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The University of Dublin

Thioacid Mediated Methods for Peptide Ligation and Cyclisation

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2025

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Submitted to the School of Chemistry
for the degree of Doctor of Philosophy

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Abstract

In recent years, peptide-based therapeutics have become the focus of intensive research due to their favourable chemical properties. Consequently, the ability to efficiently synthesise a wide range of structurally diverse peptides in high purity is essential. In particular, the orthogonal reactivity of thioacids compared to the canonical amino acids (AAs) has been previously exploited for peptide chemistry, but their full potential remains underexplored. This thesis details the initial development of novel thioacid-mediated peptide ligation and cyclisation methods to broaden the scope of synthetic peptide chemistry.

Chapter 2 outlines efforts to develop a peptide ligation reaction between a C-terminal thioester and an N-terminal γ-thioaspartic acid to form a native amide linkage *via* a transient thioanhydride intermediate. The thioacid would be reformed post ligation and could serve as a handle for late-stage diversification or could be hydrolysed under mild conditions to the native AA, aspartic acid. The reaction was successfully demonstrated on simple model substrates and attempts to extend the reaction to more complex peptide substrates is outlined.

Chapter 3 details the application of the photochemical acyl thiol-ene reaction to cyclise fully unprotected linear peptides containing both an alkene and a γ-thioaspartic acid residue to form macrocyclic peptide thiolactones under both UV and blue LED irradiation. This represents the first reported synthesis of peptide thiolactones under radical-mediated conditions.

Chapter 4 details investigation of the dethiocarboxylation of C-terminal thioacid derivatives of AAs under photochemical conditions to yield *N*-alkylamines. Furthermore, initial scoping studies were conducted to determine whether the C(sp^3) radical formed as an intermediate during the dethiocarboxylation reaction could be trapped *via* an intermolecular addition onto an alkene to form C(sp^3) linked cyclic peptides.

Abbreviations

AAs	Amino Acids
Ac	acetyl
Agl	Allylglycine
AIBN	Azobisisobutyronitrile
AIPs	Auto-Inducing Peptides
Ala; A	Alanine
All	allyl
AML	Auxiliary Mediated Ligation
APCI	Atmospheric-Pressure Chemical Ionisation
aq.	aqueous
Ar	aryl
Arg; R	Arginine
Asn; N	Asparagine
Asp; D	Aspartic Acid
ATC	Amino Thioacid Coupling
BDE	Bond Dissociation Energy
Bno	5-*n*-butyl-5-nonyl
Boc	*tert*-butyloxycarbonyl
br	broad
bs	broad singlet
Bt	benzotriazole
CDI	1,1'-Carbonyldiimidazole
COMU	(1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
COS	Carbonyl sulfide
Cys; C	Cysteine
d	doublet
DBF	dibenzofulvene
DBU	1,8-Diazabicyclo(5.4.0)undec-7-ene
Dbz	diaminobenzoyl
DCC	*N,N*'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	*N,N'*-Diisopropylcarbodiimide

DIPEA	*N,N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMES	Dimetylethylsilane
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPAP	2,2-Dimethoxy-2-phenylacetophenone
DTT	Dithiothreitol
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EPL	Expressed Protein Ligation
equiv.	equivalents
ESI	Electrospray Ionisation
Et	ethyl
Et_2O	Diethyl ether
EtOAc	Ethyl Acetate
Fm	9-fluoroenylmethyl
Fmoc	fluorenylmethoxycarbonyl
g	gram
Gln; Q	Glutamine
Glu; E	Glutamic Acid
Gly; G	Glycine
GnRH	Gonadotropin hormone-releasing hormone
h	hour
HAT	Hydrogen Atom Transfer
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HBTU	[benzotriazol-1-yloxy(dimethylamino)methylidene]-dimethylazanium;hexafluorophosphate
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His; H	Histidine
HIV	Human Immunodeficiency Virus
HMBA	4-hydroxymethyl benzamide
HMBC	Heteronuclear Multiple Bond Correlation
HOAt	1-Hydroxy-7-azabenzotriazole

HOBt	1*H*-1,2,3-Benzotriazol-1-ol
HPLC	High-Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
Hz	Hertz
Ile; I	Isoleucine
IR	Infrared
J	Coupling Constant
KAHA	α-Ketoacid-Hydroxylamine
LC-MS	Liquid Chromatography–Mass Spectrometry
LED	Light-Emitting Diode
Leu; L	Leucine
Lys; K	Lysine
m	multiplet
M	molar
m.p.	melting point
m/z	mass charge ratio
m-CPBA	*meta*-Chloroperoxybenzoic acid
MBA	4-Mercaptobenzoic acid
Me	methyl
MeCN	Acetonitrile
MeOH	Methanol
MESNa	2-Mercaptoethanesulfonate sodium salt
Met; M	Methionine
mg	milligram
min	minute
mM	millimolar
MMP	4-mercapto-4-methylpentanol
MPa	α-methylphenacyl
MPAA	4-Mercaptophenylacetic acid
MPAL	3-mercaptoproprionamide
Mpe	β-3-methylpent-3-yl
MTG	Methyl thioglycolate
MW	Molewcular Weight

Nbz	*N*-acyl benzimidazolinone
NCL	Native Chemical Ligation
nm	nanometre
nM	nanomolar
NMM	*N*-Methylmorpholine
NMP	*N*-Methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
PALA	*N*-phosphonoacetyl-L-aspartate
Pbf	2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl
PEG	Polyethyleneglycol
Ph	phenyl
Phe; F	Phenylalanine
PPIs	Protein-Protein Interactions
Pro; P	Proline
PSRC	Peptide Synthesis Research Committee
PyBOP	(Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
PyOxim	[Ethyl cyano(hydroxyimino)acetato-O^2]tri-1-pyrrolidinylphosphonium hexafluorophosphate
q	quartet
RHAT	Reverse Hydrogen Atom Transfer
RNA	Ribonucleic acid
RP-HPLC	Reverse Phase-High Performance Liquid Chromatography
rt	room temperature
s	singlet
Ser; S	Serine
SPPS	Solid Phase Peptide Synthesis
t	triplet
TAL	Thioacid Azide Ligation
TBAF	Tetra-*n*-butylammonium fluoride
tBu	*tert*-butyl
TCEP	Tris(2-carboxyethyl)phosphine
TEA	Triethylamine
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TES	Triethylsilane
TFA	Trifluoroacetic Acid

TFET	Trifluoroethanethiol
TFMS	Trifluoromethanesulfonic acid; Triflic acid
Thr; T	Threonine
TLC	Thin Layer Chromatography
Tmob	2,4,6-trimethoxybenzyl
TMS	trimethylsilyl
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
Trp; W	Tryptophan
Trt	trityl
Tyr; Y	Tyrosine
UV	Ultraviolet
VA-044	2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride
Val; V	Valine

Acknowledgments

Firstly, I would like to thank my supervisor, Prof. Eoin Scanlan, for giving me the opportunity to join his group. I greatly appreciate his support and positive outlook ever since I was a SS student in the Scanlan Group in 2019 (and especially for nurturing my passion for meteorology!). I also wish to thank the Irish Research Council for funding my PhD through the Government of Ireland Postgraduate Scholarship Programme.

I don't think there are enough words to describe my gratitude for Dr. John O'Brien for all the work he has done for these projects! I am so grateful for his extensive knowledge about NMR which saved me a lot of headaches and for all the interesting conversations in the 'chair' while he was running 100s of experiments on my samples. I apologise that 90% of my samples had a trityl though! I'd also like to thank Dr. Manuel Ruether for his knowledge on how to fix any and every technical issue to keep everything running smoothly. I wish to thank Dr. Gary Hessman for all his support with mass spectrometry which was the cornerstone for the peptide projects.

Most of all, I would like to thank all the members of the Scanlan group who I worked with, you made the last 4 years the best time of my life despite the stress from the PhD. I will always remember our time together, all the laughs and the trips together and I'm grateful to you for putting up with my rambling about the weather/rugby/LEGO/planes/hiking/running/badminton. I'd like to especially thank Lorenzo and Lucy who collaborated on projects with me, and also Laura, Nikitas and Glenna who were there for the whole 4 years! Ale-ale-jandro, Conor, Dylan, Harlei, Inés, Josh, Katie, Mark and Matthew – you all made the Scanlan group the best place to work in!

I must give a special mention to the Connons who I shared the lab with for the 4 years of my PhD. Thanks for all the good times, the best Playlists and all the weird and wonderful conversations – Ian, Lorenzo, Lee and Pierre (maybe we'll see each other in Montreal Airport again). Another special mention for the McGourans who were always with us for the ride – André, Connor, Ellen, Julie, Susie and Valerio. Also, thank you Prof. Joanna McGouran for giving me the opportunity to attend the problem sessions for the last 3 years, I really learned a lot from them.

I want to extend my gratitude to all my friends from school and college for all the breaks when needed from the chemistry. Most importantly, I want to thank my family, Mummy, Papa and Ebi for all the unconditional support over the last 4 years, I couldn't have done it without you! Finally, I have to thank Nar Nar for helping me go through all the data, they should really make you a *Paw-fessor*!

Publications

1. **Benny, A**., Scanlan E. M. Synthesis of macrocyclic thiolactone peptides *via* photochemical intramolecular radical acyl thiol–ene ligation. *Chem. Commun*., **2024**, 60, 7950-7953

2. **Benny, A †**., Di Simo L. †; Guazzelli L., Scanlan E. M. Radical Mediated Decarboxylation of Amino Acids *via* Photochemical Carbonyl Sulfide (COS) Elimination. *Molecules*, **2024**, 29(7), 1465.

3. McLean, J. T., **Benny, A**.; Nolan, M. D., Swinand, G.; Scanlan, E. M. Cysteinyl Radicals in Chemical Synthesis and in Nature. *Chem. Soc. Rev*., **2021**, 50 (19), 10857–10894.

† Denotes equal contribution.

Contents

Chapter 1

Introduction

1.1 Peptide Therapeutics

Peptides are important biological mediators that possess highly desirable pharmaceutical properties such as high specificity, efficacy, and low toxicity.[1] Due to the wide chemical space covered by the chemically diverse side chains of the 20 native amino acids (AAs), synthetic peptides could in principle be used to target almost all enzymes or receptors implicated in disease.[2] The field of peptide therapeutics was established in 1922 with the isolation of the 51-residue peptide hormone insulin from bovine pancreas by Fredrick Banting, Charles Best and John MacLeod, followed by its first therapeutic use in humans to treat type 1 diabetes.[3, 4] Banting and MacLeod were awarded the Nobel prize in Physiology or Medicine in 1923 for this discovery.[5] In the early 1950s, du Vigneaud and co-workers isolated and determined the structure of the nonapeptide hormone oxytocin **1** and subsequently accomplished its total synthesis, ushering in the era of chemically synthetised peptide therapeutics (**Figure 1.1**).[6-8]

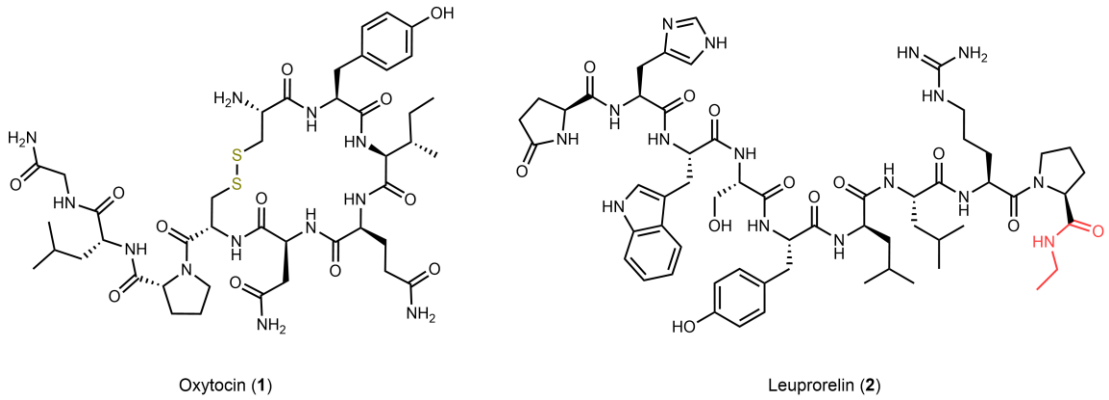


Figure 1.1 Structure of oxytocin **1** and leuprorelin **2**. The synthetic ethylamide modification at the C-terminal of leuprorelin is highlighted in red.

Early peptide drug development focused on the isolation or synthesis of known human peptide hormones for clinical use. The development of solid-phase peptide synthesis (SPPS) by Merrifield in 1963 revolutionised the field of peptide chemistry and expanded the scope of peptide therapeutics.[9] SPPS facilitated the routine synthesis of peptides in both high purity and yield, and allowed for the incorporation of non-native structural modifications, which is often required to overcome the undesirable pharmacokinetic properties of peptides such as low oral bioavailability and metabolic stability.[10, 11] In 1985, leuprorelin **2**, a synthetic analogue of gonadotropin-releasing hormone (GnRH) was introduced for the treatment of advanced prostate cancer, and subsequently for the treatment of endometriosis, uterine fibroids and precocious puberty.[12, 13] Leuprorelin became the first peptide drug which bore chemical modifications to the native peptide structure designed to improve the pharmacokinetic profile of the drug, namely an ethyl amide substitution at the C-terminal, highlighted in **Figure 1.1**.

Since the 1980s, there has been substantial investment and research into the development of peptide therapeutics in both academia and industry. As a result, approximately

80 peptide therapeutics are currently on the market for the treatment of a variety of diseases.[14, 15] Additionally, 170 peptides are in various stages of clinical trials and a further 400–600 peptides are undergoing preclinical studies.[14, 15] The majority of clinically approved peptide drugs to date have been derived from naturally occurring peptides such as human hormones or bioactive peptides from bacteria (e.g. vancomycin), fungi (e.g. cyclosporine A) or animals (e.g. exenatide).[14, 16] Another established avenue of peptide drug discovery is the screening of drug targets against vast DNA encoded peptide libraries (≈10^{10} peptides) produced *via* phage display.[17] The 60-residue peptide drug ecallantide used for the treatment of hereditary angioedema was discovered using phage display, and a significant number of antibody drugs have been discovered using this technology.[18] Over the last few decades, protein-protein interactions (PPIs) have been identified to play a critical role in many cellular functions that impact disease.[19] Due to the very nature of protein-protein interactions, peptides resembling the interaction surface could act as ideal ligands and are being investigated as potential PPI modulators.[20, 21] In recent years, the *de novo* development of peptide therapeutics is generating considerable interest through high-throughput screening of large synthetic peptide libraries, computational peptide screening and machine-learning guided peptide drug discovery.[22-24]

1.1.1 Synthetic Modifications of Peptide Therapeutics

Native peptides face several limitations which limit their widespread application as drugs. Peptides often have poor membrane permeability due to their high molecular weights, low lipophilicity and possess charged functional groups, resulting in extremely poor oral bioavailability, often less than 1%.[25] Consequently, intravenous administration of the therapeutic becomes necessary, leading to poor patient compliance.[26] Another notable limitation of peptides is poor metabolic stability due to degradation by various proteases in the body resulting in very short half-lives.[25] Finally, peptides with MW <25-60 kDa have high renal clearance since they are rapidly filtered through the glomeruli of the kidney but are not efficiently reabsorbed in the renal tubules.[27] These disadvantages can be somewhat mitigated by utilising appropriate formulations or drug delivery systems.[26] However, the main focus has been chemical modification of peptides using established medicinal chemistry strategies to improve their intrinsic properties as therapeutic agents.

The primary strategy employed to improve membrane permeability and oral bioavailability is to decrease the overall hydrophilicity of the peptide by removing hydrogen bond donors and acceptors to enable the drug to passively traverse lipid membranes. Some synthetic strategies used include (i) *N*-methylation of the amide backbone, (ii) cyclisation of the peptide to impart conformational rigidity, (iii) substitution of polar amino acids with more lipophilic natural or unnatural amino acids, or (iv) incorporation of large lipid chains.[27] This is exemplified by the orally available calcineurin inhibitor, cyclosporine A **3**, which has a cyclic peptide backbone, seven *N*-methyl groups and a lipophilic butenyl-methyl-L-threonine (Bmt) residue (**Figure 1.2**).[28] Owing

to these structural features, cyclosporine A has an excellent oral bioavailability of 57% which rises to up to 74% for microemulsion formulations.[29]

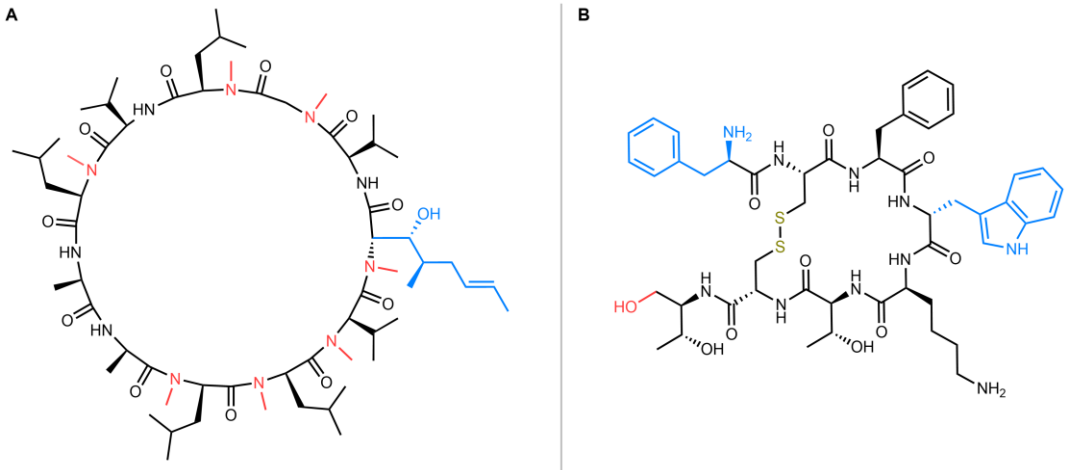


Figure 1.2. Structural features of cyclosporine A **3** and octreotide **4** which contribute to oral bioavailability and proteolytic stability.

The primary mode of peptide metabolism is the cleavage of the amide bond by proteases and peptidases, of which there are approximately 550 ubiquitously distributed throughout the tissues of the human body.[27] These enzymes generally have high affinity for the N- and/or the C-terminal of the peptide or to specific sequences of amino acids.[30] Peptides that are rich in proline (Pro), glutamic acid (Glu), serine (Ser) and threonine (Thr) residues are much more prone to proteolytic hydrolysis.[27] In order to improve metabolic stability, peptides must be modified to reduce their affinity for the active site of proteolytic enzymes while maintaining affinity for the target. Commonly employed strategies include (i) *N*-methylation, (ii) modification of the N-and C-terminal (eg. *N*-acetylation or *C*-amidation), (iii) replacement of natural L-amino acids with D-amino acids, (iv) incorporation of unnatural amino acids, (v) conjugation of the peptide to macromolecules such as polyethylene glycol (PEG) or albumin and (vi) peptide cyclisation.[27, 31, 32] For example, octreotide **4**, an analogue of the hormone somatostatin used for the treatment of gastrointestinal tumours incorporates two D-amino acids and a C-terminal which has been reduced to the primary alcohol (**Figure 2b**).[33] Due to these synthetic modifications, octreotide has an *in vivo* half-life of 1.5 h in comparison to a few minutes for somatostatin.[27]

1.2 Chemical Peptide Synthesis

Since the chemical synthesis of oxytocin by Du Vigneaud in the early 1950s, advances in peptide synthesis have enabled chemists to synthesise a wide range of peptides efficiently for use as therapeutics, even up to metric ton scale.[34] The main revolutions in chemical peptide synthesis were the development of Solid Phase Peptide Synthesis (SPPS) by Merrifield in 1963[9] and Native Chemical Ligation (NCL) by Steve Kent and co-workers in 1994.[35] However, each strategy is not without its limitations and the development of novel methodologies for peptide

synthesis continues to widen the scope of chemical peptide synthesis. In this section, the current state-of-the-art in chemical peptide synthesis is reviewed.

1.2.1 Solution Phase Peptide Synthesis

The key reaction in peptide synthesis is the formation of an amide bond between the amino group of one amino acid with the carboxylic acid of another, but this reaction is not spontaneous under ambient conditions, requiring temperatures of >200 °C.[36] To form the amide bond under milder conditions, the carboxylic acid requires activation; generally achieved by conversion to highly reactive carboxylic acid derivatives (eg. acid chlorides, anhydrides) or by the formation of an 'active ester' *in situ* using a coupling reagent. In 1902, Theodor Curtius developed the first amide coupling method using acyl azide derivatives to synthesise *N*-protected polyglycine peptides up to 5 AAs in length (**Figure 1.3a**).[37, 38] Although the acyl azide method is tolerant to many functional groups and offers unparalleled retention of chiral integrity during coupling, the instability and explosive nature of acyl azides limited its widespread application for iterative peptide synthesis.[39] The synthesis of the azide is also time-consuming and laborious as it involves three distinct steps with two purifications: converting the carboxylic acid to an ester (methyl, ethyl or benzyl), displacing the ester with hydrazine to form the hydrazide, and finally converting the hydrazide to the azide.[39] However, the acyl azide method has gained renewed interest in recent years with the advent of continuous-flow reaction systems since the explosive azide can be generated and reacted *in situ*.[40] Three years later in 1905, Emil Fischer reported another coupling method using acid chloride derivatives which he used to synthesise peptides of up to 18 AAs in length (**Figure 1.3b**).[41, 42] However, acid chlorides are known to be susceptible to various side reactions including loss of chiral integrity at the α-position, and therefore have not found widespread application in peptide synthesis.[39]

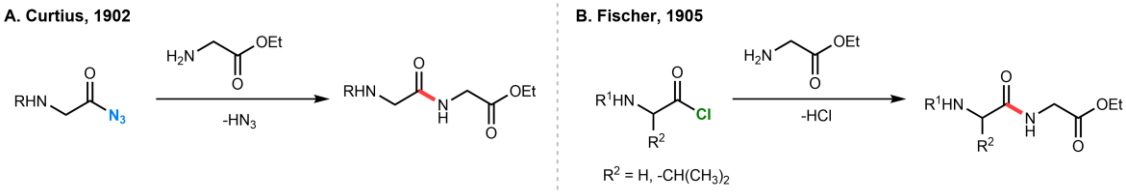


Figure 1.3. (a) Acyl azide amide coupling method reported by Curtius. **(b)** Acid chloride amide coupling method developed by Fischer.

Since the pioneering work by Curtius and Fischer, numerous amide coupling reagents have been developed as amide bond formation has become the most frequent reaction used in the pharmaceutical industry.[43] The main classes of coupling reagents that are commonly used in peptide synthesis to form the 'active ester' are carbodiimides such as *N*,*N*'-diisopropylcarbodiimide (DIC; **5**), uronium/aminium salts such as HATU **6**, and phosphonium salts such as PyBOP **7** (**Figure 1.4a**). During activation of the carboxylic acid, the α-position of the amino acid is prone to base-catalysed racemisation *via* direction enolisation (Path A), or *via* formation of an oxazolone intermediate (Path B) (**Figure 1.4b**).[39] The use of carbamate based

N^{α}-protecting groups can reduce the likelihood of oxazolone formation[44] and the use of hindered tertiary amine bases such as *N*,*N'*-diisopropylethylamine (DIPEA) or *N*-methylmorpholine (NMM) can limit deprotonation.[45]

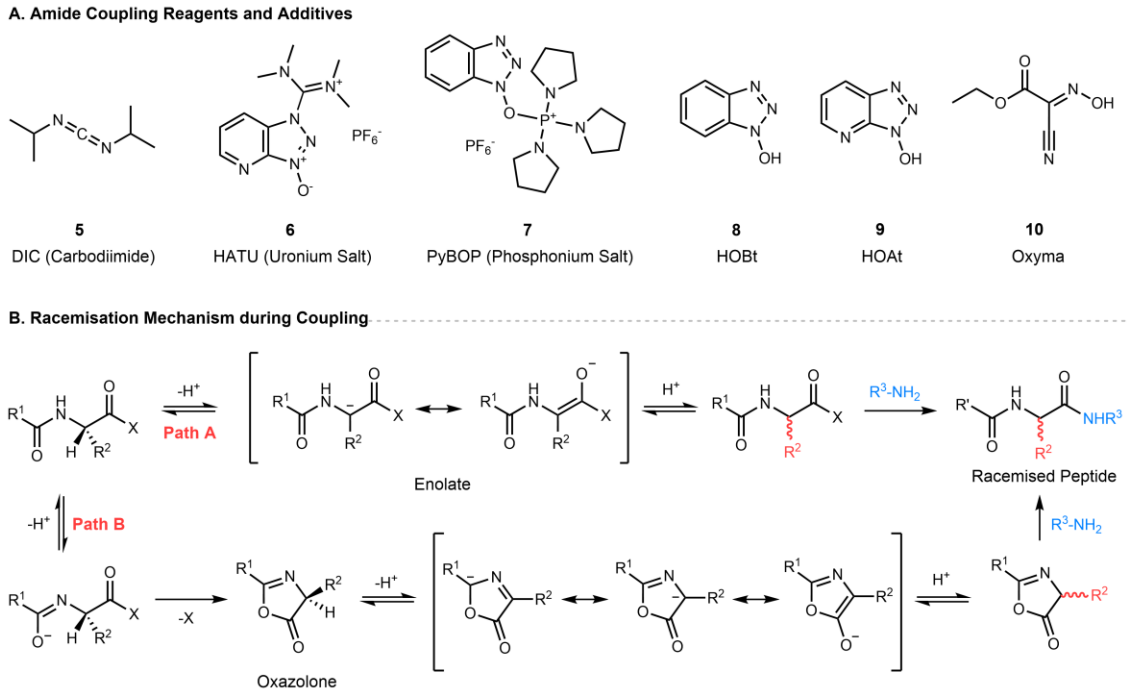


Figure 1.4. (a) Selection of commonly used amide coupling reagents and additives. **(b)** Racemisation *via* enolate formation (Path A) or oxazolone formation (Path B).

To suppress racemisation during carbodiimide coupling, König and Geiger introduced 1-hydroxybenzotriazole (HOBt; **8**) as an additive in 1970, demonstrating that racemisation levels dropped from 35% to 1.4% for the coupling of Cbz-Gly-Phe-OH to H-Val-OMe.[46] Additionally, HOBt increases yields as it reacts with the *O*-acylurea intermediate formed to yield an -OBt ester which stabilises the incoming amine *via* hydrogen bonding interactions.[47] In 1994, Carpino reported that 1-hydroxy-7-azabenzotriazole (HOAt; **9**) was even more effective, reducing racemisation levels to one third to one half of the levels of HOBt.[48] The nitrogen at the 7-position of HOAt also provides a neighbouring group effect by coordinating the incoming amine, greatly increasing reaction rates.[48] Coupling reagents such as HATU **6** and PyBOP **7** are derived from HOAt and HOBt respectively, and they release HOBt/HOAt *in situ* to suppress racemisation. However, the classification of HOBt and HOAt as potentially Class I explosive compounds in the early 2000s led to the search for safer alternatives, particularly for industrial applications.[49] Nowadays, ethyl 2-cyano-2-(hydroxyimino)-acetate (Oxyma; **10**) has become the non-explosive additive of choice, and its effectiveness at supressing racemisation is comparable to that of HOAt.[50] Coupling reagents based on Oxyma such as the uronium salt COMU® and the phosphonium salt PyOxim have been developed by Albericio.[51, 52] The relatively high cost of these coupling reagents compared to the aforementioned well-established coupling reagents has limited their widespread use.

Traditional solution-phase peptide synthesis is highly effective for the large-scale synthesis of short peptides composed of only a few amino acids and offers unparalleled flexibility in the choice of coupling chemistry available. However, the tedious purification required after each coupling/deprotection step results in low yields for longer sequences. Recent advances in solution-phase peptide synthesis include continuous flow synthesis of peptides up to 22 AAs in length and the use of auxiliary groups to simplify purification.[53, 54] Nevertheless, even highly optimised solution-phase strategies are limited to the synthesis of relatively short peptides (< 20 AAs) and the routine chemical synthesis of long peptides was only enabled by the development of solid-phase peptide synthesis.

1.2.2 Solid Phase Peptide Synthesis (SPPS)

Solid phase peptide synthesis (SPPS) was developed by Merrifield in 1963 and demonstrated through the synthesis of the model tetrapeptide, H-Leu-Ala-Gly-Val-OH, using polystyrene resin functionalised with a chloromethyl linker.[9] As recognition of his pioneering work, Merrifield was awarded the Nobel Prize in Chemistry in 1984.[55] The SPPS method involves covalent attachment of the C-terminal of an *N*-protected AA to an insoluble resin, followed by iterative deprotection of the N-terminal protecting group and coupling of activated *N*-protected AAs until the desired sequence is assembled (**Figure 1.5**).[56] Reactive side-chains are protected by orthogonal protecting groups to prevent unwanted side reactions. After each coupling/deprotection step, soluble reagents and by-products are easily removed by washing the resin with solvent, removing the need for tedious purification steps associated with traditional solution-phase peptide synthesis.[9] Once the desired AA sequence is assembled, global deprotection of AA side-chain protecting groups and cleavage from the resin yields the fully unprotected crude peptide.

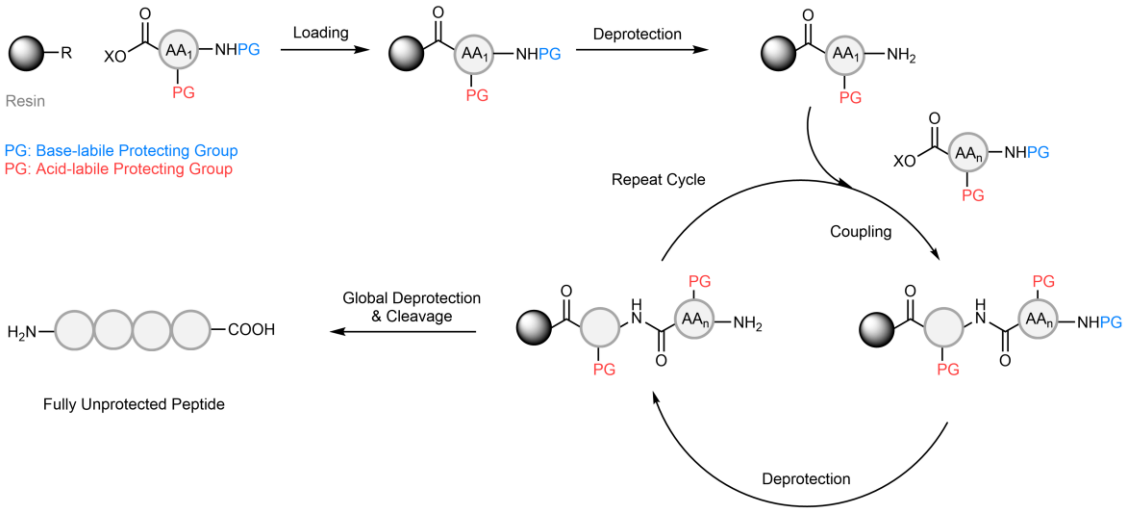


Figure 1.5. General scheme for Solid Phase Peptide Synthesis (SPPS). X = activated group, PG = Protecting Group.

Due to the iterative nature of the coupling/deprotection steps and facile purification, Merrifield noted from the outset that SPPS '*will lend itself to automation and provide a route to the synthesis of some of the higher molecular weight polypeptides which have not been accessible by conventional procedure*'.[9] Shortly after in 1965, Merrifield and Stewart engineered a fully automated peptide synthesiser and used it to synthesise the 9-AA hormone bradykinin in 32 h, approximately 4 h for each AA coupling cycle.[57] The revolutionary power of SPPS was fully realised by the automated synthesis of the 124-AA biologically active enzyme, bovine ribonuclease A, in 17% overall yield over 369 separate chemical reactions by Gutte and Merrifield in 1969.[58] In 1992, Yu *et al*. reported that microwave irradiation greatly enhanced the rate, yield and purity of SPPS, reducing the coupling cycle for each AA to just 6 min.[59] Nowadays, commercial peptide synthesisers that use microwave irradiation to reduce each coupling cycle to < 4 min and synthesise up to 2400 peptides in parallel are used widely in academia and industry.

1.2.3 Protecting Group Strategies in SPPS

The two most widely utilised SPPS strategies are defined by the choice of N^{α} protecting group; acid labile *t*-butyloxycarbonyl (Boc) or base labile 9-fluorenylethoxycarbonyl (Fmoc) (**Figure 1.6**). The conventional Boc SPPS strategy developed by Merrifield in the 1960s uses N^{α}-Boc AAs which are deprotected using trifluoracetic acid (TFA) during chain assembly and hydrofluoric acid (HF) for global deprotection and resin cleavage (**Figure 1.6a**).[60] Boc SPPS utilises benzyl (Bzl) side chain protecting groups and relies on differential acid lability for selectivity, with the N^{α}-Boc group being approximately 10^4 times more acid-labile than the Bzl groups.[61] However, the selectivity is never absolute and the accumulation of low levels of Bzl deprotection during the course of a peptide synthesis can cause significant issues with purity.[62]

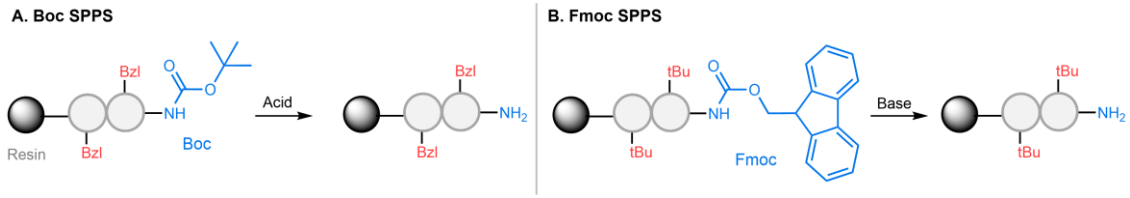


Figure 1.6. Protecting group strategy for **(a)** Boc SPPS and **(b)** Fmoc SPPS.

The major disadvantage of Boc SPPS is the extremely toxic and corrosive nature of HF which demands specialised apparatus and training for its use. Milder acids such as trifluoromethanesulfonic acid (triflic acid; TFMSA) and trimethylsilyl trifluoromethanesulfonate (TMSOTf) can be used as a substitute for HF, but they increase the risk of side reactions such as Friedel-Crafts acylation of aromatic residues.[63] In addition, HF is not compatible with sensitive modifications such as phosphorylated or glycosylated amino acids, thereby limiting the utility of Boc SPPS for the synthesis of highly functionalised peptides.[64] As a result, milder Fmoc SPPS has superseded Boc SPPS as the preferred strategy for the routine synthesis of peptides in

academic and industrial laboratories (**Figure 1.6b**). Boc SPPS still finds application in specialised cases, particularly for the direct synthesis of base sensitive C-terminal functionalities such as thioesters or for the synthesis of 'difficult sequences' since peptides are less prone to secondary structure formation and aggregation.[61, 65]

In 1970, Han and Carpino introduced the 9-fluorenylethoxycarbonyl (Fmoc) amino protecting group which can be removed through a β-elimination reaction under mild basic conditions, including by primary or secondary amines (**Figure 1.7**).[66] The Fmoc group is relatively inert to tertiary amines such as DIPEA or triethylamine (TEA) at low concentrations, permitting their use as base catalysts for amide coupling.[67] Attempts to utilise N^{α}-Fmoc AAs in solution phase peptide synthesis were largely unsuccessful as the cleavage byproduct, dibenzofulvene (DBF; **12**), can be difficult to separate chromatographically from the desired product.[64] In addition, DBF is highly electrophilic and can alkylate nucleophilic functional groups but this is easily overcome by using a nucleophilic base which can react with DBF *in situ* to form an inert adduct. In 1978, Chang *et al*. and Atherton *et al*. independently published the first use of N^{α}-Fmoc AAs in SPPS.[68, 69] In both protocols, a solution of piperidine **11**, either 50% v/v in DCM or 20% v/v in DMF, was used for Fmoc deprotection. Furthermore, AA side chains were protected using *tert*-butyl (tBu) groups which were cleaved during the final resin cleavage step using TFA.[68, 69] In comparison to Boc SPPS which relies on graduated acid lability for selectivity, tBu groups are completely stable to the basic conditions used for Fmoc deprotection, providing orthogonality. Additionally, the high UV absorbance of the N-(9-fluorenylmethyl)-piperidine adduct **13** (λ_{max} = 301 nm, ε = 6000 $M^{-1}cm^{-1}$) that is formed stoichiometrically during Fmoc deprotection enables facile *in situ* monitoring of each deprotection step, which is directly correlated to coupling efficiency.[70] The milder conditions used in Fmoc SPPS compared to Boc SPPS prompted its rapid adoption for chemical peptide synthesis. By 1994, research by the Peptide Synthesis Research Committee (PSRC) showed that of the labs surveyed, 98% were using Fmoc/tBu SPPS for routine peptide synthesis.[71]

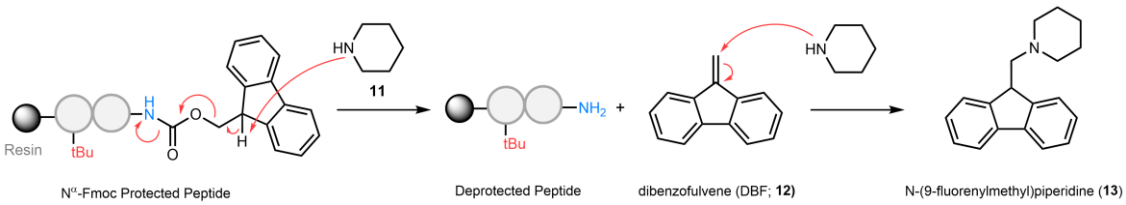


Figure 1.7. Mechanism of Fmoc deprotection using piperidine **11**.

Over the years, the range of chemistry available for Fmoc SPPS has greatly expanded, enhancing its efficiency, versatility, and applicability. There are a wide range of acid labile resin linkers available for Fmoc SPPS which release peptides bearing various functional groups at the C-terminal upon treatment with TFA; most commonly carboxylic acids (Wang, 2-chlorotrityl) and amides (Rink amide). In fact, base labile linkers such as the 4-hydroxymethyl benzamide (HMBA) linker have also been developed for Fmoc SPPS which can form C-terminal carboxylic acids,

amides, esters or thioesters upon basic nucleophilic treatment during cleavage with hydroxide, amines, alkoxides and thiolates respectively.[72] Although anchoring of the peptide *via* the C-terminal to the resin is the most common strategy, it is also possible to anchor them *via* the backbone amide[73] or nucleophilic side chains such as that of Ser, Thr, Lys, Asp, Glu and Tyr.[74]

1.2.4 Limitations of SPPS

Since the first report by Atherton *et al*., 20%(v/v) piperidine in DMF has become the standard used for Fmoc deprotection, achieving 99.99% deprotection in approximately 1.5 min ($t_{1/2}$ = 7 s) on solid phase.[75, 76] One of the most common side reactions encountered in Fmoc SPPS is base-catalysed aspartimide formation in peptides containing Asp-Xaa sequences (**Figure 1.8**).[77] The extent of aspartimide formation is highly sequence dependant with Asp-Gly, Asp-Asp, Asp-Asn, Asp-Arg, Asp-Thr and Asp-Cys sequences being more susceptible.[64] Various methods to minimise aspartimide formation have been reported including the addition of additives such as HOBt **8**[78] or Oxyma **10**[79] to the Fmoc deprotection solution, the use of bulky Asp protecting groups such as β-3-methylpent-3-yl (Mpe) [80] or 5-*n*-butyl-5-nonyl (Bno)[81] esters or amide backbone protection.[82] Another strategy to minimise aspartimide formation has been to use alternative bases to piperidine for Fmoc deprotection. Moreover, piperidine is a restricted chemical under the United Nations Convention Against Illicit Traffic in NAr Cotic Drugs and Psychotropic Substances (1988) for its illicit use in the synthesis of the hallucinogen phencyclidine (PCP).[83, 84] Some examples of alternative bases that have been successfully applied to Fmoc deprotection in SPPS include piperazine[77], morpholine[85], 4-methylpiperidine[86], *N*-methylpyrrolidine[87] and 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU).[88]

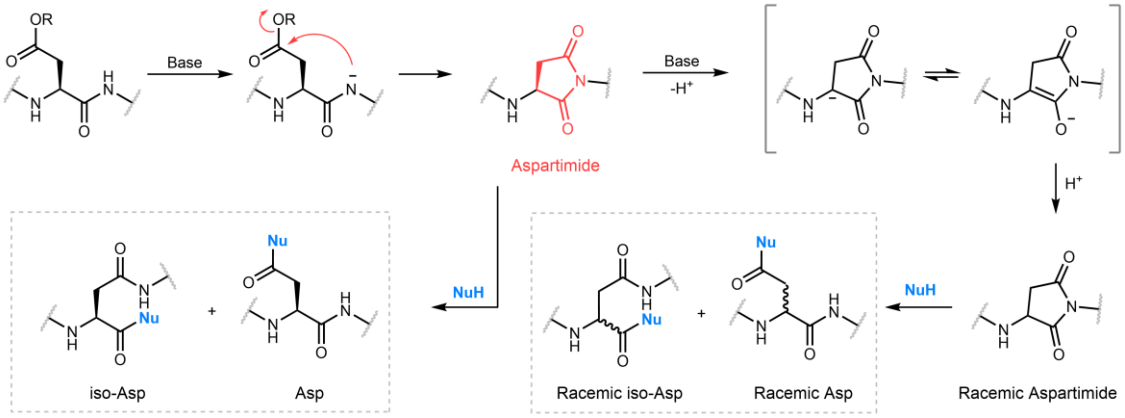


Figure 1.8. Base catalysed aspartimide formation during Fmoc SPPS.

One of the primary factors limiting the length of peptides that can be synthesised by SPPS is the aggregation of longer and more non-polar peptides due to hydrophobic non-covalent interactions, which ultimately reduces solubility and deprotection/coupling efficiency.[89] Several solutions have been developed to overcome on-resin peptide aggregation such as the use of pseudoprolines and backbone amide protection which disrupt secondary structure formation.[89] However, these solutions are highly sequence dependant and come with the disadvantage of

requiring coupling to sterically hindered secondary amines after their installation. Since SPPS is a linear synthesis, high efficiency (>99%) is required at each step to ensure a sufficiently high final yield of the desired peptide. This is often achieved by using a large excess of reagents (often 3–10 equiv.) and repeating difficult coupling reactions before N^{α}-deprotection. Nevertheless, it is impossible to avoid the accumulation of side-products due to racemisation, AA deletions, truncations, and other undesired side-reactions (e.g. aspartimide formation) over the course of a synthesis. This can complicate high performance liquid chromatography (HPLC) purification due to the close structural similarity between the desired peptide and impurities, especially for longer sequences, ultimately resulting in lower isolated yield. From a green chemistry perspective, the atom economy of the SPPS method is extremely poor due to the extensive use of protecting groups and large amounts of reagents and organic solvents required. While there is intensive research ongoing to develop more sustainable alternatives to the standard SPPS method, the adaptation of these modifications by the wider peptide synthetic community is gradual.[90, 91] Hence, convergent approaches are necessary for the synthesis of peptides >50 AAs in length or those containing particularly difficult sequences in sufficiently high yield.

1.3 Peptide Ligation

Peptide ligation is the convergent synthesis of peptides by the chemoselective coupling of smaller peptide fragments prepared by SPPS without the use of coupling reagents, often fully unprotected and in aqueous solvent. In nature, peptide synthesis occurs in the ribosome by sequential O-to-N acyl transfer of the nascent peptidyl-tRNA ester to the α-amino group of the subsequent aminoacyl tRNA. The catalytic amide bond formation is driven primarily by the reduction of the entropy of activation through solvent reorganisation, substrate positioning and the orientation of the reacting group.[92] In general, peptide ligation reactions consist of a chemoselective 'capture' step between a complementary nucleophile/electrophile pair that brings the peptides into close proximity, followed by an irreversible intramolecular acyl transfer to form a native amide bond at the ligation site (**Figure 1.9**).[93] While there have been many peptide methods developed, those that form a 'native' amide bond between the peptides are preferred.

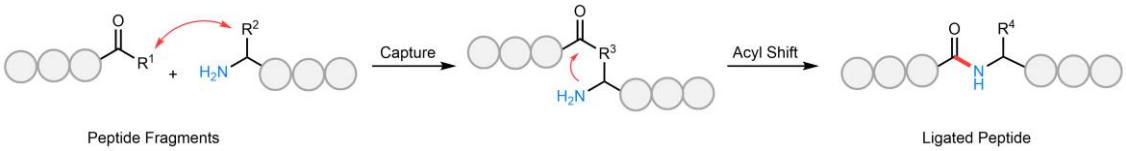


Figure 1.9. General peptide ligation strategy.

The acyl transfer concept was first applied to peptide ligation in the Prior Thiol Capture strategy by Kemp in the 1980s and demonstrated by the synthesis of the 29-AA C-terminal sequence of bovine pancreatic trypsin inhibitor (BPTI).[94] In this pioneering study, a protected peptide fragment bearing a 4-hydroxy-6-mercaptodibenzofuran (Dbf) ester template at its C-

terminal and another bearing an S-carbomethoxysulfenyl (Scm) functionalised Cys disulfide derivative at its N-terminal were used (**Figure 1.10**). The ligation is initiated by a rapid and highly efficient thiol-disulfide exchange which forms an initial disulfide linkage between the two peptides, facilitating a proximity induced intramolecular O-to-N acyl transfer to form the amide bond. Upon completion of the acyl transfer, disulfide reduction removes the Dbf template and side chain deprotection yields the unmodified target peptide. In a subsequent study by Kemp and Carey, a 39-AA peptide (26+13 ligation) and a 25-AA (13+12 ligation) peptide were synthesised using fragments where only the internal Cys residues were protected.[95] Since this seminal work, a variety of peptide ligation strategies have been developed, with the most widely used and impactful being Native Chemical Ligation (NCL).

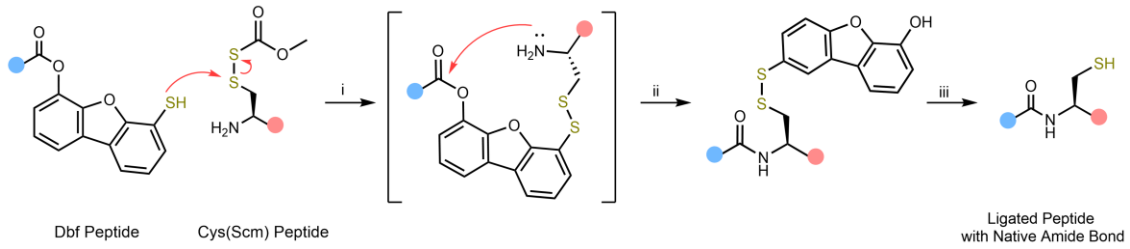


Figure 1.10. General scheme for the Prior Thiol Capture peptide ligation strategy. (i) Thiol capture (ii) O-to-N acyl transfer (iii) disulfide reduction.

1.3.1 Native Chemical Ligation

In 1953, Wieland *et al*. serendipitously discovered the foundation for NCL while investigating aryl thioesters as 'active ester' substrates for amide coupling.[96] They found that valine thiophenyl ester **14** underwent amide bond formation at a greatly accelerated rate in aqueous solution at pH >7 with unprotected Cys **15** to form Val-Cys dipeptide **16,** compared to other unprotected AAs (**Figure 1.11**). The authors postulated that the cysteine thiolate undergoes thiol-thioester exchange with the Val thioester to form a cysteinyl thioester intermediate. This facilitates an irreversible, proximity induced S-to-N acyl transfer through a 5-membered transition state to form the amide bond.[96]

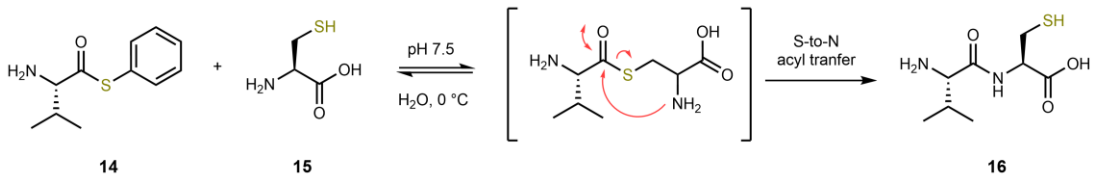


Figure 1.11 The work of Wieland *et al*. on S-to-N acyl transfer to form amide bonds.

In 1994, Kent and co-workers extended this concept to peptide ligation and demonstrated NCL with the 33+39 ligation of the 72-residue protein, [Ala33] interleukin 8 ([Ala33] IL-8).[35] The reaction uses fully unprotected peptides; one bearing a thioester at its C-terminal and another

bearing a Cys residue at the N-terminal (**Figure 1.12**). The peptides undergo reversible trans-thioesterification in aqueous solution at pH 7 to form a cysteinyl thioester intermediate, followed by a spontaneous and irreversible intramolecular S-to-N acyl transfer to furnish a native amide bond at the ligation site. The Cys residue which enables the reaction is re-formed following ligation, removing the need for further chemical modification to form a native peptide. In terms of selectivity, the acidity of the N-terminal Cys thiol ($pK_a \approx 7$) is approximately 30-100 fold less than that of internal Cys thiols, enough to ensure regioselectivity at pH 7. Since this seminal publication, NCL in combination with SPPS has become the predominant strategy for peptide ligation and recent advances have enabled the chemical synthesis of exceptionally sized peptides and proteins such as the 352-AA P2 DNA polymerase IV from *Sulfolobus solfataricus* (40.2 kDa) by Xu *et al*.[97] or tetraubiquitinated α-synuclein (48.6 kDa) by Haj-Yahya *et al*.[98]

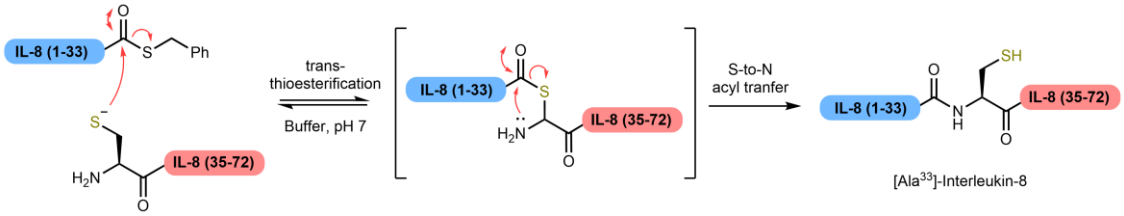


Figure 1.12. The synthesis of [Ala33] IL-8 through Native Chemical Ligation (NCL) by Dawson et al.[35]

Substrate studies have shown that NCL is feasible with thioesters of all 20 natural AAs at the C-terminal, but greatly increased reaction times of 24-48 h are necessary for hindered AAs such as Val, Thr, Ile or Pro.[99] In addition, NCL is usually performed at low concentrations (<5 mM) to increase selectively, and often at lower concentration for highly hydrophobic peptides due to poor solubility, which can also reduce reaction rates.[100] The rate-determining trans-thioesterification step of NCL can be accelerated through the addition of thiol catalysts to promote *in situ* formation of more activated thioesters.[101] Generally, aryl thiols such as thiophenol **13** and 4-mercaptophenylacetic acid (MPAA; **14**) are used due to their lower pK_a values which make them better leaving groups to facilitate efficient trans-thioesterification (**Figure 1.13**). Alkyl thiols such as 2-mercaptoethanesulfonate sodium salt (MESNa; **15**)[102], methyl thioglycolate (MTG; **16**)[103] or trifluoroethanethiol (TFET; **17**)[104] have also been used as additives in NCL, but they are more modest catalysts due to their higher pK_a values. The universal applicability of the NCL method for peptide ligation is limited by the requirement for a Cys residue at the N-terminal, confining NCL to solely Xaa-Cys ligation sites. Cysteine is not very abundant in proteins (~ 1.7% in human proteins) and is seldom centrally located in a target peptide to facilitate its convergent synthesis by NCL.[105] In some cases, analogues of peptides were synthesised by substituting a residue that is non-critical to function to cysteine to facilitate its synthesis by NCL in an attempt to overcome this limitation. This approach is exemplified by the synthesis of the 110-residue enzyme [Cys49]barnase by Dawson *et al*. where Lys49 was substituted with Cys with minimal effect on enzymatic activity.[101] However, this approach is not general and it is desirable to be able to synthesise the native sequences of the required peptide. To address this critical limitation,

several extended NCL methods have been developed including the 'ligation/desulfurisation' protocol and Auxiliary Mediated Ligation (AML) which can enable ligation at non-Cys sites.

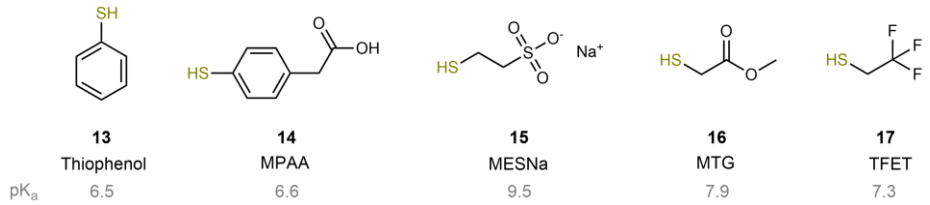


Figure 1.13. Common thiol additives used in NCL to catalyse trans-thioesterification and their corresponding pK_a values.

1.3.4 Extended Native Chemical Ligation Methods

The ligation/desulfurisation strategy which enables ligation at Xaa-Ala sites was first demonstrated by Yan and Dawson in 2001 through the synthesis of the cyclic antibiotic microcin J25, the streptococcal protein G B1 domain and [Ala49]barnase.[60] To synthesise these peptides which lack Cys residues, Yan and Dawson installed a cysteine residue in place of an Ala in the sequence of the peptides, which was then reduced or 'desulfurised' to Ala following ligation using Raney nickel and Pd/Al_2O_3 (**Figure 1.14**). This protocol significantly increased the scope of NCL since alanine is one of the most abundant AAs found in proteins (~ 9% in bacteria and 6 % in eukaryotes).[106]

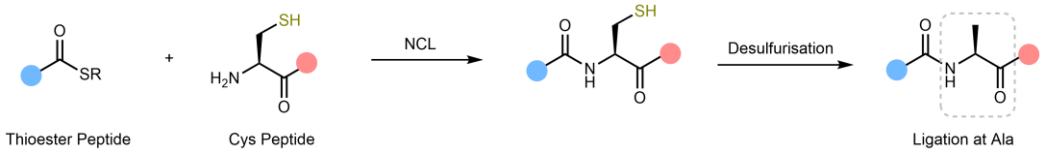


Figure 1.14. The ligation/desulfurisation strategy for ligation at Xaa-Ala junctions developed by Yan and Dawson.[107]

Difficulties in the purification of the peptide from the metal catalysts used for desulfurisation and the occurrence of metal-catalysed side reactions prompted the development of metal-free desulfurisation conditions.[100] To this end, Wan and Danishefsky reported the use of tris(2-carboxyethyl)phosphine (TCEP) in the presence of the water-soluble radical initiator VA-044 for chemoselective desulfurisation.[108] Commonly used aryl thiol additives such as thiophenol and MPAA act as radical quenchers which prevent one-pot ligation/desulfurisation, and purification of the intermediate is required.[109] Methods to remove the aryl thiol additives by liquid-liquid extraction[110] and solid-phase capture[111] have been reported to facilitate one-pot reaction. Alternatively, alkyl thiols such as TFET are not prone to radical quenching and due to its low boiling point (35-37 °C), it can also be removed easily from the reaction mixture if required.[109] Recently, Jin *et al*. demonstrated a radical and metal-free delsulfurisation protocol using sodium

borohydride ($NaBH_4$) and TCEP which is compatible with peptides bearing post-translational modifications such as phosphorylation and crotonylation.[112]

The ligation/desulfurisation strategy developed by Yan and Dawson prompted the development of derivates of other AAs incorporating β-, γ- or δ-thiols which can be used as Cys surrogates to enable NCL, followed by desulfurisation to furnish the native AA. The first such example was β-thiol Phe **18** reported independently by Crich[113] and Botti[114] (**Figure 1.15**). Since then, the ligation/desulfurisation strategy has been extended to thirteen of the twenty natural AAs.[109] Selected thiolated derivatives of valine **19**, threonine **20**, aspartic acid **21** and tryptophan **22** are shown in **Figure 1.15**. However, the limited commercial availability of required starting materials and lengthy asymmetric syntheses to access thiolated derivatives represents a considerable challenge for their general application in peptide synthesis.[105]

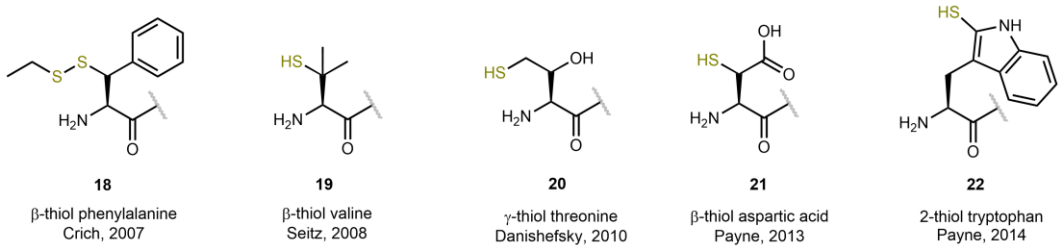


Figure 1.15. Selected thiol containing derivatives of amino acids used for ligation/desulfurisation.

Alongside the development of thiol functionalised derivatives of natural AAs, Auxiliary Mediated Ligation (AML) has further enabled ligation at non-Cys sites. The method was first demonstrated by Kent and co-workers in 1996 using an oxyethanethiol auxiliary attached to the α-amino group of Gly or Ala at the N-terminal which facilitates NCL, and could be selectively cleaved after ligation by treatment with Zn under acidic conditions to reform the natural AA.[115] However, the use of the α-amino group to anchor the auxiliary necessitates S-to-N acyl transfer to a secondary amine, which in addition to the general steric bulk introduced significantly impedes the rate of reaction, limiting this approach to less bulky residues such as Gly and Ala.[105] Since this publication, a variety of N^{α}-anchored auxiliary groups have been reported including the photocleavable 2-mercapto-1-(2-nitrophenyl)ethylamine group by Aimoto[116] and the 2-mercapto-2-phenethyl group by Seitz which is cleaved using a radical desulfurisation induced fragmentation reaction using TCEP.[117] To overcome the steric issues associated with anchoring the auxiliary to the α-amino group of the peptide, side-chain anchored auxiliaries have also been investigated. Examples include the sugar-derived auxiliary **23** developed by Wong[118] anchored *via* the side-chain of Asn to synthesise *N*-glycopeptides and the cyclohexyl-derived auxiliary **24** developed by Brik[119] anchored *via* the side-chain of Asp (**Figure 1.16**). In both cases, the S-to-N acyl transfer occurs over a large 14-membered transition state, minimising steric hindrance at the N-terminal, enabling ligation at a greater variety of AA junctions, including bulky residues such as Val.[118] For ring sizes >6, the S-to-N acyl transfer step becomes the rate-limiting step of NCL, so reaction times can be extremely long, days or weeks, when using side-chaining anchored auxiliaries.[105]

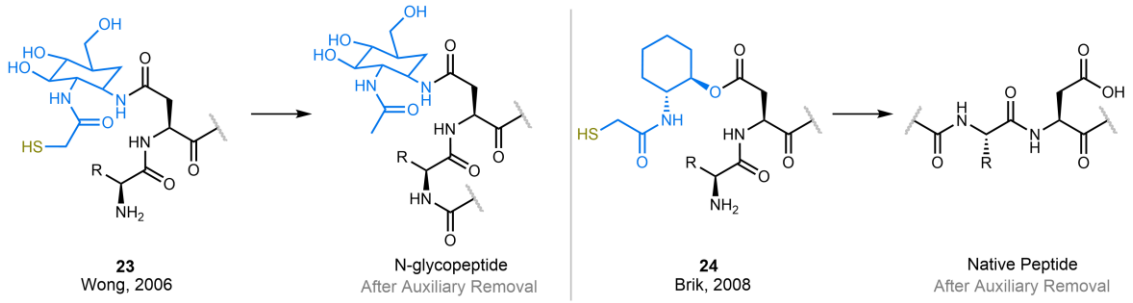


Figure 1.16. Selected auxiliaries used for NCL, and the product formed after ligation and auxiliary removal.

1.3.5 Peptide Thioester Synthesis

The invariable in all NCL reactions is the C-terminal peptide thioester which is reactive towards thiol or nitrogen nucleophiles while being relatively resistant to hydrolysis at neutral pH. The rate of thioester hydrolysis only becomes significant above pH 8.0 and is lowest at neutral pH, the pH at which NCL reactions are generally conducted.[120] Historically, peptide thioesters were synthesised *via* Boc SPPS using thioester linkers which were resistant to TFA, such as the 3-mercaptoproprionamide (MPAL) linker reported by Hojo *et al*. in 1991.[121] After peptide assembly and cleavage with HF, an MPAL peptide thioester is formed which is suitable for NCL. As mentioned earlier in **Section 1.2.3**, the use of HF for routine SPPS is not desirable and methods to synthesise thioesters using Fmoc SPPS have been developed. Typically, thioesters are not stable to the repeated piperidine treatments used for Fmoc deprotection, so they must be installed indirectly after chain assembly. Initial on-resin Fmoc SPPS strategies relied on the coupling of a thiol or a preformed AA thioester to the C-terminal of a backbone[122] or side-chain anchored[123] peptide after chain assembly, followed by deprotection/cleavage to form a fully unprotected C-terminal thioester. Another strategy is to use the Kenner safety-catch sulfonamide linker which, following chain assembly, is activated by alkylation by iodoacetonitrile[124] or TMS-diazomethane[125] and thiolysed to form the thioester.[100]

In 2008, Blanco-Canosa and Dawson reported the used of the diaminobenzoyl (Dbz) linker which after chain assembly is acylated with p-nitrophenylchloroformate and cleaved from the resin to form an unprotected *N*-acyl benzimidazolinone (Nbz) peptide (**Figure 1.17a**).[126] The Nbz peptide can be easily thiolysed at neutral pH, and the thioester is often formed *in situ* during NCL by the thiol additive. Subsequently, Blanco-Canosa *et al*. reported a second generation MeDbz linker where the para-aniline is methylated to prevent acylation during SPPS.[127] The most popular contemporary method for thioester synthesis, developed by Fang *et al*., uses a fully deprotected C-terminal peptide hydrazide which is oxidised using $NaNO_2$ to form an acyl azide which can be thiolysed to form the thioester *in situ* (**Figure 1.17b**).[128] Dbz peptides can also be oxidised by $NaNO_2$ to form C-terminal benzotriazoles which can be thiolysed to form a thioester (**Figure 1.17c**).[129] A variety of methods which take advantage of O-to-S and N-to-S acyl shifts to form thioesters have also been explored, but their use has been limited.[100] In addition to purely

chemical methods, peptide thioesters can also be formed semi-synthetically through recombinant means, first reported by Muir *et al*. in 1998 in the Expressed Protein Ligation (EPL) strategy.[130] Tan *et al*. demonstrated the enzymatic synthesis of thioesters using subtiligase, a variant of the serine protease subtilisin from *Bacillus subtilis* in which the active site Ser221 residue has been mutated to Cys, and Pro225 to Ala.[131] Using a suitable glycolate ester peptide substrate, the authors were able to thiolyse a peptide-Cys221 thioester intermediate to form the desired thioester.

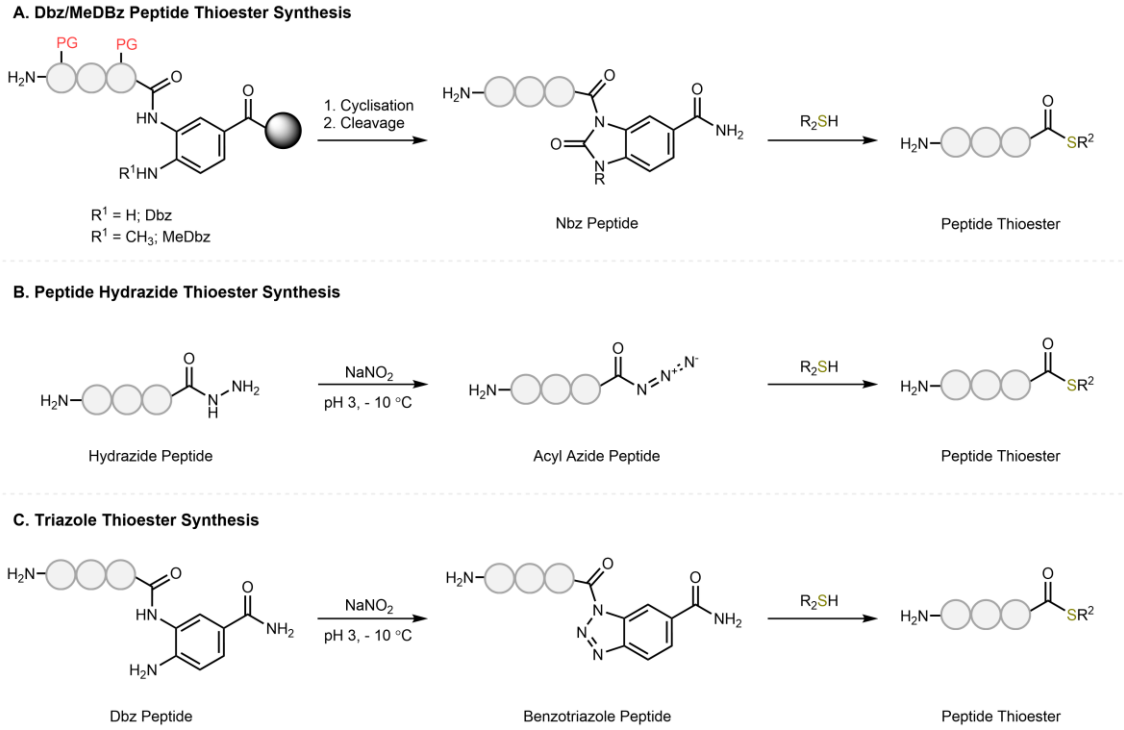


Figure 1.17. Selected methods for the chemical synthesis of peptide thioesters after Fmoc SPPS.

1.4 Thioacids

In comparison to carboxylic acids (RCOOH), their sulfur containing counterparts known as thioacids or thiocarboxylic acids (RCOSH) possess unique reactivity, which is increasingly being exploited in synthetic organic chemistry.[132] In fact, AA thioacids have been mentioned as potential intermediates in prebiotic peptide bond formation reactions through oxidative acylation.[133, 134] The simplest thioacid, thioacetic acid **26**, was first synthesised in 1854 by the German chemist August Kekulé by treating acetic acid **25** with phosphorous pentasulfide (P_4S_{10}) (**Figure 1.18a**).[135] Thioacids are more acidic than carboxylic acids: for instance, thioacetic acid (**26**, pK_a = 3.33) versus acetic acid **27**(pK_a = 4.76) or thiobenzoic acid **27** (pK_a = 2.48) versus benzoic acid **28** (pK_a = 4.20) (**Figure 1.18b**).[136] Thioacids are prone to oxidation to form diacyl disulfides[133], and can be hydrolysed in both acidic[137] or basic pH.[138] Compared to carboxylic acids, thioacids are more nucleophilic and readily react with a wide range of electrophiles

including azides[139], sulfonamides[140], isonitriles[141], aziridines[142], isocyanates[143], thiocarbamates[144], nitroso compounds[145] and Sanger and Mukaiyama reagents.[146] The use of thioacids for the formation of amide bonds has been reviewed extensively by Narendra *et al*.[132] For the purposes of this thesis, only their applications in peptide synthesis will be considered.

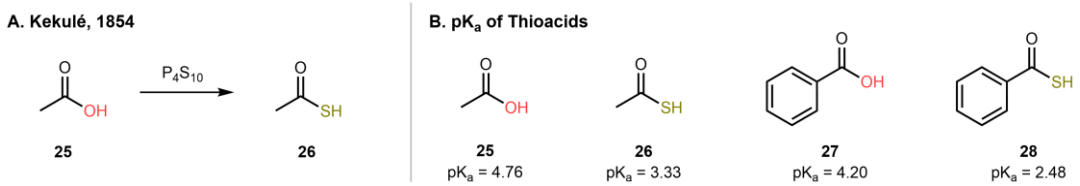


Figure 1.18. (a) Synthesis of thioacetic acid **26** by Kekulé.[135] **(b)** Comparison of the pK_a values of carboxylic acids versus thioacids.[136]

1.4.1 Synthesis of Peptide Thioacids

The classical synthesis of thioacids begins with the activation of the corresponding carboxylic acid using N-hydroxysuccinimide[147], p-nitrophenyl esters[148] or standard amide coupling reagents such as *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC)[149] or carbonyldiimidazole (CDI)[142], followed by the reaction of the active ester with sulfur sources such as H_2S, Na_2S or NaSH to furnish thioacids (**Figure 1.19**). Another protocol, developed by Rao *et al*., uses Lawesson's reagent under microwave irradiation to directly covert carboxylic acids into thioacids.[150] Matsumoto *et al*. reported another direct conversion using potassium thioacetate (AcSK) and catalytic thioacetic anhydride (Ac_2S).[151]

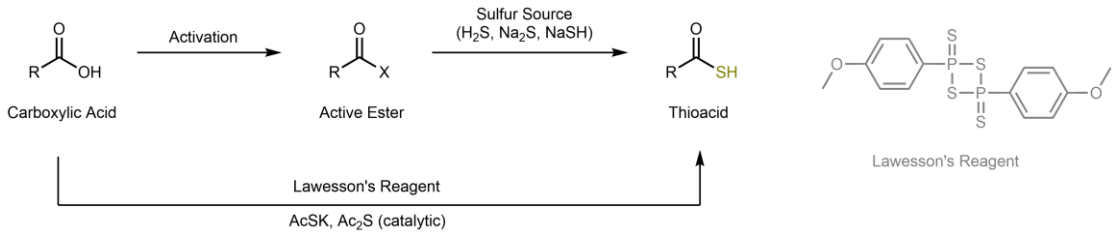


Figure 1.19. General procedure for the direct synthesis of unprotected thioacids.

