Semi-synthetic Approaches to *N*-Glycosylation:

A Story of Thiol-Ene Methodology, and Interferon-γ

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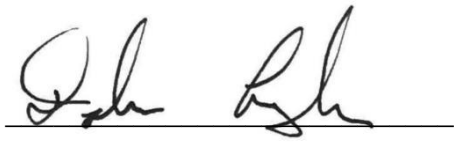

Dylan M. Lynch

Abstract

The following thesis, entitled "*Semi-synthetic Approaches to N-Glycosylation: A Story of Thiol-Ene Methodology, and Interferon-γ"* is composed of six chapters. The first chapter provides an overview of the key concepts that underpin the present work. An outline of post-translation modifications, with a focus on glycosylation, is discussed. The synthetic methods for accessing *N*-glycopeptides are thoroughly reviewed, with a highlight on radical methods that foreshadows the chapters to come. Bioconjugation and two important 'click' reactions are discussed, as is Interferon-γ – a hugely important protein which is central to this work. The thiol-ene reaction is examined in terms of mechanistic detail and versatility, as well as the behaviour of thiyl radicals in their addition to alkenes and alkynes. Finally, the research questions of each forthcoming chapter are posed, with clear aims set out.

The first part of this work, Chapter Two, investigates the use of carbohydrate moieties as molecular scaffolds for *N*-glycopeptide synthesis. A number of *S*-trityl thioesters are prepared, and reacted with two highly functionalised *N*-acetyl-D-glucosamines. The propensity of these systems to allow *S*-to-*N* acyl transfer is investigated, and a number of anomeric amides are prepared. The mechanistic detail of this reaction is thoroughly analysed, and overall an interesting synthetic methodology is depicted.

In Chapter Three, the semi-synthesis of Interferon-γ is described. The incorporation of a non-natural amino acid into the polypeptide backbone is investigated, as well as the bioconjugation of a large mammalian glycan *via* 'click' chemistry. This route is intended to provide access to glycosylated, refolded Interferon-γ in its natural dimeric form. An in-depth analysis of the purification steps, trials and tribulations associated with recombinant protein expression, and future work is discussed in this chapter.

Chapter Four introduces a thiol-ene mediated radical methodology for the cyclisation of unsaturated substrates. A serendipitously discovery in the course of supervising an undergraduate researcher is fully investigated in terms of scope, limitations, and utility. A number of unsaturated substrates are prepared, and a diverse array of thioacids are reacted with these materials. A blue-light photoreactor is constructed and investigated as an even milder form of radical initiation, with a detailed analysis of the various visible light photoinitiators that can be employed.

In Chapter Five, two collaborative projects are discussed, that the author is deeply grateful to have been part of. While these projects culminated in publications centering on the biological properties of these compounds, this brief chapter illustrates the synthetic protocols designed for their syntheses. The first of these projects involves the synthesis of a number of rhamnosides, which were later used by our immunology collaborators in studies regarding *Mycobacterium tuberculosis*. The second of these collaborations involves the synthesis of a highly functionalised cytotoxic naphthalimides drug, linked to a carbohydrate moiety *via* a 'self-immolative linker', which localises the drug release to tumour tissue.

Finally, Chapter Six described the experimental details employed in the course of this research work, and the characterisation of the compounds synthesised within this thesis. A brief appendix, Appendix A, is available at the very end of this thesis, with some select NMR spectra from Chapter Two that the reader may peruse.

Abbreviations

AA	amino acid
aaRS	aminoacyl-tRNA synthetase
Ac_2O	acetic anhydride
AIBN	azobisisobutyronitrile
Ala	alanine
AML	auxiliary mediated ligation
app	apparent
aq.	aqueous
Ar	aromatic
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BCG	Bacillus Calmette–Guérin
BDE	bond dissociation energy
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
bs	broad singlet
BSA	bovine serum albumin
C. jejuni	*Campylobacter jejuni*
cat.	catalytic
CDI	1,1'-carbonyldiimidazole
CO_2	carbon dioxide
conv.	conversion
CPET	coupled proton-electron transfer
CuAAC	copper-catalysed azide-alkyne cycloaddition
Cys	cysteine
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	*N,N'*-dicyclohexylcarbodiimide
dd	doublet of doublets

DHA	dehydroalanine
DIC	*N,N'*-diispropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMAP	4-(dimethylamino)pyridine
DMC	2-chloro-1,3-dimethylimidazolinium chloride
DMF	*N,N'*-dimethylformamide
DNA	deoxyribonucleic acid
DPAP	2,2-dimethoxy-2-acetophenone
E. coli	*Escherichia coli*
EDCI·HCl	*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride
ENGase	endo-β-*N*-acetylglucosaminidase
EPL	expressed protein ligation
eq.	equivalents
ER	endoplasmic reticulum
ESI	electrospray ionisation
EtOAc	ethyl acetate
Fm	9-fluorenylmethyl
FMO	frontier molecular orbital
Fmoc	9-fluorenylmethyloxycarbonyl
FPLC	fast protein liquid chromatography
g	gram
Gal	galactose
Gdm·Cl	guanidinium chloride
Glc	glucose
GlcA	glucuronic acid
GlcNAc	*N*-acetyl-D-glucosamine
Gln	glutamine
Glu	glutamic acid
Gly	glycine
GPI	glycosylphosphatidylinositol

GSH	glutathione
h	hour
HAT	hydrogen atom transfer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hex	hexane
His	histidine
HOBt	1-hydroxy-1*H*-benzotriazole
HOMO	highest occupied molecular orbital
HPLC	high performance liquid chromatography
HRMS	high resolution mass spectrometry
IEX	ion-exchange chromatography
IFNγ	Interferon-γ
Ile	isoleucine
Ino	inositol
IPTG	isopropylthio-β-galactoside
IR	infrared
J	coupling constant
K_2CO_3	potassium carbonate
KAHA	α-ketoacid-hydroxylamine
KCL	kinetically controlled ligation
LB	lysogeny broth/Luria broth
LED	light-emitting diode
Leu	leucine
LiOH	lithium hydroxide
LLO	lipid-linked oligosaccharide
LUMO	lowest unoccupied molecular orbital
Lys	lysine
M	mass ion or molarity (as relevant)
m	multiplet
m.p.	melting point

m/z	mass to charge ratio
MALDI	matrix assisted laser desorption ionisation
Man	mannose
MAP	4-methylacetophenone
Met	methionine
mg	milligram
MGATs	mannosyl glycoprotein *N*-acetylglucosaminytransferases
MHz	megahertz
min	minute
ml	millilitre
MOM	methoxymethyl ether
MOPS	3-(*N*-morpholino)propanesulfonic acid
Mpa	α-methylphenacyl
m-*p*a	methyl *para*-anisate
MsCl	methanesulfonyl chloride
Na_2S	sodium sulfide
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NAP	naphthalimide
NAT	*N*-acetyltransferase
NBS	*N*-bromosuccinimide
NCL	native chemical ligation
NCS	*N*-chlorosuccinimide
NEt_3	triethylamine
NGP	neighbouring group participation
nm	nanometre
NMM	4-methylmorpholine
NMR	nuclear magnetic resonance
nnAA	non-natural amino acid
NTA	nitriloacetic acid

OSTase	oligosaccharyl transferase
PAGE	polyacrylamide gel electrophoresis
PEG	poly(ethylene glycol)
*p*HBAD	*para*-hydroxybenzoic acid derivatives
Phe	phenylalanine
pI	isoelectric point
PPI	protein-protein interaction
ppm	parts per million
PrgY	propargylated tyrosine
Pro	proline
PTM	post-translational modification
pyr	pyridine
q	quartet
qC	quaternary carbon
Q-TOF	quadruple time-of-flight
RBR	Ramberg-Bäcklund reaction
R_f	retention factor
RPE	recombinant protein expression
rt	room temperature
s	singlet
SAL	sugar-assisted ligation
SDS	sodium dodecyl sulfate
SEC	size-exclusion chromatography
Ser	serine
SGP	sialylglycopeptide
Sia	sialic acid
SOMO	singly occupied molecular orbital
SPE	solid-phase extraction
SPPS	solid phase peptide synthesis
t	triplet

t-BuSH	*tert*-butyl mercaptan
TCEP	tris(2-carboxyethyl)phosphine
TEC	thiol-ene click
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TES	triethylsilane
TFA	trifluoroacetic acid
TFET	2,2,2-trifluoroethanethiol
THF	tetrahydrofuran
THPTA	tris-hydroxypropyltriazolylmethylamine
Thr	threonine
TLC	thin layer chromatography
Tmob	2,4,6-trimethoxybenzyl thiol
TMS	trimethylsilane
TNFα	tumour necrosis factor alpha
TOF	time of flight
Trp	tryptophan
Trt	trityl
TS	transition state
TYC	thiol-yne click
Tyr	tyrosine
Ub	ubiquitin
UDP	uridine diphosphate
UM	unmethylated
UV	ultraviolet
UV-vis	ultraviolet-visible
Val	valine
WT	wild-type
Xyl	xylose
μL	microlitre

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Finally, to my family. None of this would have happened without you at my back. A massive thank you to putting up with my sleep-deprived head coming through the door each evening – you three are the greatest.

I'd like to dedicate this thesis to Tessie Lynch. Fastest slipper in the west.

Publications

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Chapter One

Introduction

1.0 Introduction

The thiol-ene and thiol-yne reactions have enjoyed a privileged place among the synthetic methods available to the organic chemist, polymer chemist, and surface scientist. This 'click' reaction renders many bioconjugation methods accessible, and permits chemical transformations analogous to the post-translational modifications observed in eukaryotes. The attraction of 'click' reactions generally stems from their mild conditions, the regioselectivity of the process, and tolerance to aqueous conditions and diverse functional groups.

N-Glycosylation, a hugely important post-translational modification, generates heterogeneous 'glycoforms' of a number of mammalian proteins. While this non-templated process is somewhat understood, accessing *N*-glycopeptides displaying uniform glycans remains recalcitrant. This chapter will introduce post-translational modifications, and describe glycosylation techniques in detail. The thiol-ene and thiol-yne reactions will also be described, with a focus on their applicability to bioconjugation methodologies. Furthermore, methods of ligating peptides and glycopeptides are discussed, as is the incorporation of non-natural amino acids into proteins for further derivatisation.

1.1 Post-translational modification

The human genome has about 20,000 genes, yet due to the numerous steps in protein production (e.g. genomic recombination events and alternative transcript splicing), the human proteome comprises of between 250,000 and one million proteins.[1] Post-translational processing is where the vast majority of proteomic diversification occurs; Nature largely introduces different functionality through the post-translation modification (PTMs) of proteins. More than 200 PTMs have been discovered to date, and while some such as oxidation proceed non-enzymatically,[2] most of these modifications occur through tightly conserved enzymatic processes; it has been estimated that over 5% of the human genome is dedicated to the enzymes facilitating PTM of proteins.[3] The most common PTMs observed in eukaryotes are ubiquitination, glycosylation, phosphorylation, and acetylation, with glycosylation being of primary interest for this work. Glycosylation is the most abundant PTM found on secreted proteins, and on those which associate with extracellular membranes.[4]

PTMs are essential in maintaining the proper function of translated proteins, and are incredibly important in cell signalling and protein degradation.[5] Protein function is also tightly controlled by protein-protein interactions (PPIs), which are generally non-covalent.[6-11] As PTMs can modify the electronics and/or structural properties of the interaction sites in PPIs, it is likely that PTMs and PPIs are inextricably linked. Entries in protein databases support this hypothesis, as it has been shown that more than half of modification sites are related to the functional domains of their proteins, and have been shown to preferentially engage in PPIs.[12] This would suggest that PTMs play a central role in modulation and therein function of these interactions. The seven most common PTMs are shown below (Table 1.1).[13] A complete discussion of all known PTMs is beyond the scope and purpose of this thesis – instead, phosphorylation and acetylation are briefly discussed below, and glycosylation as a PTM is discussed later in this chapter. This thesis will focus primarily on *N*-linked glycosylation.

Table 1.1. The most common experimentally identified PTMs.

Frequency	Modification
58525	Phosphorylation
6775	Acetylation
5593	*N*-linked Glycosylation
2852	Amidation
1658	Hydroxylation
1545	Methylation
1189	*O*-linked Glycosylation

1.1.1 Phosphorylation

Protein phosphorylation is an important regulatory mechanism for many enzymes and receptor proteins, whose activation can be mediated *via* kinases (or indeed dephosphorylation *via* phosphatases). This process promotes hydrophilicity and therein facilitates conformational changes.[14] More than one third of phosphorylation events occur on the amino acids tyrosine (Tyr), threonine (Thr) or serine (Ser). Amongst these observed instances of *O*-phosphorylation, that of Ser comprises above 85% of cases.[15] *N*-Phosphorylation of histidine (His) or aspartate (Asp) has been infrequently observed, however this form of PTM is less stable.[16] Protein phosphorylation is generally facilitated

by protein kinases, which are themselves activated by phosphorylation. This activation leads to a cascade of biochemical events leading to the addition of phosphate to amino acids (AAs).[14] Studies show that almost one-third of human proteins may be transformed by the activity of kinases, and kinases are known to be heavily involved in signal transduction, as well as other mammalian pathways.[16]

1.1.2 Acetylation

As a PTM, acetylation exerts manifold effects on the metabolic level and beyond. Originating from acetyl coenzyme A (Acetyl CoA), an acetate moiety is post-translationally conjugated to the *N*-terminus of amino acids, or indeed the ε-amino side chain of lysine (Lys).[17] Protein acetylation usually manifests in one of these two forms, which together comprise the 'acetylome'. It is estimated that between 80-90% of human polypeptides are acetylated co-translationally,[18-20] but many are also transformed post-translationally. *N*-terminal acetylation is complex, and is catalyzed by a family of *N*-acetyltransferases (NATs).[21] While malfunctioning acetylation machinery can lead to cancer and severe neurodegenerative diseases, our understanding of the acetylome and the physiological consequences of this PTM remains limited.[17]

1.2 Glycosylation

1.2.1 Types of glycosylation

Glycans (that is, large polysaccharides) are reported to covalently link to either proteins or lipids, forming glycoproteins or glycolipids, respectively. Utilising the 13 different monosaccharides and eight varying AA linkers that nature has in its arsenal for this PTM, protein glycosylation can yield almost 40 different protein-carbohydrate linkages.[22] Protein glycosylation is one of the most important post-translational modifications - over half of all human proteins are estimated to be glycosylated,[4, 23] and compared to other post-translational modifications this conjugation provides the most proteomic diversity. Glycosylation is essential for many key biological processes[24-27] and is involved in the stabilisation of tertiary protein structure, aiding subsequent folding and transport, and protection of proteins from proteolysis.[26, 28-29] There are five distinct classes of glycosylation: phosphoglycosylation, *C*-glycosylation, glypiation, *O*-glycosylation and

N-glycosylation. The latter three of these are the most common, and so both glypiation and *O*-linked cases will be briefly discussed, before turning our attention to *N*-glycosylation (Figure 1.1.).

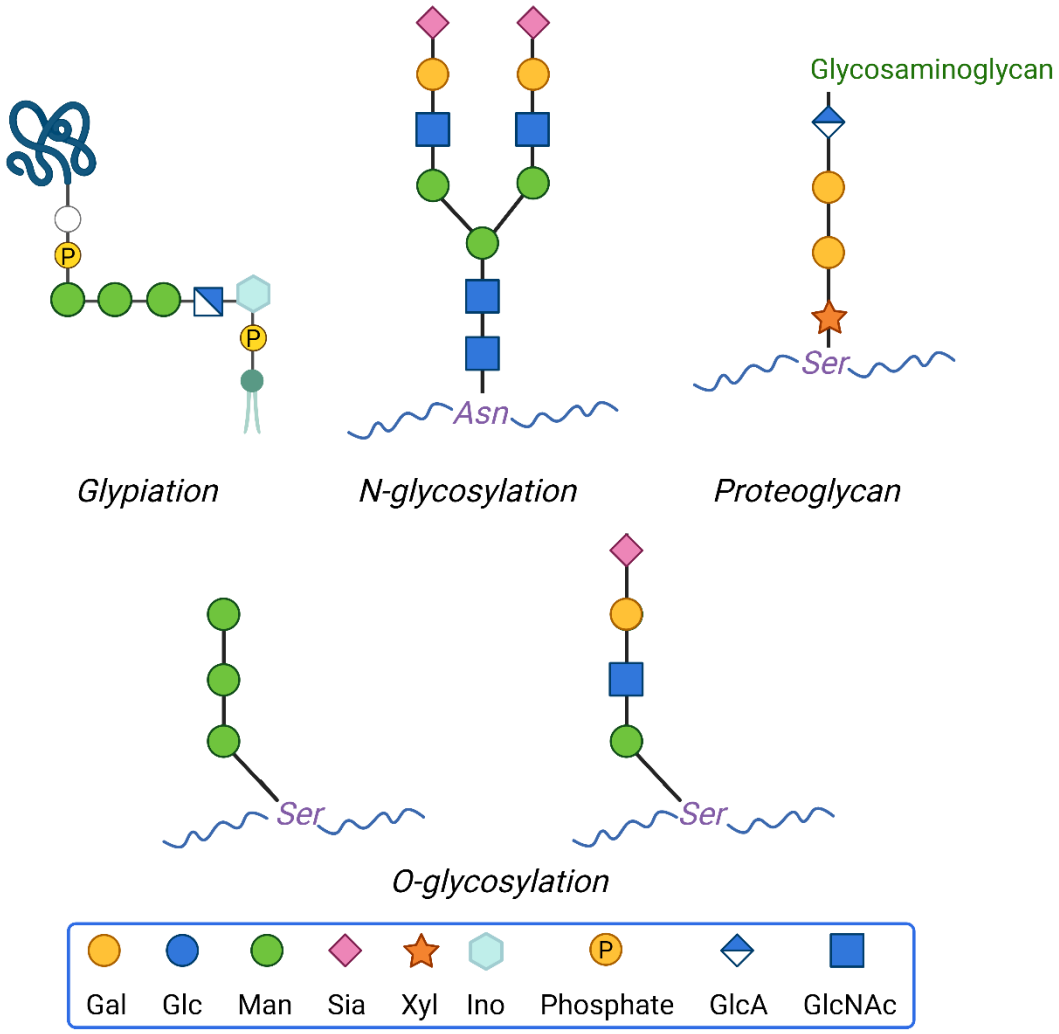


Figure 1.1. Representative structures of the major types of protein glycosylation.[30]

1.2.2 Phosphoglycosylation

As implied by the name, phosphoglycosylation involves the conjugation of a glycan and a phospholipid, a PTM which is normally limited to parasites and slime moulds. Phosphoglycosylation is characterised by the conjugation of glycans to Ser or Thr residues through phosphodiester bonds.[31] The first observed case of this rare type of glycosylation arose in the 1980s, with the modification of an endopeptidase in slime moulds. The GlcNAc-1-PO_4 motif was found linked to Ser.[32] The identification of these modifications was facilitated by the recovery of GlcNAc-1-PO_4 following base hydrolysis, and *O*-phosphorylserine after acid hydrolysis. The original findings were

reinforced more than 15 years later by Mehta *et al.*, who also employed mass spectrometry (MS) to confirm that only GlcNAc residues, and no larger groups, were linked to the polypeptide *via* phosphodiesters.[33] Indeed this is sometimes the most abundant PTM in some parasite species (*e.g. Leishmania*),[34-35] and are important in promoting parasite aggression[36] and protection against host immune system.[37] Although the glycosylation pathway and respective enzymes differ depending on species, this form of PTM proceeds with the transfer of a pre-formed phosphoglycan from a membrane-bound molecule (similar to *N*-glycosylation) *via* the action of a phosphoglycosyltransferase enzyme.[31]

1.2.3 Glypiation

Glypiation involves covalent attachment of a glycosylphosphatidylinositol (GPI) anchor to newly synthesised proteins. In this linkage, mannose (Man) is linked to the *C*-terminus of the protein through a phosphoethanolamine linker. The phospholipid tail of the bound GPI core associates with lipophilic sites and in doing so localizes the glypiated protein to the cell membrane. This PTM is widely observed in eukaryotic cell surface glycoproteins.[4, 38-40] The biosynthesis is believed to proceed *via* the action of a transamidase situated in the endoplasmic reticulum (ER). A preassembled GPI anchor is transferred to the *C*-terminus of a newly cleaved peptide, and covalently linked to the carboxyl terminus *via* the ethanolamine moiety of the glycolipid.[41-42] The conjugation occurs on the luminal side of the ER, while GPI anchor assembly occurs entirely on the cytoplasmic side.[4]

1.2.3 *O*-Glycosylation

In *O*-glycosylation, the range of monosaccharides used is more diverse, and these linkages occur through the side chain oxygen of hydroxyl amino acids such as Ser and Thr. There are five main carbohydrate residues observed in this PTM: *O*-*N*-acetylgalactosamine (*O*-GalNAc), *O*-fucose (*O*-Fuc), *O*-glucose (*O*-Glc), *O*-mannose (*O*-Man) and *O*-*N*-acetylglucosamine (*O*-GlcNAc).[4] There is no conserved sequence of AA sites where *O*-glycosylation occurs[43] (unlike for *N*-glycosylation, as detailed in the section 1.2.4); however, the identity of the carbohydrate and stereochemistry of the glycosidic bond between sugar and protein is significant in the

function of the modified protein. For example, β-linked *O*-GlcNAc modification usually modifies nuclear and cytoskeletal proteins[4, 44] and in contrast to other glycosylation types, the sugar remains as a monosaccharide and is not elaborated to form a further branched glycan.[45] In contrast, *O*-Man and *O*-GalNAc residues (which are both α-linked and display varying localisation morphology)[46-47] can be elongated to form branched, complex structures (Figure 1.2).[22, 48-49]

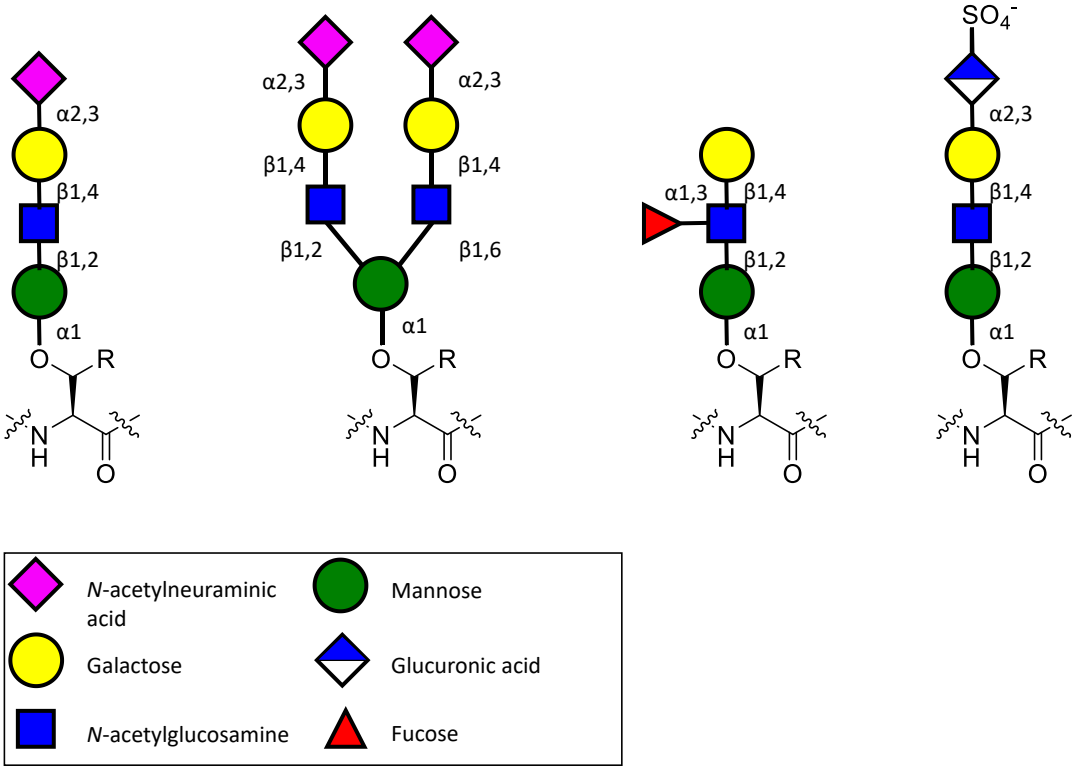
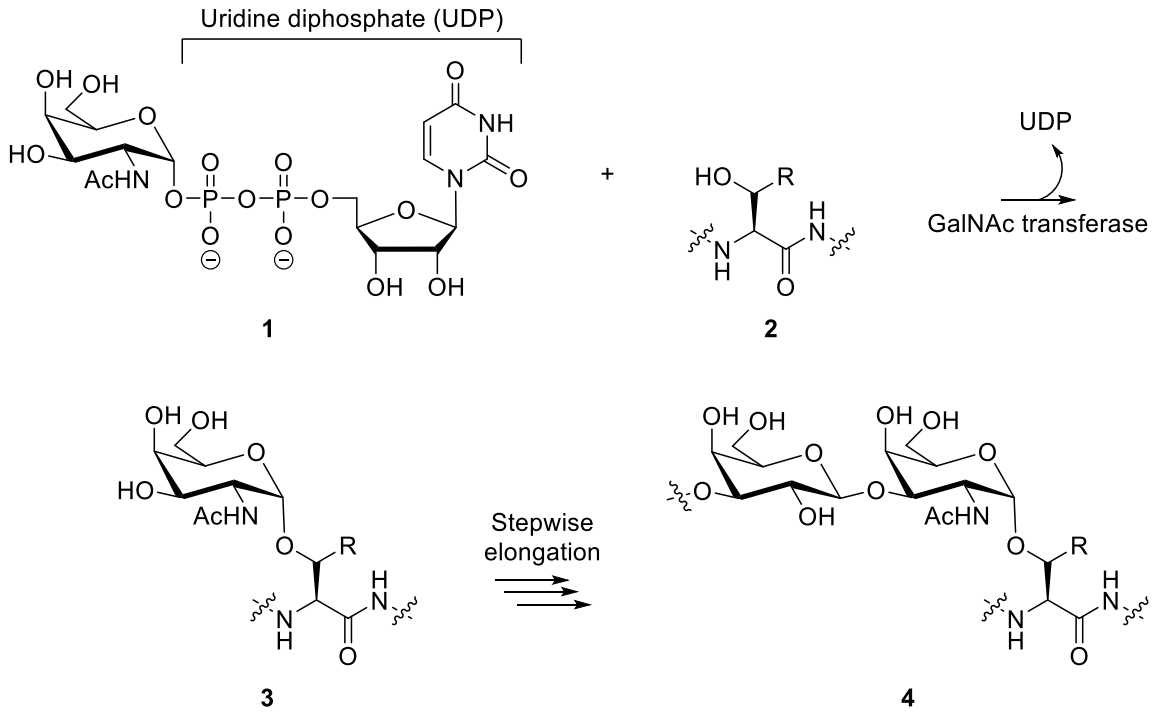


Figure 1.2. Cartoon representation of *O*-mannosyl glycan structures. The core structure Galβ1-4GlcNAcβ1-2Man-Ser/Thr is common in all mammalian *O*-mannosyl glycans. Note that where R = H the glycan is Ser linked, and R = CH_3 indicates Thr linked.

1.2.3.1 Biosynthesis of *O*-glycoproteins

O-Glycosylation occurs post-translationally and begins in the Golgi complex. Individual carbohydrate moieties are added sequentially to a Ser or Thr unit *via* glycosyltransferase enzymes. The resultant *O*-glycoproteins are significantly less complex than *N*-glycoproteins (see section 1.2.4).[50] The enzymes involved in orchestrating this PTM add sugar residues incrementally, and each nascent glycosidic bond is the product of a single enzyme from this stepwise elongation. These glycosyltransferases catalyse the transfer of the carbohydrate units from a suitable glycosyl donor onto a suitable acceptor (e.g. peptide, glycan, or lipid). In this case, the glycosyl donor is often a sugar-nucleotide

conjugate, such as UDP-GalNAc or UDP-Gal.[51] In Scheme 1.1 below, the glycosylation of a Ser/Thr residue with *N*-acetyl-D-galactosamine *via* GalNAc transferase is shown. Uridine diphosphate (UDP)-GalNAc **1** acts as the glycosyl donor for this transformation. Further elongation with related enzymes is then possible, which results in an amino acid adorned with an elaborate carbohydrate array **4**.



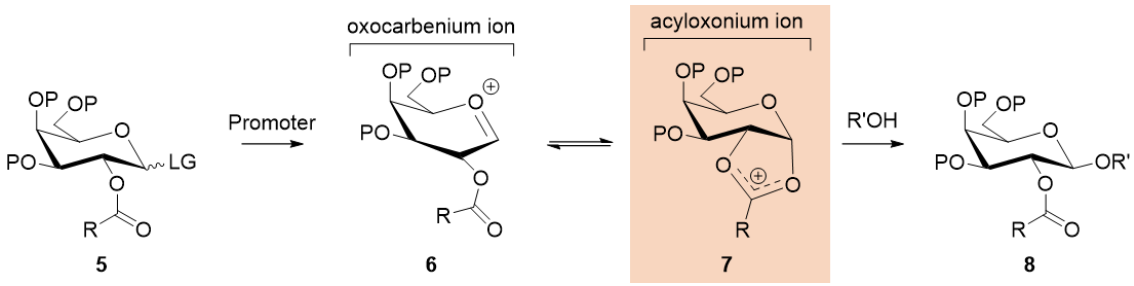
Scheme 1.1. The action of glycosyltransferases results in the glycosylation of Ser and Thr residues. For Ser, R = H, and for Thr, R = CH_3.

1.2.3.2 Synthetic Approaches to *O*-Glycosylation

In the simplest terms synthetic approaches to *O*-glycosides involve a glycosyl donor, characterised by a suitable leaving group at the anomeric centre, and an acceptor molecule, which will be conjugated to the carbohydrate through a hydroxyl group. Hence, both 1,2-*cis* and 1,2-*trans* glycosides are possible, and these are termed α-linked and β-linked respectively.

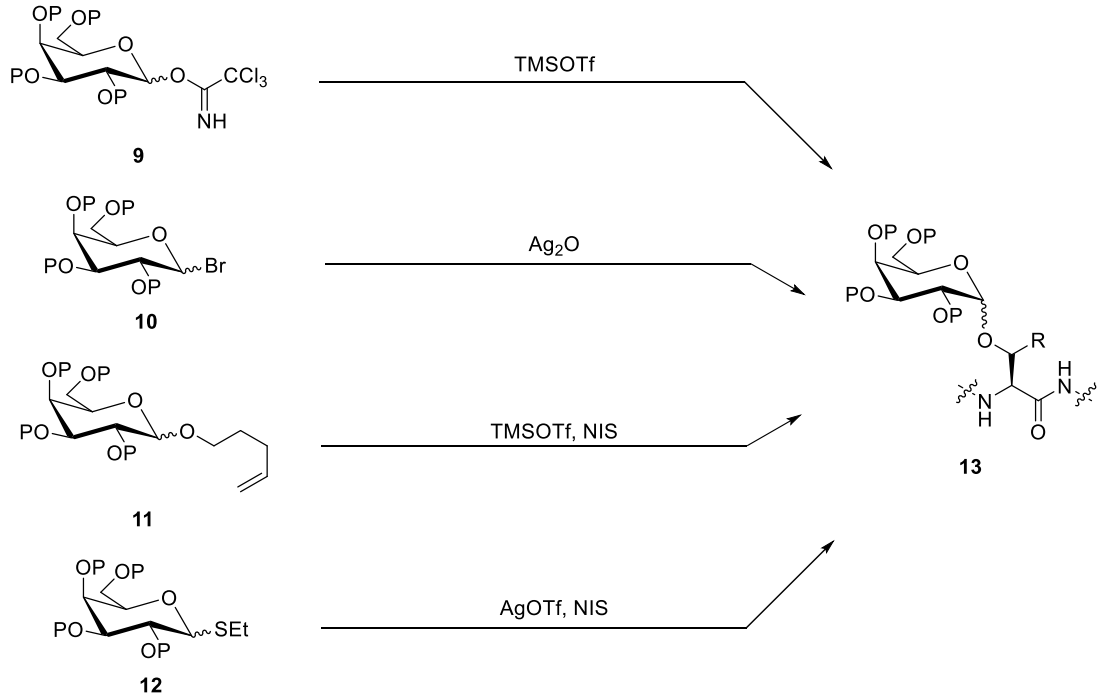
A level of control over the stereochemistry at the anomeric centre following glycosylation can be exerted by the protecting group at the C-2 alcohol. In a phenomenon known as neighbouring group participation (NGP) or 'anchimeric assistance', suitable moieties such as acetate esters can trap the nascent oxocarbenium ion from the loss of a

leaving group at the anomeric centre, and form a *cis*-acyloxonium ion **7**.[52] As there is only one face available for attack, these heterocyclic structures can then be nucleophilically opened by the acceptor alcohol to selectively furnish the 1,2-*trans* or β-linked glycoside **8**. If the α-linked glycoside is desired, this is significantly more challenging to synthesise as one requires a non-participating group on the C-2 alcohol and must instead rely on standard S_N2 chemistry if available (Scheme 1.2).[53]



Scheme 1.2. Promoter assists the loss of the anomeric leaving group. Oxocarbenium ion is stabilised as the acyloxonium ion (orange), with which the acceptor can react. P represents alcohol protecting groups.

A number of glycosyl donors are described in the literature, permitting the glycosylation of Ser and Thr residues with varying functionality on both the amino acid and the sugar itself. The range of glycosyl donors includes, but is not limited to, trichloroacetimidates, thioglycosides, *n*-pentenyl glycosides and anomeric halides (Scheme 1.3).[52, 54-55]



Scheme 1.3. Select *O*-glycosylation methods. Control over the anomeric stereochemistry of these leaving groups can be exerted, particularly for trichloroacetimidates.[56-57] NIS: *N*-iodosuccinimide. If Ser, R = H. If Thr, R = CH_3.

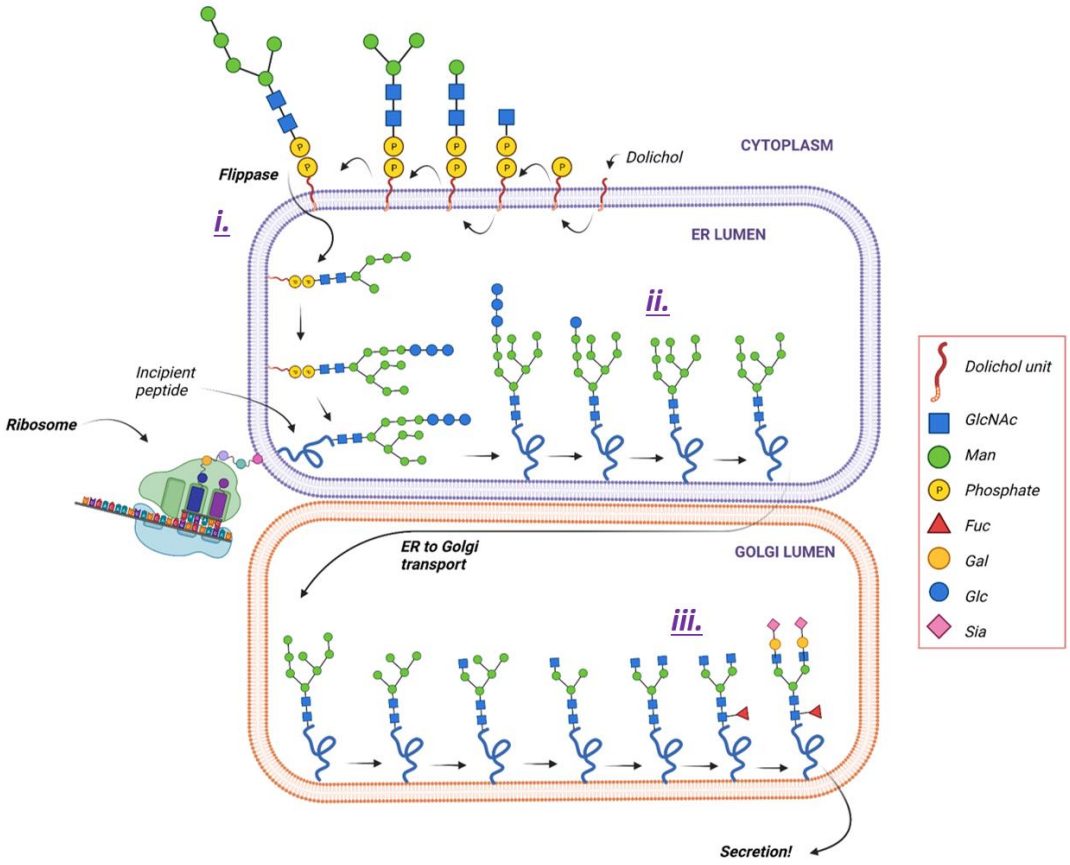
1.2.4 *N*-Glycosylation

N-Glycosylation is the most pervasive glycosylation type observed in nature, with 90% of all glycoproteins estimated to possess this PTM. *N*-glycans usually present more composite structures than *O*- or *C*-glycosylation, and this is reflected in the biosynthesis of *N*-glycosides.[58-59] *N*-Glycopeptides are conventionally bound to the amide of an asparagine (Asn) side chain through a β-GlcNAc bridgehead. Furthermore, this asparagine is typically present in the consensus sequence Asn-Xaa-Ser/Thr, where Xaa is any AA that is not proline (Pro).[30]

1.2.4.1 Biosynthesis of *N*-glycoproteins

All known *N*-linked glycans share a common pentasaccharide core: $GlcNAc_2Man_3$.[30] All *N*-linked glycans are derived from a single precursor, $Glc_3Man_9GlcNAc_2$, which is referred to as the lipid-linked oligosaccharide (LLO). This LLO is linked to a polyisoprenoid alcohol, a dolichol, through a pyrophosphate linkage. Initially, two UDP-GlcNAc units are conjugated to the dolichol residue *via* a pyrophosphate linkage. This disaccharide is then elongated by glycosyltransferases and five mannose residues which are activated as guanidine phosphate conjugates (GDP-Man). This ligation establishes the biantennary nature of the nascent *N*-glycan, and furnishes the dolichol-$GlcNAc_2$-Man_5 intermediate.[60]

The LLO is synthesised on the cytosolic face of the ER membrane, and is then flipped to the luminal side of the ER **(**Scheme 1.4, ***i***). This process is poorly understood, but is theorised to be catalysed by an enzyme known as flippase.[61] As the Asn-Xaa-Ser/Thr consensus sequence enters the ER lumen, *en bloc* conjugation of the 14-mer glycan *via* an oligosaccharyltransferase (OSTase) results in the modification of the nascent protein.[62] Trimming of glucose (Glc) residues from the glycan terminus by glucosidase I and II is believed to act as a 'quality control' step to monitor protein folding (Scheme 1.4, ***ii***). Removal of the final Glc residue by endoplasmic enzymes, and transport from the ER to the *cis*-Golgi results places the *N*-glycosylated peptide in the Golgi lumen, where it can be further diversified and elongated (Scheme 1.4, ***iii***).[59]



Scheme 1.4. Biosynthesis of *N*-glycopeptides[30] ***i.*** The action of flippase brings the nascent glycan to the luminal side of the ER. ***ii.*** Glc residues are trimmed prior to translocation to the Golgi. ***iii.*** The *N*-glycopeptide is trimmed and elaborated by glycosyltransferases before secretion.

Once glycosylation of the protein has occurred this chain is diversified with several other sugars. These elongated carbohydrates then fall into three categories: complex, hybrid and high-mannose *N*-glycans.[63] Complex *N*-glycans are termed such because they can possess any number of other carbohydrate residues, including multiple additional GlcNAc units. Hybrid glycans usually present with one arm composed of mannose, and with GlcNAc initiating a complex-type sequence on the other arm of the array. Finally, high-mannose glycans present as a disaccharide of two GlcNAc units, and multiple Man residues (Figure 1.3, *vide infra*).

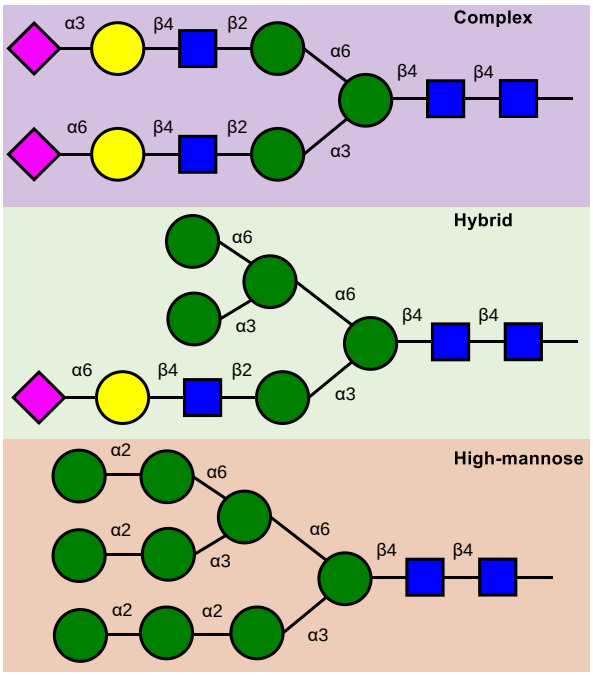


Figure 1.3. The three primary morphologies observed in *N*-glycan elaboration.

This diversification process is an enzymatically driven but non-templated process (***iii***, Figure 1.3, *vide supra*). The core pentasaccharide is modified primarily by mannosyl-glycoprotein *N*-acetylglucosaminytransferases (MGATs).[64] These enzymes lay the groundwork for transforming the core into multi-antennary oligosaccharides with the potential for distinct patterning along each branch, and a diverse global architecture (Figure 1.4).[65] Galactosyltransferases and glycosyltransferases derivatise the structure further, yielding mature *N*-glycans presenting unique epitopes. Mannosidase I and GlcNAc transferase are found in the *cis*- and medial Golgi respectively, while the *trans*-Golgi hosts galactosyltransferase and sialyltransferase.[59, 66-69]

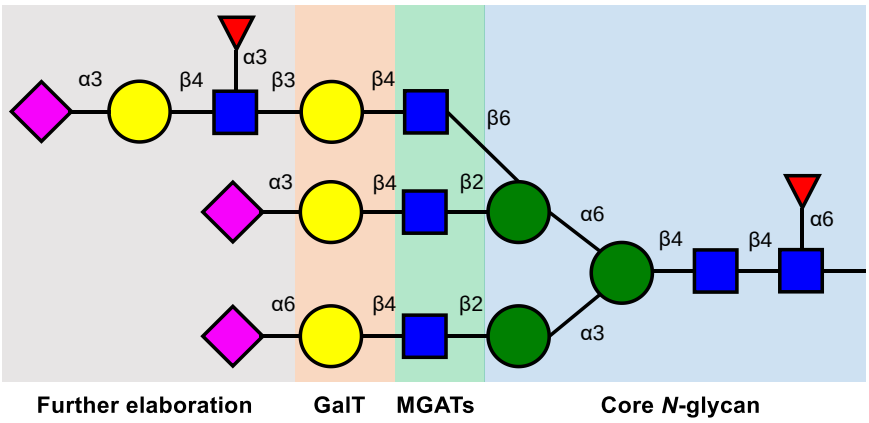


Figure 1.4. Enzyme classes involved in diversifying the architecture of *N*-glycans.

1.3 Accessing *N*-linked glycopeptides

1.3.1 Recombinant approaches

Since the *N*-glycosylation of proteins is non-templated and not controlled within the translation process itself, natural "glycoforms" are heterogeneous in nature, rendering the study of a carbohydrates effect on protein dynamics extremely difficult. Recombinant methods tend to provide non-mammalian glycoforms (e.g. if yeast or insect models are used) which can cause severe immunogenicity issues,[70-71] whereas more established models such as *Escherichia coli* (*E. coli*) lack glycosylation machinery,[72-73] and so the recombinant protein is unglycosylated. This severely affects the half-life of potential druggable proteins, including monoclonal antibodies.[74] A large amount of research into developing glycosylation methods for therapeutic glycopeptides has been carried out, however a full discussion of this is beyond the scope of this thesis – readers are directed to some of the many reviews available in the literature.[75-78]

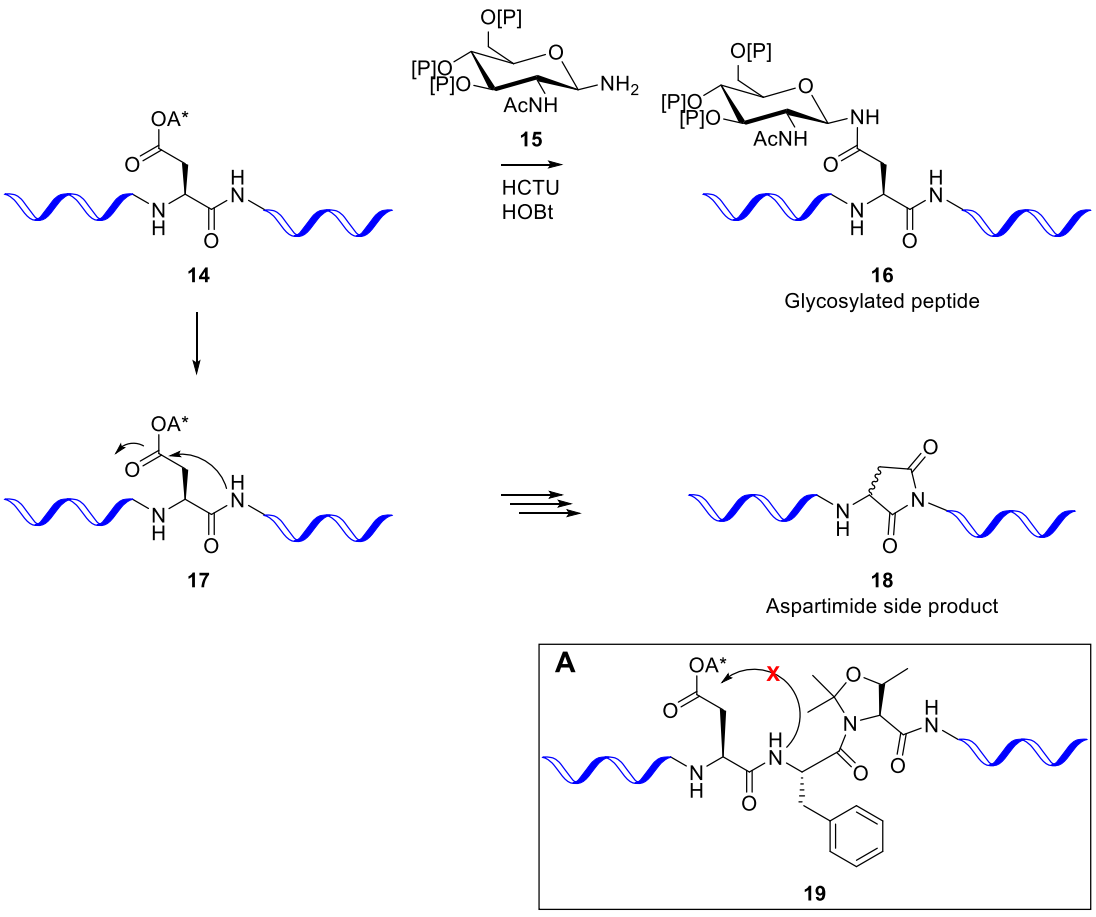
Wang and co-workers described a method to overcome the lack of glycosylation machinery in *E. coli* by coupling the glycoprotein synthesis with *in vitro* remodelling.[79] With the knowledge that *E. coli* does indeed possess a biosynthetic pathway to a lipid-conjugated GlcNAc-diphosphate precursor, the glycosylation machinery of *Campylobacter jejuni* (*C. jejuni*) was transplanted into *E. coli* and a glycoprotein was expressed.[80] The genes for the Asn-conjugated bacillosamine monosaccharide were deleted from *C. jejuni*, and thus the native OSTase from *C. jejuni* was able to use the GlcNAc-conjugated glycolipid from *E. coli* as a substrate to glycosylate the nascent target protein. The bacterial *N*-glycans were subsequently trimmed, and transglycosylation *via* the appropriate endo-β-*N*-acetylglucosaminidase (ENGase) provided access to the native eukaryotic *N*-glycoproteins.[81]

While insect *N*-glycosylation patterns rely on the same pentasaccharide precursor as the mammalian pathway, the action of fucosyltransferases conjugates fucose (Fuc) units to the bridgehead GlcNAc of the core glycan, and the action of mannosidases and GNase produces paucimannosidic glycans.[82-83] Plant *N*-glycan biosynthesis often involves the transfer of xylose (Xyl) to the nascent carbohydrate and core fucosylation, and the Lewis A structure is often observed in the final *N*-glycan. Both the β1,2-Xyl and core α1,3-Fuc moieties are speculated to be allergenic and immunogenic to humans.[84-86] In the case of

yeast expression, hypermannose structures are generated through the action of a number of mannosyltransferases and mannosyl-phosphate transferases, and can reach up to 200 mannose residues.[82-83] The use of *E. coli* in recombinant protein expression is discussed further in section 1.4.1.

1.3.2 Synthetic approaches

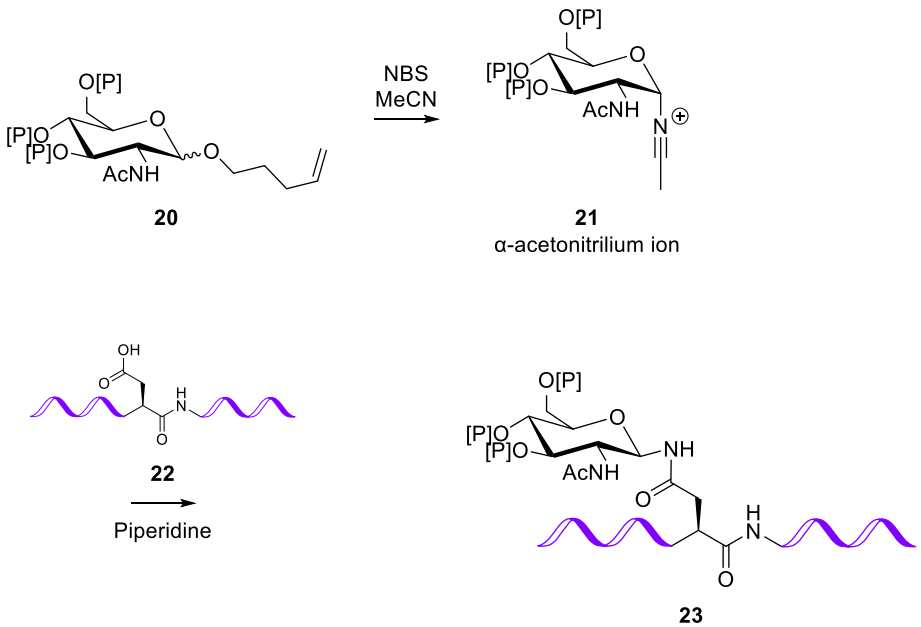
The predominant strategy for accessing *N*-linked glycopeptides synthetically is through the reaction of activated aspartic acid (Asp) side chains. One such strategy is the Lansbury Aspartylation,[87-88] in which the activated Asp residue is coupled to a glucosamine. The major drawback of this method is the competing aspartimide formation (Scheme 1.5). While this can be somewhat mitigated through the use of protecting groups (such as the pseudroproline approach, Insert **A**),[89] the extensive application of protection strategies hinders the accessibility and overall utility of these approaches.



Scheme 1.5. Strategies for chemical aspartylation with glycosylamines, where OA* indicates an activated ester of the side chain. Insert **A:** Pseudoproline approach used to minimise aspartimide formation.[90]

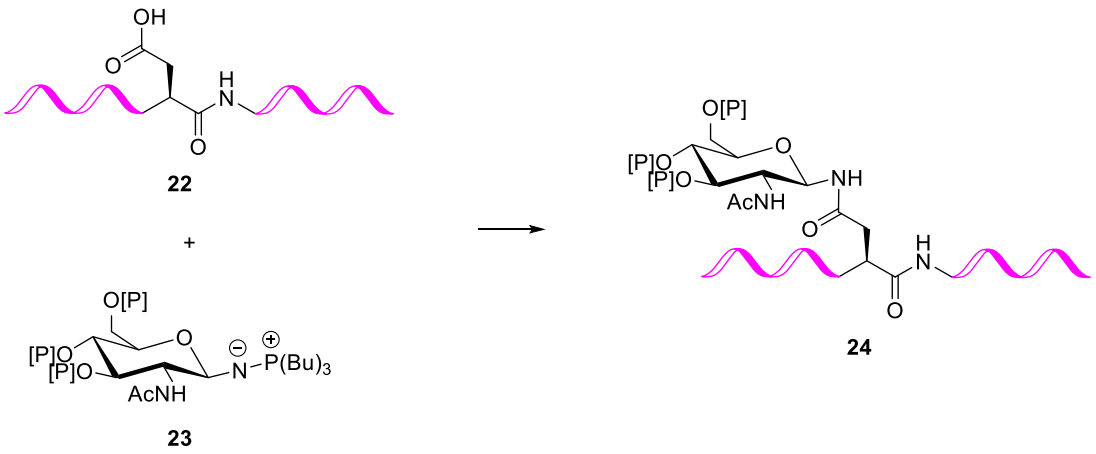
Other strategies include stepwise solid-phase peptide synthesis (SPPS) and native chemical ligation (NCL); however, both of these methods possess their own limitations. In the former, the β-GlcNAc linkage is established early with the side chain of Asp, and the glycosylated AA unit is incorporated into standard SPPS procedures. This permits the use of excess glycosyl donor to push the reaction towards completion, and has been employed to investigate small glycoproteins of interest.[91-93] However, the sensitivity of *O*-glycosidic linkages to strongly basic or acidic conditions necessitates 9-fluorenylmethyloxycarbonyl (Fmoc) based SPPS – furthermore, the increased steric bulk of the Fmoc protected glycosylated amino acids severely limits the efficiency of subsequent amide couplings in this technique, and results in long reaction times and large excesses of reagents.[94]

The previously described *n*-pentenyl glycosides (section 1.2.3.2) have also found application in the synthesis of *N*-glycopeptides.[95] By exploiting the ability of carbohydrates to stabilise a carbenium ion, Ratcliffe *et al.* employed the Ritter reaction to generate the α-acetonitrilium cation **21** *in situ*, which was subsequently trapped by the an aspartic acid side chain to furnish the acetylated amide bridgehead (Scheme 1.6). Deacetylation with piperidine furnishes the β-GlcNAc amide unit, and selectivity for the native β-GlcNAc linkage can be attained using sterically demanding protecting groups.[96]



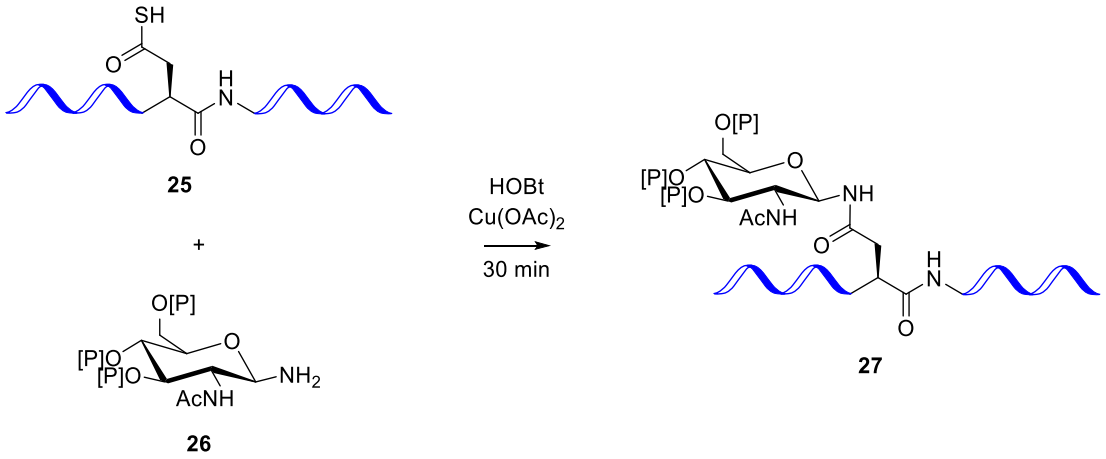
Scheme 1.6. The Ritter reaction can be employed in the synthesis of an *N*-glycopeptide **23**, starting from the *O*-pentenyl glycoside **20** and proceeding *via* the α-acetonitrilium ion **21**.

Davis and co-workers have also described an alternative synthetic method to access the native β-GlcNAc moiety, instead relying on the Staudinger reaction for ligation (Scheme 1.7).[97] The acylation of the iminophosphorane with the side chain of the peptidic Asp residue results in the formation of the native amide bond, with complete stereocontrol.



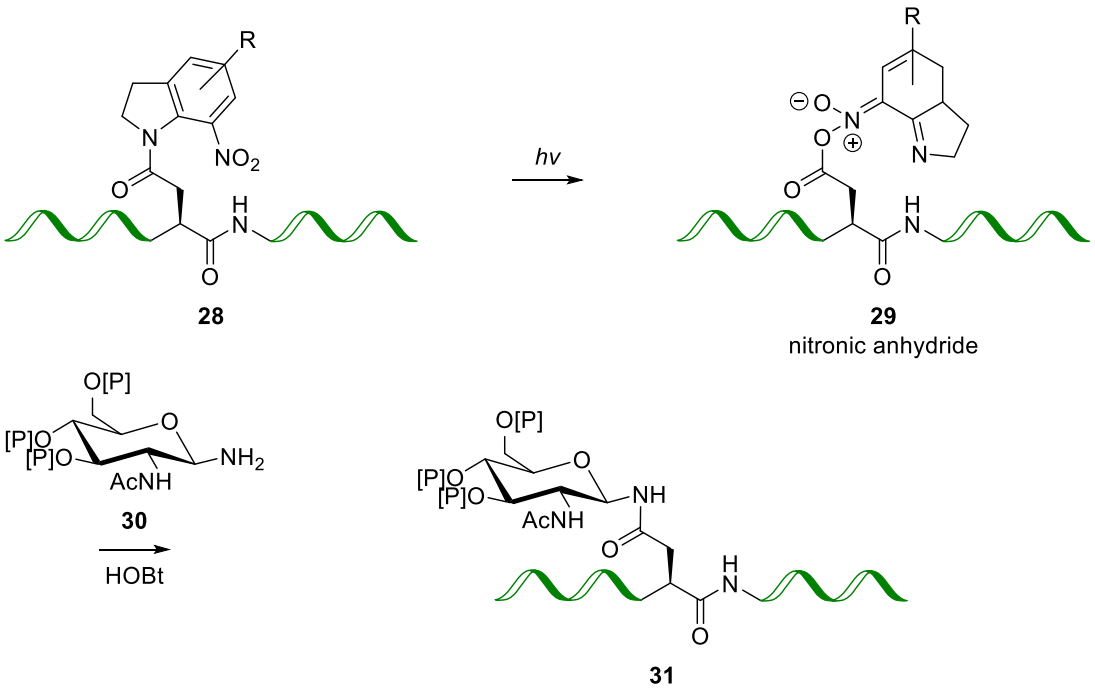
Scheme 1.7. The approach employed by Davis *et al*. in their use of the Staudinger reaction for ligation.

A further advancement by Garner *et al*. boasts a complete absence of aspartimide formation, with completion in under half an hour and compatibility with unprotected amines (e.g. Lys side chain, *N*-terminus) within the peptide.[98] In the presence of 1-hydroxybenzotriazole (HOBt) and Cu (II), peptidyl thioacids were successfully ligated *via* a currently undetermined mechanism. Danishefsky *et al*. reported a similar ligation approach relying on HOBt alone,[99] however Garner and co-workers were unsuccessful in reproducing these results, and identified the necessity of both the coupling reagent and transition metal salt (Scheme 1.8).



Scheme 1.8. Garner *et al.* furnished the *N*-linked glycopeptide *via* the coupling of an aspartyl thioacid **25** and the relevant glycosylamine **26**.

A further extension of the classical aspartylation method relies on the use of protecting groups with can be photochemically activated to promote acylation of the glycosylamine residue. Michael *et al*. employed a nitroindoline moiety as an orthogonal ester protecting group for the Asp side chain **28**, which upon *hv* activation transforms to the corresponding nitronic anhydride **29** (Scheme 1.9).[100] Acylation of the GlcNAc residue results in formation of the native linkage to the peptide chain. A particular advantage of this approach is that similar to the account by Davis *et al*., aspartimide formation is kept to a minimum due to the base-free conditions.

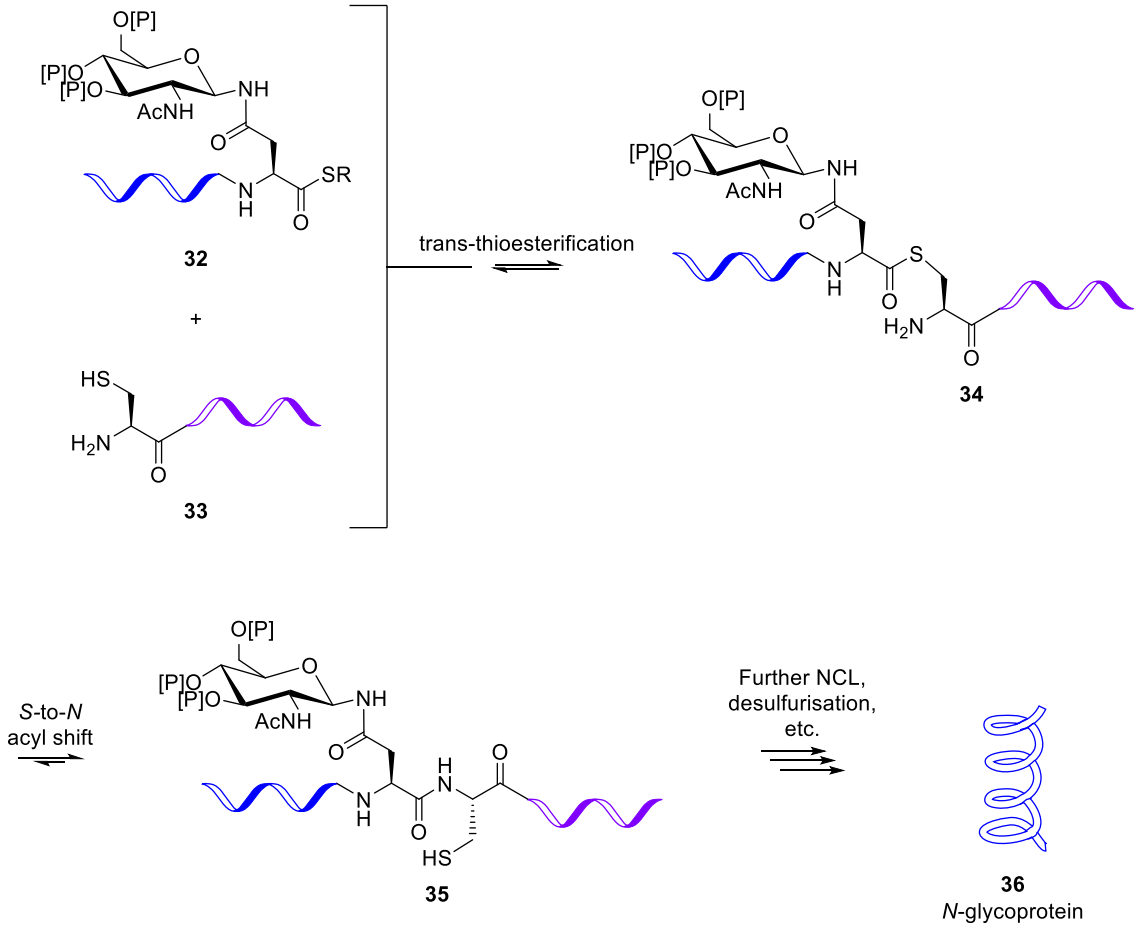


Scheme 1.9. The use of a nitroindoline protecting group **28** and subsequent activation to the nitronic anhydride **29** facilitates this base-free glycosylation described by Michael *et al*.[100]

1.3.3 Native Chemical Ligation and Auxiliary Mediated Ligation

Above the length of 50 AAs, SPPS suffers from increasing aggregation and epimerization rates, and accumulation of deletion products which renders it inefficient. Native Chemical Ligation (NCL), discovered by Kent and co-workers in 1994, can overcome some of these issues (Scheme 1.10).[101] NCL relies on the trans-thioesterification of *C*-terminal thioesters **32** with the thiol side chain of an *N*-terminal Cys residue **33**, furnishing an *S*-linked isopeptide bond **34**. In the presence of the free *N*-terminal of the

Cys, the nascent thioester can undergo a spontaneous *S*-to-*N* acyl shift through a 5-membered transition state (TS) to furnish a native amide bond between the two peptide fragments. As previously discussed, cysteine is of quite low natural abundance, and also may not be conveniently placed in the sequence for ligation to be carried out. Nevertheless, some of the most impressive glycoprotein syntheses have their roots in the application of NCL.[102-103]



Scheme 1.10. NCL between a *C*-terminal thioester glycopeptide and an unprotected *N*-terminal cysteinyl peptide fragment.

To overcome the reliance on Cys, MacMillan and Anderson searched for potential retrosynthetic disconnections at other AAs, and published one of the first papers detailing auxiliary-mediated ligation (AML)[104] – although Kent and co-workers had laid the groundwork three years previous in their total synthesis of cytochrome b562.[105] Proposed as a "cysteine-free NCL", this ligation method relies on a cleavable auxiliary group which contains a thiol functionality to mimic the Cys residue. The seminal members of this class are the TFA-cleavable 4,5,6-trimethoxy-2-mercaptobenzyl **37** and 1-(2,4-

dimethoxyphenyl)-2-mercaptoethyl linkers **38** (Figure 1.5), with which MacMillan *et al.* prepared fragments of the *O*-linked glycoprotein GlyCAM-1.[104]

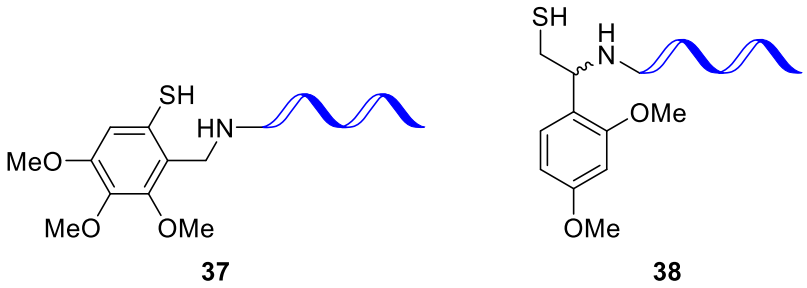


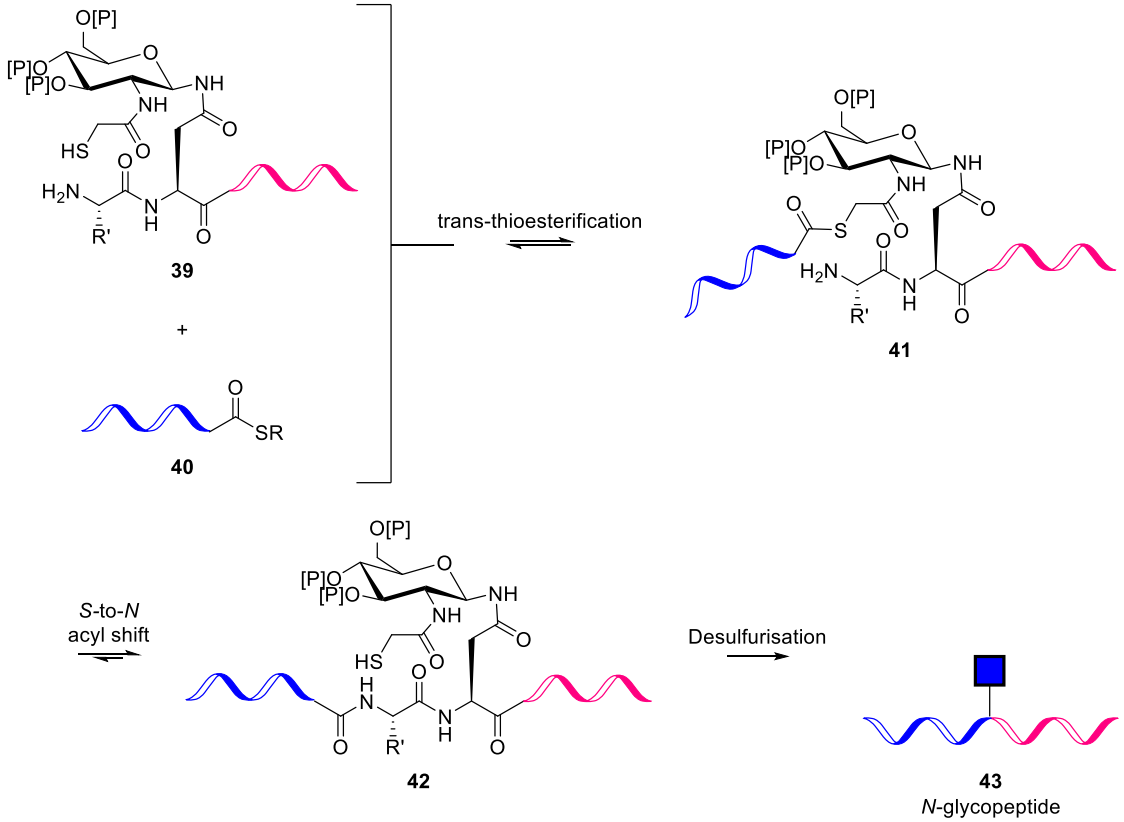
Figure 1.5. Structures of TFA-cleavable linkers used in auxiliary-mediated ligation (AML).

AML still suffers from classical problems in glycoprotein synthesis. These are in part due to harsh restrictions on scope due to the concomitant steric demands of the auxiliary, and reliance on a secondary amine for the *S*-to-*N* acyl transfer. A consequence of this is that AML is only useful when one disconnects at smaller residues such as glycine (Gly) – to offset the increased steric demand, only GlyGly or GlyAla ligation sites are useful.[104]

1.3.4 Sugar Assisted Ligation (SAL)

An additional synthetic method of note is Sugar Assisted Ligation (SAL), developed by Chi-Huey Wong and co-workers, which attempts to accomplish a convergent construction of glycopeptide fragments, without reliance on Cys. Although still centred on the dogma of NCL and AML, the thiol "auxiliary" is situated on the carbohydrate moiety rather than the peptide, at the sugar C-2[106-107] or C-3 position (Scheme 1.11).[108] This approach has been demonstrated successfully in the synthesis of both *O*-linked and *N*-linked glycopeptides.[107-109] While still reliant on trans-thioesterification and *S*-to-*N* acyl shift, the marked difference is its dependence on a 14- or 15-membered TS, which allows some flexibility to overcome the steric limitations of AML - it is not limited to just GlyGly and GlyAla junctions as a result. However this also means the thermodynamic shift at the centre of these techniques is much slower in SAL.[107-108] The authors propose that the conformational constraint exerted by the monosaccharide places the thioester **41**, formed *via* trans-thioesterification with the sulfhydryl moiety on the C-2 acetamido of the sugar, in close proximity with the *N*-terminus of the peptide.[107] While the success of SAL is encouraging for the aims of this work (which also relies on a larger TS, as discussed later in section 1.7.1), it has been called into question whether this sugar-

assisted ligation proceeds through reported intramolecular *S*-to-*N* acyl shift, or if the kinetics of the reaction are slow enough that the intermolecular shift in fact predominates.



Scheme 1.11. Sugar-assisted ligation between a *C*-terminal peptidic thioester and glycopeptide fragment. Desulfurization yields the desired β-GlcNAc decoration (shown as a blue square).[110]

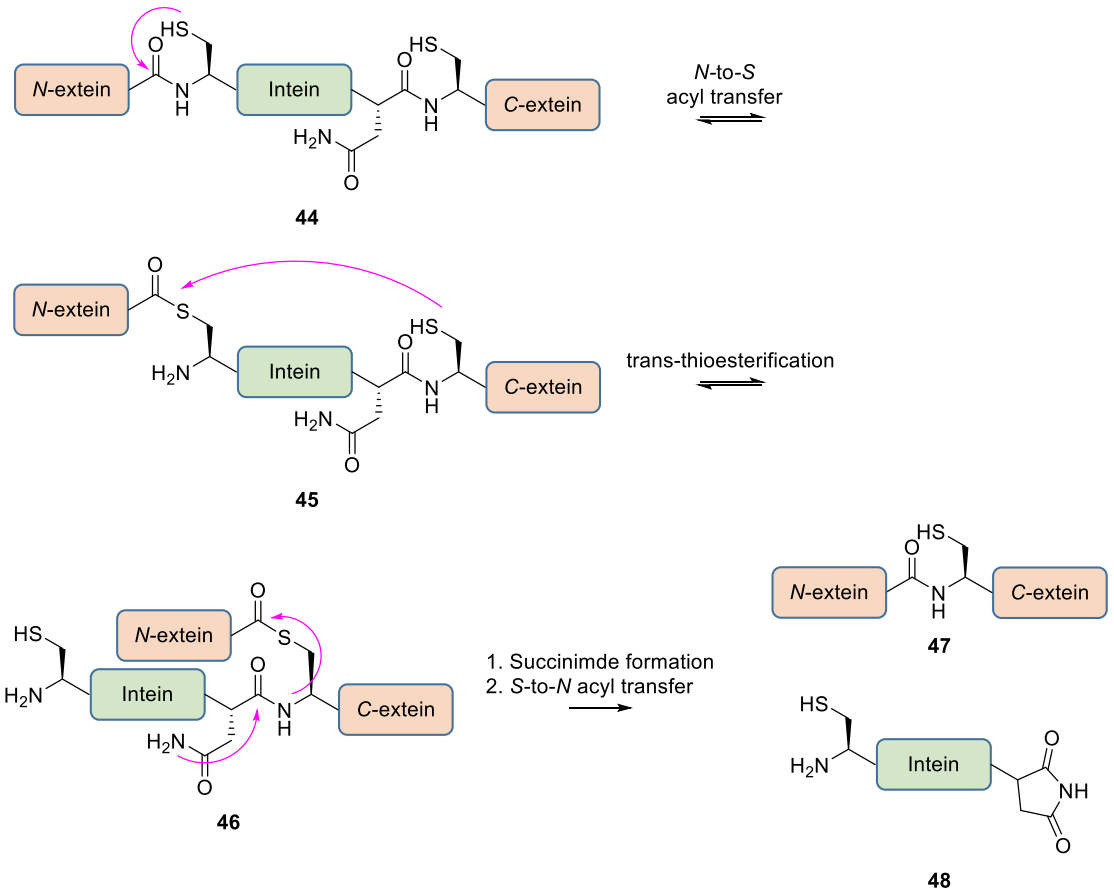
Despite the increased flexibility, thioesters at Val, Pro and Ile are known to be recalcitrant to successful application in SAL methodology. These also consist of some of the most challenging ligation sites for NCL.[107, 109, 111] In contrast to the previously discussed AML method, the range of AAs tolerated at the *N*-terminus extends beyond the facile GlyGly or GlyAla junctions. The participating proteinaceous amine in SAL is primary, whereas in AML the auxliiary amine is secondary in nature, and thus a forebearance to a variety of AA residues at the *N*-terminus is observed.[107, 110]

In a later account, Wong and co-workers described the extended SAL (exSAL) methodology, in which up to five AA residues are tolerated between the site of *N*-glycosylation and the primary amine at the terminus of the polypeptide chain.[108-109] While there remains a reliance on desulfurisation stratgeies to restore the native β-GlcNAc bridgehead after successful *S*-to-*N* acyl transfer, this may be incompatible if Cys residues are present elsewhere in the nascent glycopeptide. Instead, it has been proposed

that enzymatic cleavage of the monosaccharide following ligation offers a traceless peptide ligation strategy.[112]

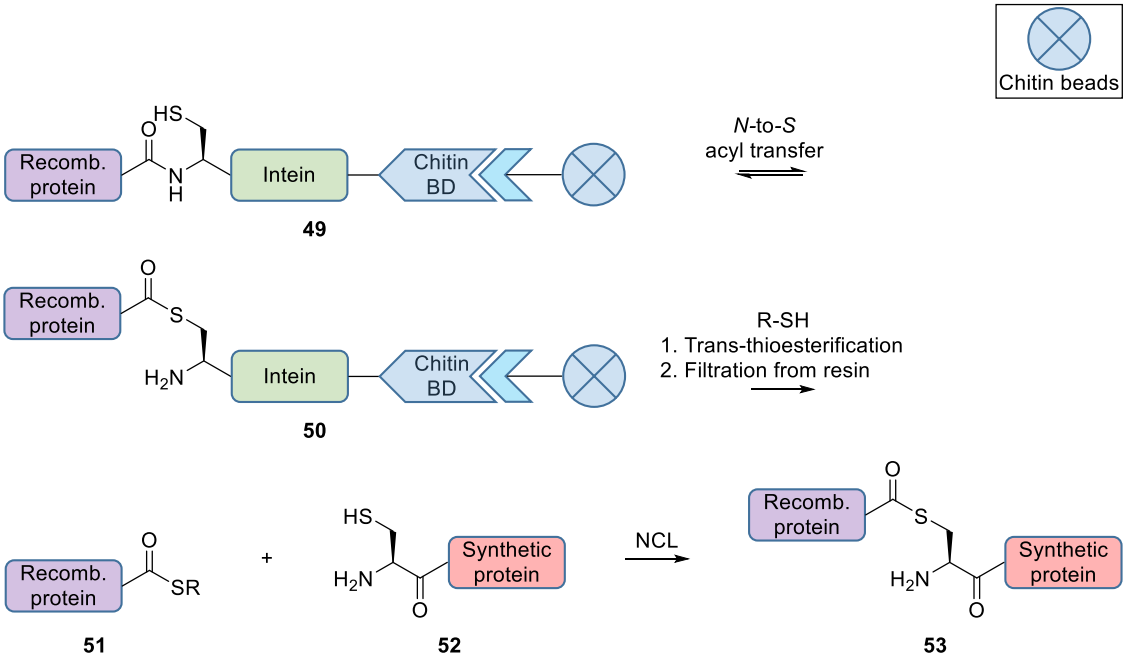
1.3.5 Expressed Protein Ligation (EPL)

Expressed protein ligation (EPL) involves the expression of a large *N*-terminal Cys-containing domain and an intact protein thioester to be used in a ligation pair, essentially combining NCL and recombinant expression. This furnishes a final protein product which is composed of a synthetic peptide component and the recombinant building block.[113] EPL methodology is based on inteins, the natural mediators of protein splicing,[114] which catalyse their own excision from a protein sequence *via* several acyl shifts (Scheme 1.12).[115] Protein splicing begins with an *N*-to-*S* acyl transfer between the Cys residue of the intein and the *N*-extein, which generates a thioester intermediate. The *N*-extein is then translocated to the sulfhydryl side chain of the *C*-extein Cys residue *via* a trans-thioesterification reaction. Finally, a conserved Asn cyclises to the respective succinimide intermediate and a concomitant *S*-to-*N* acyl shift yields the peptide bond and completes the protein splicing pathway.[113] Several mutated inteins have been described in the literature, many of which display an Ala residue at the *C*-terminus instead of the native Asn, and thus only promote the initial stage of protein splicing.[116-118]



Scheme 1.12. Protein splicing pathway as described by Muir *et al.*[114] Cyclisation of the conserved Asn residue **46** completes intein excision from the protein sequence, yielding a Cys residue flanked by the *N*- and *C*-extein **47** and the intein **48** with *N*-terminal Cys and *C*-terminal succinimide.

Beginning with an intein domain conjugated to a solid support through the *C*-terminus, the desired protein is expressed at the *N*-terminus. The previously described Asn residue, which would normally yield the succinimide intermediate in protein splicing, is mutated to an Ala residue; this prevents *C*-terminal cleavage and halts the splicing process in the initial stages. An *N*-to-*S* acyl transfer furnishes a thioester intermediate, which is then attacked by an exogenous thiol. Following this trans-thioesterification, simple filtration from the resin support (Scheme 1.13) provides the desired recombinant protein thioester **51**. The nascent thioester can then be applied to NCL with a suitable counterpart containing Cys residues **52**.[119-120]



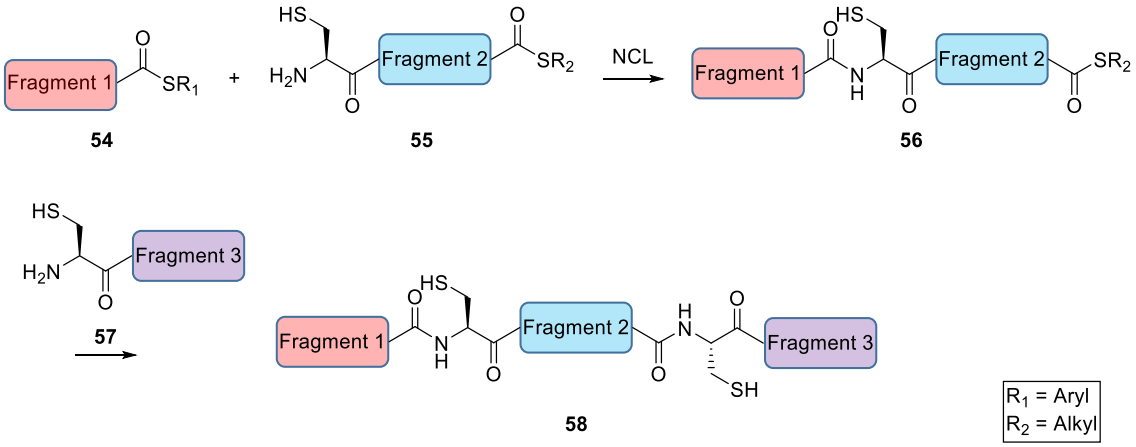
Scheme 1.13. EPL as described by Muir *et al*. [113-114] The intein is tagged with a chitin binding domain (Chitin BD, **49**), which permits conjugation to the solid support (chitin beads).

By expressing proteins which are fused to the *N*-terminus of the intein, and employing intramolecular trans-thioesterification by a suitable thiol, EPL essentially offers a traceless chemical protease which furnishes the corresponding peptide α-thioester. This thioester can then undergo NCL. Furthermore, a technique such as EPL renders structures composed of both chemical and biological origin accessible, and has been widely employed in the inclusion of fluorophores, synthetic handles and non-natural AAs in proteins of interest. [121-124]

1.3.6 Kinetically Controlled Ligation (KCL)

For the ligation of multiple peptide segments, NCL is only useful for chain growth in the *N*-terminal direction, *via* a Cys residue at the *C*-terminal. Traditionally, one would protect an *N*-terminal Cys residue to ensure that NCL proceeds between the *C*-terminus of the respective peptide and another suitable protein fragment. This sustains growth from the *C*- to *N*-terminus towards the desired polypeptide. Unfortunately, very little advancement has been recorded in the protection of the thioester moiety of peptide fragments for NCL and thus peptide growth in the *N*- to *C*-terminal direction is not accessible, nor is a fully convergent chemical synthesis using NCL. Kinetically Controlled Ligation (KCL) offers

a convergent ligation method for peptides and proteins, and exploits the varying reactivity of alkyl *versus* aryl thioesters. In an account by Kent *et al*., the authors describe the observed faster reaction times for a preformed peptide-thiophenylester over a relatively unreactive peptide-alkylthioester.[125] The authors confirmed that under competitive reactions conditions, the alkylthioester is sluggish and unreactive in comparison to the aryl-centred analogue, and hence used the approach to prepare crambin, a small protein.[125-126] Kent and co-workers performed a six-segment ligation, with NCL occurring at both terminals (Scheme 1.14). The relative reaction rates of the peptidic thioesters resulted in a controlled and organised elongation of the nascent peptide chain.



Scheme 1.14. Kinetically controlled ligation (KCL) exploits the varying reactivity of aryl *versus* alkyl thioesters. Global desulfurisation following the final NCL sequence afford the Cys-free protein.

This technique was further elaborated by Pereira and co-workers for their synthesis of madanin-1,[127-128] in which an exogenous thiol additive, 2,2,2-trifluoroethanethiol (TFET), further increased trans-thioesterification rate by forming a TFET-ester *in situ*. The nascent activated thioester undergoes the first NCL sequence, and further activation of TFET permits chain elongation in the *N*- to *C*-terminal direction. A global desulfurization strategy (see section 4.5 for a discussion of this process) afforded the completed Cys-free peptide.

1.3.7 KAHA Ligation

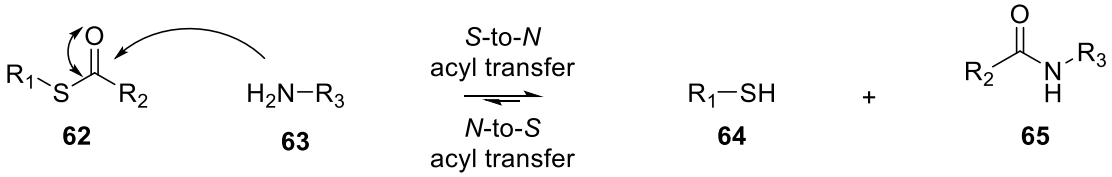
The coupling of an α-ketoacid and a hydroxylamine unit offers an aqueous approach to amide-bond formation, without the need for extensive protecting group strategies or harsh coupling reagents. This approach is referred to as the KAHA ligation, and was

detailed in an account by Bode *et al*. shortly after the turn of the millenium.[129] This chemoselective coupling of large, unprotected peptidic segments relies on the decarboxylative condensation of an *N*-terminal hydroxylamine **60** with the corresponding *C*-terminal α-ketoacid **59**, extruding CO_2 and H_2O as the only by-products (Scheme 1.15). The KAHA ligation is normally applied with either an unmodified hydroxyl group (Type I KAHA ligation) or with an *O*-substituted hydroxylamine, such as with a benzyl ether (Type II KAHA ligation). This approach has been utilised in the synthesis of a number of small proteins, namely GLP-1,[130] the Cys-free protein betatrophin,[131] nitrophorin-4,[132] and the antibacterial protein AS-48.[133]

Scheme 1.15. The KAHA ligation permits facile and protecting group-free coupling of an α-ketoacid **59** and a hydroxylamine fragment **60**, liberating CO_2 and H_2O.

1.3.8 Reversibility of *S*-to-*N* acyl transfer

Many of the ligation approaches discussed herein rely heavily on the thermodynamic shift of a sulfur-bound acyl group to a proximal amine. While this *S*-to-*N* acyl transfer forms the crux of many ligation methodologies, it is in fact a reversible process. Protein splicing, which was previously discussed in the context of EPL, encompasses both an *S*-to-*N* and *N*-to-*S* acyl shift, and thus the kinetics of acyl transfer are non-negligible when assessing ligation dynamics. In biological processes, a predilection for the amide product **65** is usually observed (Scheme 1.16). However, the reverse reaction is essential in protein splicing, wherein the extein-intein unit is transferred *en bloc* to the side chain thiol of the intein Cys residue.[117] This thermodynamically unfavourable process is catalysed by a proximal His residue, which contorts the peptide sequence into a twisted, high energy state.[134] This conformational change promotes the formation of the nascent thioester.

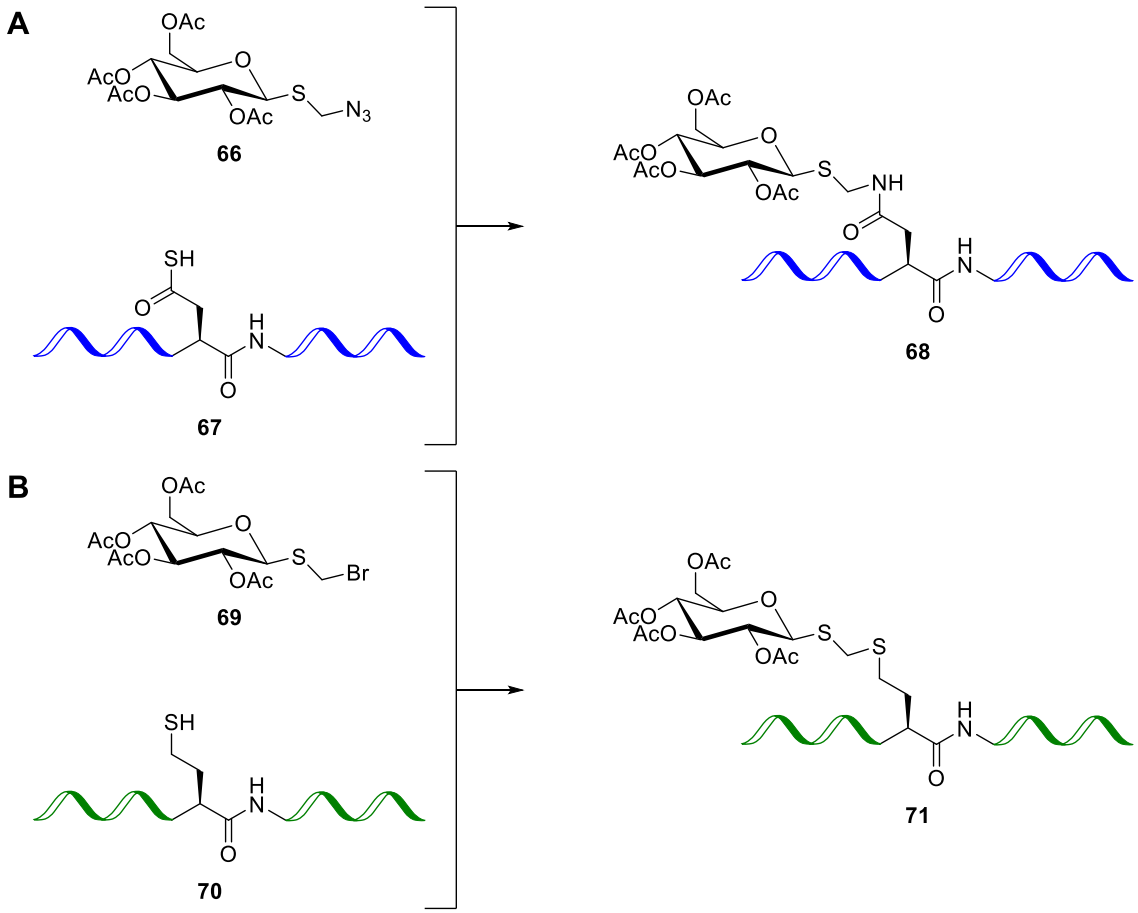


Scheme 1.16. The *S*-to-*N* acyl shift typically predominates the equilibrium to furnish the amide **65**. However this process is reversible, and *N*-to-*S* acyl transfer to generate the thioester **62** is possible.

1.3.9 Synthetic approaches non-natural glycoconjugates

Glycoconjugates which display non-native linkages to biological molecules often possess enhanced chemical and physiological stability. While natural glycoconjugates can be enzymatically hydrolysed, altering the functionality at the anomeric centre can confer resistance to hydrolysis, and as such many research groups have focused on neo-glycopeptides for their sustained biological activity.[135-136] For example, the ligation of azides and thioacids proves ready access to non-natural anomeric linkages (Scheme 1.1.7, **A**), which probably proceeds *via* a five membered intermediate with loss of nitrogen gas.

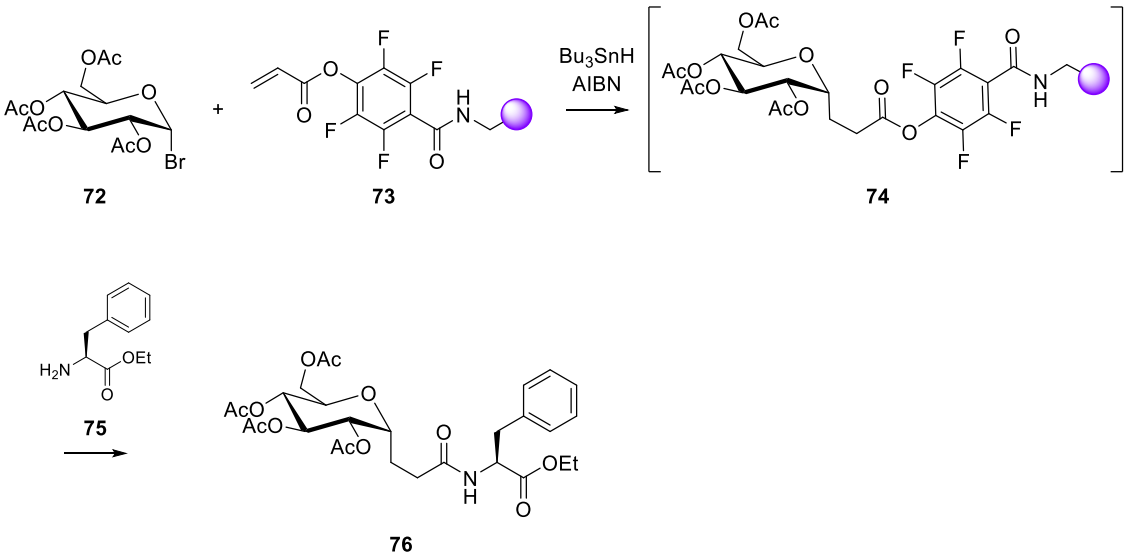
One such example by Zhu *et al*. describes the synthesis of a number of novel *S*-neoglycopeptides from the corresponding glycosylthiomethyl derivatives.[137] The authors describe the successful ligation of an array of glycosylthiomethyl azides **66** with the corresponding aspartic and glutamic thioacids to furnish glycosylthioamide derivatives**,** such as **68**. The glycosylthiomethyl halides **69** provided access to the respective *S*-(glycosylthiomethyl) peptides **71** when reacted with the thiol side chain of Cys and homocysteine **70** (homo-Cys). Representative examples of the discussed reaction scheme are shown in Scheme 1.17.



Scheme 1.17. Representative examples from an account by Zhu *et al* **A**: The thioacid-azide ligation generate *S*-linked neoglycopeptides starting from aspartic (or glutamic) thioacid. **B:** Nucleophilic displacement of a primary bromide by the homo-Cys thiol results in *S*-(glycosylthiomethyl) peptides

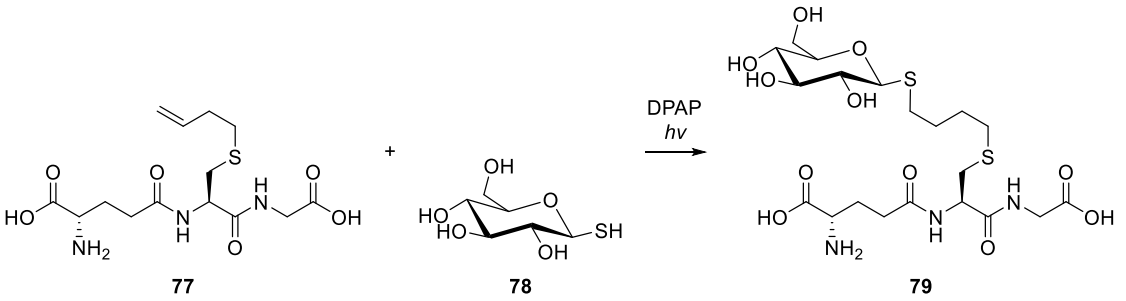
1.3.11 Radical Methods to Access Glycopeptides, and Glycopeptidomimetics

A number of examples of glycopeptide and glycopeptidomimetic syntheses involving radical mediated reactions have been reported – largely focusing on the development of exogenous glycoconjugates which are resistant to hydrolase enzymes, but which preserve their biological activity.[135, 137-138] Classical tin-based methods have been employed by Wilden and co-workers to access *C*-glycopeptide mimetics – in one report, an unnatural carbohydrate-phenylalanine linkage **76** is formed from a nascent activated ester **74** (Scheme 1.18) on solid support.[139]



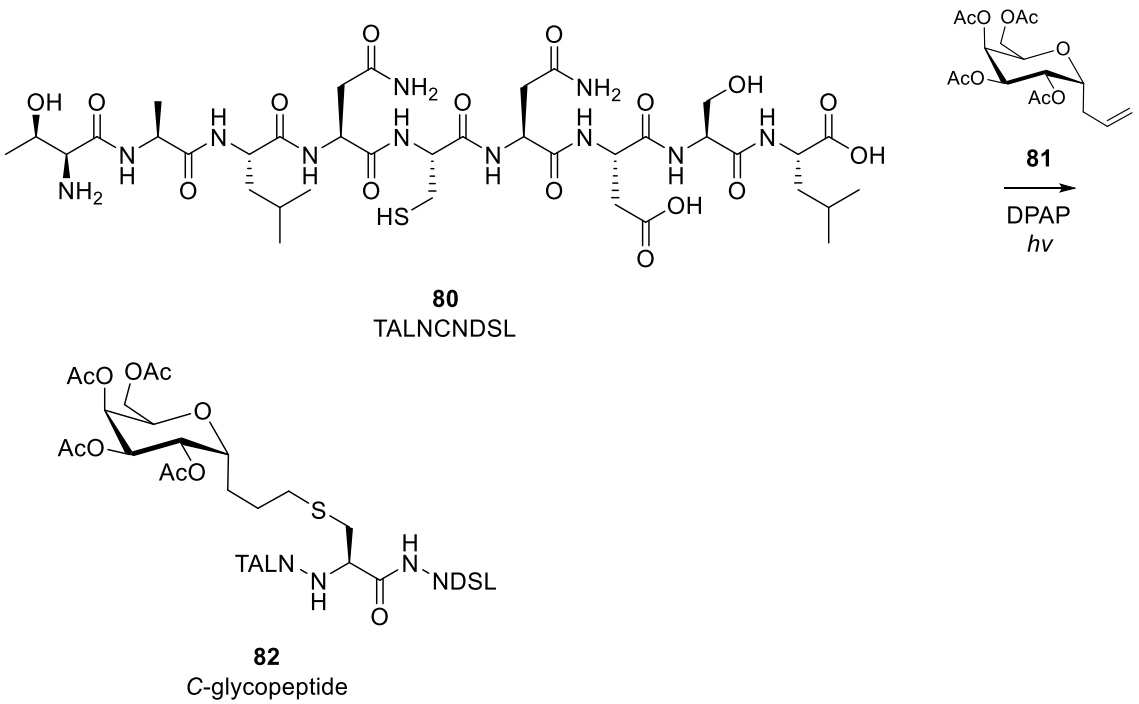
Scheme 1.18. Solid supported synthesis reported by Wilden and co-workers, in which phenylalanine ethyl ester **75** is conjugated with a protected glucose moiety **72**.

Furthermore, studies reported by Davis and Dondoni have bolstered the versatility of the thiol-ene 'click' (see section 1.6) for generating *S*-glycopeptides. Peptides displaying varying degrees of unsaturation have been demonstrated as useful substrates for hydrothiolation by the corresponding glycosyl thiol. In one such example, an alkenyl glutathione (GSH) **77** is *S*-glycosylated under photochemical conditions to give **79** (Scheme 1.19).[140]



Scheme 1.19. GSH **77** was successfully glycosylated *via* an alkene appended on its sulfahydryl side chain.

One particularly impressive example of radical mediated formation of non-canonical linkages for *C*-glycosides is shown in Scheme 1.20 below. Dondoni *et al.* published a glycopeptide library reliant on the reaction between a Cys side chain **80** in a containing sequence and an alkenyl glycoside **81**. Over a rapid reaction timescale, a *C*-galactosylated nonapeptide **82** was prepared under photochemical thiol-ene conditions.[141] Furthermore, the authors report a similar hypergalactosylation of the singular Cys residue and two thiol groups (from photolysis of a cystine residue) in bovine serum albumin (BSA).[141]



Scheme 1.20. Nonapeptide TALNCNDSL **80** was successfully modified with a glucose moiety bearing a terminal alkene, under photochemical conditions by Dondoni and co-workers.

1.4 Interferon-γ (IFNγ): An *N*-glycoprotein of interest

Interferon-γ is a cytokine which consists of 166 AAs; it is a mature protein of critical immunological function which is *N*-glycosylated at two distinct sites (Figure 1.6). This pleiotropic signalling protein is secreted by T-cells and natural killer (NK) cells in adaptive and innate immunological responses respectively.[142] IFNγ exhibits antimicrobial activity,[143] amongst a host of other functions.[144] The protein is glycosylated at Asn-48 and/or Asn-120 (often referred to as positions 25 and 97, due to a 23 amino acid signal sequence), but the exact role of the carbohydrate is poorly understood. Recombinant IFNγ is an FDA approved therapeutic for the treatment of patients with Severe Malignant Osteoporosis (SMO), a genetic disorder that affects normal bone function and also for reducing the severity of infections in patients with Chronic Granulomatous Disease (CGD), a genetic disorder that affects the functioning of cells in the immune system. Sareneva *et al.* and others have demonstrated that glycosylation at Asn-48 is important for both binding and protection from proteolysis – as observed from X-ray crystal data, the Asn-48 glycan in the IFNγ/IFNγ receptor complex is actually located in the immediate vicinity of the binding site.[143, 145] It has also been previously shown that unglycosylated IFNγ produced *via* bacterial models is biologically active, yet

the antiviral activity of the glycosylated forms is nearly double that of the unglycosylated protein.[143, 146]

Unglycosylated recombinant IFNγ is a current FDA approved therapeutic (ACTIMMUNE™, marketed by Horizon Pharmaceuticals), which predictably suffers from very poor *in vivo* half-life – small therapeutic proteins (< 50-60 kDa) often exhibit short serum half-lives due to clearance by the kidneys, and passage through the endothelial cell barrier of blood vessels.[147] The type of glycan present also plays a major role – for example the high-mannose form of human IFNγ (hIFNγ) obtained from insect models is eliminated much more rapidly than native hIFNγ.[148-149]

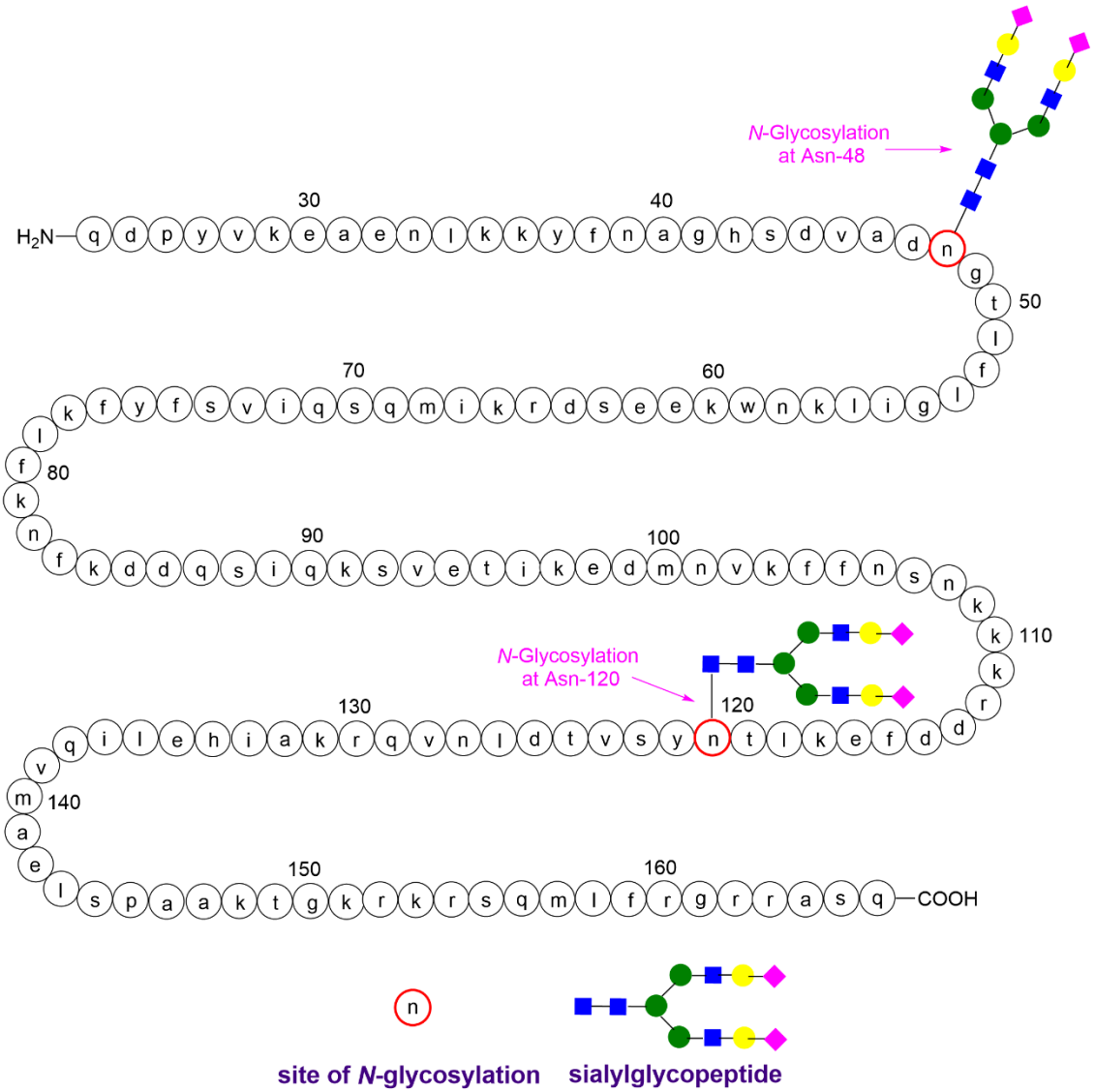


Figure 1.6. Structure of IGN-γ with *N*-glycosylation sites.

