Development of Novel Thiol-ene Ligation Strategies for Thiosugar and Peptide Synthesis

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Declaration

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Abstract

This thesis, entitled “Novel Thiol-ene Ligation Strategies for Thiosugar and Peptide Synthesis”, is composed of 6 Chapters. The first chapter gives an overview of the radical-mediated thiol-ene and thiol-yne addition reactions, including a brief outline of thiyl radicals, intra- and intermolecular addition and the applications of these reactions in chemistry, biochemistry and other areas of research. Chapter 1 also explores the area of peptide ligation, including solid phase peptide synthesis and the advances that have been made to this field since the emergence of Native Chemical Ligation. In this section, some alternative routes to ligated peptides are discussed; such as desulfurisation, auxiliary mediated ligation, kinetically controlled ligation and expressed protein ligation. Following this, *S,N*-acyl transfer, the formation of an amide from a thioester through acyl migration, is detailed. Aspects of this acyl migration such as applications in nature, synthetic applications, the reversibility of the shift and the importance of the size of the cyclic intermediate are all covered. Lastly, this Chapter deals with the topic of thioacids; their synthesis and applications in peptide and protein chemistry. This chapter concludes with a brief outline of the work described within this thesis.

The first part of this work, presented Chapter 2, details the intramolecular thiol-yne cyclisation of arabinose derivatives; a novel route to thiosugars bearing both *endo* and *exo* alkenes. This work involves the ring opening of *O*-benzyl-protected arabinose and installation of both alkyne and free thiol at either end of the open chain sugar. The methodology for this process was previously carried out in the Scanlan lab on D-arabinose and this work focuses on the same principle utilising the L-arabinose derivative. The results, which vary greatly from D- to L-glycal, depict an interesting new methodology for thioglycal synthesis, centred on the intramolecular thiol-yne coupling reaction.

The second part of this work comprises of Chapters 3 and 4 and investigates intermolecular thiol-ene addition of α-amino thioacids onto peptidyl alkenes. This novel methodology furnishes an isopeptide with a thioether linkage, which, following *S,N*-acyl transfer, leads to the formation of a native peptide bond. The entire route is intended to provide an alternative to Native Chemical Ligation and is referred to throughout this thesis as Thiol-ene Mediated Peptide Ligation and Elimination (TEMPLE). Chapter 3 describes the utilisation of radical-mediated thiol-ene coupling

onto terminal alkene moieties attached to peptides through Ser/Thr esterification. Attempts to subsequently provide a native peptide linkage are thoroughly discussed in this section. Chapter 4 explores the utilisation of dehydroalanine for TEMPLE, employing both ionic and radical-mediated pathways to provide thioether-linked isopeptides. This part of the work aims to prepare the small protein GLP-1 *via* TEMPLE from small fragments synthesised by SPPS.

Chapter 5 concludes the work presented in this thesis and gives a brief outline of future work possible for this project.

Finally, Chapter 6 describes the experimental procedures employed for this work and the characterisation of the compounds synthesised within this thesis.

Abbreviations

Ac_2O	acetic anhydride
Ace	acetone
Ala	alanine
AML	auxiliary mediated ligation
app	apparent
aq.	aqueous
Ar	aromatic
Arg	arginine
Asn	asparagine
Asp	aspartic acid
AuNP	gold nanoparticle
BDE	bond dissociation energy
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
bs	broad singlet
BSA	bovine serum albumin
C-NCA	L-cysteine-*N*-carboxyanhydrides
calcd.	calculated
cat.	catalytic
CCTP	catalytic chain transfer polymerisation
CD	cyclodextrin
CDI	1,1'-carbonyldiimidazole
CO_2	carbon dioxide
conv.	conversion
CPE	cysteine-proline ester
CSP	chiral stationary phase
CuAAC	copper-catalysed azide-alkyne cycloaddition
Cys	cysteine
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	*N,N'*-dicyclohexylcarbodiimide
dd	doublet of doublets
DHA	dehydroalanine

Dhp	dehydroproline
Dhv	dehydrovaline
DIC	*N,N'*-diispropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DIU	*N,N'*-diisopropylurea
DLP	dilauroyl peroxide
DMAP	4-(dimethylamino)pyridine
DMF	*N,N'*-dimethylformamide
Dmmb	2-mercapto-4,5-dimethoxybenzyl
DMP	2,2-dimethoxypropane
DNA	deoxyribonucleic acid
DPAP	2,2-dimethoxy-2-acetophenone
DUB	deubiquitinating enzymes
EDCI·HCl	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
EPL	expressed protein ligation
eq.	equivalents
ESI	electrospray ionisation
EtOAc	ethyl acetate
Fm	9-fluorenylmethyl
Fmoc	9-fluorenylmethyloxycarbonyl
FRET	fluorescence resonance energy transfer
g	gram
Gln	glutamine
GLP-1	glucagon-like peptide-1
Glu	glutamic acid
Gly	glycine
GOPAL	genetically encoded orthogonal protection and activated ligation
GSH	glutathione
h	hour
HATU	hexafluorophosphate azabenzotriazole tetramethyluronium
HCTU	*O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Hex	hexane
His	histidine

HIV	human immunodeficiency virus
HMPSA	hot-melt pressure-sensitive adhesives
HOBt	1-hydroxy-1*H*-benzotriazole
hOGT	human *O*-GlcNAc transferase
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
IL-8	interleukin-8
Ile	isoleucine
IPN	interpenetrating networks
IR	infrared
J	coupling constant
K_2CO_3	potassium carbonate
KCL	kinetically controlled ligation
KSAc	potassium thioacetate
L-Hag	L-homoallylglycine
LDA	lithium diisopropylamide
Leu	leucine
LiOH	lithium hydroxide
Lys	lysine
M	mass ion or molarity (as relevant)
m	multiplet
m.p.	melting point
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption ionisation
MAP	4-methylacetophenone
MBP	2-methylbenzophenone
MeLi	methyl lithium
MesNa	sodium 2-mecaptoethylsulfonate
Met	methionine
mg	milligram
MHz	megahertz
min	minute
ml	millilitre
MOM	methoxymethyl ether

Mpa	α-methylphenacyl
MsCl	methanesulfonyl chloride
Na_2S	sodium sulfide
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NCAs	*N*-carboxyanhydrides
NCL	Native Chemical Ligation
NEt_3	triethylamine
nm	nanometre
NMM	4-methylmorpholine
NMR	nuclear magnetic resonance
PCET	proton coupled electron transfer
PEG	poly(ethylene glycol)
Phe	phenylalanine
POSS	polyhedral oligosilsesquioxane
ppm	parts per million
Pro	proline
PTM	post-translational modification
PVMS	poly(vinylmethylsiloxane)
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
pyr	pyridine
q	quartet
Q-TOF	quadruple time-of-flight
qC	quaternary carbon
RAFT	reversible addition-fragmentation chain transfer
refl.	reflux
R_f	retention factor
RNRs	ribonucleotide reductases
ROP	ring opening polymerisation
ROS	reactive oxygen species
rt	room temperature
s	singlet
SAL	sugar-assisted ligation
SAMs	self-assembled monolayers

SEA	*bis*(2-sulfanylethyl)amine
Ser	serine
SPPS	solid phase peptide synthesis
SUMO	small ubiquitin-related modifier
t	triplet
t-BuSH	*tert*-butyl mercaptan
TCEP	tris(2-carboxyethyl)phosphine
TEC	thiol-ene coupling
TEMPLE	Thiol-ene Mediated Peptide Ligation and Elimination
TES	triethylsilane
TFA	trifluoroacetic acid
TFET	2,2,2-trifluoroethanethiol
TGs	Transglutaminase enzymes
THF	tetrahydrofuran
Thr	threonine
TLC	thin layer chromatography
Tmob	2,4,6-trimethoxybenzyl thiol
TMP	tris(hydroxymethyl)propane
TMS	trimethylsilane
TOF	time of flight
Trp	tryptophan
Trt	trityl
TS	transition state
TYC	thiol-yne coupling
Tyr	tyrosine
Ub	ubiquitin
UDP	uridine diphosphate
UV	ultraviolet
UV-vis	ultraviolet-visible
VA-044	2,2’-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride
Val	valine
XBPO	phenylbis(2,4,6-trimethyl benzoyl)phosphine oxide
μCP	microcontact printing
μL	microlitre

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Publications

Corcé, V., McSweeney, L., Malone A., Scanlan, E.M., "Intramolecular Thiol-yne Cyclisation as a Novel Strategy for Thioglycal Synthesis", *Chem. Commun.*, **2015**, *51* (41), 8672-8674.

McSweeney, L., Dénès, F., Scanlan, E.M., "Thiyl-Radical Reactions in Carbohydrate Chemistry: From Thiosugars to Glycoconjugate Synthesis", *Eur. J. Org. Chem.*, **2016**, *12*, 2080-2095.

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Contents

Chapter 2 – Intramolecular cyclisation of an L-arabinose derivative

Chapter 3 – Intermolecular thiol-ene coupling towards peptide ligation

Chapter 4 – Employing dehydroalanine for peptide ligation

Chapter 5 – Overall conclusions

Chapter 6 – Experimental

Chapter 7 – References

1.0 Introduction

Thiol-ene coupling (TEC) and the analogous thiol-yne coupling (TYC) have been extensively investigated and have found broad applications in many fields of research, such as chemical synthesis, bioconjugation, surface modification, polymer and supramolecular chemistry. The attraction of this "click" reaction stems mainly from the mild conditions required, tolerance of aqueous conditions, the regioselectivity of the coupling and the versatility of systems to which it can be applied.

New methods for peptide ligation have seen a significant emergence since the discovery of Native Chemical Ligation (NCL) in 1994.[1] Thioacids and thioesters have been employed to furnish isopeptides bonds, followed by acyl shift mechanisms that furnish native bonds. This chemistry has seen wide utility in protein/peptide and glycoprotein/glycopeptide ligation over the past couple of decades. This chapter will outline the basic concepts and applications associated with TEC and TYC, as well as provide an insight into the methodology and advancements that surround peptide ligation.

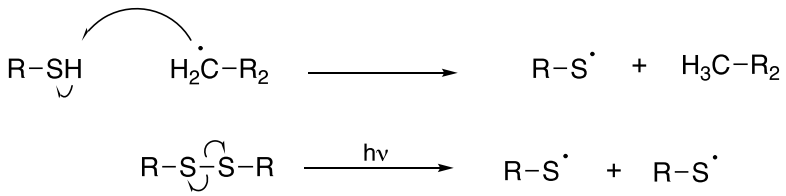
1.1 Thiol-ene coupling

1.1.1 Thiyl radicals

Sulfur is a ubiquitous element in human biology and maintains important interactions in drug design and therapeutic activity. Its valuable properties in heterocyclic structures as well as thioethers, thioesters and disulfides have been extensively reviewed within medicinal chemistry.[2] Thiyl radicals are highly reactive, versatile intermediates with a wide array of synthetic applications, primarily spanning the fields of chemistry and chemical biology. For these reasons, thiyl radicals have been widely implemented in synthetic chemistry for the preparation of biologically relevant compounds and analogues. A detailed discussion of the reactivity of thiyl radicals and their utility in chemistry and related fields is beyond the scope of thesis, however the reader is directed to recent reviews on the topic.[3, 4] This section will focus on the main aspects of hydrogen abstraction by thiyl radicals and the factors affecting this process.

Thiyl radicals are most commonly generated *via* hydrogen abstraction from a thiol by a carbon-centred radical. Thiols rapidly undergo hydrogen transfer to alkyl radicals, provided that the bond dissociation energy (BDE) of the C-H bond is higher

than that of the S-H bond. Bond dissociation energies vary with different types of thiols, however, a common alkyl thiol (RSH) has a bond dissociation energy of *ca.* 87 kcal mol^{-1}, which is notably lower than the C-H BDE of approximately 100 kcal mol^{-1} depending on the particular carbon chain.[5] Alkane thiols with varying alkyl substituents all bear similar bond dissociation energies, with the exception of cysteine, which has a weak S-H bond of 86 kcal mol^{-1} and disulfides which have weak S-S bond energies of 50 kcal mol^{-1} and 65 kcal mol^{-1} for diaryl- and dialkyldisulfides, respectively. On the other hand, hydrogen disulfide has a relatively high value of 91 kcal mol^{-1}.[6] The resonance stabilisation of thiyl radical formed from thiophenol (BDE 79 kcal mol^{-1}) makes this reagent a particularly good hydrogen atom donor, however this is dependent on the substituents on the ring.[7] The transfer of hydrogen atoms between thiols and alkyl radicals is dependent on the activation energy and enthalpy of the transfer, in addition to steric and polar factors, and the interaction of neighbouring π-systems within the transition state.[5] As a result, alkyl thiols can serve as more efficient hydrogen donors to alkyl radicals than some widely used hydrogen donors with weaker X-H bonds, such a tributyltin hydrides (BDE of 79 kcal mol^{-1}).[8]

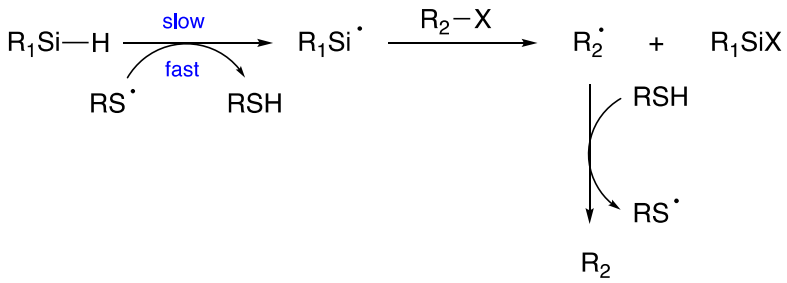


Scheme 1.1: Generation of thiyl radical by hydrogen abstraction from carbon centred radical (top) and by homolytic cleavage of a disulfide (bottom).

Thiyl radical generation (Scheme 1.1) is often initiated by hydrogen transfer to an alkoxy or alkyl radical, such as typical radical initiators under light irradiation. Single-electron oxidants such as Mn(III) compounds can also generate thiyl radicals, as well as the autoxidation of thiolates in the presence of oxygen.[9] Homolytic cleavage and substitution of disulfides are also viable methods of producing thiyl radicals; where the anionic disulfide radical formed breaks down to the corresponding thiyl radical and thiol, following protonation.[10, 11] Photosensitisers (typically ketones) are often used in the initiation step to provide a tertiary radical and ensure rapid hydrogen transfer from thiol to photosensitiser.[12]

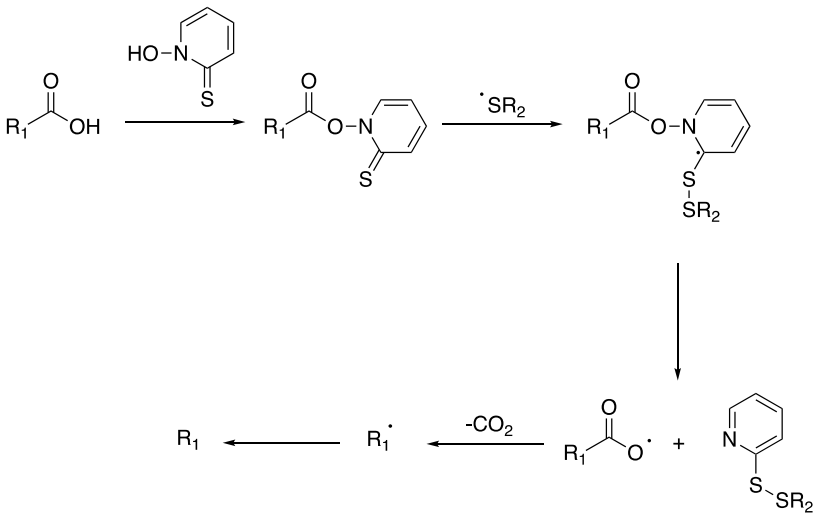
For the forward reaction of hydrogen abstraction of a thiol by an alkyl radical, primary alkyl radicals and α-alkoxy radicals react much more readily than secondary or tertiary species. However, for the reverse reaction, thiyl radical will abstract a hydrogen atom from a tertiary alkyl species faster than that of a secondary or primary.[4, 13] Roberts and co-workers have extensively explored the equilibrium of the C-H bond hydrogen abstraction by thiyl radicals and their ability to act as polarity-reversal catalysts.[14] Thiyl radicals, being electrophilic, can catalyse 'slow' radical chain reactions, such as decarbonylation,[15] as they satisfy the polar effects when nucleophilic carbon-based radicals are involved. This property of thiyl radicals has seen its utility in the catalysis of many radical reactions, including desulfurisation,[16-18] deoxygenation of methoxymethyl ether (MOM)-protected alcohols *via* β-fragmentation, silane addition onto alkenes and many more examples.[4, 19, 20]

The generation of silyl radical reactions *via* thiyl radicals encompasses many more opportunities in organic chemistry. Common alkylsilanes contain a strong Si-H bond that cannot undergo hydrogen abstraction by a carbon-centred radicals, with the exception of some silyl compounds such as tris(trimethylsilyl)silane ($(TMS)_3SiH$), which is frequently used in such reactions.[21-24] However, hydrogen abstraction from silanes by thiyl radicals have been shown to be effective in promoting radical-mediated reactions of silanes, particularly in hydrosilylation reaction of alkenes, where the use of an excess of silane can suppress the competing addition reaction of the thiyl radical onto the alkene.[20] Roberts and co-workers have also shown that dehalogenation by trialkylsilyl radicals can be promoted by the addition of a thiol to the reaction, with the possibility of the utilisation of a wide range of thiols, silanes and silanethiols (Scheme 1.2).[25, 26] Similarly, reductive alkylation of alkenes using alkylhalides was demonstrated *via* silyl radicals generated by hydrogen abstraction from silanes by thiyl radicals.[27-29] This C-C bond forming reaction again is dependent on the ability of the silyl radical to out compete the thiyl radical addition onto the alkene. Dehalogenation of the alkyl halide to form the alkyl radical leads to the addition of the alkyl radical onto the alkene. However, another possible outcome is the hydrogen atom transfer of the thiol catalyst onto the alkyl radical, hence only electron-rich alkenes and electrophilic alkyl radicals are suitable.



Scheme 1.2: Thiyl radical catalysis of dehalogenation of alkylhalides by alkylsilyl radicals.

Thiyl radicals are proficient catalysts in decarbonylation reactions due to their ability to abstract hydrogen atoms from the weak C-H bond in aldehydes.[15, 30, 31] This results in the release of CO and the formation of a carbon-centred radical capable of accepting a hydrogen atom from the newly formed thiol, thereby regenerating the thiyl radical catalyst which will propagate the reaction by hydrogen abstraction from the aldehyde. Decarboxylation is an additional feature of thiyl radicals through the use of a thiohydroxamic ester, a process known as the Barton decarboxylation.[32, 33] During this reaction, the thiyl radical adds to the C=S bond causing cleavage of the weak hydroxamic N-O bond and subsequent release of CO_2 to furnish the decarboxylated product (Scheme 1.3). Barton decarboxylation has been applied to numerous biomolecules including amino acid derivatives, peptides and carbohydrates.[34-40]



Scheme 1.3: General scheme for the Barton decarboxylation using thiohydroxamic ester.

The glycosyl thiyl radical has also been utilised by Roberts and co-workers for the purpose of desulfurisation, where the thiol acts as its own catalyst when abstracted from the sugar by a silyl radical, furnishing an anomeric carbon radical.[41] The same

authors employed the reagent to facilitate silyl radical addition onto alkenes, as previously mentioned.

The thiyl radical, however, is most established in biology in the family of enzymes responsible for deoxygenating ribonucleotides; ribonucleotase reductases (RNRs).[42, 43] The radical generation begins with a tyrosyl radical, located in one of the two subunits of a class I RNR. Proton coupled electron transfer (PCET) then occurs between this radical and Cys^{439} in the other subunit, which provides the thiyl radical. Hydrogen abstraction from the ribonucleotide 3'-hydrogen allows for deoxgygenation through the release of H_2O to follow (Figure 1.1).[44, 45] Class II and III RNRs proceed through similar pathways, however the thiyl radical generation at Cys is provided by an adenosyl radical in class II and a formate molecule in class III.[46]

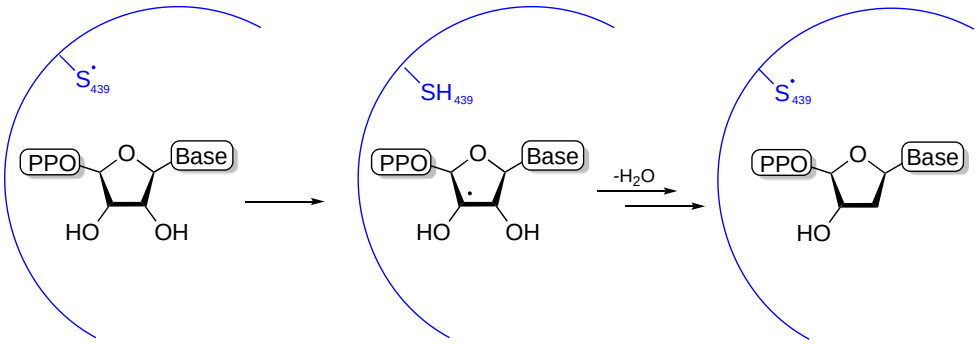


Figure 1.1: The deoxygenation of ribonucleotides by RNRs *via* thiyl radical generated in one of the enzyme's subunits.

Thiyl radicals also play a significant role in oxidative damage to DNA caused by *O*-centred radicals. Reactive oxygen species (ROS) such as OH· are reduced by endogenous thiols in Cys and Cys-containing biomolecules such as glutathione (GSH), assisting in the prevention of excessive oxidative damage by ROS.[47] However, the thiyl radical in turn can cause damage itself, often in proteins causing backbone cleavage by attacking the α-carbon centre.[48, 49, 50] Glycine, forming a secondary α-carbon centred radical due to the absence of a side group, is the most prone to attack from thiyl radicals, both intra- and intermolecularly.[51] In addition, thiyl radicals can evade their usual "repair" processes and result in addition reactions or other hydrogen atom transfers to cause mutagenesis.[52]

Despite the many associations thiyl radicals have in chemistry and related fields, the most established application of thiyl radicals, particularly in synthetic chemistry, is the addition onto unsaturated C-C bonds.

1.1.2 Intramolecular addition

1.1.2.1 Intramolecular addition onto alkenes

The intramolecular addition of thiyl radicals onto alkenes represents a fast and efficient route to sulfur-containing heterocycles. The regioselectivity of these cyclisation reactions has been thoroughly investigated by Surzur *et al*.,[53-56] where the authors determined that when a mixture of 5-*exo*-trig and 6-*endo*-trig products are formed, the reaction will have preference for the 6-membered ring. Moreover, it was shown that temperature can affect regioselectivity of the 6- or 7-membered cyclisation, where at high temperatures the 6-membered ring is preferred and by cooling the reaction to -65 °C, the 7-membered rings are the major product. The authors also found that using a mercaptan with a second heteroatom (S or O) could alter the ratio of regioisomers, without switching the result to the alternative ring size, however with a slight decrease in yield. In a similar study by the same group, the intramolecular thiyl radical addition onto a cyclic alkene almost exclusively produced thia-6-bicylco[3.2.1]octane over the alternative bicyclic structure forming two 6-memebered rings.[57] Tanaka and co-workers demonstrated a similar cyclisation with the dimerization product of an allyl mercaptan to yield 1,4-dithians in 79:21 ratio of *trans*:*cis*.[58]

Recently, Xu and co-workers have reported the synthesis of cyclobrassinin *via* intramolecular thiyl radical addition of dithiocarbamates.[59] Previous work by the same group demonstrated the synthesis of (2-alkylthiothiazolin-5-yl)-methyl dodecanoates from dithiocarbamates and using dilauroyl peroxide (DLP) as radical initiator where the 5-*exo*-trig cyclisation product was preferred over the corresponding 6-*endo*-trig.[60]

The synthesis of penicillin derivatives has been carried out using intramolecular thiol-ene coupling in an attempted synthesis of cephalosporins. Initial studies by Maki and Sako[61, 62] detailed the formation of a mixture of endocyclic and exocyclic alkenes *via* 6-*endo*-trig cyclisation when the reaction was carried out in acetonitrile at a low concentration. Interestingly, the authors found that increasing the concentration lead to the formation of an intermolecular addition as well as a 5-*exo*-trig cyclisation (Figure

1.2). It was reported that the thiyl radical **1** produced from the homolytic cleavage of the disulfide was likely to have firstly added onto the terminal alkene, which allowed for the 5-*exo*-trig cyclisation through a carbon-centred radical. The cyclisation of the desired product **3** was proposed to have gone through 6-*endo*-cyclisation after thiyl radical generation. However, it was later reported by Gordon and Cabri that the likely mechanism for the cyclisation is initially through 5-*exo*-trig cyclisation and subsequent rearrangement to form both 5- and 6-membered rings.[63] Gordon and co-workers also investigated different solvents for the reactions and found that by using a better hydrogen-donating solvent, such as MeOH, the products of the reaction included the reduced form of the penicillin derivative. Cabri *et al*. explored the metal-mediated catalysis of this reaction and reported several methods employing Mn(III), Fe(III) and Cu(II) based catalysts to carry out the radical-mediated cycloaddition.[64-66]

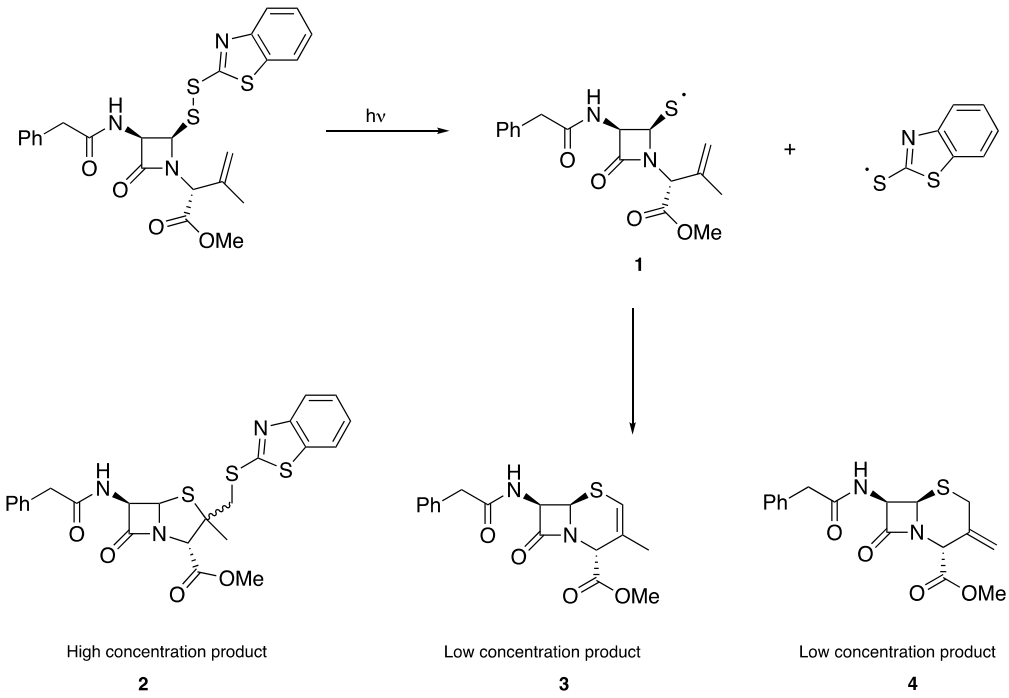
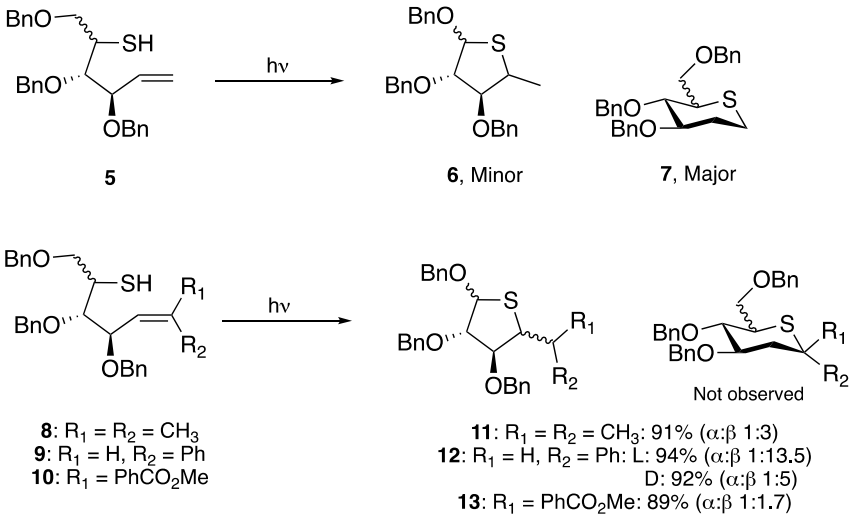


Figure 1.2: Intramolecular TEC in the synthesis of penicillin and cephalopsporin derivatives by Maki and Sako. **2** is formed through the radical reaction in high concentrations and **3** and **4** are formed in low concentration.

The Scanlan group has explored the application of intramolecular thiol-ene coupling in recent years,[67] in particular for the synthesis of thiosugars. Previously, thiyl-radical-mediated routes to such compounds were undiscovered and this novel methodology demonstrated the wide utility of the thiol-ene "click" reaction. The authors

succeeded in preparing both thiofuranose and thiopyranose products with superior control over regioselectivity from a single derivative of a commercially available sugar. *O*-benzyl protected arabinose was ring-opened, converted to the thiol **5** and functionalised with a terminal alkene. Under TEC conditions in a UV oven, the free thiol readily underwent thiol-ene addition to furnish the heterocycles **6** and **7** *via* either a 5-*exo* or 6-*endo* cyclisation. Using an unsubstituted terminal alkene, the thiopyranose **7** was obtained as the major product with both C-5 epimers.[68] The regioselectivity can be attributed to the stability of the carbon-centred radical formed after addition of the thiyl radical. With 6-*endo* cyclisation, this alkyl radical intermediate is secondary and is therefore favoured over the alternative primary alkyl radical formed in the 5-*exo* process. Subsequently, substituted thioglycosyl derivatives were employed in the same reaction in an attempt to drive the regioselectivity to the 5-*exo*-thiofuranose product.[69] Through the introduction of an isopropylidene group onto the arabinose scaffold, the cyclisation reaction of **8** was successfully directed to yield the thiofuranoside **11** exclusively, with the 1,2-*trans* product formed preferentially. In this case, in addition to steric hindrance, a tertiary carbon-centred radical provides a more stable intermediate than the alternative secondary radical intermediate and thus, the 6-*endo* heterocycle is not observed. Similar experiments with a phenyl substituent **9** and methyl benzoate substituent **10** on the alkene terminus both promoted the 5-*exo* cyclisation products **12** and **13**, but with varying degrees of diastereoselectivity. In each case the 1,2-*trans* product was observed as the major product, however, to a different degree depending on the stereochemistry of the C-5 epimer. When the L-sugar was used for cyclisation of **12**, the diastereoselectivity for 1,2-*trans* was greatly enhanced in comparison to that of the D-sugar. These findings can be rationalised in terms of this 1,2-*trans* transition state providing the most stable conformation of the thiyl radical intermediate; either "chair-like" or "boat-like". Thus, C-1 substitution bears a significant impact on the diastereoselectivity due to steric interactions. This work is further discussed in Chapter 2, Section 2.1.



Scheme 1.4: Synthesis of thiosugars *via* intermolecular thiol-ene coupling.

1.1.2.2 Intramolecular addition onto alkynes

The corresponding intramolecular thiyl radical addition onto alkynes, TYC, has seen less interest than the alkene equivalent. First demonstrated by Surzur,[70] the ring closing reaction of pent-4-yne-1-thiol yielded both 5-*exo* and 6-*endo* cyclisation products, with the major product being the 6-membered ring, similar to previous examples by Surzur. The Scanlan group further investigated the intramolecular thiol-yne reaction as a novel route to thioglycals.[71] Similar to the previous work done by the group, *O*-benzyl protected arabinose was converted to the alkyne-glycosyl thiol derivative and cyclised under radical conditions. The results were in contrast to previous reports where the 6-membered ring was formed preferentially. For the thiol-yne cyclisation the 5-*exo*-dig reaction was favoured for both C-5 epimers, and in the case of the D-sugar, the 6-*endo*-dig cyclisation was not observed at all. A detailed discussion on this work is reported in Chapter 2.

The third and final literature example of intramolecular thiol-yne coupling is the cyclisation reaction of stapled peptides, reported by Li and co-workers.[72] Stapled peptides overcome many of the issues surrounding therapeutic applications of peptides and proteins, such as solubility, proteolytic degradation and therapeutic availability.[73-76] Altering the structural complexity of proteins by introducing rigidity and stabilizing α-helical conformation is an attractive property of stapled peptides.[77] Moreover, the resistance of this type of peptide to proteases is increased due to the less exposed

peptide backbone.[78] However, the cross-link scaffold can have a significant impact on the bioavailability of the stapled peptide. While many varieties of cross-links exist in the literature, hydrocarbon-based links allow for increased lipophilicity for the penetration of the peptide into the cell membrane and the cell.[79, 80] This is often achieved by olefin metathesis,[81, 82] however the ruthenium catalyst required for this reaction is not always compatible with biological applications. Li and co-workers endeavoured to design a stapled peptide without the use of metals and that would incorporate slightly more polarity than other all-hydrocarbon links in order to avoid membrane toxicity from lipophilic aggregation (Figure 1.3).[83] Thus, stapled peptides were developed and optimised through thiol-yne coupling of short helical peptides, where the *Z*-isomer was favoured over the *E*-isomer and most products were furnished in high yields. In addition to HPLC traces at different time intervals, the reaction progress was monitored by ^{1}H NMR, where the appearance of vinyl sulfide peaks was observed. The structural helicity, cellular uptake abilities and membrane toxicity were evaluated and found to be favourable for the stapled peptides with vinyl sulfide cross-links. These compounds also display potential for tandem thiol-yne/thiol-ene functionalization of stapled peptides with biotags, a concept that will be further discussed in Section 1.1.3.2.

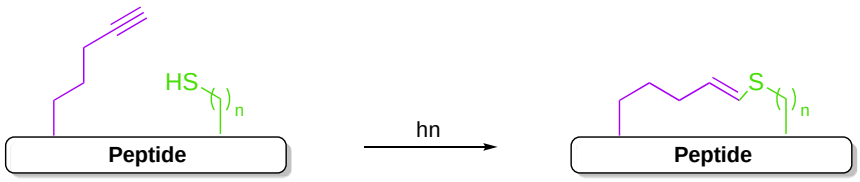


Figure 1.3: General scheme of peptide stapling *via* intramolecular thiol-yne addition.

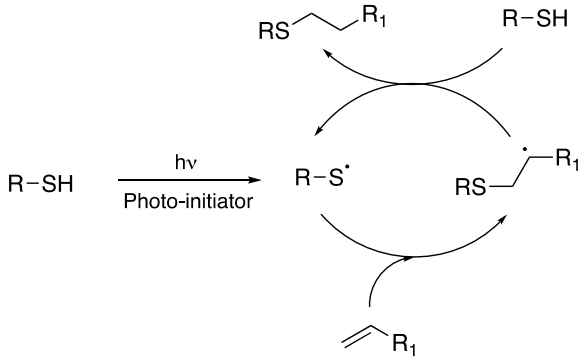
1.1.3 Intermolecular addition

1.1.3.1 Intermolecular addition onto alkenes

The most prevalent use of thiyl radical chemistry is the intermolecular thiol-ene coupling reaction between a thiol and an alkene, an efficient and rapid strategy in keeping with the characteristics of 'click' chemistry. Intermolecular thiol-ene coupling is utilised across many disciplines of chemistry, proving to be extremely valuable in polymer chemistry, surface chemistry, chemical biology and synthetic chemistry.[4] The radical-mediated addition furnishes a robust thioether linkage which can be

advantageous in overcoming issues associated with solubility and reactivity.[84] This section will include a brief overview of the main applications of intermolecular thiyl radical addition onto alkenes, however the reader is directed to a number of recent reviews for further reading.[85-94]

Initiation of the thiyl radical "donor" by conventional methods, such as UV light irradiation, is followed by anti-Markovnikov addition onto the least substituted carbon of the alkene "acceptor" (Scheme 1.5). The carbon-centred radical subsequently abstracts a hydrogen atom from another molecule of thiol, propagating the chain reaction. Many termination routes are possible, some resulting in disulfide formation or hydrogen atom transfer from another C-H bond, however the general high yields obtained with this strategy suggest that most termination options do not interfere with formation of the thioether product.



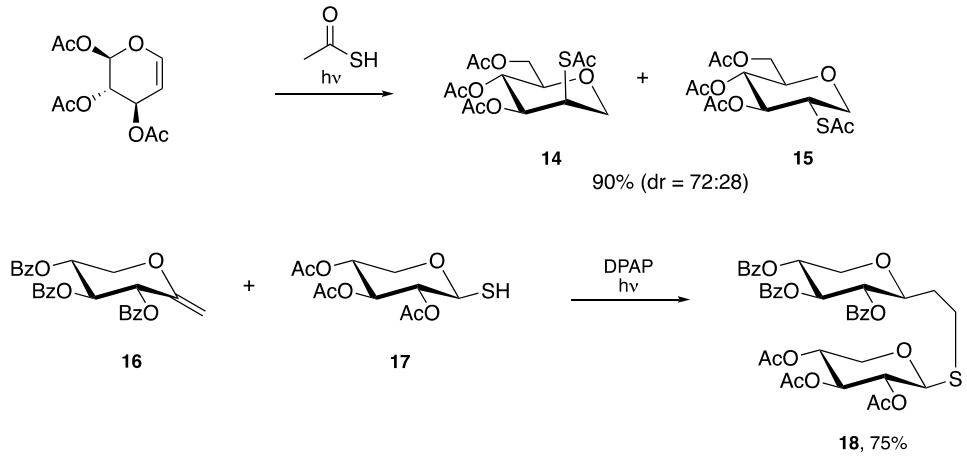
Scheme 1.5: General radical cycle of thiol-ene coupling.

Carbohydrates. The utility of TEC for the functionalization of carbohydrates and the burgeoning interest in applications for glycoconjugate chemistry has grown significantly in recent years.[86] Through both exocyclic and endocyclic glycals, a wide range of thiosugars have been synthesised *via* the fast and effective thiyl radical addition of thiols. The applications towards the addition of thiosugar to olefin have also been exploited, including the formation of *S*-linked disaccharides through TEC.[95-97]

The regioselectivity of the addition of thiols onto unsaturated bonds can often be controlled by steric and electronic factors, however the diastereoselectivity is usually less straightforward, likely due to the conformational constraints of carbohydrates. Igarashi and co-workers demonstrated the difficulty in controlling diastereoselectivity when employing TEC of thioacetic acid onto 1,2-glycals.[98] However, it was later shown that the addition onto the anomeric position could be achieved by the use of an *O*-acyl

substituent at the C-2 position (Scheme 1.6).[99] With this strategy, the thiosugars **14** and **15** were prepared in high yield and with complete α-selectivity. Borbás and co-workers also investigated TEC with 1,2-glycals with C-2 substituents, using in one instance a thiol-containing peptide, which resulted in exquisite 1,2-*cis* selectivity.[100] The authors explored the directing effects of the C-6 group, where bulky groups such as methyl or acetoxymethyl on the β-face to the carbohydrate backbone allowed for stereoselective 1,2-*cis* addition.[96] This effect was also observed when *O*-benzyl group protecting strategy was employed for the C-6 substituent. The extensive study by the same authors encompassed the TEC of glycosyl thiols onto 2,3-glycals, utilising an unsaturated *N*-acetylneuraminic acid derivative to deliver a C-3 *S*-linked disaccharide.

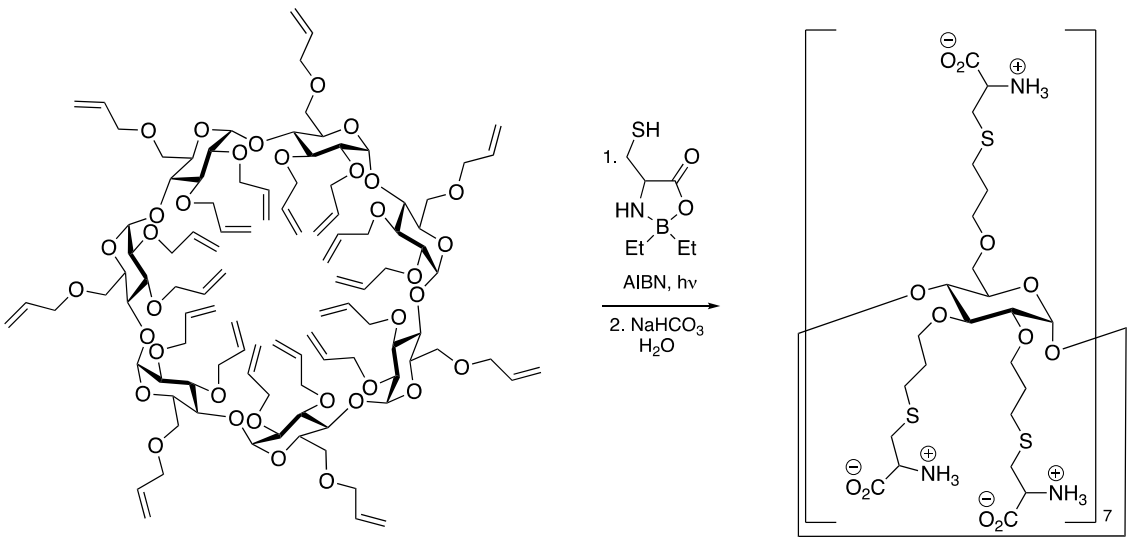
Exoglycals have been investigated as potential acceptors of intermolecular thiyl radical addition, where addition always occurs at the terminal, less substituted carbon to form a tertiary alkyl radical intermediate. Using the common thiol source of thioacetic acid, Gervay *et al*. explored the addition reaction onto unsaturated D-glucose and L-fructose derivatives and showed high equatorial stereoselectivity.[101] The same stereoselectivity was seen with D-glucose derivatives by Borbás and co-workers using ethanethiol.[96] An *exo*-xylyl derivative **16** was also employed in TEC with thiol **17** to furnish the pseudodisaccharide **18** in a high yield (Scheme 1.6).[102] Electronic effects by difluoro-*exo*-glycals were observed by Motherwell and co-workers, where the regioselectivity of the addition of thiophenol resulted in the exclusive attack of the thiyl radical at the terminal position.[103] *S*-linked disaccharides have also been prepared through intramolecular TEC by Marra, Dondoni and co-workers, again with complete regio- and stereoselectivity through addition of the thiol onto the 5,6-*exo*-glycal.[97]



Scheme 1.6: Examples of intermolecular TEC used in carbohydrate chemistry for endo- and exoglycals.

Glycodendrimer synthesis is emerging as a useful application for intramolecular TEC.[86] Multivalent glycoclusters were developed by Marra, Dondoni *et al*. from a tris(hydroxymethyl)propane (TMP) through allyl-functionalisation and subsequent thiol-ene "click" reactions.[104] This methodology was also carried out on propargyl-functionalised TMP scaffolds, which will be further discussed in the following section. The use of TEC for the synthesis of cyclodextrin (CD) dendrimer development has been of increasing interest in recent years. Stoddart and co-workers applied the thiyl radical addition of glycosyl thiols onto CDs with repeating allyl moieties.[105, 106] By using a 42-fold excess of the thiol, the authors were successful in functionalising both CD faces and in a later study, the authors managed to effectively increase the complexity of the CD dendrimers through TEC of anomeric thiodisaccharides.[107] Another level of complexity within this topic was achieved by Leydet *et al*. with the synthesis of *O*-perallylated CDs which following TEC, allowed three thiol substrates (3-mercaptopropanoic acid) per sugar molecule.[108] In addition to the anionic substrates, zwitterionic thiols were employed through the use of boroxazolidone-protected cysteine (Scheme 1.7). These dendrimers were applied to research into carbohydrate-based HIV therapeutics using anionic CDs. A range of thiols, including hydrophilic and fluorinated substrates, were applied to this same strategy, where mono-, di- or tri-functionalised sugars made up the CD macromolecule.[109] Contributions to analytical chemistry have also been made through the use of TEC for CDs in the synthesis of a chiral stationary phase (CSP) for the enantioselective purification of oppositely charged analytes.[110] Lindhorst and co-workers applied thiyl radical addition onto allylated glycosides to

form branched thioether linkages displaying a terminal alcohol, which was further functionalised with a monosaccharide unit.[111, 112] The branched glycodendrimer was used as a biomimetic for the glycocalyx, the outermost layer of the cell membrane.[113] Dondoni *et al*. have reported the use of TEC, together with copper-catalysed azide-alkyne cycloaddition (CuAAC), to prepare glycosylated calix[4]arene scaffolds.[114] In this report, the authors showed that the single and dual ligation on the upper and lower rims of the scaffold was possible with high yields and without distortion of the calix[4]arene cone conformation. Another scaffold that lead to glycodendrimers through TEC was the polyhedral oligosilsesquioxane (POSS), demonstrated by Dondoni and co-workers, a synthesis that lead to a remarkable glycoclusters effect discovered through inhibition of a specific lectin.[115]



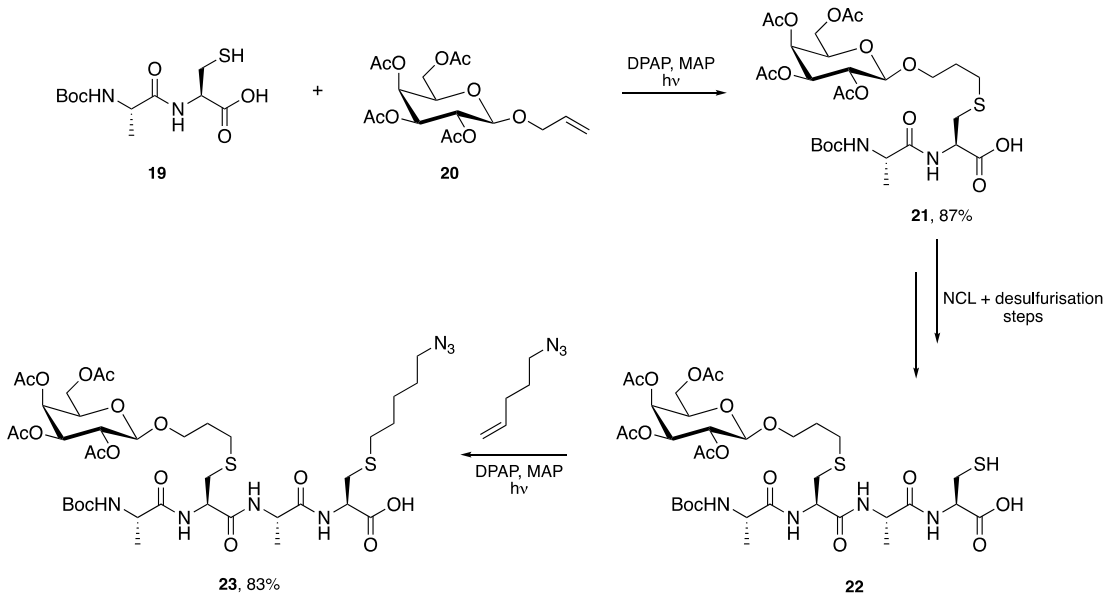
Scheme 1.7: Functionalisation of CDs *via* TEC to afford polyzwitterionic CDs.

Glycosylation though TEC is a prevalent route to surface and material modification, due to its "click-like" properties. Seeberger and co-workers have developed a solid phase poly(amidoamine) synthesis utilising thiol-ene chemistry with a continuous flow photoreactor.[116] During this novel methodology, the thiyl radical mediated addition was carried out both pre- and post-polymerisation using olefin building blocks for the former and compounds with the thioether linkage in place for the latter. Silane surface coating *via* TEC has been demonstrated by Gerrall *et al*. where trialkoxy silanes functionalised with either terminal thiols or alkenes were linked to

glycosides.[117] These reactions furnished the thioether products in > 99% conversion rate, which is a testament to the effectiveness of the thiol-ene coupling, considering the readiness of silyl radical generation from thiyl radicals (see Section 1.1.1). The Ravoo group pioneered an interesting and novel methodology to carry out microcontact printing (μCP) on an olefin-rich surface of self-assembled monolayers (SAMs) using glycosyl thiols as the "ink" which was attached to a stamp.[118] The group also carried out the reaction using thiol-functionalised SAMs and the olefin on the stamp.[119] This unusual application of thiol-ene chemistry highlights the versatility of the ligation reaction. Glycosurfactants containing a thioether linkage have been synthesised through thiol-ene chemistry as well as thiol-yne chemistry.[120] The glycosyl unit was esterified at the C-6 position with mercaptopropanoic acid, which was subjected to UV irradiation with long alkyl chains containing one or more alkene. In polymer chemistry, TEC has a substantial variety of applications (see further in this section; **Polymers**). Within the field of glycochemistry, Schubert and co-workers developed bio-responsive polymers through TEC to study binding interactions to the lectin *Ricinus communis agglutinin 120* (RCA_{120}).[121] The methodology was developed by the installation of an olefin on the monomer to be polymerised and the resulting polymer was subjected to TEC.

Glycoconjugation of peptides and proteins through thiol-ene click chemistry has been well demonstrated as a neat source of *S*-linked bioconjugates. Lee and co-workers first reported it for the synthesis of glycosides with thioether spacer arms.[122] This research was succeeded by more reports of TEC-mediated introduction of spacers, encompassing more complex sugar molecules and a wider range of thiol attachments.[123, 124] These developments led to the conjugation of cysteamine onto allylated oligosaccharides, which furnished biomimics that were recognised and transamidated by transglutaminase.[125] In anti-cancer research, vaccines have been synthesised through the TEC of glycopeptides and carrier proteins.[126] The carrier protein and synthetic glycopeptide were functionalised with either thiol or alkene (both strategies were demonstrated) and linked through UV irradiation. Another notable example of TEC in protein modification is the "tag-modify" approach reported by Davis and co-workers.[127] Protein glycosylation was carried out with thiosugars by expression of the non-natural amino acid L-homoallylglycine (L-Hag) in the protein sequence. Both alkene and thiol components were varied, including disaccharides and different proteins, to investigate the scope of steric bulk, conformation and stereochemistry for this ligation. The Scanlan

lab have reported a sequential NCL-TEC process, whereby a non-native linkage between L-Hag and a cysteine-containing dipeptide was established through TEC (Scheme 1.8).[128] Furthermore, the group extended this study to build a fluorescein-tagged glycopeptide through iterative NCL-TEC steps. Following glycosylation of cysteine in dipeptide **19** with glycosyl alkene **20** using TEC, the resulting peptide **21** was elongated to a tetrapeptide through NCL and subsequent desulfurisation to furnish Ala. An additional NCL step furnished the peptide **22**, with a second cysteine residue. This cysteine was subjected to radical-mediated addition of 5-azidopent-1-ene to provide an azide handle on **23**, which was functionalised with a fluorescein tag through CuAAC. Dondoni and co-workers have contributed greatly to research into glycopeptide/protein synthesis *via* TEC.



Scheme 1.8: Iterative NCL-TEC process developed by the Scanlan group. Following initial TEC of dipeptide **19** and glycoside **20**, the glycopeptide **21** was elongated *via* NCL and desulfurisation to provide **22**, which was subjected to another TEC to provide an azide handle for fluorescein tagging.

Anomeric allyl sugars were conjugated onto cysteine and cysteine-containing peptides such as glutathione (GSH).[129] This methodology was extended to demonstrate the glycosylation of bovine serum albumin (BSA), a post-translational modification (PTM) accomplished through intermolecular thiol-ene click chemistry. Glycosylated amino acids have been used in solid phase peptide synthesis (SPPS), where the radical-

mediated ligation to form the thioether linkage was carried out to furnish the monomer to be used for SPPS. A similar strategy was also carried out by the same authors in a one-pot, two-step approach using *S*-alkylation of GSH to attach a suitable alkene for subsequent hydrothiolation with a glycosyl thiol.[130] Deming *et al*. reported glycosylated L-cysteine-*N*-carboxyanhydride (glyco-C-NCAs) through thiol-ene ligation of an allyl-glycoside with N-protected cysteine, which was converted to the carboxyanhydride and incorporated into a glycopeptide.[131] The resulting peptides were subjected to a conformational switch from α-helix to a coil structure in order to drive water-solubility for the development of biomedical applications. In another biologically relevant example, *in vivo* cartilage regeneration has been probed by the introduction of carbohydrates onto collagen through radical thiol-ene addition, mimicking the extracellular matrix breakdown and repair mechanism *via* glycosylation of collagen.[132]

Peptides/proteins. Many examples of peptide and protein conjugation using thiol-ene click reactions have been explored, either using non-natural olefin-containing amino acids or thiol-containing amino acids (Cys) or both. Factors affecting the efficiency of thiol-ene chemistry in buffered conditions appropriate for biological studies are the size of the molecule, the thiyl radical donor and pKa of the corresponding thiol, the pH of the solution and composition of the reaction medium and the structure of the alkene acceptor molecule.[133]

Kunz *et al.* reported a major contribution to this field of research, with the conjugation of a glycopeptide to BSA for the development of vaccines.[126] Unlike the examples in Section 1.1.3, the carbohydrate component did not partake in TEC. The glycopeptide was synthesised through SPPS and was terminated with an acetyl-protected thiol linker, which was acetyl-deprotected prior to TEC. The antigen-BSA carrier was functionalised with an additional linker that provided an alkene handle. Following UV irradiation the authors were able to use MALDI-TOF mass spectrometry analysis to reveal eight glycopeptides per BSA carrier molecule. Moreover, alkene and thiol handles were incorporated onto each component, protein and glycopeptide, to show the compatibility of biomolecules with thiol-ene chemistry (Figure 1.4). More recently, S-linked UDP-peptides have been described by the van Aalten group using thiol-ene mediated conjugation of cysteine-containing peptides and allyl-UDP, which showed superior potency as inhibitors of human *O*-GlcNAc transferase (hOGT),[134] an

enzyme that is essential for embryonic development.[135, 136] Another recent publication outlined the lipidation of peptides through thiol-ene click chemistry using a radical initiator with either UV light, reflux conditions or microwave irradiation.[137, 138] The authors furnished *S*-palmitoylated peptides through the radical-mediated addition of cysteine or cysteine-containing peptides onto vinyl palmitate. Non-native isopeptide S-linkages have been reported by Strieter and co-workers to synthesise ubiquitin (Ub)-conjugated proteins, in which dimeric and trimeric Ub-conjugates were developed through TEC of multiple Cys sites on one Ub unit.[139]

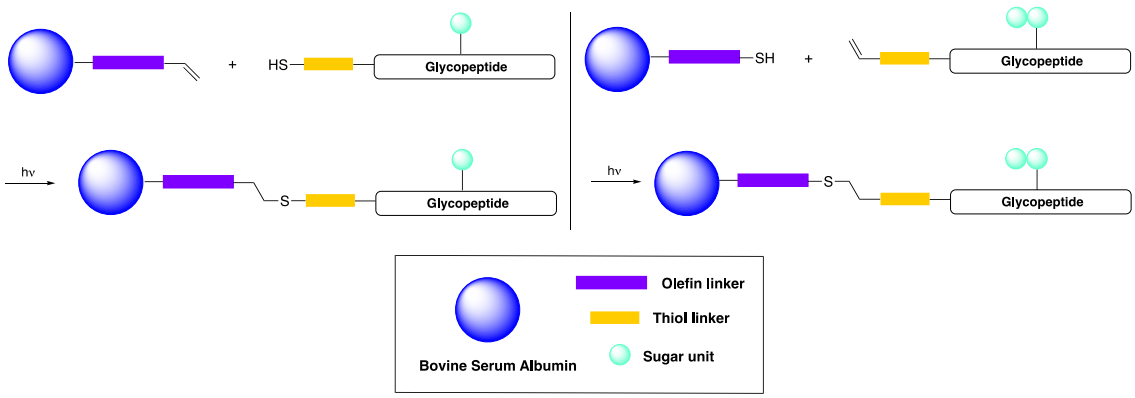


Figure 1.4: Antigen carrier BSA conjugated to tumour-associated glycopeptides for the synthesis of vaccines. The thioether linkage was forged though the alkene substituent on the protein and the thiol moiety on the glycopeptide, and vice versa.

The click-like properties of TEC provide a useful platform for the conjugation of peptides and proteins onto polymers.[140] Catalytic chain transfer polymerisation (CCTP) has been employed for the co-polymerisation of methyacrylates to provide a polymer with multiple alkene moieties.[141] An end-polymer alkene was conjugated onto the available Cys residues of α-keratin in human hair using radical-mediated addition. A branched alkene was subsequently ligated onto a thiol-containing fluorescent dye, furnishing fluorescently labelled human hair through the bioconjugation of polymers *via* TEC. The same authors accomplished a one-pot disulfide reduction and TEC to afford poly(ethylene glycol) (PEG) based polymer-protein conjugates.[142] Polymerisation of methylacrylates to furnish a polymer backbone, suitable for TEC with thiols from Cys peptides, was also applied by Klok and co-workers to deliver a tandem post-polymerisation modification process.[143] The authors later used this strategy to design

peptide-polymer conjugates for the inhibition of HIV-1 entry into the host cell by blocking the CD4 binding site on gp120.[144] The same year, the synthesis of hydrogels saw the benefit of the thiyl radical click reaction in the example of Lin *et al*., where thiol-ene cross-links established in the polymer showed significant effect on the degradation of the hydrogel, which was further tuned by the introduction of peptide cross-links, put in place by thiol-ene click reactions with the polymer.[145] Anseth and co-workers have introduced TEC to hydrogels to accomplish cytocompatible pattering of peptides within the hydrogel.[146] In materials chemistry, microarrays and surfaces have also been functionalised with biologically relevant molecules such as peptides and proteins through thiyl radical additions to alkenes.[147]

Polymers. Polymer synthesis and post-polymerisation modification has seen a major surge in the use of the intermolecular thiol-ene reaction, owing to its compatibility with many materials and fast, effective ligation under UV light.[4, 89] In particular, TEC has become popular for the formation of thioether cross-links in polymer chemistry.[85] As previously mentioned, Lin and co-workers used peptidyl cross-links for degradation studies, however the initial hydrogels were synthesised with alkyl thiol linkages.[145] Kim and co-workers developed thioether cross-links through the UV-mediated addition in order to produce hot-melt pressure-sensitive adhesives (HMPSAs).[148] The authors found that different photoinitiators and photo-cross-linkers furnished polymers with varying thermal stability and hence these variables were tested against important adhesion properties. In the synthesis of photonic crystals, a metallopolymer network was synthesised by Ozin *et al*. with thioether cross-linking of a multifunctional thiol and alkenes on a polyferrocenylsilane core.[149] Caruso and co-workers reported the functionalisation of silica nanoparticles with polymer multilayers bearing an alkene, which were further functionalised through TEC to introduce cross-linking.[150] In this example, without the cross-links in place the multilayers, held together by hydrogen bonds, would disintegrate.

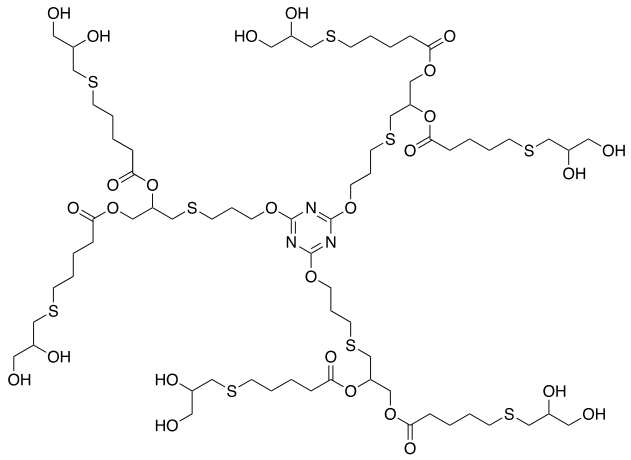


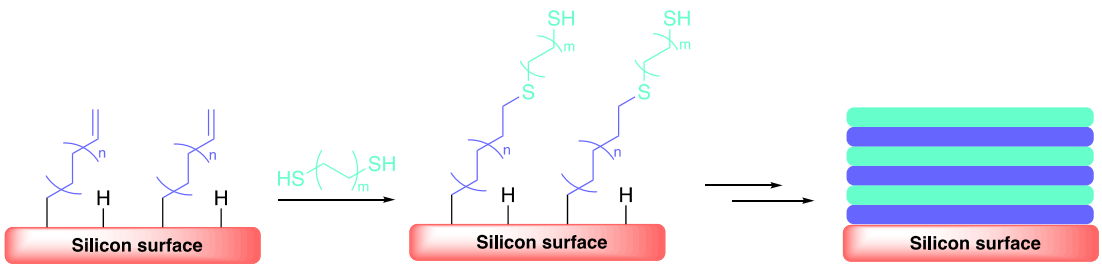
Figure 1.5: 4th generation dendrimer synthesised from the functionalisation of polymer using TEC to construct the backbone and the chain ends.

Dendrimer synthesis has utilised the radical thiol-ene addition to build the core and to modify the chain ends in the synthesis of a 4th generation dendrimer (Figure 1.5).[151] Hawker *et al*. have undertaken comprehensive research into polymerisation techniques using thiol-ene chemistry, including photochemical and thermal mediated reactions.[152] Using reversible addition-fragmentation chain transfer (RAFT) polymerisation, amongst other methods of polymerisation, the preparation of polymers functionalised with alkene moieties, which were subsequently subjected to TEC produced the resulting polymers in high yields using both thermal and photochemical radical initiation. Additionally, the sequential thiol-ene/CuAAC to polystyrene was carried out to furnish telechelic materials. The same authors have investigated the application of TEC in the preparation of stamps based on PEG and polysiloxane motifs.[153]

In dental science, ester-free resins for tooth restorative materials have been synthesised using thiol-ene step-growth reactions. The thioether linkage employed is superior to any preceding ester-linked oligomers due to their susceptibility to hydrolysis in the presence of acids/bases and certain enzymes and to swelling in aqueous environments.[154, 155]

Surface modification. Patterning of surfaces through thiol-ene chemistry is often approached through a "grafting" process, which involves the radical-mediated (or often Michael or a combination of both) addition of thiols onto alkenes, where the thiol and

alkene can be located on either the surface or the substrate.[85, 89] Thiol-ene polymerisation has been developed for forming patterned nanostructures through imprint lithography.[156] The authors used UV light irradiation to produce thiyl radicals from the thiol moieties located on the surface and carried out ligation with alkene-containing polymers. Crowe and co-workers have demonstrated the opposite ligation for the adjustment of the wettability of a surface, functionalising a poly(vinylmethylsiloxane) (PVMS) surface with a number of thiol alkanes of varying chain lengths.[157] In addition to the example with carbohydrate applications, SAMs have been subjected to thiol-ene coupling with polymers.[158, 159] Bowman and co-workers developed a gradient thiol-ene polymerised SAM through the attachment of an ultrathin film with a gradient of thiol onto the surface.[158] Hydrophobic SAMs have been prepared for microfluid devices using TEC of alkenes and thiols presented on the surface of silica or glass.[159] Buriak *et al*. have reported the molecular layer deposition of multiple layers of dithiols and dienes to create a sequential TEC process of adding layers onto three different surfaces, hydride-terminated silicon, silicon oxide and germanium (Scheme 1.9).[160]



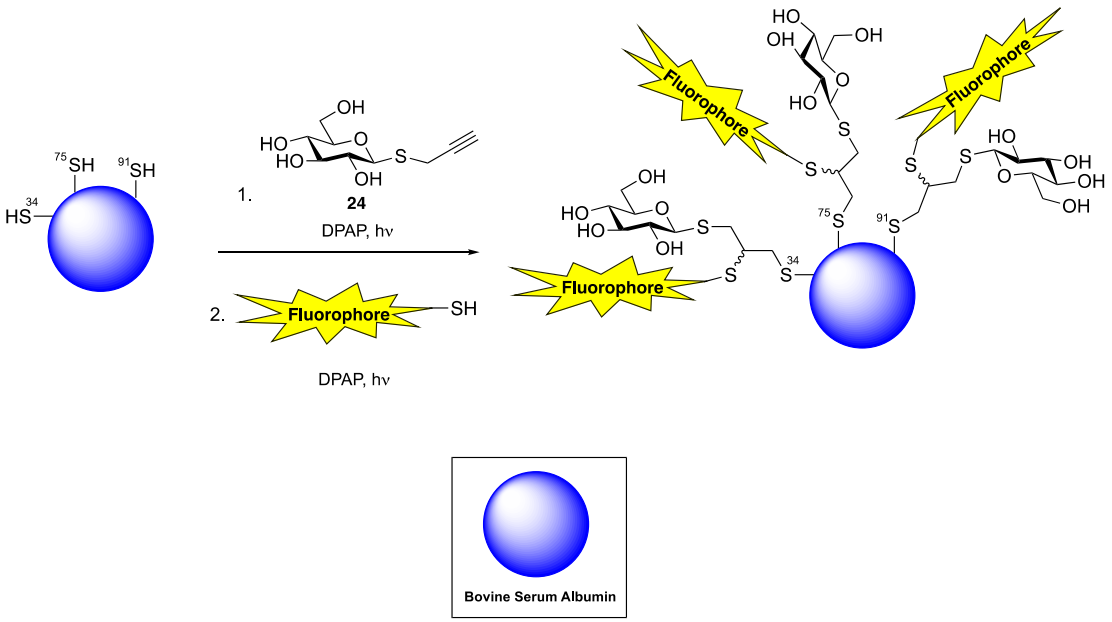
Scheme 1.9: General schematic representation of iterative TEC reactions to furnish a molecular layer deposition of multiple layers onto a silicon-based surface.

1.1.3.2 Intermolecular addition onto alkynes

The intermolecular addition of thiyl free radicals onto alkynes is comparable in utility to the most widely known click reaction involving alkynes, CuAAC. For most applications where thiol-ene coupling can be employed, an analogous thiol-yne reaction is often possible, furnishing an alkene, which can be further functionalised in a tandem TEC/TYC process. Similar to thiol-ene chemistry, intermolecular thiol-yne reactions

are exploited in a vast array of applications which is beyond the scope of this thesis and so the reader is directed to reviews on the topic.[3, 161-163]

In glycoconjugation TYC is a very attractive route to functionalised macromolecules. Borbás and co-workers reported the sequential TYC-TEC reactions on N-acetyl-neuraminic acid derivatives.[164] An *O*-propargyl galactose derivative was subjected to UV irradiation in the presence of photoiniator and sialic acid with an anomeric thiol. Unfortunately, the intended *bis*-sialic acid glycoside was not favoured, likely due to steric limitations, and the major product formed was a mixture of *E*/*Z* isomers of the mono-functionalised glycoside. The utilisation of a linker to provide space for the *bis*-addition was employed however the same results were observed with the vinyl sulfide being the major product. Marra, Dondoni and co-workers have extensively explored the use of thiol-yne chemistry for glycoconjugation.[104, 165, 166] Alkynyl peptide derivatives have been dually glycosylated through TEC-TYC reactions with anomeric glycosyl thiols.[165] Propargyl bromide was used to install the alkyne moiety onto the Cys of GSH and the resulting radical reactions furnished the biantenary glycopeptide in high yields for both the glucose and galactose analogues. Glycopeptide vinyl sulfides synthesised *via* TYC were also functionalised with a biotin affinity tag, highlighting the application of this overall process for biomolecule labelling. In a later report, the same authors described the dual functionalisation of three Cys residues in BSA with a) propargylated thioglycoside **24** and b) a thiol-containing fluorophore (Scheme 1.10).[166]



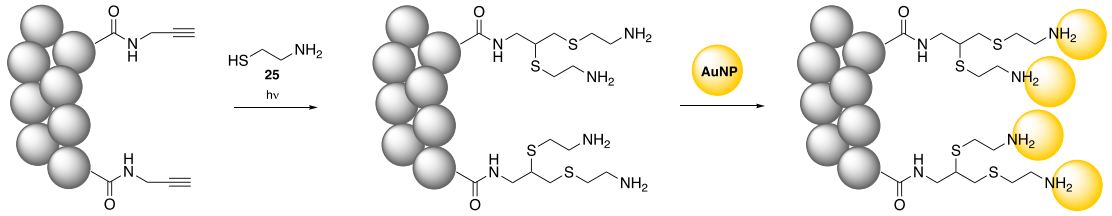
Scheme 1.10: Dual functionalisation of BSA Cys sites with i) a glycoside **24** and ii) a fluorophore. The initial TYC with the alkynyl sugar provided an alkene handle for TEC with the fluorophore.

As mentioned previously, glycosylation of dendritic clusters has been carried out using TEC, however Dondoni *et al.* have included thiol-yne chemistry in this process to furnish a higher degree of glycosylation on the scaffolds.[104] Perrier and co-workers described the co-polymerisation of alkyne acrylate monomer, which was further subjected to TYC to afford highly ordered branched glycopolymers.[167] Stenzel and co-workers used a similar method to develop co-polymeric dendritic and linear glycoconjugates which self-assembled in water due to the amphiphilic structure.[168] TYC of glycosides has been investigated for μCP by the Ravoo group in a similar fashion and in the same report as their example of TEC to prepare SAMs.[118]

Peptide and protein modifications through TYC have been widely reported. Chen *et al.* have described the synthesis of peptide-based double-hydrophilic block copolymers through TYC for their application in biomimetics. In this report the ring opening polymerisation (ROP) of NCAs furnishes the polypeptide with an alkyne moiety at each repeating unit, which undergoes thiol-yne ligation with mercaptopropionic acid.[169] Brase and co-workers have recently reported the TYC of an alkyne and a disulfide bond. In their initial report, the authors described this reaction using Cys disulfide and 6-heptynoic acid in the presence of photoinitiator, phenylbis(2,4,6-trimethyl benzoyl)phosphine oxide (XBPO), which resulted in

quantitative conversion to a mixture of diastereoisomers.[170] The study was subsequently extended to include peptides and proteins, where the authors delivered a range of alkynes to disulfide bonds in peptides cyclised by the disulfide bond present.[171] Impressively, the authors also delivered an alkyne payload to the Fab fragment of an antibody to demonstrate the potential this methodology could have in the synthesis of antibody drug conjugates. Site-specific dual labelling of a protein through thiol-yne addition was carried out by the Li group to dually functionalise the HdeA protein with two fluorescent tags.[172] The protein, bearing an alkyne-functionalised Lys residue, was subjected to radical addition of a fluorescent disulfide, *N*,*N*'-bis(dansyl)cystamine.