While thioacids are stable in their pure solid forms, they readily react with oxygen in solution or hydrolyse in both acidic or basic solutions as mentioned.[132] Consequently, synthetic strategies often employ thioacids protected as thioesters which can be easily purified, stored indefinitely and then rapidly deprotected *in situ* to release the thioacid. The process to form these thioesters involves activation of the corresponding carboxylic acid followed by the reaction of the active ester with an appropriate thiol (**Figure 1.20**). The most common thioesters used as thioacid protecting groups include 9-fluorenylmethylthiol (Fm; **29**),[143] 2,4,6-trimethoxybenzylthiol (Tmob; **30**),[152] α-methylphenacyl (MPa; **31**)[153] and triphenylmethanethiol (Trt; **32**).[146] These groups are deprotected under a range of conditions as outlined in **Figure 1.20**, permitting versatility and orthogonality during peptide synthesis.

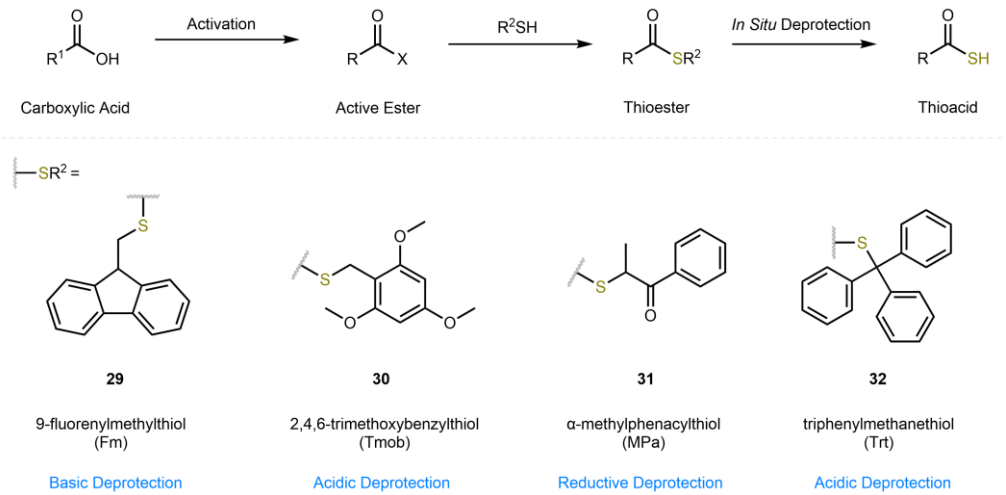


Figure 1.20. General procedure for synthesis of *S*-protected thioacids. The structures and deprotection conditions for commonly used thioacid protecting groups are shown.

The preparation of C-terminal peptide thioacids using Boc SPPS has been reported using various thioester-based linkers which form the thioacid after anhydrous HF cleavage[132], for instance the 4-(α-mercaptobenzyl)phenoxyacetic acid linker developed by Canne *et al. (***Figure 1.21a***)*.[154] To overcome the undesirable HF cleavage, Schwabacher and Maynard used the Kaiser's oxime ester resin suitable for Boc SPPS to form the C-terminal thioacid after treatment with hexamethyldisilathiane **33**/tetra-butylammonium fluoride (TBAF) (**Figure 1.21b**).[155] In this procedure, an intermediate trimethylsilyl thioester is formed which is cleaved during work-up with sodium bisulfate ($NaHSO_4$) to form the thioacid. The initial reaction to form the trimethylsilyl thioester must be conducted in anhydrous solvent such as THF to prevent thioester hydrolysis. Therefore, this method is limited to hydrophobic or fully protected peptides due to solubility issues and any side chain protecting groups must be removed in solution-phase after thioacid formation, complicating purification.

Zhang *et al*. reported the synthesis of C-terminal thioacids *via* the hydrothiolysis of thioester peptides bound to mercaptopropionylaminomethyl resin (**Figure 1.21c**).[156] The authors found that both ammonium sulfide ($(NH4)_2S$) or sodium hydrosulfide (NaSH) formed the thioacid in satisfactory yield during cleavage. On the other hand, Crich and Sana used the base labile, thiol functionalised 9-fluorenylmethyl linker in Boc SPPS which formed a C-terminal thioacid upon cleavage with piperidine *via* a β-elimination reaction (**Figure 1.21d**).[157] Although these methods that rely on Boc SPPS give relatively easy access to peptide thioacids after cleavage of the peptide from the resin, the general shift away from Boc SPPS has seen the development of alternative protocols for thioacid peptide synthesis in which the peptide can be assembled using Fmoc SPPS.

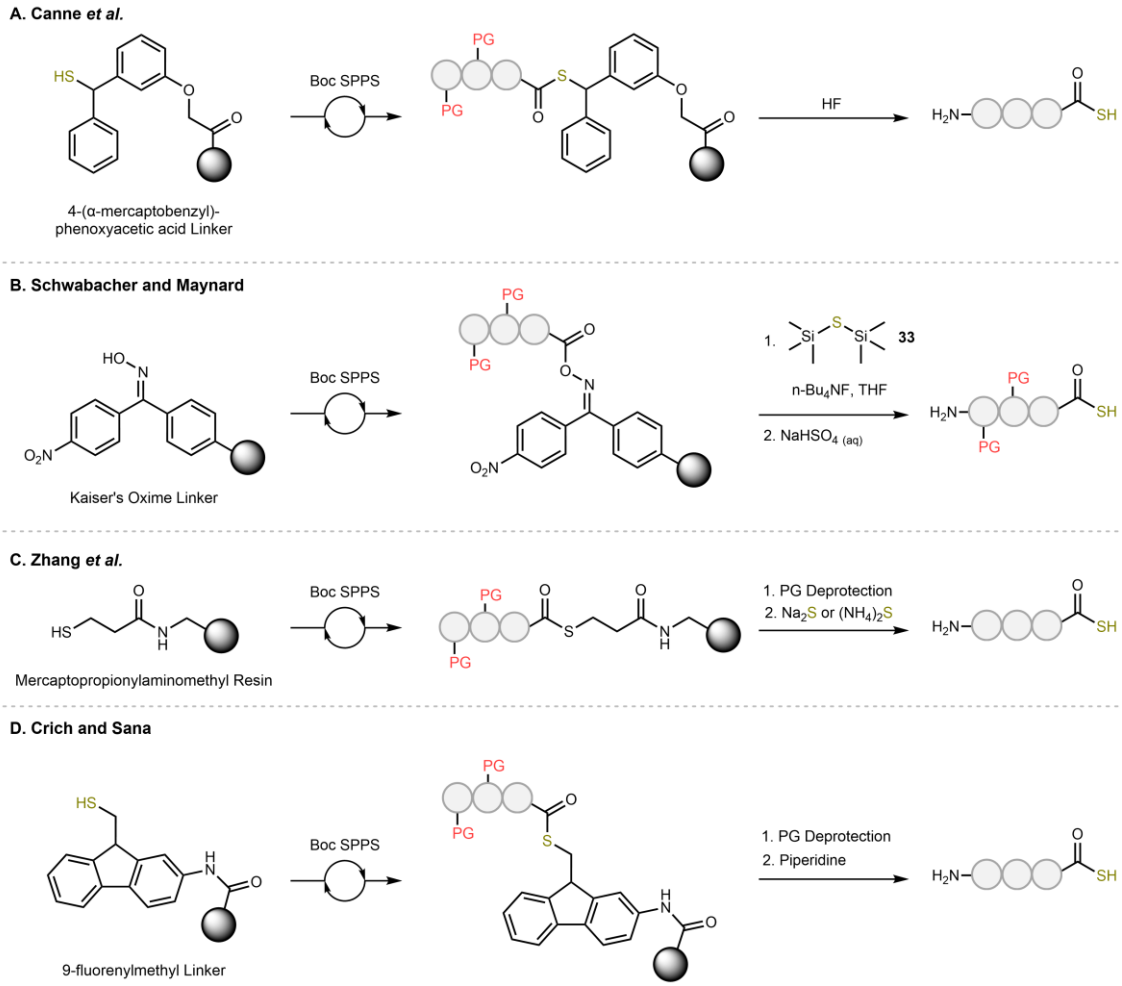


Figure 1.21. Methods for the synthesis of C-terminal peptide thioacids using Boc SPPS. PG = Protecting Group.

In general, thioester linkers are not stable to the basic conditions required for Fmoc deprotection and therefore, they cannot be used to directly synthesise thioacids *via* Fmoc SPPS. In some protocols, the thioacid is generated indirectly by forming a thioester after SPPS (see **Section 1.3.5**), followed by hydrothiolysis with NaSH[151, 158] or Na_2S.[159] Raz and Rademann developed an Fmoc SPPS strategy whereby the peptide is assembled on a 4-mercapto-4-methylpentanol (MMP) linker (**Figure 1.22**).[160] The peptide is linked to the resin through a thioester bond which is uniquely stable to Fmoc deprotection due to steric hindrance associated with two methyl groups adjacent to the thioester. Following chain assembly, nucleophilic cleavage with 3-mercaptopropionitrile **34**/sodium thiophenolate (NaSPh) forms a 2-cyanoethyl thioester intermediate which is deprotected *via* a rapid β-elimination reaction ($t_{1/2}$ < 8 min) using ammonium sulfide ($(NH_4)_2S$) to form the thioacid (**Figure 1.22a**). In another example, Pira *et al*. displace a transient thioester formed from a *bis*(2-sulfanylethyl)amide moiety with triisopropylsilylthiol **35**, and the resultant triisopropylsilyl thioester is hydrolysed to form a C-terminal thioacid (**Figure 1.22b**).[161] Finally, Tan *et al*. reported the enzymatic synthesis of C-terminal terminal thioacids using the endopeptidase, subtiligase, and suitable glycolate ester peptide substrates *via* the hydrothiolysis of a peptide-enzyme thioester intermediate using $(NH_4)_2S$ (**Figure 1.22c**).[162]

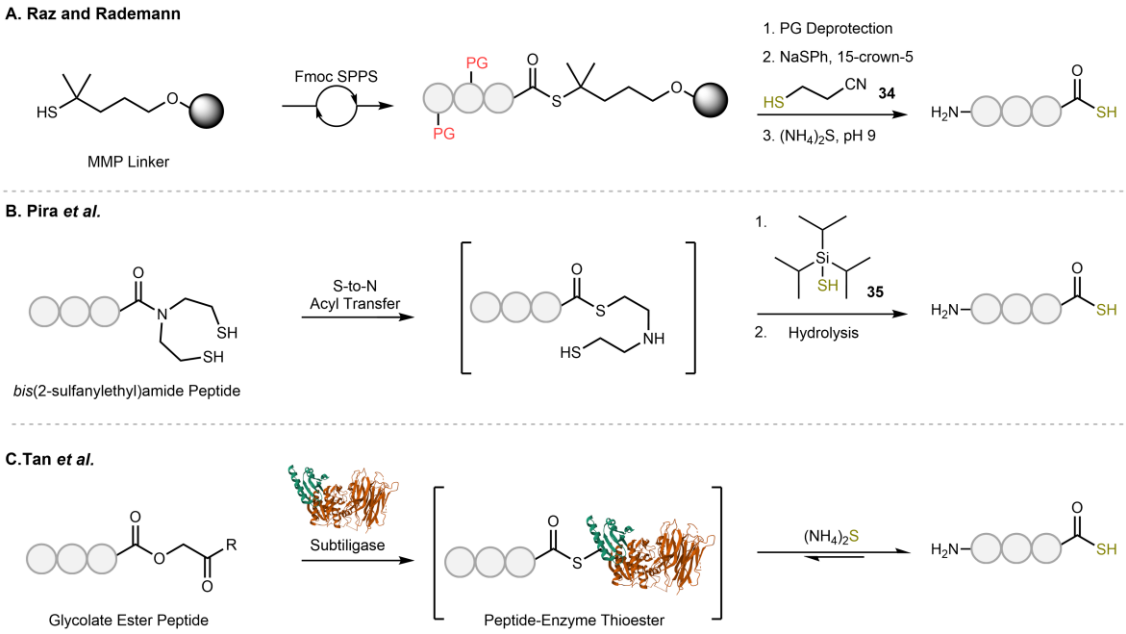


Figure 1.22c. Methods for the synthesis of C-terminal peptide thioacids compatible with Fmoc SPPS. PG = Protecting Group.

1.5 Thioacids in Peptide Ligation

The nucleophilicity of thioacids towards various electrophiles has been investigated for amide bond formation, which has been reviewed recently by Narendra *et al*.[132] In particular, peptide chemists have taken advantage of the reactivity of thioacids for the chemoselective ligation of unprotected peptides under mild conditions. Before the publication of the NCL method, Schnölzer and Kent described the synthesis of a 99-residue analogue of HIV-1 protease using a C-terminal thioacid.[163] In this approach, a fully unprotected peptide bearing a C-terminal thioacid reacts selectively with a bromoacetyl group (isostere for Gly) at the N-terminal of the other fully unprotected peptide fragment *via* an S_N2 mechanism to furnish a non-native thioester linkage (**Figure 1.23**). The analogue retained enzymatic activity and was stable under acidic conditions (pH < 2). However, the instability of the thioester bond formed under basic conditions (pH >7.5) remained a notable limitation.

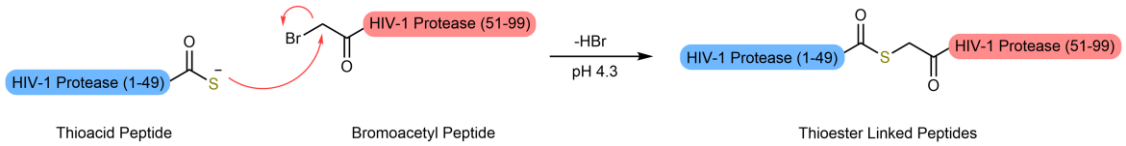


Figure 1.23. The thioacid mediated ligation strategy reported by Schnölzer and Kent.[163]

In 1995, Tam *et al*. reported a ligation reaction analogous to NCL where a C-terminal thioacid attacks an N-terminal β-bromoalanine *via* an S_N2 mechanism to form a thioester intermediate which is capable of undergoing intramolecular S-to-N acyl transfer to form a native peptide bond with Cys at the ligation site (**Figure 1.24**).[164] However, this reaction is pH-sensitive

as the β-bromoalanine is prone to 1,3-elimination of HBr to form an N-terminal aziridine intermediate at pH values >6. Subsequent ring-opening by the thioacid results in the formation of a mixture of the desired native α-peptide and its β isomer in a 6:4 ratio, with the desired product being favoured as it results from attack at the less hindered carbon of the aziridine. The authors found that this side reaction could be minimised to <3% if the ligation was conducted at pH 5, with the downside of extended reaction times due to a lower rate of S-to-N acyl transfer. The method was validated by the synthesis of the 11-residue sperm-activating peptide in 85% yield. Following on from the work of Tam *et al*., aziridine capture by thioacids has been used to form peptidomimetic reduced amide bonds by Yudin[142], and native amide bonds *via* Cu(II)-promoted coupling by Garner.[165]

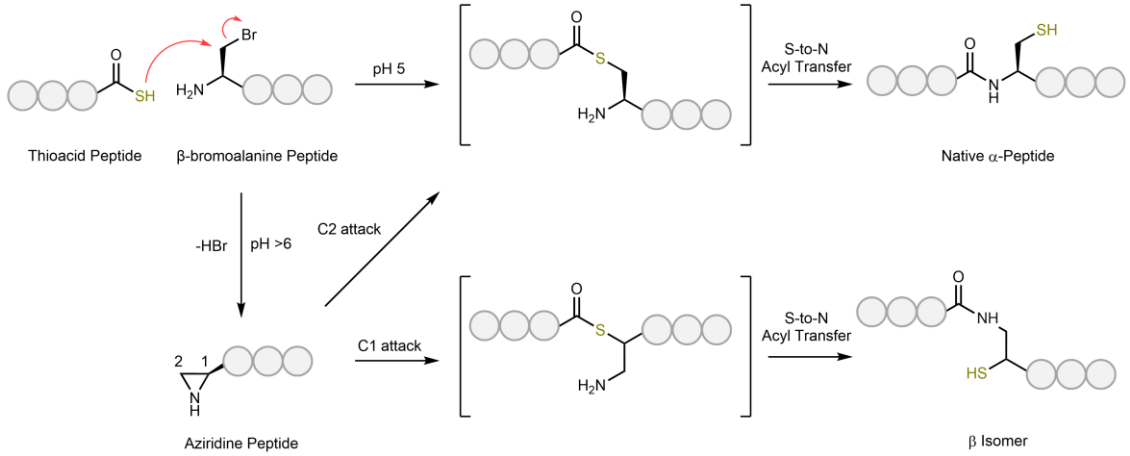


Figure 1.24. Ligation between C-terminal thioacid and N-terminal β-bromoalanine peptides reported by Tam *et al*.[164]

In a slightly modified strategy akin to the 'Prior Thiol Capture' reported by Kemp (**Section 1.3**), Tam and co-workers used a C-terminal thioacid to capture a 2-mercapto-5-nitropyridyl (Npys) modified N-terminal Cys disulfide *via* disulfide exchange (**Figure 1.25**).[166] The mixed acyl disulfide intermediate formed undergoes a rapid intramolecular S-to-N-acyl transfer (>90% in 5 min) through a 6-membered ring to form a Cys hydrodisulfide, which is reduced with dithiothreitol (DTT; **36**) to form a native linkage at Cys. The method was demonstrated by the synthesis of 32-residue model peptide, FA-32. The capture of the Npys disulfide by the thioacid occurs immediately after mixing the two peptide fragments at pH 2, attributed to the 'super-nucleophilicity' and low pK_a of the thioacid by the authors.[166] In a related strategy, the C-terminal thioacid itself can be activated by reaction with aryldisulfides such as Ellman's reagent to form a highly reactive mixed disulfide 'active ester' which can be reacted with the α-amino group of N-terminal peptide fragments to form an amide bond.[167, 168]

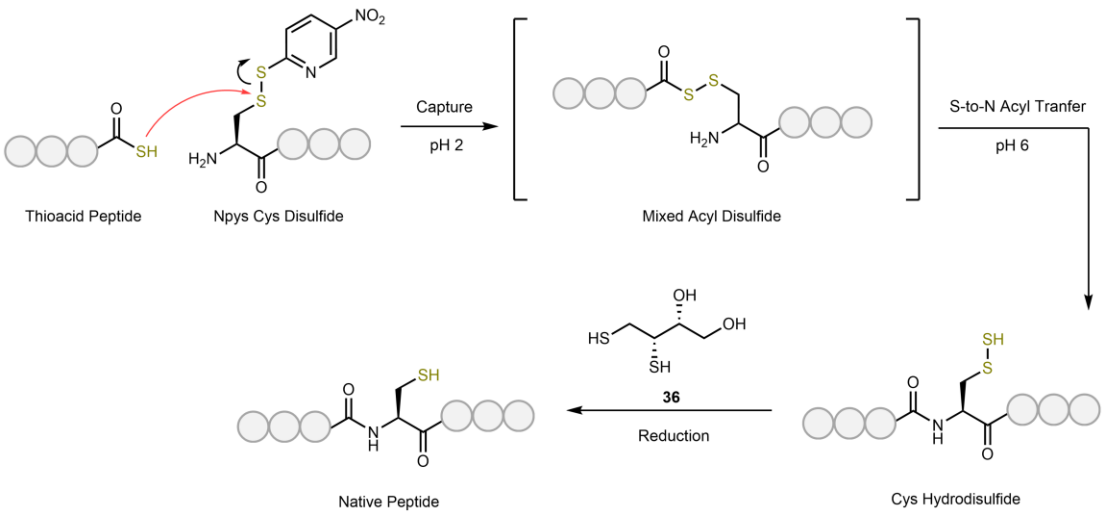


Figure 1.25. Ligation between C-terminal peptide thioacid and N-terminal 2-mercapto-5-nitropyridyl (Npys) modified peptide reported by Tam and co-workers.[166]

1.5.1 Thioacid-Sulfonamide Ligation

In 1998, Messeri *et al*. demonstrated that thioacids react with 2,4-dinitrobenzenesulfonamides *via* an S_NAr reaction in the presence of caesium carbonate (Cs_2CO_3) in organic solvent to form amide bonds.[169, 170] The authors used this procedure to prepare a series of amides using simple aliphatic and aromatic thioacids. Crich and Sharma extended this reaction for peptide ligation by reacting a C-terminal thioacid peptide with an N-terminal aryl sulfonamide modified peptide in DMF with the mild base caesium bicarbonate ($CsHCO_3$) to form a ligated peptide with a native amide bond (**Figure 1.26**). The reaction was also shown to be compatible with aqueous conditions (4:1 HEPES Buffer/*N*-methylpyrrolidone (NMP), pH 8). The reaction proceeds through a highly reactive thioester intermediate which necessitates protection of the N-terminal amine of the thioacid peptide and certain AA sidechains (e.g. Asn), a notable limitation of this method. Crich also showed that C-terminal thioacids undergo S_NAr with Sanger and Mukaiyama reagents to form highly reactive thioester intermediates which can be aminolysed with another peptide fragment to form a native peptide bond.[146] This reaction is akin to standard solution phase coupling and certain AA side chains require protection (Asp, Glu). Nevertheless, the authors demonstrated that this form of activation resulted in significantly less epimerisation compared to standard coupling reagents such as HATU and PyBOP.[146]

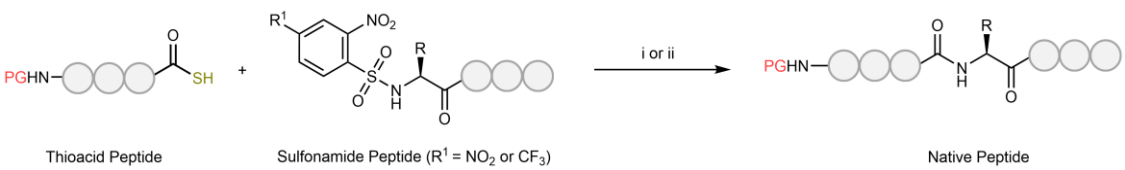


Figure 1.26. Ligation between C-terminal thioacid (**52**) and N-terminal arylsulfonamide peptides (**53**/**54**). (i) $CsHCO_3$, DMF (ii) 4:1 HEPES Buffer/NMP, pH 8. PG = Protecting Group.

1.5.2 Thioacid-Azide Ligation

In 1980, Hakimelahi and Just reported the reaction of potassium thioacetate ($KSCOCH_3$) with aryl azide **37** to form the corresponding acetamide **38** (**Figure 1.27**).[171] Subsequently in 1998, Rosen *et al*. further explored this reaction and reacted a series of aromatic and aliphatic azides with thioacetic acid to form the corresponding acetamides in excellent yields of up to 92%.[139] This reaction, often dubbed the 'Thioacid-Azide Ligation (TAL)', has been shown to be highly chemoselective and tolerant to aqueous conditions, and has found application in the labelling of peptides[172] and the synthesis of glycosylated and fluorescently labelled AAs.[173]

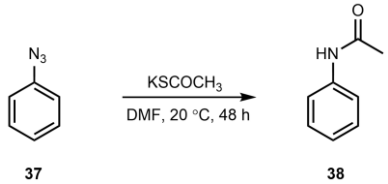


Figure 1.27. Reaction of azide **37** with potassium thioacetate ($KSCOCH_3$) to form acetamide **38**.

Owning to the chemoselectivity of the TAL reaction, Mhidia *et al*. explored its suitability for peptide ligation.[174] The authors used a C-terminal acyl azide peptide **39** which is captured by the side-chain thioacid of N^{α}-protected aspartyl peptide **40** to form an imide linked intermediate **41** (**Figure 1.28**). Following N^{α} deprotection, an N-to-N acyl shift induced by the mild electrophilicity of the imide forms a native peptide, with ligation occurring at Asn. However, a major side-reaction was identified during the initial imide capture step. It was postulated that nucleophilic attack by the thioacid of peptide **40** directly at the carbonyl of the acyl azide peptide **39** results in the formation of thioanhydride linked intermediate **42** and the release of an azide anion. Subsequent cleavage of thioanhydride by the azide anion results in the scrambling of the thioacid and azide functionalities between the peptides, leading to the formation of the symmetrical imides **43** and **44**. Despite screening of reaction conditions, the authors were unable to eliminate this side reaction, significantly limiting its utility for peptide ligation.

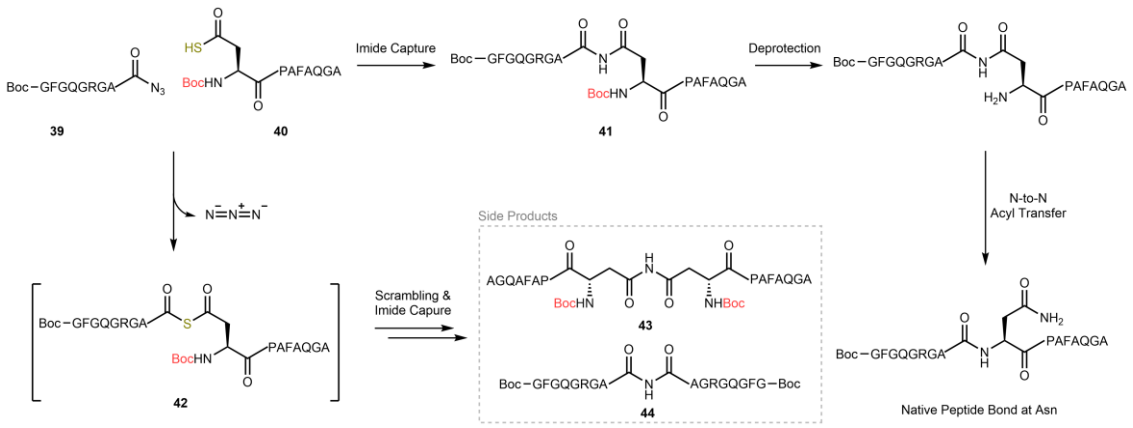


Figure 1.28. The 'Imide Capture Peptide Ligation' reported by Mhidia *et al*.[174]

1.5.3 Amino Thioacid Coupling (ATC)

Recently, Okamoto *et al*. published the chemoselective Amino Thioacid Coupling (ATC) protocol that enables amide bond formation between a C-terminal aryl thioester **45** and an α-amino thioacid **46** in DMF (**Figure 1.30**).[129] The reaction is initiated by thioacid-thioester exchange to form a transient thioanhydride intermediate, which undergoes S-to-N acyl transfer over a 5-memebred ring transition state to form a C-terminal peptide thioacid **47**. In essence, the α-amino thioacid **46** has been ligated *via* an amide bond to the C-terminal of the thioester peptide **45**. The authors demonstrated the strategy by synthesising a series of pentapeptide thioacids by coupling structurally diverse α-amino thioacids (Gly, Ala, Val, Ser, Thr, Met, Phe, Glu, propargylglycine, D-Phe, D-Leu) to the tetrapeptide thioester **45** in yields of up to 97% in 30 min. The yield was significantly lower in aqueous buffer, attributed to hydrolysis of the highly electrophilic thioanhydride intermediate. Although this reaction only enables the coupling of a singular amino acid to the C-terminal of a peptide thioester, it is noteworthy as it utilises peptide thioesters which are well established in peptide ligation protocols as one of the reaction partners.

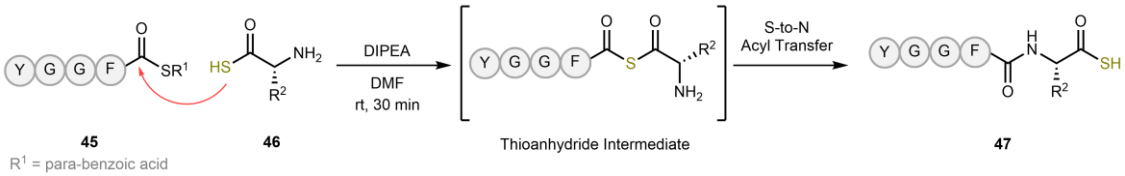


Figure 1.30. 'Amino Thioacid Coupling' developed by Okamoto *et al*.[129]

1.6 Acyl Thiol-Ene Reaction

The bond dissociation energy (BDE) of the S-H bond of thioacids is comparable to that of alkanethiols; measured at 88 kcal mol^{-1} for thioacetic acid and 87 kcal mol^{-1} for thiobenzoic acid.[175] Consequently, thioacid radicals can be easily generated with the use of radical initiators,[176] photosensitisers[177], molecular oxygen[178] or even graphene-Bi_2O_3 nanocomposities.[179] The addition of thiol radicals to alkenes and alkynes in the thiol-ene and thiol-yne reactions respectively has been applied extensively in peptide chemistry for peptide/protein labelling[180], peptide glycosylation[181], lipidation[182] and cyclisation.[183] This has been reviewed in detail by Nolan and Scanlan[184] and by Ahangarpour *et al*.[185] The analogous acyl thiol-ene reaction involving the addition of a thioacid radical onto an alkene to form a thioester has not been studied as extensively (**Figure 1.31**). The addition of the thioacid radical to the alkene occurs with *anti*-Markovnikov regioselectivity as this results in the formation of a more stable radical intermediate. In some cases, particularly with secondary or tertiary acids, the thioacid can undergo dethiocarboxylation *via* the spontaneous elimination of carbonyl sulfide (COS) to form alkyl radicals.[186]

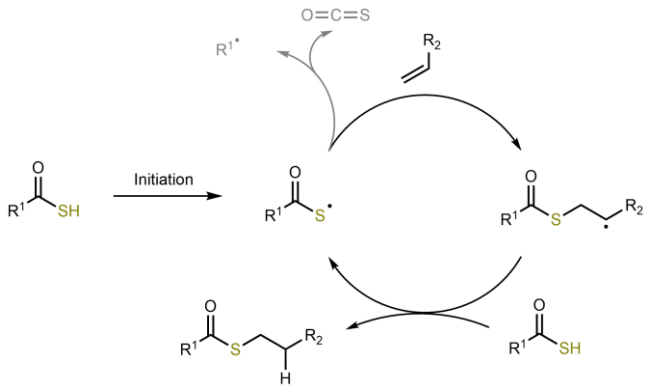


Figure 1.31. Mechanism of the photochemical acyl thiol-ene reaction, and dethiocarboxylation (grey).

1.6.1 Intermolecular Acyl Thiol-Ene

The addition of thioacetic acid to alkenes *via* the radical acyl thiol-ene reaction, followed by *S*-deacetylation of the resulting thioacetate to form the corresponding thiol (**Figure 1.32**) has been used extensively to form thiol-functionalised linkers for the synthesis of gold nanoparticles[187-190], gold surfaces[191-193], polyhedral oligomeric silsesquioxanes[194], carbosilane dendrimers[195, 196], rotaxanes[197] and fullerenes.[198] This protocol has also been used to synthesise thiol-functionalised derivatives of a variety of compounds including sugars[199, 200], steroids[201], silylalkanes[202], cyclooctadiene[203], pyridine ligands[204], para-terphenylalkanes[205] and ferrocene[206]. McCourt and Scanlan utilised this sequence to synthesise a range of δ-thiocarboxylic acids which were then condensed intramolecularly to form δ-thiolactones in up to 81% yield.[207] In a related strategy, Araújo and Mahajan utilised acyl thiol-ene to install thiols onto alkenylketones which could enable intramolecular condensation to form medium and macrocyclic ketothiolactones.[208]

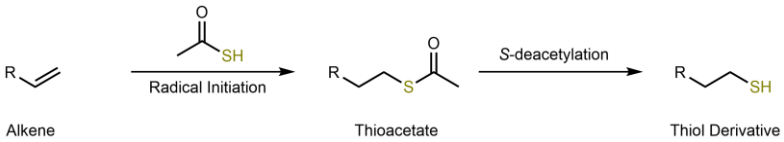


Figure 1.32. Acyl thiol-ene and *S*-deacetylation sequence used for the synthesis of thiol derivatives.

The radical acyl thiol-ene reaction has also been used to synthesise thioester derivatives. Igarashi and Honma investigated the addition of thioacetic acid **26** *via* radical acyl thiol-ene reaction to D-glucal triacetate **48** and observed exclusive addition of the thioacetic acid at the C2 position to form the equatorial **49** and axial **50** diastereomers in a 7:3 ratio in 90% yield (**Figure 1.33A**).[209] Dingwall and Tuck explored the radical addition of various compounds to the exocyclic alkene of diketene **51**, including thioacetic acid to form the corresponding thioester **52** in 75% yield (**Figure 1.33B**).[210] Nicolaou *et al*. utilised radical acyl thiol-ene to synthesise alkyl thioester modified vancomycin analogues.[211] In addition, the radical acyl thiol-ene reaction has been used for the straightforward synthesis of thioester prodrugs of anti-apicomplexa cyclic peptides[212],

histone deacetylase (HDAC) inhibitors for the treatment of latent HIV[213] and cancer[214], the pyrimidine antimetabolite *N*-phosphonoacetyl-L-aspartate (PALA)[215] and the antiviral Foscarnet[216]. In each case, the base labile thioester moiety is hydrolysed *in vivo* to release a thiol.

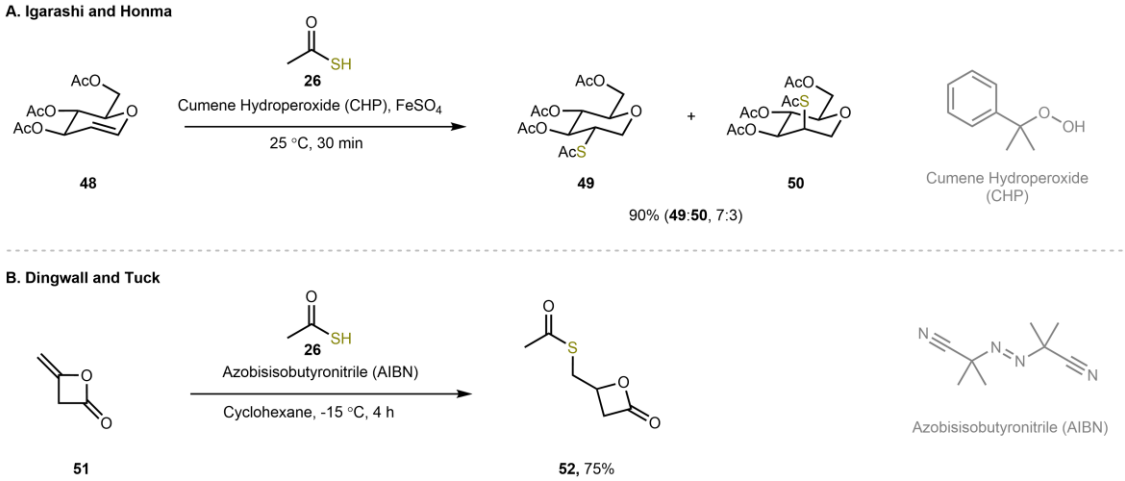


Figure 1.33. Synthesis of thioester derivatives using radical acyl thiol-ene.

Recently, Murakami *et al*. reported a three-component photocatalytic thioesterification reaction involving α-ketoacids, elemental sulfur (S_8) and alkenes.[217] The reaction is initiated by oxidation of the α-ketoacid to form an acyl radical, which is sulfurised by S_8 to form a thioacid radical that ultimately reacts with alkenes *via* acyl thiol-ene to form thioesters (**Figure 1.34**). Similarly, Tang *et al*. reported another three-component photocatalytic thioesterification reaction using aldehydes, S_8 and alkenes/alkynes, with a thioacid radical being the key intermediate which reacts *via* acyl thiol-ene/yne to furnish the thioester.

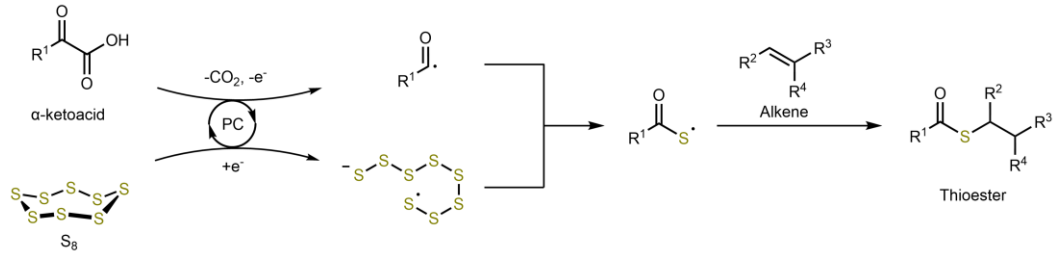


Figure 1.34. Three-component thioesterication reaction reported by Murakami et al. using α-ketoacids, elemental sulfur and alkenes to form thioesters.[217] PC = Photocatalyst.

McLean *et al*. utilised photochemical acyl thiol-ene between thioacids and alkene-functionalised peptide and sugar substrates to form thioester intermediates capable of undergoing intramolecular S-to-N acyl transfer to form amide bonds.[218] For instance, Gly thioacid **53** was reacted with β-γ-didehydrovaline dipeptide **54** to form thioester **55**, which following α-amine deprotection underwent S-to-N acyl transfer to form the thiol-functionalised tripeptide **56**

in 73% overall yield (**Figure 1.35**). This tripeptide was subjected to desulfurisation using TCEP/V-50 to form the native tripeptide **57**, with the newly formed amide bond being between Gly-Val. Similarly, Gly thioacid **53** was reacted with vinylglycine dipeptide **58** to form thioester **59**. Following α-amine deprotection, this thioester also underwent S-to-N acyl transfer to form the homocysteinyl tripeptide **60** in 66% overall yield (**Figure 1.35**). The thiol of tripeptide **60** was methylated with trimethylsilyldiazomethane to form the native tripeptide **61**, with the newly formed amide bond being between Gly-Met in this instance.

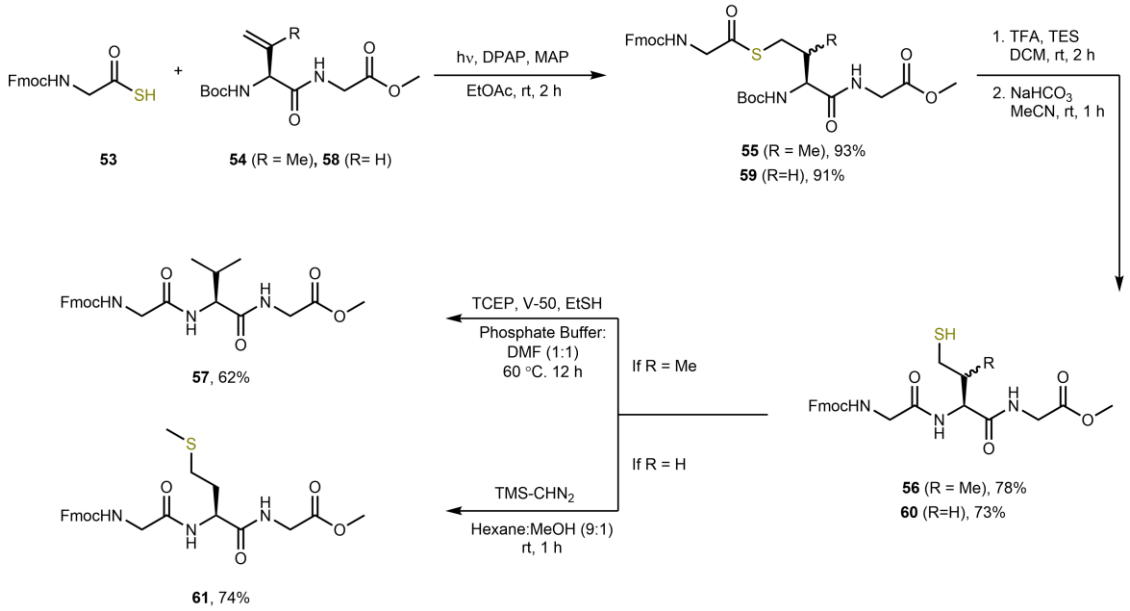


Figure 1.35. Acyl thiol-ene mediated ligation reported by McLean *et al*.[218]

1.6.2 Intramolecular Acyl Thiol-Ene/Yne

The traditional synthesis of thiolactones has relied on the intramolecular condensation of mercaptocarboxylic acids, often under reflux using activating agents which limit functional group tolerance.[219] In 1986, Toru *et al*. reported an alternative radical mediated approach for thiolactonisation.[220] The authors prepared a range of *S*-acyl phenylselenosulfides by the reaction of unsaturated thioacids with *N*-phenylselenopthalimide. Upon refluxing with 10 mol% of the radical initiator azobisisobutyronitrile (AIBN), the selenosulfides underwent radical cyclisation to form a range of seleneothiolactones (**Figure 1.36**). The seleno group was then oxidatively cleaved using an excess of m-chloroperbenzoic acid (m-CPBA) and pyridine to yield allylic or vinyl thiolactones. The reaction is initiated by homolytic cleavage of the selenosulfide S-Se bond by AIBN to form a thioacid radical which reacts *via* 5-exo-trig intramolecular acyl thiol-ene to form a thiolactone (**Figure 1.36**). The resulting C-centred radical promotes homolytic cleavage of another selenosulfide to form the selenothiolactone and another thioacid radical to propagate the reaction.

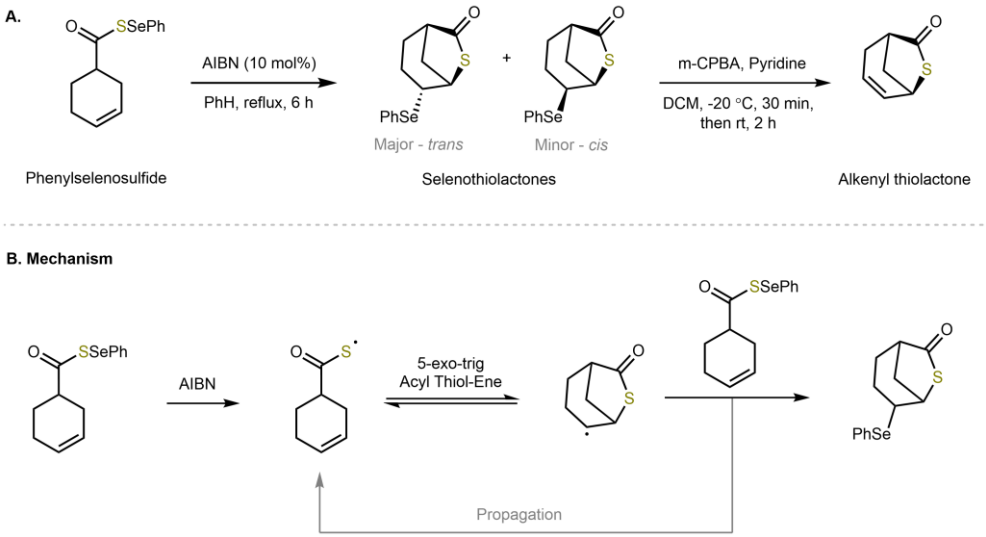


Figure 1.36. **(a)** Synthesis of alkenyl thiolactones from phenylselenosulfides *via* a selenothiolactone intermediate. **(b)** Proposed mechanism of the reaction.

In a related strategy, McCourt *et al*. reported the photochemical synthesis of thiolactones in up to 93% yield *via* the intramolecular acyl thiol-ene cyclisation of γ-alkenyl thioacids using UV irradiation (350 nm) and 2,2-dimethoxy-2-phenylacetophenone (DPAP) as photoinitiator.[176] As previously observed by Toru *et al*., it was found that 5-exo-trig acyl thiol-ene cyclisation of the γ-alkenyl thioacids to form γ-thiolactones was highly favoured over the 6-endo-trig cyclisation to form δ-thiolactones (**Figure 1.37A**). Computational studies showed that due to the stabilisation of the intermediate C-centred radical formed after 5-exo-trig cyclisation by the spin density on the sulfur, the 5-exo product was both the kinetic and thermodynamic product, accounting for the observed regioselectivity. McCourt and Scanlan also showed that γ-alkynyl thioacids can undergo cyclisation *via* intramolecular acyl thiol-yne, preferentially *via* 5-exo-dig, to form vinyl thiolactones, albeit with significantly lower yields (**Figure 1.37B**). In another study, Schierhorn *et al*. utilised a 5-exo-trig acyl thiol-ene cyclisation to form a γ-thiolactone in their enantioselective synthesis of 19,10-thio-3-epigibberellin A_1 in 72% yield using UV irradiation (220 nm).[221]

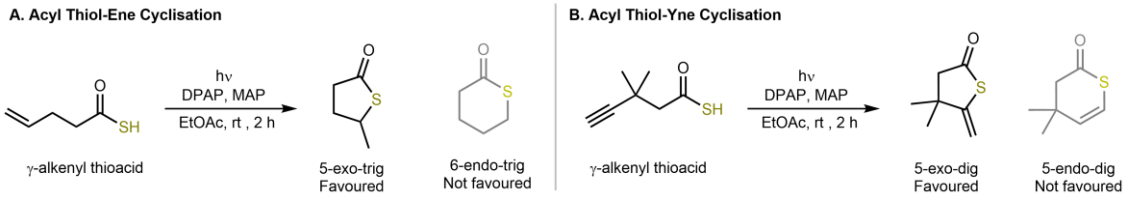


Figure 1.37. Acyl thiol-ene and thiol-yne cyclisation of γ-alkenyl and alkynyl thioacids.

1.7 Works Described in This Thesis

As discussed in this chapter, thioacids possess unique reactivity compared to the canonical amino acids which can enable chemoselective peptide modification, including peptide ligation and cyclisation. While the use of thioacids in peptide chemistry has been explored by a number of researchers to date (**Section 1.5** and **Section 1.6.1**), their potential has not been fully studied.

Chapter 2 outlines efforts to develop a peptide ligation strategy inspired by the work of Okamoto *et al*. (**Section 1.5.3**) between a fully unprotected C-terminal thioester peptide and another bearing a γ-thioaspartic acid residue, the sidechain thioacid analogue of aspartic acid. In this approach, the peptides undergo thioester-thioacid to form a thioanhydride-linked intermediate which triggers a proximity induced S-to-N acyl transfer to furnish a native amide bond between the peptide fragments. The reaction was initially tested on simple thioester and γ-thioaspartic acid substrates with excellent results and then extended to more complex peptidic substrates. The synthesis of the required N-terminal γ-thioaspartic acid peptides *via* Fmoc SPPS in high yield and purity using a novel SPPS building block is also described.

Chapter 3 describes the extension of the photochemical acyl thiol-ene reaction (**Section 1.6**) to the synthesis of macrocyclic peptide thiolactones, representing the first reported synthesis of peptide thiolactones under radical-mediated conditions. Fully unprotected linear peptides containing both an alkene and a γ-thioaspartic acid residue were synthesised by Fmoc SPPS and rapidly cyclised under UV irradiation to form the corresponding thiolactone in almost quantitative yield. The scope of the cyclisation reaction and compatibility with various AA side chains were demonstrated by the synthesis of a series of macrocyclic peptide thiolactones. Analogues of Auto Inducing Peptides (AIPs), which are naturally occurring macrocyclic peptides containing thiolactone linkages, were also synthesised. The reaction was also demonstrated under blue LED irradiation with comparable results.

Chapter 4 details investigation of the dethiocarboxylation of C-terminal thioacid derivatives of AAs under photochemical conditions to yield *N*-alkylamines. Conditions for dethiocarboxylation were optimised under both UV and blue LED irradiation and the scope of the reaction with AA substrates was explored. Furthermore, initial scoping studies were conducted to determine whether the C(sp^3) radical formed as an intermediate during the dethiocarboxylation reaction could be trapped *via* an intermolecular addition onto an alkene to form C(sp^3) linked cyclic peptides.

Chapter 5 describes experimental details and characterisation data for compounds synthesised during the course of this work.

Chapter 2

γ-Thioaspartic Acid Mediated Peptide Ligation

2.1 Introduction

As discussed in Chapter 1 (**Section 1.3**), the ligation of unprotected peptide fragments *via* Native Chemical Ligation (NCL) has become the prevailing method for the chemical synthesis of peptides >50 AAs. To overcome the requirement for a relatively rare Cys residue at the ligation junction for NCL, a variety of extended NCL methods have been developed including the ligation-desulfurisation protocol and auxiliary mediated ligation (AML). In addition, alternative peptide ligation reactions such as the Staudinger ligation[222] or the α-ketoacid-hydroxylamine (KAHA) ligation have been reported.[223] In this context, the unique reactivity of thioacids have also been explored for peptide ligation, albeit with limited applicability due to the formation of non-native linkages or significant side-reactions as discussed in Chapter 1 (**Section 1.5**). Recent work by Okamoto *et al*. has described the reaction of various α-amino thioacids with unprotected C-terminal thioesters to form a thioanhydride intermediate capable of undergoing S-to-N acyl transfer to furnish a native amide bond (see **Section 1.5.3** for details).[129] However, their exclusive use of α-amino thioacids restricted this reaction to the addition of a singular amino acid to the C-terminal of the thioester peptide.

Inspired by the work of Okamoto *et al*., we propose a Cys-free peptide ligation strategy between a C-terminal thioester peptide and another bearing a γ-thioaspartic acid residue at the N-terminal (**Figure 2.1**). In this approach, the γ-thioaspartic acid reacts with the C-terminal thioester to form a thioanhydride-linked intermediate which undergoes S-to-N acyl transfer over a 6-membered transition state to furnish a native amide bond between the peptide fragments. The γ-thioaspartic acid residue can be hydrolysed post-ligation under mild conditions to form a native Asp residue at the ligation site, an AA which is approximately 3.5 times more abundant than Cys in human proteins/peptides.[224] Alternatively, the thioacid intermediate can be used as a handle for further chemoselective peptide modification *via* established reactions, including acyl thiol-ene,[218] Cu(II) catalysed glycosylation to synthesise *N*-glycopeptides,[225] and conjugation with azides[173], sulfonamides[146] or isocyanates.[143]

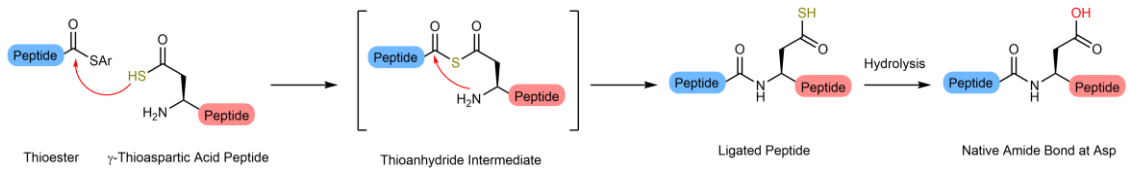


Figure 2.1 Proposed ligation of C-terminal thioester and N-terminal γ-thioaspartic acid peptides.

2.2 Aims

The aim of this project was to develop the γ-thioaspartic acid ligation for fully unprotected peptides, ideally in aqueous solvent at neutral pH. To achieve this, the development of an efficient Fmoc SPPS protocol to synthesise the required N-terminal γ-thioaspartic acid peptide fragment

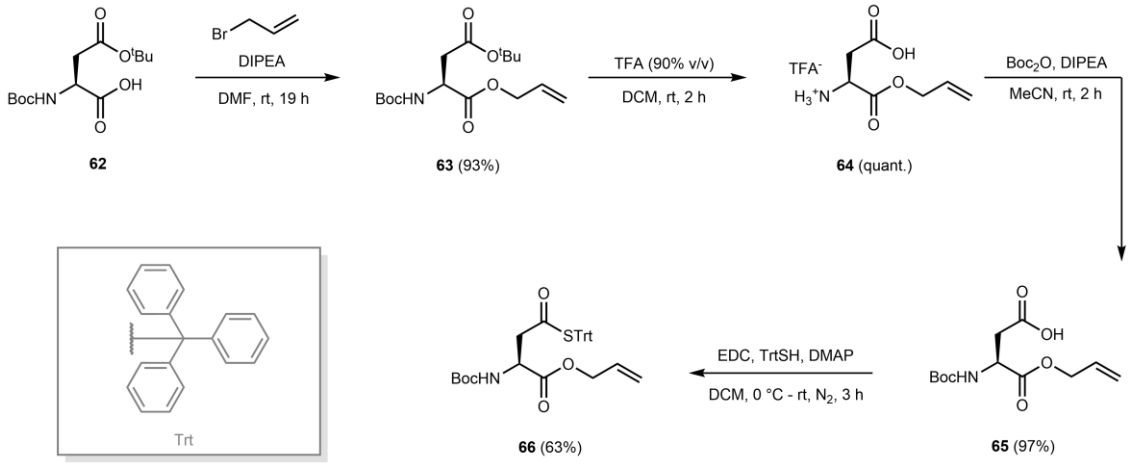
was required. Additionally, conditions for the hydrolysis of the γ-thioaspartic acid residue to the native Asp residue following ligation, ideally in one-pot, needed to be established. To demonstrate the viability of the ligation strategy, we aimed to synthesise the peptide drugs, Bivalirudin (20-AA) and Teduglutide (33-AA), both of which possess centrally located Xaa-Asp ligation sites.

2.3 Results and Discussion

In order to investigate the feasibility of the ligation reaction, we first initiated scoping studies using simple amino acid thioester and γ-thioaspartic acid substrates under conditions reported by Okamoto *et al*. given that both reactions proceed through a putative thioanhydride intermediate.[129] The thioacids were protected as acid-labile *S*-trityl (STrt) thioesters and deprotected *in situ* to furnish the desired thioacid to prevent unwanted oxidation or hydrolysis during storage. Upon completion of the initial scoping studies, we envisioned extension of the method to longer peptides synthesised by SPPS.

2.3.1 Synthesis of Boc-Asp(STrt)-OAll 66

The initial thioacid substrate investigated was Boc-Asp(STrt)-OAll **66** as both the Boc and STrt protecting groups can be rapidly deprotected in tandem using acid to furnish the α-amino and γ-thioacid groups required for ligation. In addition, the C-terminal of compound **66** was protected as an allyl ester (OAll) to enable selective removal by Pd-catalysed deallylation, a feature that would permit the thioacid substrate to be extended at the C-terminal as required.



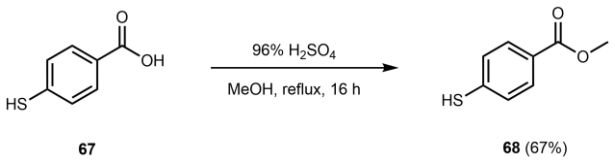
Scheme 2.1. Synthesis of Boc-Asp(STrt)-OAll **66**.

The synthesis commenced with the allylation of commercially available Boc-Asp(O*t*Bu)-OH **62** with allyl bromide to yield Boc-Asp(O*t*Bu)-OAll **63** in a yield of 93% (**Scheme 2.1**). Subsequent concomitant Boc and *tert*-butyl ester deprotection upon treatment with 90% (v/v) TFA furnished H-Asp-OAll **64** as the TFA salt in quantitative yield. The α-amino group of **64** was Boc-

protected using di-*tert*-butyl decarbonate (Boc_2O) and DIPEA to yield Boc-Asp-OAll **65** in an excellent yield of 97%. Finally, Steglich thioesterification of the side chain carboxylic acid of **65** with triphenylmethanethiol (TrtSH) and 4-dimethylaminopyridine (DMAP) as a nucleophilic catalyst furnished the required compound **66** in a yield of 63% and in sufficient quantity to proceed with our investigation. Although the late intermediate, Boc-Asp-OAll **65**, is also commercially available and would have the shortened the synthesis of compound **66** to just one step, its cost of ≈ €300/g was deemed prohibitive for our purposes.

2.3.2 Synthesis of Gly and Ala Thioesters

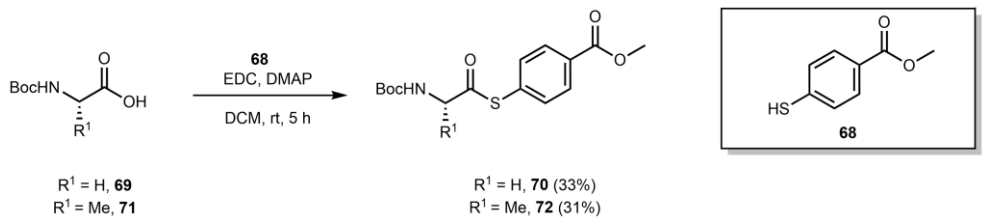
It has been well established in the literature that bulky amino residues (e.g Val, Ile, Pro) positioned at the C-terminal of the thioester fragment in NCL is detrimental to the rate of reaction.[226] In order to maximise ligation efficiency during the initial studies, we planned to synthesise aryl thioesters of the simplest canonical AAs, Gly and Ala. During their investigation of the ATC reaction, Okamoto *et al*. screened a variety of aryl thioesters and found that thioesters derived from 4-mercaptobenzoic acid (MBA; **67**) gave significantly higher yields[129] To synthesise the thioesters by coupling the thiol to the AAs in solution-phase, the *para*-carboxylic acid group of **67** required protection to prevent oligomerisation. Johnson and Kent have shown that the efficacy of a thioester as a leaving group towards thiol-thioester exchange is dependent on the pK_a of the parent thiol.[227] We reasoned that conversion of the carboxylic acid to the corresponding methyl ester would not sufficiently affect the pK_a of the thiol as to hinder thioacid-thioester exchange. Thus, Fischer esterification of 4-mercaptobenzoic acid **67** with 96% H_2SO_4 in MeOH under reflux furnished the corresponding methyl ester **68** in a yield of 67% (**Scheme 2.2**).



67 **68** (67%)

Scheme 2.2. Fisher esterification of 4-mercaptobenzoic acid **67**.

The aryl thiol **68** was subsequently coupled to Boc-Gly-OH **69** using EDC and DMAP under argon to furnish Gly thioester **70** in 33% yield after purification. Similarly, the aryl thiol **68** was coupled to Boc-Ala-OH **71** under identical conditions to give Ala thioester **72** in a comparable yield of 31%. Although the isolated yields were relatively low, the thioesters were both obtained in sufficient quantities to proceed with the study and the reactions were not further optimised. During reaction, TLC analysis indicated a spot consistent with the disulfide of thiol **68**, therefore, the yield could be increased significantly by the addition of an *in situ* disulfide reductant such as dithiothreitol (DTT) or tributylphosphine (PBu_3).



Scheme 2.3. Synthesis of Gly **70** and Ala **72** thioesters.

2.3.3 Deprotection of Boc-Asp(STrt)-OAll 66

To begin the ligation studies, the STrt protected thioacid, Boc-Asp(STrt)-OAll **66**, was treated with 90% (v/v) TFA and 5% dimethylethylsilane (DMES) as a cation scavenger in DCM for 15 min (**Figure 2.2A**). Following concentration of the reaction mixture, the product was precipitated in ice-cold diethyl ether and the resulting suspension was centrifuged to give an off-white pellet. However, detailed NMR and HRMS analysis of the deprotection product suggested that instead of the desired γ-thioaspartic acid **73**, the oligomer **74** had been formed instead. Evidence for the formation of oligomer **74** include HMBC correlation between the thioester carbonyl (δ = 195.9 ppm) and two methylene protons derived from the allyl group (δ = 2.97 and 3.28 ppm) (**Figure 2.2B**). In addition, the mass of the n = 1 and n = 2 oligomer was detected by HRMS (**Figure 2.2C**). The formation of the oligomer is hypothesised to proceed through an intermolecular radical mediated acyl thiol-ene hydrothioacylation reaction mechanism initiated by atmospheric oxygen and catalysed by TFA as reported by McCourt *et al*.[88]

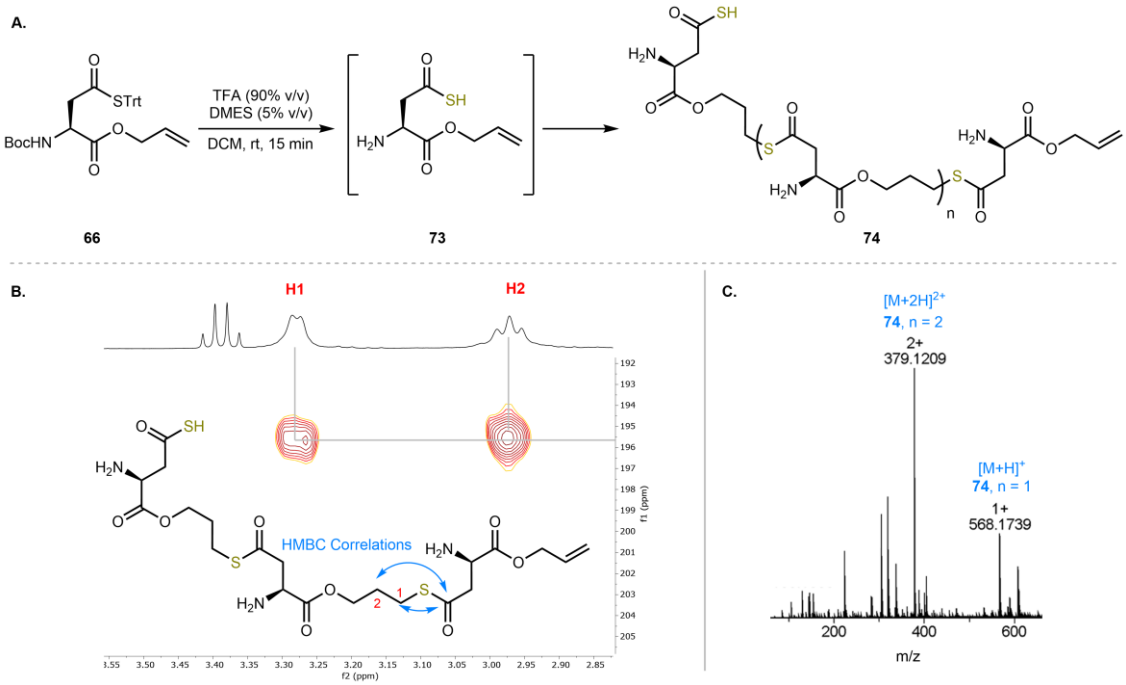


Figure 2.2. (a) Deprotection of **66** with TFA **(b)** HMBC spectrum of **74**. **(c)** HRMS spectrum of **74**.

2.3.4 Synthesis of Boc-Asp(STrt)-OMe 76

At this stage, rather than optimise conditions for the deprotection of Boc-Asp(STrt)-OAll **66** to minimise the side reaction, we decided to simplify the γ-thioaspartic acid substrate by substitution of the C-terminal allyl group to a methyl ester to completely eliminate the possibility of the oligomerisation reaction. Thus, commercially available Boc-Asp-OMe **75** was coupled to TrtSH under Steglich conditions to yield Boc-Asp(STrt)-OMe **76** in a yield of 61% (**Figure 2.3**). The STrt thioester **76** was treated with 90% (v/v) TFA with 5% (v/v) DMES as a cation scavenger for 15 min, and the product was isolated and analysed by ^{1}H NMR and ^{13}C NMR. Gratifyingly, full clean conversion to the γ-thioaspartic acid derivative H-Asp(SH)-OMe **77** was confirmed (**Figure 2.3B** & **Figure 2.3C**). With a reliable procedure to access the required γ-thioaspartic acid, we proceeded to investigate its ligation with the thioesters prepared earlier.

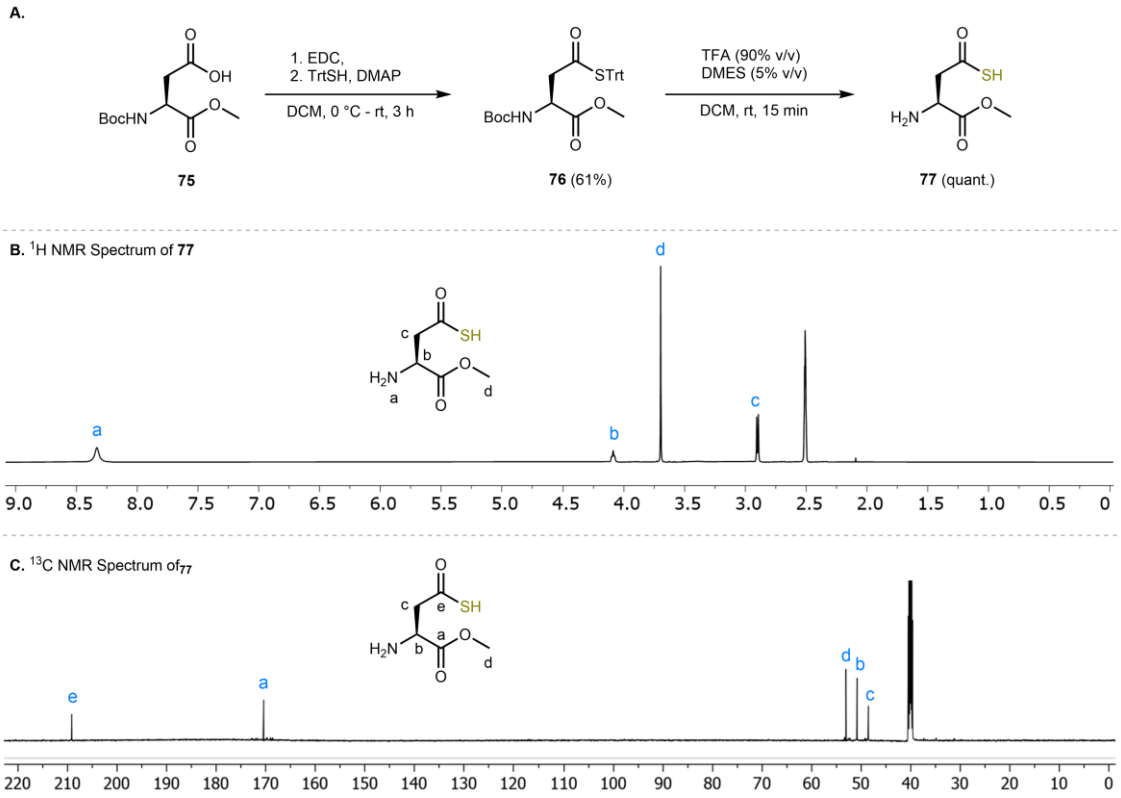


Figure 2.3. (a) Synthesis of Boc-Asp(STrt)-OMe **76**. **(b)** ^{1}H NMR (DMSO-d_6) spectrum of thioacid **77**. **(c)** ^{13}C NMR (DMSO-d_6) spectrum of thioacid **77**.

