Development of Novel Acyl Thiol-ene Mediated Peptide Ligation Strategies

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Declaration

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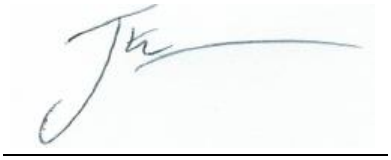

Joshua T. McLean

Abstract

This thesis, entitled 'Development of Novel Acyl Thiol-ene Mediated Peptide Ligation Strategies" is composed of six chapters. Chapter 1 provides an introductory overview of the fields of amide synthesis and peptide ligation. Particular emphasis is given to the emergence of Native Chemical Ligation and the extended methodologies developed to further the reach of this powerful synthetic technique. The limitations of these approaches, with regards to thioester formation *via* thioesterification, are also described. In addition, Chapter 1 details the application of thiol-ene chemistries to the synthesis and derivatisation of biomolecules, with specific attention given to the extent of the reaction within the field of peptide chemistry. The use of the acyl thiol-ene reaction to synthesise thioesters in organic syntheses is also detailed, along with the established chemical methods to access peptide thioacids. Chapter 1 is concluded with a brief description of the work described in this thesis and the overarching aims of this work.

Chapter 2 details efforts to develop acyl thiol-ene mediated ligation strategies *via* the synthesis and investigation of auxiliary bearing dipeptide systems. Unsaturated auxiliaries affixed to amino acid side-chains or α-amino groups of native peptides would permit the rapid formation of peptidic thioesters *via* acyl thiol-ene addition of a peptide thioacid component. S-to-N acyl transfer of the resulting thioester and subsequent auxiliary cleavage would furnish native amide products. Unfortunately, none of the auxiliary systems investigated possessed the necessary balance of synthetic accessibility, acyl transfer reliability and reaction stability to prompt their insertion into larger systems.

Chapter 3 describes the synthesis of derivatives of unsaturated unnatural amino acids vinylglycine and L-3,4-didehydrovaline inspired by literature precedent. Significant synthetic optimisation was required in the case of L-3,4-didehydrovaline. Nonetheless, routes to access gram quantities of *N-tert*-butyloxycarbonyl protected vinylglycine and L-3,4-didehydrovaline are detailed. The synthesis of unsaturated dipeptides from these precursors and investigation of their reactivity towards acyl thiol-ene addition with a range of amino acid thioacids is outlined. S-to-N acyl transfer and subsequent derivatisation of thioesters to native tripeptide products is also demonstrated.

Chapter 4 details efforts to synthesise two fragments of the therapeutically valuable peptide human parathyroid hormone. The synthesis of unsaturated decapeptides bearing vinylglycine and L-3,4-didehydrovaline functionalities *via* solid phase peptide synthesis

is described. Efforts to synthesise peptide thioacids through literature protocols are also detailed.

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

Chapter 6 describes the general experimental procedures employed in the course of this work and characterisation data of the compounds synthesised during the preparation of this thesis.

Abbreviations

AA	amino acid
Ace	acetone
Acn	acetonitrile
AcOH	acetic acid
ACTH	adrenocorticotrophic hormone
AG	allylglycine
Alloc	allyloxycarbonyl
AML	auxiliary mediated ligation
aq.	aqueous
Arg	arginine
Ar	aromatic
Asn	asparagine
Asp	aspartic acid
ATE	acyl thiol-ene
AuNP	gold nanoparticle
BAIB	bis(acetoxy)iodobenzene
BAL	backbone amide linker
BDE	bond dissociation energy
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
Boc_2O	boc anhydride
bs	broad singlet
BSA	bovine serum albumin
Bz	*N*-benzoyl
Calcd.	calculated
Cat.	catalytic
CBz	benzyloxycarbonyl
CDI	1,1'-carbonyldiimidazole
CLipPA	cysteine lipidation on peptides or amino acids
CSO	carbonyl sulfide
Cys	cysteine
d	doublet

DBF	dibenzofulvene
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCC	*N,N'*-dicyclohexylcarbodiimide
dd	doublet of doublets
DEPBT	(3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one)
DHV	L-3,4-didehydrovaline
DIC	*N,N'*-diisopropylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-dimethylaminopyridine
DME	dimethoxyethane
DMF	dimethylformamide
DMP	2,2-dimethoxypropane
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DPAP	2,2-dimethoxy-2-phenylacetophenone
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Elim.	elimination
EPL	expressed protein ligation
eq.	equivalents
ESI	electron spray ionisation
EtOAc	ethyl acetate
Et_2O	diethyl ether
Fm	9-fluoroenylmethyl
Fmoc	9-fluorenylmethyloxycarbonyl
g	gram
Gdn·HCl	guanidinium chloride
GlcNAc	*N*-acetylglucosamine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GSH	glutathione
h	hour

HBTU	*N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate
HCTU	*O*-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
Hex	hexane
His	histidine
HOBt	1-hydroxybenzotriazole
hPTH	human parathyroid hormone
HRMS	high resolution mass spectrometry
IIDQ	isobutyl 1,2-dihydro-2-isobutoxy-1-quinolinecarboxylate
IPA	isopropyl alcohol
Ile	isoleucine
IR	infrared
J	coupling constant
KAHA	ketoacid hydroxylamine
LDA	lithium diisopropylamide
Leu	leucine
Lys	lysine
M	mass ion or molarity
m	multiplet
m.p.	melting point
m/z	mass to charge ratio
MALDI	matrix assisted laser desorption ionisation
MAP	2’-methoxyacetophenone
MeOH	methanol
MESNa	sodium 2-mercaptoethylsulfonate
Met	methionine
MIC	minimum inhibitory concentration
mg	miligram
MHz	megahertz
MIM	2-methylimidazole
min	minute
mL	millilitre

mmol	milimole
Mmt	monomethoxytrityl
MPa	methylphenacyl
MPAA	4-mercaptophenylacetic acid
MsCl	methanesulfonyl chloride
MsOH	methanesulfonic acid
NaOMe	sodium methoxide
NCL	native chemical ligation
nm	nanometre
NMM	*N*-methylmorpholine
NMR	nuclear magnetic resonance
OBt	*O*-benzotriazole
PBu_3	tributylphosphine
PCC	pyridinium chlorochromate
PDC	pyridinium dichromate
PEG	polyethylene glycol
PEGA	polyethylene glycol-acrylamide
PG	protecting group
Phe	phenylalanine
ppm	parts per million
Pro	proline
PS	polystyrene
PSRC	Peptide Synthesis Research Committee
PTM	post-translational modification
p-TsOH·H_2O	*para*-toluenesulfonic acid monohydrate
	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
Pyr	pyridine
q	quartet
qC	quartenary carbon
Q-TOF	quadruple time-of-flight
RDS	rate determining step
REDOX	reduction/oxidation

R_f	retardation factor
RP-HPLC	reverse phase high performance liquid chromatography
rt	room temperature
s	singlet
SAL	sugar-assisted ligation
Ser	serine
SM	starting material
S_Ni	internal nucleophilic substitution
SPPS	solid phase peptide synthesis
t	triplet
TBAF	tetrabutylammonium fluoride
^{t}Bu	*tert*-butyl
^{t}BuOk	potassium *tert*-butoxide
TCEP	tris(2-carboxyethyl)phospine
TEA	triethylamine
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
TES	triethylsilane
TFA	trifluoroacetic acid
TFET	2,2,2-trifluoroethanthiol
TfOH	trifluoromethanesulfonic acid
THF	tetrahydrofuran
Thr	threonine
TLC	thin layer chromatography
Tmob	trimethoxybenzyl
TMS-CHN2	trimethylsilyldiazomethane
TOF	time-of-flight
Troc	2,2,2-trichloroethoxycarbonyl
Trp	tryptophan
Trt	trityl
TS	transition state
TsCl	4-toluenesulfonyl chloride
Tyr	tyrosine
UAA	unnatural amino acids

UV	ultraviolet
UV-VIS	ultraviolet-visible
VA-044	2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride
Val	valine
VG	vinylglycine

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Contents

Chapter 1

Introduction

1.0 Introduction

1.1 Therapeutic Proteins

As a fundamental component of all organisms, proteins and the myriad of functions they possess in the natural world have captivated researchers across the sciences since their discovery. Due to their role as arbiters of natural processes in the form of enzymes, receptors and ion channels, 80% of approved drugs worldwide target endogenous human proteins, with the majority of the remainder targeting their pathogenic counterparts.[1] Therefore, it is vital that protein targets of interest can be readily accessed for subsequent assay development and drug validation efforts. There is a long history of proteins and peptides being utilised as drugs, stemming from the introduction of native insulin isolated from bovine pancreas by clinicians in the 1920s.[2] The subsequent chemical synthesis of native oxytocin permitted the first therapeutic use of a fully synthetic peptide in 1962, a milestone followed by the introduction of leuprorelin, an analogue of gonadorelin, the first clinically approved synthetic peptide analogue in 1984.[3] Since the 1980s the number of peptide drugs submitted for approval has grown concomitantly with the power of chemists to effectively synthesise, purify and manufacture peptide substrates.[4] The average sequence length of peptides entering clinical development worldwide since 1980 has increased from 9 to 20 amino acids (AAs), with substrates bearing 30 – 40 AA residues now routinely submitted for clinical approval. Beyond synthetic considerations the reticence of the pharmaceutical industry to invest heavily in the development of peptide-based drugs has stemmed from the inherent therapeutic limitations of native peptides when administered *in vivo*. Particularly problematic are the poor oral bioavailability and short plasma half-life of native peptide drugs[5] due to the presence of vast numbers of peptidase enzymes present in the body for digestion and wider homeostasis.[6] Off-target effects as a result of the promiscuity of peptide sequences and poor membrane permeability also negatively impact the efficacy and increase the toxicity of drugs exhibiting native sequences.[7] However, through the advent of the new synthetic methods and purification techniques significant enhancement of the pharmacokinetics of peptidic drug compounds has proved possible. Incorporation of unnatural amino acids (UAAs) and selective modifications of the amide backbone have vastly improved the target selectivity of therapeutic peptides,[8, 9] which in addition to conjugation and cyclisation strategies increase the robustness of peptide substrates *in vivo*. This has led to

analogous peptide structures vastly outnumbering native substrates and recombinantly expressed heterologous proteins in ongoing a clinical development.[3] Scientific research continues to expand the potential range of druggable targets accessible by therapeutic proteins. Protein-protein interactions underlie most biological processes in humans and pathogens alike, disruption or stabilisation of which may provide novel modes of therapeutic action to which proteins and peptides are uniquely suited.[10] It is therefore imperative that synthetic methods continue to be realised which provide researchers and the pharmaceutical industry with the means to access novel peptides and proteins with a high degree of synthetic control.

1.2 Strategies for Chemical Protein Synthesis

1.2.1 Recombinant Protein Production

Recombinant protein expression technologies take advantage of the efficiency of natural architectures to carry out the translation and derivatisation of desired protein targets. Through the transfection of deoxyribonucleic acid (DNA) vectors in host cells, recombinant proteins can be rapidly and inexpensively produced while maintaining high translational fidelity.[11] Bacterial host cells are commonly utilised for this purpose due to their unparalleled growth kinetics.[12] *Escherichia coli* is the prevailing choice as the initial development of recombinant protein production technologies was carried out using this species.[13] However, utilisation of prokaryotic hosts to produce proteins recombinantly presents an array of problems. The accumulation of proteins resulting from natural gene expression, such as lipopolysaccharides, proteins intrinsic to the cell wall structure of Gram-negative bacteria, significantly complicates purification of the desired protein.[14] Disulfide bond formation can also prove unreliable in bacterial hosts unless further non-native reductive enzymes are expressed within the host.[15] The chief limitation of bacterial protein production is that prokaryotes cannot produce proteins bearing post-translational modifications (PTMs), naturally occurring modifications to protein topography such as glycosylation, lipidation or citrullination, which are often pivotal to therapeutic activity. Over 40% of approved peptide biologics bear at least one PTM necessitating the use of prokaryotic hosts for recombinant protein production.[16] The yeast *Saccharomyces cerevisiae* is commonly used for expression of proteins bearing PTMs along with select mammalian cell lines.[17] However, due to the non-templated nature of many PTMs proteins produced recombinantly lack homogenous patterns of modification.[18]

Glycosylation patterns, for instance, are governed by complex arrays of glycosyltransferase and glycosidase enzymes, the expression and activity of which are governed by a host of intracellular factors.[19] This general lack of homogeneity present in recombinantly derived substrates proves problematic when accurately investigating and quantifying the specific biological roles and therapeutic potential of modified proteins. Separation of proteins bearing only slight differences in PTMs is a laborious process and modified therapeutic proteins are consequently administered as mixtures in the majority of applications.[20] Works encompassing AA substrates outside of the twenty-two proteogenic AAs are also limited by recombinant methodologies, as such AAs cannot be transcribed into DNA naturally. Incorporation of certain UAAs has been accomplished through the substitution of naturally occurring AAs in the expression host media and using genetic code expansion methodologies.[21, 22] However, these techniques are possible only with select AA side-chain bioisosteres and are far from routine.[23] Due to the aforementioned shortcomings of recombinant expression it has proven necessary to develop approaches to peptide synthesis that do not rely on the apparatus of the natural world.

1.2.2 Solution Phase Peptide Synthesis

The formation of an amide bond between a carboxylic acid and an amine is the fundamental reaction in peptide synthesis. The covalent bonding of these two functional groups does not take place spontaneously, with direct condensation necessitating temperatures above 200 ºC.[24] Generally formation requires the activation of the carboxylic acid component to form a reactive intermediate, followed by subsequent acylation of the amine and concurrent loss of the activating fragment as a reaction by-product.[25] The first polypeptide synthesis carried out by Emil Fischer utilised acyl chlorides, synthesised from the parent carboxylic acid, and addition of the amine component liberated hydrochloric acid (HCl) upon acylation.[26] Classically, thionyl chloride ($SOCl_2$), oxalyl chloride ($COCl_2$) and phosphorous chlorides (PCl_3, PCl_5) are used to generate acyl chlorides for this purpose. However, the harsh oxidative nature of chlorinating species prompted the development of milder reagent such as cyanuric chloride and triphenylphosphine/trichloroacetonitrile.[27] Acid chlorides are not routinely applied to the synthesis of peptides as the generation of HCl upon amine acylation renders the methodology unsympathetic to *tert*-butyloxycarbonyl (Boc) protected amines and

other acid labile protecting groups (PGs). The propensity of acid chlorides to form ketenes under basic conditions, with concomitant loss of chiral integrity, also renders them unsuitable.[28] Acyl azides are used as intermediates in amide bond formation, in work pioneered by Curtius,[29] and provide the potential for unparalleled retention of chiral integrity but are precluded due to their explosive nature, however the development of continuous flow techniques has generated renewed interest in this area.[30]

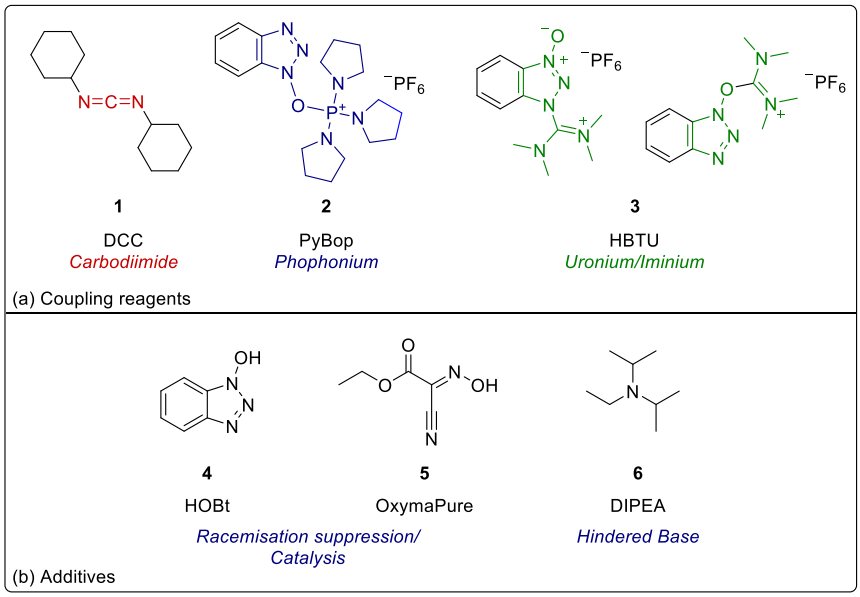


Figure 1.1: (a) Amide coupling reagents commonly utilised for peptide synthesis. Colour denotes the unique structural features of the class. (b) Additives utilised in peptide synthesis for racemisation suppresson and as non-nucleophlic bases.

Carbodiimides were the first modern amide coupling reagents to be developed specifically for peptide synthesis with the first in class, *N,N'*-dicyclohexylcarbodiimide (**1**, DCC, **Figure 1.1**), employed to effectively synthetize di- and tripeptide substrates in 1955.[31] The insoluble nature of the dicyclohexylurea by-product of DCC spurred the development of *N,N'*-diisopropylcarbodiimide (DIC) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), which produce urea by-products readily soluble in organic and aqueous (aq.) solvents, respectively.[32] Literature comparisons have demonstrated that superior yields are achieved when DIC is employed over EDC,[33] yet at laboratory scale EDC is preferred due to the simplified purification procedures it facilitates. Despite the enduring popularity of these reagents as a result of their mild and practical nature, carbodiimides are not without deficiency when applied specifically to the field of peptide synthesis. A tertiary base must be employed if the reaction is not to be prohibitively slow; *N,N*-Diisopropylethylamine (**6**, DIPEA, **Figure 1.1**) and *N*-methylmorpholine (NMM) have proven popular as their non-nucleophilic character

prohibits competition with the desired amino substrate limiting undesirable side-reactions.[34] Unfortunately, due to the electrophilic nature of the *O*-acylurea activated ester and concurrent increase in the acidity of the α-hydrogen atom upon activation, racemisation of α-carbon substituents occurs readily during amide coupling reactions. This results from either direct α-hydrogen abstraction leading to formation of enolate species **7** or, in the case of polypeptides, through formation of oxazolinone intermediate **9** through attack of an adjacent iminolate species **8** (**Figure 1.2**) leading to a racemised amide product such as **10**.[27]

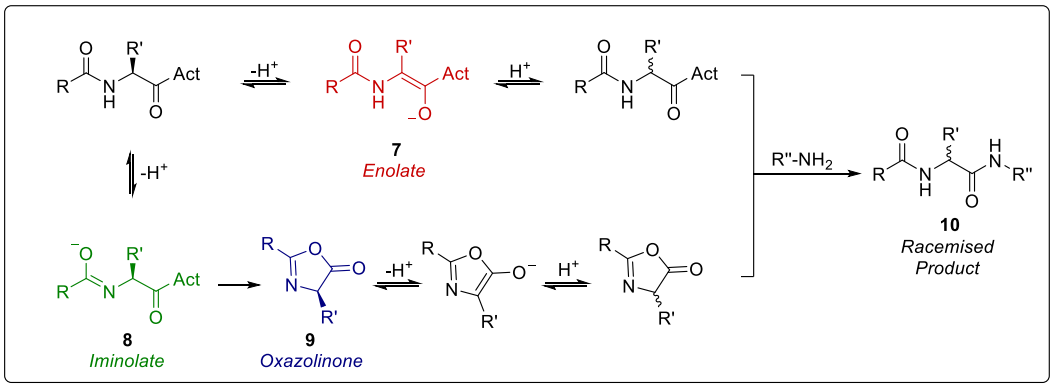


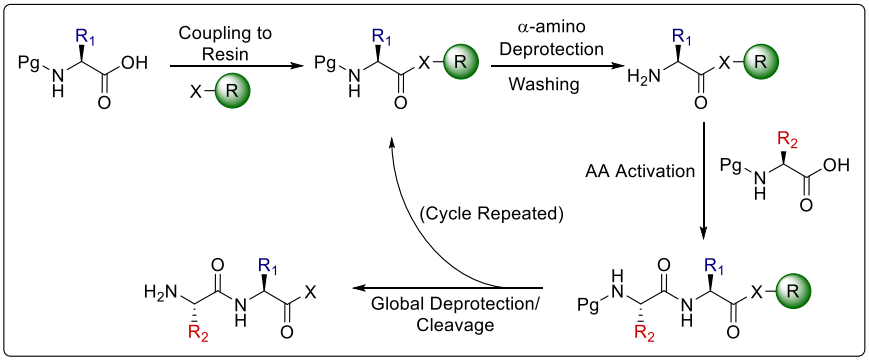
Figure 1.2: Routes by which racemisation of α-carbon substituents may occur during carboxylic acid activation during amide coupling, resulting in racemised amide product **10**. Colours indicate the key enolate (**7**, red), iminolate (**8**, green) and oxazolinone (**9**, blue) intermediates.[27]

1-Hydroxybenzotriazole (**4**, HOBt, **Figure 1.1**) was subsequently developed as an additive to increase the rate of coupling reactions and to suppress racemisation. HOBt expedites amide formation through reaction with the *O*-acylurea ester intermediate to give an *O*-benzotriazole (OBt) activated ester, the heterocyclic nitrogen atoms of which stabilise the approach of the attacking amine through hydrogen bonding interactions.[25] In addition to this, the planar aromatic structure of the OBt moiety is believed to more effectively shield the α-proton from abstraction, thereby effectively reducing racemisation. Although HOBt is widely employed within the academic community the reagent has been largely replaced by Oxyma Pure® (**5**, **Figure 1.1**) in industry, as its similarly activated *O*-hydroxyiminoester intermediate permits comparably high yields and does not possess the explosive characteristics of the former.[35] The phosphonium and uronium/iminimum type reagents (**Figure 1.1**) developed subsequently are based on the benzotriazole structural motif and are now routine in peptide synthesis. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (**2**, PyBOP, **Figure 1.1**) and *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (**3**,

HBTU, **Figure 1.1**) have proven popular due to their respective balance of coupling efficiency, mild activation conditions, racemisation suppression, safety and cost proving uniquely attractive.[27] Despite the long history of research and reagent development enabling the use of coupling reagents and additives sympathetic to peptide substrates, synthesis in solution is severely limited, simply as a function of the necessary number of coupling and protection/deprotection steps necessary to ensure sequence selectivity. Standard laboratory coupling and purification procedures, often necessitating column chromatography due to lipophilic side-chain PGs, render the syntheses of polypeptides greater than five AAs highly impractical.[36] Even highly specialised methodologies which seek to limit the number of purification steps through water soluble PG by-products have thus-far struggled to attain polypeptides greater than twenty AA residues in length.[37] The ability of the scientific community to synthesise biologically active peptides of considerable length was revolutionised upon the advent of solid phase peptide synthesis.

1.2.3 Solid Phase Peptide Synthesis

Solid phase peptide synthesis (SPPS) was first demonstrated by Merrifield in 1963, work which culminated in the synthesis of 11mer methionyl-lysyl-bradykinin and his subsequent award of the Nobel Prize in Chemistry in 1984.[38-41] Starting from an appropriately protected resin bound AA substrate, sequential *N*-terminal deprotection and amide coupling steps may be carried out, with purification between steps performed simply by washing excess reagents of the resin surface between reactions (**Scheme 1.1**).[42] This advance obviated the need for chromatographic purification steps between each individual coupling step greatly expediating syntheses. Initial syntheses were carried out using glass reaction vessels fitted with porous frits allowing vacuum filtration to quickly remove unreacted solubilised material. Latterly, inert polypropylene syringes fitted with frits are commonly used due to durability, low cost and compatibility with many automatic synthesisers on the market.[43] As a synthetic methodology SPPS is naturally suited to automation by virtue of the high number of repetitive steps carried out and straightforward purification between steps. Consequently, automated SPPS is carried out regularly in industrial and academic settings.[44] The second fundamental advantage of SPPS in comparison to its solution phase counterpart is that the resin itself imparts a high level of chemical selectivity, in terms of its use as a semi-permanent PG and as an avenue to a high number of specific *C*-terminal functionalisations.[45]



Scheme 1.1: Illustration of the principles of solid phase peptide synthesis (SPPS).

The resin employed for a particular synthesis must fulfil several criteria. The selected resin must swell adequately under the adopted coupling conditions to allow reaction to occur at the resin surface. Swelling refers to the increase in resin volume observed upon addition of solvent as solvent molecules occupy vacant regions between the cross-linked polymer within the resin structure.[46] Due to the porous nature of polymeric resin beads the majority of resin surface area, and therefore sites available for *C*-terminal attachment, are situated deep in the resin structure.[47] Poor swelling of the resin limits the number of attachment sites accessible to the solubilised substrates resulting in slower reaction times and lower conversions.[34] A myriad of resins have been developed as a result, ordinarily composed of either lipophilic polystyrene (PS), hydrophilic acrylamide-polyethylene glycol co-polymers (PEGA) or polyethylene glycol (PEG) as an intermediary choice, which provide the necessary solvation and swelling characteristics for a diverse range of polar and apolar solvents.[45] The resin must also facilitate the installation of the desired *C*-terminal functional group upon cleavage of the nascent peptide substrate. This is determined by the nature of the linker, a group which links the growing amino acid chain to the polymeric resin structure at the attachment sites.[48] Carboxylic acids and primary amides remain the simplest and most prevalent *C*-terminal moieties derived from SPPS, but resin functionalisation to access more elaborate functional groups pertinent to biological function, further augmentation and peptide ligation is now commonplace.[49] As of 2020 Novabiochem®, a subsidiary of Merck Group, provides resins which permit fourteen different *C*-terminal functionalisations after cleavage, ranging from alkyl amides and thioesters to hydroxamic acids and aldehydes.[50-53] Chiefly, the resin and linker must be compatible with the overall PG strategy employed for each respective synthesis, that is resistant to repeated *N*-terminal deprotection steps and the penultimate AA side-chain deprotection step prior to cleavage.

1.2.3.1 Protecting Group Strategies in Solid Phase Peptide Synthesis

Key to the success of SPPS in all instances is the necessity for an orthogonal PG strategy between three primary functionalities; the *C*-terminus bound directly to the resin, the side-chains of any nucleophilic residues present in the sequence and the *N*-terminal PG of the growing chain.[54] In the vast number of instances, barring syntheses in the N-to-C direction and those providing AA side-chain protected substrates, the PG lability requirement during peptide synthesis is fixed (**Figure 1.3**).[55] As SPPS is carried out in the C-to-N direction it is necessary to have *N*-terminal PGs which are cleaved prior to subsequent coupling steps. As the same PG will be present on the next AA in the sequence it must also possess adequate stability to the basic conditions necessary to carry out effective amide coupling reactions.

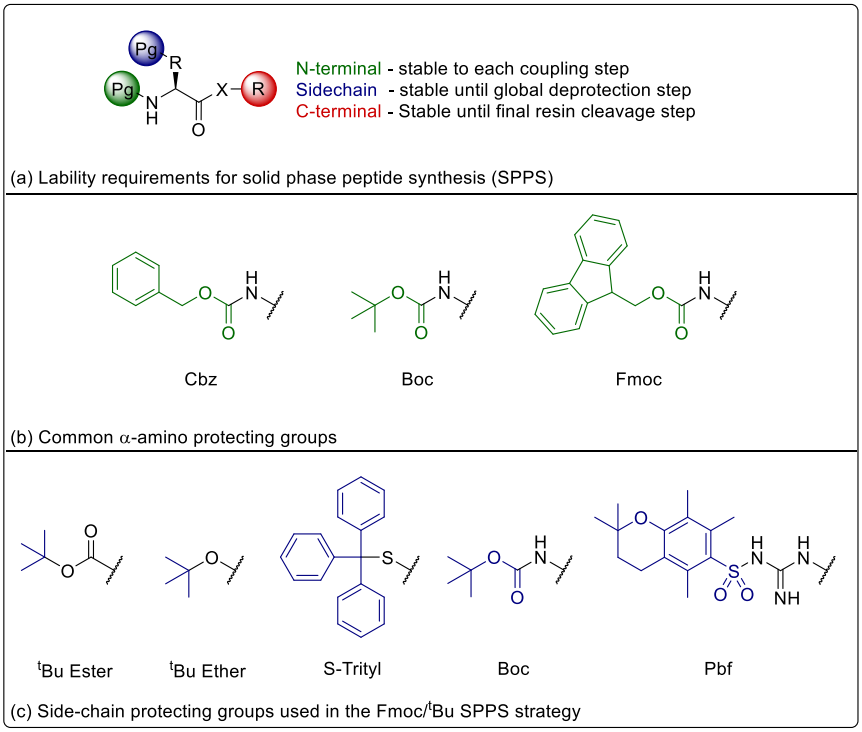


Figure 1.3: (a) Lability requirement for the synthesis of unprotected peptide fragments *via* solid phase peptide synthesis. (b) α-amino protecting groups used commonly used in solution phase and solid phase peptide synthesis. (c) Acid labile side-chain protecting groups used in the Fmoc/^{t}Bu solid phase peptide synthesis strategy.

The earliest peptide syntheses were carried out using permanent PGs such as *N*-benzoyl (Bz) which as an amide could not be removed without threatening the integrity of the newly formed peptidic bond.[26, 56] It was not until the development of the *N*-carbobenzyloxy (Cbz, **Figure 1.3**) PG in 1932 that selective removal could be demonstrated with no consequent effect on constituent amide bonds.[57] This promoted rapid progress in the field of peptide synthesis such as the solution phase the synthesis of

biologically active octapeptide oxytocin by du Vigneud in 1953, through sequential acyl chloride coupling and hydrogenation steps with Cbz protected AA substrates.[58] Synthetic oxytocin was subsequently shown to have identical biological activity to the natural isolated hormone, a landmark conclusion in the field of chemical protein synthesis at the time of publication.[59] The development of the Boc group (**Figure 1.3**) as an acid sensitive PG by McKay and Albertson provided a PG truly orthogonal to Cbz even under the harshest conditions to remove the latter, such as Birch reduction.[45] Utilising Boc as the α-amino PG and Cbz/Bz to mask nucleophilic side-chain residues permitted the synthesis of even large peptide examples, such as the synthesis of 39 residue adrenocorticotrophic hormone (ACTH) by Schwyzer and Sieber in 1963.[60] However, this necessitated the use of resin linkers stable to repeated acidic Boc deprotections and cleaved through treatment with hydrofluoric acid (HF), a significant practical deterrent for many laboratories. The introduction of 9-fluorenylmethoxycarbonyl (Fmoc, **Figure 1.3**) as the first base labile PG designed specifically for SPPS permitted the use of side-chain PGs and resin linkers less stable to acid.[61] A combination of Fmoc α-amino and *tert*-butyl (^{t}Bu) side-chain protection is now the most widely applied SPPS methodology due to the ease by which *N*-deblocking, global deprotection and resin cleavage may be carried out.[62] In 1991 the Peptide Synthesis Research Committee (PSRC) estimated a 50/50 split in American laboratories employing Boc and Fmoc PG strategies for SPPS.[63] By 1994 98% of laboratories surveyed by the same body were using Fmoc/^{t}Bu chemistries signifying the popularity of the more practicable methodology.[64] A 20% solution of piperidine in dimethylformamide (DMF) is typically used to remove the Fmoc group and the characteristic λ_{max} of the dibenzofulvene (DBF) side product liberated during the reaction can be used to monitor the success of deprotection steps.[65] The fluorenyl moiety present on the PG similarly allows monitoring of coupling reactions, a process which is typically automated to give valuable synthetic information about the growing peptide sequence.[66] Acid labile AA side-chain PGs now routinely employed in the Fmoc/^{t}Bu method typically include ^{t}Bu, trityl (Trt) and Boc group amongst others, (**Figure 1.3**) which may be removed using 90% trifluoroacetic acid (TFA) in water, with an appropriate cation scavenger. After washing cleavage from the resin at the *C*-termnius can be carried out using a solution of 95% TFA or a TFA/trifluoromethylsulfonic acid (TFMSA) mixture in the case of the most commonly used carboxylic acid yielding Wang and amide yielding Rink Amide resins, thereby eliminating the need for HF.[67, 68]

1.2.3.2 Limitations of Solid Phase Peptide Synthesis

Despite the leap forward that SPPS permitted in accessing biologically relevant target molecules, this synthetic methodology suffers from several notable disadvantages. The use of lipophilic side-chain PGs, necessary in order to guarantee reaction selectivity and effective removal at the global deprotection stage, tend to cause aggregation of the growing peptide chains preventing further coupling reactions.[69] Specific low loading resins can be utilised when synthesizing peptide targets > 30 AAs in length to mitigate aggregation through greater spacing of *C*-terminal attachment sites on the resin surface.[70] However, this results in a concurrently lower millimole (mmol) per gram loading and a substantially greater cost per mmol of product. Increases in the overall polarity of the growing peptide chain can prove unsympathetic to the apolar conditions necessary for the coupling and deprotection of protected AA substrates, leading to inefficient reaction and, at worst, precipitation.[71] Assuming a successful SPPS protocol has been realised subsequent chromatographic separation of unprotected SPPS products becomes prohibitively difficult at > 50 AAs due to the relatively small difference in polarity between desired products and deletion products of longer sequences. For the synthesis of long sequences a capping step directly following each coupling steps is routinely employed through acetylation of unreacted amino groups.[72] This prevents chains which have not undergone the desired amide coupling reaction from reacting further and, due to the acetyl capping group, permits chromatographic separation from the desired unprotected peptide products, but is not wholly effective.[72] The greatest obstacle, however, remains the sheer volume of solvents and reagents that are required to access even routine peptide targets, as well as the low overall yields in what are in essence multi-step linear syntheses.[73] In addition to the linear nature of SPPS, the need for efficient coupling reactions necessitates large excess of reagent to ensure acceptable yields of final products. Typically, 4 equivalents (eq.) of AA, 5 eq. of coupling reagent/additive and up to 8 eq. of base are used.[74] Couplings at troublesome residues, for instance those which are sterically hindered or possess lower amine nucleophilicity, are by convention repeated twice further increasing wastage. The original ribonuclease A synthesis by Merrifield and Gutte required 369 separate chemical reactions to furnish the target sequence.[75] For these reasons, methodologies which allow for the assembly of peptide targets in a convergent manner have proven essential in giving routine access to proteins longer than 50 AAs in length.

1.2.4 Ligation Methodologies

Peptide ligation is the process by which two unprotected peptide chains, usually produced using SPPS, are joined chemoselectively. Crucially, peptide synthesis through ligation is by definition convergent and the development of ligation methodologies has drastically reduced the number of steps necessary to access peptidic targets.[76] Ligation methodologies generally adhere to a two-step principle (**Figure 1.4**); an initial chemoselective capture step by which the independent peptide fragments are joined together through a non-native bond, followed by an intramolecular rearrangement coupled with a concerted or separate elimination step to furnish the native product.[77] Traceless Staudinger ligation and Ketoacid Hydroxylamine (KAHA) ligation both rely on the initial capture step occurring between suitably activated moieties at the *C*-terminus and α-nitrogen of each fragment (**Figure 1.4**).

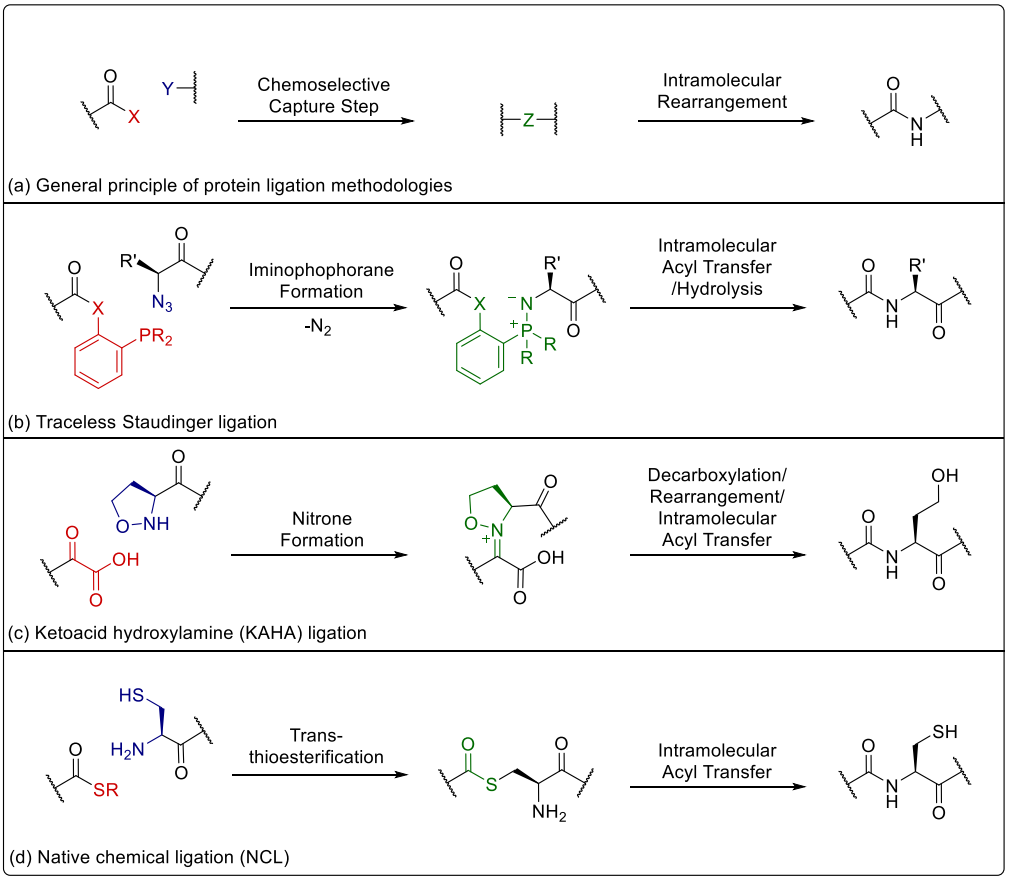


Figure 1.4: (a) The general principle of protein ligation encompassing a chemoselective fragment capture step followed by a spontaneous intramoleular rearrangement and elimination to afford the ligated product. (b) General scheme of Staudinger ligation.[84] (c) General scheme of ketoacid hydroxylamine (KAHA) ligation.[88] (d) General scheme of native chemical ligation (NCL).[94]

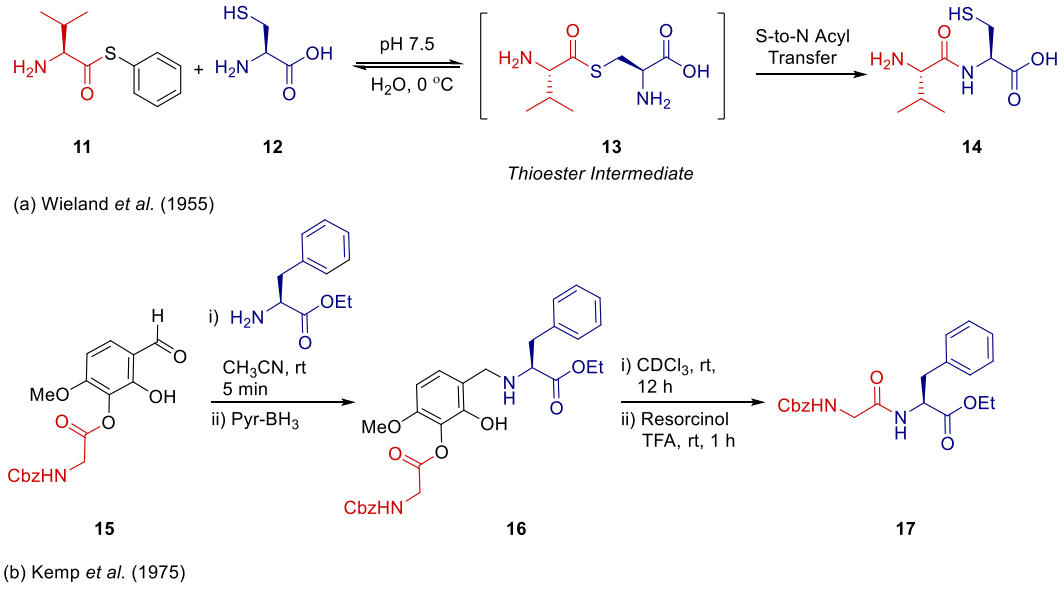
Traceless Staudinger ligation is closely related to the classical Staudinger reaction, first reported by Staudinger and Meyer in 1919,[78] in which an azide reacts with a triarylphosphine to yield an iminophosphorane. This imination reaction proceeds smoothly to give the desired products in quantitative yield under mild reaction conditions and with only nitrogen gas as a significant side product. Hydrolysis of the iminophosphorane, referred to as Staudinger reduction, yields a primary amine and remains the preeminent practical method for azide reduction.[79] However, the true utility of this reaction is demonstrated through the reaction of the iminophosphorane product with a range of electrophilic species yielding highly functionalised imines, aldimines, carbodiimides and, through intramolecular variants, cyclic azo- structures.[80] As the Staudinger reaction proceeds under mild conditions, and the reactive phosphine and azide components are not typically present within biomolecules, the reaction presented an attractive process for biomolecular conjugation.[81] However, the reactivity of the iminophosphorane intermediate with water precluded its use in biomolecular synthesis until the development of Staudinger ligation by Bertozzi and coworkers.[82] By inserting a suitably reactive electrophilic methyl ester adjacent to the phosphine group an intramolecular reaction capable of out competing the iminophosphorane hydrolysis was successfully demonstrated. This yielded a stable amide bond between the two molecules enabling cell surface functionalisation in aq. media. This methodology is widely used for the conjugation of enzyme probes, site specific DNA labelling, cell surface modification and preparation of glycan arrays.[81] The fact that azide containing proteins can now be accessed through recombinant expression methodologies has further popularized the Staudinger ligation.[83] A shortcoming of this methodology is the presence of a phosphine oxide linker in the ligated product, rendering it unsuitable for applications where native structures are essential. The traceless Staudinger reaction was demonstrated in principle by Bertozzi and coworkers shortly after the initial publication of the Staudinger ligation (**Figure 1.4**).[78] Use of a thioester containing phosphine of the prerequisite S-to-N acyl transfer transition state (TS) size as a reaction component proved capable of promoting acyl transfer to the nucleophilic nitrogen of the iminophosphorane intermediate. Hydrolysis of the resulting thioimidate yields a native amide bond between the azide/phosphine reaction components and a phosphinothiol side product.[84] The application of this methodology was impressively demonstrated by Raines and coworkers through the total synthesis of ribonuclease A in 2003.[85] In contrast to traceless Staudinger ligation, which was developed through the application and adaptation of

classical chemistry to peptide ligation, KAHA ligation was developed with peptide ligation as its primary objective. As the name implies the ligation methodology permits chemoselective ligation between peptide fragments bearing *C*-terminal ketoacids and *N*-terminal hydroxylamines to yield a native amide bond between the two.[86] The reaction proceeds *via* attack of the hydroxylamine nitrogen onto the electrophilic ketone carbonyl of the ketoacid component, forming a hemiaminal intermediate which readily dehydrates to yield the corresponding nitrone (**Figure 1.4**). The amide bond is formed from this intermediate through either decarboxylation/hydroxyl migration,[87] or through the formation and rearrangement of a series three-membered heterocycles followed by decarboxylation.[88] Due to the inherent instability of α-amino hydroxylamines to oxidation, various classes of *O*-acylated and *O*-alkylated hydroxylamines were developed but proved prone to side reactions and possessed limited reactivity, respectively.[89] The use of isoxazolidines proved fruitful, albeit at the expense of a truly native peptide as the reaction proceeds through a depsipeptide intermediate with a subsequent O-to-N acyl shift furnishing a homoserine at the ligation site (**Figure 1.4**).[90] Further additions to this methodology by the Bode group have, through effective functional group masking, permitted sequential ligations providing access to the analogues of valuable protein targets including antibacterial macrocycle AS-48 (77 residues), pancreatic hormone betatrophin (179 residues) and lipocalin nitrophorin 4 (184 residues) demonstrating the powerful potential of the methodology.[91-93] These methodologies have shown enormous potential but possess the same inherent difficulties, namely the necessity for complex, non-natural functionalities to be present on substrates prior to ligation. Despite being the pioneering ligation approach, native chemical ligation (NCL) possesses enduring popularity due to its use of readily accessible fragments.

1.3 Native Chemical Ligation

NCL, first presented in Kent and coworkers' seminal 1994 paper,[94] was the culmination of nearly half a century of research efforts to identify the role of thioesters in biosynthesis. In particular, the discovery of *S*-acetyl coenzyme A by Lynen[95] and its role in lipid biosynthesis indicated that thioesters were commonly encountered, energy-rich biosynthetic intermediates. *S*-acetyl coenzyme A's primary role as an acetyl donor inspired the systematic investigations into the role of thioesters as reactive intermediates in organic chemistry. The work of Wieland *et al.* investigated the suitability of AA

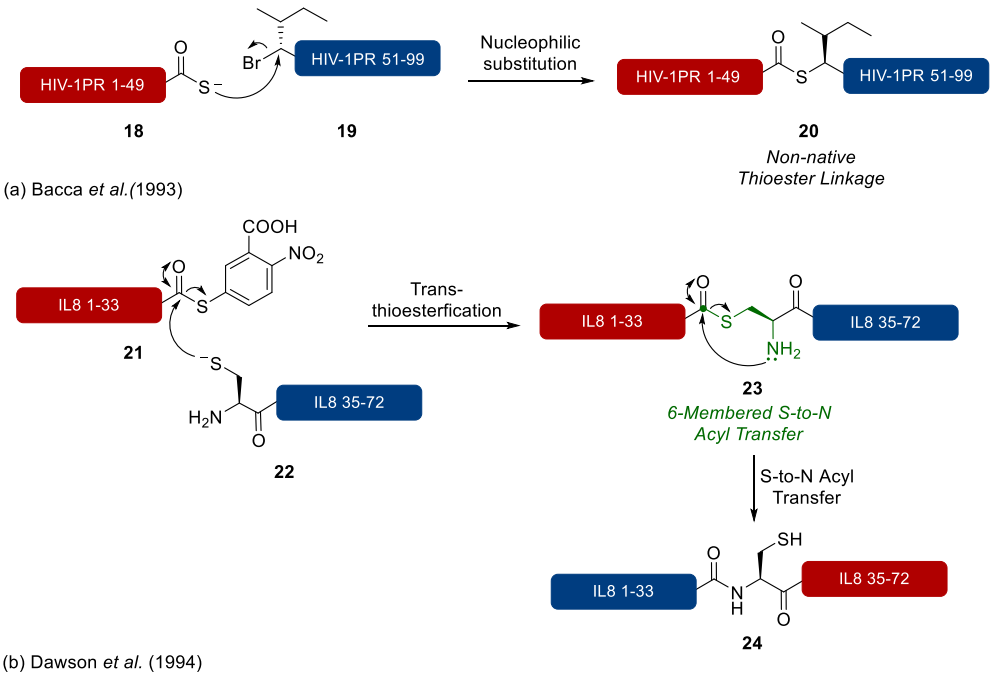
C-terminal thioesters as reagents for amide bond formation through direct reaction at AA α-amino groups.[96] Upon reaction of a range of AAs with valine (Val) aryl thioester **11** at pH 7.5, Wieland remarked on the vastly accelerated rate of amide formation when utilising unprotected cysteine (Cys) **12** to yield Cys dipeptide **14**.[96] Wieland postulated this was likely due to the formation of reactive thioester intermediate **13** *via* trans-thioesterification followed by S-to-N acyl transfer (**Scheme 1.2**), one of the hallmarks of NCL. Further work on intramolecular acyl transfer by Kemp established thioester 'amine capture' as a basis for amide bond formation within a novel class of coupling reagents further laying the groundwork for NCL.[97] Addition of Phenylalanine (Phe) ethyl ester to glycine (Gly) esterified pyrogallol derived aldehyde **15** through reductive amination yielded intermediate **16**. This intermediate permitted O-to-N acyl transfer over a seven membered TS to yield dipeptide **17** after cleavage of the coupling reagent under acidic conditions.



Scheme 1.2: (a) The pioneering work of Weiland *et al.* into aryl thioesters as activated carboxylic acids in amide synthesis.[96] (b) The work of Kemp *et al*. demonstrating a pyrogallol based amide coupling reagent, encompassing an intramolecular O-to-N acyl transfer.[97]

Work undertaken by Kent and coworkers prior to NCL utilised peptide thiolate **18** in S_N2 type reaction with brominated peptide **19** to yield HIV-1 protease analogue **20** with a non-native thioester linkage at the ligation site (**Scheme 1.3**).[98] It was demonstrated that these unnatural thioester analogues retained the biological activity of their naturally isolated counterparts, but still a general ligation protocol capable of furnishing native products proved elusive.[99] NCL ultimately provided a widely applicable chemoselective ligation methodology (**Scheme 1.3**).[100] Two peptide fragments bearing a *C*-terminal thioester and an unprotected *N*-terminal Cys are utilised for the ligation. Trans-

thioesterification ensues in which a thioester linkage is generated between the *C*-terminal thioester fragment and the *N*-terminal Cys thiol, creating a transient reactive thioester intermediate. Attack of the amine at the thioester carbonyl over a five membered cyclic TS results in spontaneous S-to-N acyl transfer to furnish a native peptide bond between the two fragments, and a Cys residue at the ligation site.[94] This was first demonstrated by Dawson *et al.* in the synthesis of native interleukin-8 **24** through ligation of activated thioester **21** and cysteinyl peptide **22** *via* reactive thioester intermediate **23** (**Scheme 1.3**).[94] The reaction possesses remarkable chemoselectivity and can be carried out with unprotected peptide fragments in neutral pH and even without the addition of additives, albeit slowly, beyond those needed to denature the peptide fragments.[101] Unprotected peptide fragments greatly aid aq. solubility, the Achilles' heel of many peptide ligation and purification methodologies. NCL has found application in peptide synthesis and beyond, chiefly in medicinal chemistry, materials science and chemical biology. In particular the synergistic combination of SPPS and NCL has revolutionised access to native proteins, with over 700 unique biologically relevant protein substrates synthesised using a combination of these techniques since 1994.[102] The current state of the art encompasses total syntheses of fully functional bacterial enzymes analogues (~ 350 AAs in length)[103, 104] and syntheses of 'mirror-image' proteins consisting of D-AAs, which are entirely inaccessible by purely biological approaches.[105]



Scheme 1.3: (a) The work of Kent preceding native chemical ligation utilising a nucleophilic substitution reaction to furnish a non-native thioester bond at the site of ligation.[98] (b) The first published example of native chemical ligation in the synthesis of interleukin 8 (IL-8).[94]

1.3.1 Thioester Fragment Synthesis

A key challenge in NCL remains the efficient formation of suitable thioester containing fragments prior to ligation. Expressed protein ligation (EPL), developed contemporaneously to NCL, takes advantage of naturally occurring classes of proteins which post-translationally modify through excising portions of their own protein backbone.[106] Proteins which modify their protein backbones in this way are referred to as auto-processing domains and the process is referred to as protein splicing. The proposed mechanism by which protein splicing occurs is shown in **Figure 1.5**.[107] An N-to-S acyl transfer followed by an intramolecular trans-thioesterifcation reaction results in the formation of a branched intermediate. Subsequent attack at the adjacent amide carbonyl by an asparagine (Asn) residue results in an N-to-S acyl transfer between the two extein portions, furnishing a native amide bond between them and leading to intein excision. Genes which code for inteins have been found in all three domains of life, and are commonly vital for host function, including in genes associated with transcription, DNA replication and an array of metabolic processes.[108]

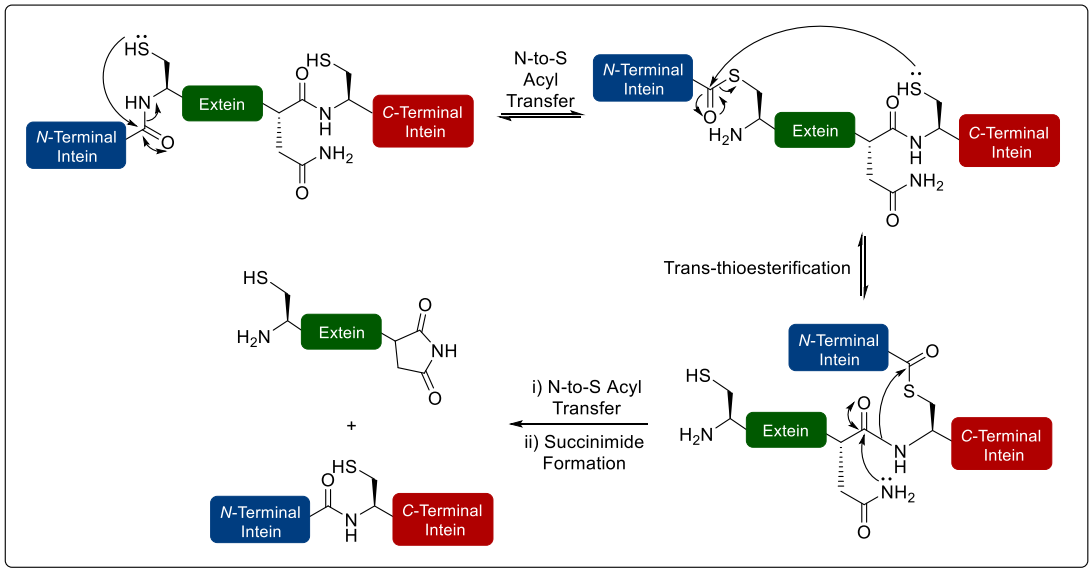
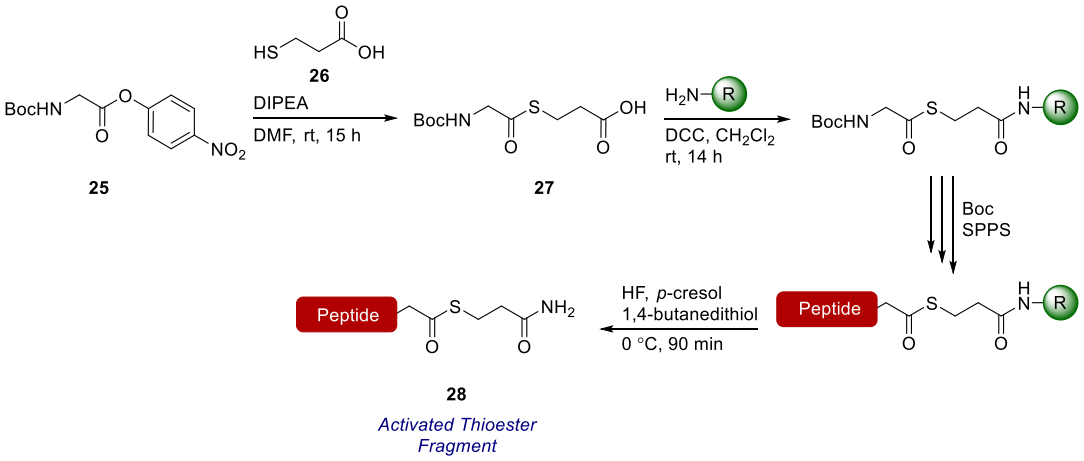


Figure 1.5: Mechanism for protein splicing *via* N to O/S acyl transfer as proposed by Xu and Perler.[107]

EPL modifies this natural process through mutation of Asn residues necessary for excision to alanine (Ala) residues. Such a change conserves the *N*-terminal extein thioester fragment in the branched intermediate, which may be simply cleaved through addition of a competing thiol.[109] Through genetic engineering thioester bearing peptide fragments for utilisation in NCL can be efficiently produced using this methodology.

Despite the success of EPL in giving access to thioester fragments research efforts are still ongoing to develop a facile chemical method to obviate the need for semi-synthesis. These synthetic methodologies can be broadly divided between those which furnish the thioester before SPPS and those which furnish the thioester after SPPS. Chief among the former type have been those utilising SPPS resins, pioneered by Hojo and Aimoto (**Scheme 1.4**).[110] Acylation of thiol **26** by activated carboxylic acid species **25** yielded thioester **27** which after attachment to an amino functionalised resin, Boc SPPS and HF cleavage provided peptide thioester **28**. This approach has been substantially limited however by the necessity for an acid labile Boc PG strategy as opposed to the more common base labile Fmoc group, in order to avoid thioester cleavage under Fmoc deprotection conditions.[109]



Scheme 1.4: Thioester fragment synthesis as pioneered by Hojo and Aimoto utilising Boc solid phase peptide synthesis.[110]

As previously mentioned, (**Section 1.2.3.1**) Boc SPPS methodologies require profligate use of TFA and final global deprotections with HF and are unsympathetic to PTM bearing fragments unable to withstand these very harsh conditions. Methodologies encompassing a more convenient Fmoc based strategy have included side-chain resin anchoring[111] and backbone amide linker (BAL) anchoring.[112] Numerous methodologies to furnish the thioester after standard SPPS have been developed, often relying on O- or N-to-S acyl transfer reactions prior to cleavage.[113, 114] All possess inherent issues, including epimerisation at the *C*-terminus and poor SPPS yields, and establishment of a truly general method has thus far proven elusive. Beyond the use of resin bound strategies, direct thioesterification of peptides in solution using the corresponding thiol and appropriate coupling agent is still used.[109] Alkyl thioesters are typically desired at the *C*-terminus as they provide greater stability prior to reaction and increase aq. solubility if a

suitable hydrophilic group is present. Alkyl thioesters are also capable of simplifying downstream desulfurisation procedures (**Section 1.3.4**), such as in the case of 4-mercaptosulfonate.[77] Aryl thioesters possess lower stability towards nucleophilic attack and hydrolysis and must be accessed *via* specialised peptide hydrazide methodologies.[115]

1.3.2 Trans-thioesterification and S-to-N Acyl Transfer in NCL

The application of thioesters in NCL, as well as an array of biological pathways, is a consequence of their unique reactivity towards nucleophilic addition. Comparison of bond lengths of amides, oxoesters and thioesters indicated that for the general structure RCO-X-R the observed bond lengths of CO-N and CO-O are ~ 0.08 Å longer than corresponding O-R and N-R bond lengths within the same molecule (**Figure 1.6**).[116, 117] The corresponding difference for thioester is ~ 0.02 Å, demonstrating the C(O)-S bond's comparatively low double bond character and the sulfur atom's poor contribution to resonance delocalisation.[117] This lack of resonance increases the electrophilic nature of the adjacent carbonyl, promoting nucleophilic attack, and justifies the thioesters moietys description as 'activated'. In synthetic terms the reactivity of thioesters towards nucleophilic addition lies above amides and oxoesters but below acyl chlorides and activated anhydrides and permits acyl transfer over convenient timescales and under mild conditions, key features of thioester reactivity exploited in NCL.[77]

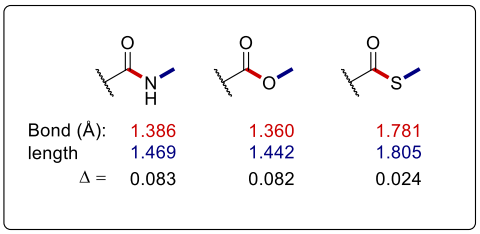


Figure 1.6: The difference in bond lengths of methyl acetamides, methyl acetates and methyl thioacetates illustrating the lack of resonance delocalisation in thioesters.[116, 117]

Thioesters are also resistant to hydrolysis, particularly at mildly basic pHs (~ 8.0) and thus are perfectly suited to use in aq. solvent in comparison to acyl chlorides and more aggressively activated substrates.[118] The exquisite chemoselectivity of NCL in comparison with other ligation methodologies stems from the inherent reversibility of the trans-thioesterfication step. Even if unprotected non-amino nucleophilic AA residues are present within the targets primary structure acyl transfer from the thioester to one of these leads to so called 'unproductive' products. These species cannot undergo

subsequent irreversible S-to-N acyl transfer and are eventually consumed by a reaction which forms the desired reactive intramolecular system.[119] The intramolecular S-to-N acyl transfer step of NCL is the thermodynamic driving force of the reaction by virtue of the formation of a stable amide bond. The activation energy of the archetypal cysteinyl S-to-N acyl transfer process has been computationally calculated as 50.6 kJ mol^{-1} in comparison to 130.1 kJ mol^{-1} for the reverse N-to-S transfer.[120] This renders the S-to-N acyl transfer irreversible over 5- or 6-membered TSs, except in cases of extensive substrate reorganisation or neighbouring residue participation, such as protein splicing (**Section 1.3.1**, **Fig 1.5.**). The 5-membered cyclic TS ensures swift kinetics, isolated reactive aryl thioesters utilised by Wieland *et al.* undergoing acyl transfer in 2 minutes (min).[121] As a result of spontaneity of S-to-N acyl transfer in classical NCL it is invariably not the rate determining step (RDS) of the reaction and proportionately there is a lack of experimental investigation in this area when compared with trans-thioesterification.

1.3.3 Limitations of Native Chemical Ligation

The trans-thioesterification step of NCL, despite conferring many advantageous characteristics, can prove problematic due to its reversibility. As trans-thioesterification is the RDS the reaction is carried out at basic pH (~ 8.0) in order to facilitate Cys thiolate formation. Even so, reaction rates can prove unsatisfactory, particularly at challenging ligation sites, primarily with *C*-terminal β-branched AAs such as Val and threonine (Thr), routinely requiring 24 – 48 hours (h) to reach completion with hindered substrates.[122] In order to expedite trans-thioesterification an array of thiol additives possessing enhanced leaving group capabilities have been developed (**Figure 1.7**).

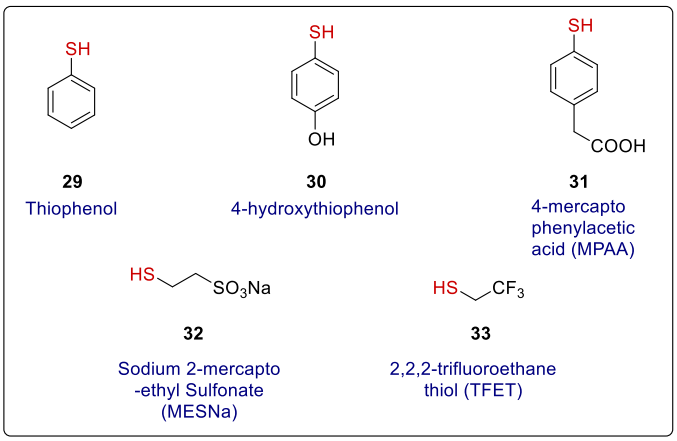


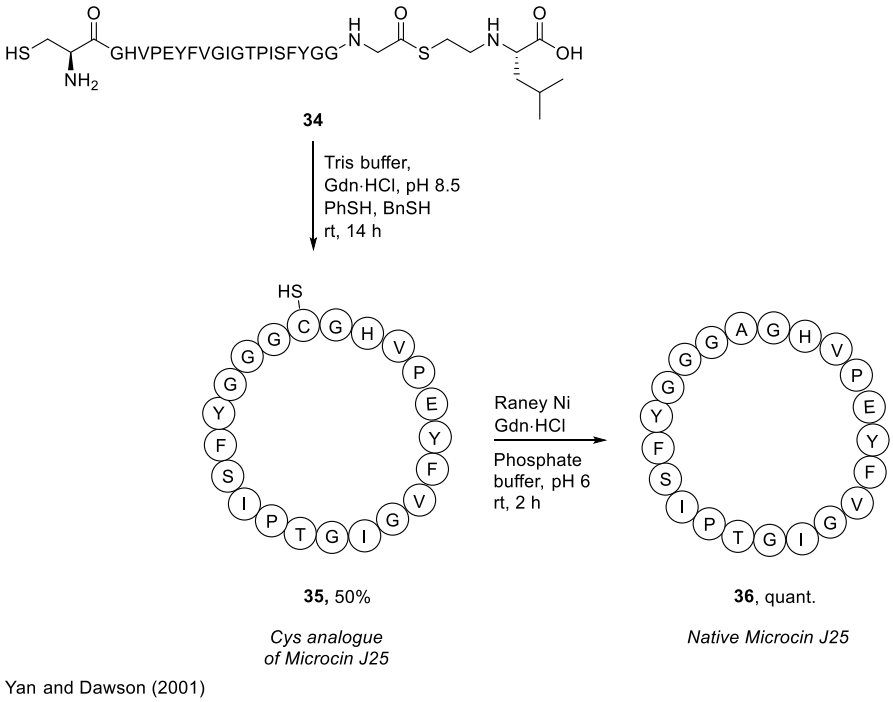
Figure 1.7: Thiol additives commonly used to enhance trans-thioesterification rates in native chemical ligation.

Aryl thiols such as **29**, **30** and **31** have proven effective trans-thioesterification catalysts as their electron rich nature enhances thiol nucleophilicity and leaving group ability in comparison to alkyl thiols, which bear greater electronic and structural similarity to Cys and therefore do not increase the rate of thioester exchange as significantly.[118] 4-Mercaptophenylacetic acid (**31**, MPAA, **Figure 1.7**) is particularly popular as it is cheap, odourless and highly soluble in aq. solution at basic pH.[123] However, alkyl thiols sodium 2-mercaptoethylsulfonate (**32**, MESNa, **Figure 1.7**) and 2,2,2-trifluoroethanthiol (**33**, TFET, **Figure 1.7**) possess pKa values compatible with catalysis and confer desirable solubility properties and aid purification, respectively.[123, 124] Fast reaction between the thiol additive and initial thioester fragment is ensured through using high concentrations of additives with up to 50 eq. generally used.[123] Higher performing fluorinated thiols such as **33** are preferred in challenging ligations or in instances in which aryl thiols preclude efficient product purification.[125] These additives have proven successful in accelerating ligation but still rely on an intrinsically irreversible process to produce the desired peptide thioesters capable of subsequent S-to-N acyl transfer. A general method capable of furnishing such thioester substrates through a rapid irreversible process is currently lacking within the protein ligation literature. Research efforts to extend NCL have primarily focussed on obviating the requirement for Cys at the site of ligation. This is particularly problematic as Cys is amongst the least abundant AAs, making up 1.5% of the translatable human genome,[126] and absent entirely from as high as 8% of translated proteins.[127] Furthermore, even if a Cys is within the primary sequence of a target its position may not be practically positioned to permit ligation. Many valuable therapeutic proteins including human parathyroid hormone (hPTH),[128] and proteins implicated in disease states such as potent oncogene YAP1[129] possess no Cys residue within their primary structure, rendering total synthesis through classical NCL impossible. Strategies to obviate the need for Cys at the ligation site have followed one of two distinct approaches; removal of the thiol moiety post ligation through desulfurisation methodologies, and the use of cleavable thiol containing auxiliaries.

1.3.4 Desulfurisation in Peptide Ligation

Desulfurisation has proven to be an important advance in the field of protein synthesis permitting ligation at AA residues beyond Cys. The seminal desulfurisation/ligation work by Yan and Dawson culminated in the synthesis of antibacterial cyclic peptide **36**

following macrocyclisation of thioester **34** and desulfurisation of the Cys analogue (**Scheme 1.5**).[130] Ala is the second most abundant AA found in human proteins (8.42%), significantly more common than Cys.[77] Therefore, this advance provided access to a considerable number of proteins hitherto inaccessible by chemical protein synthesis.



Scheme 1.5: The first demonstration of peptide ligation/desulfurisation by Wan and Dawson culminating in the synthesis of antibacterial cyclic peptide Microcin J25.[130]

Danishefsky and coworkers sought to arrest the significant volume of side reactions occurring at the metal surface under these conditions, which frequently resulted in racemisation and reduction of side-chain functional groups. This work culminated in the first metal-free desulfurisation methodology, employing tris(2-carboxyethyl)phospine (TCEP) in the presence of the water soluble radical initiator 2,2'-Azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (VA-044) (**Figure 1.8**).[131] The reaction mechanism had been known long before its application in protein synthesis as a result of the pioneering work of Walling and Hoffman on thiol reactivity towards phosphines.[132, 133] Critical to efficient desulfurisation is the inclusion of an additional thiol species, such as ethanethiol or *tert*-butylthiol, from which a hydrogen atom may be abstracted more easily in order to ensure efficient chain propagation.[134] Since Danishefsky's pioneering work a host of other metal free desulfurisation techniques have been developed, typically through varying initiation conditions and thiol additives, to render the technique sympathetic to a wide range of reaction conditions and functional groups.[134, 135]

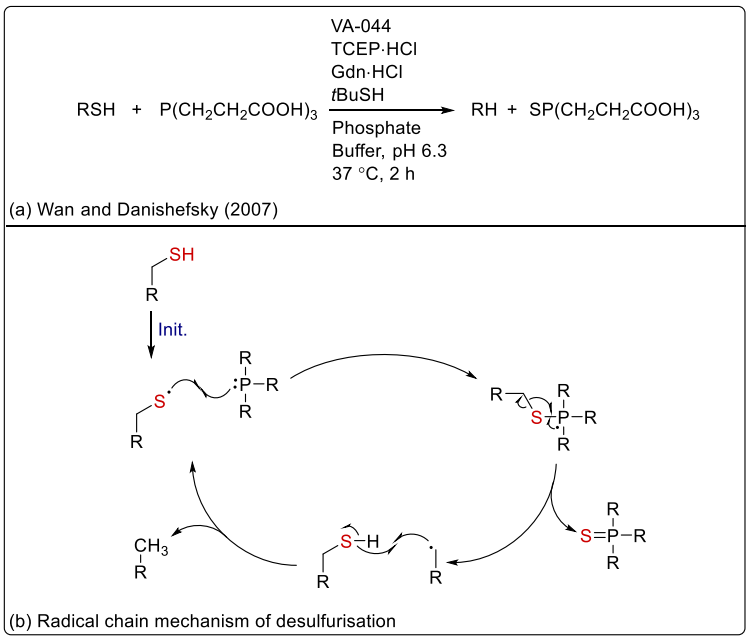


Figure 1.8: (a) Metal-free desulfurisation conditions optimised by Wan and Danishefsky.[131] (b) Proposed radical chain mechanism for desulfurisation reaction using phosphines.[132]

Following the pioneering ligation-desulfurisation work of Dawson, thiolated UAAs were developed by several groups which permitted trans-thioesterification, S-to-N acyl transfer and, through subsequent desulfurisation, transformation into their alkyl, aromatic and heteroatomic AA counterparts post-ligation. This concept has been utilised to ligate protein targets at 13 diverse AA residues (**Figure 1.9**).[134, 136-147] The synthesis of these thiolated precursors has proven a somewhat limiting factor with many requiring greater than 10 synthetic steps to access desired precursors suitable for use in SPPS. Nevertheless, deft application of the aforementioned thiol additives has proven critical in improving desulfurisation rates at sterically demanding residues and hence uptake of ligation-desulfurisation methodologies at non-Cys sites. The work of Seitz on penicillamine (i.e. β-thiol Val) type systems in particular emphasises the importance of additives which aid in the unfolding, reduction and denaturing of reactive peptide fragments.[134] The principle disadvantage of desulfurisation in chemical protein synthesis is the fact its use precludes the presence of other unprotected thiol residues within the protein structure of the target. Attempts are ongoing to probe the feasibility of selective desulfurisation conferred through intrinsic differences in reactivity[143] or peptide conformation.[148] The stability of the methionine (Met) bearing targets synthesised under these radical conditions accentuates the scope and sensitivity of desulfurisation techniques, and the potential for greater usage of radical chemistries in the synthesis of complex proteins.

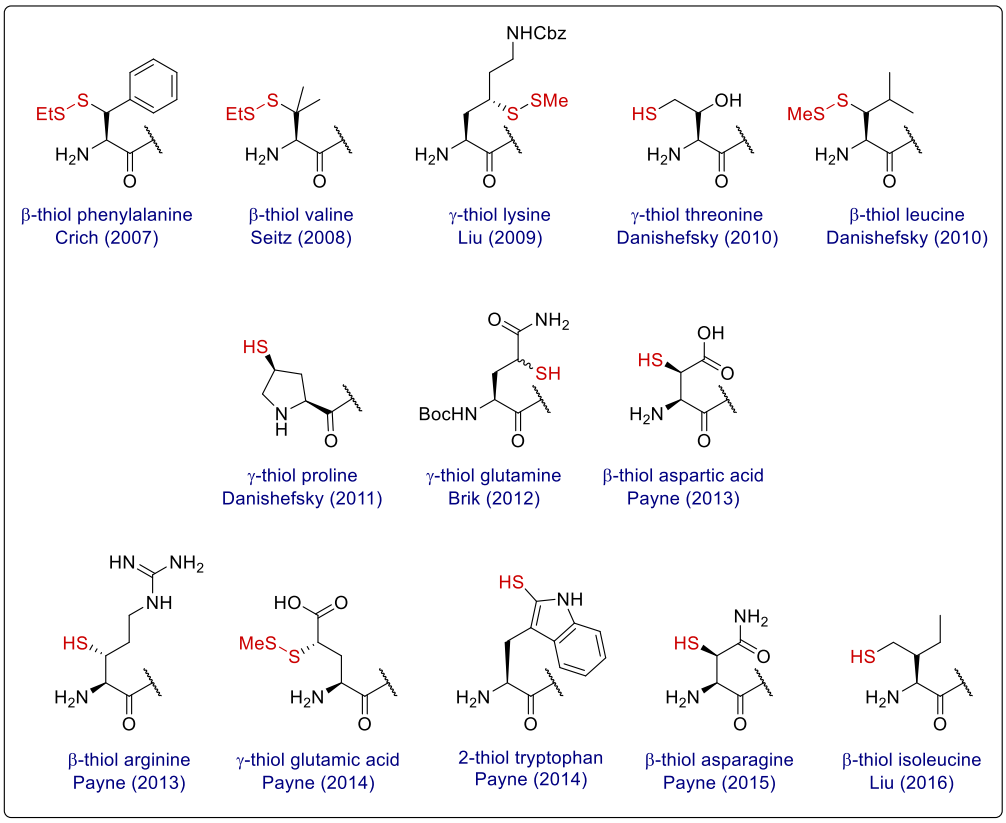


Figure 1.9: Thiolated amino acid residues synthesized used in native chemical ligation-desulfurisation protocols to extend ligation to non-cysteine sites.[134, 136-147]

1.3.5 Auxiliary Mediated Ligation

Alongside desulfurisation methodologies, the employment of thiol containing auxiliaries for auxiliary mediated ligation (AML) has attracted significant research interest. Auxiliaries in AML, affixed to the *N*-terminal of one ligation partner *via* a range of chemistries, in combination with SPPS provide both a thiol surrogate for trans-thioesterification and, once this has taken place, a system capable of undergoing S-to-N acyl transfer (**Figure 1.10**). The thiol containing auxiliary is then cleaved or elaborated post-ligation to yield the native peptide substrate. These methodologies can in principle be divided into α-amino auxiliaries utilising NCL-like S-to-N acyl transfer TSs and auxiliaries utilising side-chain assisted ligation strategies over larger, less favourable TSs. In order to ensure a thermodynamically favoured 5- or 6-membered cyclic S-to-N acyl transfer TS auxiliaries at the α-amino position have utilised scaffolds based around 2-mercaptoethyl moieties (**Figure 1.10**). AML was demonstrated in principle by Kent and coworkers shortly after the publication of NCL with a 2-mercaptoethyl auxiliary which although non-cleavable, demonstrated that despite the decreased nucleophilicity of secondary amines, S-to-N acyl transfer could take place reliably.[149]

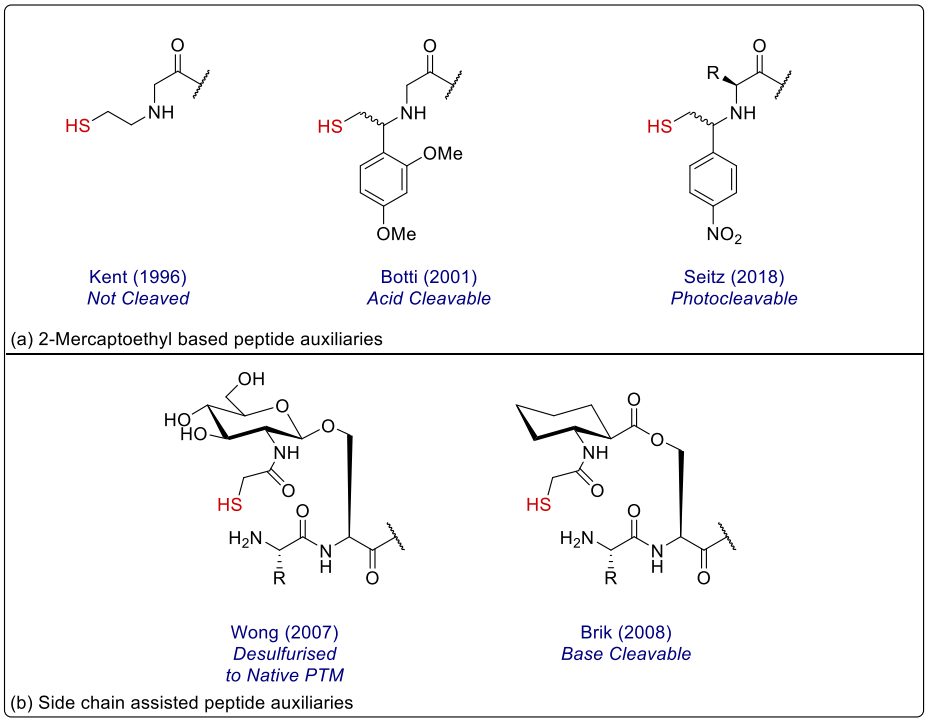
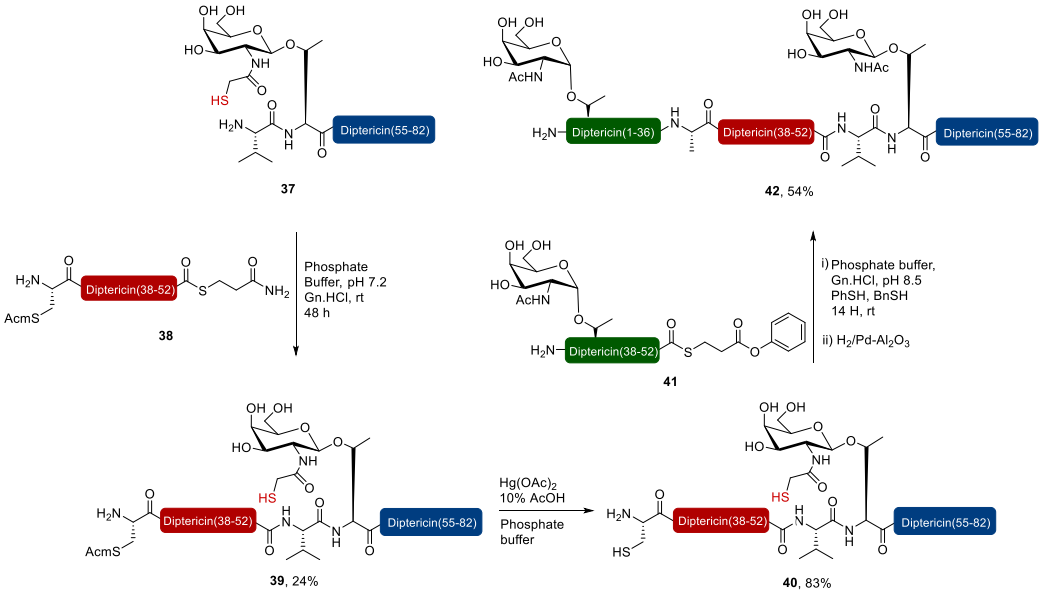


Figure 1.10: a) Structures of 2-mercaptoethyl based auxiliaries for use in auxiliary mediated ligation and their respective cleavage conditions. [149, 150, 152] b) Auxiliaries used for side-chain assisted ligation and their respective cleavage conditions.[155, 157]

Subsequent work focussed on the development of auxiliary cleavage conditions sympathetic to peptidic substrates primarily through extension of the mercaptoethyl motif to incorporate substituted aromatics. Introduction of methoxy groups by Botti *et al.* permitted removal under acidic conditions (**Figure 1.10**),[150] while addition of a nitro group by Kawakami *et al.* permitted removal under ultraviolet (UV) irradiation.[151] Ligation utilising α-amino auxiliaries was not expanded beyond Gly until the development of a modified mercaptoethyl auxiliary by Seitz which permitted ligation at Met, glutamic acid (Glu) and arginine (Arg), as well as less sterically demanding AAs.[152] The exemplary reactivity of this auxiliary was investigated and it was found that unlike in classical NCL the S-to-N acyl transfer step of the reaction was rate determining and, contrary to expectations, β-substitution of the reactive amine residue led to an increase in rate through a Thorpe-Ingold effect.[153] Cleavage of this auxiliary was undertaken under mild dealkylation conditions, utilising TCEP at mildly basic pH (8.5) through an oxidative decomposition pathway. A photocleavable variant was developed thereafter (**Figure 1.10**).[154] As the principal example of α-amino AML at ligation sites beyond Gly, this recent work suggests that there remains capacity to extend existing AML methodologies beyond the current literature precedent. AMLs utilising larger S-to-N acyl transfer TSs (> 6 membered) rely on introduction of a reactive thiol *via* auxiliary

installation at reactive residues such as aspartic acid (Asp), Glu, serine (Ser) and Thr.[155] This has served to expand the protein ligation repertoire and facilitates simple auxiliary introduction through esterification chemistry. The entropic penalty incurred through a large S-to-N acyl transfer TS is typically offset by a degree of rigidity with the design of the auxiliary (**Figure 1.10**).[156] The work of Wong and co-workers into sugar assisted ligation (SAL, **Scheme 1.7**) provided the first successful example of AML over a large S-to-N acyl transfer TS.[157, 158] Substrate **37** consisted of a thiol modified *N*-acetylglucosamine (GlcNAc) conjugated to a Thr residue penultimate to an *N*-terminal Val, mimicking a naturally occurring *O*-linked glycopeptide linkage.[158] Addition of thioester **38,** bearing an *N*-terminal protected Cys permitted isolation of ligated product **39** in a 24% yield over 48 h. Cys deprotection of **39** enabled NCL of deprotection product **40** and thioester **41**. Subsequent desulfurisation yielded native antimicrobial glycopeptide diptericin **42** in a 54% yield. Interestingly, removal of the *N*-terminal Val residue of **37** to yield a smaller ten membered S-to-N acyl transfer TS resulted in only traces of isolatable ligated product. It was suggested that interactions between the carbohydrate and peptide backbone acted to draw the unprotected terminal amine towards the thioester carbonyl facilitating S-to-N acyl transfer.[158]



Scheme 1.7: Sugar assisted ligation (SAL) as described by Yang *et al.*and used to furnish native antimicrobial glycopeptide diptericin **42**.[158]

Similarly large systems were subsequently developed for the ligation of *N*-linked glycopeptide systems utilising a glucose modified Asn residue[157] and, by Brik and co-workers, a cleavable auxiliary based on a *trans*-2-aminocyclohexanoic acid precursor (**Figure 1.10**).[155, 159] These examples demonstrate that S-to-N acyl transfer across large

cyclic TSs can be reliably undertaken if ample consideration is given to design of the auxiliary. Despite the intense attention given to NCL since the inception of the technique, leading to the establishment of many complimentary and extended methodologies, potential for improvement remains. Desulfurisation/ligation methodologies and AML have sought to address the need for Cys at the ligation site and have been successful. However, the reliance on trans-thioesterfication to furnish reactive thioester intermediates capable of ligation remains problematic particularly in systems where it is rate determining such as those bearing small S-to-N acyl transfer TSs.[77]

1.4 The Thiol-ene Reaction

The addition of thiols to unsaturated compounds has been an active area of chemical research since the early 20th century and has endured into the present, primarily in the widespread use of the radical mediated thiol-ene reaction and ionic thio-Michael type reaction. The first example of what is now commonly referred to as a thiol-ene reaction was reported in 1905 by Posner and refers to the addition of thiol to an olefin to form a saturated sulfide product.[160] The thiol-ene reaction fulfils all the criteria of a click reaction as defined by Sharpless possessing high atom economy, excellent yields, superb regio-control, and insensitivity to H_2O and O_2.[161] The reaction is also sympathetic to a striking range of unsaturated substrates including haloalkenes, vinylsulfides, acrylates and vinylphosphonates.[162] As a result of its remarkable synthetic utility, the reaction is well established throughout the fields of natural product synthesis, bioconjugation and polymerisation chemistry.

1.4.1 Mechanistic Considerations

The radical chain mechanism featuring processes and products of the radical mediated thiol-ene reaction can be seen in **Figure 1.11.** Key to the utility of the thiol-ene reaction is the ease by which thiyl radicals can be generated by hydrogen abstraction and the compatibility of the radical chain process with both organic solvents and buffered aq. conditions. The relatively low bond disassociation energy (BDE) of S-H bonds (~ 87 kcal mol^{-1} for alkyl thiols)[163] relative to the C-H bonds of initiating species provides for efficient radical formation. All commonly utilised radical initiators, including peroxides and azo-compounds, have been employed for the purpose[162] and initiation has been demonstrated using both thermally activated and photoactivated initiators and is

conducive to initiation by UV irradiation.[164] The thiol BDE is primarily dependent on the ability of the substrate to stabilise the thiyl radical formed through resonance stabilisation.[165] Therefore, aryl thiols have lower BDEs (~ 79 kcal mol^{-1}) in comparison to alkyl thiols but values are substrate dependent, ranging from 70 kcal mol^{-1} (4-aminothiophenol)[166] to 84 kcal mol^{-1} (pentafluorothiophenol).[167] After formation, thiyl radical **A** reversibly adds to the less substituted carbon across the double bond of the thiol-ene acceptor to produce a *C*-centred radical in a propagation step (**Figure 1.11**, (b)).

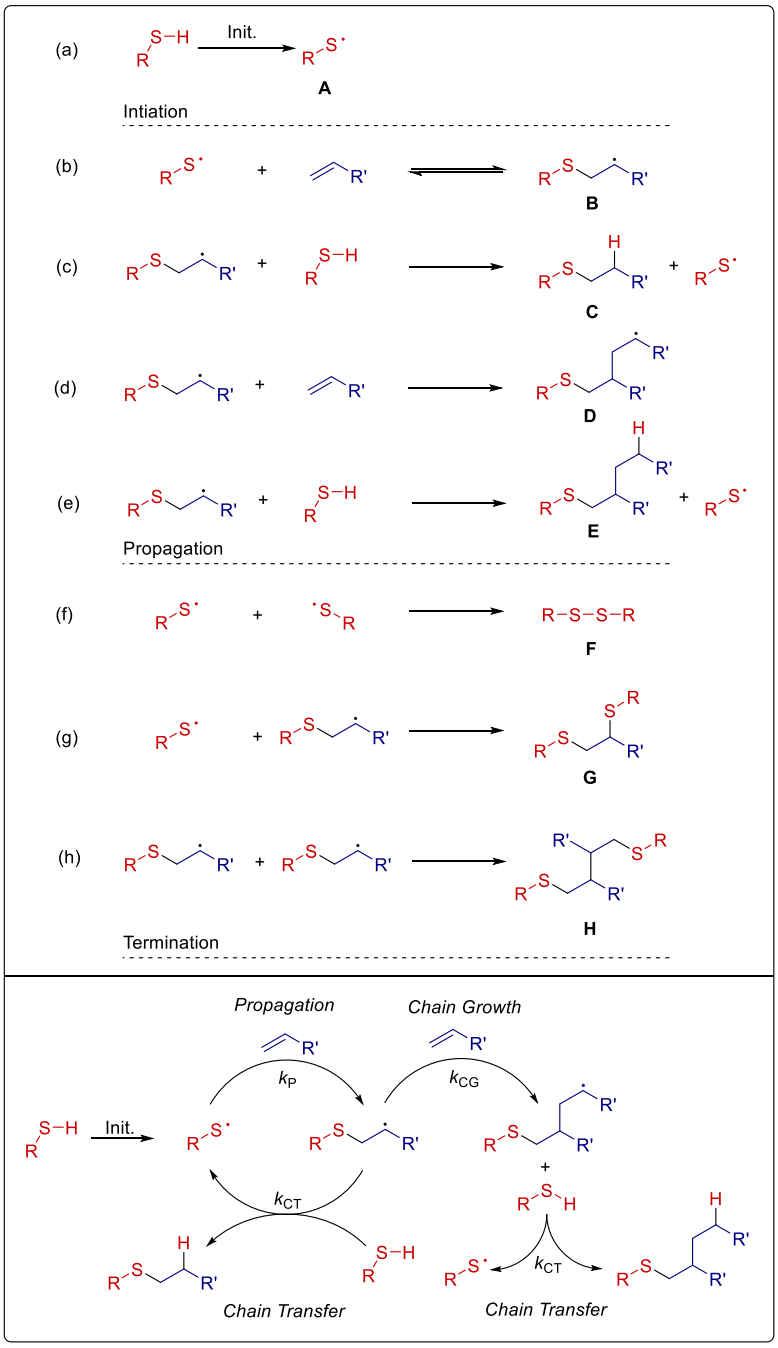


Figure 1.11: Radical chain mechanism of the radical mediated thiol-ene reaction indicating all possible processes within the cycle.[170]

The exquisite regioselectivity of the thiol-ene reaction is derived from the reversibility of this addition step, as the unstable Markovnikov product readily fragments, permitting formation of the more substituted anti-Markovnikov radical **B**.[168] However, stereoselectivity of thiol-ene addition is poor except in the case of cyclic olefinic substrates such as cyclohexenes and norbornenes.[169] After initial addition of thiyl radical **B** to the olefin, the reaction can evolve along two pathways, through a chain transfer step (**Figure 1.11**, (c)) to furnish hydrothiolated product **C** or a chain growth (**Figure 1.11**, (d)) step to yield branched *C*-centred radical **D**. A further propagation step furnishes hydrothiolated branched product **E** and another thiyl radical through chain transfer (**Figure 1.11**, (e)). Several termination steps can occur, including thiyl radical recombination (**Figure 1.11**, (f)) to yield reactive disulfide **F**, capable of homolytic cleavage, and formation of branched dithioethers **G** and **H** formed through the recombination of *C*-centred radicals (**Figure 1.11**, (g) and (h)).[162] Due to the complexity of the radical chain mechanism it is imperative to understand the kinetics of the thiol-ene reaction in order to obtain predictable product distributions. This has been an area of active research for decades, first summarised by Griesbaum in 1970.[168] Key parameters of the reaction are the relative rates of propagation (k_P) and chain transfer (k_{CT}), expressed as the ratio k_P/k_{CT}.[170] If $k_P/k_{CT} \gg 1$, a high concentration of addition radical **B** is present in the reaction mixture, thereby increasing the formation of branched products **E** and **H**. If $k_P/k_{CT} \approx 1$ or $k_P/k_{CT} \ll 1$, the initial thiol-ene addition step is the RDS and hydrogen abstraction fast, leading to a predominance of chain transfer products such as thioether **C**. k_P/k_{CT} is determined by the nature of the reaction components. The thiol component of the reaction affects overall reaction rate chiefly through its role as hydrogen donor in the chain transfer step, thereby affecting its rate (k_{CT}). Long *et al.* calculated the relative rates of hydrogen abstraction for thiols **43**, **44** and **45** and found k_{CT} reduced with proportionately with increased steric hindrance due to decreased accessibility of the thiol proton (**Figure 1.12**).[171] Munar *et al.* studied the effect of aryl substitution on the rate of k_P and k_{CT} in reactions with styrene and thiols **29**, **46**, **47** and **48**.[172] Interestingly, the electronic nature of the substituents had contrasting influence on k_P and k_{CT}. Electron poor thiols formed less stable thiyl radicals due to a lack of resonance stabilisation. However, the energy barrier to thiyl radical formation is overcome through the use of high energy *C*-centred initiating species designed specifically for the purpose and capable of forming destabilised radicals that have increased reactivity towards olefin addition, increasing k_P .[172] Conversely chain transfer requires hydrogen abstraction, therefore the

use of thiols incapable of radical stabilisation decreases k_{CT}. These investigations indicated that the nature of the thiol component does influence the reaction rate, but in a practical context low k_{CT} values can be improved through addition of a suitable proton donors to the reaction mixture, such as a silanes, acids or protic solvents, thereby limiting the effect of thiols on the overall rate of reaction.[162]

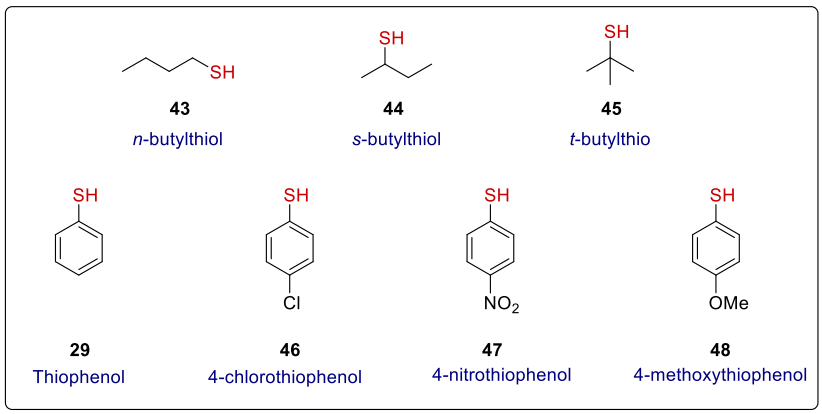
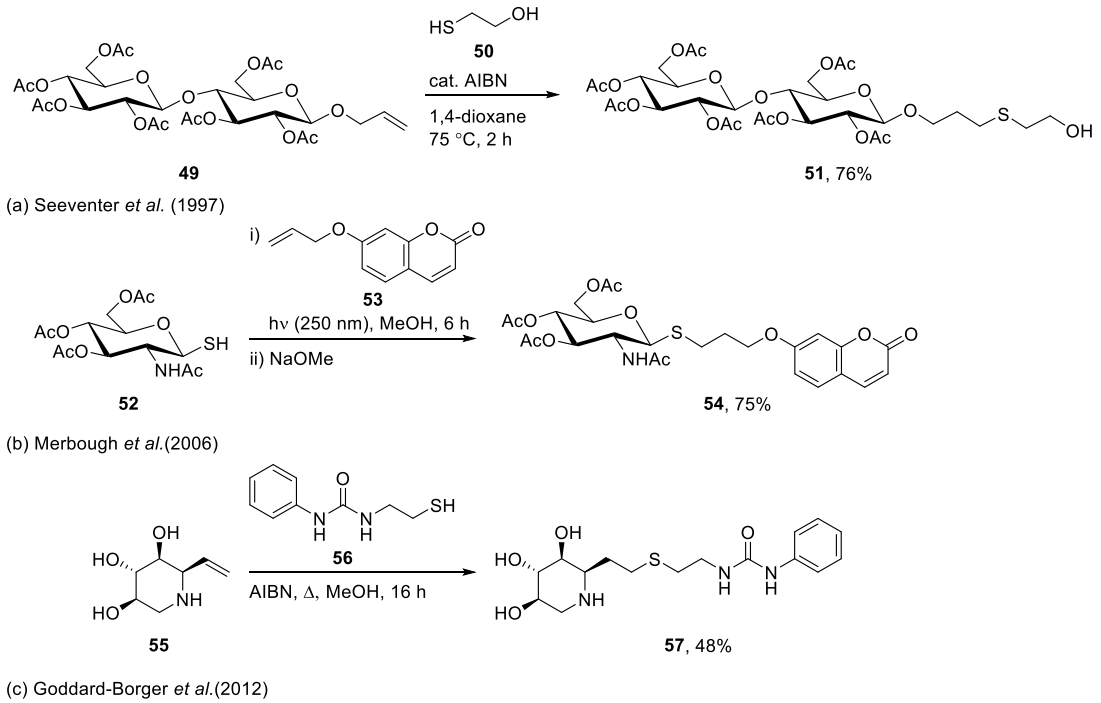


Figure 1.12: Thiols screened for effect of steric hindrance and aryl substituents on hydrogen abstraction.