In polymer and materials science, intermolecular TYC has enabled seminal contributions to the field.[161] Polymerisation through the thiol-yne click reaction has been reported by several groups, with the first literature example reported by Fairbanks and co-workers, where the authors detailed the comparison of the step-growth polymerisation of the thiyl radical addition of alkynes to that of alkenes.[173] A more recent example involves the synthesis of interpenetrating networks (IPNs) in the form of hydrogels by the polymerization of gelatin through the radical thiyl addition onto both alkenes and alkynes.[174] Post-polymerization modifications are often carried out through the use of thiol-yne chemistry. Huynh and co-workers, towards the development of polymer-bound Pt drug release, reported one such example.[175] The RAFT polymerisation of methylacrylates followed by functionalisation of the repeating unit with an alkyne provided the polymer with the TYC handle in place. The alkyne was subsequently subjected to radical thiol-yne addition of 2-mercaptosuccinic acid, which was complexed to *cis*-diamminediaqua Pt (II). Surface modifications *via* TYC are also widespread in the literature, usually involving polymer synthesis. Gold nanoparticles (AuNPs) were immobilised onto a polymer motif through linkages forged by TYC.[176] The polymer, armed with succinimide handles, was functionalised with propargyl moieties, which were subsequently subjected to TYC with cysteamine **25** to furnish branched linkers for homogeneous adsorption to the nanoparticles (Scheme 1.11). Graphene oxide surfaces were modified through thiol-yne click chemistry by the functionalisation of the surface with alkynyl-dopamine.[177] Two thiols, ferrocenethiol and perfluorinated alkenethiol, were added to the surface *via* TYC, however only the ferrocene species carried out successful double ligation, while the perfluorinated thiol favoured the mono-addition.



Scheme 1.11: Immobilisation of gold nanoparticles (AuNPs) onto a polymer surface through linkers furnished by TYC.

1.2 Peptide ligation

Peptide ligation centres on the regioselective formation of an amide bond between a *C*-terminal carboxylic acid and an *N*-terminal amine. Many routes to amide bond synthesis have been explored over the course of a number of years,[178] however the same strategies employing activating agents and coupling reagents in the presence of a suitable base continue to be used across the board in peptide chemistry.[179, 180] Amide bond formation is extremely prevalent in industrial pharmaceutical development and accounts for 16% of all reactions carried out in the industry.[178, 181] Peptide synthesis itself has become a major focus in the pharmaceutical industry since the emergence of peptides as drug targets.[182-184]

1.2.1 Solid phase peptide synthesis

Peptides, due to their polymeric nature, have limited possibilities in standard solution phase synthesis, where orthogonally protected amino acids or peptide segments are coupled in organic solvents. The solubility of such peptides becomes troublesome when sequence reaches a length that is incompatible with organic solvents, with the length of the sequences compatible being dependent on the residue-specific side chain. Moreover, extensive purification steps are required with each coupling and the entire process is considerably time consuming. Solid phase peptide synthesis (SPPS) addresses these issues in a multitude of ways, and allows polypeptides of up to 50 residues long to be prepared.[185]

The step-wise synthesis of a tetrapeptide using solid phase chemistry was reported by Merrifield in 1963 and has since revolutionised peptide chemistry.[186] The

process, depicted in Figure 1.6, begins with the coupling of the first *N*-protected amino acid in the sequence (going from *C*- to *N*-terminals) to the resin that will be used throughout the coupling, employing the activating agent, coupling reagent and base suitable for the reaction. Resins are commercially available in many different varieties with a wide range of chemical composition and linkers. The *N*-protecting group is subsequently cleaved and the coupling step is repeated with the next amino acid in the sequence. These steps are repeated until the entire sequence required is built onto the resin, with serial washing steps after each reaction using solvent to clear the resin of remaining reagents. When the sequence is finished cleavage of the *C*-terminal from the resin is carried out to deliver the peptide. The cleavage step is usually designed to universally deprotect the entire peptide from side-chain protecting groups.[187, 188]

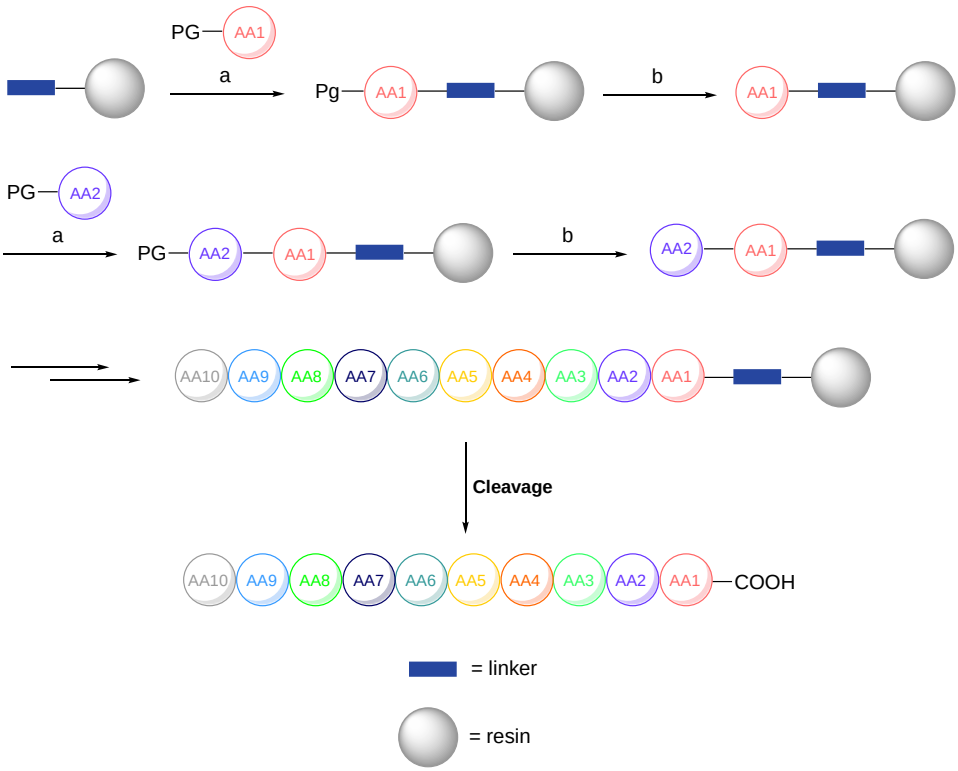


Figure 1.6: Reiterative process of solid phase peptide synthesis (SPPS). The *N*-protected amino acid (AA1) is coupled to the resin using an activating agent, coupling reagent, base and organic solvent (a). Deprotecting conditions (b) are employed to remove the protecting group and allow for another coupling of the second amino acid (AA2). These step are repeated until the entire sequence has been attached to the resin from *C*- to *N*-terminus, followed by cleavage of the peptide off the resin to furnish the globally deprotected peptide.

High concentrations of coupling reagents in respect to each amino acid drive the coupling reactions to achieve high yields and allows for a shorter reaction time than solution phase peptide coupling. However, the isolated peptide furnished is often in combination with many impurities due to the restriction of purification at each step to washes with solvent. In standard solution phase synthesis the resulting peptide could have a high purity, owing to the opportunity to carry out column chromatography after each coupling, however this leads to a significantly longer overall preparation time for the peptide.[185] In SPPS, deletion sequences are often present with the final peptide, which comprise of shorter fragments of the peptide that did not undergo coupling at one stage and are lacking one or more residues. There are various resolutions to this problem, such as dyes that show beads of resin that bear a free amine, i.e. did not undergo coupling, indicating the requirement for double-coupling of an amino acid.[189, 190] Another technique used known as "capping" involves an additional acetylation step to *N*-acetyl-protect any sequences with a free amine so they do not participate in further coupling reactions, which would render them difficult to separate from the desired peptide at the end.[191]

The SPPS method chosen is usually dependent on the protecting group required for the synthesis. Two SPPS strategies that are most commonly used are *tert*-butyloxycarbonyl (Boc)-protecting group SPPS and 9-fluorenylmethyloxycarbonyl (Fmoc)-protecting group SPPS. The appropriate resin, deprotection reagents and cleavage cocktail are determined by the choice of protecting group. The Boc protocol typically uses trifluoroacetic acid (TFA) to remove each Boc group, however the conditions to cleave from the resin usually involve very strong acid such as HF. The Fmoc-protecting group protocol is much more widely used for SPPS due its superior orthogonality, since a piperidine/DMF solution can be employed for deprotecting Fmoc groups and thus, many acid-labile protecting groups can be exploited for the side-chains of the amino acids used. Moreover, less harsh conditions required for cleaving from the resin can be employed.[192, 193]

SPPS remains the most time-efficient and well-practiced method of synthesising peptides and is consistently being enhanced in all aspects of the process.[185] Currently, automated solid phase peptide synthesis is employed in many research institutions and industrial laboratories, utilising new technologies for a faster, more efficient synthesis,

such as in-built microwave reactors to improve coupling and UV-vis monitoring of cleaved Fmoc molecules to gauge the efficiency of couplings.[194]

1.2.2 Native Chemical Ligation

Native Chemical Ligation (NCL) is a methodology first reported by Kent *et al*. in 1994 to deliver long peptide chains through the chemical ligation of two peptides.[1] It has since been applied across the board in peptide chemistry to access polypeptides and proteins of macromolecular scale.[195] The first peptide synthesis *via* NCL was employed to prepare interleukin-8 (IL-8) protein, a chemokine produced by macrophages comprising of 72-amino acid residues. Two IL-8 segments, a 33-amino acid and a 39-amino acid peptide, were successfully coupled through NCL, utilising a total of eighteen different natural amino acids including four cysteine residues. The properties of the resulting molecule (IL-8) were consistent with those of the natural protein and regular structural events, such as oxidation, disulfide bridge formation and folding, were observed. Moreover, little to no racemisation occurred during the regioselective reaction.

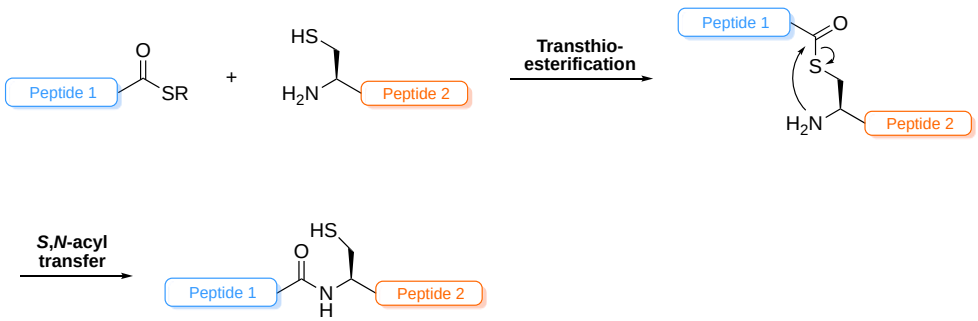


Figure 1.7: General scheme of NCL. Step 1: Transthioesterification to link peptides through thioester linkage. Step 2: *S,N*-acyl transfer to furnish peptide bond.

The ligation reaction, depicted in Figure 1.7, occurs between a *C*-terminal thioester and *N*-terminal cysteine residue. The first step is transthioesterification, targeting the thiol moiety on Cys to furnish an *S*-linked isopeptide and serves to introduce the thioester adjacent to a reactive amine. A spontaneous *S,N*-acyl transfer *via* a 5-membered cyclic transition state follows, shifting the original *C*-terminal carbonyl

onto the *N*-terminal amine and forming a native peptide bond.[1] This rapid shift is the cornerstone of NCL and its utility in peptide synthesis. The *C*-terminal thioester can determine the success of the first step, with alkyl thioesters, although straightforward to prepare by SPPS, proceeding sluggishly over a time frame of 24-48 h. It has been shown, however, that the utilisation of an aryl thiol additive in the reaction can improve the rate by reacting with the alkyl thiol first, providing a more reactive *C*-terminal thioester for transthioesterification with Cys thiol.[196, 197] Moreover, the choice of amino acid bearing the thioester can also affect the rate according to the steric bulk of the residue, where less hindered amino acids such as Gly or Ala allow the reaction to proceed at a faster rate. Dawson and co-workers investigated the steric limitations of different *C*-terminal amino acids and found that all 20 natural amino acids can partake in transthioesterification, however the reaction was significantly slower with the more hindered residues; Ile, Val and Pro.[198] This limitation of sterically hindered side-chains is typical in many aspects of peptide chemistry, however NCL carries a more restricting feature, which is the requirement for Cys at the ligation site. Cys has a particularly low abundance in nature (3.3%[199]); therefore, peptides or proteins with Cys at a suitable ligation site are not plentiful. With this limitation NCL remains a superior process, however not very applicable to most substrates. Fortunately, a vast amount of research has been focused on alternative approaches to NCL to encompass amino acids that have a higher frequency in nature.

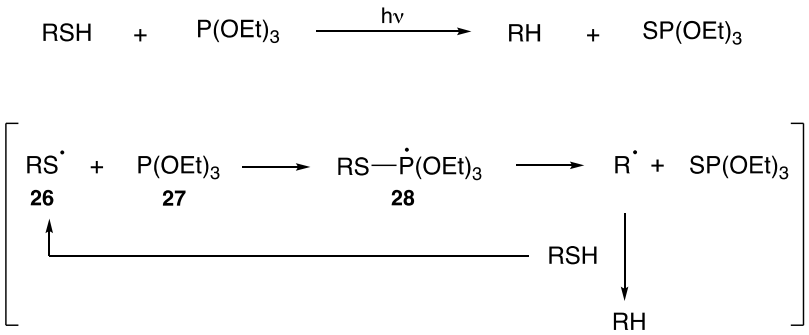
1.2.3 Alternative approaches to NCL

1.2.3.1 Desulfurisation

One noteworthy innovation in peptide ligation after NCL was the desulfurisation technique reported by Yan and Dawson in 2001.[200] The methodology involves the ligation at a *N*-terminal Cys and a *C*-terminal thioester, as in NCL, however after the *S,N*-acyl shift occurs the resulting Cys residue is reduced to Ala, which is a more abundant amino acid.[201] The possibility of ligation at Ala allows NCL-desulfurisation to be applied to many more peptides. Through the use of metal reagents, including Pd complexes and Raney nickel, both linear and cyclic proteins were prepared by the conversion of Cys to Ala post-NCL.[200] However, despite the major advancement that this process represented, the lack of selectivity seen by the use of these metals results in

the reduction of all thiol, thioethers and thioesters and the need for large quantities of Raney nickel remain a disadvantage in some circumstances.[202] Hence, alternative radical-mediated approaches to desulfurisation were explored.

The first report of a desulfurisation reaction was that of Hoffman and co-workers and was carried out under thermal and photochemical conditions between a mercaptan and a trialkylphosphite derivative.[203] Following this, Walling and Rabinowitz described a proposed mechanism for the desulfurisation (Scheme 1.12), where an alkylthiyl radical is generated and undergoes reversible addition onto the phosphite, leading to a phosphoranyl radical intermediate.[204] Cleavage of the alkyl-sulfur bond allows for formation of an alkyl radical, which is quenched by the hydrogen abstraction from the thiol, propagating the reaction. Danishefsky *et al*. investigated the desulfurisation *via* radical conditions using the radical initiator 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) and tris(2-carboxyethyl)phosphine) (TCEP) as the source of phosphine.[205] As shown in Scheme 1.12, the thiyl radical **26**, generated by the decomposition of the initiator, reversibly adds onto the phosphine **27** to generate the phosphoranyl radical **28**, which allows for cleavage of the C-S bond. Addition of a thiol additive such as *t*-butyl mercaptan (*t*-BuSH) to facilitate hydrogen abstraction gave the products as disulfides in yields of > 80%. The authors showed that the method was compatible with a variety of protecting groups and functionalities that were reduced by the methods reported by Yan and Dawson. This route has since been utilised by many research groups to provide peptides and bioconjugates by incorporating mercapto amino acids into NCL and desulfurising to afford a non-Cys ligation site.[206-208]



Scheme 1.12: Proposed general mechanism of phosphite-mediated radical desulfurisation of thiols by Walling and Rabinowitz.[204]

1.2.3.2 Auxiliary mediated ligation

Another milestone achieved in the field of peptide ligation was the development of auxiliary mediated ligation (AML), pioneered by Kent and co-workers, shortly after NCL was described, in the synthesis of cytochrome b562.[209] The implementation of an auxiliary bearing a thiol at the *N*-terminal of a peptide allows for NCL at this unnatural amino acid through transthioesterification. The resulting thioester undergoes *S,N*-acyl transfer to provide an amide bond and release the thiol on the auxiliary. Subsequent cleavage of the auxiliary should deliver the native peptide (Figure 1.8, A). Like desulfurisation, this involves modifications to the natural peptide segment, followed by NCL and then further modifications back to the natural peptide. Nevertheless, it has been applied to the synthesis of a wide range of biomolecules.[195, 210, 211] Auxiliary thiols are typically *N*-α compounds containing an accessible thiol.[212-215] A major drawback of this methodology is the steric bulk associated with the auxiliary that is at the ligation site and undergoes the transthioesterification. As discussed previously, the steric hindrance of the amino acids at the *C*-terminal thioester is important for the success of NCL. This requirement is even more significant in AML due to the bulkiness of the auxiliary and therefore only relatively small residues such as Ala and Gly at the ligation site lead to high-yielding thioesters.[216]

Sugar-assisted ligation (SAL) (Figure 1.8, B) is one example of a non-conventional auxiliary reported by Wong *et al*. for the synthesis of glycopeptides.[217, 218] The methodology centres on NCL and the acyl transfer that ensues, however the thiol "auxiliary" is located on the sugar unit of the glycopeptide. Desulfurisation then affords the native glycopeptide. The major advantage of this route is the tolerance for different sequences and ligation sites. With most auxiliary based approaches the *N*-terminal nucleophile in the acyl migration step is secondary and the transition state is sterically demanding. With SAL, the transition state formed is 14- or 15-membered, which allows more space, and the *N*-terminal nucleophile is a more reactive primary amine.[219] Both *O*-linked and *N*-linked glycopeptides were prepared through SAL, with glycosides at Ser and Asn, respectively. Furthermore, extended SAL was described, which showed the limits of the transition states during the *S,N*-acyl transfer by increasing the number of residues between the *N*-terminus and the location of the sugar.[220, 221] The authors

showed that up to five amino acids could be tolerated, increasing the transition states from 14- to 29-membered, however slower reaction rates were observed.

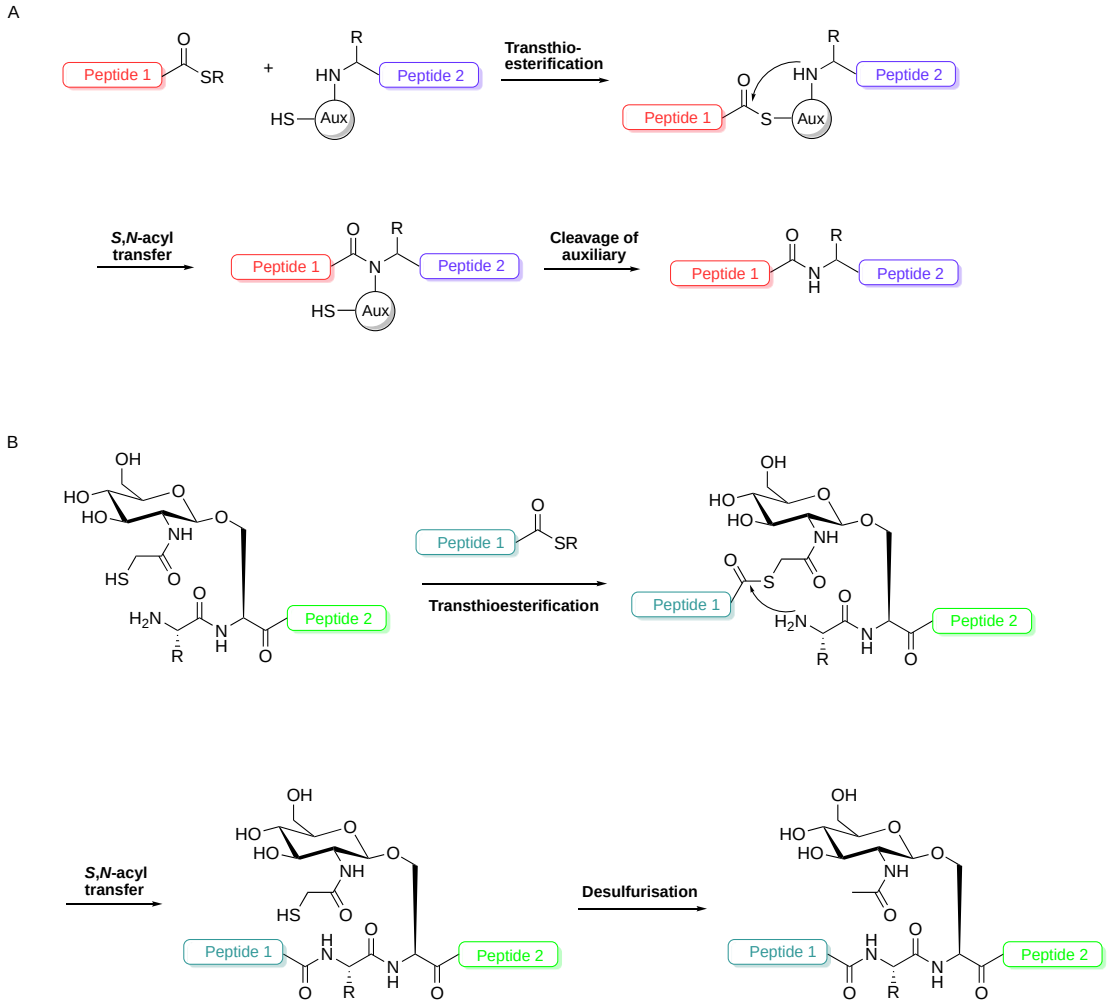
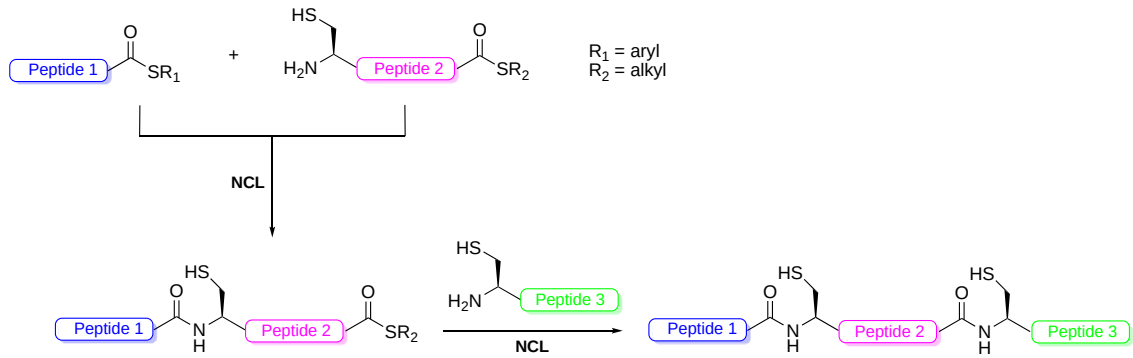


Figure 1.8: A) Auxiliary mediated ligation (AML). The *N*-α auxiliary provides the thiol for transthioesterification during NCL. Following the *S*,*N*-acyl transfer the auxiliary is removed to furnish the peptide. B) Sugar-assisted ligation (SAL). The thiol that partakes in transthioesterification is located on the sugar of the glycopeptide. Following *S*,*N*-acyl transfer, the thiol is removed from the sugar *via* desulfurisation.

1.2.3.3 Kinetically controlled ligation

Kinetically controlled ligation (KCL) facilitates the preparation of peptides in a convergent manner and from *N*- to *C*-terminals by taking advantage of the difference in reactivity of alkyl thioesters and aryl thioesters. Kent *et al*. first demonstrated this with the synthesis of small protein crambin.[222, 223] A six-segment convergent ligation was carried out, employing a bifunctional middle segment which underwent at both

terminals; at the *N*-terminal Cys with an aryl thioester of the another segment and then at the *C*-terminal alky thioester with an *N*-terminal Cys of a third segment (Scheme 1.13). These NCLs made up the first half of the protein and following the same strategy to prepare the second half, plus subsequent modifications to convert the C-terminus of one half to a thioester and reveal the Cys side chain of the N-terminus of the second half, the two peptides were ligated to form the full protein. The coupling of segments was controlled by the relative reactivity of each thioester, enabling the organised elongation of the peptide in both directions.



Scheme 1.13: Convergent synthesis of a protein *via* kinetically controlled ligation (KCL).

The methodology was further demonstrated for the one-pot synthesis of madanin-1 through three peptide segments using the thiol additive 2,2,2-trifluoroethanethiol (TFET).[224] This additive increases the rate of thioesterification by generating a reactive TFET thioester *in situ*. The first NCL reaction occurred at the TFET thioester of the first fragment, being the kinetically favoured option, with the β-thiol functionalised Asp residue at the *N*-terminus. Following this, the relatively unreactive *C*-terminal thioester of the middle fragment was converted to TFET thioester to extend the peptide in the *C*-terminal direction. The fully ligated peptide was then globally desulfurised to afford the 60 amino acid, Cys-free protein.

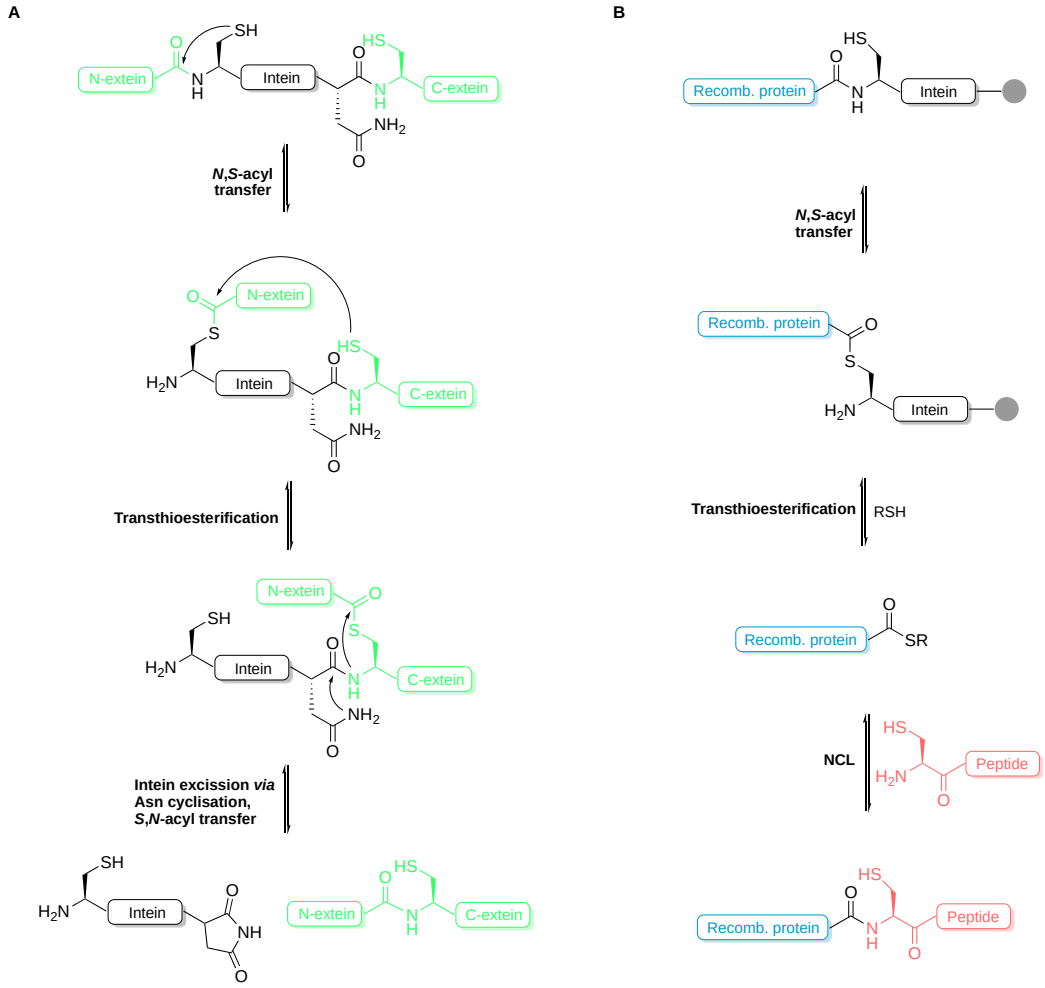
1.2.3.4 Expressed protein ligation

The utilisation of recombinant proteins in conjunction with synthetic peptides in a process termed expressed protein ligation (EPL) was discovered by Muir *et al*. in 1998.[225] The authors manipulated the inherent protein splicing mechanisms in nature to

incorporate NCL and furnish considerably larger peptide linkages than previously discussed methods.[226]

Protein splicing occurs through a series of acyl migrations between inteins and exteins, which cleave the intein from the entire scaffold.[227] Inteins are internal peptide domains that are flanked by two polypeptide units; the *N*-extein and the *C*-extein. Initially, an *N*,*S*-acyl transfer occurs at the *N*-terminal Cys of the intein and the *C*-terminal of the *N*-extein (Scheme 1.14, B). The *C*-extein bears a *N*-terminal Cys that undergoes transthioesterification to release the intein from its *N*-extein. A conserved *C*-terminal Asn residue then cyclises and facilitates the *S*,*N*-acyl transfer between the two extein units, which simultaneously excises the intein and forms a native peptide bond between the two exteins.

In EPL, the intein recombinantly expressed has a mutation at the C-terminal Asn to give Ala, which halts the self-splicing process. The protein that is expressed is located at the *N*-terminus of the intein, which is bound at its *C*-terminus to a resin. As depicted in Scheme 1.14, A, *N*,*S*-acyl transfer furnishes the thioester of the recombinant protein which is followed by intermolecular transthioesterification with an exogenous thiol. Without Asn facilitating cleavage from the exteins, the protein is cleaved from its intein on the solid support through this intermolecular transthioesterification step. Following filtration, the resulting protein bearing a *C*-terminal thioester is subjected to NCL with another peptide.[225, 228] The combination of protein expression and chemical ligation has vastly changed the way in which protein engineering can be exploited to provide bioconjugates of fluorophores, synthetic handles, unnatural amino acids and various PTMs.[229-236] In particular, a later report by Muir and co-workers described the application of EPL to furnish Crk-II with two conjugated fluorophores, a cAbl protein tyrosine kinase substrate which was then monitored through fluorescence resonance energy transfer (FRET) between the installed fluorophores.[237]



Scheme 1.14: A) General mechanism of protein splicing of an intein through multiple acyl transfer reactions. B) Expressed protein ligation (EPL)

1.3 *S,N*-acyl transfer

Thioesters, which display much more reactive properties than corresponding oxoesters, are ideal candidates for amide bond formation *via* the *S,N*-acyl transfer process.[238, 239] For this reason, the emergence of thioesters as tools for ligation reactions in both biological and chemical circumstances has been observed in recent years.[240-242] Exploited as key intermediates in many critical biological processes, thioesters facilitate the acyl transfer to *N*-, *O*- and *Se*- through inter- and intramolecular migrations.[243]

1.3.1 Applications in nature

Ubiquitination is a major PTM employed in biological processes such as DNA repair, protein degradation and inflammation.[244, 245] The formation of an isopeptide amide bond

between Ub and a protein substrate is *via* the *C*-terminal carboxylic acid and the ε-NH_2 of a lysine residue, catalysed by the utilisation of three enzymes. The first enzyme, known as the activating enzyme (E1), generates a thioester linkage between the Cys of the active site and Ub *C*-terminal Gly residue. The second enzyme, the conjugating enzyme (E2) takes the activated Ub through a *trans*-thiolation step onto a Cys in its active site and an *S*,*N*-acyl transfer from the enzyme onto the substrate Lys is catalysed by the third enzyme, ligase (E3). Two different processes can occur involving the acyl transfer, i) the thioester is cleaved from E2 by the nucleophilic attack of the ε-NH_2 and ii) a new thioester bond is formed on E3 before attack of the ε-NH_2. The occurrence of either of these options is dependent on the type of E3 enzyme, of which there are three; RING E3s, HECT E3s and RBR E3s, with the latter two forming thioesters on their own active sites before *S*,*N*-acyl shift occurs.[246, 247] Ubiquitination can involve the addition of one Ub monomer, known as monoubiquitination, or of a chain of Ubs, known as polyubiquitination, in which case many variations of Ub can be employed and can result in a divers range of molecular signals.[248] A similar pathway is exploited by the small ubiquitin-related modifier (SUMO) protein to carry out PTMs using an *S*,*N*-acyl transfer.[249]

Sortase transpeptidase enzymes are a group of enzymes that modify bacteria cell wall and surface proteins.[250] They recognise a particular sequence of amino acids (LPXTG) at the *C*-terminus of their substrates. There are four classes of sortase enzymes, which each perform a different function, however still utilise an *S*,*N*-acyl shift to carry out their function. Class A sortases produce surface proteins that become part of the cell wall due to their lipidation.[251] Class B sortases are responsible for haem-iron scavenging and cross-linking haem-containing products by recognizing a particular sorting signal. Class C are involved in covalently linking pili to enable polymerisation and Class D sortases are prevalent in spore forming microorganisms and act on molecules in the cell wall during spore formation. When a sortase cleaves its specific signal that it recognises, a thioester bond is formed in the sortase active site. This thioester is subsequently cleaved through a *S*,*N*-acyl transfer to an amino acid residue of the cell wall component.

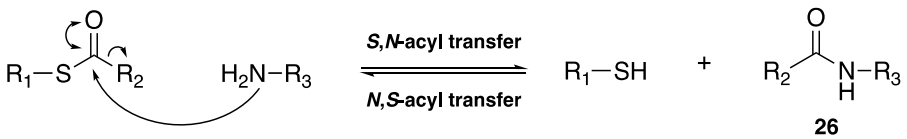
Transglutaminase enzymes (TGs) use the *S*,*N*-acyl transfer in their function to catalyse the calcium-dependent formation of inter- and intramolecular isopeptide bonds between proteins.[252] Due to their similar active site sequences and structural similarities,

TGs are known to be homologous to the papain family of proteases. During the transglutaminase process, a thioester bond is formed through the attack on a Gln side chain by the Cys of the TG active site. *S*,*N*-acyl shift then occurs onto a Lys of a second substrate, forming an isopeptide bond between the two substrates. It has been suggested recently that the specificity that TGs bear for Lys residues is reliant on the accessibility of the Lys within the protein, and not sequence dependent for the motif that holds the Lys.[253]

Another important biological process that utilises the *S*,*N*-acyl transfer is protein splicing, which was discussed in Section 1.2.3.4. The mechanism of this pathway involves both *S*,*N*- and *N*,*S*-acyl shifts, which highlights the potential reversibility of this acyl migration.

1.3.2 Reversibility of the *S*,*N*-acyl transfer

The equilibrium of the *S*,*N*- or *N*,*S*-acyl shift typically favours the amide product **26**, as is seen in many biological processes, as previously discussed. However, the reverse reaction to form a thioester bond is also utilised and therefore favoured in some instances, namely in protein splicing where the extein-intein linkage is transferred to a thioester bond at the intein Cys.[227] This process, while thermodynamically unfavourable, is catalysed by a conserved B-block His residue, which allows for conformational rearrangement of the intein causing a twisted, high-energy peptide bond, promoting thioester formation.[254]



Scheme 1.15: Reversibility of the *S*,*N*-acyl transfer reaction.

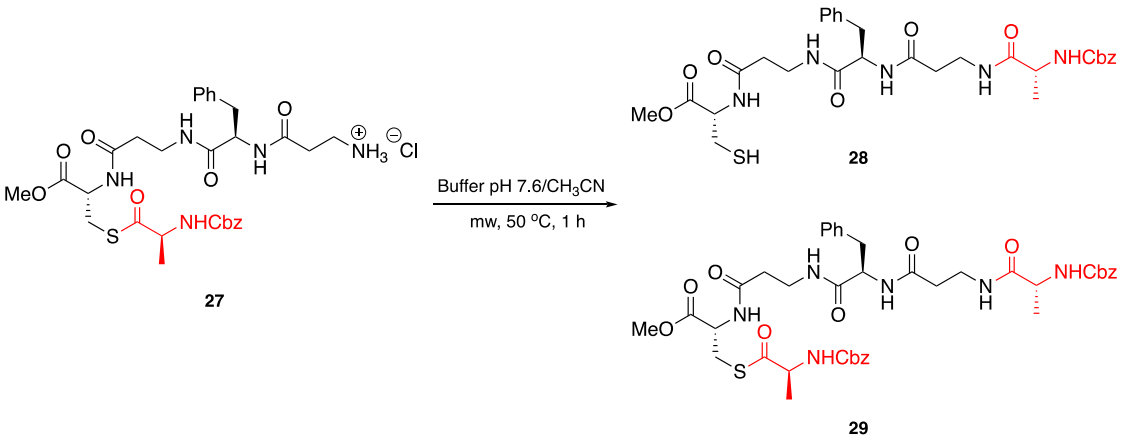
Synthetic approaches to encourage *N*,*S*-acyl transfer in the preparation of thioesters have been investigated,[255] for example in the SPPS employing a safety catch linker reported by Melynk and co-workers.[256] The same authors utilised bis(2-sulfanylethyl)amino (SEA) with a Cys or homocysteine peptide to achieve the *N*,*S*-acyl

shift.[257] *C*-terminal Cys and Cys derivatives, such as α-methylcysteine or cysteine-proline ester (CPE), have been explored for the promotion of thioester formation through acyl migration. Using a peptide with a *C*-terminal Cys containing the motif Cys/Gly/His-Cys, successful thioester formation was carried out in the presence of the thiol additive sodium 2-mercaptoethylsulfonate (MesNa).[255] The scope of this study was extended to include other peptide motifs with a *C*-terminal Cys, however it was suggested that these examples might need fine tuning of pH, temperature and peptide concentration to work as efficiently as the Cys/Gly/His-Cys sequences. Danishefsky *et al*. reported an *N*,*S*-acyl transfer when using acidic conditions to cleave an auxiliary, where the benzylic amine was protonated and drove the acyl shift.[211] A similar thioester synthesis was described by Vorherr and Aimoto while using 2-mercapto-4,5-dimethoxybenzyl (Dmmb) as an auxiliary.[258] A *C*-terminal 5-mercaptomethylated proline derivated was subjected to *N*,*S*-acyl transfer by Nakahara and co-workers using 3-mercaptopropionic acid, where it was suggested that the labile imino-amide bond in the proline derivative was the major factor promoting thioester formation.[259, 260] It is therefore prudent to suppose that the reversibility of the *S*,*N*- and *N*,*S*-acyl transfer is a feature worth exploiting for both chemical synthesis and biological research.

1.3.3 Ring size of cyclic intermediates

In most cases, the most significant factor affecting the success of the *S*,*N*-acyl transfer is the size of the cyclic transition state in the intermediate. The nucleophilic attack of an amine onto a thioester C=O leads to a cyclic intermediate, of which the stability is key to the completion of the rearrangement. A wide range of transition state (TS) sizes have been demonstrated with respect to acyl migration, from the original 5-membered rings to the much larger 32-membered rings.[261] However, in addition to ring size of the intermediate, factors contributing to the acyl shift consist of pH, proximity of amine with respect to thioester, hydrogen bonding, conformation, NH-π interactions, and relative reactivity of amine and thioester.[242] Entropic activation, which represents the proximity of amine and thioester functionalities, is commonly understood to be a major influencing aspect of the *S*,*N*-acyl transfer. Due to the complexity of the reaction and all of the features affecting its efficiency, many studies have been carried out in order to gauge whether a system is likely to undergo an *S*,*N*-acyl shift. Katritzky and co-workers have reported a number of studies undertaken by their group that demonstrate

conditions favourable and unfavourable for intramolecular *S,N*-acyl transfer. Beginning with the investigation of 5-, 8-, 11- and 14-membered cyclic TS, the authors showed that the larger ring sizes of 11- and 14-membered easily underwent acyl shift to the terminal amine, however the 8-membered only resulted in a minor amount of desired product and mostly the *bis*-acylated product due to intermolecular reactions.[262] In a following report, 13-, 15- and 16-membered rings were explored where mostly the intermolecular trans-acylated product was observed with the smaller macrocycles, however with two different isopeptides forming 16-membered cyclic TS, such as **27**, a mild preference for the desired intramolecular *N*-acylated product **28** was seen over the *bis*-acylated **29** (Scheme 1.16).[263] All of the acyl shifts were carried out in a microwave reactor at 50 °C and in phosphate buffer in order to neutralise the amine salts formed through *N*-deprotection. Furthermore, the 15-membered TS example was subjected to different pH conditions in an attempt to promote the desired acyl shift, where it was deduced that the pH range of 7.0-7.3 favoured the intramolecular rearrangement.



Scheme 1.16: *S,N*-acyl transfer carried out *via* a 16-membered transition state, furnishing both the desired product **28** and the bis-acylated product **29** through intermolecular transacylation.

The same authors carried out computational investigations into the enthalpic and geometric factors affecting the inter- and intramolecular acyl migration and the possible intramolecular hydrogen bonds formed in the TS that contributed towards the stabilisation of the ring intermediate.[264] Seitz and co-workers also explored the limitations of *S,N*-acyl transfer in the internal Cys-mediated ligation reactions that go *via* a macrocyclic TS. This study found that 8-, 11- and 14-membered cyclic TS were

more difficult to form than the larger macrocycles of 17- to 20-membered TS.[265] It is widely known that following the rate-determining step in NCL, which is transthioesterification, the *S*,*N*-acyl transfer proceeds through a favoured 5-membered cyclic TS.[266] However, if the cyclic TS is increased to > 6-membered, the acyl migration becomes the rate-determining step and the overall process is significantly dependent upon this step. In biological systems, larger macrocycles can be promoted through optimum proximity of the amine and thioester moieties, for example in ubiquitination the RING E3 is believed to carry out successful *S*,*N*-acyl transfer from the thioester in the active site of E2 to the nucleophilic Lys by the precise positioning of the reactive termini.[267]

1.3.4 Synthetic applications

The most widely known uses of the *S*,*N*-acyl transfer are those applied to methods of chemical ligation such as those previously discussed: NCL, ligation and desulfurisation, AML, KCL and EPL. One major field of research where the acyl migration from thioester to amine has been focused is the chemical ubiquitination process. Many synthetic and enzymatic pathways have been investigated in order to provide homogeneous ubiquitinated proteins due to their biological and therapeutic relevance.[248, 268, 269] Chemical and enzymatic methodologies to accomplish this both present challenges, however current options to furnish the native isopeptides bond mainly focus on NCL and EPL.[270-272] Nevertheless, routes such as these create difficulties when using Cys as it can interfere with the enzymatic thioester transfer step of ubiquitination.[273] An AML approach was recently described by Chatterjee *et al*., where an auxiliary (2-aminooxyethanethiol) was introduced to facilitate transthioesterification and subsequent *S*,*N*-acyl transfer, and then cleaved to furnish the native ubiquitinated protein.[274] This process surpasses previous attempts at employing Cys surrogates, which resulted in slow, sterically limiting reactions that require desulfurisation.[207] Similarly, Muir and co-workers developed a photolytically cleavable auxiliary, which was utilised in the formation of Ub-H2B linkages *via* two traceless orthogonal EPL reactions.[275] Poly-Ub protein conjugates have been developed in addition to monoubiquitination of substrates.[269, 276] Polymerisation of Ub-proteins is firstly carried out, followed by conjugation onto a protein substrate. Chin and co-workers reported the synthesis of Ub polymers by genetically encoded orthogonal

protection and activated ligation (GOPAL).[277] The encoded Lys residue was protected and following protection of all other amino groups and subsequent deprotection of the Lys of interest, the Ub was armed with only one reactive site for *S*,*N*-acyl transfer. This orthogonal protecting group strategy, however, brings limitations in scope due to the extensive use of protecting group manipulations.

Chemical probes for bioimaging and proteomics have also been synthesised through the chemoselective *S*,*N*-acyl transfer. Ploegh and co-workers described the preparation of Ub protease probes for molecular identification, activity profiling and structural analysis of the protease.[278] Furthermore, deubiquitinating enzymes (DUBs) have been synthesised by Ovaa and co-workers, which encompassed NCL to install a handle for the linkage of Ub thioester and a γ- or δ- thiolysine residue.[279] The development of fluorescent probes has also utilised the acyl shift mechanism. Geurink *et al*. have reported the synthesis of FRET probes, where the acyl transfer was implemented to ligate the two FRET components of a deubiquitin-based probe.[280] A dual-emission probe to distinguish between Cys/homocysteine and GSH has been developed by Strongin and co-workers.[281] This probe consists of a thioester that exchanges with Cys/homocysteine or GSH which either leads to excited-state intramolecular proton transfer *via* an *S*,*N*-acyl transfer, for the Cys/homocysteine, or no such transmission due to the lack of *S*,*N*-acyl transfer as the transition state was too large for the glutathione interaction. A probe for the binding of the same sulfur-containing biomolecules was designed by Guo and co-workers, however their probe was only activated by the *S*,*N*-acyl shift caused by the interaction with Cys, and not homocysteine or GSH.[282]

Molecular machines, the concept discovered by Sauvage, Stoddard, Feringa for which they received a Nobel prize in 2016, has also exploited the chemoselective *S*,*N*-acyl shift. Leigh and co-workers implemented the acyl transfer in molecular machines that mimic ribosomal protein synthesis.[283] Through reiterative NCL processes, the acyl migration is carried out over increasing TS ring sizes, from 11- to 14- to 17-membered rings. Each amino acid is ligated through a thiolate group connected to a rotaxane ring, which is attached to a molecular axle. The previous amino acid ligated bears a free *N*-terminus which undergoes *S*,*N*-acyl transfer to release the newly ligated amino acid from the thiolates functionality, elongating the peptide from the *C*- to *N*-terminus (Figure 1.9). DNA-templated acyl transfer has been reported by McKee and co-workers

to carry out *S*,*N*-acyl shifts between oligonucleotides modified with a thioester and a variety of amine and thiol nucleophiles.[284]

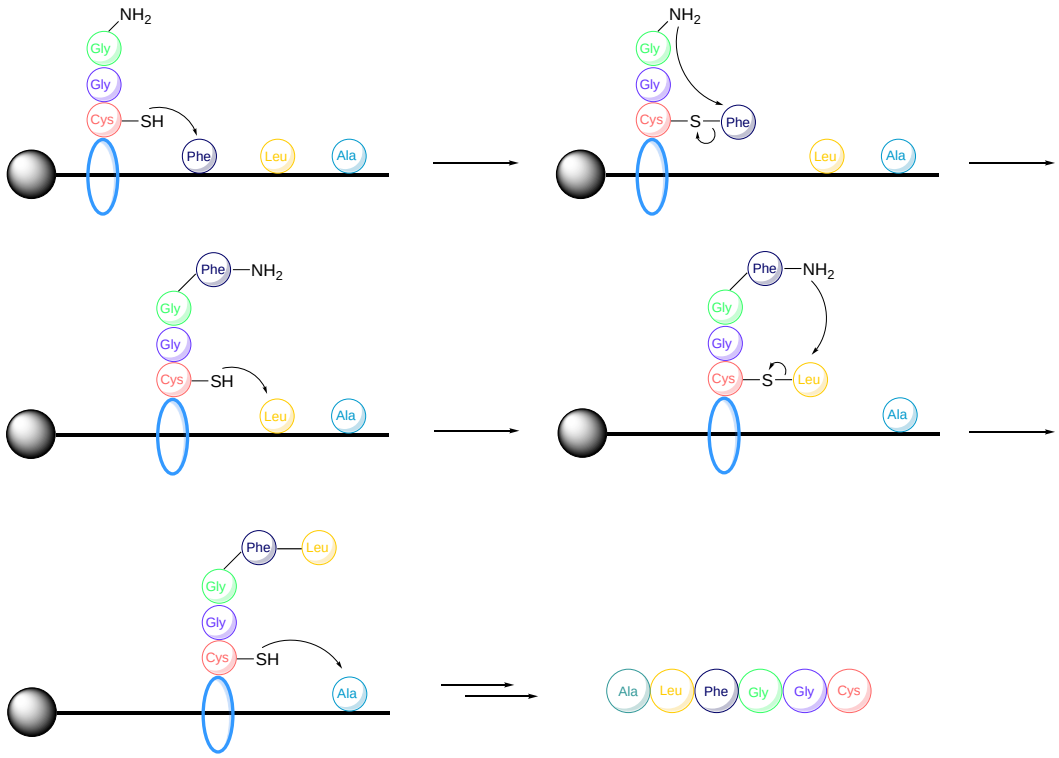


Figure 1.9: Ribosomal molecular machines for protein synthesis. The first step consists of an *O*,*S*-acyl transfer to provide a thioester which subsequently undergoes *S*,*N*-acyl transfer in the second step to add an amino acid to the chain. This process is reiterated until the sequence is completed and is cleaved from the molecular machine.

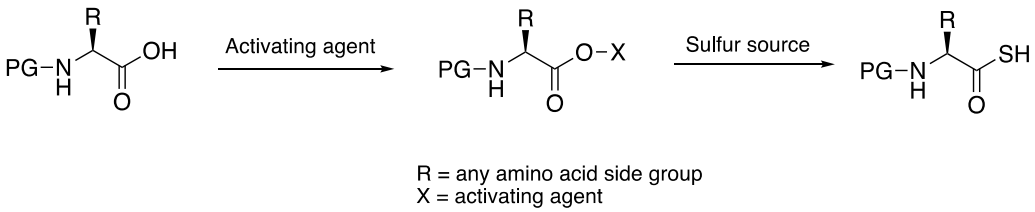
1.4 Thioacids

Thioacids, bearing a unique reactivity, represent a useful class of compounds for many aspect of chemical synthesis.[285-287] For the scope of this thesis, however, the focus of thioacid preparation and applications will be centred on α-amino thioacids and their value for peptide/protein chemistry.

1.4.1 Preparation of thioacids

Many methods for the synthesis of α-amino thioacids are available and have been reported.[288, 289] They mainly aim to provide the unprotected thioacid through various sulfur sources (Figure 1.10) or furnish the *S*-protected thioacid which can be

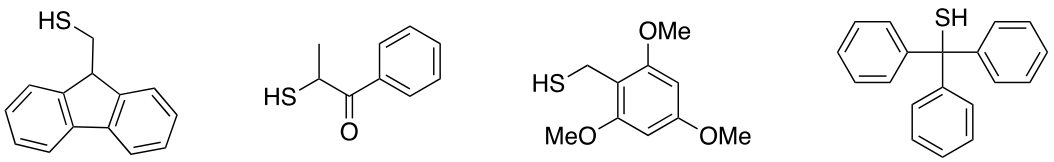
orthogonally deprotected, leaving the *N*-protecting group in place. One widely used approach is the conversion of *N*-Boc carboxylic acids to thioacids using 1,1'-carbonyldiimidazole (CDI) and a sulfur source such as NaSH.[290] This procedure carefully outlines the critical steps necessary for successful preparation of the thioacid and prevention of hydrolysis, a widely accepted obstacle within the synthesis of amino thioacids. Hydrolysis can occur rapidly in solution or in aqueous environments and at room temperature (unless in the form of a salt), however the storage of pure thioacids at 0 °C can be successful for several months.[291, 292] Another method to install the SH moiety directly is through the use of H_2S, bubbled through a solution of base in organic solvent with the addition of activated ester furnishing the desired product.[293, 294] Lawesson's reagent has been reported to provide amino thioacids successfully using microwave irradiation, however this method also requires heating to 100 °C, which can be unsuitable for peptidyl substrates.[295] A mixture NaSH and thioacetic acid has also been reported to provide the thioacid analogue of an amino acid and were successfully employed in peptide coupling reactions.[296]



Fgure 1.10: General scheme of the synthesis of thioacids through direct attack of sulfur nucleophiles such as H_2S, Lawesson's reagent and NaSH.

The preparation of *S*-protected amino thioacids has been reported with four main protecting groups, as depicted in Figure 1.11 (30-33): 9-fluorenylmethyl (Fm), 2,4,6-trimethoxybenzyl thiol (Tmob), α-methylphenacyl (MPa) and triphenylmethanethiol (Trt). The exploitation of the *S*-Fm strategy was pioneered by Crich and co-workers,[297, 298] however the first reported example was by Giralt *et al*. in 1990.[299] This route to thioacids begins with the synthesis of the Fm thiol through deprotection of an *S*-acyl Fm group. The thiol is then reacted with the activated ester of the *N*-Boc amino acid using standard coupling reagents such as *N*,*N*'-dicyclohexylcarbodiimide (DCC) or *N*,*N*'-diisopropylcarbodiimide (DIC). Following deprotection of the base-labile Fm group, the

thioacid is free for further ligation. Crich and co-workers described this synthesis as high yielding and without racemization and used the thioacids prepared for successful coupling reaction with Sanger's reagent.[289, 300] Mpa thioesters are prepared through the use of *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDCI·HCl) and phenylacyl thiol with the amino acid of interest. They are also compatible with Boc-protecting group chemistry, however the group possesses the useful feature of being cleaved by Zn-dust, a feature that introduces a new level of orthogonality to the *S*-protecting group strategy of thioacids.[301] The *S*-Tmob synthetic procedure for the preparation of thioacids was first reported by Shangguan *et al*. for Fmoc-compatible thioacid chemistry.[302, 303] Tmob thiols can be coupled to carboxylic acids through the use of typical carbodiimide reagents and are rapidly cleaved to furnish the free thioacid by acidic means, usually with a solution of TFA in CH_2Cl_2.[304] Similarly, trityl (Trt) thioesters are cleaved rapidly with TFA/CH_2Cl_2 and can be easily formed form amino acids through coupling reactions with EDC/4-(dimethylamino)pyridine (DMAP) or other carbodiimide reagents.[300] The rapid cleavage of these protecting groups can be a key attribute to these compounds as the resulting product, the α-amino thioacids, have tendencies to hydrolyse while in solution.



30 **31** **32** **33**

Figure 1.11: *S*-protecting groups for α-amino thioacid preparation. **30**: Fm, **31**: MPa, **32**: Tmob, **33**: Trt.

For the preparation of peptide thioacids, a synthesis compatible with SPPS is often preferred due to the reactivity of side groups of various residues. A wide variety of linkers have been developed in order to provide the *C*-terminal thioacid upon cleavage of the resin. Thioacids prepared by Boc-SPPS have been reported using different linkers and resins, and employing alternative cleavage cocktails to the unpopular HF method.[305-308] Hydrothiolytic cleavage has been described by Liu and co-workers using HS^- to facilitate cleavage of the thioester bond from the resin.[309, 310]

Fmoc-protected thioacids can also be prepared by SPPS *via* cleavage with NaSH or 2-mercaptopropionitrile.[311, 312]

1.4.2 Applications of thioacids in peptide/protein chemistry

Thioacids can be usefully employed in peptide chemistry as a powerful activating agent, due to the unique reactivity of the functionality. In addition, the level of racemisation associated with thioacid coupling is less than that of their carboxylic acid analogues.[288, 313] Thioacids, or peptide thioacids, are typically ligated to other peptides and amino acids by activation at either the thioacid or the incoming amine of the other reacting molecule. *C*-terminal activation can be carried by various different methods, such as metal-mediated using copper or silver salts or ruthenium complexes for photo-redox peptide coupling.[314, 315] Acyl disulfide activation has been thoroughly explored for the peptide coupling of thioacids.[316] The efficiency of this type of activation is akin to *N*-hydroxysuccinimide activation, and has accomplished the synthesis of α-inhibin-92, a protein consisting of 92 amino acid residues.[317] Reagents such as Ellman's reagent have also been reported to drive the disulfide-activation of thioacids for peptide elongation.[318, 319] Danishefsky *et al*. described the successful preparation of homogenous peptides and glycopeptides through activation of peptide thioacids with 1-hydroxy-1*H*-benzotriazole (HOBt) with negligible amounts of racemisation.[320] *N*-terminal activation has also been accomplished through the functionalisation of the unprotected *N*-terminal amine that undergoes condensation with the thioacid. Functional groups employed to do this are typically isocyanates or isothiocyanates, sulfonamides, thioanhydrides, or isonitriles. Neoglycopeptides have been prepared through the use of isocyanates and isothiocyanates, where the thiolate anion attacks isocyanate and following rearrangement, loss of COS or CS_2 furnishes the native peptide bond.[321] Neoglycoconjugates can also be prepared *via* sulfonamides derived from carbohydrates, as can amino acid counterparts to give peptides.[297, 322] The coupling of peptide thioacids and *N*-glycopeptidyl 2,4-dinitrobenzene sulfonamides has been reported for the synthesis of glycopeptides from peptide thioacids. Many more options to activate the reactive thioacids and drive peptide coupling have been elucidated and the reader is directed to a recent review on these methods.[288]

Peptide analogues employing a sulfur atom in place of oxygen on the peptide backbone are known as thioxopeptides or thiopeptides. These compounds have shown potential in improving the bioavailability of therapeutics by evading protease activity. Though multiple routes to these peptidomimic have been reported, α-amino thioacids are a widely used tool in their preparation. For example, by utilising benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) in the reaction of a thioacid and amino acid, it has been reported that the thiopeptide can be obtained in good yield.[323, 324] Furthermore, the epimerisation of this reaction has been studied, and has shown that the production of DL-isomers amounts to les than 2% using a variety of different phosphorus-containing coupling reagents.[325, 326]

Forging amide bonds through the coupling of α-amino thioacids and azides has been thoroughly evaluated for the conjugation of peptides and glycopeptides.[302, 327] This reaction is often preferred to the analogous coupling of carboxylic acids and amines when the desired amide involves complicated synthesis. Schmidt and co-workers described the preparation of *N*-linked neoglycopeptides through an anomeric azide of thioglycoside **34** and thioacid **35**, which formed the *N*-linked glycopeptide **36** in an excellent yield (Figure 1.12, A).[328] Imide bonds have also been furnished through the coupling of thioacids, utilising acyl azides.[329] This concept has been applied to synthesise peptide-drug conjugates through an imide bond (Figure 1.12, B). Consequently, the strategy was expanded to exploit an *N*,*N'*-acyl transfer to provide a native peptide bond at an Asn residue (Figure 1.12, C).[330]

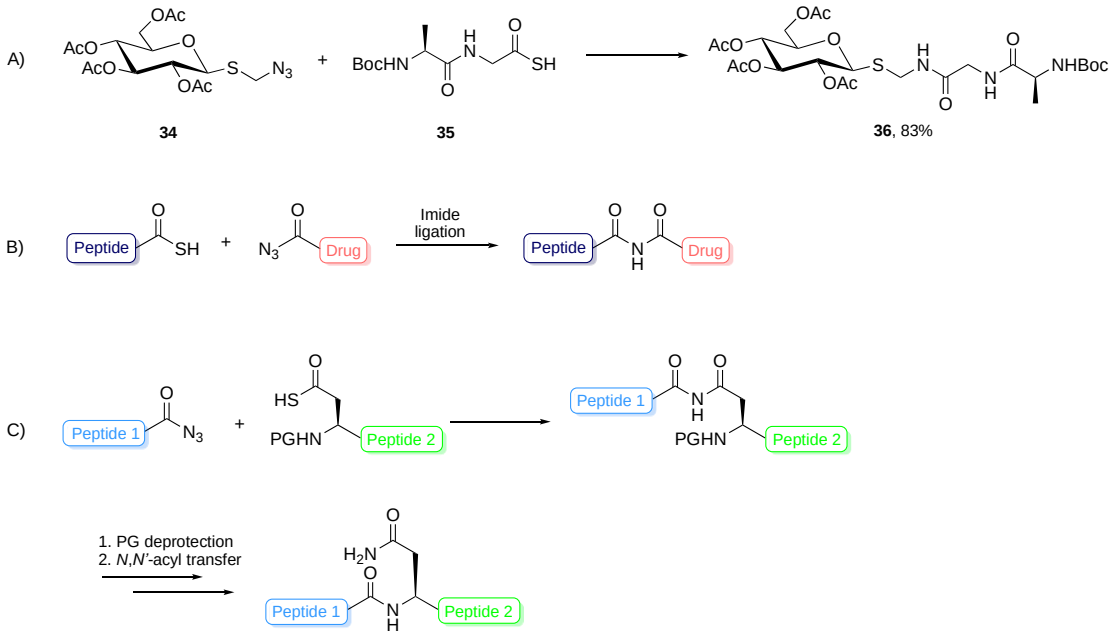


Figure 1.12: Examples of the utilisation of thioacids for the synthesis of glycopeptides and peptide conjugation: A) synthesis of *N*-linked glycopeptide **36**, B) synthesis of peptide-drug conjugates and C) synthesis of native peptide bond through *N*,*N*'-acyl transfer from Asn imide to *N*-terminus.

1.5 Work described within this thesis

As discussed throughout this chapter, the utility of thiol-ene chemistry is becoming increasingly more widespread in synthetic chemistry and bioconjugation. In addition, the radical-mediated thiol addition onto alkenes has proved to be extremely valuable in polymer chemistry, surface modification and supramolecular scaffolds. Despite the known utility of this reaction in the preparation of glycopeptides, isopeptide bonds and bioconjugates, it has not yet been the subject of interest for peptide ligation to forge native bonds. Peptide synthesis has seen massive developments in previous years, owing to the success of NCL, however there is always a need for faster, neater and milder methods to ligate peptides at sites other than Cys.

Chapter 2 outlines the application of intramolecular TYC (and previous work with intramolecular TEC) to afford endo- and exocyclic thioglycals, compounds that have shown significant utility in inhibiting glycosidase enzymes and in glycoconjugation. This methodology is an extension of previous work carried out in the Scanlan lab and provides the thioglycals through both radical and ionic means, with each route resulting in a different ratio of 5-*exo*-thioglycal and 6-*endo*-thioglycal. This work shows the effect of the sugar protecting groups in the ring closing reaction and of

the stereochemistry of the substituents on the carbohydrate backbone in providing the appropriate furanose or pyranose product through the use of L- and D-arabinose derivatives.

Chapter 3 describes the work set out to develop Thiol-ene Mediated Peptide Ligation and Elimination (TEMPLE) as an alternative to NCL. Using the widely acknowledged TEC, this methodology employs alkene-functionalised peptides and α-amino thioacids to furnish robust thioester linkages. This linkage has a specific location on the isopeptide to allow for *S,N*-acyl transfer, providing the *N*-terminal is deprotected. The acyl transfer is intended to occur through a large 14-membered cyclic transition state, which has been shown to be successful in providing amide bonds in the literature. This step leads to the formation of a native peptide bond through cleavage of the thioester and the release of a free thiol on the moiety that previously held the alkene. This moiety is an extension of either Ser or Thr and is therefore linked through an ester bond, which could subsequently be removed through mild basic conditions, restoring the natural residue in the sequence. The ligation step is expected to be neat and high yielding and could be employed on long peptides to furnish proteins, due to the mild irradiation used in TEC (365 nm), which is compatible with biomolecules. *S,N*-acyl transfer is typically a spontaneous rearrangement which should be thermodynamically favoured, provided the nucleophilic amine is available and reactive enough. This methodology represents a novel route to peptidyl thioesters through the radical reaction of thioacids and alkenes. In the final section of this chapter, β,γ-unsaturated amino acids were investigated as an alternative substrate for TEMPLE in order to examine the progress of the *S,N*-acyl transfer on a smaller cyclic intermediate.

In Chapter 4, the employment of dehydroalanine (Dha), an α,β-unsaturated amino acid residue, for TEMPLE is detailed. This residue, often used as a Michael acceptor due to its high reactivity, delivers a 5-membered cyclic transition state during *S,N*-acyl transfer. NCL, the most widely used and efficient method of peptide ligation, uses the same size cyclic intermediate and thus it is expected that this route will be successful in furnishing native peptide bonds. Following the acyl shift the residue that was Dha becomes Cys, which can be desulfurised to Ala using conventional methods. This chapter examines two different protecting group strategies for the *N*-terminus of Dha, azido and *bis*-Boc. In addition to radical-mediated TEC, ionic reactions are demonstrated to deliver thioester bonds, exploiting the Michael-acceptor nature of Dha.

The overall aim of this work is to perform TEMPLE, using *N*-terminal Dha peptide, to synthesise the small protein Glp-1 (Glucagon-like peptide amide 7-36). Glp-1 is a 29 or 30 amino acid protein involved in the regulation of insulin secretion, and is therefore of particular interest for diabetes therapeutics. The work outlined aims to prepare this protein through TEMPLE using multiple possible ligation sites, with each site containing an *N*-terminal Ala and a *C*-terminal thioacid. This methodology is carried out on small examples, employing monomer and dipeptide additions, to assess the efficiency of the process before being employed for small protein synthesis.

Chapter 5 concludes the work described in this thesis and possible future work.

Finally, Chapter 6 details the experimental procedures and characterisation for all compounds prepared within this thesis.

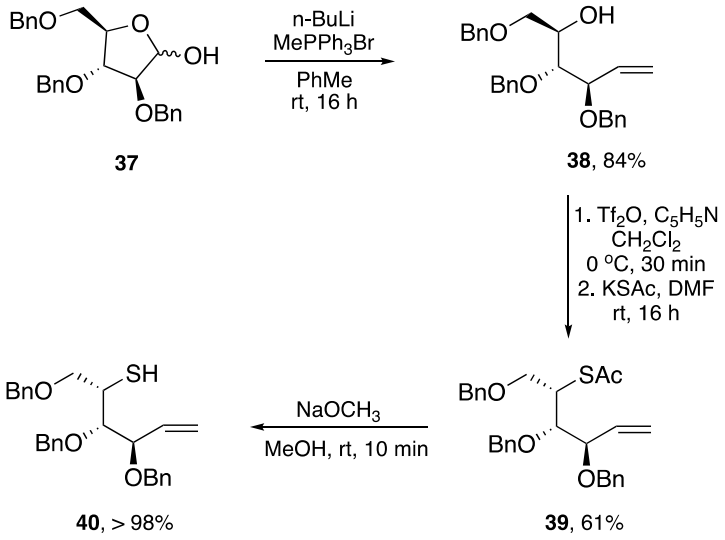
2.0 Introduction

The preparation of sulfur-containing heterocycles has gained increased importance in recent years particularly in the fields of drug design and therapeutics.[2] Perhaps the most valuable application for sulfur heterocycles is in carbohydrate chemistry, where thiosugars have shown significant biological activity as glycosidase inhibitors.[331-333] Glycosidase enzymes catalyse the hydrolysis of glycosidic bonds and are important biological targets for therapeutic design. Thiosugars have previously been exploited as glycosidase inhibitors for potential antiviral, antibacterial, anticancer and antidiabetic therapeutics.[2, 64, 334-339] Similarly, thioglycals have shown competitive glycosidase inhibition[340, 341] and have been widely utilised as a handle for glycoconjugation.[86] Examples of the synthesis of sulfur-containing heterocycles using thiyl radical-mediated methodologies are rare,[54, 56, 57, 64, 342-345] however the Scanlan group have reported the first literature examples of an intramolecular approach for accessing thiosugars and thioglycals.[68, 69, 71] Studies on these compounds utilised the highly efficient thiol-ene "click" reaction and it's alkynyl analogue; thiol-yne click, which has gained considerable interest for the intermolecular addition of thiols onto alkynes, with applications in bioconjugation, nanoscience, materials science and supramolecular chemistry.[86, 161, 346-348] In this chapter, previous work carried out by former members of the Scanlan group on the synthesis of thiosugars and thioglycals will be briefly discussed, followed by a detailed discussion on the synthesis of 5-*exo* and 6-*endo* L-arabinose thioglycals through intramolecular thiyl radical cyclisation of open chain sugar derivatives.

2.1 Previous work on intramolecular thiol-ene chemistry in the Scanlan lab

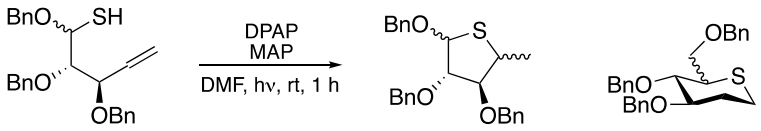
As discussed in Chapter 1, the Scanlan group has developed novel methodology for the preparation of thiosugars through radical-mediated processes.[68, 69] The synthesis of both thiofuranose and thiopyranose rings through intramolecular thiol-ene "click" has been extensively investigated, with specific focus on the regioselectivity of the ring closing reaction. To demonstrate this novel ring-closing chemistry, **40** was prepared from arabinose in three high-yielding steps, with a thiol and an alkene at either terminus (Scheme 2.1).[68] Starting from commercially available *O*-benzyl-protected arabinose, the ring was opened and terminal alkene introduced by a Wittig reaction to obtain **38**. The hydroxyl group released by this first step was subsequently converted to the triflate and

substituted with thioacetate, to afford the acetyl-protected thiol **39** in one step. Basic hydrolysis of the thioester furnished the free thiol **40** quantitatively. It is worth noting that nucleophilic substitution of the triflate by the thioacetate inverted the stereochemistry of the sugar from a D- to an L-glycan. To counteract this, the methodology was repeated with a double inversion to conserve the stereochemistry at C-5 in the original conformation. The first stereochemical inversion was introduced by the formation of a *p*-nitrobenzoate ester at the free hydroxyl using Mitsunobu conditions. Following cleavage of this ester, the secondary alcohol was formed. At this point a second inversion was carried out, using the triflate method previously described to provide the D-arabinose derivative with little impact on the overall yield, despite the additional steps. This strategy is discussed further later in this chapter.



Scheme 2.1: Synthesis of precursor **40**, carried out by previous members of the Scanlan group, starting from commercially available D-arabinose and converting to L-derivative with free thiol and alkene in place.

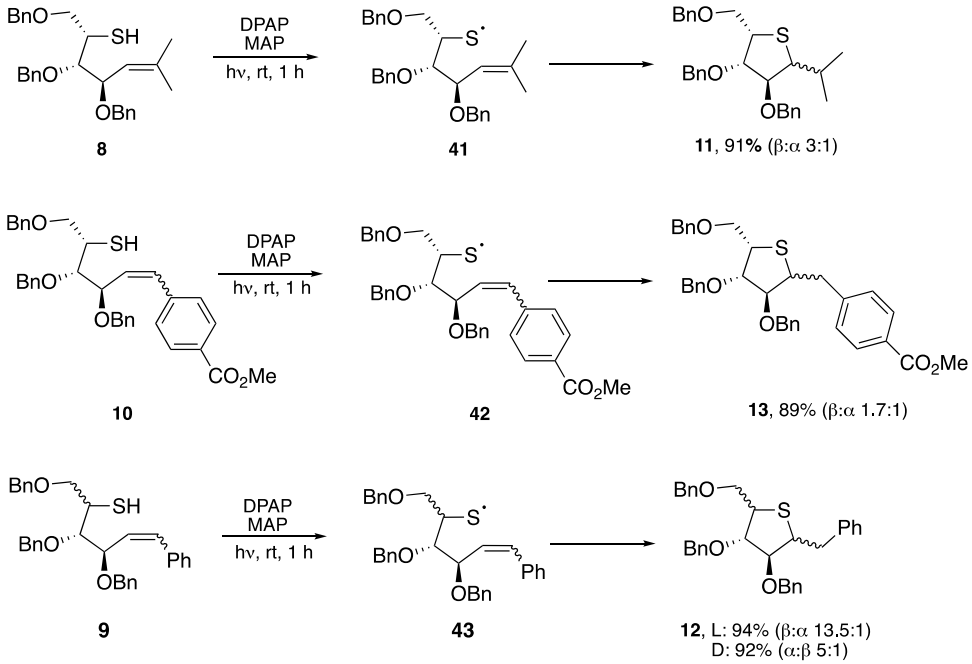
Precursor **40** was investigated under a range of different TEC conditions at 365 nm including various photoinitiators, concentrations and thermal conditions, where mixtures of endocyclic and exocyclic thiosugars were obtained in addition to the disulfide analogue of the starting material. The optimised TEC conditions were applied to **40**, which resulted again in a mixture of endocyclic and exocyclic thiosugars (Scheme 2.2). In all cases the major product of these intramolecular TEC studies was the 6-*endo* product, which was obtained in isolated yields of >70%.



