugated lactone C=O); **λ_{max}/nm (MeOH)** 277, 308, 340.

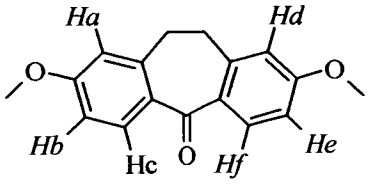
4-Methoxy-2-(3-methoxy-β-phenethyl)benzoic acid (3.14)[10]



3.13 (0.82g, 2.9mmol) was suspended in water (11.5ml) and sodium hydroxide (0.58g, 14.5mmol, 5eq) was added. The mixture was stirred and heated at 85-90°C for 2hrs. The resulting brown solution was diluted with water (9.6ml) and the pH was adjusted to 8.5 with 10%HCl. It was then cooled to room temperature and filtered. The filtrate was hydrogenated at an initial pressure of 60psi with 10% Pd/C (0.08g) at 80°C in a 250ml autoclave, for 24h. The solution was then filtered, acidified and extracted with chloroform. The organic layers were concentrated to give a white solid, which was recrystallised from ethanol.

Yield = 0.50g, 60%; **Rf** 0.6 (1:2 EtOAc/hexane); **Mp** 118-120°C (lit. 124°C[10]); **MS** EI m/z = 286 (M), 269 (M – OH), FAB m/z = 287 (MH^+); **HRMS** 287.1292, 285.1410 (MH^+ requires 287.1284); **CHN** found C 71.71%, H 6.16% ($C_{17}H_{18}O_4$ requires C 71.31%, H 6.34%, $C_{17}H_{16}O_4$ requires 71.82%, H 5.67%); **^{1}H NMR (250MHz, D^{6}DMSO, δH/ppm)** 2.90(2H, t, J = 7.3, aliphatic **CH_2**), 3.32 (2H, t, J = 7.3, aliphatic **CH_2**), 3.79 (3H, s, **CH_3O**), 3.80 (3H, s, **CH_3O**), 6.77-6.85 (5H, m, Ar-**H_a/H_b/H_d/H_e/H_g**), 7.20 (1H, t, J = 8.1, Ar-**H_f**), 8.10 (1H, d, J = 8.8, Ar-**H_c**); **^{13}C {^{1}H} NMR (63 MHz, D^{6}DMSO, δC/ppm)** 37.38 (t), 37.85 (t), 54.99 (q), 55.25 (q), 111.41 (d), 112.54 (d), 113.86 (d), 115.18 (q), 119.80 (s) 120.84 (d), 129.15 (d), 134.29 (d), 143.50 (s), 149.19 (s), 159.46 (s), 162.95 (s), 171.77 (s); **FTIR ν_{max}/cm^{-1} (KBr)** – 1738 (acid C=O), 2837, 2942 (methoxy CH), 3462 (acid OH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 255 (14400). *Impurity (3.23, 30% by nmr):* **^{1}H NMR (250MHz, D^{6}DMSO, δH/ppm)** 3.09 (1H, dd, J = 6.2, 13.5, aliphatic H-**CH**-CH_2), 3.30 (1H, dd, J = 6.2, 13.5, aliphatic H-**CH**), 3.79 (3H, s, **CH_3O**), 3.82 (3H, s, **CH_3O**), 5.59 (1H, t, J = 13.5, aliphatic **CH**-CH_2), 6.52 (1H, s, Ar-**H_d**) 6.69-6.73 (3H, m, Ar –**H_a/H_e/H_g**), 6.98 (1H, d, J = 8.4, Ar-**H_b**), 7.16 (1H, t, J = 8.1, Ar-**H_f**), 7.76 (1H, d, J = 8.8, Ar-**H_c**); **^{13}C {^{1}H} NMR (63 MHz, D^{6}DMSO, δC/ppm)** 40.87 (t), 55.11 (q), 55.59 (q), 80.33 (d), 106.23 (d), 111.42 (d), 116.49 (d), 116.57 (d), 118.34 (q), 121.95 (d), 127.05 (d), 129.42 (d), 136.55 (s), 151.75 (s), 159.54 (s), 164.16 (s), 169.90 (s);