The alkenyl component of the reaction tends to have a far greater influence on the mechanistic profile, as olefins are capable of effecting both k_P and k_{CT}. Munar *et al.* indicated that electron poor alkenes are incapable of stabilising the *C*-centered radicals resulting from addition of thiyl radicals across the olefin, decreasing k_P.[172] However, once addition has taken place the resulting unstable radical is highly reactive towards hydrogen abstraction and thus capable of enhancing k_{CT} significantly.[165] Alkenes possess greater structural variability than thiols and consequently alkene structure affects k_P and k_{CT} to a greater degree. Roper *et al.* demonstrated that sterically hindered alkenes can prevent efficient thiol addition and hydrogen abstraction, thereby reducing both k_P and k_{CT}.[165] Conversely constrained alkenes, such as cyclic or norbornene systems, reduce the entropic burden of the initial propagation step and ensure a reactive alkene confirmation prior to thiol approach. Therefore, these substrates possess considerable reactivity towards thiol-ene addition and correspondingly high values of k_P. The necessity for disrupting resonance in conjugated systems reduces k_P particularly for dienes which are not capable of forming addition products *via* competing Michael-type mechanisms.[170] However, the rate of even poorly performing thiol-ene reactions is typically in the order of 1×10^5 M^{-1} s^{-1}, which coupled with the robust nature and large substrate scope is an underlying factor in the popularity of this synthetic transformation.[162]

1.4.2 The Thiol-ene Reaction in Biomolecular Synthesis

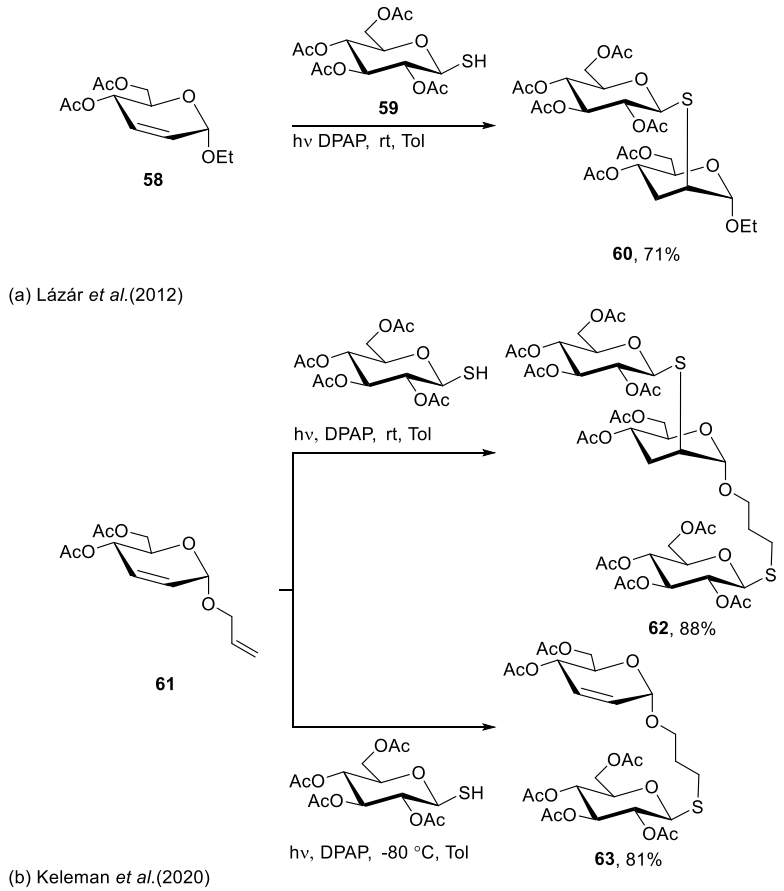
The thiol-ene reaction is eminently compatible with the organic synthesis of biomolecules due to the advantageous qualities of the reaction, including tolerance to an array of functionalities, insensitivity to ambient conditions, mild initiation conditions and exquisite regio- and chemoselectivity. As a consequence the reaction has seen widespread use in the fields of carbohydrate and peptide chemistry.[173] The reaction of allyl glycosides with alkyl thiols was first reported in 1974 by Lee *et al.*[174] and expanded upon by Van Seeventer and coworkers in the demonstration of thiol-ene addition of alkyl thiols to allylated fructose, with yields in excess of 70% in all cases.[175] Installation of mercaptoethanol **50** onto allylated disaccharide **49** furnished thiol-ene adduct **51** in a 76% yield (**Scheme 1.8**) and permitted further synthetic elaboration of the hydroxyl handle.[175] The inverse thiol-ene reaction of anomeric thiol **52** with enol ether coumarin derivative **53** to give glycoconjugate **54** in a 75% yield was demonstrated by Merbough *et al.* and permitted effective synthesis of glycosidic probes for carbohydrate modifying enzymes (**Scheme 1.8**).[176]



Scheme 1.8: (a) Installation of hydroxyl handle *via* thiol-ene addition of mercaptoethanol **50** to allyllated dissacharide **49**.[175] (b) Synthesis of glycoconjugate **54** via thiol-ene reaction of thiosugar **52** and allylated coumarin derivative **53**.[176] (c) Synthesis of iminosugar **57** *via* thiol-ene addition of urea derivative **56** to anomeric olefin **55**.[177]

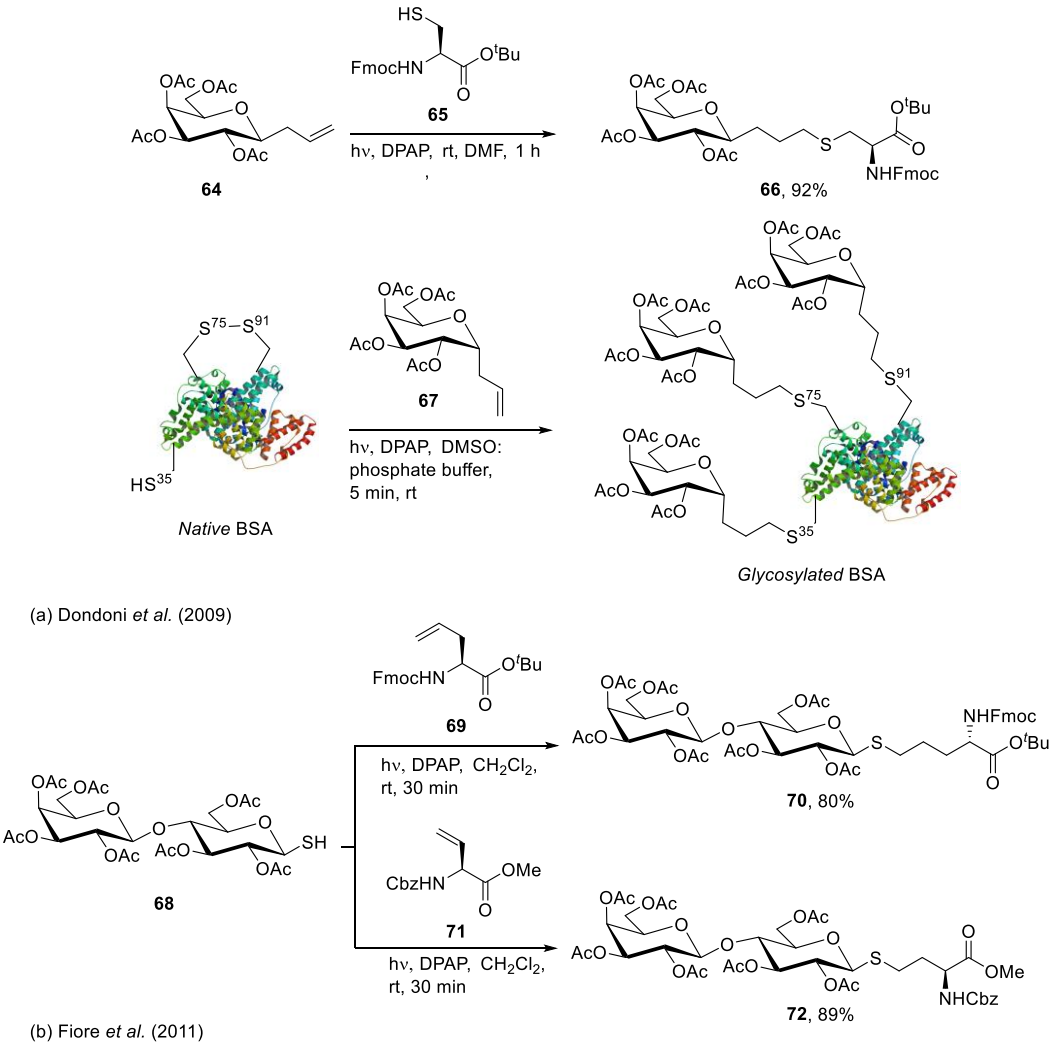
Carbohydrates directly modified at the anomeric position with vinyl groups also possess good reactivity towards thiol-ene addition as demonstrated by Goddard-Borger *et al.*

through addition of urea derivative **56** to vinylic iminosugar **55**, affording conjugate **57** in a 48% yield despite the hindered nature of the olefinic substrate (**Scheme 1.8**).[177] Modification of the thiol component provided a highly divergent synthetic route to an array of iminosugars screened for potential glucosylceramidase activity.[177] Thiol-ene addition to olefins directly within the cyclic carbohydrate structure has also been explored by Lázár *et al*. The reaction of 2,3-unsaturated glycoside **58** with thiosugar **59** demonstrated excellent regioselectivity as expected (**Section 1.4.1**) and in addition, superb axial stereoselectivity as steric repulsion of the olefin substituents permitted addition of the thiyl radical from only the less hindered face of the cycle (**Scheme 1.9**).[178] Further work has demonstrated the regioselectivity of thiol-ene addition, such as in the selective addition of thiosugar **59** to glycoside **61** at low temperature possessing both terminal and internal alkene moieties (**Scheme 1.9**).[179] The thiol-ene reaction has also proven to be convenient for the construction of supramolecular structures bearing carbohydrates demonstrated through the functionalisation of colloidal nanoparticles[180] and in the synthesis of dendritic carbohydrate systems to mimic the glycocalyx in bacterial cells.[181]



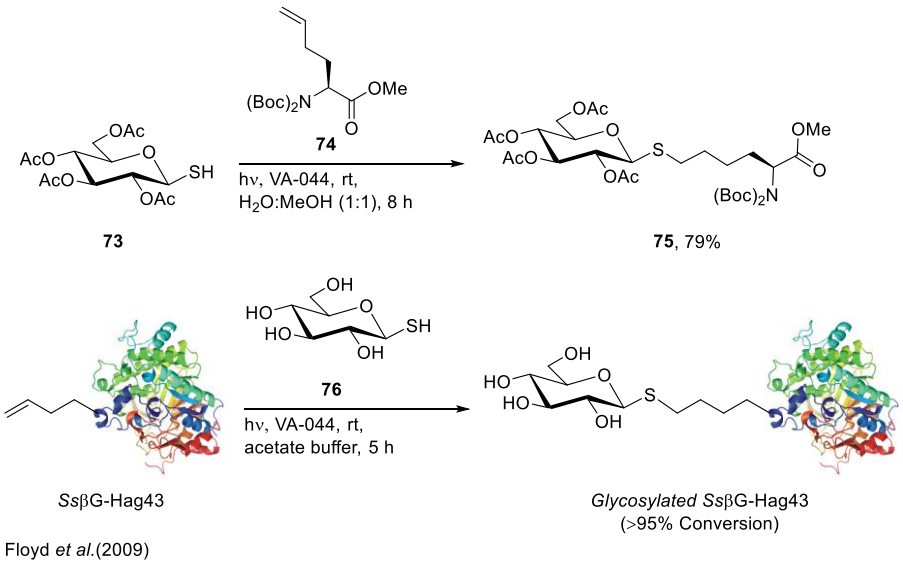
Scheme 1.9: (a) Addition of thiosugar **59** to cyclic alkene **58**.[178] (b) Thiol-ene addition at low temperature to selectively yield disaccharide **63**.[179]

The use of the thiol-ene reaction to furnish *S*-linked glycosylated AA substrates for enzymatic probes and therapeutic use has also proven a fruitful area of research. The value of these unnatural linkages lies primarily in their enhanced resistance to chemical and enzymatic degradation, and thus are capable of providing bioisosteric candidates for drug discovery.[162] Dondoni and coworkers first applied radical mediated thiol-ene to peptidic substrates through the addition of protected Cys derivative **65** to *C*-allyl glycoside **64** (**Scheme 1.10**) furnishing glycosylated Cys monomer **66** in a 92% yield.[182] This work culminated in the glycosylation of bovine serum albumin (BSA) at three Cys residues with *C*-allyl glycoside **67** (**Scheme 1.10**).[182] These optimised conditions were subsequently applied to addition of thiolated disaccharide **68** to alkene bearing UAAs protected allylglycine (AG) **69** and protected vinylglycine (VG) **71**,[183] permitting the synthesis of small glycopeptide conjugates **70** and **72** in yields of greater than 80%. This methodology proved robust enough furnish a glycoconjugate of glutathione (GSH) in two steps, the thiol-ene step proceeding quantitatively in aq. conditions.



Scheme 1.10: (a) Conjugation of allyl glycoside **64** to protected cysteine **65** to furnish glycosylated cysteine monomer **66**.[182] (b) Conjugation of thio-dissacharide **68** to allylglycine **69** and vinylglycine derivative **71**.[183]

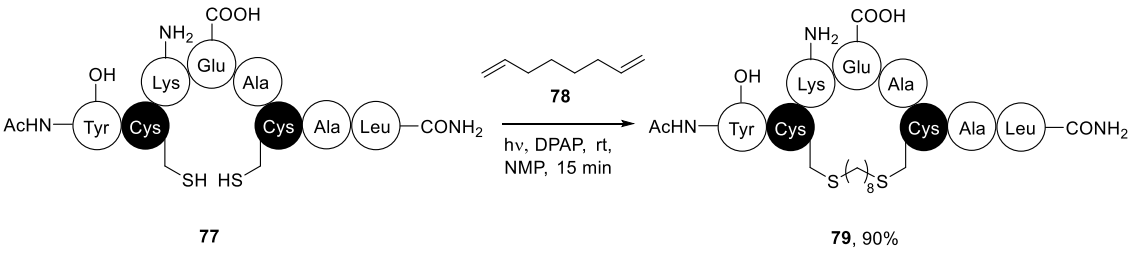
Contemporaneously Floyd *et al.* demonstrated the conjugation of thiosugars *via* thiol-ene reaction to proteins through the incorporation of UAA L-homoallylglycine in place of Met in the proteins primary structure, (**Scheme 1.11**).[184] Initial reaction optimisation was carried out utilising thiosugar **73** with protected L-homoallylglycine **74** to furnish glycoconjugate **75** in a 79% yield. Three model protein substrates Np276, *Ss*βG and the Qβ virus capsid were modified with L-homoallylglycine at a single Met site through expression of corresponding gene sequences in *Escherichia coli* bacteria. All thiol-ene conjugations with thiosugar **76** proceeded with greater than 90% conversion at pH 4, even in the presence of unprotected Cys residues. This work demonstrated the first use of the thiol-ene reaction for site specific protein modification via a 'tag-modify approach'.



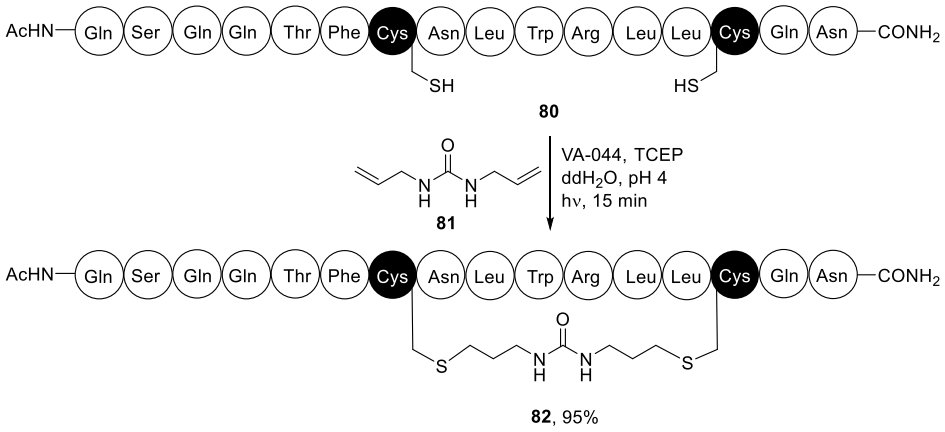
Scheme 1.11: The work of Floyd *et al.* including optimisation of the radical mediated thiol-ene reaction and demonstration of the first site specific bioconjugation utilising the thiol-ene reaction.[184]

Beyond the glycosylation of substrates, radical mediated thiol-ene has been used to carry out macrocyclisation and stapling in order to increase the durability of biologically active peptides. This was first demonstrated by Wang *et al.* using diene **78** to link together two Cys residues to furnish cyclised peptide **79** in a 90% yield under UV irradiation with 2,2-Dimethoxy-2-phenylacetophenone (DPAP).[185] Further investigation utilising diallylurea species **81** permitted the cyclisation of peptide **80** in aq. conditions with a suitable water-soluble radical initiator VA-044 to yield stapled peptide **82** in a 95% yield.[186] Developments by Aimetti *et al.* have permitted the cyclisation of protected resin bound substrates such as **83** through selective on-resin deprotection of monomethoxytrityl (Mmt) followed by thiol-ene cylclisation and global deprotection/cleavage to furnish cyclic peptide **84** in a 24% yield, from the initial resin

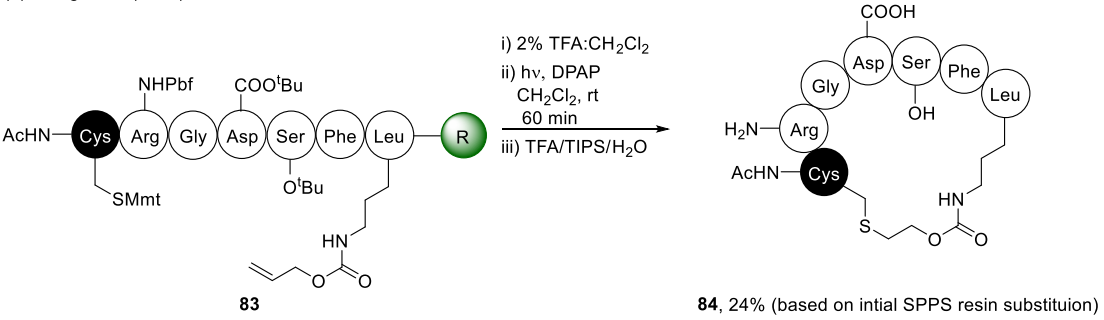
coupling prior to SPPS (**Scheme 1.12**).[187] On resin methodologies possess the distinct advantage of utilising organic solvent and thus demand lower eq. of initiator and additive than thiol-ene reactions performed in aq. solvent.[187] However, these methodologies are only capable of furnishing non-native linkages due to the introduction of exogenous olefinic linker species to permit the thiol-ene reaction.



(a) Wang *et al.* (2015)



(b) Wang *et al.* (2017)

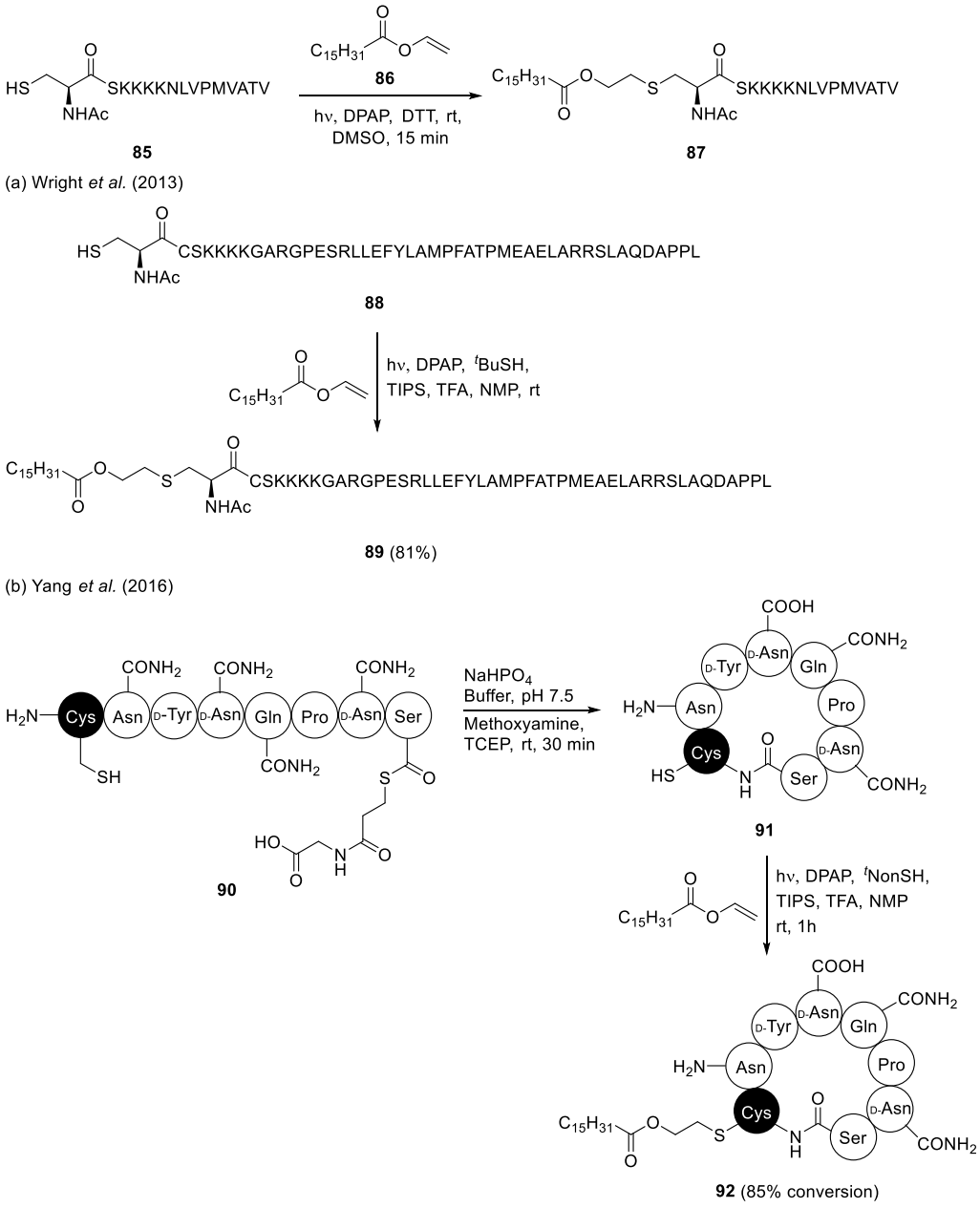


(c) Aimetti *et al.* (2010)

Scheme 1.12: (a) Thiol-ene mediated peptide stapling utilising 1,7-octadiene (**78**) to furnish **79**.[185] (b) Thiol-ene mediated peptide stapling in aqueous media using modified urea **81** yielding stapled peptide **82** quantitatively.[186] (c) On resin peptide macrocyclisation using modified lysine to yield cyclic peptide **84**.[187]

Proteins bearing Cys residues modified through lipidation are targets of particular interest to researchers due to their occurrence in the cell membrane of bacteria. Synthetic analogues may possess desirable antigenic characteristics of use in vaccine development or be used to elucidate bacterial biosynthetic pathways, thereby revealing novel antibiotic modes of action.[188] The Brimble group have pioneered the use of thiol-ene reactions for

native protein modification through the development of their 'cysteine lipidation on peptide or amino acids' (CLipPA) methodologies (**Scheme 1.13**).[189-193]



Scheme 1.13: (a) Thiol-ene mediated palmitoylation of peptide **85**.[190] (b) Thiol-ene mediated palmitoylation of peptide **88** under optimised reaction conditions.[191] (c) Sequential CLipPA and NCL to yield iturin mimetic **92**.[192]

Initial investigations permitted the synthesis of antigenic peptide **87**, lipidated at the *N*-terminus through reaction of vinyl palmitate **86** with unprotected peptide **85**, accessed through conventional Fmoc SPPS (**Scheme 1.13**).[190] Lipidation of forty-four residue peptide **88** necessitated further reaction optimisation and addition of *tert*-butylthiol and triisopropylsilane to facilitate hydrogen abstraction, resulting in an impressive 81% yield of lipidated peptide **89**.[191] Further elaboration of this methodology has expanded to

encompass reactions on SPPS resin prior to cleavage.[189] The Brimble group have also demonstrated the synergistic nature of thiol-ene based chemistries and NCL through publication of an impressive NCL/CLipPA, cyclisation/lipidation strategy.[193] Installation of a thioester at the *C*-terminus of linear peptide **90** permitted sequential NCL and CLipPA, yielding modified iturin mimetic **92** (**Scheme 1.13**). CLipPA methodologies have permitted the synthesis of a range of antibacterial lipopeptides, such as **92,** exhibiting minimum inhibitory concentrations (MICs) in the micromolar range.[192] This methodology clearly demonstrates the power, applicability and compatibility of thiol-ene chemistry to the synthesis of valuable peptide substrates.

1.4.2 Acyl Thiol-ene Reaction

Thioacids retain the chemical characteristics of their carboxylic acid counterparts but often possess greater reactivity by virtue of their increased acidity and nucleophilicity.[194] This fact has cultivated a fruitful field of research within organic chemistry and in particular in the pursuit of novel amide forging reactions. Thioacids yield amides through reactive intermediates accessed *via* azides,[195] isonitriles,[196] nitroarenes,[197] alkylnitrites,[198] isocyanates,[199] thioisocyanates,[199] aziridines,[200] silylating reagents[201] and aryl sulphonamides,[202] as well as conventional coupling reagents (**Figure 1.13**).[203]

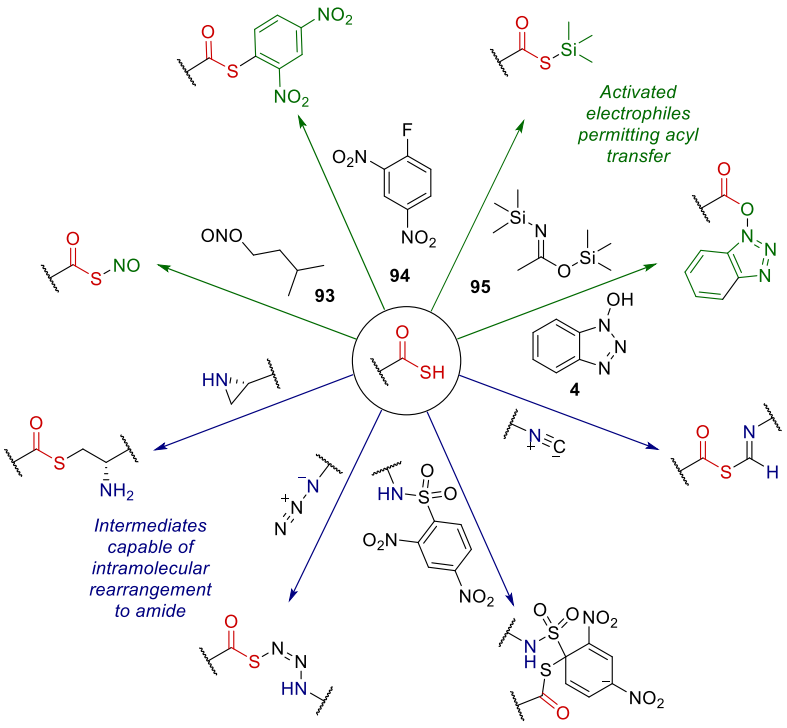
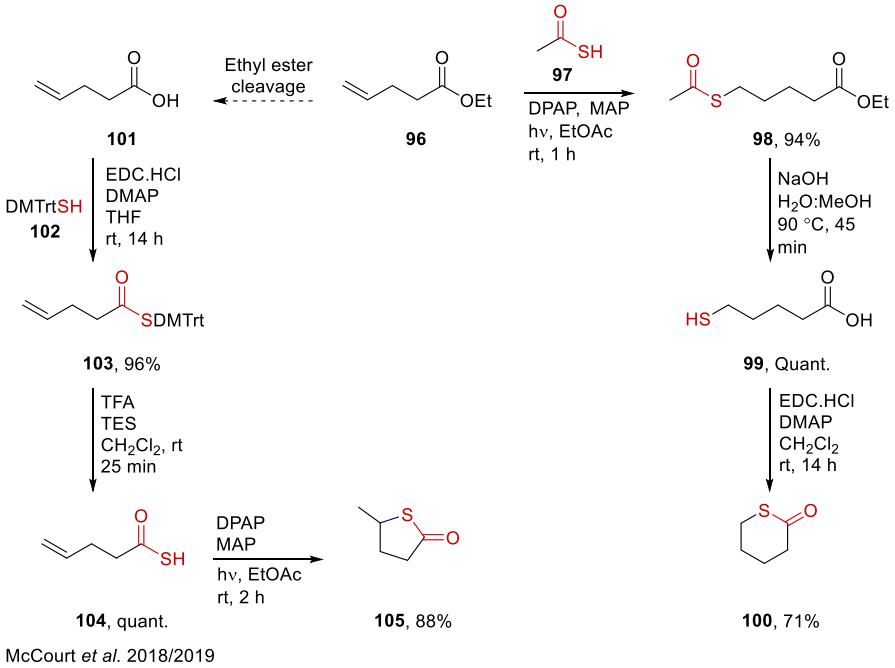


Figure 1.13: Reactive intermediates derived from thioacids permitting amide formation under mild conditons.[195-203]

Within these reaction types the thioacid component typically acts as a nucleophile to furnish either activated electrophilic species amenable to acyl transfer, or, in case of amine surrogates such as aziridines, isocyanates or isonitriles, reactive intermediates capable of intramolecular rearrangement to the target amide.[194] Thioacids also participate in acyl thiol-ene (ATE) reactions, the acyl counterpart to the thiol-ene reaction. ATE retains the covetable characteristics of the thiol-ene reaction already described (**Section 1.4**) and is believed to proceed *via* the same reaction mechanism (**Section 1.4.1**, **Figure 1.11**). Thioacids possess BDEs in the same range as alkyl thiols (87 kcal mol^{-1}) and radical formation can therefore be initiated under the same conditions.[162] However, the increased acidity of thioacids encourages deprotonation and subsequent Michael-type addition to suitable conjugated substrates by a polar mechanism, if desirable.[204] Of particular popularity in the literature is the use of radical mediated ATE to introduce thioacetate groups as thiol surrogates. Cleavage through either reductive, basic or acidic conditions furnishes the corresponding thiol in quantitative yields, enabling further synthetic elaboration.[205-207] This approach is widely utilised for surface functionalisation of gold nanoparticles (AuNPs) with thiol modified components, as sulfurs inherent 'softness' renders it an ideal ligand capable of AuNP binding.[208] ATE mediated *S*-acetylation approaches have been used to functionalise AuNPs with diverse species including carbohydrates,[209] peptides[210] and calixarene derivatives.[211] The Scanlan group have recently demonstrated the utility of this method of thiol installation as the principal reaction in a methodology permitting access to δ-thiolactones (**Scheme 1.13**).[212]



Scheme 1.14: Divergent synthetic routes to γ- and δ-thiolactones utilising the acyl thiol-ene reaction.[212, 213]

Initial reaction between thioacetic acid **97** and γ-unsaturated ethyl ester **96** in the presence of photoinitiator DPAP and photosensitiser 2'-methoxyacetophenone (MAP) furnished *S*-acetyl thioester **98** in a 94% yield after chromatographic purification. *S*-deacetylation and removal of the ethyl ester moiety of **98** in one step through treatment with sodium hydroxide yielded the corresponding free thiol compound **99** quantitatively. Steglich cyclisation of **99** under standard activation conditions furnished δ-thiolactone **100** in a 71% yield. Variation of alkyl substituents permitted access to a range of novel δ-thiolactones in moderate to excellent yields.[212] γ-Unsaturated ethyl esters permitted, to the best of our knowledge, the first demonstration of ATE utilising thioacid substrates beyond thiobenzoic and thioacetic acid (**Scheme 1.14**).[213] Steglich coupling of 4,4-dimethoxytritylthiol PG **102** provided unsaturated thioester **103**. Treatment with TFA:CH_2Cl_2 with triethylsilane (TES) cation scavenger furnished thioacid **104** *in situ* permitting intramolecular radical mediated cyclisation under ATE conditions to provide γ-thiolactone **105** in an 88% yield.

1.5 Synthesis of Peptide Thioacids

The reactivity of thioacids is ideally suited to peptide chemistry specifically due to the moiety's combination of powerful nucleophilicity and inherent 'softness'.[194] This imbues thioacids with enviable chemoselectivity in amide bond forming reactions, an issue of particular concern in the ligation reactions of unprotected peptidic fragments. However, the increased reactivities of thioacids come at the cost of their durability, as they possess dubious stability when in solution and readily react with oxygen to furnish the parent carboxylic acid.[194] Khaybullin *et al.* monitored decomposition of a thioacid derivative of Arg to the parent carboxylic acid in d_6-dimethylsulfoxide (DMSO) by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, finding a 1:1 ratio of the two compounds after 24 h.[214] Nevertheless, there exists a multitude of classical methods for the direct formation of thioacids in solution, typically through reaction of activated ester species with the sulfide anion in solution generated *via* dissolution of metallic salts or bubbling of H_2S gas through reaction media (**Figure 1.14**).[215] Carboxylic acid activation strategies demonstrated include *N*-hydroxysuccinimidyl[216] and *p*-nitrophenyl esters[217] and conventional amide coupling reagents such as EDC and carbonyldiimidazole (CDI).[218] Lawessons reagent under microwave irradiation has also been employed to furnish thioacids directly from their carboxylic acid counterparts (**Figure 1.14**).[219] However, as

a result of the thioacid groups vulnerability to oxidation, protected thioester precursors are preferred in synthetic applications. Several thioesters capable of generating thioacid species *in situ* upon deprotection have been developed, and employed specifically in the case of *N*-protected aminothioacids: 9-fluorenylmethyl (Fm),[220] *S*-Trt,[221] trimethoxybenzyl (Tmob)[195] and α-methylphenacyl (MPa)[222] thioesters (**Fig 1.14**). The Fm group developed by Sasaki and Crich permits the generation of thioacids under basic conditions, functioning analogously to the Fmoc α-amino PG and permitting orthogonality to acid cleavable groups.[220]

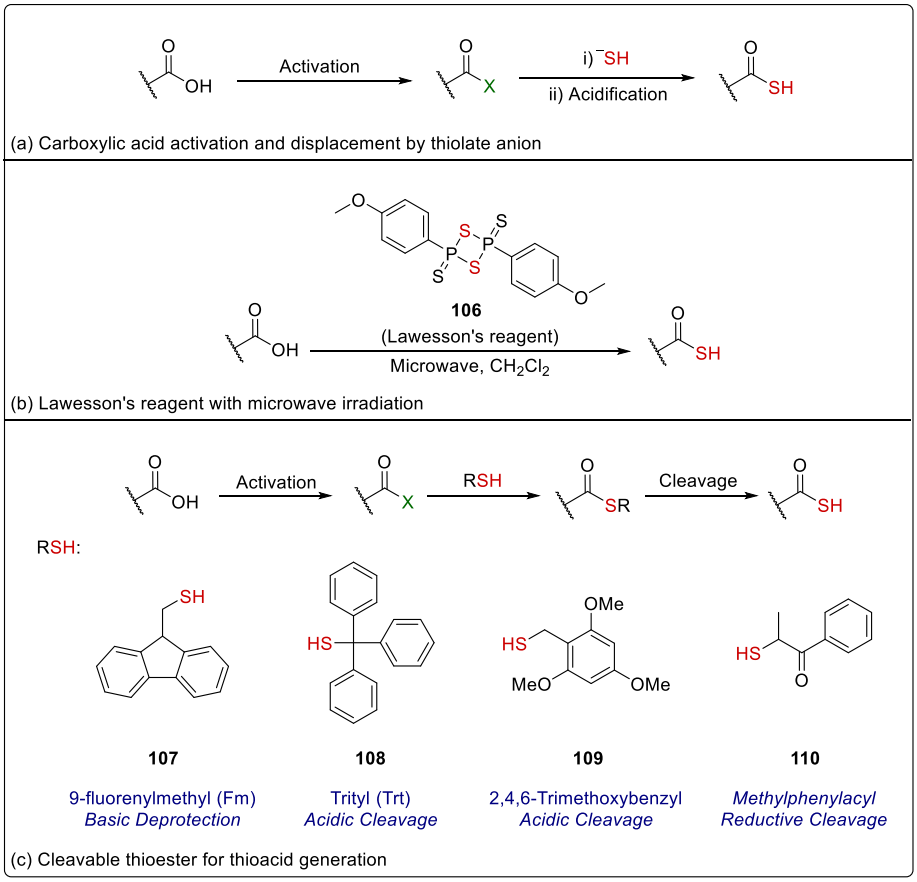


Figure 1.14: Strategies for thioacid synthesis in solution. (a) Carboxylic acid activation and thiolysis. (b) Direct thionation *via* Lawessons reagent under microwave irradiation. (c) Cleavable thioesters for *in situ* generation of thioacids.

S-Trt and Tmob are cleaved under acidic conditions and provide orthogonality to Fmoc PGs commonly required for SPPS. MPa thioesters are removed reductively through zinc and acetic acid treatment, and thereby grant orthogonality to both acid and base cleavable PGs.[222] These protected thioesters are readily prepared through reaction of a suitably activated parent carboxylic acid with a corresponding thiol (**Figure 1.14**). However, as in the preparation of peptide thioesters for NCL (**Section 1.3.1**), solubility issues can

preclude the use of these substrates to directly furnish protected thioesters in solution.[109] This has led to the development of a range of SPPS methodologies and related resins capable of furnishing peptide thioacids upon cleavage. The first resin capable of this, a phenoxyacetamidomethyl resin **(Figure 1.15)** was designed and employed by Kent to synthesise *C*-terminal peptide thioacids for use in peptide ligation *via* S_N2 type reaction with brominated *N*-terminal fragments (**Section 1.3**, **Scheme 1.3**).[98] However, resin cleavage necessitated HF treatment and, mirroring the progression of linker design for routine SPPS, linkers were sought that demanded more practical cleavage conditions. Schwaber and Maynard utilised Kaiser oxime resin capable of thioacid generation by application of hexamethyldisilathiane in the presence of tetrabutylammonium fluoride (TBAF) (**Figure 1.15**).[223] However, due to the vulnerability of the Kaiser oxime resin towards hydrolysis, or cleavage by nucleophilic AA residues, the resin has thus far proven suitable for the synthesis of small lipophilic cyclic peptides.[224]

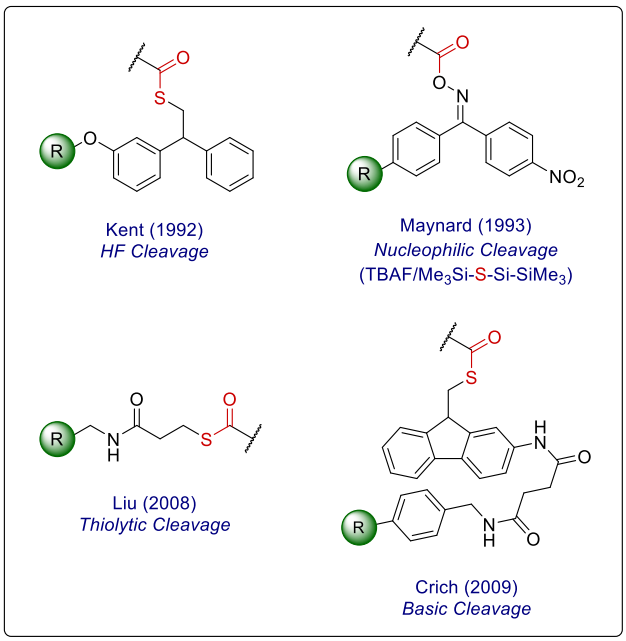


Fig 1.15: Resin used for solid phase peptide synthesis of peptide thioacids and associated cleavage condtions.[98, 223, 225, 227]

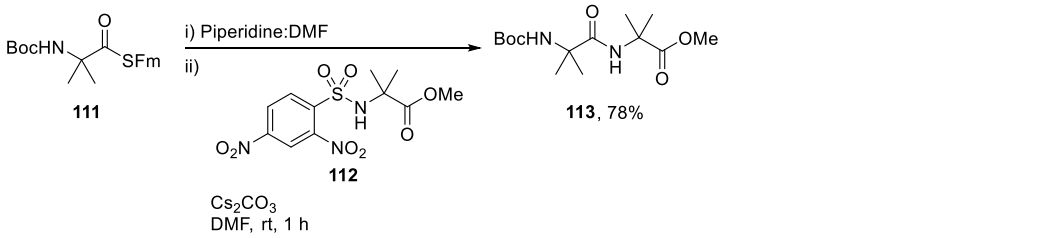
Liu pioneered the use of hydrothiolytic cleavage as a means to access peptide thioacids, providing a relatively simple approach to thioacid SPPS.[225, 226] Installation of a *S*-Trt protected mercaptopropamide linker onto an amine resin, followed by subsequent Trt deprotection and thioester coupling under standard Steglich conditions furnishes the thioester moiety. Boc SPPS is carried out and the resulting thioester bound peptide is cleaved from the resin by treatment with ammonium sulfide buffer.[226] The applicability of this methodology had been limited by the swelling characteristics of the resins accessible. PS resins are specifically designed to swell in the organic media used for

coupling and deprotection and therefore proved ill-suited to hydrothiolysis. Developments in resin synthesis, such as ChemMatrix®, a PEG based aminomethylresin, have increased the reliability and attractiveness of hydrothiolysis chemistry to furnish peptide thioacids.[226] The development by Crich of the Fm resin, based upon the Fm thioester PG, extended the applicability of acid-stable Fm thioesters from solution phase synthesis to SPPS (**Fig 1.15**). Resin bound Fm thioesters are cleavable *via* standard Fmoc removal conditions.[227] Coupled with an Fmoc side-chain PG strategy global deprotection strategy, Fm resins permit side-chain deprotection and resin cleavage in one step under mild conditions, a distinct advantage when compared with the harsh acidic conditions that have become a precondition of synthetic methodologies to access peptide thioacids *via* SPPS.

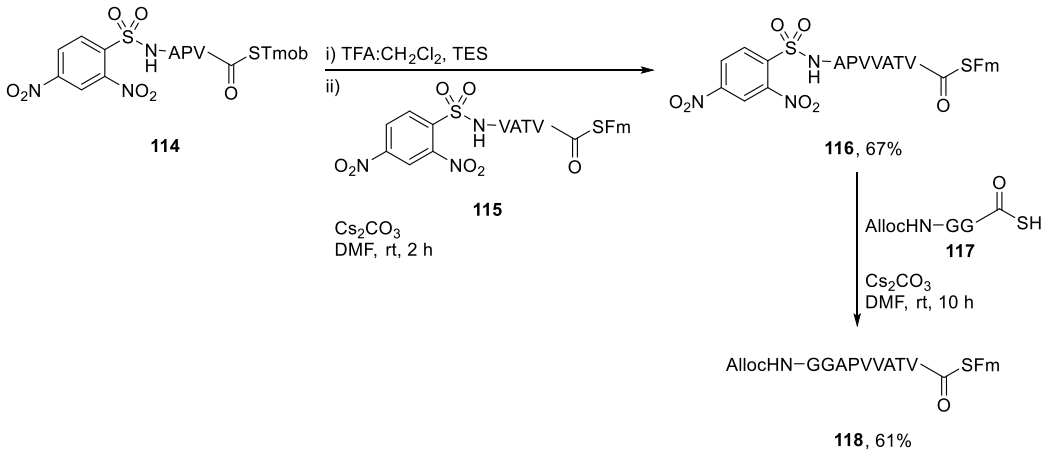
1.5.1 Application of Thioacids in Peptide Ligation

Peptide thioacids have proven amenable to peptide ligation methodologies utilising their considerable reactivity.[194] Indeed, the use of thioacids in peptide ligation predates NCL and they were utilised as key reaction components by Kent in the work preceding its publication (**Section 1.3**, **Scheme 1.3**).[98] The enhanced nucleophilicity of sulfur can increase reaction rates and permit less forceful reaction conditions. This was aptly demonstrated by Crich and Sharma through comparison of the reactivity of AAs and amino thioacids towards Mukaiyama's reagent, in which a decreased rate of *C*-terminal epimerisation was observed in products derived from the latter.[203] The Crich laboratory has been influential in developing Fm, Tmob and *S*-Trt thioesters capable of generating thioacids *in situ* (**Section 1.5**). Fm deprotection in combination with *N*-terminal dinitrobenzenesulfonamide activation permitted accesses to a range of hindered dipeptides such as **113** in excellent yields (**Scheme 1.15**).[202] The orthogonality of Fm and Tmob thioesters in combination with nitrobenzenesulfonamide coupling permitted the development of a novel a N-to-C peptide ligation approach (**Scheme 1.15**).[221] Deprotection of Tmob protected dinitrobenzenesulfonamide tripeptide **114** followed by addition of Fm protected dinitrobenzenesulfonamide tetrapeptide **115** permitted selective amide coupling to give heptapeptide **116** in a 67% yield. Addition of dipeptide thioacid **117** yielded nonapeptide **118** through reaction with the remaining dinitrobenzenesulfonamide moiety. This ligation methodology was the first demonstration of peptide synthesis utilising orthogonal thioacid generating PGs.

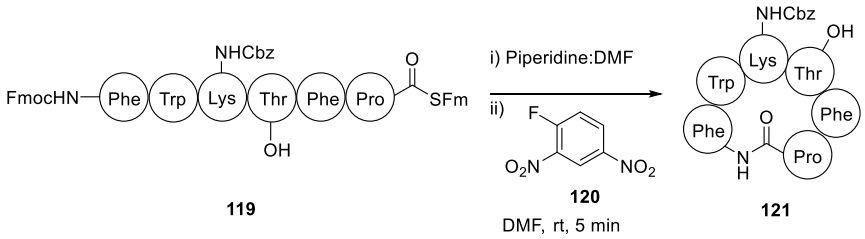
Thioacids generated *in situ* also permitted amide bond formation in high yield through activation with Sanger's reagent (**120)** even in the case of sterically hindered AA substrates.[203] This methodology was subsequently applied to the cyclisation of Fm protected endothelin hexapeptide **121** accessed *via* Boc SPPS (**Scheme 1.15**).[220]



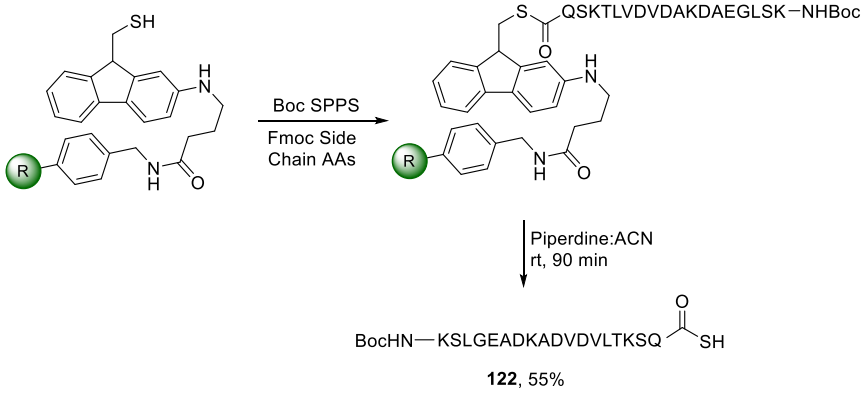
(a) Crich *et al.* (2007)



(b) Crich *et al.* (2009)



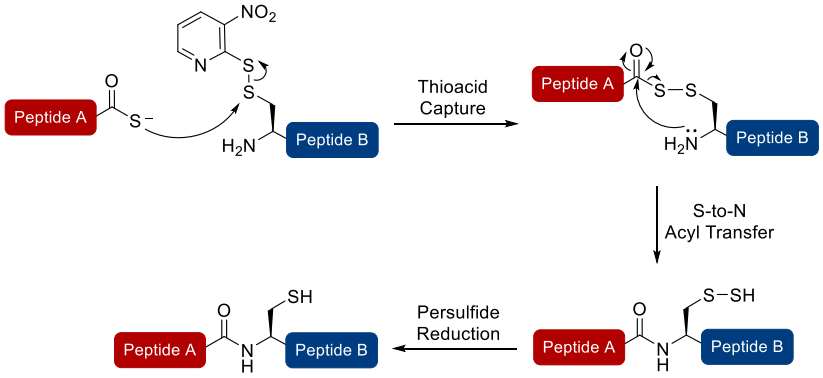
(c) Sasaki *et al.* (2010)



(d) Crich *et al.* (2009)

Scheme 1.15: Thioacid peptide ligation strategies developed by the Crich laboratory. (a) Synthesis of sterically hindered dipeptide **113** by reaction of amino acid thioacid **111** with nitrobenzenesulfonamide **112**.[202] (b) Synthesis of nonapeptide **118** through an orthogonal thioester protection strategy.[221] (c) Peptide cyclisation utilising Sangers reagent to furnish cyclic hexapeptide **122**.[220] (d) Thioacid synthesis *via* SPPS with Fm resin.[227]

Crich's Fm resin (**Section 1.5**, **Figure 1.15**) permitted the synthesis of unprotected 20 residue peptide thioacid **122** from the corresponding resin bound precursor, demonstrating that even sizeable peptide thioacids can be furnished reliably (**Scheme 1.15**).[227] The hydrothiolysis thioacid SPPS strategy of Liu also demonstrates that large peptide thioacids are synthetically attainable (**Scheme 1.15**), proving robust enough to access a 40 residue thioacid subsequently used in thioacid capture ligation (**Scheme 1.16**).[228] Peptide fragments bearing *N*-terminal Cys residues protected with 3-nitro-2-pyridinesulfenyl (Npys) are capable of trapping peptide thioacids through a process analogous to trans-thioesterification, forming a reactive acyl disulfide intermediate. Intramolecular acyl transfer followed by reduction furnishes Cys at the ligation site. This methodology has been used to furnish H3 histone proteins with remarkably short ligation times, typically < 1 h, and has been used synergistically with NCL to yield 96 residue protein monellin.[226, 228] Danishefsky *et al.* also harnessed the unique reactivity and increased solubility of peptide thioacids to directly couple unprotected peptide fragments using HOBt with supressed racemisation.[229]



Scheme 1.16: Thioacid capture ligation strategy developed by Liu.[228]

1.6 Project Aims and Work in this Thesis

The work presented in this thesis outlines efforts to develop novel ATE mediated peptide ligation methodologies. As chapter 1 details, the field of chemical protein synthesis was revolutionised upon the advent of NCL (**Section 1.3**). However, the development of novel ligation methodologies has been necessary to extend the reach of NCL beyond Cysteinyl peptides, including ligation/desulfurisation and AML (**Section 1.3.4** and **Section 1.3.5**). A fundamental limitation of these methodologies remains the need for a trans-thioesterification step to access critical thioester intermediates capable of spontaneous S-to-N acyl transfer to furnish amide products. As a fundamentally

reversible process, trans-thioesterification can be slow at challenging junctions necessitating the addition of large excesses of additives to improve reaction rates (**Section 1.3.3**). Thiol-ene chemistry has been utilised in the synthesis of biomolecules for decades and has been widely applied to the modification of thiolated peptide substrates (**Section 1.4.2**). However, the ATE reaction has not been utilised to furnish thioester intermediates suitable for peptide ligation, despite the synthetic accessibility of peptide thioacids being well described within the literature (**Section 1.5**). The aim of this work is to utilise the ATE reaction to furnish peptidyl thioesters capable of spontaneous S-to-N acyl transfer to yield valuable peptide products.

Chapter 2 details efforts to develop ATE mediated ligation *via* unsaturated auxiliary bearing substrates. As outlined in **Section 1.3.5**, auxiliaries have been used to extend the scope of classical NCL beyond Cys sites. Affixing of an unsaturated auxiliary to the *N*-terminal of a peptide fragment would permit thioester formation with a peptide thioacid. Subsequent S-to-N acyl transfer and auxiliary cleavage would furnish the desired native peptide. In principal, the auxiliary may be affixed to number of different peptidic sites, on the condition that cleavage may be carried out under conditions sympathetic to the substrate. Both side-chain residue and *N*-terminal ATE mediated AML approaches were investigated in this chapter.

Chapter 3 describes the development of ATE mediated ligation methodologies utilising β,γ-unsaturated UAAs as key reactive moieties. Unsaturated UAAs have good reactivity towards thiol-ene addition, as observed by Dondoni (**Section 1.4.2**). The work of the Brimble group has demonstrated the effective coaction of thiol-ene and protein synthesis chemistries to furnish valuable peptide products. The use of β,γ-unsaturated UAAs for ATE mediated peptide ligation would ensure that the thioester intermediate formed would possess a 6 membered S-to-N acyl transfer TS, thereby favouring subsequent amide formation (**Section 1.3.2**). In this chapter the synthesis and subsequent reactivity of unsaturated UAA bearing systems towards ATE and S-to-N acyl transfer is evaluated, culminating in the synthesis of two native tripeptide products.

Chapter 4 outlines efforts to apply ATE mediated peptide ligation to larger peptide examples. The principal drive in the development of novel ligation methodologies is to permit the expansion of NCL beyond Cys residues (**Section 1.3.3**). Many therapeutically relevant proteins do not possess a Cys residue within their primary protein structure,

thereby forgoing the possibility of classical NCL in synthetic approaches to these targets. One such protein is hPTH, administered for a variety of ailments resulting from poor calcium homeostasis. In this chapter two fragments of hPTH were selected as suitable targets for ATE mediated peptide ligation, possessing the requisite Val and Met residues at convenient junctions within the primary sequence. The synthesis of two unsaturated peptides and their corresponding thioacid counterparts is described *via* the application and optimisation of literature conditions.

Chapter 5 concludes the work in this thesis and briefly outlines potential future work.

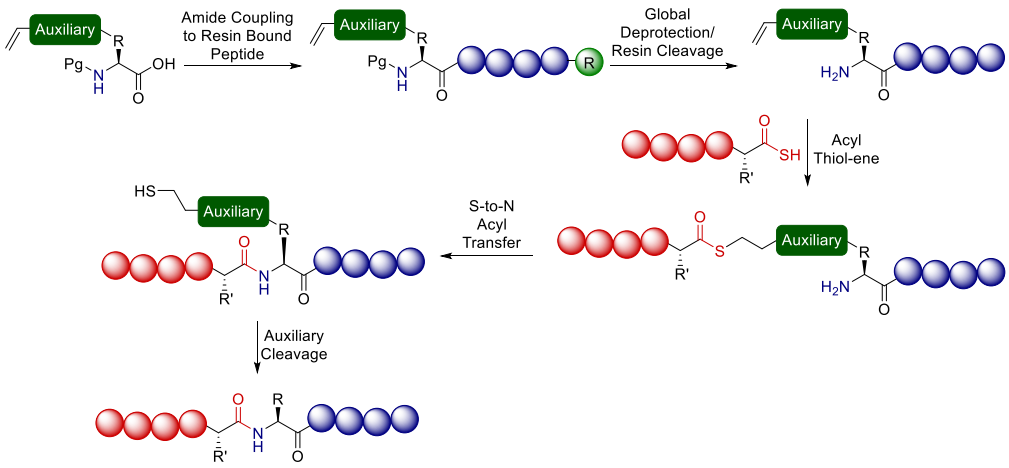
Chapter 6 describes experimental details and the characterisation data of compounds synthesised in the course of this work.

Chapter 2

Acyl Thiol-ene Mediated Ligation of Auxiliary Bearing Substrates

2.0 Introduction

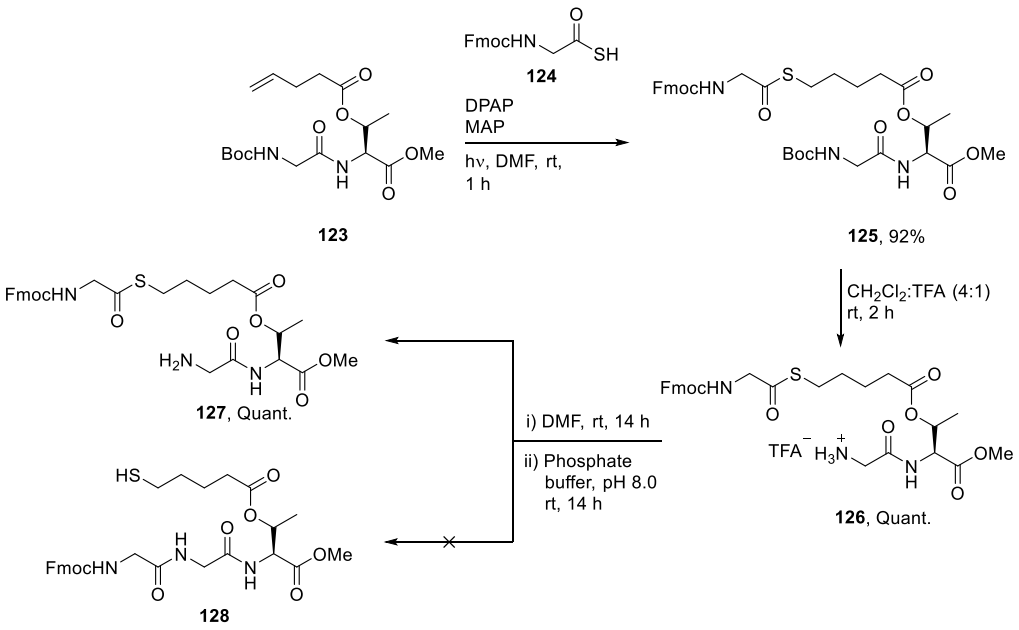
As previously discussed in Chapter 1 (**Section 1.3.5**) auxiliary mediated peptide ligation over small (5 – 8 membered) and large (9 – 14 membered) S-to-N acyl transfer TSs has been demonstrated empirically and theoretically in a number of studies, and has been observed to reliably provide access to valuable protein targets.[77, 122, 155, 157-159, 230] However, ligation auxiliaries described within the literature exclusively rely on trans-thioesterification to furnish the key reactive thioester intermediates involved in spontaneous amide bond formation.[77] Trans-thioesterification is a reversible process with efficient reaction achieved through the use of thiol additives. The process is hampered by often long reaction times at challenging junctures, resulting in reports of native chemical ligation reactions taking place over several days or weeks.[231] The radical mediated thiol-ene reaction has been deftly applied in the field of chemical protein synthesis, for cyclisation and modification of peptide substrates (**Section 1.4.2**). The ATE reaction is used throughout the literature to access thioesters for further chemical elaboration and has become an established method to reliably furnish biologically relevant thiolactone products (**Section 1.4.2**). As yet, however, the radical mediated ATE reaction has not been employed to furnish reactive thioester intermediates suitable for S-to-N acyl transfer in peptide ligation, despite the well-established usage of thioacid substrates within the field of chemical protein synthesis (**Section 1.5.1**) It was therefore envisaged that the use of a suitable unsaturated auxiliary affixed to the *N*-terminal AA of a peptide fragment would permit efficient ATE mediated thioester formation with a suitable peptidic thioacid. Spontaneous S-to-N acyl transfer, followed by auxiliary cleavage would yield the desired ligated product (**Scheme 2.1**).



Scheme 2.1: Peptide ligation methodology employing ATE mediated thioester formation followed by S-to-N acyl transfer and auxiliary elimination to yield the native ligated product.

Several factors are key when considering the synthetic design of ligation auxiliaries. Ideally, ligation auxiliaries demonstrate generality and may be utilised at as wide a variety of ligation sites as possible.[147] To enable efficient ATE to furnish the desired thioester products, the auxiliary must bear an unsaturated moiety within its structure which possesses the desired reactivity toward radical addition. The S-to-N acyl transfer characteristics of the auxiliary, particularly with regards to TS ring size, must also be considered along with the ease of synthesis and stability towards SPPS conditions. Work within this chapter details efforts to access a functional auxiliary system capable of permitting rapid thioester formation *via* ATE followed by spontaneous S-to-N acyl transfer to yield ligated products.

2.1 Previous Work within the Scanlan Group on Peptide Ligation



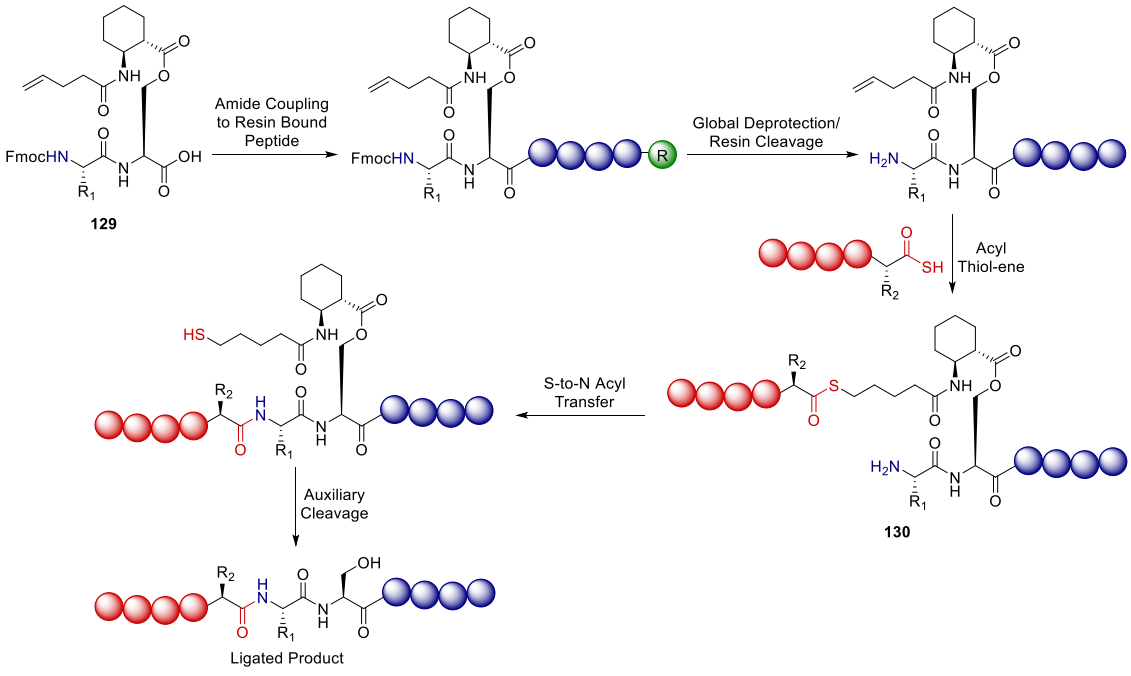
Scheme 2.2: Previous work carried out in the Scanlan group investigating the suitability of ATE substrate **123** Deprotection and freebasing of the resulting thioester **125** did not yield the desired ligation product **128**.

Previous work within the Scanlan group (**Scheme 2.2**) culminated in the synthesis of Thr modified dipeptide **123** as a potential ATE substrate for peptide ligation. In order to render the ^{1}H nuclear magnetic resonance (NMR) spectroscopic characterisation of any shifted products as simple as possible, a Thr-Gly dipeptide was employed and esterified with 4-pentenoic acid. Use of a dipeptide substrate also eliminated any possibility of competing O-to-N acyl transfer reactions.[232] Installation of the thioester moiety by radical mediated ATE with Fmoc-Gly thioacid **124** proceeded smoothly to give thioester **125** in a 92% yield. Subsequent Boc deprotection yielded trifluoroacetate salt **126**

quantitatively. Unfortunately, stirring of **126** over 14 h, in both DMF and phosphate buffer (pH 8.0) yielded only free amine starting material (SM) **127**, indicating no intra- or intermolecular S-to-N acyl transfer had occurred to give desired ligation product **128**. This finding was rationalised based on work of Katritzky *et al.* and Brik outlining the feasibility of the S-to-N acyl transfer reaction over larger transition intermediates and emphasised the importance of structural pre-organisation.[155, 157-159, 233] In order to produce an auxiliary system capable of spontaneous S-to-N acyl transfer a new synthetic strategy was desired which necessitated the incorporation of a moiety capable of decreasing the N to S-C=O distance critical to S-to-N acyl transfer. The work of Brik *et al.*, building upon Wong's seminal SAL methodology, describes the synthesis of a cyclohexyl based ligation auxiliary, the rigidity of which permitted efficient S-to-N acyl transfer.[155, 159] Insertion of this auxiliary in addition to the alkenyl chain present in **123** formed the basis of our new synthetic approach.

2.2. Rationalisation of Auxiliary Design

It was anticipated that the cyclohexyl auxiliary described by Brik and coworkers could be inserted into the existing ATE mediated ligation strategy (**Scheme 2.3**) through the synthesis of carboxylic acid **129**, which could subsequently be installed into a resin linked peptide accessed *via* SPPS.

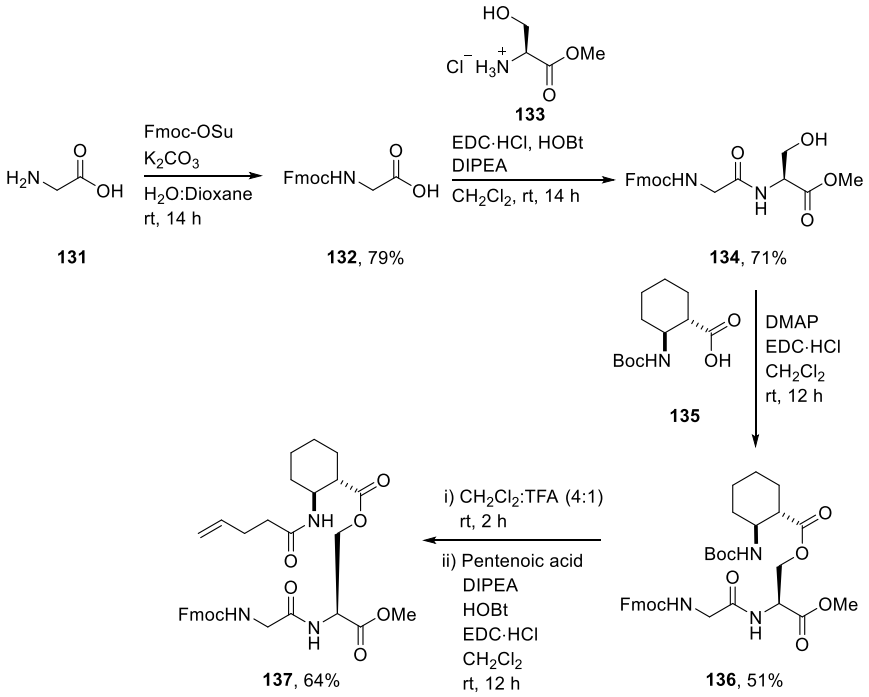


Scheme 2.3: Use of auxiliary modified dipeptide acid **129** in ATE mediated peptide ligation.

Global deprotection and resin cleavage would yield the modified *N*-terminal amino acid. Addition of a peptidyl thioacid, synthesised according to literature procedures outlined in Chapter 1 (**Section 1.5**), and UV irradiation under ATE conditions would yield substrate **130** capable of intramolecular S-to-N acyl transfer. Subsequent auxiliary cleavage under basic conditions (> pH 10) would furnish the native protein target featuring Ser adjacent to the ligation site. The designed auxiliary aimed to maximise generality, is capable of installation at Ser and Thr residues and, with inversion of the cyclohexyl ester group, Asp and Glu residues. The pentenoyl alkene moiety was selected as it had shown to have reactivity towards ATE when present in substrate **123**. Variation of alkene chain length would also provide a degree of optimisation if the auxiliary did not prove amenable to S-to-N acyl transfer. As with **123**, initial synthesis of an auxiliary bearing dipeptide was undertaken in order to access the key characteristics of the system; ATE reactivity and S-to-N acyl transfer feasibility and ease of synthesis. The synthesis of larger examples *via* SPPS could then be undertaken according to literature procedure.

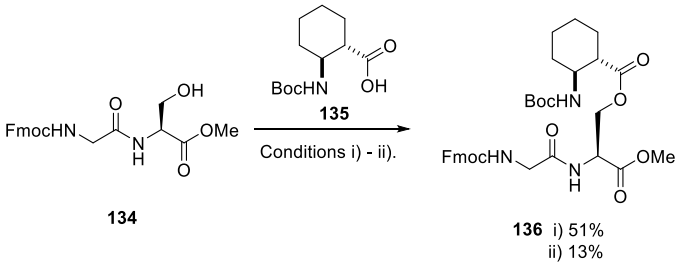
2.2.1 Synthesis of *trans*-2-(Boc-amino)-cyclohexanecarboxylic Acid Acceptor 137

A synthetic strategy was devised to incorporate the racemic *trans*-2-(Boc-amino)-cyclohexanecarboxylic acid precursor **135** into the pre-existing substrate **123** to furnish model dipeptide **137** (**Scheme 2.4**)



Scheme 2.4: The synthetic route to auxiliary modified dipeptide **137** incorporating the *trans*-2-(Boc-amino)-cyclohexanecarboxylic acid precursor.

The racemate was selected over the enantiopure SM due to cost (Sigma-Aldrich; racemate - € 75.00 g^{-1}, enantiopure - € 730.00 g^{-1}), with Brik and coworkers not reporting any significant impact on S-to-N acyl transfer characteristics.[155] An orthogonal amino PG strategy, allowing selective amide formation from **136** to **137**, was necessary. An Fmoc PG on the terminal amine was used in order to ensure compatibility with SPPS conditions. Addition of the Fmoc group *via* reaction of Fmoc-succinimide and Gly **131** provided Fmoc protected Gly **132** in high yield upon simple aq. work up. Following this, amide coupling of **132** with Ser methyl ester **133** utilising DIC, HOBt and DIPEA in anhydrous CH_2Cl_2 was undertaken. It was anticipated that a highly polar solvent, such as DMF, would be necessary to solubilise the carboxylic acid bearing SM **131**. This was not necessary, likely due to the non-polar Fmoc group aiding solvation, greatly simplifying subsequent separation. However, the *N,N'*-diisopropylurea by-product of the reaction proved difficult to remove by chromatographic means, possessing a similar retardation factor (R_f) to the desired dipeptide product **134** (0.33 and 0.29 respectively, CH_2Cl_2:methanol (MeOH) – 98:2). A change to *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC·HCl) assisted purification greatly owing to the water solubility of the urea by-product formed. This change did not have any appreciable effect on yield. Esterification of **134** with *trans*-2-(Boc-amino)-cyclohexanecarboxylic acid **135** was carried out under Steglich conditions with EDC·HCl and catalytic 4-dimethylaminopyridine (DMAP) in anhydrous CH_2Cl_2. Even at small scale (0.2 mmol of SM **134**) yields of product **136** proved stubbornly low (40% – 50%, **Scheme 2.5**).



Scheme 2.5.: Scheme of the esterification step to give intermediate **136** attempted under Steglich conditions i) DMAP, EDC·HCl, CH_2Cl_2, rt, 12 h and Mitsunobu conditions ii) PPh_3, DIAD, CH_2Cl_2, rt, 10 h.

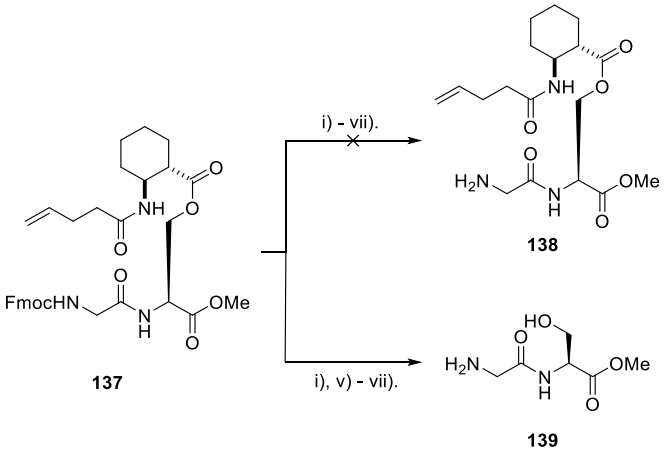
This was likely due to a combination of the Boc group adjacent to the esterification site providing significant steric hindrance, and unintended cleavage of the Fmoc PG by DMAP over the course of the reaction. This was evidenced by the presence of the Fmoc deprotection by-product DBF in the reaction mixture, isolated as a bright yellow fraction following chromatographic separation and identified by ^{1}H NMR spectroscopy.[234] Due

to the role of DMAP as a nucleophilic catalyst it was reasoned that increasing the eq. of the reagent would expedite the formation of **136**. The consumption of carboxylic acid **135** observed by thin layer chromatography (TLC) was indeed increased after doubling the eq. of DMAP used (0.1 to 0.2), however this did not outweigh the deleterious effect of higher concentrations of base on the stability of the Fmoc PG. Mitsunobu esterification conditions were explored (**Scheme 2.5**), but yields were comparatively low (13%) and chromatographic separation considerably more difficult due to the presence of triphenylphosphine oxide in the reaction mixture. Formation of an acyl chloride precursor was not attempted, primarily due to the perceived sensitivity of the Boc group to acidic conditions during acyl chloride generation and subsequent coupling. Sequential Boc deprotection with TFA solution (CH_2Cl_2:TFA – 4:1) and amide coupling with optimised EDC·HCl/HOBt/DIPEA conditions in anhydrous CH_2Cl_2 progressed smoothly to give the target auxiliary modified dipeptide **137** in 64% yield. It was decided that due to the likely instability of the thioester linkage under basic conditions,[62] deprotection of the Fmoc PG would be attempted prior to radical mediated thiol-ene addition and subsequent S-to-N acyl transfer.

2.2.2 Fmoc Deprotection of *trans*-2-(Boc-amino)-cyclohexanecarboxylic Acid Thiol-ene Acceptor (137)

A variety of reaction conditions were screened for the Fmoc deprotection of **137 (Scheme 2.6**). Standard Fmoc deprotection conditions in SPPS methodologies employ the piperidine:DMF couple.[42] Attempts to deprotect **137** under these conditions were successful by TLC analysis, with total consumption of SM observed, coupled with formation of a DBF by-product. However, isolation of the desired free amine **138** proved difficult. Product isolation through workup produced a considerable loss of product due to the propensity of DMF in solution to keep **138** in the aq. layer. Removal of DMF *in vacuo* prior to workup resulted in the cleavage of the serine ester moiety of **138** through nucleophilic attack of piperidine. It was postulated that this undesired cleavage occurred as a result of the concentration of piperidine *in vacuo,* likely assisted by the heating required to remove DMF (~ 50 °C). This was surprising considering the relative boiling points of DMF and piperidine (152 °C and 106 °C, respectively). Nevertheless, isolation by column chromatography and subsequent ^{1}H NMR spectroscopy confirmed the presence of the serine ester cleavage product **139**. Use of a piperidine:CH_2Cl_2 couple,

which has seen limited use in the literature,[235] resulted in the formation of a crystalline piperidine chloride salt upon addition and severely limited the efficiency of the reaction. Beyond the piperidine couples utilised for SPPS, little literature precedent exists for Fmoc removal in solution phase likely due to its lability. In the synthesis of dipeptide **137**, even under mild basic conditions, undesired Fmoc deprotection was unavoidable. In spite of this, various methods have been established in the literature[236] and were attempted for the Fmoc deprotection of **137**. Treatment overnight with resin bound piperidine and solubilisation in DMF followed by heating, respectively, yielded only unreacted SM **137**. Treatment of **137** with various bases exploited in the literature including diethylamine,[237] 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU)[238] and Cs_2CO_3 resulted in direct ester cleavage alongside Fmoc deprotection, the presence of cleavage product **139** confirmed by TLC and ^{1}H NMR spectroscopy of the crude mixture.



Scheme 2.6: Scheme of the attempted Fmoc deprotection of **137** under conditions i) DMF:Piperidine, rt, 1 h; ii) CH_2Cl_2:Piperidine, rt, 1 h; iii) Polymer resin bound piperidine, rt, 14 h; iv). DMF, 80 °C, 1 h; v) Diethylamine, CH_2Cl_2, rt, 30 min; vi) DBU, CH_2Cl_2, rt, 30 min; vii) Cs_2CO_3, DMF, rt, 1h.

2.3 Simplification of Synthetic Approach

Following consideration of the synthesis and subsequent difficulties in the Fmoc deprotection of **137** a reassessment of the auxiliary structure was undertaken. Despite the sound synthetic rational behind the formulation of **137** it was determined that the synthesis itself, in particular the crucial deprotection step, would undermine the utility of such an auxiliary, particular when used in conjunction with valuable peptide fragments. More simplistic synthetic targets were called for that would ideally eliminate the reliance on orthogonal PG strategy and could be accessed from readily available SMs. The system would be realistically capable of S-to-N acyl transfer. Acryloylated dipeptide **140** satisfied a number of criteria; a seemingly facile synthesis unreliant on orthogonal PG strategy, an alkene moiety reactive towards thiol-ene chemistry and a feasible S-to-N

acyl transfer TS (**Figure 2.1**).[233] Cleavage post ligation would be accomplished under basic conditions, as for the cyclohexyl acceptor of Brik. The use of an acryloyl group also served to broaden the scope of the ligation methodology to encompass ionic Michael addition type reactions. Radical mediated acyl thiol-ene reactions of peptidic thioacids with acryloylated substrates such as **140** have not been investigated in the literature. A Boc PG strategy was utilised, primarily to avoid the use of the Fmoc PG, the removal of which had proven troublesome in the unsuccessful synthesis of free amine **138**. This additionally allowed for deprotection under acidic conditions to which the thioester moiety is stable, enabling optimisation of ATE with fully protected substrates, aiding product isolation and purification.

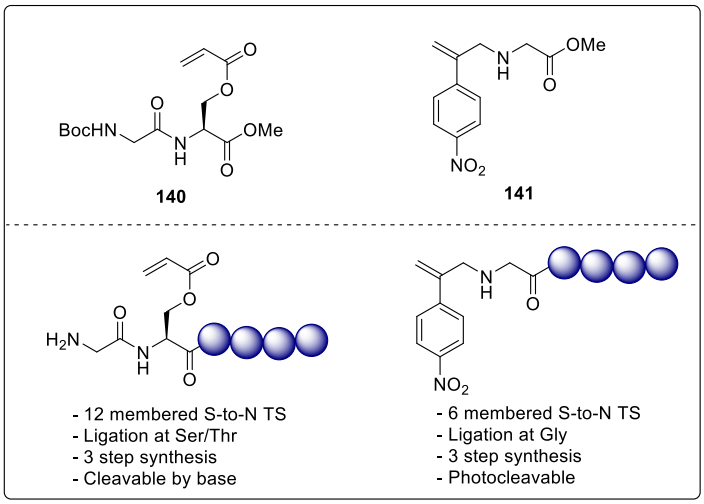


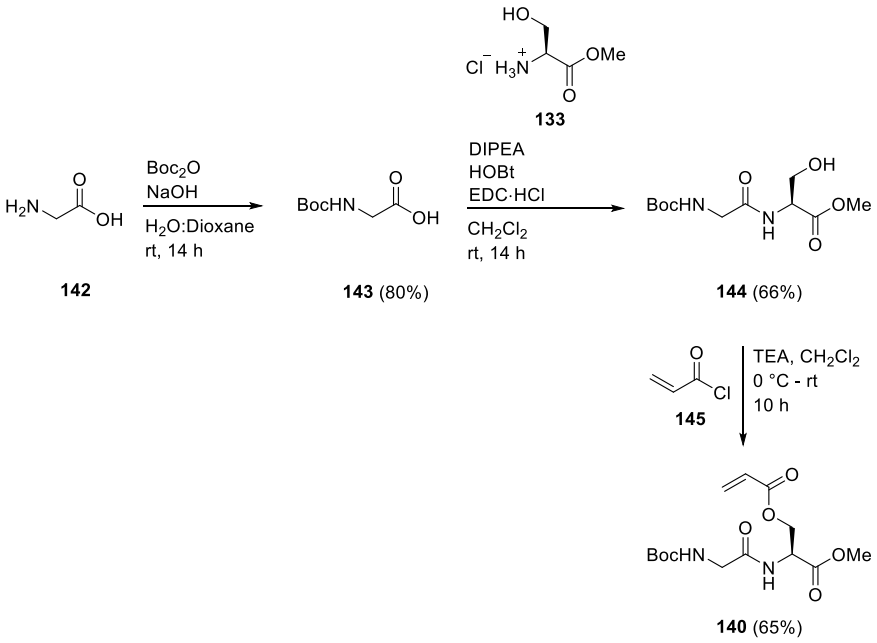
Figure 2.1: Auxiliary bearing targets 1**40** and **141** and their respective characteristics.

Auxiliary **141** (**Figure 2.1**) was fortuitously identified as a synthetic intermediate to a pre-existing ligation auxiliary developed by Seitz.[154] Peptide ligation utilizing trans-thioesterification chemistry to access the reactive thioester intermediate capable of S-to-N acyl transfer had already been demonstrated by Seitz. The reactivity of benzylic terminal alkenes such as **141** under ATE conditions with thioacetic acid had also been demonstrated.[212] Seitz performed cleavage of the auxiliary after ligation under UV irradiation, although addition of TCEP as a reduction/oxidation (REDOX) couple proved critical in permitting lysis of the C-N bond linking the auxiliary at the ligation site.[154] Contingent on the success of the ATE reaction, and the auxiliary possessing the necessary stability towards ATE conditions, the potential for a one-pot ligation/cleavage methodology under photoactive conditions could be anticipated. Synthesis was undertaken to access targets **140** and **141** bearing the respective auxiliaries to evaluate

the systems reactivity towards ATE and respective S-to-N acyl transfer characteristics, prior to the construction of large auxiliary bearing peptides *via* SPPS.

2.3.1 Synthesis of Acryloylated Acyl Thiol-ene Acceptor 140

ATE acceptor **140** was accessed through a straightforward three-step synthesis (**Scheme 2.7**). Boc protection of glycine using di-*tert*-butyl carbonate (Boc_2O) gave Boc-Gly **142** in a satisfactory yield (80%). Following this amide coupling between **143** and serine methyl ester **133**, utilising amide coupling conditions (EDC·HCl/HOBt/DIPEA) previously optimised in the synthesis of **134**, gave dipeptide **144** in a 66% yield. Extraction required the use of a more polar organic solvent (EtOAc) than the comparative Fmoc protected dipeptide **134** due to absence of the fluorenyl group decreasing solubility in organic solvent, clarifying the slight disparity in yield between dipeptides **134** and **144**.

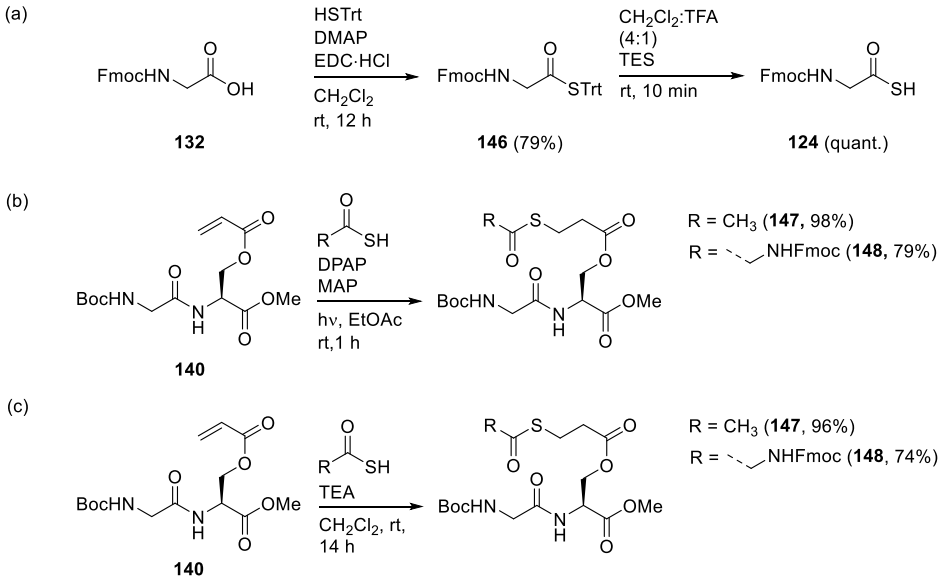


Scheme 2.7: Three step synthesis of acryloylated ATE acceptor dipeptide **140**.

Acryloylation of Ser dipeptide **144**, carried out with acryloyl chloride **145** and triethylamine (TEA) in anhydrous CH_2Cl_2, proceeded smoothly to give the target thiol-ene acceptor **140** in 65% yield. An excess of TEA was used in order to ensure immediate neutralisation of the HCl by-product, with no possibility of concomitant loss of Fmoc PG as in the synthesis of **137**, emphasising the advantage of employing the Boc PG strategy in solution phase.

2.3.2 Acyl Thiol-ene Reactions of Acryloylated Acceptor (140)

ATE addition to acceptor **140** was investigated under both radical mediated and ionic conditions. Radical mediated reactions of **140** were undertaken with thioacetic acid **97** and Fmoc-Gly thioacid **124** (**Scheme 2.8**).



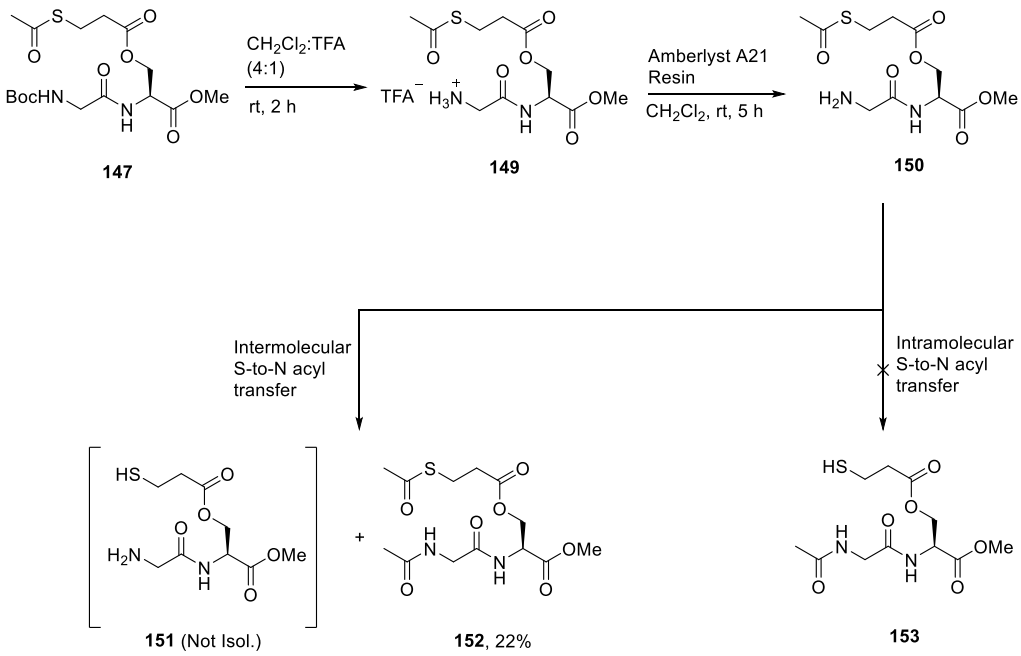
Scheme 2.8. (a) Synthesis of trityl protected glycine thioacid **146** by direct thioesterfication and subsequent trityl deprotection prior to ATE reactions. (b) Scheme of radical mediated ATE reactions to furnish thioesters **147** and **148**. (c) Scheme of ionic Michael addition reactions to furnish thioesters **147** and **148.**

The synthesis of Trt protected thioacid substrates by direct thioesterification has been previously optimised within the Scanlan group and was inspired by the work of Crich on cleavable thioester PGs.[203, 221] This strategy permits deprotection of *S*-Trt precursors *in situ* by treatment with TFA. The corresponding thioacid is generated quantitatively and, after concentration *in vacuo* to remove solvent, used without further purification, avoiding any substantial thioacid hydrolysis/oxidation. Deprotection of Trt thioester **146** was carried out by treatment with CH_2Cl_2:TFA (4:1) and TES as a cation scavenger to avoid alkylative side-reactions. Radical mediated ATE reactions were carried out utilising the radical photoinitiator DPAP in the presence of the photosensitiser MAP solubilised in EtOAc and irradiated for 1 h (**Scheme 2.8**). 3 Eq. of Gly thioacid were required in order to ensure consumption of the alkene, due to elimination of carbonyl sulfide (CSO), established in earlier work prior to the commencement of this thesis. The reaction progressed smoothly in 1 h under these conditions, quantitatively in the case of **147**, and provided a respectable 79% yield of **148**. The ionic pathway provided thioacetate **147** quantitatively after 12 h, and the yield of **148** was also comparable to that

achieved through the radical-mediated pathway (**Scheme 2.8**). Comparison of reaction conversion of acceptor **140** with thioacetic acid after 1 h through both radical mediated and ionic pathways was undertaken, as determined by ^{1}H NMR spectroscopy. Total conversion was observed under radical conditions, while a 3:1 conversion ratio of SM **140** to product **147** was seen under ionic conditions, underscoring the slower kinetics of this reaction type. Repetition of the reaction of **147** with thioacetic acid **97** under ionic conditions was repeated in EtOAc, to ensure unanticipated solvent effects were not culpable for the disparity in yields between radical mediated and ionic reactions. No appreciable difference in conversion of **140** to **147** in EtOAc under ionic reaction conditions was observed by ^{1}H NMR spectroscopy. The high yielding nature of these reactions outlines the potential of thiol-ene chemistries to furnish thioesters efficiently by either radical or ionic means.