5 **6**, L: 9% D: 12% **7**, L:73% D:72%

Scheme 2.2: Intramolecular thiol-ene cyclisation to form 5-*exo*-thiofuranose and 6-*endo*-thiopyranose structures under optimised conditions: 2,2-dimethoxy-2-phenylacetophenone (DPAP) (10%), 4-methoxyacetophenone (MAP) (10%), degassed solution of DMF under hν for 1 h at rt.

Following the discovery of this novel route to access thiosugars, it was envisaged that the yield of the 5-*endo*-thiofuranose ring could be improved by altering the nature at the alkene group. According to the work of Surzur et. al.,[55] in the presence of an unsubstituted terminal alkene, 6-membered rings are formed preferentially in the intramolecular process, which is in agreement with the studies of the Scanlan group. An important factor in determining the regioselectivity of the cyclisation is the stability of the carbon-centred radical formed upon addition of the thiol onto the alkene. Substituting the terminal alkene in order to provide a more stable carbon-centred radical was found to promote the formation of the 5-*exo*-heterocycle. A study of intramolecular TEC for the formation of thiosugars was expanded to investigate the addition of the thiol onto a range of substituted alkenes (Scheme 2.3).[69] As expected, the introduction of an isopropylidene group onto the arabinose scaffold led to the exclusive formation of the thiofuranoside **11**. Here, the 1,2-*trans* product was obtained as the major isomer, with a 3:1 ratio to the *cis* product.



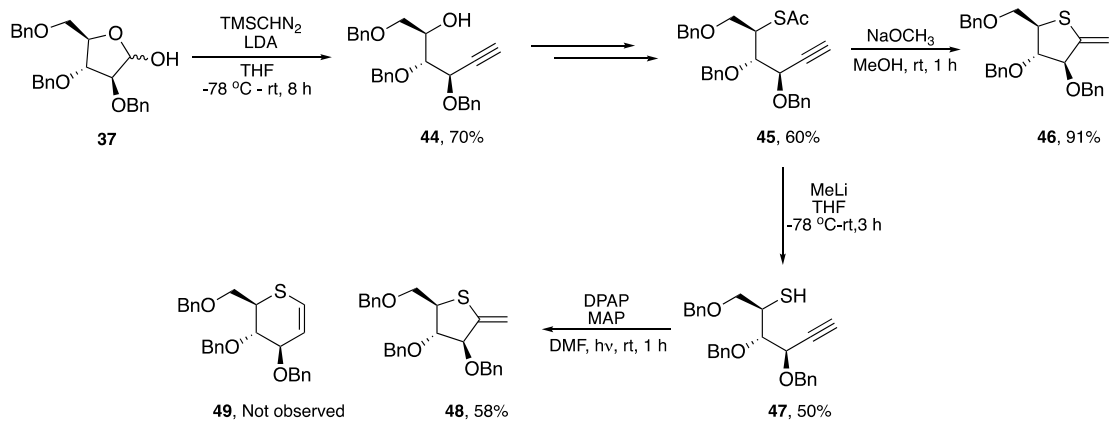
Scheme 2.3: 5-*exo*-trig cyclisations of substituted arabinose derivatives. The *trans*:*cis* ratios obtained were greatly dependent on olefinic substituents and relative stereochemistry of the C-5 substituent.

Similar experiments with varying substituents on the arabinose starting material were also carried out. When a methyl benzoate was introduced onto the terminal alkene the regioselectivity again favoured the 5-*exo* cyclisation product **13**, with a slight majority of 1,2-*trans* thiopyranose. It was found that use of an electron-withdrawing substituent greatly diminished the diastereoselectivity of the reaction. Investigations were conducted using both L- and D-epimers of a phenyl-substituted arabinose derivative, which both resulted in formation of the 5-*exo* product **12**, however the preference for the 1,2-*trans* thiopyranose was far greater with the L-sugar. With this example the diastereoselectivity was far superior with either epimer compared to the previous methylbenzoate example. It was concluded from these extensive experiments that the presence of an olefinic substituent greatly increased the regioselectivity of the 5-*exo* cyclisation, in which the diastereoselectivity is dependent on both the orientation of the C-5 epimer and the stereo-electronic nature of the olefinic substituent.

2.2 Aims of this work

As outlined previously, the intramolecular addition of thiyl radicals onto unsaturated substrates provides a valuable route to access biologically interesting thioglycals and thiosugars[68, 69] The following work details the application of this methodology to

acyclic acetylene substrates in order to furnish thioglycals. Recent work by Dr. Corcé in the Scanlan group described the synthesis of *O*-benzyl protected D-arabinose derivatives functionalised with both free thiol and terminal alkyne moieties (Scheme 2.4).[71] The synthesis, similar to that reported for the precursors for thiol-ene cyclisations, involved cleavage of the carbohydrate backbone at the anomeric position in order to install an alkyne moiety at this site. This was carried out by the utilisation of the Colvin rearrangement,[349, 350] which is described in detail later in this chapter. Following introduction of the alkyne, the double inversion strategy employing a *p*-nitrobenzoate ester was utilized to introduce the reactive ester. Following cleavage of this ester, the free hydroxyl group of the open chain sugar was converted to the thioacetate, which was subsequently deprotected to furnish the free thiol for radical addition onto the alkyne.

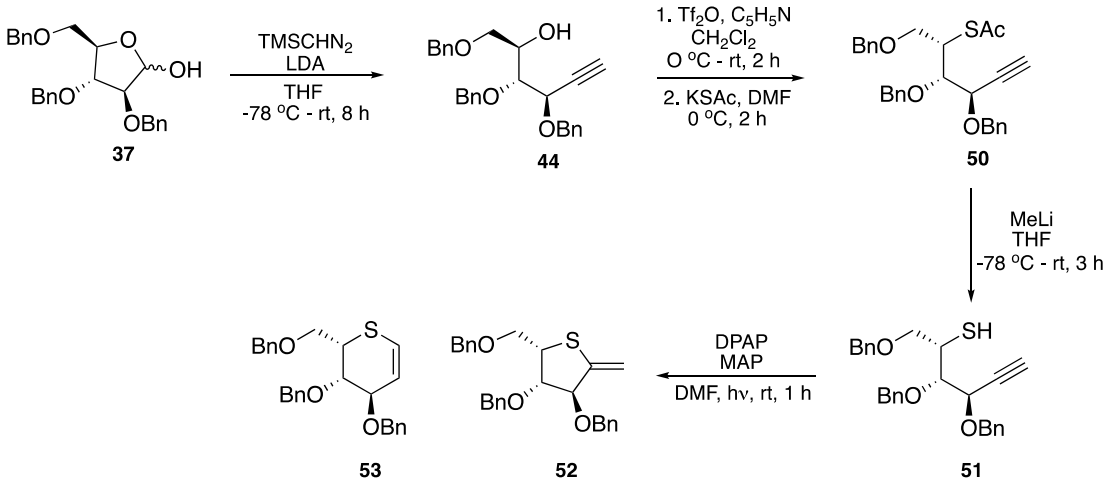


Scheme 2.4: Work carried out by Dr. Corcé in the Scanlan Lab: the novel route to thioglycal **48** through both intramolecular thiol-yne cyclisation and ionic cyclisation under basic conditions.

The intramolecular thiol-yne coupling was successfully utilised in this instance for the preparation of the thioglycal. The thiol-yne coupling was carried out with 365 nm UV irradiation, in the presence of photoinitiator and photosensitiser, for 1 h at rt. This method afforded the 5-*exo*-glycal **48** exclusively in a 58% conversion with no 6-*endo*-glycal **49** formation observed, which was shown by ^{1}H NMR. This result compounded the hypothesis that the relative stability of the carbon-centred radical could control the regioselectivity of the reaction. In addition, the analogous ionic reaction was also carried out to compare the efficiency of each method. Here, the acetyl protecting group was deprotected under basic conditions, providing the thiolate anion, which very readily cyclised onto the alkyne to form the 5-membered ring. In summary, both radical

and ionic routes led to the formation of a 5-*exo*-thioglycal in only 1 h, with the latter affording the product in a superior yield.

While this was an interesting and applicable result, the formation of the 6-*endo*-glycal was still a high priority for this work. It was believed that the same carbohydrate scaffold, but with different stereochemistry, could alter the outcome of the thiol-yne coupling and furnish the 6-endo cyclisation product. We anticipated that the stereochemistry of substituents on the sugar backbone could cause steric hindrance during the formation of the 6-*endo*-glycal, therefore leading to the *exo* glycal as the exclusive product. In order to probe this hypothesis, we aimed to modify the synthesis of the thiol substrate to explore the possibilities of the same thiol-yne cyclisation process on the L-arabinose analogue. As shown in Scheme 2.5, we envisaged that the L-sugar could be treated with an identical procedure to furnish the homologous epimer product **51**. A single S_N2 inversion of the C-5 position through displacement of the triflate was used to introduce a stereochemical inversion. This strategy is detailed in the following section.



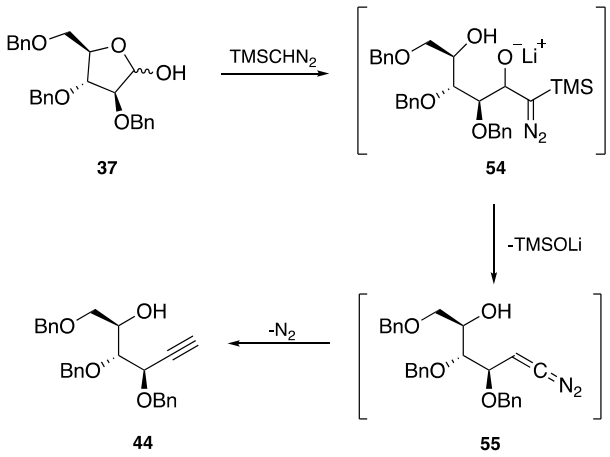
Scheme 2.5: The synthetic route to L-arabinose derivative **51** and the subsequent intramolecular cyclisation *via* TYC.

2.3 Synthesis of (2*R*,3*S*,4*R*)-1,3,4-tris(benzyloxy)hex-5-yne-2-thiol (51)

2.3.1 Synthesis of the alkyne terminal

The synthesis of **51** began with the ring opening of *O*-benzyl protected arabinose **37** and the introduction of an alkyne at the anomeric terminus. At this point of the original

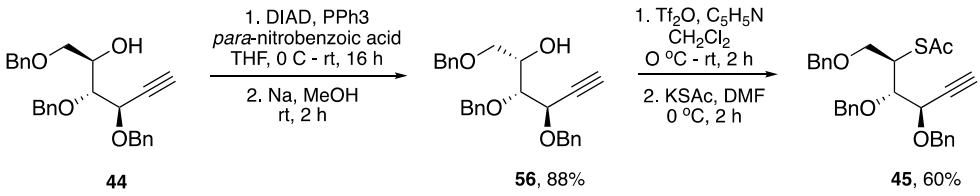
synthesis carried out by Dr. Corcé, other methods for this step were attempted, such as the Corey-Fuchs method.[351] From the hemiacetal, the Corey-Fuchs route was intended to convert the anomeric C-OH group to the corresponding alkyne. However, this strategy only afforded the degradation products of the reaction. The Bestmann-Ohira reagent was employed in a similar approach, which furnished the desired product in a 25% yield. Disappointingly, the product was obtained as an inseparable mixture with the C-2 epimerization by-product. The Colvin rearrangement was subsequently chosen as the method to afford the homologous alkyne (Scheme 2.6) and was therefore used for the L-sugar. The substrate **37** was treated with trimethylsilyldiazomethane and lithium diisopropylamide (LDA) to cleave the bond between the cyclic oxygen and anomeric carbon of the sugar and subsequently introduce the alkyne. During this reaction, the formation of a lithiated trimethylsilyldiazomethane intermediate **54** allowed for nucleophilic attack onto the carbohydrate backbone. For this step, trimethylsilyldiazomethane was solubilised in anhydrous THF, LDA was added at 0 °C and the lithiated species was formed over 1 h. The reaction was cooled further to -78 °C and a solution of tri-*O*-benzyl-D-arabinofuranose in anhydrous THF was added. Nucleophilic attack, to form the lithiated trimethylsilyldiazomethane arabinose intermediate, followed by the elimination of $TMSO^-Li^+$, provided the diazoalkene **55**. This intermediate underwent rearrangement to furnish the alkyne **44**, accompanied by the expulsion of N_2. Subsequent treatment of the crude product with KF was carried out to remove any remaining trimethylsilyl contaminants. Following column chromatography, the pure product was furnished in a 70% overall yield.



Scheme 2.6: Installation of the terminal alkyne on the ring-opened sugar *via* Colvin rearrangement.

2.3.2 Synthesis of the free thiol

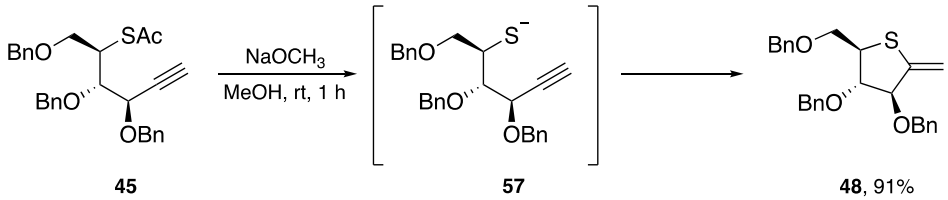
Following successful preparation of **44** through the Colvin rearrangement approach, the secondary alcohol was to be converted into a thiol moiety. It was envisaged that a simple S_N2 reaction of an acetyl thiolate to remove the hydroxyl group on the carbohydrate backbone would provide the desired product. In order to retain the stereochemistry at C-5, a double inversion strategy was used in the initial synthesis on the D-epimer. For this, the Mitsunobu reaction was employed to provide the *p*-nitrobenzoate ester with inverted stereochemistry, which was then cleaved under basic conditions to provide the hydroxyl group (Scheme 2.7). Subsequent triflation using triflic anhydride was carried out to introduce a suitable leaving group, followed by nucleophilic displacement using potassium thioacetate (KSAc) to afford **45**.



Scheme 2.7: Double inversion strategy carried out by Dr. Corcé to prepare the D-arabinose derivative and retain the stereochemistry.

In order to repeat the process using the opposite epimer, the L-sugar derivative was formed *via* nucleophilic displacement using KSAc. Dr. Corcé previously explored a number of different options for the deprotection of the *S*-acetyl group to furnish the free thiol. Firstly, as depicted in Scheme 2.8, Zemplén conditions were applied to the

thioacetate **45**, which resulted in spontaneous cyclisation due to the formation of the thiolate intermediate **57**. This ionic demonstration of thiol addition onto an alkyne produced the 5-*exo*-glycan **48** exclusively in a 91% yield.

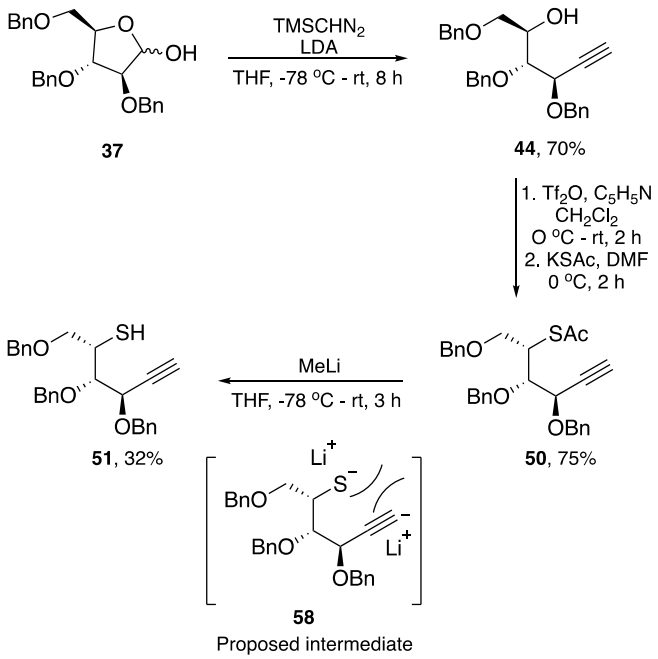


45 **57** **48**, 91%

Scheme 2.8: Deprotection of acetyl group under basic conditions and spontaneous cyclisation.

While the ionic ring-closing reaction proved to be effective for the preparation of the 5-*exo*-glycal of D-arabinose, we endeavoured to prepare the free thiol **51** in an effort to explore radical methods as a potential means for the production of the 6-*endo*-glycal **53**. As stated, basic conditions for acetyl deprotection of **50** would result in cyclisation; therefore, other routes were investigated for the D-sugar including cleavage of the acetyl group by acidic conditions and by hydride sources. Unfortunately, these methods only gave mixtures of products including starting material, the 5-*exo*-glycal and trace amounts of **51**. In addition, other reagents were employed to introduce the unprotected thiol moiety, such as H_2S and NaSH, which resulted in the formation of a mixture of products, or no reaction at all took place. Fortunately, after extensive screening, it was found that by utilising methyl lithium (MeLi) negative charges on both terminals of the molecule were created, which in turn stabilised the thiolate anion **58** due to repulsive forces between the two charges (Scheme 2.9). This allowed for the cleavage of the acetate and protonation of the thiolate before cyclisation could occur. However, when this procedure was used for the L-glycan, discrepancies in the success of this reaction between the two epimers of the arabinose derivatives were observed. For the D-epimer, the cleavage of the ester was carried out at -78 °C and warmed to rt furnishing the product in a 50% yield. Contrastingly, the L-epimer achieved a diminished yield of 32% using the same procedure. In an attempt to drive the deprotection, separate aliquots of MeLi were used, however the progress of the reaction did not improve and the yield remained low. In addition, the L-arabinose derivative resulted in a sluggish reaction, which did not proceed to completion, and proved to be unstable on silica gel. Where the MeLi caused the acetate of the D-sugar to cleave and

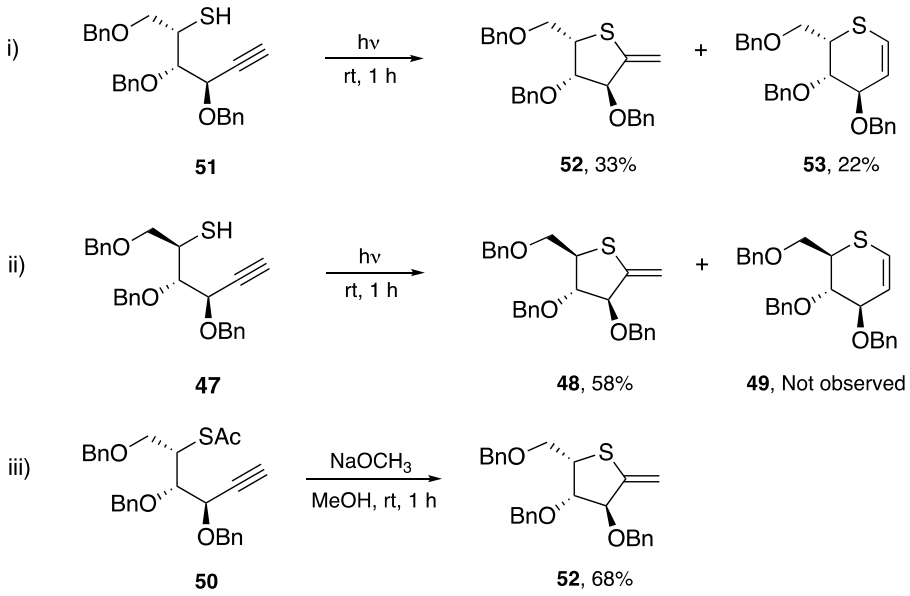
afford either thiol **47** (in majority) or 5-*exo*-glycal **48**, only thiol **51** (in a moderate yield) was obtained for the L-sugar.



Scheme 2.9: Synthesis of the acyclic substrate **51**.

2.4 Intramolecular thiyl radical cyclisation of arabinose derivatives

Due to the unstable nature of free thiols and their tendency to form the corresponding disulfide, the L-arabinose derivative **51** was used either immediately after preparation or stored under argon and at -20 °C for short periods prior to use. For the cyclisation, **51** was irradiated under UV at 365 nm at room temperature for 1 h. Various photoinitiators and photosensitisers had previously been screened and optimised for intramolecular thiol-ene reactions by the Scanlan lab,[68] and these optimised conditions were employed for the thiol-yne radical reactions. In the case of this work, DPAP and 2-methylbenzophenone (MBP) were utilized as a photoinitiator and photosensitiser, respectively. The reaction mixture of **51**, DPAP and MBP in anhydrous DMF was degassed and the solution was irradiated for 1 h without agitation. Gratifyingly, this reaction furnished both *endo* and *exo* products **53** and **52**, with the *exo*-glycal **52** formed as the major product (Scheme 2.10).



Scheme 2.10: i) Intramolecular thiol-yne cyclisation of L-arabinose derivative ii) intramolecular thiol-yne cyclisation with D-arabinose derivative iii) intramolecular ionic cyclisation of L-arabinose derivative.

The presence of both products was observed by thin layer chromatography (TLC), where the *exo* isomer **52** had a slightly higher retention factor (R_f) than the *endo* isomer **53**. Additionally, the two species were clearly visible by ^{1}H NMR due to the different chemical environments of the alkene moieties. The chemical shifts of the alkene CH/CH_2 protons were used to determine the conversion of starting material **51** to either 5-*exo* or 6-*endo* products, **52** or **53**, respectively. This result was in stark contrast to the analogous reaction with the D-glycan substrate, carried out by Dr. Corcé, which gave the *exo*-glycal **48** exclusively. It is therefore possible to deduce that the stereochemistry at the C-5 substituent plays a vital role in the regioselectivity of the cyclisation. Similarly, the nature of the protecting groups on the molecule could have an effect on the regioselectivity of the thiol-yne intramolecular addition. By changing the protecting group to a smaller, less bulky appendage, a small proportion of the D-arabinose free thiol could cyclise at the terminal CH to furnish the *endo*-cyclic product. In addition, by using a different protecting group strategy, the yield of cyclised L-arabinose could be increased, with a higher percentage of endocyclic product present.

2.5 Conclusion

In summary, the intramolecular thiyl radical cyclisation to furnish thiosugars in both furanose and pyranose forms has been comprehensively demonstrated. We have extended the study of previous work in this field from the robust and reliable thiol-ene

reaction to form thiosugars to the analogous thiol-yne coupling to furnish thioglycals. We have shown that the C-5 stereochemistry and the substituents and/or protecting groups of the sugar play a prominent role in directing either the 5-*exo*-trig or the 6-*endo*-dig cyclisation.

O-benzyl-protected arabinose was converted to the suitable precursors **47** and **51** *via* a short and efficient synthesis during which an alkyne and thiol moiety were introduced to the molecule. Using the Colvin rearrangement the arabinose starting material was ring-opened and the alkyne moiety was installed at the anomeric position in one step. The ensuing secondary alcohol was then converted to the triflate to be replaced by a thioacetate through nucleophilic substitution using KSAc. This step reversed the stereochemistry of the C-5 substituent, providing the L-epimer of the arabinose derivative. A double inversion strategy previously used by the Scanlan group could have been employed prior to this step to provide the D-epimer, however as Dr. Corcé had previously explored this, we aimed to demonstrate this methodology on the L-arabinose derivative and hence, the double inversion approach was excluded. The deprotection of the thioacetate **50** was carried out by two means, the first employed basic conditions to explore the results of the ionic cyclisation. Here, the 5-*exo*-glycal **52** was observed exclusively. The second method of deprotection involved the treatment of **50** with MeLi to, firstly, cleave the acetate group and secondly, to deprotonate the terminal alkyne. Due to formation of a repulsion of negative charges, the thiolate anion was protonated before cyclisation with the alkyne could occur. Through this route we furnished the precursor **51** in a moderate yield, which was used immediately to avoid dimerization to the disulfide or spontaneous cyclisation. Cyclisation was carried out using thiol-yne conditions to provide a mixture of both *exo* and *endo* products, which was observed by ^{1}H NMR and TLC.

In comparison to the parallel study of the D-epimer, where the radical thiol-yne reaction afforded the 5-*exo*-glycal exclusively, we can conclude that the stereochemical conformation of the C-5 substituent, and the nature of the substituent itself, can drive the regioselectivity of the cyclisation. When we compare these experiments to those carried out previously in the Scanlan lab, it is interesting to note that the addition of the thiyl radical onto an alkyne can behave substantially differently than the addition of the same radical onto and alkene. Where the 6-*endo*-dig cyclisation occurs so readily with an alkene, we see very little of it when using a different degree of unsaturation on an identical substrate. Similarly, in order to promote the 5-*exo*-trig cyclisation using thiol-

ene addition, the alkene must be terminally substituted, however we have demonstrated that it occurs very rapidly through both ionic and radical routes.

Given the bulky nature of the benzyl protecting group, which is the only protecting group used in these intramolecular thiyl radical cyclisation studies, it would be significant to try these experiments on the sugar with a protecting group of a different nature. For example, an acetate group would greatly decrease the overall size of the molecule and could lead to even more varying results, provided that selective deprotection of the thioacetate could be accomplished without exposing free hydroxyl groups on the sugar.

3.0 Introduction

Thiol-ene ligation has been employed for a wide range of synthetic applications across many fields of study such as polymer science, heterocycle synthesis, supramolecular chemistry, organocatalysis and bioconjugation.[4, 85, 86] The mild reaction conditions, high yields and tolerance to a large variety of functional groups make this chemistry attractive for many areas of research. In bioconjugation, thiol-ene and thiol-yne ligation techniques have been used to provide glycopeptides and glycoproteins, biotin tag and fluorophore labelling, biopolymers and nanoconjugates.[86, 143, 352]

The use of thioacids to deliver thioesters *via* TEC has limited precedent, especially with α-amino thioacids. Current reports are limited to those using thioacetic acid or thiobenzoic acid, in which case they can provide a very efficient route to thiols following deacetylation.[353-356] Thioacids are frequently used to afford thioesters, however, the usual protocol would involve ionic reaction such as the Michael addition to activated alkenes and again, only example of this using thioacetic acid or thiobenzoic acid have been explored.[357, 358]

3.1 Peptide ligation

As described in Chapter 1, peptide ligation has expanded vastly over the previous two decades following the ground breaking development of Native Chemical Ligation (NCL) by Kent *et al*.[1] Owing to the limitations of SPPS, peptides longer than fifty amino acids cannot be readily prepared through sequential amino acid coupling. NCL employs a transthioesterification step between cysteine and a *C*-terminal thioester to furnish a proximal thioester suitable for *S*,*N*-acyl transfer to form a native peptide bond (Scheme 1.7). The *S*,*N*-acyl transfer, is critical as it irreversibly forms the native peptide bond between the two ligated fragments at the cysteine *N*-terminus. Hence, two long chain peptides can be conjugated through a native peptide bond as long as one of them has an *N*-terminal cysteine. Criticisms of this process are mainly associated with the low natural abundance of cysteine. Accordingly, methods have been developed to convert the resulting cysteine residue in the peptide or protein to other residues, which occur more frequently in nature through desulfurization.[200, 205, 359] In addition, auxiliary thiol-containing moieties have been attached to natural amino acids to install the appropriate thiol for transthioesterification to occur and following *S*,*N*-acyl transfer, the

auxiliary is removed to reveal the natural amino acid.[209] Another method to avoid the use of cysteine is the "traceless" Staudinger reaction for amide bond synthesis developed by Bertozzi and co-workers which involves the formation of an amide bond through the reaction between an azide and a phosphine functionalised with a carbonyl, in which the authors converted an azido-nucleoside to the *N*-acetyl analogue.[360] While these methods provide the native peptide, they involve extra synthetic steps, more harsh reagents and generally diminish the simplicity and efficiency of peptide ligation in comparison to NCL.

3.1.1 Intramolecular *S,N*-acyl transfer and the size of the cyclic transition state

During NCL, the acyl transfer process forms the natural peptide bond through migration of the acyl moiety from the cysteinyl sulfur residue to the terminal amine. Nucleophilic attack from the amine drives this migration, where a cyclic transition state is formed during this intramolecular process. The ability of the molecule to form a stable intermediate is key to the acyl transfer and therefore, the size of the transition state is a significant factor to consider. The work of Katritsky *et al.*[261-263, 361] outlines the specific intermediate ring sizes that are likely to drive acyl migration and similarly, those that may hinder it. By building isopeptides with a thioester situated on the backbone and a free *N*-terminus, the acyl shift was carried out and monitored at room temperature and in a microwave reactor at 50 °C. These studies found that the ideal transition state is 5-membered where the reaction proceeds at room temperature to form the target peptide in good yields. For larger transition states, microwave assistance at 50 °C was needed to drive the conversion. 11- and 14-membered intermediates underwent acyl migration in 1 h under these conditions, however, some formation of the intermolecular transacylated products was observed. Other transition states, such as 8-, 13- and 15-membered formed mainly the transacylated products and required longer reaction times. The 16-membered transition state, however, behaved more like the smaller ring sizes and the major product was the desired intramolecular acyl migration.

3.2 Aims of this work

To our knowledge, the utility of α-amino thioacids for the synthesis of thioesters is still unexplored. α-Amino thioacids, as outlined in Chapter 1, Section 1.4, are used in many

areas of chemistry and biochemistry, however they are yet to be involved in thiol-ene ligation and moreover, they are yet to be used for the synthesis of peptidyl thioesters. Herein, we report a novel methodology for the chemical ligation of peptides through the thiol-ene ligation of thioacids onto peptidyl alkenes, in order provide an alternative approach to NCL.

The first step of our method involves a thiol-ene mediated coupling reaction between a thioacid-derived thioacyl radical species and an unsaturated radical acceptor. Thioacids are yet to be thoroughly explored as potential thiyl radical donors, however we intended to study the efficiency of thiyl radical generation of thioacids for this purpose. The synthesis of α-amino thioacids is outlined in detail later in this chapter. Since no natural amino acid bears an alkene, it was decided to functionalise one amino acid in a peptide, near the *N*-terminus, with an alkene moiety to serve as a radical acceptor. Initially, Ser was chosen as the modified amino acid residue due to the accessible primary alcohol situated on the side chain, which could be esterified with an olefin-containing carboxylic acid or acid chloride. The most straightforward route to the donor was to esterify the alcohol with 4-pentenoyl chloride in order to install an alkyl chain with a terminal alkene that would not be hindered by structural rigidity, as the close proximity of the amine and *S*-acyl group is paramount to the *S*,*N*-acyl transfer.[242] Following the formation of a thioester *via* thiol-ene ligation of thioacid and alkenyl peptide, spontaneous acyl transfer would deliver the new peptide bond. Elimination of the modification on Ser through cleavage of the ester would then afford the peptide in its native state (Figure 3.1). This process is known in the Scanlan group as TEMPLE: Thiol-ene Mediated Peptide Ligation and Elimination.

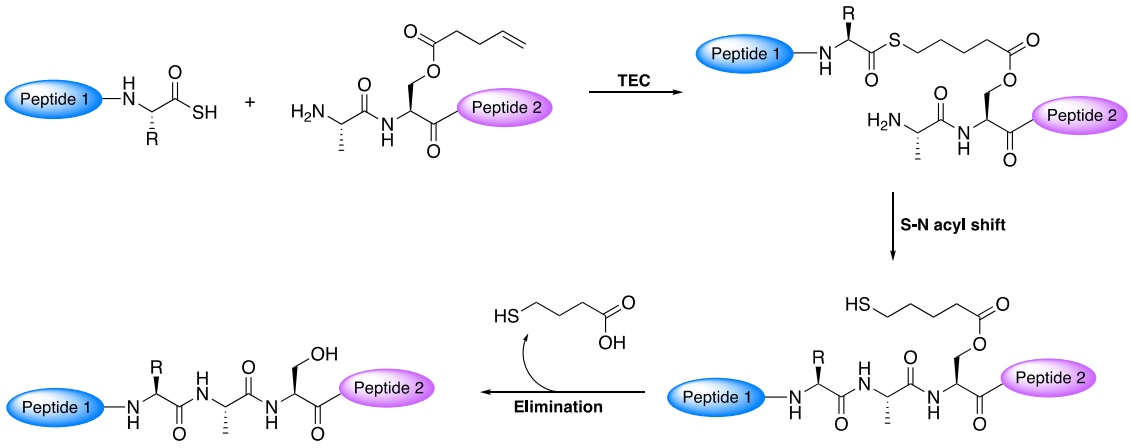


Figure 3.1: TEMPLE strategy: Thiol-ene Mediated Peptide Ligation and Elimination.

We set out to identify a suitable target protein that could potentially be assembled either in full or in part using the TEMPLE methodology. The transmembrane protein shown in Figure 3.2, the most abundant lipoprotein produced by *Pseudomonas aeruginosa*, is a protein consisting of 83 amino acids, with a diacylglycerol component situated on cysteine at location 20. As this group is introduced post-translationally onto the protein, access to this lipoprotein is very challenging through recombinant expression. Therefore, it was considered an ideal target for the TEMPLE process. The possible ligation sites and modifiable residues (Thr) are highlighted in Figure 3.2.

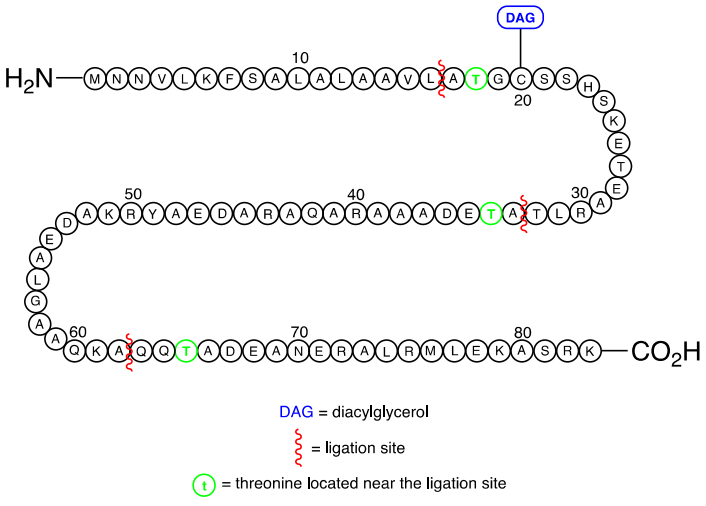
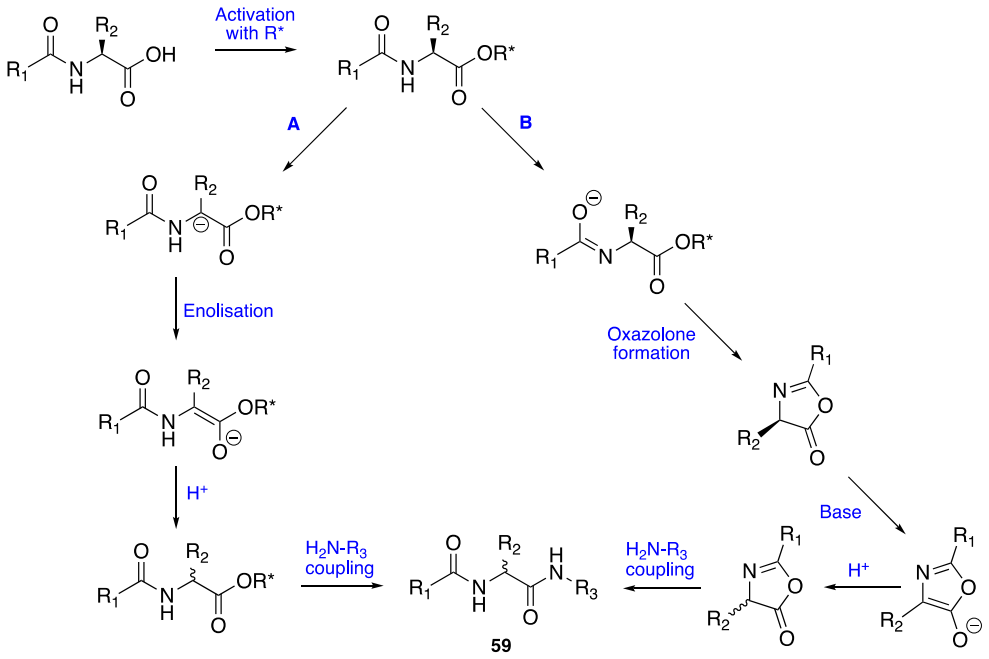


Figure 3.2: The transmembrane protein Oprl, which contains a diacylglycerol group on the cysteine, preventing its synthesis through recombinant methods. It is the target protein for TEMPLE.

This strategy eliminates the requirement of a Cys residue for peptide ligation and utilises Ser or Thr, which are both more naturally abundant than Cys. The thiol-terminated ester remaining on the peptide after acyl transfer can be cleaved through standard ester hydrolysis using acid or base, depending on the tolerance of the other residues on the peptide. Alternatively, it is hypothesised that a slight change of pH using buffer could drive thiolate formation, which could cyclise at the ester carbonyl, eliminating itself from the peptide as a 6-membered thiolactone.

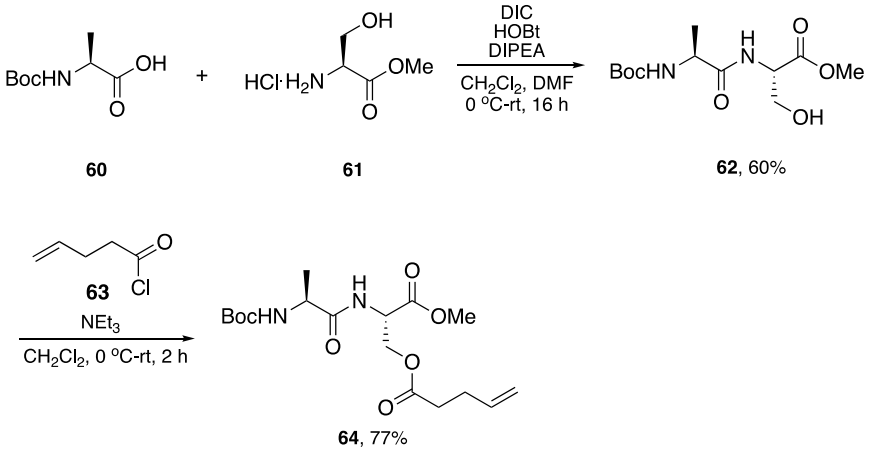
3.3 Synthesis and preliminary TEC of dipeptide acceptor

Initial model substrates for investigating the TEMPLE strategy were based on a dipeptide with Ala-Ser sequence, where the alkene appendage was introduced onto the Ser primary alcohol. The first step in this synthesis involved the peptide coupling of Ala and Ser. Many coupling reagents are commercially available and are widely used for the general coupling of amino acids.[185] *N,N'*-diisopropylcarbodiimide (DIC) is among those most commonly used and was chosen as reagent for this synthesis. It is well known that activation of the carboxylic acid of amino acids can lead to racemisation at the α-carbon. Two possible pathways have been identified for this side reaction, the first involves the formation of an oxazolone and the second is through direct enolisation (Scheme 3.1). Either route is detrimental to the chiral purity of the peptide, and so the reaction is supplemented with specific reagents to minimise racemisation. 1-Hydroxybenzotriazole hydrate (HOBt) is generally used to reduce racemisation in most peptide couplings; therefore it was used for this synthesis.



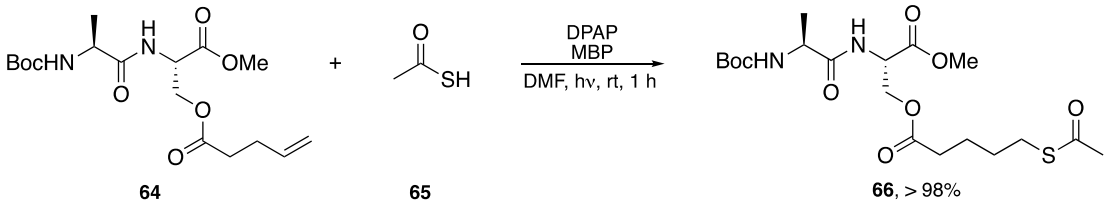
Scheme 3.1: Two pathways leading to racemic peptide. Path A: deprotonation of α-carbon forms enolate, which can be reprotonated from either face of the molecule, giving racemate **59**; Path B: formation of oxyanion drives cyclisation to oxazolone, which in turn can be deprotonated and reprotonated at the α-carbon to give the racemate **59**.

The coupling was carried out between the commercially available Boc-Ala-OH **60** and HCl·H_2N-Ser-OMe **61**, with additives DIC and HOBt, to form **62** in 60% yield with no racemisation observed (Scheme 3.2). **62** was then subjected to esterification of the free hydroxyl functionality using 4-pentenoyl chloride and NEt_3, which afforded the alkenyl dipeptide acceptor **64** in a 77% yield.



Scheme 3.2: Synthesis of dipeptide acceptor **64**.

In order to investigate the viability of the dipeptide **64** as a thiyl radical acceptor, thiol-ene coupling was carried out with thioacetic acid (Scheme 3.3). It was envisaged that this reagent would form a reactive thiyl radical (donor) and had previously been used in the Scanlan lab for this purpose. The conversion of alkene to acylated thioester would demonstrate the first step of the TEMPLE methodology and give an indication of the efficiency of thiol-ene coupling on alkenyl peptides. As the *N*-terminus of the peptide was still protected with a Boc group, the spontaneous *S*,*N*-acyl transfer could not occur therefore halting process after the first step to determine the yield of thioester product. The coupling of **64** and thioacetic acid **65** was carried out in a UV oven at 365 nm for 1 h. Similar to standard thiol-ene conditions in the Scanlan lab, DPAP and MBP were added to the degassed reaction flask and the components were all solubilised in DMF.



Scheme 3.3: Thiol-ene ligation of **64** with thioacetic acid **65**.

Gratifyingly, thioester **66** was furnished in a quantitative yield and the product was purified by column chromatography. ^{1}H NMR was used to probe the outcome of the reaction, as the crude product clearly showed no alkene CH or CH_2 peaks in the respective shift regions. In addition, the disappearance of the alkene starting material was apparent by TLC, along with the appearance of a new compound. This result confirmed our assumption that a peptide displaying an alkene on the side chain would fully partake in TEC reactions, providing a reactive thiyl radical donor was present.

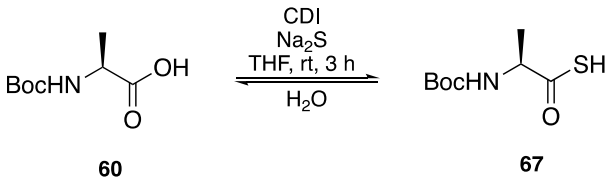
3.4 Thioacid synthesis

Following on from the successful preparation of thioester **66** through TEC, it was paramount that the same reaction was carried out with an α-amino thioacid derivatives

to ensure that the amino acid analogues could behave as thiyl radical donors. As previously discussed, α-amino thioacids have the potential to rapidly become hydrolysed to the corresponding carboxylic acid.[362] Therefore, several attempts were made to prepare the compounds while avoiding hydrolysis.

3.4.1 Synthesis of thioacids using Na_2S and CDI

The synthesis of Boc-protected thioacids through a nucleophilic sulfur source (such as sodium sulfide (Na_2S)), together with an activating agent (such as CDI), has been reported, with specific detail into the avoidance of hydrolysis back to the carboxylic acid.[290] The protocol involved a number of critical points for reducing hydrolysis, including cooling the reaction to 0 °C for quenching, prohibiting the acidity to drop below pH 4 and ensuring that all CO_2 had evolved before adding Na_2S.



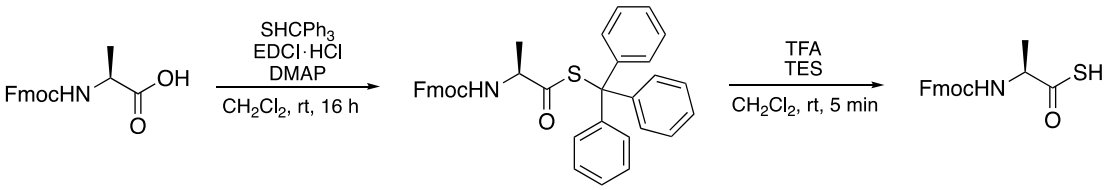
60 **67**

Scheme 3.4: Preparation of Boc-protected thioalanine **67** using CDI and Na_2S.

HRMS analysis of the crude product showed the presence of both thioacid **67** and carboxylic acid **60**. Due to the sensitive nature of the reaction and subsequent difficulties with monitoring the reaction, it is unknown as to whether the reaction was successful and the resulting thioacid was subsequently hydrolysed during the work-up, or if the reaction did not go to completion, which lead to the mixture of products. It is also likely that both of these outcomes occurred. Furthermore, given that the difference in ^{1}H NMR of the thioacid **67** and carboxylic acid **60** is negligible, the ratio of **67**:**60** was unknown, meaning any subsequent thiol-ene reactions would not depict a true yield. Hence, it was decided to prepare the donor through deprotection of a stable, protected thioacid, which could then be used directly without any work-up or purification.

3.4.2 Synthesis of *S*-trityl protected thioacids

Simultaneously, another member of the Scanlan group (Dr. Helen Burke) was investigating the utility of the trityl (Trt) group on α-amino thioacids, which has been reported by Crich and co-workers.[298] By using this protecting group, we could introduce the thiol onto the amino acid through standard coupling conditions and deprotect the thiol rapidly, rendering the free thiol available for direct TEC (Scheme 3.5).



68 **69**, 65% **70**

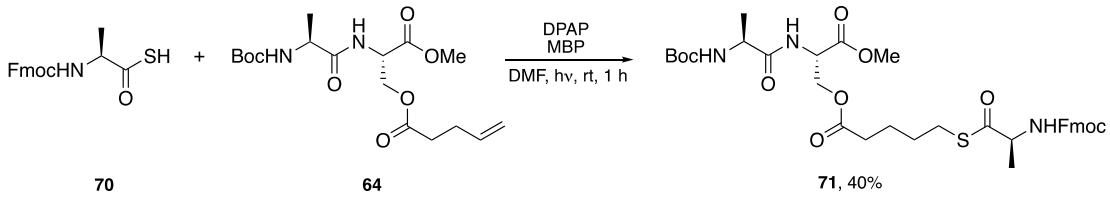
Scheme 3.5: Synthesis of *S*-Trityl protected thioacids and deprotection to afford free thioacid **70**.

In order to cleave the trityl protecting group with acid and leave the amino protecting group in place, a base-labile protecting group was required for the amino functionality. It was decided to use the Fmoc protecting group, which is ubiquitous in peptide chemistry and orthogonal to any acid-labile protecting groups on the molecule. Accordingly, Fmoc-Ala-OH **68** was coupled with SH-Trt using EDCI·HCl and DMAP, which furnished the trityl thioester **69** in a 65% yield (Scheme 3.5). The trityl group was subsequently cleaved using trifluoroacetic acid (TFA) (25%) in CH_2Cl_2, with triethylsilane (TES) used as a scavenger for the trityl cation. With the addition of the TFA there was a colour change to bright yellow, indicating the presence of the trityl cation. Upon addition of TES, the bright yellow colour was instantly quenched, indicating that all of the trityl cation was consumed by TES. After 5 min TLC showed full deprotection of the starting material **69** and the crude mixture was concentrated *in vacuo* as quickly as possible and at a moderate temperature to avoid hydrolysis. The products, including trityl-TES by-products, were re-dissolved in DMF and used directly for thiol-ene ligation.

3.5 TEC of thioalanine and alkenyl dipeptide

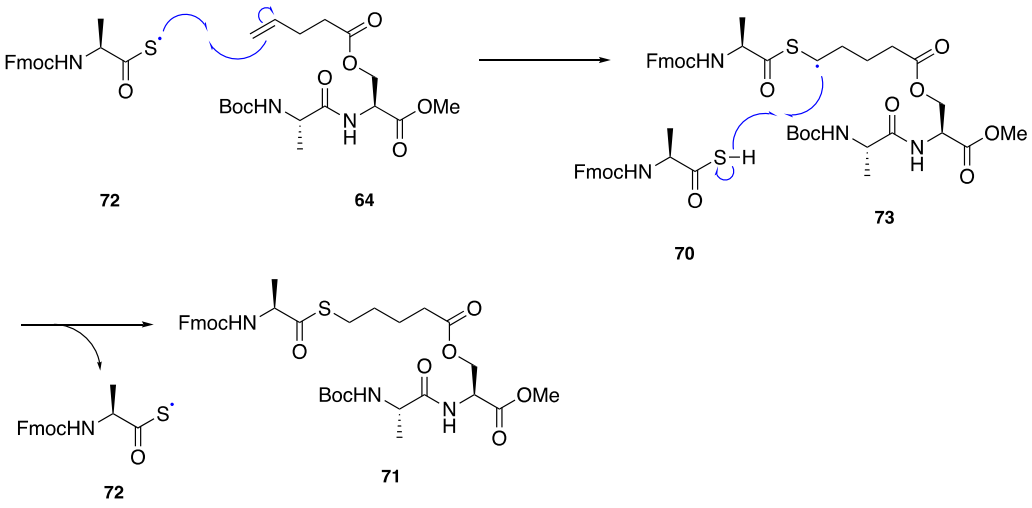
3.5.1 Thiol-ene ligation of thioalanine and alkenyl dipeptide

With **70** in hand, TEC was carried out immediately with dipeptide **64** in DMF under UV conditions for 1 h. Conditions usually employed for thiyl radical reactions in our lab were continued, with additional focus on the anhydrous environment necessary to ensure that the thioacid **70** did not hydrolyse. DPAP and MBP were utilised as photoinitiator and photosensitiser and the reaction was conducted at 365 nm without agitation (Scheme 3.6).



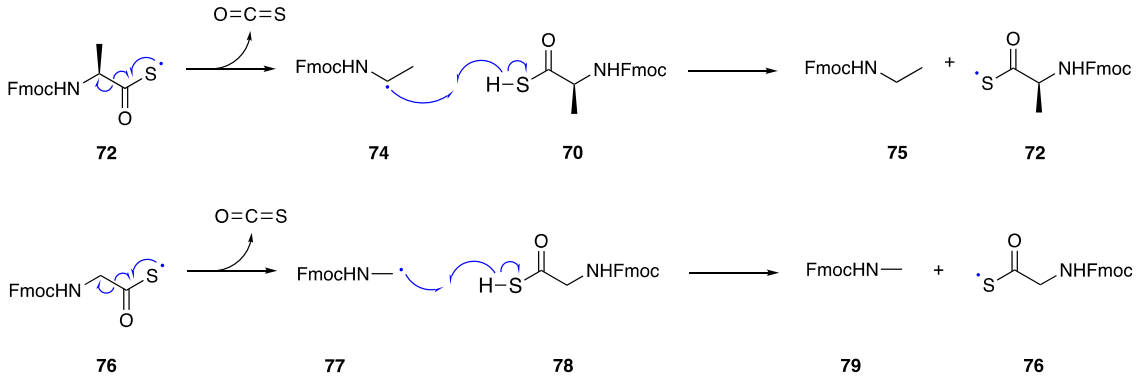
Scheme 3.6: Thiol-ene ligation of thioalanine and dipeptide **64**.

Disappointingly, the thioester **71** was furnished in a modest yield of 40%. It was evident from ^{1}H NMR analysis that not all of the alkene starting material had been consumed, which was surprising given the success of the model reaction with thioacetic acid, where **66** was afforded in a quantitative yield. The postulated mechanism of the radical addition was considered further (Scheme 3.7) with particular attention to the thiyl radical.



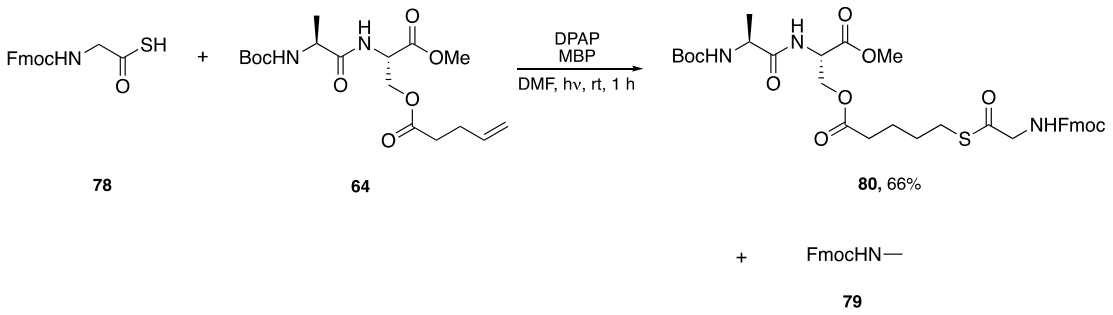
Scheme 3.7: Radical mechanism for the intermolecular addition of thiyl radical **72** onto alkene **64**, including propagation of thiyl radical **72** through H-abstraction of thioacid.

It was deduced that due to the remaining proportion of alkene acceptor **64** left after the reaction, the thiyl radical donor was consumed through a competing reaction with TEC. It was then theorised that carbonyl sulfide (CSO) elimination could be occurring on the thioacid upon radical initiation, producing an alkyl radical which could be similarly propagated in the reaction through H-abstraction (Scheme 3.8). Indeed, byproducts **75** and **79** were isolated and characterised, proving that CSO elimination was occuring.



Scheme 3.8: CSO elimination during thiyl radical formation on α-amino thioacids of Ala (above) and Gly (below), where Fmoc-protected amines **75** and **79** were formed as by-products.

It was further postulated that the stability of the alkyl radical formed during CSO elimination was dependent on the substituents of the carbon-centred radical. A simple solution, therefore, was to use a less substituted thioacid, which would de-stabilise the alkyl radical and diminish the competing side-reaction. Consequently, thioglycine was prepared as per Scheme 3.5 with the analogous thioalanine and TEC was carried out as previously described (Scheme 3.9). As expected, thioester **80** was afforded in an increased yield of 66%, with a reduced amount of CSO by-product **79** isolated and less alkene starting material recovered.

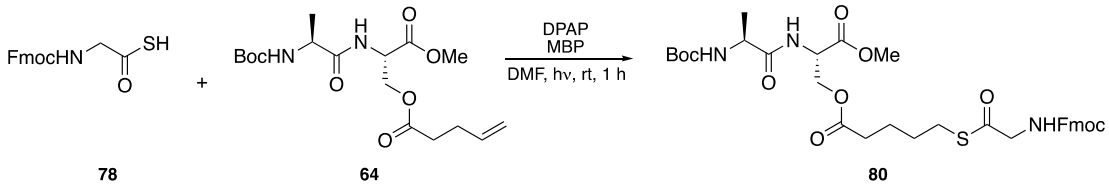


78 **64** **80**, 66% **79**

Scheme 3.9: TEC using thioglycine to reduce CSO elimination and furnish **80** in a 66% yield.

However, this result was still not satisfactory in comparison to the test reaction with thioacetic acid to furnish **66** quantitatively. Hence, an optimisation strategy was designed using thioglycine **78** and alkene **64**, where the variables being studied included concentration and equivalents of both thioacid donor and alkene acceptor.

Table 3.1. Optimisation of thiol-ene ligation of thioglycine donor and dipeptide acceptor.



78 **64** **80**

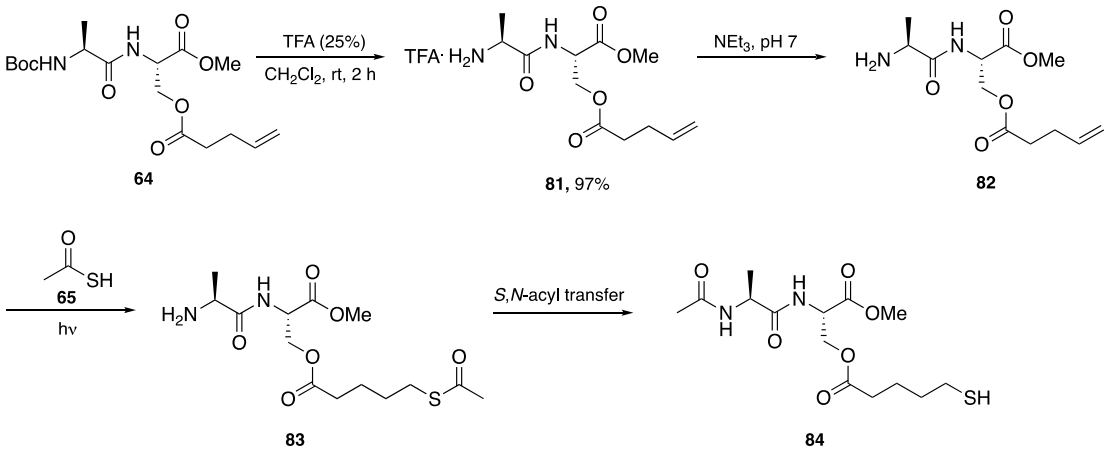
Entry	**78** eq.	**64** eq.	DPAP eq.	MBP eq.	**[78]** mmol/mL	Isolated Yield
1	1.5	1	0.2	0.2	0.268	66%
2	1	3	0.2	0.2	0.106	78%
3	1	1	0.2	0.2	0.067	34%
4	1	1	0.2	0.2	0.134	52%
5	3	1	0.2	0.2	0.268	99%
6	2	1	0.2	0.2	0.179	76%
7	2	1	0.2	0.2	0.268	93%

It was envisaged that the concentration of reactants and volume of solvent could promote TEC over the competing CSO elimination. The major aim of this optimisation

was to minimise the excessive use of either thioacid or alkene, as the main application for this methodology would see both components as modifications on long peptides. As evident from Table 3.1, varying these conditions of the reaction had a significant effect on thioester formation. Entry 3 shows that using equimolar amounts of both reactants gave a low yield of 34%, however by concentrating the reaction mixture the yield was increased to 52% (Entry 4). By using a three-fold excess of alkene a yield of 78% was obtained (Entry 2), and by changing that excess to the thioacid, the thioester **80** was formed quantitatively (Entry 5). As previously stated, we aimed to find conditions that would not lead to poor atom economy and so by decreasing the equivalents of thioacid **78** to 2 and using solvent volumes of 3 mL and 2 mL, we obtained the product in 76% and 93%, respectively. This initial study demonstrated for the first time that thioacid derived amino acids could be employed in thiol-ene ligation to furnish thioester derivatives with potential applications for chemical ligation. This represents one of the major findings of this thesis.

3.5.2 Driving the *S*,*N*-acyl transfer to furnish a tripeptide

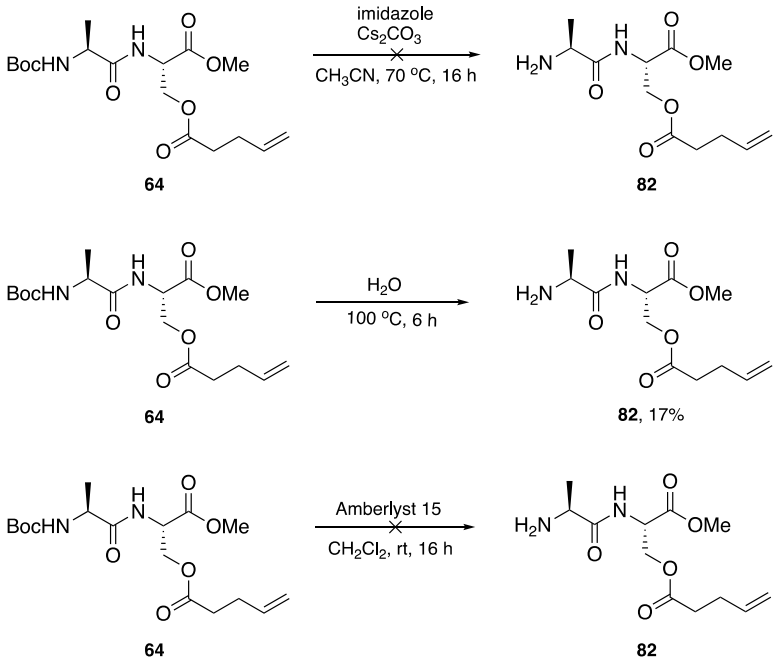
With the first step of the TEMPLE process optimised, the *S*,*N*-acyl transfer was subsequently investigated. Initially, the strategy that we adopted was to carry out the TEC on dipeptide alkene acceptor with a free terminal amine (Scheme 3.10). Hence, following formation of the thioester **83**, the acyl functionality could undergo *S*,*N*-acyl transfer in a one-pot process to form a native peptide bond. This was carried out by treatment of the alkene acceptor **64** with TFA (25%) in CH_2Cl_2, until TLC showed no starting material present. The solvent and majority of the TFA was then evaporated and the product was re-solubilised in CH_2Cl_2. NEt_3 was added until the solution reached neutral pH in order to free-base the peptidyl amine from the analogous TFA salt. The solution was then concentrated *in vacuo*, solubilised in DMF and used for TEC with thioacetic acid, due to the known success of this reaction. The solution was then left to stir in DMF overnight to allow for the spontaneous *S*,*N*-acyl transfer to occur. These three steps were carried out without isolating the intermediates in order to demonstrate the process as a one-pot approach.



Scheme 3.10: Route to amide bond formation *via* thiol-ene ligation and subsequent *S*,*N*-acyl transfer.

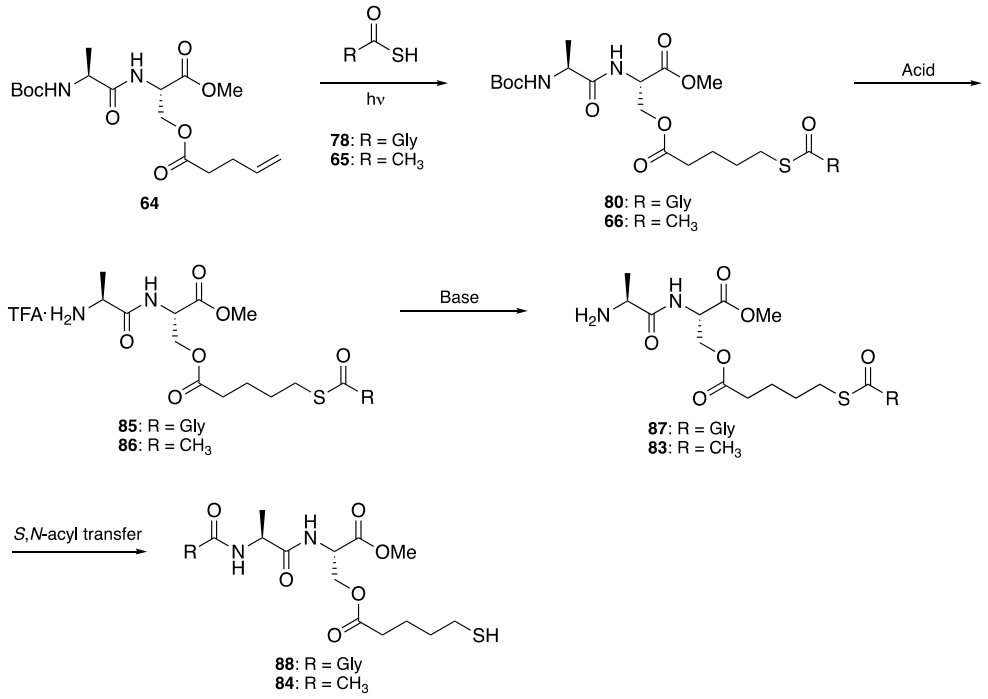
Unfortunately, after stirring overnight ^{13}C NMR analysis showed the presence of the thioester through a peak at 195 ppm. This peak is evident in all previously made thioesters and is a valuable tool for tracking the progress of TEMPLE. Assuming the thiol-ene ligation was successful, the lack of acyl shift was accredited to the peptidyl amine being unavailable. It was theorised that this was due to the lack of consumption of TFA and the peptide remaining as a TFA salt. Therefore, it was decided to halt the process after the deprotection of the amine to ensure the amine was available.

Several different methods to free-base the TFA salt were investigated, including using different bases (DIPEA, NEt_3, $NaHCO_3$, NaOH). Despite the pH being brought to neutral each time, none of these resulted in formation of the free amine, which was determined by ^{19}F NMR. It was then envisaged that different methods to remove Boc protecting groups could be employed (Scheme 3.11). Many of these methods required harsh conditions which are unfavourable for peptides, however one such procedure involved the use of imidazole and Cs_2CO_3 in CH_3CN at 70 °C.[363] This resulted in a mixture of products, most of which were too polar to isolate and were assumed to be hydrolysis products of the methyl ester on the dipeptide. Another method that was explored was the deprotection of Boc using water[364] at 100 °C for 6 h, which gave the product in a 17% yield. After increasing the duration of the reaction to 16 h with the expectation that the yield would improve, only more by-products were observed by TLC. The use of acidic ion-exchange resin Amberlyst 15 was also employed to deprotect the peptide, as reported by Romo and co-workers,[365] however in our hands no reaction occurred.



Scheme 3.11: Alternative approaches to the deprotection of Boc-protected dipeptide **64** to avoid salt formation of the peptide.

An additional strategy was also ventured, where TEC was carried out on the Boc-protected dipeptide **64** and only when the resulting thioester was isolated and purified was the Boc group cleaved and the amine made available for acyl transfer. This route was explored using both thioacetic acid and Fmoc-protected thioglycine and despite both thioesters furnished in high yields and high purity, the desired products **88** and **84** were not obtained (Scheme 3.12). This was due to the same problem surrounding salt formation and hence, the peptidyl amine was not available for acyl transfer.



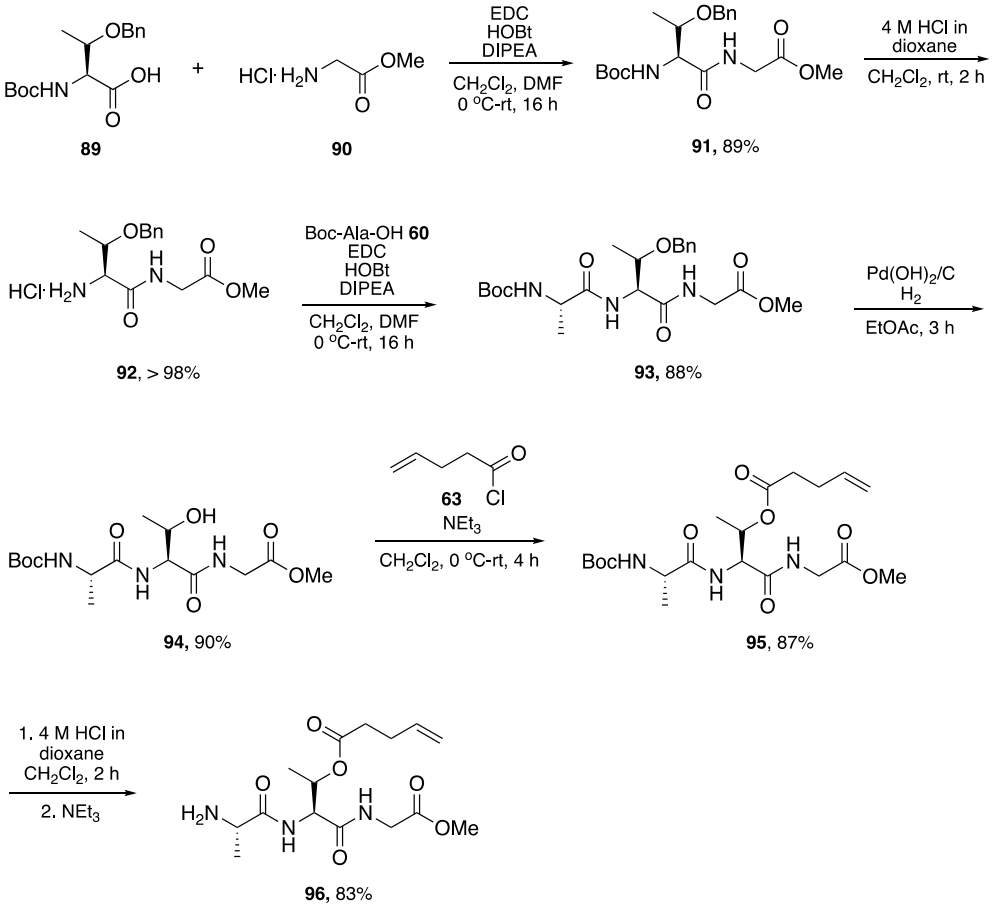
Scheme 3.12: Proposed route to amide bond formation. Synthesis of thioesters **80** and **66** through TEC, Boc-deprotection using TFA followed by addition of base to obtain the free amine. Subsequent attempts at *S*,*N*-acyl transfer did not furnish the expected products **88** and **84**.

It is notable that the use of HCl instead of TFA was also employed, in the possibility that basifying the HCl salt of the peptide would be more successful than the corresponding TFA salt. One such example of this was the use of NEt_3 to neutralise the product and the precipitation of the $HCl{\cdot}NEt_3$ by-product in THF. Disappointingly, not all of the salt by-product was precipitated and the ensuing acyl transfer following TEC did not occur. Given that the presence of HCl is not as identifiable as TFA (using ^{19}F NMR), the use of this acid was not continued.

3.6 Synthesis of tripeptide alkene acceptor

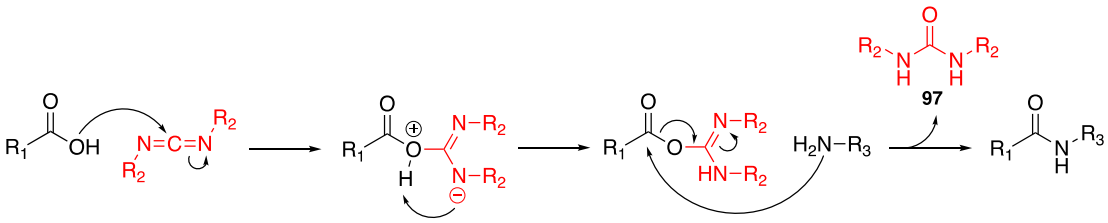
In the interest of time and progress, we endeavoured to move on to the synthesis of the tripeptide alkene acceptor that would mimic the target ligation site in Figure 3.2. It was postulated that a different peptide sequence could perhaps be more easily handled and that neutralisation of this tripeptide salt may be more operationally simple. The sequence on the acceptor side of the site was Ala-Thr-Gly, hence, for our TEMPLE

substrate we required the compound **96**, of which the synthesis is depicted in Scheme 3.13.



