UNIVERSITY OF SOUTHAMPTON

New Linkers for the Direct Biological Assay of Combinatorial Libraries

by

Jennifer Kathleen Susan Britton

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Department of Chemistry
Faculty of Science

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ABSTRACT

FACULTY OF SCIENCE

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Solid phase combinatorial libraries are primarily synthesised for screening against a biological target. This thesis describes the synthesis and evaluation of three safety-catch linkers, which allow cleavage of amines and alcohols from the solid phase in biologically compatible conditions.

The ability of these linkers to release alcohols was demonstrated through the synthesis and evaluation of models in solution and on the solid phase. Two of these linkers were shown to successfully release amines through the synthesis and evaluation of model compounds tested in solution. However these linkers were not demonstrated to be useful for cleaving hydroxamic acids from the solid phase.

The purpose of this technology was to facilitate the development of a novel high throughput screening technique known as the zone diffusion assay. This assay was designed to isolate active compounds rapidly from a combinatorial library. Studies towards this new assay are described, using gelatinase (the enzyme), gelatin (the substrate) and marimastat (a known hydroxamic acid inhibitor) and the principle was conclusively demonstrated. Due to the lack of the utility of these linkers to the cleavage of hydroxamic acids from the solid phase, the linkers were not successfully applied to the development of this assay.

Contents

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Last but not least a big thanks to Dave for everything, I owe you my sanity!

Abbreviations

Ala	Alanine
APMA	Aminoparamercuric acetate
Boc_2O	Di-*tert*-butyl dicarbonate
BOP	1-Benzotriazolyloxy-tris-dimethylaminophosphonium hexafluorophosphate
Bn	Benzyl
Cys	Cysteine
DCC	Dicyclohexylcarbodiimide
DCU	Dicyclohexylurea
DCM	Dichoromethane
DIBAL-H	Diisobutylaluminum hydride
DIC	Diisopropylcarbodiimide
DIPEA	Diisopropylethylamine
DMAP	Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
Fmoc	Fluoren-9-ylmethoxycarbonyl
Glu	Glutamic acid
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HMPA	Hexamethylphosphoric triamide
IR	Infra red
min	minutes
MMP	Matrix metalloproteinase enzyme
MS	Mass spectrometry
NBS	N-bromosuccinimde
Nu	Nucleophile
NMR	Nuclear magnetic resonance
NPM	([(2*S*)-1-(nitrophenyl)pyrrolidin-2-yl]methan-1-ol)
PCC	Pyridinium chlorochromate

PDC	Pyridinium dichromate
PPTS	Pyridinium p-toluene sulfonate
Pro	Proline
PyBOP	Benzotriazolyloxy-tris[pyrrolidino]-phosphonium hexafluorophosphate
RP-HPLC	Reverse phase high pressure liquid chromatography.
Su	N-Hydroxy-succinimide
TBAF	Tetrabutylammonium fluoride
TBS/TBDMS	*tert*butyldimethylsilyl
TBTU	2-(1H-triazole-1-yl)-1,1,3,3-tetramehtyluronium tetrafluoroborate
TIMP	Tissue inhibitor of matrix metalloproteinase enzymes
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS-Cl	Trimethylsilylchloride
UV	Ultra violet
Z group	Benzyloxycarbonyl

NMR data

δ	Chemical shift (ppm)
s	singlet
d	doublet
q	quartet
br	broad
J	coupling constant (Hz)
Ar	Aromatic

IR data

s	strong
w	weak
m	medium
br.	broad

Chapter One Introduction

1.1] Solid phase combinatorial chemistry

With so many diseases still without an effective cure, and the increasing occurrence of pathogens, which are resistant to existing treatments, the search for new and more effective drugs has never been so intense. One of the most powerful techniques to have emerged in recent years is the preparation and screening of compound libraries.[1] The application of chemical libraries in drug discovery is two fold; firstly the synthesis of libraries with no rational design, the aim being to generate a new lead and secondly the synthesis of analogues of a known lead compound to optimise biological activity. Although the combinatorial approach has been used to generate arrays of individual compounds in parallel using solution chemistry, the application of solid phase synthesis has been revolutionary.

1.1.1] Solid phase synthesis

Merrifield[2] was a pioneer in the field of solid phase synthesis, and his visionary ideas revolutionised peptide synthesis. The technique has since been adapted for the synthesis of other oligomeric compounds such as oligonucleotides[3] and more recently oligosaccharides.[4] Although solid phase peptide synthesis has been comprehensively studied,[5] there has recently been a much greater focus on the adaptations of other synthetic techniques to the solid phase to generate small non-oligomeric molecules.[6]

Solid phase synthesis has many advantages over standard solution based chemistry, most notably;

- High concentrations of reagents can be used leading to increased yields and lower reaction times.
- Purification is simply washing and filtering the resin.
- The experimental protocol is simple and amenable to automation.

Solid phase synthesis however, is not compatible with traditional solution based analytical techniques available to the synthetic chemist, which is the most significant disadvantage. Cleavage and traditional analysis is often the most reliable method of determining the progress of a solid phase reaction. Quantitative colourmetric tests such as the Kaiser test[7] and Fmoc test[8] are widely used, although their application is limited to specific functional groups. The use of gel phase ^{13}C NMR[9] is increasing and enriched samples improve sensitivity. The advent of magic angle spinning technology has made the use of ^{1}H gel phase NMR possible.[10] IR spectroscopy is now regularly applied to solid phase product analysis, although it is limited in scope and requires expensive equipment for single bead analysis.[11]

Resins for solid phase synthesis

Resins commonly utilised for solid phase chemistry are small insoluble particles. The resin must be both physically and chemically robust and swell in the solvents used in the synthetic procedure. Traditionally the resin of choice is polystyrene based, made with 1-2% divinylbenzene as a cross-linking agent. This resin type is very useful for chemistry in organic solvents, and has a typical loading of 1-2 mmol/g. Polystyrene resin however, will not swell in polar protic solvents. TentaGel,[12] polyethylene co-grafted polystyrene will to swell equally well in protic and aprotic solvents and is probably the most versatile resin available. However, the substitution level attainable is typically 0.3 mmol/g, considerably lower than polystyrene resin.

1.1.2] Linkers

The role of the linker in solid phase synthesis is to attach the initial substrate to the solid support and allow quantitative cleavage of the target compound from the solid phase on completion of the synthesis, under specified conditions. The substrate is attached to the linker via a bond labile to the cleavage conditions and the linker is attached to the solid phase via an inert bond (*figure 1.1*). The linker is central to the design of the synthetic strategy and the screening techniques, which can be applied.

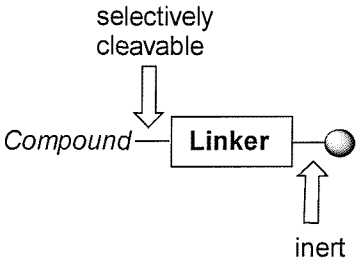


Figure 1.1: Linker properties

1.1.2.1] The Wang linker

The Wang linker is an alkoxy-benzyl alcohol derivative, developed initially to enable anchored peptides to be cleaved under much milder conditions than those required to effect cleavage from chloromethyl polystyrene resin (Merrifield resin). The Wang linker is one of the most widely used linkers for immobilising carboxylic acids.[13] This can be achieved in a variety of ways, but DIC/DMAP is often the method of choice. 50% TFA/DCM is sufficient to effect cleavage (*figure 1.2*). The Sasrin linker is more acid labile because of the additional electron donating methoxy group, and cleavage from the resin can be effected with 1% TFA/DCM.[14]

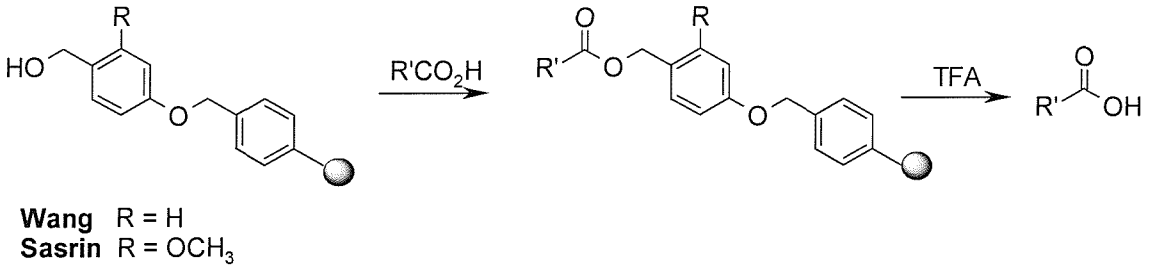


Figure 1.2: Wang and Sasrin linkers

Cleavage of the Wang linker can be achieved without acid. Under basic conditions attack by methoxide[15] yields the methyl ester. To extend the utility of the Wang linker, halide derivatives[16] and trichloroacetimidate derivatives[17] have been synthesised which can then be derivatised with a range of nucleophiles. Sulfonamides, ureas, amides and alcohols have all been attached to the solid phase using these Wang linker derivatives.

1.1.2.2] The Rink linker

The Rink linker was originally designed for the synthesis of peptide amides, but it is now widely used in solid phase organic chemistry.[18] The amine can be acylated with a variety of reagents and cleavage is effected with 20-50% TFA/DCM[19] (*figure 1.3*).

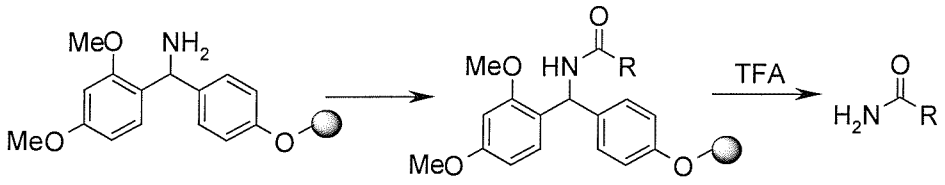
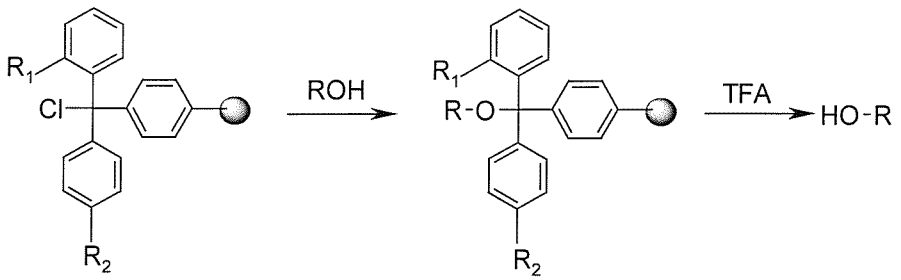


Figure 1.3: Rink Linker

The chloride derivative of this linker has been prepared by reacting Rink alcohol with 1% HCl in DCM/THF or PPh_3/C_2Cl_6.[20] Many functionalities can be immobilised on this variant including amines, alcohols, phenols, thiols and acids.

1.1.2.3] Trityl linkers

Alcohols can be attached to the trityl linker in the presence of pyridine[21] (*figure 1.4*). Cleavage can be effected using 5% TFA/DCM although the use of higher concentrations has been documented.[21] A chlorinated variant has been reported and a higher concentration of TFA is required to effect cleavage.[22]



Trityl $R_1 = H,\ R_2 = H$
Chlorotrityl $R_1 = Cl,\ R_2 = H$
Methoxytrityl $R_1 = H,\ R_2 = OCH_3$

Figure 1.4: Trityl linkers

A more activated methoxy variant requiring only 1% TFA/DCM to achieve cleavage has also been reported.[23] Other functionalities can be attached to the trityl linkers including amines[24] and carboxylic acids.[25]

1.1.2.4] Photolabile linkers

Photolabile linkers are useful because they allow the direct assay of the substrates cleaved. However, cleavage of photolabile linkers tends to require longer reaction times in comparison to other linkers, due to shadowing and this can be damaging for the substrates. The most common type is based on the nitrobenzyl functionality[26] (*figure 1.5*).

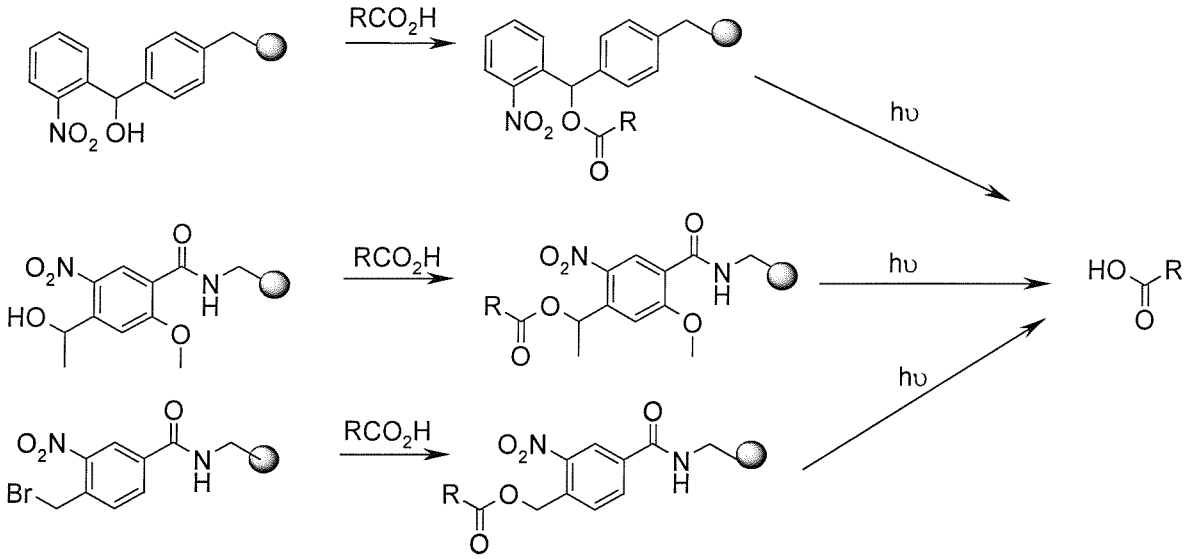


Figure 1.5: Photolabile linkers

1.1.2.5] Traceless linkers

Most linkers leave a functional group on the cleaved molecule, which was used to attach that molecule to the linker. Linkers that leave no obvious residue on the cleaved molecule have been termed *traceless*. The most common linkers in this category are aryl silyl linkers that cleave forming a new carbon hydrogen bond.

One example is the synthesis of benzodiazepine derivatives using an aryl silyl linker[27] (*figure 1.6*). Cleavage is effected by exposure to HF, which results in cleavage of the aryl

silicon bond releasing an aromatic compound with no obvious memory of resin attachment.

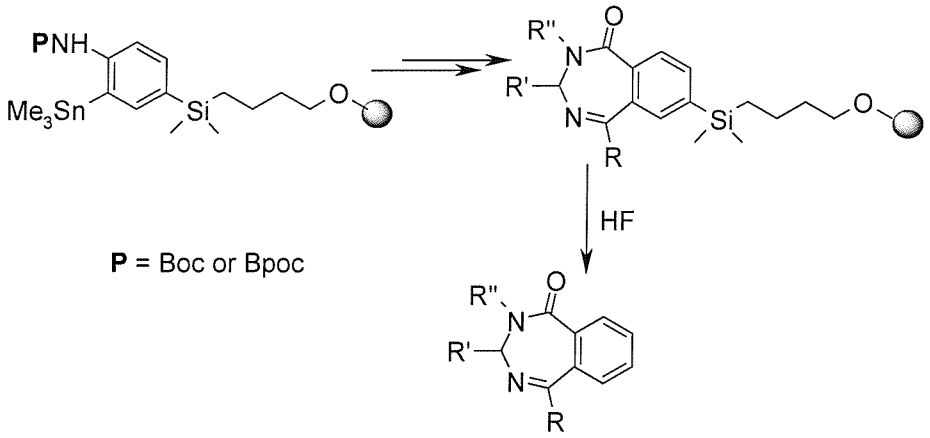


Figure 1.6: Benzodiazepine synthesis using a traceless linker

1.1.3] Safety-catch linkers

By definition, the safety-catch principle involves a process based upon *the conversion of a relatively stable form of a linker into a labile and cleavable one.*[28] This principle has been applied to solid phase synthesis and combinatorial chemistry, with the design of several linkers. The use of safety-catch linkers adds an extra element of orthogonality, which allows the potential use of a larger repertoire of organic reactions. In addition it allows deprotection and final cleavage from the resin to be two distinct steps, thus allowing the final cleavage from the solid support in mild biologically compatible conditions and the removal of by-products and protecting groups prior to cleavage. This is useful since in most cases the purpose of a synthetic library is to screen for biological activity, and common synthetic reagents and solvents are generally harmful to biological entities. The linkers described are categorised by the conditions in which they perform the final cleavage step after activation.

1.1.3.1] Safety-catch linkers sensitive to nucleophilic attack.

Kenner[29] developed the first safety-catch linker and employed it in solid phase peptide chemistry (*figure 1.7*). It was based on the acyl sulfonamide functionality, which is stable to both strong acid and base. N-methylation activates the linker and renders it susceptible to cleavage by NaOH, ammonia, amines or hydrazine.

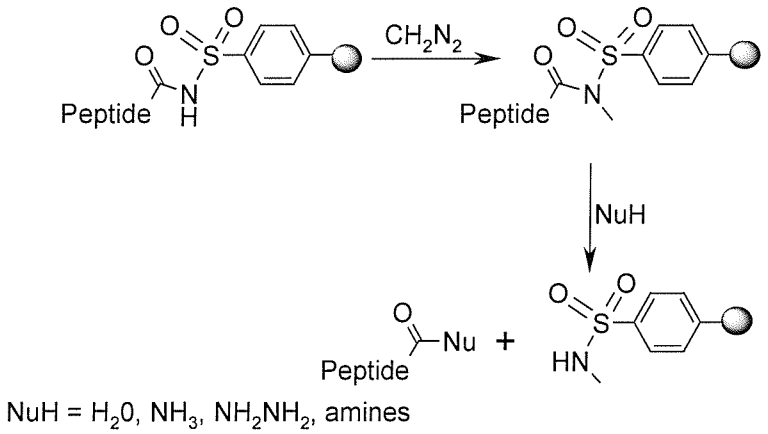


Figure 1.7: Kenner's safety-catch linker

Ellman[30] demonstrated that Kenner's linker was amenable to solid phase organic synthesis by performing Suzuki couplings and enolate alkylations in the synthesis of aryl acetic acid derivatives. However limitations included poor loading efficiencies, racemisation in the loading step and poor reactivity of the N,N-methylacylsulfonamide. To address these problems the linker was adapted. It was initially discovered that haloacetonitrile activation dramatically increased the reactivity of the acylsulfonamide towards nucleophiles and other amines (*figure 1.8*). However when carboxylic acids containing α electron-withdrawing groups were used incomplete alkylation of the acylsulfonamide linker was noted. It transpired that by using an alkyl variant (*figure 1.8*) nucleophilicity of the acyl sulfonamide anion was increased and solved this problem.[31] Using PyBOP in the presence of DIPEA to couple the first amino acid to the linker gave good loading efficiencies and no detectable racemisation.[32]

Figure 1.8: Variants of Kenner's linker

An oxidative safety-catch linker was designed by Marshall[33] and has been used in the synthesis of protected peptide fragments, cyclic and linear peptides. The linker was stable to acid and aminolysis during synthesis. Activation by oxidation to the sulfone was facile and nucleophilic attack by amines or amino acids released peptidic compounds from the solid phase. (*figure 1.9*).

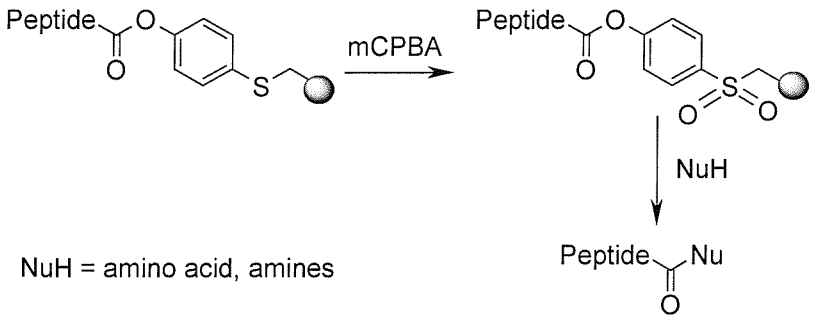


Figure 1.9: Marshall's sulfur based linker

A limitation of this linker was that oxidation sensitive amino acids such as cysteine and tryptophan could not be included. This linker was used in the synthesis of tetrahydro-β-carboline-3-carboxamides[34] and piperazine-2-carboxamides[35] where an excess of amine was used as the nucleophile, with products cleaved from the linker without prior activation by oxidation.

A new thiopyrimidine linker was developed by Hoffmann-La Roche for the synthesis of new pyrimidines.[36] Oxidation to the sulfone resulted in a species that was susceptible to attack by secondary amines and other nucleophiles (*figure 1.10*).

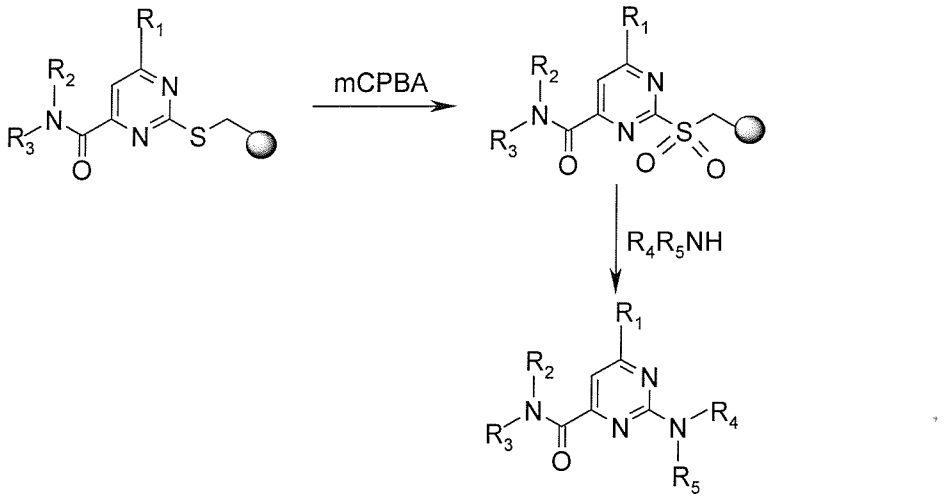


Figure 1.10: Thiopyrimidine linker

Suto[37] developed an analogous linker for applications requiring a limiting amount of nucleophile to cleave (*figure 1.11*). By subjecting this linker to a number of conditions including acid chloride formation, ester to alcohol reduction and Mitsunobu alkylation the stability and utility of the linker was demonstrated.

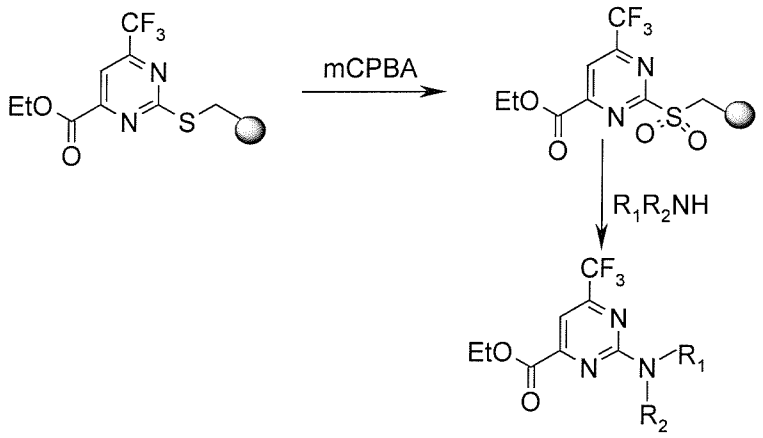


Figure 1.11: Suto's sulfur based linker

Hulme[38] developed a novel linker that utilised an Ugi multi component condensation strategy to gain access to a number of cyclic compounds (*figure 1.12*). The isocyanide component of the reaction was resin bound, generating a resin bound product on completion of the Ugi reaction. Boc activation of the benzamide carbonyl promotes facile

cleavage from the resin with methoxide or hydroxide. This linker was used to synthesise diketopiperazines, 1,4 benzodiazepines, ketopiperazines and dihydroquinoxalinones to demonstrate the utility of this strategy.

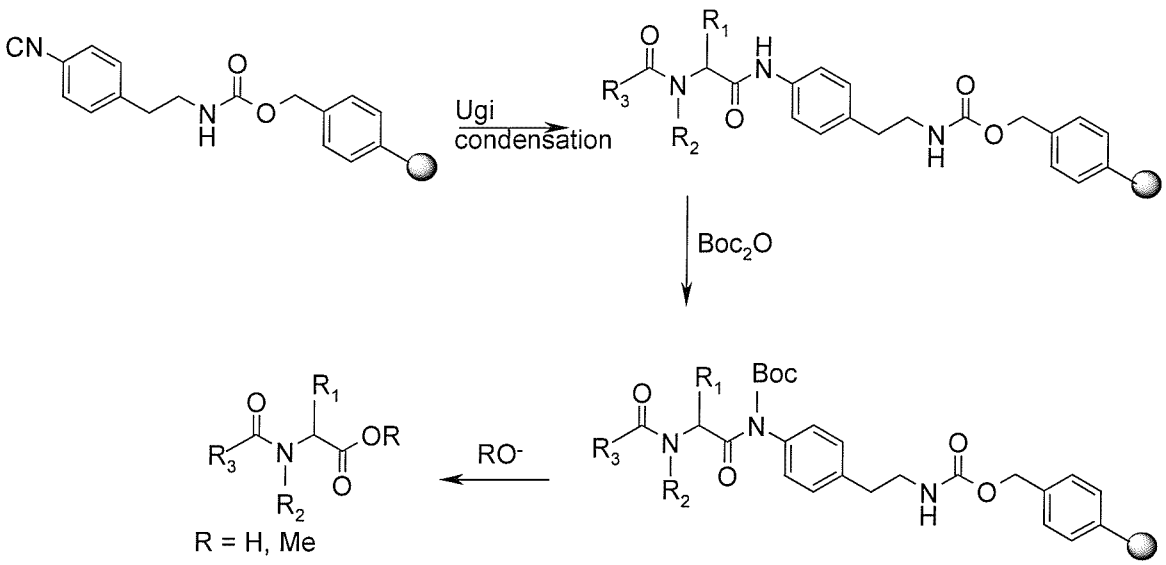


Figure 1.12: Ugi/De-Boc/Cyclisation linker

The Dpr(Phoc) linker[39] is anchored via an amide bond, and thus is stable to the conditions used for peptide synthesis and is also stable under acidic conditions (*figure 1.13*).

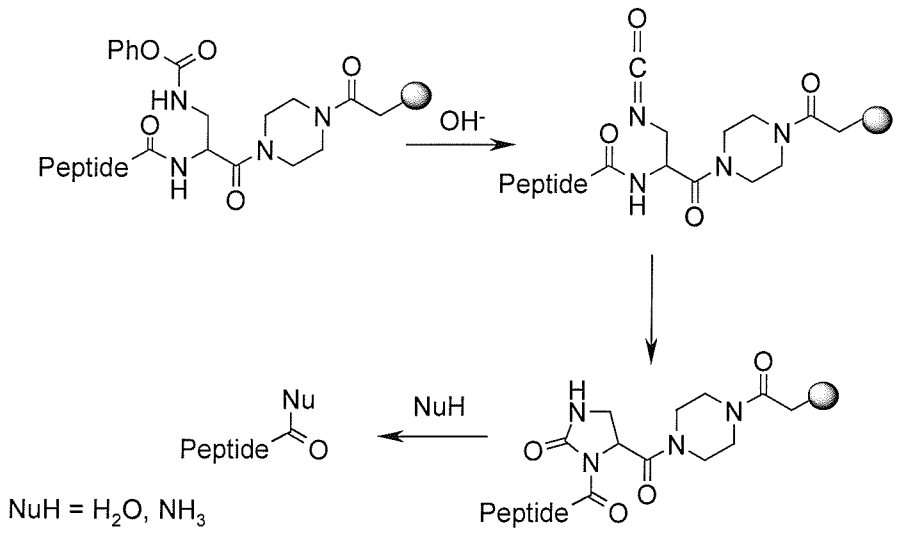


Figure 1.13: The Dpr(Phoc) linker

Its development was prompted by the lack of linkers that are amenable to the use of water. Activation was achieved through the exposure of the linker to alkaline conditions, which resulted in the generation of an isocyanate, which was primed to undergo a fast intramolecular cyclisation. The resultant species was susceptible to aminolysis and hydrolysis, leading to product release. The Dpr(Phoc) linker is compatible with both Boc and Fmoc[40] peptide synthesis strategies.

Wieland[41] designed a linker based on a 2,2-diphenyl-2-hydroxy-ethyl ester (*figure 1.14*). Activation was achieved through exposure to aqueous acid, which dehydrated the 2-hydroxy ethyl ester generating a reactive enol ester. Reaction with amines resulted in the isolation of secondary peptide amide products.

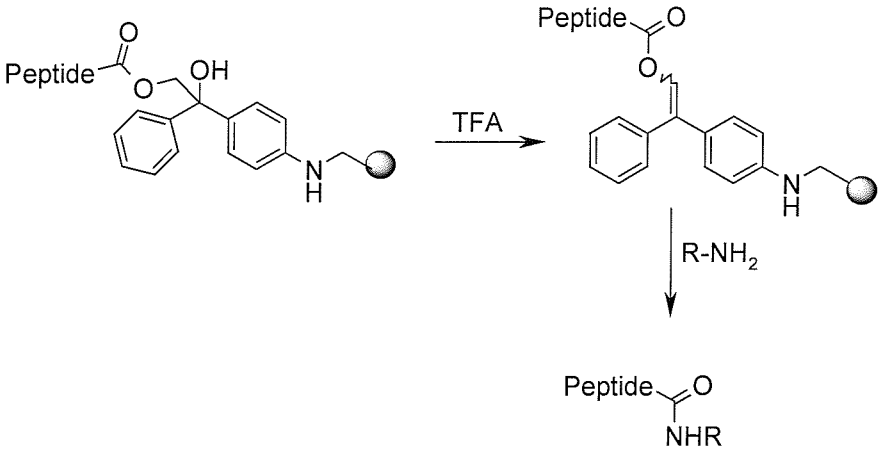


Figure 1.14: 2,2-Diphenyl-2-hydroxy-ethyl ester linker

Another safety-catch linker which was developed by Wieland,[42] was based on benzyl hydrazide (*figure 1.15*).

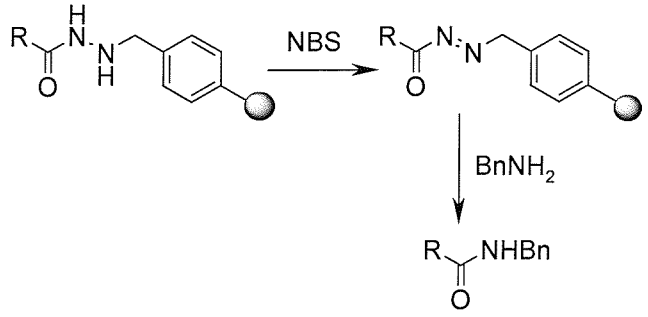


Figure 1.15: Wieland's safety-catch linker

Oxidation generated a hydrazide, and subsequent addition of amine nucleophiles released the corresponding acyl derivatives.

A phenylhydrazide linker reported by Semenov[43] decomposes *in situ* following oxidation, to release tripeptides into solution (*figure 1.16*). A different strategy was suggested by Lowe,[44] and subsequently demonstrated by Waldmann.[45] Using a different phenylhydrazide linker, aromatic compounds were released into solution by using $Cu(OAc)_2$ or NBS in the presence of a nucleophile (*figure 1.16*).

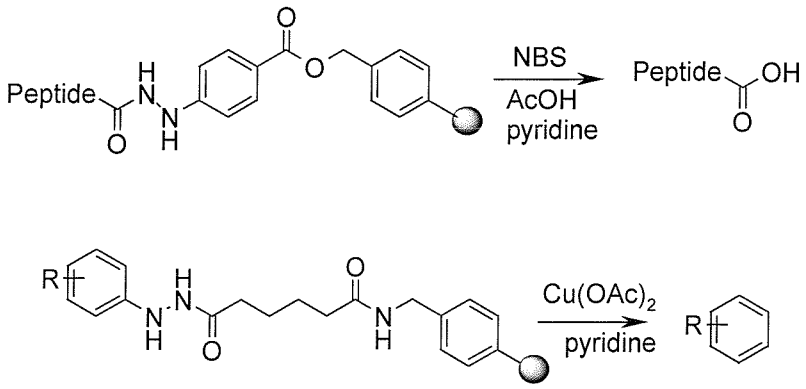


Figure 1.16: Phenylhydrazide linkers

1.1.3.2] Base labile safety-catch linkers

The novel linker strategy was designed for the clean synthesis of tertiary amines on the solid phase[46] (*figure 1.17*). Michael addition of a secondary amine onto the acrylate terminal generated a resin bound tertiary amine.

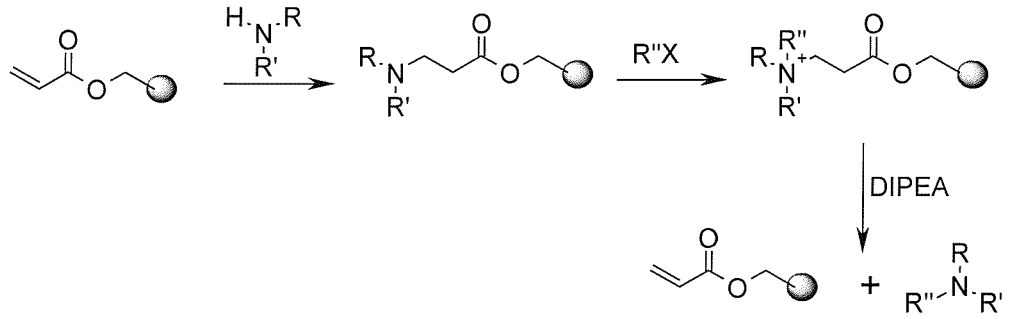


Figure 1.17: REM Resin

Quaternisation of this amine with an alkyl halide is the activation step. Hoffman elimination with a base regenerated the original linker and released the tertiary amine product from the resin. The functionised resin was termed REM resin, because it was regenerated by a Michael addition. The synthesis of an array of amines demonstrated the utility of the linker.

1.1.3.3] Acid labile safety-catch linkers

Based on the p-(methylsufinyl)benzyl protecting group[47] the SCAL (safety-catch acid labile linker) was developed for the synthesis of peptide amides, which are present in many naturally occurring peptides.[48] In the oxidised form the linker is stable to acids and bases and is amenable to peptide synthesis using either Fmoc or Boc protection strategies. The linker was activated by reductive acidolysis, with $PPh_3/Me_3SiCl/DCM$ or $(EtO)_2P(S)SH/DMPU$ converting the sulfoxide to a thioether, thus making the sulphur electron donating (*figure 1.18*). Peptide amides were released from the linker in the presence of TFA/H_2O.

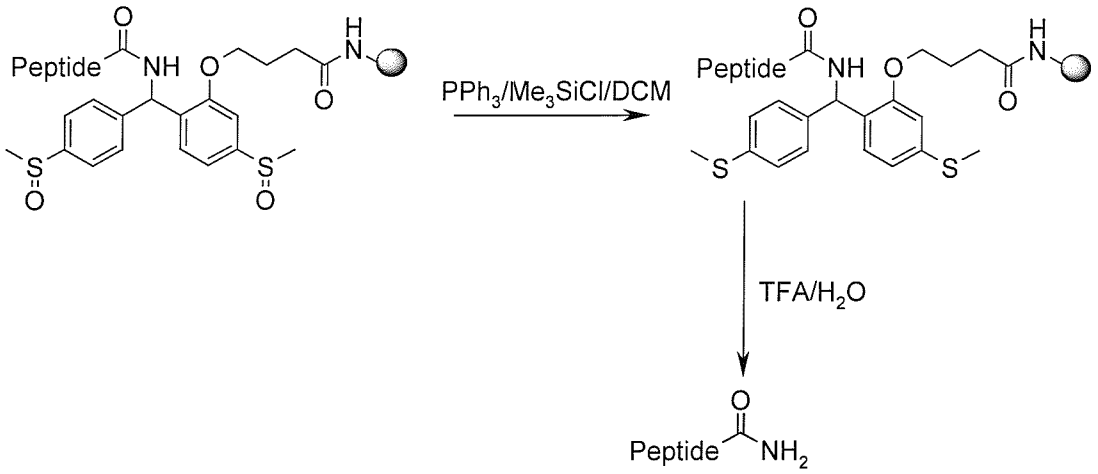


Figure 1.18: The SCAL linker

The DSB linker is based on 4-(2,5-dimethyl-4-methylsulfonyl)-4-hydroxybutanoic acid skeleton (*figure 1.19*). It illustrates a two-dimensional protection strategy.[49] Boc groups are employed as acid labile temporary protecting groups. Side chain protection was

effected using a series of acid stable but reductive acidolysis labile safety-catch protecting groups based on 4-methylsulfinylbenzyl (Msob) group.[50]

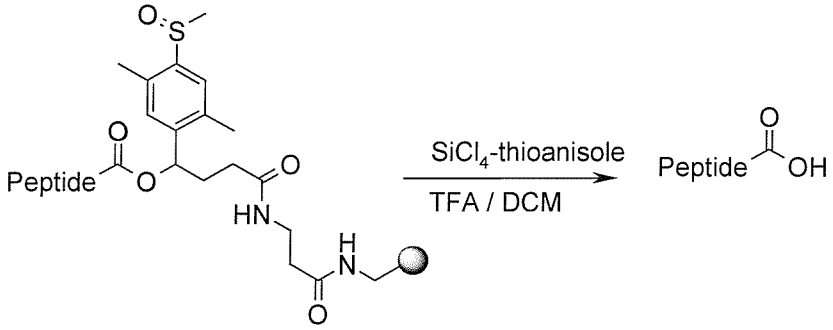


Figure 1.19: DSB linker

The first amino acid was attached via an ester bond and peptide synthesis was subsequently performed using Boc chemistry. On exposure to reductive acidolysis conditions, the cleavage from the resin is effected with concomitant deprotection of the side chains. The utility of this linker was demonstrated by the synthesis of γ endorphin. A similar linker was described by the same authors, based on dialkoxyalkylsulfinyl-benzhydrylamine (DSA), however this linker is less stable to strong acid[51] (*figure 1.20*).

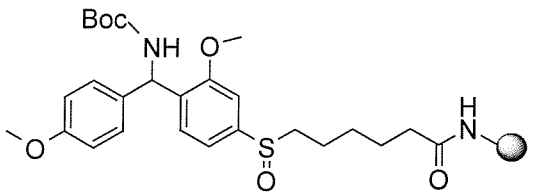


Figure 1.20: DSA linker

1.1.3.4] Photolabile safety-catch linkers

Chan[52] described a safety-catch cleavage strategy based on the dithiane protected 3-alkoxy benzoin, and demonstrated the practicability of the strategy in solution. Balasubramanian[53] demonstrated the utility of this strategy on the solid phase (*figure 1.21*). Activation of the linker was performed by exposure to mercury (II) perchlorate which removed the dithiane protection. Irradiation at 350 nm effected photolytic cleavage.

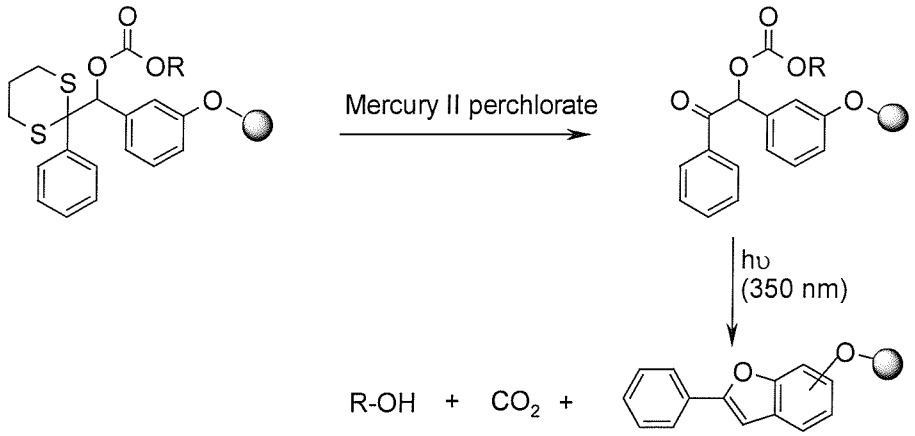


Figure 1.21: Photolabile safety-catch linker

1.1.3.5] Safety-catch linkers which cleave in aqueous buffer

Hoffman[54] amended a previously documented glycolic acid linker[55] by incorporating an imidazole ring (*figure 1.22*). The imidazole ring has significantly reduced basicity due to the Boc protection. Activation of this linker occured in acidic conditions, removing the Boc group and other acid labile protection. On exposure to buffer the imidazole became deprotonated and assisted the hydrolysis of the ester by intramolecular catalysis.

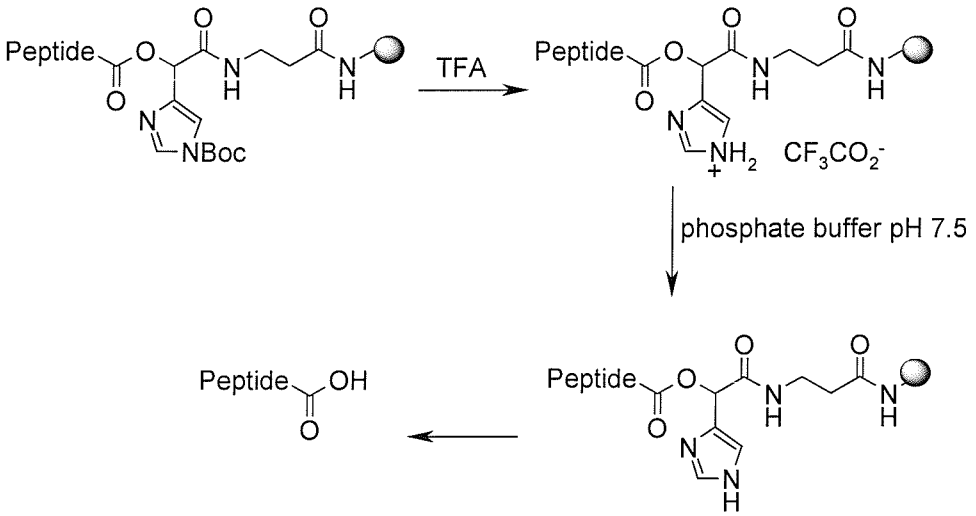


Figure 1.22: Imidazole based linker

A diketopiperazine forming linker was utilised in the synthesis of thousands of discrete peptides formed on polyethylene pins[56] (*figure 1.23*). Diketopiperazine formation was originally noted as a side reaction that occurs during peptide synthesis at the dipeptide stage.[57] Acid deprotection of the Boc group activated the linker generating the amine salt, which was then washed to remove contaminants. Placing the pins into a buffered biological assay resulted in the release of the peptide C-terminally modified with the diketopiperazine.

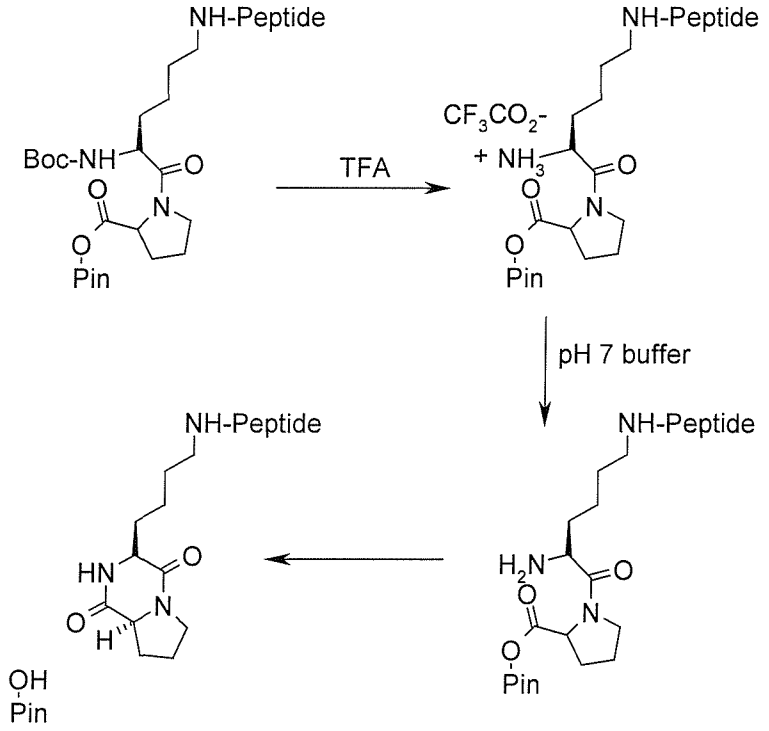


Figure 1.23: Diketopiperazine forming linker

Bradley[58] developed another linker based on this mechanism (*figure 1.24*). The products were released without C-terminal modification. Following acidic activation, the linker cleaved rapidly in buffered agarose gel. The cleavage occured via diketopiperazine formation followed by a facile 1,6 elimination, releasing a stoichiometric amount of quinone methide in addition to the carboxylic acid product.

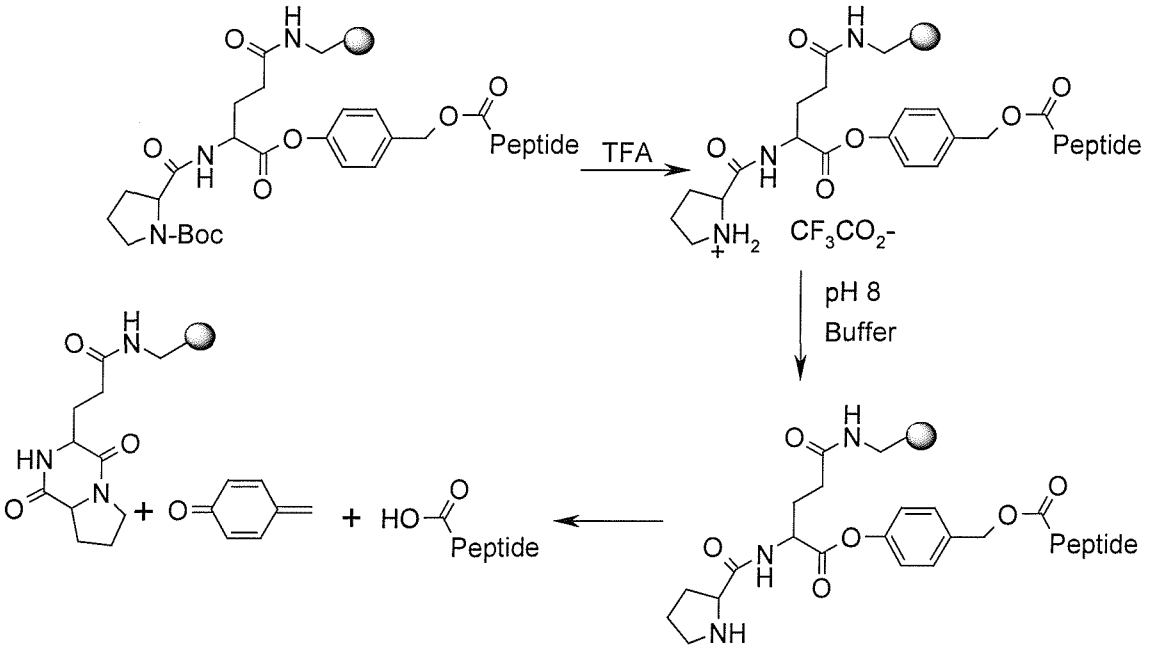


Figure 1.24: Diketopiperazine forming linker

1.1.3.6] Multiple cleavable linker strategies

The multiple cleavable linker strategy allows the release of a fraction of the organic compound synthesised from the individual solid support particle, screening of its activity, isolation of the relevant particle and confirmation of its activity after the release of a second portion from the bead. This is of immense use in screening one-bead-one-compound combinatorial libraries.[59] Safety-catch linkers have been used as part of two multiple release linkers. Salmon[66b] devised a multiple release linker which releases two aliquots of peptide for use in a two stage bio-assay, with a third aliquot remaining attached to the bead for Edman sequencing (*figure 1.25*). The two-stage bio-assay described enabled the identification of active peptide sequences from a large split and mix library rapidly and efficiently. The peptide library was synthesised on TentaGel. The first aliquot of peptide was released by deprotection of the Boc group and subsequent diketopiperazine formation in the buffered assay conditions. The beads were tested for activity in an ELISA assay in 96 well plates, 500 beads per well. The wells that contained "hits" were then redistributed into 500 wells, one bead per well and the second aliquot

was released by exposure to NaOH. Active beads were isolated and the third portion of the active peptide retained on the bead was sequenced.

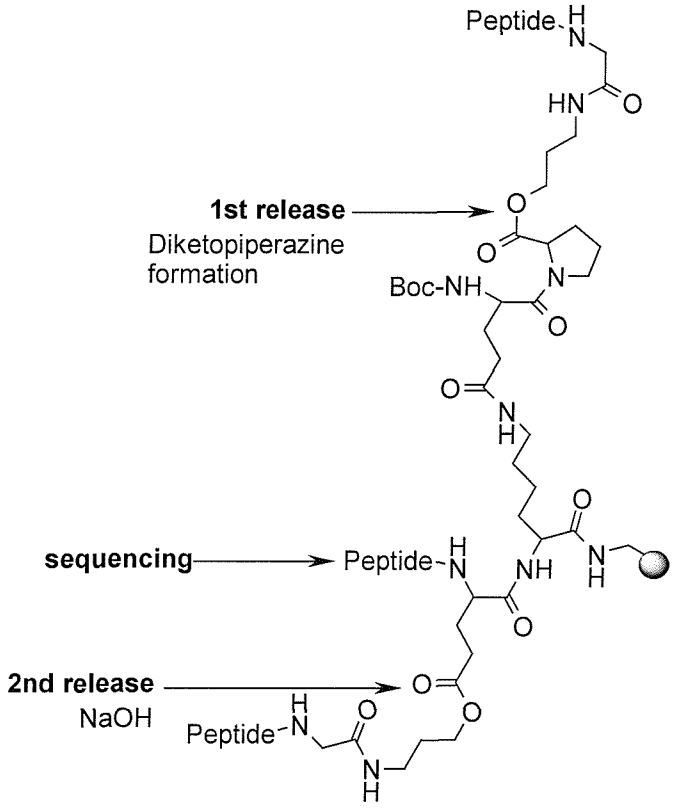


Figure 1.25: Multiple cleavable linker with three levels of cleavability

A similar linker based on iminodiacetic acid which releases N-terminally modified peptides was subsequently reported.[66d] A linker with five levels of cleavability was designed for peptide synthesis[66c] (*figure 1.26*). Leu-Enk was synthesised initially as a model peptide. The Boc group was cleaved using TFA then exposing to buffer released the first portion **A** by diketopiperazine formation. Deprotection of the Nps group resulted in the release of the second portion **B** again via diketopiperazine formation. NaOH released the third portion **C**. The next portion **D** was released by the application of UV light. The remaining peptide could be retained on the solid support for sequencing or released by oxidation of the SCAL safety-catch linker resulting in a species labile to acid. There were several imperfections in this system, but it demonstrates the potential of multiply cleavable linkers.

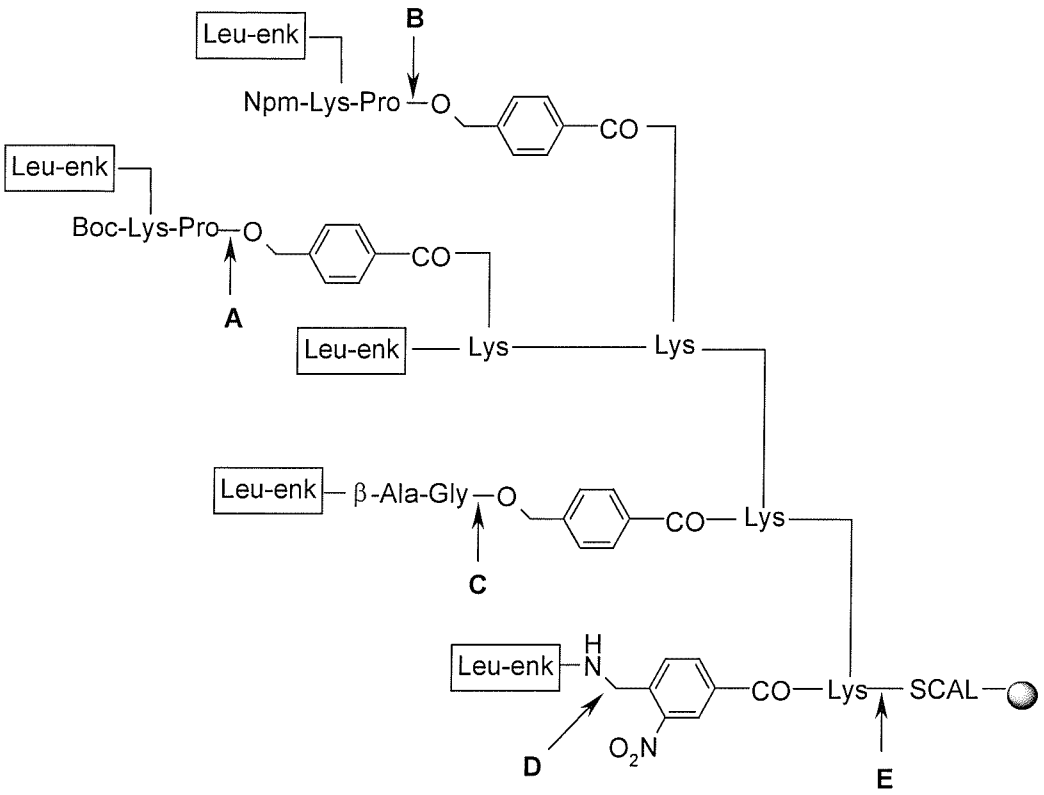


Figure 1.26: Multiply cleavable linker with five levels of cleavability.

The interest in safety-catch linkers is ever increasing, as their utility has been widely recognised and it is expected that this area will expand rapidly in the years to come.

1.1.4] Split and mix synthesis

Furka[60] first described the "split and mix" synthesis technique as an efficient method of producing libraries of peptides as mixtures (*figure 1.27*). To perform split and mix synthesis the resin is separated into aliquots, and each reacted with a different monomer. The resin is then combined and split into aliquots and each reacted with a different monomer. This process gives a library of compounds comprised of sub-libraries, where the last monomer is defined and the others randomised. Repeated iteratively split and mix synthesis can produce vast numbers of compounds, rapidly and efficiently. The compounds can then be tested either in solution or on the solid phase.

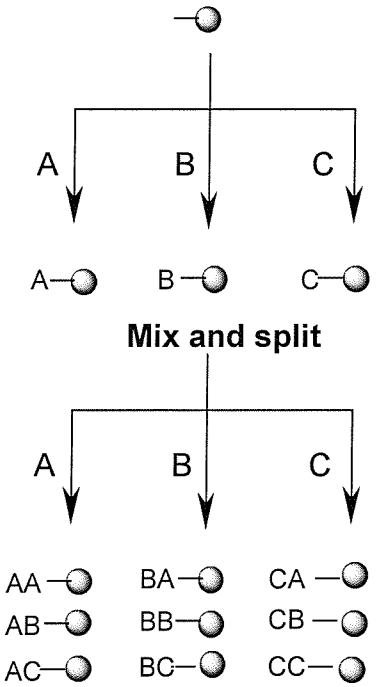


Figure 1.27: Split and mix synthesis

Split and mix synthesis is more efficient than reacting a resin bound compound with a mixture of monomers as each will react at a different rate and so an unequal representation of all the possible permutations will result. Houghten,[61] however did successfully overcome this problem by adding variable quantities of each monomer, which were comparable to their relative reactivity. The "one-bead-one-compound" concept was first recognised by Lam[62] noting that during a spilt and mix synthesis one bead only encountered one set of reagents at each cycle, and thus each bead will display only one compound.

1.1.5] Solid phase combinatorial library screening and the identification of active compounds

There are several major strategies, which have been used to synthesise solid phase compound libraries, evaluate their activity against a biological target, and isolate any active compounds.

1.1.5.1] Screening solid phase libraries in solution

Most of the available screening techniques have been developed for solution phase testing and when compound mixtures are tested in solution there must be a reliable method of identifying the active component from the active mixture (deconvolution). A common method of deciphering the active component from compound libraries generated by split and mix synthesis is known as *recursive deconvolution.*[63] When a split and mix peptide library is prepared samples of resin from each coupling step are retained. When each mixture is screened the last residue is known, so on the identification of an active mixture the last residue in the active compound is easily deduced. All the compounds in the active mixture can be re-synthesised using the precursor resin which was retained in aliquots where all the previous residues are known, and these mixtures can the be re-screened. Through this process of iterative re-synthesis and re-screening the active compound can be successfully identified.

Houghten devised two iterative methods of screening solid phase peptide libraries; dual positional synthetic peptide combinatorial libraries[64] and positional scanning peptide libraries.[61] Dual positional synthetic peptide libraries are represented by the formula O_1O_2XXXX.[65] The first two positions in each peptide were defined and the last four consisted of equimolar mixtures of 18 natural amino acids. These mixtures were screened using an ELISA assay to ascertain which mixture contained any peptide(s) that had an interaction with a monoclonal antibody. The peptide mixture defined by DVXXXX caused the greatest inhibition, thus twenty new peptide mixtures were synthesised represented by DVOXXX, and screened in the same assay. Repeating this process iteratively determined the sequence of the most active peptide.

Positional scanning is a related technique, which is based on screening a set of mixtures represented by the formulas O_1XXXXX, XO_2XXXX, XXO_3XXX, $XXXO_4XX$, $XXXXO_5X$, $XXXXXO_6$. The screening of each library determines the most active amino acid at that position. A potent hexapeptide opiate receptor ligand was identified by this technique.

Multiply cleavable linkers with two or more levels of orthogonality incorporated in the construction of a library allows biological testing to be performed repetitively on the

same library of compounds.[66] This facilitates the identification of active compounds without the need for deconvolution.

Jayawickreme[67] reported the use of the same solid phase peptide libraries in several screens. This screening technique involved the use of the MBHA linker, which has slow release kinetics for gaseous TFA, allowing partial amounts to be cleaved from the beads. The beads were then used in repetitive studies. Neutralisation with NH_3/H_20 following cleavage with gaseous TFA allows the released peptide to remain attached to its source bead. The utility of this concept was demonstrated by screening a peptide library for agonist activity against the bombesin receptor. Bombesin is a biologically active peptide, which is a growth factor for small cell lung carcinoma cells. The assay involved immobilising the beads in agarose gel containing melanophore cells expressing the bombesin receptor, and melatonin. 500 positive beads were identified from the initial screen of 5000 beads by the appearance of pigment darkening and were isolated and applied to a second screen. This process was repeated and the seven isolated agonists sequenced. This technique was similarly applied to the isolation of α MSH receptor antagonists[68] and anticancer agents. [69]

1.1.5.2] Spatially addressable compound libraries

Spatially addressable compound libraries contain many compounds synthesised in parallel, and identifiable by their location. Thus the structure of any "hits" can be easily determined. Geysen[70] [in] the search for the identification of an epitope (a small region important for recognition) in the antibody-binding region of the foot and mouth virus, synthesised 208 hexapeptides on a rack of discrete polyethylene rods. One compound only was attached to each rod and the synthetic history of every rod was known. Assaying the immobilised peptides using an ELISA type assay, lead to the identification of the rods containing the active peptides.

The "tea-bag" method developed by Houghten[71] enables the simultaneous preparation of hundreds of peptides. Aliquots of resin were encapsulated in separate solvent permeable polyethylene packets. These "tea bags" were then immersed in individual solutions of activated amino acids, and combined for deprotection and washing steps. A label on each

bag recorded its synthetic history. 248 x 13-mer peptides were synthesised and tested for antibody binding in the influenza hemagglutinin protein. Fodor[72] described a novel strategy of light-directed synthesis allowing the construction of spatially addressable libraries. The initial residue was anchored to a glass plate, then masks were used to cover parts of the plate and only deprotect the photolabile N^{α} protecting group from selected positions. On exposure of the plate to activated amino acids, reaction only occurred at deprotected sites. An array of 1024 peptides was synthesised and interaction with a monoclonal antibody was monitored using fluorescence spectroscopy.