It is clear that access to homogenous samples of glycosylated IFNγ are of great importance in both academic research and for clinical applications, and that access to highly defined glycoforms would enable deeper investigation into the structure, stability and activity of this human cytokine. The importance of IFNγ to the present work is outlined in section 1.7.2.

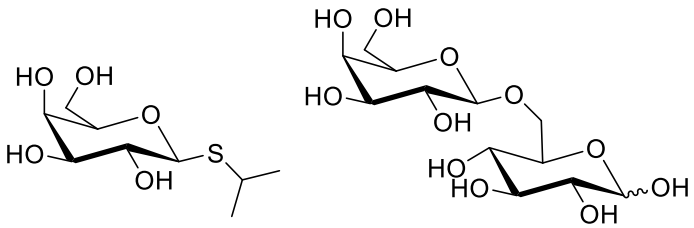
1.4.1 Recombinant protein expression

The Gram-negative bacterium *Escherichia coli* (*E. coli*) is one of the most attractive hosts for recombinant protein expression, due to the low cost of its required media, ease of handling, and its ability to grow rapidly to a high density under a range of growth conditions. The wide variety of cloning vectors and mutant host strains has further bolstered the utility of this organism as a host for protein expression and isolation.[150]

To begin recombinant protein expression, heterologous complementary DNAs (cDNAs) are first cloned into plasmids. Plasmid loss can severely hamper the effectiveness of expression, and this problem is especially apparent when cell cultivation is performed at high cell density, or with a very high copy number plasmids. Thankfully, a means of preventing this plasmid loss and the concomitant expression aberrations is available; by supplementing the growth media with antibiotics (typically kanamycin or spectinomycin) and making use of the plasmid-encoded antibiotic-resistance markers, plasmid-free cells are killed and do not interfere with the incipient colonies on the growth plate.[151] Albeit, these antibiotics can become degraded or inactivated in the growth medium, and thus the prevention of plasmid-free cells from taking over the culture often calls for the use of another approach. Cloning vectors can be engineered so that cell death occurs upon plasmid loss, by introducing repressors or genes into the cells. While this does offer a workaround, these additional genes often pose an increased metabolic burden on the cells, and can also restrict the growth media conditions.

At the turn of the millennium, Williams and co-workers disclosed a method for conservation of multiple copy plasmids.[152] This ‘repressor titration’ approach involves an essential gene which is under control of the *lac* operator/promoter region, and a multicopy plasmid which possess the *lac* operator. When the LacI repressor protein is titrated with *lac* operators (which are plasmid encoded), the activation and expression of

a gene for conveying resistance to the antibiotic kanamycin is observed. This results in the growth of only plasmid-containing cells in the antibiotic-dosed media. In the absence of an inducer (allolactose or IPTG, Figure 1.7), no growth is observed in kanamycin-doped media. Both allolactose and IPTG bind to the lac repressor, and release it from the *lac* operator *via* an allosteric process, which permits transcription of the genes in the *lac* operon. IPTG is often preferred as a non-hydrolysable alternative to allolactose.[152]



83 IPTG **84** Allolactose

Figure 1.7. The structures of the *lac* operon inducers IPTG (left) and allolactose (right).

1.4.2 Non-natural amino acid incorporation

The enormous diversity of structure and variance in function of human proteins boils down to only 20 amino acids. This relatively constrained repertoire of amino acids is encoded by the ubiquitous genetic code of all organisms. Two additional amino acids in Life's toolbox are selenocysteine and pyrrolysine, which are incorporated into nascent protein in response to the UGA and UAG stop codons respectively.[153-154] Given the enormous role of protein structure and function in biochemical processes, and the inherent therapeutic value of modified proteins, methods to judiciously modify their structure are of considerable interest.

Protein translation is a ribosomal-mediated process in which the amino acid sequence of the nascent polypeptide chain is dictated by the nucleotide sequence of mRNA. The 20 canonical amino acids are encoded by triplets, or three-base codons. Three of the 64 codons are nonsense or 'stop' codons: UAG (amber), UGA (opal) and UAA (ochre). mRNA is the basis for the polypeptide sequence, and is complemented by tRNA charged with an amino acid corresponding to its anticodon – this tRNA is referred to as the 'adapter' molecule. This tRNA charging process or aminoacylation is a two-step process officiated by the respective aminoacyl-tRNA synthetase (aaRS), which yields the

aminoacylated-tRNA (aa-tRNA), and relies upon ATP activation of the amino acid in question. These aaRSs are hence specific for their own unique amino acid substrate.[155] aaRSs are often specific for one particular species, and do not use tRNAs from other species as substrates in their aminoacylation processes (Figure 1.8).

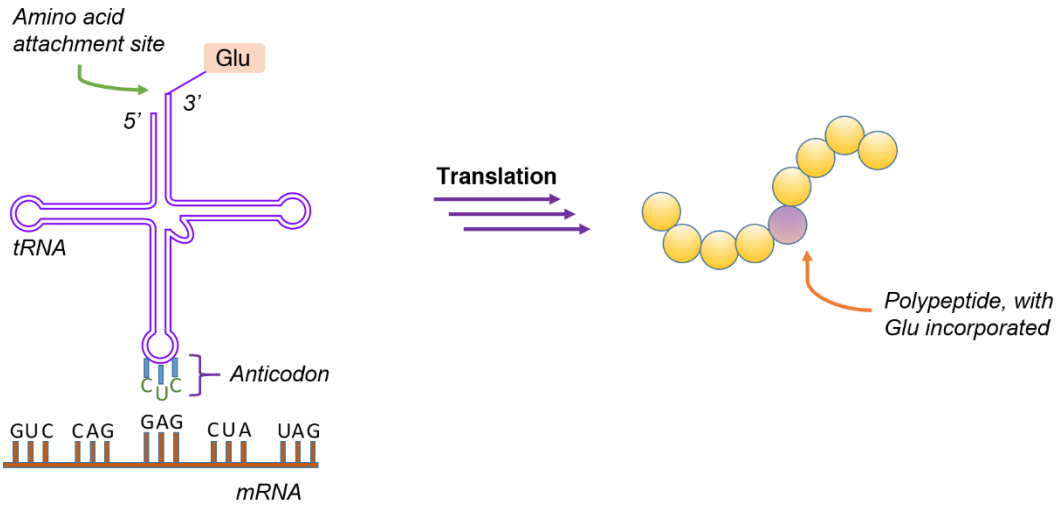


Figure 1.8. tRNA charged with glutamic acid, and the representative codon/anticodons.

E. coli has enjoyed a long-standing tenure as the protein scientist's workhorse. The use of its translational machinery was identified as a possible means to expand the genetic code, and furnish recombinant proteins bearing the required synthetic handles for non-canonical derivatisation and post-translational modification. The selection of *E. coli* as a host organism resides in its simplistic genetic manipulation, and the wealth of knowledge present in the literature about its versatile applications.[72] Over twenty years ago, a general method was devised for the co-translational incorporation of non-natural amino acid (nnAA) residues into proteins of interest. The orthogonal pairing of a non-endogenous aminoacyl tRNA synthetase/tRNA directs incorporation of the nnAA, with respect to the position of the amber stop-codon, UAG. In a general sense, the genetic encoding and positioning of UAG in the gene sequence results in site-specific insertion of ones desired amino acid. This approach has revolutionised the fields of protein engineering and synthetic biology, and to date more than 200 nnAAs have been successfully incorporated into a diverse array of protein backbones. This expansion of chemical space offers huge versatility in the fine-tuning of physicochemical and biological properties of proteins, expressed *via* mammalian, bacterial, and yeast cells.[156]

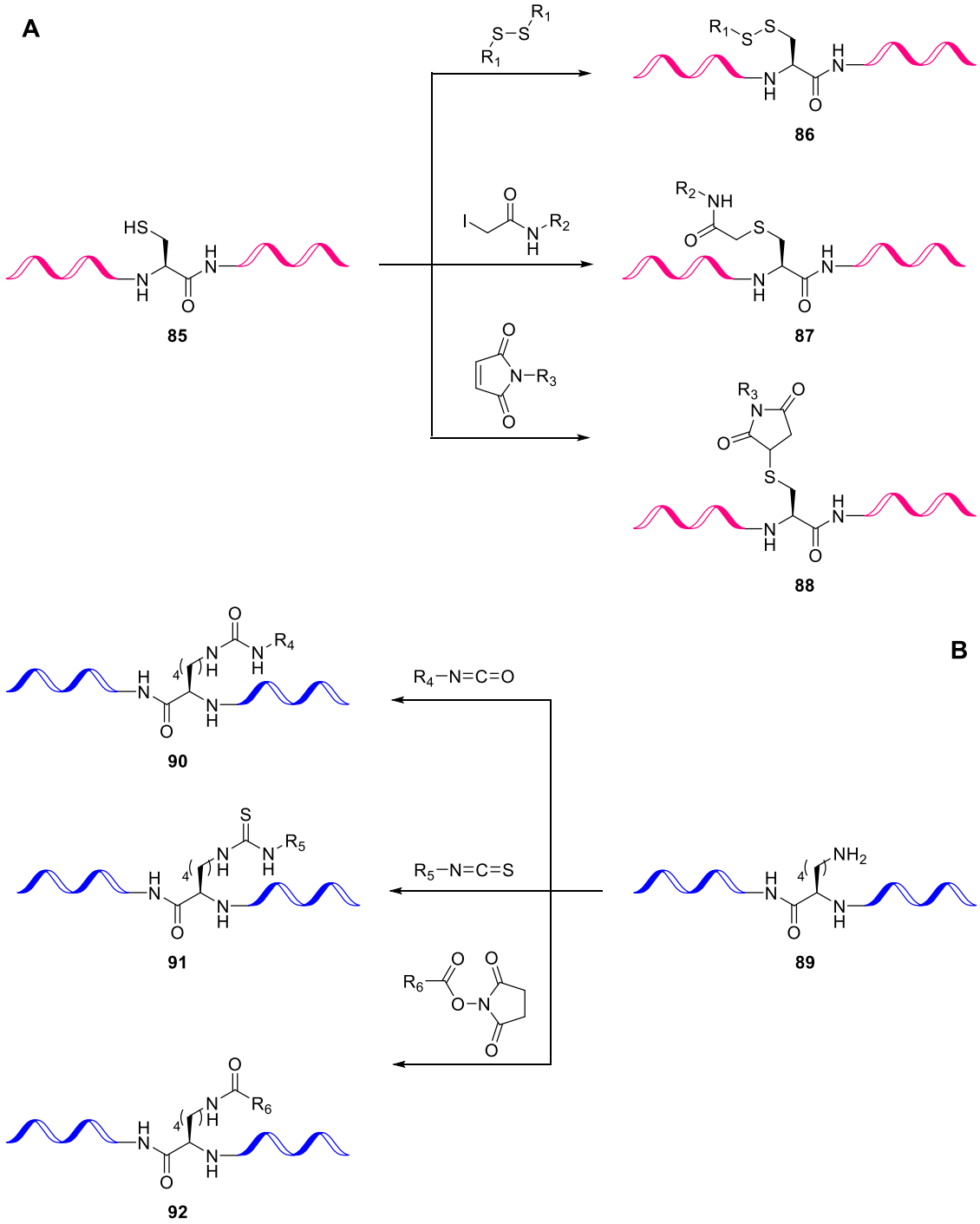
In order for this orthogonal pair and codon to successfully incorporate an nnAA into an incipient protein, there should ideally not be any recognition of the pair by an endogenous synthetase, and it should only 'decode' the orthogonal codon. The orthogonal synthetase component should ideally only aminoacylated the orthogonal tRNA with the nnAA, which one would hope is metabolically stable and has sufficient cellular bioavailability.

1.5 Bioconjugation

In order to study the effect that synthetic modifications have on the function of a protein, access to homogeneously modified proteins is required, as is the ability to modify biomolecules with the high degree of selectivity and specificity observed in nature. The concept of conjugation with biomolecules to relay information, or as a strategy to improve the bioavailability of therapeutics, has been widely employed.[76] For example, it was originally reported in 1929 that immunogenicity of carbohydrates could be enhanced by conjugation to a protein, a notion that is now well established.[157] Conversely, a peptides biological activity can also be enhanced by conjugation to carbohydrates.[158] For the purpose of this work, and in the context of the in-depth analysis of ligation methodology previoulsy described, we will consider bioconjugation methods to access *N*-linked glycoproteins.

Bioconjugation is the process of covalent derivatisation to form biomolecules, that possess different properties.[159] This coupling involves at least two molecules, one of which is a biomolecule such as a carbohydrate, nucleic acid or peptide/protein. Bioconjugation is a diverse process, utilising a relatively simple set of functional groups, and the influence of these functionalities on proteins provides the mechanical diversity we see in silk and feathers,[160] allows efficient attack and neutralization of hostile microorganisms,[161] and expedites the vast majority of operations required for cell signal and function. The difficulty in accessing synthetic biomolecular protein conjugates is largely dependent on the requirement for site-specificity – simple, general functionalisation may be possible, but selectivity at a single amino acid residue at a single site on a wild-type protein surface requires more elegant methods. For this reason, there has been much investigation into recombinant expression methods, through which AAs possessing orthogonal reactivity to the rest of the protein may be incorporated.[72]

Many bioconjugation approaches proceed at Cys and Lys residues, which possess thiol and amino containing side chain residues respectively (Scheme 1.21). Cys in particular is widely utilised for conjugation due to the potent nucleophilicity of the thiolate anion, which is present in aqueous reaction conditions at neutral pH.[162] One major caveat of relying on Cys however, is its rarity – it is the second least abundant amino acid found in natural proteins, with an abundance of *ca.* 1.7%.[163]

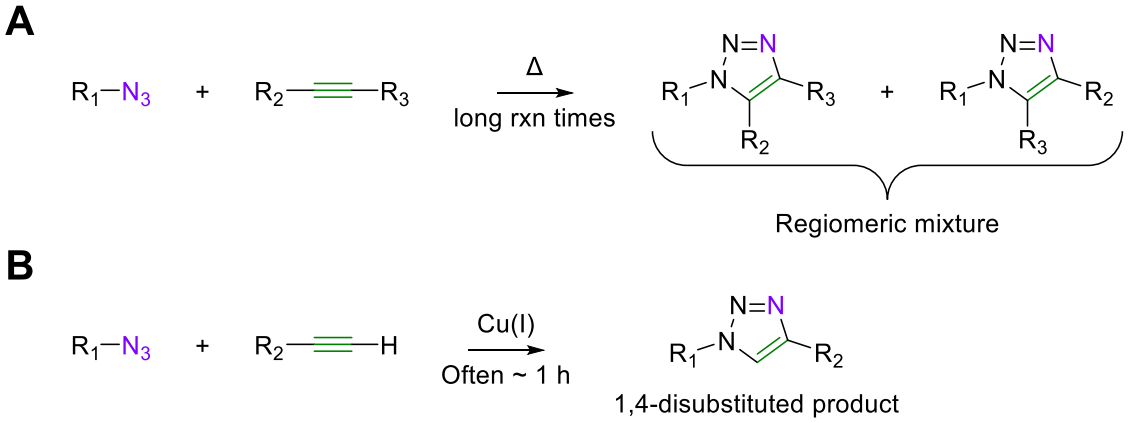


Scheme 1.21 The nucleophilicity of thiol (**A**) and amino (**B**) side chains can be exploited for bioconjugation with different reagents, yielding glycopeptides.

Side chain functionalisation approaches are challenging when one attempts bioconjugation *in vivo* or in cell lysate –covalent bond formation will occur at many residues, including those present in any untargeted proteins present. For this strategy to be successful, a biologically orthogonal functional group is required, which can be selectively ligated without risk of competing nonspecific bioconjugation. These approaches largely rely on enzymatic modification of amino acid sequences and on small protein domains. These methods are used to provide a synthetic handle, ordinarily unreactive within biological systems, such as azides,[164-167] or alkynes.[168-171] Further derivatisation utilising these handles furnishes the biomolecule of interest, albeit often with a non-native linker.

1.5.1 Copper-catalysed azide-alkyne cycloaddition (CuAAC)

One bioconjugative method of particular interest to this work is the copper-catalysed azide-alkyne cycloaddition (CuAAC). CuAAC is the stalwart, prototypical example of transition metal catalysis in chemical biology and synthetic organic chemistry. The non-catalysed variant, designed by the late Prof. Rolf Huisgen, requires high temperatures and suffers from a lack of selectivity and sluggish reaction kinetics. The Huisgen 1,3-dipolar cycloaddition produces an equimolar ratio of regiomers, furnishing both 1,4- and 1,5-disubstituted 1,2,3-triazole linkages from this concerted thermal addition (Scheme 1.22, **A**).[172-173] Gratifyingly, the addition of catalytic quantities of copper (I) salts to the reaction boasts a striking and complete selectivity for the 1,4-disubstituted regiomers, with up to a seven-fold acceleration of the reaction kinetics (Scheme 1.22, **B**).[174]

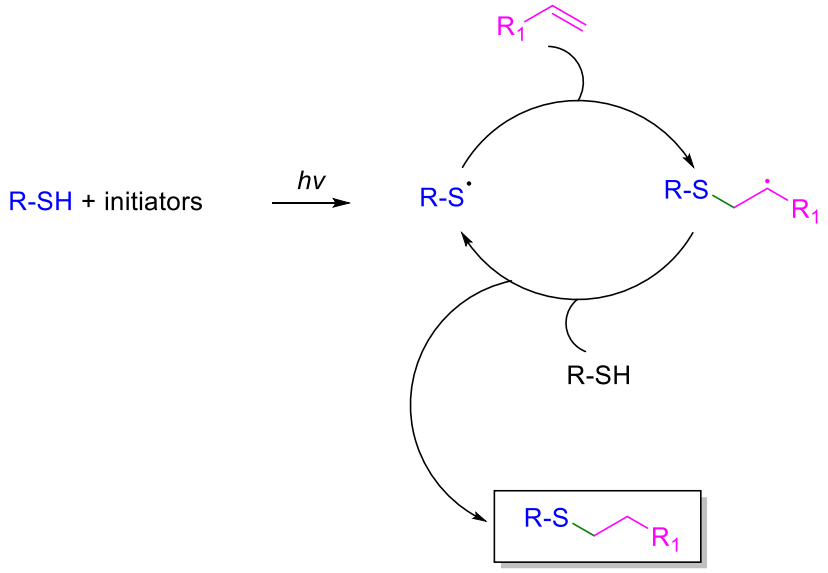


Scheme 1.22. The Huisgen 1,3-dipolar cycloaddition (**A**), and the copper-catalysed azide-alkyne cycloaddition (**B**),

Under the lens of bioconjugation, biomolecules possessing an alkyne or azide functionality may react with a cargo molecule possessing the opposite coupling partner. The 1,2,3-triazole linkage is explicitly non-natural, however it can somewhat be considered as 'biologically inert' – it is recalcitrant to oxidation, reduction, and hydrolysis, making it an invaluable bioisostere of the ubiquitous amide bond. Additionally, these traizole moieties participate in both dipole-diploe interactions and H-bond formation.[175-177] A further "click" method often employed for bioconjugation is the thiol-ene click, which we will examine below in section 1.6.

1.6 The Thiol-ene 'Click' (TEC) Reaction

Sulfur, in its -2 oxidation state, can act as either a nucleophile or a radical. The S-H bond is generally considered weak, with a bond dissociation energy (BDE) of 365 kJ/mol,[178] and as such both alkyl thiols and thiocarboxylic acids (thioacids) are precursors to thiyl/acyl thiyl radicals through facile homolytic cleavage. Thiyl radicals may be generated by homolytic bond breakage, and indeed by one-electron redox processes. In the former of these two, a carbon centred radical (for example, from the radical initiator azobisisobutyronitrile (AIBN)) will abstract a hydrogen atom from the thiol. Rate constants for this homolytic breakdown are of the magnitude 10^8 M^{-1} sec^{-1}, which is below the diffusion limit for this reaction.[179] In the case of redox processes, single electron oxidants such as Mn (III) complexes or other metal sources can be used to accomplish radical formation.[180-182] Both radiolysis and direct photolysis of the S-H bond under UV irradiation are also valuable pathways to thiyl radical formation.[183-184] Furthermore, it is compatible with both aqueous and oxygenated conditions, and thus bears the hallmarks of a click reaction.[185] The thiol-ene click is commonly utilised in surface science, chemical biology, and organic synthesis.[184] In the presence of a suitable photosensitiser and photoinitiator, ultraviolet (UV) light irradiation of a thiol or thioacid yields a thiyl radical, which adds onto alkene-containing substrates in an anti-Markovnikov manner. The analogous thiol-yne reaction involves the hydrothiolation on alkyne moieties.



Scheme 1.23. The thiol-ene 'click' (TEC) pathway, showing propagation of the cascade and product formation.

1.6.1 Intermolecular addition to alkenes and alkynes

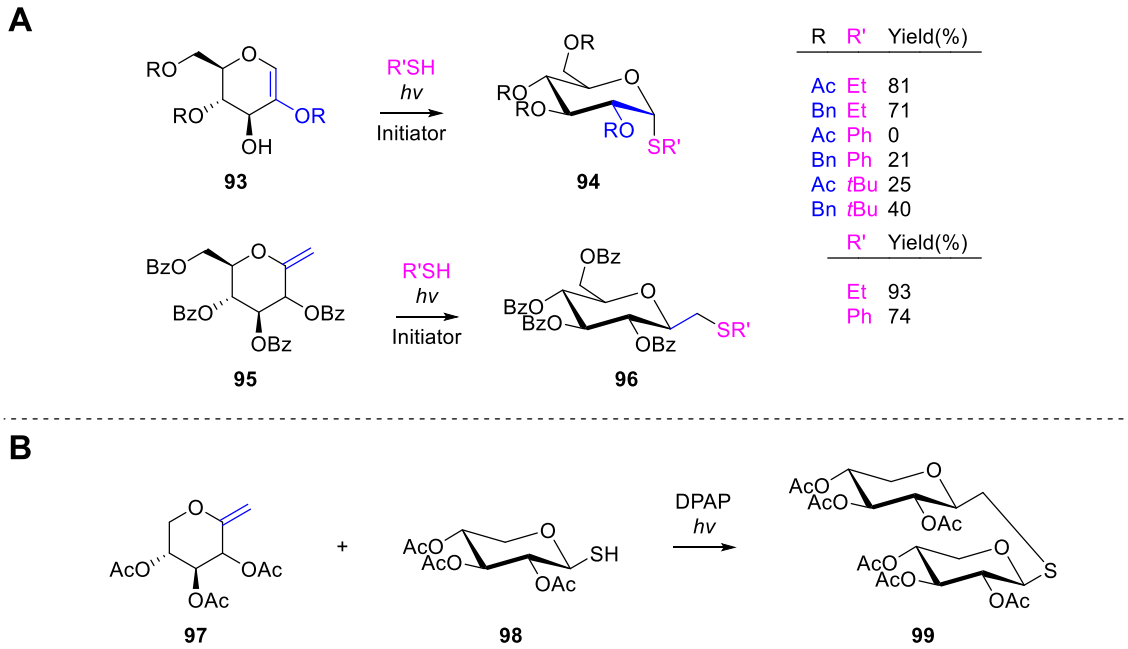
Possibly the most widespread use of thiyl radical based chemistry is focused on the intermolecular hydrothiolation of an alkene. The synthetic versatility of this intermolecular addition has been suitably employed in chemical biology, surface science and polymer chemistry.[184] In the case of thiols, a robust thioether linkage is furnished, which may undergo further transformation; for example *via* the Ramberg-Bäcklund reaction, in which the thioether is first oxidised to a sulfone.[186] The following subsection highlights a selection of the many applications of intermolecular thiyl radical addition; however, the author recommends a number of recent review articles.[184-185, 187-189]

Thiol-ene reactions are generally initiated using conventional methods (UV light, photosensitizer/photoinitiator pairs, and/or thermal initiators). The incipient thiyl radical exerts an anti-Markovnikov addition upon the unsaturated substrate at the least substituted carbon, after which the carbon-centred radical abstracts a proton from a remaining thiol molecule, which propagates the chain of the TEC. A number of termination routes are available in this process, and the general high yields observed throughout the literature suggest that the final abstraction does not directly interfere or consume the thioether product.

The use of intermolecular thiyl radical addition offers a valuable alternative to classical organotin-based radical reactions, and has been routinely employed in the construction

of complex molecular architectures for a number of decades. The synthetic utility of this radical conjugation for carbohydrate functionalisation has grown exponentially in present times.[188, 190] Employing both endocyclic and exocyclic glycals, a number of thiosugars have been furnished and characterised. In contrast, the converse pair of thiosugar and unsaturated substrates has also been examined for the formation of *S*-linked disaccharides.[191-193] The control of these reactions is not always straightforward, and while the regioselectivity can often be fine-tuned by the steric and electronic character of the substrates, diastereoselectivity is somewhat more complex. An account by Igarishi *et al.*[194] highlighted the challenges in controlling the diastereoselectivity of the TEC for carbohydrate transformation. Both Kushida and co-workers, and work by Borbás *et al*. found that C-2 substituents offered some selectivity in this process (Scheme 1.24, **A**).[191, 195-196] Borbás and co-workers further explored the selectivity exerted by the C-6 substituent of the endoglycal, and identified that steric bulk upon the β-face of the sugar facilitated stereoselective radical transformation in a 1,2-*cis* fashion.[191] Glycals presenting an *O*-benzyl on this primary alcohol displayed the same selectivity in this process.

Similarly, exoglycals have been investigated as substrates for the intermolecular TEC. In this case, thiyl radical addition is always expected to occur at the terminal carbon of the alkene, which yields a tertiary radical intermediate. An account by Gervay *et al.* explored the use of thioacetic acid on unsaturated glucose and fructose derivatives, and observed excellent stereoselectivity.[197] Pseudodisaccharides have also been obtained *via* exoglycals, notably in work disclosed by József *et al.*, in their TEC between an *exo*-xylyl derivative **97** and thiol **98** (Scheme 1.24, **B**).[198]



Scheme 1.24. (A) An account published by Borbás and co-workers with unsaturated monosaccharides. **(B)** Pseudodisaccharide synthesis by József *et al*.

1.6.2 Thioacids

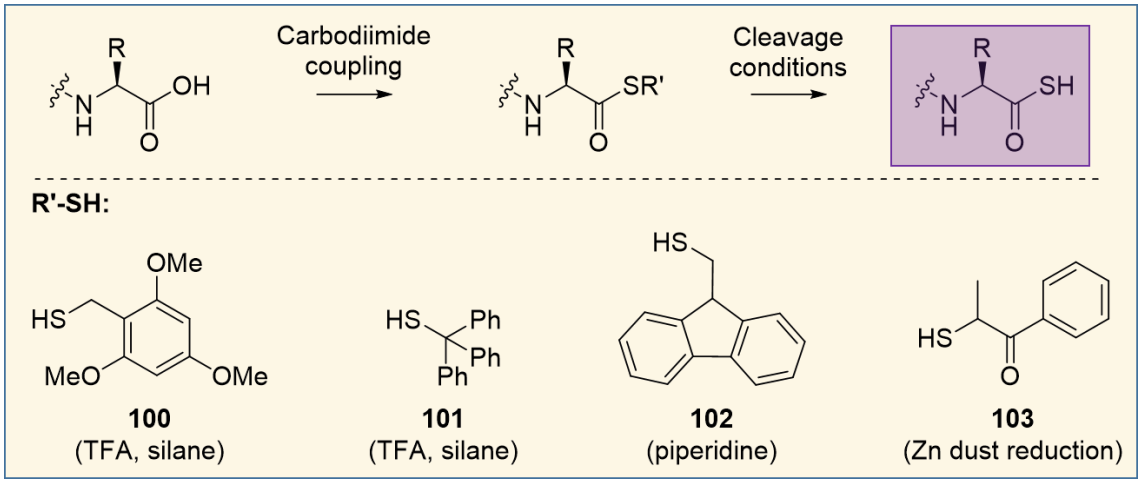
Thioacids offer an increasingly important source of acyl thiyl radicals for building thioesters. Thioacids boast powerful nucleophilicity, and relatively low pK_a (*e.g.* thioacetic acid p$K_a \approx 3.4$). Thioacids have earned their place as a privileged functional group in the construction of glycopeptides and challenging peptidic junctions and thus, reliable and reproducible conditions for their use must be established. A wealth of research has gone into understanding the utility of these amide synthons.[199]

The synthesis of *N*-protected amino thioacids is of interest to the present work, as the availability of such substrates forms the basis of the STING project (Chapter Two). Furthermore, the general transformation of commercially available carboxylic acids to their corresponding sulfur analogues is a prerequisite for many of the examples discussed in Chapter Four, regarding the TRACE cyclisation project.

There are a number of established synthetic methods to furnish thioacids from suitably protected amino acid precursors. AAs can be treated with Lawesson's reagent under microwave irradiation.[200] Another method for thioacid generation which boasts concomitant stereochemical retention involves the use of H_2S gas, and mixed anhydrides

of the amino acid precursor. Active esters such as *N*-hydroxysuccimidyl esters and *p*-nitrophenyl esters also have regularly been employed in this approach.[201-203] Na_2S and $NaSH{\cdot}H_2O$ can also be employed as sulfur sources for this reaction.

Beyond the traditional 'sulfur-lab', the synthesis of bench stable thioacid precursors often takes the form of Steglich esterification to furnish the required thioester; 2,4,6-trimethoxybenzyl (Tmob) **100**, trityl (Trt) **101**, 9-fluorenylmethyl (Fm) **102**, and α-methylphenacyl (MPa) **103** thioesters are all regularly employed for this purpose (Scheme 1.25). Standard carbodiimide coupling conditions generate isolable quantities of these intermediates, which liberate the hydrolytically sensitive thioacid under acidic or basic conditions (depending on the blocking functionality present).[204-207]



Scheme 1.25. Carbodiimide coupling offers access to thioester intermediates, which can be liberate thioacids under the correct cleavage conditions (in parentheses).

Disappointingly, peptidic and glycopeptidic thioacids suffer from extensive instability to atmospheric oxygen and sensitivity to hydrolysis. Storage in solid form at low temperatures, or as the K^+ or Na^+ salt, can alleviate some of the decomposition issues. Additionally, these salts are often more soluble in organic solvents.[208-209]

As evidenced in this introduction, synthetic chemistry has made major contributions to our understanding of biology and glycoscience, and permitted elucidation of the many complex functions of biomolecules. However, further methodology development is essential to improve and enhance existing techniques. In particular, the application of thiol-ene ligation remains somewhat underutilised in chemical biology, despite its high compatibility with biomolecules such as carbohydrates and proteins.

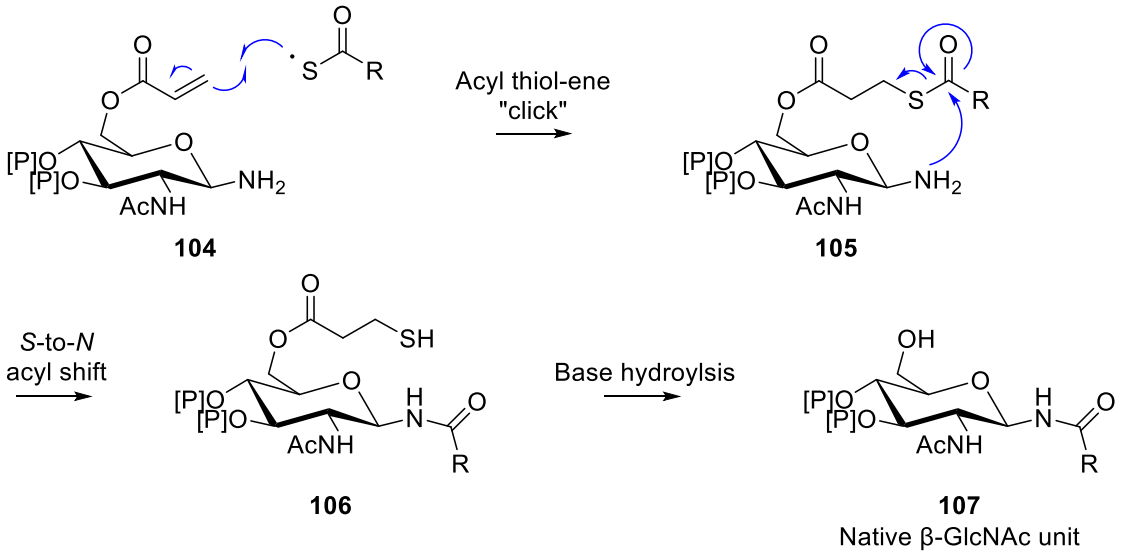
1.7 Work outlined in this thesis

The first portion of this work outlines studies towards achieving single glycoforms of IFNγ, for study of the biological effects of different glycosylation patterns. Two approaches are investigated in this work – a semisynthetic approach based on *S*-to-*N* acyl transfer, and a non-natural glycosylation with a glycan isolated from a natural source.

1.7.1 Sugar Templated Intramolecular *N*-Glycosylation (STING)

Through the preceding overview of the many methods to access *N*-glycopeptides, it is evident that the significant drawbacks associated with each technique warrants further investigation, through the design of novel methodologies. In the course of this work, the production of *N*-linked glycopeptides using a thiol-ene based thioesterification and subsequent *S*-to-*N* acyl shift to the *N*-glycan is investigated.

Our general strategy for *N*-glycosylation is outlined below in Scheme 1.26. A thioacid derivative of an amino acid or peptide functions as a thiyl radical donor for hydrothiolation of a terminal alkene located on the 6-*O* primary alcohol of a modified *N*-acetylglucosamine to furnish a thioester intermediate. Spontaneous *S*-to-*N* transfer of the peptide onto the anomeric amine furnishes a native glycan bond. Using the natural carbohydrate stereochemistry, the templating effect of the cyclic sugar should offset the reduced kinetics of the larger ring size.[107, 210] This Sugar Templated Intermolecular *N*-Glycosylation ('STING') approach is investigated for the synthesis of native linked glycopeptides.



Scheme 1.26. General concept of the 'STING' project.

1.7.2 Synthesis of Glycosylated IFNγ

In parallel with the development of the STING methodology, a more recombinant expression strategy to prepare homogenously glycosylated samples of IFNγ was investigated. In collaboration with Professor Marina Rubini (Centre for Synthesis and Chemical Biology, University College Dublin), it is a major goal of this research to express biorthogonal handles at the *N*-glycosylation sites of IFNγ, and to prepare complex mammalian glycans with which to "tag" the IFN- γ.

The production of IFNγ with defined glycans involves the expression of the cytokine through the *E. coli* model, but with non-natural amino acids (nnAAs) in place of the Asn residue usually found in the consensus sequence, as discussed previously in section 1.4.2. To summarise the previously described approach in sections 1.4.1 and 1.4.2, this is performed through nonsense suppression, in which amber suppressor tRNAs are chemically aminoacylated (an enzymatic ligation of an aminoacyl-dinucleotide with a tRNA, in place of the terminal dinucleotide unit) with the desired nnAA. The resulting tRNAs are added to the *E. coli* system possessing an mRNA strand containing the amber stop codon (UAG). The stop codon is then suppressed by the nnAA-tRNA conjugate, and position specific incorporation of the non-natural amino acid into the protein of interest is achieved.[211-212] These nnAAs possess modified side chains presenting alkynes, with which a classical chemical bioconjugation approach can be used – the copper-based azide-alkyne cycloaddition (CuAAC).[213]

In order to prepare a suitable azide bearing partner for semi-synthetic glycosylation of IFNγ, access to a reliable source of homogenous complex glycan is required. Hen egg yolks are widely reported to contain an undecasaccharide possessing a short hexapeptide fragment, known as egg yolk sialylglycopeptide (SGP).[214-217] This glycan in turn has been proven to be a mammalian glycan.[143, 218] Using the wealth of literature available on isolating the carbohydrate, the second portion of this project aims to devise an updated isolation procedure, and derivatise the glycan for use in CuAAC and potentially for further use in chemical protein synthesis. In Figure 1.9 below, a general scheme of the ligation between the "taggable" IFNγ and the derivatised azido-glycan is shown.

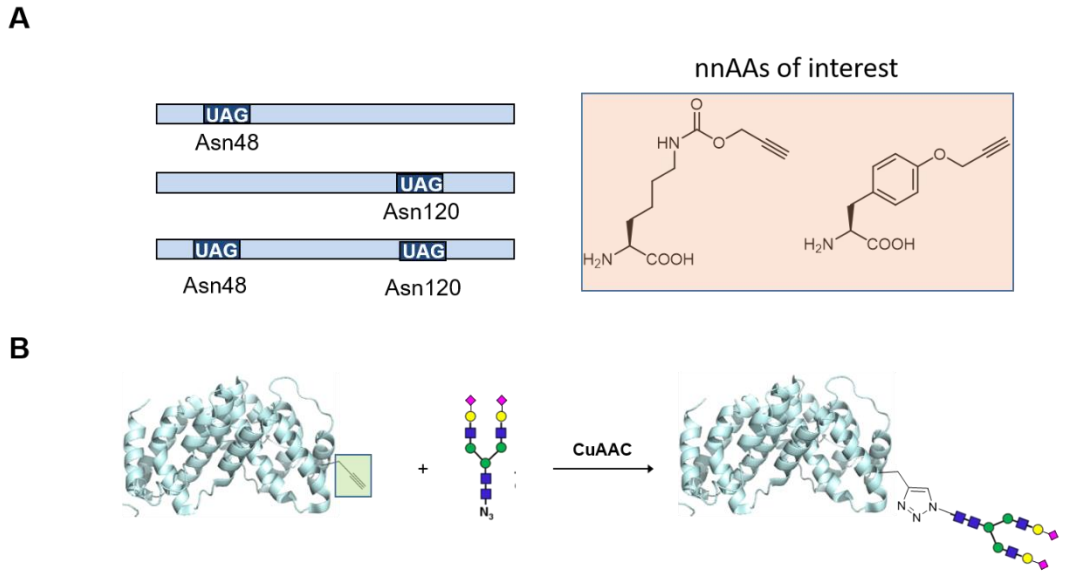
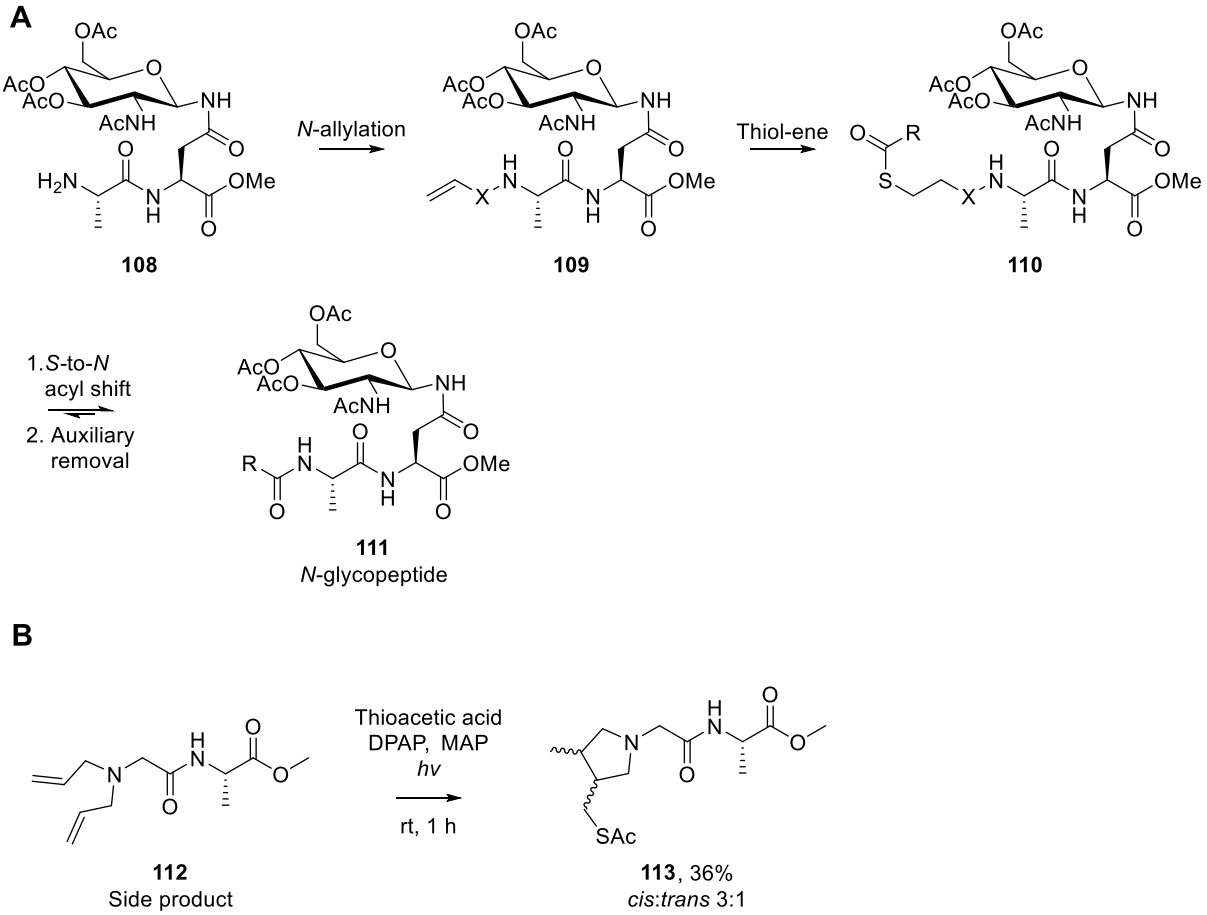


Figure 1.9. (**A**) The gene sequence of IFNγ, where the non-natural amino acids (nnAAs) will be inserted. (**B**) Chemoselective ligation between IFNγ (see alkynyl side chain highlighted in green) and the desired derivative of egg yolk sialylglycopeptide, sialyloligosaccharyl azide.

1.7.3 Radical Thiol-Ene Methodology for Cyclisation

One can argue that serendipity is key in scientific discovery, and that many collaborations and excellent accounts in the chemical literature originate from outliers in the data. Furthermore, some of the greatest feats of synthetic methodology have their roots in attempted total synthesis and natural product derivatisations.[219-221] In the course of supervising an undergraduate sophister project for *N*-glycopeptide ligation, an interesting radical thiol-ene mediated cyclisation methodology was discovered. During attempts to employ this auxiliary-mediated ligation on a basic dipeptide, the undergraduate student

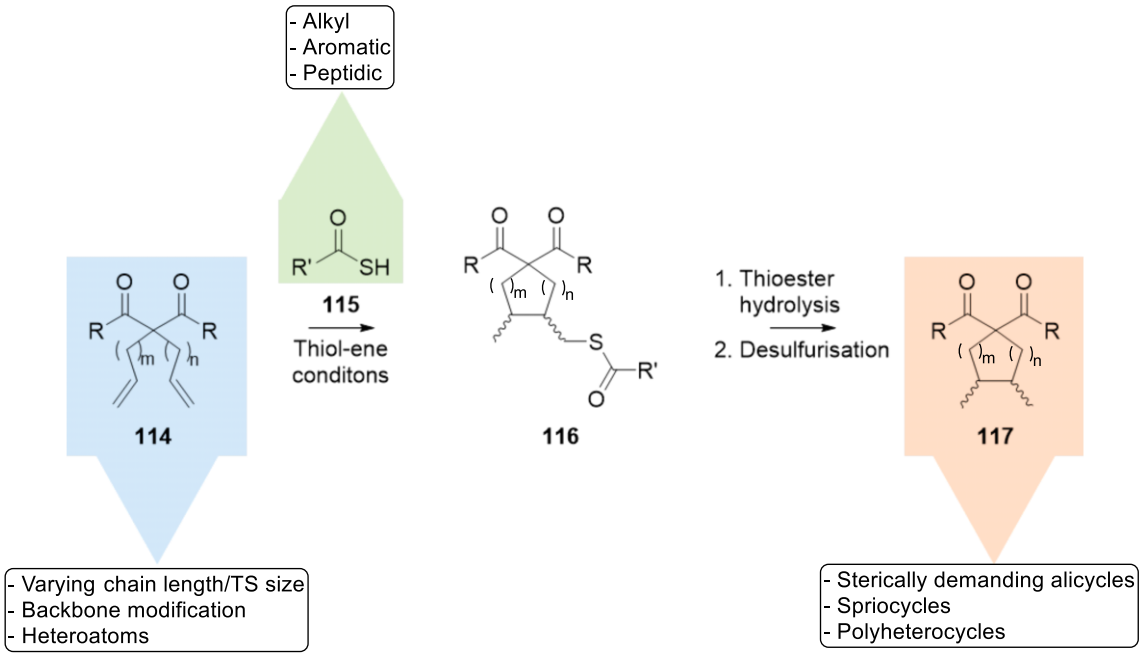
under the author's supervision instead observed a range of cyclised products for their test case. The intended ligation methodology (Scheme 1.27, **A**) and observed side reaction on a simple diallylated dipeptide **112** is shown below. While the principle of this reaction was discovered in the late 1970s/early 1980s,[222-223] the scope was not fully explored. Furthermore, in the course of this work, temperate and facile conditions were developed for the transformation in question, and reaction time was reduced by over 95%.



Scheme 1.27. A: The proposed AML strategy for furnishing *N*-glycopeptides. X = O, C. **B**: Di-functionalised dipeptide, isolated as an impurity and tested under thiol-ene conditions, gave cyclised product **113**.

Using thiyl radicals, an array of 1,6-dienes were imagined cyclizable *via* a **T**hiyl **R**adical **A**ddition **C**yclisation and **E**limination (TRACE) cascade. One envisioned a scope of viable thioacid precursors from which to generate thiyl radicals, and a slew of both heterocyclic and alicyclic products were proposed. The aim of this work was to optimise the reaction conditions and select the best thioacid to facilitate the proposed cyclisation, whilst tolerating varying functionality and substitution. Hydrolysis of the resultant

thioester and desulfurisation would therein provide a traceless approach to the cyclisation of dienes, diynes, enynes and ynamides. A representative example **114** possessing a 1,3-dicarbonyl backbone is shown in Scheme 1.28.



Scheme 1.28. The archetypal TRACE reaction for a 1,3-dicarbonyl type system. Varying types of thioacids will be examined, as will tolerance to heteroatoms and chain length in the unsaturated substrate. R = ester, amide, etc. $(\ )_m$ and $(\ )_n$ indicate varying chain lengths

1.7.4 Collaboration – Glycosylated Naphthalimides

A collaboration with the Gunnlaugsson group in Trinity College Dublin and the Elmes research group in Maynooth University is briefly discussed. The synthesis of a glycosylated cytotoxic drug, possessing a self-immolative linker is disclosed. Additionally, the real-time tracking and bio-imaging of this prodrug upon enzyme-triggered release is evaluated.

The chapter in question contains results contributed by Dr. Elena Calatrava-Pérez, and the synthetic detail of experiments conducted by Dr. Pérez and the present author.

1.7.5 Collaboration – Rhamnosides for Immunological Studies

In collaboration with the Lavelle group in Trinity College Dublin, this chapter examines mycobacterial *para*-hydroxybenzoic acid derivatives (*p*HBADs) and their viability in the

induction of macrophage innate memory. The synthetic detail of a number of rhamnosides is disclosed, and their prospects in reducing macrophage bactericidal mechanisms over varying time periods are evaluated.

The chapter in question contains biological results contributed by Dr. Mimmi E. Lundahl, and the synthetic detail of experiments conducted by the present author, Dr. Danielle Barnes, and Dr. Lauren McSweeney.

Chapter Two

STING

2.0 Sugar Templated Intramolecular *N*-Glycosylation (STING)

The *S*-to-*N* acyl shift (often referred to as *S,N*-acyl transfer) represents a chemoselective and high-yielding process for amide bond formation. It has found widespread application in synthetic organic chemistry for peptide and protein synthesis, chemical modification of proteins, protein-protein ligation and the development of probes and molecular machines.[224]

Of particular recent interest are 'templated' *S,N*-acyl transfer processes whereby a molecular scaffold enables spontaneous *S,N*-acyl transfer over a macromolecular ring-size transition state.[225-226] Within the context of templated *S,N*-acyl transfer processes, carbohydrates exist as privileged molecular scaffolds for enabling amide bond formation. The most widely utilised example of this approach is Sugar Assisted Ligation (SAL), a form of Auxiliary Mediated Ligation (AML) introduced and developed by Wong and co-workers.[106-107, 110] In SAL, a thiosugar auxiliary is prepared as a glycosyl amino acid building block and installed *via* SPPS adjacent to the ligation site, where it mediates amide bond formation between two peptide fragments to furnish a glycopeptide product. The residual thiol auxiliary can be efficiently removed using established desulfurisation methods. A notable aspect of SAL is the size of the cyclic transition state; with SAL proceeding *via* a 14-membered transition state, the large transition state believed to be facilitated by the sugar moiety which acts as a rigid scaffold for the reacting groups.

The templating effect of the carbohydrate unit was later employed by Brik and co-workers using a cyclohexane auxiliary to mimic the carbohydrate unit in SAL, to facilitate acyl transfer over an 11-membered transition state for use in peptide ligation.[106-107, 110] Recently, Guo *et al.* have reported an efficient *N*-glycosylation reaction between glycosylamines and *p*-nitrophenyl thioester peptides for the synthesis of glycopeptides using the protocol of native chemical ligation.[227] Admittedly, the possibility of intermolecular *versus* intramolecular *S*-to-*N* acyl transfer can limit the application of a methodology which boasts the use of templating scaffolds. This is challenging to address, as dilution studies often 'shut-down' any sort of reactivity, even that which is unrelated to the acyl transfer.

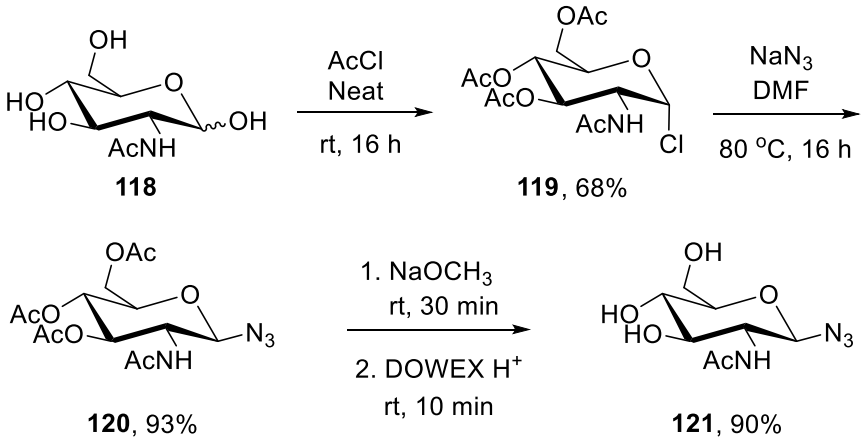
Inspired by the concept of SAL and the intermolecular *N*-glycosylation approach of Guo, we set out to investigate if a **S**ugar **T**emplated **I**ntramolecular ***N*-G**lycosylation (**STING**)

methodology could be developed to facilitate chemoselective *N*-glycosylation. The critical step involves *S*-to-*N* acyl transfer of an acyl group located on the 6-*O* primary alcohol of a modified *N*-acetyl-D-glucosamine, to the anomeric amine to yield a native glycan bond. The initial glycothioesters are prepared using thiol-ene "click" (TEC), a photochemical or thermal induced hydrothiolation of an alkene which boasts high efficiency, short reaction times and orthogonality to a wide range of functional groups. This chapter discloses the first examples, scope and limitations of this synthetic approach.

2.1 Synthesis of the thiol-ene acceptor sugar

In order to assess the scope of the TEC for *N*-glycosylation methodology, access to a modified GlcNAc displaying an ester with a terminal alkene on the 6-*O* position and a protected amine at the anomeric centre was required. It was decided to first synthesise the β-azide sugar, which could be subsequently reduced to the amine, either before or after the TEC.

Commercially available *N*-acetyl-D-glucosamine **118** was peracetylated using neat acetyl chloride. Portionwise addition of the monosaccharide to the acetylating reagent gave a strong exothermic reaction with a visible colour change to a red/pink syrup observed after 6 h and the release of white fumes of hydrochloric acid (HCl). After stirring at rt for 16 h, the desired α-chloride donor **119** was isolated as a white powder through an aqueous workup followed by precipitation with cold diethyl ether (Et_2O), in good yield of 80% (Scheme 2.1). Successful installation of the α-chloride donor was confirmed by the ^1H NMR spectrum, which showed a doublet at 6.18 ppm with a *J* value equal to 3.7 Hz. This coupling is consistent with the expected small vicinal H1-H2 coupling, as is the downfield shift of the anomeric proton H1.



Scheme 2.1. Synthesis of β-azide GlcNAc unit.

An α-configuration at the anomeric centre was expected due to the anomeric effect, a trend in which the α-anomer of substrates bearing electronegative substituents at C1 is favoured. This is a generally observed phenomenon in the field of carbohydrate chemistry, where electronegative substituents at C1 prefer the axial orientation ('α') rather than the equatorial orientation ('β'). This effect was first observed by J. T. Edward in 1955 at Trinity College Dublin, and was later designated the *anomeric effect* by R. U. Lemieux in 1958.[228-229] Letting 'X' equal some electronegative substituent, the interaction and delocalisation of the endocyclic oxygen long pair (**n**) to the C1-X antibonding orbital (**σ***) offers stabilisation – the bond to the anomeric substituent is lengthened while the C-O bond within the sugar ring is shortened, as one would expect in the case of hyperconjugation. Conversely, this effect is not observed in the case of the β-anomer, and thus this epimer is less favoured (Figure 2.1).[230]

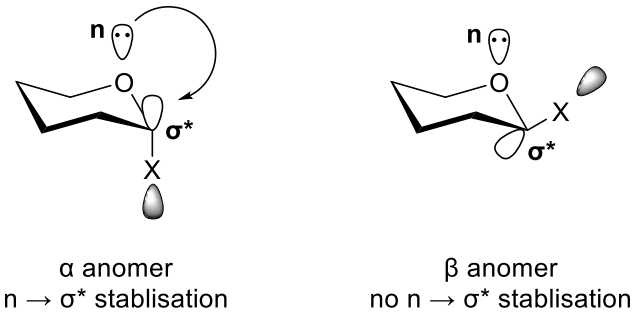


Figure 2.1. The anomeric effect, with X signifying an electron withdrawing substituent.

In an effort to prevent unwanted hydrolysis of the anomeric centre, the α-chloride donor was immediately dissolved in anhydrous *N,N'*-dimethylformamide (DMF) and sodium azide was added. The chloride was subsequently displaced in an S_N2 process during

stirring of the suspension at 80 °C for 16 h. An aqueous workup and purification by column chromatography gave the peracetylated anomeric azide **120** in a good yield of 82%. The identity of isolated sugar was confirmed through FTIR spectroscopy, with the recorded spectrum displaying a characteristic azide peak at 2104 cm^{-1}. Confirmation of the installation and successful stereochemical inversion at the anomeric centre was confirmed by the upfield shift of the H1 doublet resonance to 4.76 ppm, and a *J* value of 9.3 Hz — the larger H1-H2 vicinal coupling constant is consistent with stereochemical inversion to the β-anomer.

Following Zémplen deacetylation upon addition of freshly prepared catalytic $NaOCH_3$ in anhydrous methanol, the *O*-acetyl groups were selectively removed without affecting the C2 amide. Upon quenching this reaction, the removal of the sodium alkoxide salt with acidic resin (DOWEX 50wx8 H^+ form) and evaporation of solvent *in vacuo* gave **121** in excellent yield of 92%.

2.1.1 Ester Installation and Limitations

In our initial approach, the aim was to protect the free hydroxyl groups of the sugar with base-labile protecting groups. This would facilitate global deprotection under alkaline conditions to furnish the native β-GlcNAc unit following TEC and *S*-to-*N* acyl transfer. Both acryloyl and 4-pentenoyl chloride were employed separately to furnish the 6-*O* decorated TEC substrates, which would allow the author to probe different TS ring sizes. Gratifyingly, the reaction with 4-pentenoyl chloride furnished the desired glycoester **126** in a good yield of 72%. This was then peracetylated with acetic anhydride and pyridine, achieving near quantitative yields of substrate **127**. The product was stable when protected from light at -20 °C for *ca*. three months.

Unfortunately, the use of acryloyl chloride (AcrCl) did not give the desired 6-*O* ester in good yield; only 34% isolated yield of the desired product was achieved and this proved difficult to separate from starting material and triethylamine hydrochloride. Furthermore, this reaction did not scale well beyond the milligram scale. A variety of temperatures, reaction times and equivalents of acid chloride were explored and are outlined in Table 2.1. The presence of free C3 and C4 hydroxyl caused the compound to streak during purification, and separation of the desired material from the concomitantly formed triethylamine hydrochloride posed a challenge. Furthermore, scaling this reaction above

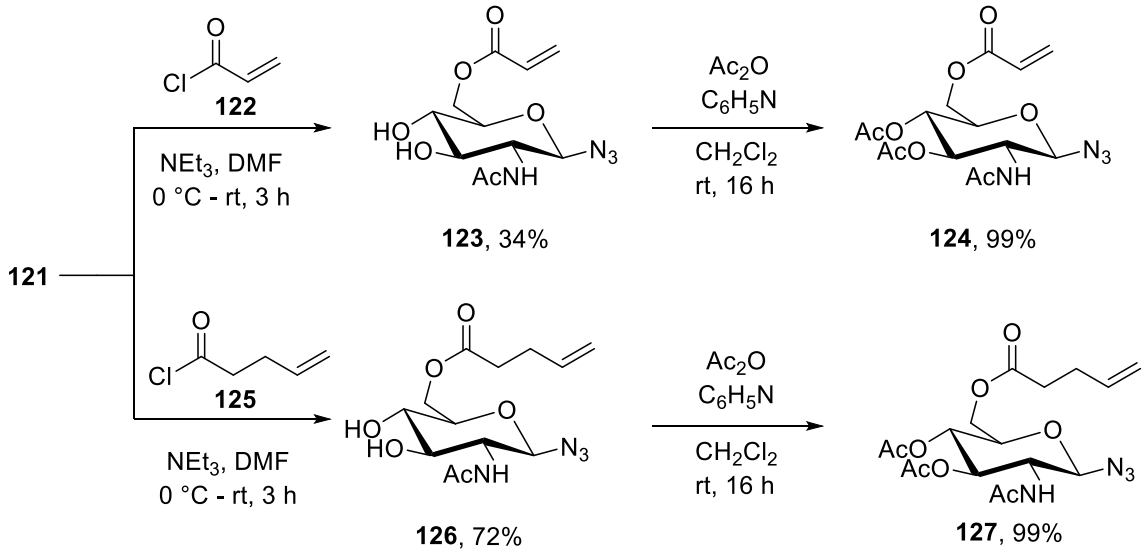
0.8 mmol resulted in no conversion to the desired ester. Despite purification challenges, a slow gradient elution from a silica column did eventually prove successful in isolating **123**, which was subsequently peracetylated under standard conditions with acetic anhydride and pyridine to give **124** in 99% yield (Scheme 2.2).

Table 2.1. Summary of acryloyl ester formation conditions.

Entry	AcrCl (eq.)	Solvent	Base[a]	T (°C)[b]	Time (h)	Yield (%)
1	1.2	DMF	TEA	0 °C → rt	3	10
2	**1.2**	**DMF**	**TEA**	**-20 °C → rt**	**3**	**34**
3	1.4	DMF	TEA	-20 °C → rt	16	11
4	1.2	DMF	DIPEA	0 °C → rt	3	12
5	1.2	DMF	DIPEA	-20 °C → rt	3	29
6	1.4	DMF	DIPEA	-20 °C → rt	16	23

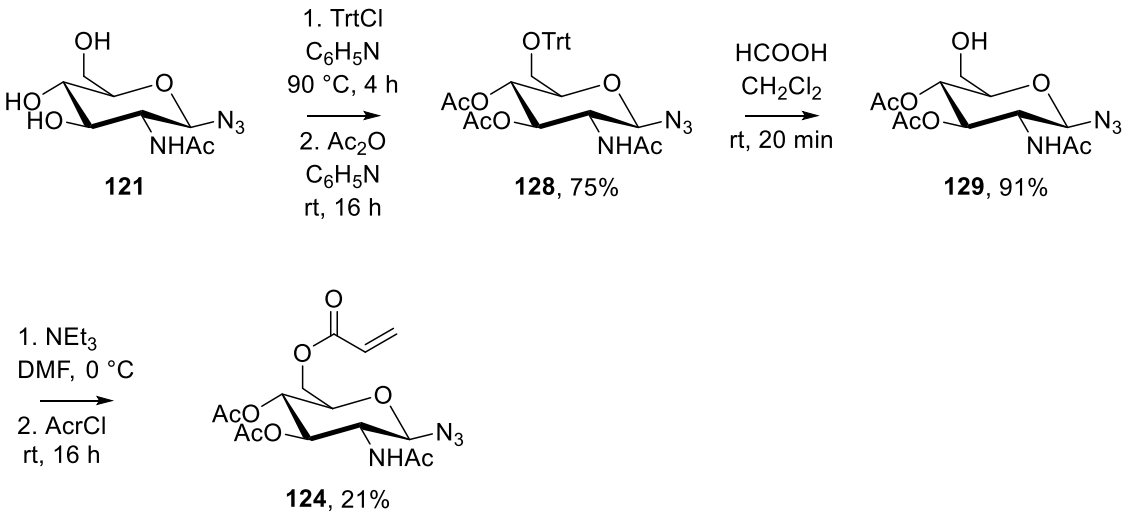
[a] Equivalents of base added were equal to those of the acryloyl chloride. [b] Addition of the acid chloride at low temperature, and reaction allowed to warm to rt upon completion of addition.

It was hypothesised that either i) residual methanol from the Zémplen deacetylation was quenching the acid chloride, or ii) incomplete desalting with DOWEX had occurred, leaving the sugar with sodium salts of its alcohols. However, repeated azeotropic distillation with cyclohexane to remove methanol did not improve yields of **123**, nor did the use of varying resin mesh-sizes/resin batches. The inconsistencies in yield may originate from differing batches of AcrCl, or some additional unidentified experimental factor.



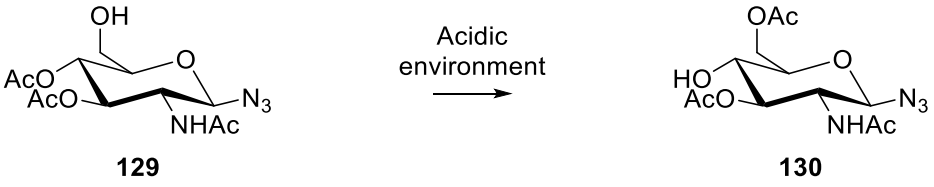
Scheme 2.2. Synthesis of TEC acceptor sugars.

The problems associated with the use of acryloyl chloride necessitated the development of a further synthetic route to the TEC acceptor **127**. Selective protection of the 6-*O* alcohol with a group orthogonal to the acetate protecting groups of the secondary alcohols was investigated (Scheme 2.3). A triphenylmethane (trityl) ether was installed using trityl chloride suspended in anhydrous pyridine for 4 h at 90 °C. Aqueous workup followed by acetylation of the crude tritylated material in a mixture of acetic anhydride and pyridine gave the fully protected sugar **128** in good yield of 75%. This reaction was scaled up, allowing the synthesis of up to 20 g of **128** per iteration as a bench stable intermediate.



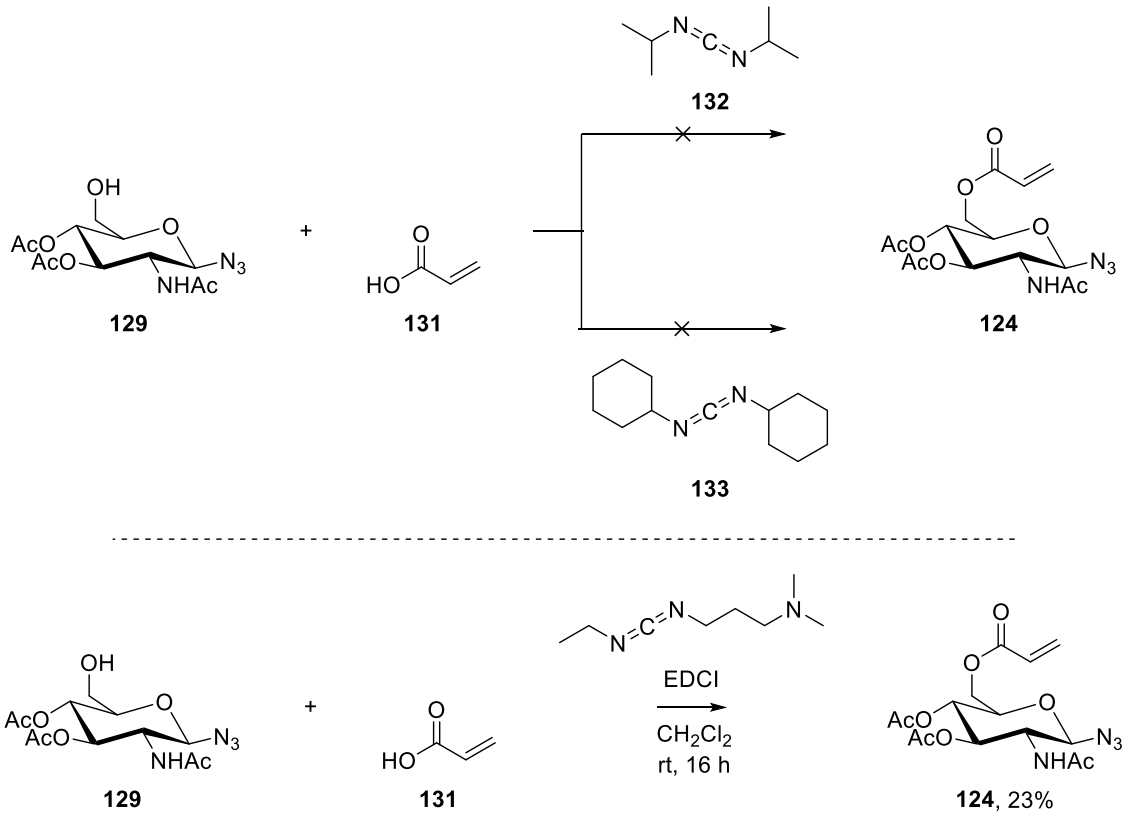
Scheme 2.3. Synthesis of TEC acceptor through trityl protecting group strategy.

The trityl ether was selectively deprotected to the primary alcohol with a solution of formic acid in less than 30 minutes, after which time co-evaporation of the reaction mixture with toluene and purification of the residue gave **129** in excellent yield of 91%. This material was quickly subjected to the next step, as the 4→6 acetate migration is a significant caveat of this approach (Scheme 2.4).[231]



Scheme 2.4. A 4→6 acetate migration is observed when **129** is left in a weakly acidic environment (e.g., silica gel) over hours to days.

Disappointingly, attempts to install the acrylic ester using more forceful conditions proved futile, as the reaction was again non-scalable past 100 mg. In an effort to pinpoint the exact problem, a Steglich esterification of **129** with acrylic acid using a variety of coupling reagents in the presence of catalytic 4-dimethylaminopyridine (DMAP) was tested (Scheme 2.5). In the case of *N*,*N'*-diisopropylcarbodiimide (DIC) and *N*,*N'*-dicyclohexylcarbodiimide (DCC), no product formation was observed at rt or 40 °C. EDCI·HCl gave a yield of 23% at 40 °C, using 1.2 eq. of both the acrylic acid and coupling reagent.

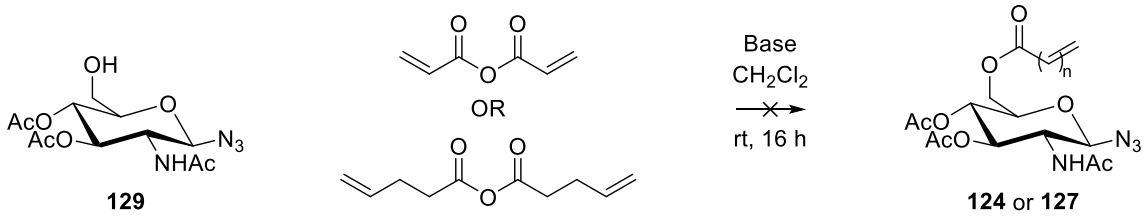


Scheme 2.5. Attempts at using carbodiimide reagents. Only EDCI (bottom) gave the product, albeit in low yield.

2.1.2 Scaling Attempts

As a final attempt to optimise and scale-up the synthesis of the thiol-ene acceptors, acrylic anhydride and 4-pentenoic anhydride were purchased from commercial sources. Both reagents possess a considerable predilection for polymerisation, evidenced by the exponentially increasing viscosity of the material as days passed from its delivery. **128** was again deprotected with formic acid to yield **129**, which was immediately reacted with

either 4 eq. of acrylic anhydride (Acr_2O) or 4-pentenoic anhydride in CH_2Cl_2, with an additional 4 eq. of pyridine (Scheme 2.6). In the case of Acr_2O, the presence of **any** base resulted in a trace of product formation, and polymerisation throughout. Changing the base to triethylamine, or diluting the reaction media ten-fold did not alleviate the ostensible reactivity of this active anhydride. Instead, the use of 4-pentenoic anhydride was considered as a scalable alternative to the synthesis of glycosyl azide **127**.



Scheme 2.6. Attempted ester formation with anhydrides. Base was either NEt_3 or pyridine.

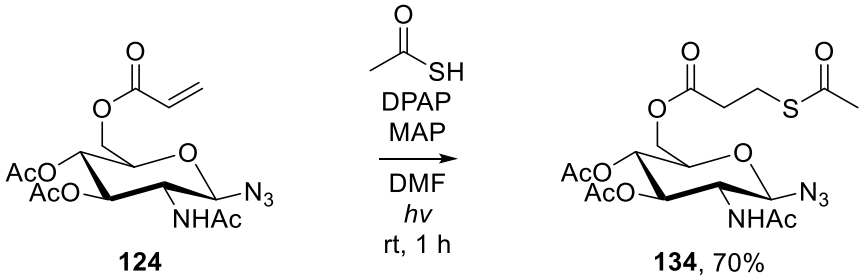
The use of pyridine or NEt_3 alone did not generate the desired thiol-ene acceptor in any perceptible amount when 4-pentenoic anhydride was used — precipitate was consistently observed in the reaction flask. Some moderate success was observed when the combination of 4-pentenoic anhydride, pyridine, and catalytic DMAP was employed, however there seemed to be some migration of the acetates from C4 of the sugar over the 16 h reaction timescale.

We decided to rely on the non-scalable reaction of 4-pentenoic chloride with **121** to furnish **127**. Selective esterification at the 6-*O* of **129** was not achieved in appreciable yield with any of the classical carbodiimide coupling conditions or anhydride conditions, and in conjunction with the previous acid chloride results this indicates that the formation of an acrylic ester at the 6-*O* linkage is difficult to achieve. Nevertheless, the small quantities of **127** available were subjected to thiol-ene conditions to test the viability of this substrate.

2.2 Initial thiol-ene click, and reduction of β-azide

With material combined from the various optimisation attempts of acrylic ester formation, the ability of **124** to function as an acceptor substrate for thiol-ene ligation was investigated. As the Scanlan group has explored thiol-ene chemistry for bioconjugation over several years, prototypical reactions with a photoinitiator/photosensitiser pair were investigated. The combination of photoinitiator 2,2-dimethoxy-2-phenylacetophenone (DPAP) and photosensitiser

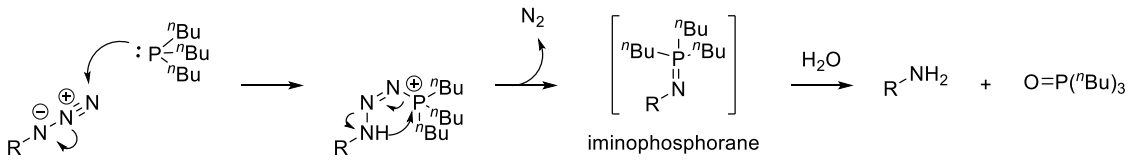
4’-methoxyacetophenone (MAP) were initially tested for ligation under UV irradiation — Yagci and co-workers have identified that the use of DPAP as initiator often provides the highest efficiencies under ultraviolet (UV) irradiation.[232] UV irradiation of glycosyl azide **124** with this DPAP/MAP pair and thioacetic acid (1.2 eq.) in DMF gave the product **134** in an isolated yield of 70%, with no aberrant side products observed, and with 0.1 eq. loadings of the photoinitiator and photosensitiser (Scheme 2.7). The NMR spectrum indicated quantitative consumption of the alkene after 1 h of irradiation. Furthermore, a control experiment was prepared with a solution of **124** in DMF and thioacetic acid, DPAP and MAP, placed in the dark for 1 h. No thia-Michael addition was observed on this timescale. Additionally, the 4-pentenoyl ester analogue **127** was subjected to the same conditions, and furnished the thioacetate ester **127a** in a 93% yield following isolation by column chromatography.



Scheme 2.7. TEC of acceptor **124** and thioacetic acid.

2.2.1 Azide Reduction

Following successful formation of the thioester, azide reduction of **134** was attempted under a range of conditions. While azide chemistry has a longstanding history with innumerable synthetic uses,[233-234] anomeric azides are recalcitrant to some of the most established reductive conditions, and stereochemical retention is often difficult to achieve. In tandem with the need to leave protecting groups and other functionalities intact, only a handful of anomeric azide reductions have been reported.[235-239] In the course of the present work, classical Staudinger reduction[240] was attempted with both triphenylphosphine and tributylphosphine. The Staudinger reduction was first described at the beginning of the 20th century, and has been widely employed due its versatility and mild reaction conditions. A tertiary phosphine, such as *n*-tributylphosphine, reacts with an azide to form a phosphazide intermediate, and N_2 gas is extruded as the driving force of this reaction. The loss of nitrogen generates an iminophosphorane which is hydrolysed to yield the desired amine and the respective phosphine oxide (Scheme 2.8).

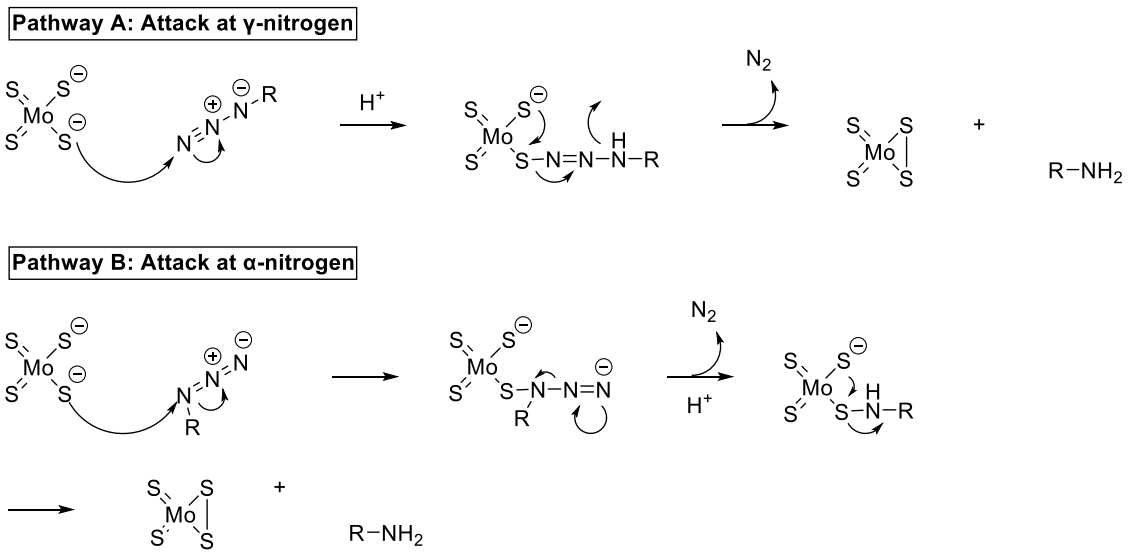


Scheme 2.8. Mechanism of the Staudinger reduction with *n*-tributylphosphine.

Both triphenylphosphine and *n*-tributylphosphine were employed in the course of this work. In both cases, an iminophosphorane intermediate was observed, which was extremely resistant to hydrolysis. Other classic approaches to azide reduction such as catalytic hydrogenation were tested, yet failed to reduce the anomeric azide. Furthermore a novel approach by Suthagar *et al.* was tested in which the glycosyl azide was concentrated on a rotary evaporator at 200 mbar, in the presence of NaI and a strongly acidic resin (Amberlite-IR120 H^+ form).[241] This led only to decomposition in our hands.

Catalytic hydrogenation offers high yield of the anomeric amine, but this approach is rendered unsuitable by the lack of stereoselectivity and the failure of the reaction once the thioester is in place. This is likely due to poisoning of the Pd surface by the presence of sulfur.[242-243] Even with equimolar amount of Pd/C or $Pd(OH)_2/C$ in a range of solvents, anomeric hydrolysis prevailed, instead of the desired azide reduction.

Reduction of the azido group prior to thiol-ene ligation, while not ideal due to the labile nature of anomeric amines, provided opportunity to test alternative reducing conditions. One methodology of particular interest is the use of a tetrathiomolybdate reduction, reported by Chandrasekaran and co-workers for the stereospecific reduction of anomeric azides in the presence of many functional groups, including acetyl (Scheme 2.9).[239]



Scheme 2.9. Reduction with tetrathiomolybdate ion, as reported by Sridhar *et al*.[239] Two distinct pathways of attack are possible.

Under sonochemical conditions, tetrathiomolybdate salts were investigated for the reduction of azides **124** and **127**. Unfortunately, the acrylic ester derivative **124** decomposed under these conditions, even when the reaction was simply stirred overnight and not subject to sonication. The reduction of the glycosyl azide post-TEC once again suffered from redox problems due to the thioester functionality — Stiefel and co-workers reported that the tetrathiomolybdate ion (MoS_4^{2-}) is readily oxidised to the $Mo_2S_8^-$ dimer, which would attribute the lack in reactivity to this dimerisation process.[243] Following these studies, we decided to focus on investigating TEC on substrates displaying an anomeric amine, as post-ligation azido reduction had proved fruitless.

Gratifyingly, this method worked well for pre-ligation reduction of the anomeric azide **127**, furnishing **135** in a 53% yield over 3 h. The azide **127** was dissolved in CH_3CN:EtOH and treated with $(NH_4)_2MoS_4$ in a small sonicator bath (Scheme 2.10). The azide reduction was visualised in real-time with the use of FT-IR, with 500 μL aliquots of the reaction media taken at defined time points to track the disappearance of the azide stretch. (Figure 2.2, *vide infra*).

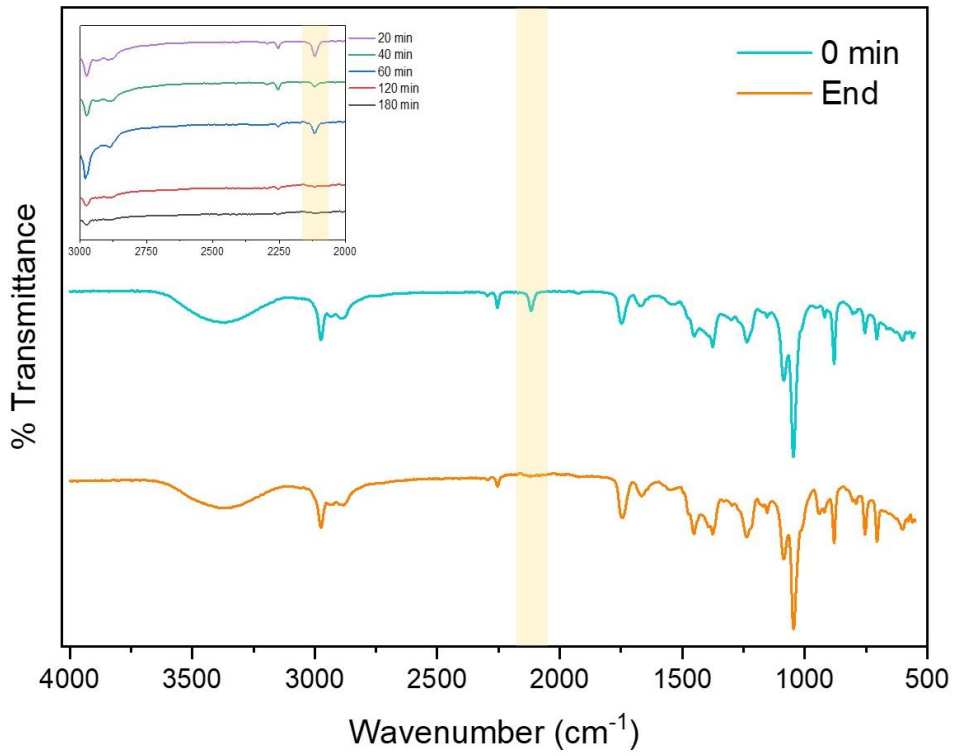
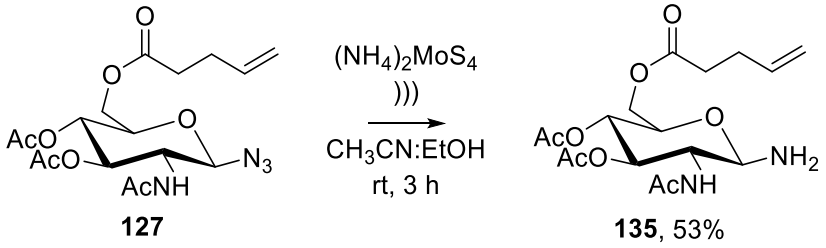


Figure 2.2. IR timescale experiment showing reduction of azide functionality over 180 min. **Insert:** Individual time points, with azido peak highlighted.

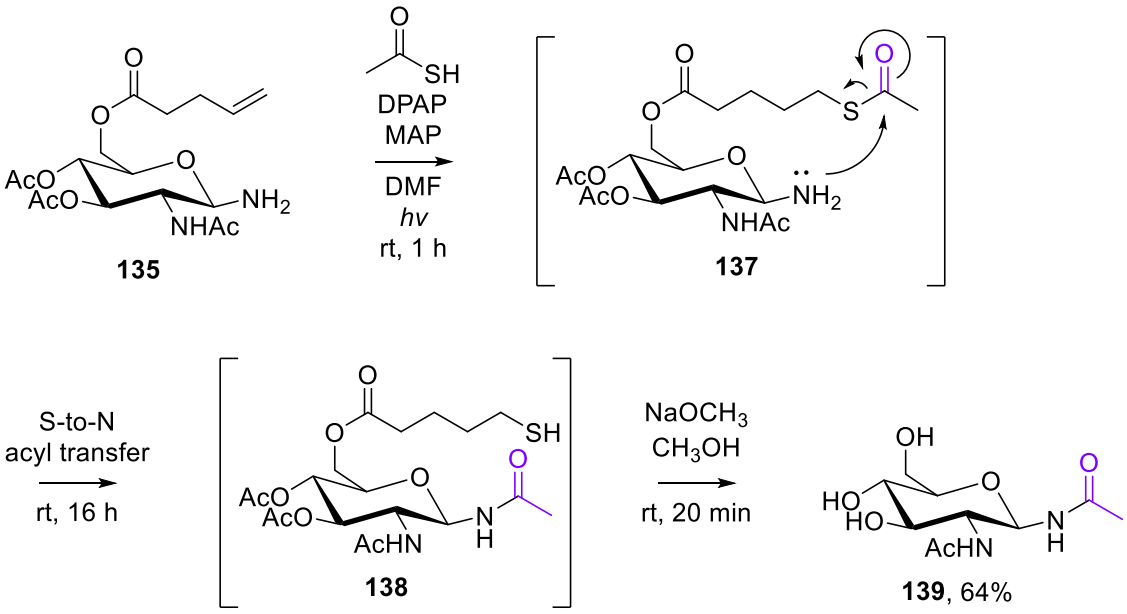
Once the IR spectra indicated the complete consumption of the azide, the reaction was repetitively filtered to obtain **135** as a colourless oil in a good yield of 53%. The loss in yield is attributed to the continual filtration through Celite® required to remove all traces of dimerised $Mo_2S_8^-$ and persulfido species. ^{1}H NMR analysis revealed the presence of exclusively the β-anomer of **135**, and the identity of the amine was confirmed *via* HRMS and NMR analysis. Gratifyingly, the alkene functionality remained intact throughout this reduction. Another molybdenum species, benzyltriethylammonium tetrathiomolybdate $[BnEt_3N]_2MoS_4$, was also investigated in a further iteration of this experiment. No appreciable improvement in isolated yield was identified, as the workup of this reaction required several filtration steps to remove the brick-red colour. Retention of yield with this organic derivative was observed.



Scheme 2.10. The reduction of glycosyl azide **127** proceeds well under sonochemical conditions.

With a viable substrate for TEC and a suitable anomeric azide reduction methodology in hand, the viability of STING was investigated by attempting to 'shift' thioacetic acid to the anomeric locus. Photochemical thiol-ene was performed on glycosylamine **135** with thioacetic acid under the previously described conditions. Following completion of the alkene hydrothiolation, the reaction mixture was removed from the photochemical reactor and allowed to stir at rt for 16 h. Reports of *S*-to-*N* over comparable ring sizes to our 13-membered transition state indicate reaction times of 8 and 5 h at sterically unhindered ligation sites.[107, 244] Following the elapsed time, base hydrolysis of the acetate protecting groups ensued, with concomitant removal of the ester at the 6-*O* alcohol (Scheme 2.11).

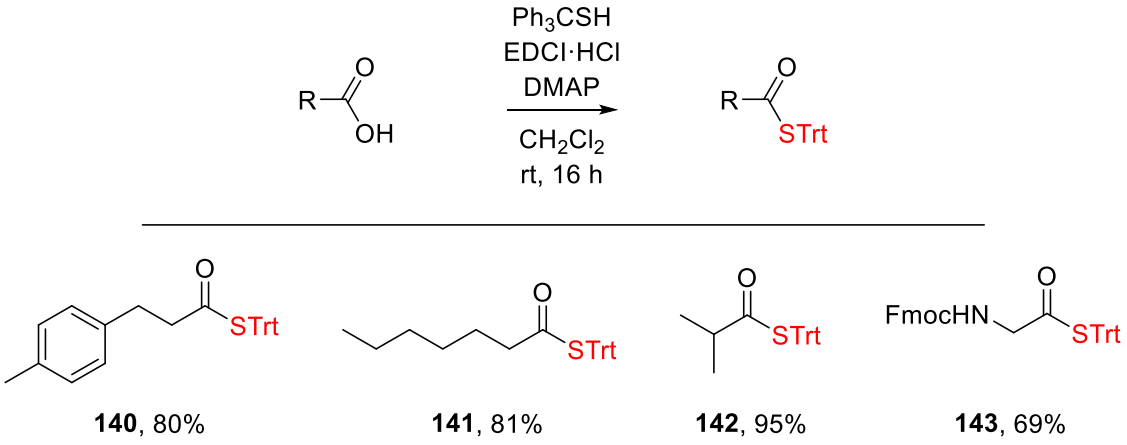
Gratifyingly, following concentration *in vacuo* and chromatographic purification, ^{1}H NMR analysis indicated that the C-1 acetamide had indeed formed with complete selectivity for the β-anomer, as hoped. The large vicinal coupling constant of 9.4 Hz, due to the dihedral angle of H1-H2, was used to confirm the presence of the β-anomer **135**.



Scheme 2.11. *S*-to-*N* acyl transfer furnishes the anomeric acetamide **139** in good yield, following TEC ligation.

2.3 Synthesis of thioacid precursors, and TEC

In tandem with preparation of the TEC acceptor **135**, diverse thioacids for use in TEC were prepared for reaction scope studies. The Scanlan group has explored a number of methods to prepare thioacids, and most notable are those used to prepare amino acid thioesters which are bench stable. The use of *S*-tritylthioesters as a thioacid precursor protects the sensitive thioacid from hydrolysis, and furthermore the acidic deprotection conditions furnish the protonated thioacid rather than the thiolate anion — an important factor for TEC ligation. In preparation for ligation studies, a number of *S*-tritylthioesters were prepared, using EDCI·HCl as a coupling reagent. All of these proceeded with excellent yields, and are summarised below in Scheme 2.12.

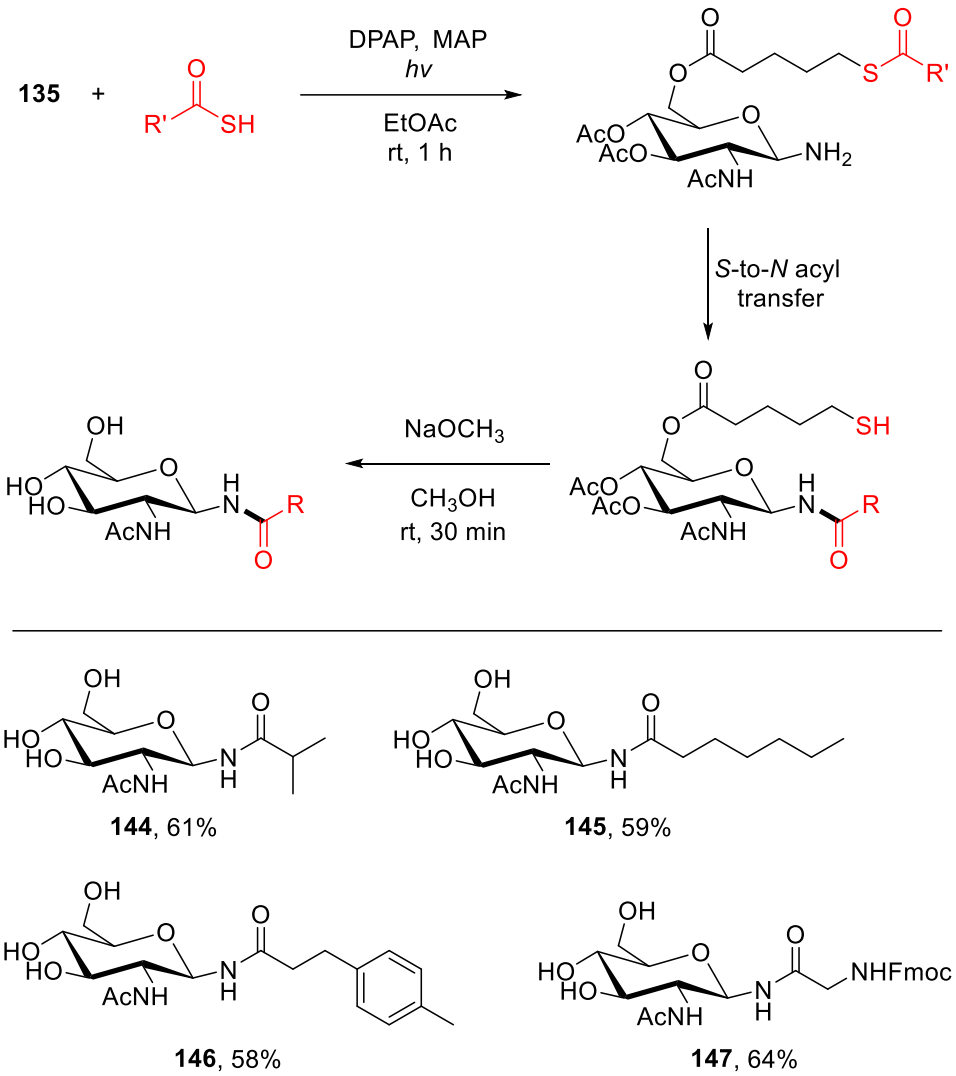


Scheme 2.13. Steglich esterification between triphenylmethanethiol and commercially available acids.

With the above thioesters in hand, we set out to test their viability in the radical mediated hydrothiolation of the alkene-appended monosaccharides. To release the desired thioacid, cleavage of the trityl thioesters was envisioned *via* an acidic mixture, and a silane scavenger present to 'mop up' the trityl cation. Initial experiments relied upon triethylsilane or triisopropylsilane as the scavenger, but the high boiling points of these reagents was not ideal and permitted hydrolysis of the nascent thioacids while on the rotary evaporator. The thioacid generated from **142**, the sulphurous analogue of isobutyric acid, proved quite volatile and evaporated during the concentration of the cleavage mixture. To combat this, the use of ethyldimethylsilane (boiling point ~ 40 °C at atmospheric pressure) was implemented.

Generally, the thiol-ene 'click' of the glycosylamine acceptor **135** proceeded as follows. Cleavage of the required *S*-trityl thioester proceeded in CH_2Cl_2 with TFA (25%) and ethyldimethylsilane (20 eq.) under anhydrous conditions. Due to the low density of this silane (*ca*. 0.67 g/cm^3), the volume of scavenger must be included in the calculation of final TFA percentage. An intense yellow colour is observed upon addition of the acidic mixture, which quickly dissipates as the trityl cation is reduced to triphenylmethane. Concentration *in vacuo* after the elapsed time gives an oily yellow residue which is immediately injected into a flask containing the alkene acceptor, DPAP/MAP and degassed solvent. This mixture is the irradiated for 1 h, after which it is stirred for 16 h in the dark to permit the *S*-to-*N* acyl transfer to occur. Base hydrolysis with catalytic sodium methoxide then furnishes the deprotected and 'native' β-amide. The one exception is for the Fmoc-glycine analogue, which was instead deprotected with triethylamine (20 eq.) to retain the Fmoc functionality. This also prevents unwanted derivatisation and scrambling due to the dibenzofulvene moiety released upon Fmoc deprotection.

Gratuitously, the one-pot TEC, shift and deprotection process furnished a number of anomeric amides. The hydrothiolation proceeded with complete consumption of the alkene, and following flash chromatography isolation the shifted products were obtained in yield of 58-64%, with some loss attributed to the highly polar nature of the products, and their ability to interact with the silica surface (Scheme 2.14).



Scheme 2.14. The one-pot STING furnished five different anomeric amides, with the correct stereochemistry.

Encouraged by the apparent success of the STING cascade, attention was focused on developing this approach further. As will be discussed in the next chapter, Interferon-γ (IFNγ) is of huge interest to this project, and so it was endeavoured to synthesise one of the tripeptide consensus sequences where this invaluable human cytokine is *N*-glycosylated.

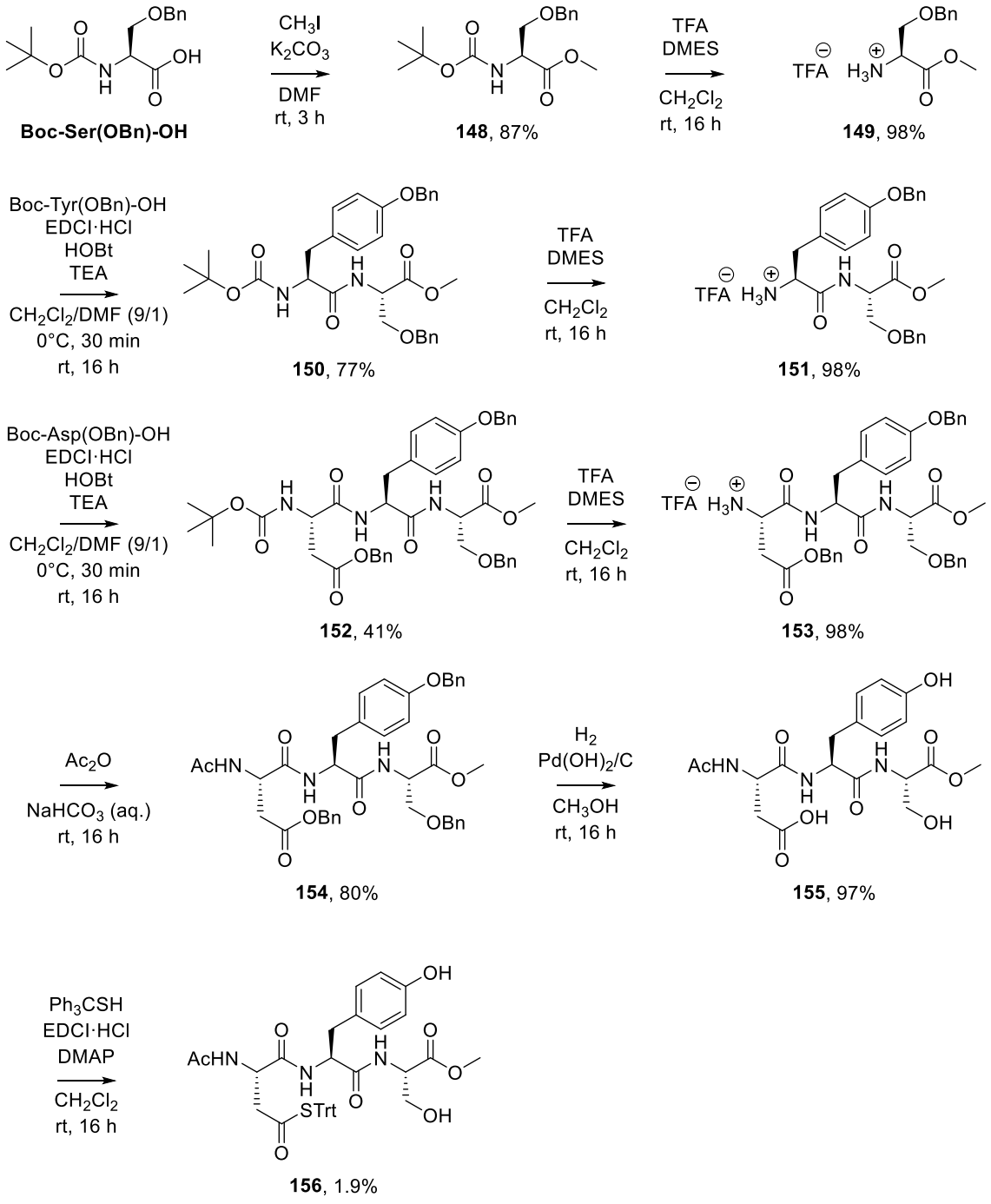
2.4 Synthesis of IFNγ tripeptide and ligation

IFNγ is glycosylated at an Asn-Gly-Thr and at an Asn-Tyr-Ser consensus sequence. The second of these tripeptides was targeted as a substrate for STING. As the sequence is short, solid-phase peptide synthesis (SPPS) was deemed unnecessary, and thus solution-phase chemistry was envisioned.

To begin, Boc-Ser(OBn)-OH was methylated under standard conditions to give Boc-Ser-OMe **148** in excellent yield of 87% on the gram scale. This was easily deprotected with TFA (25% in CH_2Cl_2) and ethyldimethylsilane as cation scavenger, to give the trifluoroacetate salt **149** in near quantitative yield of 98%. This was followed by a carbodiimide coupling with EDCI·HCl, DMAP, and HOBt as a racemisation suppressant. The dipeptide **150** was isolated in 77% yield following silica gel chromatography. Reiterating the TFA cleavage and coupling conditions furnished the Boc protected tripeptide **151**. A final deprotection step followed by crude acetylation of the TFA salt gave **152** as a bench stable intermediate, with no evidence of racemisation at any position, based on the ^{1}H NMR.

Removal of the benzyl protecting groups by catalytic hydrogenation may seem somewhat straightforward, but the correct source of Pd must be considered — unpublished results from the Scanlan group have often shown that benzyl protecting groups can in fact be hydrogenated to the cyclohexyl species when catalytic Pd/C is used on long reaction timescales. While at first this seems nonobvious, Crawford *et al.* recently disclosed the same, identifying that certain catalysts have a propensity to inflict unsaturation rather than global hydrogenolysis upon organic frameworks.[245] With respect to the present work, the IFNγ tripeptide did not undergo the desired hydrogenation with Pd/C. A quick catalyst screen identified $Pd(OH)_2/C$ as the cheapest and most viable catalyst for this transformation. Over a 16 h reaction time, the tripeptide **155** was generated in a 97% yield, which was used without further purification (Scheme 2.15, *vide infra*).