Scheme 3.13 Synthesis of alkene tripeptide acceptor **96**.

To begin with, the first amino acid coupling of Boc-Thr(OBn)-OH and Gly-OMe hydrochloride was carried using previously discussed peptide coupling conditions, DIC and HOBt. Following Boc deprotection of the resulting dipeptide, Boc-Ala-OH was coupled using the same procedure and coupling reagents. However, carbodiimides are known to form a urea by-product during activation of carboxylic acids (Scheme 3.14), which can be extremely challenging to remove. The DIC urea (DIU) by-product formed in this reaction co-eluted with **91** during column chromatography and was visible in ^{1}H NMR. In order to circumvent this, EDCI hydrochloride was employed as a coupling reagent as it forms a water-soluble urea by-product, which can be removed during an aqueous work-up. Despite the change in reagent, the dipeptide **91** was produced in a very high yield of 89%.



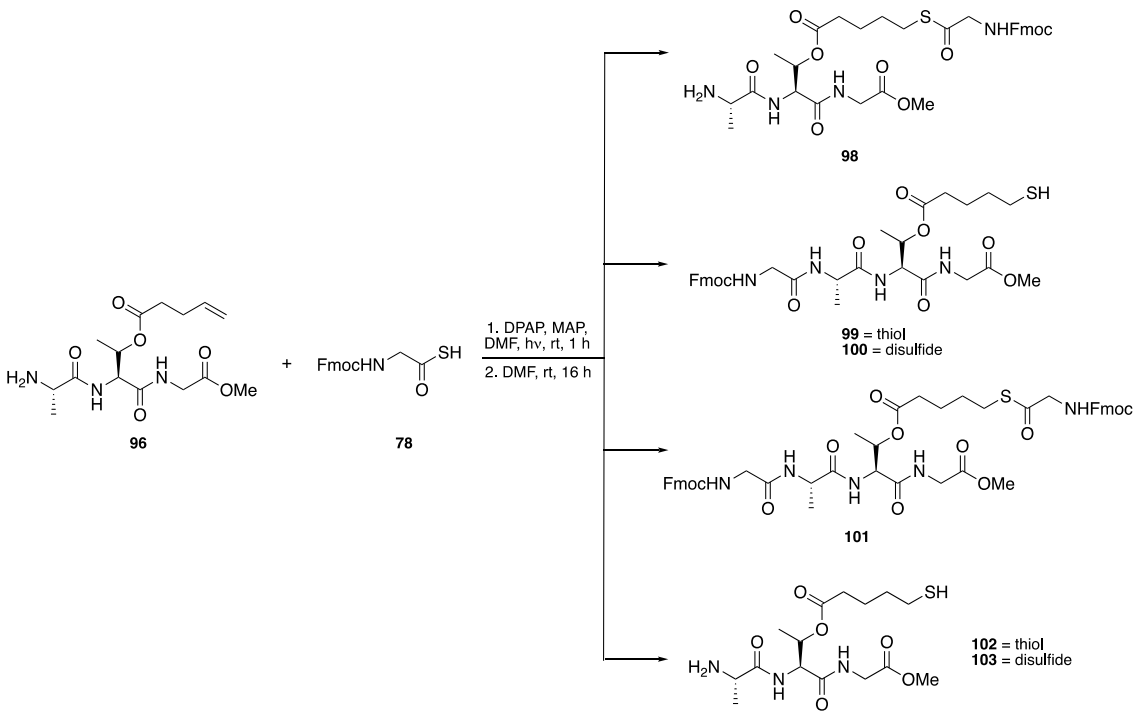
Scheme 3.14: General mechanism of the formation of the urea by-product during the activation of carboxylic acids through carbodiimides. When R_2 was an isopropyl group, the urea **97** was inseparable from the peptide product.

Following Boc cleavage from the dipeptide using 4 M HCl in dioxane, **92** was converted to the tripeptide **93** *via* the same coupling conditions as previous discussed. The benzyl-protecting group on the side chain of Thr was subsequently removed by catalytic hydrogenolysis under an atmosphere of hydrogen to furnish **94** in 90% yield. The free alcohol was then converted to the ester **95** using 1.2 eq. of 4-pentenoyl chloride and NEt_3. With the alkene handle in place, the Boc group was removed, again using HCl, and NEt_3 was attempted to free-base the amine HCl salt.

3.6.1 Driving the *S,N*-acyl transfer to furnish a tetrapeptide

Initially, similar to the dipeptide example in Section 3.5.2, a one-pot procedure for thiol-ene ligation and *S,N*-acyl transfer was conducted. Following standard TEC of **96** and thioglycine **78**, the crude material was stirred in DMF at rt for 16 h. The progress of the reaction was monitored by TLC, which showed a mixture of inseparable compounds after 16 h. This result was unexpected as ^{1}H NMR of the crude mixture showed full consumption of the alkene starting material. Assuming the TEC was successful and the multiple products arose from the second step, the acyl transfer, it is likely that the mixture was composed of all or some of the inter- and intramolecular *S,N*-acyl transfer products shown in Scheme 3.15. Theoretically, it would not be expected for all of these possible compounds to have such similar R_f, excluding perhaps amine **98** and thiol **99**, however due to the readiness and rapidity of thiol-ene chemistry and the lack of starting material in the crude mixture, it is assumed that this was the case. Compounds **101** and **102** are unexpected (see Section 1.3.2 on the reversibility of the *S,N*-acyl transfer) as the nucleophilicity of the free amine at the *N*-terminus of a peptide should be greater than

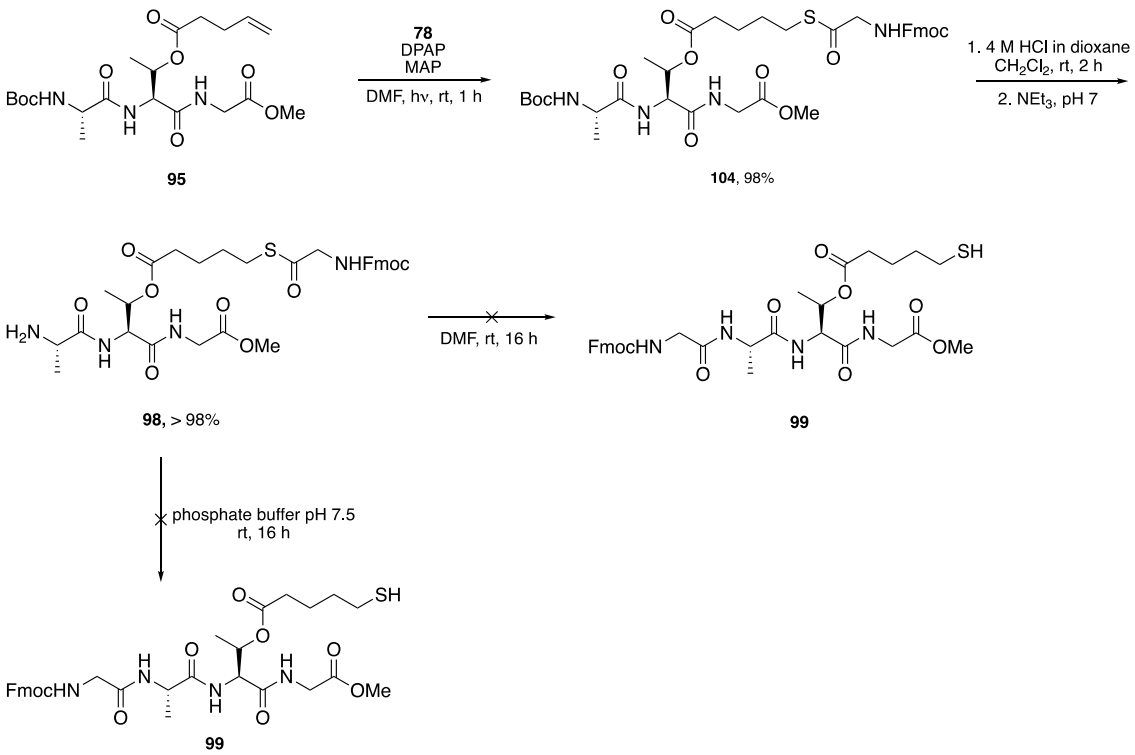
the stability of the thioester and the nucleophilicity of the thiol, especially if the disulfide has formed, however, in certain cases in the Scanlan group it has been observed. Additionally, if the HCl salt of the peptide remained after treatment with NEt_3, the amine would not have been available for acyl transfer. This was also likely as TLC showed a prominent, polar compound that was consistent with salt formation. In order to decipher the components of the mixture, it was treated with freshly prepared NaOMe and stirred for one hour. It was anticipated that this would cleave any thioester present and the peptide fragment could be isolated. Unfortunately this resulted in an insoluble mixture of products that were not identifiable.



Scheme 3.15: Possible products of one-pot TEC and the inter- and intramolecular *S*,*N*-acyl transfer. **98**: product of thiol-ene ligation with no acyl transfer. **99**/**100**: product of TEC and intramolecular acyl transfer in one-pot. **101** and **102**/**103**: products of the dynamic intermolecular acyl transfer.

The complicated nature and ambiguity surrounding the one-pot reaction with tripeptide **96** lead us to believe that a more step-wise approach would be needed for this methodology. Accordingly, we aimed to carry out the thiol-ene ligation on the *N*-protected tripeptide to break up the process and gain further understanding of the *S*,*N*-acyl transfer. **95** was subjected to thiol-ene ligation under standard conditions in DMF

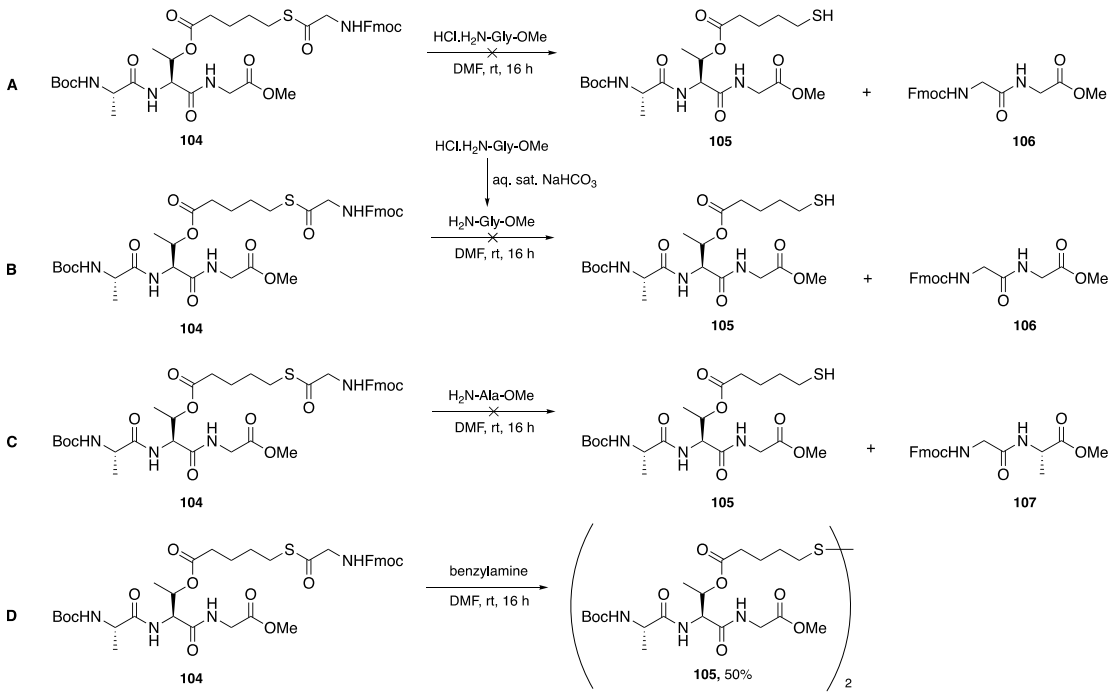
and thioester **104** was obtained in 98% yield (Scheme 3.16). **104** was then treated with HCl to afford the HCl salt and the solution was neutralised to pH 7 with NEt_3. The product was concentrated *in vacuo* with additional MeOH to remove any remaining HCl or NEt_3 and the crude product was used without further purification. After stirring in neutral pH in DMF for 16 h, we deduced from ^{13}C NMR that the thioester was still present and conversion to **99** had not occurred. The same procedure was then carried out using phosphate buffer at pH 7.5 in place of DMF, however again, no acyl transfer occurred.



Scheme 3.16: Synthesis of thioester **104** through TEC of **95** and thioglycine **78,** followed by Boc-deprotection to furnish **98** using HCl and subsequent attempts to drive the *S*,*N*-acyl transfer by stirring at rt in either DMF or phosphate buffer.

At this point, the theory that the *S*,*N*-acyl transfer was not occurring due to the salt formation of the peptide needed to be investigated further. It was decided to demonstrate an intermolecular *S*,*N*-acyl transfer using **104** as the *S*-acyl component and the HCl salt of an amino acid as the amine. As depicted in Scheme 3.17, **104** and Gly-OMe hydrochloride **90** were stirred in a 1:1 mixture of DMF:phosphate buffer and after 16 h only starting materials were observed. This result confirmed our hypothesis that

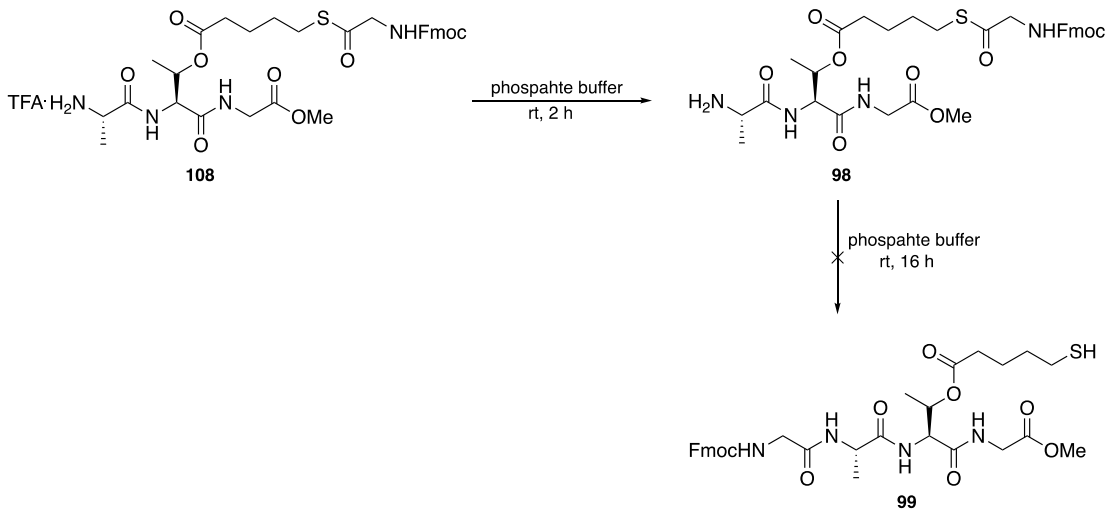
the amine could not be in the form of a salt for an acyl transfer to occur. Additionally, we endeavoured to demonstrate a successful intermolecular *S,N*-acyl transfer on the thioester **104** to prove that the peptidyl thioesters prepared using our methodology could undergo acyl transfer. Therefore, **90** was washed with aq. $NaHCO_3$ and extracted into EtOAc and used for the same reaction. Disappointingly, only unreacted starting materials were obtained. We postulated that this result was due to the amine still existing as a HCl salt, despite our efforts to free-base it. In a similar vein, H_2N-Ala-OMe was tested using the same procedure, however again no reaction occurred. Benzylamine was then used as it is a more nucleophilic amine than neutral amino acids and, gratifyingly, the peptide thiol was isolated as the disulfide dimer in a 50% yield.



Scheme 3.17: Intermolecular *S,N*-acyl shift attempts. No reaction took place when using A: HCl·H_2N-Gly-OMe, B: H_2N-Gly-OMe or C: H_2N-Ala-OMe, possibly due to the unavailable amine that is hindered by HCl salt. D: Utilisation of benzylamine furnished **105** in a 50% yield.

Another approach that was explored was the Boc-deprotection of **104** using TFA (25%) in CH_2Cl_2. This was the preferred method due to the useful tool of ^{19}F NMR, as previously mentioned. Finally, through the use of phosphate buffer pH 8.5, the free amine **98** was obtained which was visible by TLC as well as NMR techniques. With the free amine in our hands, we left the product to stir in phosphate buffer pH 8.5 for 16 h

with the intent of driving the acyl transfer. Unfortunately, no change in TLC and the presence of the thioester ^{13}C NMR shift meant that the acyl transfer had not occurred (Scheme 3.18). This process was repeated and the reaction was left to stir at rt in phosphate buffer for 3, 5 and 7 d, however still no acyl transfer occurred. It has also been reported in the literature that the use of microwaves can drive the reaction to completion in a shorter time. Unfortunately this, too, resulted in no change from the thioester.



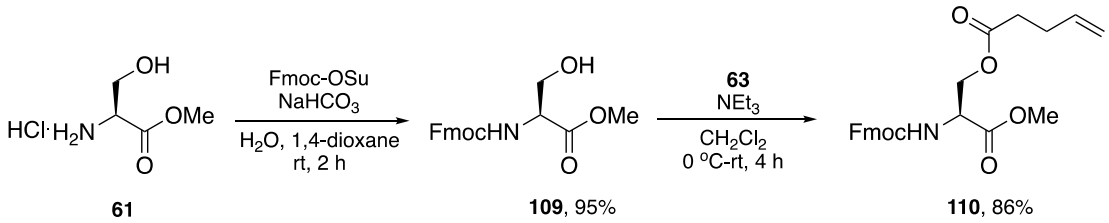
Scheme 3.18: Successful conversion of TFA salt **108** to free amine **98** using phosphate buffer pH 8.5, followed by unsuccessful *S*,*N*-acyl transfer to **99**.

Having verified that the substrate **104** could undergo intermolecular *S*,*N*-acyl transfer with an amine and successfully obtaining the tripeptide with unprotected amine, it was no longer evident that the failure of the intramolecular transfer could be attributed to the presence of the HCl/TFA salt. More likely, we had discovered that the ring size for the cyclic intermediate formed during *S*,*N*-acyl transfer was more significant and more complex than we originally anticipated, which is in contrast to the reported work of Katritsky and co-workers, albeit with more flexible substrates. As previously mentioned, the efficiency of the *S*,*N*-acyl transfer is largely dependent on the ring size of the intermediate. The system that has been designed in this project has afforded a 14-membered intermediate, which is suitable for acyl transfer and favours the intramolecular process, according to the literature. However, in our hands it has not

been possible to achieve this and therefore, there is potential for a different model affording a smaller ring size that may drive the reaction.

3.7 Building peptidyl thioesters through thiol-ene ligation

The success of the preparation of thioacids through *S*-trityl protection and subsequent thiol-ene ligation of these thioacids and alkenyl peptides encouraged the exploration of the scope of this reaction. It was envisaged that a wide range of peptidyl and non-peptidyl thioesters could be synthesised using this methodology. Hence, we endeavoured to utilise a simple olefin acceptor to demonstrate TEC on a variety of different thioacids, prepared as in Scheme 3.5. Accordingly, Ser-OMe hydrochloride was converted, in a very satisfactory yield of 95%, to the Fmoc-protected analogue **109**,[366] to which the alcohol side chain was esterified using 4-pentenoyl chloride to afford the acceptor compound **110** (Scheme 3.19).



Scheme 3.19: Synthesis of alkene ester **110** from commercially available Ser-OMe hydrochloride.

3.7.1 Synthesis of *S*-trityl-protected thioacid donors

The α-amino thioacids were prepared from the corresponding commercially available Fmoc-protected amino acids. Following standard coupling of carboxylic acid and trityl thiol using EDCI·HCl and DMAP (Scheme 3.20), an extensive array of thioacids were developed from amino acids, peptides, sugars and conventional carboxylic acids (Figure 3.3).

Scheme 3.20: *S*-trityl esterification of carboxylic acids using $SHCPh_3$, EDCI·HCl, and DMAP in CH_2Cl_2 at rt for 16 h.

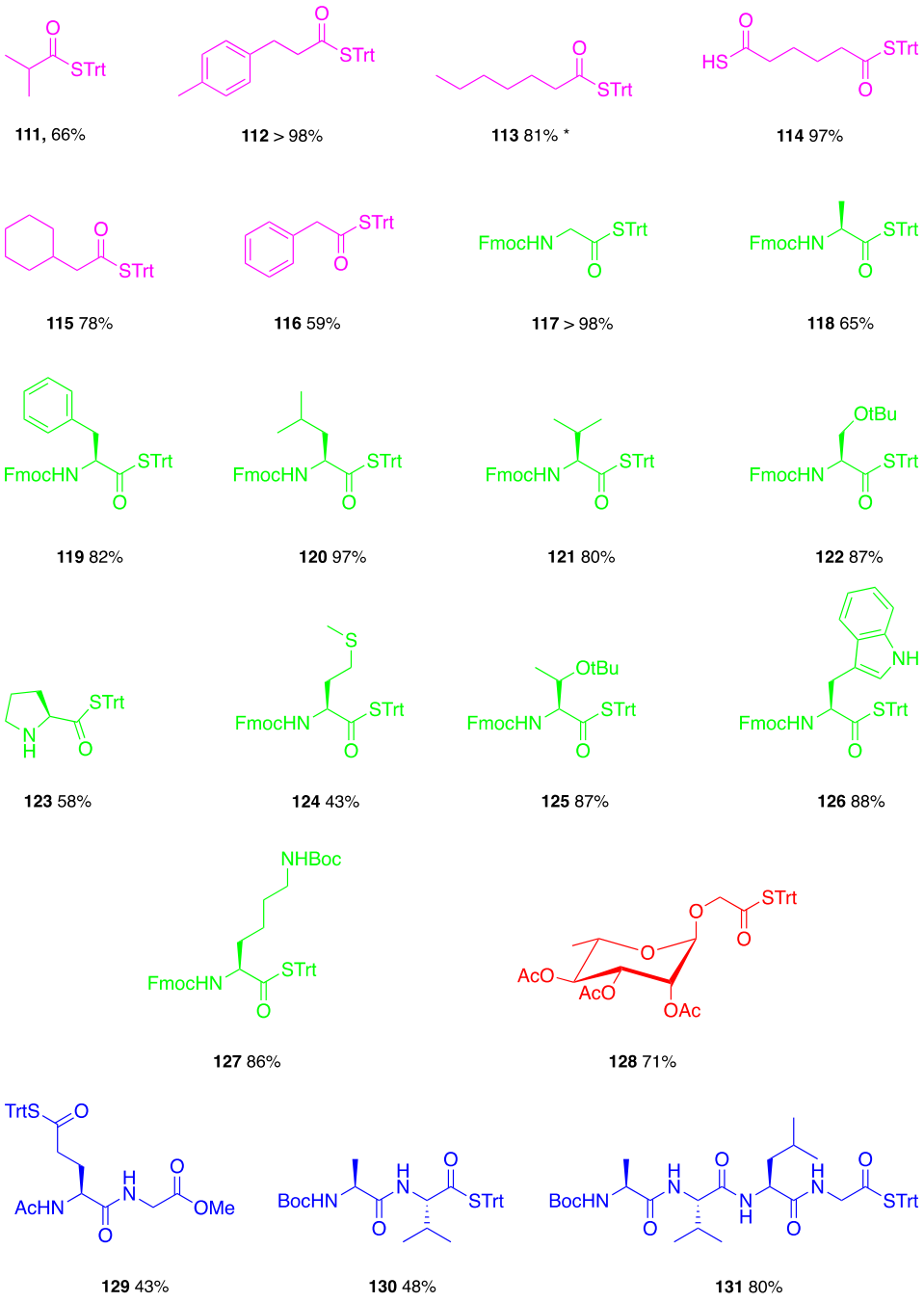


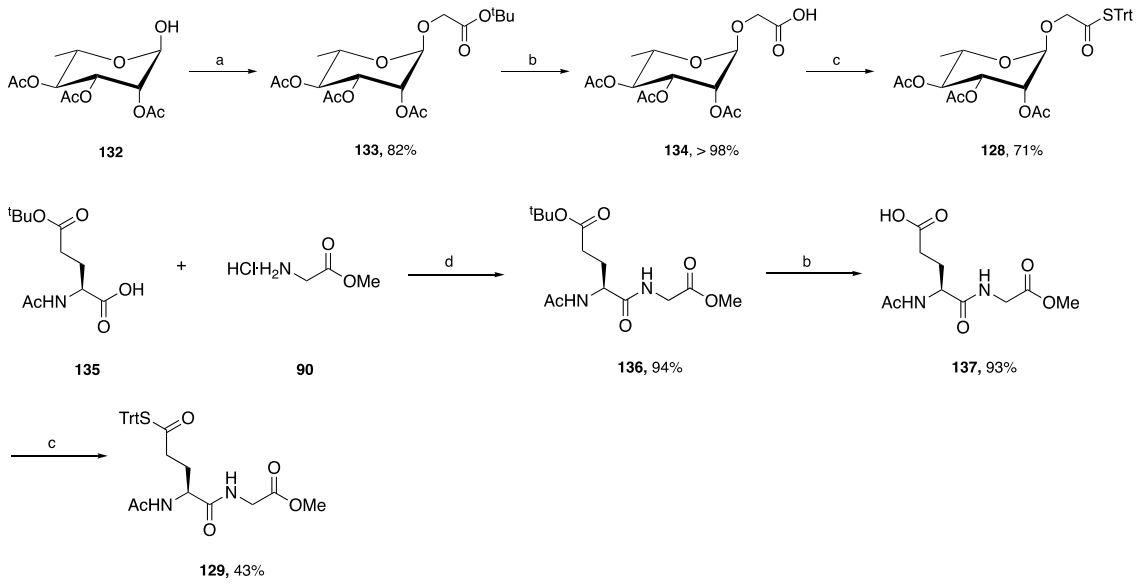
Figure 3.3: Synthetic scope of the preparation of *S*-trityl thioacids. Compounds **111-116** (pink) consist of common aliphatic and aromatic carboxylic acids. Compounds 11**7-131** demonstrate the utility of the reaction on biomolecules, including amino acids (green), L-rhamnose derivative **128** (red) and di- and tripeptides (blue), where the *S*-trityl group was installed at both the side chain and *C*-terminus. *Compound **113** was synthesised by another member of the Scanlan group, Dr. Helen Burke.

It is evident from Figure 3.3 that the thioesterification of carboxylic acids using trityl thiol is a high yielding, robust and general route to protected thioacids. Superior yields were obtained using many of the carboxylic acid starting materials, in particular

for the substrates **111-115**. Likewise, most of the peptidyl precursors were converted to *S*-trityl protected thioacids with great success, with some of the lower yields pertaining to the peptides or amino acids with slightly more steric hindrance, leading to less accessible carboxylic acids. Moreover, the method does not require a work-up and the products are exceptionally suitable for column chromatography.

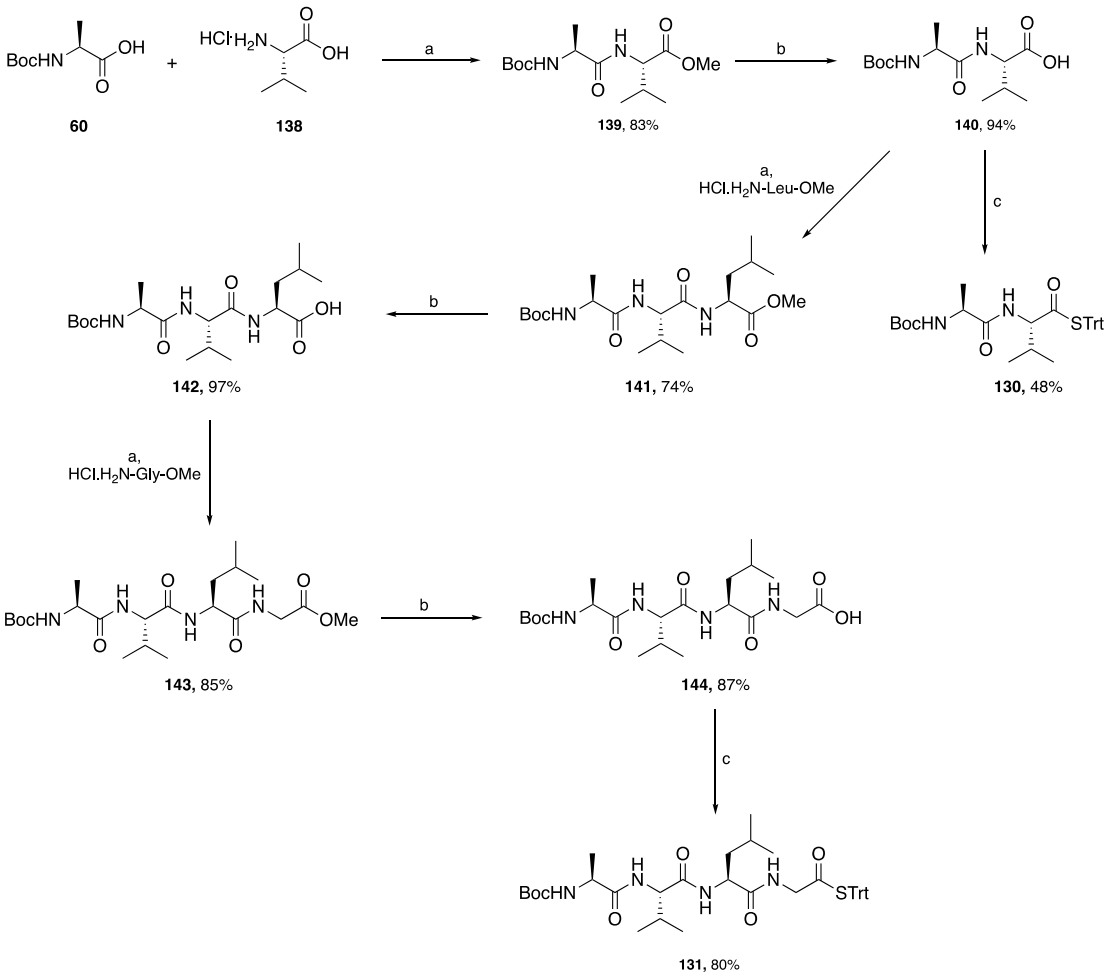
3.7.2 Synthesis of the precursors to compounds 128-131

In the case of compounds **128-131**, precursors were synthesised in minimal steps from commercially available materials (Scheme 3.21). **128** was prepared from partially acetylated L-rhamnose, which was treated with *tert*-butyl 2-bromoacetate and K_2CO_3 to afford **133**. The *tert*-butyl protecting group of the carboxylic acid was then cleaved using TFA and the free acid **134** was converted to **128**. For the synthesis of dipeptide **129**, Ac-Glu(O^{t}Bu)-OH was coupled with Gly-OMe hydrochloride using standard coupling conditions, followed by the deprotection of the *tert*-butyl protecting group with TFA and subsequent coupling with trityl thiol to furnish **129**.



Scheme 3.21 Synthesis of *S*-trityl precursors **128** and **129**; a) 2-bromo-*tert*-butylacetate, K_2CO_3, DMF, rt, 48 h; b) TFA (25%), CH_2Cl_2, rt, 2 h; c) $SHCPh_3$, EDCI·HCl, DMAP, CH_2Cl_2, rt, 16 h; d) EDCI·HCl, HOBt, DIPEA, CH_2Cl_2, DMF, 0 °C-rt, 16 h.

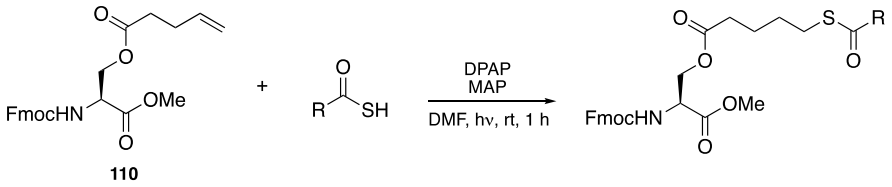
Compounds **130** and **131** were synthesised using standard coupling conditions (Scheme 3.22), starting with the coupling of Boc-Ala-OH **60** and Val-OMe hydrochloride **138**, **139** was formed in 83% yield and hydrolysed to the carboxylic acid using Cs_2CO_3 in MeOH:H_2O to furnish **140**, which was then converted to the *S*-trityl protected dipeptide **130**. **140** was also used to synthesise **141** through coupling with Leu-OMe hydrochloride. **141** was subsequently hydrolysed to **142** and coupled to Gly-OMe hydrochloride to afford the tetrapeptide **143** in 85% yield. **143** was hydrolysed using Cs_2CO_3 in MeOH:H_2O and coupled to trityl thiol to furnish the tetrapeptide **131**. Attempts were also made to convert the tripeptide **142** to the *S*-trityl protected analogue, however coupling reactions with **142** and tritylthiol resulted in very poor yields, possibly due to steric hindrance of the two bulky groups. In addition, the tetrapeptide Boc-Ala-Val-Leu-Ala-*S*-Trt was also produced, using the same procedures as for **131**, but in a disappointing yield and due to its extremely poor solubility it was not brought through for further experiments. The use of a mixture of DMF and CH_2Cl_2 as the reaction solvent allowed for a much higher yield for **131** than for the corresponding dipeptides **129** and **130**.



Scheme 3.22: Synthetic route for the preparation of *S*-trityl thioacids **130** and **131**; a) EDCI·HCl, HOBt, DIPEA, CH_2Cl_2, DMF, 0 °C-rt, 16 h; b) Cs_2CO_3, MeOH:H_2O (1:1), rt, 16 h; c) $SHCPh_3$, EDCI·HCl, DMAP, CH_2Cl_2, rt, 16 h.

3.7.3 Synthesis of thioesters

Thioacids **111-131** were then employed in TEC with **110** to form the corresponding thioesters (Scheme 3.23). These reactions were carried out under standard thiol-ene ligation conditions utilised in the Scanlan group. Accordingly, thioacid donors were deprotected using TFA and TES for 5 min and the crude products were concentrated *in vacuo* to dryness. Given the instability previously experienced with thioacids, the crude products were used without further purification. The alkene acceptor **110**, DPAP and MAP were added to the degassed flask and solubilised in DMF. The reaction flask was irradiated with 365 nm UV light for 1 h at rt. The solvent was removed *in vacuo* or through multiple brine washes and the products were purified by column chromatography.



Scheme 3.23: Intermolecular thiol-ene addition of thioacids onto alkene **110**.

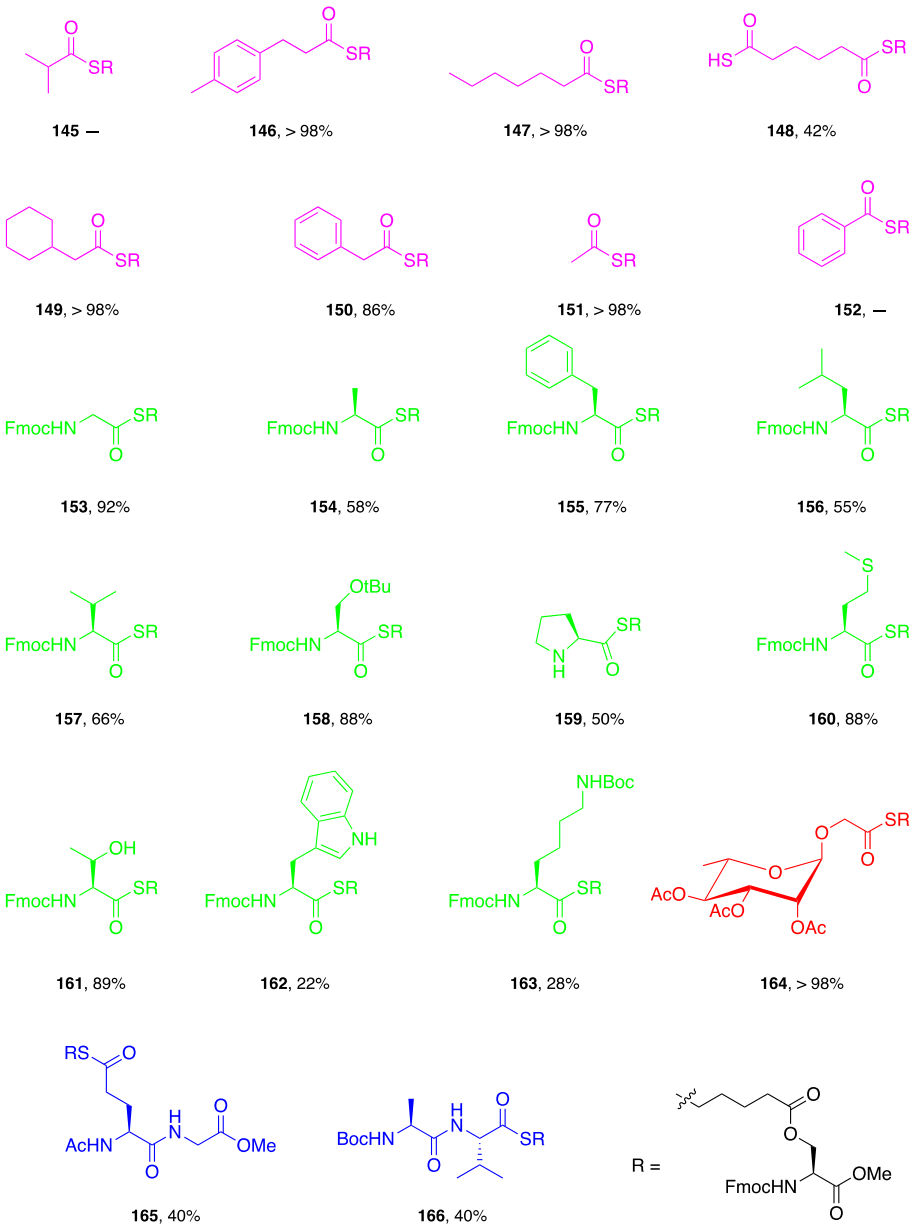


Figure 3.4: Synthetic scope of thioesters prepared *via* TEC. Compounds **145-152** (pink) consist of common aliphatic and aromatic carboxylic acids. Compounds **153-166** demonstrate the utility of the reaction on biomolecules, including amino acids (green), L-rhamnose derivative **164** (red) and di- and tripeptides (blue), where the *S*-trityl group was installed at both the side chain and *C*-terminus.

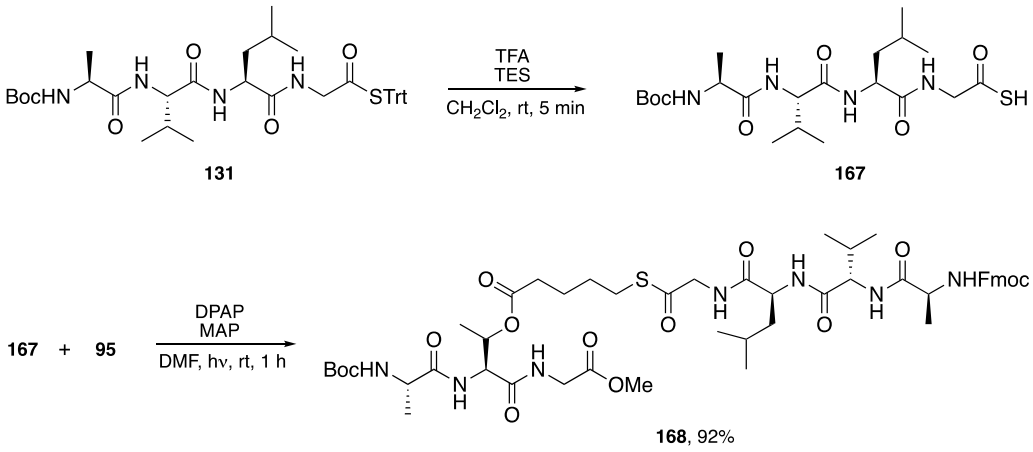
The utility of this reaction has shown remarkable success for the preparation of thioesters through a mild and high-yielding reaction. The majority of the non-biomolecular examples (pink) were obtained in very high yield or quantitatively, with the exceptions being the smaller molecules **145** and **152**. In the crude reaction mixture of the TEC with **111**, only alkene starting material was observed. Upon purification of this mixture, no isobutyric derivatives were found and it was therefore concluded that the thioacid furnished after trityl-deprotection of **145** was volatile and was lost during rotary evaporation prior to TEC. Similarly, the product of the thiobenzoic TEC was also not found following purification techniques and the alkene starting material was the only compound recovered. In this case, however, it is likely that the CSO elimination previously experienced was favoured during this reaction, as it would create a very stable benzyl radical which would diminish the thiol-ene ligation. A modest yield of 42% was obtained for the addition of thioadipic acid, which was deemed as satisfactory considering this was a dual-ligation reaction.

Each α-amino thioacid behaved differently during TEC. This was due to the side-chain, which largely depicts the properties of the amino acid. For example, it was observed that the residues that contain a heteroatom on the side-chain, yet close to the peptide backbone (**158**, **160**, **161**), were excellent thiyl radical donors. It is possible that the heteroatom disfavours the elimination of CSO and hence, drives thiol-ene ligation. Since these examples were carried out with 3 eq. of thioacid, Ser ligation was repeated with only 1 eq. of Ser-SH and the product was furnished in 38% yield. Modest yields were obtained when larger amino acids were used, such as Leu, Trp and Pro (**156**, **159**, **162**), which have bulky side-chains and in the case of Pro, a more rigid structure. Given the small size of Ala and in comparison to the yield of the Gly ligation, it was surprising that Ala resulted in 58% yield. After further investigation, it was observed that increasing the equivalents of thioacid to 5 eq. led to a higher yield of 81%, and decreasing to 2.5 eq. gave a 47% yield. It is therefore evident that by altering equivalents, this reaction can be fine-tuned to maximise product depending on the specific residue of thioacid.

Dipeptide examples **165** and **166** demonstrated again the effect of steric hindrance on this model reaction. The Ac-protected dipeptide example showed the utility of the side chain as an *S*-ligation site, however in a modest yield of 40%. The *C*-terminal thioacid was also a significant example, as this would demonstrate the overall

purpose of the process, which is to form a native peptide bond. **166** was furnished in a 40% yield, however the terminal Val must be considered here, as it also contains a bulky side chain. Gratifyingly, the carbohydrate example **164** was high-yielding and shows potential for a viable route to carbohydrate conjugation onto other biomolecules.

Encouraged by the success of the scope of TEC on amino acids and peptides, it was envisaged that the tetrapeptide **131**, prepared as part of the range of the thioacids in Figure 3.3 could be utilised to demonstrate this methodology on larger substrates, including a larger alkene acceptor. Therefore, we carried out thiol-ene ligation with **131** and **95** to form the largest thioester in this study, comprising of 7 amino acids.



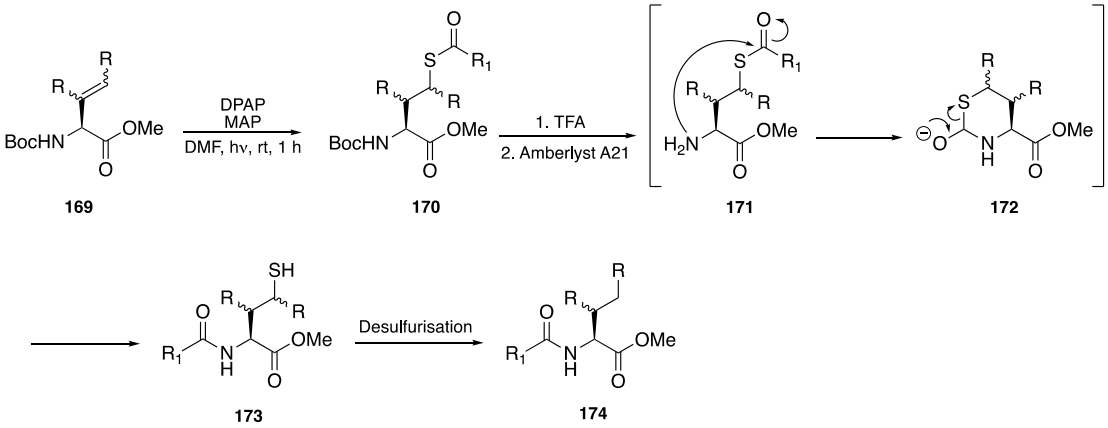
Scheme 3.24: Synthesis of **168** through the intermolecular thiol-ene ligation of **167** and **95**.

Pleasingly, this reaction proceeded well and the desired product **168** was obtained in a 92% yield. Given the relatively low yields obtained for the dipeptide examples in Figure 3.4, the yield of **168** was exceedingly high. It is possible that the side chain of the residue bearing the thioacid can hinder the addition onto the alkene, even when the alkene is easily accessible. For the formation of thioester **166**, the residue bearing the thioacid was Val, which contains a bulky isopropyl side chain. By using Gly to provide the thioacid, the steric hindrance was reduced and the efficiency of thiol-ene ligation was greatly improved. In addition, it is possible that the improvement in yield was due to lack of either hydrolysis of the thioacid or CSO elimination. As previously mentioned in Chapter 1, hydrolysis can occur through cyclisation of the peptide, where H_2S is released and the addition of water or base can ring-open the by-product to the

hydrolysed peptide or amino acid. However, if the cyclisation was hindered, perhaps by the length of the peptide and steric bulk of the residues, hydrolysis may not be as prevalent. This particular topic was not explored during this project, however more research into the hydrolysis of thioacids is on-going in the Scanlan group.

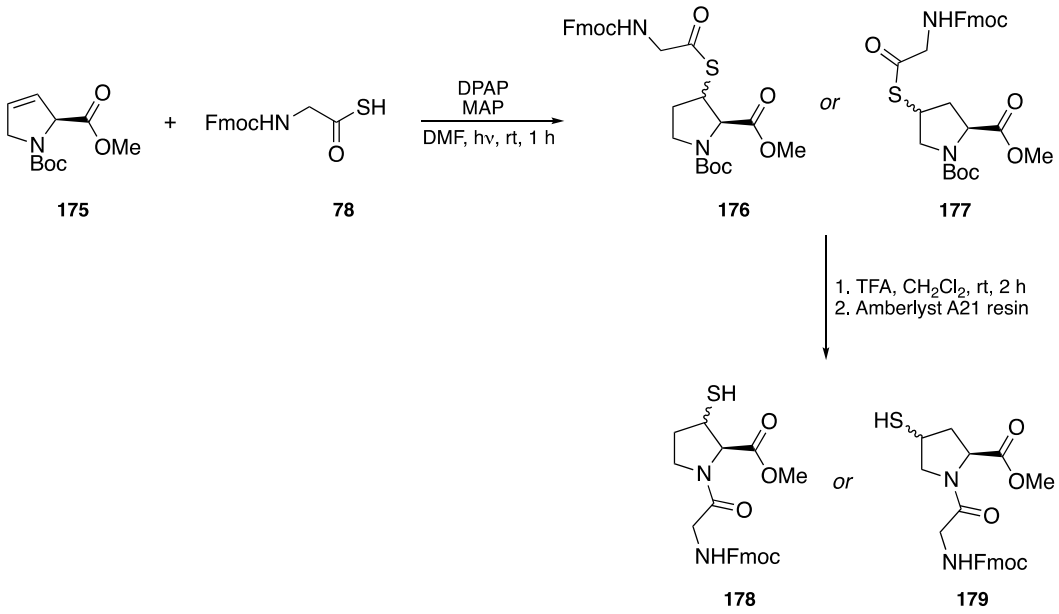
3.8 β-γ unsaturated acceptors

As described in Section 3.6.1, the *S,N*-acyl transfer was prohibited by the formation of a 14-membered cyclic transition state. Despite the reports on ring size and the possibilities of larger rings undergoing *S,N*-acyl transfer, it was decided to focus our efforts on a scaffold that would induce a smaller, more efficient ring size.



Scheme 3.25 Proposed route to native peptides through TEMPLE using β,γ-unsaturated amino acids.

Accordingly, we sought to develop an alkene acceptor that following thioester formation, would lead to a 6-membered intermediate and drive the acyl transfer. It was therefore postulated that β,γ-unsaturated amino acids would provide the olefin in a suitable position for the thioester to undergo the acyl transfer (Scheme 3.25). Unfortunately, reported examples of the synthesis of a range of β,γ-unsaturated amino acids did not exist and the process seemed undiscovered. It is likely that the reluctance to prepare these compounds in the past has arisen from the challenges presented in retaining the stereochemical integrity of amino acids. After extensive research into the literature, it was found that two amino acids were viable for inexpensive synthesis or were commercially available: dehydroproline (Dhp) and dehydrovaline (Dhv).

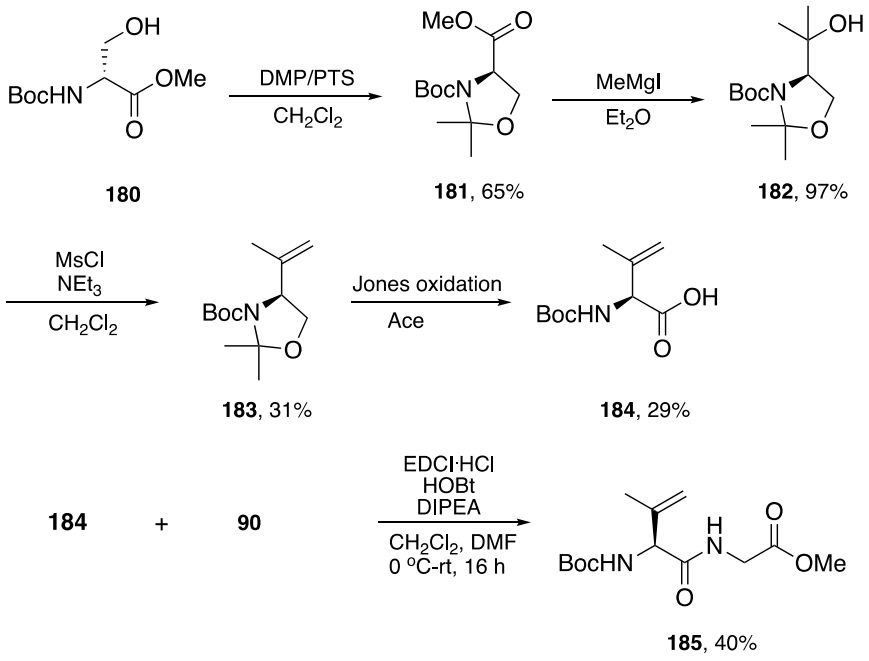


Scheme 3.26: Proposed strategy for the synthesis of dipeptides **178** and **179**.

It was hypothesised that the commercially available 3,4-dehydro-L-proline would easily undergo thiol-ene ligation with an Fmoc-protected thioacid (**78**) and following deprotection of the amine, acyl migration would occur. Having encountered previous trouble with the HCl salts of various alkene acceptors, Amberlyst A21 free base resin was utilised by other members of the Scanlan group and was successful in providing the free amine of amino acids and peptides. For the synthesis of the Boc-protected methyl ester, 3,4-dehydro-L-proline was stirred for 16 h with AcCl and MeOH to form the methylester and was subsequently reacted with base and Boc_2O to protect the amine. The free amine of **175** was initially intended as the acceptor for the process, however the HCl salt formed after the methylation step required could not be isolated. The mixture was therefore taken and reacted with Boc_2O and NEt_3 to furnish **175** in a 75% yield, which was purified by column chromatography and irradiated in a UV oven for 1 h with Fmoc-Gly-SH **78**, DPAP and MAP. ^{1}H NMR analysis of the crude material showed that not all of the alkene had been consumed and there was a ratio of 2:1 thioester:alkene. The mixture was treated with TFA (25%) until TLC showed deprotection of the Boc protecting group. It was then stirred with Amberlyst A21 free base resin for 2 h, during which TLC showed the disappearance of a polar, base line spot and the appearance of a more non-polar compound, presumably the free

amine of the thioester. Unfortunately, after allowing time for the *S*,*N*-acyl transfer to occur, ^{13}C NMR still showed the presence of a thioester carbonyl. After several more attempts and still no progress in obtaining dipeptides **178** or **179**, this route was abandoned. The structure and rigidity of Pro means that the amine is on the opposite side of the ring as the *S*-acyl group, hence, it is possible that the transfer is not favoured as the location of the acyl-donor and acyl-acceptor are not close enough. Moreover, the route to the starting material **175** was not atom economic or cost-efficient.

Simultaneously, other members of the Scanlan group were pursuing the synthetic route to Dhv (Scheme 3.29), which was reported by Shin and co-workers.[367] This route was the only example found in literature to afford the β,γ-unsaturated amino acid without the use of expensive chiral catalysts.



Scheme 3.27: Synthesis of dipeptide containing Dhv.

To begin with, D-Ser-OMe was converted to the DMP-protected analogue to tie up the Boc-amine and the free alcohol on the side chain. Grignard reaction with MeMgI introduced two methyl groups in high yield, followed by the β-elimination of the hydroxyl to install the unsaturated side chain using methanesulfonyl chloride (MsCl) and NEt_3. Jones oxidation converted **183** to the free carboxylic acid **184** through deprotection of the DMP group and subsequent oxidation of the primary alcohol to

carboxylic acid. The procedure reported by Shin *et al*. delivered the free carboxylic acid of Dhv, however we required the product with the protected carboxylic acid. Due to the Boc-protected amine, the use of AcCl in MeOH to afford the methyl ester was not applicable. In addition, the presence of the alkene restricted other methods of installing the methyl group, such as MeI and a suitable base. To avoid this, **184** was coupled to Gly-OMe hydrochloride **90** in order to mask the free carboxylic acid.

While this route to Dhv was efficient in some steps, other steps were not as successful when scaled-up. The β-elimination using MsCl presented problems such as a low yield and by-products being formed. The chromium source used for the Jones oxidation was difficult to remove on larger scale, which created purification issues with chromium contaminants brought through to the amino acid coupling.

It was therefore decided that other members of the Scanlan group would continue the research into the synthesis of Dhv with the intent of improving the yields of the cumbersome steps involved and providing large amounts of Dhv-peptides for further research into the TEMPLE methodology.

3.9 Conclusions

Thiol-ene mediated peptide ligation has been thoroughly studied in this work. The thiol-ene radical reaction has been extensively utilised to provide thioesters on peptides and amino acids. This method explores the capacity of α-amino thioacids to act as thiyl radical donors in order to furnish thioester isopeptides, a possibility that has never been examined before in peptide ligation. We have outlined successful synthetic steps towards a wide range of biomolecular and non-biomolecular thioacids and moreover, we have effectively converted the thioacids to peptidyl thioesters through radical means and mild conditions. Notably, a 7-mer isopeptide was assembled in a 95% yield through the thiol-ene ligation of a tetrapeptide thioacid and an alkene-containing tripeptide. This example authenticates the radical thiol-ene reaction as a neat, high yielding route to thioesters from simple alkenes and trityl-protected thioacids.

Despite the high success of thiol-ene ligation of thioacids onto di- and tripeptide alkenes, unfortunately native peptide bond formation was not achieved. To begin with, TEC onto the alkene acceptor with a free terminal amine was carried out. Unfortunately, owing to the counter ions of the salt of the peptide, the amine was not available for

intramolecular *S*,*N*-acyl transfer. Attempts to neutralise the amine and remove the salt were plentiful, both pre- and post-TEC, however they were unsuccessful and the acyl migration did not occur. This was the case using both dipeptide and tripeptide alkene acceptors, where only the thioester was observed. In addition, intermolecular acyl migrations were attempted, where an amino acid with a free *N*-terminus was introduced to the thioester previously formed through TEC, in order to furnish a free thiol on the peptide. To our dismay, no reaction took place and these experiments did not provide the peptidyl thiol. To ensure that the thioester was cleavable by an amine, it was stirred with benzyl amine and, pleasingly, the acyl functionality was migrated onto the benzyl amine. Following on from this result, it was clear that the thioester was capable of undergoing acyl migration, provided that the incoming amine was nucleophilic enough. Phosphate buffer at pH 8.5 was then employed to free base the *N*-terminus of the thioester, which successfully afforded the free amine for the first time. Disappointingly, however, still no *S*,*N*-acyl transfer occurred, according to the ^{13}C NMR, which continued to show the thioester carbonyl peak. This result led to the conclusion that the 14-membered cyclic transition state that would be formed during *S*,*N*-acyl transfer was not favoured and could not be driven to form the native peptide bond in our hands.

Moving forward, it was hypothesised that peptides with different positions of unsaturation would subsequently form smaller cyclic transition states for intramolecular *S*,*N*-acyl transfer. β,γ-unsaturated amino acids we explored as potential alkene acceptors for TEMPLE. 3,4-dehydro-L-proline was obtained through commercial sources and converted to the corresponding *N*-Boc-protected methylester and subjected to TEC with thioglycine. Unfortunately, following treatment with TFA and Amberlyst A21 basic resin, no acyl migration was observed. It is possible that this is due to the rigid structure of Pro and the inadequate positioning of the amine to the acyl group. Dhv was prepared from D-Ser-OMe and was coupled to Gly-OMe hydrochloride to furnish a Dhv-containing dipeptide. However, the synthesis of the dehydrovaline monomer is in need of optimisation to procure more starting material in order to investigate its potential as a TEMPLE acceptor.

Due to the success of the *S*,*N*-acyl transfer carried through 14-member cyclic transition states in the literature,[261-263, 361] future work should endeavour to further evaluate the factors affecting this reaction and the factors that could be exploited to drive the rearrangement. For example, computational analysis could be sought to gain

further understanding of the molecule and its conformational freedom. Moreover, future work in this subject should focus on the preparation of alkene substrates that will allow a small enough cyclic transition state to form during intramolecular acyl transfer. This would encompass many possibilities, including the development of new synthetic routes to β,γ-unsaturated amino acids, the investigation of the scope of thiol-ene ligation of thioamino acids onto these acceptors and the formation of native peptide bonds through intramolecular *S*,*N*-acyl transfer.

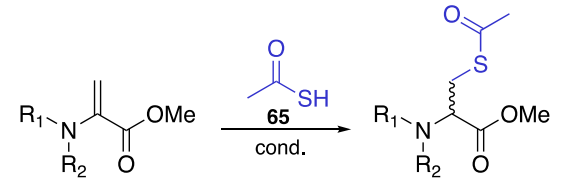
4.0 Introduction

As discussed in Chapter 3, the ring size and flexibility of the *transition state* intermediate is essential to the success of the intramolecular *S*,*N*-acyl transfer. The work of Katritsky *et al*.[261-264, 361] described the ring sizes of the intermediate that allow for acyl transfer to occur, from conventional 5- to 19-membered transition states macrocycles. Despite these encouraging reports, in the context of our TEC mediated thioester synthetic methodology, the 14-membered transition state intermediate did not successfully promote intramolecular acyl migration from the sulfur atom to the amine. Although the thioester residue could be readily installed in high yield, the formation of the cyclic intermediate obtained during the acyl migration step appeared to be kinetically and thermodynamically unfavoured, resulting in a mixture of intra- and intermolecular adducts. The formation of the amidic bond was, in this case, not favoured, therefore this strategy showed its limitation in forging novel isopeptide bonds. Based on these considerations and experimental results, the insertion of the double bond directly on the alpha carbon of the desired amino acid was envisaged as an alternative strategy; following the thioesterification step, the α,β-unsaturation would allow the formation of a 5-membered cyclic intermediate, which would be expected to undergo rapid *S*,*N*-acyl transfer. Indeed, NCL, which represents the gold standard for chemical protein synthesis, proceeds through the formation of such a 5-membered cyclic intermediate. To this aim, the α,β-unsaturated amino acid dehydroalanine (Dha) was evaluated as an unsaturated electrophilic residue for the development of a novel ligation methodology. Dha is a non-natural amino acid, widely explored for Cys-linked conjugation and general protein modification.[368-373] Its ionic Michael acceptor-like properties allow for a very reactive alkene, which could react with several sulfur-containing nucleophiles, resulting in the generation of alkyl cysteine analogues. Moreover, Dha could potentially offer an excellent substrate to develop a new ionic-mediated TEMPLE methodology. Notably, the process would render a Cys residue post-acyl migration, which could be easily desulfurised into the more naturally abundant Ala in a manner analogous to the NCL-desulfurisation route.

4.1 Previous work on Dha in the Scanlan lab

Within the Scanlan group, Dr. Rita Petracca has recently investigated the use of Dha as a Michael acceptor for thioester synthesis. As the first step, several *N*-protecting groups and reaction conditions to drive the ionic reaction were evaluated (Table 4.1). Thioacetic acid was selected as the nucleophilic thiol for this optimization process, it being commercially available and easily removed by evaporation *in vacuo* from the reaction mixture. As showed in Table 4.1, no conjugate addition was observed with the mono-protected substrates (Entries 1-5, 8, 12 and 15). However, when dual *N*-protection was introduced, the desired thioester **187** was obtained in good to excellent yields (Entries 6-7, 9-11, and 13-14). Addition of a catalytic quantity of an organic base resulted in a marked acceleration of the reaction time to achieve full conversion at room temperature. Optimum results were obtained using THF as a solvent at room temperature for 5 hours in the presence of catalytic NEt_3, with quantitative recovery of the thioester as the only product after purification (Entry 14). Among the protecting groups screened, the *bis*-Boc-protection (**186:d**) undoubtedly fulfilled our requirements for peptide ligation and was therefore selected for further methodology development and the related scope expansion. The plausible role of the second *N*-protecting group in assisting the thia-Michael addition could be envisaged and it is currently under investigation in our laboratory.

Table 4.1: Optimisation of the ionic thiol-ene addition of thioacetic acid onto Dha



186
a: R_1 = Boc, R_2 = H
b: R_1 = Boc, R_2 = Ac
c: R_1 = Ac, R_2 = H
d: R_1 = Boc, R_2 = Boc
e: $R_1 = N_2$

187
a: R_1 = Boc, R_2 = H
b: R_1 = Boc, R_2 = Ac
c: R_1 = Ac, R_2 = H
d: R_1 = Boc, R_2 = Boc
e: $R_1 = N_2$

Entry	Dha (186)	SAc (eq.)	Base	Solvent	Time (h)	Temp. (°C)	Yield (%)
1	**186:a**	4.0	-	$CHCl_3$	12	rt	no conv.[a]
2	**186:a**	4.0	K_2CO_3	$CHCl_3$	24	rt	no conv.[a]
3	**186:a**	1.2	NEt_3 cat.	DMF	96	rt	no conv.[a]
4	**186:a**	4.0	NEt_3 (4 eq.)	H_2O	96	rt	no conv.[a]
5	**186:a**	10	-	toluene	16	reflux	no conv.[b]
6	**186:b**	10	-	toluene	16	reflux	50[c]
7	**186:b**	20	-	toluene	20	reflux	87[c]
8	**186:b**	10	NEt_3 cat.	DMF	16	140	no conv.[a]
9	**186:b**	4.0	NEt_3 cat.	DMF	16	rt	84[d]
10	**186:b**	6.0	NEt_3 (6 eq)	H_2O	16	rt	53[d]
11	**186:b**	4.0	NEt_3 cat.	THF	2	rt	89[c]
12	**186:c**	4.0	NEt_3 cat.	DMF	16	rt	no conv.[b]
13	**186:d**	10	NEt_3 cat.	toluene	16	reflux	99[d]
14	**186:d**	4.0	NEt_3 cat.	THF	4	rt	99[d]
15	**186:e**	4.0	NEt_3 cat.	THF	4	rt	no conv.[b]

[a] Only the presence of the starting Dha was observed by crude ^{1}H-NMR. [b] Conversion of the starting Dha into a degradation product observed by crude ^{1}H-NMR. [c] Inseparable mixture with the starting Dha. [d]Isolated yield.

Following on from these results, the *S,N*-acyl transfer was tested in order to confirm the potential of the Dha-mediated strategy in delivering new native amide bonds. Following thioacid addition onto the Dha, the deprotection of the Boc protecting groups on **188** was carried out to afford the corresponding TFA salt, which was subsequently stirred in CH_2Cl_2 at rt in the presence of Amberlyst A21 basic resin to neutralise the amine thus promoting the acyl transfer. Gratifyingly, the *S*-acetyl group migrated to the free amine to furnish **193** in only 20 min, as confirmed by the different chemical shift values of the Cys methylene and the methyl of the acetyl group in the ^1H NMR spectrum. As reported in the Section 3.7.1, a range of trityl protected α-amino thioacids could be easily obtained from the carboxylic acid derivatives (Figure 3.3). A selection of these were converted into thioesters upon treatment with the protected Dha (Figure 4.1) and then subjected to the *S,N*-acyl transfer process in order to investigate the scope of the methodology for peptide bond formation (Scheme 4.1).

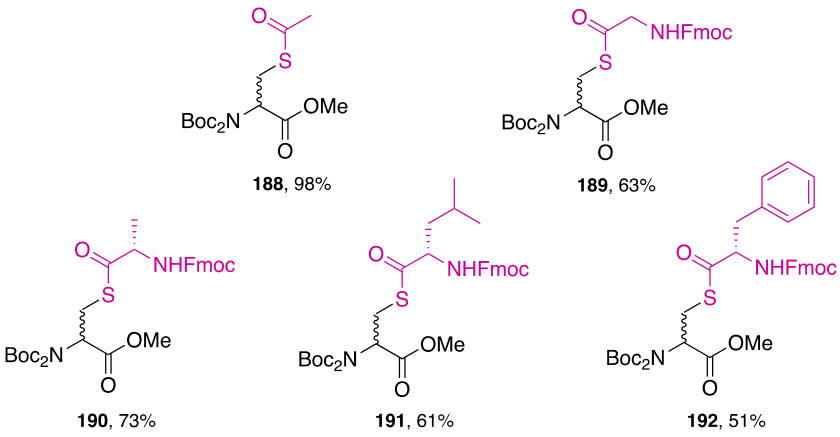
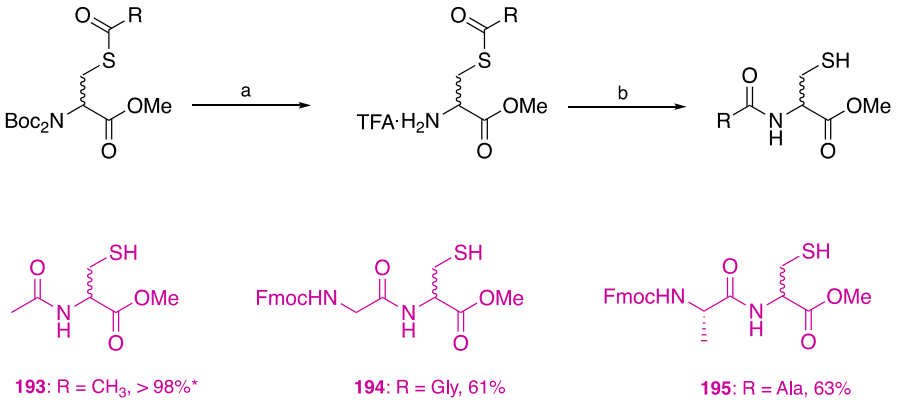
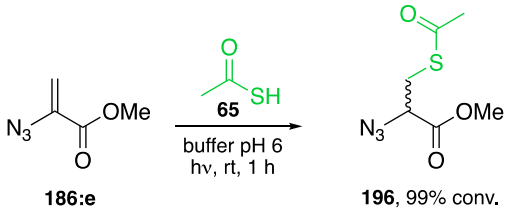


Figure 4.1: Thioesters prepared *via* thia-Michael addition onto protected Dha.



Scheme 4.1: Deprotection of *bis*-Boc-thioesters prepared through ionic methods and subsequent *S*,*N*-acyl transfer to produce **193** and dipeptides **194** and **195**. a) TFA/CH_2Cl_2, rt, 1 h; b) Amberlyst A21, rt, 20 min. *Conversion by ^{1}H NMR.

In a related study, the radical addition of thioacetic acetic onto an azido-protected Dha monomer was carried out, following optimisation of possible *N*-protecting groups, and demonstrated significant potential for a radical-mediated TEMPLE approach to deliver native peptide bonds. Commercially available thioacetic acid was subjected to UV irradiation under a nitrogen environment with **186:e** and solubilised in aq. ammonium buffer at pH 6 (Scheme 4.2). Surprisingly, despite the absence of any of conversion in the ionic example (Table 4.1, entry 15), the thioester **196** was afforded with 99% conversion, determined by ^{1}H NMR. This example of TEC onto Dha could provide a useful alternative to thia-Michael addition and was further explored in this chapter.



Scheme 4.2: Preparation of thioester **196** *via* radical-medtiated thiol-ene of azido-protected Dha and thioacetic acid.

4.2 Aims of this work

Owing to the success of the steps taken so far to introduce thioesters through the employment of Dha, the extension of this strategy for chemical protein synthesis was envisaged. Unfortunately, due the challenges encountered with the *S,N*-acyl transfer in Chapter 3, the investigation into macrocyclic transition states was not carried forward. Gratifyingly, the smaller 5-membererd cyclic transition state intermediate circumvented these challenges, thus providing an effective route to native peptide bonds through acyl migration. In this chapter, we further explored the utility of Dha as an acceptor towards the completion of the TEMPLE protocol (Figure 4.2).

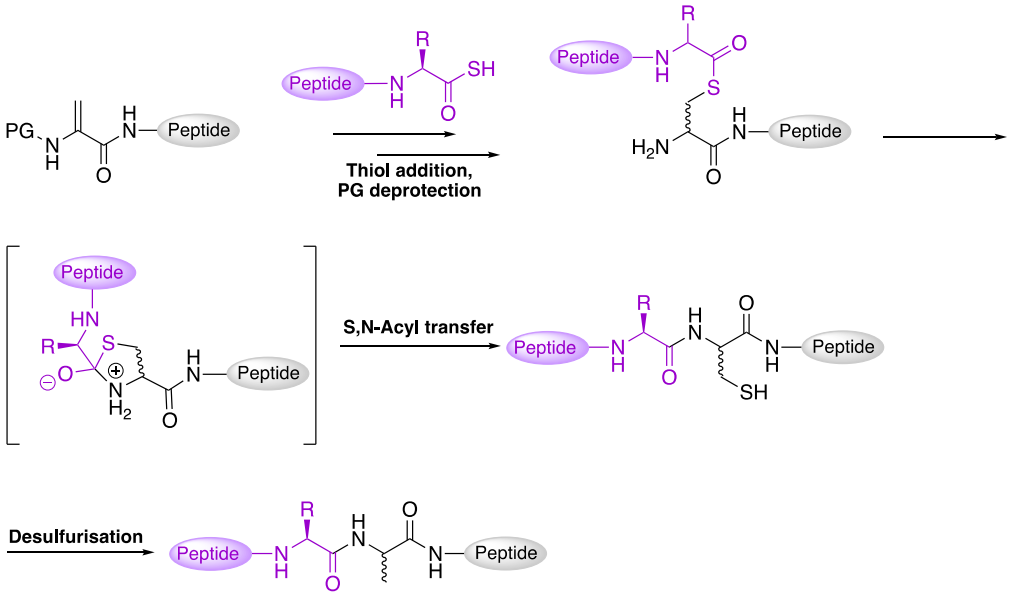


Figure 4.2: Aim of this work: thiol addition of α-amino thioacid onto *N*-protected Dha to furnish a thioester. Subsequent conversion to the free amine allows for *S,N*-acyl transfer to afford the native peptide bond. Subsequent desulfurisation of Cys to Ala provides a more naturally abundant amino acid at the ligation site.