1.1.5.3] On bead screening

On bead screening is a useful way of rapidly screening combinatorial libraries generated by spilt and mix synthesis. Lam[62] reported the first example of an on bead screening technique. A library of pentapeptides was synthesised and screened to determine if any would bind to an antibody whose native epitope was YGGFL. The antibody (the acceptor molecule) was coupled to the enzyme alkaline phosphatase (AP) which reacts with the additional reagents nitroblue tetrazolium (NBT) and 5-bromo, chloro-3-indolyl phosphate (BCIP) resulting in the production of a coloured dye. The immobilised library was placed in a solution of the acceptor bound alkaline phosphatase and the additional reagents. The peptides, which bound to the antibody, were identified by the intense staining of their parent bead, which was visible to the naked eye. These beads were removed and the peptide sequenced. A problem with this technique was the isolation of false positives through non-specific binding interactions. To minimise the number of false positives, a dual colour detection system was subsequently developed.[73]

Chen[74] used a fluorescently labelled enzyme, phosphatidylinositol–3-kinase to identify peptide ligands that bound to the SH_3 domain of the enzyme. This enzyme was incubated with an immobilised library of more than two million peptides and positive beads were isolated with a fluorescent microscope. Still[75] used a dye labelled substrate to screen a peptide library containing over 117, 000 14-mers for binding to a monoclonal antibody. Kassarjian[76] used anti-β-endorphin monoclonal antibody radiolabelled with ^{125}I. A resin bound pentapeptide library containing three million members, was incubated with the

antibody then immobilised in an agarose gel. The radioactivity was recorded on a x-ray film placed over the gel. Positive beads were identified, extracted from the gel and sequenced.

Meldal[77] devised a novel fluorogenic quenching assay for the identification of a proteolytic substrate motif from a bead bound peptide library. A fluorescent molecule was attached to the carboxyl terminus of each peptide and a fluorescence-quenching molecule was attached to the amino terminus. After incubating with the endoprotease subtilisin Carlsberg, the peptide sequences cleaved by the enzyme resulted in the quencher being released and the beads became highly fluorescent, thus enabling isolation of active beads and the identification of the remaining peptide fragments. This technique allowed the complete mapping of the enzyme rapidly.

On bead screening offers many advantages over solution phase screening, as many thousands of compounds can be screened rapidly and identified readily. On bead screening has been applied to many peptide libraries so far, as the technology exists for identifying peptide sequences from the small amounts of compound present on one bead. The identification of small molecules from the amount of compound on one bead is not so straightforward.

1.1.5.4] Tagging and encoding

The use of encoding strategies in the synthesis of libraries enables the identification of nonsequenceable compounds and increases the speed and throughput of compound identification. The use of polynucleotides to code for combinatorial libraries has the advantage that small quantities of nucleic acids can be reliably amplified by the polymerase chain reaction (PCR).[78] Nucleic acid encoding has been applied to many peptide libraries successfully.[79] Its application to encoding for organic libraries is limited, as nucleic acid chemistry is incompatible with many organic reactions. Coding peptide libraries with amino acids has also been documented.[80] Secondary amines are chemically robust tags that have been used by a number of groups to tag libraries.[81] Still[75] developed a binary encoding system using various halogenated aromatic compounds as tags. These compounds, he demonstrated, were compatible with general synthetic chemistry

protocols and were unlikely to interfere with receptor binding. Following the isolation of active beads the tags were removed and analysed by electron capture capillary gas chromatography. Mass spectrometry as a method for identifying compounds from single beads has also been documented.[82]

On bead screening is a useful technique, but the limitations are that the chosen screen must be specific and reliable, and it is essential that the presence of the linker, resin, or tagging strand does not have a significant effect on binding affinity.

The field of combinatorial chemistry has revolutionised the search and optimisation of lead drugs and it is likely that interest in the field will only increase in the years to come.

1.2] Matrix Metalloproteinase enzymes

1.2.1] Matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc dependent proteolytic enzymes that are responsible for the degradation of the extracellular matrix and play an important role in both normal and pathological tissue remodelling. The components of the extracellular matrix degraded by MMPs are summarised in table 1.1.[83]

	Structural features	Function	Location
Collagen	Stiff, triple-stranded helices.	Major structural component	Skin, tendons, bone, blood vessels, heart.
Elastin	Random cross-linked coil	Gives elasticity to tissues	Lungs, skin, blood vessels.
Proteoglycan	Core protein surrounded by glycosaminoglycan chains	Resistance to compression	Cartilage in joints
Fibronectin	Glycoprotein	Promotes cell adhesion	Connective tissues, blood.
Laminin	Glycoprotein	Barrier between organ types	Basal laminae

Table 1.1: The components of the extracellular matrix degraded by MMPs.

The breakdown of these components and subsequent tissue remodelling is important during growth, embryonic development[84] and wound healing.[85]

The mammalian MMP family contains more than 20 enzymes (*table 1.2*).[86]

MMP No	Enzyme	Principal substrates
MMP-1	Fibroblast collagenase	Fibrillar and non fibrillar collagens (types I, II, III, VI, X), gelatins
MMP-2	Gelatinase A	Basement membrane and nonfibrillar collagens (types IV, V, VII, X), fibronectin and elastin.
MMP-3	Stromelysin-1	Proteoglycan, laminin, fibronectin, collagen (types III, IV, V, IX), gelatins, pro-MMP-1
MMP-7	Matrilysin	Proteoglycan, fibronectin, gelatins, pro-MMP-1
MMP-8	Neutrophil collagenase	Fibrillar collagens (types I, II, III)
MMP-9	Gelatinase B	Basement membrane collagens (types IV, V), gelatins
MMP-10	Stromelysin-2	Fibronectin, collagen (types III, IV), gelatins, pro-MMP-1
MMP-11	Stromelysin-3	Sorption
MMP-12	Metalloelastase	Elastin
MMP-13	Collagenase-3	Fibrillar collagens (type I, II, III), gelatins
MMP-14	Membrane type 1	Pro-MMP-2
MMP-15	Membrane type 2	To be determined
MMP-16	Membrane type 3	Pro-MMP-2
MMP-17	Membrane type 4	To be determined
MMP-18	Collagenase-4	To be determined
MMP-19	Novel MMP (RASI 1)	Gelatin
MMP-20	Enamelysin	Amelogenin (dentine), gelatin
MMP-21	MMP identified on chromosome 1	To be determined
MMP-22	MMP identified on chromosome	To be determined
MMP-23	From human ovary cDNA	To be determined

Table 1.2: Matrix Metalloproteinase enzymes

Substrate specificity of this class of enzymes is broad, and there is considerable crossover.

1.2.2] Structure of MMPs

There is a high degree of homology in the structures of this enzyme family (*figure 1.28*). The catalytic domain of the MMPs contains two zinc atoms, one providing a catalytic role and the other a structural role. The catalytic zinc is co-ordinated to three histidine residues present in a highly conserved sequence -VAAHEXGHXXGXXH- in the catalytic site. -PRCGXPD- is another conserved sequence from the propeptide domain. The thiol group from the cysteine residue binds to the catalytic zinc atom and confers latency. The enzyme must be activated resulting in loss of this propeptide region before it becomes catalytically active.

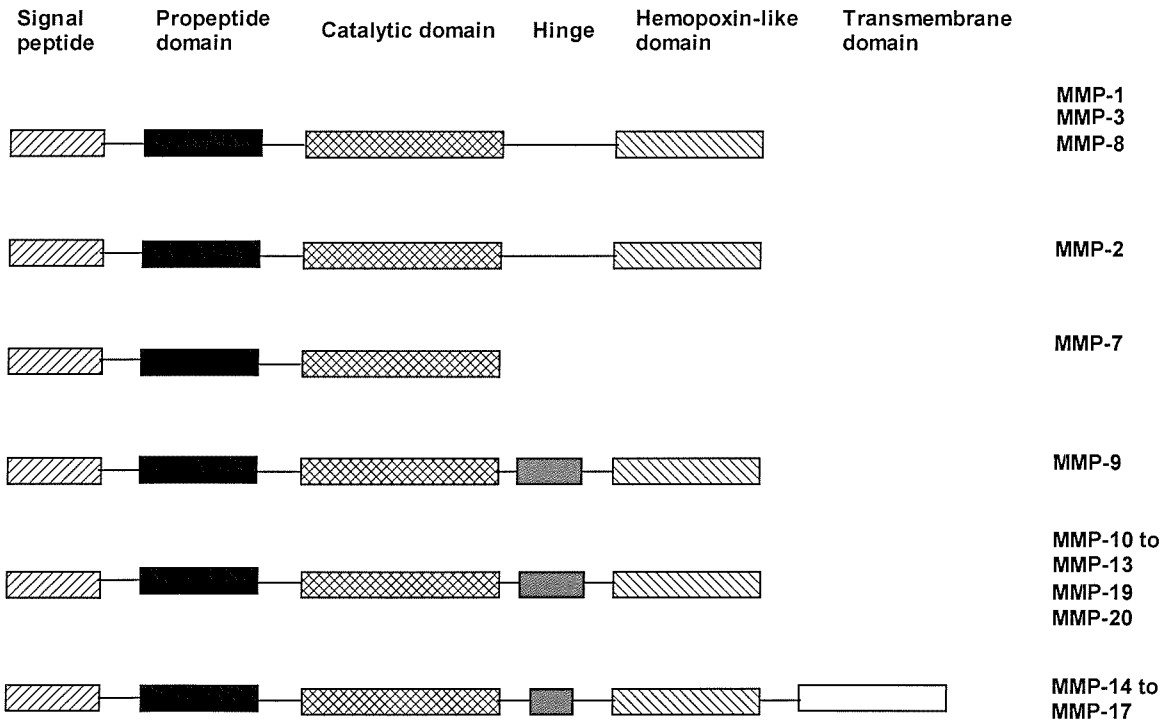


Figure 1.28 General topology of matrix metalloproteinase enzymes

A domain which has homology to hemopoxin is also highly conserved (except in MMP-7 and MMP-23) and this domain is believed to be important in substrate binding and binding to natural tissue inhibitors of matrix metalloproteinases (TIMPs).[87] In addition the two gelatinases contain what is believed to be a gelatin-binding region.[88]

The believed mechanism of proteolysis[89] has been rationalised based on structural information[90] (*figure 1.29*). The scissile amide carbonyl co-ordinates to the active site zinc ion, and is subsequently attacked by a water molecule held in place by interactions with the conserved Glu and the zinc ion. A proton from the water molecule is donated to the Glu and is transferred to the nitrogen of the scissile bond. The shuttling of the remaining proton from the water molecule to the nitrogen via the Glu results in the bond cleavage.

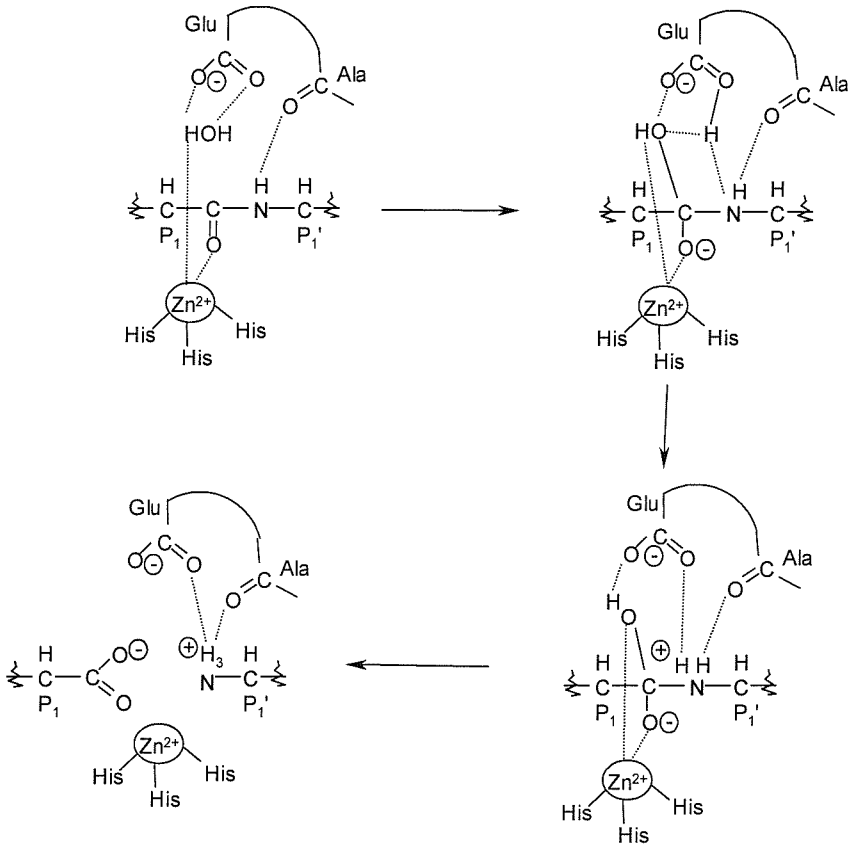


Figure 1.29: Mechanism of Proteolysis

1.2.3] Regulation of MMP activity

Due to the destructive potential of these enzymes their activity requires tight regulation. This is achieved by regulation of gene transcription, secretion of inactive proenzymes, and the presence of endogenous inhibitors TIMPs.

1.2.3.1] Regulation of gene transcription

The genes encoding MMPs are not constitutively expressed in most cells, but may be induced by a variety of agents including growth factors, cytokines, oncogene products or tumour promoters.[91]

1.2.3.2] Regulation of MMP activation

All MMPs are secreted as inactive proenzymes. A conserved cysteine residue from the proenzyme region binds to the active site zinc ion conferring inactivity. If this interaction is disturbed subsequent cleavage of the proenzyme region results in an active enzyme of lower molecular weight.[92] The interaction and subsequent activation is known as the *cysteine switch* (*figure 1.30*). Mercuric compounds disturb this strong interaction and are used to activate these enzymes *in vitro.*[93] This activation step is less well understood *in vivo*. It is believed that a proteolytic cascade occurs, resulting in the production of plasmin and this will activate pro-MMPs.[94] In addition activated MMPs can also feedback and activate other pro-MMPs.[95]

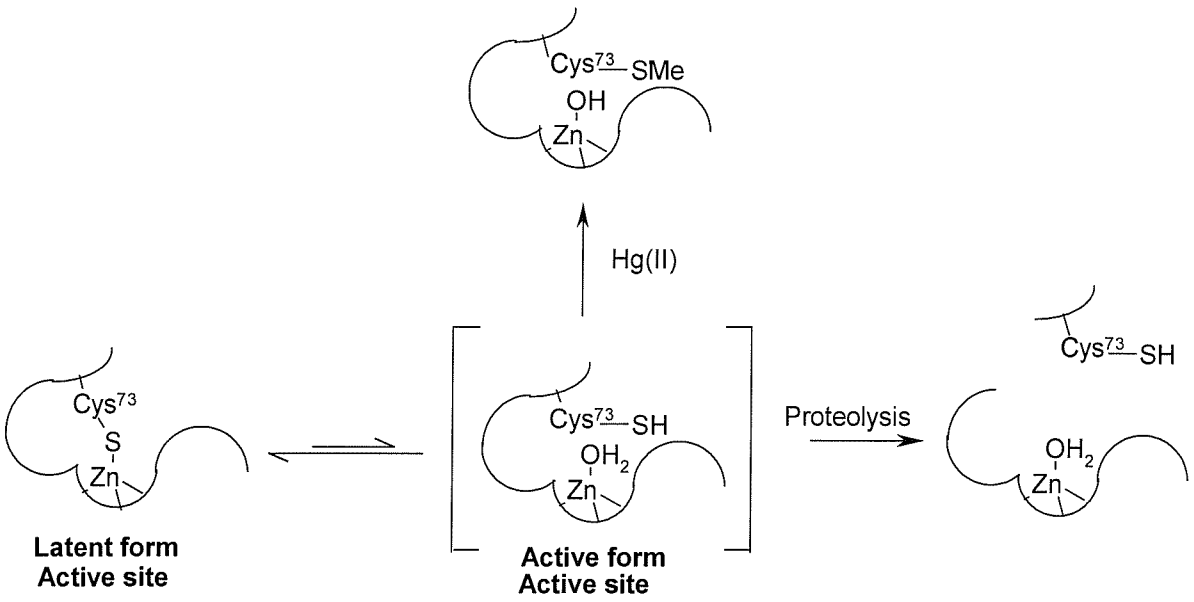


Figure 1.30: Activation of MMPs

1.2.3.3] Inhibition of active MMPs by TIMPs

The final negative regulatory control is the presence of specific tissue inhibitors (TIMPs). There are four recognised TIMPs; TIMP-1,[96] TIMP-2,[97] TIMP-3,[98] and TIMP-4.[99] The TIMPs are small proteins between 184-194 amino acids, and they share between 37-50% sequence homology. TIMPs bind to active MMPs non-covalently in a stoichiometric 1:1 ratio. The resultant proteolytic activity within the matrix will depend on the balance between MMPs and TIMPs and small changes in either will lead to a significant alteration in the overall MMP activity.

1.2.4] The role of MMPs in cancer

The disruption of normal MMP activity has been implicated in the pathology of many disease states. The unhealthy destruction of bone and cartilage in rheumatoid and osteoarthritis,[100] degradation of myelin in neuroinflammatory diseases,[101] tissue degradation in gastric ulceration[102] and breakdown of connective tissue in peridontal disease.[103]

The role of matrix metalloproteinases in cancer pathology has been extensively studied in recent years, due to the potential of inhibitors as therapeutic agents. There is considerable evidence that MMPs can be exploited by malignant cells in three aspects of tumour growth and spread.[104]

Metastasis: MMPs will break down the extracellular matrix and allow primary tumour cells to invade neighbouring blood vessels and thus be transported to start secondary tumours in other organs.

Local invasion: For these secondary tumours to grow MMPs are required to break down existing tissue at these sites.

Angiogenesis: For tumours to grow above a certain size they need to generate their own blood supply. MMP activity facilitates the invasive in-growth of these new blood vessels.

Excessive MMP activity has been documented in a range of solid tumours. Elevated levels of MMP-1, MMP-9 and MMP-11 in colorectal cancer have been demonstrated in several studies.[105] A good correlation was found between the levels of MMP-2 and MMP-9 and the invasiveness of cervical cancer.[106] Increased levels of MMPs have been

demonstrated in lung cancer,[107] breast cancer,[108] bladder cancer,[109] prostate cancer[110] and brain tumours.[111] It has also been demonstrated that the levels of MMPs correlated with the invasiveness of the tumour.[107a, 110]

1.2.5] Inhibiting MMPs

The design of inhibitors was originally achieved by the substrate analogue approach, screening libraries of peptides and peptide analogues. More recently some inhibitors have been identified using structure based approach. The requirements for a molecule to be an effective inhibitor of MMPs are:

- A zinc binding group (ZBG), a functional group which chelates to the active site zinc (e.g., carboxylic acid, hydroxamic acid, or sulfhydryl).
- At least one functional group which provides a hydrogen bond with the enzyme backbone.
- One or more side chains which will undergo effective Van der Waals interactions with the enzyme subsites.

Early work in inhibitor design was structured around the amino acid sequence at the cleavage site of human triple helical collagen (*figure 1.31*).

This was substrate-based design, which included a zinc-binding group attached to peptide derivatives. Three classes developed; combined inhibitors, left hand and right hand inhibitors, based on the position of the peptidyl fragment. It was determined that compounds which mimic the right hand side of the active site and incorporated an hydroxamic acid as the zinc binding group showed most potency.

Studies were performed to determine the most effective zinc binding group and using MMP-1 the following preference was reported: hydroxamate >> formylhydroxylamine > sulfhydryl > phosphinate > aminocarboxylate > carboxylate.[112] X-ray crystal structures have shown that the hydroxamate acts as a bidentate ligand with each oxygen at the optimal distance (1.9-2.3 Å) from the active site zinc ion. The position of the hydroxamate nitrogen suggests that it is in its protonated form and is hydrogen bonding to the carbonyl oxygen in the enzyme backbone.

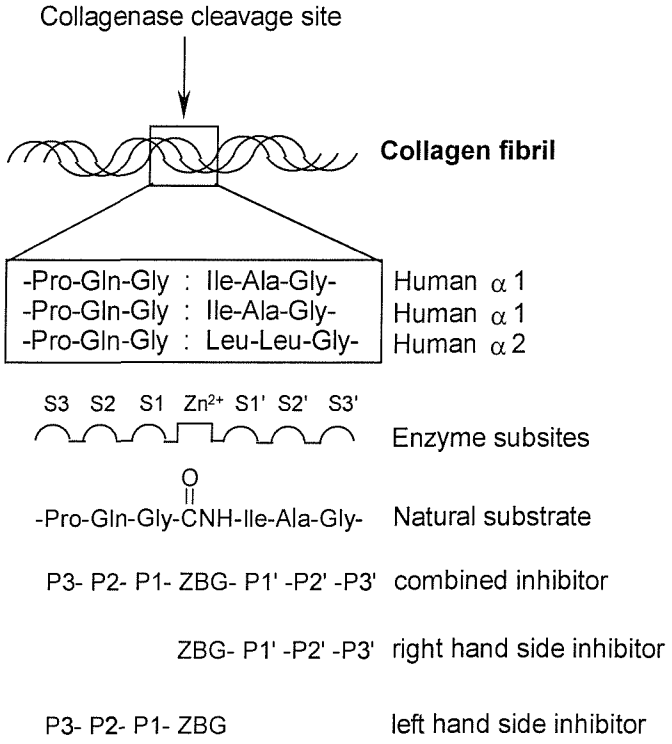
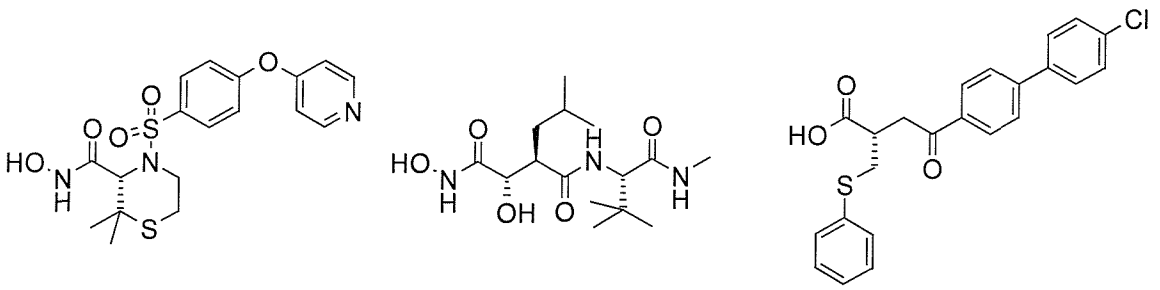


Figure 1.31: Design of MMP inhibitors based on the sequence of the collagen substrate cleavage site.

From the hundreds of inhibitors synthesised to date three have reached phase III clinical trials, (*figure 1.32*) AG3340 (Agouron), BAY 12-9566 (Bayer) and marimastat (BB2516, British Biotech). These orally available treatments are presently in placebo controlled studies in patients with advanced pancreatic, gastric, lung, breast, and ovarian cancer.



AG3340 **Marimastat (BB2516)** **BAY 12-9566**

Figure 1.32: MMP inhibitors in late stage clinical development.

The field of MMPs and their inhibitors has grown rapidly in recent years and there are

many potential drugs being developed for MMP mediated diseases, notably cancer. The clinical results generated in the next few years will demonstrate the true clinical utility of MMP inhibitors.

1.3] Solid phase synthesis of hydroxamic acids

Interest in the synthesis of hydroxamic acids increased substantially once their ability to inhibit MMP enzymes was discovered. Many approaches to the solid phase synthesis of hydroxamate derivatives have been devised in recent years to meet the increasing demand for combinatorial libraries of hydroxamic acids.

Floyd[113] developed the first documented approach, based on the Wang linker, derivatised to release hydroxamic acids instead of carboxylic acids (*figure 1.33*).

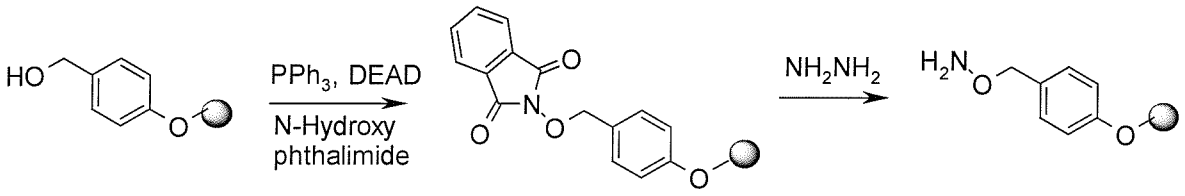


Figure 1.33: Synthesis of hydroxylamine resin

The hydroxylamine resin was synthesised by reacting N-hydroxyphthalimide with the Wang linker immobilised on polystyrene resin, under Mitsunobu conditions and subsequent exposure to hydrazine. To demonstrate the utility of this modified resin a tripeptide Z-Pro-Leu-Ala-NHOH was synthesised using standard Fmoc peptide synthesis protocols.[114] Cleavage from the resin with 70% TFA in DCM led to the desired hydroxamic acid as the only peptide product, with no detectable racemisation. A series of tripeptide hydroxamic acids were synthesised, with the only problem being that a varying amount of a non-peptidic product was produced. Using the more acid labile linker HMPB instead of Wang reduced the percentage of TFA required to effect cleavage and produced uncontaminated products in higher yield. Another method of preparing hydroxylamine resin was described subsequently (*figure 1.34*).[115]

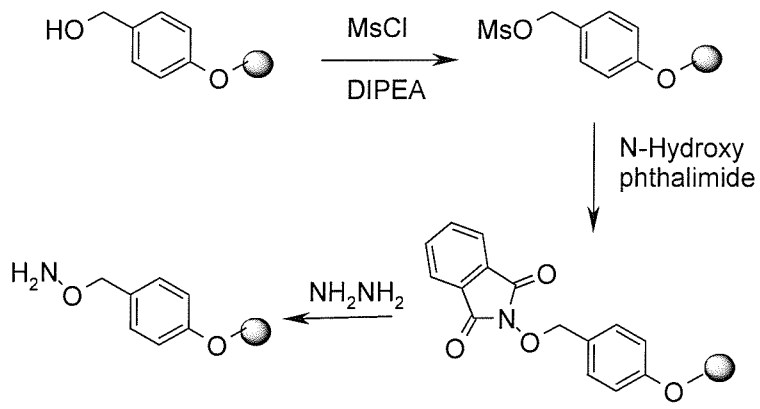


Figure 1.34: Synthesis of hydroxylamine resin

The alcohol of the immobilised Wang linker was transformed into a good leaving group by mesylation, and subsequently displaced with N-hydroxyphthalimide. Reaction with hydrazine generated hydroxylamine resin. This procedure was also applied to polystyrene immobilised Sasrin and Rink linkers.

Bauer[116] developed a modified trityl linker which was devised for the synthesis of peptidyl, succinyl, and urea type hydroxamic acids. N-hydroxyphthalimide was reacted with trityl chloride resin, and subsequently transformed to a hydroxylamine by exposure to hydrazine.

Chan[117] developed another strategy based on the trityl linker (*figure 1.35*).

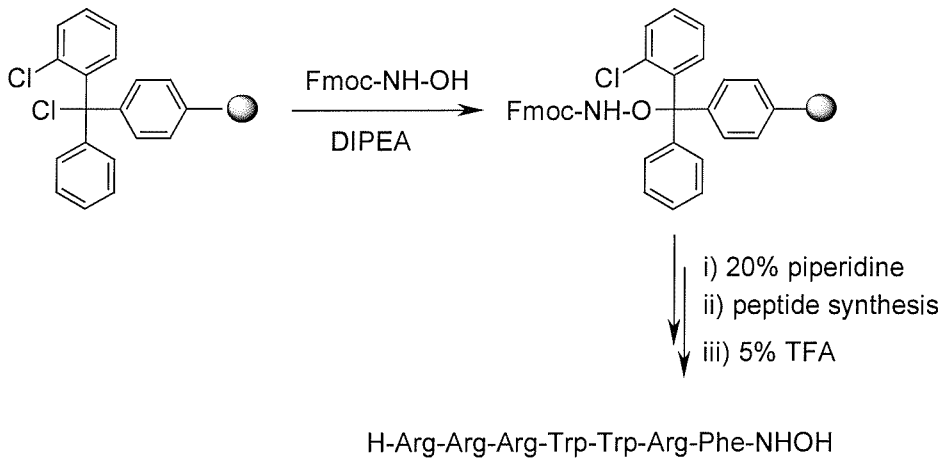


Figure 1.35: Synthesis of peptidic hydroxamic acids using Chan's trityl linker

N-Fmoc hydroxylamine was attached to the chlorotrityl resin in the presence of DIPEA. A heptapeptide was synthesised on this resin using TBTU and HOBt activation conditions. Cleavage from the resin with 5% TFA gave the hydroxamic acid in 80% yield and >90% purity.

The same group reported a strategy, which could be used to synthesise N-alkyl hydroxamic acids[118] (*figure 1.36*). This strategy was based on the Rink linker as it is a highly acid labile linker with less steric bulk than the trityl linker. The immobilised Rink linker was transformed to the cationic derivative by exposure to 1% HCl and this species was readily reacted with Fmoc-N(R^1)-OH in the presence of DIPEA, the same method used by Rink[18] to immobilise primary amines.

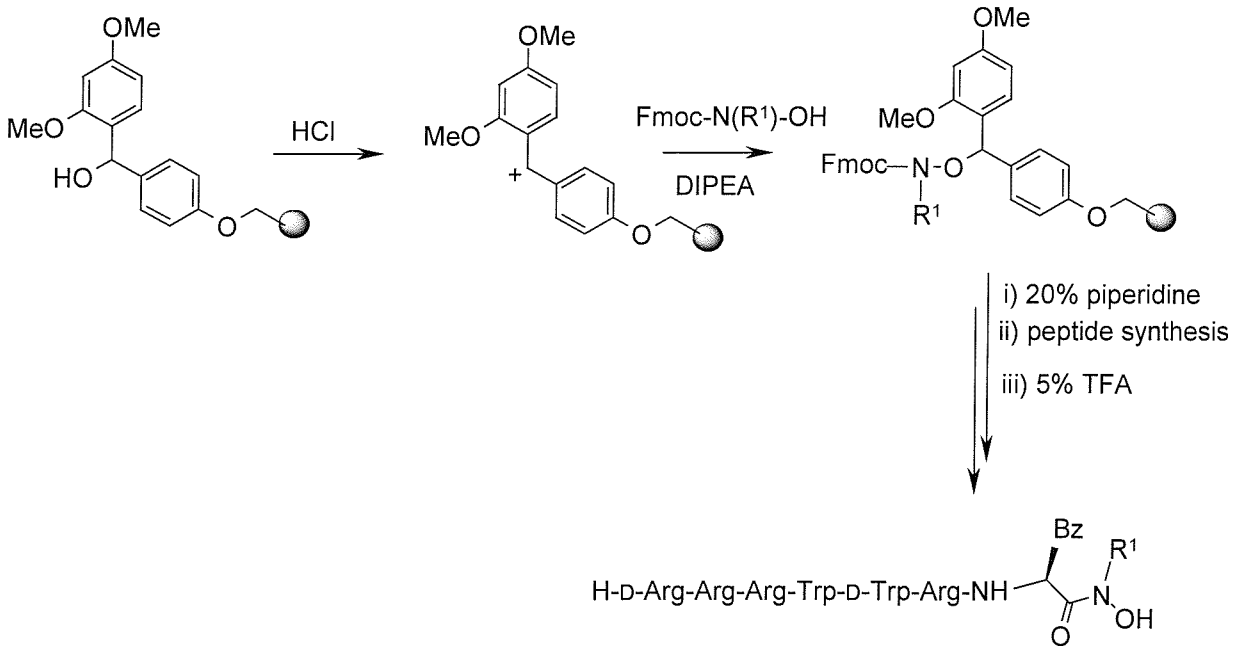


Figure 1.36: Synthesis of N-alkyl hydroxamic acids using modified Rink resin.

To demonstrate the utility of this strategy a number of heptapeptide N-alkyl hydroxamic acids were synthesised, using standard solid phase Fmoc peptide chemistry and cleaved from the resin in excellent yield and purity (80-90%).

Ngu[119] proposed a novel approach where the linker was attached to the nitrogen of the hydroxamate functionality and serves as a protecting group for the hydroxamate.

The hyper acid sensitive acid labile HAL (tris(alkoxy)benzyl ester) linker was chosen.[120] Hydroxylamine O-protected with the acid labile tetrahydropyran group (THP) was attached to the HAL linker via a reductive alkylation in solution. The alkoxyamine was protected with Fmoc and the linker attached to aminoethyl TentaGel. Fmoc deprotection lead to the generation of alkoxyamine resin, which could be applied to solid phase hydroxamic acid synthesis (*figure 1.37*).

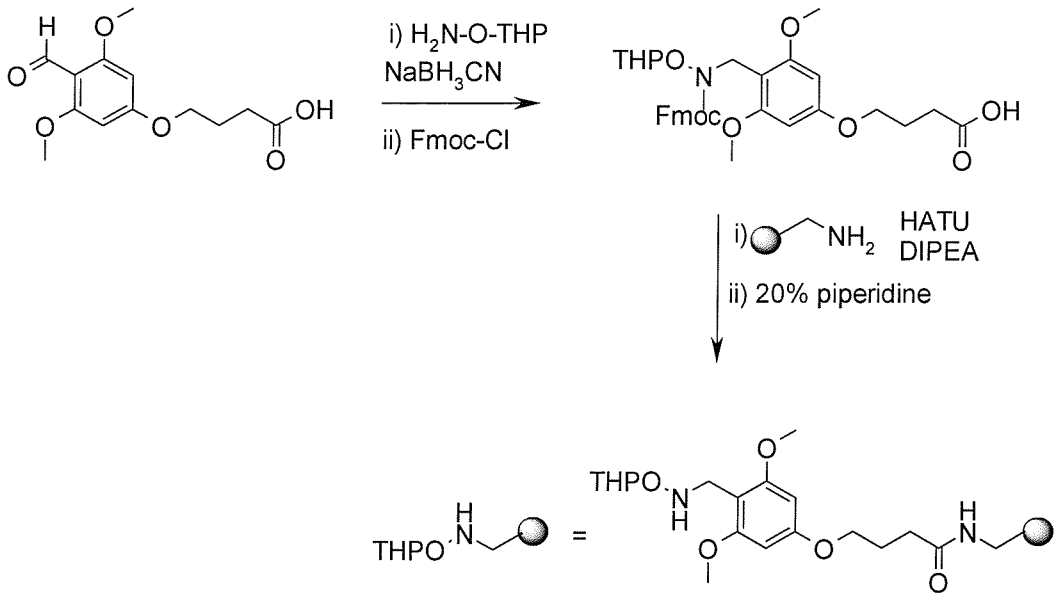


Figure 1.37: Synthesis of N-tethered, Alkoxyamine linker resin

The versatility of this alkoxyamine resin was demonstrated by the synthesis of a number of different types of hydroxamic acids (*figure 1.38*). A simple hydroxamic acid was initially synthesised by treatment with an acid chloride. Deprotection of the THP was accomplished using 2.5 % TFA to deprotect the THP group and followed by 50% TFA to cleave the hydroxamate from the solid phase. A simple succinyl analogue was synthesised simply by acylation with succinic anhydride followed by coupling with benzylamine, and treatment with acid as before, resulting in the release of the deprotected derivative from the resin. Similarly the synthesis of a phenylalanine based α-acetamido hydroxamate was also easily accomplished. The synthesis of a known MMP inhibitor described by Ciba-Geigy CGS27023A was undertaken as a demonstration of the utility of this strategy. The synthesis of this compound demonstrated that several challenging

transformations could be performed, N-acylation of a hindered amino acid (valine), N-sulfonylation of an amine, and N-alkylation of a sulfonamide group on the solid phase. DIC mediated coupling of Fmoc-Val-OH was quantitative, and subsequent sulfonylation of the deprotected amine was also successful. The final N-alkylation of the sulfonamide required some investigation. The reaction was successfully performed using Tsunoda's modification[121] of the standard Mitsunobu conditions.

Thus it was demonstrated that this novel strategy could provide routes to many and varied combinatorial libraries of hydroxamate derivatives.

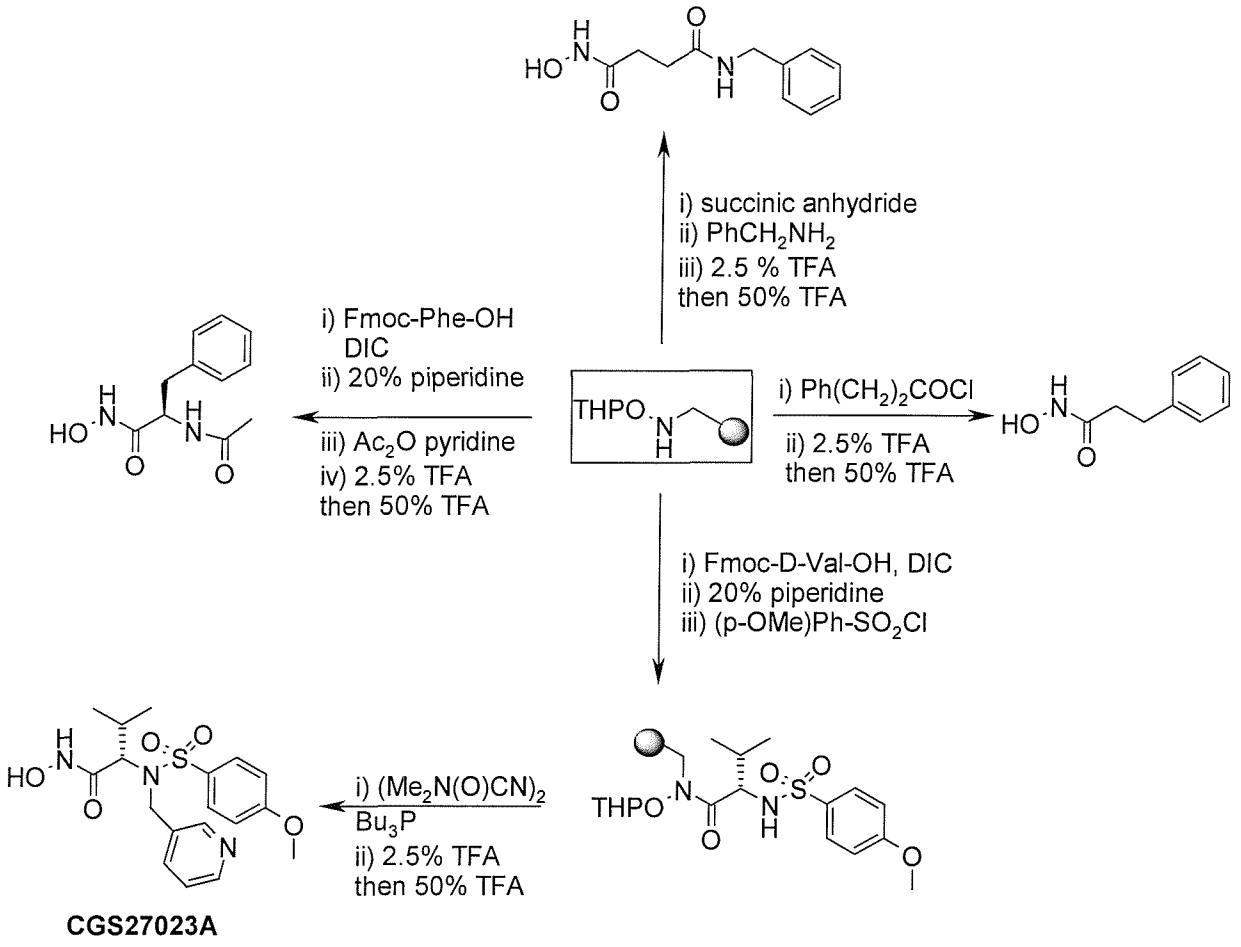


Figure 1.38: Preparation of hydroxamic acids using alkoxyamine resin

Klopfenstein[122] noted that all the previously published strategies did not allow for the use of acid labile protecting groups in the synthetic protocol. In addition strategies using O-hydroxylamine bound resin were prone to by-product formation deriving from the functionisation of the hydroxamate nitrogen. A new synthetic strategy was developed which did not have these disadvantages (*figure 1.39*).

Kaiser's oxime resin[123] was coupled with a carboxylic acid under standard conditions. Exposure to *tert*-butyldimethylsilyl hydroxylamine resulted in cleavage from the resin of crude protected hydroxamate. This crude product was purified by column chromatography and subsequent exposure to TFA afforded the pure hydroxamic acid. Synthesis was performed using a number of different acids all leading to the production of hydroxamic acids in good yields and purity.

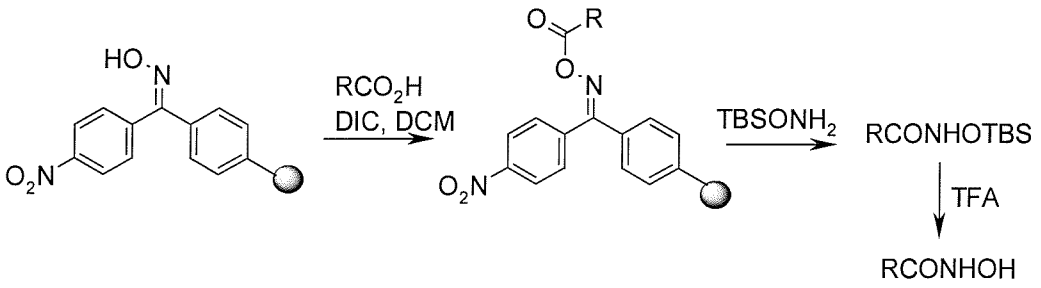


Figure 1.39: Synthesis of hydroxamic acids using Kaiser's oxime resin.

Barlaam[124] used hydroxylamine derivatised immobilised Sasrin linker to synthesise a variety of analogues of marimastat (*figure 1.40*).

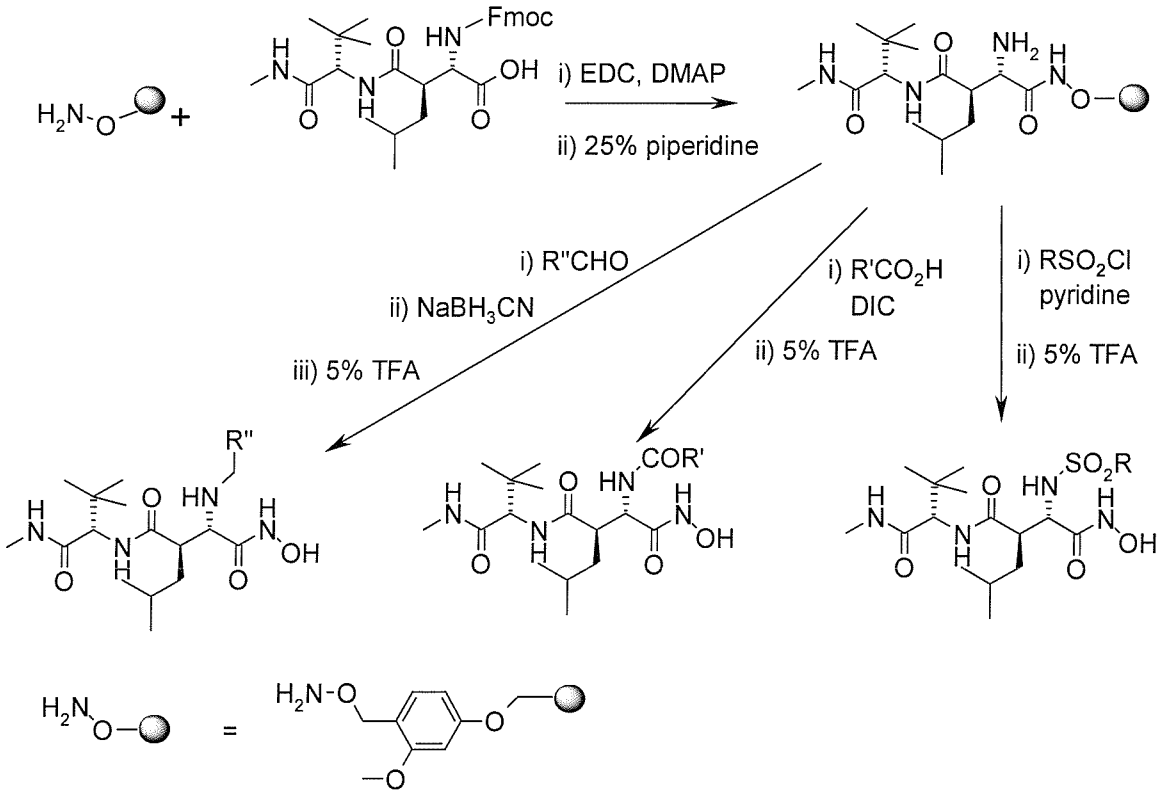


Figure 1.40: Synthesis of marimastat analogues using derivatised Sasrin resin.

The linker was derivatised using the conditions that Floyd[113] optimised for Wang resin. A derivative of marimastat was coupled to this resin and the amine deprotected. The amino group was then derivatised using a range of conditions. A range of sulfonamides were synthesised by reacting the resin bound amine with sulfonyl chlorides. Subsequent treatment with TFA released the sulfonamides from the resin in good yields and purity. Carboxamides were similarly synthesised by reacting the resin bound amine with a range of acids in classical conditions. These were released in good yields and purity. The versatility of this strategy was further demonstrated by the synthesis of amine derivatives by reductive alkylation.

1.4] Aims for the project

The aims of this project were to develop novel safety-catch linkers, which would enable the cleavage of compounds from the solid phase under mild biologically compatible conditions. The purpose of these linkers was to contribute to the development of a novel high throughput screening technique, the *zone diffusion assay.*

The initial linker was designed to attach/release carboxylic acids and hydroxamic acids to/from the solid phase. Three further linkers were designed to act as safety-catch linkers and attach/release amines, alcohols and hydroxamic acids to/from the solid phase. Acid is the activation mechanism of all the linkers resulting in the generation of a stable amine salt. Exposure to aqueous buffer would deprotonate this salt and facilitate cleavage. (*figure 1.41*).

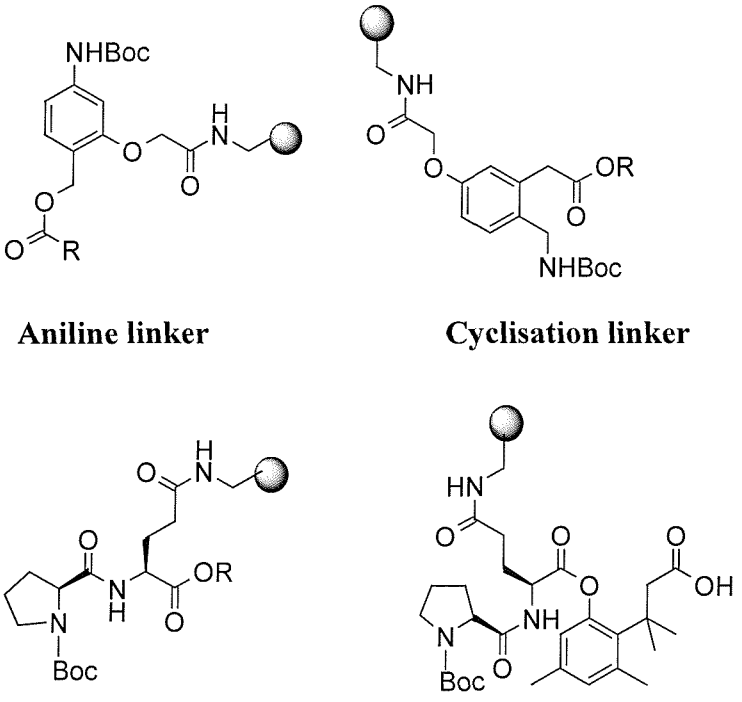


Figure 1.41: Safety-catch linkers for the development of zone diffusion assays.

1. Aniline based linker: Release of compounds would occur via a facile 1,6 elimination.

2. Cyclisation linker: Compounds would be released via lactamisation, which is thermodynamically and kinetically favoured

3. Diketopiperazine linker: Release of the substrates would occur following diketopiperazine formation.

4. Trimethyl lock linker: Trimethyl lock facilitated lactonisation has been used as a prodrug delivery system and a redox based linker. The cleavage of this linker would be via initial diketopiperazine formation, which would release the trimethyl lock substrate and thus release the compound attached.

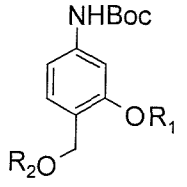
The activity and cleavage kinetics of these linkers were analysed by the synthesis of models in solution and on the solid phase. By the analysis of amide and ester models the feasibility of attachment of hydroxamic acids via the oxygen or the nitrogen was examined. It was hoped that it would be demonstrated that these linkers would be useful for the synthesis of hydroxamic acids on the solid phase.

Studies into the feasibility of zone diffusion assays were performed using gelatinase A (MMP-2), its substrate gelatin and a known potent inhibitor marimastat. Studies were initially performed to demonstrate the practicality of this concept. The ultimate aim was to demonstrate the utility of this screening technique by the synthesis of a small peptidyl hydroxamic acid library on the solid phase using the new linkers described. The identification of marimastat as an inhibitor from this library would demonstrate the ability of this novel technology to isolate new inhibitors from a combinatorial library.

Chapter Two Aniline based linkers

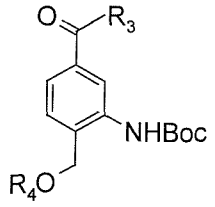
2.1] Introduction.

The aim of this project was to design, synthesise and evaluate safety-catch linkers that cleave in a two-stage process allowing the final cleavage to occur under biologically compatible conditions. The first step is acid *activation* of the linker followed by treatment with aqueous buffer to induce *cleavage* of the compound from the linker. Three anilines **[1]**, **[3]** and **[5]** were designed to act as safety-catch linkers which, when attached to the solid phase release compounds attached to the hydroxymethyl terminal into solution (*figure 2.1*). Model compounds **[2]**, **[4]** and **[6]** were synthesised and analysed in solution in order to examine the properties of these compounds and to determine their potential as safety-catch linkers.



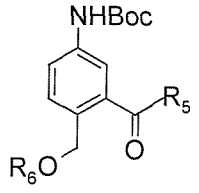
[1] : $R_1 = R_2 = H$

[2] : R_1= Bn, R_2 = Z-Ala



[3] : R_3 = OH, R_4 = H

[4] : R_3= OBn, R_4 = Z-Ala



[5] : R_5 = OH, R_6 = H

[6] : R_5 = OBn, R_6 = Z-Ala

Figure 2.1: Aniline based linkers

2.2] Synthesis and analysis of aniline based linker model [2]

Aniline **[1]** was designed to act as a safety-catch linker, releasing compounds with acid termini from the solid phase via a two-step mechanism (*figure 2.2*). Exposure to acid would remove the Boc protection from the linker resulting in a protonated amine **[7]**, which was presumed to be a stable species in acidic media. On addition to mildly basic aqueous buffer, it was expected that the linker would undergo a facile 1,6-elimination.

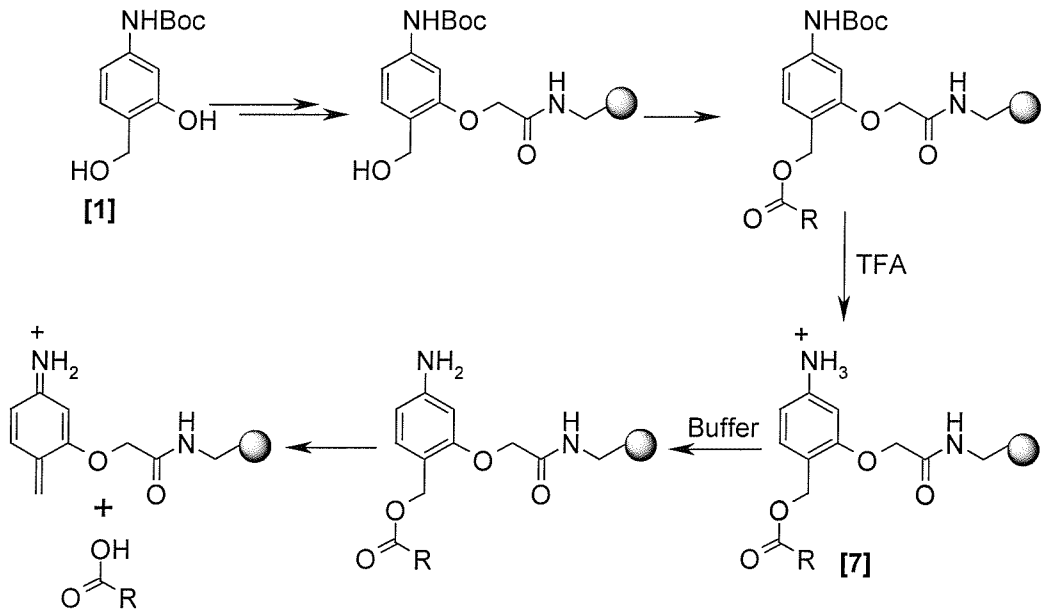


Figure 2.2: Proposed mechanism of the safety-catch linker derived from **[1]**

To assess the properties of this linker, model compound **[2]** was synthesised. 4-Amino salicyclic acid was protected with Boc_2O followed by simultaneous protection of the phenol and the acid with benzyl bromide to afford **[9]**. Reduction of the ester and subsequent esterification with Z-Ala-OH led to the isolation of the desired model compound **[2]** (*figure 2.3*).

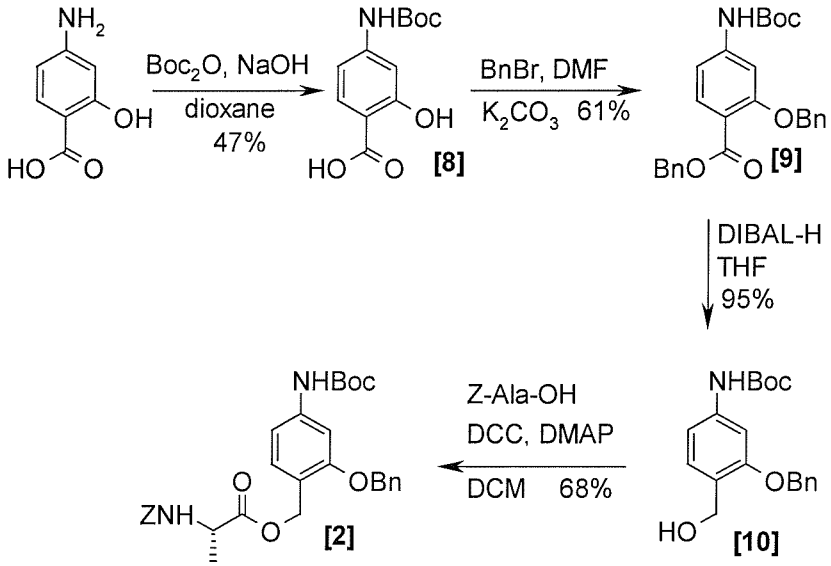


Figure 2.3: Synthesis of linker model **[2]**

On addition of **[2]** to 50% TFA/ DCM it was evident by TLC that Z-Ala-OH was released. In addition a yellow, very polar, component was released. Electrospray MS analysis suggested that it was the cation **[11]** (*figure 2.4*).

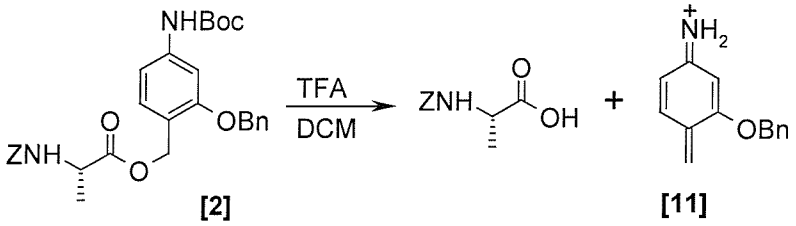


Figure 2.4: Analysis of linker model **[2]**

It was believed that the ether was contributing to the lability of this linker model. The ether has an electron donating effect, which could stabilise the transition state of the 1,6-elimination. Also in the presence of acid the ether oxygen could donate electrons and cause the cleavage of Z-Ala-OH by 1,4-elimination. In addition it has an activating resonance effect, which will also increase reactivity. A new model **[13]** that did not contain this ether was synthesised in order to examine this hypothesis (*figure 2.5*).

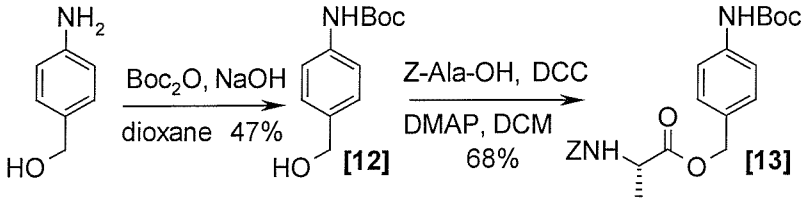


Figure 2.5: Synthesis of linker model **[13]**.

4-Amino benzyl alcohol was protected with Boc_2O, followed by esterification with Z-Ala-OH to generate model **[13]**. On exposure to acid the linker model **[13]** decomposed completely, analogous to model **[2]** (*figure 2.6*). Therefore the resonance effects of the ether did not significantly affect the lability of the linker model **[2]**. It was concluded that on treatment with acid the linker model decomposes completely making it an efficient acid labile linker but disappointingly not a safety-catch linker.

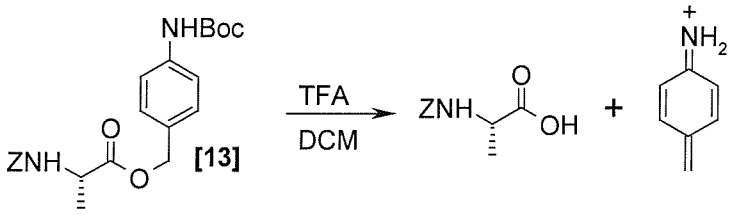


Figure 2.6: Analysis of linker model **[13]**.

2.3] Synthesis and analysis of aniline based linker model [4]

It was decided to investigate the behaviour of another aniline **[3]**, which was analogous to **[1]**. On exposure to acid it was uncertain whether the deprotected species **[14]** would be stable or decompose analogously to model **[2]**. Additionally assuming that it was stable in acidic and neutral media, it was uncertain whether on exposure to mildly basic buffer it would decompose via 1,4-elimination pathway **[A]** or via pathway **[B]** to generate a new amide species **[15]** (*figure 2.7*).

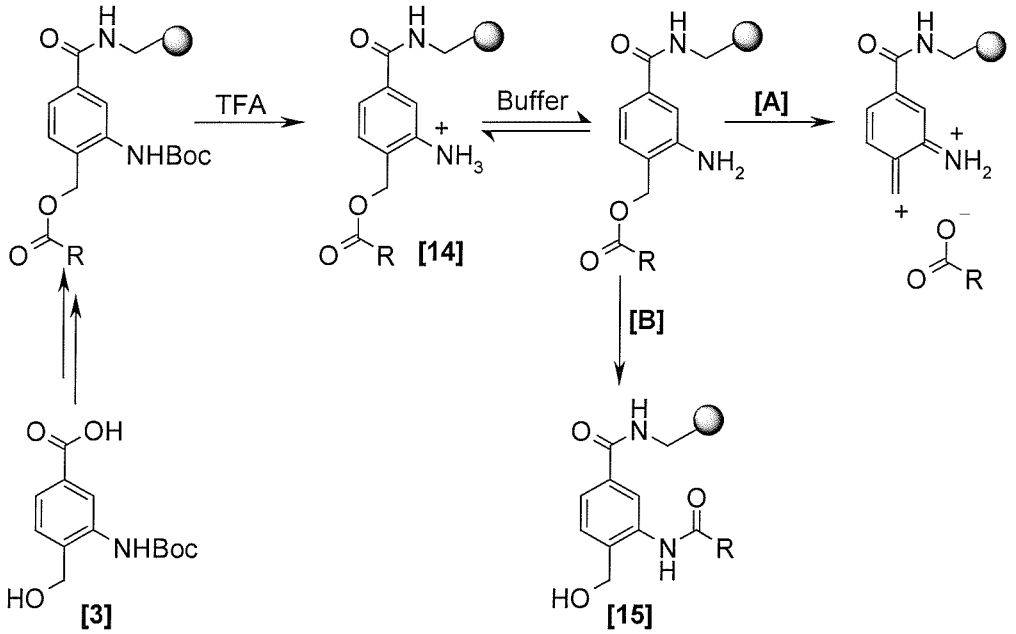


Figure 2.7: Proposed mechanism of the safety-catch linker derived from **[3]**.