2.3.5 Ligation Scoping Study with Thioacid 77

Following Boc and STrt deprotection of **76**, the resulting thioacid, H-Asp(SH)-OMe **77** (100 mM, 4 equiv.), was immediately reacted with Gly thioester **70** (25 mM, 1 equiv.) in DMF with DIPEA as a base (**Figure 2.4A**). The reaction was monitored by analytical RP-HPLC. After 30 min, full consumption of the Gly thioester **70** was observed with 85% conversion to a new peak which was isolated and confirmed to be the ligated dipeptide Boc-Gly-Asp(SH)-OMe **78** by HRMS

(**Figure 2.4B**). The reaction was then repeated with Ala thioester **72** under identical conditions, with 80% conversion to the corresponding dipeptide Boc-Ala-Asp(SH)-OMe **79** as confirmed by HRMS (**Figure 2.4C**). The reaction with the Ala thioester proceeded at a lower rate compared to the Gly analogue with a reaction time of 1 h being required for full consumption, likely due to the increased steric bulk of the Ala side chain. The dipeptide **79** was then isolated by semi-preparative RP-HPLC in 42% yield and its structure was confirmed by NMR. Importantly, the NMR showed no significant epimerisation of either residue, consistent with the results obtained by Okamoto *et al*.[129] Interestingly, when the sample of the dipeptide **79** dissolved in DMSO-d_6 was reanalysed after two days, complete hydrolysis of the thioacid to form the corresponding carboxylic acid Boc-Ala-Asp-OMe **81** was observed by HRMS (**Figure 2.4D**). The hydrolysis likely proceeded *via* oxidation of the thioacid by DMSO to form a highly activated diacyl disulfide intermediate **80** which was hydrolysed by residual H_2O in the DMSO to form the carboxylic acid. The oxidation of thioacids to diacyl disulfides by DMSO has been previously reported by both Nomura *et al*.[228] and Wang and Danishefsky in their studies into the application of diacyl disulfides as potent acyl donors for amide bond formation.[229]

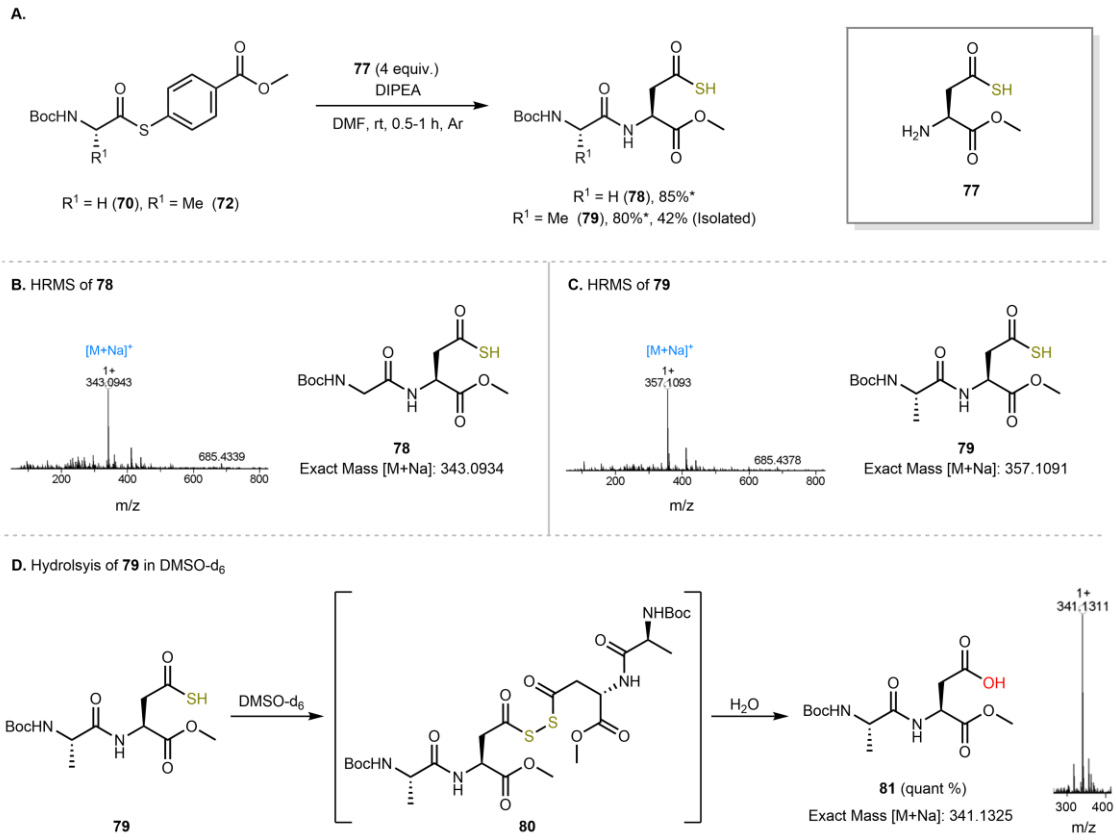
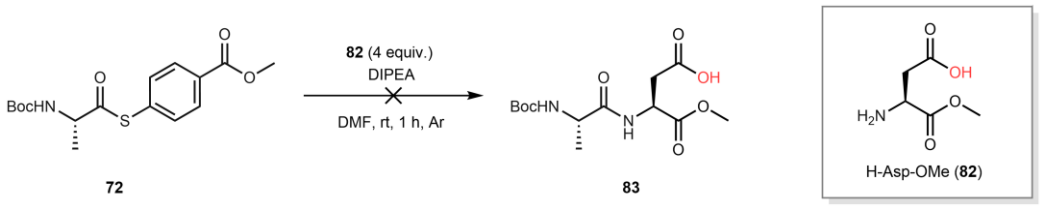


Figure 2.4 (a) Ligation of γ-thioaspartic acid **77** with Gly **70** and Ala **72** thioesters. *HPLC conversion **(b)** HRMS of dipeptide **78**. **(c)** HRMS of dipeptide **79**. **(d)** Hydrolysis of thioacid dipeptide **79** *via* a proposed diacyl disulfide intermediate **80** to the dipeptide carboxylic acid **81** in DMSO-d_6. HRMS of **81** is shown as an inset.

To confirm that we were not simply observing direct amide coupling of the α-amino group of the thioacid with the thioester, the reaction with Ala thioester **72** was repeated with H-Asp-OMe

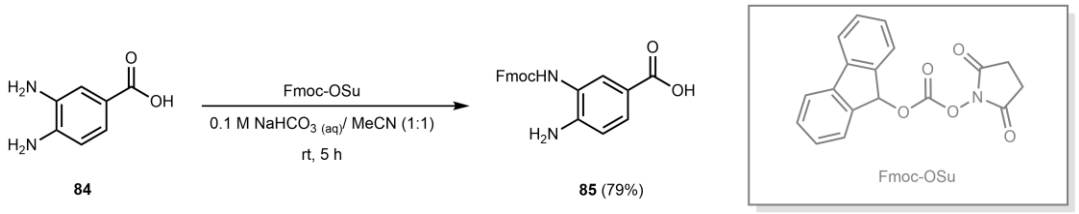
82, the carboxylic acid analogue of H-Asp(SH)-OMe **77** (**Scheme 2.3**). After 1 h, no formation of the dipeptide Boc-Ala-Asp-OMe **83** was observed in this control reaction. This strongly suggests that the thioacid is necessary for ligation and the mechanism for amide bond formation involves thioacid-thioester exchange followed by S-to-N acyl transfer. After establishing the feasibility of the γ-thioaspartic mediated ligation reaction on the model small molecules substrates, we set out to investigate the reaction on longer peptides synthesised by SPPS.



Scheme 2.3. Control reaction of Ala thioester **72** with the carboxylic acid, H-Asp-OMe **82**.

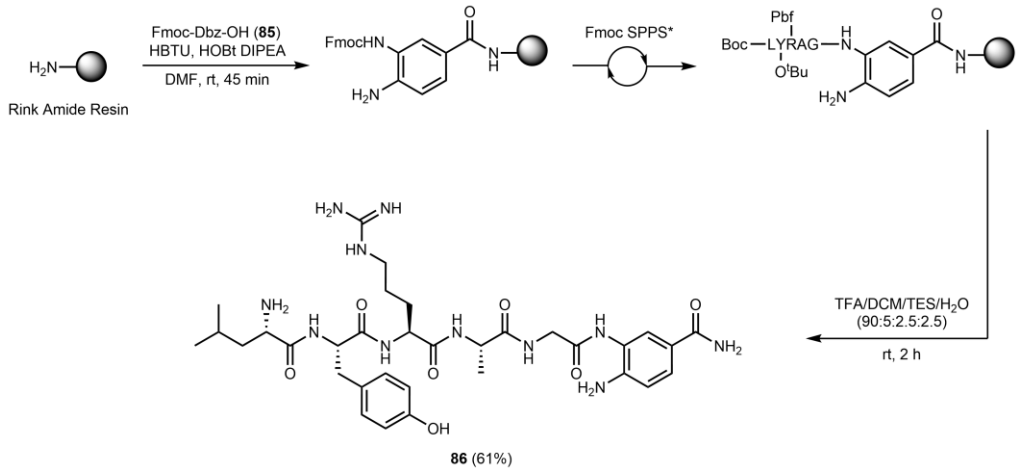
2.3.6 Synthesis of Peptide Thioester LYRAG-MBA 88

To synthesise peptide thioesters indirectly *via* Fmoc SPPS, we utilised the triazole method discussed in **Chapter 1**, **Section 1.3.5**. In this protocol, a diaminobenzoyl (Dbz) linker at the C-terminal of the peptide is oxidised with sodium nitrite ($NaNO_2$) to form a triazole which can be thiolysed to form the thioester. To install the linker *via* Fmoc SPPS, Fmoc-Dbz-OH **85** was synthesised using the original procedure reported by Blanco-Canosa and Dawson.[126] This involved the selective Fmoc protection of the meta-amino group of 3,4-diaminobenzoic acid **84** with *N*-(9-fluorenyl- methyloxycarbonyloxy)succinimide (Fmoc-OSu) to furnish Fmoc-Dbz-OH **85** in 79% yield (**Scheme 2.4**).



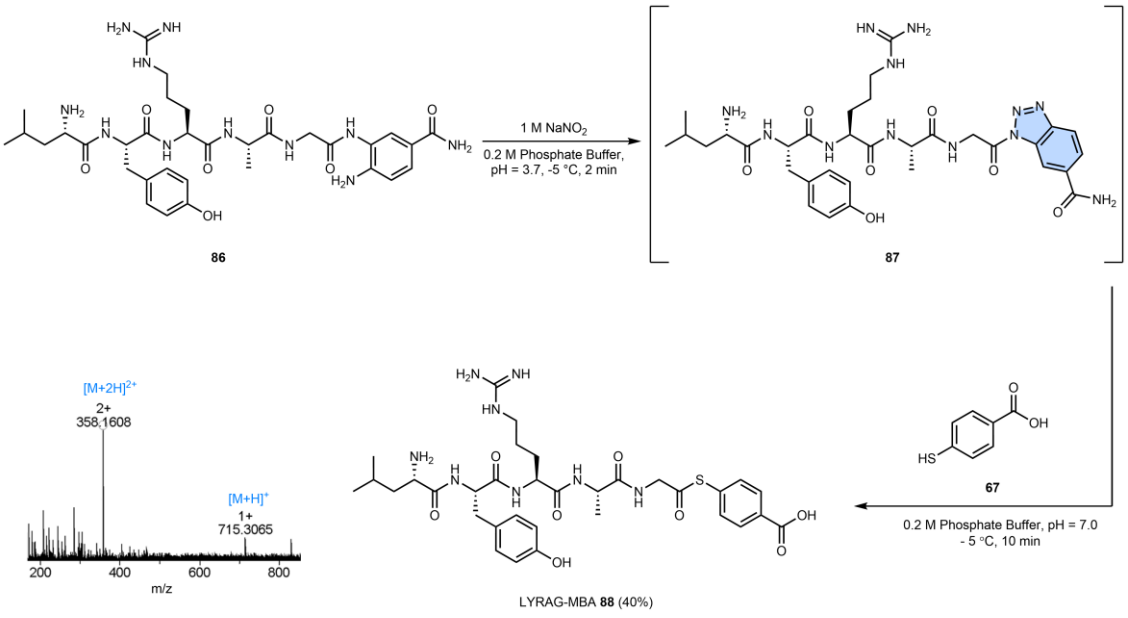
Scheme 2.4. Synthesis of Fmoc-Dbz-OH **85**.

For the peptide thioester, the sequence Lys-Tyr-Arg-Ala-Gly (LYRAG) was chosen because it is frequently encountered as a model peptide in ligation studies. The synthesis began with the coupling of the linker Fmoc-Dbz-OH **85** to Rink Amide resin on 0.2 mmol scale using HBTU (4 equiv.), HOBt (4 equiv.) and DIPEA (6 equiv.) (**Scheme 2.5**). Thereafter, the peptide was assembled *via* Fmoc SPPS using HBTU/HOBt/DIPEA (1:1:1.5) for the initial coupling and PyBOP/NMM (1:2) for the remaining residues. The final residue (Leu) was introduced as Boc-Leu. The peptide was then deprotected and cleaved from the resin upon treatment with TFA to yield LYRAG-Dbz **86** in 61% yield and 90% purity.



Scheme 2.5. Synthesis of LYRAG-Dbz **86**. *Initial Coupling: Fmoc-Gly-OH (4 equiv.), HBTU (4 equiv.), HOBt (4 equiv.), DIPEA (6 equiv.), DMF, 0.2 M, rt, 45 min. Remainder: AA (3 equiv.), PyBOP (3 equiv.), NMM (6 equiv.), DMF, 0.2 M, rt, 45 min. Fmoc Deprotection: 20% (v/v) piperidine/DMF, rt, 2 x 10 min. AAs from Arg (R) were double coupled.

To synthesise the thioester, H-LYRAG-Dbz **86** was dissolved in phosphate buffer at pH 3.7 and treated with a solution of 1 M $NaNO_2$ at -5 °C for 2 min to oxidise the Dbz linker to the C-terminal triazole **87** (**Scheme 2.6**). Immediately afterwards, the triazole was thiolysed by the addition of solution of 4-mercaptobenozic acid (MBA; **67**) in phosphate buffer/MeCN at pH 7.0. Following semi-preparative RP-HPLC purification and lyophilisation, the pentapeptide thioester LYRAG-MBA **88** was isolated in 40% yield and >95% purity.



Scheme 2.6. Synthesis of LYRAG-MBA **88**. HRMS spectrum of **88** is shown as an inset.

2.3.7 Ligation of LYRAG-MBA 88 and Thioacid 77

With the pentapeptide thioester **88** in hand, the ligation reaction with thioacid H-Asp(SH)-OMe **77** was conducted to investigate its reactivity with a more complex thioester substrate under the previously successful conditions in DMF (**Section 2.3.5**). Therefore, LYRAG-MBA **88** (25 mM, 1 equiv.) and DIPEA were dissolved in DMF and added to a freshly prepared sample of H-Asp(SH)-OMe **77** (100 mM) (**Figure 2.5A**). Gratifyingly, full consumption of the thioester was observed after 30 min, with 95% conversion to the ligated hexapeptide LYRAGD(SH)-OMe **89** (**Figure 2.5B**). The mass of the ligated hexapeptide **89** was also confirmed by HRMS (**Figure 2.5C**). With this promising result, we set out to extend the ligation reaction to longer N-terminal thioacid peptides synthesised by SPPS.

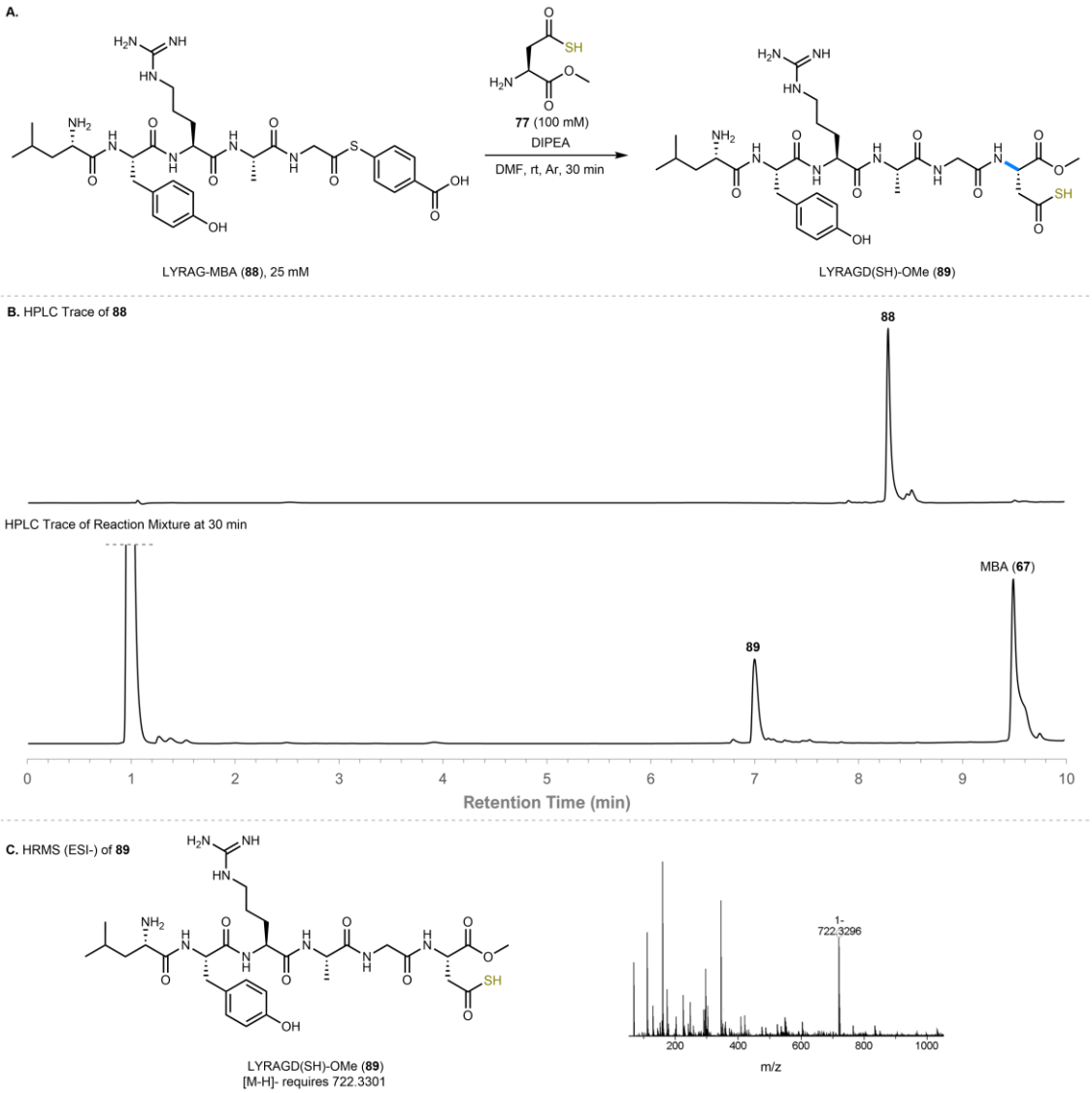


Figure 2.5 (a) Ligation of thioacid **77** with peptide thioester LYRAG-MBA **88**. (b) Comparison of the HPLC trace (254 nm, 5-95% MeCN/H_2O + 0.1% TFA) of thioester **88** (top) and the reaction mixture at 30 min. **(c)** HRMS (ESI-) confirming formation of hexapeptide **89**.

2.3.8 Synthesis of Thioacid Peptides

In order to easily access the necessary N-terminal γ-thioaspartic acid peptides, we required a synthetic protocol whereby the thioacid could be installed directly *via* standard Fmoc SPPS through the coupling of a pre-synthesised AA monomer. Recently, Schöwe *et al*. reported a protocol for the synthesis of γ-thioaspartic acid peptides through the coupling of a pre-synthesised (over 9 steps) tripeptide building block **90** in Fmoc SPPS (**Figure 2.6A**).[230] The tripeptide was required because the single AA building block, Fmoc-Asp(Tmob)-OH **91**, was highly susceptible to an intramolecular, base-catalysed elimination reaction resulting in the formation of cyclic anhydride **92** (**Figure 2.6B**). To minimise aspartimide (Asi) formation during SPPS, the n-2 Thr residue of tripeptide **90** was protected as a Mutter pseudoproline, and a modified deprotection solution consisting of piperazine 6% (w/v)/DMF was used for Fmoc deprotection. While this approach is adequate for limited cases where only a few tripeptide building blocks must be synthesised, it is not a general solution. In fact, 400 (1x20x20) tripeptide building blocks would be required for all combinations of just the canonical AAs.

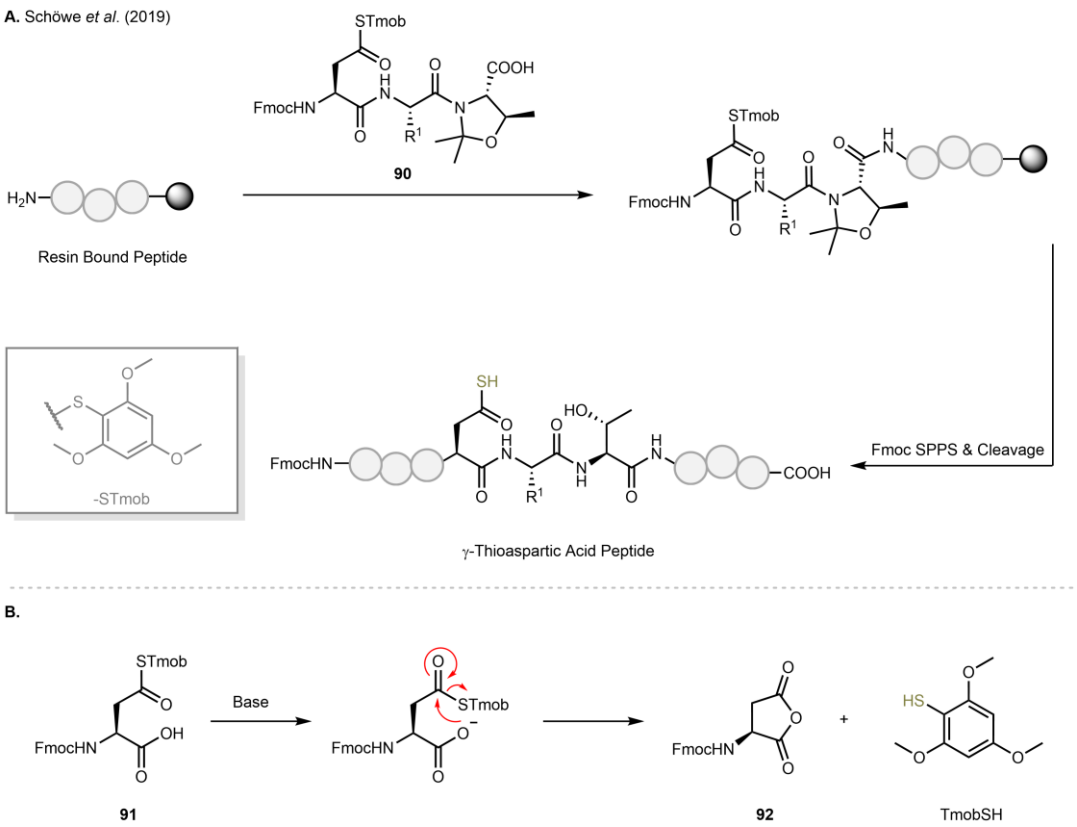


Figure 2.6. (a) Synthesis of γ-thioaspartic acic containing peptides reported by Schöwe *et al*. using the tripeptide building block **90**. **(b)** Base catalysed elimination reaction of single AA building block **91** to form the cyclic anhydride **92**.

Joseph *et al*. reported an alternative strategy for the incorporation of γ-thioaspartic acid into peptides *via* Fmoc SPPS using Fmoc-Asp(STrt)-OTMS **93** as the single AA building block (**Figure 2.7A**).[231] Similar to the aforementioned study, Joseph *et al*. also encountered issues with

base-catalysed anhydride formation when the carboxylic acid Fmoc-Asp(STrt)-OH was used in SPPS. However, the authors found that silylation of the carboxylic acid to form the corresponding trimethylsilyl (TMS) ester resulted in elimination of this side-reaction. In the proposed mechanism, the silyl group acts as a proton surrogate and is sufficiently reactive to be transferred to the coupling reagent, *N*,*N*-diisopropylcarbodiimide (DIC; **5**), to form the active ester **94** (**Figure 2.7B**). Nevertheless, the reported protocol had a notable disadvantage. Significant aspartimide formation of up to 20% was detected during Fmoc deprotection after Asp(STrt) installation, even when a Thr pseudoproline was positioned at the n-2 position and a modified deprotection solution consisting of 6% (w/v) piperazine/DMF was used. Nevertheless, we decided to synthesise the γ-thioaspartic acid peptides using this established route in anticipation that we could optimise the deprotection conditions to minimise aspartimide formation.

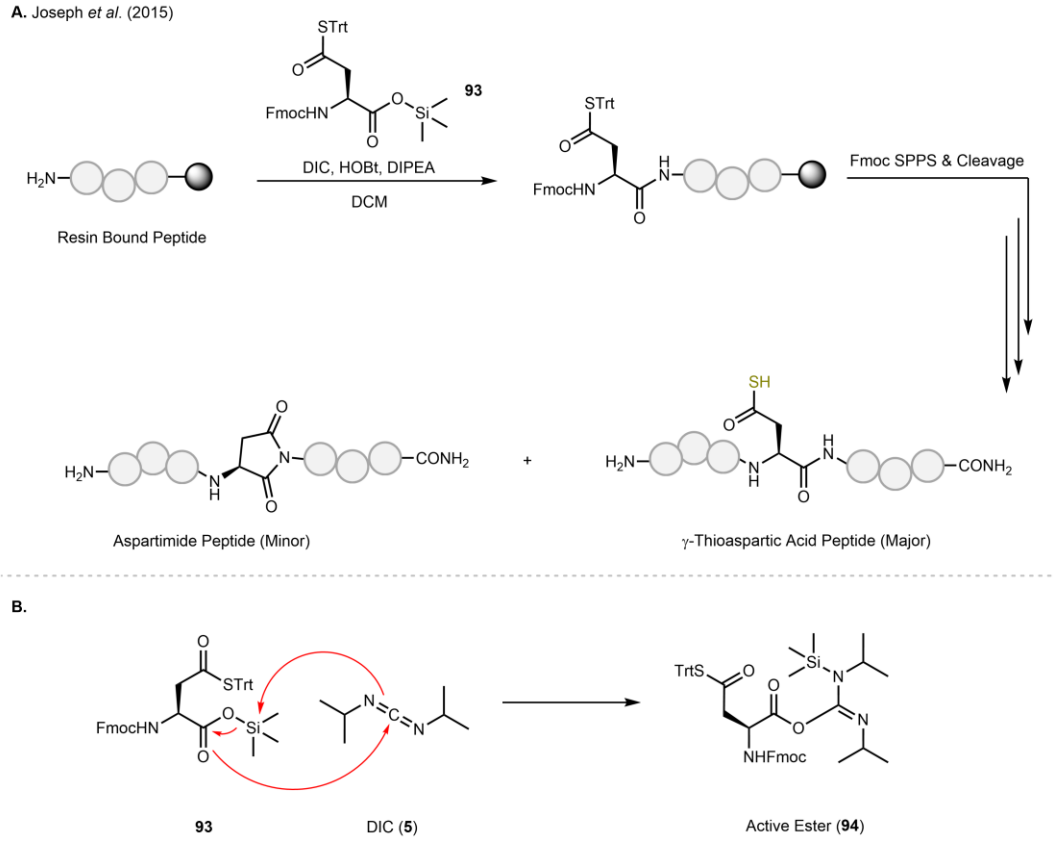
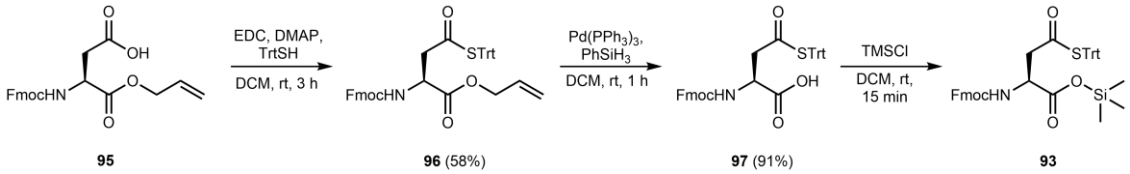


Figure 2.7 (a) Synthesis of γ-thioaspartic acic containing peptides reported by Joseph *et al*. using Fmoc-Asp(STrt)-OTMS **93**. **(b)** Proposed mechanism of coupling of **93** with DIC **5** involving silyl transfer to form the active ester **94**.

2.3.8.1 Synthesis of Fmoc-Asp(STrt)-OTMS 93

To begin, Fmoc-Asp(STrt)-OTMS **93** was synthesised following the procedure reported by Joseph *et al* (**Scheme 2.7**).[231] Thus, Steglich thioesterification of commercially available Fmoc-Asp-OAll **95** formed the thioester Fmoc-Asp(STrt)-OAll **96** in 58% yield. Selective Pd-

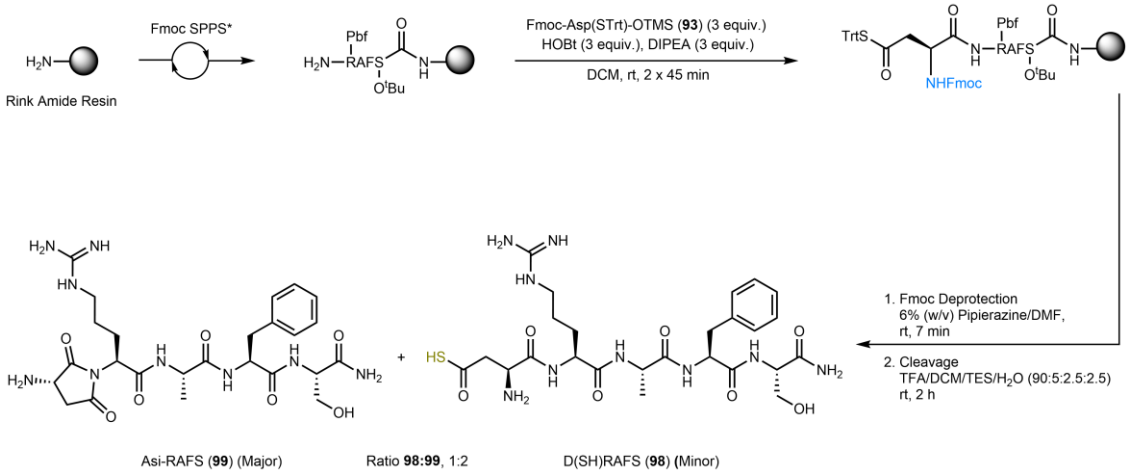
mediated deallylation of the C-terminal using $Pd(PPh_3)_3$ and phenylsilane ($PhSiH_3$) in 1 h furnished Fmoc-Asp(STrt)-OH **97** in 91% yield. Finally, treatment of compound **97** with trimethylsilylchloride (TMSCl) for 15 min in DCM formed the desired trimethylsilyl ester **93** in quantitative yield. Overall, the required SPPS building block, Fmoc-Asp(STrt)-OTMS **93**, was synthesised in 53% yield over 3 steps, requiring two chromatographic purification steps.



Scheme 2.7. Synthesis of Fmoc-Asp(STrt)-OTMS **93**.

2.3.8.2 Synthesis of Peptides Using Fmoc-Asp(STrt)-OTMS 93

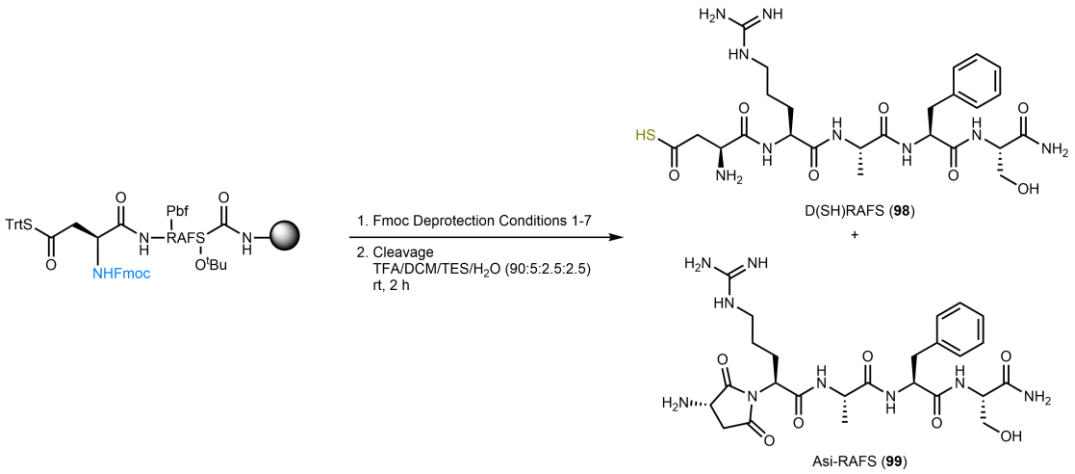
With the SPPS building block in hand, we planned to synthesise the model N-terminal γ-thioaspartic acid pentapeptide, D(SH)RAFS **98**. Using Rink Amide resin on 0.1 mmol scale, the peptide was assembled up to the penultimate residue using PyBOP/NMM in DMF for coupling and 20% (v/v) piperidine/DMF (2 x 10 min) for Fmoc deprotection (**Scheme 2.8**). The final coupling of Fmoc-Asp(Trt)-OTMS **93** was conducted with DIC/HOBt/DIPEA in DCM and the N-terminal Fmoc was deprotected with 6% (w/v) piperazine/DMF (1 x 7 min) as reported by Joseph *et al*.[231] Following cleavage with TFA, the crude peptide was precipitated in cold diethyl ether, dried and analysed by RP-HPLC and HRMS. Unfortunately, the major product was found to be C-terminal aspartimide peptide, Asi-RAFS **99**, rather than the desired thioacid D(SH)RAFS **98** in a ratio of 2:1 as determined by RP-HPLC.



Scheme 2.8. Synthesis of D(SH)RAFS **98** using Fmoc-Asp(STrt)-OTMS **93** and 6% (w/v) piperazine/DMF for the final Fmoc deprotection. * Coupling: AA (3 equiv.), PyBOP (3 equiv.), NMM (6 equiv.), DMF, 0.2 M, rt, 45 min. Fmoc Deprotection: 20% (v/v) piperidine/DMF, rt, 2 x 10 min. AAs from Arg (R) were double coupled.

Next, we screened conditions for the final Fmoc deprotection in the synthesis of **98** in an attempt to minimise or eliminate aspartimide formation. The results are summarised in **Table 2.1**. Unfortunately, the aspartimide peptide, Asi-RAFS **99**, was detected as the major product in each case, including with piperidine (**Entry 1**), piperazine (**Entry 2**), DBU (**Entry 3**) and Aimotos's cocktail (**Entry 4**). Addition of 0.1 M Oxyma **10** to the deprotection solution, reported in the literature as a method to reduce base-catalysed aspartimide formation was also attempted.[79] However, Asi-RAFS **99** was once again the major product with solutions of piperidine (**Entry 5**), piperazine (**Entry 6**) and DBU (**Entry 7**) containing 0.1 M Oxyma. These results , taken together with the previous results obtained by Schowe *et al*. and Joseph *et al.* suggest that γ-thioesters of Asp are highly unstable under basic conditions.[230, 231] The acceptable levels of aspartimide formation detected by both in their synthesis of γ-thioaspartic acid peptides could be attributed to the use of the pseudoproline protected Thr residue at the n-2 position. However, pseudoproline derivatives are only available for Ser, Thr and Cys residues and we required a general synthetic strategy where any AA could be installed at any position.[232]

Table 2.1 Screening of final Fmoc deprotection conditions in the synthesis of D(SH)LYRAG **98** using Fmoc-Asp(STrt)-OTMS **93**.

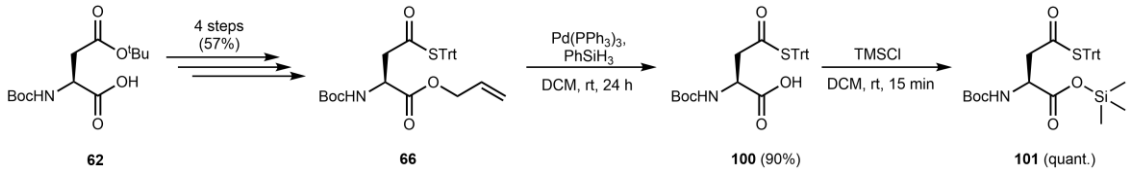


Entry	Deprotection Conditions	Major Product	
		98	99
1	5% (v/v) piperidine/DMF (1 x 5 min)		x
2	6% (w/v) piperazine/DMF (1 x 7 min)[231]		x
3	2% (v/v) DBU/DMF (1 x 5 min)[233]		x
4	Aimoto's Cocktail* (2 x 7 min)[87]		x
5	5% (v/v) piperidine + 0.1 M Oxyma Pure/DMF (1 x 5 min)		x
6	6% (w/v) piperazine + 0.1 M Oxyma Pure/DMF (1 x 7 min)		x
7	2% (v/v) DBU + 0.1 M Oxyma Pure/DMF (1 x 5 min)		x

Synthesis conducted on 0.05 mmol scale on Rink Amide resin. 1 mL of deprotection solution was used. The crude peptide was analysed after cleavage by RP-HPLC and HRMS. The major product for each entry is marked with an 'x'. *Aimoto's Cocktail: 2.5 mL 1-methylpyrrolidine, 0.2 mL azepane, 0.2 g HOBt, 3.65 mL DMSO, 3.65 mL *N*-Methyl-2-pyrrolidone.

2.3.8.3 Synthesis of Boc-Asp(STrt)-OTMS 101

To completely eliminate the requirement for a basic deprotection following installation of the γ-thioaspartic acid residue which introduced the risk of aspartimide formation, we decided to retool the synthesis by using Boc-Asp(STrt)-OTMS **101** as the SPPS building block. In this instance, the N-terminal Boc group can be deprotected during the final acidic resin cleavage step in TFA. To begin, Boc-Asp(STrt)-OAll **66** was synthesised from Boc-Asp(O*t*Bu)-OH **62** *via* the previously established route discussed in **Section 2.3.1** in 4 steps and 57% overall yield. Subsequently, Pd-mediated deallylation of **66** furnished the acid, Boc-Asp(STrt)-OH **100**, in 90% yield (**Scheme 2.9**). Compound **100** was then reacted with TMSCl in DCM for 15 min to form the required silyl ester, Bos-Asp(STrt)-OTMS **101**, in quantitative yield. Overall, **101** was prepared in a yield of 51% over 6 steps. Although the synthetic route is 3 steps longer than that of the Fmoc building block **93**, the overall isolated yield is comparable, and both require just two chromatographic purification steps. We found that Boc-Asp(STrt)-OTMS **101** is stable for several months at 0 °C if stored under a layer of inert gas and care is taken to exclude moisture, making it a suitable building block for SPPS.



Scheme 2.9. Synthesis of Boc-Asp(STrt)-OTMS **101**.

2.3.8.4 Synthesis of Peptides Using Boc-Asp(STrt)-OTMS 101

The SPPS of D(SH)RAFS **98** was then repeated. Using Rink Amide resin at 0.1 mmol scale, the peptide was assembled up to the penultimate residue using PyBOP/NMM in DMF (**Figure 2.8A**). Then, Boc-Asp(STrt)-OTMS **101** was double coupled with DIC/Oxyma Pure/DIPEA in dry DCM. In comparison to the conditions reported by Joseph *et al*. for coupling, we substituted HOBt **8** for Oxyma **10** as it has been shown to be superior additive for coupling efficiency and inhibition of racemisation.[50] Following cleavage with TFA, the crude peptide was precipitated, dried and analysed by analytical RP-HPLC and HRMS. Gratifyingly, the desired N-terminal γ-thioaspartic acid peptide, D(SH)RAFS **98**, was found to be the major product in a crude yield of 79% and 75% purity (**Figure 2.8B**). The two main impurities were found to be the aspartimide Asi-RAFS **99** (≈10%) and the γ-thioaspartic acid deletion peptide, RAFS (≈10%). Following purification by semi-preparative RP-HPLC (5-95% MeCN/H_2O + 0.1% TFA, 60 min) and lyophilisation, D(SH)RAFS **98** was isolated in 24% yield and >95% purity (**Figure 2.8C**). While the purified yield is significantly lower than the crude yield, it could be improved by optimisation of the semi-preparative HPLC method in the future.

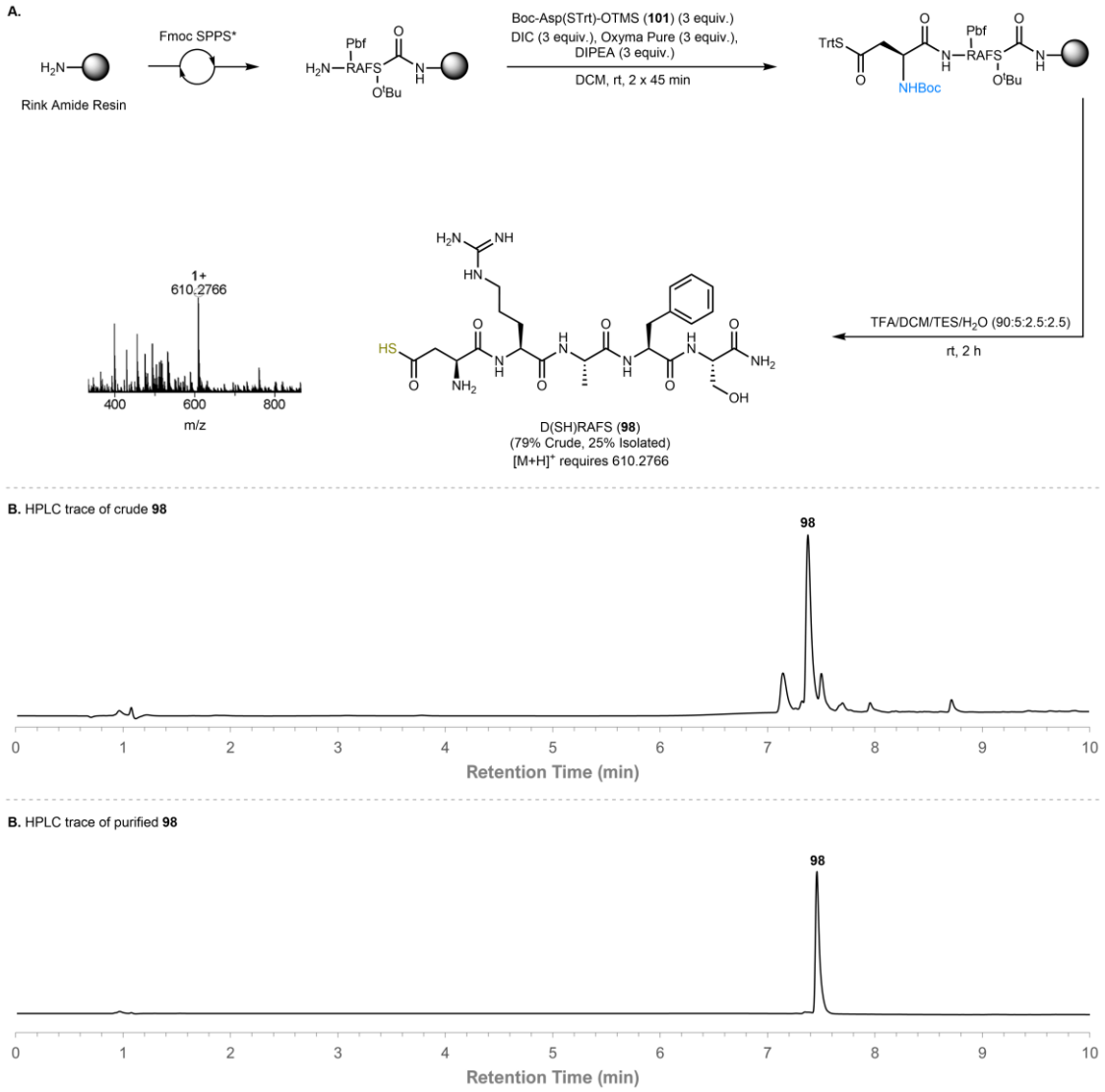


Figure 2.8. (a) Synthesis of D(SH)RAFS **98** using Boc-Asp(STrt)-OTMS **101**. HRMS (ESI+) of **98** is shown as an inset. *Coupling: AA (3 equiv.), PyBOP (3 equiv.), NMM (6 equiv.), DMF, 0.2 M, rt, 45 min. Fmoc Deprotection: 20% (v/v) piperidine/DMF, rt, 2 x 10 min. AAs from Arg (R) were double coupled. **(b)** HPLC trace (254 nm, 5-95% MeCN/H_2O + 0.1% TFA) of crude **98**. **(c)** HPLC trace (254 nm, 5-95% MeCN/H_2O + 0.1% TFA) of **98** after semi-preparative RP-HPLC purification.

To validate the protocol for the synthesis of N-terminal γ-thioaspartic acid peptides using Boc-Asp(STrt)-OTMS **101** as the SPPS building block, we synthesised another example, D(SH)FEE **102**, a tetrapeptide fragment of the peptide drug bivalirudin at the prospective ligation site. As before, the peptide was assembled on Rink Amide resin at 0.1 mmol using PyBOP/NMM up to the penultimate AA, followed by coupling of Boc-Asp(STrt)-OTMS **101** using DIC/Oxyma Pure/DIPEA (**Figure 2.9A**). Following cleavage of the peptide in TFA, precipitation in cold ether and drying, crude D(SH)FEE **102** was obtained in 71% yield and 85% purity (**Figure 2.9B**). In this instance, the main impurities identified were the γ-thioaspartic acid deletion peptide, FEE, and the hydrolysed thioacid peptide, DFEE. The crude was subjected to purification by semi-preparative RP-HPLC (5-95% MeCN/H_2O + 0.1% TFA, 60 min) and **102** isolated in 44% yield and >95% purity (**Figure 2.9C**).

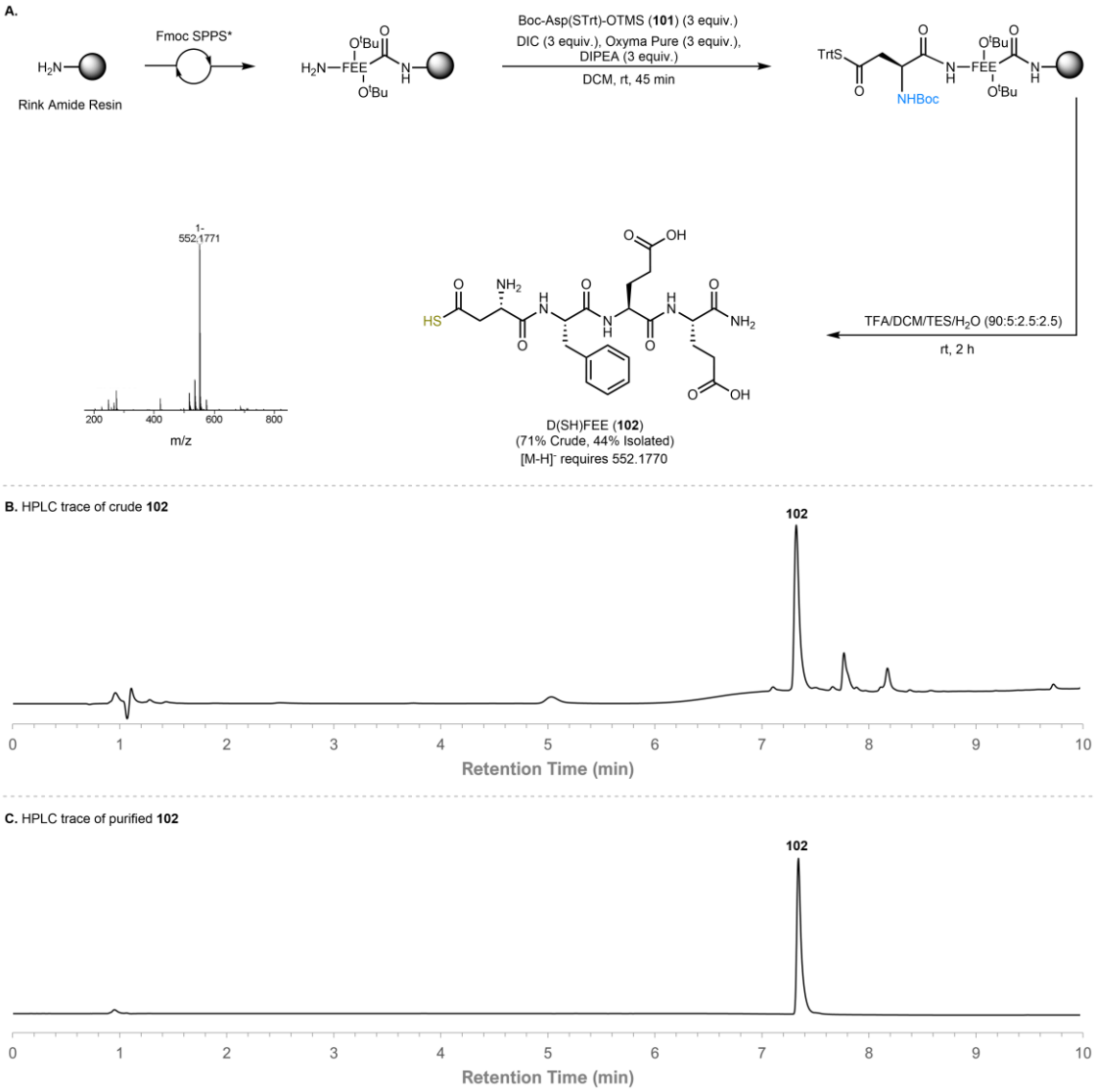


Figure 2.9. (a) Synthesis of D(SH)FEE **102** using Boc-Asp(STrt)-OTMS **101**. HRMS (ESI-) of **102** is shown as an inset. *Coupling: AA (3 equiv.), PyBOP (3 equiv.), NMM (6 equiv.), DMF, 0.2 M, rt, 45 min. Fmoc Deprotection: 20% (v/v) piperidine/DMF, rt, 2 x 10 min. **(b)** HPLC trace (254 nm, 5-95% MeCN/H_2O + 0.1% TFA) of crude **102**. **(c)** HPLC trace (254 nm, 5-95% MeCN/H_2O + 0.1% TFA) of **102** after semi-preparative RP-HPLC purification.

2.3.9 Ligation of Peptide Thioester 88 & Thioacid 102 in DMF

With both the required peptide thioester and thioacid in hand, we commenced our investigation of the ligation reaction between these peptidic substrates. To begin, we attempted the ligation of the thioester LYRAG-MBA **88** with the thioacid D(SH)FEE **102** in DMF under previously established conditions. Thus, LYRAG-MBA **88** (25 mM, 1 equiv.) was dissolved in dry DMF with DIPEA and added to D(SH)FEE **102** (100 mM, 4 equiv.) and stirred under an inert atmosphere (**Figure 2.10A**). Aliquots were taken at 30 min intervals to be analysed by RP-HPLC.

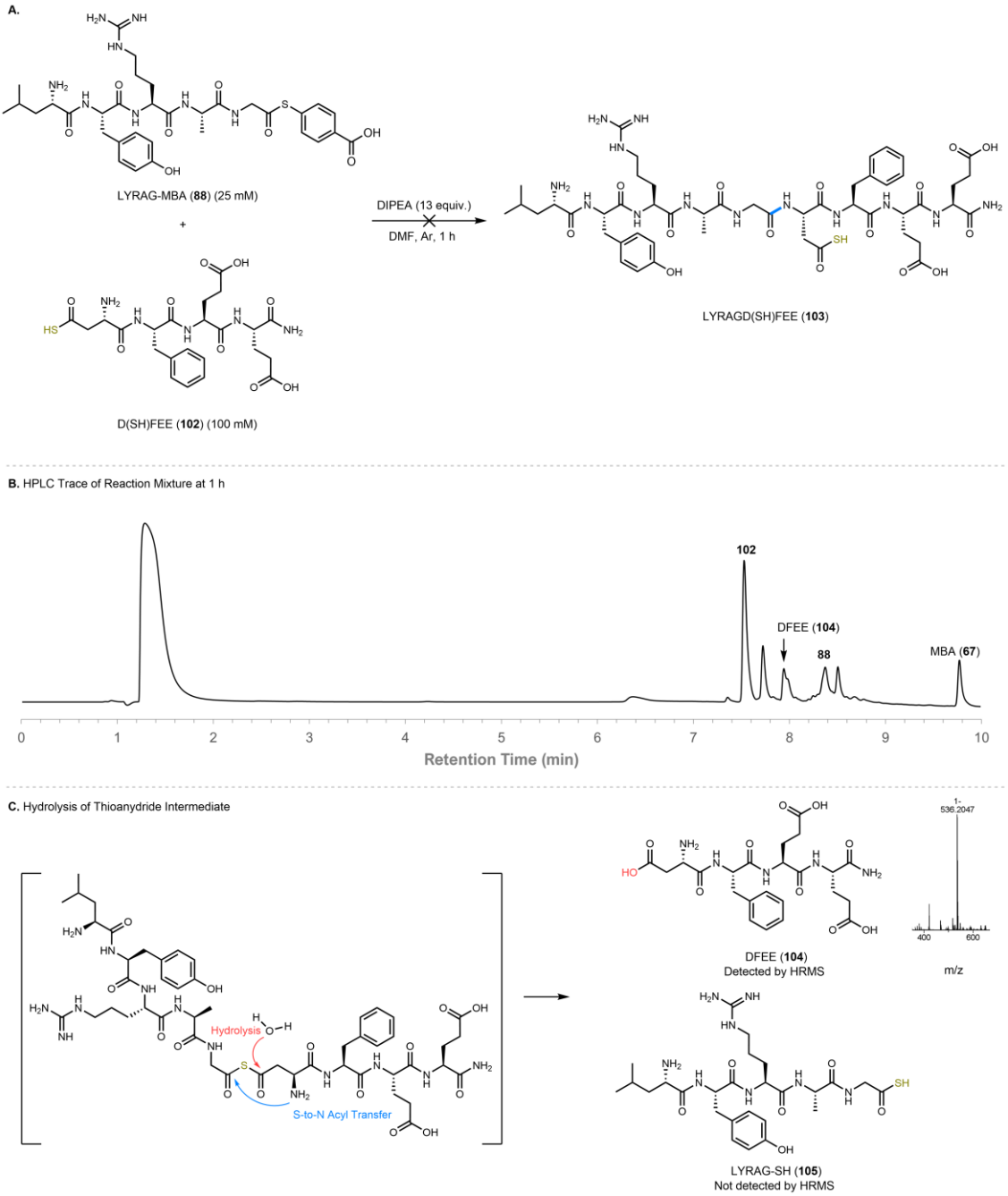


Figure 2.10 (a) Ligation of LYRAG-MBA **88** and D(SH)FEE **102** in DMF. **(b)** HPLC trace (220 nm, 5-95% MeCN/H_2O + 0.1% TFA) of the reaction mixture at 1 h. **(c)** Proposed mechanism for hydrolysis of the thioanhydride intermediate leading to formation of DFEE **104** detected by HRMS (ESI-).

After 1 h, a complex mixture had formed without full consumption of the limiting thioester **88** (**Figure 2.10B**). Each newly formed peak was isolated and analysed by HRMS (ESI +/-), however the mass corresponding to the desired ligated peptide, LYRAGD(SH)FEE **103**, was not detected. The only detected mass that could be assigned was the hydrolysed thioacid, DFEE **104**, at t_R = 7.95 min. Stability studies of the thioacid, D(SH)FEE **102**, in DMF with just DIPEA demonstarted that the thioacid is resistant to hydrolysis under these conditions. Therefore, the formation of DFEE **104** suggests that the thioanhydride intermediate could be undergoing

hydrolysis rather than S-to-N acyl transfer to form DFEE **104** and the C-terminal thioacid, LYRAG-SH **105** (**Figure 2.10C**). Indeed, the hydrolysis could also occur at the other carbonyl of the thioanhydride to reform the original thioacid. Since the reaction of LYRAG-MBA **88** with a simple thioacid proceeded cleanly with 95% conversion to the ligated peptide (**Section 2.3.7**), the additional steric bulk of the peptide thioacid may adversely affect the rate of S-to-N acyl transfer.

2.3.10 Ligation of Peptide Thioester 88 & Thioacid 102 in Buffer

While Okamoto *et al*. reported that the thioanhydrides intermediates were highly unstable in aqueous buffer, we sought to attempt the ligation reaction in buffer to confirm their findings.[129] Thus, LYRAG-MBA **88** (10 mM) and D(SH)FEE **102** (40 mM) were dissolved in a denaturing phosphate buffer containing 6 M guanidinium chloride at pH 7 and stirred under an inert atmosphere (**Figure 2.11A**). Aliquots were taken at 30 min intervals to be analysed by RP-HPLC.

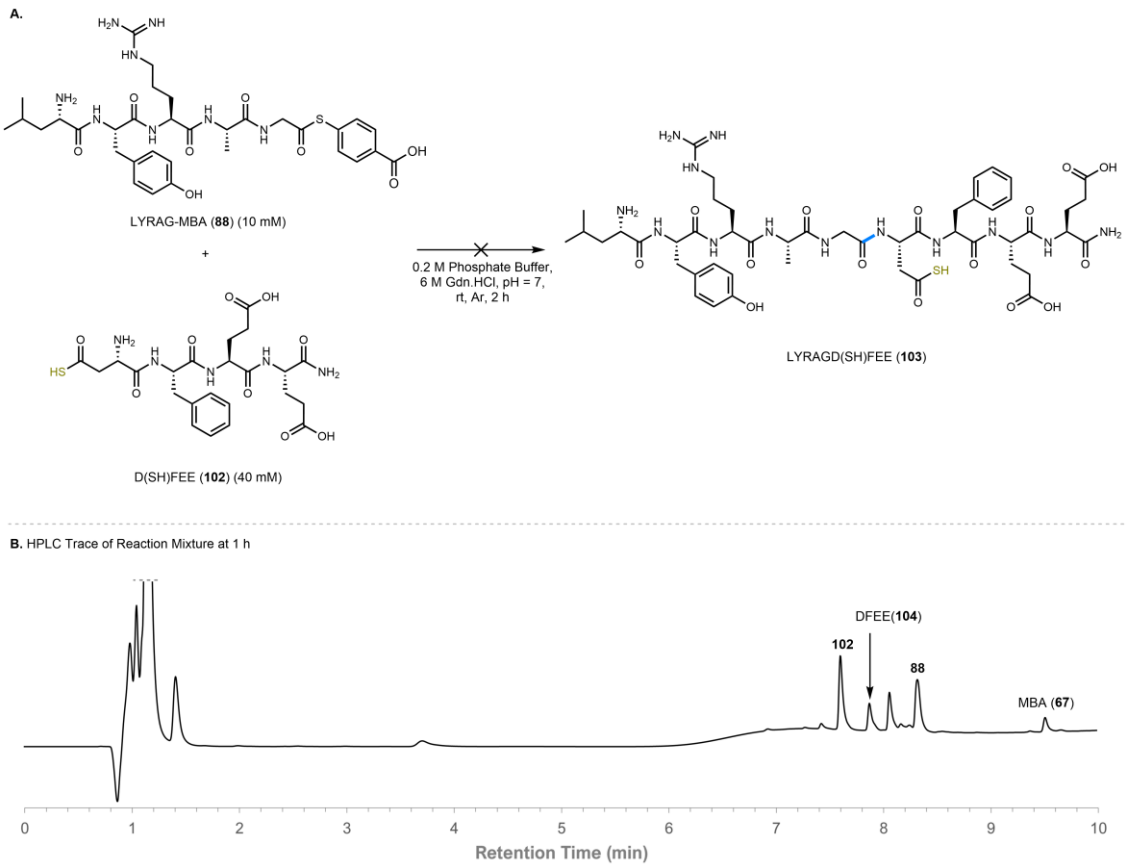


Figure 2.11 (a) Ligation of LYRAG-MBA **88** and D(SH)FEE **102** in Buffer at pH = 7. Gdn.HCl = guanidinium chloride. **(b)** HPLC trace (220 nm, 5-95% MeCN/H_2O + 0.1% TFA) of the reaction mixture at 1 h.

As before, new peaks had formed after 1 h without full consumption of the limiting thioester **88** (**Figure 2.11B**). In addition, consumption of the starting thioester **88** was significantly lower in the aqueous buffer compared to the reaction in DMF. The reduced rate of thioacid-

thioester exchange could be explained by the lower concentration of the peptides in the buffer due to reduced solubility, or the effective solvation of the thiocarboxylate by water reducing its nucleophilicity.[129] In any case, the newly formed peaks were isolated and analysed by HRMS, however, the mass corresponding to the desired ligated product **103** was not detected. The hydrolysed thioacid, DFEE **104**, was detected once again suggesting that the thioanhydride intermediate was being hydrolysed before S-to-N acyl transfer to form the product. This hypothesis was strengthened by the finding that the thioacid, D(SH)FEE **102**, was stable to hydrolysis for up to 24 h at pH 7 in the absence of the thioester. With these disappointing results which were consistent with that reported by Okamoto *et al.*, we decided to refocus our attention towards optimising the ligation of peptide thioacids in organic solvent.

2.3.11 Ligation of Boc-Gly-MBA 70 and Peptide Thioacid 98

To simplify the reaction and focus exclusively on the reactivity of the peptide thioacid, the simple Gly thioester substrate **70** prepared for the initial scoping studies was revisited. Thus, thioester **70** (25 mM) and D(SH)RAFS **98** (100 mM) were dissolved in DMF with DIPEA and stirred under an inert atmosphere (**Figure 2.12A**). Aliquots were taken at 30 min intervals to be analysed by RP-HPLC.

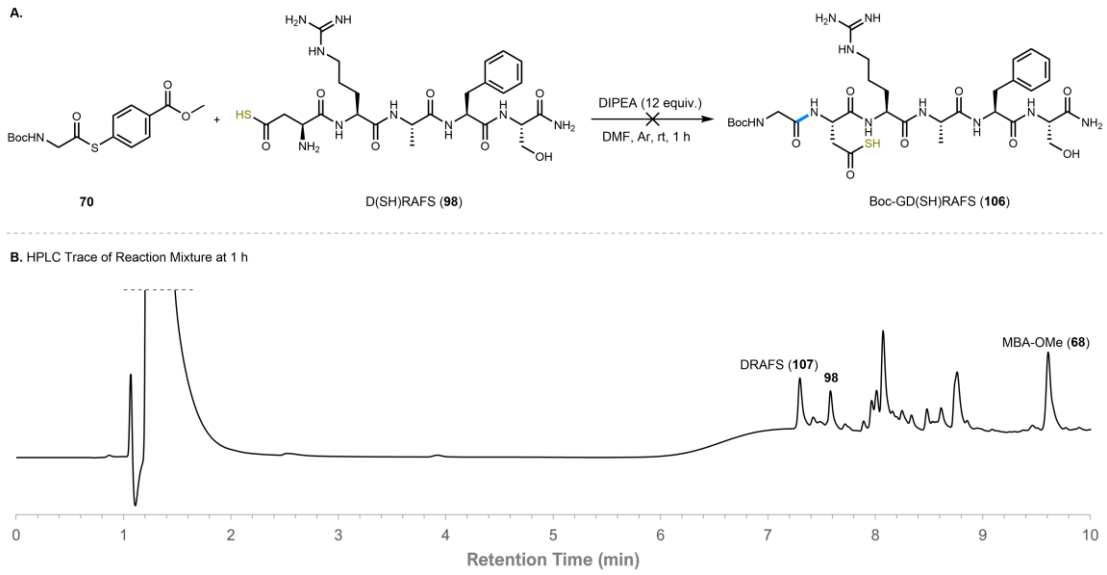


Figure 2.12 (a) Ligation Gly thioester **70** with D(SH)RAFS **98** in DMF. **(b)** HPLC trace (220 nm, 5-95% MeCN/H_2O + 0.1% TFA) of the reaction mixture at 1 h.

After 1 h, full consumption of the thioester **70** was observed with the concomitant formation of a multitude of peaks (**Figure 2.12B**). In particular, the prominent peak at t_R = 8.07 min seemed to be consistent with the expected retention time for the ligated product. However, the mass of the ligated hexapeptide, Boc-GD(SH)RAFS **106**, was not detected by HRMS in any of the peaks. The hydrolysed thioacid, DRAFS **107**, was identified suggesting that the hydrolysis of the thioanhydride intermediate was being favoured over S-to-N acyl transfer. These results

imply that the structure of the γ-thioaspartic acid peptide is critical for ligation efficiency and further work is needed to explore the structural requirements. Another limitation of our study was the lack of *in situ* LC-MS monitoring of the reaction which could have facilitated detection of key transient intermediates to fully understand the reaction process. We were reliant on isolation of each peak and analysis by *ex situ* HRMS, often up to 48 h after sample preparation.

2.4 Conclusions and Future Work

This chapter outlines the initial development of a peptide ligation method utilising C-terminal thioester and N-terminal γ-thioaspartic acid peptides *via* a mechanism involving thioester-thioacid exchange to form a putative thioanhydride linked intermediate, which subsequently undergoes S-to-N acyl transfer to furnish an amide bond. In an initial scoping study, the reaction was successfully demonstrated through the '1+1' ligation of simple thioester derivatives of Gly **70** and Ala **72** with γ-thioaspartic acid **77** in DMF to form dipeptides.

To extend the reaction to more complex peptide substrates, a pentapeptide model thioester, LYRAG-MBA **88** was synthesised. The '5+1' ligation of this thioester with γ-thioaspartic acid **77** in DMF proceeded efficiently with 95% conversion to the ligated hexapeptide within 30 min. To synthesise N-terminal γ-thioaspartic peptides *via* Fmoc SPPS, we initially turned to the previously reported SPPS building block, Fmoc-Asp(STrt)-OTMS **93**. However, aspartimide formation was found be to be a major side-reaction during the final Fmoc deprotection, even after screening of a variety of Fmoc deprotection conditions. In order to mitigate this issue, the SPPS protocol was retooled to use the novel building block, Boc-Asp(STrt)-OTMS **101**, which enabled successful synthesis of the model N-terminal γ-thioaspartic peptides, D(SH)RAFS **98** and D(SH)FEE **102**, in good yields and at least 75% crude purity.

However, extending the method to the more complex thioacid peptides proved challenging. The '5+4' ligation of LYRAG-MBA **88** and D(SH)FEE **102** in both DMF and aqueous buffer at pH 7 resulted in no detectable product formation. In both cases, the hydrolysed thioacid, DFEE **104**, was identified which suggested that the thioanhydride intermediate underwent hydrolysis rather than productive S-to-N acyl transfer. Simplification of the thioester substrate to the single AA Gly derivative **70** also failed to produce any ligated product. The results obtained suggest that the structure of the thioacid peptide is critical to ligation efficiency, though further exploration was limited by time constraints.

Future work will focus on the synthesis of simplified γ-thioaspartic acid peptide substrates, such as those bearing Gly or Ala adjacent to the N-terminal γ-thioaspartic acid residue to probe side-chain steric effects. In addition, an unnatural D-amino acid could be incorporated adjacent to the γ-thioaspartic acid residue to determine whether the orientation of the residue was preventing S-to-N acyl transfer. Future investigations could be greatly aided by *in situ* LC-

MS monitoring to identify key transient intermediates. Ultimately, the reaction must be demonstrated at ligation junctions of all 20 naturals AAs, both at the C-terminal of the thioester and adjacent to the γ-thioaspartic acid residue, for this protocol to be considered a universally applicable peptide ligation strategy. It is anticipated that with further optimisation of the reaction conditions and development of one-pot thioacid hydrolysis conditions, native peptide drugs such as Bivalirudin and Teduglutide could be synthesised using this approach, offering a new method for peptide ligation with mild desulfurisation.

Chapter 3

Photochemical Acyl Thiol-Ene Thiolactonisation of Peptides

3.1 Introduction

Macrocyclic thiolactones are found in several naturally occurring peptides such as the depsipeptides verrucosamide[234] and thiocoraline[235] produced by marine organisms, and the auto-inducing peptides (AIPs) produced by Gram positive bacteria such as *Staphylococcus aureus* (**Figure 3.1A**).[236] In *S. aureus*, AIPs are secreted by bacteria in the late exponential phase of bacterial growth in an intercellular process known as quorum sensing which promotes biofilm dispersion to spread infection and the production of virulence factors.[237] As such, analogues of AIPs have been investigated as modulators of the quorum sensing systems of *S. aureus* to attenuate and to treat infection (**Figure 3.1B**).[238-240]

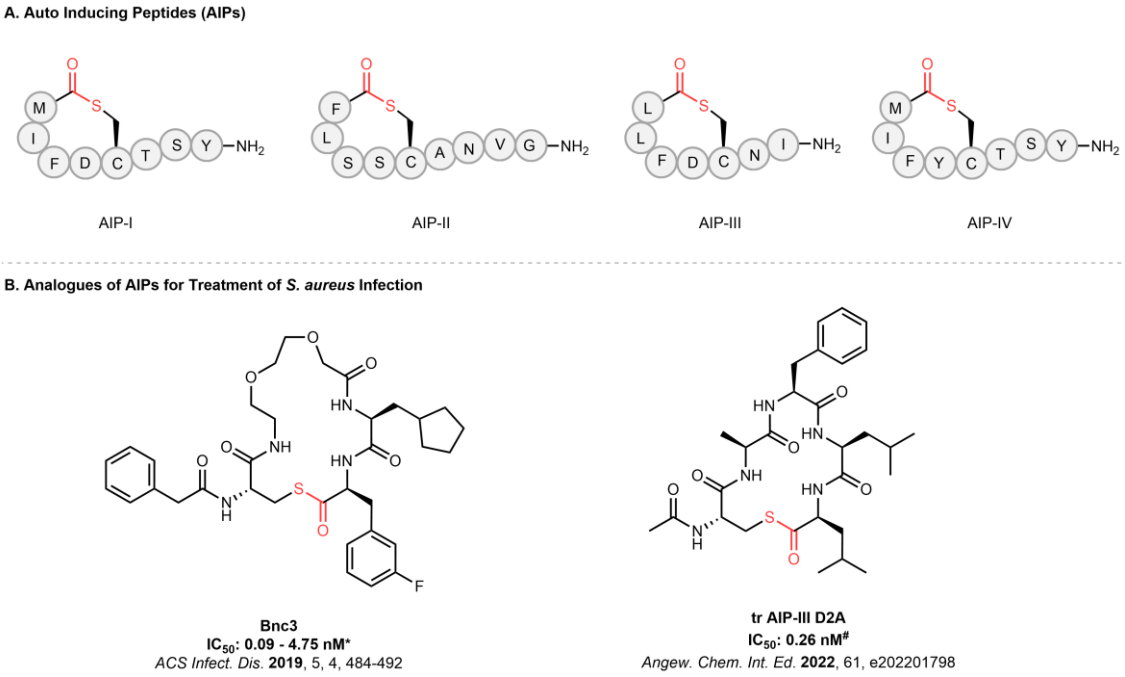


Figure 3.1 (a) Structures of the autoinducing peptides (AIPs) from *S. aureus*. **(b)** Selected analogues of AIPs containing thiolactone bonds which have been investigated for the treatment of *S. aureus* infection. **agr* reporter assay in wild-type *S. aureus* (Group I-IV), # *agr* reporter assay in wild-type Group I *S. aureus*.