The major goal of this project was the preparation of a small protein by using TEMPLE methodology. For this purpose, we required a small protein that is compatible with SPPS, to build the peptide fragments, and that contains Ala residues for potential ligation sites. The latter requirement is not a challenge given the natural frequency of Ala, however as many small proteins are involved in transmembrane roles, they are not compatible with SPPS due to their lipophilicity. To this end, GLP-1 (Glucagon-like

peptide amide 7-36) (Figure 4.3) was selected as the ideal target protein. GLP-1 is a processed segment from pre-proglugagon, a molecule that promotes the secretion of insulin upon specific receptor activation;[374, 375] it is therefore an important target of research for the treatment of diabetes and obesity.[376, 377] It is noteworthy that the beginning His-Ala sequence is a possible ligation site. This region of the peptide is frequently studied and altered, as the first 4 residues are responsible for receptor activation and binding.[378] By using our methodology, the first residue (His) can be easily modified for receptor binding and activation studies by simply using a different α-amino thioacid for thiol-ene ligation.

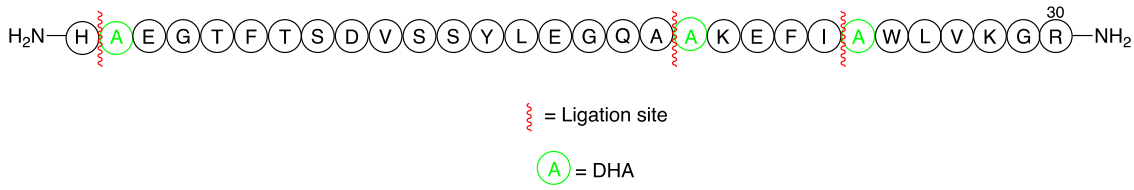
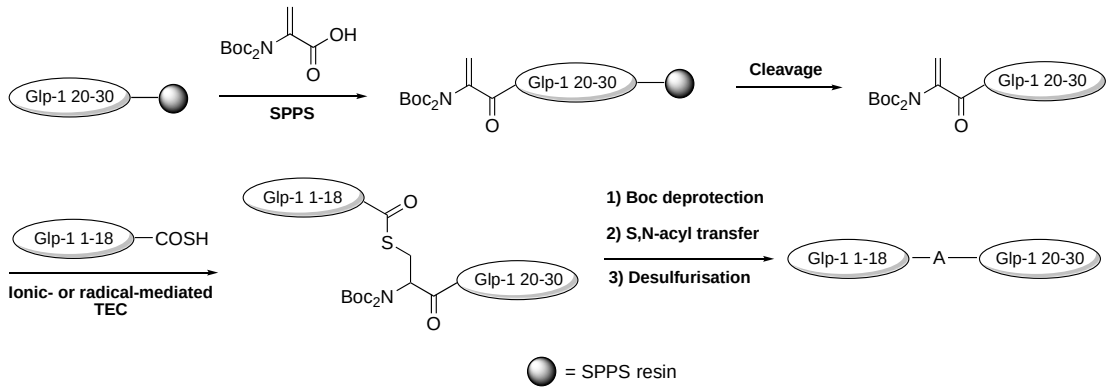


Figure 4.3: Glp-1 sequence including possible ligation sites and locations of Dha that will consequently be converted to Ala.

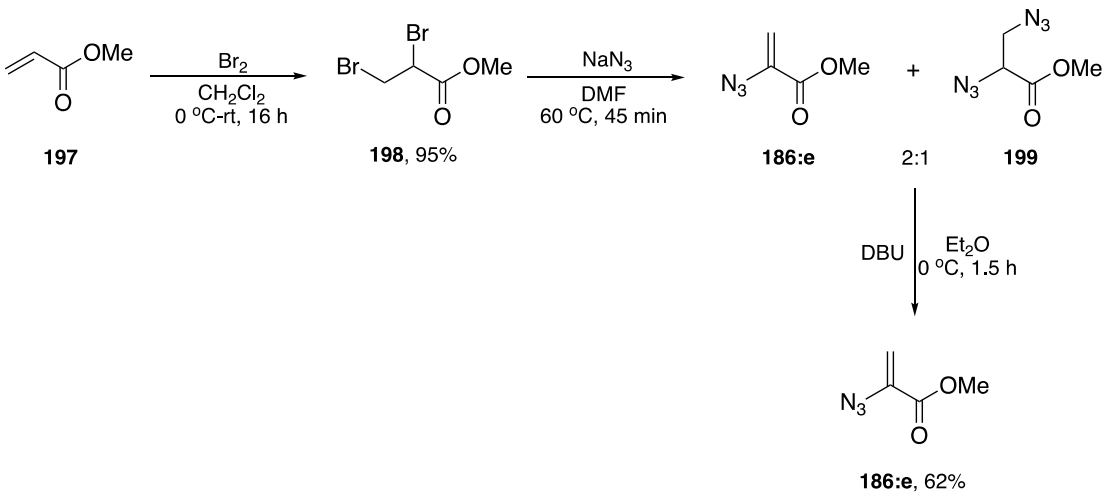
We therefore set out to investigate the synthesis of GLP-1 using the modified TEMPLE ligation methodology at a number of varying ligation sites, however first demonstrating the methodology on smaller ligations using monomers and dipeptides.



Scheme 4.4: Synthetic work plan towards the chemical ligation of Glp-1 by the novel TEMPLE strategy.

4.3 Synthesis and TEC of azido-Dha

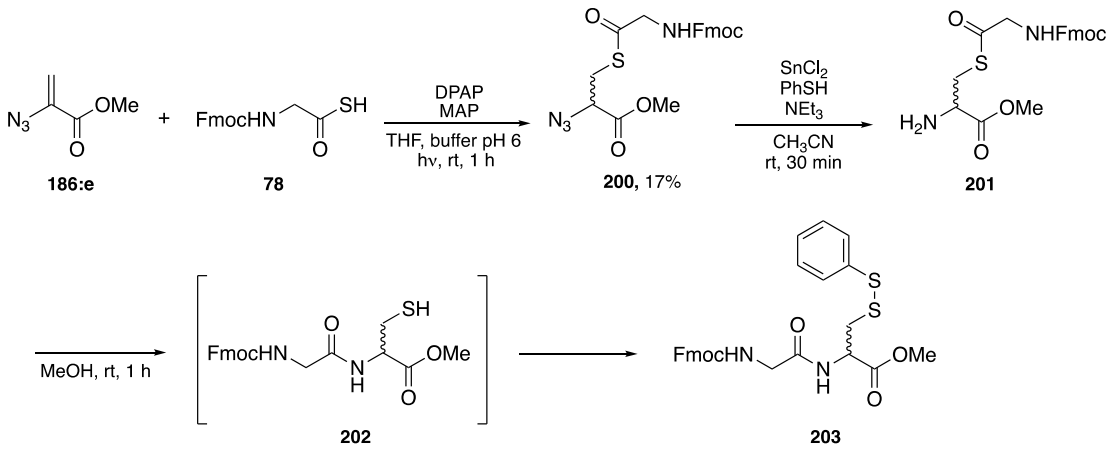
Previous work carried out on the addition of thiols onto Dha showed that both *N*-acetyl- and azido-Dha were successful in radical-mediated ligation reactions. In addition, the azido-Dha provided new opportunities in the orthogonality of the methodology. The azido-Dha acceptor was prepared in 3 steps from the commercially available methyl acrylate (Scheme 4.3). To furnish the di-bromo compound **198** in a very high yield, methyl acrylate **197** was treated with Br_2 at 0 °C and warmed to rt for 18 h. This compound was volatile and it required rotary evaporation at 20 °C during the work-up. **198** was subsequently heated to 60 °C and NaN_3 was added to install the azide and promote elimination to the alkene. Unfortunately, the product of this step was a 2:1 mixture of Dha and di-azide **199**, and a subsequent step to convert any di-azide to Dha was implemented. The mixture was therefore treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) at 0 °C for 1.5 h and the final product **186:e** was obtained in a good yield over the two steps.



Scheme 4.3: Synthesis of *N*-azido-Dha methyl ester.

With the azido-Dha in hand, a test TEC was carried out with Gly-SH. Gly-STrt was deprotected upon treatment with TFA and TES as per standard trityl deprotection and the thioacid **78** was added directly to Dha, DPAP and MAP in a mixture of THF and aq. ammonia buffer pH 6 (Scheme 4.4). While the ^{1}H NMR of the crude reaction mixture showed full conversion, the thioester **200** was only afforded in a 17% yield.

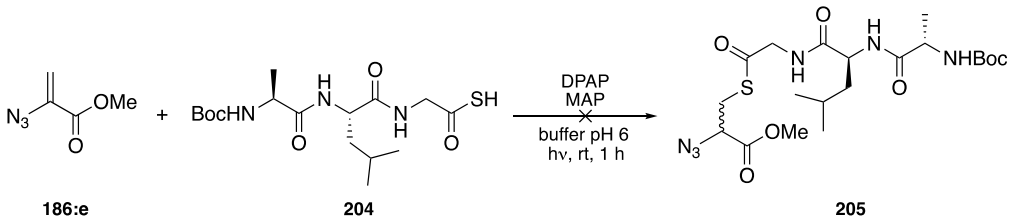
Moreover, during column chromatography **200** co-eluted with a substantial amount of Fmoc-Gly-COOH. It is therefore likely that the thioacid hydrolysed quickly due to the aqueous buffer and hence, was not available for TEC, or that the Dha acceptor degraded quickly under UV irradiation and the thioacid hydrolysed thereafter. Regardless of the poor yielding thiol-ene ligation, the *S*,*N*-acyl transfer was still of major interest. Accordingly, **200** was treated with SnCl, thiophenol and NEt_3 for 30 min to reduce the azide to amine. **201** was subsequently concentrated *in vacuo*, re-dissolved in MeOH and stirred at rt for 1 h to drive acyl migration. Interestingly, the dipeptide **202** was not isolated after column chromatography, however a small amount of the product of disulfide formation of **202** and thiophenol was found.



Scheme 4.4: Thiol-ene ligation of azido-Dha and thioglycine for afford thioester **200**. Subsequent reduction of the azide to furnish the free amine **201**, followed by *S*,*N*-acyl transfer and disulfide formation of dipeptide **202** with thiophenol.

Although the product obtained in Scheme 4.4 was not the desired outcome, it showed that the method was successful and the *S*,*N*-acyl transfer occurred by stirring in MeOH for just 1 h. However, the low yield of **200** and the possible degradation of the Dha acceptor led to a control reaction being conducted with no thioacid present. **186:e**, DPAP and MAP were irradiated in a UV oven for 1 h in ammonia buffer pH 6, after which TLC analysis confirmed that some **186:e** had been consumed, however no by-product was isolated. A second thiol-ene ligation was then carried out with a longer peptidyl thioacid in order to determine whether solubility issues contributed to the poor yield of **200**. Boc-protected tripeptide **204** provided a less hydrophobic thioacid, which

was anticipated to have better solubility in aq. buffer (Scheme 4.5). Unfortunately, increased solubility was not observed and ^{1}H NMR analysis of the crude product showed no conversion to **205**.

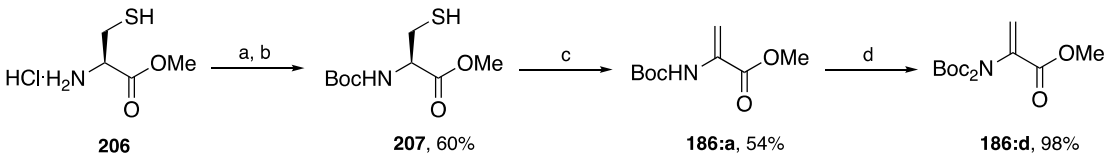


Scheme 4.5: Unsuccessful TEC of azido-Dha and tripeptide **204**.

4.4 Synthesis and TEC of *N,N'-bis*-Boc-Dha

4.4.1 Synthesis of *bis*-Boc-Dha from Cys

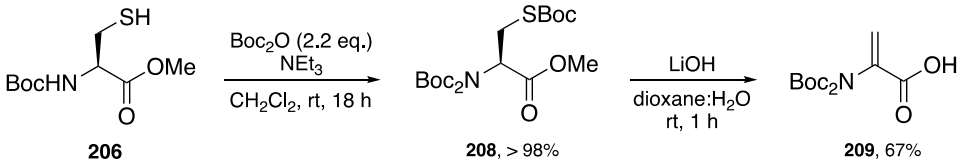
Bis-Boc-Dha **186:d**, was synthesised in high yields over 4 steps (Scheme 4.6). Firstly, the commercially available H_2N-Cys-OMe hydrochloride **206** was *N*-Boc protected, followed by direct treatment with tributylphosphine to reduce the resulting disulfide. **207** was then converted to dehydroalanine **186:a** through an alkylation-elimination reaction employing the commercially available 1,4-diiodobutane.[369] The second *N*-Boc protection was catalysed by DMAP, purification for each step consisted of either standard aqueous work-up or a short plug of silica.



Scheme 4.6: Synthetic route to *bis*-Boc-Dha; a) Boc_2O, NEt_3, CH_2Cl_2, rt, 18 h; b) $P(Bu)_3$, MeOH, H_2O, rt, 4 h; c)1,4-diiodobutane, K_2CO_3, DMF, rt, 2 h; d) Boc_2O, DMAP, CH_3CN, rt, 3 h.

The synthesis of the monomer of Dha, protected at the *N*-terminus by double-Boc and at the *C*-terminus by methyl ester, was straightforward, however challenges arose when the carboxylic acid analogue was investigated. Incorporating *bis*-Boc-Dha into peptides presented two obstacles: 1) the second Boc group is particularly labile in a

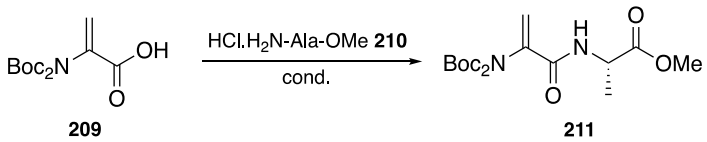
wide range of conditions and 2) the alkene is a very reactive electrophile (Michael acceptor) which unfortunately is not conducive to high-yielding peptide coupling as side reactions involving the amine addition on to the alkene can take place. Initially, the desired carboxylic acid was obtained by saponification of the corresponding methyl ester derivative. The treatment of **208** with LiOH (2.5 eq.) furnished the acid in modest yield (75%) and in a short time (1 h). Interestingly, it was serendipitously discovered that the treatment of the *N*,*N*,*S*-tri-Boc protected Cys with LiOH both eliminated the thiol to form Dha and hydrolysed the ester (Scheme 4.7). Using this route, multi gram quantities of *bis*-Boc-Dha were be obtained in only two synthetic steps and did not require further purification.



Scheme 4.7: Synthesis of **209** through the *tris*-Boc protection of Cys-OMe hydrochloride **206** and subsequent treatment with LiOH.

Once **209** was available, its reactivity in the amidic coupling with other amino acids was investigated. In the first instance, the coupling of *bis*-Boc-Dha and Ala-OMe hydrochloride using hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) and HOBt as coupling reagents was carried out. Unfortunately, together with the desired dipeptide, several byproducts were obtained, including the mono-Boc protected dipeptide and the addition product of H_2N-Ala-OMe onto the Dha alkene. We therefore set out to screen different coupling reagents for the amidic coupling (Table 4.2).

Table 4.2: Screening of coupling reagents for the amino acid coupling reaction between Dha and Ala-OMe.



209 **211**

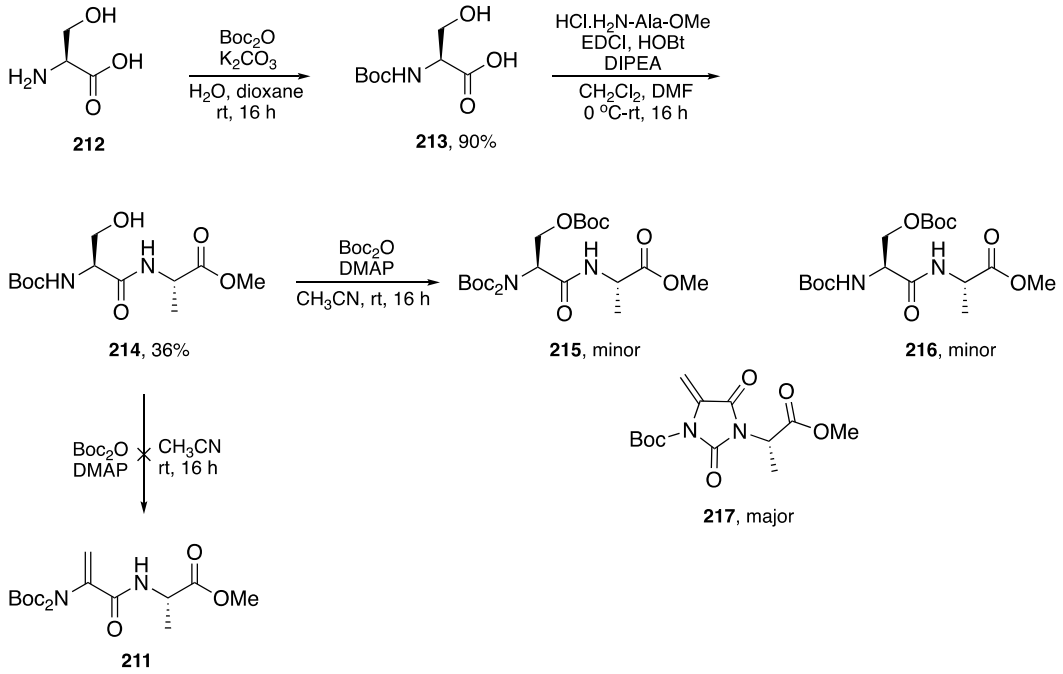
Entry	Coupling reagents	Base	Solvent	Yield (%)
1	HATU	DIPEA	CH_2Cl_2:DMF	26
2	HATU + HOBt	DIPEA	CH_2Cl_2:DMF	27
3	PyBop	NMM	CH_2Cl_2:DMF	24
4	DIC + HOBt	DIPEA	CH_2Cl_2:DMF	36
5	3-phosgene	DIPEA	CH_2Cl_2:DMF	/
6	DCC	DIPEA	CH_2Cl_2:DMF	~ 30
7	HCTU	DIPEA	CH_2Cl_2:DMF	> 40

The low yields obtained for the Dha amidic couplings were not unexpected, Dha being a very electrophilic, highly-reactive alkene. Despite the acceptable results obtained using *O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) as coupling reagent, we endeavoured to explore another route to *bis*-Boc-Dha containing peptides.

4.4.2 Synthesis of *bis*-Boc-Dha from Ser

The conversion of Boc-protected Ser to Dha was evaluated as a means of installing the alkene onto a peptide, instead of onto an amino acid monomer, in both solution and solid phase. The aim of this study was to provide more routes to Dha-containing peptides for different varieties of peptide synthesis. Beginning with solution phase, the procedure outlined by Ferreira *et al.*[379] was implemented, where Boc-Ser-OH was coupled with another amino acid to form a dipeptide **214**. **214** was further treated with Boc_2O and DMAP in CH_3CN to afford the alkene bearing two Boc groups protecting the amine. Disappointingly, when the reported reaction conditions were used, a mixture

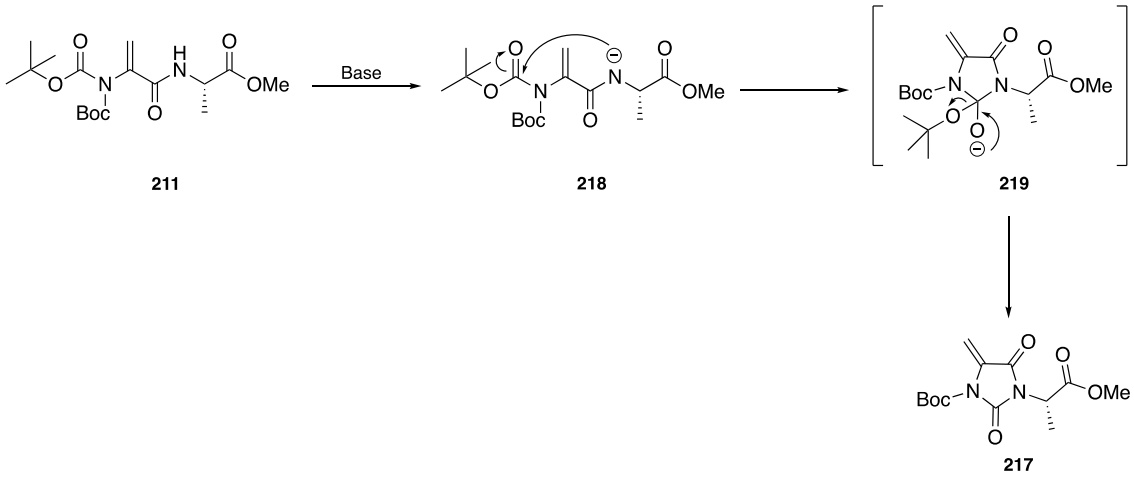
of three products were found: tri-Boc-protected Ser, *N*,*S*-di-Boc-Ser and a hydantoin cyclisation product (Scheme 4.8).



Scheme 4.8: Synthesis of Boc-protected dipeptide **214** and subsequent treatment with Boc_2O and DMAP to afford by-products **215**, **216** and **217**.

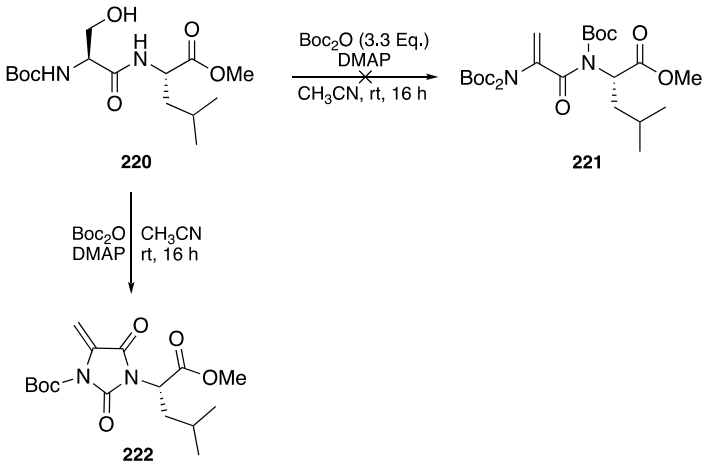
To convert byproduct **215** into Dha-containing dipeptide **211**, **215** was treated with DBU in CH_3CN, however unfortunately, the use of DBU resulted in hydantoin formation. The major by-product of the reaction depicted in Scheme 4.8, hydantoin, is formed though a competing side-reaction.[380] This occurs through the mechanism outlined in Scheme 4.9 and can be driven in either solution or solid phase peptide synthesis. Many derivatives of hydantoin are desired for therapeutic applications, and therefore sought out through chemical synthesis,[381] however for the peptide chemist, hydantoin formation must be minimised. Several factors influencing this side-reaction consist of solvent, type of base, nucleophilicity of the amino acid for coupling and steric hindrance. For example, it was discovered that hydantoin formation can be driven by using DMF as solvent and the use of THF may drive the peptide formation.[382] Additionally, the use of amines with high basicity and large steric hindrance, such as tertiary amines, promote the cyclisation. The *C*-terminal amino acid can also have an effect on the probability of hydantoin formation depending on steric hindrance. Gly, due

to its lack of side-chain, can readily undergo intramolecular cyclisation to hydantoin, whereas a residue with a bulkier side-chain may couple more efficiently.[383]



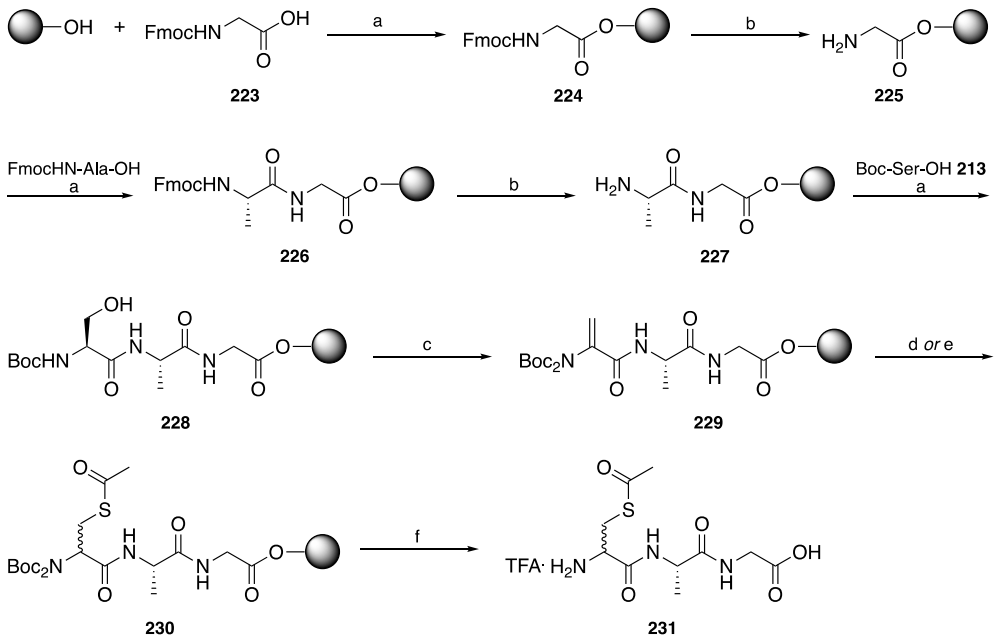
Scheme 4.9: Hydantoin formation mechanism

Following these preliminary studies, it was decided to attempt the Boc-protection and elimination on a more sterically hindered dipeptide, **220** (Scheme 4.10). Through the use of the bulky Leu residue it was anticipated that the side-reaction would be suppressed and the desired peptide **221** would be furnished as major product. Additionally, 3.3 eq. of Boc_2O was utilised with the intent of Boc-protecting the amide NH of the peptide bond. This additional protection was thought to prevent the amide NH from nucleophilically attacking the carbamate carbonyl. Unfortunately, only the Ser amine and thiol were Boc-protected and the reaction again resulted in hydantoin formation.



Scheme 4.10: Attempted synthesis of Dha-containing dipeptide **221** through further Boc-protection of amide NH to avoid hydantoin formation. Disappointingly, Dha-containing hydantoin **222** was the sole product.

Manual solid phase synthesis of Dha-containing peptides was also explored to investigate whether the hydantoin side-product would out-compete amino acid coupling, as occurred in solution phase. Using Wang resin, PyBOP and 4-methylmorpholine (NMM), Gly and Ala were coupled, in that order, to furnish the dipeptide **226** on the solid support (Scheme 4.11). *N*-Boc-Ser was then coupled as the third amino acid to afford **228** and the resin was suspended in DMF and treated with Boc_2O (2.5 eq.) and DMAP (cat.). The suspension was shaken for 16 h at rt to allow sufficient time for the secondary amine and Ser hydroxyl group Boc-protection and subsequent elimination to Dha.



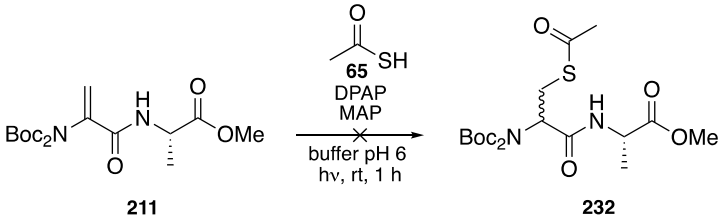
Scheme 4.11: Solid phase synthesis of tripeptide Ser-Ala-Gly, followed by conversion of Ser to Dha and thiol-ene ligation with thioacetic acid using the following conditions: a) PyBOP, NMM, DMF, rt, 45 min; b) piperidine (20%), DMF, rt, 5 mins (x 3); c) Boc_2O, DMAP, DMF, rt, 18 h; d) thioacetic acid, DPAP, MAP, DMF, hν, rt, 1 h; e) thioacetic acid, NEt_3, THF, rt, 4 h; f) TFA/TES/H_2O (95%/2.5%/2.5%), rt, 1.5 h.

Initially, the thiol-ene coupling was carried out under radical conditions, however crude ^{1}H NMR analysis of the cleaved peptide did not contain and acetyl CH_3 peak from the thioester and instead showed three distinct residues; Ser-Ala-Gly. Therefore, unfortunately, the conversion of Boc-Ser to *bis*-Boc-Dha did not occur on the solid support and the alkene was never presented for the subsequent TEC. In an attempt to force the elimination to Dha, more equivalents of Boc_2O were added to the resin and the reaction was repeated again for 18 h. One half of this resin was then cleaved and analysed by NMR to determine whether Dha had been afforded on the beads. Disappointingly, only hydantoin by-product was isolated.

Unfortunately, at this stage hydantoin formation was too prevalent to continue research into the synthesis of Dha from Boc-Ser. Due to the simple and efficient route to Dha from Boc-Cys, it was concluded that the low yield obtained for amino acid coupling of Dha (Table 4.2) would suffice in order to further the study of *bis*-Boc-Dha TEC.

4.4.3 Radical mediated thiol-ene ligation of Dha

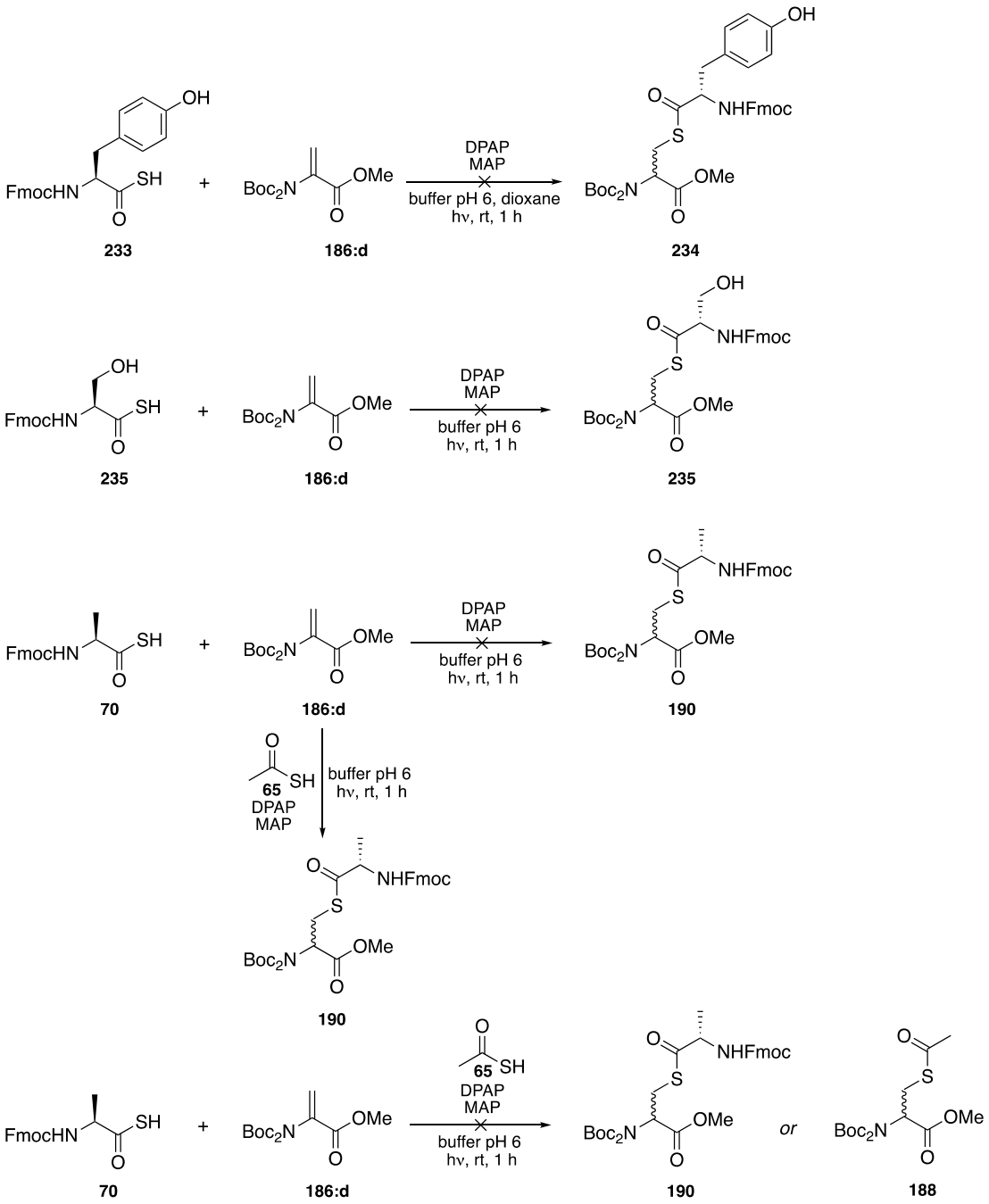
Owing to the attractive nature and "click"-like properties of thiol-ene chemistry, it was envisaged that radical-mediated thiol addition onto Dha would be most advantageous for the TEMPLE protocol. Undertaking the previously optimised methodology developed by Dr. Petracca, the dipeptide **211**, synthesised using DIC and HOBt, was irradiated with thioacetic acid, DPAP and MAP in aq. NH_4OOCH_3 buffer at pH 6. Surprisingly, this reaction did not furnish the desired peptide, as ^{1}H NMR showed no evidence of the Cys CH_2. A notable observation of this reaction was that neither the Fmoc-protected thioacid nor the Dha-dipeptide was completely soluble in aq. buffer. In particular, the thioacid precipitated out as a white solid when the buffer was added to the reaction vessel.



Scheme 4.12: Attempted radical thiol-ene ligation of Dha-dipeptide **211** and thioacetic acid, which did not furnish the desired thioester **232**.

It was subsequently decided to carry out some examples of simple 1 + 1 additions of α-amino thioacids onto the Dha monomer (Scheme 4.13). To begin with, Fmoc-Tyr-SH **233** was subjected to TEC with *bis*-Boc-Dha-OMe **186:d** in a mixture of buffer and dioxane. The use of dioxane was expected to improve the solubility of the reaction mixture, while keeping the pH constant with the aq. buffer. Unfortunately, the thiol-ene ligation did not occur as crude ^{1}H NMR again showed no evidence for formation of the Cys CH_2. Fmoc-Ser-SH **235** was also utilised in an attempt to provide the thioester **236**, however without the use of any organic solvent, but again no thioester was formed and only the CSO elimination by-product was isolated. Fmoc-Ala-SH **70** was then subjected to the same conditions and after 1 h no reaction had occurred. This mixture was then treated with a base wash and more DPAP and MAP were added, along with thioacetic acid. This time, Cys CH_2 peaks were observed in crude ^{1}H NMR, meaning that after the removal of the α-amino thioacid (or just the amino acid if

hydrolysis had taken place), the thioacetic acid was reactive enough to form the thioester. In light of these results, a TEC was carried out with *bis*-Boc-Dha-OMe, Fmoc-Ala-SH and thioacetic acid, using previous conditions. Interestingly, no reaction took place. It is possible that the α-amino thioacid is quenching the radical reaction in these particular conditions using aq. NH_4COOCH_3 at pH 6. At this pH the thioacid should be predominantly in the form of the thiolate anion, which may inhibit the formation of the thiyl radical. However, if this was possible for the α-amino thioacid, it would also be expected for thioacetic acid. Additionally, the precipitation of the thioacid out of solution before the reaction has begun is a possible contributing factor to the failure of the reaction.



Scheme 4.13: Attempted radical TEC reactions with Dha using Thr-SH and Ser-SH, which did not afford the thioesters **234** and **235**. Additional attempts included using both thioacetic acid and Ala-SH to afford either thioester **190** or **188**, however only **190** was furnished when no α-amino thioacid was present in the reaction.

With all of these factors considered, it was decided to investigate the ionic β,γ-*C,S* thia-Michael addition of thioacids onto Dha. If the quenching of the radical generated in the radical TEC was the cause of the complete absence of formation, then it is possible that the ionic route would have a different outcome.

4.4.4 Ionic α,β-*C,S*-Thia-Michael addition onto Dha

The previously screened conditions described in Table 4.1 to drive the ionic thiol-ene addition of thioacetic acid to Dha clearly showed that the most efficient route to the *bis*-Boc-thioester was through the use of 4 eq. thioacid and cat. NEt_3 in THF for 4 h at rt. However, subsequent searches of the literature led to the use of different conditions reported by Bernardes *et al.*,[368] where the authors carried out aza-Michael addition of amines onto Dha residues in proteins to accomplish selective *N*-linked modifications on proteins. The reactions were conducted at 37 °C in a mixture of DMF and phosphate buffer at pH 8. In this instance, the basic phosphate buffer employed facilitated nucleophilic attack of the amine onto Dha, with particular success when using a more nucleophilic amine, such as benzylamine. It was anticipated that the phosphate buffer would also drive thiolate formation in our system and that the warmer reaction temperatures would assist in solubility issues. We therefore set out to use these conditions to furnish a family of thioesters from Dha. Table 4.3 outlines the range of thioesters prepared *via* this methodology.

Table 4.3: Experimental scope of the ionic thiol-ene ligation of a range of thioacids with Dha.

Entry	Thioacid	Dha	% Conv. (^{1}H NMR)
1	Fmoc-Gly-SH	Dha-OMe **186:d**	86
2	Fmoc-Ala-SH	Dha-OMe **186:d**	79
3	Fmoc-Val-SH	Dha-OMe **186:d**	68
4	Fmoc-Ile-SH	Dha-OMe **186:d**	76
5	Fmoc-His-SH	Dha-OMe **186:d**	68
6	Fmoc-Tyr-SH	Dha-OMe **186:d**	91
7	Fmoc-Ala-Gly-SH	Dha-OMe **186:d**	43
8	Fmoc-Ala-Gly-SH	Dha-Leu-OMe	71

All reactions were carried out at 37 °C, in a 1:1 mixture of DMF:phosphate buffer for 18 h.

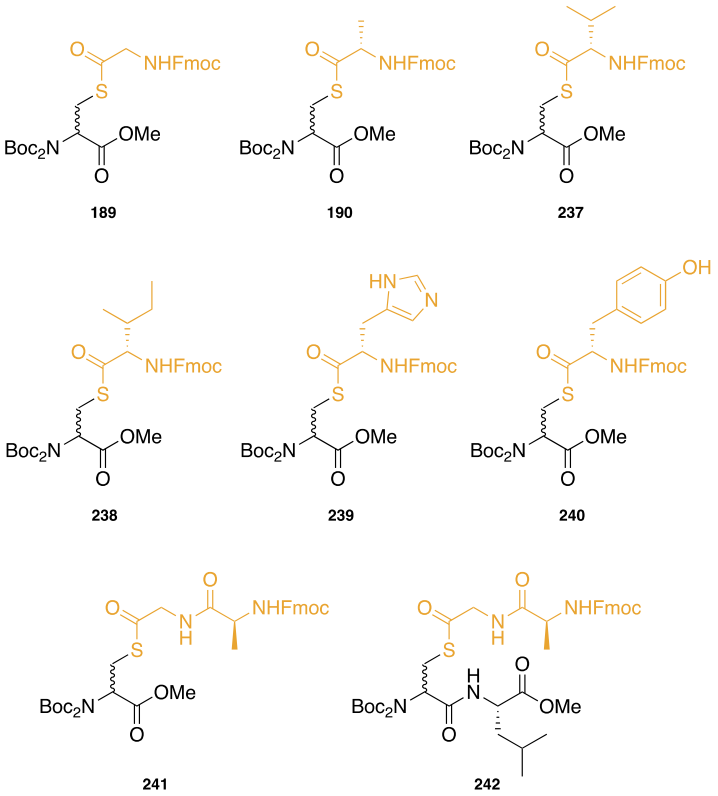
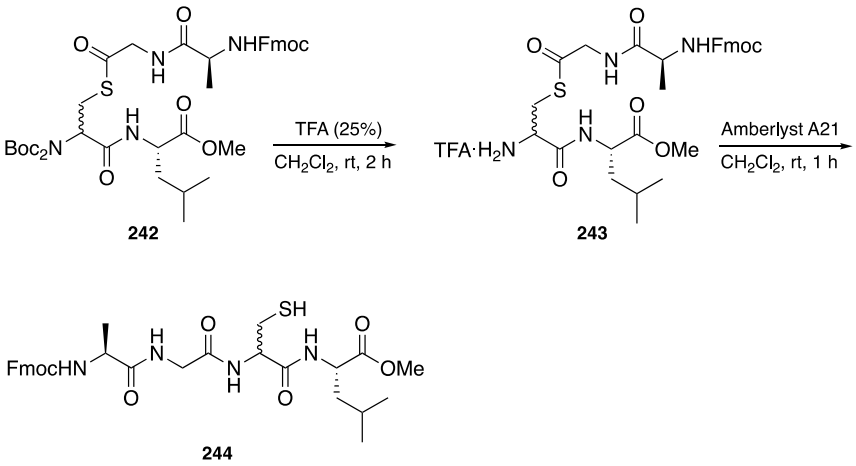


Figure 4.5: Structures of the thioesters prepared through ionic thiol-ene ligation using the different amino and peptide thioacids (orange) with Dha-monomer and Dha-dipeptide.

From this table it is evident that the ionic thiol-ene ligation of amino and peptide thioacids onto both Dha-monomers and Dha-dipeptides provides thioesters with high conversions. Moreover, the reaction is mild, fast and requires only a short work-up of an extraction into organic solvent. Several thioesters were purified using flash column chromatography on silica gel (20-40% EtOAc/Hex).

As the 2 + 2 addition of the dipeptide thioacid onto Dha-dipeptide (Entry 8) furnished the largest peptidyl thioester, **242**, we next endeavoured to cleave the Boc groups of the *N*-terminus and drive the *S*,*N*-acyl transfer, confident that the cyclic transition state would be of an ideal size; 5-membered. The deprotection was carried out with TFA (25%) in CH_2Cl_2 at rt for 2 h (Scheme 4.14). The TFA salt **243** was then stirred with Amberlyst A21 basic resin for 1 h, at which point the ^{19}F NMR analysis showed no presence of TFA, either as a salt or the reagent itself. Gratifyingly, ^{1}H NMR displayed a change in the Cys-CH_2 chemical shift, indicating that the acyl migration onto the free amine had occurred and the native peptide bond was present.



Scheme 4.14 Cleavage of Boc groups on thioester **242** to afford the TFA salt **243**. After stirring with Amberlyst A21 resin the conversion to tetrapeptide **244** was confirmed by ^{1}H NMR.

4.5 Ionic α,β-*C,S*-Thia-Michael addition towards the synthesis of GLP-1

Members of the Scanlan lab have continued efforts towards the synthesis of the small protein Glp-1. Following the promising results obtained with small ligations, β,γ-C,S thia-Michael addition, *S,N*-acyl transfer and desulfurisation were endeavoured to synthesise the entire protein at different ligation points outlined in Figure 4.3. To date, successful thioester formation and subsequent *S,N*-acyl transfer has been carried out on Ile-*S*-Trt and a peptide containing the last 7 residues of the protein with an *N*-terminal Dha (Dha-WLVHGR) (Figure 4.6). This reaction was carried out using the same conditions described in Section 4.4.4; DMF:phosphate buffer pH 8 (1:1), 37 °C for 18 h. Mass spectrometry analysis confirmed the presence of the octapeptide **248**. In addition, thioacetic acid has been attached to peptide fragments of GLP-1, followed by removal of Boc groups and *S,N*-acyl shift, to furnish *N*-acetyl peptide fragments of GLP-1. All peptide fragments were assembled by manual SPPS.

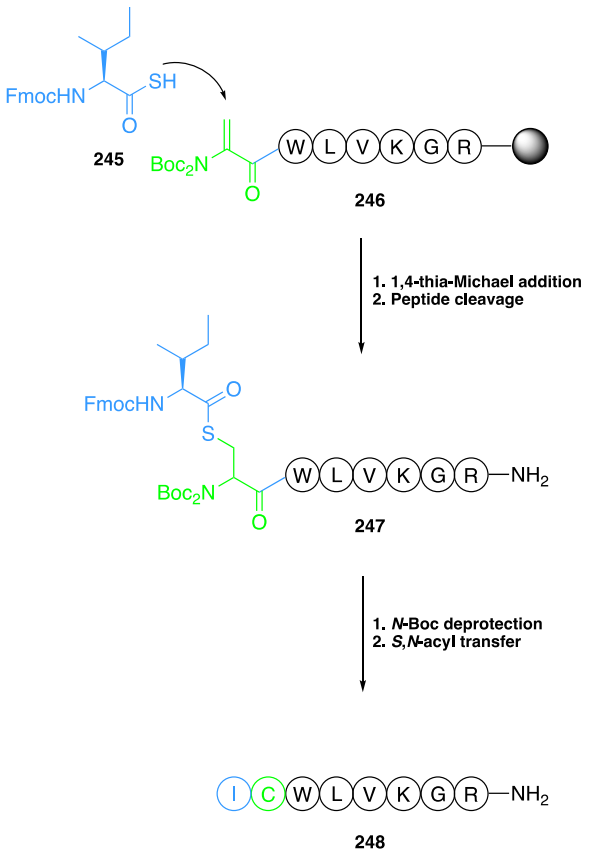


Figure 4.6: Successful thioacid addition of Ile onto a Dha residue of a peptide fragment of GLP-1, followed by acyl migration to furnish a native peptide bond.

Currently, methods to install thioacids onto the C-terminus of peptides prepared by SPPS are being explored. The most successful route to these compounds is through the solution phase amino acid coupling of the *S*-Trt protected thioacid residue onto a peptide that has been cleaved from the resin. However, this process requires optimisation before being utilised for TEMPLE in the synthesis of GLP-1.

4.6 Conclusions

In summary, a novel route to peptidyl thioesters has been developed through the use of Dha and α-amino thioacids. Both ionic and radical-mediated addition of thioacids have been demonstrated on azido-Dha and *bis*-Boc-Dha monomer and dipeptide. Previous methods to carry out TEMPLE on larger systems failed to undergo the *S*,*N*-acyl transfer to provide a native peptide bond, however this was circumvented by utilising Dha, due to the 5-membered cyclic transition state formed during acyl migration.

Initially, the azido-Dha monomer was investigated as a potential alkene acceptor for the thiyl radical donor. Following a straightforward synthesis, the azido-Dha was delivered in 4 steps and with good yields. Unfortunately, despite the successful radical TEC of thioacetic acid previously carried out, the thiol-ene ligation of Gly-SH did not furnish a sufficient yield of the desired thioester product. Nevertheless, the small amount of azido-thioester obtained was reduced to free amine and the native peptide bond was afforded rapidly and with little treatment through acyl migration. This result confirmed the hypothesis that the 5-membered transition state is ideal for driving the *S*,*N*-acyl transfer. Moving forward, the *bis*-Boc-Dha substrate was examined as a TEMPLE acceptor. The Dha methyl ester was synthesised in high yield from the commercially available Cys-OMe hydrochloride and the corresponding carboxylic acid was provided through elimination of the Boc-protected thiol of Cys using LiOH. However, subsequent amino acid coupling of Dha and other residues were low yielding, which led to the investigation of the synthesis of Dha-containing peptides *via* dehydration of Ser. This method was carried out by the tri-Boc protection of Ser residue and elimination of the Boc-protected hydroxyl to furnish the alkene at the α-position. Unfortunately, these attempts mainly led to the formation of hydantoin, a cyclic by-product commonly formed in certain peptide reactions. The difficulties associated with this line of synthesis, both in solution and in solid phase, encouraged us to return to the synthesis of Dha from Cys.

Radical TEC was attempted using ammonia buffer at pH 6, which had previously shown success with thioacids. Disappointingly, this method did not provide the desired thioesters and hence, an alternative route was examined. Using a variety of different thioacids, eight thioesters were developed through ionic thiol-ene ligation onto a Dha residue and a Dha-containing dipeptide. These thioesters were afforded with good conversion and with minimal work-up. The thioesters that were purified were isolated using simple column chromatography on silica gel. The 2 + 2 addition of a dipeptide thioacid onto a Dha-containing dipeptide was subsequently carried forward in the TEMPLE process, where it was deprotected and neutralised to allow acyl migration to occur. The presence of the tetrapeptide was confirmed by ^{1}H NMR, which clearly showed the change from thioester to free thiol/disulfide. This compound was isolated and fully characterised.

Significant steps have been made towards the synthesis of Glp-1, using SPPS and our TEMPLE methodology. A short peptide fragment of Glp-1 has been prepared *via* SPPS, by members of the Scanlan lab, and has been reacted with thioacetic acid and with Ile-thioacid to form isopeptides through Dha residue. Following *N*-Boc deprotection, *S*,*N*-acyl transfer has been carried out to convert these isopeptides to natural amide/peptide bonds, providing Cys at the ligation site, which can be desulfurised, similar to NCL. Having demonstrated that a peptide bearing an *N*-terminal Dha residue can undergo thia-Michael addition, the most imminent work to be carried out on this project is the preparation of suitable peptide thioacids, corresponding to fragments of Glp-1, to be implemented in the same process in order to furnish the complete protein. This work is currently being undertaken within the Scanlan lab.

5.0 Overall conclusions and future work

The work described in this thesis outlines our recent investigations into the ligation of biologically relevant compounds through thiol-ene and thiol-yne chemistry. The first part of the thesis details the cyclisation of open chain carbohydrate derivatives for the synthesis *exo*- and *endo*-thioglycals *via* TYC. The second part of the thesis describes our studies towards the development of the Thiol-Ene Mediated Peptide Ligation and Elimination (TEMPLE) methodology, involving the chemical ligation of thioacids and alkenyl peptides through TEC, followed by *S*,*N*-acyl transfer and elimination of the sulfur-component to furnish native peptide bonds at naturally occurring residues.

Chapter 1 provides a broad introduction to thiol-ene ligation and recent developments in chemical ligation of peptides. Chapter 2 outlines the continuation of work previously carried out by Dr. Vincent Corcé in the Scanlan lab. Previously, *O*-benzyl-D-arabinose was used as the substrate to investigate the TYC-mediated synthesis of thioglycals. Therefore, the L-arabinose derivative was subsequently tested and this work is described in Chapter 2. *O*-benzyl-L-arabinose was ring-opened and an alkyne moiety was inserted at the anomeric position in one step. This was carried out using the Colvin rearrangement, a reaction involving a lithiated trimethylsilyldiazomethane intermediate, which introduced the alkyne with the expulsion of N_2. The free hydroxyl that was released during this reaction was then converted to the triflate and treated with KSAc to provide a terminal acetyl-protected thiol in close proximity to the alkyne. Initially, multiple methods were tested to remove the *S*-acetyl group, however most attempts failed to provide the free thiol or resulted in ionic ring-closing to give the 5-*exo* product. While the basic removal of the acetyl group to furnish the 5-*exo*-glycal exclusively was a good comparison, the radical-mediated reaction was of significant interest, as the aim was to provide both 5-*exo* and 6-*endo* thioglycals. Gratifyingly, the use of MeLi to remove the acetyl protecting group afforded the free thiol, which was immediately irradiated with UV light in the presence of photoinitiator and photosensitiser. Both the 5-*exo* and 6-*endo* thioglycal were obtained through this route in a ratio of 3:2, respectively, and with a 55% overall yield. This result was particularly interesting as the same radical reaction using the D-epimer only gave the 5-*exo* product and no 6-*endo* product was obtained. This suggested that the stereochemistry of the C-5 substituent was very important in promoting either cyclisation, which is likely due to the steric hindrance of the bulky benzyl protecting group. It is therefore possible that a

smaller, less sterically demanding protecting group may not influence the cyclisation as much and may allow the formation of the 6-*endo* thioglycal as the major product.

In Chapter 3, thiyl radical addition onto alkenes was extended to peptide ligation, in an endeavour to develop TEMPLE methodology. This chapter described the overall peptide ligation plan; thiol-ene ligation, *N*-terminal deprotection and *S*,*N*-acyl shift and finally, elimination of thiol-containing moiety. The end goal of this project was to use this route to build the lipoprotein Oprl, which is the most abundant lipoprotein in *pseudomonas aeruginosa* and is currently unobtainable by standard recombinant approaches due to the diacylglycerol unit on the Cys in the protein. To begin with, a terminal alkene was attached to a Ser residue on the *C*-terminus of a dipeptide through esterification. This was subjected to UV irradiation at 365 nm with thioacetic acid in the presence of photoinitiator and photosensitiser. Gratifyingly, this reaction went to completion in just 1 h to prove that the thiol-ene ligation onto peptidyl alkene was highly efficient. The reaction was also carried out with α-amino thioacids, which required a three-fold excess of thioacid but resulted in high yields of thioesters. The scope of the thiol-ene route to thioesters was investigated across a range of thioacids, including α-amino, alkyl, aryl and a carbohydrate derivative, using one simple Ser-modified alkene. A tripeptide from the protein of interest was functionalised with an alkene and also underwent successful thiol-ene ligation, including one with a tetrapeptide *C*-terminal thioacid to afford a 7-mer isopeptide. Specific thioesters were *N*-terminally deprotected to afford the free amine, which required the use of phosphate buffer at pH 8.5, in order to promote the *S*,*N*-acyl transfer. Unfortunately, after many unsuccessful attempts at intramolecular *S*,*N*-acyl transfer it was decided that the 14-membered cyclic transition state that occurs during the rearrangement of this particular system was not favoured for the intramolecular process. This conclusion was reached following the successful intermolecular *S*,*N*-acyl transfer carried out by the thioester and benzylamine. This disappointing result lead to the investigation of β,γ-unsaturated amino acids, such as dehydroproline (Dhp) and dehydrovaline (Dhv), which would employ a 6-membered cyclic intermediate during acyl migration. Unfortunately, following TEC, the Pro thioester formed through TEC did not undergo acyl transfer, presumable due to the conformational restraints associated with that particular residue. The unnatural amino acid Dhv would appear to be more promising as it does not employ a more rigid structure, however the procedure to acquire the starting Dhv was

inefficient and low-yielding, hence that project is currently being optimised within the Scanlan lab.

Chapter 4 details the investigation into dehydroalanine (Dha), an α,β-unsaturated amino acid, for TEMPLE. The motive for using this alkene was to utilise a 5-membered cyclic transition state during the *S,N*-acyl transfer. The size of this intermediate is widely understood to be the most favourable for the rearrangement. Previous work in the Scanlan lab by Dr. Rita Petracca provided ample precedent into the utilisation of Dha for furnishing peptide thioesters that could undergo *S,N*-acyl transfer. The goal of this project was to synthesise GLP-1, a small protein involved in the regulation of insulin, through the Dha-TEMPLE process. Both radical and ionic methods of affording thioesters were explored, encompassing an azide protecting group and a *bis*-Boc protecting group for the *N*-terminus of Dha. Using the *N*-azido-Dha acceptor, the thiol-ene reaction was successful with thioacetic acid, however with α-amino thioacids the ligation gave a very poor yield. Ionic β,γ-*C,S* thia-Michael ligations using *Bis*-Boc-Dha and α-amino thioacids were more successful, and thus the methodology was continued using this route. A range of 1 + 1 TEC reactions were carried out in addition to 2 + 1 and 2 + 2 ligations using a dipeptide thioacid and a Dha-containing dipeptide. In the case of the 2 + 2 additions, Dha was *N*-terminally deprotected and the *S,N*-acyl transfer was carried to afford a tetrapeptide with Cys at the ligation site. Following these promising results, a fragment of GLP-1 was synthesised by manual SPPS with a Dha residue in place at the *N*-terminus. This peptide was subjected to thia-Michael addition of Ile-SH to furnish an isopeptide through a thioester linkage. Boc deprotection of the *N*-terminus then afforded the native peptide bond, giving an octapeptide with sequence ICWLVKGR, which was confirmed by mass spectrometry. This work is ongoing in the Scanlan lab, with efforts being made to synthesise fragments of GLP-1 with a *C*-terminal thioacid. This peptide will be ligated *via* a Dha residue to the rest of the peptide, and following acyl shift and desulfurisation, provide the entire small protein through SPPS and our methodology.

In conclusion, this thesis presents novel findings in the application of thiol-ene and thiol-yne ligation reactions for the preparation of carbohydrate and peptide derivatives. Both intra- and intermolecular reactions were investigated and a highly novel approach that utilises thiol-ene ligation but delivers a sulfur-free ligation called

TEMPLE was developed. This research advances the current state-of-the-art in radical mediated reactions and peptide chemistry.

6.0 Experimental details

6.1 General experimental details

All commercial chemicals used were supplied by Sigma Aldrich (Merck), Fluorochem, VWR Carbosynth and Tokyo Chemical Industry and used without further purification unless otherwise stated. Deuterated solvents for NMR were purchased from Sigma Aldrich (Merck) or VWR. Solvents for synthesis purposes were used at GPR grade. Anhydrous CH_2Cl_2, THF, CH_3CN and Et_2O were obtained from a PureSolv MD-4EN Solvent Purification System. All UV reactions were carried out in a Luzchem photoreactor, LZC-EDU (110 V/ 60 Hz) containing 10 UVA lamps centred at 365 nm. Silica gel 60 (Merck, 230-400 mesh) was used for flash column chromatography and all compounds were subject to purification using silica gel, unless otherwise stated. Analytical thin layer chromatography (TLC) was carried out with silica gel 60 (fluorescence indicator F254; Merck) and visualised by UV irradiation or molybdenum staining [ammonium molybdate (5.0 g) and concentrated H_2SO_4 (5.3 mL) in 100 mL H_2O]. 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 600 (600.13 MHz for ^{1}H NMR and 150.90 MHz for ^{13}C NMR), Bruker AV 400 (400.13 MHz for ^{1}H NMR and 100.61 MHz for ^{13}C NMR) or Agilent MR400 (400.13 MHz for ^{1}H NMR and 100.61 MHz for ^{13}C NMR) instruments. Chemical shifts, δ, are in ppm and referenced to the internal solvent signals. NMR data was processed using Bruker TopSpin software. The assignment of the signals was confirmed by 2D spectra (COSY, HMBC, HSQC). Melting points are uncorrected and were measured with a Stuart SP-10 melting point apparatus. MALDI time of flight (TOF) spectra were acquired using a Waters MALDI Q-Tof Premier in positive or negative mode with DCTB (trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile) as the MALDI matrix. ESI mass spectra were acquired in positive and negative modes as required, using a Micromass TOF mass spectrometer, interfaced to a Waters 2690 HPLC or a Bruker micrOTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC. 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 modes. Specific rotation was recorded in a Rudolph research autopol IV polarimeter with a D-line sodium lamp (589 nm) at 20 °C and are quoted as deg cm^3 g^{-1} dm^{-1}. Infrared spectra (IR) spectra were recorded on a

Perkin-Elmer Spectrum 100 FT-IR spectrometer.

6.2 General experimental procedures

Procedure A: Peptide coupling

To the amino acid-COOH in anhydrous CH_2Cl_2:DMF (8:2) was added the coupling reagent (EDCI·HCl or DIC) (1.1 eq.) and HOBt (1.1 eq.). The solution was cooled to 0 °C and stirred under N_2 for 45 min. To this was added the amino acid-NH_2 (1.0 eq.) and DIPEA (4.0 eq.) and the solution was stirred at rt for 18 h. The crude material was extracted with EtOAc (2 x 20 mL) and washed with 1 M HCl solution (2 x 20 mL), sat. aq. $NaHCO_3$ solution (2 x 20 mL) and brine (20 mL). The organic layers were dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The product was purified by flash chromatography.

Procedure B: Esterification of Ser/Thr

To a stirred solution of peptide (1.0 eq.) in anhydrous CH_2Cl_2 was added NEt_3 (2.0 eq.) under N_2. The solution was cooled to 0 °C and 4-Pentenoyl chloride (1.5 eq.) was added dropwise. The reaction was warmed to rt and left to stir for 3 h. The crude product was washed with sat. aq. $NaHCO_3$ solution (2 x 20 mL) and brine (2 x 20 mL). The solution was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The product was subjected to chromatographic purification.

Procedure C: Removal of the Boc protecting group

To a solution of Boc-protected peptide was added TFA:CH_2Cl_2 (1:4) or HCl, 4 M in dioxane:CH_2Cl_2 (1:9) and the solution was stirred at rt for 2 h. The solvent was removed *in vacuo* to furnish the product as the TFA salt and was used without further purification.

Procedure D: Intermolecular radical-mediated thiol-ene coupling

To a mixture of alkene (1.0 eq.), DPAP (0.2 eq.) and MAP (0.2 eq.) in either anhydrous DMF or buffered aq. Solution and under N_2 was added the thioacid (0.3 eq.). The mixture was irradiated at 365 nm at rt for 1 h. The reaction mixture was diluted with

H_2O, extracted with EtOAc (2 x 10 mL) and washed with brine (10 mL). The crude product was dried over $MgSO_4$, filtered, concentrated *in vacuo* and subjected to chromatographic purification.

Procedure E: Preparation of *S*-Trityl thioesters

To a strirred solution of the amino acid-COOH in anhydrous CH_2Cl_2 and under N_2 was added DMAP (0.1 eq.), triphenylmethanethiol (1.0 eq.) and EDCI·HCl (1.2 eq.). The solution was stirred for 18 h at rt. The solvent was removed *in vacuo* and purified by column chromatography.

Procedure F: Removal of the *S*-Trityl protecting group

To a solution of trityl-protected thioacid was added TFA:CH_2Cl_2 (1:3) followed by triethylsilane (20.0 eq.) under N_2. The reaction was stirred for 5 min at rt, concentrated *in vacuo* and used directly without further purification.

Procedure G: Preparation of *N*-Fmoc amino acids

To a stirred solution of amino acid-NH_2 (1.0 eq.) and $NaHCO_3$ (2.5 eq.) in H_2O was added a solution of Fmoc-OSu (1.0 eq.) in 1,4-dioxane and the reaction was stirred at rt for 2 h. H_2O (20 mL) was added and the product was extracted with EtOAc (2 x 15 mL). The organic layers were washed with brine (2 x 20 mL), dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*.

Procedure H: Preparation of *N*-Boc amino acids

To a solution of amino acid-NH_2 in dioxane was added Boc_2O (1.2 eq.) and NEt_3 (2.0 eq.) and the solution was stirred at rt for 18 h. The solvent was evaporated *in vacuo*, diluted with H_2O (20 mL) and acidified with 1 M HCl solution. The product was extracted with EtOAc (2 x 15 mL), washed with brine (20 mL), dried over $MgSO_4$ and filtered. The solvent was evaporated *in vacuo* and the product was used without further purification.

Procedure I: Hydrolysis of methyl ester protected peptides

To a stirred solution of the methyl ester protected peptide in MeOH:H_2O (1:1) was added Cs_2CO_3 (4.0 eq.) and the reaction mixture was stirred at rt for 18 h. MeOH was removed *in vacuo* and the crude product was acidified with 1 M HCl solution and extracted with EtOAc (2 x 15 mL). The solution was washed with brine (20 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The product was used without further purification.

Procedure J: Removal of *tert*-butyl protecting group

To a solution of *tert*-butyl protected carboxylic acid was added TFA:CH_2Cl_2 (1:4). The reaction was stirred at rt for 2 h and concentrated *in vacuo*. The product was used without further purification.

Procedure K: Preparation of thioesters 145-166 through intermolecular radical TEC

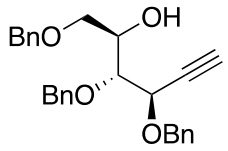
To a flask under N_2 was added **110** (0.1 g, 0.236 mmol), DPAP (0.0127 g, 0.0427 mmol) and MAP (0.0071 g, 0.0427 mmol), followed by a solution of thioacid (0.708 mmol), prepared as per Procedure F and used immediately, in anhydrous DMF (2 mL). The mixture was irradiated in a UV oven at 365 nm for 1 h at rt. The crude product was concentrated and subjected to chromatographic purification.

Procedure L: Preparation of thioesters 189-242 through intermolecular ionic TEC

To a flask under N_2 was added **186:d** or Dha-Leu dipeptide (1.0 eq.) and phosphate buffer pH 8. To this mixture was added a solution of the thioacid (1.0 eq.) in DMF and the reaction was stirred at 37 °C for 18 h. The crude mixture was diluted with H_2O (5 mL), and extracted with EtOAc (2 x 10 mL). The organic layers were combined, washed with brine (15 mL), dried over $MgSO_4$ and filtered. The crude product was concentrated *in vacuo* and the conversion rate was determined by ^{1}H NMR.

6.3 Experimental details for Chapter 2

(2*R*,3*R*,4*R*)-1,3,4-Tris(benzyloxy)hex-5-yn-2-ol (44)



To a stirred solution of trimethylsilyldiazomethane (3.56 mL, solution 2 M in hexane, 7.13 mmol) under N_2 and in anhydrous THF (60 mL) was added lithium diisopropylamide (14 mL, solution 1.02 M in THF/heptanes/ethylbenzene, 14.25 mmol) and the solution was stirred for 1 h at -20 °C. The mixture was cooled to -78 °C and a solution of 2,3,5-tri-*O*-benzyl-D-arabinofuranose (2.0 g, 4.75 mmol) in anhydrous THF (40 mL) was added *via* syringe. The resulting mixture was allowed to warm to rt over a period of 8 h. The reaction was quenched by the addition of H_2O (10 mL) and diethyl ether (200 mL) was added. The organic layer was extracted with diethyl ether (3 x 200 mL) and the ethereal layers were combined, dried over $MgSO_4$, and evaporated *in vacuo*. The residue was dissolved in MeOH (20 mL) and KF was added (2.76 g, 47.50 mmol). After being stirred at rt for 2 h, the solvent was removed *in vacuo* and the residue was partitioned between CH_2Cl_2 (100 mL) and H_2O (100 mL). The organic phase was washed with H_2O (2 x 50 mL), dried over $MgSO_4$ and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel (10% EtOAc:Hex) to afford **44** as a colorless oil (1.4 g, 70%).

$[\alpha]_D^{23}$: -29.8 (deg cm^3 g^{-1} dm^{-1}) (c = 1.1, $CHCl_3$).

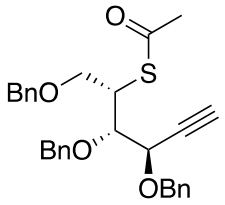
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 – 7.40 (15H, m, Ph), 4.95-4.89 (2H, m, Bn CH_2), 4.61-4.44 (4H, m, Bn CH_2), 4.41 (1H, dd, *J* = 3.7 Hz, *J* = 2.2 Hz, H-4), 4.08 – 4.16 (1H, m, H-2), 3.75 (1H, dd, *J* = 7.3 Hz, *J* = 3.7 Hz, H-3), 3.64 (1H, dd, *J* = 9.9 Hz, *J* = 3.3 Hz, H-1), 3.59 (1H, dd, *J* = 9.9 Hz, *J* = 5.1 Hz, H-1'), 2.55 (1H, d, *J* = 2.2 Hz, H-6) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 138.2, 138.1, 137.3 (Ar-qC), 128.6, 128.5, 128.45, 128.4, 128.3 128.1, 128.0, 127.9, 127.8 (Ar-CH), 80.5 (C-5), 80.1 (C-3), 76.2 (C-6), 74.5 (Bn CH_2), 73.5 (Bn CH_2), 71.1 (Bn CH_2), 70.7 (C-1), 70.3 (C-2), 68.8 (C-4) ppm.

HRMS (*m/z*) calculated for $C_{27}H_{28}NaO_4$ $[M + Na]^+$, calcd. 439.1880, found 439.1880.

ν_{max} (film)/cm^{-1} 3282 (OH), 3031 (Ar CH), 1348 (CO).

_S_-((2_S_,3_S_,4_R_)-1,3,4-Tris(benzyloxy)hex-5-yn-2-yl) ethanethioate (50)



To a stirred solution of **44** (975 mg, 2.43 mmol) in anhydrous CH_2Cl_2 (25 mL) was added pyridine (393 µL, 4.86 mmol). The reaction mixture was cooled to 0 °C and trifluoromethanesulfonic anhydride (614 µL, 3.65 mmol) was added dropwise. The reaction mixture was stirred at rt for 2 h and the solution was filtered over a pad of silica and eluted with CH_2Cl_2. The filtrate was evaporated under reduced pressure and dried under high vacuum. The crude material was solubilized in anhydrous DMF (5 mL) and cooled to 0 °C. KSAc (554 mg, 4.86 mmol) was added and the reaction mixture was stirred for 2 hours at 0 °C. The reaction mixture was diluted with diethyl ether (50 mL), washed with H_2O (2 x 25 mL), brine (2 x 25 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The product was purified by flash column chromatography on silica gel (10% EtOAc:Hex) to afford **50** as pale yellow oil (868 mg, 75%).

$[\alpha]_D^{23}$: -1.6 (deg cm^3 g^{-1} dm^{-1}) (c = 0.1, $CHCl_3$).

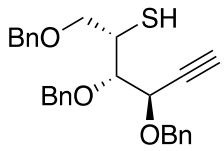
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32-7.40 (15H, m, Ph), 5.01-4.92 (2H, m, Bn CH_2), 4.67-4.60 (4H, m, Bn CH_2), 4.49-4.43 (3H, m, H-2, H-3, H-4), 4.32 (1H, d, *J* = 8.4 Hz, H-6), 3.74 (1H, td, *J* = 9.7 Hz, *J* = 2.9 Hz, H-1), 3.65-3.60 (1H, m, H-1'), 2.65 (1H, s, H-6), 2.36 (3H, s, CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) 194.5 (C=O), 138.4, 137.9, 137.5 (Ar-qC), 128.3, 128.2, 128.1, 127.8, 127.6, 127.5, 127.4 (Ar-CH), 79.6 (C-5), 79.1 (C-3), 76.7 (C-6), 75.4 (Bn CH_2), 73.1 (C-4), 72.5 (Bn CH_2), 71.4 (Bn CH_2), 69.6 (C-1), 45.2 (C-2) ppm.

HRMS (*m/z*) calculated for $C_{29}H_{30}NaO_4S$ $[M + Na]^+$, calcd. 497.1763, found 497.1756.

ν_{max} (film)/cm^{-1} 3031 (Ar CH), 1693 (C=O), 1258 (CO).