If pathway **[B]** was favoured variant **[16]** could be investigated as a safety-catch linker for alcohol release (*figure 2.8*).

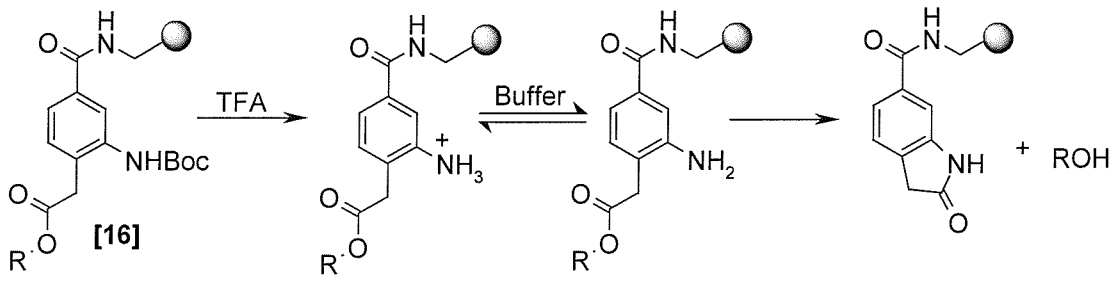


Figure 2.8: Possible linker variant based on linker **[3].**

To investigate the properties of this linker in solution, model **[4]** was synthesised. 4-Methyl benzoic acid was successfully brominated using NBS and benzoyl peroxide, generating **[17]**, which upon exposure to classical nitration conditions afforded the nitro derivative **[18]**.[125] S_N2 displacement with H_2O generated **[19]** (*figure 2.9*).

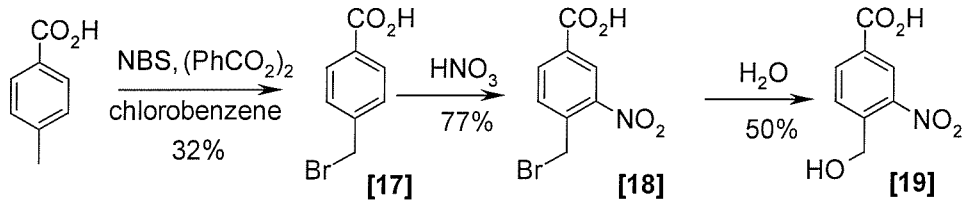


Figure 2.9: Synthesis of linker model precursor **[19].**

The subsequent reduction was attempted with Pd/C[126] in an atmosphere of H_2, which successfully reduced the nitro group, but also reduced the hydroxymethyl group. Using Pd/C poisoned with $BaSO_4$ in a H_2 atmosphere afforded the desired amine derivative **[20]** (*figure 2.10*).

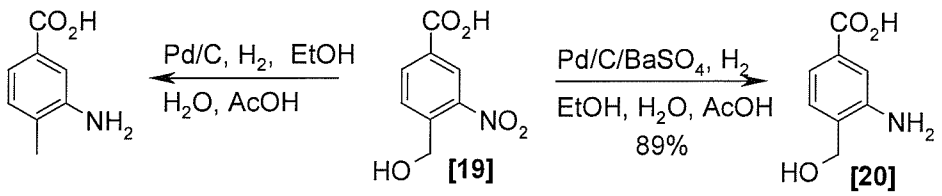


Figure 2.10: Synthesis of linker model precursor **[20].**

Protection of the amine group with Boc_2O and the acid with benzyl bromide resulted in the isolation of **[22]**. Subsequent esterification with Z-Ala-OH onto the hydroxymethyl group gave **[4]** (*figure 2.11*).

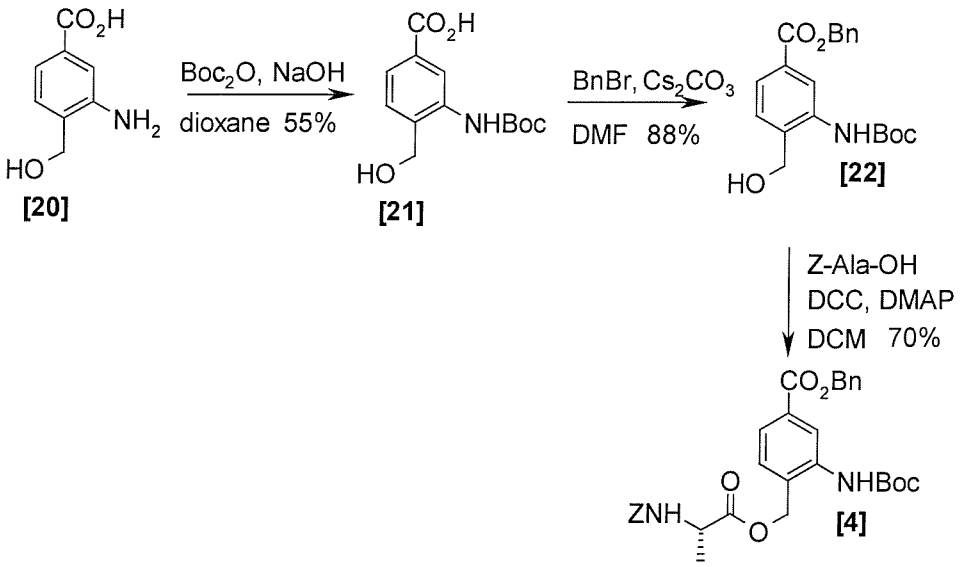


Figure 2.11: Synthesis of linker model **[4].**

RP-HPLC analysis indicated two products were produced upon exposure of **[4]** to acid. Both of these products had identical masses that corresponded to **[23]** and **[24]** (*figure 2.12*). Addition to buffer and analysis by RP-HPLC periodically indicated that **[23]** was being consumed at a rate inversely proportional to the increase of **[24]** (*figure 2.13* and *figure 2.14*).

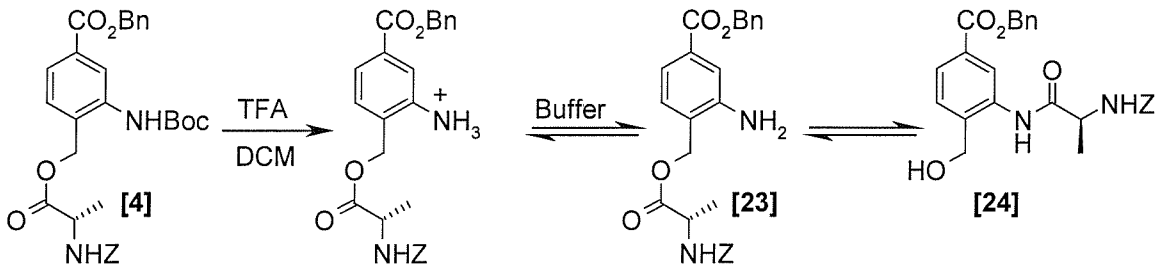


Figure 2.12: Analysis of linker model **[4].**

There was no evidence of the release of Z-Ala-OH. Thus it was concluded that the 1,4-elimination reaction was unfavoured and that the rearrangement from amine **[23]** to the amide **[24]** was promoted with or without the presence of buffer.

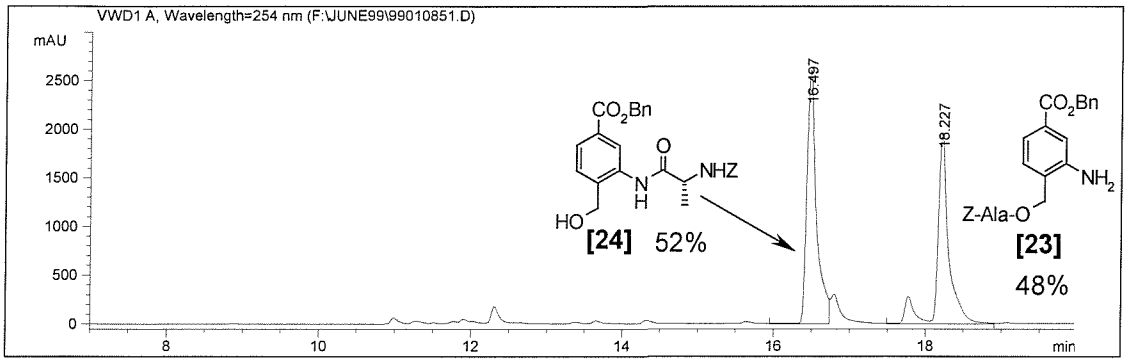


Figure 2.13: HPLC trace depicting reaction composition at start.

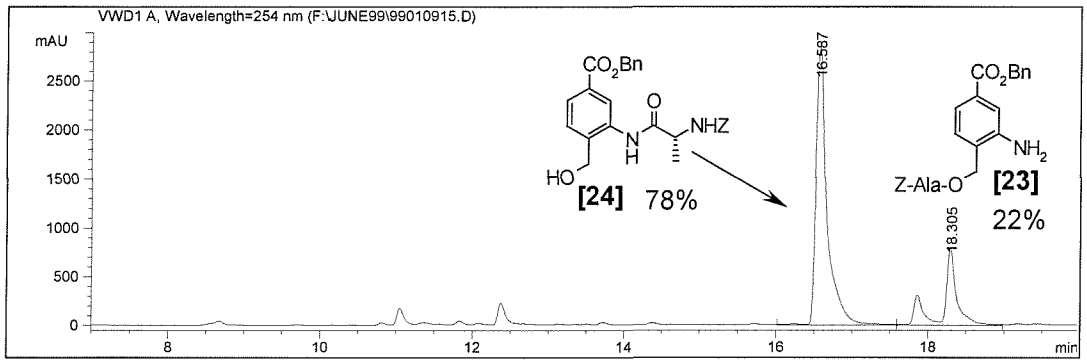


Figure 2.14: HPLC trace depicting reaction composition after 43 hours.

To confirm the assignment of these compounds **[23]** and **[24]** were synthesised by a direct route (*figure 2.15*).

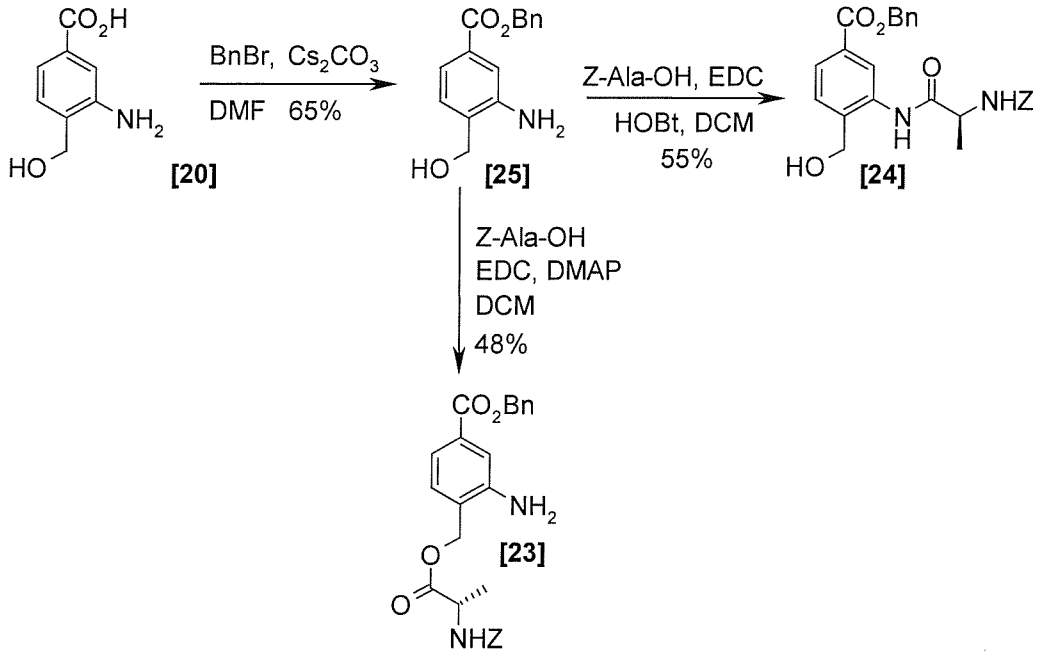


Figure 2.15: Synthesis of standards **[23]** *and* **[24]**.

It was concluded that the rearrangement reaction takes place under both acidic and basic conditions. However it did not appear to go to completion even after extended times. This may be due to the reaction approaching equilibrium. Thus it was considered unsuitable to be utilised in any further linker variants. Interestingly, there was no evidence for the 1,4-elimination occurring.

2.4] Synthesis and analysis of aniline based linker model [6]

Another pre-loaded linker **[5]** was designed analogous to the original aniline **[1]**. It was unclear how it would behave on exposure to acid, whether it would decompose via a 1,6-elimination reaction analogous to pre-loaded linker **[1]** or if it would have increased stability analogous to pre-loaded linker **[3]** (*figure 2.16*).

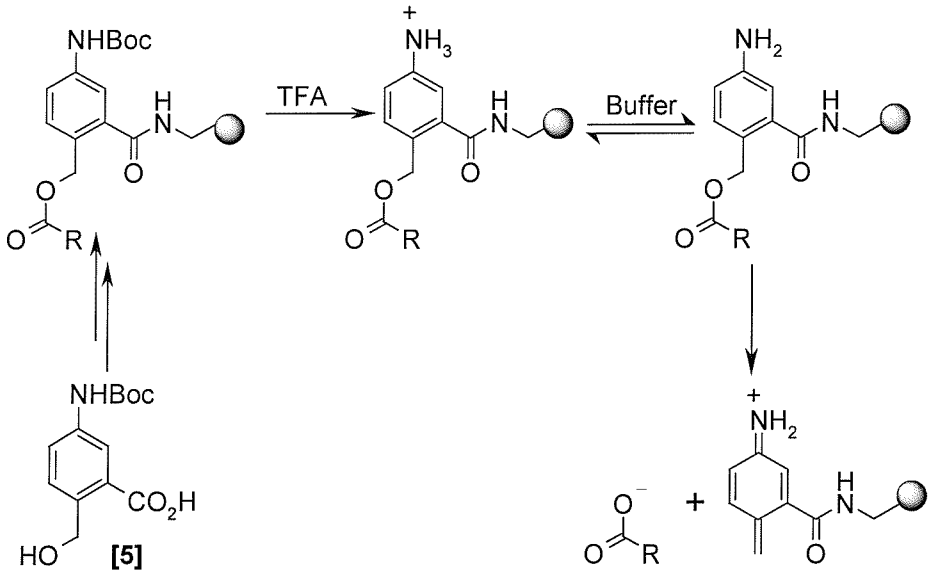


Figure 2.16: Proposed mechanism of safety-catch linker derived from **[5].**

Model **[6]** was synthesised to study the properties of this linker (*figure 2.17*).

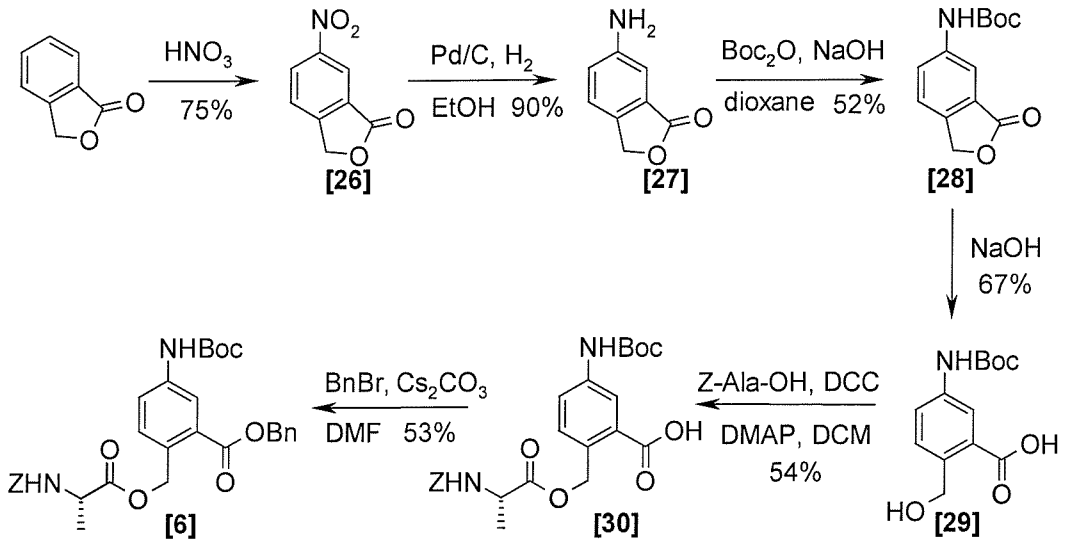


Figure 2.17: Synthesis of linker model **[6].**

3-Hydroisobenzofuran-1-one was nitrated, reduced and the amine protected with Boc_2O generating **[28]**. The lactone was opened using NaOH and the alcohol trapped by esterifying with Z-Ala-OH. Finally the acid was alkylated to give **[6]**.

Upon exposure of **[6]** to acid, the stable compound **[31]** was exclusively produced, characterised by HPLC and MS and showed no evidence of elimination (*figure 2.18* and *figure 2.19*). On addition of **[31]** to buffer, analysis by RP-HPLC showed the presence of Z-Ala-OH. MS analysis provided evidence that the cation **[32]** was also present. However many other side products were also observed *(figure 2.20)*, although their amounts may not be very significant.

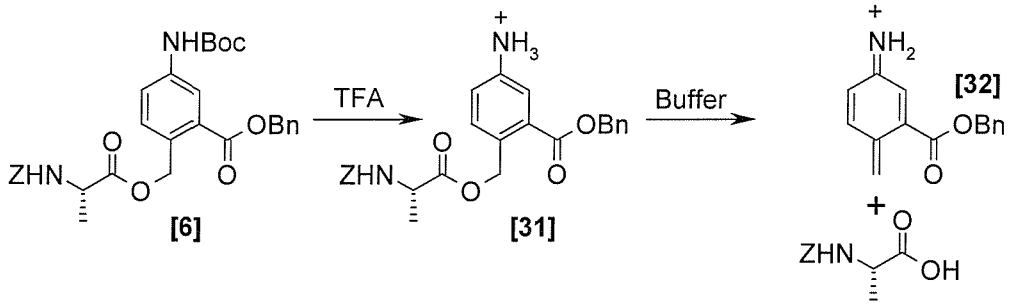


Figure 2.18: Analysis of linker model **[6].**

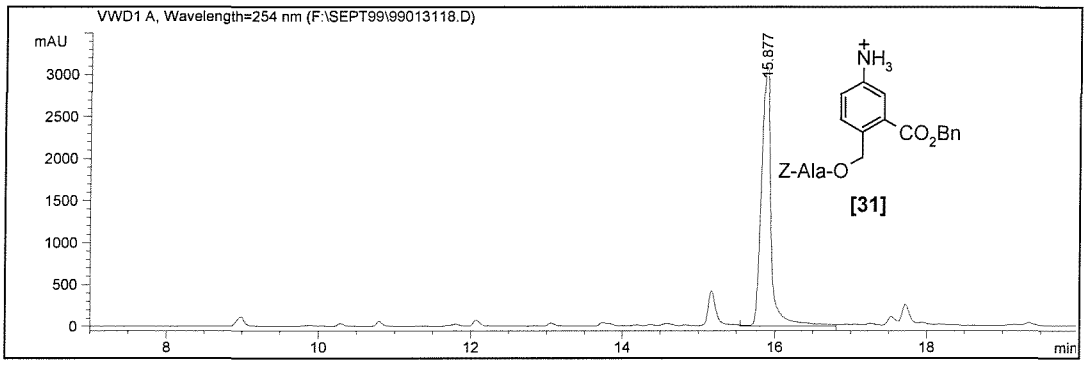


Figure 2.19: HPLC trace depicting reaction composition before buffer addition.

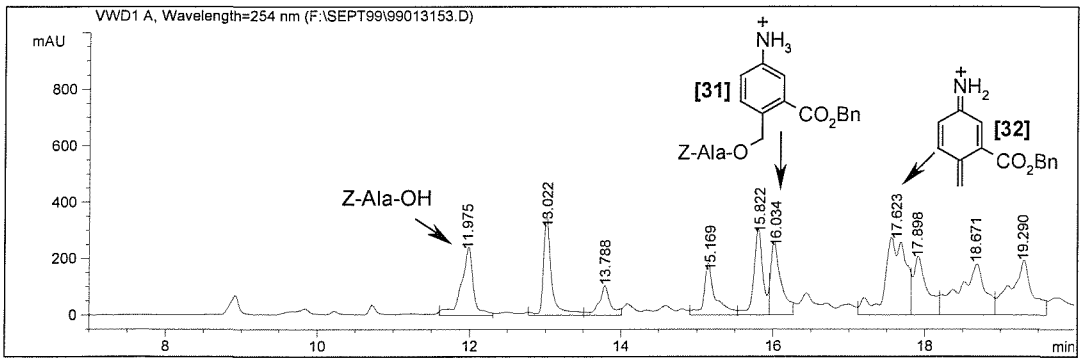


Figure 2.20: HPLC trace depicting reaction composition after 48 hours.

Since decomposition was slow, the aniline **[31]** can undergo various intermolecular reactions in basic conditions, which compete with the 1,6-elimination. The esters are vulnerable to transesterfication and if the hydroxymethyl group could be freed through an intermolecular amidation reaction, analogous to the previous model, it will cyclise rapidly to give the lactone. In addition it is not unreasonable to expect that the electrophilic cation released from the elimination will be susceptible to attack. Thus it is easy to imagine that there are many permutations of these reactions which will result in the formation of multiple side products (*figure 2.21*). Many of these reactions however could be avoided by the attachment of the acid to the solid phase via a stable amide bond, or at least the side reactions would not be observed because the products would be resin bound.

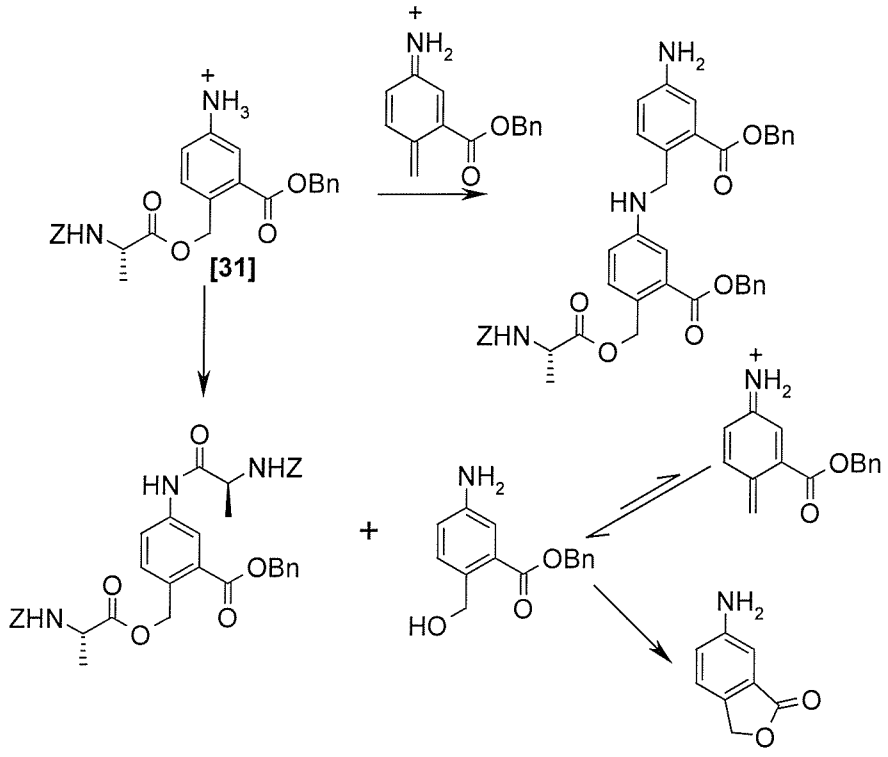


Figure 2.21: Possible side reactions resulting from the decomposition of linker model **[6]**.

2.5] Conclusions

It was unexpected that the linker model **[2]** would be decompose under acidic conditions. This eventuality can be rationalised by the low pKa of anilines (3-5) allowing for the deprotonated species to be present in small amounts, which decompose rapidly. This was not envisaged as a problem, as it was believed that the acids used were acidic enough to prevent this occurring. The linker model **[6]** once deprotected is stable under acidic conditions. This change in reactivity can only be attributed to the carboxylic ester group that is present in this linker model but not in the original model **[2]**. This group has an electron withdrawing field effect, which causes the ΔG of the cation **[31]** to be reduced relative to the analogous cation derived from model **[2]**. In addition this group also has a deactivating resonance effect, removing electron density from the aromatic ring, which contributes further to the reduction in reactivity. It is possible that pre-loaded linker **[5]** could function as a safety-catch linker, as attaching the **[5]** to the solid phase via a stable amide bond would prevent many of the side reactions that were believed to occur on the testing of model **[6]**. However the decomposition was not very rapid so it is unlikely that without modification this system could be of practical use. The behaviour of linker model **[4]** was also unexpected. The deprotected linker model does not undergo an elimination reaction under acidic or buffered conditions unlike other models. This change in reactivity can again be attributed to the presence of the carboxylic ester group. However the reason for the elimination reaction being unfavoured is not clear.

To conclude none of the aniline based linkers investigated would be efficient safety-catch linkers. Pre-loaded linker **[1]** would be a very efficient acid labile linker, but this is not a useful discovery as there is no advantage to using this linker over any previously documented acid labile linkers.

Chapter three Cyclisation linker

3.1] Introduction

Safety-catch linker **[33]** was designed to enable the release of alcohols, amines or hydroxamic acids into aqueous buffered conditions. By examining the rate of release of alcohols and amines the utility of this linker for the solid phase synthesis of hydroxamic acids could be determined. Exposure to acid was envisaged as the *activation* step, which would remove the Boc protecting group from **[34]**, resulting in salt **[35]**, which was expected to be stable under acidic conditions *(figure 3.1)*.

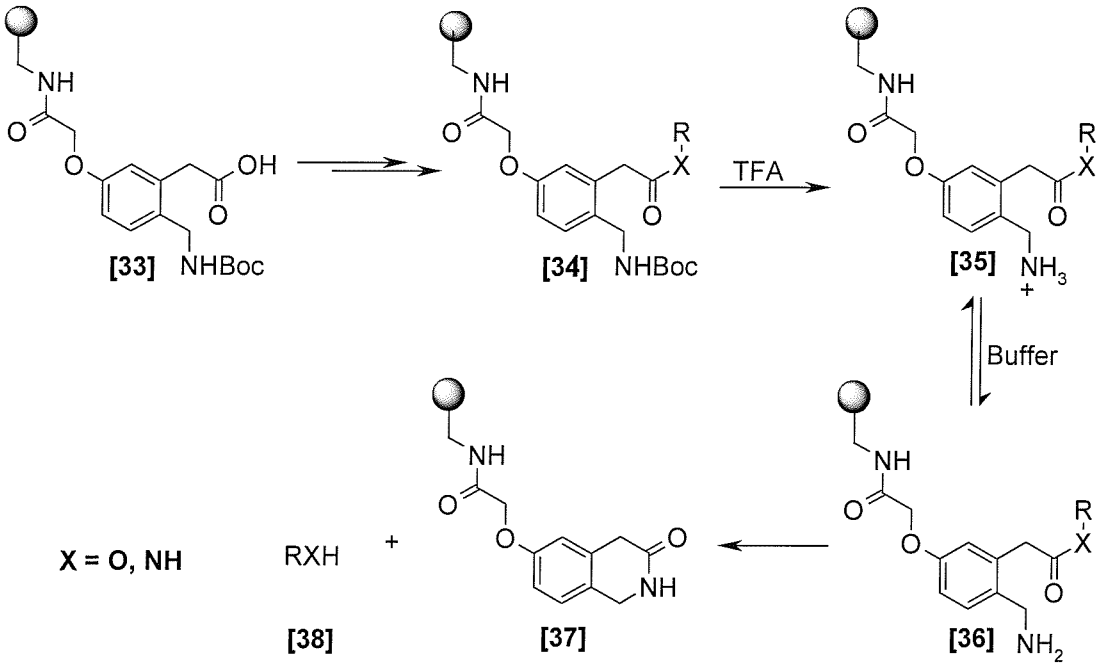


Figure 3.1: Proposed mechanism of safety-catch linker **[33]**.

On addition to pH 7.5 buffer, (the *cleavage* step), partial deprotonation of the amine would occur and promote a fast intramolecular cyclisation reaction releasing the desired compound **[38]**. The cyclisation is both kinetically and thermodynamically favoured. Models of this linker were synthesised in solution and on the solid phase in order to examine the properties of the linker, including the cleavage kinetics and thus determine the utility as a safety-catch linker.

3.2] Synthesis of the linker

The initial strategy proposed for the synthesis of linker **[33]** used a literature based photocyclisation to perform the cyclisation step.[127] Commercially available 3-cyanophenol was protected using allyl bromide, and subsequently reduced using $LiAlH_4$ to give **[40].**

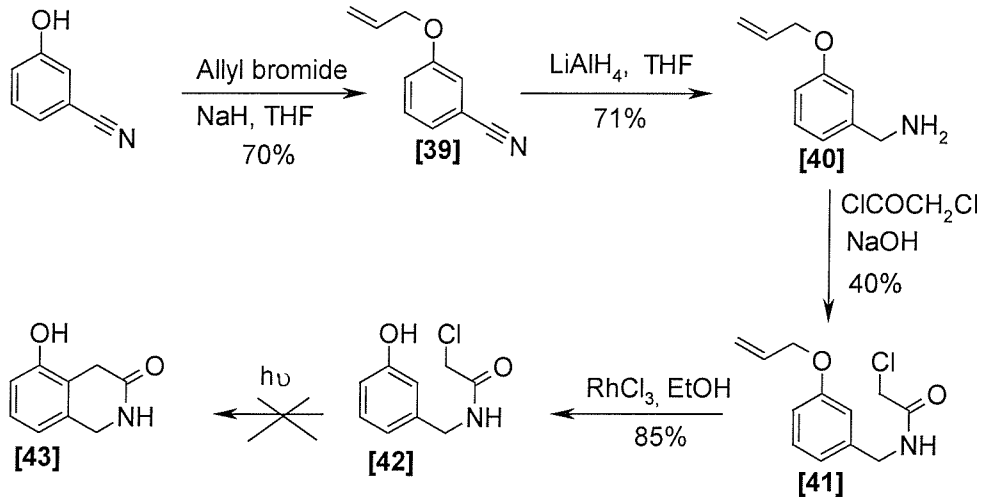


Figure 3.2: Attempted synthesis of linker precursor **[43]**.

A Schotten Baumnn reaction performed with chloroacetyl chloride gave **[41]** and the phenol was simply deprotected using $RhCl_3$ resulting in the isolation of the photocyclisation precursor **[42]** *(figure 3.2)*. All attempts to perform the cyclisation either by photochemistry or Lewis acid mediated cyclisation and obtain **[43]** proved unsuccessful. Another strategy was subsequently proposed. Chromanone **[44]** was synthesised from commercially available 3-methoxy phenyl acetic acid by reaction with HCl/ AcOH and CH_2O.[128] Treatment of **[44]** with HCl/ EtOH generated **[45]** and subsequent aminolysis gave the lactam **[46]**. The lactam was hydrolysed in refluxing HCl and subsequent treatment with Boc_2O generated **[48]** *(figure 3.3)*.

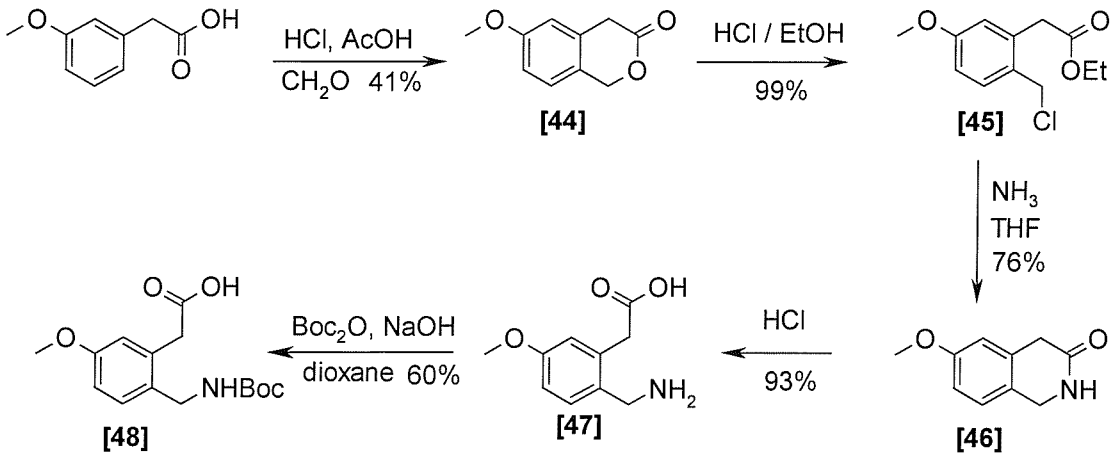


Figure 3.3: Synthesis of linker intermediate **[48]**.

From intermediate **[48]** the phenol **[50]** was theoretically accessible in one step by a simple deprotection. However no successful method was found to deprotect a methoxy group in the presence of a Boc group. Another strategy was employed starting from lactam **[46]** a precursor in the synthesis of **[48]**. The methoxy group was easily removed with boron tribromide, and the crude product subjected to refluxing HCl which opened the lactam to give the non proteogenic amino acid **[49]**. This intermediate was protected with Boc_2O and the acid group protected with a methyl ester to generate **[51]** *(figure 3.4)*.

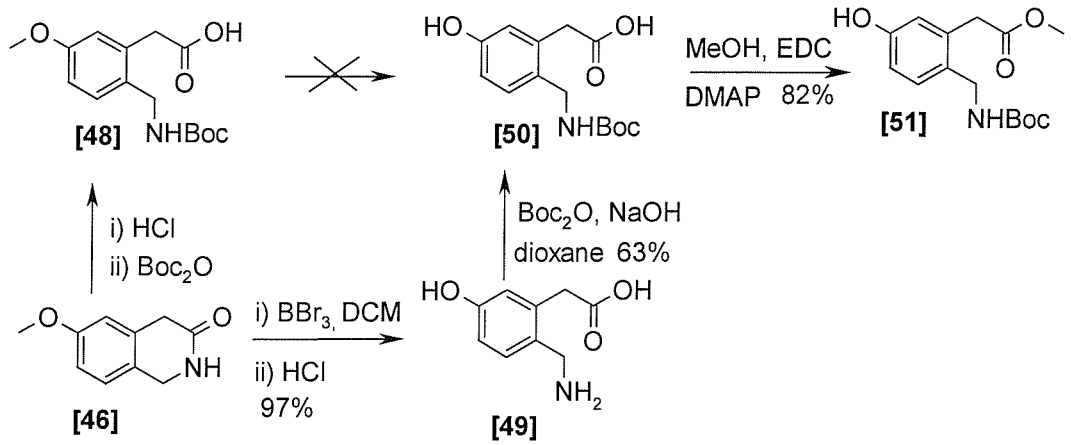


Figure 3.4: Synthesis of linker intermediate **[51].**

Bromoethyl TentaGel resin **[52]** was synthesised by reacting bromoacetic acid with aminoethyl TentaGel resin in the presence of DIC and HOBt, and the success of this reaction was confirmed by a qualitative ninhydrin test.[7] Alkylation with **[51]** in the

presence of Cs_2CO_3 in refluxing MeCN generated **[53]**. Subsequent deprotection on the solid phase with $KOSiMe_3$[129] afforded linker **[33]** *(figure 3.5)*. The success of this reaction was confirmed by a qualitative BCG test.[130]

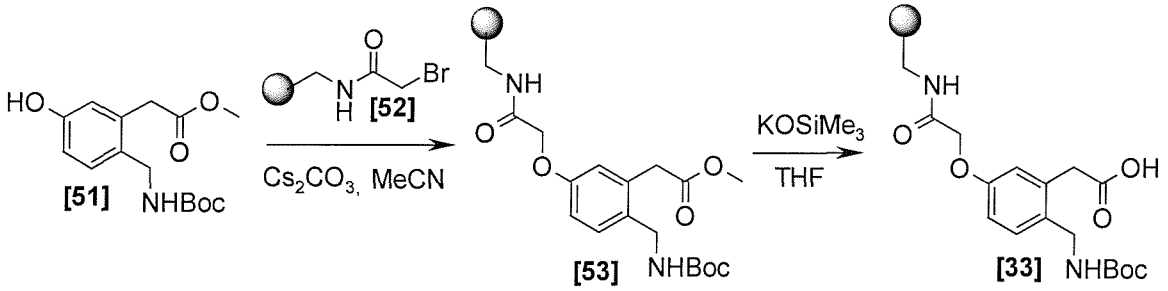


Figure 3.5: Synthesis of resin bound linker **[33]**.

3.3] Evaluation of linker models in solution

3.3.1] Synthesis of linker models

To evaluate the utility of the linker two models were synthesised and their cleavage was analysed in solution. By comparing the relative rates of ester and amide cleavage the possibility of using this strategy to synthesise hydroxamic acids on the solid phase using this linker could be determined.

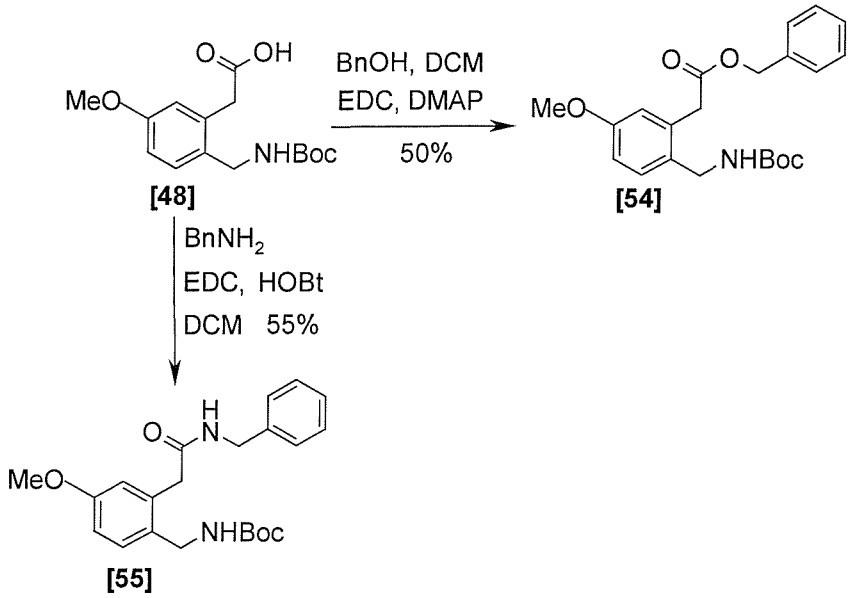


Figure 3.6: Synthesis of linker models **[54]** *and* **[55]**.

The models were synthesised by reacting **[48]** with benzyl alcohol and benzylamine to give **[54]** and **[55]** respectively *(figure 3.6)*.

Exposing **[54]** and **[55]** to acid removes the Boc group and generates amine salts **[56]** and **[57]**. Exposure to pH 7.5 phosphate buffer promotes lactam formation and release of the model substrate *(figure 3.7)*.

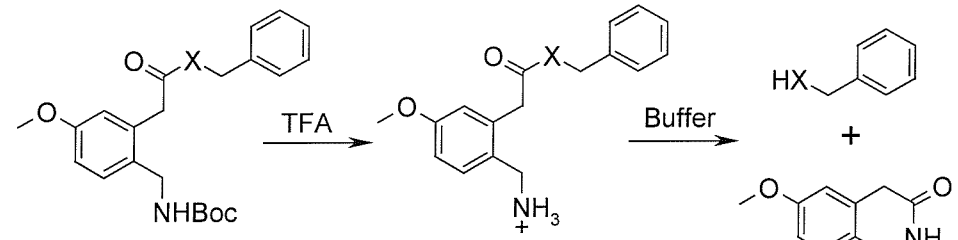


Figure 3.7: Analysis of solution phase models

3.3.2] Analysis using RP-HPLC

The kinetics of cleavage were studied by removing aliquots at various time points, quenching with 2% TFA and analysing by RP-HPLC.

3.3.2.1] Analysis of benzyl ester model **[54]**.

Treatment of the protected benzyl ester **[54]** with TFA gave the expected amine **[56]** as the exclusive product, which appeared to be stable in neutral and acidic conditions. This product was fully characterised. Treatment with buffer (50 mM phosphate, pH 7.5) promotes cyclisation and RP-HPLC analysis showed the presence of two new compounds corresponding to lactam **[46]** and benzyl alcohol *(figure 3.8)*. With time the proportion of these compounds increased inversely with the decrease of the starting material.

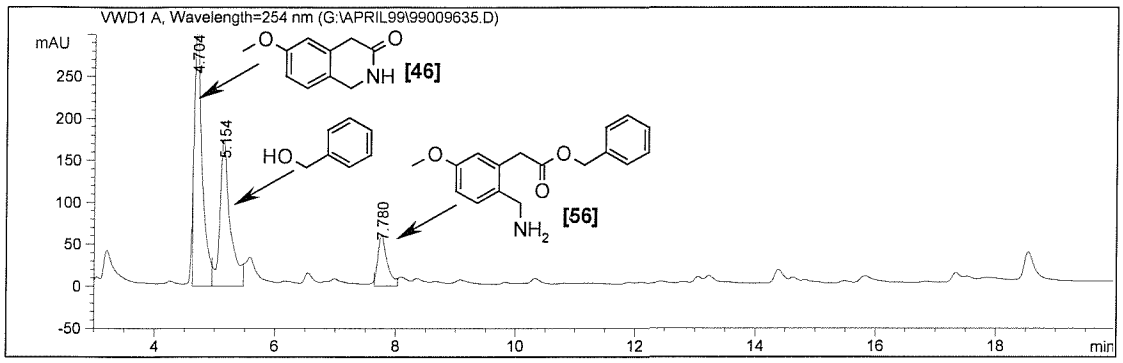
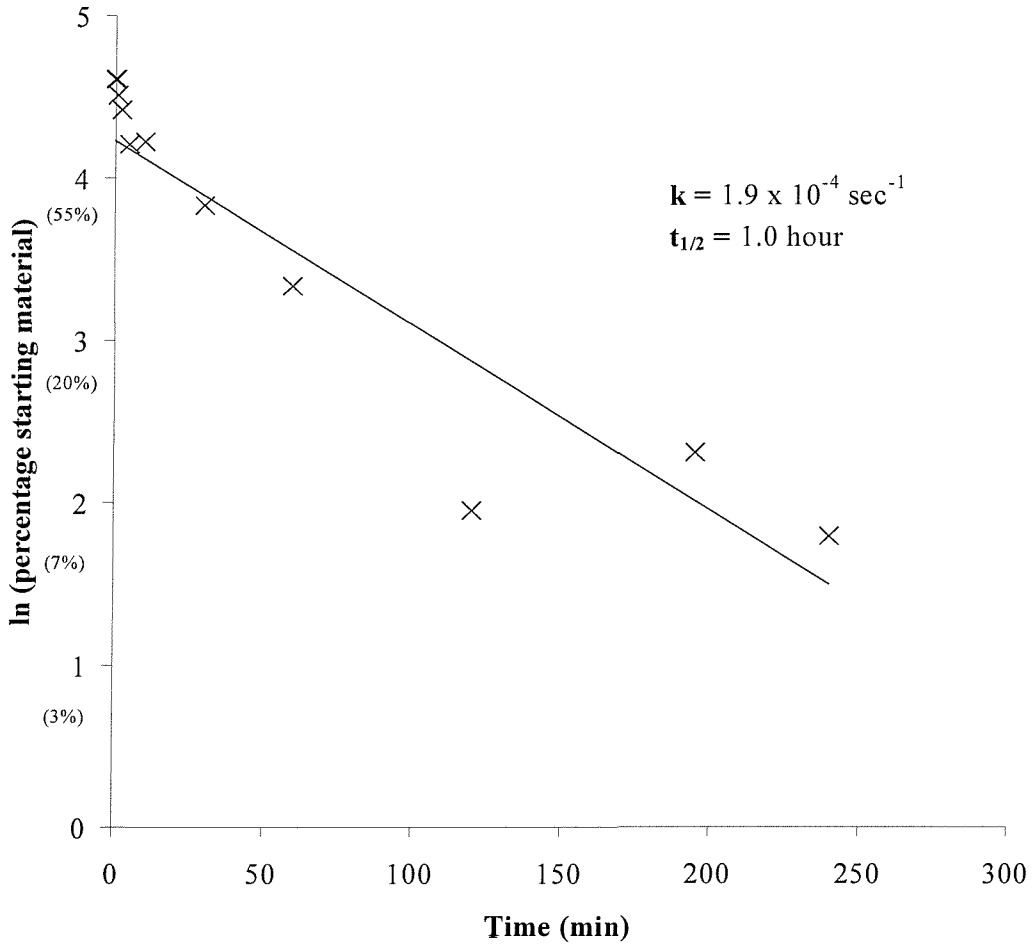


Figure 3.8: HPLC trace depicting reaction composition after 300 min

The cleavage of the linker is depicted in graph 3.1.



Graph 3.1: Fragmentation of benzyl ester model in pH 7.5 phosphate buffer.

The decay of the linker model adheres to first order reaction kinetics as expected, which can be expressed:

$$-d[\mathbf{A}]/dt = k[\mathbf{A}]$$

integrating between limits t = 0 and t = t, gives:

$$\ln [\mathbf{A}] = -kt + \ln \mathbf{A_0}$$

where

[**A**] = Concentration of starting material at time t, t = time, $\mathbf{A_0}$ = initial concentration of starting material, k = rate constant.

Thus if a plot of ln [**A**] against time is linear the reaction is first order, and k can be calculated from the gradient.

3.3.2.2] Analysis of benzyl amide model **[55]**.

Treatment of the protected benzyl amide **[55]** with TFA gave the expected amine **[57]** as the exclusive product, which appeared to be stable in neutral and acidic conditions. The product was fully characterised. Treatment with buffer (50 mM phosphate, pH 7.5) and subsequent RP-HPLC analysis showed the presence of two new compounds corresponding to the lactam **[46]** and benzylamine (*figure 3.9*).

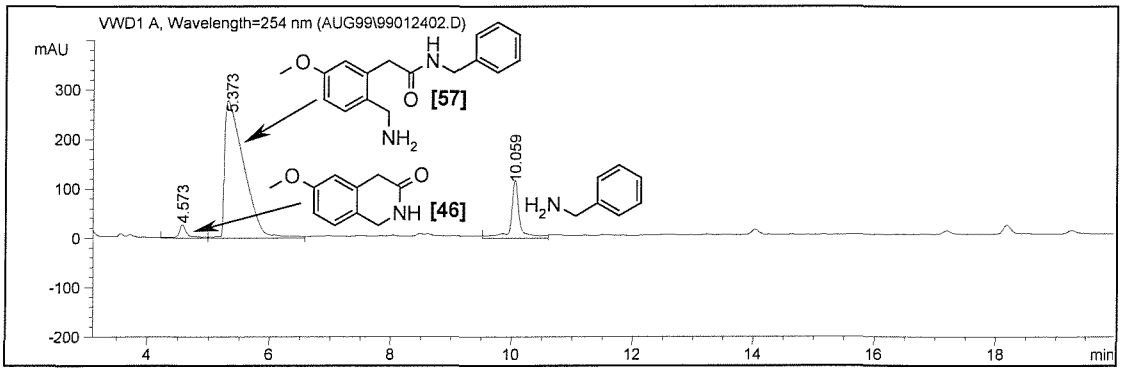
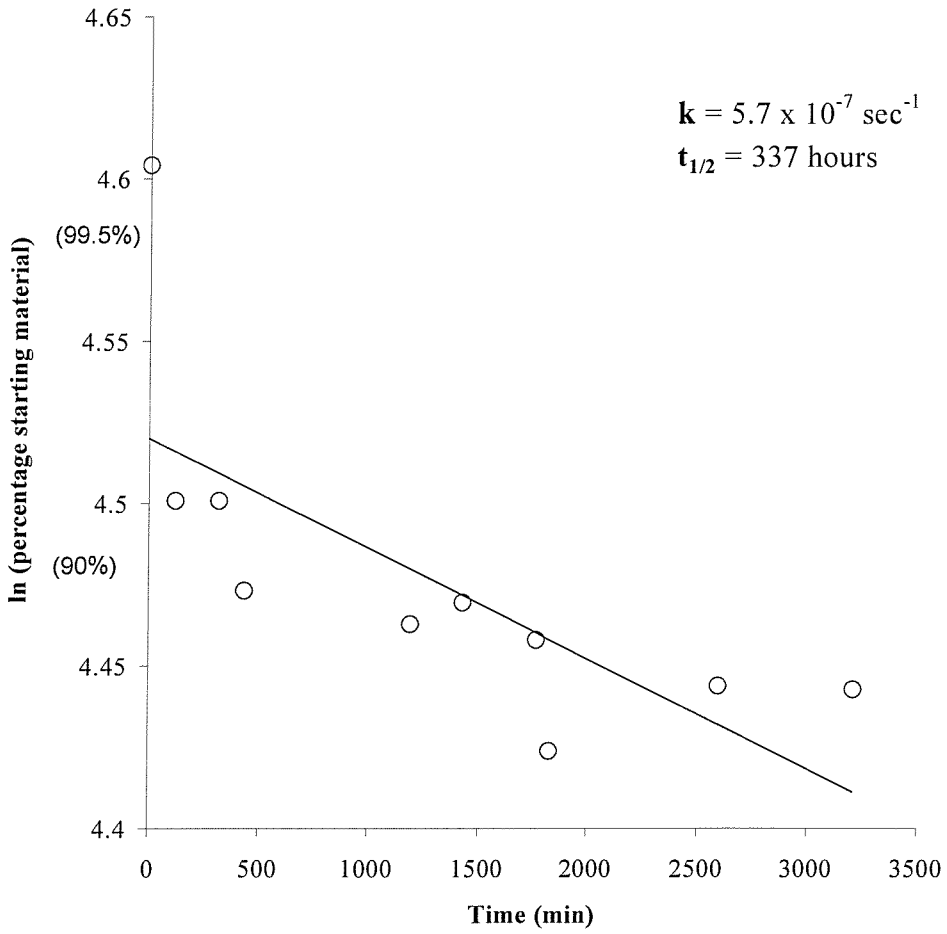


Figure 3.9: HPLC trace depicting reaction composition after 102 hours.

The cleavage of the linker model is depicted in graph 3.2.



Graph 3.2: Fragmentation of benzyl amide model in pH 7.5 phosphate buffer.

The decay of this model was very slow and the degree of correlation to first order kinetics is not as high as the previous model. HPLC analysis, has an associated error and the witnessed error will be more evident with lower sample concentration. Thus due to the slow progress of this reaction, HPLC analysis revealed only small changes in the composition of the reaction mixture over time and hence the degree of error was expected to be relatively large.

3.4] Analysis using NMR

The cleavage of the benzyl ester model **[56]** was monitored using NMR. The safety-catch was removed with TFA and ^{1}H NMR spectra in pD 7.5 50 mM phosphate buffer (made with D_2O instead of H_2O) were recorded (*figure 3.10*)

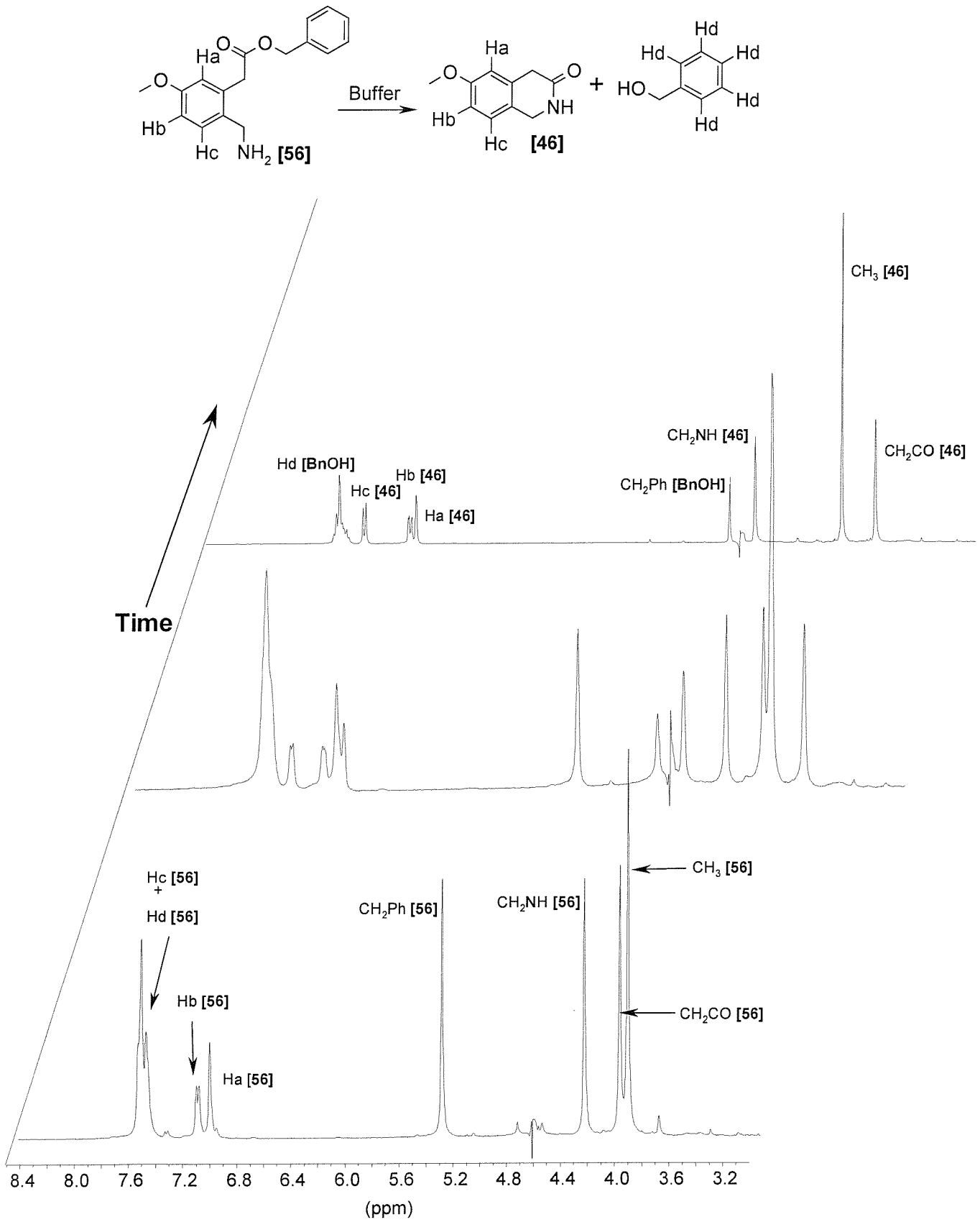


Figure 3.10: NMR analysis of the decomposition of the benzyl ester model ***[56]***

3.5] Comparison of the cleavage rates

It is evident from studying the HPLC data that the linker will cleave alcohols substantially faster than amines. In fact, amine cleavage is so sluggish that only 10% was released after 4 days. This outcome is not unexpected since the amide bond is much stronger than that of an ester and alcohols are better leaving groups than amines.

Model type	Substrate	k (sec^{-1})	$t_{1/2}$ (hours)
Ester	Benzyl alcohol	1.9×10^{-4}	1
Amide	Benzylamine	5.7×10^{-7}	337

Table 3.1: Rate constants and half-lives of the solution models.

From studying the rate constants (*table 3.1)* the relationship can be quantified demonstrating that alcohols were released over 300 times faster than amines. The cleavage of the amide was too slow to make this linker useful for releasing amines from the solid phase. Thus this system is suitable for releasing alcohols but not amines. This means that it is possible that the linker could be used for releasing hydroxamic acids from the solid phase via the associated alcohol.

3.6] Conclusions

A novel safety-catch linker has been successfully synthesised and the ability of this linker to release alcohols was demonstrated by the analysis of models in solution. This linker was shown to release amines, but at a rate too slow to be of practical use.

Chapter Four Diketopiperazine linker

4.1] Introduction

Another safety-catch linker **[57]** was designed to enable the release of alcohols, amines or hydroxamic acids into aqueous buffered conditions. Analogous to linker **[33]**, exposure to acid was envisaged as the *activation* step, which would remove the Boc protecting group from **[58]**. The resulting salt **[59]** should be stable under acidic conditions. Addition to pH 7.5 buffer, (the *cleavage* step), partial deprotonation of the amine would occur and promote diketopiperazine formation, releasing the desired compound **[61]** (*figure 4.1*).

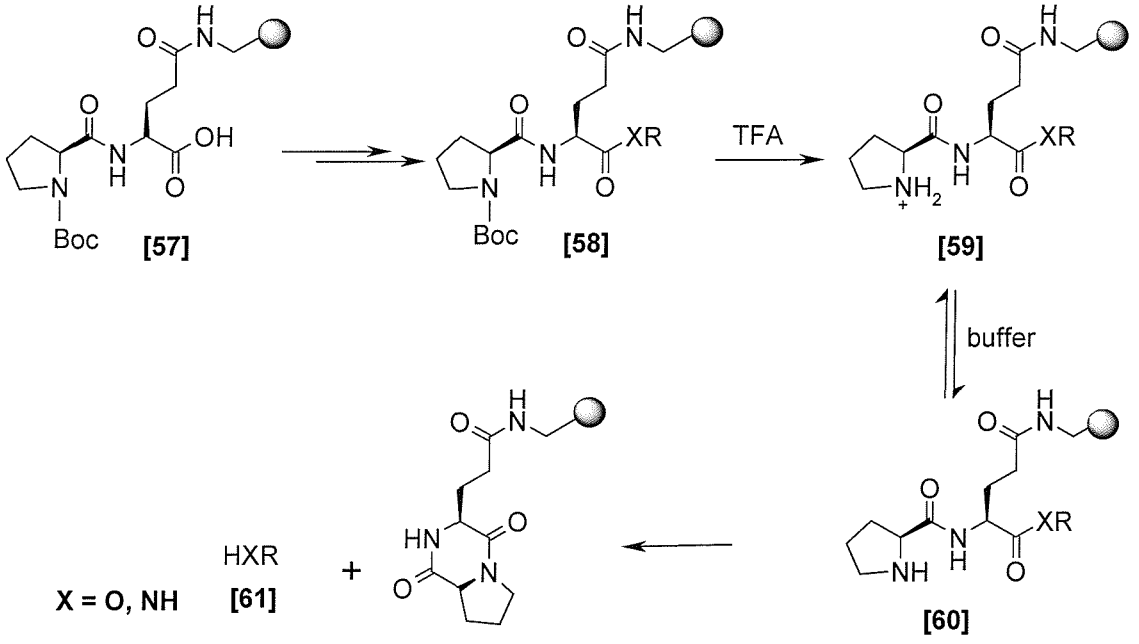


Figure 4.1: Proposed mechanism of the safety-catch linker.

Diketopiperazine formation was originally noted as a side reaction that occurs during peptide synthesis at the dipeptide stage. Rate of diketopiperazine formation is dependent on the N-alkyl amino acid[131] and the type of bond broken for the diketopiperazine to form.[132] This process has been used to develop a number of safety-catch linkers and as part of two multiple release linkers. The utility of the linker

[57] was examined by the synthesis and analysis models in solution and on the solid phase.