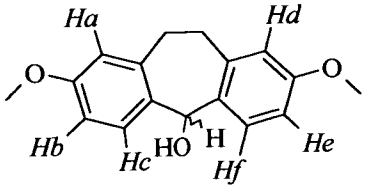
10,11-Dihydro-2,8-dimethoxy-5H-dibenzo[a,d]cyclohepten-5-one (3.15)[11]



To **3.14** (1.12g, 3.9mmol) was added PPA (8.6g, 30mmol, 8-10eq). The mixture was heated at 130°C, under nitrogen. After 1.5h, an excess of ice/water mixture (200ml) was added to the reaction and the organic components were extracted with ethyl acetate (3x200ml). The combined organic extracts were washed with dilute NaOH (1M, 100ml) and brine (100ml), dried ($NaSO_4$) and concentrated. The product was then recrystallised from ethanol, resulting in off-white prisms.

Yield = 0.49g, 47%; **Rf** = 0.7 (EtOAc/Hex, 1:2); **HPLC** (Aquapore RP18 C_{18}, λ = 214nm) Rt 18 min, 56% MeCN; **Mp** = 117-120°C (lit. 120°C[10]); **MS** FAB m/z = 269 (MH^+); **HRMS** 268.1177 (MH^+ requires 268.1099); **^{1}H NMR (250MHz, D^{6}DMSO, δH/ppm)** 3.10 (4H, s, **CH_2-CH_2**), 3.83 (6H, s, 2x**CH_3O**), 6.88 (2H, d, J = 2.6, Ar-**H_a/ H_d**), 6.93 (2H, dd, J = 2.6, 8.71, Ar-**H_b/ H_e**), 7.99 (2H, d, J = 8.7, Ar-**H_c/H_f**); **^{13}C {^{1}H} NMR (63 MHz, D^{6}DMSO, δC/ppm)** 34.96 (t), 55.56 (q), 112.64 (d), 113.96 (d), 130.59 (s), 133.49 (d), 145.22 (s), 162.46 (s), 189.88 (s); **FTIR ν_{max}/cm^{-1} (KBr)** - 1597 (diaryl ketone C=O), 2841, 2947 (methoxy CH); **λ_{max}/nm (MeOH, ε/dm^{3}mol^{-1}cm^{-1})** 313 (21600).

10,11-Dihydro-2,8-dimethoxy-5H-dibenzo[a,d]cyclohepten-5-ol (3.16)[11]



To **3.15** (104mg, 0.37mmol) in isopropyl alcohol (2ml) was added $NaBH_4$ (68mg, 0.18mmol, 0.5eq) and TEA (24μL, 0.37mmol, 1eq). The reaction was heated at reflux, under nitrogen, and monitored by HPLC. The starting material had disappeared after 2h. The solvent was removed *in vacuo* and water was added, dropwise, to yield a white precipitate which was recovered by filtration and dried in a vacuum dessicator. The product was then recrystallised from isopropyl alcohol, to yield white needle-like crystals.

Yield = 0.94g, 90%; **Rf** = 0.8 (1:2 EtOAc/Hexane); **HPLC** (Aquapore RP18 C_{18}, λ = 214nm) Rt 14.6 min, 49% MeCN; **Mp** = 113°C; **MS** FAB m/z = 253 (M – OH), 270 (MH^+); **HRMS** 270.1256 (MH^+ requires 270.1256); **CHN** found C 75.70%, H 6.31% ($C_{17}H_{18}O_3$ requires C 75.53%, H 6.71%); **^{1}H NMR (250MHz, $CDCl_3$, δH/ppm)** 1.57 (1H, s, **CH**-OH), 2.82 (2H, dd, J = -14.0, 7.9, **HCH**-**HCH**), 3.57 (2H, dd, J = -14.0, 7.9, **HCH**-**HCH**), 3.77 (6H, s, 2x**CH_3**O), 5.04 (1H, broad, **OH**), (6.66 (4H, m, Ar-**H_a**/**H_b**/**H_d**/**H_e**), 6.97 (2H, d, J = 7.8, Ar-**H_c**/ **H_f**); **^{13}C {^{1}H} NMR (63 MHz, $CDCl_3$, δC/ppm)** 32.40 (t), 55.22 (q), 110.43 (d), 115.64 (d), 129.82 (d), 130.84 (s), 132.73 (d), 141.84 (s), 159.05 (s); **FTIR ν_{max}/cm^{-1} (AcCN)** – 1042 (C-O), 2948, 3010 (methoxy CH), 3162, sharp, (OH,); **λ_{max}/nm (MeOH)** 238, 274.