Early synthetic strategies for the chemical synthesis of macrocyclic thiolactone peptides focused on the intramolecular coupling of Cys residues of fully protected peptides with the C-terminal carboxylic acid using coupling reagents such as DCC, EDC or PyBOP, followed by deprotection to yield the fully unprotected thiolactone peptide.[241] However, the high dilution required to prevent formation of dimers or oligomers and the steric bulk associated with side-chain protecting groups lead to reaction times of up to 72 h and the necessity for large excess of coupling reagents (up to 5 equiv.) and catalysts such as DMAP and HOAt.[241] Subsequently, methods compatible with unprotected peptides were developed whereby the Cys residue selectively displaces an activated group (e.g. thioester, Dbz or MeDbz) at the C-terminal to form the thiolactone. However, these methods require elevated temperatures of 50 °C and reaction

times of 2–12 h for sufficient yield, often with crude purities <80%. For further detail, the prevailing methods for the synthesis of AIP peptides have been reviewed recently by Gordon.[241]

3.2 Aims

The aim of this project was to develop a methodology to synthesise macrocyclic thiolactone peptides *via* the photochemical acyl thiol-ene reaction (**Figure 3.2**). Recent work by the Scanlan group has demonstrated the utility of the acyl thiol-ene reaction for the synthesis of 5- and 6-membered thiolactones.[176] However, the reaction has not been explored for the synthesis of highly functionalised thiolactones or macrocycles, nor has it been applied to the synthesis of peptide thiolactones.

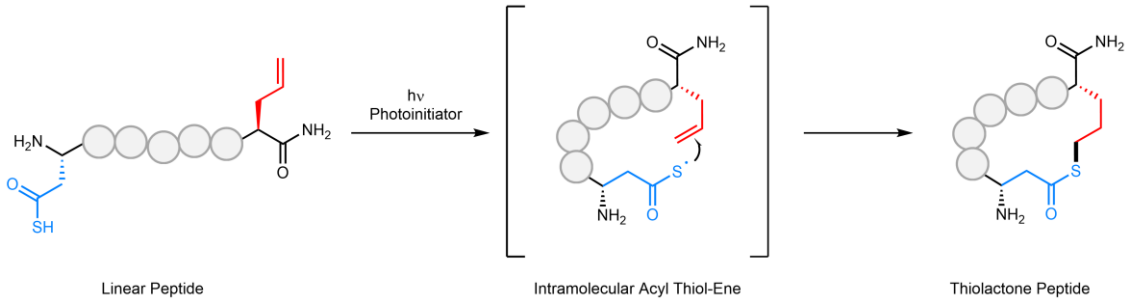


Figure 3.2 Project Aim. Synthesis of macrocyclic peptide thiolactone *via* photochemical acyl thiol-ene.

3.3 Results and Discussion

3.3.1 Synthesis of Linear Model Alkene/Thioacid Peptide 108

To facilitate intramolecular acyl thiol-ene cyclisation, a peptide containing both an alkene and thioacid functionality is required. The unsaturated amino acid allylglycine (Agl) was selected since Fmoc-Agl-OH is commercially available and stable to the standard conditions used in Fmoc SPPS. In addition, anti-Markovnikov addition of the thiyl radical to the terminal alkene is highly favoured, which should result in the formation of a single isomer of the thiolactone. Previous work by Shimuzu *et al.* demonstrated that C-terminal peptide thioacid radicals undergo dethiocarboxylation *via* carbonyl sulfide (COS) elimination to form *N*-alkylamides.[186] In order to avoid this established side reaction during acyl thiol-ene macrocyclisation, we utilised the previously described γ-thioaspartic acid residue as dethiocarboxylation should be unfavourable due to the requisite formation of a high-energy primary alkyl radical intermediate. In addition, this thioacid can be introduced in SPPS as the N-terminal AA using the STrt protected derivative Boc-Asp(STrt)-OTMS **101** as previously described in **Chapter 2**.

To commence the initial investigation, the model tetrapeptide H-Asp(SH)-Ala-Ala-Agl-NH_2 **108** was synthesised using manual Fmoc SPPS on Rink Amide resin, with Fmoc deprotection achieved using 20%(v/v) piperidine in DMF (2 x 10 min) (**Figure 3.3A**). The initial three AAs were coupled using a solution of Fmoc-AA-OH (4 equiv.), HATU (3.9 equiv.) and DIPEA (8 equiv.) in DMF for 45 min. HATU can react with the unprotected N-terminal of the growing peptide to form guanido derivatives which prevents chain elongation.[242] Therefore, a limiting amount of HATU was used and the coupling solution was also preactivated by mixing for 2 min prior to addition to the resin. The thioacid was double coupled as the final residue using Boc-Asp(STrt)-OTMS **101** (3 equiv.), DIC (3 equiv.), Oxyma (3 equiv.) and DIPEA (3 equiv.) in dry DCM for 45 min. Following chain assembly, the peptide was cleaved from the resin using a cleavage cocktail consisting of TFA (90% v/v), DCM (5% v/v), triethylsilane (TES, 2.5% v/v) and H_2O (2.5% v/v) for 2 h to furnish the linear peptide **108** in a yield of 83%. The isolated crude peptide was >90% pure by analytical RP-HPLC (t_R = 7.60 min) which was sufficient for investigation of the acyl thiol-ene cyclisation without further purification (**Figure 3.3B).**

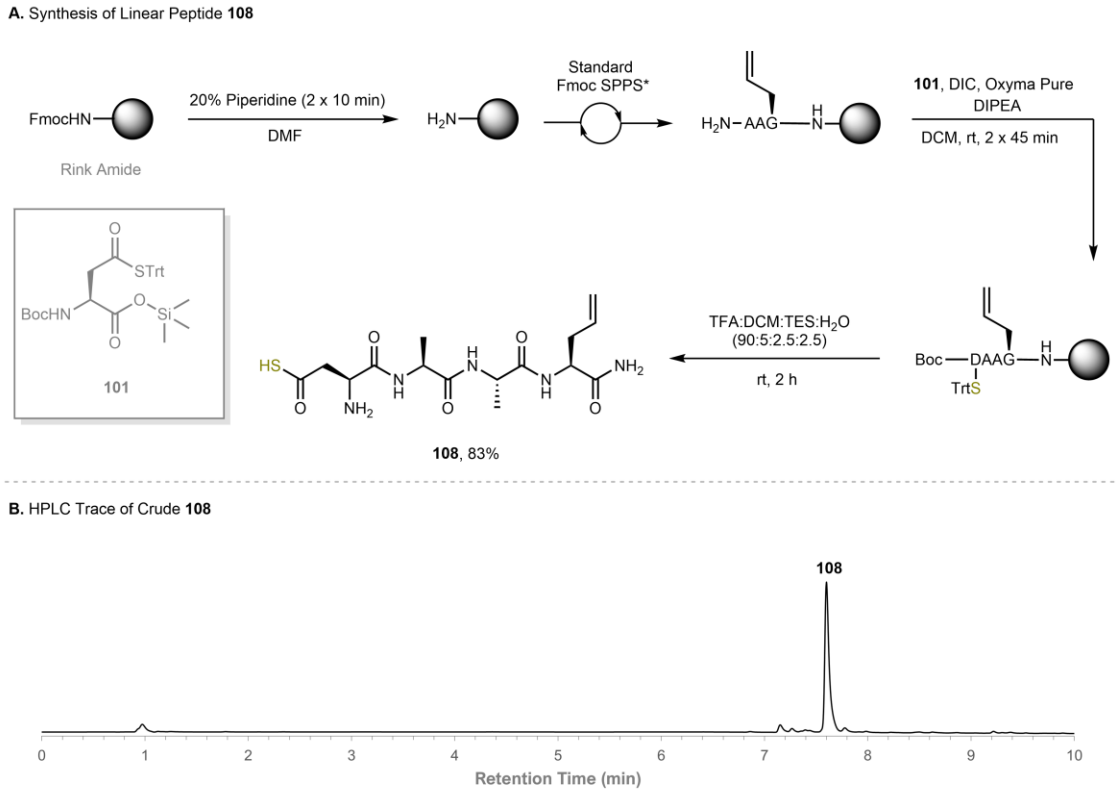


Figure 3.3 (a) Synthesis of the linear peptide **108**. *Coupling: Fmoc-AA-OH (4 equiv.), HATU (3.9 equiv.), DIPEA (8 equiv.) in DMF. Deprotection: 20% (v/v) piperidine in DMF **(b)** RP-HPLC trace of crude **108** (220 nm). Gradient: 5-95% MeCN in H_2O (+0.1% TFA) over 10 min.

3.3.2 Acyl Thiol-Ene Macrocyclisation of 108

For the initial investigation of the photochemical acyl thiol-ene macrocyclisation reaction, conditions previously reported by Nolan *et al*. within the Scanlan group for the thiol-ene

cyclisation of oxytocin analogues were adapted.[243] The linear peptide **108** was dissolved at 5 mM concentration in a solvent mix of H_2O/MeCN (1:1) containing 0.1% TFA and one equiv. of the photoinitiator, 2,2-dimethoxy-2-phenylacetophenone **109** (DPAP) in a sealed *vial*. The reagents were fully soluble, forming a clear solution which could ensure even penetration of light during the photoreaction. The reaction mixture was then irradiated with UV light (354 nm) in a Luzchem photoreactor (LZC-EDU,110 V/60 Hz) for 15 min using magnetic stirring (**Figure 3.4A**). After completion of the reaction, an aliquot (250 µL) of the reaction mixture was analysed by RP-HPLC. Gratifyingly, full consumption of the linear peptide and a conversion of 90% to the macrocyclic thiolactone **110** (t_R = 6.89 min) was observed by analytical HPLC after 15 min (**Figure 3.4B**). Following semi-preparative RP-HPLC purification and lyophilisation, the product **110** was isolated in 54% yield.

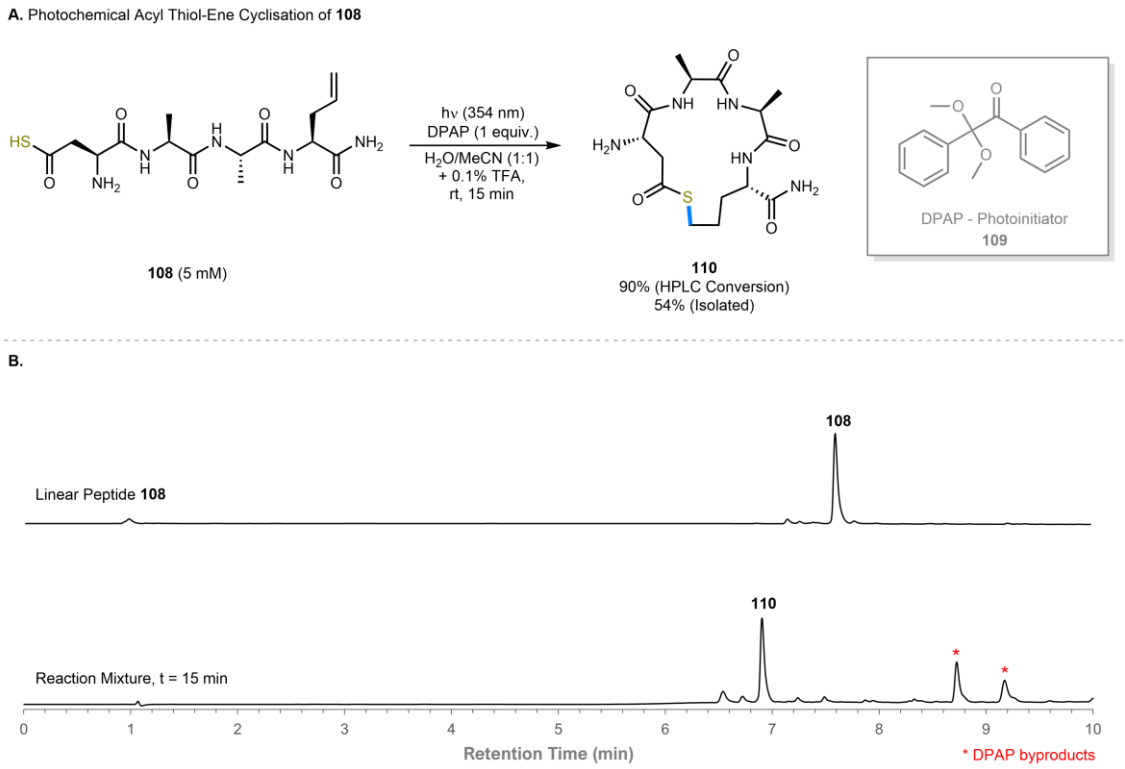


Figure 3.4 (a) Photochemical acyl thiol-ene cyclisation of **108**. **(b)** Comparison of the HPLC trace (5-95% MeCN in H_2O + 0.1% TFA, 220 nm) of the linear peptide **108** and the reaction mixture at 15 min showing formation of the thiolactone **110**.

Since the acyl thiol-ene macrothiolactonisation reaction is an intramolecular hydrothioacylation reaction, the mass of the linear and cyclised peptides are identical. Consequently, HR-MS which is the standard analytical method for the characterisation of peptides cannot be used to unambiguously prove formation of the cyclic product. Indirect evidence for the formation of the thiolactone include the difference in retention time of -0.71 min between the linear peptide **108** (t_R = 7.80 min) and the putative thiolactone **110** (t_R = 6.89 min) (**Figure 3.4B**). Although cyclisation could be expected to increase retention time in RP-HPLC, peptides can often adopt more polar conformations after cyclisation which leads to less retention.

Furthermore, the ^{1}H NMR spectrum of **110** showed complete disappearance of alkene resonances and the formation of diastereotopic methylene protons consistent with hydrothioacylation of the alkene. Ultimately, the Heteronuclear Multiple Bond Correlation (HMBC) spectrum of **110** provided definitive evidence for the formation of the thiolactone linkage (**Figure 3.5**). The HMBC spectrum shows long range through bond ^{13}C-^{1}H correlations, typically two- to three-bond correlations, but occasionally up to five bonds.[244] The HMBC spectrum of **110** shows a three-bond correlation between the thiolactone carbonyl at position #2 (^{13}C δ194.8 ppm) and the diastereotopic methylene protons at position #3 (^{1}H δ3.13–3.07 and δ2.59–2.51 ppm).

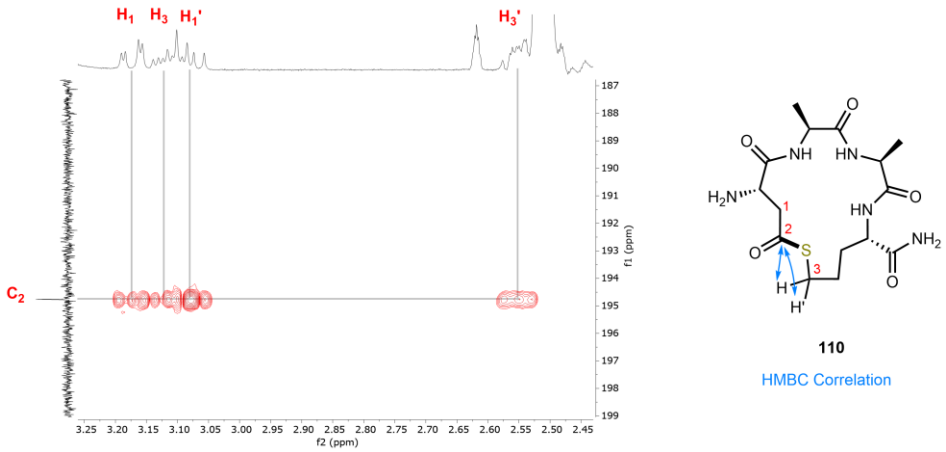


Figure 3.5 HMBC spectrum of **110** confirming formation of the thiolactone linkage.

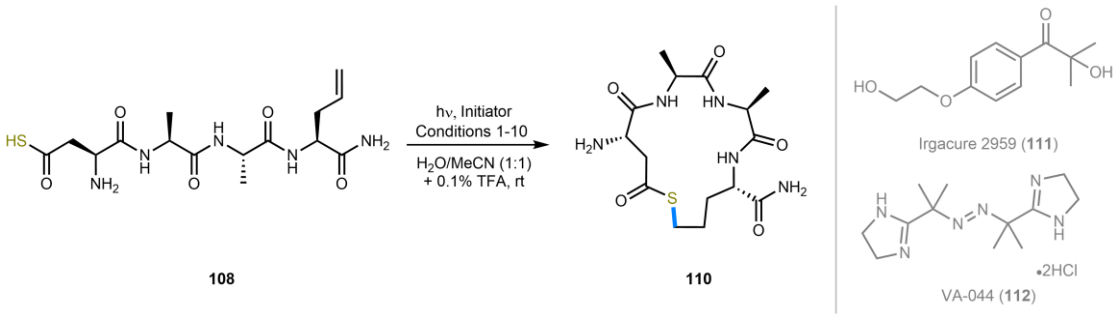
3.3.3 Investigation of Acyl Thiol-Ene Cyclisation Conditions

Subsequently, the reaction conditions for the photochemical acyl thiol-ene cyclisation of the model linearpeptide **108** were explored, and the results are summarised in **Table 3.1**. The reaction without the addition of 0.1% (v/v) TFA to the solvent system resulted in a significantly reduced conversion of 35% to the thiolactone **110** after 15 min (**Entry 2**). As previously observed for the analogous thiol-ene reaction, an acidic pH is crucial to ensure that the thioacid remains protonated for efficient hydrogen atom transfer (HAT) to form the requisite thiyl radical to initiate the reaction.[243] For the thiol-ene reaction, Colak *et al*. found that a pH range of 4–7 was best depending on the thiol, with the optimal pH for each thiol being correlated to its pK_a (typically 6-10).[245] Since thioacids have pK_a values in the range of 3–5, the optimal pH for acyl thiol-ene should be pH 1-2 to ensure that >99% of the thioacid remains protonated for efficient HAT. The addition of 0.1% (v/v) TFA to the reaction mixture results in a pH of ≈2.

The photoinitiator was shown to be necessary as a negligible conversion of 2% was observed after 15 min when the photoinitiator DPAP **109** was omitted from the reaction mixture under UV irradiation (**Entry 3**). The minimal conversion to the thiolactone may be accounted for by direct homolytic cleavage of the thioacid S-H bond to form the thiyl radical[203], or free radical generation by dissolved molecular oxygen.[178] Screening of reaction time showed a moderate

conversion of 59% at just 1 min irradiation time (**Entry 4**), and up to 80% at 5 min (**Entry 5**). Variation of the stoichiometry of the photoinitiator DPAP showed excellent conversion of 81% using just 0.25 equiv. of DPAP (**Entry 6**) which dropped significantly to 10% using 0.1 equiv. (**Entry 7**). Increasing the concentration of the linear peptide five-fold to 25 mM resulted in only a slightly reduced conversion of 82% compared to the standard conditions, but the analytical HPLC showed the appearance of small peaks consistent with the formation of dimers or oligomers arising from intermolecular reaction (**Entry 8**).

Table 3.1. Investigation of reaction conditions for acyl thiol-ene macrocyclisation of **108**.



Entry	108 Conc. (mM)	Time (min)	Radical Initiator	Notes	Conversion* (HPLC)
1	5	15	DPAP (1 equiv.)	Standard	90%
2	5	15	DPAP (1 equiv.)	No TFA	35%
3	5	15	-	No Initiator	2%
4	5	1	DPAP (1 equiv.)		59%
5	5	5	DPAP (1 equiv.)		80%
6	5	15	DPAP (0.25 equiv.)		81%
7	5	15	DPAP (0.1 equiv.)		10%
8	25	15	DPAP (1 equiv.)		82%
9	5	15	Irgacure 2959 (1 equiv.)		67%
10	5	15	VA-044 (1 equiv.)	No UV, 37 °C	11%#

UV irradiation at 354 nm. *Conversion to **110** as determined by HPLC. # Major product is hydrolysed thioacid of **108**.

The reaction was also attempted with the photoinitiator Irgacure 2959 **111** under UV irradiation and a moderate conversion of 67% was obtained after 15 min (**Entry 9**). DPAP **109** and Irgacure 2959 **111** are both Type I photoinitiators that undergo homolytic cleavage to form a benzoyl and either a benzyl (DPAP) or alkyl (Irgacure 2959) radical, both of which can initiate the radical reaction (**Figure 3.6**).[246] Irgacure 2959 has become a popular water-soluble UV initiator, but its relatively low UV absorbance compared to DPAP and slower kinetics of homolytic cleavage require longer reaction times to obtain comparable yields.[247] An attempt at thermal radical initiation using VA-044 **112** at 37 °C resulted in a poor 11% conversion to the desired thiolactone, with the major product observed being the linear hydrolysed thioacid (**Entry 10**). Ultimately, the

standard conditions proved to be the most efficient and were carried forward for further investigation of the scope of the cyclisation reaction (**Entry 1**).

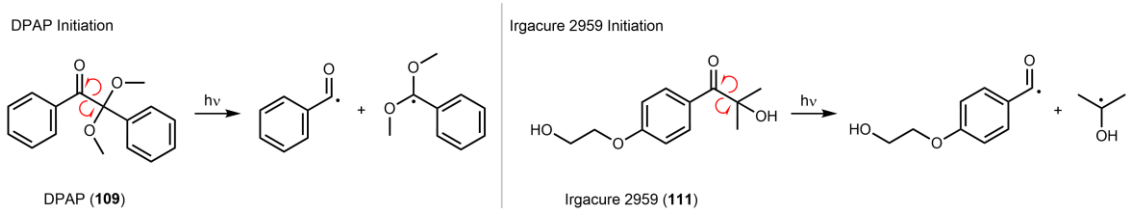


Figure 3.6. Homolytic cleavage of DPAP **109** and Irgacure 2959 **11** to generate the corresponding radicals which initiate reaction.

3.3.4 Scope: Synthesis of Linear Peptides 113-118

Thereafter, a series of structurally diverse linear peptides **113**–**118** containing an allylglycine residue at the C-terminal and a γ-thioaspartic acid residue at the N-terminal were synthesised *via* Fmoc SPPS at 0.1 mmol scale (**Table 3.2**). The crude peptides were obtained in yield ranging from 42-95%, and the crude purities of all peptides were sufficient to use without further purification (>80%). The major impurities identified by HRMS were the deletion peptide arising from incomplete coupling of the γ-thioaspartic acid (*m/z* = M-131) and the carboxylic acid derivative of the peptide arising from thioacid hydrolysis (*m/z* = M-16). In all cases, the impurities were either co-eluting or eluting close to the desired peptide by analytical RP-HPLC which indicated that semi-preparative purification of the linear peptides would have been inefficient and resulted in a large loss of material. Since the impurities do not contain thioacids, they should be inert under the photochemical acyl thiol-ene conditions, and it was anticipated that a shift in retention time after cyclisation could aid in separation.

Table 3.2. Linear alkene/thioacid peptides synthesised.

Compound No.	**Sequence**	**Yield** (%)	**Purity** (%)
113	H-Asp(SH)-Leu-Tyr-Agl-NH_2	65	90
114	H-Asp(SH)-Lys-Leu-Agl-NH_2	93	90
115	H-Asp(SH)-Asp-Phe-Ile-Agl-NH_2	42	85
116	H-Asp(SH)-Ser-Ser-Leu-Agl-NH_2	66	90
117	H-Asp(SH)-Gln-Trp-Met-Agl-NH_2	46	80
118	H-Asp(SH)-Asp-Phe-Leu-Leu-Agl-NH_2	95	85

Refer to **Chapter 5, Section 5.3** for details of Fmoc SPPS of the peptides. Yields are crude isolated yields after lyophilisation. Purity determined by HPLC.

3.3.5 Scope: Acyl Thiol-Ene Thiolactonisation of 113-118

The linear peptides were then cyclised to afford the macrocyclic thiolactone peptides **119**–**124** in low to moderate isolated yields (22-61%) following semi-preparative RP-HPLC

purification (**Figure 3.7**). In all cases, the isolated yields of the thiolactones were significantly lower than the conversion by analytical HPLC (typically >80%) and the yields could be significantly improved by optimisation of the semi-preparative RP-HPLC methods. However, each peptide may require a bespoke method to maximise yield and in this initial investigation, we focused on isolation of the peptides using standard methods. In the case of thiolactone **122**, no cyclisation was observed under the standard conditions. We theorised that the linear peptide **116** was adopting an unreactive conformation in solution due to intramolecular hydrogen bonding of the thioacid to the adjacent serine residues, preventing proton abstraction by the photoinitiator to initiate the reaction. Therefore, the reaction was repeated with the addition of the chaotropic agent, 6 M guanidinium chloride, to the solvent mixture. Gratifyingly, under these modified conditions, full consumption of the linear peptide was observed by HPLC, and thiolactone **122** was isolated in a yield of 61%.

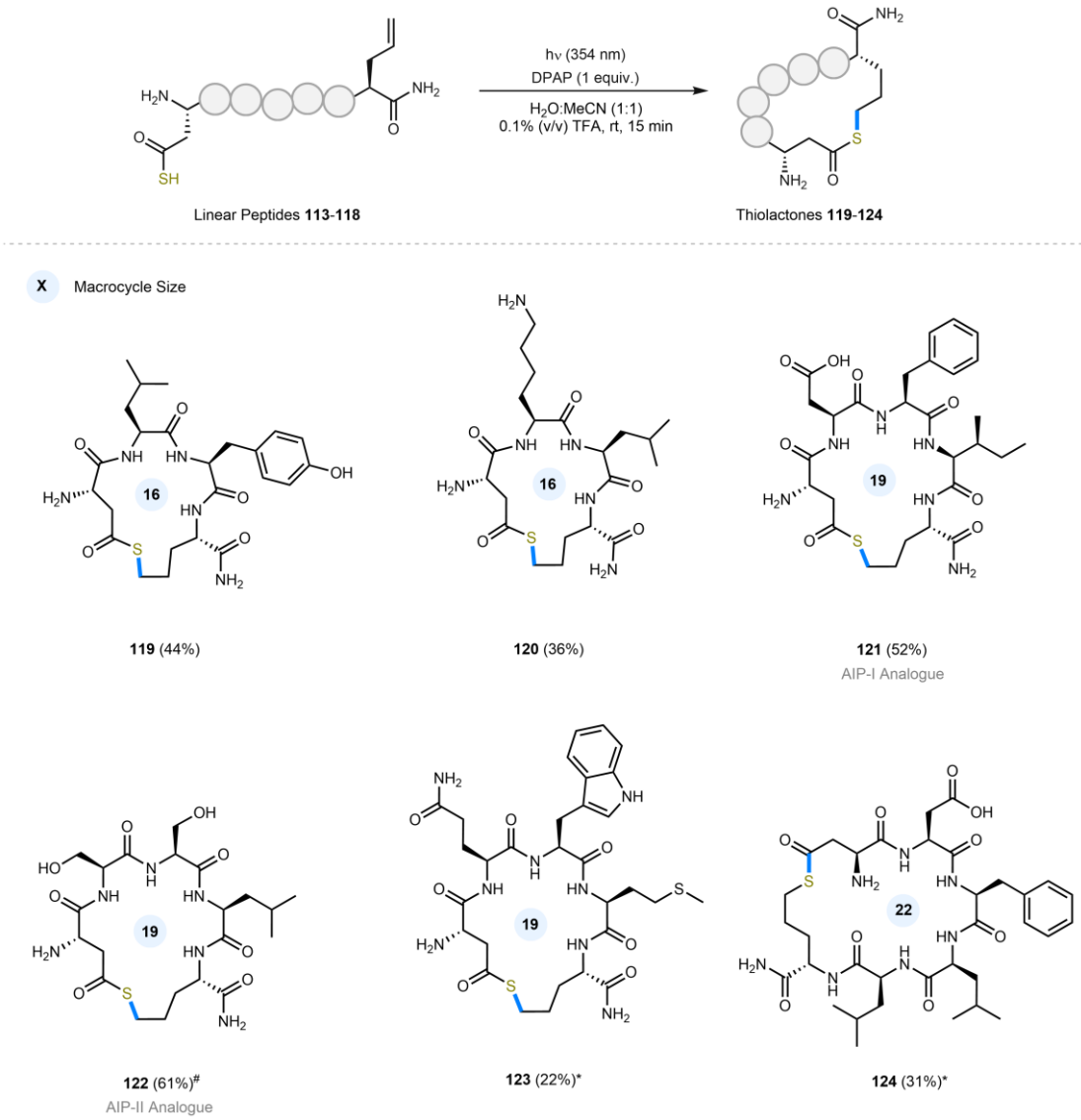


Figure 3.7. Scope of the acyl thiol–ene cyclisation. Reported yields are isolated yields after semi-preparative HPLC purification and lyophilisation. # 6 M guanidinium chloride was added to reaction mixture. *Isolated with approximately 20% of an inseparable impurity as determined by ^{1}H NMR spectroscopy.

The thiolactone peptides **123** and **124** were isolated with approximately 20% of an inseparable coeluting impurity as determined by ^{1}H NMR spectroscopy, and confirmed by HRMS to be the γ-thioaspartic acid deletion peptide (*m/z* = M-131) carried through from Fmoc SPPS of the linear peptides. Attempts to separate the deletion impurity from the products by using a shallower gradient, extended runtimes and/or lower injection volumes during semi-preparative RP-HPLC purification proved unsuccessful. Following cyclisation, the retention time of the linear and cyclic peptides were often not significantly different from each other, or to the linear impurities which complicated purification. For example, even with an extended RP-HPLC analytical run (5-95% MeCN/H_2O with 0.1% TFA, 30 min), the difference in retention time between the linear peptide **118** (t_R = 12.30 min) and the corresponding thiolactone **124** (t_R =12.22 min) was just 0.08 min over a 30 min run (see **Chapter 5**, **Section 5.3.2**). However, thiolactones **123** and **124** are still of sufficient purity (>70%) for initial, high-throughput drug screening studies.[248] Importantly, side-chain functional groups including phenols (**119**), amines (**120**), carboxylic acids (**121**), alcohols (**122**), indoles (**123**) and sulfides (**123**) were all compatible with the acyl thiol-ene macrocyclisation protocol described. The reaction was highly efficient for all ring sizes attempted, ranging from 16 to 22. Furthermore, the sequence of thiolactone peptides **121** and **122** were based on the structure of the autoinducing peptides AIP-I and AIP-II respectively, highlighting the utility of the reaction for the synthesis of biologically relevant peptides.

3.3.6 Removal of Unreacted Impurities

While the photochemical acyl thiol-ene reaction was highly efficient for the macrocyclisation of peptides, the presence of inseparable impurities in examples such as **123** and **124** was a notable limitation of this protocol. Levels of γ-thioaspartic acid deletion peptide impurities could be reduced by repeating the coupling of the monomer Boc-Asp(STrt)-OTMS **101** during Fmoc SPPS of the linear peptide. However, this was deemed uneconomical since **101** must be synthesised *via* a 6-step route and was already being double coupled with 3 equivalents of each time. To remove the alkene-containing deletion peptide impurity prior to RP-HPLC purification, we planned a solid-phase extraction protocol whereby a thiol-ene reaction on the crude reaction mixture after cyclisation using 3-mercaptopropyl functionalised silica gel **125** could be used to form an insoluble silica-peptide conjugate that could be easily filtered (**Figure 3.8**).

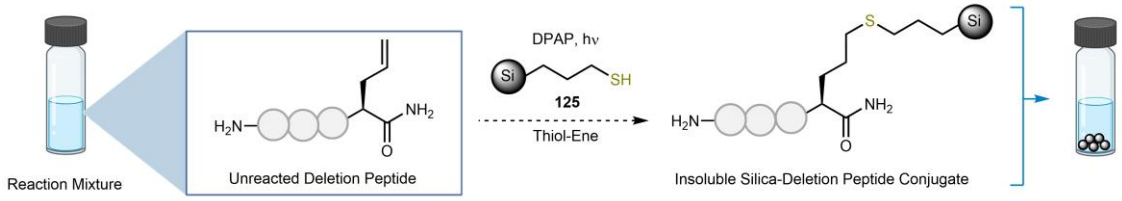


Figure 3.8. Proposed removal of alkene-containing impurities from the reaction mixture after cyclisation using 3-mercaptopropyl functionalised silica **125** which would form an insoluble silica-peptide conjugate.

To investigate the feasibility of this purification protocol, the linear peptide H-Asp(SH)-Gln-Trp-Met-Agl-NH_2 **117** was cyclised *via* acyl thiol-ene using the standard conditions, resulting in full consumption of the linear peptide and formation of the thiolactione **123** (**Figure 3.9A**). Notably in the HPLC trace of the reaction mixture, a shoulder can be seen on the thiolactone peak at t_R = 8.22 min which was identified as the linear 4-mer γ-thioaspartic acid deletion peptide **126** by HRMS (**Figure 3.9B**). Immediately after completion of the acyl thiol-ene reaction, another equivalent of DPAP and 3-mercaptopropyl functionalised silica gel **125** was added to the reaction mixture and irradiated for 1 h. The reaction mixture was then filtered to remove any silica and subjected to semi-preparative RP-HPLC purification. The isolated product was analysed by ^{1}H NMR spectroscopy, but was still found to contain approximately 20% of the deletion peptide **126**. In an effort to drive the thiol-ene reaction with the silica thiol **125** to completion, the process was repeated with 2 equivalents of both DPAP and **125**, but similar results were obtained after purification. Finally, the process was repeated with 3 equivalents of both DPAP and **125** which resulted in complete disappearance of all peptide-related peaks, including the deletion peptide **126** and thiolactone **123** (**Figure 3.9C**).

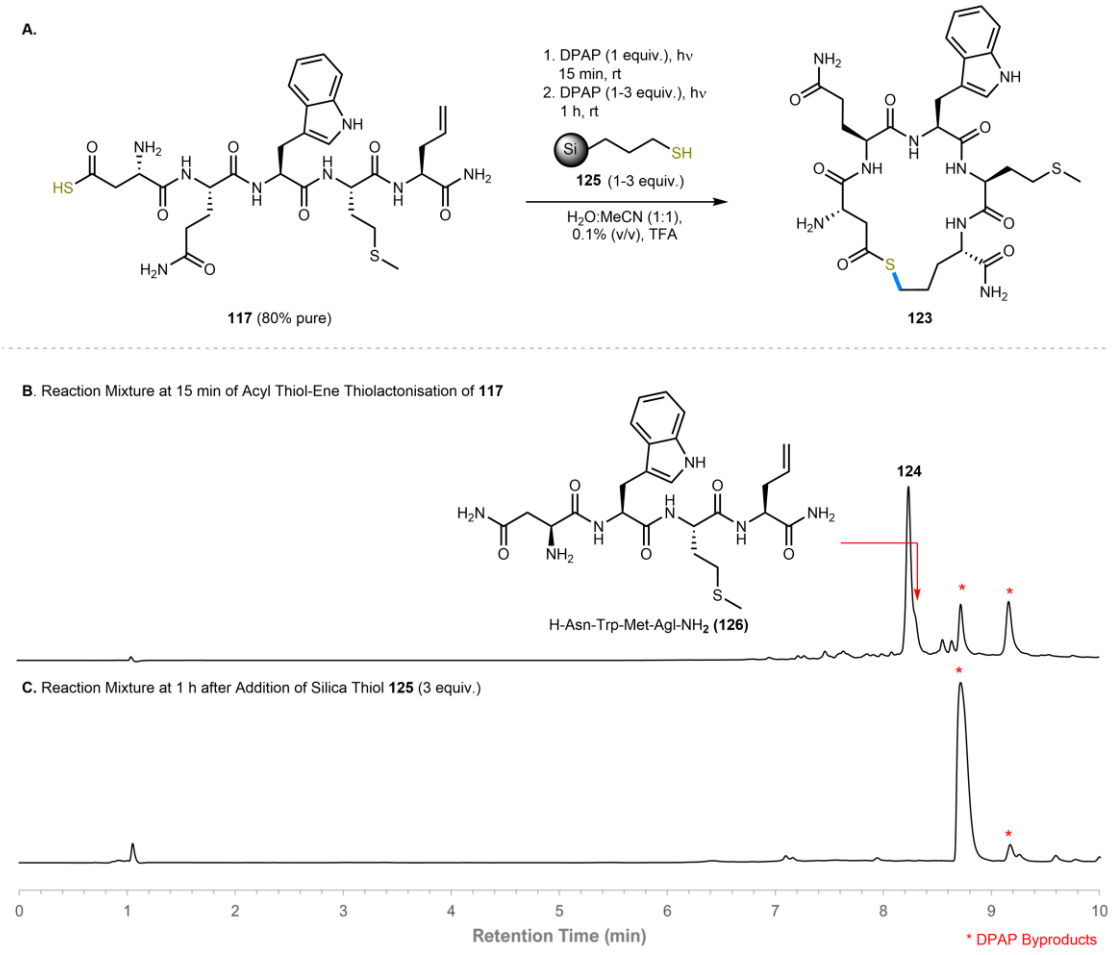


Figure 3.9. (a) Acyl thiol-ene cyclisation of **117** followed by reaction with 3-mercaptopropyl functionalised silica gel **125** to remove unreacted impurities. **(b)** RP-HPLC trace (220 nm) of the acyl thiol-ene cyclisation of **117** to form thiolactone **123**, with deletion impurity **126** highlighted. **(b)** RP-HPLC trace (220 nm) after addition of 3 equiv of silica thiol **125** showing complete removal of peptide-related peaks.

We theorised that the thiolactone **123** could be undergoing thiol-thioester exchange with the 3-mercaptopropyl functionalised silica gel and becoming attached to the silica. However, extensive kinetic studies by Bracher *et al*. have shown that the rate of thiol-thioester exchange is favoured at pH 6-10 and is negligible at pH values <4.[249] For our study, all reactions were conducted in a H_2O:MeCN solvent mixture containing 0.1% (v/v) of TFA with a pH value of <2. The thiolactone **123** should be unreactive towards thiol-ene reaction under these conditions, therefore, it is likely that both the thiolactone **123** and the deletion peptide **126** were being strongly adsorbed onto the surface of the silica due to hydrogen bonding interactions and being removed during filtration. As a result, this purification protocol was abandoned because the deletion peptide **126** could not be selectively removed. Even if thiol-ene reaction was possible, the results suggest that extensive optimisation will be required for each sequence and is not a practical general solution for the removal of alkene-containing impurities. Non-polar (e.g. polystyrene) thiol-functionalised resins could prevent issues with surface binding but were not initially considered since these resins will not swell sufficiently in the aqueous solvent system used. Further work is required to address the presence of inseparable impurities in some of the thiolactones, including optimisation of the Fmoc SPPS synthesis of the linear peptides of these difficult sequences to minimise deletion/hydrolysis peptide formation in the first place.

3.3.7 Acyl Thiol-Ene Thiolactonisation with Blue LED

The acyl thiol-ene thiolactonisation of peptides was also investigated under blue LED (440 nm) irradiation. While UV irradiation was highly efficient for macrocyclisation, it is not compatible with modifications such as disulfide linkages and peptides can undergo degradation or side-reactions with prolonged UV exposure. For example, Tyr-Gly sequences are particularly prone to photodegradation,[250] and Tyr can form tyrosyl radicals (Tyr-O$^{\bullet}$) which can induce dityrosine formation and disulfide cleavage.[251] By comparison, the inability of native peptide sequences to absorb visible light prevents photo-induced side reactions. Furthermore, blue LED irradiation is less energy intensive, safer and can be conducted using inexpensive light sources.

To investigate the blue LED reaction, the linear peptide H-Asp(SH)-Ser-Ser-Leu-Agl-NH_2 **116** was dissolved at 5 mM concentration in a 1:1 mix of H_2O/MeCN containing 0.1%(v/v) TFA and 6 M guanidinium chloride. The blue LED compatible photosensitiser Eosin Y **127** was added and the solution was irradiated using blue LEDs centred at 440 nm for 15 min (**Figure 3.10A**). Eosin Y **127** is excited by blue light to form a diradical (*Eosin Y) which undergoes a HAT with the thioacid to form the thiyl radical which initiates the acyl thiol-ene reaction (**Figure 3.10B**).[252] The protonated Eosin Y (Eosin Y-H) subsequently undergoes reverse HAT (RHAT) to regenerate Eosin Y. After 15 min, full consumption of the linear peptide **116** was observed by analytical RP-HPLC with 80% conversion to the corresponding thiolactone **122** (**Figure X.Xc**). Following semi-preparative HPLC purification and lyophilisation, the thiolactone **122** was isolated in a yield of 40% which is comparable to the UV conditions.

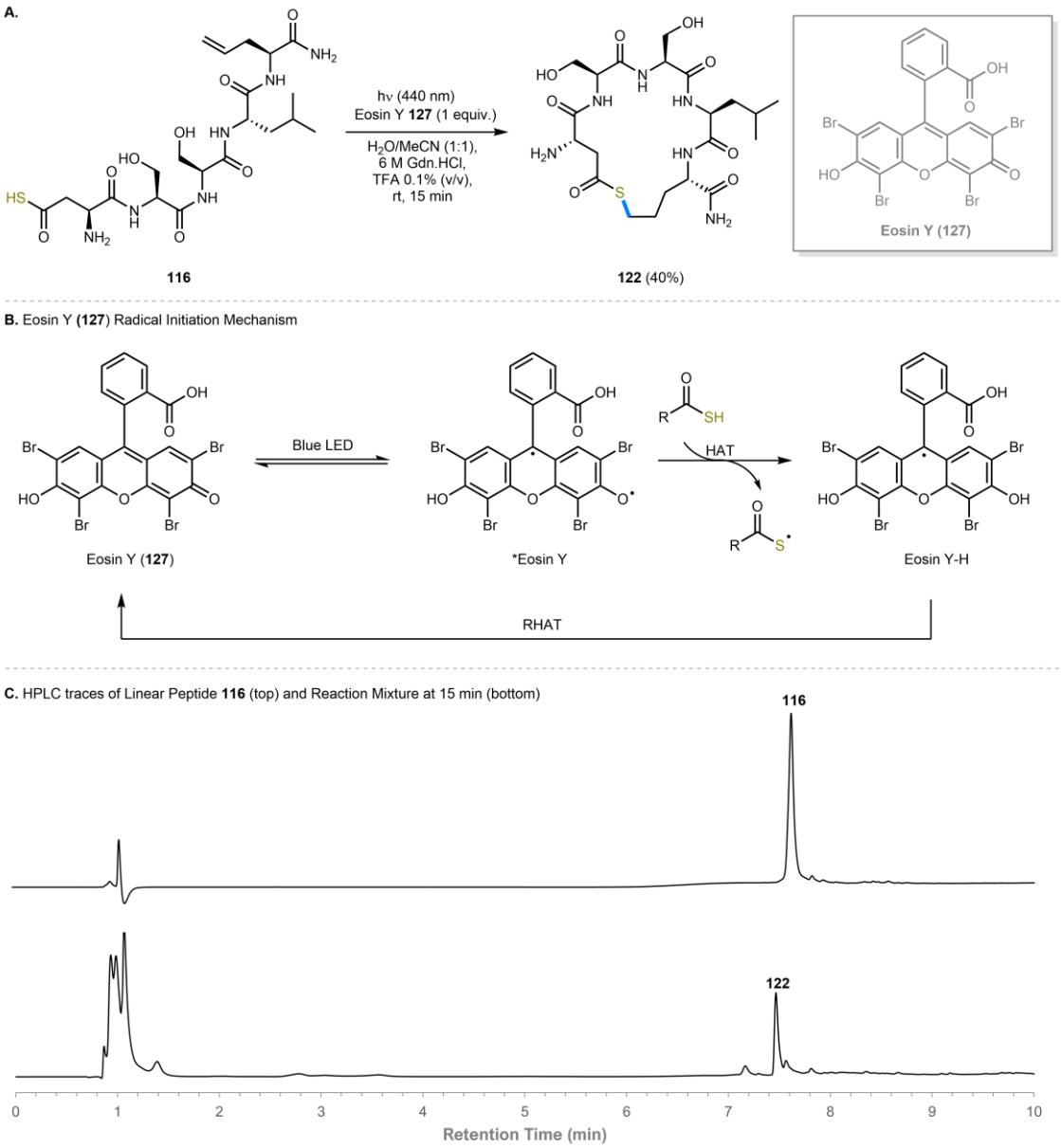


Figure 3.10 (a) Acyl thiol-ene thiolactonisation of **116** under blue LED irradiation using Eosin Y **127**. Isolated yield after semi-preperative purification reported. Gdn.HCl = guanidinium chloride. **(b)** Radical initiation mechanism of Eosin Y. **(c)** Comparison of the RP-HPLC traces (220 nm) of the linear peptide **116** and the reaction mixture at 15 min showing formation of thiolactone **122**.

3.4 Conclusions and Future Work

This chapter summarises the development of a photochemical acyl thiol-ene methodology for the synthesis of macrocyclic peptide thiolactones.[253] The linear peptides incorporating both an allylglycine (alkene) and γ-thioaspartic acid (thioacid) residues were synthesised *via* Fmoc SPPS in good to excellent yields (42-95%) and at least 80% purity. These peptides were cyclised using UV irradiation (354 nm) in optimised conditions consisting of a solvent mixture of 1:1 H_2O/MeCN containing 0.1% (v/v) TFA and the photoinitiator DPAP for 15

mins. The cyclisation reaction was highly efficient, with conversions measured by analytical RP-HPLC typically >80%. Thiolactones bearing a variety of functional groups, including analogues of AIPs, with ring sizes ranging from 16-22 atoms were synthesised and isolated using semi-preparative RP-HPLC in moderate to good yields (22-61%). We identified one peptide which was not reactive under the optimised conditions, but addition of the chaotropic agent 6 M guanidinium chloride resulted in excellent conversion to the thiolactone.

For some of the thiolactones, an inseparable impurity corresponding to the γ-thioaspartic acid deletion peptide carried through from the SPPS of the linear peptide was identified. Attempts to selectively remove the impurity by solid-phase extraction prior to RP-HPLC purification using thiol-functionalised silica was unsuccessful. The conditions resulted in removal of all peptides from solution including the desired product, attributed to adsorption of the peptides to the surface of the silica. Future work on the solid-phase extraction protocol should focus on the use of non-polar polystyrene thiol functionalised resins. Alternatively, the Fmoc SPPS of the linear peptides could be optimised for difficult sequences by incorporating microwave heating to increase the efficiency of γ-thioaspartic acid coupling to minimise the deletion side-product. The purification could also be conducted using chiral RP-HPLC columns to achieve better resolution between the impurities and the thiolactone.

The acyl thiol-ene thiolactonisation reaction was also conducted under blue LED (440 nm) irradiation using Eosin Y as a photosensitiser with comparable results to UV irradiation at the same reaction time. In this study, a stoichiometric amount of Eosin Y was used. Future work using blue LED irradiation should focus on identifying catalytic conditions for the cyclisation. The reaction could also be attempted using green and red LED irradiation to further widen the scope of the reaction.

In our studies, the γ-thioaspartic acid was restricted to the N-terminal of the linear peptide during Fmoc SPPS due to its propensity for based-catalysed aspartimide formation during Fmoc deprotection, which prevents chain elongation after its incorporation (discussed in **Chapter 2**, **Section 2.3.8**). However, the analogous glutamic acid variant, δ-thioglutamic acid, may be less prone to this side-reaction and could enable incorporation of the thioacid at internal positions of the peptide (**Figure 3.11A**). Furthermore, the alkene residue can be varied, including its position within the peptide. Recent work by Nolan *et al*. has demonstrated the modification of peptides using UV-initiated thiol-ene reaction at nanomole-scale in 1536-well plates.[254] Similarly, the chemoselectivity and fast kinetics of the photochemical acyl thiol-ene thiolactonisation reaction would be amenable to the rapid synthesis of peptide thiolactone libraries at ambient conditions in microplates. The peptide thiolactones could also serve as useful intermediates, for the synthesis of branched iso-peptides by NCL (**Figure 3.11B**) or the synthesis of peptide lactams *via* intramolecular S-to-N acyl transfer (**Figure 3.11C**).

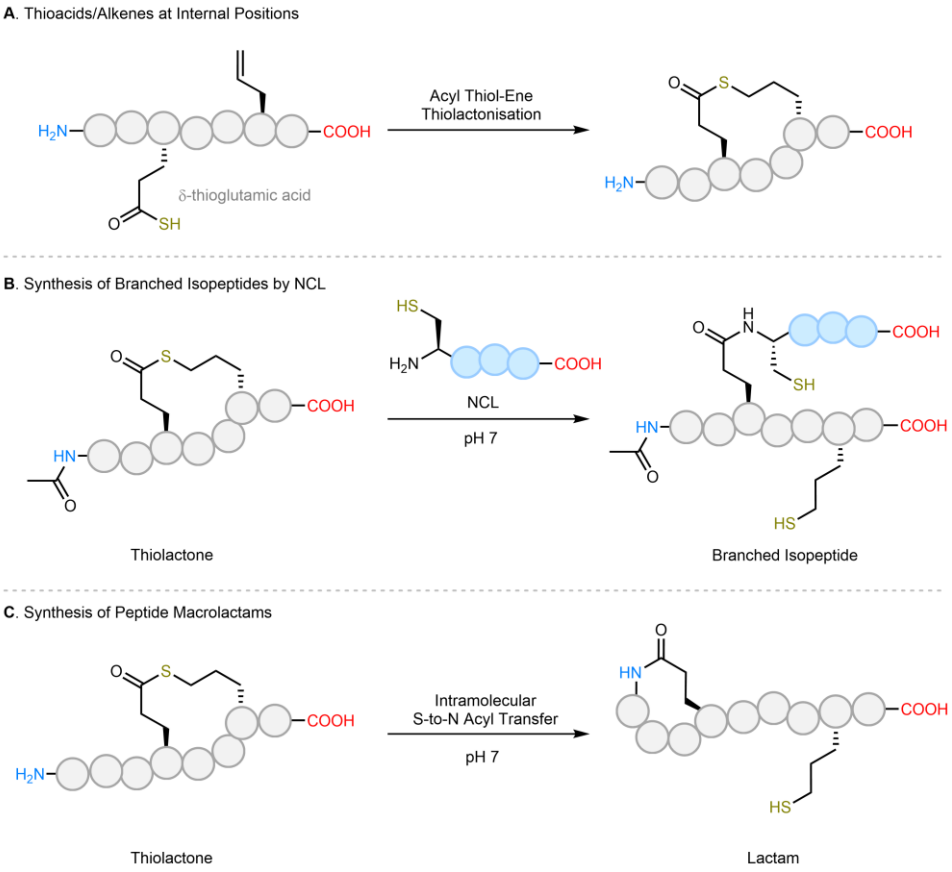


Figure 3.11 Future Work. **(a)** Incorporation of thioacid and alkenes at internal positions. **(b)** Synthesis of branched isopeptides from thiolactones by NCL. **(c)** Intramolecular S-to-N acyl transfer of thiolactones to form lactams.

Chapter 4

Dethiocarboxylation of Thioacids

Part of this work was conducted by Lorenzo Di Simo during his secondment to the Scanlan Group

4.1 Introduction

As discussed in **Chapter 1**, **Section 1.6**, thioacid radicals can undergo dethiocarboxylation *via* elimination of gaseous carbonyl sulfide (COS) to form carbon-centred radicals. During their investigation of photochemical acyl thiol-ene mediated peptide ligation detailed in **Section 1.6.1**, McLean *et al*. found that an excess of the α-thioacid substrate was required for non-Gly examples due to competing dethiocarboxylation to form *N*-alkylamide derivatives.[218] The authors hypothesised that due to the requirement for the formation of a high-energy primary radical intermediate, α-thioacids of Gly were not prone to dethiocarboxylation. This is corroborated by our own work detailed in **Chapter 3** since we did not observe dethiocarboxylation of γ-thioaspartic acid, which would also proceed *via* a primary radical intermediate.

To the best of our knowledge, only one further example of radical-mediated dethiocarboxylation exists in the literature, whereby Shimizu *et al*. demonstrated the thermally induced radical dethiocarboxylation of C-terminal thioacid peptides using the water-soluble initiator VA-044 to furnish *N*-alkylamide peptides (**Figure 4.1A**).[186] In both of the aforementioned reports, the intermediate C(sp^3) radical was simply reduced by Hydrogen Atom Transfer (HAT) to form *N*-alkylamines or *N*-alkylamides. However, the C(sp^3) radical could potentially be intercepted by other reactive functional groups to form a diverse range of products, akin to the well-established Barton decarboxylation.[255] Of particular interest to peptide chemistry is the interception of the carbon-centred radical *via* an intramolecular reaction with alkenes, enabling C(sp^3)-C(sp^3) bond formation to form robust, hydrocarbon linked macrocyclic peptides (**Figure 4.1B**). Recently, Smith *et al*. has demonstrated a similar cyclisation protocol whereby a C(sp^3) radical formed *via* desulfurisation of Cys is intercepted in an intramolecular fashion to form hydrocarbon linked macrocyclic peptides.[256]

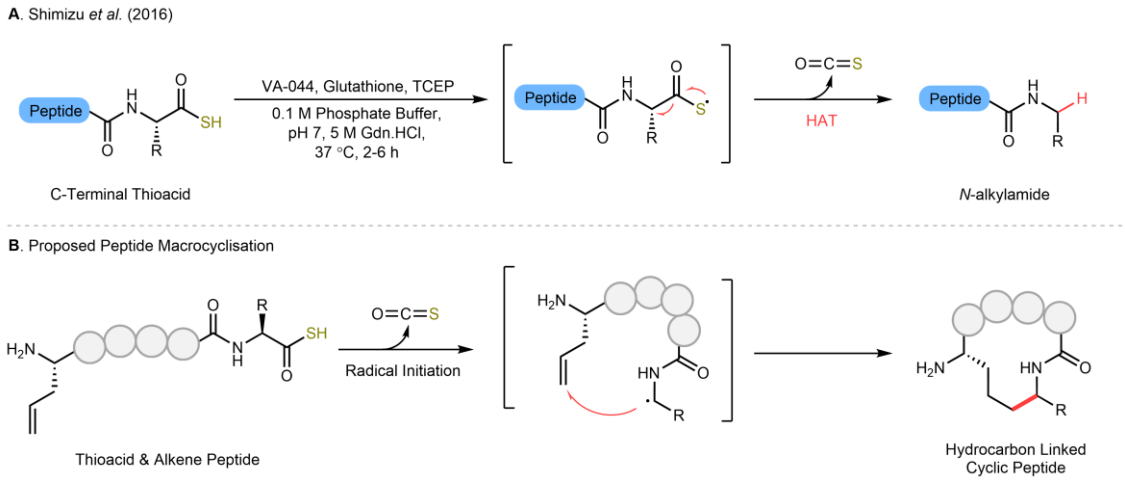


Figure 4.1 (a) Dethiocarboxylation of C-terminal thiacid peptides to form N-alkylamide peptides reported by Shimizu *et al*.[186] **(b)** Intramolecular interception of the carbon-centred radical intermediate to form hydrocarbon-linked cyclic peptides.

4.2 Aims

The aim of this project was to develop mild, metal-free, photochemical conditions for the dethiocarboxylation of amino acids, and explore the influence of various amino acid side chains on the reaction. To date, the study of the radical-mediated dethiocarboxylation reaction has been limited to thermal radical initiation.[186] Subsequently, we planned to synthesise a model system containing a C-terminal thioacid and an alkene to study whether the intermediate C(sp^3) radical formed during radical-mediated dethiocarboxylation could be trapped to form hydrocarbon-linked macrocycles. If the model proved to be successful, we hoped to extend the reaction to peptides to form hydrocarbon-linked cyclic peptides as depicted in **Figure 4.1B**.

4.3 Results and Discussion

4.3.1 Optimisation of Dethiocarboxylation Reaction

In order to simplify the analysis during the initial optimisation of the dethiocarboxylation reaction, a simple N^{α}-Fmoc thioacid derivative of Phe which could be easily synthesised by solution phase chemistry on large scale was selected as a model substrate. Thus, Steglich thioesterification of Fmoc-Phe-OH **128** furnished Fmoc-Phe-STrt **129** in 87% yield. Upon treatment of **129** with 25% (v/v) TFA/DCM for 5 min, the desired C-terminal thioacid, Fmoc-Phe-SH **130**, was formed in quantitative yield (**Figure 4.1A**). Comparison of the ^{13}C-NMR spectra confirmed quantitative deprotection, with clear disappearance of the thioester carbonyl of **129** at δ = 197.2 ppm and the appearance of the thioacid carbonyl of **130** at δ = 199.3 ppm.

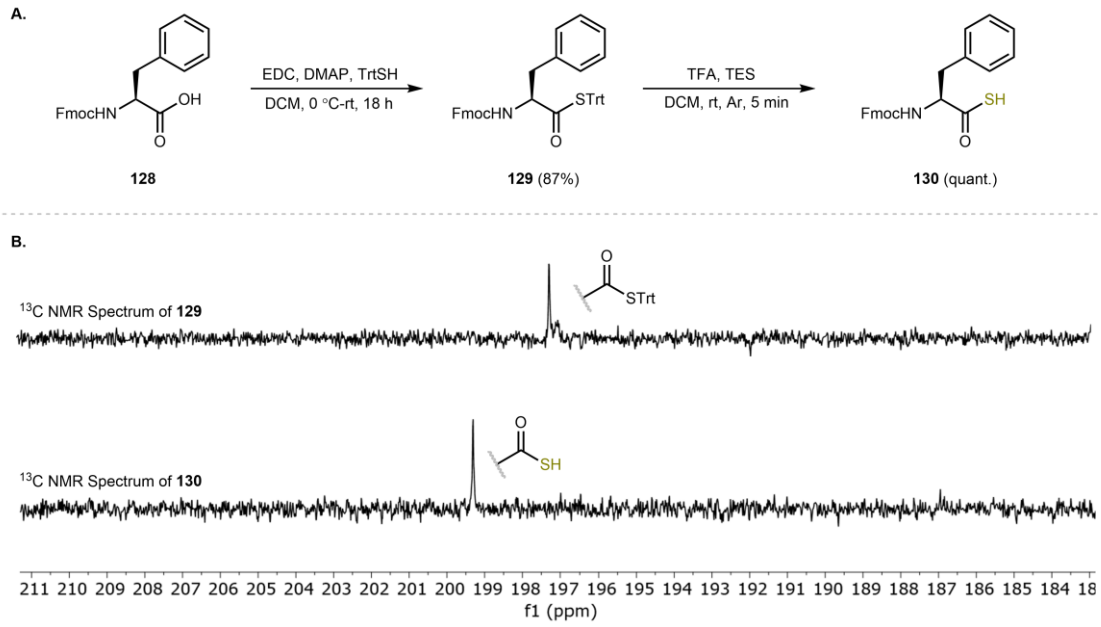
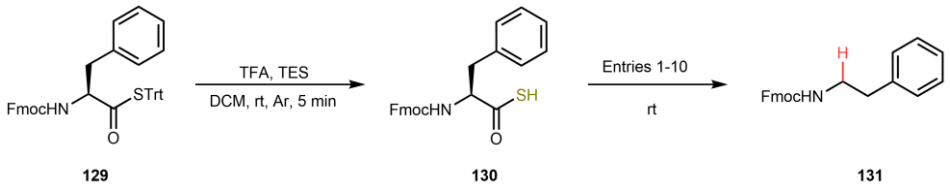


Figure 4.1 (a) Synthesis of Fmoc-Phe-SH **130**. **(b)** Comparison of the ^{13}C-NMR spectra (101 MHz, DMSO-d_6) of the thioester **129** (top) and thioacid **130** (bottom) showing the shift in the thiocarbonyl.

Thereafter, conditions for the dethiocarboxylation of Fmoc-Phe-SH **130** to form *N*-Fmoc-protected phenethylamine **131** were screened (**Table 4.1**). High yields of the dethiocarboxylated product **131** were achieved under both UV light irradiation (354 nm) using 2,2-dimethoxy-2-phenylacetophenone **109** (DPAP) as a photoinitiator (**Entries 1–3**) and blue LED light irradiation (440 nm) with Eosin Y **127** as a photoredox catalyst (**Entries 4–7**).

Table 4.1 Optimisation of dethiocarboxylation reaction conditions on Fmoc-Phe-SH **130**.



129 **130** **131**

Entry	Time	Solvent	Photoinitiator &	Light Source	Yield[a]
1	5 min	EtOAc	DPAP (20 mol%.)	UV	84%
2	15 min	EtOAc	DPAP (20 mol%)	UV	96%
3	60 min	EtOAc	DPAP (20 mol%)	UV	90%
4	15 min	EtOAc	Eosin Y (25 mol%.)	Blue LED	85%
5	60 min	EtOAc	Eosin Y (25 mol%.)	Blue LED	97% (72%[b])
6	60 min	MeCN	Eosin Y (25 mol%.)	Blue LED	80%
7	60 min	EtOAc	Eosin Y (1 mol%)	Blue LED	72%
8	60 min	EtOAc	No Initiator	Blue LED	6%
9	60 min	EtOAc	Eosin Y (0.25 equiv.)	Dark	nr
10	60 min	EtOAc	Eosin Y (0.25 equiv.), TEMPO (2 equiv.)	Blue LED	nr

[a]Yield determined by quantitative ^{1}H NMR using 1,3,5-trimethoxybenzene as an internal standard, with the yield reported being the overall yield of STrt deprotection and dethiocarboxylation. [b]Isolated Yield. UV = 354 nm, Blue LED = 440 nm. Nr = No reaction. Reactions were conducted with 100 mM concentration of **130**.

Under UV irradiation, a yield of 84% was obtained after just 5 min with 20 mol% of DPAP in EtOAc (**Entry 1**). Increasing the reaction time to 15 min resulted in a near quantitative yield of 96% (**Entry 2**), which dropped slightly to 90% with 1 h reaction time suggesting photodegradation of the product with prolonged UV exposure (**Entry 3**). Using blue LED light, a yield of 85% was obtained with 25 mol% of Eosin Y in EtOAc in 15 min (**Entry 4**). Under the same conditions for an extended reaction time of 1 h, the NMR yield increased to 97% and the dethiocarboxylated product **131** was obtained in 72% isolated yield after purification (**Entry 5**). Since both light sources yielded comparable results, we chose to focus on blue LED irradiation as it is a milder initial energy source, minimising the risk of product photodegradation.

To further explore the reaction conditions, an attempt was made with a catalytic amount of Eosin Y (1 mol%) which gave a satisfactory yield of 72% in 1 h (**Entry 7**). Conducting the reaction in the absence of Eosin Y resulted in only a negligible yield of 6% (**Entry 8**), with the low conversion likely arising from the formation of thiyl radicals from molecular O_2-derived free radicals in solution.[178] When the reaction was conducted with Eosin Y (25 mol%) in the dark, no

reaction was observed (**Entry 9**). Finally, the radical nature of the dethiocarboxylation reaction was confirmed by addition of 2 equiv. of the stable radical TEMPO capable of capturing radical intermediates to the reaction mixture, resulting in no reaction (**Entry 10**). In summary, the optimal conditions were identified as: 100 mM thioacid, 25 mM Eosin Y in EtOAc under blue LED (440 nm) irradiation at room temperature for 1 h.

The proposed mechanism for dethiocarboxylation based on the obtained results involves the abstraction of a proton from the thioacid (**A**) by the photoinitiator/photocatalyst to form an initial thioacid radical (**B**) (**Figure 4.2**). In the absence of functional groups that can trap the thiyl radical, spontaneous COS elimination occurs to form a C(sp^3) radical intermediate (**C**). This species can abstract a proton from another thioacid to furnish the dethiocarboxylated product (**D**) and another thioacid radical to propagate the reaction.

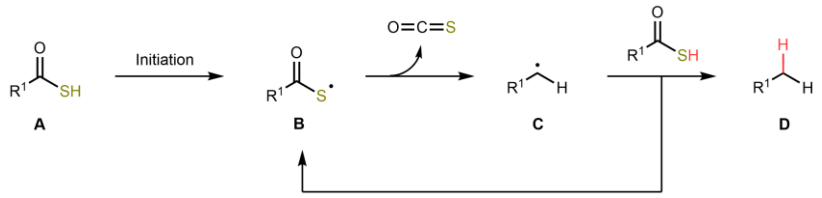
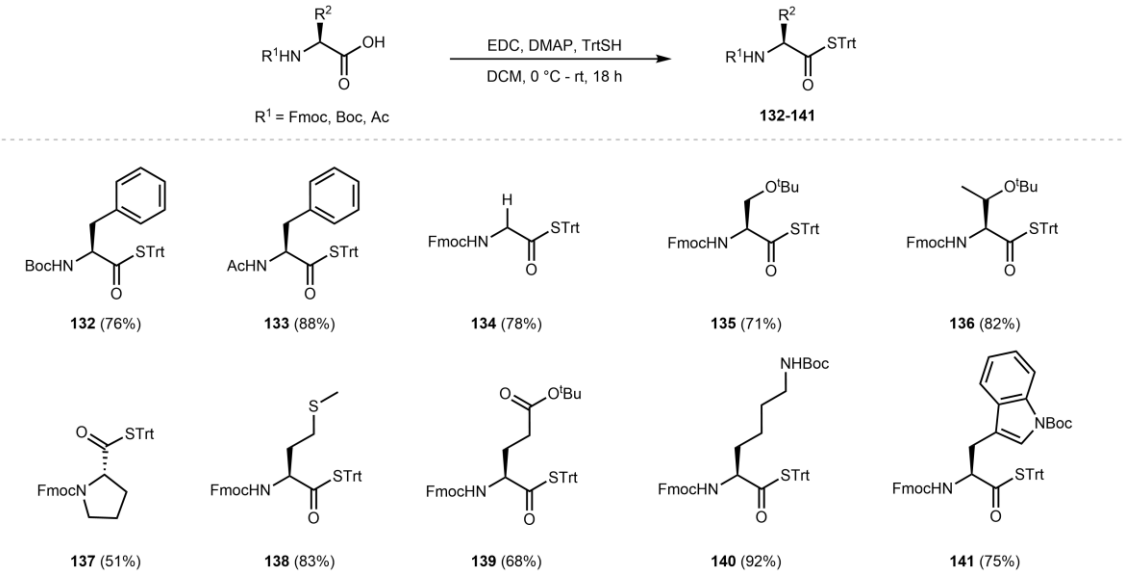


Figure 4.2. Proposed mechanism for the radical mediated dethiocarboxylation of thioacids.

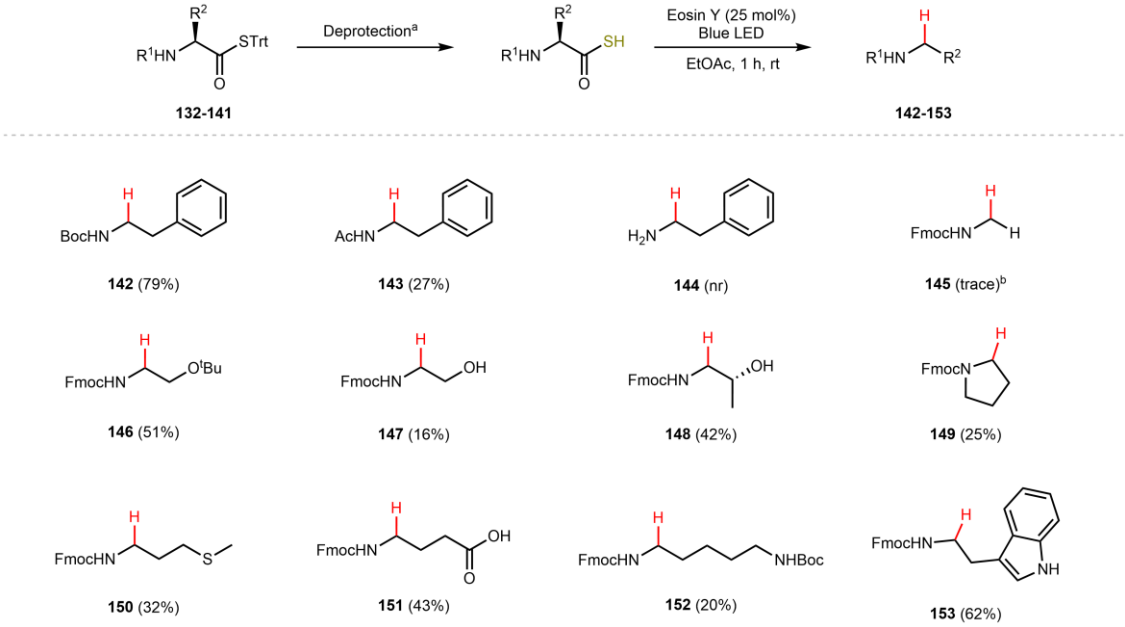
4.3.2 Scope of Dethiocarboxylation of AAs

With optimised conditions established, C-terminal STrt thioesters **132**–**141** of various AAs were synthesised in moderate to excellent yields (51–92%) using the standard Steglich thioesterification conditions (**Scheme 4.1**).



Scheme 4.1. Isolated yields of C-terminal STrt thioesters.

The STrt thioesters **132**–**141** were then deprotected with TFA, with the concentration of TFA dependant on whether concomitant sidechain deprotection was also needed to produce the desired thioacids (see **Chapter 5, Section 5.4** for details). Following deprotection, the thioacids were concentrated *in vacuo* and subjected to the optimised dethiocarboxylation conditions without further purification (**Scheme 4.2**).