(2*S*,3*S*,4*R*)-1,3,4-Tris(benzyloxy)hex-5-yne-2-thiol (51)



To a stirred solution of **50** (200 mg, 0.42 mmol) in anhydrous THF (4 mL) was added methyllithium (1 mL, 0.16 M solution in diethyl ether, 1.35 mmol) at -78 °C. The reaction mixture was warmed to -50 °C and stirred for 3 h. The reaction was cooled to -78 °C, quenched by addition of aq. 1 M HCl (3 mL) and diluted with Et_2O (30 mL). The organic layer was washed with H_2O (2 x 10 mL), dried over $MgSO_4$ and solvent was removed *in vacuo*. The product was purified by flash column chromatography on silica gel (5% Et_2O:Hex) to afford **51** as a colourless oil and was used immediately (58 mg, 32%).

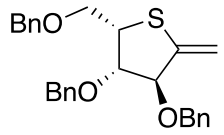
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24-7.36 (15H, m, Ph), 4.96-4.85 (2H, m, Bn CH_2), 4.62-4.56 (2H, m, Bn CH_2), 4.42 (2H, q, *J* = 12.0 Hz, Bn CH_2), 4.10 (1H, dd, *J* = 8.1 Hz, *J* = 2.0 Hz, H-3), 3.44-3.55 (4H, m, H-1, H-1', H-2, H-4), 2.57 (1H, d, *J* = 2.3 Hz, H-6), 1.68 (1H, d, *J* = 10.2 Hz, SH) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 138.6, 137.9, 137.7 (Ar-qC), 128.4, 128.3, 128.2, 127.9, 127.8, 127.7, 127.6, 127.5 (Ar-CH), 80.2 (C-5), 79.2 (C-3), 76.5 (C-6), 75.3 (Bn CH_2), 73.6 (C-4), 72.8 (Bn CH_2), 72.7 (Bn CH_2), 71.6 (C-1), 40.1 (C-2) ppm.

HRMS (*m/z*) calculated for $C_{27}H_{28}NaO_3S$ $[M + Na]^+$, calcd. 455.1651 ; found 455.1673.

1-Deoxy-1-methylene-2,3,5-tri-*O*-benzyl-4-thio-L-arabinofuranose

(2*S*,3*S*,4*S*)-3,4-Bis(benzyloxy)-2-((benzyloxy)methyl)-5-methylenetetrahydrothiophene (52)



To a solution of **51** (100 mg, 0.21 mmol) in MeOH (1 mL) was added a freshly prepared solution of sodium methoxide in MeOH (1 mL) and the reaction was stirred at rt for 1 h. The solution was neutralized with Dowex® H+ form. The resin was filtered, washed thoroughly with MeOH and the solvent was removed *in vacuo*. The product was

purified by flash column chromatography on silica gel (5% EtOAc:Hex) to afford **52** as a pale yellow oil (50 mg, 55%).

$[\alpha]_D^{22}$: -0.8 (deg cm^3 g^{-1} dm^{-1}) (c = 0.1, $CDCl_3$).

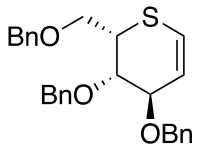
^1H NMR (400 MHz, $CDCl_3$) δ 7.29-7.38 (15H, m, PhCH), 5.31 (1H, s, C=CH_2), 5.27 (1H, s, C=CH_2), 4.71 (1H, d, *J* = 11.7 Hz, Bn CH_2), 4.63-4.50 (5H, m, Bn CH_2), 4.37 (1H, d, *J* = 3.1 Hz, H-4), 4.22-4.17 (1H, m, H-2), 4.12 (1H, t, *J* = 3.1 Hz, H-3), 3.91 (1H, dd, *J* = 9.5 Hz, *J* = 7.0 Hz, H-1), 3.71 (1H, dd, *J* = 9.5 Hz, *J* = 7.0 Hz, H-1') ppm

^{13}C NMR (100 MHz, $CDCl_3$) δ 145.3 (C-5), 137.9, 137.8, 137.6 (Ar-qC), 128.3, 128.2, 127.8, 127.7, 127.6, 127.5, 127.5, 127.4 (Ar-CH), 108.1 (C=$\underline{C}H_2$), 82.2 (C-3), 73.2 (Bn CH_2), 72.3 (Bn CH_2), 70.0 (C-4), 68.9 (C-1), 50.2 (C-2) ppm.

HRMS (*m/z*) calculated for $C_{27}H_{28}NaO_3S$ $[M + Na]^+$, calcd. 455.1651, found 455.1652.

ν_{max} (film)/cm^{-1} 3030 (Ar CH), 1720 (C=C), 1266 (CO).

(2*S*,3*S*,4*R*)-3,4-Bis(benzyloxy)-2-((benzyloxy)methyl)-3,4-dihydro-2*H*-thiopyran (53)



To a degassed solution of thiol **51** (57 mg, 0.13 mmol) in anhydrous DMF (264 μL) was added DPAP (6 mg, 26.4 μmol) and MBP (4 μL, 26.4 μmol). The solution was placed in a UV oven and irradiated at rt for 1 h without agitation to furnish a mixture of products **52** and **53**. The solvent was removed *in vacuo* and the products were isolated by flash column chromatography on silica gel (5% EtOAc:Hex). **53** was afforded as a pale yellow oil (12 mg, 22%).

$[\alpha]_D^{22}$: -0.3 (deg cm^3 g^{-1} dm^{-1}) (c = 0.1, $CDCl_3$)

^1H NMR (400 MHz, $CDCl_3$) δ 7.39-7.29 (15H, m, Ph), 6.37 (1H, d, *J* = 10.1 Hz, H-6), 5.85 (1H, dd, *J* = 10.1 Hz, *J* = 4.9 Hz, H-5), 4.71-4.50 (6H, m, Bn CH_2), 3.99-3.95 (1H, m, H-3), 3.91 (1H, t, *J* = 4.7 Hz, H-4), 3.76 (1H, dd, *J* = 9.0 Hz, *J* = 6.1 Hz, H-1), 3.67 (1H, td, *J* = 6.1 Hz, *J* = 2.1 Hz, H-2), 3.63 (1H, dd, *J* = 9.0 Hz, *J* = 6.1 Hz, H-1') ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 138.3, 137.9, 137.8 (Ar-qC), 128. 2, 127.9, 127.7, 127.6 (Ar-CH), 124.5 (C-5), 118.3 (C-6), 72.9 (Bn CH_2), 72.4 (C-3), 72.1 (Bn CH_2), 70.8 (Bn CH_2), 70.1 (C-4), 68.7 (C-1), 40.4 (C-2) ppm.

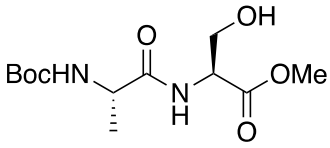
HRMS (*m/z*) calculated for $C_{27}H_{28}NaO_3S$ $[M + Na]^+$, calcd. 455.1651, found 455.1652.

ν_{max} (film)/cm^{-1} 3030 (Ar CH), 1720 (C=C), 1256 (CO).

6.4 Experimental details for Chapter 3

Methyl-(*tert*-butoxycarbonyl)-L-alanyl-L-serinatemethyl (*tert*-butoxycarbonyl)-L-alanyl-L-serinate (62)

Boc-Ala-Ser-OMe



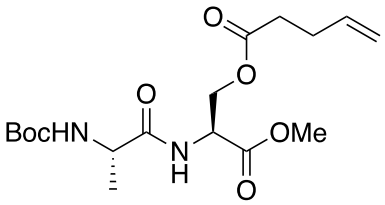
Prepared as per Procedure A using Boc-Ala-OH (244 mg, 1.29 mmol) and H_2N-Ser-OMe·HCl (200 mg, 1.29 mmol) in CH_2Cl_2 (8 mL) and DMF (2 mL). The crude product was purified by flash column chromatography on silica gel (2% MeOH:CH_2Cl_2) to afford **62** as an off-white oil (248 mg, 66%). The isolated compound was in good agreement with literature.[384]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.00 (1H, d, *J* = 7.2 Hz, N-H), 5.12 (1H, d, *J* = 5.4 Hz, N-H), 4.70-4.66 (1H, m, Ser αCH), 4.17 (1H, m, Ala αCH), 3.98 (2H, d, *J* = 7.9 Hz, Ser CH_2), 3.82 (3H, s, OCH_3), 1.47 (9H, s, $C(CH_3)_3$), 1.41 (3H, d, *J* = 7.0 Hz, Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{12}H_{21}N_2O_6$ $[M - H]^-$, calcd. 289.1401, found 289.1400.

(*S*)-2-((*S*)-2-((*tert*-Butoxycarbonyl)amino)propanamido)-3-methoxy-3-oxopropyl pent-4-enoate (64)

Boc-Ala-Ser-*O*-(4-pentenoate)-OMe



Prepared as per Procdure B using **62** (760 mg, 2.62 mmol) in anhydrous CH_2Cl_2 (25 mL). The crude product was purified by flash column chromatography on silica gel (40% EtOAc:Hex) to afford **64** as a colourless oil (798 mg, 82%).

R_f (40 % EtOAc:Hex): 0.36

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.87 (1H, d, *J* = 6.6 Hz, N-H), 5.84-5.80 (1H, m, C<u>H</u>=CH_2), 5.07-5.02 (3H, m, CH=C<u>H</u>$_2$, N-H), 4.87-4.83 (1H, m, Ser αCH), 4.48 (1H, dd, *J* = 11.4 Hz, *J* = 4.0 Hz, Ser CH_2), 4.39 (1H, dd, *J* = 11.4 Hz, *J* = 4.0 Hz, Ser CH_2), 4.24-2.40 (1H, m, Ala αCH), 3.79 (3H, s, OCH_3), 2.44 (2H, t, *J* = 7.1 Hz, C<u>H</u>$_2$$CH_2$CH=$CH_2$), 2.37 (2H, dd, *J* = 13.9 Hz, *J* = 7.1 Hz, CH_2C<u>H</u>$_2$CH=CH_2), 1.48 (9H, s, C(CH_3)$_3$), 1.39 (3H, d, *J* = 7.1 Hz, Ala CH_3) ppm.

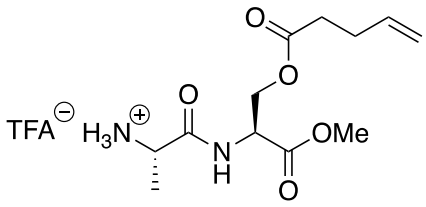
^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 171.2, 169.7, 155.4 (C=O), 136.5 (<u>C</u>H=CH_2), 115.9 (CH=<u>C</u>H_2), 80.3 (qC, Boc), 63.7 (Ser CH_2), 52.8 (OCH_3), 51.7 (Ser αCH), 50.4 (Ala αCH), 33.2 (<u>C</u>$H_2$$CH_2$CH=$CH_2$), 28.7 ($CH_2$<u>C</u>$H_2$CH=$CH_2$), 28.3 (C($CH_3$)$_3$), 18.2 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{17}H_{28}N_2NaO_7$ $[M + Na]^+$: calcd. 395.1788 ; found 395.1789.

ν_{max} (film)/cm^{-1} 3320 (NH), 2979 (C-H stretch), 1742 (C=O).

(*S*)-2-((*S*)-2-Aminopropanamido)-3-methoxy-3-oxopropyl pent-4-enoate (trifluoroacetate) (81)

TFA·H_2N-Ala-Ser-*O*-(4-pentenoate)-OMe



Prepared as per Procedure C using **64** (115 mg, 0.31 mmol) in CH_2Cl_2 (3 mL) the product **81** was obtained as a yellow oil (110 mg, 97%).

R_f (5% MeOH/CH_2Cl_2): 0.2

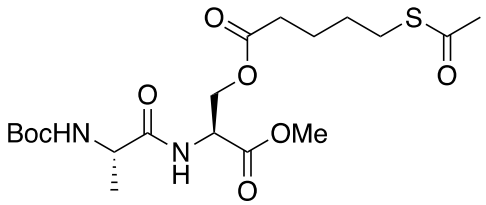
^{1}H NMR (400 MHz, MeOD) δ 5.89-5.79 (1H, m, C<u>H</u>=CH_2), 5.07 (1H, dq, *J* = 17.3 Hz, *J* = 3.2 Hz, *J* = 1.6 Hz, CH=C<u>H</u>$_2$), 5.00 (1H, dq, *J* = 10.4 Hz, *J* = 2.8 Hz, *J* = 1.6 Hz, CH=C<u>H</u>$_2$), 4.38 (2H, app. qd, *J* = 20.5 Hz, *J* = 11.2 Hz, *J* = 3.4 Hz, Ser CH_2), 4.27 (1H, td, *J* = 3.4 Hz, *J* = 1.3 Hz, Ser αCH), 4.08 (1H, qd, *J* = 13.9 Hz, *J* = 7.0 Hz, *J* = 1.2 Hz, Ala αCH), 3.78 (3H, s, OCH_3), 2.49-2.34 (4H, m, C<u>H</u>$_2$C<u>H</u>$_2$CH=CH_2), 1.51 (3H, d, *J* = 7.0 Hz, Ala CH_3) ppm.

^{13}C NMR (100 MHz, MeOD) δ 172.0, 171.6, 170.0 (C=O), 136.4 (<u>C</u>H=CH_2), 114.7 (CH=<u>C</u>H_2), 64.0 (Ser CH_2), 54.3 (Ser αCH), 52.2 (OCH_3) 50.5 (Ala αCH), 32.8 (<u>C</u>H_2CH_2CH=CH_2), 22.8 (CH_2<u>C</u>H_2CH=CH_2), 19.9 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{12}H_{21}N_2NaO_5$ $[M + Na]^+$, calcd. 273.1450, found 273.1449.

ν_{max} (film)/cm^{-1} 3330 (NH), 2898 (C-H stretch), 1705 (C=O) cm^{-1}.

(*S*)-2-((*S*)-2-(*tert*-Butoxycarbonyl)amino)propanamido)-3-methoxy-3-oxopropyl 5-(acetylthio)pentanoate (66)



Prepared as per Procedure D using dipeptide **64** (50.0 mg, 0.13 mmol) and thioacetic acid (14 μL, 0.20 mmol) in anhydrous DMF (2 mL) and following flash chromatography on silica gel (40% EtOAc:Hex) the product **66** was obtained as a yellow gum (59 mg, > 98%).

R_f (40% EtOAc:Hex): 0.29

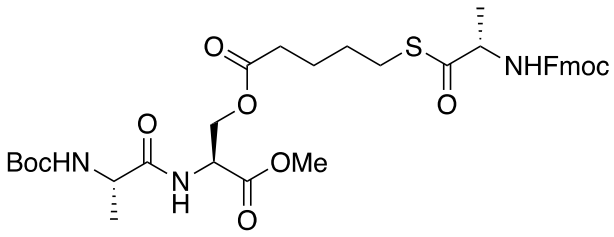
^{1}H NMR (400 MHz, $CDCl_3$) δ 6.94 (1H, d, *J* = 6.9 Hz, NH), 5.11 (1H, bs, NH), 4.86-4.83 (1H, m, Ser αCH), 4.48 (1H, dd, *J* = 11.3 Hz, *J* = 3.4 Hz, Ser CH_2), 4.39 (1H, dd, *J* = 11.3 Hz, *J* = 3.4 Hz, Ser CH_2), 4.24, (1H, bs, Ala αCH), 3.79 (3H, s, OCH_3), 2.89 (2H, t, *J* = 7.2 Hz, CH_2S), 2.36 (5H, m, $COCH_2CH_2CH_2CH_2S$, $SCOCH_3$), 1.72-1.67 (2H, m, $COCH_2CH_2CH_2CH_2S$), 1.60-1.64 (2H, m, $COCH_2CH_2CH_2CH_2S$), 1.47 (9H, s, $C(CH_3)_3$), 1.40 (3H, d, *J* = 7.1 Hz, Ala CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 196.1, 172.8, 172.6, 169.7, 155.4 (C=O), 79.6 (qC), 63.7 (Ser CH_2), 52.9 (Ala αCH), 51.8 (OCH_3), 50.2 (Ser αCH), 33.3 ($COCH_2CH_2CH_2CH_2S$), 30.7 (SAc CH_3), 28.9, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.5 $C(CH_3)_3$), 28.3 (CH_2S), 18.3 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{19}H_{32}N_2NaO_8S$ $[M + Na]^+$, calcd. 471.1777, found 471.1786

ν_{max} (film)/cm^{-1} 3329 (NH), 1685 (C=O), 1245 (C-O)

(*S*)-2-((*S*)-2-((*tert*-Butoxycarbonyl)amino)propanamido)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-alanyl)thio)pentanoate (71)



Prepared as per Procedure F using **118** (230 mg, 0.40 mmol) to give the crude thioacid, which was immediately subjected to Procedure D using dipeptide **64** (100 mg, 0.27 mmol) and purified by flash chromatography on silica gel (40% EtOAc:Hex) to furnish **71** as a yellow gum (75 mg, 40%).

R_f (40% EtOAc:Hex): 0.35

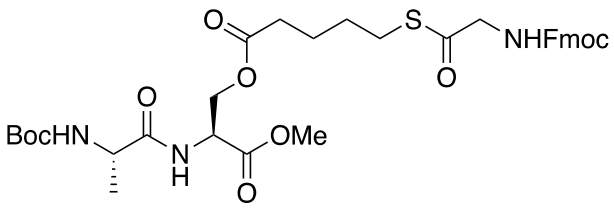
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.69 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.33 (2H, t, *J* = 7.5 Hz, Ar-CH), 6.96 (1H, d, *J* = 6.5 Hz, N-H), 5.27 (1H, bs, N-H), 5.14 (1H, d, *J* = 5.6 Hz, N-H), 4.87-4.80 (1H, m, Ser αCH), 4.50-4.46 (3H, m, Ser CH_2, Fmoc CH_2, Fmoc CH), 4.43-4.36 (2H, m, Ser CH_2, Fmoc CH_2), 4.30-4.24 (2H, m, Ala αCH, Ala αCH), 3.77 (3H, s, OCH_3), 2.93-2.88 (2H, m, $COCH_2CH_2CH_2CH_2S$), 2.40 (2H, t, *J* = 7.1 Hz, CH_2S), 1.74-1.63 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.46 (9H, s, $C(CH_3)_3$), 1.41, 1.39 (6H, d, *J* = 7.1 Hz, Ala CH_3, Ala CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 200.2, 172.9, 172.7, 169.7, 155.7, 155.5 (C=O), 143.9, 140.1 (Ar-qC), 127.8, 127.1, 126.6, 120.1 (Ar-CH), 80.9 (qC), 67.2 (Fmoc CH_2), 63.7 (Ser CH_2), 57.0 (Ser αCH), 52.9 (OCH_3), 51.8 (Ala αCH), 49.8 (Ala αCH), 47.2 (Fmoc CH), 38.0 ($COCH_2CH_2CH_2CH_2S$), 33.9 (CH_2S), 28.8 ($COCH_2CH_2CH_2CH_2S$), 28.3 ($C(CH_3)_3$), 23.3 ($COCH_2CH_2CH_2CH_2S$), 18.7 (Ala CH_3), 18.4 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{25}H_{45}N_3NaO_{10}S$ $[M + Na]^+$, calcd. 722.2723, found 722.2721.

ν_{max} (film)/cm^{-1} 3330 (NH), 1678 (C=O stretch), 1450 (Ar C-C), 1248 (CO).

(*S*)-2-((*S*)-2-((*tert*-Butoxycarbonyl)amino)propanamido)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)pentanoate (80)



Prepared as per Procedure F using **117** (446 mg, 0.80 mmol) to give the crude thioacid, which was immediately subjected to Procedure D using dipeptide **64** (100 mg, 0.27 mmol) and purified by flash chromatography on silica gel (40% EtOAc:Hex) to furnish **80** as a yellow gum (180 mg, > 98%).

R_f (30% EtOAc:Hex): 0.26

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.60 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.39 (2H, t, *J* = 7.4 Hz, Ar-CH), 7.29 (2H, t, *J* = 7.4 Hz, Ar-CH), 6.96 (1H, d, *J* = 6.6 Hz, N-H), 5.82 (1H, bs, N-H), 5.18 (1H, bs, N-H), 4.84-4.87 (1H, m, Ser αCH), 4.40-4.51 (4H, m, Ser CH_2, Fmoc CH_2), 4.30-4.26 (2H, m, Ala αCH, Fmoc CH), 4.18 (2H, m, Gly CH_2), 3.75 (3H, s, OCH_3), 2.92-2-88 (2H, m, CH_2S), 2.33 (2H, t, *J* = 7.1 Hz, $COCH_2CH_2CH_2CH_2S$), 1.64-1.72 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.43 (s, 9H, $C(CH_3)_3$), 1.36 (3H, d, *J* = 7.1 Hz, Ala CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.2, 172.7, 171.2, 169.7, 156.4, 155.5 (C=O), 143.8, 141.3 (Ar-qC), 127.6, 127.1, 125.2, 120.0 (Ar-CH), 80.2 (Boc qC), 67.4 (Fmoc CH_2), 63.7 (Ser CH_2), 53.0 (OCH_3), 51.9 (Ser αCH), 50.7 (Gly CH_2), 50.1 (Ala αCH), 47.3 (Fmoc CH), 33.3 ($COCH_2CH_2CH_2CH_2S$), 29.3 ($COCH_2CH_2CH_2CH_2S$), 28.4 ($C(CH_3)_3$), 28.0 (CH_2S), 23.6 ($COCH_2CH_2CH_2CH_2S$), 18.6 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{34}H_{43}N_3NaO_{10}S$ $[M + Na]^+$, calcd. 708.2567, found 708.2566.

ν_{max} (film)/cm^{-1} 3327 (NH), 1676 (C=O), 1449 (Ar C-C), 1244 (CO).

(9*H*-Fluoren-9-yl)methyl ethylcarbamate (75)

Isolated as a byproduct from the synthesis of **71**.

M.p. 140-144 °C.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.63 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.2 Hz, Ar-CH), 7.35 (2H, t, *J* = 7.2 Hz, Ar-CH), 4.69 (1H, bs, N-H), 4.43 (2H, d, *J* = 6.9 Hz, Fmoc CH_2), 4.25 (1H, t, *J* = 6.9 Hz, Fmoc CH), 3.28 (2H, t, *J* = 6.4 Hz, $C\underline{H}_2CH_3$), 1.19 (3H, t, *J* = 6.4 Hz, $CH_2C\underline{H}_3$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 143.6 (C=O), 141.3, 140.9 (Ar-qC), 127.6, 127.0, 124.8, 119.7 (Ar-CH), 66.2 (Fmoc CH_2), 47.1 (Fmoc CH), 35.6 ($\underline{C}H_2CH_3$), 15.0 ($CH_2\underline{C}H_3$) ppm.

HRMS (*m/z*) calculated for $C_{16}H_{15}NNaO_2$ $[M + Na]^+$, calcd. 290.1157, found 290.1149.

ν_{max} (film)/cm^{-1} 3333 (NH), 2975 (C-H), 1690 (C=O), 1254 (CO).

(9*H*-Fluoren-9-yl)methyl methylcarbamate (79)

FmocHN—

Isolated as a byproduct from the synthesis of **80**.

M.p. 140-143 °C.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.63 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.34 (2H, t, *J* = 7.5 Hz, Ar-CH), 4.76 (1H, bs, N-H), 4.44 (2H, d, *J* = 6.9 Hz, Fmoc CH_2), 4.25 (1H, t, *J* = 6.9 Hz, Fmoc CH), 2.86 (3H, d, *J* = 5.1 Hz, CH_3) ppm.

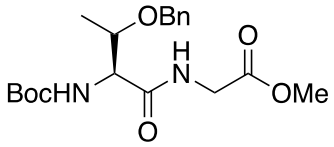
^{13}C NMR (100 MHz, $CDCl_3$) δ 157.1 (C=O), 144.1, 141.4 (Ar-qC), 127.7, 127.1, 125.0, 120.0 (Ar-CH), 66.7 (Fmoc CH_2), 47.3 (Fmoc CH), 27.6 (CH_3) ppm.

HRMS (*m/z*) calculated for $C_{16}H_{15}NNaO_2$ $[M + Na]^+$, calcd. 276.1000, found 276.1003.

ν_{max} (film)/cm^{-1} 3339 (NH), 1695 (C=O), 1543 (Ar C-C), 1271 (CO).

Methyl-*O*-benzyl-*N*-(*tert*-butoxycarbonyl)-L-allothreonylglycinate (91)

Boc-Thr(OBn)-Gly-OMe



Prepared as per Procedure A using Boc-Thr(OBn)-OH (2.5 g, 7.96 mmol) and Gly-OMe·HCl (1.0 g, 7.96 mmol) in anhydrous CH_2Cl_2 (70 mL) and anhydrous DMF (10mL). Following column chromatography (2% MeOH:CH_2Cl_2), **91** was obtained as a white solid (2.1 g, 89%).

R_f (2% MeOH:CH_2Cl_2): 0.30

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.30 (5H, m, Ar-CH), 7.10-7.05 (1H, m, NH), 5.53 (1H, d, *J* = 6.8 Hz, NH), 4.65-4.61 (2H, m, Bn CH_2), 4.38-4.33 (1H, m, Thr αCH), 4.25-4.22 (1H, m, Thr CH), 4.07 (2H, t, *J* = 5.5 Hz, Gly αCH_2), 3.76 (3H, s, OCH_3), 1.48 (9H, s, $C(CH_3)_3$), 1.23 (3H, d, *J* = 6.3 Hz, Thr CH_3) ppm.

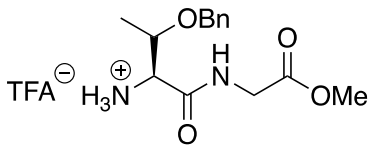
^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3, 170.2, 170.0 (C=O), 138.0 (Ar-qC), 128.5, 127.9 (Ar-CH), 80.2 (qC), 74.7 (Thr CH), 71.8 (Bn CH_2), 57.8 (Thr αCH), 52.5 (OCH_3), 41.2 (Gly αCH_2), 28.5 ($C(CH_3)_3$), 15.4 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{19}H_{28}N_2O_6Na$ $[M + H]^+$, calcd. 403.1845, found 403.1851.

ν_{max} (film)/cm^{-1} 3408 (NH), 1695 (C=O).

Methyl-*O*-benzyl-L-allothreonylglycinate (trifluoacetate) (91)

TFA·H_2N-Thr(OBn)-Gly-OMe



Prepared as per Procedure C using **91** (961 mg, 2.53 mmol) in CH_2Cl_2 (20 mL) and the product **92** was obtained as a colourless oil (784 mg, 98%).

R_f (5% EtOAc:Hex): 0.2

^{1}H NMR (400 MHz, MeOD) δ 7.36-7.25 (5H, m, Ar-CH), 4.64-4.48 (3H, m, Thr αCH, Bn CH_2), 4.20 (1H, m, Thr CH), 3.94 (2H, m, Gly αCH_2), 3.59 (3H, s, OCH_3), 1.42 (3H, d, *J* = 5.5 Hz, Thr CH_3) ppm.

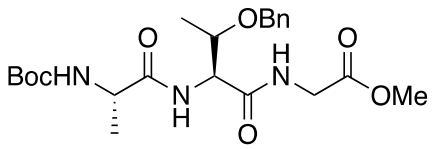
^{13}C NMR (100 MHz, MeOD) δ 170.1, 167.8 (C=O), 137.3 (Ar-qC), 128.5, 128.2, 127.9 (Ar-CH), 73.6 (Thr CH), 71.6 (Bn CH_2), 57.4 (Thr αCH), 52.5 (OCH_3), 41.4 (Gly αCH_2), 16.2 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{14}H_{21}N_2O_4$ $[M + H]^+$, calcd. 281.1501, found 281.1494.

ν_{max} (film)/cm^{-1} 2923 (NH), 1749, 1659 (C=O).

Methyl-*O*-benzyl-*N*-((*tert*-butoxycarbonyl)-L-alanyl)-L-allothreonylglycinate (93)

Boc-Ala-Thr(OBn)-Gly-OMe



Prepared as per Procedure A using Boc-Ala-OH (1.3 g, 6.95 mmol) and **92** (2.2 g, 6.95 mmol) in anhydrous CH_2Cl_2 (60 mL) and DMF (10 mL). Following column chromatography (60% EtOAc/Hex) **93** was obtained as a white solid (2.8 g, 89%).

R_f (60% EtOAc:Hex): 0.30

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.39-7.31 (6H, m, Ar-CH, NH), 7.00 (1H, d, *J* = 7.3 Hz, NH), 4.9 (1H, bs, NH), 4.64 (2H, s, Bn CH_2), 4.54 (1H, d, *J* = 7.3 Hz, Thr αCH), 4.37-4.32 (1H, m, Thr CH), 4.19-4.12 (1H, m, Ala αCH), 4.05 (2H, dd, *J* = 23.7, *J* = 5.3, Gly CH_2), 3.73 (3H, s, OCH_3), 1.43 (9H, s, $C(CH_3)_3$), 1.21 (6H, d, *J* = 6.4 Hz, Ala CH_3, Thr CH_3) ppm.

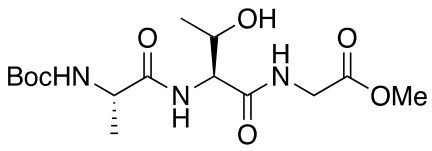
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 170.4, 169.8, 156.2 (C=O), 138.3 (Ar-qC), 128.4, 127.9, 127.8 (Ar-CH), 80.9 (qC), 73.9 (Thr CH), 71.9 (Bn CH_2), 56.5 (Thr αCH), 52.2 (OCH_3), 51.3 (Ala αCH), 41.5 (Gly CH_2), 28.2 ($C(CH_3)_3$), 23.2 (Thr CH_3, Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{22}H_{33}N_3NaO_7$ $[M + Na]^+$, calcd. 474.2224, found 474.2216.

ν_{max} (film)/cm^{-1} 3309 (NH), 1751, 1644 (C=O).

Methyl-(*tert*-butoxycarbonyl)-L-alanyl-L-allothreonylglycinate (94)

Boc-Ala-Thr(OH)-Gly-OMe



To a stirred solution of **93** (2.8 g, 6.19 mmol) in EtOAc (10 mL) was added $Pd(OH)_2/C$ (164 mg, 1.55 mmol) and the mixture was deoxygenated with N_2 for 10 min. The solution was then stirred at rt for 3 h under a balloon of H_2. The product was filtered and the solvent was removed *in vacuo* to give **94** as a white solid (1.9 g, 79%).

R_f (60% EtOAc:Hex): 0.15

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55-7.50 (1H, m, NH), 7.11 (1H, d, *J* = 7.4 Hz, NH), 5.05 (1H, d, *J* = 3.3 Hz, NH), 4.48-4.41 (2H, m, αThr CH, Thr CH), 4.22-4.14 (2H, m, Ala αCH, Gly αCH_2), 3.92-3.86 (1H, m, Gly αCH_2), 3.75 (3H, s, OCH_3), 1.43-1.41 (12H, m, $C(CH_3)_3$, Ala CH_3), 1.21 (3H, d, *J* = 6.4 Hz, Thr CH_3) ppm.

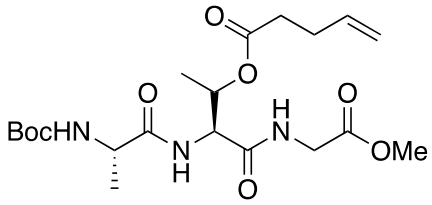
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.5, 171.7, 170.8, 156.0 (C=O), 81.0 (qC), 67.0 (Thr αCH), 58.0 (Thr CH), 52.5 (OCH_3), 51.2 (Ala αCH), 41.0 (Gly αCH_2), 28.2 ($C(CH_3)_3$), 18.7 (Thr CH_3), 17.9 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{15}H_{27}N_3NaO_7Na$ $[M + Na]^+$, calcd. 384.1748, found 384.1747.

ν_{max} (film)/cm^{-1} 3316 (NH), 1754, 1636 (C=O).

Methyl-(6*S*,9*S*)-2,2,6-trimethyl-4,7,10-trioxo-9-((*S*)-1-(pent-4-enoyloxy)ethyl)-3-oxa-5,8,11-triazatridecan-13-oate (95)

Boc-Ala-Thr-*O*-(4-pentenoate)-Gly-OMe



Prepared as per Procedure B using **94** (32 mg, 0.09 mmol) in CH_2Cl_2 (2 mL) and following column chromatography (60% EtOAc:Hex) gave the product **95** as a white solid (35 mg, 87%).

R_f (60% EtOAc:Hex): 0.28

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.05-6.99 (2H, m, 2 x NH), 5.89-5.80 (1H, m, C<u>H</u>=CH_2), 5.55-5.50 (1H, m, Thr CH), 5.11-4.98 (3H, m, CH=C<u>H</u>$_2$, NH), 4.61 (1H, dd, *J* = 8.6 Hz, *J* = 3.2 Hz, Thr αCH), 4.21-4.08 (2H, m, Ala αCH, Gly CH_2), 3.94 (1H, m, Gly CH_2), 3.75 (3H, s, OCH_3), 2.50-2.36 (4H, m, CH_2CH_2), 1.45-1.43 (12H, m, $C(CH_3)_3$, Ala CH_3), 1.26 (3H, d, *J* = 5.9 Hz, Thr CH_3) ppm.

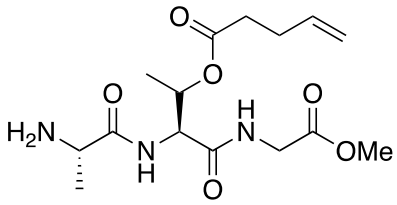
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 171.7, 169.7, 169.0 (C=O), 136.7 (CH_2=<u>C</u>H), 115.6 (<u>C</u>H_2=CH, NH), 80.9 (qC), 69.8 (Thr CH), 56.5 (Thr αCH), 52.2 (OCH_3), 51.0 (Ala αCH), 41.0 (Gly CH_2), 33.4, 28.8 (CH_2CH_2), 28.2 ($C(CH_3)_3$), 17.8 (Ala CH_3), 16.6 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{20}H_{33}N_3O_8Cl$ $[M - H]^-$, calcd. 478.1956, found 478.1955.

ν_{max} (film)/cm^{-1} 3301 (NH), 1737, 1645 (C=O).

(2*S*,3*S*)-3-((*S*)-2-Aminopropanamido)-4-((2-methoxy-2-oxoethyl)amino)-4-oxobutan-2-yl pent-4-enoate (96)

H_2N-Ala-Thr-*O*-(4-pentenoate)-Gly-OMe



Prepared as per Procedure C using **95** (35 mg, 0.07 mmol) in CH_2Cl_2 (2 mL), the crude product was basified with NEt_3 until pH 7 and concentrated *in vacuo*. The product **96** was obtained as a yellow oil (24 mg, > 98%).

R_f (10% MeOH:CH_2Cl_2): 0.22

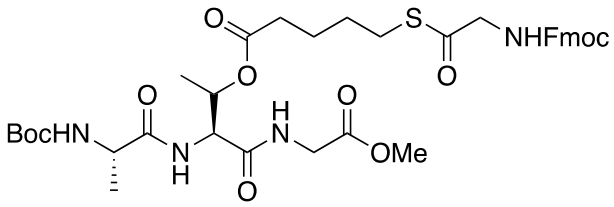
^{1}H NMR (400 MHz, MeOD) δ 5.88-5.75 (1H, m, C<u>H</u>=CH_2), 5.38-5.32 (1H, m, Thr CH), 5.06-4.95 (2H, m, CH=C<u>H</u>$_2$), 4.69 (1H, dd, *J* = 13.0 Hz, *J* = 4.3 Hz, Thr αCH), 4.14-4.06 (1H, m, Ala αCH), 4.01-3.88 (2H, m, Gly CH_2), 3.71 (3H, s, OCH_3), 2.46-2.31 (4H, m, CH_2CH_2), 1.54 (3H, d, *J* = 7.0 Hz, Ala CH_3), 1.28 (3H, d, *J* = 6.2 Hz, Thr CH_3) ppm.

^{13}C NMR (100 MHz, MeOD) δ 170.8, 168.7, 168.4, 168.3 (C=O), 135.5 (<u>C</u>H=CH_2), 113.0 (CH=<u>C</u>H_2), 68.5 (Thr CH), 55.2 (Thr αCH), 49.8 (OCH_3), 47.4 (Ala αCH), 39.1 (Gly CH_2), 31.4, 26.9 (CH_2CH_2), 14.8 (Ala CH_3), 14.1 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{15}H_{26}N_3O_6$ $[M + H]^+$, calcd. 344.1816, found 344. 1821.

ν_{max} (film)/cm^{-1} 3288 (NH), 1660 (C=O).

Methyl-(6*S*,9*S*)-9-((*S*)-1-(9*H*-fluoren-9-yl)-3,6,12-trioxo-2,13-dioxa-7-thia-4-azapentadecan-14-yl)-2,2,6-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate (104)



Prepared as per Procedure F using **117** (376 mg, 0.68 mmol) to give the crude thioacid, which was immediately subjected to Procedure D using **95** (100 mg, 0.22 mmol) in anhydrous DMF (2 mL). Following column chromatography (40-80% EtOAc:Hex) the product **104** was obtained as a white solid (162 mg, 95%).

R_f (60% EtOAc:Hex): 0.28

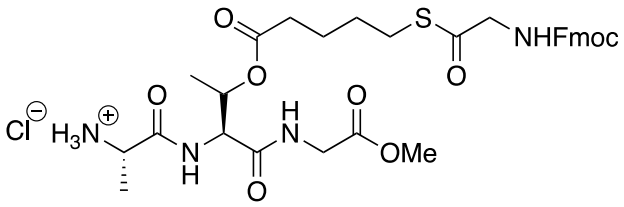
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.63 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.6 Hz, Ar-CH), 7.33 (2H, t, *J* = 7.6 Hz, Ar-CH), 7.01-6.97 (2H, m, 2 x NH), 5.52 (1H, d, *J* = 38.8 Hz, NH), 5.50-5.45 (1H, m, Thr CH), 5.11 (1H, d, *J* = 3.73 Hz, NH), 4.58 (1H, dd, *J* = 8.5 Hz, *J* = 3.2 Hz, Thr αCH), 4.42 (2H, d, *J* = 6.9 Hz, Fmoc CH_2), 4.24-3.87 (6H, m, Fmoc CH, Gly CH_2, Gly CH_2, Ala αCH), 3.74 (3H, s, OCH_3), 2.94-2.89 (2H, m, CH_2S), 2.36-2.31 (2H, m, $COCH_2CH_2CH_2CH_2S$), 1.71-1.58 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.44-1.40 (12H, m, $C(CH_3)_3$, Ala CH_3), 1.26 (3H, d *J* = 6.4 Hz, Thr CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.1, 173.4, 172.0, 169.8, 169.2, 156.4 (C=O), 143.8, 141.3 (Ar-qC), 127.8, 127.2, 125.1, 120.0 (Ar-CH), 80.7 (qC), 69.8 (Thr αCH), 67.2 (Fmoc CH_2), 55.9 (Thr CH), 52.4 (OCH_3), 50.9 (Ala αCH), 50.7 (Gly' CH_2), 47.1 (Fmoc CH), 41.3 (Gly CH_2), 33.7 (CH_2S), 28.9, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.0 ($COCH_2CH_2CH_2CH_2S$), 28.1 ($C(CH_3)_3$), 17.9 (Ala CH_3), 16.7 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{37}H_{48}N_4O_{11}S_2$ $[M + H]^+$, calcd. 779.2946, found 779.2938.

ν_{max} (film)/cm^{-1} 3316 (NH), 1659 (C=O).

(2*S*,3*S*)-3-((*S*)-2-Aminopropanamido)-4-((2-methoxy-2-oxoethyl)amino)-4-oxobutan-2-yl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)pentanoate hydrochloride (108)



Prepared as per Procedure C using **104** (135 mg, 0.18 mmol) in CH_2Cl_2 gave the product **108** as a yellow oil (136 mg, > 98%).

R_f (5% MeOH:CH_2Cl_2): 0.25

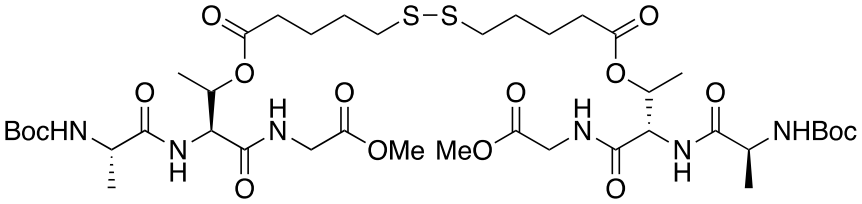
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.63 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.33 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.13 (1H, bs, NH), 7.05 (1H, d, *J* = 8.7 Hz, NH), 5.68 (1H, bs, NH), 5.54-5.50 (1H, m, Thr CH), 5.21 (1H, d, *J* = 5.0 Hz, NH), 4.63 (1H, dd, *J* = 8.7 Hz, *J* = 3.5 Hz, Thr αCH), 4.45 (2H, d, *J* = 7.5 Hz, Fmoc CH_2), 4.26 (1H, t, *J* = 7.5 Hz, Fmoc CH), 4.22-4.05 (4H, m, Ala αCH, Gly CH_2, Gly CH_2), 3.95-3.92 (1H, m, Gly CH_2), 3.72 (3H, s, OCH_3), 2.98 (2H, m, CH_2S), 2.40 (2H, m, $COCH_2CH_2CH_2CH_2S$), 1.75-1.60 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.47-1.44 (3H, m, Ala CH_3), 1.28-1.26 (3H, m, Thr CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.1, 184.9, 173.2, 69.8, 169.2 (C=O), 143.7, 141.3 (Ar-qC), 127.8, 127.1, 125.1, 120.1 (Ar-CH), 70.1 (Thr CH), 67.5 (Fmoc CH_2), 56.3 (Thr αCH), 52.3 (OCH_3), 51.0 (Ala αCH), 47.3 (Fmoc CH), 41.2 (2 x Gly CH_2), 33.5 (CH_2S), 28.8, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.1 ($COCH_2CH_2CH_2CH_2S$), 17.9 (Ala CH_3), 16.5 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{32}H_{41}N_4O_9S$ $[M + H]^+$, calcd. 657.2568, found 657.2588.

ν_{max} (film)/cm^{-1} 3320 (NH), 1685 (C=O).

Dimethyl-9,9'-((1*S*,1'*S*)-((5,5'-disulfanediylbis(pentanoyl))bis(oxy))bis(ethane-1,1-diyl))(6*S*,6'*S*,9*S*,9'*S*)-bis(2,2,6-trimethyl-4,7,10-trioxo-3-oxa-5,8,11-triazatridecan-13-oate) (105)



To a solution of **104** (150 mg, 0.20 mmol) in DMF (5 mL) was added benzylamine (60 μL, 0.59 mmol) and the reaction mixture was stirred at rt for 18 h. The crude material was diluted with H_2O (15 mL) and extracted with EtOAc (2 x 10 mL). The organic layer was washed with brine (2 x 20 mL), dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The residue was purified by flash column chromatography (80% EtOAc:Hex) to give the disulfide product **104** as a yellow oil (46 mg, 50%).

R_f (100% EtOAc): 0.45

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (1H, t, d, *J* = 4.9 Hz, NH), 7.15 (1H, d, *J* = 8.1 Hz, NH), 5.28 (1H, bs, NH), 5.45 (1H, app. bs, Thr CH), 4.69 (1H, dd, *J* = 8.4, *J* = 3.3 Hz, Thr αCH), 4.19 (1H, app. t, *J* = 6.0 Hz, Ala αCH), 4.01 (2H, qd, *J* = 40.0 Hz, *J* = 18.0 Hz, *J* = 5.4 Hz, Gly CH_2), 3.73 (3H, s, OCH_3), 2.68 (2H, t, *J* = 6.3 Hz, CH_2S), 2.40-2.31 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.73-1.70 (2H, m, $COCH_2CH_2CH_2CH_2S$), 1.45 (9H, s, $C(CH_3)_3$), 1.40 (3H, d, *J* = 7.1 Hz, Ala CH_3), 1.27 (3H, d, *J* = 6.5 Hz, Thr CH_3) ppm.

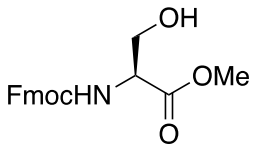
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4, 172.2, 169.9, 169.2, 156.0 (C=O), 80.7 (qC), 69.9 (Thr CH), 56.3 (Thr αCH), 52.3 (OCH_3), 50.9 (Ala αCH), 41.2 (Gly CH_2), 38.5 (CH_2S), 33.7 ($COCH_2CH_2CH_2CH_2S$), 28.5, 23.5 ($COCH_2CH_2CH_2CH_2S$), 28.4 ($C(CH_3)_3$), 17.9 (Ala CH_3), 16.6 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{40}H_{68}N_6NaO_{16}S_2$ $[M + Na]^+$, calcd. 975.4031, found 975.4003.

ν_{max} (film)/cm^{-1} 1677 (C=O), 737, 697 (CH).

Methyl-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-serinate (109)

Fmoc-Ser-OMe



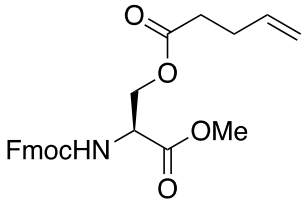
To a stirred solution of HCl·H_2N-Ser-OMe (1.0 g, 6.45 mmol) in H_2O (20 mL) was added $NaHCO_3$ (2.2 g, 16.13 mmol). To this was added (slowly) a solution of Fmoc-OSu (2.2 g, 6.45 mmol) in 1,4-Dioxane (20 mL) and the reaction was stirred at rt for 2 h. The mixture was diluted with H_2O (20 mL) and the product was extracted with EtOAc (2 x 20 mL). The organic phase was washed with brine (2 x 20 mL), dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure to yield the product **109** as a white solid (2.1 g, 95%). The compound was in good agreement with the literature.[366]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (2H, d, *J* = 7.5 Hz, Ar-CH_2), 7.58 (2H, d, *J* = 7.5 Hz, Ar-CH_2), 7.38 (2H, t, *J* = 7.5 Hz, Ar-CH_2), 7.29 (2H, t, *J* = 7.5 Hz, Ar-CH_2), 5.85 (1H, d, *J* = 7.5 Hz, NH), 4.45-4.39 (3H, m, Fmoc CH_2, Ser αCH), 4.20 (1H, t, *J* = 6.8 Hz, Fmoc CH), 4.01-3.88 (2H, m, Ser CH_2), 3.75 (3H, s, OCH_3) ppm.

HRMS (*m/z*) calculated for $C_{19}H_{20}NO_5$ $[M + H]^+$, calcd. 342.1335, found 342.1340.

(*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl pent-4-enoate (110)

Fmoc-Ser-(*O*-pentenoate)-OMe



As per Procedure B using **109** (100 mg, 0.29 mmol) in CH_2Cl_2 (3 mL) and following flash chromatography (30% EtOAc:Hex) the product **110** was obtained as a pale yellow solid (106 mg, 86%).

R_f (40% EtOAc:Hex): 0.38

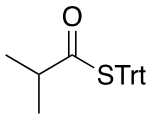
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.63 (2H, dd, *J* = 7.5 Hz, *J* = 2.2 Hz, Ar-CH), 7.44 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.35 (2H, t, *J* = 7.5 Hz, Ar-CH), 5.88-5.78 (1H, m, C<u>H</u>=CH_2), 5.59 (1H, d, *J* = 8.1 Hz, NH), 5.11-5.02 (2H, m, CH=C<u>H_2</u>), 4.68-4.64 (1H, m, Ser αCH), 4.54-4.40 (4H, m, Ser CH_2, Fmoc CH_2), 4.27 (1H, t, J = 7.0 Hz, Fmoc CH), 3.82 (3H, s, OCH_3), 2.49-2.36 (4H, m, CH_2CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 172.6, 172.0, 155.8 (C=O), 143.9, 143.7, 141.4 (Ar-qC), 127.8, 127.2, 125.1, 120.0 (Ar-CH), 136.4 (<u>C</u>H=CH_2), 115.8 (CH=<u>C</u>H_2), 67.3 (Fmoc CH_2), 63.9 (Ser CH_2), 53.5 (Ser αCH), 53.0 (OCH_3), 47.2 (Fmoc CH), 33.3, 28.8 (CH_2CH_2) ppm.

HRMS (*m/z*) calculated for $C_{24}H_{25}NNaO_6$ $[M + Na]^+$, calcd. 446.1561, found 446.1574.

ν_{max} (film)/cm^{-1} 3328 (NH), 2949, 1740, 1703 (C=O).

_S_-Trityl 2-methylpropanethioate (111)



Prepared as per Procedure E using isobutyric acid (0.4 mL, 4.62 mmol) in CH_2Cl_2 (50 mL) and following flash column chromatography (5% EtOAc:Hex) gave the product **111** as a yellow oil (969 mg, 60%).

R_f (5% EtOAc:Hex): 0.30

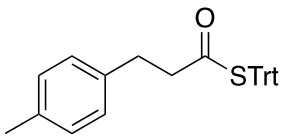
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.26 (15H, m, Ar-CH), 2.82-2.76 (1H, m, CH), 1.15 (6H, d, *J* = 6.8 Hz, 2 x CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 201.3 (C=O), 144.4 (Ar-qC), 129.9, 127.8, 127.2 (Ar-CH), 69.8 (qC), 43.1 (CH), 19.4 (2 x CH_3) ppm.

HRMS (*m/z*) calculated for $C_{23}H_{22}NaOS$ $[M + Na]^+$, calcd. 369.1283, found 369.1288.

ν_{max} (film)/cm^{-1} 1690 (C=O), 734 (CH).

3-(*p*-Tolyl)propanethioic *S*-acid (112)



Prepared as per Procedure E using 3-(*p*-tolyl)propanoic acid (290 mg, 1.77 mmol) in CH_2Cl_2 (18 mL) and following flash column chromatography (2% EtOAc:Hex) gave the product **112** as a yellow oil (742 mg, 99%).

R_f (5% EtOAc:Hex): 0.41

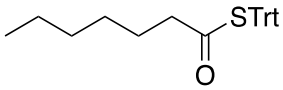
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.24 (15H, m, Ar-CH), 7.12 (4H, dd, *J* = 27.9 Hz, *J* = 7.9 Hz, 2 x Ar-CH), 2.88 (4H, m, 2 x CH_2), 2.39 (3H, s, CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 196.2 (C=O), 143.9 (Ar-qC), 137.0, 135.9, 129.8, 129.2, 128.4, 127.8, 127.1 (Ar-CH), 70.4 (qC), 45.3 (αCH_2), 30.9 (CH_2), 21.1 (CH_3) ppm.

HRMS (*m/z*) calculated for $C_{29}H_{26}NaOS$ [M + Na]+, calcd. 445.1704, found 445.1724.

ν_{max} (film)/cm^{-1} 1695 (C=O), 734, 697 (CH).

***S*-Trityl heptanethioate (113)**



Prepared as per Procedure E by Dr. Helen Burke.

R_f (20% EtOAc:Hex): 0.42

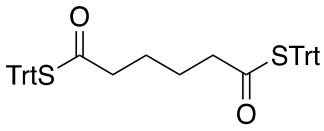
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35-7.25 (15H, m, Ar-CH), 2.53 (2H, t, *J* = 7.3 Hz, CH_2), 1.65-1.58 (2H, m, CH_2), 1.34-1.26 (6H, m, 3 x CH_2), 0.91 (3H, t, *J* = 7.0 Hz, CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 197.2, 144.1 (Ar-qC), 129.9, 127.8, 127.1 (Ar-CH), 70.4 (qC), 44.0, 31.4, 28.4, 25.6, 22.5 (5 x CH_2), 14.1 (CH_3) ppm.

HRMS (*m/z*) calculated for $C_{26}H_{28}NaOS$ [M + Na]$^+$, calcd. 411.1740, found 411.1753.

ν_{max} (film)/cm^{-1} 1694 (C=O), 738 (CH).

Hexanebis(thioic) *S*,*S*-acid (114)



Prepared as per Procedure E using adipic acid (219 mg, 1.50 mmol) in CH_2Cl_2 (15 mL) and following flash column chromatography gave the product **114** (10% EtOAc:Hex) as a white solid (960 mg, 97%).

R_f (20% EtOAc:Hex): 0.38

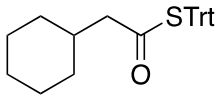
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29-7.23 (15H, m, Ar-CH), 2.48 (4H, t, *J* = 5.7 Hz, 2 x CH_2), 1.59-1.53 (4H, m, 2 x CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 196.8 (C=O), 144.1 (Ar-qC), 129.8, 127.8, 127.2 (Ar-CH), 70.4 (qC), 43.4, 24.8 (4 x CH_2) ppm.

HRMS (*m/z*) calculated for $C_{44}H_{38}NaO_2S_2$ $[M + Na]^+$, calcd. 685.2239, found 685.2205.

ν_{max} (film)/cm^{-1} 1677 (C=O), 697 (CH).

2-Cyclohexylethanethioic *S*-acid (115)



Prepared as per Procedure E using cyclohexaneacetic acid (400 mg, 2.81 mmol) in CH_2Cl_2 (30 mL) and following column chromatography (1% EtOAc:Hex) gave the product **115** as a white crystalline solid (875 mg, 78%).

M.p. 79-81 °C.

R_f (1% EtOAc:Hex): 0.28

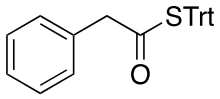
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.26 (15H, m, Ar-CH), 2.41 (2H, d, *J* = 6.9 Hz, αCH_2), 1.86-1.77 (1H, m, CH), 1.72-1.64, 1.31-1.10, 1.01-0.90 (10H, m, 5 x CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 196.4 (C=O), 144.0 (Ar-qC), 129.9, 127.8, 127.1 (Ar-CH), 70.4 (qC), 51.4 (αCH_2), 35.7 (CH), 32.8, 26.1, 26.0 (5 x CH_2) ppm.

HRMS (*m/z*) calculated for $C_{27}H_{28}NaOS$ $[M + Na]^+$, calcd. 423.1735, found 423.1753.

ν_{max} (film)/cm^{-1} 1686 (C=O), 738, 694 (CH).

_S_-Trityl 2-phenylethanethioate (116)



Prepared as per Procedure E using phenylacetic acid (241 mg, 1.77 mmol) in CH_2Cl_2 (18 mL) and following column chromatography (5% EtOAc:Hex) gave the product **116** as a white crystalline solid (413 mg, 59%).

M.p. 84-86 °C.

R_f (5% EtOAc:Hex): 0.30

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35-7.19 (20H, m, Ar-CH), 3.78 (2H, s, αCH_2) ppm.

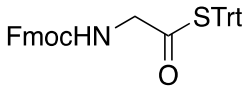
^{13}C NMR (100 MHz, $CDCl_3$) δ 194.3 (C=O), 143.7, 133.6 (Ar-qC), 129.8, 129.4, 128.7, 127.8, 127.1, 127.3 (Ar-CH), 70.7 (qC), 50.4 (αCH_2) ppm.

HRMS (*m/z*) calculated for $C_{27}H_{22}NaOS$ $[M + Na]^+$, calcd. 417.1273, found 417.1284.

ν_{max} (film)/cm^{-1} 3027 (NH), 1699 (C=O), 693 (CH).

_S_-Triphenylmethane 2-[((_S_)-9-fluorenylmethoxycarbonyl)amino] ethanethioate (117)

Fmoc-Gly-STrt



Prepared as per Procedure E using Fmoc-Gly-OH (1.40 g, 4.71 mmol), the product **117** was obtained following flash column chromatography on silica gel (10% EtOAc:Hex) to afford white crystals (2.3 g, 88%)

M.p. 65-70 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.55 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.37 (2H, t, *J* = 7.6 Hz, Ar-CH), 7.33-7.25 (17H, m, Ar-CH), 5.30 (1H, t, *J* = 5.8 Hz, NH), 4.42 (2H, d, *J* = 6.8 Hz, Fmoc CH_2), 4.24 (1H, t, *J* = 6.8 Hz, Fmoc CH), 4.15 (2H, d, *J* = 5.6 Hz, Gly CH_2) ppm.

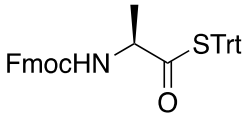
^{13}C NMR (100 MHz, $CDCl_3$) δ 194.6, 156.0 (C=O), 143.8 (qC, Ar-CH), 143.4 (Ar-CH), 141.4 (Ar-qC), 129.8, 127.9 (Ar-CH), 127.7 (Ar-CH), 127.4 (Ar-CH) 127.1, 125.1, 120.0 (Ar-CH), 70.0 (qC), 67.3 (Fmoc CH_2), 50.5 (Gly CH_2), 47.1 (Fmoc-CH) ppm.

HRMS (*m/z*) calculated for $C_{36}H_{29}NNaO_3S$ $[M + Na]^+$, calcd. 578.1766, found 578.1760.

ν_{max} (film)/cm^{-1} 1696 (C=O), 1444 (Ar C-C), 1239 (CO).

_S_-Triphenylmethane 2-[((*S*)-9-fluorenylmethoxycarbonyl)amino] propanethioate (118)

Fmoc-Ala-STrt



Prepared as per Procedure E using Fmoc-Ala-OH (1.00 g, 3.21 mmol), the product **118** was obtained following flash column chromatography (10% EtOAc:Hex) as white crystals (1.00 g, 55%).

M.p. 72-75 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.55 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.37 (2H, t, *J* = 7.6 Hz, Ar-CH), 7.33-7.25 (17H, m, Ar-CH), 5.28 (1H, t, *J* = 7.8 Hz, NH), 4.54-4.46 (2H, m, Ala αCH, Fmoc CH_2), 4.40-4.35 (1H, m, Fmoc CH_2), 4.24 (1H, t, *J* = 7.3 Hz, Fmoc CH), 1.36 (3H, d, *J* = 7.1 Hz, Ala CH_3) ppm.

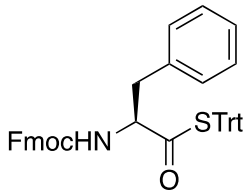
^{13}C NMR (100 MHz, $CDCl_3$) δ 198.1, 155.4 (C=O), 143.5, 141.3 (Ar-qC), 129.8, 127.9, 127.7, 127.3, 127.1, 125.2, 125.1, 120.0 (Ar-CH), 70.7 (qC), 67.1 (Fmoc CH_2), 56.7 (Ala αCH), 47.2 (Fmoc CH), 19.2 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{37}H_{31}NNaO_3S$ $[M + Na]^+$, calcd. 592.1919, found 592.1917.

ν_{max} (film)/cm^{-1} 3330 (NH), 1729 (C=O), 1239 (CO)

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-phenylpropanethioate (119)

Fmoc-Phe-_S_-Trt



Prepared as per Procedure E using Fmoc-Phe-OH (628 mg, 1.62 mmol) in CH_2Cl_2 (15 mL) and following column chromatography (10% EtOAc:Hex) the product **119** was obtained as a white crystalline solid (823 mg, 82%).

M.p. 73-75 °C.

R_f (20% EtOAc:Hex): 0.48

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.56 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.4 Hz, Ar-CH), 7.32-7.23 (20H, m, Ar-CH), 7.05 (2H, d, *J* = 7.4 Hz, Ar-CH), 5.12 (1H, d, *J* = 9.0 Hz, NH), 4.76-4.71 (1H, m, Phe αCH), 4.43-4.35 (2H, m, Fmoc CH_2), 4.22 (1H, t, *J* = 7.23 Hz, Fmoc CH), 3.07-2.96 (2H, m, Phe CH_2) ppm.

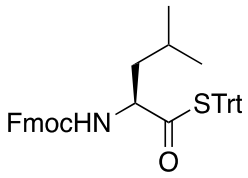
^{13}C NMR (100 MHz, $CDCl_3$) δ 197.2, 155.7 (C=O), 143.5, 141.5, 135.4 (Ar-qC), 129.9, 129.6, 128.7, 127.8, 127.7, 127.2, 127.1, 125.1, 120.0 (Ar-CH), 70.7 (qC), 66.9 (Fmoc CH_2), 61.3 (Phe αCH), 47.2 (Fmoc CH), 38.2 (Phe CH_2) ppm.

HRMS (*m/z*) calculated for $C_{43}H_{35}NNaO_3S$ $[M + Na]^+$, calcd. 668.2218, found 668.2229.

ν_{max} (film)/cm^{-1} 3316 (NH), 1688 (C=O), 737, 695 (CH).

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-4-methylpentanethioate (120)

Fmoc-Leu-_S_-Trt



Prepared as per Procedure E using Fmoc-Leu-OH (600 mg, 1.69 mmol) in CH_2Cl_2 (30 mL) and following flash column chromatography (15% EtOAc:Hex) gave the product **120** as a white crystalline solid (1.0 g, 97%).

M.p. 60-63 °C

R_f (15% EtOAc:Hex): 0.32

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.62 (2H, d, *J* = 7.7 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.2 Hz, Ar-CH), 7.32-7.24 (17H, m, Ar-CH), 5.06 (1H, d, *J* = 8.5 Hz, NH), 4.53-4.36 (3H, m, Fmoc CH_2, Leu αCH), 4.26 (1H, t, *J* = 7.0 Hz, Fmoc CH), 1.61-1.55 (2H, m, Leu CH, Leu CH_2), 1.42-1.33 (1H, m, Leu CH_2), 0.92, (6H, t, *J* = 4.6 Hz, 2 x Leu CH_3) ppm.

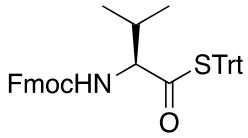
^{13}C NMR (100 MHz, $CDCl_3$) δ 198.8, 156.5 (C=O), 144.3, 142.1 (Ar-qC), 130.6, 128.6, 128.0, 127.9, 125.9, 125.8, 120.8 (Ar-CH), 71.4 (qC), 68.0 (Fmoc CH_2), 60.3 (Leu αCH), 48.1 (Fmoc CH), 42.8 (Leu CH_2), 25.4 (Leu CH), 23.7, 22.4 (2 x Leu CH_3) ppm.

HRMS (*m/z*) calculated for $C_{40}H_{37}NNaO_3S$ $[M + Na]^+$, calcd. 634.2375, found 634.2386.

ν_{max} (film)/cm^{-1} 3315 (NH), 1681 (C=O), 739 (CH).

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanethioate (121)

Fmoc-Val-_S_-Trt



Prepared as per Procedure E using Fmoc-Val-OH (700 mg, 2.06 mmol) in CH_2Cl_2 (20 mL) and following flash column chromatography (15% EtOAc:Hex) gave the product **121** as a white crystalline solid (987 mg, 80%).

M.p. 65-68 °C.

R_f (15% EtOAc:Hex): 0.31

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80 (2H, d, *J* = 7.3 Hz, Ar-CH), 7.62 (2H, d, *J* = 7.3 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.3 Hz, Ar-CH), 7.34-7.28 (17H, m, Ar-CH), 5.22 (1H, d, *J* = 9.4 Hz, NH), 4.52-4.48 (1H, dd, *J* = 10.5 Hz, *J* = 7.1 Hz, Fmoc CH_2), 4.43-4.39 (2H, m, Fmoc CH_2, Val αCH), 4.28 (1H, t, J = 7.1 Hz, Fmoc CH), 2.24-2.16 (1H, m, Val CH), 0.92 (3H, d, *J* = 6.6 Hz, Val CH_3), 0.75 (3H, d, *J* = 6.6 Hz, Val CH_3) ppm.

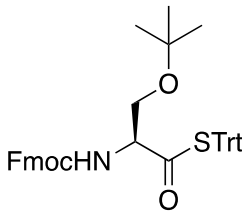
^{13}C NMR (100 MHz, $CDCl_3$) δ 197.5, 156.2 (C=O), 143.6, 141.5 (Ar-qC), 129.9, 127.8, 127.7, 127.2, 127.1, 125.2, 125.1, 120.1 (Ar-CH), 70.8 (qC), 67.5 (Fmoc CH_2), 65.5 (Val αCH), 47.2 (Fmoc CH), 31.6 (Val CH), 19.5 (Val CH_3), 16.9 (Val CH_3) ppm.

HRMS (*m/z*) calculated for $C_{39}H_{35}NNaO_3S$ $[M + Na]^+$, calcd. 620.2225, found 620.2338.

ν_{max} (film)/cm^{-1} 3325 (NH), 1686 (C=O), 738, 696 (CH).

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-(_tert_-butoxy)propanethioate (122)

Fmoc-Ser(_O_^{t}Bu)-_S_-Trt



Prepared as per Procedure E using Fmoc-Ser(O^{t}Bu)-OH (678 mg, 1.77 mmol) in CH_2Cl_2 (18 mL) and following flash column chromatography (10% EtOAc:Hex) the product **122** was obtained as a white crystalline solid (987 mg, 80%).

M.p. 65-70 °C.

R_f (20% EtOAc:Hex): 0.45

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80-7.77 (2H, m, Ar-CH), 7.65 (2H, t, *J* = 7.9 Hz, Ar-CH), 7.41 (2H, t, *J* = 7.9 Hz, Ar-CH), 7.32-7.24 (17H, m, Ar-CH), 5.78 (1H, d, *J* = 5.8 Hz, NH), 4.57-4.53 (1H, dd, *J* = 10.0 Hz, *J* = 6.4 Hz, Fmoc CH_2), 4.50-4.46 (1H, m, Ser αCH), 4.37-4.28 (2H, m, Fmoc CH_2, Fmoc CH), 3.87 (1H, dd, *J* = 8.4 Hz, *J* = 2.2 Hz, Ser CH_2), 3.49 (1H, dd, *J* = 8.4 Hz, *J* = 3.2 Hz, Ser CH_2), 1.22 (9H, s, $(CH_3)_3$) ppm.

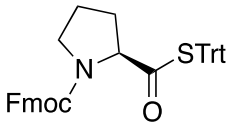
^{13}C NMR (100 MHz, $CDCl_3$) δ 197.5, 155.8 (C=O), 143.6, 141.3 (Ar-qC), 129.9, 127.8, 127.1, 125.1, 125.2, 120.0 (Ar-CH), 73.5, 70.1 (qC), 67.5 (Fmoc CH_2), 62.5 (Ser CH_2), 61.0 (Ser αCH), 47.3 (Fmoc CH), 27.5 ($(CH_3)_3$) ppm.

HRMS (*m/z*) calculated for $C_{41}H_{39}NNaO_4S$ $[M + Na]^+$, calcd. 664.2509, found 664.2492.

ν_{max} (film)/cm^{-1} 3420 (NH), 1690 (C=O), 738, 696 (CH).

(9*H*-Fluoren-9-yl)methyl-(*S*)-2-((tritylthio)carbonyl)pyrrolidine-1-carboxylate (123)

Fmoc-Pro-*S*-Trt



Prepared as per Procedure E using Fmoc-Pro-OH (597 mg, 1.77 mmol) in CH_2Cl_2 (18 mL) and following flash column chromatography (10% EtOAc:Hex), the product **123** was obtained as a white crystalline solid (610 mg, 58%).

M.p. 75-77 °C.

R_f (10% EtOAc:Hex): 0.28

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83-7.80 (2H, m, Ar-CH), 7.70-7.65 (2H, m, Ar-CH), 7.46-7.41 (2H, m, Ar-CH), 7.35-7.22 (17H, m, Ar-CH), 4.60-4.28 (4H, m, Fmoc CH_2, Fmoc CH, Pro αCH), 3.69-3.48 (2H, m, Pro CH_2), 2.26-1.82 (4H, m, 2 x Pro CH_2) ppm.*

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.5, 198.2, 154.9, 154.5 (C=O), 143.6, 143.7, 141.4, 141.2 (Ar-qC), 129.9, 129.8, 127.7, 127.2, 127.0, 125.5, 125.2, 120.0 (Ar-CH), 70.2, 70.1 (qC), 67.9, 67.7 (Fmoc CH_2), 66.4, 66.5 (Pro αCH), 47.4, 47.3 (Fmoc CH), 47.2, 46.9 (Pro CH_2), 31.3, 30.2 (Pro CH_2), 24.2, 23.2 (Pro CH_2) ppm.

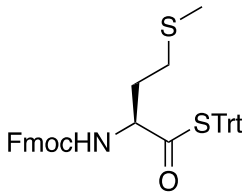
HRMS (*m/z*) calculated for $C_{39}H_{33}NNaO_3S$ $[M + Na]^+$, calcd. 618.2088, found 618.2073.

ν_{max} (film)/cm^{-1} 3052 (NH), 1696 (C=O) 737, 697 (CH).

*multiple signals account for inseparable mixture of cis/trans isomers

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-4-(methylthio)butanethioate (124)

Fmoc-Met-_S_-Trt



Prepared as per Procedure E using Fmoc-Met-OH (657 mg, 1.77 mmol) in CH_2Cl_2 (18 mL) and following flash column chromatography (10% EtOAc:Hex) gave the product **124** as off white solid (475 mg, 43%).

R_f (10% EtOAc:Hex): 0.35

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.62 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.6 Hz, Ar-CH), 7.35-7.28 (17H, m, Ar-CH), 5.24 (1H, d, *J* = 8.7 Hz, NH), 4.62-4.40 (3H, m, Fmoc CH_2, Met αCH), 4.26 (1H, t, *J* = 7.0 Hz, Fmoc CH), 2.35 (2H, t, *J* = 7.2 Hz, Met CH_2), 2.09-2.06 (4H, m, Met CH_2, Met CH_3), 1.91-1.82 (1H, m, Met CH_2) ppm.

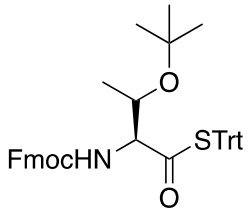
^{13}C NMR (100 MHz, $CDCl_3$) δ 197.2, 155.4 (C=O), 143.4, 141.4 (Ar-qC), 130.2, 129.8, 127.9, 127.8, 127.3, 127.1, 125.1, 120.0 (Ar-CH), 70.9 (qC), 67.3 (Fmoc CH_2), 60.3 (Met αCH), 47.3 (Fmoc CH), 32.3 (Met CH_2), 29.5 (Met CH_2), 15.5 (Met CH_3) ppm.

HRMS (*m/z*) calculated for $C_{39}H_{35}NO_3S_2$ $[M + Na]^+$, calcd. 652.1950, found 652.1959.

ν_{max} (film)/cm^{-1} 3271 (NH), 1683 (C=O), 696 (CH).

_S_-Trityl (2_S_,3_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-(*tert*-butoxy)butanethioate (125)

Fmoc-Thr(O^{t}Bu)-_S_-Trt



Prepared as per Procedure E using Fmoc-Thr(O^{t}Bu)-OH (703 mg, 1.77 mmol) in CH_2Cl_2 (18 mL) and following flash column chromatography (10% EtOAc:Hex) gave the product **125** as a white crystalline solid (1.0 g, 87%).