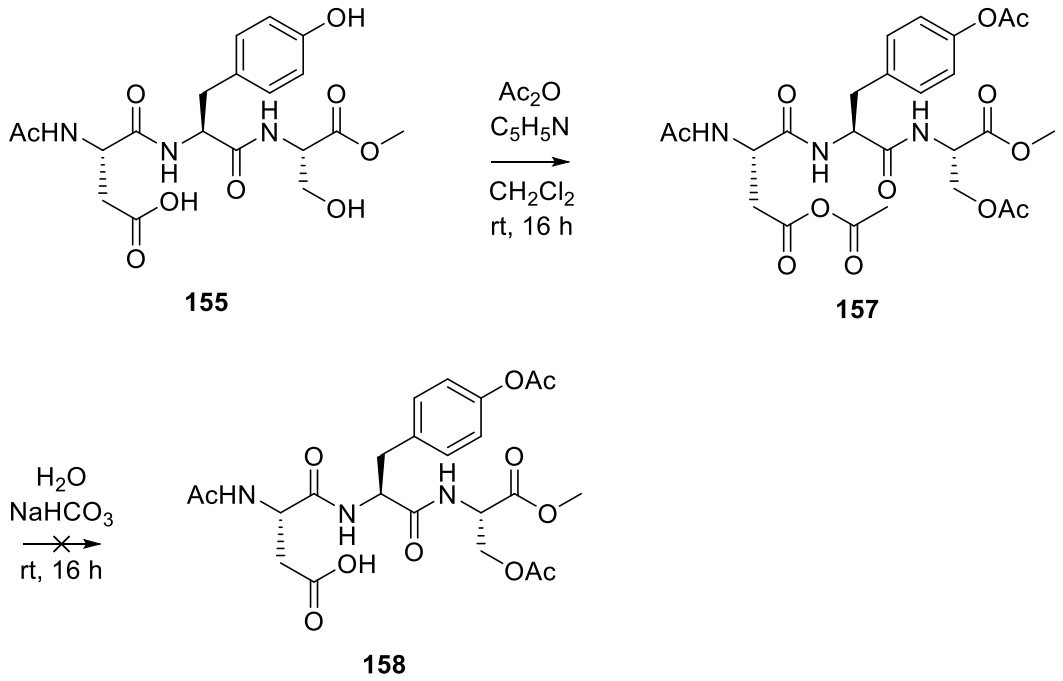
Unfortunately, the unprotected amino acid side chains interfered with the final synthetic step. The final carbodiimide coupling to furnish the *S*-tritylthioester on the Asp side chain was hampered by self-condensations/polymerisations, which rendered the starting material a deep red polymeric gum. Following column chromatography, the desired tripeptide *S*-tritylthioester **156** was isolated in only a 1.9% yield (approx. 20 mg in this case). Repetition of this reaction at more dilute concentrations did not alleviate the formation of this deep red polymeric material.



Scheme 2.15. Synthesis of tripeptide consensus sequence from IFNγ.

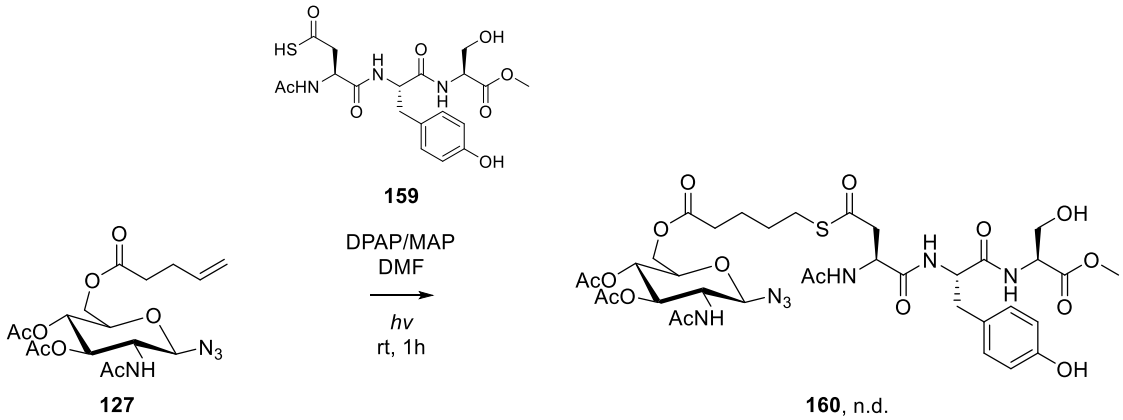
It was envisaged that a selective protection of the alcohol side chains, without blocking of the aspartyl side chain would circumvent this issue. Peracetylation of **155** to give the protected tyrosine and serine side chains worked well, with concomitant formation of a mixed anhydride on the aspartyl side chain (Scheme 2.16). Disappointingly, hydrolysis of this anhydride proved fruitless under multiple conditions, including aqueous alkaline hydrolysis. Fmoc protection of side chains was attempted using Fmoc-Cl and pyridine in

anhydrous CH_2Cl_2, but unfortunately this also did not circumvent the lack of orthogonality in this final tripeptide.



Scheme 2.16. Attempted protection and mixed anhydride hydrolysis. None of **158** was identified following hydrolysis.

With the small amount of material in hand, the thiol-ene 'click' with glycosyl azide **127** was attempted. The tripeptide ligation proceeded with trityl deprotection as previously outlined, with a large excess of silane scavenger present. This gave peptidic thioacid **159**. A deep orange colour persisted in the solution throughout this procedure. Subsequent concentration *in vacuo* and irradiation under UV light with **127** ensued. Disappointingly, no appreciable alkene consumption was observed by ^{1}H NMR, and glycothioester **160** was not generated in any tangible amount (Scheme 2.17). One could in theory ascribe this to steric constraints.



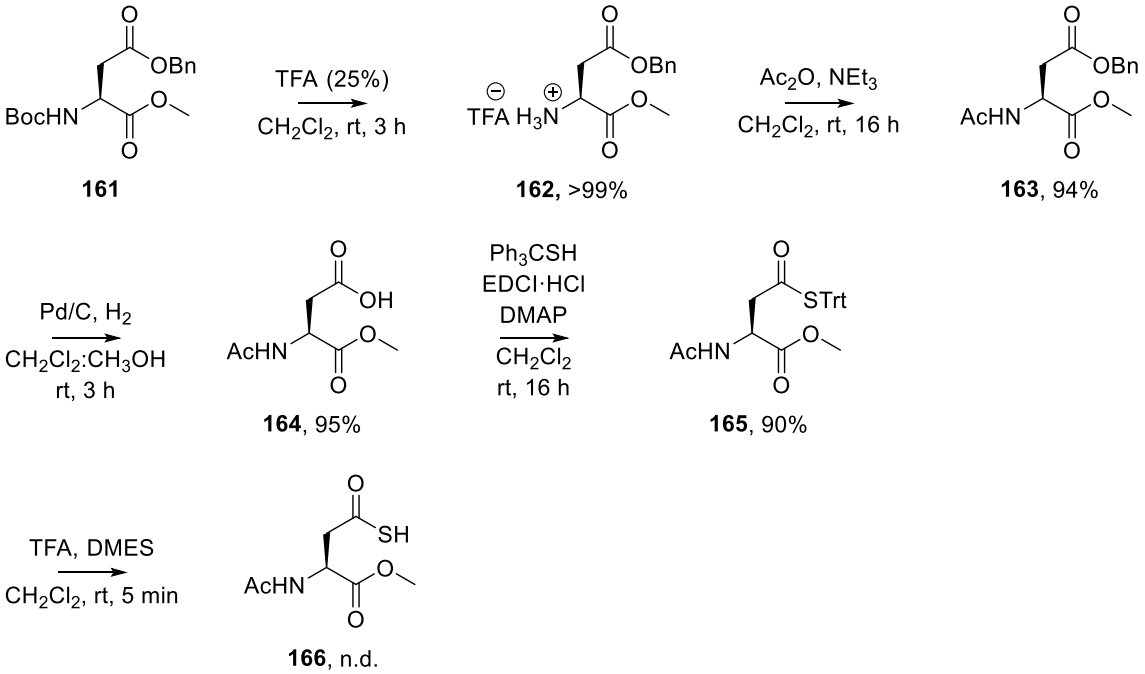
Scheme 2.17. No determinable amount of ligation occurred between glycosyl azide **127** and thioacid **160**.

2.4 Ligation with aspartyl/glutamyl species

Following the absence of product formation from the tripeptide ligation, the reaction was next investigated with single amino acids, to investigate whether they could be ligated under the established conditions, and if the shift observed with the small thioacid examples could be replicated. Considering that the common element in all *N*-glycopeptides is the linkage of a β-GlcNAc to an asparagine, aspartyl thioacids were proposed.

2.4.1 Aspartyl Species

Initially, the simplest Asp thioester **165** was synthesised, with the *N*-terminus capped as the acetamide. Boc-Asp(OBn)-OMe was deprotected to the trifluoroacetate salt in quantitative yield, and the secondary amine then peracetylated with acetic anhydride and triethylamine in CH_2Cl_2. Palladium-catalysed hydrogenolysis liberated the carboxylic acid side chain in a 95% yield, after which Steglich esterification under standard conditions with triphenylmethanethiol gave the *S*-trityl protected species, **165** (Scheme 2.18). This isolable compound served as a bench stable precursor to thioacid **166**.



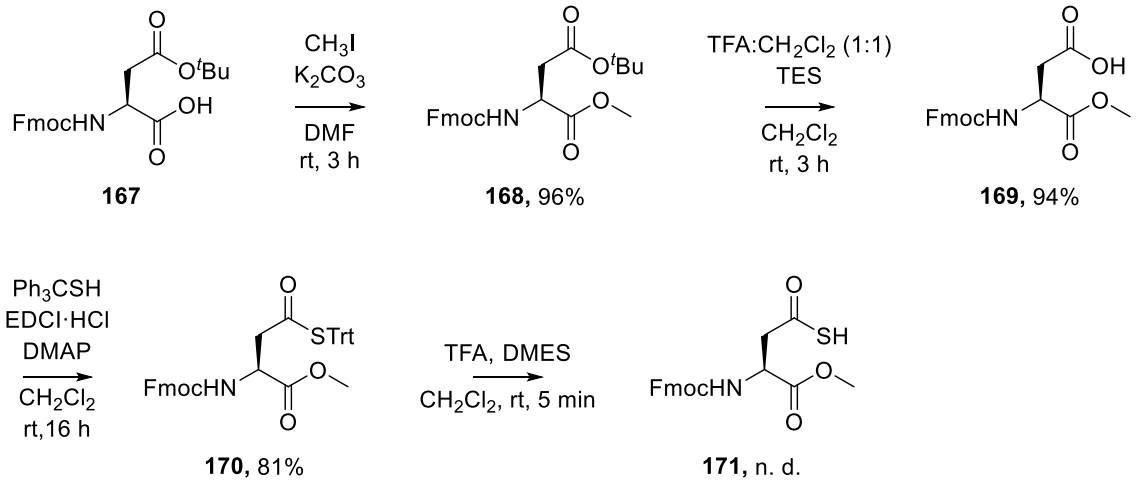
161 **162,** >99% **163**, 94%

164, 95% **165**, 90%

166, n.d.

Scheme 2.18. Synthesis of aspartic *S*-trityl protected thioacid **166**.

Separately, the Fmoc-protected thioacid was also synthesised. This proceeded with carboxylic acid methylation of Fmoc-Asp(O^{t}Bu)-OH with methyl iodide and potassium carbonate at rt for 3 h in DMF. Fully protected compound **168** was obtained in excellent yield of 96%, after which the *tert*-butyl protecting group was removed in acidic conditions. Carbodiimide coupling with tritylthiol gave the *S*-tritylthioester **170** in a yield of 81%, with partial loss as expected due to some DMAP-catalysed deprotection of the Fmoc moiety. Trityl deprotection proceeded smoothly to yield the desired thioacid **171** (Scheme 2.19).

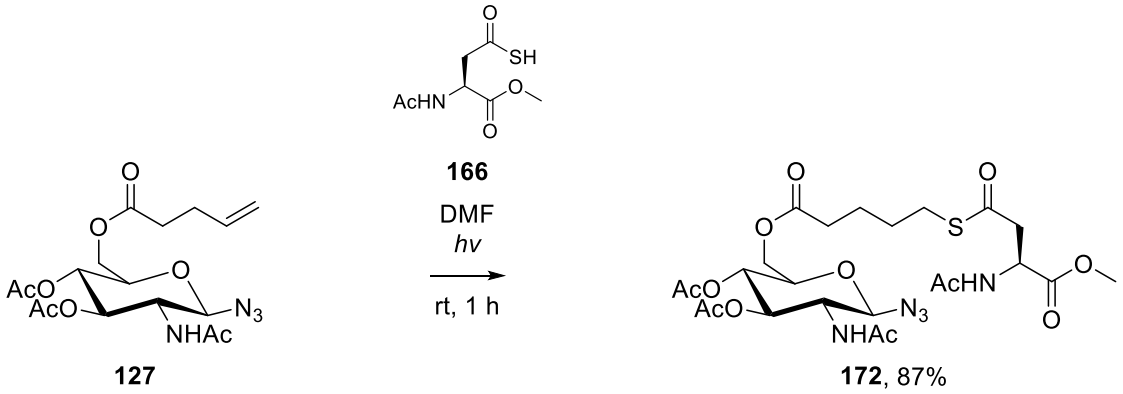


167 **168,** 96% **169,** 94%

170, 81% **171,** n. d.

Scheme 2.19. Synthesis of Fmoc-Asp(SH)-OMe.

With the thioacid precursors in hand, their viability as thiols in thiol-ene ligation was evaluated. Ac-Asp(STrt)-OMe **165** was subjected to trityl deprotection to yield thioacid **166**, and immediately reacted with glycosyl azide **127**, to yield glycothioester **172** in excellent isolated yield of 87% (Scheme 2.20).

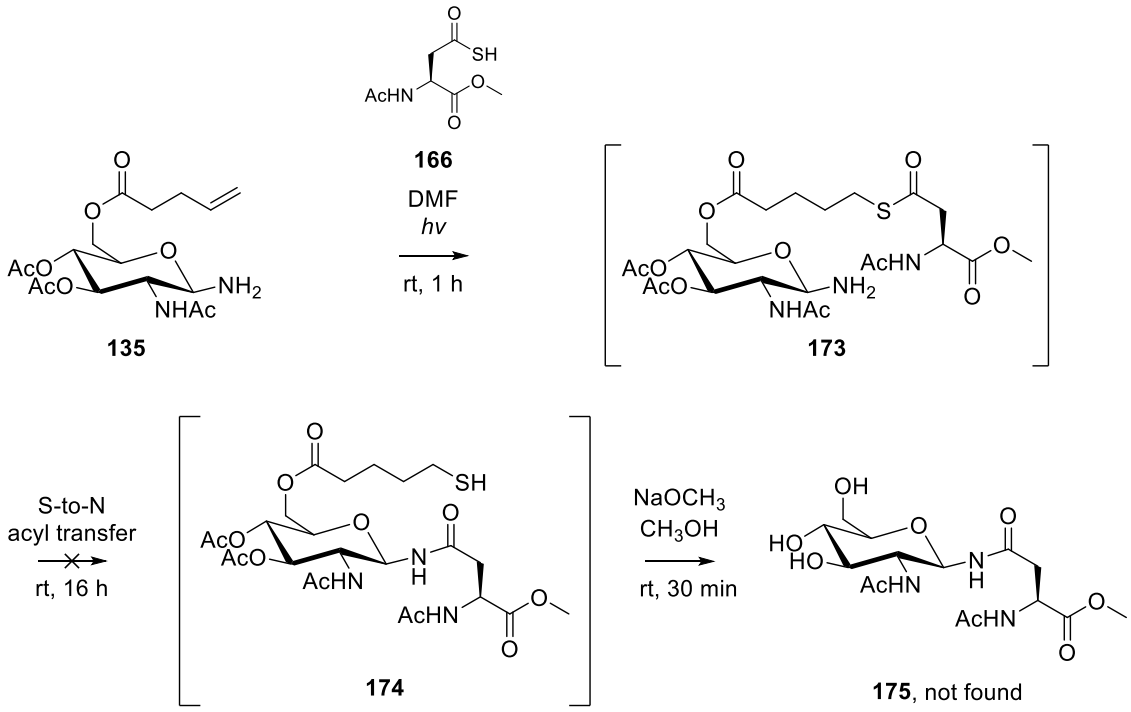


Scheme 2.20. Ligation between thioacid **166** and TEC acceptor **127** proceeds smoothly, with an isolated yield of 87%.

2.4.1 One-pot Process

Having verified that this material is suitable for thiol-ene 'click', both **166** and the Fmoc-protected derivative **171** were reacted in a one-pot reaction with glycosylamine **135**, to determine if *S*-to-*N* acyl transfer was possible (Scheme 2.21). If ligation and the desired shift was observed with the Fmoc-bearing analogue, a variety of glycopeptides could be rendered accessible *via* SPPS.

While disappearance of the starting material was clearly seen *via* TLC after UV irradiation, the attempted purification of shifted β-amides such as **175** proved ineffectual. Neither anomeric amides were obtained, with decomposition of the reactants observed. It was hypothesised that steric constraint around the transition state, especially in the case of the bulky Fmoc substituent, was responsible for the lack of acyl shift to the anomeric position.

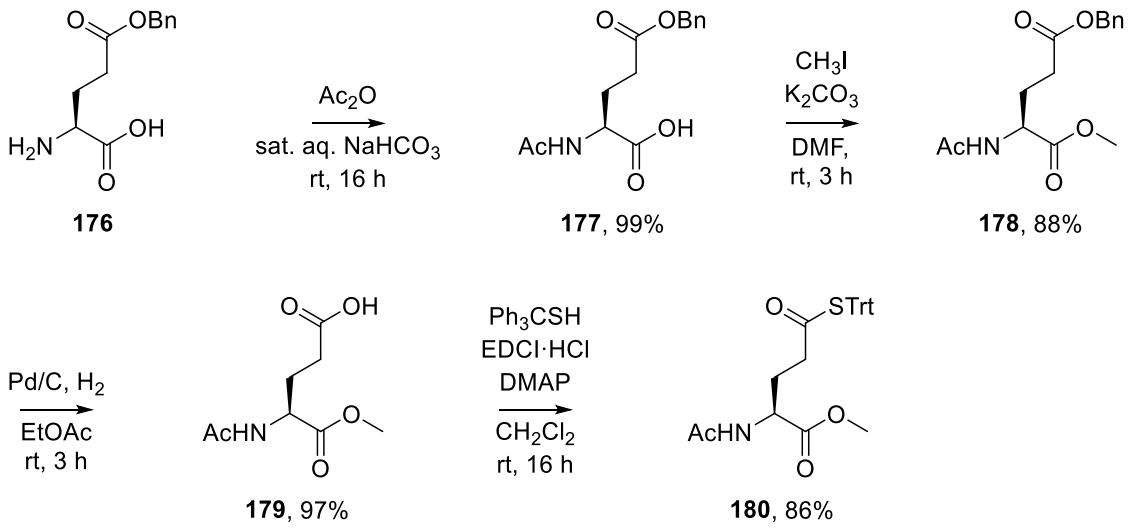


Scheme 2.21. While ligation proceeds smoothly with glycosylamine **135**, no shift is observed, and none of **175** found after alkaline hydrolysis.

2.4.2 Glutamic acid derivative

Frustrated by this failure, we considered that the recalcitrance of these species to yielding the desired amide stemmed from steric impedance around the incipient thioester. Furthermore, one could imagine some hydrogen bonding between the anomeric amine and the thioacid backbone following ligation. An effective way of testing both these parameters was to synthesise the corresponding glutamic acid derivative **180** — with an additional CH_2 in the amino acid side chain, the physical space around the thioester site would be less constricted. Additionally, the increased degrees of freedom would hopefully limit the hydrogen bonding interactions with the thioacid backbone.

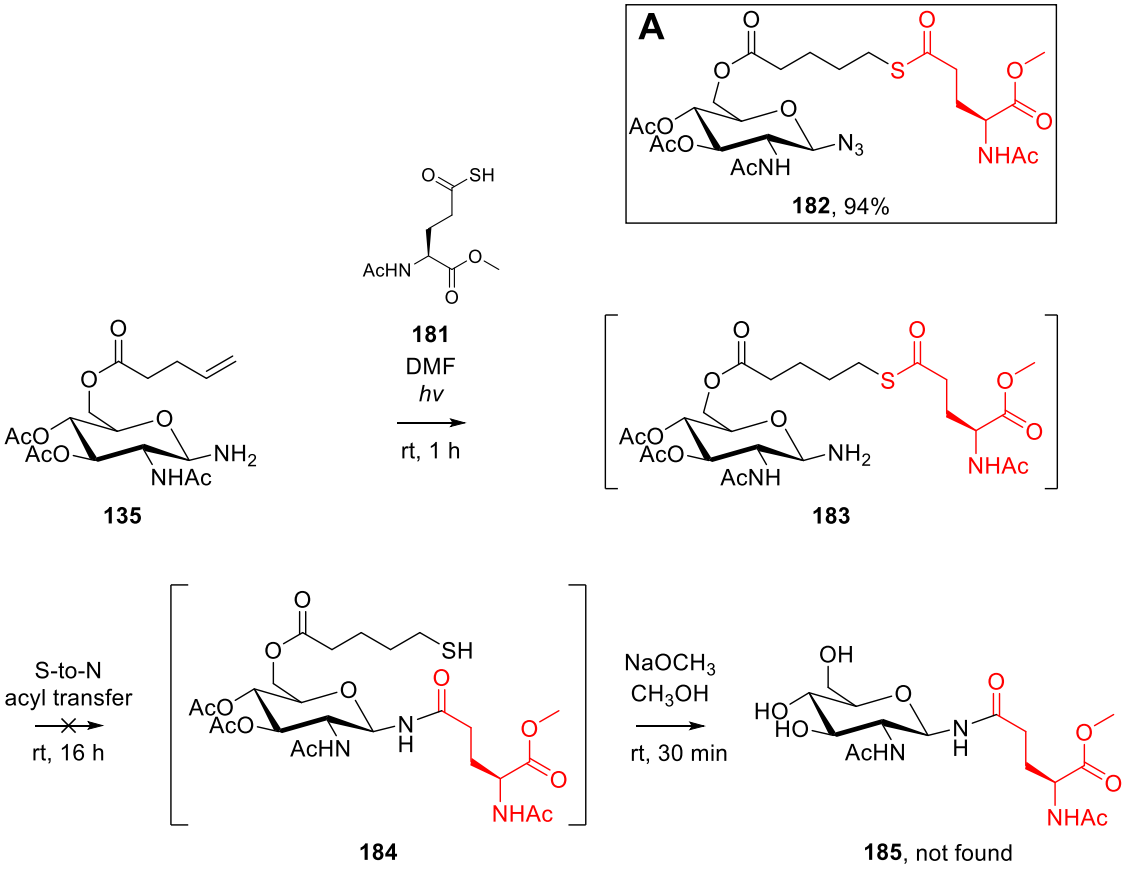
Freebased glutamic acid, bearing a benzyl protecting group on its side chain **176** was peracetylated under aqueous alkaline conditions with acetic anhydride, to give **177** in excellent yield of 99%. The methylation of the *C*-terminal acid proceeded with an isolated yield of 88%, furnishing compound **178**. Deprotection of the amino acid side chain gave **179**, which was subjected to an EDCI·HCl coupling to furnish thioester **180** in 86% isolated yield (Scheme 2.22).



Scheme 2.22. Synthesis of glutamyl derivative **180**, for use in STING.

180 was deprotected to the corresponding thioacid, and photochemically ligated with glycosylazide **127**. Gratifyingly, improved ligation yields were observed compared to the aspartyl species, adding weight to the hypothesis that steric constraint around the thioacid inhibits (or at least renders sluggish) the thiyl-radical reaction with the unsaturated moiety. This provided glycothioester **182** in excellent yield of 94% (Scheme 2.23, **A**).

Once again, the thioacid was combined with glycosylamine in an attempted one-pot process for thiol-ene 'click' followed by *S*-to-*N* acyl transfer (Scheme 2.23). Unfortunately, although consumption of the starting material was observed during the photochemical reaction stage, no discernible shift of the thioester carbonyl to the anomeric position was observed.



Scheme 2.23. Attempted one-pot ligation of glutamyl thioacid and glycosylamine **135**. **Insert A:** Isolated glycothioester, with anomeric azide.

2.5 Conclusions and Future Work

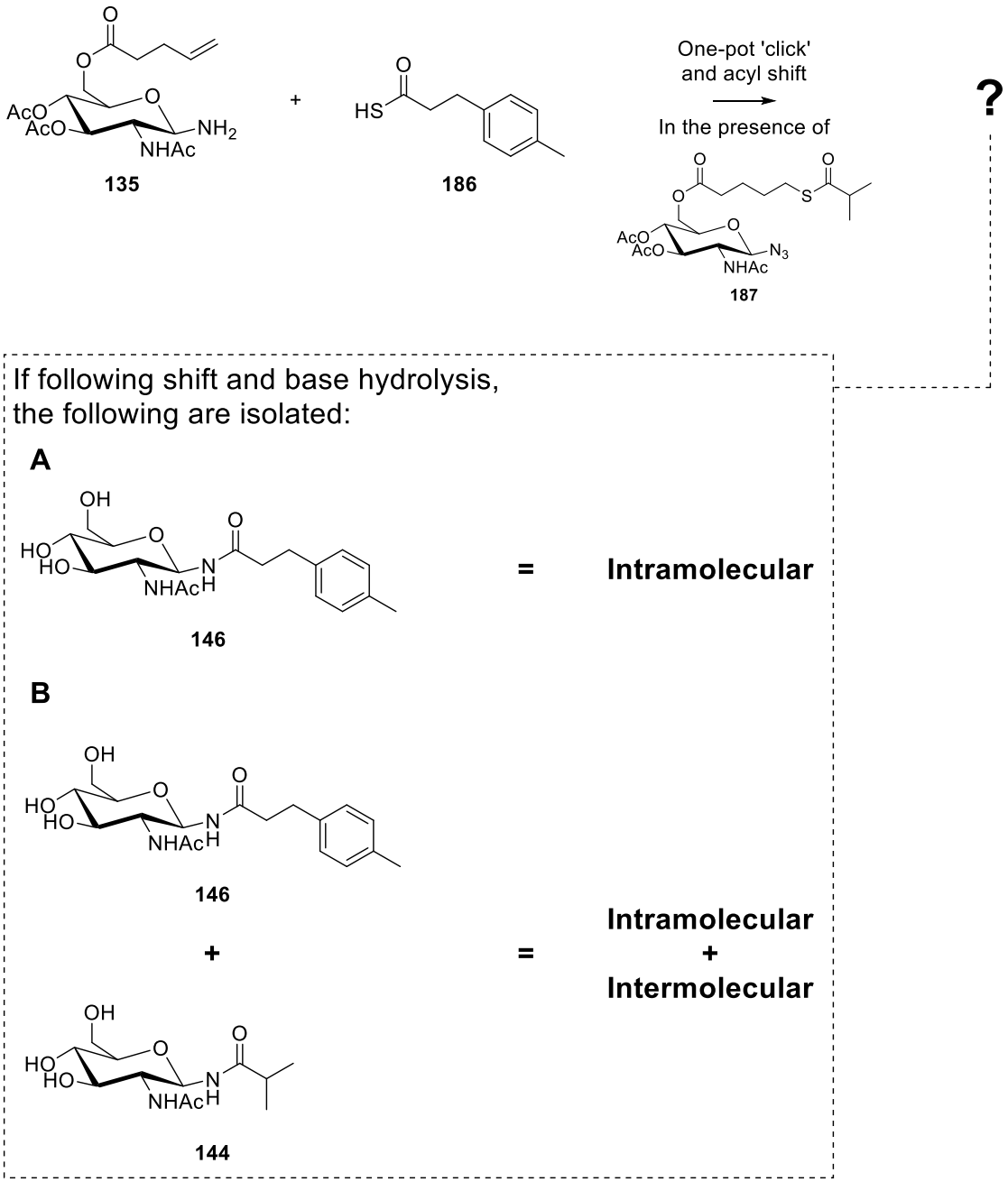
Two novel thiol-ene acceptors, on a valuable carbohydrate scaffold were synthesised herein. While the synthesis of these acceptors was anything but facile, the scope and limitations of such an approach were explored. A number of novel *S*-tritylthioesters were synthesised as bench-stable thioacid precursors, and were liberated to their thioacid form for use in thiol-ene 'click' methodology. The tolerance of glycosyl azide acceptors **124** and **127** to hydrothiolation by a number of thioacids, bearing diverse functionality, was studied. While these acceptors, bearing alkene termini appended *via* an ester on the 6-*O* alcohol of *N*-acetyl-D-glucosamine, were excellent substrates for various thioacids, their anomeric azides were intractable to reduction, and required significant optimisation.

Admittedly, the **S**ugar **T**emplated **I**ntramolecular ***N*-G**lycosylation (STING) methodology proposed herein was not as straightforward as hoped. While a number of glycothioesters were able to undergo *S*-to-*N* acyl transfer to furnish anomeric β-amides, the more biologically relevant examples did not proceed as desired. Aspartyl thioacids

with varying protecting groups **166** and **171** were suitable participants in the thiol-ene reaction with acceptor **135** however no determinable acyl transfer was observed. Expansion to the glutamyl species increased the ligation yields somewhat, possibly due to reduced steric demand around the thioacid environment, but this increased entropic freedom of the amino acid side chain did not alleviate the predicted hydrogen bonding interactions, which may have prevented a shift to the anomeric centre.

Despite the numerous setbacks of this approach, we have theorised a number of potential future experiments. Firstly, the intramolecular nature of the *S*-to-*N* acyl transfer must be confirmed. This could be achieved in several ways, the most straightforward of which is outlined below (Scheme 2.24). Glycosyl azide **127** is first ligated with one thioacid, purified, to yield a 'blocked' or 'non-shifting' glycothioester **187**. Glycosylamine **135** is then added, followed by the thioacid **186** of a separate, distinct thioester, and thiol-ene ligation is initiated at defined concentration. Following irradiation, the reaction media is then stirred overnight in the dark. *If the reaction is truly intramolecular*, then only the anomeric amide of thioacid **186** should be found, as no intermolecular shift from 'blocked' glycothioester **187** should occur. Base hydrolysis should give only one shifted product — anomeric amide **146** (Scheme 2.24, **A**). If any other anomeric amides are identified (Scheme 2.24, **B**), then the reaction is <u>not</u> truly intramolecular.

Additionally, further investigation into the anomeric azide reduction is necessary. The author postulates that if the required tetrathiomolybdate species could be solid-supported on some sort of polymer, as many classical reagents now are, the low yields following repeated filtration could be mitigated. Resin functionalisation is no easy feat, and so this would likely require careful planning and optimisation. Heterogeneous azide reduction would hopefully offer increased surface area and therein shortened reaction time, the reusability of the molybdenum source, and easy filtration to furnish the glycosylamine.



Scheme 2.24. Proposed experiment to discern intra- *vs* intermolecular processes. If only **A** has formed, then none of the thioester from **187** has shifted to glycosylamine **135**. If **B** is the case, then there is scrambling between inter- and intramolecular processes.

Finally, computational modelling should be immediately employed before further reactions are under this project. The likelihood of some form of intramolecular hydrogen bonding, which may hinder the acyl transfer step in this approach, should be investigated thoroughly.

Chapter Three

Interferon-γ

3.0 Introduction

As outlined in section 1.4, interferon-γ (IFNγ) is an important human glycoprotein with diverse biological function. The human form of the protein is glycosylated at Asn-25 and/or Asn-97, referred to as position 25 and position 97 for our purposes. Recombinant IFNγ is an FDA approved therapeutic for the treatment of genetic disorders affecting bone function and the immune system. An account by Sareneva *et al.* and others have demonstrated that glycosylation of IFNγ is required for protection from proteolysis and receptor binding; for example, the glycan at Asn-25 in the IFNγ/IFNγ receptor complex is located in the immediate vicinity of the binding site. The protein exists as a homodimer, which is usually accessed by refolding of denatured monomeric strands[143, 145]

In this chapter, a semisynthetic approach for accessing glycosylated form of IFNγ is presented. Access to the wild-type (WT) protein was first established through recombinant protein expression, before the protein was refolded into its homodimeric form *via* a rapid dilution approach. The incorporation of a non-natural amino acid (nnAA) was attempted with the amber stop codon (UAG) supression system, after which the use of CuAAC to bioconjugate a suitable mammalian glycan is reported. The significant challenges associated with protein purification are discussed in detail.

The author wishes to acknowledge the contributions of Dr. Marina Rubini (M.R.) and Kirti Sharma (K.S.) to the results disclosed in this chapter.

3.1 Recombinant Protein Expression

Recombinant protein expression (RPE) of human interferon-γ was deemed the most viable for obtaining sufficient quantities of protein for immunological testing. Firstly, an expression system for the desired protein was required. Reflecting upon results obtained by our collaborators, it was determined that expression of IFNγ in a completely insoluble form and liberation from inclusion bodies would be most amenable to purification. Alternative expression systems, such as using the pTXB1 plasmid vector, had caused their own problems in the amber suppression method; spontaneous intein splicing resulted in considerable protein loss, and the nnAA incorporation yields were very poor for this expression system, when compared to the wild type.

A new plasmid was constructed for the expression of IFNγ. Plasmid pETDuet was employed for cloning of the required gene and expression in *E. coli*, which utilises a

highly efficient bacteriophage T7 promoter. In initial expression experiments, a hexahistidine (His_6) tag was not employed in order to limit the number of variables that could have inimical effects on expression and incorporation, however the opportunity to employ affinity chromatography was soon required, and thus the His_6 tag was included in the expression system design. In the hands of our collaborators (M.R. and K.S.), access to a competent pETDuet expression system was obtained for the expression of wild-type (WT) IFNγ, and with nnAA incorporation at glycosylation sites 25 and/or 97.

A typical protein expression proceeded as follows. Chemically competent *E. coli* strain ER2566 cells were transformed with the previously prepared pETDuet plasmid vector, which contained the DNA sequence for WT-IFNγ-His_6 , and grown to a suitable optical density (*ca*. 0.6 at 600 nm) in lysogeny broth (LB) media. Expression was induced using IPTG (see Chapter One, section 1.4.1), with a non-induced control stored as a reference. Ampicillin and spectinomycin were employed to provide selective pressure in the expression culture and stave off plasmid loss. Centrifugation and separation of the supernatant provided a cell pellet, which was then resuspended in a suitable buffer. The cells were disrupted using ultrasonication following which, repeated centrifugation and resuspension as before gave a pellet of inclusion bodies.

Denaturation of these inclusion bodies with guanidinium chloride (Gdm·Cl, 7.4 M) as a chaotropic agent was conducted overnight at room temperature. A gradual dilution with H_2O to 1 M Gdm·Cl and subsequent centrifugation provided a supernatant with crude WT-IFNγ-His_6.

3.1.1 Nickel-NTA Affinity Chromatography

Nickel-NTA chromatography is a type of immobilized metal affinity chromatography. The immobilized nickel resin of interest to this work is nitrilotriacetic acid (NTA), which forms a coordination compound with metal ions. The hexahistidine tag at the IFNγ *C*-terminus strongly coordinates to Ni^{2+}, whereas other proteins lacking this affinity tag will have low or no binding to the resin.[246] Albeit, some electrostatic attraction may result in non-specific binding, which one can offset *via* the addition of NaCl to the wash and elution buffer, or by washing with low imidazole concentrations which will not elute the target protein but will disrupt other interactions. Drastically lowering pH to protonate the His_6 tag is a common method of releasing the desired protein from the column.

Alternatively, increasing concentrations of imidazole can be employed to sequentially release the protein (Figure 3.1). A major advantage of Ni^{2+} affinity chromatography to the present work is the independence of the approach to secondary structure; thus, denatured proteins can be purified.

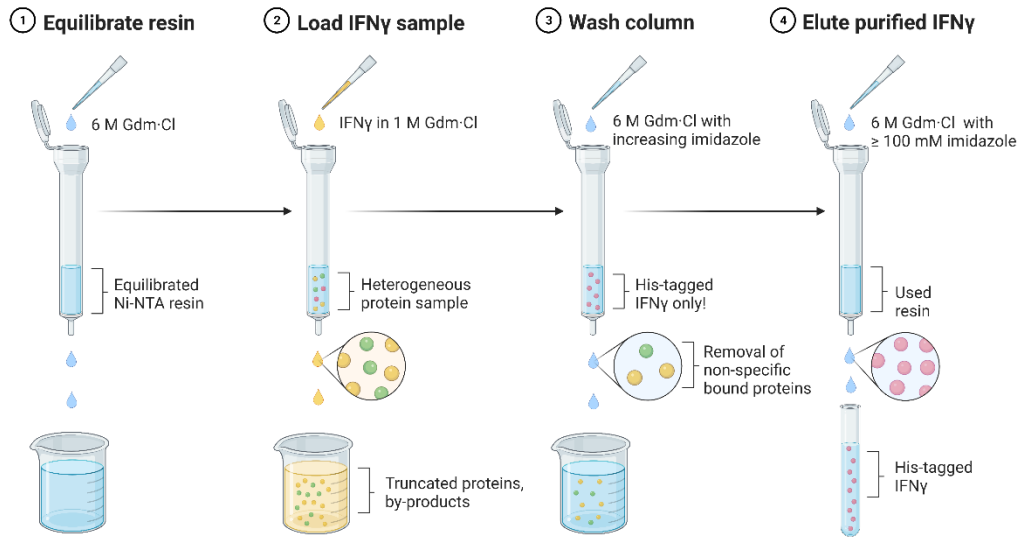


Figure 3.1. The steps in Ni-NTA affinity chromatography of IFNγ.[247]

The supernatant from the previous denaturation of inclusion bodies was flowed through a short Ni-NTA column (*ca*. 3 mL) to bind the His_6 tag to the resin. It was desirable to keep the protein denatured, and hence gradual washing of the column with Gdm·Cl (6 M) and increasing concentration of imidazole was employed. Small fractions were collected once imidazole concentration reached 100 mM, and continued until the final elution with 1 M imidazole. SDS-PAGE analysis was used to determine concentrated and reasonably-pure fractions. It is noteworthy that Gdm·Cl is incompatible with SDS-PAGE, and thus the produced gel often presents diffuse and potentially unclear bands of protein (Figure 3.2). A particular advantage of the His_6 affinity tag approach is the absence of a His_6 tag in truncated proteins – since protein expression occurs in an *N*-to-*C* terminal direction, proteins which are not fully expressed will not possess the tag at their *C*-terminus.

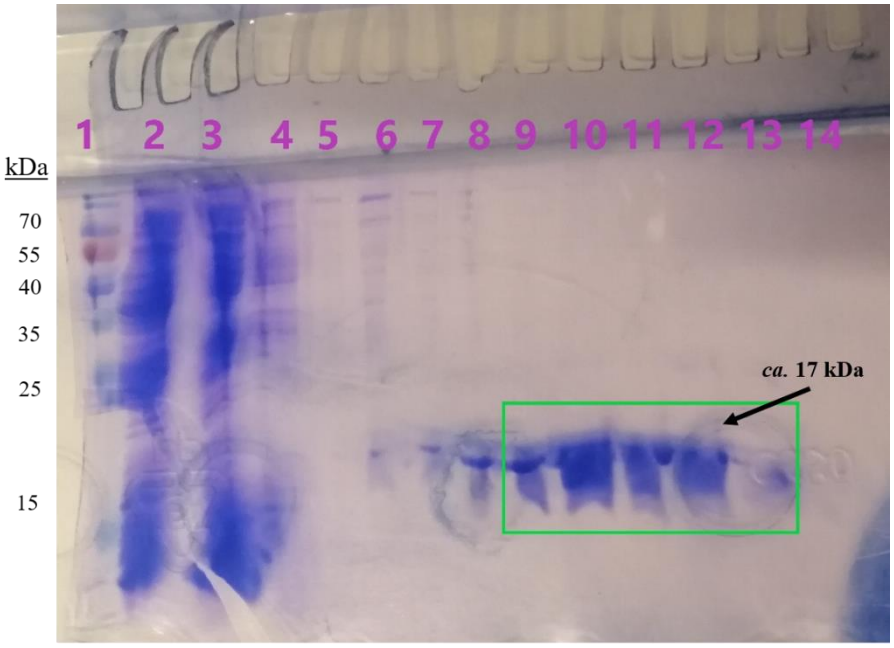


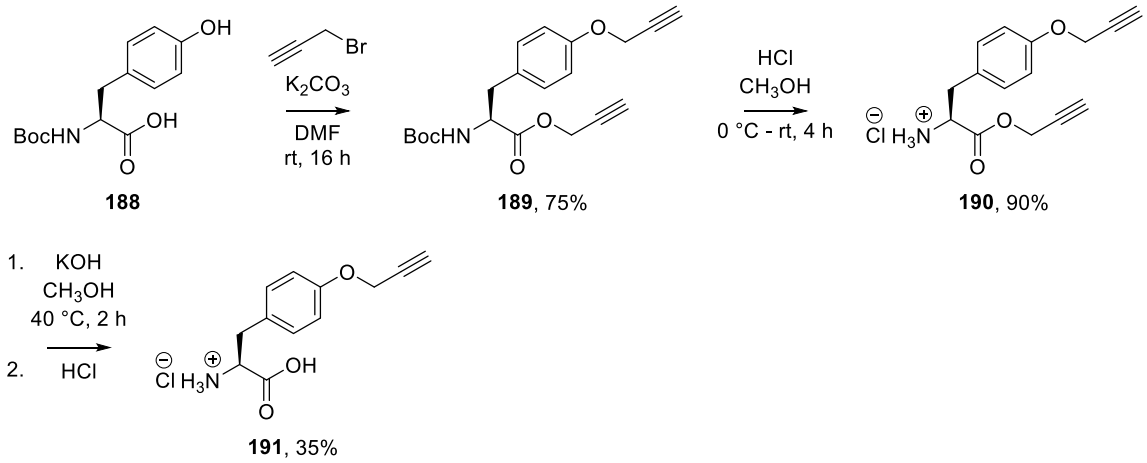
Figure 3.2. SDS-PAGE following Ni^{2+} affinity chromatography. Highlighted lanes are those imidazole fractions deemed to be of sufficient purity.

3.1.2 nnAA Synthesis and Incorporation

The expression of non-glycosylated WT-IFNγ-His_6 has been outlined hitherto. The procedure does not change significantly when nnAA incorporation is required. The primary difference is that prior to induction of protein expression with IPTG (as previously discussed), the cells must be incubated with the desired nnAA added to the culture medium, to allow it to enter the cells. *O*-Propargyltyrosine was envisioned as a suitable nnAA for incorporation, and was prepared as the hydrochloride salt.

Boc-Tyr-OH **188** was propargylated under alkaline conditions in anhydrous DMF. Aqueous workup following dilution in diethyl ether gave an organic phase containing Boc-Tyr(OPrg)-OPrg **189**, which was concentrated and used without further purification. An anhydrous solution of methanolic HCl was prepared by controlled esterification of acetyl chloride with anhydrous methanol, which was utilised to effect Boc deprotection. The hydrochloride salt **190** was isolated in excellent yield of 90%, and once again the residue was applied directly to the next step. The final synthetic step involved alkaline hydrolysis of the amino acid *C*-terminus, to remove the unwanted propargyl ester. This was conducted with a slurry of methanolic KOH, followed by slow acidification to precipitate the desired hydrochloride salt **191** (Scheme 3.1). The relatively poor yield of 35% originates from the use of precipitation as a means of purification – aqueous

work-up is not possible, and column chromatography of the organic residue proved ineffectual.



Scheme 3.1. Synthesis of nnAA Tyr(OPrg)-OH **191**.

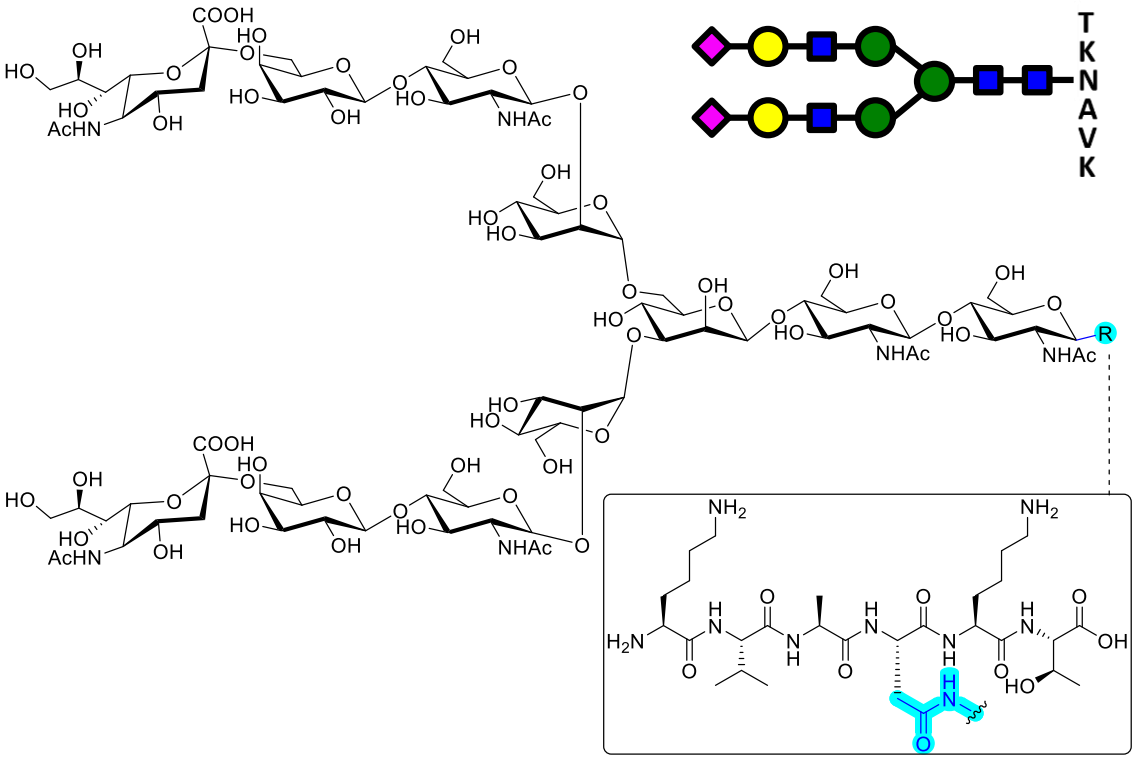
With the nnAA (PrgY) **191** in hand, incorporation at position 97 was first attempted. *E. coli* ER2566 cells were transformed with *pETDuet-hIFNγ-(N97PrgY)-His*$_6$ and *pULTRA-CNF*, a highly efficient suppressor plasmid which encodes for the required tyrosyl aaRS to effect nnAA incorporation.[248] Prior to induction with IPTG, *O*-propargyltyrosine hydrochloride **191** (PrgY) was dissolved in aqueous acetic acid (80% v/v), and added to a suspensions of cells in LB media such that a nnAA concentration of 2.5 mM was attained. The cells were incubated with the solution to allow the nnAA to enter the cells. IPTG was then added to induce recombinant DNA expression, and the procedure was followed as before. Ni-NTA affinity chromatography provided purified IFNγ-His$_6$ with PrgY at position 97. Incorporation of the nnAA was confirmed by virtue of a full length protein with a *ca.* 17 kDa band similar to WT-IFNγ-His$_6$ observed *via* SDS-PAGE analysis. Once again, protein expression halts if the nnAA is not incorporated; in this event, the amber stop codon (UAG) terminates expression, and a truncated peptide lacking the polyhistidine tag is formed. A slight reduction in protein yield was observed over the Ni-NTA fractions when incorporation of nnAA at position 97 was desired, which can be expected due to inefficient uptake when compared to the WT expression.

IFNγ-His$_6$ with PrgY at position 97 (PrgY97-IFNγ-His$_6$) was stored in its denatured form in order to both prevent precipitation of the polypeptide, and ensure that the alkyne

moiety of **191** would be available for click chemistry – one cannot be sure if the refolded protein surface would sufficiently expose the unsaturated moiety for conjugation with an azide-bearing click partner. With the protein in hand, a large mammalian glycan with an azide at the anomeric locus to glycosylate IFNγ was required.

3.2 Egg yolk sialylglycopeptide: Extraction and derivatisation

Sialylglycopeptide (SGP) is a biantennary sialyloligosaccharide, bearing a short hexapeptide fragment (Lys-Val-Ala-Asn-Lys-Thr) through the β-GlcNAc bridgehead of its chitobiose core (Figure 3.3). The first isolation of this undeccasaccharylpeptide was reported in 1994, in which Seko and co-workers isolated the glycopeptide from hen egg yolk using phenol, gel filtration and successive chromatographic techniques.[214] Since this original paper, numerous isolation protocols have been developed and refined, increasing the extraction yield and switching to greener chemical approaches.[215-217, 249-251]



192

Figure 3.3. The structure of SGP. Peptide sequence shown in insert.

As SGP is also a mammalian glycan[143, 218] it was endeavoured to isolate, purify and derivatise this glycopeptide for chemical protein synthesis and the glycosylation of IFNγ. Initially, freeze-dried egg yolk powder was purchased from a gym supplement company,

as ready access to a freeze-drier was unavailable. However, initial attempts to isolate the glycan failed, likely due to the pasteurisation and spray-drying process involved in preparing the commercial powder. This presumably caused glycosidic cleavage of the sialic acid residues, which are sensitive to self-catalysed hydrolysis above 40° C.[250]

Through testing the numerous reported procedures and combining the most favourable aspects of each one, an optimised protocol was developed. Fresh hen egg yolks were separated from the whites by hand, and stirred in H_2O to break the yolks, before being lyophilised. The egg yolk powder was then repeatedly delipidated with diethyl ether and filtered, giving a bright yellow organic phase which was discarded, and an off white filter cake. Successive washes of the compressed cake with aqueous acetone solutions removed many of the impurities, after which repeated extraction with aqueous acetone (40% v/v) gave a pale-green solution containing SGP.

Upon concentration of this solution, the extract was circulated through a column of charcoal and Celite®, employing the principles of large-scale solid-phase extraction (SPE). Elution of this column with increasing concentrations of acetonitrile in water was used to remove some of the more hydrophilic contaminants, while a final elution of aqueous acetonitrile (25% v/v) released the glycan from the column. Lyophilisation of this solution gave crude SGP, which was further purified through low-temperature centrifugation.

A one-metre long size-exclusion column was used to separate the mono-desialyl and fully sialylated glycans, giving the purified glycopeptide in a yield of *ca*. 120 mg per 50 eggs. The exact details of the *Scanlan lab protocol* are outlined in the experimental section of this thesis, and relevant NMR spectra of interest can be found in Appendix A. Details of size-exclusion chromatography are discussed in section 3.4.2. NMR studies aided structural elucidation and confirmation of the exact glycan isolated. Figure 3.4 shows an overlaid spectrum with a literature ^{1}H NMR from Liu *et al*.[252]

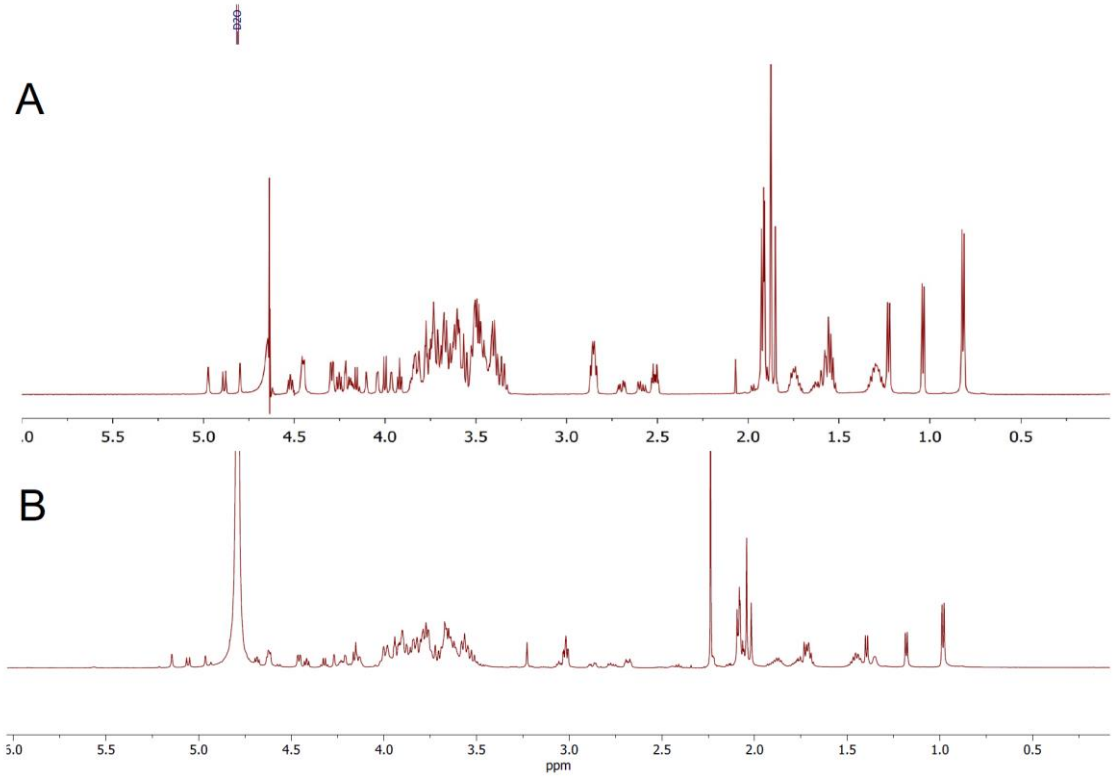


Figure 3.4. Overlaid 1H NMR. **A**: ^{1}H NMR reported by Liu *et al*. **B**: ^{1}H NMR spectrum of SGP, obtained through the course of this work.

HSQC studies were used to count and confirm the six anomeric carbons, which were in good agreement with the literature from Liu *et al*. (Figure 3.5).[252] Mass spectrometry was employed to confirm the presence of SGP, as well as the absence of the partially desialylated forms.

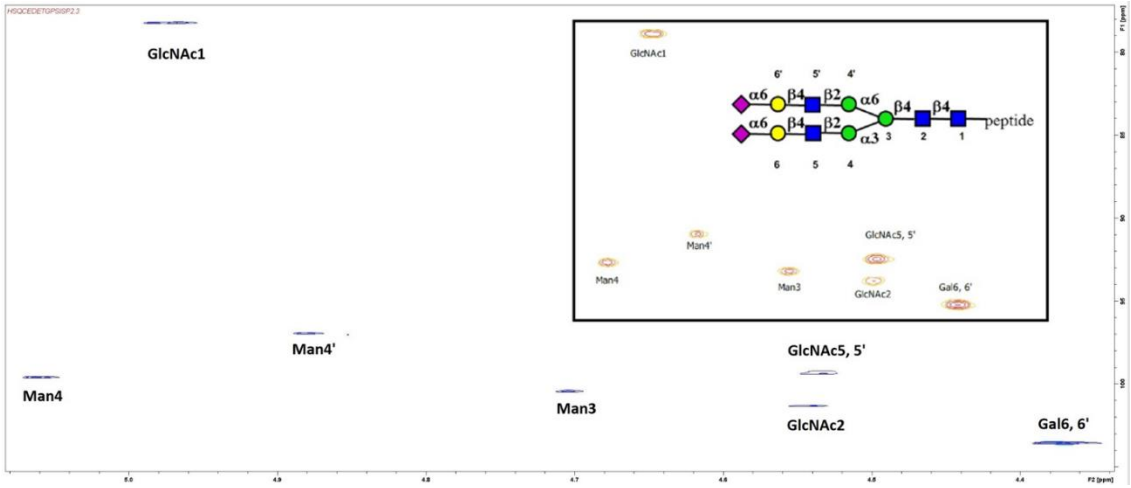
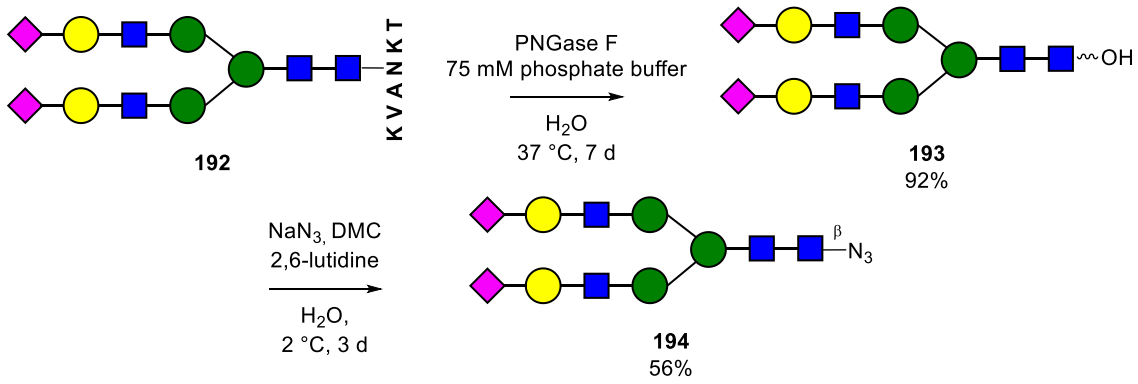


Figure 3.5. HSQC of isolated SGP. **Insert**: HSQC data from Liu *et al*. for the same compound, confirming the anomeric resonances.

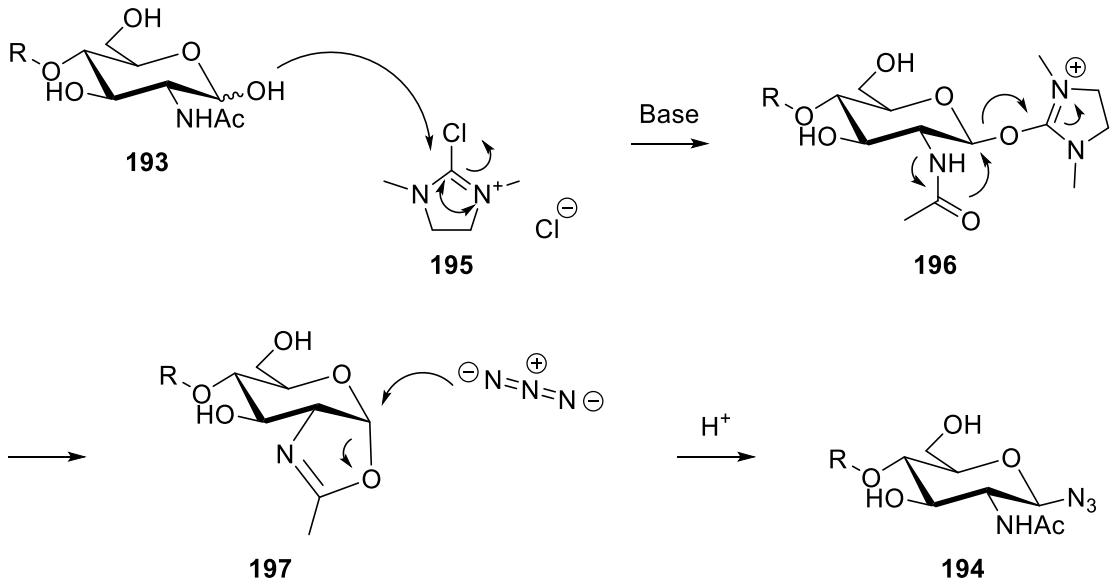
3.2.1 Derivatisation for glycosylation of IFNγ

In order to prepare the azide bearing glycan for glycosylation of IFNγ, it was necessary to first remove the peptide and access the reducing sugar. This was accomplished with PNGase F (E.C. 3.5.1.52), an enzyme used for removing *N*-linked oligosaccharides from glycoproteins. Digestion of native SGP with PNGase F gave the reducing sugar **193** as a mixture of anomers in excellent yield of 92% (Scheme 3.2). The identity of the reducing sugar was confirmed primarily through MS data, which showed no remaining native SGP after digestion. In order to stereospecifically install the β-azide functionality, an oxazoline was formed using 2,6-lutidine and 2-chloro-1,3-dimethylimidazolinium chloride (DMC) (Scheme 3.2). With DMC functioning as an activating agent for the anomeric centre, the C2 amide of the GlcNAc bridgehead forms the desired oxazoline. Due to the expected vulnerability of this intermediate to hydrolysis, the oxazoline was not isolated at this time.



Scheme 3.2. Derivatisation of SGP **192** to SOS-N_3 **194**.

The oxazoline **197** was subsequently transformed into the anomeric azide upon treatment with a large excess of sodium azide (685 eq.) and stirred for 3 days at 2 °C (Scheme 3.3). Following size-exclusion chromatography, this gave the β-azide **194** in good yield of 52%. This sialyloligosaccharyl azide (SOS-N_3) was protected from light, and could reliably be stored for 24 months at -20 °C without any appreciable decomposition.



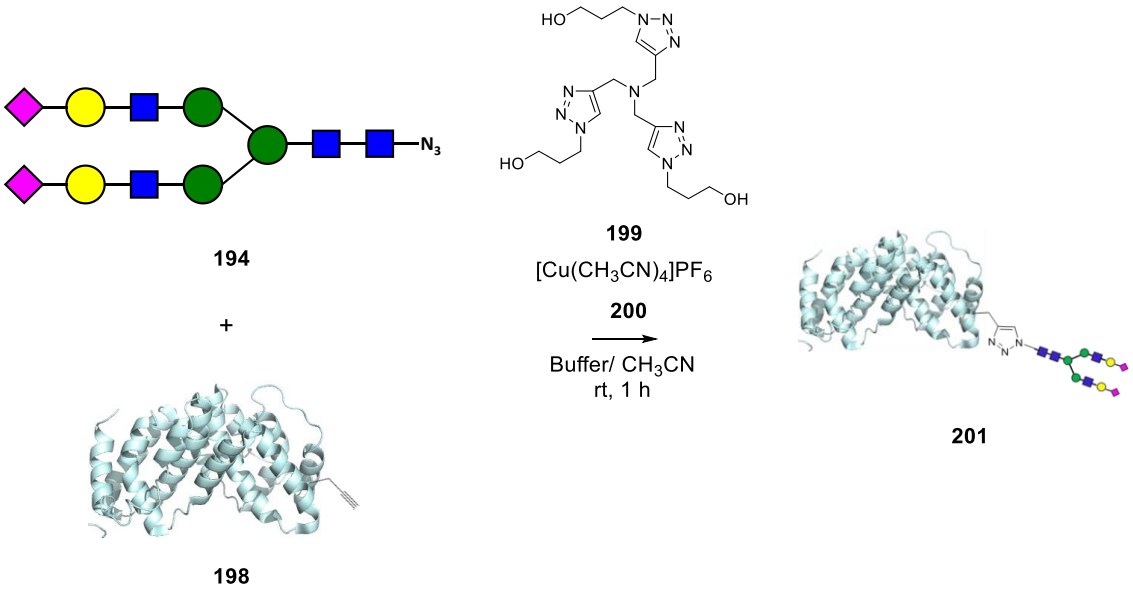
Scheme 3.3. Mechanism for the transformation of oligosaccharide **193** into sugar oxazoline **197**, and subsequent ring opening to yield SOS-N_3 **194**.

3.3 Click Chemistry

3.3.1 Click at position 97 and Refolding

The bioconjugation of azidoglycan **194** and IFNγ-His_6 with PrgY at position 97 (hereafter, referred to as PrgY97-IFNγ-His_6) was first attempted in a number of buffer systems. Imidazole from the Ni-NTA was removed *via* buffer exchange, as some chelation of the copper complexes employed for CuAAC was postulated. This buffer exchange gave solutions of denatured protein in 6 M Gdm·Cl, 3 M Gdm·Cl, and 6 M urea, each with 10 mM Tris·HCl. The click experiments were conducted at a physiological pH of 7.5.

A typical click reaction proceeds as follows. The glycan **194** and protein **198** are combined with a stabilising ligand, tris((1-hydroxy-propyl-1H-1,2,3-triazol-4-yl)methyl)amine (THPTA) **199**, which coordinates to the metal centre and maintains the Cu(I) oxidation state. Oxygen is excluded from this buffer premix by bubbling inert gas (N_2) through the solution. Tetrakis(acetonitrile)copper(I) tetrafluoroborate **200** was dissolved in degassed CH_3CN and added to the buffered solution under N_2 with the reaction mixture and then sealed for 1 h at rt (Scheme 3.4). A blue colour persists towards the end of the reaction, attributed to small quantities of Cu^{2+} ions forming as a by-product.



Scheme 3.4. A typical CuAAC reaction between protein **198** and glycan **194**.

The use of 6 M Gdm·Cl returned unreacted IFNγ, with a small amount of conjugation product observed for 3 M Gdm·Cl by SDS-PAGE. Good CuAAC yields were observed for 6 M urea – however, over time, a lower molecular weight band (*ca*. 14 kDa) appeared in samples 'clicked' in urea. This arose from the storage of the clicked protein in urea, which may result in some carbamylation of the protein *N*-terminus and/or lysine and arginine residues, and general decomposition.[253] Optimisation of the click conditions resulted in a reduction of urea concentration to 3 M, which is still well above the denaturing/unfolding midpoint for IFNγ.[254] The buffer was quickly changed to 3 M Gdm·Cl following bioconjugation to prevent unwanted carbamylation. The use of phosphate buffer did not permit any click reaction to proceed, however 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) and 3-morpholinopropane-1-sulfonic acid (MOPS) were somewhat tolerated, albeit with some decomposition (Figure 3.6).

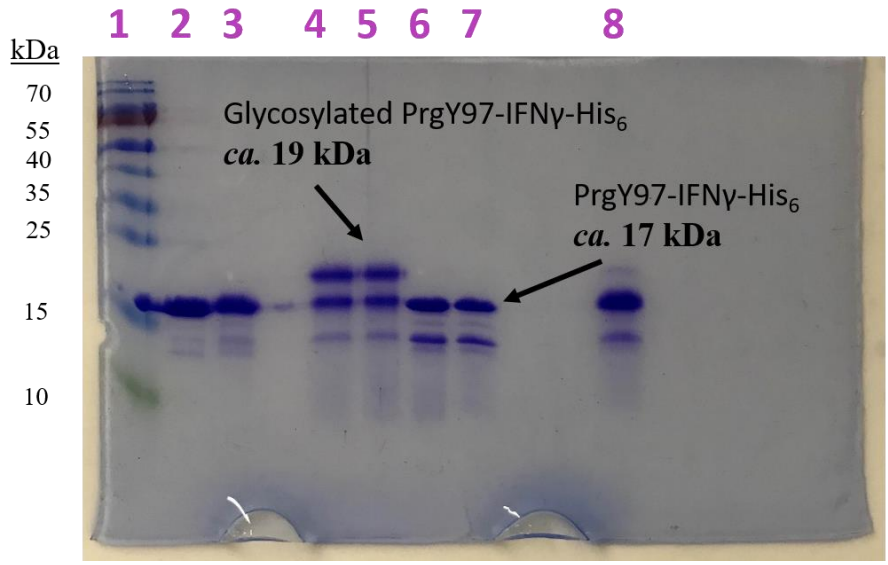


Figure 3.6. Optimisation attempts. Gel with lanes 1) marker, 2) control, 3) control of the control, 4) click in urea/HEPES pH 7.2, 5) click in urea/MOPS pH 7.2, 6) click in urea/phosphate pH 7.2, 7) click in urea/phosphate pH 7.5, and 8) week-old click reaction.

Reliable access to glycosylated PrgY97-IFNγ-His_6 had now been established, and efforts to refold this protein commenced. A refolding buffer with L-Arginine (0.5 M) was determined the most suitable for refolding, based on literature accounts.[255-256] Refolding was effected by placing a 1 mL globule of a 1-2 mg/mL buffered solution containing glycosylated PrgY97-IFNγ-His_6 at the bottom of a beaker. Rapid dilution (100x) with refolding buffer at 4 °C and storage of the resulting solution at 4 °C for 24 – 48 h without agitation ensued. Concentration of this refolding solution to *ca*. 1 mL and buffer exchange gave a solution of refolded PrgY97-IFNγ-His_6 in Tris·HCl buffer for further purification.

Significant protein losses were recorded for modification and bioconjugation at position 97. Following refolding, protein quantities recovered were near negligible, likely due to precipitation of the refolded protein in the centrifugal concentrators. Repeated click reactions and a number of refolding iterations were required to access enough of the dimeric protein such that an accurate concentration could be measured *via* UV. It was postulated that the specific domain in which position 97 is located may contribute to its recalcitrance to modification with a hydrophobic amino acid residue (PrgY) and the subsequent bioconjugation with a large glycan, and the subsequent refolding.

3.3.2 Click at position 25 and Refolding

In tandem with our investigation of this protein derivative, the analogous IFNγ-His_6 with PrgY at position 25 (hereafter, referred to as PrgY25-IFNγ-His_6) was investigated. It was theorised that the protein losses and expected precipitation problems for PrgY97-IFNγ-His_6 were due to the environment surrounding the consensus sequence at that site.

Incorporation of PrgY at position 25 was accomplished *via* the methodology outlined for PrgY97-IFNγ-His_6. Once again, Ni-NTA affinity chromatography was employed, and CuAAC mediated glycosylation reaction was inflicted upon the denatured protein. Refolding proceeded as outlined previously, and gratifyingly, the stability of the refolded protein was greatly increased. Following a number of click-refold-concentrate iterations, concentrations of 0.5 mg/mL were achieved that were stable at 4 °C (Figure 3.7). It should be noted that freezing protein samples following refolding is not advised, as any sort of freeze-thaw action resulted in a number of misfolded protein states, which were visible in purification attempts (see section 3.4.1). Additionally, it should be clarified that the desired dimer is not visible *via* SDS-PAGE analysis as this is a denaturing technique in its own right due the detergent present.

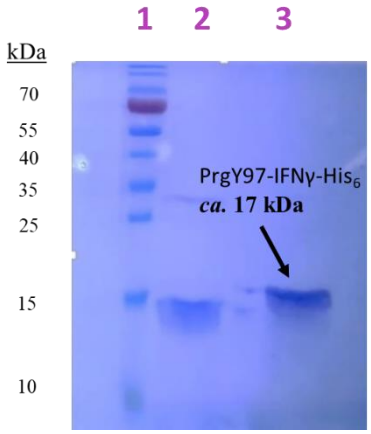


Figure 3.7. Gel showing that PrgY25-IFNγ-His_6 stays in solution following refolding. Lanes are 1) marker, 2) denatured WT-IFNγ-His_6, and 3) refolded PrgY25-IFNγ-His_6.

3.4 Purification of glycosylated IFNγ

3.4.1 Ion-exchange chromatography (IEX)

By definition, ion exchange chromatography (IEX) functions on the principle of separation by total charge. Similar, ionisable molecules which would be difficult to separate *via* other chromatographic methods can be routinely purified through

modulation of the buffer pH. Shifting the lens to biomolecules, the zwitterionic amino acids which form the basis for most biomolecules can carry net positive, negative or neutral charges. The point at which this biomolecule has no net charge as the isoelectric point (pI). Once one has access to the polypeptide sequence of the protein of interest, pI can be calculated, and whichever buffer is employed will dictate the net charge of this protein in solution.

There are two broad classes of IEX: cation exchange chromatography, and anionic exchange chromatography. If one intends to use a buffer pH higher than the pI of the desired protein to be purified, then the protein or biomolecule will be negatively charged; thus, a positively charged anion exchange resin must be employed. Conversely, should one use a buffer with a pH lower than the pI, the primary structure of the protein will carry a positive net charge, and hence a negatively charged cation exchange resin is required. While IEX boasts high yields and purification under non-denaturing conditions, clusters of positively charged residues can cause a net-negative protein to bind incorrectly, and vice versa. Furthermore, infinitesimal changes in pH can skew the binding profile to the IEX resin dramatically. The protein of interest can be eluted with either pH modulation, or changes in salt gradient (Figure 3.8).[257]

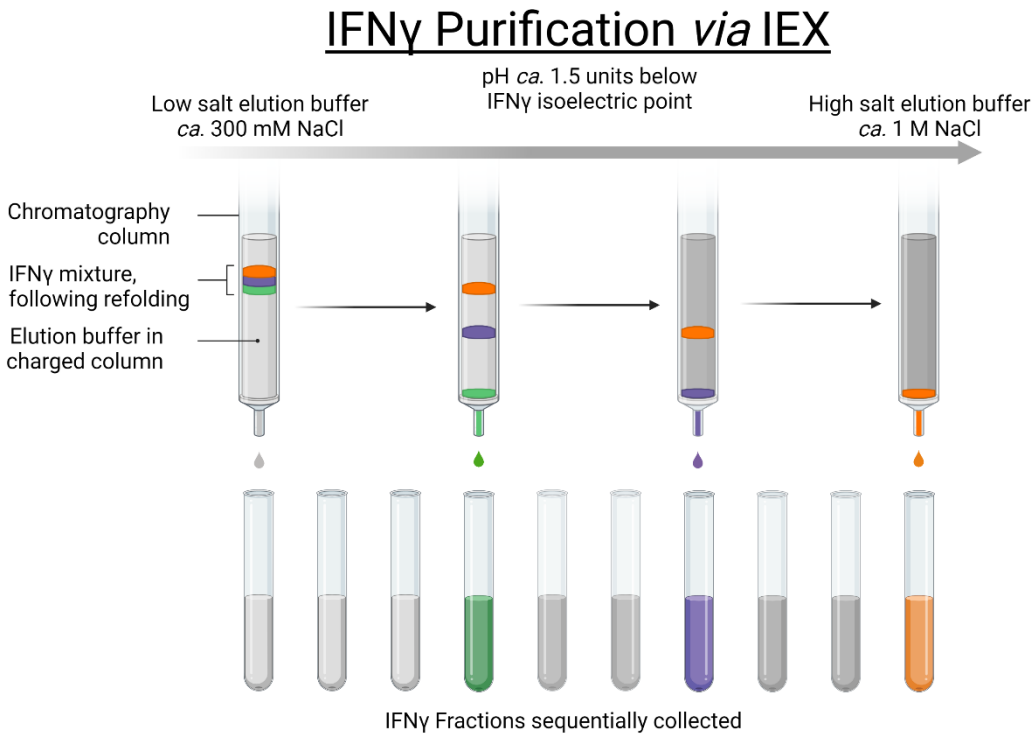


Figure 3.8. General procedure of purifying IFNγ *via* IEX.[247]

In the present account, two IEX resins were employed for the purification of WT-IFNγ-His_6 and the glycosylated IFNγ derivatives. If one computes the pI of WT-IFNγ-His_6 (and includes the polyhistidine tag in the calculation), the pI is *ca*. 9.70.[258] Cation IEX works best with a buffer pH of 0.5 – 1.5 units less than the pI of the desired biomolecule, and this was factored in accordingly in the choice of purification parameters.

The weak cation exchanger carboxymethyl (CM) Sepharose, supported on cross-linked agarose, was first employed for this purification. A fast-protein liquid chromatography (FPLC) system was employed for this purpose, with initial runs used to pack and equilibrate the column. A 1 mg/mL solution of WT-IFNγ-His_6 was applied to the column, and the column washed with Tris·HCl buffer (pH 8.2) containing NaCl (200 mM). Fractionation began once NaCl concentration reached 400 mM, as this is the specified range in which IFNγ is expected to elute, according to Petrov and co-workers.[259] Disappointingly, no discernible protein peaks were eluted from this column. Repeated attempts with longer, linear salt gradient were ineffectual.

The IEX media was changed to a pre-packed sulofpropyl (SP) Sepharose column, which is a strong cation exchanging resin. Following the same procedure as previously outlined, purification of a 1 mg/mL solution of WT-IFNγ-His_6 was attempted using identical gradient and fractionation range as before. Unfortunately, in our hands this procedure furnished only misfolded forms of IFNγ (Figure 3.9). The chromatogram below shows three diffuse peaks (labelled **A**, **B**, and **C**), all of which were assigned as WT-IFNγ-His_6 following SDS-PAGE analysis. As the electrophoresis bands were identical under the denaturing effect of the SDS detergent, one can hypothesise that the different elution dynamics originates from secondary structure. A number of IEX attempts were made, with varying conditions, but frustratingly the results were inconclusive.

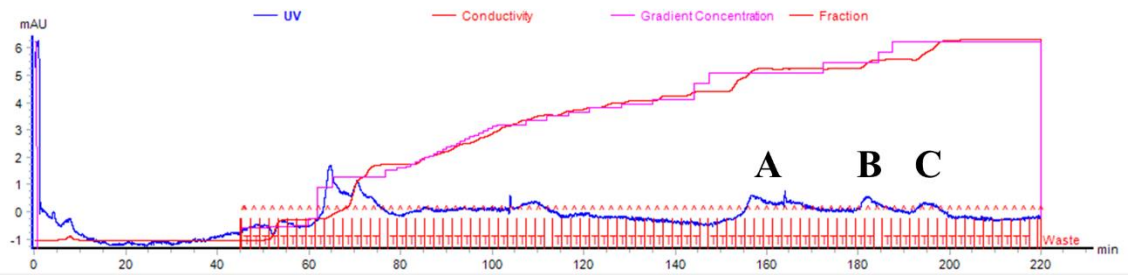


Figure 3.9. Chromatogram of a 1 mg/mL purification on SP-Sepharose. A, B, and C are all WT-IFNγ-His_6.

3.4.2 Size-exclusion chromatography (SEC)

At this juncture it was elected to switch to size-exclusion chromatography (SEC) purification. Rather than purification based on net charge, SEC offers purification based on molecular weight *via* gel filtration. Generally, this method relies on molecules of different sizes interacting with the pores in the gel matrix. Small molecules enter the pores of the gel 'beads' and interact with an increased surface area of the stationary phase – this causes them to be retained in the column. Molecules of sufficient size such that they do not enter the pores pass through the media faster, and thus one can elute biomolecules in order of decreasing molecular weight (Figure 3.10).

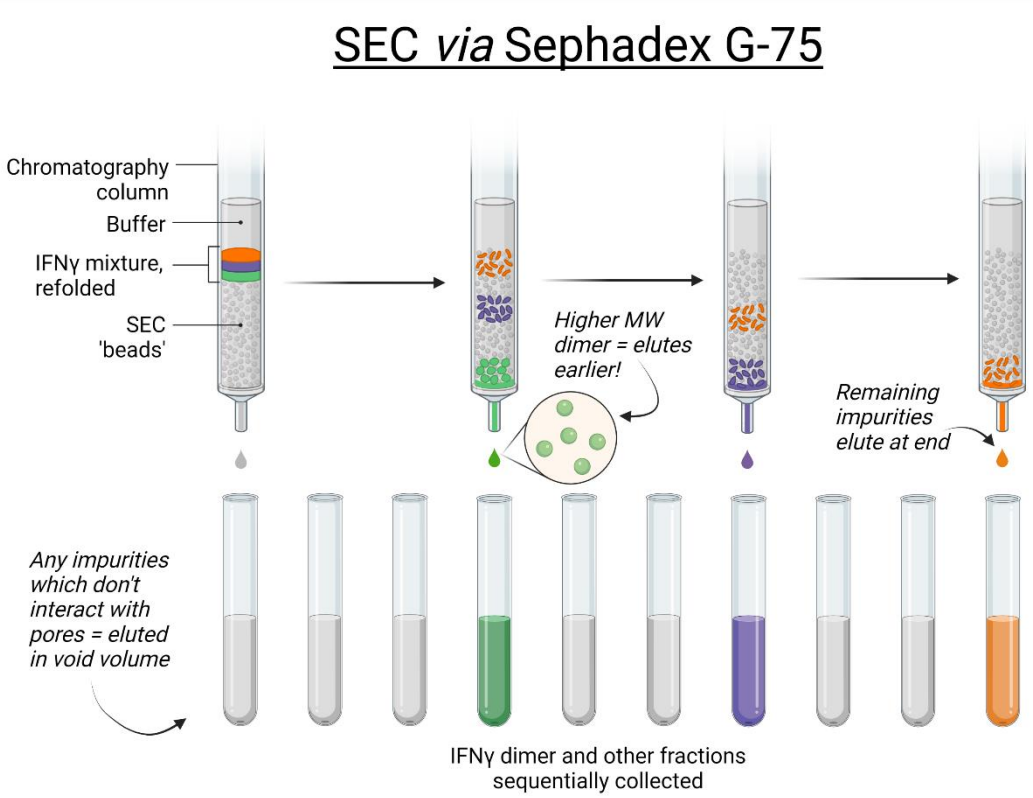


Figure 3.10. SEC of IFNγ following refolding. Smaller impurities are retained longer, and higher MW biomolecules pass through based on size.

Superdex G-75 was chosen as a suitable fractionation system, as the molecular weight of WT-IFNγ-His_6 (*ca*. 17 kDa) and the glycosylated derivative of PrgY25-IFNγ-His_6 fall within its usable range. A pre-packed HiLoad Superdex column, suitable for preparative grade purification, was acquired. A 1 mg/mL solution was purified during the first run, and much to our delight, the WT-IFNγ-His_6 was easily purified by this method with the peak eluting at *ca.* 55 min (Figure 3.11).

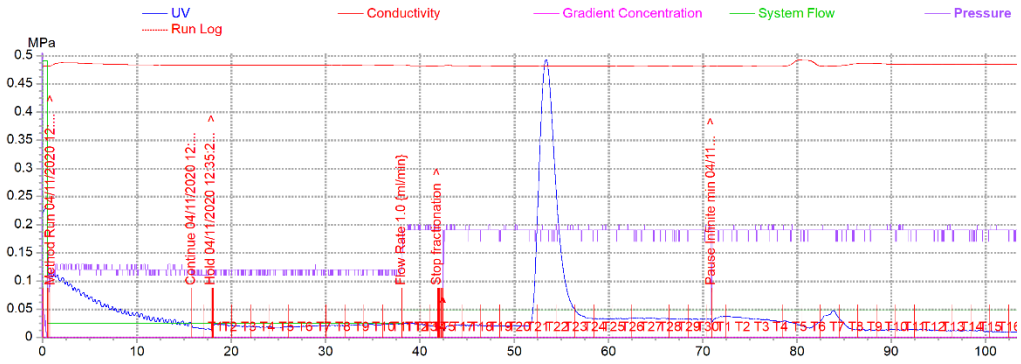


Figure 3.11. Chromatogram for the SEC purification of WT-IFNγ-His_6.

However, significant protein losses occurred throughout this purification. From the 1 mg of refolded protein loaded onto the column, 60-70% was lost, presumably due to precipitation. The author validated the column by separately purifying a 1 mg/mL sample of Bovine Serum Albumin (BSA), which showed recovery of over 85%. Having validated the column, it was theorised that IFNγ possesses a predilection for precipitation in the absence of chaotropic agents, which may explain the low recovery of *ca.* 35%. Nevertheless, the purification of glycosylated PrgY25-IFNγ-His_6 was attempted – however, obtaining more than 0.5 mg of material at one time was not possible in this stage of the project. While there was some evidence that the purification was similar to the WT-IFNγ-His_6 trials, the high rate of protein precipitation and general disappearance on the column rendered this approach moot.

Finally, a Superdex G-75 column with a significantly lower diameter and lower bed volume was purchased. Theoretically a smaller column operating under higher pressure, and somewhat akin to a HPLC purification, would yield a usable protein sample. Unfortunately, this approach was quickly thwarted by the necessity for a specific pump/automatic purification system in order to use this Superdex Increase column, which was not accessible to the author at the time of writing.

3.5 Conclusions and Future Work

In conclusion, the critical human cytokine IFNγ has been synthesised *via* recombinant expression in chemically competent *E. coli* ER2566 cells. The incorporation of *O*-propargyltyrosine at two *N*-glycosylation sites (positions 25 and 97) was accomplished using the amber codon suppression framework. This provided access to samples of

reasonable purity (assessed by SDS-PAGE), possessing a hexahistidine tag at the *C*-terminus. Ni-NTA affinity chromatography was routinely used for crude purification of protein samples, and copper-catalysed azide-alkyne cycloaddition reactions were conducted on denatured protein samples following this affinity purification. Refolding presented the first major bottleneck of the approach, with large protein losses recorded following concentration of the refolding mixture. Additionally, the refolded samples were intractable under all ion-exchange chromatography procedures investigated. Size-exclusion chromatography offered the best approach for obtaining high quality, refolded IFNγ and glycosylated IFNγ samples, but was marred by lack of access to the required instruments for small-scale purification (primarily due to pandemic restrictions).

In terms of future work, a number of suggestions are presented, the collaborative study is ongoing. Firstly, the smaller Superdex Increase column has already been purchased, and must be validated with a suitable instrument and reference protein. An FPLC system such as the ÄKTA Go, ÄKTA Prime, or ÄKTA Pure must be used to reach the required operating pressure of the column. Until access to a suitable system has been established, there is little point in continuing this project. If access to these systems cannot be secured, it is possible to connect the column to a HPLC system and use finely controlled pressure limits and a blast shield around the column. Routine purification of glycosylated samples ranging from 0.25 mg/mL to 0.5 mg/mL should be optimised, perhaps by first validating the smaller column with a protein such as BSA.

In terms of further synthesis, the problems posed by modification of position 97 must be addressed. Since this site is glycosylated in mammalian systems, it is possibly the hydrophobic nnAA that is problematic. Our collaborators have made initial steps to alternatively incorporate a cysteine residue at this position (there is no other Cys residues present in IFNγ), so that desulfurisation to dehydroalanine (DHA) can be effected. Separately, we have performed initial experiments to generate the anomeric thiol of egg yolk sialyloligosaccharide, so that radical thiol-ene can be inflicted upon the DHA residue for bioconjugative purposes instead. Hypothetically, this reduction in hydrophobicity and steric bulk at position 97 would alleviate some of the issues associated with protein refolding following glycosylation.

Should the purification issues be resolved and the incorporation of two different nnAA's at positions 25 (PrgY) and position 97 (DHA), the possibility of conjugating two different

glycans arises. A wealth of other complex and hybrid glycans are available *via* enzymatic modification of the glycans discussed herein, or indeed by extraction from other natural sources such as locust bean gum. Derivatisation of these glycans to the suitable click partner would render accessible a diverse array of glycoforms.

Finally, once the synthesis of purification of refolded dimers has been accomplished, immunological studies are of key importance. The Scanlan lab has enjoyed a long standing collaboration with the Lavelle group in Trinity College Dublin, who have agreed to perform immunological experiments once IFNγ samples are available in sufficient purity. This would provide an invaluable insight into the activity of recombinantly expressed, and bioconjugated IFNγ glycoforms.

Chapter Four

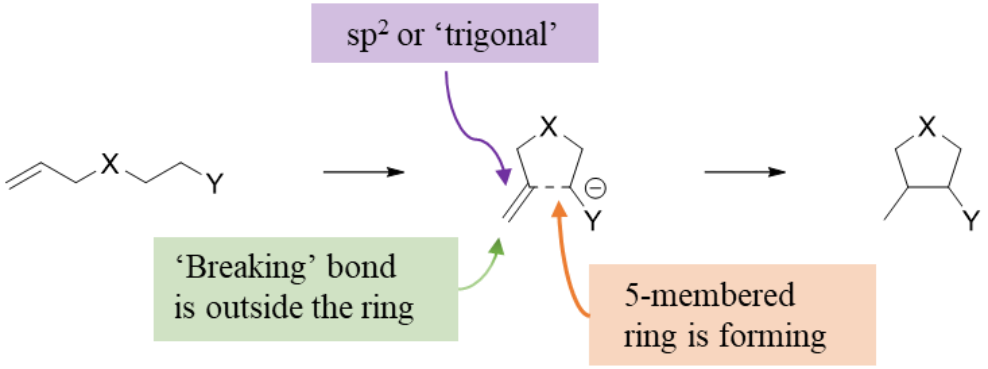
TRACE

4.0 Thiyl Radical Addition, Cyclisation, and Elimination

Sulfur centred radicals have enjoyed a privileged position in the fields of medicinal chemistry, polymer science and chemical biology over the past number of decades, particularly since the turn of the millennium.[260-266] For the purposes of this chapter, we will consider that sulfur in its -2 oxidation state can act both as an ionic nucleophile, and as a radical. The S-H bond is somewhat weak, with a bond dissociation energy (BDE) of *ca*. 365 kJ/mol[178] – therefore, thiocarboxylic acids and aliphatic thiols can be employed to generate both acyl-thiyl and thiyl radicals respectively, *via* homolytic cleavage or by single electron processes. In the case of a redox process, oxidants such as Mn (III) complexes can be used to furnish the desired sulfur-centred radical.[180-182] If a homolytic cleavage approach is employed, usually a carbon-centred radical from a suitable initiator will abstract a proton from the S-H moiety and furnish the desired radical (RS$^{\bullet}$). Thiyl radicals are routinely employed to perform hydrothiolation at sites of unsaturation. The thiol-ene and thiol-yne reactions represent the reactions of primary interest to this chapter. A full analysis of the utility of these reactions is beyond the scope of this chapter, however the reader is directed to a number of publications in the area.[184, 188, 267-269]

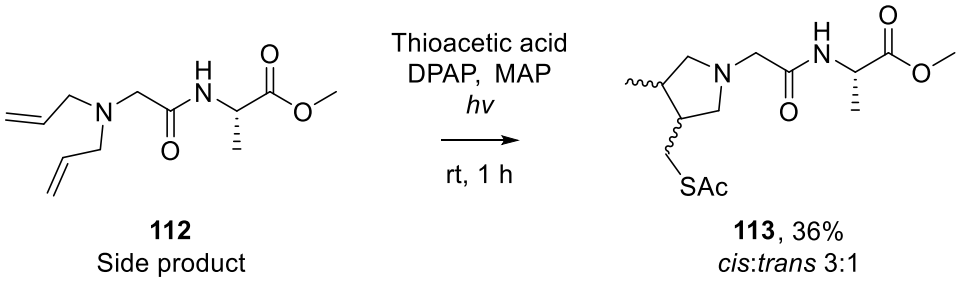
One cannot discuss ring forming reactions without paying tribute the Jack Baldwin. In 1976, Baldwin generated the seminal publication on 'rules' for ring closures.[270] These governances on the favourability of certain ring closures do not describe the absolute probability of one cyclisation mode over the other, and rather offer a general sense of which cyclisation mode is obtained through ionic or radical processes.[271] Generally speaking, Baldwin's hypothesis was that ring closure can occur *"when the length and nature of linking chain enables the terminal atoms to achieve the required trajectories"*.[270] Conversely, those closures which would require harsh contortion of bond angles and long atom chains or 'tethers' in order to facilitate closure are termed disfavoured, and thus cannot compete with much faster favoured reactions. While an extensive description of Baldwin's Rules will not be outlined here, the general naming conventions are of particular relevance. Baldwin separated differing cyclisation modes based on three factors; i) the number of atoms in the incipient ring, ii) the position of the breaking bond relative to the ring, and iii) the hybridisation at the ring closing point. In practice, the cyclisation of a simple alkene could be termed 5-*exo*-trig, in which a five membered ring is formed, with the 'breaking' alkene ending up outside the ring as a

methyl group, and the cyclisation occurring on an alkene (trigonal, or 'trig'). This is outlined in Scheme 4.1 below.



Scheme 4.1. An example of a 5-*exo*-trig cyclisation, with annotations to indicate how terminology arises.

The origins of this project lie in the supervision of an undergraduate SS project student. The student, Susannah H. Calvert (S.H.C.), conducted research under my supervision on auxiliary mediated ligation methodology for glycopeptides. Through the synthesis of their auxiliary, a number of 1,6-dienes were synthesised as by-products from amine over-alkylation. However, upon subjecting these undesired side products to photochemical thiol-ene, an interesting cyclization product was observed. One such peptidic example is shown below (Scheme 4.2).



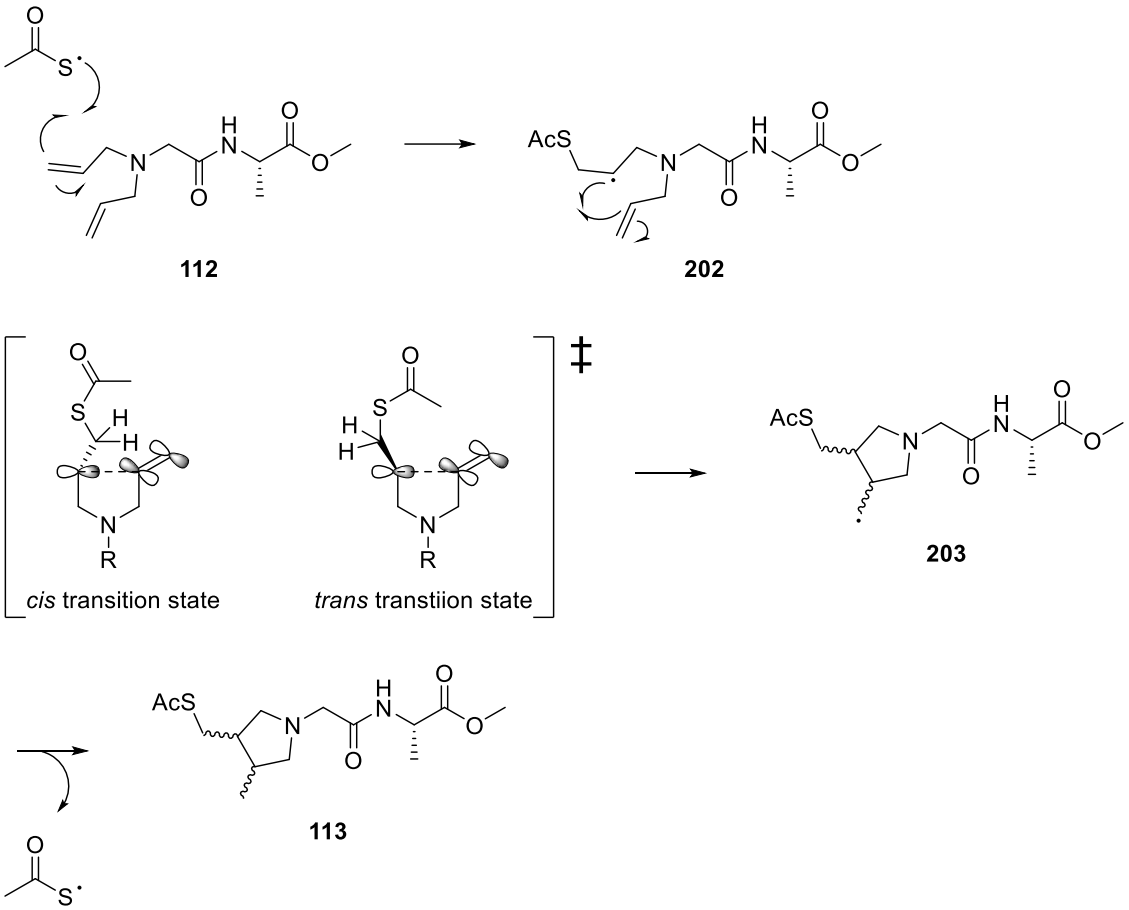
112
Side product

113, 36%
cis:*trans* 3:1

Scheme 4.2. Cyclisation identified by hydrothiolating peptide **112**.

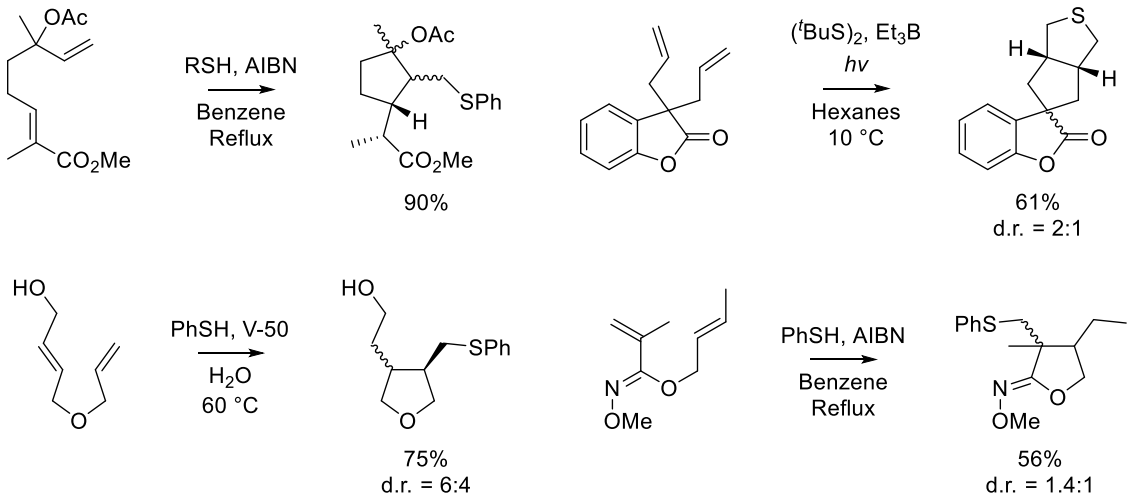
This 5-*exo*-trig cyclisation appears to have some predilection for the *cis* isomer, where both ring substituents end up on the same face of the pyrrolidine. While the *trans* isomer is the thermodynamic product with fewer steric repulsion effects, orbital symmetry considerations show a clear preference for the *cis* isomer. Hyperconjugative mixing of the half-filled p orbital and the C-H σ and σ* orbitals interact to generate a partly delocalised orbital of similar symmetry to the remaining alkene π*. This frontier molecular orbital (FMO) interaction between the radical singly occupied molecular orbital (SOMO) and lowest unoccupied molecular orbital (LUMO) of the π-bond can be

used to rationalise the stereo- and regiochemistry of the reaction. Therein, the formation of the *cis* isomer is characterised by further stabilisation in its transition state.[272-273] This reaction is outlined in the below mechanism (Scheme 4.3).



Scheme 4.3. Radical addition to dipeptide **112** gives a 5-*exo*-trig cyclisation, with two primary transition states.

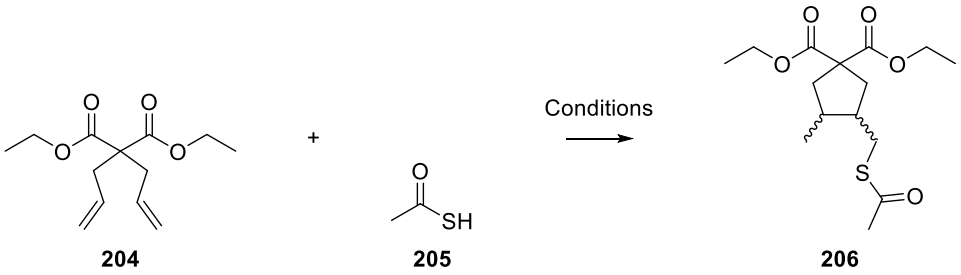
While this class of radical-mediated cyclisation is far from novel (Scheme 4.4),[184, 188] the comparably mild conditions to the reported thermal initiation approaches, characterised by long reaction times and poor yields, were seen as synthetically valuable. Furthermore, very little research has been conducted into the full scale of possibilities for this class of synthetic transformation. This **T**hiyl **R**adical **A**ddition, **C**yclisation and **E**limination (**TRACE**) approach was envisioned as a versatile approach to the cyclisation of enynes, dienes, and heteroatomic species: after which the sulfur atom could be removed or used for further derivatisation. This chapter will detail the initial study of reaction scope, the expansion to more complex unsaturated systems, the use of different thioacids to effect cyclisation, and the employment of heteroatom centred species. Additionally, desulfurisation approaches towards sulfur-free products will be explored.



Scheme 4.4. Assorted literature examples of thiyl radical mediated cyclisation.[184]

4.1 Initial Reaction Scope

Following from the success of dipeptide **112** as a substrate for cyclisation, commercially available diethyl diallylmalonate **204** was employed to probe the initial reaction scope. A number of reaction conditions (Table 4.1) were employed to identify the optimum conditions under UV irradiation (Scheme 4.5).



Scheme 4.5. Archetypal 1,6-diene cyclisation employed for optimisation.

Table 4.1. Optimisation attempts.

Entry	t (min)	DPAP/MAP (eq)	Thioacid (eq)	Solvent	Conc (M)	Inert atm.	UV irr. (nm)	Conversion (NMR, %)
1	120	0.1/0.1	1.2	EtOAc	0.1	-	0[1]	0
2	120	0/0	1.2	$CDCl_3$	0.1	+	0	0
3	120	0/0	1.2	EtOAc	0.1	-	0	68
4	180	0/0	1.2	$CDCl_3$	0.1	+	365	70
5	120	0.1/0.1	1.2	EtOAc	0.1	-	365	>99
6	120	0/0	1.2	EtOAc	0.1	-	365	>99

[1] *1.2 eq of NEt_3 added to form thiolate/prevent radical formation*

As evidenced by Table 4.1, the acyl thiol-ene reaction does not appear to initiate in the dark or under an inert atmosphere. However, control experiments lacking the DPAP/MAP photoinitiator/photosensitiser pair (entries **2**-**3**, and **6**) show that while there appears to be little dependence on the presence of oxygen for the overall yield, the reaction can initiate with just UV irradiation and even without an inert atmosphere. It was hypothesised that these results are more so a product the reactivity of thioacetic acid **205**, rather than an indication of the dynamics of the overall reaction scheme Entry **1** involved the preparation of the corresponding thiolate prior to the reaction initiation, to show that indeed the process is radical mediated. This is further bolstered by the use of a 'TEMPO test' – a simple colorimetric test in which the bench stable radical (2,2,6,6-tetramethylpiperidin-1-yl)oxyl or TEMPO is added to a reaction prior to radical initiation to give a rich red colour. Loss of this colour indicates some form of radical generation, and the 'trapped' adducts can often be characterised by mass spectrometry methods. A TEMPO test for this process showed clear evidence of radical intermediates.

4.1.1 NMR Timescale Experiments

In order to further understand this process, NMR timescale experiments were performed. These involved performing the UV initiated thiol-ene in deuterated solvent, and taking reaction aliquots at defined intervals. Diethyl diallylmalonate was dissolved in $CDCl_3$ (final substrate concentration = 0.1 M). Thioacetic acid (1.2 eq.) was added, and the reaction media irradiated at 365 nm for 2 h. Aliquots were taken at 15 min, 30 min, 45 min, 60 min, 90 min, and finally 120 min. Disappearance of the alkene signals at 5.63 and 5.07 ppm, and formation of the characteristic doublet at 0.90 ppm for the methyl coming off the pyrrolidine ring are used as indicators to track this process (Figure 4.1).