4.2] Synthesis of the linker

L-Glutamic acid was selectively protected on the γ carboxylic acid with allyl alcohol in the presence of chlorotrimethylsilane to generate **[62]**.[159] This amino acid was reacted with the succinimide active ester of Boc-Pro-OH to give dipeptide **[63].** Protection of the α carboxylic acid with MeOH was essential so the two acids were differentiable and this reaction proceeded in good yield to give **[64]**. Deprotection of the allyl ester generated **[65]** (*figure 4.2*).

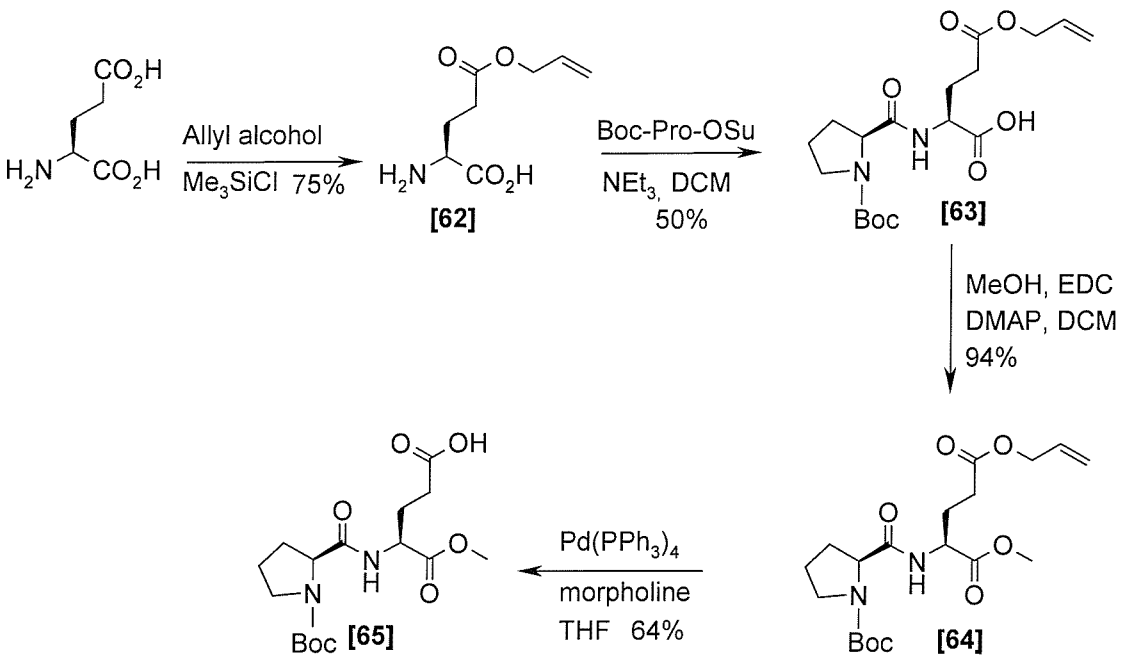


Figure 4.2: Synthesis of linker intermediate **[65]**

Dipeptide **[65]** was attached to aminoethyl TentaGel using standard coupling conditions. The reaction was monitored using the qualitative ninhydrin test. Deprotection on the solid phase using $KOSiMe_3$ gave the linker **[57]**, and the success of this reaction was confirmed by a qualitative BCG test (*figure 4.3*).

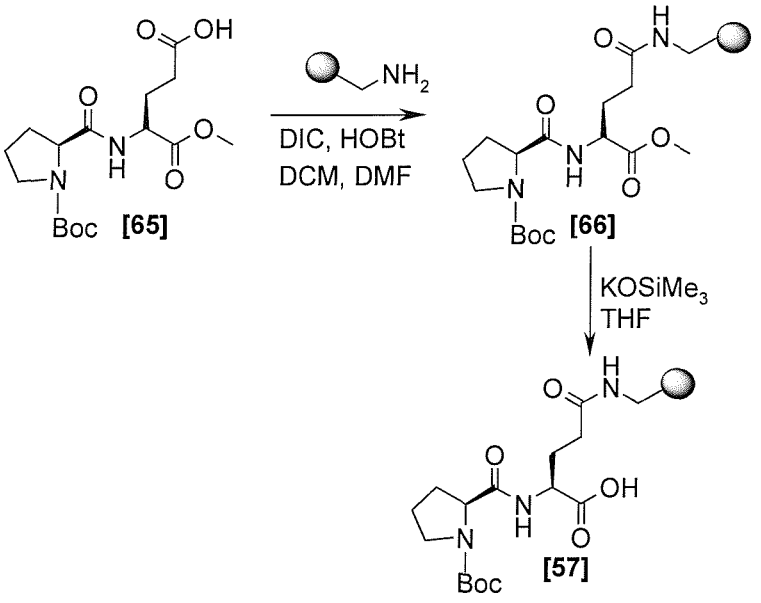


Figure 4.3: Synthesis of the resin bound linker **[57]**

4.3] Evaluation of linker models in solution

4.3.1] Synthesis of the linker models

To evaluate the utility of the linker, ester and amide models were synthesised by reacting acid **[63]** with benzyl alcohol and benzylamine respectively under standard conditions to give **[67]** and **[68]** (*figure 4.4*).

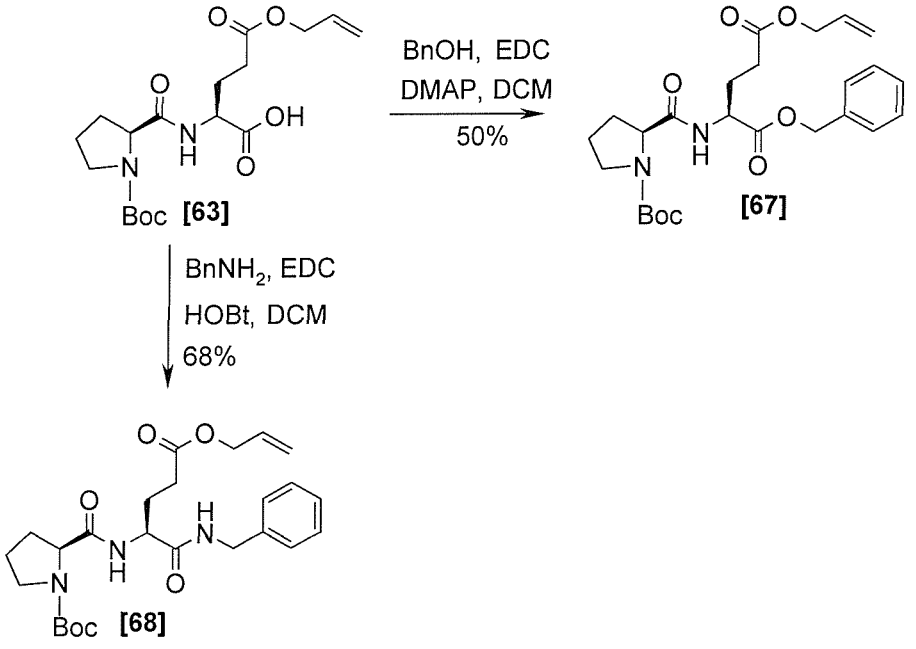


Figure 4.4: Synthesis of linker models **[67]** *and* **[68]**.

Exposing these compounds to acid removes the Boc group and activates the safety-catch to generate salts **[69]** and **[70]**. Exposing these compounds to buffer induces diketopiperazine formation and release of the model substrate benzyl alcohol or benzylamine in addition to the diketopiperazine **[71]** (*figure 4.5*).

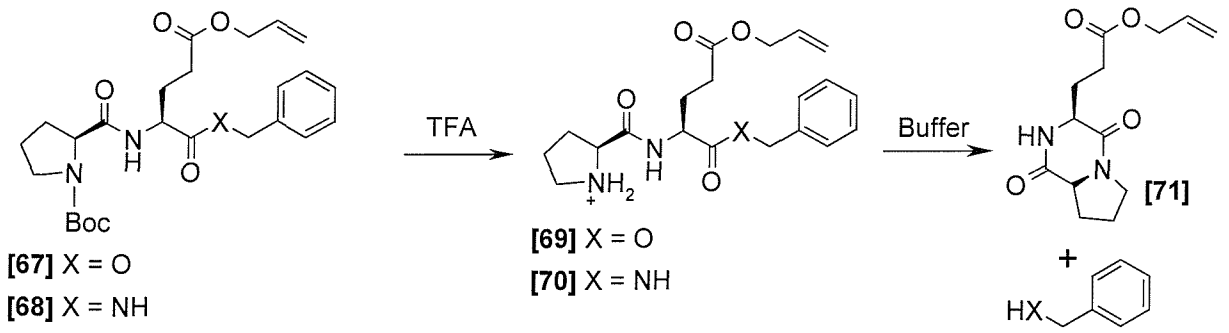


Figure 4.5: Analysis of solution phase models.

4.3.2] Analysis using RP-HPLC

The kinetics of cleavage were studied using RP-HPLC analysis, as described in the previous chapter.

4.3.2.1] Analysis of benzyl ester model **[67]**.

Treatment of the protected benzyl ester **[67]** with TFA gave the expected amine **[69]** as the exclusive product, which appeared to be stable under neutral and acidic conditions. This product was fully characterised. Treatment with buffer (50 mM phosphate, pH 7.5) promotes diketopiperazine formation and subsequent RP-HPLC analysis showed the presence of three new compounds. With time the proportion of these compounds increased inversely with the decrease of the starting material. Benzyl alcohol was unambiguously identified by a standard. MS analysis of both the other two new peaks gave masses that corresponded to the diketopiperazine expected. This phenomenon can be explained by the existence of two diastereomers of the diketopiperazine, caused by epimerisation at the α CH Glu centre (*figure 4.6*).

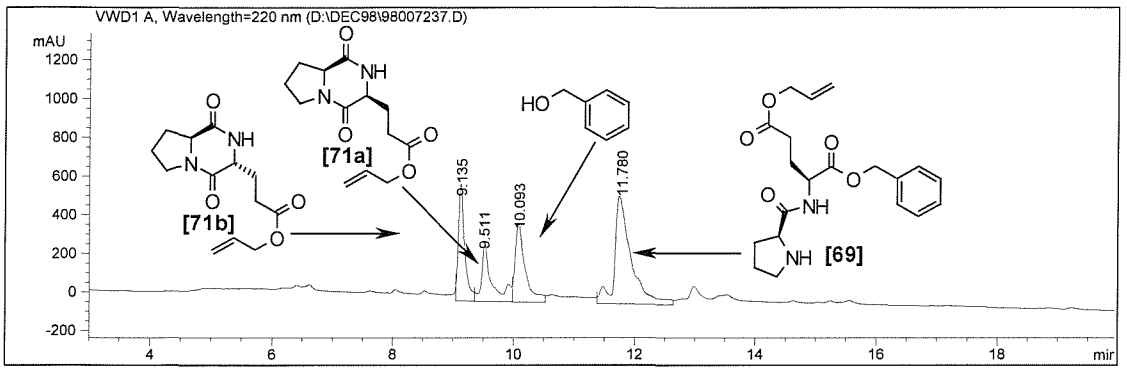


Figure 4.6: HPLC trace depicting reaction composition after 85 hours.

This epimerisation was believed to have occurred during the esterification reaction used to synthesise the model **[67]**. After the carboxylic acid was activated by EDC and DMAP, oxazalone formation could occur to generate **[72]**. The oxazalone could then isomerise easily to produce the two diastereomers **[72]** and **[74]**. This would result in the isolation of epimers of the benzyl ester **[67]** (*figure 4.7*).

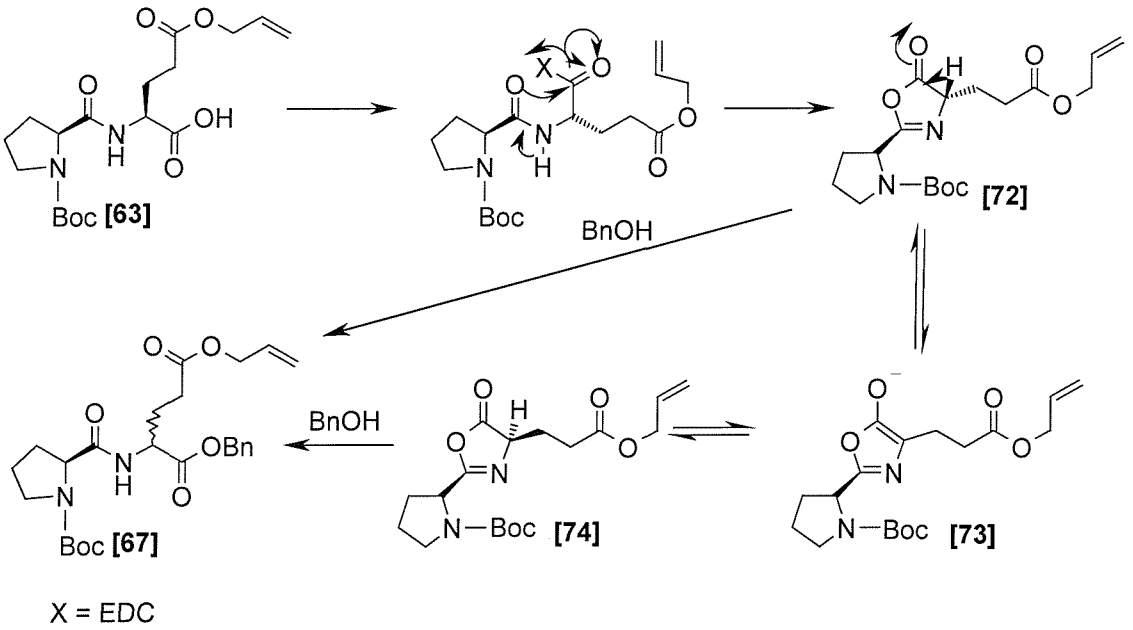


Figure 4.7: Mechanism of epimerisation by oxazalone formation

Further evidence for this phenomenon was generated by studying a different model **[75]**. Commercially available H-Glu(OAllyl)-OAllyl, was coupled to Boc-Pro-OH to generate dipeptide **[75]** (*figure 4.8*). Following Boc deprotection and exposure to pH 8, 50 mM phosphate buffer, analysis by RP-HPLC showed that only one diastereomer of the diketopiperazine **[71a]** was produced (*figure 4.9*).

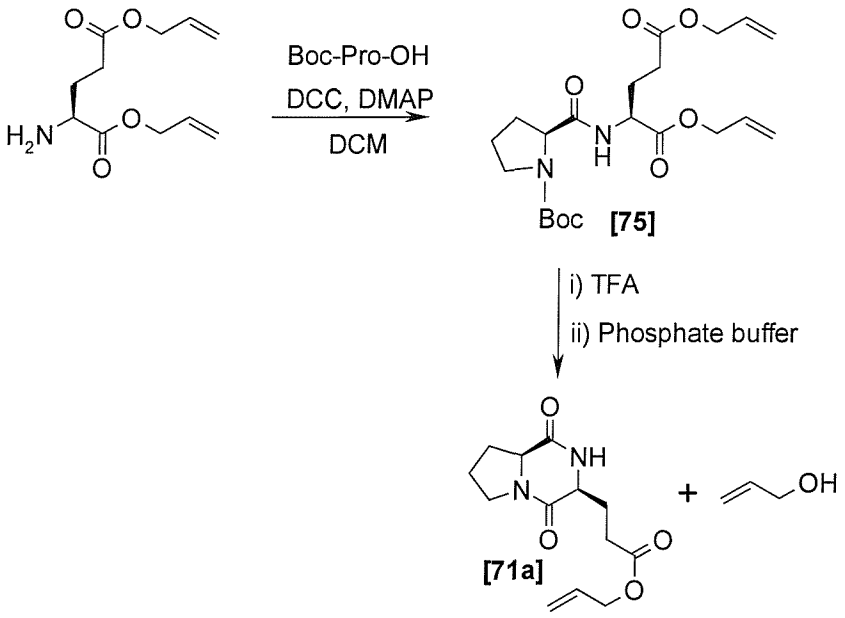


Figure 4.8: Synthesis and evaluation of linker model **[75]**.

This model cannot epimerise because at no stage in the synthesis is oxazalone formation possible. This evidence supports the believed epimerisation mechanism. In addition the only diketopiperazine formed in this reaction contains both L-amino acid residues and thus was a valuable aid to assigning the HPLC data.

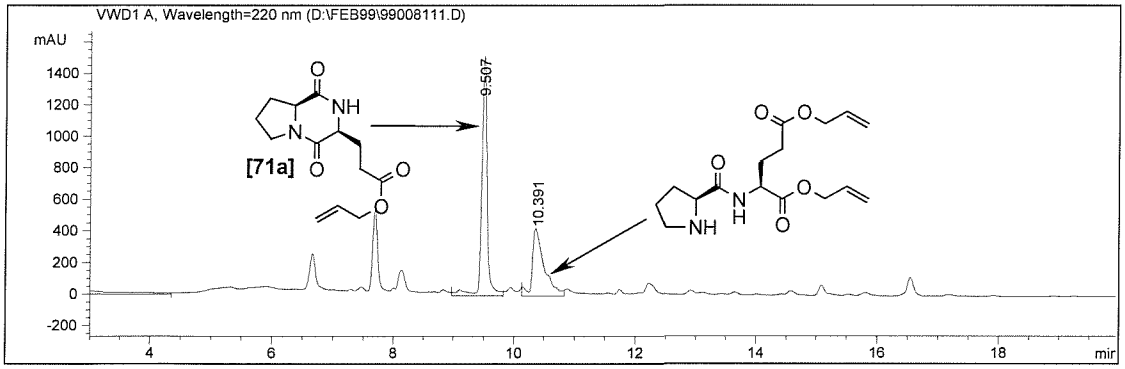
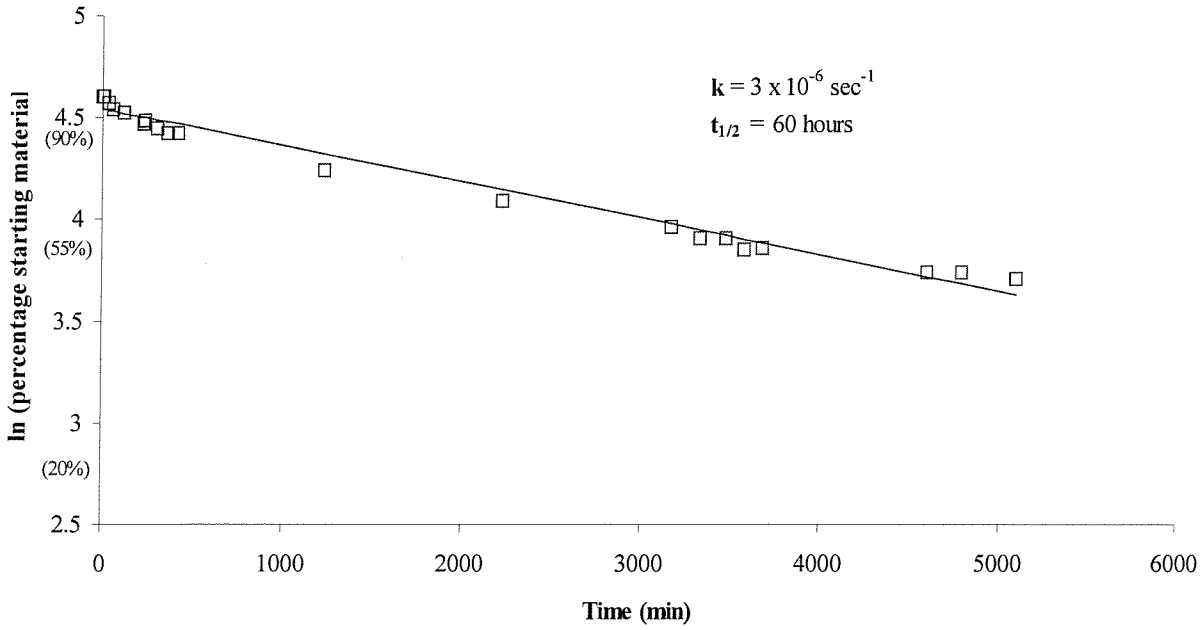


Figure 4.9: HPLC trace depicting reaction composition after 85 hours.

It is also feasible however that that the two diastereomers of the diketopiperazine were generated *in situ* during the cleavage reaction. The precursor compounds were not conclusively demonstrated to exist as both expected diastereomers and thus this explanation for the observed phenomenon must also be considered.

The cleavage kinetics of the benzyl ester model adheres to first order kinetics (*graph 4.1*).



Graph 4.1: Fragmentation of benzyl ester model in pH 7.5 phosphate buffer.

4.3.2.2] Analysis of benzyl amide model **[71]**.

Treatment of the protected benzyl amide **[71]** with TFA gave the expected amine **[73]** as the exclusive product, which appeared to be stable in neutral and acidic conditions. The product was fully characterised. On treatment with buffer (50 mM phosphate, pH 7.5) this compound did not appear to decompose significantly, even after extended reaction times.

4.4] Analysis using NMR

The cleavage of the benzyl ester model **[69]** was monitored using NMR. The safety-catch was removed with TFA and then HOD suppressed ^1H NMR spectra in pD 7.5 50 mM phosphate buffer (made with D_2O instead of H_2O) were recorded (*figure 4.10*).

The chemical shift of several components change subtly on the transformation.

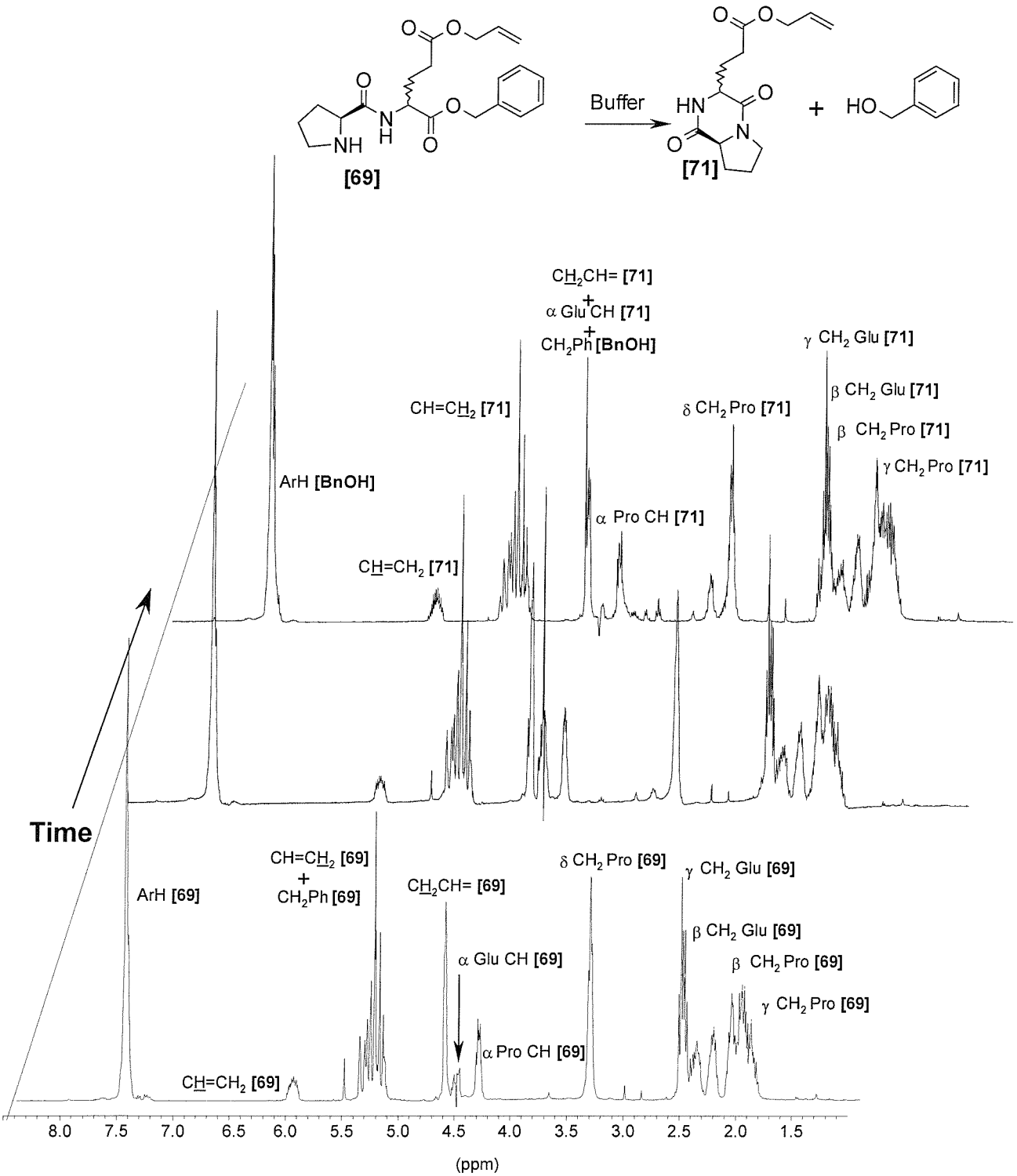


Figure 4.10: NMR analysis of the decomposition of the benzyl ester model.

The aliphatic protons do not change significantly. The δ CH_2 Pro chemical shift is moved downfield from 3.3 ppm to 3.5 ppm. Also the α protons are both shifted downfield. The CH_2Ph chemical shift moves upfield, from 5.5 ppm to 4.8 ppm.

4.5] Comparison of the cleavage rates

It was evident from studying the HPLC data that the linker will cleave alcohols, although at a slow rate. No amine cleavage was witnessed, even after extended reaction times. The rate of diketopiperazine formation is strongly sequence dependent and is promoted by the inclusion of amino acids, which encourage β turns, such as proline and glycine. It is disappointing that the cleavage kinetics of this linker were too slow to be of practical use for releasing alcohols. This may be for a number of reasons. Primary amines are substantially better nucleophiles than secondary amines, so the cleavage rate could be improved by using glycine instead of proline. The linker model itself is a relatively hydrophobic molecule and not very soluble in aqueous buffers, so this lack of solubility combined with the lack of solubility of benzyl alcohol, the released substrate, may have contributed to the apparently slow reaction rate. The reaction is promoted by base and pH 7.5 buffer is only mildly basic. Thus it can be concluded that this linker system will release alcohols but not amines. This outcome means that it is possible that the linker could be used for releasing hydroxamic acids attached via the alcohol from the solid phase.

4.6] Conclusions

A safety-catch linker has been synthesised and its ability to release of alcohols into solution demonstrated. It has also been proven by studying a solution model that this system would not be useful for the release of amines into solution from the solid phase.

Chapter Five Trimethyl lock based linker

5.1] Introduction

5.1.1] Trimethyl lock facilitated lactonisation

Cohen[133] first reported the phenomenon of *trimethyl lock facilitated lactonisation* when he studied of the rate of lactonisation of several hydroxyhydrocinnamic acid derivatives. He reported that the derivative with three methyl groups (**[B]** *figure 5.1*) lactonises 10^{11} times faster than the derivative with no methyl groups (**[A]** *figure 5.1*).

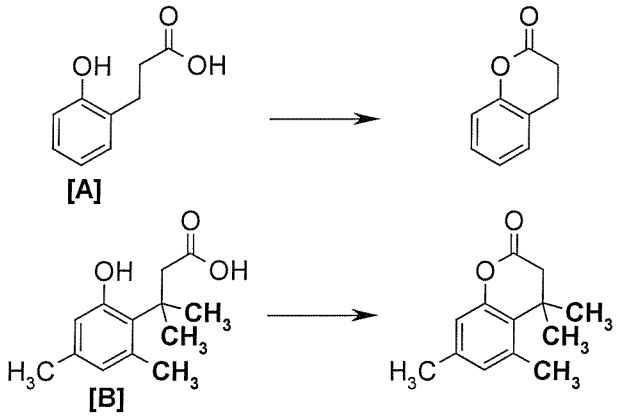


Figure 5.1: Trimethyl lock facilitated lactonisation (the methyl groups involved in the trimethyl lock are shown in bold)

Comprehensive structural analysis suggested that the increase in rate was due to the interaction of the three methyl groups, resulting in the considerable increase in the population of a conformer highly favourable to the formation of the tetrahedral intermediate.[134] Any restriction on rotational freedom, which serves to narrow the distribution of conformational populations, ideally by eliminating non-productive conformers, is known as *stereopopulation control.* Crystal structures of the related quinones have determined that the trimethyl lock forces the side chain to fold back putting the side chain carbonyl carbon in close proximity to the quinone carbonyl oxygen.[135] The relative directionality between these two atoms also favours the reaction, similar to what has been termed *near-attacking conformation.*[136] Due to its short half-life (100 secs) in aqueous solution, it has been used in the preparation of redox,[137] esterase[138] and phosphatase[139] sensitive prodrugs. The trimethyl lock has

also recently been applied to the design of a redox sensitive protecting group for amines.[140]

Wang[141] reported a redox sensitive linker, which utilises the trimethyl lock. C-terminally modified peptides were synthesised using this linker immobilised on polystyrene resin, and cleaved in good yields and purity. The reductive cleavage conditions ($Na_2S_2O_4$) are relatively mild (*figure 5.2*).

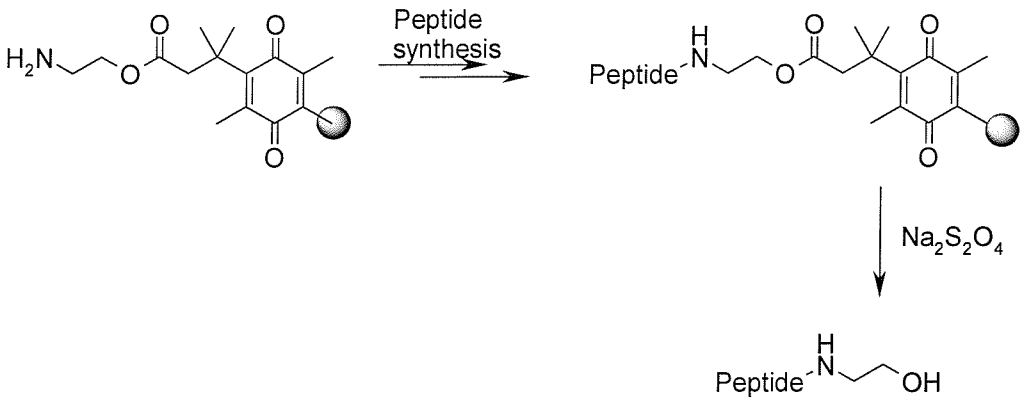


Figure 5.2: Trimethyl lock based redox linker

5.1.2] Trimethyl lock based linker

A safety-catch linker **[76]** was designed using both the previously documented diketopiperazine formation and trimethyl lock facilitated lactonisation to instigate the cleavage of alcohols, amines or hydroxamic acids from the solid phase into aqueous buffered conditions. The cleavage mechanism was envisaged to be comparable to the cyclisation linker **[33]** and the diketopiperazine linker **[57]** (*figure 5.3*). The acid *activation* step would cause the deprotection of the Boc group from **[77]** producing salt **[78]**. Subsequent addition to pH 7.5 buffer (the *cleavage* step) would deprotonate the amine and promote diketopiperazine formation, releasing anion **[80]** which is primed to undergo trimethyl lock facilitated lactonisation releasing the desired compound in addition to lactone **[81]**. The utility of the linker **[76]** was examined by the synthesis and analysis of models in solution and on the solid phase.

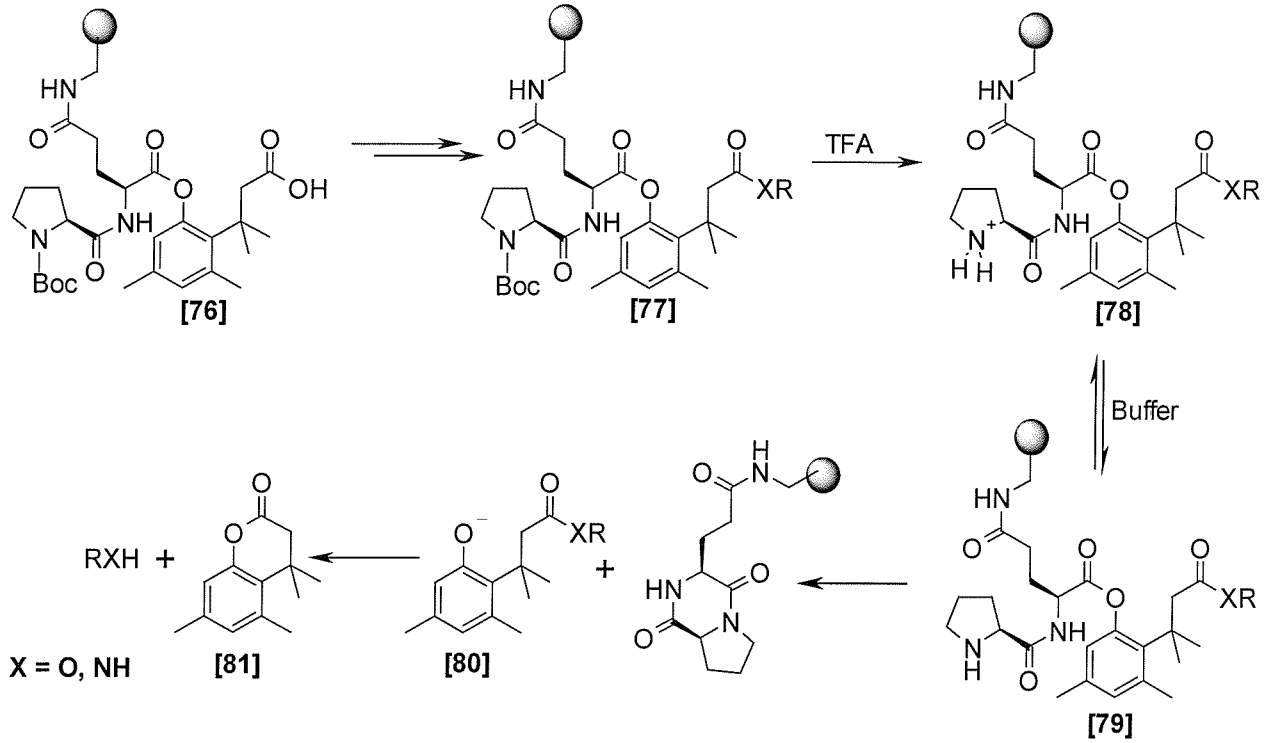


Figure 5.3: Proposed mechanism of the trimethyl lock safety-catch linker.

5.2] Synthesis of the trimethyl lock linker

The synthesis of the protected phenol **[83]** was based on literature precedent.[142] Reacting commercially available 3,5 dimethylphenol with methyl 3,3 dimethylacrylate in acidic conditions produced lactone **[81]**. Reduction of this lactone followed by selective protection of the primary alcohol generated the phenol **[83]** (*figure 5.4*).

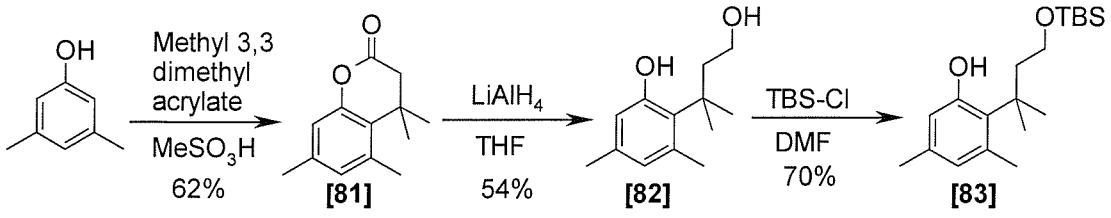


Figure 5.4: Synthesis of linker intermediate **[83].**

Coupling this phenol to Boc-Pro-Glu-(OAllyl)-OH **[63]** afforded the intermediate **[84]** (*figure 5.5*), however the reaction proceeded with a low yield of 30%. Although this is

not surprising considering the steric bulk of the reactants and the relatively low nucleophilicity of the phenol, it was decided to ascertain if other coupling reagents could improve this yield.

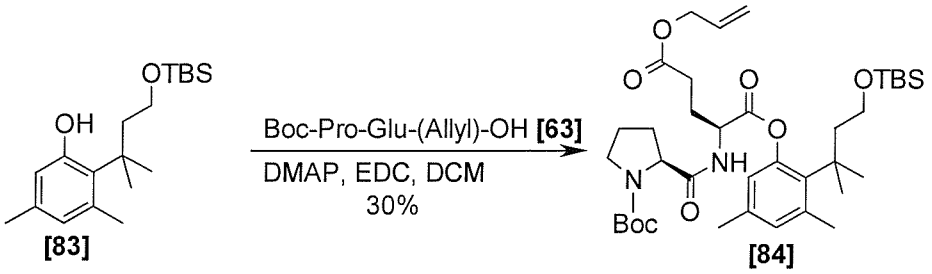


Figure 5.5: Synthesis of intermediate **[84]**.

Using DCC instead of the EDC had no effect on the yield. PyBOP, a popular coupling reagent that has superseded BOP in its comparable efficiency without the production of the carcinogenic HMPA, was also used with DMAP and the yield in this case was 25%. Thus it was decided to accept this low yield, since un-reacted starting materials were easily re-isolated and recycled. The deprotection of the alcohol was initially attempted using AcOH in THF/H_2O and resulted in the isolation of two products. It has been reported that compounds similar to **[85]** containing the trimethyl lock can be susceptible to rearrangement to give the free phenol[135] **[86]** (*figure 5.6*).

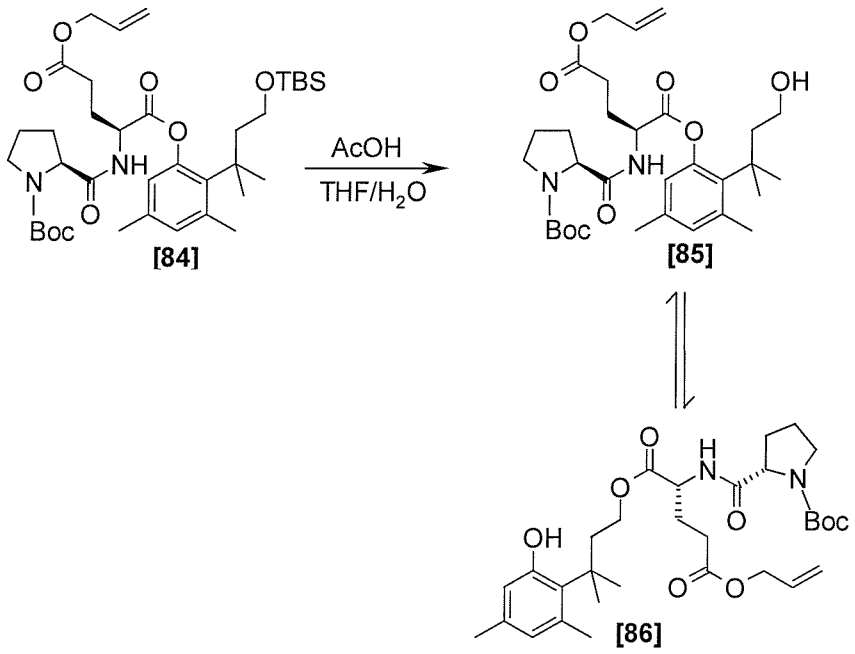


Figure 5.6: Rearrangement reaction producing side product **[86]**.

MS and NMR analysis provided evidence to support this hypothesis. To reduce the probability of this rearrangement occuring, which is undoubtly acid catalysed other methods of deprotection were investigated. TBAF (tetrabutylammonium fluoride)[143] often the method of choice, proved ineffective giving the phenol almost exclusively. PPTS (pyridinium p-toluene sulphonate)[144] a reagent used primarily for the selective deprotection of tetrabutyldimethylsilyl protection in the presence of other types of silicon protection, proved to be the optimal reagent giving the alcohol exclusively in good yield (*figure 5.7*).

The selection of oxidising agents, which were available to perform the subsequent oxidation to the carboxylic acid were limited. The oxidant needed to be active in the absence of acid and base and to oxidise a relatively hindered alcohol selectively in the presence of a double bond. Both PCC (pyridinium chlorochromate)[145] and Dess Martin periodinane[146] were used successfully to oxidise the alcohol to the aldehyde. The periodinane was vastly superior giving the aldehyde in better than 92% yield.

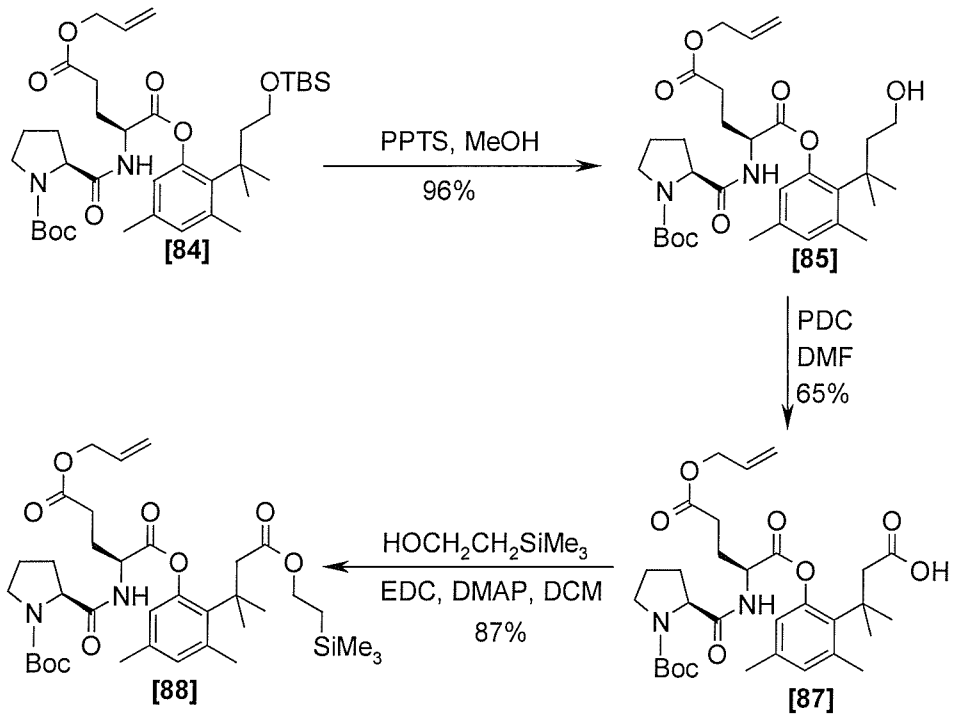


Figure 5.7: Synthesis of linker intermediate **[88]**.

After examining other oxidants AgO and $RuCl_3$ /$NaIO_4$,[147] it was discovered that pyridinium dichromate[148] (PDC) would successfully oxidise the alcohol **[85]** and the intermediate aldehyde to the desired acid **[87]**. The acid was subsequently protected

using 2-trimethylsilanyl-ethanol under standard esterification conditions to give **[88]** (*figure 5.7*).

The γ carboxylic acid was deprotected using $Pd(PPh_3)_4$ in good yield to give **[89]** and then coupled to aminoethyl TentaGel using DIC and HOBt. This reaction was monitored by the qualitative ninhydrin test. The silyl protection was removed using TBAF to give linker **[76]** and the success of this reaction was confirmed using the qualitative BCG test (*figure 5.8*).

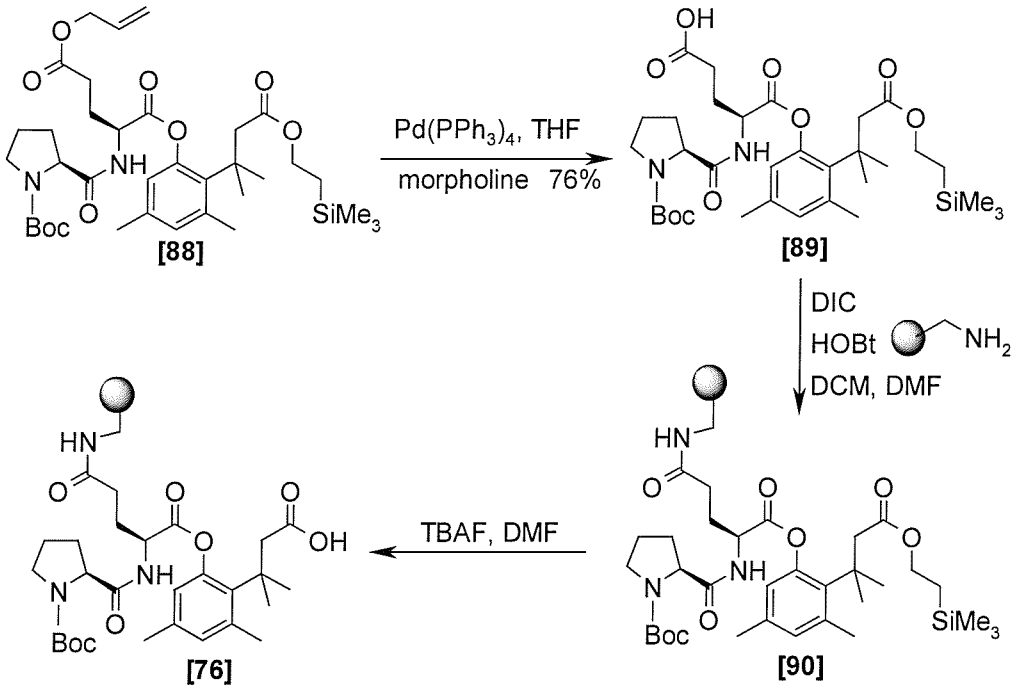


Figure 5.8: Synthesis of linker **[76]**.

5.3] Evaluation of linker models in solution.

5.3.1] Synthesis of the linker models

To evaluate the utility of the linker, ester and amide models were synthesised by reacting acid **[87]** with benzyl alcohol and benzylamine respectively under standard conditions (*figure 5.9*).

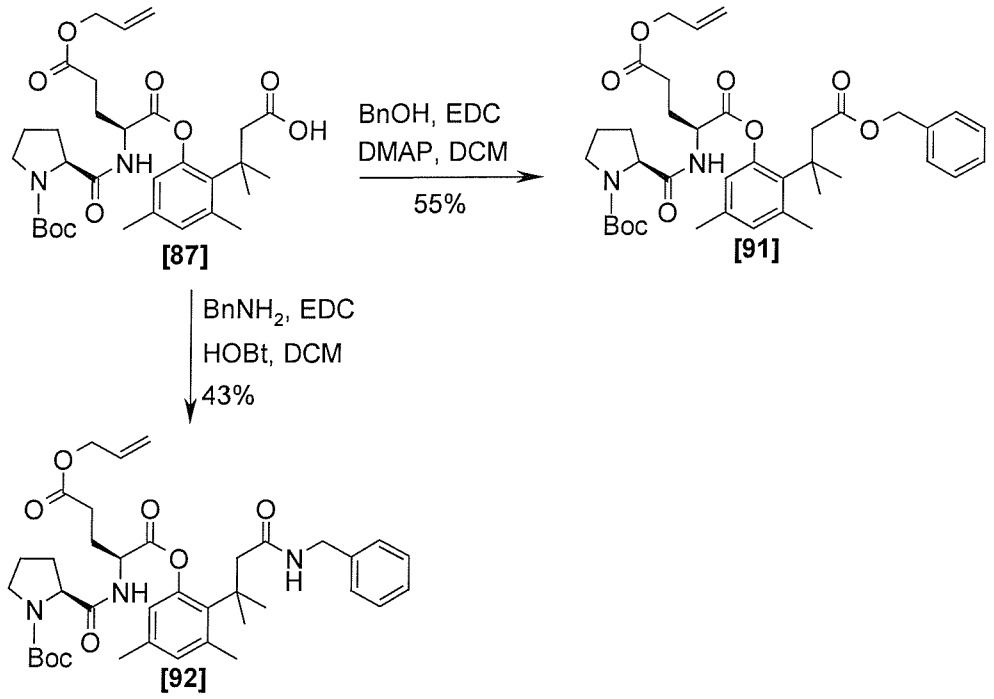


Figure 5.9: Synthesis of linker models **[91]** *and* **[92].**

The cleavage kinetics of this linker were studied using these model compounds. Exposing **[91]** and **[92]** to acid removes the Boc group and releases the safety-catch to generate amine salts **[93]** and **[94]**. Exposing these compounds to pH 7.5, 50 mM phosphate buffer promotes diketopiperazine formation and subsequent lactonisation and release of the model substrate (*figure 5.10*).

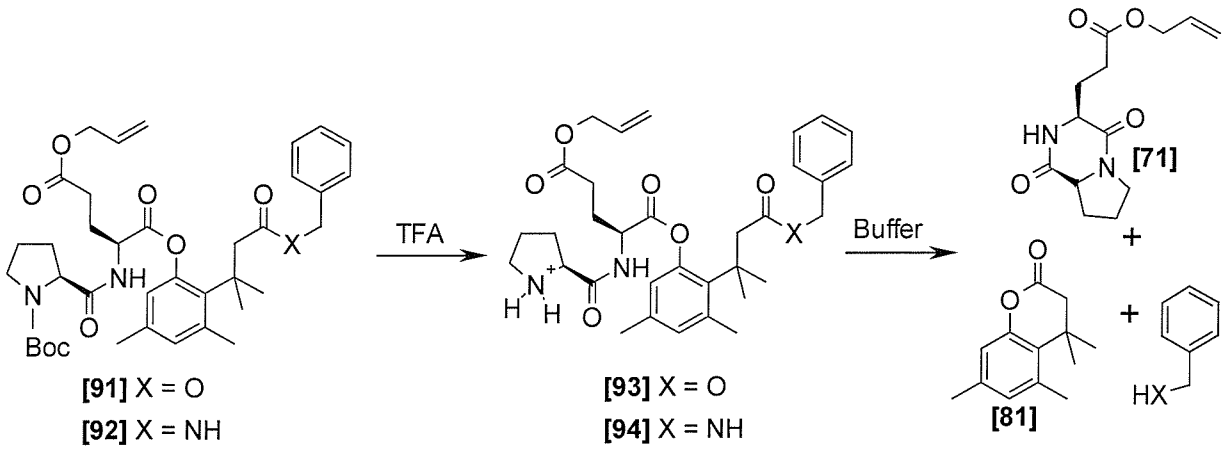


Figure 5.10: Analysis of solution phase models.

5.3.2] Analysis using RP-HPLC

The kinetics of cleavage were studied as described in chapter three.

5.3.2.1] Analysis of benzyl ester model **[91]**.

Treatment of the protected benzyl ester **[91]** with TFA gave the expected amine **[93]** as the exclusive product, which appeared to be stable in neutral and acidic conditions. This product was fully characterised. Treatment with buffer (50 mM phosphate, pH 7.5) promotes diketopiperazine formation, which releases an anion prone to trimethyl lock facilitated lactonisation. RP-HPLC analysis showed the presence of four new compounds. Benzyl alcohol and lactone **[81]** were unambiguously identified by comparing to standards (*figure 5.11*). The other two peaks were identified as the two diastereomers of the expected diketopiperazine, by comparison with the results from the cleavage of the analogous diketopiperazine linker model **[69]**.

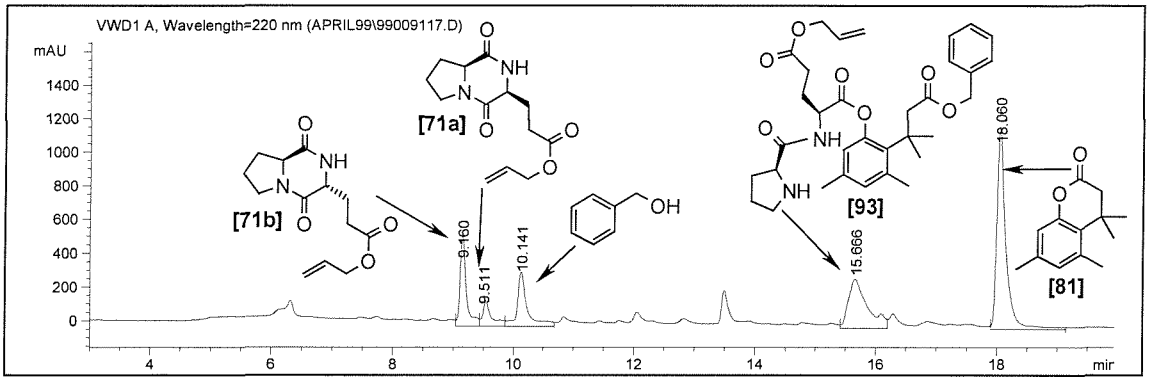
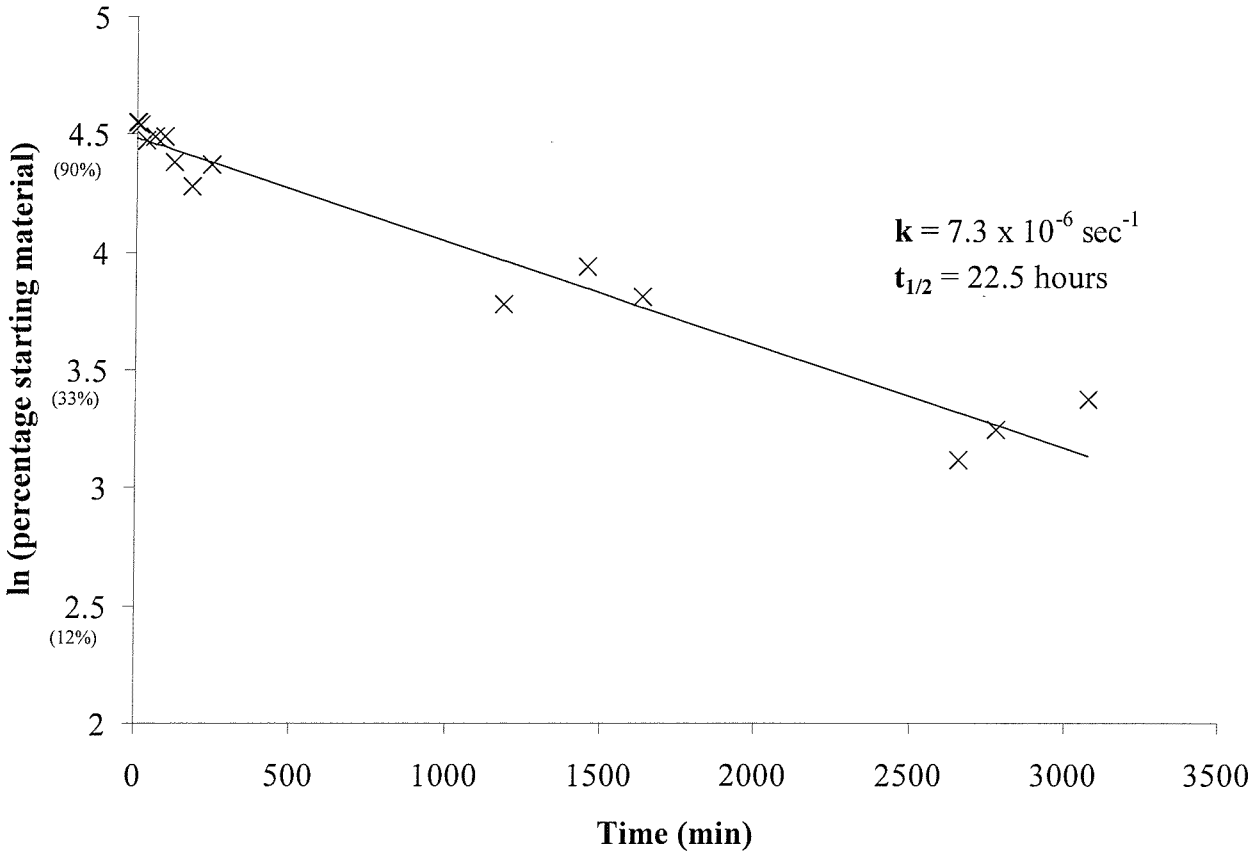


Figure 5.11: HPLC trace depicting reaction composition after 50 hours.

The cleavage of the benzyl ester model adheres to first order kinetics (*graph 5.1*).



Graph 5.1: Fragmentation of benzyl ester model in pH 7.5 phosphate buffer

5.3.2.2] Analysis of benzyl amide model **[92]**.

Treatment of the protected benzyl amide **[92]** with TFA gave the expected amine **[94]** as the exclusive product, which appeared to be stable in neutral and acidic conditions. This product was characterised by RP-HPLC and MS. Treatment with buffer (pH 7.5, phosphate) and periodic analysis by RP-HPLC showed that the linker model was decomposing into the expected fragments (*figure 5.12*).

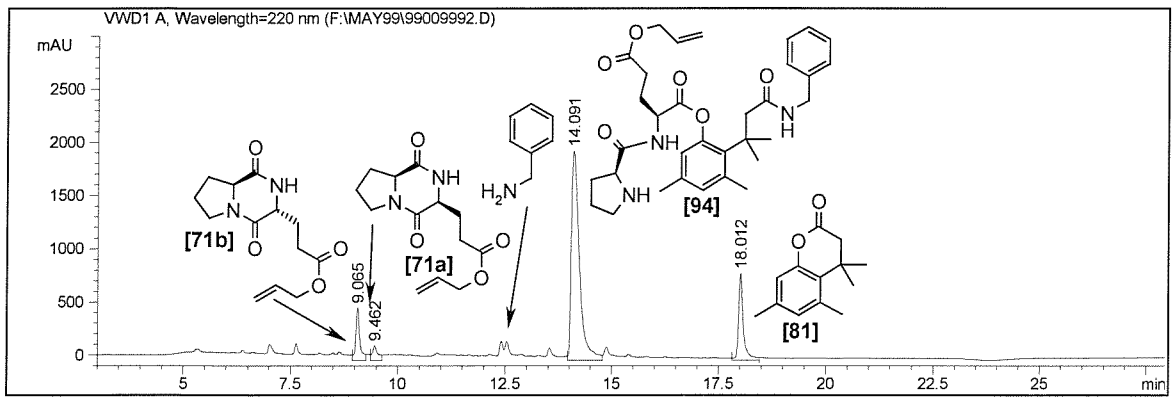
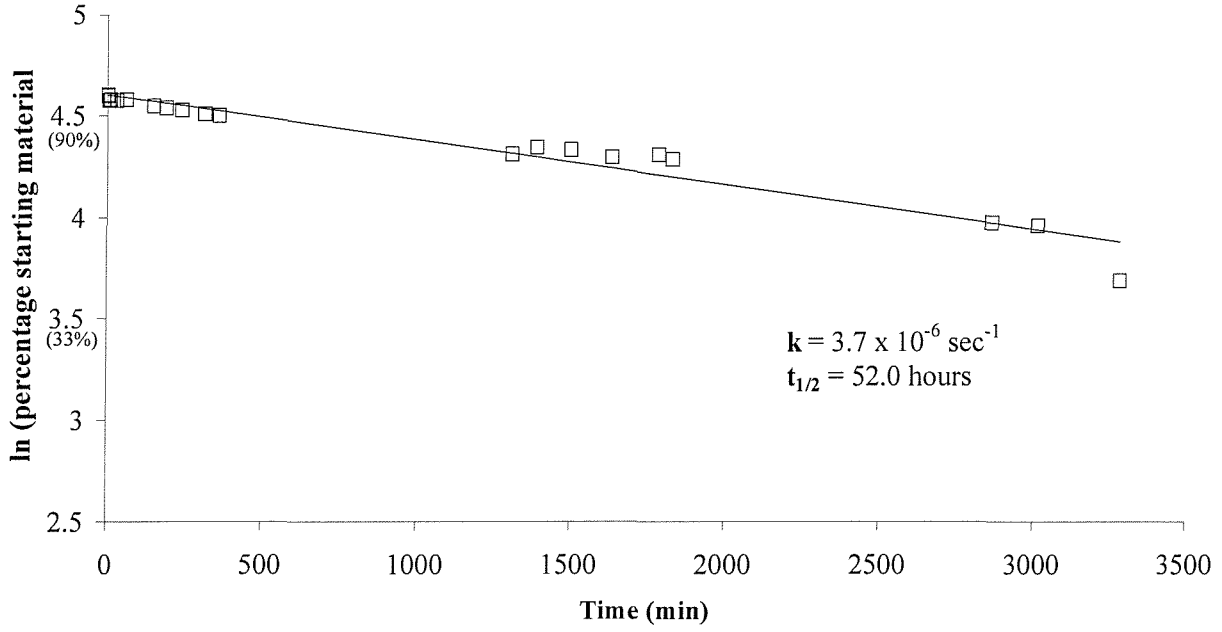


Figure 5.12: HPLC trace depicting reaction composition after 50 hours

The fragmentation of the benzyl amide model adheres to first order kinetics (*graph 5.2*).