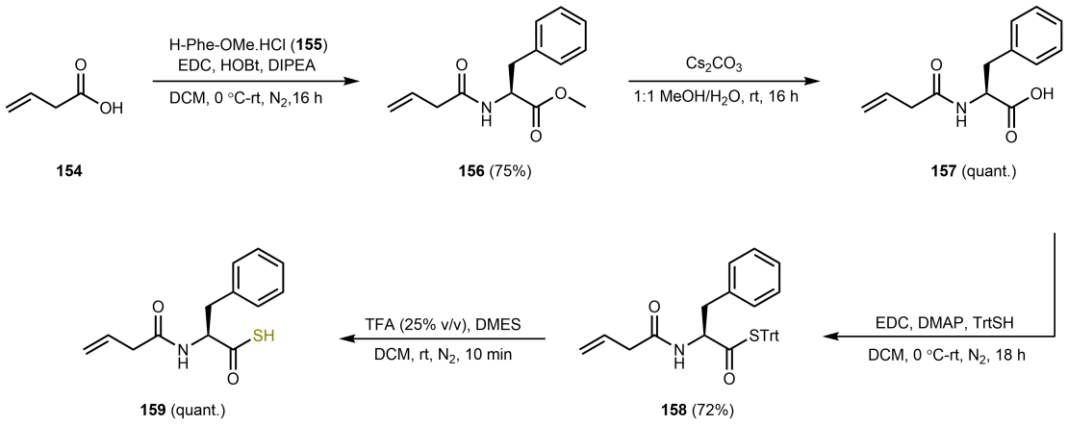
Scheme 4.2. Isolated yields of dethiocarboxylated products. [a]Deprotection conditions for each compound is outlined in **Chapter 5, Section 5.4**. [b]Yield was determined by analysis of the crude ^{1}H NMR (400 MHz, DMSO-d_6) using triphenylmethane formed during STrt deprotection as an internal reference. Nr = No reaction.

High isolated yields of *N*-Boc phenethylamine **142** (79%) were obtained by the dethiocarboxylation of the corresponding Phe α-thioacid. Along with the 72% isolated yield obtained for the Fmoc derivative **131** previously, the results demonstrate the compatibility of the process towards the most prevalent α-amino protecting groups used for AAs. However, the *N*-Ac derivative **143** was obtained in a poor yield of 27%, primarily due to an unidentified side-reaction during the deprotection of Ac-Phe-STrt **133**. The free α-amino derivative **144** did not undergo reaction. Since the dethiocarboxylation reaction requires a protonated thioacid ($pK_a \approx 3$) for proton abstraction to form the thiyl radical, the α-amino group ($pK_a \approx 9$) must be in the protonated alkylammonium ($R\text{-}NH_4^+$) form under these conditions. The strongly electron withdrawing ammonium group could destabilise the adjacent carbon-centred radical that would form upon COS elimination, thus preventing dethiocarboxylation. Comparable findings have been reported by Nolan *et al*., where an α-amino group adjacent to the intermediate carbon-centred radical was demonstrated to inhibit thiol-ene reaction on vinylglycine peptide substrates.[243]

The dethiocarboxylated Gly derivative **145** was only detected in trace amounts in the ^{1}H NMR spectrum of the crude reaction mixture. The low rate of dethiocarboxylation can be rationalised by the relatively high-energy primary radical intermediate that must formed. The fully protected Ser derivative **146** was isolated in a moderate yield of 51%. Deprotection of the side chain to the hydroxy group **147** resulted in a notable drop in yield to 16%, which increased to 42% with the more substituted Thr derivative **148**. *N*-alkyl derivatives of Pro **149**, Met **150**, Glu **151**, Lys **152** and Trp **153** were isolated in low to moderate yields. In the case of **146**–**153**, incomplete consumption of the corresponding α-thioacids were observed by TLC, indicating that the yields could be increased with longer reaction times. Based on these results, no obvious general trends in reactivity were noted. Although the isolated yields obtained were generally moderate, we were confident that the dethiocarboxylation method was broadly compatible with AA substrates and wanted to proceed to investigate if the intermediate C(sp^3) radical could be trapped.

4.3.3 Synthesis of All-Phe-SH 159

For the initial investigation, we utilised a model Phe α-thioacid derivative containing an allyl moiety, since Phe derivatives were previously shown to readily undergo dethiocarboxylation under the optimised conditions. The synthesis of the model substrate began with the amide coupling of 3-butenoic acid **154** with H-Phe-OMe.HCl **155** using EDC/HOBt to form the allylated derivative, All-Phe-OMe **156**, in 75% yield (**Scheme 4.3**). The C-terminal methyl ester was then hydrolysed using Cs_2CO_3 in a solvent mixture of 1:1 MeOH/H_2O to quantitatively form the corresponding C-terminal carboxylic acid, All-Phe-OH **157**. Steglich thioesterification furnished the C-terminal STrt thioester, All-Phe-STrt **158**, in 72% yield. Deprotection of the thioester with 25% (v/v) TFA and 5% (v/v) DMES as a cation scavenger for 10 min quantitatively furnished the desired thioacid, All-Phe-SH **159**. In this model system, the intermediate C(sp^3) radical formed after dethiocarboxylation is primed for cyclisation *via* either a 5- or 6-membered transition state, both of which should be highly favoured kinetically under Baldwin's rules for cyclisation.[257]



Scheme 4.3. Synthesis of the model thioacid, All-Phe-SH **159**.

4.3.4 Intramolecular Trapping

For successful trapping of the intermediate C(sp^3) radical, there are several considerations. Firstly, the reaction must be conducted at high dilution to prevent intermolecular acyl thiol-ene (see **Chapter 1, Section 1.6.1**). Secondly, the rate of dethiocarboxylation must be significantly faster than the rate of intramolecular acyl thiol-ene to prevent the formation of cyclic thioesters (thiolactones). Finally, once dethiocarboxylation has occurred, the rate of intramolecular cyclisation of the C(sp^3) radical with the alkene must be favoured over reduction of the radical by HAT to form *N*-alkylamide products. To address some of these concerns, we decided to conduct the reaction using UV irradiation as dethiocarboxylation was shown to be faster under these conditions compared to blue LED irradiation (**see Table 4.1**). Thus, All-Phe-SH **159** was dissolved in EtOAc at 5 mM concentration with 0.2 equiv. of the photoinitiatior DPAP and stirred under UV irradiation for 15 min (**Figure 4.3A**). After concentration *in vacuo*, the crude reaction mixture was analysed by ^{1}H NMR which showed >80% consumption of the alkene and the formation of a complex mixture (**Figure 4.3B**). In particular, the ^{1}H NMR spectrum showed no characteristic peaks corresponding to the potential cyclised products 5-benzyl-4-methylpyrrolidin-2-one **160** and 6-benzylpiperidin-2-one **161** as reported in the literature.[258, 259]

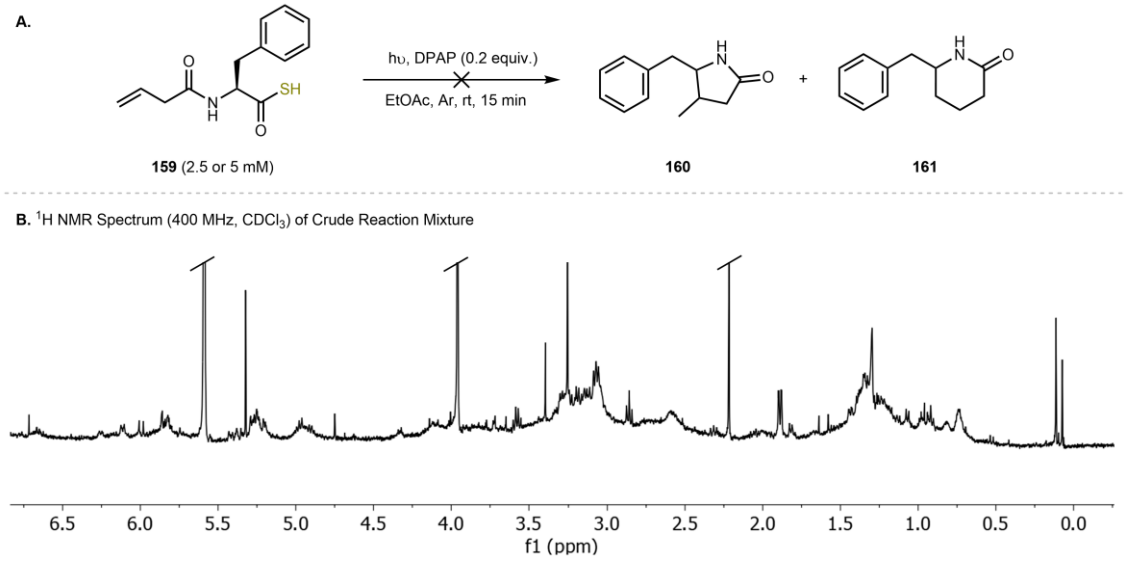


Figure 4.3. (a) Attempted dethiocarboxylation of thioacid **159**, followed by intramolecular interception of the resulting C(sp^3) radical to form cyclic products **160** and **161**. **(b)** ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of the crude reaction mixture showing the complex mixture that was formed.

Based on these results, we proposed that intramolecular acyl thiol-ene, which was shown to be highly efficient for the synthesis of macrocyclic thiolactones in the previous chapter (see **Chapter 3**, **Section 3.3.5**), was the predominant reaction. To discount intermolecular acyl thiol-ene, the reaction was repeated with 2.5 mM concentration of the thioacid **159**, but a similar result was obtained. In the similar reaction process reported by Smith *et al*. whereby a C(sp^3) radical

formed by the desulfurisation of Cys is intercepted by an alkene, the equilibrium is shifted strongly towards the formation of the C(sp^3) radical by the addition of a large excess of TCEP (**Figure 4.4A**).[260] Therefore, the risk of the initial Cys thiyl radical undergoing intramolecular thiol-ene is minimised. In our case, the equilibrium appears to favour the thioacid thiyl radical rather than dethiocarboxylation to form the C(sp^3) radical, resulting in intramolecular acyl thiol-ene reactivity (**Figure 4.4B**). Therefore, extensive work will be needed to develop conditions where dethiocarboxylation can be induced almost immediately after thioacid radical formation to prevent intramolecular acyl thiol-ene from becoming the dominant reaction. Having said that, the rate of dethiocarboxylation is dependant primarily on the nature of the substrate and it may be the case that intramolecular acyl thiol-ene would always be favoured over dethiocarboxylation except in exceptional circumstances.

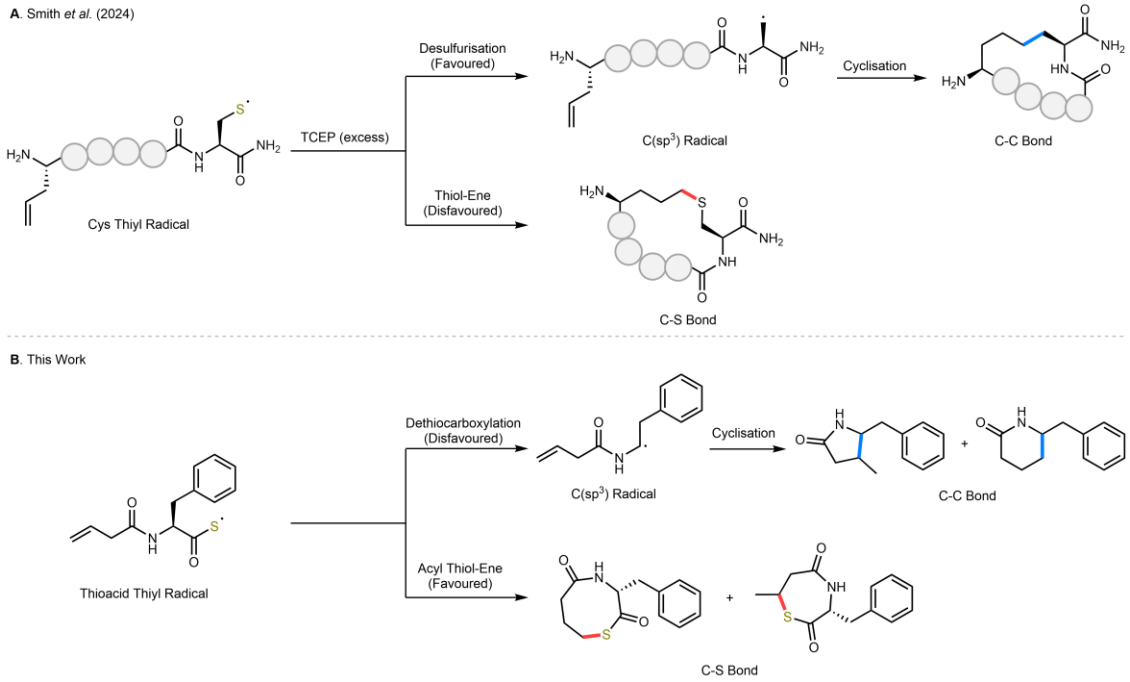


Figure 4.4. (a) The work of Smith *et al*.[260] Excess of TCEP favours desulfurisation of Cys thiyl radicals to form the C(sp^3) radical which prevents intramolecular thiol-ene reactivity. **(b)** In this work, dethiocarboxylation of the thioacid thiyl radical is disfavoured resulting in intramolecular acyl thiol-ene reactivity.

4.4 Conclusions and Future Work

This chapter details the exploration of the dethiocarboxylation of C-terminal thioacids *via* the elimination of carbonyl sulfide (COS) under photochemical conditions. Mild, metal-free conditions for the dethiocarboxylation of a model AA substrate was optimised resulting in almost quantitative (>95%) conversion to the *N*-alkylamine derivative. The reaction was highly efficient with both UV irradiation with DPAP initiation and blue LED irradiation using Eosin Y as a photocatalyst.

Subsequently, C-terminal thioacids of various AAs were synthesised and subjected to the optimised dethiocarboxylation conditions, and generally low to moderate isolated yields (16-79%) of the corresponding N-alkylamines were obtained. Importantly, it was shown that strong electron withdrawing groups such as an alkylammonium ($R\text{-}NH_3^+$) adjacent to the thioacid can prevent dethiocarboxylation. Furthermore, the Gly thioacid which would undergo dethiocarboxylation *via* a relatively high-energy primary C(sp^3) radical only exhibited trace conversion under these conditions. Future work should focus on the expansion of the dethiocarboxylation reaction to more complex peptide substrates and non-peptide substrates. Ultimately, conversion of carboxylic acids to thioacids followed by dethiocarboxylation could serve as a mild, highly selective and metal-free method for the reduction of carboxylic acids to alkanes alongside established reactions such as the Barton-McCombie deoxygenation[255] or catalytic hydrogenation.[261]

We also investigated if the intermediate C(sp^3) radical formed after COS elimination could be trapped by an intramolecular addition onto an alkene to form a C(sp^3)- C(sp^3) bond. However, preliminary results indicated that intramolecular acyl thiol-ene was the predominant reaction, but further investigation was limited by time constraints. Extensive additional work is needed to determine if conditions can be identified to kinetically-fast induce dethiocarboxylation immediately after thioacid radial formation to form the required C(sp^3) radical, thereby eliminating the undesired side reaction between the thioacid radical and the alkene.

Chapter 5

Experimental

5.1 General Experimental Details

All commercially available chemicals were purchased from Merck KGaA, Fluorochem Ltd or Tokyo Chemical Industry (TCI) and used without further purification unless otherwise stated. Solvents for synthesis and purification were supplied by Sigma-Aldrich at HPLC grade. Dry solvents were obtained from a PureSolv MD-4EN solvent purification system. Room-temperature (rt) is classified in this work as 18 – 21 °C. Thin Layer Chromatography (TLC) was performed using Merck 60 F254 silica gel plates (pre-coated, 0.2 mm thick) and visualised by UV illumination (254 nm), ninhydrin staining (1.5 g ninhydrin, 3 mL acetic acid in 100 mL n-butanol), permanganate staining (1.6 g potassium permanganate, 10 g potassium carbonate and 1.25 mL 10% NaOH in 200 mL H_2O) or molybdenum staining (5.0 g ammonium molybdate, 5.3 mL concentrated H_2SO_4 in 100 mL H_2O). Melting points are uncorrected and were measured with a Stuart SP-10 melting point apparatus. Infrared spectra (IR) were recorded on a Perkin Elmer Spectrum 100 FT-IR spectrometer. All UV reactions were carried out in a Luzchem photoreactor, LZC-EDU (110 V/60 Hz), containing 14 UVA lamps centred at 354 nm. All blue LED reactions were carried out using 2 Kessil PR160-440 LED lamps centered at 440 nm. Solid Phase Peptide Synthesis (SPPS) was performed manually in polypropylene syringe reaction vessels (10 mL; Torviq, MI, USA) fitted with a polystyrene frit. All reactions were performed at room temperature under continuous agitation on an LBX Orb-B2 shaker set to 180 rpm.

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra were recorded using Bruker DPX 400 (400.13 MHz for ^{1}H NMR and 100.61 MHz for ^{13}C NMR), Bruker AV 400 (400.13 MHz for ^{1}H NMR and 100.61 MHz for ^{13}C NMR), Agilent MR400 (400.13 MHz for ^{1}H NMR and 100.61 MHz for ^{13}C NMR) or Bruker AV 600 (600.13 MHz for ^{1}H NMR and 150.90 MHz for ^{13}C NMR) instruments. Deuterated solvents for NMR were purchased from Sigma Aldrich (Merck). Chemical shifts, δ, are in ppm and referenced to the internal solvent signals for chloroform (δH 7.26 and δC 77.0) or DMSO (δH 7.26 and δC 39.7). NMR descriptions: s, singlet; d, doublet; t, triplet; m, multiplet; br, broad. Coupling constants, J, are reported in Hertz (Hz). NMR data was processed using MestReNova software (version 14.1.1-24571) by MestReNova Lab ReseAr Ch S. L.

High Resolution Mass Spectrometry (HRMS)

ESI mass spectra were acquired using a Bruker micrOTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC in positive and negative modes as required. Agilent ESI-L Low Concentration Tuning Mix was used to calibrate the system, and this was also used as an internal lock mass. Masses were recorded over the range 100 – 2000 *m/z*. Operating conditions were as follows: end-plate offset 500 V, capillary 4500 V, nebulizer 2.0 bar, dry gas 8.0 L min^{-1}, and dry temperature 180 °C. APCI experiments were carried out on a Bruker micrOTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC or direct insertion probe in positive or negative mode as required. Agilent APCI-TOF Tuning Mix was used to calibrate the system.

Masses were recorded over a range of 100–2000 *m/z*. Operating conditions were as follows: capillary voltage 4000 V, corona 4000 nA, nebulizer gas 2.0 bar, dry gas 3.0 L min^{-1}, dry gas temperature 100–200 °C, vap. temperature 100–400 °C. Compass HyStar version 3.2 and micrOTOF Control version 3.2 software were used to analyse the data.

High-performance Liquid Chromatography (HPLC)

HPLC was carried out using a Shimadzu Nexera lite Low Pressure Gradient system. For analytical HPLC, an Ascentis® Express 90 Å C18 100 mm x 4.6 mm, 5 µm column with a flow rate of 1.0 mL min^{-1} was used. For semi-preparative HPLC purification, an Ascentis® C18 250 mm x 10 mm, 5 µm column with a flow rate of 5 mL min^{-1} was used. Gradient elution with Solvent A (0.1% TFA in H_2O) and Solvent B (0.1% TFA in MeCN) was used for both analytical and semi-preparative HPLC. Eluting compounds were detected with an SPD-M40 PDA detector at 220 or 254 nm.

5.2 Experimental Details for Chapter 2

5.2.1 General Procedures

Procedure 2A: *S*-Trityl (STrt) Deprotection

To a solution of STrt thioester (1 equiv.) dissolved in DCM (25 µL) was added TFA (450 µL) and DMES (25 µL) and stirred at rt for 15 min. The reaction mixture was concentrated to dryness under a stream of N_2 and ice-cold diethyl ether was added to the residue to form a suspension. The suspension was centrifuged at 6000 rpm for 2 min and the solvent was decanted. This procedure was repeated twice, and the pellet was then dried under a stream of N_2 to give the desired thioacid as a solid which was used without further purification.

Procedure 2B: Ligation in DMF

Following *S*-trityl deprotection as per General Procedure A, the thioacid (100mM) was dissolved in DMF with DIPEA (1.2 M) and added to the thioester (25 mM). The solution was stirred at rt under argon for 0.5-1 h. To monitor the reaction by analytical HPLC, a 100 µL aliquot of the reaction mixture was taken at the required time point and diluted with 1 mL of 1:1 H_2O/MeCN + 0.1% TFA. The analytical method consisted of a gradient of 5-95% MeCN/H_2O + 0.1% TFA over 10 min.

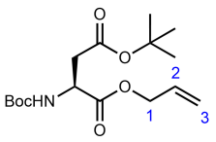
Procedure 2C: Ligation in Buffer

Peptide thioester (1.5 mg, 10 mM) and peptide thioacid (4.4 mg, 40 mM) were dissolved in 0.2 M phosphate buffer containing 6 M guanidinium chloride adjusted to pH 7. The mixture was stirred at rt under argon for 1 h. To monitor the reaction by analytical HPLC, a 100 µL aliquot of the reaction mixture was taken at the required time point and diluted with 100 µL of 1:1

H_2O/MeCN + 0.1% TFA. The analytical method consisted of a gradient of 5-95% MeCN/H_2O + 0.1% TFA over 10 min.

5.2.2 Characterisation Data

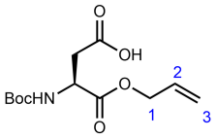
1-allyl 4-(tert-butyl) (tert-butoxycarbonyl)-L-aspartate (63)



To a solution of Boc-Asp(O*t*Bu)-OH **62** (5.00 g, 17.3 mmol) in MeCN (50 mL) was added DIPEA (4.6 mL, 25.8 mmol) and allyl bromide (2.30 mL, 25.8 mmol) and the solution was stirred at rt for 19 h. The reaction mixture was concentrated *in vacuo*, diluted with H_2O (150 mL) and extracted with EtOAc (2 x 150 mL). The organic extract was washed with brine (2 x 150 mL), dried over $MgSO_4$ and the solvent was evaporated *in vacuo* to give the product as a yellow oil (5.30 g, 93%). The isolated product was in good agreement with literature.[262]

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.94 – 5.81 (m, 1H, H2), 5.47 (d, *J* = 8.8 Hz, 1H, NH), 5.30 (dd, *J* = 17.2, 1.0 Hz, 1H, H3), 5.21 (dd, *J* = 10.3, 1.0 Hz, 1H, H3), 4.68 – 4.56 (m, 2H, H1), 4.55 – 5.48 (m, 1H, Asp αCH), 2.88 (dd, *J* = 16.8, 4.7 Hz, 1H, Asp βCH_2), 2.70 (dd, *J* = 16.7, 4.7 Hz, 1H, Asp βCH_2), 1.43 (s, 9H, *t*Bu), 1.41 (s, 9H, *t*Bu). **HRMS** APCI *(m/z)*: $[M+H]^+$ calculated for $C_{16}H_{28}NO_6$ 330.1911; found 330.1920.

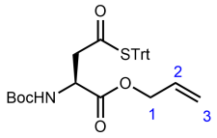
(S)-4-(allyloxy)-3-((tert-butoxycarbonyl)amino)-4-oxobutanoic acid (65)



To a solution of **63** (5.00 g, 7.60 mmol) suspended in DCM (2 mL) was added TFA (18 mL) and the mixture was stirred at rt for 2 h. The solvent was removed *in vacuo* and the resulting residue wad dried under high vacuum to give H-Asp-OAll.TFA **64** as a white solid which was used without further purification. To a solution of **64** (4.36 g, 15.2 mmol) in MeCN (40 mL) was added DIPEA (7.95 mL, 45.6 mmol) and di-*tert*-butyl dicarbonate (3.93 g, 18.2 mmol) and the mixture was stirred at rt for 2 h. The solvent was then removed *in vacuo* and the residue was diluted with 1 M $HCl_{(aq)}$ solution (100 mL) and extracted with EtOAc (2 x 50 mL). The combined organic extraxts were washed with brine (100 mL), dried over $MgSO_4$ and the solvent was evaporated *in vacuo* to give the product as a yellow oil (4.02 g, 97%). The isolated product was in good agreement with literature.[263]

^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.47 (brs, 1H, COOH), 5.95 – 5.83 (m, 1H, H2), 5.53 (d, *J* = 8.5 Hz, 1H, NH), 5.33 (dd, *J* = 17.2, 1.0 Hz, 1H, H3), 5.25 (dd, *J* = 10.6, 1.0 Hz, 1H, H3), 4.67 – 4.57 (m, 3H, Asp αCH, H1), 3.07 (dd, *J* = 17.4, 4.6 Hz, 1H, Asp βCH2), 2.88 (dd, *J* = 17.4, 4.6 Hz, 1H, Asp βCH_2), 1.45 (s, 9H, *t*Bu). **HRMS** APCI *(m/z)*: $[M-H]^-$ calculated for $C_{12}H_{18}NO_6$ 272.1140; found 272.1139.

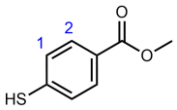
allyl (S)-2-((tert-butoxycarbonyl)amino)-4-oxo-4-(tritylthio)butanoate (66)



To a stirring solution of **65** (2.00 g, 7.32 mmol) in DCM (60 mL) at 0 °C was added EDC.HCl (2.10 g, 11.0 mmol) and the mixture was stirred for 5 min. Then DMAP (0.134 g, 1.10 mmol) and triphenylmethanethiol (2.02 g, 7.32 mmol) were added and the mixture was stirred at rt under nitrogen for 3 h. The reaction mixture was diluted with DCM (100 mL) and water (100 mL) and the organic layer was extracted, washed with brine (100 mL) and dried over $MgSO_4$. The solvent was then removed *in vacuo*. The residue was dry-loaded on Celite and purified by silica gel flash chromatography (0–20% (v/v) EtOAc/Hexane). Fractions containing pure product were combined and the solvent was evaporated *in vacuo* to give the product as a white foam (2.45 g, 63%).

R_f (30:70 EtOAc:Hex): 0.63. **m.p.**. 92–94 °C. **^{1}H-NMR** (400 MHz, DMSO-d_6): δ 7.34 – 7.25 (m, 10 H, Ph, NH), 7.15 – 7.10 (m, 6 H, Ph), 5.86 (ddt, *J* = 17.2, 10.3, 5.2 Hz, 1H, H2), 5.30 (dd, *J* = 17.2, 1.9 Hz, 1H, H3), 5.19 (dd, *J* = 10.4, 1.9 Hz, 1H, H3), 4.55 (d, *J* = 5.2 Hz, 2H, H1), 4.33 (m, 1 H, Asp αCH), 3.01 (dd, *J* = 15.8, 5.7 Hz, 1H, Asp βCH_2), 2.94 (dd, *J* = 15.8, 8.2 Hz, 1H, Asp βCH_2), 1.38 (s, 9H, *t*Bu). **^{13}C-NMR** (101 MHz, DMSO-d_6): δ 193.4 (SC=O), 170.7 (C=O), 155.3 (Boc C=O), 143.4 (Ar qC), 132.4 (C2), 129.5 (Ar C), 128.0 (Ar C), 127.4 (Ar C), 117.8 (C3), 78.7 (tBu qC), 70.1 (Asp αC), 65.3 (C1), 50.3 (Trt qC), 44.3 (Asp βC), 28.3 (*t*Bu CH_3). **HRMS ESI** *(m/z)*: $[M+Na]^+$ calculated for $C_{31}H_{33}NNaO_5S$ 554.1972; found 554.1972

methyl-4-mercaptobenzoate (68)

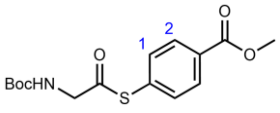


To a suspension of 4-mercaptobenzoic acid **67** (0.50 g, 3.2 mmol) in MeOH (6 mL) was added 96% H_2SO_4 (0.17 mL, 3.3 mmol) dropwise and the mixture was refluxed for 16 h. The reaction mixture was quenched with addition of saturated aqueous $NaHCO_3$ (20 mL). The resulting aqueous solution was acidified to pH 3 with dropwise addition of 1 M $HCl_{(aq)}$ solution and then extracted with EtOAc (3 x 15 mL). The combined organic extracts were dried over

$MgSO_4$ and the solvent was evaporated *in vacuo* to give the product as yellow crystals (0.36 g, 67%). The isolated compound was in good agreement with the literature.[264]

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.89 (m, 2H, H2), 7.28 (m, 2H, H1), 3.89 (s, 3H, OCH_3), 3.60 (s, 1H, SH). **HRMS** ESI *(m/z)*: $[M-H]^-$ calculated for $C_8H_7NO_2S$ 167.0172; found 167.0170.

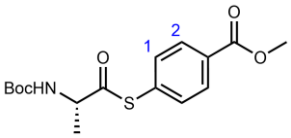
methyl-4-(((tert-butoxycarbonyl)glycyl)thio)benzoate (70)



To a stirring solution of Boc-Gly-OH **69** (0.310 g, 1.78 mmol) in DCM (4 mL) was added EDC.HCl (0.513 g, 2.68 mmol), DMAP (0.024 g, 0.20 mmol) and thiol **68** (0.300 g, 1.78 mmol). The mixture was stirred at rt under argon for 5 h. The reaction mixture was then diluted with H_2O (10 mL) and extracted with DCM (3 x 10 mL). The combined organic extracts were dried over $MgSO_4$ and the solvent was removed *in vacuo*. The residue was purified by silica gel flash chromatography (5–10% (v/v) EtOAc/Hexane). Fractions containing pure product were combined and the solvent evaporated *in vacuo* to give the product as a white powder (0.192 g, 33%).

R_f (30:70 EtOAc:Hex): 0.39. **^{1}H-NMR** (400 MHz, $CDCl_3$): δ 8.06 (d, *J* = 8.4 Hz, 2H, H2), 7.48 (d, *J* = 8.4 Hz, 2H, H1), 5.20 (t, *J* = 6.3 Hz, 1H, NH), 4.15 (d, *J* = 6.3 Hz, 2H, Gly αCH_2), 3.92 (s, 3H, OCH_3), 1.48 (s, 9H, *t*Bu). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 195.7 (SC=O), 166.4 (C=O), 155.5 (Boc C=O), 134.4 (C2), 132.6 (Ar qC), 131.0 (Ar qC), 130.2 (C1), 80.7 (*t*Bu qC), 52.4 (OCH_3), 50.4 (Gly αC), 28.3 (*t*Bu CH_3). **ν_{max} (film)/cm^{-1}**: 3328 (NH), 2978, 2953, 1695 (C=O), 1515, 1268, 1168, 1110, 857, 764. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{15}H_{19}NNaO_5S$ 348.0876; found 348.0877.

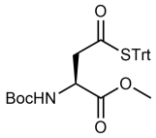
methyl 4-(((tert-butoxycarbonyl)-L-alanyl)thio)benzoate (72)



To a stirring solution of Boc-Ala-OH **71** (0.507 g, 2.68 mmol) in DCM (10 mL) was added EDC.HCl (0.770 g, 4.02 mmol), DMAP (0.077 g, 0.63 mmol) and thiol **68** (0.300 g, 1.78 mmol). The mixture was stirred at rt under argon for 5 h. The solvent was removed *in vacuo* and the residue was purified by silica gel flash chromatography (20% v/v EtOAc/Hexane). Fractions containing pure product were combined and the solvent evaporated *in vacuo* to give the product as a white powder (0.280 g, 31%).

R_f (30:70 EtOAc:Hex): 0.49. **^{1}H-NMR** (400 MHz, $CDCl_3$): δ 8.03 (d, *J* = 8.4 Hz, 2H, H2), 7.46 (d, *J* = 8.1 Hz, 2H, H1), 5.18 (d, *J* = 8.2 Hz, 1H, NH), 4.54 – 4.44 (m, 1H, Ala αCH), 3.90 (s, 3H, OCH_3), 1.47 (s, 9H, *t*Bu), 1.42 (d, *J* = 7.2 Hz, 3H, Ala βCH_3). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 199.3 (SC=O), 166.5 (C=O), 155.0 (Boc C=O), 134.3 (C2), 133.4 (Ar qC), 130.8 (Ar qC), 130.1 (C1), 80.6 (*t*Bu qC), 56.5 (Ala αC), 52.3 (OCH_3), 28.4 (*t*Bu CH_3), 18.4 (Ala βC). **ν_{max} (film)/cm^{-1}**: 3383 (NH), 2976, 1718 (C=O), 1688 (C=O), 1503, 1277, 1162, 764. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{16}H_{21}NNaO_5S$ 362.1038; found 362.1045.

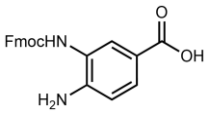
methyl (S)-2-((tert-butoxycarbonyl)amino)-4-oxo-4-(tritylthio)butanoate (76)



To a solution of Boc-Asp-OMe **75** (2.50 g, 10.1 mmol) in DCM (50 mL) was added EDC.HCl (2.90 g, 15.2 mmol) and the mixture was stirred at 0 ºC for 5 min. Then DMAP (0.186 g, 1.52 mmol) and triphenylmethanethiol (2.80 g, 10.1 mmol) were added and the mixture was stirred at rt under argon for 3 h. The reaction mixture was concentrated *in vacuo* and the residue was purified by silica gel flash chromatography (5–30% v/v EtOAc:Hexane). Fractions containing pure product were combined and the solvent evaporated was *in vacuo* to give the product as an off-white powder (3.11g, 61%).

R_f (30:70 EtOAc:Hex): 0.51**. ^{1}H-NMR** (400 MHz, $CDCl_3$): δ 7.32 – 7.18 (m, 15H, Trt), 5.27 (d, *J* = 8.7, 1H, NH), 4.45 (dt, *J* = 8.7, 4.8, 1H, Asp αCH), 3.66 (s, 3H, OCH_3), 3. 19 (dd, *J* = 16.2, 4.8 Hz, 1H, Asp βCH2), 3.03 (dd, *J* = 16.2, 4.8 Hz, 1H, Asp βCH_2), 1.43 (s, 9H, *t*Bu). **^{13}C-NMR** (100 MHz, $CDCl_3$): δ 195.1 (SC=O), 171.2 (C=O), 155.4 (Boc C=O), 143.4 (Ar qC), 129.9 (Trt Ar-C), 128.0 (Trt Ar C), 127.4 (Trt Ar C), 80.3 (*t*Bu qC), 71.3 (Asp αC), 52.8 (OCH_3), 50.7 (Trt qC), 45.0 (Asp βC), 28.4 (*t*Bu CH_3). **ν_{max} (film)/cm^{-1}**: 3328 (NH), 2978, 1696 (C=O), 1516, 1269, 1169, 1110, 1046, 764. **HRMS** APCI *(m/z)*: $[M+H]^+$ calculated for $C_{28}H_{31}NNaO_5S$ 528.1821; found 528.1820.

3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-aminobenzoic acid (85)

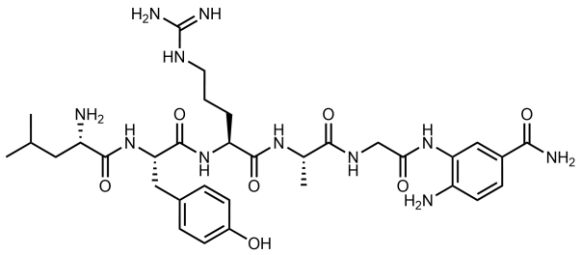


To a solution of 3,4-diaminobenzoic acid **84** (5.00 g, 33.0 mmol) dissolved in 0.1 M $NaHCO_{3(aq)}$/MeCN (1:1, 100 mL) was added N-(9-fluorenylmethyloxycarbonyloxy)succinimide (11.1 g, 33.0 mmol) in small portions over 30 min. After addition, the mixture was stirred for a further 4.5 h. The reaction mixture was then acidified with 1 M $HCl_{(aq)}$ and the resulting precipitate was filtered, washed with cold ether (250 mL), hexane (250 mL) and MeOH (250 mL). The solid

was dried under high vacuum to give the product as a pale wine-coloured solid (9.7 g, 79%). The isolated product was in good agreement with the literature.[126] **^{1}H-NMR** (400 MHz, DMSO-d_6): δ 12.12 (bs, 1H, COOH), 8.77 (bs, 1H, NH), 7.91 (m, 2H, ArH), 7.84 – 7.67 (m, 3H, ArH),7.50 (d, *J* = 7.9 Hz, 1H, ArH), 7.43 (t, *J* = 7.5 Hz, 2H, ArH), 7.38 – 7.29 (m, 2H, ArH), 6.71 (d, *J* = 8.4 Hz, 1H), 4.53 – 4.34 (m, 3H, Fmoc CH, CH_2), 3.44 (bs, NH_2). **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{22}H_{19}N_2O_4$ 375.1339; found 375.1341.

LYRAG-Dbz

4-amino-3-((5S,8S,11S,14S)-14-amino-8-(3-guanidinopropyl)-11-(4-hydroxybenzyl)-5,16-dimethyl-4,7,10,13-tetraoxo-3,6,9,12-tetraazaheptadecanamido)benzamide (86)



Rink amide aminomethyl resin 100-200 mesh (0.257 g, 0.2 mmol) was swollen in DMF (5 mL) for 20 min and then drained. The resin bound Fmoc group was removed using 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL) and the resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). Fmoc-Dbz-OH **85** (0.300g, 4 equiv.), HBTU (0.303 g, 4 equiv.), HOBt (0.108 g, 4 equiv.) and DIPEA (0.209 mL, 6 equiv.) were dissolved in DMF (0.2 M) and transferred immediately to the syringe containing the resin and agitated for 45 min. Excess reagents were drained and the resin was washed with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL). The Fmoc group was removed using 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL) and the resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). Fmoc-Gly-OH (0.238 g, 4 equiv.), HBTU (0.303 g, 4 equiv.), HOBt (0.108 g, 4 equiv.) and DIPEA (0.209 mL, 6 equiv.) were dissolved in DMF (0.2 M) and transferred immediately to the syringe containing the resin and agitated for 45 min. The Fmoc group was removed using 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL) and the resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). Subsequent coupling cycles consisted of **(i)** Coupling: AA (3 equiv.), PyBOP (0.312 g, 3 equiv.) and NMM (0.131 mL, 6 equiv.) in DMF (0.2 M) for 45 min; **(ii)** Wash: DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL); **(iii)** Fmoc Deprotection: 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL) and **(iv)** Wash: DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). The AAs used in order were Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH. Fmoc-Tyr(O^{t}Bu)-OH and Boc-Leu-OH. All AAs from Arg onwards were double coupled and Fmoc deprotection was not required after the coupling of the final residue.

Following assembly of the peptide sequence, the resin was washed with DCM (3 x 5 mL), dried under reduced pressure and then treated with the cleavage cocktail (TFA:DCM:H_2O:TES; 90:5:2.5:2.5 v/v, 6 mL) for 2 h. The syringe was then drained, and the filtrate was collected. The resin was washed with another portion of the cleavage cocktail (1 mL) and the washings were combined with the initial filtrate. The combined filtrate was concentrated under a stream of N_2 to form an oily residue which was precipitated with ice-cold diethyl ether (10 mL). The suspension was allowed to rest at 0 °C for 10 min, centrifuged and the supernatant was decanted. The pellet was resuspended in ice-cold diethyl ether (10 mL), centrifuged and the supernatant was decanted twice. After the final wash, the pellet was dried under a stream of N_2 and lyophilised to furnish the desired peptide as a solid (0.087 g, 61%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% (v/v) TFA over 10 min, 220 nm): 7.51 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{33}H_{50}N_{11}O_7$ 712.3889; found 712.3887.

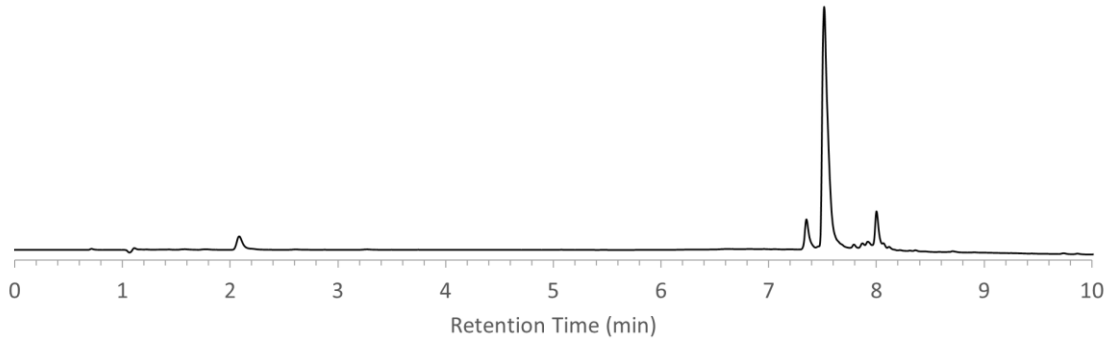
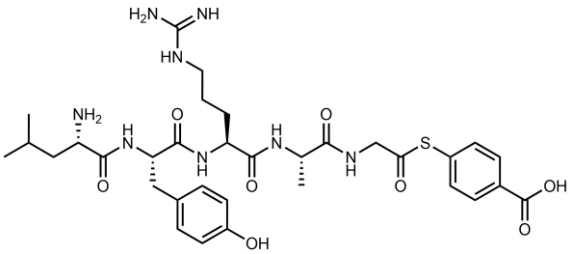


Figure 5.1. HPLC trace (220 nm) of crude LYRAG-Dbz **86**.

LYRAG-MBA

4-(((5S,8S,11S,14S)-14-amino-8-(3-guanidinopropyl)-11-(4-hydroxybenzyl)-5,16-dimethyl-4,7,10,13-tetraoxo-3,6,9,12-tetraazaheptadecanoyl)thio)benzoic acid (88)



To a solution of LYRAG-Dbz **86** (20.0 mg, 28.1 µmol) dissolved in 0.2 M phosphate buffer (pH 3.7, 2.8 mL) chilled to –5 °C was added 1sodium $nitrite_{(aq)}$ (1 M, 0.28 mL) and stirred for 2 min. Then 4-mercaptobezoic acid **67** (44 mg, 285 µmol) dissolved in 0.2 M phosphate buffer (pH 7.0, 2.8 mL) and MeCN (0.1 mL) was added and the solution was stirred at –5 °C for 10 min. The reaction mixture was washed with diethyl ether (2 x 10 mL) and the resulting aqueous solvent

was lyophilised. The dried powder was dissolved in 3:7 MeCN/H_2O with 0.1% (v/v) TFA and purified by semi-preparative HPLC (5–95% MeCN/H_2O with 0.1% (v/v) TFA, 60 min) over multiple injections. Fractions containing pure product were combined and the solvent was lyophilised to give the desired product as a white solid (8.0 mg, 40%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% (v/v) TFA over 10 min, 254 nm): 8.30 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{33}H_{47}N_8O_8S$ 715.3232; found 715.3065.

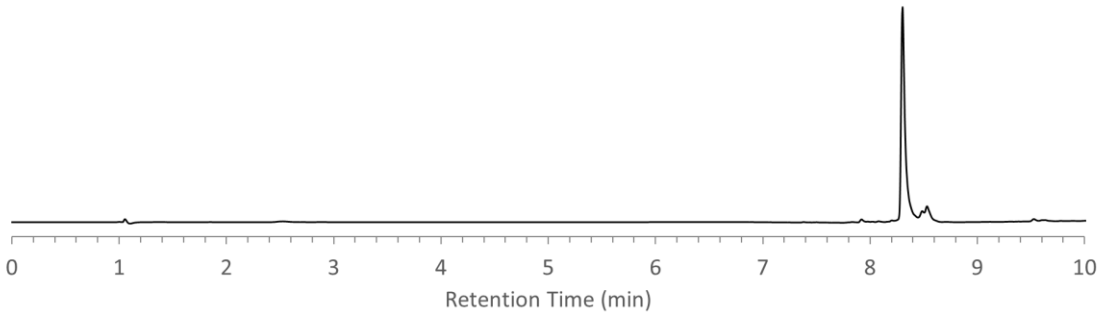
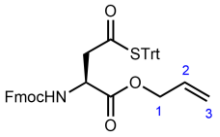


Figure 5.2. HPLC trace (254 nm) of purified LYRAG-MBA **88**.

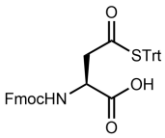
allyl (S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylthio)butanoate (96)



To a solution of Fmoc-Asp-OAll **95** (2.00 g, 5.06 mmol) in DCM (40 mL) was added EDC.HCl (0.97 g, 5.06 mmol) and the mixture was stirred at rt for 5 min. Then DMAP (0.094 g, 0.77 mmol) and triphenylmethanethiol (1.40 g, 5.06 mmol) were added and stirred at rt for 3 h. The reaction mixture was concentrated *in vacuo* and the residue was purified directly by silica gel flash chromatography (0–30% EtOAc/Hexane). Fractions containing pure product were combined and the solvent evaporated was *in vacuo* to furnish the product as a white foam (1.90 g, 58%). The isolated compound was in good agreement with the literature.[231]

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.79 (dd, *J* = 7.6, 5.1 Hz, 2H, Fmoc ArH), 7.61 (d, *J* = 7.5 Hz, 2H, Fmoc ArH), 7.46 – 7.39 (m, 2H, Fmoc ArH), 7.36 – 7.20 (m, 17H, Fmoc ArH, Trt ArH), 5.86 (ddt, *J* = 16.5, 11.0, 5.8 Hz, 1H, H2), 5.59 (d, *J* = 8.6 Hz, 1H, NH), 5.31 (dd, *J* = 17.1, 1.6 Hz, 1H,H3), 5.25 (dd, *J* = 10.5, 1.3 Hz, 1H, H3), 4.68 – 4.53 (m, 3H, Asp αCH, H1), 4.49 – 4.34 (m, 2H, Fmoc CH_2), 4.25 (t, *J* = 7.1 Hz, 1H, Fmoc CH), 3.30 (dd, *J* = 16.4, 4.8 Hz, 1H, Asp βCH_2), 3.11 (dd, *J* = 16.4, 4.5 Hz, 1H Asp βCH_2). **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{16}H_{28}N_3NaO_6$ 676.2128; found 676.2128.

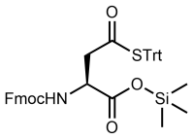
(S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylthio)butanoic acid (97)



To a solution of **96** (1.80 g, 2.76 mmol) in DCM (120 mL) was added phenylsilane (1.20 mL, 11.1 mmol) and the mixture was stirred at rt under N_2 for 1 h. The reaction mixture was concentrated *in vacuo* and the residue was purified directly by silica gel flash chromatography (0-7% DCM/MeOH). Fractions containing pure product were combined and the solvent evaporated was *in vacuo* to furnish the product as brown foam (1.54 g, 91%). The isolated compound was in good agreement with the literature.[231]

^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.77 (t, *J* = 7.1 Hz, 2H, Fmoc ArH), 7.72 – 7.52 (m, 2H, Fmoc ArH), 7.51 – 7.37 (m, 2H, Fmoc ArH), 7.35 – 7.18 (m, 17H, Fmoc ArH, Trt ArH), 5.53 (d, *J* = 8.4 Hz, 1H, NH), 4.60 (dt, *J* = 8.8, 4.8 Hz, 1H, Asp αCH), 4.51 – 4.36 (m, 2H, Fmoc CH_2), 4.24 (t, *J* = 7.0 Hz, 1H, Fmoc CH), 3.28 (dd, *J* = 16.4, 4.9 Hz, 1H, Asp βCH_2), 3.11 (dd, *J* = 16.6, 4.8 Hz, 1H, Asp βCH_2). **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{38}H_{31}NNaO_5S$ 636.1821; found 636.1808.

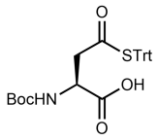
trimethylsilyl(S)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylthio)butanoate (93)



To a solution of **97** (1.50 g, 2.44 mmol) in DCM (10 mL) was added trimethylsilyl chloride (3.17 mL, 25.0 mmol) and the mixture was stirred at rt under N_2 for 15 min. The reaction mixture was concentrated *in vacuo* and dried under high vacuum until a constant weight was achieved to furnish the product as a tan foam (1.68 g, quant.). The isolated compound was in good agreement with the literature.[231]

m.p. 82 – 84 ºC. **^{1}H-NMR** (400 MHz, $CDCl_3$): δ 7.75 (t, *J* = 6.7 Hz, 2H, Fmoc ArH), 7.57 (d, *J* = 8.0 Hz, 2H, Fmoc ArH), 7.42 – 7.35 (m, 2H, Fmoc ArH), 7.32 – 7.16 (m, 17 H, Fmoc ArH, Trt ArH), 5.53 (d, *J* = 8.3 Hz, 1H, NH), 4.61 – 4.51 (m, 1H, Asp αCH), 4.49 – 4.34 (m, 2H, Fmoc CH_2), 4.21 (t, *J* = 6.9 Hz, 1H, Fmoc CH), 3.28 (dd, *J* = 16.6, 4.8 Hz, 1H, Asp βCH_2), 3.11 (dd, *J* = 16.6, 4.8 Hz, 1H, Asp βCH_2), 0.09 (s, 9H, TMS).

(S)-2-((tert-butoxycarbonyl)amino)-4-oxo-4-(tritylthio)butanoic acid (100)

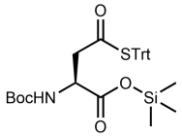


To a stirring solution of Boc-Asp-OAll **66** (2.0 g, 3.76 mmol) in DCM (60 mL) was added tetrakis(triphenylphosphine)palladium(0) (0.020 g, 17.3 µmol) and phenylsilane (2.2 mL, 17.8 mmol) and the mixture was stirred under nitrogen at rt for 24 h. The reaction mixture was concentrated *in vacuo* and the residue was dry loaded on Celite and purified by silica gel flash chromatography (0 to 7% v/v DCM/MeOH). Fractions containing pure product were combined and the solvent was evaporated *in vacuo* to give the product as a pale-yellow foam (1.66 g, 90%).

R_f (80:20 EtOAc:Hex): 0.40. **m.p.** 172 – 174 °C. **^{1}H-NMR** (400 MHz, $CDCl_3$:CD_3OD 98:2 v/v)*: δ 7.23 – 7.09 (m, 15 H, Trt), 5.62 (d, *J* = 6.9 Hz, 1H, NH), 4.16 – 4.07 (m, 1H, Asp αCH), 3.14 (dd, *J* = 15.5, 5.2 Hz, 1H, Asp βCH_2), 2.98 (dd, *J* = 15.5, 5.2 Hz, 1H, Asp βCH_2), 1.37 (s, 9H, *t*Bu). **^{13}C-NMR** (101 MHz, $CDCl_3$:CD_3OD 98:2 v/v): δ 196.5 (SC=O), 176.0 (COOH), 155.6 (Boc C=O), 143.4 (Ar qC), 129.6 (Ar C), 127.6 (Ar C), 127.0 (Ar C), 79.6 (*t*Bu qC), 70.7 (Asp αC), 51.7 (Trt qC), 44.8 (Asp βC), 28.1 (*t*Bu CH_3). **HRMS** ESI-MS *(m/z)*: $[M+Na]^+$ calculated for $C_{28}H_{29}NNaO_5S$ 514.1659; found 514.1655.

*The compound was found to aggregate in $CDCl_3$, acetone-d_6 and toluene-d_8 resulting in very broad peaks. In solvents such as DMSO-d_6 and CD_3OD, the compound readily decomposed *via* the elimination of triphenylmethanethiol. Thus, the NMR was conducted in $CDCl_3$ with 2% v/v CD_3OD to prevent aggregation.

trimethylsilyl (S)-2-((tert-butoxycarbonyl)amino)-4-oxo-4-(tritylthio)butanoate (101)

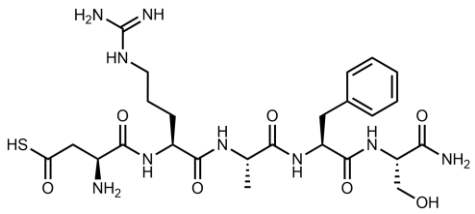


To a stirring solution of Boc-Asp(STrt)-OH **100** (1.5 g, 3.05 mmol) in dry DCM (10 mL) with 4 Å molecular sieves under nitrogen was added trimethylsilyl chloride (4 mL) slowly. The mixture was stirred at rt for 30 min. The solvent was removed in vacuo and the residue was dried under high vacuum until a constant weigh was achieved. The product was isolated as a tan-coloured foam (1.72 g, quant %). Used without further purification.

m.p. 155 – 157 °C **^{1}H-NMR** (400 MHz, $CDCl_3$): δ 7.30 – 7.17 (m, 15H, Ph), 5.29 (d, *J* = 8.0 Hz, 1H, NH), 4.52 – 4.32 (m, 1H, Asp αCH), 3.20 (dd, *J* = 17.2, 5.5 Hz, 1H, Asp βCH_2), 3.05 (dd, *J* = 16.3, 5.1 Hz, 1H, Asp βCH_2), 1.44 (s, 9H, ^{t}Bu), 0.07 (s, 9H, $Si(CH_3)_3$). **^{13}C-NMR** (101 MHz, $CDCl_3$): δ 195.3 (SC=O), 174.3 (C=O), 155.6 (Boc C=O), 143.3 (Ar qC), 129.7 (Ar C), 127.9 (Ar C), 127.3 (Ar C), 80.6 (^{t}Bu qC), 71.1 (Asp αC), 50.5 (Trt qC), 44.2 (Asp βC), 28.3 (^{t}Bu CH_3), 1.9 ($Si(CH_3)_3$).

D(SH)RAFS

(2S,5S,8S,11S,14S)-1,14-diamino-5-benzyl-11-(3-guanidinopropyl)-2-(hydroxymethyl)-8-methyl-1,4,7,10,13-pentaoxo-3,6,9,12-tetraazahexadecane-16-thioic S-acid (98)



Rink amide aminomethyl resin 100-200 mesh (0.130 g, 0.1 mmol) was swollen in DMF (2.5 mL) for 20 min and then drained. The resin bound Fmoc group was removed using 20% (v/v) piperidine in DMF (2 x 10 min; 2.5 mL) and the resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). Coupling cycles until the final sequence consisted of **(i)** Coupling: Fmoc-AA-OH (3 equiv.), PyBOP (0.156 g, 3 equiv.) and NMM (0.066 mL, 6 equiv.) in DMF (0.2 M) for 45 min; **(ii)** Wash: DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL); **(iii)** Fmoc Deprotection: 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL) and **(iv)** Wash: DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). The AAs used in order were Fmoc-Ser(O*t*Bu)-OH, Fmoc-Phe-OH, Fmoc-Ala-OH and Fmoc-Arg(Pbf)-OH. The AAs from Arg onwards were double coupled. Final Coupling: Boc-Asp(STrt)-OTMS **101** (3 equiv.), DIC (0.047 mL, 3 equiv.), Oxyma Pure (0.043 g, 3 equiv.) and DIPEA (0.0525 mL, 3 equiv.) in dry DCM (0.2 M) for 45 min. The resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL).

Following assembly of the peptide sequence, the resin was washed with DCM (3 x 5 mL), dried under reduced pressure and then treated with the cleavage cocktail (TFA:DCM:H_2O:TES; 90:5:2.5:2.5 v/v, 3 mL) for 2 h. The syringe was then drained, and the filtrate was collected. The resin was washed with another portion of the cleavage cocktail (1 mL) and the washings were combined with the initial filtrate. The combined filtrate was concentrated under a stream of N_2 to form an oily residue which was precipitated with ice-cold diethyl ether (10 mL). The suspension was allowed to rest at 0 °C for 10 min, centrifuged and the supernatant was decanted. The pellet was resuspended in ice-cold diethyl ether (10 mL), centrifuged and the supernatant was decanted twice. After the final wash, the pellet was dried under a stream of N_2 and lyophilised to furnish the crude peptide as a solid (48.2 mg, 79%).

The crude peptide was dissolved in H_2O with 0.1% (v/v) TFA and purified by semi-preparative HPLC (5–95% MeCN/H_2O with 0.1% (v/v) TFA, 60 min) over multiple injections. Fractions containing pure product were combined and the solvent was lyophilised to give the desired product as a white solid (15.3 mg, 40%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% (v/v) TFA over 10 min, 254 nm): 7.46 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{25}H_{40}N_9O_7S$ 610.2766; found 610.2766.

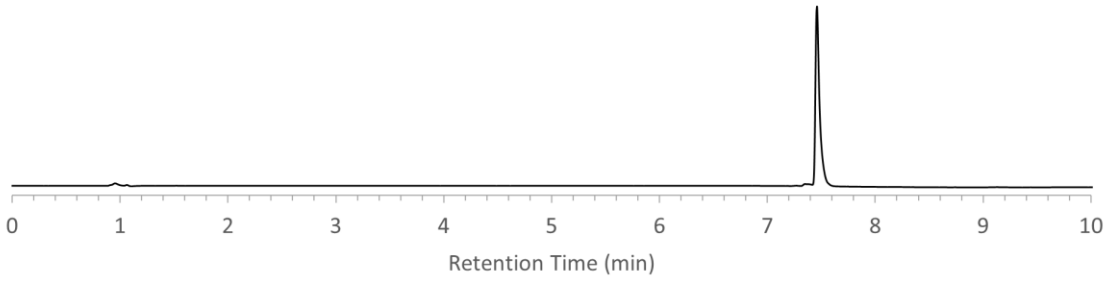
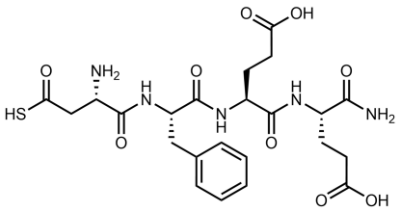


Figure 5.3. HPLC trace (254 nm) of purified D(SH)RAFS **98**.

D(SH)FEE

(S)-5-amino-4-((S)-2-((S)-2-((S)-2-amino-3-thiocarboxypropanamido)-3-phenylpropanamido)-4-carboxybutanamido)-5-oxopentanoic acid (102)



Rink amide aminomethyl resin 100-200 mesh (0.130 g, 0.1 mmol) was swollen in DMF (2.5 mL) for 20 min and then drained. The resin bound Fmoc group was removed using 20% (v/v) piperidine in DMF (2 x 10 min; 2.5 mL) and the resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). Coupling cycles until the final sequence consisted of **(i)** Coupling: Fmoc-AA-OH (3 equiv.), PyBOP (0.156 g, 3 equiv.) and NMM (0.066 mL, 6 equiv.) in DMF (0.2 M) for 45 min; **(ii)** Wash: DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL); **(iii)** Fmoc Deprotection: 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL) and **(iv)** Wash: DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). The AAs used in order were Fmoc-Glu(O*t*Bu)-OH and Fmoc-Phe-OH. Final Coupling: Boc-Asp(STrt)-OTMS **101** (3 equiv.), DIC (0.047 mL, 3 equiv.), Oxyma Pure (0.043 g, 3 equiv.) and DIPEA (0.0525 mL, 3 equiv.) in dry DCM (0.2 M) for 45 min. The resin was then washed with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL).

Following assembly of the peptide sequence, the resin was washed with DCM (3 x 5 mL), dried under reduced pressureand then treated with the cleavage cocktail (TFA:DCM:H_2O:TES; 90:5:2.5:2.5 v/v, 3 mL) for 2 h. The syringe was then drained, and the filtrate was collected. The resin was washed with another portion of the cleavage cocktail (1 mL) and

the washings were combined with the initial filtrate. The combined filtrate was concentrated under a stream of N_2 to form an oily residue which was precipitated with ice-cold diethyl ether (10 mL). The suspension was allowed to rest at 0 °C for 10 min, centrifuged and the supernatant was decanted. The pellet was resuspended in ice-cold diethyl ether (10 mL), centrifuged and the supernatant was decanted twice. After the final wash, the pellet was dried under a stream of N_2 and lyophilised to furnish the crude peptide as a solid (39.2 mg, 71%).

The crude peptide was dissolved in 1:1 H_2O/MeCN with 0.1% (v/v) TFA and purified by semi-preparative HPLC (5–95% MeCN/H_2O with 0.1% (v/v) TFA, 60 min) over multiple injections. Fractions containing pure product were combined and the solvent was lyophilised to give the desired product as a white solid (24.3 mg, 44%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% (v/v) TFA over 10 min, 254 nm): 7.36 min. **HRMS** ESI *(m/z)*: $[M-H]^-$ calculated for $C_{23}H_{30}N_5O_9S$ 552.1770; found 552.1771.

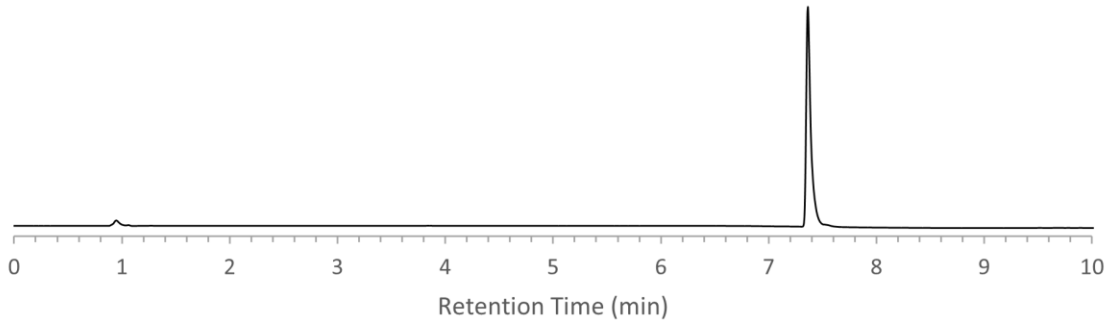


Figure 5.4 HPLC trace (254 nm) of purified D(SH)FEE **102**.

5.3 Experimental Details for Chapter 3

5.3.1 General Procedures

Procedure 3A: SPPS of Linear Peptides

Coupling of First Amino Acid, Fmoc-allylglycine-OH (Fmoc-Agl-OH): Rink amide aminomethyl resin 100-200 mesh (0.13 g, 0.1 mmol) was swollen in DMF (5 mL) for 20 min and then drained. The resin bound Fmoc group was removed using 20% v/v piperidine in DMF (2 x 10 min; 5 mL) and the resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). Fmoc-Agl-OH (4 equiv.), HATU (3.9 equiv.) and DIPEA (8 equiv.) were dissolved in DMF (0.2 M) and transferred immediately to the syringe containing the resin and agitated for 45 min. Excess reagents were drained and the resin was washed with DMF (3 x 5 mL), DCM (3 x 5 mL)

and DMF (3 x 5 mL). The synthesis was continued *via* the following protocols, unless otherwise stated.

Coupling up to Penultimate Amino Acid: Subsequent amino acid coupling cycles consisted of: **(i)** Fmoc deprotection with 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL). **(ii)** Resin washes with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL). **(iii)** Coupling with a solution of Fmoc-AA-OH (4 equiv.), HATU (3.9 equiv.) and DIPEA (8 equiv.) in DMF (0.2 M) for 45 min. The coupling was repeated for all subsequent amino acids in the sequence after the incorporation of Leu or Ile. The resin was washed with DMF (3 x 5 mL) between the repeat coupling steps to remove excess reagents. **(iv)** Resin washes with DMF (3 x 5 mL), DCM (3 x 5 mL) and DMF (3 x 5 mL).

Coupling of Final Amino Acid, Boc-Asp(STrt)-OTMS 101: The Fmoc group was removed using 20% (v/v) piperidine in DMF (2 x 10 min; 5 mL) and the resin was then washed with DMF (3 x 5 mL), DCM (3 x 5mL) and DMF (3 x 5 mL). The resin was subsequently washed with dry DCM (3 x 5 mL). Boc-Asp(STrt)-OTMS **101** (3 equiv.) was added to a solution containing DIC (3 equiv.), Oxyma Pure (3 equiv.) and DIPEA (3 equiv.) in dry DCM (0.2 M), and the resulting mixture was immediately added to the resin and agitated for 45 min. The coupling procedure was then repeated after washing the resin with dry DCM (3 x 5 mL) to remove any excess reagent. Following coupling, the resin was washed with DCM (3 x 5 mL), DMF (3 x 5 mL) and DCM (3 x 5 mL) and dried under reduced pressure.

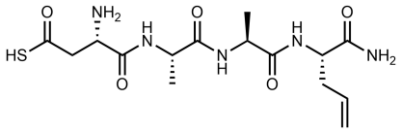
Resin Cleavage: The dried resin was immersed in the cleavage cocktail (TFA:DCM:H_2O:TES; 90:5:2.5:2.5 v/v, 3 mL) unless otherwise stated and agitated for 2 h. The syringe was then drained, and the filtrate was collected. The resin was washed with another portion of the cleavage cocktail (1 mL) and the washings were combined with the initial filtrate. The combined filtrate was concentrated under a stream of N_2 to form an oily residue which was precipitated with ice-cold diethyl ether (10 mL). The suspension was allowed to rest at 0 °C for 10 min, centrifuged and the supernatant was decanted. The pellet was resuspended in ice-cold diethyl ether (10 mL), centrifuged and the supernatant was decanted twice. After the final wash, the pellet was dried under a stream of N_2 and lyophilised to furnish the peptide.

General Procedure 3B: Cyclisation of Linear Peptides

Linear peptide (10 mg) and 2,2-dimethoxy-2-phenylacetophenone (DPAP, 1 equiv.) were dissolved in 1:1 H_2O:MeCN containing 0.1% TFA at 5 mM concentration in a *via*l and the resulting solution was stirred at rt under UV light illumination for 15 min. 200 µL of the reaction mixture was taken and analysed by analytical HPLC (5 – 95% MeCN/H_2O + 0.1% TFA v/v over 10 min) to confirm complete consumption of the linear peptide. The reaction mixture was then purified by semi-preparative HPLC as described in the procedure for each peptide followed by lyophilisation to yield the desired cyclic peptide.

5.3.2 Characterisation Data

H-Asp(SH)-Ala-Ala-Agl-NH_2
(S)-3-amino-4-(((S)-1-(((S)-1-(((S)-1-amino-1-oxopent-4-en-2-yl)amino)-1-oxopropan-2-yl)amino)-1-oxopropan-2-yl)amino)-4-oxobutanethioic S-acid (108)



Prepared using General *Procedure 3A* using Fmoc-Agl-OH, Fmoc-Ala-OH and Boc-Asp(STrt)-OTMS **101**. Isolated yield: 32 mg (83%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 7.60 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{15}H_{26}N_5O_5S$ 388.1649, found 388.1650.

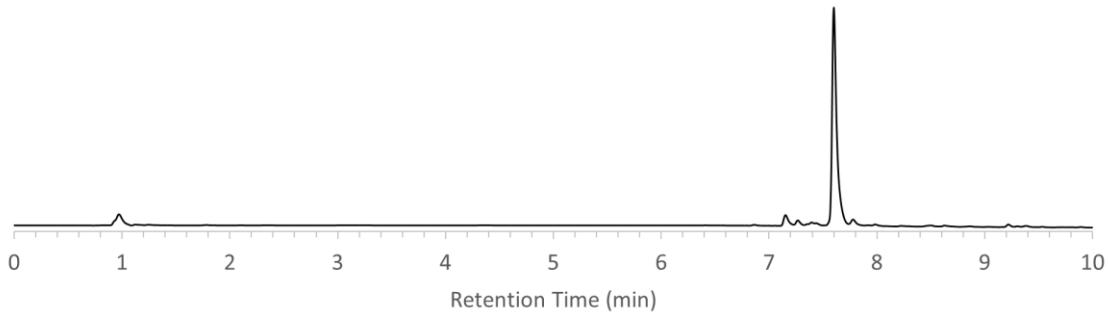
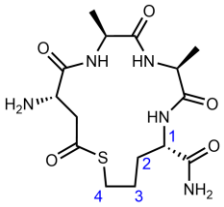


Figure 5.5. HPLC trace (220 nm) of crude H-Asp(SH)-Ala-Ala-Agl-NH_2 **108**.

(5S,8S,11S,14S)-14-amino-8,11-dimethyl-7,10,13,16-tetraoxo-1-thia-6,9,12-triazacyclohexadecane-5-carboxamide (110)



Prepared following General *Procedure 3B* from linear peptide **108** (10.0 mg). Purified by semi-preparative HPLC (5 – 15% MeCN in H_2O with 0.1% TFA over 60 min). After lyophilisation, the desired product was isolated as a fluffy white powder (5.4 mg, 54%). Purity: >95%.

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 6.89 min. **^{1}H-NMR** (600 MHz, DMSO-d_6): δ 8.58 (d, *J* = 8.1 Hz, 1H, Ala NH), 8.27 (bs, 2H, amino NH_2), 7.87 (d, *J* = 9.2 Hz, 1H, NH), 7.68 (d, *J* = 6.2 Hz, 1H, Ala NH), 7.30 (s, 1H, amide NH_2), 7.03 (s, 1H, amide NH_2),

4.40 – 4.33 (m, 1H, H1), 4.26 – 4.21 (m, 1H, Ala αCH), 4.15 – 4.09 (m, 1H, Ala αCH), 4.09 – 4.04 (m, 1H, Asp αCH), 3.17 (dd, J =16.3, 3.9 Hz, 1H, Asp βCH$_2$), 3.14 – 3.04 (m, 2H, Asp βCH$_2$, H4), 2.58 – 2.51 (m, 1H, H4), 1.75 – 1.68 (m, 1H, H3), 1.59 – 1.45 (m, 2H, H2, H3), 1.45 – 1.37 (m, 1H, H2), 1.28 (d, *J* = 7.3 Hz, 3H, Ala CH$_3$), 1.23 (d, *J* = 6.6 Hz, 3H, Ala CH$_3$). **^{13}C-NMR** (151 MHz, DMSO-d$_6$): δ 194.8 (SC=O), 173.6 (C=O), 171.1 (C=O), 170.8 (C=O), 166.6 (C=O), 50.5 (C1) ,49.3 (Ala αC), 48.9 (Asp αC), 43.5 (Asp βC), 30.7 (C2), 27.6 (C4), 25.9 (C3), 18.4 (Ala CH$_3$), 16.9 (Ala CH$_3$). **HRMS** ESI *(m/z)*: [M+H]$^+$ calculated for $C_{15}H_{26}N_5O_5S$ 388.1649, found 388.1653.