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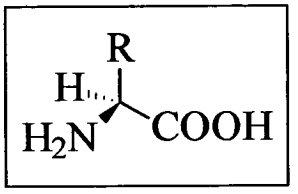
[1] Kaiser, E., Colescott R.L., Bossinger C.D., Cook, P.I., *Anal. Biochem.*, 1970, **34**, 595-598
[2] Gosden A., Macrae R. and Young G.T., *J. Chem. Soc. Perkin Trans. I*, 1977, **8**, 317-323.
[3] Bland L., *Ph.D. Thesis*, University of Edinburgh, 1999.
[4] Ramage, R., Biggin G.W., Brown A.R., Comer A.J., Davidson A., Draffan L.C., Jiang L., Morton G.H., Robertson N., Shaw K.T., Tennant, G., Urqhart, K. and Wilken J., *Proc. 4th Int. Symp. In Solid Phase Synth. and Combinatorial Chemical Libraries*, Edinburgh, 1995.
[5] Zhang, R.E., Cao, Y.-L. and Hearn, M.W., *Anal. Biochem.*, 1991, **195**, 160-170.
[6] Patterson, J.A. and Ramage, R., *Tetrahedron Lett.*, 1999, **40**, 6121.
[7] Robertson, N., *PhD Thesis*, University of Edinburgh, 1996.
[8] McAlees, *J.Chem Soc.Perkin I*, 1977, 2030.
[9] Chakravarti S.N., Perkin W.H., *J.Chem. Soc.*, 1929, 197-201.
[10] Humber L.G., *United States Patent no. 3697581*, 1972.
[11] Noda .M., Yamaguchi M., Ando E., Takeda K. and Nokihara K., *J. Org. Chem.*, 1994, **59**, 7968-7975.

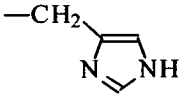
Chemically Synthesised Endostatin Fragments

Endostatin Fragments

Peptide		
1	*Tbfmoc*-QLQPGARIFSFDGRDVLRHPAWPQKSVWHGSDPSGRRLMESYCETWRTETTGATGQAS SLLSGRLLEQKAASCHNSYIVLCIENSFMTSFSK-*OH*	93aa
2	*Fmoc*-QLQPGARIFSFDGRDVLRHPAWPQKSVWHGSDPSGRRLMESYCETWRTETTGATGQAS SLLSGRLLEQKAASCHNSYIVLCIENSFMTSFSK-*OH*	93aa
3	*Fmoc*-CETWRTETTGATGQASSLLSGRLLEQKAAS-*OH*	30aa
4	*Fmoc*-CHNSYIVLCIENSFMTSFSK-*Resin*	20aa
5	*Fmoc*-CFQQARAVGLSGTFRAFLSSRLQDLYSIVRRADRGSVPIVNLKDEVLSPSWDSLFSGSQGQLQPGARIFSFDGRDVLRHPAWPQKSVWHGSDPSGRRLMESY-*OH*	102aa
6	ATGQASSLLSGRLLEQKAASCHNSYIVLCIENSFMTSFSK-*OH*	40aa
7	*Fmoc*-ARIFSFDGRDVLRHPAWPQKSVWHGSDPSGRRLMESYCETWRTETTG-*NHNHCONH2*	47aa
8	*Fmoc*-PIVNLKDEVLSPSWDSLFSGSQGQLQPG-*NHNHCONH2*	28aa
9	*Fmoc*-VGLSGTFRAFLSSRLQDLYSIVRRADRGSV-*NHNHCONH2*	30aa
10	*Fmoc*-GMRGIRGADFQCFQQARA-*NHNHCONH2*	18aa
11	*Fmoc*-HTHQDFQPVLHLVALNTPLSG-*NHNHCONH2*	21aa
12	*Fmoc*-TETTG-*NHNHCONH2*	5aa
13	ATGQA-*OH*	5aa
14	*Fmoc*-TETTG-*NHNH2*	5aa
15	ATGQA-*Resin*	5aa
16	*Fmoc*-GQLQPG-*NHNH2*	5aa
17	ARIFS-*Resin*	6aa
18	*Fmoc*-ETWRTETTGA-*NHNH2*	10aa
19	*Fmoc*-GSQGQLQPG-*NHNH2*	9aa
20	*Fmoc*-GSQGQLQP-*NHNH2*	8aa
21	ARIFSFDGRD-*Resin*	10aa
22	ASCHNSYIVLCIENSFMTSFSK-*Resin*	22aa
23	*Fmoc*-ATGQASSLLSGRLLEQKA-*NHNH2*	18aa
	Coupled Products	
24	*Fmoc*-TETTGATGQA-*OH*	10aa
25	*Fmoc*-TETTGATGQA-*OH*	10aa
26	*Fmoc*-TETTGATGQA-*OH*	10aa
27	GQLQPGARIFS-*Resin*	10aa
28	*Fmoc*-TETTGGQLQPGARIFS-*OH*	16aa
29	*Fmoc*-GSQGQLQPGARIFSFDGRD-*OH*	19aa
30	*Fmoc*-GSQGQLQPGARIFSFDGRD-*OH*	19aa
31	*Fmoc*-GSQGQLQPGARIFSFDGRD-*OH*	19aa
32	*Fmoc*-GSQGQLQPARIFSFDGRD-*OH*	19aa
33	GSQGQLQPGARIFSFDGRD-*Resin*	18aa
34	*Fmoc*-ETWRTETTGAGSQGQLQPGARIFSFDGRD-*OH*	29aa
35	*Fmoc*-ATGQASSLLSGRLLEQKAASCHNSYIVLCIENSFMTSFSK-*OH*	40aa