Graph 5.2: Fragmentation of the benzyl amide model in pH 7.5 phosphate buffer.

5.4] Comparison of the cleavage rates.

Studying the rate constants demonstrated that alcohols were released twice as fast as amines (*table 5.1*).

Model Type	Substrate	k (sec^{-1})	$t_{1/2}$ (hours)
Ester	Benzyl alcohol	7.3×10^{-6}	22.0
Amide	Benzylamine	3.7×10^{-6}	52.0

Table 5.1: Rate constants and half lives of the solution models.

This result means that potentially this linker could be used for the release of hydroxamic acids from either the associated alcohol or amine. The release of hydroxamic acids from the amine would be more desirable as N-acylhydroxamates are more stable than O-acylhydroxamates, and thus the derived linker would be more versatile.

5.5] Comparison of the cleavage rates of the linkers in solution.

It was evident from the analysis of the solution models that the cyclisation linker releases alcohols the most rapidly, and the diketopiperazine linker will release alcohols at the slowest rate. The diketopiperazine linker does not release amines at a detectable rate, even after extended reaction times. Both the trimethyl lock linker and the cyclisation linker will release amines, but it is likely that only the trimethyl lock linker does so at a rate that is fast enough to be practically useful. These relationships can be quantified by studying the rate constants, and related half lives (*table 5.2*).

Linker	Model type	Substrate	k (sec^{-1})	$t_{1/2}$ (hours)
Cyclisation	Ester	Benzyl alcohol	1.9×10^{-4}	1.0
	Amide	Benzylamine	5.7×10^{-7}	337.0
Diketopiperazine	Ester	Benzyl alcohol	3.0×10^{-6}	60.0
	Amide	Benzylamine	No cleavage	No cleavage
Trimethyl lock	Ester	Benzyl alcohol	7.3×10^{-6}	22.5
	Amide	Benzylamine	3.7×10^{-6}	52.0

Table 5.2: Comparison of the rate constants and half lives of the solution models of all the linkers described.

Analysing the solution models of these linkers showed that the trimethyl lock linker released benzyl alcohol at over twice the speed of the analogous solution model of the diketopiperazine linker. Since the same cyclisation is the first and only step in the trimethyl lock and diketopiperazine linker respectively, the reasons for the difference in rate constants must be considered. The most likely reason is that more MeCN was added to the buffer on analysis of the trimethyl lock linker than the comparable analysis of the diketopiperazine linker. The reason for this difference was the anticipated lack of solubility of the released lactone **[81]**, and the benzyl ester model **[93]**, in aqueous buffer. The trimethyl lock linker however did release amines, albeit slowly and this result was not witnessed using the solution model of the

diketopiperazine linker. This result can be rationalised because trimethyl lock facilitated lactonisation is a greater driving force than diketopiperazine formation.

5.6] Conclusions

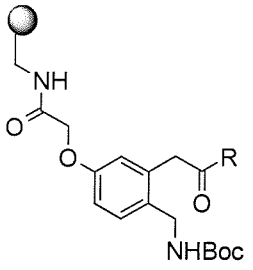
A novel safety-catch linker based on diketopiperazine formation and trimethyl lock facilitated lactonisation, has been synthesised and its ability to release alcohols and amines into solution was demonstrated by analysing models in solution

Chapter Six Evaluation of the linkers on the solid phase

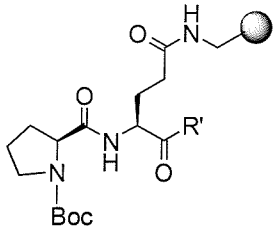
6.1] Synthesis of the solid phase models

Following the demonstration that the linkers were effective at liberating alcohols in solution, it was important to demonstrate that this cleavage could be performed on the solid phase.

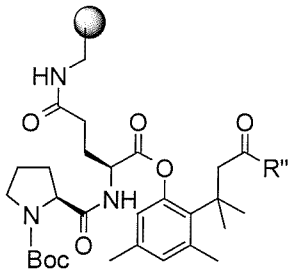
Models were synthesised by esterifying two dyes, disperse red and NPM ([(2*S*)-1-(nitrophenyl)pyrrolidin-2-yl]methan-1-ol) onto the resin bound linkers using DIC and HOBt (*figure 6.1*).



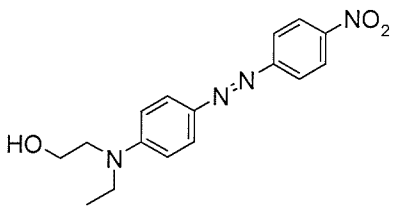
[33] R = OH
[95] R = Disperse red
[96] R = NPM



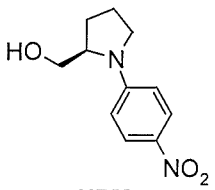
[57] R' = OH
[97] R' = Disperse red
[98] R' = NPM



[76] R" = OH
[99] R" = Disperse red
[100] R" = NPM



Disperse red

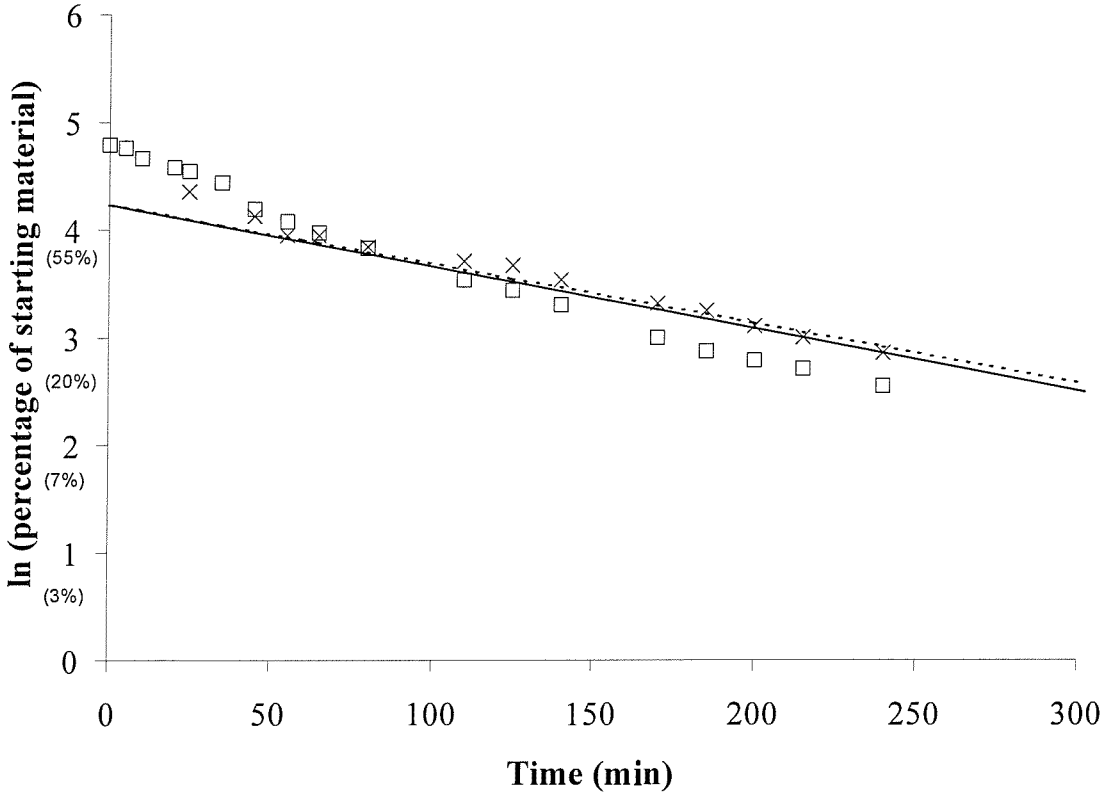


NPM

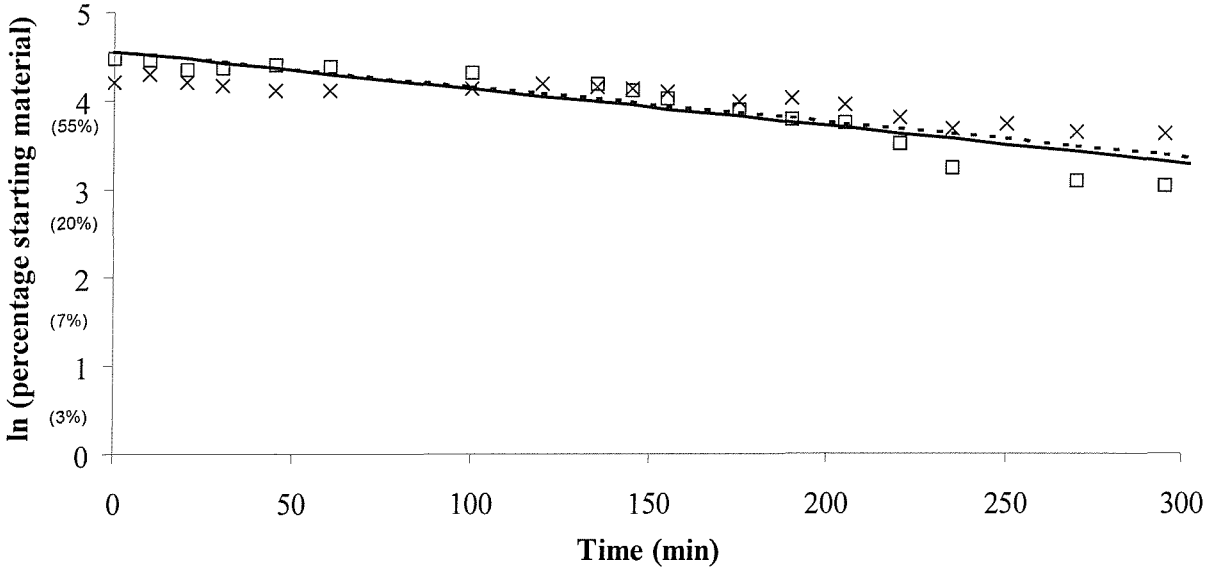
Figure 6.1: Synthesis of solid phase models.

6.2] Analysis of solid phase models

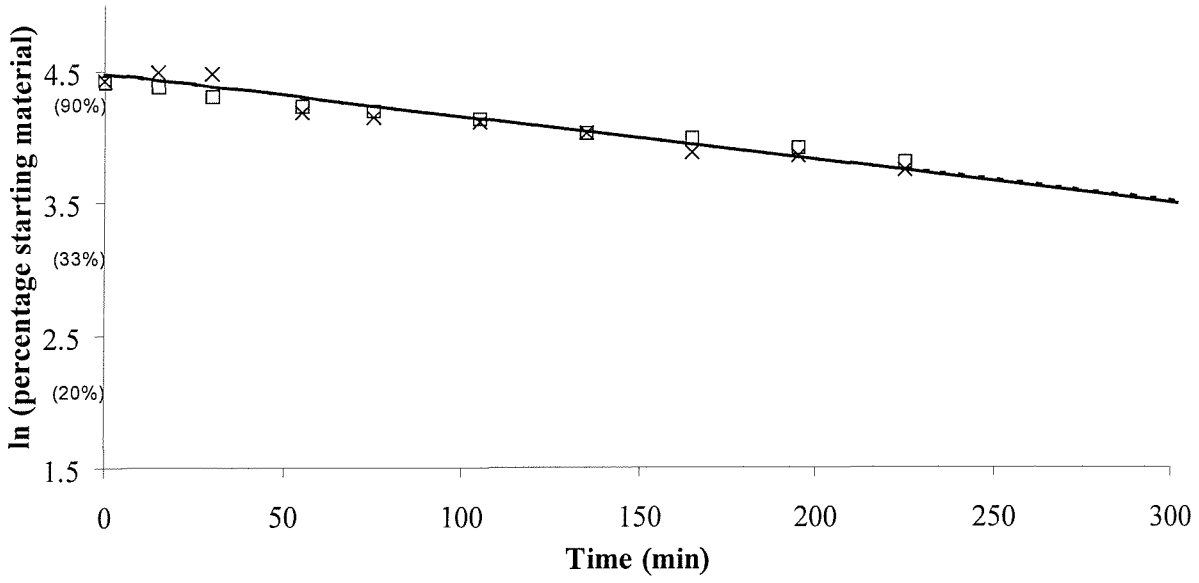
The loaded linkers ([95]-[100]) were exposed to 50% TFA to remove the safety-catch. After thorough washing and drying the resin was added to buffer (pH 7.5, 50mM, phosphate) and the release of the dyes was monitored by UV spectroscopy in real time. The release of the dyes followed first order reaction kinetics and is depicted in graphs 6.1-6.3.



Graph 6.1: Fragmentation of solid phase models of the cyclisation linker **[95]** *and* **[96]** *in pH 7.5 phosphate buffer.* **[Disperse red: X; ---- : NPM: □, ——]**



Graph 6.2: Fragmentation of solid phase models of the diketopiperazine linker **[97]** *and* **[98]** *in pH 7.5 phosphate buffer.* **[Disperse red: X; ---- : NPM: □, ——]**



Graph 6.3: Fragmentation of solid phase models of the trimethyl lock linker **[99]** *and* **[100]** *in pH 7.5 phosphate buffer.* **[Disperse red: X; ---- : NPM: □, ——]**

6.2.1] Comparison of the rates of cleavage of the dyes from the solid phase

The rate constants and corresponding half lives were calculated (*table 6.1*)

Linker	Dye	k (sec^{-1})	$t_{1/2}$ (hours)
Cyclisation	Disperse red	9.2×10^{-5}	2.1
	NPM	9.6×10^{-5}	2.0
Diketopiperazine	Disperse red	6.7×10^{-5}	2.5
	NPM	7.0×10^{-5}	2.5
Trimethyl lock	Disperse red	5.4×10^{-5}	2.9
	NPM	5.5×10^{-5}	2.9

Table 6.1: Rate constants and half lives of the solid phase models.

The rate constants calculated for the release of these two substrates were remarkably similar for each linker confirming that differing substrates could be released from each linker with comparable rates (*table 6.1*). Disperse red and NPM have similar hydrophobic character and thus it was expected that the release of these compounds into an aqueous assay would occur at similar rates.

If diketopiperazine formation is a slower step than trimethyl lock facilitated lactonisation then it would be the rate-determining step of the cleavage of alcohols from the trimethyl lock linker in addition to the diketopiperazine linker. The rate constants calculated for the release of these dyes from the resin are very similar for the trimethyl lock linker and the diketopiperazine linker. This result therefore suggests that diketopiperazine formation is indeed the rate-determining step in the cleavage of the trimethyl lock linker.

The purity of the compounds released from the solid phase for all the linkers was >99% as demonstrated by RP-HPLC analysis. (Representative samples are shown in *figure 6.2* and *figure 6.3*).

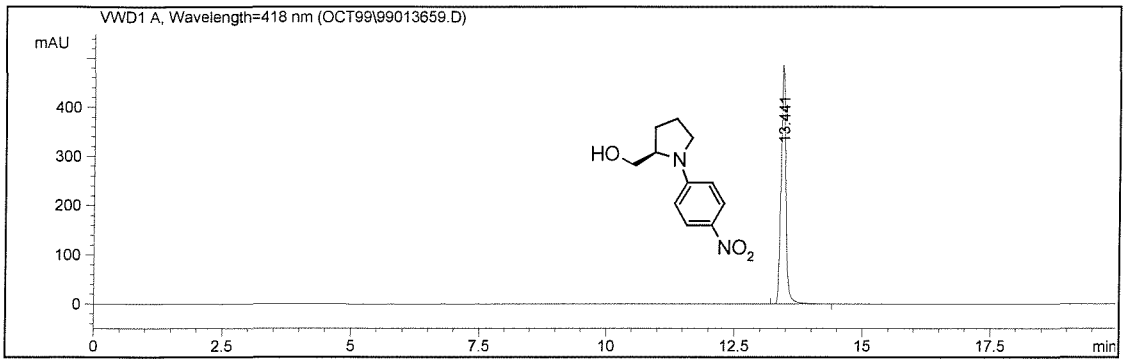


Figure 6.2: NPM cleaved from solid phase model **[96]**.

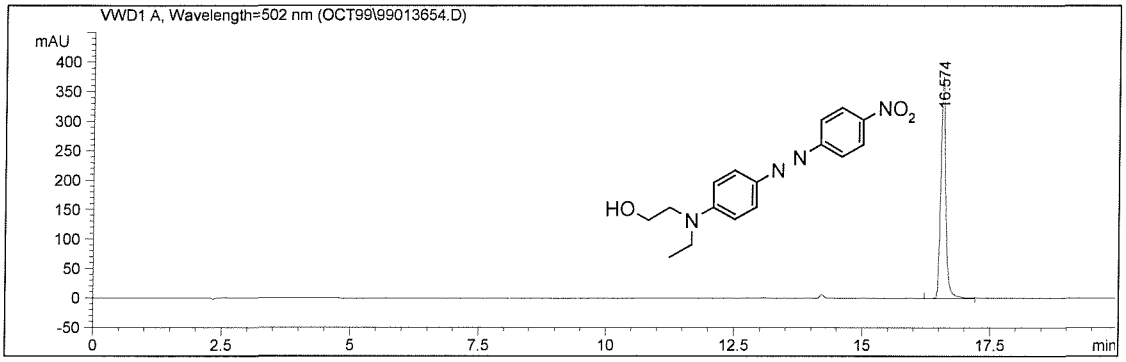


Figure 6.3: Disperse red cleaved from solid phase model **[99]**.

6.2.2] Comparison of the cleavage rates of the solution and solid phase models.

The rate constants calculated for the cleavage of alcohols from the linkers differed dramatically depending on whether they were calculated using the solution or solid phase models (*table 6.2).*

A difference in the rate constants calculated for these reactions in solution and on the solid phase was not unexpected because the substrates released are not the same and therefore the results are not directly comparable. Additionally, the rate constant for the reaction was not expected to be independent of the substrate released. However, the variation of $t_{1/2}$ = 60 hours (solution) and $t_{1/2}$ = 2.5 (solid phase) for the diketopiperazine linker was unexpectedly extreme.

Linker	Model type	Substrate	k (sec^{-1})	$t_{1/2}$ (hours)
Cyclisation	Solution	Benzyl alcohol	1.9×10^{-4}	1.0
	Solid phase	Disperse red	9.2×10^{-5}	2.1
	Solid phase	NPM	9.6×10^{-5}	2.0
Diketopiperazine	Solution	Benzyl alcohol	3.0×10^{-6}	60.0
	Solid phase	Disperse red	6.7×10^{-5}	2.5
	Solid phase	NPM	7.0×10^{-5}	2.5
Trimethyl lock	Solution	Benzyl alcohol	7.3×10^{-6}	22.5
	Solid phase	Disperse red	5.4×10^{-5}	2.9
	Solid phase	NPM	5.5×10^{-5}	2.9

Table 6.2: Comparison of the rate constants and half lives for solution and solid phase models

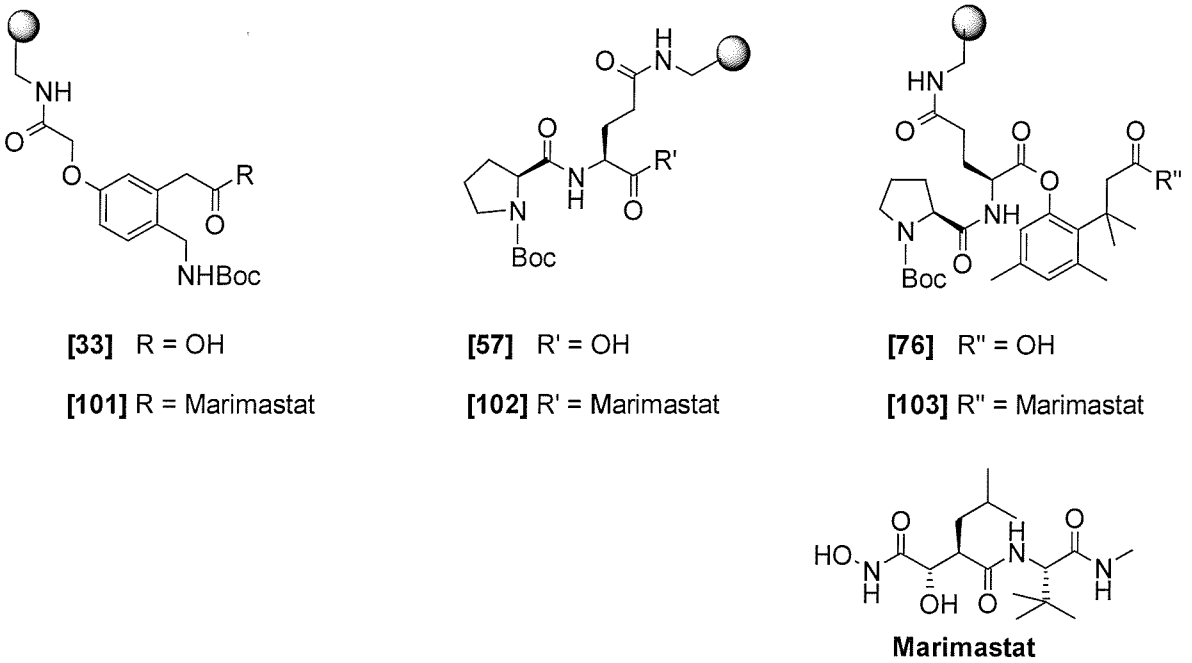
The leaving group ability of the alcohols would effect the rate constant for the reaction. In addition, the presence, or absence of the resin particle would have an effect on the rate constant for the reaction. Czarnik[149] suggested that the effect of the polymer matrix on reaction rate may be similar to the effect of solvent on the rate of a solution reaction. The presence of the polymer matrix and any associated spacer will pose important effects on the diffusion of incoming reagents and reactants depending on their molecular properties, such as polarity. Although it is generally considered that reactions have more solution-like properties when performed on TentaGel, as opposed to polystyrene, due to the long flexible PEG spacers distancing the reaction from the polystyrene core. Another factor to consider was the slight differences in the protocols of the two types of experiments. A different proportion of MeCN was used in the solid phase experiments. This solvent aids the solvation of organic compounds in the aqueous buffer and it was very important that any compound released from the resin was completely solvated for accurate results. This would have the effect of increasing the rate of release slightly because of the increased solvation of organic compound.

The magnitude of the difference between the solid phase and solution experiments for the diketopiperazine and trimethyl lock linkers was rather surprising. The reason for this discrepancy was likely to be due to solubility issues. The solution models **[69]** and **[93]**

were not very soluble in aqueous media, and although a proportion of MeCN was added to aid solubility, it was still apparent that complete dissolution was not obtained. This could lead to the reaction measured in solution to appear to progress at a slower rate, as starting material, which was undissolved was measured, in addition to the soluble componants of the reaction mixture. The solid phase models were completely solvated, thus the rate constants calculated from these reactions were likely to be more accurate.

6.3] Evaluation of the linkers in the zone diffusion assay

It has been demonstrated that the linkers will release alcohols from the solid phase. It now had to be determined if they would release hydroxamic acids. To evaluate this marimastat was attached to the solid phase via the linkers by reacting them with marimastat, DIC, HOBt and DMAP. *(figure 6.3).*



[33] R = OH

[101] R = Marimastat

[57] R' = OH

[102] R' = Marimastat

[76] R" = OH

[103] R" = Marimastat

Marimastat

Figure 6.3: Marimastat models of the linkers.

It was documented that in the presence of carbodiimide reagents *O*-acyl hydroxamic acids will undergo the Lossen rearrangement[150] generating isocyanates. To avoid this eventuality DIC, HOBt and DMAP were added to the acid resin and allowed to react before the addition of marimastat. In addition 5 equivalents of marimastat were used as opposed to 2 equivalents of DIC.

The marimastat models **[101]**, **[102]** and **[103]** were then exposed to TFA followed by NEt_3 to ascertain whether this system could be used to determine the utility of the zone diffusion assays.

Disappointingly, it was believed that marimastat was released upon exposure to acid (50% TFA/DCM for 10 minutes) determined by RP-HPLC analysis. Exposure to milder acidic conditions (5% TFA/DCM for five minutes) also released marimastat from the resin. This observation can be attributed to the fact that this *O*-acylhydroxamate is more activated than in the ester models, and thus more labile to hydrolysis. In addition hydroxamic acids are better leaving groups than alcohols, which explains the increased reactivity of *O*-acylhydroxamates compared to esters. This eventually could possibly be prevented by investigating other methods of deprotecting Boc groups, or investigating other protection strategies. It can be deduced however that these safety catch linkers, without modification, cannot be used to release hydroxamic acids via the alcohol.

6.4] Conclusions

Three successful safety-catch linkers were synthesised and their ability to release alcohols into solution was demonstrated using both solution and solid phase models. The ability to release amines was demonstrated using solution models for the trimethyl lock and cyclisation linker, although more work would be required to realise this observation into something of practical use. Unfortunately, none of the three linkers were demonstrated to be useful for the release of hydroxamic acids from the solid phase and so the utility of these linkers in the zone diffusion assay could not to be determined. However with further work the ability to release hydroxamic acids from the trimethyl lock linker via the associated amine could be realised as a useful development in the solid phase synthesis of hydroxamic acids.

Chapter Seven Studies towards the development of protease based zone diffusion assays

7.1] Introduction

Zone diffusion assays have been used to find compounds with antibacterial action. The safety-catch linkers previously described were designed to study the feasibility of zone diffusion assays for the discovery of new enzyme inhibitors. To determine the feasibility of this assay, studies were performed using gelatinase A (MMP-2, a typical matrix metalloprotease enzyme),[86] gelatin (the enzyme substrate) and marimastat (a known inhibitor developed by British Biotech). In recent years there has been much interest in this class of enzyme because of the evidence of their involvement in the pathology of a number of diseases including cancer.[104]

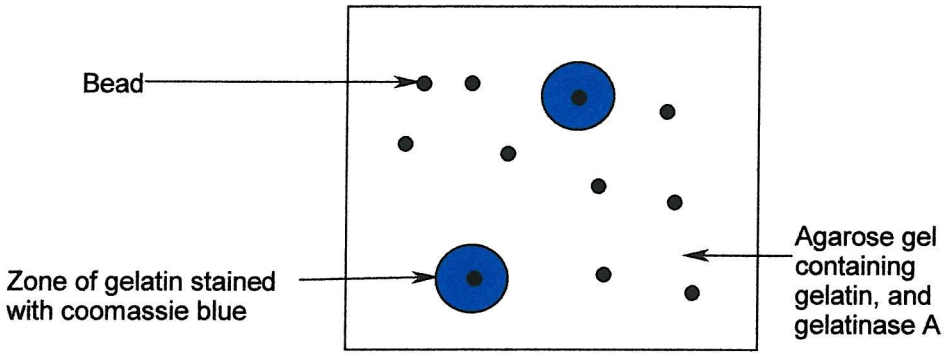


Figure 7.1: Zone diffusion assay

The aim was to develop these assays so that potential inhibitors of gelatinase, could be synthesised using one of the safety-catch linkers described. On completion of the synthesis the beads could be exposed to acid (to activate the linker) and then added to buffered molten agarose gel containing gelatin and gelatinase A. It was hoped that on exposure to the mildly basic buffered conditions the linker would release the compounds attached slowly, generating a **zone** around the parent bead. An inhibitor of gelatinase would prevent the gelatin surrounding the parent bead being degraded by enzyme action and this can be easily detected by staining the gel with coomassie blue[154] on completion of the assay (*figure 7.1*). Thus the beads from which inhibitors

originated can be identified. Many different compounds could be screened at one time and this would be a very useful high throughput screening technique. Any "hits" could be identified by a variety of documented techniques. The strategy that was proposed was to use the linkers described to synthesise hydroxamic acids on the solid phase. The inhibitor marimastat is a hydroxamic acid and it was envisaged that a library of hydroxamic acids could be synthesised including marimastat. If marimastat could be identified as an inhibitor from a library then this would provide evidence that this was a viable assay technique.

There are two possible strategies, which could be implemented (*figure 7.2*). Hydroxylamine could be attached to the carboxyl terminus of one of the linkers, either protected on the hydroxyl to give **[104]** (strategy **[B]**) or the amine to give **[105]** (strategy **[A]**). Synthesis could then be performed on the secondary amine. On completion of the synthesis the safety-catch activation and deprotection of the protecting groups could be performed in one step by treating with TFA. On exposure to buffer the linker would then cleave releasing the hydroxamic acid product **[106]** directly into the assay. The feasibilty of these two strategies was determined by the synthesis of benzyl ester and benzyl amide models of the diketopiperazine linker, trimethyl lock facilitated linker and the cyclisation linker as described in previous chapters. The cleavage of amines from the diketopiperazine and cyclisation linker models was slow or negligible. The implication of this result was that releasing hydroxamic acids from these linkers would only be viable using strategy **[A]**. The trimethyl lock linker was shown to release amines, so the release of hydroxamic acids from this linker may be viable using strategy **[B]**.

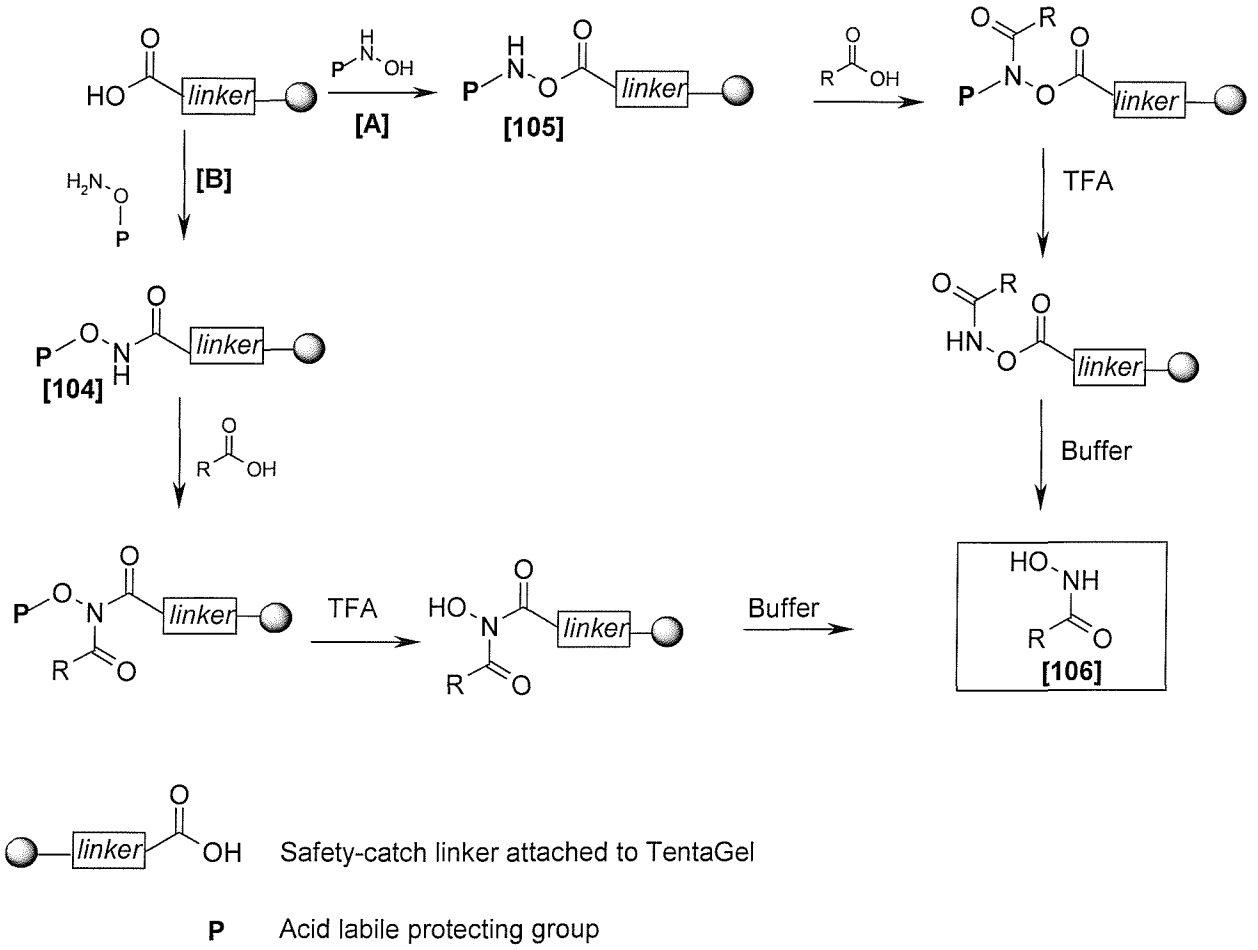


Figure 7.2: Strategies for the solid phase synthesis of hydroxamic acids.

7.2] Initial enzyme studies

7.2.1] Activation of Progelatinase A

Matrix metalloprotease enzymes are secreted as inactive proenzymes, which require activation. Exposure to mercuric compounds induces activation of these proenzymes *in vitro*. Human recombinant progelatinase A[151] was activated by incubating at 37°C for one hour with 0.1 M APMA (aminoparamercuric acetate).[152] The activated enzyme was diluted in 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.02% NaN_3 pH 7.5 buffer and stored in 200 μL aliquots each containing 2.5 μg/ mL of enzyme.

7.2.2] Gel Preparation

Agar gels were prepared by dissolving agarose in 100 mM Tris·HCl/ 10 mM NaCl/ 10 mM $CaCl_2$/ 0.02% NaN_3 pH 7.5 at 100°C. The molten agarose was allowed to cool to 60°C and gelatin and Brij 35 were added. The molten agarose was poured onto GelBond in a mini protean II.[153] The composition of the gels were 1% agarose, 0.1% gelatin, in 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.05% Brij 35/ 0.02% NaN_3 pH 7.5 buffer with dimensions of 82 mm x 82 mm x 2.25 mm (*figure 7.3*). Wells were created by piercing the gel with a Pasteur pipette.

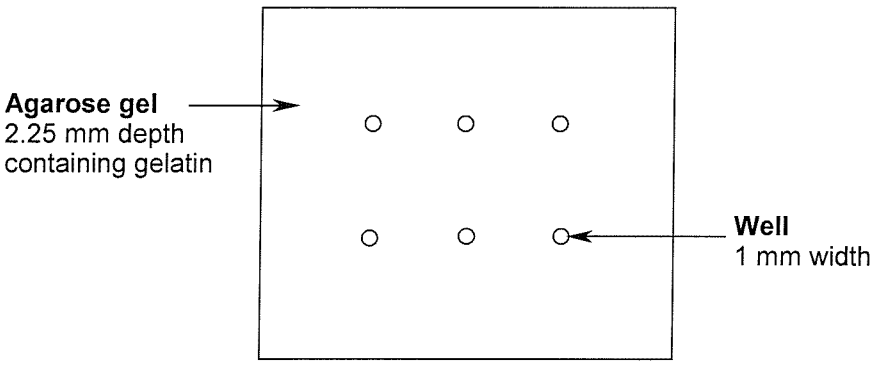


Figure 7.3: Agar Gel

7.2.3] Enzyme concentration

The initial enzyme assays were performed by adding gelatinase to the wells in the gel and allowing the enzyme to degrade the gelatin by incubating at 37°C for 15 hours. Following the incubation the gels were washed with copious amounts of water to remove inorganic salts and then dried. Staining with coomassie blue,[154] a non-specific protein dye, resulted in any remaining gelatin being stained blue. The enzyme was added to the wells in different concentrations to assess the activity of the enzyme in the gel described.

	Amount of enzyme (ng)	Concentration of enzyme (ng/ mL)	Diameter of zone (mm)
1	10	2500	6
2	1	250	0
3	0.1	25	0

Table 7.1: Comparison of three enzyme concentrations in the gel assay.

Three concentrations of enzyme were used (*table 7.1*). 4 μL of each enzyme concentration were added to separate wells in the gels described. The diameter of any clear zones were measured after staining with coomassie blue. The clear zones resulted from the enzymatic degradation of gelatin surrounding the well (*figure 7.4*). The optimum amount of enzyme determined for use in further assays was 10 ng, 149 fmol (4 μL of 2.5 μg/ mL solution). Over time the activity of the gelatinase decreases as it degrades itself, thus this assay was also used to monitor the activity of different batches of the enzyme and ensure that the activity of each was comparable.

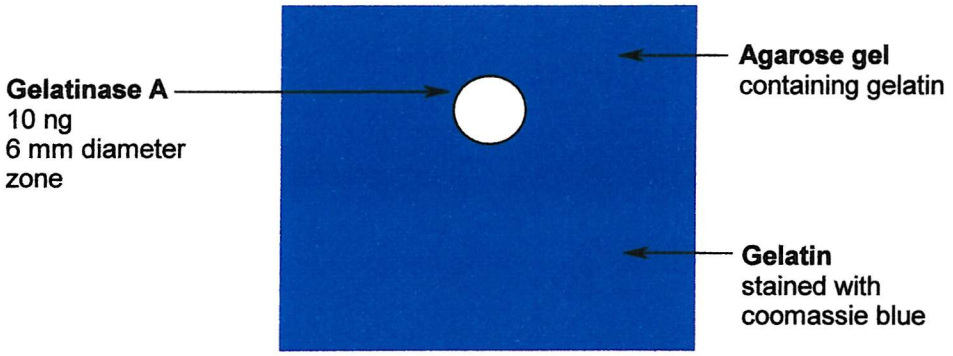


Figure 7.4: Evidence of enzyme activity identified in the gel assay.

7.3] Inhibition studies

7.3.1] Marimastat concentration

The enzyme activity was tested in the presence of marimastat, a known inhibitor of gelatinase A. Marimastat was dissolved in 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.05% Brij 35/ 0.02% NaN_3 pH 7.5 buffer to generate a stock solution of marimastat (2.5 μg/ mL). Lower concentration solutions of marimastat were generated by serial dilution. A mixture of enzyme (4 μL of 2.5 μg/ mL solution) and 4 μL of marimastat solution of variable concentrations were added to the wells in the gel. A control was included by adding the enzyme mixed with 4 μL of 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.05% Brij 35/ 0.02% NaN_3 pH 7.5 buffer. From the results (*table 7.2*) it can be seen that marimastat is an extremely potent inhibitor of gelatinase. No enzyme activity was detected until the amount of inhibitor was as low as 3 fmol. The effect of inhibitor is undetectable when the amount is 0.3 fmol.

	Amount of marimastat	**Concentration of marimastat**	**Diameter of zone (mm)**
Control	0	0	6
1	10 ng, 30 pmol	2.5 μg/ mL	0
2	1 ng, 3 pmol	250 ng/ mL	0
3	100 pg, 300 fmol	25 ng/ mL	0
4	10 pg, 30 fmol	2.5 ng/ mL	0
5	1 pg, 3 fmol	250 pg/ mL	3
6	100 fg, 0.3 fmol	25 pg/ mL	6

Table 7.2: Comparison of different marimastat concentrations in the gel assay.

7.3.2] Single bead studies

To test the principle of the assay an experiment was performed using PAMAM dendrimer functionalised aminomethyl polystyrene resin (0.72 mmol/ g).[155] These beads were swollen in a solution of marimastat dissolved in 100 mM Tris· HCl/ 100 mM NaCl/ 10 mM $CaCl_2$ /0.05% Brij 35/ 0.02% NaN_3 pH 7.5 buffer (2.5 μg/ mL) and then allowed to dry. Single beads were selected with the aid of a microscope. Standard agar gels were prepared and single beads added to each well in addition to the enzyme. The gels were incubated, washed, dried and stained as previously described. The size of each zone of gelatin degradation was measured on completion of the assay.

	Average diameter of zone (mm)	**Percentage of control**
Control	6	100
Single bead assay	4.4	73

Table 7.3: Detection of enzyme inhibition using a single bead screen.

From the results (*table 7.3*) it can be seen enough marimastat is adsorbed onto a bead to cause a significant reduction in the size of the zone generated by the enzyme degradation.

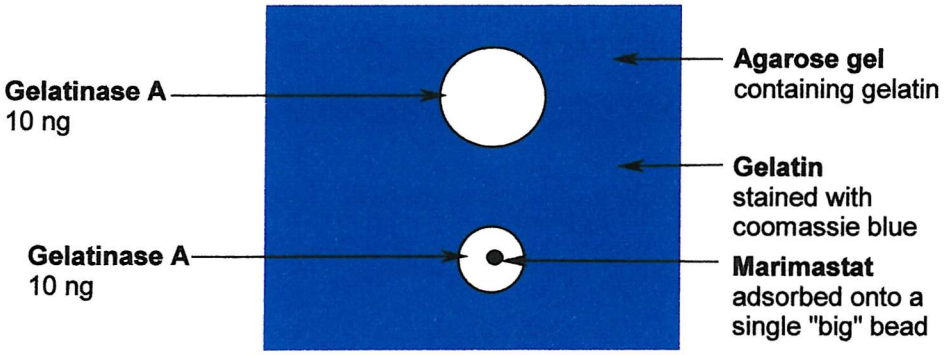


Figure 7.5: Inhibition of enzyme activity detected in the gel assay.

7.3.3] Gelatinase gels

The next variable to probe was the amount of enzyme required to visibly degrade all the gelatin in the gel. The molton agarose was prepared as before and then allowed to cool to 40°C and maintained at this temperature in an incubator. Differing concentrations of gelatinase were added to the molton agarose gel and cast as previously described. The gels were incubated, washed, dried and stained as before. The intensity of the blue colouration of the resulting gels was noted with the naked eye (*table 7.4*).

	Concentration of gelatinase (ng/ mL)	**Concentration of gelatinase (pmol/ mL)**	**Appearance of stained gel**
Control	0	0	Deep blue
1	160	2.4	Pale blue
2	240	3.6	Colourless
3	320	4.8	Colourless

Table 7.4: Optimisation of enzyme concentration in gelatinase gels.

From the concentrations of enzyme tested there are two that visibly degrade the gelatin in the gel, 3.6 pmol/ mL and 4.8 pmol/ mL.

7.3.4] Single bead studies with gelatinase gels

Gelatinase gels were prepared as described in the previous section and a single "big bead" with marimastat adsorbed onto its surface was placed on the gel. The gels were incubated, washed, dried and stained as before. The size of each zone of enzyme inhibition was measured on completion of the assay (*table 7.5*). It can be seen that if a higher concentration of enzyme is used then the zone generated by the inhibitor is smaller. This was expected, since a higher concentration of enzyme will be require a higher concentration of inhibitor to cause the same degree of inhibition

	Concentration of gelatinase ng/ mL	Concentration of gelatinase pmol/ mL	Zone of inhibition (mm)
Control 1	240	3.6	0
Control 2	320	4.8	0
Single bead assay 1	240	3.6	20
Single bead assay 2	320	4.8	18

Table 7.5: Detection of enzyme inhibition in gelatinase gels using a single bead screen

A scanned image of a genuine gel is shown in figure 7.6. The original position of the parent bead is indicated by the arrow.

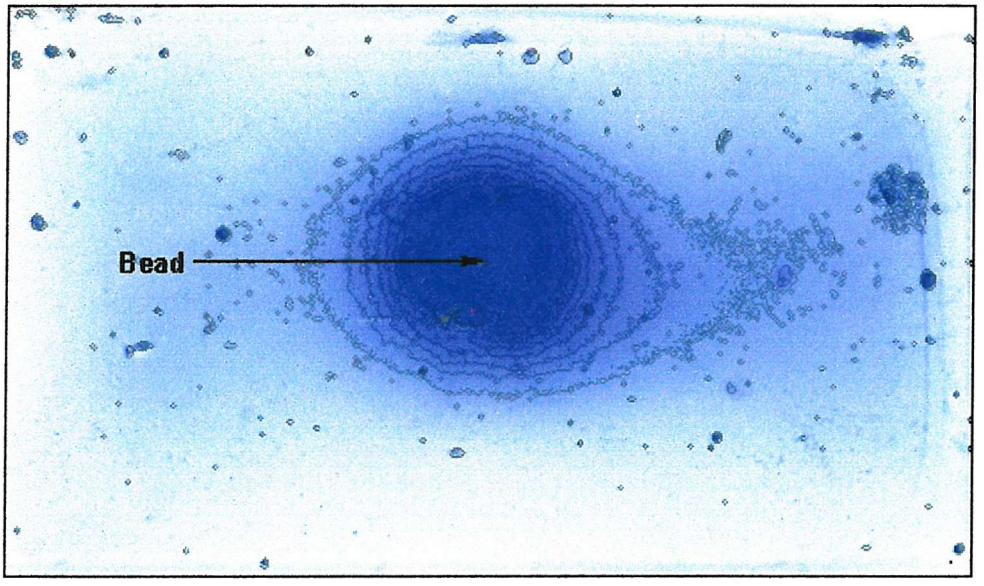
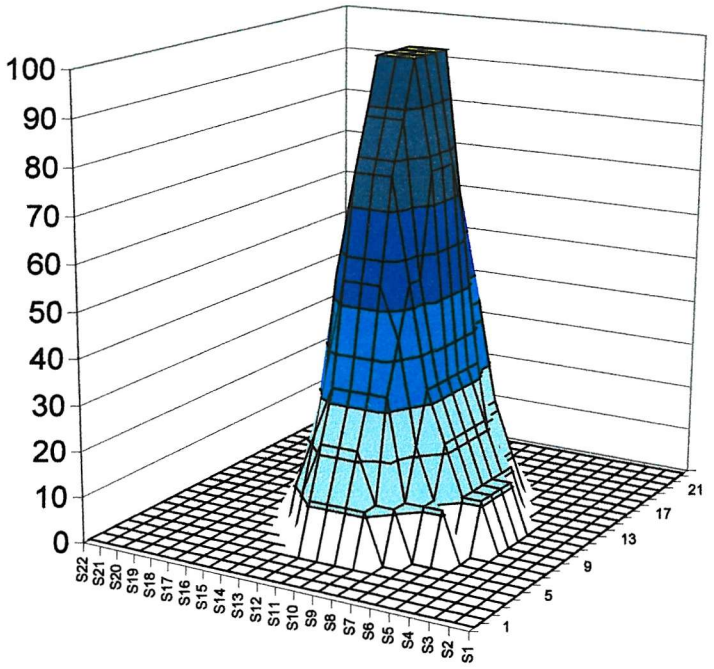


Figure 7.6: Scanned image of the single bead assay

It can be seen that the intensity of the blue colouration is greatest nearest the site of the bead and it decreases as the distance from the parent bead increases. The intensity of the blue colouration was quantified using the scanning software and a graphical representation of this relationship is depicted in graph 7.1.



Graph 7.1: Graphical depiction of the zone surrounding single bead.

7.4] Conclusions

Using gelatinase, gelatin and marimastat the preliminary studies towards the development of this new assay technique have been performed. By deriving various parameters it was demonstrated that the small quantity of inhibitor adsorbed onto a bead could generate a visible zone and thus the principal of the assay was shown to be viable for the detection of inhibitors. With further work this technique could be applied to finding new inhibitors for a variety of enzymes, thus the potential scope of this assay is wide. It was hoped that these assays could have been performed using marimastat attached to the linkers described and that the proof of the utility of these assays in conjunction with the linkers described would be conclusively demonstrated.

Chapter Eight Experimental

8.1] General Information

NMR spectra were recorded using a Bruker AC 250 spectrometer operating at 250 MHz for ^{1}H and 62.5 MHz for ^{13}C, a Bruker AC300 spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C, or a Bruker AC400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. Coupling constants (*J* values) were measured in Hz. ESI mass spectra were recorded using a VG Platform Quadrupole Electrospray Ionisation mass spectrometer, measuring monoisotopic masses. FAB mass spectra were recorded on a VG Analytical 70-250-SE normal geometry double focusing mass spectrometer, using argon as the bombarding gas. High resolution accurate mass measurements were carried out at 10 000 resolution mixtures of polyethylene glycols and / or polyethylene glycolmethyl ethers as mass calibrants for FAB. Infra red spectra were recorded on a BioRad Golden gate FTS 135. All samples were run neat as solids or oils. Melting points were determined using a Gallenkamp melting point apparatus and are uncorrected.

UV-VIS spectra were recorded using a 8452A Diode Array Spectrophotometer.

Analytical HPLC was accomplished using a Hewlett Packard HP1100 Chemstation.

Details of the columns, solvents and gradients are given below.

Gradient 1

Column – C_{18} size 150 mm x 3 mm (Analytical flow 0.5 mL/ min)

A = H_2O, 0.1% TFA; B = MeCN, 0.1% TFA

t = 0 min, 100% A; t= 20 min, 100% B; t = 25 min, 100% B

Gradient 2

Column – C_{18} ODS, size 150 mm x 3 mm (Analytical flow 0.5 mL/ min)

A = H_2O, 0.1% TFA, B = MeCN, 0.1% TFA

t = 0 min, 75% A; t= 20 min, 25% A; t = 25 min, 25% A

8.2] General Resin Procedures

8.2.1] Quantitative Ninhydrin test[7]

2-5 mg dry resin was weighed into a test tube. Reagent A (6 drops) and reagent B (2 drops) was added, mixed and heated to 100°C for 10 min. As a control the reagents were added in the same proportions as above without any resin. The tubes were then cooled, and 60% ethanol (2 mL) was added and the solutions mixed thoroughly. The solutions were filtered through a pasteur pipette containing a tight plug of glass wool, washed twice with 0.5 M Et_4NCl (0.5 mL) and diluted to 25 mL with 60% ethanol. The solution was measured against a blank at 570nm.

$mmol/g = A_{570} \times V \times 10^3 / \varepsilon m$

where V = volume (mL)

ε = extinction coeffient ($1.5 \times 10^4 M^{-1} cm^{-1}$)

m = mass of resin (mg)

8.2.2] Quantitative Fmoc analysis[8]

5-7 mg of dry resin was weighed into eppendorf tubes and subjected to 10 min agitation with 20% piperidine in DMF (1.5 mL). The resin was filtered through a pasteur pipette containing a tight plug of glass wool, washed twice with 20% piperidine in DMF. The solution was diluted to 10 mL with 20% piperidine in DMF. The solution was measured against a blank at 302 nm.

$mmol/g = A_{302} \times V \times 10^3 / \varepsilon m$

where V = volume (mL)

ε = extinction coeffient ($7800 M^{-1} cm^{-1}$)

m = mass of resin (mg)

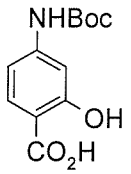
8.2.3] Qualitative Bromocresol Green test[130]

2-5 mg dry resin was weighed into a test tube and 2 drops of CH_2Cl_2 was added. 2 drops of bromocresol green indictor (0.04% w/v bromocresol green in EtOH and a few drops of aqueous NaOH) was added, and the mixture was shaken. A resultant yellow solution indicates the presence of free carboxyl groups on the resin.

8.3] Chapter Two

8.3.1] Preparation of aniline based linker model [2]

Preparation of 4-[(tert-Butoxy)carbonylamino]-2-hydroxybenzoic acid **[8]**



4-Amino salicylic acid (4.0 g, 26 mmol) was dissolved in dioxane (50 mL) and 1 M NaOH (24 mL) was added. The solution was cooled to 0°C and Boc_2O (5.0 g 26 mmol) dissolved in dioxane (20 mL) was added. The mixture was allowed to reach room temperature and stirred for 24 hours. The dioxane was removed *in vacuo*, H_2O (150 mL) was added and the mixture was extracted with EtOAc (3 x 200 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (200 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by recrystallisation from EtOAc and hexane to give the title compound as white prisms.

Yield: 2.6 g, 40%

R_f: 0.39 (7.5: 2: 0.5 hexane: EtOAc: AcOH)

δ_H (300 MHz, CD_3SOCD_3): 1.45 (9H, s, $C(C\underline{H}_3)_3$); 7.00 (1H, dd, $ArC^5\underline{H}$, J = 2 Hz, 9 Hz); 7.15 (1H, d, $ArC^3\underline{H}$, J = 2 Hz); 7.66 (1H, d, $ArC^6\underline{H}$, J = 9 Hz)

δ_C (75 MHz, CD_3SOCD_3): 28.7 ($C(\underline{C}H_3)_3$); 79.71 ($\underline{C}(CH_3)_3$); 104.5 ($Ar\underline{C}^3H$); 106.5 ($Ar\underline{C}^1$); 109.1 ($Ar\underline{C}^5H$); 130.9 ($Ar\underline{C}^6H$); 146.2 ($Ar\underline{C}^4$); 152.3 ($\underline{C}O_2C(CH_3)_3$); 162.2 ($Ar\underline{C}^2$); 171.7 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3367 (s) O-H; 1705 (s) C=O acid; 1624 (s) C=O carbamate

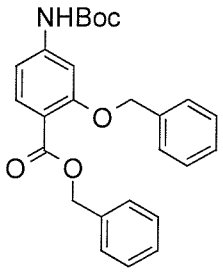
MP: 163-165°C

UV (EtOH) λ_{max}: 264 nm

RP-HPLC (λ_{254}): 14.7 min (gradient 1)

m/z (ES -ve): 252.2 $[M-H]^-$ (20%), 366.2 $[M-H+TFA]^-$ (100%)

Preparation of Phenylmethyl 4-[(tert-butoxy)carbonylamino]-2-(phenylmethoxy) benzoate **[9]**



4-[(*tert*-Butoxy)carbonylamino]-2-hydroxybenzoic acid (500 mg, 2.0 mmol) was dissolved in DMF (5 mL). Benzyl bromide (513 mg, 3.0 mmol) was added followed by K_2CO_3 (1.1 g, 8.0 mmol) and KI (3 mg, 0.018 mmol). The mixture was stirred at room temperature overnight. H_2O (10 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with H_2O (6 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (2: 8 EtOAc: hexane) to give the desired product as a white solid.

Yield: 525 mg, 61%

R_f: 0.90 (1: 1 hexane: EtOAc)

δ_H (300 MHz, $CDCl_3$): 1.53 (9H, s, $C(C\underline{H}_3)_3$); 5.19 (2H, s, $ArOC\underline{H}_2Ph$); 5.33 (2H, s, $CO_2C\underline{H}_2Ph$); 6.65 (1H, d, $ArC^3\underline{H}$, J = 2 Hz); 6.73 (1H, dd, $ArC^5\underline{H}$, J = 2 Hz, 8 Hz); 7.31-7.48 (10H, m, $Ar\underline{CH}$); 7.84 (1H, d, $ArC^6\underline{H}$, J = 8 Hz)

δ_C (75 MHz, $CDCl_3$): 28.3 ($C(\underline{C}H_3)_3$); 66.4 ($CO_2\underline{C}H_2Ph$); 70.5 ($ArO\underline{C}H_2Ph$); 81.2 ($\underline{C}(CH_3)_3$); 102.7 ($Ar\underline{C}^3H$); 109.5 ($Ar\underline{C}^5H$); 114.1 ($Ar\underline{C}^1$); 127.2 ($Ar\underline{C}H$); 127.7 ($Ar\underline{C}H$); 128.0 ($Ar\underline{C}H$); 128.4 ($Ar\underline{C}H$); 133.4 ($Ar\underline{C}^6H$); 136.3 ($Ar\underline{C}CH_2$); 136.5 ($Ar\underline{C}CH_2$); 143.7 ($Ar\underline{C}^4$); 152.1 ($\underline{C}O_2C(CH_3)_3$); 159.9 ($Ar\underline{C}^2$); 165.6 ($Ar\underline{C}O_2$)

IR υ cm^{-1}: 1730 (s) C=O (ester); 1685 (s) C=O (carbamate); 1584 (s) N-H

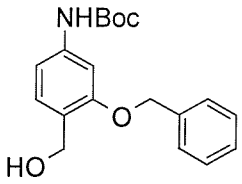
MP: 76-78°C

UV (EtOH) λ_{max}: 270 nm

RP-HPLC (λ_{254}): 20.8 min (gradient 1)

m/z (ES +ve): 434.2 $[M+H]^+$ (100%)

Preparation of (tert-Butoxy)-N-[4-(hydroxymethyl)-3-(phenylmethoxy)phenyl] carboxamide **[10]**



A solution of phenylmethyl 4-[(*tert*-butoxy)carbonylamino]-2-(phenylmethoxy) benzoate (450 mg, 1.0 mmol) in CH_2Cl_2 (5 mL) was cooled to 0°C and treated with DIBAL-H (3 mL, 1 M toluene solution). The resultant mixture was stirred for 1 hour at 0°C. The reaction was quenched by the addition of MeOH (1 mL). The resultant mixture was poured into 30% aq K-Na-tartrate (35 mL) and stirred at room temperature for 30 min. The mixture was then extracted with CH_2Cl_2 (3 x 10 mL). The combined organic layers were washed with 5% $NaHCO_3$ (20 mL), brine (20 mL) and dried with $MgSO_4$. The product was purified by column chromatography using silica gel (2: 1 hexane: EtOAc) to give the title compound as a yellow solid.

Yield: 325 mg, 95%

R_f: 0.45 (2: 1 hexane: EtOAc)

δ_H (300 MHz, $CDCl_3$): 1.54 (9H, s, $C(C\underline{H}_3)_3$); 4.66 (2H, s, $C\underline{H}_2OH$); 5.07 (2H, s, $OC\underline{H}_2Ph$); 6.73 (1H, dd, $ArC^6\underline{H}$, J = 2 Hz, 8 Hz); 6.84 (1H, d, $ArC^2\underline{H}$, J = 2 Hz); 7.17 (1H, d, $ArC^5\underline{H}$, J = 8 Hz); 7.39-7.41 (5H, m, $ArC\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 28.4 ($C(\underline{C}H_3)_3$); 61.5 ($\underline{C}H_2OH$); 70.0 ($O\underline{C}H_2Ph$); 80.5 ($\underline{C}(CH_3)_3$); 102.7 ($Ar\underline{C}^2H$); 110.3 ($Ar\underline{C}^6H$); 124.0 ($Ar\underline{C}^4$); 127.4 ($Ar\underline{C}H$); 128.0 ($Ar\underline{C}H$); 128.6 ($Ar\underline{C}H$); 128.7 ($Ar\underline{C}H$); 129.1 ($Ar\underline{C}^5H$); 136.6 ($Ar\underline{C}CH_2$); 139.4 ($Ar\underline{C}^1$); 152.8 ($\underline{C}O_2C(CH_3)_3$); 157.1 ($Ar\underline{C}^3$)

IR υ cm^{-1}: 3477 (s) O-H; 1695 (s) C=O carbamate; 1599 (s) N-H

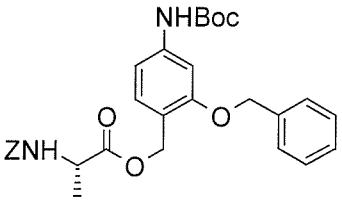
MP: 76-78°C

UV (EtOH) λ_{max}: 264 nm

RP-HPLC (λ_{254}): 16.7 min (gradient 1)

m/z (ES -ve): 331.2 $[M+H]^+$ (100%)

Preparation of {4-[(tert-butoxy)carbonylamino]-2-(phenylmethoxy)phenyl}methyl (benzyloxycarbonyl) alanoate **[2]**



(*tert*-Butoxy)-N-[4-(hydroxymethyl)-3-(phenylmethoxy)phenyl]carboxamide (400 mg, 1.2 mmol) was dissolved in DMF (5 mL) and Z-L-Ala-OH (300 mg, 1.3 mmol) was added, followed by DMAP (30 mg, 0.2 mmol) and EDC (250 mg, 1.3 mmol). The mixture was stirred at room temperature overnight. H_2O (20 mL) was added and the mixture was extracted with Et_2O (3 x 20 mL). The combined organic layers were washed with H_2O (6 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product purified by column chromatography on silica gel (2: 8 EtOAc: hexane) to give the desired product as a yellow solid.