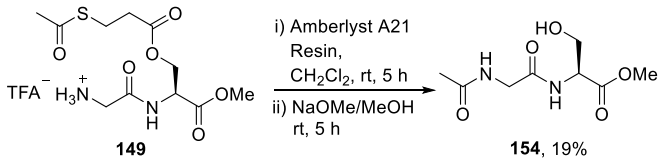
2.3.3 S-to-N Acyl Transfer Studies of Acyl Thiol-ene Products 147 and 148

With thioester products **147** and **148** in hand the S-to-N acyl transfer characteristics of each were investigated. Thioacetate **147** was examined first (**Scheme 2.9**) primarily due to the anticipated simplicity of ^{1}H NMR spectroscopic interpretation and associated reaction spectra, as the chemical shifts of SM acyl thioester **147** and acyl amide **153**, resulting from S-to-N acyl transfer, would likely be straightforward to discern. Deprotection of the Boc PG of the Gly terminal amine in CH_2Cl_2:TFA (20%) proceeded quantitively by TLC analysis. ^{19}F NMR spectroscopy confirmed the presence of **149** as a TFA salt, preventing S-to-N acyl transfer. Free amine **150** was obtained by treatment with Amberlyst® A21 ion-exchange resin, upon which TLC analysis indicated the formation of a complex mixture. Consultation of the literature suggested the mixture may have resulted from competing inter- and intramolecular reactions, as discussed briefly in the work of Katritzky.[233] ^{1}H NMR spectroscopy of the crude mixture showed the presence of two distinct acyl methyl singlets at 2.36 parts per million (ppm) and 2.07 ppm, in a ratio of 1:2 indicating that some S-to-N acyl transfer was occurring. Purification by silica gel flash chromatography was undertaken (CH_2Cl_2:MeOH – 95:5) and yielded pure fractions of bis-acylated intramolecular product **152** in a 22% yield and starting material **150**. However, fractions of free amine **150** proved unstable in solution with TLC analysis and ^{1}H NMR spectroscopy indicating formation of intermolecular products **151** and **152**.



Scheme 2.9: Scheme detailing the Boc deprotection of thioacetate **147** to yield trifluoroacetate salt **149**. Subsequent treatment of **149** with Amberlyst® A21 yielded intermolecular S-to-N acyl transfer products **151** and **152**, and not the desired acetamide **153.**

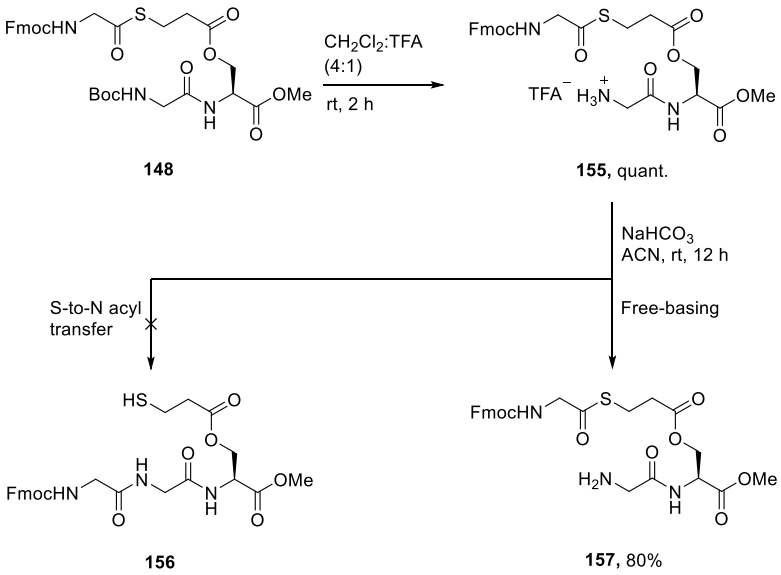
The yield of intermolecular product **152**, along with deacylated **151**, the yield of which must be equal to **152** within the reaction mixture, indicated a high predominance of the intermolecular S-to N-acyl transfer pathway over the desired intramolecular pathway in this instance. None of the desired intramolecular monoacylated product **153** was isolated from the reaction even after multiple attempts. Repetition of the reaction at low concentration increased reaction time (> 12 h) while yielding a similar product distribution. Nevertheless, cleavage of the Ser auxiliary was carried out in order to assess the total ligation yield (**Scheme 2.10**).



Scheme 2.10: Scheme detailing the serine ester cleavage of reaction mixture to yield product **154**.

This was undertaken with sodium methoxide (NaOMe)/MeOH, conditions too harsh for cleavage within peptidic systems due to likely α-carbon racemisation, utilised here in order to preserve the methyl ester PG of **154** to aid in isolation. Upon cleavage, extraction, and subsequent chromatographic purification the desired acylated product **154** was recovered in a 19% yield. This low yield indicated the unfavourable nature of both intra- and intermolecular S-to-N acyl transfer in this system. The relative rates of

inter- and intramolecular transfer are scarcely mentioned in the literature with regards to auxiliary systems bearing large S-to-N TS sizes. It was therefore surmised that the small size of shifting acyl group, although assisting spectroscopic interpretation, may in fact promote the intermolecular reaction to furnish bis-acylated **152** through the acyl groups lack of steric bulk. Investigations were continued with Gly thioester **148** (**Scheme 2.11**). Boc deprotection of Gly thioester **148** under optimised conditions proceeded smoothly to give trifluoroacetate salt **155** quantitively. Use of Amberlyst® A21 resin to furnish S-to-N acyl transfer products was complicated by the presence of the Fmoc PG. Significant removal of the PG was observed by TLC analysis even over short reaction times (< 1 h). Amberlyst® A21 resin is exclusively manufactured by Merck and is described as possessing alkyl amine functionality and therefore well capable of deprotonating the fluorenyl moiety necessary for deprotection.[239] Tertiary amines TEA and DIPEA were considered but would likely complicate NMR interpretation of the resulting reaction mixtures. It was determined that a heterogeneous mixture of the weak inorganic base $NaHCO_3$ in organic solvent would not be sufficiently basic to instigate Fmoc deprotection but, if sufficiently basic to free base trifluoroacetate salt **155**, could be removed through filtration. Acetonitrile (ACN) proved proficient in solubilising **155** while permitting facile separation of $NaHCO_3$. After stirring for 12 h in the basic solution TLC analysis indicated the formation of one compound which upon chromatographic separation provided free amine **157** in an 80% yield (**Scheme 2.11**).

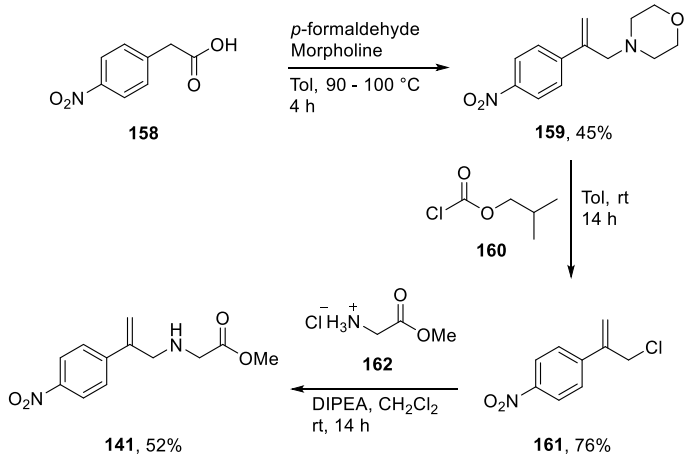


Scheme 2.11: Scheme detailing the Boc deprotection of thioester **148** to yield trifluoroacetate salt **155**. Subsequent treatment of **155** with $NaHCO_3$ in ACN yielded free amine **157** and no ligation product **156**.

None of the desired S-to-N acyl transfer product **156** was isolated from the reaction mixture and the high yield of free amine **157** indicated that intramolecular acyl transfer was not favoured within the system. The presence of free amine **157** was conclusively verified through 2D ^{1}H NMR spectroscopy and ^{15}N NMR spectroscopy. ^{19}F NMR spectroscopy indicated no fluorine remained within the isolated sample. Interestingly, no bis-acylated product comparable to **152** was isolated indicating that intermolecular S-to-N acyl transfer was inhibited for this system, likely due to the increased steric bulk around the thioester moiety of **157** or due to free rotation around the ester linkage, underlining the importance of directing effects. The use of a less bulky orthogonal PGs such as 2,2,2-trichloroethoxycarbonyl (Troc) or allyloxycarbonyl (Alloc) for the thioester α-amino group were considered but these necessitate harsh reductive conditions for their removal.[54] It was concluded that if the S-to-N acyl transfer was not observed for the transfer of a single AA in this system it is unlikely it would be suitable for larger peptide substrates. Attention was therefore directed to the synthesis of auxiliary **141**, the S-to-N acyl transfer characteristics of which had already been appraised by Seitz and demonstrated to reliably provide amide products over a 6-membered cyclic TS.[152, 154]

2.4 Aromatic Photo-Cleavable Auxiliary

2.4.1 Synthesis of para-Nitrophenyl Allyl Acceptor 141



Scheme 2.12.: Three step synthesis of *p*-nitrophenyl ATE acceptor **141.**

The synthesis of *p*-nitrophenyl ATE acceptor **141** was executed according to the previously published route developed by Seitz (**Scheme 2.12**), allylchloride **161** being a synthetic intermediate.[154] *p*-Nitrophenylacetic acid **158** was converted to allylamine **159** through an unusual decarboxylative double Mannich/elimination reaction (**Figure 2.2**) in a 45% yield, comparative to the literature precedent. Addition of

isobutylchloroformate **160** to allylamine **159** furnished allylchloride **161** in a 76% yield, likely through an internal nucleophilic substitution (S_Ni) mechanism comparable to chlorination of alcohols with thionyl chloride.[240] Nucleophilic substitution of the allylchloride **161** with Gly methyl ester **162** yielded the target alkene **141** in a modest 52% yield. With the auxiliary bearing compound **141** in hand the auxiliary's reactivity towards ATE addition could be appraised.

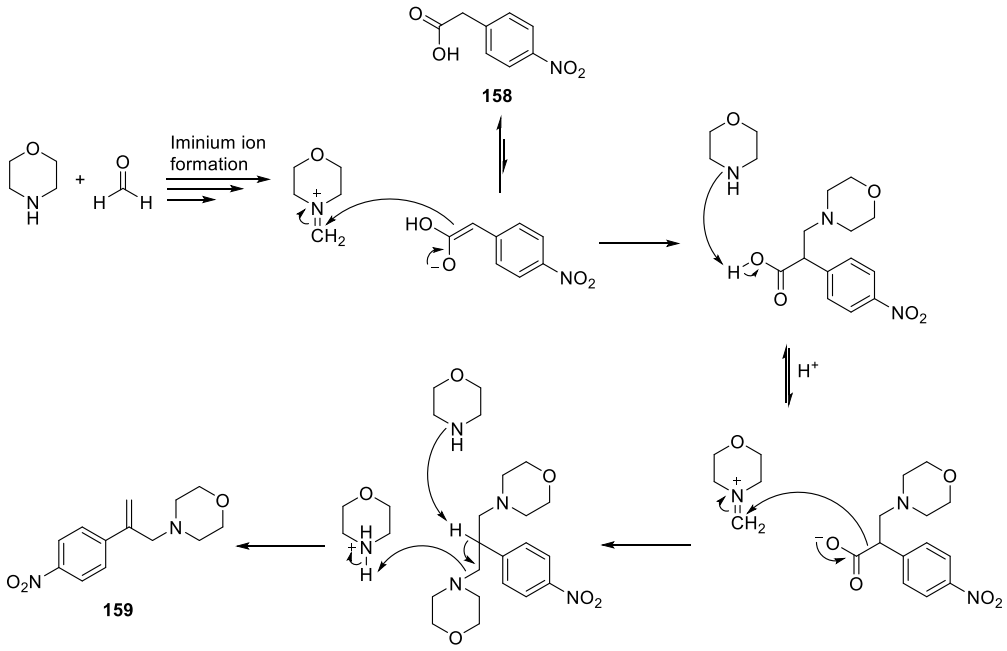
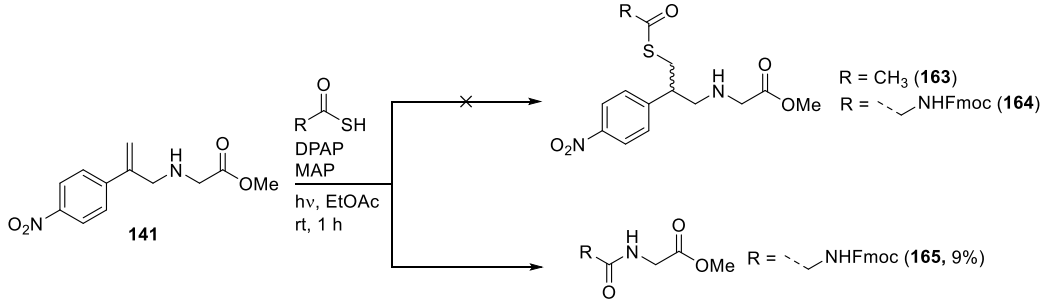


Figure 2.2: Proposed mechanism for the decarboxylative double Mannich/elimination reaction of morpholine, formaldehyde and *p*-nitrophenylacetic acid **158** to yield benzylic alkene **159.**

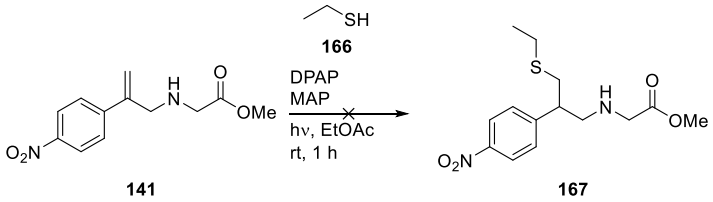
2.4.2. Acyl Thiol-ene Reactions and Auxiliary Bearing Substrate 141

ATE reactions were carried out under optimised conditions with thioacetic acid **97** and Gly thioacid **124** (**Scheme 2.13**). Full consumption of the alkene was observed upon inspection of the crude ^{1}H NMR spectrum after 1 h of UV irradiation, singlet peaks at 5.48 and 5.60 ppm corresponding to each alkenyl proton were no longer present. However, TLC analysis of the reaction mixture indicated a complex product distribution.



Scheme 2.13. Scheme of the product distribution observed after reaction of *p*-nitrophenyl allylic acceptor **141** with thioacetic acid **97** and Gly thioacid **124**.

Following chromatographic separation, it was determined that neither reaction furnished expected thioester products **163** or **164**. Ligated dipeptide **165** was isolated from the ATE reaction of auxiliary **141** and Gly thioacid **124** in a 9% yield. This was an indication that thioester formation and spontaneous S-to-N acyl transfer had taken place sequentially, a possibility due to the unprotected secondary amine present in thioester **164**. However, cleavage of the aryl nitro fragment was not anticipated under these conditions. Further work was necessary to elucidate the mechanism of amide bond formation, particularly as cleavage of *p*-nitroaryl auxiliaries had previously only been demonstrated in the presence of reducing agent TCEP.[154] In order to ensure auxiliary **141** was capable of furnishing the desired thiol-ene addition products, a test reaction with ethanethiol **166** in place of Gly thioacid **124** was undertaken (**Scheme 2.14**). The presence of thioether **167** in the reaction mixture would confirm the amenability of alkene **141** towards thiol-ene addition, as thioether **167** would be incapable of subsequent S-to-N acyl transfer.



Scheme 2.14: Reaction of auxiliary **141** with ethanethiol **166** under optimised acyl thiol-ene conditions.

Inspection of the crude ^{1}H NMR spectrum of the reaction mixture indicated full consumption of the alkene after 1 h, yet thioether **167** could not be isolated from the complex reaction mixture. This suggested that consumption of the alkene observed was not as a result of thiol-ene addition in this case, or in prior ATE reactions. The stability of alkene **141** towards ATE conditions was therefore called into question.

2.4.3 Stability of Auxiliary System 141 to Acyl Thiol-ene Conditions

The stability of auxiliary system **141** under ATE conditions was probed through addition of DPAP and MAP in the absence of thiyl radical forming species. TLC analysis of the reaction mixture after 1 h indicated full consumption of alkene **141**. Comparison of the ^{1}H NMR spectrum of auxiliary system **141**, MAP, DPAP and the crude ^{1}H NMR spectrum of the reaction mixture clearly conveyed the degree of degradation under these conditions (**Figure 2.3**). As indicated by TLC analysis, alkene protons at 5.60 and 5.48 ppm (**Figure 2.3**, H_c and H_d, respectively) were no longer present in the crude mixture.

However, the disappearance of the signal at 3.45 ppm corresponding to the glycinyl protons of **141** (**Figure 2.3**, H_f) was surprising and suggested significant fragmentation of **141** under UV conditions. Interestingly, doublet signals at 8.20 and 7.64 ppm indicative of the aromatic protons of *p*-nitroaryl moiety (**Figure 2.3**, H_a and H_b, respectively) were not present in the crude mixture, perhaps indicative of a change in proton environment as a result of cleavage.

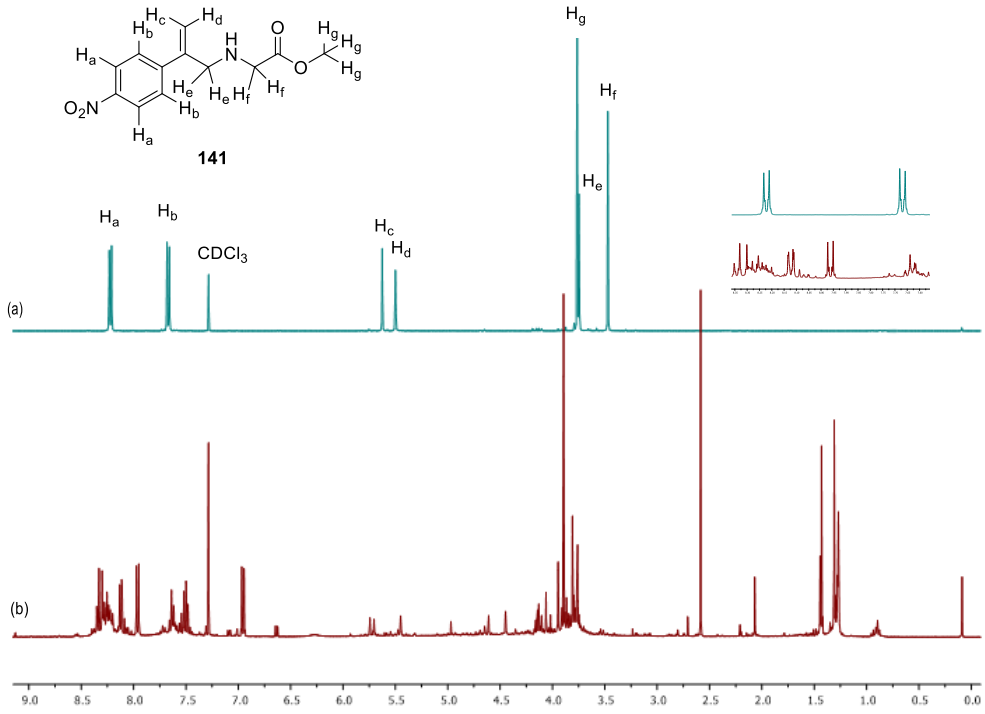


Figure 2.3: (a) ^{1}H NMR spectrum of auxiliary system **141**. (b) Crude ^{1}H NMR spectrum of auxiliary system **141** after UV irradiation for 1 h with DPAP/MAP, showing substantial degradation.

It was therefore clear that auxiliary system **141** did not possess the stability required under thiol-ene conditions to warrant further investigation as a substrate for ATE mediated peptide ligation. The test reaction utilising ethanethiol **166** indicated the isolation of dipeptide product **165** in a 9% yield after reaction of Fmoc-Gly-SH **124** and auxiliary system **141** under ATE conditions was not likely to have resulted from amide formation *via* a thioester intermediate. As briefly mentioned in Chapter 1 (**Section 1.4.2**), thioacids act as acyl donors in a wide range of amide bond forming reactions. The work of Tang *et al.* into direct amidation of thioacids described disulfide formation *via* thiyl radical intermediates permitting efficient nucleophilic acyl substitution to furnish substituted amides (**Figure 2.4**).[241] Potentially the formation of dipeptide **165** under ATE conditions in the presence of Gly thioacid **124** could have resulted from a similar mechanism, assuming the amine moiety of **141** possessed adequate nucleophilicity. The

use of 3 eq. of thioacid may have also served to increase the concentration of disulfide within the reaction mixture. The low yield of dipeptide **165** (9%) in comparison with that observed by Tang (63%) may have resulted from CSO elimination consuming the thiyl radical species.

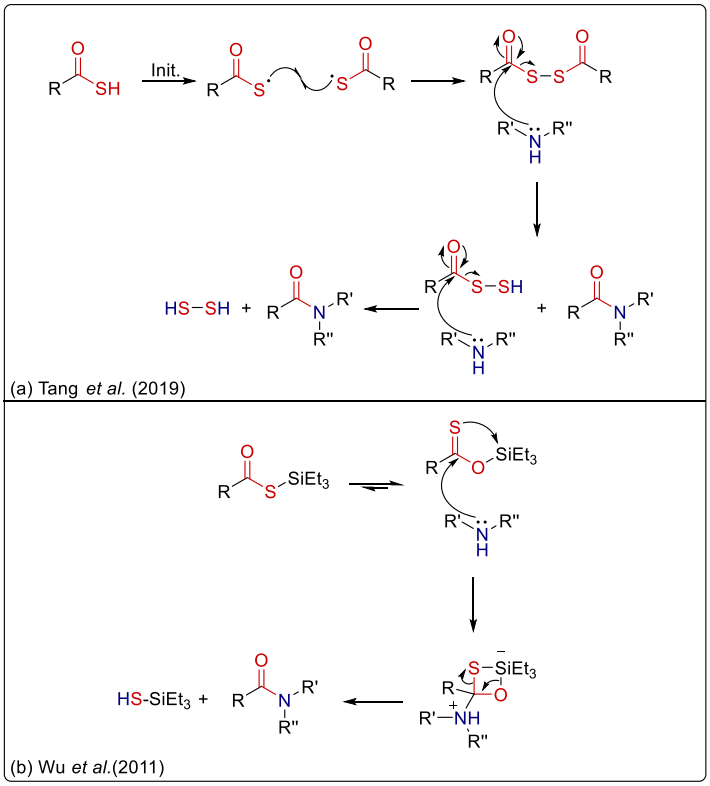


Fig 2.4: (a) Mechanism of amide bond formation *via* thiyl radical intermediate published by Tang *et al.*[241] (b) Mechanism of amide bond formation *via* silylation pathway described by Wu *et al.*[201]

Thioacids have been demonstrated to participate in amide bond forming reactions that proceed *via* conventional polar mechanisms. Thioacids can act as activated electrophiles, as demonstrated in the work of Crich[203] and Pan *et al*.[198] among others. The work of Wu demonstrated amide bond formation through thioacid activation *via* *O*-silylthionoesters.[201] The presence of residual TES and related trifluoroacetate salts, from the Trt deprotection of thioester **146** (**Scheme 2.8**), in the reaction mixture provides a mechanistic pathway by which thioacid activation could occur (**Figure 2.4**). However, initial proton transfer from thioacid to trifluoroacetate salt enabling thiolate silylation would be highly disfavoured due to the respective pKas of the species. Activation of the amine component through fragmentation of auxiliary system **141** is also feasible, as azides[242] and isocyanates[199] permit direct amide bond formation in reaction with thioacid nucleophiles. However, work to elucidate the precise mechanisms of amide bond formation to yield dipeptide **165** and fragmentation of auxiliary system **141** was deemed beyond the scope of this thesis.

2.5 Conclusions

Evaluation of the suitability of auxiliary systems **137**, **140** and **141** towards ATE mediated peptide ligation was undertaken. Cyclohexyl auxiliary **137** was designed in order to improve on previously examined auxiliary system **123** which had proven incapable of S-to-N acyl transfer upon thioester formation and *N*-terminal deprotection (**Scheme 2.2**). However, the sensitivity of auxiliary **137** to Fmoc PG deprotection conditions prior to ATE reaction halted further synthetic optimisation and served to accentuate the point that in order for broad adoption of a ligation strategy, any requisite synthetic targets must be easily attainable. The synthesis of acryloylated compound **140** and subsequent thiol-ene reactions under radical mediated and ionic conditions underscored the versatility of ATE chemistry in producing thioester linkages in the high yields necessary for a useable ligation methodology. Unfortunately, when considering its facile and reliable synthesis, the characteristics of S-to-N acyl transfer, in the instance of thioesters **147**, were not favourable, with the intermolecular reaction to give bis-acylated product **152** preferred over the desired intramolecular product **153**. Gly thioester **148** did not undergo S-to-N acyl transfer to furnish the desired product, with only amine SM **157** isolated after freebasing. Synthesis of *p*-nitroaryl based auxiliary system **141**, demonstrated to be capable of S-to-N acyl transfer by Seitz, provided the desired ATE substrate in gram quantities. Reaction of Gly thioacid **124** and alkene **141** under ATE conditions permitted the isolation of dipeptide **165**, albeit in a low yield of 9%. Unfortunately, further investigation suggested amide formation was taking place by a mechanism unrelated to ATE, and significant degradation of **141** was observed under ATE conditions. These results serve to emphasise the vast number of underlying factors which must be reconciled in order to establish a methodology capable of ATE mediated peptide ligation. Substrates must be easily accessible through synthesis, capable of efficient ATE reactions to furnish thioester products in high yield, possess the necessary characteristics to ensure efficient S-to-N acyl transfer and prove sympathetic to the constraints of SPPS. Having identified the considerable limitations of auxiliaries in thiol-ene mediated ligation of peptides, Chapter 3 details our efforts to fulfil these criteria.

Chapter 3

Acyl Thiol-ene Mediated Ligation of Unnatural Amino Acids

3.0 Introduction

Chapter 2 outlined the characteristics of ligation auxiliary systems **137**, **140** and **141** (**Figure 3.1**) in terms of their synthetic accessibility, reactivity towards ATE and amenity to S-to-N acyl transfer processes to furnish amide products. A summary of the key findings from this chapter is shown in **Figure 3.1** below.

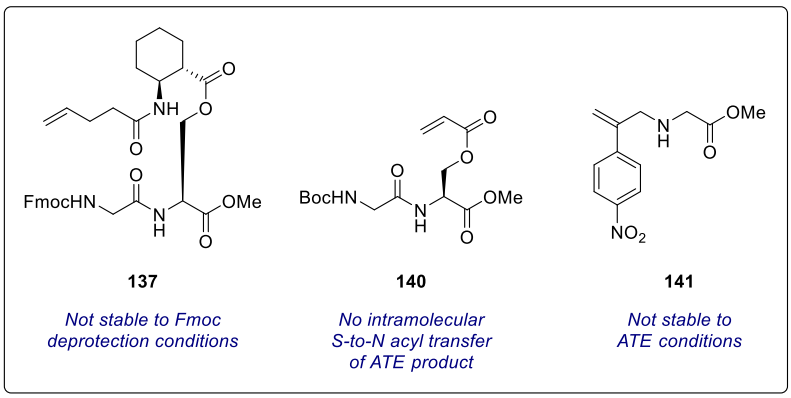


Figure 3.1: Ligation auxiliary systems **137**, **140** and **141** investigated in Chapter 2 for use in ATE mediated peptide ligation.

The synthesis of auxiliary system **137**, based on the work of Brik,[155, 159] proved challenging to work with and necessitated the use of an orthogonal PG strategy to permit selective amide formation at the cyclohexyl moiety. Unfortunately, the ester of moiety of **137** did not exhibit the necessary stability to the numerous Fmoc deprotection conditions screened. A simplification of this synthetic approach was undertaken leading to the design of auxiliary system **140** accessed through the acryloylation of Ser containing dipeptide **144**. Acryloylated dipeptide **140** possessed good reactivity towards ATE reaction with thioacetic acid **97** and Fmoc-Gly thioacid **124**, generated *in situ* through acidic deprotection of the corresponding Trt thioester as described by Crich.[203] Thioesters **147** and **148** were furnished in 98% and 79% yield respectively, demonstrating the capability of the ATE reaction to generate valuable AA based thioesters. Unfortunately, Boc deprotection and subsequent freebasing in order to promote spontaneous S-to-N acyl transfer did not furnish the desired amide products in either case. Auxiliary **141**, inspired by the work of Seitz,[154] was accessed synthetically *via* the published route but did not possess the necessary stability towards ATE conditions. Alkene **141** underwent an unspecified degradation pathway under UV irradiation in the presence of radical initiator DPAP. NCL remains the gold standard among ligation methodologies, in part due to the 5-membered S-to-N acyl transfer TS ensuring swift formation of the native amide bond

upon trans-thioesterification. In the case of ligation auxiliary **140**, ATE proved capable of furnishing thioesters in good yields but relied on an S-to-N acyl transfer TS too large to enable efficient amide bond formation upon deprotection. In order to achieve acyl transfer TSs of comparable size to NCL, which would promote efficient amide formation while maintaining desirable ATE reactivity, it was necessary to consider insertion of the alkene moiety into the fundamental structure of the AA substrate. Sacrificing the degree of generality that AML offered was deemed a necessary cost to ensure efficient S-to-N acyl transfer. A literature review was undertaken in order to uncover suitable substrates which promised to fulfil the selected S-to-N acyl transfer criteria and as facile a synthetic route as possible. β,γ-unsaturated UAA VG **168** provided such a substrate (**Figure 3.2**).

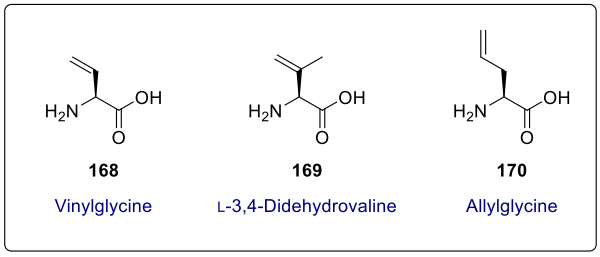
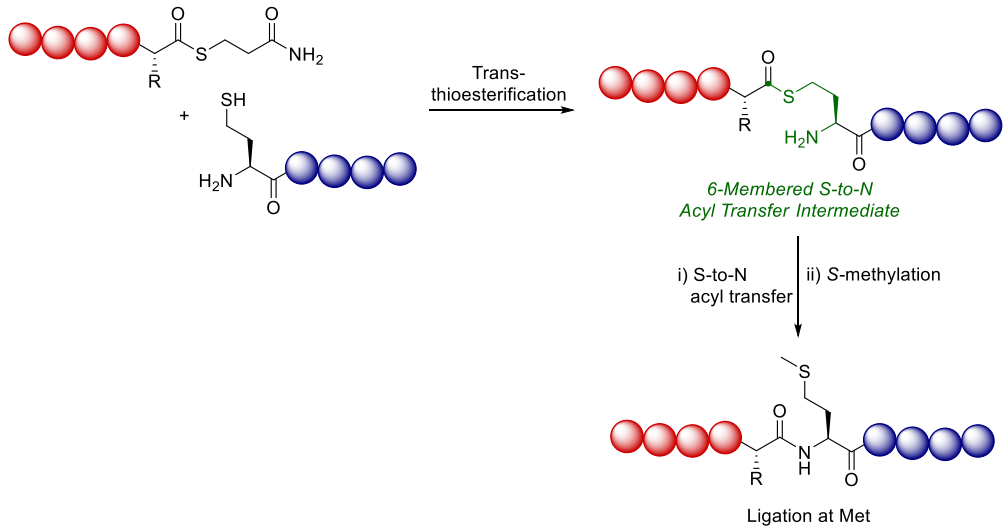
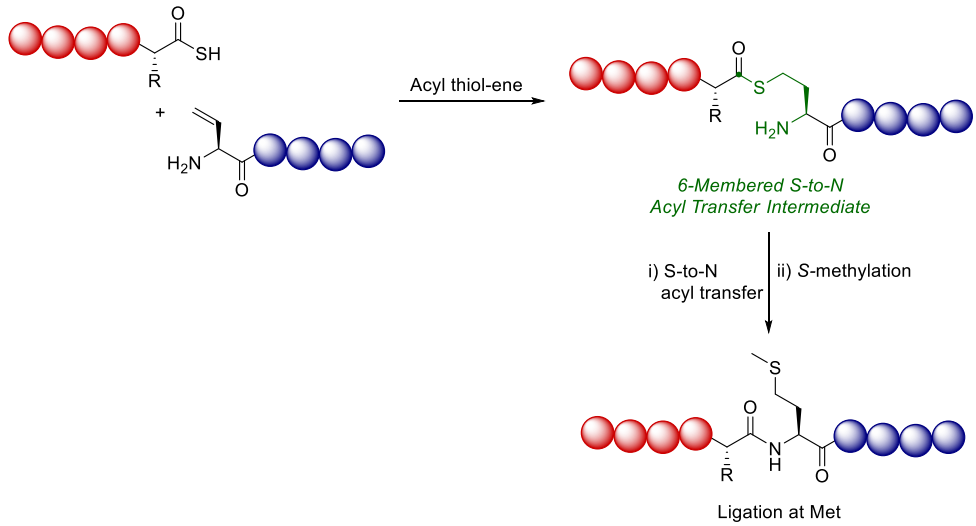


Figure 3.2: Unsaturated unnatural amino acids vinylglycine **168**, L-3,4-didehydrovaline **169** and allylglycine **170** investigated as potential substrates for acyl thiol-ene mediated peptide ligation.

The syntheses and applications of VG compounds are well described throughout the fields of peptide synthesis and modification, including as components in thiol-ene reactions by Dondoni *et al*.[182, 183] The Dondoni group demonstrated the synthesis of thioglycoside **72** in an 89% isolated yield from VG derivative **71**, thereby demonstrating both the stability and reactivity of VG substrates towards radical mediated thiol-ene addition under photochemical initiation conditions (**Section 1.4.2**, **Scheme 1.10**). S-to-N acyl transfer over 6-membered TSs has been observed by Liu,[137] Payne,[144] and Danishefsky,[138] among others, in their development of ligation/desulfurisation methodologies (**Section 1.3.4.**). One such example was in the ligation/methylation methodology of Tam and Yu, in which homocysteine was used in place of Cys in the synthesis of human parathyroid hormone.[243] S-to-N acyl transfer of the thioester intermediate followed by methylation of the homocysteine thiol yielded a Met residue at the ligation site (**Scheme 3.1**).[243]



(a) NCL utilsing homocysteine,Tam and Yu (1998)

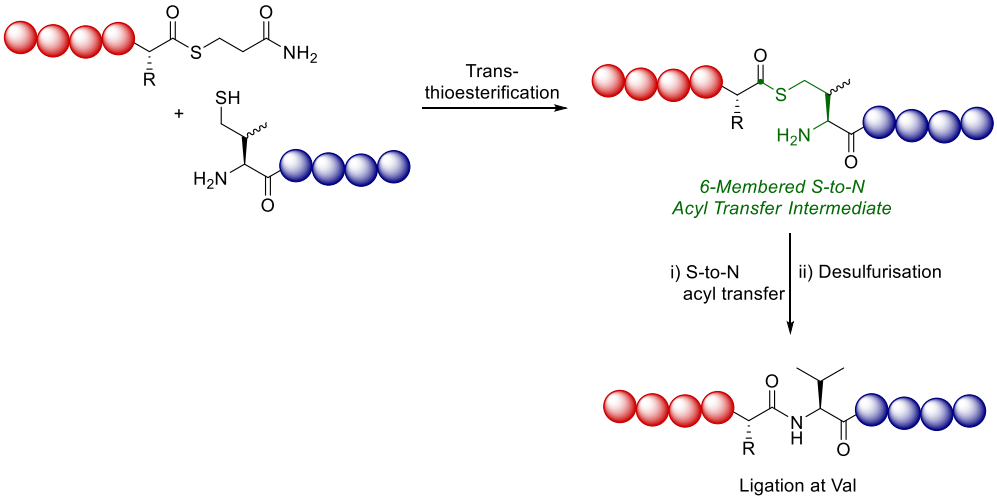


(b)Acyl thiol-ene mediated peptide ligation at Met (This work)

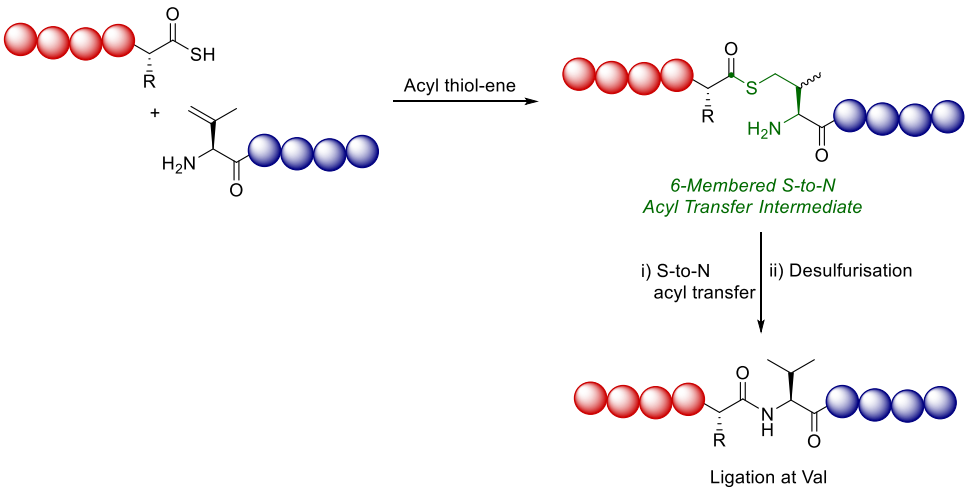
Scheme 3.1: (a) NCL/methylation at homocysteine to yield Met at the ligation site as demonstrated by Tam and Yu.[243] (b) Acyl thiol-ene mediated peptide ligation at vinylglycine followed by S-to-N acyl transfer and methylation to yield Met at the ligation site.

The novel ATE mediated ligation methodology utilising VG was inspired by this precedent. The addition of a *C*-terminal peptide thioacid *via* ATE to a peptide substrate bearing an *N*-terminal VG residue would furnish a reactive thioester intermediate capable of spontaneous S-to-N acyl transfer. Methylation of the resulting homocysteine residue would provide a native product with Met at the ligation site as in the case of Tam and Yu.[243] In addition to VG, UAA L-3,4-Didehydrovaline (DHV) **169** provided another promising substrate for ATE mediated peptide ligation (**Figure 3.2**). Addition of a *C*-terminal peptide thioacid to an *N*-terminal DHV residue of another peptide fragment under ATE conditions would furnish a reactive thioester intermediate capable of S-to-N acyl transfer. Desulfurisation of the resulting thiolated Val residue (**Section 1.3.4**) would furnish Val at the ligation site (**Scheme 3.2**). As in the case of VG, the reactive valine

thioester intermediate had already been proven to undergo spontaneous S-to-N acyl transfer by Danishefsky (**Scheme 3.2**).[244]



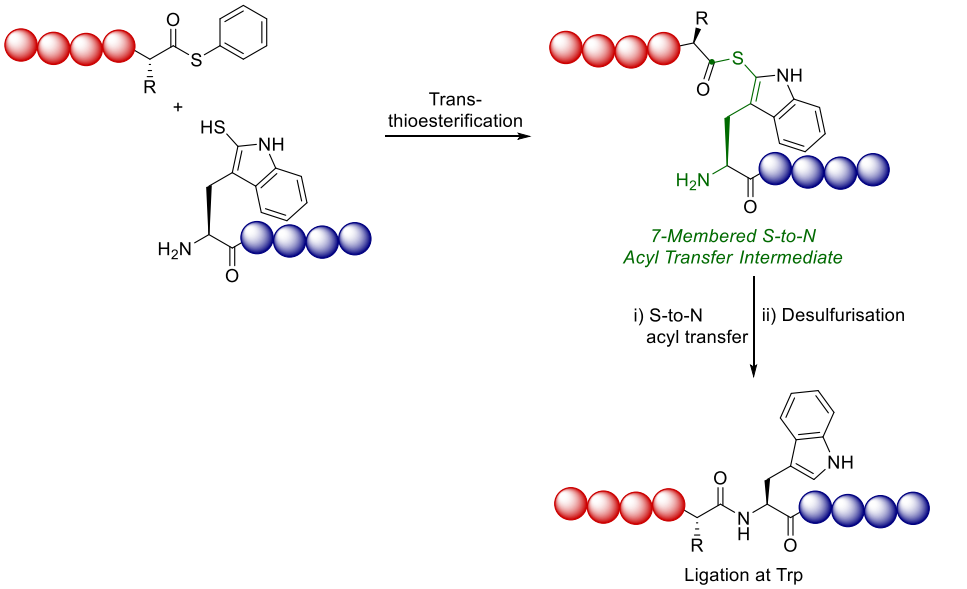
(a) Ligation/desulfurization utilsing γ-thiol Val, Danishefsky (2008)



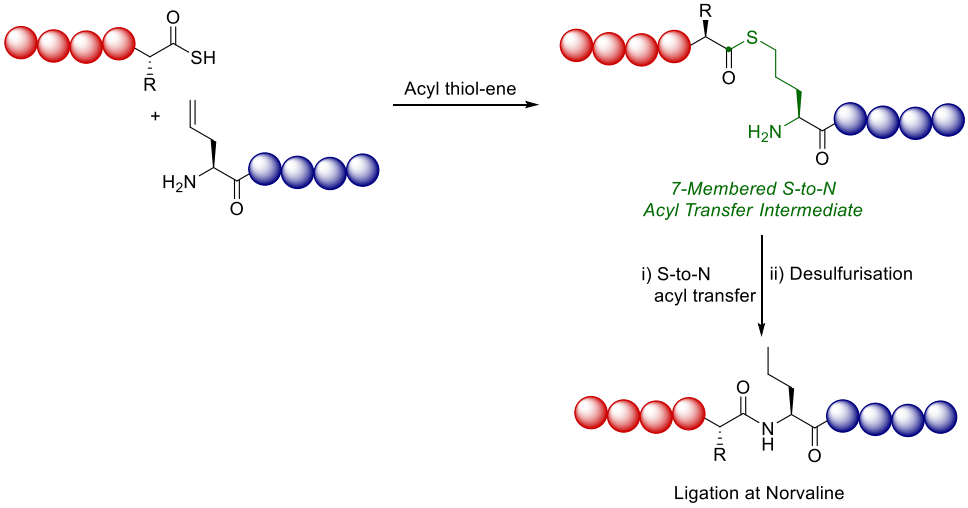
(b) Acyl thiol-ene mediated peptide ligation at Val (This work)

Scheme 3.2: (a) Ligation/desulfurisation at γ-thiol Val to yield Val at the ligation site as demonstrated by Danishefsky.[244] (b) Acyl thiol-ene mediated peptide ligation at L-3,4-didehydrovaline followed by S-to-N acyl transfer and desulfurisation to yield Val at the ligation site.

UAA allylglycine (AG) **170** was also identified as a potential substrate for ATE mediated peptide ligation (**Figure 3.2**). The compound had already been identified as an excellent thiol-ene acceptor by Dondoni *et al.*[183] and S-to-N acyl transfer over a 7 membered TS had been reported by Payne, albeit with a more rigid thiolated tryptophan (Trp) substrate (**Scheme 3.3**).[145] ATE mediated peptide ligation, in this instance, would yield UAA residue norvaline at the site of ligation through subsequent desulfurisation. However, AG derivatives could be readily purchased and therefore little synthetic work would be necessary to access a suitable substrate for ATE and test the viability of the ligation in AG derived systems.



(a) Ligation/desulfurization utilsing 2-thiol Trp, Payne (2014)



(b) Acyl thiol-ene mediated peptide ligation at norvaline (This work)

Scheme 3.3: (a) Ligation/desulfurisation at 2-thiol Trp to yield Trp at the ligation site as demonstrated by Payne.[145] (b) Acyl thiol-ene mediated peptide ligation at allylglycine followed by S-to-N acyl transfer and desulfurisation to yield norvaline at the ligation site.

As with the synthesis of auxiliary systems **137**, **140** and **141** proof of concept was to be established using dipeptide SMs to ensure the necessary reactivity of each system towards ATE and facilitate facile S-to-N acyl transfer. Assessment of the ATE reaction was to be carried out on Boc protected dipeptide precursors **174**, **175** and **176**, synthesised through solution phase amide coupling of Boc protected UAAs **171**, **172** and **173** (**Figure 3.3**). Deprotection and freebasing of the resulting Gly thioesters would then be undertaken to investigate the S-to-N acyl transfer characteristics of each UAA system. If the desired amide products could be reliably accessed, methylation or desulfurisation of the resulting thiolated products could be carried out to yield characterizable tripeptide substrates prior to installation of the UAAs in larger peptide fragments *via* SPPS.

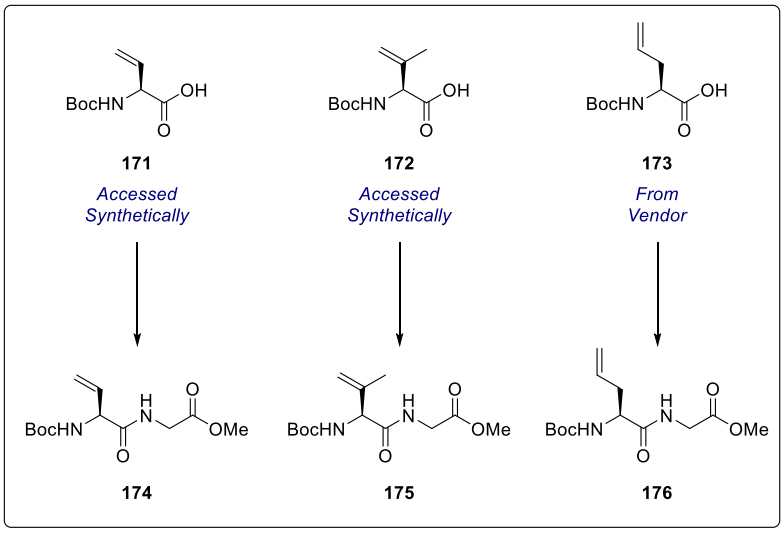
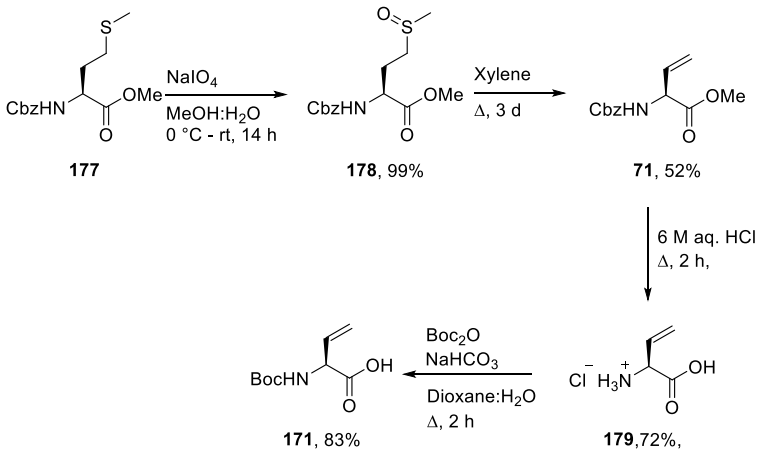


Figure 3.3: Boc protected unnatural amino acid synthetic targets Boc vinylglycine **171**, Boc L-3,4-didehydrovaline **172** and Boc allylglycine **173** and corresponding dipeptides **174**, **175** and **176** to be used in ATE mediated peptide ligation.

3.1 Synthesis of Boc-Vinylglycine (171)

VG has been a compound of interest across the fields of organic synthesis and biochemistry for decades and was first isolated as a natural product from the mushroom *Rhodophyllus nidorosus* in 1974.[245] L-VG is generated as a reactive intermediate in a considerable number of enzymatic conversions including the conversion of homoserine to Thr by threonine synthase and the synthesis of α-ketobutyrate by cystathionine γ-synthase.[246] VG and VG derivatives have also been demonstrated to possess a broad spectrum of transaminase inhibition.[246] In organic synthesis VG derivatives possess associated reactivity to other olefins and are amenable to epoxidation,[247, 248] metathesis[249, 250] and thiol-ene addition reactions.[183] As a result of the biologically active nature of VG and its derivatives, as well as the vast synthetic potential that olefinic UAAs possess, investigation into the synthesis of VG has been an active area of research since the 1970s. The first synthesis of racemic VG was carried out by Friis *et al.* in 1974 *via* the Strecker reaction.[251] Afzali-Ardakani and Rapoport published the first synthesis of optically pure VG in 1980, obtained *via* the oxidation and subsequent thermolysis of protected Met, followed by global deprotection under acidic conditions to furnish the hydrochloride salt of VG.[252] Boc protection of the hydrochloride salt was demonstrated in the same publication thereby permitting access to target UAA Boc-VG **171**. Subsequent literature has sought to render the synthesis more convenient, particularly due to the original papers significant use of Kugelrohr distillation for thermolysis and purification. The synthetic route selected to access Boc-VG was a combination of Afzali-Ardakani and Rapoport's

original publication and a variation by Sicherl *et al.* permitting thermolysis through reflux in xylene (**Scheme 3.4**).[253]



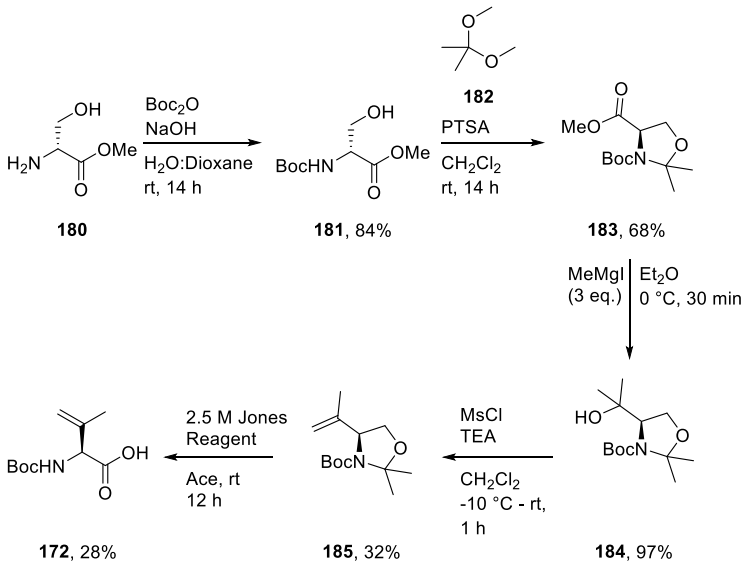
Scheme 3.4: Synthesis of Boc-VG **171** undertaken, inspired by that of Afzali-Ardakani and Rapoport.[252]

Oxidation of *N*-Cbz protected Met methyl ester **177** with sodium metaperiodate proceeded smoothly to give the desired sulfoxide **178** in quantitative yield. The pure sulfoxide product was isolated through simple filtration and work up and the reaction proved highly scalable. The product was confirmed through the appearance of a methyl signal at 2.53 ppm in ^{1}H NMR spectrum of the product, consistent with the formation of the methyl sulfoxide moiety and distinct from that of Met derivative **177**. Thermolysis of sulfoxide **178** was carried out in refluxing xylenes over 3 days. TLC analysis indicated full starting material consumption, but generation of a significant number of side products with comparable R_f to that of VG derivative **71**. Consultation of the literature revealed the side products to be the *cis* and *trans* isomers of the corresponding conjugated internal alkene, and sequential iterations of column chromatography proved sufficient to remove all side-products. Synthetic routes with greater selectivity have been published, including the photolysis route of Griesbeck *et al.*,[254] but the 52% isolated yield of the desired terminal alkene **71** was deemed satisfactory in this case. Eliminations carried out at large scale (> 10 mmol) suffered comparably lower yields (~ 30%) and subsequent reactions to gain access to larger quantities of terminal alkene **71** were performed in parallel prior to collective purification. Simultaneous deprotection of *N*-Cbz and methyl ester groups of terminal alkene **71** in 6 M aq. HCl under reflux, followed by recrystallisation from acetone (Ace) furnished the hydrochloride salt of VG **179** in a 72% yield. Boc protection with Boc_2O and $NaHCO_3$ under reflux gave the desired Boc-VG

171 in an 83% yield after washing with organic solvent, acidification and extraction of the reaction mixture.

3.2 Synthesis of Boc-L-3,4-Didehydrovaline (172)

Interest in the synthesis of DHV stems chiefly from the presence of the molecule's structural motif in phomopsin B, a hepatotoxic cyclic mycoprotein produced by *Diaporthe toxica* responsible for lupinosis in livestock.[255] Various literature syntheses to access Boc-DHV **172** were considered. However, excluding those of Woiwode and Wandless,[255] and Yonezawa *et al.*,[256] all of the reported procedures required chiral resolution, which was to be avoided if possible. The synthetic route of Woiwode and Wandless necessitated the use of an Evans type chiral auxiliary in tandem with phenyl sulfoxide elimination to yield the desired terminal alkene.[255] Yonezawa *et al.* reported a synthetic route which required no stereochemical control. Starting from Boc-D-Ser methyl ester **180** the route consisted of sequential protection steps, followed by two FGIs at the methyl ester moiety to introduce the requisite alkene, followed by a final tandem deprotection/oxidation step using Jones reagent to furnish Boc-DHV **172**.[256] This route was deemed the most straightforward of those considered and carried out (**Scheme 3.5**).



Scheme 3.5: Synthesis of Boc-DHV **172** carried out under conditions described by Yonezawa *et al*.[255]

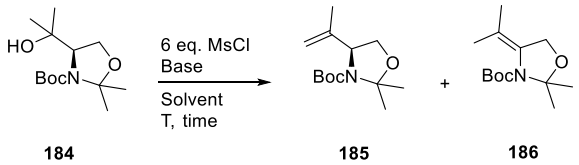
Boc protection of D-Ser methyl ester **180** furnished Boc protected product **181** in an 84% yield after column chromatography. The second protection step of Ser derivative **181** with 2,2-dimethoxypropane (DMP) **182** also proceeded smoothly to furnish oxazolidine **183** in a 68% yield. This yield was lower than that indicated by the crude ^{1}H NMR spectrum of the reaction mixture and likely due to the necessity for product isolation by

vacuum distillation, as poor chromatographic separation of starting alcohol **181** and oxazolidine **183** on TLC precluded column chromatography as a means of purification. Grignard reaction of **183** with methylmagnesium iodide furnished tertiary alcohol **184** quantitatively and did not require further purification after acidic quench and aq. work up to remove iodide salts present in the reaction mixture, as determined by ^{1}H NMR spectroscopy.

3.2.1. Optimisation of Mesylation/Elimination Step to Access Unsaturated Oxazolidine Intermediate (185)

Yonezawa *et al.* reported the facile mesylation/elimination of alcohol **184** with methanesulfonyl chloride (MsCl) in the presence of TEA to furnish alkene **185** in one step, providing a 76% yield of product in their hands.[256] Repetition of the reaction under these conditions was undertaken and TLC analysis indicated the full consumption of tertiary alcohol **184** after 1 h. However, subsequent aq. workup and column chromatography furnished the desired product in a disappointing 32% yield, with a significant number of side-products (**Table 3.1**, Entry i). To ensure the purity of the starting material was not culpable, tertiary alcohol **184** was resynthesised and purified by column chromatography in addition to aq. work up, but repetition of the reaction resulted in a comparable yield of 27%. The reaction was also repeated taking care to prevent the internal reaction temperature from rising above -10 ºC upon addition of MsCl to the reaction mixture as dictated by the original publication. This produced a cleaner reaction mixture by TLC analysis, but no appreciable effect on yield was observed after purification (32%). It was noted that upon analysis of the mass balance of the reaction a considerable percentage of the reaction mixture mass, relative to the mass of tertiary alcohol SM **184**, was lost through isolation *via* aq. work up, indicating the formation of water-soluble by-products. Column chromatography permitted the isolation of internal alkene **186** in an 11% yield (**Table 3.1**), but other side products proved elusive. This suggested that the low yield of alkene **185** was due to a combination of unselective elimination yielding both the desired terminal alkene **185** and internal alkene **186**, coupled with deleterious deprotection of acid labile Boc and pseudoproline PGs by acidic reaction by-products HCl and hydrolysed MsCl. As oxazolidine **185** was a key intermediate in the synthesis of Boc-DHV **172** necessary for the synthesis of ATE substrate dipeptide **175** optimisation of the mesylation/elimination was undertaken (**Table. 3.1**).

Table 3.1: Optimisation of mesylation/elimination reaction of alcohol **184** to furnish terminal alkene **185** inspired by reaction conditions of Yonezawa *et al.*[256] *Yield determined by analysis of ^{1}H NMR spectrum of the crude reaction mixture after aq. work up.



184 **185** **186**

Entry	Eq. base	Base	Solvent	T (ºC)	Time (h)	Conc. (M)	Yield **185** (%)
i	10	TEA	CH_2Cl_2	-10 to rt	1	0.35	32
ii.	20	TEA	CH_2Cl_2	-10 to rt	1	0.35	27
iii.	30	TEA	CH_2Cl_2	-10 to rt	1	0.35	29
iv.	10	DIPEA	CH_2Cl_2	-10 to rt	1	0.35	30
v.	10	DBU	CH_2Cl_2	-10 to rt	1	0.35	27
vi.	10 + 6	TEA + Pyr	CH_2Cl_2	-10 to rt	1	0.35	29
vii.	10	TEA	DME	-10 to rt	1	0.35	28
viii.	10	TEA	CH_2Cl_2	-10	1	0.35	4
ix.	10	TEA	CH_2Cl_2	rt	1	0.35	21
x.	10	TEA	DME	-10 to 85	1	0.35	Trace*
xi.	10	DBU	DME	-10 to 85	1	0.35	Trace*
xii.	10	TEA	CH_2Cl_2	-10 to rt	14	0.35	31
xiii	10	TEA	CH_2Cl_2	-10 to rt	1	0.10	19
xiv.	10	TEA	CH_2Cl_2	-10 to rt	1	0.50	28

As it was speculated that the low yield of alkene **185** may have resulted from unwanted deprotection of Boc or pseudoproline PGs the effect of increases in base on the reaction was investigated first. The 10 eq. of TEA utilised in the conditions of Yonezawa *et al.* should have proven sufficient to neutralise HCl evolved from 6 eq. of MsCl in the reaction medium. Nevertheless, the reaction was repeated with 20 eq. and 30 eq. of TEA but failed to produce a change in yield beyond that accountable through loss of product during chromatographic separation (**Table 3.1,** Entry ii – iii). Variation of the non-nucleophilic basic component in the reaction in an effort to promote more selective and efficient elimination of the mesylate and reduce PG deprotection also did not foment any appreciable changes in yield (**Table 3.1**, Entry iv – v). 6 eq. of pyridine (Pyr) in addition to 10 eq. TEA was added in an effort neutralise the acidic side products of the reaction while maintaining efficient elimination, but little effect on the yield of terminal alkene **185** was discerned (**Table 3.1**, Entry vi). Likewise, the use of solvent

1,2-dimethoxyethane (DME), commonly employed for elimination reactions,[257] had little effect on the yield of the reaction (**Table 3.1**, Entry vii). Overall, attempts to diminish deprotection of the Boc and pseudoproline PGs of tertiary alcohol **184** through variation of the reactions basic components were not successful (**Table 3.1**, Entry i – vii). Of note also was that analysis of the ^{1}H NMR spectrums of the crude reaction mixtures (**Table 3.1**, entry i – vii) indicated little variation in the product distribution of terminal alkene **185** and internal alkene **186** (3:1 ratio). Efforts to improve the selectivity of the elimination step entailed consideration of the reaction mechanism. Establishment of the precise elimination mechanism by varying concentrations of tertiary alcohol **184** and base was deemed impracticable due to the volume of competing deprotective side-reactions. However, the reaction could be assumed to proceed *via* an E2 type mechanism, as the presence of hindered base TEA, a good mesylate leaving group and apolar reaction solvent (CH_2Cl_2) all favour this elimination mechanism (**Figure 3.4**).[258] The use of a hindered organic base in the reaction ensured that hydrogen abstraction to furnish terminal alkene **185** was faster than abstraction at the more hindered proton of the oxazolidine cycle (**Figure 3.4**), accounting for the 3:1 ratio of terminal alkene **185** and internal alkene **186** isolated. Internal alkene **186**, as the more substituted alkene, was thermodynamically more stable due to greater resonance contribution of the alkylic alkene substituents. Therefore, terminal alkene **185** was identified as the kinetic product and internal alkene **186** the thermodynamic product of the reaction (**Figure 3.4**).

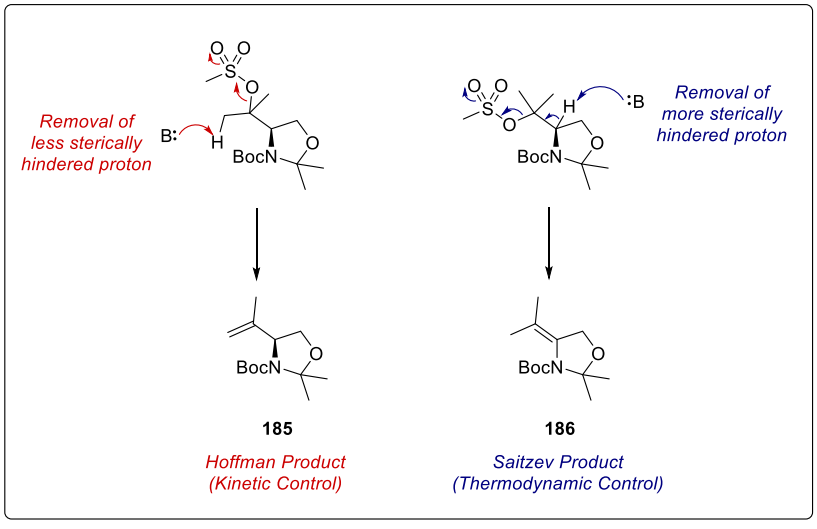
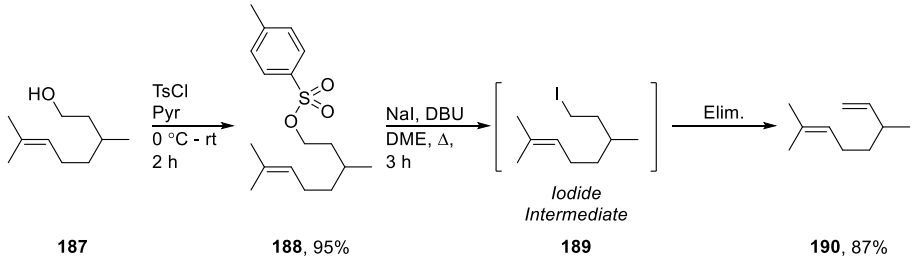


Figure 3.4: Mesylate elimination mechanisms resulting in the formation of kinetic Hoffman product **185** and thermodynamic Saitzev product **186.**

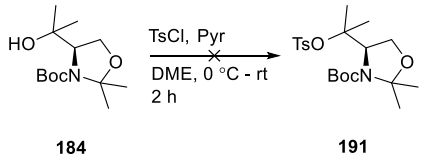
If this was indeed the case, increasing selectivity for terminal alkene **185** could be accomplished through use of more hindered bases or by ensuring low internal reaction

temperature was maintained throughout the elimination step.[258] However, as already observed, addition of sterically hindered bases DIPEA and DBU had failed to achieve increased selectivity for terminal alkene **185 (Table 3.1**, Entry iv – v). More aggressive hindered bases such as potassium *tert*-butoxide (^{t}BuOK) and lithium diisopropylamide (LDA) would likely provide greater selectivity and more efficient proton abstraction.[258] However, the presence of MsOH and HCl in the reaction mixture, generated by formation of the intermediate mesylate, would be liable to neutralise these highly basic reactants. As attempts involving altering the base had proved unsuccessful, other modifications to the reaction were considered, with the aim to increase reaction selectivity. Due to the higher energy barrier to abstraction of the hindered oxazolidine proton maintaining low reaction temperature would increase selectivity and favour the kinetic product, desired terminal alkene **185**. Therefore, the elimination/mesylation reaction was undertaken at -10 ºC for the duration of the reaction (1 h) prior to aq. work up. Unfortunately, purification by column chromatography yielded just 4% of the desired terminal alkene **185** under these conditions, likely as a result of low temperature inhibiting elimination almost entirely (**Table 3.1**, Entry viii). Addition of MsCl to the mixture at room temperature (rt) led to a decrease in yield to 21% of desired alkene **185** but a considerably more difficult purification process due to greater quantities of side products (**Table 3.1**, Entry ix). Heating the reaction to reflux in DME after addition of MsCl at -10 ºC with TEA and DBU in an effort to increase the yield of terminal alkene **185** and internal alkene **186** at the expense of selectivity, yielded only trace amounts of both by ^{1}H NMR spectroscopic analysis of the crude reaction mixtures (**Table 3.1**, Entry x – xi). This indicated that these substrates were not stable to this degree of heating, possibly as a result of PG sensitivity. Stirring the reaction mixture overnight had no effect on the yield of alkene **185** (**Table 3.1**, Entry xii). Likewise, variation of the reaction concentration also had a negligible effect on the yield of terminal alkene **185** (**Table 3.1**, Entry xiii – xiv). Upon consideration of these results, it was concluded that the reaction conditions published by Yonezawa *et al.* were not capable of furnishing the desired terminal alkene **185** in a satisfactory yield. Consideration of other literature conditions applicable to the key elimination step was therefore necessary. Facile dehydration of acid sensitive alcohols was reported by Maier *et al.*, tosylation with 4-toluenesulfonyl chloride (TsCl) in the presence of Pyr yielded the corresponding tosylates in quantitative yields.[257] Elimination promoted by the addition of NaI and DBU, proceeding through an iodide intermediate *via* a pseudo-Finkelstein mechanism, yielded the corresponding alkenes in

excellent yields (**Scheme 3.6**).[257] However, application of these literature conditions, in one pot, for the elimination of tertiary alcohol **184** yielded only unreacted tertiary alcohol SM **184** after stirring for 8 h (**Scheme 3.6**).



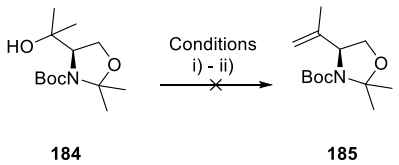
(a) Maier *et al.* (2003)



(b) This work:

Scheme 3.6: (a) Tosylate elimination to yield terminal alkenes under pseudo-Finkelstein conditions as described by Maier *et al.*[257] (b) Attempted tosylation of tertiary alcohol **XX** to yield tosylate **XX**.

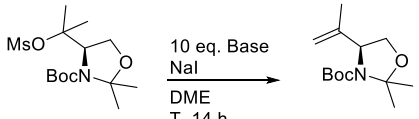
The reaction was repeated after purification of TsCl through organic washing and filtration through activated charcoal, but still no tosylate formation was observed by TLC analysis, likely as a result of the increased steric hindrance of the reagent in comparison to MsCl. Elimination of the intermediate mesylate formed under the conditions of Yonezawa *et al.* was attempted under the conditions described by Maier *et al.* However, attempted purification of the mesylate intermediate, as was carried out with Maier's tosylate **188**, was not successful as the compound was not stable to silica gel column chromatography. It was speculated that the presence of excess MsCl in the reaction mixture after mesylate formation had the potential to consume NaI added to the reaction through a nucleophilic acyl substitution mechanism, to form methylsulfonyl iodide and a chloride ion incapable of mesylate displacement. Eq. of MsCl relative to tertiary alcohol **184** and NaI were reduced accordingly (2:1:3, respectively) (**Scheme 3.7**).



Scheme 3.7: Attempted mesylation/elimination of tertiary alcohol **184** under condition adapted from Maier *et al.*[257] i) 2 eq. MsCl, 1 eq. tertiary alcohol **184**, 3 eq. NaI, CH_2Cl_2, 0 ºC – rt, 8 h. ii) 6 eq. MsCl, 1 eq. tertiary alcohol **184**, 3 eq. NaI, CH_2Cl_2, 0 ºC – rt, 8 h.

Unfortunately, ^{1}H NMR spectroscopic analysis of the crude reaction mixture revealed low consumption of tertiary alcohol **184** and trace quantities of terminal alkene **185** after stirring for 14 h, likely due to insufficient eq. of MsCl. Repetition of the reaction with additional eq. of MsCl relative to alcohol **184** and NaI (6:1:3, respectively) led to incomplete reaction conversion and a complex reaction mixture which was not purified further (**Scheme 3.7**). It was speculated that partial isolation of the mesylate intermediate and concurrent removal of MsCl through aq. work up might enable a cleaner reaction and prevent the side product formation observed. Elimination of tertiary alcohol **184** was repeated under the original conditions of Yonezawa *et al.* (**Table 3.1** entry i) and after full conversion of tertiary alcohol **184** was observed, remaining eq. of MsCl were quenched by addition of water followed by extraction into CH_2Cl_2 and washing with 1 M aq. HCl solution, sat. aq. $NaHCO_3$ solution, and brine. The mixture was subsequently dried over $MgSO_4$ and concentrated *in vacuo*. Analysis of the crude ^{1}H NMR spectrum of the resulting residue indicated the presence of internal alkene **185**, mesylate intermediate **192**, MsOH and triethylamine hydrochloride salt. Presence of mesylate **192** was confirmed by the presence of a characteristic methyl peak at 2.80 ppm in the ^{1}H NMR spectrum, distinct from that of methanesulfonic acid (MsOH) at 2.87 ppm and MsCl at 3.67 ppm.[259] This mixture was redissolved in DME prior to the addition of NaI and DBU, followed by heating under reflux for 14 h, as described by Maier *et al.* (**Table 3.2**).

Table 3.2: Optimisation of mesylation/elimination reaction of alcohol **184** to furnish terminal alkene **185** inspired by reaction conditions of Maier *et al.*[257] through isolation of intermediate mesylate **192** *via* aq. work up. *Yield determined by analysis of ^{1}H NMR spectrum of the crude reaction mixture after aq. work up.



(*Isolated via aq. work up)*

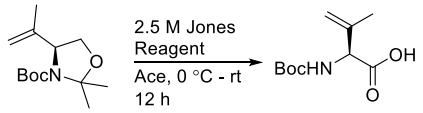
192 **185**

Entry	Base	T (ºC)	Yield **185** (%)
i	DBU	-10 to 85	Trace*
ii.	TEA	-10 to 85	Trace*
iii.	DBU	-10 to rt	38
iv.	TEA	-10 to rt	40

As with reaction under the conditions of Yonezawa *et al.* heating under reflux in DME (**Table 3.1**, Entry x – xi) in the presence of NaI and TEA yielded only trace of the desired terminal alkene product in a complex reaction mixture (**Table 3.2**, Entry i). The use of DBU in place of TEA again resulted in only trace of the desired terminal alkene **185** (**Table 3.2**, Entry ii). The reaction was therefore carried out at rt in the presence of TEA and DBU, respectively (**Table 3.2**, Entry iii – iv). Gratifyingly, this led to the first increase in yield over the initial mesylation/elimination conditions employed by Yonezawa *et al.*, albeit only providing a 40% yield of the desired terminal alkene **185**. These reaction conditions also provided far greater selectivity for terminal alkene **185** over internal alkene **186**, with only trace of the latter observed by ^{1}H NMR analysis of the crude reaction mixture prior to purification. This is likely due to iodide substitution enhancing the rate of reaction, and thereby ensuring only formation of terminal alkene **185**, the kinetic product of the reaction. The increase in yield of **185** from 32% to 40% under these conditions could be accounted for through the increase in selectivity, as a 3:1 ratio of terminal alkene **185** to internal alkene **186** was previously isolated in all cases compared to only a trace amount of internal alkene in this case (**Table 3.2**). However, the overall yield of elimination products was unchanged due to deleterious acidic deprotection reactions of the Boc and pseudoproline groups of oxazolidine **184** and **185** over the course of the mesylation reaction. This necessitated investigation into PG manipulation, work undertaken by postdoctoral research fellow within the Scanlan Group, Dr Pierre Milbeo. Unfortunately, this work was not successful in improving on the optimised conditions based on those of Maier *et al.* above in furnishing the desired in terminal alkene **185** in a yield greater than 40%.

3.2.2. Optimisation of Deprotection/Oxidation Step to Access Boc-L-3,4-Didehydrovaline (172)

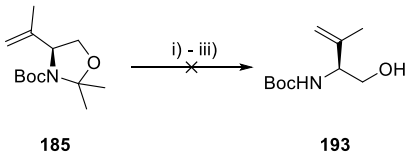
The one step deprotection and oxidation of oxazolidine **185** to furnish Boc-DHV **172** was carried out under reaction conditions described by Yonezawa *et al.* (**Scheme 3.8**).[256]



185 **172**, 29%

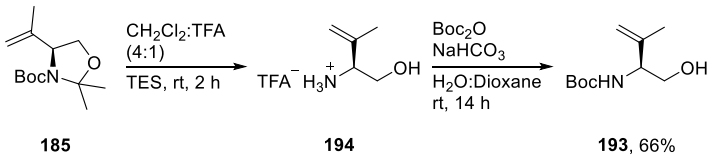
Scheme 3.8: Tandem deprotection and oxidation of oxazolidine **185** to Boc-DHV **172** carried out under conditions described by Yonezawa *et al.*[256]

Freshly prepared 2.5 M Jones reagent was added dropwise to oxazolidine **185** in Ace at 0 ºC and the mixture allowed to reach rt, before stirring for 12 h. Aggregation of chromium salts was observed over this time and after quenching with isopropyl alcohol (IPA), filtration over Celite® and aq. work up, the reaction furnished acid **172** in a disappointing 29% yield. The optimisation of the previous mesylation/elimination step to synthesise terminal alkene **185** had clearly demonstrated the considerable sensitivity of the Boc and pseudoproline PGs to acidic conditions. Deprotection of the Boc group, resulting in water soluble deprotection products being lost through aq. work up, was therefore a likely cause of the low yield of oxidation product **172**. Therefore, a selective deprotection protocol capable of deprotecting the pseudoproline moiety while conserving the Boc group was necessary or, failing the discovery of such a preparation, a global deprotection reaction with TFA followed by Boc protection could be undertaken. A literature preparation capable of selective deprotection was not forthcoming and a selection of acids were screened for selective deprotection of oxazolidine **185** to amino alcohol **193** (**Scheme 3.9**).



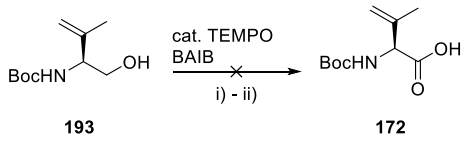
Scheme 3.9: Failed selective deprotection of the pseudoproline group of **185** i) *para*-Toluenesulfonic acid monohydrate, CH_2Cl_2, rt, 3 h. ii) 1 M AcOH, Ace, rt, 3 h. iii) Citric Acid, Ace, rt, 14 h.

Unfortunately, the acids screened for this purpose resulted in either no deprotection of either Boc or pseudoproline, as in the case of citric acid, or full deprotection, as in the case of acetic acid (AcOH) and *para*-toluenesulfonic acid monohydrate (*p*-TsOH·H_2O), as determined by ^{1}H NMR spectroscopy after organic extraction of the respective reaction mixtures. Therefore, global deprotection of oxazolidine **185** to trifluoroacetate intermediate **194** was undertaken using TFA in the presence of cation scavenger TES. TLC analysis after 2 h indicated full consumption of oxazolidine SM **185** (**Scheme 3.10**).



Scheme 3.10: Global deprotection of oxazolidine **185** to yield trifluoroacetate salt **194** and subsequent Boc protection to yield protected amino alcohol **193**.

The crude mixture was redissolved and Boc protection carried out under optimised conditions to yield the desired amino alcohol **193** after chromatographic purification in a 66% yield over two steps, albeit inelegantly (**Scheme 3.10**). With amino alcohol **193** in hand a variety of oxidation conditions inspired by literature precedent could be evaluated (**Scheme 3.11**). Consideration of the sensitivity of the substrate precluded permanganate oxidation and conditions employing bleach based cooxidants.[260, 261] Use of a (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO)/bis(acetoxy)iodobenzene (BAIB) oxidative couple for the oxidation of sensitive primary alcohols including those bearing Boc groups and alkene moieties appeared widespread within the literature.[262] Amino alcohol **193** was treated with catalytic TEMPO and stoichiometric BAIB after dissolution in ACN (**Scheme 3.11**).

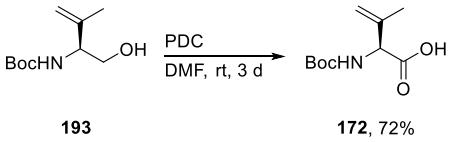


193 **172**

Scheme 3.11: Attempted oxidation of amino alcohol **193** to Boc-DHV **172** with the TEMPO/BAIB oxidation couple. i) TEMPO/BAIB, ACN, rt, 14 h. ii) TEMPO/BAIB, ACN:Phosphate buffer (1:1), pH 7, 14 h.

TLC analysis indicated full consumption of starting amino alcohol **193** but after aq. work up analysis of the ^{1}H NMR spectrum of the crude reaction mixture indicated only the presence of oxidation by-products, predominantly iodobenzene. The absence of alkene signals corresponding to amino alcohol **193** or Boc-DHV **172** in the crude ^{1}H NMR spectrum suggested that Boc deprotection of amino alcohol **193** has occurred, thereby transferring water soluble deprotection products into the aq. layer upon work up. This likely occurred due to liberation of 2 eq. of AcOH in the reaction mixture by BAIB upon re-oxidation of TEMPO. It had been demonstrated that a mixture of ACN and phosphate buffer can be used to maintain reaction pH when oxidising acid sensitive substrates, as in the total synthesis of Largamide B by Qu *et al.*[263] Repetition of the oxidation of amino alcohol **193** with TEMPO/BAIB in ACN:Phosphate buffer (pH 7) prevented deprotection as determined by ^{1}H NMR spectroscopy of the crude reaction mixture after work up. However, subsequent attempts to isolate Boc-DHV **172** proved troublesome. Boc-DHV **172** was not amenable to isolation through aq. work up as even under basic conditions the carboxylate salt of **172** could not be effectively drawn into the aq. layer to permit removal of non-polar reaction products, such as iodobenzene, by organic wash. Chromatographic separation was undertaken but did not prove successful as streaking of carboxylic acid **172** across the column precluded efficient purification. Addition of acid

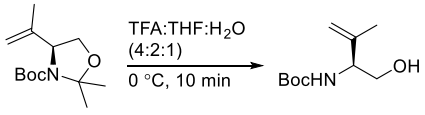
to the mobile phase to aid isolation was considered, but not carried out due to the perceived sensitivity of Boc-DHV **172** towards deprotection under acidic conditions. Oxidation conditions were required that would permit facile separation of reagents and by-products from acid **172** which, as demonstrated through attempted isolation from TEMPO/BAIB, would likely only be feasible when utilising water-soluble oxidants. Chromium oxidants pyridinium chlorochromate (PCC) and pyridinium dichromate (PDC) had been developed expressly for oxidation under milder, less acidic reaction conditions than Jones reagent.[264] PDC has been widely demonstrated to oxidise primary alcohols to carboxylic acids when the reaction is undertaken in 'wet' DMF.[265] Addition of amino alcohol **193** to 5 eq. of PDC in DMF was carried out and stirred for 14 h. Disappointingly, amino alcohol **193** was not fully consumed upon analysis of the reaction mixture by TLC. Addition of another 5 eq. of PDC led to full consumption after a further 48 h. Aq. work up to remove PDC furnished the desired carboxylic acid product **172** in a 72% yield (**Scheme 3.12**).



193 **172**, 72%

Scheme 3.12: Oxidation of amino alcohol **193** to Boc-DHV **172** with PDC in DMF.

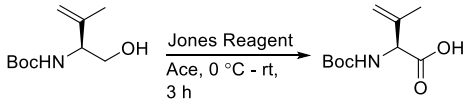
Gratifyingly, this development allowed substantial quantities of Boc-DHV **172** to be accessed permitting investigation and optimisation of subsequent peptide coupling and ATE reactions. However, the 3 days necessary for the oxidation to reach completion was not entirely practical and further oxidation conditions were sought by literature screen. Fortuitously, this led to the discovery of selective pseudoproline deprotection conditions to furnish amino alcohol **193** in one step from oxazolidine **185**, utilised by Yamakawa *et al.* in the total synthesis of tryprostatins A and B.[266] Application of TFA:Tetrahydrofuran (THF):H_2O in 4:2:1 ratio at 0 ºC for 10 min prior to basic quench at 0 ºC was reported to remove the pseudoproline group in the presence of Boc with almost total selectivity. Under these conditions amino alcohol **193** was furnished in a 92% yield (**Scheme 3.13**).



185 **193**, 92%

Scheme 3.13: Selective deprotection of oxazolidine **185** to yield amino alcohol **193** under conditions described by Yamakawa *et al.*[266]

With large quantities of amino alcohol **193** now accessible, oxidation with 2.5 M Jones reagent was again attempted. As tandem deprotection of the pseudoproline group and oxidation was no longer necessary a titration type oxidation approach as described by Berkowitz and Maiti was undertaken.[267] Slow addition of Jones reagent at 0 ºC and high dilution (0.025 M) of amino alcohol **193** followed by stirring at rt was carried out until TLC analysis indicated no amino alcohol **193** remained. Aggregation of chromium salts was not observed and aq. work up in this case furnished Boc-DHV **172** in a 96% yield (**Scheme 3.14**).



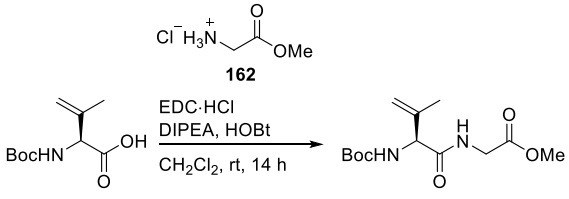
193 **172**, 96%

Scheme 3.14: Oxidation of amino alcohol **193** to Boc-DHV **172** with Jones reagent in Ace as escribed by Berkowitz and Maiti.[267]

Despite the failure to optimise the mesylation and elimination step to access protected terminal alkene **185** optimisation of the following deprotection and oxidation steps ensured Boc-DHV **172** could be accessed in gram quantities.

3.3 Synthesis of Dipeptide Acyl Thiol-ene Substrates (174, 175 and 176)

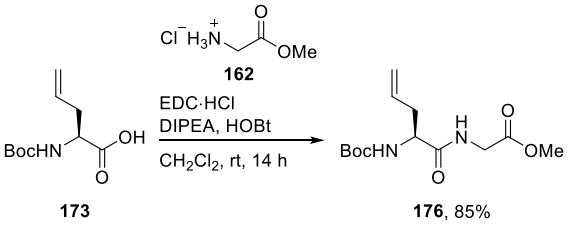
With alkenyl Boc protected UAAs Boc-VG **171** and Boc-DHV **172** in hand, the synthesis of dipeptide substrates suitable for assessing the reactivity of these UAAs towards ATE could now be undertaken. HCl·Gly-OMe **162** was chosen as the amide coupling partner to furnish Boc-UAA-Gly-OMe in all cases, primarily to simplify analysis of concurrent ^{1}H NMR spectra obtained. Boc-DHV **172** had been reported to readily undergo amide coupling under standard carbodiimide mediated conditions in literature syntheses.[255, 268] The coupling of Boc-DHV **172** and HCl·Gly-OMe **162** was carried out with EDC·HCl in the presence of DIPEA and racemisation suppression additive HOBt in CH_2Cl_2 for 14 h to yield dipeptide **175** in a satisfactory 62% yield (**Scheme 3.15**).



172 **175**, 62%

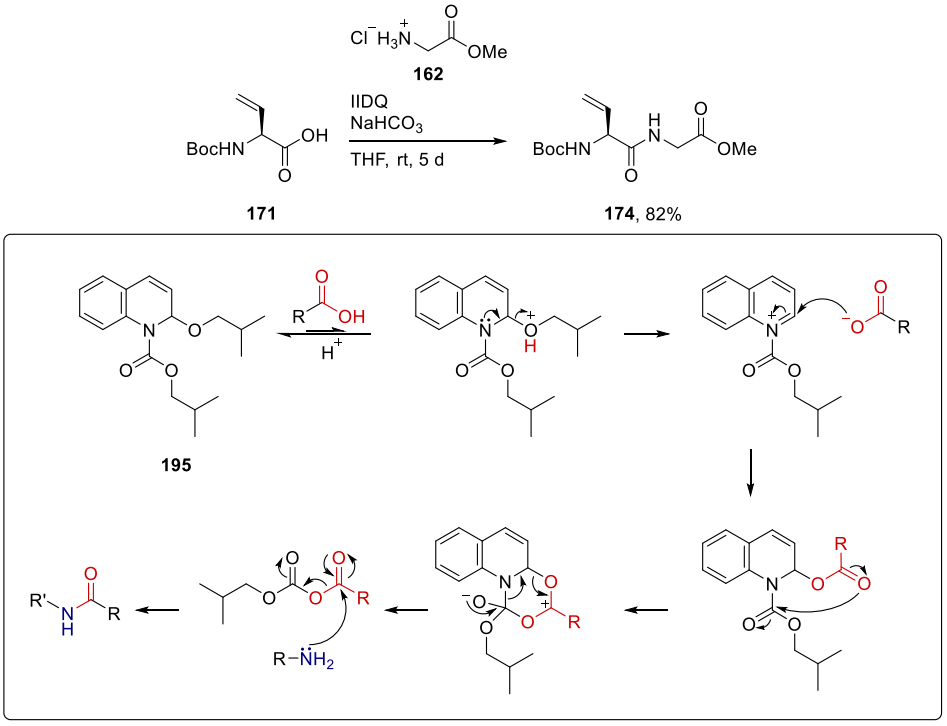
Scheme 3.15: Amide coupling of Boc-DHV **172** and HCl·Gly-OMe **162** to yield unsaturated dipeptide **175** under optimised carbodiimide mediated coupling conditions.

Boc-AG **173** was purchased and used without further purification and under the identical coupling conditions to furnish dipeptide Boc-AG-Gly-OMe **176** in an 85% yield (**Scheme 3.16**).



Scheme 3.16: Amide coupling of Boc-AG **173** and HCl·Gly-OMe **162** to yield unsaturated dipeptide **176** under optimised carbodiimide mediated coupling conditions.

As reported by Afzali-Ardakani and Rapoport, VG and its derivatives require certain precautions to ensure that neither formation of the corresponding conjugated isomer nor racemisation occur upon activation of the carboxylic acid moiety.[252] This has been accomplished in the literature by use of specialist coupling reagents such as (3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one) (DEPBT)[269] and isobutyl 1,2-dihydro-2-isobutoxy-1-quinolinecarboxylate (IIDQ).[270] IIDQ **195** was selected for our studies due to the slightly lower cost of the reagent and, as a liquid, was deemed to be simpler to manipulate under anhydrous conditions. IIDQ **195** ensures low rates of racemisation by generating the activated acid as a mixed anhydride slowly, with only a low concentration of activated acid in solution (**Scheme 3.17**).

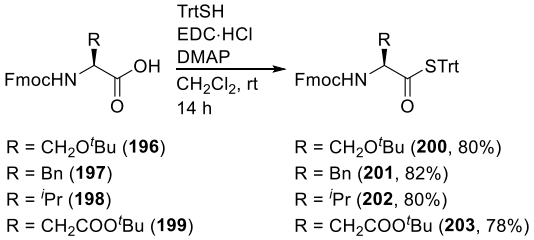


Scheme 3.17: Amide coupling of Boc-VG **171** and HCl·Gly-OMe **162** with IIDQ **195** to yield dipeptide **174**. The reaction mechanism for IIDQ coupling may also be seen.

The high relative concentration of amine in the reaction mixture therefore ensures the activated acid is consumed quickly, diminishing the opportunity for α-proton abstraction and concurrent racemisation. However, as the initial protonation of the reagent and subsequent anhydride generation is slow, long reaction times are necessary. Treatment of Boc-VG **171** and HCl·Gly-OMe **162** in the presence of IIDQ **195** and $NaHCO_3$ over 3 days furnished the desired unsaturated dipeptide **174** in an 83% yield (**Scheme 3.17**). The reaction is amenable to gram scale synthesis and long reaction times did not delay access to dipeptide **174** significantly. The disparate reactivity between Boc-DHV **172** and Boc-VG **171** in this instance is likely a result of increased steric hindrance around the α-carbon of Boc-DHV preventing efficient hydrogen abstraction and concomitant isomerisation.

3.4 Synthesis of *S*-Trityl Thioesters (200 – 203)

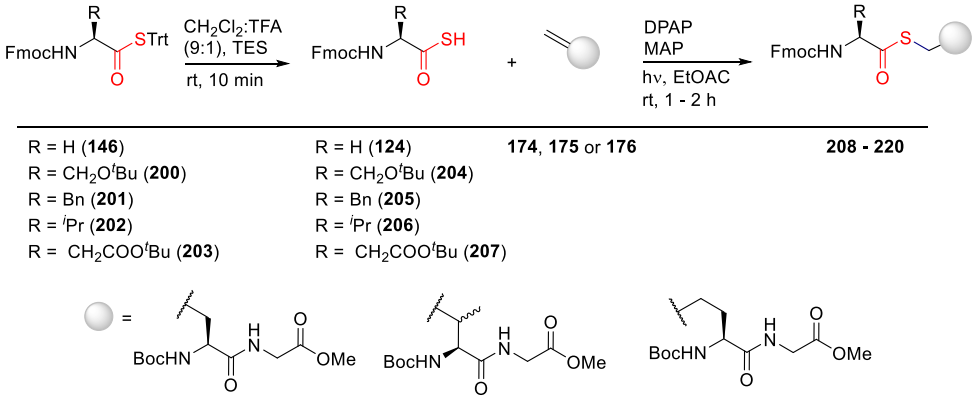
The synthesis of *S*-Trt thioesters was undertaken using conditions first described by Crich and Sharma, already applied successfully to the synthesis of Fmoc-Gly-STrt **146** for ATE reactions with auxiliary systems **140** and **141** (**Chapter 1**). In order to demonstrate the general nature and functional group tolerance of ATE ligation a variety of Fmoc protected AAs were selected (**Scheme 3.18**). After stirring of their carboxylic acid counterparts **196 - 199** overnight with EDC·HCl and stoichiometric DMAP, Ser thioester **200**, Phe thioester **201**, Val thioester **202** and Asp thioester **203** were isolated in > 78% yields after column chromatography (**Scheme 3.18**). Little difference was observed in yield between each thioester indicating that the stereo-electronic properties of each side-chain residue did not significantly affect thioester formation. The yields obtained were slightly lower than those reported by Crich and Sharma in the synthesis of Cbz protected thioesters.[221] This was likely due to loss of Fmoc through deprotection by catalytic DMAP as observed in the synthesis of auxiliary ATE acceptor **137**.



Scheme 3.18: Synthesis of Fmoc protected AA *S*-Trt thioesters **200 - 203** synthesized according to conditions described by Crich and Sharma.[221]

3.5 Acyl Thiol-ene Reactions of Unsaturated Dipeptides (174, 175 and 176)

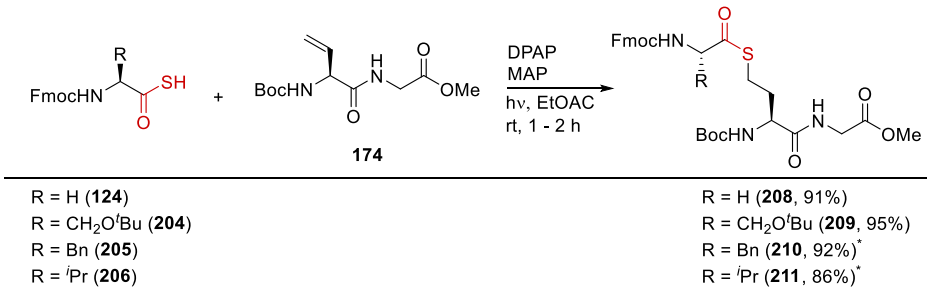
With UAA bearing dipeptides **174**, **175** and **176** and a range of Fmoc protected *S*-Trt AAs (**146**, **200 – 203**) in hand, evaluation of the reactivity of each acceptor towards ATE addition could be undertaken. The reactions were carried out under optimised conditions employed for ATE of auxiliary system **140** (**Section 2.3.2**). The thioacids were generated *in situ* through the treatment of 3 eq. of the corresponding Trt thioester with TFA in CH_2Cl_2 in the presence of cation scavenger TES. After stirring for 10 min this mixture was concentrated *in vacuo*, DPAP, MAP and the unsaturated dipeptide added, and the mixture redissolved in EtOAc prior to UV irradiation over 1 h. (**Scheme 3.19**)



Scheme 3.19: General scheme of ATE reaction to be undertaken with UAA containing dipeptides **174**, **175** and **176**.