M.p. 65-68 °C.

R_f (20% EtOAc:Hex): 0.42

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, dd, *J* = 7.4 Hz, *J* = 2.5 Hz, Ar-CH), 7.67 (2H, dd, *J* = 10.2 Hz, *J* = 7.4 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.4 Hz, Ar-CH), 7.35-7.22 (17H, m, Ar-CH), 5.77 (1H, d, *J* = 9.3 Hz, NH), 4.61-4.56 (1H, dd, *J* = 10.2 Hz, *J* = 6.2 Hz, Fmoc CH_2), 4.39-4.21 (4H, m, Fmoc CH_2, Fmoc CH, Thr αCH, Thr CH), 1.12-1.09 (12H, m, $(CH_3)_3$, Thr CH_3) ppm.

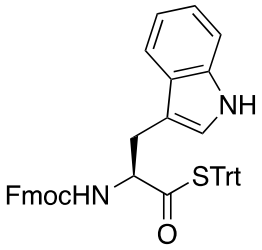
^{13}C NMR (100 MHz, $CDCl_3$) δ 198.2, 156.6 (C=O), 146.6, 144.0, 143.7, 141.3 (Ar-qC), 130.0, 128.0, 127.7, 127.2, 127.1, 127.0, 125.3, 125.2, 120.0 (Ar-CH), 74.2, 70.1 (qC), 67.4 (Fmoc CH_2), 66.8 (Thr CH), 66.4 (Thr αCH), 47.2 (Fmoc CH), 28.3 ($(CH_3)_3$), 20.6 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{42}H_{41}NNaO_4S$ $[M + Na]^+$, calcd. 678.2655, found 678.2648.

ν_{max} (film)/cm^{-1} 3429 (NH), 1692 (C=O), 737, 696 (CH).

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-(1_H_-indol-3-yl)propanethioate (126)

Fmoc-Trp-_S_-Trt



Prepared as per Procedure E using Fmoc-Trp-OH (853 mg, 2.00 mmol) in CH_2Cl_2 (20 mL) and following flash column chromatography (20% EtOAc:Hex) gave the product **126** as a yellow oil (1.2 g, 88%).

R_f (20% EtOAc/Hex): 0.28

^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (1H, s, NH), 7.78 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.62-7.52 (3H, m, Ar-CH), 7.43-7.38 (3H, m, Ar-CH), 7.30-7.23 (19H, m, Ar-CH), 7.17 (1H, t, *J* = 7.5 Hz, Ar-CH), 5.72 (1H, d, *J* = 8.7 Hz, NH), 4.82 (1H, m, Trp αCH), 4.38 (2H, m, Fmoc CH_2), 4.21 (1H, t, *J* = 7.2 Hz, Fmoc CH), 3.21 (2H, d, *J* = 5.7 Hz, Trp CH_2) ppm.

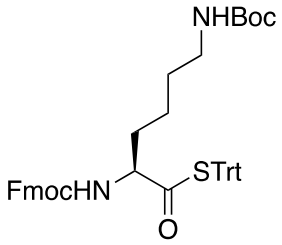
^{13}C NMR (100 MHz, $CDCl_3$) δ 198.0, 155.9 (C=O), 143.9, 143.7, 143.5, 141.3 (Ar-qC), 129.9, 127.8, 127.7, 127.1, 125.2, 125.1, 123.3, 122.4, 120.0, 119.9, 111.3, 109.5 (Ar-CH), 70.4 (qC), 67.3 (Fmoc CH_2), 61.4 (Trp αCH), 47.3 (Fmoc CH), 27.8 (Trp CH_2) ppm.

HRMS (*m/z*) calculated for $C_{45}H_{36}ClN_2O_3S$ $[M - Cl]^-$, calcd. 719.2135, found 719.2141.

ν_{max} (film)/cm^{-1} 3391, 3292 (NH), 1687 (C=O).

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-6-((_tert_-butoxycarbonyl)amino)hexanethioate (127)

Fmoc-Lys(NHBoc)-_S_-Trt



Prepared as per Procedure E using Fmoc-Lys(NHBoc)-OH (1.0 g, 2.20 mmol) in CH_2Cl_2 (20 mL) and following column chromatography (20% EtOAc:Hex) gave the product **127** as a white crystalline solid (1.4 g, 86%).

M.p. 78-80 °C.

R_f (20% EtOAc:Hex): 0.32

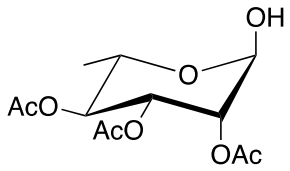
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.62 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.4 Hz, Ar-CH), 7.33-7.24 (17H, m, Ar-CH), 4.54 (1H, d, *J* = 7.4 Hz, NH), 4.54-4.33 (4H, m, Fmoc CH_2, Lys αCH, NH), 4.25 (1H, t, *J* = 6.8 Hz, Fmoc CH), 3.10-3.09 (2H, m, Lys CH_2), 1.82-1.74 (1H, m, Lys CH_2), 1.68-1.57 (1H, m, Lys CH_2), 1.46-1.41 (11H, m, $(CH_3)_3$, Lys CH_2), 1.25-1.15 (2H, m, Lys CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 197.7, 156.1, 155.8 (C=O), 143.9, 143.7, 143.4, 141.3 (Ar-qC), 129.9, 127.8, 127.7, 127.3, 127.1, 125.1, 120.1 (Ar-CH), 79.3 (qC), 70.7 (qC), 67.2 (Fmoc CH_2), 60.6 (Lys αCH), 47.2 (Fmoc CH), 39.9 (Lys CH_2), 32.6 (Lys CH_2), 29.6 (Lys CH_2), 28.3 ($(CH_3)_3$), 22.0 (Lys CH_2) ppm.

HRMS (*m/z*) calculated for $C_{45}H_{46}N_2NaO_5S$ $[M + Na]^+$, calcd. 749.3042, found 749.3019.

ν_{max} (film)/cm^{-1} 3309 (NH), 1687 (C=O), 738, 696 (CH).

(2*R*,3*R*,4*R*,5*S*,6*S*)-2-Hydroxy-6-methyltetrahydro-2*H*-pyran-3,4,5-triyl triacetate 2,3,4-tri-*O*-acetyl-L-rhamnopyranoside (132)

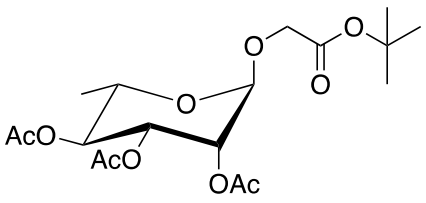


To a solution of 1,2,3,4-tetra-*O*-acetyl-α-L-rhamnopyranoside (20.0 g 0.06 mol) in THF (150 mL) was added benzylamine (13 mL, 0.12 mol) and the mixture was stirred at rt for 18 h. The solvent was removed *in vacuo* and the crude material was re-dissolved in CH_2Cl_2. The solution was washed with 1M HCl solution (100 mL), sat. aq. $NaHCO_3$ solution (100 mL) and brine (100 mL). The organic layer was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The product **132** was recrystallised from EtOAc/Hex to give white crystals (16.7 g, 96%). The isolated compound was in good agreement with the literature.[385]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.30 (1H, dd, *J* = 10.1 Hz, *J* = 3.1 Hz, H-3), 5.28 (1H, app. s, H-2), 5.17 (1H, app. s, H-1), 4.14 (1H, m, H-4), 2.17, 2.07, 2.01 (3H, s, CO$\underline{C}H_3$), 1.23 (3H, d, *J* = 6.2 Hz, H-6) ppm.

HRMS (*m/z*) calculated for $C_{12}H_{18}NaO_8$ $[M + Na]^+$, calcd. 313.0899, found 313.0897.

(2*R*,3*R*,4*R*,5*S*,6*S*)-2-(2-(*tert*-Butoxy)-2-oxoethoxy)-6-methyltetrahydro-2*H*-pyran-3,4,5-triyl triacetate (133)



To a solution of partially acetylated rhamnose **132** (870 mg, 3.00 mmol) in anhydrous DMF (75 mL) was added K_2CO_3 (1.7 g, 12.00 mmol) followed by *tert*-butyl bromoacetate (1.8 g, 9.00 mmol). The reaction mixture was stirred at rt for 48 h. The precipitate was filtered, the solvent was removed *in vacuo* and the crude material was re-dissolved in CH_2Cl_2. The organic phase was washed with water (2 x 20 mL), dried

over $MgSO_4$ and concentrated *in vacuo*. Following column chromatography (25% EtOAc:Hex) the product **133** was obtained as a colourless oil (995 mg, 82%).

R_f (30% EtOAc:Hex): 0.41

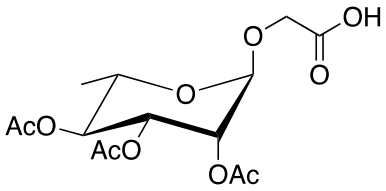
^{1}H NMR (400 MHz, $CDCl_3$) δ 5.37-5.31 (2H, m, H-2, H-3), 5.08 (1H, t, *J* = 9.7 Hz, H-4), 4.88 (1H, d, *J* = 1.1 Hz, H-1), 4.16-3.99 (3H, m, H-5, CH_2), 2.15, 2.05, 1.99 (3H, s, CO$\underline{C}H_3$), 1.48 (9H, s, $(CH_3)_3$), 1.22 (3H, d, *J* = 6.2 Hz, H-6) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 170.0, 169.9, 169.8, 168.4 (C=O), 97.5 (C-1), 82.2 (qC), 70.9 (C-4), 69.6 (C-2), 68.9 (C-3), 67.0 (C-5), 64.7 (CH_2), 28.3 $(CH_3)_3$), 20.9, 20.8, 20.7 (3 x CO$\underline{C}H_3$), 17.5 (C-6) ppm.

HRMS (*m/z*) calculated for $C_{18}H_{28}NaO_{10}$ $[M + Na]^+$, calcd. 427.1586, found 427.1575.

ν_{max} (film)/cm^{-1} 1743 (C=O).

2-(((2*R*,3*R*,4*R*,5*S*,6*S*)-3,4,5-Triacetoxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)acetic acid (134)



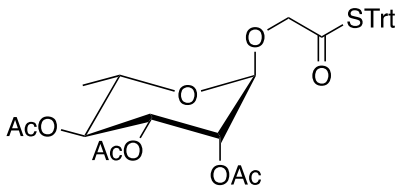
Prepared as per Procedure J using **133** (606 mg, 1.50 mmol) in CH_2Cl_2 (15 mL) to give the product **134** as a colourless oil (502 mg, 96%). The compound was in good agreement with the literature.[386]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.38-5.31 (2H, m, H-2, H-3), 5.12 (1H, t, *J* = 9.6 Hz, H-4), 4.89 (1H, bs, H-1), 4.33-4.21 (2H, m, CH_2), 4.02-3.96 (1H, m, H-5), 2.17, 2.08, 2.01 (3H, s, CO$\underline{C}H_3$), 1.24 (3H, m, H-6) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 173.4,170.3, 170.2, 170.1 (C=O), 97.7 (C-1), 69.4 (C-2), 68.9 (C-3), 67.2 (C-5), 63.9 (CH_2), 20.9, 20.8, 20.7 (3 x CO$\underline{C}H_3$), 17.3 (C-6) ppm.

HRMS (*m/z*) calculated for $C_{14}H_{20}NaO_{10}$ $[M + Na]^+$, calcd. 371.0950, found 371.0949.

(2*S*,3*S*,4*R*,5*R*,6*R*)-2-methyl-6-(2-oxo-2-(tritylthio)ethoxy)tetrahydro-2*H*-pyran-3,4,5-triyl triacetate (128)



Prepared as per Procedure E using **134** (500 mg, 1.13 mmol) in CH_2Cl_2 (12 mL) and following flash column chromatography (10-15% EtOAc:Hex), the product **128** was obtained as a colourless oil (486 mg, 71%).

R_f (20% EtOAc:Hex): 0.36

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33-7.25 (15H, m, Ar-CH), 5.38-5.32 (2H, m, H-2, H-3), 5.09 (1H, t, *J* = 9.9Hz, H-4), 4.83 (1H, m, H-1), 4.19 (2H, m, CH_2), 3.98-3.91 (1H, m, H-5), 2.17, 2.06, 2.01 (3H, s, CO$\underline{C}H_3$), 1.21 (3H, d, *J* = 6.1 Hz, H-6) ppm.

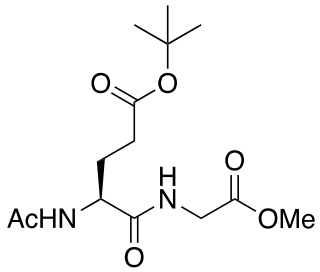
^{13}C NMR (100 MHz, $CDCl_3$) δ 194.6, 170.0, 169.9, 169.8 (C=O), 143.5 (Ar-qC), 129.8, 127.9, 127.2 (Ar-CH), 98.2 (C-1), 71.7 (C-7), 70.8 (C-4), 70.5 (qC), 69.4 (C-2), 68.8 (C-3), 67.2 (C-5), 20.9, 20.8, 20.7 (3 x CO$\underline{C}H_3$), 17.9 (C-6) ppm.

HRMS (*m/z*) calculated for $C_{33}H_{34}NaO_9S$ $[M + Na]^+$, calcd. 629.1822, found 629.1816.

ν_{max} (film)/cm^{-1} 3505 (NH), 1745 (C=O).

***tert*-Butyl-(*S*)-4-acetamido-5-((2-methoxy-2-oxoethyl)amino)-5-oxopentanoate (136)**

Ac-Glu(O^{t}Bu)-Gly-OMe



Prepared as per Procedure A using Ac-Glu(O^{t}Bu)-OH (450 mg, 1.84 mmol) and HCl·H_2N-Gly-OMe (230 mg, 1.84 mmol) in anhydrous CH_2Cl_2 (16 mL) and DMF (2

mL). Following column chromatography (10% EtOAc:Hex) the product **136** was obtained as a colourless oil (545 mg, 94%).

R_f (10% EtOAc:Hex): 0.30

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21 (1H, bs, NH), 6.78 (1H, d, *J* = 5.8 Hz), 4.56-4.51 (1H, dd, *J* = 13.6 Hz, *J* = 7.4 Hz, Glu αCH), 4.05 (2H, qd, *J* = 18.4 Hz, *J* = 5.0 Hz, Gly CH_2), 3.77 (3H, s, OCH_3), 2.62-2.43 (2H, m, Glu CH_2), 2.22-1.96 (5H, m, Glu CH_2, $CO\underline{C}H_3$), 1.47 (9H, s, $C(CH_3)_3$) ppm.

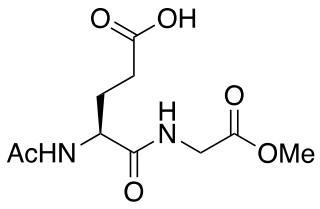
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.3, 171.8, 170.7, 170.1 (C=O), 81.1 (qC), 52.6 (Glu αCH), 52.4 (OCH_3), 41.2 (Gly CH_2), 31.8 (Glu CH_2), 28.1 ($C(CH_3)_3$), 27.6 (Glu CH_2), 23.2 ($CO\underline{C}H_3$) ppm.

HRMS (*m/z*) calculated for $C_{14}H_{25}N_2O_6$ $[M + H]^+$, calcd. 317.1700, found 317.1707.

ν_{max} (film)/cm^{-1} 3289 (NH), 1727, 1650 (C=O).

(*S*)-4-Acetamido-5-((2-methoxy-2-oxoethyl)amino)-5-oxopentanoic acid (137)

Ac-Glu(OH)-Gly-OMe



Prepared as per Procedure J using **136** (500 mg, 1.58 mmol) in CH_2Cl_2 (9 mL), the product **137** was obtained as off-white crystals (383 mg, 93%).

M.p. 83-85 °C.

R_f (30% EtOAc:Hex): 0.25

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.20 (1H, bs, NH), 6.75 (1H, d, *J* = 5.8 Hz, NH), 4.59 (1H, m, Glu αCH), 4.05 (2H, qd, *J* = 18.2 Hz, *J* = 5.4 Hz, Gly CH_2), 3.77 (3H, s, OCH_3), 2.62-2.43 (2H, m, Glu CH_2), 2.22-2.13 (1H, m, Glu CH_2), 2.05-1.96 (4H, m, Glu CH_2, Ac CH_3) ppm.

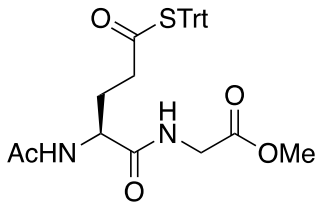
^{13}C NMR (100 MHz, $CDCl_3$) δ 174.2, 171.6, 170.8, 170.0 (C=O), 52.5 (OCH_3), 52.3 (Glu αCH), 41.2 (Gly CH_2), 30.3 (Glu CH_2), 27.8 (Glu CH_2), 23.1 (Ac CH_3) ppm.

HRMS (*m/z*) calculated for $C_{10}H_{15}N_2O_6$ $[M + H]^+$, calcd. 259.0935, found 259.0936.

ν_{max} (film)/cm^{-1} 3269 (NH), 3075 (OH), 1739, 1633 (C=O).

Methyl (*S*)-(2-Acetamido-5-oxo-5-(tritylthio)pentanoyl)glycinate (129)

Ac-Glu(*S*-Trt)-Gly-OMe



Prepared as per Procedure E using **137** (300 mg, 1.15 mmol) in CH_2Cl_2 (12 mL) and following flash column chromatography (20% EtOAc:Hex) gave the product **129** as a yellow oil (257 mg, 43%).

R_f (30% EtOAc/Hex): 0.40

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.32-7.23 (15H, m, Ar-CH), 6.85 (1H, m, NH), 6.43 (1H, m, NH), 4.42-4.37 (1H, m, Glu αCH), 4.04-3.89 (2H, m, Gly CH_2), 3.74 (3H, s, OCH_3), 2.82-2.61 (2H, m, Glu CH_2), 2.12-1.93 (5H, m, Glu CH_2, $COCH_3$) ppm.

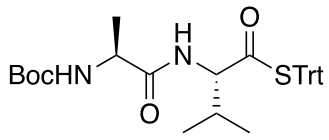
^{13}C NMR (100 MHz, $CDCl_3$) δ 197.9, 171.4, 170.6, 169.8 (C=O), 143.5 (Ar-qC), 129.9, 127.8, 127.3 (Ar-CH), 70.8 (qC), 52.4 (OCH_3), 52.2 (Glu αCH), 41.1 (Gly CH_2), 39.6, 27.8 (2 x Glu CH_2), 23.2 ($CO\underline{C}H_3$) ppm.

HRMS (*m/z*) calculated for $C_{29}H_{30}N_2NaO_5S$ $[M + Na]^+$, calcd. 541.1765, found 541.1767.

ν_{max} (film)/cm^{-1} 3297 (NH), 1735 (C=O).

_S_-Trityl (_S_)-2-((_S_)-2-((_tert_-butoxycarbonyl)amino)propanamido)-3-methylbutanethioate (130)

Boc-Ala-Val-_S_-Trt



Prepared as per procedure E using **140** (100 mg, 0.35 mmol) in CH_2Cl_2 (4 mL) and following flash column chromatography (10-15% EtOAc/Hex) the product **130** was obtained as a yellow oil (90 mg, 48%).

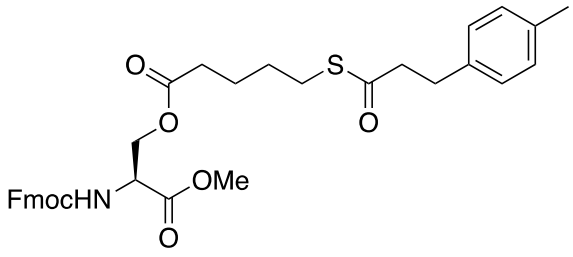
R_f (20% EtOAc/Hex): 0.29

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28-7.21 (15H, m, Ar-CH), 6.68 (1H, bs, NH), 4.95 (1H, bs, NH), 4.60 (1H, q, *J* = 4.4 Hz, Ala αCH), 4.21-4.13 (1H, m, Val αCH), 2.21-2.13 (1H, m, Val CH), 1.42-1.34 (12H, m, $C(CH_3)_3$, Ala CH_3), 0.85 (3H, m, Val CH_3), 0.71 (3H, t, *J* = 6.9 Hz, Val CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 200.7, 172.6, 155.9 (C=O), 143.4, 141.6 (Ar-qC), 130.1, 129.9, 127.9, 127.3 (Ar-CH), 73.4 (qC), 63.0 (Ala αCH), 50.1 (Val αCH), 30.9 (Val CH), 28.4 ($C(\underline{C}H_3)_3$), 19.3 (Val CH_3), 17.0 (Ala CH_3), 16.8 (Val CH_3) ppm.

ν_{max} (film)/cm^{-1} 3345 (NH), 1667 (C=O), 699 (CH).

(_S_)-2-((((9_H_-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-((3-(_p_-tolyl)propanoyl)thio)pentanoate (146)



Prepared as per Procedure K using **112** and following column chromatography (10% EtOAc:Hex) **146** was obtained as a pale yellow solid (140 mg, 99%).

R_f (20% EtOAc:Hex): 0.45

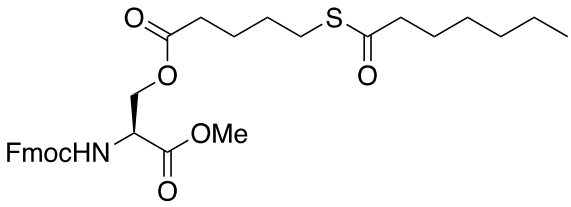
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.63 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.42 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.35 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.14-7.06 (4H, m, Ar-CH), 5.66 (1H, d, *J* = 7.8 Hz, NH), 4.69-4.65 (1H, m, Ser αCH), 4.54-4.39 (4H, m, Fmoc CH_2, Ser CH_2), 4.25 (1H, t, *J* = 6.9 Hz, Fmoc CH), 3.80 (3H, s, Ser OCH_3), 2.97-2.83 (6H, m, 3 x CH_2), 2.39-2.33 (5H, m, 2 x CH_2, CH_3), 1.73-1.58 (4H, m, 2 x CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.7, 172.8, 170.0, 155.8 (C=O), 143.8 (Ar-qC), 141.4 (Ar-qC), 137.1 (Ar-qC), 136.0 (Ar-qC), 128.2 (Ar-CH), 129.2 (Ar-CH), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.1 (Ar-CH), 67.4 (Fmoc CH_2), 64.0 (Ser CH_2), 53.5 (Ser αCH), 53.0 (Ser OCH_3), 47.2 (Fmoc CH), 45.7 (CH_2), 33.2 (CH_2), 31.1 (CH_2), 29.0 (CH_2), 28.3 (CH_2), 23.8 (CH_2), 21.0 (CH_3) ppm.

HRMS (*m/z*) calculated for $C_{34}H_{37}NNaO_7S$ $[M + Na]^+$, calcd. 626.2187, found 626.2183.

ν_{max} (film)/cm^{-1} 3357 (NH), 1733 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(heptanoylthio)pentanoate (147)



Prepared as per Procedure K using **113** and following column chromatography (20% EtOAc:Hex) **147** was obtained as a white solid (133 mg, 99%).

R_f (20% EtOAc:Hex): 0.35

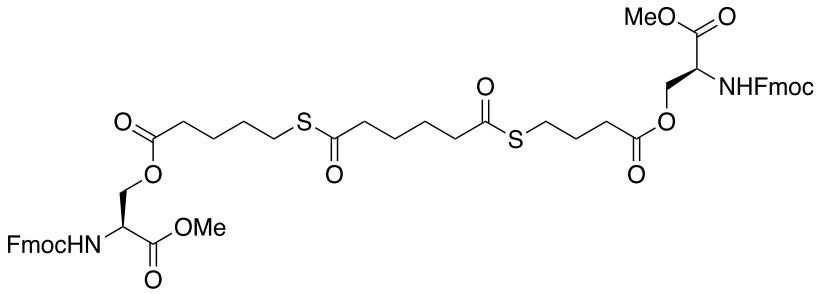
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.5 Hz, Ar-CH), 7.63 (4H, d, *J* = 7.5 Hz, Ar-CH), 7.43 (4H, t, *J* = 7.5 Hz, Ar-CH), 7.35 (4H, t, *J* = 7.5 Hz, Ar-CH), 5.66 (2H, d, *J* = 7.9 Hz, NH), 4.68-4.64 (2H, m, Ser αCH), 4.53-4.39 (4H, m, Fmoc CH_2, Ser CH_2), 4.27 (1H, t, *J* = 6.8 Hz, Fmoc CH), 3.82 (3H, s, OCH_3), 2.88 (2H, t, *J* = 7.1 Hz, CH_2S), 2.55 (2H, t, *J* = 7.3 Hz, CH_2), 1.75-1.58 (6H, m, 3 x CH_2), 1.36-1.27 (6H, m, 3 x CH_2), 0.90 (3H, t, *J* = 6.8 Hz, CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 199.8, 172.9, 170.0, 155.8 (C=O), 143.7 (Ar-qC), 141.3 (Ar-qC), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.1 (Ar-CH), 67.3 (Fmoc CH_2), 63.9 (Ser CH_2), 53.4 (Ser αCH), 52.9 (OCH_3), 47.3 (Fmoc CH), 44.2 (CH_2), 33.4 (CH_2), 31.4 (CH_2), 29.0 (CH_2), 23.9 (CH_2), 28.6 (CH_2), 28.2 (CH_2S), 25.7 (CH_2), 22.5 (CH_2), 14.0 (CH_3) ppm.

HRMS (*m/z*) calculated for $C_{31}H_{39}NNaO_7S$ $[M + Na]^+$, calcd. 592.2344, found 592.2339.

ν_{max} (film)/cm^{-1} 3357 (NH), 1726, 1688 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((*S*)-1-(9*H*-fluoren-9-yl)-5-(methoxycarbonyl)-3,8,13-trioxo-2,7-dioxa-12-thia-4-azaoctadecan-18-oyl)thio)pentanoate (148)



Prepared as per Procedure K using **114** and following column chromatography (30-40% EtOAc:Hex) **148** was obtained as a yellow oil (102 mg, 42%).

R_f (40% EtOAc:Hex): 0.35

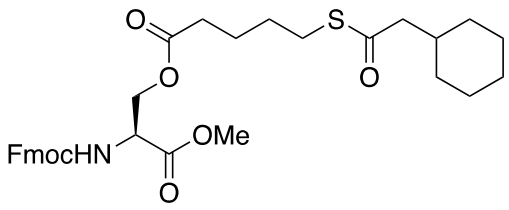
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.5 Hz, Ar-CH), 7.63 (4H, d, *J* = 7.5 Hz, Ar-CH), 7.43 (4H, t, *J* = 7.5 Hz, Ar-CH), 7.34 (4H, t, *J* = 7.5 Hz, Ar-CH), 5.66 (2H, d, *J* = 8.3 Hz, NH), 4.68-4.64 (2H, m, Ser αCH), 4.53-4.39 (8H, m, 2 x Fmoc CH_2, 2 x Ser CH_2), 4.27 (2H, t, *J* = 6.8 Hz, 2 x Fmoc CH), 3.81 (3H, s, OCH_3), 2.88 (4H, t, *J* = 6.8 Hz, CH_2S), 2.55 (4H, t, *J* = 5.7 Hz, 2 x CH_2), 2.37 (4H, t, *J* = 6.8 Hz, 2 x CH_2), 1.73-1.57 (12H, m, 6 x CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 199.0, 172.9, 170.2, 155.8 (C=O), 143.8 (Ar-qC), 141.4 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.1 (Ar-CH), 120.1 (Ar-CH), 67.4 (Fmoc CH_2), 63.9 (Ser CH_2), 53.6 (Ser αCH), 52.9 (OCH_3), 47.2, 47.1 (Fmoc CH), 43.6 (2 x CH_2), 33.3 (CH_2), 28.9 (CH_2), 23.9 (CH_2), 28.3 (CH_2S), 24.7 (2 x CH_2) ppm.

HRMS (*m/z*) calculated for $C_{54}H_{60}N_2NaO_{14}S_2$ $[M + Na]^+$, calcd. 1047.3378, found 1047.3378.

ν_{max} (film)/cm^{-1} 2949 (NH), 1737, 1682 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-((2-cyclohexylacetyl)thio)pentanoate (149)



Prepared as per Procedure K using **115** and following column chromatography (30-40% EtOAc:Hex) **149** was obtained as a yellow oil (135 mg, 99%).

R_f (40% EtOc:Hex): 0.35

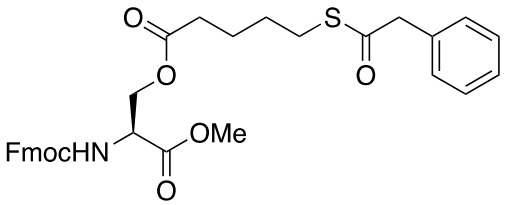
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.5 Hz, Ar-CH), 7.62 (4H, d, *J* = 7.5 Hz, Ar-CH), 7.43 (4H, t, *J* = 7.5 Hz, Ar-CH), 7.35 (4H, t, *J* = 7.5 Hz, Ar-CH), 5.65 (1H, d, *J* = 7.78 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.53-4.38 (4H, m, Fmoc CH_2, Ser CH_2), 4.27 (1H, t, *J* = 6.9 Hz, Fmoc CH), 3.80 (3H, s, OCH_3), 2.88 (2H, t, *J* = 6.4 Hz, CH_2S), 2.43 (2H, d, *J* = 6.4 Hz, CH_2), 2.37 (2H, t, *J* = 6.4 Hz, CH_2), 1.91-1.80 (1H, m, CH), 1.74-1.58 (9H, m, 5 x CH_2), 1.32-1.14 (3H, m, 2 x CH_2), 1.02-0.92 (2H, m, CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 199.0, 172.9, 170.0, 155.8 (C=O), 143.8 (Ar-qC), 141.3 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 67.4 (Fmoc CH_2), 64.0 (Ser CH_2), 53.5 (Ser αCH), 52.9 (OCH_3), 51.8 (CH_2) 47.2 (Fmoc CH), 35.6 (CH), 33.4 (CH_2), 32.9, (CH_2), 29.2 (CH_2), 23.9 (CH_2), 28.5 (CH_2S), 26.1 (CH_2), 26.0 (CH_2) ppm.

HRMS (*m/z*) calculated for $C_{32}H_{39}NNaO_7S$ $[M + Na]^+$, calcd. 604.2362, found 604.2339.

ν_{max} (film)/cm^{-1} 3335 (NH), 1768 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-((2-phenylacetyl)thio)pentanoate (150)



Prepared as per Procedure K using **116** and following column chromatography (20% EtOAc:Hex) **150** was obtained as a colourless oil (117 mg, 86%).

R_f (30% EtOAc:Hex): 0.40

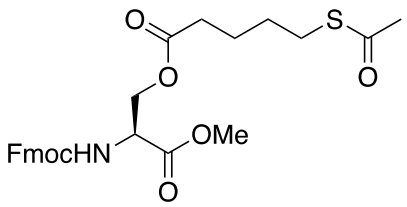
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.7 Hz, Ar-CH), 7.63 (4H, d, *J* = 7.7 Hz, Ar-CH), 7.42 (4H, t, *J* = 7.7 Hz, Ar-CH), 7.35 (4H, t, *J* = 7.7 Hz, Ar-CH), 5.68 (1H, d, *J* = 8.0 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.53-4.38 (4H, m, Fmoc CH_2, Ser CH_2), 4.26 (1H, t, *J* = 6.9 Hz, Fmoc CH), 3.83 (2H, s, C$\underline{H}_2$Ph), 3.80 (3H, s, OCH_3), 2.87 (2H, t, *J* = 7.0 Hz, CH_2S), 2.35 (2H, t, *J* = 7.0 Hz, COC$\underline{H}_2CH_2CH_2CH_2S$), 1.73-1.56 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 197.5, 173.7, 170.3, 156.1 (C=O), 143.8 (Ar-qC), 141.4 (Ar-qC), 133.8 (Ar-qC), 129.5 (Ar-CH), 128.7 (Ar-CH), 127.8 (Ar-CH), 127.4 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.1 (Ar-CH), 67.4 (Fmoc CH_2), 63.9 (Ser CH_2), 53.5 (Ser αCH), 52.9 (OCH_3), 50.6 ($\underline{C}H_2$Ph), 47.2 (Fmoc CH), 33.3 (CO$\underline{C}H_2CH_2CH_2CH_2S$), 28.8, 23.8 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.7 ($CH_2S$) ppm.

HRMS (*m/z*) calculated for $C_{32}H_{33}NNaO_7S$ $[M + Na]^+$, calcd. 598.1857, found 598.1869.

ν_{max} (film)/cm^{-1} 2950 (NH), 1690 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(acetylthio)pentanoate (151)



Prepared as per Procedure K using thioacetic acid (50 μL, 0.708 mmol) and following column chromatography (30% EtOAc:Hex) **151** was obtained as a colourless oil (104 mg, 88%).

R_f (30% EtOAc:Hex): 0.32

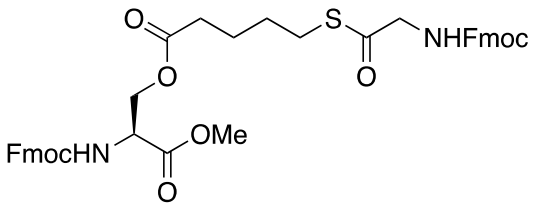
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.7 Hz, Ar-CH), 7.63 (2H, d, *J* = 7.7 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.7 Hz, Ar-CH), 7.35 (2H, t, *J* = 7.7 Hz, Ar-CH), 5.68 (1H, d, *J* = 8.2 Hz, NH), 4.69-4.65 (1H, m, Ser αCH), 4.54-4.39 (4H, m, Ser CH_2, Fmoc CH_2), 4.27 (1H, t, *J* = 7.1 Hz, Fmoc CH), 3.82 (3H, s, OCH_3), 2.89 (2H, t, *J* = 7.1 Hz, CH_2S), 2.39-2.36 (5H, m, $COC\underline{H}_2CH_2CH_2CH_2S$, SAc CH_3), 1.75-1.50 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 195.9, 172.7, 170.0, 155.8 (C=O), 143.8, 141.4 (Ar-qC), 127.8, 127.1, 125.1, 120.1 (Ar-CH), 67.4 (Fmoc CH_2), 63.9 (Ser CH_2), 53.4 (Ser αCH), 53.0 (OCH_3), 47.1 (Fmoc CH), 33.3 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 30.6 (SAc CH_3), 28.9, 23.8 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.5 ($CH_2S$) ppm.

HRMS (*m/z*) calculated for $C_{26}H_{29}NNaO_7S$ $[M + Na]^+$, calcd. 522.1557, found 522.1548.

ν_{max} (film)/cm^{-1} 3423 (NH), 1730, 1674 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)pentanoate (153)



Prepared as per Procedure K using **117** and following column chromatography (30-40% EtOAc:Hex) **153** was obtained as a colourless oil (159 mg, 92%).

R_f (40% EtOAc:Hex): 0.38

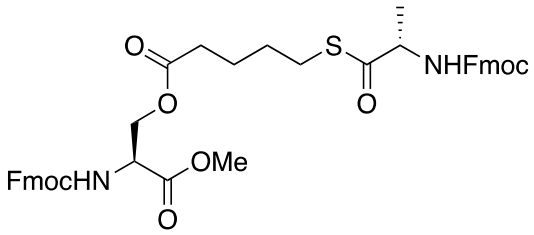
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.2 Hz, Ar-CH), 7.62 (4H, d, *J* = 7.2 Hz, Ar-CH), 7.42 (4H, t, *J* = 7.2 Hz, Ar-CH), 7.33 (4H, t, *J* = 7.2 Hz, Ar-CH), 5.81 (1H, d, *J* = 8.2 Hz, NH), 5.58 (1H, t, *J* = 6.3 Hz, NH), 4.70-4.67 (1H, m, Ser αCH), 4.53-4.41 (6H, m, 2 x Fmoc CH_2, Ser CH_2), 4.28-4.23 (2H, dd, *J* = 7.1 Hz, 2 x Fmoc CH), 4.11 (2H, d, *J* = 6.0 Hz, Gly CH_2), 3.80 (3H, s, OCH_3), 2.92 (2H, t, *J* = 6.8 Hz, CH_2S), 2.37 (2H, t, *J* = 6.8 Hz, $COCH_2CH_2CH_2CH_2S$), 1.75-1.61 (4H, m, $COCH_2CH_2CH_2CH_2S$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 197.6, 172.7, 170.1, 156.2, 155.7 (C=O), 143.8 (Ar qC), 143.7 (Arq C), 141.3 (Ar qC), 127.8 (Ar-CH), 127.2 (Ar-CH), 125.1 (Ar-CH), 120.1 (Ar-CH), 67.3 (Fmoc CH_2), 64.1 (Ser CH_2), 53.4 (Ser αCH), 52.9 (OCH_3), 50.7 (Gly CH_2), 47.1 (Fmoc CH), 33.3 ($COCH_2CH_2CH_2CH_2S$), 28.7, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.0 (CH_2S) ppm.

HRMS (*m/z*) calculated for $C_{41}H_{40}N_2NaO_9S$ $[M + Na]^+$, calcd. 759.2333, found 759.2347.

ν_{max} (film)/cm^{-1} 3345 (NH), 1712 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-alanyl)thio)pentanoate (154)



Prepared as per Procedure K using **118** and following column chromatography (30-40% EtOAc:Hex) **154** was obtained as a pale yellow oil (103 mg, 58%).

R_f (40% (EtOAc:Hex): 0.32

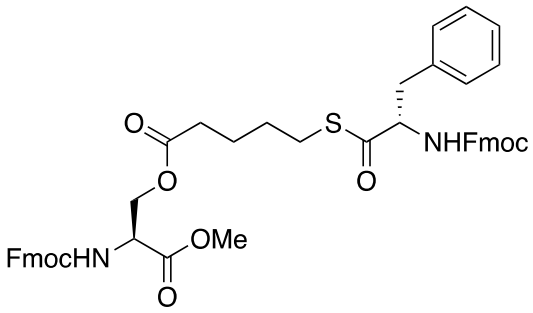
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.62 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.42 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.33 (4H, t, *J* = 7.4 Hz, Ar-CH), 5.69 (1H, d, *J* = 8.4 Hz, NH), 5.31 (1H, d, *J* = 8.0 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.52-4.37 (7H, m, 2 x Fmoc CH_2, Ser CH_2, Ala αCH), 4.28-4.24 (2H, m, 2 x Fmoc CH), 3.80 (3H, s, OCH_3), 2.91 (2H, t, *J* = 6.5 Hz, CH_2S), 2.38 (2H, t, *J* = 6.5 Hz, $COC\underline{H}_2CH_2CH_2CH_2S$), 1.74-1.61 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$), 1.43 (3H, d, *J* = 6.5 Hz, Ala CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 201.3, 172.7, 170.0, 155.8, 155.6 (C=O), 143.9 (Ar-qC), 143.8 (Ar-qC), 143.7 (Ar-qC), 141.3 (Ar-qC), 127.8 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 67.3, 67.1 (Fmoc CH_2), 64.0 (Ser CH_2), 56.6 (Ala αCH), 53.5 (Ser αCH), 52.9 (OCH_3), 47.2, 47.1 (Fmoc CH), 33.3 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 28.8, 23.7 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.1 ($CH_2S$), 18.9 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{42}H_{42}N_2NaO_9S$ $[M + Na]^+$, calcd. 773.2521, found 773.2503.

ν_{max} (film)/cm^{-1} 3330 (NH), 1729 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-phenylalanyl)thio)pentanoate (155)



Prepared as per Procedure K using **119** and following column chromatography (30% EtOAc:Hex) **155** was obtained as a pale yellow solid (151 mg, 77%).

R_f (30% EtOAc:Hex): 0.33

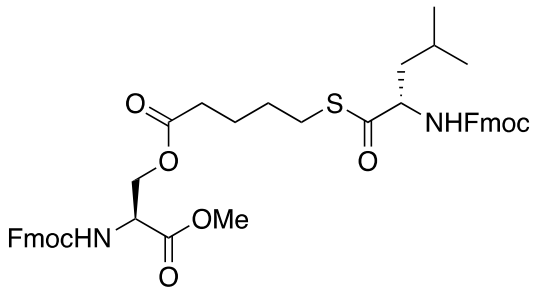
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.8 Hz, Ar-CH), 7.66-7.54 (4H, m, Ar-CH), 7.44 (4H, t, *J* = 7.8 Hz, Ar-CH), 7.37-7.28 (9H, m, Ar-CH, Phe Ar-CH), 5.76 (1H, d, *J* = 7.8 Hz, NH), 5.35 (1H, d, *J* = 8.9 Hz, NH), 4.78-4.68 (2H, m, Ser αCH, Phe αCH), 4.55-4.40 (6H, m, 2 x Fmoc CH_2, Ser CH_2), 4.29-4.20 (2H, m, 2 x Fmoc CH), 3.81 (3H, s, OCH_3), 3.24-3.05 (2H, m, Phe CH_2), 2.92 (2H, t, *J* = 6.8 Hz, CH_2S), 2.38 (2H, t, *J* = 6.8 Hz, $COCH_2CH_2CH_2CH_2S$), 1.75-1.61 (4H, m, $COCH_2CH_2CH_2CH_2S$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 200.2, 172.7, 170.0, 155.8, 155.6 (C=O), 143.8 (Ar-qC), 143.7 (Ar-qC), 141.3 (Ar-qC), 135.5 (Phe Ar-qC), 129.4 (Phe Ar-CH), 128.7 (Phe Ar-CH), 127.8 (Ar-CH),127.3 (Phe Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 67.3, 67.2 (Fmoc CH_2), 64.0 (Ser CH_2), 61.6 (Phe αCH), 53.5 (Ser αCH), 53.0 (OCH_3), 47.2 (Fmoc CH), 38.4 (Phe CH_2), 33.3 ($COCH_2CH_2CH_2CH_2S$), 28.7, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.4 (CH_2S) ppm.

HRMS (*m/z*) calculated for $C_{48}H_{46}N_2O_9S$ $[M + Na]^+$, calcd. 849.2822, found 849.2816.

ν_{max} (film)/cm^{-1} 3332 (NH), 1728 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-*L*-leucyl)thio)pentanoate (156)



Prepared as per Procedure K using **120** following column chromatography (30% EtOAc:Hex) **156** was obtained as a yellow oil (103 mg, 55%).

R_f (30% EtOAc:Hex): 0.36

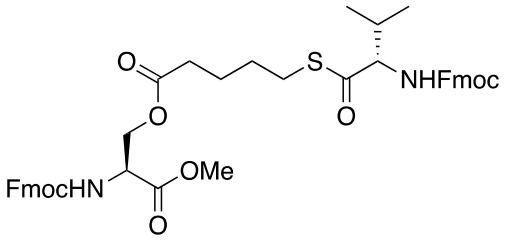
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.8 Hz, Ar-CH), 7.62 (4H, d, *J* = 7.8 Hz, Ar-CH), 7.42 (4H, t, *J* = 7.8 Hz, Ar-CH), 7.33 (4H, t, *J* = 7.8 Hz, Ar-CH), 5.66 (1H, d, *J* = 7.5 Hz, NH), 5.12 (1H, d, *J* = 7.5 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.52-4.38 (7H, m, 2 x Fmoc CH_2, Ser CH_2, Leu αCH), 4.25 (2H, t, *J* = 6.9 Hz, 2 x Fmoc CH), 3.80 (3H, s, OCH_3), 2.88 (2H, t, *J* = 6.4 Hz, CH_2S), 2.37 (2H, t, *J* = 6.4 Hz, $COCH_2CH_2CH_2CH_2S$), 1.72-1.60 (7H, m, $COCH_2CH_2CH_2CH_2S$, Leu CH_2, Leu CH), 0.96 (6H, d, *J* = 2.5 Hz, 2 x Leu CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 201.5, 172.7, 170.0, 155.8, 155.7 (C=O), 143.8 (Ar-qC), 143.7 (Ar-qC), 141.4 (Ar-qC), 141.3 (Ar-qC), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 67.5, 67.1 (Fmoc CH_2), 64.0 (Ser CH_2), 59.8 (Leu αCH), 53.5 (Ser αCH), 53.0 (OCH_3), 47.3, 47.2 (Fmoc CH), 42.2 (Leu CH_2), 33.3 ($COCH_2CH_2CH_2CH_2S$), 28.8, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.2 (CH_2S), 24.8 (Leu CH), 23.2, 21.5 (Leu CH_3) ppm.

HRMS (*m/z*) calculated for $C_{45}H_{48}N_2O_9S$ $[M + Na]^+$, calcd. 815.2973, found 815.2972.

ν_{max} (film)/cm^{-1} 3331 (NH), 1724 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-valyl)thio)pentanoate (157)



Prepared as per Procedure K using **121** and following column chromatography (20-30% EtOAc:Hex) **157** was obtained as a yellow oil (122 mg, 66%).

R_f (30% EtOAc:Hex): 0.29

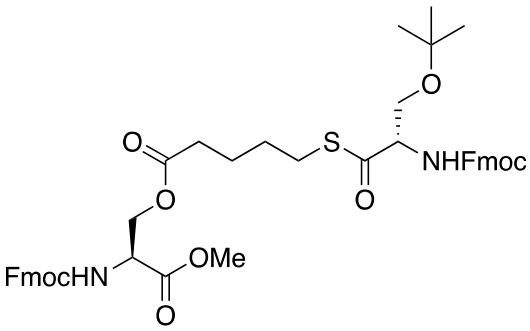
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.63 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.42 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.34 (4H, t, *J* = 7.4 Hz, Ar-CH), 5.76 (1H, d, *J* = 8.0 Hz, NH), 5.41 (1H, d, *J* = 9.3 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.52-4.36 (7H, m, 2 x Fmoc CH_2, Ser CH_2, Val αCH), 4.25 (2H, t, *J* = 7.1 Hz, 2 x Fmoc CH), 3.80 (3H, s, OCH_3), 2.91 (2H, t, *J* = 6.9 Hz, CH_2S), 2.36-2.32 (2H, m, $COCH_2CH_2CH_2CH_2S$, Val CH), 1.74-1.59 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.01 (3H, d, *J* = 6.6 Hz, Val CH_3), 0.89 (3H, d, *J* = 6.6 Hz, Val CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 200.4, 172.7, 170.0, 156.2, 155.8 (C=O), 143.9 (Ar-qC), 143.8 (Ar-qC), 141.4 (Ar-qC), 141.3 (Ar-qC), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 67.3, 67.1 (Fmoc CH_2), 66.1 (Val αCH), 64.0 (Ser CH_2), 53.5 (Ser αCH), 53.0 (OCH_3), 47.2, 47.1 (Fmoc CH), 33.3 ($COCH_2CH_2CH_2CH_2S$), 31.3 (Val CH), 28.8, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.4 (CH_2S), 19.5, 17.0 (2 x Val CH_3) ppm.

HRMS (*m/z*) calculated for $C_{44}H_{46}N_2NaO_9S$ $[M + Na]^+$, calcd. 801.2805, found 801.2816.

ν_{max} (film)/cm^{-1} 3338 (NH), 1716 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-((*N*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-L-seryl)thio)pentanoate (158)



Prepared as per Procedure K using **122** and following column chromatography (25% EtOAc:Hex) **158** was obtained as a yellow oil (172 mg, 88%).

R_f (30% EtOAc:Hex): 0.36

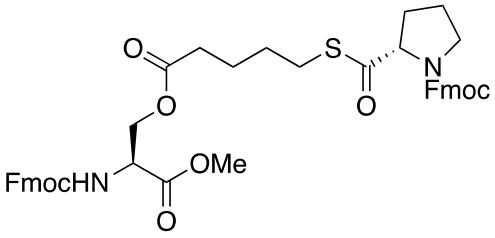
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.65 (4H, m, Ar-CH), 7.42 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.34 (4H, t, *J* = 7.4 Hz, Ar-CH), 5.76 (1H, d, *J* = 8.8 Hz, NH), 5.67 (1H, d, *J* = 8.1 Hz, NH), 4.67-4.64 (1H, m, Ser αCH), 4.52-4.36 (7H, m, 2 x Fmoc CH_2, Ser CH_2, Ser αCH), 4.32-4.24 (2H, m, 2 x Fmoc CH), 3.92 (1H, dd, *J* = 2.5 Hz, *J* = 9.1 Hz, Ser CH_2), 3.80 (3H, s, OCH_3), 3.56 (1H, dd, *J* = 2.5 Hz, *J* = 9.1 Hz, Ser CH_2), 2.91 (2H, t, *J* = 6.8 Hz, CH_2S), 2.37 (2H, t, *J* = 6.8 Hz $COC\underline{H}_2CH_2CH_2CH_2S$), 1.73-1.62 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$), 1.18 (9H, s, $C(CH_3)_3$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 200.2, 172.8, 170.0, 155.8, 156.1 (C=O), 143.9 (Ar-qC), 143.7 (Ar-qC), 141.4 (Ar-qC), 127.8 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 73.7 (Ser qC), 67.3 (Fmoc CH_2), 63.9 (Ser CH_2), 61.9 (Ser CH_2), 61.2 (Ser αCH), 53.5 (Ser αCH), 53.0 (OCH_3), 47.2, 47.1 (Fmoc CH), 33.3 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 28.8, 23.8 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.4 ($CH_2S$), 27.4 ($C(CH_3)_3$) ppm.

HRMS (*m/z*) calculated for $C_{46}H_{50}N_2NaO_{10}S$ $[M + Na]^+$, calcd. 845.3082, found 845.3078.

ν_{max} (film)/cm^{-1} 3338 (NH), 1719 (C=O).

(9*H*-Fluoren-9-yl)methyl (*S*)-2-((S)-1-(9*H*-fluoren-9-yl)-5-(methoxycarbonyl)-3,8-dioxo-2,7-dioxa-13-thia-4-azatetradecan-14-oyl)pyrrolidine-1-carboxylate (159)



Prepared as per Procedure K using **123** and following column chromatography (40% EtOAc:Hex) **159** was obtained as a yellow oil (92 mg, 50%).

R_f (40% EtOAc:Hex): 0.36

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81-7.78 (4H, m, Ar-CH), 7.65-7.61 (4H, m, Ar-CH), 7.46-7.41 (4H, m, Ar-CH), 7.38-7.33 (4H, m, Ar-CH), 5.67 (1H, dd, *J* = 8.0 Hz, *J* = 39.1 Hz, NH), 4.67-4.62 (1H, m, Ser αCH), 4.57-4.24 (9H, m, 2 x Fmoc CH_2, 2 x Fmoc CH, Ser CH_2, Pro αCH), 3.79 (3H, s, OCH_3), 3.71-3.50 (2H, m, Pro CH_2), 2.92-2.80 (2H, m, CH_2S), 2.39-2.28 (2H, m, $COC\underline{H}_2CH_2CH_2CH_2S$), 2.26-1.90 (4H, m, 2 x Pro CH_2), 1.72-1.53 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$) ppm.*

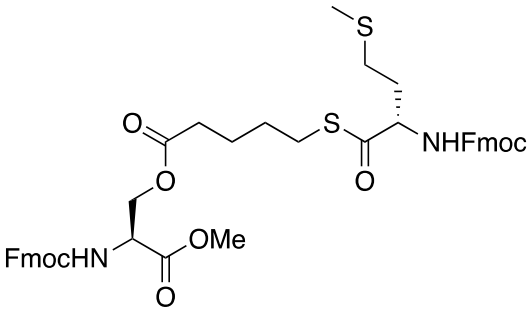
^{13}C NMR (100 MHz, $CDCl_3$) δ 202.1, 201.6, 172.8, 172.7, 169.9, 155.8, 155.1 154.7 (C=O), 144.2, 143.8, 141.4 (Ar-qC), 127.8 (Ar-CH), 127.7 (Ar-CH), 127.1 (Ar-CH), 127.0 (Ar-CH), 125.2 (Ar-CH), 125.1 (Ar-CH), 120.0 (Ar-CH), 67.4 (Fmoc CH_2), 66.2 (Pro αCH), 63.9 (Ser CH_2), 53.5 (Ser αCH), 52.9 (OCH_3), 47.1 (Fmoc CH), 46.9 (Pro CH_2), 33.2 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 31.8 (Pro CH_2), 28.8, 23.8 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.2 ($CH_2S$), 24.2 (Pro CH_2) ppm.*

HRMS (*m/z*) calculated for $C_{44}H_{44}N_2NaO_9S$ $[M + Na]^+$, calcd. 799.2659, found 799.2642.

ν_{max} (film)/cm^{-1} 3332 (NH), 1704 (C=O).

*multiple signals account for inseparable mixture of cis/trans isomers

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-methionyl)thio)pentanoate (160)



Prepared as per Procedure K using **124** and following column chromatography (30% EtOAc:Hex) **160** was obtained as yellow crystals (167 mg, 88%).

R_f (30% EtOAc:Hex): 0.31

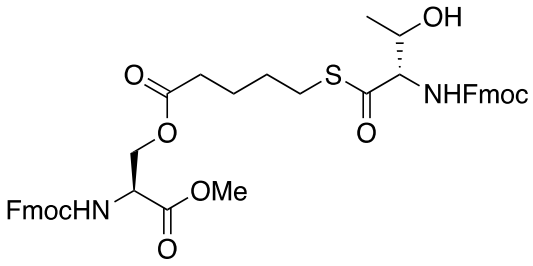
M.p. 33-35°C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.62 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.42 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.33 (4H, t, *J* = 7.4 Hz, Ar-CH), 5.69 (1H, d, *J* = 8.3 Hz, NH), 5.51 (1H, d, *J* = 9.0 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.60-4.40 (7H, m, 2 x Fmoc CH_2, Ser CH_2, Met αCH), 4.25 (2H, t, *J* = 6.9 Hz, 2 x Fmoc CH), 3.80 (3H, s, OCH_3), 2.90 (2H, t, *J* = 6.6 Hz, CH_2S), 2.58-2.50 (5H, m, Met CH_3, Met CH_2), 2.37 (2H, t, *J* = 6.6 Hz, $COC\underline{H}_2CH_2CH_2CH_2S$), 2.14-2.09 (2H, m, Met CH_2), 1.72-1.58 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 200.5, 172.7, 170.2, 155.8 (C=O), 143.7, 143.6, 141.4, 141.3 (Ar-qC), 127.8, 127.1, 125.1, 120.0 (Ar-CH), 67.3, 67.1 (Fmoc CH_2), 63.9 (Ser CH_2), 60.3 (Met αCH), 53.4 (Ser αCH), 52.9 (OCH_3), 47.2, 47.1 (Fmoc CH), 38.4 (Met CH_3), 33.2 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 32.0 (Met CH_2), 30.0 (Met CH_2), 28.7, 23.8 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.3 ($CH_2S$) ppm.

ν_{max} (film)/cm^{-1} 3325 (NH), 1726 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-*L*-threonyl)thio)pentanoate (161)



Prepared as per Procedure K using **125** and following column chromatography (30% EtOAc:Hex) **161** was obtained as a yellow oil (176 mg, 89%).

R_f (30% EtOAc:Hex): 0.28

M.p. 60-62 °C

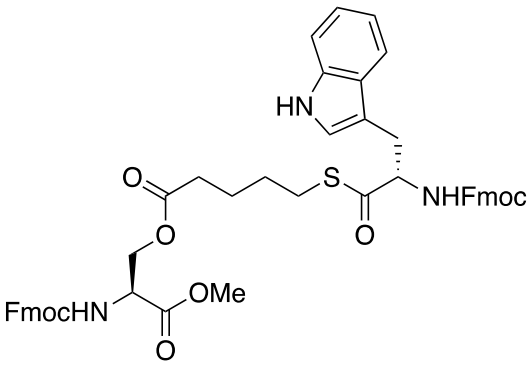
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.65-7.61 (4H, m, Ar-CH), 7.42 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.33 (4H, t, *J* = 7.4 Hz, Ar-CH), 5.76-5.73 (2H, m, 2 x NH), 4.67 (1H, m, Ser αCH), 4.53-4.36 (8H, m, 2 x Fmoc CH_2, Thr αCH, Thr CH, Ser CH_2), 4.28-4.25 (2H, m, 2 x Fmoc CH), 3.80 (3H, s, OCH_3), 2.89 (2H, t, *J* = 7.1 Hz, CH_2S), 2.39-2.36 (2H, m, $COCH_2CH_2CH_2CH_2S$), 1.72-1.60 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.24 (3H, d, *J* = 6.4 Hz, Thr CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 201.2, 172.3, 170.2, 156.7, 155.9 (C=O), 143.7, 143.8, 141.4, 141.3 (Ar-qC), 127.8, 127.7, 127.1, 127.0, 125.1, 120.0 (Ar-CH), 67.5 (Thr CH), 67.4, 67.3 (2 x Fmoc CH_2), 65.7 (Thr αCH), 63.8 (Ser CH_2), 53.5 (Ser αCH), 53.1 (OCH_3), 47.2, 47.2 (2 x Fmoc CH), 33.3 ($COCH_2CH_2CH_2CH_2S$), 28.6, 23.5 ($COCH_2CH_2CH_2CH_2S$), 28.3 (CH_2S), 19.9 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{43}H_{45}N_2O_{10}S$ $[M + H]^+$, calcd. 781.2779, found 781.2789.

ν_{max} (film)/cm^{-1} 3385 (NH), 1705 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-tryptophyl)thio)pentanoate (162)



Prepared as per Procedure K using **126** and following column chromatography (30% EtOAc:Hex) **162** was obtained as a yellow oil (46 mg, 22%).

R_f (30% EtOAc:Hex): 0.36

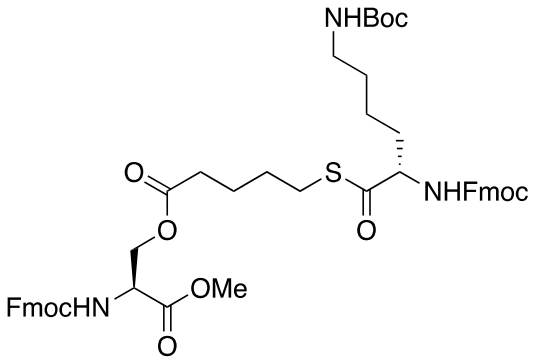
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79-7.69 (4H, m, Ar-CH), 7.62-7.53 (5H, m, Ar-CH), 7.44-7.38 (5H, m, Ar-CH), 7.35-7.29 (5H, m, Ar-CH), 7.25-7.15 (2H, m, Ar-CH), 7.00 (1H, bs, Ar-NH), 5.67 (1H, d, *J* = 8.2 Hz, NH), 5.35 (1H, d, *J* = 8.5 Hz, NH), 4.83-4.78 (1H, m, Trp αCH), 4.69-4.67 (1H, m, Ser αCH), 4.53-4.39 (6H, m, Ser CH_2, 2 x Fmoc CH_2), 4.27-4.19 (2H, m, 2 x Fmoc CH), 3.80 (3H, s, OCH_3), 3.44-3.39 (1H, m, Trp CH_2), 3.32-3.27 (1H, m, Trp CH_2), 2.94-2.81 (2H, m, CH_2S), 2.33 (2H, t, *J* = 7.1 Hz, $COC\underline{H}_2CH_2CH_2CH_2S$), 1.65-1.53 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 201.2, 172.9, 170.2, 155.9 (C=O), 143.8, 143.7, 141.6, 141.3, 141.2, 136.4 (Ar-qC), 127.8, 127.7, 127.1, 127.0, 125.2, 125.1, 123.4, 122.3, 120.0, 119.9, 119.8, 118.6, 111.3 (Ar-CH), 67.3, 67.2 (2 x Fmoc CH_2), 64.0 (Ser CH_2), 61.3 (Trp αCH), 53.5 (Ser αCH), 52.9 (OCH_3), 47.2, 47.1 (2 x Fmoc CH), 33.4 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 28.6, 23.7 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.5 (Trp CH_2), 28.1 (CH_2S) ppm.

HRMS (*m/z*) calculated for $C_{50}H_{48}N_3O_9S$ $[M + H]^+$, calcd. 866.3077, found 866.3106

ν_{max} (film)/cm^{-1} 3375 (NH), 1723 (C=O), 741 (CH).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-((*N*^{2}-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-*N*^{6}-(*tert*-butoxycarbonyl)-*L*-lysyl)thio)pentanoate (163)



Prepared as per Procedure K using **127** and following column chromatography (40% EtOAc:Hex) **163** was obtained as white crystals (60 mg, 28%).

R_f (40% EtOAc:Hex): 0.32

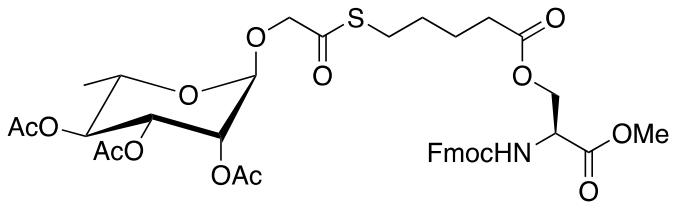
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.80-7.75 (4H, d, *J* = 7.7 Hz, Ar-CH), 7.65-7.61 (4H, m, Ar-CH), 7.55 (1H, bs, NH), 7.42 (4H, t, *J* = 7.7 Hz, Ar-CH), 7.33 (4H, t, *J* = 7.7 Hz, Ar-CH), 5.72 (1H, d, *J* = 6.9 Hz, NH), 5.52 (1H, d, *J* = 6.6 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.54-4.36 (7H, m, 2 x Fmoc CH_2, Ser CH_2, Lys αCH), 4.25 (2H, t, *J* = 6.9 Hz, 2 x Fmoc CH), 3.80 (3H, s, OCH_3), 3.14-3.10 (2H, m, Lys CH_2), 2.89 (2H, t, *J* = 6.2 Hz, CH_2S), 2.33 (2H, t, *J* = 6.2 Hz, $COC\underline{H}_2CH_2CH_2CH_2S$), 1.94-1.86 (1H, m, Lys CH_2), 1.73-1.58 (5H, m, Lys CH_2, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$), 1.54-1.29 (13H, m, $C(CH_3)_3$, 2 x Lys CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 201.1, 172.7, 170.0, 156.0, 155.8 (C=O), 143.7, 141.3 (Ar-qC), 127.8, 127.1, 125.2, 120.0 (Ar-CH), 67.3, 67.1 (Fmoc CH_2), 63.9 (Ser CH_2), 61.0 (Lys αCH), 53.4 (Ser αCH), 52.9 (OCH_3), 47.2, 47.1 (Fmoc CH), 40.0 (Lys CH_2), 33.3 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 32.2, 29.7 (2 x Lys CH_2), 28.7, 23.8 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.4 ($C(CH_3)_3$), 28.3 ($CH_2S$), 22.4 (Lys CH_2) ppm.

HRMS (*m/z*) calculated for $C_{50}H_{57}N_3NaO_{11}S$ $[M + Na]^+$, calcd. 930.3609, found 930.3606

ν_{max} (film)/cm^{-1} 3351 (NH), 1712 (C=O)

(2*R*,3*R*,4*R*,5*S*,6*S*)-2-(((*S*)-1-(9*H*-Fluoren-9-yl)-5-(methoxycarbonyl)-3,8,14-trioxo-2,7-dioxa-13-thia-4-azapentadecan-15-yl)oxy)-6-methyltetrahydro-2*H*-pyran-3,4,5-triyl triacetate (164)



Prepared as per Procedure K using **128** and following column chromatography (40% EtOAc:Hex) **164** was obtained as a yellow oil (184 mg, 99%).

R_f (40% EtOAc:Hex): 0.33

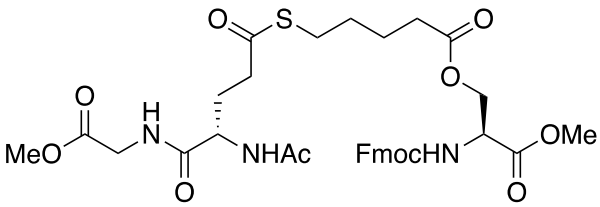
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (4H, d, *J* = 7.7 Hz, Ar-CH), 7.64-7.60 (4H, m, Ar-CH), 7.42 (4H, t, *J* = 7.7 Hz, Ar-CH), 7.34 (4H, t, *J* = 7.7 Hz, Ar-CH), 5.70 (1H, d, *J* = 8.7 Hz, NH), 5.42-5.36 (2H, m, H-3, H-2), 5.11 (1H, t, *J* = 9.7 Hz, H-4), 4.84 (1H, s, H-1), 4.68-4.64 (1H, m, Ser αCH), 4.53-4.39 (4H, m, Fmoc CH_2, Ser CH_2), 4.28-4.25 (3H, m, Fmoc CH, $COCH_2O$), 3.97-3.90 (1H, m, H-5), 3.81 (3H, s, OCH_3), 2.92 (2H, t, *J* = 7.2 Hz, CH_2S), 2.38 (2H, t, *J* = 7.2 Hz, $COC\underline{H}_2CH_2CH_2CH_2S$), 2.17, 2.07, 2.02 (3H, s, $CO\underline{C}H_3$), 1.75-1.61 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$), 1.23 (3H, d, *J* = 6.1 Hz, H-6) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 198.1, 172.7, 170.0, 155.9 (C=O), 143.8, 141.4 (Ar-qC), 127.9, 127.1, 125.2, 120.1 (Ar-CH), 97.8 (C-1), 71.6 ($CO\underline{C}H_2O$), 70.8 (C-4), 69.5 (C-2), 68.9 (C-3), 67.4 (Fmoc CH_2), 67.2 (C-5), 63.9 (Ser CH_2), 53.5 (Ser αCH), 53.0 (OCH_3), 47.2 (Fmoc CH), 33.4 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 28.8, 23.9 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 27.5 ($CH_2S$), 20.9, 20.8, 20.7 ($CO\underline{C}H_3$), 17.3 (C-6).

HRMS (*m/z*) calculated for $C_{38}H_{45}NNaO_{15}S$ $[M + Na]^+$, calcd. 810.2397, found 810.2402.

ν_{max} (film)/cm^{-1} 3365 (NH), 1741 (C=O).

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl 5-(((*S*)-4-acetamido-5-((2-methoxy-2-oxoethyl)amino)-5-oxopentanoyl)thio)pentanoate (165)



Prepared as per Procedure K using **129** and following column chromatography (100% EtOAc) **165** was obtained as a white solid (44 mg, 40%).

R_f (100% EtOAc): 0.26

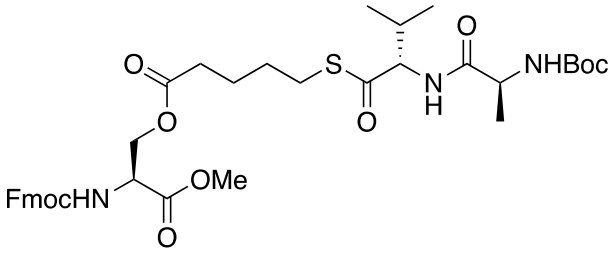
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.632 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.41 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.32 (4H, t, *J* = 7.4 Hz, Ar-CH), 6.90 (1H, d, *J* = 5.7 Hz, NH), 5.83 (1H, d, *J* = 8.2 Hz, NH), 4.68-4.64 (1H, m, Ser αCH), 4.58-4.37 (5H, Fmoc CH_2, Ser CH_2, Glu αCH), 4.25 (1H, t, *J* = 6.8 Hz, Fmoc CH), 4.01 (2H, d, *J* = 5.7 Hz, Gly CH_2), 3.80-3.72 (6H, m, Gly OCH_3, Ser OCH_3), 2.87 (2H, t, *J* = 6.8 Hz, CH_2S), 2.75-2.65 (2H, m, Glu CH_2), 2.36 (2H, t, *J* = 6.8 Hz, $COC\underline{H}_2CH_2CH_2CH_2S$), 2.17-2.00 (5H, m, Glu CH_2, Ac CH_3), 1.70-1.53 (4H, m, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 199.4, 172.9, 171.4, 170.3, 170.1, 169.9, 155.9 (C=O), 143.9, 141.3 (Ar-qC), 127.9, 127.2, 125.2, 120.1 (Ar-CH), 67.3 (Fmoc CH_2), 63.8 (Ser CH_2), 53.4 (Ser αCH), 52.9 (Ser OCH_3), 52.5 (Gly OCH_3), 47.1 (Fmoc CH), 41.2 (Gly CH_2), 39.8 (Glu CH_2), 33.2 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 28.9, 23.8 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.3 ($CH_2S$), 27.8 (Glu CH_2), 22.9 (Ac CH_3) ppm.

HRMS (*m/z*) calculated for $C_{34}H_{41}N_3NaO_{11}S$ $[M + Na]^+$, calcd. 722.2365, found 722.2354

ν_{max} (film)/cm^{-1} 3306 (NH), 1739, 1660 (C=O)

(*S*)-2-((((9*H*-Fluoren-9-yl)methoxy)carbonyl)amino)-3-methoxy-3-oxopropyl (6*S*,9*S*)-9-isopropyl-2,2,6-trimethyl-4,7,10-trioxo-3-oxa-11-thia-5,8-diazahexadecan-16-oate (166)



Prepared as per Procedure K using **110** (19 mg, 0.05 mmol) and dipeptide thioacid **130** (0.14 mmol) in DMF (0.5 mL). Following column chromatography (50-80% Et_2O:petroleum ether) **166** was obtained as a yellow oil (14 mg, 41%).

R_f (80% EtOAc:Hex): 0.42

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.63 (4H, d, *J* = 7.4 Hz, Ar-CH), 7.41 (4H, t, *J* = 7.4 Hz, Ar-CH), 7.32 (4H, t, *J* = 7.4 Hz, Ar-CH), 6.89 (1H, bs, NH), 5.68 (1H, d, *J* = 6.7 Hz, NH), 4.98 (1H, bs, NH), 4.67-4.59 (2H, m, Ser αCH, Val αCH), 4.52-4.49 (4H, m, Fmoc CH_2, Ser CH_2), 4.28-4.20 (2H, m, Fmoc CH, Ala αCH), 3.80 (3H, s, OCH_3), 2.89 (2H, t, *J* = 6.9 Hz, CH_2S), 2.37-2.30 (3H, m, $COCH_2CH_2CH_2CH_2S$, Val CH), 1.72-1.58 (4H, m, $COCH_2CH_2CH_2CH_2S$), 1.47 (9H, s, $C(CH_3)_3$), 1.39 (3H, app. t, *J* = 6.5 Hz, Ala CH_3), 0.99-0.88 (6H, m, 2 x Val CH_3) ppm.