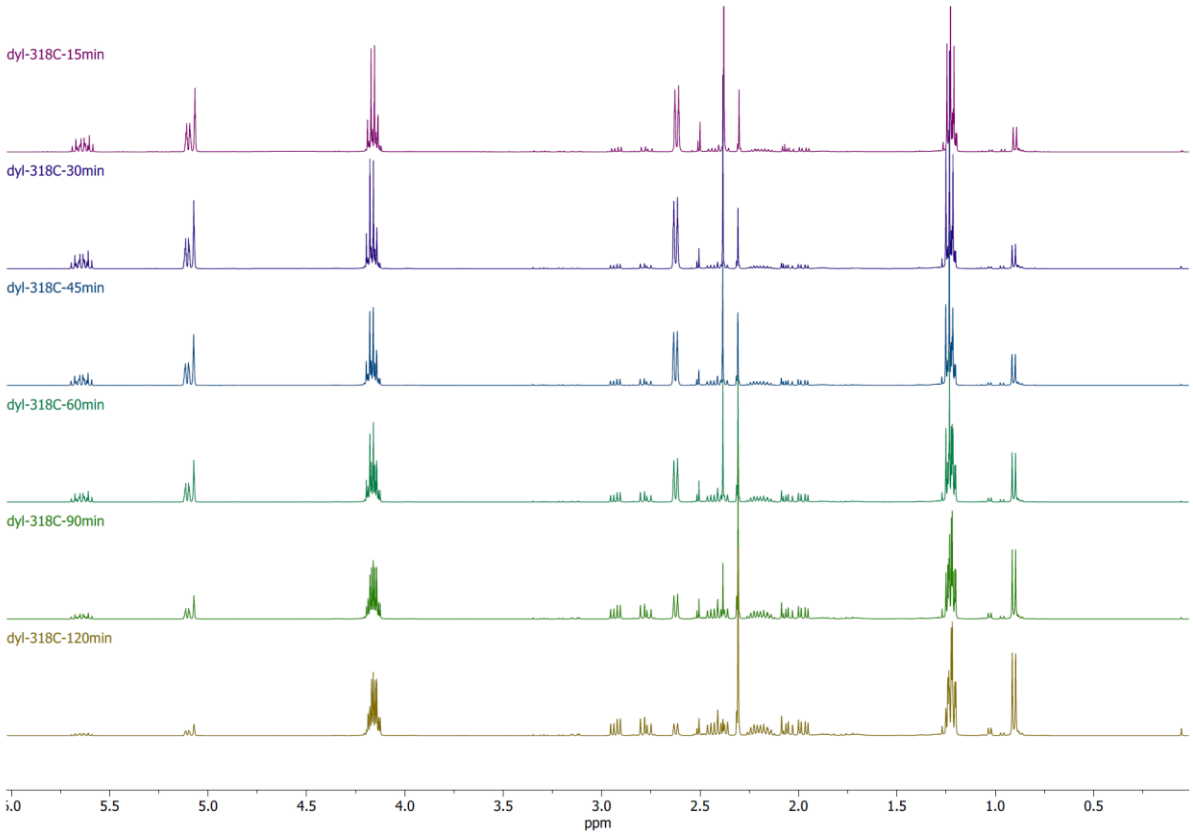


Figure 4.1. Timescale ^{1}H NMR experiment in $CDCl_3$ to analyse the formation of **206**.

Having visualised this process on an appropriate timescale, the same reaction was employed as a control, but without UV irradiation, and with the reaction flask wrapped in tinfoil to exclude light. As the below timescale control shows (Figure 4.2), there was no appreciable consumption of the alkene observed over a 2 h timescale. With these results in hand, we then turned our attention to expanding scope of thioacids utilised in this approach.

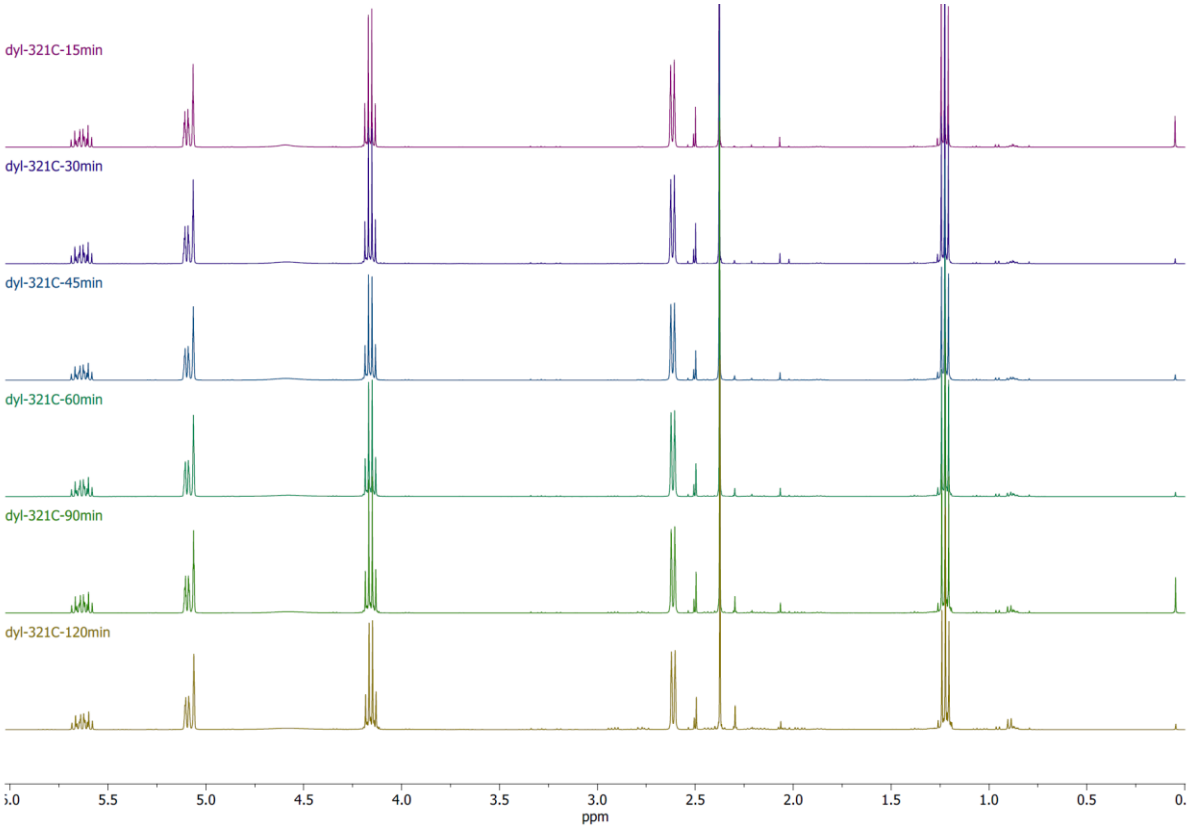
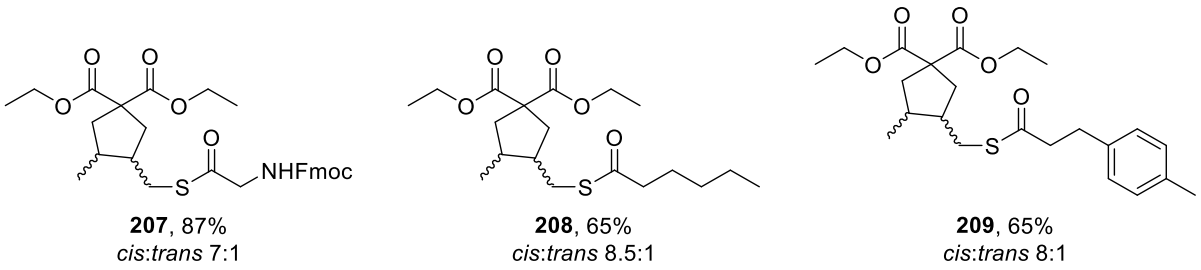


Figure 4.2. Control experiment showing no consumption of the alkene over the same time period.

4.1.2 Alternative thioacids

Some of the previously described *S*-tritylthioesters from Chapter Two were resynthesized, and applied to the cyclisation process. Fmoc-Gly thioacid (from **143**), heptanoic thioacid (from **141**), and aromatic thioacid (from **140**) were all employed to give the corresponding cyclised products **207** – **209**. Isobutyric thioacid (from **142**) was omitted, as the volatility of this donor gave non-reproducible yields between reactions. These reactions proceeded as follows; the corresponding *S*-trityl thioester was deprotected with TFA in CH_2Cl_2 (25% v/v), in the presence of ethyldimethylsilane as a radical scavenger. Upon removal of the TFA and solvent *in vacuo*, the thioacid was resuspended in degassed EtOAc and immediately added to diene **204**, DPAP, and MAP. The resulting solution was irradiated for 2 h after which the remaining thioacid was removed with an alkali wash, and the organic phase concentrated *in vacuo*. The residue was then purified to isolate the cyclised thioester conjugates (Figure 4.3).



207, 87%
cis:*trans* 7:1

208, 65%
cis:*trans* 8.5:1

209, 65%
cis:*trans* 8:1

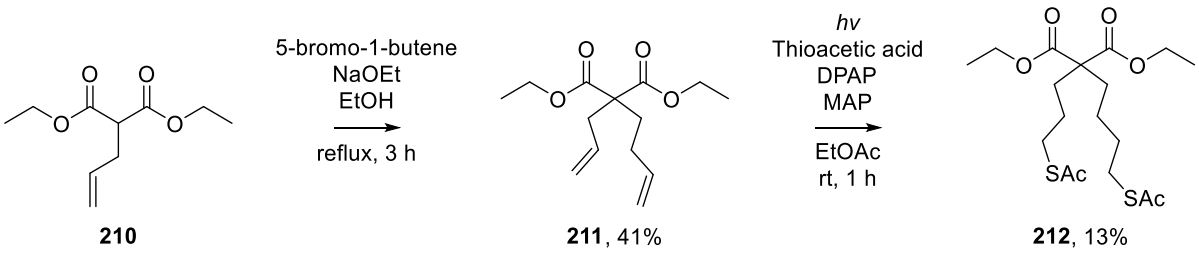
Figure 4.3. The employment of other thioacids for this cyclisation still presents good yields.

The good yields and moderate diastereoselectivity are encouraging in this case, as these results hint that TRACE is not totally reliant on the specific steric properties of thioacetic acid.

4.2 Expansion to further unsaturated substrates

To develop the substrate scope for the cyclisation reaction, a number of unsaturated substrates were prepared. It was envisioned that an expansion of the cyclisation transition state beyond 5-membered would significantly reduce the cyclisation yield as bis-hydrothiolation would begin to predominate; the concomitant increase in degree of freedom with each additional CH_2 centre would likely cut off cyclisation dynamics. The stereoelectronic effects of substitution on one or both alkene termini would also be expected to change the reaction dynamics significantly.

Diene **211** was synthesised to investigate cyclisation towards a larger ring size. Sodium metal was added to anhydrous EtOH and following complete dissolution, diethyl allylmalonate **210** was added dropwise. An alkyl halide, 5-bromo-1-butene (homoallyl bromide) was then added dropwise and the reaction heated to reflux. **211** was isolated after a simple workup and column purification. Radical acyl thiol-ene was initiated as previously described with thioacetic acid, however no appreciable amount of cyclisation *via* a 6-*endo* process or otherwise was identified for this 1,7-diene species. Instead, only bis-hydrothiolated compound **212** was isolated in a 13% yield (Scheme 4.6).

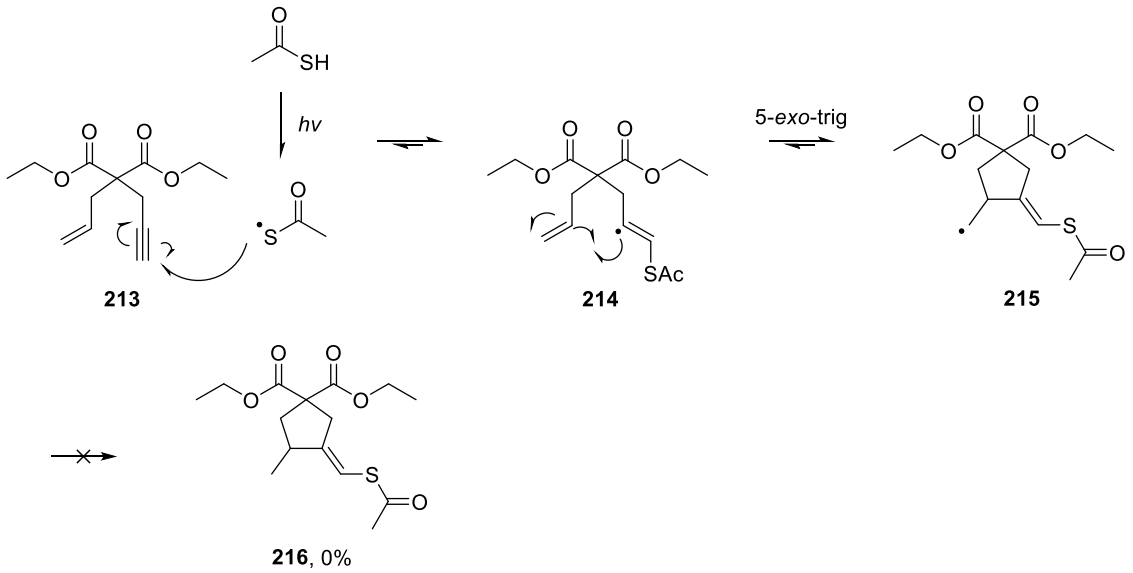


210 **211**, 41% **212**, 13%

Scheme 4.6. Failed cyclisation of 1,7-diene **211**.

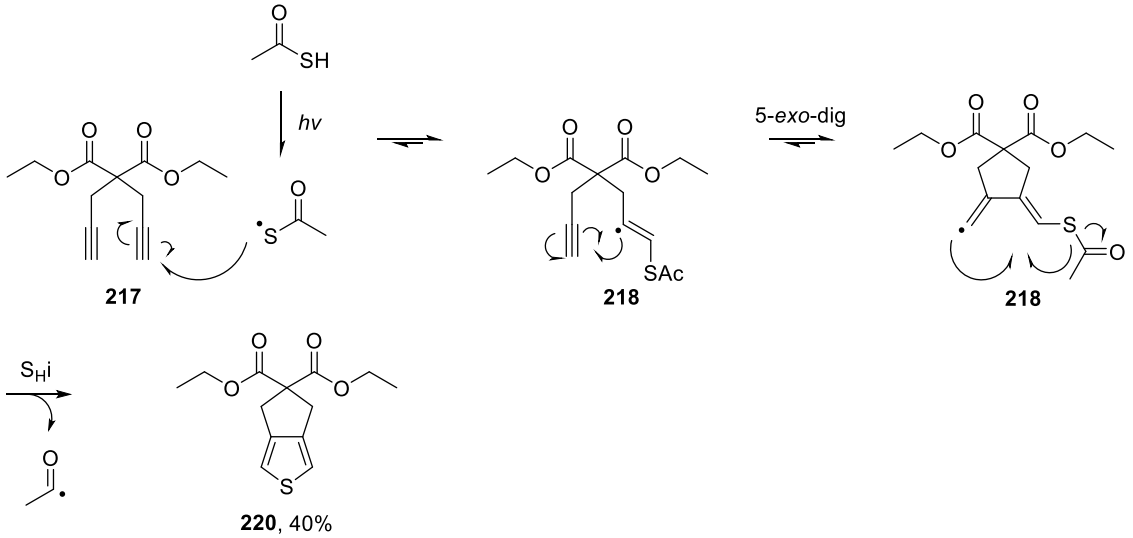
Enynes are an attractive substrate for this class of cyclisation; however, they are often difficult to cyclise due to the high-energy and critically unstable intermediates formed upon radical additions.[184, 274] Enyne **213** was selected to test this hypothesis, and was synthesised analogously to diene **211** – with sodium metal, and the propargyl bromide in this instance. Cyclisation of 1,6-enynes was previously reported by Broka and co-workers, with relatively simple systems and low concentrations of thiophenol as the radical source. They achieved moderate yields by the slow addition of their thiol to a refluxing solution of the enyne substrate and thermal initiator AIBN. However, any substitution at the alkene terminus was not well tolerated, and bis-substitution shut down the reactivity completely.[274] There have been no reported acyl thiol-ene mediated cyclizations of enynes to our knowledge. Broka and co-workers exclusively identified addition to the triple bond, followed by 5-*exo*-trig cyclisation.[274]

Trials with equimolar quantities of thioacetic acid provided some minor amounts of alkene consumption, but no discernible cyclisation product was observed (Scheme 4.7). When the thioacid is used in excess (*ca*. 3 eq.), complete consumption of both alkyne and alkene is observed, however, upon concentration *in vacuo* attempts at purifying any identifiable compound proved futile with TLC analysis showing multiple products formed.



Scheme 4.7. Failed cyclisation of enyne **213**.

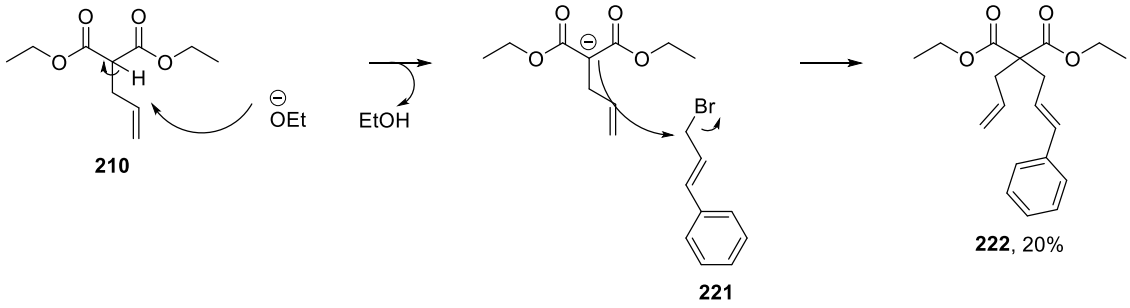
In a final step of increased unsaturation, diyne **217** was synthesised from diethyl malonate and a large excess of propargyl bromide, with sodium ethoxide solution again acting to generate the desired species. Interestingly, diyne species such as **217** have a propensity to undergo intramolecular homolytic substitution (S_Hi) reactions, which can generate bicyclic unsaturated products such as thiophenes.[222] Once again, the same procedure for thiyl radical mediated cyclisation was applied, with thioacetic acid as the radical donor. Gratifyingly, thiophene **220** was identified as a major product, showing that S_Hi chemistry is permitted under these reaction parameters (Scheme 4.8).



Scheme 4.8. Thiophene **220** is formed *via* S_Hi chemistry, during the course of the thiol-yne reaction.

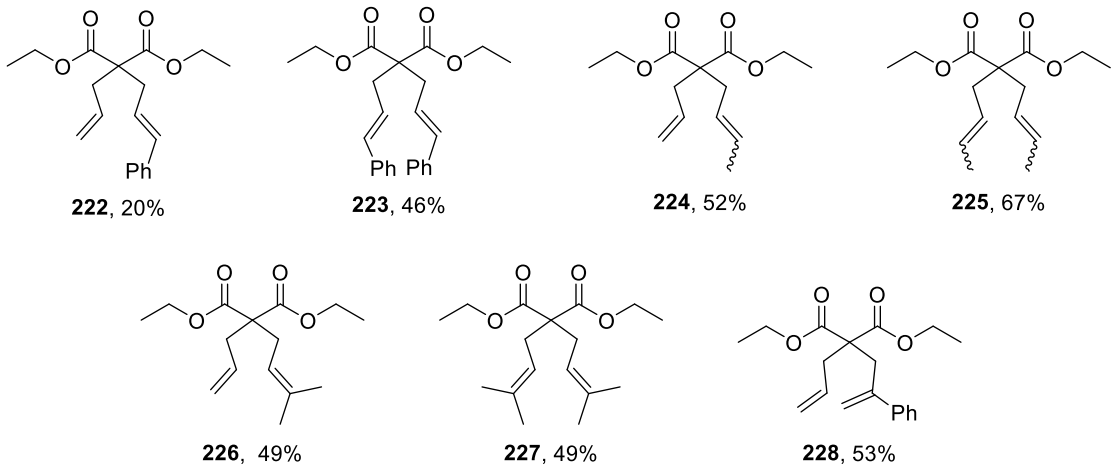
4.2.1 Substituted 1,6-diene systems

A complete expansion of the 1,6-diene space was conducted. A number of varying substitution patterns were employed, with examples shown below in Figure 4.6. In the case of **222** – **228**, the corresponding ester was functionalised using freshly prepared sodium ethoxide and the desired alkyl bromide, with varying equivalents based on whether mono- or disubstitution was required. A typical functionalisation was performed as follows. A solution of the required malonate backbone with sodium ethoxide was stirred for 10 min; the use of the ethoxide species prevented unwanted trans-thioesterification of the malonate esters, and formed the salt of the methine group (Scheme 4.9). Dropwise addition of the corresponding halide (**221**, Scheme 4.9) and subsequent reflux brings the alkylation reaction to completion, after which precipitation of sodium bromide and ensuing chromatographic purification yields the desired diene.



Scheme 4.9. The alkylation of diethyl allylmalonate **210** to give 1,6-diene **222**.

These resulting 1,6-diene species were obtained in moderate to good yield, ranging from 20 – 67% (Figure 4.6). This was largely due to the requirement of synthesising some of the strong lachrymatory precursors which suffer poor bench stability.

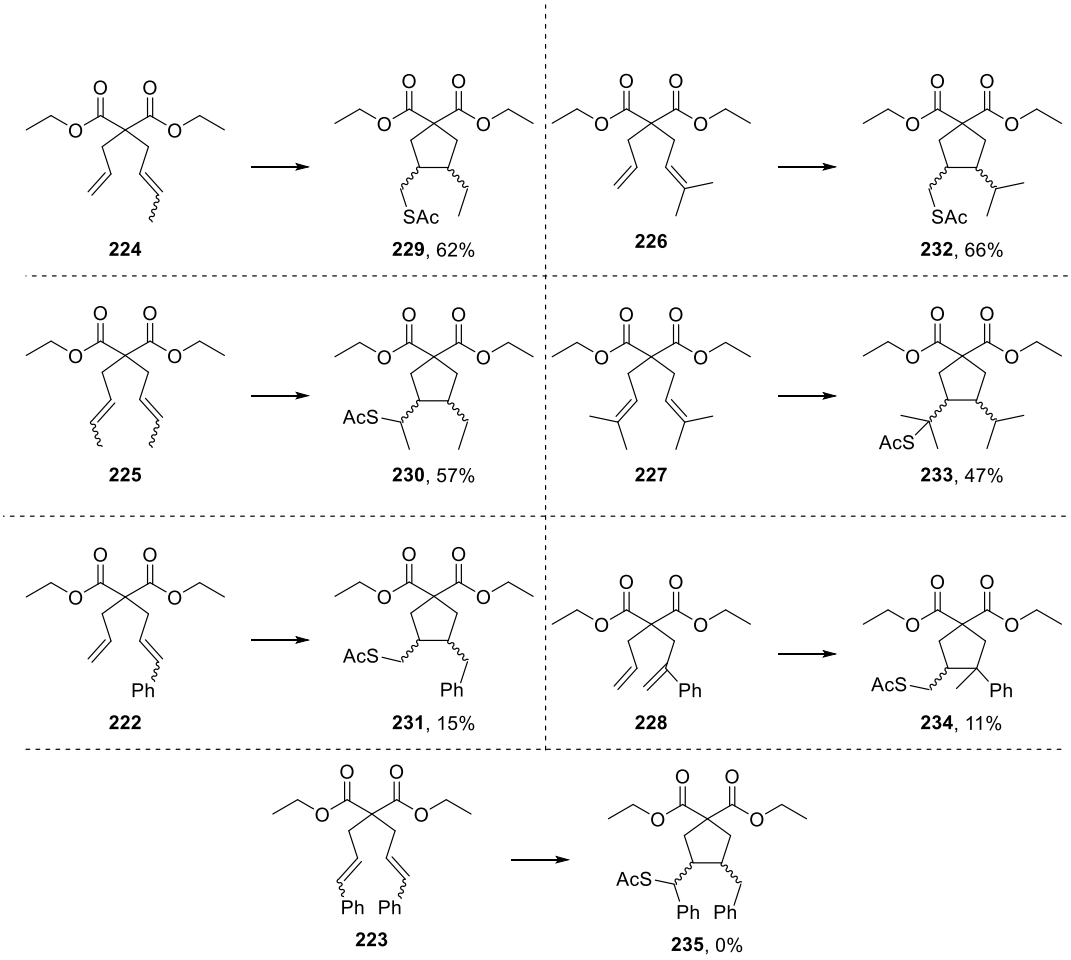


222, 20% **223**, 46% **224**, 52% **225**, 67%

226, 49% **227**, 49% **228**, 53%

Figure 4.6. A range of 1,6-diene substrates were prepared for the TRACE methodology.

With these substrates in hand, the thiyl radical mediated cyclisation of each diene was investigated under TEC conditions. Each substrate was subject to radical hydrothiolation with a slight excess (1.2 eq.) of thioacetic acid, in the presence of DPAP and MAP, under UV irradiation at 365 nm. As expected, those substrates with less steric bulk on the alkene termini were better cyclisation partners, giving higher yields of the 5-*exo*-trig product. Substitution of both alkene termini saw a decrease in cyclisation (**230** and **233**), as in the case of geminal substitution on one arm of the malonate-derived species (**232**). As expected, the addition of phenyl rings to any position resulted in significant decreases in yield (**231**), with reactivity completely shut off once both termini were substituted with a phenyl substituent (**235**) (Scheme 4.10). A detailed discussion of the observed yields is outlined below.



Scheme 4.10. Cyclizations with varying methyl and phenyl substitution.

4.2.2 Rationale

In terms of a rationale for the observed yields, one can consider the following. In the first example (transformation of **224** → **229**), the thiyl radical addition occurs at an unhindered alkene, and cyclises onto an internal alkene with a methyl substituent. There is a small increase in yield when one trigonal centre is dimethylated, such as in **226** – the radical formed following 5-*exo*-trig cyclisation is tertiary, rather than secondary in **224**. However, the steric penalty supersedes this stabilisation when both sites of unsaturation are dimethylated as in **227**. Equally, when both alkenes are internal and symmetric with a CH_3 substituent at one end (e.g. **225**), the yield drops from that observed for **230**.

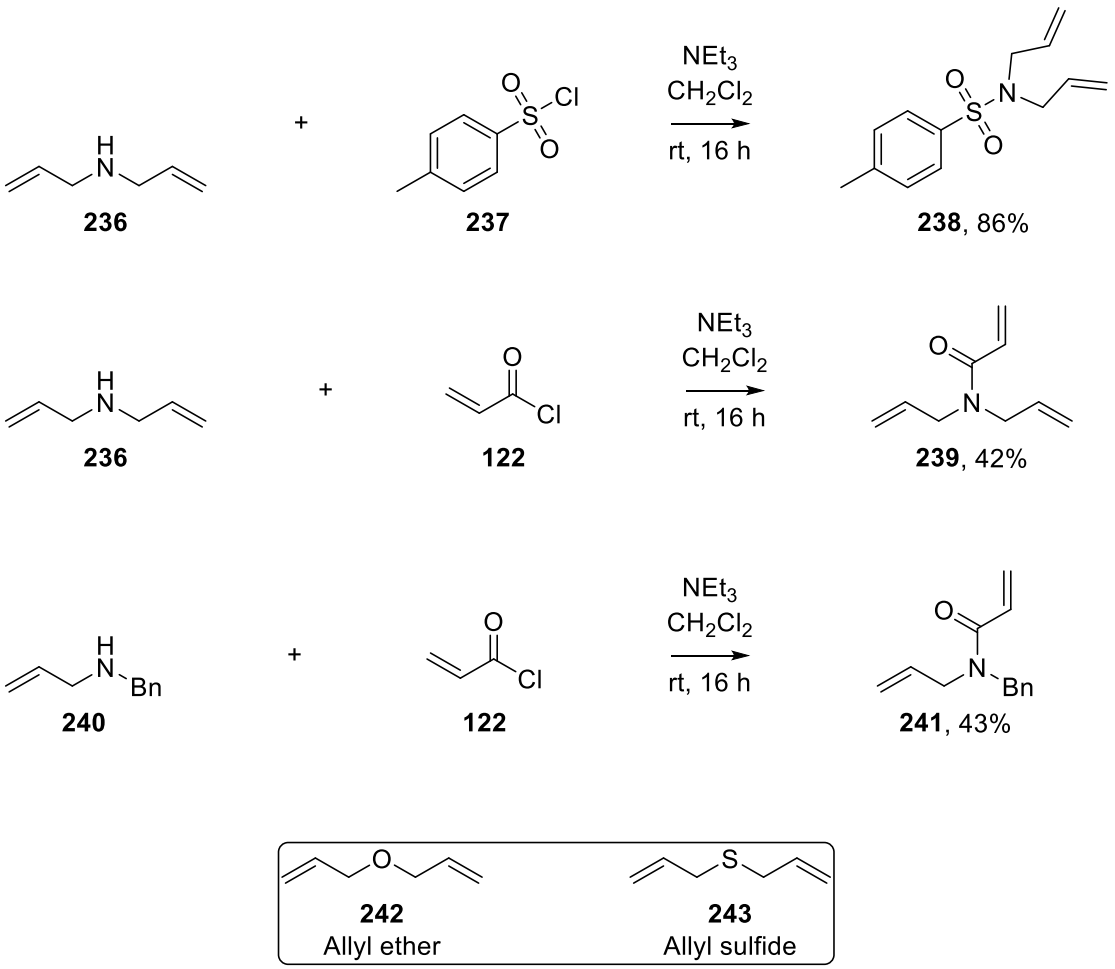
When the alkenes are instead substituted for styrene moieties, such as in **222**, **223** and **228**, significant decreases in yield are observed, at one stage completely closing off the reaction (**223**). One might assume that the increased bulk of the phenyl substituent is the major contributing factor, however, the hydrothiolation of the naked terminal alkene in

222 would be expected to proceed with good yield and this is not the case. It is possible however that the 5-*exo*-trig cyclisation does in fact take place, but proceeds *via* a benzylic radical, which is stabilised, delocalised, and long-lived. Styrene and its radical polymerisation has enjoyed a privileged role in the field of polymer synthesis,[275] and in theory the benzylic radicals formed herein instead prefer to polymerise with each other, rather than chain terminate to furnish the desired cyclic products shown in Scheme 4.10. This hypothesis is bolstered by the intense brick red to deep brown colours that appeared and persisted following radical hydrothiolation of the phenyl-decorated substrates **222**, **223** and **228**; this often signifies to the radical chemist that one's reaction is closer to an amorphous polymeric mixture than the desired product.

4.3 Heteroatomic substrates

Hitherto we have only examined the utility of the TRACE methodology for the generation of alicyclic frameworks. Next, we investigated systems including heteroatomic centres. A number of substrates featuring nitrogen, oxygen and sulfur centres were envisioned, and synthesised. Admittedly, some of the desired substrates could not be synthesised in any appreciable yield, which hindered expansion of the full the scope of this study.

Initially, a number of tertiary amines were envisioned (Scheme 4.11). Tosyl chloride **237** and triethylamine were added to a mixture a solution of diallylamine **236** in anhydrous CH_2Cl_2, which when reacted overnight gave the desired diallyl species **238** in an excellent yield of 86%. Acrylamides **239** and **241** were synthesised with acryloyl chloride **122** and the required secondary amine **236** or **240**, in the presence of triethylamine. This gave **239** in a moderate yield of 42%, and the benzylamine derivative **241** in a similar yield of 43%. Commercial 1,6-dienes **242** and **243** were purchased, and included in the heteroatomic trials.



Scheme 4.11. Synthesis of heteroatomic 1,6-dienes. **Insert**: Commercially available substrates.

Compound **238**, possessing a tosylamine backbone, was subject to the previously optimised thiol-ene cyclisation conditions. In the presence of thioacetic acid, DPAP and MAP, this substrate was irradiated for 1 h, which gave the desired pyrrolidine in an excellent yield of 90% following column chromatography. Unfortunately, the low diastereoselectivity of 2.5:1 *cis:trans* detracts somewhat from this otherwise excellent result.

The next two amine-centred systems investigated possess the unique functionality of an acrylamide. The acyl thiyl radical generated from thioacetic acid likely displays somewhat both nucleophilic and electrophilic reactivity.[276] Therefore, the thiyl radical SOMO should exhibit more favourable overlap with the terminus of the electron-deficient alkene in the acrylamide appendage, due to its lower-energy LUMO. Furthermore, the radical formed *via* anti-Markovnikov addition to the acrylamide terminus resides α- to a carbonyl, and thus benefits from additional stabilisation. Since the thiol-ene reaction is reversible (see Chapter One, section 1.3.8): this stabilisation

fortifies the mode of addition – addition to the allylic terminus lacks this stabilisation and thus the thiol-ene equilibrium leans towards the starting substrate amide.[188, 277] A simplified FMO analysis is provided in Figure 4.5 below – for the allyl substituent, the HOMO-LUMO gap of the substrate would likely be increased.

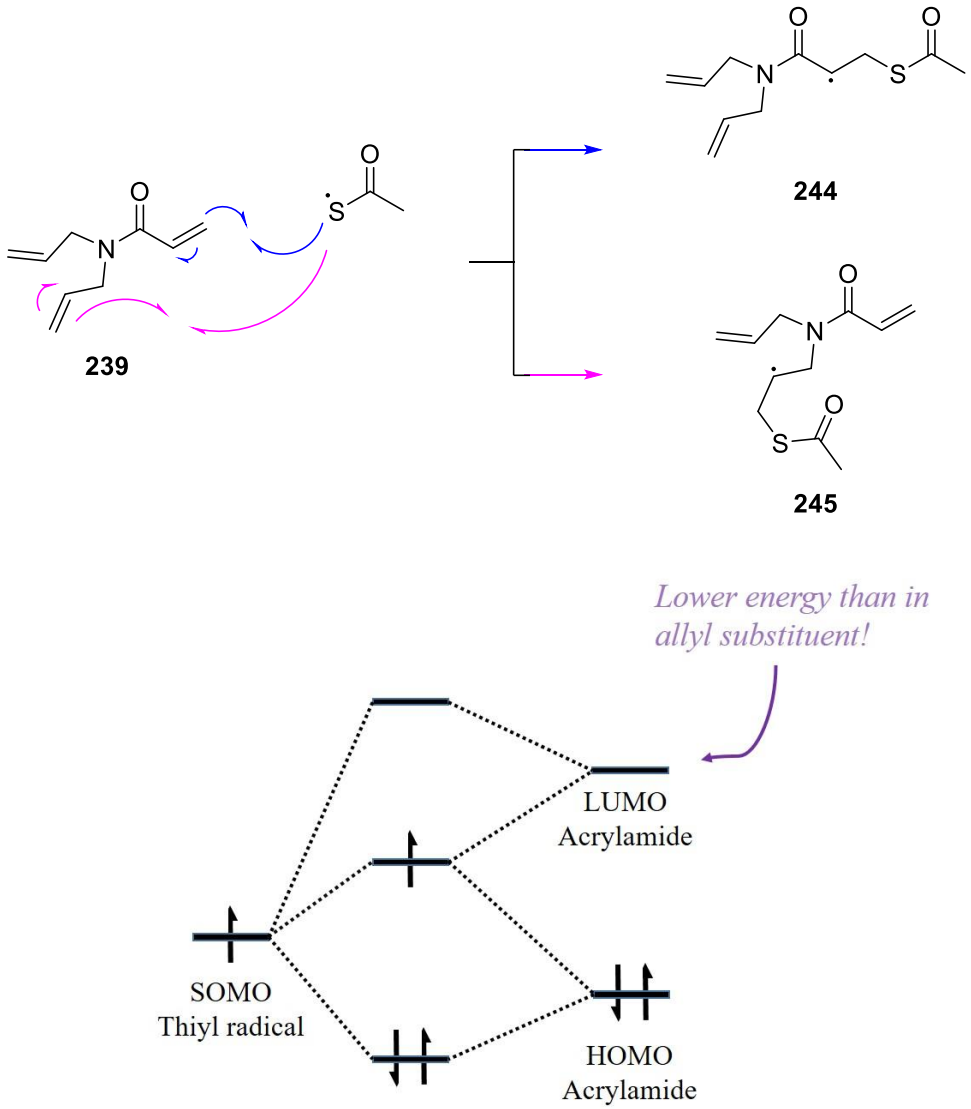
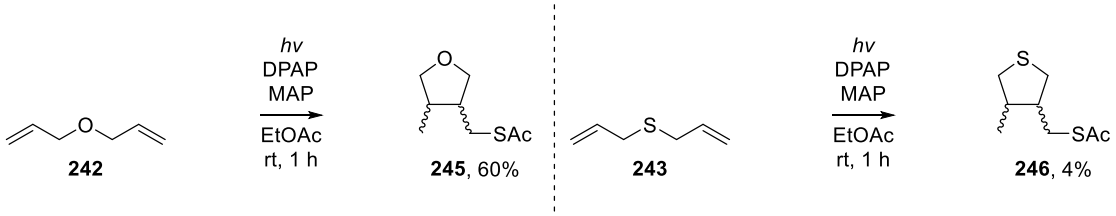


Figure 4.5. The two sites of attack for the thiyl radical are shown in pink and blue. The acrylamide constitutes an electron-deficient alkene, with a lower energy LUMO (FMO diagram).

Repeated attempts at effecting 5-*exo*-trig cyclisation of these amides were met only with hydrothiolation in some form of thia-Michael process; it is postulated that some residual base from the synthesis of the starting amides may be responsible, or that the thioacetic acid is forming the quaternary salt of the tertiary nitrogen and resulting in a reactive thiolate which undergoes ionic addition at the acrylamide terminus. Column chromatography of the starting material did not serve to eliminate these problems, and

therefore it was endeavoured to maintain substrate concentration to a minimum, without interfering with the thiol-ene radical chain process. Thioacetic acid, DPAP and MAP were dissolved in ethyl acetate and irradiated with stirring. A stock solution of benzylamide **241** in ethyl acetate was prepared, and aliquots of this solution were injected into the irradiated flask every 10 minutes, such that 10% of the total required amount of **241** was added with each injection. This continued until the complete stock solution was added, after which the reaction was irradiated for a further 30 minutes. Disappointingly, this approach did not generate any of the desired product despite the expectation that the high dilution factor would permit the cyclisation process to take place.

Thiolane and tetrahydrofuran derivatives were envisioned *via* the cyclisation of diallyl ether **242** and allyl sulfide **243**. Allyl sulfide **243** did not tolerate the TEC, instead returning either starting material or small quantities of uncyclized thioacetate adducts. Even with optimisation studies, no more than 4% of the desired thiolane **246** was isolated. Gratifyingly, the cyclisation of allyl ether **242** to tetrahydrofuran **245** proceeded with excellent yield of 60% in l h (Scheme 4.12).



Scheme 4.12. Cyclization of commercially available dienes.

One could potentially rationalise the success of ether **242** *vs*. the failure of sulfide **243** as follows. As has been previously outlined, sulfides can undergo S_Hi chemistry, and so it is not unreasonable that the action of a radical upon **243** may generate some inseparable mixture of radical origin. Alternatively, perhaps the increased size of the sulfur atom in **243** compared to the oxygen in **242** is sufficient to disrupt the cyclisation process. Computational modelling studies may prove valuable in illuminating the dynamics of this reaction.

4.4 BucketOfBlue (BoB) – 'Illuminating' milder conditions

4.4.1 Reactor Concept, and Components

One of the primary advantages of the TRACE methodology is the mildness of the reaction conditions and shortened timescales. However, an obvious limitation is the use of UV mediated conditions which are not suitable for some classes of organic molecules or for scale-up. Furthermore, due to the hydrophobic nature of the ultraviolet photosensitiser and photoinitiator pair, column chromatography is required in each case and the concomitant excesses of solvent for purification are costly and wasteful. As milder irradiation methods become prevalent in the literature,[278-280] the author envisioned a 'home-built' blue light photoreactor, which would rely on water soluble photoinitiators/photosensitisers. This would permit simple aqueous workup to furnish cyclised products, with less light intensity required overall.

Blue light emitting diodes (LEDs) have spearheaded a new age of visible light irradiation methodology, and were chosen as a suitable construct for this photoreactor. A common misconception with photoreactor design is that certain bulbs run 'cooler' than others, however the physics of heat transfer remain the same – if one generates 10 watts of light, one has 10 'watts' of heat to deal with also. A small desktop fan was utilised to dissipate the generated heat energy, with thermometers scattered throughout the reactor body to ensure homogeneity of the internal temperature.

Two meters of blue LEDs were acquired form a domestic supplier, backed with adhesive tape. Flex wire from broken home appliances was severed and rewired to the blue LED strip. The LED strip was wired into an LED driver, which manages the incoming power to ensure reliable light output from the LEDs, and sufficient lifetime. Further experimental detail regarding the reactor components is available in Chapter 6. This formed the 'BucketOfBlue or '*BoB*', which was investigated for the facile initiation of the thiol-ene reaction (Figure 4.6).

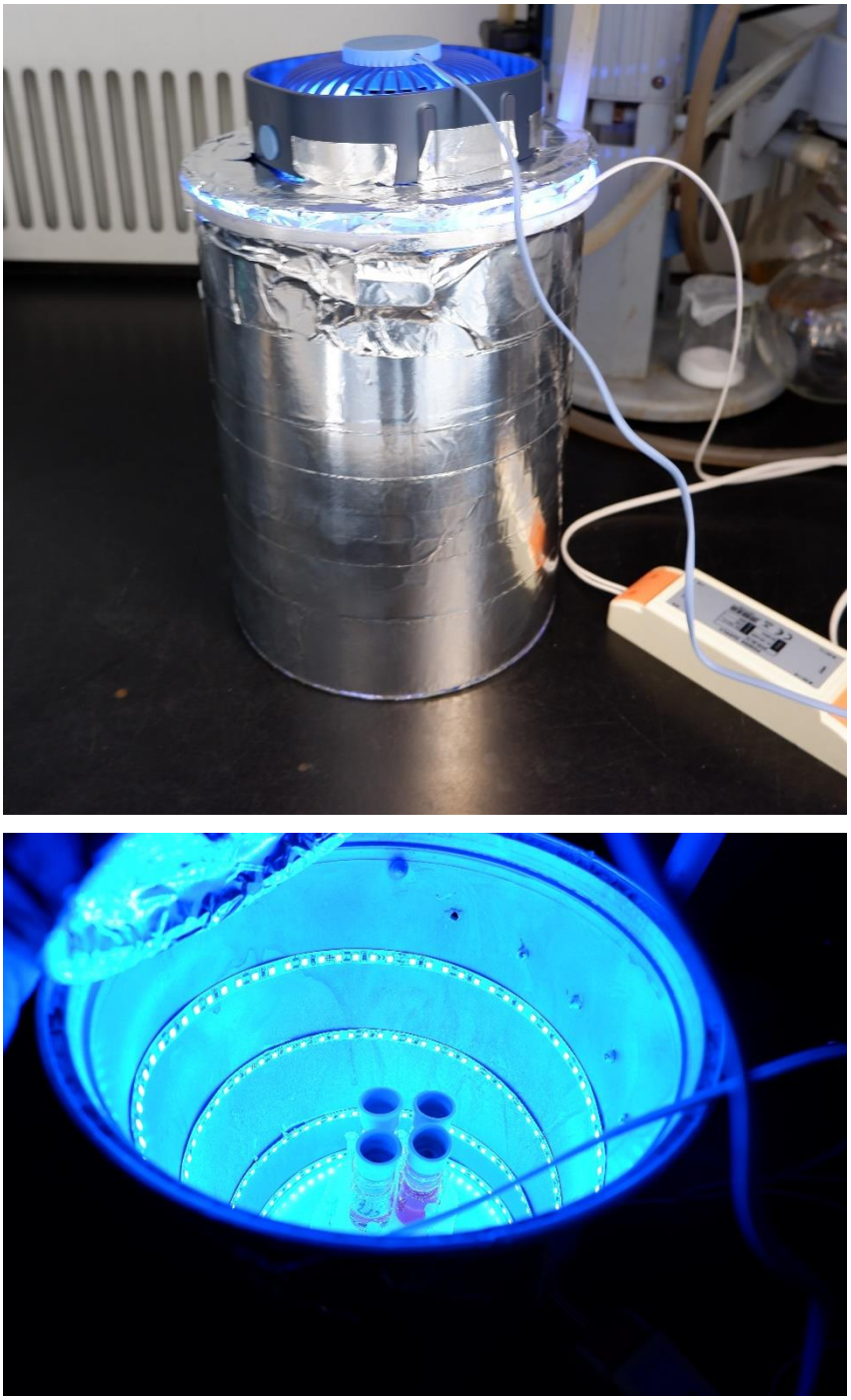
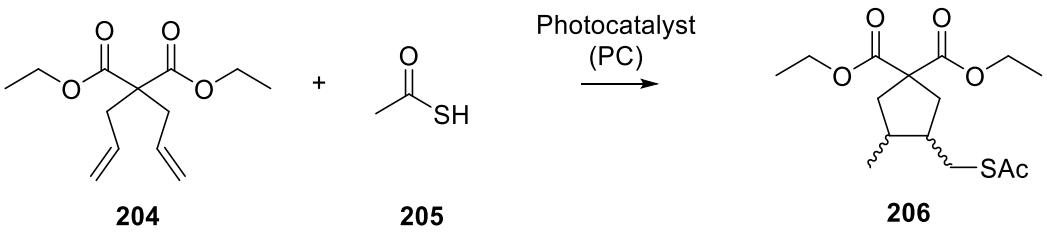


Figure 4.6. The BucketOfBlue (*BoB*) photoreactor.

4.4.2 Initial screening

In the first instance, the prototypical reaction as shown in Scheme 4.13 was employed in the *BoB*. For each entry, the alkene **204** was dissolved in degassed solvent in the presence of the photocatalyst. Thioacetic acid was added *via* syringe, and the solution subsequently irradiated at *ca*. 400 – 450 nm for 1 to 2 h.



Scheme 4.13. The prototypical cyclisation diethyl diallylmalonate with thioacetic acid.

Following completion of this reaction, the solution was washed with either aqueous acidic or alkaline solutions to remove the chosen photocatalyst, and yield a mixture of alicycle **206** and/or unreacted starting material. These results are summarised in Table 4.2. The classical DPAP/MAP pair gave no consumption of the alkene, as expected, due to the lack of initiation without UV light (entries 2 and 3, Table 4.2). Eosin Y, the tetrabromo derivative of fluorescein furnished good to very good yields in a number of solvents, with degassed CH_3CN proving optimal. A clear solvatochromic effect was observed in different solvents, and a bright orange emission was observed upon irradiation in *BoB*. Control experiments (entries 7 and 8, Table 4.2) revealed that blue light irradiation was required for radical initiation, and the diastereoselectivity resided approximately between 9:1 and 10:1 *cis*:*trans*. This represents a slight increase with respect to the observed diastereoselectivity for the UV-mediated reactions. One could propose that the decrease in incident light intensity permits the intramolecular radical addition step to equilibrate to a more favoured orientation, resulting in a slight increase in selectivity.

Next, the free base of acridine orange was employed as a means of radical initiation. Poor solubilisation hampered the use of this bright orange solid, which seemed to prefer suspensions rather than dissolution, except when cyclohexane was employed as the reaction solvent. Nevertheless, moderate yields of 47-57% were achieved when CH_2Cl_2 and cyclohexane were employed respectively. A slight reduction in diastereoselectivity was observed (entries 9 and 10, Table 4.2).

The final initiation mode of note is that of 9*H*-thioxanthen-9-one, which overall provided the highest yields coupled with a promising diastereomeric ratio. Excellent yields of up to 90% were achieved over 1 h of irradiation, which did not improve upon lengthening of the reaction time. Ratios of 9:1 and 10:1 *cis*:*trans* were achieved, however removal of the initiator was not as straightforward. A short silica column was necessary to isolate the cyclic product **206**.

Table 4.2. Reaction data for *BucketOfBlue* (BoB) optimisation.

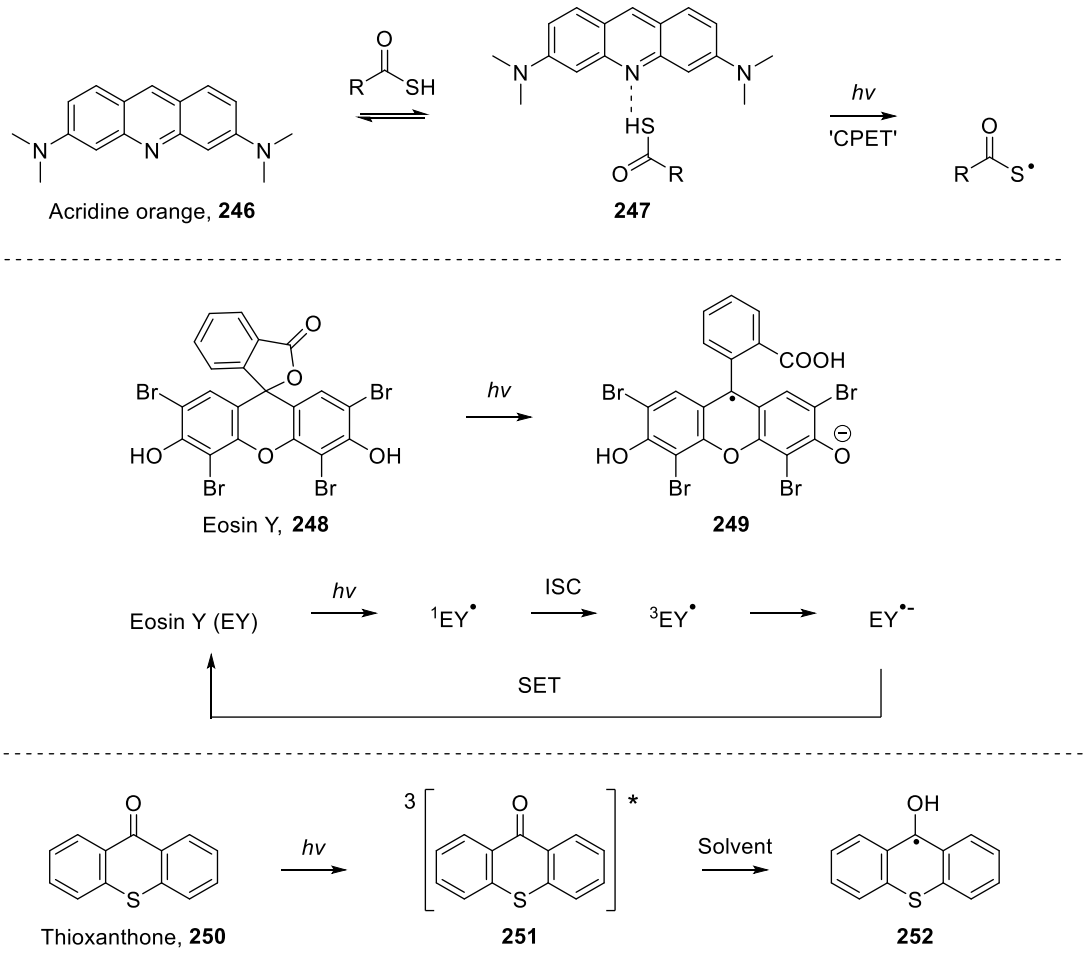
Entry	Photocatalyst (PC)	PC Eq.	Solvent	hν	Rxn time (h)	Yield	Dias. Ratio (cis:trans)
1	None	0	CH_2Cl_2	+	1	0%	N/A
2	DPAP*	0.1	$CHCl_3$	+	1	0%	N/A
3	DPAP*	0.1	EtOAc	+	1	0%	N/A
4	Eosin Y	0.1	EtOAc	+	1	60%	8.9:1
5	Eosin Y	0.1	CH_3CN	+	1	88%	8.7:1
6	Eosin Y	0.1	CH_2Cl_2	+	1	42%	9.8:1
7	Eosin Y	0.1	EtOAc	-	1	0%	N/A
8	Eosin Y	0.1	CH_2Cl_2	-	1	0%	N/A
9	Acridine Orange	0.1	CH_2Cl_2	+	1	41%	8.4:1
10	Acridine Orange	0.1	Cyclohexane	+	1	57%	7.4:1
11	Acridine Orange	0.1	CH_2Cl_2	-	1	0%	N/A
12	Acridine Orange	0.1	Cyclohexane	-	1	0%	N/A
13	9*H*-thioxanthen-9-one	0.1	CH_2Cl_2	+	1	86%	10:1
14	9*H*-thioxanthen-9-one	0.1	CH_2Cl_2	-	1	0%	N/A
15	**9*H*-thioxanthen-9-one**	**0.1**	**PhMe**	**+**	**1**	**90%**	**9:1**
16	9*H*-thioxanthen-9-one	0.1	CH_2Cl_2	+	2	87%	10:1

All reactions done in degassed solvent, and under Ar. * DPAP (2,2'-dimethoxypheylacetophenone) operates as a pair with photosensitiser MAP (4'-methoxyacetophenone).

4.4.3 Mechanistic Aspects

The photoinitiators employed in *BoB* (Table 4.2) show huge structural diversity. Indeed, while yields vary greatly, one can choose the correct initiator based on what aqueous work-up works best for the synthesis. The structure of acridine orange **246** is easily protonated and washed out in solutions of aqueous acid, while the product of Eosin Y **248** initiation, acid **249**, is readily base soluble, and can be separated from organic intermediates with ease. Finally, the highest yielding photoinitiator, thioxanthone **250**, can be easily removed with a silica plug. This requires small volumes of solvent, and obviates the requirement for column chromatography.

Acridine orange is a Lewis basic photocatalyst, which furnishes a thiyl radical through a proton-coupled electron transfer. Given that the reactions with this photocatalyst proceeded best in a non-polar solvent, cyclohexane, is a direct indicator that the formation of charged intermediates seems unfavourable.[281] According to an account by Levin *et al*., the mechanism for thiols and thioacids likely proceeds *via* a concerted proton-electron transfer (CPET),[281] although this is yet to be fully confirmed. A tentative mechanism is postulated in Scheme 4.14. In the case of Eosin Y **248**, excitation with visible light and subsequent intersystem crossing gives the excited form **249**, which completes its photoredox cycle back to Eosin Y *via* single electron transfer (SET).[282] Finally, thioxanthone **250** possesses its own initiation pathway; in polar, protic solvents, thioxanthone is excited by visible light to a triplet state **251**, following which direct hydrogen atom transfer (HAT) from the solvent (e.g. H_2O) yields the thioxanthone radical **252**.[283]



Scheme 4.14. The three visible light photoinitiators employed with *BoB*.

4.5 Desulfurisation and Extrusion

Generally, the goal of the sulfur-based methodologies discussed in this thesis is to effect the desired chemical transformation using the thiyl radical, and eventually to remove the sulfur atom (see Chapter Two, STING) in an overall traceless approach. The following section details the sulfur elimination portion of the TRACE methodology to furnish sulfur-free cyclic products from acyclic starting materials.

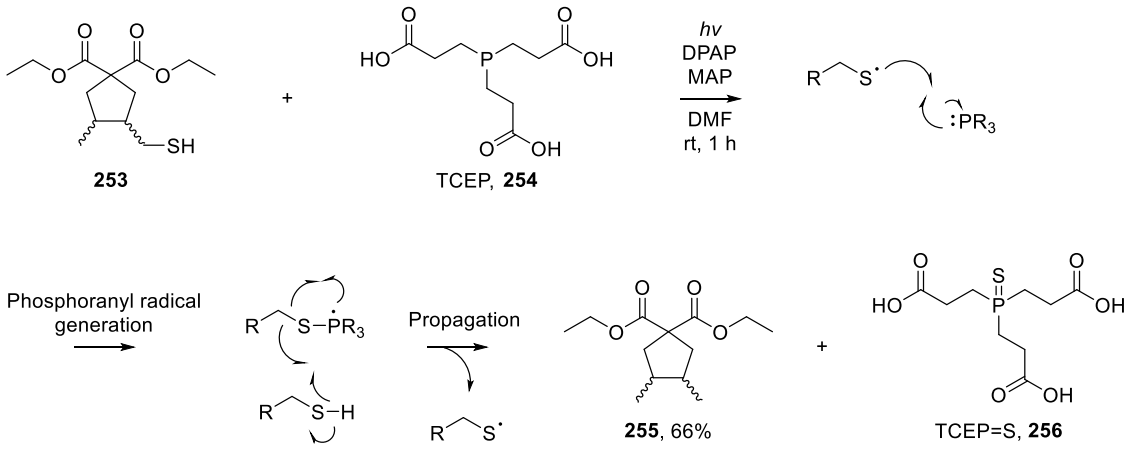
4.5.1 Tris(2-carboxyethyl)phosphine mediated approach

Tris(2-carboxyethyl)phosphine, or TCEP **254**, is a reducing agent often employed in biochemistry and peptide science for the desulfurisation of cysteine side chains to alanine

residues following NCL. The use of TCEP offers a welcome departure from the archetypal metal-based desulfurisation approaches, such as Raney nickel.[284]

A photochemical desulfurisation approach was envisioned, and was validated as follows. Thioester **206** was first deprotected to the corresponding thiol with the use of freshly prepared sodium ethoxide. The ethoxide base is used to prevent scrambling of the malonic ester backbone. Following completion of the reaction and the quenching of remaining alkaline species, the thiol was isolated in an excellent yield. A solution of this thiol with an excess of TCEP was solubilised in DMF, and DPAP/MAP was added (0.1 eq. of each). The solution was then irradiated at 365 nm under argon, and after 1 h was concentrated *in vacuo*. A brief aqueous work-up and chromatographic purification gave the desired, sulfur-free compound **255** in good yield of 66%.

The reaction likely proceeds as follows. DPAP and MAP serve to generate the thiyl radical from **253**, which then reacts with TCEP to generate the corresponding phosphoranyl radical. Following homolytic cleavage of the C-S bond, the nascent alkyl radical abstracts a proton from remaining thiol, and serves to propagate the reaction.[284] This is shown in Scheme 4.15.



Scheme 4.15. Proposed mechanism for the desulfurisation of **253** with TCEP.

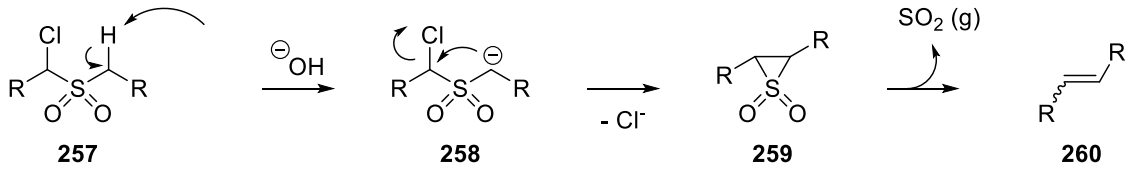
Despite its widespread use, TCEP is reasonably expensive at *ca*. €133 per gram (hydrochloride salt, in good purity). The primary benefit of TCEP is its water solubility, permitting easy extraction and removal following completion of the reaction. Tributylphosphine is significantly more affordable, and so was tested under the same conditions for TCEP desulfurisation. While NMR analysis indicated the successful removal of sulfur from **253**, repeated column chromatography was necessary to remove

remaining tributylphosphine. If one were to work on significantly large scale, it may be beneficial to employ tributylphosphine for this reduction, however TCEP is the clear forerunner for standard laboratory scale reductions.

4.5.2 The Ramberg-Bäcklund Reaction

The Ramberg-Bäcklund reaction (RBR) is a base-mediated transformation of α-halosulfones into the corresponding alkenes, often as a regio-defined process. The reaction was first described by Ludwig Ramberg and Birger Bäcklund in 1940 and has proved a versatile rearrangement of huge synthetic value.[285-286] The RBR offers a number of attractive aspects from a synthetic standing, such as the unambiguous location of the incipient alkene, the availability of all substitution patterns (including tetra-substituted alkenes) *via* this process, and the availability of deuterated alkenes when the RBR is conducted in deuterated solvent.[286]

An anionic mechanism is generally accepted, which proceeds with deprotonation of the α-proton of the sulfone **257**, followed by a cyclisation to the thiirane dioxide intermediate **259**. Extrusion of SO_2 by a not yet elucidated mechanism generates the alkene **260**. When weak base is employed for most substrates, a predilection for the *Z* alkene is observed (Scheme 4.16).



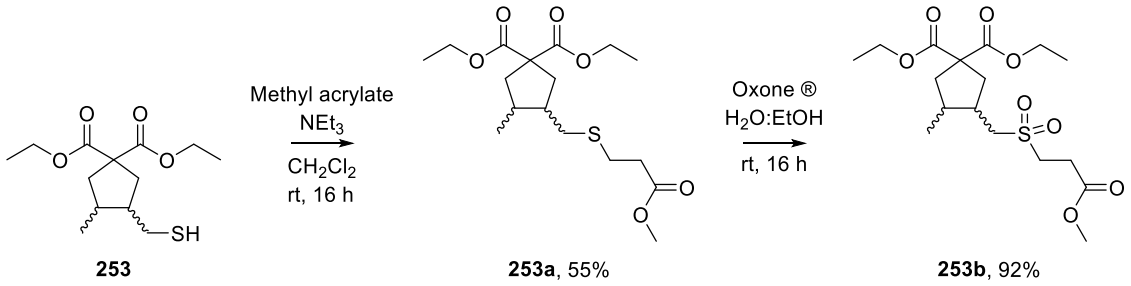
Scheme 4.16. The generally accepted mechanism of the Ramberg-Bäcklund mechanism.

With respect to the required starting materials, sulfones can be used directly and deprotonated with strong alkyllithium bases for subsequent halogenation. Electrophilic halide sources such as *N*-bromosuccinamide (NBS) or *N*-chlorosuccinamide (NCS) are routinely employed. As one might expect, α-iodosulfones react faster as RBR substrates than the corresponding α-bromo and α-chloro species; the loss of halide *via* the 1,3-cyclisation pathway (with concomitant thiirane dioxide formation) proceeds such that $k_I > k_{Br} > k_{Cl}$. Alternatively, sulfides can first be halogenated and then oxidised.[286]

One particular modification of this reaction is Myers' procedure, in which carbon tetrachloride can be used as both a solvent and halogenating reagent. Following

successful chlorination, olefin formation proceeds *in situ*. However, the use of CCl_4 often requires the use of strong base and high temperatures, which liberates small quantities of dichlorocarbene and facilitates insertion chemistry at the nascent alkene. This reaction with electron rich alkenes provides *gem*-dichlorocyclopropane adducts, which can be staved off by the addition of phenol or another unsaturated species as a carbene scavenger. Additionally, the Chan modification employs CBr_2F_2 in place of CCl_4, as the **:**CF_2 carbene is much less reactive than **:**CCl_2.[287]

The RBR was envisioned as a viable route to the desulfurisation of thiol-ene product **206**, which would first be derivatised to form the thioether bridge. Thiol **253** was prepared as outlined previously, and immediately applied to the next step before disulfide formation proceeded. Thia-Michael addition ensued following addition of methyl acrylate and an excess of triethylamine, which gave the expected sulfide **253a** in near quantitative yield (Scheme 4.17). At this juncture, two options present themselves – should one first prepare the sulfone and attempt late-stage halogenation, or should careful oxidation of the α-halosulfide be employed?



Scheme 4.17. Attempts at yielding an α-halosulfone began with thia-Michael addition, and oxidation.

Electing the former of these two options, the sulfide **253a** was oxidised with *meta*-chloroperoxybenzoic acid (*m*-CPBA) in anhydrous CH_2Cl_2. Incomplete oxidation was observed, and the remaining unreacted peroxy acid was difficult to fully remove following purification. Oxone® was instead investigated as an oxidant. This triple salt of $2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$ is a versatile oxidant, often employed in the pool and spa industry.[288] To a solution of sulfide **253a** in EtOH was added a slight excess of Oxone® in an equal volume of H_2O, which exothermically produced a white slurry. Stirring overnight in air generated the desired sulfone **253b** in excellent yield following aqueous workup, with no further purification required (Scheme 4.17). Myers' modified RBR was attempted, with a solution of the incipient sulfone, sodium ethoxide and CCl_4 heated to 60 °C for 16 h. However, this resulted in widespread decomposition, with no discernible

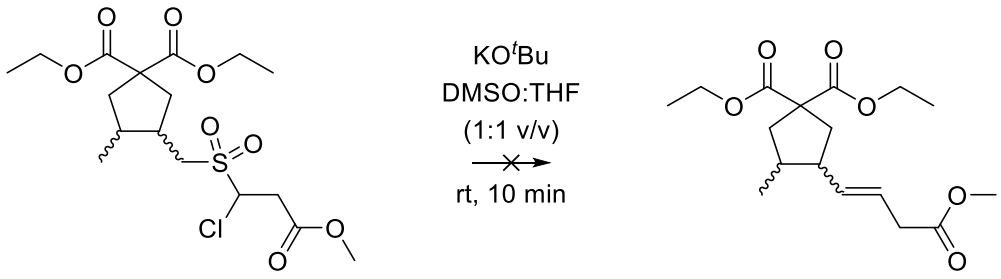
signals in the proton NMR following concentration. Furthermore, the septum of the reaction flask disintegrated, and the round bottom flask was damaged.

Instead, a new approach was designed. Literature precedent for the α-chlorination of unactivated sulfides was set by MaGee *et al.* in 2000, in their total synthesis of manzamine C.[289] The authors reported that NCS in CCl_4 was heated to reflux with the alkyl sulfide and subsequently oxidised to give the substrate for the RBR. With this knowledge in hand, the same protocol was applied to the present work. Sulfide was dissolved in CCl_4 with equimolar NCS and heated to reflux for 3 h, after which it was cooled to 0 °C. The resulting precipitate was filtered and the filtrate concentrated to yield an α-chlorosulfide. This intermediate was then immediately oxidised with Oxone® as previously described. Once again, this electrophilic chlorination strategy failed to yield the desired α-chlorosulfone.

To investigate the possibility that the reaction was impeded by residual H_2O, we conducted a final trial, in which CCl_4 was freshly distilled and dried over 4Å molecular sieves. The starting material was dissolved in CH_2Cl_2 and washed with brine, before drying over $MgSO_4$. The same drying prep was applied to freshly recrystallized NCS, and thus the reaction was made as anhydrous as possible. Addition of NCS to a solution of sulfide in CCl_4 at 0 °C gave a white slurry, which was slowly warmed to rt over 1 h. It was postulated that the electrophilic chlorination occurs in a short timescale, and that previous experiments had failed due to formation and subsequent decomposition of the desired chloride. TLC analysis of this reaction showed a new compound at an R_f of 0.53 (8:2 hexanes:EtOAc), compared to the starting material at 0.43 in the same solvent system. Furthermore, this new spot stained in potassium permanganate, as one would expect for the α-chlorosulfide in its oxidation to sulfoxide and/or sulfone. Upon completion of this reaction, the precipitated succinimide was filtered and the solution concentrated *in vacuo*. Crude ^{1}H NMR analysis displayed a shifting of signals in the CH/CH_2 region. The crude residue was immediately oxidised under standard Oxone® conditions but in anhydrous DMF, and once again crude ^{1}H NMR analysis gave some hope that the α-chlorosulfone might be in hand.

The RBR was attempted on this crude material **261**, once again relying on conditions from MaGee *et al.*, in which powdered potassium *tert*-butoxide was dissolved in DMSO, and added to a solution of the suspected α-chlorosulfone in anhydrous THF (Scheme

4.18). This slurry was stirred at rt for 10 min, after which it was heavily diluted in diethyl ether and an aqueous work-up was employed. Over the reaction timescale, a colour change from pale yellow, through orange to deep brown was observed, as was faint bubbling in the solution. Unfortunately, the isolated material once again did not resemble the desired product **262**, with no olefinic signals visible in the ^{1}H NMR spectrum.



261 **262**

Scheme 4.18. Unsuccessful application of conditions by MaGee *et al*. The identity of starting material **261** was, albeit, not fully confirmed.

4.6 Conclusions and Future Work

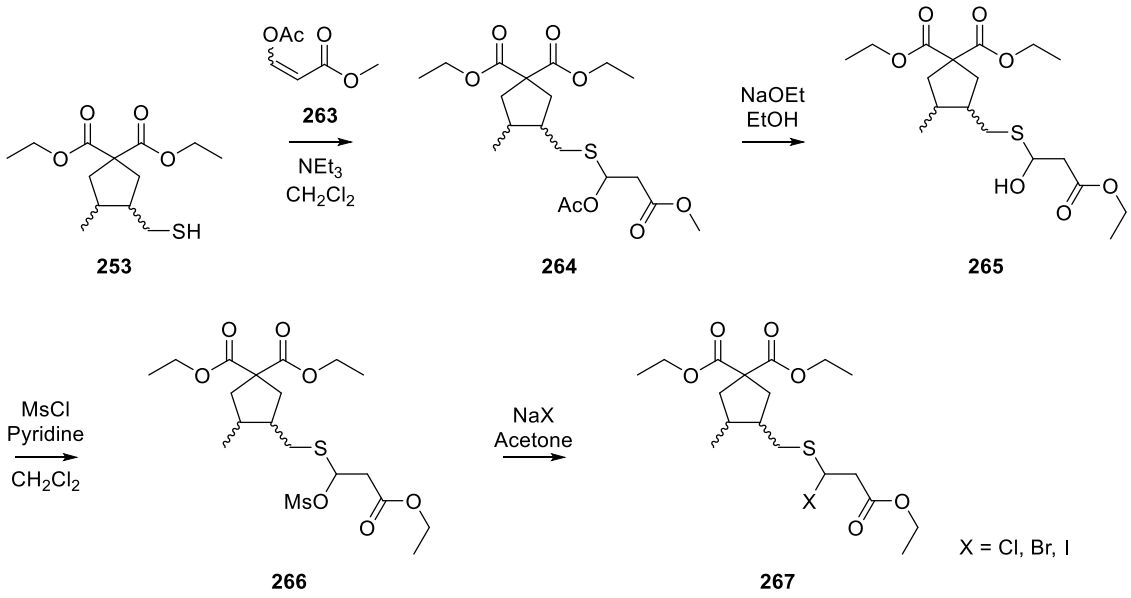
This chapter describes an ostensibly facile approach for the thiyl-radical mediated cyclisation of 1,6-dienes. A diverse plethora of malonic ester-based systems, decorated with varying substitution patterns, were investigated over the course of this methodology, and reasons for the relative cyclisation yields were postulated. The presence of at least one terminal alkene appears to be optimum for the 5-*exo*-trig cyclisation. Indeed as both alkene termini become more sterically complex, the effectiveness of this anti-Markovnikov hydrothiolation begins to shut down. Substituents with both steric and additionally electronic effects, such as benzylic alkenes, displayed poor efficacy as cyclisation substrates.

Desulfurisation approaches were explored, including the use of phosphines such as TCEP, and the Ramberg-Bäcklund reaction. The use of phosphines proved powerful in the removal of the sulfur atom left over from thiol-ene induced cyclisation, and the potential of the RBR was clearly outlined. Therein, the TRACE methodology has been realised – A selective **T**hiyl **R**adical **A**ddition, **C**yclisation and **E**limination scheme was established, which provided access to otherwise elusive alicyclic compounds.

In terms of future work, the author has a number of ideas, which unfortunately could not be realised before their departure from the lab. Firstly, while the diastereoselectivity is somewhat interesting, it could greatly be improved. Transesterification of the malonic backbone with a suitable chiral species would be an interesting avenue through which increased selectivity could be achieved. Alternatively, the use of a chiral thioacid may prove viable in altering the cyclisation transition state enough to effect the desired increase in *cis*:*trans* ratio.

One element clearly lacking from the work discussed herein is temperature control. Admittedly the author had attempted to cool the *BoB* reactor down below room temperature, but this proved much more difficult than initially anticipated. Thermometers were wired into *BoB* following the initial experiments, and the internal temperature following one hour of irradiation was *ca*. 30 °C. Some mechanism to control the internal temperature at *ca*. 10 °C or lower might provide some increase in diastereomeric ratio. Further substrates and heteroatomic-centres species should be applied in this methodology. A divergence form the classical malonic ester backbone is required, and a number of 1,6-dienes are envisioned. Interested parties' need only venture into the realm of ring-closing metathesis literature to identify interesting substrates.

Finally, despite the failure to execute the Ramberg-Bäcklund reaction during the course of this project, the author has a number of suggestions. The oxidation of the generated sulfide has proved high yielding and facile. The electrophilic chlorination at a relatively unactivated site would require extensive optimisation, however, there may be a workaround. Consider Michael acceptor **263** below (Scheme 4.19) – once again, thia-Michael addition with thiol **253** is straightforward. However following this transformation, deprotection of the acetate moiety **264** to the corresponding alcohol **265** would offer access to a wealth of useful derivatisations. Mesylation and S_N2 displacement with an ionic halide source is one avenue to access the α-halo motif. Additionally, while not widely reported, the use of mesylate **266** as a 'pseudo-halide' and direct RBR on this substrate would be an interesting prospect. The author speculates that this mesylate would be too bulky to participate in the reaction cascade – however, a Finkelstein reaction could be easily employed to displace the mesylate with a suitably reactive halide. Sulfone formation could occur before the selective deprotection of the acetate moiety in **264** (Scheme 4.19), or once mesylate formation has been accomplished, depending on the stability of these intermediates.



Scheme 4.19. Suggested future work for halogenation *via* Finkelstein chemistry. Sulfone formation could occur before acetate deprotection of **264**, or prior to mesylate displacement.

Chapter Five

Collaborations

5.0 Introduction

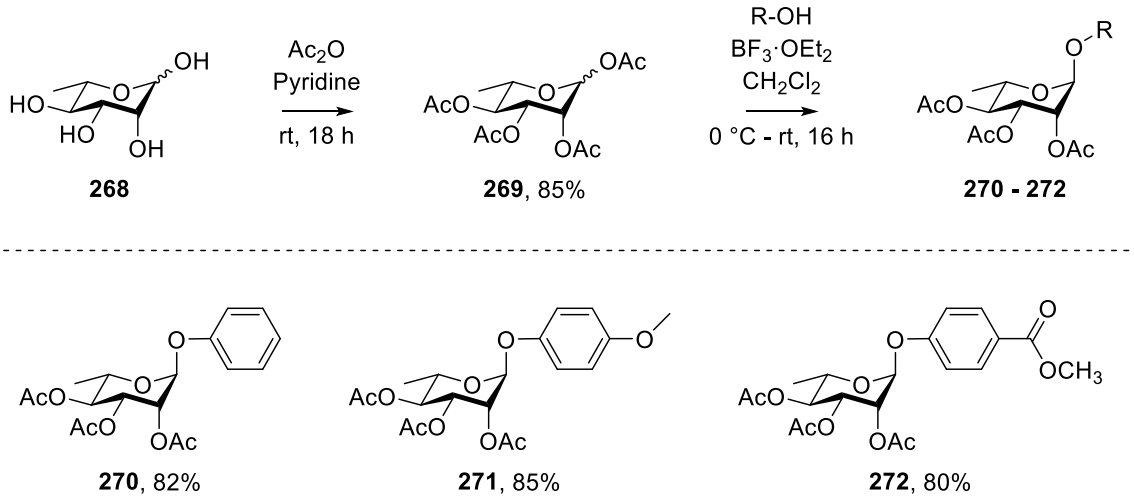
This chapter serves to briefly outline two collaborative projects undertaken through the course of the present work. The following sections will focus on synthetic detail, with a brief mention of the resulting biological results from testing these compounds. Additionally, characterisation for each of these compounds forms part of the final section of Chapter 6 – Experimental.

5.1 *Para*-hydroxybenzoic acid derivatives (*p*HBADs)

Para-hydroxybenzoic acid derivatives (*p*HBADs) were synthesised for a collaboration with Dr. Mimmi Lundahl (M. L.). Macrophages are immune cells with a special role in the detection and destruction of pathogenic bacteria. They can also present antigens to T cells, and can activate other cells *via* the release of cytokines. In the scope of the present work, they are key fighters against *Mycobacterium tuberculosis* (*M. tuberculosis*). Unfortunately, this bacterium possesses elegant methods to evade these immune cells and their bactericidal measures, such as the secretion of *p*HBADs.[290] These compounds acutely inhibit the macrophage response to *M. tuberculosis* infection, but little is known about their effect on the induction of long-term innate memory. Innate memory is a phenomenon in which future immune responses by macrophages and monocytes can be augmented by a previous 'challenge'. One such example is the Bacillus Calmette-Guérin (BCG) vaccine which offers protection against *M. tuberculosis* infection; this vaccine enhances the secretion of IFNγ (see chapter 3) in response to the bacterium, and it is believed that this innate response contributes to protection in immunised individuals.[291-292]

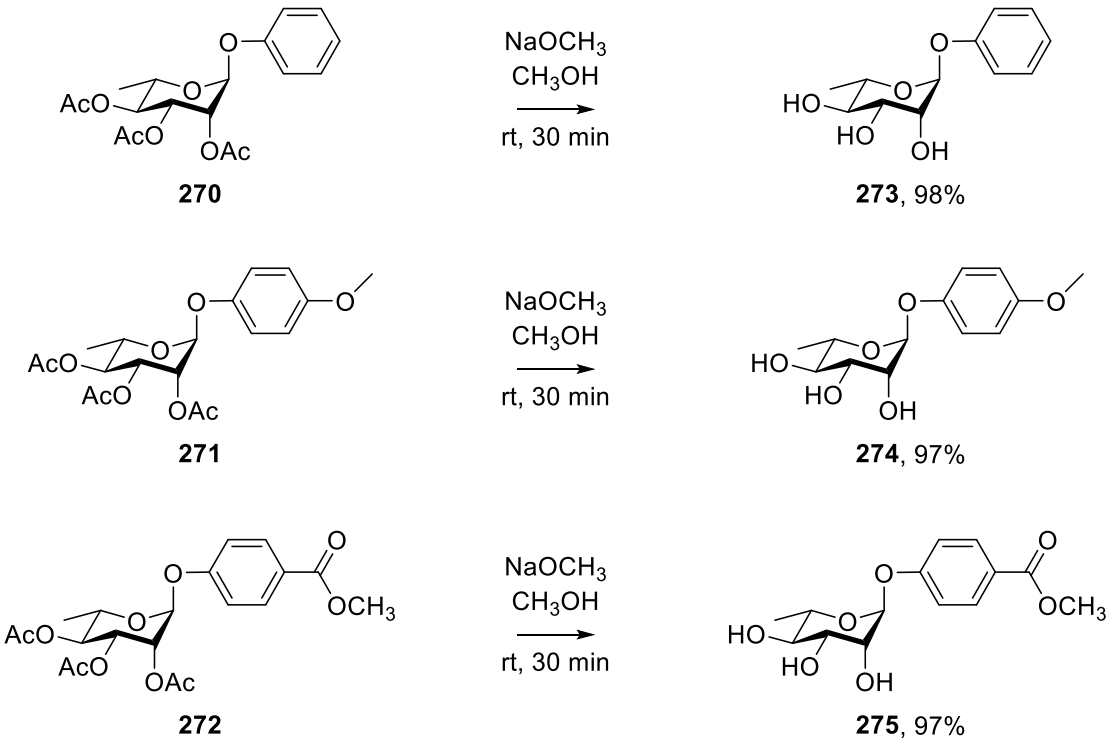
A number of compounds relating to *M. tuberculosis* pathogenesis were synthesised. All of the compounds are related to the biosynthesis of *p*HBAD 1, with the exception of methyl *para*-anisate (m-*p*a), which is believed to be secreted by *M. tuberculosis*, and has been isolated from cultures of this bacterium.[293] In order to better understand the importance of the m-*p*a motif, a number of synthetic rhamnosides were prepared, glycosylated with m-*p*a, and derivatives with slight modifications. Peracetylated rhamnose was first prepared from L-rhamnose *via* peracetylation under standard conditions, in a solution of pyridine and acetic anhydride. Column chromatography gave **269** in excellent yield of 85% following column chromatography. This peracetylated intermediate was then easily transformed in each of the compounds required for

screening *via* Lewis acid mediated glycosylation. $BF_3{\cdot}OEt_2$ was employed as the glycosylating agent, in the presence of the desired alcohol. Following column chromatography, this gave compound **270** – **272** in yield ranging from 80-92% (Scheme 5.1).



Scheme 5.1. *O*-Glycosylation of peracetylated rhamnose **269**.

Following the successful *O*-glycosylation to generate compounds **270** – **272**, each compound was deacetylated with freshly prepared $NaOCH_3$ in anhydrous CH_3OH. This gave compounds **273 – 275** in yields of 97-98%, which were then used without further purification (Scheme 5.2).



Scheme 5.2. Zémplen deacetylation gave **273 – 275** in excellent yield.

One notable exception to these syntheses was the synthesis of the anomeric methoxy derivative, Methyl-α-L-rhamnopyranoside. Previously employed glycosylation conditions were refractory when methanol was employed as the alcohol. Instead, a solution of methanolic HCl was prepared by the controlled esterification of acetyl chloride with an excess of anhydrous methanol. This solution was then added to L-rhamnose and stirred for 16 h at rt, to furnish a mixture of anomers. These anomers were separated by numerous iterations of slow, controlled column chromatography, which eventually gave the desired α-anomer **275a** in moderate yield of 42%.

With these compounds in hand, they were transferred to our immunology collaborators, and tested in tandem with a number of other highly modified *p*HBADs. While a fully analysis of the results is beyond the scope of this thesis, it was concluded that compound **275** (often termed unmethylated (UM) *p*HBAD 1) reduced expression of IFNγ, interleukins IL-17, and IL-10 in splenocytes, and also reduced expression of Tumour Necrosis Factor α (TNFα) in bone-marrow derived macrophages. This work demonstrated that *p*HBAD 1 induces innate immune memory that hinders bactericidal function. This account further the understanding of rhamnosides and related molecule sin *M. tuberculosis* pathogenesis, and may be used to improve TB vaccine development in the future.

A full analysis of the results, with further immunological discussion, can be found at https://doi.org/10.1021/acschembio.0c00378.

5.2 Glycosylated Theranostic Prodrugs

The following brief account outlines the synthesis of a number of highly functionalised carbohydrates. β-glucuronidase activated prodrugs are of significant interest for anticancer treatment.[294-297] As elevated levels of this enzyme are observed in tumour tissue, it is a potentially valuable target for glycosylated prodrugs, which would be selectively activated on the extracellular level.[295, 298-301] Amonafide is a 3-amino-1,8-naphthalimide (Nap) derivative with excellent anticancer activity, which acts as a topoisomerase II inhibitor.[300-301] A number of compounds with this naphthalimide backbone have been identified as excellent candidates for anticancer therapeutics and cellular probes, however their use is severely limited but their side effects and dose limiting bone-marrow toxicity.[302]

In collaboration with the Gunnlaugsson group (Trinity College Dublin) and Elmes group (Maynooth University), a number of molecular probes were designed. These probes were designed to undergo negligible cellular uptake when conjugated to a carbohydrate moiety, but upon enzymatic hydrolysis at the tumour surface would be rapidly internalised. Removal of the carbohydrate moiety would result in a self-immolation of the nitrobenzene linker, with release of Amonafide. The present account details the synthetic approach to two novel glycosylated naphthalimides, a β-galactosyl **276** and a β-glucuronyl derivative **277**, which were then supplied to collaborators for biological evaluation (Figure 5.1). Initially these compounds were prepared by Dr. Elena Calatrava-Pérez, who generated and optimised the protocols followed herein. Prior to publication more material was required for biological testing, and thus we prepared additional quantities of these glycosylated prodrugs. This section will focus mainly on the synthetic transformations and intermediates formed, rather than an in-depth analysis of the biological outcomes.

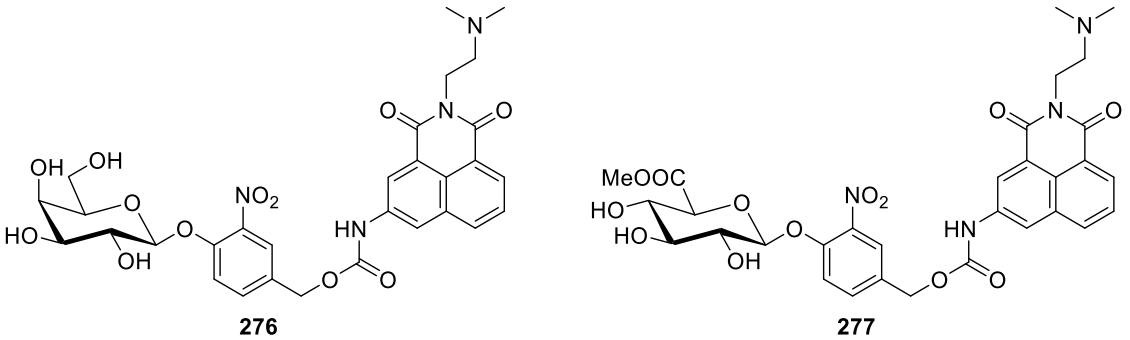
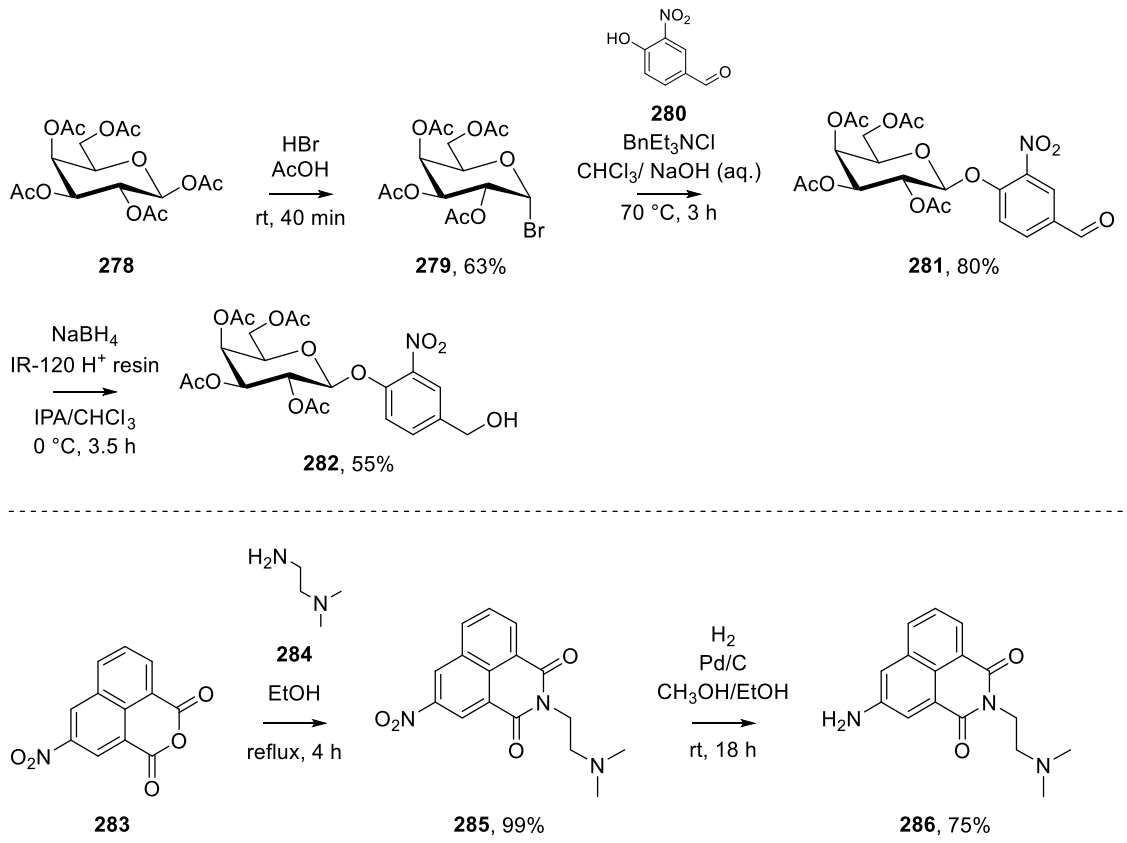


Figure 5.1. Structures of β-galactosyl and a β-glucuronyl derivatives, with self-immolative linker.

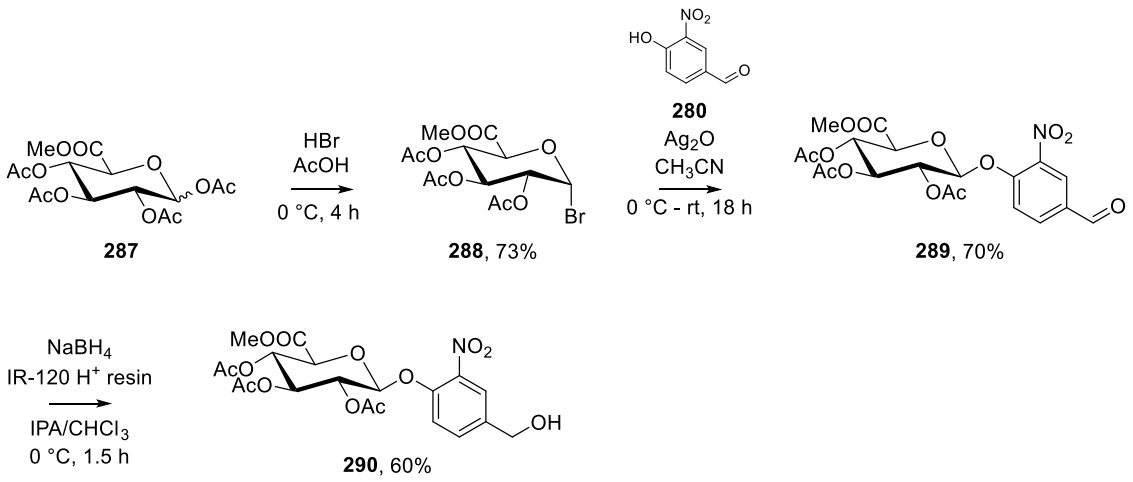
Synthesis of β-galactosyl derivative **276** began as follows. β-D-galactose pentaacetate **278** was converted to the anomeric α-bromide using HBr in acetic acid (33% v/v). Glycosylation of 4-hydroxy-3-nitrobenzaldehyde **280** was then accomplished under biphasic conditions, which provided **281** in excellent yield of 80% following column chromatography. Reduction of the aldehyde **281** was realised using $NaBH_4$ in the presence of an acidic resin, which gave primary alcohol **282** in moderate yield of 55% (Scheme 5.3).

Separately, commercially available 3-ntrio-1,8-napthalic anhydride **283** was converted to amonafide **285** by refluxing in the presence of excess *N*,*N*-dimethylethylenediamine **284**. Following a brief aqueous work-up, amonafide **285** was isolated in quantitative yield, and used without further purification. Conversion of the aryl nitro moiety to the desired amine was accomplished *via* hydrogenolysis with H_2 in the presence of Pd/C. Crystallisation from boiling methanol gave pure amonafide **286**. as a yellow powder in good yield of 75% (Scheme 5.3).



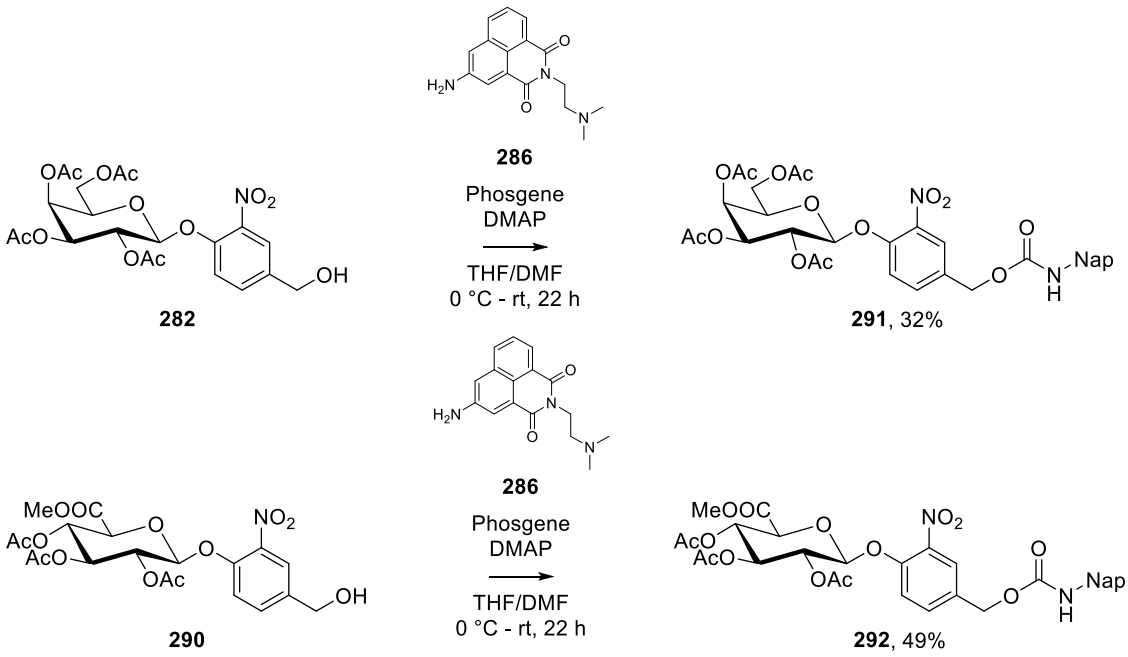
Scheme 5.3. Synthesis of β-galactosyl derivative **282** and amonafide **286**.

The analogous glucuronic acid derivative was also desired, and thus the α-bromoglycoside **279** was synthesised as before. β-D-glucuronic acid tetraacetate **287** was dissolved in a solution of HBr in acetic acid, and following reaction over 4 h at 0 °C, bromide **288** was obtained in good yield of 73%. Deviating from the glycosylation procedure for **279**, instead **288** and 4-hydroxy-3-nitrobenzaldehyde **280** were dissolved in anhydrous CH_3CN and shielded from light. A source of Ag (I) was employed for conjugation of the carbohydrate and benzaldehyde derivative, to prevent unwanted reactions with the methyl ester of **288**. Following repeated filtration to remove trace silver species, **289** was obtained in a yield of 70%. Finally, reduction with $NaBH_4$ over silica gel, as before, furnished the primary alcohol **290** in good yield of 60% (Scheme 5.4).



Scheme 5.4. Synthesis of GlcA derivative **290**, with nitrobenzene linker.

Finally, the conjugation of the carbohydrate-linker compounds **282** and **290** with amonafide **286** was attempted. Carbodiimide couplings failed in every permutation, with the sluggish reactivity of the aryl amine likely the culprit. Triphosgene also failed to furnish the desired carbamate linkage. Instead, Amonafide was dissolved in anhydrous THF with an excess of DMAP, and a solution of phosgene in toluene was added. This suspension was stirred for 4 h at rt, following which a solution of **282** or **290** in dry DMF was added dropwise at 0 °C. After much optimisation, this furnished galactose-bearing (Gal-AM) and glucuronic acid-bearing (GlcA-AM) prodrugs as **291** and **292** respectively. Deacetylation of Gal-AM was conducted with freshly prepared sodium methoxide in methanol, whereas the deacetylation of GlcA-AM was conducted in the presence of NaOH. HPLC purification provided **276** in 98% yield, and **277** in a 49% yield in the hands of our collaborators (Scheme 5.5).



Scheme 5.5. Phosgene-mediated coupling of Nap and carbohydrate derivatives **291** and **292**.

These compounds were provided to our collaborators for further testing, which formed the basis of a publication on theranostic prodrugs. The uptake and cellular viability of conjugates **291** and **292** were tested in a number of cancer cell lines. Amonafide was released and underwent cellular uptake following enzymatic treatment, with a significant increase in uptake rate with respect to the glycosylated conjugates. Enhanced toxicity upon enzymatic activation was also recorded, which correlates this effect. Confocal fluorescence microscopy was employed to verify the activation of the derivatives by endogenous enzymes, and the emission profile of the naphthalimide moiety was studied in real time. For a fully analysis of the results, the publication can be read at https://doi.org/10.1002/chem.202103858.

Chapter Six

Experimental

6.0 Experimental

6.0.1 General Experimental Detail

All commercial chemicals used were supplied by Sigma Aldrich, Fluorochem, VWR, Carbosynth, New England Biolabs (NEB) or 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 HPLC 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-4 (110 V/ 60 Hz) containing 14 UVA lamps centred at 365 nm. Silica gel 60 (Merck, 230-400 mesh) was used for flash column chromatography. All compounds were subject to purification using silica gel, unless otherwise stated. Analytical thin layer chromatography was carried out with silica gel 60 (fluorescence indicator F_{254}; Merck) and visualized by UV irradiation, $KMnO_4$, H_2SO_4 (10% in EtOH), or molybdenum staining (ammonium molybdate (5.0 g) and concentrated H_2SO_4 (5.3 mL) in H_2O (100 mL)). Melting points are uncorrected and were measured with a Stuart SP-10 melting point apparatus. NMR spectra were recorded using 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 are given in ppm and referenced to the internal solvent signals. The assignment of the signals was confirmed by 2D spectra (COSY, HMBC, HSQC). MALDI time of flight (TOF) spectra were acquired using a Waters MALDI Q-TOF Premier in positive or negative mode with *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) as the MALDI matrix. ESI mass spectra were acquired in positive and negative modes as required, using a Micromass TOFmass 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. IR spectra were recorded on a Perkin-Elmer Spectrum 100 FT-IR spectrometer.

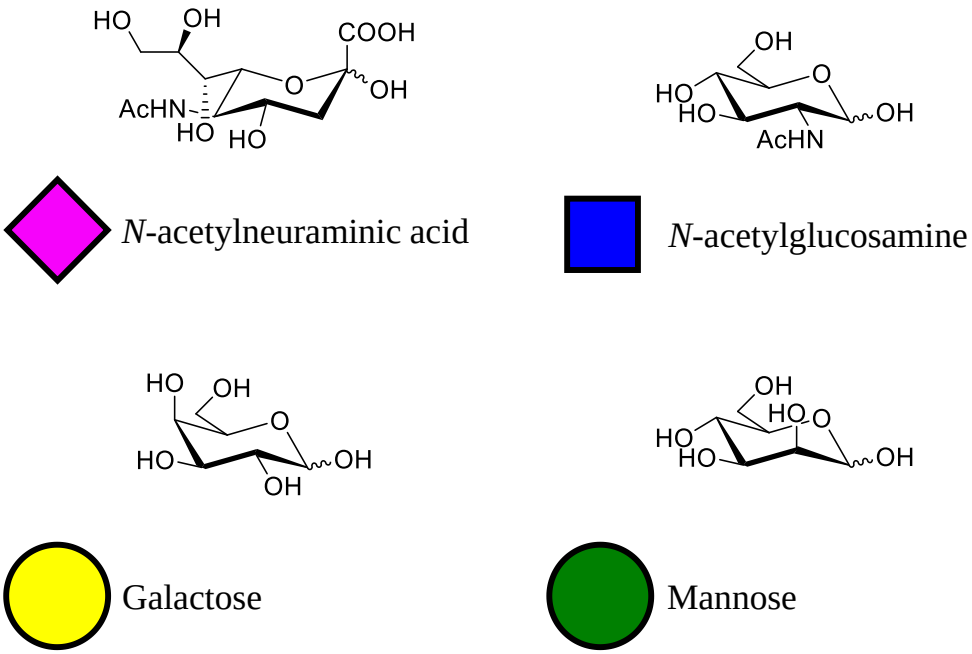


Figure 6.1. Key for carbohydrate units.

6.1 Chapter 2 – Experimental Detail

6.1.1 General Procedures

Procedure 1: Carboxylic acid methylation

To a solution of the amino acid (1.0 eq.) in anhydrous DMF was added K_2CO_3 (1.5 eq.) and iodomethane (2.0 eq.) and the solution was stirred at rt for 3 h. H_2O (50 mL) was added and the product was extracted with EtOAc (3 x 30 mL). The combined organic layers were washed with saturated aqueous $NaHCO_3$ solution (50 mL), and brine (50 mL). The organic layer was dried over $MgSO_4$, filtered, and solvent was removed *in vacuo* and the product was used without further purification.

Procedure 2: Synthesis of *S*-trityl thioesters

To a stirred solution of the starting carboxylic acid (1.0 eq.), triphenylmethanethiol (1.1 eq.) and DMAP (0.1 eq.) in anhydrous CH_2Cl_2 was added EDCI·HCl (1.1 eq.) and the solution was stirred at rt for 18 h. The solvent was removed *in vacuo* and the mixture was subjected to chromatographic purification.

Procedure 3: Removal of *S*-trityl group

To a solution of the *S*-trityl thioester (1.0 eq.) in anhydrous CH_2Cl_2 was added TFA (to a final concentration of 25% v/v in CH_2Cl_2) and ethyldimethylsilane (20.0 eq.) and the mixture was stirred for 10 min. Toluene was added and the solvent was removed *in vacuo* to give the crude thioacid which was applied immediately to the next step.

Procedure 4: Thiol-ene ligation

To a mixture of the alkene (1.0 eq.), DPAP (0.1 eq.) and MAP (0.1 eq.) in anhydrous DMF was added the crude thioacid (1.2 eq.) from Procedure **3** and the reaction mixture was irradiated in an UV oven at rt without agitation for 1 h. The solvent was removed *in vacuo* and the mixture was subject to chromatographic purification.

Procedure 5: *N*-Acetylation

To a stirred solution of the amino acid (1.0 eq.) in sat. aq. $NaHCO_3$ solution was added Ac_2O (5.0 eq.) and the mixture was stirred at rt for 16 h. The mixture was acidified to pH 2 with aq. HCl solution (3 M), extracted with EtOAc (2 x 15 mL), dried over $MgSO_4$, filtered, and the solvent was removed *in vacuo.* The product was used without further purification.

Procedure 6: Removal of benzyl group

The benzyl protected amino acid (1.0 eq.) was dissolved in an appropriate solvent and $Pd(OH)_2/C$ (0.1 eq.) was added and the suspension deoxygenated with a N_2 balloon for 10 min. The solution was then placed under a H_2 atmosphere using a balloon and stirred at rt for 16 h. The mixture was filtered through a plug of Celite® and the solvent was removed *in vacuo.* Product used without further purification unless otherwise specified.

Procedure 7: Removal of Boc protecting group

To a solution of the Boc protected amino acid (1.0 eq.) in CH_2Cl_2 was added TFA (to a final concentration of 25% v/v in CH_2Cl_2) and ethyldimethylsilane (20.0 eq.), and the solution stirred at rt for 16 h. The solvent was removed *in vacuo* to yield the product as the trifluoroacetate salt which was used without further purification.

Procedure 8: Peptide coupling

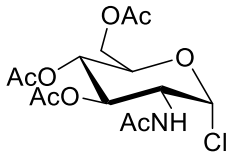
To the *N*-protected amino acid (1.0 eq.) in anhydrous CH_2Cl_2:DMF (9:1) was added EDCI·HCl (1.1 eq.) and HOBt (1.1 eq.) and the solution stirred under an argon atmosphere at 0 °C for 30 min. To this was added a solution of the *C*-protected amino acid (1.0 eq.) and NEt_3 (2.0 eq.) in anhydrous CH_2Cl_2:DMF (9:1) and the solution was stirred at rt for 16 h. The reaction mixture was diluted with H_2O (20 mL), extracted with CH_2Cl_2 (2 x 15 mL). The combined organic layers were washed with brine (2 x 20 mL), dried over $MgSO_4$, filtered, the solvent was removed *in vacuo* and the mixture subjected to chromatographic purification.

Procedure 9: Thiol-ene ligation, *S*-to-*N* acyl shift, base hydrolysis

To a mixture of the alkene (1.0 eq.), DPAP (0.1 eq.) and MAP (0.1 eq.) in anhydrous solvent was added the crude thioacid (1.1 or 1.5 eq.) from **General Procedure 3** and the reaction mixture was irradiated in an UV oven at rt without agitation for 1 h, followed by stirring at rt for 16 h outside of the UV oven. The solvent was removed *in vacuo* and the mixture was re-dissolved in CH_3OH:H_2O (10 mL, 8:2). NEt_3 (25.0 eq.) was added and the solution was stirred at rt for 4 h. The mixture was neutralised by the addition of DOWEX H^+ resin and then filtered. The solvent was removed *in vacuo* and the reaction mixture was subjected to chromatographic purification.