3.5.1 Acyl Thiol-ene Reactions of Vinylglycine Dipeptide (174)

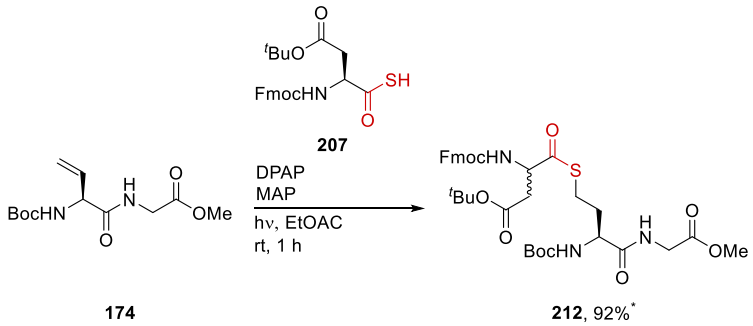
ATE reactions were first undertaken with VG dipeptide **174** and thioacids **124,** and **204 – 207**, generated *in situ* from the corresponding thioesters, under optimised ATE conditions (**Scheme 3.20**). Reaction with 3 eq. Gly thioacid **124** proceeded quantitatively to yield peptide thioester **208** in a 91% yield. Reaction of protected Ser thioacid **204** also yielded the corresponding thioester **209** in a quantitative yield (95%) after purification. However, the ATE reaction of Phe thioacid **205** with VG dipeptide **174** did not go to completion by TLC analysis and no new spot signifying product formation was observed. Crude ^{1}H NMR spectroscopy indicated VG dipeptide **174** had not been fully consumed in the reaction mixture. Integration of alkene ddd at 5.90 ppm of VG dipeptide **174** against the newly formed multiplet corresponding to the γCH_2 of Phe thioester **210** at 1.89 ppm showed a 4:1 ratio of product to SM.



Scheme 3.20: ATE reactions of VG dipeptide **174** with Fmoc-AA thioacids **124** and **204 - 206,** generated *in situ* by deprotection of Fmoc-AA Trt thioesters **146**, **200 - 202**, under optimised conditions. *5 eq. of thioacid used to fully consume VG dipeptide **174**.

Unfortunately, this signified that the R_f of VG dipeptide **174** and newly formed Phe thioester **210** were identical, precluding isolation and subsequent characterisation of the product **210** by column chromatography. TLC analysis of the reaction of VG dipeptide **174** with Val thioacid **206** suggested the same issue with the reaction of VG dipeptide **174** and formation of Val thioester **211**. ^{1}H NMR spectroscopy of the crude reaction mixture indicated a product to SM ratio of 3:1, and therefore a lower reaction conversion than that observed in the case of Phe thioacid **205**. Incomplete reaction conversion in these cases was judged to be due to either the increased steric bulk of the phenyl and valyl residues preventing efficient addition to the alkene moiety of VG dipeptide **174**, or due to an increased rate of CSO elimination observed with Phe thioacid **205** and Val thioacid **206** in comparison with Gly thioacid **124** and Ser thioacid **204**. These results could be better contextualised when compared with the results of the ATE reactions of DHV dipeptide **175** and AG dipeptide **176** in order to ensure the chemical characteristics of dipeptide **174** was not affecting the outcome of these reactions. In order for the characterisation of Phe thioester **210** and Val thioester **211** to be carried out it was necessary to consume excess VG dipeptide **174** remaining in the reaction mixtures. This was first attempted by addition of thioacetic acid **97**, DPAP and MAP to the reaction mixtures followed by UV irradiation for 1 h. It was expected that the thioacetate formed in this reaction would consume the remaining VG dipeptide and permit isolation of the desired peptide thioesters **210** and **211**. ^{1}H NMR spectroscopy of the crude reaction mixtures indicated VG dipeptide **174** had been fully consumed due to the absence of alkene signals at 5.92 and 5.40 ppm. A singlet characteristic of a thioacetate, distinct to that of thioacetic acid **97**, was observed at 2.33 ppm. However, TLC analysis of both reaction mixtures indicated no change in the reaction mixture beyond the addition of thioacetic acid **97**. Unfortunately, the thioacetate formed possessed the same R_f as Phe

thioester **210** and Val thioester **211**. This again precluded purification by column chromatography to obtain the pure thioester products. It was decided that the reactions would be repeated under the optimised ATE conditions and after 1 h of irradiation 2 eq. of thioacid added to consume the remaining VG dipeptide **174** in the reaction mixture and permit purification (**Scheme 3.20**). This permitted the isolation of Phe thioester **210** and Val thioester **211** in 95% and 82% yields, respectively. This prevented direct comparison between the isolated yields of these thioesters with those requiring only 3 eq. of thioacid to reach completion. However, these results still demonstrated the power of the ATE to furnish peptide thioesters reliably and in high yields. The final ATE reaction undertaken with VG dipeptide **174** was that with protected Asp thioacid **207** under optimised conditions (**Scheme 3.21**).

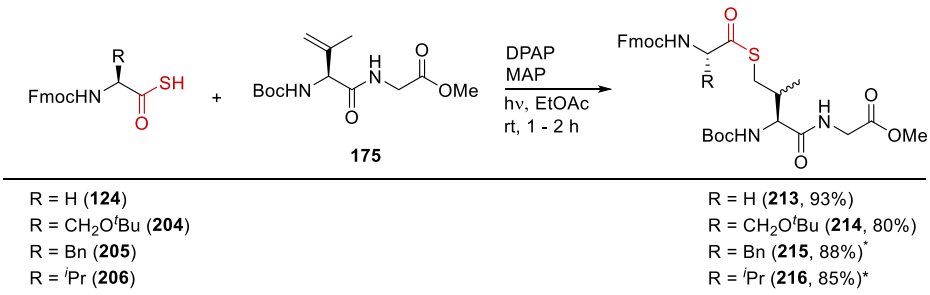


Scheme 3.21: ATE reactions of VG dipeptide **174** with Fmoc thioacid **207**, generated *in situ* by deprotection of Fmoc-AA trityl thioester **203**, under optimised conditions. *Isolated as a mixture of diastereoisomers.

This reaction proceeded with a 92% yield of Asp thioester **212** and full consumption of starting material **174** with 3 eq. of thioacid **207** after 1 h. Unfortunately, analysis of the ^{1}H NMR and ^{13}C NMR spectra of the product indicated racemisation of the Asp α-hydrogen, with substantial peak doubling, in a 3:1 ratio of epimers. This was likely to have occurred in the synthesis of Asp Trt thioester **203** through Steglich esterification with TrtSH (**Scheme 3.18**), the highly activated 4-dimethylaminopyridinium ester intermediate capable of facilitating α-hydrogen abstraction resulting in loss of optical purity. This had been reported in the literature in the synthesis of aspartate and glutamate esters under these conditions.[271] However, it was reasoned that in the case of solid phase peptide thioacid synthesis described by Liu *et al.*, among others, racemisation at the *C*-terminus would not occur due to the milder condition employed for resin coupling.[226] Therefore, the optimisation of thioesterification conditions to supress racemisation was not undertaken in this instance and Asp Trt thioester **203** was not utilised in subsequent ATE reaction with DHV dipeptide **175** and AG dipeptide **176**.

3.5.2 Acyl Thiol-ene Reactions of L-3,4-Didehydrovaline Dipeptide (175)

ATE reactions of DHV dipeptide **175** were carried out under optimised conditions with AA thioacids **124** and **204 - 206** (**Scheme 3.22**). Reaction of Gly thioacid **124** and DHV dipeptide **175** yielded Gly thioester **213** in a 93% yield (**Scheme 3.22**).

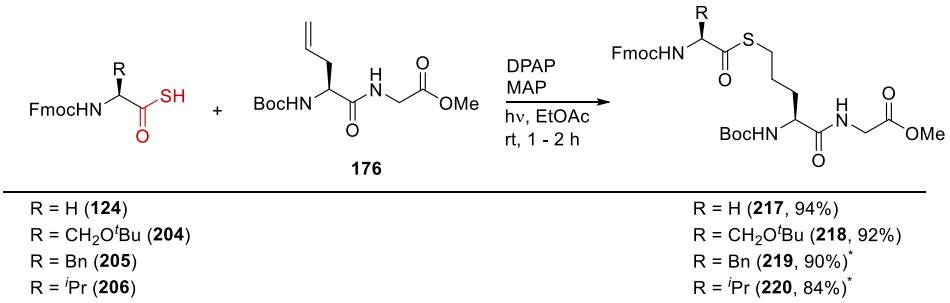


Scheme 3.22: ATE reactions of DHV dipeptide **175** with Fmoc-AA thioacids **124** and **204 - 206,** generated *in situ* by deprotection of Fmoc-AA Trt thioesters **146**, **200 - 202**, under optimised conditions. *5 eq. of thioacid used to fully consume DHV dipeptide **175**.

Under the same conditions, reaction of protected Ser thioacid **204** and DHV dipeptide **175** yielded Ser thioester **214** in an 80% yield, lower than that observed in the corresponding reaction with VG dipeptide **174** (**Scheme 3.22**). DHV dipeptide **175** was not fully consumed in the reaction mixture, but addition of the polar ^{t}Bu ether moiety permitted purification of Ser thioester **214** by column chromatography. As in the case of VG dipeptide **174**, ATE reactions with both Phe thioacid **205** and Val thioacid **206** did not go to completion, necessitating the addition of 2 eq. more of the corresponding thioacids in each reaction and irradiation for 1 h longer (**Scheme 3.22**) to aid purification. This permitted the isolation of Phe thioester **215** and Val thioester **216** in 88% and 85% yields, respectively. Reactions with DHV dipeptide **175** resulted in diastereomeric products due to the formation of a new stereogenic centre at the β Val position. The assignment of ^{1}H NMR spectra was therefore more challenging in the case of these substrates. The ratio of diastereoisomers was 1:1 to in all cases. This was expected (**Section 1.4.1**), as thiol-ene reactions typically possess poor stereoselectivity apart from in cyclic systems where facial selectivity may be enhanced with steric influence from neighbouring groups.[169, 272]

3.5.3 Acyl Thiol-ene Reactions of Allylglycine Dipeptide (176)

ATE reactions of AG dipeptide **176** were also carried out under optimised conditions with Fmoc thioacids **124** and **204 - 206** (**Scheme 3.23**).



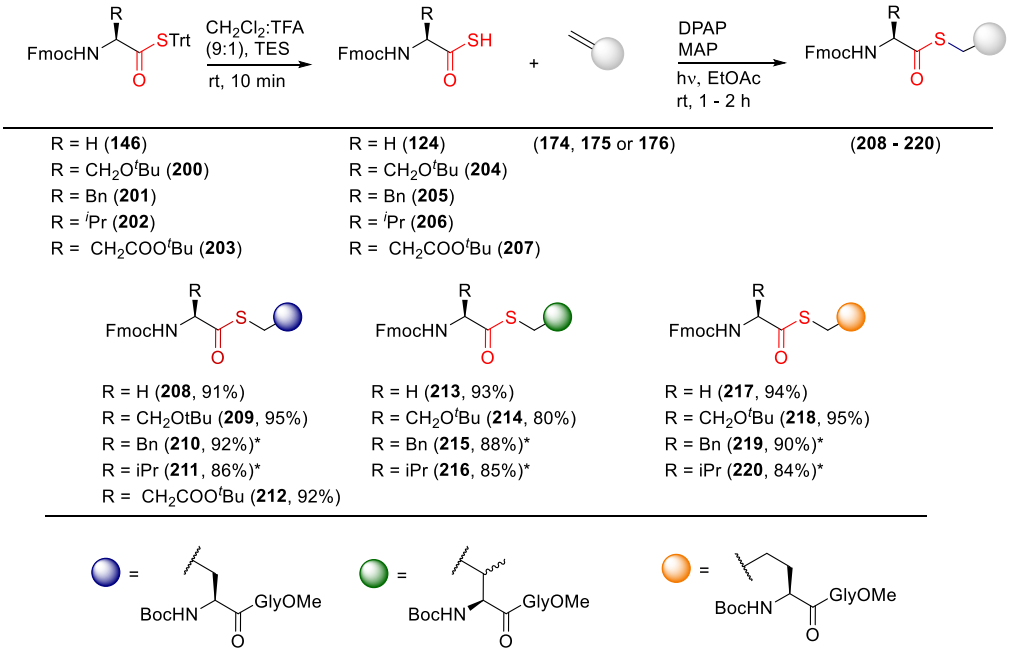
Scheme 3.23: ATE reactions of AG dipeptide **176** with Fmoc-AA thioacids **124** and **204 - 206,** generated *in situ* by deprotection of Fmoc-AA Trt thioesters **146**, **200 - 202**, under optimised conditions. *5 eq. of thioacid used to fully consume AG dipeptide **176**.

Again, the reaction of Gly thioacid **124** and AG dipeptide **176** under these conditions proved high yielding, furnishing the desired Gly thioester product in a 94% yield after purification (**Scheme 3.23**). Equally, the ATE of protected Ser thioacid **204** and AG dipeptide **176** furnished the Ser thioester product **218** quantitatively, in a 95% yield (**Scheme 3.23**). As in the previous ATE reactions with dipeptides **174** and **175** reactions between Phe thioacid **205** or Val thioacid **206** and AG dipeptide **176** did not go to completion with 3 eq. of thioacid. Addition of a further 2 eq. of thioacid coupled with 1 h of UV irradiation proved sufficient to consume AG dipeptide **176** in both cases and permit efficient separation of the reaction mixtures (**Scheme 3.23**). Phe thioester **219** and Val thioester **220** were isolated in 90% and 84% yields, respectively. With the results of these ATE reactions with UAA containing dipeptides **174**, **175** and **176** in hand the results could be rationalised in terms of the contributions of both alkene and thioacid components.

3.5.4 Effect of Dipeptide Acceptors on Acyl Thiol-ene Reactions

The results of ATE reaction between UAA dipeptide acceptors **174**, **175** and **176** and a variety of Fmoc thioacids are summarised in **Scheme 3.24.** Generally, acceptors **174**, **175** and **176** all possessed good reactivity towards ATE addition with the Fmoc protected thioacids screened. 3 eq. of Gly thioacid **124** reacted with acceptors **174**, **175** and **176** quantitatively in each case, the low steric demands of the substrate likely contributing to the high isolated yields. (**Scheme 3.24**) More variability was observed in the isolated

yields of thioesters **209**, **214** and **218** resulting from the reaction of Ser thioacid **204** and the UAA dipeptides **174**, **175** and **176**. ATE reaction of VG dipeptide **174** and AG dipeptide **176** yielded Ser thioesters **XX** and **XX** in a 95% yield in both cases (**Scheme X.X**).



Scheme 3.24: Summary of the results of ATE reactions of UAA containing dipeptides **174**, **175** and **176** and Fmoc thioacids **124** and **204 - 207,** generated *in situ* by deprotection of Fmoc-AA trityl thioesters **146** and,**200 – 203** under optimised conditions. *5 eq. of Fmoc thioacid used to fully consume UAA dipeptides **174, 175** or **176**.

The isolated yield of Ser thioester **214** resulting from reaction of Ser thioacid **204** and DHV dipeptide **175** was lower at 80% (**Scheme 3.24**). This was likely as a result of increased steric hindrance around the alkene moiety of DHV dipeptide **175** in comparison with dipeptides **174** and **176**. The ^{t}Bu ether group would not only disrupt efficient approach of the thioacid towards the reactive alkene centre but also form a higher energy *C*-centred radical intermediate upon addition. As the first addition step of the ATE reaction is reversible (**Section 1.4.1**) this high energy hindered intermediate would be more prone to fragmentation, reforming dipeptide **175** and the corresponding acyl thiyl radical. This has the effect of reducing the rate of formation of the desired thioester product and increasing the likelihood of CSO elimination. In the reaction of Phe thioacid **205** with dipeptides **174**, **175** and **176** a slight decrease in yield from VG thioester **210** to AG thioester **219** was observed from 92% to 90%, respectively (**Scheme 3.24**). The yield of DHV thioester **215** isolated after reaction of dipeptide **175** and Phe thioacid **205** was slightly lower at 88%, again likely as a result of the more demanding sterics at the alkene

moiety of DHV derived dipeptide **175**. The yields of Val thioesters **211**, **216** and **220** obtained through reaction of dipeptides **174**, **175** and **176** with Val thioacid **206** were almost identical (86%, 85% and 84%, respectively). As in the case of Phe thioacid **205**, the ATE reactions with Val thioacid **206** required the addition of 2 eq. to ensure full consumption of alkene starting materials. The greater similarity between the isolated yields of thioesters obtained in reactions with Phe thioacid **205** and Val thioacid **206** suggested that the addition of 2 eq. of thioacid to the reaction mixture in these cases may have concealed the influence of the alkene component on ATE reaction yields. However, consultation of the crude ^{1}H NMR spectra of the ATE reactions prior to addition of 2 eq. of thioacid allowed some general trends to be ascertained. ATE reactions of Phe thioacid **205** and Val thioacid **206** with VG dipeptide **174** and AG dipeptide **176** displayed higher alkene conversions than corresponding ATE reactions with DHV dipeptide **175** after 1 h**.** This observation is correlates well with the isolated yields obtained for ATE with Ser thioacid **204**, indicating that the increased steric hindrance around the alkene moiety of DHV dipeptide **175** impeded thioester formation. These results highlight the importance of alkene availability when considering the viability of ATE reactions.

3.5.5 Effect of Fmoc Thioacids on Acyl Thiol-ene Reactions

As previously discussed (**Section 2.3.2**), work within the Scanlan group by Dr Lauren McSweeney had identified CSO elimination as a significant side reaction within ATE addition. Until recently, little work had been carried out within the literature to study dethiocarboxylation beyond that employing high energy radiolysis.[273] However, in 2016 Shimizu *et al.* published a novel synthetic methodology to access peptidic alkyl amides. Peptide thioacids were synthesised with the desired *C*-terminal UAA bearing an alkylic sidechain.[274] Cleavage from SPPS to generate the peptide thioacids (**Section 1.5**) followed by addition of VA-044 radical initiator and heating to 37 ºC furnished a range of alkylated amide products *via* dethiocarboxylation.[274] However, the mechanism of the reaction is not explored in any detail. A proposed mechanism of the ATE reaction and concurrent CSO elimination may be seen in **Figure 3.5**. Upon radical initiation forming thiyl radical **B** from thioacid **A**, spontaneous elimination of CSO **C** forms *C*-centred radical **D (Figure 3.5**). Hydrogen abstraction from another thioacid **A** results in dethiocarboxylated product **E** and another thiyl radical **B** (**Figure 3.5**). Alkylamide **XX**

resulting from the CSO elimination of Fmoc-Ala thioacid **XX** was isolated by Dr Lauren McSweeney providing evidence for this mechanism (**Figure 3.5**).[275]

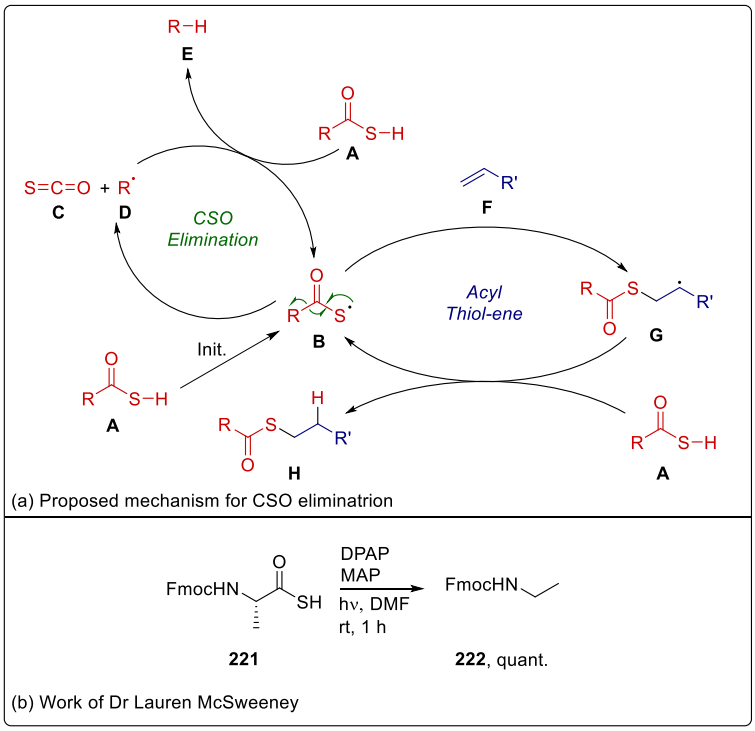


Figure 3.5: (a) Proposed mechanism for CSO elimination and ATE addition. (b) Isolation of alkylamine **222** from CSO elimination of Ala thioacid **221** under optimised ATE conditions.

Key to understanding the results of the reaction of thioacids **124** and **204 – 207** with UAA containing dipeptides **174**, **175** and **176** is the prevalence of CSO elimination as opposed to ATE addition in these reactions. The rate of hydrogen abstraction necessary to form dethiocarboxylation product **E** and thioester product **H** is high as a result of the excess of thioacid **A** in the reaction mixture and presence of residual TES from thioester deprotection to generate **A** *in situ* (**Figure 3.5**). Therefore, these chain transfer processes are unlikely to be the RDS in either the ATE or CSO elimination mechanism. The relative rates of alkene addition and CSO elimination will therefore determine the efficiency of thioester formation. The rate of CSO elimination is determined by the stability of the *C*-centred radical formed after elimination. Destabilised *C*-centred radicals formed during thiol-ene reactions form at slower rates as a consequence of the high energy barrier to their formation (**Section 1.4.1**). In the case of competing CSO elimination and ATE addition, the formation of more a stable radical *via* CSO elimination will consume the thioacid component of the reaction at a greater rate, thereby slowing down ATE addition. Comparison of the *C*-centred radicals formed by CSO elimination of thiyl radicals of

thioacids **124** and **204 – 207** was undertaken to better understand the trends seen during ATE reactions with dipeptides **174**, **175** and **176** (**Figure 3.6**).

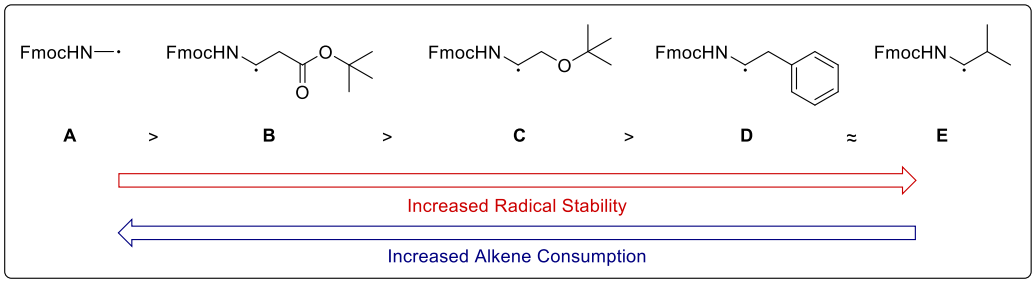


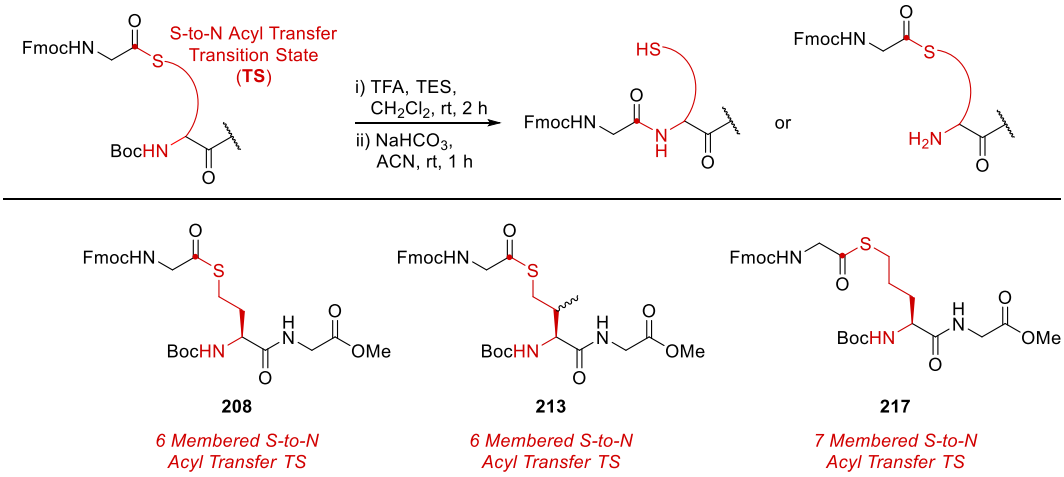
Figure 3.6: Relative stability of *C*-centred radicals resulting from the CSO elimination of thioacid **124** and **204** - **207** under ATE conditions and relationship to alkene consumption.

Consultation of the literature permitted comparisons of the radical stabilisation energies (RSEs) of *C*-centred radicals **A – E** (**Figure 3.6**). The precise RSEs of these species has not been calculated thus far in the literature. However, assuming the contribution towards radical stability of the NHFmoc constituent is the same in each case, comparisons may be drawn from *ab initio* calculation of RSEs of monosubstituted *C*-centred radicals within the literature. Radical **A**, as a primary *C*-centred radical, is the least stable relative to radicals **B – E** as the lack of substituents precludes radical stabilisation through hyperconjugation.[276] Radical **B**, although a secondary *C*-centred radical, is destabilised by the electron withdrawing nature of the ester group. Radical **C** is comparatively more stable than radical **B** due to the electron donating nature of the former's ether moiety. The relative effect of these substituents on RSE has been well explored in the literature.[277] The relative RSEs of **D** and **E** are similar (0.6 kJ mol^{-1} difference) and are well characterised, as these types of alkylic and aromatic secondary radicals are of interest in the development of novel polymerisation methodologies.[278] In the case of the performed ATE reactions, a link can be found between the number of eq. of thioacid necessary for full alkene conversion and the relative stability radicals **A – B** and **D** – **E**. CSO elimination is not favoured in the case of **A – B** as a result of low stability of the resulting *C*-centred radical. A higher concentration of thiyl radical for ATE addition is therefore available, leading to the higher alkene consumption. The inverse is true for **D – E**, the increased stability of the *C*-centred radicals formed by CSO elimination leads to increased consumption of thiyl radicals by this pathway, and less efficient ATE addition. RSEs of **C** and **D/E** have not been directly compared in the literature using the same *ab initio* methodologies. However, the higher yields resulting from ATE reactions of Ser thioacid **204** and dipeptides **174**, **175** and **176** would suggest radical **C** possesses less

stability than radical **D** or **E**. Full reaction conversion was observed between dipeptides **174**, **175** and **176** with 3 eq. of Ser thioacid **204** in all cases, suggesting that CSO elimination is less favourable, as with Gly thioacid **124** and Asp thioacid **207**. However, this assumes that the ^{t}Bu ether moiety of thioacid **204**, benzyl moiety of thioacid **205** and *iso*-propyl group of thioacid **206** have similar steric characteristics with regards to ATE addition. In reality, the efficiency of the ATE reactions of thioacids **204**, **205** and **206** is a function of both the RSE of radicals formed through CSO elimination and the degree of side-chain steric hindrance upon alkene addition. More work is required to independently assess the contributions of these factors in ATE reactions.

3.5 Studies of S-to-N Acyl Transfer of Thioester Products (208, 213 and 217)

With the ATE reaction having proven capable of reliably furnishing thioester products, the S-to-N acyl transfer characteristics of each UAA based system could be evaluated. It was decided that thioesters derived from the reaction of Fmoc-Gly thioacid **124** and UAA containing dipeptides **174**, **175** and **176** would be evaluated, as the ^{1}H NMR spectrums of these products would enable more straightforward analysis (**Scheme 3.25**).



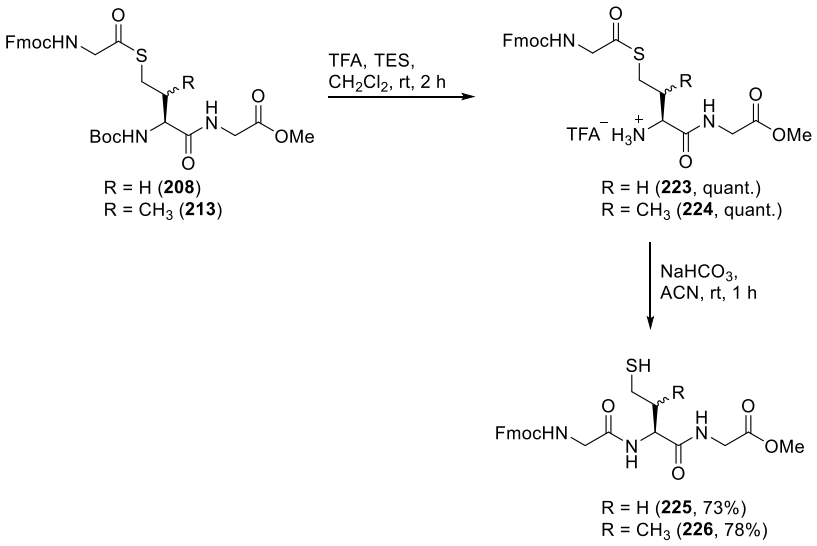
Scheme 3.25: General scheme of the deprotection and freebasing of Gly thioesters **208**, **213** and **217** to investigate the S-to-N acyl transfer characteristics of each.

Deprotection of the Boc group of thioesters **208**, **213** and **217** was to be carried out under the conditions optimised for the attempted S-to-N acyl transfer of auxiliary bearing Ser dipeptide **140** (**Section 2.3.3**). This engendered the deprotection of the Boc group with TFA in the presence of radical scavenger TES, prior to stirring in ACN with heterogenous $NaHCO_3$. TLC analysis would be utilised to ensure reaction conversion prior to inspection of the crude ^{1}H NMR spectra of the reaction mixtures. Column

chromatography would then permit access to the pure products of the reactions for characterisation by NMR spectroscopy.

3.6.1 Boc Deprotection and Freebasing of Boc Protected Thioesters (208 and 213)

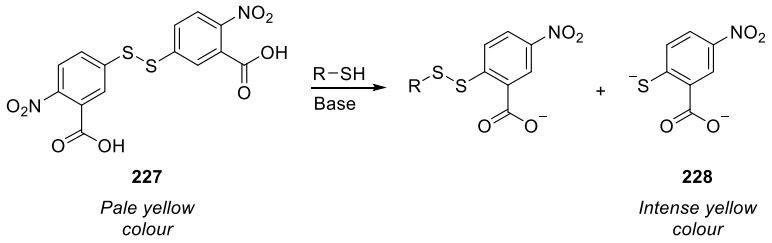
The Boc deprotections of VG dipeptide derived thioester **208** and DHV derived thioester **213** were undertaken first (**Scheme 3.26**). As already discussed (**Section 3.0)**, S-to-N acyl transfer of ligation intermediates possessing 6 membered homocysteinyl and valyl TSs had been demonstrated independently by Tam[243] and Danishefsky.[244] Therefore, deprotection and freebasing could be carried out with confidence that the desired amide product would be isolated and that any issues encountered would likely be as a result of the deprotection/freebasing conditions employed rather than the innate characteristics of the systems in question. These conditions could then be further optimised prior to deprotection of AG derived thioester **217** which had not been demonstrated in the literature to furnish amide products through S-to-N acyl transfer. Boc deprotection of thioesters **208** and **213** proceeded smoothly to yield the corresponding trifluoroacetate salts **223** and **224** quantitatively (**Scheme 3.26**).



Scheme 3.26: Boc deprotection and freebasing of VG derived thioester **208** and DHV derived thioester **213** to yield thiolated tripeptides **225** and **226** *via* S-to-N acyl transfer.

^{1}H NMR and ^{19}F NMR spectroscopy of the crude reaction mixtures indicated that upon removal of TFA and CH_2Cl_2 *in vacuo* the trifluoroacetate salts had been obtained. Residual TES and associated silyl salt signals were also present in the crude NMR, yet as these by-products were unlikely to impede S-to-N acyl transfer, trifluoroacetates **223** and **224** were freebased without further purification. Dissolution of trifluoroacetate salts

223 and **224** in ACN was carried out followed by addition of an excess of $NaHCO_3$. The heterogeneous mixtures were stirred until TLC analysis indicated full consumption of trifluoroacetates **223** and **224** within 1 h. In the case of the freebasing of VG derived trifluoroacetate **223**, TLC analysis indicated the formation of two new products at R_f values of 0.4 and 0.2 (EtOAc). Ninhydrin stain in the absence of heating did not indicate the presence of a free amine in the mixture. A solution of 5,5′-dithio-bis(2-nitrobenzoic acid) **227** (Ellman's reagent) was utilised in order to verify the presence of a free thiol in the reaction mixture. Ellman's reagent **227** was introduced by Ellman in 1959 in order to quantitatively measure the presence of Cys residues within isolated proteins.[279] Addition of the reagent and a hindered base to a solution containing a nucleophilic thiol results in the generation of 1 eq. of thiolate species **228**, which possesses an intense yellow colour, the intensity of which is proportional to concentration of thiol in solution (**Scheme 3.27**).



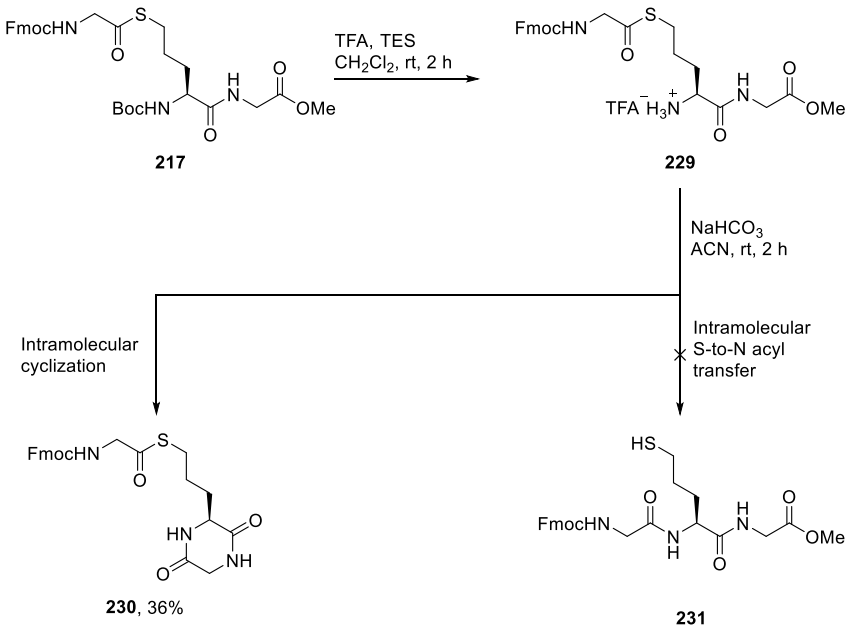
Scheme 3.27: Reaction of Ellman's reagent **227** in reaction with a thiol to produce bright yellow anion **228.**

Ellman's reagent **227** can also be employed qualitatively. Addition of a thiol containing solution to Ellman's reagent and base dissolved in MeOH instigates a colour change from pale to bright yellow. An aliquot of the reaction mixture resulting from the freebasing of trifluoroacetate **223** was treated with a solution of Ellman's reagent and gratifyingly changed from colourless to bright yellow, indicative of the presence of a free thiol. Subsequent column chromatography of the reaction mixture yielded thiol **225** in a 73% yield (**Scheme 3.26**). 2D ^{1}H NMR, ^{13}C NMR and ^{15}N NMR and spectroscopy were utilised to characterise thiol **225** and ensure S-to-N acyl transfer had taken place. In particular, the disappearance of a signal at 198.1 ppm in the ^{13}C NMR spectrum corresponding to the thioester carbonyl of Gly thioester **208** and the appearance of a fourth carbonyl signal at 169.6 ppm indicated successful amide bond formation. The loss of yield may have been as a result of disulfide formation, or more likely simply through the synthetic manipulation necessary to remove heterogenous $NaHCO_3$. Freebasing of trifluoroacetate **224** resulting from Boc deprotection of DHV thioester **213** was carried

out under the same conditions (**Scheme 3.26**). TLC analysis of the reaction mixture indicated the formation of two products of comparable R_f value (0.4 and 0.5, EtOAc). Treatment of an aliquot of the reaction mixture with Ellman's reagent indicated the presence of a thiol in the mixture and following column chromatography thiol **226** was isolated as a mixture of diastereoisomers in a 1:1 ratio in a 78% yield. Gratifyingly these results provided the first amide products furnished through ATE mediated peptide ligation. With these amide products in hand the S-to-N acyl transfer characteristics of AG derived thioester **217** could be evaluated.

3.6.2 Boc Deprotection and Freebasing of Allylglycine Derived Thioesters (217 and xx)

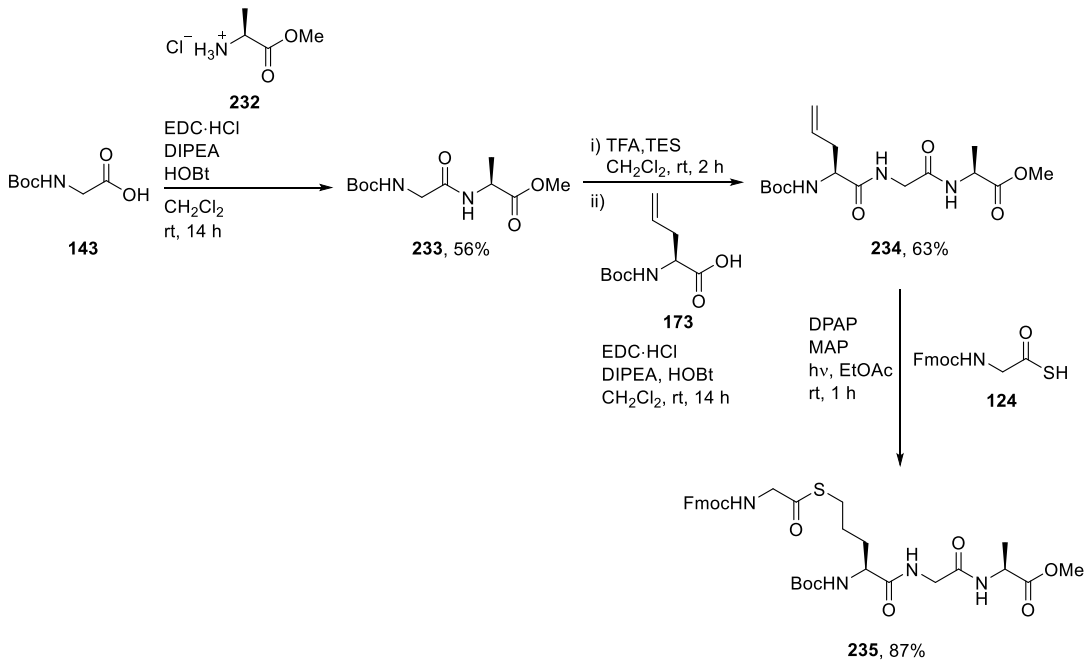
Boc deprotection of AG derived peptide thioester **217** was carried out under optimised conditions (**Scheme 3.28**). ^{1}H NMR and ^{19}F NMR spectroscopy of the crude reaction mixture indicated quantitative conversion to trifluoroacetate **229**. Dissolution of the reaction mixture in ACN and addition of $NaHCO_3$ was subsequently carried out (**Scheme 3.28**).



Scheme 3.28: Boc deprotection and freebasing of AG derived thioester **217** to yield cyclised substituted 2,5-diketopiperazine product **230**.

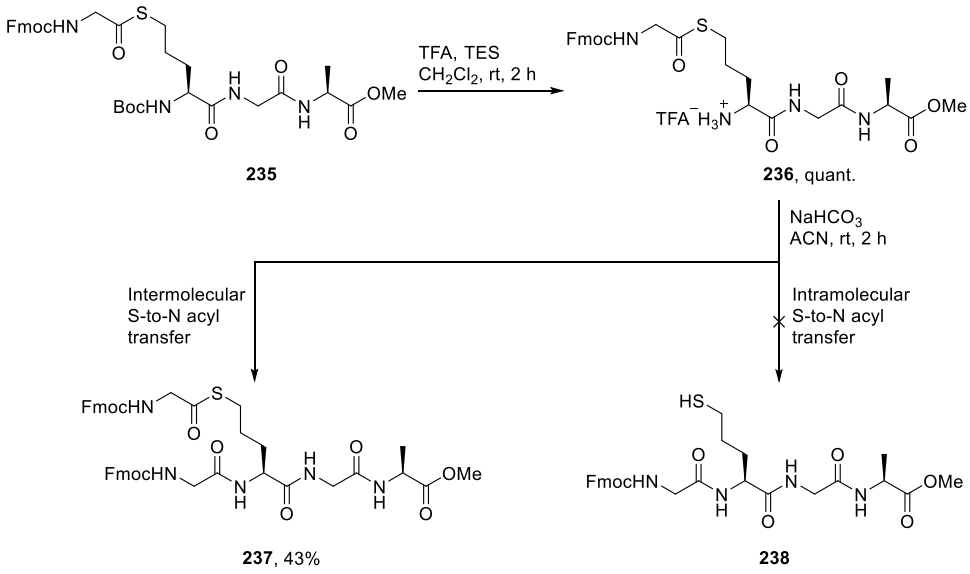
TLC analysis indicated the consumption of trifluoroacetate **229**. However, treatment of an aliquot of the reaction mixture with a solution of Ellman's reagent **227** and DIPEA did not indicate the presence of a thiol in the resulting solution. TLC analysis of the reaction mixture indicated no products had formed of similar polarity to free thiols **225**

and **226** resulting from S-to-N acyl transfer of VG and DHV derived trifluoroacetates **223** and **224** but two products of significantly higher polarity (R_f 0.5 and 0.7, CH_2Cl_2:MeOH, 15%). Filtration to remove $NaHCO_3$ and removal of ACN *in vacuo* yielded material insoluble in $CDCl_3$, D_2O and CD_3OD. Dissolution in d_6-dimethylsulfoxide (d_6-DMSO) through sonication permitted ^{1}H NMR spectroscopic analysis of the mixture and indicated the presence of two major products. Column chromatography furnished substituted 2,5-diketopiperazine **230**, as the major product of the reaction, in 36% yield. The absence of a methyl ester signal in the ^{1}H NMR spectrum and mass spectroscopy confirmed the identity of the product. The free amine intermediate of the reaction was also isolated in a trace amount, albeit impurely. Free thiol **231** was not isolated from the reaction mixture. Consultation of the literature indicated that the formation of 2,5-diketopiperazines from dipeptides is a known phenomenon, capable of occurring under basic conditions such as during Fmoc deprotection.[280] The low yield of substituted 2,5-diketopiperazine **230** was likely due to the difficulty in purification of the crude reaction mixture by column chromatography. Unprotected 2,5-diketopiperazine species bearing organic sidechains such as **230** are notoriously difficult to solvate, as the high capacity for hydrogen bonding of the diamide cyclic moiety prevents dissolution in organic solvents, and the organic side-chain prevents effective dissolution in polar solvents.[280] This resulted in problematic column loading and very slow elution, leading to band broadening and concomitant loss of product. Isolation of 2,5-diketopiperazine **230** from the deprotection and freebasing of thioester **217** was problematic, indicating the likelihood of furnishing amide products via S-to-N acyl transfer of AG based systems, at reasonable rates, was low. These results suggested that O-to-N acyl transfer over a 6 membered TS was favoured over S-to-N acyl transfer over a 7 membered TS, despite the relative leaving group abilities of methoxide and thiolate components favouring the latter transformation. However, 2,5-diketopiperazine formation may have only been possible as a result of the slower kinetics of S-to-N acyl transfer over a 7 membered, and the difficult purification of the reaction mixture may have concealed the presence of the quantities of the desired amide product **231**. Therefore, a redesign of AG derived dipeptide **176** was necessary to prevent 2,5-diketopiperazine formation. Tripeptide substrate **235** was designed to impede 2,5-diketopiperazine formation by creating a larger and more unfavourable O-to-N acyl transfer TS, thereby promoting S-to-N acyl transfer. The synthesis was carried out in the same manner as dipeptide **176**, but with the inclusion of an additional *C*-terminal Ala coupling step (**Scheme 3.29**)



Scheme 3.29: Synthesis of AG tripeptide **235** and subsequent ATE reaction with Gly thioacid **124** under optimised conditions to yield thioester **235.**

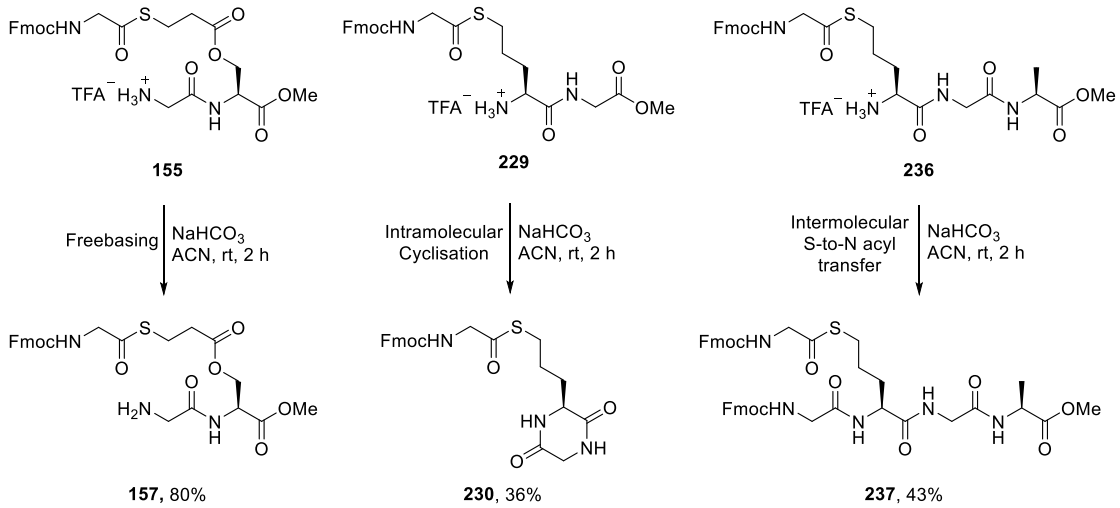
Carbodiimide mediated peptide coupling of Boc-Gly **143** and HCl·Ala-OMe **232** under optimised conditions proceeded smoothly to furnish dipeptide **233** in a moderate yield of 56%. Sequential Boc deprotection of dipeptide **233** and coupling of Boc-AG **173** furnished the desired AG tripeptide **234** in a 63% yield. ATE reaction of Gly thioacid **124**, generated through deprotection of Gly Trt thioester **146**, and AG tripeptide **234** under optimised conditions furnished the desired thioester **235** in an 87% yield. Gratifyingly, complete consumption of AG tripeptide **234** was observed in 1 h, as in the case of ATE reactions with acceptors **174**, **175** and **176** with Gly thioacid **124**. Investigation into the S-to-N acyl transfer characteristics of tripeptide thioester **235** could now be undertaken without competing O-to-N acyl transfer. Deprotection of thioester **235** by treatment with TFA and TES gave the corresponding trifluoroacetate **236** in quantitative yield (**Scheme 3.30**). Dissolution of trifluoroacetate **236** in ACN was followed by addition of $NaHCO_3$ and stirring. TLC analysis of the crude indicated the formation of a complex mixture of products. However, ^{1}H NMR spectroscopy of the crude reaction mixture obtained after filtration and removal of solvent *in vacuo* produced a spectrum similar in appearance to thioester starting material **235**. Subsequent column chromatography yielded bis-acylated product **237** in a 43% yield, as the major reaction component (**Scheme 3.30**).



Scheme 3.30: Boc deprotection and freebasing of AG derived tripeptide thioester **235** to yield substituted bis-acylated product **237**.

This was confirmed by the presence of a characteristic thioester carbonyl peak at 199.0 ppm in the ^{13}C NMR spectrum of the product, indicating S-to-N acyl transfer to furnish free thiol **238** had not occurred. Two signals at 156.5 and 156.4 ppm in the ^{13}C NMR spectrum of the product corresponding to two carbamate carbonyls within the molecule in addition to doubling of Fmoc proton integration in the ^{1}H NMR spectrum confirmed bis-acylation had occurred. This signified that intramolecular S-to-N acyl transfer was not favoured in this instance. However, unlike in the case of acryloylated trifluoroacetate **155** (**Section 2.3.3**) and dipeptide trifluoroaceate **229,** intermolecular acylation was observed upon freebasing (**Scheme 3.30**). The difference in reactivity between the trifluoroacetates **155**, **229** and **236** upon freebasing cannot be justified electronically, as the thioester carbonyl and amine moieties within each molecule possess very similar electronic environments, and hence similar electro- and nucleophilicity. The isolation of free amine **157** from the freebasing of trifluoroacetate **155** in high yield suggests that the latter compound adopts a conformation in solution which prohibits both intra- and intermolecular acyl transfer. The presence of the Ser ester moiety in trifluoroacetate **155** also introduces the possibility of intramolecular hydrogen bond type interactions, possibly reducing the nucleophilicity of the free amine upon freebasing. However, this would necessitate hydrogen bonding interactions over unfavoured 7 or 9 membered cyclic TS. Therefore, these interactions would not likely lead to the complete inhibition

2,5-diketopiperazine formation over a more thermodynamically favoured 6 membered TS, as observed during freebasing of dipeptide trifluoroacetate **229**.



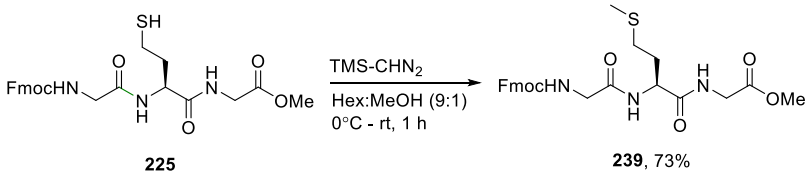
Scheme 3.31: Comparison of the reactivity of trifluoroacetates **155**, **229** and **236** upon freebasing.

As mentioned previously, intramolecular S-to-N acyl transfer over a 7 membered TS has been demonstrated in the case of a thiolated Trp derivative for ligation/desulfurisation in the work of Malins *et al.* (**Section 1.3.4**).[145] However, AG derived trifluoroacetates **229** and **236** did not possess the desired S-to-N acyl transfer characteristics when freebased. This is justifiable, as the intrinsic rigidity of the Trp residue is capable of ensuring a reactive conformation for acyl transfer upon thioester formation. Bis-acylation suggests that the conformation of tripeptide **237** prevents intramolecular S-to-N acyl transfer. However, the free amine formed after freebasing of trifluoroacetate **236** possesses ample nucleophilicity to react intermolecularly. This suggests that the conformation adopted by the free amine prevents efficient nucleophilic attack. The two AG derived systems synthesised proved incapable of reliable S-to-N acyl transfer to form the desired amide products in our hands. However, the efficient S-to-N acyl transfer to furnish thiols **225** and **226** over a 6 membered TS permitted further investigation into the synthetic elaboration of these substrates into their native counterparts.

3.7 Methylation of Thiolated Tripeptide (225)

Methylation of homocysteine residues had been reported in the literature by Tam *et al.* in the development of a novel ligation strategy at Met.[243] This was undertaken using methyl 4-nitrobenzenesulfonate in a fiftyfold excess, conditions that were deemed impractical in obtaining sufficient quantities of methylation products for characterisation

by NMR spectroscopy. Trimethylsilyldiazomethane (TMS-CHN_2), although likely too indiscriminate a methylating agent for use on unprotected peptides bearing nucleophilic residues, had been used to effectively methylate at variety of nucleophilic sites within the literature. Although thiols have not been among these, diazomethane, the less stable precursor of TMS-CHN_2, had been demonstrated to effectively methylate thiol groups.[281] The methylation of free thiol **225** was undertaken under conditions previously used with the Scanlan lab for the methylation of carboxylic acids, adopted from Hashimoto *et al.*[282] (**Scheme 3.32**).



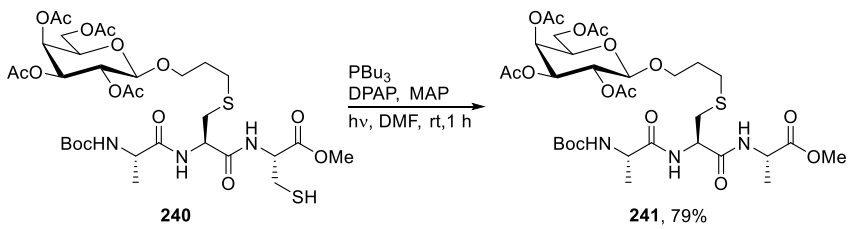
225 **239**, 73%

Scheme 3.32: Methylation of thiolated tripeptide **225** to yield tripeptide **239** under conditions adapted from Hashimoto *et al*.[282]

Dissolution of free thiol **225** in MeOH:Hexane (Hex), prior to slow addition of TMS-CHN_2 at 0 ºC, stirring at rt, and quenching with AcOH permitted isolation of Met tripeptide **239** in a 73% yield after column chromatography. The appearance of a characteristic Met methyl signal at 15.3 ppm in the ^{13}C NMR spectrum confirmed the identity of the product.

3.8 Desulfurisation of Thiolated Tripeptide (226)

As previously discussed (**Section 1.3.4**), desulfurisation utilising phosphine reagents is now common place across chemical protein synthesis in ligation/desulfurisation methodologies. However, these protocols typically rely on thermally activated initiators and water-soluble phosphines. As the aq. solubility of free thiol **226**, isolated from the Boc deprotection and freebasing of DHV derived thioester **213**, was likely to be poor, conditions in organic solvent were sought. Fortuitously, such conditions had previously been employed within the Scanlan group to access glycosidic tripeptide **241** (**Scheme 3.33**).[161]

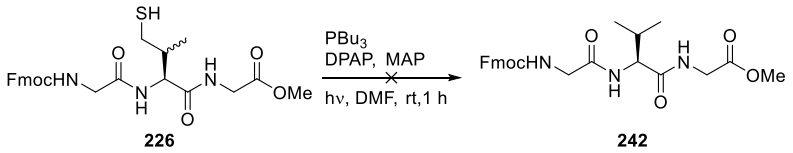


240 **241**, 79%

(a) Markey *et al.* (2013)

Scheme 3.33: The work of Markey *et al.* describing desulfurisation in organic media with tributyl phosphine, used to furnish thiolated glycopeptide **240**.[161]

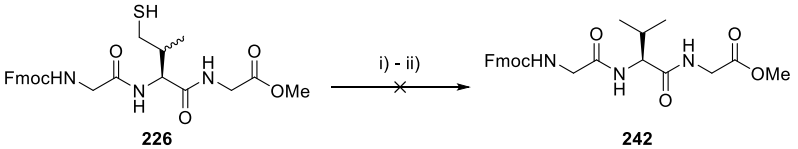
Glycopeptide **240** was irradiated for 1 h in the presence of tributylphosphine (PBu_3), DPAP and MAP in DMF. This yielded the desulfurised tripeptide product **241** in a 79% yield after column chromatography. Desulfurisation of DHV derived free thiol **226** was attempted under these conditions (**Scheme 3.34**).



226 **242**

Scheme 3.34 Attempted desulfurisation of thiolated tripeptide **226** to yield tripeptide **242** under conditions described by Markey *et al.*[161]

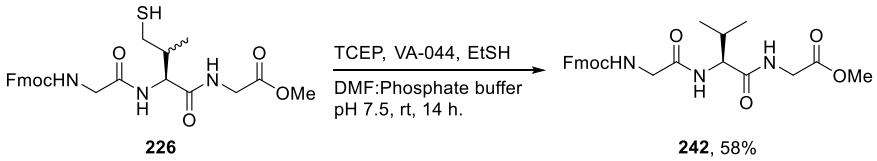
TLC analysis indicated full consumption of thiol **226**. ^{1}H NMR spectroscopy of the crude reaction mixture proved inconclusive and subsequent purification by column chromatography did not furnish tripeptide product **242** purely. In particular, the tributylphosphine sulfide by-product of the reaction proved prone to streaking on silica. Trituration with Hex and diethyl ether (Et_2O) proved ineffective in removing phosphine impurities from the reaction mixture. Water soluble phosphine TCEP is the phosphine reagent of choice within the literature for desulfurisation of peptides. It was decided that a cosolvent system utilising an organic solvent in tandem with aq. buffer would permit the ready removal of water-soluble phosphine TCEP, thereby simplifying product isolation. Conditions optimised with TCEP may also be applicable to larger peptide examples and could therefore be screened during the desulfurisation of free thiol **226**. Conditions published by Chisholm *et al.* described the desulfurisation of thiolated peptides under UV irradiation.[283] This was deemed more desirable than the thermally activated methodologies usually employed, as it permits the possibility of sequential ATE mediated ligation and desulfurisation under UV irradiation. Free thiol **226** was solubilised in DMF:HEPES buffer (pH 7.5) prior to the addition of TCEP, GSH as a hydrogen abstraction source, and water-soluble radical initiator VA-044, and the resulting mixture irradiated for 1 h (**Scheme X.X**).



226 **242**

Scheme 3.35: Attempted desulfurisation of free thiol **226** to tripeptide **242** under conditions of Chisholm *et al.*[283] and Haase *et al.*[134] i) TCEP, VA-044, GSH, hν, DMF:HEPES buffer, pH 7.5, rt, 1 h. ii) TCEP, VA-044, GSH, hν, DMF:HEPES buffer, pH 7.5, 50 ºC, 1 h.

Although used by Chisholm *et al.*, guanidinium chloride (Gdn·HCl) and 2-methylimidazole (MIM) were excluded from the reaction mixture as their roles to regulate folding and enhance the rate of NCL, respectively, were not needed in this application.[283] Organic extraction of the resulting aq. solution permitted facile product isolation as expected. However, only free thiol SM **226** was isolated from the mixture indicating that no desulfurisation had occurred. Conditions described by Haase *et al.* utilised the identical desulfurisation cocktail but under thermally activated conditions.[134] However, heating at 50 ºC in place of UV irradiation for 1 h again yielded only free thiol **226** upon organic extraction of the reaction mixture (**Scheme 3.35**). The radical mediated metal-free desulfurisation conditions first described by Wan and Danishefsky remain popular within the literature but had been avoided in our case due to the necessity for malodorous ethanethiol as a hydrogen abstraction source.[131] Nevertheless, free thiol **226** was solubilised in DMF:Phosphate buffer prior to the addition of TCEP, VA-044 and ethanethiol and heated at 50 ºC for 1 h. (**Scheme 3.36**).



226 **242**, 58%

Scheme 3.36: Desulfurisation of free thiol **226** to tripeptide **242** under conditions of Wan and Danishefsky.[131]

Organic extraction of the reaction mixture and subsequent ^{1}H NMR spectroscopy of the residue obtained indicated the presence of desulfurised tripeptide **242** in 1:5 ratio with free thiol **226**. This was indicated by the appearance of characteristic valine methyl doublets at 0.99 and 0.96 ppm. The residue was resolubilised in DMF:Phosphate buffer and the requisite reagents added and the mixture left heating at 50 ºC for 14 h. Extraction of this reaction mixture and ^{1}H NMR spectroscopy of the crude residue obtained indicated full reaction conversion, by disappearance of γCH_2 signals of both diastereoisomer of **226** at 3.51 and 3.23 ppm. Column chromatography permitted isolation of the desired tripeptide **242** in a 58% yield. The low yield is likely attributable to inefficient organic extraction of the product from the DMF:Phosphate buffer solution used within the reaction. The success of desulfurisation in the case of the conditions reported by Wan and Danishefsky as opposed to those by Chisolm *et al.* and Haase *et al.* is likely as a result of utilising ethanethiol as a proton abstraction source in place of GSH. In the conditions of Haase *et al.* only 5 eq. of GSH relative to thiolated peptide. The

conditions of Danishefsky *et al*. utilise 20 eq. of ethanethiol relative to thiolated peptide, thereby permitting more effective hydrogen abstraction and rapid product formation.

3.9 Conclusions

The synthesis of Boc protected derivatives of the UAAs, VG and DHV, was undertaken inspired by literature precedent. In the case of Boc-VG **171**, syntheses as described by Afzali-Ardakani and Rapoport[252], and Sicherl *et al.*[253] provided a reliable template, permitting the synthesis of the target compound in gram quantities. The synthesis of Boc DHV **172** as described by Yonezawa *et al.* was undertaken but required significant optimisation to afford DHV in the volumes necessary for further use. This was accomplished in part through increasing the selectivity of the key mesylation/elimination step by application of pseudo-Finkelstein conditions described by Maier *et al.*[257] Additionally, a new deprotection/oxidation strategy was optimised utilising highly selective pseudoproline deprotection conditions described by Yamakawa *et al.*[266] These modifications permitted the synthesis of Boc-DHV **172** in gram quantities. These results illustrate that unsaturated UAAs are accessible *via* synthesis in quantities suitable for peptide ligation methodologies.

With optimised syntheses in hand, Boc-VG **171** and Boc-DHV **172** could be inserted into dipeptides suitable for appraisal in terms of their reactivity towards ATE and S-to-N acyl transfer processes. Boc-AG **173** was investigated in tandem, as the compound was readily purchasable. Peptide coupling was undertaken under conditions described in the literature and readily furnished unsaturated dipeptides **174**, **175** and **176**. These substrates were then utilised in ATE reactions with a range of peptide thioacids, synthesised *in situ* from the deprotection of the apposite Trt thioesters as described by Crich and Sharma.[221] These reaction furnished the corresponding thioesters (**208** – **220**) in good yields, however in some cases necessitated the use of an excess of thioacid to ensure full consumption of alkene SMs. These results were justified with reference to both the contributions of the unsaturated dipeptide **174**, **175** and **176**, and the characteristics of the thioacids employed. Yields with respect to the alkene component revealed a trend relative to the availability of the alkene moiety. VG derived dipeptide **174** and AG derived dipeptide **176** gave relatively higher yields than DHV derived dipeptide **175**. This was likely as a result of the greater steric hindrance around the alkene moiety of the latter compound. The contribution of the thioacid component to yield was slightly more

complex, as both the steric effects of the thioacid side-chain residue and propensity of each thioacid to undergo CSO elimination had to be considered. Nevertheless, the necessity for addition of 5 eq. of Phe thioacid **205** and Val thioacid **206** in ATE reactions was justified by the greater stability of the *C*-centred radicals formed upon loss of CSO promoting elimination and slowing down ATE addition.

Finally, the S-to-N acyl transfer characteristics of Gly thioesters **208**, 2**13** and **217** were evaluated under optimised Boc deprotection and freebasing conditions. Gratifyingly, VG derived thioester **208** and DHV derived thioester **213**, both of which undergo S-to-N acyl transfer via a 6 membered TS, furnished the corresponding amide products in good isolated yields. These thiolated tripeptides **225** and **226** presented the first amide products formed *via* an ATE mediated ligation process. Subsequent methylation and desulfurisation of the thiolated products gave access to native tripeptide products. Boc deprotection and freebasing of AG derived thioesters was not successful. In the case of AG derived thioester **217** O-to-N acyl transfer over a 6 membered TS was preferred to S-to-N acyl transfer over a 7 membered TS. Synthesis of thioester substrate **235**, incapable of O-to-N acyl transfer, and subsequent Boc deprotection and freebasing yielded bis-acylated product **237**, indicating intramolecular S-to-N acyl transfer was not favourable in this case. As demonstrated by Malins *et al.*,[145] S-to-N acyl transfer over a 7 membered TS must be undertaken with rigidified substrates to ensure efficient amide formation. With the first amide products of ATE mediated ligation in hand, investigation into the synthesis of larger peptides could commence. These investigations will be discussed in the next chapter.

Chapter 4

Synthesis of Unsaturated Peptides and Peptide Thioacids

4.0 Introduction

Chapter 3 outlined the synthesis of the UAAs Boc-VG and Boc-DHV. The synthesis of unsaturated dipeptides **174**, **175** and **176** permitted access to a range of peptide thioesters in high yield *via* ATE. These substrates were capable of spontaneous S-to-N acyl transfer to form amide products, demonstrated in the case of Gly thioesters **208** and **213**. Synthetic elaboration in the form of desulfurisation/methylation furnished native tripeptide products **239** and **242** derived from the ATE mediated ligation (**Figure 4.1**).

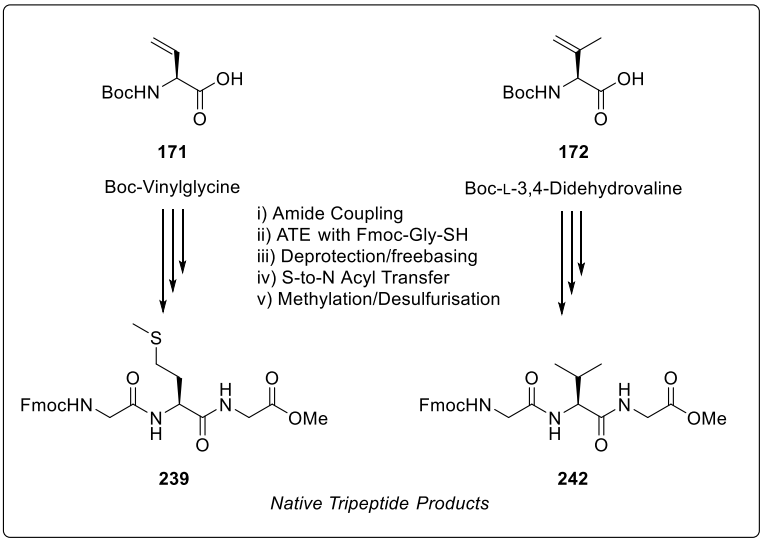


Figure 4.1: Summary of the successful ATE mediated ligation strategy employed for the synthesis of native tripeptides **239** and **242** from UAA starting materials **171** and **172**.

With proof of concept for ATE mediated ligation established on small molecules, our attention turned to larger targets. Within the literature, total synthesis of an array of peptides and native proteins have been achieved using chemical ligation methodologies. The chosen target would ideally satisfy several criteria. The peptide would be therapeutically relevant, thereby demonstrating the utility of ATE mediated ligation in furnishing useful protein substrates. The absence of a Cys residue in the proteins primary structure would also be desirable, as this would preclude the use of NCL in the synthesis of the target thereby demonstrating the value of ATE mediated ligation in accessing challenging synthetic targets. Prior demonstration of the attainability of the target by chemical protein synthesis within the literature would also be desirable. Pivotally, the target would have to present Val and Met residues at viable positions in its primary structure to permit application of ATE mediated peptide ligation. With these caveats in mind, two hPTH fragments, 20 AA residues in length, were selected as peptidic targets in the first instance, with the complete protein being a final target if fragment synthesis

proved successful. hHPT is an 84 residue protein secreted by the parathyroid gland and is a systemic regulator of calcium ion concentrations in the blood.[284] Recombinant hPTH and synthetic hPTH fragments are administered as therapeutics for the treatment of disorders resulting from errant serum calcium levels, such as osteoporosis and hypoparathyroidism.[285] There are no Cys residues within the primary protein structure of hPTH. This fact, coupled with the proteins considerable therapeutic value, has contributed to its use as a model synthetic target for novel ligation methodologies. Tam and Yu successfully synthesised hPTH through ligation/methylation to furnish valuable peptide targets beyond those containing Cys residues.[243] The onset of ligation/desulfurisation methodologies enabled the first successful total synthesis of hPTH through ligation at Leu and Ile residues by Dong *et al*.[128] Inspired by these literature precedents, two fragments of hPTH conducive to ATE mediated ligation at Met and Val were selected (**Figure 4.2**).

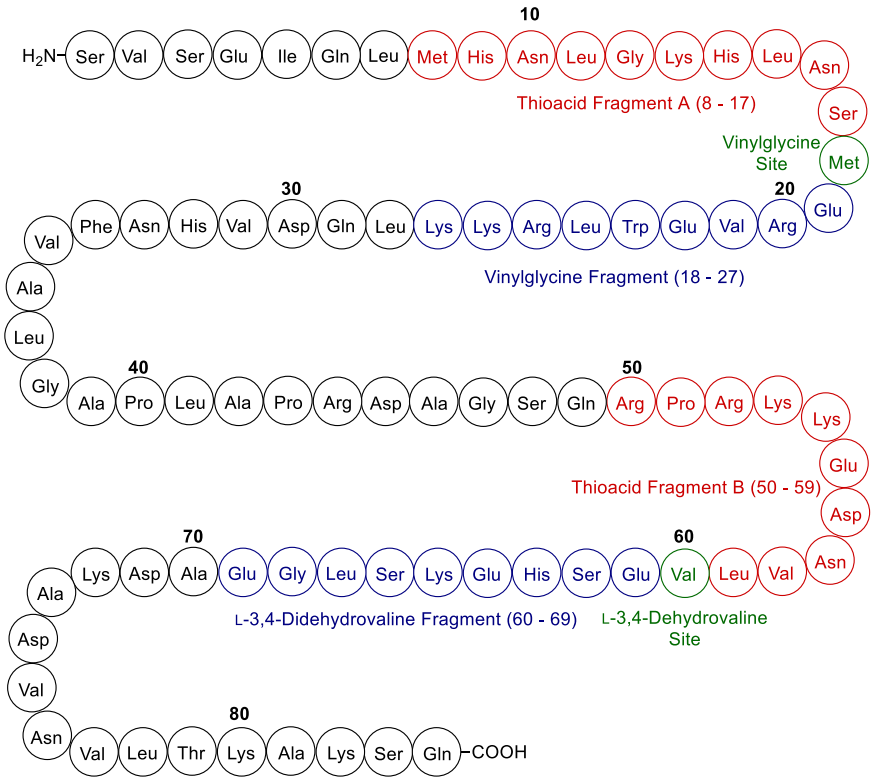


Figure 4.2: Structure of human parathyroid hormone (hPTH) annotated with fragment 8 – 17 and 50 – 69 identified as synthetic targets for acyl thiol-ene mediated peptide ligation. The peptide thioacid and unsaturated peptide components of the ligation reaction are coloured red and blue, respectively.

The synthesis of unsaturated peptides fragments and peptide thioacids for each ATE mediated ligation would be carried out using SPPS methodologies described in the literature. Once these reaction components were accessed, ATE reactions would be carried out under conditions sympathetic towards unprotected peptide fragments, such as

those optimised by the Brimble group for use in CLipPA type chemistries (**Section 1.4.2**).[189, 191-193] Crude isolation of the resulting 20 AA thioester ATE product and subsequent dissolution in ligation buffer amenable to S-to-N acyl transfer would furnish the corresponding thiolated peptide fragment with desulfurisation or methylation under literature conditions furnishing the target hPTH fragments. The synthesis of UAA containing peptide fragments **243** and **244** was to be undertaken prior to the synthesis of the corresponding thioacids **245** and **246** (**Figure 4.3**).

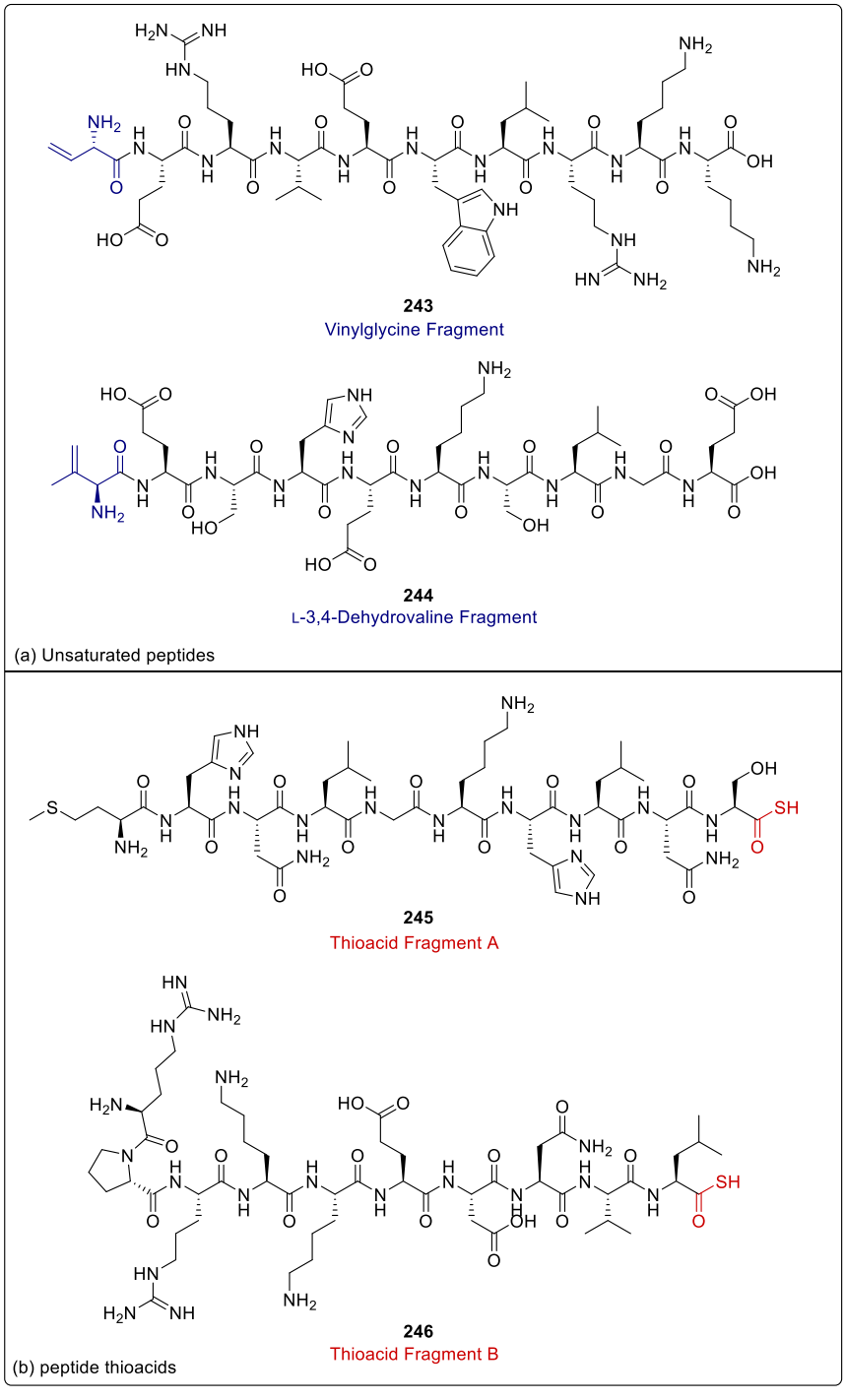
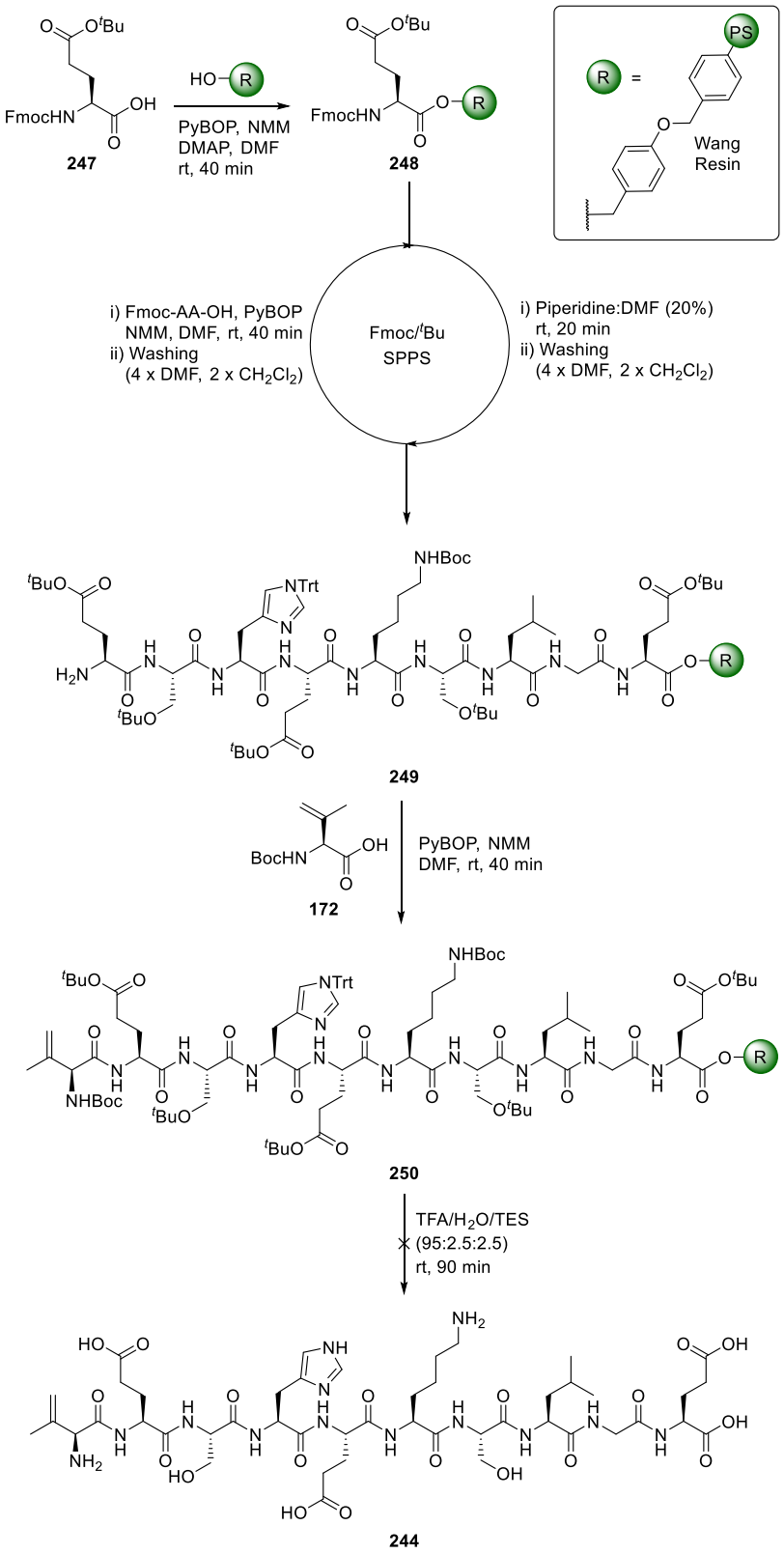


Figure 4.3: (a) Unsaturated peptide targets **243** and **244** for use in ATE mediated ligation. (b) Peptide thioacid targets **245** and **246** for use in ATE mediated ligation.

Consultation of the literature suggested that the syntheses of unsaturated peptide fragments **243** and **244** would likely be less complex than those of the corresponding peptide thioacids **245** and **246**, since the former could be accessed with standard Fmoc SPPS up to the addition of Boc-VG **171** and Boc-DHV **172**. Coupling of UAAs Boc-VG **171** and Boc-DHV **172** would be optimised to ensure the stability of alkene moiety prior to global deprotection, resin cleavage and purification *via* reverse-phase high-performance liquid chromatography (RP-HPLC). RP-HPLC would be carried out with the assistance of our collaborators from the research group of Prof. Marc Devocelle at the Royal College of Surgeons in Ireland (RCSI). Synthesis of DHV peptide fragment **244** was undertaken first due to the higher reported stability of Boc-DHV **172** towards amide coupling conditions in comparison to Boc-VG **171**.[255, 268] Conditions optimised in the synthesis of DHV peptide fragment **244** could then be applied to the synthesis of VG peptide fragment **243**.

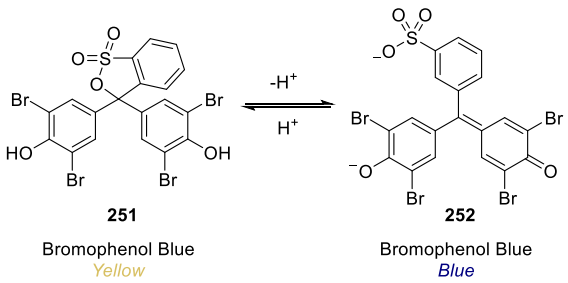
4.1 Synthesis of L-3,4-Didehydrovaline Peptide Fragment (244)

The synthesis of DHV fragment **244** was first attempted using manual standard Fmoc SPPS protocols inspired by literature precedent (**Scheme 4.1**).[286]



Scheme 4.1: Attempted synthesis of peptide fragment **244** *via* SPPS on Wang resin utilising an Fmoc/O^{t}Bu protecting group strategy.

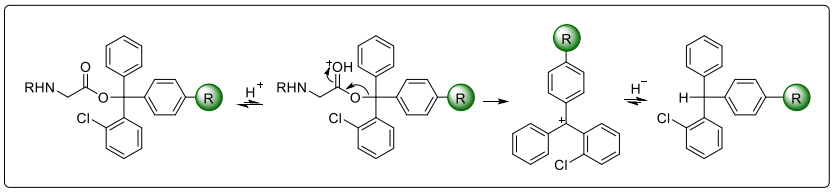
Fritted syringes were utilised as reaction vessels enabling facile manipulation of the resin during sequential coupling, washing and deprotection cycles. An Fmoc/^{t}Bu PG strategy was employed necessitating the use of Fmoc-AAs suitably protected at nucleophilic moieties. These included Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Ser(O^{t}Bu)-OH, Fmoc-Lys(Boc)-OH and Fmoc-His(Trt)-OH. Wang resin was used as it permitted cleavage without HF treatment to furnish a *C*-terminal carboxylic acid. Coupling of the first protected Glu residue **247** to the resin was carried out with 4 eq. of Fmoc-Glu(O^{t}Bu)-OH, 4 eq. of PyBOP, 0.1 eq. of nucleophilic catalyst DMAP and 8 eq. of non-nucleophilic base NMM in DMF. The addition of DMAP was necessary to ensure efficient addition to the hydroxyl moiety of the resin *via* a Steglich type esterification mechanism. Subsequent couplings were accomplished with 3 eq. Fmoc-AA, 3 eq. of PyBOP and 4 eq. of NMM in DMF. The reaction progress of coupling steps was monitored qualitatively by treatment of a sample of resin beads with 0.15 mmol bromophenol blue indicator in CH_2Cl_2 as described by Krchňák *et al.*[287] Unreacted resin bound amino groups are capable of deprotonating bromophenol blue **251** to form the extensively conjugated anionic species **252**, with a concurrent change in colour from pale yellow to deep blue (**Scheme 4.2**).[288]



Scheme 4.2: Action of bromophenol blue indicator used for monitoring coupling reactions during SPPS.

If treatment of a sample of resin beads with bromophenol blue solution does not promote this colour change then few unreacted amine sites remain and coupling is judged to have reached completion. If a colour change is observed the coupling may be repeated under the same conditions to ensure efficient amide formation, although this was not necessary during the synthesis of fragment **244**. Fmoc deprotection between coupling steps was achieved with piperidine in DMF (20%). After coupling of Boc-DHV **172** at the *N*-terminus of resin bound peptide **249**, global deprotection of AA side-chain PGs and cleavage of unsaturated resin bound peptide **250** was undertaken in one step by treatment with a solution of TFA/H_2O/TES (95:2.5:2.5) for 90 min at rt. The resulting mixture was

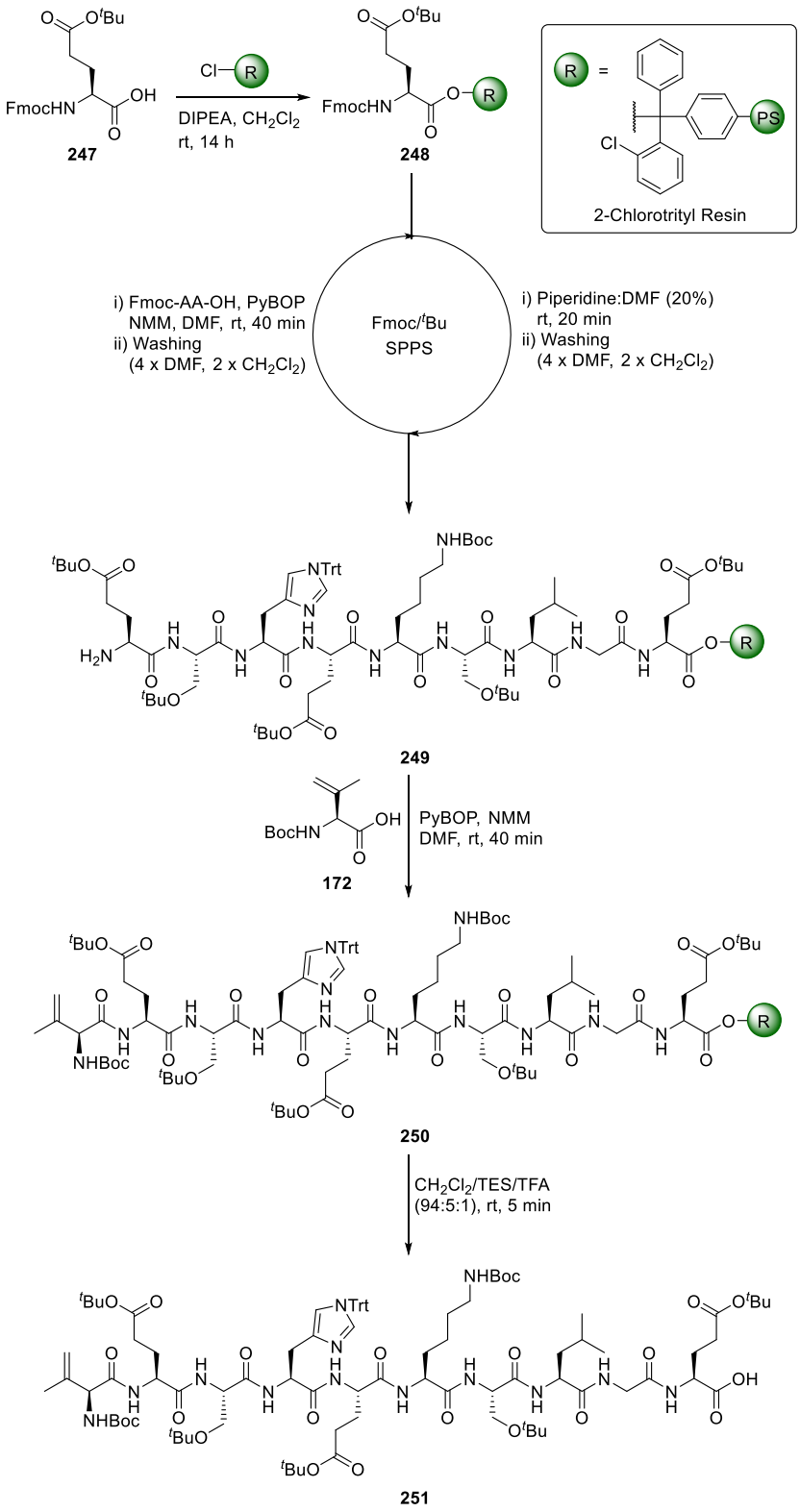
filtered to remove the remnants of the resin beads and concentrated under a stream of N_2. Addition of cold Et_2O led to precipitation of the crude peptide which was isolated by decantation and lyophilised. Unfortunately, analysis of the crude ^{1}H NMR spectrum and mass spectrum of the isolated residue indicated that the synthesis of fragment **244** had not been successful. In particular, the absence of characteristic DHV signals in the alkene region (4.50 – 6.50 ppm)[289] of the ^{1}H NMR spectrum acquired suggested the desired peptide fragment **244** had not been obtained. However, the crude ^{1}H NMR spectrum and bromophenol blue tests were indicative of successful peptide synthesis in this case. It was therefore likely that unsuccessful coupling of Boc-DHV **172** to the resin bound peptide **249** or alkene degradation under the harsh global PG deprotection/resin cleavage conditions employed had prevented isolation of unsaturated fragment **244**. As, the coupling of Boc-DHV had been monitored, albeit qualitatively, with bromophenol blue solution it was deemed more likely that the harsh deprotection conditions had led to alkene degradation. However, the alkene functionality of UAA VG had previously proven stable to reflux in 6 M aq. HCl during the deprotection of protected VG derivative **71** to yield HCl·VG **179** (**Section 3.1**). Nevertheless, an SPPS resin that would permit milder global deprotection and cleavage conditions was required to ensure the stability of the alkene moiety of unsaturated peptide **244** under acidic conditions. 2-Chlorotrityl chloride resin was first described by Athanassopoulos *et al.* and has proven the resin of choice when mildly acidic cleavage conditions are necessary.[290] The resin functions analogously to the Trt group in solution phase synthesis, permitting resin cleavage through treatment with a solution of CH_2Cl_2/TES/TFA (94:5:1) (**Scheme 4.3**).



Scheme 4.3: Mechanism of peptide cleavage from 2-chlorotrityl SPPS resin under acidic conditions. Silanes are employed as a source of H^- in the reaction.

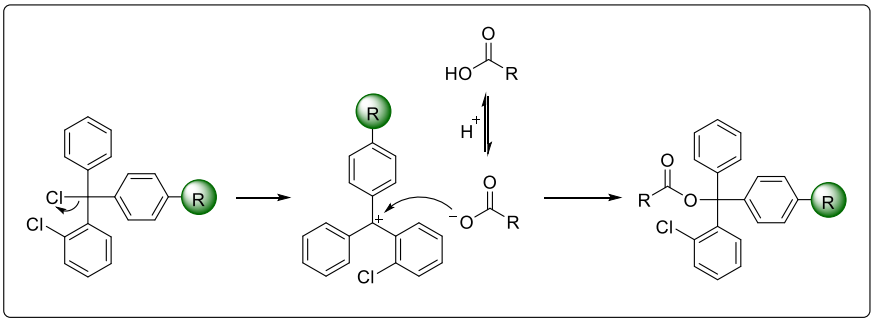
Global deprotection may then be carried out in solution under more forceful conditions prior to purification. This renders monitoring of deprotection progress facile in comparison to on-resin strategies, and consequently offers a greater degree of reaction control.[291] 2-Chlorotrityl chloride resin is primarily utilised for the synthesis of fully protected peptide fragments or those bearing acid sensitive components and thus was ideally suited to the synthesis of DHV fragment **244**.[292] The manual SPPS of fragment

244 was undertaken utilising 2-chlorotrityl chloride resin in place of Wang resin (**Scheme 4.4**).



Scheme 4.4: Attempted synthesis of peptide fragment **244** *via* SPPS on 2-chlorotrityl resin utilising an Fmoc/O^{t}Bu protecting group strategy.

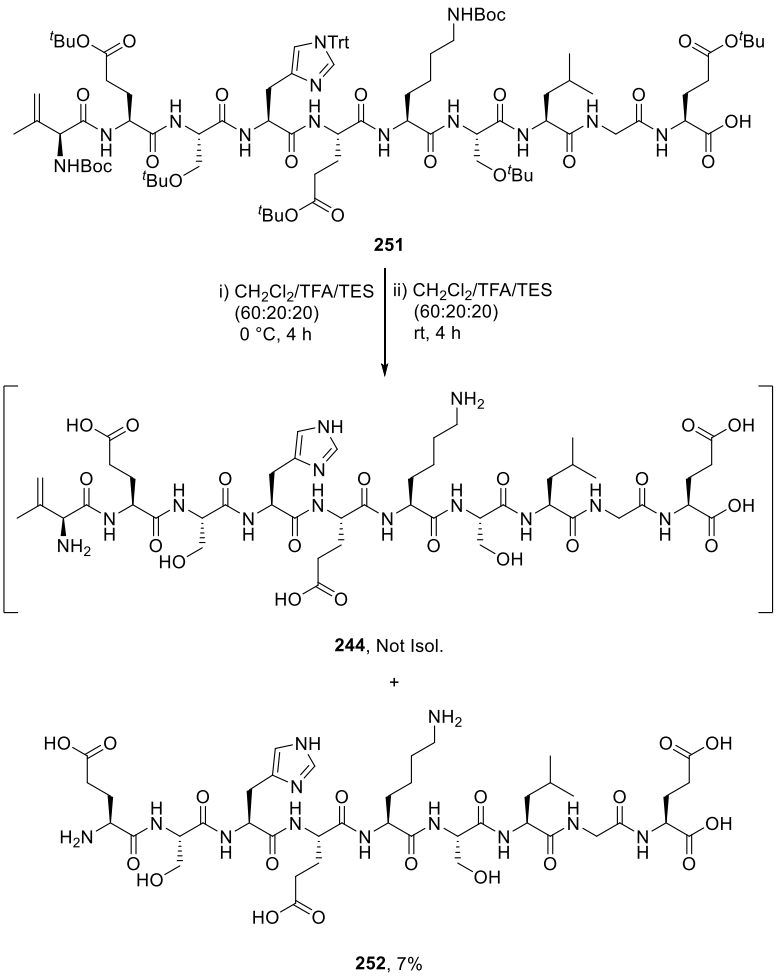
As the attachment sites of 2-chlorotrityl chloride resin are significantly more hindered than those of other commonly utilised SPPS resins, the resin surface must be more sufficiently activated to ensure efficient coupling of the first Fmoc-AA. The resin was purchased as the moisture sensitive 2-chlorotrityl chloride precursor and the first protected Glu residue **247** affixed to the resin in the presence of DIPEA in dry CH_2Cl_2 under Ar, *via* an S_N1 type substitution mechanism (**Scheme 4.5**). As resin bound 2-chlorotrityl chloride is moisture sensitive, and the site of AA attachment significantly hindered, steps must be taken to ensure effective loading of the first Fmoc-AA has been achieved prior to subsequent couplings.