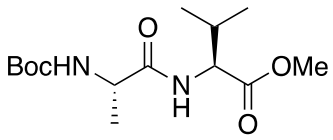
^{13}C NMR (100 MHz, $CDCl_3$) δ 200.0, 172.8, 172.7, 170.0, 155.9, 155.8 (C=O), 143.8, 141.4 (Ar-qC), 127.9, 127.1, 125.2, 120.1 (Ar-CH), 80.4 (qC), 67.3 (Fmoc CH_2), 63.9 (Ser CH_2), 63.8 (Ala αCH), 53.5 (Ser αCH), 53.0 (OCH_3), 50.1 (Thr αCH), 47.1 (Fmoc CH), 33.4 ($COCH_2CH_2CH_2CH_2S$), 31.8 (Thr CH), 28.7, 23.8 ($COCH_2CH_2CH_2CH_2S$), 28.4 (3 x CH3), 28.3 (CH_2S), 19.4, 16.8 (2 x Thr CH_3), 17.2 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{37}H_{49}N_3NaO_{10}S$ $[M + Na]^+$, calcd. 750.3045, found 750.3031.

ν_{max} (film)/cm^{-1} 3325 (NH), 1683 (C=O).

Methyl (*tert*-butoxycarbonyl)-L-alanyl-L-valinate (139)

Boc-Ala-Val-OMe



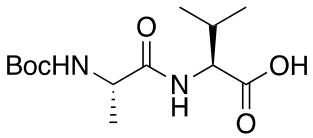
Prepared as per Procedure A using Boc-Ala-OH (2.0 g, 10.57 mmol) and HCl·H_2N-Val-OMe (1.8 g, 10.58 mmol) in anhydrous CH_2Cl_2 (85 mL) and DMF (15 mL). Following column chromatography (20% EtOAc:Hex), **139** was obtained as a yellow oil (2.6 g, 83%). The compound was in good agreement with the literature.[387]

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.70 (1H, bs, NH), 5.03 (1H, bs, NH), 4.54 (1H, dd, *J* = 8.8 Hz, *J* = 4.9 Hz, Val αCH), 4.21 (1H, t, *J* = 6.8 Hz, Ala αCH), 3.75 (3H, s, OCH_3), 2.23-2.15 (1H, m, Val CH), 1.46 (9H, s, $C(CH_3)_3$), 1.37 (3H, d, *J* = 6.8 Hz, Ala CH_3), 0.94 (3H, d, *J* = 6.8 Hz, Val CH_3), 0.92 (3H, d, *J* = 6.8 Hz, Val CH_3) ppm.

HRMS (*m/z*) calculated for $C_{14}H_{26}N_2NaO_5$ [M + Na]+, calcd. 325.1741, found 325.1734.

(*tert*-Butoxycarbonyl)-L-alanyl-L-valine (140)

Boc-Ala-Val-OH



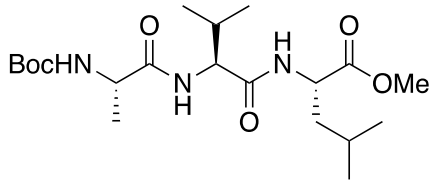
Prepared as per Procedure I using **139** (1.3 g, 4.30 mmol) in MeOH:H_2O (1:1, 40 mL) **140** was obtained as a white solid (1.2 g, 94%). The compound was in good agreement with the literature.[388]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.01 (1H, d, *J* = 7.0 Hz, NH), 5.28 (1H, m, NH), 4.59-4.54 (1H, m, Val αCH), 4.30-4.26 (1H, m, Ala αCH), 2.31-2.23 (1H, m, Val CH), 1.46-1.36 (12H, m, $C(CH_3)_3$, Ala CH_3), 0.99-0.91 (6H, m, 2 x Val CH_3) ppm.

HRMS (*m/z*) calculated for $C_{13}H_{24}N_2NaO_5$ [M + Na]$^+$, calcd. 311.1579, found 311.1577.

Methyl-(*tert*-butoxycarbonyl)-L-alanyl-L-valyl-L-leucinate (141)

Boc-Ala-Val-Leu-OMe



Prepared as per Procedure A using **140** (1.2 g, 4.16 mmol) and HCl·H_2N-Leu-OMe (754 mg, 4.16 mmol) in anhydrous CH_2Cl_2 (35 mL) and DMF (7 mL). Following flash column chromatography (30% EtOAc:Hex) **141** was obtained as a white solid (1.3 g, 74%). The compound was in good agrememt with the literature.[389]

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.91-6.68 (2H, m, 2 x NH), 5.27-5.16 (1H, m, NH), 4.63-4.57 (1H, m, Leu αCH), 4.34-4.29 (1H, m, Val αCH), 4.26-4.20 (1H, m, Ala αCH), 3.74 (3H, s, OCH_3), 2.21-2.13 (1H, m, Val CH), 1.70-1.55 (3H, m, Leu CH_2, Leu CH), 1.45 (9H, s, $C(CH_3)_3$), 1.36 (3H, d, *J* = 6.8 Hz, Ala CH_3), 0.97-0.91 (12H, m, 2 x Val CH_3, 2 x Leu CH_3) ppm.

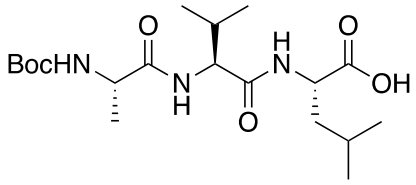
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.2, 172.8, 170.8, 155.6 (C=O), 80.5 (qC), 58.4 (Val αCH), 52.2 (OCH_3), 50.8 (Leu αCH), 50.3 (Ala αCH), 41.2 (Leu CH_2), 30.9 (Val CH), 28.5 ($C(CH_3)_3$), 24.8 (Leu CH), 22.8, 21.8 (2 x Leu CH_3), 19.2 (Val CH_3), 18.0 (Ala CH_3), 17.9 (Val CH_3) ppm.

HRMS (*m/z*) calculated for $C_{20}H_{37}N_3NaO_6$ $[M + Na]^+$, calcd. 438.2577, found 438.2575.

ν_{max} (film)/cm^{-1} 3293 (NH), 1728, 1641 (C=O).

(*tert*-Butoxycarbonyl)-L-alanyl-L-valyl-L-leucine (142)

Boc-Ala-Val-Leu-OH



Prepared as per Procedure I using **141** (820 mg, 1.97 mmol) in MeOH:H_2O (1:1, 20 mL) **142** was obtained as a white solid (766 mg, 97%).

R_f (5% MeOH:CH_2Cl_2): 0.27

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.47 (1H, bs, NH), 7.26 (1H, bs, NH), 5.43 (1H, bs, NH), 4.62-4.55 (1H, m, Leu αCH), 4.39-4.26 (2H, m, Val αCH, Ala αCH), 2.15-2.09 (1H, m, Val CH), 1.77-1.60 (3H, m, Leu CH_2, Leu CH), 1.45 (9H, s, $C(CH_3)_3$), 1.34 (3H, d, *J* = 6.9 Hz, Ala CH_3), 0.97-0.91 (12H, m, 2 x Val CH_3, 2 x Leu CH_3) ppm.

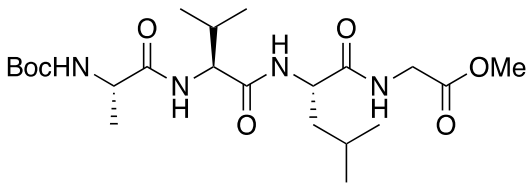
^{13}C NMR (100 MHz, $CDCl_3$) δ 175.2, 173.5, 171.7, 155.8 (C=O), 80.3 (qC), 58.8 (Val αCH), 51.0 (Leu αCH), 50.3 (Ala αCH), 41.0 (Leu CH_2), 31.0 (Val CH), 28.4 ($C(CH_3)_3$), 24.8 (Leu CH), 22.8, 21.9 (2 x Val CH_3), 19.2 (Leu CH_3), 18.4 (Ala CH_3), 18.1 (Leu CH_3) ppm.

HRMS (*m/z*) calculated for $C_{19}H_{35}N_3NaO_6$ $[M + Na]^+$, calcd. 424.2426, found 424.2418.

ν_{max} (film)/cm^{-1} 3295 (NH), 2962 (OH), 1710, 1641 (C=O).

Methyl-(*tert*-butoxycarbonyl)-L-alanyl-L-valyl-L-leucylglycinate (143)

Boc-Ala-Val-Leu-Gly-OMe



Prepared as per Procedure A using **142** (760 mg, 1.89 mmol) and HCl·H_2N-Gly-OMe (238 mg, 1.89 mmol) in anhydrous CH_2Cl_2 (15 mL) and DMF (3 mL) **143** was obtained

as a white solid (758 g, 85%) and was used without purification due to solubility difficulties.

R_f (80% EtOAc:Hex): 0.29

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81-7.69 (2H, m, 2 x NH), 7.49 (1H, d, *J* = 7.9 Hz, NH), 5.62 (1H, d, *J* = 6.0 Hz, NH), 4.69-4.34 (3H, m, Leu αCH, Val αCH, Ala αCH), 4.12-3.90 (2H, m, Gly CH_2), 3.72 (3H, s, OCH_3), 2.12-2.10 (1H, m, Val CH), 1.79-1.57 (3H, m, Leu CH_2, Leu CH), 1.44-1.33 (12H, m, $C(CH_3)_3$, Ala CH_3), 0.98-0.89 (12H, m, 2 x Val CH_3, 2 x Leu CH_3) ppm.

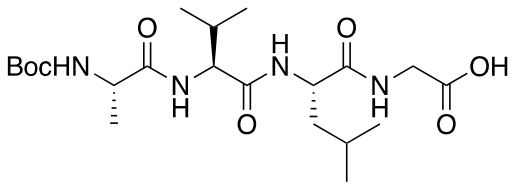
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.5, 172.7, 171.4, 170.1, 155.9 (C=O), 80.2 (qC), 58.8 (Val αCH), 51.6 (Leu αCH), 50.4 (Ala αCH), 40.7 (Leu CH_2), 41.1 (Gly CH_2), 30.9 (Val CH), 28.6 ($C(CH_3)_3$), 24.7 (Leu CH), 22.8 (Leu CH_3), 21.9 (Leu CH_3), 19.2 (Val CH_3), 19.0 (Ala CH_3), 18.1 (Val CH_3) ppm.

HRMS (*m/z*) calculated for $C_{22}H_{40}N_4NaO_7$ $[M + Na]^+$, calcd. 495.2790, found 495.2789.

ν_{max} (film)/cm^{-1} 3279 (NH), 1634 (C=O).

(*tert*-Butoxycarbonyl)-*L*-alanyl-*L*-valyl-*L*-leucylglycine (144)

Boc-Ala-Val-Leu-Gly-OH



Prepared as per Procedure I using **143** (730 mg, 1.55 mmol) in MeOH:H_2O (1:1, 16 mL) **144** was obtained as a pale pink solid (614 mg, 87%).

R_f (100% EtOAc): 0.20

^{1}H NMR (400 MHz, MeOD) δ 4.51-4.41 (1H, m, Leu αCH), 4.21-4.08 (2H, m, Val αCH, Ala αCH), 4.00-3.83 (2H, m, Gly CH_2), 2.16-2.05 (1H, m, Val CH), 1.77-1.61 (3H, m, Leu CH_2, Leu CH), 1.46 (9H, s, $C(CH_3)_3$), 1.31 (3H, d, *J* = 7.2 Hz, Ala CH_3), 1.01-0.92 (12H, m, 2 x Val CH_3, 2 x Leu CH_3) ppm.

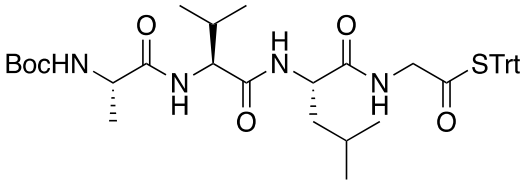
^{13}C NMR (100 MHz, MeOD) δ 174.8, 173.5, 172.0, 171.2, 156.4 (C=O), 79.3 (qC), 58.8 (Val αCH), 51.6 (Leu αCH), 50.4 (Ala αCH), 40.4 (Leu CH_2), 40.3 (Gly CH_2), 30.6 (Val CH), 27.4 ($C(CH_3)_3$), 24.3 (Leu CH), 22.1 (Leu CH_3), 20.5 (Leu CH_3), 18.3 (Val CH_3), 17.2 (Val CH_3), 16.5 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{21}H_{38}N_4O_7$ $[M + Na]^+$, calcd. 481.2630, found 481.2632.

ν_{max} (film)/cm^{-1} 3324 (NH), 2963 (OH), 1635 (C=O).

_S_-Trityl (6*S*,9*S*,12*S*)-12-isobutyl-9-isopropyl-2,2,6-trimethyl-4,7,10,13-tetraoxo-3-oxa-5,8,11,14-tetraazahexadecane-16-thioate (131)

Boc-Ala-Val-Leu-Gly-*S*-Trt



Prepared as per Procedure E using **144** (360 mg, 0.78 mmol) in CH_2Cl_2 (6 mL) and DMF (1 mL) and following column chromatography (80-100% Et_2O:petroleum ether) gave the product **131** as a white solid (452 mg, 80%).

R_f (100% Et_2O/petroleum ether): 0.32

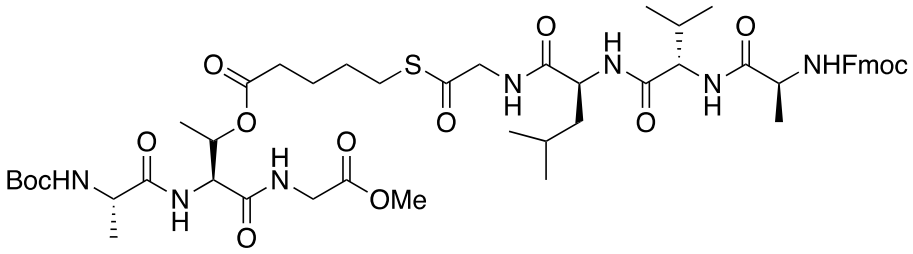
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.90 (1H, bs, NH), 7.63 (1H, bs, NH), 7.41 (1H, d, *J* = 7.3 Hz, NH), 5.47 (1H, d, *J* = 5.3 Hz, NH), 4.68-4.63 (1H, m, Leu αCH), 4.40-4.36 (2H, m, Val αCH, Ala αCH), 4.03 (2H, ddd, *J* = 29.5 Hz, *J* = 17.6 Hz, *J* = 5.5 Hz, Gly CH_2), 2.16-2.09 (1H, m, Val CH), 1.83-1.59 (1H, m, Leu CH, Leu CH_2), 1.43 (9H, s, $C(CH_3)_3$), 1.27 (3H, d, *J* = 7.0 Hz, Ala CH_3), 0.92-0.85 (12H, m, 2 x Val CH_3, 2 x Leu CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 194.6, 173.5, 172.7, 171.4, 155.9 (C=O), 143.7 (Ar-qC), 129.8, 127.8, 127.1 (Ar-CH), 80.2, 70.1 (qC), 58.7 (Leu αCH), 51.7 (Val αCH), 50.3 (Ala αCH), 49.3 (Gly CH_2), 40.5 (Leu CH_2), 30.8 (Val CH), 28.4 ($C(CH_3)_3$), 24.8 (Leu CH), 22.9 (Leu CH_3), 21.7 (Leu CH_3), 19.3 (Val CH_3), 19.2 (Ala CH_3), 18.0 (Val CH_3) ppm.

HRMS (*m/z*) calculated for $C_{40}H_{52}N_4NaO_6S$ $[M + Na]^+$, calcd. 739.3502, found 739.3500

ν_{max} (film)/cm^{-1} 3285 (NH), 1738, 1639 (C=O)

(2*S*,3*S*)-3-((*S*)-2-((*tert*-Butoxycarbonyl)amino)propanamido)-4-((2-methoxy-2-oxoethyl)amino)-4-oxobutan-2-yl (5S,8S,11S)-1-(9*H*-fluoren-9-yl)-11-isobutyl-8-isopropyl-5-methyl-3,6,9,12,15-pentaoxo-2-oxa-16-thia-4,7,10,13-tetraazahenicosan-21-oate (168)



Prepared as per Procedure F using **131** (84 mg, 0.12 mmol) to give the crude thioacid, which was immediately subjected to Procedure D with **95** (17 mg, 0.04 mmol) in anhydrous DMF (2 mL). Following column chromatography (5-7% MeOH:CH_2Cl_2) the product **168** was obtained as a white solid (33 mg, 92%).

R_f (7% MeOH/CH_2Cl_2): 0.33

M.p. 84-86 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63-7.59 (2H, m, 2 x NH), 7.45-7.42 (2H, m, 2 x NH), 7.36 (1H, bs, NH), 5.71 (1H, bs, NH), 5.64 (1H, d, *J* = 6.3 Hz, NH), 5.55 (1H, d, *J* = 5.8 Hz, NH), 5.41-5.38 (1H, m, Thr CH), 4.83-4.81 (1H, m, Thr αCH), 4.69-4.65 (1H, m, Ala αCH), 4.60-4.56 (1H, m, Ala αCH), 4.44-4.39 (2H, m, Val αCH, Leu αCH), 4.25 (1H, d, *J* = 17.0 Hz, Gly CH_2), 4.05-3.95 (3H, m, Gly CH_2, Gly CH_2), 3.72 (3H, s, OCH_3), 2.86 (2H, t, *J* = 6.2 Hz, CH_2S), 2.36-2.24 (2H, m, $COC\underline{H}_2CH_2CH_2CH_2S$), 2.18-2.14 (1H, m, Val CH), 1.79-1.54 (7H, m, Leu CH, Leu CH_2, $COCH_2C\underline{H}_2C\underline{H}_2CH_2S$), 1.44 (18H, m, 2 x $C(CH_3)_3$), 1.35-1.32 (6H, m, 2 x Ala CH_3), 1.27 (3H, d, *J* = 6.5 Hz, Thr CH_3), 0.96-0.88 (12H, m, 2 x Val CH_3, 2 x Leu CH_3) ppm.

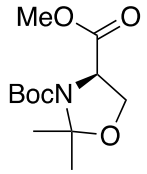
^{13}C NMR (100 MHz, $CDCl_3$) δ 198.1, 173.6, 173.2, 172.8, 171.2, 169.9, 169.4, 162.5, 156.1, 155.9 (C=O), 80.3, 80.2 (qC), 70.2 (Thr CH), 58.8 (Val αCH), 56.2 (Thr αCH), 52.3 (OCH_3), 51.8 (2 x Ala αCH), 50.7 (Leu αCH), 49.3 (Gly CH_2), 41.3 (Gly CH_2), 40.5 (Leu CH_2), 33.7 ($CO\underline{C}H_2CH_2CH_2CH_2S$), 30.8 (Val CH), 28.6, 23.7 ($COCH_2\underline{C}H_2\underline{C}H_2CH_2S$), 28.3 (2 x $\underline{C}(CH_3)_3$), 27.9 (CH_2S), 24.9 (Leu CH), 22.8 (Leu

CH_3), 21.7 (Leu CH_3), 19.3 (Val CH_3), 18.9 (2 x Ala CH_3), 17.9 (Val CH_3), 16.5 (Thr CH_3) ppm.

HRMS (*m/z*) calculated for $C_{41}H_{70}N_7O_{14}S$ $[M - H]^-$, calcd. 916.4707, found 916.4680.

ν_{max} (film)/cm^{-1} 3284 (NH), 1638 (C=O).

3-(*tert*-Butyl) 4-methyl (*R*)-2,2-dimethyloxazolidine-3,4-dicarboxylate (181)

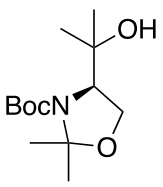


To a solution of Boc-D-Ser-OMe (11.9 g, 54.46 mmol) in CH_2Cl_2 (65 mL) were added DMP (33 mL, 272.31 mmol) and PTSA.H_2O (1.0 g, 5.45 mmol) at 0 °C. The reaction mixture was warmed to rt and stirred for 14 h. The mixture was quenched with sat. aq. $NaHCO_3$ solution (65 mL) and extracted with Et_2O (3 x 40 mL). The combined organic extracts were washed with sat. aq. $NaHCO_3$ solution (2 x 40 mL), brine (2 x 40 mL) and dried over $MgSO_4$. Solvent was removed *in vacuo* to and the crude product was distilled *in vacuo* to give **181** as a colourless oil (9.23 g, 65%). The isolated compound was in good agreement with the literature.[390]

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.48 (1H, dd, *J* = 7.0 Hz, *J* = 2.8 Hz, Ser αCH), 4.13 (1H, td, *J* = 9.2 Hz, *J* = 7.0 Hz, Ser CH_2), 4.03 (1H, td, *J* = 9.2, *J* = 2.8 Hz, Ser CH_2), 3.75 (3H, s, OCH_3), 1.66 (3H, s, CH_3), 1.53 (3H, s, CH_3), 1.40 (9H, s, $C(CH_3)_3$) ppm.

HRMS (*m/z*) calculated for $C_{12}H_{21}NO_5Na$ $[M + Na]^+$, calcd. 282.1314, found 282.1308.

***tert*-Butyl-(*R*)-4-(2-hydroxypropan-2-yl)-2,2-dimethyloxazolidine-3-carboxylate (182)**



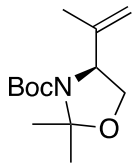
To a solution of MeMgI (24.7 g, 148.95 mmol) in anhydrous Et_2O (49 mL) was added dropwise a solution of protected amino acid **181** (6.4 g, 24.82 mmol) in anhydrous Et_2O

(10 mL) at 0 °C under an atmosphere of N_2. The reaction was allowed to proceed for 30 min at 0 °C and was quenched with dropwise addition of sat. aq. NH_4Cl solution (70 mL). The reaction mixture was extracted with EtOAc (3 x 70 mL) and the organic layers were washed with brine (3 x 30 mL) and dried over $MgSO_4$. The crude product was concentrated *in vacuo* and subjected to column chromatography (25% EtOAc:Hex) to give **182** as a colourless oil (6.27 g, 97%). The isolated compound was in good agreement with the literature.[390]

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.02-3.95 (2H, m, Ser CH_2), 3.79 (1H, bs, Ser αCH), 1.59 (3H, s, CH_3), 1.51 (3H, s, CH_3), 1.50 (9H, s, $C(CH_3)_3$), 1.18 (3H, s, CH_3), 1.17 (3H, s, CH_3) ppm.

HRMS (*m/z*) calculated for $C_{13}H_{26}NO_4$ $[M + H]^+$, calcd. 260.1856, found 260.1854.

***tert*-Butyl-(*S*)-2,2-dimethyl-4-(prop-1-en-2-yl)oxazolidine-3-carboxylate (183)**

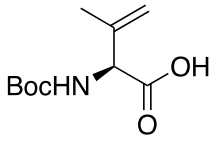


To a solution of **182** (0.4 g, 1.66 mmol) and NEt_3 (2 mL, 16.55 mmol) in anhydrous CH_2Cl_2 (5 mL) was added MsCl (640 μL, 8.28 mmol) dropwise at -10 °C under an atmosphere of N_2. The solution was warmed to rt and stirred for 1 h. The reaction mixture was diluted with Et_2O (25 mL) and H_2O (15 mL) and washed with aq. 1 M 10% citric acid solution (3 x 12 mL), sat. aq. $NaHCO_3$ solution (3 x 12 mL), brine (3 x 12 mL) and dried over $MgSO_4$. Solvent was removed *in vacuo* and the crude product was purified by column chromatography (15% EtOAc:Hex) to give **183** as a yellow oil (0.12 g, 31%). The isolated compound was in good agreement with the literature.[390]

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.89 (1H, s, C=CH_2), 4.83 (1H, s, C=CH_2), 4.38-4.21 (1H, m, Ser αCH), 4.06 (1H, dd, J = 8.9 Hz, J = 7.1 Hz, Ser CH_2), 3.73 (1H, dd, J = 8.9 Hz, J = 3.0 Hz, Ser CH_2), 1.71 (3H, s, CH_3), 1.66-1.60 (3H, m, CH_3), 1.49 (9H, s, $C(CH_3)_3$), 1.39 (3H, s, CH_3) ppm.

HRMS (*m/z*) calculated for $C_{13}H_{23}NO_3Na$ $[M + Na]^+$, calcd. 282.1573, found 264.1569.

(*S*)-2-((*tert*-Butoxycarbonyl)amino)-3-methylbut-3-enoic acid (184)



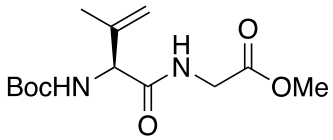
To a solution of alkene **183** (113 mg, 0.47 mmol) in Ace (1 mL) was added freshly prepared Jones reagent (350 μL, 1.07 mmol) at 0 °C. The mixture was allowed to stir at rt for 12 h before the addition of IPA (500 μL) and celite (100 mg). The reaction mixture was filtered, the filtrate basified to pH 9 with sat. aq. $NaHCO_3$ solution (2 mL) and concentrated *in vacuo*. The mixture was washed with Et_2O (2 x 2 mL), acidified to pH 3 with aq. 10% 1 M citric acid solution and extracted with EtOAc (3 x 2 mL). The organic layers were washed with brine (2 x 2 mL) and dried over $MgSO_4$. Concentration *in vacuo* gave **184** as an orange oil (31 mg, 29%). The isolated compound was in good agreement with the literature.[390]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.16 (1H, bs, NH), 5.12 (1H, s, C=CH_2), 5.05 (1H, bs, C=CH_2), 4.60-4.56 (1H, m, αCH), 1.82 (3H, s, CH_3), 1.44 (9H, s, C(CH_3)$_3$) ppm.

HRMS (*m/z*) calculated for $C_{10}H_{17}NO_4Na$ $[M + Na]^+$, calcd. 238.1049, found 238.1048.

Methyl((*S*)-2-((*tert*-butoxycarbonyl)amino)-3-methylbut-3-enoyl)-L-alaninate (185)

Boc-Dhv-Ala-OMe



Prepared as per Procedure A using **184** (100 mg, 0.46 mmol) and HCl·H_2N-Gly-OMe (70 mg, 0.56 mmol) and following column chromatography (30% EtOAc/Hex) the product **185** was obtained as a white solid (82 mg, 62%).

R_f (40% EtOAc/Hex): 0.42

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.38 (1H, bs, NH), 5.60 (1H, bs, NH), 5.19 (1H,s, C=CH_2), 5.10 (1H, s, C=CH_2), 4.67 (1H, s, Dhv αCH), 4.05 (2H, m, Gly CH_2), 3.76 (3H, s, OCH_3), 1.72 (3H, s, CH_3), 1.44 (9H, s, C(CH_3)$_3$) ppm.

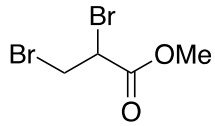
^{13}C NMR (100 MHz, $CDCl_3$) δ 178.2, 170.0, 156.7 (C=O), 118.9 (<u>C</u>=CH_2), 112.2 (C=<u>C</u>H_2), 79.9 (qC), 63.8 (Dhv αCH), 52.6 (OCH_3), 41.5 (Gly CH_2), 28.4 ($C(CH_3)_3$), 20.6 (CH_3) ppm.

HRMS (*m/z*) calculated for $C_{13}H_{22}N_2NaO_5$ $[M + Na]^+$, calcd. 309.1421, found 309.1420.

ν_{max} (film)/cm^{-1} 3364, 2979, 1696

6.5 Experimental details for Chapter 4

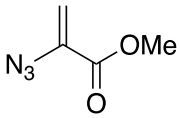
Methyl-2,3-dibromopropanoate (198)



To anhydrous CH_2Cl_2 (100 mL) under a nitrogen atmosphere was added methyl acrylate (3 mL, 34.85 mmol). Br_2 (2 mL, 34.85 mmol) was added to the solution dropwise at 0 °C and the solution was stirred for 20 min. The reaction mixture was warmed to rt and stirred for 18 h. The crude material was poured into aq. $NaHSO_4$ and the organic layer was collected and washed with brine. The product was dried over $MgSO_4$, filtered and concentrated *in vacuo* (at max. 20 °C) to furnish **198** as a yellow oil (8.1 g, 95%), which was used without further purification. The compound was in good agreement with the literature.[391]

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.45 (1H, dd, *J* = 11.3 Hz, *J* = 4.3 Hz, CH), 3.93 (1H, dd, *J* = 11.3 Hz, *J* = 9.9 Hz, CH_2), 3.68 (1H, dd, *J* = 9.9 Hz, *J* = 4.3 Hz, CH_2), 3.85 (3H, s, OCH_3) ppm.

Methyl-2-azidoacrylate (186:e)



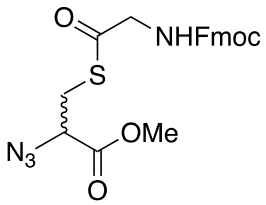
To a stirred solution of **198** (2.9 g, 11.72 mmol) in DMF (60 mL) was added NaN_3 (1.1 g, 17.58 mmol) at 60 °C. The mixture was stirred for 20 min and more NaN_3 (762 mg, 11.72 mmol) was added and stirred for a further 25 min. The reaction was cooled to rt

and H_2O (60 mL) and cold hexane (60 mL) was added. The organic layer was collected and washed with brine, dried over $MgSO_4$, filtered and solvent was removed *in vacuo*. The product was re-dissolved in Et_2O (5 mL) and DBU (33 mg, 0.22 mmol) was added. The solution was stirred at 0 °C for 1.5 h and the solvent was removed *in vacuo* to obtain **186:e** as a yellow oil (918 mg, 62%), which was used without further purification. The product was in good agreement with the literature.[391]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.86 (1H, d, *J* = 1.5 Hz, CH_2), 5.36 (1H, d, *J* = 1.5 Hz, CH_2), 3.84 (3H, s, OCH_3) ppm.

HRMS (*m/z*) calculated for $C_4H_4N_3O_2$ $[M - H]^-$, calcd. 126.0390, found 126.0304.

Methyl-3-(((((9*H*-Fluoren-9-yl)methoxy)carbonyl)glycyl)thio)-2-azidopropanoate (200)



To a solution of **186:e** (100 mg, 0.79 mmol), DPAP (40 mg, 0.16 mmol) and MAP (23 mg, 0.16 mmol) in aq. ammonia buffer pH 6 (20% buffer in H_2O, 4 mL) and THF (4 mL) was added Gly-SH (2.36 mmol), freshly prepared as per procedure F using **117**. The reaction mixture was irradiated at 365 nm in a UV oven at rt for 1 h. The mixture was diluted with H_2O (5 mL) and extracted with EtOAc (2 x 10 mL). The organic layers were combined and washed with brine (15 mL), dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. Purification by column chromatography (30-50% EtOAc:Hex) afforded **200** as a colourless oil (62 mg, 17%).

R_f (50% EtOAc:Hex): 0.34

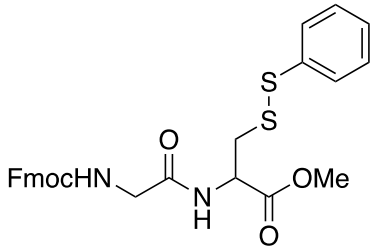
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.63 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.44 (2H, t, *J* = 7.2 Hz, Ar-CH), 7.35 (2H, t, *J* = 7.2 Hz, Ar-CH), 5.38 (1H, t, *J* = 5.6 Hz, NH), 4.49 (2H, d, *J* = 7.2 Hz, Fmoc CH_2), 4.27 (1H, t, *J* = 7.2 Hz, Fmoc CH), 4.19-4.12 (3H, m, Cys αCH, Cys CH_2), 3.84 (3H, s, OCH_3), 3.45 (1H, dd, *J* = 13.9 Hz, *J* = 5.6 Hz, Gly CH_2), 3.23 (1H, dd, *J* = 13.9 Hz, *J* = 5.6 Hz, Gly CH_2) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 196.8, 169.1, 156.2 (C=O), 143.6, 141.5 (Ar-qC), 127.8, 127.1, 125.0, 120.1 (Ar-CH), 67.5 (Fmoc CH_2), 61.2 (Cys αCH), 53.2 (OCH_3), 50.6 (Cys CH_2), 47.1 (Fmoc CH), 29.8 (Gly CH_2) ppm.

HRMS (*m/z*) calculated for $C_{21}H_{21}N_4O_5S$ $[M + H]^+$, calcd. 441.1215, found 441.1227.

ν_{max} (film)/cm^{-1} 3490 (NH), 2110 (N_3), 1702 (C=O).

Methyl-*N*-((((9*H*-fluoren-9-yl)methoxy)carbonyl)glycyl)-*S*-(phenylthio)cysteinate (203)



Isolated as a byproduct from the synthesis of **200**.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.62 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.52 (2H, d, *J* = 7.3 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.38-7.32 (4H, m, Ar-CH), 7.29-7.27 (1H, m, Ar-CH), 6.70 (1H, d, *J* = 7.1 Hz, NH), 5.31 (1H, bs, NH), 4.94-4.90 (1H, m, Cys αCH), 4.46 (2H, d, *J* = 6.7 Hz, Fmoc CH_2), 4.26 (1H, t, *J* = 6.7 Hz, Fmoc CH), 3.78 (3H, s, OCH_3), 3.86 (2H, d, *J* = 7.3 Hz, Cys CH_2), 3.33-3.22 (2H, m, Gly CH_2) ppm.

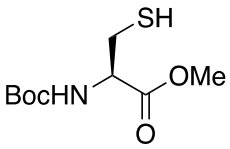
^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 168.7 (C=O), 143.8, 141.3, 136.6 (Ar-qC), 129.3, 128.4, 127.8, 127.6, 127.1, 125.2, 120.2 (Ar-CH), 67.5 (Fmoc CH_2), 53.1 (OCH_3), 52.0 (Cys αCH), 47.2 (Fmoc CH), 43.4 (Cys CH_2), 40.3 (Gly CH_2) ppm.

HRMS (*m/z*) calculated for $C_{27}H_{27}N_2O_5S_2$ $[M + H]^+$, calcd. 523.1363, found 523.1355.

ν_{max} (film)/cm^{-1} 2923 (NH), 1703 (C=O).

Methyl-(*tert*-butoxycarbonyl)-L-cysteinate (207)

Boc-Cys-OMe



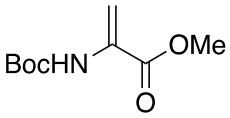
Prepared as per Procedure H using HCl·H_2N-Cys-OMe (4.2 g, 24.70 mmol) to obtain the disulfide, which was directly re-dissolved in MeOH (220 mL). To this solution was added tributylphosphine (6 mL, 24.70 mmol) in H_2O (30 mL) and the mixture was stirred at rt for 4 h.[392] The solvent was evaporated *in vacuo* and the crude material was diluted with H_2O (100 mL) and extracted with EtOAc (2 x 75 mL). The organic layers were combined and washed with brine, dried over $MgSO_4$, filtered and concentrated *in vacuo* to furnish **207** as a colourless oil (3.5 g, 60%). The compound was in good agreement with the literature.[393]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.44 (1H, bs, NH), 4.64-4.60 (1H, m, Cys αCH), 3.80 (3H, s, OCH_3), 3.00-2.96 (2H, m, Cys CH_2), 1.47 (9H, s, $C(CH_3)_3$) ppm.

HRMS (*m/z*) calculated for $C_9H_{17}NNaO_4S$ $[M + Na]^+$, calcd. 258.0769, found 258.0770.

Methyl-2-((*tert*-butoxycarbonyl)amino)acrylate (186:a)

Boc-Dha-OMe

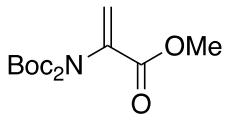


To a solution of **207** (3.5 g, 14.70 mmol) in DMF (100 mL) was added 1,4-diiodobutane (3 mL, 22.05 mmol) and K_2CO_3 (4.1g, 29.40 mmol). The reaction mixture was stirred at rt for 18 h. The solution was diluted with H_2O (100 mL) and extracted with EtOAc (2 x 75 mL). The organic layers were combined and washed with brine (3 x 100 mL), dried over MgSO4, filtered and the solvent was removed *in vacuo*. Purification by silica plug (2-5% EtOAc:Hex) afforded **186:a** as a white wax (1.6 g, 54%). The compound was in good agreement with the literature.[369]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.0 (1H, bs, NH), 6.15 (1H, s, C=CH_2), 5.72 (1H, d, J = 1.5 Hz, C=CH_2), 3.83 (3H, s, OCH_3), 1.48 (9H, s, $C(CH_3)_3$) ppm.

Methyl-2-(di(*tert*-butoxycarbonyl)amino) acrylate (186:d)

Boc_2-Dha-OMe



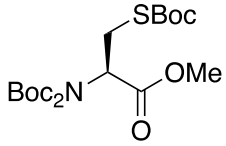
To a solution of **186:a** (1.6 g, 7.80 mmol) in CH_3CN (80 mL) was added Boc_2O (3.7 g, 17.17 mmol) and DMAP (190 mg, 1.56 mmol). The reaction was stirred at rt for 3 h. The solvent was removed *in vacuo* and the crude material was purified by silica plug (10% EtOAc:Hex) to furnish **186:d** as a white solid (2.3 g, 98%). The compound was in good agreement with the literature.[394]

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.32 (1H, s, C=CH_2), 5.62 (1H, s, C=CH_2), 3.78 (3H, s, OCH_3), 1.44 (18H, s, C(CH_3)$_3$) ppm.

HRMS (*m/z*) calculated for $C_{14}H_{23}NNaO_6$ $[M + Na]^+$, calcd. 324.1422, found 324.1417.

Methyl-*N*,*N*,*S*-tris(*tert*-butoxycarbonyl)-L-cysteinate (208)

Boc_2-Cys(*S*-Boc)-OMe



Prepared as per Procedure H using Boc-Cys-OMe (500 mg, 2.12 mmol) and Boc_2O (2.2 eq.) the product **208** was obtained as a white powder (694 mg, > 98%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.19 (1H, q, *J* = 4.9 Hz, Cys αCH), 3.75 (3H, s, OCH_3), 3.61 (1H, dd, *J* = 14.5 Hz, *J* = 4.8 Hz, Cys CH_2), 3.31 (1H, dd, *J* = 14.5 Hz, *J* = 9.7 Hz, Gly CH_2), 1.51 (18H, s, 2 x C(CH_3)$_3$), 1.49 (9H, s, C(CH_3)$_3$) ppm.

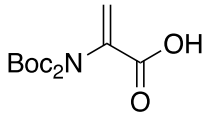
^{13}C NMR (100 MHz, $CDCl_3$) δ 170.2, 168.5, 151.9 (C=O), 85.0, 83.5 (qC), 57.7 (Cys αCH), 52.4 (OCH_3), 31.7 (Cys CH_2), 28.2 (C($\underline{C}H_3$)$_3$), 28.0 (C($\underline{C}H_3$)$_3$) ppm.

HRMS (*m/z*) calculated for $C_{19}H_{33}NNaO_8S$ $[M + Na]^+$, calcd. 458.1819, found 458.1828.

ν_{max} (film)/cm^{-1} 1751, 1688 (C=O).

2-(Di(*tert*-butoxycarbonyl)amino)acrylic acid (209)

Boc$_2$-Dha-OH



To a solution of **208** (6.8 g, 15.65 mmol) in dioxane:H_2O (1:1, 150 mL) was added LiOH (936 mg, 39.12 mmol) and the mixture was stirred at rt for 1 h. The solvent was removed *in vacuo* and the crude material was re-dissolved in H_2O and extracted into EtOAc (2 x 50 mL). The organic layers were combined and washed with brine, dried over $MgSO_4$, filtered and concentrated to furnish **209** as a white solid (3.0 g, 67%).

R_f (10% EtOAc/Hex): 0.35

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.46 (1H, s, C=CH_2), 5.77 (1H, s, C=CH_2), 1.47 (18H, s, C(CH_3)$_3$) ppm.

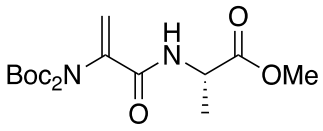
^{13}C NMR (100 MHz, $CDCl_3$) δ 168.2, 150.8 (C=O), 135.6 (<u>C</u>=CH_2), 126.7 (C=<u>C</u>H_2), 83.7 (qC), 28.1 (C(CH_3)$_3$) ppm.

HRMS (*m/z*) calculated for $C_{13}H_{21}NNaO_6$ $[M + Na]^+$, calcd. 310.1268, found 310.1261.

ν_{max} (film)/cm^{-1} 1723 (C=O), 2981 (C=C).

2-(Di(*tert*-butoxycarbonyl)amino)acrylic-L-alaninate (211)

Boc$_2$-Dha-Ala-OMe



Prepared as per Procedure A using **209** (100 mg, 0.35 mmol) and HCl·H_2N-Ala-OMe (48 mg, 0.35 mmol) in CH_2Cl_2 (4 mL) and DMF (500 μL). Following flash column chromatography (15-20% EtOAc:Hex) **211** was afforded as a colourless oil (38 mg, 26%).

R_f (20% EtOAc:Hex): 0.35

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.55 (1H, d, *J* = 7.4 Hz, NH), 6.21 (1H, s, C=CH_2), 5.50 (1H, s, C=CH_2), 4.72-4.65 (1H, m, Ala αCH), 3.77 (3H, s, OCH_3), 1.49-1.47 (21H, m, 2 x $C(CH_3)_3$, Ala CH_3) ppm.

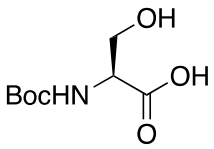
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 162.9, 150.6 (C=O), 138.6 (<u>C</u>=CH_2), 121 (C=<u>C</u>H_2), 83.6 (qC), 53.5 (OCH_3), 48.5 (Ala αCH), 27.8 ($C(CH_3)_3$), 18.8 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{17}H_{28}N_2NaO_7$ [M + Na]+, calcd. 395.1796, found 395.1789.

ν_{max} (film)/cm^{-1} 3344 (NH), 1790, 1744 (C=O).

(*tert*-Butoxycarbonyl)-L-serine (213)

Boc-Ser-OH



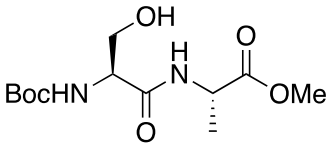
Prepared as per Procedure H using L-Serine (3.0 g, 28.54 mmol) in H_2O:dioxane (1:1, 200 mL) furnished the product **213** as a colourless oil (5.3 g, 90%). The compound was in good agreement with the literature.[395]

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.85 (1H, d, *J* = 7.0 Hz, NH), 4.36 (1H, m, Ser αCH), 4.06-3.98 (1H, m, Ser CH_2), 3.86-3.79 (1H, m, Ser CH_2), 1.44 (9H, s, $C(CH_3)_3$) ppm.

HRMS (*m/z*) calculated for $C_8H_{14}NO_5$ [M – H]$^-$, calcd. 204.0905, found 204.0877.

Methyl-(*tert*-butoxycarbonyl)-L-seryl-L-alaninate (214)

Boc-Ser-Ala-OMe



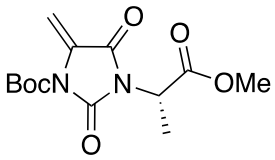
Prepared as per procedure A using **213** (300 mg, 1.46 mmol) and HCl·H_2N-Ala-OMe (203 mg, 1.46 mmol) in CH_2Cl_2 (13 mL) and DMF (2 mL) and after column

chromatography to obtain **214** as a colourless oil (153 mg, 36%). The compound was in good agreement with the literature.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.08 (1H, bs, NH), 5.55 (1H, d, *J* = 6.9 Hz, NH), 4.61-4.53 (1H, m, Ala αCH), 4.22-4.18 (1H, m, Ser αCH), 3.75 (3H, s, OCH_3), 1.45 (9H, s, $C(CH_3)_3$), 1.42 (3H, d, *J* = 6.9 Hz, Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{12}H_{21}N_2O_6$ $[M - H]^-$, calcd. 289.1402, found 289.1405.

tert-Butyl (*S*)-3-(1-methoxy-1-oxopropan-2-yl)-5-methylene-2,4-dioxoimidazolidine-1-carboxylate (217)



Isolated as a byproduct from the synthesis of **211**.

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.08 (1H, s, C=CH_2), 5.91 (1H, d, *J* = 0.9 Hz, C=CH_2), 4.91 (1H, q, *J* = 7.3 Hz, Ala αCH), 3.78 (3H, s, OCH_3), 1.68 (3H, d, *J* = 7.3 Hz, Ala CH_3), 1.63 (9H, s, $C(CH_3)_3$) ppm.

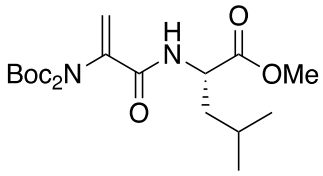
^{13}C NMR (100 MHz, $CDCl_3$) δ 169.4, 160.3, 149.5, 148.0 (C=O), 131.8 (<u>C</u>=CH_2), 105.8 (C=<u>C</u>H_2), 85.6 (qC), 53.1 (OCH_3), 48.5 (Ala αCH), 28.0 ($C(CH_3)_3$), 14.6 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{13}H_{18}N_2NaO_6$ $[M + Na]^+$, calcd. 321.1049, found, 321.1057.

ν_{max} (film)/cm^{-1} 2924 (CH alkene), 1750 (C=O).

2-(Di(*tert*-butoxycarbonyl)amino)acrylic-L-leucinate

Boc$_2$-Dha-Leu-OMe



Prepared as per Procedure A using **209** (200 mg, 0.700 mmol) and HCl·H$_2$N-Leu-OMe (127 mg, 0.700 mmol) in CH_2Cl_2 and following column chromatography (15-20% EtOAc:Hex) the product was furnished as a coloueless oil (52 mg, 18%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.65 (1H, d, *J* = 7.9 Hz, NH), 6.27 (1H, s, C=CH_2), 5.51 (1H, s, C=CH_2), 4.67-4.61 (1H, m, Leu αCH), 4.49-4.43 (1H, m, Leu CH_2), 4.26-4.21 (1H, m, Leu CH_2), 3.74 (3H, s, OCH_3), 1.69-1.64 (1H, m, Leu CH), 1.49 (18H, s, 2 x $C(CH_3)_3$), 0.96-0.93 (6H, m, 2 x Leu CH_3) ppm.

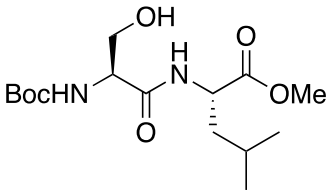
^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 169.0, 153.2 (C=O), 138.7 (C=CH_2), 121.6 (C=CH_2), 83.1 (qC), 66.0 (Leu CH_2), 52.3 (OCH_3), 50.9 (Leu αCH), 28.4 ($C(CH_3)_3$), 24.8 (Leu CH), 21.9 (2 x Leu CH_3) ppm.

HRMS (*m/z*) calculated for $C_{20}H_{34}N_2NaO_7$ $[M + Na]^+$, calcd. 437.2258, found 437.2253.

ν_{max} (film)/cm^{-1} 3339 (NH), 1740 (C=O), 1677 (C=C).

Methyl-(*tert*-butoxycarbonyl)-L-seryl-L-leucinate

Boc-Ser-Leu-OMe

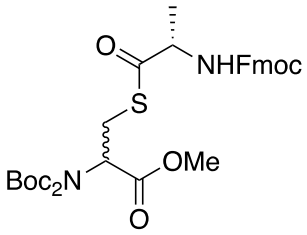


Prepared as per Procedure A using **213** (700 mg, 3.41 mmol) and HCl·H$_2$N-Leu-OMe (620 mg, 3.41 mmol) in CH_2Cl_2 (31 mL) and DMF (3 mL). Following flash column chromatography (50% EtOAc:Hex) the product was obtained as a colourless oil (471 mg, 36%). The compound was in good agreement with the literature.[396]

^{1}H NMR (400 MHz, $CDCl_3$) δ 6.99 (1H, bs, NH), 5.59 (1H, d, *J* = 7.3 Hz, NH), 4.60-4.55 (1H, m, Leu αCH), 4.20-4.16 (1H, m, Ser αCH), 3.73 (3H, s, OCH_3), 3.66-3.60 (1H, m, Ser CH_2), 3.41-3.37 (1H, m, Ser CH_2), 1.67-1.54 (3H, m, Leu CH, Leu CH_2), 1.44 (9H, s, $C(CH_3)_3$), 0.91 (6H, d, *J* = 3.7 Hz, 2 x Leu CH_3) ppm.

HRMS (*m/z*) calculated for $C_{15}H_{27}N_2O_6$ $[M - H]^-$, calcd. 331.1880, found 331.1875.

Methyl-*S*-((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-alanyl)-*N*,*N*-bis(*tert*-butoxycarbonyl)cysteinate (190)



To a solution of **186:d** (100 mg, 0.33 mmol), DPAP (17 mg, 0.06 mmol) and MAP (10 mg, 0.06 mmol) in aq. ammonia buffer pH 6 (20% buffer in H_2O, 3 mL) was added Gly-SH (0.99 mmol), freshly prepared as per procedure F using **118**. The reaction mixture was irradiated at 365 nm in a UV oven at rt for 1 h. Upon crude ^{1}H NMR analysis, the mixture was diluted with H_2O (3 mL), extracted with EtOAc (5 mL) and washed with sat. aq. $NaHCO_3$ (5 mL). The organic layer was concentrated *in vacuo* and DPAP (17 mg, 0.06 mmol), MAP (10 mg, 0.06 mmol) and thioacetic acid (70 μL, 0.99 mmol) were added. The mixture was diluted with aq. ammonia buffer pH 6 (20% buffer in H_2O, 3 mL) and the reaction mixture was again irriadiated at 365 m, in a UV oven at rt for 1 h. The mixture was diluted with H_2O (5 mL) and extracted with EtOAc (2 x 10 mL). The organic layers were combined and washed with brine (15 mL), dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. Purification by column chromatography (30-50% EtOAc:Hex) afforded **190** as a colourless oil (10 mg, 5%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.62 (2H, app. t, *J* = 7.2 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.2 Hz, Ar-CH), 7.34 (2H, t, *J* = 7.2 Hz, Ar-CH), 5.32-5.26 (1H, m, NH), 5.09 (1H, dd, *J* = 9.1 Hz, *J* = 5.4 Hz, Cys αCH), 4.53-4.38 (3H, m, Fmoc CH_2, Ala αCH), 4.25 (1H, t, *J* = 7.2 Hz, Fmoc CH), 3.77-3.68 (4H, m, OCH_3, Cys CH_2), 3.48 (1H, dd, *J* = 14.2 Hz, *J* = 9.1 Hz, Cys CH_2), 1.52 (18H, s, $C(CH_3)_3$), 1.44 (3H, d, *J* = 6.5 Hz, Ala CH_3) ppm.

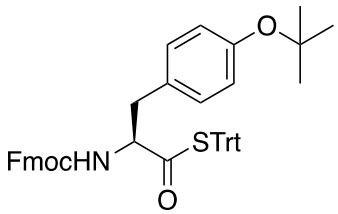
^{13}C NMR (100 MHz, $CDCl_3$) δ 200.2, 169.9, 155.5, 151.9 (C=O), 143.8, 141.3 (Ar-qC), 127.8, 127.7, 125.1, 120.1 (Ar-CH), 83.7 (qC), 67.2 (Fmoc CH_2), 57.5 (Cys αCH), 56.5 (Ala αCH), 52.7 (OCH_3), 47.4 (Fmoc CH), 29.4 (Cys CH_2), 28.0 ($C(CH_3)_3$), 19.1 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{32}H_{40}N_2NaO_9S$ $[M + Na]^+$, calcd. 651.2347, found 651.2365.

ν_{max} (film)/cm^{-1} 3420 (NH), 1700 (C=O), 741 (CH).

_S_-Trityl-(_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-(_tert_-butoxy)phenyl)propanethioate

Fmoc-Tyr(O^{t}Bu)-_S_-Trt



Prepared as per Procedure E using Fmoc-Tyr-O^{t}Bu (2.0 g, 4.35 mmol) in CH_2Cl_2 (40 mL). Following flash column chromatography (5-10% EtOAc:Hex) the product was afforded as white crystals (2.7 g, 87%).

M.p. 80-82 °C

R_f (10% EtOAc/Hex): 0.30

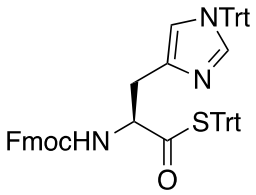
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.57 (2H, app. t, *J* = 7.2 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.2 Hz, Ar-CH), 7.33-7.23 (17H, m, Ar-CH), 6.96-6.89 (2H, m, Ar-CH), 5.14 (1H, d, *J* = 8.9 Hz), 4.73-4.68 (1H, m, Tyr αCH), 4.46-4.35 (2H, m, Fmoc CH_2), 4.23 (1H, t, *J* = 7.2 Hz, Fmoc CH), 3.01-2.92 (2H, m, Tyr CH_2), 1.34 (9H, s, $C(CH_3)_3$) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 197.3, 155.6 (C=O), 143.8, 143.7, 143.4, 141.4 (Ar-qC), 130.1,129.9, 127.9, 127.8, 127.2, 127.1, 125.2, 125.1, 120.0 (Ar-CH), 78.4 (qC), 70.7 (qC), 67.2 (Fmoc CH_2), 61.5 (Tyr αCH), 47.2 (Fmoc CH), 37.6 (Tyr CH_2), 28.9 ($C(CH_3)_3$) ppm.

ν_{max} (film)/cm^{-1} 3332 (NH), 1690 (C=O), 738, 696 (CH).

_S_-Trityl (_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-(1-trityl-1_H_-imidazol-4-yl)propanethioate

Fmoc-His(_N_-Trt)-_S_-Trt



Prepared as per Procedure E using Fmoc-His(*N*-Trt)-OH (2.0 g, 3.23 mmol) in CH_2Cl_2 (30 mL) and following flash column chromatography (7% EtOAc:Hex), the product was obtained as white crystals (947 mg, 46%).

M.p. 82-85 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.65 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.41-7.10 (35H, m, Ar-CH), 6.98 (1H, d, *J* = 7.9 Hz, NH), 6.63 (1H, s, Ar-CH), 4.63-4.58 (1H, His αCH), 4.49-4.46 (1H, m, Fmoc CH_2), 4.29-4.23 (2H, m, Fmoc CH_2, Fmoc CH), 2.97 (2H, d, *J* = 5.5 Hz, His CH_2) ppm.

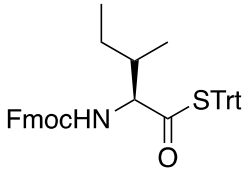
^{13}C NMR (100 MHz, $CDCl_3$) δ 198.2, 156.1 (C=O), 143.8, 142.4, 141.3 (Ar-qC), 138.9, 136.1, 129.9, 129.8, 128.1, 127.7, 127.1, 127.0, 126.9, 125.3, 119.8 (Ar-CH), 67.3 (Fmoc CH_2), 61.1 (His αCH), 47.2 (Fmoc CH), 29.7 (His CH_2) ppm.

HRMS (*m/z*) calculated for $C_{59}H_{47}N_3NaO_3S$ $[M + Na]^+$, calcd. 900.3230, found 900.3223.

ν_{max} (film)/cm^{-1} 3287 (NH), 1655 (C=O), 737, 696 (CH).

_S_-Trityl-(2_S_,3_S_)-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylpentanethioate

Fmoc-Ile-_S_-Trt



Prepared as per Procedure E using Fmoc-Ile-OH (2.0 g, 5.66 mmol) in CH_2Cl_2 (50 mL) and following flash column chromatography (15% EtOAc:Hex) the product was afforded as white crystals (3.1g, 90%).

M.p. 60-62 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.63 (2H, d, *J* = 7.4 Hz, Ar-CH), 7.43 (2H, t, *J* = 7.4 Hz, Ar-CH), 7.34-7.25 (17H, m, Ar-CH), 5.20 (1H, d, *J* = 9.3 Hz, NH), 4.53-4.48 (1H, m, Fmoc CH_2), 4.43-4.38 (2H, m, Fmoc CH_2, Ile αCH), 4.28 (1H, t, *J* = 7.0 Hz, Fmoc CH), 1.94-1.88 (1H, m, Ile CH), 1.26-1.18 (1H, m, Ile CH_2), 1.05-0.85 (7H, m, Ile CH_2, 2 x Ile CH_3) ppm.

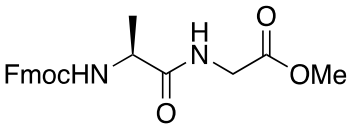
^{13}C NMR (100 MHz, $CDCl_3$) δ 197.4, 156.0 (C=O), 143.9, 143.7, 143.5, 141.4 (Ar-qC), 129.8, 127.8, 127.2, 127.1, 125.1, 125.0, 120.0 (Ar-CH), 70.9 (qC), 67.3 (Fmoc CH_2), 65.1 (Ile αCH), 47.3 (Fmoc CH), 38.2 (Ile CH), 24.0 (Ile CH_2), 15.7, 11.7 (2 x Ile CH_3) ppm.

HRMS (*m/z*) calculated for $C_{40}H_{41}N_2O_3S$ $[M + H]^+$, calcd. 629.2803, found 629.2932.

ν_{max} (film)/cm^{-1} 1686 (C=O), 738, 696 (CH).

Methyl-(((9_H_-fluoren-9-yl)methoxy)carbonyl)-L-alanylglycinate

Fmoc-Ala-Gly-OMe



Prepared as per Procedure A using Fmoc-Ala-OH (100 mg, 0.321 mmol) and HCl·H_2N-Gly-OMe (40 mg, 0.321 mmol) in anhydrous CH_2Cl_2 (4 mL) and DMF (1 mL) and

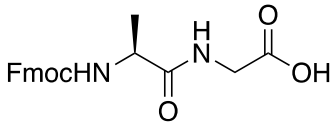
following column chromatography (20% EtOAc:Hex), the product was obtained as a colourless oil (95 mg, 78%). The compound was in good agreement with the literature.[397]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.58 (2H, d, *J* = 7.6 Hz, Ar-CH), 7.40 (2H, t, *J* = 7.6 Hz, Ar-CH), 7.31 (2H, t, *J* = 7.6 Hz, Ar-CH), 6.45 (1H, bs, NH), 5.26 (1H, bs, NH), 4.44-4.39 (2H, m, Fmoc CH_2), 4.31-4.28 (1H, m, Ala αCH), 4.22 (1H, t, *J* = 6.5 Hz, Fmoc CH), 4.03 (2H, d, *J* = 3.5 Hz, Gly CH_2), 3.75 (3H, s, OCH_3), 1.41 (3H, d, *J* = 6.1 Hz, Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{21}H_{22}N_2NaO_5$ $[M + Na]^+$, calcd. 405.1421, found 405.1425.

(((9*H*-Fluoren-9-yl)methoxy)carbonyl)-L-alanylglycine

Fmoc-Ala-Gly-OH

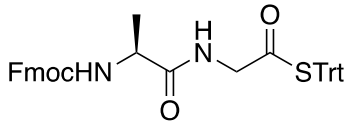


Prepared as per procedure I using Fmoc-Ala-Gly-OMe (80 mg, 0.21 mmol) in MeOH:H_2O (1:1, 2 mL) the product was obtained as a white solid (70 mg, 90%). The compound was in good agreement with the literature.[398]

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.58 (2H, d, *J* = 7.5 Hz, Ar-CH), 7.40 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.31 (2H, t, *J* = 7.5 Hz, Ar-CH), 7.15 (1H, bs, NH), 5.89 (1H, bs, NH), 4.40 (2H, d, *J* = 6.5 Hz, Fmoc CH_2), 4.28-4.32 (1H, m, Ala αCH), 4.20 (1H, t, *J* = 6.5 Hz, Fmoc CH), 4.01 (2H, s, Gly CH_2), 1.37 (3H, d, *J* = 6.6 Hz, Ala CH_3) ppm.

_S_-Trityl-(_S_)-2-(2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)propanamido)ethanethioate

Fmoc-Ala-Gly-_S_-Trt



Prepared a per Procedure E using Fmoc-Ala-Gly-OH (840 mg, 2.28 mmol) in CH_2Cl_2 (22 mL) and following flash chromatography (30% EtOAc:Hex) the product was obtained as an off-white solid (1.0 g, 72%).

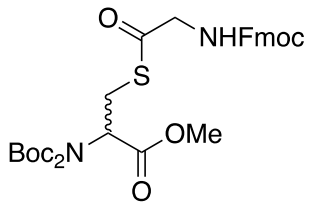
^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, *J* = 7.7 Hz, Ar-CH), 7.58 (2H, app. t, *J* = 7.7 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.7 Hz, Ar-CH), 7.33-7.23 (17H, m, Ar-CH), 6.66 (1H, bs, NH), 5.43 (1H, d, *J* = 7.0 Hz, NH), 4.41 (2H, d, *J* = 6.6 Hz, Fmoc CH_2), 4.29-4.26 (1H, m, Ala αCH), 4.21-4.15 (3H, m, Fmoc CH, Gly CH_2), 1.38 (3H, d, *J* = 5.9 Hz, Ala CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 193.7, 172.5, 156.1 (C=O), 143.4, 143.1, 141.4 (Ar-qC), 129.8, 127.9, 127.8, 127.3, 127.1, 125.1, 125.0, 120.0 (Ar-CH), 70.9 (qC), 67.1 (Fmoc CH_2), 50.5 (Ala αCH), 49.1 (Gly CH_2), 47.2 (Fmoc CH), 18.5 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{39}H_{33}N_2O_4S$ $[M - H]^-$, calcd. 625.2144, found 625.2167.

ν_{max} (film)/cm^{-1} 3294 (NH), 1710, 1656 (C=O), 737, 696 (CH).

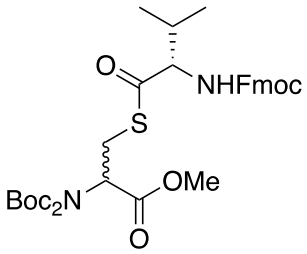
Methyl-_S_-((((9_H_-fluoren-9-yl)methoxy)carbonyl)glycyl)-_N_,_N_-bis(*tert*-butoxycarbonyl)cysteinate (189)



Prepared as per Procedure L using **186:d** (100 mg, 0.33 mmol), and Gly-SH (0.33 mmol), freshly prepared as per Procedure F using **117**, in 0.2 M phosphate buffer pH 8 (1.5 mL) and DMF (1.5 mL). Conversion determined by ^{1}H NMR: 86%.

HRMS (*m/z*) calculated for $C_{31}H_{38}N_2NaO_9S$ $[M + Na]^+$, calcd. 637.2190, found 637.2176.

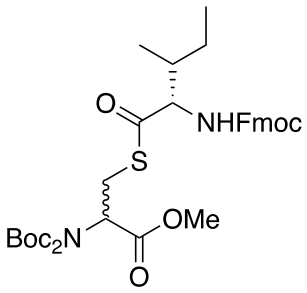
Methyl-*S*-((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-valyl)-*N*,*N*-bis(*tert*-butoxycarbonyl)cysteinate (237)



Prepared as per Procedure L using **186:d** (30 mg, 0.10 mmol), and Val-SH (1.5 eq., 0.15 mmol), freshly prepared as per Procedure F using **121**, in 0.2 M phosphate buffer pH 8 (0.5 mL) and DMF (0.5 mL). Conversion determined by ^{1}H NMR: 68%

HRMS (*m/z*) calculated for $C_{34}H_{44}N_2NaO_9S$ $[M + Na]^+$, calcd. 679.2659, found 679.2663.

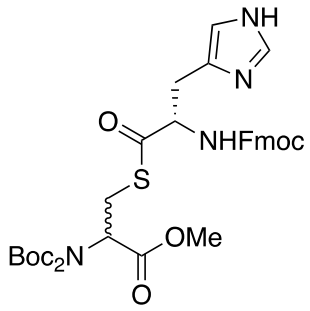
Methyl-*S*-((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-isoleucyl)-*N*,*N*-bis(*tert*-butoxycarbonyl)cysteinate (238)



Prepared as per Procedure L using **186:d** (30 mg, 0.10 mmol), and Ile-SH (1.5 eq., 0.15 mmol), freshly prepared as per Procedure F using Ile-*S*-Trt, in 0.2 M phosphate buffer pH 8 (0.5 mL) and DMF (0.5 mL). Conversion determined by ^{1}H NMR: 76%

HRMS (*m/z*) calculated for $C_{35}H_{46}N_2NaO_9S$ $[M + H]^+$, calcd. 693.2816, found. 693.2826.

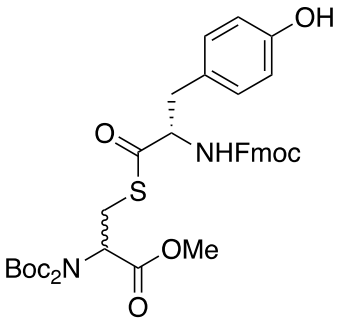
Methyl-*S*-((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-histidyl)-*N*,*N*-bis(*tert*-butoxycarbonyl)cysteinate (239)



Prepared as per Procedure L using **186:d** (30 mg, 0.10 mmol), and His-SH (1.5 eq., 0.15 mmol), freshly prepared as per Procedure F using His-*S*-Trt, in phosphate buffer (0.5 mL) and DMF (0.5 mL). Conversion determined by ^{1}H NMR: 68%

HRMS (*m/z*) calculated for $C_{35}H_{43}N_4O_9S$ $[M + H]^+$, calcd. 695.2745, found 695.2725.

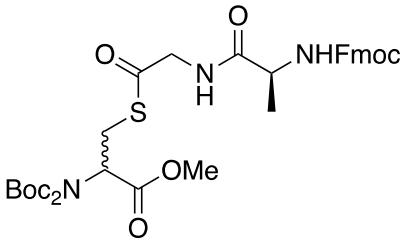
Methyl-*S*-((((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-tyrosyl)-*N*,*N*-bis(*tert*-butoxycarbonyl)cysteinate (240)



Prepared as per Procedure L using **186:d** (30 mg, 0.10 mmol), and Tyr-SH (1.5 eq., 0.15 mmol), freshly prepared as per Procedure F using Tyr-*S*-Trt, in 0.2 M phosphate buffer pH 8 (0.5 mL) and DMF (0.5 mL). Conversion determined by ^{1}H NMR: 91%

HRMS (*m/z*) calculated for $C_{38}H_{44}N_2NaO_{10}S$ $[M + Na]^+$, calcd. 743.2609, found 743.2604.

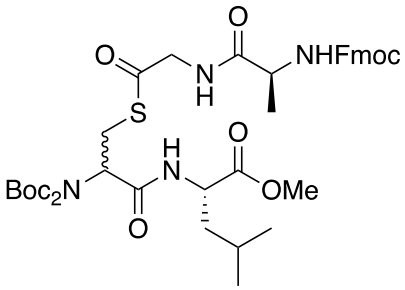
Methyl-*S*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-alanylglycyl-*N*,*N*-bis(*tert*-butoxycarbonyl)cysteinate (241)



Prepared as per Procedure L using **186:d** (50 mg, 0.17 mmol), and Fmoc-Gly-Ala-SH (0.17 mmol), freshly prepared as per Procedure F using Fmoc-Gly-Ala-*S*-Trt, in 0.2 M phosphate buffer pH 8 (2 mL) and DMF (2 mL). Conversion determined by ^{1}H NMR: 43%

HRMS (*m/z*) calculated for $C_{34}H_{43}N_3NaO_{10}S$ $[M + Na]^+$, calcd. 708.2561, found 708.2579.

Methyl-*S*-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-alanylglycyl-*N*,*N*-bis(*tert*-butoxycarbonyl)cysteinyl-L-leucinate (242)

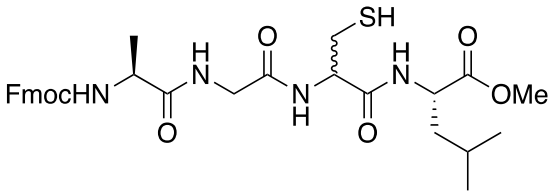


Prepared as per Procedure L using *bis*-Boc-Dha-Leu-OMe (100 mg, 0.24 mmol), and Fmoc-Ala-Gly-SH (0.24 mmol), freshly prepared as per Procedure F using Fmoc-Gly-Ala-*S*-Trt, in 0.2 M phosphate buffer pH 8 (0.8 mL) and DMF (0.8 mL). Conversion determined by ^{1}H NMR: 73%

HRMS (*m/z*) calculated for $C_{40}H_{54}N_4NaO_{11}S$ $[M + Na]^+$, calcd. 821.3402, found 821.3425.

Methyl-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-L-alanylglycylcysteinyl-L-leucinate (244)

Fmoc-Ala-Gly-Cys-Leu-OMe



To a solution of **242** (44 mg, 0.06 mmol) in CH_2Cl_2 (4 mL) was added TFA (1 mL) and the solution was stirred at rt for 2 h. The mixture was concentrated *in vacuo* and re-dissolved in CH_2Cl_2 (5 mL). Amberlyst A21 free base resin was added and the suspension was stirred for 1 h. The resin was filtered and the filtrate was concentrated *in vacuo* to furnish the tetrapeptide **244** as a colourless oil (12 mg, 27%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.78 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.61 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.42 (2H, t, *J* = 7.2 Hz, Ar-CH), 7.32 (2H, d, *J* = 7.2 Hz, Ar-CH), 7.10 (1H, bs, NH), 7.05 (1H, bs, NH), 6.96 (1H, d, *J* = 8.7 Hz, NH), 5.99 (1H, d, *J* = 6.1 Hz, NH), 4.91 (1H, t, *J* = 7.6 Hz, Cys αCH), 4.75-4.70 (1H, m, Ile αCH), 4.48-4.36 (4H, m, Gly CH_2, Fmoc CH_2, Ala αCH), 4.23 (1H, t, *J* = 6.7 Hz, Fmoc CH), 4.04 (1H, d, *J* = 17.8 Hz, Gly CH_2), 3.77 (3H, s, OCH_3), 3.63-3.60 (2H, m, Cys CH_2), 1.71-1.63 (3H, m, Ile CH, Ile CH_2), 0.97-0.94 (6H, m, 2 x Ile CH_3) ppm.

^{13}C NMR (100 MHz, $CDCl_3$) δ 173.8, 173.2, 172.9, 170.6, 156.9 (C=O), 143.8, 143.7, 141.3, 141.2 (Ar-qC), 127.8, 127.1, 125.1, 120.0 (Ar-CH), 77.8 (Cys αCH), 67.4 (Fmoc CH_2), 52.4 (OCH_3), 50.5 (Ile αCH), 50.1 (Ala αCH), 47.2 (Fmoc CH), 42.2 (Gly CH_2), 41.3 (Ile CH_2), 37.1 (Cys CH_2), 25.0 (Ile CH), 22.8, 21.9 (2 x Ile CH_3), 16.9 (Ala CH_3) ppm.

HRMS (*m/z*) calculated for $C_{30}H_{38}N_4NaO_7S$ $[M + Na]^+$, calcd. 621.2353, found 621.2356.

ν_{max} (film)/cm^{-1} 3328 (NH), 1749, 1650 (C=O), 734 (CH).

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