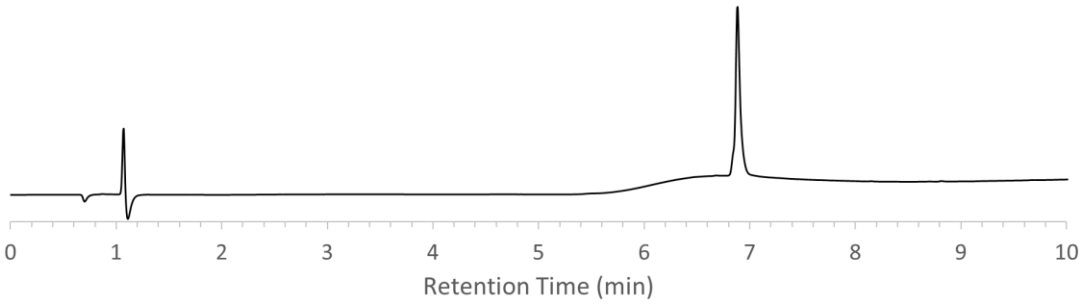
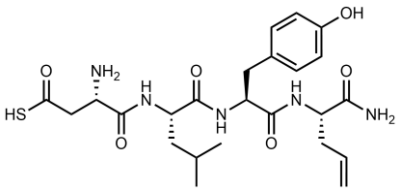


Figure 5.12 HPLC trace (220 nm) of purified **110**.

H-Asp(SH)-Leu-Tyr-Agl-NH$_2$
(S)-3-amino-4-(((S)-1-(((S)-1-(((S)-1-amino-1-oxopent-4-en-2-yl)amino)-3-(4-hydroxyphenyl)-1-oxopropan-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-4-oxobutanethioic S-acid (113)



Prepared using General *Procedure 3A* using Fmoc-Agl-OH, Fmoc-Tyr(O*t*Bu)-OH, Fmoc-Leu-OH and Boc-Asp(STrt)-OTMS **101**. Isolated yield: 34 mg (65%).

HPLC t_R (5 – 95% MeCN in H$_2$O with 0.1% TFA over 10 min, 220 nm): 8.13 min. **HRMS** ESI *(m/z)*: [M+H]$^+$ calculated for $C_{24}H_{36}N_5O_6S$ 522.2381, found 522.2387.

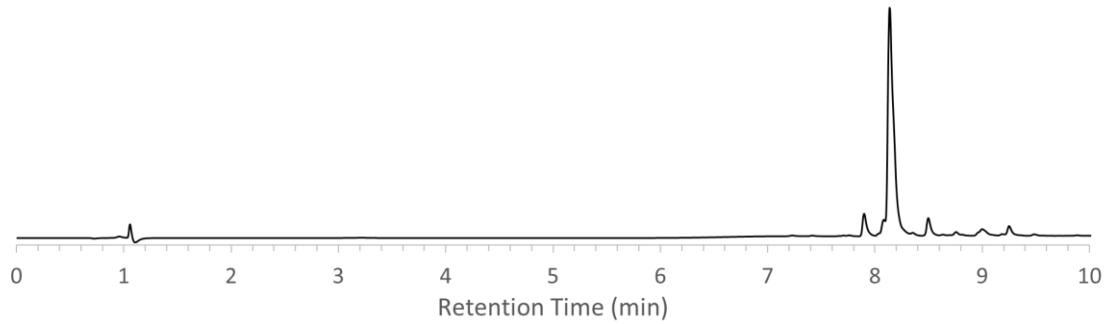
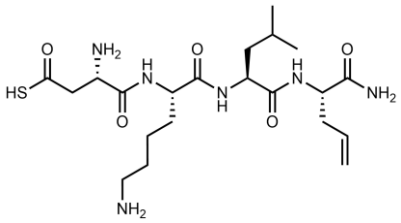


Figure 5.6 HPLC trace (220 nm) of crude H-Asp(SH)-Leu-Tyr-Agl-NH_2 **113**.

H-Asp(SH)-Lys-Leu-Agl-NH_2
(S)-3-amino-4-(((S)-6-amino-1-(((S)-1-(((S)-1-amino-1-oxopent-4-en-2-yl)amino)-4-methyl-1-oxopentan-2-yl)amino)-1-oxohexan-2-yl)amino)-4-oxobutanethioic S-acid (114)



Prepared using General *Procedure 3A* using Fmoc-Agl-OH, Fmoc-Leu- OH, Fmoc-Lys(Boc)-OH and Boc-Asp(STrt)-OTMS **101**. Isolated yield: 45 mg (93%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 7.32 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{21}H_{39}N_6O_5S$ 487.2697, found 487.2694.

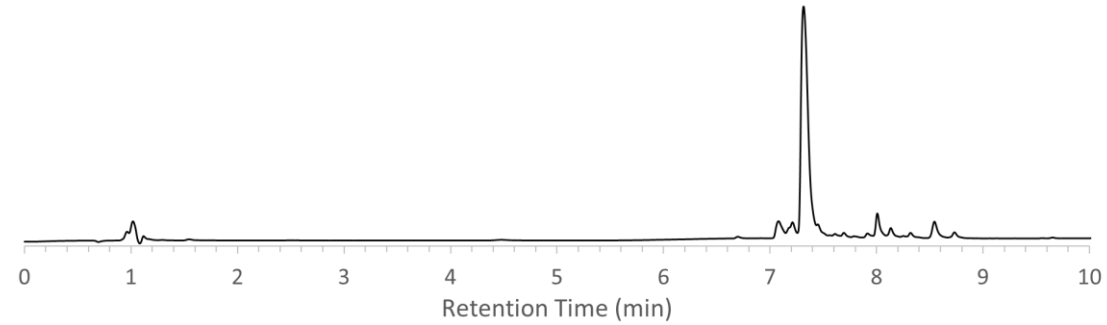
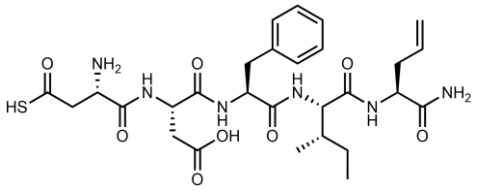


Figure 5.7 HPLC trace (220 nm) of crude H-Asp(SH)-Lys-Leu-Agl-NH_2 **114**.

H-Asp(SH)-Asp-Phe-Ile-Agl-NH_2
(S)-4-(((S)-1-(((2S,3S)-1-(((S)-1-amino-1-oxopent-4-en-2-yl)amino)-3-methyl-1-oxopentan-2-yl)amino)-1-oxo-3-phenylpropan-2-yl)amino)-3-((S)-2-amino-3-thiocarboxypropanamido)-4-oxobutanoic acid (115)



Prepared using General *Procedure 3A* using Fmoc-Agl-OH, Fmoc-Ile-OH, Fmoc-Phe-OH, Fmoc-Asp(O*t*Bu)-OH and Boc-Asp(STrt)-OTMS **101.** Isolated yield: 26 mg (42%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 8.66 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{28}H_{41}N_6O_8S$ 621.2701, found 621.2698.

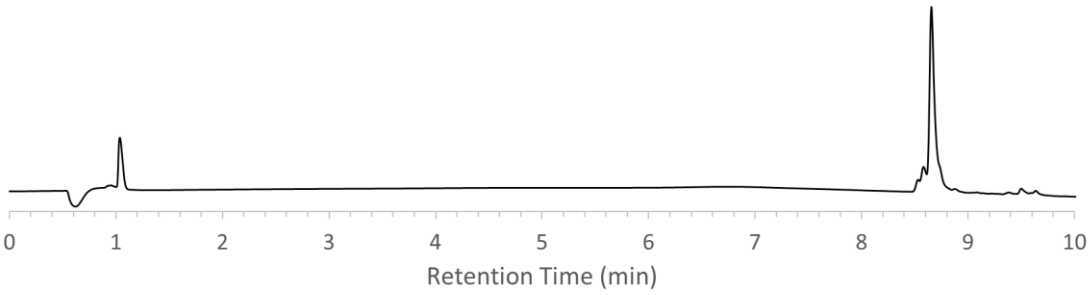
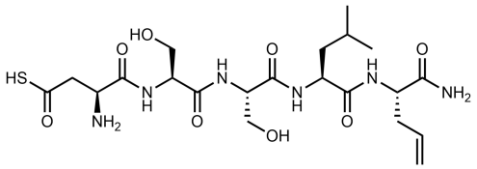


Figure 5.8 HPLC trace (220 nm) of crude H-Asp(SH)-Asp-Phe-Ile-Agl-NH_2 **115**.

H-Asp(SH)-Ser-Ser-Leu-Agl-NH_2
(3S,6S,9S,12S,15S)-3-amino-15-carbamoyl-6,9-bis(hydroxymethyl)-12-isobutyl-4,7,10,13-tetraoxo-5,8,11,14-tetraazaoctadec-17-enethioic S-acid (116)



Prepared using General *Procedure 3A* using Fmoc-Agl-OH, Fmoc-Leu-OH, Fmoc-Ser(O*t*Bu)-OH and Boc-Asp(STrt)-OTMS **101**. Isolated yield: 35 mg (66%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 7.62 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{21}H_{37}N_6O_8S$ 533.2388, found 533.2388.

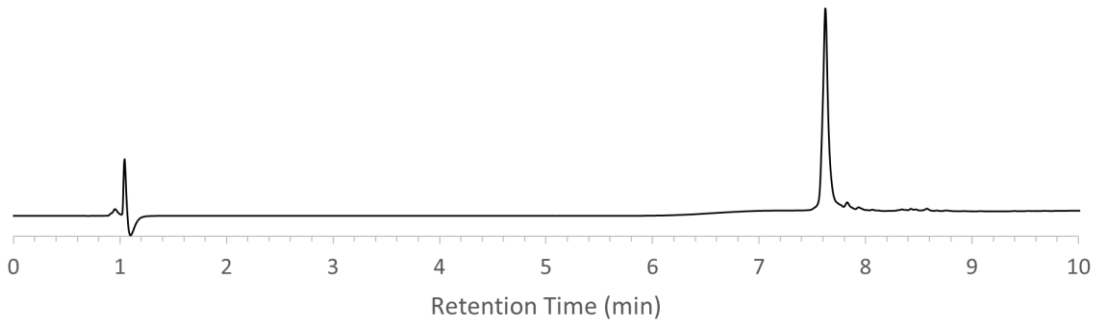
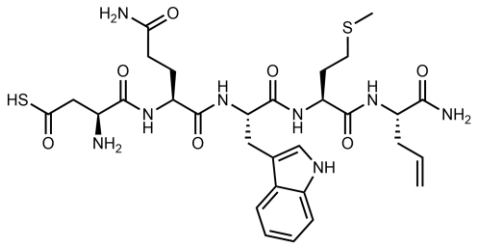


Figure 5.9 HPLC trace (220 nm) of crude H-Asp(SH)-Ser-Ser-Leu-Agl-NH_2 **116**.

H-Asp(SH)-Gln-Trp-Met-Agl-NH_2

(3S,6S,9S,12S,15S)-9-((1H-indol-3-yl)methyl)-3-amino-6-(3-amino-3-oxopropyl)-15-carbamoyl-12-(2-(methylthio)ethyl)-4,7,10,13-tetraoxo-5,8,11,14-tetraazaoctadec-17-enethioic S-acid (117)



Prepared using the SPPS general procedures using Fmoc-Agl-OH, Fmoc-Met-OH, Fmoc-Trp(Boc)-OH, Fmoc-Gln(Trt)-OH and Boc-Asp(STrt)-OTMS **101**. The cleavage cocktail consisted of TFA:EDT:TES:H_2O 94:2.5:2.5:1 v/v. Isolated yield: 32 mg (46%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 8.30 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{30}H_{43}N_8O_7S_2$ 691.2691, found 691.2691.

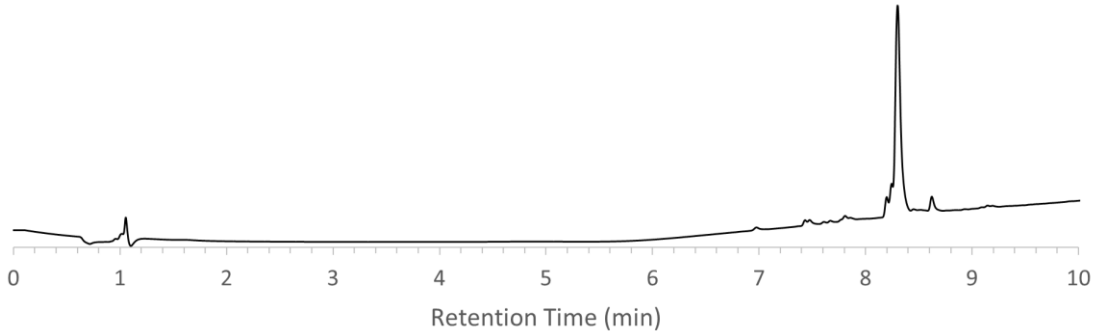
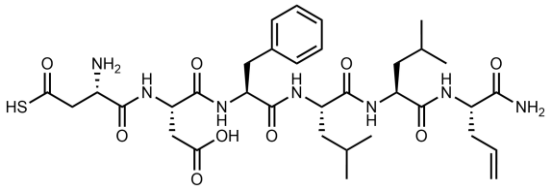


Figure 5.10 HPLC trace (220 nm) of crude H-Asp(SH)-Gln-Trp-Met-Agl-NH_2 **117**.

H-Asp(SH)-Asp-Phe-Leu-Leu-Agl-NH_2
(3S,6S,9S,12S,15S)-3-((S)-2-amino-3-thiocarboxypropanamido)-6-benzyl-15-carbamoyl-9,12-diisobutyl-4,7,10,13-tetraoxo-5,8,11,14-tetraazaoctadec-17-enoic acid (118)



Prepared using the SPPS general procedures using Fmoc-Agl-OH, Fmoc-Leu-OH, Fmoc-Phe-OH, Fmoc-Asp(O*t*Bu)-OH and Boc-Asp(STrt)-OTMS **101**. Isolated yield: 69 mg (95%).

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 30 min, 220 nm): 12.30 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{34}H_{53}N_7O_9S$ 734.3541, found 734.3546.

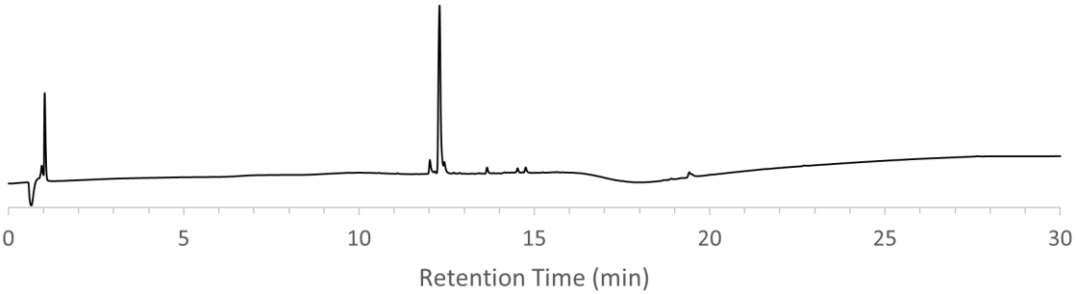
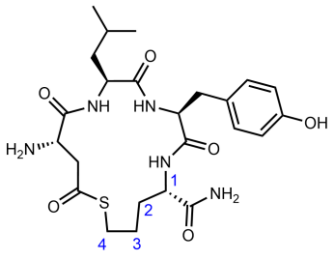


Figure 5.11 HPLC trace (220 nm) of crude H-Asp(SH)-Asp-Phe-Leu-Leu-Agl-NH_2 **118**.

(5S,8S,11S,14S)-14-amino-8-(4-hydroxybenzyl)-11-isobutyl-7,10,13,16-tetraoxo-1-thia-6,9,12-triazacyclohexadecane-5-carboxamide (119)



Prepared following General *Procedure 3B* from linear peptide **113** (10.0 mg). Purified by semi-preparative HPLC (5 – 25% MeCN in H_2O with 0.1% TFA over 60 min). After lyophilisation, the desired product was isolated as a fluffy white powder (4.4 mg, 44%). Purity: >90%.

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 8.05 min**. ^{1}H-NMR** (600 MHz, DMSO-d_6): δ 9.13 (s, 1H, OH), 8.46 (d, *J* = 8.8 Hz, 1H, Leu NH), 8.26 (bs, 2H, amino NH_2), 7.80 (d, *J* = 8.7 Hz, 1H, NH), 7.63 (d, *J* = 7.1 Hz, 1H, Tyr NH), 7.04 (s, 1H, amide NH_2), 6.99 (s, 1H, amide NH_2), 6.93 (d, *J* = 8.1 Hz, 2H, Tyr Ar H), 6.62 (d, *J* = 8.1 Hz, 2H, Tyr Ar H), 4.38 – 4.32 (m, 1H, H1), 4.32 – 4.27 (m, 1H, Tyr αCH), 4.26 – 4.20 (m, 1H, Leu αCH), 4.10 – 4.00 (m, 1H, Asp αCH), 3.18 (dd, *J* = 16.6, 3.8 Hz, 1H, Asp βCH_2), 3.10 – 3.00 (m, 2H, Asp βCH_2, H4), 2.94 (dd, *J* = 13.7, 6.6 Hz, 1H, Tyr βCH_2), 2.83 (dd, *J* = 13.7, 7.7 Hz, 1H, Tyr βCH_2), 2.64 – 2.55 (m, 1H, H4), 1.73 – 1.66 (m, 1H, H2), 1.58 – 1.31 (m, 5H, Leu γCH, H2, H3, Leu βCH_2), 1.28 – 1.21 (m, 1H, Leu βCH_2), 0.84 (d, *J* = 6.4 Hz, 3H, Leu CH_3), 0.79 (d, *J* = 6.4 Hz, 3H, Leu CH_3).**^{13}C-NMR** (151 MHz, DMSO-d_6): δ 194.7 (SC=O), 173.4 (C=O), 171.0 (C=O), 170.1 (C=O), 166.8 (C=O), 156.0 (Ar qC), 130.3 (Ar C), 127.0 (Ar C), 115.0 (Ar qC), 54.9 (Tyr αC), 52.4 (Leu αC), 50.8 (C1), 49.2 (Asp αC), 43.7 (Asp βC), 42.1 (Leu βC), 35.7 (Tyr βC), 30.9 (C2), 27.8 (C4), 25.9 (C3), 23.8 (Leu γC), 23.0 (Leu δC), 21.7 (Leu δC). **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{24}H_{36}N_5O_6S$ 522.2381, found 522.2387.

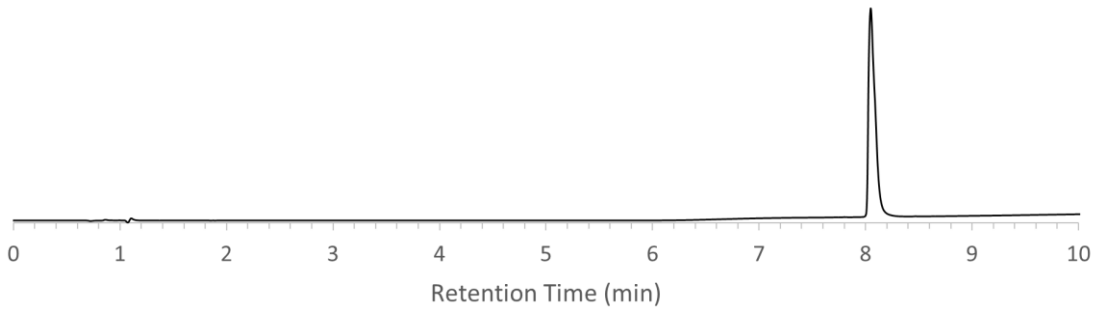
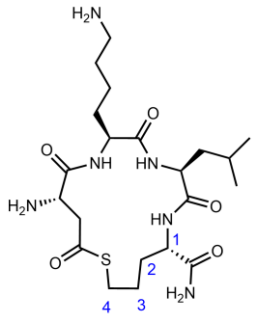


Figure 5.13 HPLC trace (220 nm) of purified **119**.

(5S,8S,11S,14S)-14-amino-11-(4-aminobutyl)-8-isobutyl-7,10,13,16-tetraoxo-1-thia-6,9,12-triazacyclohexadecane-5-carboxamide (120)



Prepared following General *Procedure 3B* from linear peptide **114** (10.0 mg). Purified by semi-preparative HPLC (5 – 25% MeCN in H_2O with 0.1% TFA over 60 min). After lyophilisation, the desired product was isolated as a fluffy white powder (3.6 mg, 36%). Purity: >90%.

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 7.10 min. **^{1}H-NMR** (600 MHz, DMSO-d_6): δ 8.47 (dd, *J* = 8.5 Hz, 1H, Leu NH), 8.33 (bs, 1H, amino NH_2), 7.95 (dd, *J* = 9.0 Hz, 1H, NH), 7.73 – 7.60 (m, 3H, Lys NH, Lys NH_2), 7.26 (s, 1H, amide NH_2), 7.02 (s, 1H, amide NH_2), 4.42 – 4.27 (m, 1H, H1), 4.25 – 4.19 (m, 1H, Lys αCH), 4.18 – 4.13 (m, 1H, Leu αCH), 4.09 – 4.03 (m, 1H Asp αCH), 3.21 – 3.15 (m, 1H, Asp βCH_2), 3.13 – 3.05 (m, 2H, Asp βCH_2, H4), 2.78 (m, 2H, Lys εCH_2), 2.58 – 2.52 (m, 1H, H4), 1.75 – 1.65 (m, 1H, H2), 1.62 – 1.27 (m, 12H, H2, H3, Leu βCH_2, Leu γCH, Lys βCH_2, Lys γCH_2, LysδCH_2), 0.91 (d, *J* = 6.5 Hz, 3H, Leu CH_3), 0.84 (d, *J* = 6.5 Hz, 3H, Leu CH_3). **^{13}C-NMR** (151 MHz, DMSO-d_6): δ 194.8 (SC=O), 173.6 (C=O), 170.5 (C=O), 170.3 (C=O), 53.6 (Lys αC), 52.1 (Leu αC), 50.6 (C1), 49.2 (Asp αC), 43.6 (Asp βC), 38.8 (Lys εC), 31.9 (Lys βC), 30.8 (C2), 27.7 (C4), 26.7 (Lys δC), 26.0 (C3), 24.5 (Leu γC), 22.8 (Leu δC), 22.6 (Leu δC), 22.0 (Lys γC). **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{21}H_{39}N_6O_5S$ 487.2697, found 487.2699.

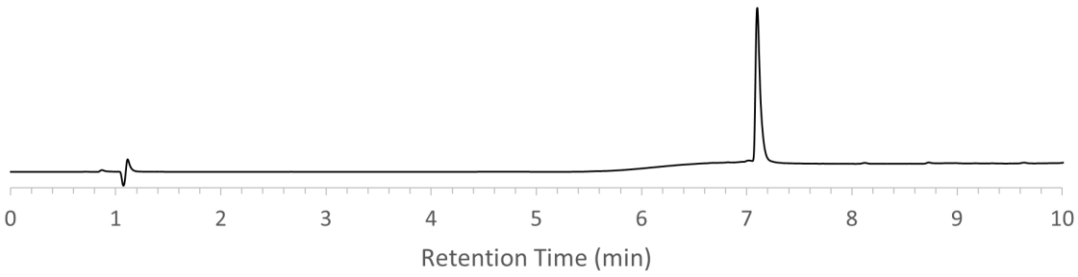
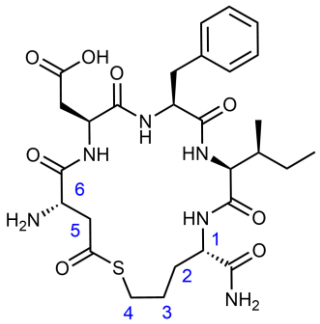


Figure 5.14 HPLC trace (220 nm) of purified **120**.

2-((4S,7S,10S,13S,16S)-4-amino-10-benzyl-13-((S)-sec-butyl)-16-carbamoyl-2,5,8,11,14-pentaoxo-1-thia-6,9,12,15-tetraazacyclononadecan-7-yl)acetic acid (121)



Prepared following General *Procedure 3B* from linear peptide **115** (10.0 mg). Purified by semi-preparative HPLC (5 – 95% MeCN in H_2O with 0.1% TFA over 60 min). After lyophilisation, the desired product was isolated as a fluffy white powder (5.2 mg, 52%). Purity: >95%.

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 8.43 min. **^{1}H NMR** (600 MHz, DMSO-d_6): δ 12.41 (bs, 1H, COOH), 8.94 (d, *J* = 7.6 Hz, 1H, Asp NH), 8.33 (m, 3H, NH_2, Ile NH), 7.71 (d, *J* = 9.2 Hz, 1H, NH), 7.35 (d, *J* = 9.1 Hz, 1H, Phe NH), 7.32 – 7.17 (m, 5H, Phe Ar H), 7.14 (s, 1H, amide NH_2), 7.09 (s, 1H, amide NH_2), 4.61 (td, *J* = 9.4, 9.1, 3.5 Hz, 1H, Phe αCH), 4.49 (td, *J* = 8.1, 7.6, 4.3 Hz, 1H, Asp αCH), 4.25 (td, *J* = 9.2, 9.1, 3.4 Hz, 1H, H1), 4.11-4.07 (m, 1H, H6), 3.91 (dd, *J* = 10.2, 7.7 Hz, 1H, Ile αCH), 3.25 – 3.12 (m, 3H, H5, H4), 3.0 (dd, *J* = 13.9, 3.5 Hz, 1H, Phe βCH_2), 2.73 – 2.65 (m, 1H, Phe βCH_2), 2.59 – 2.52 (m, 1H, H4), 2.44 (dd, *J* = 16.9, 4.3 Hz, Asp βCH_2), 2.33 (dd, *J* = 16.9, 8.1 Hz, 1H, Asp βCH_2), 1.82 – 1.74 (m, 1H, Ile βCH), 1.67 – 1.49 (m, 4H, Ile γCH_2, H2, H3), 1.42 – 1.43 (m, 1H, H3), 1.18 – 1.04 (Ile γCH_2), 0.85 (t, *J* = 7.5 Hz, 3H, Ile δCH_3), 0.81 (d, *J* = 6.7 Hz, 3H, Ile γCH_3). **^{13}C NMR** (151 MHz, DMSO-d_6): δ 196.1 (SC=O), 173.4 (C=O), 171.6 (COOH), 170.9 (C=O), 169.2 (C=O), 169.0 (C=O), 168.1 (C=O), 137.5 (Phe qC), 129.6 (Phe ortho Ar C), 128.2 (Phe meta Ar C), 126.4 (Phe para Ar C), 58.4 (Ile αC), 52.4 (Phe αC), 52.2 (C1), 50.4 (Asp αC), 48.5 (C6), 43.5 (C5), 38.3 (Phe βC), 35.8 (Asp βC), 33.9 (Ile βC), 31.8 (C2), 28.7 (C4), 25.9 (C3), 24.9 (Ile γCH_2), 15.3 (Ile γCH_3), 10.5 (Ile δCH_3). **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{28}H_{41}N_6O_8S$ 621.2701, found 621.2704.

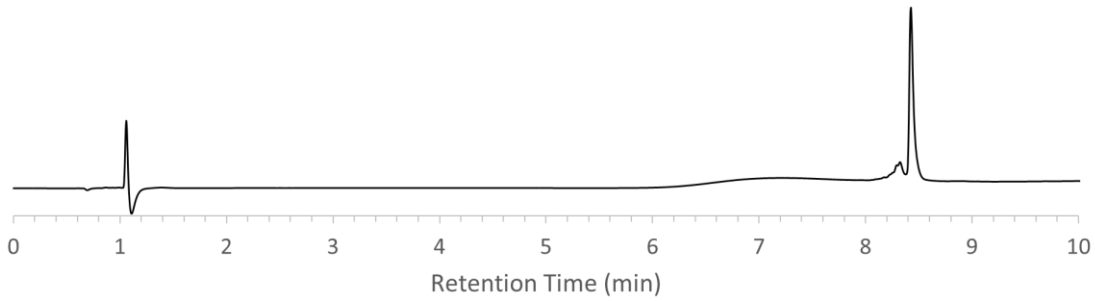
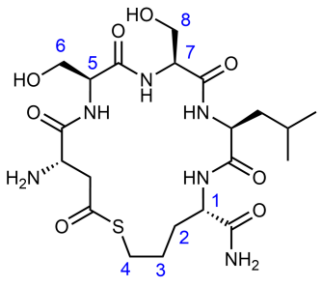


Figure 5.15 HPLC trace (220 nm) of purified **121**.

(5S,8S,11S,14S,17S)-17-amino-11,14-bis(hydroxymethyl)-8-isobutyl-7,10,13,16,19-pentaoxo-1-thia-6,9,12,15-tetraazacyclononadecane-5-carboxamide (122)



Linear peptide **116** (10.0 mg) and DPAP (1 equiv.) were dissolved in 1:1 H_2O:MeCN containing 0.1% TFA and 6 M guanidinium hydrochloride at 5 mM concentration in a *via*l. The

resulting solution was stirred at rt under UV light illumination for 15 min. The reaction mixture was purified by semi-preparative HPLC (5 – 95% MeCN in H_2O with 0.1% TFA over 60 min). After lyophilisation, the desired product was isolated as a fluffy white powder (6.1 mg, 61%). Purity: >95%.

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 7.50 min. **^{1}H NMR** (600 MHz, DMSO-d_6): δ 8.28 (bs, 2H, amino NH_2), 8.23 (d, *J* = 7.1 Hz, 1H, Ser NH), 8.16 (d, *J* = 6.2 Hz, 1H, Ser NH), 7.84 (d, *J* = 6.9 Hz, 1H, Leu NH), 7.45 (d, *J* = 7.8 Hz, 1H, NH), 7.06 (s, 1H, amide NH_2), 6.99 (s, 1H, Amide NH_2), 5.46 (t, *J* = 5.5 Hz, 1H, Ser OH), 4.98 (m, 1H, Ser OH), 4.38 (dt, *J* = 7.1, 5.0 Hz, 1H, H5), 4.27 – 4.21 (m, 1H, Asp αCH), 4.17 (dt, *J* = 6.2, 4.0 Hz, 1H, H7), 4.11 – 4.01 (m, 2H, H1, Leu αCH), 3.98 – 3.92 (m, 1H, Ser H6), 3.72 – 3.61 (m, 2H, Ser H8), 3.61 – 3.55 (m, 1H, H6), 3.28 (dd, *J* = 17.2, 3.0, 1H, Asp βCH_2), 3.17 – 3.07 (m, 2H, Asp βCHb, H2), 2.75 – 2.69 (m, 1H, H2), 1.68 – 1.55 (m, 4H, H3, Leu γCH, Leu βCH_2), 1.54 – 1.41 (m, 3H, H4, Leu βCH_2), 0.88 (d, *J* = 6.6 Hz, 3H, Leu CH_3), 0.82 (d, *J* = 6.5 Hz, 3H, Leu CH_3). **^{13}C NMR** (151 MHz, DMSO-d_6): δ 195.5 (SC=O), 173.2 (C=O), 171.8 (C=O), 170.4 (C=O), 170.2 (C=O), 167.4 (C=O), 61.6 (C6), 61.1 (C8), 56.2 (C7), 55.1 (C5), 52.9 (C1), 52.2 (Leu αC), 48.3 (Asp αC), 43.6 (Asp βC), 38.8 (C4), 30.6 (C3), 28.5 (C2), 25.5 (Leu βC), 24.3 (Leu γC), 22.8 (Leu δC), 21.4 (Leu δC). **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{21}H_{37}N_6O_8S$ 533.2388, found 533.2390.

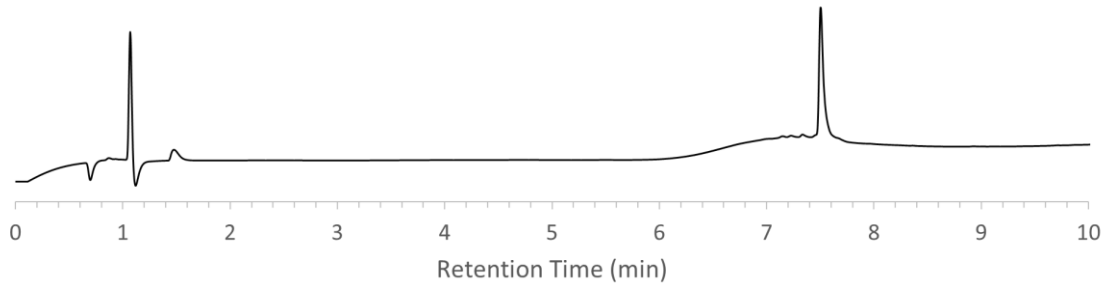
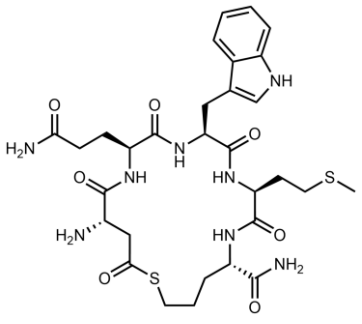


Figure 5.16 HPLC trace (220 nm) of purified **122**.

(5S,8S,11S,14S,17S)-11-((1H-indol-3-yl)methyl)-17-amino-14-(3-amino-3-oxopropyl)-8-(2-(methylthio)ethyl)-7,10,13,16,19-pentaoxo-1-thia-6,9,12,15-tetraazacyclononadecane-5-carboxamide (123)



Prepared following General *Procedure 3B* from linear peptide **117** (10.0 mg). Purified by semi-preparative HPLC (5 – 15% MeCN in H_2O with 0.1% TFA over 60 min). After lyophilisation, the desired product was isolated as a fluffy white powder (2.2 mg, 22%). Purity: >75%.

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 10 min, 220 nm): 8.28 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{30}H_{43}N_8O_7S_2$ 691.2691, found 691.2688.

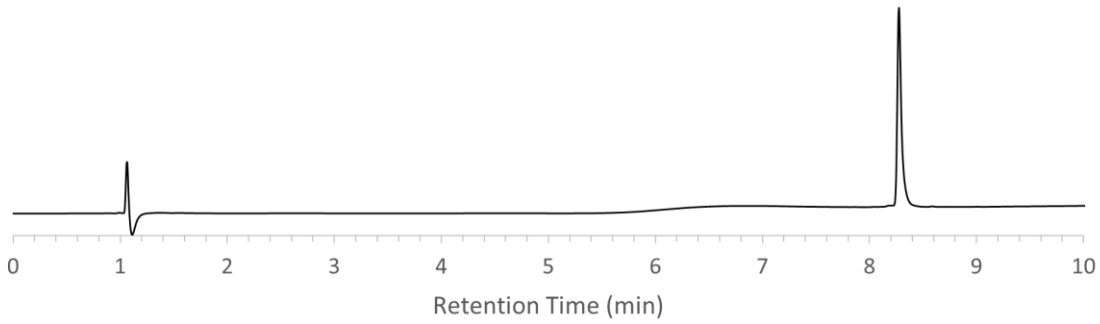
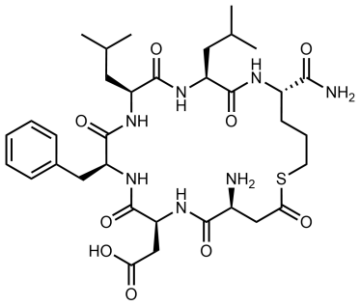


Figure 5.17 HPLC trace (220 nm) of purified **123**.

2-((4S,7S,10S,13S,16S,19S)-4-amino-10-benzyl-19-carbamoyl-13,16-diisobutyl-2,5,8,11,14,17-hexaoxo-1-thia-6,9,12,15,18-pentaazacyclodocosan-7-yl)acetic acid (124)



Prepared following General *Procedure 3B* from linear peptide **118** (10.0 mg). Purified by semi-preparative HPLC (5 – 15% MeCN in H_2O with 0.1% TFA over 80 min). After lyophilisation, the desired product was isolated as a fluffy white powder (3.1 mg, 31%). Purity: >75%

HPLC t_R (5 – 95% MeCN in H_2O with 0.1% TFA over 30 min, 220 nm): 12.2 min. **HRMS** ESI *(m/z)*: $[M+H]^+$ calculated for $C_{34}H_{53}N_7O_9S$ 734.3454, found 734.3546.

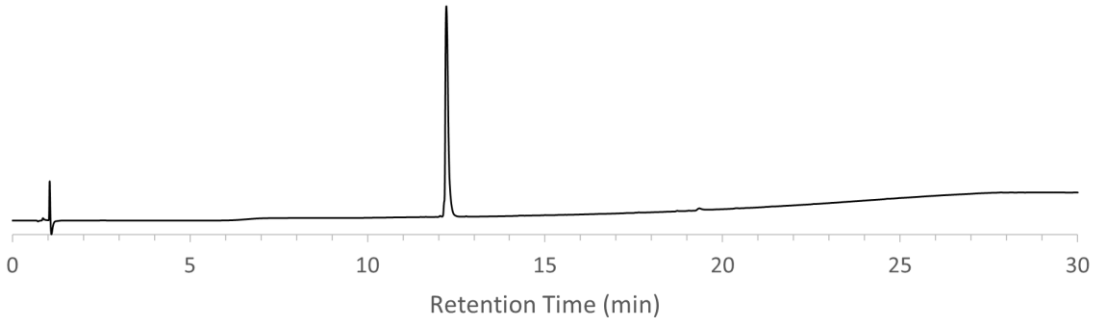


Figure 5.18 HPLC trace (220 nm) of purified **124**.

Purification Attempts with 3-Mercaptopropyl Functionalised Silica

Linear peptide **117** (10.0 mg, 14.5 μmol) and DPAP (3.7 mg, 14.5 μmol) were dissolved in 1:1 H_2O:MeCN (2.9 mL) containing 0.1% TFA. The resulting solution was stirred at rt under UV irradiation for 15 min. Then, 200-400 mesh 3-mercaptopropyl functionalised silica gel (1–3 equiv.) and DPAP (1–3 equiv.) were added and the mixture was stirred at rt for 1 h under UV irradiation. The reaction mixture was then filtered using a syringe filter to remove the silica, and the filtrate was analysed by analytical HPLC (5 – 95% MeCN/H_2O + 0.1% TFA v/v over 10 min). If required, the filtrate was purified by semi-preparative HPLC (5 – 15% MeCN in H_2O with 0.1% TFA over 60 min).

Cyclisation Using Blue LED

Linear peptide **116** (10.0 mg, 18.8 μmol) and Eosin Y (13.0 mg, 18.8 μ mol) were dissolved in 1:1 H_2O:MeCN (3.75 mL) containing 0.1% v/v TFA and 6 M guanidinium hydrochloride in a *via*l. The resulting solution was stirred at rt under blue LED irradiation for 15 min. The reaction mixture was purified by semi-preparative HPLC (5 – 95% MeCN in H_2O with 0.1% TFA over 60 min). After lyophilisation, the cyclised product **122** was isolated as a fluffy white powder (4.0 mg, 40%). Purity: >95%.

5.4 Experimental Details for Chapter 4

5.4.1 General Procedures

Procedure 4A: Synthesis of STrt Thioesters

To a solution of carboxylic acid (1 equiv., 0.2 M) in anhydrous DCM under argon was added EDC.HCl (1.2 equiv.) and the solution was stirred at 0 °C for 10 min. Then, DMAP (0.2 equiv.) and triphenylmethanethiol (1 equiv.) were added and the mixture was stirred at rt for 18 h. The reaction mixture was concentrated *in vacuo* and the residue was purified directly by silica

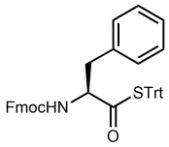
gel flash chromatography (EtOAc/Hexane gradient). Fraction containing pure product were combined and the solvent was removed *in vacuo* to afford the thioester.

Procedure 4B: Dethiocarboxylation Using Blue LED Irradiation

Thioacid (1 equiv.) and Eosin Y (0.25 equiv.) were dissolved in EtOAc (0.1 M) and stirred at rt under blue LED irradiation for 1 h. The reaction mixture was concentarted *in vacuo* and the residue was purified by silica gel flash chromatography (EtOAc/Hexane gradient). Fractions containing pure product were combined and the solvent was removed *in vacuo* to afford the dethiocarboxylated product.

5.4.2 Characterisation Data

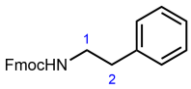
S-trityl (*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-phenylpropanethioate (129)



Synthesised from Fmoc-Phe-OH (1.00 g, 2.58 mmol) using *Procedure 4A*. Isolated as a white foam (1.46 g, 87%). Isolated compound was in good agreement with the literature.[218]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.7 Hz, 2H, Fmoc ArH), 7.53 (t, *J* = 7.7 Hz, 2H, Fmoc ArH), 7.39 (t, *J* = 7.7 Hz, 2H, Fmoc ArH), 7.31 – 7.15 (m, 20H, Fmoc ArH, Trt ArH, Phe ArH), 7.02 (d, *J* = 7.7 Hz, 2H, Phe ArH), 5.09 (d, *J* = 9.0 Hz, 1H, NH), 4.73 – 4.68 (m, 1H, Phe αCH), 4.40 – 4.32 (m, 2H, Fmoc CH_2), 4.19 (t, *J* = 7.2 H, 1H, Fmoc CH), 3.04 – 2.93 (m, 2H, Phe βCH_2). **HRMS** ESI *(m/z):* [M+Na]$^+$calculated for $C_{43}H_{35}NNaO_3S$; 668.2229; found 668.2230.

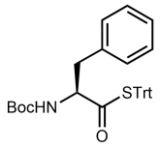
(9*H*-fluoren-9-yl)methyl phenethylcarbamate (131)



Thioester **129** (0.40 g, 0.62 mmol) was dissolved in DCM (6.2 mL) and deprotected to the required thioacid with triethylsilane (1.98 mL, 12.4 mmol) and TFA (2.73 mL, 25% v/v) at rt for 5 min under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised using *Procedure 4B*. Isolated as a pale-yellow oil (0.153 g, 72%). Isolated compound was in good agreement with the literature.[265]

^{1}H NMR (400 MHz, $CDCl_3$, mixture of two rotamers) δ 7.76 (d, *J* = 7.6 Hz, 2H, Fmoc Ar H), 7.57 (d, *J* = 7.6 Hz, 2H, Fmoc Ar H), 7.46 – 7.39 (m, 2H, Fmoc Ar H), 7.37 – 7.30 (m, 4H, Fmoc Ar H, Ar H), 7.29 – 7.24 (m, 1H, Ar H), 7.23 – 7.16 (m, 2 H, Ar H), 4.77 (bs, 1H, NH), 4.43 (d, *J* = 7.0 Hz, 2H, Fmoc CH_2), 4.24 (t, *J* = 7.0 Hz, 1H, Fmoc CH), 3.36 (q, *J* = 6.8 Hz, 2H, $H1_{major}$), 3.34 (bs, 2H, $H1_{minor}$), 2.84 (t, *J* = 6.9 Hz, 2H, $H2_{major}$), 2.67 (bs, 2H, $H2_{minor}$). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.4 (C=O), 144.1 (Ar qC), 141.5 (Ar qC), 138.9 (Ar qC), 129.0 (Ar H), 128.8 (Ar H), 127.8 (Ar H), 127.2 (Ar H), 126.6 (Ar H), 125.2 (Ar H), 120.1 (Ar H), 66.7 (Fmoc CH_2), 47.5 (Fmoc CH), 42.4 (C1), 36.3 (C2). **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{23}H_{21}NNaO_2$ 366.1465; found 366.1464.

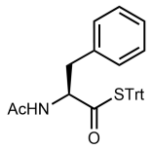
S-trityl (*S*)-2-((tert-butoxycarbonyl)amino)-3-phenylpropanethioate (132)



Synthesised from Boc-Phe-OH (1.00 g, 3.76 mmol) using *Procedure 4A*. Isolated as a pale yellow foam (1.55 g, 87%). Ioslated compound was in good agreement with the literature.[266]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29 – 7.20 (m, 18H, Trt ArH, Phe ArH), 7.04 – 6.98 (m, 2H, Phe ArH), 4.80 (d, *J* = 8.8 Hz, 1H, NH), 4.60 (m, 1H, Phe αCH), 2.97 (dd, *J* = 14.1, 5.9 Hz, 1H, Phe βCH_2), 2.88 (dd, *J* = 14.1, 6.9 Hz, 1H, Phe βCH_2), 1.41 (s, 9H, *t*Bu). **HRMS** ESI *(m/z):* $[M+Na]^+$ calculated for $C_{33}H_{33}NNaO_3S$ 546.2079; found 546.2081.

S-trityl (*S*)-2-acetamido-3-phenylpropanethioate (133)

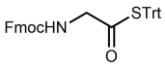


Synthesised from Ac-Phe-OH (1.50 g, 7.24 mmol) using *Procedure 4A*. Isolated as a white foam (2.96 g, 88%).

R_f (50:50 EtOAc:Hex): 0.48. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.31 – 7.06 (m, 18H. Trt ArH, Phe ArH), 6.98 – 6.85 (m, 2H, Phe ArH), 5.60 (d, *J* = 8.5 Hz, 1H, NH), 4.92 (dt, *J* = 8.5, 6.2 Hz, 1H, Phe αCH), 3.03 – 2.82 (m, 2H, Phe βCH_2), 1.85 (s, 3H, CH_3). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 196.9 (SC=O), 169.7 (C=O), 143.4 (Ar qC), 135.5 (Ar qC), 130.0 (Ar C), 129.7 (Ar C), 128.7 (Ar C), 127.9 (Ar C), 127.3 (Ar C), 127.3 (Ar C), 70.9 (Phe αC), 59.5 (Trt qC), 38.1 (Phe βC), 23.3 (CH_3).

ν_{max} (film)/cm^{-1}: 3268 (NH), 3058, 3030, 1689 (C=O), 1652 (C=O), 1492, 1443, 1265, 735, 695. **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{30}H_{27}NNaO_2S$ 488.1663; found 488.1655.

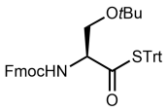
S-trityl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)ethanethioate (134)



Synthesised from Fmoc-Gly-OH (1.00 g, 3.36 mmol) using *Procedure 4A*. Isolated as a white foam (1.14 g, 78%). Isolated compound was in good agreement with the literature.[218]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H, Fmoc ArH), 7.57 (d, *J* = 7.5 Hz, 2H, Fmoc ArH), 7.39 (t, *J* = 7.5 Hz, 2H, Fmoc ArH), 7.32 – 7.20 (m, 17H, Fmoc ArH, Trt ArH), 5.27 (t, *J* = 5.7 Hz, 1H, NH), 4.38 (d, *J* = 7.2 Hz, 2H, Fmoc CH_2), 4.20 (t, *J* = 7.2 Hz, 1H, Gly αCH_2), 4.12 (d, *J* = 5.7 Hz, 2H, Fmoc CH). **HRMS** ESI-MS (*(m/z)*): $[M+Na]^+$ calculated for $C_{36}H_{29}NNaO_3S$ 578.1764; found 578.1760.

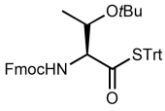
S-trityl(*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(tert-butoxy)propanethioate (135)



Synthesised from Fmoc-Ser(O*t*Bu)-OH (1.00 g, 2.61 mmol) using *Procedure 4A*. Isolated as a white foam (1.19 g, 71%). Isolated compound was in good agreement with the literature.[218]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 – 7.77 (m, 2H, Fmoc ArH), 7.65 (t, *J* = 7.9 Hz, 2H, Fmoc ArH), 7.41 (t, *J* = 7.9 Hz, 2H, Fmoc ArH), 7.32 – 7.24 (m, 17H, Fmoc ArH, Trt ArH), 5.78 (d, *J* = 5.8 Hz, NH), 4.57 – 4.46 (m, 2H, Fmoc CH_2), 4.37 – 4.28 (m, 2H, Fmoc CH, Ser αCH), 3.87 (dd, *J* = 8.4, 2.2 Hz, 1H, Ser βCH_2), 3.49 (dd, *J* = 8.4, 3.2 Hz, 1H, Ser βCH_2), 1.22 (s, 9H, *t*Bu). **HRMS** ESI *(m/z)* calculated for $C_{41}H_{39}NO_4S$ $[M+Na]^+$; 664.2492, found 664.2491.

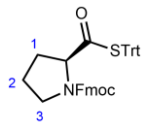
S-trityl(2*S*,3*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-(tert-butoxy)butanethioate (136)



Synthesised from Fmoc-Thr(O*t*Bu)-OH (1.00 g, 2.52 mmol) using *Procedure 4A*. Isolated as a white foam (1.35 g, 82%). Isolated compound was in good agreement with the literature.[218]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (dd, *J* = 7.4, 2.5 Hz, 2H, Fmoc ArH), 7.64 (dd, *J* = 10.2, 7.4 Hz, 2H, Fmoc ArH), 7.39 (t, *J* = 7.4 Hz, 2H, Fmoc ArH), 7.32 – 7.16 (m, 17H, Fmoc ArH, Trt ArH), 5.72 (d, *J* = 9.4 Hz, 1H, NH), 4.56 (dd, *J* = 10.2, 6.5 Hz, 1H, Fmoc CH_2), 4.41 – 4.15 (m, 4H, Fmoc CH_2, Fmoc CH, Thr αCH, Thr βCH), 1.14 – 1.00 (m, 12H, Thr CH_3, *t*Bu). **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{42}H_{41}NNaO_4S$ 678.2648; found 678.2655.

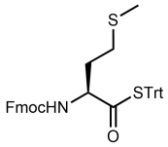
(9H-fluoren-9-yl)methyl (*S*)-2-((tritylthio)carbonyl)pyrrolidine-1-carboxylate (137)



Synthesised from Fmoc-Pro-OH (1.00 g, 2.96 mmol) using *Procedure 4A*. Isolated as a white foam (0.91 g, 51%). Isolated compound was in good agreement with the literature.[218]

^{1}H NMR (400 MHz, $CDCl_3$, mixture of cis/trans isomers) δ 7.83 – 7.77 (m, 2H, Fmoc ArH), 7.70 – 7.61 (m, 2H, Fmoc ArH), 7.46 – 7.38 (m, 2H, Fmoc ArH), 7.35 – 7.18 (m, 17H, Fmoc ArH, Trt ArH), 4.60 – 4.37 (m, 3H, Fmoc CH_2, Fmoc CH), 4.36 – 4.24 (m, 1H, Pro αCH), 3.69 – 3.69 (m, 1H, H3), 3.59 – 3.45 (m, 1H, H3), 2.24 – 2.01 (m, 1H, H1), 2.00 – 1.79 (m, 3H, H1, H2). **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{39}H_{33}NNaO_3S$ 618.2079; found 618.2077.

S-trityl(*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-(methylthio)butanethioate (138)

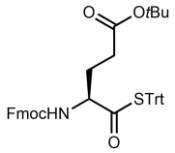


Synthesised from Fmoc-Met-OH (1.00 g, 2.69 mmol) using *Procedure 4A*. Isolated as a white foam (1.41, 83%).

R_f (30:50 EtOAc:Hex): 0.62. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 7.6 Hz, 2H, Fmoc ArH), 7.61 (dd, *J* = 7.6, 2.3 Hz, 2H, Fmoc ArH), 7.42 (t, *J* = 7.5 Hz, 2H, Fmoc ArH), 7.34 – 7.21 (m, 17H, Fmoc ArH, Trt ArH), 5.41 (d, *J* = 8.4 Hz, 1H, NH), 4.59 (td, *J* = 8.4, 4.8 Hz, 1H, Met αCH), 4.54 – 4.38 (m, 2H, Fmoc CH_2), 4.25 (t, *J* = 7.0 Hz, 1H, Fmoc CH), 2.35 (t, *J* = 7.0 Hz, 2H, Met γCH_2), 2.15 – 2.00 (m, 4H, Met CH_3, Met βCH_2), 1.92 – 1.79 (m, 1H, Met βCH_2). **^{13}C NMR** (101

MHz, $CDCl_3$) δ 197.1 (SC=O), 155.7 (C=O), 143.3 (Ar qC), 141.3 (Ar qC), 129.8 (Ar C), 127.8 (Ar C), 127.8 (Ar C), 127.3 (Ar C), 127.1 (Ar C), 125.1 (Ar C), 120.0 (Ar C), 70.9 (Met αC), 67.2 (Fmoc CH_2), 60.1 (Trt qC), 47.2 (Fmoc CH), 32.3 (Met βC), 29.6 (Met γC), 15.4 (Met CH_3). **ν_{max} (film)/cm^{-1}**: 3328 (NH), 3060, 2917, 1726 (C=O), 1696 (C=O), 1496, 1445, 1244, 1035, 740, 699. **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{39}H_{35}NNaO_3S_2$ 652.1956; found 692.1951.

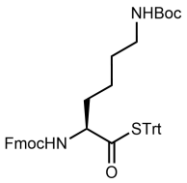
tert-butyl (*S*)-4-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-oxo-5-(tritylthio)pentanoate (139)



Synthesised from Fmoc-Glu(O*t*Bu)-OH (1.00 g, 2.35 mmol) using *Procedure 4A*. Isolated as a white foam (1.10 g, 68%).

R_f (30:50 EtOAc:Hex): 0.59. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.78 (d, *J* = 7.4 Hz, 2H, Fmoc ArH), 7.61 (dd, *J* = 7.6, 3.0 Hz, 2H, Fmoc ArH), 7.41 (t, *J* = 7.5 Hz, 2H, Fmoc ArH), 7.35 – 7.21 (m, 17H, Fmoc ArH, Trt ArH), 5.58 (d, *J* = 8.2 Hz, 1H, NH), 4.53 – 4.40 (m, 2H, Fmoc CH_2), 4.37 – 4.29 (m, 1H, Glu αCH), 4.25 (t, *J* = 7.1 Hz, 1H, Fmoc CH), 2.26 – 1.99 (m, 3H, Glu βCH_2, Glu γCH_2), 1.92–1.76 (m, 1H, Glu βCH_2), 1.46 (s, 9H, *t*Bu). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 197.4 (SC=O), 172.2 (C=O), 155.7 (Fmoc C=O), 143.4 (Ar qC), 141.3 (Ar qC), 129.8 (Ar C), 127.8 (Ar C), 127.8 (Ar C), 127.2 (Ar C), 127.1 (Ar C), 125.1 (Ar C), 120.0 (Ar C), 80.9 (*t*Bu qC), 70.7 (Glu αC), 67.2 (Fmoc CH_2), 60.4 (Trt qC), 47.1 (Fmoc CH), 31.1 (Glu βC), 28.1 (*t*Bu CH_3), 27.8 (Glu γC). **ν_{max} (film)/cm^{-1}**: 3327 (NH), 3061, 2977, 1723 (C=O), 1699 (C=O), 1494, 1446, 1245, 1151, 737, 697. **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{43}H_{41}NO_5S$ 706.2603; found 706.2591.

S-trityl(*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-6-((tert-butoxycarbonyl)amino)hexanethioate (140)

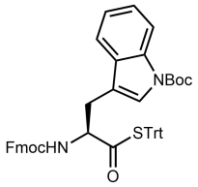


Synthesised from Fmoc-Lys(Boc)-OH (1.00 g, 2.13 mmol) using *Procedure 4A*. Isolated as a white foam (1.42 g, 92%). Isolated compound was in good agreement with the literature.[218]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.7 Hz, 2H, Fmoc Ar H), 7.59 (d, *J* = 7.4 Hz, 2H, Fmoc Ar H), 7.39 (t, *J* = 7.5 Hz, 2H, Fmoc Ar H), 7.31 – 7.18 (m, 17H, Fmoc Ar H, Trt Ar H), 5.33 (d, *J* =

8.2 Hz, 1H, NH), 4.53 – 4.30 (m, 4H, NH, Fmoc CH_2, Lys αCH), 4.23 (t, *J* = 7.0 Hz, 1H, Fmoc CH), 3.13 – 2.97 (m, 2H, Lys εCH_2), 1.81 – 1.70 (m, 1H, Lys βCH_2), 1.65 – 1.53 (m, 1H, Lys βCH_2), 1.49 – 1.35 (m, 11H, *t*Bu, Lys δCH_2), 1.25–1.08 (m, 2H, Lys γCH_2). **HRMS** ESI *(m/z)*: $[M+Na]^+$ calculated for $C_{45}H_{46}N_2NaO_5S$ 749.3025; found 749.3021

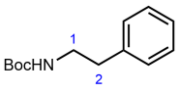
tert-butyl(*S*)-3-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-oxo-3-(tritylthio)propyl)-1H-indole-1-carboxylate (141)



Synthesised from Fmoc-Trp(Boc)-OH (1.00 g, 1.90 mmol) using *Procedure 4A*. Isolated as a white foam (1.12 g, 75%).

R_f (30:50 EtOAc:Hex): 0.62. **^{1}H NMR** (400 MHz, $CDCl_3$, mixture of rotamers) δ 8.16 (d, *J* = 8.1 Hz, 1H, Trp Ar H), 7.77 (d, *J* = 7.5 Hz, 2H, Fmoc Ar H), 7.60 – 7.45 (m, 4H, Fmoc Ar H, Trp Ar H), 7.43 – 7.33 (m, 3H, Fmoc Ar H, Trp Ar H), 7.30 – 7.18 (m, 18 H, Fmoc Ar H, Trt Ar H, Trp Ar H), 5.39 (d, *J* = 8.7 Hz, 1H, NH), 4.94 – 4.77 (m, 1H, Trp αCH), 4.47 – 4.29 (m, 2H, Fmoc CH_2), 4.25 – 4.11 (m, 1H, Fmoc CH), 3.19 (dd, *J* = 15.0, 5.8 Hz, 1H, Trp βCH_2), 3.08 (dd, *J* = 15.0, 7.0 Hz, 1H, Trp βCH_2), 1.68 (s, 9H, *t*Bu). **^{13}C NMR** (101 MHz, $CDCl_3$, mixture of rotamers, minor rotamer*) δ 197.2 (SC=O), 155.6 (C=O), 149.6 (C=O), 143.7 (Ar qC), 143.4 (Ar qC), 141.3 (Ar qC), 135.3 (Ar qC), 130.3 (Ar qC), 129.8 (Ar C), 127.8 (Ar C), 127.7* (Ar C), 127.2 (Ar C), 127.1* (Ar C), 125.2 (Ar C), 125.1* (Ar C), 124.7 (Ar C), 124.5* (Ar C), 122.8 (Ar C), 120.0 (Ar C), 119.0 (Ar C), 115.4 (Ar C), 114.6 (Ar qC), 83.8 (*t*Bu qC), 70.9 (Trp αC), 67.4 (Fmoc CH_2), 60.6 (Trt qC), 47.1 (Fmoc CH), 28.2 (*t*Bu CH_3), 27.9 (Trp βCH_2). **ν_{max} (film)/cm^{-1}**: 3366 (NH), 3060, 2978, 1726 (C=O), 1694 (C=O), 1450, 1369, 1253, 1154, 1086, 737, 698. **HRMS** ESI *(m/z)*: calculated for $C_{50}H_{44}N_2O_5S$ $[M+Na]^+$; 807.2869, found 807.2863.

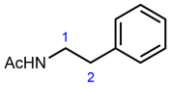
tert-butyl phenethylcarbamate (142)



Thioester **132** (0.370 g, 0.71 mmol) was dissolved in DCM (7.1 mL) and deprotected to the required thioacid with triethylsilane (2.27 mL, 14.2 mmol) and dropwise addition of TFA (0.49 mL, 5% v/v) at 0 °C for 5 min under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised using *Procedure 4B*. Isolated as a pale yellow oil (0.124 g, 79%). Isolated compound was in good agreement with the literature.[265]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.19 (m, 5H, Ar H), 4.57 (bs, 1H, NH), 3.44 – 3.34 (m, 2H, H1), 2.82 (t, *J* = 7.0 Hz, 2H, H2), 1.46 (s, 9H, *t*Bu). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 155.9 (C=O), 139.0 (Ar qC), 128.8 (Ar C), 128.6 (Ar C), 126.4 (Ar C), 79.1 (*t*Bu qC), 41.9 (C1), 36.3 (C2), 28.4 (*t*Bu CH_3). **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{13}H_{19}NNaO_2$ 244.1313; found 244.1312.

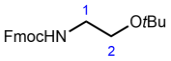
_N_-phenethylacetamide (143)



Thioester **133** (0.42 g, 0.90 mmol) was dissolved in DCM (5 mL) and deprotected to the required thioacid with triethylsilane (2.88 mL, 18.0 mmol) and TFA (2.63 mL, 25% v/v) at rt for 5 min under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised using *Procedure 4B*. Isolated as a white solid (0.040 g, 27%). Isolated compound was in good agreement with the literature.[267]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 – 7.02 (m, 5H, Ar H), 5.37 (s, 1H, NH), 3.78 – 3.26 (m, 2H, H1), 2.75 (t, *J* = 6.9 Hz, 2H, H2), 1.88 (s, 3H, CH_3). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 170.1 (C=O), 139.0 (Ar qC), 128.8 (Ar C), 128.7 (Ar C), 126.7 (Ar C), 40.8 (C1), 35.8 (C2), 23.5 (CH_3). **HRMS** ESI (*m*/*z*): $[M+H]^+$ calculated for $C_{10}H_{14}NO$ 164.1064; found 164.1069.

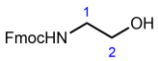
(9*H*-fluoren-9-yl)methyl (2-(tert-butoxy)ethyl)carbamate (146)



Thioester **135** (0.32 g, 0.50 mmol) was dissolved in DCM (2.5 mL) and deprotected to the required thioacid with triethylsilane (1.60 mL, 10.0 mmol) and TFA (1.37 mL, 25% v/v) at rt for 5 min under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised using *Procedure 4B*. Isolated as a pale-yellow oil (0.087 g, 51%).

R_f (50:50 EtOAc:Hex): 0.64. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.5 Hz, 2H, Fmoc Ar H), 7.60 (dd, *J* = 7.5, 1.1 Hz, 2H, Fmoc Ar H), 7.44 – 7.36 (m, 2H, Fmoc Ar H), 7.31 (td, *J* = 7.5, 1.1 Hz, 2H, Fmoc Ar H), 5.14 (d, *J* = 7.1 Hz, 1H, NH), 4.39 (d, *J* = 7.1 Hz, 2H, Fmoc CH_2), 4.24 (t, *J* = 7.1 Hz, 1H, Fmoc CH), 3.47 – 3.42 (m, 2H, H2), 3.39 – 3.33 (m, 2H, H1),1.20 (s, 9H, *t*Bu). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.6 (C=O), 144.2 (Ar qC), 141.5 (Ar qC), 127.8 (Ar H), 127.2 (Ar H), 125.2 (Ar H), 120.1 (Ar H), 73.3 (*t*Bu qC), 66.9 (Fmoc CH_2), 60.8 (C2), 47.4 (Fmoc CH), 41.8 (C1), 27.7 (*t*Bu CH_3). **ν_{max} (film)/cm^{-1}**: 3344 (NH), 2973, 1702 (C=O), 1515, 1450, 1363, 1250, 1194, 1090, 758, 740. **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{21}H_{25}NNaO_3S$ 362.1727; found 362.1733.

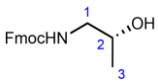
(9*H*-fluoren-9-yl)methyl (2-hydroxyethyl)carbamate (147)



Thioester **135** (0.38 g, 0.59 mmol) was dissolved in DCM (2.5 mL) and deprotected to the required thioacid with triethylsilane (1.89 mL, 11.8 mmol) and TFA (4.40 mL, 50% v/v) at rt for 2 h under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesized using *Procedure 4B*. Isolated as a pale-yellow oil (26 mg, 16%). Isolated compound was in good agreement with the literature.[268]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (d, *J* = 7.5 Hz, 2H, Fmoc Ar H), 7.59 (d, *J* = 7.4 Hz, 2H, Fmoc Ar H), 7.47 – 7.38 (m, 2H, Fmoc Ar H), 7.33 (td, *J* = 7.5, 1.2 Hz, 2H, Fmoc Ar H), 5.21 (s, 1H, NH), 4.45 (d, *J* = 6.8 Hz, 2H, Fmoc CH_2), 4.23 (t, *J* = 6.8 Hz, 1H, Fmoc CH), 3.72 – 3.68 (m, 2H, H1), 3.38 – 3.32 (m, 2H, H2), 2.20 (bs, 1H, OH). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 157.1 (C=O), 143.9 (Ar qC), 141.4 (Ar qC), 127.7 (Ar C), 127.1 (Ar C), 125.0 (Ar C), 120.0 (Ar C), 66.8 (Fmoc CH_2), 62.3 (C2), 47.3 (Fmoc CH), 43.5 (C1). HRMS ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{17}H_{17}NNaO_3$ 306.1101; found 306.1105.

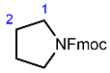
(9*H*-fluoren-9-yl)methyl (*R*)-(2-hydroxypropyl)carbamate (148)



Thioester **136** (0.44 g, 0.67 mmol) was dissolved in DCM (2.5 mL) and deprotected to the required thioacid with triethylsilane (2.15 mL, 13.4 mmol) and TFA (4.65 mL, 50% v/v) at rt for 2 h under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised using *Procedure 4B*. Isolated as pale-yellow oil (0.052 g, 42%).

R_f (50:50 EtOAc:Hex): 0.27. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.77 (dt, *J* = 7.6, 1.0 Hz, 2H, Fmoc Ar H), 7.59 (dt, *J* = 7.5, 0.9 Hz, 2H, Fmoc Ar H), 7.41 (tt, *J* = 7.5, 1.0 Hz, 2H, Fmoc Ar H), 7.32 (td, *J* = 7.5, 1.2 Hz, 2H, Fmoc Ar H), 5.12 (bs, 1H, NH), 4.44 (d, *J* = 6.8 Hz, 2H, Fmoc CH_2), 4.22 (t, *J* = 6.8 Hz, 1H, Fmoc CH), 3.95 – 3.91 (m, 1H, H2), 3.37 – 3.32 (m, 1H, H1), 3.09 – 3.02 (m, 1H, H1), 2.00 (bs, 1H, OH), 1.19 (d, *J* = 6.3 Hz, 3H, H3). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 157.3 (C=O), 144.1 (Ar qC), 141.5 (Ar qC), 127.9 (Ar C), 127.2 (Ar C), 125.2 (Ar C), 120.1 (Ar C), 67.6 (C2), 66.9 (Fmoc CH_2), 48.4 (C1), 47.4 (Fmoc CH), 20.9 (C3). **ν_{max} (film)/cm^{-1}**: 3358 (OH), 2968, 1698 (C=O), 1548, 1450, 1269, 1152, 760, 741. **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculate for $C_{18}H_{19}NNaO_3$ 320.1257; found 320.1258.

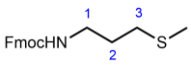
(9*H*-fluoren-9-yl)methyl pyrrolidine-1-carboxylate (149)



Thioester **137** (0.50 g, 0.84 mmol) was dissolved in DCM (2.5 mL) and deprotected to the required thioacid with TES (2.70 mL, 16.8 mmol) and TFA (1.73 mL, 25% *v*/*v*) at rt for 5 min under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised *Procedure 4B*. Isolated as an off-white solid (0.062 g, 25%). Isolated compound was in good agreement with the literature.[269]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (dd, *J* = 7.5, 1.0 Hz, 2H, Fmoc Ar H), 7.63 (dd, *J* = 7.5, 1.0 Hz, 2H, Fmoc Ar H), 7.42 – 7.38 (m, 2H, Fmoc Ar H), 7.32 (td, *J* = 7.4, 1.2 Hz, 2H), 4.39 (d, *J* = 7.1 Hz, 2H, Fmoc CH_2), 4.25 (t, *J* = 7.1 Hz, 1H, Fmoc CH), 3.48 – 3.36 (m, 4H, H1), 1.95 – 1.85 (m, 4H, H2). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 155.0 (C=O), 144.2 (Ar qC), 141.3 (Ar qC), 127.6 (Ar C), 127.0 (Ar C), 125.2 (Ar C), 120.0 (Ar C), 67.1 (Fmoc CH_2), 47.4 (Fmoc CH), 46.1 (C1), 25.4 (C2). **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{19}H_{19}NNaO_2$ 316.1313; found 316.1312.

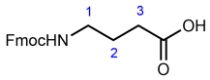
(9*H*-fluoren-9-yl)methyl (3-(methylthio)propyl)carbamate (150)



Thioester **138** (0.50 g, 0.79 mmol) was dissolved in DCM (2.5 mL) and deprotected to the required thioacid with triethylsilane (2.54 mL, 15.8 mmol) and TFA (1.68 mL, 25% v/v) at rt for 5 min under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised using *Procedure 4B*. Isolated as a pale-yellow oil (0.083 g, 32%).