6.1.2 Methods and Characterisation

2-Deoxy-2-acetamido-3,4,6-tri-*O*-acetyl-α-D-glucopyranosyl chloride (119)[303]



An oven-dried flask was charged with acetyl chloride (19.9 mL, 0.28 mol). To this was added portionwise *N*-acetyl-D-glucosamine **118** (10.0 g, 0.05 mol), and the resulting solution stirred at rt for 16 h. After the elapsed time, the solution was poured into H_2O (250 mL, 0 °C) and immediately extracted with $CHCl_3$ (3 x 50 mL). The organic extracts were combined and washed with sat. aq. $NaHCO_3$ solution (250 mL), after which they were again immediately separated. The organic phase was then dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The resulting syrup was solubilised in CH_2Cl_2 (10 mL), and an excess of ice cold Et_2O (100 mL, 0 °C) was added to precipitate the product

as a fine white powder (11.2 g, 68%). The isolated compound was in good agreement with the literature.

M.p: 124-126 °C (from $CHCl_3$). Literature value: 124-126 °C.

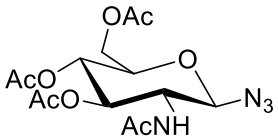
δ_H (400 MHz, $CDCl_3$) 6.18 (d, *J* = 3.7 Hz, 1H, H-1), 5.78 (d, *J* = 8.7 Hz, 1H, NH), 5.36 – 5.26 (m, 1H, H-3), 5.25 – 5.16 (m, 1H, H-4), 4.57 – 4.48 (m, 1H, H-2), 4.32 – 4.21 (m, 2H, H-6 and H-5), 4.17 – 4.10 (m, 1H, H-6'), 2.12 – 2.07 (s, 3H, CH_3), 2.05 (s, 6H, 2 x CH_3), 1.97 (s, 3H, CH_3)

δ_C (101 MHz, $CDCl_3$) 178.5 (C=O), 169.8 (C=O), 93.8 (C-1), 71.1 (C-3), 70.3 (C-5), 67.1 (C-4), 61.3 (C-6), 53.7 (C-2), 23.3 (CH_3), 20.8 (CH_3), 20.7 (CH_3)

HRMS: $C_{14}H_{21}ClNO_8$ requires 366.0956. APCI: Found 366.0943 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 2850, 2541, 1737 (C=O), 762 (C-Cl)

2-Deoxy-2-acetamido-3,4,6-tri-*O*-acetyl-β-D-glucopyranosyl azide (120)[303]



To a solution of **119** (10.43 g, 0.029 mol) in anhydrous DMF (30 mL) was added NaN_3 (5.56 g, 0.086 mol) and the resulting solution stirred at 80 °C for 16 h. The solution was then cooled to rt, and diluted with EtOAc (80 mL). The solution was washed with brine (3 x 50 mL), dried over $MgSO_4$ and concentrated *in vacuo* to yield an orange oil. This material was then subject to chromatographic purification (6:4 EtOAc:hexanes), which furnished **120** as a colourless oil (9.89 g, 93%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 5.78 (d, *J* = 8.8 Hz, 1H, NH), 5.29 – 5.19 (m, 1H, H-3), 5.09 (t, *J* = 9.7 Hz, 1H, H-4), 4.76 (d, *J* = 9.3 Hz, 1H, H-1), 4.26 (dd, *J* = 12.5, 4.8 Hz, 1H, H-6), 4.16 (dd, *J* = 12.5, 2.3 Hz, 1H, H-6'), 3.94 – 3.87 (m, 1H, H-2), 3.8 – 3.76 (m, 1H, H-5), 2.09 (s, 3H, CH_3), 2.04 (m, 6H, 2 x CH_3), 1.97 (s, 3H, CH_3)

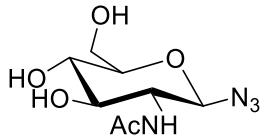
δ_C (101 MHz, $CDCl_3$) 171.2 (C=O), 170.8 (C=O), 170.5 (C=O), 169.4 (C=O), 88.6 (C-1), 74.2 (C-3) , 72.3 (C-5), 68.1 (C-4), 61.9 (C-6), 54.4 (C-2), 23.4 (CH_3), 20.9 (CH_3), 20.7 (CH_3)

HRMS: $C_{14}H_{21}N_4O_8$ requires 373.1359. ESI: Found 373.1355 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 2945, 2542, 2104 (N=N=N), 1734 (C=O)

***R*$_f$:** 0.3 (EtOAc:Hexanes 60:40)

2-Deoxy-2-acetamido-β-D-glucopyranosyl azide (121)[304]



To a solution of **120** (0.8 g, 2.15 mmol) in anhydrous CH_3OH (10 mL) was added a catalytic amount of freshly prepared $NaOCH_3$. The resulting solution was stirred at rt for 30 min, after which it was neutralised by the addition of DOWEX 50wx8 H^+ resin. The suspension was filtered and the solvent removed *in vacuo*. The oil obtained was subject to an azeotropic distillation with hexanes to remove residual CH_3OH to yield **121** as a colourless oil (476 mg, 90%). The isolated compound was in good agreement with the literature.

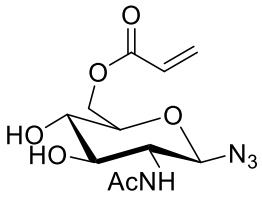
δ_H (400 MHz, CD_3OD) 4.50 (d, *J* = 9.3 Hz, 1H, H-1), 3.90 (dd, *J* = 12.1, 1.7 Hz, 1H, H-6), 3.73 – 3.69 (m, 1H, H-6'), 3.68 – 3.64 (m, 1H, H-2), 3.45 (t, *J* = 10.1 Hz, 1H, H-3), 3.37 – 3.35 (m, 1H, H-4), 3.37 - 3.35 (m, 1H, H-5), 1.99 (s, 3H, CH_3) ppm

δ_C (101 MHz, CD_3OD) 173.8 (NH<u>C</u>=OCH_3), 90.2 (C-1), 80.4 (C-5), 75.8 (C-3), 71.7 (C-4), 62.6 (C-6), 56.7 (C-2), 22.9 (CH_3) ppm

HRMS: $C_8H_{14}N_4NaO_5$ requires 269.0862. ESI: Found 269.0855 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3298 (OH), 2981 (NH), 2117 (N=N=N), 1650 (C=O)

2-Deoxy-2-acetamido-6-*O*-prop-2-enoate-β-D-glucopyranosyl azide (123)



To a solution of **121** (300 mg, 1.22 mmol) in anhydrous DMF (5 mL) was added anhydrous NEt_3 (0.2 mL, 1.46 mmol) and the solution cooled to -20 °C. Acryloyl chloride **122** (0.12 mL, 1.46 mmol) was added dropwise. Upon completion of addition the solution was warmed to rt and stirred under argon for 3 h. After the elapsed time the solution was concentrated *in vacuo*, using heptanes to assist in the removal of DMF. The resulting orange residue was purified by column chromatography (CH_2Cl_2 to CH_2Cl_2:CH_3OH 88:12) to yield **123** as a yellow oil (160 mg, 34%).

δ_H (400 MHz, $CDCl_3$) 6.44 (dd, *J* = 17.3, 1.5 Hz, 1H, CH_2=CH), 6.23 (dd, *J* = 17.3, 10.4 Hz, 1H, CH_2=CH), 5.93 (dd, , *J* = 10.4, 1.5 Hz, 1H, CH_2=CH), 4.56 – 4.51 (m, 2H, H-6 and H-1), 4.31 (dd, *J* = 12.1, 5.8 Hz, 1H, H-6'), 3.67 (t, *J* = 9.7 Hz, 1H, H-2), 3.64 – 3.59 (m, 1H, H-5), 3.51 – 3.45 (t, *J* = 10.4 Hz, 1H, H-3), 3.41 – 3.35 (m, 1H, H-4) 1.99 (s, 3H, CH_3)

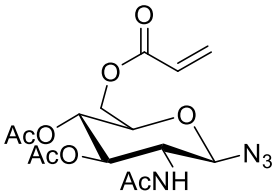
δ_C (151 MHz, $CDCl_3$) 173.8 (C=O), 167.5 (C=O), 131.9 (CH_2=CH), 129.3 (CH_2=CH), 89.9 (C-1), 77.5 (C-5), 75.5 (C-3), 71.7 (C-4), 64.6 (C-6), 56.6 (C-2), and 22.9 (CH_3)

HRMS: $C_{11}H_{16}N_4NaO_6$ requires 323.0968. ESI: Found 323.0959 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2981 (NH), 2901, 2215, 2117 (N=N=N), 1644 (C=C)

***R*f:** 0.27 (CH_2Cl_2:CH_3OH 92:8)

2-Deoxy-2-acetamido-6-*O*-prop-2-enoate-3,4,-di-*O*-acetyl-β-D-glucopyranosyl azide (124)



To a solution of **123** (170 mg, 0.52 mmol) in anhydrous CH_2Cl_2 (5 mL) was added anhydrous pyridine (0.12 mL, 1.54 mmol) followed by acetic anhydride (0.15 mL, 1.54

mmol). The resulting solution was stirred at rt for 16 h under Ar. After the elapsed time, the solvent was removed *in vacuo* and the product used without further purification, as a yellow oil (196 mg, 99%).

δ_H (400 MHz, CD_3OD) 6.45 – 6.40 (m, 1H, $CH=CH_2$), 6.23 – 6.15 (m, 1H, $CH_2=CH$), 5.95 – 5.91 (m, 1H, $CH_2=CH$), 5.29 – 5.19 (m, 1H, H-3), 5.03 (t, *J* = 9.9 Hz, 1H, H-4), 4.83 (d, *J* = 9.3 Hz, 1H, H-1) 4.33 (m, 2H, H-6, H-6'), 4.01 – 3.94 (m, 1H, H-5), 3.90 – 3.83 (m, 1H, H-2), 2.03 – 2.01 (m, 3H, CH_3), 2.00 – 1.97 (m, 3H, CH_3), 1.92 (s, 3H, CH_3)

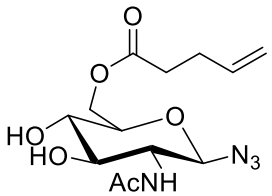
δ_C (101 MHz, CD_3OD) 173.5 (C=O), 171.7 (C=O), 171.1 (C=O), 167.1 (C=O), 132.2 ($CH=CH_2$), 129.0 ($CH=CH_2$), 89.4 (C-1), 75.0 (C-5), 73.8 (C-3), 69.9 (C-4), 63.2 (C-6), 54.8 (C-2), 22.7 (CH_3), 20.6 (CH_3), 20.5 (CH_3)

HRMS: $C_{15}H_{20}N_4NaO_8$ requires 407.1179. ESI: Found 407.1165 $[M + Na]^+$

ν_{max} (ATR/cm^{-1}): 2981 (NH), 2119 (N=N=N), 1748 (C=O), 1663 (C=C)

***R*f:** 0.38 (EtOAc:Hexanes 7:3)

2-Deoxy-2-acetamido-6-*O*-pent-4-enoate-β-D-glucopyranosyl azide (126)



To a solution of **121** (0.49 g, 2.00 mmol) in anhydrous DMF (8 mL) was added NEt_3 (0.33 mL, 2.40 mmol) and the solution was cooled to 0 °C. To this was added 4-pentenoyl chloride (0.26 mL, 2.40 mmol) and the solution was allowed to warm to rt and was stirred for 2 h under argon. The solvent was removed *in vacuo* and the product was purified by flash chromatography (CH_2Cl_2 to CH_2Cl_2:CH_3OH - 88:12) to yield the product as a yellow oil (0.47 g, 72%).

δ_H (600 MHz, CD_3OD) 5.88 (m, 1H, $CH=CH_2$), 5.10 (m, 1H, $CH=CH_2$), 5.02 (m, 1H, $CH=CH_2$), 4.61 (d, *J* = 8.6 Hz, 1H, H-1), 4.51 (dd, *J* = 2.0, *J* = 12.1 Hz, 1H, H-6), 4.28 (dd, *J* = 5.8, *J* = 12.1 Hz, 1H, H-6'), 3.73 (t, *J* = 9.7 Hz, 1H, H-2), 3.65 (m, 1H, H-5), 3.57 (t, *J* = 10.2 Hz, 1H, H-3), 3.36 (m, 1H, H-4), 2.52 (m, 1H, CH_2), 2.41 (m , 1H, CH_2), 2.05 (s, 3H, CH_3)

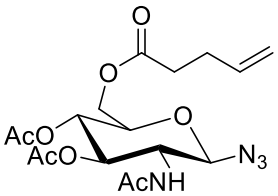
δ_C (150 MHz, CD_3OD) 173.0 (C=O), 172.3 (C=O), 136.6 ($CH=CH_2$), 114.5 ($CH=CH_2$), 88.4 (C-1), 75.9 (C-5), 74.2 (C-3), 70.5 (C-4), 62.9 (C-6), 55.2 (C-2), 33.0 (CH_2), 28.5 (CH_2), 21.4 (CH_3)

HRMS: $C_{13}H_{20}N_4NaO_6$ requires 351.1275. ESI: Found 351.1281 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3658 (NH), 3268 (OH), 2981 (CH), 2114 (N_3), 1733 (C=O)

***R*f:** 0.28 (CH_2Cl_2:CH_3OH 90:10)

2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-pent-4-enoate-β-D-glucopyranosyl azide (127)



To a solution of **126** (0.18 g, 0.55 mmol) in CH_2Cl_2 (5 mL) was added pyridine (0.3 mL, 3.30 mmol) and Ac_2O (0.3 mL, 3.30 mmol) and the resulting solution was stirred at rt for 16 h. The solvent was removed *in vacuo* to yield the product as a yellow oil (0.23 g, 99%).

δ_H (600 MHz, $CDCl_3$) 7.02 (d, *J* = 9.0 Hz, 1H, NH), 5.82 – 5.78 (m, 1H, $CH=CH_2$), 5.33 (t, *J* = 9.9 Hz, 1H, H-3), 5.05 – 5.01 (m, 2H, $CH=CH_2$), 5.03 – 5.01 (m, 1H, H-4), 5.03 – 5.02 (m, 1H, H-1), 4.22 (dd, *J* = 4.6, *J* = 12.4 Hz, 1H, H-6), 4.16 (dd, *J* = 1.1, 12.4 Hz, 1H, H-6'), 3.94 (q, *J* = 9.4 Hz, 1H, H-2), 3.83 – 3.80 (m, 1H, H-5), 2.47 – 2.44 (m, 1H, CH_2), 2.37 – 2.34 (m, 1H, CH_2), 1.99 (s, 3H, CH_3), 1.99 (s, 3H, CH_3), 1.96 (s, 3H, CH_3)

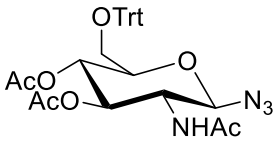
δ_C (150 MHz, $CDCl_3$) 172.5 (C=O), 170.5 (C=O), 170.4 (C=O), 169.1 (C=O), 136.6 ($CH=CH_2$), 115.7 ($CH=CH_2$), 88.4 (C-1), 73.9 (C-5), 72.6 (C-3), 68.6 (C-4), 61.8 (C-6), 54.0 (C-2), 33.1 (CH_2), 28.7 (CH_2), 23.0 (CH_3), 20.5 (CH_3), 20.4 (CH_3)

HRMS: $C_{17}H_{24}N_2NaO_8$ requires 435.1486. ESI: Found 435.1482 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3285 (NH), 2602, 2497 (CH), 2117 (N_3), 1747 (C=O)

***R*f:** 0.40 (EtOAc:Hexanes 7:3)

2-Deoxy-2-acetamido-3,4,-di-*O*-acetyl-6-trityl-β-D-glucopyranosyl azide (128)



Trityl chloride (0.93 g, 3.34 mmol) and **121** (0.57 g, 2.28 mmol) were suspended in anhydrous pyridine (7 mL) and stirred at 90 °C for 4 h under Ar. After the elapsed time the solution was concentrated *in vacuo*, diluted in CH_2Cl_2 (20 mL) and washed with H_2O (2 x 40 mL). The organic phase was separated, dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was then acetylated with a mixture of Ac_2O (1.60 mL, 16.72 mmol) and pyridine (1.36 mL, 16.72 mmol) in anhydrous CH_2Cl_2 (10 mL), in which it was stirred at rt for 16 h. The reaction mixture was then concentrated *in vacuo* and the resulting yellow oil subject to chromatographic purification (EtOAc:Hexanes 6:4). This furnished **128** as a white solid (1.2 g, 75%).

δ_H (400 MHz, $CDCl_3$) 7.47 – 7.44 (m, 5H, Ar-CH), 7.30 (t, *J* = 7.5 Hz, 5H, Ar-CH), 7.25 – 7.20 (m, 2H, Ar-CH), 5.46 (d, *J* = 9.0 Hz, 1H, NH), 5.26 (t, *J* = 9.7 Hz, 1H, H-3), 5.16 – 5.08 (m, 1H, H-4), 4.69 (d, *J* = 9.2 Hz, 1H, H-1), 4.03 (dd, *J* = 19.6, 9.2 Hz, 1H, H-2), 3.65 (ddd, *J* = 10.1, 4.4, 2.2 Hz, 1H H-5), 3.31 (dd, *J* = 10.7, 2.1 Hz, 1H, H-6), 3.08 (dd, *J* = 10.7, 4.5 Hz, 1H, H-6').

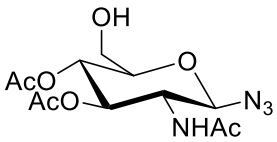
δ_C (101 MHz, $CDCl_3$) 170.4 (C=O), 168.8 (C=O), 143.6 (quaternary Ar-C), 128.8 (Ar-C), 128.0 (Ar-C), 127.2 (Ar-C), 88.4 (C-1), 77.2 (C-5), 75.8 (C-3), 68.3 (C-4), 61.9 (C-6), 54.5 (C-2), 23.5 (CH_3), 20.8 (CH_3), 20.5 (CH_3).

HRMS: $C_{31}H_{32}N_4NaO_7$ requires 595.2169. ESI: Found 595.2165 $[M+Na]^+$.

ν_{max} (ATR/cm^{-1}): 2981(Ar-CH), 2884 (Ar-CH), 2114 (N=N=N), 1747 (C=O)

***R*f:** 0.35 (EtOAc:Hexanes 6:4).

2-Deoxy-2-acetamido-3,4,di-*O*-acetyl-β-D-glucopyranosyl azide (129)[127]



To a solution of **128** (300 mg, 0.524 mmol) in anhydrous CH_2Cl_2 (7.5 mL) was added HCOOH (7.5 mL, 198.8 mmol) and the resulting solution stirred at room temperature for

20 min under Ar. After the elapsed time toluene (20 mL) was added and the solution concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc:hexanes 7:3) to yield **129** as a colourless oil (160 mg, 91%). The compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 5.52 (d, *J* = 8.8 Hz, 1H, NH), 5.27 (dd, *J* = 10.6, 9.4 Hz, 1H, H-3), 5.06 (t, *J* = 9.6 Hz, 1H, H-4), 4.76 (d, *J* = 9.3 Hz, 1H, H-1), 3.94 – 3.87 (m, 1H, H-2), 3.80 – 3.76 (m, 1H, H-5), 3.67 – 3.57 (m, 2H, H-6, 6'), 2.06 (app. s, 6H, 2 x CH_3), 1.98 (s, 3H, CH_3)

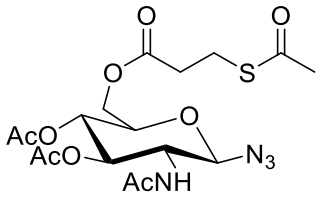
δ_C (101 MHz, $CDCl_3$) 173.5 (C=O), 171.2 (C=O), 89.4 (C-1), 77.8 (C-5), 73.8 (C-3), 69.9 (C-4), 62.4 (C-6), 54.9 (C-2), 22.7 (CH_3), 20.5 (CH_3)

HRMS: $C_{12}H_{17}N_4O_7$ requires 329.1097. ESI: Found 329.1110 $[M-H]^-$

ν_{max} (ATR/cm^{-1}): 2981 (NH), 2119 (N=N=N), 1748 (C=O), 1663 (C=C)

***R*f:** 0.3 (EtOAc:Hexanes 7:3)

2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-acetylthiopropanoate-β-D-glucopyranosyl azide (134)



To a mixture of alkene **124** (100 mg, 0.26 mmol,) DPAP (6.6 mg, 0.026 mmol) and MAP (4 mg, 0.026 mmol) in anhydrous DMF (3 mL) was added thioacetic acid (22 μL, 0.31 mmol) and the reaction mixture was irradiated in an UV oven at rt without agitation for 1 h. The solvent was removed *in vacuo* and following flash chromatography (EtOAc:hexanes 7:3), the product was obtained as a colourless oil (80 mg, 70%).

δ_H (400 MHz, CD_3OD) 5.25 (app. t, *J* = 9.4 Hz, 1H, H-3), 5.07 (t, *J* = 9.7 Hz, 1H, H-4), 4.33 (dd, *J* = 12.4, 4.5 Hz, 1H), 4.27 (dd, *J* = 12.4, 2.3 Hz, 1H, H-6), 3.95 (app. q, *J* = 9.6, 1H, H-2), 3.89 – 3.86 (m, 1H, H-5), 3.14 (t, *J* = 6.9 Hz, 2H, CH_2), 2.70 (t, *J* = 6.9 Hz, 2H, CH_2), 2.35 (s, 3H, CH_3), 2.04 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 1.95 (s, 3H, CH_3)

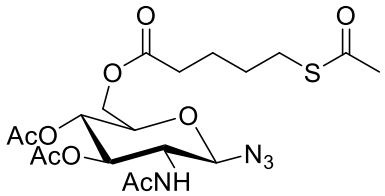
δ_C (101 MHz, CD_3OD) 196.1 (C=O), 172.5 (C=O), 171.8 (C=O), 170.8 (C=O), 170.2 (C=O), 88.4 (C-1), 74.0 (C-5), 72.84 (C-3), 68.7 (C-4), 62.2 (C-6), 53.8 (C-2), 34.1 (CH_2), 29.4 (CH_2), 24.1 (CH_3), 21.6 (CH_3), 19.6 (CH_3), 19.5 (CH_3)

HRMS: $C_{17}H_{24}N_4NaO_9S$ requires 483.1162. ESI: Found 483.1168 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2988 (NH), 2119 (N=N=N), 2071, 1750 (C=O), 1693 (C=O), 1378

***R*$_f$:** 0.24 (EtOAc:Hexanes 7:3)

2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-acetylthiopentanoate-β-D-glucopyranosyl azide (134b)



To a mixture of the alkene **127** (50 mg, 0.13 mmol,) DPAP (3.3 mg, 0.013 mmol) and MAP (2 mg, 0.013 mmol) in anhydrous DMF (3 mL) was added thioacetic acid (15 μL, 0.14 mmol) and the reaction mixture was irradiated in an UV oven at rt without agitation for 1 h. The solvent was removed *in vacuo* and following flash chromatography (EtOAc:hexanes 7:3), the product was obtained as a colourless oil (56 mg, 93%).

δ_H (600 MHz, $CDCl_3$) 5.80 (d, *J* = 9.4 Hz, 1H, NH), 5.24 (t, *J* = 9.8 Hz, 1H, H- 3), 5.07 (t, *J* = 9.8 Hz, 1H, H-4), 4.76 (d, *J* = 9.4 Hz, 1H, H-1), 4.24 (dd, *J* = 4.9, *J* = 12.2 Hz, 1H, H-6), 4.17 (dd, *J* = 1.5, *J* = 12.2 Hz, 1H, H-6'), 3.92 (q, *J* = 9.7 Hz, 1H, H-2), 3.90 – 3.78 (m, 1H, H-5), 2.87 (t, *J* = 7.5 Hz, 2H, CH_2), 2.37 (t, *J* = 7.5 Hz, 2H, CH_2), 2.31 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.97 (s, 3H, CH_3), 1.72 – 1.69 (m, 2H, CH_2), 1.62 – 1.58 (m, 2H, CH_2)

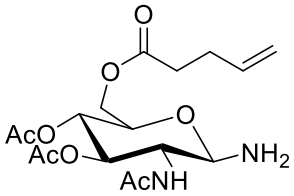
δ_C (150 MHz, $CDCl_3$) 196.0 (C=O), 173.0 (C=O), 171.1 (C=O), 170.6 (C=O), 169.4, (C=O), 88.5 (C-1), 74.1 (C-5), 72.3 (C-3), 68.2 (C-4), 61.9 (C-6), 54.3 (C-2), 33.5 (CH_2), 30.7 (CH_2), 29.1 (CH_2), 28.7 (CH_2), 23.9 (CH_3), 23.4 (CH_3), 20.8 (CH_3), 20.7 (CH_3)

HRMS: $C_{19}H_{28}N_4O_9S$ requires 511.1475. ESI: Found 511.1478 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3289 (NH), 2117 (N_3), 1748, 1689 (C=O), 1233 (CH)

***R*f:** 0.23 (EtOAc:Hexanes 7:3)

2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-pent-4-enoate-β-D-glucopyranosyl amine (135)



To a solution of **127** (0.35 g, 0.85 mmol) in CH_3CN:EtOH (1:1, 15 mL) was added ammonium tetrathiomolybdate (0.22 g, 0.85 mmol) and the reaction flask was placed in a sonication bath at rt for 3 h. The solvent was removed *in vacuo* and the black residue was dissolved in CH_2Cl_2:Et_2O (1:10, 50 mL) and the mixture filtered. The solvent was removed *in vacuo* and the black residue was re-dissolved in CH_2Cl_2:Et_2O (1:10, 50 mL) and the filtering was repeated (x 4) to yield the product as a colourless oil (0.17 g, 53%).

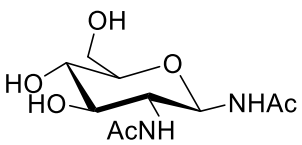
δ_H (400 MHz, $CDCl_3$) 5.95 – 5.71 (m, 1H, C<u>H</u>=CH_2), 5.15 – 4.97 (m, 4H, H-4, CH=C<u>H</u>$_2$, H-3), 4.25 – 3.96 (m, 3H, H-6, H-6', H-1), 3.67 – 3.59 (m, 1H, H-5), 2.51 – 2.33 (m, 4H, CH_2, CH_2), 2.10 – 1.95 (m, 9H, 3 x CH_3)

δ_C (151 MHz, $CDCl_3$) 172.9 (C=O), 171.7 (C=O), 170.9 (C=O), 169.4 (C=O), 136.7 (CH=CH_2), 115.6 (CH=CH_2), 86.5 (C-1), 73.6 (C-5), 72.9 (C-3), 68.7 (C-4), 62.4 (C-6), 55.0 (C-2), 33.3 (CH_2), 28.7 (CH_2), 23.4 (CH_3), 20.8 (CH_3), 20.7 (CH_3)

HRMS: $C_{17}H_{26}N_2O_8$ requires 387.1767. ESI: Found 387.1766 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 3628, 3398 (NH), 2968 (NH_2), 2968 (CH), 1971, 1730 (C=O)

2-Deoxy-2-acetamido-β-D-glucopyranosylacetamide (139)[305]



To a solution of **135** (50 mg, 0.13 mmol), DPAP (10 mg, 0.01 mmol) and MAP (10 mg, 0.01 mmol) in anhydrous DMF (3 mL) was added thioacetic acid (15 μL, 0.14 mmol) and the reaction mixture was irradiated in a UV oven at rt without agitation for 1 h

followed by stirring at rt for 16 h. The solvent was removed *in vacuo* and the mixture was re-dissolved in CH_3OH (5 mL) and to this was added a catalytic amount of freshly prepared $NaOCH_3$ and the solution was stirred at rt for 20 min. The mixture was neutralised by the addition of DOWEX 50wx8 H^+ resin and then filtered. The solvent was removed *in vacuo* and the product was purified by flash chromatography (CH_2Cl_2 to CH_2Cl_2:CH_3OH 9:1) to yield the product as a colourless oil (22 mg, 64%). The isolated compound was in good agreement with the literature.

δ_H (600 MHz, DMSO-d_6) 4.79 (t, *J* = 9.4 Hz, 1H, H-1), 3.64 (dd, *J* = 11.9, 1.8 Hz, 1H, H-6), 3.51 (m, 1H, H-2), 3.41 (dd, *J* = 11.8, 5.0 Hz, 1H, H-6'), 3.32 – 3.27 (m, 1H, H-3), 3.09 – 3.03 (m, 2H, H-4. H-5), 1.79 (s, 3H, CH_3), 1.78 (s, 3H, CH_3)

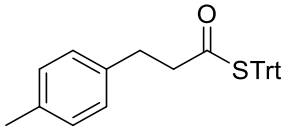
δ_C (151 MHz, DMSO-d_6) 169.8 (C=O), 169.6 (C=O), 78.8 (C-1), 78.7 (C-5), 74.6 (C-3), 70.5 (C-4), 60.9 (C-6), 54.5 (C-2), 22.9 (CH_3), 22.8 (CH_3).

HRMS: $C_{10}H_{17}N_2O_6$ requires 261.1087. ESI: Found 261.1091 $[M - H]^-$

ν_{max} (ATR/cm^{-1}): 3269 (NH), 2924 (OH), 1646 (C=O), 1314 (CH)

***R*$_f$:** 0.24 (CH_2Cl_2:CH_3OH 9:1)

S-trityl-3-(*p*-tolyl)propanethioate (140)[306]



Prepared as per **Procedure 2** using 3-(*p*-tolyl)propanoic acid (0.25 g, 1.52 mmol) in CH_2Cl_2 (10 mL) and following flash chromatography (EtOAc:hexanes 5:95), the product was obtained as a yellow oil (0.51 g, 80%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.33 – 7.17 (m, 15H, Ar-CH), 7.12 (d, *J* = 7.5 Hz, 2H, Ar- CH), 7.04 (d, *J* = 7.5 Hz, 2H, Ar-CH), 2.90 – 2.78 (m, 4H, CH_2), 2.34 (s, 3H, CH_3)

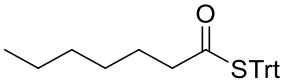
δ_C (100 MHz, $CDCl_3$) 196.1 (C=O), 143.8 (qC), 136.9 (qC), 135.8 (qC), 129.8 (Ar- CH), 129.2 (Ar-CH), 128.4 (Ar-CH), 127.8 (Ar-CH), 127.1 (Ar-CH), 70.4 (C=OS), 45.3 (CH_2), 30.9 (CH_2), 21.1 (CH_3)

HRMS: $C_{29}H_{26}NaOS$ requires 445.1602. ESI: Found 445.1603 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 1693 (C=O), 1443 (CH)

***R*f:** 0.26 (EtOAc:Hexanes 5:95)

S-tritylheptanethioate (141)[306]



Prepared as per **Procedure 2** using heptanoic acid (0.3 mL, 2.30 mmol) in CH_2Cl_2 (6 mL) and following flash chromatography (EtOAc:hexanes - 5:95), the product was obtained as a yellow oil (1.59 g, 81%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.34 – 7.21 (m, 15H, Ar-CH), 2.52 (t, *J* = 7.3 Hz, 2H, CH_2), 1.64 – 1.55 (m, 2H, CH_2), 1.37 – 1.23 (m, 2H, CH_2), 0.96 – 0.86 (t, *J* = 7.1 Hz, 3H, CH_2)

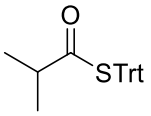
δ_C (101 MHz, $CDCl_3$) 197.1 (C=O), 144.0 (qC), 129.8 (Ar-CH), 127.8 (Ar-CH), 127.1 (Ar-CH), 70.2 (SqC), 43.8 (CH_2), 31.4 (CH_2), 28.5 (CH_2), 25.7 (CH_2), 22.5 (CH_2), 14.0 (CH_3)

HRMS: $C_{26}H_{28}NaOS$ requires 411.1759. ESI: Found 411.1772 $[M+Na]^+$

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

***R*f:** 0.31 (EtOAc:hexanes 5:95)

S-trityl-2-methylpropanethioate (142)



Prepared as per **Procedure 2** using isobutyric acid (0.62 mL, 6.81 mmol), triphenylmethanethiol (2.07 g, 7.49 mmol) and DMAP (84 mg, 0.68 mmol) in anhydrous CH_2Cl_2 (10 mL), with EDCI·HCl (1.44 g, 7.491 mmol). Crude residue subject chromatographic purification (EtOAc:hexanes 5:95), which gave **142** as a fine white powder (2.24 g, 95%).

δ_H (400 MHz, $CDCl_3$) 7.26 (s, 15H, Trt-CH), 2.76 (sept., *J* = 8.0 Hz, 1H, CH), 1.11 (s, 3H, CH_3), 1.10 (s, 3H, CH_3)

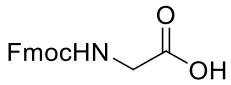
δ_C (101 MHz, $CDCl_3$) 201.7 (C=O), 144.5 (Ar-qC), 130.3 (Ar-CH), 128.2 (Ar-CH), 127.5 (Ar-CH), 70.3 (Trt-C-S), 43.5 (CH), 19.7 (CH_3)

HRMS: $C_{23}H_{22}NaOS$ requires 369.1289. ESI: Found 369.1285 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2972 (Ar-CH), 1689 (S-C=O)

***R*f:** 0.35 (EtOAc:Hexanes 5:95)

Fmoc-Gly (143a)[307]

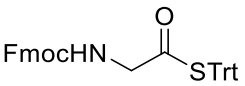


To glycine (1.0 g, 13.32 mmol) in H_2O:dioxane (1:1, 20 mL) was added K_2CO_3 (2.95 g, 21.31 mmol) and Fmoc-OSu (4.94 g, 14.65 mmol) and mixture was stirred at rt for 16 h. The dioxane was removed *in vacuo* and the mixture was washed with Et_2O (2 x 10 mL). The aqueous layer was acidified to pH 2 with aq. HCl solution and extracted with Et_2O (2 x 20mL), dried over $MgSO_4$, filtered, and solvent was removed *in vacuo* to yield the product as a white solid (3.42 g, 86%). M.p.: 165-167 °C, Literature value: 166-167 °C. The isolated compound was in good agreement with the literature.

δ_H (400 MHz, CD_3OD) 7.77 (d, *J* = 7.2 Hz, 2H, Ar-CH), 7.65 (d, *J* = 7.6 Hz, 2H, Ar-CH), 7.36 (t, *J* = 7.2 Hz, 2H, Ar-CH), 7.28 (t, *J* = 7.2 Hz, 2H, Ar-CH), 4.32 (d, *J* = 7.1 Hz, 2H, C<u>H</u>$_2$CH), 4.21 (t, *J* = 7.0 Hz, 1H, CH_2C<u>H</u>), 3.82 (s, 2H, CH_2C=O)

HRMS: $C_{17}H_{15}NNaO_4$ requires 320.0899. ESI: Found 320.0900 $[M+Na]^+$

Fmoc-Gly-*S*Trt (143)[308]



Prepared as per **Procedure 2** using Fmoc-Gly (1.45 g, 4.88 mmol) in CH_2Cl_2 (20 mL) and following flash chromatography (EtOAc:hexanes - 10:90), the product was obtained as a yellow solid (1.87 g, 69%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.79 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.60 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.42 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.35 – 7.23 (m, 17H, Ar-CH), 5.32 (d, *J* = 5.4 Hz, 2H, NH), 4.42 (d, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH$), 4.23 (t, *J* = 7.2 Hz, 1H, $CH_2C\underline{H}$), 4.14 (m, 2H, $CH_2C{=}O$)

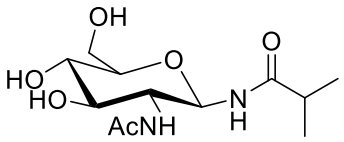
δ_C (101 MHz, $CDCl_3$) 194.4 (C=O), 156.0 (C=O), 143.7 (qC), 143.4 (qC), 141.3 (qC), 129.8 (Ar-CH), 127.9 (Ar-CH), 127.8 (Ar-CH), 127.1 (Ar-CH), 125.1 (Ar-CH), 120.0 (Ar-CH), 70.9 (SqC), 67.0 (CH_2CH), 50.6 ($CH_2C{=}O$), 47.3 (CH_2CH)

HRMS: $C_{36}H_{29}NNaO_3S$ requires 578.1766. ESI: Found 578.1779 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3410 (NH), 1697 (C=O), 1184 (CH)

***R*f:** 0.34 (EtOAc:Hexanes 1:9)

2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-isobutyramide (144)



Prepared as per **Procedure 9** using **135** (70 mg, 0.18 mmol) and **142** (90 mg, 0.27 mmol) in DMF (6 mL) and following flash chromatography (CH_2Cl_2:CH_3OH – 91:9), the product was obtained as a colourless oil (31 mg, 61%).

δ_H (600 MHz, DMSO-d_6) 4.77 (t, *J* = 9.4 Hz, 1H, H-1), 3.66 (dd, *J* = 11.8, 1.9 Hz, 1H, H-6), 3.54 (td, *J* = 9.8, 8.7 Hz, 1H, H-2), 3.42 (dd, *J* = 11.8, 5.3 Hz, 1H, H-6'), 3.33 – 3.27 (m, 1H, H-3), 3.12 – 3.01 (m, 2H, H-4, H-5), 2.39 – 2.28 (m, 1H, CH), 1.77 (s, 3H, CH_3), 0.95 (dd, *J* = 6.9, 3.2 Hz, 6H, 2 x CH_3)

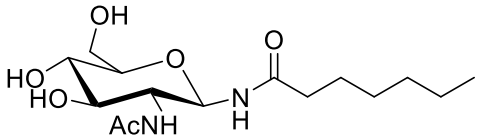
δ_C (151 MHz, DMSO-d_6) 174.4 (C=O), 171.9 (C=O), 79.1 (C-1), 78.8 (C-5), 74.4 (C-3), 70.6 (C-4), 60.9 (C-6), 54.6 (C-2), 33.8 (CH), 22.8 (CH_3), 19.7, 18.5 ($C\underline{H}CH_3$)

HRMS: $C_{12}H_{21}N_2O_6$ requires 289.1400. ESI: Found 289.1396 $[M-H]^-$

ν_{max} (film/cm^{-1}): 3301 (NH), 2982 (OH), 1666 (C=O)

***R*f:** 0.24 (CH_2Cl_2:CH_3OH 9:1)

2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-heptaneamide (145)



Prepared as per **Procedure 9** using **135** (50 mg, 0.13 mmol) and **141** (80 mg, 0.2 mmol) in DMF (5 mL) and following flash chromatography (CH_2Cl_2:CH_3OH 91:9), the product was obtained as a colourless oil (25 mg, 58%).

δ_H (400 MHz, CD_3OD) 4.97 (d, *J* = 9.7 Hz, 1H, H-1), 3.90 – 3.82 (m, 1H, H-6), 3.77 (t, *J* = 10.0 Hz, 1H, H-2), 3.73 – 3.65 (m, 1H, H-6'), 3.52 – 3.41 (m, 1H, H-3), 3.36 – 3.33 (m, 2H, H-4, H-5) 2.27 – 2.13 (m, 2H, C=OC$\underline{H}_2$), 1.67 – 1.53 (m, 2H, CH_2), 1.40 – 1.27 (m, 6H, 3 x CH_2), 1.00 – 0.89 (m, 3H, CH_3)

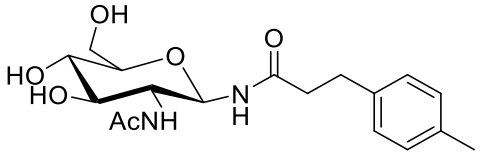
δ_C (101 MHz, CD_3OD) 176.8 (C=O), 174.3 (C=O), 80.4 (C-1), 79.8 (C-5), 76.3 (C-3), 71.8 (C-4), 62.7 (C-6), 56.1 (C-2), 37.3(C=OCH_2), 32.7 (CH_2), 29.9 (CH_2), 26.7 (CH_2), 23.6 (CH_2), 22.8 ($C\underline{H}_2CH_3$, NHAc CH_3), 14.4 ($CH_2C\underline{H}_3$)

HRMS: $C_{15}H_{28}N_2NaO_6$ requires 355.1845. ESI: Found 355.1841 $[M+Na]^+$

ν_{max} (film/cm^{-1}): 3309 (NH), 2949 (OH), 1654 (C=O)

***R*f:** 0.3 (CH_2Cl_2:CH_3OH 91:9)

2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-(*p*-tolyl)propanamide (146)



Prepared as per **Procedure 9** using **135** (50 mg, 0.13 mmol) and **140** (82 mg, 0.2 mmol) in DMF (5 mL) and following flash chromatography (CH_2Cl_2:CH_3OH – 9:1), the product was obtained as a colourless oil (28 mg, 59%).

δ_H (600 MHz, DMSO-d_6) 8.07 (dd, *J* = 9.2, 1.9 Hz, 1H, NH), 7.79 (d, *J* = 8.9 Hz, 1H, NH), 7.06 (s, 4H, Ar-CH), 4.84 – 4.78 (m, 1H, H-1), 3.65 (dd, *J* = 11.8, 1.9 Hz, 1H, H-6), 3.57 – 3.49 (m, 1H, H-2), 3.42 (dd, *J* = 11.8, 5.3 Hz, 1H, H-6), 3.33 – 3.27 (m, H-3), 3.12 – 2.99 (m, 2H, H-4, H-5), 2.77 – 2.65 (m, 2H, CH_2), 2.41 – 2.27 (m, 2H, CH_2), 2.24 (s, 3H, CH_3), 1.75 (s, 3H, CH_3)

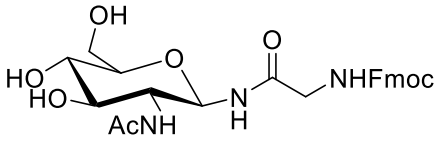
δ_C (151 MHz, DMSO-d_6) 171.7 (C=O), 169.8 (C=O), 138.1 (qC), 134.7 (qC), 128.8 (Ar-CH), 128.0 (Ar-CH), 78.9 (C-1), 78.7 (C-5), 74.5 (C-3), 70.5 (C-4), 60.9 (C-6), 54.6 (C-2), 37.1 (CH_2), 30.3 (CH_2), 22.8 (CH_3), 20.6 (CH_3)

HRMS: $C_{18}H_{26}NNa_2O_6$ requires 389.1689. ESI: Found 389.1708 $[M+Na]^+$

ν_{max} (film/cm^{-1}): 3301 (NH), 2981 (OH), 2981 (CH), 1655 (C=O)

***R*$_f$:** 0.32 (CH_2Cl_2:CH_3OH 9:1)

2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-Fmoc-Gly amide (147)



Prepared as per **Procedure 9** using **135** (50 mg, 0.13 mmol) and **143** (110 mg, 0.2 mmol) in DMF (5 mL). Deprotection conducted with excess triethylamine, and following flash chromatography (CH_2Cl_2:CH_3OH 92:8), the product was obtained as a colourless oil (41 mg, 64%).

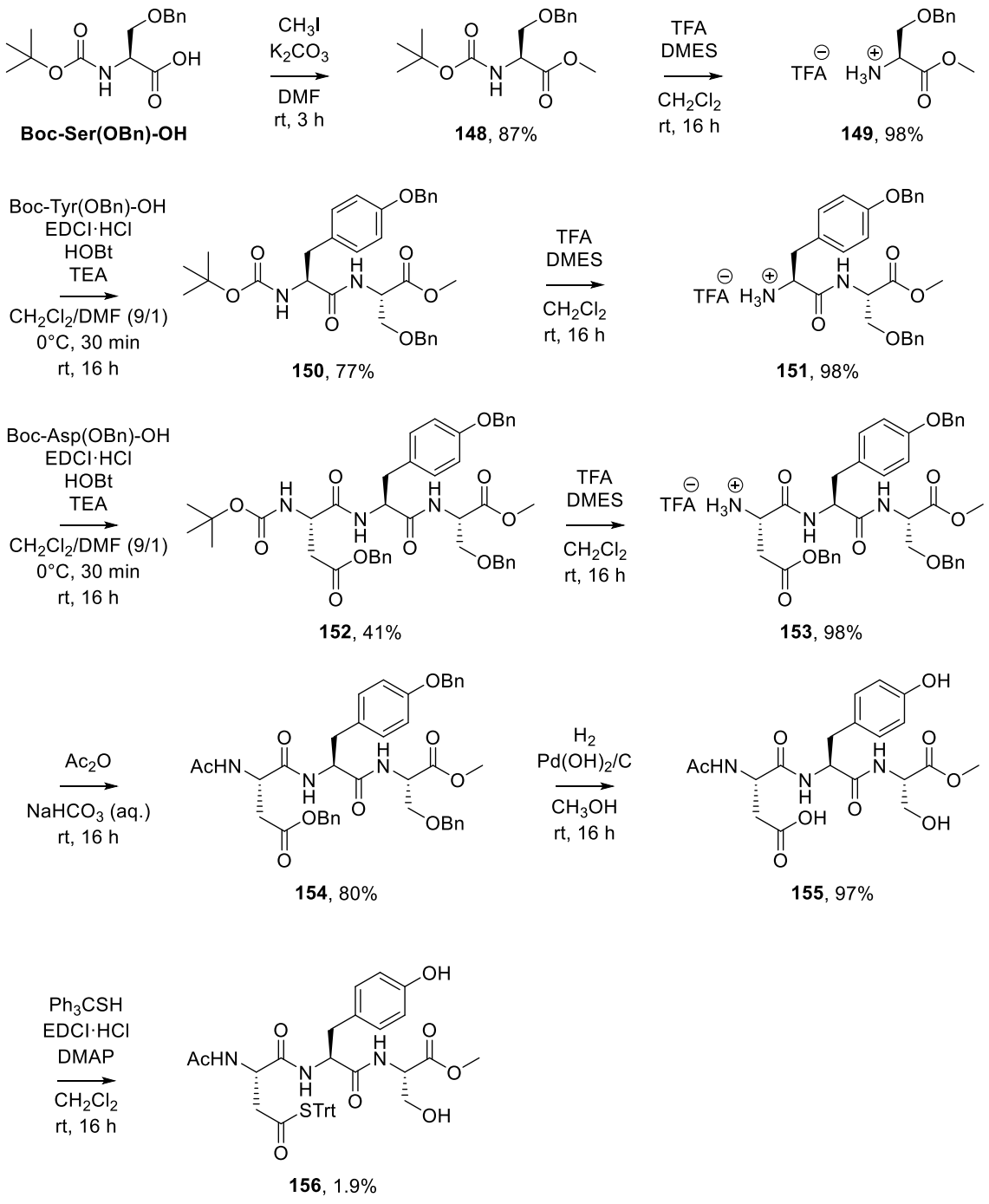
δ_H (600 MHz, CD_3OD) 7.82 (d, *J* = 7.5 Hz, 2H, Ar-CH), 7.71 (dd, *J* = 11.2, 7.5 Hz, 2H, Ar-CH), 7.41 (t, *J* = 7.5 Hz, 2H, Ar-CH), 7.34 (t, *J* = 7.5 Hz, 2H, Ar-CH), 4.97 (d, *J* = 9.7 Hz, 1H, H-1), 4.45 – 4.36 (m, 2H, Fmoc-CH_2), 4.28 (t, *J* = 7.0 Hz, 1H, Fmoc-CH), 3.89 – 3.66 (m, 4H, H-2, H-6, H-6', α-CH_2), 3.53 – 3.46 (m, 1H, H-3), 3.43 – 3.34 (m, 2H, H-4, H-5), 2.01 (d, *J* = 10.3 Hz, 3H, NHC=O$\underline{CH_3}$)

δ_C (151 MHz, CD_3OD) 174.7 (C=O), 173.1 (C=O), 159.1 (Gly-NH$\underline{C=O}$), 145.4 (qC), 145.2 (qC), 142.6 (qC), 142.6 (qC), 128.8 (Ar-CH), 128.8 (Ar-CH), 128.2 (Ar-CH), 126.3 (Ar-CH), 126.2 (Ar-CH), 120.9 (Ar-CH), 80.8 (C-1), 79.7 (C-5), 76.0 (C-3), 71.9 (C-4), 68.3 (Fmoc-CH_2), 62.6 (C-6), 55.9 (C-2), 47.9 (Fmoc-CH), 45.0 (α-CH_2), 22.8 (NHC=O$\underline{C}H_3$)

HRMS: $C_{14}H_{17}NNaO_5$ requires 302.1004. ESI: Found 302.1009 $[M+Na]^+$

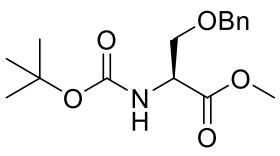
ν_{max} (film/cm^{-1}): 3600 (OH), 1867 (C=O), 1300 (CH)

***R*$_f$:** 0.34 (CH_2Cl_2:CH_3OH 9:1)



Scheme 2.15. Synthesis of *S*-trityl protected Interferon-γ tripeptide *S*-tritylthioester.

Boc-Ser(OBn)-OMe (148)[309]

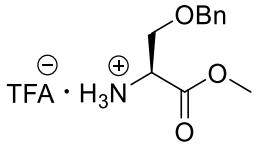


To a solution of Boc-Ser(OBn)-OH (1.53 g, 5.1812 mmol) in anhydrous DMF (10 mL) was added K_2CO_3 (1.07 g, 7.7718 mmol) and CH_3I (0.64 mL, 16.3624 mmol). The resulting suspension was stirred at rt for 3 h. Following the elapsed time, H_2O (20 mL) was added and the solution extracted with EtOAc (2 x 15 mL). The combined organic layers were washed with sat. aq. $NaHCO_3$ (20 mL), brine (20 mL), and dried over $MgSO_4$. The solution was filtered and concentrated *in vacuo* to give **148** as a pale yellow oil (1.4 g, 87%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, CD_3OD) 7.41 – 7.26 (m, 5H, Ar-CH), 4.62 – 4.48 (m, 2H, OC$\underline{H_2}$Bn), 4.40 (t, *J* = 4.5 Hz, 1H, α-CH), 3.82 (dd, *J* = 9.7, 5.0 Hz, 1H, β-CH_2), 3.75 (s, 3H, OCH_3), 3.74 – 3.69 (m, 1H, β-CH_2), 1.48 (s, 9H, ^{t}Bu CH_3)

HRMS: $C_{16}H_{23}NNaO_5$ requires 332.1474. ESI: Found 332.1473 $[M+Na]^+$

H-Ser(OBn)-OMe (149)[310]

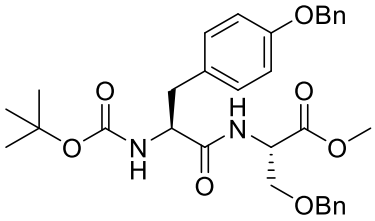


To a solution of **148** (1.31 g, 5.983 mmol) in anhydrous CH_2Cl_2 (4 mL) was added ethyldimethylsilane (DMES) (15.8 mL, 119.7 mmol). TFA (2 mL) was added, and the resulting solution stirred at rt for 16 h. Following the elapsed time, toluene (10 mL) was added and the solution was concentrated *in vacuo*. Removal of the solvent furnished **149** as a white solid (1.8 g, 98%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, CD_3OD) 7.40 – 7.25 (m, 5H, Ar-CH), 4.62 (d, *J* = 12.1 Hz, 1H, OC$\underline{H_2}$Bn), 4.54 (d, *J* = 12.1 Hz, 1H, OC$\underline{H_2}$Bn), 4.30 – 4.24 (m, 1H, α-CH), 3.94 – 3.87 (m, 1H, β-CH_2), 3.84 – 3.80 (m, 4H, β-CH_2, OCH_3)

HRMS: $C_{11}H_{16}NO_3$ requires 210.1130. ESI: Found 210.1123 $[M+H]^+$

Boc-Tyr(OBn)-Ser(OBn)-OMe (150)



To Boc-Tyr(OBn)-OH (2.07 g, 5.57 mmol) in anhydrous CH_2Cl_2:DMF (9/1 v/v) was added EDCI·HCl (1.18 g, 6.13 mmol) and HOBt (828 mg, 6.13 mmol), and the resulting solution stirred under Ar at 0 °C for 30 min. Upon completion of this pre-activation, solution of **149** (1.71 g, 5.57 mmol) and triethylamine (1.56 mL, 11.15 mmol) in anhydrous CH_2Cl_2:DMF (9:1 v/v) was added dropwise, and the resulting solution stirred at rt for 16 h. Following the elapsed time, H_2O (20 mL) was added, and the reaction mixture was extracted with CH_2Cl_2 (2 x 15 mL). The combined organic layers were washed with brine (2 x 20 mL), dried over $MgSO_4$, and filtered. The solution was concentrated *in vacuo* to yield a yellow oily solid. The residue was purified by column chromatography (EtOAc:hexanes 6:4) to give **150** as a pale yellow solid (2.4 g, 77%).

δ_H (600 MHz, CD_3OD) 7.48 – 6.88 (m, 14H, Ar-CH), 5.06 (s, 2H, Tyr-O$\underline{CH_2}$Bn), 4.68 (t, *J* = 4.1 Hz, 1H, Ser α-CH), 4.57 (d, *J* = 12.0 Hz, 1H, Ser-O$\underline{CH_2}$Bn), 4.50 (d, *J* = 12.0 Hz, 1H, Ser-O$\underline{CH_2}$Bn), 4.35 (dd, *J* = 9.1, 5.3 Hz, 1H, Tyr α-CH), 3.87 (dd, *J* = 9.7, 4.6 Hz, 1H, Ser β-CH_2), 3.72 (s, 4H, Ser β-CH_2, OCH_3), 3.07 (dd, *J* = 14.0, 5.4 Hz, 1H, Tyr β-CH_2), 2.79 (dd, *J* = 14.0, 9.1 Hz, 1H, Tyr β-CH_2), 1.38 (s, 9H, ^{t}Bu CH_3)

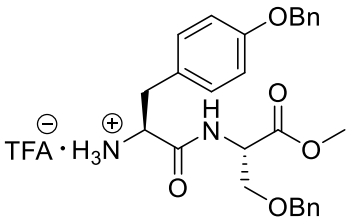
δ_C (151 MHz, CD_3OD) 174.4 (C=O), 171.7 (C=O), 159.1 (Ar qC), 139.2 (Ar qC), 138.9 (Ar qC), 131.5 (Ar qC), 130.8 (Ar qC), 129.5 (Ar CH), 129.5 (Ar CH), 129.4 (Ar CH), 128.9 (Ar CH), 128.8 (Ar CH), 128.5 (Ar CH), 115.9 (Ar CH), 80.7 (^{t}Bu qC), 74.2 (Ser-O$\underline{C}H_2$Bn), 71.0 (Tyr-O$\underline{C}H_2$Bn), 70.5 (Ser β-CH_2), 57.3 (Tyr α-CH), 54.1 (Ser α-CH), 52.9 (OCH_3), 38.4 (Tyr β-CH_2), 28.7 (^{t}Bu CH_3)

HRMS: $C_{32}H_{38}N_2NaO_7$ requires 585.2577. ESI: Found 585.2572 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3328 (NH), 1648 (C=O)

***R*$_f$:** 0.28 (EtOAc:Hexanes 6:4)

H-Tyr(OBn)-Ser(OBn)-OMe (151)



Prepared using the same procedure previously described for **149**, with **150** (400 mg, 0.71 mmol), ethyldimethylsilane (1.9 mL, 14.22 mmol), and TFA (2 mL) in anhydrous CH_2Cl_2 (6 mL). This gave **151** as a yellow oil (390 mg, 98%). This isolated compound was used without further purification.

δ_H (400 MHz, CD_3OD) 7.40 – 7.10 (m, 14H, Ar-CH), 5.07 (d, *J* = 3.8 Hz, 2H, Tyr-O$\underline{C}H_2$Bn), 4.70 (dd, *J* = 4.8, 3.6 Hz, 1H, Ser α-CH), 4.56 (d, *J* = 12.0 Hz, 1H, Ser-O$\underline{C}H_2$Bn), 4.47 (d, *J* = 12.0 Hz, 1H, Ser-O$\underline{C}H_2$Bn), 4.21 – 4.07 (m, 1H, Tyr α-CH), 3.87 (dd, *J* = 9.8, 4.8 Hz, 1H, Ser β-CH_2), 3.70 (s, 4H, Ser β-CH_2, OCH_3), 3.08 (dd, *J* = 14.6, 8.0 Hz, 1H, Tyr β-CH_2), 2.97 (dd, *J* = 14.6, 8.0 Hz, 1H, Tyr β-CH_2)

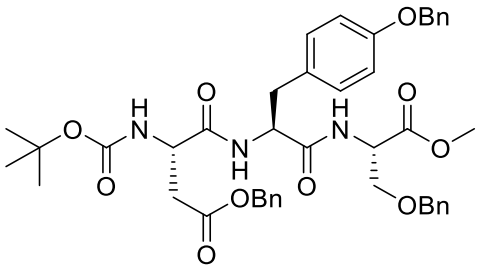
δ_C (101 MHz, CD_3OD) 171.3 (C=O), 169.9 (C=O), 159.9 (Ar qC), 139.0 (Ar qC), 138.7 (Ar qC), 131.7 (Ar CH), 131.6 (Ar CH), 129.5 (Ar CH), 129.4 (Ar CH), 128.9 (Ar CH), 128.9 (Ar CH), 128.5 (Ar CH), 127.6 (Ar CH), 116.5 (Ar CH), 116.5 (Ar CH), 74.3 (Ser O$\underline{C}H_2$Bn), 70.9 (Tyr O$\underline{C}H_2$Bn), 70.4 (Ser β-CH_2), 55.6 (Tyr α-CH), 54.4 (Ser α-CH), 53.0 (OCH_3), 37.7 (Tyr β-CH_2)

HRMS: $C_{27}H_{31}N_2O_5$ requires 463.2233. APCI: Found 463.2230 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 3319 (NH), 1647 (C=O)

***R*$_f$:** 0.1 (EtOAc:Hexanes 1:1)

Boc-Asp(OBn)-Tyr(OBn)-Ser(OBn)-OMe (152)



Prepared using the same procedure previously described for **150**. Pre-activation took place as before with Boc-Asp(OBn)-OH (213 mg, 0.66 mmol), EDCI·HCl (139 mg, 0.72 mmol), and HOBt (98 mg, 0.72 mmol). Reaction then took place with **151** (395 mg, 0.6588 mmol) and triethylamine (0.18 mL, 1.32 mmol) over 16 h at rt. Column chromatography (EtOAc:hexanes 1:1) gave **152** as a white powder (207 mg, 41%).

δ_H (400 MHz, CD_3OD) 7.59 – 6.84 (m, 19H, Ar CH), 5.11 (s, 2H, Tyr OCH_2Bn), 5.04 (d, *J* = 7.0 Hz, 2H, Asp OCH_2Bn), 4.72 – 4.44 (m, 5H, Asp α-CH, Ser OCH_2Bn, Tyr α-CH, Ser α-CH), 3.84 (dd, *J* = 9.8, 4.9 Hz, 1H, Ser β-CH_2), 3.77 – 3.65 (m, 4H, Ser β-CH_2, OCH_3), 3.10 (dd, *J* = 14.1, 5.6 Hz, 1H, Tyr β-CH_2), 2.97 – 2.76 (m, 2H, Tyr β-CH_2, Asp β-CH_2), 2.65 (dd, *J* = 16.7, 7.8 Hz, 1, Asp β-CH_2), 1.51 – 1.34 (m, 9H, ^{t}Bu CH_3)

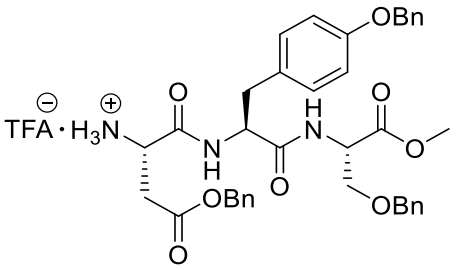
δ_C (101 MHz, CD_3OD) 173.6 (C=O), 173.2 (C=O), 171.7 (C=O), 159.2 (C=O), 139.2 (Ar qC), 138.8 (Ar qC), 137.3 (Ar qC), 131.6 (Ar qC), 131.4 (Ar qC), 131.2 (Ar qC), 130.2 (Ar CH), 129.5 (Ar CH), 129.5 (Ar CH), 129.4 (Ar CH), 129.4 (Ar CH), 129.3 (Ar CH), 129.2 (Ar CH), 128.9 (Ar CH), 128.8 (Ar CH), 128.8 (Ar CH), 128.6 (Ar CH), 128.5 (Ar CH), 128.5 (Ar CH), 128.3 (Ar CH), 128.0 (Ar CH), 116.1 (Ar CH), 115.9 (Ar CH), 74.2 (Ser OCH_2Bn), 71.0 (Asp OCH_2Bn), 70.4 (Ser β-CH_2), 67.7 (Tyr OCH_2Bn), 55.7 (Tyr α-CH), 54.2 (Ser α-CH), 52.9 (OCH_3), 38.0 (Tyr β-CH_2), 28.7 (Asp β-CH_2), 28.6 (^{t}Bu CH_3).

HRMS: $C_{43}H_{49}N_3NaO_{10}$ requires 790.3316. ESI: Found 790.3316 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3314 (NH), 1647 (C=O)

***R*f:** 0.31 (EtOAc:Hexanes 1:1)

H-Asp(OBn)-Tyr(OBn)-Ser(OBn)-OMe (153)



Prepared using the same procedure previously described for **149**, with **152** (606 mg, 0.78 mmol), ethyldimethylsilane (2 mL, 15.78 mmol), and TFA (2 mL) in anhydrous CH_2Cl_2 (6 mL). This gave **153** as a yellow oil (587 mg, 98%). The isolated compound was used without further purification.

δ_H (400 MHz, CD_3OD) 7.59 – 6.82 (m, 16H, Ar-CH), 5.25 – 5.12 (m, 2H, Asp OCH_2Bn), 5.03 (s, 2H, Tyr OCH_2Bn), 4.71 (dd, *J* = 9.0, 5.4 Hz, 1H, Tyr α-CH), 4.64 (dd, *J* = 4.8, 3.8 Hz, 1H, Ser α-CH), 4.54 (d, *J* = 12.0 Hz, 1H, Ser OCH_2Bn), 4.46 (d, *J* = 12.0 Hz, 1H, Ser OCH_2Bn), 4.18 (dd, *J* = 9.0, 4.0 Hz, 1H, Asp α-CH), 3.82 (dd, *J* = 9.8, 4.9 Hz, 1H, Ser β-CH_2), 3.69 (s, 4H, Ser β-CH_2), 3.18 – 3.01 (m, 2H, Tyr β-CH_2, Asp β-CH_2), 2.92 – 2.82 (m, 2H, Tyr β-CH_2, Asp β-CH_2)

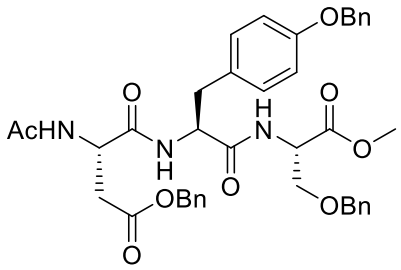
δ_C (101 MHz, CD_3OD) 173.2 (C=O), 171.7 (C=O), 171.2 (C=O), 168.9 (C=O), 159.3 (Ar-qC), 139.1 (Ar-qC), 138.8 (Ar-qC), 136.9 (Ar-qC), 131.4 (Ar-CH), 130.2 (Ar-CH), 129.6 (Ar-CH), 129.5 (Ar-CH), 129.5 (Ar-CH), 129.4 (Ar-CH), 128.9 (Ar-CH), 128.9 (Ar-CH), 128.5 (Ar-CH), 116.0 (Ar-CH), 74.2 (Ser O$\underline{C}H_2Bn$), 71.0 (Tyr O$\underline{C}H_2Bn$), 70.4 (Ser β-CH_2), 68.4 (Asp O$\underline{C}H_2Bn$), 56.3 (Tyr α-CH), 54.2 (Ser α-CH), 52.9 (OCH_3), 50.6 (Asp α-CH), 38.0 (Tyr β-CH_2), 36.4 (Asp β-CH_2)

HRMS: $C_{38}H_{42}N_3O_8$ requires 668.2972. APCI: Found 668.2966 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 1643 (C=O)

***R*f:** 0.1 (EtOAc:Hexanes 1:1)

Ac-Asp(OBn)-Tyr(OBn)-Ser(OBn)-OMe (154)



To a stirred solution of **153** (590 mg, 0.78 mmol) sat. aq. $NaHCO_3$ (5 mL) was added Ac_2O (0.4 mL, 3.91 mmol) and the resulting solution stirred at rt for 16 h. Following the elapsed time, the reaction mixture was acidified to pH 2 with HCl (3 M, aq.) and the aqueous phase extracted with EtOAc (2 x 15 mL). The combined organic layers were dried over $MgSO_4$, filtered, and the solution concentrated *in vacuo* to give **154** as a white solid (441 mg, 80%). The crude compound was used without further purification.

δ_H (600 MHz, DMSO) 7.46 – 7.09 (m, 14H, Ar-CH), 6.97 – 6.82 (m, 2H, Ar-CH), 5.21 – 5.01 (m, 4H, Asp OCH_2Bn, Tyr OCH_2Bn), 4.67 – 4.60 (m, 1H, Asp α-CH), 4.58 – 4.45 (m, 4H, Ser OCH_2Bn, Ser α-CH, Tyr α-CH), 3.74 (ddd, *J* = 9.8, 6.8, 5.4 Hz, 1H, Ser β-CH_2), 3.66 – 3.59 (m, 4H, Ser β-CH_2, OCH_3), 3.05 – 2.71 (m, 3H, Tyr β-CH_2, Asp β-CH_2), 1.77 (s, 3H, Ac CH_3)

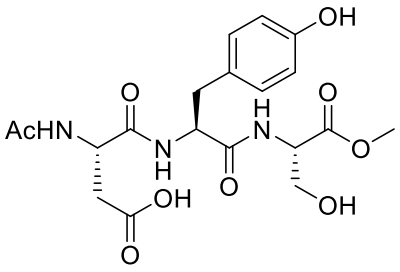
δ_C (151 MHz, DMSO) 171.0 (C=O), 170.4 (C=O), 170.1 (C=O), 170.0 (C=O), 169.3 (C=O), 156.9 (Ar-qC), 137.8 (Ar-qC), 137.2 (Ar-qC), 136.0 (Ar-qC), 130.3 (Ar-CH), 130.2 (Ar-CH), 129.5 (Ar-qC), 128.5 (Ar-CH), 128.4 (Ar-CH), 128.4 (Ar-CH), 128.4 (Ar-CH), 128.3 (Ar-CH), 128.3 (Ar-CH), 128.2 (Ar-CH), 127.9 (Ar-CH), 127.8 (Ar-CH), 127.8 (Ar-CH), 127.6 (Ar-CH), 127.6 (Ar-CH), 127.6 (Ar-CH), 127.5 (Ar-CH), 114.4 (Ar-CH), 114.3 (Ar-CH), 72.2 (Ser OCH_2Bn), 69.1 (Tyr OCH_2Bn), 69.0 (Ser β-CH_2), 66.4 (Asp OCH_2Bn), 53.7 (Tyr α-CH), 52.3 (Ser α-CH), 52.1 (OCH_3), 52.0 (Asp α-CH), 36.6 (Tyr β-CH_2), 35.9 (Asp β-CH_2), 22.4 (Ac CH_3)

HRMS: $C_{40}H_{44}N_3O_9$ requires 710.3078. APCI: Found 710.3074 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 3283 (NH), 1640 (C=O)

***R*$_f$:** 0.4 (EtOAc:Hexanes 1:1)

Ac-Asp-Tyr-Ser-OMe (155)



A suspension of **154** (750 mg, 1.06 mmol) and $Pd(OH)_2/C$ in CH_2Cl_2:CH_3OH (1:1, 10 mL) was degassed *via* argon balloon for 15 minutes. The suspension was placed under a H_2 atmosphere at rt for 16 h. Following the elapsed time the suspension was filtered through Celite® and the solution concentrated *in vacuo* to give **155** as a colourless oil (451 mg, 97%), which was used without further purification.

δ_H (600 MHz, DMSO-d_6) 7.11 – 6.57 (m, 4H, Ar-CH), 4.54 – 4.42 (m, 2H, Asp α-CH, Ser α-CH), 4.34 (dt, *J* = 7.6, 4.9 Hz, 1H, Tyr α-CH), 3.76 – 3.68 (m, 1H, Ser β-CH_2), 3.63 (s, 4H, OCH_3, Ser β-CH_2), 2.95 – 2.86 (m, 1H, Tyr β-CH_2), 2.71 – 2.65 (m, 1H, Tyr β-CH_2), 2.61 – 2.56 (m, 1H, Asp β-CH_2), 2.39 – 2.32 (m, 1H, Asp β-CH_2), 1.80 (s, 3H, OCH_3)

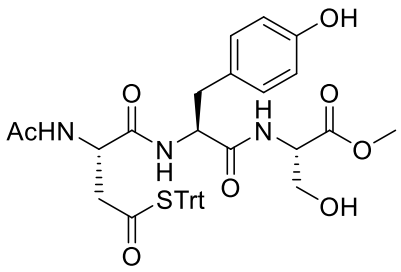
δ_C (151 MHz, DMSO-d_6) 171.7 (C=O), 171.0 (C=O), 170.9 (C=O), 170.4 (C=O), 169.5 (C=O), 155.8 (Tyr qC-OH), 130.2 (Ar-CH), 130.1 (Ar-CH), 127.4 (Tyr Ar-qC), 114.9 (Ar-CH), 114.8 (Ar-CH), 61.2 (Ser β-CH_2), 54.8 (Tyr α-CH), 53.7 (Ser α-CH), 51.9 (OCH_3), 49.4 (Asp α-CH) 36.6 (Tyr β-CH_2), 35.7 (Asp β-CH_2), 22.4 (Ac CH_3)

HRMS: $C_{19}H_{24}N_3O_9$ requires 438.1513. ESI: Found 438.1519 $[M-H]^-$

ν_{max} (ATR/cm^{-1}): 3288 (NH), 1644 (C=O)

***R*f:** 0.1 (EtOAc:Hexanes 1:1)

Ac-Asp(*S*Trt)-Tyr-Ser-OMe (156)



Prepared using **Procedure 2** with **155** (673 mg, 1.53 mmol), EDCI·HCl (352 mg, 1.84 mmol), DMAP (19 mg, 0.15 mmol) and Ph_3CSH (508 mg, 1.84 mmol) in anhydrous CH_2Cl_2 (10 mL) at rt for 16 h. Following the elapsed time, the solution was concentrated *in vacuo* and the residue purified by flash column chromatography (EtOAc) to remove the red polymeric material, and furnish **156** as a white solid (20 mg, 1.9%).

δ_H (600 MHz, CD_3OD) 7.28 – 7.16 (m, 15H, Trt Ar -CH), 7.04 – 6.99 (m, 2H, Tyr Ar-CH), 6.69 – 6.65 (m, 2H, Tyr Ar-CH), 4.65 (dd, *J* = 9.0, 4.9 Hz, 1H, Asp α-CH), 4.55 (dd, *J* = 8.6, 5.5 Hz, 1H, Tyr α-CH), 4.47 (t, *J* = 4.5 Hz, 1H, Ser α-CH)), 3.86 – 3.79 (m, 1H, Ser β-CH_2), 3.78 – 3.70 (m, 4H, Ser β-CH_2, OCH_3), 3.06 (dd, *J* = 14.1, 5.5 Hz, 1H, Tyr β-CH_2), 2.96 (dd, *J* = 15.9, 4.9 Hz, 1H, Asp β-CH_2), 2.85 – 2.79 (m, 2H, Tyr β-CH_2, Asp β-CH_2), 1.89 (s, 3H, Ac CH_3)

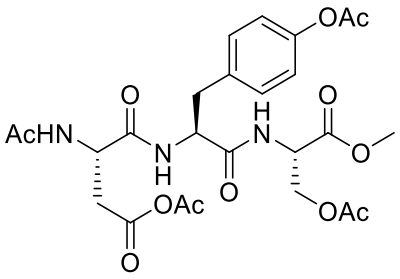
δ_C (151 MHz, CD_3OD) 195.3, (C=OS) 173.3 (C=O), 173.2 (C=O), 172.2 (C=O), 171.9 (C=O), 157.3 (Ar-qC), 145.0 (Ar-CH), 131.5 (Ar-CH), 131.0 (Ar-CH), 131.0 (Ar-CH), 129.8 (Ar-CH), 128.8 (Ar-CH), 128.7 (Ar-CH), 128.7 (Ar-CH), 128.2 (Ar-CH), 116.3 (Ar-CH), 116.2 (Ar-CH), 71.8 (Trt Ar-qC), 62.8 (Ser β-CH_2), 56.3 (Tyr α-CH), 56.1 (Ser α-CH), 52.8 (OCH_3), 51.4 (Asp α-CH), 45.2 (Asp β-CH_2), 37.8 (Tyr β-CH_2), 22.6 (Ac CH_3)

HRMS: $C_{38}H_{38}N_3NaO_8S$ requires 720.2356. ESI: Found 720.2359 $[M+Na]^+$

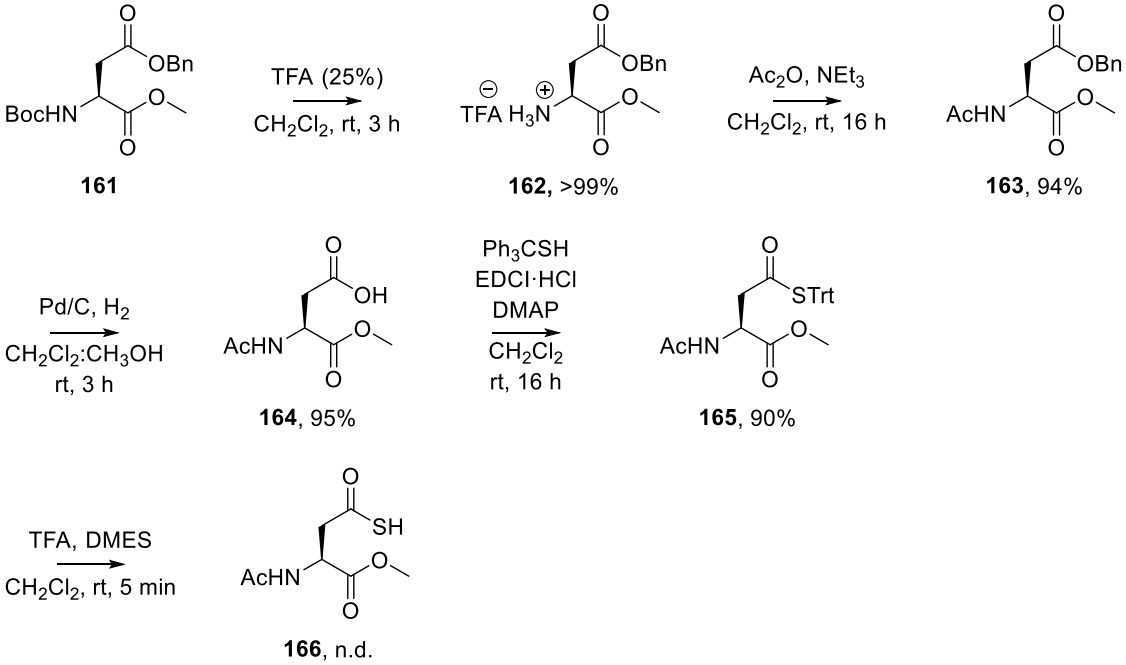
ν_{max} (ATR/cm^{-1}): 2980 (Trt), 1658 (C=O)

***R*f:** 0.23 (EtOAc)

Ac-Asp-Tyr(OAc)-Ser(OAc)-OMe (157)

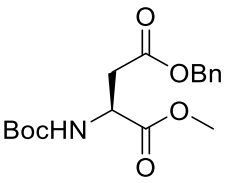


To a solution of **155** (87 mg, 0.198 mmol) in anhydrous CH_2Cl_2/DMF (6:1, 3.5 mL) was added pyridine (0.24 mL, 2.97 mmol) and acetic anhydride (0.19 mL, 1.98 mmol). The resulting solution was stirred at rt for 16 h, after which H_2O (0.5 mL) and toluene (2 mL) were added, and the solution concentrated *in vacuo*. The residue was triturated with heptane (10 mL), and the crude **157** without isolation to the next step.



Scheme 2.18. Synthesis of aspartic *S*-trityl protected thioacid.

Boc-Asp(OBn)-OMe (161)[311]



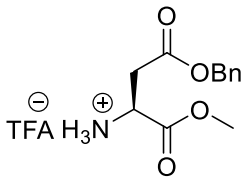
Prepared using **Procedure 1** using Boc-Asp(OBn)-OH (1.399 g, 4.33 mmol), K_2CO_3 (897 mg, 6.49 mmol) and iodomethane (0.55 mL, 8.65 mmol). This gave the product **161**

as a yellow oil (1.38 g, 95%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.39 – 7.28 (m, 5H, Ar-CH), 5.48 (d, *J* = 8.0 Hz, 1H, NH), 5.17 – 5.08 (m, 2H, OCH_2Ph), 4.59 – 4.57 (m, 1H, α-CH) 3.69 (s, 3H, CH_3), 3.04 (dd, *J* = 17.0, 4.4 Hz, 1H, $CHCH_2$), 2.86 (dd, *J* = 17.0, 4.4 Hz, 1H, $CHCH_2$), 1.44 (s, 3H, CH_3)

HRMS: $C_{17}H_{23}NNaO_6$ requires 360.1423. ESI: Found 360.1425 $[M+Na]^+$

H-Asp(OBn)-OMe (162)[311]



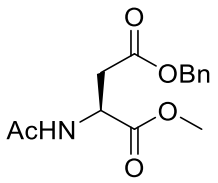
To a solution of **161** (800 mg, 2.3713 mmol) in anhydrous CH_2Cl_2 (4 mL) was added TFA (2 mL) and the solution was stirred at rt for 3 h. Following completion of the reaction, the solvent was removed i*n vacuo* to yield the **162** as a pale yellow solid (634 mg, 99%). The compound was used without further purification, and was in good agreement with the literature.

M.p.: 67-69 °C (from CH_2Cl_2). Literature value: 67-68 °C.

δ_H (400 MHz, $CDCl_3$) 8.65 (s, 3H, NH_3^+), 7.27-7.06 (m, 5H, Ar-CH), 5.04 – 5.02 (m, 2H, OCH_2Ph), 4.61 – 4.59 (m, 1H, CH), 3.54 (s, 3H, OCH_3), 3.2 – 3.22 (m, 2H, $CHCH_2$)

HRMS: $C_{12}H_{16}NO_4$ requires 238.1079. ESI: Found 238.1074 $[M+H]^+$

Ac-Asp(OBn)-OMe (163)[312]



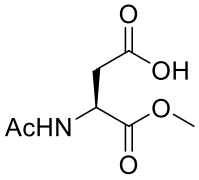
To a solution of H-Asp(OBn)-OMe **162** (1.81 g, 5.398 mmol) in anhydrous CH_2Cl_2 (15 mL) was added NEt_3 (1.5 mL, 10.8 mmol) followed by acetic anhydride (1.02 mL, 10.8 mmol). The solution was stirred at rt for 16 h. After the elapsed time the reaction was

quenched with H_2O (100 mL), and extracted with CH_2Cl_2 (2 x 100 mL). The combined organic layers were washed with 1 M aq. HCl solution (50 mL), brine (50 mL), dried over $MgSO_4$ and filtered. The solvent was removed *in vacuo* to give the product **163** as a yellow oil (1.18 g, 78%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.34 – 7.28 (m, 5H, Ar-CH), 5.13 – 5.11 (m 2H, OCH_2Ph), 4.89 – 4.83 (m, 1H, α-CH), 3.67 (s, 3H, OCH_3), 3.10 – 2.87 (m, 1H, $CHCH_2$), 2.91 – 2.81 (m, 1H, $CHCH_2$), 1.97 (s, 3H, CH_3)

HRMS: $C_{14}H_{17}NNaO_5$ requires 302.1004. ESI: Found 302.1005 $[M+Na]^+$

Ac-Asp(OH)-OMe (164)[313]



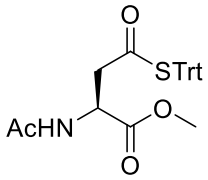
To a solution of **163** (600 mg, 2.15 mmol) in degassed EtOAc (10 mL) was added $Pd(OH)_2/C$ (23 mg, 0.215 mmol). The flask was placed under a H_2 atmosphere (balloon) and stirred at rt for 3 h, refiling the balloon as necessary. Following completion of the reaction as indicated by TLC, the solution was filtered through Celite® and the solvent removed *in vacuo*. The residue was purified by column chromatography (EtOAc:hexanes 7:3) to yield the product as a colourless oil (368 mg, 91%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) δ 4.58 (s, 1H, α-CH), 3.75 (s, 3H, OCH_3), 3.07 (dd, J = 17.2, 4.5 Hz, 1H, CH_2COOH), 2.90 (dd, J = 17.2, 4.5 Hz, 1H, CH_2COOH), 2.00 (s, 3H, CH_3)

HRMS: $C_7H_{11}NNaO_5$ requires 212.0535. ESI: Found 212.0529 $[M+Na]^+$

***R*f:** 0.2 (EtOAc:Hexanes 7:3)

Ac-Asp(*S*Trt)-OMe (165)



Prepared as per **Procedure 2** using **164** (492 mg, 2.601 mmol), triphenylmethanethiol (791 mg, 2.861 mmol) and DMAP (32 mg, 0.2601 mmol) in anhydrous CH_2Cl_2 (10 mL), with EDCI·HCl (549 mg, 2.861 mmol). Purification of the crude residue by column chromatography (EtOAc:hexanes 2:8) gave **165** as a colourless oil (544 mg, 47%).

δ_H (400 MHz, CD_3OD) 7.41 – 7.11 (m, 15H, Trt CH), 4.43 (t, *J* = 6.4 Hz, 1H, α-CH), 3.70 (s, 3H, OCH_3), 3.04 (t, *J* = 6.3 Hz, 2H, β-CH_2), 1.46 (s, 3H, Ac CH_3).

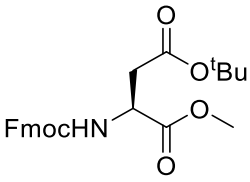
δ_C (101 MHz, CD_3OD) 195.3 (TrtS-C=O), 145.0 (Trt Ar-qC), 131.0 (Trt Ar-CH), 131.0 (Trt Ar-CH), 128.8 (Trt Ar-CH), 128.3 (Trt Ar-CH), 53.0 (OCH_3), 51.8 (α-CH), 45.6 (β-CH_2), 28.7 (Ac CH_3).

HRMS: $C_{26}H_{25}NNaO_4S$ requires 470.1402. ESI: Found 470.1404 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2956 (Ar-CH), 1721 (S-C=O), 1689 (C=O)

***R*f:** 0.32 (EtOAc:Hexanes 2:8)

Fmoc-Asp(O^{t}Bu)-OMe (168)[314]



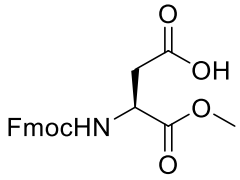
Prepared as per **Procedure 1** using Fmoc-Asp(O^{t}Bu)-OH (1.00 g, 2.43 mmol), K_2CO_3 (500 mg, 3.62 mmol) and iodomethane (0.3 mL, 4.82 mmol). The product presented as white needles (997 mg, 96%), and was used without further purification. The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.77 (d, *J* = 7.4 Hz, 2H, Fmoc-ArCH), 7.61 (s, 2H, Fmoc-ArCH), 7.40 (t, *J* = 7.4 Hz, 2H, Fmoc-ArCH), 7.32 (t, *J* = 7.4 Hz, 2H, Fmoc-ArCH), 4.62 (m, 1H α-CH), 4.39 (m, 2H, Fmoc-CH_2), 4.25 (t, *J* = 7.1 Hz, 1H, Fmoc-CH), 3.78 (s, 3H, C-

terminus CH_3), 2.95 (dd, *J* = 16.8, 4.2 Hz, 1H, β-CH_2), 2.82 – 2.72 (m, 1H, β-CH_2), 1.46 (s, 9H, ^{t}Bu CH_3)

HRMS: $C_{24}H_{28}NO_6$ requires 426.1917. ESI: Found 426.1902 $[M+H]^+$

Fmoc-Asp(OH)-OMe (169)[315]



To a solution of Fmoc-Asp(O^{t}Bu)-OMe **168** (797 mg, 1.873 mmol) in anhydrous CH_2Cl_2 (5 mL) was added triethylsilane (0.98 mL, 6.182 mmol), followed by TFA (5.88 mL, 76.80 mmol). The solution was stirred at rt for 3 h, after which it was quenched by addition of H_2O (60 mL). The organic phase was separated, washed with H_2O (3 x 30 mL), dried over $MgSO_4$ and filtered. The solution was concentrated *in vacuo*, and purification of a residue by column chromatography (CH_2Cl_2:Et_2O 9:1) gave **169** as a colourless oil (650 mg, 94%). The isolated compound was in good agreement with the literature.

δ_H (600 MHz, $CDCl_3$) 7.76 (d, *J* = 7.4 Hz, 2H, Fmoc Ar-C5), 7.59 (d, *J* = 7.4 Hz, 2H, Fmoc Ar-C2), 7.40 (t, *J* = 7.4 Hz, 2H, Fmoc Ar-C4), 7.31 (t, *J* = 7.4 Hz, 2H, Fmoc Ar-C3), 4.70-4.61 (m, 1H, α-CH), 4.46-4.36 (m, 2H, Fmoc-CH_2), 4.23 (app. t, *J* = 7.02 Hz, 1H, Fmoc-CH), 3.79 (s, 3H, C-term CH_3), 3.11 (ddd, *J* = 16.0 Hz, 7.2 Hz, 7.1 Hz, 2H, β-CH_2)

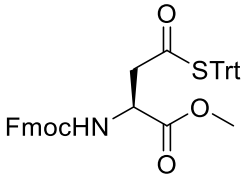
δ_C (151 MHz, $CDCl_3$) 174.6 (C=OOH), 171.0 (C=OOMe), 156.0 (Fmoc C=O), 143.7 (Fmoc Ar-C1), 141.3 (Fmoc Ar-C6), 127.8 (Fmoc Ar-C4), 127.1 (Fmoc Ar-C3), 125.1 (Fmoc Ar-C5), 120.0 (Fmoc Ar-C2), 67.4 (Fmoc-CH_2) 53.0 (α-CH), 50.2 (C-terminus CH_3), 47.1 (Fmoc-CH), 36.2 (β-CH_2)

HRMS: $C_{20}H_{19}NNaO_6$ requires 392.1110. ESI: Found 392.1095 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 3329 (OH), 1754, 1721, 1681 (C=O)

***R*$_f$:** 0.3 (CH_2Cl_2:Et_2O 9:1)

Fmoc-Asp(*S*Trt)-OMe (170)



Prepared as per **Procedure 2** using **169** (100 mg, 0.27 mmol), triphenylmethanethiol (82.3 mg, 0.30 mmol) and DMAP (3.3 mg, 0.027 mmol) in anhydrous CH_2Cl_2 (2 mL), and EDCI·HCl (57.1 mg, 0.230 mmol). Crude residue was subject to chromatographic purification (EtOAc:Hexanes 20:80). This yielded **170** as a pale yellow oil (138 mg, 81%).

δ_H (400 MHz, $CDCl_3$) 7.76 (dd, *J* = 6.8, 6.1 Hz, 2H, Fmoc-ArCH), 7.59 (d, *J* = 7.5 Hz, 2H, Fmoc-ArCH), 7.41 (dt, *J* = 11.8, 6.0 Hz, 2H, Fmoc-ArCH), 7.33 – 7.20 (m, 17H, Trt-CH Fmoc-ArCH) 5.55 (d, *J* = 8.5 Hz, 1H, NH), 4.56 (m, 1H, α-CH), 4.40 (m, 2H, Fmoc-CH_2), 4.22 (t, *J* = 7.0 Hz, 1H, Fmoc-CH), 3.68 (s, 3H, C-terminal CH_3), 3.25 (dd, *J* = 16.4, 4.8 Hz, 1H, β-CH_2), 3.06 (dd, *J* = 16.4, 4.5 Hz, 1H, β-CH_2)

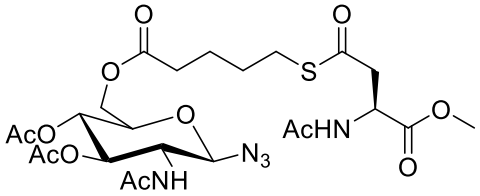
δ_C (101 MHz, $CDCl_3$) 195.1 (S-C=O), 170.8 (C=OOCH_3), 156.0 (Fmoc-C=O), 143.8 (ArC), 143.5 (ArC), 141.5 (ArC), 129.9 (ArCH), 128.0 (ArCH), 127.9 (ArCH), 127.4 (ArCH), 127.2 (ArCH), 125.3 (ArCH), 120.1 (ArCH), 71.4, 67.3 (Fmoc-CH_2), 60.5 (TrtC), 52.9 (α-CH) , 51.0 ($COOCH_3$), 47.3 (Fmoc-CH), 45.0 (β-CH_2)

HRMS: $C_{39}H_{33}NNaO_5S$ requires 650.1977. ESI: Found 650.1979 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2956 (Ar-CH), 1721 (S-C=O), 1689 (C=O)

***R*f:** 0.4 (EtOAc:Hexanes 2:8)

2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-Ac-L-Asp(OMe)thio-pentanoate-β-D-glucopyranosyl azide (172)



Prepared as per **Procedure 3** using **165** (0.54 g, 1.21 mmol) in CH_2Cl_2 (15 mL) to give the crude thioacid which was then immediately subjected to **Procedure 4** using **127** (0.35

g, 0.86 mmol) in DMF (3 mL) and following flash chromatography (CH_2Cl_2:CH_3OH – 8:2), the product was obtained as a yellow oil (0.46 g, 87%).

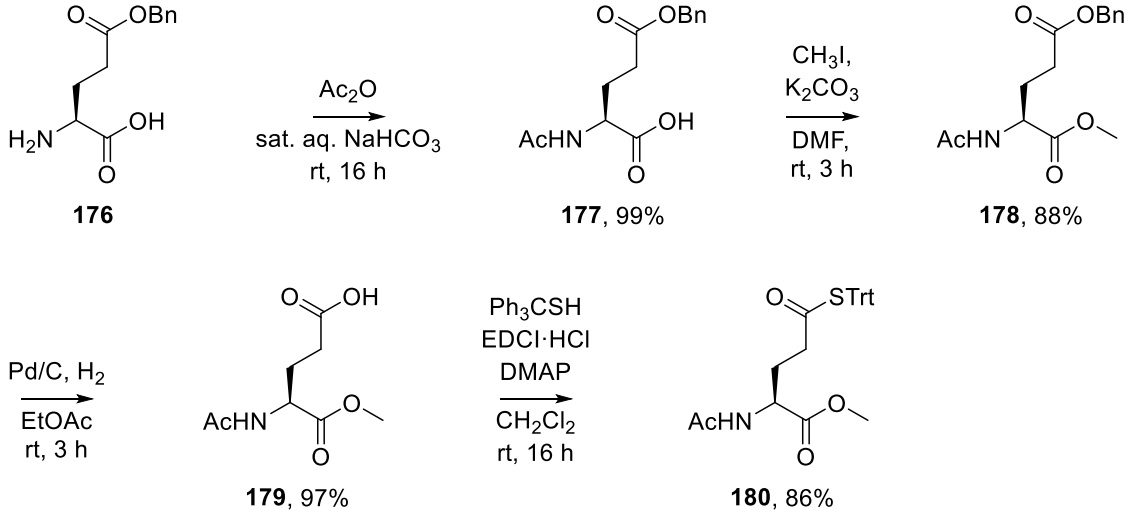
δ_H (600 MHz, $CDCl_3$) 6.68 (d, *J* = 7.8 Hz, 1H, NH), 6.50 (d, *J* = 8.5 Hz, 1H, NH), 5.27 (t, *J* = 9.9 Hz, 1H, H-3), 5.03 (t, *J* = 9.7 Hz, 1H, H-4), 4.84 (m, 1H, H-1), 4.82 (m, 1H, C<u>H</u>CH_2), 4.26 (dd, *J* = 1.5, *J* = 12.5 Hz, 1H, H-6), 4.17 (dd, *J* = 5.3, *J* = 12.5 Hz, 1H, H-6'), 3.97 (q, *J* = 9.7 Hz, 1H, H-2), 3.83 (m, 1H, H-5), 3.74 (s, 3H, OCH_3), 3.19 (dd, *J* = 4.6, 16.5 Hz, 1H, CHC<u>H</u>$_2$), 3.15 (dd, *J* = 4.6, 16.5 Hz, 1H, CHC<u>H</u>$_2$), 2.97– 2.81 (m, 2H, CH_2), 2.38 – 2.35 (m, 2H, CH_2), 2.02 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 2.01 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 1.69 – 1.67 (m, 2H, CH_2), 1.61 – 1.58 (m, 2H, CH_2)

δ_C (150 MHz, $CDCl_3$) 197.3 (C=O), 172.8 (C=O), 171.5 (C=O), 170.9 (C=O), 170.7 (C=O), 170.3 (C=O), 169.5, (C=O), 88.4 (C-1), 74.1 (C-5), 72.3 (C-3), 68.6 (C-4), 62.1 (C-6), 53.9 (C-2), 49.0 (<u>CH</u>CH_2), 45.1 (CH<u>CH</u>$_2$), 33.4 (CH_2), 28.8 (CH_2), 28.6 (CH_2), 23.4 (CH_2), 23.2 (CH_3), 23.1 (CH_3), 20.7 (CH_3), 20.7 (CH_3)

HRMS: $C_{24}H_{35}N_2O_{12}S$ requires 640.1901. ESI: Found 640.1899 $[M+Na]^+$

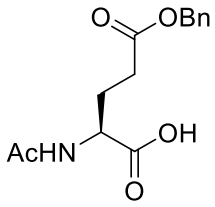
ν_{max} (ATR/cm^{-1}): 3288 (NH), 2117 (N_3), 1743, 1660 (C=O), 1217 (CH)

***R*$_f$:** 0.38 (CH_2Cl_2:CH_3OH 98:2)



Scheme 2.22. Synthesis of glutamic *S*-trityl protected thioacid

Ac-Glu(OBzl)-OH (177)[316]



Prepared as per **Procedure 5** using Glu(OBzl)-OH **176** (1.5 g, 6.32 mmol) in sat. aq. $NaHCO_3$ solution (15 mL) to give the product as a colourless oil (1.75 g, 99%). The isolated compound was in good agreement with the literature.

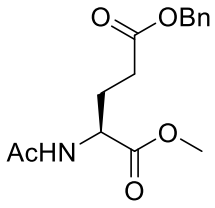
δ_H (600 MHz, $CDCl_3$) 7.38 – 7.28 (m, 5H, Ar-CH), 6.59 (d, *J* = 6.1 Hz, 1H, NH), 5.11 (s, 2H, OCH_2Ph), 4.55 – 4.52 (m, 1H, α-CH), 2.61 (m, 1H, CH_2), 2.47 (m, 1H, CH_2), 2.22 (m, 1H, CH_2), 2.07 – 1.99 (m, 4H, CH_3, CH_2)

δ_C (150 MHz, $CDCl_3$) 173.7 (C=O), 171.6 (C=O), 170.2 (C=O), 135.7 (qC), 128.7 (Ar-CH), 128.5 (Ar-CH), 128.4 (Ar-CH), 66.9 (OCH_2Ph), 52.4 (αCH), 30.5 (CH_2), 26.4 (CH_2), 22.9 (CH_3)

HRMS: $C_{14}H_{17}NNaO_5$ requires 302.1004. ESI: Found 302.1010 $[M+Na]^+$

ν_{max} (film/cm^{-1}): 3665 (NH), 2772 (OH), 1849, 17528 (C=O)

Ac-Glu(OBzl)-OMe (178)[316]



Prepared as per **Procedure 1** using **177** (1.73 g, 6.19 mmol) in DMF (12 mL) to give the product as a colourless oil (1.61 g, 88%). The isolated compound was in good agreement with the literature.

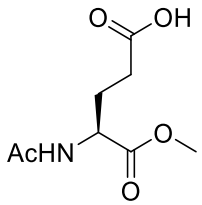
δ_H (400 MHz, CD_3OD) 7.41 – 7.29 (m, 5H, Ar-CH), 5.15 (d, *J* = 3.0 Hz, 2H, OCH_2Ph), 4.48 (dd, *J* = 9.0, 5.4 Hz, 1H, α-CH), 3.73 (s, 3H, OCH_3), 2.50 (t, *J* = 7.4 Hz, 2H,CH_2), 2.27 – 2.13 (m, 1H, CH_2), 2.05 – 2.03 (m, 1H, CH_2), 1.99 (s, 3H, Ac CH_3)

δ_C (101 MHz, CD_3OD) 173.9 (C=O), 173.6 (C=O), 173.4 (C=O), 137.5 (Ar qC), 129.8 (Ar-CH), 129.5 (Ar-CH), 129.3 (Ar-CH), 129.0 (Ar-CH), 67.4 (CH_2OBn), 52.9 (OCH_3), 52.6 (αCH), 31.2 (CH_2), 27.6 (CH_2), 22.3 (CH_3)

HRMS: $C_{15}H_{19}NNaO_5$ Requires: 316.1161. ESI: Found 316.1147 $[M+Na]^+$

ν_{max} (film/cm^{-1}): 3661 (NH), 1722, 1578 (C=O)

Ac-Glu(OH)-OMe (179)[313]



Prepared as per **Procedure 6** using **178** (0.90 g, 3.07 mmol) in EtOAc (15 mL) to give the product as a colourless oil (0.60 g, 97%). The isolated compound was in good agreement with the literature.

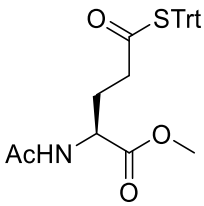
δ_H (400 MHz, CD_3OD) 4.46 (s, 1H, α-CH), 3.73 (s, 3H, OCH_3), 2.45 – 2.42 (m, 2H, CH_2), 2.18 – 2.16 (m, 1H, CH_2), 1.96 – 1.93 (m, 1H, CH_2), 2.00 (s, 3H, CH_3)

δ_C (100 MHz, CD_3OD) 173.5 (C=O), 172.5 (C=O), 172.4 (C=O), 52.0 (α-CH), 51.8 (OCH_3), 30.0 (CH_2), 26.5 (CH_2), 21.2 (CH_3)

HRMS: $C_8H_{13}NNaO_5$ requires 226.0691. ESI: Found 226.0699 $[M+Na]^+$

ν_{max} (film/cm^{-1}): 3288 (NH), 2954 (OH), 1831, 1738 (C=O), 1210 (CH)

Ac-Glu(*S*Trt)-OMe (180)



Prepared as per **Procedure 2** using **179** (0.5 g, 2.46 mmol) in CH_2Cl_2 (15 mL) and following flash chromatography (EtOAc:hexanes - 2:8), the product was obtained as a yellow oil (0.98 g, 86%).

δ_H (400 MHz, CD_3OD) 7.34 – 7.17 (m, 15H, Ar-CH), 4.36 (dd, *J* = 9.1, 5.2 Hz, 1H, α-CH), 3.71 (s, 3H, OCH_3), 2.75 – 2.57 (m, 2H, CH_2), 2.12 – 2.02 (m, 1H, CH_2), 1.98 (s, 3H, Ac CH_3), 1.92 – 1.78 (m, 1H, CH_2)

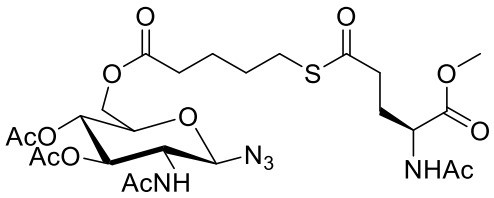
δ_C (101 MHz, CD_3OD) 197.4 (TrtS-C=O), 173.5 (C=O), 145.1 (Trt Ar-qC), 131.0 (Trt Ar-CH), 128.8 (Trt Ar-CH), 128.2 (Trt Ar-CH), 52.8 (OCH_3) 52.8 (α-CH), 40.4 (β-CH_2), 27.8 (γ-CH_2), 22.3 (Ac CH_3)

HRMS: $C_{27}H_{27}NNaO_4S$ requires 484.1559. ESI: Found 484.1553 $[M+Na]^+$

ν_{max} (film/cm^{-1}): 3287 (NH), 1742, 1691 (C=O), 1211 (CH)

***R*f:** 0.31 (EtOAc:Hexanes 2:8)

2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-Ac-Glu(OMe)-thio-pentanoate-β-D-glucopyranosyl azide (182)



Prepared as per **Procedure 3** using **180** (0.17 g, 0.36 mmol) in CH_2Cl_2 (5 mL) to give the crude thioacid which was then immediately subjected to **Procedure 4** using **127** (0.1 g, 0.24 mmol) in DMF (5 mL) and following flash chromatography (CH_2Cl_2:CH_3OH – 99:1), the product was obtained as a yellow oil (0.14 g, 94%).