Scheme 4.5: Mechanism of 2-chlorotrityl chloride SPPS resin loading.

Quantification of loading to the 2-chlorotrityl chloride resin was undertaken using the method described by Gordeev *et al.*[293] After initial coupling of protected Glu **247** to the resin, 5 mg of resin beads were weighed out and washed thoroughly prior to treatment with 1 mL of piperidine/DMF solution (20%). After 20 min the mixture was filtered and 0.10 mL of the filtrate diluted in 10 mL of DMF. The absorbance of the resulting dilute solution at 301 nm was measured by ultraviolet-visible (UV-Vis) spectroscopy against a DMF blank. 301 nm corresponds to the maximum absorbance (λ_{max}) of the dibenzofulvene-piperidine adduct formed upon Fmoc deprotection.[294] The measurements were repeated in triplicate and the average absorbance used to calculate resin substitution using the formula described by Gude *et al.* derived from the Beer-Lambert law.[294] Resin substitution of Glu **247** to yield resin bound Glu **248** was calculated as 0.50 mmol g^{-1} using this method. This resin loading was substantially lower than the maximum resin substitution obtainable of 1.33 mmol g^{-1}, as stated by the resin vendor. This indicated that coupling of Glu **247** to yield resin bound Glu **248** had been only moderately successful. This could be attributed to the water-sensitive nature of the 2-chlorotrityl chloride precursor. However, as the resin was obtained fresh from the supplier and water-free laboratory techniques were used over the course of the coupling reaction it is likely the

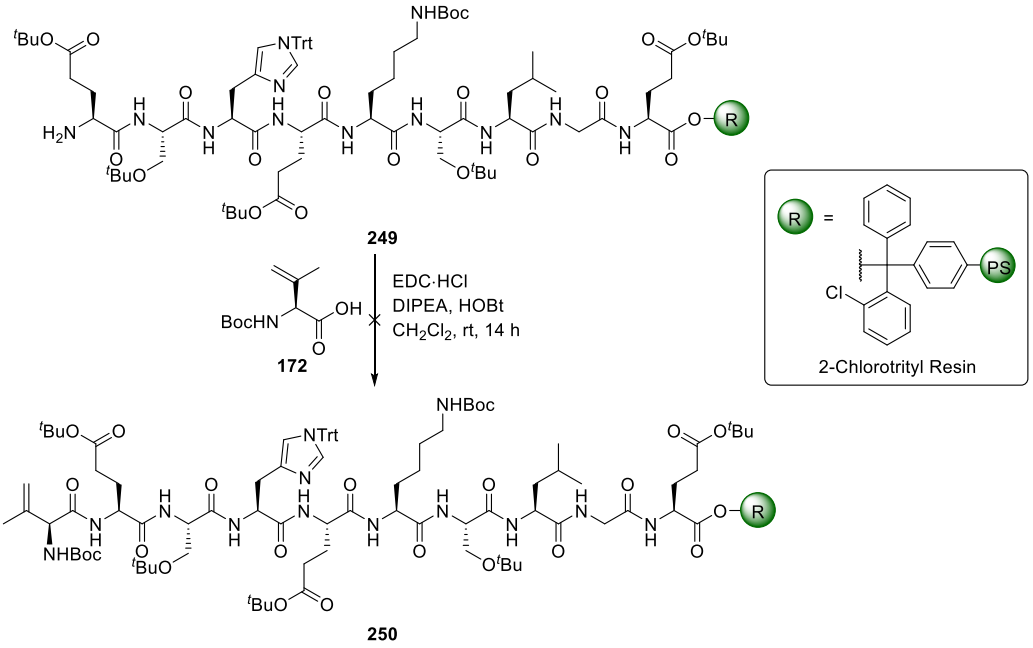
adverse sterics of the substitution also inhibited efficient loading. Subsequent resin loading quantifications yielded values in the same range, further indicating this was the case. Nevertheless, standard Fmoc/^{t}Bu SPPS conditions were used after this first coupling step to synthesise the protected resin bound peptide **250** (**Scheme 4.4**). The nascent protected peptide fragment **251** was then cleaved from the resin by treatment with CH_2Cl_2/TES/TFA (94:5:1) at 0 °C. The mixture was dried under a stream of N_2 to isolate the crude peptide. Characteristic DHV peaks observed at 5.54 and 5.47 ppm in the ^{1}H NMR spectrum of the crude peptide in CD_3OD indicated the presence of an alkene, and therefore protected peptide fragment **251**, within the isolated mixture albeit at very low intensity. The purification and characterisation of protected peptides such as **251** are notoriously difficult on account of their poor solubility and reduced ionisation potential in mass spectromety.[295] Therefore, global deprotection was attempted under conditions that were compatible with the alkene moiety of **244** prior to characterisation (**Scheme 4.6**).



Scheme 4.6: Global deprotection of crude mixture of **XX** to yield truncated 9mer **XX** as the major product after purification by RP-HPLC.

The crude peptide **251** was dissolved in a solution of CH_2Cl_2/TFA/TES (60:20:20) at 0 °C. The resulting mixture was stirred at 0 °C for 4 h, prior to concentration under a stream of N_2, and subsequently triturated in Et_2O to remove non-polar deprotection by-products. Analysis of the crude ^{1}H NMR spectrum of the mixture in CD_3OD indicated the presence of ^{t}Bu and *N*-Trt PGs. However, no signals corresponding to the Boc groups of Boc-DHV **172** or protected Lys were observed suggesting effective deprotection of these PGs had been accomplished. A second trituration in Et_2O and further analysis by ^{1}H NMR did not alter the signals observed to any significant degree, indicating that the characteristic ^{t}Bu and Trt signals were not deprotection artefacts, but resulted from incomplete side-chain deprotection. The residue was treated with fresh deprotection cocktail at rt for 4 h (**Scheme 4.6**). After concentration, trituration in Et_2O and decantation, analysis of the ^{1}H NMR of the crude peptide mixture indicated that these deprotection conditions had been successful in removing the outstanding ^{t}Bu and *N*-Trt PGs. The characteristic alkene peaks of DHV were also observed indicating the deprotection conditions employed had not led to substantial degradation. The crude peptide mixture was purified by semi-preparative RP-HPLC (ACN/H_2O + 0.1% TFA, 5 – 65% ACN). Unfortunately, DHV peptide fragment **244** was not isolated from the crude. The fact that characteristic alkene peaks were present in the crude ^{1}H NMR spectrum of the mixture, although not conclusive evidence, strongly suggests that **244** was present as a minor product and was stable to the global deprotection conditions employed. However, the major product of the synthesis was 9mer **252** indicating that coupling of Boc-DHV **172** to form resin bound unsaturated peptide **250** was not successful under the conditions employed. PyBop had been employed as a coupling reagent with Alloc protected DHV in the total synthesis of phomopsin B by Grimley *et al.*, and reported without issue in their hands.[255] The Alloc group may have conferred slightly less steric hindrance than the corresponding Boc group of Boc-DHV **172**. However, Fmoc AAs were demonstrated to be well capable of coupling under these conditions to yield 9mer **252** despite being significantly more sterically demanding than Alloc. Nevertheless, coupling of Boc-DHV **172** to resin bound peptide **249** was attempted under carbodiimide mediated coupling conditions employed for the synthesis of DHV dipeptide **175** (**Section 3.3**). Resin bound peptide **249** was synthesised under optimised Fmoc SPPS conditions that furnished 9mer **252** after RP-HPLC purification (**Scheme 4.4**). After the last cycle of Fmoc deprotection and washing a preactivated solution of Boc-DHV **172**, EDC·HCl, HOBt and DIPEA in

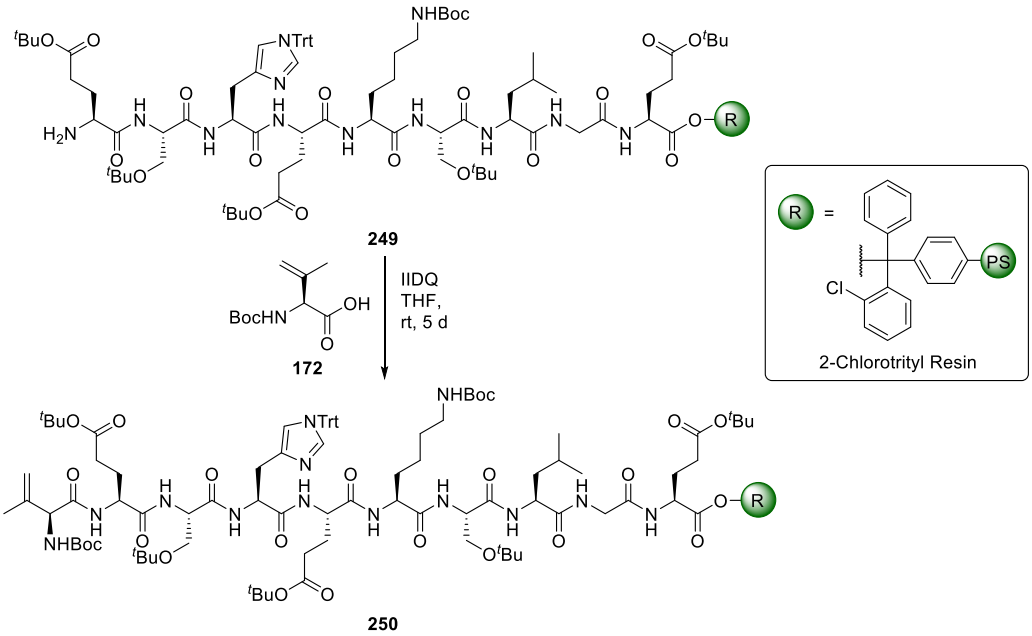
DMF was added to the resin and shaken for 14 h at rt (**Scheme X.X**). After washing, a 30 mg portion of resin was treated with CH_2Cl_2/TES/TFA (60/20/20) cleavage cocktail.



Scheme 4.7: Attempted amide coupling of Boc-DHV **172** and resin bound peptide fragment **249** under carbodiimide mediated conditions.

The supernatant was decanted and dried under a stream of N_2 and the ^{1}H NMR spectrum of the crude peptide analysed. Coupling of Boc-DHV **172** to resin bound fragment **249** had not been successful, as indicated by the lack of alkene peaks within the spectrum obtained. This may have been a consequence of the lack of availability of the amine functionality of the resin bound peptide **249**, thereby impeding effective amide formation and necessitating long reaction times with carbodiimide type reagents. IIDQ was employed to good effect in the synthesis of VG dipeptide **174** and succeeded in preventing racemisation and isomerisation of the sensitive substrate Boc-VG **171** (**Section 3.3**). As the synthesis of VG peptide fragment **243** would likely require the application of this reagent it was reasoned that its use in coupling Boc-DHV **172** to resin bound peptide **249** would, if successful, obviate the need for optimisation of this crucial coupling step in the synthesis of VG fragment **243**. Consultation of the literature revealed that IIDQ had not been used as a reagent in SPPS, likely as a result of the long reaction times necessary for acceptable reaction conversions. Nevertheless, the coupling of resin bound peptide **249** and Boc-DHV **172** was carried out under optimised IIDQ mediated coupling conditions (**Scheme 4.8**). THF is the recommended solvent for IIDQ mediated

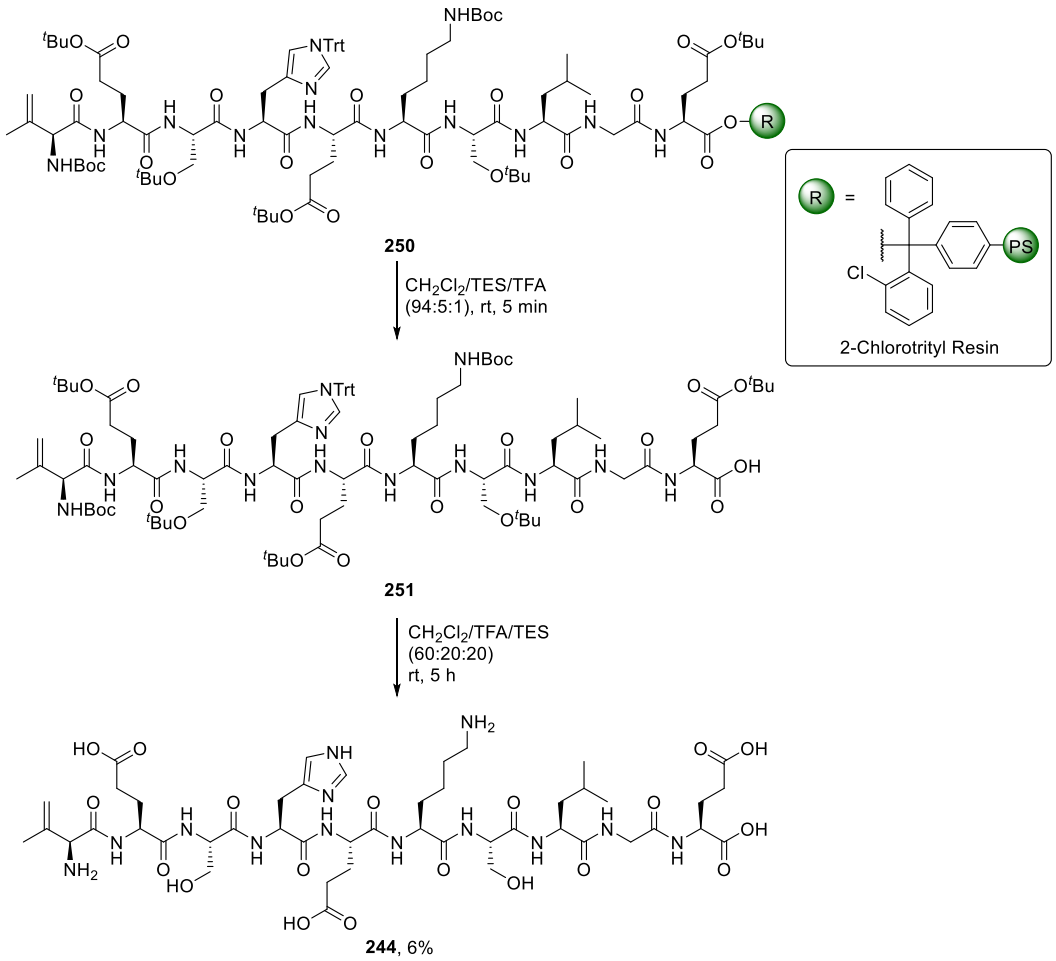
couplings as the ether moiety is capable of stabilising the cationic intermediates of the reaction.[296]



Scheme 4.8: IIDQ mediated amide coupling of Boc-DHV **172** and resin bound peptide fragment **249**.

Consultation of the literature described SPPS protocols employing THF and 2-chlorotrityl resin for a variety of reaction types, suggesting resin swelling would not be adversely affected by this choice of solvent.[297] Resin bound peptide **249** was shaken with a solution of Boc-DHV **172** and IIDQ in THF for 5 d at rt. The addition of $NaHCO_3$, as in the case of VG dipeptide **174** (**Section 3.3**), was not necessary in this case as the Fmoc deprotection step prior to coupling ensured the *N*-terminal of resin bound peptide **249** was freebased. After 5 days the resin beads were washed, and a 30 mg portion cleaved under optimised conditions to monitor the progress of the reaction. Gratifyingly, the ^{1}H NMR spectrum of the residue obtained after drying indicated that coupling of Boc-DHV **172** to resin bound peptide **249** had been successful. Characteristic DHV alkene signals at 5.54 ppm and 5.47 ppm were present, and at a greater intensity than in the ^{1}H NMR spectrum obtained from the cleavage of peptide fragment **250** after PyBOP/NMM mediated amide coupling. Satisfied that a sufficient quantity of resin bound peptide fragment **250** had been furnished to enable purification by semi-preparative RP-HPLC, the remaining resin was subjected to optimised cleavage and global deprotection conditions (**Scheme 4.9**) After cleavage and global deprotection crude peptide **244** was subjected to purification by RP-HPLC (ACN/H_2O + 0.1% TFA, 5 – 65% ACN) and

unprotected DHV peptide fragment **244** found to be the major synthetic product, isolated in a 6% yield over the entire SPPS, cleavage and global deprotection steps.



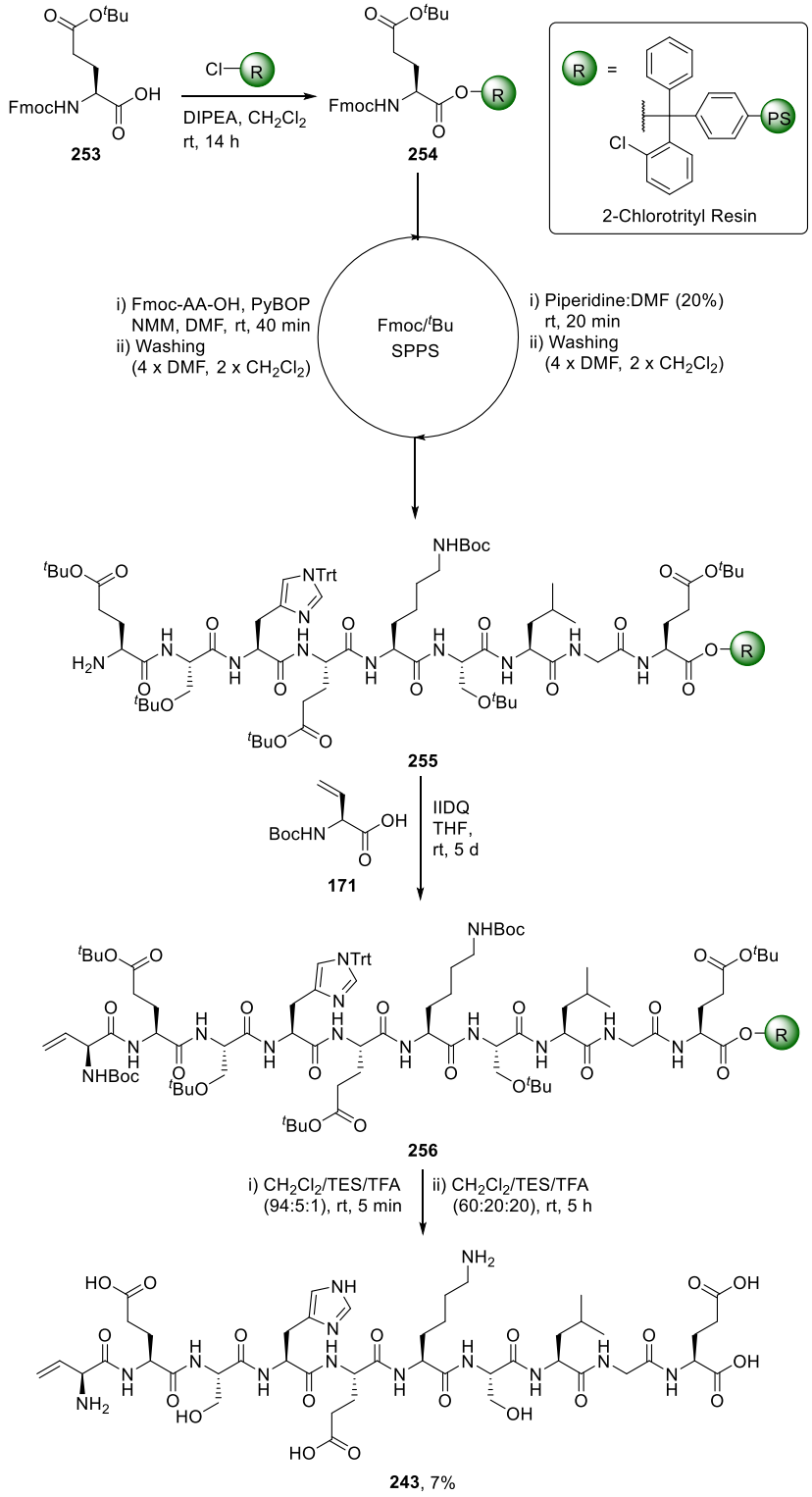
Scheme 4.9: Resin cleavage and global deprotection of resin bound peptide fragment **249** to yield target DHV peptide fragment **244** after purification by RP-HPLC.

The identity of **244** was confirmed by ^{1}H, ^{13}C and 2D NMR spectroscopy and mass spectrometry. With UAA containing DHV peptide fragment **244** in hand these optimised SPPS conditions could be applied to the synthesis of VG peptide fragment **243**.

4.2 Synthesis of Vinylglycine Peptide Fragment (243)

The synthesis of VG peptide fragment **243** was carried out utilising optimised 2-chlorotrityl chloride resin Fmoc SPPS conditions, followed by IIDQ coupling with Boc-VG **171**, resin cleavage and global deprotection (**Scheme 4.10**). The ^{1}H NMR spectrum of the crude cleavage of resin bound peptide **256** displayed characteristic VG alkene peaks at 5.85 ppm, 5.45 ppm and 5.41 ppm, suggesting synthesis had been successful. After global deprotection and cleavage the crude peptide was purified by RP-HPLC (ACN/H_2O + 0.1% TFA, 5 – 65% ACN**)** to give VG peptide fragment **243** in a

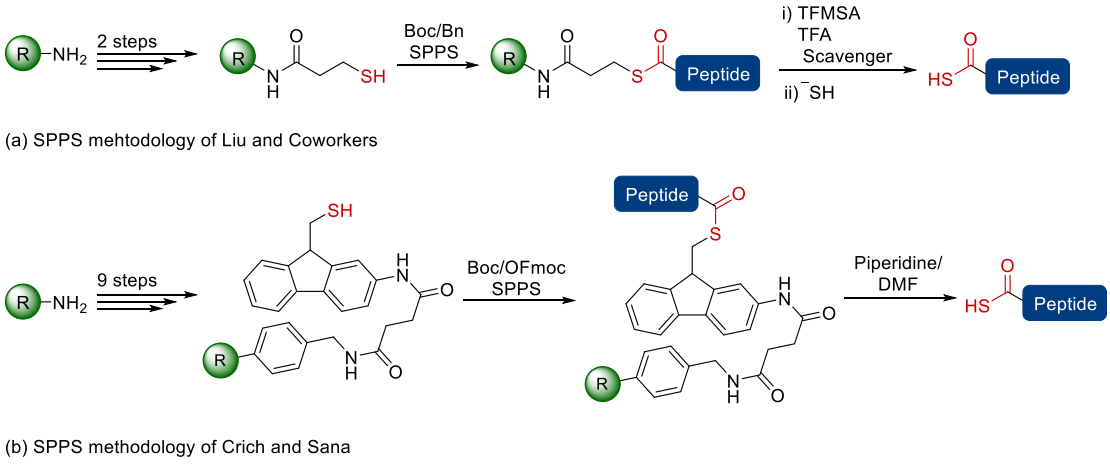
7% yield (**Scheme 4.10**). The identity of the product was confirmed with ^{1}H, ^{13}C and 2D NMR spectroscopy, and mass spectrometry. With both DHV and VG modified hPTH peptide obtained our attention turned to the synthesis of the corresponding thioacid fragments *via* SPPS methodologies.



Scheme 4.10: Resin cleavage and global deprotection of resin bound peptide fragment **256** to yield target VG peptide fragment **243** after purification by RP-HPLC.

4.3 Solid Phase Synthesis of Peptide Thioacid Fragment (245)

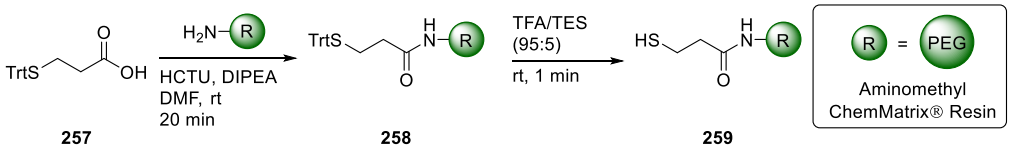
SPPS of peptide thioacids is well established within the field of peptide chemistry (**Section 1.5**). Several literature methodologies were considered as potential approaches to access thioacid fragments **245** and **246**. Ideally, the selected methodology would enable SPPS from readily accessible SMs and not entail the use of HF for resin cleavage. The most promising routes were those described by Liu and coworkers,[226, 298] and by Crich and Sana (**Scheme 4.11**).[227]



Scheme 4.11: SPPS methodologies to access peptide thioacids. (a) Hydrothiolytic methodology of Liu and coworkers.[266, 298] (b) Fm resin mediated methodology of Crcih and Sana.[227]

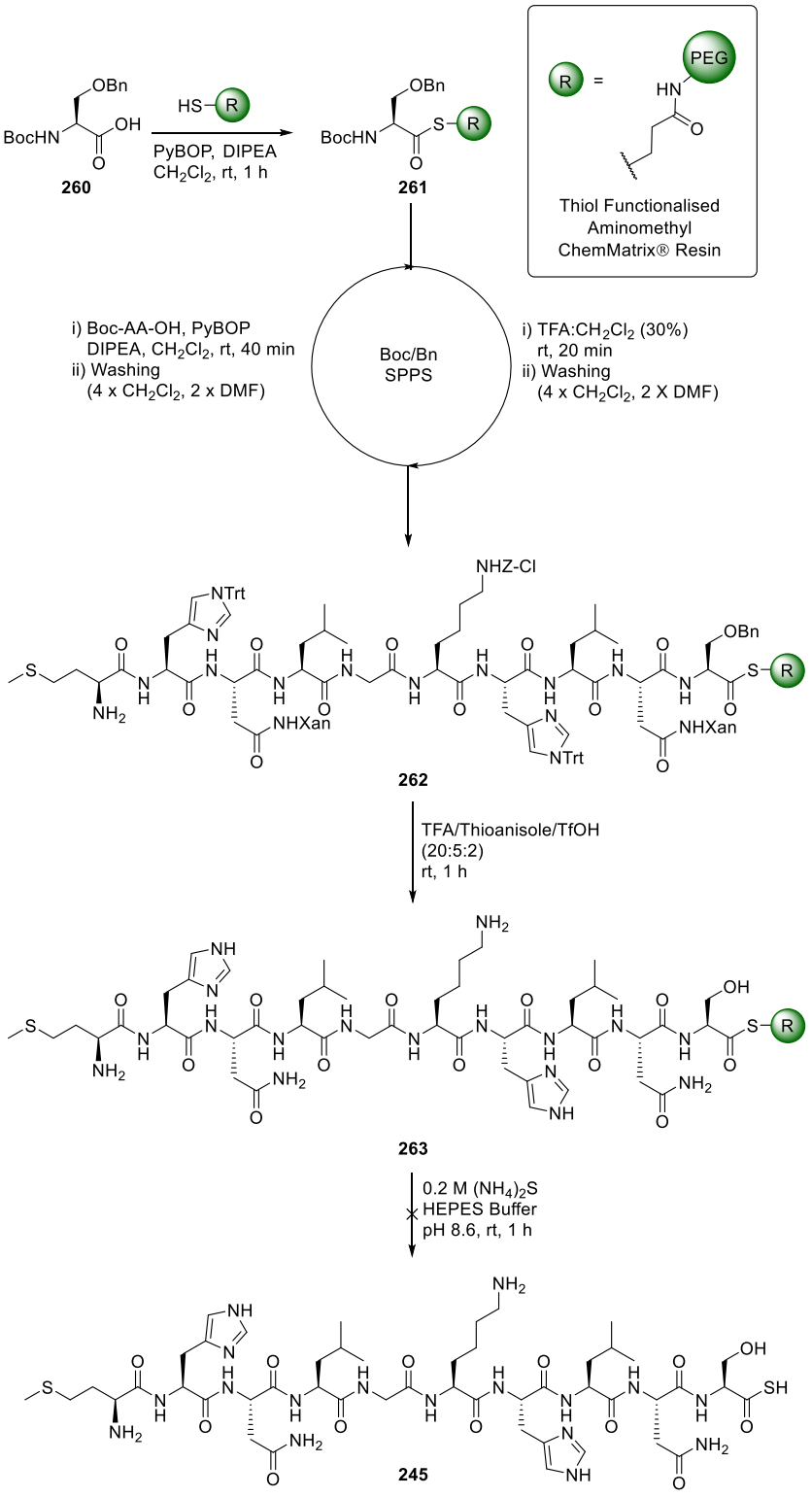
These two strategies both employed Boc SPPS chemistries, thereby ensuring the stability of the nascent thioester resin linkage during peptide elongation (**Section 1.5**) and resin cleavage was accomplished in both cases without the need for HF (**Scheme 4.11**) However, the method of Crich and Sana required Boc AAs bearing OFmoc and Alloc side-chain PGs, the majority of which are not readily available from vendors.[227] In addition, the Fm resin of Crich and Sana required a nine-step synthesis to yield a thiolated resin suitable for thioesterification and subsequent Boc SPPS. On the other hand, the SPPS methodology of Liu and coworkers permitted the use of readily available Boc AAs, protected as in standard Boc/Bn SPPS chemistries. Resin preparation was carried out simply through addition of *S*-Trt 3-mercaptopropionic acid to the amino functionalised resin followed by *S*-Trt deprotection. Alternative conditions for resin preparation, SPPS, global deprotection and resin cleavage, had also been recently reported by Okamoto *et al.* for the methodology of Liu and coworkers, thereby permitting optimisation if necessary.[299] Ultimately, the more readily available nature of the Boc AA SMs necessary

for the SPPS methodology of Liu and coworkers led us to believe the feasibility of this approach could be appraised more quickly. The synthesis of peptide thioacid **245** was attempted first as the primary sequence of the fragment possessed fewer AA residues associated with problematic amide couplings, such as arginine (Arg) and proline (Pro), than fragment **246**.[300] Resin functionalisation was carried out as described by Okamoto *et al.* (**Scheme 4.12**).[299]



Scheme 4.12: Preparation of thiol modified aminomethyl ChemMatrix® resin under conditions described by Okamoto *et al.*[299]

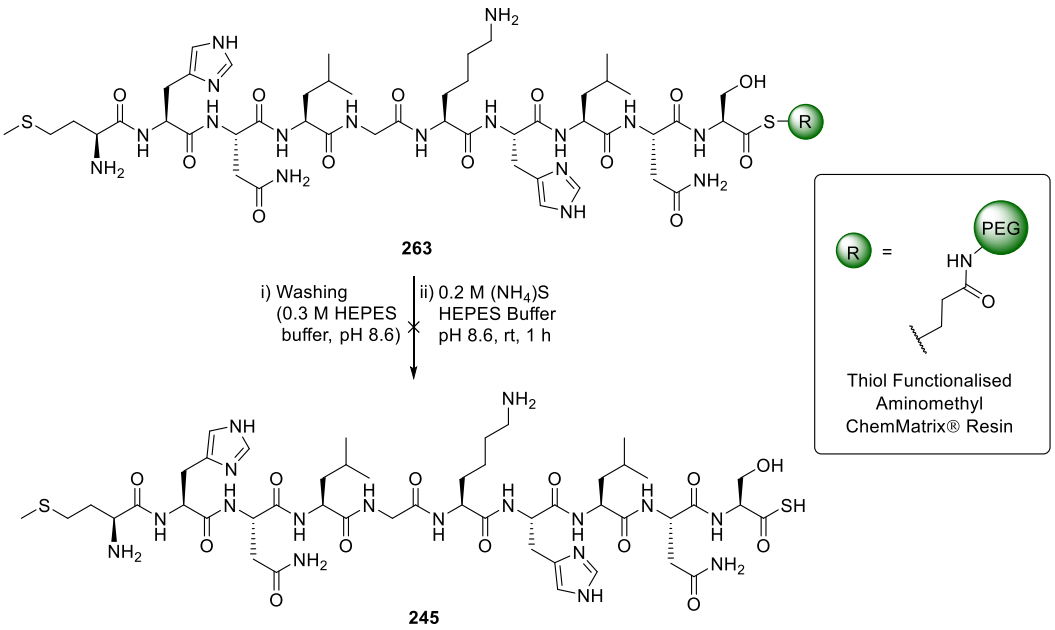
S-Trt 3-mercaptopropanoic acid **257** was coupled to aminomethyl ChemMatrix® resin with aminimum coupling reagent *O*-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU) in the presence of DIPEA to yield resin bound Trt thioester **258**. ChemMatrix® resin is a proprietary PEG-based resin bead permitting superior swelling in aq. media and was necessary for efficient hydrothiolysis (**Section 1.5**). Deprotection of the *S*-Trt group in TFA/TES (95:5) proceeded smoothly to yield resin bound thiol **259** suitable for thioesterification. The observed colour change of the deprotection cocktail from colourless to intense yellow indicated that the coupling of **257** to the resin had been successful. Boc/Bn SPPS was carried out as described by Liu and coworkers (**Scheme 4.13**).[225, 226] Thioesterification of the functionalised resin was undertaken with 4 eq. of Boc-Ser(OBn)-OH **260**, 4 eq. of PyBop and 8 eq. of DIPEA for 1 h. Treatment of a sample of resin beads with Ellman's reagent confirmed that efficient coupling had been achieved. Boc deprotections were carried out with 30% TFA in CH_2Cl_2 with TES for 20 min. Subsequent couplings were undertaken with 4 eq. of Boc-AA, 4 eq. of PyBop and 8 eq. of DIPEA. Unfortunately, monitoring of the progress of amide couplings by treatment of a sample of resin beads with bromocresol blue was not possible, yielding false negatives due to the residual surface amino groups of the aminomethyl ChemMatrix® resin. After coupling of the final AA to the resin and subsequent *N*-terminal Boc deprotection, global deprotection was undertaken with triflic acid (TfOH), TFA and thioanisole as a radical scavenger at rt for 1 h to yield resin bound unprotected peptide **263** (**Scheme 4.13**). The resin beads were carefully washed with TFA and ACN prior to treatment with the hydrothiolysis cocktail comprising 0.2 M $(NH_4)_2S$ in 0.3 M HEPES buffer at pH 8.6 (**Scheme 4.13**).



Scheme 4.13: Attempted synthesis of peptide thioacid fragment **245** *via* SPPS on thiol functionalised aminomethyl ChemMatrix® resin utilising an Boc/Bn protecting group strategy.

Upon addition of the hydrothiolysis buffer a colour change was observed from yellow to opaque white and the mixture was shaken for 2 h prior to freeze-drying and analysis of the crude peptide by ^{1}H NMR spectroscopy and mass spectrometry. Upon freeze-drying

the ^{1}H NMR spectroscopy indicated that resin cleavage had been unsuccessful. The crude ^{1}H NMR spectrum appeared to consist of only buffer and salt species obtained by neutralisation of the hydrothiolysis buffer, with no peptidic products such as thioacid fragment **245** observed. It was surmised that washing with TFA and ACN between global deprotection and hydrothiolysis steps may have been insufficient to neutralise the resin surface. Addition of the basic thiolate buffer to the acidic resin may then have resulted in quenching of the $^{-}$SH species necessary for cleavage from the resin. This would have resulted in the generation of H_2S gas, although pressure build up in the reaction vessel was not observed. Neutralisation may have occurred immediately upon addition of the buffer prior to sealing the reaction vessel and manipulation of the malodourous nature of the $(NH_4)_2S$ buffer well capable of masking H_2S generation. Hydrothiolysis of resin bound unprotected peptide **263** was repeated with the inclusion of a neutralising 0.3 M HEPES buffer washing step before the final resin cleavage step (**Scheme 4.14**).



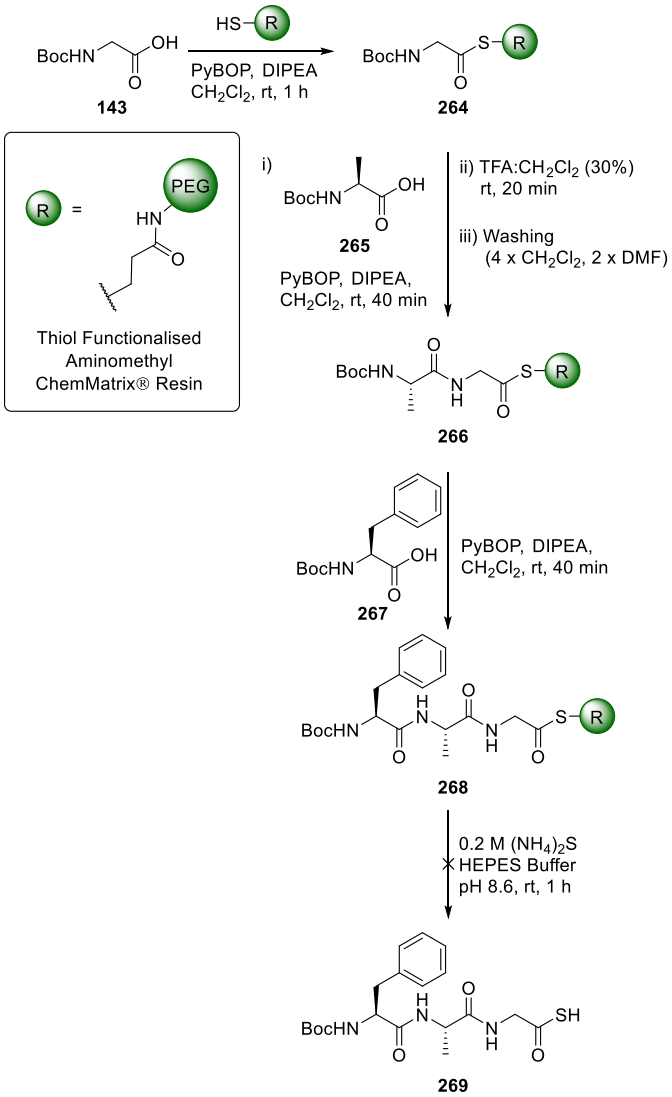
Scheme 4.14: Attempted hydrothiolysis of unprotected peptide thioacid fragment **245** from thiol functionalised aminomethyl ChemMatrix® including a resin neutralisation step.

Addition of the hydrothiolysis buffer in this case did not initiate a colour change within the reaction mixture, suggesting that washing had been effective in impeding neutralisation. However, freeze-drying and subsequent ^{1}H NMR spectroscopy again revealed no peaks indicative of a peptide product. Unfortunately, the nature of SPPS renders direct recognition of problematic chemical steps difficult. Possible causes of failure in the synthesis of thioacid fragment **245** were identified and constituted; premature cleavage of the thioester linkage due to repetitive basic treatment, ineffective

Boc deprotection during coupling cycles, ineffective global deprotection or simply poor amide formation efficiency due to the primary sequence of fragment **245**.

4.3.1 Synthesis of Tripeptide Thioacid (269)

In order to investigate the feasibility of the SPPS/hydrothiolysis methodology employed a small tripeptide was synthesised consisting of AA residues which would not require side-chain deprotection. Boc-Phe-Ala-Gly-SH **269** was selected, and the synthesis attempted using the conditions already described omitting the unnecessary global deprotection step (**Scheme 4.15**).



Scheme 4.15: Boc SPPS of tripeptide **269** under conditions described by Liu and coworkers.

After freeze-drying ^{1}H NMR spectroscopic analysis of the resulting residue indicated that the synthesis had been partially successful, as signals corresponding to the methyl group of Ala and phenyl group of Phe could be identified within the spectrum at 1.36 ppm and

7.20 ppm, respectively. However, relative integration of these signals and the appearance of the spectrum generally suggested an almost equal mixture of truncated products. Mass spectrometry of the mixture yielded evidence for the corresponding carboxylic acid exclusively, likely from oxidation of the desired thioacid. The unsuccessful synthesis of tripeptide thioacid **269** suggested that the Boc deprotection conditions employed may not be sufficient for effective deblocking in this case.

4.3.2 Synthesis of Tetrapeptide Thioacid (270) and Pentapeptide Thioacid (271)

Okamoto *et al.* described the synthesis of tetrapeptide thioacid **270** used to investigate the possible role of thioacids in the abiogenetic synthesis of simple proteins (**Figure 4.4**).[299]

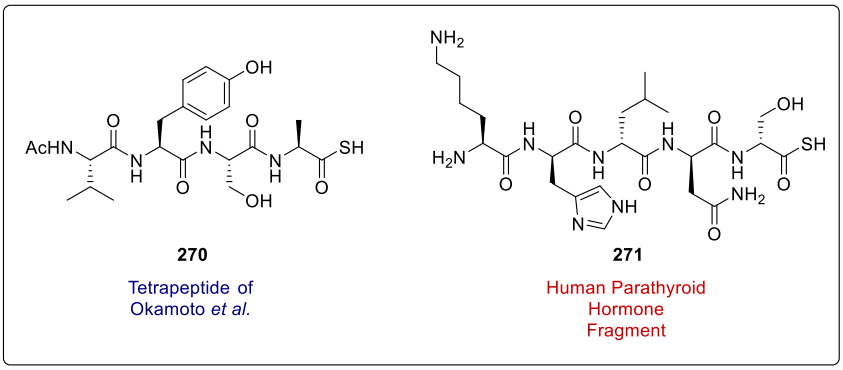
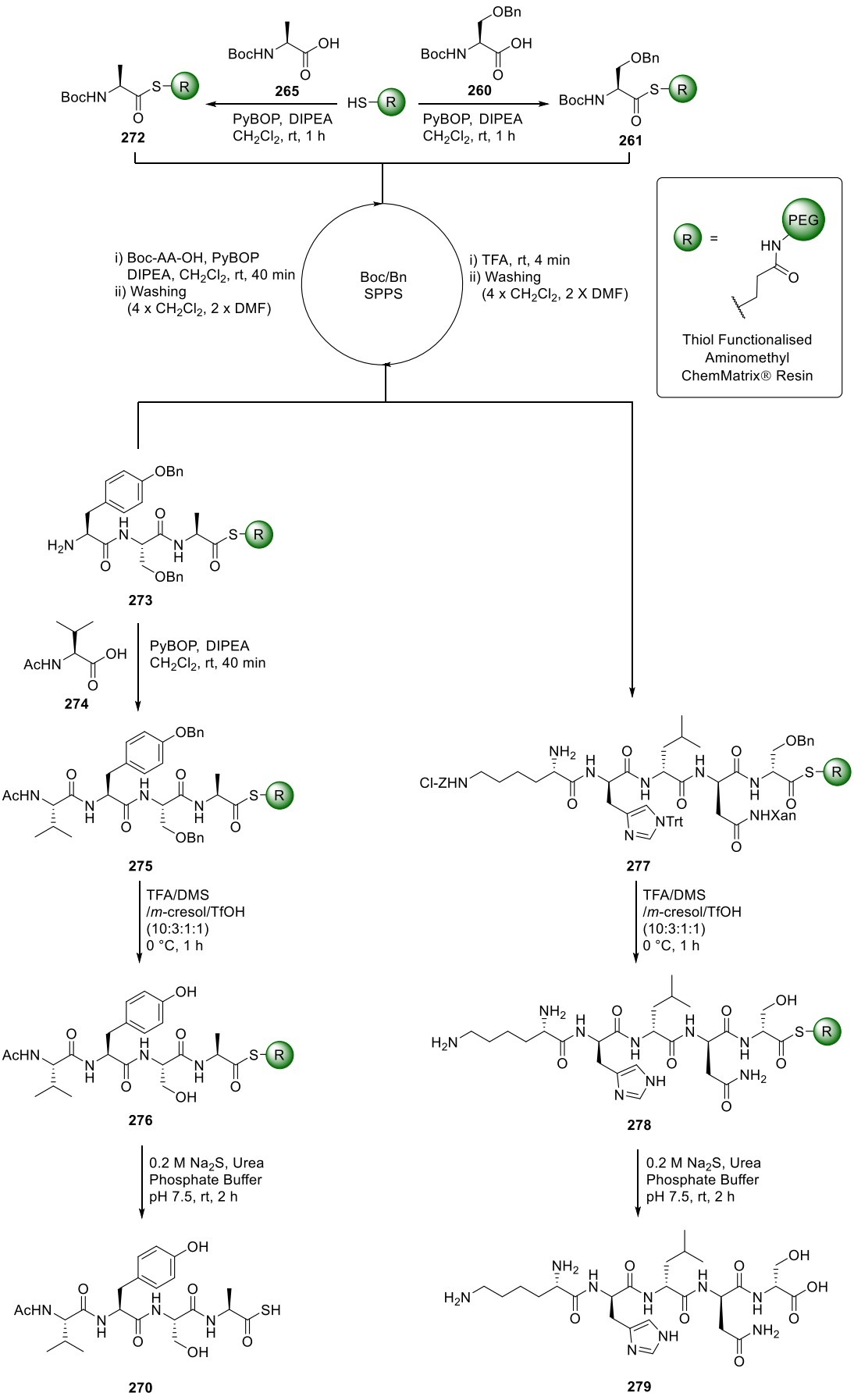


Figure 4.4: Synthetic targets tetrapeptide **270** synthesized by Okamoto *et al.*[299] and human parathyroid hormone pentapeptide fragment **271**.

It was decided that pentapeptide thioacid **271**, a smaller fragment of the desired 10mer **245**, would be synthesised in parallel to the tetrapeptide **270** described by Okamoto *et al.* (**Figure 4.4**) This would provide valuable information about the feasibility of synthesizing our selected sequence and success in applying the synthetic protocol. The optimised conditions described by Okamoto *et al.* constituted more aggressive Boc deprotection conditions, in neat TFA for 4 min, suggesting that the 30% TFA solution utilised by Liu and coworkers may have not been sufficient in the hands of Okamoto *et al.*[299] However, milder global deprotection conditions were utilised at 0 ºC, again suggesting the conditions originally published by Liu and coworkers elicited optimisation in the hands of Okamoto *et al.* The syntheses of literature tetrapeptide thioacid **270** and hPTH pentapeptide thioacid **271** were undertaken in parallel (**Scheme 4.16**). Resin functionalisation was carried out under conditions already described by Okamoto *et al.* (**Scheme 4.16**).



Scheme 4.16: Parallel Boc/Bn SPPS of literature tetrapeptide **271** and human parathyroid hormone pentapeptide fragment **279** under conditions described by Okamoto *et al*.[299]

Coupling of the first Boc-AA and subsequent amide couplings were carried out with PyBop and DIPEA under the same conditions as those employed by Liu and coworkers (**Scheme 4.16**). Boc deprotections between couplings were carried out with neat TFA for 4 min at rt. While rendering manipulation of reaction mixtures difficult, these conditions removed any doubt regarding the effective removal of the *N*-terminal PG and concurrent loss of sequence selectivity, as observed in the attempted synthesis of tripeptide **269**. Resin bound thioesters **275** and **277** were treated with a deprotection cocktail of TFA, *m*-cresol, dimethylsulfide (DMS) and TfOH (10:3:1:1) at 0 °C for 1 h (**Scheme 4.16**). This was followed by multiple washes of the resin beads with Et_2O, CH_2Cl_2, DMF and phosphate buffer at pH 7.5 for neutralisation of the acidic resin. Resin cleavage was carried out under modified conditions, utilizing phosphate buffer and Na_2S as a source of ^-SH.[299] Okamoto *et al.* specified that RP-HPLC must be performed immediately following hydrothiolysis on the supernatant solution obtained without isolation through freeze-drying or evaporation, suggesting that lyophilisation of the thioacid product results in formation of the corresponding carboxylic acid.[299] Hydrothiolysis was undertaken with 0.2 M Na_2S in phosphate buffer at pH 7.5 with the addition of 8 M urea at rt for 2 h. High concentrations of Gdn·HCl are routinely added to ligation reactions in order to prevent protein folding capable of inhibiting the chemical activity of active moieties within the primary protein structure.[77] However, in this case, the addition of high concentration of urea was likely to increase solubility of the crude peptide aiding in RP-HPLC purification. After hydrothiolysis, the resulting mixtures were purified directly by RP-HPLC. The analytical RP-HPLC traces obtained for tetrapeptide **270** by Okamoto *et al.* and in the course of this work may be seen in **Figure 4.5**. Gratifyingly, the analytical RP-HPLC trace obtained by Okamoto *et al.* tetrapeptide **270** (**Figure 4.5**, **A**) bore a close resemblance to the analytical RP-HPLC trace obtained in the course of this work (**Figure 4.5**, **B**). Differences in the retention times of the purified products was due to a difference in flow rate (2.5 mL min^{-1} vs 1 mL min^{-1}) during purification due to the limitations of the HPLC instrument used in our case. The presence of thioacid **270** was confirmed by mass spectrometry, along with the corresponding carboxylic acid **280**. The peak observed during purification at a retention time of 23.9 min likely corresponded to a benzylated product of incomplete deprotection of tetrapeptide **270** (**Fig 4.5**, **B**).

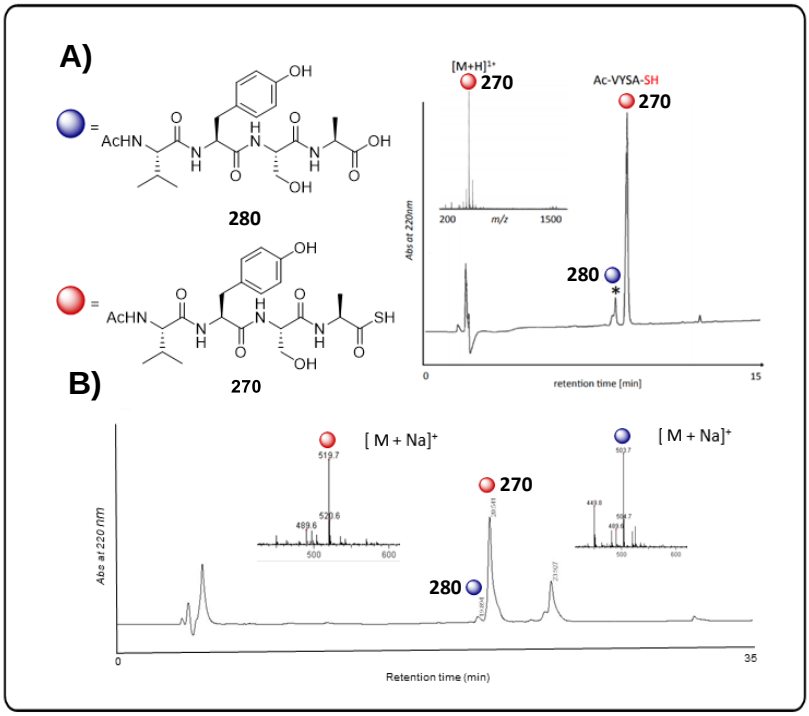


Fig 4.5: A) Analytical RP-HPLC trace and mass spectrum obtained by Okamoto *et al.* of tetrapeptide thioacid **270** and carboxylic acid **280** adapted from Okamoto *et al.*[299] B) Analytical RP-HPLC trace and mass spectrum obtained during the course of this work of tetrapeptide thioacid **270** and carboxylic acid **280**. RP-HPLC performed on C4 Φ4.6 × 250 mm, linear gradient of buffer A (0.1% aq. formic acid):buffer B (90% aq. ACN) from 90:10 to 50:50 over 60 min at a flow rate of 2.5 mL min^{-1} (i) and 1 mL min^{-1} (ii).

However, purification of the reaction mixture following the attempted synthesis of pentapeptide thioacid **271** yielded only the corresponding pentapeptide acid **279** as confirmed by mass spectrometry (**Figure 4.6**). The desired thioacid fragment **271** was not observed in the mass spectrum of the crude sample, even after multiple synthetic attempts. Unfortunately, this suggested that the methodology described by Okamoto *et al.* was not suitable for the synthesis of pentapeptide thioacid **271** in our hands.

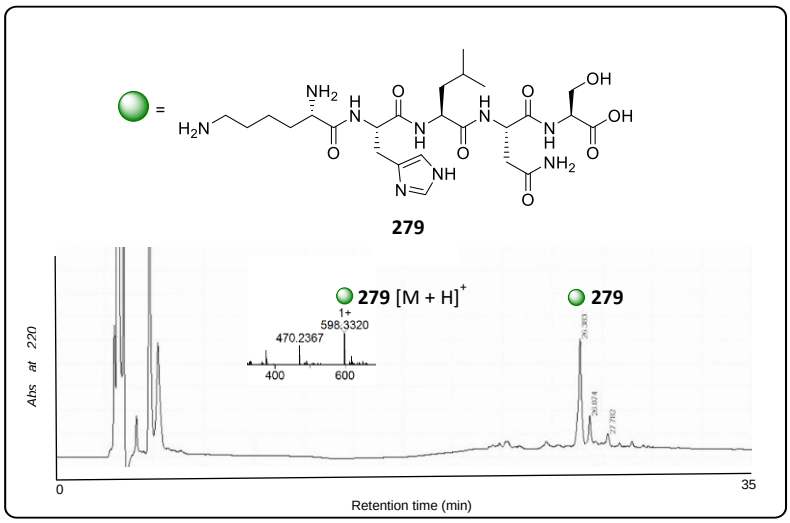


Fig 4.6. Analytical RP-HPLC trace and mass spectrum obtained of pentapeptide **279** RP-HPLC performed on C18 Φ4.6 × 250 mm, linear gradient of buffer A (0.1% aq. formic acid):buffer B (90% aq. ACN) from 90:10 to 50:50 over 60 min at a flow rate of 1 mL min^{-1}.

The fact that pentapeptide acid **279** was isolated as the main product of the synthesis confirms that the synthesis of resin bound unprotected thioester peptide **278** was successful. However, it can be inferred that hydrothiolysis produced an unstable thioacid which rapidly oxidised to the corresponding carboxylic acid product **279**. The discrepancy in the outcomes of the parallel syntheses of tetrapeptide thioacid **270** and pentapeptide thioacid **271** proved difficult to rationalise chemically. The acetylated *N*-terminus of tetrapeptide thioacid **270** was introduced in the case of Okamoto *et al.* in order to ensure selectivity in subsequent iron catalysed coupling reaction with free amine components.[299] It is unlikely that the unprotected terminal amine of pentapeptide thioacid **270** would impact the molecules stability towards oxidation in solution. Likewise, it was not clear how the variation in AA side-chain residues between **270** and **271** would impact their respective stabilities. It was deemed more conceivable that the practical limitations of the SPPS methodology employed had rendered the isolation of pentapeptide thioacid **271** challenging in this case. The hydrothiolysis reactions were carried out as quickly as possible in degassed buffer as described prior to direct analysis by analytical RP-HPLC. However, the dependence for RP-HPLC analysis at the Royal College of Surgeons in Ireland generated an unavoidable delay, in terms of sample relocation, which was presumably sufficient to permit the degradation of pentapeptide thioacid **271** to carboxylic acid **279**. The power of this SPPS methodology had been demonstrated in the literature by Liu and coworkers, culminating in the synthesis of 40 residue thioacid, purified, and used as a component in subsequent peptide ligation.[226] The SPPS of peptide thioacids through alternative methodologies, including in work predating NCL,[98] suggested that isolation and utilisation of these sensitive substrates in valuable amide bond forming reactions is possible. Work is currently ongoing within the Scanlan group to investigate various SPPS and solution phase strategies to access peptide thioacids.

4.4 Conclusions

Unsaturated peptide fragments **243** and **244** and peptide thioacids **245** and **246** were identified as suitable targets to permit the synthesis by ATE mediated peptide ligation of fragments of hPTH, a valuable peptide therapeutic. The synthesis of unsaturated peptides **243** and **244**, incorporating UAAs VG and DHV, was successfully undertaken. Initial syntheses utilizing standard Fmoc SPPS procedures on Wang resin did not furnish the desired unsaturated peptide **244**, likely as a result of the harsh acidic conditions necessary for global peptide deprotection and resin cleavage. The use of 2-chlorotrityl chloride

resin permitted mild resin cleavage to yield protected crude peptide prior to optimisation of global deprotection conditions in solution phase. Deprotection under optimised conditions enabled purification of the resulting crude peptide by semi-preparative RP-HPLC. However, isolation indicated unsuccessful coupling of Boc-DHV **172** to resin bound peptide **249**, yielding truncated 9mer **252** as the major product. Screening of amide coupling conditions permitted successful on-resin coupling of Boc-DHV **172** utilising IIDQ, a coupling reagent used to good effect in the synthesis of VG dipeptide **174** (**Section 3.3**). Subsequent resin cleavage, global deprotection and semi-preparative RP-HPLC, yielded the desired unsaturated peptide **244** in a 6% yield. These optimised SPPS, resin cleavage and deprotection conditions were then applied the synthesis of unsaturated peptide **243**, permitting isolation of the target in a 7% yield without further optimisation. The incorporation of β,γ-unsaturated moieties into peptides *via* SPPS had not been demonstrated prior to this work, likely as a result of their perceived sensitivity towards harsh resin cleavage conditions. The synthesis of these unsaturated compounds paves the way for a litany of interesting chemistries beyond peptide ligation, including post-translational modification and peptide stapling.

However, the syntheses of peptide thioacids **245** and **246**, in our hands, have not been successful as of yet. The methodology of Liu and coworkers employing thiol modified ChemMatrix® aminomethyl resin to permit thioacid synthesis *via* Boc SPPS and hydrothiolytic cleavage did not yield thioacid products. Application of the methodology to the synthesis of simple tripeptide thioacid **269**, obviating the need for harsh side-chain deprotection conditions, yielded a mixture of truncated products upon cleavage. Okamoto *et al.* utilised more forceful *N*-Boc deprotection conditions in the synthesis of tetrapeptide thioacid **270**. In order to ensure the synthetic protocol was being followed accurately tetrapeptide thioacid **270** and pentapeptide thioacid **271**, a smaller fragment of initial target **245**, were synthesised in parallel under these conditions. Gratifyingly, the synthesis of tetrapeptide thioacid **270** was successful, albeit with side-products indicating incomplete deprotection. Unfortunately, the corresponding synthesis of pentapeptide thioacid **271** yielded only carboxylic acid **279**. This was deemed an issue of timely purification and analysis in our hands, as the SPPS of thioacids has been well established in the literature. Future work on this project will aim to establish reliable methodologies for thioacid isolation.

Chapter 5

Conclusions

5.0 Overall Conclusions

The work in this thesis describes efforts to develop ATE mediated peptide ligation methodologies capable of furnishing native products. Several potential systems were explored which were deemed to possess the desired combination of ATE reactivity S-to-N acyl transfer capability and synthetic accessibility.

Chapter 2 details investigation into three unsaturated auxiliary systems for use in ATE mediated peptide ligation. Previous work within the Scanlan group indicated that a level of molecular preorganisation would be necessary to ensure efficient S-to-N acyl transfer over large cyclic TS. Auxiliary system **137**, inspired by the work of Brik and coworkers, was designed in order to facilitate acyl transfer through intramolecular hydrogen bonding interactions. The synthesis of protected cyclohexyl auxiliary bearing dipeptide **137** was carried out smoothly, albeit with a low yielding esterification step likely as a result of steric effects. However, Fmoc deprotection of **137** was unsuccessful when attempted under a wide range of literature conditions, leading to auxiliary cleavage. A simplified acryloylated system was next investigated. Acryloylated dipeptide **140** was accessed synthetically in high yield. The reactivity of the acryloyl group towards ATE was good, permitting the isolation of acyl thioester **147** and Gly thioester **148** in good to excellent yields under either conjugate addition or ATE mediated conditions. Unfortunately, subsequent *N*-Boc deprotection and freebasing under optimised conditions did not result in the isolation of the desired products. *N*-Boc deprotection and freebasing of acyl thioester **147** induced intermolecular S-to-N acyl transfer to yield bis-acylated product **152**. Treatment of Gly thioester **148** under the same conditions permitted the isolation of free amine **157**, indicating no inter- or intramolecular acyl transfer had occurred in this case. This result was justified with regards to both the lack of preorganisation within the system and the considerable steric bulk of the Fmoc PG directing the reactive thioester moiety away from the freebased amine. This conclusion led to the investigation of auxiliary system **141** inspired by the work of Seitz and coworkers. An intermediate in the synthesis of the auxiliary of Seitz, benzylic alkene **141** had been demonstrated to possess the necessary S-to-N acyl transfer characteristics to enable reliable amide formation. The auxiliary was synthesised in yields comparable to those in the literature. Unfortunately, ATE reactions with Gly thioacid **124** yielded no thioester products and a low yield of dipeptide **165**. Suspecting that this dipeptide product was not a result of sequential thioester formation, S-to-N acyl transfer and auxiliary cleavage in one pot, further

investigations into the stability of **141** under ATE conditions were carried out. Auxiliary system **141** was found to be unstable under UV irradiation in the presence of radical initiator DPAP, and that the isolation of dipeptide **165** likely through a thioacid activation mechanism resulting from decomposition of **141**.

Chapter 3 details the development of ATE mediated ligation utilising unsaturated UAAs Boc-VG **171** and Boc-DHV **172**. As larger S-to-N acyl transfer cyclic TS sizes had proven unreliable in furnishing amide products in our hands, a different approach was sought. The use of β,γ-unsaturated AAs, providing they proved synthetically accessible, would permit S-to-N acyl transfer over 6-membered TSs already demonstrated in the literature. VG and DHV were identified as ideal candidates for ATE mediated ligation as their synthesis was established and, in the case of VG, reactivity towards thiol-ene chemistries also well characterised. The synthesis of VG was carried out according to a combination of literature procedures originating from the work of Afzali-Ardakani and Rapoport. Boc-VG **171** suitable for insertion into peptidic systems was accessed in gram quantities in comparable yields to the literature. However, the synthesis of Boc-DHV **172** required considerable optimisation.

Particularly troubling was the critical mesylation/elimination step to form the unsaturated oxazolidine **185**. A combination of poor PG resilience and elimination selectivity limited yields to ~30% under a myriad of conditions. The application of pseudo-Finkelstein conditions described by Maier *et al.* permitted total selectivity for the desired terminal alkene **185** increasing the yield of the reaction to 40%. However, the loss of PG upon mesylate formation could not be prevented under these conditions. Further optimisation was required to yield Boc-DHV **172** from oxazolidine **185**. This was accomplished through application of selective pseudoproline deprotection and Jones reagent titration, to permit access to Boc-DHV **172** in gram quantities.

With UAAs Boc-VG **171** and Boc-DHV **172** in hand the synthesis of unsaturated dipeptides **174** and **175** was carried out according to literature procedures. The synthesis of VG dipeptide **174** required the use of base-free coupling reagent IIDQ to ensure the suppression of isomerising side-reactions, as described by Afzali-Ardakani and Rapoport. Boc-AG **173** was purchased and used to furnish dipeptide **176**, in order to concurrently investigate the feasibility of S-to-N acyl transfer over a 7-membered TS. This intramolecular transfer had been described by Malins *et al.*, albeit in the case of a

rigid thiolated Trp derivative, and the characteristics of the AG dipeptide **176** appraised quickly due to the accessibility of Boc-AG **173**. The reactivity of unsaturated dipeptides **174**, **175** and **176** towards ATE addition was investigated with a range of Fmoc protected amino thioacids. ATE reactions with 3 eq. of Gly thioacid **124** and Fmoc-Ser(O^{t}Bu)-SH **204** proceeded in high yield in all cases. 3 eq. of thioacid was necessary to ensure full consumption of the alkene starting material as radical mediated CSO elimination was found to be a competing side-reaction. Unfortunately, ATE reactions with Phe thioacid **205** and Val thioacid **206** necessitated 5 eq. of thioacid and longer reaction times to permit isolation of thioester products by column chromatography. The yields of ATE reactions of unsaturated dipeptides **174**, **175** and **176** were justified with regards to the steric contribution of the dipeptide, and the stability of the corresponding *C*-centered radicals formed by thioacids **124** and **204** – **207** upon CSO elimination. The S-to-N acyl transfer characteristics of Gly thioesters **208**, **213** and **217** furnished in these reactions were subsequently evaluated. Boc deprotection of AG derived Gly thioester **217** yielded 2,4-diketopiperazine **230** due to the sluggish rate of S-to-N acyl transfer in this case. Gratifyingly, Boc deprotection and freebasing of VG and DHV derived Gly thioesters **208** and **213** yielded amide products **225** and **226** in good yield. Further synthetic elaboration of **225** and **226** by methylation and desulfurisation, respectively, yielded native tripeptides **239** and **242**, the first amide products obtained *via* ATE mediated ligation. This demonstrated the considerable potential of ATE mediated ligation to yield thioesters capable of S-to-N acyl transfer quickly and in high yields.

Chapter 4 describes efforts to apply the ATE mediated ligation methodology demonstrated in Chapter 3 to larger peptide substrates accessed through SPPS. Fragments of hPTH were selected as targets accessible *via* the ATE mediated ligation unsaturated VG decapeptide **243** and thioacid fragment **245**, and unsaturated DHV decapeptide **244** and thioacid fragment **246**. Attempts to synthesise unsaturated peptide **244** under literature SPPS conditions did not yield alkene containing products, likely as a result of harsh deprotection conditions. However, the use of 2-chlorotrityl chloride resin permitted far greater control of global deprotection and revealed that incomplete coupling of Boc-DHV **172** to resin bound peptide **249** had prevented product isolation by semi-preparative RP-HPLC. The use of IIDQ furnished the desired amide linkage, albeit over a long reaction time of 5 days. Global deprotection, resin cleavage and purification by semi-preparative RP-HPLC yielded the desired unsaturated peptide **244** in a 6% yield.

Application of these optimised conditions to the synthesis of unsaturated peptide **243** proceeded smoothly to yield 7% of the desired fragment after purification. However, the SPPS of thioacid fragment **245** proved troublesome. Conditions described by Liu and coworkers proved attractive due to the readily available SMs and facile resin functionalisation. In our hands, the methodology proved insufficient to synthesise even simple tripeptide **269**, instead yielding truncated products indicating the fundamental amide bond forming reactions were not successful. Application of the synthetic conditions described by Okamoto *et al.* permitted the synthesis of tetrapeptide **270** that they had described. Unfortunately, the methodology did not furnish desired thioacid fragments of hPTH necessary for ATE mediated ligation of the target peptide.

Further work would focus on the application of other literature protocols, such as those described by Crich and Sana, to the synthesis of thioacid fragments **245** and **246**. Once this has been achieved ligation of the unsaturated peptides **243** and **244** *via* ATE mediated thioester formation may be undertaken with the corresponding thioacids to afford the target hPTH fragments.

Chapter 6

Experimental

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 silica gel flash 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. Infrared spectra (IR) spectra were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer. Reverse phase HPLC was performed on a Shimadzu Prominence system. For analytical HPLC, a C18 Phenomenex

Gemini 5 µm, 110 Å, 250 x 4.6 mm LC column was used, with a flow rate of 1 mL/min. For semi-preparative HPLC a C18 Phenomenex Gemini 5 µm, 110 Å, 250 x 10 mm LC column was used with a flow rate of 4 mL/min. UV absorption signals were detected with a PDA detector at wavelengths of 214 nm. UV-Vis spectra were recorded by means of a Varian CARY 50 spectrophotometer.

6.2 General Experimental Procedures

Procedure A: Carbodiimide Mediated Amide Coupling

To a stirred solution of carboxylic acid in anhydrous CH_2Cl_2 under Ar was added the EDCI·HCl (2.0 eq.) and HOBt (1.2 eq.) at rt. The solution was cooled to 0 °C and stirred under Ar for 1 h. Amine (1.0 eq.) and DIPEA (4.0 eq.) were added, the solution allowed to reach rt and stirred for 18 h. The crude mixture was washed with 1 M aq. 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 resulting mixture was purified by silica gel flash chromatography.

Procedure B: Removal of *N*-Boc Protecting Groups

To a stirred solution of Boc protected amine in CH_2Cl_2 under Ar was added TES (10.0 eq.). TFA (20% v/v) was added and the reaction mixture stirred for 2 h at rt. Solvent was removed *in vacuo* to give the corresponding TFA salt.

Procedure C: Intermolecular Acyl Thiol-ene

To a stirred solution of alkene acceptor in EtOAc was added DPAP (0.2 eq.), MAP (0.2 eq.) and thioacid (3 eq.) dissolved in a minimum volume of the same solvent. The reaction mixture was subjected to UV irradiation for 1 - 2 h, solvent removed *in vacuo* and the resulting mixture purified by silica gel flash chromatography.

Procedure D: Acyl Thia-Michael Addition

To a mixture of alkene (1.0 eq.) and TEA (0.1 eq.) in anhydrous CH_2Cl_2 was added thioacid (1 eq.) and the reaction mixture stirred at rt under Ar for 12 h. Solvent was removed *in vacuo* and the resulting mixture purified by silica gel flash chromatography.

Procedure E: Preparation of *S*-Trityl thioesters

To a stirred solution of carboxylic acid in anhydrous CH_2Cl_2 under Ar 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 under Ar. The solvent was removed *in vacuo* and the resulting residue purified by silica gel flash chromatography.

Procedure F: Removal of *S*-Trityl protecting groups

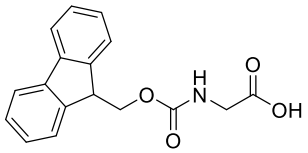
To a stirred solution of *S*-Trt thioester in CH_2Cl_2 under Ar was added triethylsilane (20.0 eq.). TFA (20% v/v) was added and the reaction mixture stirred for 5 min at rt, concentrated *in vacuo* and the resulting crude thioacid used directly without further purification.

Procedure G: Freebasing of TFA salts

To a stirred solution of TFA salt in ACN (0.1 M) was added a sufficient quantity of $NaHCO_3$ to saturate the solution. The reaction mixture was stirred for 2 h at rt and following filtration solvent was removed *in vacuo* and the resulting mixture purified by silica gel flash chromatography.

6.3 Experimental Details for Chapter 2

(((9H-Fluoren-9-yl)methoxy)carbonyl)glycine (132)

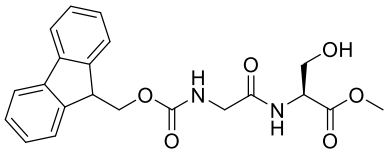


To a solution of glycine (1.00 g, 13.32 mmol) in H_2O (25 mL) was added K_2CO_3 (4.49 g, 13.32 mmol) at rt. A solution of FmocOSu (2.95 g, 21.36 mmol) in dioxane (25 mL) was added and the reaction mixture stirred at rt for 14 h. Dioxane was removed *in vacuo* and the mixture washed with Et_2O (3 x 10 mL), acidified to pH 2 with aq. 1 M HCl solution and extracted with EtOAc (3 x 15 mL). The combined extracts were dried over $MgSO_4$, gravity filtered and concentrated *in vacuo* to yield the product as a white powder (3.47 g, 79%). The isolated compound was in good agreement with the literature.[301]

δ_H (400 MHz, D_6-Acetone): 7.83 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.70 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.39 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.30 (t, *J* = 7.5 Hz, 2H, Ar-CH), 6.79 (bs, 1H, NH), 4.33 (d, *J* = 7.3 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.23 (t, *J* = 7.3 Hz, 1H, Fmoc C$\underline{H}$CH_2), 3.93 (d, *J* = 6.1 Hz, 2H, Gly αCH_2)

HRMS (*m/z* ESI^+): Found: 320.0892 ($[M + Na]^+$, $C_{17}H_{15}NO_4Na$ requires: 320.0893)

Methyl (((9H-fluoren-9-yl)methoxy)carbonyl)glycyl-L-serinate (134)



Prepared as per general procedure A using **132** (1.00 g, 3.36 mmol) and methyl-L-serinate hydrochloride **133** (0.40 g, 3.36 mmol). The resulting off-white solid was purified by silica gel flash chromatography (CH_2Cl_2:MeOH – 98:2) to yield the product as a white solid (0.96 g, 71%).

δ_H (400 MHz, D_6-Acetone): 7.75 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.57 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.39 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.30 (t, *J* = 7.4 Hz, 2H, Ar-CH), 6.78 (d, *J* =

6.8 Hz, 1H, NH), 5.40 (bs, 1H, NH), 4.68 – 4.64 (m, 1H, Ser αCH), 4.42 (d, *J* = 6.8 Hz, 2H, Fmoc CHC<u>H</u>$_2$), 4.22 (t, *J* = 6.8 Hz, 1H, Fmoc C<u>H</u>CH$_2$), 3.99 (dd, *J* = 11.2, 3.6 Hz, 1H, Ser βCH$_2$), 3.96 – 3.87 (m, 3H, Ser βCH$_2$, Gly αCH$_2$) 3.78 (s, 3H, OCH$_3$)

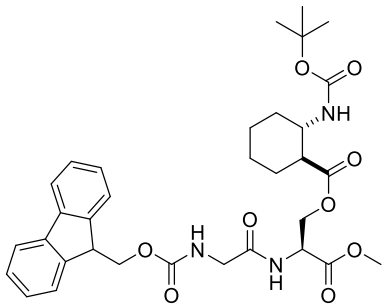
δ_C (100 MHz, $CDCl_3$): 170.7 (C=O), 169.2 (C=O), 156.6 (C=O), 144.1 (Ar-CH), 141.2 (Ar-CH), 127.6 (Ar-CH), 127.1 (Ar-CH), 125.3 (Ar-CH), 119.9 (Ar-CH), 66.5 (Fmoc CH<u>C</u>H$_2$), 62.1 (Ser βCH$_2$), 54.7 (Ser αCH$_2$), 51.5 (OCH$_3$), 47.1 (Fmoc <u>C</u>HCH$_2$), 43.9 (Gly αCH$_2$)

HRMS (*m/z* ESI$^+$): Found: 421.1364 ([M + Na]$^+$, $C_{21}H_{22}N_2O_6Na$ requires: 421.1370)

ν_{max} (film)/cm^{-1}: 3314 (OH, NH), 1663 (C=O), 1440 (Ar C-C), 1239 (C-O)

Mp: 129 – 130 °C

(*S*)-2-(2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)acetamido)-3-methoxy-3-oxopropyl (1S*,2S*)-2-((*tert*-butoxycarbonyl)amino)cyclohexane-1-carboxylate (136)



To a solution of EDC·HCl (0.80 g, 5.15 mmol) and DMAP (0.025 g, 0.20 mmol) in anhydrous CH_2Cl_2 (20 mL) was added *trans*-2-(Boc-amino)-cyclohexanecarboxylic acid (0.50 g, 2.06 mmol) and the reaction mixture stirred at 0 °C for 1 h under Ar. Dipeptide **134** (0.82 g, 2.06 mmol) solubilised in anhydrous CH_2Cl_2 (5 mL) was added and the reaction stirred at rt for 16 h under Ar. The reaction mixture was washed with aq. 1 M HCl solution (2 x 10 mL), sat. aq. $NaHCO_3$ solution (2 x 10 mL) and brine (2 x 10 mL). The mixture was dried over $MgSO_4$, gravity filtered and concentrated *in vacuo* to give an off-white powder which was purified by silica gel flash chromatography (CH_2Cl_2 to CH_2Cl_2:MeOH – 90:10) to yield a white powder (0.66 g, 51%).

δ_H (600 MHz, $CDCl_3$): 7.78 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.64 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.42 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.32 (t, *J* = 7.5 Hz, 2H, Ar-CH), 5.92 (bs, 1H, NH), 4.82 (bs, 1H, Ser αCH), 4.73 (bs, 1H, NH), 4.39 (d, *J* = 7.3 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.31 – 4.21 (m, 1H, Fmoc C$\underline{H}CH_2$), 4.13 – 4.01 (m, 2H, Ser βCH_2), 3.86 (d, *J* = 6.5 Hz, 2H, Gly αCH_2), 3.80 (s, 3H, OCH_3), 2.23 – 2.11 (m, 1H, $CH_2C\underline{H}NH$), 2.03 – 1.96 (m, 2H, $CH_2C\underline{H}C$=O), 1.95 – 1.88 (m, 2H, $CH_2C\underline{H}_2CH$), 1.80 – 1.67 (m, 2H, $CH_2C\underline{H}_2CH_2$), 1.62 – 1.51 (m, 2H, $CH_2C\underline{H}_2CH_2$), 1.45 (s, 9H, $C(CH_3)_3$), 1.32 – 1.25 (m, 2H, $CH_2C\underline{H}_2CH$) (mixture of diastereoisomers)

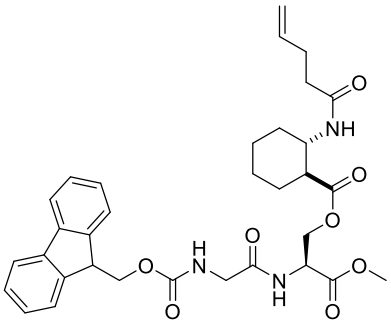
δ_C (151 MHz, $CDCl_3$): 175.1 (C=O), 169.5 (C=O), 169.5 (C=O), 155.4 (C=O), 151.6 (C=O), 143.9 (Ar-C), 141.3 (Ar-C), 127.7 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 120.0 (Ar-CH), 80.1 ($\underline{C}(CH_3)_3$, 67.2 (Fmoc CH$\underline{C}H_2$), 64.3 (Ser βCH_2), 52.8 (Ser αCH), 52.5 ($CH_2\underline{C}HNH$), 51.5 (OCH_3), 51.1 ($CH_2\underline{C}HC$=O), 47.1 (Fmoc $\underline{C}HCH_2$), 42.2 (Gly αCH_2), 33.2 ($CH_2\underline{C}H_2CH$), 28.4 ($C(\underline{C}H_3)_3$, 24.9 ($CH_2\underline{C}H_2CH_2$), 24.8 ($CH_2\underline{C}H_2CH_2$), 24.2 ($CH_2\underline{C}H_2CH$) (mixture of diastereoisomers)

HRMS (*m/z* ESI^+): Found: 646.2731 ($[M + Na]^+$, $C_{33}H_{41}N_3O_9Na$ requires: 646.2735)

ν_{max} (film)/cm^{-1}: 3331 (NH), 1681 (C=O), 1435 (Ar C-C), 1242 (C-O)

Mp: 103 – 105 °C

(*S*)-2-(2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)acetamido)-3-methoxy-3-oxopropyl (1S*,2S*)-2-(pent-4-enamido)cyclohexane-1-carboxylate (137)



Trifluororacetate salt of **136** was prepared by *N*-Boc deprotection of **136** (150 mg, 0.29 mmol) as per general procedure B and used immediately without further purification as per general procedure A with 4-pentenoic acid. The resulting brown oil was purified by

silica gel flash chromatography (Hex:EtOAc – 60:40) to give the product as an orange oil (167 mg, 64%).

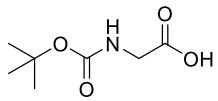
δ_H (600 MHz, $CDCl_3$): 7.77 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.64 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.41 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.32 (t, *J* = 7.5 Hz, 2H, Ar-CH), 6.35 (bs, 1H, NH), 5.89 – 5.65 (m, 2H, $\underline{H}_2C$=CH), 5.12 – 4.97 (m, 3H, H_2C=C$\underline{H}$, Ser αCH), 4.87 (bs, 2H, NH), 4.43 – 4.37 (m, 2H, Fmoc CHC$\underline{H}_2$), 4.31 – 4.24 (m, 1H, Fmoc C$\underline{H}$CH_2), 4.23 – 4.00 (m, 3H, Ser βCH_2, Gly αCH_2), 3.78 (s, 3H, OCH_3), 2.49 – 2.30 (m, 3H, CH_2C$\underline{H}$NH,CH_2C$\underline{H}_2$CH), 2.27 – 2.16 (m, 3H, CH_2C$\underline{H}$C=O, CH_2C$\underline{H}_2CH_2$), 1.93 (t, *J* = 13.8 Hz, 2H, CH_2=CHC$\underline{H}_2CH_2$), 1.76 (t, *J* = 13.8 Hz, 2H, CH_2=CHCH_2C$\underline{H}_2$), 1.58 – 1.34 (m, 2H, CH_2C$\underline{H}_2CH_2$), 1.23 – 1.08 (m, 2H, CH_2C$\underline{H}_2$CH) (mixture of diastereoisomers)

δ_C (151 MHz, $CDCl_3$): 176.5 (C=O), 173.5 (C=O), 169.8 (C=O), 169.5 (C=O), 156.5 (C=O), 144.0 (Ar-CH), 141.3 (Ar-CH), 137.0 (H_2C=$\underline{C}$H), 127.7 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 120.0 (Ar-CH), 115.9 ($H_2\underline{C}$=CH), 67.2 (Fmoc CH$\underline{C}H_2$), 64.7 (Ser βCH_2), 52.7 (Ser αCH), 51.8 ($CH_2\underline{C}$HNH), 51.2 (OCH_3), 49.3 ($CH_2\underline{C}$HC=O), 47.2 (Fmoc $\underline{C}$HCH_2), 44.1 (Gly αCH_2), 35.8 (CH_2=CH$CH_2\underline{C}H_2$), 33.1 ($CH_2\underline{C}H_2$CH), 29.5 (CH_2=CH$\underline{C}H_2CH_2$), 28.7 ($CH_2\underline{C}H_2CH_2$), 24.7 ($CH_2\underline{C}H_2CH_2$), 24.3 ($CH_2\underline{C}H_2$CH) (mixture of diastereoisomers)

HRMS (*m/z* MALDI): Found: 628.2650 ($[M + Na]^+$, $C_{33}H_{39}N_3O_8Na$ requires: 628.2635)

ν_{max} (film)/cm^{-1}: 3314 (OH, N-H), 2932 C-H) 1698 (C=O), 1645 (C=C), 1448 (Ar C-C), 1246 (C-O)

(*tert*-Butoxycarbonyl)glycine (143)



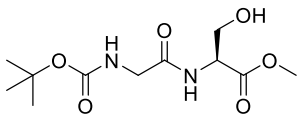
To a solution of glycine (2.00 g, 26.64 mmol) in dioxane (20 mL) was added aq. 1 M NaOH solution (40 mL) at 0 °C. Following this Boc_2O (6.98 g, 31.97 mmol) (amount) was added slowly while stirring at 0 °C. The reaction mixture was stirred at 0 °C for 15 min before being allowed to reach rt and stirred for 16 h. Dioxane was evaporated *in vacuo* and the aq. mixture washed with Et_2O (2 X 20 mL), diluted with EtOAc (60 mL) and acidified to pH 2 with aq. 1 M HCl solution. The aq. layer was saturated with NaCl

and extracted with EtOAc (5 x 60 mL). The combined extracts were dried over $MgSO_4$ and concentrated *in vacuo* to give a white powder (3.75 g, 80%). The isolated compound was in good agreement with the literature.[302]

δ_H (600 MHz, $CDCl_3$): 5.01 (bs, 1H, NH), 3.98 – 3.86 (m, 2H, Gly αCH_2), 1.44 (s, 9H, $C(CH_3)_3$).

HRMS (*m/z* APCI$^-$): Found: 174.0768 ([M - H]$^-$, $C_7H_{12}NO_4$ requires: 174.0771)

Methyl (*tert*-butoxycarbonyl)glycyl-L-serinate (144)

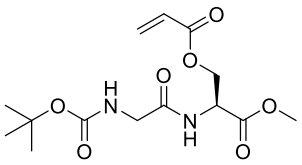


Prepared as per general procedure A using **143** (1.00 g, 5.71 mmol) and methyl-L-serinate hydrochloride **133** (0.40 g, 3.36 mmol). The resulting brown oil was purified by silica gel flash chromatography (Hex:EtOAc– 90:10) to yield the product as a colourless oil (1.51 g, 66%). The isolated compound was in good agreement with the literature.[303]

δ_H (400 MHz, $CDCl_3$): 7.24 (s, 1H, NH), 5.55 (bs, 1H, NH), 4.66 – 4.61 (m, 1H, Ser αCH), 3.95 (dd, *J* = 11.6, 3.6 Hz, 1H, Ser βCH_2), 3.90 (dd, *J* = 11.6, 3.1 Hz, 1H, Ser βCH_2), 3.83 (d, *J* = 3.9 Hz, 2H, Gly αCH_2), 3.76 (s, 3H, OCH_3), 1.43 (s, 9H, $C(CH_3)_3$).

HRMS (*m/z* APCI$^+$): Found: 277.1385 ([M + H]$^+$, $C_{11}H_{21}N_2O_6$ requires: 277.1394)

(*S*)-2-(2-((*tert*-Butoxycarbonyl)amino)acetamido)-3-methoxy-3-oxopropyl acrylate (140)



To a solution of dipeptide **144** (0.56 g, 2.04 mmol) in anhydrous CH_2Cl_2 (25 mL) was added TEA (0.76 mL, 6.11 mmol) and acryloyl chloride **145** (0.36 mL, 4.48 mmol) at

0 °C and the reaction mixture stirred at rt for 16 h under Ar. The reaction was quenched with MeOH (2 mL), concentrated *in vacuo* and the resulting red oil purified by silica gel flash chromatography (Hex:EtOAc – 50:50) to yield the product as a yellow oil (0.43 g, 65%).

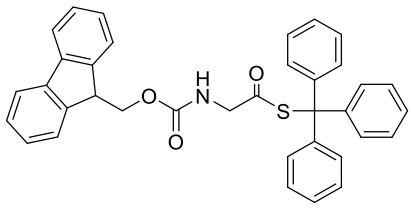
δ_H (400 MHz, $CDCl_3$): 6.93 (s, 1H, NH), 6.40 (dd, *J* = 17.3, 1.2 Hz, 1H, CH=C$\underline{H}_2$), 6.08 (dd, *J* = 17.3, 10.5 Hz, 1H, C$\underline{H}$=CH_2), 5.86 (dd, *J* = 10.5, 1.2 Hz, 1H, CH=C$\underline{H}_2$), 5.18 (bs, 1H, NH), 4.91 – 4.85 (m, 1H, Ser αCH), 4.53 (dd, *J* = 11.5, 3.9 Hz, 1H, Ser βCH_2), 4.45 (dd, *J* = 11.5, 3.5 Hz, 1H, Ser βCH_2), 3.84 (t, *J* = 5.8 Hz, 2H, Gly αCH_2), 3.77 (s, 3H, OCH_3), 1.44 (s, 9H, $C(CH_3)_3$)

δ_C (100 MHz, $CDCl_3$): 169.6 (C=O), 169.5 (C=O), 165.5 (C=O), 155.9 (C=O), 131.9 (CH=$\underline{C}H_2$), 127.4 ($\underline{C}$H=CH_2), 80.4 ($\underline{C}(CH_3)_3$), 63.8 (Ser βCH_2), 52.9 (Ser αCH), 51.7 (OCH_3), 44.3 (Gly αCH_2), 28.2 ($C(\underline{C}H_3)_3$)

HRMS (*m/z* ESI^+): Found: 353.1314 ($[M + Na]^+$, $C_{14}H_{22}N_2O_7Na$ requires: 353.1319)

ν_{max} (film)/cm^{-1}: 3321 (N-H), 1685 (C=O), 1652 (C=C), 1255 (C-O)

_S_-Trityl 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)ethanethioate (146)



Prepared as per general procedure E using Fmoc-Gly-OH **132** (1.00 g, 3.36 mmol). The resulting yellow residue was purified by silica gel flash chromatography (Hex:EtOAc – 99:1) to yield the product as a yellow foam (1.46 g, 79%).

δ_H (400 MHz, $CDCl_3$): 7.76 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.57 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.39 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.33 – 7.20 (m, 17H, Ar-CH), 5.27 (bs, 1H, NH), 4.39 (d, *J* = 7.1 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.21 (t, *J* = 7.1 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.11 (d, *J* = 5.6 Hz, 2H, Gly αCH_2)

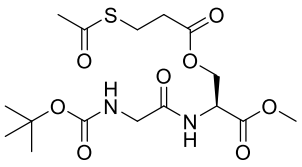
δ_C (100 MHz, $CDCl_3$): 194.3 (SC=O), 155.9 (C=O), 143.7 (Ar-CH), 143.4 (Ar-CH), 141.3 (Ar-CH), 129.8 (Ar-CH), 127.9 (Ar-CH), 127.7 (Ar-CH), 127.3 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.0 (Ar-CH), 70.9 (Fmoc $CH\underline{C}H_2$), 67.3 $\underline{C}(Ar)_3$, 50.5 (αCH_2), 47.1 (Fmoc $\underline{C}HCH_2$)

HRMS (*m/z* ESI^+): Found: 578.1779 ($[M + Na]^+$, $C_{36}H_{29}NO_3SNa$ requires: 578.1766)

ν_{max} (film)/cm^{-1}: 1691 (C=O), 1442 (Ar C-C), 1238 (C-O)

Mp: 121 – 123 °C

Methyl *O*-(3-(acetylthio)propanoyl)-*N*-((*tert*-butoxycarbonyl)glycyl)-L-serinate (147)



Prepared using acryloylated dipeptide **140** and thioacetic acid **97** as per general procedure C (50 mg, 0.17 mmol) and D (130 mg, 0.40 mmol). The resulting yellow residue was purified by silica gel flash chromatography (Hex:EtOAc – 1:1) to yield the product as a yellow film (63 mg, 98% and 160 mg, 96%, respectively).

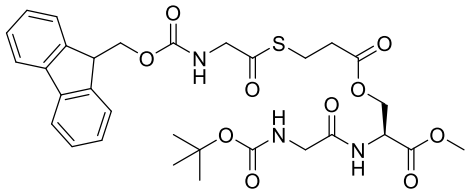
δ_H (600 MHz, $CDCl_3$): 6.87 (s, 1H, NH), 5.18 (bs, 1H, NH), 4.87 – 4.82 (m, 1H, Ser αCH), 4.47 (dd, *J* = 11.4, 3.9 Hz, 1H, Ser βCH_2), 4.41 (dd, *J* = 11.4, 3.5 Hz, 1H, Ser βCH_2), 3.85 (d, *J* = 5.7 Hz, 2H, Gly αCH_2), 3.78 (s, 3H, OCH_3), 3.09 (t, *J* = 6.9 Hz, 2H, $SC\underline{H}_2CH_2$), 2.62 (t, *J* = 6.9 Hz, 2H, $SCH_2C\underline{H}_2$), 2.33 (s, 3H, $COCH_3$), 1.46 (s, 9H, $C(CH_3)_3$)

δ_C (151 MHz, $CDCl_3$): 195.5 (SC=O), 171.0 (C=O), 169.5 (C=O), 169.4 (C=O), 156.0 (C=O), 80.3 ($\underline{C}(CH_3)_3$), 63.9 (Ser βCH_2), 52.9 (Ser αCH), 51.6 (OCH_3), 44.2 (Gly αCH_2), 34.2 ($SCH_2\underline{C}H_2$), 30.5 ($CO\underline{C}H_3$), 28.3 ($C(\underline{C}H_3)_3$), 24.0 ($S\underline{C}H_2CH_2$)

HRMS (*m/z* ESI+): Found: 429.1306 ($[M + Na]^+$, $C_{16}H_{26}N_2O_8SNa$ requires: 429.1302)

ν_{max} (film)/cm^{-1}: 3314 (N-H), 2961 (C-H), 1735 (C=O), 1249 (C-O)

Methyl *O*-(3-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)propanoyl)-*N*-((*tert*-butoxycarbonyl)glycyl)-L-serinate (148)



Following *S*-Trt deprotection of **146** as per procedure F, crude Fmoc-Gly thioacid **124** was reacted with acryloylated dipeptide **140** immediately as per procedures C (61 mg, 0.18 mmol) and D (58 mg, 0.18 mmol). The resulting yellow residue was purified by silica gel flash chromatography (MePh:Ace – 95:5) to yield the product as a yellow oil (94 mg, 79% and 84 mg, 74%, respectively).