The Naturally Occurring Amino Acids



Amino Acid	3 Letter Code	1 Letter Code	R Group
Alanine	Ala	A	$-CH_3$
Arginine	Arg	R	$-(CH_2)_3NHC(NH)NH_2$
Asparagine	Asn	N	$-CH_2CONH_2$
Aspartic Acid	Asp	D	$-CH_2COOH$
Cysteine	Cys	C	$-CH_2SH$
Glutamic Acid	Glu	E	$-(CH_2)_2COOH$
Glutamine	Gln	Q	$-(CH_2)_2CONH_2$
Glycine	Gly	G	-H
Histidine	His	H	
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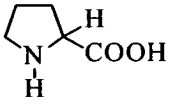
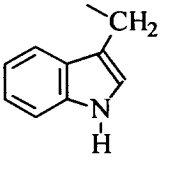
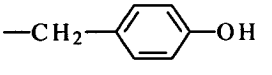
Leucine	Leu	L	$-CH_2CH(CH_3)_2$
Lysine	Lys	K	$-(CH_2)_4NH_2$
Methionine	Met	M	$-CH_2CH_2SCH_3$
Phenylalanine	Phe	F	$-CH_2(C_6H_5)$
Proline	Pro	P	
Serine	Ser	S	$-CH_2OH$
Threonine	Thr	T	$-CH(CH_3)OH$
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	$-CH(CH_3)_2$

Lectures & Conferences Attended

Organic Section Seminars (1997-2000): various speakers

Departmental Colloquia (1997-2000): various speakers

Current Awareness in Organic Chemistry (5 lectures p.a.) (1997-2000); sponsored by Zeneca.

Walker Memorial Lectures (1997-2000): Prof J.M.Lehn (Strasbourg), Dr.T.McKillop (AstraZeneca Ltd.), Prof. G. Guilbalt (University College, Cork).

Synthons in Organic Chemistry (5 lectures) (1998): Prof. Dr. E.Villsmaier (Kaiserslautern)

Amino Acids in Organic Chemistry (5 lectures) (1999): Dr.J.Podlech (Stuttgart)

Romanes Symposium (1998): Prof.K.C.Nicolau (Scripps Research Institute), Prof.K.J.Hale (University College London), Prof.P.J.Kocienski (Glasgow).

RSC Scottish Perkin Division Meetings: Strathclyde (1997), St.Andrews (1998), Aberdeen (1999).

SCI Graduate Symposium on Novel Organic Chemistry: Edinburgh (1998), Glasgow(1999).

15th International Conference in Medicinal Chemistry (1998), Edinburgh.

6th Solid Phase Synthesis Symposium (1999), York.

10th RSC - SCI Medicinal Chemistry Symposium (1999), Cambridge.