^{1}H NMR (400 MHz, DMSO-d_6, mixture of rotamers) δ 7.89 (d, *J* = 7.4 Hz, 2H, Fmoc Ar H), 7.68 (d, *J* = 7.4 Hz, 2H, Fmoc Ar H), 7.41 (td, *J* = 7.5, 1.2 Hz, 2H, Fmoc Ar H), 7.36 – 7.28 (m, 3H, Fmoc Ar H, NH), 4.31 (d, *J* = 6.8 Hz, 2H, Fmoc CH_2), 4.21 (t, *J* = 6.8 Hz, 1H, Fmoc CH), 3.09 – 3.01 (m, 1.7H, H1), 2.96 – 2.85 (m, 0.3 H, H1), 2.43 (t, *J* = 7.3 Hz, 1.7H, H3), 2.34 – 2.27 (m, 0.3H, H3), 2.02 (s, 3H, CH_3), 1.65 (p, *J* = 7.0 Hz, 1.7H, H2), 1.51 – 1.44 (m, 0.3H, H2). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 156.1 (C=O), 143.9 (Ar qC), 140.7 (Ar qC), 127.6 (Ar C), 127.0 (Ar C), 125.1 (Ar C), 120.1 (Ar C), 65.1 (Fmoc CH_2), 46.8 (Fmoc CH), 39.2 (C1), 30.4 (C3), 28.8 (C2), 14.6 (CH_3). **HRMS** ESI (*m*/*z*): $[M+H]^+$ calculated for $C_{19}H_{22}NO_2S$ 328.1366; found 328.1364.

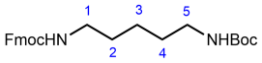
4-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)butanoic acid (151)



Thioester **139** (0.50 g, 0.73 mmol) was dissolved in DCM (0.5 mL) and deprotected to the required thioacid with TES (0.50 mL, 3.11 mmol) and TFA (9 mL, 90% v/v) at rt for 3 h under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesized using *Procedure 4B*. Purified by silica gel flash chromatography using 7% MeOH/DCM. Isolated as a white powder (0.102 g, 43%). Isolated compound was in good agreement with the literature.[270]

^{1}H NMR (400 MHz, DMSO-d_6) δ 11.88 (bs, 1H, COOH), 7.89 (d, *J* = 7.5 Hz, 2H, Fmoc Ar H), 7.68 (d, *J* = 7.6 Hz, 2H, Fmoc Ar H), 7.41 (t, *J* = 7.4 Hz, 2H, Fmoc Ar H), 7.36 – 7.28 (m, 3H, Fmoc Ar H, NH), 4.29 (d, *J* = 6.9 Hz, 2H, Fmoc CH_2), 4.21 (t, *J* = 6.9 Hz, 1H, Fmoc CH), 3.04 –2.81 (m, 2H, H1), 2.24 – 2.05 (m, 2H, H3), 1.68 – 1.41 (m, 2H, H2). **^{13}C NMR** (101 MHz, DMSO-d_6) δ 174. 2 (C=O), 156. 1 (C=O), 143.9 (Ar qC), 140.7 (Ar qC), 127.6 (Ar H), 127.0 (Ar H), 125.1 (Ar H), 120.1 (Ar H), 65.2 (Fmoc CH_2), 46.7 (Fmoc CH), 39.6 (C1), 30.9 (C3), 24.8 (C2). **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{19}H_{19}NNaO_4$ 348.1212; found 348.1207.

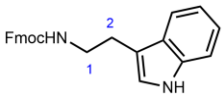
(9*H*-fluoren-9-yl)methyl tert-butyl pentane-1,5-diyldicarbamate (152)



Thioester **140** (0.50 g, 0.69 mmol) was dissolved in DCM (5 mL) and deprotected to the required thioacid with TES (2.22 mL, 13.8 mmol) and TFA (0.38 mL, 5% v/v) at 0 °C for 5 min under argon. Following concentration and drying of the reaction mixture *in vacuo*, the product was synthesised using *Procedure 4B*. Isolated as a pale-yellow oil (0.058 g, 20%). Isolated compound was in good agreement with the literature.[271]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, *J* = 7.6 Hz, 2H, Fmoc Ar H), 7.69 (d, *J* = 7.5 Hz, 2H, Fmoc Ar H), 7.40 (t, *J* = 7.5 Hz, 2H. Fmoc Ar H), 7.31 (t, *J* = 7.5 Hz, 2H, Fmoc Ar H), 4.79 (bs, 1H, NH), 4.61 – 4.36 (m, 3H, Fmoc CH_2, NH), 4.21 (d, *J* = 7.2 Hz, 1H, Fmoc CH), 3.24 – 2.99 (m, 4H, H1, H5), 1.67 – 1.23 (m, 15H, H2, H3, H4, *t*Bu). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.5 (C=O), 156.1 (C=O), 144.0 (Ar qC), 141.3 (Aq qC), 127.7 (Ar C), 127.0 (Ar C), 125.1 (Ar C), 120.0 (Ar C), 77.2 (*t*Bu qC), 66.5 (Fmoc CH_2), 47.3 (Fmoc CH), 40.9 (C5), 40.3 (C1), 29.8 (C2), 29.6 (C4), 28.4 (*t*Bu CH_3), 23.8 (C3). **HRMS** ESI (*m*/*z*) calculated for $C_{25}H_{32}N_2O_4$ $[M+Na]^+$; 447.2260, found 447.2259.

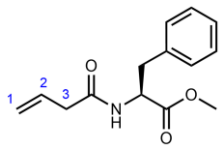
(9*H*-fluoren-9-yl)methyl (2-(1H-indol-3-yl)ethyl)carbamate (153)



Thioester **141** (0.50 g, 0.73 mmol) was dissolved in DCM (2.5 mL) and deprotected to the required thioacid with TES (2.35 mL, 14.6 mmol) and TFA (1.62 mL, 25% v/v) at rt for 2 h under argon. Following concentration and drying of the reaction mixture in vacuo, the product was synthesised using *Procedure 4B*. Isolated as a pale-yellow solid (0.151 g, 62%). Isolated compound was in good agreement with the literature.[272]

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (bs, 1H, Indole NH), 7.79 (d, *J* = 7.6 Hz, 2H, Fmoc Ar H), 7.65 (d, *J* = 7.9 Hz, 1H, Ar H), 7.59 (d, *J* = 7.5 Hz, 2H, Fmoc Ar H), 7.46 – 7.39 (m, 3H, Fmoc Ar H, Ar H), 7.32 (td, *J* = 7.5, 1.2 Hz, 2H, Fmoc Ar H), 7.26 – 7.22 (m, 1H, Ar H), 7.18 – 7.13 (m, 1H, Ar H), 7.08 (bs, 1H, Ar H) 4.87 (bs, 1H, NH), 4.43 (d, *J* = 7.0 Hz, 2H, Fmoc CH_2), 4.23 (t, *J* = 7.0 Hz, 1H, Fmoc CH), 3.63 – 3.45 (m, 2H, H1), 3.06 – 2.84 (m, 2H, H2). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 156.4 (C=O), 144.0 (Ar qC), 141.3 (Ar qC), 136.4 (Ar qC), 127.7 (Ar qC), 127.1 (Ar C), 127.0 (Ar C), 125.1 (Ar C), 122.3 (Ar C), 122.1 (Ar C), 120.0 (Ar C), 119.6 (Ar C), 118.8 (Ar C), 113.0 (Ar qC), 111.2 (Ar C), 66.6 (Fmoc CH_2), 47.3 (Fmoc CH), 41.3 (C1), 25.8 (C2). **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{25}H_{22}N_2NaO_2$ 405.1579, found 405.1580.

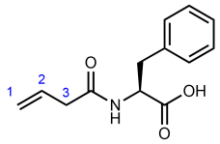
methyl but-3-enoyl-L-phenylalaninate (156)



To a solution of 3-butenoic acid **154** (1.50 mL, 17.7 mmol) in DCM (100 mL) was added EDC (6.77 g, 35.4 mmol) and HOBt (2.86 g, 21.2 mmol) and the mixture was stirred at 0 °C for 20 min under nitrogen. Then, H-Phe-OMe.HCl **155** (3.80 g, 17.6 mmol) and DIPEA (3.08 mL, 17.6 mmol) dissolved in another portion of DCM (50 mL) was added to the reaction mixture and stirred at rt under nitrogen for 16 h. The reaction mixture was concentrated *in vacuo*, diluted with EtOAc (150 mL) and washed with H_2O (150 mL), saturated aqueous $NaHCO_3$ (150 mL) and 1 M $HCl_{(aq)}$ (150 mL). The organic layer was then washed with brine (150 mL), dried over anhydrous magnesium sulfate concentrated *in vacuo*. The residue was purified by silica gel flash chromatography (20% v/v EtOAc:Hexane).Fractions containing the pure product were combined and the solvent was removed *in vacuo* to furnish the product as a pale yellow oil (3.62 g, 75%).

R_f (30:70 EtOAc:Hex): 0.20. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.33 – 7.24 (m, 3H, Ar H), 7.11 – 7.07 (m, 2H, Ar H), 6.02 (d, *J* = 7.8 Hz, 1H, NH), 6.89 (ddt, *J* = 17.2, 10.1, 7.1 Hz, 1H, H2), 5.25 – 5.18 (m, 2H, H1), 4.90 (dt, *J* = 7.8, 5.8 Hz, 1H, Phe αCH), 3.75 (s, 3H, CH_3), 3.17 (dd, *J* = 13.8, 5.8 Hz, 1H, Phe βCH_2), 3.11 (dd, *J* = 13.8. 5.8 Hz, 1H, Phe βCH_2), 3.01 (dt, *J* = 7.2, 1.2 Hz, 2H, H3). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 172.1 (C=O), 170.2 (C=O), 135.9 (Ar qC), 130.9 (C2), 129.4 (Ar C), 128.7 (Ar C), 127.3 (Ar C), 120.2 (C1), 53.2 (Phe αC), 52.5 (CH_3), 41.6 (C3), 38.0 (Phe βC). **ν_{max} (film)/cm^{-1}**: 3358 (OH), 2968, 1698 (C=O), 1548, 1450, 1269, 1152, 760, 741. **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{14}H_{17}NNaO_3$ 270.1101; found 270.1108.

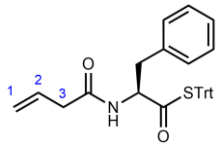
but-3-enoyl-L-phenylalanine (157)



To a solution of **156** (3.50 g, 14.2 mmol) in 1:1 MeCN/H_2O (100 mL) was added caesium carbonate (6.92 g, 21.2 mmol) and the mixture was stirred at rt for 16 h. The reaction mixture was concentrated *in vacuo*, diluted with water (50 mL) and then washed with EtOAc (100 mL). The remaining aqueous extract was acidified to pH 1 with dropwise addition of 37% HCl and then extracted with EtOAc (2 x 100 mL). The combined organic extracts were washed with brine (100 mL), dried under magnesium sulfate and then concentrated *in vacuo* to give the product as a pale-yellow oil (3.30 g, quant.).

R_f (10:90 MeOH:DCM): 0.30. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 9.32 (bs, 1H, COOH), 7.31 – 7.22 (m, 3H, Phe Ar H), 7.16 – 7.11 (m, 2H, Phe Ar H), 6.21 (d, *J* = 7.7 Hz, NH), 5.82 (ddt, *J* = 17.2, 10.2, 7.2 Hz, 1H, H2), 5.24 – 5.13 (m, 2H, H1), 4.87 (dt, 1H, *J* = 7.7, 5.7 Hz, Phe αCH), 3.23 (dd, 1H, *J* = 14.0, 5.7 Hz, Phe βCH_2), 3.13 (dd, 1H, *J* = 14.0, 6.0 Hz, Phe βCH_2), 3.00 (d, 2H, *J* = 7.2 Hz, H3).**^{13}C NMR** (101 MHz, $CDCl_3$) δ 174.5 (C=O), 171.5 (C=O), 135.6 (Ar qC), 130.4 (C2), 129.5 (Ar C), 128.7 (Ar C), 127.4 (Ar C), 120.7 (C1), 53.3 (Phe αCH), 41.2 (C3), 37.3 (Phe βC). **δν_{max} (film)/cm^{-1}**: 3322 (NH, OH), 3032, 2974, 1725 (C=O), 1630 (C=O), 1532, 1199, 701. **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{13}H_{15}NNaO_3$ 256.0944; found 256.0958.

S-trityl (S)-2-(but-3-enamido)-3-phenylpropanethioate (158)



To a solution of **157** (3.00 g, 12.9 mmol) in DCM (150 mL) was added EDC.HCl (2.96 g, 15.4 mmol) and the mixture was stirred at 0 °C for 5 min. Then DMAP (0.157 g, 1.28 mmol) and triphenylmethanethiol (4.29 g, 15.5 mmol) were added and the mixture was stirred under nitrogen at rt for 18 h. The reaction mixture was concentrated *in vacuo* and the residue was purified by silica gel flash chromatography (20% v/v EtOAc/Hexane). Fractions containing pure product were combined and the solvent was evaporated *in vacuo* to give the product as a pale-yellow oil (4.57 g, 72%).

R_f (30:70 EtOAc:Hex): 0.40. **^{1}H NMR** (400 MHz, $CDCl_3$) δ 7.33 – 7.20 (m, 18H, Trt Ar H, Phe Ar H), 7.01 (dd, *J* = 7.30, 2.2 Hz, 2H, Phe Ar H), 5.92 – 5.76 (m, 2H, NH, H2), 5.23 – 5.13 (m, 2H, H1), 5.01 (dt, *J* = 8.6, 6.3 Hz, 1H, Phe αCH), 3.06 (dd, *J* = 14.0, 6.3 Hz, 1H, Phe βCH_2), 3.00 – 2.92 (m, 3H, Phe βCH_2, H3). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 196.6 (C=O), 169.9 (C=O), 143.3 (Ar qC), 135.2 (Ar qC), 130.6 (C2), 129.8 (Ar C), 129.5 (Ar C), 128.5 (Ar C), 127.8 (Ar C), 127.1 (Ar C), 127.1 (Ar C), 120.1 (C1), 70.7 (Phe αC), 59.2 (Trt qC), 41.3 (C3), 37.9 (Phe βC). **ν_{max} (film)/cm^{-1}**: 3279 (NH), 3060, 3030, 1688 (C=O), 1652 (C=O), 1493, 1443, 909, 731, 696. **HRMS** ESI (*m*/*z*): $[M+Na]^+$ calculated for $C_{32}H_{29}NNaO_2S$ 514.1811; found 514.1810.

General Procedure for Cyclisation

To a solution of **158** (0.100 g, 0.204 mmol) in DCM (6 mL) was added DMES (0.08 mL) and TFA (2 mL) and the mixture was stirred at rt under nitrogen for 10 min. The reaction mixture was then concentrated to dryness *in vacuo* and used without further purification. The residue was dissolved in EtOAc (2.5 mM or 5 mM based on the amount of **158**) and DPAP (0.0104 g, 0.0408 mmol) was added. The mixture was stirred under UV irradiation for 15 min. The reaction mixture was concentrated *in vacuo* and analysed.

Chapter 6

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Appendix

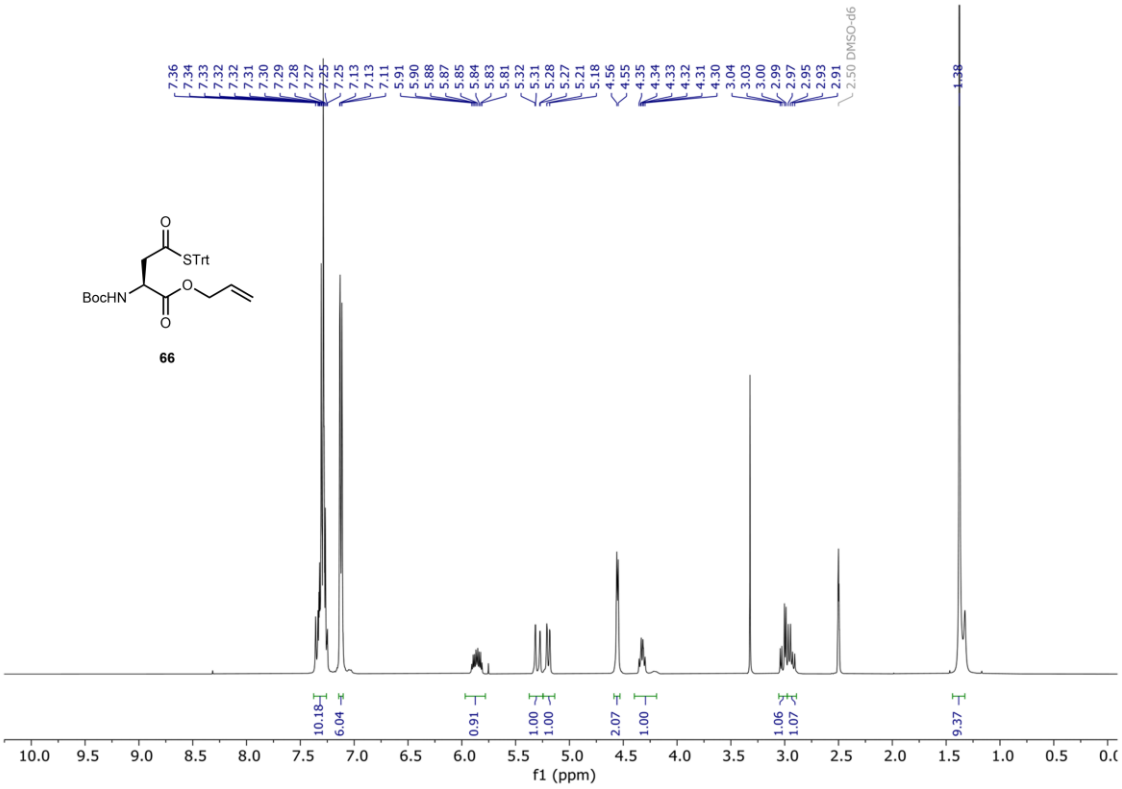

Figure A1. ^{1}H NMR spectrum of **66** (400 MHz, DMSO-d_6).

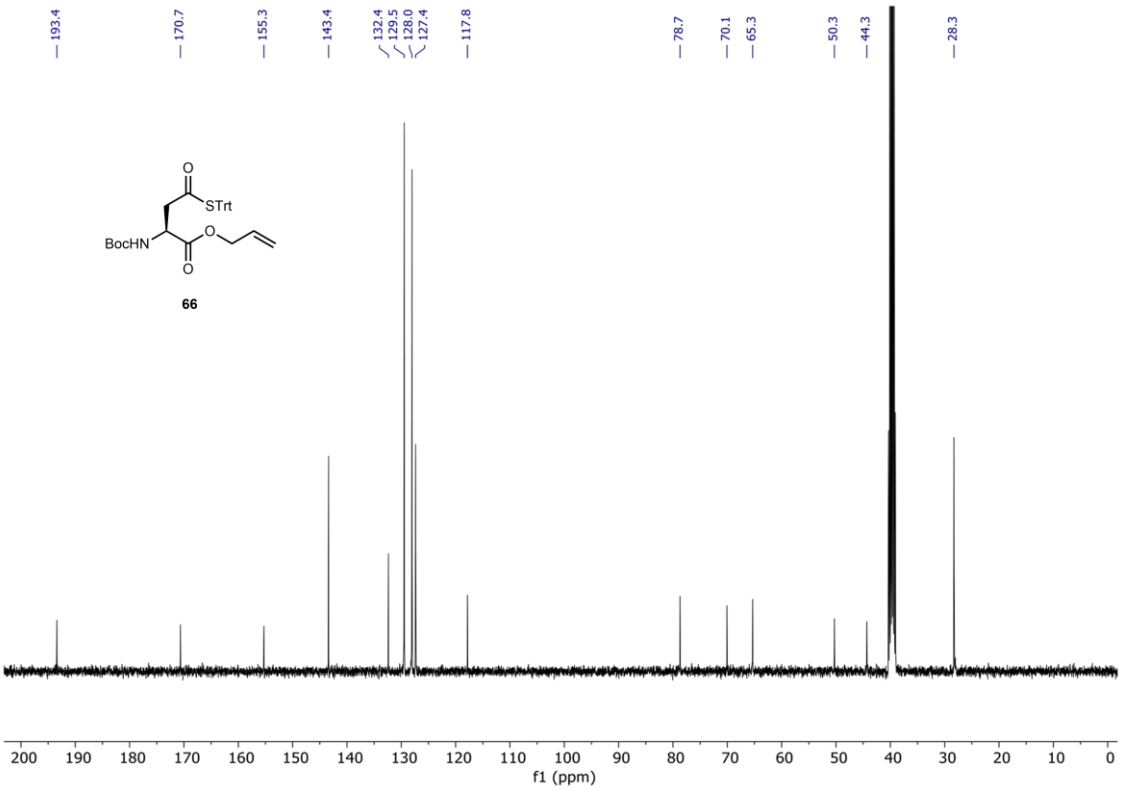

Figure A2. ^{13}C NMR spectrum of **66** (101 MHz, DMSO-d_6).

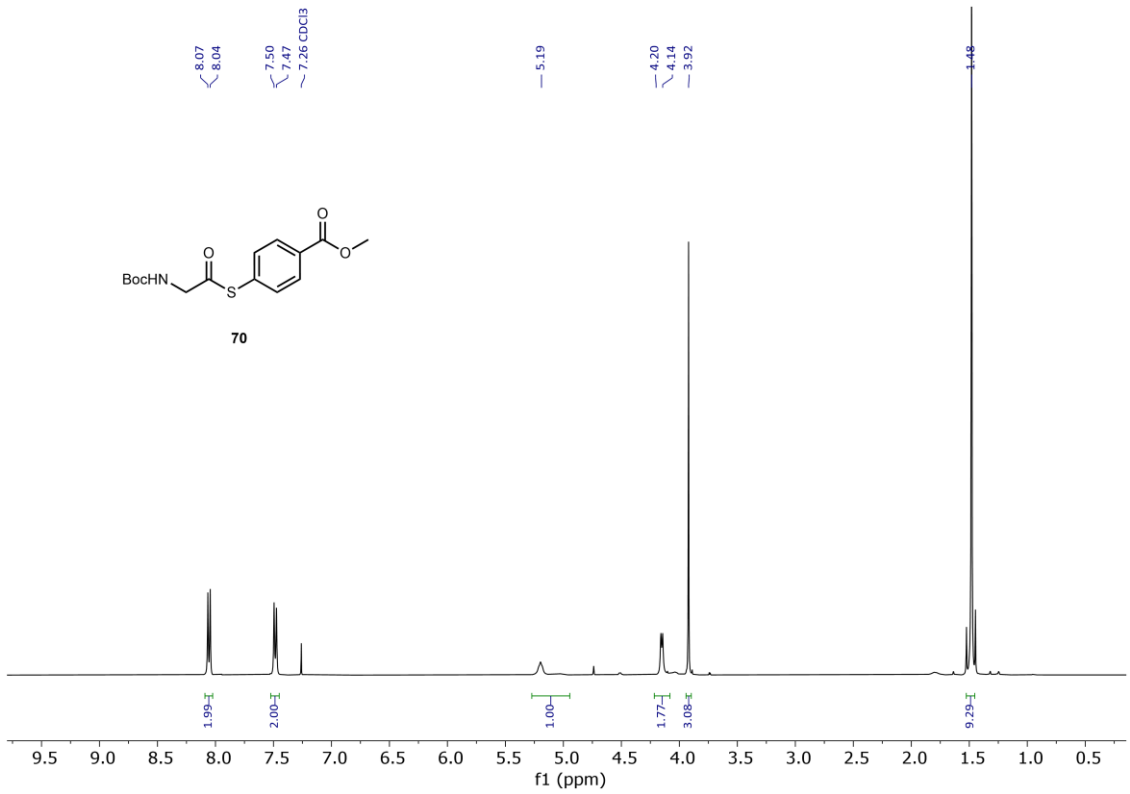

Figure A3. ^{1}H NMR spectrum of **70** (400 MHz, $CDCl_3$).

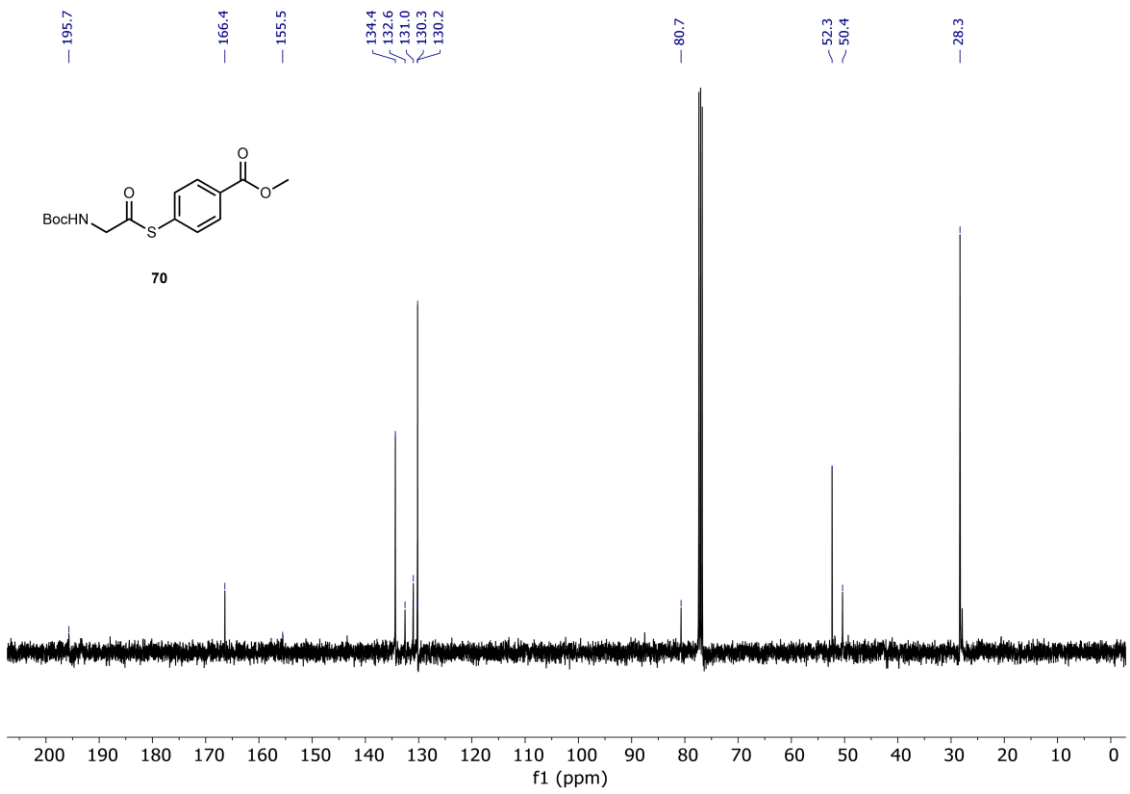

Figure A4. ^{13}C NMR spectrum of **70** (101 MHz, $CDCl_3$).

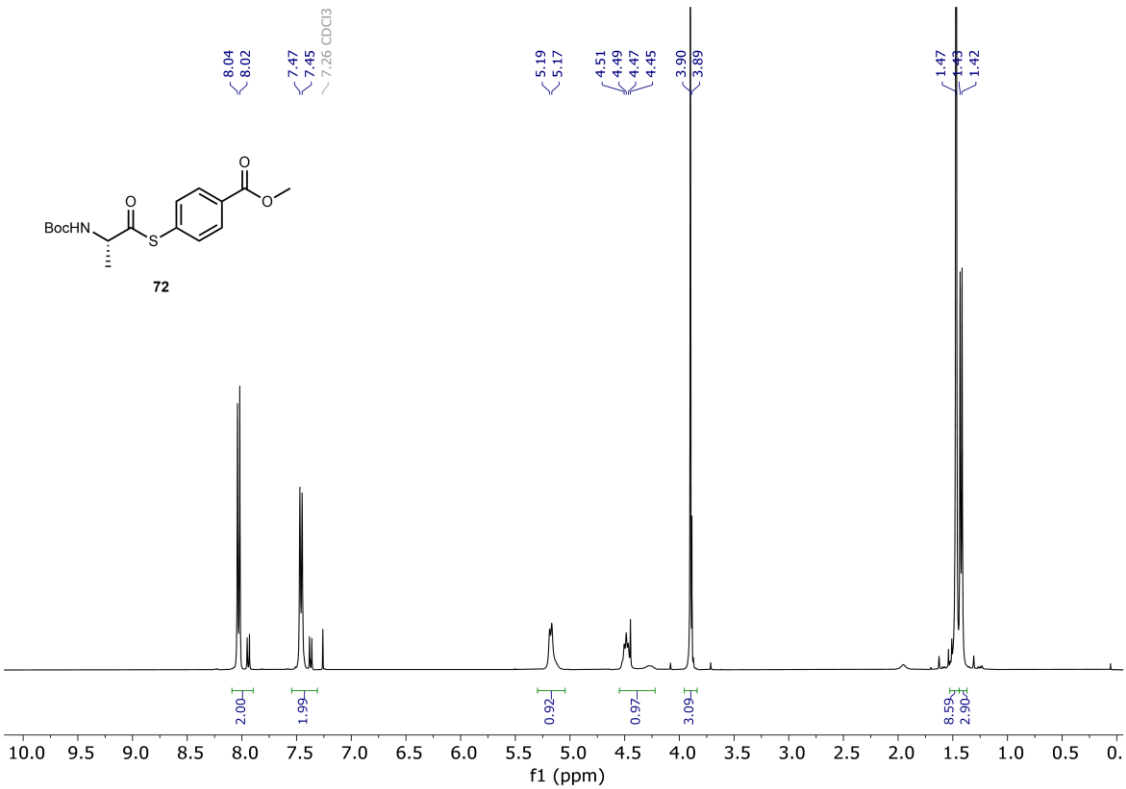

Figure A5. ^{1}H NMR spectrum of **72** (400 MHz, CDCl3).

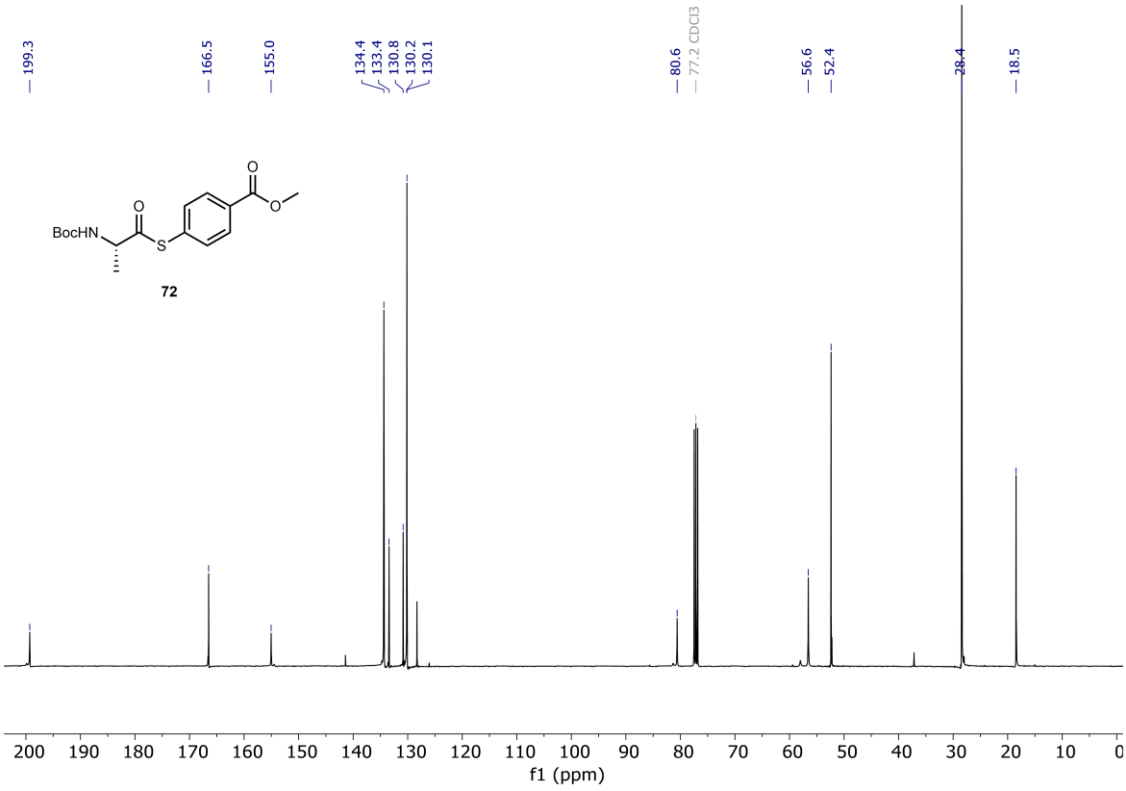

Figure A6. ^{13}C NMR spectrum of **72** (101 MHz, $CDCl_3$).

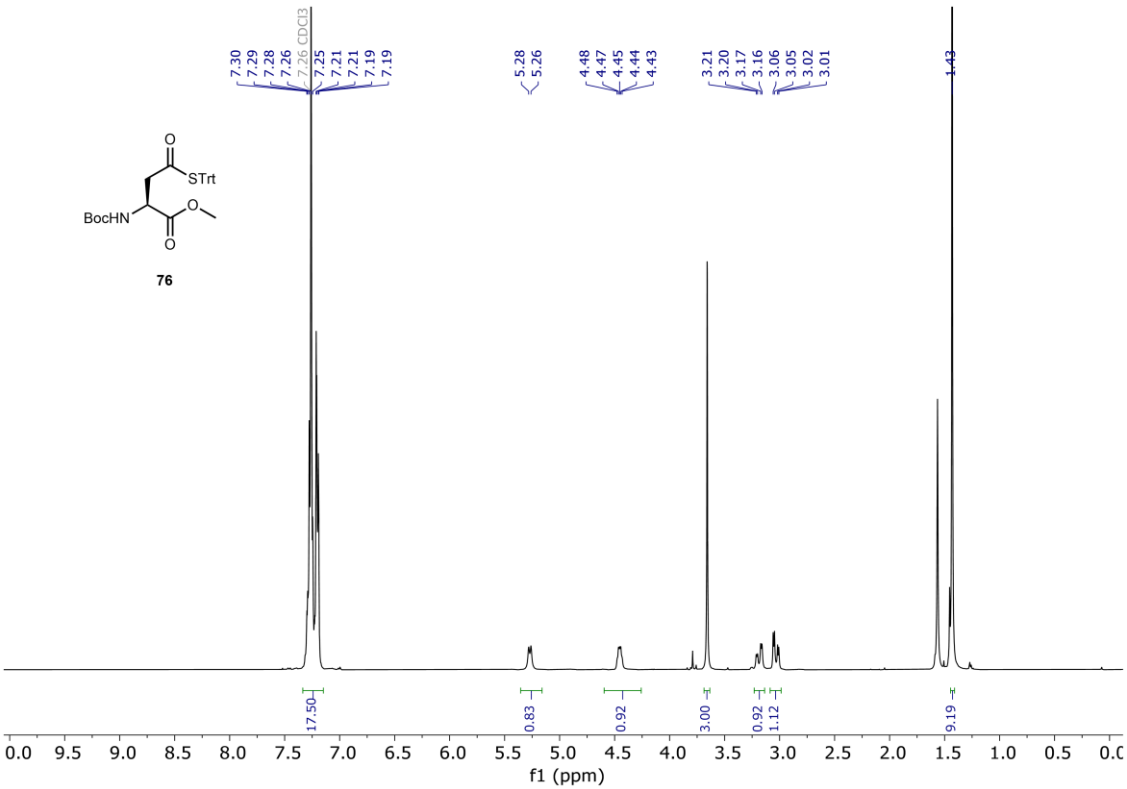

Figure A7. ^{1}H NMR spectrum of **76** (400 MHz, $CDCl_3$)

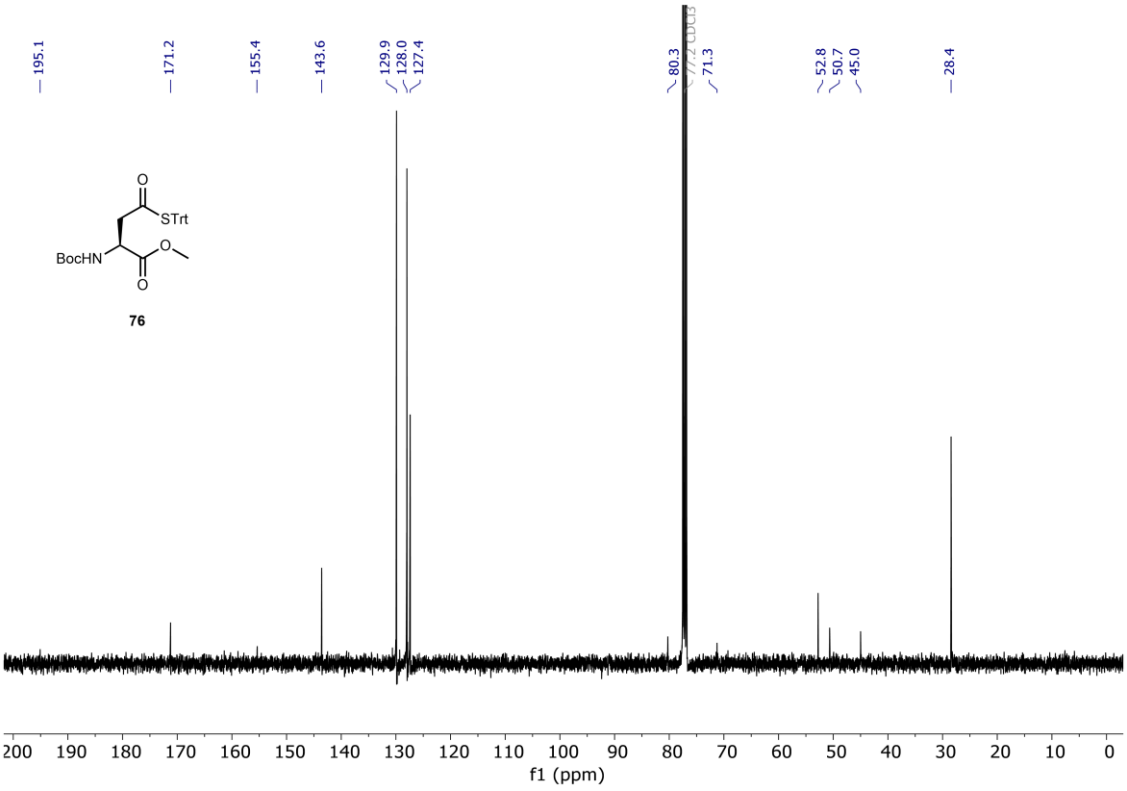

Figure A8. ^{13}C NMR spectrum of **76** (101 MHz, $CDCl_3$).

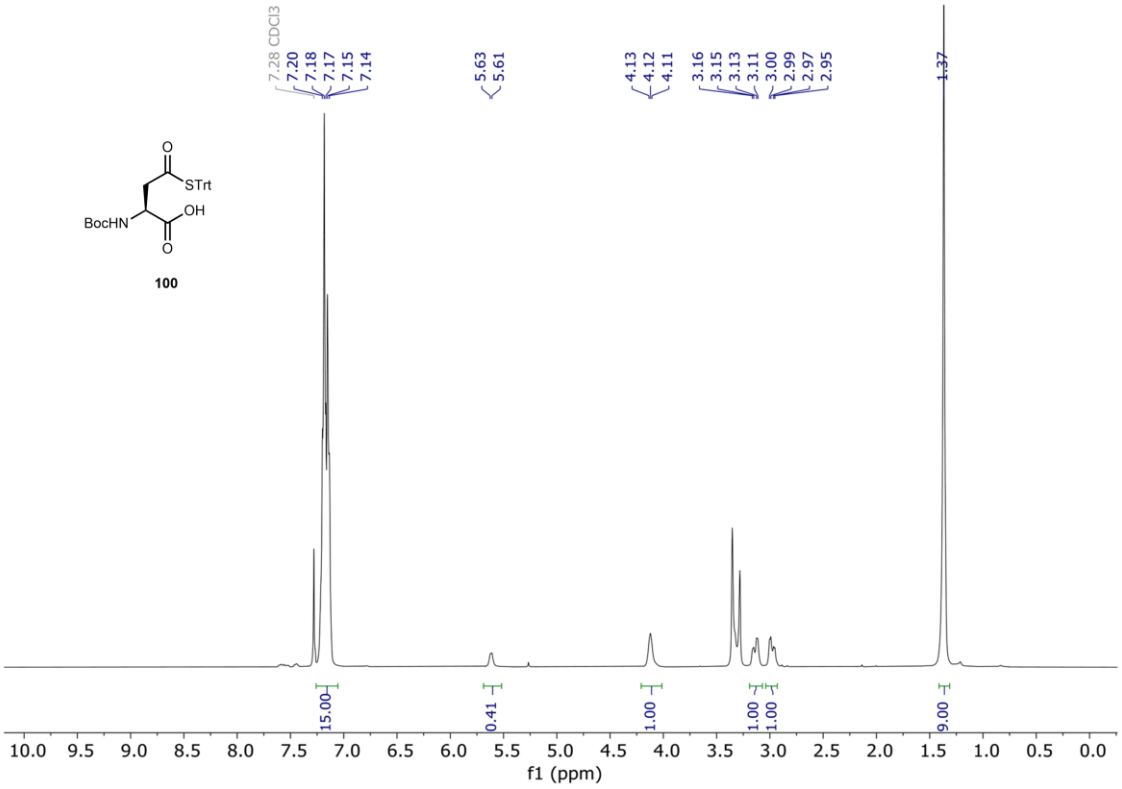

Figure A9. ^{1}H NMR spectrum of **100** (400 MHz, $CDCl_3/CD_3OD$ 98:2).

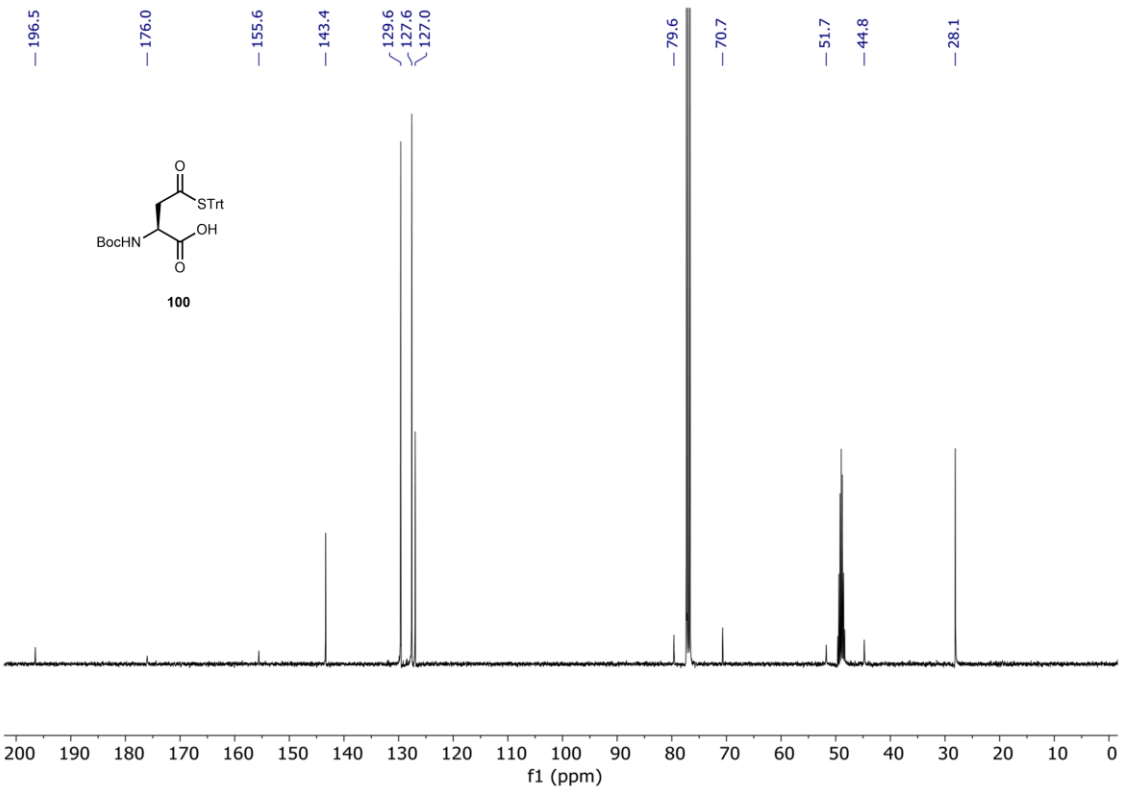

Figure A10. ^{13}C NMR spectrum of **100** (101 MHz, $CDCl_3/CD_3OD$ 98:2).

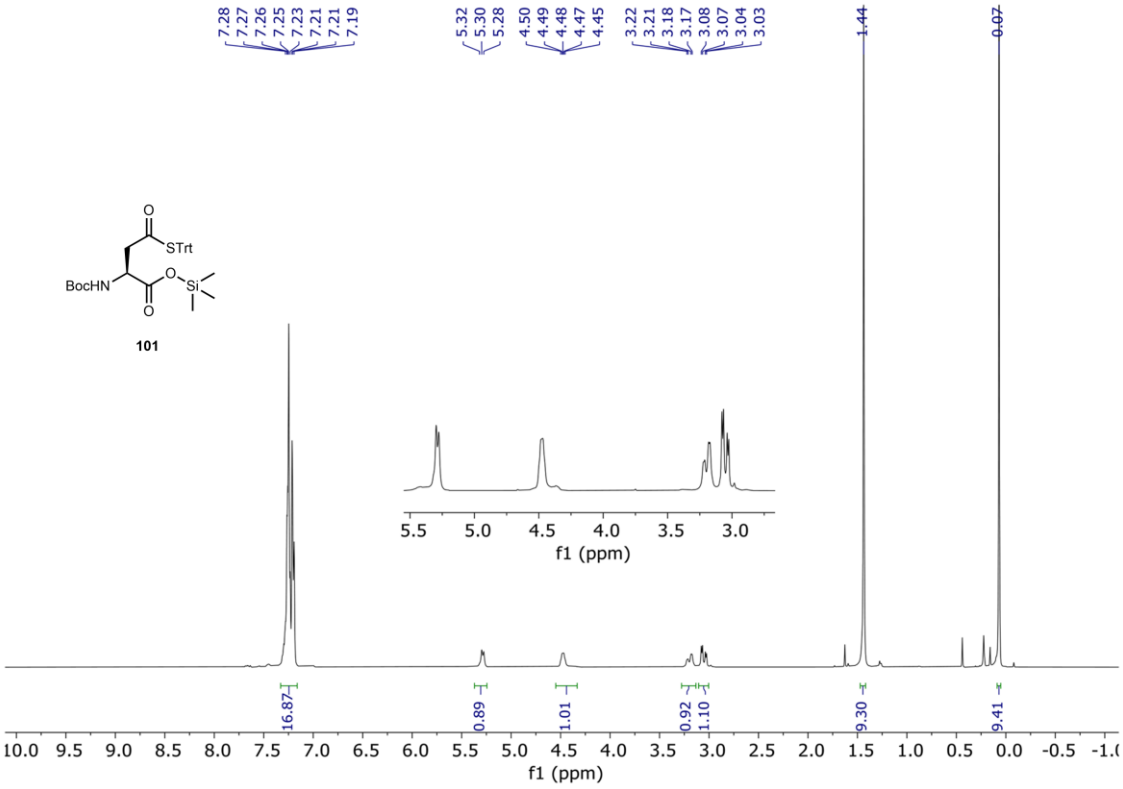

Figure A11. ^{1}H NMR spectrum of **101** (400 MHz, $CDCl_3$).

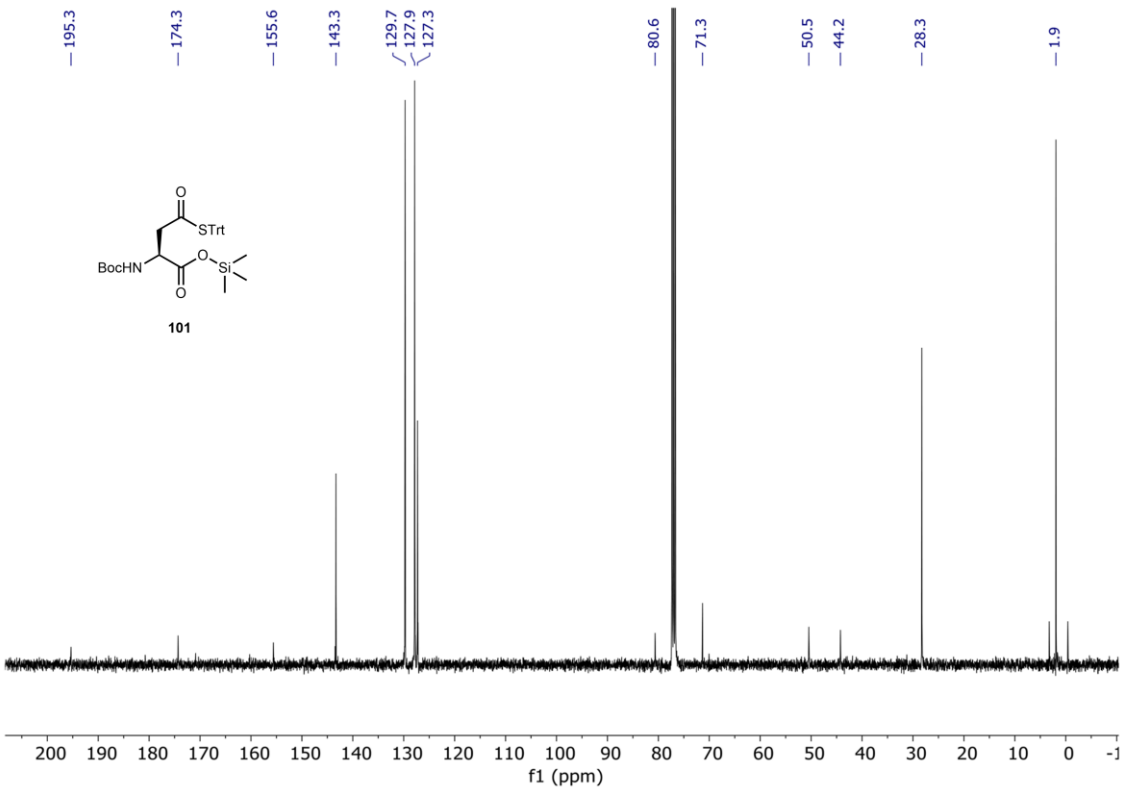

Figure A12. ^{13}C NMR spectrum of **101** (101 MHz, $CDCl_3$).

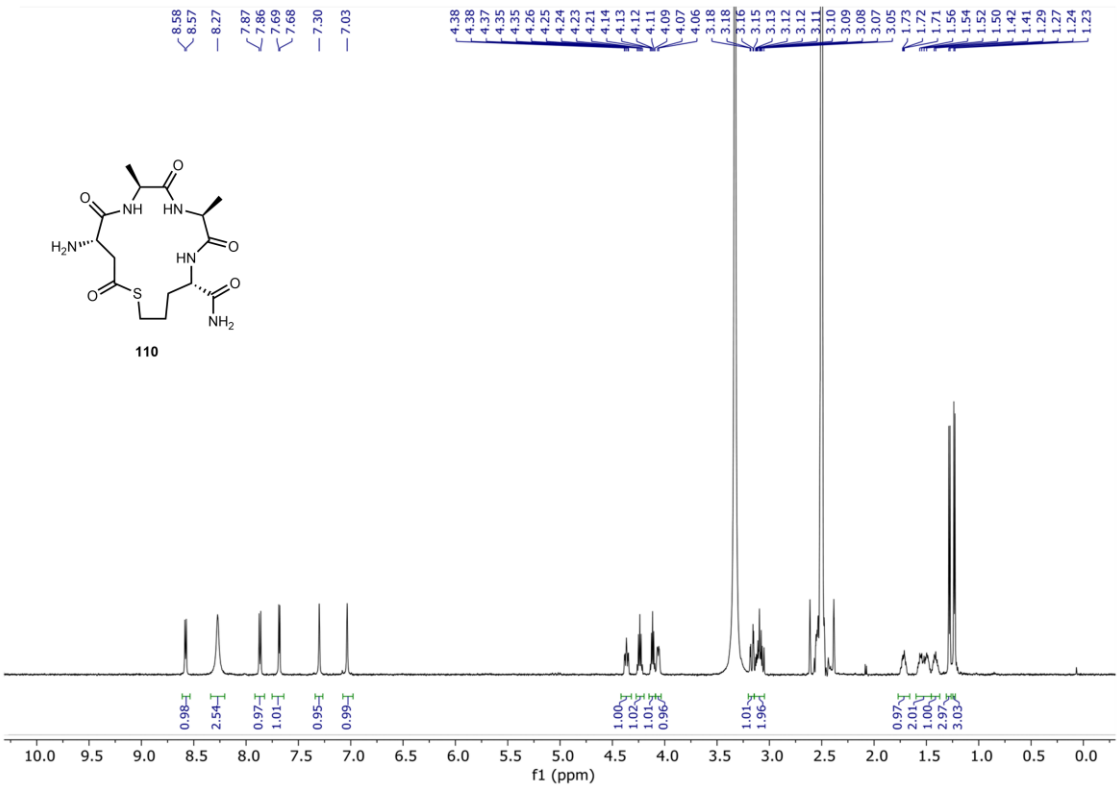

Figure A13. ^{1}H NMR spectrum of **110** (600 MHz, DMSO-d_6).

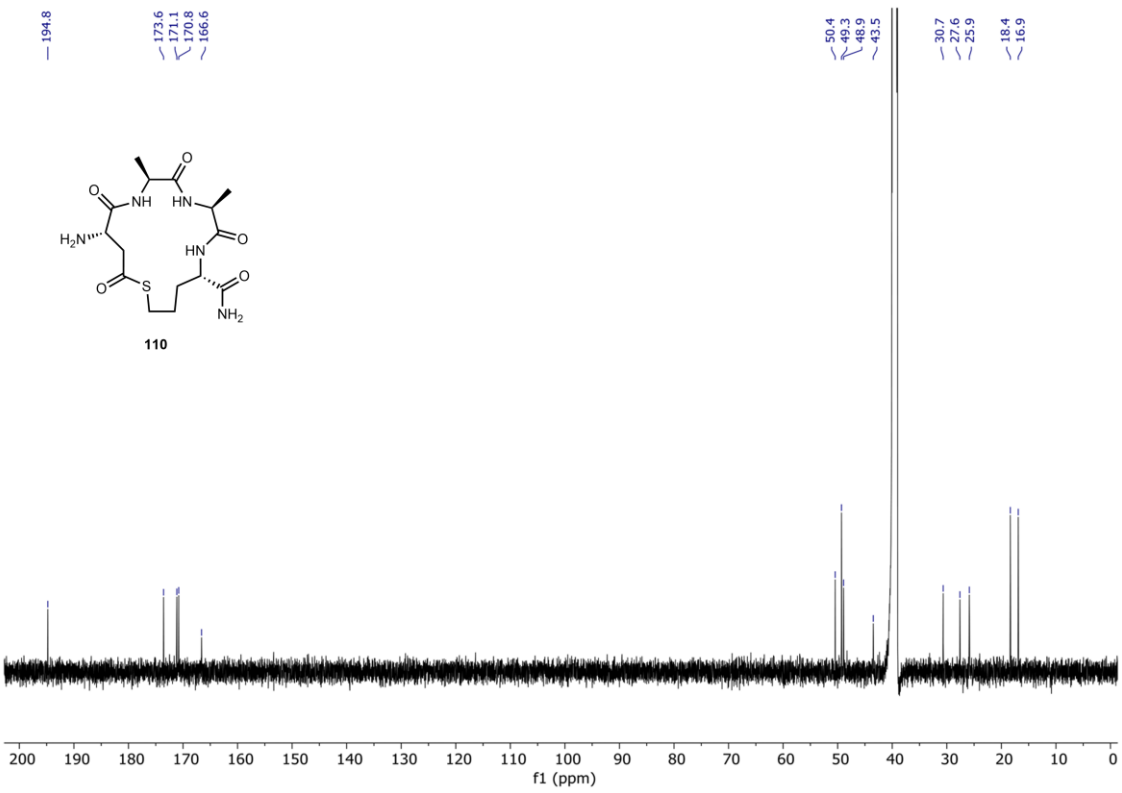

Figure A14. ^{13}C NMR spectrum of **110** (151 MHz, DMSO-d_6).

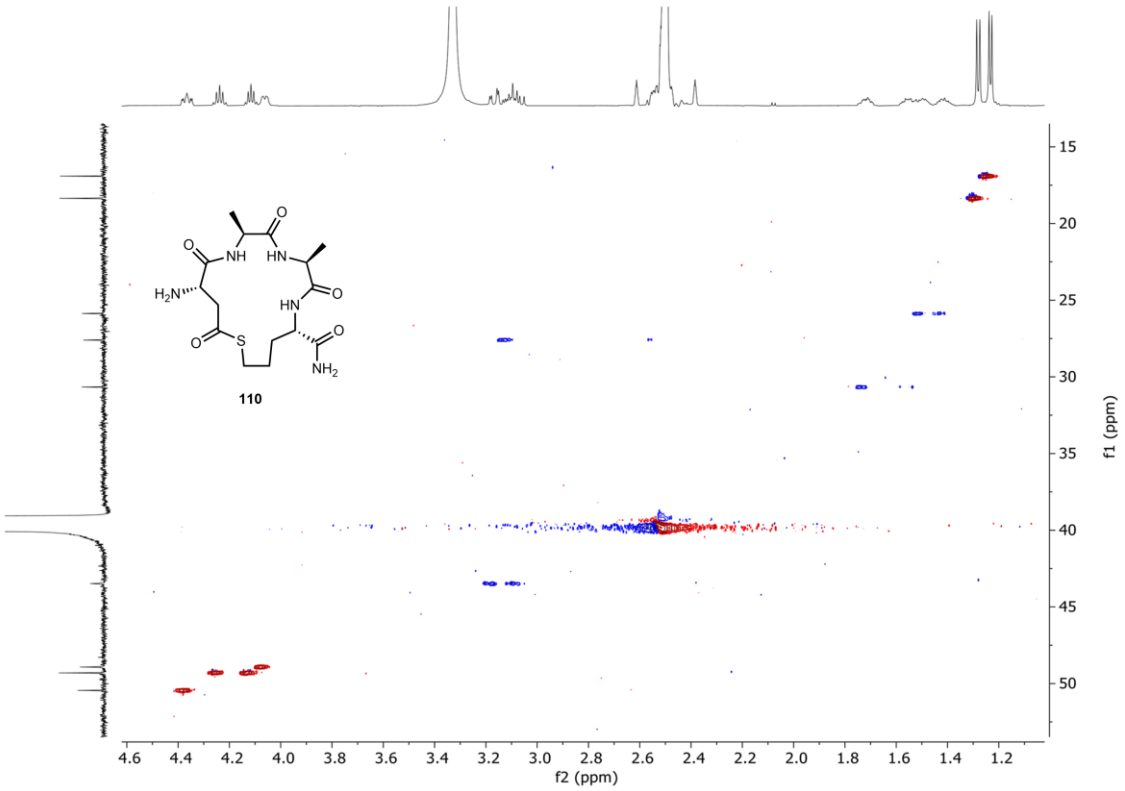

Figure A15. HSQC spectrum of **110**.

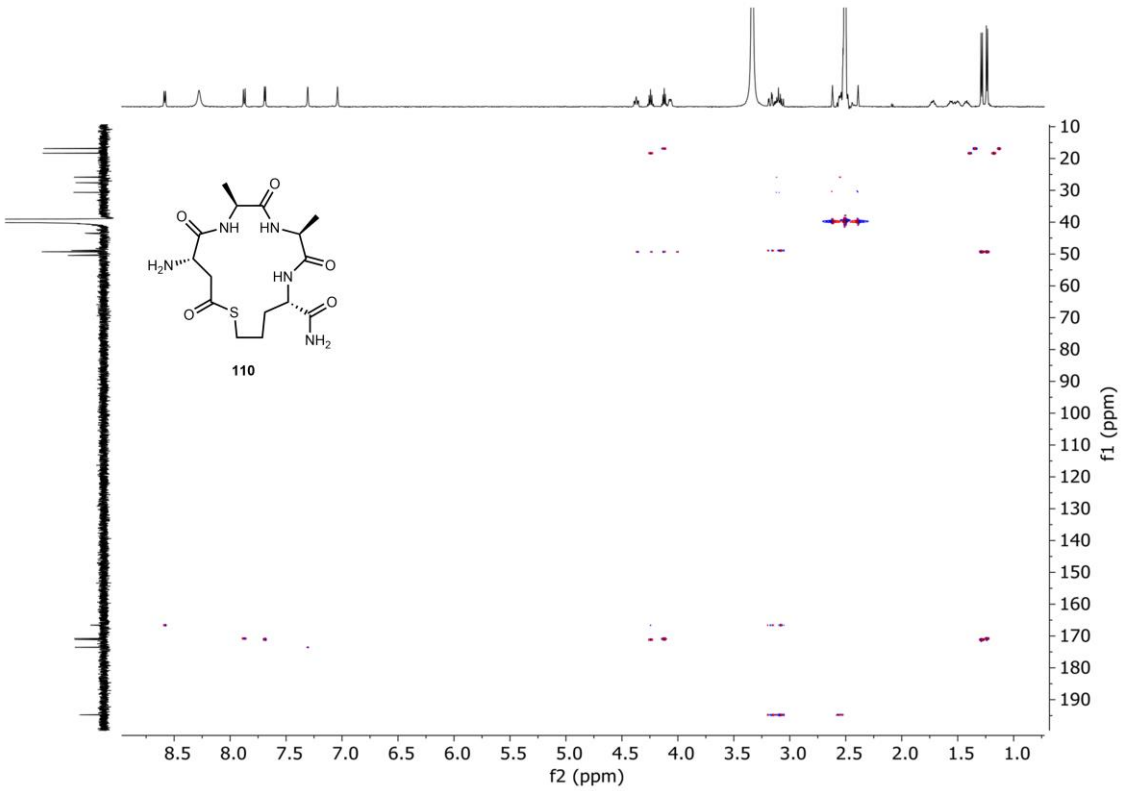

Figure A16. HMBC spectrum of **110**.

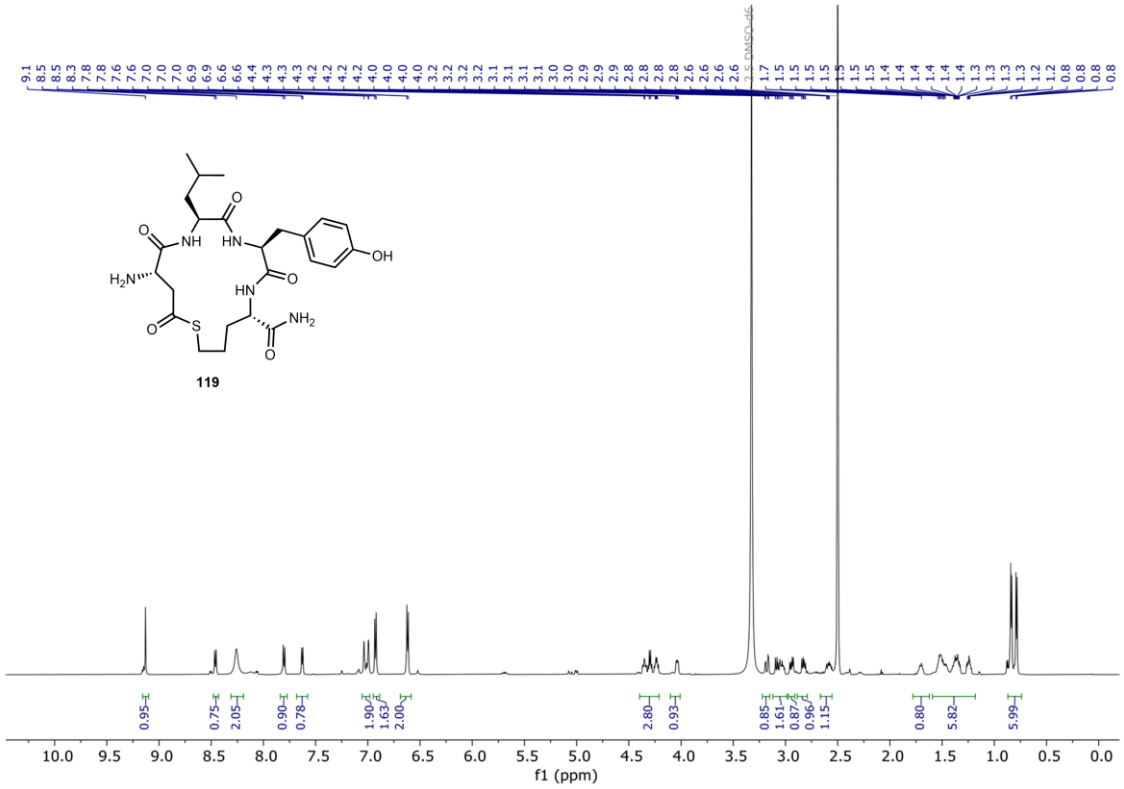

Figure A17. ^{1}H NMR spectrum of **119** (600 MHz, DMSO-d_6).

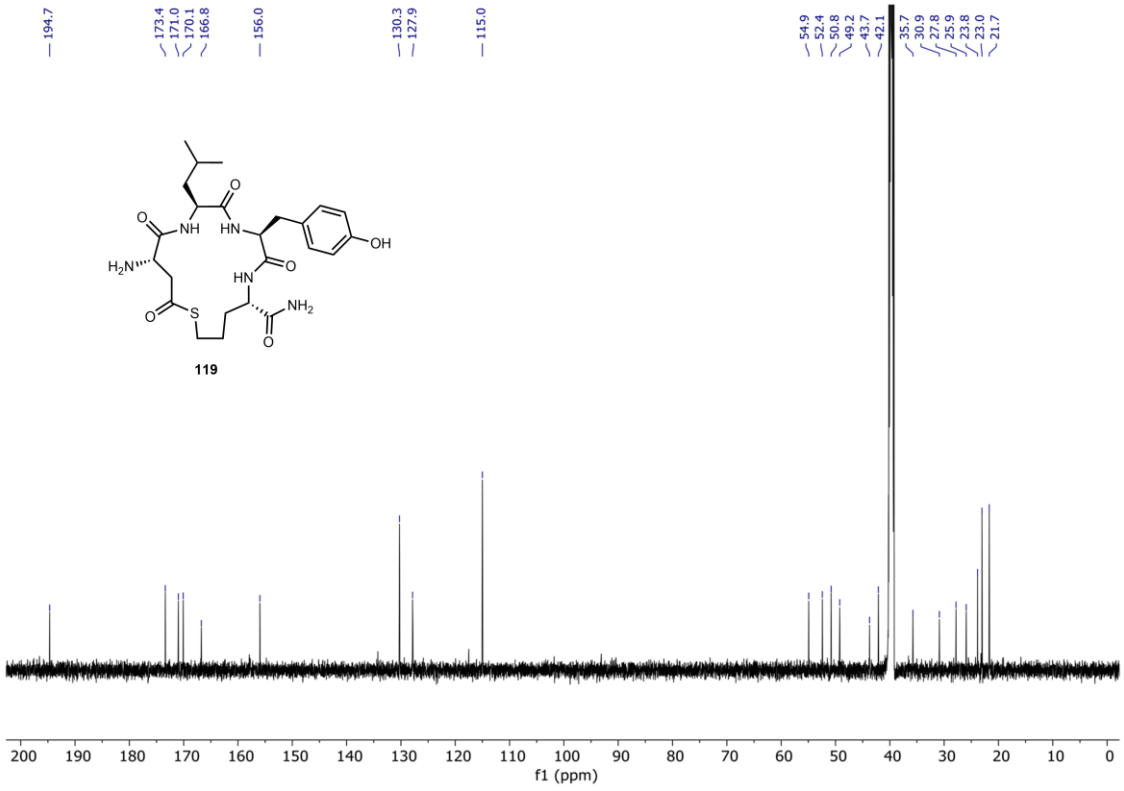

Figure A18. ^{13}C NMR spectrum of **119** (151 MHz, DMSO-d_6).

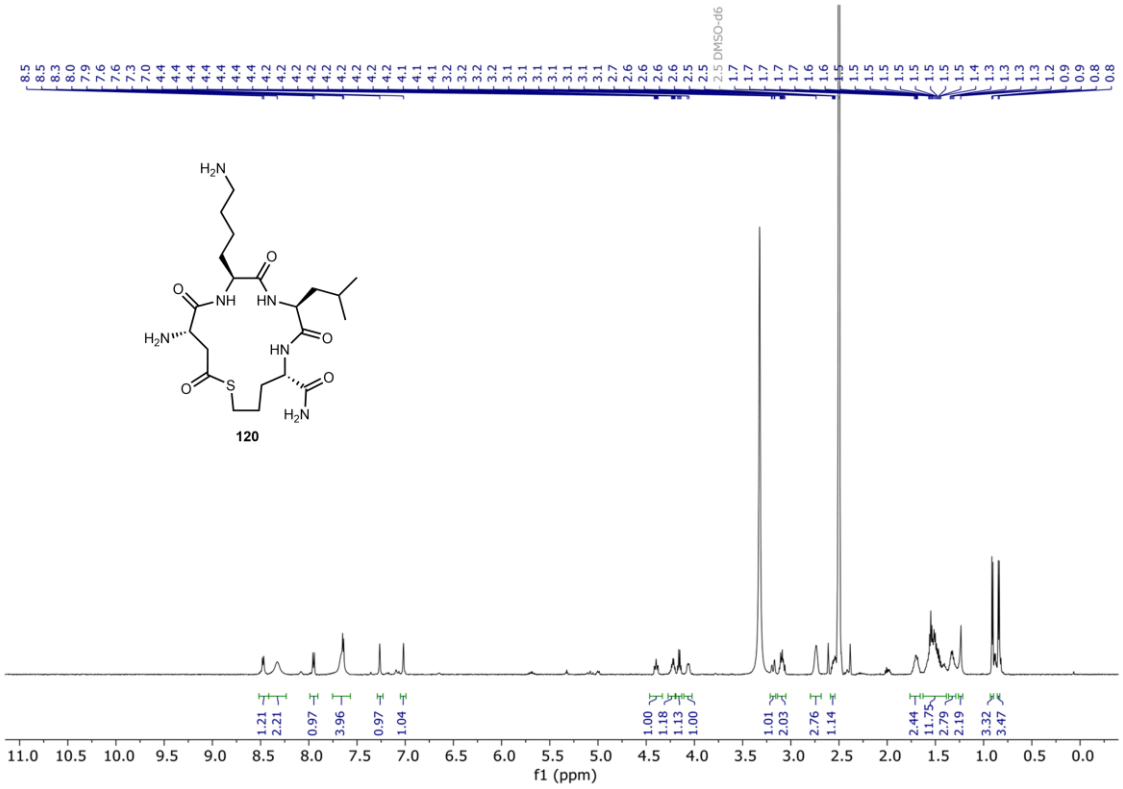

Figure A19. ^{1}H NMR spectrum of **120** (600 MHz, DMSO-d_6).