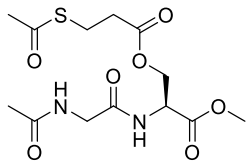
δ_H (400 MHz, $CDCl_3$): 7.74 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.57 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.40 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.30 (t, *J* = 7.5 Hz, 2H, Ar-CH), 6.87 (s, 1H, NH), 5.71 (bs, 1H, NH), 5.40 (bs, 1H, NH), 4.92 – 4.85 (m, 1H, Ser αCH), 4.49 (dd, *J* = 11.3, 3.9 Hz, 1H, Ser βCH_2), 4.46 – 4.37 (m, 3H, Fmoc CHC$\underline{H}_2$, Ser βCH_2), 4.22 (t, *J* = 6.7 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.13 (d, *J* = 5.4 Hz, 2H, Gly αCH_2), 3.92 (d, *J* = 5.6 Hz, 2H, Gly αCH_2), 3.76 (s, 3H, OCH_3), 3.10 (t, *J* = 7.1 Hz, 2H, SC$\underline{H}_2CH_2$), 2.63 (t, *J* = 7.1 Hz, 2H, $SCH_2C\underline{H}_2$), 1.45 (s, 9H, $C(CH_3)_3$)

δ_C (100 MHz, $CDCl_3$): 196.2 (SC=O), 172.2 (C=O), 170.51 (C=O), 169.9 (C=O), 158.7 (C=O), 157.9 (C=O), 144.2 (Ar-CH), 141.6 (Ar-CH), 128.9 (Ar-CH), 126.9 (Ar-CH), 124.3 (Ar-CH), 121.7 (Ar-CH), 80.4 ($\underline{C}(CH_3)_3$, 69.8 (Fmoc CH$\underline{C}H_2$), 63.7 (Ser βCH_2), 52.9 (Ser αCH), 51.8 (OCH_3), 48.1 (Gly αCH_2), 47.3 (Fmoc $\underline{C}HCH_2$), 44.2 (Gly αCH_2), 35.1 ($SCH_2\underline{C}H_2$), 28.4 ($C(\underline{C}H_3)_3$, 23.8 ($S\underline{C}H_2CH_2$)

HRMS (*m/z* ESI^+): Found: 666.2088 ($[M + Na]^+$, $C_{31}H_{37}N_3O_{10}SNa$ requires: 666.2096)

ν_{max} (film)/cm^{-1}: 3311 (N-H), 2957 (C-H), 1742 (C=O), 1450 (Ar C-C), 1244 (C-O)

Methyl *N*-(acetylglycyl)-*O*-(3-(acetylthio)propanoyl)-L-serinate (152)



Trifluororacetate salt **149** was prepared by *N*-Boc deprotection of thioester **147** (0.45g, 1.38 mmol) as per general procedure B. The resulting brown oil was redissolved in CH_2Cl_2, Amberlyst® A21 resin (2.00 g) added and the reaction mixture stirred for 5 h at rt. The reaction mixture was gravity filtered and the filtrate concentrated *in vacuo* to give a white residue which was purified by silica gel flash chromatography (CH_2Cl_2:MeOH - 92:8) to give the product as a yellow oil (0.086 g, 22%).

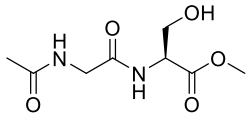
δ_H (600 MHz, $CDCl_3$): 7.08 (d, *J* = 7.6 Hz, 1H, NH), 6.49 (s, 1H, NH), 4.97 – 4.80 (m, 1H, αCH), 4.48 (dd, *J* = 12.0, 3.6 Hz, 2H, βCH_2), 4.02 (d, *J* = 5.2 Hz, 2H, αCH_2), 3.80 (s, 3H, OCH_3), 3.11 (t, *J* = 6.7 Hz, 2H, SC$\underline{H}_2CH_2$), 2.65 (t, *J* = 7.2 Hz, 2H, $SCH_2C\underline{H}_2$), 2.36 (s, 3H, $COCH_3$), 2.07 (s, 3H, $COCH_3$)

δ_C (151 MHz, $CDCl_3$): 195.7 (SC=O), 171.2 (C=O), 170.8 (C=O), 169.4 (C=O), 169.1 (C=O), 63.7 (Ser βCH_2), 53.0 (Ser αCH), 51.8 (OCH_3), 43.1 (Gly αCH_2), 34.3 ($SCH_2C\underline{H}_2$), 29.4 ($COCH_3$), 24.0 ($SCH_2C\underline{H}_2$), 22.9 ($COCH_3$)

HRMS (*m/z* ESI^+): Found: 371.0891 ($[M + Na]^+$, $C_{13}H_{20}N_2O_7SNa$ requires: 371.0883)

ν_{max} (film)/cm^{-1}: 3326 (N-H), 2954 (C-H), 1732 (C=O), 1243 (C-O)

Methyl acetylglycyl-L-serinate (154)



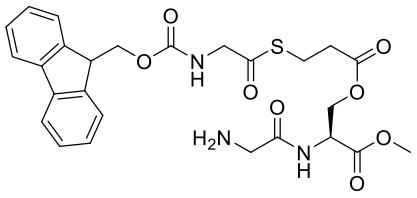
Trifluororacetate salt **149** was prepared by *N*-Boc deprotection of thioester **147** (68 mg, 0.16 mmol) as per general procedure B. The resulting brown oil was redissolved in CH_2Cl_2, Amberlyst® A21 resin (0.50 g) added and the reaction mixture stirred for 5 h at rt. The reaction mixture was gravity filtered and the filtrate concentrated *in vacuo* to give a white residue which was treated with 0.1 M NaOMe/MeOH solution (1 mL) while

stirring at rt for 30 min. The solution was neutralised with AcOH and solvent evaporated *in vacuo.* The resulting white solid was redissolved in H_2O (1 mL) and extracted with EtOAc (3 x 0.5 mL) and the combined extracts washed with brine (2 x 0.5 mL) dried over $MgSO_4$, gravity filtered and concentrated *in vacuo* to give a white powder which was purified by silica gel flash chromatography (CH_2Cl_2:MeOH – 90:10) to give the product as a white powder (18 mg, 19%). The isolated compound was in good agreement with the literature.[304]

δ_H (400 MHz, $CDCl_3$): 4.89 - 4.78 (m, 1H, Ser αCH), 4.51 (dd, *J* = 12.0, 3.6 Hz, 2H, βCH_2), 4.41 (dd, *J* = 11.4, 3.7 Hz, 1H, βCH_2), 3.87 (m, 2H, Gly αCH_2), 3.76 (s, 3H, $COCH_3$), 2.10 (s, 3H, OCH_3)

HRMS (*m/z* APCI$^+$): Found: 298.0986 ($[M + H]^+$, $C_8H_{15}N_2O_5$ requires: 298.0979)

Methyl *O*-(3-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)propanoyl)-*N*-glycyl-L-serinate (157)



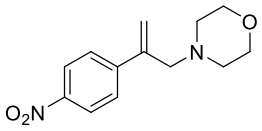
Trifluororacetate salt **155** was prepared by *N*-Boc deprotection of thioester **148** (60 mg, 0.093 mmol) as per general procedure B. Trifluoroacetate salt **155** was immediately freebased as per general procedure G. Purification by silica gel flash chromatography (10 - 30% MeOH: CH_2Cl_2) gave the product as a colourless oil (40 mg, 80%).

δ_H NMR (400 MHz, $CDCl_3$): 7.79 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.59 – 7.52 (m, 2H, Ar-CH), 7.43 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.33 (t, *J* = 7.0 Hz, 2H, Ar-CH), 6.78 (bs, 1H, NH), 6.68 (bs, 1H, NH), 5.33 (d, *J* = 6.6 Hz, 1H, NH), 4.74 – 4.65 (m, *J* = 12.5, 7.1 Hz, 1H, Ser αCH), 4.49 – 4.33 (m, 3H, Fmoc CHC$\underline{H}_2$, Ser βCH_2), 4.21 (t, *J* = 6.8 Hz, 1H, Fmoc C$\underline{H}CH_2$), 3.98 (m, 3H, Ser βCH_2, Gly αCH_2), 3.73 (s, 3H, OCH_3), 3.12 (bs, 2H, $SCH_2C\underline{H}_2$), 2.54 (d, *J* = 7.0 Hz, 1H, S$\underline{C}H_2CH_2$) 2.15 – 1.97 (m, 2H, Gly αCH_2)

δ_C NMR (151 MHz, $CDCl_3$): 199.9 (SC=O), 171.2 (C=O), 170.8 (C=O), 170.1 (C=O), 156.4 (C=O), 143.9 (Ar-qC), 141.5 (Ar-qC), 129.4 (Ar-CH), 128.0 (Ar-CH), 125.1 (Ar-CH), 120.2 (Ar-CH), 67.4 (Fmoc CH<u>C</u>H_2), 56.6 (Ser βCH_2), 52.5 (OCH_3), 52.0 (Ser αCH), 47.2 (Fmoc <u>C</u>HCH_2) 41.2 (Gly αCH_2), 38.2 (S<u>C</u>H_2CH_2), 35.8 (Gly αCH_2) 20.9 (SCH_2<u>C</u>H_2)

ν_{max} (film)/cm^{-1}: 3317 (N-H), 3063 (C-H), 1746 (C=O), 1716 (C=O), 1688 (C=O)

4-(2-(4-Nitrophenyl)allyl)morpholine (159)

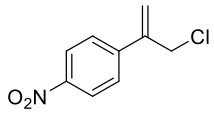


p-Nitrophenylacetic acid **158** (10.00 g, 55.20 mmol) and *p*-formaldehyde (3.98 g, 132.52 mmol) were heated in toluene (Tol, 25 mL) at 90 ºC. Upon dissolution, morpholine (4.72 mL, 55.20 mmol) was added dropwise and the reaction mixture stirred at 90 ºC for 1 h and at 100 ºC for a further 4 h. The mixture was concentrated *in vacuo* before being redissolved in CH_2Cl_2 (50 mL), washed with H_2O (2 x 25 mL), dried over $MgSO_4$ and concentrated *in vacuo*. The resulting residue was purified by silica gel flash chromatography (Hex:EtOAc - 75:25) to yield the product as a yellow crystalline solid (5.86 g, 45%). The isolated compound was in good agreement with the literature.[154]

δ_H NMR (400 MHz, $CDCl_3$): 8.25 – 8.20 (m, 2H, Ar-CH), 7.74 – 7.64 (m, 2H, Ar-CH), 5.67 (s, 1H, C=CH_2), 5.44 (s, 1H, C=CH_2), 3.69 (bs, 4H, CH_2C<u>H</u>$_2$NC<u>H</u>$_2CH_2$), 3.38 (s, 2H, H_2C =CC<u>H</u>$_2$N), 2.48 (bs, 4H, CH_2C<u>H</u>$_2$OC<u>H</u>$_2CH_2$)

δ_C NMR (151 MHz, $CDCl_3$): 153.0 (Ar-qC), 146.5 (Ar-qC), 142.2 (<u>C</u>=CH_2), 127.2 (Ar-CH), 119.3 (Ar-CH), 116.6 (C=<u>C</u>H_2) 67.0 (CH_2<u>C</u>H_2O<u>C</u>H_2CH_2), 64.8 (H_2C=C<u>C</u>H_2N), 53.4 (CH_2<u>C</u>H_2N<u>C</u>H_2CH_2)

1-(3-Chloroprop-1-en-2-yl)-4-nitrobenzene (161)

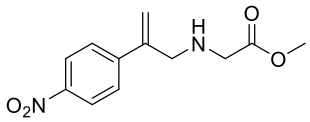


Morpholine derivative **159** (2.31 g, 9.30 mmol) was dissolved in Tol (7 mL) and isobutyl chloroformate **160** (1.45 mL, 11.20 mmol) added dropwise and the reaction stirred for 14 h at rt. Solvent was removed *in vacuo* and the resulting orange residue purified by silica gel flash chromatography (Hex:EtOAc - 80:20) to yield the product as a yellow oil (1.39 g, 76%). The isolated compound was in good agreement with the literature.[154]

δ_H NMR (400 MHz, $CDCl_3$): 8.26 (d, *J* = 8.9 Hz, 2H Ar-CH), 7.67 (d, *J* = 8.9 Hz, 2H Ar-CH), 5.74 (s, 1H, C=CH_2), 5.67 (s, 1H, C=CH_2), 4.52 (s, 2H, CH_2Cl)

δ_C NMR (151 MHz, $CDCl_3$): 146.0 (Ar-qC), 144.0 (Ar-qC), 142.4 (Allyl-qC), 127.1 (Ar-CH), 123.9 (Ar-CH), 120.3 (C=$\underline{C}H_2$), 45.9 (CH_2Cl)

Methyl (2-(4-nitrophenyl)allyl)glycinate (141)



Allyl chloride **161** (0.30 g, 1.52 mmol) was dissolved in CH_2Cl_2 (15 mL), DIPEA (1.40 mL, 8.05 mmol) and HCl·Gly-OMe **162** (0.57 g, 4.55 mmol) added and the resulting mixture stirred for 16 h at rt. The mixture was washed with H_2O (2 x 10 mL), dried over $MgSO_4$, and concentrated *in vacuo*. The resulting red residue was purified by silica gel flash chromatography (Hex:EtOAc – 80:20) yield the product as a yellow oil (0.19 g, 52 %).

R_f (30% EtOAc:Hex): 0.35

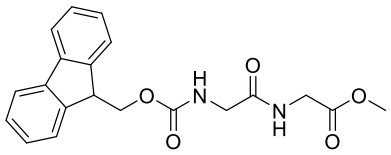
δ_H NMR (400 MHz, $CDCl_3$): 8.21 (d, *J* = 9.1 Hz, 2H, Ar-CH), 7.68 (d, *J* = 9.1 Hz, 2H, Ar-CH), 5.61 (s, 1H, C=C$\underline{H}_2$), 5.48 (s, 1H, C=C$\underline{H}_2$), 3.76 (s, 3H, OCH_3), 3.72 (s, 2H, H_2C=CC$\underline{H}_2$NH), 3.46 (s, Gly αCH_2, 2H)

δ_C NMR (151 MHz, $CDCl_3$): 172.8 (C=O), 146.2 (Ar-qC,), 146.2 (Ar-qC), 144.1 ($\underline{C}=CH_2$), 127.1 (Ar-CH), 123.7 (Ar-CH), 117.7 ($C=\underline{C}H_2$), 52.8 ($H_2C=CC\underline{H}_2NH$), 51.9 (OCH_3), 49.7 (Gly αCH_2)

HRMS (*m/z* APCI$^+$): Found: 251.1024 ($[M + H]^+$, $C_{12}H_{15}N_2O_4$ requires: 251.1026)

ν_{max} (film)/cm^{-1}: 2951 (C-H), 1736 (C=O), 1639 (C=C), 1574 (N-O)

Methyl (((9H-fluoren-9-yl)methoxy)carbonyl)glycylglycinate (165)



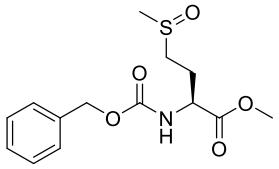
Following *S*-Trt deprotection of thioester **146** as per procedure F, crude Fmoc-Gly thioacid **124** was reacted with *para*-Nitrophenyl alkene **141** immediately as per procedure C (80 mg, 0.32 mmol). The resulting brown residue was purified by silica gel flash chromatography (EtOAc:Hex – 70:30) to yield the product as a white solid (12 mg, 9%). The isolated compound was in good agreement with the literature.[305]

δ_H NMR (400 MHz, $CDCl_3$): 7.80 (d, *J* = 8.7 Hz, 2H, Ar-CH), 7.63 (d, *J* = 6.1 Hz, 2H, Ar-CH), 7.45 (t, *J* = 7.3 Hz, 2H, Ar-CH), 7.33 (t, *J* = 7.3 Hz, 2H, Ar-CH), 6.40 (bs, 1H, NH), 5.41 (s, 1H, NH), 4.49 (d, *J* = 7.26, 2H, Fmoc CHC$\underline{H}_2$), 4.28 (t, *J* = 6.05 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.10 (d, *J* = 5.24 Hz, 2H, Gly αCH_2, 2H), 3.96 (d, *J* = 6.05 Hz, 2H, Gly αCH_2), 3.80 (s, 3H, OCH_3).

HRMS (*m/z* APCI$^+$): Found: 369.1444 ($[M + H]^+$, $C_{20}H_{20}N_2O_5$ requires: 369.1372)

6.4 Experimental Details for Chapter 3

Methyl (2*S*)-2-(((Benzyloxy)carbonyl)amino)-4-(methylsulfinyl)butanoate (178)

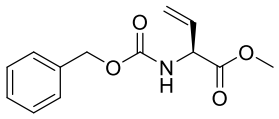


To stirred solution of Cbz-Met-OMe (5.00 g, 16.81 mmol) in MeOH (25 mL) at 0 °C was added dropwise a solution of $NaIO_4$ (3.96 g, 18.60 mmol) in H_2O (25 mL) after which the reaction was allowed to reach rt and stirred for 14 h. The resulting mixture was filtered through elite to remove precipitated solids and the filtrate extracted with $CHCl_3$ (3 x 25 mL). Combined extracts were washed with brine (2 x 30 mL) and dried over $MgSO_4$ to yield the product as a pale yellow oil (5.20 g, 99%). The isolated compound was in good agreement with the literature.[252]

δ_H NMR (400 MHz, $CDCl_3$): 7.37 – 7.28 (m, 5H, Ar-CH), 5.74 (dd, *J* = 27.9, 7.7 Hz, 1H, NH), 5.08 (s, 2H, Ar-CH_2CO), 4.52 – 4.42 (m, 1H, αCH), 3.74 (s, 3H, OCH_3), 2.81 – 2.63 (m, 2H, γCH_2), 2.53 (d, *J* = 3.0 Hz, 3H, $SOCH_3$), 2.44 – 2.27 (m, 1H, βCH_2), 2.21 – 2.03 (m, 1H, βCH_2)

HRMS (*m/z* ESI^+): Found: 336.0879 ($[M + Na]^+$, $C_{14}H_{19}NO_5Na$ requires: 336.0876)

Methyl *(S)*-2-(((benzyloxy)carbonyl)amino)but-3-enoate (71)

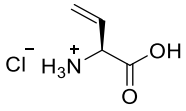


A stirred solution of **178** (5.00 g, 15.96 mmol) in xylene (50 mL) was heated under reflux for 72 h. Solvent was evaporated *in vacuo* and the resulting brown residue was purified by silica gel flash chromatography (Hex:EtOAc – 90:10 to 80:20) to yield the product as a colourless oil (2.08 g, 52%). The isolated compound was in good agreement with the literature.[252]

δ_H NMR (400 MHz, $CDCl_3$): 7.38 – 7.28 (m, 1H, Ar-CH), 5.94 – 5.84 (m, 1H, C<u>H</u>=CH_2), 5.48 (bs, 1H, αCH), 5.35 (d, *J* = 17.1 Hz, 1H, CH=C<u>H</u>$_2$), 5.26 (d, *J* = 10.3 Hz, 1H, CH=C<u>H</u>$_2$), 5.11 (s, 2H, Ar-C<u>H</u>$_2$), 4.93 (bs, 1H, NH), 3.75 (s, 1H, OC<u>H</u>$_3$)

HRMS (*m/z* ESI^+): Found: 272.0899 ($[M + Na]^+$, $C_{13}H_{15}NO_4Na$ requires: 272.0893)

(S)-2-Aminobut-3-enoic acid hydrochloride (179)

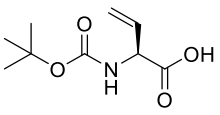


A solution of **71** (1.00 g, 4.01 mmol) in 6 M aq. HCl solution (20 mL) was heated under reflux for 1.5 h. After cooling, the resulting mixture was washed with $CHCl_3$ (2 x 15 mL) and solvent removed *in vacuo* to yield the crude product. Recrystallisation from acetone gave the product as a white crystalline solid (0.39 g, 72%). The isolated compound was in good agreement with the literature.[252]

δ_H NMR (400 MHz, H_2O): 5.85 (ddd, *J* = 17.5, 10.4, 7.4 Hz, 1H, C<u>H</u>=CH_2), 5.45 (dd, *J* = 2.5, 1.0 Hz, 1H, CH=C<u>H</u>$_2$), 5.41 (dd, *J* = 4.2, 1.0 Hz, 1H, CH=C<u>H</u>$_2$), 4.43 (d, *J* = 7.4 Hz, 1H, αCH)

HRMS (*m/z* ESI^+): Found: 102.0552 ($[M + H]^+$, $C_4H_8NO_2$ requires: 102.0550)

(S)-2-((*tert*-Butoxycarbonyl)amino)but-3-enoic acid (171)



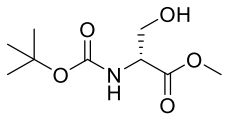
To a solution of **179** (0.30 g, 2.18 mmol) and $NaHCO_3$ (0.33 g, 4.36 mmol) in H_2O (12 mL) was added a solution of Boc_2O (0.63 g, 2.40 mmol) in dioxane (12 mL) and the resulting mixture heated under reflux for 2 h. Dioxane was removed *in vacuo* and the mixture acidifed with 1 M aq. HCl solution to pH 2 and extracted with $CHCl_3$ (3 x 15 mL). The combined organic extracts were washed with brine (2 x 15 mL), dried over

$MgSO_4$ and solvent removed *in vacuo* to yield the product as a white waxy solid (0.36 g, 83%). The isolated compound was in good agreement with the literature.[252]

δ_H NMR (400 MHz, $CDCl_3$): 7.01 (s, 0.5H, NH), 5.92 (m, 1H, C<u>H</u>=CH_2), 5.37 (d, *J* = 17.1 Hz, 1H, CH=C<u>H</u>$_2$), 5.27 (d, *J* = 10.3 Hz, 1H, CH=C<u>H</u>$_2$), 5.21 (bs, 0.5H, NH), 4.88 (bs, 0.6H, αCH), 4.66 (s, 0.4H, αCH), 1.43 (s, 9H, C(C<u>H</u>$_3$)$_3$) (mixture of rotamers at rt).

HRMS (*m/z* ESI^-): Found: 200.0930 ($[M - H]^-$, $C_9H_{14}NO_4$ requires: 200.0928)

Methyl (*tert*-butoxycarbonyl)-D-serinate (181)

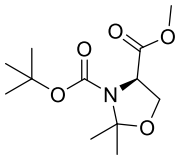


To a stirred solution of Boc_2O (16.83 g, 77.13 mmol) and TEA (13.02 mL, 128.55 mmol) in CH_2Cl_2 (130 mL) was added methyl-D-serinate **180** (10.00 g, 64.28 mmol) and the reaction mixture left stirring at rt for 16 h. Upon completion the reaction mixture was washed with 1 M aq. HCl solution (2 x 50 mL), brine (2 x 50 mL) and dried over $MgSO_4$. Solvent was removed *in vacuo* to yield a colourless oil which was purified by silica gel flash chromatography (Hex:EtOAc – 70:30) to give the product as a colourless oil (11.94 g, 84%). The isolated compound was in good agreement with the literature.[256]

δ_H NMR (400 MHz, $CDCl_3$): 5.46 (bs, 1H, NH), 4.38 (bs, 1H, Ser αCH), 3.96 (dd, *J* = 11.2, 3.9 Hz, 1H, Ser βCH_2), 3.89 (dd, *J* = 11.2, 3.6 Hz, 1H, Ser βCH_2), 3.78 (s, 3H, OCH_3), 1.45 (s, 9H, $C(CH_3)_3$)

HRMS (*m/z* ESI^+): Found: 257.0744 ($[M + K]^+$, $C_9H_{17}NO_5K$ requires 257.0742)

3-(*tert*-Butyl) 4-methyl (*R*)-2,2-dimethyloxazolidine-3,4-dicarboxylate (183)

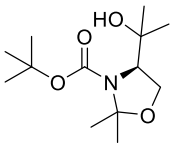


To a stirred solution of protected serine **181** (23.90 g, 109.01 mmol) in CH_2Cl_2 (155 mL) were added DMP (66.79 mL, 545 mmol) and PTSA.H_2O (2.07 g, 5.45 mmol) at 0 °C. The reaction mixture was stirred at rt for 16 h and upon completion was quenched with sat. aq. $NaHCO_3$ solution (65 mL) and extracted 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 give a colourless oil which was distilled *in vacuo* to give the product as a colourless oil (24.82 g, 88%). The isolated compound was in good agreement with the literature.[256]

δ_H NMR (400 MHz, $CDCl_3$): 4.48 (dd, *J* = 7.0, 2.8 Hz, 0.4H, Ser αCH), 4.37 (dd, *J* = 7.0, 2.8 Hz, 0.6H, Ser αCH), 4.13 (td, *J* = 9.2, 7.0 Hz, 1H, Ser βCH_2), 4.03 (td, *J* = 9.2, 2.8 Hz, 1H, Ser βCH_2), 3.75 (s, 3H, OCH_3), 1.66 (s, 2H, CH_3), 1.63 (s, 1H, CH_3), 1.53 (s, 2H, CH_3), 1.49 (s, 3H, $C(CH_3)_3$), 1.45 (s, 1H, CH_3), 1.40 (s, 6H, $C(CH_3)_3$) (Isolated as a mixture of rotamers at rt)

HRMS (*m/z* ESI^+): Found: 282.1308.($[M + Na]^+$, $C_{12}H_{21}NO_5Na$ requires 282.1314)

***tert*-Butyl (*R*)-4-(2-hydroxypropan-2-yl)-2,2-dimethyloxazolidine-3-carboxylate (184)**



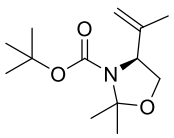
To a stirred solution of MeMgI (24.76 g, 148.95 mmol) in anhydrous Et_2O (49 mL) was added dropwise a solution of protected amino acid **183** (6.44 g, 24.82 mmol) solubilised in anhydrous Et_2O (10 mL) at 0 °C under Ar. After addition the reaction was allowed to proceed for 30 min at 0 °C and following this 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 combined extracts washed with brine (3 x 30 mL) and dried over $MgSO_4$. Concentration *in vacuo* gave a colourless oil which was purified by silica gel flash chromatography (Hex:EtOAc – 75:25) to give the product as a colourless oil (6.27 g, 97%). The isolated compound was in good agreement with the literature.[256]

δ_H NMR (400 MHz, $CDCl_3$): 4.02 – 3.95 (m, 2H, Ser βCH_2), 3.79 (bs, 1H, Ser αCH), 1.59 (s, 3H, CH_3), 1.51 (s, 3H, CH_3), 1.50 (s, 9H, $C(CH_3)_3$), 1.18 (s, 3H, CH_3), 1.17 (s, 3H, CH_3).

HRMS (*m/z* APCI$^+$): Found: 260.1854 ($[M + H]^+$, $C_{13}H_{26}NO_4$ requires 260.1856)

tert-Butyl *(S)*-2,2-dimethyl-4-(prop-1-en-2-yl)oxazolidine-3-carboxylate (185)

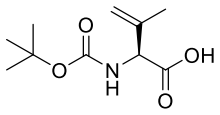


To a stirred solution of tertiary alcohol **184** (0.42 g, 1.66 mmol) and TEA (2.31 mL, 16.55 mmol) in anhydrous CH_2Cl_2 (5 mL) was added MsCl (0.64 mL, 8.28 mmol) dropwise at – 10 °C under Ar. After addition the reaction was allowed to reach rt and stirred for 1 h under Ar. The reaction mixture was 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* to give an orange oil which was purified by silica gel flash chromatography (Hex:EtOAc – 85:15) to give the product as a yellow oil (0.12 g, 31%). The isolated compound was in good agreement with the literature.[256]

δ_H NMR (400 MHz, $CDCl_3$): 4.89 (bs, 1H, $C=CH_2$), 4.83 (bs, 1H, $C=CH_2$), 4.38 – 4.21 (m, 1H, Ser αCH), 4.06 (dd, J = 8.9, 7.1 Hz, 1H, Ser βCH_2), 3.73 (dd, J = 8.9, 3.0 Hz, 1H, Ser βCH_2), 1.71 (s, 3H, CH_3), 1.66 – 1.60 (m, 3H, CH_3), 1.49 (s, 9H, $C(CH_3)_3$), 1.39 (s, 3H, CH_3)

HRMS (*m/z* ESI$^+$): Found: 264.1569 ($[M + Na]^+$, $C_{13}H_{23}NO_3Na$ requires 282.1573)

_(S)_-2-((_tert_-Butoxycarbonyl)amino)-3-methylbut-3-enoic acid (172)

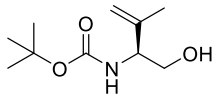


To a stirred solution of amino alcohol **193** (250 mg, 1.24 mmol) in acetone (45 mL) was added freshly prepared 2.5 M Jones reagent (1.5 mL) while stirring at 0 °C. The mixture was allowed to stir at 0 °C for 3 h and quenched with IPA (10 mL). Precipitated solids were dissolved by addition of H_2O and acetone and the organic solvent was removed *in vacuo* at 20 °C. The aq. mixture was extracted with EtOAc (3 x 20 mL) and the combined organic extracts washed with brine (5 x 10 mL) and dried over $MgSO_4$. Removal of solvent *in vacuo* gave the product as a colourless oil (256 mg, 96%). The isolated compound was in good agreement with the literature.[256]

δ_H NMR (400 MHz, $CDCl_3$): 7.16 (s, 0.5H, NH), 5.32 (s, 0.5H, NH), 5.12 (s, 1H, C=CH_2), 5.05 (bs, 1H, C=CH_2), 4.78 (bs, 0.5H, αCH), 4.59 (bs, 0.5H, αCH), 1.82 (s, 3H, CH_3), 1.44 (s, 9H, $C(CH_3)_3$) (mixture of rotamers at rt)

HRMS (*m/z* ESI^+): Found: 238.1048 ($[M + Na]^+$, $C_{10}H_{17}NO_4Na$ requires 238.1049)

***tert*-Butyl *(S)*-(1-hydroxy-3-methylbut-3-en-2-yl)carbamate (193)**

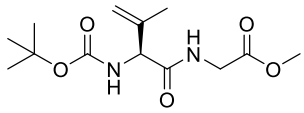


To a stirred solution of **185** (1.70 g, 7.04 mmol) in THF (9.5 mL) and H_2O (4.8 mL) at 0 °C was added ice-cold TFA (19.0 mL). The reaction mixture was stirred for 10 min and quenched by pouring into ice-cold sat. aq. $NaHCO_3$ solution. The resulting mixture was stirred for a further 20 min at 0 °C until effervescence ceased and extracted with EtOAc (3 x 10 mL). The combined organic extracts were washed with sat. aq. $NaHCO_3$ solution (3 x 10 mL), brine (2 x 10 mL) and dried over $MgSO_4$. Solvent was removed *in vacuo* to yield a pale yellow oil which was purified by silica gel flash chromatography (Hex:EtOAc – 80:20) to give the product as a colourless oil which solidified to a white waxy solid upon standing (1.31 g, 92 %). The isolated compound was in good agreement with the literature.[256]

δ_H NMR (400 MHz, $CDCl_3$): 4.98 (s, 1H, C=CH_2), 4.94 (s, 1H, C=CH_2), 4.11 (bs, 1H, αCH), 3.70 (d, 2H, *J* = 4.7 Hz, βCH_2), 1.79 (s, 3H, CH_3), 1.45 (s, 9H, C(CH_3)$_3$)

HRMS (*m/z* ESI^+): Found: 224.1260 ($[M + Na]^+$, $C_{10}H_{19}NO_3Na$ requires 224.1263)

Methyl *(S)*-(2-((*tert*-butoxycarbonyl)amino)-3-methylbut-3-enoyl)glycinate (175)



Prepared as per procedure E using Boc-DHV **172** (194 mg, 0.94 mmol) and glycine methyl ester hydrochloride (142 mg, 1.13 mmol) in CH_2Cl_2 (10 mL) which after purification by silica gel flash chromatography (Hex:EtOAc – 1:1) gave the product as a white solid (135 mg, 50%).

R_f (50% EtOAc:Hex): 0.45

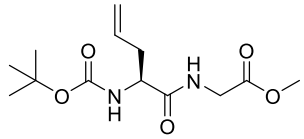
δ_H NMR (400 MHz, $CDCl_3$): 6.38 (bs, 1H, NH), 5.60 (bs, 1H, NH), 5.19 (s, 1H, C=CH_2), 5.10 (s, 1H, C=CH_2), 4.67 (s, 1H, αCH), 4.05 (m, 2H, Gly αCH_2), 3.76 (s, 3H, OCH_3), 1.72 (s, 3H, CH_3), 1.44 (s, 9H, C(CH_3)$_3$)

δ_C NMR (151 MHz, $CDCl_3$): 170.0 (C=O), 169.9 (C=O), 155.3 (C=O), 142.2 (<u>C</u>=CH_2), 116.2 (<u>C</u>=CH_2), 80.0 (<u>C</u>(CH_3)$_3$), 60.6 (αCH), 52.5 (OCH_3), 41.4 (Gly αCH_2), 28.4 (C(<u>C</u>H_3)$_3$), 17.9 (CH_3)

HRMS (*m/z* ESI^+): Found: 309.1420 ($[M + Na]^+$, $C_{13}H_{22}N_2O_5Na$ requires 309.1421)

ν_{max} (film)/cm^{-1}: 3364 (N-H), 2979 (C-H), 1696 (C=O)

Methyl *(S)*-(2-((tert-butoxycarbonyl)amino)pent-4-enoyl)glycinate (176)



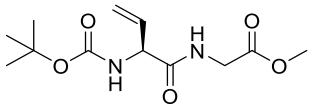
Prepared as per procedure E using (*S*)-Boc allylglycine **173** (500 mg, 2.32 mmol) and glycine methyl ester hydrochloride (350 mg, 2.79 mmol) in CH_2Cl_2 (23 mL) which after

purification by silica gel flash chromatography (Hex:EtOAc – 1:1) gave the product as a white solid (560 mg, 85%). The isolated compound was in good agreement with the literature.[306]

δ_H NMR (400 MHz, $CDCl_3$): 6.71 (bs, 1H, NH), 5.76 (m, 1H, C<u>H</u>=CH_2), 5.21 – 5.11 (m, 2H, CH=C<u>H</u>$_2$), 5.01 (s, 1H, NH), 4.21 (s, 1H, αCH), 4.04 (dd, *J* = 9.2, 5.4 Hz, 2H, αCH_2), 3.75 (s, 3H, OCH_3), 2.61 – 2.44 (m, 2H, CH_2), 1.44 (s, 9H, $C(CH_3)_3$)

HRMS (*m/z* ESI^+): Found: 309.1421 ($[M + Na]^+$, $C_{13}H_{22}N_2O_5Na$ requires 309.1420)

Methyl *(S)*-(2-((*tert*-butoxycarbonyl)amino)but-3-enoyl)glycinate (174)

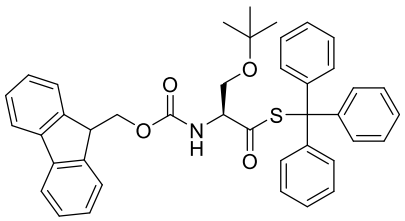


Prepared as per procedure F with Boc-VG **171** (50 mg, 0.25 mmol) and glycine methyl ester hydrochloride (31 mg, 0.25 mmol) in THF (2 mL). Purification by silica gel flash chromatography (Hex:EtOAc – 60:40) gave the product as a waxy grey solid (53 mg, 82%). The isolated compound was in good agreement with the literature.[307]

δ_H NMR (400 MHz, $CDCl_3$): 6.74 (bs, 1H, NH), 5.90 (ddd, *J* = 17.1, 10.3, 6.4 Hz, 1H, C<u>H</u>=CH_2), 5.38 (d, *J* = 17.1 Hz, CH=C<u>H</u>$_2$), 5.28 (d, *J* = 10.3 Hz, 1H, CH=C<u>H</u>$_2$), 4.72 (bs, 1H, NH) 4.03 (d, *J* = 5.4 Hz, 1H), 3.73 (s, 1H, OCH_3), 1.42 (s, 1H, $C(C\underline{H}_3)_3$)

HRMS (*m/z* $APCI^-$): Found: 271.1295 ($[M - H]^-$, $C_{12}H_{19}N_2O_5$ requires: 271.1299)

_S_-Trityl _(S)_-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-(_tert_-butoxy)propanethioate (200)



Prepared as per general experimental procedure E using Fmoc-Ser(O^tBu)-OH (678 mg, 1.77 mmol) in CH_2Cl_2 (18 mL) and following silica gel flash chromatography (Hex: EtOAc – 90:10) the product was obtained as a white crystalline solid (987 mg, 80%).

R_f (20% EtOAc:Hex): 0.45

δ_H NMR (400 MHz, $CDCl_3$): 7.80-7.77 (m, 2H, Ar-CH), 7.65 (t, *J* = 7.9 Hz, 2H, Ar-CH), 7.41 (t, *J* = 7.9 Hz, 2H, Ar-CH), 7.32-7.24 (m, 17H, Ar-CH), 5.78 (d, *J* = 5.8 Hz, 1H, NH), 4.57-4.53 (dd, *J* = 10.0, 6.4 Hz, 1H, Fmoc CHC$\underline{H}_2$), 4.50-4.46 (m, 1H, Ser αCH), 4.37-4.28 (m, 2H, Fmoc CHC$\underline{H}_2$, Fmoc C$\underline{H}CH_2$), 3.87 (dd, *J* = 8.4, 2.2 Hz, 1H, Ser βCH$_2$), 3.49 (dd, *J* = 8.4 Hz, 3.2 Hz, 1H, Ser βCH$_2$), 1.22 (s, 9H, $(CH_3)_3$)

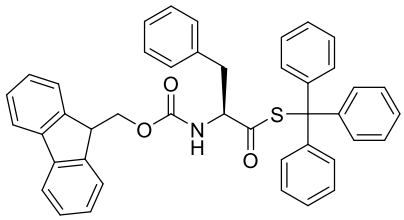
δ_C NMR (100 MHz, $CDCl_3$): 197.5 (SC=O), 155.8 (C=O), 143.6 (C=O), 141.3 (Ar-qC), 129.9 (Ar-CH), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 125.2 (Ar-CH), 120.0 (Ar-CH), 73.5 ($\underline{C}(CH_3)_3$) , 70.1 ($C(Ar)_3$), 67.5 (Fmoc CH$\underline{C}H_2$), 62.5 (Ser βCH$_2$), 61.0 (Ser αCH), 47.3 (Fmoc $\underline{C}HCH_2$), 27.5 ($C(\underline{C}H_3)_3$)

HRMS (*m/z* ESI+) Found: 664.2492 ($[M + Na]^+$, $C_{41}H_{39}NNaO_4S$ requires 664.2509)

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

M.p. 65-70 °C

_S_-Trityl _(S)_-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-phenylpropanethioate (201)



Prepared as per general experimental procedure E using Fmoc-Phe-OH (628 mg, 1.62 mmol) in CH_2Cl_2 (15 mL) and following silica gel flash chromatography (Hex:EtOAc – 90:10) the product was obtained as a white crystalline solid (823 mg, 82%).

R_f (20% EtOAc:Hex): 0.48

δ_H NMR (400 MHz, $CDCl_3$): 7.78 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.56 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.42 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.32-7.23 (m, 20H, Ar-CH), 7.05 (d, *J* = 7.4 Hz, Ar-CH, 2H), 5.12 (d, *J* = 9.0 Hz, 1H, NH), 4.76-4.71 (m, 1H, Phe αCH), 4.43-4.35 (m, 2H, Fmoc CHC$\underline{H}_2$), 4.22 (t, *J* = 7.23 Hz, 1H, Fmoc C$\underline{H}CH_2$), 3.07-2.96 (m, 2H, Phe βCH_2)

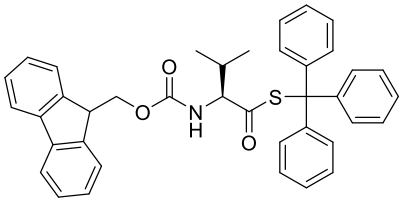
δ_C NMR (100 MHz, $CDCl_3$): 197.2 (SC=O), 155.7 (C=O), 143.5 (C=O), 141.5 (C=O), 135.4 (Ar-qC), 129.9 (Ar-CH), 129.6 (Ar-CH), 128.7 (Ar-CH), 127.8 (Ar-CH), 127.7 (Ar-CH), 127.2 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.0 (Ar-CH), 70.7 ($C(CH_3)_3$), 66.9 (Fmoc CH$\underline{C}H_2$), 61.3 (Phe αCH), 47.2 (Fmoc $\underline{C}HCH_2$), 38.2 (Phe βCH_2)

HRMS (*m/z* ESI^+): Found 668.2229 ($[M + Na]^+$, $C_{43}H_{35}NNaO_3S$ requires 668.2218)

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

M.p. 73-75 °C

_S_-Trityl _(S)_-2-((((9_H_-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanethioate (202)



Prepared as per general experimental procedure E using Fmoc-Val-OH (700 mg, 2.06 mmol) in CH_2Cl_2 (20 mL) and following silica gel flash chromatography (Hex:EtOAc – 85:15) gave the product as a white crystalline solid (987 mg, 80%).

R_f (15% EtOAc:Hex): 0.31

δ_H NMR (400 MHz, $CDCl_3$): 7.80 (d, *J* = 7.3 Hz, 2H, Ar-CH), 7.62 (d, *J* = 7.3 Hz, 2H, Ar-CH), 7.43 (t, *J* = 7.3 Hz, 2H, Ar-CH), 7.34-7.28 (m, 17H, Ar-CH), 5.22 (d, *J* = 9.4 Hz, 1H, NH), 4.52-4.48 (dd, *J* = 10.5, 7.1 Hz, 1H, Fmoc CHC<u>H</u>$_2$), 4.43-4.39 (m, 2H, Fmoc CHC<u>H</u>$_2$, Val αCH), 4.28 (t, *J* = 7.1 Hz, 1H, Fmoc C<u>H</u>CH_2), 2.24-2.16 (m, 1H, Val βCH), 0.92 (d, *J* = 6.6 Hz, 3H, Val CH_3), 0.75 (d, *J* = 6.6 Hz, 3H, Val CH_3)

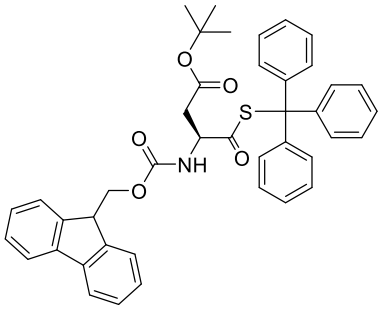
δ_C NMR (100 MHz, $CDCl_3$): 197.5 (SC=O), 156.2 (C=O), 143.6 (C=O), 141.5 (Ar-qC), 129.9 (Ar-CH), 127.8 (Ar-CH), 127.7 (Ar-CH), 127.2 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 125.1 (Ar-CH), 120.1 (Ar-CH), 70.8 ($C(Ar)_3$), 67.5 (Fmoc CH<u>C</u>H_2), 65.5 (Val αCH), 47.2 (Fmoc <u>C</u>HCH_2), 31.6 (Val βCH), 19.5 (Val CH_3), 16.9 (Val CH_3)

HRMS (*m/z* ESI^+): Found: 620.2338 ($[M + Na]^+$, $C_{39}H_{35}NNaO_3S$ requires 620.2235)

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

M.p. 65-68 °C

tert-Butyl(S)-3-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-4-oxo-4-(tritylthio)butanoate (203)



Prepared as per general experimental procedure E using Fmoc-Asp(O^{t}Bu)-OH (1.00 g, 3.36 mmol) in CH_2Cl_2 (20 mL) and following silica gel flash chromatography (10% EtOAc:Hex), the product was obtained as a yellow foam (1.46 g, 79%).

R_f (20% EtOAc:Hex): 0.48

δ_H (400 MHz, $CDCl_3$): 7.80 (dd, *J* = 7.5, 4.0 Hz, 2H, Ar-CH), 7.68 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.43 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.36 – 7.25 (m, 17H, Ar-CH), 6.20 (d, *J* = 10.0 Hz, 1H, NH), 4.70 – 4.56 (m, 2H, Fmoc CHC<u>H</u>$_2$), 4.40 – 4.28 (m, 3H, Fmoc C<u>H</u>CH$_2$, Asp αCH), 3.02 (dd, *J* = 17.3, 4.4 Hz, 1H, Asp βCH$_2$), 2.61 (dd, *J* = 17.3, 4.4 Hz, 1H, Asp βCH$_2$), 1.53 (s, 9H, C(CH$_3$)$_3$)

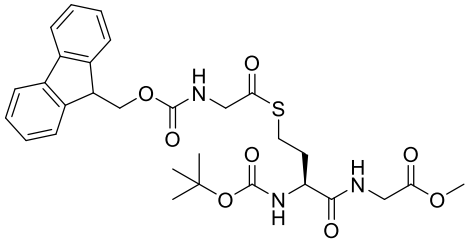
δ_C (100 MHz, $CDCl_3$): 197.8 (SC=O), 170.8 (C=O), 155.8 (C=O), 143.9 (Ar-qC), 143.6 (Ar-qC), 141.35 (Ar-qC), 130.0 (Ar-CH), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.3 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 82.1 (C(Ar)$_3$), 70.3 (<u>C</u>(CH$_3$)$_3$), 67.5 (Fmoc CH<u>C</u>H$_2$), 57.5 (Asp αCH), 47.2 (Fmoc <u>C</u>HCH$_2$), 37.6 (Asp βCH$_2$), 28.1 (C(<u>C</u>H$_3$)$_3$)

HRMS (*m/z* ESI$^+$): Found: 692.2440 ([M + Na]$^+$, $C_{42}H_{39}NNaO_5S$ requires 692.2447)

ν_{max} (film)/cm^{-1}: 3318 (NH), 1678 (C=O), 740, 700 (CH)

M.p. 72-74 °C

Methyl *S*-((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)-*N*-(*tert*-butoxycarbonyl)-L-homocysteinylglycinate (208)



Following *S*-Trt deprotection of **146** (183 mg, 0.33 mmol) as per procedure C, crude Gly thioacid **124** was reacted with thiol-ene acceptor **174** (30 mg, 0.11 mmol) immediately as per procedure D in EtOAc. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the product as a yellow foam (59 mg, 91%).

R_f (50% EtOAc/Hex): 0.30

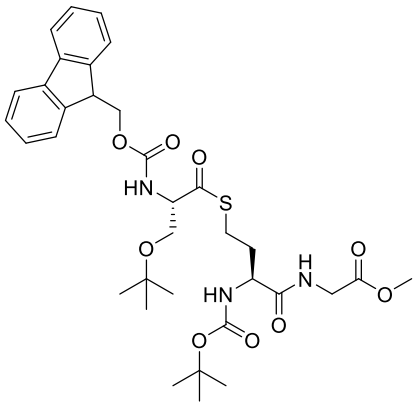
δ_H NMR (400 MHz, $CDCl_3$): 7.79 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.64 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.43 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.34 (t, *J* = 7.4 Hz, 2H, Ar-CH), 6.87 (bs, 1H, NH), 5.50 (t, *J* = 5.5 Hz, 1H, NH), 5.27 (d, *J* = 7.9 Hz, 1H, NH), 4.47 (d, *J* = 7.0 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.27 (m, 2H, Fmoc C$\underline{H}$CH$_2$, αCH), 4.15 (d, *J* = 6.0 Hz, 2H, Gly αCH$_2$), 4.06 (d, *J* = 5.5 Hz, 2H, Gly αCH$_2$), 3.75 (s, 3H, OCH_3), 3.11 (dt, *J* = 14.0, 7.1 Hz, 1H, SC$\underline{H}_2$CH$_2$), 2.95 (dt, *J* = 14.0, 7.1 Hz, 1H, SC$\underline{H}_2$CH$_2$), 2.16 (dt, *J* = 21.1, 7.1 Hz, 1H, SCH$_2$C$\underline{H}_2$), 1.94 (dt, *J* = 21.1, 7.1 Hz, 1H, SCH$_2$C$\underline{H}_2$), 1.47 (s, 9H, C(CH_3)$_3$)

δ_C NMR (151 MHz, $CDCl_3$): 198.1 (SC=O), 171.6 (C=O), 170.0 (C=O), 156.3 (C=O), 155.7 (C=O), 143.7 (Ar-qC), 141.3 (Ar-qC), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.0 (Ar-CH), 80.5 (C($\underline{C}H_3$)), 67.4 (Fmoc CH$\underline{C}$H$_2$), 53.2 (αCH), 52.4 (OCH_3), 50.7 (Gly αCH$_2$), 47.1 (Fmoc $\underline{C}$HCH$_2$), 41.2 (Gly αCH$_2$), 32.6 (SCH$_2\underline{C}$H$_2$), 28.3 (C($\underline{C}H_3$)), 24.9 (S$\underline{C}$H$_2$CH$_2$)

HRMS (*m/z* APCI$^+$): Found: 586.2225 ([M + H])$^+$, $C_{29}H_{36}N_3O_8S$ requires 586.2145)

ν_{max} (film)/cm^{-1}: 3318 (N-H), 2949 (C-H), 1708 (C=O), 1675 (C=O)

Methyl *S*-(*N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-L-seryl)-*N*-(tert-butoxycarbonyl)-L-homocysteinyl glycinate (209)



Following *S*-Trt deprotection of **200** (212 mg, 0.32 mmol) as per procedure F, crude Ser thioacid **204** was reacted with thiol-ene acceptor **174** (30 mg, 0.11 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the product as a yellow foam (70 mg, 95%).

R_f (50% EtOAc/Hex): 0.35

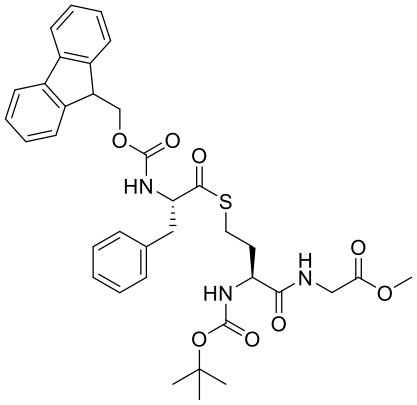
δ_H NMR (400 MHz, $CDCl_3$): 7.76 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.64 (dd, *J* = 10.8, 7.6 Hz, 2H,Ar-CH), 7.39 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.31 (t, *J* = 7.4 Hz, 2H, Ar-CH), 6.81 (bs, 1H, NH), 5.75 (d, *J* = 8.7 Hz, 1H, NH), 5.20 (d, *J* = 7.6 Hz, 1H, NH), 4.57 – 4.43 (m, 3H, Ser αCH, Fmoc CHC$\underline{H}_2$), 4.36 (t, *J* = 7.1 Hz, 1H, αCH), 4.30-4.11 (m, 3H, Fmoc C$\underline{H}$CH_2, Gly αCH_2), 4.01 (d, *J* = 5.3 Hz, 2H, Gly αCH_2), 3.87 (dd, *J* = 9.1, 3.1 Hz, 1H, Ser βCH_2), 3.70 (s, 3H, OCH_3), 3.55 (dd, *J* = 9.1, 3.1 Hz, 1H, Ser βCH_2), 3.05 – 2.99 (m, 1H, SC$\underline{H}_2$CH_2), 2.91 – 2.85 (m, 1H, SC$\underline{H}_2$CH_2), 2.13-2.05 (m, 1H, SCH_2C$\underline{H}_2$), 1.88 (dt, *J* = 14.3, 7.7 Hz, 1H, SCH_2C$\underline{H}_2$), 1.43 (s, 9H, C(CH_3)$_3$), 1.15 (s, 9H, OC(CH_3)$_3$) (mixture of rotamers at rt)

δ_C NMR (151 MHz, $CDCl_3$): 200.7 (SC=O), 171.8 (C=O), 170.0 (C=O), 156.3 (C=O), 155.8 (C=O) 143.8 (Ar-qC), 141.5 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.7 ($\underline{C}$(CH_3)$_3$), 80.5 ($\underline{C}$(CH_3)$_3$), 73.9 (Ser αCH), 70.4 (Ser αCH), 67.5 (Fmoc CH$\underline{C}H_2$), 61.9 (Ser βCH_2), 61.4 (αCH), 52.5 (OCH_3), 47.3 (Fmoc $\underline{C}$HCH_2), 41.3 (Gly αCH_2), 34.3 (SC$H_2\underline{C}H_2$), 32.0 (SC$H_2\underline{C}H_2$), 28.4 (C($\underline{C}H_3$)$_3$), 27.4 (C($\underline{C}H_3$)$_3$), 25.5 (S$\underline{C}H_2CH_2$) (mixture of rotamers at rt)

HRMS (*m/z* APCI$^+$): Found: 672.2949 ([M + H]$^+$, $C_{34}H_{46}N_3O_9S$ requires 672.2949)

ν_{max} (film)/cm^{-1}: 3323 (N-H), 2949 (C-H), 1728 (C=O), 1676 (C=O)

Methyl *S*-((((9H-fluoren-9-yl)methoxy)carbonyl)-L-phenylalanyl)-*N*-(*tert*-butoxycarbonyl)-L-homocysteinylglycinate (210)



Following *S*-Trt deprotection of **201** (237 mg, 0.36 mmol) as per procedure F, crude Phe thioacid **205** was reacted with thiol-ene acceptor **174** (20 mg, 0.07 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (Hex:EtOAc – 60:40) gave the product as a yellow foam (46 mg, 92%).

R_f (50% EtOAc/Hex): 0.40

δ_H NMR (400 MHz, $CDCl_3$): 7.75 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.53 (dd, *J* = 14.1, 7.5 Hz, 2H, Ar-CH), 7.39 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.33 – 7.23 (m, 5H, Ar-CH), 7.16 (d, *J* = 7.0 Hz, 2H, Ar-CH), 6.90 (bs, 1H, NH), 5.40-5.28 (m, 1H, NH), 4.68 (dd, *J* = 13.5, 8.0 Hz, 1H, Phe αCH), 4.38 (d, *J* = 7.0 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.20 (m, 2H, αCH, Fmoc C$\underline{H}$CH$_2$), 4.02 (d, *J* = 5.2 Hz, 2H, Gly αCH$_2$), 3.71 (s, 3H, OCH$_3$), 3.19 (dd, *J* = 14.1, 5.2 Hz, 1H, Phe βCH$_2$), 3.08 – 2.98 (m, 2H, Phe βCH$_2$, SCH$_2$C$\underline{H}_2$), 2.96 – 2.87 (m, 1H, SCH$_2$C$\underline{H}_2$), 2.16 – 2.05 (m, 1H, SC$\underline{H}_2$CH$_2$), 1.94 – 1.83 (m, 1H, SC$\underline{H}_2$CH$_2$), 1.45 (s, 9H, C(CH$_3$)$_3$)

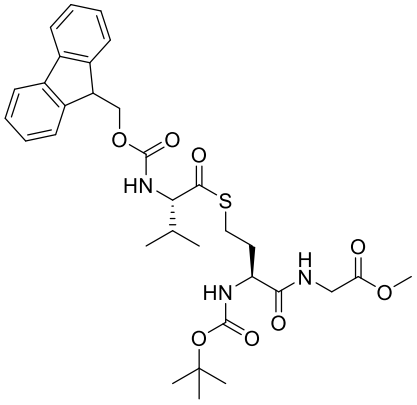
δ_C NMR (151 MHz, $CDCl_3$) δ 200.7 (SC=O), 171.8 (C=O), 170.1 (C=O), 155.9 (C=O) 154.8 (C=O), 143.7 (Ar-qC), 141.4 (Ar-qC), 135.6 (Ar-qC), 129.4 (Ar-CH), 128.9 (Ar-

CH), 127.8 (Ar-CH), 127.4 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.6 (C(CH$_3$)$_3$), 67.3 (Fmoc CHCH$_2$), 61.8 (Phe αCH), 53.4 (αCH), 52.5 (OCH$_3$), 47.2 (Fmoc CHCH$_2$), 41.3 (Gly αCH$_2$), 38.3 (Phe βCH$_2$), 32.6 (SCH$_2$CH$_2$), 28.4 (C(CH$_3$)$_3$), 25.3 (SCH$_2$CH$_2$)

HRMS (*m/z* ESI$^+$): Found: 698.2507 ([M + Na]$^+$, $C_{36}H_{41}N_3O_8SNa$ requires 698.2507)

ν_{max} (film)/cm^{-1}: 2922 (C-H), 1766 (C=O), 1636 (C=O)

Methyl *S*-((((9H-fluoren-9-yl)methoxy)carbonyl)-L-valyl)-*N*-(*tert*-butoxycarbonyl)-L-homocysteinylglycinate (211)



Following *S*-Trt deprotection of **202** (219 mg, 0.36 mmol) as per procedure F, crude Val thioacid **206** was reacted with thiol-ene acceptor **174** (20 mg, 0.07 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (Hex:EtOAc– 60:40) gave the product as a yellow foam (40 mg, 86%).

R_f (50% EtOAc/Hex): 0.40

δ_H NMR (400 MHz, $CDCl_3$): 7.81 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.66 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.45 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.36 (t, *J* = 7.4 Hz, 2H, Ar-CH), 6.89 (bs, 1H, NH), 5.52 (t, *J* = 5.5 Hz, 1H, NH), 5.28 (d, *J* = 7.9 Hz, 1H, NH), 4.49 (d, *J* = 7.0 Hz, 2H, Fmoc CHCH$_2$), 4.29 (m, 3H, Fmoc CHCH$_2$, Val αCH, αCH), 4.17 4.08 (d, *J* = 5.5 Hz, 2H, Gly αCH$_2$), 3.77 (s, 3H, OCH$_3$), 3.13 (dt, *J* = 14.0, 7.1 Hz, 1H, SCH$_2$CH$_2$), 2.97 (dt, *J* = 14.0, 7.1 Hz, 1H, SCH$_2$CH$_2$), 2.31-2.29 (m, 1H, Val βCH) 2.18 (dt, *J* = 21.1, 7.1.

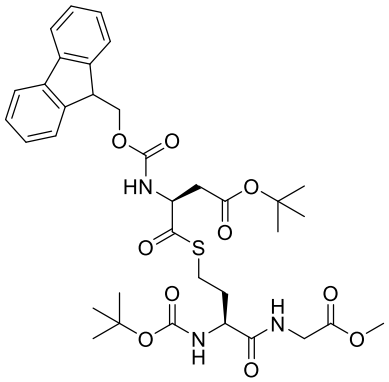
Hz, 1H, $SCH_2C\underline{H}_2$), 1.96 (dt, *J* = 21.1, 7.1 Hz, 1H, $SCH_2C\underline{H}_2$), 1.49 (s, 9H, $C(CH_3)_3$), 1.01-0.88 (m, 6H, Val CH_3, Val CH_3)

δ_C NMR (151 MHz, $CDCl_3$): 197.9 (SC=O), 171.4 (C=O), 169.8 (C=O), 156.1 (C=O), 155.6 (C=O), 143.5 (Ar-qC), 141.2 (Ar-qC), 127.6 (Ar-CH), 126.9 (Ar-CH), 124.9 (Ar-CH), 119.8.0 (Ar-CH), 80.3 ($\underline{C}(CH_3)$), 67.2 (Fmoc $CH\underline{C}H_2$), 58.8 (Val αCH), 53.0 (αCH), 52.2 (OCH_3), , 46.9 (Fmoc $\underline{C}HCH_2$), 41.0 (Gly αCH_2), 32.4 ($SCH_2\underline{C}H_2$), 30.9 (Val βCH), 28.1 ($C(\underline{C}H_3)$), 24.7 ($S\underline{C}H_2CH_2$), 19.3 (Val CH_3) 16.8 (Val CH_3)

HRMS (*m/z* ESI^+): Found: 650.2507 ($[M + Na]^+$, $C_{32}H_{41}N_3O_8SNa$ requires 650.2507)

ν_{max} (film)/cm^{-1}: 3318 (N-H), 2911 (C-H), 1752 (C=O), 1676 (C=O)

Methyl (5*S*, 10*S*)-5-(2-(*tert*-butoxy)-2-oxoethyl)-10-((*tert*-butoxycarbonyl)amino)-1-(9H-fluoren-9-yl)-3,6,11-trioxo-2-oxa-7-thia-4,12-diazatetradecan-14-oate (212)



Following *S*-Trt deprotection of **203** (148 mg, 0.22 mmol) as per procedure F, crude Asp thioacid **207** was reacted with thiol-ene acceptor **174** (20 mg, 0.07 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (Hex:EtOAc– 60:40) gave the product as a yellow foam (47 mg, 92%).

R_f (50% EtOAc/Hex): 0.35

δ_H NMR (400 MHz, $CDCl_3$): δ 7.79 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.69 – 7.58 (m, 2H, Ar-CH), 7.43 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.34 (t, *J* = 7.4 Hz, 1H, Ar-CH), 6.83 (bs, 1H, NH), 6.02 (d, *J* = 9.4 Hz, 1H, NH), 5.26 (d, *J* = 7.9 Hz, NH), 4.72 – 4.64 (m, 1H, Asp αCH), 4.62 – 4.53 (m, 1H, Fmoc $CHC\underline{H}_2$), 4.44 – 4.35 (m, 1H, Fmoc $CHC\underline{H}_2$), 4.29 (t, *J*

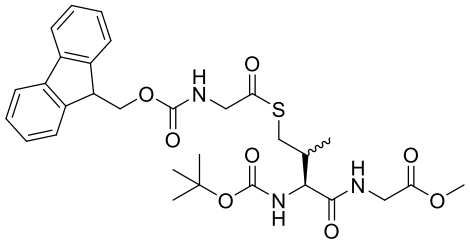
= 7.0 Hz, 1H, Fmoc C<u>H</u>CH$_2$), 4.23 (bs, 1H, αCH) 4.05 (d, *J* = 5.4 Hz, 2H, Gly αCH$_2$), 3.75 (s, *J* = 3.1 Hz, 3H, OCH$_3$), 3.07 – 2.98 (m, 1H, Asp βCH$_2$), 2.93 (m, 2H, SC<u>H</u>$_2$CH$_2$), 2.73 (dd, *J* = 16.9, 4.4 Hz, 1H, Asp βCH$_2$), 2.13 (m, 1H, SCH$_2$C<u>H</u>$_2$), 1.96 – 1.84 (m, 1H, SCH$_2$C<u>H</u>$_2$), 1.47 (s, 18H, C(CH$_3$)$_3$, C(CH$_3$)$_3$) (mixture of diastereoisomers)

δ_C NMR (151 MHz, CDCl$_3$): 200.3 (SC=O), 171.6 (C=O), 170.1 (C=O), 169.9 (C=O), 155.9 (C=O), 155.7 (C=O), 143.9 (Ar-qC)*, 143.6 (Ar-qC)*, 141.3 (Ar-qC), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH)*, 125.1 (Ar-CH)*, 120.0 (Ar-CH), 82.3 (<u>C</u>(CH$_3$)$_3$), 82.2 (<u>C</u>(CH$_3$)$_3$), 68.0 (Fmoc CH<u>C</u>H$_2$)*, 67.5 (Fmoc CH<u>C</u>H$_2$)*, 57.4 (Asp αCH), 52.4 (OCH$_3$), 50.9 (αCH), 47.2 (Fmoc <u>C</u>HCH$_2$), 41.2 (Gly αCH$_2$), 37.6 (Asp βCH$_2$), 32.4 (SCH$_2$<u>C</u>H$_2$), 28.3 (C(<u>C</u>H$_3$)$_3$), 28.1 (C(<u>C</u>H$_3$)$_3$), 25.6 (S<u>C</u>H$_2$CH$_2$)*, 25.4 (S<u>C</u>H$_2$CH$_2$) (mixture of diastereoisomers)

HRMS (*m/z* APCI$^+$): Found: 700.2902 ([M + H]$^+$,C$_{35}$H$_{46}$N$_3$O$_{10}$S requires 700.2898)

ν_{max} (film)/cm^{-1}: 3325 (N-H), 2902 (C-H), 1746 (C=O), 1671 (C=O)

Methyl *(S)*-(4-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoyl)glycinate (213)



Following *S*-Trt deprotection of **146** (116 mg, 0.21 mmol) as per procedure F, crude G;y thioacid **124** was reacted with thiol-ene acceptor **175** (20 mg, 0.07 mmol) in EtOAc (2 mL) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (Hex:EtOAc – 1:1) gave the product as a yellow oil (39 mg, 93%).

R_f (50% EtOAc:Hex): 0.35

δ_H NMR (400 MHz, CDCl$_3$): 7.78 (d, *J* = 7.5 Hz, 4H, Ar-CH), 7.64 (d, *J* = 7.5 Hz, 4H, Ar-CH), 7.42 (t, *J* = 7.5 Hz, 4H, Ar-CH), 7.34 (t, *J* = 7.5 Hz, 4H, Ar-CH), 6.94 (bs, 1H,

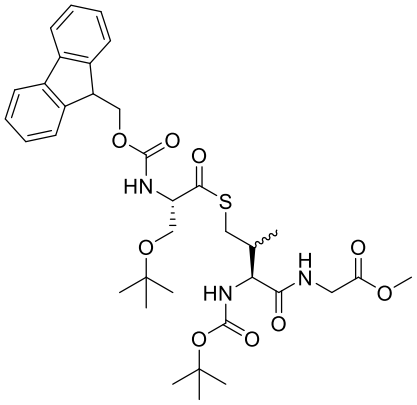
NH), 6.74 (bs, 1H, NH), 5.62 (bs, 1H, NH), 5.57 (bs, 1H, NH), 5.49 (d, *J* = 8.4 Hz, 1H, NH), 5.29 (d, *J* = 8.4 Hz, 1H, NH), 4.49 – 4.44 (m, 4H, Fmoc CHC$\underline{H}_2$), 4.40 (m, 1H, αCH), 4.27 (t, *J* = 7.0 Hz, 2H, Fmoc C$\underline{H}$CH_2), 4.19 – 4.00 (m, 9H, Gly αCH_2, Gly αCH_2, αCH), 3.75 (s, 3H, OCH_3), 3.72 (s, 3H, OCH_3), 3.26 (dd, *J* = 14.0, 6.6 Hz, 1H, γCH_2), 2.97 (d, *J* = 6.6 Hz, 2H, γCH_2), 2.79 (dd, *J* = 14.0, 6.6 Hz, 1H, γCH_2), 2.25 (d, *J* = 6.2 Hz, 1H, βCH), 1.46 (s, 18H, C(CH_3)$_3$), 1.04 (d, *J* = 6.9 Hz, 3H, CH_3), 1.00 (d, *J* = 6.9 Hz, 3H, CH_3) (mixture of diastereoisomers)

δ_H NMR (151 MHz, $CDCl_3$): 198.9 (SC=O), 197.7 (SC=O), 171.4 (C=O), 170.8 (C=O), 170.1 (C=O), 170.0 (C=O), 156.4 (C=O), 156.3 (C=O), 155.9 (C=O), 155.7 (C=O), 143.8 (Ar-qC), 143.7 (Ar-qC), 141.3 (Ar-qC), 141.3 (Ar-qC), 127.8 (Ar-CH), 127.1 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.0 (Ar-CH), 80.3 ($\underline{C}$(CH_3)), 67.4 (Fmoc CH$\underline{C}H_2$), 67.3 (Fmoc CH$\underline{C}H_2$), 57.3 (αCH), 56.4 (αCH), 52.4 (OCH_3), 52.4 (OCH_3), 50.8 (Gly CH_2), 50.7 (Gly CH_2), 47.1 (Fmoc $\underline{C}$HCH_2), 47.1 (Fmoc $\underline{C}$HCH_2), 41.2 (Gly CH_2) 41.2 (Gly CH_2), 37.1 (βCH), 36.7 (βCH), 32.3 (γCH_2), 31.3 (γCH_2), 28.3 (C($\underline{C}H_3$)), 28.3 (C($\underline{C}H_3$)), 16.3 (CH_3), 14.4 (CH_3) (mixture of diastereoisomers)

HRMS (*m/z* APCI$^+$): Found: 600.2375 ([M + H]$^+$, $C_{30}H_{38}N_3O_8S$ requires 600.2374)

ν_{max} (film)/cm^{-1}: 3321 (NH), 2978 (C-H), 1682 (C=O)

Methyl (*(S)*-4-((*N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-L-seryl)thio)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoyl)glycinate (214)



Following *S*-Trt deprotection of **200** (134 mg, 0.21 mmol) as per procedure F, crude Ser thioacid **204** was reacted with thiol-ene acceptor **175** (20 mg, 0.07 mmol) immediately

as per procedure C in EtOAc. Purification by silica gel flash chromatography (Hex:EtOAc – 1:1) gave the product as a yellow foam (39 mg, 80%).

R_f (50% EtOAc:Hex): 0.35

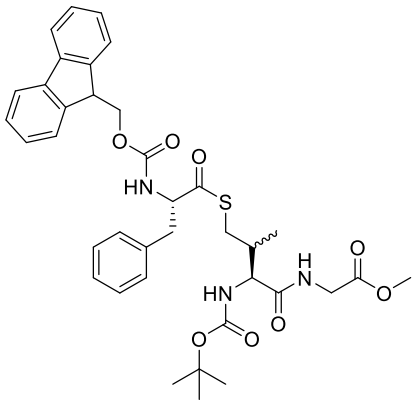
δ_H NMR (600 MHz, $CDCl_3$): 7.79 (bs, 1H, NH) 7.69-7.62 (m, 8H, Ar-CH), 7.40 (t, *J* = 7.4 Hz, 4H, Ar-CH), 7.34-7.28 (m, 4H, Ar-CH), 7.05 (bs, 2H, NH), 6.18 (app. t, 2H, NH), 5.74 (d, *J* = 8.9 Hz, 2H, NH), 5.27-5.18 (m, 2H, NH), 5.26 (d, *J* = 8.5 Hz, 1H, NH), 4.89 (bs, 1H, Ser αCH), 4.73 – 4.65 (m, 2H, Val αCH, Val αCH), 4.42-4.38 (m, 2H, Fmoc CHC$\underline{H}_2$), 4.16 – 3.91 (m, 5H, Ser αCH, Fmoc CHC$\underline{H}_2$, Fmoc C$\underline{H}$CH$_2$, Fmoc C$\underline{H}$CH$_2$), 3.85 – 3.72 (m, 4H, Gly αCH$_2$, Gly αCH$_2$), 3.68-3.66 (m, 6H, OCH$_3$, OCH$_3$), 3.14 (d, *J* = 11.0 Hz, 1H, Val γCH$_2$), 3.06 – 2.93 (m, 4H, Ser βCH$_2$, Val γCH$_2$, Ser βCH$_2$), 2.86-2.80 (m, 1H Ser βCH$_2$), 2.78-2.71 (m, 2H, Val γCH$_2$), 2.22-2.12 (m, 2H, Val αCH, Val αCH), 1.46-1.44 (m, 18H, C(CH$_3$)$_3$, C(CH$_3$)$_3$), 1.37-1.24 (m, 18H, OC(CH$_3$)$_3$, OC(CH$_3$)$_3$), 1.02-0.95 (m, 6H, Val CH$_3$, Val CH$_3$) (mixture of diastereoisomers)

δ_C NMR (151 MHz, $CDCl_3$): 200.2 (SC=O), 199.9 (SC=O), 171.7 (C=O), 171.1 (C=O), 170.1 (C=O), 170.0 (C=O), 156.9 (C=O), 156.7 (C=O), 156.1 (C=O), 155.8 (C=O), 146.1 (Ar-qC), 146.1 (Ar-qC), 145.8 (Ar-qC), 145.6 (Ar-qC), 139.9 (Ar-qC), 139.9 (Ar-qC), 139.7 (Ar-qC), 139.7 (Ar-qC), 129.8 (Ar-CH), 129.7 (Ar-CH), 128.2 (Ar-CH), 128.1 (Ar-CH), 125.3 (Ar-CH), 124.5, (Ar-CH) 120.3 (Ar-CH), 120.3 (Ar-CH), 82.5 (C(CH$_3$)$_3$), 82.3 (C(CH$_3$)$_3$), 81.1 (C(CH$_3$)$_3$), 81.0 (C(CH$_3$)$_3$), 80.5 (C(CH$_3$)$_3$), 80.4 (C(CH$_3$)$_3$), 70.2 (Fmoc CHCH$_2$), 70.1 (Fmoc CHCH$_2$), 58.0 (Ser αCH), 57.6 (Ser αCH), 57.1 (Val αCH), 55.7 (Val αCH), 52.4 (OCH$_3$), 52.4 (OCH$_3$), 41.3 (Fmoc CHCH$_2$), 41.2 (Fmoc CHCH$_2$), 41.1 (Gly αCH$_2$), 41.1 (Gly αCH$_2$), 37.6 (Ser βCH$_2$), 37.5 (Ser βCH$_2$), 37.4 (Val βCH$_2$), 36.3 (Val βCH$_2$), 32.6 (Val γCH$_2$), 31.7 (Val γCH$_2$), 28.4 (C(CH$_3$)$_3$), 28.3 (C(CH$_3$)$_3$), 15.8 (Val CH$_3$), 14.8 (Val CH$_3$) (mixture of diastereoisomers)

HRMS (*m/z* ESI$^+$): Found: 690.2850 ([M+H]$^+$, $C_{37}H_{44}N_3O_8S$ requires 690.2844)

ν_{max} (film)/cm^{-1}: 3331 (N-H), 2976 (C-H), 1708 (C=O), 1682 (C=O)

Methyl ((*S*)-4-(((((9H-fluoren-9-yl)methoxy)carbonyl)-L-phenylalanyl)thio)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoyl)glycinate (215)



Following *S*-Trt deprotection of **201** (563 mg, 0.87 mmol) as per procedure F, crude Phe thioacid **205** was reacted with thiol-ene acceptor **175** (50 mg, 0.17 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (Hex:EtOAc – 1:1) gave the product as a yellow foam (106 mg, 88%).

R_f (50% EtOAc/Hex): 0.35

δ_H NMR (600 MHz, $CDCl_3$): 7.82 – 7.73 (m, 4H, Ar-CH), 7.61 – 7.48 (m, 4H, Ar-CH), 7.41 (t, *J* = 7.2 Hz, 4H, Ar-CH), 7.36 – 7.25 (m, 10H, Ar-CH), 7.19 (t, *J* = 7.2 Hz, 4H, Ar-CH), 6.92-6.72 (m, 2H, NH), 5.45-5.25 (m, 4H, NH), 4.80 – 4.63 (m, 2H, Phe αCH), 4.51 – 4.33 (m, 5H, Fmoc CHC$\underline{H}_2$, Val αCH), 4.25-4.17 (m, 2H, Fmoc C$\underline{H}$CH_2), 4.15-3.95 (m, 5H, Gly αCH_2, Val αCH), 3.78-3.68 (m, 6H, OCH_3), 3.27 – 3.15 (m, 3H, Phe βCH_2, Val γCH_2), 3.06 (dt, *J* = 21.7, 10.7 Hz, 2H, Phe βCH_2), 2.98 – 2.89 (m, 2H, Val γCH_2), 2.79 (dd, *J* = 13.4, 7.6 Hz, 1H, Val γCH_2), 2.29 – 2.12 (m, 2H, Val βCH), 1.46 (s, 18H, C(CH_3)$_3$), 0.99 (dd, *J* = 19.9, 6.7 Hz, 6H, Val γCH_3) (mixture of diastereoisomers)

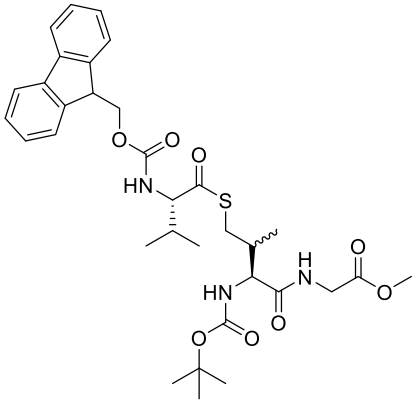
δ_C NMR (151 MHz, $CDCl_3$): 200.7 (SC=O), 200.6 (SC=O), 171.6 (C=O), 171.0 (C=O), 170.1 (C=O), 170.0 (C=O), 156.1 (C=O), 156.3 (C=O), 156.0 (C=O), 155.7 (C=O), 143.9 (Ar-qC), 143.8 (Ar-qC), 143.7 (Ar-qC), 141.4 (Ar-qC), 135.6 (Ar-qC), 129.5 (Ar-CH), 129.4 (Ar-CH), 128.9 (Ar-CH), 128.9 (Ar-CH), 127.8 (Ar-CH), 127.4 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.4 ($\underline{C}$(CH_3)), 80.3 ($\underline{C}$(CH_3)), 67.4 (Fmoc CH$\underline{C}H_2$), 67.3 (Fmoc CH$\underline{C}H_2$), 62.0 (Phe αCH), 61.8 (Phe αCH), 57.8 (Val αCH), 56.4 (Val αCH), 52.6 (OCH_3), 52.5 (OCH_3), 52.4 (OCH_3), 47.2 (Fmoc $\underline{C}$HCH_2), 47.2 (Fmoc

$\underline{C}HCH_2$), 41.5 (Gly αCH_2) 41.3 (Gly αCH_2), 38.4 (Phe βCH_2), 38.3 (Phe βCH_2), 37.3 (Val βCH), 36.6 (βCH), 32.6 (γCH_2), 31.9 (γCH_2), 28.4 (C($\underline{C}H_3$)), 28.4 (C($\underline{C}H_3$)), 16.0 (CH_3), 14.4 (CH_3) (mixture of diastereoisomers)

HRMS (*m/z* ESI^+): Found: 690.2844 ($[M+H]^+$, $C_{37}H_{44}N_3O_8S$ requires 690.2850)

ν_{max} (film)/cm^{-1}: 3323 (N-H), 2963 (C-H), 1731 (C=O), 1690 (C=O)

Methyl ((*S*)-4-(((((9H-fluoren-9-yl)methoxy)carbonyl)-L-valyl)thio)-2-((*tert*-butoxycarbonyl)amino)-3-methylbutanoyl)glycinate (216)



Following *S*-Trt deprotection of **202** (365 mg, 0.61 mmol) as per procedure F, crude Val thioacid **206** was reacted with thiol-ene acceptor **175** (35 mg, 0.12 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (Hex:EtOAc – 1:1) gave the product as a yellow foam (67 mg, 85%).

R_f (50% EtOAc/Hex): 0.37

δ_H NMR (600 MHz, $CDCl_3$): 7.76 (d, *J* = 7.5 Hz, 4H, Ar-CH), 7.72 – 7.59 (m, 4H, Ar-CH), 7.45-7.37 (m, 4H, Ar-CH), 7.36 – 7.28 (m, 4H, Ar-CH), 6.85-6.63 (m, 2H, NH), 5.37-5.15 (m, 4H, NH), 4.55 – 4.30 (m, 6H, Fmoc CHC$\underline{H}_2$, Fmoc Val αCH, Boc Val αCH), 4.32-4.19 (m, 3H, Fmoc C$\underline{H}CH_2$, Fmoc Val αCH), 4.14-3.93 (m, 5H, Gly αCH_2, Boc Val αCH), 3.74-3.65 (m, 6H, OCH_3), 3.20 (dd, *J* = 13.8, 5.4 Hz, 1H, Boc Val γCH_2), 2.91 (d, *J* = 6.7 Hz, 2H, Boc Val γCH_2), 2.77 (dd, *J* = 13.8, 7.6 Hz, 1H, Boc Val γCH_2), 2.40 – 2.08 (m, 1H, Boc Val βCH, Fmoc Val βCH) , 1.43 (s, 18H, $C(CH_3)_3$), 1.03-0.95

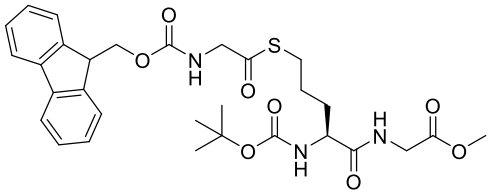
(m, 12H, Boc Val γCH_3, Fmoc Val γCH_3), 0.90 (dd, *J* = 13.3, 6.8 Hz, 6H, Fmoc Val γCH_3) ppm. (mixture of diastereoisomers)

δ_C NMR (151 MHz, $CDCl_3$): 201.0 (SC=O), 200.8 (SC=O), 171.5 (C=O), 171.0 (C=O), 170.0 (C=O), 169.9 (C=O), 156.6 (C=O), 156.3 (C=O), 156.0 (C=O), 155.7 (C=O), 144.1 (Ar-qC), 143.8 (Ar-qC), 143.7 (Ar-qC), 141.5 (Ar-qC), 127.9 (Ar-CH), 127.3 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.3 (<u>C</u>(CH_3)), 67.4 (Fmoc CH<u>C</u>H_2), 67.3 (Fmoc CH<u>C</u>H_2), 66.4 (Fmoc Val αCH), 66.1 (Fmoc Val αCH), 57.9 (Val αCH), 56.4 (Val αCH), 52.5 (OCH_3), 52.3 (OCH_3), 47.4 (Fmoc <u>C</u>HCH_2), 41.3 (Gly αCH_2), 37.3 (Boc Val βCH), 36.6 (Boc Val βCH), 32.5 (Boc Val γCH_2), 31.8 (Boc Val γCH_2), 31.2 (Fmoc Val βCH), 31.0 (Fmoc Val βCH), 28.4 (C(<u>C</u>H_3)), 19.6 (CH_3), 17.4 (CH_3), 17.1 (CH_3), 16.0 (CH_3), 15.6 (CH_3), 14.5 (CH_3), 14.3 (CH_3) (mixture of diastereoisomers)

HRMS (*m/z* ESI^+): Found: 642.2847 ($[M+H]^+$, $C_{33}H_{44}N_3O_8S$ requires 642.2844)

ν_{max} (film)/cm^{-1}: 3323 (N-H), 2927 (C-H), 1670 (C=O)

Methyl *(S)*-(5-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)-2-((*tert*-butoxycarbonyl)amino)pentanoyl)glycinate (217)



Following *S*-Trt deprotection of **146** (128 mg, 0.23 mmol) as per procedure F, crude Gly thioacid **124** was reacted with thiol-ene acceptor **176** (22 mg, 0.08 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the product as a yellow foam (43 mg, 94%).

R_f (50% EtOAc/Hex): 0.12

δ_H NMR (600 MHz, $CDCl_3$): 7.77 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.61 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.40 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.32 (t, *J* = 7.4 Hz, 2H, Ar-CH), 6.64 (bs, 1H, NH), 5.51 (bs, 1H, NH), 5.07 (bs, 1H, NH), 4.45 (d, *J* = 7.0 Hz, 2H, Fmoc CHC<u>H</u>$_2$),

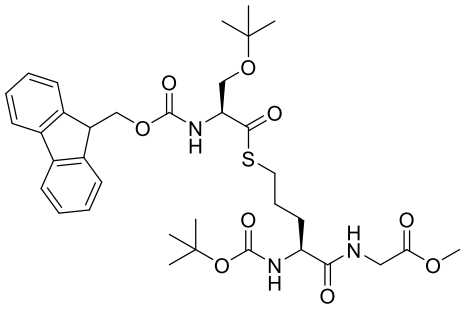
4.31-4.23 (m, 2H, Fmoc C<u>H</u>CH$_2$, αCH), 4.22-4.09 (m, 3H, Gly αC<u>H</u>$_2$), 3.95 (dd, *J* = 18.1, 4.4 Hz, 1H, Gly αC<u>H</u>$_2$), 3.72 (s, 3H, OCH$_3$), 3.16-3.08 (m, 1H, SC<u>H</u>$_2$CH$_2$CH$_2$), 2.94-2.88 (m, 1H, SC<u>H</u>$_2$CH$_2$CH$_2$), 1.89-1.81 (m, 1H, SCH$_2$CH$_2$C<u>H</u>$_2$), 1.80-1.72 (m, 1H, SCH$_2$C<u>H</u>$_2$CH$_2$), 1.68-1.60 (m, 2H, SCH$_2$C<u>H</u>$_2$C<u>H</u>$_2$), 1.44 (s, 9H, C(CH$_3$)$_3$)

δ_C NMR (151 MHz, CDCl$_3$): 198.4 (SC=O), 172.3 (C=O), 170.4 (C=O), 156.7 (C=O), 156.4 (C=O), 143.9 (Ar-qC), 141.5 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.2 (Ar-CH), 80.4 (<u>C</u>(CH$_3$)), 67.4 (Fmoc CH<u>C</u>H$_2$), 53.4 (αCH), 52.6 (OCH$_3$), 50.8 (Gly αCH$_2$), 47.3 (Fmoc <u>C</u>HCH$_2$), 41.3 (Gly αCH$_2$), 31.6 (<u>C</u>H$_2$), 31.1 (<u>C</u>H$_2$), 28.5 (C(<u>C</u>H$_3$)$_3$), 27.8 (<u>C</u>H$_2$), 25.7 (CH$_2$)

HRMS (*m/z* ESI$^+$): Found: 600.2305 ([M+H]$^+$ $C_{30}H_{38}N_3O_8S$ requires 600.2301)

ν_{max} (film)/cm^{-1}: 3329 (N-H), 2927 (C-H), 1741 (C=O), 1683.92 (C=O)

Methyl ((*S*)-5-((*N*-(((9H-fluoren-9-yl)methoxy)carbonyl)-*O*-(*tert*-butyl)-L-seryl)thio)-2-((*tert*-butoxycarbonyl)amino)pentanoyl)glycinate (218)



Following *S*-Trt deprotection of **200** (148 mg, 0.23 mmol) as per procedure F, crude Ser thioacid **204** was reacted with thiol-ene acceptor **176** (22 mg, 0.08 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the product as a white foam (50 mg, 95%).

R_f (50% EtOAc/Hex): 0.17

δ_H NMR (600 MHz, CDCl$_3$): 7.77 (d, *J* = 7.6 Hz, 2H, Ar-CH), 7.65 (d, *J* = 7.7 Hz, 1H, Ar-CH), 7.63 (d, *J* = 7.5 Hz, 1H, Ar-CH), 7.40 (t, *J* = 7.6 Hz, 2H, Ar-CH), 7.32 (t, *J* = 7.4 Hz, 2H, Ar-CH), 6.74 (bs, 1H, NH), 5.74 (d, *J* = 8.8 Hz, 1H, NH), 5.15 (d, *J* = 7.5, 1H, NH), 4.54-4.46 (m, 2H, Ser αCH, Fmoc CHC<u>H</u>$_2$), 4.38-4.34 (m, 1H, Fmoc CHC<u>H</u>$_2$),

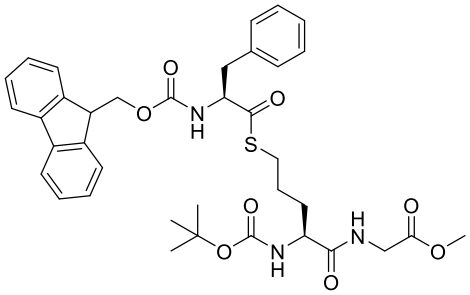
4.28 (t, *J* = 7.3 Hz, 1H, Fmoc C<u>H</u>CH$_2$), 4.22 (bs, 1H, αCH), 4.00-3.98 (m, 2H, Gly αC<u>H</u>$_2$), 3.89 (dd, *J* = 9.1, 2.3 Hz, 1H, Ser βC<u>H</u>$_2$), 3.70 (s, 3H, OC<u>H</u>$_3$), 3.56 (dd, *J* = 9.1, 3.4 Hz, 1H, Ser βC<u>H</u>$_2$), 3.02-2.96 (m, 1H, SC<u>H</u>$_2$CH$_2$CH$_2$), 2.93-2.86 (m, 1H, SC<u>H</u>$_2$CH$_2$CH$_2$), 1.92-1.84 (m, 1H, SCH$_2$CH$_2$C<u>H</u>$_2$), 1.76-1.60 (m, 3H, SCH$_2$C<u>H</u>$_2$CH$_2$, SCH$_2$C<u>H</u>$_2$C<u>H</u>$_2$), 1.43 (s, 9H, Boc C(C<u>H</u>$_3$)$_3$), 1.14 (s, 9H, Ser C(C<u>H</u>$_3$)$_3$)

δ_C NMR (151 MHz, $CDCl_3$): 200.5 (SC=O), 172.3 (C=O), 170.1 (C=O), 156.2 (C=O), 155.8 (C=O), 144.0 (Ar-qC), 141.4 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.2 (Boc <u>C</u>(CH$_3$)$_3$), 73.8 (Ser <u>C</u>(CH$_3$)$_3$), 67.4 (Fmoc CH<u>C</u>H$_2$), 61.9 (Ser-βCH$_2$), 61.2 (Ser-αCH), 53.7 (αCH), 52.4 (OCH$_3$), 47.3 (Fmoc <u>C</u>HCH$_2$), 41.3 (Gly αCH$_2$), 31.6 (<u>C</u>H$_2$), 28.4 (Boc C(<u>C</u>H$_3$)), 28.3 (<u>C</u>H$_2$), 27.4 (Ser C(<u>C</u>H$_3$)$_3$), 25.6 (<u>C</u>H$_2$)

HRMS (*m/z* ESI$^+$): Found: 686.3104 ([M+H]$^+$, $C_{35}H_{48}N_3O_9S$ requires 686.3106)

ν_{max} (film)/cm^{-1}: 3330 (N-H), 2974 (C-H), 1676 (C=O), 1243 (C-O)

Methyl ((*S*)-5-(((((9H-fluoren-9-yl)methoxy)carbonyl)-L-phenylalanyl)thio)-2-((*tert*-butoxycarbonyl)amino)pentanoyl)glycinate (219)



Following *S*-Trt deprotection of **205** (473 mg, 0.73 mmol) as per procedure F, crude Phe thioacid **205** was reacted with thiol-ene acceptor **176** (41 mg, 0.15 mmol) immediately as per procedure C in EtOAc. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the product as a yellow foam (92 mg, 90%).

R_f (50% EtOAc/Hex): 0.25

δ_H NMR (400 MHz, $CDCl_3$): 7.75 (d, *J* = 7.6 Hz, 2H, Ar-CH), 7.55 (d, *J* = 7.4 Hz, 1H, Ar-CH), 7.52 (d, *J* = 7.3 Hz, 1H, Ar-CH), 7.40 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.32-7.24 (m,

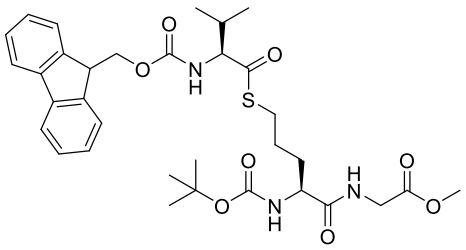
5H, Ar-CH), 7.15 (d, *J* = 7.5 Hz, 2H, Ar-CH), 6.66 (bs, 1H, NH), 5.37 (d, *J* = 8.2 Hz, 1H, NH), 5.08 (bs, 1H, NH), 4.71 (dd, *J* = 14.0, 7.5 Hz, 1H, Phe αCH), 4.43-4.35 (m, 2H, Fmoc CHC$\underline{H}_2$), 4.26 (bs, 1H, αCH), 4.19 (t, *J* = 7.5 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.06 (dd, *J* = 18.5, 5.6 Hz, 1H, Gly αC$\underline{H}_2$), 3.97 (dd, *J* = 18.3, 5.0 Hz, 1H, Gly αC$\underline{H}_2$), 3.69 (s, 3H, OCH_3), 3.17 (dd, *J* = 14.3, 5.6 Hz, 1H, Phe- βC$\underline{H}_2$), 3.09-2.99 (m, 2H, Phe- βC$\underline{H}_2$, SC$\underline{H}_2CH_2CH_2$), 2.90-2.85 (m, 1H, SC$\underline{H}_2CH_2CH_2$), 1.89-1.80 (m, 1H, $SCH_2CH_2C\underline{H}_2$), 1.77-1.68 (m, 1H, $SCH_2C\underline{H}_2CH_2$), 1.67-1.57 (m, 2H, $SCH_2C\underline{H}_2C\underline{H}_2$), 1.45 (s, 9H, $C(CH_3)_3$)

δ_C NMR (151 MHz, $CDCl_3$): 200.9 (SC=O), 172.2 (C=O), 170.3 (C=O), 155.8 (C=O, C=O), 143.9 (Ar-qC), 141.4 (Ar-qC), 135.7 (Ar-qC), 129.5 (Ar-CH), 128.9 (Ar-CH), 127.9 (Ar-CH), 127.4 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.3 ($\underline{C}(CH_3)$), 67.3 (Fmoc CH$\underline{C}H_2$), 61.6 (Phe-α$\underline{C}$H), 53.5 (αCH), 52.5 (OCH_3), 47.3 (Fmoc $\underline{C}HCH_2$), 41.3 (Gly αCH_2), 38.4 (Phe-β$\underline{C}H_2$), 31.6 ($\underline{C}H_2$), 28.5 ($C(\underline{C}H_3)_3$), 28.2 ($\underline{C}H_2$), 25.6 ($\underline{C}H_2$)

HRMS (*m/z* ESI^+): Found: 690.2840 ($[M+H]^+$, $C_{37}H_{44}N_3O_8S$ requires 690.2844)

ν_{max} (film)/cm^{-1}: 3325 (N-H), 2967 (C-H), 1732 (C=O), 1681 (C=O)

Methyl ((*S*)-5-(((((9H-fluoren-9-yl)methoxy)carbonyl)-L-valyl)thio)-2-((*tert*-butoxycarbonyl)amino)pentanoyl)glycinate (220)



Following *S*-Trt deprotection of **202** (427 mg, 0.72 mmol) as per general procedure F, crude Val thioacid **206** was reacted with thiol-ene acceptor **176** (41 mg, 0.14 mmol) immediately as per general procedure C in EtOAc. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the product as a yellow foam (77 mg, 84%).