Yield: 548 mg, 84%

R_f: 0.35 (7: 3 EtOAc: hexane)

δ_H (300 MHz, CD_3OD): 1.33 (3H, d, $C\underline{H}_3$ Ala, J = 7 Hz); 1.55 (9H, s, $C(C\underline{H}_3)_3$); 4.21-4.28 (1H, br s, α $C\underline{H}$ Ala); 5.07 (2H, s, $ArOC\underline{H}_2Ph$); 5.09 (2H, s, $C\underline{H}_2Ph$ (Z)); 5.14 (1H, d, $ArC\underline{H}_2O$, J = 12 Hz); 5.23 (1H, d, $ArC\underline{H}_2O$, J = 12 Hz); 6.95 (1H, dd, $ArC^5\underline{H}$, J = 8 Hz, 2 Hz); 7.23 (1H, d, $ArC^6\underline{H}$, J = 8 Hz); 7.31-7.35 (11H, m, $ArC^3\underline{H}$, $Ar\underline{CH}$)

δ_C (75 MHz, CD_3OD): 17.7 ($\underline{C}H_3$ Ala); 28.7 ($C(\underline{C}H_3)_3$); 51.4 (α $\underline{C}H$ Ala); 63.6 ($Ar\underline{C}H_2O$); 67.5 ($\underline{C}H_2Ph$ (Z)); 71.0 ($\underline{C}H_2Ph$ (Bn)); 81.0 ($\underline{C}(CH_3)_3$); 103.8 ($Ar\underline{C}^3H$); 111.9 ($Ar\underline{C}^6H$); 119.4 ($Ar\underline{C}^4$); 128.5 ($Ar\underline{C}H$); 128.8 ($Ar\underline{C}H$); 128.9 ($Ar\underline{C}H$); 129.4 ($Ar\underline{C}H$); 131.6 ($Ar\underline{C}^5H$); 138.5 ($Ar\underline{C}^1$); 138.6 ($Ar\underline{C}CH_2$); 142.5 ($Ar\underline{C}^2$); 155.0 ($\underline{C}O_2C(CH_3)_3$); 158.6 ($NH\underline{C}O_2CH_2Ph$); 174.7 ($ArCH_2O\underline{C}O$)

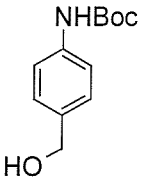
IR υ cm^{-1}: 1724 (s) C=O ester; 1690 (s) C=O carbamates; 1519 (s) N-H

UV (EtOH) λ_{max}: 252 nm

RP-HPLC (λ_{254}): 19.9 min (gradient 1)

m/z (ES +ve): 552.1 $[M+NH_4]^+$ (100%), 557.1 $[M+Na]^+$ (30%)

Preparation of (tert-Butoxy)-N-[4-(hydroxymethyl)phenyl]carboxamide **[12]**



4 Amino benzyl alcohol (2.0 g, 16.4 mmol) was dissolved in dioxane (30 mL) and NaOH (16 mL), and cooled to 0°C. A solution of Boc_2O (3.5 g, 16.4 mmol) in dioxane (10 mL) was added and the reaction was stirred at room temperature overnight. The solvent was removed *in vacuo,* H_2O (30 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with H_2O (3 x 50 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a yellow solid.

Yield: 2.2 g, 61 %

R_f: 0.52 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.51 (9H, s, $C(C\underline{H}_3)_3$); 4.55 (2H, s, $ArC\underline{H}_2O$); 6.82 (1H, s, $N\underline{H}$); 7.22 (2H, d, $ArC^3\underline{H}$, $ArC^5\underline{H}$, J = 8 Hz); 7.30 (2H, d, $ArC^2\underline{H}$, $ArC^6\underline{H}$, J = 8 Hz)

δ_C (75 MHz, $CDCl_3$): 28.4 ($C(\underline{C}H_3)_3$); 64.5 ($\underline{C}H_2OH$); 81.2 ($\underline{C}(CH_3)_3$); 118.7 ($Ar\underline{C}^2H$, $Ar\underline{C}^6H$); 127.8 ($Ar\underline{C}^3H$, $Ar\underline{C}^5H$); 135.6 ($Ar\underline{C}^4$); 137.7 ($Ar\underline{C}^1$); 153.0 ($\underline{C}O_2C(CH_3)_3$)

IR υ cm^{-1}: 3244 (br) OH; 1689 (s) C=O carbamate; 1524 (s) N-H

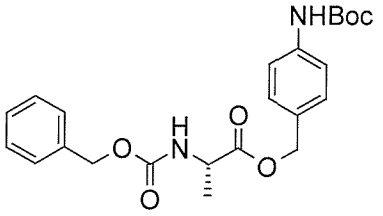
MP: 61-63°C

UV (EtOH) λ_{max}: 240 nm

RP-HPLC (λ_{254}): 12.9 min (gradient 1)

m/z (ES +ve): 241.2 $[M+NH_4]^+$ (100%)

Preparation of 4-[(tert-butoxy)carbonylamino]phenyl}methyl (benzyloxycarbonyl) alanoate **[13]**



To a solution of (*tert*-butoxy)-N-[4-(hydroxymethyl)phenyl]carboxamide (2.0 g, 9.0 mmol) and DMAP (60 mg, 0.3 mmol) in CH_2Cl_2 (50 mL), Z-L-Ala-OH (2.0 g, 9.0 mmol) was added followed by EDC (1.7 g, 9.0 mmol). The reaction was stirred at room temperature overnight. The solvent was removed *in vacuo*, the residue was taken up in CH_2Cl_2 (50 mL), washed with H_2O (2 x 50 mL), 5% $NaHCO_3$ (2 x 50 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography using silica gel (9: 1 CH_2Cl_2: EtOAc) to give the title compound as a pale yellow solid.

Yield: 3.3 g, 86%

R_f: 0.79 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.41 (3H, s, $C\underline{H}_3$ Ala, J = 7 Hz); 1.45 (9H, s, $C(C\underline{H}_3)_3$); 4.37-4.44 (1H, br s, α $C\underline{H}$ Ala); 5.11 (2H, s, $C\underline{H}_2Ph$ (Z)); 5.33 (2H, s, $ArC\underline{H}_2O$); 6.58 (1H, br s, $N\underline{H}$); 7.25-7.38 (9H, m, $ArC^2\underline{H}$, $ArC^3\underline{H}$, $ArC^5\underline{H}$, $ArC^6\underline{H}$, $ArC\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 18.7 ($\underline{C}H_3$ Ala); 28.5 ($C(\underline{C}H_3)_3$); 49.7 (α $\underline{C}H$ Ala); 67.0 ($\underline{C}H_2O$), ($\underline{C}H_2Ph$ (Z)); 80.7 ($\underline{C}(CH_3)_3$); 118.6 ($Ar\underline{C}^2H$, $Ar\underline{C}^6H$); 128.2 ($Ar\underline{C}^3H$, $Ar\underline{C}^5H$); 128.3 ($Ar\underline{C}H$); 128.7 ($Ar\underline{C}H$); 129.4 ($Ar\underline{C}H$); 129.8 ($Ar\underline{C}H$); 136.4 ($Ar\underline{C}^4$); 137.1 ($Ar\underline{C}^1$); 153.0 ($\underline{C}O_2CH_2Ph$); 155.9 ($\underline{C}O_2C(CH_3)_3$); 173.1 ($CH_2O\underline{C}O$)

IR υ cm^{-1}: 1752 (s) C=O ester; 1692 (s) C=O carbamate; 1519 (s) N-H

MP: 86-88°C

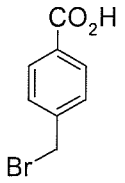
UV (EtOH) λ_{max}: 244 nm

RP-HPLC (λ_{254}): 18.0 min (gradient 1)

m/z (ES +ve): 451.1 $[M+Na]^+$(100%)

8.3.2] Preparation of aniline based linker model [4]

Preparation of 4-(Bromomethyl)benzoic acid **[17]**[156]



A solution of 4-tolunic acid (27.2 g, 20.0 mmol) and benzoyl peroxide (2.0 g, 8.2 mmol) in chlorobenzene (250 mL) was heated to reflux, and NBS (39.6 g, 22.0 mmol) was added portionwise over 30 min. The mixture was heated at reflux for 1.5 hours and then cooled in an ice bath. The resultant precipitate was collected by filtration and washed with petroleum ether (3 x 50 mL). The resulting white solid was added to H_2O (300 mL) and stirred for 30 min, filtered, and washed with H_2O (3 x 50 mL) and petroleum ether (3 x 50 mL). Recrystallisation from EtOAc gave the title compound as white needles.

Yield 14.1 g, 32%

R_f: 0.58 (6.5: 3: 0.5 hexane: EtOAc: AcOH)

δ_H (300 MHz, $CDCl_3$): 4.74 (2H, s, $C\underline{H}_2Br$); 7.55 (2H, d, $ArC^3\underline{H}$, $ArC^5\underline{H}$, J = 8 Hz); 7.93 (2H, d, $ArC^2\underline{H}$, $ArC^6\underline{H}$, J = 8 Hz)

δ_C (75 MHz, $CDCl_3$): 33.3 ($\underline{C}H_2Br$); 129.2 ($Ar\underline{C}^3H$, $Ar\underline{C}^5H$); 129.6 ($Ar\underline{C}^2H$, $Ar\underline{C}^6H$); 130.6 ($Ar\underline{C}^1$); 142.8 ($Ar\underline{C}^4$); 166.9 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3414 (br) OH; 1672 (s) C=O acid

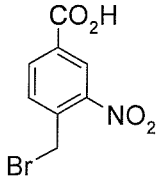
MP: 204-206°C (lit 220-222°C)[156]

UV (EtOH) λ_{max}: 244 nm

RP-HPLC (λ_{254}): 13.4 min (gradient 1)

m/z (APCI -ve): 212.8 $[M-H]^-$ (65%) (^{79}Br); 214.9 $[M-H]^-$(100%) (^{81}Br)

Preparation of 4-(Bromomethyl)-3-nitrobenzoic acid **[18]**[156]



4-(Bromomethyl)benzoic acid (13.5 g, 65.0 mmol) was added over one hour to fuming nitric acid (50 mL) at -10°C with stirring. After a further hour at 0°C, the reaction mixture was poured into crushed ice (20 g). The resultant precipitate was collected by filtration, washed with cold H_2O (250 mL) and dried *in vacuo*. Recrystallisation from CH_2Cl_2 and pentane gave the title compound as pale yellow needles.

Yield 12.6 g, 77%

R_f: 0.52 (6.5: 3: 0.5 hexane: EtOAc: AcOH)

δ_H (300 MHz, D_3CSOCD_3): 4.88 (2H, s, $C\underline{H}_2Br$); 7.74 (1H, d, $ArC^5\underline{H}$, J = 8 Hz); 8.32 (1H, dd, $ArC^6\underline{H}$, J = 8 Hz, 2 Hz); 8.75 (1H, d, $ArC^2\underline{H}$, J = 2 Hz)

δ_C (75 MHz, D_3CSOCD_3): 27.8 ($\underline{C}H_2Br$); 127.2 ($Ar\underline{C}^2H$); 130.9 ($Ar\underline{C}^1$); 133.1 ($Ar\underline{C}^5H$); 134.8 ($Ar\underline{C}^6H$); 138.0 ($Ar\underline{C}^4$); 148.1 ($Ar\underline{C}^3$); 168.8 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3423 (br) OH; 1700 (s) C=O acid; 1533 (s) NO_2

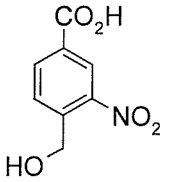
MP: 118-120°C (lit 128-130°C)[156]

UV (EtOH) λ_{max}: 266 nm

RP-HPLC (λ_{254}): 13.8 min (gradient 1)

m/z (APCI -ve): 257.9 $[M-H]^-$ (65%)(^{79}Br); 259.9 $[M-H]^-$ (100%)(^{81}Br)

Preparation of 4-(Hydroxymethyl)-3-nitrobenzoic acid **[19]**



4-(Bromomethyl)-3-nitrobenzoic acid (12.0 g, 46.0 mmol) was suspended in H_2O (360 mL) and heated at reflux for 1.5 hours. The solution was allowed to cool, and the resultant precipitate was collected by filtration. Purification by column

chromatography on silica gel (eluting with petroleum ether: EtOAc: AcOH 6.5: 3: 0.5) gave the title material as a yellow solid.

Yield: 4.5 g, 50%

R_f: 0.31 (6.5: 3: 0.5 hexane: EtOAc: AcOH)

δ_H (300 MHz, D_3CSOCD_3): 4.89 (2H, s, $C\underline{H}_2OH$); 7.98 (1H, d, $ArC^5\underline{H}$, J = 8 Hz); 8.28 (1H, dd, $ArC^6\underline{H}$, J = 8 Hz, 2 Hz); 8.48 (1H, d, $ArC^2\underline{H}$, J = 2 Hz)

δ_C (75 MHz, D_3CSOCD_3): 59.9 ($\underline{C}H_2OH$); 124.9 ($Ar\underline{C}^2H$); 128.7 ($Ar\underline{C}^1$); 128.7 ($Ar\underline{C}^5H$); 133.8 ($Ar\underline{C}^6H$); 143.1 ($Ar\underline{C}^4$); 146.5 ($Ar\underline{C}^3$); 165.5 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3584 (br) OH; 1692 (s) C=O acid

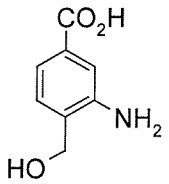
MP: 164-165°C

UV (EtOH) λ_{max}: 260 nm

HPLC (λ_{254}): 11.6 min (gradient 1)

m/z (APCI-ve): 196.0 $[M-H]^-$ (100%)

Preparation of 3-Amino-4-(hydroxymethyl)benzoic acid **[20]**



4-(Hydroxymethyl)-3-nitrobenzoic acid (1.0 g, 5 mmol) was dissolved in EtOH: H_2O: AcOH (49.5: 49.5: 1, 30 mL). $Pd/BaSO_4$ (250 mg, 0.025 mol percent) was suspended in the solution and the flask was flushed with N_2 and then stirred at room temperature under an atmosphere of H_2 for 3.5 hours. The catalyst was removed by filtration and the solvent removed *in vacuo*. The resultant yellow solid was purified by column chromatography on silica gel (5: 4.5: 0.5 EtOAc: hexane: AcOH) to give the title compound as a yellow solid.

Yield: 744 mg, 89%

R_f: 0.3 (5: 4.5: 0.5 EtOAc: hexane: AcOH)

δ_H (300 MHz, CD_3OD): 4.40 (2H, s, $C\underline{H}_2OH$); 7.11 (1H, d, $ArC^2\underline{H}$, J = 2 Hz); 7.15 (1H, dd, $ArC^6\underline{H}$, J = 8 Hz, 2 Hz); 7.20 (1H, d, $ArC^5\underline{H}$, J = 8 Hz)

δ_C (75 MHz, CD_3OD): 60.6 ($\underline{C}H_2OH$); 115.2 (Ar$\underline{C}^2H$); 117.0 (Ar$\underline{C}^6H$); 127.0 (Ar$\underline{C}^5H$); 130.0 (Ar$\underline{C}^1$); 131.2 (Ar$\underline{C}^4$); 146.0 (Ar$\underline{C}^3$); 168.0 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3372 (s) N-H; 3291 (br) OH; 1689 (s) C=O acid

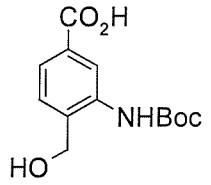
MP: 155-156°C

UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{254}): 9.65 min (gradient 1)

m/z (ES +ve): 190.8 $[M+Na]^+$(100%)

Preparation of 3-[(tert-Butoxy)carbonylamino]-4-(hydroxymethyl)benzoic acid **[21]**



3-Amino-4-(hydroxymethyl)benzoic acid (450 mg, 2.7 mmol) was dissolved in dioxane (1 mL) and NaOH (1 M, 5.4 mL). The solution was cooled to 0°C and Boc_2O (196 mg, 0.9 mmol) dissolved in dioxane (4.8 mL) was added. The mixture was the stirred at room temperature for 48 hours. The dioxane was removed *in vacuo* and H_2O (30 mL) was added. The aqueous solution was acidified to pH 1 with 2 M $KHSO_4$ and extracted with EtOAc (3 x 20 mL). The combined organic fractions were dried with $MgSO_4$ and the solvent was removed *in vacuo*. The resulting solid was purified using column chromatography on silica gel (5: 4.5: 0.5 EtOAc: hexane: AcOH) to give the desired compound as a yellow solid.

Yield: 402 mg, 55%

R_f: 0.64 (5: 4.5: 0.5 EtOAc: hexane: AcOH)

δ_H (300 MHz, CD_3OD): 1.56 (9H, s, $C(C\underline{H}_3)_3$); 4.70 (2H, s, $C\underline{H}_2OH$); 7.43 (1H, d, Ar$C^5\underline{H}$, J = 8 Hz); 7.77 (1H, dd, Ar$C^6\underline{H}$, J = 2 Hz, 8 Hz); 8.3 (1H, br s, Ar$C^2\underline{H}$)

δ_C (75 MHz, CD_3OD): 28.5 ($C(\underline{C}H_3)_3$); 62.7 ($\underline{C}H_2OH$); 81.4 ($\underline{C}(CH_3)_3$); 124.4 (Ar$\underline{C}^2H$); 126.1 (Ar$\underline{C}^6H$); 128.9 (Ar$\underline{C}^5H$); 131.7 (Ar$\underline{C}^1$); 138.0 (Ar$\underline{C}^3$); 138.1 (Ar$\underline{C}^4$); 155.2 ($\underline{C}O_2C(CH_3)_3$); 169.6 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3318 (br) OH; 1689 (s) C=O acid, carbamate

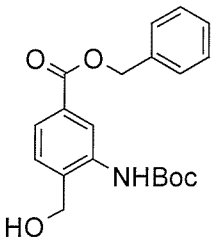
MP: 144-146°C

UV (EtOH) λ_{max}: 240 nm

RP-HPLC (λ_{254}): 12.2 min (gradient 1)

m/z (ES +ve): 268.9 $[M+H]^+$(100%), 285.9 $[M+NH_4]^+$ (70%)

Preparation of Phenylmethyl 3-[(tert-butoxy)carbonylamino]-4-(hydroxymethyl) benzoate **[22]**



3-[(*tert*-Butoxy)carbonylamino]-4-(hydroxymethyl)benzoic acid (200 mg, 0.75 mmol) was dissolved in DMF (5 mL). Benzyl bromide (130 mg, 0.75 mmol) was added followed by Cs_2CO_3 (180 mg, 1.50 mmol) and KI (3 mg, 0.018 mmol). The mixture was stirred at room temperature overnight. H_2O (20 mL) was added and the mixture extracted with Et_2O (3 x 20 mL). The combined organic fractions were washed with H_2O (6 x 20 mL), 5% $NaHCO_3$ (2 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* to give the title compound as a yellow oil.

Yield: 236 mg, 88%

R_f: 0.33 (7: 3 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.56 (9H, s, $C(C\underline{H}_3)_3$); 4.70 (2H, s, $C\underline{H}_2OH$); 5.36 (2H, s, $C\underline{H}_2Ph$); 7.23 (1H, d, $ArC^5\underline{H}$, J = 8 Hz); 7.32-7.46 (5H, m, $ArC\underline{H}$); 7.66 (1H, br s, $ArC^2\underline{H}$); 7.70 (1H, dd, $ArC^6\underline{H}$, J = 2 Hz, 8 Hz); 8.51 (1H, s, $N\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 28.5 ($C(\underline{C}H_3)_3$); 63.9 ($\underline{C}H_2OH$); 66.9 ($\underline{C}H_2Ph$); 80.9 ($\underline{C}(CH_3)_3$); 122.4 ($Ar\underline{C}^2H$); 124.6 ($Ar\underline{C}^6H$); 128.3 ($Ar\underline{C}H$); 128.7 ($Ar\underline{C}H$); 128.9 ($Ar\underline{C}^5H$); 130.8 ($Ar\underline{C}^1$); 133.9 ($Ar\underline{C}^3$); 136.2 ($Ar\underline{C}^4$); 138.0 ($Ar\underline{C}CH_2$); 153.4 ($\underline{C}O_2C(CH_3)_3$); 166.3 ($\underline{C}O_2Bn$)

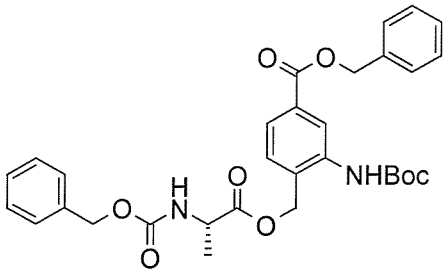
IR υ cm^{-1}: 3333 (br) OH; 1751 C=O (s) ester; 1718 (s) C=O carbamate; 1527 (s) N-H

UV (EtOH) λ_{max}: 240 nm

RP-HPLC (λ_{254}): 17.8 min (gradient 1)

m/z (ES +ve): 375.5 $[M+NH_4]^+$ (100%)

Preparation of {2-[(tert-butoxy)carbonylamino]-4-[benzyloxycarbonyl][phenyl}methyl (benzyloxycarbonyl) alanoate **[4]**



Phenylmethyl 3-[(*tert*-butoxycarbonyl)amino]-4-(hydroxymethyl)benzoate (128 mg, 0.4 mmol) was dissolved in DMF (5 mL) and Z-L-Ala-OH (240 mg, 1.1 mmol) was added followed by DMAP (44 mg, 0.3 mmol) and DCC (74 mg, 0.4 mmol). The mixture was stirred at room temperature overnight. H_2O (20 mL) was added and extracted with Et_2O (3 x 20 mL). The combined organic layers were washed with H_2O (6 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo.* The residue was triturated with hexane and filtered to give the title compound as a white solid.

Yield: 138 mg, 70%

R_f: 0.35 (7: 3 EtOAc: hexane)

δ_H (250 MHz, $CDCl_3$): 1.39 (3H, d, $C\underline{H}_3$ Ala, J = 7 Hz); 1.52 (9H, s, $C(C\underline{H}_3)_3$); 4.38-4.44 (1H, br s, α $C\underline{H}$ Ala); 5.09 (2H, s, $C\underline{H}_2Ph$ (Z)); 5.15 (1H, d, $ArC\underline{H}_2O$, J = 13 Hz); 5.23 (1H, d, $ArC\underline{H}_2O$, J = 13 Hz); 5.37 (2H, s, $C\underline{H}_2Ph$ (Bn)); 7.09 (1H, s, $N\underline{H}$); 7.32-7.46 (11H, m, $ArC^6\underline{H}$, $ArC\underline{H}$); 7.77 (1H, dd, $ArC^5\underline{H}$, J = 8 Hz, 2 Hz); 8.44 (1H, br s, $ArC^3\underline{H}$)

δ_C (75 MHz, $CDCl_3$):18.4 ($\underline{C}H_3$ Ala); 28.3 ($C(\underline{C}H_3)_3$); 49.6 (α $\underline{C}H$ Ala); 64.2 ($Ar\underline{C}H_2O$); 66.8 ($\underline{C}H_2Ph$ (Z)); 67.1 ($\underline{C}H_2Ph$ (Bn)); 81.1 ($\underline{C}(CH_3)_3$); 124.4 ($Ar\underline{C}^3H$); 125.3 ($Ar\underline{C}^5H$); 128.1 ($Ar\underline{C}^4$); 128.4 ($Ar\underline{C}H$); 130.4 ($Ar\underline{C}^6H$); 131.5 ($Ar\underline{C}^2$); 136.0 ($Ar\underline{C}^1$); 137.3 ($Ar\underline{C}CH_2$); 153.0 ($NH\underline{C}O_2CH_2Ph$); 155.6 ($\underline{C}O_2C(CH_3)_3$); 165.7 ($\underline{C}O_2CH_2Ph$); 173.0 ($ArCH_2O\underline{C}O$)

IR υ cm^{-1}: 3333 (br) OH; 1753 C=O (s) benzyl ester; 1718 (s) C=O amide; 1696 (s) C=O carbamates; 1527 (s) N-H

MP: 129-131°C

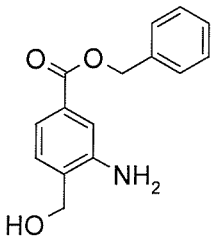
UV (EtOH) λ_{max}: 234 nm

RP-HPLC (λ_{254}): 20.4 min (gradient 1)

m/z (ES +ve): 580.4 (45%) $[M+NH_4]^+$, 585.4 (100%) $[M+Na]^+$

HRMS: [M+H]: $C_{31}H_{35}N_2O_8$ (calc) 562.2295; (found) 562.2315

Preparation of Phenylmethyl 3-amino-4-(hydroxymethyl)benzoate **[25]**



3-Amino-4-(hydroxymethyl)benzoic acid (100 mg, 0.6 mmol) was dissolved in DMF (20 mL). Benzyl bromide (105 mg, 0.6 mmol) was added followed by Cs_2CO_3 (390 mg, 1.2 mmol) and KI (3 mg, 0.018 mmol). The mixture was stirred at room temperature overnight. H_2O (20 mL) was added and the mixture was extracted with Et_2O (3 x 30 mL). The combined organic fractions were washed with H_2O (6 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* to give the title compound as a yellow solid.

Yield: 102 mg, 65%

R_f: 0.45 (1: 1 EtOAc: hexane)

δ_H: (300 MHz, CD_3OD): 4.63 (2H, s, $C\underline{H}_2OH$); 5.34 (2H, s, $C\underline{H}_2Ph$); 7.24 (1H, d, $ArC^5\underline{H}$, J = 8 Hz); 7.37-7.45 (7H, m, $ArC^2\underline{H}$, $ArC^6\underline{H}$, $Ar\underline{C}H$)

δ_C: (75 MHz, CD_3OD): 62.7 ($\underline{C}H_2OH$); 67.5 ($\underline{C}H_2Ph$); 117.4 ($Ar\underline{C}^2H$); 119.8 ($Ar\underline{C}^6H$); 129.0 ($Ar\underline{C}H$); 129.1 ($Ar\underline{C}H$); 129.2 ($Ar\underline{C}H$); 129.5 ($Ar\underline{C}^5H$); 131.1 ($Ar\underline{C}^1$); 131.8 ($Ar\underline{C}^4$); 137.6 ($Ar\underline{C}CH_2$); 147.4 ($Ar\underline{C}^3$); 168.1 ($\underline{C}O$)

IR υ cm^{-1}: 3382 (s) OH; 1698 (s) C=O acid

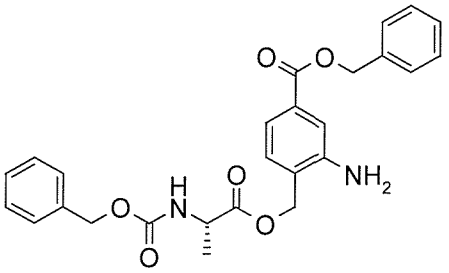
MP: 132-134°C

UV (EtOH) λ_{max}: 234 nm

RP-HPLC (λ_{254}): 12.1 min (gradient 1)

m/z (ES +ve): 258.2 $[M+H]^+$ (100%)

Preparation of {2-amino-4-[benzyloxycarbonyl]phenyl}methyl (benzyloxycarbonyl) alanoate **[23]**



Phenylmethyl 3-amino-4-(hydroxymethyl)benzoate (50 mg, 0.2 mmol) was dissolved in CH_2Cl_2 (10 mL). Z-L-Ala-OH (54 mg, 0.2 mmol) was added, followed by DMAP (24 mg, 0.2 mmol) and EDC (74 mg, 0.2 mmol). The mixture was stirred at room temperature overnight. H_2O (40 mL) was added and the mixture was extracted with Et_2O (3 x 30 mL). The combined organic fractions were washed with H_2O (6 x 20 mL), 5% $NaHCO_3$ (20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (96: 4 CH_2Cl_2: MeOH) to give the title compound as a yellow oil.

Yield: 54 mg, 48%

R_f: 0.48 (96: 4 CH_2Cl_2: MeOH)

δ_H (250 MHz, $CDCl_3$): 1.40 (3H, d, C$\underline{H}_3$ Ala, J = 7 Hz); 2.79 (2H, s, N$\underline{H}_2$); 4.37 (1H, dq, α C$\underline{H}$ Ala, J = 5 Hz, 7 Hz); 5.08 (2H, s, C$\underline{H}_2$Ph (Z)); 5.14 (2H, s, ArC$\underline{H}_2$O); 5.23 (1H, d, N$\underline{H}$, J = 5 Hz); 5.33 (2H, s, C$\underline{H}_2$Ph (Bn)); 7.22 (1H, d, ArC$^6\underline{H}$, J = 8 Hz); 7.23-7.44 (12H, m, ArC$^3\underline{H}$, ArC$^5\underline{H}$, ArC$\underline{H}$)

δ_C (62.5 MHz, $CDCl_3$): 18.8 (C$\underline{H}_3$ Ala); 49.8 (α C$\underline{H}$ Ala); 65.1 (ArC$\underline{H}_2$O); 67.2 (C$\underline{H}_2$Ph (Z)); 67.5 (C$\underline{H}_2$Ph (Bn)); 117.6 (ArC$^3\underline{H}$); 119.9 (ArC$^5\underline{H}$); 124.4 (ArC^4);128.5 (ArC$\underline{H}$); 128.6 (ArC$\underline{H}$); 128.7 (ArC$\underline{H}$); 129.0 (ArC$\underline{H}$); 131.6 (ArC$^6\underline{H}$); 132.2 (ArC^1); 136.5 (ArC$\underline{C}$H$_2$); 146.3 (ArC^2); 166.6 (C$\underline{O}_2$CH$_2$Ph); 173.4 (ArCH$_2$O$\underline{C}$O)

IR υ cm^{-1}: 1698 C=O (s) esters

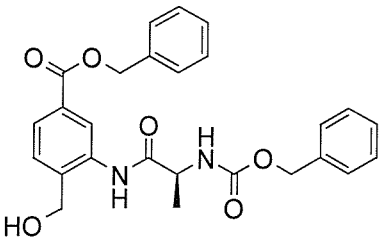
UV (EtOH) λ_{max}: 234 nm

RP-HPLC (λ_{254}): 18.3 min (gradient 1)

m/z (ES +ve): 463.0 $[M+H]^+$ (100%)

HRMS: [M+H]: $C_{26}H_{27}O_6N_2$: (calc) 463.1869; (found) 463.1864

Preparation of Phenylmethyl 3-{[(benzyloxycarbonyl)alanyl]amino}-4-(hydroxymethyl) benzoate **[24]**



Phenylmethyl 3-amino-4-(hydroxymethyl)benzoate (50 mg, 0.2 mmol) was dissolved in CH_2Cl_2 (5 mL). Z-L-Ala-OH (54 mg, 0.2 mmol) was added followed by HOBt (27 mg, 0.2 mmol) and EDC (37 mg, 0.2 mmol). The mixture was stirred at room temperature overnight. H_2O (20 mL) was added and the mixture was extracted with Et_2O (3 x 20 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (20 mL), 5% $NaHCO_3$ (20 mL), H_2O (6 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* to give the title compound as a colourless oil.

Yield: 62 mg, 55%

R_f: 0.63 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.49 (3H, d, C$\underline{H}_3$ Ala, J = 7 Hz); 4.36-4.39 (1H, br s, α C$\underline{H}$ Ala); 4.58 (2H, s, C$\underline{H}_2$Ph (Z)); 5.09-5.14 (2H, m, ArC$\underline{H}_2$O); 5.36 (2H, s, C$\underline{H}_2$Ph (Bn)); 7.33-7.44 (12H, m, ArC$^2\underline{H}$, ArC$^6\underline{H}$, ArC$\underline{H}$); 7.77 (1H, d, ArC$^5\underline{H}$, J = 8 Hz)

δ_C (75 MHz, $CDCl_3$): 17.1 (C$\underline{H}_3$ Ala); 48.7 (α C$\underline{H}$ Ala); 62.6 (ArC$\underline{H}_2$O); 65.7 (C$\underline{H}_2$Ph (Z)); 66.3 (C$\underline{H}_2$Ph (Bn)); 116.4 (ArC$^2\underline{H}$); 118.8 (ArC$^6\underline{H}$); 122.8 (ArC^1); 125.0 (ArC$^5\underline{H}$); 127.1 (ArC$\underline{H}$); 127.2 (ArC$\underline{H}$); 127.3 (ArC$\underline{H}$); 127.5 (ArC$\underline{H}$); 129.8 (ArC^4); 134.9 (ArC$\underline{C}$H$_2$); 135.9 (ArC^3); 155.3 (NH$\underline{C}$O$_2$CH$_2$Ph); 169.9 ($\underline{C}$O$_2$CH$_2$Ph); 172.0 (ArCH$_2$O$\underline{C}$O)

IR υ cm^{-1}: 3317 (br) OH; 1706 C=O (s) esters; 1530 (s) N-H

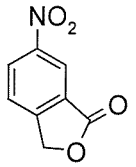
UV (EtOH) λ_{max}: 234 nm

RP-HPLC (λ_{254}): 16.6 min (gradient 1)

m/z (ES +ve): 463.0 $[M+H]^+$ (100%)

8.3.3] Preparation of aniline based linker model [6]

Preparation of 6-Nitro-3-hydroisobenzofuran-1-one **[26]**[157]



3-Hydroisobenzofuran-1-one (20 g, 0.15 mol) was added over one hour to fuming nitric acid (50 mL) at -10°C. After stirring for a further hour at 0°C, the reaction mixture was poured into crushed ice (20 g). The resultant orange solid was collected by filtration, washed with cold H_2O (250 mL) and dried *in vacuo*. Recrystallisation from EtOAc and hexane gave the title compound as pale yellow needles.

Yield 20 g, 75%

R_f: 0.45 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 5.48 (2H, s, $C\underline{H}_2$); 7.75 (1H, d, $ArC^4\underline{H}$, J = 9 Hz); 8.58 (1H, dd, $ArC^5\underline{H}$, J = 9 Hz, 2 Hz); 8.75 (1H, d, $ArC^7\underline{H}$, J = 2 Hz)

δ_C: (75 MHz, $CDCl_3$): 69.6 ($\underline{C}H_2$); 121.4 ($Ar\underline{C}^7H$); 123.7 ($Ar\underline{C}^5H$); 127.6 ($Ar\underline{C}^{7a}$); 128.9 ($Ar\underline{C}^4H$); 149.0 ($Ar\underline{C}^6$); 151.9 ($Ar\underline{C}^{3a}$); 168.6 ($\underline{C}O$)

IR υ cm^{-1}: 1748 (s) C=O lactone; 1603 (s) NO_2

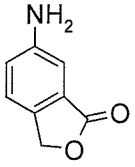
MP: 144-146°C (lit 141-142°C)[157]

UV (EtOH) λ_{max}: 254 nm

RP-HPLC (λ_{254}): 11.7 min (gradient 1)

m/z (APCI -ve): 177.9 $[M-H]^-$ (70%)

Preparation of 6-Amino-3-hydroisobenzofuran-1-one **[27]**[158]



6-Nitro-3-hydroisobenzofuran-1-one (6.0 g, 33.6 mmol) was dissolved EtOAc (200 mL), Pd/C (150 mg, 0.025 mol percent) was suspended in the solution and the flask was flushed with N_2. The suspension was allowed to stir at room temperature under a

H_2 atmosphere for 40 hours. The catalyst was removed by filtration and the solvent was removed *in vacuo* to give the title compound as a yellow solid.

Yield 4.5 g, 90%

R_f: (0.29 1: 1 EtOAc: hexane)

δ_H (300 MHz, CD_3OD): 5.28 (2H, s, $C\underline{H}_2$); 7.09-7.13 (2H, m, $ArC^4\underline{H}$, $ArC^7\underline{H}$); 7.34 (1H, dd, $ArC^5\underline{H}$, J = 1 Hz, 8 Hz)

δ_C (75 MHz, CD_3OD): 71.9 ($\underline{C}H_2$); 110.7 ($Ar\underline{C}^7H$); 123.6 ($Ar\underline{C}^5H$); 124.6 ($Ar\underline{C}^4H$); 128.9 ($Ar\underline{C}^{7a}$); 137.6 ($Ar\underline{C}^{3a}$); 151.5 ($Ar\underline{C}^6$); 174.4 ($\underline{C}O$)

IR υ cm^{-1}: 1744 (s) C=O lactone

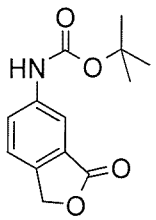
MP: 108-111°C

UV (EtOH) λ_{max}: 256 nm

RP-HPLC (λ_{254}): 11.6 min (gradient 1)

m/z (ES +ve): 150.0 $[M+H]^+$ (50%), 191.0 $[M+MeCN]^+$ (75%)

Preparation of 6-[(tert-butoxy)carbonylamino]-3-hydroisobenzofuran-1-one **[28]**



6-Amino-3-hydroisobenzofuran-1-one (2.3 g, 15.4 mmol) was dissolved in dioxane (50 mL) and 1 M NaOH (15.4 mL) was added. The solution was cooled to 0°C and a solution of Boc_2O (3.4 g, 15.4 mmol) in dioxane (15 mL) was added. The mixture was allowed to reach room temperature and stirred for 48 hours. The dioxane was removed *in vacuo*, H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 200 mL). The combined organic fractions were washed with H_2O (2 x 100mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by recrystallisation from EtOAc and hexane to afford the title compound as white needles.

Yield: 1.8 g, 52%

R_f: 0.61 (1: 1 EtOAc: hexane)

δ_H (300 MHz, CD_3OD): 1.49 (9H, s, $C(C\underline{H}_3)_3$); 5.24 (2H, s, $C\underline{H}_2$); 7.36 (1H, d, $ArC^4\underline{H}$, J = 8 Hz); 7.75 (1H, dd, $ArC^5\underline{H}$, J = 2 Hz, 8 Hz); 7.89 (1H, d, $ArC^7\underline{H}$, J = 2 Hz)

δ_C (75 MHz, CD_3OD): 28.6 ($C(\underline{C}H_3)_3$); 70.2 ($\underline{C}H_2O$); 81.2 ($\underline{C}(CH_3)_3$); 114.7 ($Ar\underline{C}^7H$); 123.8 ($Ar\underline{C}^5H$); 125.1 ($Ar\underline{C}^4H$); 127.1 ($Ar\underline{C}^{7a}$); 141.9 ($Ar\underline{C}^6$); 142.3 ($Ar\underline{C}^{3a}$); 154.9 ($\underline{C}O_2C(CH_3)_3$); 173.3 ($CH_2O\underline{C}O$)

IR υ cm^{-1}: 3341 (s) NH; 1744 (s) C=O lactone, 1713 (s) C=O carbamate

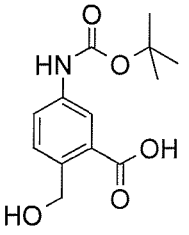
MP: 157°C-160°C

UV (EtOH) λ_{max}: 256 nm

RP-HPLC (λ_{254}): 14.6 min (gradient 1)

m/z (ES +ve): 267.2 $[M+NH_4]^+$ (100%)

Preparation of 5-[(tert-Butoxy)carbonylamino]-2-(hydroxymethyl) benzoic acid **[29]**



6-[(*tert*-Butoxy)carbonylamino]-3-hydroisobenzofuran-1-one (190 mg, 1.6 mmol) was added to a solution of NaOH (64 mg, 1.6 mmol) in H_2O (4 mL) and the suspension was heated at 70°C for 15 hours. The solvent was removed *in vacuo* and the residue was redissolved in H_2O (10 mL), acidified with 2 M $KHSO_4$ and extracted with EtOAc (3 x 50 mL). The combined organic extracts were dried with $MgSO_4$ and the solvent removed *in vacuo* to yield the title compound as a yellow solid.

Yield: 200 mg, 67%

R_f: 0.25 (6.5: 3: 0.5 EtOAc: hexane: AcOH)

δ_H (300 MHz, CD_3OD): 1.57 (9H, s, $C(C\underline{H}_3)_3$); 4.89 (2H, s, $C\underline{H}_2OH$); 7.55 (1H, d, $ArC^3\underline{H}$, J = 8 Hz); 7.64 (1H, dd, $ArC^4\underline{H}$, J = 2 Hz, 8 Hz); 8.1 (1H, d, $ArC^6\underline{H}$, J = 2 Hz)

δ_C (75 MHz, CD_3OD): 28.7 ($C(\underline{C}H_3)_3$); 63.6 ($\underline{C}H_2O$); 81.1 ($\underline{C}(CH_3)_3$); 122.0 ($Ar\underline{C}^6H$); 123.2 ($Ar\underline{C}^4H$); 129.8 ($Ar\underline{C}^3H$); 130.5 ($Ar\underline{C}^1$); 138.4 ($Ar\underline{C}^5$); 139.8 ($Ar\underline{C}^2$); 155.2 ($\underline{C}O_2C(CH_3)_3$); 170.7 ($Ar\underline{C}O_2H$)

IR υ cm^{-1}: 3331 (s) O-H; 1694 (s) C=O acid, carbamate

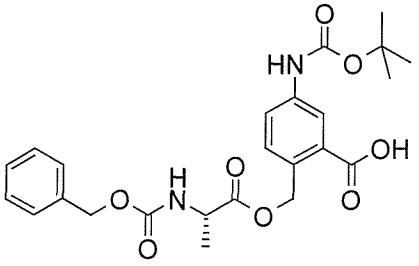
MP: 148-150°C

UV (EtOH) λ_{max}: 254 nm

RP-HPLC (λ_{254}): 12.3 min (gradient 1)

m/z (ES +ve): 285.3 $[M+NH_4]^+$ (100%), 535.5 $[2M+H]^+$ (100%)

Preparation of 2-{[(benzyloxy)carbonyl]alanylmethyl}-5-[(tert-butoxy)carbonyl amino] benzoic acid **[30]**



5-[(*tert*-Butoxy)carbonylamino]-2-(hydroxymethyl) benzoic acid (200 mg, 0.78 mmol) was dissolved in DMF (5 mL). NEt_3 (140 mg, 1.5 mmol) was added followed by Z-L-Ala-OSu (440 mg, 1.5 mmol) and the reaction was stirred at room temperature overnight. H_2O (20 mL) was added, acidified to pH 1 with 2 M $KHSO_4$ and the mixture was extracted with EtOAc (3 x 30 mL). The combined organic fractions were washed with H_2O (6 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (6.5: 3: 0.5 EtOAc: hexane: AcOH) to give the title compound as a transparent oil.

Yield: 200 mg, 54%

R_f: 0.26 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.43 (3H, s, C$\underline{H}_3$ Ala, J = 7 Hz); 1.54 (9H, s, C(C$\underline{H}_3$)$_3$); 4.43-4.48 (1H, br s, α C$\underline{H}$ Ala); 5.10 (2H, s, C$\underline{H}_2$Ph (Z)); 5.50 (2H, s, C$\underline{H}_2$O); 7.33-7.40 (6H, m, ArC$^3\underline{H}$, ArC$\underline{H}$); 7.68 (1H, m, ArC$^4\underline{H}$); 8.00 (1H, br s, ArC$^6\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 18.9 (C$\underline{H}_3$ Ala); 28.7 (C(C$\underline{H}_3$)$_3$); 50.2 (α C$\underline{H}$ Ala); 65.9 (C$\underline{H}_2$O); 67.4 (C$\underline{H}_2$Ph); 80.1 (C$\underline{(CH_3)_3}$); 121.9 (ArC$^6\underline{H}$); 123.0 (ArC$^4\underline{H}$); 128.5 (ArC$\underline{H}$); 128.6 (ArC$\underline{H}$); 128.9 (ArC$\underline{H}$); 129.2 (ArC$\underline{H}$); 130.7 (ArC$^3\underline{H}$); 131.9 (ArC^1); 136.6 (ArC^5); 139.1 (ArC^2, ArC$\underline{C}H_2$); 156.2 ($\underline{C}O_2C(CH_3)_3$, $\underline{C}O_2CH_2Ph$); 170.6 ($CH_2O\underline{C}O$); 173.3 (Ar$\underline{C}O_2H$)

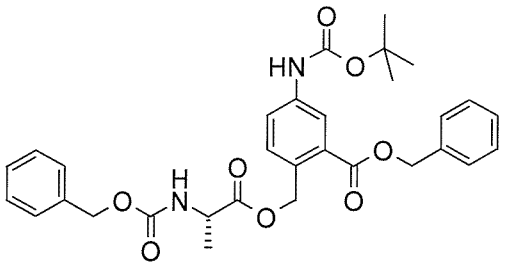
IR υ cm^{-1}: 3341 (s) N-H; 1738 (s) C=O ester; 1713 (s) C=O acid; 1673 C=O carbamate

UV (EtOH) λ_{max}: 256 nm

RP-HPLC (λ_{254}): 16.8 min (gradient 1)

m/z (ES +ve): 490.4 $[M+NH_4]^+$ (100%)

Preparation of Phenylmethyl-2-{[(benzyloxy)carbonyl]alanylmethyl}-5-[(tert-butoxy) carbonylamino]benzoate **[6]**



2-{[(benzyloxy)carbonyl]alanylmethyl}-5-[(*tert*-butoxy)carbonylamino] benzoic acid (200 mg, 0.4 mmol) was dissolved in DMF (10 mL). Benzyl bromide (80 mg, 0.4 mmol) was added followed by $CsCO_3$ (280 mg, 0.8 mmol, 2equ) and KI (3 mg, 0.018 mmol). The mixture was stirred at room temperature overnight. H_2O (20 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with H_2O (6 x 20 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (2:8 EtOAc: hexane) to give the desired product as a white solid.

Yield: 125 mg, 53%

R_f: 0.92 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.38 (3H, s, C$\underline{H}_3$ Ala, J = 7 Hz); 1.52 (9H, s, C(C$\underline{H}_3$)$_3$); 4.39-4.44 (1H, br s, α C$\underline{H}$ Ala); 5.11 (2H, s, C$\underline{H}_2$Ph (Z)); 5.33 (2H, s, C$\underline{H}_2$Ph (Bn)); 5.46 (1H, d, ArC$\underline{H}_2$O, J = 13 Hz); 5.55 (1H, d, ArC$\underline{H}_2$O, J = 13 Hz); 6.75 (1H, br s, N$\underline{H}$); 7.35-7.44 (11H, m, ArC$^3\underline{H}$, ArC$\underline{H}$); 7.67 (1H, m, ArC$^4\underline{H}$); 7.90 (1H, br s, ArC$^6\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 18.7 (C$\underline{H}_3$ Ala); 28.3 (C(C$\underline{H}_3$)$_3$); 49.7 (α C$\underline{H}$ Ala); 65.3 (C$\underline{H}_2$O); 66.9 (C$\underline{H}_2$Ph (Z)); 67.0 (C$\underline{H}_2$Ph (Bn)); 81.2 (C(CH$_3$)$_3$); 120.7 (ArC^{6}H); 122.1 (ArC^{4}H); 128.1 (ArCH); 128.6 (ArCH); 128.7 (ArCH); 128.8 (ArCH); 129.5 (ArC^1);

130.2 (Ar$\underline{C}^3$H); 131.1 (Ar$\underline{C}^5$); 136.2 (Ar$\underline{C}^2$); 138.6 (Ar$\underline{C}CH_2$); 152.5 ($\underline{C}O_2CH_2Ph$); 155.6 ($\underline{C}O_2C(CH_3)_3$); 166.0 ($CH_2O\underline{C}O$); 172.6 (Ar$\underline{C}O_2$)

IR υ cm^{-1}: 1715 C=O (s) esters; 1692 (s) C=O carbamates; 1530 (s) N-H

UV (EtOH) λ_{max}: 254 nm

RP-HPLC (λ_{254}): 20.3 min (gradient 1)

m/z (ES +ve): 580.2 $[M+NH_4]^+$ (100%)

HRMS [M+H]: $C_{31}H_{35}O_8N_2$: (calc) 563.2394; (found) 563.2401

8.3.4] Evaluation of the aniline based linker models

Evaluation of aniline based linker model **[2]**

{4-[(*tert*-butoxy)carbonylamino]-2-(phenylmethoxy)phenyl}methyl (benzyloxy-carbonyl) alanoate (5 mg, 9 μmol) was added to 50% TFA/ DCM (1 mL) and stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* and the resultant residue analysed by MS and TLC.

Evaluation of aniline based linker model **[13]**

4-[(*tert*-butoxy)carbonylamino]phenyl}methyl (benzyloxycarbonyl) alanoate (5 mg, 12 μmol) was added to 50% TFA/ DCM (1 mL) and stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* and the resultant residue analysed by MS and TLC.

Evaluation of aniline based linker model **[4]**

{2-[(*tert*-butoxy)carbonylamino]-4-[benzyloxycarbonyl]phenyl}methyl (benzyloxycarbonyl) alanoate (5 mg, 9 μmol) was added to 50% TFA/ DCM (1 mL) and stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo*, and the residue added to 50 mM phosphate buffer pH 7.5 (3.75 mL) and MeCN (1.25 mL). The solution was stirred thoroughly and left to stand at room temperature. Aliquots (150 μL) were removed periodically, the reaction quenched by adding to 2% TFA (MeCN: H_2O 25: 75, 100 μL). The samples were analysed by RP-HPLC (gradient 1).

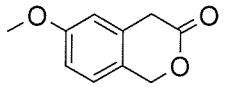
Evaluation of aniline based linker model **[6]**

Phenylmethyl-2-{[(benzyloxy)carbonyl]alanylmethyl}-5-[(tert-butoxy) carbonylamino]benzoate (5 mg, 9 μmol) was added to 50% TFA/ DCM (1 mL) and stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo*, and the residue added to 50 mM phosphate buffer pH 7.5 (7.5 mL) and MeCN (2.5 mL). The solution was stirred thoroughly and left to stand at room temperature. Aliquots (300 μL) were removed periodically, the reaction quenched by adding to 2% TFA (MeCN: H_2O 25: 75, 100 μL). The samples were analysed by RP-HPLC (gradient 1).

8.4] Chapter Three

8.4.1] Linker preparation and synthesis of model compounds

Preparation of 6-Methoxyisochroman-3-one **[44]**[128]



A solution of 3-methoxyphenyl acetic acid (10.0 g 66 mmol), glacial acetic acid (45 mL), formaldehyde (37% in H_2O, 15 mL) and concentrated HCl (3 mL) was stirred at room temperature for 5 days. The solution was poured into H_2O (250 mL) and extracted with $CHCl_3$ (3 x 75 mL). The combined organic extracts were washed with 5% $NaHCO_3$ (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product purified by column chromatography on silica gel (7: 3 hexane: EtOAc) to give the desired compound as white prisms.

Yield: 4.43 g, 41%

R_f: 0.29 (7: 3 hexane: EtOAc)

δ_H (250 MHz, $CDCl_3$): 3.65 (2H, s, $C\underline{H}_2CO$); 3.80 (3H, s, $OC\underline{H}_3$); 5.23 (2H, s, $C\underline{H}_2O$); 6.73 (1H, d, $ArC^5\underline{H}$, J = 2 Hz); 6.80 (1H, dd, $ArC^7\underline{H}$, J = 2 Hz, 8 Hz); 7.13 (1H, d, $ArC^8\underline{H}$, J = 8 Hz)

δ_C (62.5 MHz, $CDCl_3$): 36.8 ($\underline{C}H_2CO$); 56.0 ($O\underline{C}H_3$); 70.2 ($\underline{C}H_2O$); 113.0 ($Ar\underline{C}^5H$); 113.2 ($Ar\underline{C}^7H$); 124.1 ($Ar\underline{C}^{8a}$); 126.3 ($Ar\underline{C}^8H$); 133.0 ($Ar\underline{C}^{4a}$); 160.5 ($Ar\underline{C}^6$); 171.2 ($CH_2\underline{C}O$)

IR υ cm^{-1}: 1731 (s) C=O lactone

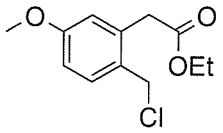
MP: 72-74°C (Lit 74-78°C)[128]

UV (EtOH) λ_{max}: 236 nm

RP-HPLC (λ_{254}): 12.0 min (gradient 1)

m/z (ES +ve): 179.2 $[M+H]^+$ (100%)

Preparation of Ethyl-2-[2-(chloromethyl)-5-methoxyphenyl] acetate **[45]**



Acetyl chloride (35.2 g, 0.56 mol) was added dropwise to ethanol (400 mL) cooled to 0°C. The solution was warmed to room temperature and a solution of 6-methoxyisochroman-3-one (4.0 g, 22.4 mmol) was added portionwise over 30 min. The resultant solution was left to stir at room temperature overnight. The solvent was removed *in vacuo* to give the desired product as a pale yellow oil.

Yield: 5.8 g, 99%

R_f: 0.89 (7: 3 hexane: EtOAc)

δ_H (250 MHz, $CDCl_3$): 1.25 (3H, t, $OCH_2C\underline{H}_3$, J = 7 Hz); 3.75 (2H, s, $C\underline{H}_2CO$); 3.79 (3H, s, $OC\underline{H}_3$); 4.15 (q, 2H, $OC\underline{H}_2CH_3$, J = 7 Hz); 4.64 (2H, s, $C\underline{H}_2Cl$); 6.76-6.83 (2H, m, $ArC^6\underline{H}$, $ArC^4\underline{H}$); 7.27 (1H, d, $ArC^3\underline{H}$, J = 8 Hz)

δ_C (62.5 MHz, $CDCl_3$): 14.6 ($OCH_2\underline{C}H_3$); 38.9 ($\underline{C}H_2CO$); 44.9 ($O\underline{C}H_2CH_3$); 56.2 ($O\underline{C}H_3$); 61.2 ($\underline{C}H_2Cl$); 113.4 ($Ar\underline{C}^4H$); 117.2 ($Ar\underline{C}^6H$); 128.8 ($Ar\underline{C}^2$); 132.3 ($Ar\underline{C}^3H$); 135.4 ($Ar\underline{C}^1$); 160.4 ($Ar\underline{C}^5$); 171.5 ($\underline{C}O$)

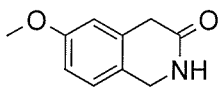
IR υ cm^{-1}: 1728 (s) C=O ester

UV (EtOH) λ_{max}: 240 nm

RP-HPLC (λ_{254}): 25.9 min (gradient 1)

m/z (EI +ve): 242.1 $[M]^+$ (100%)

Preparation of 6-Methoxy-1,2,4-trihydroisoquinolin-3-one **[46]**



Ammonia (sp. gr = 0.88, 400 mL) was added to ethyl-2-[2-(chloromethyl)-5-methoxyphenyl] acetate (4.0 g, 32 mmol) in THF (160 mL). The resultant yellow solution was stirred at room temperature for 15 hours. The THF was removed *in vacuo* and the aqueous residue was acidified to pH 1 with 2 M $KHSO_4$ and extracted with EtOAc (3 x 300 mL). The combined organic fractions were washed with H_2O (300 mL), brine (300 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo*

and the product was purified by recrystallisation from MeOH to give the title compound as yellow platlets.

Yield: 4.3 g, 76%

R_f: 0.1 (2: 1 EtOAc: hexane)

δ_H (250 MHz, $CDCl_3$): 3.55 (2H, s, $C\underline{H}_2CO$); 3.80 (3H, s, $OC\underline{H}_3$); 4.44 (2H, d, $C\underline{H}_2NH$, J = 2 Hz); 6.68 (1H, d, $ArC^5\underline{H}$, J = 2 Hz); 6.78 (1H, dd, $ArC^7\underline{H}$, J = 2 Hz, 8 Hz); 7.07 (1H, d, $ArC^8\underline{H}$, J = 8 Hz); 7.85 (1H, br s, $N\underline{H}$)

δ_C (62.5 MHz, $CDCl_3$): 36.8 ($\underline{C}H_2CO$); 44.8 ($\underline{C}H_2NH$); 55.5 ($O\underline{C}H_3$); 112.6 ($Ar\underline{C}^5H$); 113.0 ($Ar\underline{C}^7H$); 123.4 ($Ar\underline{C}^{4a}$); 126.6 ($Ar\underline{C}^8H$); 133.2 ($Ar\underline{C}^{8a}$); 159.0 ($Ar\underline{C}^6$); 172.4 ($CH_2\underline{C}O$)

IR υ cm^{-1}: 1654 (s) C=O lactam

MP: 156-157°C

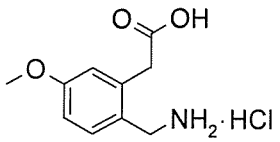
UV (EtOH) λ_{max}: 278 nm

RP-HPLC (λ_{220}): 9.9 min (gradient 1)

m/z (ES +ve)**:** 178.2 $[M+H]^+$ (100%)

HRMS $[M^+]$: $C_{10}H_{11}NO_2$ (calc) 177.0786; (found) 177.0789

Preparation of 2-[2-(Aminomethyl)-5-methoxyphenyl]acetic acid·HCl **[47]**



A mixture of 6-methoxy-1,2,4-trihydroisoquinolin-3-one (1.0 g, 5.7 mmol) and 6 N HCl (50 mL) was heated at reflux for 4 hours. The reaction mixture was evaporated to dryness to give the title compound as a white solid.

Yield: 1.23 g, 93%

R_f: 0.63 (5: 3: 1 $CHCl_3$: MeOH: AcOH)

δ_H (250 MHz, CD_3OD): 3.80 (3H, s, $OC\underline{H}_3$); 3.84 (2H, s, $C\underline{H}_2CO$); 4.90 (2H, s, $C\underline{H}_2NH_2$); 6.88-6.96 (2H, m, $ArC^4\underline{H}$, $ArC^6\underline{H}$); 7.42 (1H, d, $ArC^3\underline{H}$, J = 8 Hz)

δ_C (62.5 MHz, CD_3OD): 39.8 ($\underline{C}H_2CO$); 41.8 ($\underline{C}H_2NH_2$); 56.3 ($O\underline{C}H_3$); 115.1 ($Ar\underline{C}^4H$); 118.3 ($Ar\underline{C}^6H$); 125.9 (ArC^1); 133.7 ($Ar\underline{C}^3H$); 137.0 ($Ar\underline{C}^2$); 162.3 ($Ar\underline{C}^5$); 174.8 ($CH_2\underline{C}O$)

IR υ cm^{-1}: 3828 (br) OH; 1687 (s) C=O acid

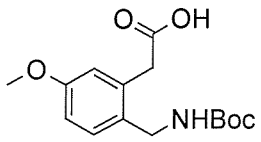
MP: 177-180°C

UV (EtOH) λ_{max}: 280 nm

RP-HPLC (λ_{254}): 7.5 min (gradient 1)

m/z (ES +ve)**:** 179 $[M\text{-}OH+H]^+$ (100%)

Preparation of 2-(tert-Butoxycarbonylaminomethyl)-5-methoxyphenyl]acetic acid **[48]**



Boc_2O (910 mg, 4.3 mmol) in dioxane (30 mL) was added to a mixture of 2-[2-(aminomethyl)-5-methoxyphenyl]acetic acid·HCl (1 g, 4.3 mmol) and NaOH (1 M, 10 mL) in dioxane (10 mL) cooled to 0°C. The reaction mixture was allowed to stir at room temperature overnight. The dioxane was removed *in vacuo*. The aqueous residue was acidified to pH 1 with 2 M $KHSO_4$ and extracted with EtOAc (3 x 100 mL). The combined organic fractions were washed with H_2O (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by recrystallisation from EtOAc to give the desired product as white prisms.

Yield: 700 mg, 60%

R_f: 0.4 (7: 3 hexane: EtOAc)

δ_H (250 MHz, CD_3OD): 1.43 (9H, s, $C(C\underline{H}_3)_3$); 3.66 (2H, s, $C\underline{H}_2CO$); 3.76 (3H, s, $OC\underline{H}_3$); 4.20 (2H, s, $C\underline{H}_2NH$); 6.67-6.81 (2H, m, $ArC^4\underline{H}$, $ArC^6\underline{H}$); 7.20 (1H, d, $ArC^3\underline{H}$, $J = 8$ Hz)

δ_C (62.5 MHz, CD_3OD): 29.2 ($C(\underline{C}H_3)_3$); 39.5 ($\underline{C}H_2CO$); 42.9 ($\underline{C}H_2NH$); 56.1 ($O\underline{C}H_3$); 80.6 ($\underline{C}(CH_3)_3$); 113.8 ($Ar\underline{C}^4H$); 117.8 ($Ar\underline{C}^6H$); 131.5 ($Ar\underline{C}^3H$); 136.0 ($Ar\underline{C}^1$); 136.1 ($Ar\underline{C}^2$); 158.6 ($\underline{C}O_2C(CH_3)_3$); 162.3 ($Ar\underline{C}^5$); 175.7 ($CH_2\underline{C}O$)

IR υ cm^{-1}: 3343 (s) OH; 1685 (s) C=O carbamate, acid

MP: 119-120°C

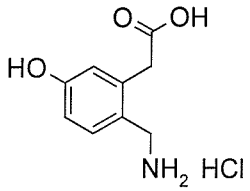
UV (EtOH) λ_{max}: 264 nm

RP-HPLC (λ_{254}): 13.8 min (gradient 1)

m/z (ES -ve)**:** 294.0 $[M-H]^-$ (100%)

HRMS [M+H]: $C_{15}H_{22}O_5N$ (calc) 296.1519; (found) 296.1497

Preparation of 2-[2-(Aminomethyl)-5-hydroxyphenyl]acetic acid•HCl **[49]**



6-Methoxy-1,2,4-trihydroisoquinolin-3-one (2.0 g, 11 mmol) was dissolved in anhydrous CH_2Cl_2 (100 mL) and the solution was cooled to –10°C. BBr_3 (1 M in CH_2Cl_2, 43 mL, 0.11 mmol) in CH_2Cl_2 was added dropwise with stirring. Stirring was continued at –10°C for 1 hour and at room temperature for 2 hours. The reaction was terminated by dropwise addition of H_2O (100 mL). The layers were separated and the organic phase was washed with H_2O (3 x 20 mL) The aqueous layers were combined and concentrated HCl (100 mL) was added and the resulting mixture was heated at reflux for 4 hours. The reaction mixture was evaporated to dryness to give the title compound as a white solid.