δ_H (600 MHz, DMSO-d_6) 8.31 (d, *J* = 7.4 Hz, 1H, NH), 8.12 (d, *J* = 9.2 Hz, 1H, NH), 5.10 (t, *J* = 10.2 Hz, 1H, H-3), 4.90 – 4.85 (m, 2H, H-1, H-4), 4.25 – 4.20 (m, 1H, CHCH$_2$), 4.18 (dd, *J* = 4.6, *J* = 12.8 Hz, 1H, H-6), 4.12 (dd, *J* = 1.2, *J* = 12.1 Hz, 1H, H-6'), 4.00 – 3.97 (m, 1H, H-5), 3.81 (q, *J* = 9.9 Hz, 1H, H-2), 2.85 – 2.82 (m, 2H, CH_2), 2.65 – 2.61 (m, 2H, CH_2), 2.51 (s, 3H, OCH_3), 2.34 – 2.32 (m, 2H, CH_2), 2.01 - .99 (m, 2H, CH_2), 1.97 (s, 3H, CH_3), 1.92 (s, 3H, CH_3), 1.85 (s, 3H, CH_3), 1.81 (s, 3H, CH_3), 1.87 – 1.85 (m, 2H, CH_2), 1.55 (m, 2H, CH_2)

δ_C (150 MHz, DMSO-d_6) 198.3 (C=O), 172.7 (C=O), 172.4 (C=O), 170.2 (C=O), 170.2 (C=O), 170.0 (C=O), 169.6 (C=O), 87.7 (H-1), 73.2 (H-5), 72.6 (H-1), 68.3 (H-4), 61.6

(C-6), 52.9 (C-2), 51.2 (αCH), 39.5 (OCH_3), 39.5 (CH_2), 33.1 (CH_2), 28.6 (CH_2), 30.0 (CH_2), 26.5 (CH_2), 23.6 (CH_2), 22.8 (CH_3), 22.4 (CH_3), 20.6 (CH_3), 20.5 (CH_3)

HRMS: $C_{25}H_{38}N_5O_{12}S$ requires 632.2238. ESI: Found 632.2237 $[M + H]^+$

ν_{max} (film/cm^{-1}): 1737, 1684 (C=O), 1222 (CH)

***R*f:** 0.35 (CH_2Cl_2:CH_3OH 99:1)

6.2 Chapter 3 – Experimental Detail

6.2.1 General Experimental Detail

Ampicillin and Spectinomycin prepared as 100 mg/mL and 50 mg/mL solutions respectively. IPTG (isopropyl-β-D-1-thiogalactopyranoside) was prepared as a 0.5 M solution. Optical density measurements (OD_{600}) were preceded by taking a blank with lysogeny broth (LB) media in disposable plastic cuvettes, followed by decanting a small aliquot of the incubated media/cells into a fresh cuvette. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to qualitatively assess the respective protein expression, with ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) present in both stacking and separation gels.

Table 6.1. Recipes for SDS-PAGE components.

Separation gel
ddH_2O (3.7 mL)
Acrylamide (30%, 8 mL)
Tris·HCl (4 mL, 1.5 M, pH 8.8)
SDS (160 μL, 10%)
APS (160 μL, 10%)
TEMED (16 μL)

Stacking gel
ddH_2O (5.3 mL)
Acrylamide (30%, 2 mL)
Tris·HCl (4 mL, 0.5 M, pH 6.8)
SDS (100 μL, 10%)
APS (100 μL, 10%)
TEMED (10 μL)

Loading Dye (6 X)
Glycerol (5 mL)
SDS (500 mg)
Bromophenol blue (5 mg)
β-mercaptoethanol (1.25 mL)
Tris·HCl (2.25 mL, 1 M, pH 6.8)
Adjust to 10 mL with ddH_2O

Running Buffer
Glycine (14.42 g)
Tris (3.099 g)
SDS (10 mL, 10%)
Adjust to 1 L with ddH_2O

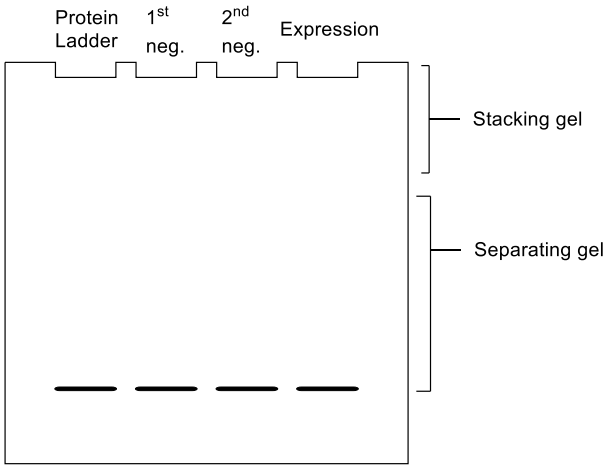


Figure 6.2. General SDS-PAGE gel.

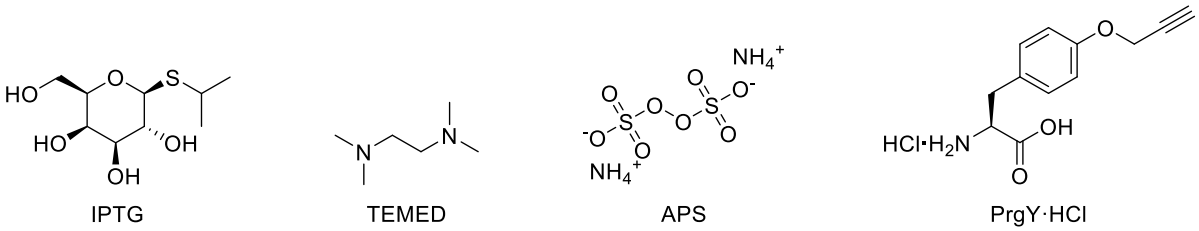
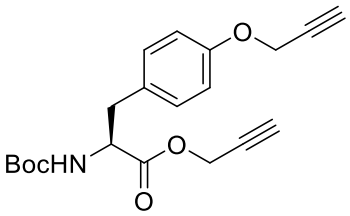


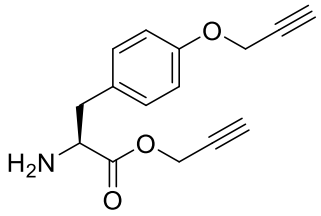
Figure 6.3. Structures of relevant reagents.

2-*tert*-butoxycarbonylamino-3-[4-(prop-2-ynyloxy)phenyl] propionic acid propargyl ester (189)



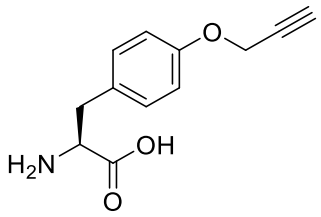
N-tert-Butoxycarbonyl-tyrosine **188** (5.0 g, 17.8 mmol, 1 eq.) and K_2CO_3 (7.4 g, 53.3 mmol, 3 eq.) were suspended in anhydrous DMF (30 mL). Propargyl bromide (4.75 mL, 53.3 mmol, 3 eq., 80 wt.% in toluene) was added dropwise over 10 min, and the reaction mixture was stirred at rt for 16 h. H_2O (100 mL) and Et_2O (100 mL) were added, the layers separated, and the aq. phase then extracted with Et_2O (3 × 50 mL). The combined organic layers were dried over $MgSO_4$ and the solution concentrated *in vacuo*. This yielded intermediate **189** (4.8 g, 75% yield) as a yellow oil. The product was used without further purification.

2-amino-3-[4-(prop-2-ynyloxy) phenyl]-propionic acid propargyl ester (190)



Ice cold acetyl chloride (22.74 mL) was carefully added to anhydrous methanol (195 mL) at 0 °C to give a 5 M solution of anhydrous HCl in CH_3OH. The methanolic HCl solution was added to compound **189** (4.8 g, 13.4 mmol) at 0 °C *via* cannula, and the reaction mixture was warmed to room temperature over 4 h with stirring. Following the elapsed time, the solution was concentrated *in vacuo*, which gave the hydrochloride salt of intermediate **190** as a pale yellow solid (3.54 g, 90% yield), which was used without further purification.

2-amino-3-[4-(prop-2-ynyloxy) phenyl] propionic acid (191)



Intermediate **190** (6.80 g, 23.14 mmol) was dissolved in CH_3OH, and the solution stirred at 40 °C. KOH (14 g, 239 mmol) was added to the mixture, and the resulting suspension stirred for 2 h. Following the elapsed time, the reaction was quenched by addition of H_2O (20 mL), and the unreacted ester **190** removed by extraction with Et_2O (2 x 10 mL). The aqueous phase was acidified to pH 2.0 with 6 M HCl, after which the yellow solution was allowed to stand overnight. This precipitated the hydrochloride salt of **191** as a pale yellow powder (2.1 g, 35%), which was dried under vacuum and used without further purification. The obtained compound was in good agreement with the literature.[317-318]

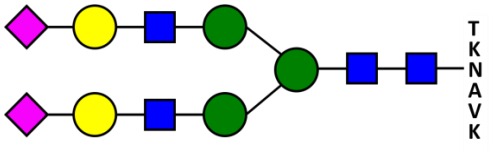
δ_H (400 MHz, CD_3OD) 7.22 (d, *J* = 8.8 Hz, 2H, Ar-CH), 6.97 (d, *J* = 8.8 Hz, 2H, Ar-CH), 4.73 (d, J = 2.4 Hz, 2H, $\underline{CH_2}C{\equiv}CH$), 4.22 (dd, *J* = 7.6, 5.4 Hz, 1H, α-CH) 3.29 – 3.24 (dd, *J* = 14.6, 5.4 Hz, 1H, β-CH_2), 3.15 – 3.09 (dd, *J* = 14.6, 7.6 Hz, 1H, β-CH_2), 2.94 (t, *J* = 2.5 Hz, 1H, $CH_2C{\equiv}\underline{CH}$)

δC (100 MHz, CD_3OD) 171.2 (COOH), 158.8 (Ar-qC-O-CH_2)), 131.6 (Ar-CH), 128.1 (Ar-qC), 116.6 (Ar-CH), 79.7 (CH_2C≡CH), 76.8 (CH_2C≡CH), 56.6 (CH_2C≡CH), 55.2 (α-CH), 36.5 (β-CH_2)

HRMS: $C_{12}H_{14}NO_3$ requires 220.0974. ESI: Found 220.0969 $[M + H]^+$.

6.2.2 Glycan and nnAA Synthesis, and Characterisation

α-2,6-Sialylglycopeptide (192)



Fifty medium-sized free range eggs were bought from the local Tesco, the morning they were delivered. The yolks were separated from the white and set aside. H_2O (250 mL) was added and the yolks stirred at rt for 30 min. The mixture was then lyophilised, yielding approximately 375 g of egg yolk powder. Egg yolk powder (200 g) was washed with diethyl ether (600 mL x 2) for 30 m. The mixture was filtered and the yellow filtrate was discarded. The filter cake was then washed with a 70:30 acetone:H_2O solution (600 mL x 2) for 30 minutes, filtered, and again the filtrate was discarded. The cake was then extracted with a 40:60 acetone:water solution (600 mL x 2) for 1 h. The filtrate was concentrated *in vacuo* to an approx. volume of 500 mL – the temperature of the rotary evaporator bath was kept below 35 °C at all times.

A 1:1 charcoal:Celite® mixture (200 g, thoroughly mixed) was loaded into a column (8.5 cm x 20 cm), and prewashed with H_2O (1 L). Columns thinner than this were avoided due to the significant backpressure generated. The concentrated 4:6 acetone:H_2O extract was circulated through the column three times. The column was then washed successively with H_2O (2 L), 5:95 CH_3CN:H_2O (2 L) and 1:9 CH_3CN:H_2O (2 L). These washes were then discarded.

The column was then eluted two times with 25:75 CH_3CN:H_2O (2L). The eluents were pooled and concentrated *in vacuo* below 35 °C to an approx. volume of 500 mL and lyophilised to yield crude egg yolk sialylglycopeptide (300 – 400 mg).

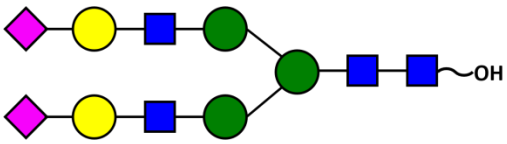
The crude sialylglycopeptide was then dissolved in H_2O (1.25 mL) and subject to centrifugation (3,200 g at 4 °C) for 30 minutes. The supernatant from this centrifugation step was immediately loaded onto a Sephadex G-25 column (2.6 x 80 cm). This column was eluted with H_2O at a rate of 0.5 ml min^{-1}, and the sialylglycopeptide fractions identified using thin layer chromatography plates, stained with 10% (v/v) H_2SO_4 in EtOH. The relevant fractions were combined and lyophilised. This furnished the isolated egg yolk sialylglycopeptide **192** (approx. 100 – 125 mg, purity > 95 %). The isolated compound was in good agreement with the literature.[249]

δ_H (400 MHz, D_2O) 5.05 (s, 1H, H1 of Man-4), 4.97 (d, *J* = 9.4 Hz, 1H, H1 of GlcNAc1), 4.86 (s, 1H, H1 of Man4'), 4.52 (s, 1H, H1 of Man3), 4.39 - 4.29 (m, 3H, H1 of GlcNAc 2, 5 and 5'), 4.26 - 4.17, (m, 2H, H1 of Gal-6 and Gal-6'), 4.24 (d, *J* = 7.2 Hz, 1H, Ala α-CH), 4.14 (m, 1H, Thr β-CH), 4.07 (m, 1H, Thr α-CH), 4.05 (d, 1H, *J* = 7.5 Hz, Val α-CH), 2.93 (m, 4H, Lys-CH_2), 2.83 – 2.67 (m, 2H, Asn β-CH), 2.59 (m, 2H, $H3_{eq}$ of NeuAc), 2.03 – 1.89 (m, 18H, Ac x 6), 1.74 – 1.54 (m, 10H, Lys-CH_2 and $H3_{ex}$ of NeuAc), 1.31 (d, *J* = 7.2 Hz, 3H, Ala-CH_3), 1.09 (d, *J* = 6.4 Hz, 3H, Thr-CH_3), 0.89 (d, *J* = 6.7 Hz, 6H, Val-CH_3)

δ_C (101 MHz, D_2O) 103.5 (C1 of Gal6, 6'), 101.3 (C1 of GlcNAc2), 100.4 (C1 of Man3), 99.6 (C1 of Man4), 99.3 (C1 of GlcNAc5, 5'), 97.0 (C1 of Man4'), 78.3 (C1 of GlcNAc1), 76.6 (Thr-βC), 60.6 (Thr-αC), 59.2 (Val-αC), 49.4 (Ala-αC), 38.8 (Lys-CH_2), 22.1 (Ac x 18) 19.3 (Thr-CH_3), 17.8 (Val-CH_3), 6.7 (Ala-CH_3)

HRMS: $C_{112}H_{189}N_{15}O_{70}$ requires 2864.1691. ESI: *z* = 2 requires 1433.0933; found 1433.0900 $[M-2H]^{2-}$. *z* = 3 requires 955.7308; found 955.7304 $[M-3H]^{3-}$

Reducing sialyloligosaccharide (193)[319]



To a solution of **192** (50 mg, 0.017 mmol) in phosphate buffer (4 mL, 75 mM, pH 7.5) was added PNGase F (1000 U, NEB units, E.C. 3.5.1.52). The solution as then incubated with shaking at 37 °C for 72 h. Following the elapsed time, the solution was immediately loaded onto a Sephadex G-25 column (2.6 x 80 cm). This column was eluted with H_2O at a rate of 0.5 ml min^{-1}, and the sugar positive fractions identified using thin layer

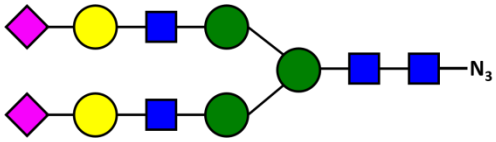
chromatography plates, stained with 10% (v/v) H_2SO_4 in EtOH. The relevant fractions were combined and lyophilised. This furnished **193** as a white powder (35.6 mg, 92%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, D_2O) 5.20 (s, 1H, H-1, α-anomer, GlcNAc1), 5.14 (s, 1H, H-1 of Man4), 4.96 (s, 1H, H-1 of Man4'), 4.61 (s, 3H, H-1 of GlcNAc2,5,5'), 4.46 (d, *J* = 8.3 Hz, 2H, H-1 of Gal6,6'), 4.27 (s, 1H, H-3 of Man3), 4.21 (s, 1H, H-2 of Man4'), 4.13 (s, 1H, H-2 of Man4), 2.68 (d, *J* = 9.8 Hz, 2H, H-3_{eq} of NeuAc7,7'), 2.12 – 1.99 (m, 18H, 6 x Ac), 1.73 (app. dd, *J* = 12.2 Hz, 2H, H-3_{ax} of NeuAc7,7')

HRMS: $C_{84}H_{138}N_6O_{62}$ requires 2222.7830. ESI: : *z* = 2 requires 1110.3837; found 1110.8860 $[M-2H]^-$

R_f: 0.81 (1 M NH_4OAc:isopropyl alcohol 1:1)

Sialyloligosachharyl azide (194)



To a solution of the reducing sugar **193** (35 mg, 0.0156mmol), 2,6-lutidine (0.07 mL, 0.622 mmol) and NaN_3 (693 mg, 10.659 mmol) in H_2O (1 mL) was added 2-chloro-1,3-dimethylimidazolinium chloride (53 mg, 0.311 mmol). The resulting solution was stirred for 3 days at 2 °C. Following the elapsed time, the solution was immediately loaded onto a Sephadex G-25 column (2.6 x 80 cm). This column was eluted with H_2O at a rate of 0.5 mL min^{-1}, and the sugar positive fractions identified using thin layer chromatography plates, stained with 10% (v/v) H_2SO_4 in EtOH. The relevant fractions were combined and lyophilised. This gave **194** as a white powder (20 mg, 56%).

δ_H (600 MHz, D_2O) δ 5.15 (s, 1H, H-1, GlcNAc1), 4.95 (d, *J* = 10.9 Hz, 1H, H-1 of Man4'), 4.63-4.61 (m, 3H, H-1 of GlcNAc2,5,5'), 4.46 (d, *J* = 8.3 Hz, 2H, H-1 of Gal6,6'), 4.27 (s, 1H, H-3 of Man3), 4.21 (s, 1H, H-2 of Man4'), 4.13 (s, 1H, H-2 of Man4), 2.68 (d, *J* = 10.1 Hz, 2H, H-3_{eq} of NeuAc7,7'), 2.10-2.04 (m, 18H, 6 x Ac), 1.73 (t, *J* = 12.1 Hz, 2H, H-3_{ax} of NeuAc7,7')

HRMS: $C_{84}H_{136}N_9O_{61}$ requires 2246.7817. ESI: Found 2246.7689 $[M-H]^-$.

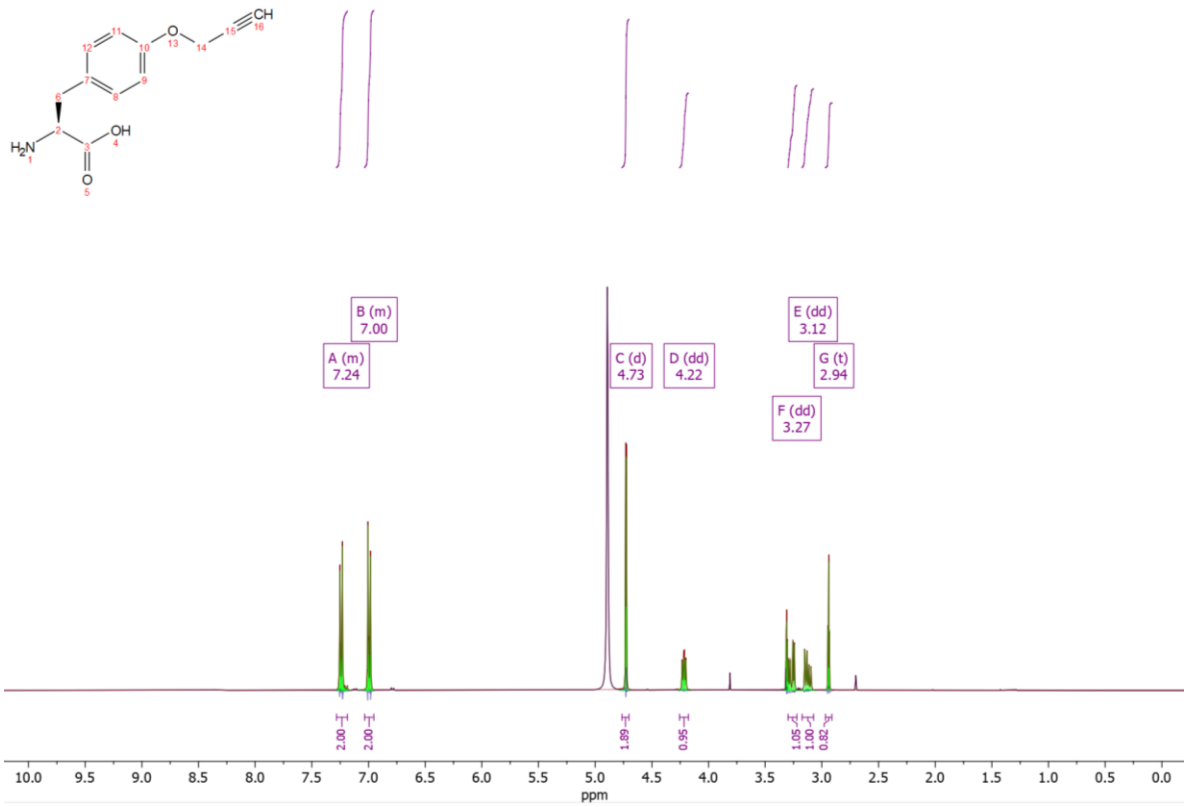


Figure 6.4. ^{1}H NMR spectrum of nnAA **191**.

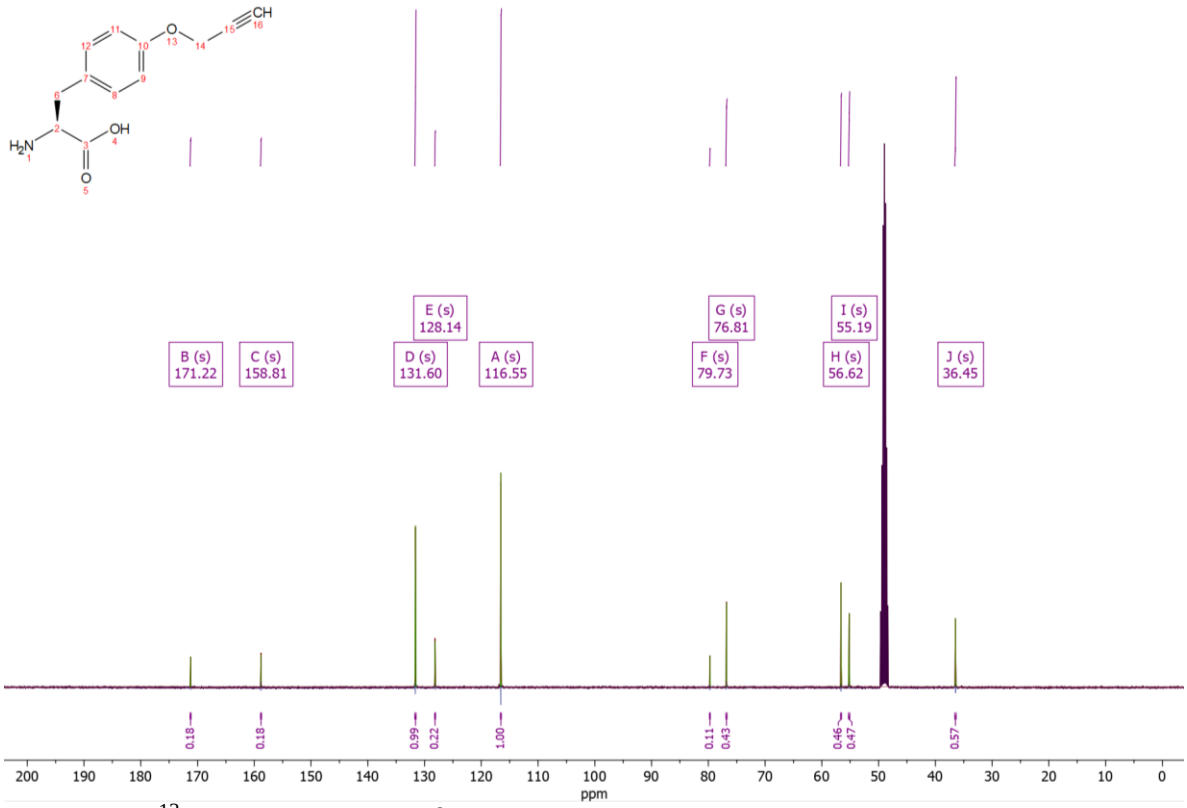
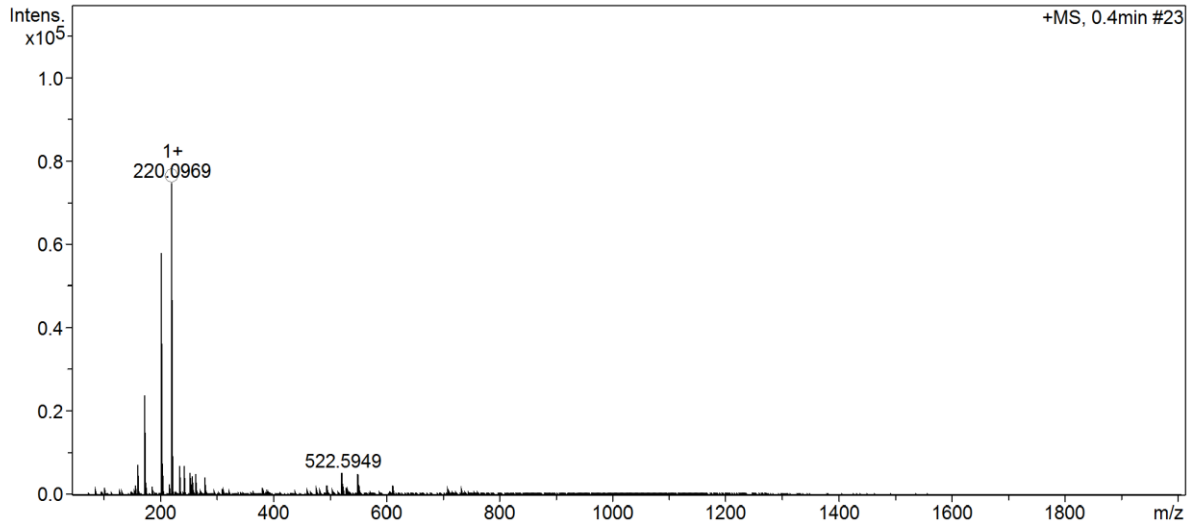


Figure 6.5. ^{13}C NMR spectrum of nnAA **191**.



Meas. m/z	#	Ion Formula	m/z	err [mDa]	err [ppm]	rdb	N-Rule	e⁻ Conf	mSigma
220.096885	1	C12H14NO3	220.096820	-0.1	-0.3	6.5	ok	even	1.1

Figure 6.6. ESI mass spectrum of nnAA **191**.

6.2.3 IFNγ Expression and Purification

Cell transformation with the plasmid of interest and ER2566 cells in LB agar (lysogeny broth) yielded a number of *Escherichia coli* (*E. coli)* colonies, two of which were selected for overnight incubation in LB media. Following overnight incubation, an aliquot of these cells was added to fresh LB media (5 mL per 500 mL media), in a conical flask with a metal aeration coil. Previously prepared solutions of ampicillin and spectinomycin were added (500 μL of each per 500 mL LB media). The solutions were then incubated at 30 °C and agitated at 200 rpm for a period, with the OD_{600} measurements taken every 90 min. Once the optical density reached approx. 0.6, the first negative control was taken (non-induced, no amino acid present). A separate aliquot was taken to later form the second negative control.

The incubated solutions were centrifuged at 4000 rpm for 20 min, and the supernatant removed. The cell pellet was resuspended in fresh LB media, at the same concentration as previously described. Ampicillin and spectinomycin were added (500 μL of each per 500 mL LB media). 4-propargyloxyphenylalanine hydrochloride was dissolved in acetic acid and water (80% v/v) with the aid of a mechanical stirrer. The amino acid solution was added to the LB media containing the cells and antibiotics, such that a 2.5 mM concentration was attained in each culture flask. The pH of these cultures was then corrected to pH 7.5 by adding aliquots of NaOH (5 M).

The resultant cultures were incubated with shaking at 30 °C and 200 rpm for 10 min, to allow the amino acid to enter the cells. Following the elapsed time, aliquots of IPTG (500 μL per 500mL media) were added to each flask to induce protein expression. The induced cultures were then incubated for 16 h at 30 °C and 200 rpm. The second negative control (induced, no amino acid) was then induced with IPTG, and incubated under the same conditions. Following overnight incubation, an aliquot (1 mL) of the overnight culture was taken. This aliquot, along with the first and second negative control were centrifuged at 4000 rpm for 15 min. The supernatant was decanted, and the pellets set aside for later SDS-PAGE analysis. The bulk overnight culture was then centrifuged at 4000 rpm for 20 minutes, and the supernatant discarded. The cells were placed in the freezer for later lysis and purification.

The two control cell pellets and expressed pellet (i.e. non-induced and no amino acid, induced with no amino acid, and induced with the amino acid present) were suspended in Tris·HCl buffer (100 μL, 20 mM, pH 6.8). Loading dye (20 μL, 6 X) was added, and each pellet sonicated (0 °C, 40% amplitude, 1 minute, 1 sec on/1 sec off). A small sample (10-15 μL) of each cell mixture was loaded onto a previously prepared electrophoretic gel, with a commercial protein ladder loaded in another lane. Once loading was complete, this gel was run at 30 A for approx. 45 minutes in freshly prepared running buffer, after which the apparatus was disassembled. The gel and plates were carefully washed with ddH_2O.

The gel was stained with Coomassie blue (0.25 g, dissolved in 90 mL MeOH:H_2O (1:1 v/v) and acetic acid (10 mL)). Destaining of the background was accomplished using the same solvent mixture in the absence of Coomassie blue. The gel was stored in H_2O and photographed.

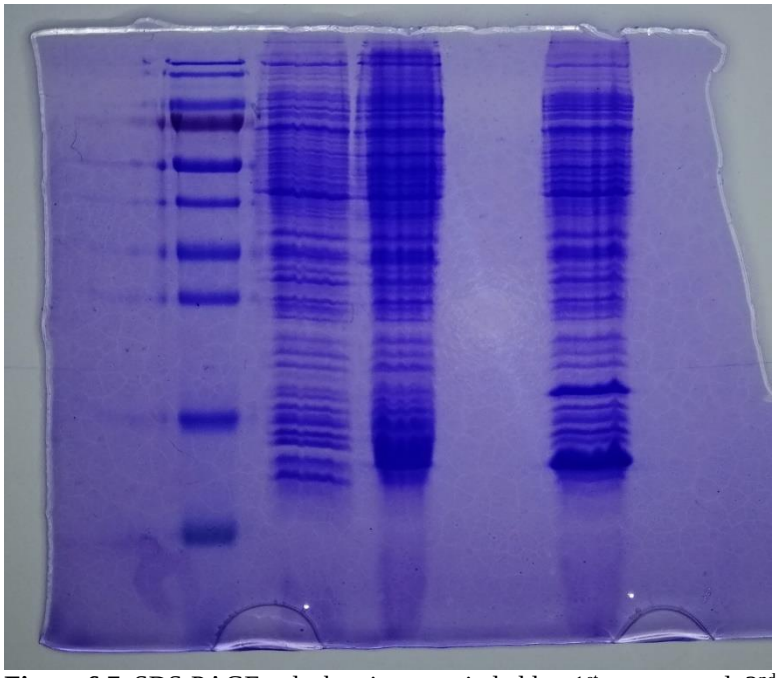


Figure 6.7. SDS-PAGE gel, showing protein ladder, 1st neg. control, 2nd neg. control, and expressed trial.

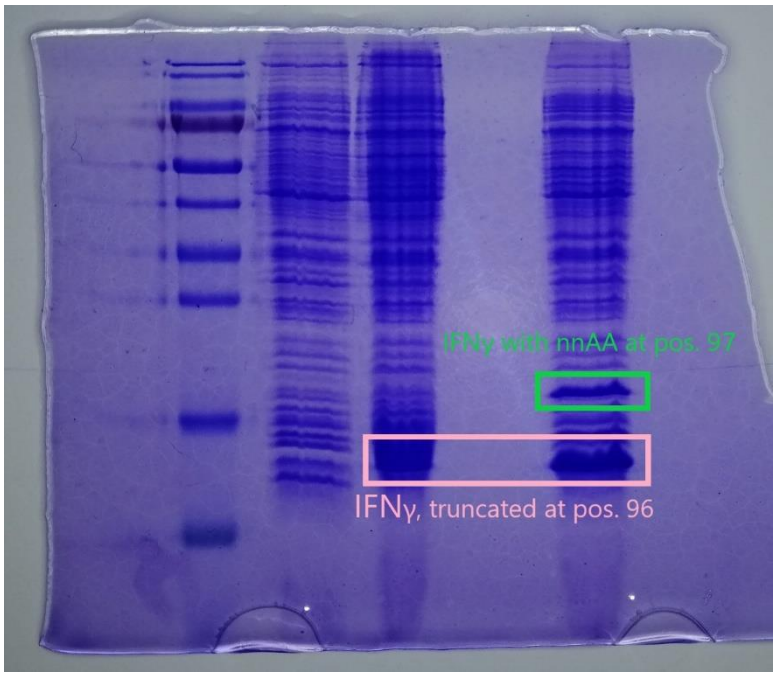


Figure 6.8. Gel with relevant highlights.

Frozen cells were resuspended in 10 mM Tris·HCl, 50 mM NaCl, pH 7.2 buffer, and centrifuged at 4000 rpm for 40 minutes. Supernatant was discarded, the pellet resuspended, and subject to the same conditions again, with the supernatant again discarded. The wet biomass was resuspended in 200 ml of 1 M urea, 0.4 M guanidinium hydrochloride, 20 mM Tris·HCl, pH 8.8 and sonicated for on ice with an ultrasonic disintegrator (0 °C, 40% amplitude, 1 min, 1 sec on/1 sec off) for 5 min total.

The disrupted cell suspension was centrifuged at 9000 rpm for 45 min. The supernatant was discarded and the crude pellet of inclusion bodies was further suspended in 7.4 M GdmCl, pH 7.0. The suspension was stirred overnight at rt, after which water was added dropwise with continuous stirring, until the concentration of GdmCl was approximately 1 M. The suspension was then centrifuged for 1 h at 4000 rpm. The supernatant was set aside to be purified by Ni^{2+} chromatography, and the pellet containing cell membranes and insoluble proteins was discarded.

Purification of the IFNγ presenting 4-propargyloxy-Phe at position 97 was achieved by the use of Ni^{2+} affinity chromatography (HisPur Ni-NTA spin column, 3 mL, product 88226). The His_6 tag of the expressed protein C-terminus was chelated to the Ni^{2+} surface by flowing the inclusion body supernatant from the previous step through the column bed. Washing away of impurities left the protein immobilised within the Ni^{2+} matrix. Elution from the affinity column proceeded with the following elution buffers: **1)** 6 M GdmCl, 20 mM imidazole (20 mL), **2)** 6 M GdmCl, 40 mM imidazole (50 mL), **3)** 6 M GdmCl, 100 mM imidazole (20 mL), **4)** 6 M GdmCl, 200 mM imidazole (20 mL), **5)** 6 M GdmCl, 300 mM imidazole (20 mL), and finally **6)** 6 M GdmCl, 1 M mM imidazole (10 mL). Fractions (3 mL) were collected from whence the 100 mM imidazole buffer was added to the column.

SDS-PAGE of each fraction (Figure 6.9) was performed, with the first four lanes being the protein marker, positive control, flow through, and wash respectively. Lanes 9-13 (highlighted, green) were identified as ‘clean’ fractions of the desired protein.

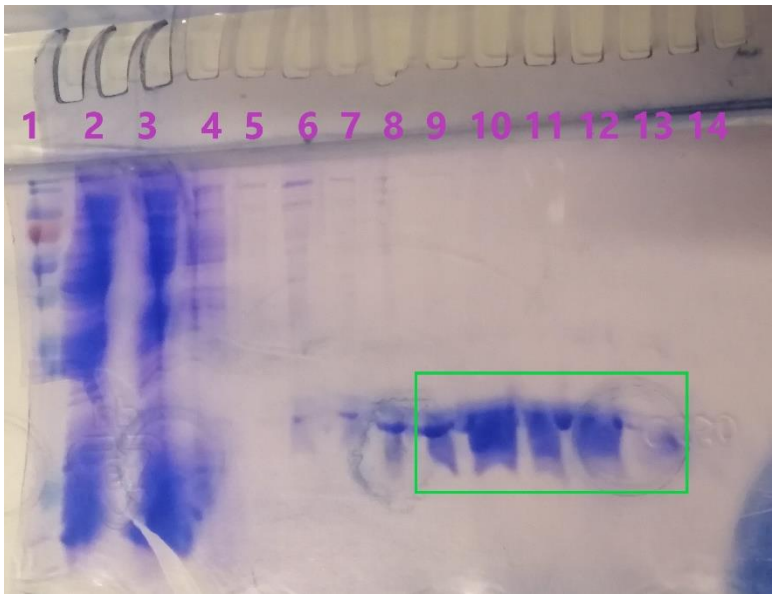


Figure 6.9. SDS-PAGE following Ni^{2+} affinity chromatography.

6.2.4 Click, Refolding, and SEC

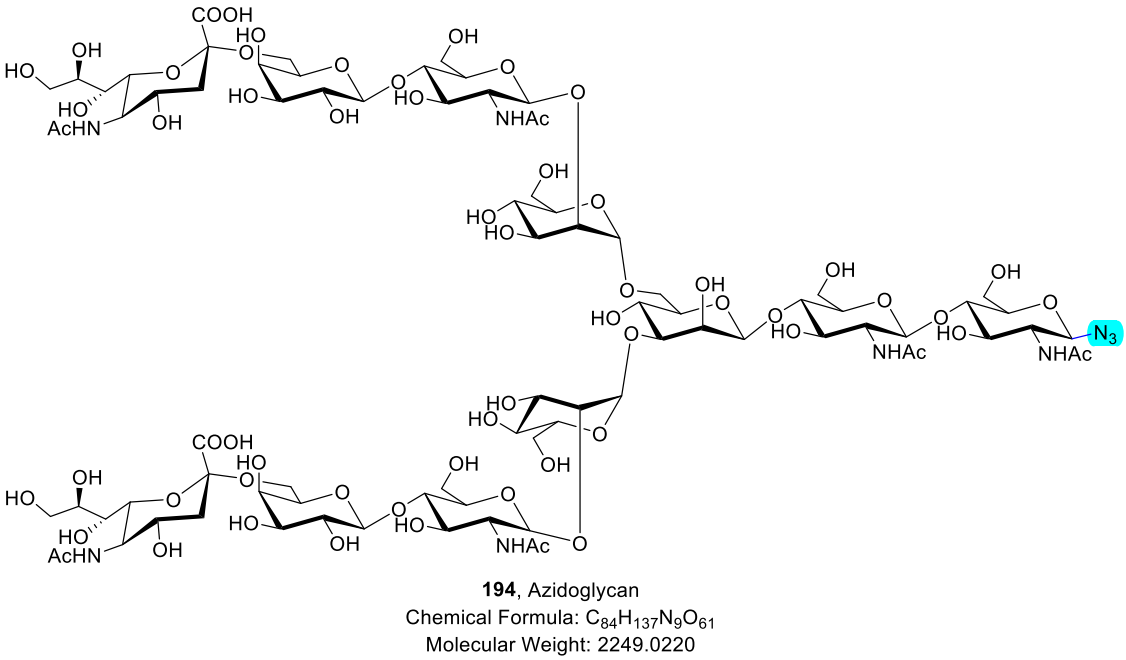


Figure 6.10. Glycan with azide functionality for CuAAC reaction.

Imidazole was removed from the purified fractions by performing a buffer exchange with Amicon Ultra-4 10K protein concentrators (product UFC801024). Repetitive centrifugation was used to continually dilute the imidazole concentration to a negligible quantity. Buffer exchange gave a solution of the protein in **1)** 6 M GdmCl, 10 mM Tris-HCl pH 7.5, **2)** 3 M GdmCl, 10 mM Tris-HCl pH 7.5, and **3)** 6 M urea, 10 mM Tris-HCl, pH 7.5.

THPTA was prepared as an 80 mM stock solution in the desired buffer, whilst Tetrakis(acetonitrile)copper(I) tetrafluoroborate was prepared as an 80 mM solution in acetonitrile. The glycan azide was prepared as a 960 μM solution in ddH_2O. Initial reactions proceeded on a 14 μM scale of protein with a 1:20 ratio of protein to glycan, and with 1.4 mM of Cu^{I} and 2.8 mM of THPTA. Control reactions omitted the glycan in place of additional buffer solution (Figure 6.11).

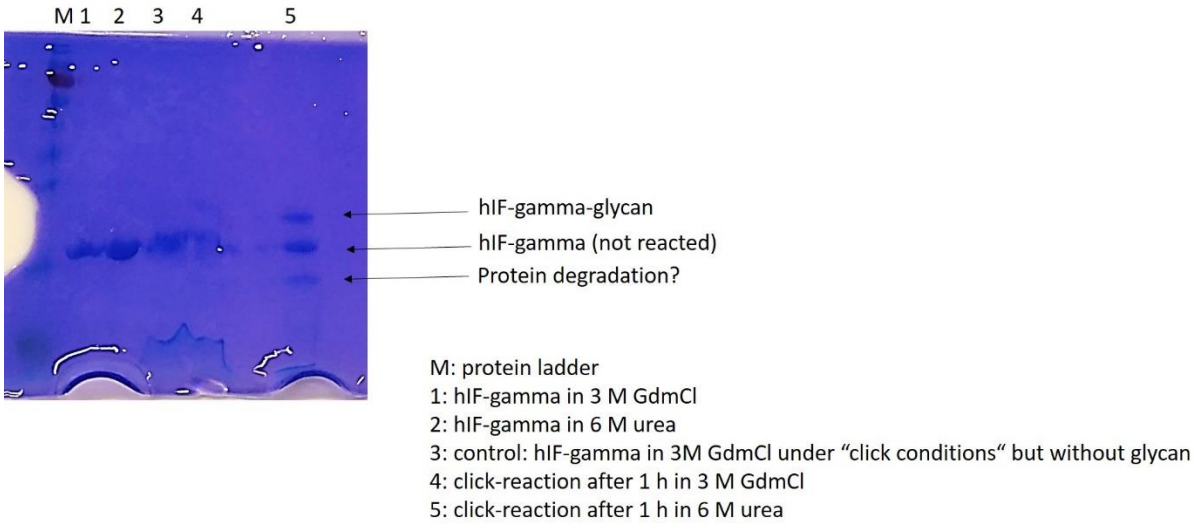


Figure 6.11. SDS-PAGE of click reaction, with annotations.

Optimal conditions were identified as the following: A premix of protein (14 μM), glycan (280 μM), THPTA (2.5 mM) in 6 M urea, 10 mM Tris·HCl, pH 7.2 buffer was degassed by nitrogen flow *via* a needle. The Eppendorf was repeatedly flushed with nitrogen, and the reaction volume kept between 50 – 100 μL. Separately, a degassed solution of tetrakis(acetonitrile)copper(I) tetrafluoroborate was dissolved in degassed CH_3CN, and sparged briefly with nitrogen so as to not affect the concentration. To initiate the reaction, an aliquot of the copper solution was added to the reaction tube (effective concentration 1.4 mM), and the final combined solution degassed once more. The Eppendorf was then sealed and placed in a two neck flask, through which a gentle nitrogen flow was maintained for 1 h.

Following the elapsed time, the reactions were analysed with SDS-PAGE (Figure 6.12). The buffer was exchanged and copper was removed with a PD MidiTrap G-25 column, yielding a solution of the protein-glycan conjugate in 3 M guanidinium chloride (Gdm·Cl), 10 mM Tris·HCl, pH 7.2.

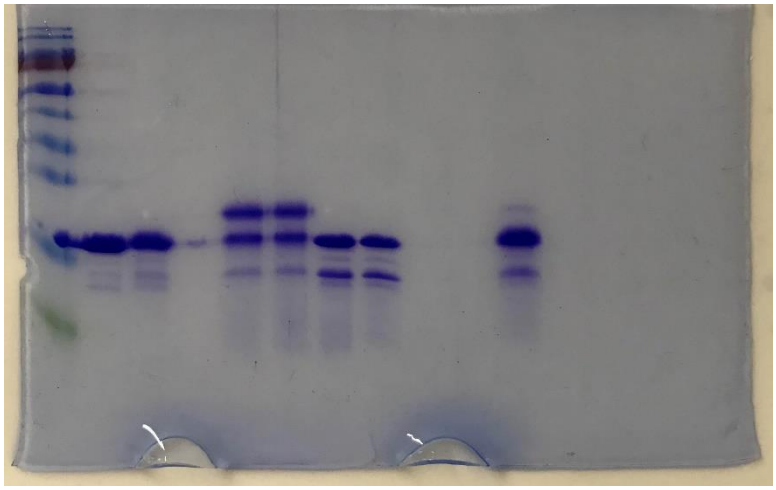


Figure 6.12. Optimisation attempts. Gel with lanes 1) marker, 2) control, 3) control of the control, 4) click in urea/HEPES pH 7.2, 5) click in urea/MOPS pH 7.2, 6) click in urea/phosphate pH 7.2, 7) click in urea/phosphate pH 7.5, and 8) week-old click reaction.

Refolding of the wild-type IFNγ (WT-IFNγ), singly-clicked IFNγ (SC-IFNγ) and doubly-clicked IFNγ (DC-IFNγ) was conducted as follows. Buffer composed of 100 mM Tris·HCl (pH 8.2), 0.02 mM EDTA, and 0.5 M L-Arginine was passed through a 0.22 μM filter. The previously concentrated protein samples (1 mL) from the PD MidiTrap G-25 column were placed in a beaker, and refolding buffer (100 mL) was added. The solution was covered and incubated without shaking at 4 °C for 4 days. Following the elapsed time, the refolding solution was concentrated to approx. 1 mL in a centrifuge at 4 °C, *via* an Amicon Ultra-4 10K protein concentrator. The gel with marker, unfolded SC-IFNγ, and refolded SC-IFNγ in two different buffers (marked 2 and 3) is shown below (Figure 6.13).

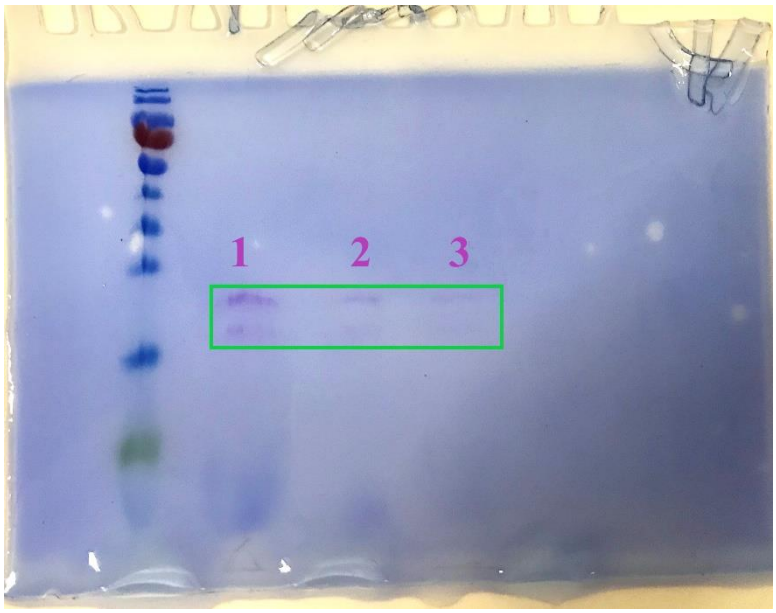


Figure 6.13. SDS-PAGE gel with protein marker, clicked unfolded protein (1), 20 mM Tris·HCl (pH 8.2) refolding buffer (2), and refolding buffer with 0.5 M L-Arg (3).

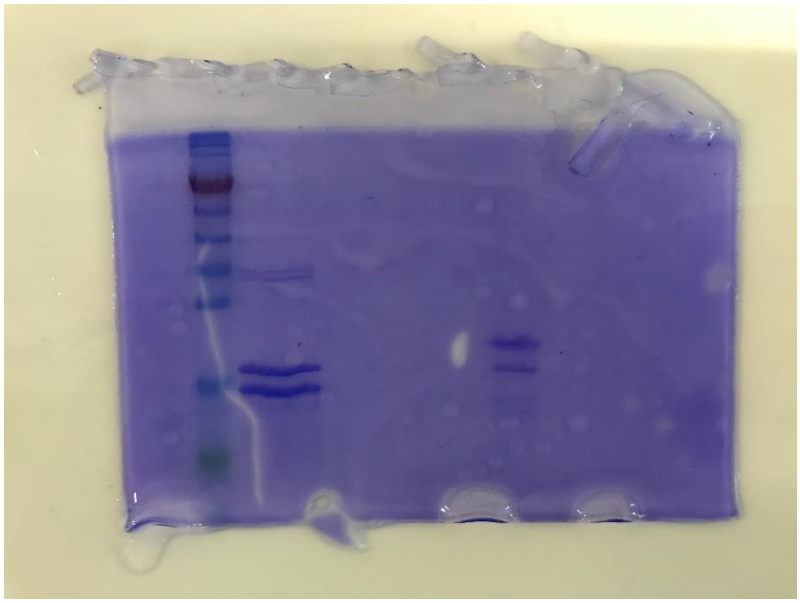


Figure 6.14. SDS-Page with protein marker, WT-IFNγ and SC@25-IFNγ.

The WT-IFNγ, SC-IFNγ, and DC-IFNγ were all purified in a similar fashion. Purification was conducted using a HiLoad® 16/600 Superdex® 75 pg column, with a column volume (CV) of approx. 120 mL. The chromatographic parameters were controlled using an ÄKTA™ start. The size-exclusion chromatography (SEC) column was first washed with one CV of a low ionic strength buffer, 20 mM Tris·HCl (pH 8.2), at a flow rate of 1 mL/min. Following the completion of this wash, two CV of 20 mM Tris·HCl, 300 mM NaCl (pH 8.2) buffer were passed through the column at the same flow rate for equilibration, ensuring that the internal pressure did not exceed 0.5 MPa. Both buffers were previously filtered under vacuum through a 0.22 μM cellulose acetate filter.

A sample loop (1 mL) was primed with the buffer to be used (5 mL), with the injection valve in the **load sample** position. To load the refolded protein sample (volume not exceeding 1 mL), a syringe was filled with the refolding buffer containing the concentrated protein, and connected to the injection port. The sample was slowly loaded into the loop. To prevent siphoning, the syringe was left in the injection port until the sample had been injected onto the column by the ÄKTA™ start. A method run was initiated *via* the UNICORN software, with the flow rate set to 1 mL/min and fractionating volume set to 2 mL. The injection valve was switched between **load sample** and **inject to column** as indicated by the display within the UNICORN software. The column was eluted isocratically with one CV of freshly filtered 20 mM Tris·HCl, 300 mM NaCl (pH 8.2) buffer. Chromatograms were collected to analyse the UV absorbance of each

fraction, and are presented below (Figures 6.15, 6.16, and 6.17). The SEC column was stored in aqueous EtOH (20% v/v) between uses.

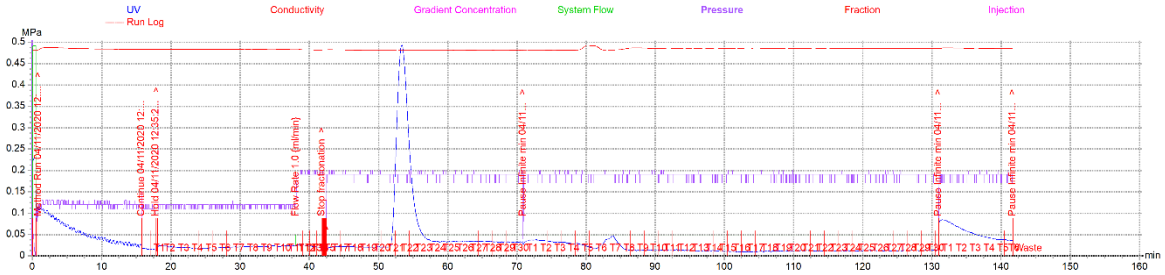


Figure 6.15. Chromatogram from SEC of refolded WT-IFNγ.

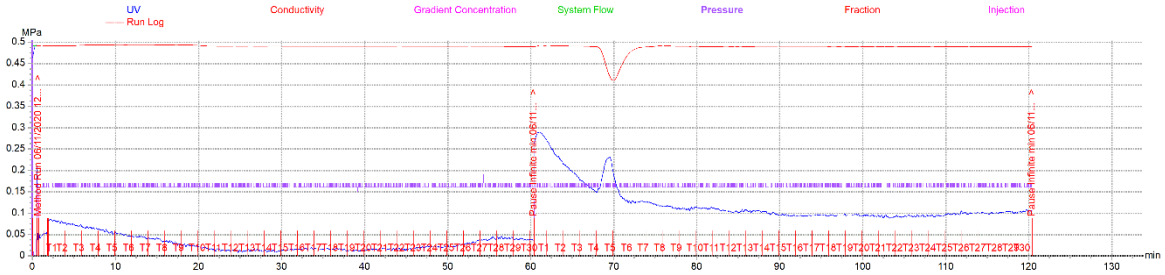


Figure 6.16. Chromatogram from SEC of refolded SC-IFNγ, first attempt.

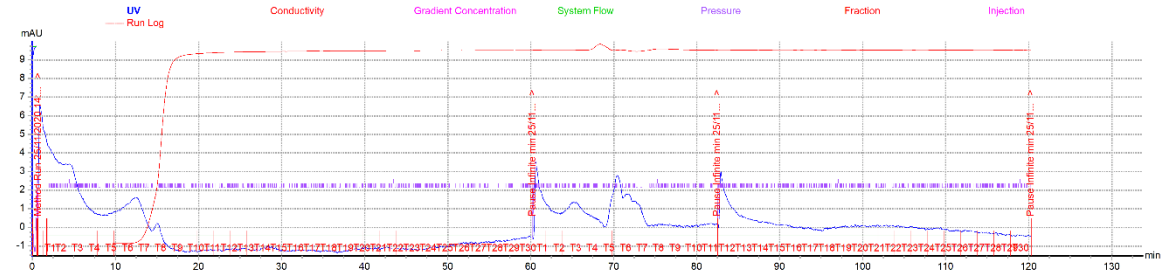


Figure 6.17. Chromatogram from SEC of refolded SC-IFNγ, second attempt.

With respect to the WT-IFNγ, fractions 20-23 were concentrated to approx. 1 mL and subjected to SDS-PAGE. The removal of an impurity (see lane 2, < 15 kDa) *via* SEC was successful. Both lanes 2 and 3 appear to have an additional band at ~ 34 kDa – this can be attributed to the desired dimer of the refolded protein, which was not completely denatured by the SDS-PAGE loading dye (Figure 6.18).

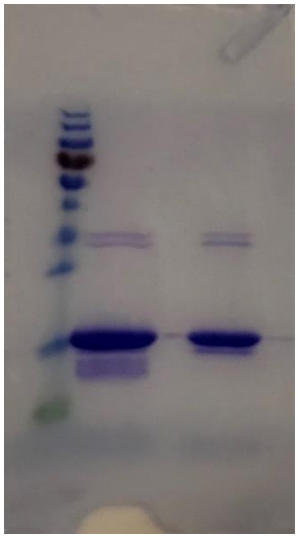


Figure 6.18. Lanes are marker, unpurified refolded WT-IFNγ, and refolded WT-IFNγ which had been purified by SEC.

6.3 Chapter 4 – General Experimental Procedures

General Procedure 1: Thiol-ene cyclisation

To a solution of DPAP (0.1 eq.) and MAP (0.1 eq.) in degassed EtOAc was added diene/enyne/diyne (1.0 eq.), followed by the relevant thioacid (1.2 eq.) The resulting solution was swirled to attain homogeneity, and thereafter irradiated (*hv*, 365 nm) under Ar for 1 h without agitation. Following the completion of the reaction, the solution was washed with sat. aq. $NaHCO_3$, brine, and dried over $MgSO_4$. This suspension was filtered, the filtrate concentrated *in vacuo*, and subject to chromatographic purification with SiO_2 as the stationary phase.

General Procedure 2: Synthesis of *S*-trityl thioesters

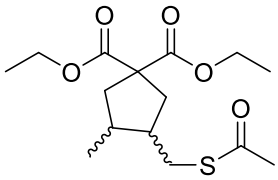
To a stirred solution of the starting carboxylic acid (1.0 eq.), triphenylmethanethiol (1.1 eq.) and DMAP (0.1 eq.) in anhydrous CH_2Cl_2 was added EDCI·HCl (1.1 eq.) and the solution was stirred at rt for 18 h. The solvent was removed *in vacuo* and the mixture was subjected to chromatographic purification

General Procedure 3: Removal of *S*-trityl group

To a solution of the *S*-trityl thioester (1.0 eq.) in anhydrous CH_2Cl_2 was added TFA (25% v/v in CH_2Cl_2) and ethyldimethylsilane (10.0 eq.) and the mixture was stirred for 10 min. Toluene was added and the solvent was removed *in vacuo* to give the crude thioacid which was applied immediately to the next step.

6.3.1 Initial Thioacid Scope

Diethyl 3-((acetylthio)methyl)-4-methylcyclopentane-1,1-dicarboxylate (206)



Prepared as per **Procedure 1** using diethyl diallylmalonate (0.3 mL, 1.24 mmol), thioacetic acid (0.11 mL, 1.49 mmol) DPAP (32 mg, 0.124 mmol) and MAP (19 mg, 0.124 mmol) in EtOAc (12 mL). Purified by column chromatography (hexanes:EtOAc 9:1), which gave a colourless oil (385 mg, 98%).

Dias.ratio: *cis:trans* 8:1

δ_H (400 MHz, $CDCl_3$) 4.19 – 4.14 (m, 4H, ester $CH_3\underline{CH_2}$ x 2), 2.94 (dd, *J* = 13.2, 6.5 Hz, 1H, CH_2), 2.82 – 2.74 (dd, *J* = 13.2, 6.5 Hz , 1H, CH_2), 2.47 – 2.37 (m, 2H, ring CH_2), 2.32 (s, 3H, thioester $\underline{CH_3}C{=}OS$), 2.27 – 2.14 (m, 2H, ring CH_2), 2.09-2.04 (m, 1H, ring $\underline{CH}CH_2SC{=}O$), 2.01-1.96 (m, 1H, ring $\underline{CH}CH_3$), 1.26 – 1.20 (t, *J* = 7.1 Hz, 6H, ester $\underline{CH_3}CH_2$ x 2), 0.92 (d, *J* = 6.9 Hz, 3H, ring $CH\underline{CH_3}$ (*cis*))

If *trans*, 1.04 (d, *J* = 5.9 Hz, 3H, ring $CH\underline{CH_3}$ (*trans*)) ppm.

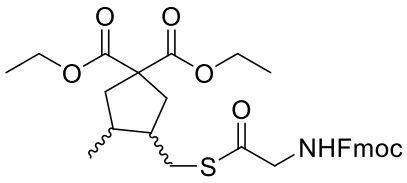
δ_C (101 MHz, $CDCl_3$) 195.8 (thioester $CH_3\underline{C{=}O}S$), 172.8 (ester C=O), 172.7 (ester C=O), 61.6 (ester $CH_3\underline{CH_2}$), 58.9 (qC), 42.6 ($\underline{CH}CH_2SC{=}O$), 41.3 ($qC\underline{CH_2}$), 38.3 ($CH\underline{CH_2}SC{=}O$), 36.2 ($qC\underline{CH_2}$), 30.8 ($\underline{CH}CH_3$), 29.9 ($\underline{CH_3}C{=}OS$), 14.9 ($CH\underline{CH_3}$), 14.2 ($CH_2\underline{CH_3}$)

HRMS: $C_{15}H_{25}O_5S$ requires 317.1423. APCI: Found 317.1420 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 2937 (C-H stretch), 2970 (C-H stretch), 2876 (C-H stretch), 1726 (C=O stretch), 1692 (C=O stretch)

***R*f:** 0.23 (Hexanes:EtOAc 95:5)

Diethyl 3-(((Fmoc-glycyl)thio)methyl)-4-methylcyclopentane-1,1-dicarboxylate (207)



Prepared as per **Procedure 1** using diethyl diallyl malonate (0.15 mL, 0.62 mmol) and the crude thioacid obtained from **Procedure 3** using Fmoc-Gly-*S*Trt **143** (414 mg, 0.74 mmol), with DPAP (16 mg, 0.062 mmol) and MAP (9.5 mg, 0.062 mmol) in EtOAc (6 mL). Column chromatography (hexanes:EtOAc 9:1) gave a colourless oil (300 mg, 87%).

Dias.ratio: *cis:trans* 7:1

δ_H (400 MHz, $CDCl_3$) 7.77 (d, *J* = 7.5 Hz, 2H,Ar$\underline{CH}$), 7.61 (d, *J* = 7.4 Hz, 2H, Ar$\underline{CH}$), 7.40 (t, *J* = 7.4 Hz, 2H, Ar$\underline{CH}$), 7.32 (t, *J* = 7.2 Hz, 2H, Ar$\underline{CH}$), 4.44 (d, *J* = 7.0 Hz, 2H, Fmoc $\underline{CH_2}$), 4.25 (t, *J* = 7.0 Hz, 1H, Fmoc $\underline{CH}$), 4.20 – 4.11 (m, 6H, ester $CH_3\underline{CH_2}$ x 2,

Gly$\underline{CH_2}$), 3.02-2.97 (m, 1H, CH_2), 2.87-2.82 (m, 1H, CH_2), 2.48-2.37 (m, 2H, ring CH_2), 2.28 – 2.15 (m, 2H, ring CH_2), 2.11-2.05 (m, 1H, ring CH), 2.01-1.96 (m, 1H, ring CH), 1.27 – 1.20 (m, 6H, ester $\underline{CH_3}CH_2$ x 2), 0.92 (d, *J* = 6.9 Hz, 3H, ring CH$\underline{CH_3}$ (*cis*))

If *trans*, 1.05 (d, *J* = 6.0 Hz, 3H, ring CH$\underline{CH_3}$ (*trans*))

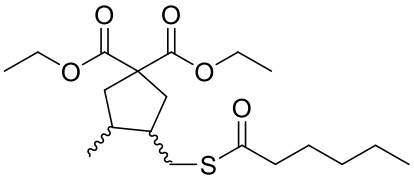
δ_C (101 MHz, $CDCl_3$) 197.4 (thioester C=O), 172.7 (ester C=O), 172.7 (ester C=O), 156.3 (Fmoc C=O), 143.9 (Ar-qC), 141.5 (Ar-qC), 127.9 (Ar-qC), 127.2 (Ar-qC), 125.2 (Ar-qC), 120.1 (Ar-qC), 67.5 (Fmoc CH_2), 61.7 (ester $CH_3\underline{CH_2}$), 58.9 (qC), 50.8 (Gly$\underline{CH_2}$), 47.3 (Fmoc CH), 42.5 ($\underline{CH}CH_2SC=O$), 41.3 (qC$\underline{CH_2}$), 38.3 (CH$\underline{CH_2}SC=O$), 36.3 (qC$\underline{CH_2}$), 29.4 ($\underline{CH_3}C=OS$), 14.9 (CH$\underline{CH_3}$), 14.2 ($CH_2\underline{CH_3}$)

HRMS: $C_{30}H_{36}NO_7S$ requires 554.2212. APCI: Found 554.2209 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 3315 (N-H stretch), 2936 (C-H stretch), 1711 (C=O)

***R*$_f$:** 0.29 (Hexanes:EtOAc 9:1)

Diethyl 3-((heptanoylthio)methyl)-4-methylcyclopentane-1,1-dicarboxylate (208)



Prepared as per **Procedure 1** using diethyl diallyl malonate (0.15 mL, 0.62 mmol) and the crude thioacid obtained from **Procedure 3** using *S*-tritylheptanethioate **141** (286 mg, 0.74 mmol), with DPAP (16 mg, 0.062 mmol) and MAP (9.5 mg, 0.062 mmol) in EtOAc (6 mL). Column chromatography (hexanes:EtOAc 98:2 → 95:5) gave a colourless oil (150 mg, 65%).

Dias.ratio: *cis:trans* 8.5:1

δ_H (400 MHz, $CDCl_3$) 4.17 (m, 4H, ester $CH_3\underline{CH_2}$), 2.94 (dd, *J* = 13.2, 6.5 Hz, 2H, CH_2), 2.78 (dd, *J* = 13.2, 8.6 Hz, 2H, CH_2), 2.56 – 2.50 (m, 4H, CH_2 x 2), 2.48 – 2.35 (m, 4H, CH_2 x 2), 2.28 – 2.13 (m, 4H, CH_2, ring CH x 2), 2.07 (dd, *J* = 13.2, 8.6 Hz, 2H, CH_2), 1.99 (dd, *J* = 13.8, 5.7 Hz, 2H, CH_2), 1.64 (dt, *J* = 15.0, 7.7 Hz, 4H, CH_2 x 2), 1.36 – 1.20 (m, 12H, ester $\underline{CH_3}CH_2$ x 2, hept CH_2 x 3), 1.04 (d, *J* = 5.9 Hz, 3H, ring $CHCH_3$ (*trans*)), 0.92 (d, *J* = 6.9 Hz, 6H, ring $CHCH_3$ (*cis*)), 0.88 (t, *J* = 6.9 Hz, 3H, hept CH_3)

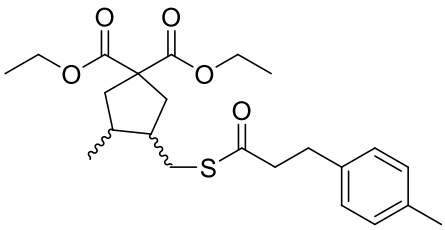
δ_C (101 MHz, $CDCl_3$) 199.6 (thioester C=O), 172.7 (ester C=O), 61.6 (ester $CH_3\underline{CH_2}$), 58.9 (qC), 44.3 (ring $\underline{CH}CH_2S$), 42.7 (hept $SC{=}O\underline{CH_2}$), 41.3 (ring CH_2), 38.3 ($CH_2SC{=}O$), 36.2 (ring CH_2), 31.6 (ring $\underline{CH}CH_3$), 29.5 (hept CH_2), 28.8 (hept CH_2), 25.8 (hept CH_2), 22.6 (hept CH_2), 14.9 (ring $CH\underline{CH_3}$), 14.2 (ester $\underline{CH_3}CH_2$, hept CH_3)

HRMS: $C_{20}H_{35}O_5S$ requires 387.2205. APCI: Found 387.2199 $[M+H]^+$.

ν_{max} (ATR/cm^{-1}): 2958 (C-H stretch), 2931 (C-H stretch), 2873 (C-H stretch), 1729 (C=O), 1690 (C=O)

***R*f:** 0.31 (Hexanes:EtOAc 96:4)

Diethyl 3-methyl-4-(((3-(p-tolyl)propanoyl)thio)methyl)cyclopentane-1,1-dicarboxylate (209)



Prepared as per **Procedure 1** using diethyl diallyl malonate (0.15 mL, 0.62 mmol) and the crude thioacid obtained from **Procedure 3** using *S*-trityl-3-(p-tolyl)propanethioate **140** (315 mg, 0.74 mmol), with DPAP (16 mg, 0.062 mmol) and MAP (9.5 mg, 0.062 mmol) in EtOAc (6 mL). Column chromatography (hexanes:EtOAc 9:1) gave a colourless oil (150 mg, 65%).

Dias.ratio: *cis:trans* 8:1

δ_H (400 MHz, $CDCl_3$) 7.11 – 7.05 (m, 4H, ArCH), 4.17 (qd, *J* = 7.2, 2.3 Hz, 4H, ester $\underline{CH_2}CH_3$ x 2), 2.96-2.91 (m, 3H, ArqC-$\underline{CH_2}$, thioester CH_2), 2.86 – 2.76 (m, 3H, ArqC-$CH_2\underline{CH_2}$, thioester CH_2), 2.47-2.36 (m, 2H, ring CH_2), 2.31 (s, 3H, ArqC-CH_3), 2.25 – 2.12 (m, 2H, ring CH_2), 2.06 (dd, *J* = 13.3, 8.7 Hz, 1H, ring CH), 1.99 (dd, *J* = 13.8, 5.7 Hz, 1H, ring CH), 1.24 (td, *J* = 7.2, 1.2 Hz, 6H, ester $CH_2\underline{CH_3}$ x 2), 0.91 (d, *J* = 6.9 Hz, 3H, ring $CH\underline{CH_3}$ (*cis*)).

If *trans*, 1.03 (d, *J* = 5.9 Hz, 3H, ring $CH\underline{CH_3}$ (*trans*))

δ_C (101 MHz, $CDCl_3$) 198.6 (thioester C=O), 172.7 (ester C=O), 129.3 (ArqC-CH_2CH_2), 128.3 (ArqC-CH_3), 61.6 (ArCH), 58.9 (ArCH), 45.8 (thioester SC=OCH_2), 42.6 (ring CH), 41.3 (ring CHCH_2), 38.3 (ring CH_2), 36.2 (ring CH), 31.2 (ArqC-CH_2CH_2), 29.6, 21.1 ($ArCH_3$), 14.9 (ester CH_2CH_3), 14.2 (ring CHCH_3).

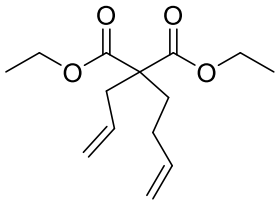
HRMS: $C_{23}H_{33}O_5S$ requires 421.2049. APCI: Found 421.2049 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 2963 (C-H stretch), 1723 (C=O stretch)

***R*f:** 0.27 (Hexanes:EtOAc 9:1)

6.3.2 Aliphatic and Aromatic Substrate Expansion

Diethyl 2-allyl-2-(but-3-en-1-yl)malonate (211)[320]



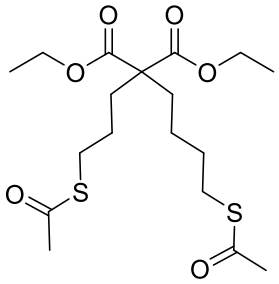
Sodium metal (117 mg, 5.07 mmol) was added to dry EtOH (5 mL). Following complete dissolution, diethyl allylmalonate (1 mL, 5.07 mmol) was added dropwise, and the resulting solution stirred at rt for 10 min. 5-bromo-1-butene (0.72 mL, 6.08 mmol) was added dropwise at rt, and the solution was then heated to reflux for 3 h. After the elapsed time the reaction was cooled, acidified with DOWEX 50wx8 H^+ resin and filtered. After concentration *in vacuo*, the residue was subject to chromatographic purification (hexanes → hexanes:EtOAc 98:2) to yield **211** as a colourless oil (532 mg, 41%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 5.82 – 5.71 (m, 1H, CH=CH_2), 5.70 – 5.58 (m, 1H, CH=CH_2), 5.12 – 4.93 (m, 4H, CH=CH_2 x 2), 4.18 (q, *J* = 7.1 Hz, 4H, CH_3CH_2), 2.64 (d, *J* = 7.4 Hz, 2H, CH_2CH=CH_2), 2.05 (q, *J* = 7.2 Hz, 2H, CH_2CH_2CH=CH_2), 1.24 (t, *J* = 7.1 Hz, 6H, ester CH_3CH_2 x 2)

δ_c (101 MHz, $CDCl_3$) 171.4 (ester C=O), 138.25 (CH_2CH_2CH=CH_2), 132.7 (CH_2CH=CH_2), 118.9 (CH=CH_2), 115.1 (CH=CH_2), 61.3 (ester CH_3CH_2 x 2), 57.5 (ester qC), 37.1 (CH_2CH=CH_2), 31.8 (CH_2CH_2CH=CH_2), 23.4 (CH_2CH_2CH=CH_2), 14.3 (ester CH_3CH_2)

HRMS: $C_{14}H_{22}NaO_4$ requires 277.1410. ESI^+: Found 277.1409 $[M+Na]^+$

Diethyl 2-(4-(acetylthio)butyl)-2-(3-(acetylthio)propyl)malonate (212)



Prepared as per **Procedure 1** using **211** (177 mg, 0.70 mmol) and thioacetic acid (64 mg, 0.84 mmol), with DPAP (18 mg, 0.07 mmol) and MAP (11 mg, 0.07 mmol) in EtOAc (7 mL). Column chromatography (hexanes:EtOAc 95:5) gave **212** as a colourless oil (37.9 mg, 13%).

δ_H (400 MHz, $CDCl_3$) 4.17 (q, J = 7.11 Hz, 4H, ester $\underline{CH_2}CH_3$ x 2), 2.88 – 2.82 (m, 4H, *S*-$\underline{CH_2}CH_2$ x 2), 2.32 – 2.30 (m, 6H, thioester CH_3 x 2), 1.94 – 1.89 (m, 2H, short arm qC$\underline{CH_2}CH_2$), 1.86 – 1.81 (m, 2H, long arm qC$\underline{CH_2}CH_2$), 1.61 – 1.52 (m, 2H, long arm $SCH_2\underline{CH_2}$), 1.49 – 1.41 (m, 2H, short arm $SCH_2\underline{CH_2}$), 1.28 – 1.18 (m, 8H, ester $CH_2\underline{CH_3}$ x 2, long arm $qCCH_2\underline{CH_2}$)

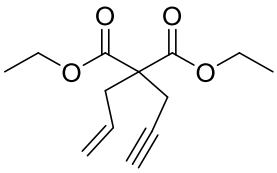
δ_C (101 MHz, $CDCl_3$) 195.8 (long arm thioester C=O), 195.5 (short arm thioester C=O), 171.4 (ester C=O x 2), 61.2 (ester $CH_3\underline{CH_2}$ x 2), 57.1 (ester qC), 31.9 (long arm qC$\underline{CH_2}$), 31.6 (short arm qC$\underline{CH_2}$), 30.6 (thioester CH_3), 29.6 (long arm $SCH_2\underline{CH_2}$), 29.2 (short arm S$\underline{CH_2}$), 28.7 (long arm S$\underline{CH_2}$), 24.4 (short arm $SCH_2\underline{CH_2}$), 23.3 (long arm $qCCH_2\underline{CH_2}$), 14.1 (ester $CH_2\underline{CH_3}$)

HRMS: $C_{18}H_{30}NaO_6S_2$ requires 429.1376. ESI^+: Found 429.1380 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2981 (C-H stretch), 1725 (C=O stretch), 1688 (C=O) stretch

***R*f:** 0.28 (Hexanes:EtOAc 95:5)

Diethyl 2-allyl-2-(prop-2-yn-1-yl)malonate (213)[321]

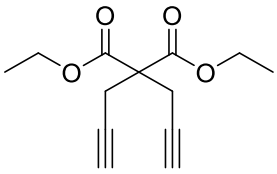


Diethyl allylmalonate (1.0 g, 5.0 mmol) was added to dry Et_2O (10 mL) and cooled to 0 °C. Separately, sodium metal (115 mg, 5 mmol) was dissolved in dry EtOH (5 mL). Once dissolved, the freshly prepared sodium ethoxide solution was added dropwise to the ethereal solution, and the reaction stirred vigorously for 10 min at rt. Propargyl bromide (80% in toluene, 0.65 mL, 6 mmol) was added dropwise to the reaction. Following completion of addition, the resulting solution was stirred at rt for 1 h. After 1 h, brine (100 mL) was added, and the aq. phase extracted with Et_2O (3 x 100 mL). Organic layers were combined, dried over $MgSO_4$ and the magnesium salts filtered. This solution was concentrated *in vacuo* to yield **213** as a colourless oil (1.096 g, 92%), which was used without further purification. The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 5.69 – 5.57 (m, 1H, $CH_2\underline{CH}=CH_2$), 5.22 – 5.10 (m, 2H ($CH=\underline{CH_2}$), 4.24 – 4.17 (m, 4H, ester $CH_3\underline{CH_2}$), 2.83 – 2.77 (m, 4H, $\underline{CH_2}CH=CH_2$, $\underline{CH_2}C\equiv CH$), 2.01 (s, 1H, $CH_2C\equiv\underline{CH}$), 1.25 (t, J = 7.5 Hz, 6H, ester $\underline{CH_3}CH_2$)

HRMS: $C_{13}H_{19}O_4$ requires 239.1283. APCI: Found 239.1280 $[M+H]^+$

Diethyl 2,2-di(prop-2-yn-1-yl)malonate (217)[322]



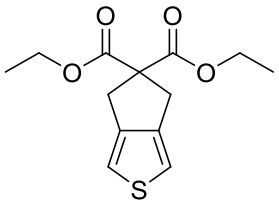
Diethyl malonate (0.76 mL, 5 mmol) was added to dry Et_2O (20 mL) and cooled to 0 °C. Separately, sodium metal (230 mg, 10 mmol) was dissolved in dry EtOH (50 mL). The freshly prepared sodium ethoxide solution was added dropwise to the ethereal reaction mixture, and stirred vigorously for 10 min. Propargyl bromide (80% in toluene, 1.3 mL, 12 mmol) was added dropwise while the reaction temperature was kept at 0 °C.

Following completion of addition, the resulting solution was stirred at rt for 1h. Brine (100 mL) was added and the aq. phase extracted with Et_2O (3 x 100 mL). Organic layers were combined, dried over $MgSO_4$ and the magnesium salts filtered. This solution was concentrated *in vacuo* to yield a yellow residue, which was distilled under vacuum to give **217** as a colourless oil (827 mg, 70%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 4.23 (q, *J* = 7.1 Hz, 4H, ester $CH_3\underline{CH_2}$), 2.99 (d, *J* = 2.6 Hz, 4H, $\underline{CH_2}CCH$), 2.02 (t, *J* = 2.6 Hz, 2H), 1.26 (t, *J* = 7.2 Hz, 6H, ester $\underline{CH_3}CH_2$)

HRMS: $C_{13}H_{16}NaO_4$ requires 259.0946. ESI: Found 259.0938 $[M+Na]^+$

Diethyl 4H-cyclopenta[c]thiophene-5,5(6H)-dicarboxylate (220)[323]

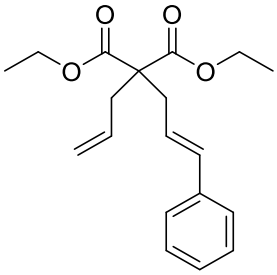


Prepared as per **Procedure 1** using **217** (100 mg, 0.4232 mmol), thioacetic acid (36 μL, 0.5078 mmol), DPAP (11 mg, 0.042 mmol) and MAP (6.4 mg, 0.042 mmol) in EtOAc (4 mL). Subject to chromatographic purification (hexanes → hexanes:EtOAc 98:2) to yield **220** as a colourless oil (45 mg, 40%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 6.81 (s, 2H, C=$\underline{CH}$S x 2), 4.29 – 4.12 (m, 4H, $CH_3\underline{CH_2}$), 3.34 (s, 4H, $\underline{CH_2}C$=CH), 1.28 (t, *J* = 7.1 Hz, 6H, $\underline{CH_3}CH_2$)

HRMS: $C_{13}H_{16}NaO_4S$ requires 291.0667. ESI: Found 291.0661 $[M+Na]^+$

Diethyl 2-allyl-2-cinnamylmalonate (222)[324]

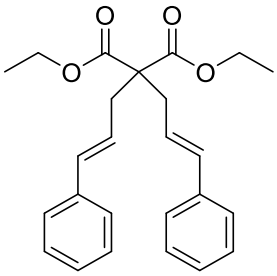


To a solution of NaOEt in EtOH (0.1 M, 5 mL) was added diethyl allyl malonate (1.00 g, 5.00 mmol) and the solution was stirred vigorously for 10 min. The solution was cooled to 0 °C over ice and cinnamyl bromide (1.28 g, 6 mmol) was added dropwise. The resulting solution was heated to reflux and stirred for 2 h. The reaction mixture was cooled over ice and filtered to remove the formed ppt. The crude filtrate was concentrated *in vacuo* and subject to chromatographic purification (hexanes:EtOAc 95:5) to yield a colourless oil (305 mg, 20%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.33 – 7.18 (m, 5H, Ar-CH), 6.47 – 6.42 (m, 1H, CH<u>CH</u>-Ar), 6.09 – 6.00 (m, 1H, CH_2<u>CH</u>CH), 5.77 – 5.65 (m, 1H, CH_2<u>CH</u>CH_2), 5.17 – 5.09 (m, 2H, CH_2CH<u>CH_2</u>), 4.19 (q, *J* = 7.1 Hz, 4H, ester CH_2 x 2), 2.81 – 2.77 (m, 2H, cinnamyl CH_2), 2.71 – 2.67 (m, 2H, allyl CH_2), 1.25 (t, J = 7.1 Hz, 6H, ester CH_3)

HRMS: $C_{19}H_{24}NaO_4$ requires 339.1572. ESI: Found 339.1567 $[M+Na]^+$

Diethyl 2,2-dicinnamylmalonate (223)[325]



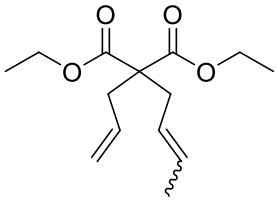
To a solution of NaOEt in EtOH (0.1 M, 10 mL) was added diethyl malonate (801 mg, 5.00 mmol) and the solution was stirred vigorously for 10 min. The solution was cooled to 0 °C over ice and cinnamyl bromide (2.56 g, 12 mmol) was added dropwise. The resulting solution was heated to reflux and stirred for 2 h. The reaction mixture was cooled over ice and filtered to remove the formed ppt. The crude filtrate was concentrated *in vacuo* and subject to chromatographic purification (hexanes:EtOAc 95:5) to yield a

colourless oil (900 mg, 46%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.35 – 7.20 (m, 10H, Ar-CH), 6.50 – 6.44 (m, 2H, CHCH-Ar x 2), 6.14 – 6.06 (m, 2H, CH_2CHCH x 2), 4.22 (q, *J* = 7.1 Hz, 4H, ester CH_2 x 2), 2.85 (dd, *J* = 7.5, 1.1 Hz, 4H, cinnamyl CH_2 x 2), 1.26 (t, *J* = 7.1 Hz, 6H, ester CH_3)

HRMS: $C_{25}H_{28}NaO_4$ requires 415.1885. ESI: Found 415.1882 $[M+Na]^+$

Diethyl-2-allyl-2-(but-2-en-1-yl)malonate (224)[324]



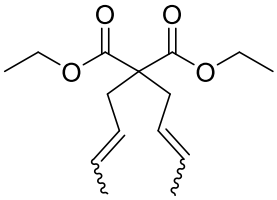
To a solution Na metal in EtOH (1 M, 6 mL)) was added dropwise diethyl allylmalonate (0.99 mL, 5 mmol). Crotyl bromide (0.62 mL, 6 mmol) was carefully added at 0 °C, after which the reaction flask was heated to reflux for 2 h. Following the elapsed time, the solution was cooled and acidified with glacial acetic acid. The acidic solution was then cooled over ice, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexanes:EtOAc 95:5) to afford **224** as a pale yellow oil (660 mg, 52%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 5.73 – 5.44 (m, 2H, CH_2=CH, CH_3CH=CH_2), 5.32 – 5.18 (m, 1H, CH_3CH=CH), 5.14 – 5.03 (m, 2H, CH_2=CH), 4.17 (q, *J* = 7.1 Hz, 4H, ester CH_2 x 2), 2.68 – 2.52 (m, 4H, qC-CH_2 x 2), 1.67 – 1.57 (m, 3H, CH_3CH=CH), 1.28 – 1.19 (m, 6H, ester CH_3 x 2).

HRMS: $C_{14}H_{22}NaO_4$ requires 277.1416. ESI: Found 277.1410 $[M+Na]^+$

***R*f:** 0.46 (hexanes:EtOAc 9:1)

Diethyl 2,2-di(but-2-en-1-yl)malonate (225)[326]



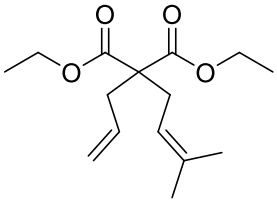
To a solution Na metal in EtOH (1 M, 6 mL) was added dropwise diethyl malonate (0.76 mL, 5 mmol). Crotyl bromide (0.62 mL, 12 mmol) was carefully added at 0 °C, after which the reaction flask was heated to reflux for 2 h. Following the elapsed time, the solution was cooled and acidified with glacial acetic acid. The acidic solution was then cooled over ice, filtered and concentrated *in vacuo.* The residue was purified by flash column chromatography (hexanes:EtOAc 95:5) to afford **225** as a colourless oil (890 g, 67%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 5.58 – 5.43 (m, 2H, $CH_3\underline{CH}$=CH x 2), 5.32 – 5.17 (m, 2H CH_3CH=$\underline{CH}$ x 2), 4.16 (q, *J* = 7.1 Hz, 4H, ester CH_2), 2.66 – 2.50 (m, 4H, qC-CH_2 x 2), 1.67 – 1.60 (m, 6H, $\underline{CH_3}CH$=CH x 2), 1.29 – 1.19 (m, 6H, ester CH_3 x 2).

HRMS: $C_{15}H_{24}NaO_4$ requires 291.1572. ESI: Found 291.1570 $[M+Na]^+$

***R*f:** 0.60 (hexanes:EtOAc 9:1)

Diethyl 2-allyl-2-(3-methylbut-2-en-1-yl)malonate (226)[324]



To a solution Na metal in EtOH (1 M, 6 mL) was added dropwise diethyl allylmalonate (0.99 mL, 5 mmol)). Prenyl bromide (0.69 mL, 6 mmol) was carefully added at 0 °C, after which the reaction flask was heated to reflux for 2 h. Following the elapsed time, the solution was cooled and acidified with glacial acetic acid. The acidic solution was then cooled over ice, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexanes:EtOAc 95:5) to afford **226** as a pale yellow oil (657 mg, 49%). The isolated compound was in good agreement with the literature.

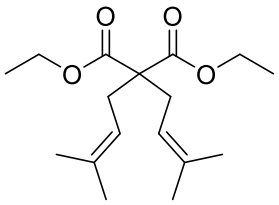
δ_H (400 MHz, $CDCl_3$) 5.73 – 5.58 (m, 1H, CH_2=<u>CH</u>), 5.15 – 5.02 (m, 2H, <u>CH_2</u>=CH), 5.01 – 4.91 (m, 1H, prenyl C=<u>CH</u>), 4.24 – 4.08 (m, 4H, ester CH_2 x 2), 2.65 – 2.55 (m, 4H, C=OC-CH_2 x 2), 1.68 (q, *J* = 1.4 Hz, 3H, prenyl CH_3), 1.60 (q, *J* = 1.4 Hz, 3H, prenyl CH_3),), 1.23 (t, *J* = 7.1 Hz, 6H, ester CH_3 x 2).

HRMS: $C_{15}H_{24}NaO_4$ requires 291.1572. ESI: Found 291.1569 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2975 (C-H stretch), 2912 (C-H stretch), 1723 (C=O stretch), 1177 (ester C-O stretch)

***R*f:** 0.62 (Hexanes:EtOAc 9:1)

Diethyl 2,2-bis(3-methylbut-2-en-1-yl)malonate (227)[327]



To a solution Na metal in EtOH (1 M, 6 mL) was added dropwise diethyl malonate (0.61 mL, 4 mmol). Prenyl bromide (1.11 mL, 9.6 mmol) was carefully added at 0 °C, after which the reaction flask was heated to reflux for 2 h. Following the elapsed time, the solution was cooled and acidified with glacial acetic acid. The acidic solution was then cooled over ice, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexanes:EtOAc 95:5) to afford **227** as a pale yellow oil (581 mg, 49%). The isolated compound was in good agreement with the literature.

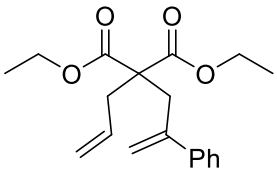
δ_H (400 MHz, $CDCl_3$) 5.00 – 4.91 (m, 2H, prenyl CH x 2), 4.15 (q, *J* = 7.1 Hz, 4H, ester CH_2 x 2), 2.60 – 2.54 (m, 4H, C=OC<u>CH_2</u> x 2), 1.67 (q, *J* = 1.4 Hz, 6H, prenyl CH_3CCH_3), 1.58 (d, *J* = 1.4 Hz, 6H, prenyl CH_3CCH_3), 1.22 (t, *J* = 7.1 Hz, 6H, ester CH_3 x 2).

HRMS: $C_{17}H_{28}NaO_4$ requires 319.1885. ESI: Found 319.1879 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2972 (C-H stretch), 2912 (C-H stretch), 1727 (C=O stretch), 1174 (ester C-O stretch)

***R*f:** 0.61 (Hexanes:EtOAc 95:5)

Diethyl 2-allyl-2-(2-phenylallyl)malonate (228)[328]



To a solution Na metal in EtOH (1 M, 6 mL) was added dropwise diethyl allylmalonate (0.99 mL, 5 mmol). Cinnamyl bromide (0.86 mL, 6 mmol) was carefully added at 0 °C, after which the reaction flask was heated to reflux for 2 h. Following the elapsed time, the solution was cooled and acidified with glacial acetic acid. The acidic solution was then cooled over ice, filtered and concentrated *in vacuo*. The residue was purified by flash column chromatography (hexanes:EtOAc 95:5) to afford **228** as a pale yellow oil (840 mg, 53%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.37 – 7.23 (m, 5H, Ar-CH), 5.63 (ddt, *J* = 17.5, 10.2, 7.3 Hz, 1H, CH_2=<u>CH</u>), 5.28 (d, *J* = 1.7 Hz, 1H, <u>CH_2</u>=CPh), 5.18 (d, *J* = 1.7, 1H, PhC(=<u>CH_2</u>)CH_2), 5.12 – 5.04 (m, 1H, <u>CH_2</u>=CH), 5.06 – 4.96 (m, 1H, <u>CH_2</u>=CH), 3.96 (dq, *J* = 10.7, 7.2 Hz, 2H, ester CH_2), 3.83 (dq, *J* = 10.8, 7.2 Hz, 2H, ester CH_2), 3.18 (s, 2H, PhC(=CH_2)<u>CH_2</u>), 2.60 (dt, *J* = 7.4, 1.3 Hz, 2H, CH_2=CH<u>CH_2</u>), 1.16 (t, *J* = 7.1 Hz, 6H, ester CH_3 x 2).

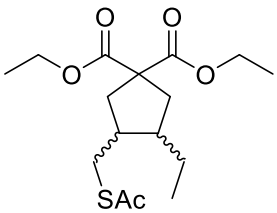
HRMS: $C_{19}H_{24}NaO_4$ requires 339.1572. ESI: Found 339.1568 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2979 (C-H stretch), 1727 (C=O stretch), 1185 (ester C-O stretch)

***R*f:** 0.49 (Hexanes:EtOAc 9:1)

6.3.3 Alicylic Products

Diethyl 3-((acetylthio)methyl)-4-ethylcyclopentane-1,1-dicarboxylate (229)



Prepared as per **Procedure 1** using **224** (254 mg, 1 mmol), thioacetic acid (85 μL, 1.2 mmol), DPAP (25 mg, 0.1 mmol) and MAP (15 mg, 0.1 mmol) in EtOAc (2.6 mL).

Purified *via* column chromatography (hexanes:EtOAc 98:2 → hexanes:EtOAc 95:5) to yield **229** as a colourless oil (204 mg, 62%).

Dias.ratio: n.d.

δ_H (400 MHz, $CDCl_3$) 4.23 – 4.10 (m, 4H, ester CH_2 x 2), 3.02 (dd, *J* = 13.2, 5.4 Hz, 1H, S$\underline{CH_2}$CH), 2.62 (dd, *J* = 13.2, 10.1 Hz, 1H, S$\underline{CH_2}$CH), 2.53 – 2.49 (m, 1H, SCH$_2\underline{CH}$), 2.42 – 2.28 (m, 5H, thioester CH_3, ring $\underline{CH_2}$), 2.28 – 2.17 (m, 1H, SCH$_2\underline{CH}$), 2.12 (dd, *J* = 13.6, 6.1 Hz, 1H, $\underline{CH_2}$), 2.07 – 1.89 (m, 2H, ring $\underline{CH_2}$, $CH_3CH_2\underline{CH}$), 1.52 – 1.37 (m, 1H, $CH_3\underline{CH_2}CH$) 1.36 – 1.15 (m, 7H, ester CH_3 x 2, $CH_3\underline{CH_2}CH$), 0.97 – 0.83 (m, 3H, $\underline{CH_3}CH_2CH$).

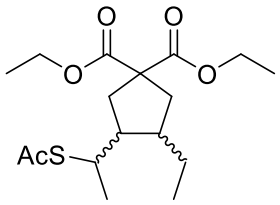
δ_C (101 MHz, $CDCl_3$) 195.8 (thioester C=O), 172.8 (ester C=O), 61.6 (ester CH_2), 58.8 (ester-qC), 44.5 ($CH_3CH_2\underline{CH}$), 41.8 (SCH$_2\underline{CH}$), 38.7 (ring $\underline{CH_2}$), 38.3 (ring $\underline{CH_2}$), 30.7 (S$\underline{CH_2}$CH), 29.6 (thioester CH_3), 22.2 ($CH_3\underline{CH_2}CH$), 14.1 (ester CH_3), 12.8 ($\underline{CH_3}CH_2CH$).

HRMS: $C_{16}H_{26}NaO_5S$ requires 353.1399. ESI: Found 353.1396 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2972 (C-H stretch), 2874 (C-H stretch), 1725 (C=O stretch), 1689

***R*f:** 0.28 (EtOAc:Hexanes 1:9)

Diethyl 3-(1-(acetylthio)ethyl)-4-ethylcyclopentane-1,1-dicarboxylate (230)



Prepared as per **Procedure 1** using **225** (268 mg, 1 mmol), thioacetic acid (85 μL, 1.2 mmol), DPAP (25 mg, 0.1 mmol) and MAP (15 mg, 0.1 mmol) in EtOAc (3.3 mL). Purified *via* column chromatography (hexanes:EtOAc 98:2 → hexanes:EtOAc 95:5) to yield **230** as a colourless oil (197 mg, 57%).

Dias.ratio: n.d.

δ_H (400 MHz, $CDCl_3$) 4.25 – 4.07 (m, 4H, ester CH_2 x 2), 3.63 – 3.41 (m, 1H, S$\underline{CH}$(CH_3)CH), 2.55 – 2.32 (m, 2H, ring $\underline{CH_2}$), 2.32 – 2.27 (m, 3H, thioester CH_3), 2.18

(dtd, *J* = 14.4, 7.2, 1.1 Hz, 1H, ring $\underline{CH_2}$), 2.13 – 2.01 (m, 1H, SCH(CH_3)$\underline{CH}$), 1.98 – 1.88 (m, 2H, ring $\underline{CH_2}$, $CH_3CH_2\underline{CH}$), 1.36 – 1.30 (m, 3H, SCH($\underline{CH_3}$)CH), 1.30 – 1.18 (m, 6H, ester CH_3 x 2), 0.96 – 0.80 (m, 3H, $\underline{CH_3}CH_2CH$).

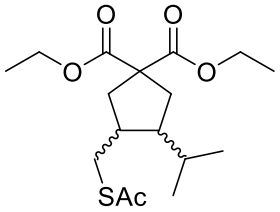
δ_C (101 MHz, $CDCl_3$) 195.7 (thioester C=O), 172.7 (ester C=O), 61.6 (ester CH_2), 58.4 (ester qC), 48.8 (SCH(CH_3)$\underline{CH}$), 43.1 ($CH_3CH_2\underline{CH}$), 40.4 (S$\underline{CH}$(CH_3)CH) 37.6, 37.4, 37.1, 36.9, 31.0, 30.9 (thioester CH_3), 22.1 (ring CH_2), 21.6 (CH_3), 14.1 (CH_3), 12.3 (CH_3)

HRMS: $C_{17}H_{28}NaO_5S$ requires 367.1555. ESI: Found 367.1544 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2968 (C-H stretch), 2871 (C-H stretch), 1726 (C=O stretch), 1688

***R*$_f$:** 0.33 (EtOAc:Hexanes 1:9), 0.15 (EtOAc:Hexanes 5:95)

Diethyl 3-((acetylthio)methyl)-4-isopropylcyclopentane-1,1-dicarboxylate (232)



Prepared as per **Procedure 1** using **226** (268 mg, 1 mmol), thioacetic acid (85 μL, 1.2 mmol), DPAP (25 mg, 0.1 mmol) and MAP (15 mg, 0.1 mmol) in EtOAc (2.5 mL). Purified *via* column chromatography (hexanes:EtOAc 98:2 → hexanes:EtOAc 95:5) to yield **232** as a colourless oil (228 mg, 66%).

Dias.ratio: n.d.

δ_H (400 MHz, $CDCl_3$) δ 4.26 – 4.08 (m, 4H, ester CH_2), 3.17 (ddd, *J* = 13.0, 3.1, 1.5 Hz, 1H, S$\underline{CH_2}$CH), 2.55 – 2.41 (m, 1H, ring CH_2), 2.39 – 2.33 (m, 1H, S$\underline{CH_2}$CH) 2.31 (s, 3H, thioester CH_3), 2.29 – 2.21 (m, 1H, $SCH_2\underline{CH}$), 1.99 – 1.83 (m, 1H, ring CH_2), 1.73 – 1.52 (m, 1H, CH$\underline{CH}$(CH_3)$_2$), 1.23 (td, *J* = 7.1, 3.9 Hz, 6H, ester CH_3 x 2), 1.04 (d, *J* = 6.2 Hz, 3H, CH$\underline{CH}$(CH_3)$_2$), 0.94 (dd, *J* = 6.2 Hz, 3H, CH$\underline{CH}$(CH_3)$_2$).

δ_C (101 MHz, $CDCl_3$) δ 196.1 (thioester C=O), 173.0 (ester C=O), 61.7 (ester CH_2), 58.5 (ester qC), 52.0 ($\underline{CH}$CH(CH_3)$_2$), 41.0 ($SCH_2\underline{CH}$), 38.6 (ring CH_2), 37.2 (ring CH_2), 30.7

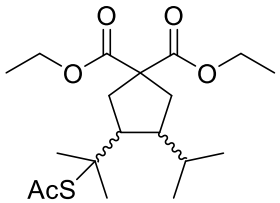
(thioester CH_3), 29.1 ($S\underline{CH_2}CH$), 28.8 ($CH\underline{CH}(CH_3)_2$), 22.2 ($CHCH(\underline{CH_3})_2$), 21.8 ($CHCH(\underline{CH_3})_2$), 14.2 (ester CH_3).

HRMS: $C_{17}H_{28}NaO_5S$ requires 367.1555. ESI: Found 367.1541 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2963 (C-H stretch), 2160 (C-H stretch), 1722 (C=O stretch), 1683

***R*f:** 0.41 (EtOAc:Hexanes 1:9)

Diethyl 3-(2-(acetylthio)propan-2-yl)-4-isopropylcyclopentane-1,1-dicarboxylate (233)



Prepared as per **Procedure 1** using **227** (296 mg, 1 mmol), thioacetic acid (85 μL, 1.2 mmol), DPAP (25 mg, 0.1 mmol) and MAP (15 mg, 0.1 mmol) in EtOAc (2 mL). Purified *via* column chromatography (hexanes:EtOAc 98:2 → hexanes:EtOAc 95:5) to yield **223** as a colourless oil (173 mg, 47%).

Dias.ratio: n.d.

δ_H (400 MHz, $CDCl_3$) δ 4.21 – 4.05 (m, 4H, ester CH_2 x 2), 2.71 – 2.60 (m, 1H, $SC\underline{H}(CH_3)_2CH$), 2.42 (ddd, *J* = 13.5, 3.9, 2.5 Hz, 1H, ring CH_2), 2.31 (s, 3H, thioester CH_3), 2.17 – 2.09 (m, 2H, ring CH_2), 2.08 – 1.90 (m, 2H, ring CH_2), 1.71 – 1.55 (m, 1H, $\underline{CH}(CH_3)_2CH$), 1.33 – 1.19 (m, 6H, $SCH(C\underline{H_3})_2C$), 1.04 – 0.76 (m, 6H, ester CH_3 x 2).

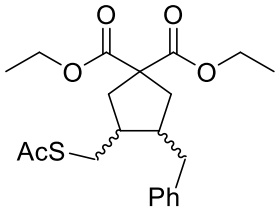
δ_C (101 MHz, $CDCl_3$) δ 199.8 (thioester C=O), 171.6 (ester C=O), 61.7 (ester CH_2), 55.8 (ester qC), 51.4 ($S\underline{CH}(CH_3)_2CH$), 48.5 (ring CH), 34.9 (ring CH_2), 30.9 (thioester CH_3), 30.6 (ring CH_2) 26.7 (ring CH_2) , 26.6 ($\underline{CH}(CH_3)_2CH$), 24.6 (CH_3), 18.4 (CH_3), 16.9 (CH_3), 14.2 (CH_3), 14.0 (CH_3).

HRMS: $C_{19}H_{32}NaO_5S$ requires 395.1868. ESI: Found 395.1853 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2964 (C-H stretch), 1727 (C=O stretch), 1693

***R*f:** 0.39 (EtOAc:Hexanes 1:9)

Diethyl 3-((acetylthio)methyl)-4-benzylcyclopentane-1,1-dicarboxylate (231)



Prepared as per **Procedure 1** using **222** (153 mg, 0.48 mmol), thioacetic acid (41 μL, 0.58 mmol), DPAP (12.8 mg, 0.05 mmol) and MAP (7.5 mg, 0.05 mmol) in EtOAc (10 mL). Purified *via* column chromatography (hexanes:EtOAc 98:2 → hexanes:EtOAc 95:5) to yield **231** as a colourless oil (28 mg, 15%).

Dias.ratio: n.d.

δ_H (600 MHz, $CDCl_3$) 7.28 (dd, *J* = 8.3, 7.0 Hz, 2H, Ar CH)), 7.21 – 7.18 (m, 3H, Ar CH)), 4.22 – 4.10 (m, 4H, ester CH_2), 3.11 (dd, *J* = 13.2, 6.3 Hz, 1H, S$\underline{CH_2}$CH), 2.86 (dd, *J* = 13.2, 9.5 Hz, 1H, S$\underline{CH_2}$CH), 2.80 (d, *J* = 8.4 Hz, 1H, Ph$\underline{CH_2}$CH), 2.48 – 2.39 (m, 3H, Ph$\underline{CH_2}$CH, SCH$_2\underline{CH}$, ring CH_2), 2.34 (d, *J* = 2.0 Hz, 3H, thioester CH_3), 2.33 – 2.27 (m, 1H, PhCH$_2\underline{CH}$) 2.27 – 2.15 (m, 2H, ring CH_2), 2.14 – 2.03 (m, 1H, ring CH_2), 1.24 (t, *J* = 7.1 Hz, 3H, ester CH_3), 1.20 (t, *J* = 7.1 Hz, 3H, ester CH_3).

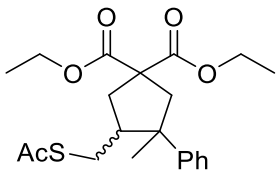
δ_C (151 MHz, $CDCl_3$) 195.7 (thioester C=O), 172.8 (ester C=O), 172.6 (ester C=O), 140.7 (Ar qC), 129.1 (Ar CH), 128.5 (Ar CH), 126.1 (*para* Ar CH), 61.7 (ester CH_2), 61.6 (ester CH_2), 58.6 (ester qC), 44.0 (SCH$_2\underline{CH}$), 42.4 (PhCH$_2\underline{CH}$), 38.6 (ring CH_2), 37.9 (ring CH_2), 35.1 (Ph$\underline{CH_2}$CH), 30.8 (thioester CH_3), 29.7 (S$\underline{CH_2}$CH), 14.2 (ester CH_3), 14.1 (ester CH_3).