R_f (50% EtOAc/Hex): 0.27

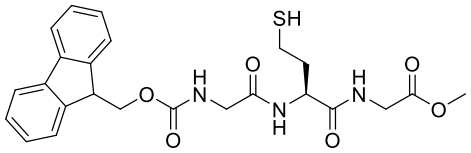
δ_H NMR (600 MHz, $CDCl_3$): 7.75 (d, *J* = 7.6 Hz, 2H, Ar-CH), 7.60 (t, *J* = 8.0 Hz, 2H, Ar-CH), 7.39 (t, *J* = 7.6 Hz, 2H, Ar-CH), 7.30 (t, *J* = 7.6 Hz, 2H, Ar-CH), 6.84 (bs, 1H, NH), 5.51 (bs, 1H, NH), 5.21 (bs, 1H, NH), 4.47-4.40 (m, 2H, Fmoc CHC$\underline{H}_2$), 4.33 (dd, *J* = 9.3, 5.0 Hz, 1H, Val αCH), 4.29 (bs, 1H, αCH), 4.23 (t, *J* = 6.8 Hz, 1H, Fmoc C$\underline{H}$CH_2), 4.22 (bs, 1H, αCH_2), 4.04 (dd, *J* = 18.1, 4.9 Hz, 1H, Gly αC$\underline{H}_2$), 3.96 (dd, *J* = 18.3, 4.7 Hz, 1H, Gly αC$\underline{H}_2$), 3.69 (s, 3H, OCH_3), 3.02 (bs, 1H, SC$\underline{H}_2$CH_2CH_2), 2.91-2.83 (m, 1H, SC$\underline{H}_2$CH_2CH_2), 2.31-2.21 (m, 1H, Val βCH), 1.89-1.81 (m, 1H, SCH_2CH_2C$\underline{H}_2$), 1.76-1.67 (m, 1H, SCH_2C$\underline{H}_2$CH_2), 1.67-1.58 (m, 2H, SCH_2C$\underline{H}_2$C$\underline{H}_2$), 1.43 (s, 9H, C(CH_3)$_3$), 0.98 (d, *J* = 6.6 Hz, 3H, Val CH_3), 0.88 (d, *J* = 6.7 Hz, 3H, Val CH_3)

δ_C NMR (151 MHz, $CDCl_3$): 201.0 (SC=O): 172.3 (C=O), 170.2 (C=O), 156.4 (C=O), 155.8 (C=O), 144.0 (Ar-qC), 141.4 (Ar-qC), 127.8 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.2 (C(CH_3)), 67.2 (Fmoc CHCH_2), 66.0 (Val-αCH), 53.4 (αCH), 52.4 (OCH_3), 47.3 (Fmoc CHCH_2), 41.2 (Gly αCH_2), 31.7 (CH_2), 31.1 (Val-βCH, 28.4 (C(CH_3)$_3$), 28.1 (CH_2), 25.6 (CH_2), 19.5 (Val CH_3, Val CH_3)

HRMS (*m/z* ESI^+): Found: 642.2855 ($[M+H]^+$, $C_{33}H_{44}N_3O_8S$ requires 642.2844)

ν_{max} (film)/cm^{-1}: 3313 (N-H), 2964 (C-H), 1736 (C=O), 1685 (C=O).

Methyl (((9H-fluoren-9-yl)methoxy)carbonyl)glycyl-L-homocysteinylglycinate (225)



Following Boc deprotection of thioester **208** (65 mg, 0.11 mmol) as per general procedure B, crude TFA salt **223** was freebased as per general procedure G. Purification by silica gel flash chromatography (EtOAc) gave the product as a colourless wax (39 mg, 73%).

R_f (100% EtOAc): 0.4

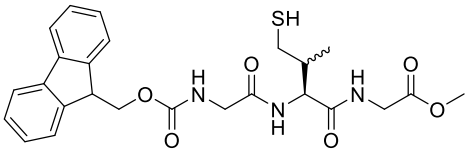
δ_H NMR (600 MHz, $CDCl_3$): 7.79 (d, *J* = 7.6 Hz, 2H, Ar-CH), 7.61 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.43 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.34 (t, *J* = 7.5 Hz, 2H, Ar-CH), 6.95 – 6.87 (m, 2H, NH, NH), 5.53 (bs, 1H, NH), 4.78 (dd, *J* = 13.7, 8.0 Hz, 1H, αCH), 4.46 (d, *J* = 6.9 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.25 (t, *J* = 6.8 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.13 – 3.96 (m, 2H, Gly αCH_2), 3.92 (d, *J* = 4.9 Hz, 2H, Gly αCH_2), 3.75 (s, 3H, OCH_3), 2.73 – 2.57 (m, 2H, γCH_2), 2.22 – 2.11 (m, 1H, βCH_2), 2.05 (m, 1H, βCH_2)

δ_C NMR (151 MHz, $CDCl_3$): 171.4 (C=O), 170.2 (C=O), 169.6 (C=O), 157.0 (C=O), 143.8 (Ar-qC), 141.4 (Ar-qC), 127.9 (Ar-CH), 127.3 (Ar-CH), 125.2 (Ar-CH), 120.2 (Ar-CH), 67.5 (Fmoc CH$\underline{C}H_2$), 52.6 (OCH_3), 51.9 (αCH), 47.2 (Fmoc $\underline{C}HCH_2$), 44.7 (Gly αCH_2), 41.3 (Gly αCH_2), 36.3 (βCH_2), 21.0 (γCH_2)

HRMS (*m/z* ESI^+): Found 991.2960 ($[2M-2H+Na]^+$, $C_{48}H_{52}N_6O_{12}S_2Na$ requires 991.2976)

ν_{max} (film)/cm^{-1}: 3298 (N-H), 2922 (C-H), 1631 (C=O)

Methyl (S)-(2-(2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)acetamido)-4-mercapto-3-methylbutanoyl)glycinate (226)



Following Boc deprotection of thioester **213** (70 mg, 0.12 mmol) as per general procedure B, crude TFA salt **224** was freebased as per general procedure G. Purification by silica gel flash chromatography (EtOAc) gave the product as a colourless wax (46 mg, 78%).

Diastereoisomer **226a**:

R_f (100% EtOAc): 0.5

δ_H NMR (400 MHz, $CDCl_3$): 7.91 (s, 1H, NH), 7.79 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.64 (d, *J* = 7.5 Hz, 2H), 7.42 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.33 (t, *J* = 7.5 Hz, 2H, Ar-CH), 5.43 (s, 1H, NH), 4.49 – 4.43 (m, 2H, Fmoc CHC$\underline{H}_2$), 4.28 (t, *J* = 7.1 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.16 (dd, *J* = 18.2, 5.7 Hz, 1H, Gly αCH_2), 4.11 (s, 2H, Gly αCH_2), 4.03 (dd, *J* = 18.1, 5.3 Hz, 1H, Gly αCH_2), 3.86 (s, 1H, αCH), 3.73 (s, 3H, OCH_3), 3.51 (dd, *J* = 12.2, 4.6 Hz, 1H, γCH_2), 2.91 (d, *J* = 2.9 Hz, 1H, βCH), 2.81 (d, *J* = 12.2 Hz, 1H, γCH_2), 0.91 (d, *J* = 6.6 Hz, 3H, CH_3)

δ_C NMR (151 MHz, $CDCl_3$): 171.8 (C=O), 170.3 (C=O), 162.3 (C=O), 156.5 (C=O), 144.0 (Ar-qC), 141.5 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.2 (Ar-CH), 67.3 (Fmoc CH$\underline{C}H_2$), 62.6 (αCH), 52.4 (OCH_3), 48.5 (Gly αCH_2), 47.3 (Fmoc $\underline{C}HCH_2$), 41.0 (Gly αCH_2), 33.8 (γCH_2), 24.0 (βCH), 12.3 (CH_3)

Diastereoisomer **226b**:

R_f (100% EtOAc): 0.4

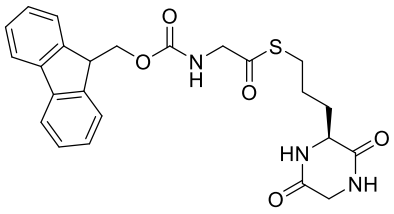
δ_H NMR (400 MHz, $CDCl_3$): 7.79 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.63 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.43 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.33 (t, *J* = 7.5, 2H, Ar-CH), 7.09 (s, 1H, NH), 5.52 (s, 1H, NH), 4.42 (d, *J* = 5.4 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.26 (t, *J* = 7.1 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.19 (s, 1H, αCH), 4.10 (dd, *J* = 17.5, 5.9 Hz, 1H, Gly αCH_2), 4.04 (dd, *J* = 16.7, 5.5 Hz, 2H Gly αCH_2), 3.94 (dd, *J* = 17.9, 5.5 Hz, 1H, Gly αCH_2), 3.68 (s, 3H, OCH_3), 3.25 – 3.20 (m, 1H, γCH_2), 2.70 (dd, *J* = 12.6, 5.3 Hz, 1H, γCH_2), 2.53 (s, 1H, βCH), 1.19 (d, *J* = 6.9 Hz, 3H, Val CH_3)

δ_C NMR (151 MHz, $CDCl_3$): 171.7 (C=O), 170.2 (C=O), 162.2 (C=O), 157.1 (C=O), 144.0 (Ar-qC) 141.4 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.2 (Ar-CH), 67.4 (Fmoc CH$\underline{C}H_2$), 64.7 (αCH), 52.4 (OCH_3), 49.0 (Gly αCH_2), 47.3 (Fmoc $\underline{C}HCH_2$), 41.2 (Gly αCH_2), 29.8 (γCH_2), 24.8 (βCH), 17.8 (CH_3)

HRMS (*m/z* ESI^+): Found: 504.1569 ($[M\text{-}H_2O+Na]^+$, $C_{25}H_{27}N_3O_5SNa$ requires 504.1675)

ν_{max} (film)/cm^{-1}:3320 (N-H), 2920 (C-H), 1634 (C=O)

(*S*)-*S*-(3-(3,6-dioxopiperazin-2-yl)propyl) 2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)ethanethioate (230)



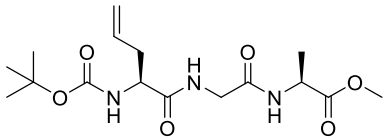
Following Boc deprotection of **217** (35 mg, 0.07 mmol) as per general procedure B, crude TFA salt **229** was freebased as per general procedure G. Purification by silica gel flash chromatography (EtOAc – CH_2Cl_2:MeOH, 30%) gave the product as a white solid (10 mg, 36%).

R_f (15% MeOH/CH_2Cl_2): 0.52

δ_H NMR (600 MHz, DMSO): 8.20 (bs, 1H, NH), 8.04 (bs, 1H, NH), 8.01 (bs, 1H, NH), 7.90 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.73 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.43 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.34 (t, *J* = 7.4 Hz, 2H, Ar-CH), 4.36 (d, *J* = 6.9 Hz, 2H, Fmoc CHC<u>H</u>$_2$), 4.25 (t, *J* = 6.8 Hz, 1H, Fmoc C<u>H</u>CH$_2$), 3.90 (d, *J* = 6.1 Hz, 2H, Gly αCH$_2$), 3.81 – 3.63 (m, 3H, DKP αCH, DKP αCH$_2$), 2.83 (t, *J* = 7.2 Hz, 2H, (S<u>C</u>H$_2$CH$_2$CH$_2$), 1.75 – 1.67 (m, 2H, SCH$_2$CH$_2$<u>C</u>H$_2$), 1.61 – 1.50 (m, 2H, SCH$_2$<u>C</u>H$_2$CH$_2$)

δ_C NMR (151 MHz, DMSO): 199.0 (SC=O), 167.7 (C=O), 166.0 (C=O), 156.4 (C=O), 143.7 (Ar-qC), 140.7 (Ar-qC), 127.7 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 65.8 (Fmoc CH<u>C</u>H$_2$), 53.8 (DKP αCH), 50.4 (Gly αCH$_2$), 46.6 (Fmoc <u>C</u>HCH$_2$), 44.2 (DKP αCH$_2$), 32.0 (SCH$_2$CH$_2$<u>C</u>H$_2$), 27.3 (S<u>C</u>H$_2$CH$_2$CH$_2$), 24.6 (SCH$_2$<u>C</u>H$_2$CH$_2$)

Methyl ((*S*)-2-((*tert*-butoxycarbonyl)amino)pent-4-enoyl)glycyl-L-alaninate (234)



Boc-Gly-OH (200 mg, 1.14 mmol) was coupled to Ala-OMe hydrochloride **232** (191 mg, 1.37 mmol) as per general procedure A to obtain the resulting dipeptide (168 mg, 0.64

mmol, 56%). Upon deprotection of the Boc PG following general procedure G, the dipeptide was coupled to Boc-Allylglycine **173** (116 mg, 0.54 mmol) as per general procedure E. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the pure tripeptide as a colourless oil (121 mg, 63 %).

R_f (10% MeOH/CH_2Cl_2): 0.62

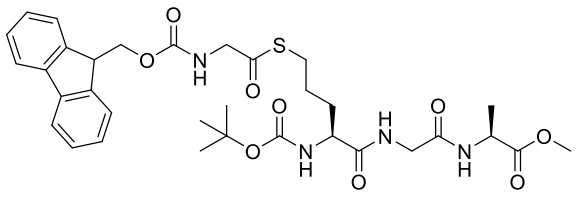
δ_H NMR (400 MHz, $CDCl_3$): 7.06 - 6.97 (m, 1H, NH), 5.74 (dd, *J* = 17.1, 10.0 Hz, 1H, $\underline{H}C=CH_2$), 5.21 – 5.06 (m, 3H, $HC=C\underline{H_2}$ and NH), 4.61 – 4.49 (m, 1H, Ala αCH), 4.19-4.12 (m, 1H, Alg αCH), 4.06 (dd, *J* = 16.8, 5.8 Hz, 1H, Gly αCH_2), 3.91 (dd, *J* = 16.8, 5.2 Hz, 1H, Gly αCH_2), 3.73 (s, 3H, OCH_3), 2.51 (ddd, *J* = 40.0, 14.2, 7.3 Hz, 2H, Alg βCH_2), 1.50 – 1.37 (m, 12H, $C(CH_3)_3$ and Ala CH_3)

δ_C NMR (150 MHz, $CDCl_3$): 173.14 (C=O), 172.07 (C=O), 168.46 (C=O), 155.73 (C=O), 132.89 ($H\underline{C}=CH_2$), 119.28 ($HC=\underline{C}H_2$), 80.42 ($C(CH_3)_3$), 54.16 (Alg αCH), 52.48 (OCH_3), 48.16 (Ala αCH), 43.00 (Gly αCH_2), 36.50 (Alg βCH_2), 28.28 ($C(CH_3)_3$), 17.97 (Ala CH_3)

HRMS (*m/z* ESI^+): Found 358.1979 $(M+H)^+$, $C_{16}H_{28}N_3O_6$ requires 358.1973)

ν_{max} (film)/cm^{-1}: 3308 (N-H), 2975 (C-H), 1750 (C=O), 1692 (C=O), 1641 ($HC=CH_2$)

Methyl ((*S*)-5-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)-2-((*tert*-butoxycarbonyl)amino)pentanoyl)glycyl-L-alaninate (235)



Following *S*-Trt deprotection of **146** (466 mg, 0.84 mmol) as per general procedure F, crude Gly thioacid **124** was reacted with thiol-ene acceptor **234** (100 mg, 0.28 mmol) immediately as per general procedure C in EtOAc. Purification by silica gel flash chromatography (EtOAc:Hex – 60:40) gave the product as a white foam (163 mg, 87%).

R_f (10% MeOH CH_2Cl_2): 0.62

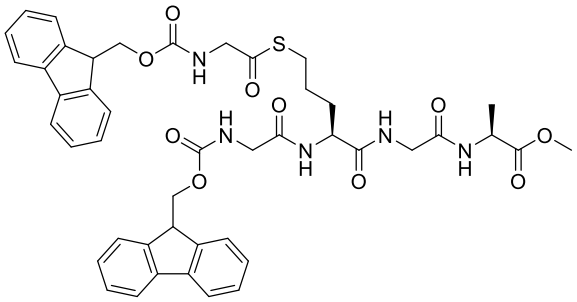
δ_H NMR (400 MHz, $CDCl_3$): 7.76 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.61 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.40 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.31 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.00 (bs, 1H, NH), 6.83 (bs, 1H, NH), 5.79 (bs, 1H, NH), 5.21 (d, *J* = 7.6 Hz, 1H, NH), 4.56 (p, *J* = 7.2 Hz, 1H, Fmoc C<u>H</u>CH_2), 4.43 (d, *J* = 7.0 Hz, 2H, Fmoc CHC<u>H</u>$_2$), 4.28 – 4.11 (m, 4H, Ala αCH, Alg αCH, Gly αCH_2), 3.99 (m, 2H, Gly αCH_2), 3.71 (s, 3H, OCH_3), 3.14-2.85 (m, 2H, Alg δCH_2), 2.04 – 1.54 (m, 4H, Alg βCH_2 Alg γCH_2), 1.49 – 1.35 (m, 12H, C(CH_3)$_3$, Ala βCH_3)

δ_C NMR (151 MHz, $CDCl_3$): 198.6 (SC=O), 173.3 (C=O), 172.7 (C=O), 168.5 (C=O), 156.6 (C=O), 156.0 (C=O), 143.9 (Ar-qC), 141.4 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 80.4 (<u>C</u>(CH_3)$_3$), 67.4 (Fmoc CH<u>C</u>H_2), 53.8 (Ala αCH), 52.7 (OCH_3), 50.9 (Gly αCH_2), 48.3 (Fmoc <u>C</u>HCH_2), 47.2 (Alg αCH), 43.1 (Gly αCH_2), 31.3 (Alg CH_2), 28.4 (C(<u>C</u>H_3)$_3$), 27.7 (Alg δCH_2), 25.8 (Alg CH_2), 18.2 (Ala βCH_3)

HRMS (*m/z* ESI^+): Found 671.2740 ($[M+H]^+$, $C_{33}H_{43}N_4O_9S$ requires 671.2745)

ν_{max} (film)/cm^{-1}: 3309 (N-H), 1738 (C=O), 1684 (C=O)

Methyl ((*S*)-5-(((((9H-fluoren-9-yl)methoxy)carbonyl)glycyl)thio)-2-aminopentanoyl)glycyl-L-alaninate (237)



Following Boc deprotection of **235** (50 mg, 0.07 mmol) as per general procedure B, crude TFA salt **237** was freebased as per general procedure G. Purification by silica gel flash

chromatography (CH_2Cl_2:MeOH – 90:10) gave the product as a colourless oil (27 mg, 43%).

R_f (10% MeOH/ CH_2Cl_2): 0.30

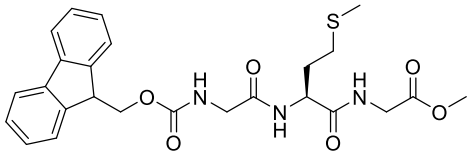
δ_H NMR (600 MHz, DMSO-d_6): 8.29 (t, *J* = 5.6 Hz, 1H, NH), 8.15 (d, *J* = 6.7 Hz, 1H, NH), 8.08 (d, *J* = 7.3 Hz, 1H, NH), 8.03 (t, *J* = 6.0 Hz, 1H, NH), 7.92 – 7.86 (m, 4H, Ar-CH), 7.71 (t, *J* = 7.5 Hz, 4H, Ar-CH), 7.49 (t, *J* = 5.8 Hz, 1H, HN), 7.42 (dd, *J* = 7.1, 5.1 Hz, 4H, Ar-CH), 7.33 (dd, *J* = 12.5, 7.1 Hz, 4H, Ar-CH), 4.36 (d, *J* = 6.9 Hz, 2H, Fmoc CHC<u>H</u>$_2$), 4.3 – 4.19 (m, 6H, Fmoc CHC<u>H</u>$_2$, Fmoc C<u>H</u>CH$_2$, Ala αCH, Alg αCH), 3.89 (d, *J* = 6.0 Hz, 1H, Gly αCH$_2$), 3.72 (d, *J* = 5.5 Hz, 2H, Gly αCH$_2$), 3.69 – 3.65 (m, 2H, Gly αCH$_2$), 3.61 (s, 3H, OCH$_3$), 2.852.78 (m, 2H, Alg δCH$_2$), 1.71 (s, 1H, Alg βHCH), 1.62 – 1.48 (m, 3H, Alg βHC<u>H</u> and γCH$_2$), 1.31-1.17 (m, 3H, Ala CH$_3$)

δ_C NMR (151 MHz, DMSO-d_6): 199.0 (SC=O), 173.0 (C=O), 171.6 (C=O), 169.3 (C=O), 168.6 (C=O), 156.5 (C=O), 156.4 (C=O), 143.8 (Ar-qC), 143.7 (Ar-qC), 140.8 (Ar-qC), 140.7 (Ar-qC), 127.7 (Ar-CH), 127.7 (Ar-CH), 127.1 (Ar-CH), 127.1 (Ar-CH), 125.2 (Ar-CH), 125.2 (Ar-CH), 120.1 (Ar-CH), 120.1 (Ar-CH), 65.9 (Fmoc CH<u>C</u>H$_2$), 65.7 (Fmoc CH<u>C</u>H$_2$), 52.3 (Alg αCH), 51.9 (OCH$_3$), 50.4 (Gly αCH$_2$), 47.6 (Ala αCH), 46.6 (Fmoc <u>C</u>HCH$_2$, Fmoc <u>C</u>HCH$_2$), 43.3 (Gly αCH$_2$), 41.6 (Gly αCH$_2$), 31.1 (Alg βCH$_2$), 27.4 (Alg δCH$_2$), 25.4 (Alg γCH$_2$), 16.9 (Ala CH$_3$)

HRMS (*m/z* ESI$^+$): Found: 849.3042 ([M+H]$^+$, $C_{45}H_{48}N_5O_{10}S$ requires 849.3044)

ν_{max} (film)/cm^{-1}: 3340 (N-H), 3931 (C-H), 1664 (C=O)

Methyl (((9H-fluoren-9-yl)methoxy)carbonyl)glycyl-L-methionylglycinate (239)



To a solution of thiol bearing tripeptide **225** (28 mg, 0.06 mmol) in Hex:MeOH (0.35 mL, 7:2) was added trimethylsilyldiazomethane in Hex (35 µL, 0.07 mmol) dropwise at

0 °C under Ar. The reaction was allowed to reach rt and stirred for 1 h. The reaction was subsequently quenched by addition of acetic acid (35 µL) and concentrated *in vacuo* to yield a yellow oil which was purified by silica gel flash chromatography (EtOAc) to yield the product as a white solid (21 mg, 73 %).

R_f (EtOAc): 0.4

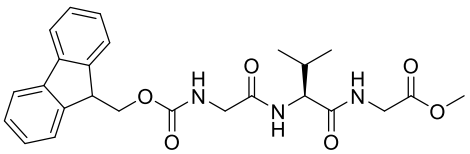
δ_H NMR (400 MHz, $CDCl_3$): 7.79 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.61 (d, *J* = 7.4 Hz, 2H, Ar-CH), 7.42 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.33 (t, *J* = 7.4 Hz, 2H, Ar-CH), 7.07 (d, *J* = 6.9 Hz, 1H, NH), 7.02 (bs, 1H, NH), 4.74 (dd, *J* = 14.3, 6.9 Hz, 1H, Met αCH), 4.44 (d, *J* = 7.0 Hz, 2H, Fmoc CHC$\underline{H}_2$), 4.24 (t, *J* = 6.9 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.05 (d, *J* = 5.1 Hz, 2H, Gly αCH_2), 3.93 (d, *J* = 3.5 Hz, 2H, Gly αCH_2), 3.74 (s, 3H, OCH_3), 2.63 (d, *J* = 6.8 Hz, 2H, Met γCH_2), 2.18 – 2.03 (m, 5H, Met βCH_2, SCH_3)

δ_C NMR (151 MHz, $CDCl_3$): 171.3 (C=O), 170.1 (C=O), 169.3 (C=O), 156.9 (C=O), 143.9 (Ar-qC), 143.8 (Ar-qC), 141.4 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.2 (Ar-CH), 67.5 (Fmoc CH$\underline{C}H_2$), 52.5 (Gly αCH_2), 52.3 (Met αCH), 47.2 (Fmoc $\underline{C}HCH_2$), 41.3 (Gly αCH_2), 30.9 (Met βCH_2), 30.2 (Met γCH_2), 15.3 (SCH_3)

HRMS (*m/z* ESI^+): Found: 522.1671 ($[M+Na]^+$, $C_{25}H_{29}N_3O_6SNa$ requires 522.1669)

ν_{max} (film)/cm^{-1}: 3342 (N-H), 1781 (C=O), 1687 (C=O)

Methyl (((9H-fluoren-9-yl)methoxy)carbonyl)glycyl-L-valylglycinate (242)



To a solution of **226** (17 mg, 0.034 mmol) in a minimum volume of DMF (10 µL) was added a solution of V-50 radical initiator (92 mg, 0.34 mmol), TCEP.HCl (487 mg, 1.70 mmol) and 1,2-ethanethiol (50 µL, 0.68 mmol) in phosphate buffer (3.5 mL, pH 6.0) which had previously been sparged with Ar and the resulting solution heated at 60 ºC for 16 h. The solution was subsequently extracted with EtOAc and the combined extracts washed with 1 M aq. HCl, sat. aq. $NaHCO_3$ solution and brine, dried over $MgSO_4$ and

concentrated *in vacuo*. Purification by silica gel flash chromatography (EtOAc:Hex – 70:30 to EtOAc) gave the product as a transparent solid (10 mg, 58%).

R_f (70% EtOAc:Hex): 0.25

δ_H NMR (400 MHz, $CDCl_3$): 7.78 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.61 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.42 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.33 (t, *J* = 7.5 Hz, 2H, Ar-CH), 6.79 (m, 2H, NH, NH), 5.69 (s, 1H, NH), 4.47 – 4.36 (m, 3H, Fmoc CHC$\underline{H}_2$, Val αCH), 4.24 (t, *J* = 7.0 Hz, 1H, Fmoc C$\underline{H}CH_2$), 4.05 (d, *J* = 5.3 Hz, 2H, Gly αCH_2), 3.95 (t, *J* = 4.8 Hz, 2H, Gly αCH_2), 3.74 (s, 3H, OCH_3) 2.26 – 2.14 (m, 1H, Val βCH), 0.99 (d, *J* = 6.8 Hz, 3H, Val CH_3), 0.96 (d, *J* = 6.8 Hz, 3H, Val CH_3)

δ_C NMR (151 MHz, $CDCl_3$): 171.4 (C=O), 170.2 (C=O), 169.4 (C=O), 156.9 (C=O), 143.8 (Ar-qC), 141.4 (Ar-qC), 127.9 (Ar-CH), 127.2 (Ar-CH), 125.2 (Ar-CH), 120.2 (Ar-CH), 67.5 (Fmoc CH$\underline{C}H_2$), 58.6 (Val αCH), 52.6 (OCH_3), 47.2 (Fmoc $\underline{C}HCH_2$), 44.7 (Gly αCH_2), 41.2 (Gly αCH_2), 30.1 (Val βCH), 19.3 (Val CH_3), 18.1 (Val CH_3)

HRMS (*m/z* ESI^+): Found: 490.1950 ($[M+Na]^+$, $C_{25}H_{29}N_3O_6Na$ requires 490.1948)

ν_{max} (film)/cm^{-1}: 3320 (N-H), 2820 (C-H), 1770 (C=O), 1740 (C=O)

6.5 Experimental Details for Chapter 4

First Amino Acid Coupling to 2-Chlorotrityl Chloride Resin

To a polypropylene syringe fitted with a polypropylene frit was added 2-chlorotrityl chloride resin (1.1 mmol/g, 100 - 200 mesh, 1.0 equiv.) and anhydrous CH_2Cl_2 (5 mL) under Ar. The syringe was agitated for 20 min, then drained. To a solution of Fmoc-AA-OH (3 eq., 0.2 M) in anhydrous CH_2Cl_2 was added DIPEA (7.5 eq.) under Ar. This solution was added to the resin and agitated for 14 h under Ar. Excess reagents were drained from the syringe and the resin was washed with CH_2Cl_2 (3 x 5 mL), DMF (3 x 5 mL) and CH_2Cl_2 (5 mL). A capping solution of CH_2Cl_2:DIPEA:MeOH (17:2:1 v/v/v, 5 mL) was transferred to the syringe and agitated for 10 min. The resin was washed with CH_2Cl_2 (3 x 5 mL) and DMF (3 x 5 mL) prior to addition of fresh capping solution and agitation for a further 10 min, followed by washing with two rounds of CH_2Cl_2 (3 x 5 mL), DMF (3 x 5 mL).

Qualitative Monitoring of Coupling Reactions with Bromophenol Blue Solution

Progression of amide coupling reaction on 2-chlorotrityl chloride resin was monitored qualitatively by treatment of ~ 10 resin beads with a solution of bromophenol blue in CH_2Cl_2 (0.15 mM, 0.1 mL).

Second to Penultimate Amino Acid Fmoc deprotection and coupling

Each amino acid coupling cycle consisted of i) Fmoc deprotection of the resin bound peptide by the addition of 20% (v/v) piperidine in DMF (5 mL) to the resin for 10 min. Following this the solution was expelled from the syringe and replaced by fresh deprotection cocktail, ii) resin washes with DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL) and DMF (3 x 5 mL), iii) peptide coupling with addition of PyBOP (3.0 eq.), NMM (6.0 eq.) and N-protected amino acid (3.0 eq, 0.2 M) in DMF to the peptide resin for 45 min, (iv) resin washes with DMF (3 x 5 mL), CH_2Cl_2 (3 x 5 mL) and DMF (3 x 5 mL), (v) qualitative monitoring of reaction progress with bromophenol blue. Following the final coupling, the resin was treated with 20% (v/v) piperidine in DMF twice for 10 min and the resin was washed with DMF (3 x 5 mL) and CH_2Cl_2 (3 x 5 mL) and dried *in vacuo*.

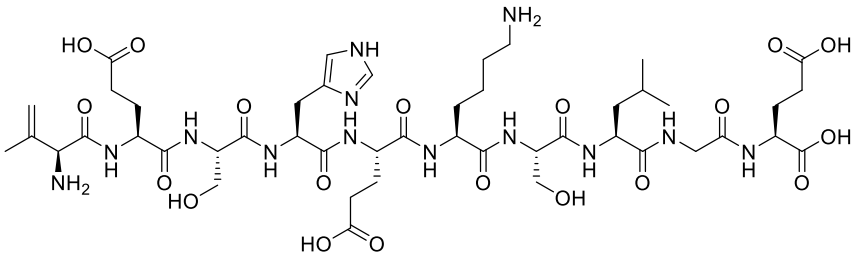
Coupling of Unsaturated Amino Acids to Resin bound Peptide

To the dry resin bound peptide was added anhydrous THF (5 mL) and the syringe agitated for 20 min, then drained. A solution of Boc-UAA-OH (2 eq., 0.2 M) and IIDQ (2 eq.) in anhydrous THF was added to the to the resin, the syringe capped and the resulting mixture agitated for 5 days. Ecess reagents were drained from the reaction vessel and the resin washed with THF (3 x 5 mL), DMF (3 x 5 mL) and CH_2Cl_2 (3 x 5 mL) prior to drying *in vacuo*.

2-Chlorotrityl Chloride Resin Cleavage and Global Deprotection

To the dry resin bound peptide was added CH_2Cl_2 (5 mL) and the syringe agitated for 20 min, then drained. The cleavage cocktail (CH_2Cl_2:TES:TFA, 94:5:1 v/v/v, 5 mL) was added to the syringe which was tightly capped and agitated for 5 min. The syringe was drained, and the filtrate was collected. The resin was washed with cleavage cocktail (2 x 2.5 mL) and washings combined with the initial filtrate. The solution was concentrated under a stream of N_2, followed by precipitation of the peptide with Et_2O (10 mL) at 0 °C. The crude peptide suspension was centrifuged and the supernatant decanted. The peptide pellet was washed again with Et_2O (2 x 10 mL) at 0 °C, centrifuged and collected. The crude solid was dried *in vacuo*. The crude protected peptide pellet was dissolved in deprotection cocktail (CH_2Cl_2/TFA/TES, 60:20:20, v/v/v, 5 mL) and stirred for 5 h at rt. The resulting solution was concentrated under a stream of N_2 and the resulting residue triturated with Et_2O (3 x 5 mL) at 0 °C. ^{1}H NMR spectroscopy was used to ensure full deprotection had been achieved after which the crude peptide was purified by RP-HPLC.

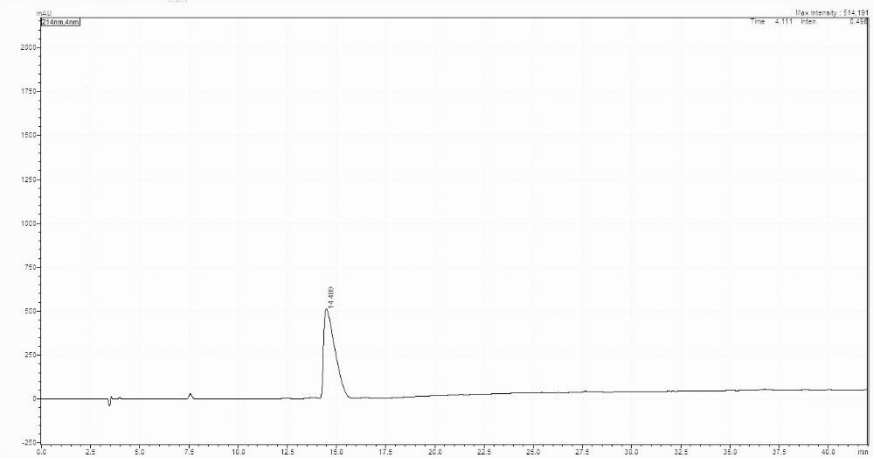
((*S*)-2-amino-3-methylbut-3-enoyl)-L-glutamyl-L-seryl-L-histidyl-L-glutamyl-L-lysyl-L-seryl-L-leucylglycyl-L-glutamic acid (244)



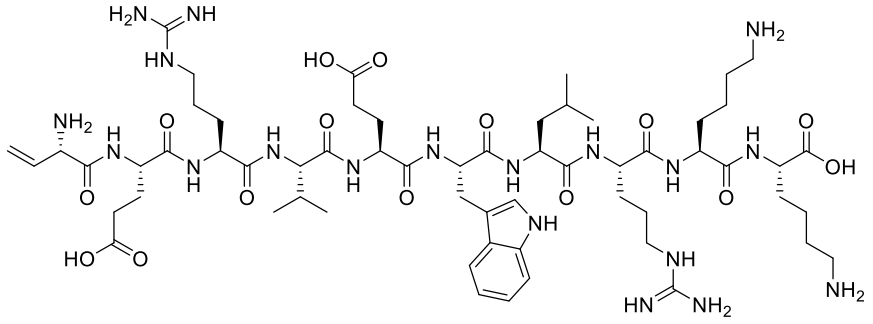
Prepared on 2-chlorotrityl chloride resin using general procedures above utilising Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Ser(O^{t}Bu)-OH, Fmoc-Lys(Boc)-OH and Fmoc-His(Trt)-OH and Boc-DHV **172**. RP-HPLC performed on C18 Φ4.6 × 250 mm column, 1 mL min^{-1} flow rate.

Analytical HPLC: R_t 14.5 min (5 - 65% ACN over 42 min, 0.1% FA, λ = 214 nm)

HRMS (*m/z* ESI$^+$): Found: 1112.5229 ([M+H]$^+$, $C_{46}H_{74}N_{13}O_{19}$ requires 1112.5224)



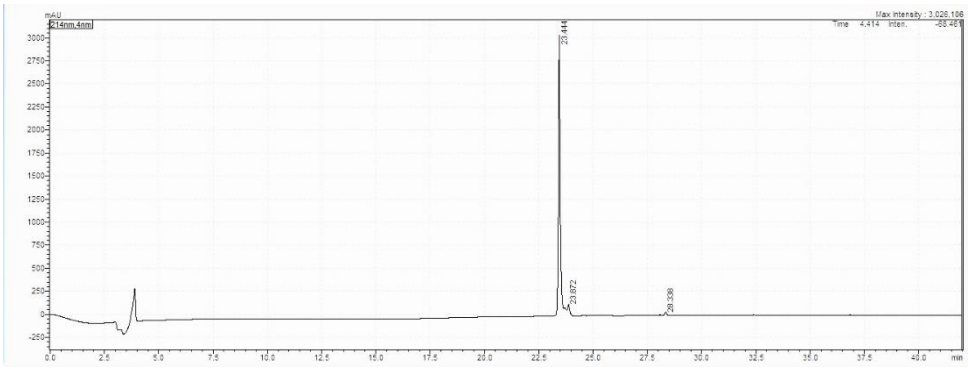
((*S*)-2-aminobut-3-enoyl)-L-glutamyl-L-arginyl-L-valyl-L-glutamyl-L-tryptophyl-L-leucyl-L-arginyl-L-lysyl-L-lysine (243)



Prepared on 2-chlorotrityl chloride resin using general procedures above utilising Fmoc-Lys(Boc)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Ile-OH, Fmoc-Trp-OH, Fmoc-Glu(O^{t}Bu)-OH, Fmoc-Val-OH and Boc-VG **171**. RP-HPLC performed on C18 Φ4.6 × 250 mm column, 1 mL min^{-1} flow rate.

HPLC: R_t 23.4 min (5 - 65% ACN over 42 min, 0.1% FA, λ = 214 nm)

LRMS (*m/z* ESI$^+$): Found: 664.2 $[M+2H]^{2+}$, $C_{46}H_{74}N_{13}O_{19}$ requires 664.2)



Chapter 7

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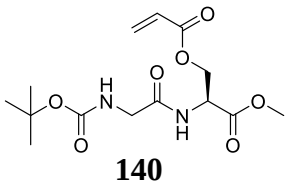
Appendix



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4.89
4.88
4.87
4.86
4.52
4.52
4.46
3.85
3.84
3.82
3.77
1.44
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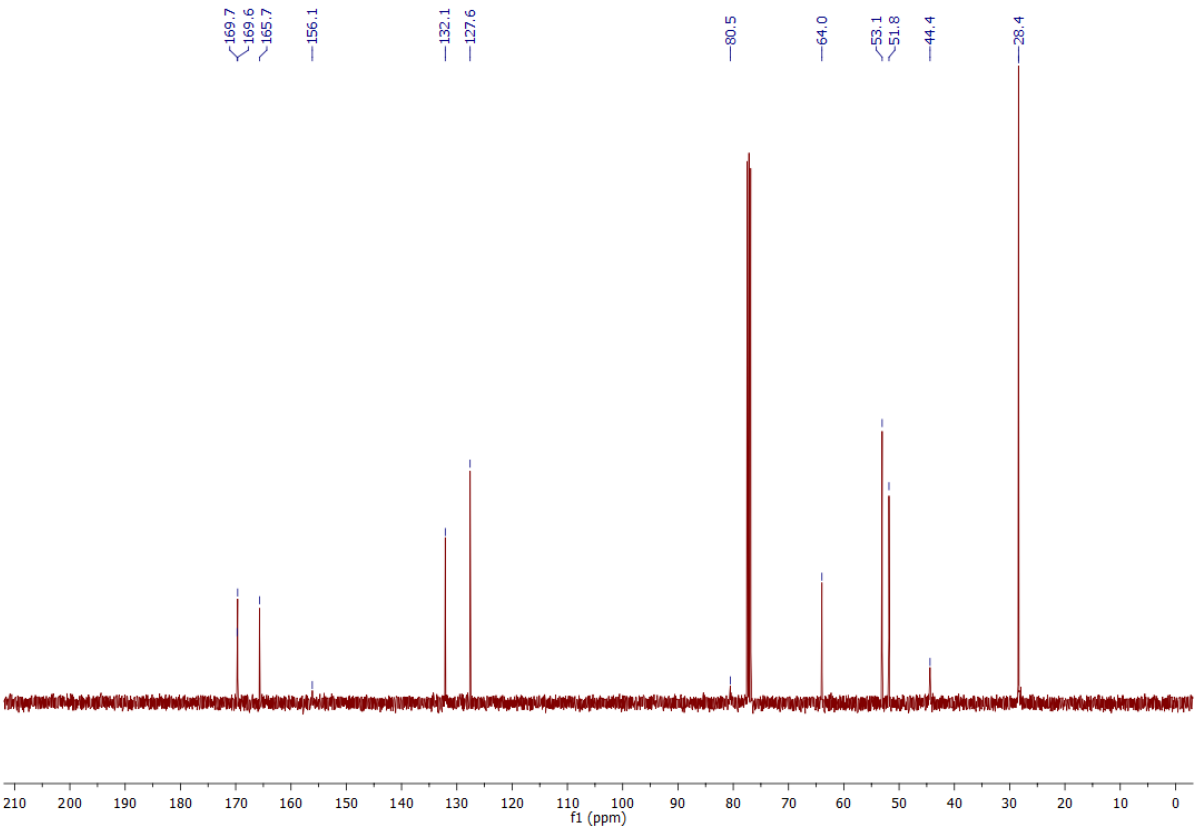
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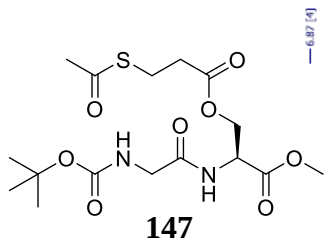
f1 (ppm)



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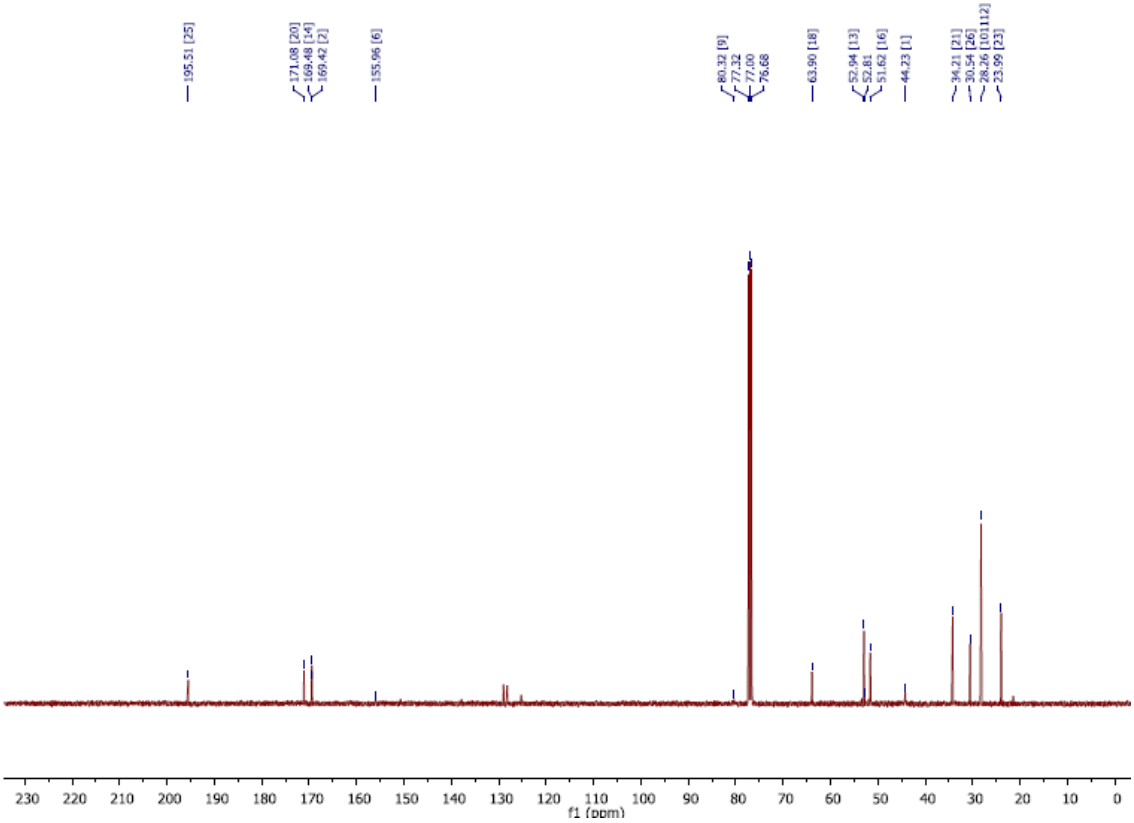

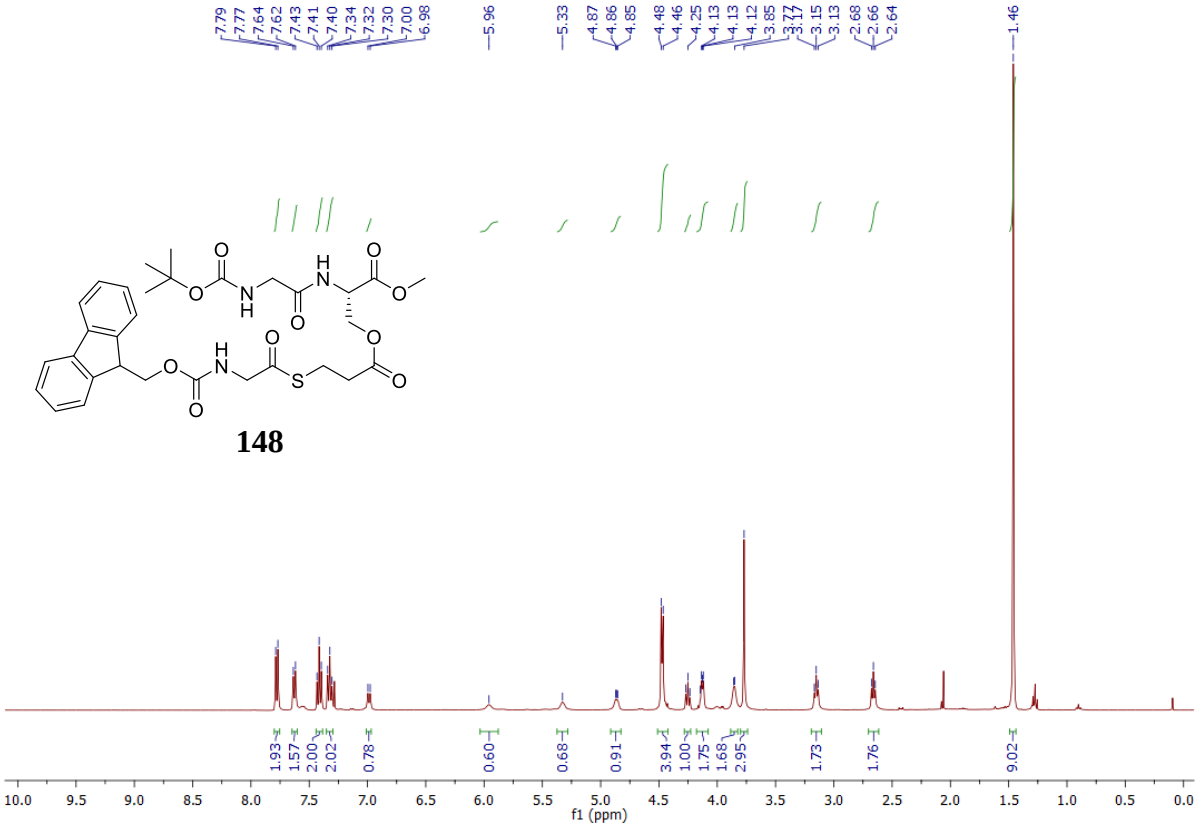

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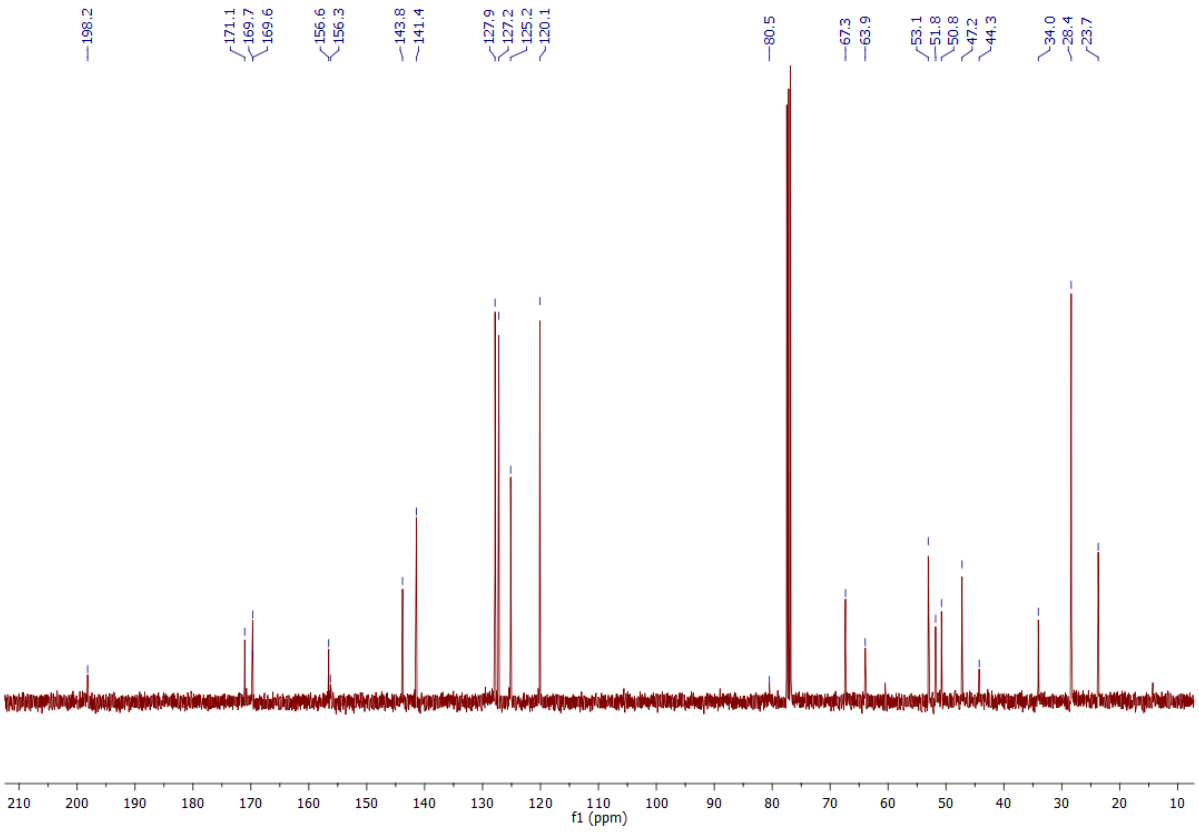

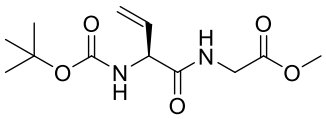

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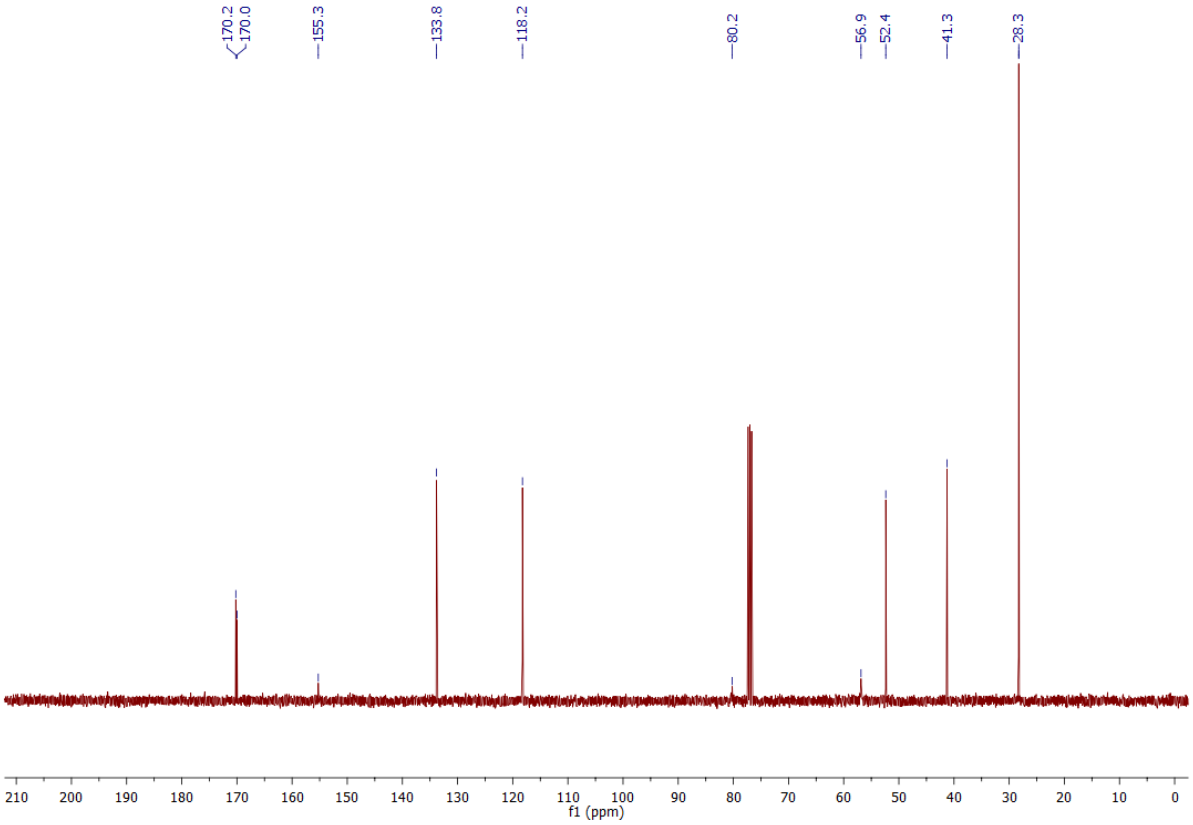
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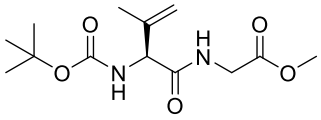

175

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3.97
3.76
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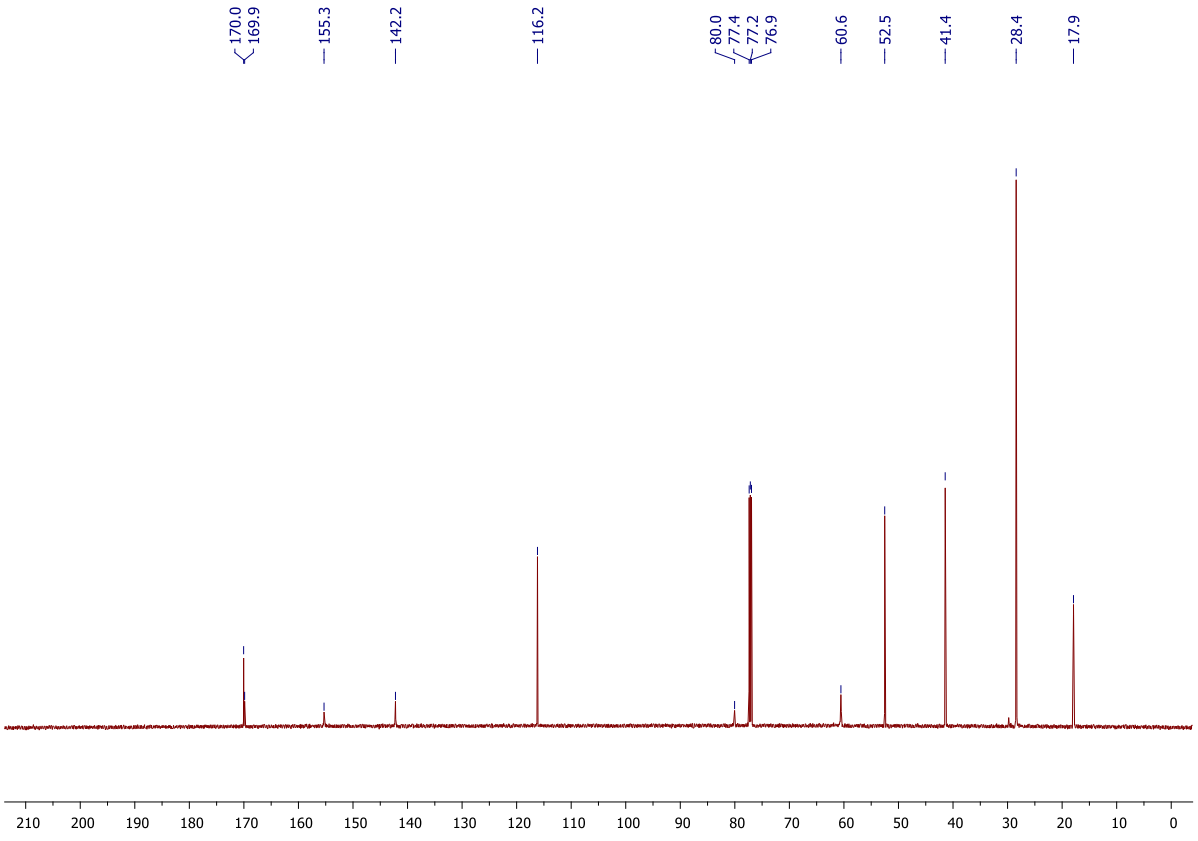
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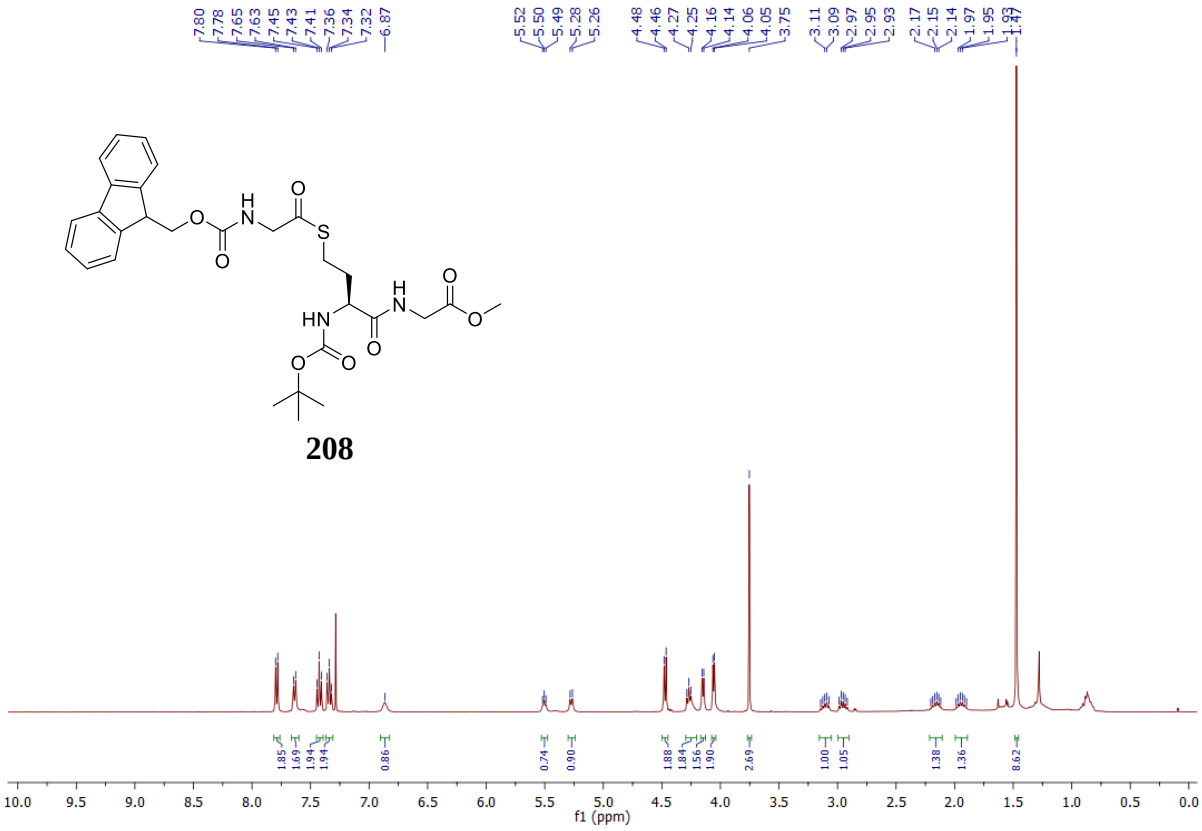
28.4
17.9



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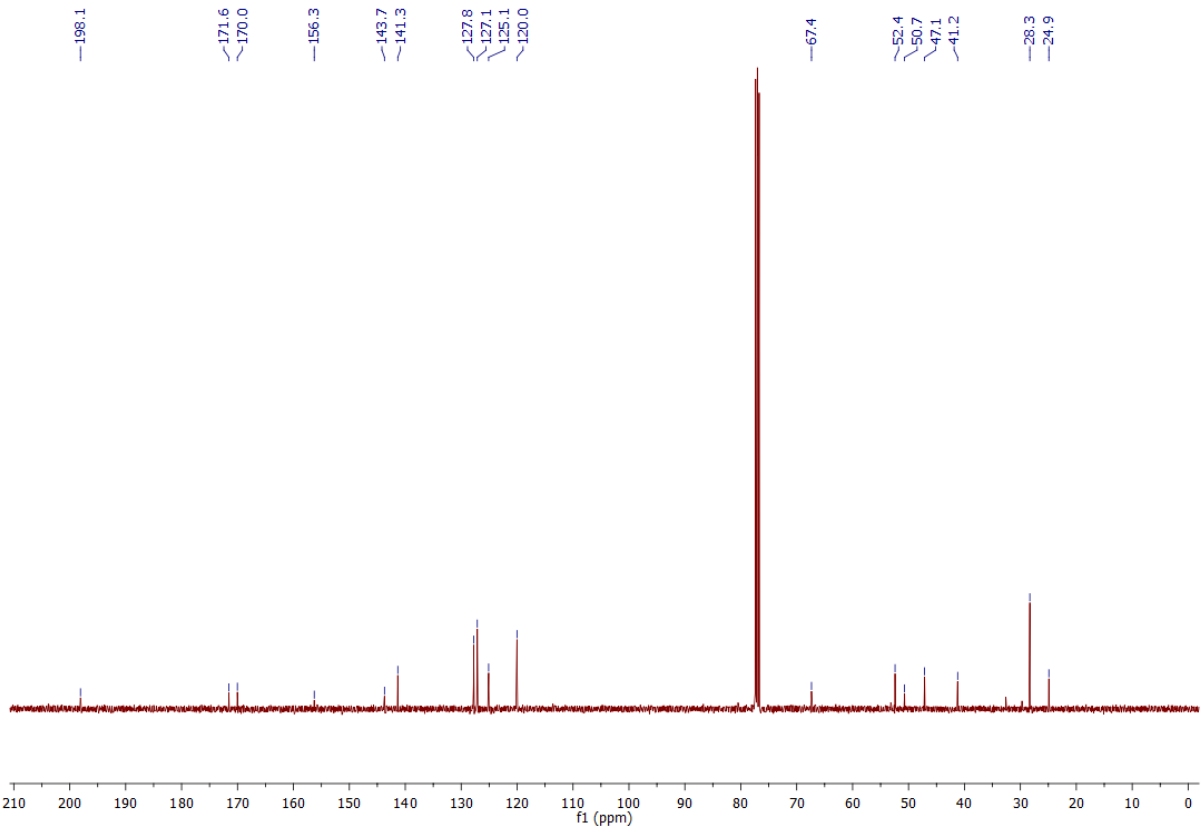
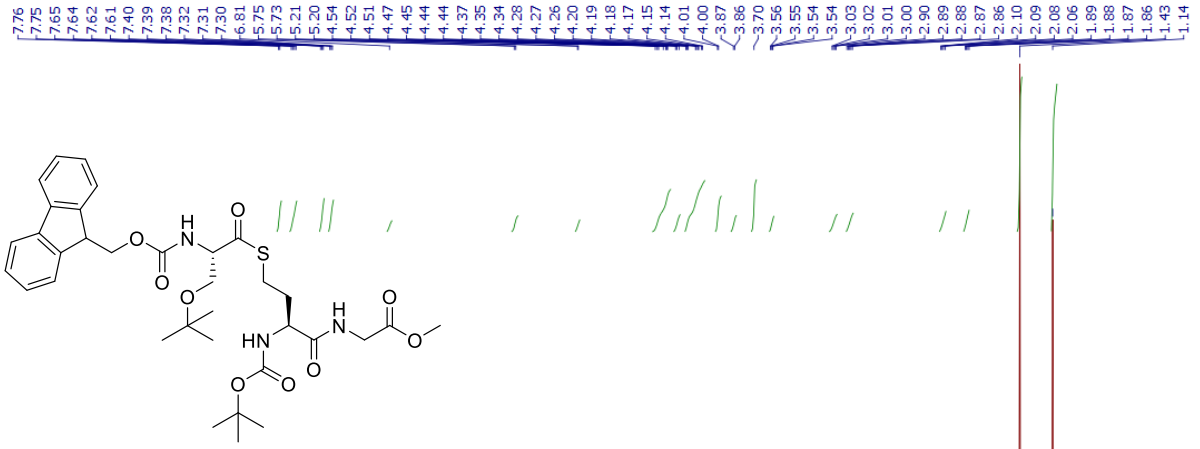
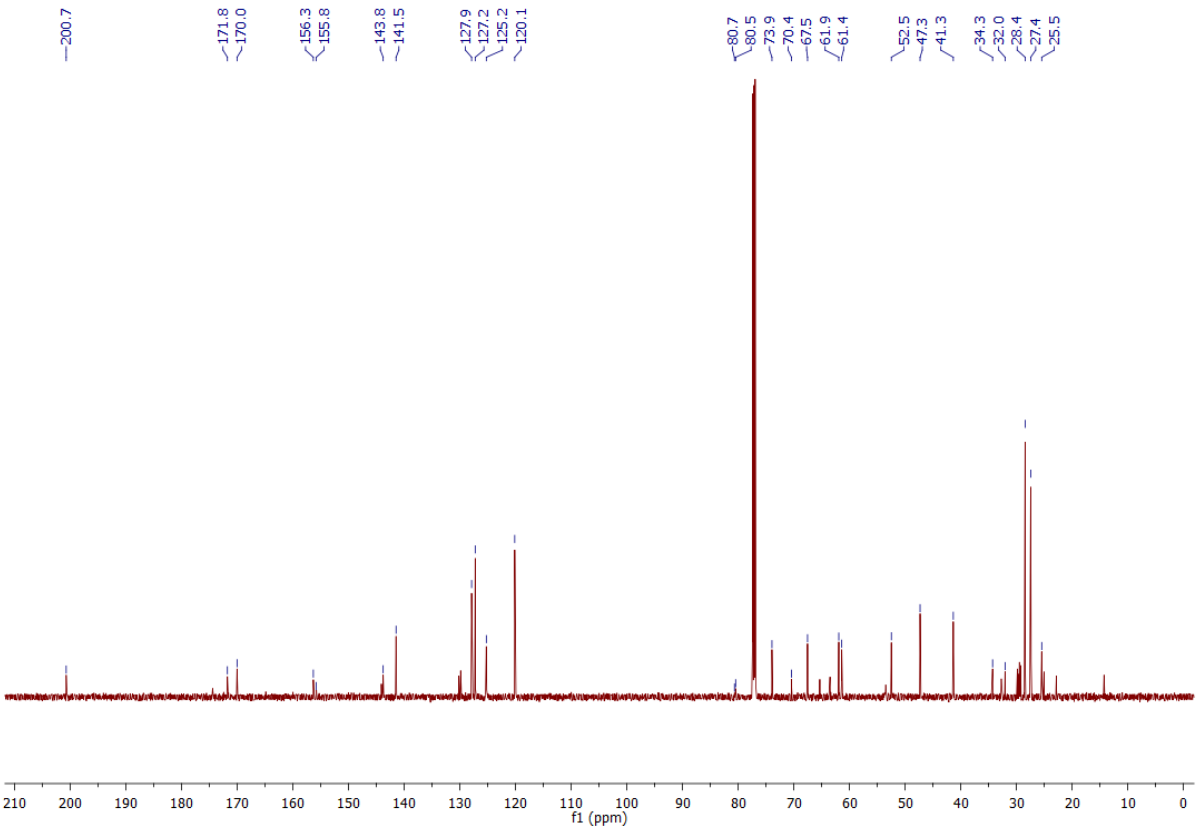
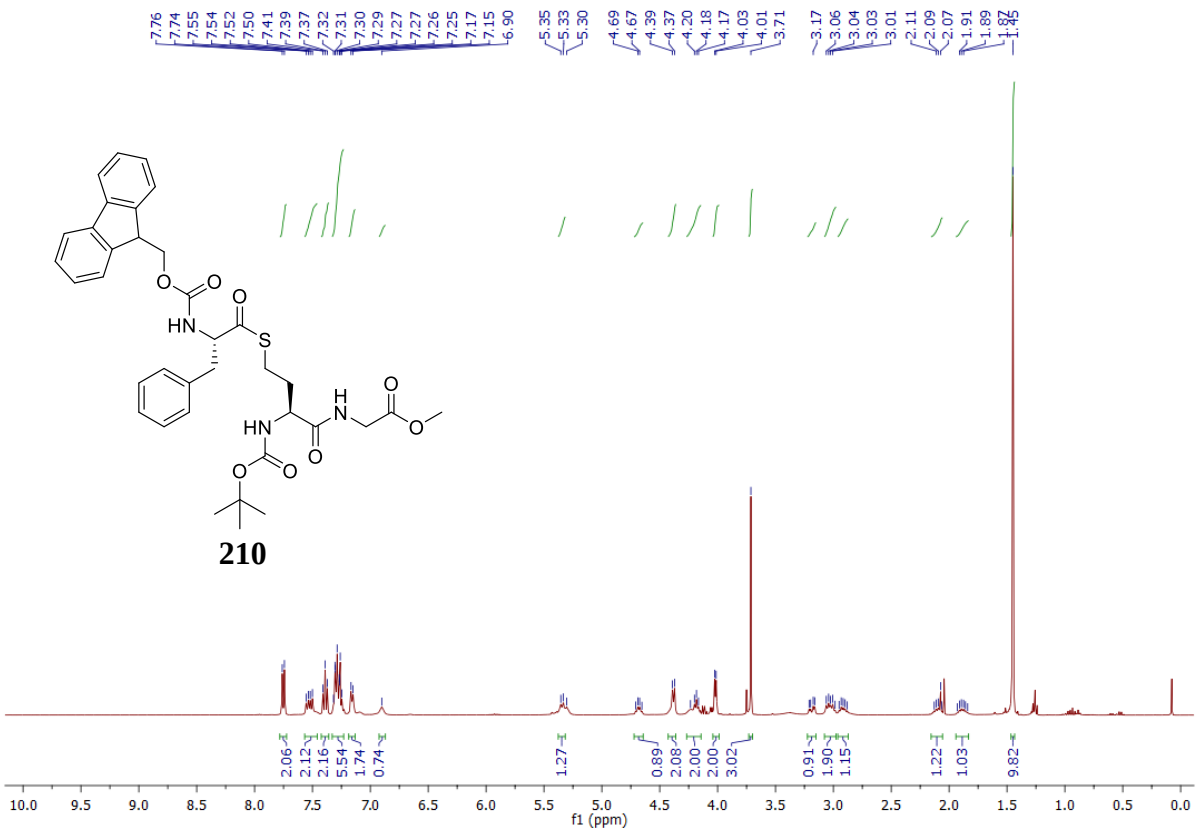




209

7.76 7.75 7.65 7.64 7.62 7.61 7.40 7.39 7.38 7.32 7.31 7.30 6.81 5.75 5.73 5.21 5.20 4.54 4.52 4.51 4.47 4.45 4.44 4.44 4.37 4.35 4.34 4.28 4.27 4.26 4.20 4.19 4.18 4.17 4.15 4.14 4.01 4.00 3.87 3.86 3.70 3.56 3.55 3.54 3.54 3.03 3.02 3.01 3.00 2.90 2.89 2.88 2.87 2.86 2.10 2.09 2.08 2.06 1.89 1.88 1.87 1.86 1.43 1.14
1.78 1.78 1.92 1.83 0.65 0.93 0.70 2.44 1.00 2.94 2.06 0.95 2.99 0.91 1.02 1.09 1.19 1.26 8.85 8.45
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


200.7
171.8 170.0 156.3 155.8 143.8 141.5 127.9 127.2 125.2 120.1
80.7 80.5 73.9 70.4 67.5 61.9 61.4 52.5 47.3 41.3 34.3 32.0 28.4 27.4 25.5
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


7.76
7.74
7.55
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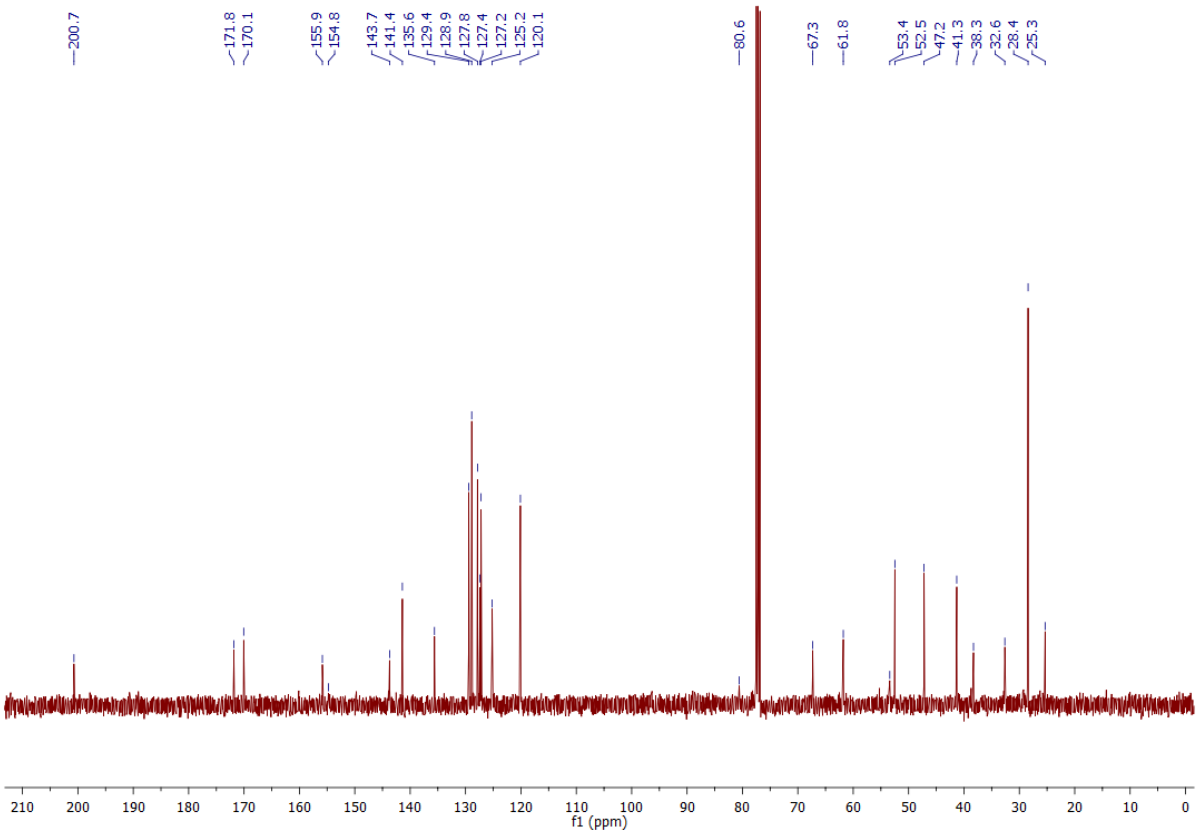
7.50
7.41
7.39
7.37
7.32
7.31
7.30
7.29
7.27
7.27
7.26
7.25
7.17
7.15
6.90
5.35
5.33
5.30
4.69
4.67
4.39
4.37
4.20
4.18
4.17
4.03
4.01
3.71
3.17
3.06
3.04
3.03
3.01
2.11
2.09
2.07
1.91
1.89
1.87
1.45
2.06
2.12
2.16
5.54
1.74
0.74
1.27
0.89
2.08
2.00
2.00
3.02
0.91
1.90
1.15
1.22
1.03
9.82
f1 (ppm)

210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155.9
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141.4
135.6
129.4
128.9
127.8
127.4
127.2
125.2
120.1
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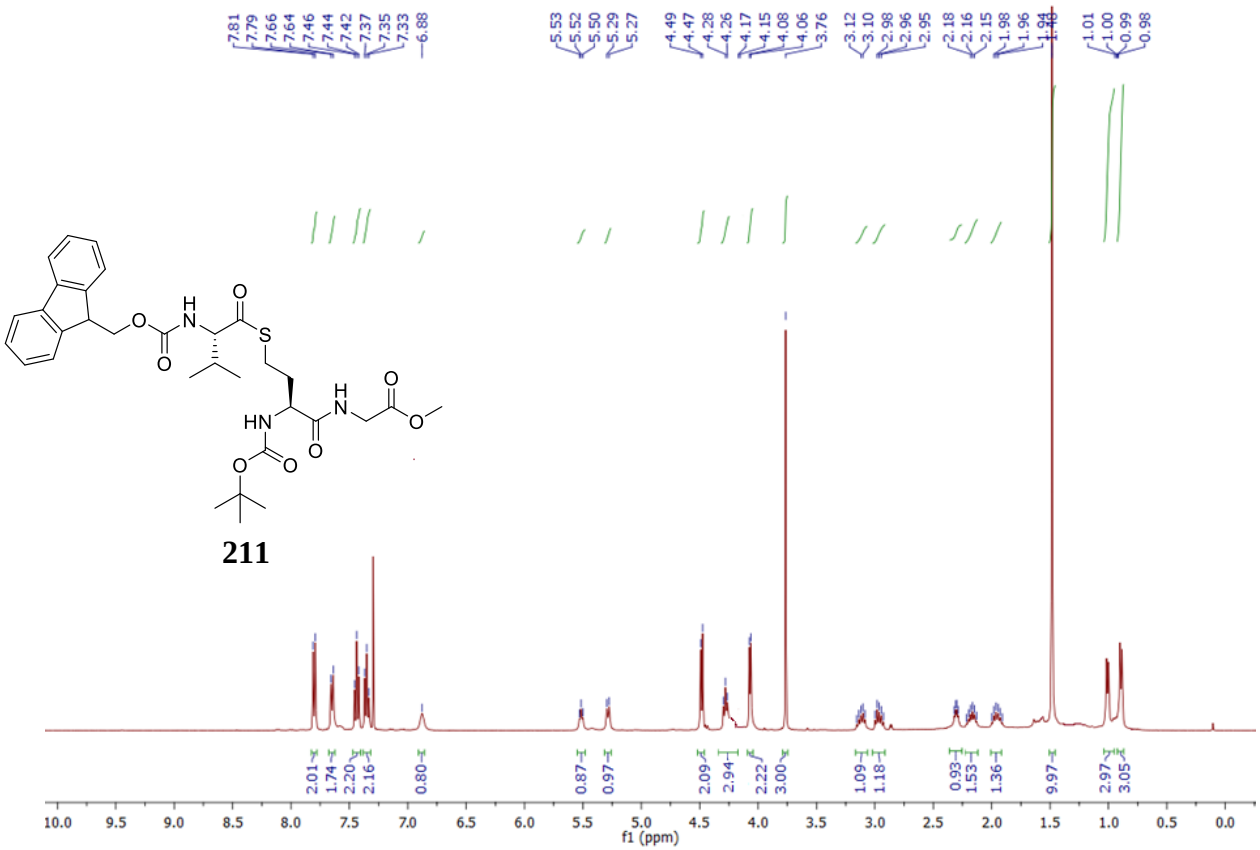
67.3
61.8
53.4
52.5
47.2
41.3
38.3
32.6
28.4
25.3
f1 (ppm)


7.81
7.79
7.66
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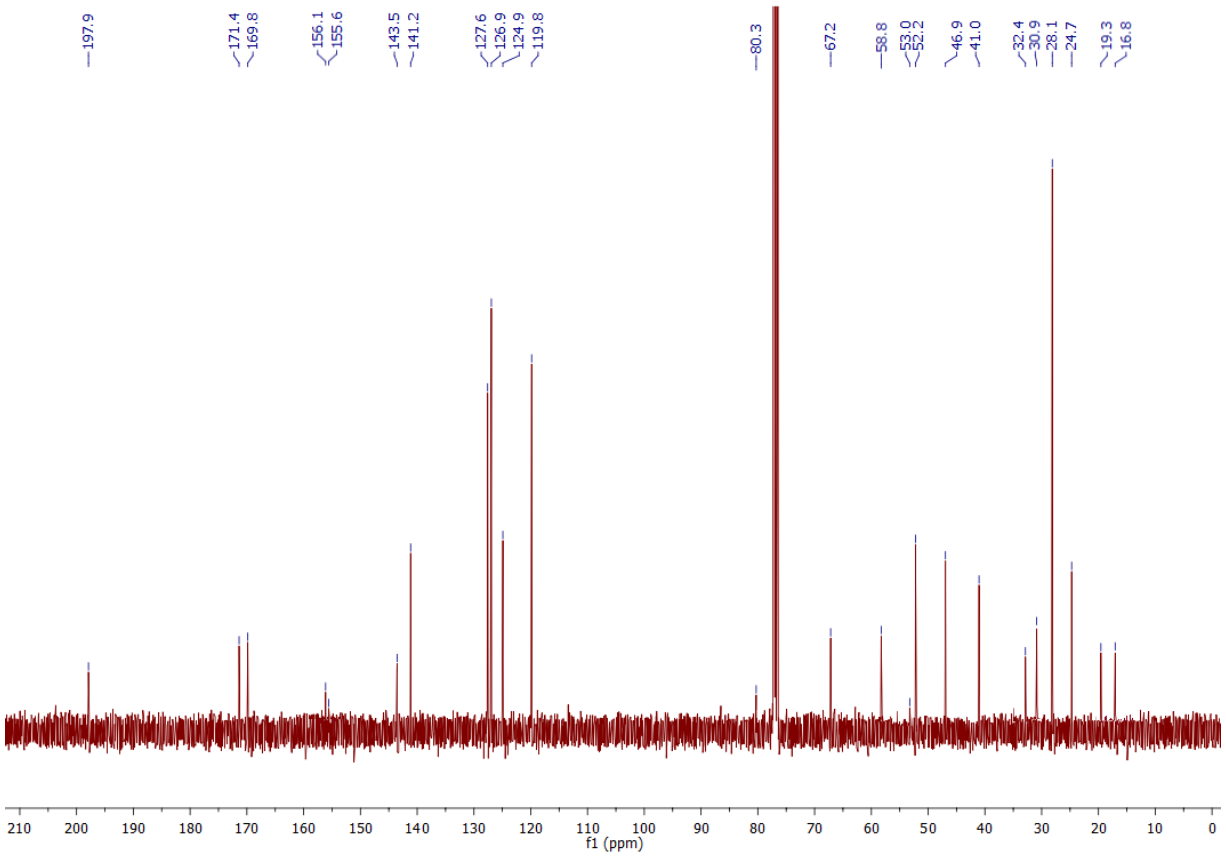
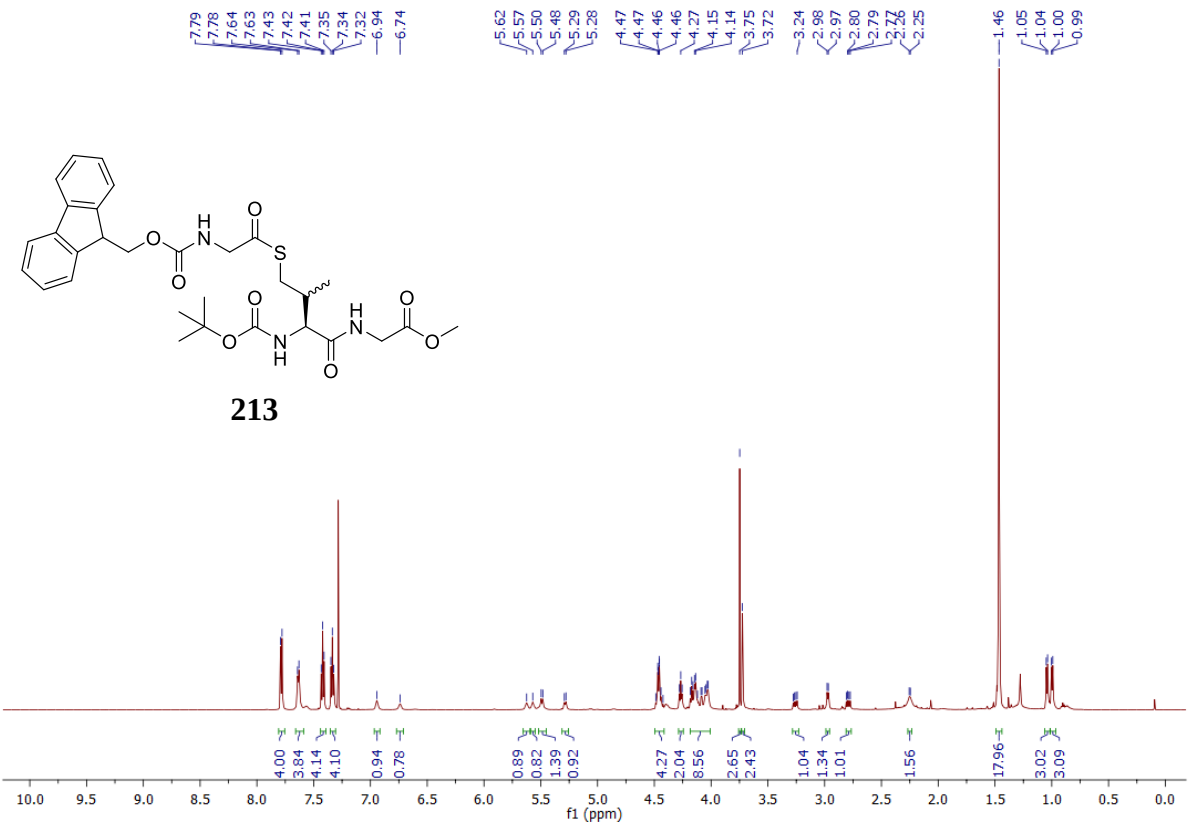
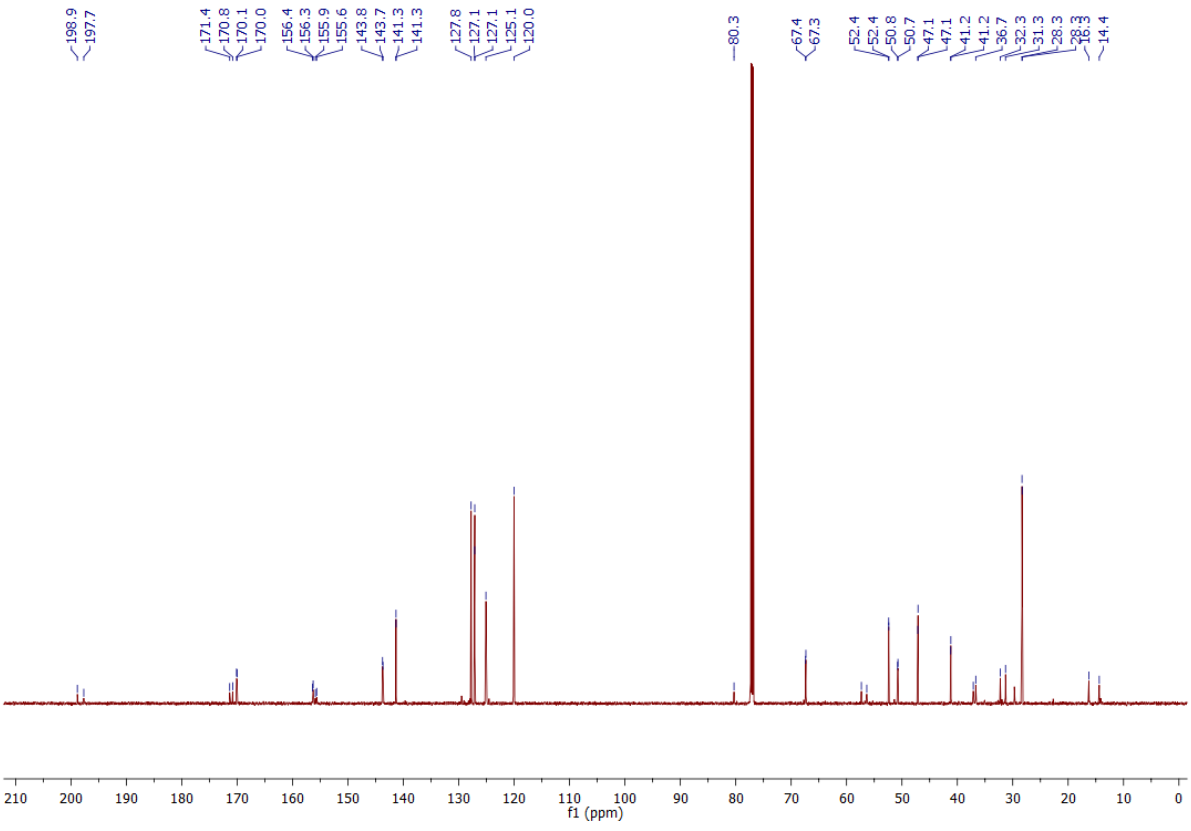
7.42
7.37
7.35
7.33
6.88
5.53
5.52
5.50
5.29
5.27
4.49
4.47
4.28
4.26
4.17
4.15
4.08
4.06
3.76
3.12
3.10
2.98
2.96
2.95
2.18
2.16
2.15
1.98
1.96
1.94
1.48
1.01
1.00
0.99
0.98
2.01
1.74
2.20
2.16
0.80
0.87
0.97
2.09
2.94
2.22
3.00
1.09
1.18
0.93
1.53
1.36
9.97
2.97
3.05
f1 (ppm)

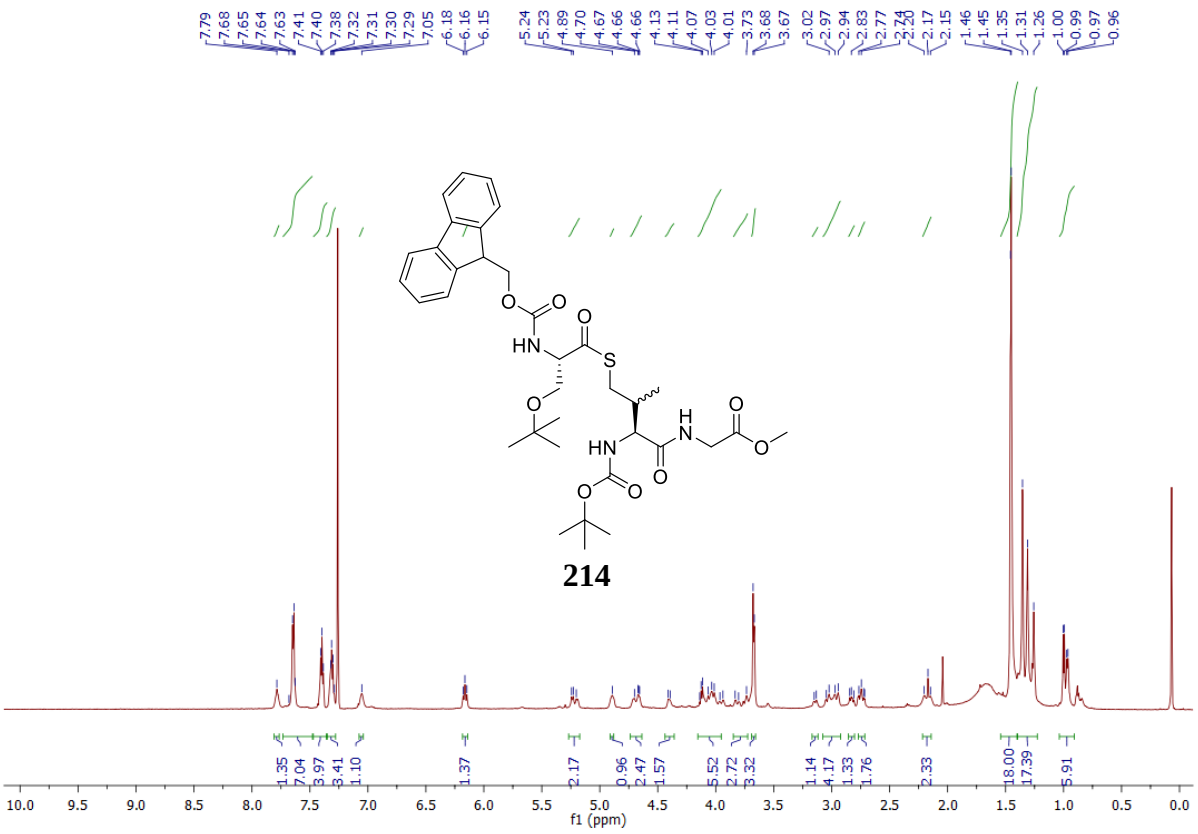
211


197.9
171.4
169.8
156.1
155.6
143.5
141.2
127.6
126.9
124.9
119.8
80.3
67.2
58.8
53.0
52.2
46.9
41.0
32.4
30.9
28.1
24.7
19.3
16.8
f1 (ppm)