Yield: 2.0 g, 97%

R_f : 0.28 (5: 3: 1 $CHCl_3$: MeOH: AcOH)

δ_H (300 MHz, D_2O): 3.67 (2H, s, $C\underline{H}_2CO$); 4.03 (2H, s, $C\underline{H}_2NH_2$); 6.71 (1H, d, $ArC^6\underline{H}$, J = 2 Hz); 6.76 (1H, dd, $ArC^4\underline{H}$, J = 2 Hz, 8 Hz); 7.22 (1H, d, $ArC^3\underline{H}$, J = 8 Hz)

δ_C (75 MHz, D_2O): 40.7 ($\underline{C}H_2CO$); 42.5 ($\underline{C}H_2NH_2$); 117.8 ($Ar\underline{C}^6H$); 120.6 ($Ar\underline{C}^4H$); 126.0 ($Ar\underline{C}^1$); 134.7 ($Ar\underline{C}^3H$); 137.8 ($Ar\underline{C}^2$); 159.0 ($Ar\underline{C}^5$); 178.8 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3211 (br) OH; 1673 (s) C=O acid

MP: 146-149°C

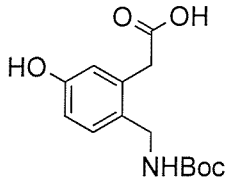
m/z (ES +ve)**:** 182.1 $[M+H]^+$ (100%)

UV (EtOH) λ_{max}: 278 nm

RP-HPLC (λ_{254}): 6.0 min (gradient 1)

HRMS [M+H]: $C_9H_{12}O_3N$ (calc) 182.0825; (found) 182.0817

Preparation of 2-(2-{[(tert-Butoxy)carbonylamino]methyl}-5-hydroxyphenyl)acetic acid **[50]**



Boc_2O (2.5 g, 11 mmol) in dioxane (25 mL) was added to a mixture of 2-[2-(aminomethyl)-5-hydroxyphenyl]acetic acid (2.0 g, 11.0 mmol) and NaOH (34 mL 1M solution) in dioxane (75 mL) cooled to 0°C. The reaction mixture was stirred at room temperature for 15 hours. The dioxane was removed *in vacuo*, the aqueous residue was acidified to pH 1 with 2 M $KHSO_4$ and extracted with EtOAc (3 x 100 mL). The combined organic fractions were washed with H_2O (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (5: 4.5: 0.5 hexane: EtOAc: AcOH) to give the title compound as a pale yellow solid.

Yield: 1.95 g, 63%

R_f: 0.24 (5: 4.5: 0.5 hexane: EtOAc: AcOH)

δ_H (300 MHz, CD_3OD): 1.45 (9H, s, $C(C\underline{H}_3)_3$); 3.65 (2H, s, $C\underline{H}_2CO$); 4.21 (2H, s, $C\underline{H}_2NH$); 6.68-6.71 (2H, m, $ArC^6\underline{H}$, $ArC^4\underline{H}$); 7.14 (1H, d, $ArC^3\underline{H}$, $J = 8$ Hz)

δ_C (75 MHz, CD_3OD): 28.7 ($C(\underline{C}H_3)_3$); 39.3 ($\underline{C}H_2CO$); 42.6 ($\underline{C}H_2NH$); 80.1 ($\underline{C}(CH_3)_3$); 115.1 ($Ar\underline{C}^4H$); 118.4 ($Ar\underline{C}^6H$); 129.7 ($Ar\underline{C}^1$); 131.2 ($Ar\underline{C}^3H$); 135.8 ($Ar\underline{C}^2$); 157.7 ($\underline{C}O_2C(CH_3)_3$); 158.1 ($Ar\underline{C}^5$); 175.7 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3336 (s) OH; 1694 (s) C=O acid; 1639 (s) C=O carbamate

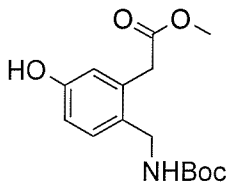
MP: 137-139°C

UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{254}): 11.8 min (gradient 1)

m/z (ES +ve)**:** 282.2 $[M+H]^+$ (80%)

Preparation of Methyl 2-(2-{[(tert-Butoxycarbonyl)amino]methyl}-5-hydroxyphenyl)acetate **[51]**



EDC (688 mg, 3.6 mmol) was added to a mixture of 2-(2-{[(*tert*-butoxycarbonyl)amino]methyl}-5-hydroxyphenyl)acetic acid (1.0 g, 3.6 mmol) and DMAP (440 mg, 3.6 mmol) dissolved in methanol (25 mL). The reaction was stirred at room temperature for 16 hours. The solvent was removed *in vacuo*, H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 100 mL). The combined organic extracts were washed with H_2O (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (7: 3 hexane: EtOAc) followed by recrystallisation from EtOAc and hexane to give the desired compound as white solid.

Yield: 860 mg, 82%

R_f: 0.57 (1: 1 hexane: EtOAc)

δ_H (300 MHz, $CDCl_3$): 1.45 (9H, s, $C(C\underline{H}_3)_3$); 3.58 (2H, s, $C\underline{H}_2CO$); 3.65 (3H, s, $CO_2C\underline{H}_3$); 4.21 (2H, d, $C\underline{H}_2NH$); 6.67-6.69 (2H, m, $ArC^6\underline{H}$, $ArC^4\underline{H}$); 7.10 (1H, d, $ArC^3\underline{H}$, $J = 8$ Hz); 7.6 (1H, s, $O\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 28.7 ($C(\underline{C}H_3)_3$); 39.4 ($\underline{C}H_2CO$); 42.4 ($\underline{C}H_2NH$); 52.5 ($CO_2\underline{C}H_3$); 80.1 ($\underline{C}(CH_3)_3$); 115.1 ($Ar\underline{C}^4H$); 118.4 ($Ar\underline{C}^6H$); 128.3 ($Ar\underline{C}^1$); 131.1 ($Ar\underline{C}^3H$); 133.8 ($Ar\underline{C}^2$); 158.1 ($Ar\underline{C}^5$, $\underline{C}O_2C(CH_3)_3$); 175.2 ($CH_2\underline{C}O_2H$)

IR υ cm^{-1}: 3413 (s) OH; 1726 (s) C=O ester; 1687 (s) C=O carbamate

MP: 94-96°C

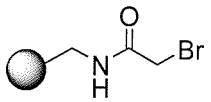
UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{254}): 13.6 min (gradient 1)

m/z (ES +ve): 296.2 $[M+H]^+$ (100%), 313.2 $[M+NH_4]^+$ (90%)

HRMS [M+H]:$C_{15}H_{22}O_5N$ (calc) 296.1489; (found) 296.1497

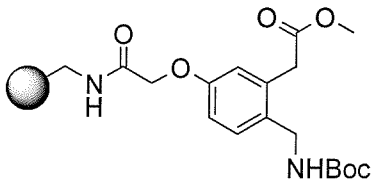
Preparation of 2-Bromo-N-(TentaGel resin)acetamide **[52]**



HOBt (92 mg, 0.675 mmol) was added to a solution of bromoacetic acid (93 mg, 0.675 mmol) in DMF (2 mL) and stirred for 10 min. DIC (85 mg, 0.675 mmol) was added and the mixture was stirred for a further 10 min. The mixture was added to NH_2-Tentagel resin (500 mg, 0.135 mmol, 0.27 mmol/ g NH_2 Rapp Polymere, 130 μM, S 30 132), preswollen in CH_2Cl_2 (3 mL) and the suspension was agitated overnight. The resin was filtered and washed with DMF (3 x 10 mL), CH_2Cl_2 (3 x 10 mL), MeOH (3 x 10 mL) and Et_2O (5 x 10 mL) and dried *in vacuo*. A qualitative ninhydrin test was negative.

IR υ cm^{-1}: 1669 (s) C=O amide

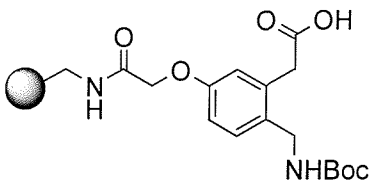
Preparation of Methyl 2-(2-{[(tert-butoxy)carbonylamino]methyl-5-{[(N-TentaGel resin) carbamoyl]methoxy}phenyl)acetate **[53]**



A solution of methyl 2-(2-{[(*tert*-Butoxycarbonyl)amino]methyl}-5-hydroxyphenyl) acetate (200 mg, 0.7 mmol) in MeCN (2 mL) was added to a suspension of Cs_2CO_3 (500 mg, 1.6 mmol) and KI (2 mg, 0.01 mmol) in MeCN (4 mL). 2-Bromo-N-(Tentagel resin)acetamide (600 mg, 0.16 mmol) was added to the reaction and the suspension was heated at reflux overnight with gentle stirring. The resin was filtered and washed with H_2O (5 mL), DMF (3 x 10 mL), CH_2Cl_2 (3 x 10 mL), MeOH (3 x 10 mL) and Et_2O (5 x 10 mL) and dried *in vacuo*.

IR υ cm^{-1}: 1709 (s) C=O ester; 1659 (s) C=O amide, carbamate

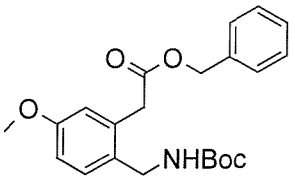
Preparation of 2-(2-{[(tert-butoxy)carbonylamino]methyl-5-{[(N-TentaGel resin) carbamoyl]methoxy}phenyl)acetic acid **[33]**



A solution of $KOSiMe_3$ (96 mg, 0.75 mmol) in anhydrous THF (2 mL) was added to methyl 2-(2-{[(*tert*-butoxy)carbonylamino]methyl-5-{[(N-Tentagel resin)carbamoyl] methoxy}phenyl)acetate resin (600 mg, 0.15 mmol) preswollen in anhydrous THF (4 mL) and agitated overnight under nitrogen. The resin was filtered and washed with 5% citric acid in DMF (3 x 10 mL), DMF (3 x 10 mL), CH_2Cl_2 (3 x 10 mL), MeOH (3 x 10 mL) and Et_2O (3 x 10 mL) and dried *in vacuo*. A qualitative BCG test was positive.

IR υ cm^{-1}: 1703 (s) C=O acid; 1673 (s) C=O amide, carbamate

Phenylmethyl 2-(2-{[(tert-butoxy)carbonylamino]methyl}-5-methoxyphenyl)acetate **[54]**



EDC (71 mg, 0.37 mmol) was added to a solution of [2-(*tert*-butoxy-carbonylaminomethyl)-5-methoxyphenyl]acetic acid (100 mg, 0.34 mmol), DMAP (45 mg, 0.37 mmol) and benzyl alcohol (40 mg, 0.37 mmol) in CH_2Cl_2 (30 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*, H_2O (50 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with H_2O (100 mL), 2 M $KHSO_4$ (50 mL), brine (50 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a yellow oil.

Yield: 54 mg, 50%

R_f: 0.42 (1: 1 EtOAc: hexane)

δ_H (250 MHz, $CDCl_3$): 1.45 (9H, s, $C(C\underline{H}_3)_3$); 3.73 (2H, s, $C\underline{H}_2CO$); 3.78 (3H, s, $OC\underline{H}_3$); 4.27 (2H, d, $C\underline{H}_2NH$, J = 6 Hz); 4.96 (1H, br s, N$\underline{H}$); 5.15 (2H, s, $C\underline{H}_2Ph$); 6.78 (1H, dd, $ArC^4\underline{H}$, J = 8 Hz, 2 Hz); 6.81 (1H, d, $ArC^6\underline{H}$, J = 2 Hz); 7.25-7.36 (6H, m, Ar$C\underline{H}$, $ArC^3\underline{H}$)

δ_C (62.5 MHz, $CDCl_3$): 28.2 ($C(\underline{C}H_3)_3$); 38.7 ($\underline{C}H_2CO$); 42.2 ($\underline{C}H_2NH$); 55.4 ($O\underline{C}H_3$); 67.0 ($\underline{C}H_2Ph$); 79.5 ($\underline{C}(CH_3)_3$); 112.7 ($Ar\underline{C}^4H$); 116.3 ($Ar\underline{C}^6H$); 128.3 (Ar$\underline{C}$H); 128.5 (Ar$\underline{C}$H); 128.7 (Ar$\underline{C}$H); 129.6 ($Ar\underline{C}^1$); 131.2 ($Ar\underline{C}^3H$); 133.9 ($Ar\underline{C}^2$); 135.7 ($Ar\underline{C}CH_2$); 155.7 ($\underline{C}O_2C(CH_3)_3$); 159.2 ($Ar\underline{C}^5$); 171.6 ($CH_2\underline{C}O$)

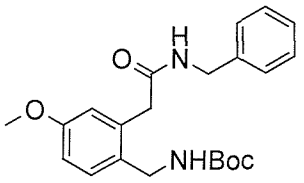
IR υ cm^{-1}: 1710 (s) C=O ester; 1685 (s) C=O carbamate

UV (EtOH) λ_{max}: 264 nm

RP-HPLC (λ_{220}): 18.5 min (gradient 1)

m/z (ES +ve)**:** 386.4 $[M+H]^+$ (60%), 403.4 $[M+NH_4]^+$ (100%), 408.3 $[M+Na]^+$ (40%)

Preparation of 2-(2 {[(tert-butoxy)carbonylamino]methyl}-5-methoxyphenyl) N-benzylacetamide **[55]**



EDC (71 mg, 0.37 mmol) was added to a solution of 2-(*tert*-butoxy-carbonylaminomethyl)-5-methoxyphenyl]acetic acid (100 mg, 0.34 mmol), HOBt (50 mg, 0.37 mmol) and benzylamine (40 mg, 0.37 mmol) in CH_2Cl_2 (30 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*, H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with H_2O (50 mL), 2 M $KHSO_4$ (50 mL), brine (50 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a white solid.

Yield: 60 mg, 55%

R_f: 0.23 (1: 1 EtOAc: hexane)

δ_H (250 MHz, $CDCl_3$): 1.42 (9H, s, $C(C\underline{H}_3)_3$); 3.61 (2H, s, $C\underline{H}_2CO$); 3.77 (3H, s, $OC\underline{H}_3$); 4.25 (2H, d, $C\underline{H}_2NH$, J = 6 Hz); 4.41 (2H, d, $C\underline{H}_2Ph$, J = 6 Hz); 5.21 (1H, br

s, CH_2NH); 6.57 (1H, br s, CONH); 6.78 (1H, dd, ArC^4H, J = 2 Hz, 8 Hz); 6.82 (1H, d, ArC^6H, J = 2 Hz); 7.21-7.32 (6H, m, ArCH, ArC^3H)

δ_C (62.5 MHz, $CDCl_3$): 28.5 ($C(CH_3)_3$); 40.9 (CH_2CO); 42.1 (CH_2NH); 43.8 (CH_2Ph); 55.4 (OCH_3); 79.8 ($C(CH_3)_3$); 113.3 (ArC^4H); 116.3 (ArC^6H); 127.5 (ArCH); 127.7 (ArCH); 128.8 (ArCH); 129.5 (ArC^1); 131.2 (ArC^3H); 135.0 (ArC^2); 138.4 ($ArCCH_2$); 156.2 ($CO_2C(CH_3)_3$); 159.3 (ArC^5); 170.7 (CH_2CONH)

IR υ cm^{-1}: 1655 (s) C=O amide; 1686 (s) C=O carbamate

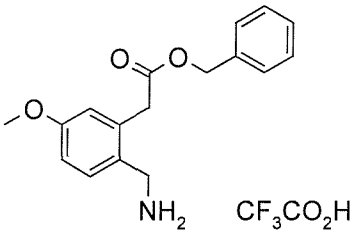
MP: 124-126°C

UV (EtOH) λ_{max}: 254 nm

RP-HPLC (λ_{254}): 16.4 min (gradient 1)

m/z (ES +ve)**:** 385.6 $[M+H]^+$ (100%), 407.6 $[M+Na]^+$ (60%)

Preparation of Phenylmethyl 2-[2-(aminomethyl)-5-methoxyphenyl] acetate•TFA **[56]**



Phenylmethyl 2-(2-{[(*tert*-butoxy)carbonylamino]methyl}-5-methoxyphenyl)acetate (5.0 mg, 13 μmol) was dissolved in CH_2Cl_2 (500 μL) and TFA (500 μL) was added dropwise. The solution was stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* to give the title compound as a colourless oil.

Yield: 5.3 mg, 100%

R_f: 0.19 (5: 3: 1: $CHCl_3$: MeOH: AcOH)

δ_H (400 MHz, $CDCl_3$): 3.60 (2H, s, CH_2CO); 3.70 (3H, s, OCH_3); 4.09 (2H, s, CH_2NH); 5.06 (2H, s, CH_2Ph); 6.69 (1H, br s, ArC^6H); 6.81 (dd, 1H, ArC^4H, J = 2 Hz, 8 Hz); 7.20-7.29 (5H, m, ArCH); 7.39 (1H, d, ArC^3H, J = 8 Hz) 8.28 (2H, s, NH_2)

δ_C (100 MHz, $CDCl_3$): 39.5 (CH_2CO); 41.5 (CH_2NH); 55.7 (OCH_3); 68.6 (CH_2Ph); 114.1 (ArC^4H); 117.5 (ArC^6H); 124.2 (ArC^1); 128.9 (ArCH); 129.1 (ArCH); 129.2 (ArCH); 134.1 (ArC^3H); 135.0 (ArC^2); 143.6 ($ArCCH_2$); 159.2 (ArC^5); 173.9 (CH_2CO)

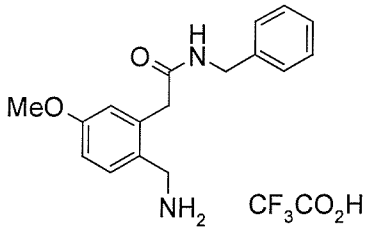
IR υ cm^{-1}: 1675 (s) C=O ester

UV (EtOH): λ_{max}: 258 nm

RP-HPLC (λ_{254}): 7.46 min (gradient 2)

m/z (ES +ve)**:** 286.2 $[M+H]^+$ (100%)

Preparation of 2-[2-(aminomethyl)-5-methoxyphenyl] N-phenylmethyl acetamide•TFA **[57]**



2-(2-{[(*tert*-Butoxy)carbonylamino]methyl}-5-methoxyphenyl)*N*-phenylmethylacetamide (5.0 mg, 13 μmol) was dissolved in CH_2Cl_2 (500 μL) and TFA (500 μL) was added dropwise. The solution was stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* to give the title compound as a colourless oil.

Yield: 5.3 mg, 100%

R_f: 0.70 (5: 3: 1: $CHCl_3$: MeOH: AcOH)

δ_H (300 MHz, $CDCl_3$): 3.55 (2H, s, $C\underline{H}_2CO$); 3.71 (3H, s, $OC\underline{H}_3$); 4.0 (2H, s, $C\underline{H}_2NH$); 4.28 (2H, s, $C\underline{H}_2Ph$); 6.69 (1H, s, N$\underline{H}$); 6.78-6.82 (2H, m, $ArC^6\underline{H}$, $ArC^4\underline{H}$); 7.16-7.27 (6H, m, Ph$C\underline{H}$, $ArC^3\underline{H}$); 8.14 (2H, s, $N\underline{H}_2$)

δ_C (75 MHz, $CDCl_3$): 40.8 ($\underline{C}H_2CO$); 41.7 ($\underline{C}H_2NH$); 44.3 ($\underline{C}H_2Ph$); 55.4 ($O\underline{C}H_3$); 113.5 ($Ar\underline{C}^4H$); 116.9 ($Ar\underline{C}^6H$); 123.7 ($Ar\underline{C}CH_2$); 127.8 ($Ar\underline{C}H$); 128.0 ($Ar\underline{C}H$); 128.9 ($Ar\underline{C}H$); 133.5 ($Ar\underline{C}^3H$); 136.2 ($Ar\underline{C}^2$); 136.9 ($Ar\underline{C}^1$); 160.9 ($Ar\underline{C}^5$); 172.4 ($CH_2\underline{C}O$)

IR υ cm^{-1}: 1639 (s) C=O amide

UV (EtOH): λ_{max}: 258 nm

RP-HPLC (λ_{254}): 5.44 min (gradient 2)

m/z (ES +ve)**:** 285.2 $[M+H]^+$ (100%)

8.4.2] Evaluation of the linker in solution

Evaluation of the benzyl ester model **[56]**

50 mM phosphate buffer pH 7.5 (5 mL) was added to the phenylmethyl 2-[2-(aminomethyl)-5-methoxyphenyl] acetate•TFA (5 mg, 13 μmol) and the solution was stirred thoroughly and left to stand at room temperature. Aliquots (160 μL) were removed periodically, the reaction quenched by adding to 2% TFA (MeCN: H_2O 25: 75, 40 μL) and the samples were analysed by RP-HPLC (gradient 2).

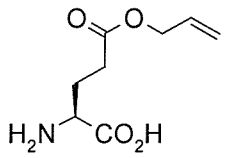
Evaluation of the benzyl amide model **[57]**

50 mM phosphate buffer pH 7.5 (5 mL) was added to 2-[2-(aminomethyl)-5-methoxyphenyl] N-phenylmethyl acetamide•TFA (5 mg, 13 μmol) and the solution was stirred thoroughly and left to stand at room temperature. Aliquots (160 μL) were removed periodically, the reaction quenched by adding to 2% TFA (MeCN: H_2O 25: 75, 40 μL) and the samples were analysed by RP-HPLC (gradient 2).

8.5] Chapter Four

8.5.1] Linker preparation and synthesis of model compounds

Preparation of H-Glu-(OAllyl)-OH **[62]** [159]



Chlorotrimethylsilane (54.0 mL, 0.4 mmol) was added dropwise to a stirred suspension of H-Glu-(OH)-OH (25 g, 0.16 mol) in anhydrous allyl alcohol (750 mL, 11.0 mol) under N_2. The resulting solution was stirred for eighteen hours at room temperature. Diethyl ether (1 L) was added to the solution and the resultant white precipitate was collected by filtration and dried *in vacuo.*

Yield: 24 g, 75.5%

R_f: 0.75 (5: 3: 1 $CHCl_3$: CH_3OH: AcOH)

δ_H (300 MHz, D_2O): 2.17 (2H, m, β $C\underline{H}_2$); 2.62 (2H, t, γ $C\underline{H}_2$, J = 7 Hz); 4.04 (1H, t, α $C\underline{H}$, J = 7 Hz); 4.55 (2H, dd, $C\underline{H}_2$-CH=CH_2, J = 1 Hz, 6 Hz); 5.22 (1H, dd, CH_2-CH=C$\underline{H}$, *cis*, J = 11 Hz, 2 Hz); 5.23 (1H, ddd, CH_2-CH=C$\underline{H}$, *trans*, J = 17 Hz, 2 Hz, 1 Hz); 5.86 (1H, ddt, CH_2-C$\underline{H}$=CH_2, J = 6 Hz, 11 Hz, 17 Hz)

δ_C (75 MHz, D_2O): 27.4 (β $\underline{C}H_2$); 32.2 (γ $\underline{C}H_2$); 54.7 (α $\underline{C}H$); 68.8 ($\underline{C}H_2$-CH=CH_2); 121.3 (CH_2-CH=$\underline{C}H_2$); 134.3 (CH_2-$\underline{C}$H=CH_2); 174.1 ($\underline{C}O_2CH_2$-CH=CH_2); 176.7 ($\underline{C}O_2H$)

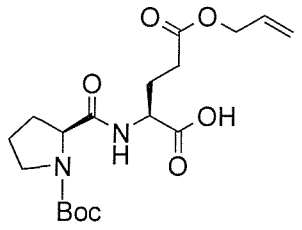
IR υ cm^{-1}: 3151 (s) OH; 2976 (s) aliphatic C-H; 2911 (s) aliphatic C-H; 1741 (s) C=O ester; 1713 (s) C=O acid

MP: 129-131°C [lit mp 130-132][159]

RP-HPLC (λ_{220}): 6.74 min (Gradient 1)

m/z (ES +ve): 187.8 $[M+H]^+$ (80%)

Preparation of Boc-Pro-Glu-(OAllyl)-OH **[63]**



H-Glu-(OAllyl)-OH (5.0 g, 26.7 mmol) was dissolved in CH_2Cl_2 (50 mL), and triethylamine (7.4 mL, 53 mmol) was added. Boc-Pro-OSu (8.3 g, 26.7 mmol) was added and the reaction was stirred at room temperature for 48 hours. H_2O (500 mL) was added and acidified to pH 1 with 2 M $KHSO_4$ (100 mL) and the mixture was extracted with CH_2Cl_2 (3 x 100 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (6.5: 3: 0.5 hexane: EtOAc: AcOH) to give the desired product as a pale yellow oil.

Yield: 4.90 g, 50%

R_f: 0.23 (6.5: 3: 0.5 hexane: EtOAc: AcOH)

δ_H (300 MHz, $CDCl_3$): 1.43 (9H, s, $C(C\underline{H}_3)_3$); 1.87-2.19 (4H, m, γ $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Pro); 2.19-2.27 (2H, m, β $C\underline{H}_2$ Glu); 2.41-2.48 (2H, m, γ $C\underline{H}_2$ Glu); 3.36-3.48 (2H, m, δ $C\underline{H}_2$ Pro); 4.20-4.29 (1H, br s, α C<u>H</u> Pro); 4.55-4.57 (3H, m, $C\underline{H}_2$-CH=CH_2, α C<u>H</u> Glu); 5.22 (1H, d, CH_2-CH=C<u>H</u>, *cis*, J = 11 Hz); 5.28 (1H, d, CH_2-CH=C<u>H</u>, *trans*, J = 17 Hz); 5.86 (1H, ddt, CH_2-C<u>H</u>=CH_2, J = 6, 11, 17 Hz); 9.41(1H, s, $CO_2\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 23.7 (γ $\underline{C}H_2$ Pro); 24.5 (γ $\underline{C}H_2$ Pro); 27.2 (β $\underline{C}H_2$ Glu); 28.3 ($C(\underline{C}H_3)_3$); 30.2 (β $\underline{C}H_2$ Pro); 32.2 (γ $\underline{C}H_2$ Glu); 47.1 (δ $\underline{C}H_2$ Pro); 51.5 (α $\underline{C}H$ Glu); 59.8 (α $\underline{C}H$ Pro); 61.0 (α $\underline{C}H$ Pro); 65.4 ($\underline{C}H_2$-CH=CH_2); 80.8 ($\underline{C}(CH_3)_3$); 118.4 (CH_2-CH=$\underline{C}H_2$); 132.1 (CH_2-$\underline{C}H$=CH_2); 155.2 ($\underline{C}O_2C(CH_3)_3$); 172.6 ($\underline{C}O_2CH_2$-CH=$CH_2$); 173.6, 174.0 ($\underline{C}ONH$, $\underline{C}O_2H$)

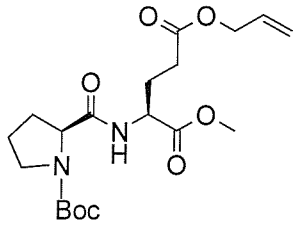
IR υ cm^{-1}: 3147 (br) OH; 2976 (s) aliphatic C-H; 2900 (s) aliphatic C-H; 1713 (s) C=O ester, acid; 1659 (s) C=O carbamate, amide

RP-HPLC (λ_{220}): 12.7 min (Gradient 1)

m/z (ES +ve): 385.1 $[M+H]^+$ (30%), 786.2 $[2M+NH_4]^+$ (100%), 791.2 $[2M+Na]^+$ (90%)

HRMS $[M+H]^+$: $C_{18}H_{29}O_7N_2$ (calc) 385.1975; (found) 385.1974

Preparation of Boc-Pro-Glu-(OAllyl)-OCH_3 **[64]**



DCC (270 mg, 1.3 mmol) was added to a solution of Boc-Pro-Glu-(OAllyl)-OH (500 mg, 1.3 mmol), DMAP (158 mg, 1.3 mmol) and methanol (41 mg, 1.3 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*, H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with H_2O (100 mL), 2 M $KHSO_4$ (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a colourless oil.

Yield: 490 mg, 94%

R_f: 0.24 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.57 (9H, s, $C(C\underline{H}_3)_3$); 1.62-2.17 (4H, m, γ $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Pro); 2.18-2.25 (2H, m, β $C\underline{H}_2$ Glu); 2.41-2.44 (2H, m, γ $C\underline{H}_2$ Glu); 3.20-3.51 (2H, m, δ $C\underline{H}_2$ Pro); 3.74 (3H, s, $CO_2C\underline{H}_3$); 4.21-4.31 (1H, br s, α $C\underline{H}$ Pro); 4.40-4.65 (3H, m, $C\underline{H}_2CH=CH_2$, α $C\underline{H}$ Glu); 5.22 (2H, d, $CH_2CH=C\underline{H}$, *cis*, J = 10 Hz); 5.30 (2H, d, $CH_2CH=C\underline{H}$, *trans*, J = 17 Hz); 5.86 (1H, ddt, $CH_2C\underline{H}=CH_2$, J = 6 Hz, 10 Hz, 17 Hz); 6.74 (1H, s, N$\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 23.6 (γ $\underline{C}H_2$ Pro); 27.1 (β $\underline{C}H_2$ Glu); 28.2 ($C(\underline{C}H_3)_3$); 29.9 (β $\underline{C}H_2$ Pro); 31.1 (γ $\underline{C}H_2$ Glu); 47.0 (δ $\underline{C}H_2$ Pro); 51.4 ($OC\underline{H}_3$); 52.4 (α $\underline{C}H$ Glu); 61.2 (α $\underline{C}H$ Pro); 65.2 ($\underline{C}H_2CH=CH_2$); 80.7 ($\underline{C}(CH_3)_3$); 118.2 ($CH_2CH=\underline{C}H_2$); 132.0 ($CH_2\underline{C}H=CH_2$); 155.8 ($\underline{C}O_2C(CH_3)_3$); 171.9 ($\underline{C}O_2CH_2CH=CH_2$); 172.2 ($\underline{C}OCH_3$); 172.8 ($\underline{C}ONH$)

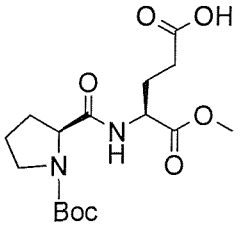
IR υ cm^{-1}: 1734 (s) C=O allyl ester, methyl ester; 1684 (s) C=O carbamate, amide

RP-HPLC (λ_{220}): 14.4 min (Gradient 1)

m/z (ES +ve): 399.1 $[M+H]^+$(90%), 421.4 $[M+Na]^+$ (100%)

HRMS $[M+H]^+$: $C_{19}H_{31}O_7N_2$ (calc) 399.2153; (found) 399.2131

Preparation of Boc-Pro-Glu-(OH)-OCH_3 **[65]**



$Pd(PPh_3)_4$ (145 mg, 1.25 mmol) was added to a solution of Boc-Pro-Glu-(OAllyl)-OCH_3 (500 mg, 1.25 mmol), in anhydrous THF (30 mL) under nitrogen. Morpholine (110 mg, 1.25 mmol) was added dropwise and the mixture was stirred at room temperature for 1 hour. The solvent was removed *in vacuo*, and the residue was redissolved in CH_2Cl_2 (100 mL), washed with 2 M $KHSO_4$ (2 x 50 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a colourless oil.

Yield: 313 mg, 64%

R_f: 0.24 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.45 (9H, s, $C(C\underline{H}_3)_3$); 1.85-2.12 (6H, m, γ $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Glu); 2.35-2.38 (2H, m, γ $C\underline{H}_2$ Glu); 3.30-3.45 (2H, m, δ $C\underline{H}_2$ Pro); 3.72 (3H, s, $CO_2C\underline{H}_3$); 4.20-4.31 (1H, br s, α $C\underline{H}$ Pro); 4.39-4.45 (1H, br s, α $C\underline{H}$ Glu)

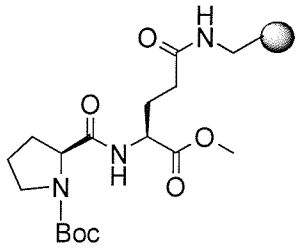
δ_C (75 MHz, $CDCl_3$): 23.5 (γ $\underline{C}H_2$ Pro); 24.5 (β $\underline{C}H_2$ Glu); 26.5 (β $\underline{C}H_2$ Pro); 28.4 ($C(\underline{C}H_3)_3$); 31.2 (γ $\underline{C}H_2$ Glu); 46.9 (δ $\underline{C}H_2$ Pro); 52.0 (α $\underline{C}H$ Glu); 52.4 ($CO_2\underline{C}H_3$); 61.0 (α $\underline{C}H$ Pro); 81.4 ($\underline{C}(CH_3)_3$); 155.5 ($\underline{C}O_2C(CH_3)_3$); 171.5 ($\underline{C}O_2CH_3$); 172.5, 177.9 ($\underline{C}ONH$, $\underline{C}O_2H$)

IR υ cm^{-1}: 3291 (br) OH; 1737 (s) C=O allyl ester, acid; 1673 (s) C=O amide, carbamate

RP-HPLC (λ_{220}): 10.9 min (Gradient 1)

m/z (ES +ve): 359.3 $[M+H]^+$ (100%), 734.5 $[2M+NH_4]^+$ (40%)

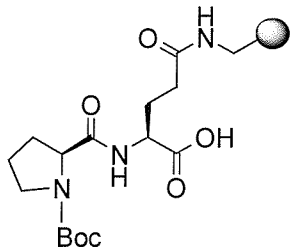
Preparation of Boc-Pro-Glu-(NH-TentaGel resin)-OMe **[66]**



HOBt (50 mg, 0.37 mmol) was added to a solution of Boc-Pro-Glu-(OH)-OMe (75 mg, 0.2 mmol) in DMF (1 mL) and the mixture was stirred for 10 min. DIC (50 mg, 0.4 mmol) was added and the mixture was stirred for a further 10 min. The mixture was added to NH_2-TentaGel resin (500 mg, 0.135mol, 0.27 mmol/g NH_2 Rapp Polymere 130 μM S 30 132) preswollen in CH_2Cl_2 (4 mL) and agitated overnight. The resin was filtered and washed with DMF (3 x 10 mL), CH_2Cl_2 (3 x 10 mL), MeOH (3 x 10 mL) and Et_2O (3 x 10 mL) and dried *in vacuo*. A qualitative ninhydrin test was negative.

IR υ cm^{-1}: 1740 (s) C=O ester; 1655 (s) C=O amides, carbamate

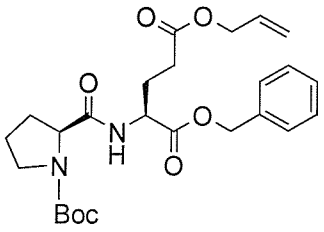
Preparation of Boc-Pro-Glu-(NH-TentaGel resin)-OH **[57]**



A solution of $KOSiMe_3$ (86 mg, 0.675 mmol) in anhydrous THF (2 mL) was added to Boc-Pro-Glu-(NH-TentaGel)-OMe (500 mg, 0.135 mmol) preswollen in anhydrous THF (3 mL) and agitated overnight under nitrogen. The resin was filtered and washed with 5% citric acid in DMF (3 x 10 mL), DMF (3 x 10 mL), CH_2Cl_2 (3 x 10 mL), MeOH (3 x 10 mL) and Et_2O (5 x 10 mL) and dried *in vacuo.* A qualitative BCG test was positive.

IR υ cm^{-1}: 1735 (s) C=O acid; 1655 (s) C=O amides, carbamate

Preparation of Boc-Pro-Glu-(OAllyl)-OBn **[67]**



DCC (53 mg, 0.26 mmol) was added to a solution of Boc-Pro-Glu-(OAllyl)-OH (100 mg, 0.26 mmol), DMAP (32 mg, 0.26 mmol) and benzyl alcohol (31 mg, 0.26 mmol) in CH_2Cl_2 (50 mL) The reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo* and H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed H_2O (100 mL), 2 M $KHSO_4$ (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the desired compound as a colourless oil.

Yield: 62 mg, 50%

R_f: 0.55 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.43 (9H, s, $C(C\underline{H}_3)_3$); 1.64-1.92 (4H, m, γ $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Pro); 2.17-2.22 (2H, m, β $C\underline{H}_2$ Glu); 2.25-2.43 (2H, m, γ $C\underline{H}_2$ Glu); 3.23-3.43 (2H, m, δ $C\underline{H}_2$ Pro); 4.20-4.29 (1H, br s, α $C\underline{H}$ Pro); 4.54 (2H, d, $C\underline{H}_2CH{=}CH_2$, J = 6 Hz); 4.60-4.63 (1H, br s, α $C\underline{H}$ Glu); 5.15 (2H, s, $C\underline{H}_2Ph$); 5.20 (1H, dd, CH_2-CH=$C\underline{H}$, *cis*, J = 11 Hz, 2 Hz); 5.27 (1H, dd, CH_2-CH=$C\underline{H}$, *trans*, J = 17 Hz, 2 Hz); 5.86 (1H, ddt, $CH_2C\underline{H}{=}CH_2$, J = 6 Hz, 11 Hz, 17 Hz); 7.31-7.38 (5H, m, Ar$C\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 24.7 (γ $\underline{C}H_2$ Pro); 27.3 (β $\underline{C}H_2$ Glu); 28.5 ($C(\underline{C}H_3)_3$); 30.1 (β $\underline{C}H_2$ Pro); 33.9 (γ $\underline{C}H_2$ Glu); 47.2 (δ $\underline{C}H_2$ Pro); 51.7 (α $\underline{C}H$ Glu); 60.0 (α $\underline{C}H$ Pro); 65.2 ($\underline{C}H_2CH{=}CH_2$); 67.3 ($\underline{C}H_2Ph$); 80.8 ($\underline{C}(CH_3)_3$); 118.5 ($CH_2CH{=}\underline{C}H_2$); 127.1 (Ar$\underline{C}$H); 127.6 (Ar$\underline{C}$H); 128.4 (Ar$\underline{C}$H); 128.6 (Ar$\underline{C}$H); 132.1 ($CH_2\underline{C}H{=}CH_2$); 135.4 (Ar$\underline{C}CH_2$); 155.8 ($\underline{C}O_2C(CH_3)_3$); 171.5 ($\underline{C}O_2CH_2CH{=}CH_2$); 171.5 ($\underline{C}O_2CH_2Ph$); 172.4 ($\underline{C}$ONH)

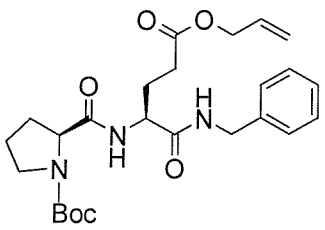
IR: υ cm^{-1}: 1737 (s) C=O allyl ester; 1697 (s) benzyl ester; 1670 (s) C=O amide, carbamate

RP-HPLC (λ_{220}): 17.1 min (Gradient 1)

m/z (ES +ve): 475.4 $[M+H]^+$ (100%), 497.4 $[M+Na]^+$ (70%)

HRMS $[M+H]^+$:$C_{25}H_{35}O_7N_2$ (calc) 475.2442; (found) 475.2444

Preparation of Boc-Pro-Glu-(OAllyl)-NHBn **[68]**



DCC (110 mg, 0.52 mmol) was added to a solution of Boc-Pro-Glu-(OAllyl)-OH (200 mg, 0.52 mmol), HOBt (70.2 mg, 0.52 mmol) and benzylamine (60 mg, 0.52 mmol) in CH_2Cl_2 (50 mL). The mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*, H_2O (100 mL) was added and the mixture wasextracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with H_2O (100 mL), 2 M $KHSO_4$ (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a yellow oil.

Yield: 70 mg, 68%

R_f: 0.52 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.34 (9H, s, $C(C\underline{H}_3)_3$); 1.83-1.85 (2H, m, γ $C\underline{H}_2$ Pro); 2.05-2.14 (4H, m, β $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Glu); 2.45-2.50 (2H, m, γ $C\underline{H}_2$ Glu); 3.35-3.51 (2H, m, δ $C\underline{H}_2$ Pro); 4.16-4.18 (1H, br s, α $C\underline{H}$ Pro); 4.34-4.49 (3H, m, α $C\underline{H}$ Glu, $C\underline{H}_2Ph$); 4.51 (2H, d, $C\underline{H}_2CH=CH_2$, J = 6 Hz); 5.22 (1H, dd, $CH_2CH=C\underline{H}$, *cis*, J =2 Hz, 10 Hz); 5.28 (1H, dd, $CH_2CH=C\underline{H}$, *trans*, J = 2 Hz, 17 Hz); 5.86 (1H, ddt, $CH_2C\underline{H}=CH_2$, J = 6 Hz, 10 Hz, 17 Hz); 7.22-7.27 (5H, m, Ar$C\underline{H}$); 7.50-7.57 (1H, br s, N$\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 24.7 (γ $\underline{C}H_2$ Pro); 26.5 (β $\underline{C}H_2$ Glu); 28.3 ($C(\underline{C}H_3)_3$); 29.7 (β $\underline{C}H_2$ Pro); 30.6 (γ $\underline{C}H_2$ Glu); 43.5 ($\underline{C}H_2Ph$); 47.3 (δ $\underline{C}H_2$ Pro); 53.3 (α $\underline{C}H$ Glu); 61.0 (α $\underline{C}H$ Pro); 65.6 ($\underline{C}H_2CH=CH_2$); 80.8 ($\underline{C}(CH_3)_3$); 118.7 ($CH_2CH=\underline{C}H_2$); 127.5 (Ar$\underline{C}H$); 127.5 (Ar$\underline{C}H$); 128.6 (Ar$\underline{C}H$); 128. (Ar$\underline{C}H$); 131.9 ($CH_2\underline{C}H=CH_2$); 138.5 ($Ar\underline{C}CH_2$); 155.8 ($\underline{C}O_2C(CH_3)_3$); 171.0 ($\underline{C}O_2CH_2CH=CH_2$); 172.7, 174.2 ($\underline{C}$ONH, $\underline{C}ONHCH_2Ph$)

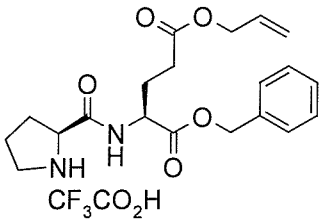
IR: υ cm^{-1}:1737 (s) C=O ester; 1649 (s) C=O amides, carbamate

RP-HPLC (λ_{220}): 15.1 min (Gradient 1)

m/z (ES +ve): 474.6 $[M+H]^+$ (100%), 947.9 $[2M+H]^+$ (40%)

HRMS $[M+H]^+$: $C_{25}H_{36}O_6N_3$ (calc) 474.2634; (found) 474.2604

Preparation of Pro-Glu-(OAllyl)-OBn•TFA **[69]**



Boc-Pro-Glu-(OAllyl)-OBn (7 mg, 15 μmol) was dissolved in CH_2Cl_2 (500 μL) and TFA (500 μL) was added dropwise. The solution was stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* to give the title compound as a colourless oil.

Yield: 7.5 mg, 100%

R_f: 0.23 (90: 8: 2 $CHCl_3$: MeOH: AcOH)

δ_H (300 MHz, $CDCl_3$): 1.90-2.21 (6H, m, γ C$\underline{H}_2$ Pro, β C$\underline{H}_2$ Pro, β C$\underline{H}_2$ Glu); 2.39-2.41 (2H, m, γ C$\underline{H}_2$ Glu); 3.40-3.44 (2H, m, δ C$\underline{H}_2$ Pro); 4.54-4.68 (4H, m, α C$\underline{H}$ Pro, α C$\underline{H}$ Glu, $C\underline{H}_2CH=CH_2$); 5.09-5.32 (4H, m, $C\underline{H}_2Ph$, $CH_2CH=C\underline{H}_2$); 5.85 (1H, ddt, $CH_2C\underline{H}=CH_2$, J = 6 Hz, 10 Hz, 17 Hz); 7.27-7.34 (5H, m, ArC$\underline{H}$); 7.78 (1H, d, N$\underline{H}$, J = 7 Hz); 8.01 (1H, d, N$\underline{H}$, J = 7 Hz)

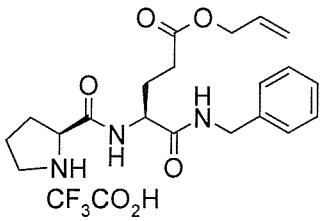
δ_C (75 MHz, $CDCl_3$): 24.4 (γ $\underline{C}H_2$ Pro); 25.9 (β $\underline{C}H_2$ Glu); 26.4 (β $\underline{C}H_2$ Pro); 30.2 (γ $\underline{C}H_2$ Glu); 47.1 (δ $\underline{C}H_2$ Pro); 52.7 (α $\underline{C}$H Glu); 59.7 (α $\underline{C}$H Pro); 65.8 ($\underline{C}H_2CH=CH_2$); 67.8 ($\underline{C}H_2Ph$); 118.6 ($CH_2CH=\underline{C}H_2$); 128.2 (Ar$\underline{C}$H); 128.4 (Ar$\underline{C}$H); 128.6 (Ar$\underline{C}$H); 131.6 ($CH_2\underline{C}H=CH_2$); 134.6 (Ar$\underline{C}CH_2$); 169.0 ($\underline{C}O_2CH_2CH=CH_2$); 170.5 ($\underline{C}O_2CH_2Ph$); 173.1 ($\underline{C}$ONH)

IR υ cm^{-1}: 1735 (s) C=O esters; 1699 (s) C=O amide

RP-HPLC (λ_{220}): 11.8 min (Gradient 1)

m/z (ES +ve): 375.4 $[M+H]^+$ (100%)

Preparation of Pro-Glu-(OAllyl)-NHBn•TFA **[70]**



Boc-Pro-Glu-(OAllyl)-NHBn (7 mg, 15 μmol) was dissolved in CH_2Cl_2 (500 μL) and TFA (500 μL) was added dropwise. The solution was stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* to give the title compound a colourless oil.

Yield: 7.5 mg, 100%

R_f: 0.38 (5: 3: 1: $CHCl_3$: MeOH: AcOH)

δ_H (300 MHz, $CDCl_3$): 1.94-2.01 (6H, m, γ C$\underline{H}_2$ Pro, β C$\underline{H}_2$ Pro, β C$\underline{H}_2$ Glu); 2.36-2.39 (2H, m, γ C$\underline{H}_2$ Glu); 3.29-3.35 (2H, m, δ C$\underline{H}_2$ Pro); 4.34-4.64 (6H, m, α C$\underline{H}_2$ Pro, α C$\underline{H}$ Glu, C$\underline{H}_2$Ph, C$\underline{H}_2$CH=CH$_2$); 5.19-5.29 (2H, m, CH$_2$CH=C$\underline{H}_2$); 5.85 (1H, ddt, CH$_2$C$\underline{H}$=CH$_2$, J = 6 Hz, 10 Hz, 17 Hz); 7.23-7.29 (5H, m, ArC$\underline{H}$); 8.00 (1H, br s, N$\underline{H}$); 8.23 (1H, br s, N$\underline{H}$); 8.46 (1H, br s, N$\underline{H}$)

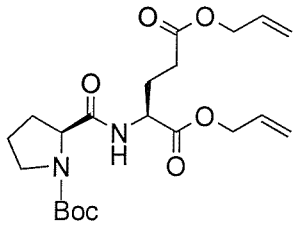
δ_C (75 MHz, $CDCl_3$): 24.4 (γ $\underline{C}H_2$ Pro); 27.8 (β $\underline{C}H_2$ Glu); 29.9 (β $\underline{C}H_2$ Pro); 30.1 (γ $\underline{C}H_2$ Glu); 43.6 ($\underline{C}H_2$Ph); 46.6 (δ $\underline{C}H_2$ Pro); 53.2 (α $\underline{C}$H Glu); 59.7 (α $\underline{C}$H Pro); 65.7 ($\underline{C}H_2$CH=CH$_2$); 118.7 (CH$_2$CH=$\underline{C}H_2$); 127.6 (Ar$\underline{C}$H); 128.1 (Ar$\underline{C}$H); 128.7 (Ar$\underline{C}$H); 131.9 (CH$_2\underline{C}$H=CH$_2$); 138.0 (COOCH$_2\underline{C}$Ar); 169.0 ($\underline{C}$ONH); 171.3 ($\underline{C}$OOCH$_2$CH=CH$_2$); 173.1 ($\underline{C}$OOCH$_2$CAr).

IR υ cm^{-1}: 1724 (s) C=O allyl ester; 1653 (s) C=O amides

RP-HPLC (λ_{220}): 10.7 min (Gradient 1)

m/z (ES +ve): 374.4 $[M+H]^+$ (100%)

Preparation of Boc-Pro-Glu-(OAllyl)-OAllyl **[75]**



DCC (260 mg, 1.25 mmol) was added to a solution of Boc-Pro-OH (270 mg, 1.25 mmol), HOBt (170 mg, 1.25 mmol) and H-Glu-(OAllyl)-OAllyl (500 mg, 1.25 mmol) in CH_2Cl_2 (50 mL), and DMF (10 mL). The reaction mixture was stirred at room temperature overnight. The solvent was removed *in vacuo*, H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (100 mL), H_2O (6 x 100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a colourless oil.

Yield: 250 mg, (60%)

R_f: 0.60 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.43 (9H, s, $C(C\underline{H}_3)_3$); 1.88-2.26 (6H, m, γ $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Glu); 2.38-2.48 (2H, m, γ $C\underline{H}_2$ Glu); 3.31-3.45 (2H, m, δ $C\underline{H}_2$ Pro); 4.26-4.28 (1H, br s, α $C\underline{H}$ Pro); 4.55-4.63 (5H, m, $C\underline{H}_2CH=CH_2$, α $C\underline{H}$ Glu); 5.21-5.35 (4H, m, $CH_2CH=C\underline{H}_2$); 5.83 (2H, m, $CH_2C\underline{H}=CH_2$)

δ_C (75 MHz, $CDCl_3$): 24.3 (γ $\underline{C}H_2$ Pro); 26.2 (β $\underline{C}H_2$ Glu); 27.2 (β $\underline{C}H_2$ Pro); 28.2 ($C(\underline{C}H_3)_3$); 30.1 (γ $\underline{C}H_2$ Glu); 47.1 (δ $\underline{C}H_2$ Pro); 51.6 (α $\underline{C}H$ Glu); 60.0 (α $\underline{C}H$ Pro); 65.2 ($\underline{C}H_2CH=CH_2$); 66.1 ($\underline{C}H_2CH=CH_2$); 80.6 ($\underline{C}(CH_3)_3$); 118.3 ($CH_2CH=\underline{C}H_2$); 119.0 ($CH_2CH=\underline{C}H_2$); 131.4 ($CH_2\underline{C}H=CH_2$); 131.9 ($CH_2\underline{C}H=CH_2$); 155.6 ($\underline{C}O_2C(CH_3)_3$); 169.8 ($\underline{C}O_2CH_2CH=CH_2$); 170.6 ($\underline{C}O_2CH_2CH=CH_2$); 172.4 ($\underline{C}ONH$)

IR υ cm^{-1}: 1737 (s) C=O allyl ester; 1679 (s) C=O amide, carbamate

RP-HPLC (λ_{220}): 15.7 min (Gradient 1)

m/z (ES +ve): 425.2 $[M+H]^+$ (100%), 447.2 $[M+Na]^+$ (30%)

8.5.2] Evaluation of the linker in solution

Evaluation of benzyl ester model **[69]**

MeCN (500 μL) and 50 mM phosphate buffer pH 7.5 (9.5 mL) were added to Pro-Glu-(OAllyl)-OBn•TFA (7.5 mg, 15 μmol) and the solution was stirred thoroughly and left to stand at room temperature. Aliquots (300 μL) were removed periodically, quenched with 2% TFA (MeCN: H_2O 25: 75, 100 μL) and analysed by RP-HPLC (gradient 1).

Evaluation of benzyl amide model **[70]**

MeCN (500 μL) and 50mM phosphate buffer pH 7.5 (9.5 mL) were added to Pro-Glu-(OAllyl)-NHBn•TFA (7.5 mg, 15 μmol) and the solution was stirred thoroughly and left to stand at room temperature. Aliquots (300 μL) were removed periodically, quenched with 2% TFA (MeCN: H_2O 25: 75, 100 μL) and analysed by RP-HPLC (gradient 1).

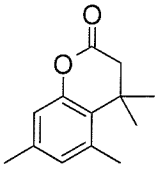
Evaluation of allyl ester model **[75]**

Boc-Pro-Glu-(OAllyl)-OAllyl (10 mg, 23 μmol) was dissolved in CH_2Cl_2 (1 mL) and TFA (1 mL) was added dropwise. The solution was stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo*. The residue was added to 50 mM phosphate buffer pH 8 (4.5 mL) and MeCN (500 μL) and the solution was shaken thoroughly and left to stand at room temperature. Aliquots (300 μL) were removed periodically, quenched with 2% TFA (MeCN: H_2O 25: 75, 100 μL) and analysed by RP-HPLC (gradient 1).

8.6] Chapter Five

8.6.1] Linker preparation and synthesis of model compounds

Preparation of 1,1,6,8-tetramethylisochroman-3-one **[81]**[142]



3,5-Dimethylphenol (20 g, 164 mmol) was added to a solution of methyl 3,3-dimethylacrylate (20 g, 196 mmol) in methane sulfonic acid (20 mL) and stirred for 12 hours at 70°C. After addition of H_2O (500 mL) the mixture was extracted with EtOAc (3 x 400 mL). The combined organic fractions were washed with 5% aqueous $NaHCO_3$ (3 x 200 mL), brine (300 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by recrystallisation from EtOAc and hexane to give the title compound as white prisms.

Yield: 20 g, 60%

R_f: 0.71 (2: 1 hexane: EtOAc)

δ_H (300 MHz, $CDCl_3$): 1.44 (6H, s, $(C\underline{H}_3)_2$); 2.27 (3H, s, $ArC^8C\underline{H}_3$); 2.47 (3H, s, $ArC^6C\underline{H}_3$); 2.59 (2H, s, $C\underline{H}_2$); 6.7 (2H, s, $ArC^5\underline{H}$, $ArC^7\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 20.7 ($ArC^8\underline{C}H_3$); 23.2 ($ArC^6\underline{C}H_3$); 27.9 ($C(\underline{C}H_3)_2$); 35.4 ($\underline{C}(CH_3)_2$); 45.9 ($\underline{C}H_2$); 116.3 ($Ar\underline{C}^5H$); 126.8 ($Ar\underline{C}^6$); 129.6 ($Ar\underline{C}^7H$); 136.2 ($Ar\underline{C}^8$); 137.8 ($Ar\underline{C}^{4a}$); 151.7 ($Ar\underline{C}^{8a}$); 168.6 ($\underline{C}O$)

IR υ cm^{-1}: 1769 (s) C=O (lactone)

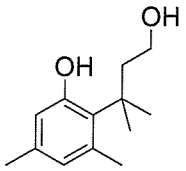
MP: 86-87°C (lit 89-90°C)[142]

UV (EtOH) λ_{max}: 236 nm

RP-HPLC (λ_{254}): 17.3 min (gradient 1)

m/z (APCI +ve): 205.0 $[M+H]^+$(50%)

Preparation of 2-(3-hydroxy-1,1-dimethylpropyl)-3,5-dimethylphenol **[82]**[143]



1,1,6,8-tetramethylisochroman-3-one (20 g, 97.6 mmol) was dissolved in anhydrous THF (200 mL), and added dropwise to a suspension of $LiAlH_4$ (4 g, 98 mmol) in anhydrous THF (200 mL) and stirred for 1 hour at room temperature. Excess $LiAlH_4$ was quenched by the dropwise addition of a saturated aqueous solution of NH_4Cl (200 mL) and the insoluble white precipitate was removed by filtration through celite. After the addition of H_2O (200 mL) the mixture was extracted with Et_2O (3 x 400 mL). The combined organic fractions were dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by recrystallisation from EtOAc and hexane, to give the desired compound as white platelets.

Yield 10 g, 49%

R_f: 0.2 (3: 7 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.55 (6H, s, $(C\underline{H}_3)_2$); 2.17 (3H, s, $ArC^3C\underline{H}_3$); 2.26 (2H, t, $C\underline{H}_2CH_2OH$, J = 7 Hz); 2.49 (3H, s, $ArC^5C\underline{H}_3$); 3.65 (2H, t, $CH_2C\underline{H}_2OH$, J = 7 Hz); 6.34 (1H, s, $ArC^6\underline{H}$); 6.49 (1H, s, $ArC^4\underline{H}$)

δ_C: (75 MHz, $CDCl_3$): 20.3 ($ArC^3\underline{C}H_3$); 25.6 ($ArC^5\underline{C}H_3$); 31.9 ($C(\underline{C}H_3)_2$); 39.52 ($\underline{C}(CH_3)_2$); 44.9 ($\underline{C}H_2CH_2OH$); 61.5 ($CH_2\underline{C}H_2OH$); 116.3 ($Ar\underline{C}^6H$); 126.8 ($Ar\underline{C}^4H$); 128.5 ($Ar\underline{C}^2$); 136.2 ($Ar\underline{C}^3$); 137.8 ($Ar\underline{C}^5$); 155.5 ($Ar\underline{C}^1$)

IR υ cm^{-1}: 3508 (br) OH

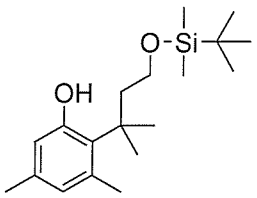
MP: 106-107°C (lit 112-114°C)[142]

UV (EtOH) λ_{max}: 238 nm

RP-HPLC (λ_{254}): 17.5 min (gradient 1)

m/z (APCI -ve): 207.0 $[M-H]^-$ (50%)

Preparation of 2-[1,1-Dimethyl-3-(1,1,2,2-tetramethyl-1-silapropoxy)propyl]-3,5-dimethylphenol **[83]**[142]



2-(3-Hydroxy-1,1-dimethylpropyl)-3,5-dimethylphenol (9.2 g, 44 mmol) was added to a solution of imidazole (7.2 g, 110 mmol) and *tert*-butyldimethylsilyl chloride (8 g, 52.8 mmol) in DMF (100 mL). The mixture was stirred at room temperature for 2 hours. After the addition of H_2O (300 mL), the mixture was extracted with Et_2O (3 x 200 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (200 mL), H_2O (6 x 200 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product purified by column chromatography on silica gel (3: 7 EtOAc: hexane) to give the title compound as a white solid.