HRMS: $C_{21}H_{28}NaO_5S$ requires 415.1555. ESI: Found 415.1553 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2979 (C-H stretch), 1723 (C=O stretch),

***R*f:** 0.16 (EtOAc:Hexanes 5:95)

Diethyl 4-((acetylthio)methyl)-3-methyl-3-phenylcyclopentane-1,1-dicarboxylate (234)



Prepared as per **Procedure 1** using **228** (316 mg, 1 mmol), thioacetic acid (85 μL, 1.2 mmol), DPAP (25 mg, 0.1 mmol) and MAP (15 mg, 0.1 mmol) in EtOAc (2.65 mL). Purified *via* column chromatography (hexanes:EtOAc 98:2 → hexanes:EtOAc 95:5) to yield **234** as a colourless oil (43 mg, 11%).

Dias.ratio: *cis*:*trans* 1.8:1

δ_H (400 MHz, $CDCl_3$) 7.37 – 7.27 (m, 3H, Ar-CH), 7.24 – 7.17 (m, 2H, Ar-CH), 4.29 – 4.09 (m, 4H, ester CH_2), 3.42 (dd, *J* = 13.4, 1.3 Hz, 1H S$\underline{CH_2}$CH), 3.23 (dd, *J* = 16.3, 13.4 Hz, 1H, S$\underline{CH_2}$CH), 3.03 – 2.86 (m, 1H, ring CH_2), 2.73 – 2.59 (m, 1H, ring CH_2), 2.58 – 2.48 (m, 1H, ring CH_2), 2.47 – 2.24 (m, 2H, $SCH_2\underline{CH}$, ring CH_2), 2.25 – 2.19 (m, 3H, thioester CH_3) 1.35 – 1.13 (m, 6H, ester CH_3), 1.01 (d, *J* = 6.8 Hz, 3H, $\underline{CH_3}$C(Ph)),

If *trans*, 0.62 (d, *J* = 7.0 Hz, 3H, $\underline{CH_3}$C(Ph)).

δ_C (101 MHz, $CDCl_3$) 195.3 (thioester C=O), 172.8 (ester C=O), 172.3 (ester C=O), 144.7 (Ar qC), 128.4 (Ar-CH), 128.1 (Ar-CH), 127.9 (Ar-CH), 126.5 (Ar-CH), 61.8 (ester CH_2), 58.1 (ester qC), 52.4 ($CH_3\underline{C}$(Ph)), 45.7 ($SCH_2\underline{CH}$), 45.0 (C(Ph)$\underline{CH_2}$), 41.1 (ring CH_2), 34.0 (S$\underline{CH_2}$CH), 30.7 (thioester CH_3), 30.6 ($\underline{CH_3}$C(Ph)), 14.2 (ester CH_3)

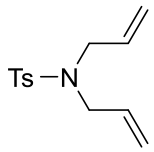
HRMS: $C_{21}H_{28}NaO_5S$ requires 415.1555. ESI: Found 415.1553 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2979 (C-H stretch), 1724 (C=O stretch), 1690

***R*f:** 0.3 (EtOAc:Hexanes 1:9)

6.3.4 Heteroatomic Species

N,*N*-diallyl-4-methylbenzenesulfonamide (238)[329]

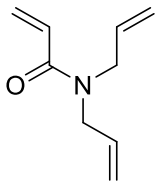


Triethylamine (1.5 mL, 10.68 mmol) and tosyl chloride **237** (2.0 g, 10.48 mmol) were added to a solution of diallylamine **236** (1.27 mL, 10.29 mmol) in CH_2Cl_2 (35 mL). The resulting solution was stirred at rt for 18 h. Following completion of the reaction, the organic phase was washed with aq. $KHSO_4$ (10%, 2 x 100 mL), sat. aq. $NaHCO_3$ (2 x 100 mL), and H_2O (100 mL). The organic phase was then dried over $MgSO_4$, filtered, and concentrated *in vacuo* to yield **238** as a pale yellow oil (2.24 g, 86%), which was used without further purification. The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.73 – 7.68 (d, *J* = 8.0 Hz, 2H, ArCH x 2), 7.29 (d, *J* = 8.0 Hz, 2H, ArCH x 2), 5.67 – 5.55 (m, 2H, $NCH_2\underline{CH}$), 5.18 – 5.10 (m, 4H, $NCH_2CH\underline{CH_2}$ x 2), 3.80 (d, *J* = 6.3 Hz, 4H, $N\underline{CH_2}CH$ x 2), 2.43 (s, 3H, Tosyl CH_3)

HRMS: $C_{13}H_{18}NO_2S$ requires 252.1058. APCI: Found 252.1053 $[M+H]^+$

N,*N*-diallylacrylamide (239)[330]



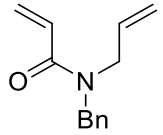
To a solution of diallylamine **236** (0.62 mL, 5.00 mmol) in toluene (10 mL) at 0 °C was added triethylamine (0.84 mL, 6.00 mmol) and acryloyl chloride **122** (0.49 mL, 6.00 mmol). The resulting solution was stirred at 0 °C for 3 h, during which triethylamine hydrochloride was seen to precipitate. Following the elapsed time, the solution was filtered, the precipitate washed with ice-cold Et_2O and concentrated *in vacuo*. The residue was subject to chromatographic purification (hexanes:EtOAc 8:2) to yield 239 as a colourless oil (316 mg, 42%).

δ_H (600 MHz, DMSO) 6.66 (dd, *J* = 16.7, 10.3 Hz, 1H, CH_2=<u>CH</u>C=O), 6.17 (dd, *J* = 16.6, 2.5 Hz, 1H, <u>CH_2</u>=CHC=O), 5.91 – 5.72 (m, 2H, CH_2=<u>CH</u>CH_2N x 2), 5.69 (dd, *J* = 10.3, 2.4 Hz, 1H, <u>CH_2</u>=CHC=O), 5.22 – 5.08 (m, 4H, <u>CH_2</u>=CHCH_2N x 2), 3.98 (ddt, *J* = 23.8, 5.9, 1.7 Hz, 4H, CH_2=CH<u>CH_2</u>N).

δ_C (151 MHz, DMSO) 165.1 (N<u>C</u>=O), 134.2 (CH_2=<u>CH</u>CH_2N), 133.6 (<u>CH_2</u>=<u>CH</u>CH_2N), 128.3 (CH_2=<u>CH</u>C=O), 127.6 (<u>CH_2</u>=CHC=O), 117.0 (<u>CH_2</u>=CHCH_2N), 116.1 (<u>CH_2</u>=CHCH_2N), 48.8 (CH_2=CH<u>CH_2</u>N), 47.8 (CH_2=CH<u>CH_2</u>N).

HRMS: $C_9H_{13}NNaO$ requires 174.0889. ESI^+: Found 174.0887 $[M+Na]^+$

N-allyl-*N*-benzylacrylamide (241)[277]

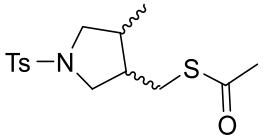


To a solution of *N*-allylbenzylamine **240** (0.78 mL, 5.00 mmol) in toluene (10 mL) at 0 °C was added triethylamine (0.84 mL, 6.00 mmol), followed by acryloyl chloride **122** (0.49 mL, 6.00 mmol). The resulting solution was stirred at 0 °C for 3 h, during which triethylamine hydrochloride was seen to precipitate. Following the elapsed time, the solution was filtered, the precipitate washed with ice-cold Et_2O and concentrated *in vacuo*. The residue was subject to chromatographic purification (hexanes:EtOAc 9:1 → hexanes:EtOAc 7:3) to yield **241** as a colourless oil (430 mg, 43%).

δ_H (400 MHz, $CDCl_3$) 7.39 – 7.16 (m, 5H), 6.55 (dd, J = 16.7, 10.2 Hz, 1H, NC=O<u>CH</u>), 6.43 (dd, J = 16.7, 2.2 Hz, 1H, NC=OCH<u>CH_2</u>), 5.87 – 5.66 (m, 1H, allyl <u>CH</u>CH_2), 5.25 – 5.10 (m, 2H, NCH_2CH<u>CH_2</u>), 4.65 (s, 1H, Ar<u>CH_2</u>), 4.58 (s, 1H, $ArCH_2$), 4.06 (d, J = 5.9 Hz, 1H, N<u>CH_2</u>$CHCH_2$), 3.89 (dd, J = 8.0, 5.3 Hz, 1H, N<u>CH_2</u>$CHCH_2$)

HRMS: $C_{13}H_{15}NNaO$ requires 224.1046. ESI^+: Found 224.1050 $[M+Na]^+$

S-((4-methyl-1-tosylpyrrolidin-3-yl)methyl) ethanethioate (238a)[222]



Prepared as per **Procedure 1** using **238** (200 mg, 0.79 mmol), thioacetic acid (68 μL, 0.95 mmol), DPAP (20 mg, 0.08 mmol) and MAP (12 mg, 0.08 mmol) in EtOAc (8 mL). Purified *via* column chromatography (hexanes:EtOAc 9:1) to yield **238a** as a pale yellow oil (233 mg, 90%). The isolated compound was in good agreement with the literature.

Dias.ratio: *cis*:*trans* 2.5:1

δ_H (400 MHz, $CDCl_3$) 7.69 (dd, *J* = 8.2, 2.6 Hz, 2H, *ortho* Ar-CH), 7.31 (dd, *J* = 8.4, 2.2 Hz, 2H, *meta* Ar-CH), 3.53 – 3.32 (m, 2H, NCH_2), 3.09 – 2.74 (m, 3H, NCH_2, SCH_2CH), 2.62 (m, 1H, SCH_2CH), 2.42 (d, *J* = 2.5 Hz, 3H, Ar-CH_3), 2.30 (d, *J* = 4.2 Hz, 3H, thioester CH_3), 2.28 – 2.11 (m, 1H, CH_3CH), 1.90 – 1.74 (m, 1H, SCH_2CH), 0.81 (d, *J* = 6.5 Hz, 3H, $CHCH_3$).

If *trans*¸ 0.95 (d, *J* = 5.7 Hz, 3H, $CHCH_3$),

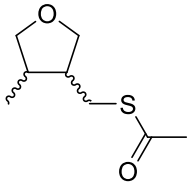
δ_C (101 MHz, $CDCl_3$) 195.2 (thioester C=O), 143.6 (CH_3-Ar-qC), 134.0 (Ar qC), 133.8 (Ar CH), 129.8 (Ar CH), 127.6 (Ar CH), 127.5 (Ar CH), 54.8 (NCH_2), 54.5 (NCH_2), 41.9 (SCH_2CH), 35.6 (CH_3CH), 30.7 (SCH_2), 27.8 (thioester CH_3), 21.6 (Ar CH_3), 13.2 (CH_3CH).

HRMS: $C_{15}H_{21}NNaO_3S_2$ requires 350.0861. ESI: Found 350.0868 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2884 (C-H stretch), 1688 (C=O)

***R*$_f$:** 0.34 (EtOAc:Hexanes 1:9)

S-((4-methyltetrahydrofuran-3-yl)methyl) ethanethioate (245)



Prepared as per **Procedure 1** using **242** (200 mg, 2.04 mmol), thioacetic acid (0.17 mL, 2.45 mmol), DPAP (52 mg, 0.2 mmol) and MAP (31 mg, 0.2 mmol) in EtOAc (20 mL). Purified *via* column chromatography (hexanes:EtOAc 98:2 → hexanes:EtOAc 95:5) to yield **245** as a colourless oil (212 mg, 60%).

Dias.ratio: *cis*:*trans* 3:1

δ_H (400 MHz, $CDCl_3$) 4.04 – 3.86 (m, 2H, OCH_2), 3.59 – 3.41 (m, 2H, OCH_2), 3.15 – 2.76 (m, 2H, $CHCH_2S$), 2.51 (s, 1H, ring CH), 2.43 – 2.36 (m, 1H, ring CH), 2.34 (s, 3H, thioester CH_3), 1.01 (d, *J* = 6.8 Hz, 3H, $CHCH_3$ (*cis*)).

If *trans*, 1.07 (d, *J* = 6.4 Hz, 1H, ring $CHCH_3$ (*trans*)).

δ_C (101 MHz, $CDCl_3$) 195.6 (thioester C=O), 75.0 (CH_3CHCH_2), 71.9 (SCH_2CHCH_2), 42.4 (SCH_2CHCH_2), 36.3 (CH_3CHCH_2), 30.8 (SCH_2CHCH_2), 27.8 (thioester CH_3), 12.9 (methyl CH_3).

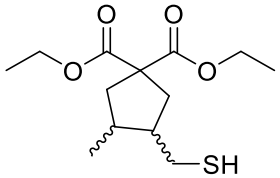
HRMS: $C_8H_{15}O_2S$ requires 175.0793. APCI: Found 175.0796 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 2971 (C-H stretch), 2933 (C-H stretch), 1689 (C=O stretch)

***R*f:** 0.47 (Hexanes:EtOAc 8:2)

6.3.5 Ramberg-Bäcklund Chemistry

Diethyl 3-(mercaptomethyl)-4-methylcyclopentane-1,1-dicarboxylate (253)



To a solution of **206** (200 mg, 0.63 mmol) in dry EtOH (10 mL) was added a catalytic amount of freshly prepared sodium ethoxide. The resulting solution was stirred for 30 m at rt, after which it was quenched by addition of DOWEX 50wx8 H^+ resin, filtered, and the filtrate concentrated *in vacuo*. This gave **253** as a colourless oil (175 mg, 100%), which was used without further purification.

δ_H (400 MHz, $CDCl_3$) 4.17 (q, *J* = 7.1 Hz, 4H, ester CH_2), 2.62 – 2.50 (m, 1H, $CHCH_2SH$), 2.49 – 2.37 (m, 2H, ring CH_2, $CHCH_2SH$), 2.35 – 2.21 (m, 1H, $CHCH_3$), 2.19 – 2.07 (m, 2H, $CHCH_2SH$, ring CH_2), 2.03 – 1.92 (m, 1H, ring CH_2), 1.24 (t, *J* = 7.1, 6H, ester CH_3), 0.89 (dd, *J* = 7.1, 2.6 Hz, 3H, $CHCH_3$).

δ_C (101 MHz, $CDCl_3$) 172.9 (ester C=O), 61.6 (ester CH_2), 58.9 (ester qC), 46.7, ($CHCH_2SH$), 41.3 (ring CH_2), 37.9 (ring CH_2), 35.8 ($CHCH_3$), 25.1 ($CHCH_2SH$), 14.6 ($CHCH_3$), 14.2 (ester CH_3).

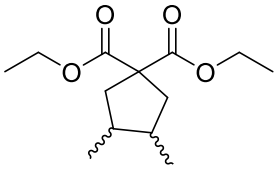
HRMS: $C_{13}H_{23}O_4S$ requires 275.1317. APCI: Found 275.1310 $[M+H]^+$.

Disulfide: $C_{26}H_{43}O_8S_2$ requires 547.2399. APCI: Found 547.2424 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 2979 (C-H stretch), 1725 (C=O stretch), 1679 (C=O stretch)

***R*f:** 0.59 (Hexanes:EtOAc 8:2). Disulfide at *ca.* 0.32 in same system.

Diethyl 3,4-dimethylcyclopentane-1,1-dicarboxylate (255)[331]



To a solution of thiol **253** (434 mg, 1.58 mmol) and TCEP **254** (1.186 g, 4.7406 mmol) in DMF (15 mL) was added DPAP (40 mg, 0.158 mmol) and MAP (24 mg, 0.158 mmol). The resulting solution was irradiated under Ar for 1 h. Upon completion of the reaction, the solution was concentrated *in vacuo*, and suspended in EtOAc (100 mL). The organic phase was washed with HCl (1M, 1 x 100 mL), sat. aq. $NaHCO_3$ (1 x 100 mL), brine (1 x 100 mL), and dried over $MgSO_4$. This suspension was filtered, concentrated *in vacuo*, and subject to chromatographic purification (hexanes:EtOAc 99:1 → 95:5) to yield **255** as a colourless oil (245 mg, 66%). The isolated compound was in good agreement with the literature.

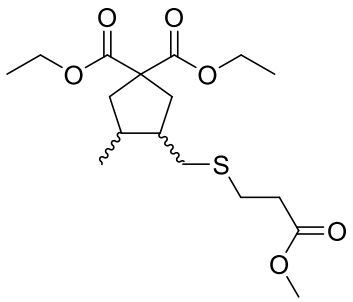
Dias.ratio: *cis:trans* 8:1

δ_H (400 MHz, $CDCl_3$) 4.20 – 4.13 (m, 4H, ester $CH_3\underline{CH_2}$ x 2), 2.38 (dd, *J* = 13.6, 7.0 Hz, 2H, ring CH_2), 2.10 (dq, *J* = 11.7, 6.8 Hz, 2H, ring CH), 1.94 (dd, *J* = 13.6, 7.0 Hz, 2H, ring CH_2), 1.27 – 1.20 (m, 6H, ester $\underline{CH_3}CH_2$), 0.89 – 0.84 (m, 6H, ring $CH\underline{CH_3}$ (*cis*))

If *trans*, 0.98 – 0.95 (m, 6H, ring $CH\underline{CH_3}$ (*trans*))

HRMS: $C_{13}H_{22}NaO_4$ requires 265.1409. ESI^+: Found 265.1410 $[M+Na]^+$

Diethyl 3-(((3-methoxy-3-oxopropyl)thio)methyl)-4-methylcyclopentane-1,1-dicarboxylate (253a)



Thiol **253** (336 mg, 1.21 mmol) was dissolved in anhydrous CH_2Cl_2 (10 mL), and NEt_3 (0.34 mL, 2.42 mmol) was added. The resulting solution was stirred at rt for 5 min. To this solution was added methyl acrylate (0.16 mL, 1.82 mmol) dropwise, and the resulting solution was stirred at rt for 16 h. Following completion of the reaction, the organic phase was washed with aq. HCl (1 M, 100 mL), sat. aq. $NaHCO_3$ (100 mL), and brine (100 mL). The organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo* to give **253a** as a yellow oil (238 mg, 55%). The isolated compound was used without further purification.

δ_H (400 MHz, $CDCl_3$) 4.17 (q, *J* = 7.1, 4H, ester CH_2), 3.69 (s, 3H, $\underline{CH_3}OC=O$), 2.81 – 2.72 (m, 2H, $S\underline{CH_2}CH_2$), 2.63 – 2.54 (m, 2H, $SCH_2\underline{CH_2}$), 2.50 – 2.38 (m, 2H, $CH\underline{CH_2}S$), 2.29 – 1.95 (m, 3H, $\underline{CH}CH_3$, $\underline{CH}CH_2S$, ring $\underline{CH_2}$), 1.23 (t, *J* = 7.1, 6H, ester CH_3), 0.89 (dd, *J* = 7.0, 3.2 Hz, 3H, $CH\underline{CH_3}$).

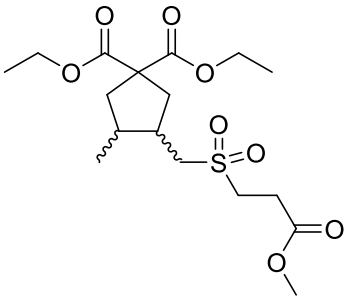
δ_C (101 MHz, $CDCl_3$) 172.9 ($CH_3O\underline{C}=O$), 172.8 (ester C=O), 61.6 (ester CH_2), 59.0 (ester qC), 51.9 ($\underline{CH_3}OC=O$), 42.3 ($\underline{CH}CH_2S$), 41.4 (ring CH_2), 38.3 (ring CH_2), 35.9 ($\underline{CH}CH_3$), 34.9 ($CH\underline{CH_2}S$), 33.0 ($SCH_2\underline{CH_2}$), 27.5 ($S\underline{CH_2}CH_2$), 14.8 ($CH\underline{CH_3}$), 14.2 (ester CH_3).

HRMS: $C_{17}H_{28}NaO_6S$ requires 383.1504. ESI: Found 383.1509 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2957 (C-H stretch), 1724 (C=O stretch)

***R*f:** 0.55 (EtOAc:Hexanes 2:8)

Diethyl 3-(((3-methoxy-3-oxopropyl)sulfonyl)methyl)-4-methylcyclopentane-1,1-dicarboxylate (253b)



To a solution of sulfide **253a** (211 mg, 0.59 mmol) in EtOH (2 mL) was added a solution of Oxone ® (720 mg, 22.34 mmol) in H_2O (2 mL). An exothermic reaction was observed upon addition, and the resulting white slurry was stirred at rt for 16 h. Following the elapsed time, the solution was diluted with H_2O (10 mL), and extracted with $CHCl_3$ (3 x 50 mL). The organic phase was washed with H_2O (100 mL) and brine (100 mL). The organic phase was then dried over $MgSO_4$, filtered, and concentrated *in vacuo*. This gave **253b** as a pale yellow oil (214 mg, 92%). The isolated compound was used without further purification.

δ_H (400 MHz, $CDCl_3$) 4.18 (q, *J* = 7.1 Hz, 4H, ester CH_2), 3.74 (s, 3H $\underline{CH_3}OC=O$), 3.38 – 3.26 (m, 2H, $SO_2\underline{CH_2}CH_2$), 3.12 – 2.96 (m, 2H, $CH\underline{CH_2}SO_2$), 2.94 – 2.83 (m, 2H, $SO_2CH_2\underline{CH_2}$), 2.71 – 2.22 (m, 4H, ring CH_2, $\underline{CH}CH_3$, $\underline{CH}CH_2SO_2$), 1.99 (dd, *J* = 13.8, 5.2 Hz, 1H, ring CH_2), 1.24 (t, *J* = 7.1 Hz, 6H, ester CH_3), 0.91 (d, *J* = 7.1 Hz, 3H, $CH\underline{CH_3}$).

δ_C (101 MHz, $CDCl_3$) 172.3 ($CH_3C=O$), 171.6 (ester C=O), 61.8 (ester CH_2), 58.7 (ester qC), 53.8 ($CH\underline{CH_2}SO_2$), 52.6 ($\underline{CH_3}OC=O$), 49.2 ($SO_2\underline{CH_2}CH_2$), 41.1 (ring CH_2), 38.2 (ring CH_2), 36.6 ($\underline{CH}CH_2SO_2$), 36.5 ($\underline{CH}CH_3$), 26.9 ($SO_2CH_2\underline{CH_2}$), 15.1 ($\underline{CH}CH_3$), 14.1.

HRMS: $C_{17}H_{28}NaO_8S$ requires 415.1403. ESI: Found 415.1409 $[M+Na]^+$

ν_{max} (ATR/cm^{-1}): 2982 (C-H stretch), 1723 (C=O stretch), 1311 (S=O stretch), 1122 (S=O stretch)

***R*f:** 0.45 (EtOAc:Hexanes 2:8)

6.4 Chapter 5 – General Experimental Procedures

Procedure 1: Preparation of per-*O*-acetylated carbohydrates

To a solution of the starting sugar (1.0 equiv.) in pyridine (15.0 equiv.), Ac_2O (13.0 equiv.) was added and the mixture was stirred at rt for 18 h. The reaction was quenched with the addition of H_2O (100 mL) and diluted with EtOAc (100 mL). The organic layer was collected and washed with sat. aq. $CuSO_4$ (2 x 50 mL), dried over $MgSO_4$, filtered and the solvent removed *in vacuo*. The crude material was purified by silica column chromatography.

Procedure 2: *O*-Glycosylation of peracetylated rhamnose

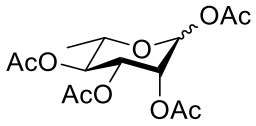
Peracetylated L-rhamnose (1 eq.) was dissolved in anhydrous CH_2Cl_2 over 4Å molecular sieves. The relevant alcohol (1.5 eq.) was added, and the solution cooled to 0 °C. $BF_3{\cdot}OEt_2$ (2 eq.) was added dropwise, and following completion of addition the resulting solution was allowed to warm to rt and stir for 16 h. Following the elapsed time, the reaction was quenched with ice-cold sat. aq. $NaHCO_3$ solution (40 mL). The organic phase was separated, washed with H_2O (2 x 60 mL) and brine (60 mL), and dried over $MgSO_4$. The suspensions was filtered, and concentrated *in vacuo*. Purification of the residue by column chromatography furnished the peracetylated glycosides in good yield.

Procedure 3: *O*-Deacetylation of protected rhamnosides

To a solution of the peracetylated glycoside (1 eq.) in CH_3OH (10 mL) was added a catalytic amount of freshly prepared $NaOCH_3$. The resulting solution was stirred at rt for 30 min, after which the reaction was quenched by addition of DOWEX H^+ 50wx8 resin. The suspension was filtered, and the filtrate concentrated *in vacuo*. This gave the deacetylated *O*-rhamnosides in excellent yield, which were used without further purification.

6.4.1 Chapter 5.1 – Experimental Detail

1,2,3,4-Tetra-*O*-acetyl-α/β-L-rhamnopyranose (269)[332]



Prepared as per **Procedure 1** with L-rhamnose **268** (2.00 g, 12.2 mmol), pyridine (14.74 mL, 182 mmol) and Ac_2O (15.64 mL, 158 mmol). Resulting solution stirred at rt for 18 h. Purification by column chromatography (EtOAc:hexanes 1:1) to yield **269** as a colourless oil (3.45 g, 85%). The isolated compound was in good agreement with the literature.

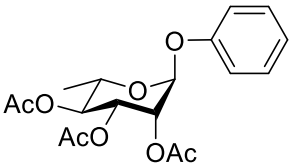
δ_H (400 MHz, $CDCl_3$) 6.00 (s, H-1, 1H), 5.29 – 5.27 (m, H-2, 1H), 5.24 (d, H-3, 1H), 5.10 (app. t, H-4, 1H), 3.93 (dq, *J* = 9.9, 6.2 Hz, H-5, 1H), 2.15 (s, Ac, 3H), 2.14 (s, Ac, 3H), 2.08 (s, Ac, 3H)

HRMS: $C_{14}H_{20}NaO_9$ requires 355.1005. ESI: Found 355.1008 $[M+H]^+$

ν_{max} (ATR/cm^{-1}): 2971 (C-H stretch), 1749 (C=O stretch)

***R_f*:** 0.6 (Hexanes:EtOAc 1:1)

Phenyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside (270)



Prepared as per **Procedure 2** with peracetylated rhamnose **269** (2.17 g, 6.53 mmol), phenol (1.23 g, 13.06 mmol), and $BF_3{\cdot}OEt_2$ (4.04 mL, 32.65 mmol) in CH_2Cl_2 (75 mL). Resulting solution stirred at rt for 18 h. Purification by column chromatography (hexanes:EtOAc 8:2) to yield **270** as a colourless oil (1.96 g, 82%).

δ_H (400 MHz, $CDCl_3$) 7.30 (t, *J* = 7.9 Hz, 2H, *meta* Ar-CH), 7.07 (d, *J* = 7.9 Hz, 2H, *ortho* Ar-CH), 7.05 (t, *J* = 7.4 Hz, 1H, *para* Ar-CH), 5.52 (dd, *J* = 10.1, 3.5 Hz, 1H, H-3), 5.46 (d, *J* = 1.8 Hz, 1H, H-1), 5.43 (dd, *J* = 3.3, 1.8 Hz, 1H, H-2), 5.15 (t, *J* = 10.0 Hz, 1H, H-4) 4.00 (dq, *J* = 12.5, 6.2 Hz, 1H, H-5), 2.19 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.03 (s, 3H, Ac), 1.20 (d, *J* = 6.2 Hz, 1H, H-6)

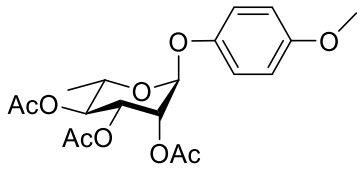
δ_C (101 MHz, $CDCl_3$) 170.2 (Ac C=O), 156.0 (Ar-qC), 129.8 (Ar-C), 122.9 (Ar-C), 116.5 (Ar-C), 95.8 (C-1), 71.2 (C-3), 69.9 (C-4), 69.1 (C-5), 67.3 (C-2), 21.1 (Ac-CH_3), 21.0 (Ac-CH_3), 20.9 (Ac-CH_3), 17.6 (C-6)

HRMS: $C_{18}H_{21}O_8$ requires 365.1236. APCI: Found 365.1242 $[M-H]^-$

ν_{max} (ATR/cm^{-1}): 2946 (C-H stretch), 2834 (C-H stretch), 1736 (C=O stretch), 1019 (ArC-H stretch)

***R*f:** 0.31 (Hexanes:EtOAc 8:2)

4-methoxyphenyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranoside (271)[333]



Prepared as per **Procedure 2** with peracetylated rhamnose **269** (2.32 g, 6.98 mmol), 4-methoxyphenol (1.73 g, 13.96 mmol), and $BF_3{\cdot}OEt_2$ (4.31 mL, 34.9 mmol) in CH_2Cl_2 (75 mL). Resulting solution stirred at rt for 18 h. Purification by column chromatography (hexanes:EtOAc 8:2) to yield **271** as a colourless oil (2.35 g, 85%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.00 (d, *J* = 9.2 Hz, 2H, 2 x *ortho* Ar-CH), 6.83 (d, *J* = 9.2 Hz 2H, 2 x *meta* Ar-CH), 5.50 (dd, *J* = 10.1, 3.5 Hz, 1H, H-3), 5.42 (dd, *J* = 3.5, 1.8 Hz, 1H, H-2), 5.33 (d, *J* = 1.8 Hz, 1H, H-1), 5.13 (t, *J* = 10.1 Hz, 1H, H-4), 4.02 (dq, *J* = 9.9, 6.2 Hz, 1H, H-5), 3.77 (s, 3H, Ar-OCH_3), 2.18 (s, 3H, Ac-CH_3), 2.06 (s, 3H, Ac-CH_3), 2.02 (s, 3H, Ac-CH_3), 1.21 (d, *J* = 6.3 Hz, 3H, CH_3)

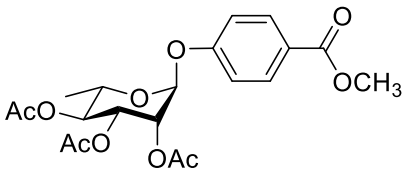
δ_C (101 MHz, $CDCl_3$) 170.2 (Ac C=O), 155.4 (Ar-qC), 150.1 (Ar-qC), 117.8 (Ar-C), 114.8 (Ar-C), 96.6 (C-1), 71.2 (C-3), 69.9 (C-4), 69.1 (C-5), 67.1 (C-2), 55.8 (Ar-OCH_3), 21.1 (Ac-CH_3), 21.0 (Ac-CH_3), 20.9 (Ac-CH_3), 17.6 (C-6)

HRMS: $C_{19}H_{23}O_9$ requires 395.1342. APCI: Found 395.1345 $[M-H]^-$

ν_{max} (ATR/cm^{-1}): 2945 (C-H stretch), 2834 (C-H stretch), 1749 (C=O stretch), 1025 (ArC-H stretch)

***R*f:** 0.3 (hexanes:EtOAc 8:2)

2,3,4-Tri-*O*-acetyl-1-*O*-(4-(methoxycarbonyl)phenoxy)-α-L-rhamnopyanoside (272)[334]



Prepared as per **Procedure 2** using peracetylated rhamnose **269** (2.42 g, 7.28 mmol), methyl-4-hydroxybenzoate (2.15 g, 14.6 mmol) and $BF_3 \cdot OEt_2$ (4.52 mL, 36.4 mmol) in CH_2Cl_2 (73 mL). Resulting solution stirred at rt for 18 h. Purification by column chromatography (hexanes:EtOAc 8:2) to yield **272** as a colourless oil (2.47 g, 80%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 8.00 (d, *J* = 9.2 Hz, 2H, *ortho* Ar-CH), 7.11 (d, *J* = 9.2 Hz, 2H, *meta* Ar-CH), 5.53 (d, *J* = 1.8 Hz, 1H, H-1), 5.50 (dd, *J* = 10.1, 3.5 Hz, 1H, H-3), 5.43 (dd, *J* = 3.5, 1.8 Hz, 1H, H-2), 5.16 (t, *J* = 10.8 Hz, 1H, H-4), 3.97-3.90, (m, 1H, H-5), 3.89 (s, 3H, Ar-$COCH_3$), 2.20 (s, 3H Ac-CH_3), 2.05 (s, 3H, Ac-CH_3), 2.03 (s, 3H, Ac-CH_3), 1.20 (d, *J* = 6.3 Hz, 3H, CH_3)

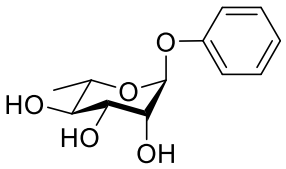
δ_C (101 MHz, $CDCl_3$) 170.2 (C=O), 166.7 (C=O), 159.4 (C=O), 131.8 (Ar-C), 124.7 (Ar-qC), 116.0 (Ar-C), 95.5 (C-1), 90.8 (Ar-qC), 70.9 (C-4), 69.6 (C-2), 68.9 (C-3), 67.6 (C-5), 52.2 (OCH_3), 21.0 (Ac-CH_3), 20.9 (Ac-CH_3), 20.9 (Ac-CH_3), 17.6 (C-6)

HRMS: $C_{20}H_{23}O_{10}$ requires 423.1291. APCI: Found 423.1306 $[M-H]^-$

ν_{max} (ATR/cm^{-1}): 2947 (C-H stretch), 2835 (C-H stretch), 1735 (C=O stretch), 1071 (ArC-H stretch)

***R_f*:** 0.32 (hexanes:EtOAc 8:2)

Phenyl-α-L-rhamnopyranoside (273)[290]



Prepared as per **Procedure 3** using **270** (141 mg, 0.385 mmol). Resulting solution stirred at rt for 30 min. Compound **273** obtained as a yellow oil (88 mg, 98%). The isolated compound was in good agreement with the literature.

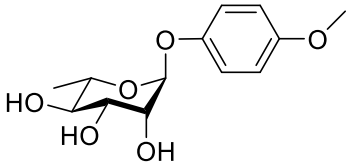
δ_H (400 MHz, $CDCl_3$) 7.25 – 7.19 (m, 2H, *meta* Ar-CH), 7.02 – 6.94 (m, 3H, *ortho* and *para* Ar-CH), 5.50 (s, 1H, H-1), 4.17 (s, 1H, H-2), 4.03 (dd, *J* = 9.3, 2.7 Hz, 1H, H-3), 3.79-3.72 (m, 1H, H-5), 3.64 – 3.57 (m, 1H, H-4), 1.26 (d, *J* = 6.1 Hz, 1H, H-6)

δ_C (101 MHz, $CDCl_3$) 156.2 (Ar-C), 129.7 (*meta* Ar-C), 122.5 (*para* Ar-C), 116.5 (*ortho* Ar-C), 97.9 (C-1), 73.1 (C-5), 71.7 (C-4), 71.1 (C-2), 69.0 (C-3), 17.7 (C-6)

HRMS: $C_{12}H_{15}O_5$ requires 239.0919. APCI: Found 239.0931 $[M-H]^-$

ν_{max} (ATR/cm^{-1}): 2943 (C-H stretch), 2833 (C-H stretch) 1750 (C=O stretch), 1224 (ArC-H stretch)

4-methoxyphenyl-α-L-rhamnopyranoside (274)[290]



Prepared as per **Procedure 3** using **271** (212 mg, 0.535 mmol). Resulting solution stirred at rt for 30 min. Compound **274** obtained as a yellow oil (140 mg, 97%). The isolated compound was in good agreement with the literature.

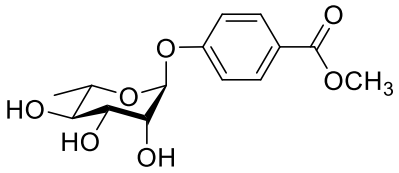
δ_H (400 MHz, $CDCl_3$) 6.96 (d, *J* = 9.2 Hz, 2H, *ortho* Ar-CH)), 6.81 (d, *J* = 9.2 Hz, 2H, *meta* Ar-CH), 5.40 (s, 1H, H-1), 4.15 (s, 1H, H-2), 3.99 (dd, *J* = 9.3, 3.0 Hz, 1H, H-3), 3.85 – 3.77 (m, 1H, H-5), 3.76 (s, 3H, OCH_3), 3.57 (t, *J* = 9.7 Hz, 1H, H-4), 1.29 (d, *J* = 6.2 Hz, 1H, CH_3)

δ_C (101 MHz, $CDCl_3$) 155.1 (Ar-qC), 150.3 (Ar-qC), 117.8 (*ortho* Ar-C), 114.8 (*meta*-Ar-C), 98.7 (C-1), 73.4 (C-5), 71.7 (C-4), 71.1 (C-2), 68.7 (C-3), 55.8 (OCH_3), 17.7 (C-6)

HRMS: $C_{13}H_{17}O_6$ requires 269.1025. APCI: Found 269.1037 $[M-H]^-$

ν_{max} (ATR/cm^{-1}): 2943 (C-H stretch), 2833 (C-H stretch), 1508 (ArC=C stretch), 1024 (ArC-H stretch)

4-(Methoxycarbonyl)phenyl-α-L-rhamnopyranoside (275)[334]

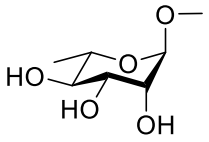


Prepared as per **Procedure 3** using **272** (2.47 g, 5.82 mmol). Resulting solution stirred at rt for 3 h. Compound **275** obtained as a yellow oil (1.68 g, 97%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.98 (d, *J* = 8.9 Hz, 2H, *ortho* Ar-CH), 7.07 (d, *J* = 8.9 Hz, 2H, *meta* Ar-CH), 5.59 (s, 1H, H-1), 4.17 (s, 1H, H-2), 4.03-3.97 (m, 1H, H-3), 3.89 (s, 3H, OCH_3), 3.71 (dq, *J* = 12.1, 6.1 Hz, 1H, H-5), 3.58 (t, *J* = 9.4 Hz, 1H, H-4), 1.27 (d, *J* = 6.1 Hz, 3H, H-6)

HRMS: $C_{14}H_{19}O_7$ requires 299.1131. APCI: Found 299.1123 $[M+H]^+$

Methyl-α-L-rhamnopyranoside (275a)[335]



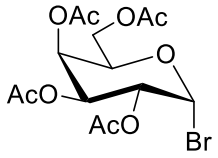
To a solution of anhydrous CH_3OH (0.67 mL, 16.47 mmol) was added dropwise acetyl chloride (0.39 mL, 5.49 mmol) at 0 °C. Following completion of this addition, L-rhamnose (0.5 g, 2.747 mmol) was added and the resulting solution stirred for 16 h at rt. The reaction was quenched with solid $NaHCO_3$, and the solution concentrated *in vacuo*. Purification of the residue by flash column chromatography (CH_2Cl_2:CH_3OH 9:1) gave the α-anomer **275a** as a colourless oil (206 mg, 42%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 4.65 (d, *J* = 1.0 Hz, 1H, H-1), 3.92 (dd, *J* = 3.2, 1.4 Hz, 1H, H-2), 3.73 (dd, *J* = 9.4, 3.2 Hz, 1H, H-3), 3.64 – 3.55 (m, 1H, H-5), 3.49 – 3.42 (m, 1H, H-4), 3.35 (s, 3H, OCH_3), 1.31 (d, *J* = 6.2 Hz, 3H, H-6)

HRMS: $C_7H_{13}O_5$ requires 177.0763. APCI: Found 177.0768 $[M-H]^-$

6.4.2 Chapter 5.2 – Experimental Detail

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide (279)[336]

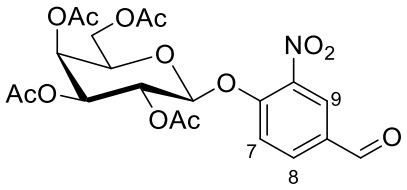


To a stirred solution of HBr in acetic acid (33% v/v, 18 mL) was added β-D-galactose pentaacetate **278** (3.0 g, 7.69 mmol) portionwise. The resulting syrup was stirred at rt for 40 min. Following completion of the reaction, the reaction was diluted in CH_2Cl_2 (100 mL) and poured onto ice. Organic phase was washed repeatedly with ice-water until neutral pH was obtained. The organic phase was separated, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was purified by column chromatography (hexanes:EtOAc 7:3) to obtain **279** as a white powder (2.01 g, 63%). The furnished compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 6.69 (d, *J* = 4.0 Hz, 1H, H-1), 5.51 (dd, *J* = 3.3, 1.0 Hz, 1H, H-4), 5.40 (dd, *J* = 10.6, 3.3 Hz, 1H, H-3), 5.04 (dd, *J* = 10.6, 4.0 Hz, 1H, H-2), 4.48 (t, *J* = 6.6 Hz, 1H, H-5), 4.18 (dd, *J* = 11.4, 6.4 Hz, 1H, H-6), 4.14 – 4.07 (m, 1H, H-6'), 2.15 (s, 3H, Ac CH_3), 2.11 (s, 3H, Ac CH_3), 2.05 (s, 3H, Ac CH_3), 2.01 (s, 3H, Ac CH_3)

HRMS: Not obtained due to instability of compound.

3-Nitro-4-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-benzaldehyde (281)[337]



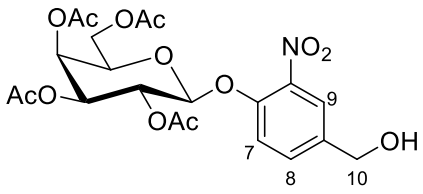
To a solution of **280** (1.51 g, 3.67 mmol), 4-hydroxy-3-nitrobenzaldehdye **280** (1.10 g, 6.601 mmol) and $BnEt_3NCl$ (668 mg, 2.93 mmol) in $CHCl_3$ (15 mL) was added a solution of NaOH (1.25 M, 5.3 mL). The resulting biphasic mixture was heated to 70 °C and stirred for 3 h. Following the elapsed time, the solution was cooled and diluted in $H_2O/CHCl_3$ (2:1 v/v, 50 mL). The organic phase was separated, washed consecutively with NaOH (2 x 100 mL) and brine (1 x 100 mL), and dried over $MgSO_4$. The suspension was filtered, and concentrated *in vacuo*. The residue was purified by column

chromatography (hexanes:EtOAc 7:3) to obtain **281** as a white powder (1.46 g, 80%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 9.98 (s, 1H, Ar-CHO), 8.30 (d, *J* = 2.0 Hz, 1H, H-9), 8.07 (dd, *J* = 8.6, 2.0 Hz, 1H, H-8), 7.49 (d, *J* = 8.6 Hz, 1H, H-7), 5.59 (dd, *J* = 10.4, 7.9 Hz, 1H, H-2), 5.51-5.47 (m, 1H, H-4), 5.21 (d, *J* = 7.9 Hz, 1H, H-1), 5.13 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3), 4.30-4.23 (m, 1H, H-6), 4.20 – 4.13 (m, 2H, H-5, H-6'), 2.19 (s, 3H, Ac CH_3), 2.13 (s, 3H, Ac CH_3), 2.08 (s, 3H, Ac CH_3), 2.03 (s, 3H, Ac CH_3)

HRMS: $C_{21}H_{23}NNaO_{13}$ requires 520.1067. ESI: Found 520.1074 $[M+Na]^+$

2,3,4,6-Tetra-*O*-acetyl-(2-nitro-4-(hydroxymethyl)phenyl)-β-D-galactopyranoside (282)[337]

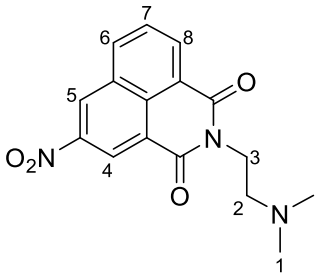


To a solution of **281** (600 mg, 1.312 mmol) in degassed $CHCl_3$ (6 mL) was added isopropyl alcohol (4 mL), silica gel (1.32 g) and IR-120 H^+ ion-exchange resin (14 mg). The resulting suspension was cooled to 0 °C and stirred for 30 min. Following the elapsed time, $NaBH_4$ (200 mg, 5.27 mmol) was added portionwise, and stirring was continued at 0 °C for 3 h. The reaction was quenched by the addition of ice-cold sat. aq. NH_4Cl (100 mL) and extracted with CH_2Cl_2 (100 mL x 2). The organic layers were combined, washed with H_2O (1 x 100 mL) and dried over $MgSO_4$. The suspension was filtered, and he solvent removed *in vacuo* to give **282** as a white powder (362 mg, 55%) which was used without further purification. The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 7.81 (d, *J* = 2.0 Hz, 1H, H-9), 7.52 (dd, *J* = 8.6, 2.1 Hz, 1H, H-8), 7.35 (d, *J* = 8.6 Hz, 1H, H-7), 5.54 (dd, *J* = 10.5, 8.0 Hz, 1H, H-2), 5.49 – 5.45 (m, 1H, H-4), 5.10 (dd, *J* = 10.5, 3.4 Hz, 1H, H-3), 5.05 (d, *J* = 8.0 Hz, 1H, H-1), 4.73 (s, 2H, H-10), 4.26 (dd, *J* = 11.3, 7.0 Hz, 1H, H-6), 4.17 (dd, *J* = 11.3, 6.2 Hz, 1H, H-6'), 4.06 (t, *J* = 6.6 Hz, 1H, H-5), 2.19 (s, 3H, Ac CH_3), 2.13 (s, 3H, Ac CH_3), 2.07 (s, 3H, Ac CH_3), 2.02 (s, 3H, Ac CH_3)

HRMS: $C_{21}H_{25}NNaO_{13}$ requires 522.1224. ESI: Found 522.1224 $[M+Na]^+$

Mitonafide (285)[338]

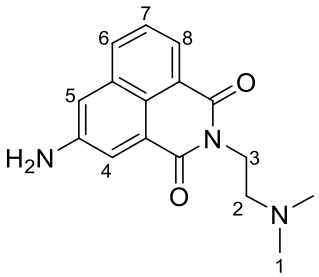


To a solution of 3-nitro-1,8-napthalic anhydride **283** (700 mg, 2.88 mmol) in EtOH (30 mL) was added *N*,*N*-dimethylethylenediamine **284** (0.4 mL, 3.46 mmol). The resulting solution was heated to reflux for 4 h, after which it was cooled to rt, and diluted in CH_2Cl_2 (200 mL). The organic phase was separated, and washed with brine (100 mL x 2) and H_2O (100 mL). The organic layer was dried over $MgSO_4$, the suspension filtered, and the solvent removed *in vacuo* to give **285** as a brown powder (892 mg, 99%) which was used without further purification. The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 9.32 (d, *J* = 2.2 Hz, 1H, H-4), 9.13 (d, *J* = 2.2 Hz, 1H, H-5), 8.78 (dd, *J* = 7.5, 1.0 Hz, 1H, H-6), 8.41 (dd, *J* = 8.2, 1.0 Hz, 1H, H-8), 7.94 (dd, *J* = 8.2, 7.5 Hz, 1H, H-7), 4.36 (t, *J* = 6.7 Hz, 2H, H-3), 2.70 (broad s, 2H, H-2), 2.35 (s, 6H, H-1)

HRMS: $C_{16}H_{16}N_3O_4$ requires: 314.1142. ESI: Found 314.1139 $[M+H]^+$

Amonafide (286)[339]



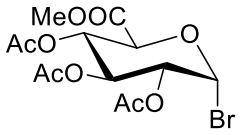
To a solution of **285** (780 mg, 2.489 mmol) in degassed CH_3OH/EtOH (7:3, 10 mL) was added palladium on carbon (10 wt. % 53 mg, 0.498 mmol). H_2 was added *via* balloon,

and the solution was stirred under H_2 for 18 h at rt. Following the elapsed time, the solution was filtered through Celite®, the solvent removed *in vacuo*, and the residue recrystallized from boiling CH_3OH to give **286** as a yellow powder (529 mg, 75%). The isolated compound was in good agreement with the literature.

δ_H (400 MHz, CD_3OD) 8.21 (d, *J* = 7.2 Hz, 1H, H-8), 8.05 (s, 1H, H-5), 8.00 (d, *J* = 8.3 Hz, 1H, H-6), 7.61 (t, *J* = 7.8 Hz, 1H, H-7), 7.37 (s, 1H, H-4), 4.31 (t, *J* = 7.1 Hz, 2H, H-3), 2.72 – 2.67 (m, 2H, H-2), 2.38 (s, 6H, H-1)

HRMS: $C_{16}H_{16}N_3O_2$ requires 282.1243. ESI: Found 282.1249 $[M-H]^-$.

1-Bromo-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronic acid, methyl ester (288)[340]

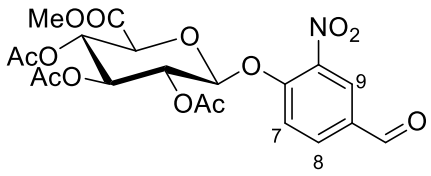


To a stirred solution of HBr in acetic acid (33% v/v, 6 mL) at 0 ° was added β-D-glucuronic acid tetraaacetate **287** (1.5 g, 3.98 mmol) portionwise. The resulting syrup was stirred at 0 °C for 4 h. Following completion of the reaction, the reaction was diluted in CH_2Cl_2 (100 mL) and poured onto ice. Organic phase was washed repeatedly with ice-water until neutral pH was obtained. The organic phase was separated, dried over $MgSO_4$ and concentrated *in vacuo*. This gave **288** as a white foam (1.16 g, 73%) which was applied directly to the next step. The furnished compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 6.64 (d, *J* = 4.1 Hz, 1H, H-1), 5.61 (t, *J* = 9.7 Hz, 1H, H-3), 5.31 – 5.20 (m, 1H, H-4), 4.85 (dd, *J* = 10.0, 4.1 Hz, 1H, H-2), 4.58 (d, *J* = 10.3 Hz, 1H, H-5), 3.76 (s, 3H, $COOCH_3$), 2.09 (s, 3H, Ac CH_3), 2.05 (s, 6H, Ac CH_3)

HRMS: Not obtained due to instability of compound.

Methyl-1-(4-formyl-2-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyronuronate (289)[341]

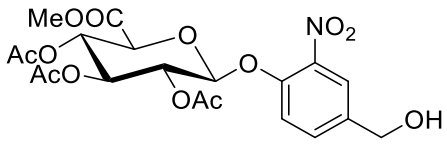


A solution of **288** (1.46 g, 3.68 mmol) and 4-hydroxy-3-nitrobenzaldehyde **280** (921 mg, 5.51 mmol) in anhydrous CH_3CN (30 mL) over 4Å MS was cooled to 0 °C, and shielded from light. Ag_2O (3.1 g, 9.19 mmol) was added and the suspension stirred for 30 min. Following the elapsed time, the solution as warmed to rt and stirred for 18 h in the dark under Ar. Following completion of the reaction, the suspension was filtered through Celite®, and the solvent removed *in vacuo*. H_2O (100 mL) was added, and the aqueous suspension extracted with EtOAc (100 mL x 2). The organic layers were combined, dried over $MgSO_4$ and filtered. The filtrate was concentrated, and the residue purified *via* column chromatography (hexanes:EtOAc 1:1) to yield **289** (1.24 g, 70%) as a white powder. The isolated compound was in good agreement with the literature.

δ_H (400 MHz, $CDCl_3$) 9.98 (s, 1H, Ar-CHO), 8.31 (d, *J* = 2.0 Hz, 1H, H-9), 8.09 (dd, *J* = 8.7, 2.0 Hz, 1H, H-8), 7.50 (d, *J* = 8.7 Hz, 1H, H-7), 5.45 – 5.40 (m, 2H, H-1, H-3), 5.35 – 5.30 (m, 2H, H-2, H-4), 4.32 (d, *J* = 8.4 Hz, 1H, H-5), 3.71 (s, 3H, $COOCH_3$), 2.13 (s, 3H, Ac CH_3), 2.08 (s, 6H, Ac CH_3)

HRMS: $C_{20}H_{20}NO_{13}$ requires 482.0940. ESI: Found 482.0904 $[M-H]^-$

Methyl-1-(4-hydroxymethyl-2-nitrophenyl)-2,3,4-tri-*O*-acetyl-β-D-glucopyronuronate (290)[341]



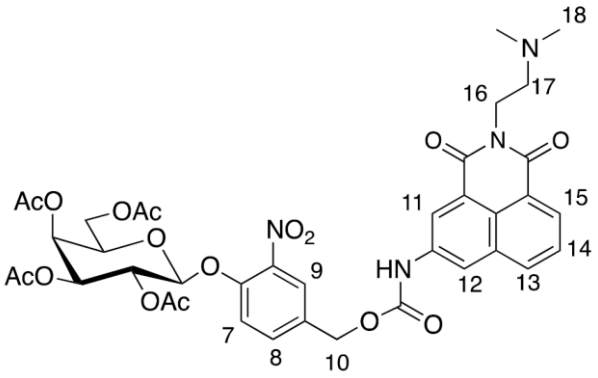
To a solution of **289** (620 mg, 1.28 mmol) in degassed $CHCl_3$:isopropylalcohol (2:1, 4.5 mL) was added silica gel (895 mg), and the solution cooled to 0 °C. After stirring for 30 min, $NaBH_4$ (97 mg, 2.57 mmol) was added portionwise, and the stirring continued for 1 h at 0 °C. Following completion of the reaction, the solution was filtered through

Celite®, quenched with ice-cold sat. aq. NH_4Cl (100 mL) and extracted with CH_2Cl_2 (100 mL x 2). Organic extracts were combined, washed with H_2O (100 mL), brine (100 mL) and dried over $MgSO_4$. The suspension was filtered, and the solvent removed *in vacuo*. Recrystallization from boiling Et_2O gave **290** as a white powder (374 mg, 60%). The isolated compound was in good agreement with the literature.

δ_H (600 MHz, $CDCl_3$) 7.82 (s, 1H, Ar-H), 7.54 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.37 (d, *J* = 8.5 Hz, 1H, Ar-H), 5.39 – 5.27 (m, 3H, H-2, H-3, H-4), 5.19 (d, *J* = 6.7 Hz, 1H, H-1), 4.73 (s, 2H, Ar-$\underline{CH_2}$OH), 4.20 (d, *J* = 8.8 Hz, 1H, H-5), 3.75 (s, 3H, $COOCH_3$), 2.13 (s, 3H, Ac CH_3), 2.08-2.04 (m, 6H, Ac CH_3 x 2)

HRMS: $C_{20}H_{23}NNaO_{13}$ requires 508.1067. ESI: Found 508.1065 $[M+Na]^+$

(2R,3S,4S,5R,6S)-2-(acetoxymethyl)-6-(4-((((2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl)carbamoyl)oxy)methyl)-2-nitrophenoxy)-tetrahydro-2H-pyran-3,4,5-triyl triacetate (**291**)



286 (100 mg, 0.35 mmol) and DMAP (128 mg, 1.05 mmol) were dissolved in anhydrous THF (25 mL) under an N_2 atmosphere, at rt. The reaction mixture was cooled to 0 °C for 10 min before the slow addition of phosgene (1.4 mL, 2.10 mmol). The reaction was stirred for 4 h at rt. N_2 was bubbled through the reaction mixture for 1 h to remove the phosgene and THF. **282** (192 mg, 0.385 mmol) was dissolved in anhydrous DMF (15 mL) under a nitrogen atmosphere, and added to the reaction mixture at 0 °C. The reaction was stirred for further 18 h at rt followed by washed with 0.1 M HCl (20 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. The residue was purified by column chromatography (EtOAc:hexanes 2:1) to yield the product **291** as a white wax (91 mg, 32%).

δ_H (400 MHz, CD_3OD) 8.73 (d, $J_{15\text{-}13}$ = 2.2 Hz, 1H, H-15), 8.52 – 8.47 (m, 2H, H-9, H-11), 8.29 (d, *J* = 8.0 Hz, 1H, H-12), 8.08 (s, 1H, NH), 7.96 (d, $J_{13\text{-}15}$ = 2.2 Hz, 1H, H-13), 7.81 (app. t, 1H, H-14), 7.75 (dd, $J_{8\text{-}7}$ = 8.6 Hz, $J_{8\text{-}9}$ = 2.2 Hz, 1H, H-8), 7.50 (d, $J_{7\text{-}8}$ = 8.6 Hz, 1H, H-7), 5.50 – 5.47 (m, 1H), 5.43 – 5.38 (m, 2H, H-1), 5.31 (s, 2H, H-10), 5.27 (dd, *J* = 9.3 Hz, *J* = 4.7 Hz, 1H), 4.56 (t, $J_{16\text{-}17}$ = 5.9 Hz, 2H, H-16), 4.37 (t, *J* = 6.5 Hz, 1H), 4.25 – 4.21 (m, 2H, H-6), 3.57 (t, $J_{17\text{-}16}$ = 5.9 Hz, 2H, H-17), 3.06 (s, 6H, H-18), 2.20, 2.10, 2.06, 1.99 (s, 12H, OAc).

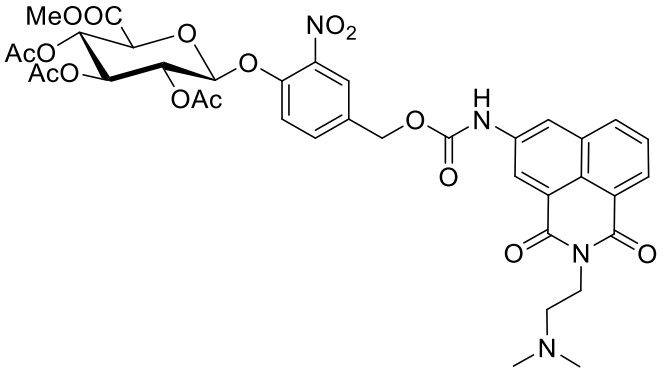
δ_C (100 MHz, CD_3OD) 171.9, 171.4, 166.0, 165.8, 161.2, 155.3, 142.5, 139.5, 135.2 (C-12), 134.5 (C-8), 130.8 (C-9 or C-11), 128.7 (C-14), 125.9 (C-13), 125.5 (C-15), 123.2, 121.9 (C-9 or C-11), 119.8 (C-7), 101.0 (C-1), 72.6, 72.0, 69.6, 68.6, 66.28 (C-10), 62.6 (C-6), 57.7 (C-17), 44.3 (C-18), 36.6 (C-16), 20.6, 20.6, 20.4 (OAc).

HRMS: $C_{38}H_{39}N_4O_{16}$ requires 807.2367. ESI: Found 807.2425 $[M\text{-}H]^-$.

ν_{max} (ATR/cm^{-1}): 2972 (arC-H), 1748 (C=O), 1666 (NO2), 1629, 1601, 1536, 1511 (NH), 1468, 1431, 1370, 1231 (NCOO)

***R*f:** 0.28 (EtOAc:Hexanes 2:1)

(2S,3S,4S,5R,6S)-6-(4-((((2-(2-(dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[de]isoquinolin-5-yl)carbamoyl)oxy)methyl)-2-nitrophenoxy)-3,4,5-trihydroxy-tetrahydro-2H-pyran-2-carboxylic acid (292)



286 (65 mg, 0.19 mmol) and DMAP (69 mg, 0.56 mmol) were dissolved in anhydrous THF (15 mL) under a nitrogen atmosphere, at rt. The reaction mixture was cooled to 0 °C for 10 min before the slow addition of phosgene (1.7 mL, 1.69 mmol). The reaction was stirred for 4 h at rt. N_2 was bubbled through the reaction mixture for 1 h to remove the

phosgene and THF. **290** (100 mg, 0.206 mmol) was dissolved in anhydrous DMF (10 mL) under an N_2 atmosphere, and added to the reaction mixture at 0 °C. The reaction was stirred for further 18 h at rt followed by washed with 0.1 M HCl (20 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure. This furnished **292** as a white wax (80 mg, 49%), which was used without further purification.

δ_H (400 MHz, CD_3OD) 8.76 (d, $J_{11\text{-}12}$ = 2.2 Hz, 1H, H-11), 8.55 - 8.51 (m, 2H, H-9, H-15), 8.32 (dd, $J_{13\text{-}14}$ = 8.4 Hz, $J_{13\text{-}15}$ = 1.1 Hz, 1H, H-13), 7.99 (d, $J_{12\text{-}11}$ = 2.2 Hz, 1H, H-12), 7.83 (app. t, 1H, H-14), 7.76 (dd, $J_{8\text{-}7}$ = 8.6 Hz, $J_{8\text{-}9}$ = 2.2 Hz, 1H, H-8), 7.53 (d, $J_{7\text{-}8}$ = 8.6 Hz, 1H, H-7), 5.56 (d, $J_{1\text{-}2}$ = 7.6 Hz, 1H, H-1), 5.46 (app. t, 1H), 5.32 (s, 2H, H-10), 5.31 – 5.23 (m, 2H,), 4.60 – 4.54 (m, 3H, H-16), 3.76 (s, 3H, COOMe), 3.48 (app. t, 2H, H-17), 2.99 (s, 6H, H-18), 2.11, 2.07, 2.05 (3s, 9H, OAc)

δ_C (100 MHz, CD_3OD) 171.2, 170.9, 168.8, 149.9, 135.3 (C-12), 134.6 (C-13), 130.8 (C-9 or C-11), 128.7 (C-14), 125.6 (C-7), 124.1 (C-15), 123.2, 121.9 (C-9 or C-11), 119.9 (C-8), 100.4 (C-1), 73.2, 72.8, 71.8, 70.4, 66.3 (C-10), 57.7 (C-17), 53.4 (COOMe), 44.3 (C-18), 36.6 (C-16), 20.5, 20.5, 20.4.

HRMS: $C_{37}H_{37}N_4O_{16}$ requires 793.2210. ESI: Found 793.2191 $[M\text{-}H]^-$.

ν_{max} (ATR/cm^{-1}): 2983 (arC-H), 1688 (C=O), 1380, 1208 (NCOO)

***R*f:** 0.32 (EtOAc:Hexanes 2:1)

7.0 References

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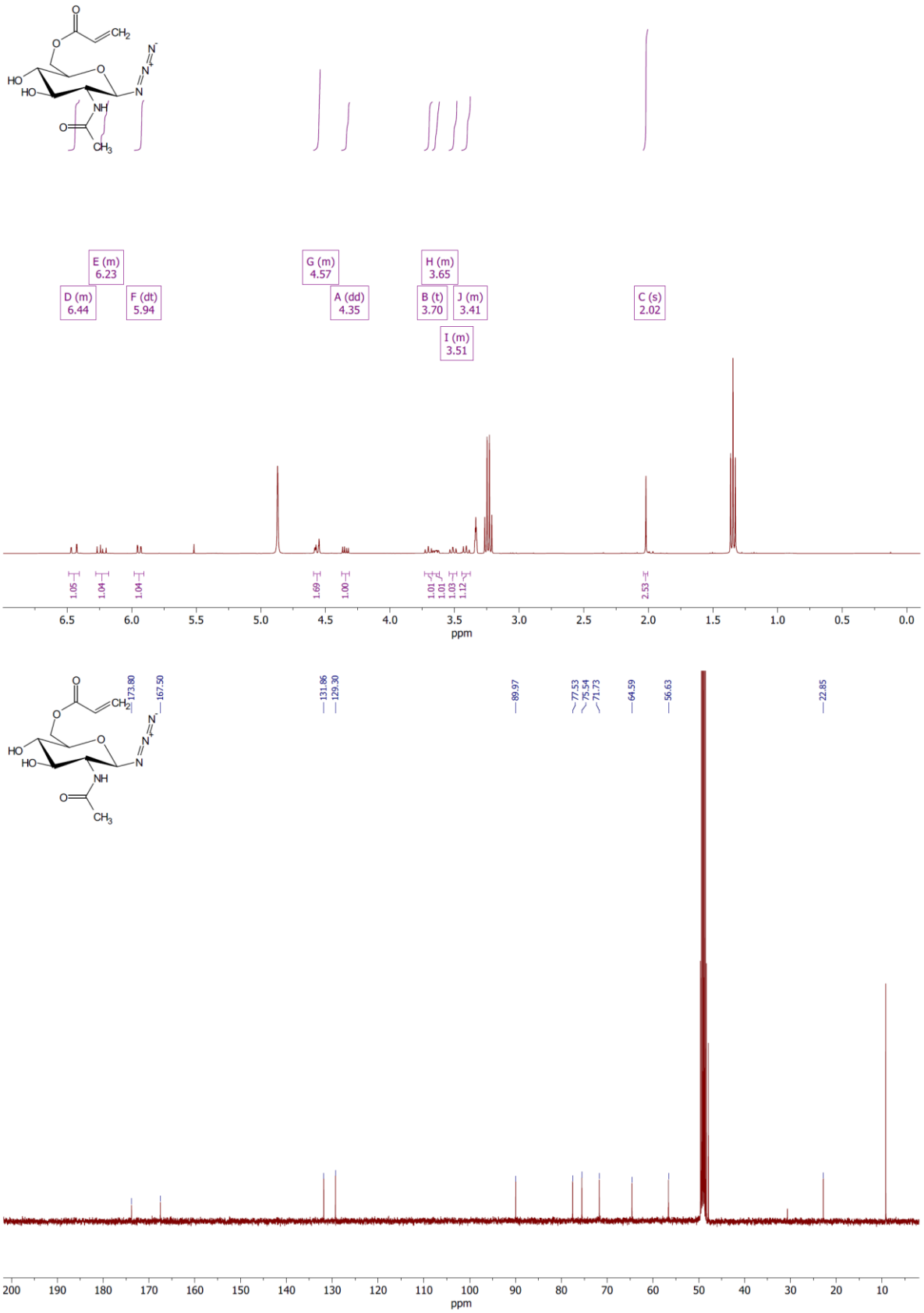
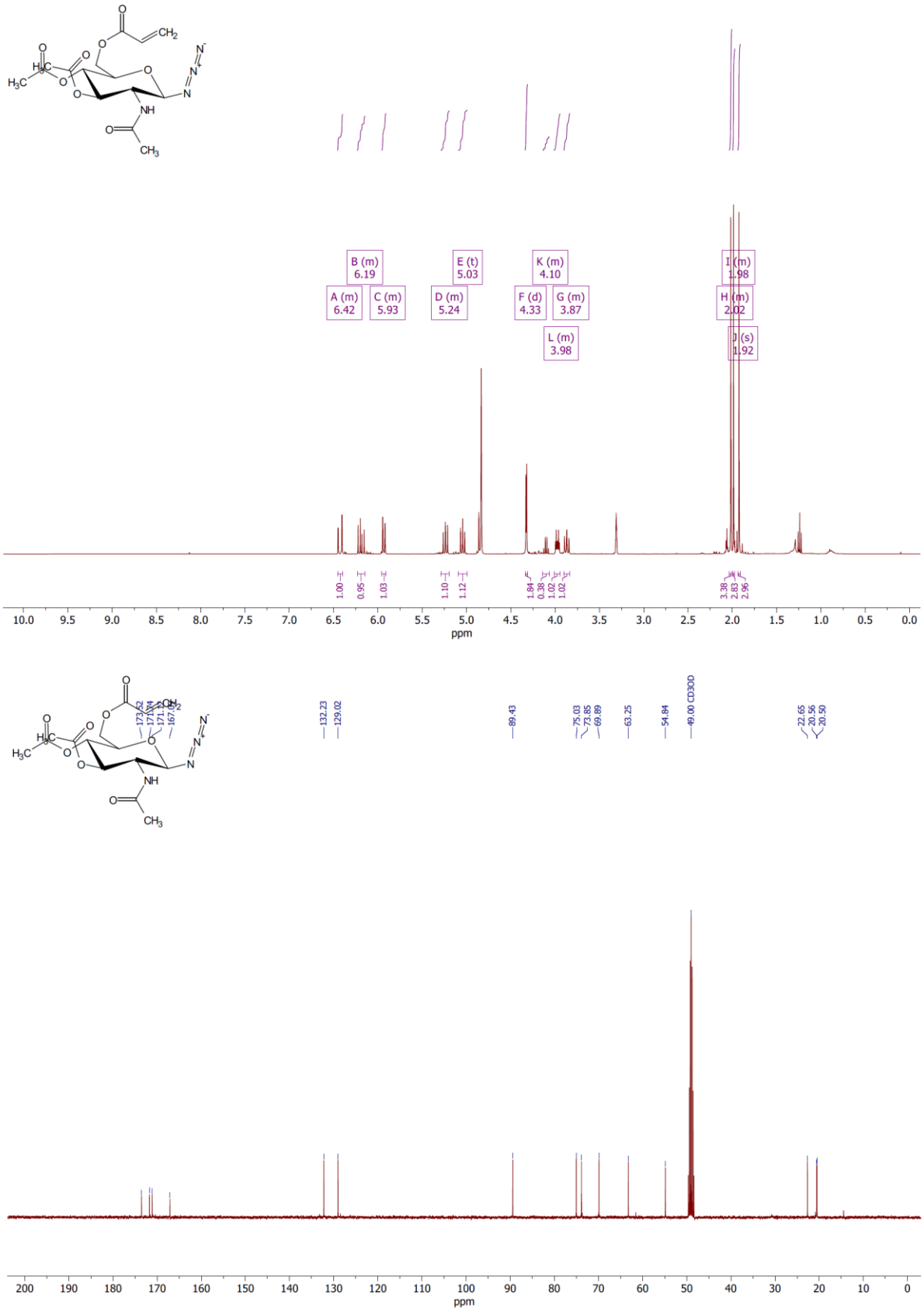
Appendix A
Selected NMR Data

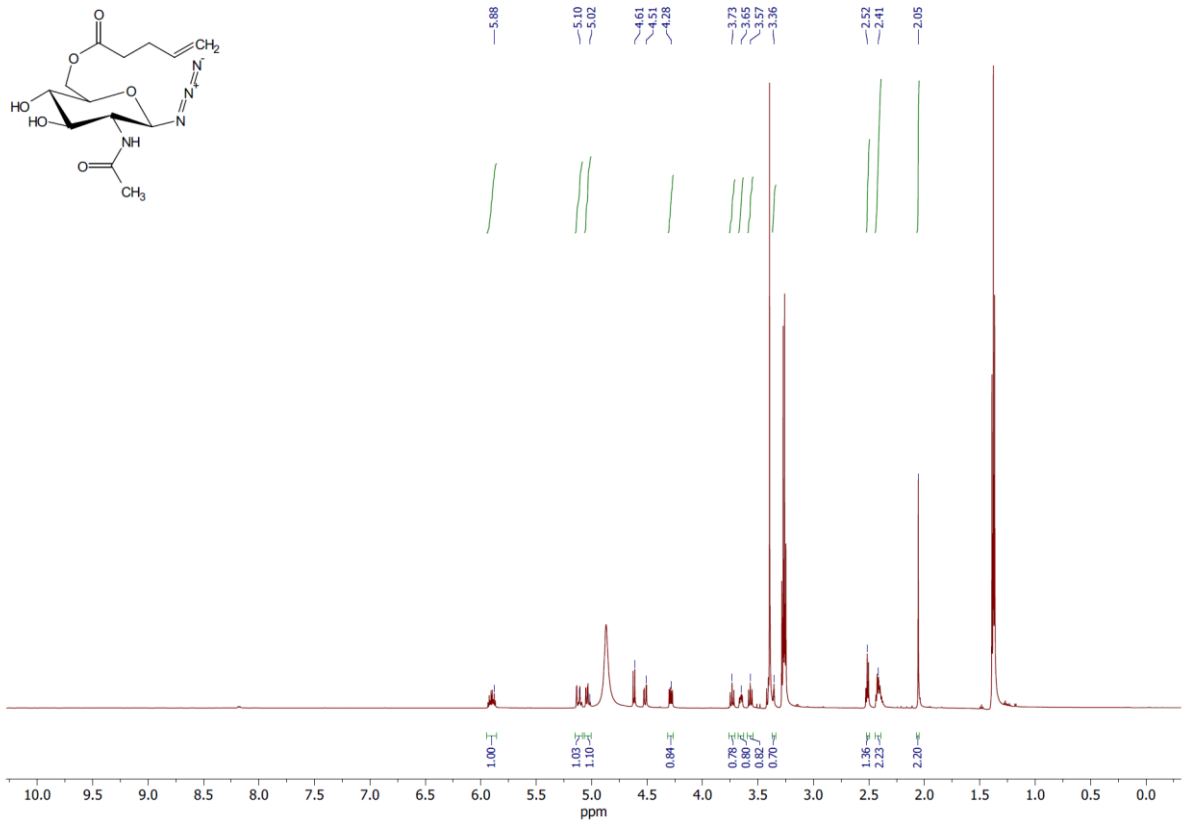
2-Deoxy-2-acetamido-6-*O*-pent-4-enoate-β-D-glucopyranosyl azide (123)

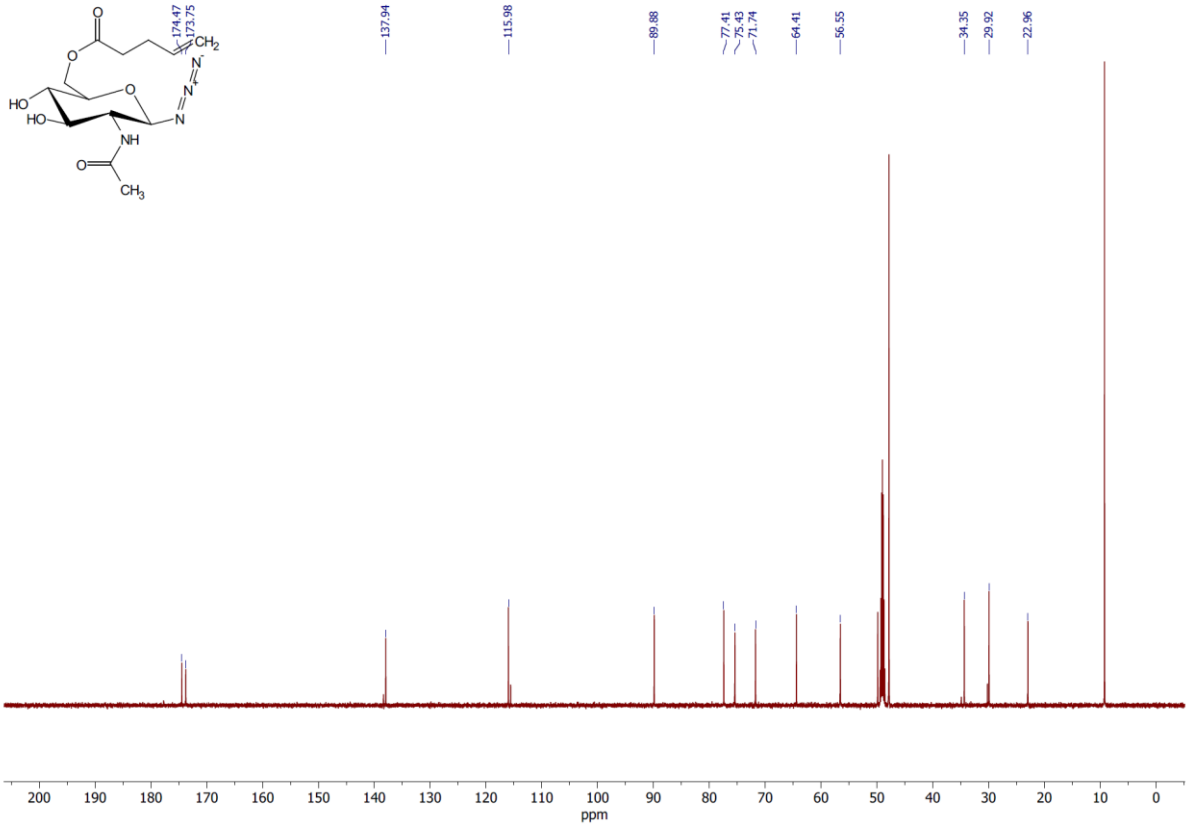


2-Deoxy-2-acetamido-6-*O*-prop-2-enoate-3,4,-di-*O*-acetyl-β-D-glucopyranosyl azide (124)

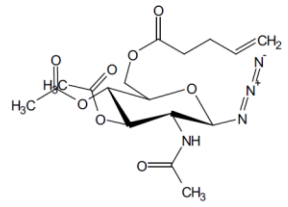


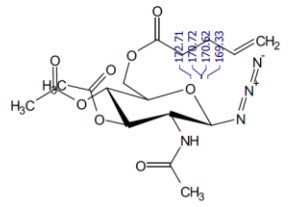
2-Deoxy-2-acetamido-6-*O*-pent-4-enoate-β-D-glucopyranosyl azide (126)





2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-pent-4-enoate-β-D-glucopyranosyl azide (127)





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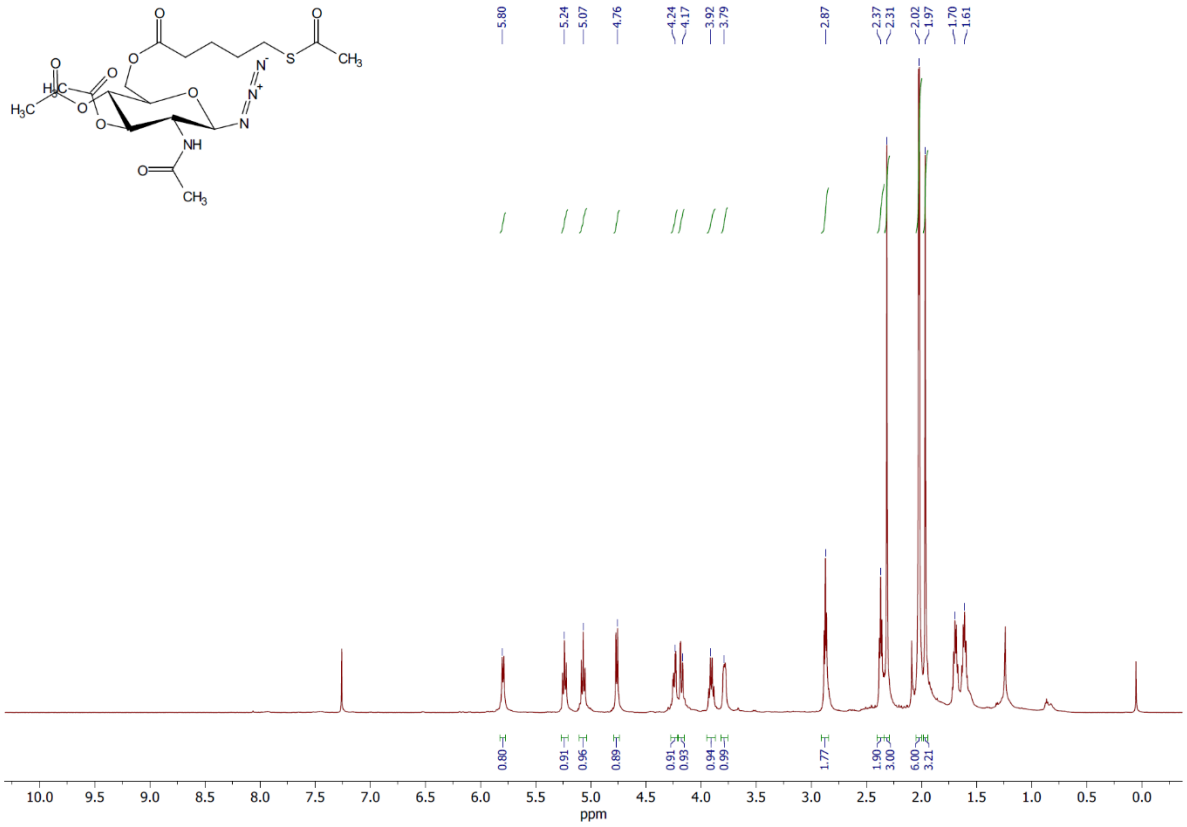
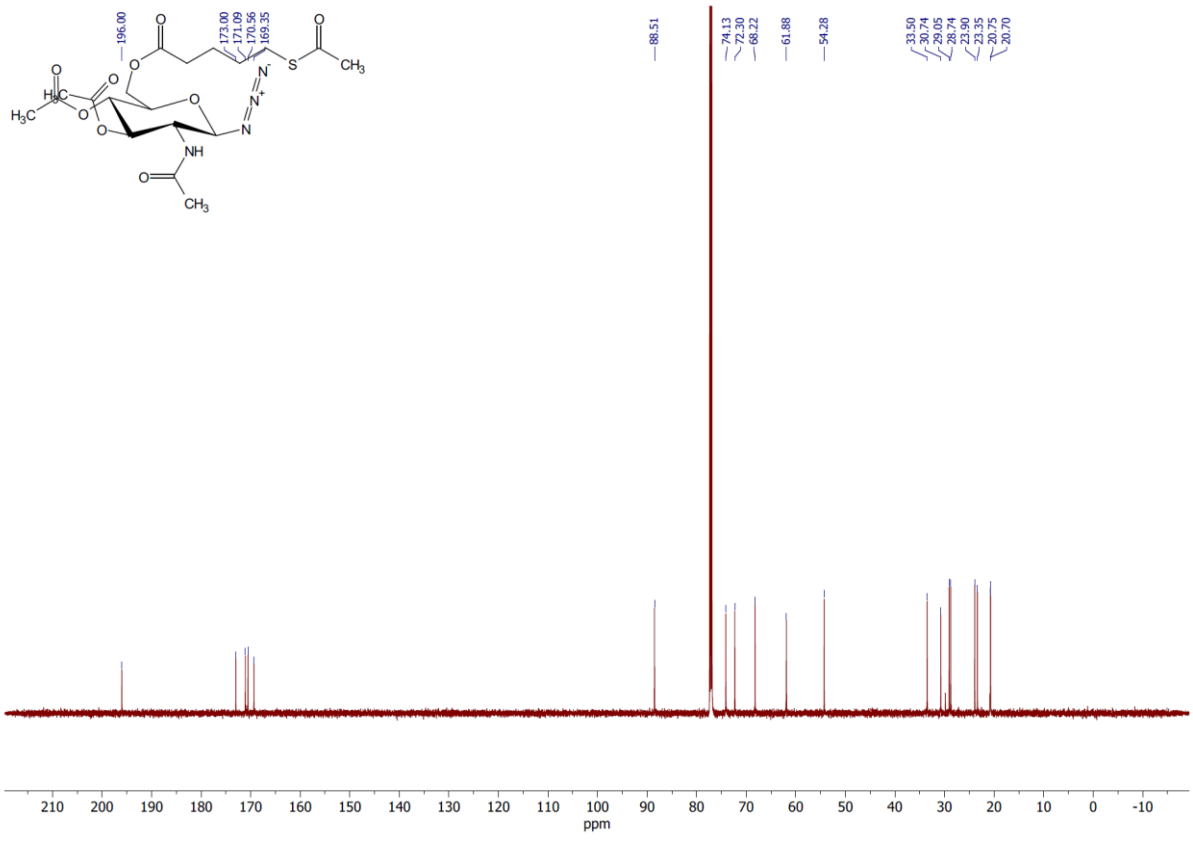
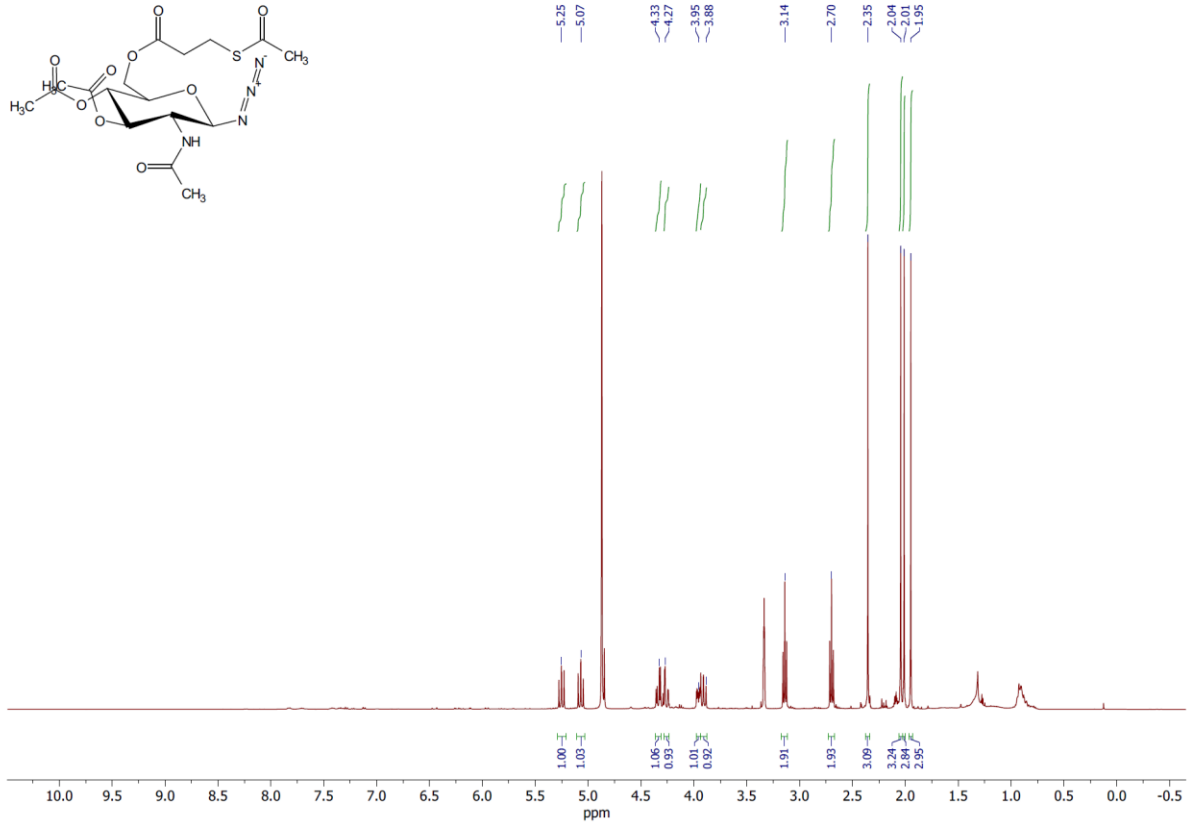
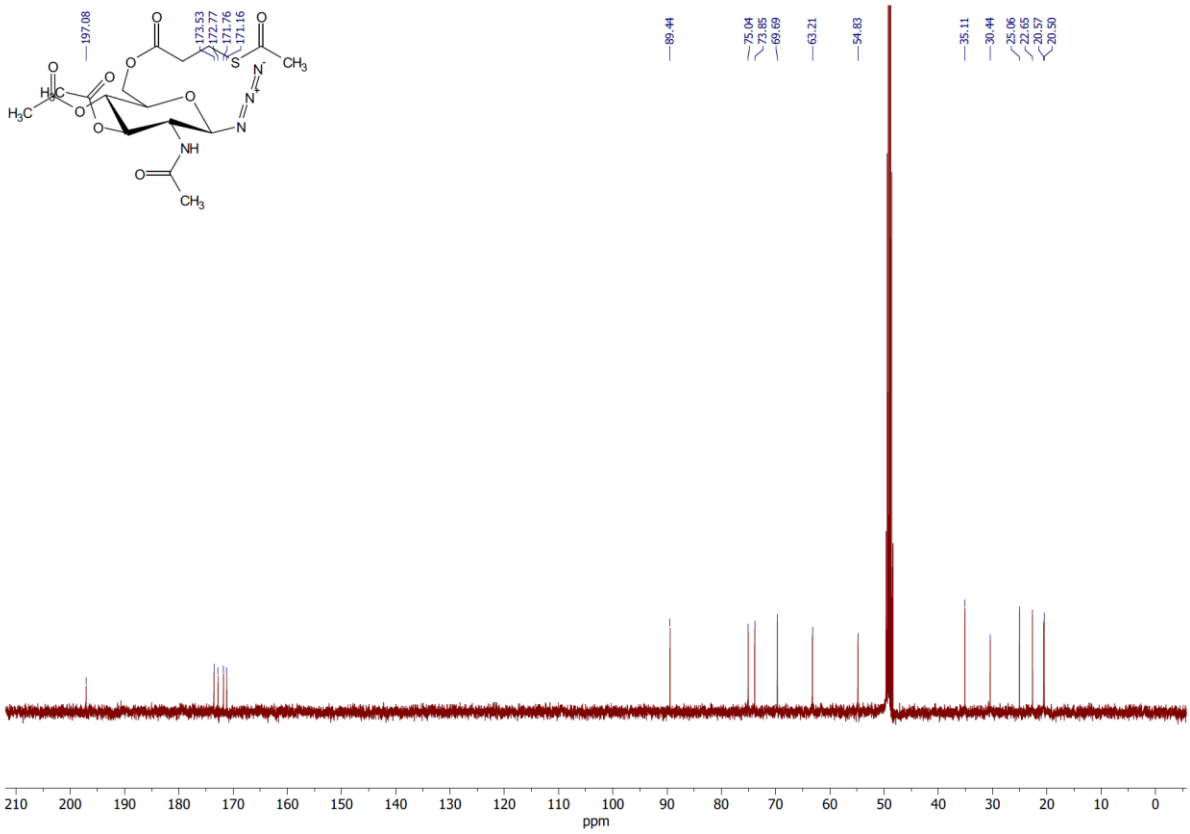


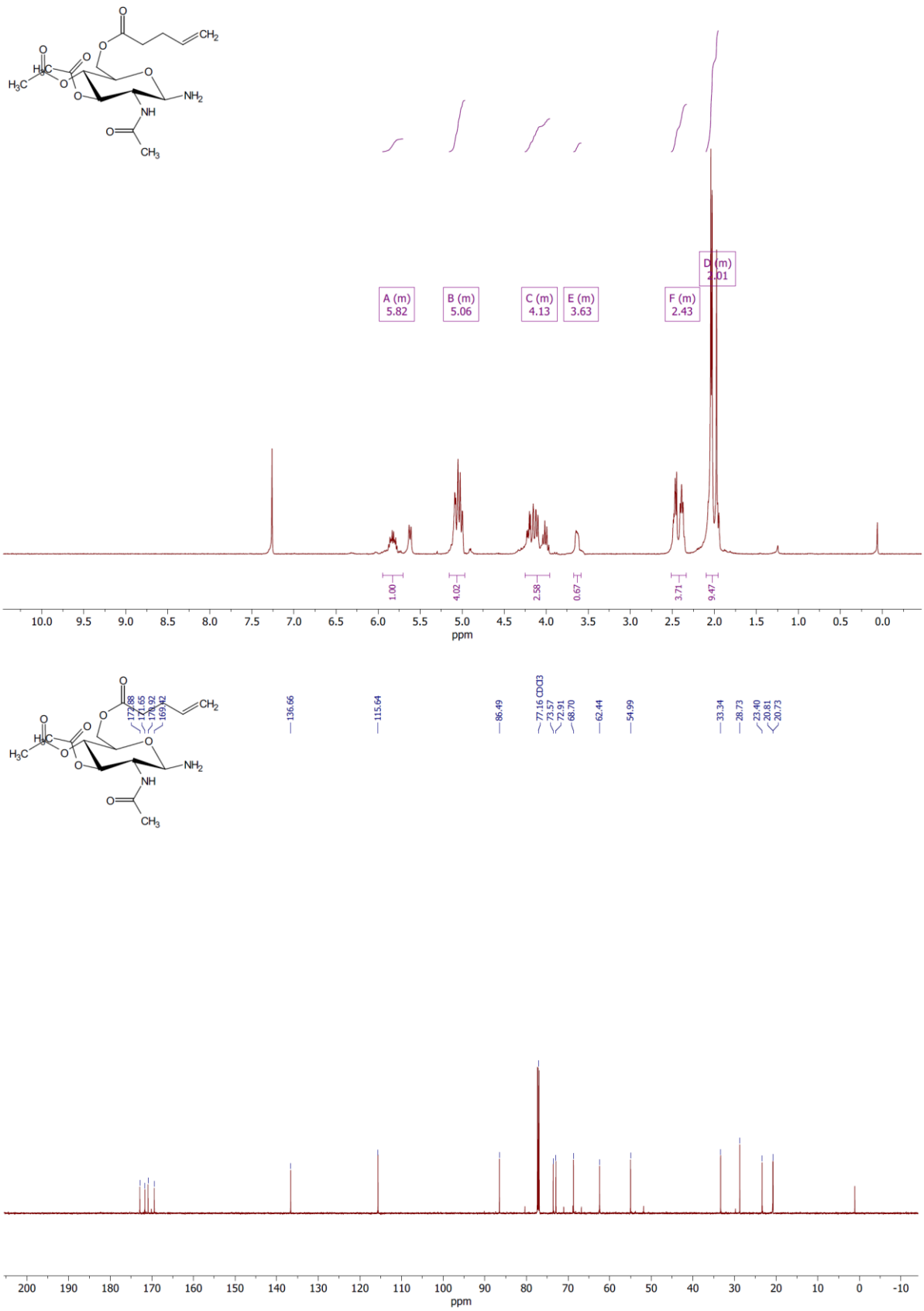


2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-acetylthiopropanoate-β-D-glucopyranosyl azide (134)

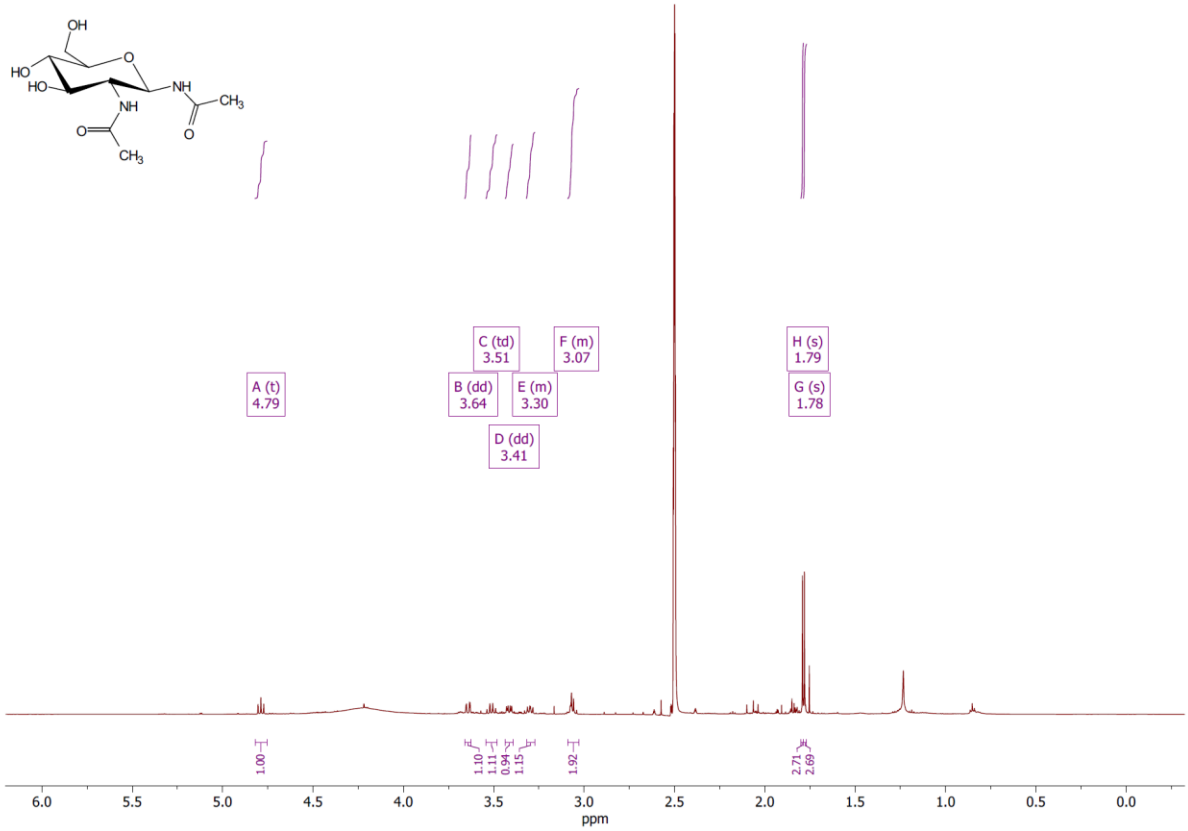


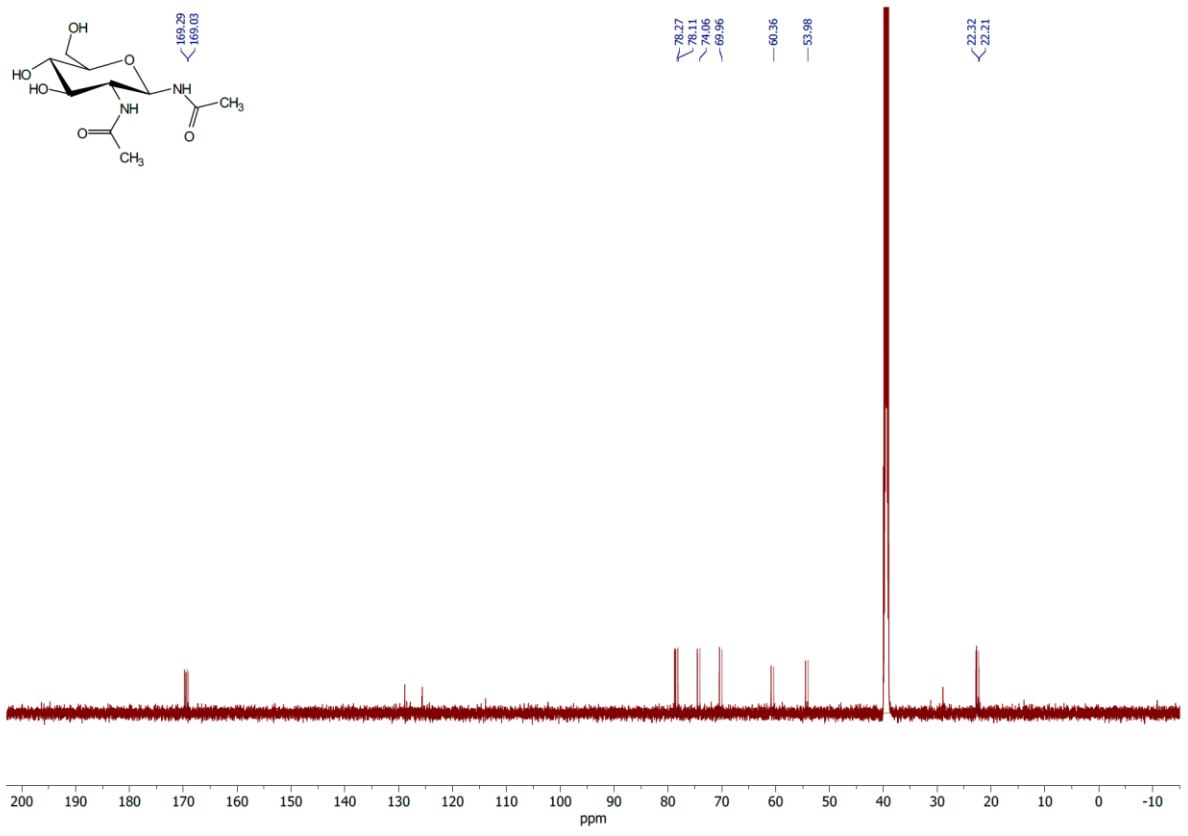


2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-pent-4-enoate-β-D-glucopyranosyl amine (135)

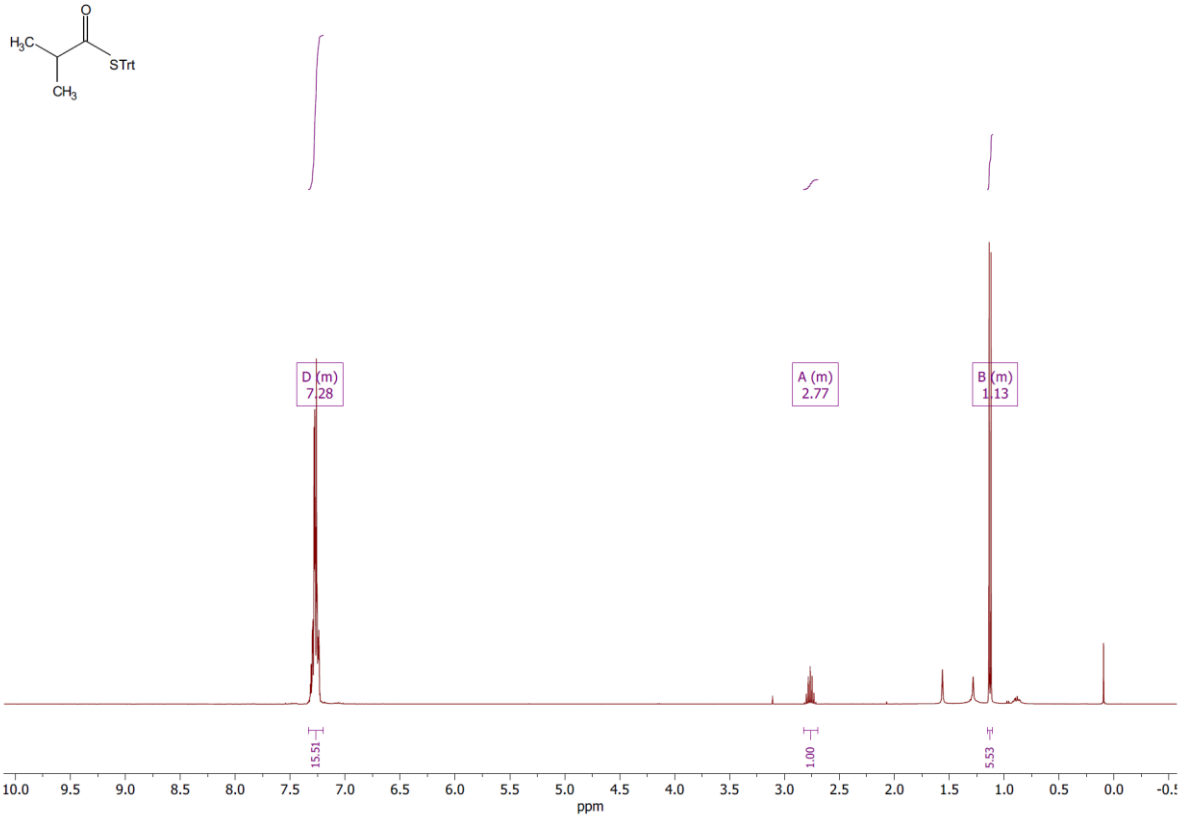


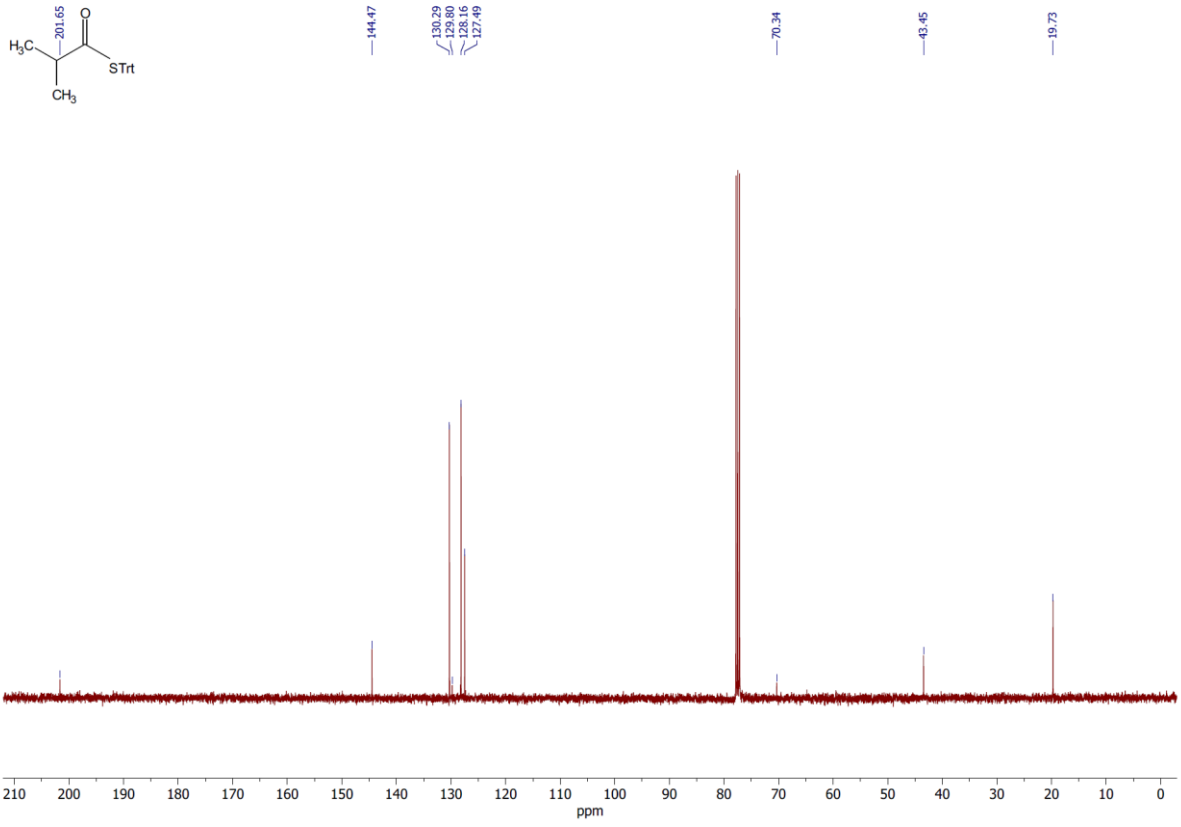
2-Deoxy-2-acetamido-β-D-glucopyranosylacetamide (139)