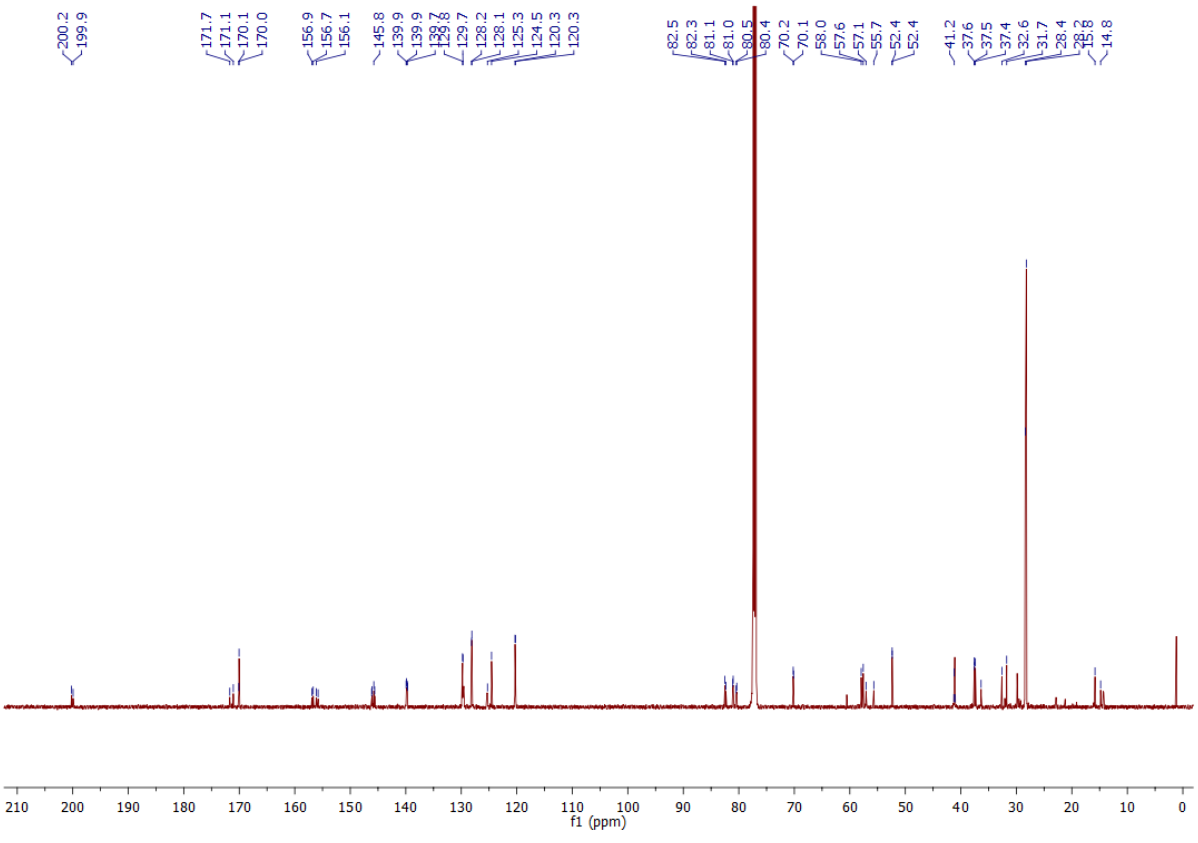


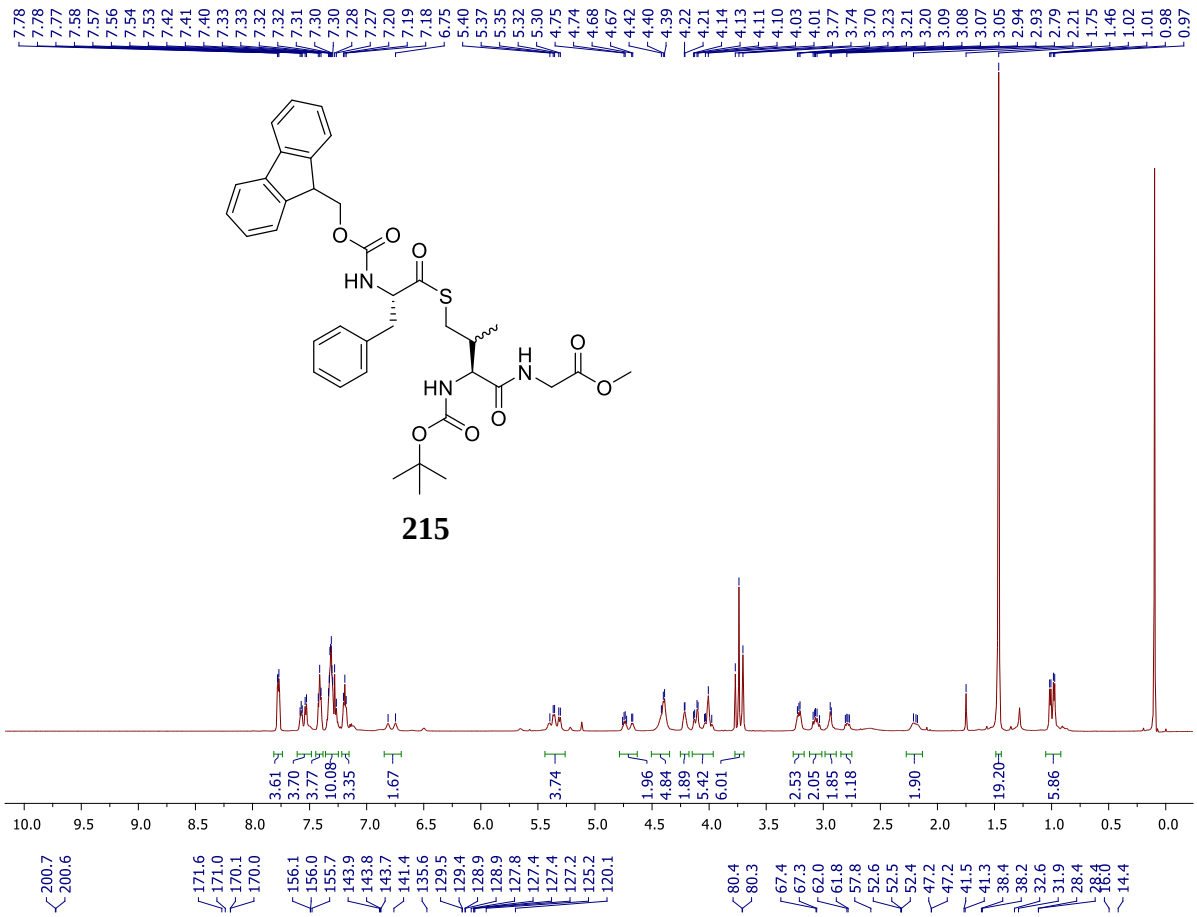
213



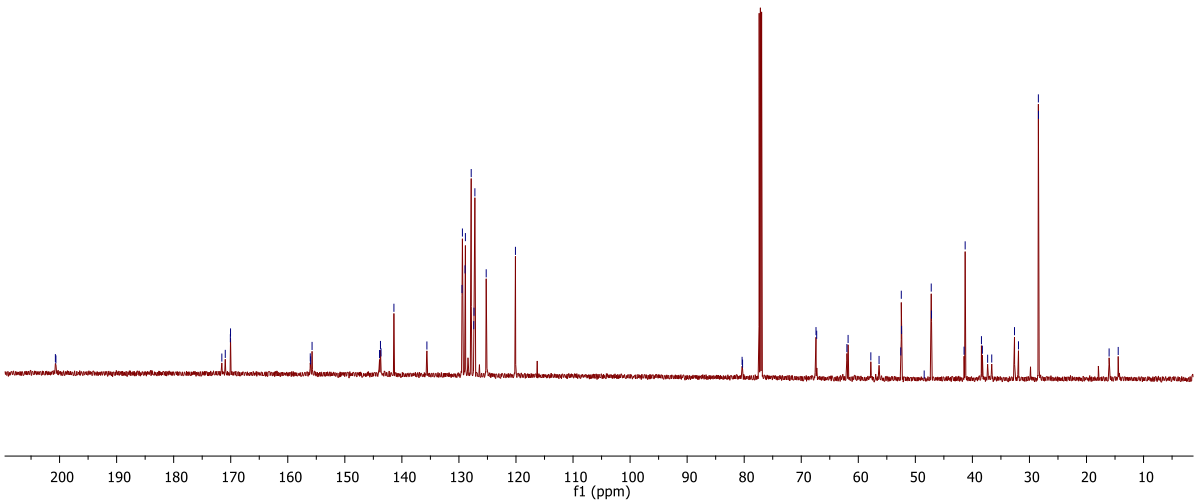


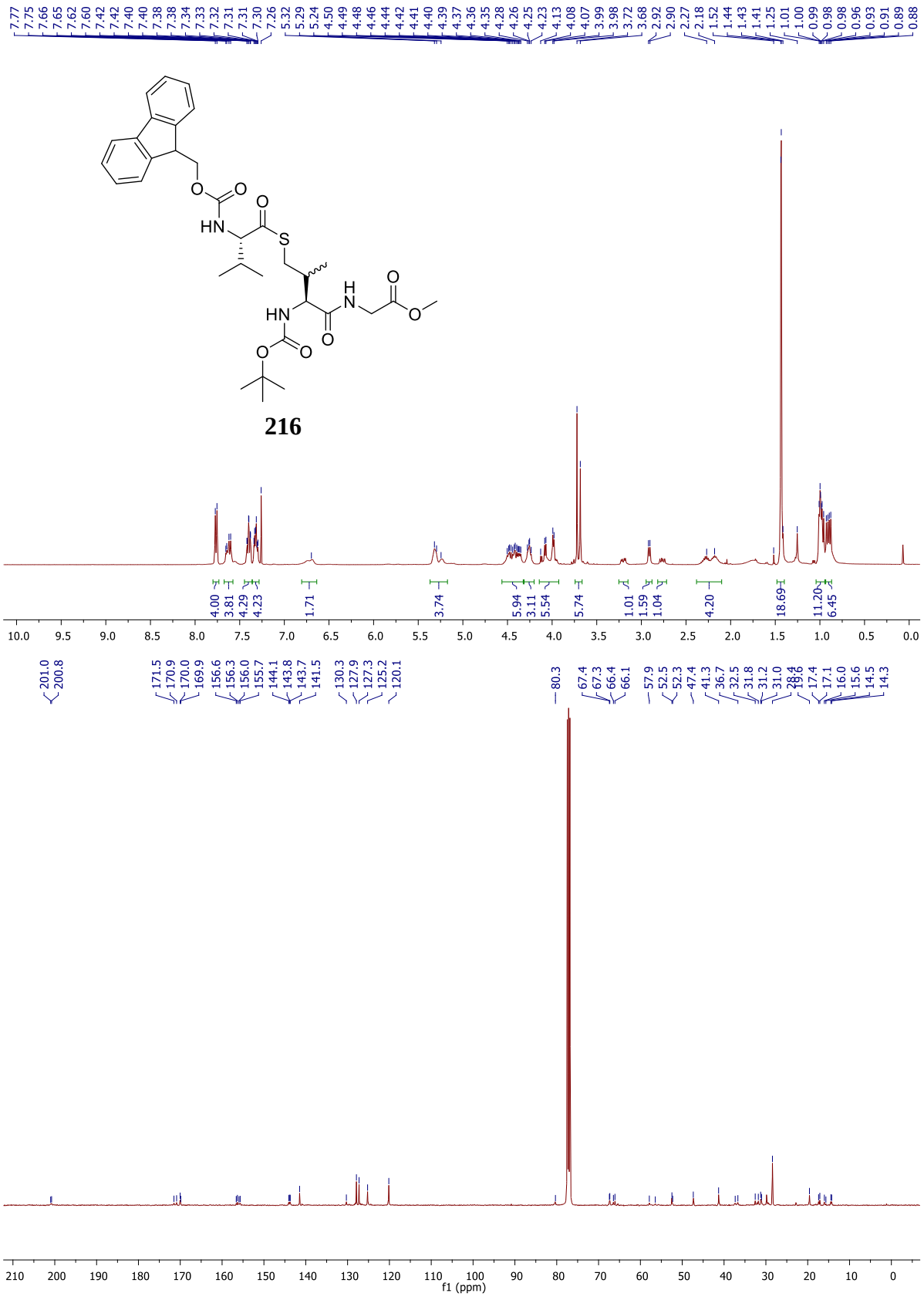
214



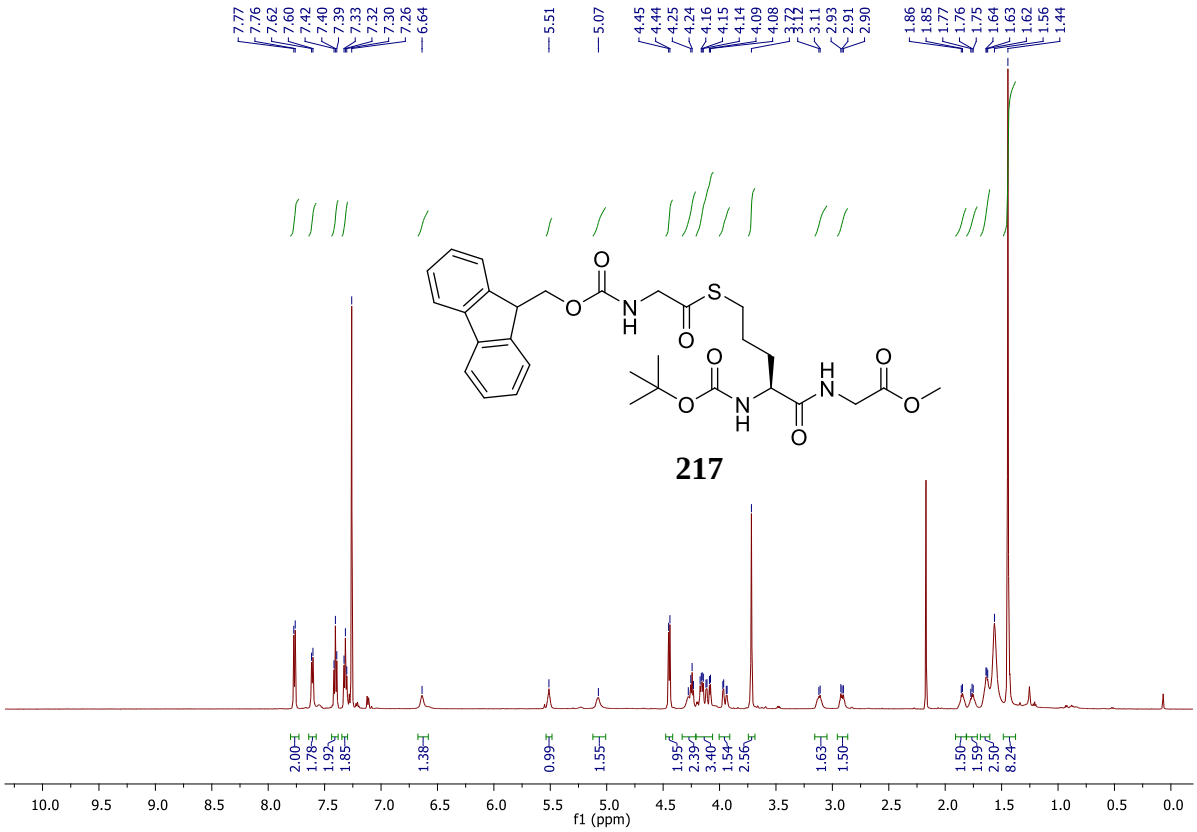


215

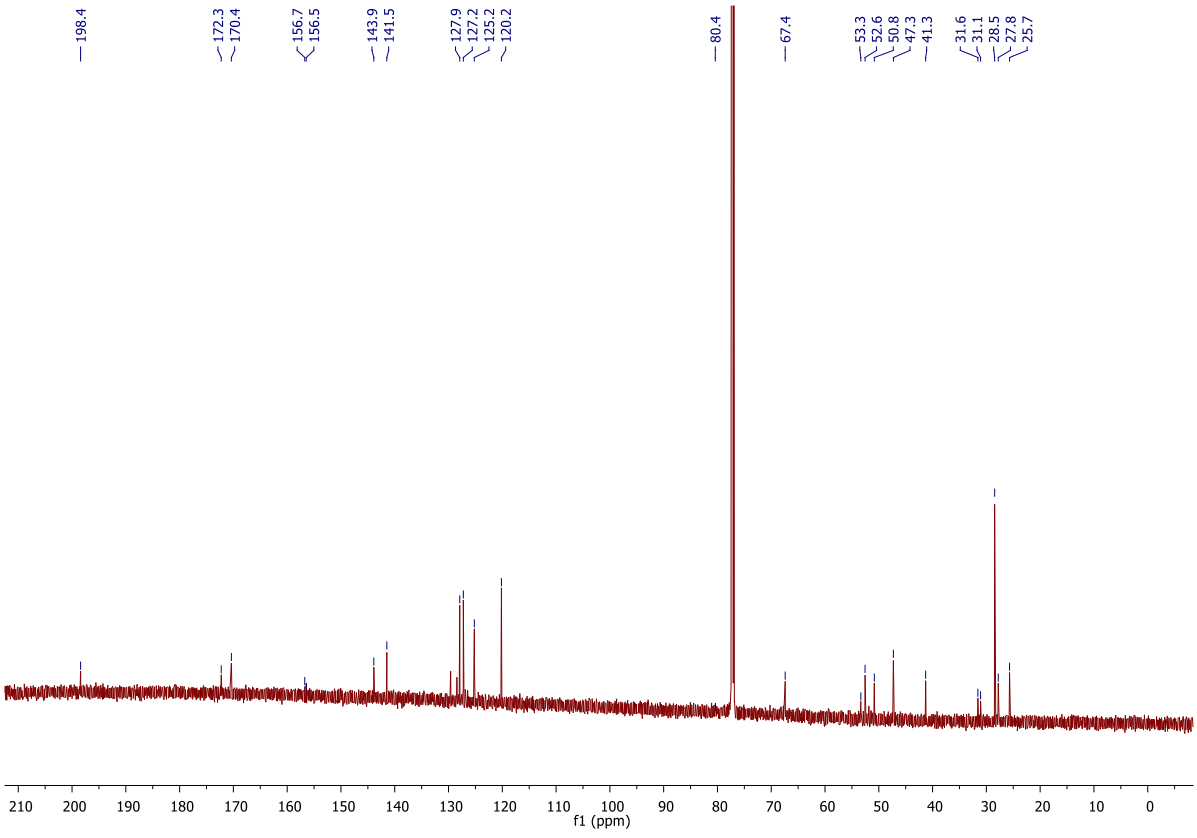


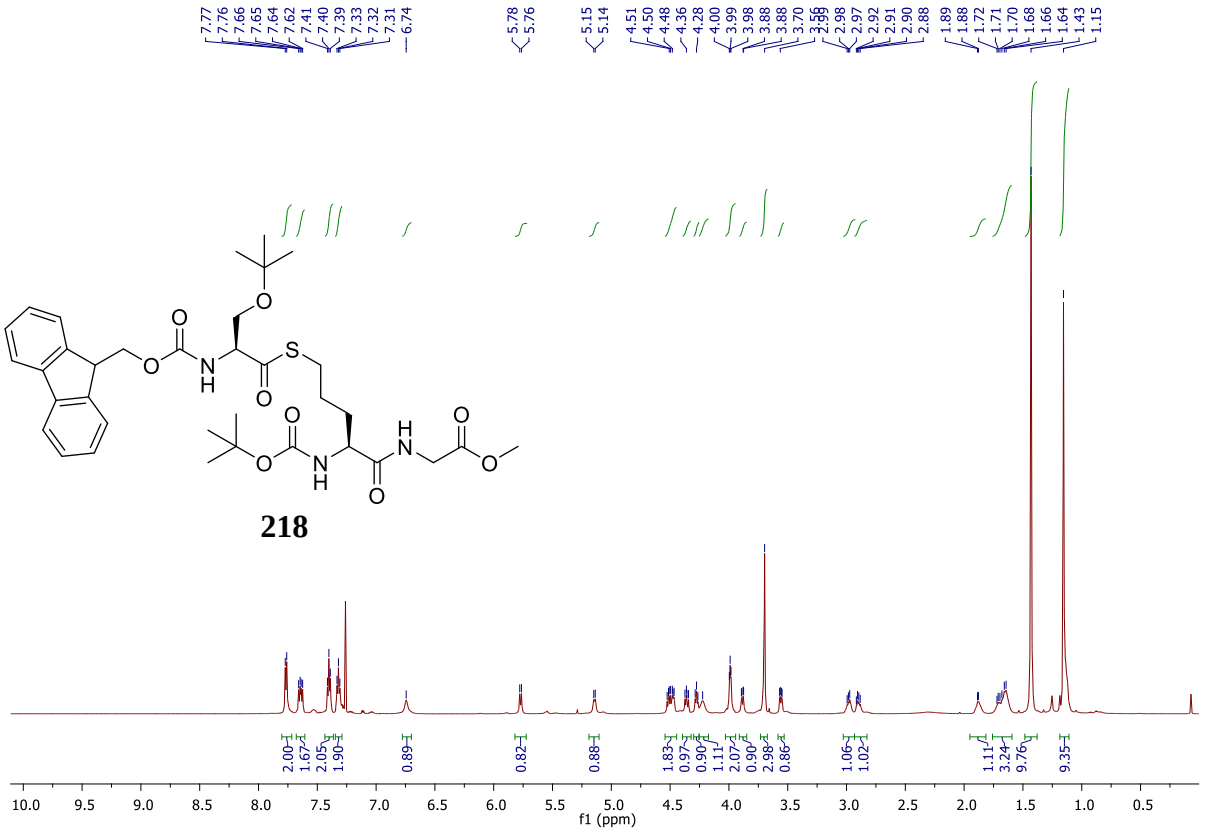


216

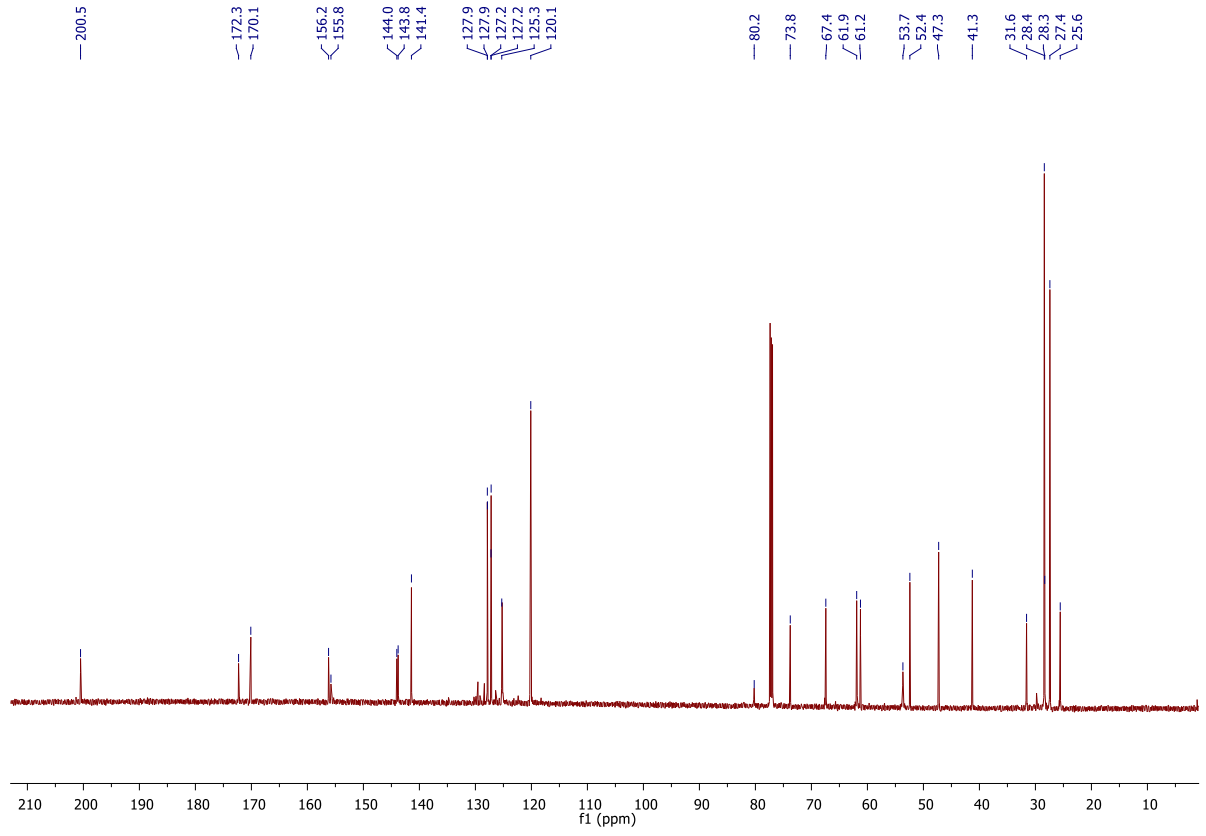

7.77
7.76
7.62
7.60
7.42
7.40
7.39
7.33
7.32
7.30
7.26
6.64
5.51
5.07
4.45
4.44
4.25
4.24
4.16
4.15
4.14
4.09
4.08
3.12
3.11
2.93
2.91
2.90
1.86
1.85
1.77
1.76
1.75
1.64
1.63
1.62
1.56
1.44
2.00
1.78
1.92
1.85
1.38
0.99
1.55
1.95
2.39
3.40
1.54
2.56
1.63
1.50
1.50
1.59
2.50
8.24
f1 (ppm)

217

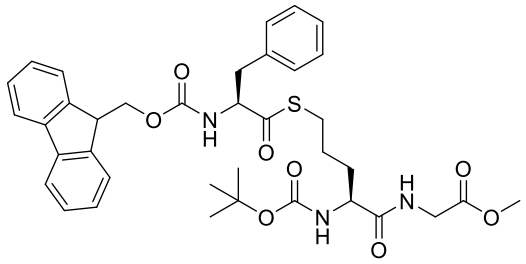

198.4
172.3
170.4
156.7
156.5
143.9
141.5
127.9
127.2
125.2
120.2
80.4
67.4
53.3
52.6
50.8
47.3
41.3
31.6
31.1
28.5
27.8
25.7
f1 (ppm)


7.77
7.76
7.66
7.65
7.64
7.62
7.41
7.40
7.39
7.33
7.32
7.31
6.74
5.78
5.76
5.15
5.14
4.51
4.50
4.48
4.36
4.28
4.00
3.99
3.98
3.88
3.88
3.70
2.98
2.97
2.92
2.91
2.90
2.88
1.89
1.88
1.72
1.71
1.70
1.68
1.66
1.64
1.43
1.15
2.00
1.67
2.05
1.90
0.89
0.82
0.88
1.83
0.97
0.90
1.11
2.07
0.90
2.98
0.86
1.06
1.02
1.11
3.24
9.76
9.35
f1 (ppm)

218

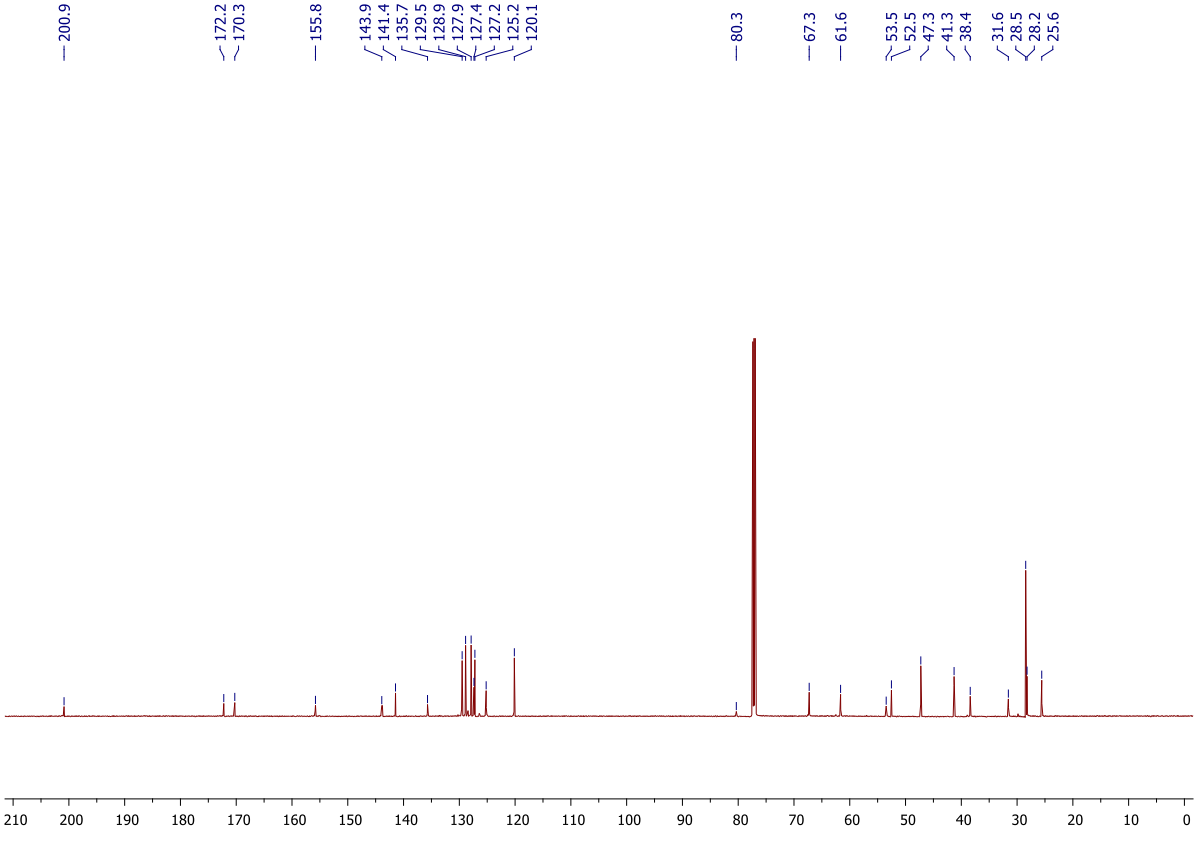

200.5
172.3
170.1
156.2
155.8
144.0
143.8
141.4
127.9
127.9
127.2
127.2
125.3
120.1
80.2
73.8
67.4
61.9
61.2
53.7
52.4
47.3
41.3
31.6
28.4
28.3
27.4
25.6
f1 (ppm)


7.76
7.75
7.55
7.54
7.52
7.51
7.41
7.40
7.38
7.31
7.30
7.29
7.28
7.26
7.25
7.24
7.16
7.15
6.66
5.38
5.36
5.08
4.39
4.38
4.37
4.37
4.19
4.04
3.99
3.98
3.70
3.17
3.07
3.06
3.05
3.04
3.03
1.85
1.73
1.72
1.71
1.62
1.45


O
O
N
H
S
O
O
O
O
N
H
H
N
O
O

219

1.91
1.94
2.04
3.73
1.58
1.20
0.97
1.19
1.00
2.29
1.01
1.10
2.36
2.78
1.01
2.01
1.13
1.08
1.16
3.15
8.61
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0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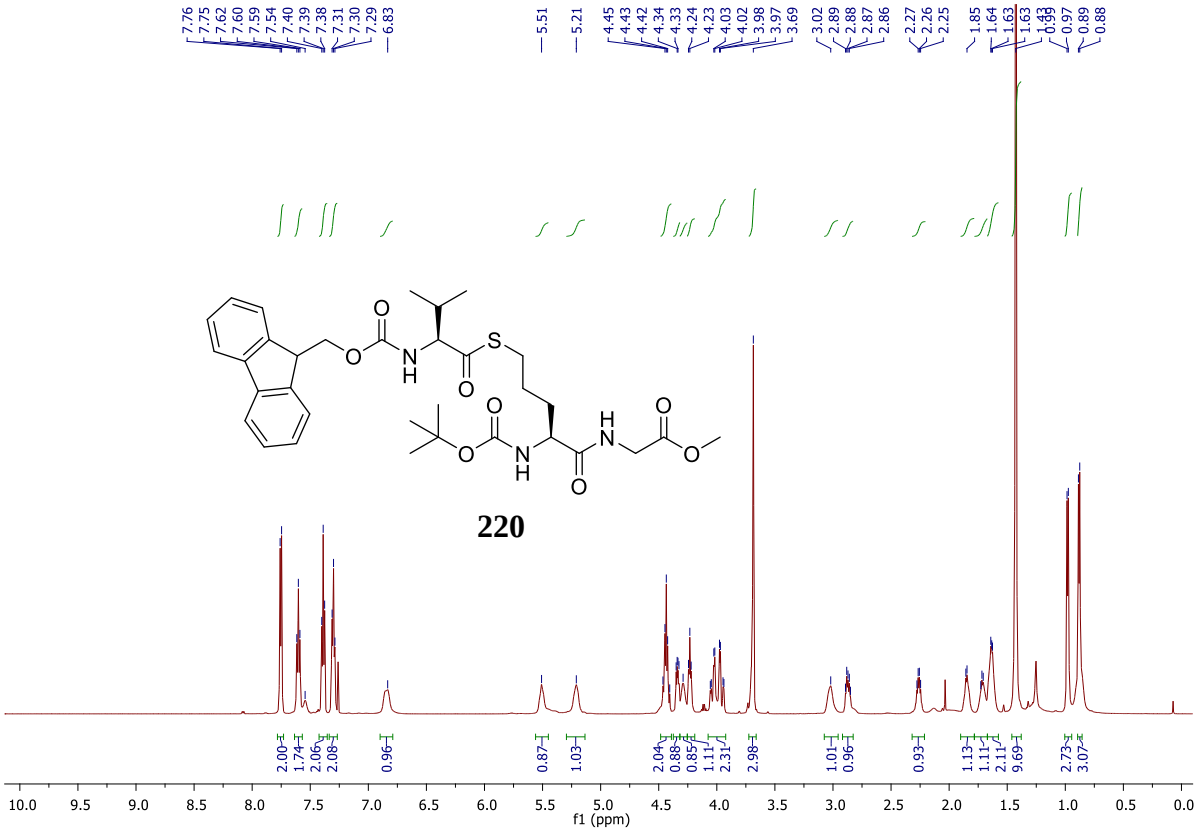
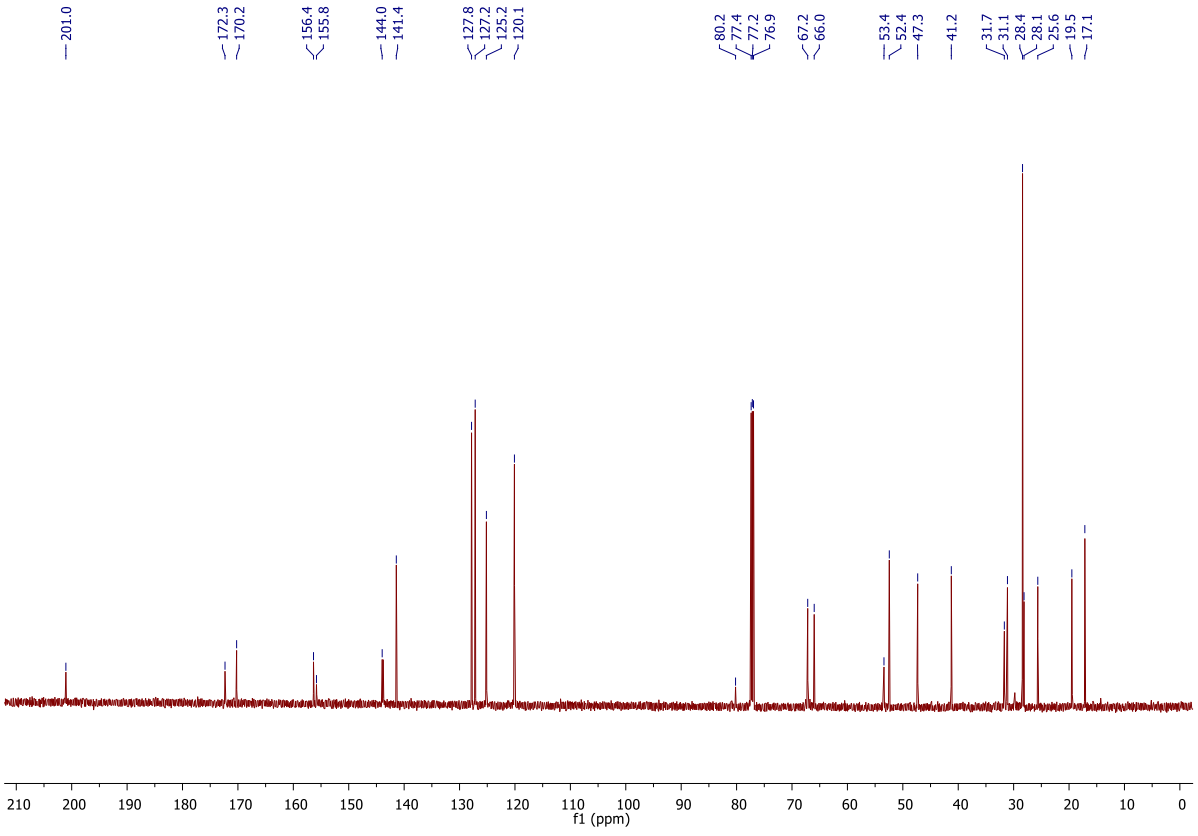
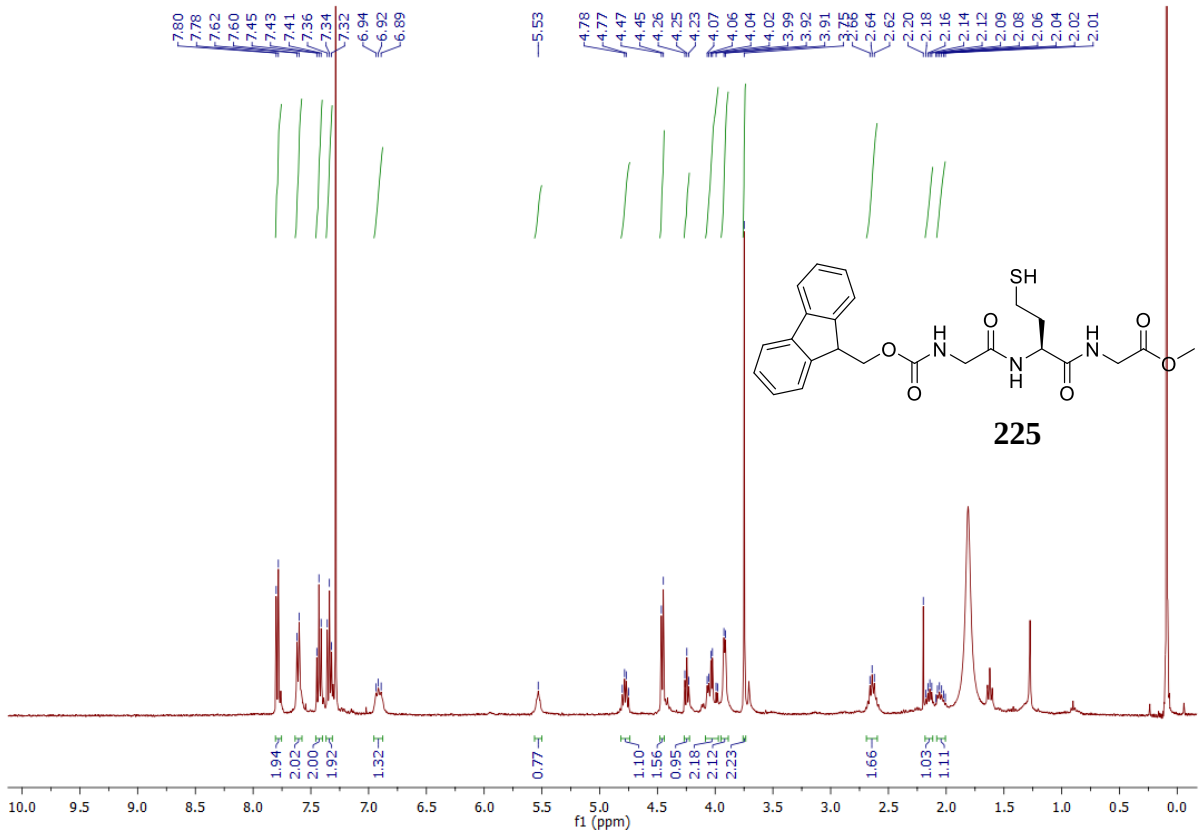
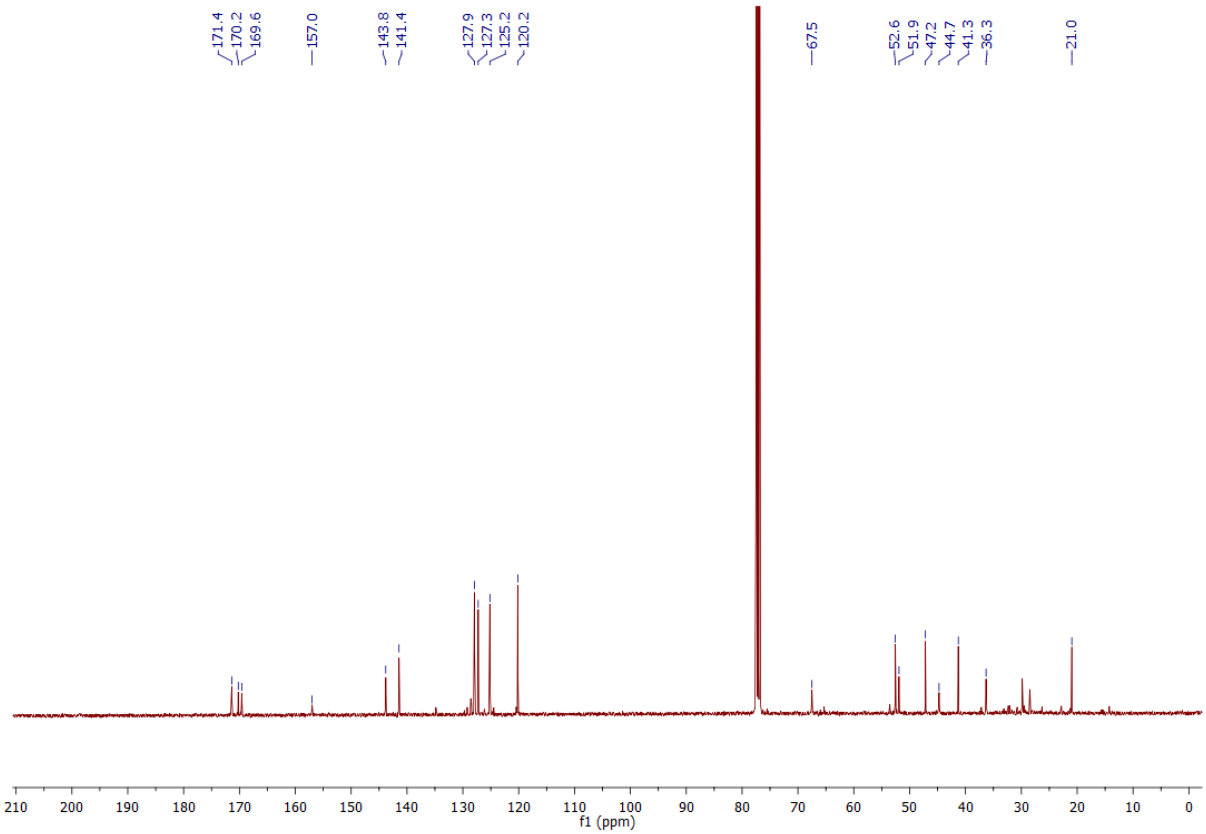
172.2
170.3
155.8
143.9
141.4
135.7
129.5
128.9
127.9
127.4
127.2
125.2
120.1
80.3
67.3
61.6
53.5
52.5
47.3
41.3
38.4
31.6
28.5
28.2
25.6
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0

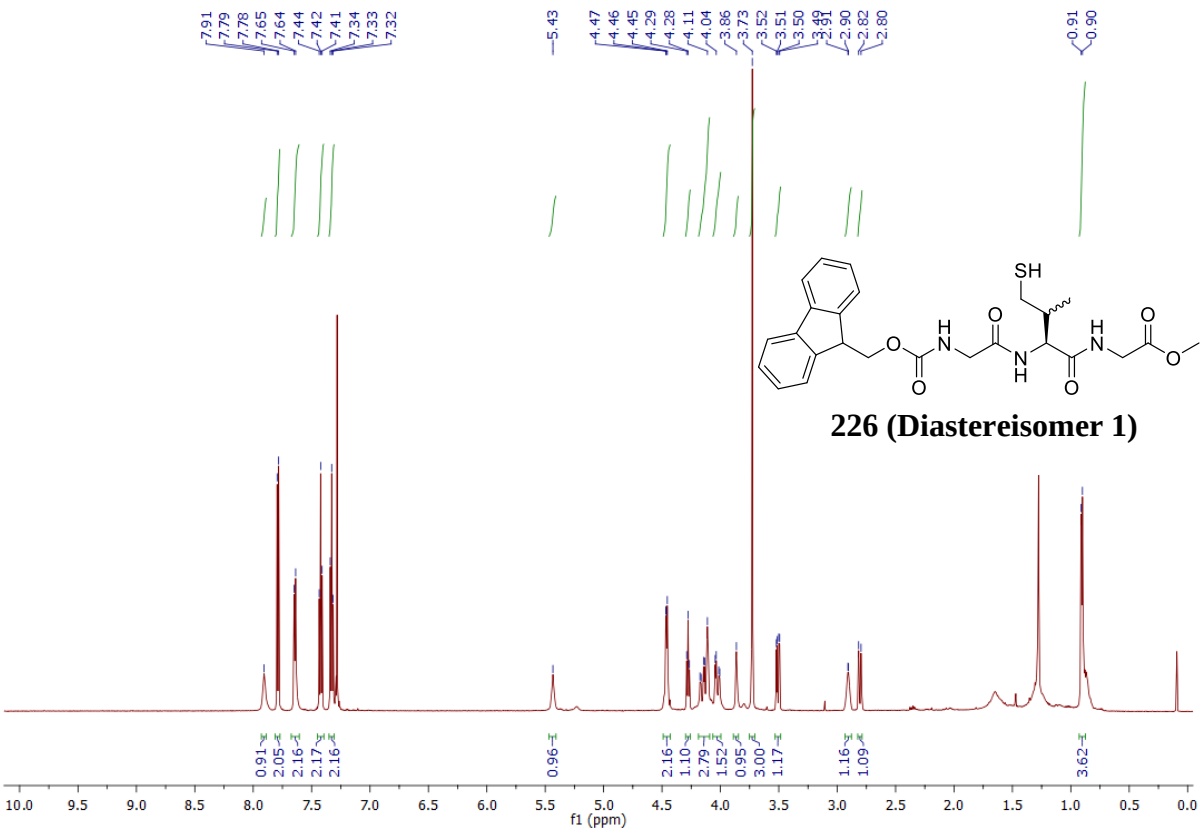


220

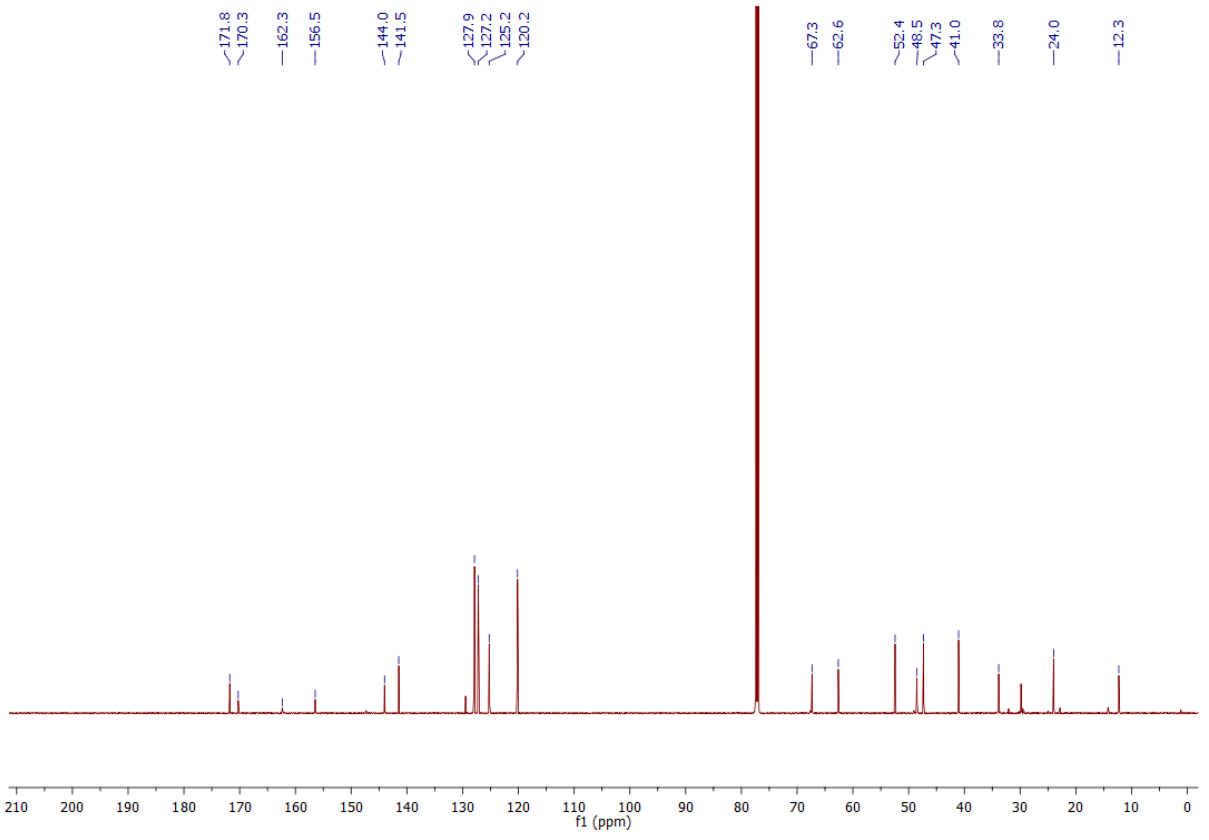


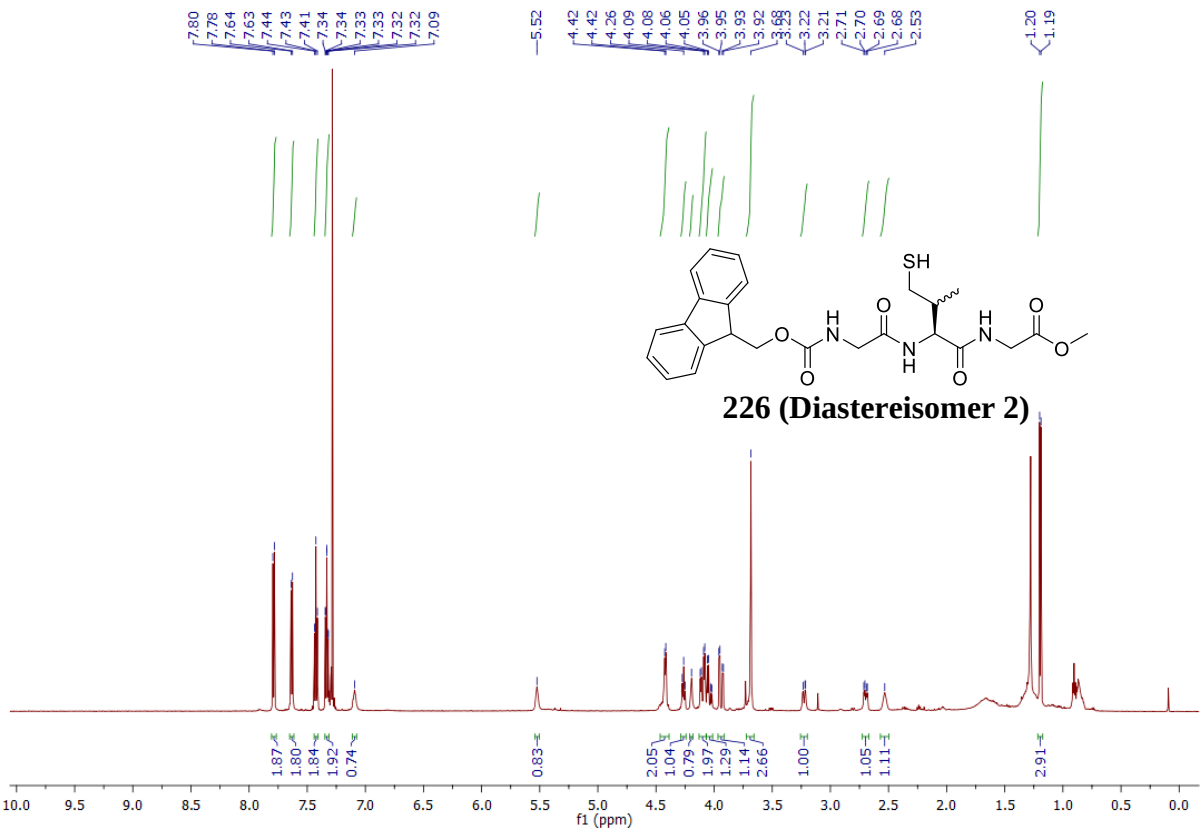

7.80
7.78
7.62
7.60
7.45
7.43
7.41
7.36
7.34
7.32
6.94
6.92
6.89
5.53
4.78
4.77
4.47
4.45
4.26
4.25
4.23
4.07
4.06
4.04
4.02
3.99
3.92
3.91
3.75
3.66
2.64
2.62
2.20
2.18
2.16
2.14
2.12
2.09
2.08
2.06
2.04
2.02
2.01
SH
H
N
O
O
H
N
O
H
N
O
O
O
225
1.94
2.02
2.00
1.92
1.32
0.77
1.10
1.56
0.95
2.18
2.12
2.23
1.66
1.03
1.11
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


171.4
170.2
169.6
157.0
143.8
141.4
127.9
127.3
125.2
120.2
67.5
52.6
51.9
47.2
44.7
41.3
36.3
21.0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)



226 (Diastereisomer 1)

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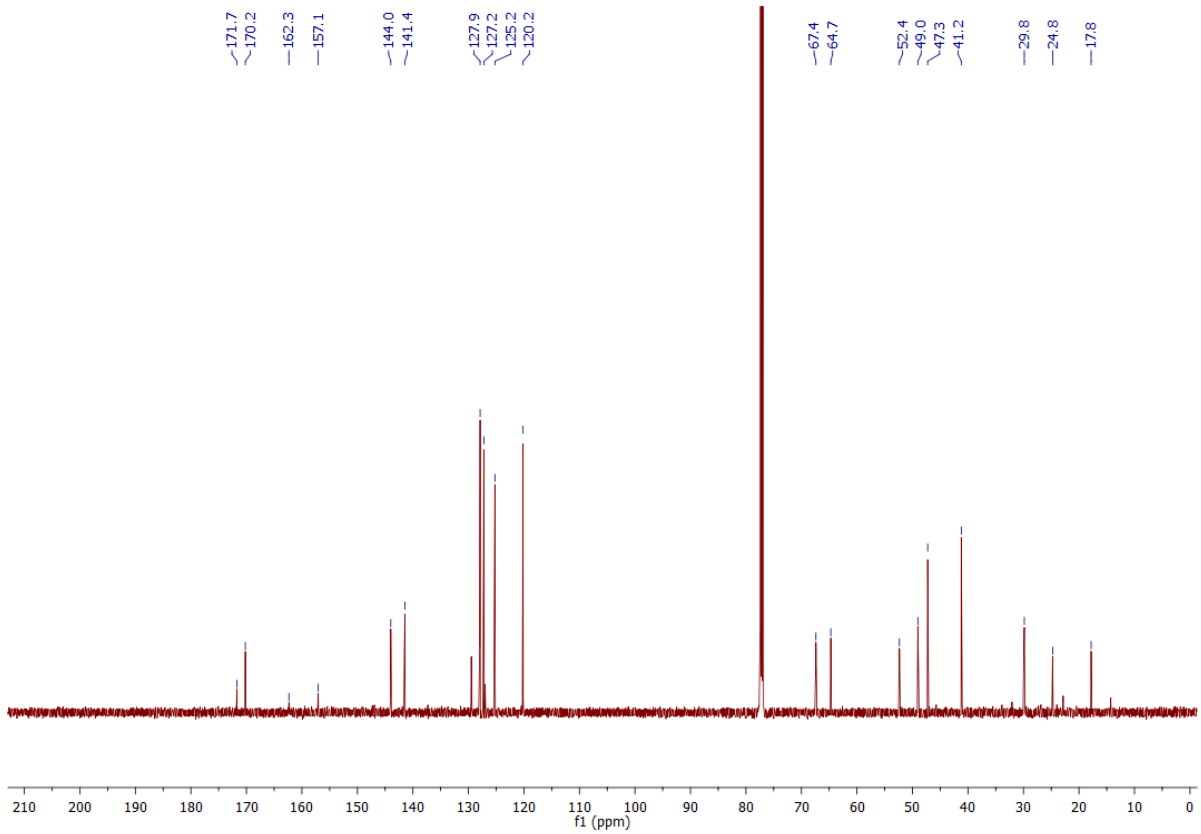
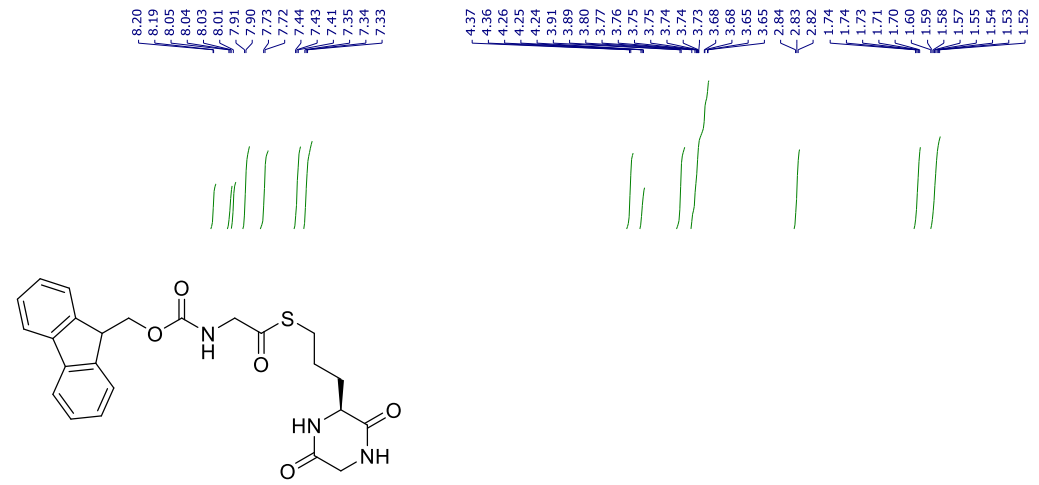
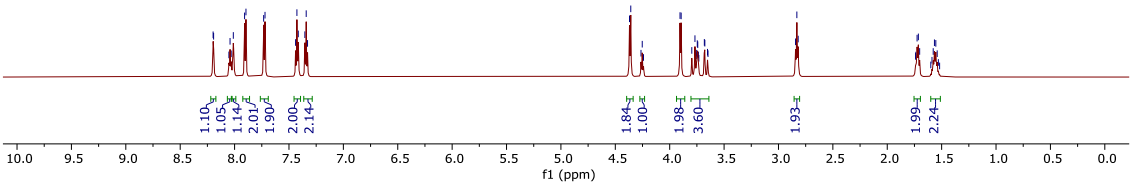
7.78
7.64
7.63
7.44
7.43
7.41
7.34
7.34
7.33
7.33
7.32
7.32
7.09
5.52
4.42
4.42
4.26
4.09
4.08
4.06
4.05
3.96
3.95
3.93
3.92
3.68
3.23
3.22
3.21
2.71
2.70
2.69
2.68
2.53
1.20
1.19
SH
O
H
N
O
N
H
O
H
N
O
O
O
O
226 (Diastereisomer 2)
1.87
1.80
1.84
1.92
0.74
0.83
2.05
1.04
0.79
1.97
1.29
1.14
2.66
1.00
1.05
1.11
2.91
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


171.7
170.2
162.3
157.1
144.0
141.4
127.9
127.2
125.2
120.2
67.4
64.7
52.4
49.0
47.3
41.2
29.8
24.8
17.8
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
f1 (ppm)


8.20
8.19
8.05
8.04
8.03
8.01
7.91
7.90
7.73
7.72
7.44
7.43
7.41
7.35
7.34
7.33
4.37
4.36
4.26
4.25
4.24
3.91
3.89
3.80
3.77
3.76
3.75
3.75
3.74
3.74
3.73
3.68
3.68
3.65
2.84
2.83
2.82
1.74
1.74
1.73
1.71
1.70
1.60
1.59
1.58
1.57
1.55
1.54
1.53
1.52

O
O
N
H
S
O
HN
O
NH
O

230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1.05
1.14
2.01
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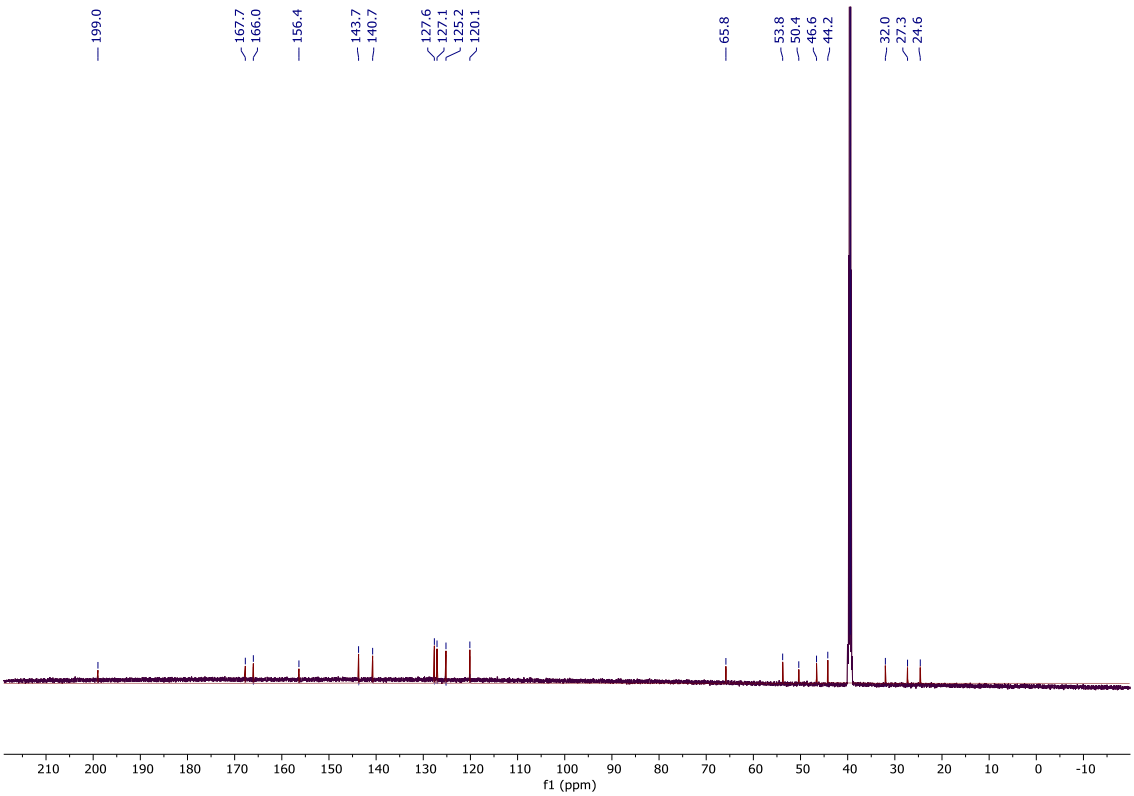
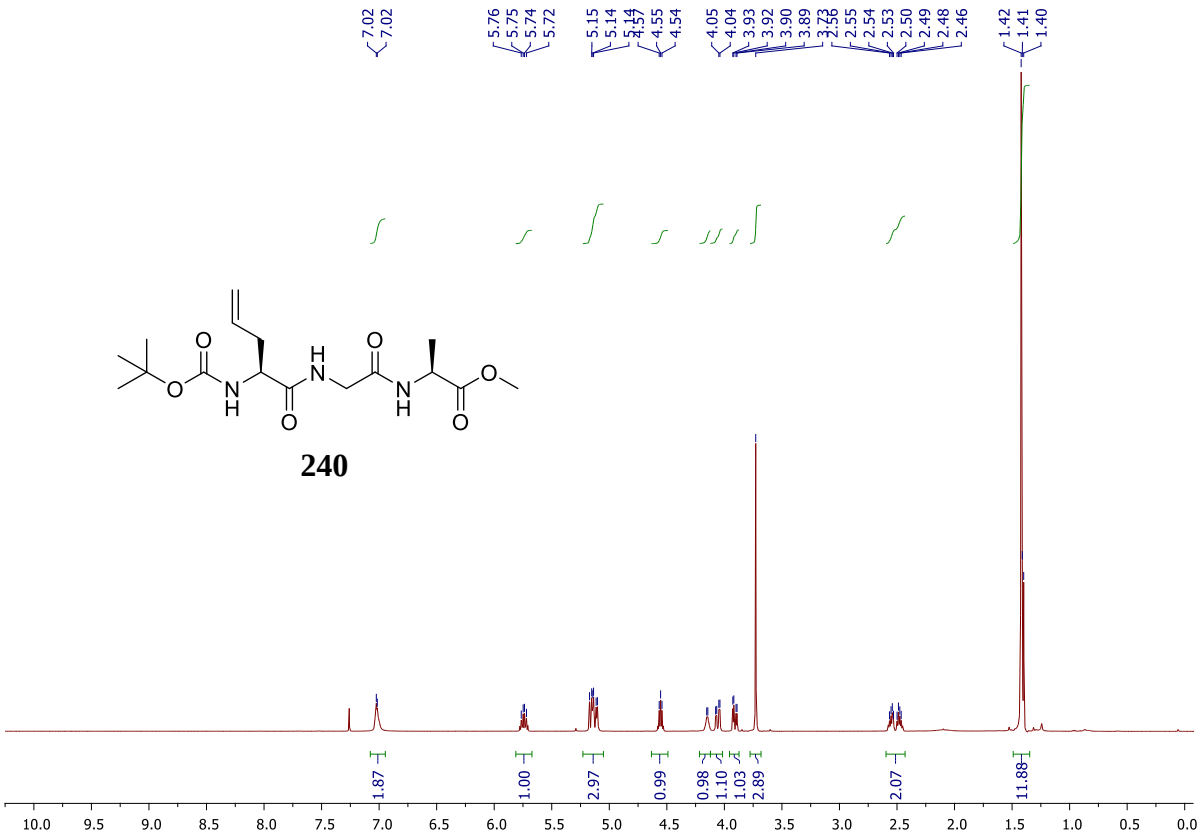
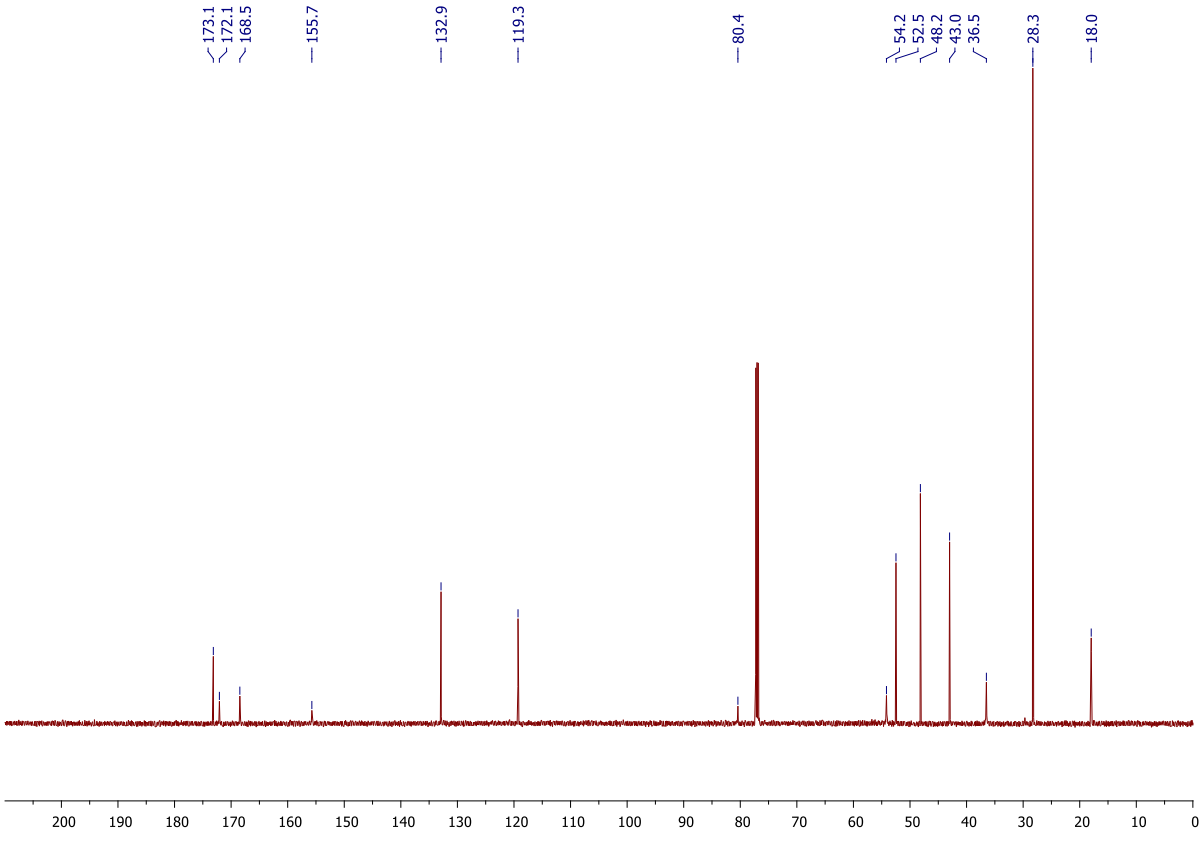
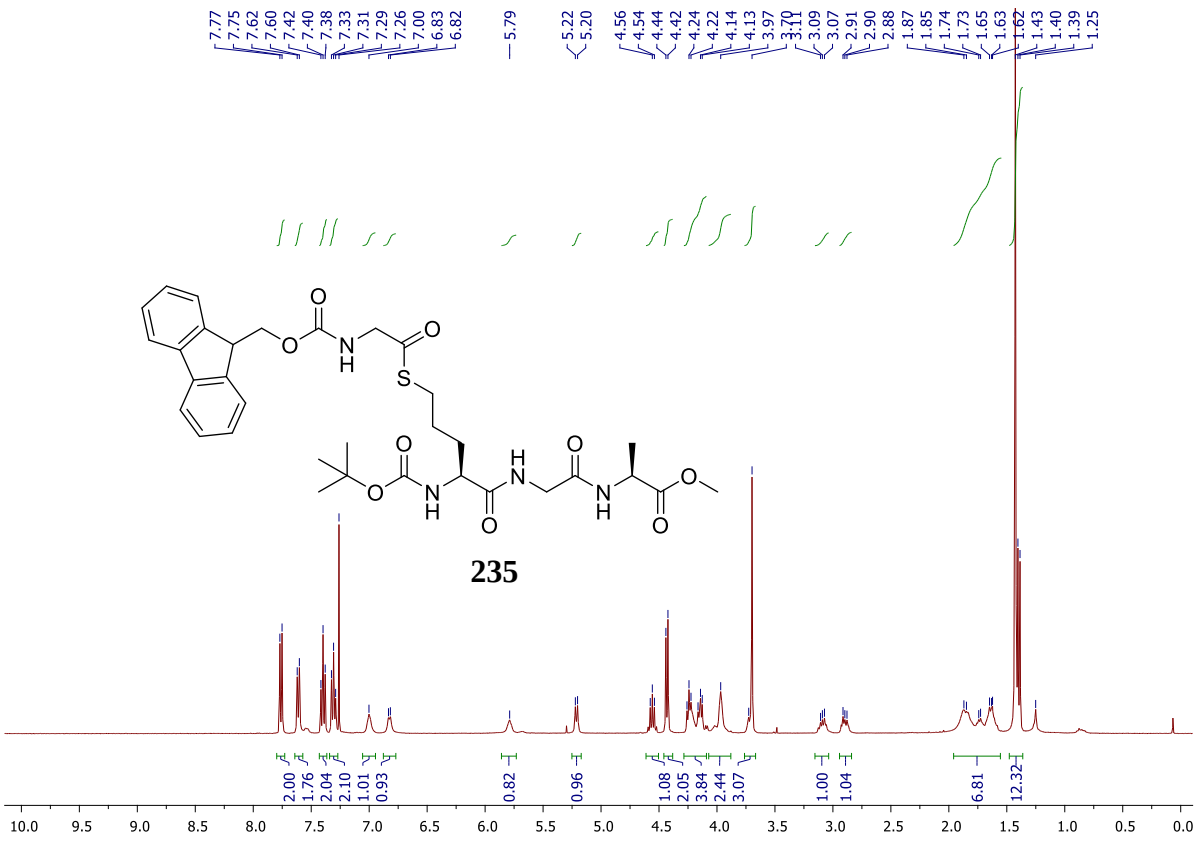
2.00
2.14
1.84
1.00
1.98
3.60
1.93
1.99
2.24
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
f1 (ppm)


199.0
167.7
166.0
156.4
143.7
140.7
127.6
127.1
125.2
120.1
65.8
53.8
50.4
46.6
44.2
32.0
27.3
24.6
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

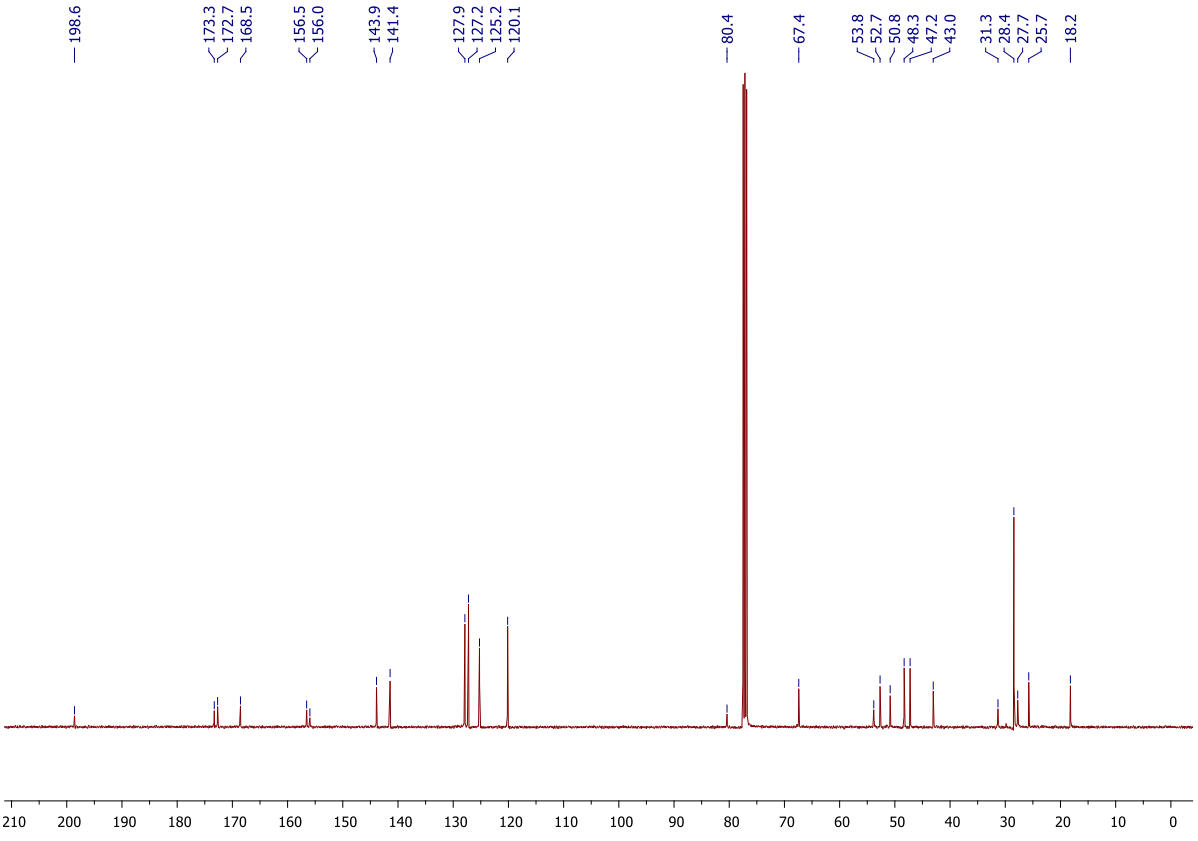

7.02
7.02
5.76
5.75
5.74
5.72
5.15
5.14
4.55
4.54
4.05
4.04
3.93
3.92
3.90
3.89
2.55
2.54
2.53
2.50
2.49
2.48
2.46
1.42
1.41
1.40
1.87
1.00
2.97
0.99
0.98
1.10
1.03
2.89
2.07
11.88
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

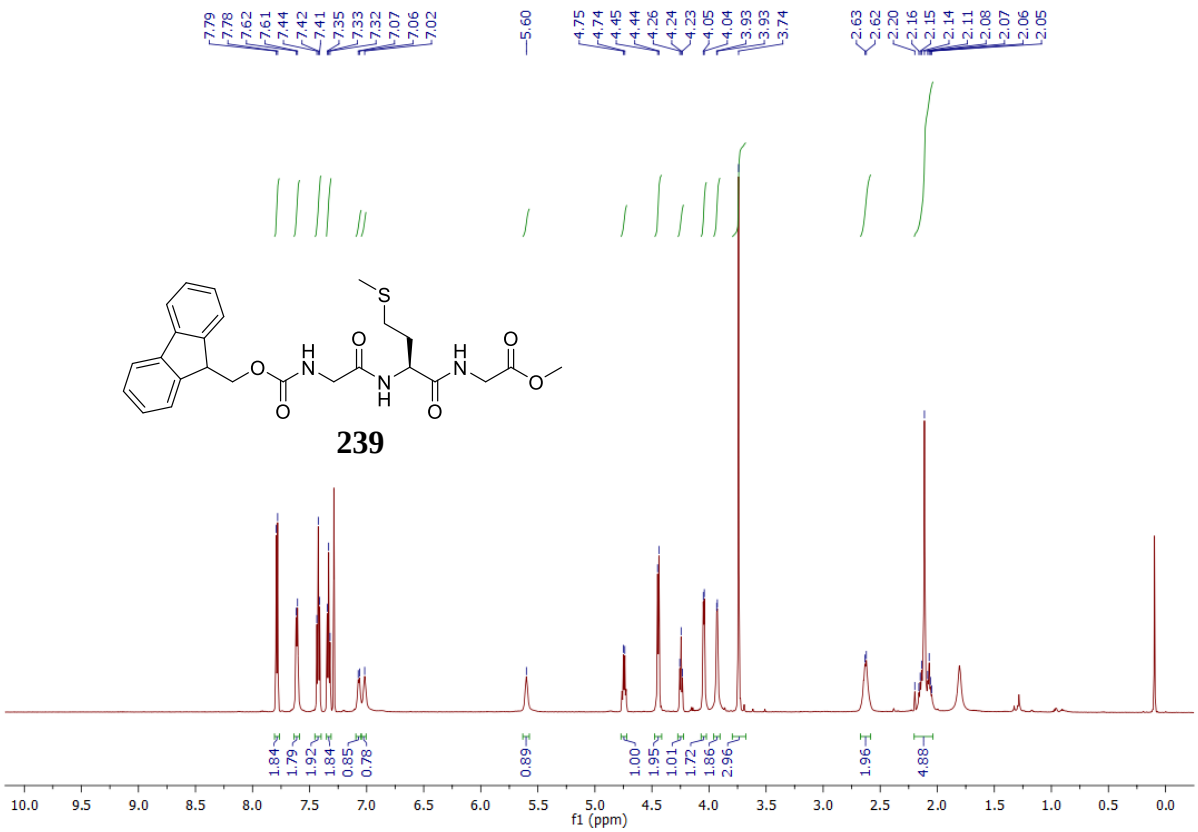
240

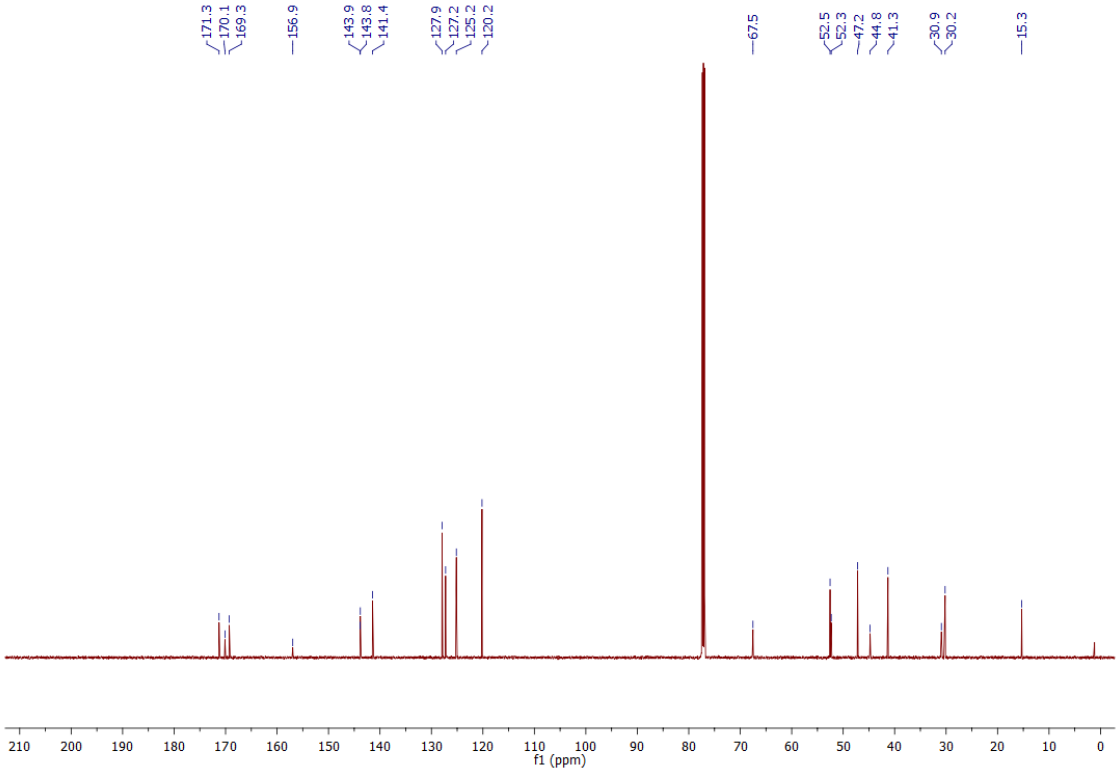

173.1
172.1
168.5
155.7
132.9
119.3
80.4
54.2
52.5
48.2
43.0
36.5
28.3
18.0
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0

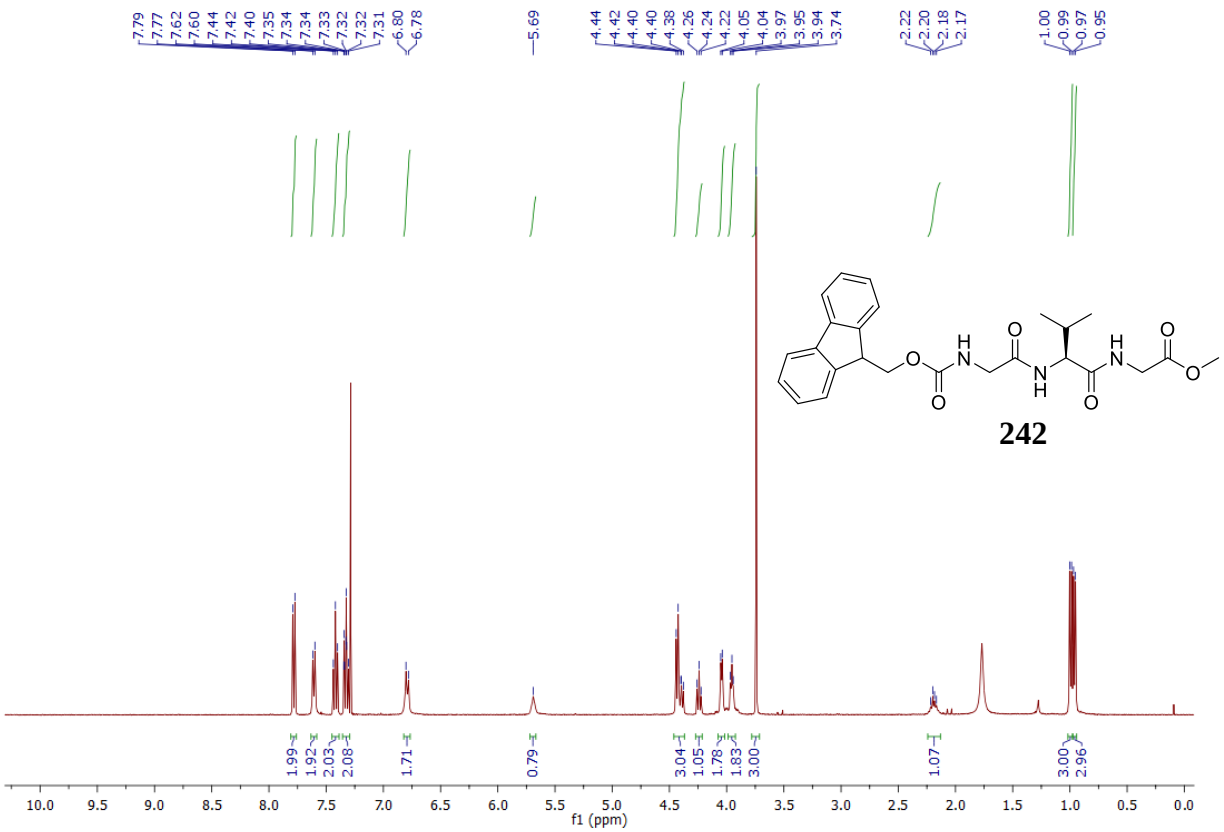

7.77
7.75
7.62
7.60
7.42
7.40
7.38
7.33
7.31
7.29
7.26
7.00
6.83
6.82
5.79
5.22
5.20
4.56
4.54
4.44
4.42
4.24
4.22
4.14
4.13
3.97
3.70
3.11
3.09
3.07
2.91
2.90
2.88
1.87
1.85
1.74
1.73
1.65
1.63
1.62
1.43
1.40
1.39
1.25
2.00
1.76
2.04
2.10
1.01
0.93
0.82
0.96
1.08
2.05
3.84
2.44
3.07
1.00
1.04
6.81
12.32
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0

235

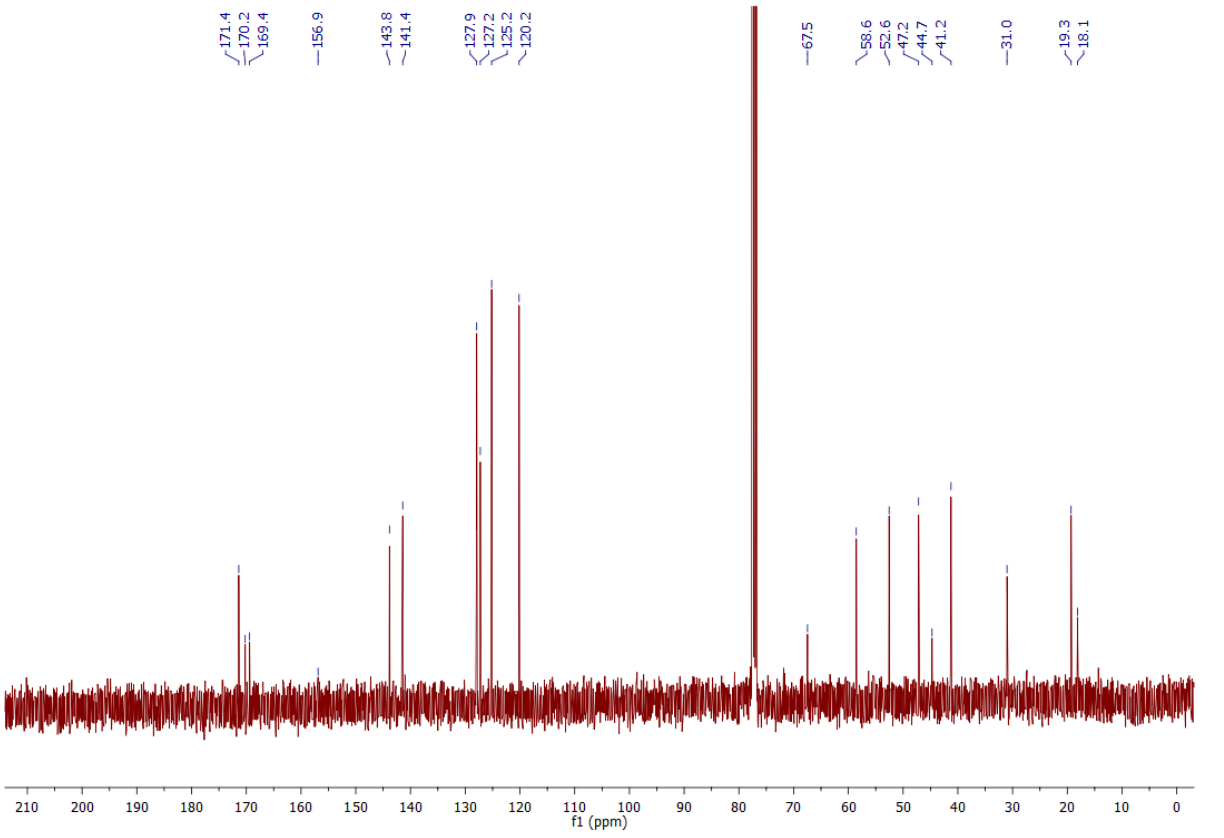

198.6
173.3
172.7
168.5
156.5
156.0
143.9
141.4
127.9
127.2
125.2
120.1
80.4
67.4
53.8
52.7
50.8
48.3
47.2
43.0
31.3
28.4
27.7
25.7
18.2
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0


7.79
7.78
7.62
7.61
7.44
7.42
7.41
7.35
7.33
7.32
7.07
7.06
7.02
5.60
4.75
4.74
4.45
4.44
4.26
4.24
4.23
4.05
4.04
3.93
3.93
3.74
2.63
2.62
2.20
2.16
2.15
2.14
2.11
2.08
2.07
2.06
2.05
S
O
H
N
O
O
H
N
O
O
O
239
1.84
1.79
1.92
1.84
0.85
0.78
0.89
1.00
1.95
1.01
1.72
1.86
2.96
1.96
4.88
f1 (ppm)


171.3
170.1
169.3
156.9
143.9
143.8
141.4
127.9
127.2
125.2
120.2
67.5
52.5
52.3
47.2
44.8
41.3
30.9
30.2
15.3
f1 (ppm)


7.79
7.77
7.62
7.60
7.44
7.42
7.40
7.35
7.34
7.34
7.33
7.32
7.32
7.31
6.80
6.78
5.69
4.44
4.42
4.40
4.40
4.38
4.26
4.24
4.22
4.05
4.04
3.97
3.95
3.94
3.74
2.22
2.20
2.18
2.17
1.00
0.99
0.97
0.95
1.99
1.92
2.03
2.08
1.71
0.79
3.04
1.05
1.78
1.83
3.00
1.07
3.00
2.96
f1 (ppm)

242


171.4
170.2
169.4
156.9
143.8
141.4
127.9
127.2
125.2
120.2
67.5
58.6
52.6
47.2
44.7
41.2
31.0
19.3
18.1
f1 (ppm)