Yield 10.1 g, 70%

R_f: 0.85 (3: 7 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 0.05 (6H, s, $Si(C\underline{H}_3)_2$); 0.9 (9H, s, $SiC(C\underline{H}_3)_3$); 1.57 (6H, s, $C(C\underline{H}_3)_2$); 2.14-2.19 (5H, s + t, $ArC^3C\underline{H}_3$, $C\underline{H}_2CH_2O$, J = 7 Hz); 2.48 (3H, s, $Ar^5C\underline{H}_3$); 3.62 (2H, t, $CH_2C\underline{H}_2O$, J = 7 Hz); 5.93 (1H, s, O$\underline{H}$); 6.41 (1H, s, $ArC^6\underline{H}$); 6.49 (1H, s, $ArC^4\underline{H}$)

δ_C (75 MHz, $CDCl_3$): -5.3 ($Si(\underline{C}H_3)_2$); 18.3 ($\underline{C}(CH_3)_3$); 20.4 ($ArC^3\underline{C}H_3$); 25.6 ($ArC^5\underline{C}H_3$); 26.12 ($C(\underline{C}H_3)_3$); 32.1 ($C(\underline{C}H_3)_2$); 39.49 ($\underline{C}(CH_3)_2$); 45.1 ($\underline{C}H_2CH_2O$); 62.0 ($CH_2\underline{C}H_2O$); 116.7 ($Ar\underline{C}^6H$); 126.7 ($Ar\underline{C}^4H$); 129.1 ($Ar\underline{C}^2$); 135.9 ($Ar\underline{C}^3$); 137.8 ($Ar\underline{C}^5$); 155.7 ($Ar\underline{C}^1$)

IR υ cm^{-1}: 3307 (br) OH

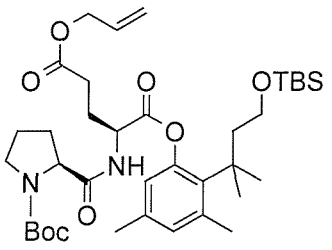
MP: 93-94°C

UV (EtOH) λ_{max}: 264 nm

RP-HPLC (λ_{254}): 14.5 min (gradient 1)

m/z (APCI -ve): 321.1 $[M-H]^-$ (50%)

Preparation tert-Butoxycarbonyl-L-Prolyl -L-Glutamyl-(γ-Allyl)-2-[1,1-dimethyl-3-(1,1,2,2-tetramethyl-1-silapropoxy)propyl]-3,5-dimethylphenyl ester **[84]**



EDC (4.0 g, 20.8 mmol) in CH_2Cl_2 (100 mL) was added to a solution of Boc-Pro-Glu-(OAllyl)-OH (8.0 g, 20.8 mmol), 2-[1,1-dimethyl-3-(1,1,2,2-tetramethyl-1-silapropoxy)propyl]-3,5-dimethylphenol (6.8 g, 20.8 mmol) and DMAP (2.4 g, 20.8 mmol) in CH_2Cl_2 (200 mL). The mixture was stirred at room temperature for 48 hours. The solvent was removed *in vacuo*, H_2O (300 mL) was added and the mixture was extracted with EtOAc (3 x 200 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (200 mL), 5% $NaHCO_3$, (200 mL), H_2O (200 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (3: 7 EtOAc: hexane) to give the desired product as a pale yellow oil.

Yield: 4.32 g, 30%

R_f: 0.25 (3: 7 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 0.04 (6H, s, $Si(C\underline{H}_3)_2$); 0.83 (9H, s, $SiC(C\underline{H}_3)_3$); 1.44 (15H, s, $CO_2C(C\underline{H}_3)_3$, $(C\underline{H}_3)_2$); 1.80-2.03 (6H, m, β $C\underline{H}_2$ Pro, γ $C\underline{H}_2$ Pro, $C\underline{H}_2CH_2O$); 2.04-2.22 (5H, s + m, $ArC^3C\underline{H}_3$ + β $C\underline{H}_2$ Glu); 2.40-2.53 (5H, s + m, $ArC^5C\underline{H}_3$ + γ $C\underline{H}_2$ Glu); 3.36-3.47 (5H, m, δ $C\underline{H}_2$ Pro, $CH_2C\underline{H}_2O$); 4.21-4.39 (1H, br s, α $C\underline{H}$ Pro); 4.60 (2H, d, $C\underline{H}_2$-CH=CH_2, J = 5 Hz); 4.72-4.83 (1H, br s, α $C\underline{H}$ Glu); 5.22 (1H, d, CH_2-CH=$C\underline{H}_2$, *cis*, J = 11 Hz); 5.31 (1H, dd, CH_2-CH=$C\underline{H}$, *trans*, J = 17 Hz, 2 Hz); 5.86 (1H, ddt, CH_2-$C\underline{H}$=CH_2, J = 5 Hz, 11 Hz, 17 Hz); 6.49 (1H, s, $ArC^6\underline{H}$); 6.81 (1H, s, $ArC^4\underline{H}$); 7.47 (1H, s, N$\underline{H}$)

δ_C (75 MHz, $CDCl_3$): -1.05 ($Si(\underline{C}H_3)_2$); 18.2 ($Si\underline{C}(CH_3)_3$); 20.1 ($ArC^3\underline{C}H_3$); 23.6 (γ $\underline{C}H_2$ Pro); 24.5 (β $\underline{C}H_2$ Glu); 25.2 ($ArC^5\underline{C}H_3$); 25.9 ($SiC(\underline{C}H_3)_3$); 26.4 (β $\underline{C}H_2$ Pro); 28.4 ($C(\underline{C}H_3)_3$); 30.1 (γ $\underline{C}H_2$ Glu); 31.9 ($C(\underline{C}H_3)_2$); 39.2 ($\underline{C}(CH_3)_2$); 45.9 ($\underline{C}H_2CH_2O$);

47.7 (δ $\underline{C}H_2$ Pro); 52.1 (α $\underline{C}H$ Glu); 60.3 (α $\underline{C}H$ Pro); 62.0 ($CH_2\underline{C}H_2O$); 65.4 ($\underline{C}H_2CH=CH_2$); 80.4 ($C(\underline{C}H_3)_3$); 118.3 ($CH_2CH=\underline{C}H_2$); 122.6 ($Ar\underline{C}^6H$); 132.1 ($Ar\underline{C}^4H$, $CH_2\text{-}\underline{C}H=CH_2$); 134.1 ($Ar\underline{C}^3$); 136.0 ($Ar\underline{C}^5$); 138.5 ($Ar\underline{C}^2$); 149.8 ($Ar\underline{C}^1$); 155.6 ($\underline{C}O_2C(CH_3)_3$); 170.8 (ArO$\underline{C}$O); 171.1 ($\underline{C}O_2CH_2\text{-}CH=CH_2$); 172.3 ($\underline{C}$ONH)

IR υ cm^{-1}: 1754 (s) C=O phenolic ester; 1739 C=O allyl ester; 1695 (s) C=O carbamate, amide

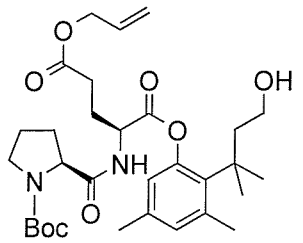
UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{220}): 15.6 min (gradient 1)

m/z (ES +ve): 689.0 $[M+H]^+$ (90%), 706.1 $[M+NH_4]^+$ (100%)

HRMS [M+H]: $C_{37}H_{61}O_8N_2Si$ (calc) 689.4141; (found) 689.4197

Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-(3-Hydroxy-1,1-dimethylpropyl)-3,5-dimethylphenyl ester **[85]**



tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-[1,1-dimethyl-3-(1,1,2,2-tetra-methyl-1-silapropoxy)propyl]-3,5-dimethylphenyl ester (1.6 g, 2.3 mmol) was dissolved in MeOH (30 mL). After the addition of PPTS (580 mg, 2.3 mmol) the reaction was stirred at room temperature for 4.5 hours. The solvent was removed *in vacuo* and the residue was taken up in H_2O (100 mL) and extracted with EtOAc (3 x 100 mL). The combined organic fractions were washed with H_2O (3 x 100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* to give the title compound as a colourless oil.

Yield: 1.3 g, 96%

R_f: 0.3 (EtOAc: hexane 2: 1)

δ_H (300 MHz, $CDCl_3$): 1.44 (9H, s, $C(C\underline{H}_3)_3$); 1.49 (6H, s, $C(C\underline{H}_3)_2$); 1.86-2.04 (4H, m, β $C\underline{H}_2$ Pro, γ $C\underline{H}_2$ Pro); 2.13-2.22 (7H, s + m, $ArC^3C\underline{H}_3$ + β $C\underline{H}_2$ Glu, $C\underline{H}_2CH_2O$); 2.46-2.59 (5H, s + m, $ArC^5C\underline{H}_3$ + γ $C\underline{H}_2$ Glu); 3.31-3.58 (5H, m, δ $C\underline{H}_2$ Pro,

$CH_2C\underline{H}_2O$); 4.21-4.4 (1H, br s, α C<u>H</u> Pro); 4.62 (2H, d, $C\underline{H}_2$-CH=CH_2, J = 5 Hz); 4.72-4.76 (1H, br s, α C<u>H</u> Glu); 5.25 (1H, dd, CH_2-CH=C<u>H</u>, *cis*, J = 10 Hz, 1 Hz); 5.33 (1H, dd, CH_2-CH=C<u>H</u>, *trans*, J = 17 Hz, 1 Hz); 5.91 (1H, ddt, CH_2-C<u>H</u>=CH_2, J = 5 Hz, 10 Hz, 17 Hz); 6.52 (1H, s, $ArC^6\underline{H}$); 6.81(1H, s, $ArC^4\underline{H}$); 7.67 (1H, s, N<u>H</u>)

δ_C (75 MHz, $CDCl_3$): 20.1 ($ArC^3\underline{C}H_3$); 23.5 (γ $\underline{C}H_2$ Pro); 24.5 ($ArC^5\underline{C}H_3$); 25.9 (β $\underline{C}H_2$ Glu); 28.3 ($C(\underline{C}H_3)_3$); 30.2 (β $\underline{C}H_2$ Pro); 31.0 (γ $\underline{C}H_2$ Glu); 31.7 ($C(\underline{C}H_3)_2$); 31.9 ($C(\underline{C}H_3)_2$); 39.2 ($\underline{C}(CH_3)_2$); 45.9 ($\underline{C}H_2CH_2OH$); 47.0 (δ $\underline{C}H_2$ Pro); 52.4 (α $\underline{C}H$ Glu); 59.7 (α $\underline{C}H$ Pro); 60.0 ($CH_2\underline{C}H_2OH$); 65.5 ($\underline{C}H_2$-CH=CH_2); 80.6 ($\underline{C}(CH_3)_3$); 118.5 (CH_2-CH=$\underline{C}H_2$); 122.5 ($Ar\underline{C}^6H$); 131.8 (CH_2-$\underline{C}H$=CH_2); 132.6 ($Ar\underline{C}^4H$); 134.3 ($Ar\underline{C}^3$); 136.0 ($Ar\underline{C}^5$); 138.3 ($Ar\underline{C}^2$); 149.7 ($Ar\underline{C}^1$); 155.4 ($\underline{C}O_2C(CH_3)_3$); 170.8 (ArO<u>C</u>O); 171.0 ($\underline{C}O_2CH_2$-CH=CH_2); 172.4 (<u>C</u>ONH)

IR υ cm^{-1}: 3300 (br) O-H; 1735 (s) C=O phenolic ester, allyl ester; 1668 (s) C=O carbamate, amide

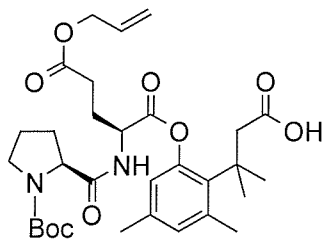
UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{220}): 17.4 min (gradient 1)

m/z (ES +ve): 575.4 $[M+H]^+$ (100%)

HRMS $[M+H]^+$: $C_{31}H_{47}O_8N_2$ (calc) 575.3317; (found) 575.3332

Preparation of 3-[N-tert-Butoxycarbonyl-L-Prolyl- L-Glutamyl-(γ-Allyl)-4,6-dimethylphenyl ester]-3,-methylbutanoic acid **[87]**



tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-(3-Hydroxy-1,1-dimethylpropyl)-3,5-dimethylphenyl ester (1 g, 1.7 mmol) in anhydrous DMF (10 mL) was added to a solution of PDC (2 g, 5 mmol) in anhydrous DMF (20 mL) under N_2. The mixture was stirred at room temperature for 48 hours. After the addition of H_2O (100 mL) the mixture was extracted with EtOAc (3 x 100 mL). The organic fractions were combined and washed with H_2O (6 x 100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified

by column chromatography on silica gel (3: 6.5: 0.5 EtOAc: hexane: AcOH) to give the title compound as a pale yellow oil.

Yield: 660 mg, 65%

R_f: 0.3 (1: 1 EtOAc: hexane)

δ_H (300 MHz, $CDCl_3$): 1.41 (9H, s, $C(C\underline{H}_3)_3$); 1.50 (3H, s, $C(C\underline{H}_3)_2$); 1.52 (3H, s, $C(C\underline{H}_3)_2$); 1.84-2.04 (4H, m, β $C\underline{H}_2$ Pro, γ $C\underline{H}_2$ Pro); 2.14-2.24 (5H, s + m, $ArC^6C\underline{H}_3$ + β $C\underline{H}_2$ Glu); 2.37-2.57 (5H, s + m, $ArC^4C\underline{H}_3$ + γ $C\underline{H}_2$ Glu); 2.77 (2H, s, $C\underline{H}_2CO_2H$); 3.25-3.50 (2H, m, δ $C\underline{H}_2$ Pro); 4.21-4.39 (1H, br s, α $C\underline{H}$ Pro); 4.57 (2H, d, $C\underline{H}_2$-$CH{=}CH_2$, J = 5 Hz); 4.72-4.75 (1H, br s, α $C\underline{H}$ Glu); 5.21 (1H, d, CH_2-$CH{=}C\underline{H}$, *cis*, J = 10 Hz); 5.27 (1H, d, CH_2-$CH{=}C\underline{H}$, *trans*, J = 17 Hz); 5.88 (1H, ddt, CH_2-$C\underline{H}{=}CH_2$, J = 5 Hz, 10 Hz, 17 Hz); 6.50 (1H, s, $ArC^3\underline{H}$); 6.78 (1H, s, $ArC^5\underline{H}$)

δ_C (75 MHz, $CDCl_3$): 20.1 ($ArC^6\underline{C}H_3$); 23.7 (γ $\underline{C}H_2$ Pro); 24.6 (β $\underline{C}H_2$ Pro); 25.5 ($ArC^4\underline{C}H_3$); 26.2 (β $\underline{C}H_2$ Glu); 26.4 (β $\underline{C}H_2$ Glu); 28.4 ($C(\underline{C}H_3)_3$); 30.3 (γ $\underline{C}H_2$ Glu); 31.5 ($C(\underline{C}H_3)_2$); 38.5 ($\underline{C}(CH_3)_2$); 47.2 ($\underline{C}H_2CO_2H$); 47.7 (δ $\underline{C}H_2$ Pro); 52.6 (α $\underline{C}H$ Pro); 60.0 (α $\underline{C}H$ Glu); 61.0 (α $\underline{C}H$ Glu); 65.7 ($\underline{C}H_2$-$CH{=}CH_2$); 80.9 ($\underline{C}(CH_3)_3$); 118.6 (CH_2-$CH{=}\underline{C}H_2$); 122.5 ($Ar\underline{C}^3H$); 132.1 (CH_2-$\underline{C}H{=}CH_2$); 132.8 ($Ar\underline{C}^5H$); 133.9 ($Ar\underline{C}^6$); 136.4 ($Ar\underline{C}^4$); 138.5 ($Ar\underline{C}^1$); 149.5 ($Ar\underline{C}^2$); 155.4 ($\underline{C}O_2C(CH_3)_3$); 171.1 ($ArO\underline{C}O$); 172.6 ($\underline{C}O_2CH_2$-$CH{=}CH_2$); 174.1 ($\underline{C}ONH$); 174.9 ($\underline{C}O_2H$)

IR υ cm^{-1}: 3332 (br) O-H; 1716 (s) C=O phenolic ester, allyl ester, 1696 (s) C=O carbonate, amide, acid.

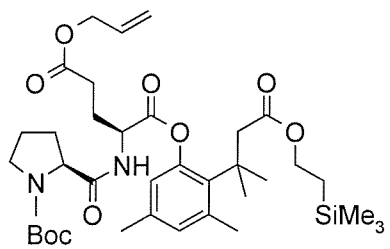
UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{220}): 17.2 min (gradient 1)

m/z (ES +ve): 589.3 $[M+H]^+$ (100%), 611.3 $[M+Na]^+$ (50%)

HRMS [M+H]: $C_{31}H_{45}O_9N_2$ (calc) 589.3163; (found) 589.3165

Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{2-[(3,3-dimethyl-3-silabutyl)oxycarbonyl]-tert-butyl}-3,5-dimethylphenyl ester **[88]**



EDC (81 mg, 0.43 mmol) was added to a solution of 3-[2-*tert*-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-4,6-dimethylphenyl ester]-3-methylbutanoic acid (250 mg, 0.43 mmol), DMAP (51 mg, 0.43 mmol) and 2-trimethylsilanyl ethanol (55 mg, 4.7 mmol) in CH_2Cl_2 (25 mL). The mixture was stirred at room temperature for 15 hours. The solvent was removed *in vacuo*, H_2O (100 mL) was added, and the mixture was extracted with EtOAc (3 x 100 mL). The combined organic fractions were washed with H_2O (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a colourless oil.

Yield: 257 mg, 87%

R_f: 0.71 (1: 1 hexane: EtOAc)

δ_H (300 MHz, $CDCl_3$): -0.02 (9H, s, $Si(C\underline{H}_3)_3$); 0.82 (2H, t, $CH_2C\underline{H}_2Si$, J = 9 Hz); 1.44 (9H, s, $C(C\underline{H}_3)_3$); 1.50 (3H, s, $C(C\underline{H}_3)_2$); 1.57 (3H, s, $C(C\underline{H}_3)_2$); 1.85-1.98 (4H, m, β $C\underline{H}_2$ Pro, γ $C\underline{H}_2$ Pro); 2.14-2.30 (5H, s + m, $ArC^3C\underline{H}_3$ + β $C\underline{H}_2$ Glu); 2.41-2.57 (5H, s + m, $ArC^5C\underline{H}_3$ + γ $C\underline{H}_2$ Glu); 2.72 (1H, d, $C\underline{H}_2CO_2$, J = 15 Hz); 2.86 (1H, d, $C\underline{H}_2CO_2$, J = 15 Hz); 3.34-3.46 (2H, m, δ $C\underline{H}_2$ Pro); 3.99 (2H, t, $C\underline{H}_2CH_2Si$, J = 9 Hz); 4.19-4.28 (1H, br s, α C<u>H</u> Pro); 4.59 (2H, d, $C\underline{H}_2$-CH=CH_2, J = 4 Hz); 4.77-4.79 (1H, br s, α C<u>H</u> Glu); 5.22 (1H, d, CH_2-CH=C<u>H</u>, *cis*, J = 10 Hz); 5.30 (1H, d, CH_2-CH=C<u>H</u>, *trans*, J = 17 Hz); 5.88 (1H, ddt, CH_2-C<u>H</u>=CH_2, J = 4 Hz, 10 Hz, 17 Hz); 6.53 (1H, s, $ArC^6\underline{H}$); 6.80 (1H, s, $ArC^4\underline{H}$)

δ_C: (75 MHz, $CDCl_3$): -1.40 ($Si(\underline{C}H_3)_3$); 17.3 ($CH_2\underline{C}H_2Si$); 20.4 ($ArC^3\underline{C}H_3$); 23.8 (γ $\underline{C}H_2$ Pro); 24.7 (β $\underline{C}H_2$ Pro); 25.4 ($ArC^5\underline{C}H_3$); 26.8 (β $\underline{C}H_2$ Glu); 26.9 (β $\underline{C}H_2$ Glu); 28.5 ($C(\underline{C}H_3)_3$); 30.3 (γ $\underline{C}H_2$ Glu); 31.7 ($C(\underline{C}H_3)_2$); 31.2 ($C(\underline{C}H_3)_2$); 39.2 ($\underline{C}(CH_3)_2$); 47.2 (δ $\underline{C}H_2$ Pro); 47.8 ($C\underline{H}_2CO_2$); 52.3 (α $\underline{C}H$ Glu); 52.4 (α $\underline{C}H$ Glu); 61.3 (α $\underline{C}H$ Pro); 62.3 ($\underline{C}H_2CH_2Si$); 65.7 ($\underline{C}H_2$-CH=CH_2); 80.7 ($\underline{C}(CH_3)_3$); 118.6 (CH_2-CH=$\underline{C}H_2$); 122.6 ($Ar\underline{C}^6H$); 132.1 (CH_2-$\underline{C}H$=CH_2); 132.8 ($Ar\underline{C}^4H$); 133.7 ($Ar\underline{C}^3$); 136.4 ($Ar\underline{C}^5$); 138.4 ($Ar\underline{C}^2$); 149.7 ($Ar\underline{C}^1$); 155.4 ($\underline{C}O_2C(CH_3)_3$); 170.8 (ArO<u>C</u>O); 171.1 ($\underline{C}O_2CH_2$-CH=CH_2); 172.0 ($\underline{C}O_2CH_2CH_2Si$); 172.5 (<u>C</u>ONH)

IR υ cm^{-1}: 1739 (s) C=O phenolic ester, allyl ester, aliphatic ester; 1692 (s) C=O carbamate, amide

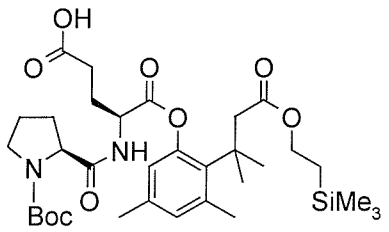
UV (EtOH) λ_{max}: 258 nm

RP-HPLC (λ_{220}): 22.6 min (gradient 1)

m/z (ES +ve): 689.8 $[M+H]^+$ (100%)

HRMS [M+H]: $C_{36}H_{57}O_9N_2Si$ (calc) 689.3833; (found) 689.3877

Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamic acid-2-{2-[(3,3-dimethyl-3-silabutyl)oxycarbonyl]-tert-butyl}-3,5-dimethylphenyl ester **[89]**



$Pd(PPh_3)_4$ (25 mg, 20 μmmol) was added to a solution of of *tert*-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{2-[(3,3-dimethyl-3-silabutyl)oxycarbonyl]-*tert*-butyl}-3,5-dimethylphenyl ester (144 mg, 0.2 mmol), in anhydrous THF (30 mL) under nitrogen. Morpholine (18 mg, 0.2 mmol) was added dropwise and the mixture was stirred for 1 hour at room temperature. The solvent was removed *in vacuo*, and the residue was redissolved in CH_2Cl_2 (50 mL), washed with 2 M $KHSO_4$ (2 x 50 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (97: 3 CH_2Cl_2: MeOH) to give the title compound as a pale yellow oil.

Yield: 102 mg, 76%

R_f: 0.3 (6.5: 3: 0.5 hexane: EtOAc: AcOH)

δ_H (400 MHz, $CDCl_3$): -0.0 (9H, s, $Si(C\underline{H}_3)_3$); 0.8 (2H, t, $CH_2C\underline{H}_2Si$, J = 9 Hz); 1.46 (9H, s, $C(C\underline{H}_3)_3$); 1.56 (6H, s, $C(C\underline{H}_3)_2$); 1.83-2.01 (4H, m, β $C\underline{H}_2$ Pro, γ $C\underline{H}_2$ Pro); 2.12-2.26 (5H, s + m, $ArC^3C\underline{H}_3$ + β $C\underline{H}_2$ Glu); 2.40-2.54 (5H, s + m, ArC^5CH_3 + γ $C\underline{H}_2$ Glu); 2.84-2.89 (2H, m, $C\underline{H}_2CO_2$); 3.34-3.46 (2H, m, δ $C\underline{H}_2$ Pro); 4.02 (2H, t, $C\underline{H}_2CH_2Si$, J = 9 Hz); 4.17-4.28 (1H, br s, α C<u>H</u> Pro); 4.40-4.52 (1H, br , α C<u>H</u> Glu); 6.54 (1H, s, $ArC^6\underline{H}$); 6.79 (1H, s, $ArC^4\underline{H}$)

δ_C (100 MHz, $CDCl_3$): 0.0 ($Si(\underline{C}H_3)_3$); 18.7 ($CH_2\underline{C}H_2Si$); 21.7 ($ArC^3\underline{C}H_3$); 25.1 ($ArC^5\underline{C}H_3$); 26.8 (γ $\underline{C}H_2$ Pro); 29.7 ($C(\underline{C}H_3)_3$); 30.1 (γ $\underline{C}H_2$ Glu); 32.5 (β $\underline{C}H_2$ Pro); 33.0 (β $\underline{C}H_2$ Glu); 33.1 (β $\underline{C}H_2$ Glu); 34.5 ($C(\underline{C}H_3)_2$); 40.6 ($\underline{C}(CH_3)_2$); 48.1 (δ $\underline{C}H_2$ Pro); 49.3 ($C\underline{H}_2CO_2$); 51.9 (α $\underline{C}H$ Glu); 62.8 (α $\underline{C}H$ Pro); 63.6 ($\underline{C}H_2CH_2Si$); 80.9 ($\underline{C}(CH_3)_3$); 124.1 ($Ar\underline{C}^6H$); 133.9 ($Ar\underline{C}^4H$); 135.3 ($Ar\underline{C}^3$); 137.7 ($Ar\underline{C}^5$); 139.6 ($Ar\underline{C}^2$);

151.3 (Ar$\underline{C}^1$); 157.2 ($\underline{C}O_2C(CH_3)_3$); 172.6 (ArO$\underline{C}$O); 172.7 ($\underline{C}O_2CH_2CH_2Si$); 173.7, 174.7 ($\underline{C}$ONH, $\underline{C}O_2H$)

IR υ cm^{-1}: 3331 (br) O-H; 1754 (s) C=O phenolic ester, silabutyl ester; 1719 (s) C=O acid; 1668 (s) C=O carbamate, amide

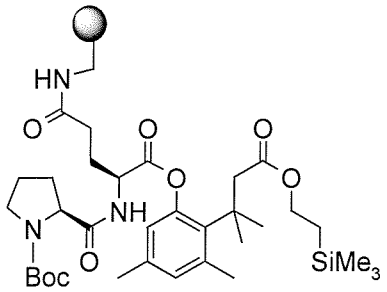
UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{220}): 20.4 min (gradient 1)

m/z (ES +ve): 649.2 $[M+H]^+$ (100%)

HRMS [M+H]: $C_{33}H_{53}O_9N_2Si$: (calc) 649.3520; (found) 649.3545

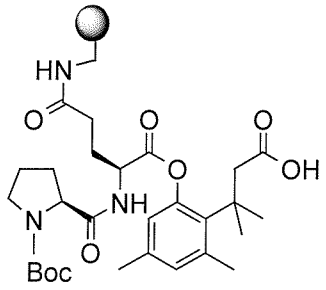
Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-NH TentaGel resin)-2-{2-[(3,3-dimethyl-3-silabutyl)oxycarbonyl]-tert-butyl}-3,5-dimethylphenyl ester **[90]**



HOBt (22 mg, 0.16 mmol) was added to a solution of *tert*-Butoxycarbonyl-L-Prolyl-L-Glutamic acid-2-{2-[(3,3-dimethyl-3-silabutyl)oxycarbonyl]-*tert*-butyl}-3,5-dimethylphenyl ester (52 mg, 80 μmol) in DMF (1 mL) and stirred for 10 min at room temperature. DIC (52 mg, 0.16 mmol) was added and the mixture was stirred for a further 10 min. The mixture was added to Tentagel-NH_2 resin (200 mg, 54 μmol, 0.27 mmol/ g NH_2 Rapp Polymere 130 μM S 30 132), preswollen in CH_2Cl_2 (1 mL) and the suspention was agitated for 15 hours. The resin was filtered and washed with DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL) and Et_2O (5 x 5 mL) and dried *in vacuo*. A qualitative ninhydrin test was negative.

IR υ cm^{-1}: 1724 (s) C=O esters; 1664 (s) C=O amides, carbamate

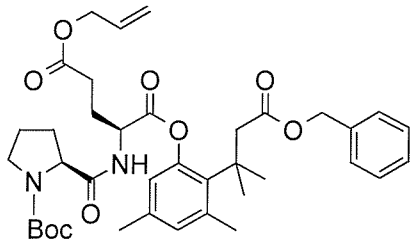
Preparation of 3-[2-tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-NH TentaGel resin)-4,6-dimethylphenyl ester]-3-methylbutanoic acid **[76]**



TBAF (70 mg, 0.27 mmol) in DMF (1 mL) was added to *tert*-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-NH-TentaGel-resin)-2-{2-[(3,3-dimethyl-3-silabutyl)oxy-carbonyl]-*tert*-butyl}-3,5-dimethylphenyl ester (235 mg, 54 μmol), preswollen in DMF (1 mL) and agitated overnight. The resin was filtered and washed with 5% citric acid in DMF (3 x 5 mL), DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL) and Et_2O (5 x 5 mL) and dried *in vacuo*. A qualitative BCG test was positive.

IR υ cm^{-1}: 1709 (s) C=O acid; 1679 (s) C=O amides, carbamate

Preparation of tert-Butoxycarbonyl-L-Prolyl-L- Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxycarbonyl]ethyl}-3,5-dimethylphenyl ester **[91]**



EDC (20 mg, 68 μmol) was added to a solution of 3-[2-*tert*-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-4,6-dimethylphenyl ester]-3-methylbutanoic acid (40 mg, 68 μmol), DMAP (8 mg, 68 μmol) and benzyl alcohol (15 mg, 68 μmol) in CH_2Cl_2 (30 mL). The mixture was stirred at room temperature for 15 hours. The solvent was removed *in vacuo*, H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (100 mL), H_2O (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a colourless oil.

Yield: 60 mg, 55%

R_f: 0.23 (1: 1 EtOAc: hexane)

δ_H (250 MHz, $CDCl_3$): 1.37 (9H, s, $C(C\underline{H}_3)_3$); 1.43 (3H, s, $C(C\underline{H}_3)_2$); 1.50 (3H,s, $C(C\underline{H}_3)_2$); 1.75-1.98 (4H, m, β $C\underline{H}_2$ Pro, γ $C\underline{H}_2$ Pro); 2.03-2.27 (5H, s + m, $ArC^3C\underline{H}_3$ + β $C\underline{H}_2$ Glu); 2.31-2.47 (5H, s + m, $ArC^5C\underline{H}_3$ + γ $C\underline{H}_2$ Glu); 2.75 (1H, d, $C\underline{H}_2CO_2Bn$, J = 15 Hz); 2.88 (1H, d, $C\underline{H}_2CO_2Bn$, J = 15 Hz); 3.25-3.40 (2H, m, δ $C\underline{H}_2$ Pro); 4.18-4.22 (1H, br s, α $C\underline{H}_2$ Pro); 4.49-4.53 (2H, m, $C\underline{H}_2CH{=}CH_2$); 4.56-4.75 (1H, br s, α C<u>H</u> Glu); 4.89 (2H, s, $C\underline{H}_2Ph$); 5.12 (1H, dd, $CH_2CH{=}C\underline{H}$, *cis*, J = 2 Hz, 10 Hz); 5.23 (1H, dd, $CH_2CH{=}C\underline{H}$, *trans*, J = 2 Hz, 17 Hz); 5.84 (1H, ddt, $CH_2C\underline{H}{=}CH_2$, J = 6 Hz, 10 Hz, 17 Hz); 6.45 (1H, s, $ArC^6\underline{H}$); 6.70 (1H, s, $ArC^4\underline{H}$); 7.07-7.41 (5H, m, Ar<u>H</u>)

δ_C (62.5 MHz, $CDCl_3$): 20.7 ($ArC^3\underline{C}H_3$); 25.1 (γ $\underline{C}H_2$ Pro); 25.6 ($ArC^5\underline{C}H_3$); 27.1 (β $\underline{C}H_2$ Glu); 27.2 (β $\underline{C}H_2$ Glu); 27.8 (β $\underline{C}H_2$ Pro); 28.8 ($C(\underline{C}H_3)_3$); 31.8 (γ $C\underline{H}_2$ Glu); 32.0 (γ $C\underline{H}_2$ Glu); 32.1 ($C(\underline{C}H_3)_2$); 39.6 ($\underline{C}(CH_3)_2$); 47.5 ($\underline{C}H_2CO_2Bn$); 48.2 (δ $\underline{C}H_2$ Pro); 51.9 (α $\underline{C}H$ Glu); 52.0 (α $\underline{C}H$ Glu); 52.6 (α $\underline{C}H$ Pro); 66.1 ($\underline{C}H_2CH{=}CH_2$); 67.7 ($CO_2\underline{C}H_2Ph$); 81.0 ($\underline{C}(CH_3)_3$); 118.9 ($CH_2CH{=}\underline{C}H_2$); 122.9 ($Ar\underline{C}^6H$); 128.6 (Ar<u>C</u>H); 128.8 (Ar<u>C</u>H); 128.9 (Ar<u>C</u>H); 132.4 ($CH_2\underline{C}H{=}CH_2$); 133.1 ($Ar\underline{C}^4H$); 133.9 ($Ar\underline{C}^3$); 136.4 ($Ar\underline{C}^5$); 136.7 ($Ar\underline{C}^2$); 138.6 ($Ar\underline{C}CH_2$); 149.9 ($Ar\underline{C}^1$); 155.8 ($\underline{C}O_2C(CH_3)_3$); 171.1 (ArO<u>C</u>O); 171.4 ($\underline{C}O_2Bn$); 172.0 ($\underline{C}O_2CH_2CH{=}CH_2$); 172.8 (<u>C</u>ONH)

IR υ cm^{-1}: 1738 (s) C=O allyl ester, benzyl ester; 1693 (s) C=O amide, carbamate

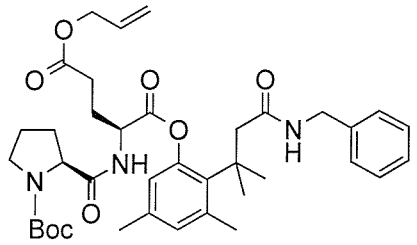
UV (EtOH) λ_{max}: 262 nm

RP-HPLC (λ_{220}): 21.5 min (gradient 1)

m/z (ES +ve)**:** 679.4 $[M+H]^+$ (100%)

HRMS [M+H]: $C_{38}H_{51}O_9N_2$ (calc) 679.3624; (found) 679.3594

Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxycarbamoyl]ethyl}-3,5-dimethylphenyl ester **[92]**



EDC (36 mg, 0.17 mmol) was added to a solution of 3-[2-*tert*-Butoxycarbonyl-L-Prolyl- L-Glutamyl-(γ-Allyl)-4,6-dimethylphenyl ester]-3-methylbutanoic acid (100 mg, 0.17 mmol), DMAP (25 mg, 0.17 mmol) and benzylamine (20 mg, 0.17 mmol) in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 15 hours. The solvent was removed *in vacuo*, H_2O (100 mL) was added and the mixture was extracted with EtOAc (3 x 50 mL). The combined organic fractions were washed with 2 M $KHSO_4$ (100 mL), H_2O (100 mL), brine (100 mL) and dried with $MgSO_4$. The solvent was removed *in vacuo* and the product was purified by column chromatography on silica gel (1: 1 hexane: EtOAc) to give the title compound as a colourless oil.

Yield: 50 mg, 43%

R_f: 0.48 (1: 1 EtOAc: hexane)

δ_H (250 MHz, $CDCl_3$): 1.43 (9H, s, $C(C\underline{H}_3)_3$); 1.63 (3H, s, $ArC(C\underline{H}_3)_2$); 1.67 (3H, s, $ArC(C\underline{H}_3)_2$); 1.79-1.96 (4H, m, β $C\underline{H}_2$ Pro, γ $C\underline{H}_2$ Pro); 2.32-2.45 (5H, s + m, $ArC^3C\underline{H}_3$ + β $C\underline{H}_2$ Glu); 2.48-2.68 (7H, s + m, $ArC^5C\underline{H}_3$ + γ $C\underline{H}_2$ Glu, $C\underline{H}_2CONH$); 3.35-3.51 (2H, m, δ $C\underline{H}_2$ Pro); 4.27-4.31 (3H, m, α $C\underline{H}$ Pro, $NHC\underline{H}_2Ph$); 4.58-4.69 (3H, m, $C\underline{H}_2CH{=}CH_2$, α $C\underline{H}$ Glu); 5.24 (1H, dd, $CH_2CH{=}C\underline{H}$, *cis*, J = 2 Hz, 10 Hz); 5.33 (2H, dd, $CH_2CH{=}C\underline{H}$, *trans*, J = 2 Hz, 17 Hz); 5.88 (1H, ddt, $CH_2C\underline{H}{=}CH_2$ J = 5 Hz, 10 Hz, 17 Hz); 6.49 (1H, s, $ArC^6\underline{H}$); 6.75 (1H, s, $ArC^4\underline{H}$); 7.0-7.20 (5H, m, $Ar\underline{CH}$)

δ_C (62.5 MHz, $CDCl_3$): 20.6 ($ArC^3\underline{C}H_3$); 26.1 ($ArC^5\underline{C}H_3$); 26.4 (γ $\underline{C}H_2$ Pro); 27.8 (β $\underline{C}H_2$ Glu); 28.8 ($C(\underline{C}H_3)_3$); 30.6 (γ $C\underline{H}_2$ Glu, β $\underline{C}H_2$ Pro); 32.5 ($C(\underline{C}H_3)_2$); 40.4 ($\underline{C}(CH_3)_2$); 43.6 ($\underline{C}H_2CONH$); 47.4 (δ $\underline{C}H_2$ Pro); 49.2 ($NH\underline{C}H_2Ph$); 53.1 (α $\underline{C}H$ Pro, α

CH Glu); 66.1 ($\underline{C}H_2CH=CH_2$); 81.1 ($\underline{C}(CH_3)_3$); 119.1 ($CH_2CH=\underline{C}H_2$); 122.1 ($Ar\underline{C}^6H$); 127.2 (Ar$\underline{C}$H); 128.1 (Ar$\underline{C}$H);128.6 (Ar$\underline{C}$H); 132.2 ($CH_2\underline{C}H=CH_2$); 133.4 ($Ar\underline{C}^4H$); 134.8 ($Ar\underline{C}^3$); 136.8 ($Ar\underline{C}^5$);139.2 ($Ar\underline{C}^2$); 139.5 (Ar$\underline{C}CH_2$); 150.2 (ArC^1); 171.6 (ArO$\underline{C}$O); 171.7 ($\underline{C}OOCH_2CH=CH_2$); 171.9 ($\underline{C}$ONH)

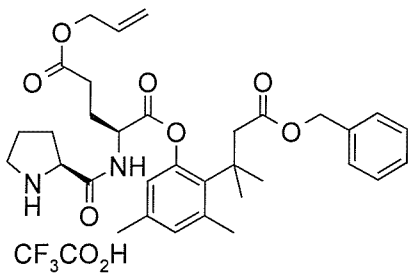
IR υ cm^{-1}: 1738 (s) C=O esters; 1697 (s) C=O amides, 1650 (s) carbamate

UV (EtOH) λ_{max}: 260 nm

RP-HPLC (λ_{220}): 19.9 min (gradient 1)

m/z (ES +ve)**:** 678.4 $[M+H]^+$ (100%)

Preparation of L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxycarbonyl]ethyl}-3,5-dimethylphenyl ester•TFA **[93]**



tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxy-carbonyl]ethyl}-3,5-dimethylphenyl ester (4 mg, 8 μmol) was dissolved in CH_2Cl_2 (500 μL) and TFA (500 μL) was added dropwise. The solution was stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* to give the title compound as a colourless oil.

Yield: 4.5 mg, 100%

R_f: 0.95 (5: 3: 1: $CHCl_3$: MeOH: AcOH)

δ_H (300 MHz, $CDCl_3$): 1.26-1.80 (8H, m, $C(C\underline{H}_3)_2$, γ $C\underline{H}_2$ Pro); 1.97-2.24 (7H, m, β $C\underline{H}_2$ Pro, β $C\underline{H}_2$ Glu, $ArC^3C\underline{H}_3$); 2.45-2.54 (5H, m $ArC^5C\underline{H}_3$, γ $C\underline{H}_2$ Glu); 2.84 (1H, d, $C\underline{H}_2CO_2$, J = 15 Hz); 2.85 (1H, d, $C\underline{H}_2CO_2$, J = 15 Hz); 3.38-3.42 (2H, m, δ $C\underline{H}_2$ Pro); 4.61 (2H, d, $C\underline{H}_2CH=CH_2$, J = 6 Hz); 4.78-4.97 (4H, m, α $C\underline{H}$ Glu, α $C\underline{H}$ Pro $C\underline{H}_2$Ph); 5.18 (1H, d, $CH_2CH=C\underline{H}$, *cis*, J = 10 Hz); 5.33 (1H, d, $CH_2CH=C\underline{H}$, *trans*, J = 17 Hz); 5.90 (1H, ddt, $CH_2C\underline{H}=CH_2$, J = 6 Hz, 10 Hz, 17 Hz); 6.50 (1H, s, $ArC^6\underline{H}$); 6.82 (1H, s, $ArC^4\underline{H}$); 7.13-7.35 (5H, m, Ar$\underline{C}$H)

δ_C (75 MHz, $CDCl_3$): 20.5 ($ArC^3\underline{C}H_3$); 24.9 (γ $\underline{C}H_2$ Pro); 25.6 ($ArC^5\underline{C}H_3$); 25.8 (β $\underline{C}H_2$ Glu); 26.5 (β $\underline{C}H_2$ Pro); 30.2 (γ $\underline{C}H_2$ Glu); 33.5 ($C(\underline{C}H_3)_2$); 39.6 ($\underline{C}(CH_3)_2$); 47.5 ($\underline{C}H_2CO_2Bn$); 48.3 (δ $\underline{C}H_2$ Pro); 53.4 (α $\underline{C}H$ Glu); 60.0 (α $\underline{C}H$ Pro); 66.1 ($\underline{C}H_2CH=CH_2$); 66.9 ($CO_2\underline{C}H_2Ph$); 119.1 ($CH_2CH=\underline{C}H_2$); 122.7 ($Ar\underline{C}^6H$); 128.5 ($Ar\underline{C}H$); 128.8 ($Ar\underline{C}H$); 129.1 ($Ar\underline{C}H$); 132.0 ($CH_2\underline{C}H=CH_2$); 133.3 ($Ar\underline{C}^4H$); 133.9 ($Ar\underline{C}^3$); 135.8 ($Ar\underline{C}^5$); 137.1; ($Ar\underline{C}^2$); 138.6 ($Ar\underline{C}CH_2$); 149.7 ($Ar\underline{C}^1$); 169.3 ($ArO\underline{C}O$); 170.3 ($\underline{C}O_2Bn$); 172.7 ($\underline{C}O_2CH_2CH=CH_2$); 173.7 ($\underline{C}ONH$)

IR υ cm^{-1}: 1739 (s) C=O esters; 1679 (s) C=O amide

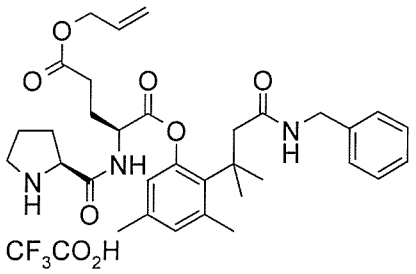
UV (EtOH): λ_{max}: 250 nm

RP-HPLC (λ_{220}): 15.5 min (gradient 1)

m/z (ES +ve)**:** 579.5 $[M+H]^+$

HRMS [M+H]: $C_{33}H_{43}O_7N_2$ (calc) 579.3070; (found) 579.3057

Preparation of L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxycarbamoyl]ethyl}-3,5-dimethylphenyl ester•TFA **[94]**



tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxycarbamoyl]ethyl}-3,5-dimethylphenyl ester (4 mg, 8 μmol) was dissolved in CH_2Cl_2 (500 μL) and TFA (500 μL) was added dropwise. The solution was stirred at room temperature for 10 min. The acid and the solvent were removed *in vacuo* to give the title compound as a colourless oil.

Yield: 4.5 mg, 100%

R_f: 0.79 (5: 3: 1: $CHCl_3$: MeOH: AcOH)

IR υ cm^{-1}: 1740 (s) C=O esters; 1669 (s) C=O amides

UV (EtOH): λ_{max}: 250 nm

RP-HPLC (λ_{220}): 14.8 min (gradient 1)

m/z (ES +ve)**:** 578.5 $[M+H]^+$ (100%)

8.6.2] Evaluation of the linker in solution

Evaluation of the benzyl ester model **[93]**

MeCN (1 mL) and 50 mM phosphate buffer pH 7.5 (9.0 mL) were added to L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxycarbonyl]ethyl}-3,5-dimethylphenyl ester•TFA (4 mg, 8 μmol) and the solution stirred thoroughly and left to stand at room temperature. Aliquots (300 μL) were removed periodically, quenched with 2% TFA (MeCN: H_2O 25: 75, 100 μL) and analysed by RP-HPLC (gradient 1).

Evaluation of the benzyl amide model **[94]**

MeCN (1 mL) and 50mM phosphate buffer pH 7.5 (9.0 mL) were added to L-Prolyl-L-Glutamyl-(γ-Allyl)-2-{1,1-dimethyl-2-[benzyloxycarbamoyl]ethyl}-3,5-dimethylphenyl ester•TFA (4 mg, 8 μmol) and the solution stirred thoroughly and left to stand at room temperature. Aliquots (300 μL) were removed periodically, quenched with 2% TFA (MeCN: H_2O 25: 75, 100 μL) and analysed by RP-HPLC (gradient 1).

8.7] Chapter Six

8.7.1] Synthesis of solid phase models

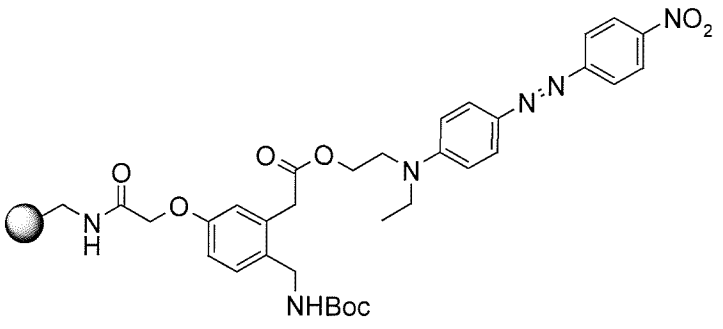
Solid phase synthesis of disperse red and NPM linker models

A solution of HOBt (5 eq) and DMAP (5 eq) in DMF was added to the resin (**[33]**, **[57]** or **[76]**) preswollen in CH_2Cl_2 and agitated for 5 min. DIC (5 eq) was added and the reaction was agitated for a further 10 min. A solution of dye (5 eq) in DMF was added and the suspension was agitated overnight. The resin was filtered and washed with DMF (x 3), CH_2Cl_2 (x 3), MeOH (x 3) and Et_2O (x 5) and dried *in vacuo.*

Solid phase synthesis of marimastat linker models

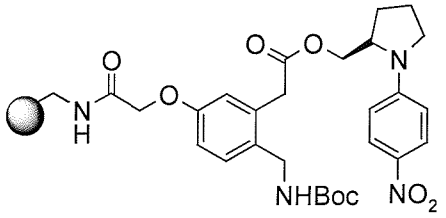
A solution of HOBt (2 eq) and DMAP (2 eq) in anhydrous DMF was added to the resin (**[33]**, **[57]** or **[76]**) preswollen in anhydrous DMF and agitated for 5 min. DIC (2 eq) was added and the reaction was agitated for a further 10 min. A solution of marimastat (5 eq) in anhydrous DMF was added and the reaction was agitated overnight. The resin was filtered and washed with DMF (x 3), CH_2Cl_2 (x 3), MeOH (x 3) and Et_2O (x 5) and dried *in vacuo.*

Preparation of 2-(ethyl{4-[(4-nitrophenyl)diazenyl]phenyl}amino)ethyl 2-(2-{[(tert-butoxy)carbonylamino]methyl-5-{[(N-TentaGel resin)carbamoyl]methoxy}phenyl) acetate **[95]**



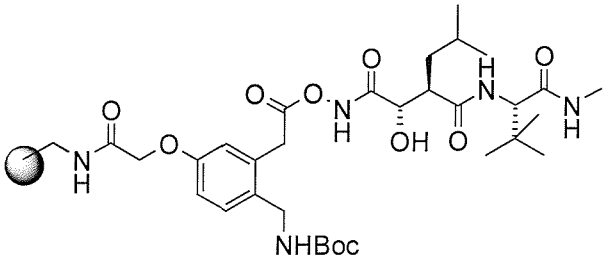
IR υ cm^{-1}: 1709 (s) C=O ester; 1673 (s) carbamate, amide; 1603 (s) NO_2

Preparation of [(2S)-1-(4-nitrophenyl)pyrrolidin-2-yl]methyl 2-(2-{[(tert-butoxy)carbonylamino]methyl-5-{[(N-TentaGel resin)carbamoyl] methoxy}phenyl)acetate **[96]**



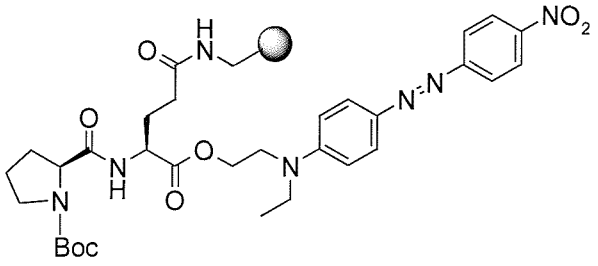
IR υ cm^{-1}: 1705(s) C=O ester; 1675 (s) carbamate, amide; 1604 (s) NO_2

Preparation of 2-(2-{[(tert-butoxy)carbonylamino]methyl-5-{[(N-TentaGel resin) carbamoyl]methoxy}phenyl)acetohydroxamic-[2R-isobutyl-3S-hydroxysuccinyl-L-tert-leucine-N-methylamide] anhydride **[101]**



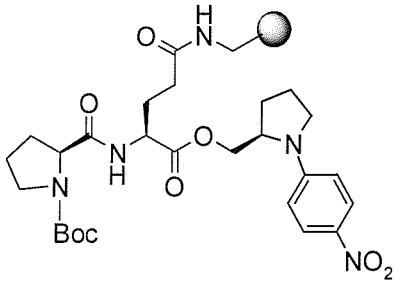
IR υ cm^{-1}: 1700 (s) C=O ester; 1675 (s) C=O amides, carbamate

Preparation of Boc-Pro-Glu-(NH-TentaGel resin)-O-2-[4-(4-nitrophenylazo)-phenyl]ethyl ester **[97]**



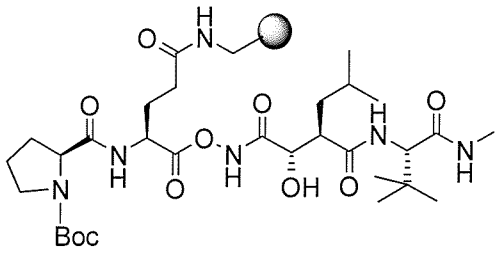
IR υ cm^{-1}: 1734 (s) C=O ester; 1675 (s) C=O amides, carbamate; 1599 (s) NO_2

Preparation of Boc-Pro-Glu-(NH-TentaGel resin)-O-1-(4-nitro-phenyl)-pyrrolidin-2-ylmethyl ester **[98]**



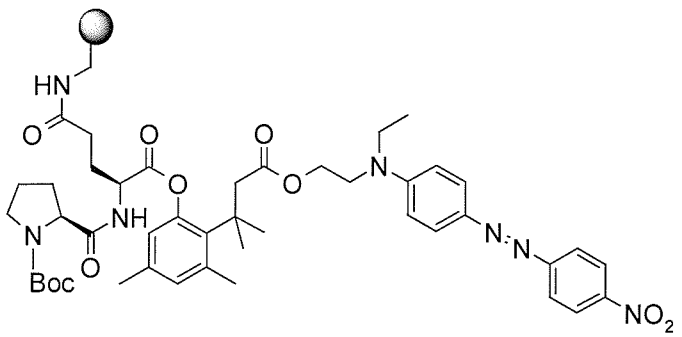
IR υ cm^{-1}: 1739 (s) C=O ester; 1672 (s) C=O amides, carbamate; 1597 (s) NO_2

Preparation of [tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-NH TentaGel resin)] hydroxamic-[2R-isobutyl-3S-hydroxysuccinyl-L-tert-leucine-N-methylamide] anhydride **[102]**



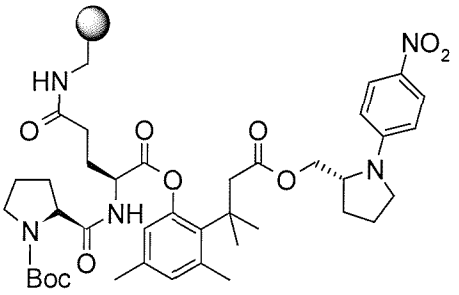
IR υ cm^{-1}: 1730 (s) C=O ester; 1649 (s) C=O amides, carbamate

Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-NH TentaGel resin)-2-(2-{[2-(ethyl{4(-nitrophenyl)diazenyl]phenyl}amino)ethyl] oxycarbonyl} -tert-butyl)-3,5-dimethylphenyl ester **[99]**



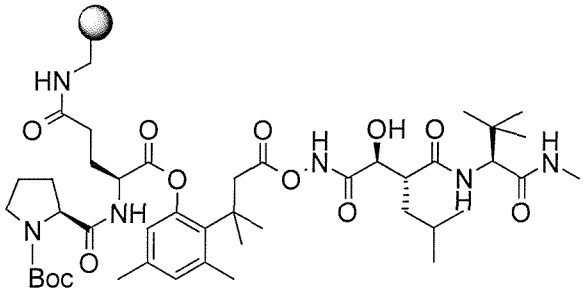
IR υ cm^{-1}: 1739 (s) C=O ester; 1669 (s) C=O amides, carbamate; 1594 (s) NO_2

Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-NH TentaGel resin)-2-[2-({[(2S)-1-(4-nitrophenyl)pyrrolidin-2-yl]methyl}oxycarbonyl)-tert-butyl]-3,5-dimethylphenyl ester **[100]**



IR υ cm^{-1}: 1740 (s) C=O ester; 1675 (s) C=O amides, carbamate; 1595 (s) NO_2

Preparation of tert-Butoxycarbonyl-L-Prolyl-L-Glutamyl-(γ-NH TentaGel resin)-2-{[hydroxamic-(2R-isobutyl-3S-hydroxysuccinyl-L-tert-leucine-N-methylamide) anhydride]tert-butyl}-3,5-dimethylphenyl ester **[103]**



IR υ cm^{-1}: 1740 (s) C=O ester; 1665 (s) C=O amides, carbamate

8.7.2] Evaluation of the linkers on the solid phase

Monitoring the release of disperse red

50% TFA in CH_2Cl_2 (500 μL) was added to the resin (**[95]**, **[97]** or **[99]**,10 mg) and agitated at room temperature for 10 min. The resin was filtered and washed with DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL) and Et_2O (5 x 5 mL) and dried *in vacuo.*

The resin was then placed in a 1.5 mL cuvette and 50 mM phosphate buffer pH 7.5 (750 μL) was added followed by MeCN (250 μL) and the contents mixed thoroughly. UV absorbance readings were noted periodically at λ_{max} = 502 nm.

Monitoring the release of [(2S)-1-(4-nitrophenyl)pyrrolidin-2-yl]methanol

50% TFA in CH_2Cl_2 (500 μL) was added to the resin (**[96]**, **[98]** or **[100]**,10 mg) and agitated at room temperature for 10 min. The resin was filtered and washed with DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL) and Et_2O (5 x 5 mL) and dried *in vacuo.*

The resin was then placed in a 1.5 mL cuvette and 50 mM phosphate buffer pH 7.5 (750 μL) was added followed by MeCN (250 μL) and the contents mixed thoroughly UV absorbance readings were noted periodically at λ_{max} = 418 nm.

Monitoring the release of marimastat.

50% TFA in CH_2Cl_2 (500 μL) was added (**[101]**, **[102]** or **[103]**) (50 mg) and agitated at room temperature for 10 min. The resin was filtered and washed with DMF (1 x 5 mL), CH_2Cl_2 (3 x 5 mL), MeOH (3 x 5 mL) and Et_2O (5 x 5 mL) and dried *in vacuo.* The resin was added to 50% NEt_3 in CH_2Cl_2 (500 μL) and agitated at room temperature overnight. The resin was filtered and washed with DMF (1 x 5 mL) and CH_2Cl_2 (3 x 5 mL). The solvent from the acid and basic washings was removed *in vacuo* and the residue analysed by RP-HPLC.

8.8] Chapter Seven

8.8.1] Materials

Human recombinant Progelatinase A 72 KDa (MMP 2) (160 μg/ mL in 50 mM Tris·HCl/ 5 mM $CaCl_2$/ 0.05% Brij 35/ 0.02% NaN_3 pH 7.5 buffer): supplied by British Biotech Pharmaceuticals Ltd.

Agarose: SeaKem® LE Agarose (Flowgen)

GelBond: Gel support film sheets 0.2 mm thick (Flowgen)

Gelatin: porcine skin (Sigma)

Marimastat (BB2516): supplied by British Biotech Pharmaceuticals Ltd.

Aminoparamercuric acetate: (Sigma)

8.8.2] Methods

Gel Preparation

The gel was cast by dissolving agarose (500 mg) in 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.02% NaN_3 pH 7.5 buffer (50 mL) at 100°C. The solution was cooled to 60°C and gelatin (50 mg) and Brij 35 (160 μL, 30% w/v) were added. The molton agarose was poured onto GelBond film in a Bio-Rad Mini Protean II and allowed to cool. The resulting gel was 82 mm x 82 mm x 2.25 mm.

Enzyme activation

Progelatinase (100 μL, 160 μg/ mL) was diluted in 100 mM Tris·HCl/ 10 mM $CaCl_2$/ 100 mM NaCl/ 0.05% Brij 35/ 0.02% NaN_3 pH 7.5 buffer (540 μL) to give a final concentration of 25 μg/ mL. Freshly prepared 0.1 M AMPA in DMSO (6.4 μL) was added and the enzyme solution was incubated at 37°C for 1 hour. 100 mM Tris·HCl/ 10 mM $CaCl_2$/ 100 mM NaCl/ 0.05% Brij 35/ 0.02% NaN_3 pH 7.5 buffer (5.76 mL) was added to give a final concentration of activated gelatinase 2.5 μg/ mL, which was stored at –70°C in 200 μL aliquots.

Control Gel

Wells were punched in the prepared gel using the tip of a pasteur pipette. Gelatinase (4 μL) was added to the wells using a gilson pipette. The gel was placed into a robust plastic box and buffer was added so it was surrounding the gel. A large filter paper was placed on the gel and into the surrounding buffer. The gel was incubated at 37°C for 15 hours. The gel was washed with copious H_2O to remove the inorganic salts and allowed to dry. The gel was agitated in copious coomassie blue stain (0.5% w/v in 4: 1: 5 MeOH: AcOH: H_2O) for 20 min. The stain was decanted from the gel. The gel was then agitated in copious destain (3: 1: 6 MeOH: AcOH: H_2O) for 20 min. The destain was decanted from the gel and it was allowed to dry.

Single bead studies

Wells were punched in the prepared gel using the tip of a pasteur pipette. High loading aminomethyl PAM-AM dendrimer polystyrene beads (0.72 mmol/ g)[160] were allowed to swell in a solution of marimastat in 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.02% NaN_3 pH 7.5 buffer (2.5 μg/ mL) and then allowed to dry resulting in beads with marimastat adsorbed onto the surface. Single beads were selected with the aid of a microscope, and added to the wells in the gel. The enzyme (4 μL, 2.5 μg/ mL) was added carefully to each well in addition to 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.02% NaN_3 pH 7.5 buffer (1 μL). The gel was incubated, dried and stained as described previously.

Gelatinase gel

The gel was cast by dissolving agarose (500 mg) in 100 mM Tris·HCl/ 100 mM NaCl/ 10 mM $CaCl_2$/ 0.02% NaN_3 pH 7.5 buffer (50 mL) at 100°C. The solution was cooled to 60°C and gelatin (50 mg) and Brij 35 (80 μL, 30% w/v) were added. The molton agarose was allowed to cool to 40°C and maintained at this temperature in an oven. Gelatinase was added and the molton agarose and the gel was cast as described previously. The gel was incubated, dried and stained as before.

Single bead studies using gelatinase gel

Gelatinase gels were cast and wells were then punched in the gels using the tip of a pasteur pipette. Single beads with marimastat adsorbed onto the surface were prepared as before and placed into the wells. The gels were incubated, dried and stained as before.

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