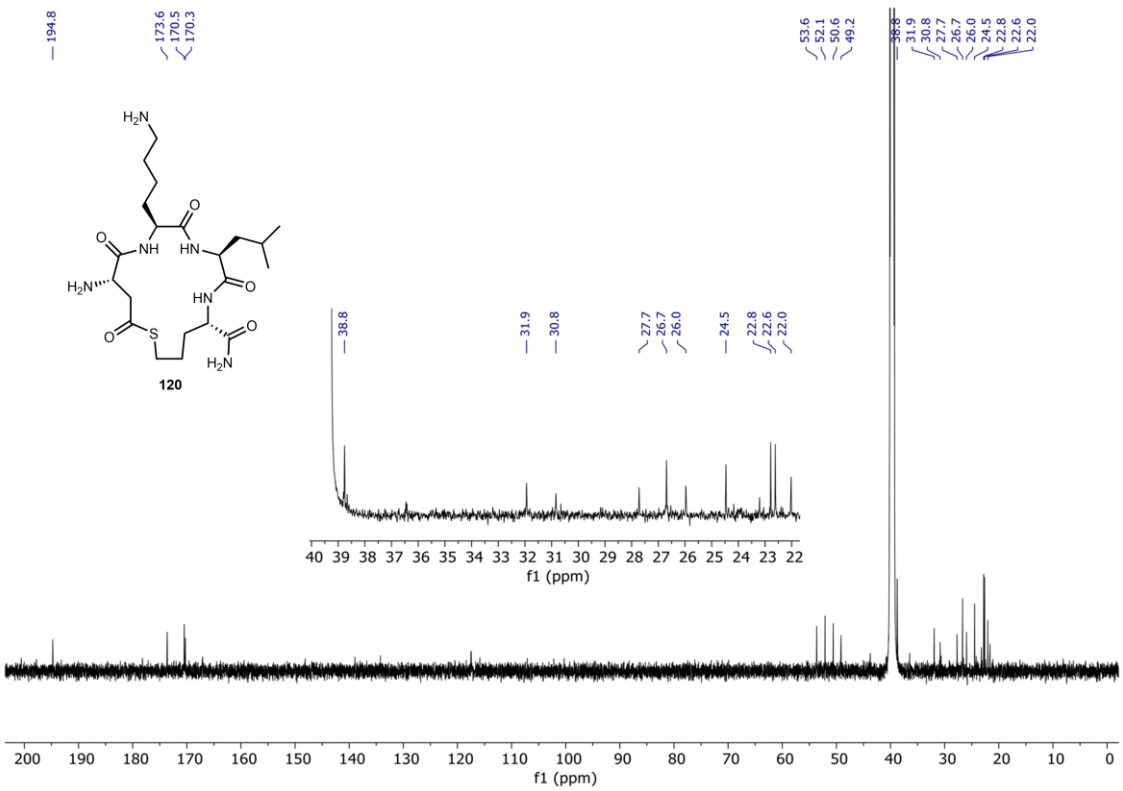

Figure A20. ^{13}C NMR spectrum of **120** (151 MHz, DMSO-d_6).

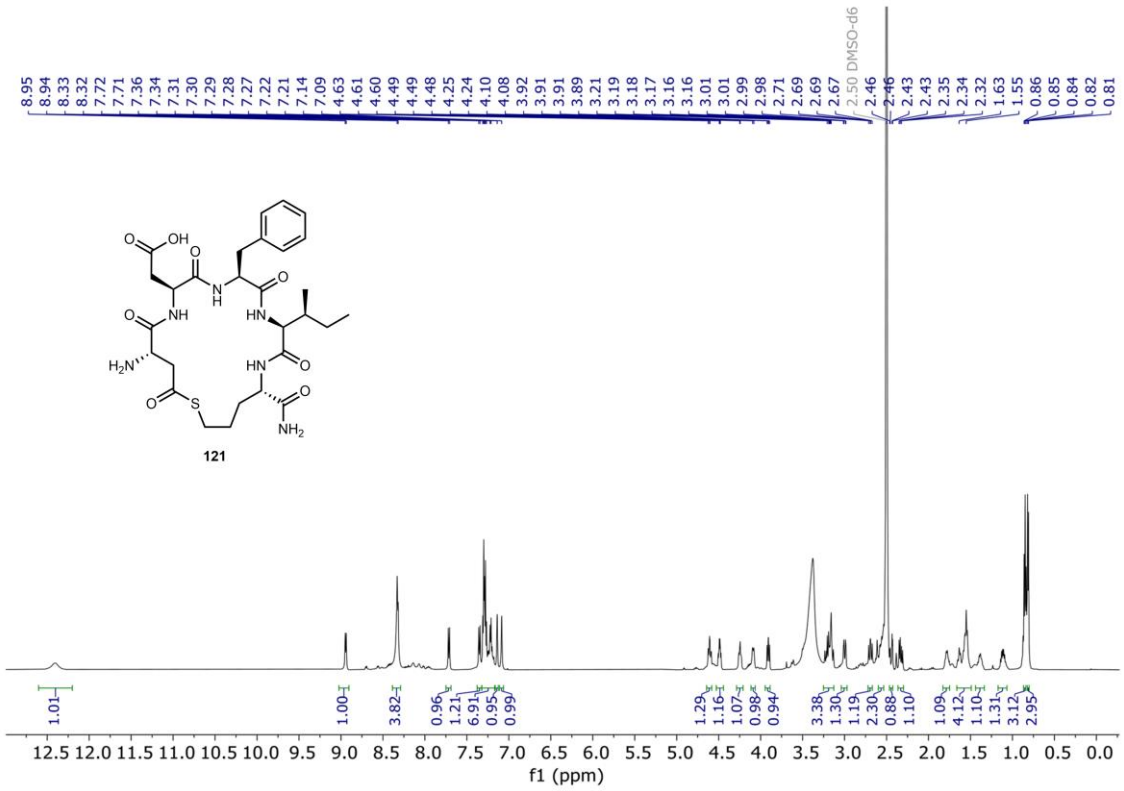

Figure A21. ^{1}H NMR spectrum of **121** (600 MHz, DMSO-d_6).

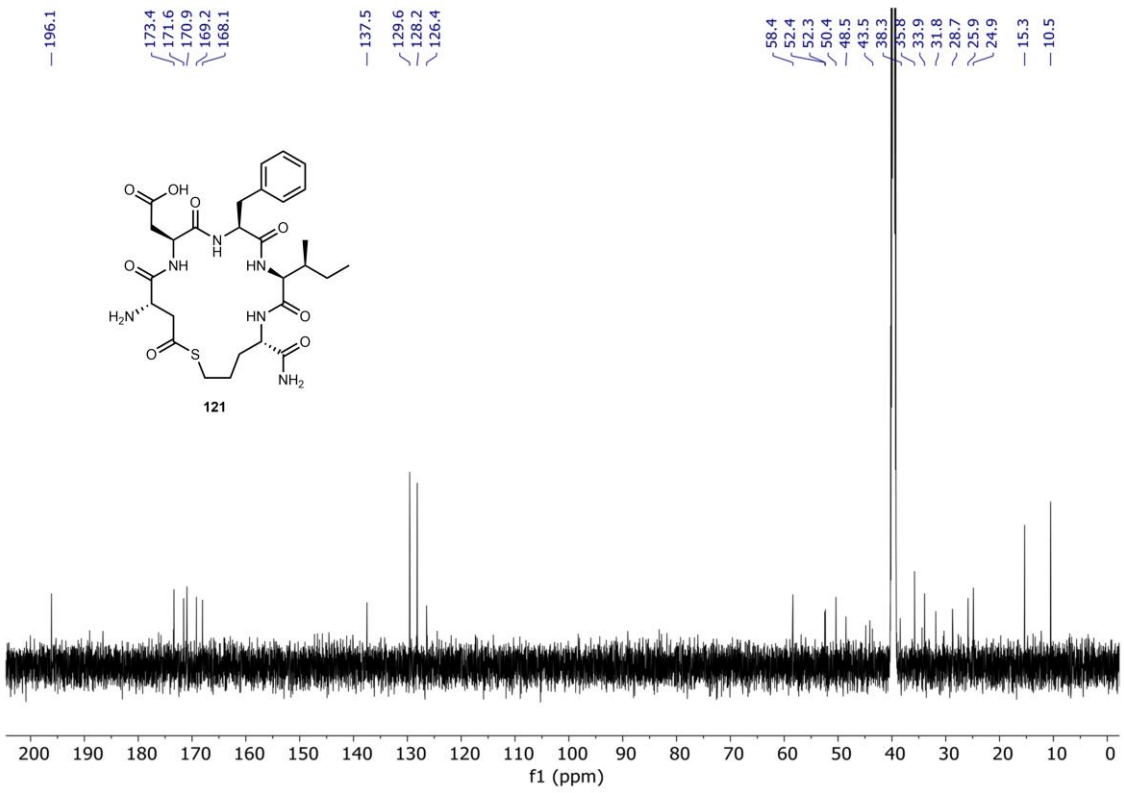

Figure A22. ^{13}C NMR spectrum of **121** (151 MHz, DMSO-d_6).

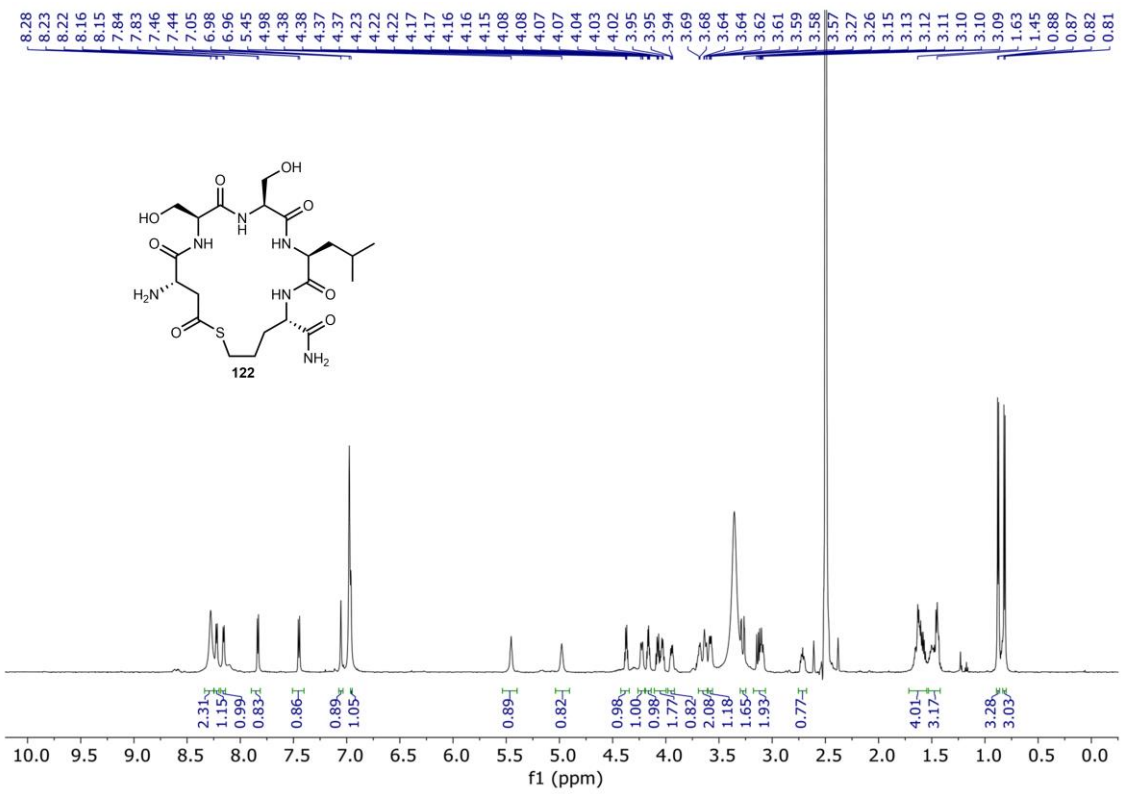

Figure A23. ^{1}H NMR spectrum of **122** (600 MHz, DMSO-d_6).

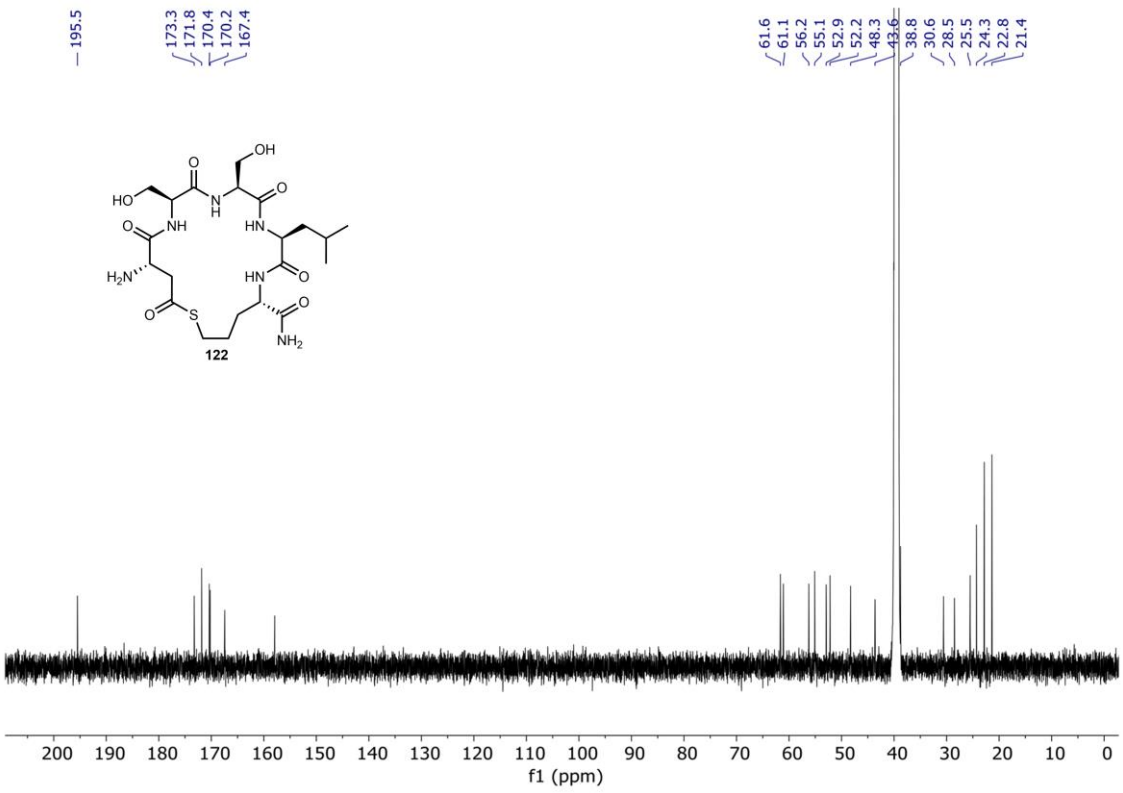

Figure A24. ^{13}C NMR spectrum of **122** (151 MHz, DMSO-d_6).

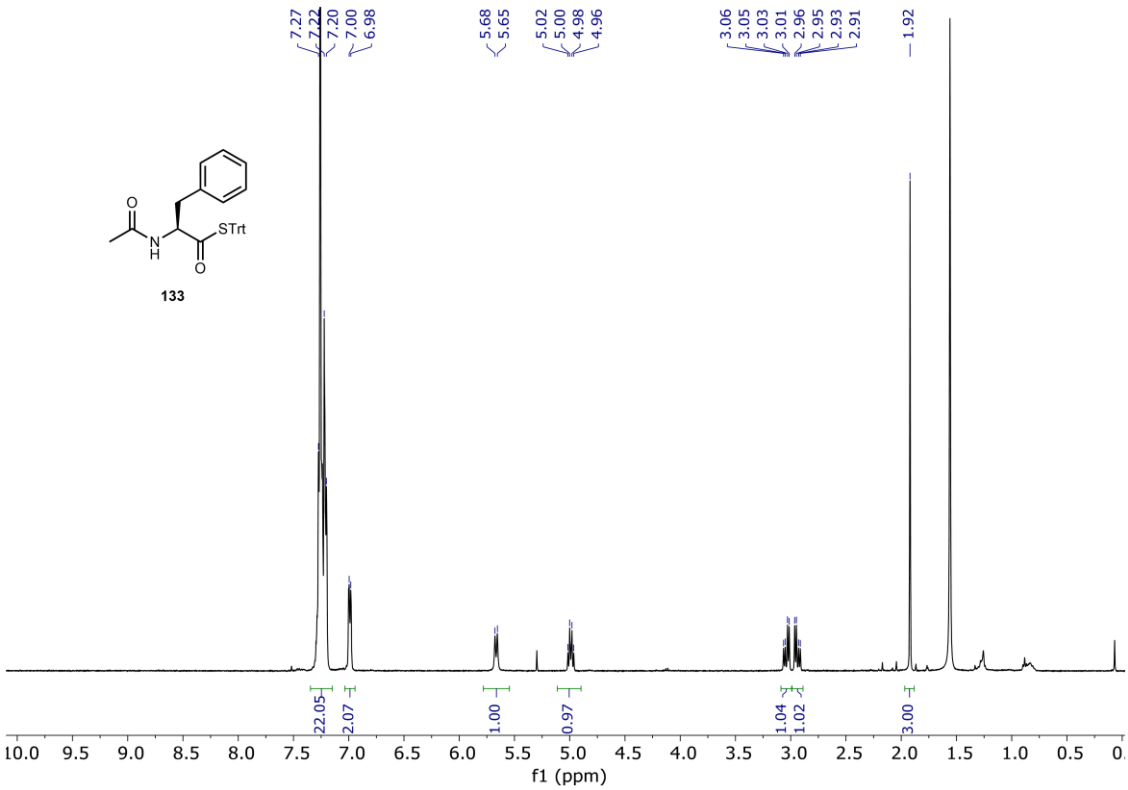

Figure A25. ^{1}H NMR spectrum of **133** (400 MHz, $CDCl_3$).

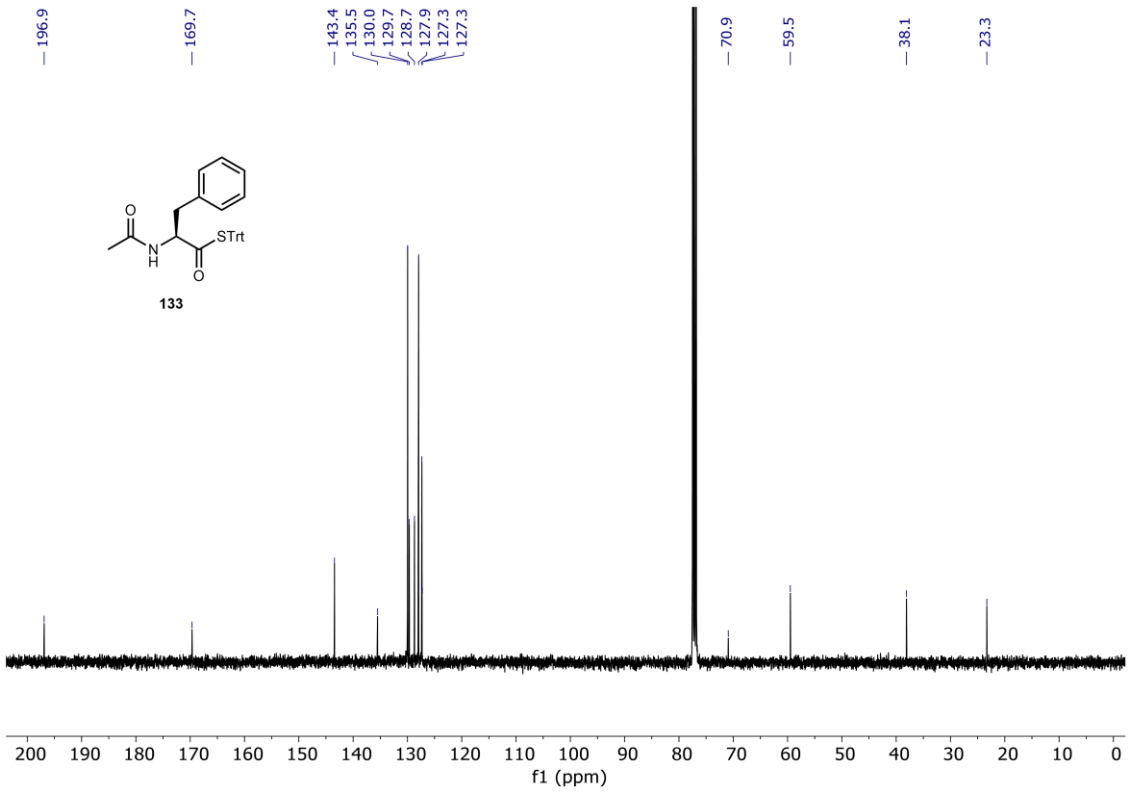

Figure A26. ^{13}C NMR spectrum of **133** (101 MHz, $CDCl_3$).

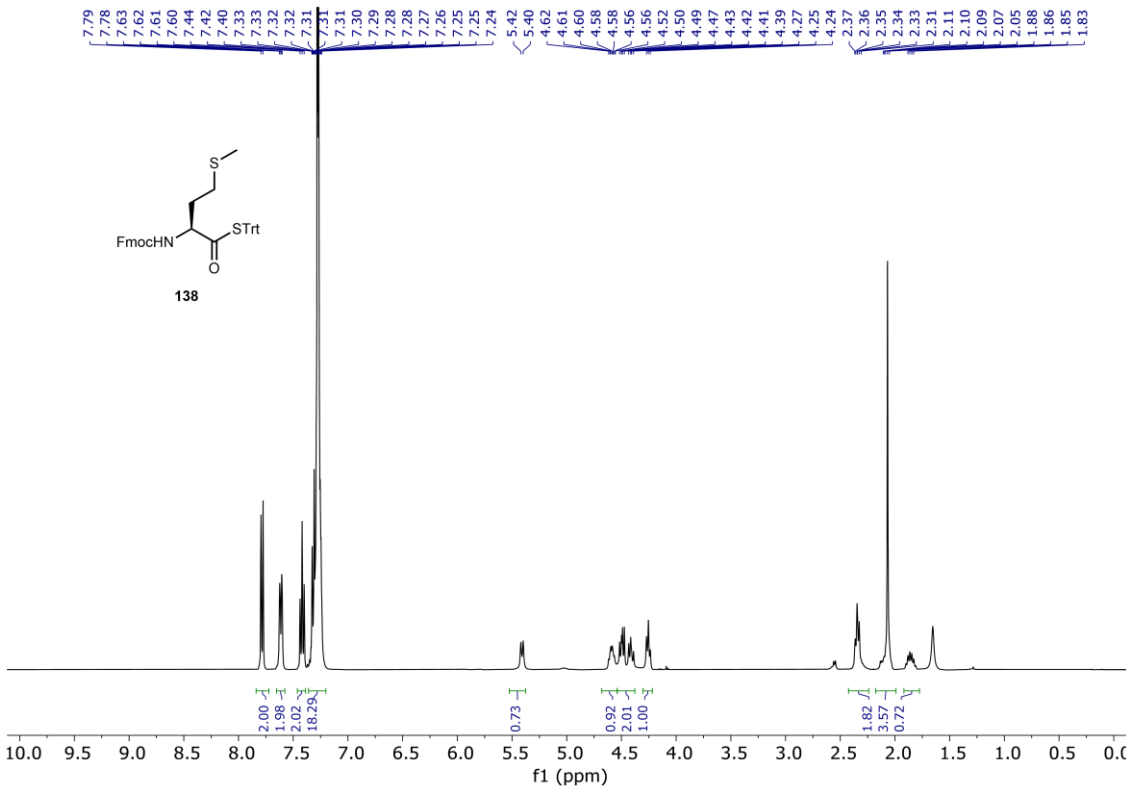

Figure A27. ^{1}H NMR spectrum of **138** (400 MHz, $CDCl_3$).

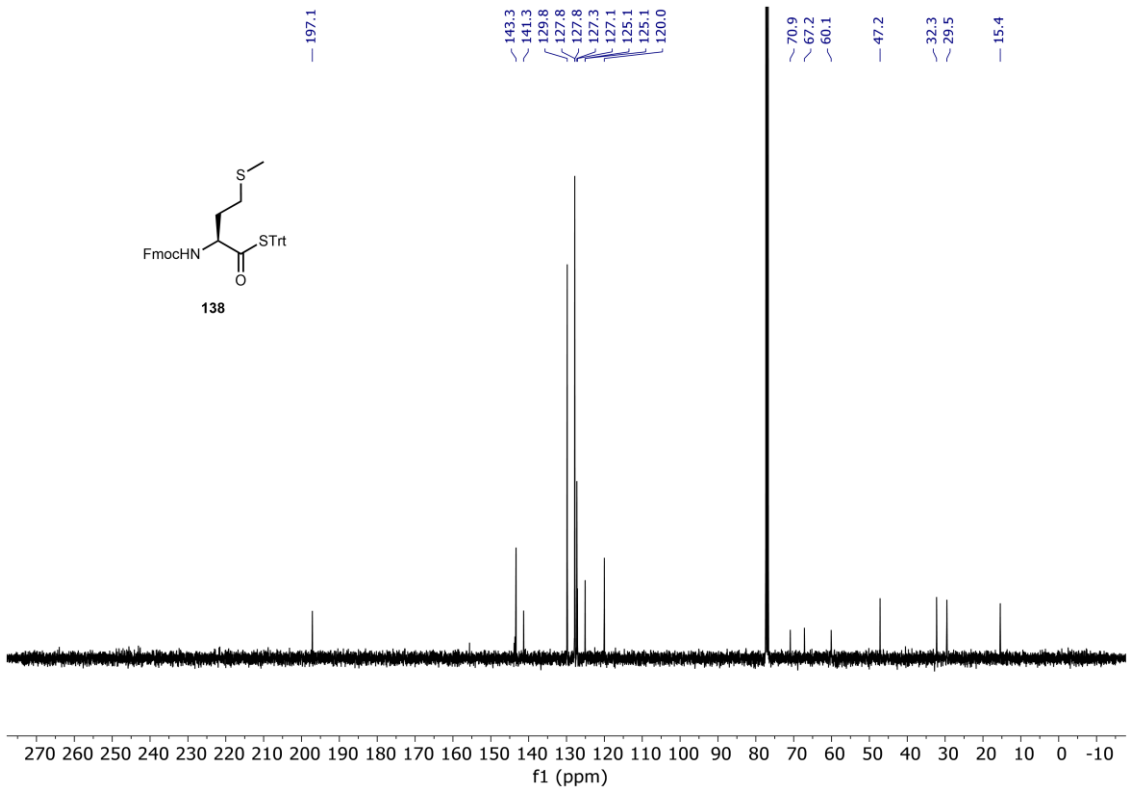

Figure A28. ^{13}C NMR spectrum of **138** (101 MHz, $CDCl_3$).

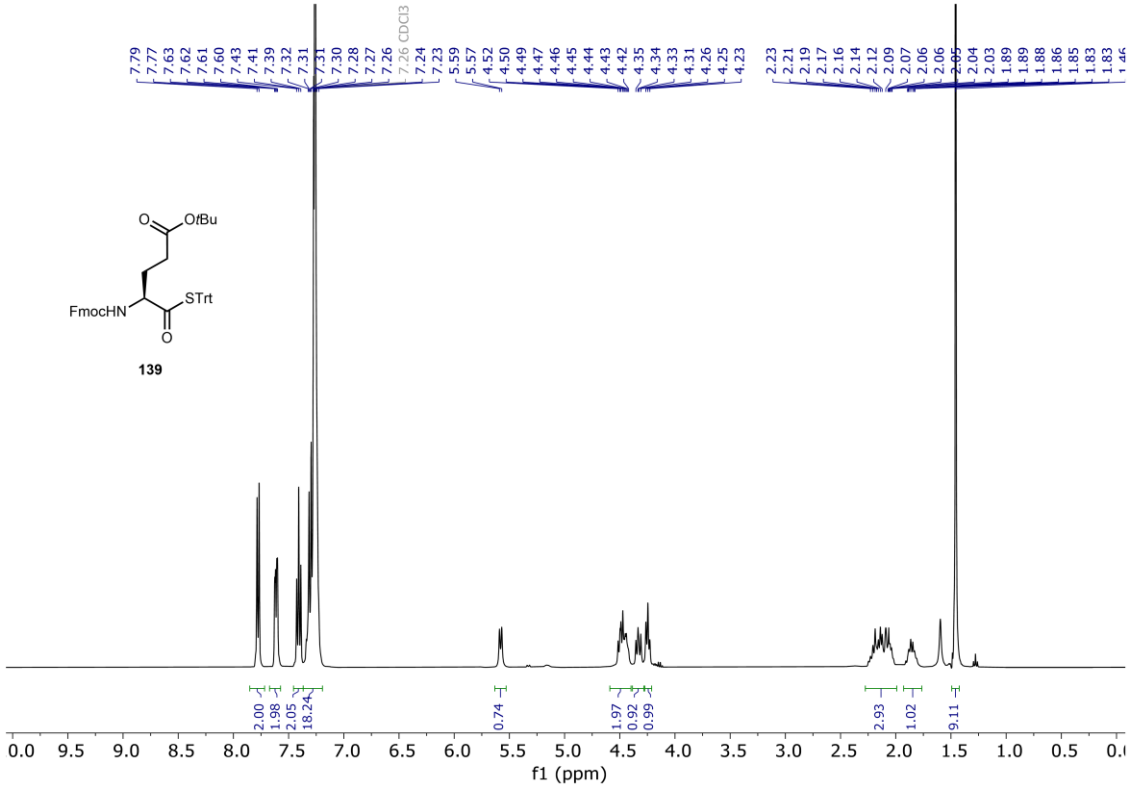

Figure A29. ^{1}H NMR spectrum of **139** (400 MHz, $CDCl_3$).

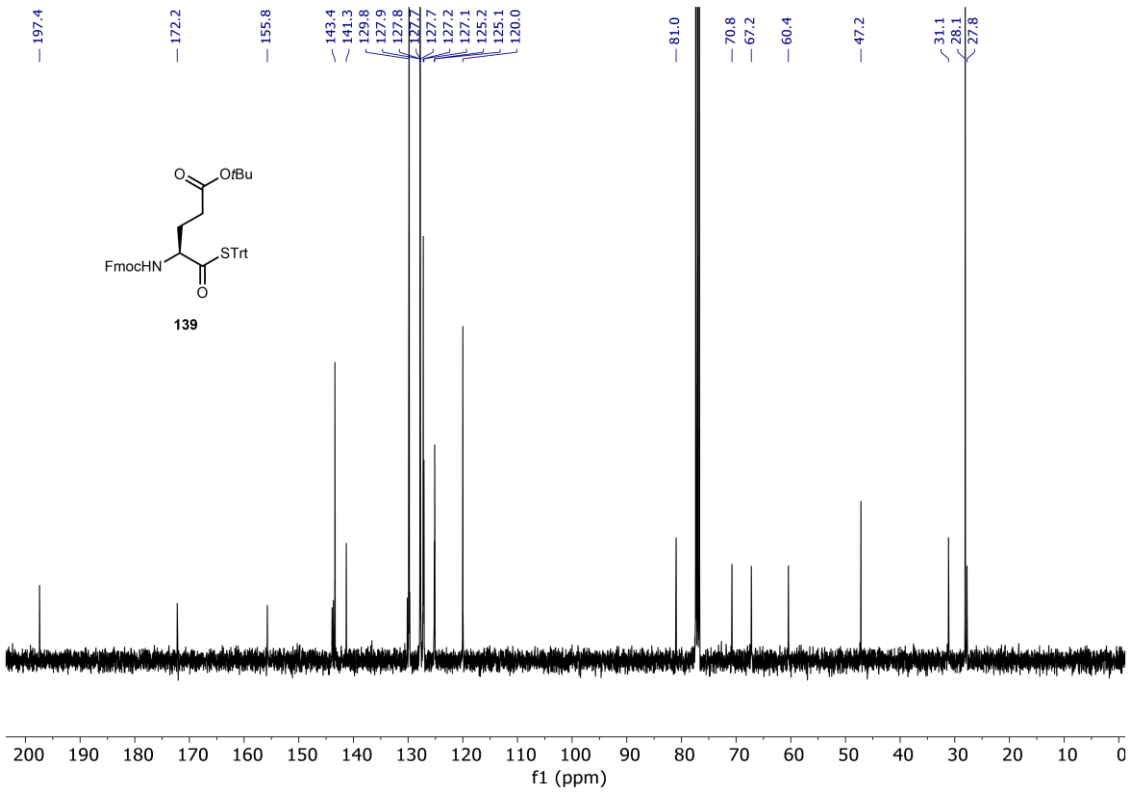

Figure A30. ^{13}C NMR spectrum of **139** (101 MHz, $CDCl_3$).

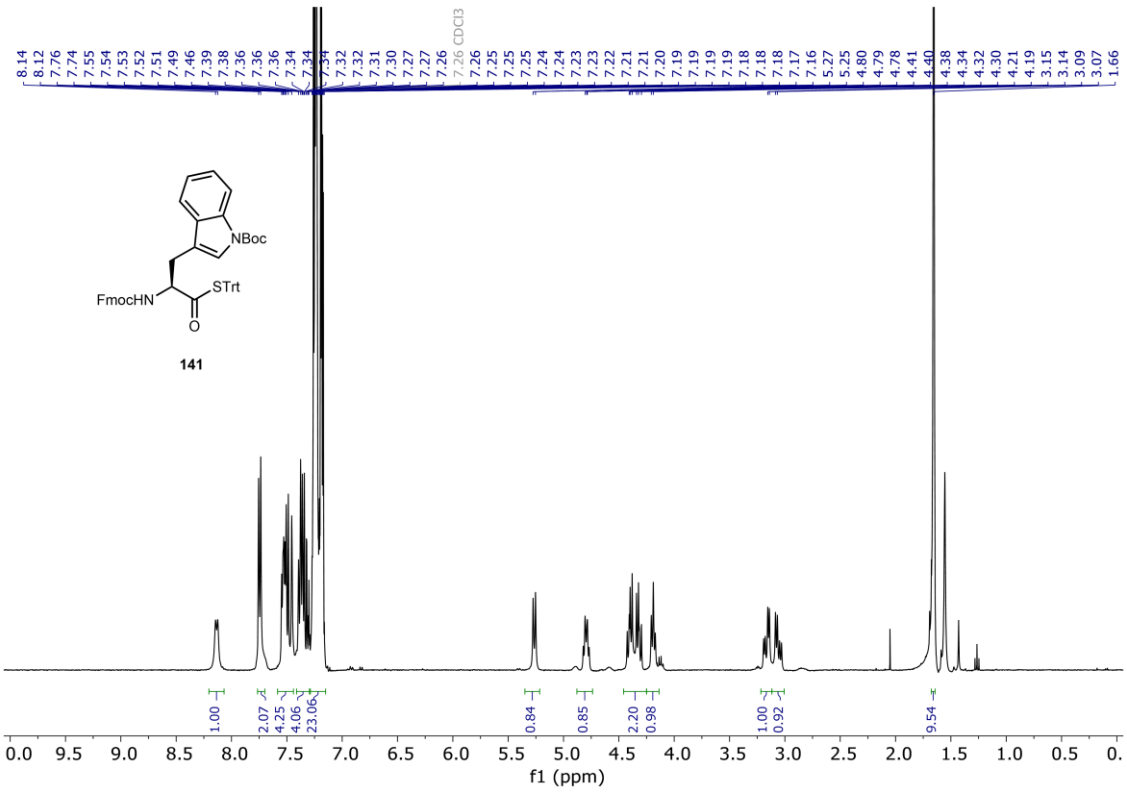

Figure A31. ^{1}H NMR spectrum of **141** (400 MHz, $CDCl_3$).

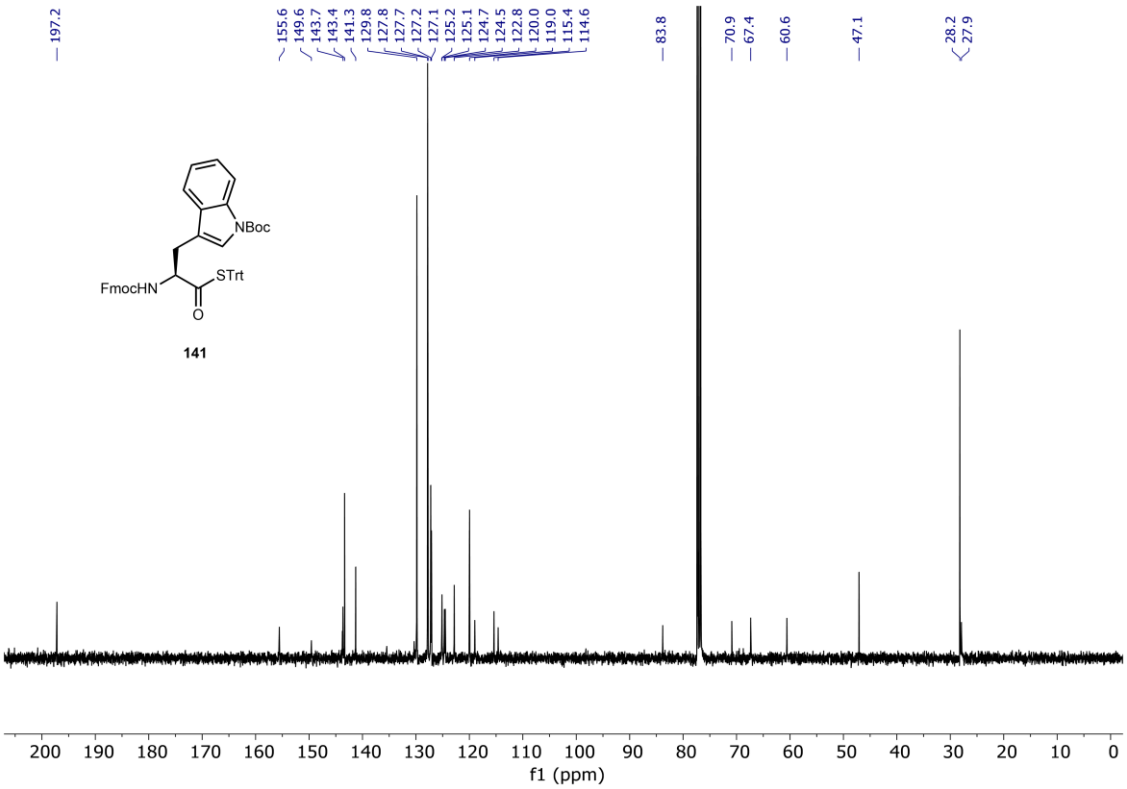

Figure A32. ^{13}C NMR spectrum of **141** (101 MHz, $CDCl_3$).

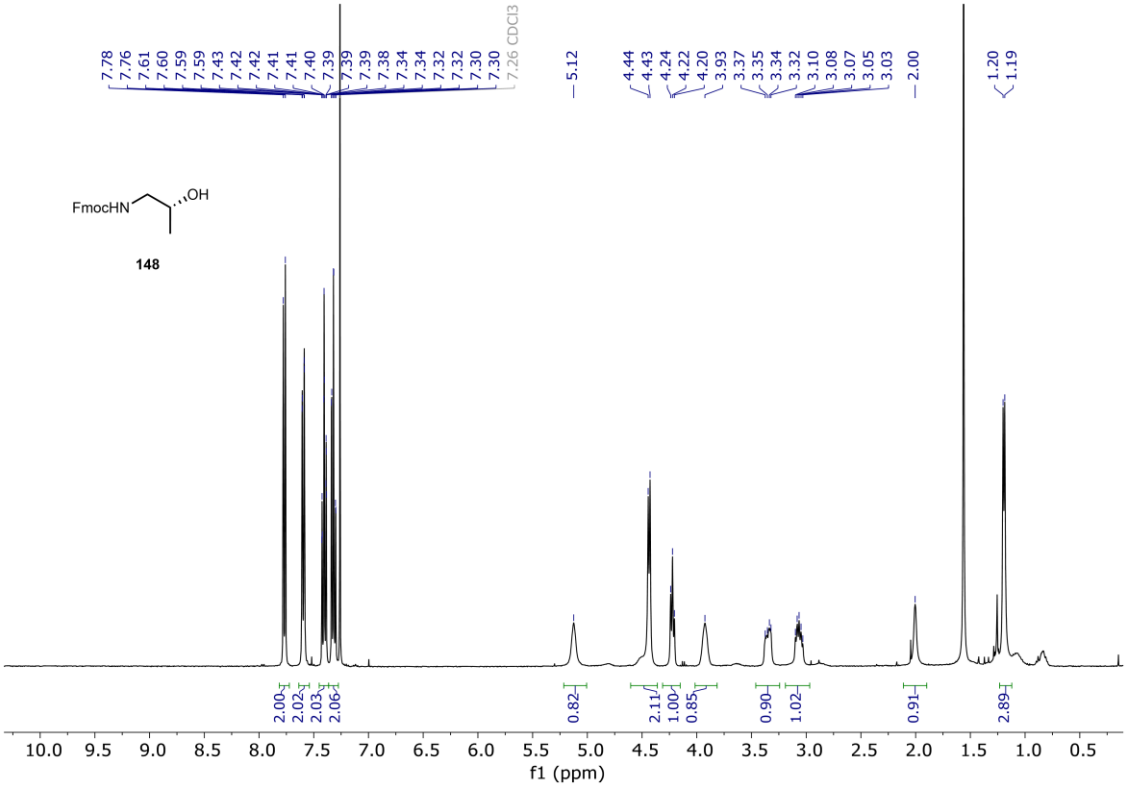

Figure A33. ^{1}H NMR spectrum of **148** (400 MHz, $CDCl_3$).

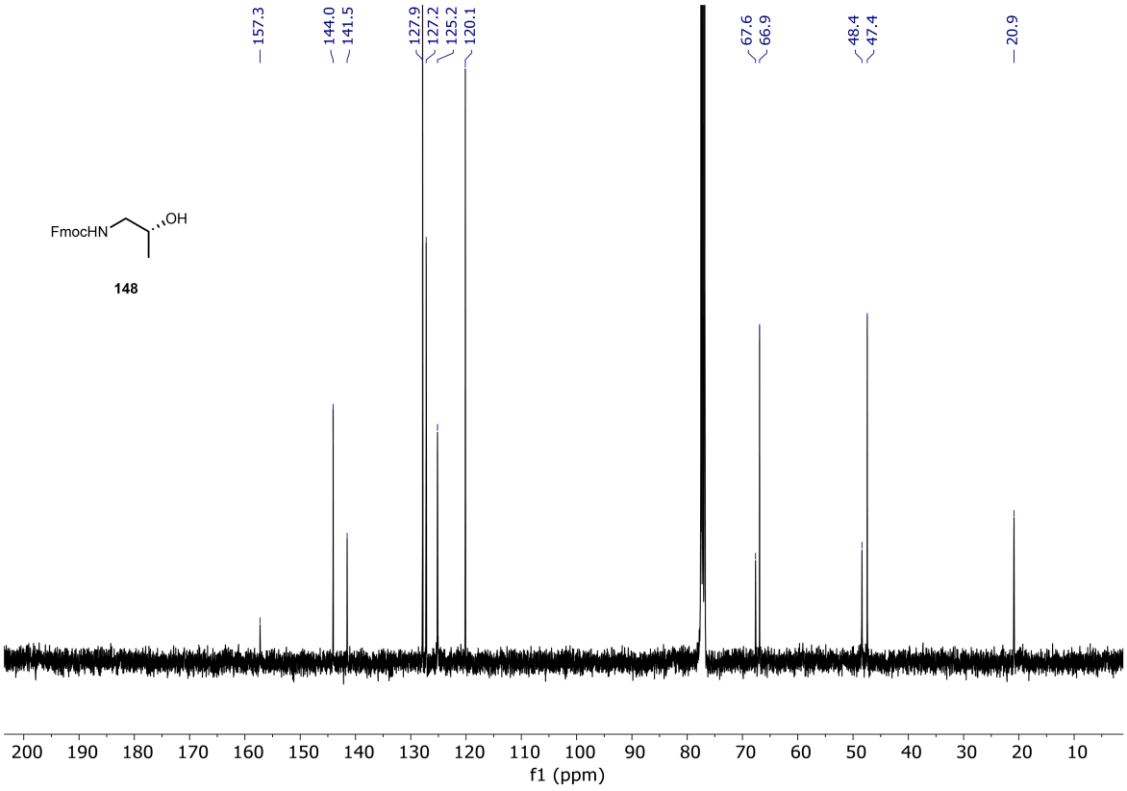

Figure A34. ^{13}C NMR spectrum of **148** (101 MHz, $CDCl_3$).

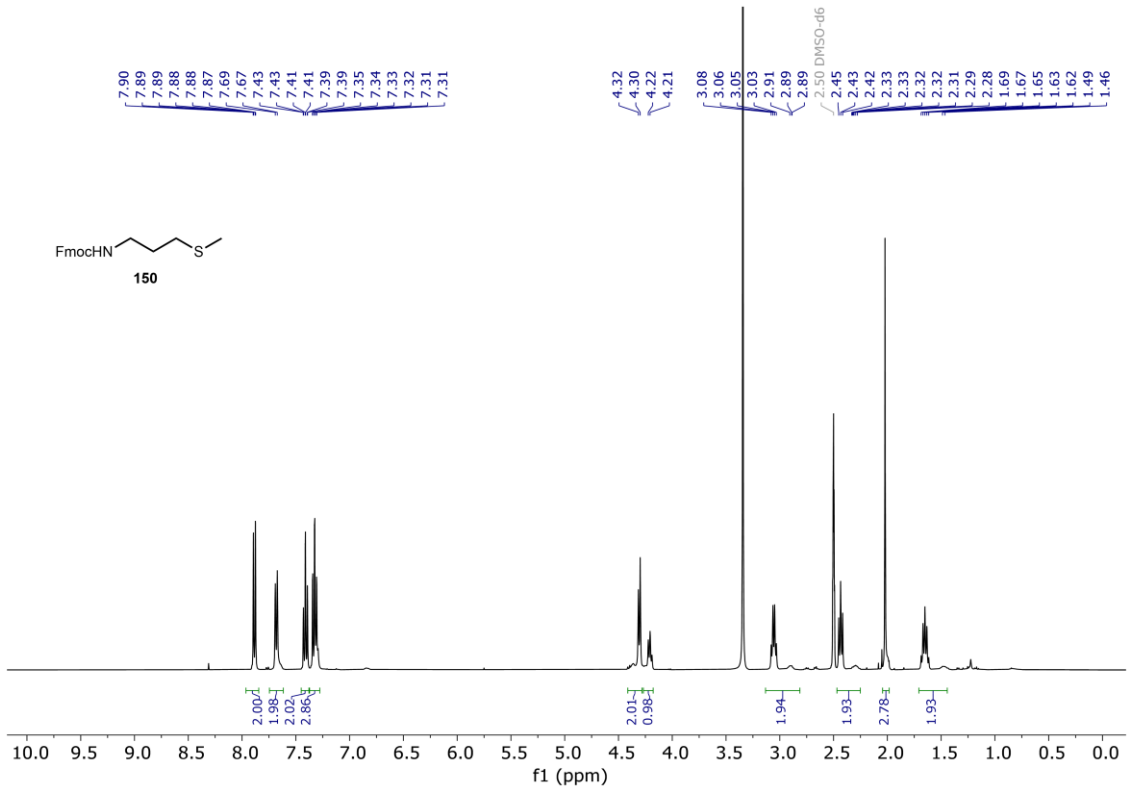

Figure A35. ^{1}H NMR spectrum of **150** (400 MHz, DMSO-d_6).

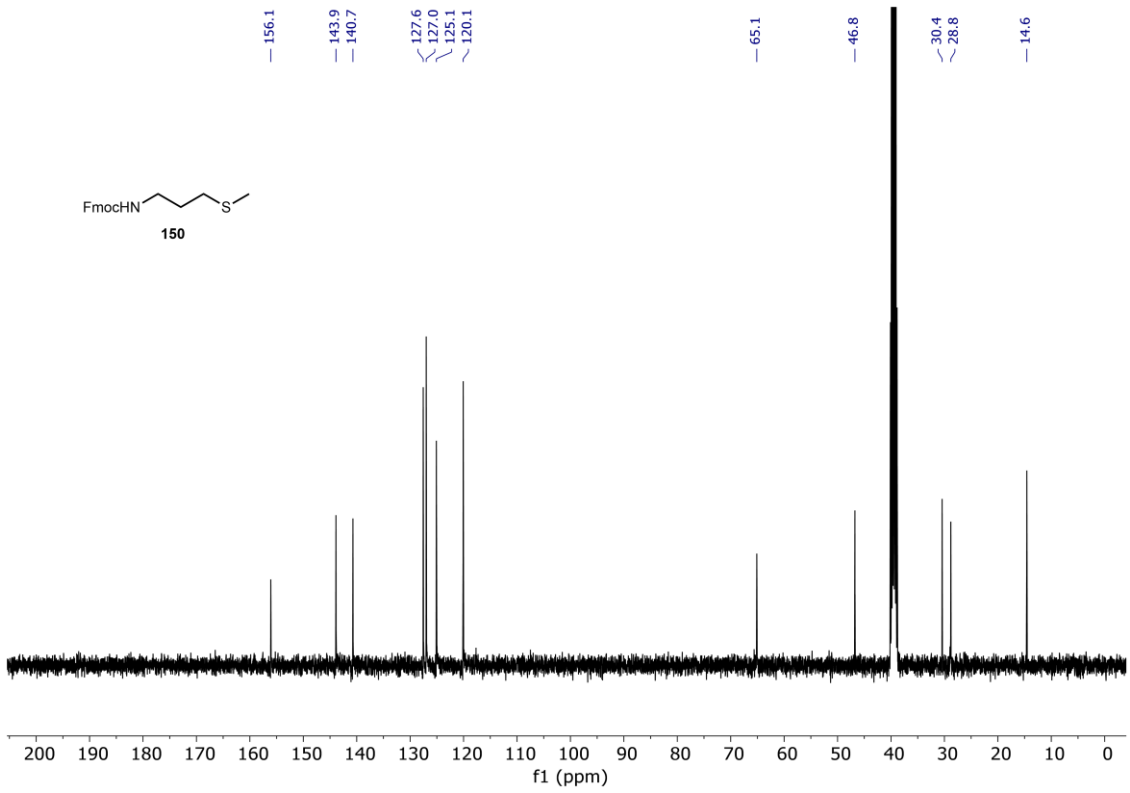

Figure A36. ^{13}C NMR spectrum of **150** (101 MHz, DMSO-d_6).

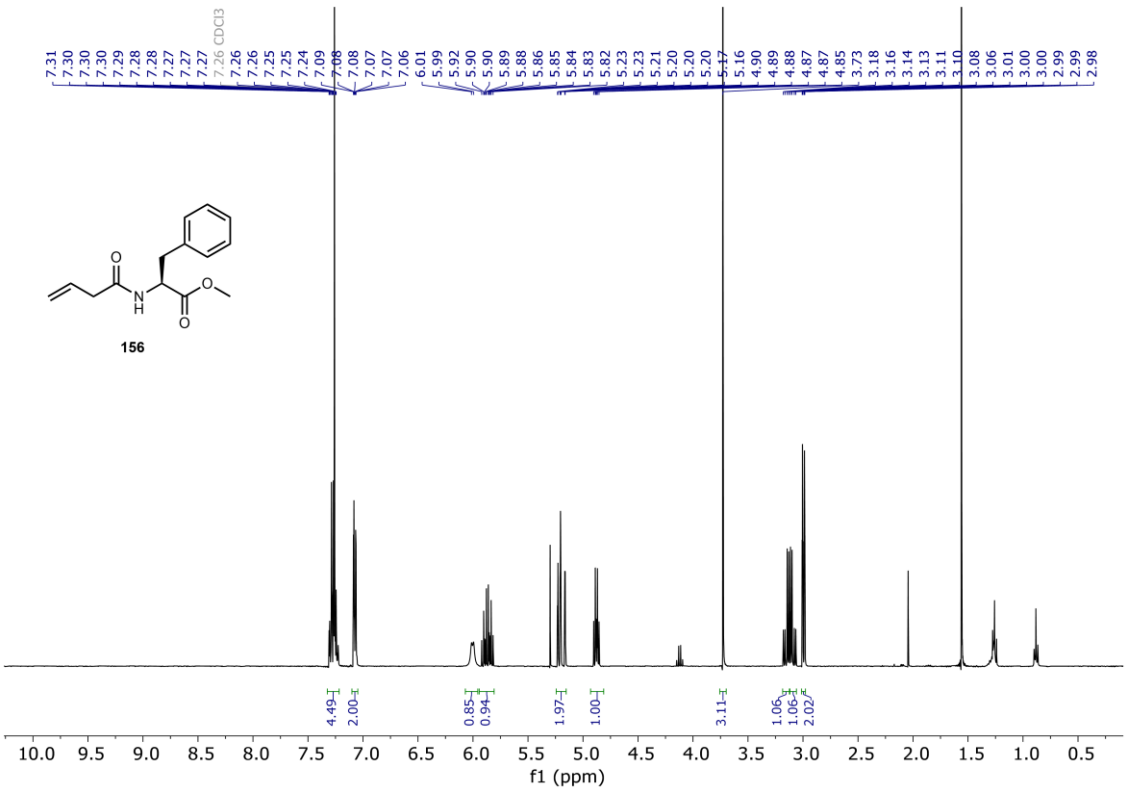

Figure A37. ^{1}H NMR spectrum of **156** (400 MHz, $CDCl_3$).

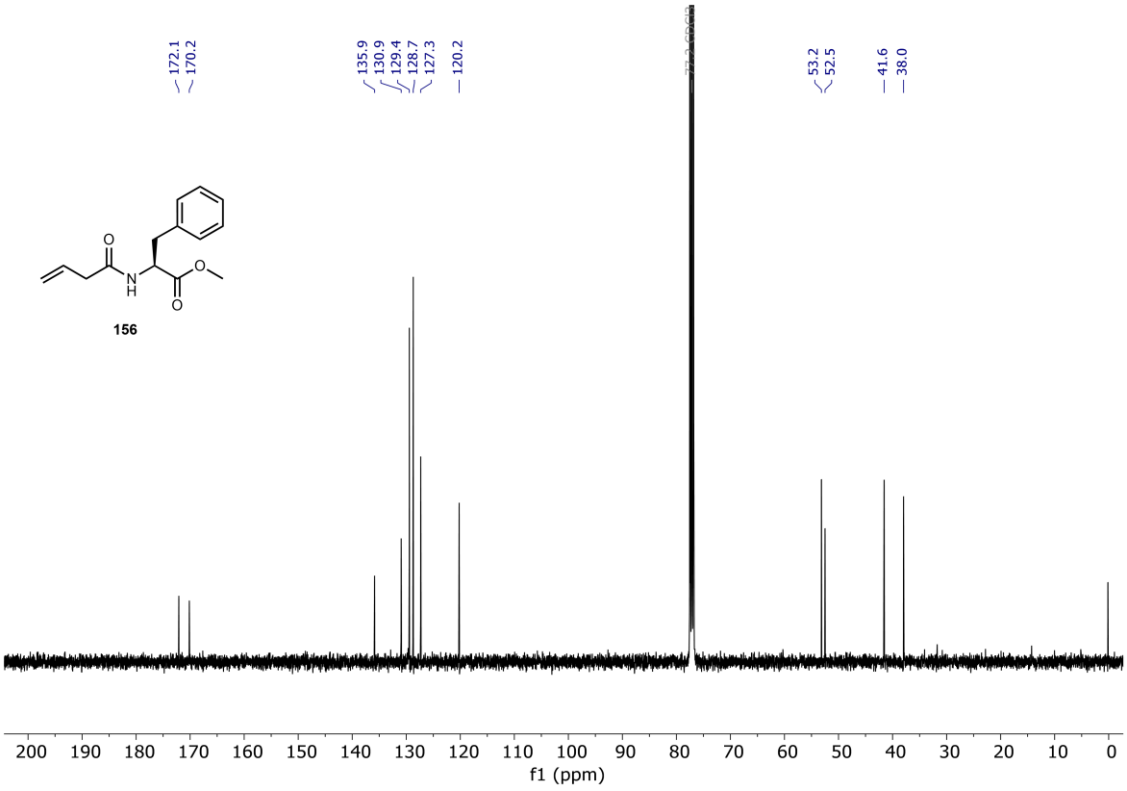

Figure A38. ^{13}C NMR spectrum of **156** (101 MHz, $CDCl_3$).

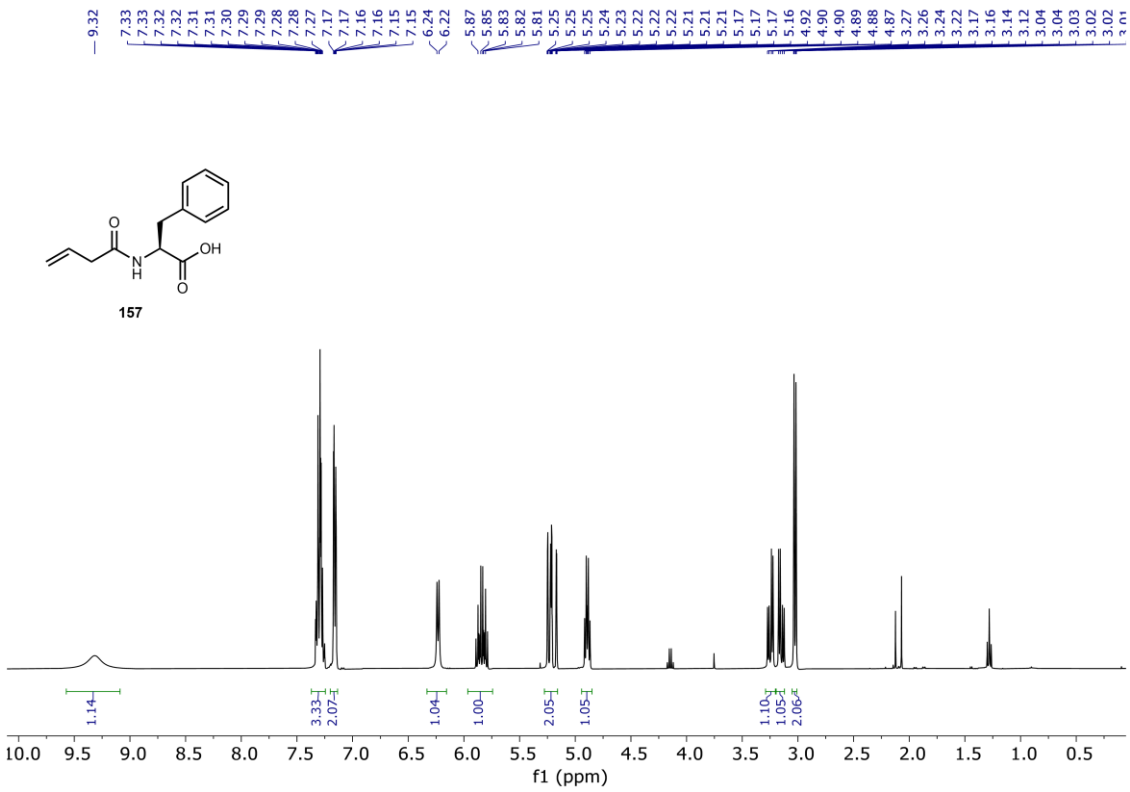

Figure A39. ^{1}H NMR spectrum of **157** (400 MHz, $CDCl_3$).

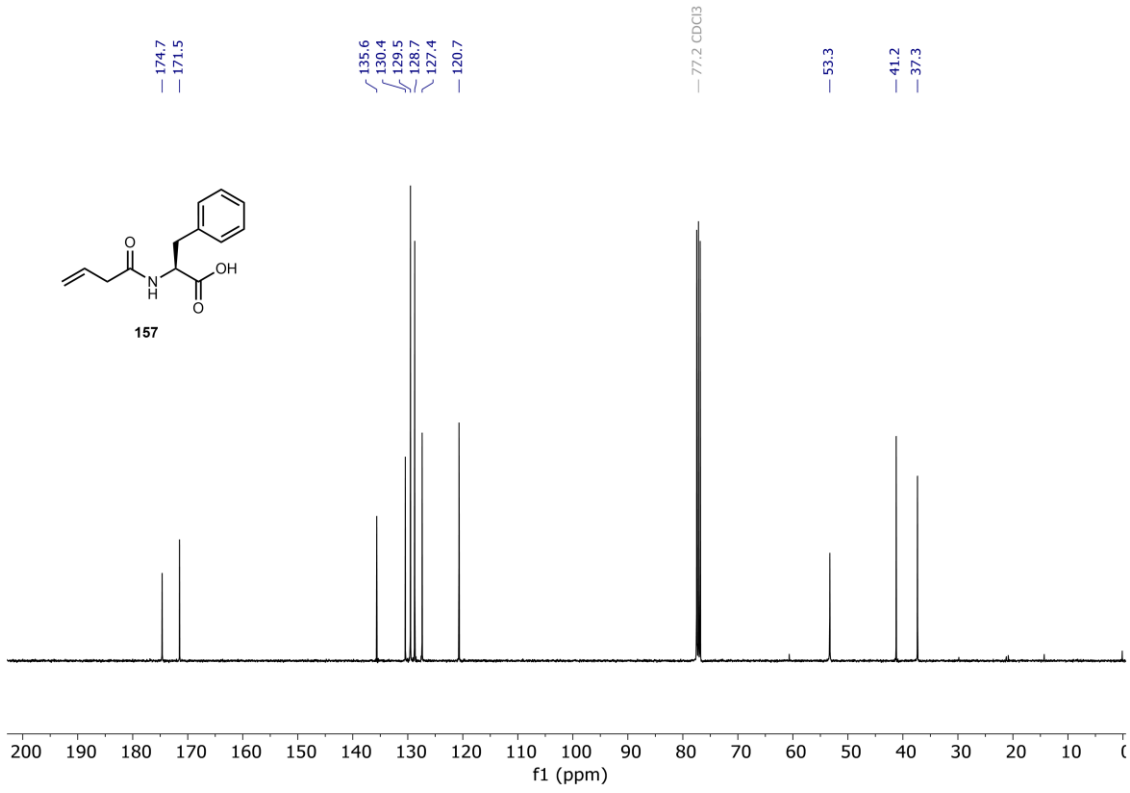

Figure A40. ^{13}C NMR spectrum of **157** (101 MHz, $CDCl_3$).

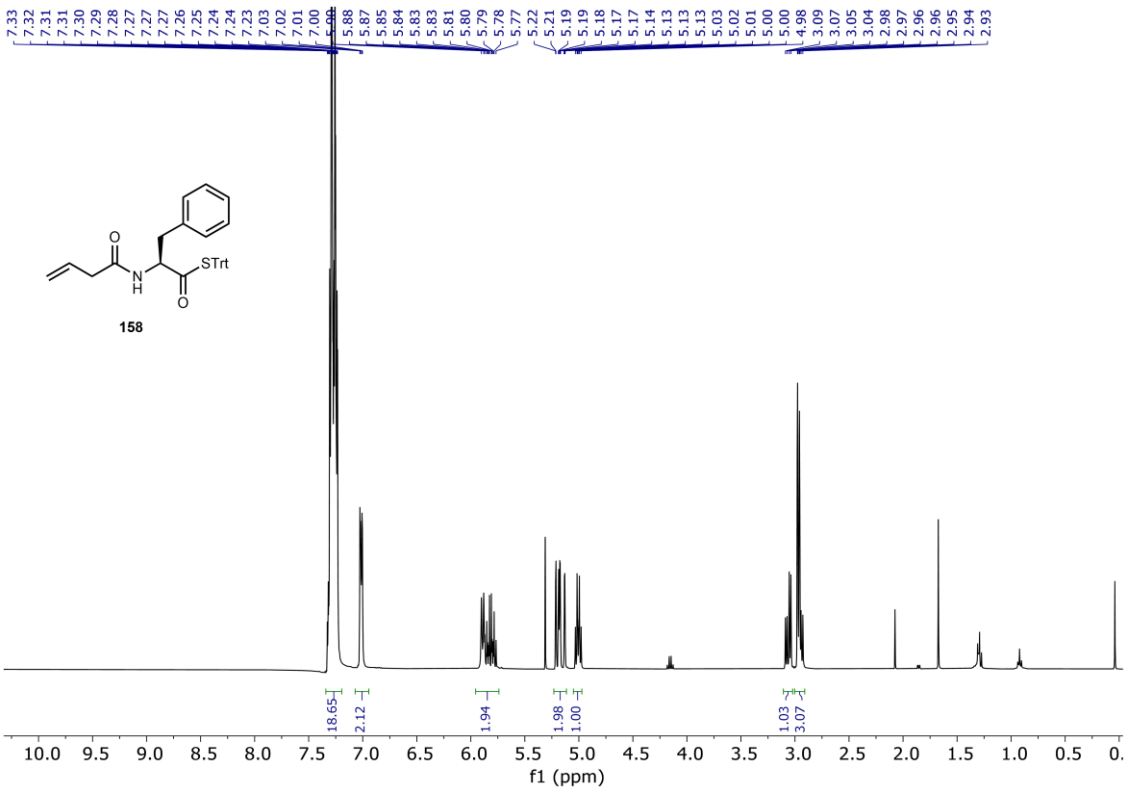

Figure A41. ^{1}H NMR spectrum of **158** (400 MHz, $CDCl_3$).

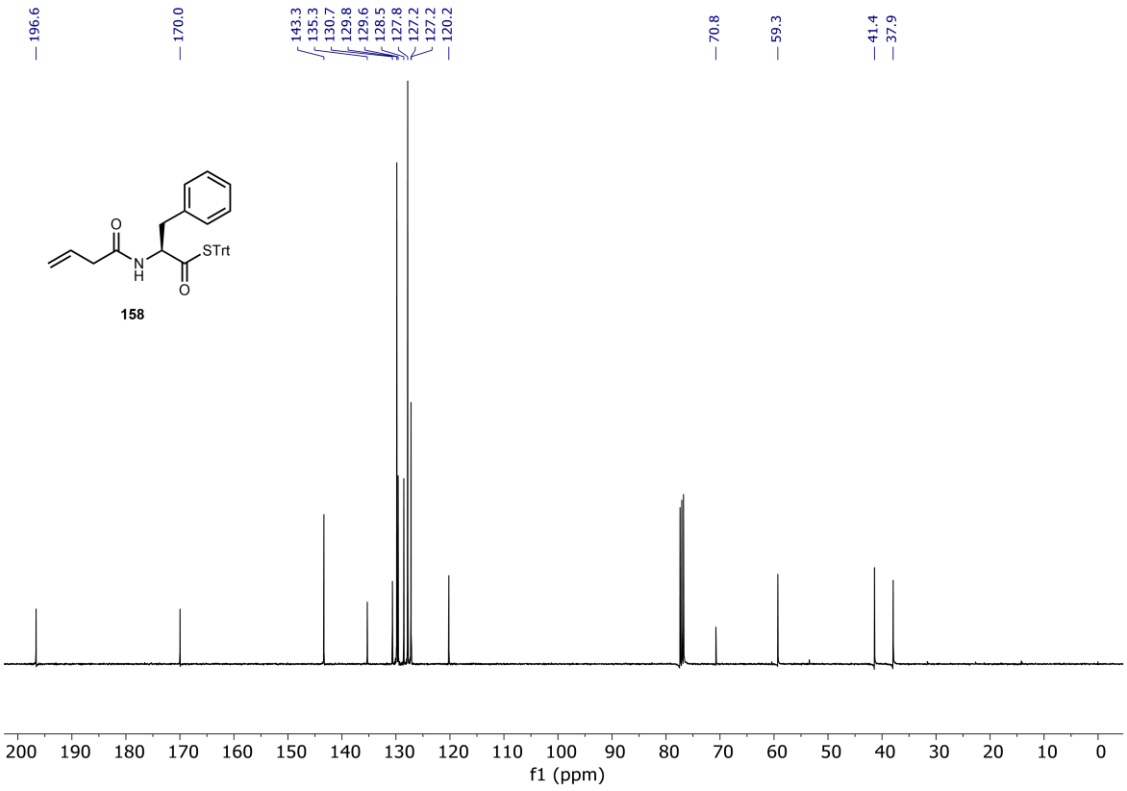

Figure A42. ^{13}C NMR spectrum of **158** (101 MHz, $CDCl_3$).