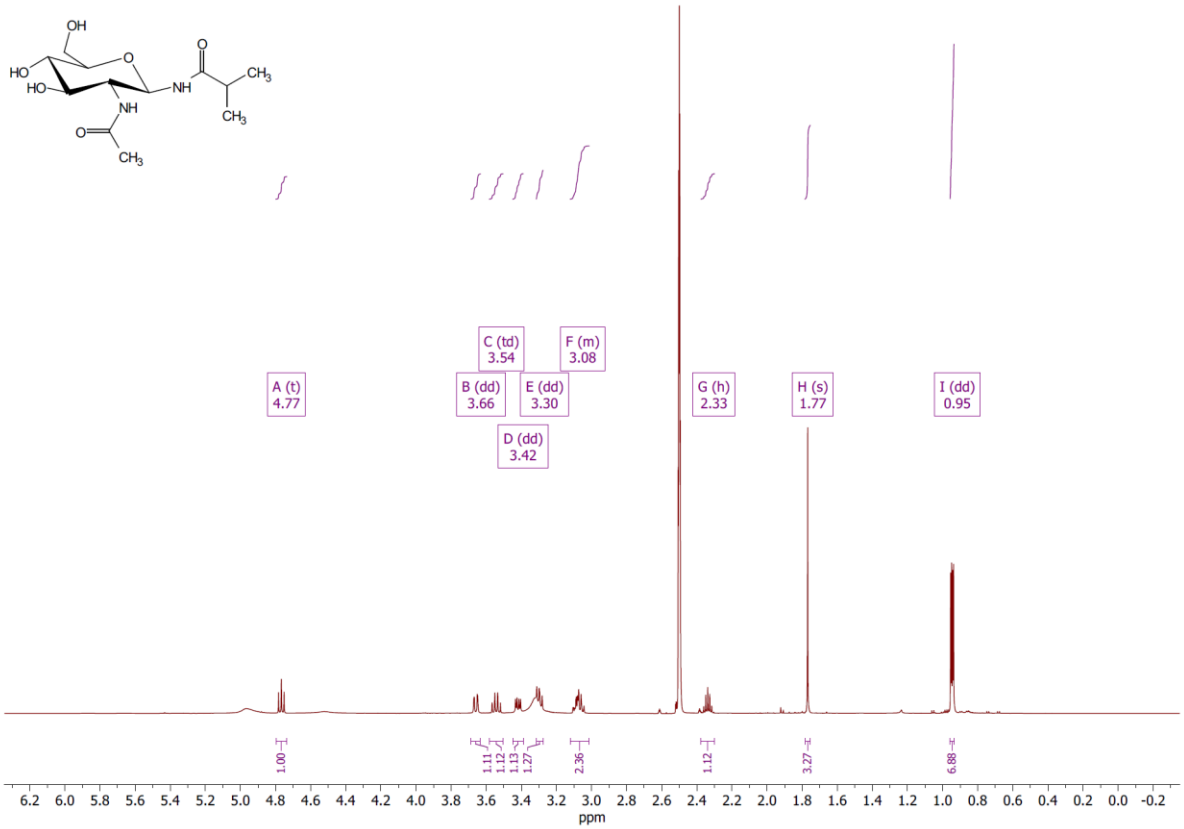


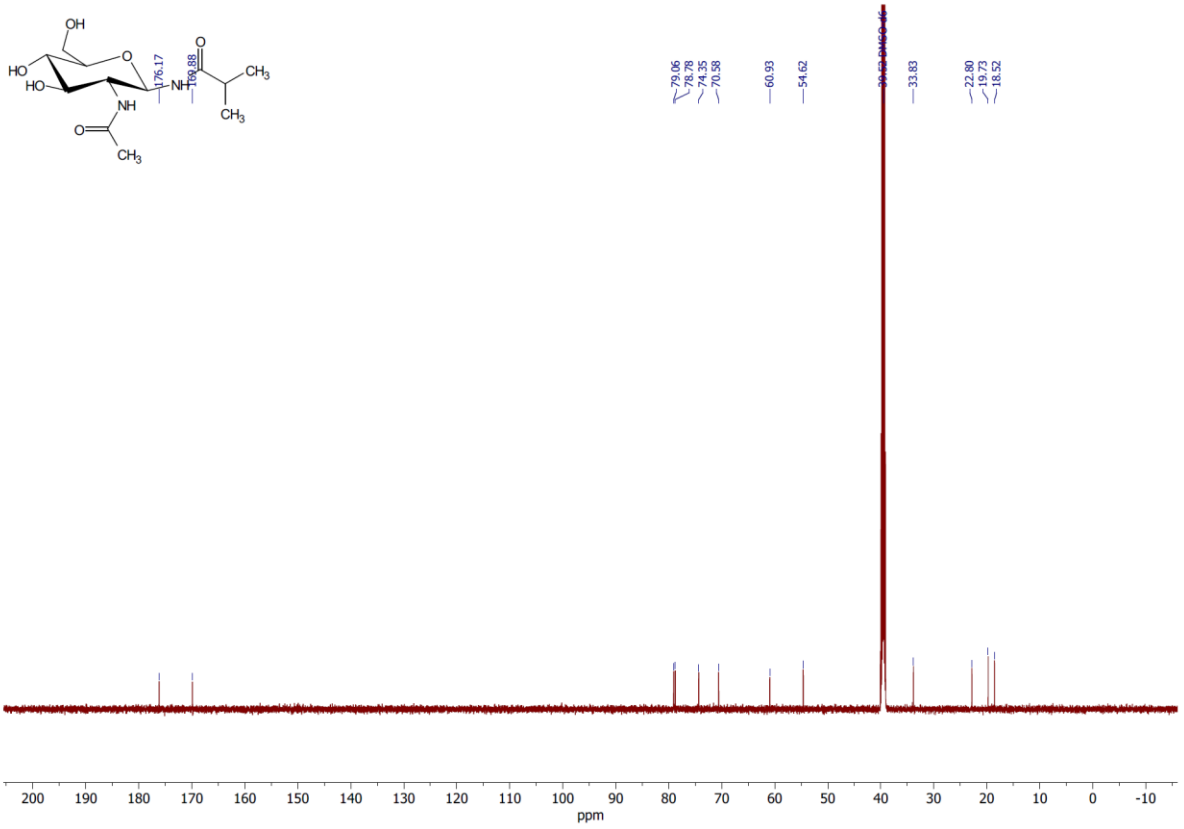
S-trityl-2-methylpropanethioate (142)



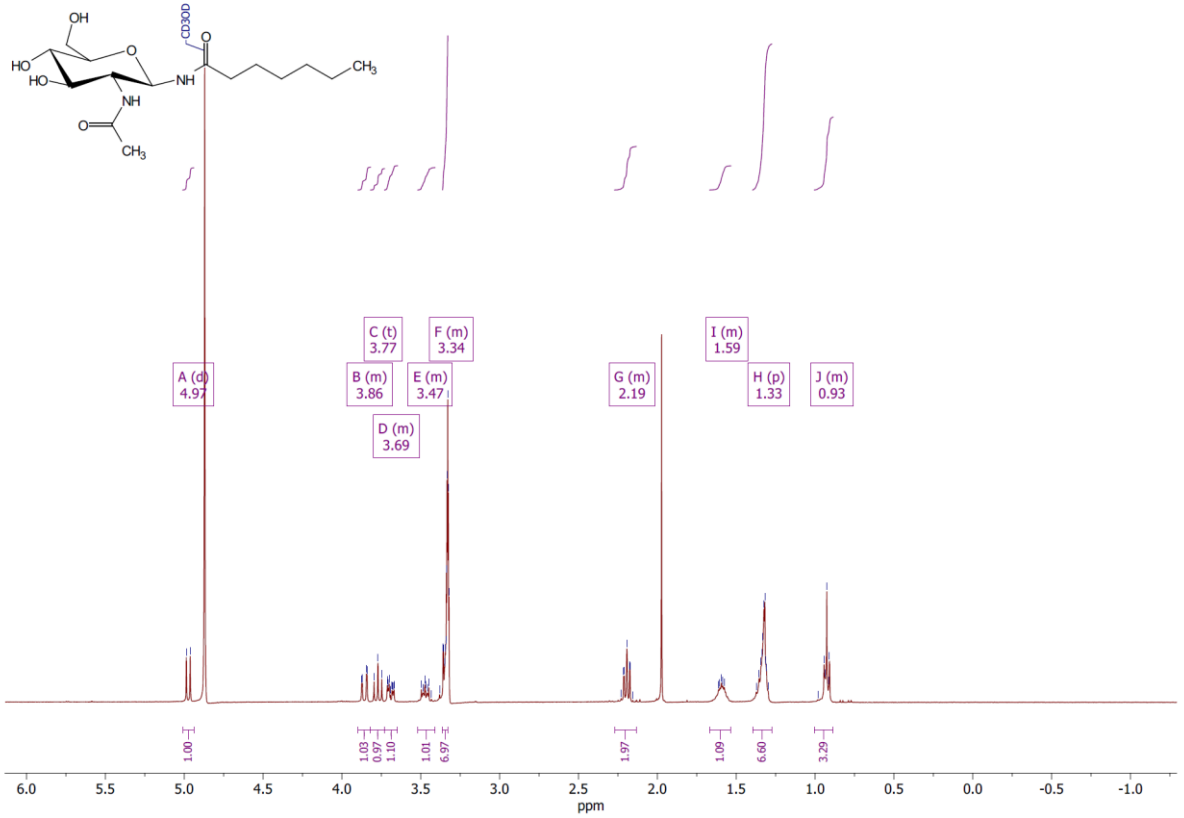


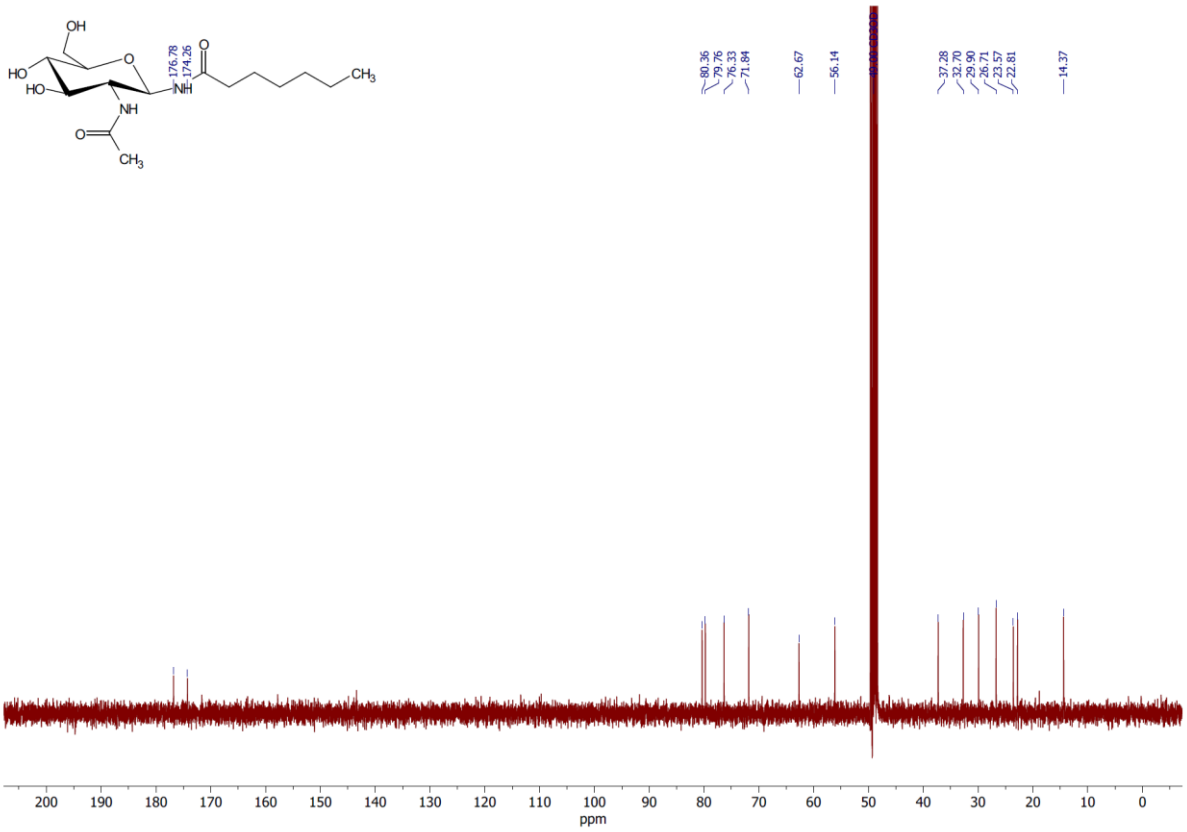
2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-isobutyramide (144)



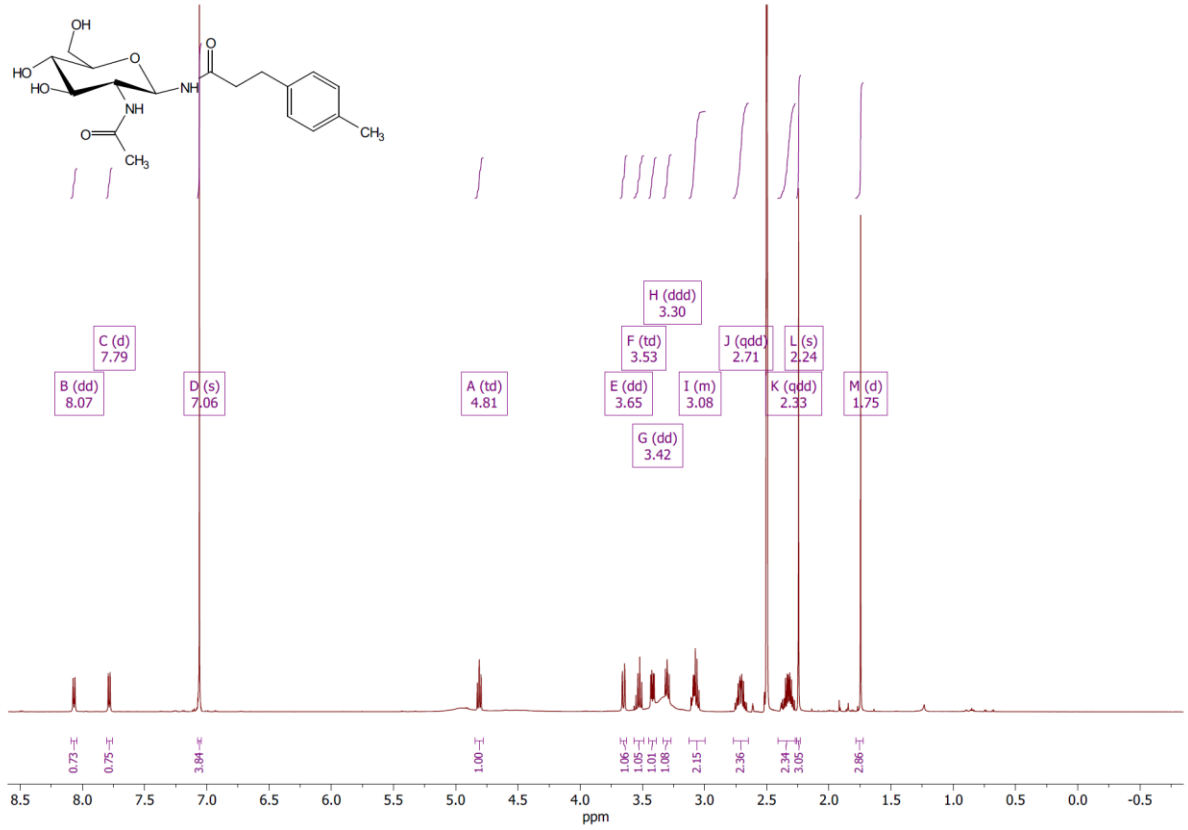


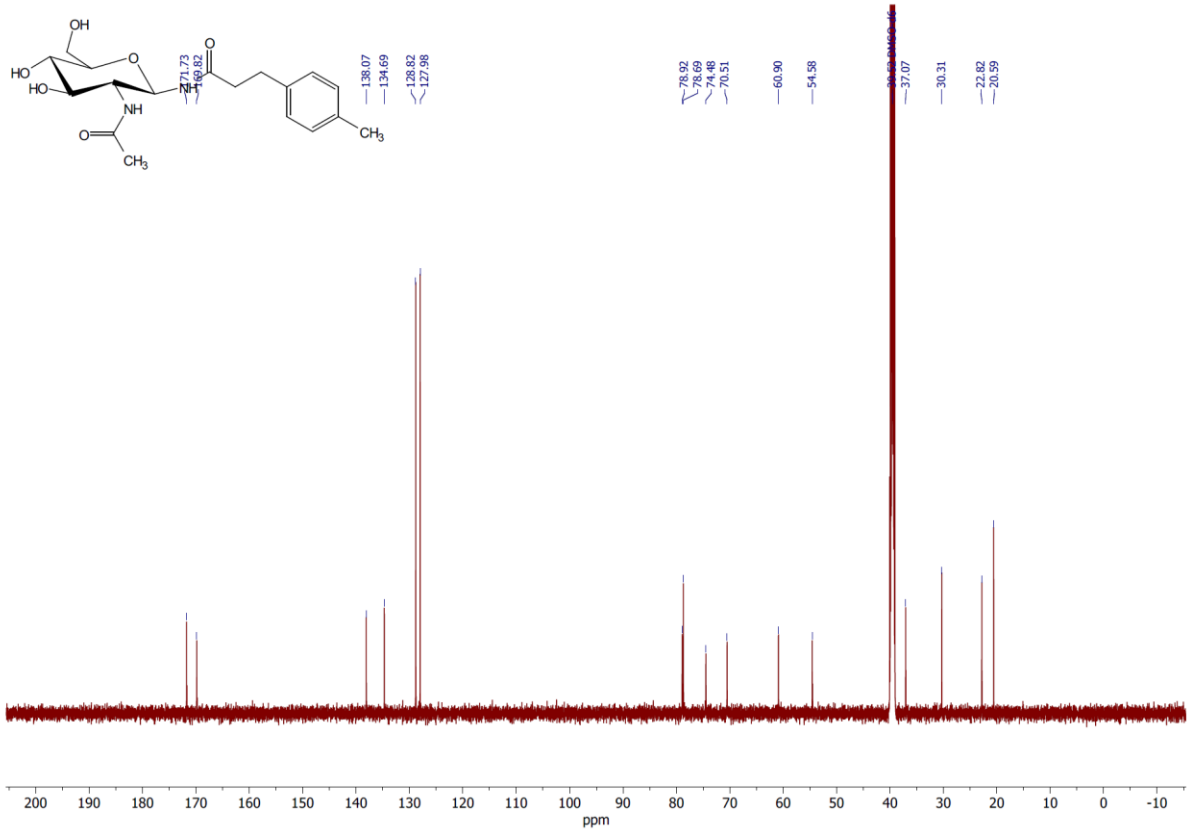
2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-heptaneamide (145)



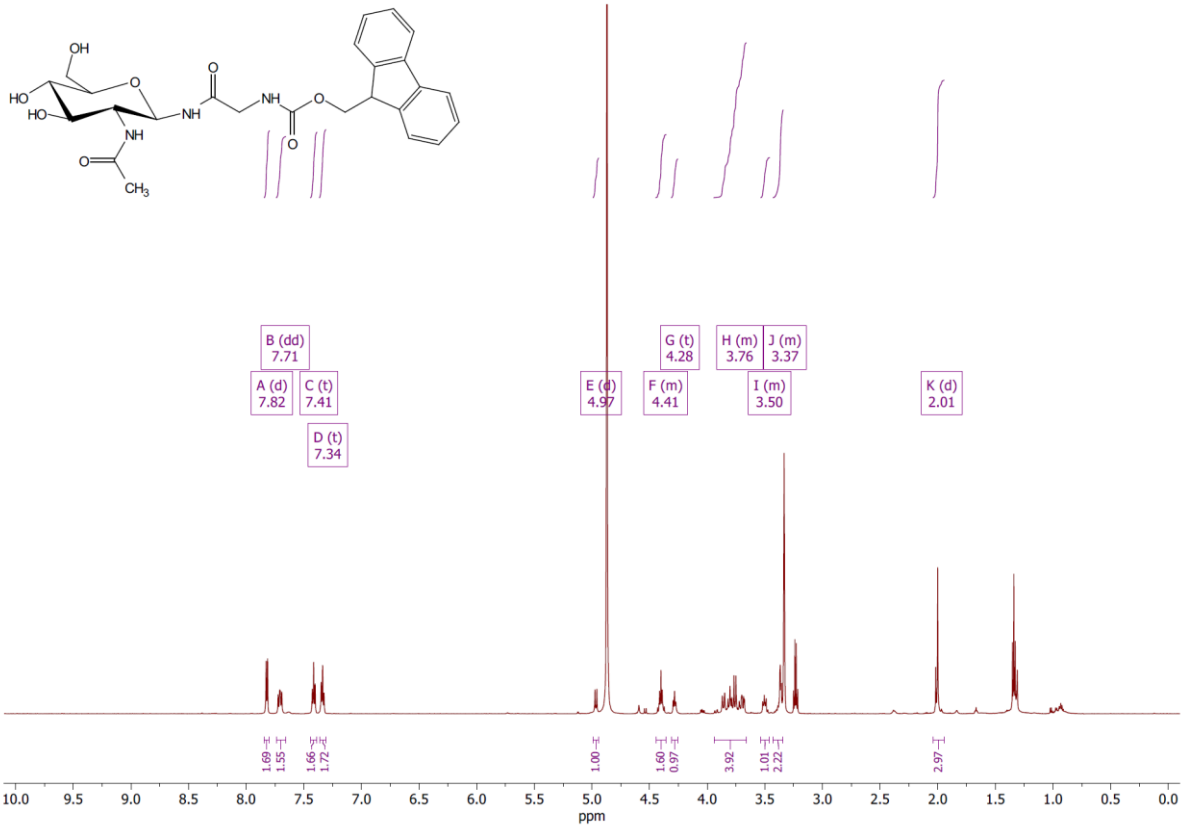


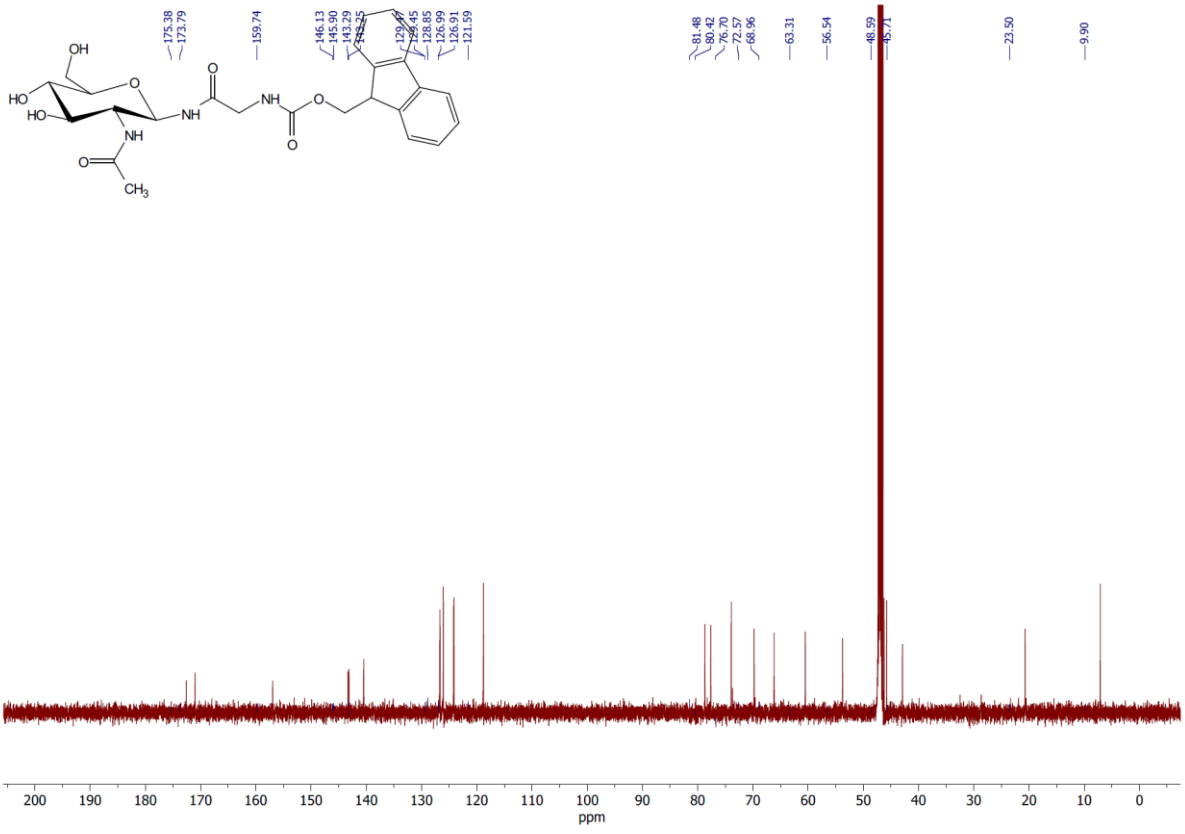
2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-(*p*-tolyl)propanamide (146)



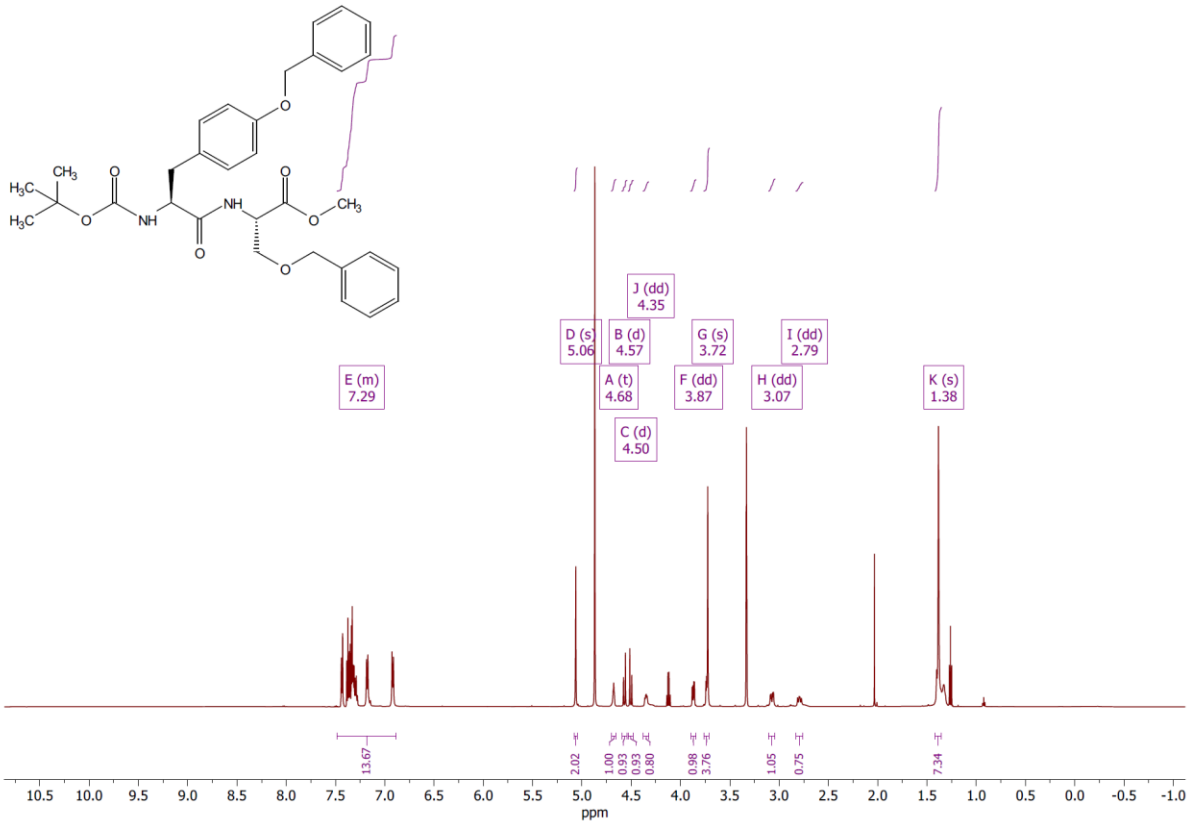


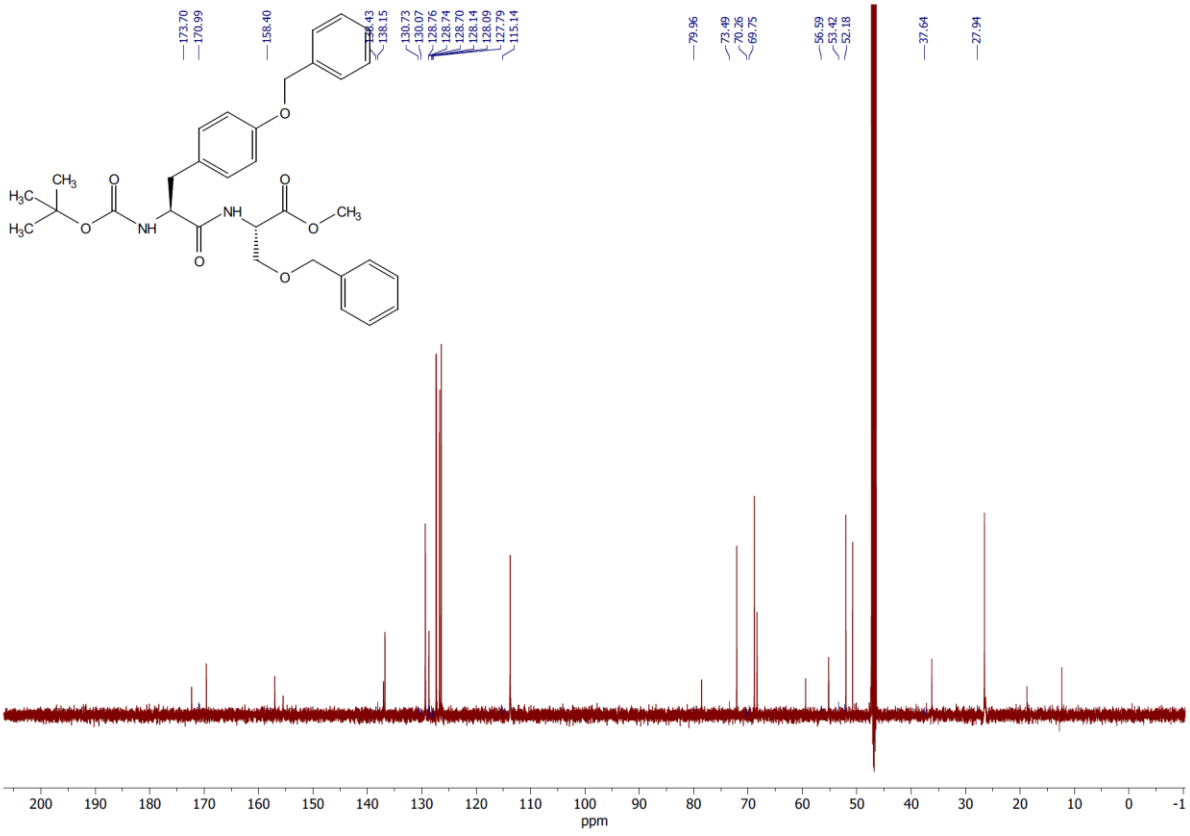
2-Deoxy-2-acetamido-β-D-glucopyranosyl-1-*N*-Fmoc-Gly amide (147)



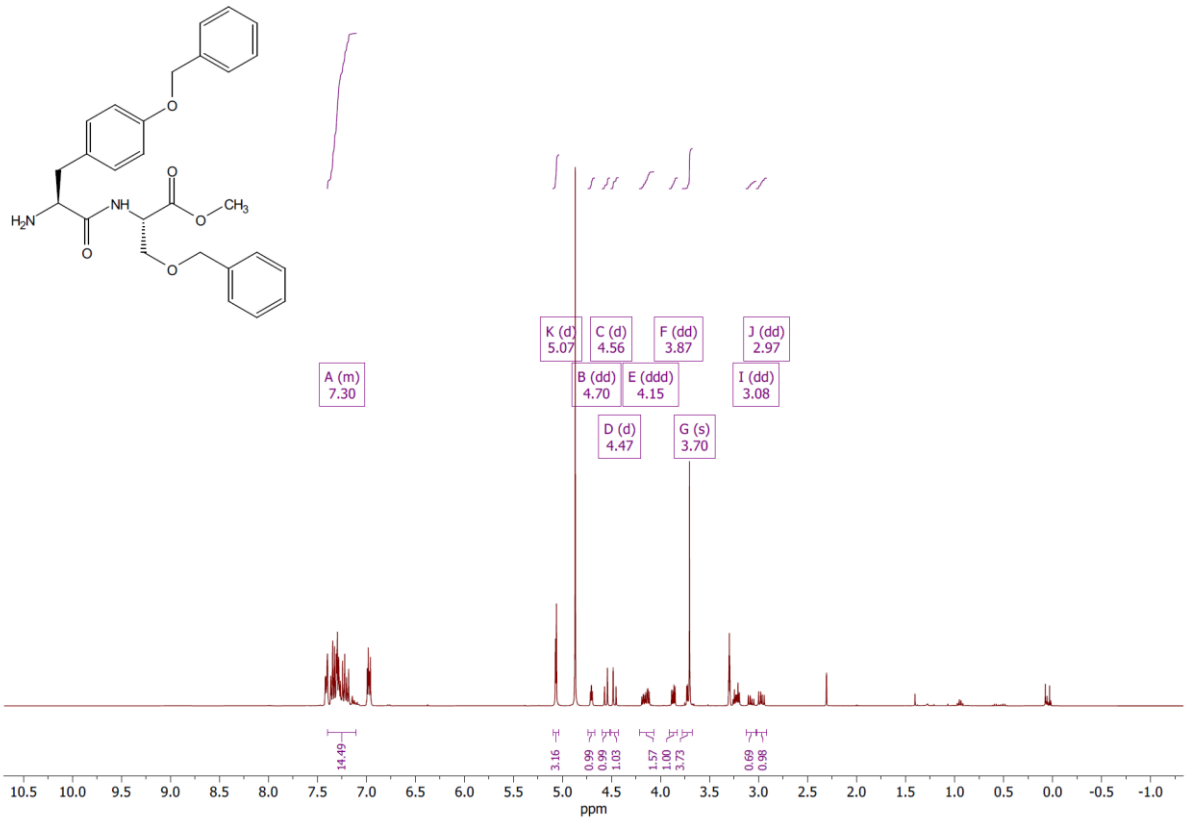


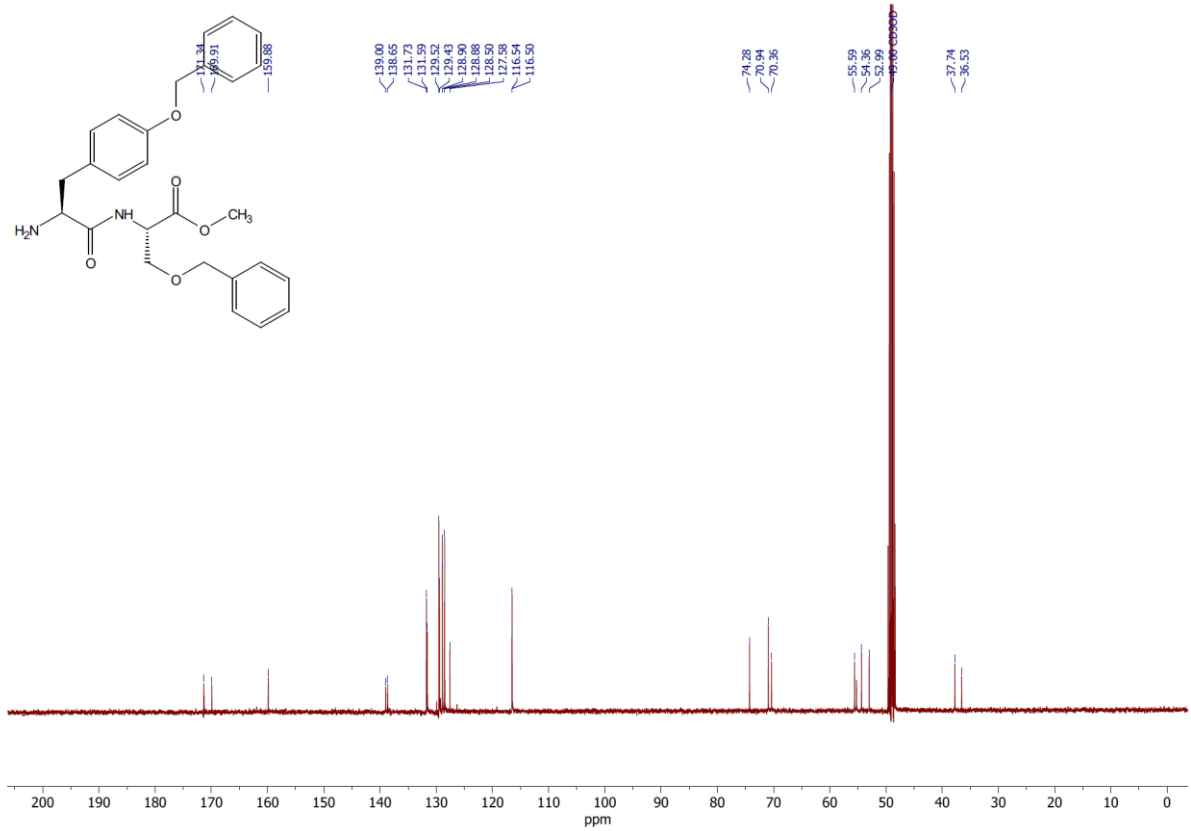
Boc-Tyr(OBn)-Ser(OBn)-OMe (150)



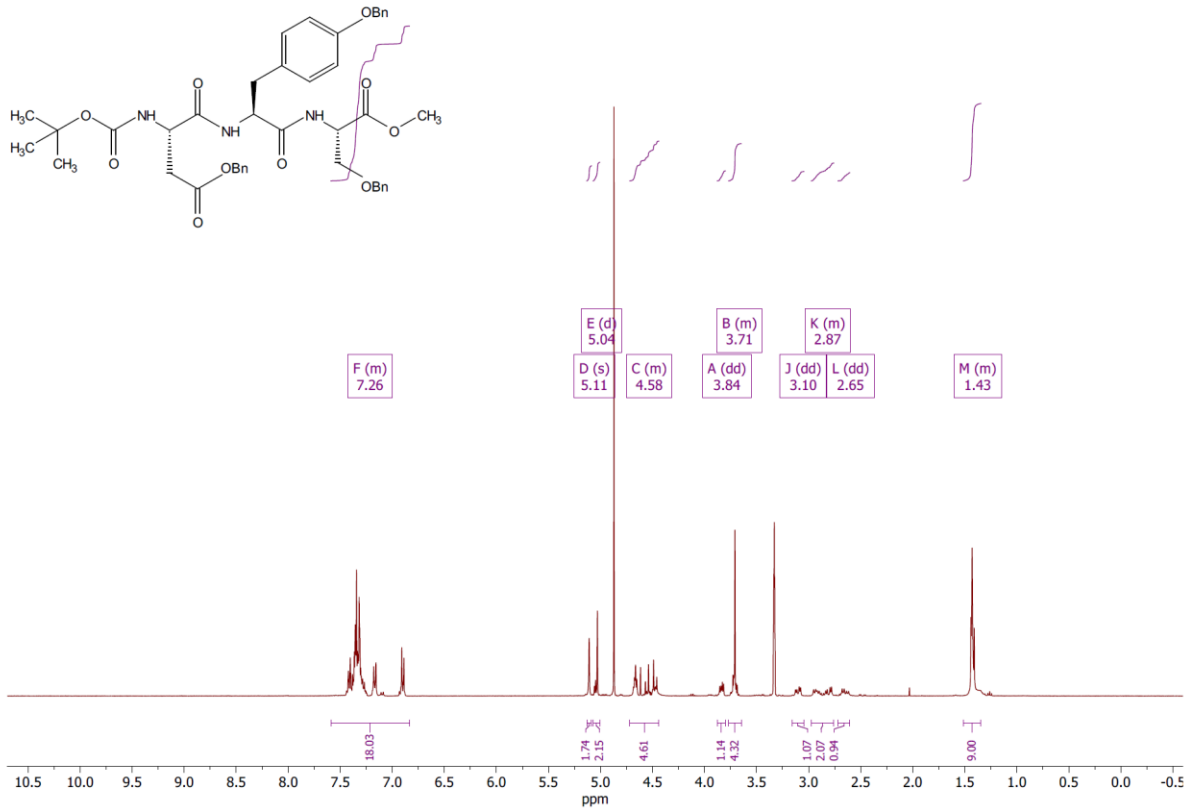


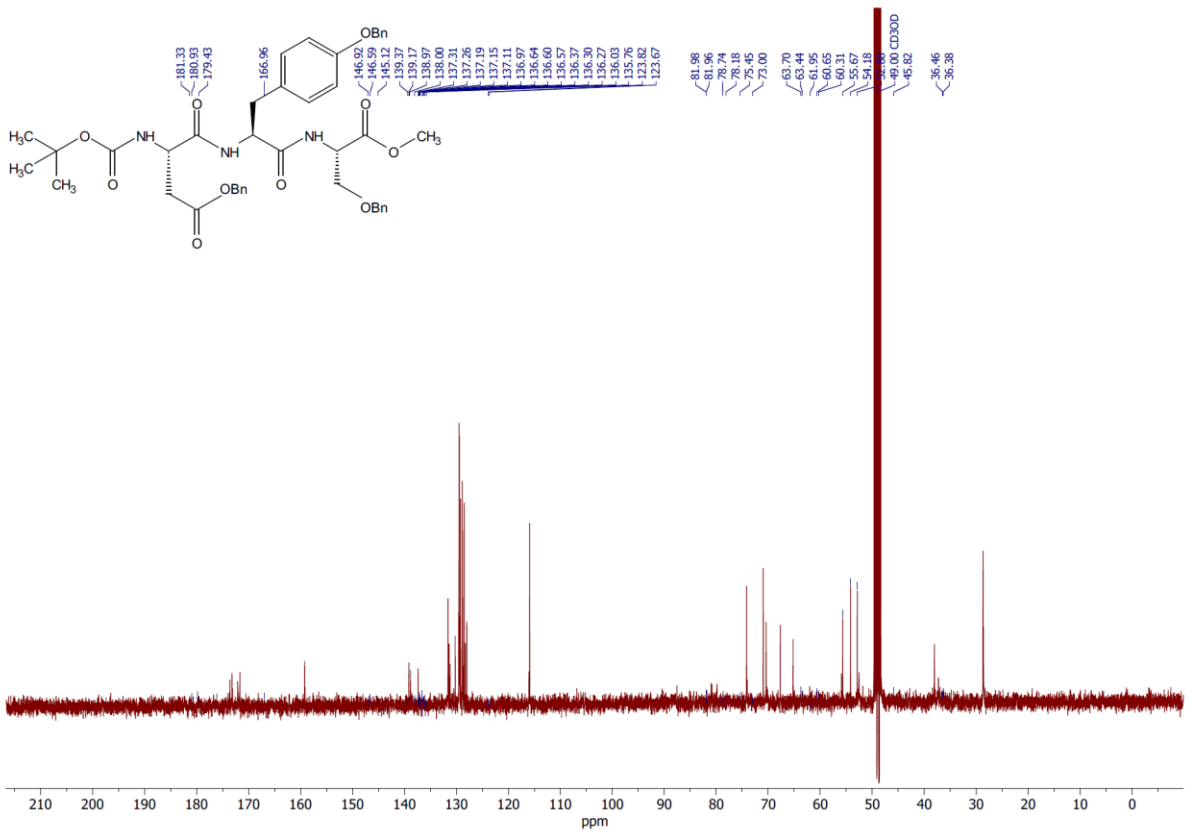
H-Tyr(OBn)-Ser(OBn)-OMe (151)



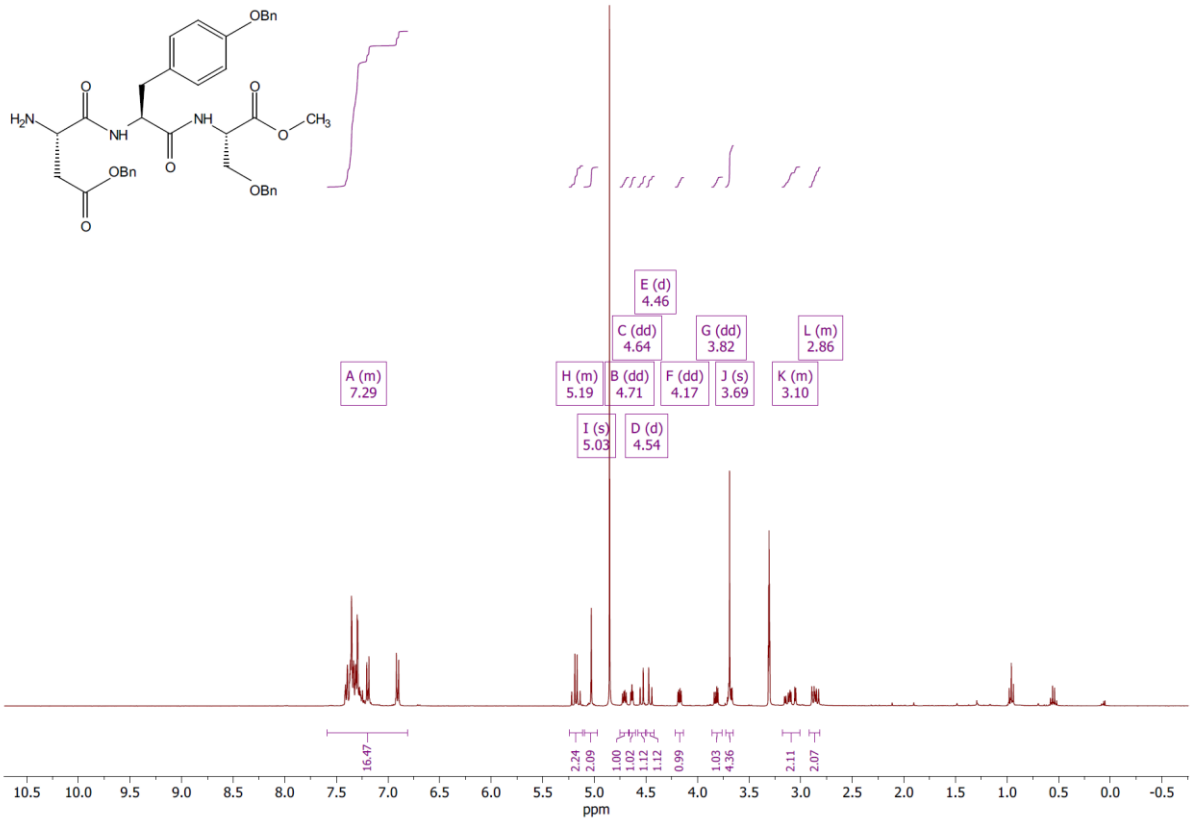


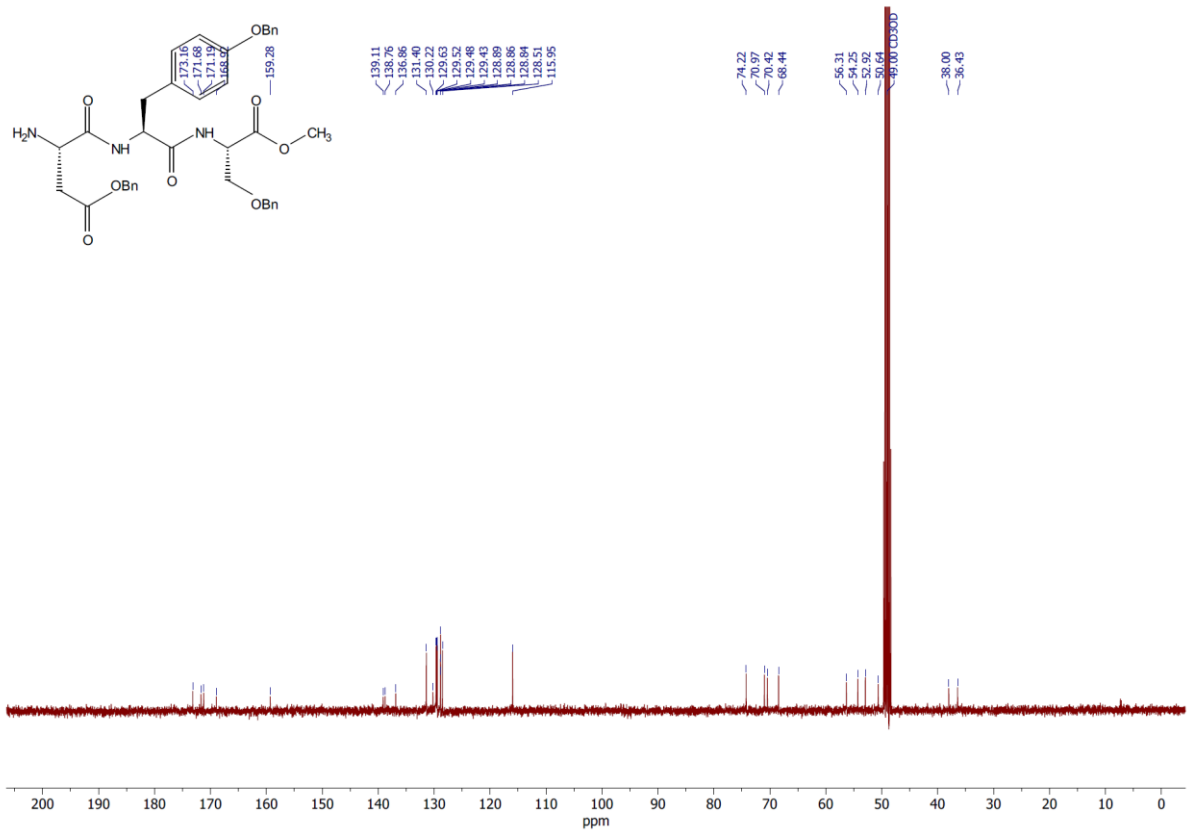
Boc-Asp(OBn)-Tyr(OBn)-Ser(OBn)-OMe (152)



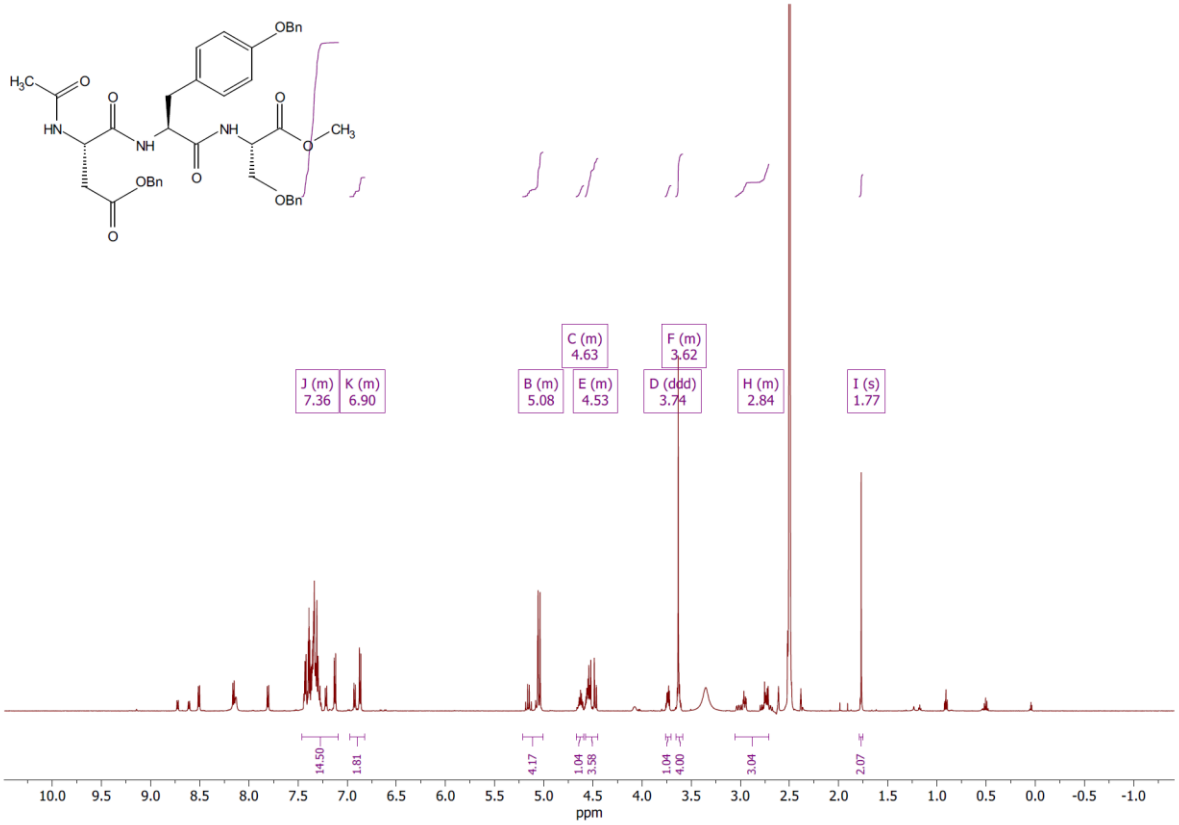


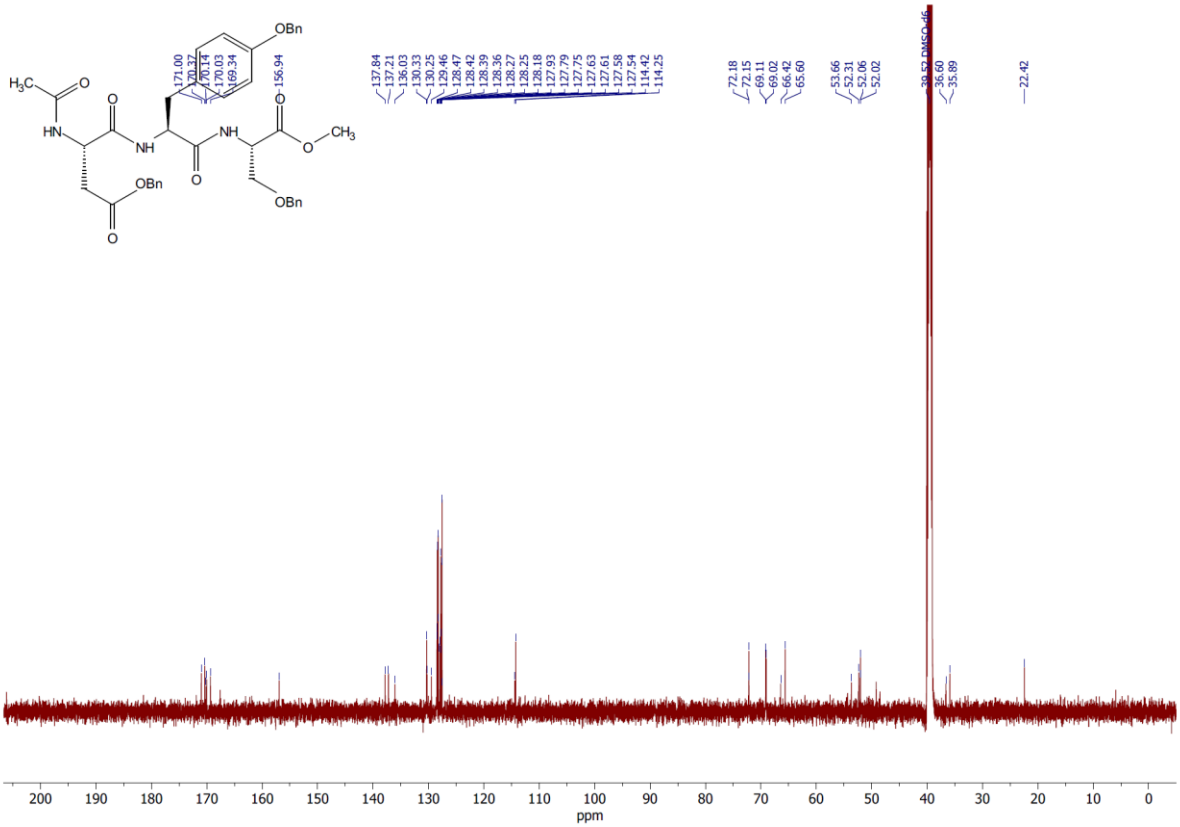
H-Asp(OBn)-Tyr(OBn)-Ser(OBn)-OMe (153)



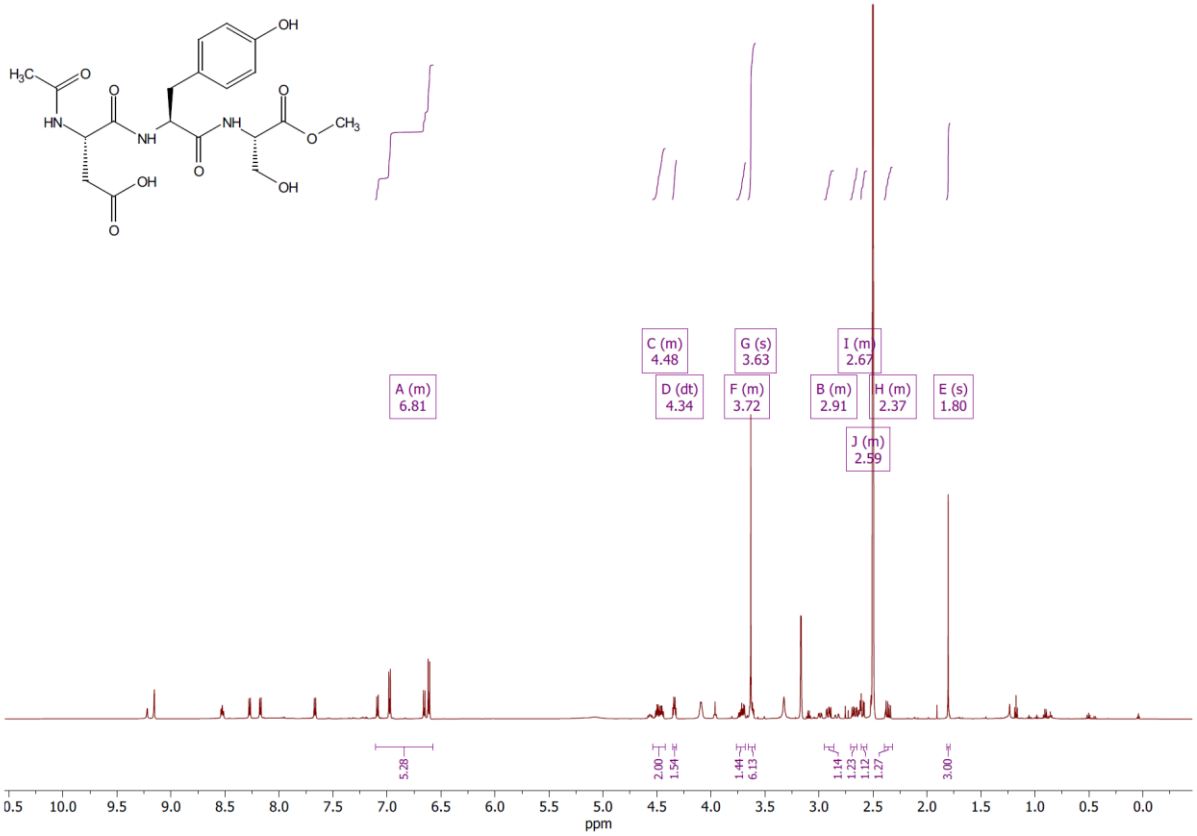


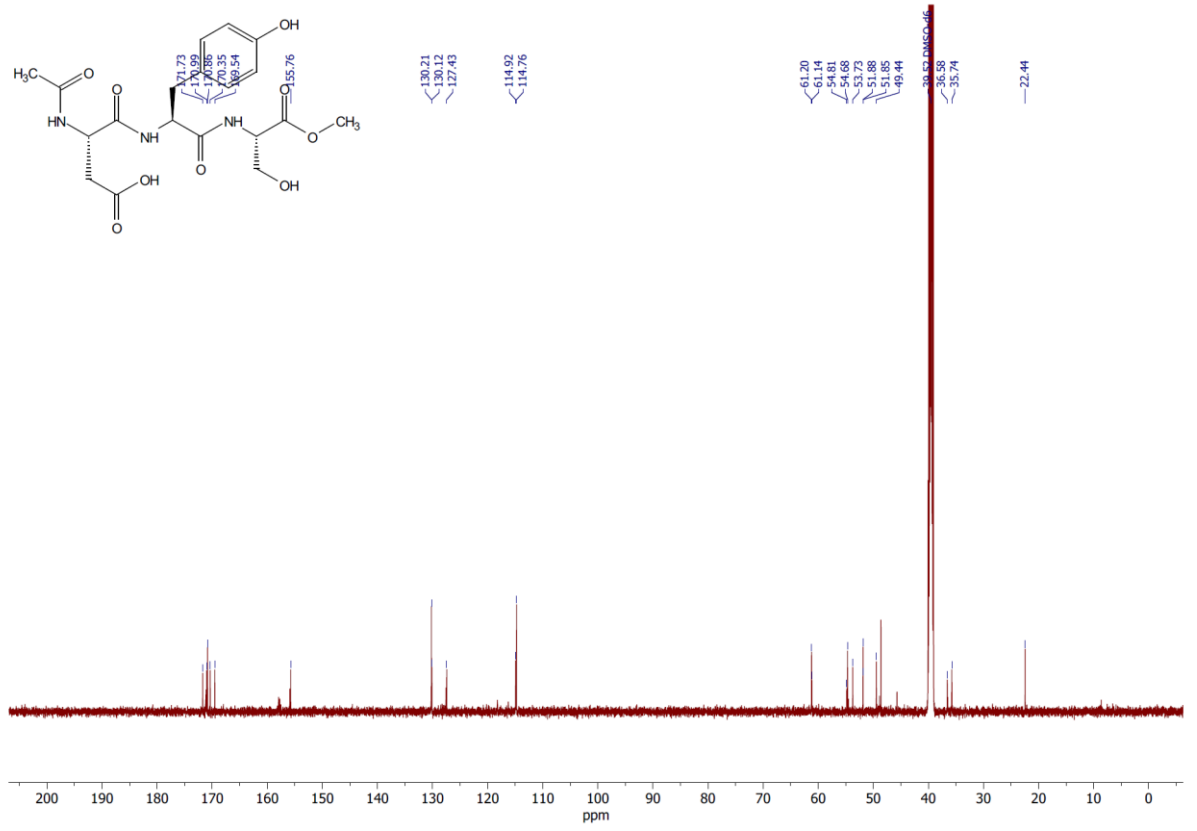
Ac-Asp(OBn)-Tyr(OBn)-Ser(OBn)-OMe (154)



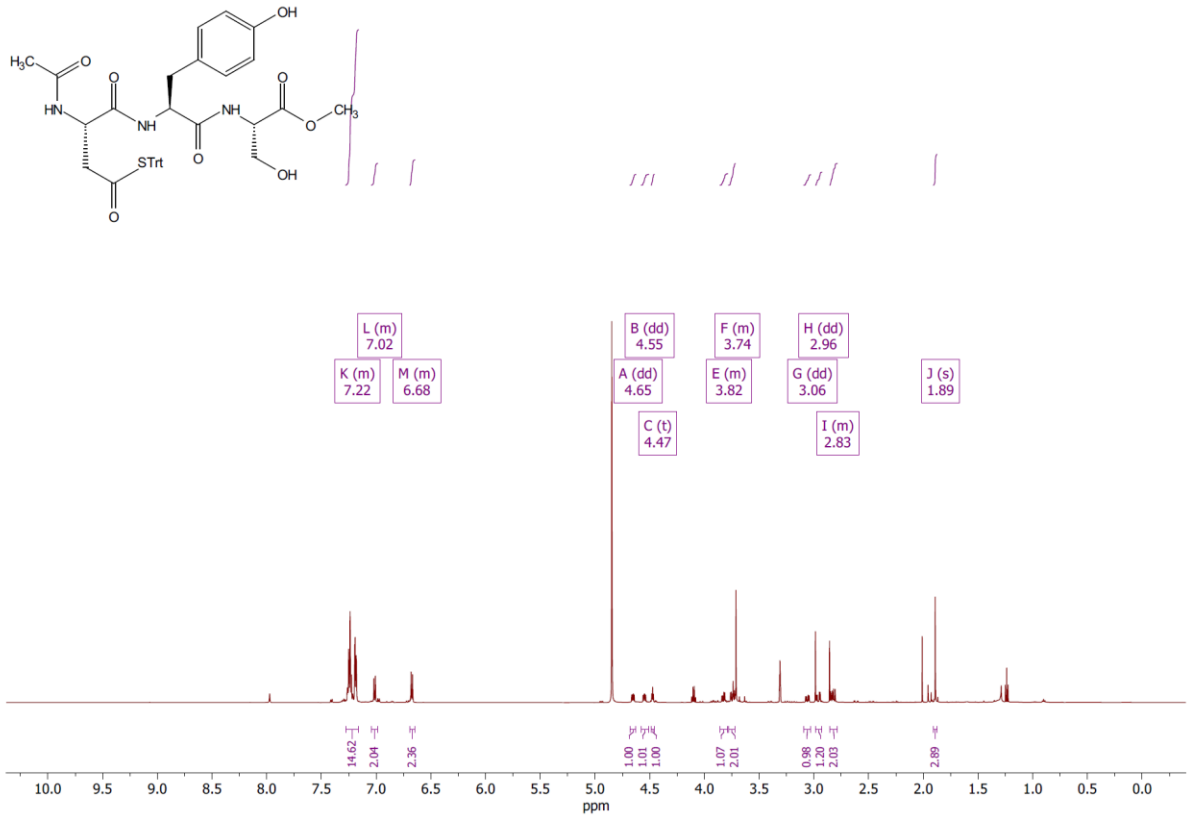


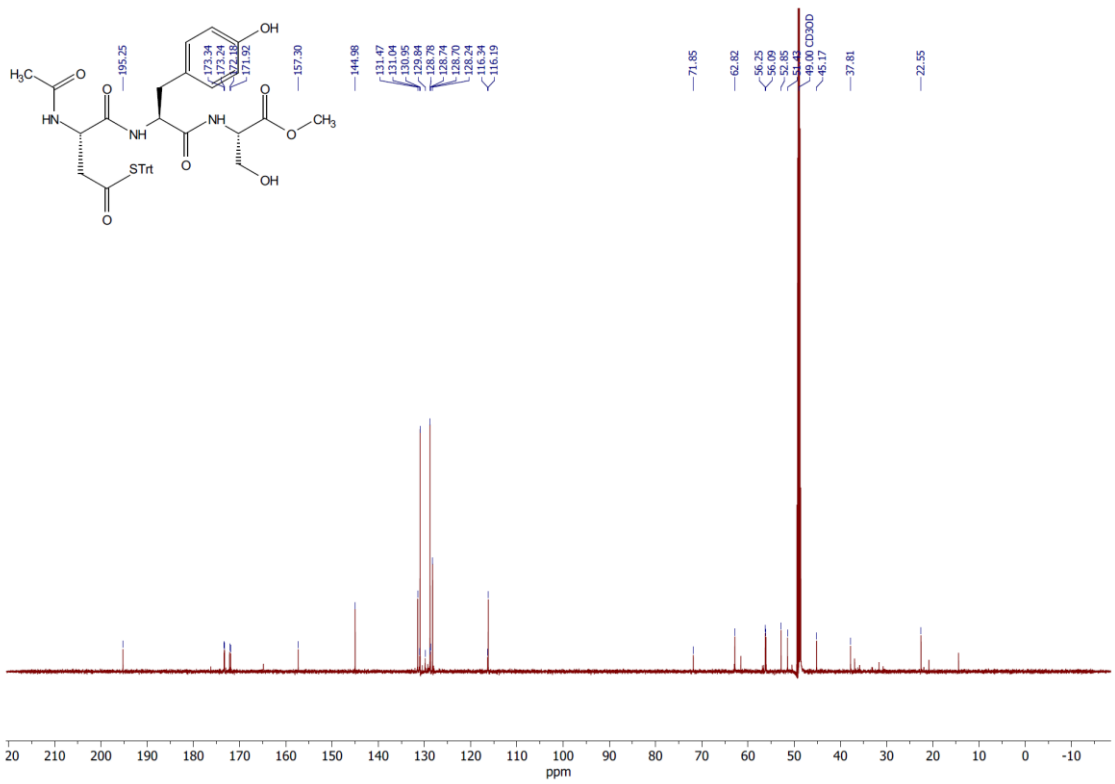
Ac-Asp-Tyr-Ser-OMe (155)



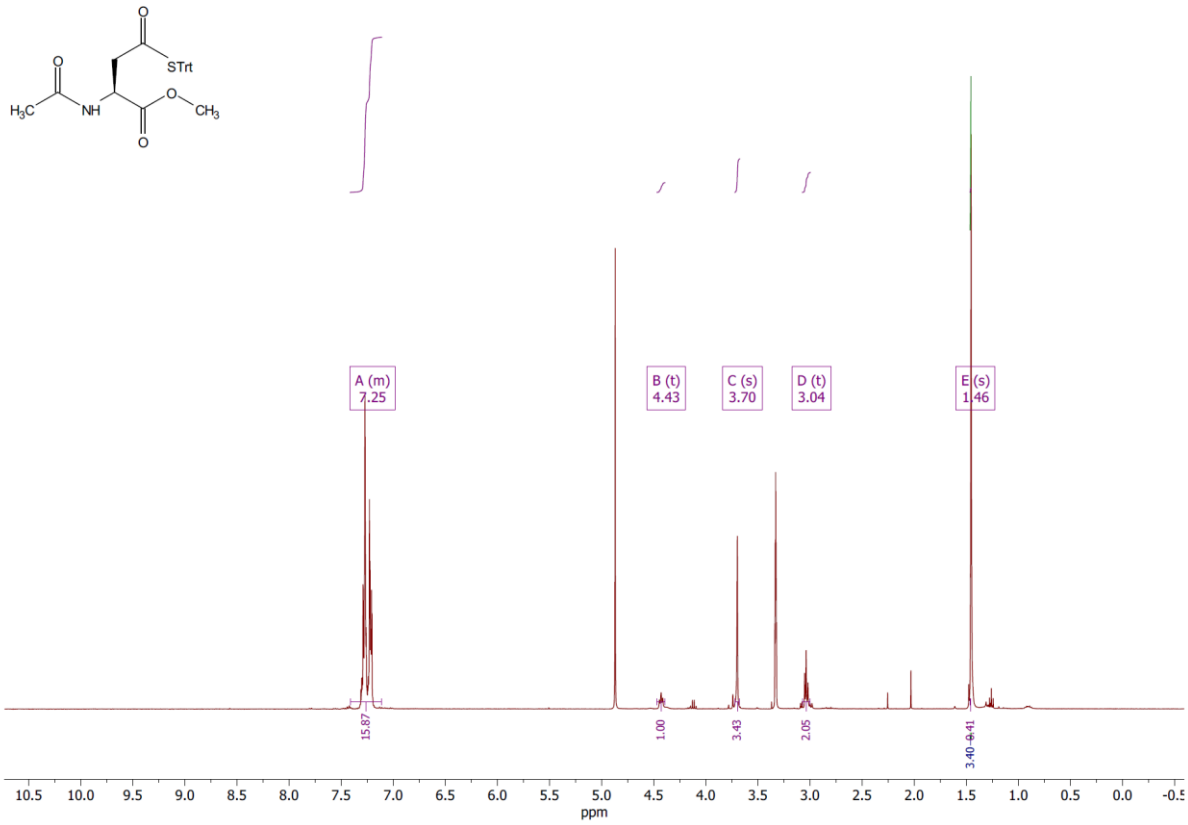


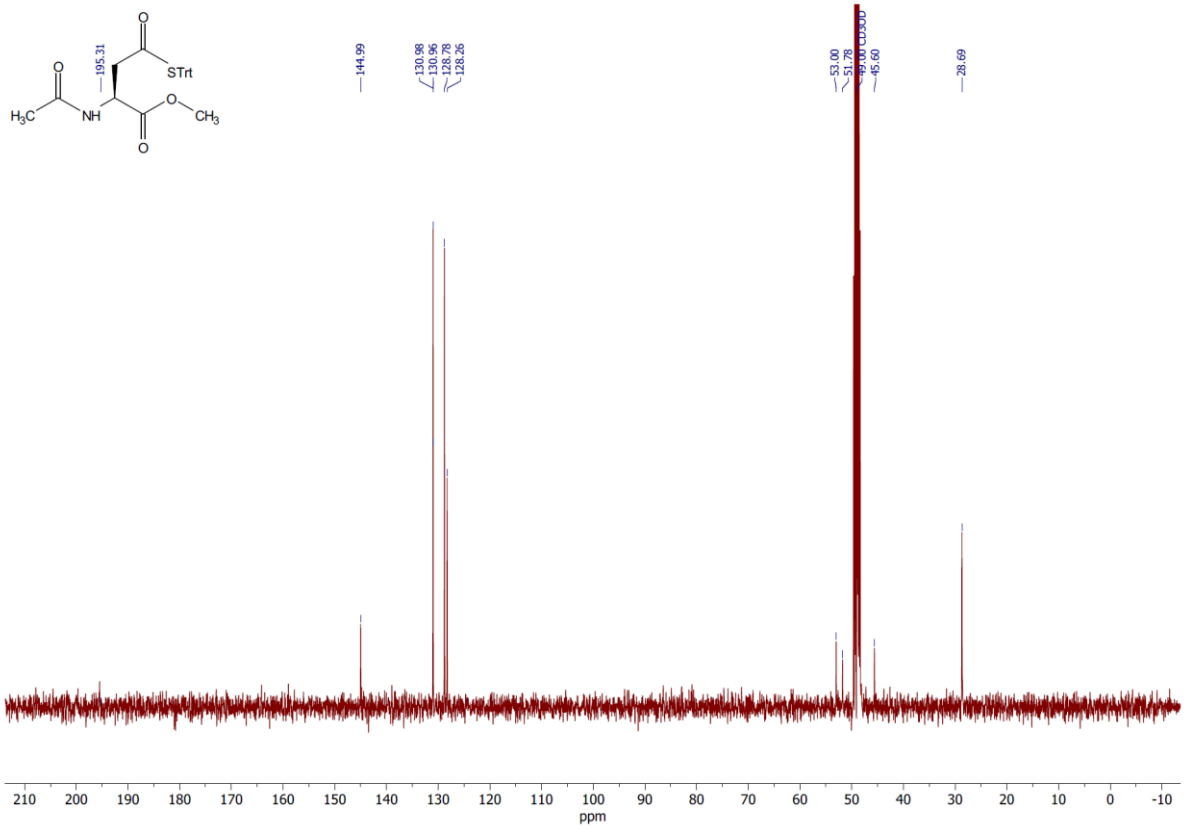
Ac-Asp(STrt)-Tyr-Ser-OMe (156)



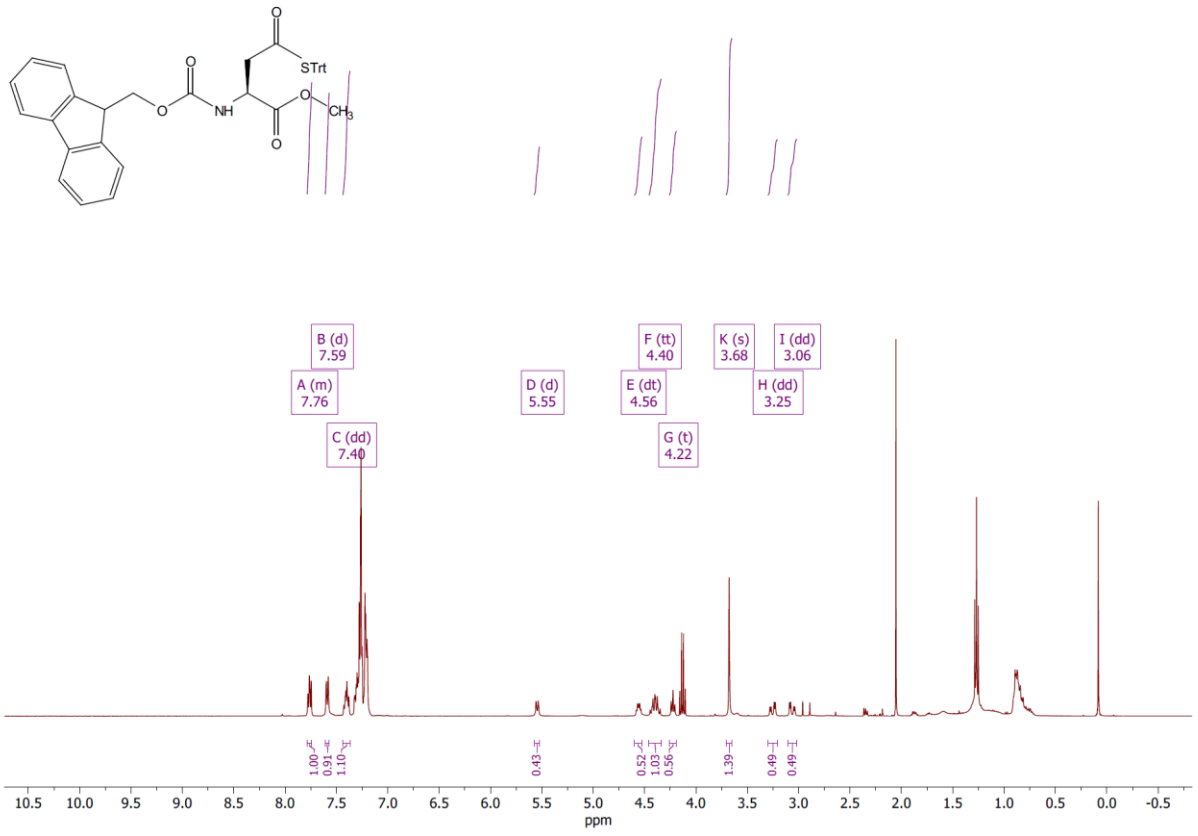


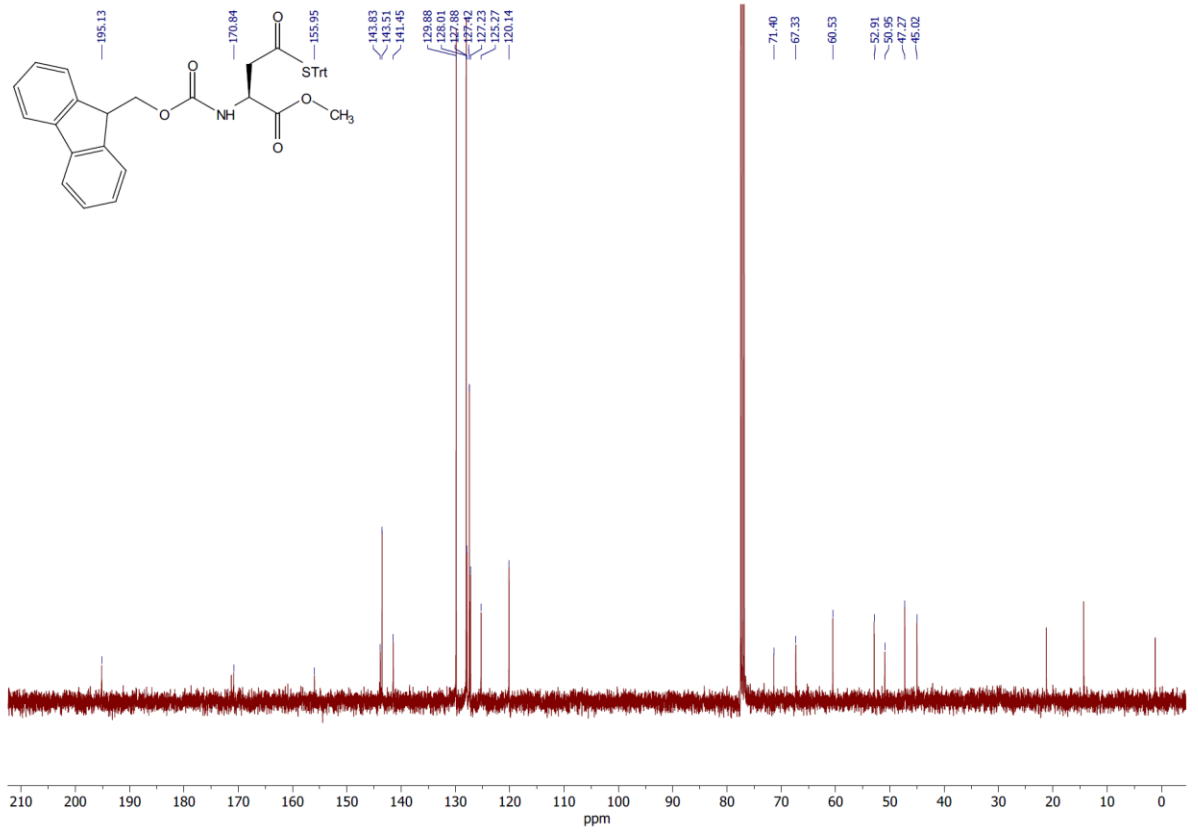
Ac-Asp(STrt)-OMe (165)



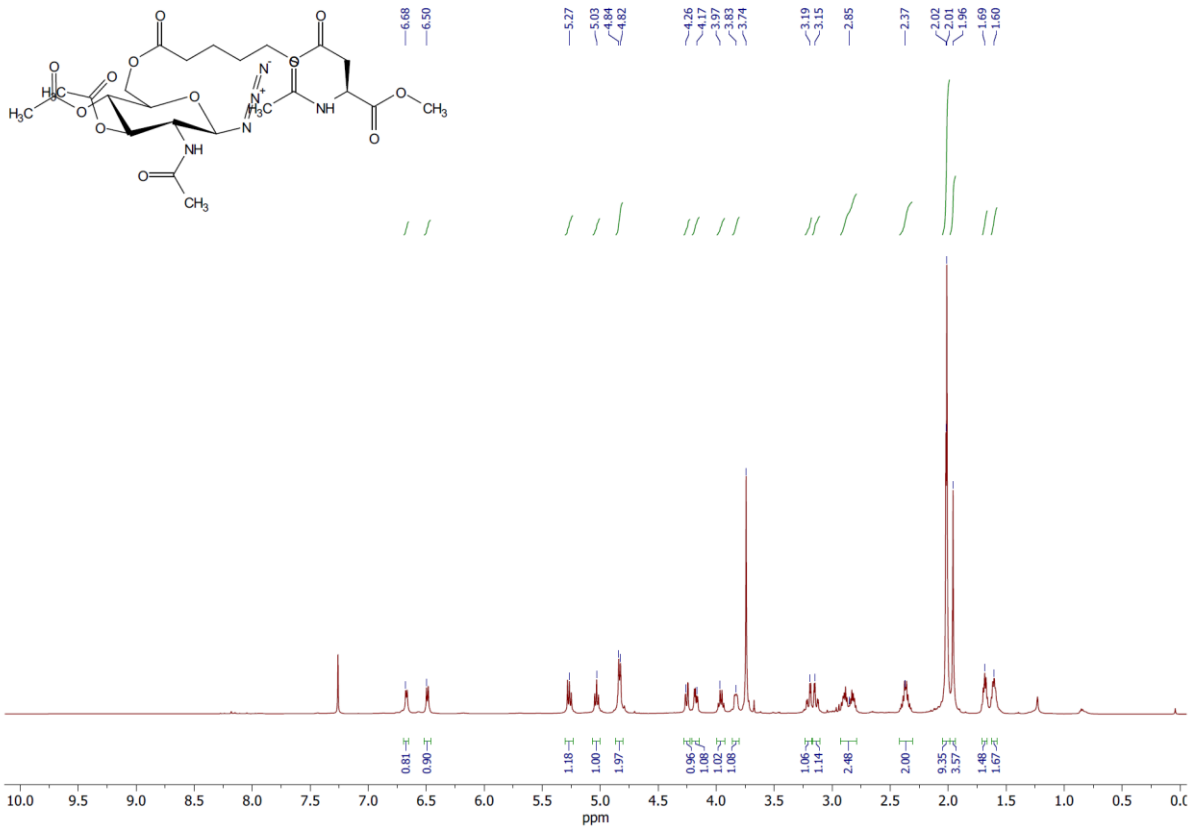


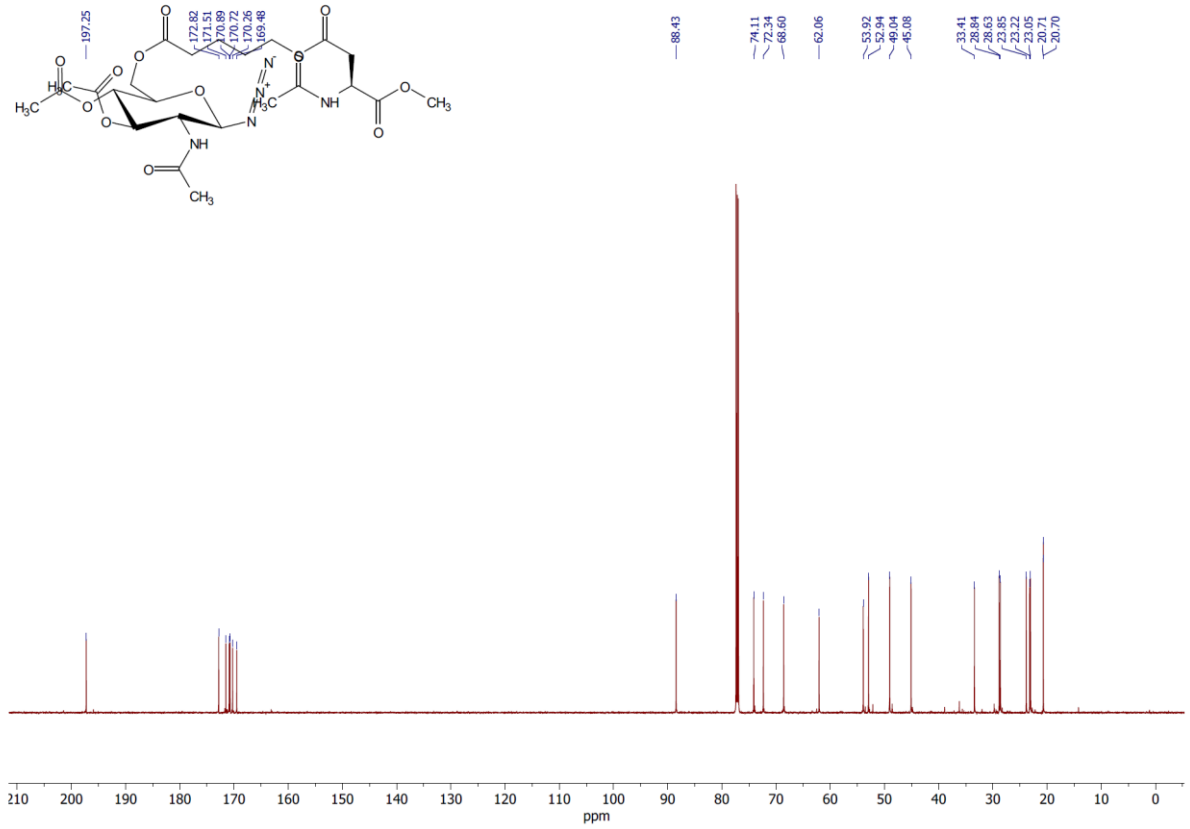
Fmoc-Asp(STrt)-OMe (170)



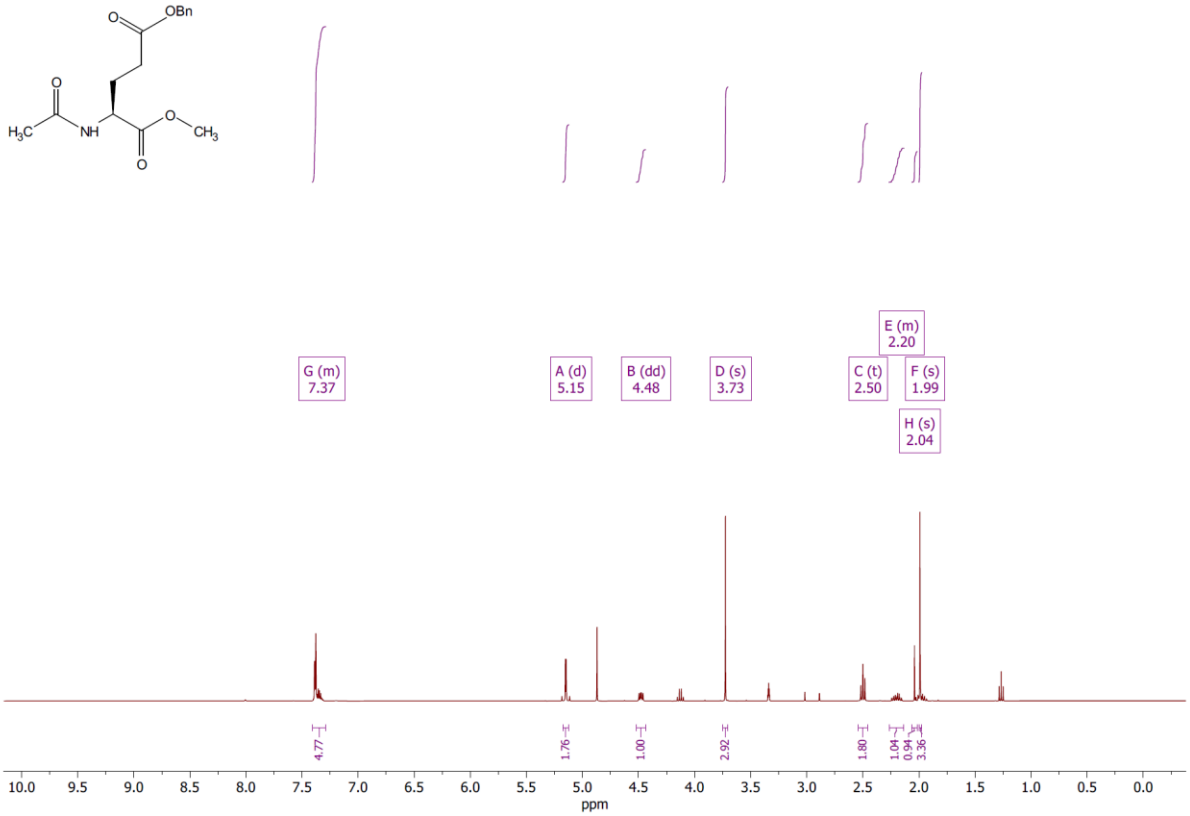


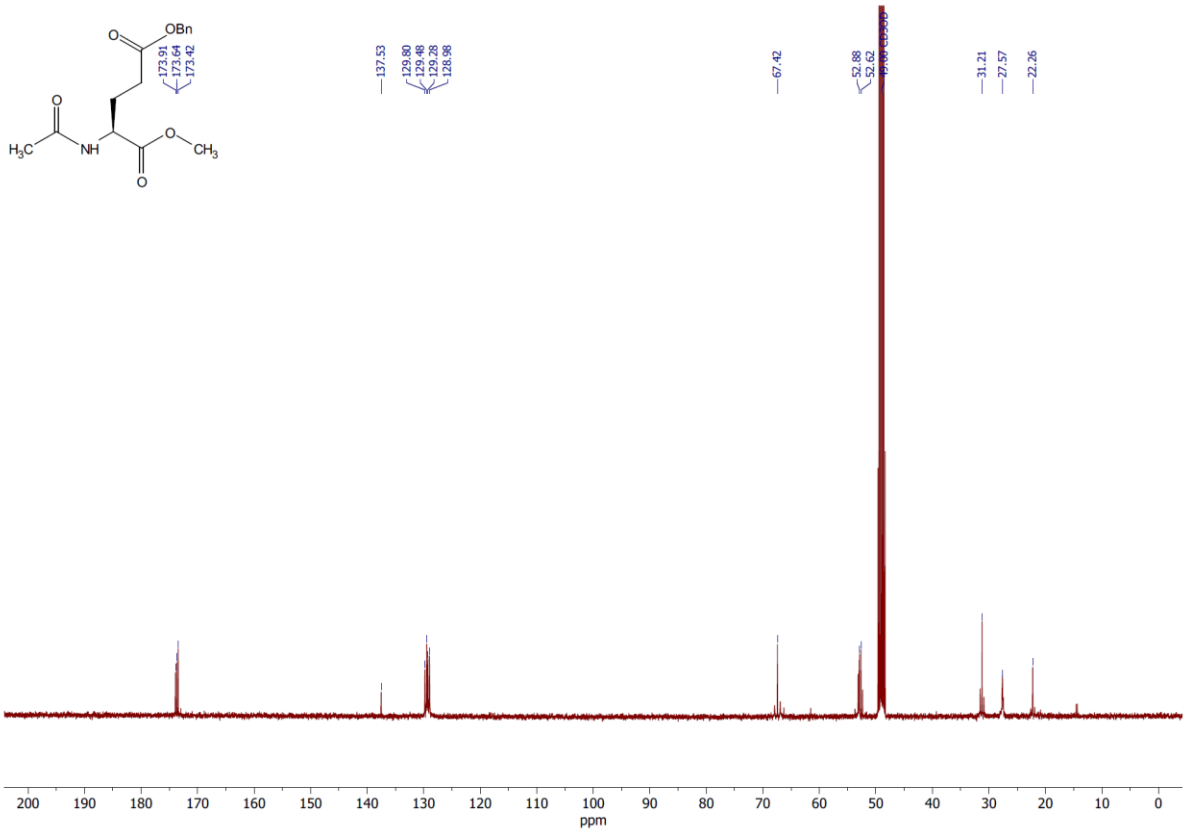
2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-Ac-L-Asp(OMe)thio-pentanoate-β-D-glucopyranosyl azide (172)



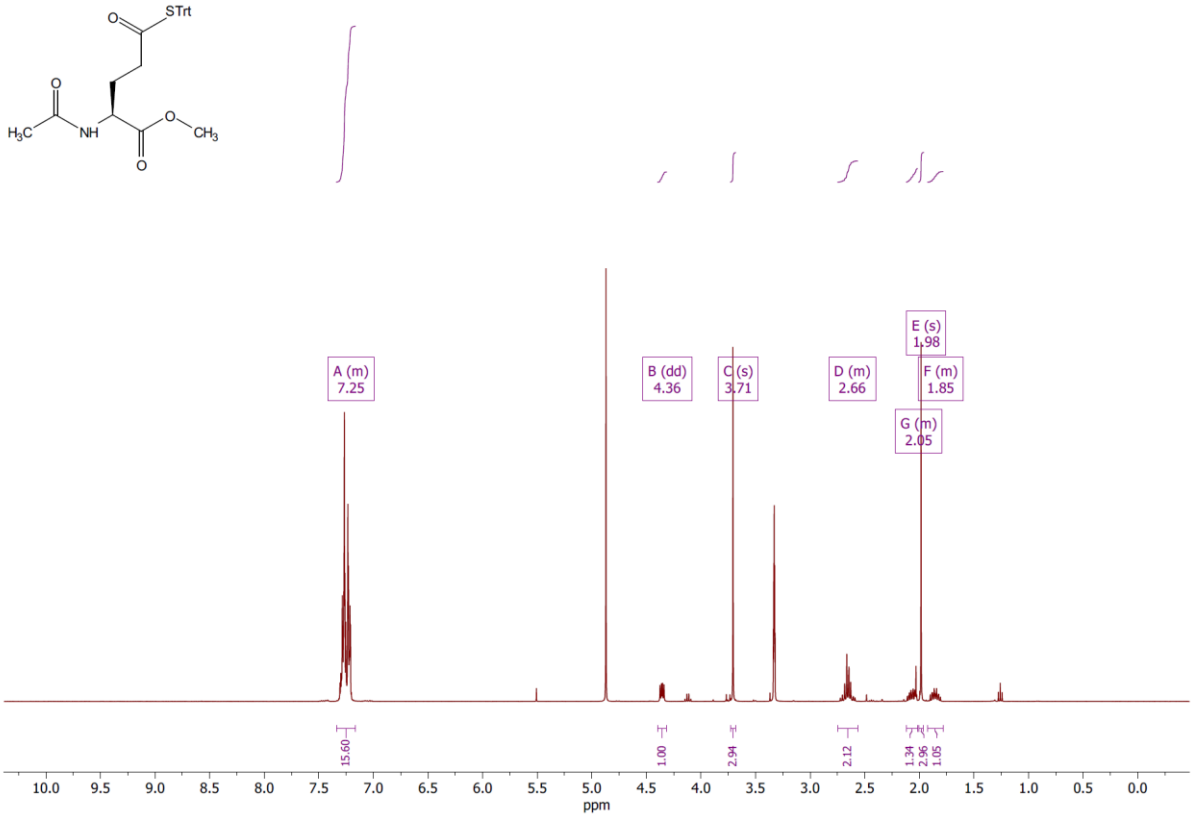


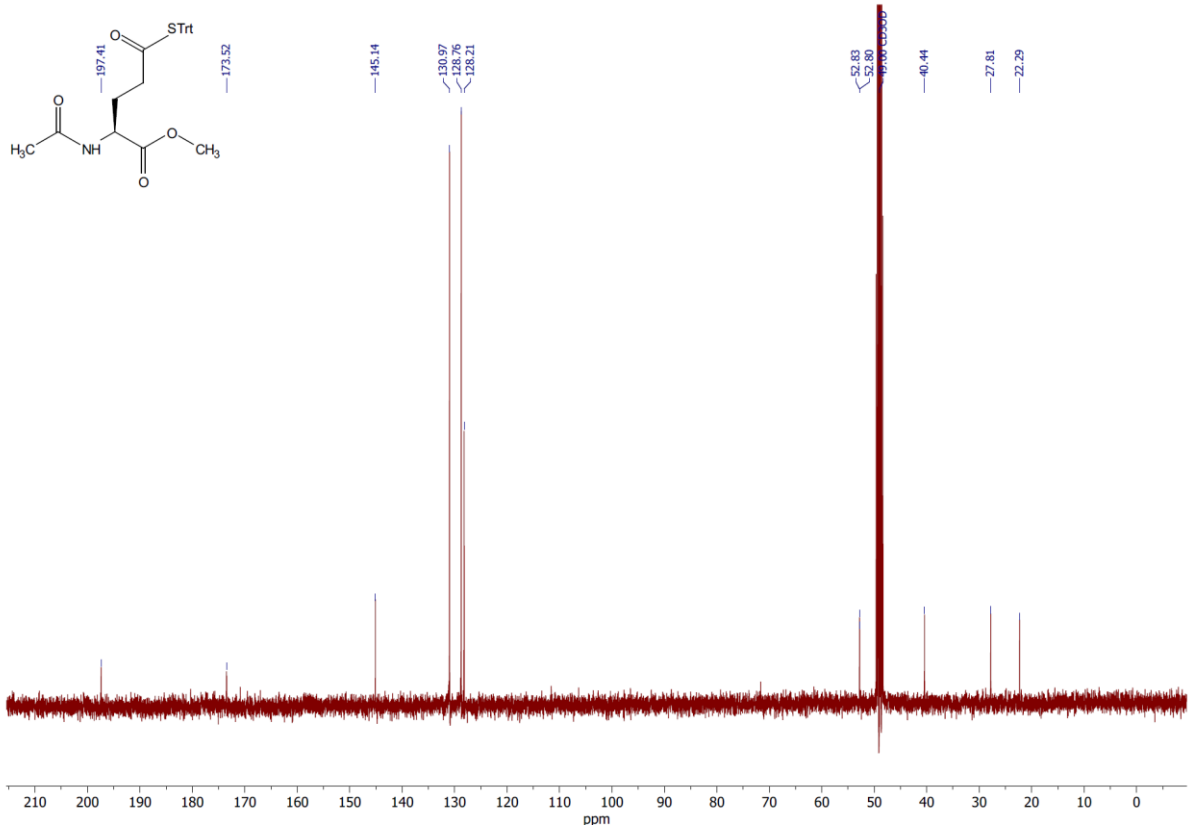
Ac-Glu(OBn)-OMe (177)





Ac-Glu(STrt)-OMe (180)





2-Deoxy-2-acetamido-3,4-di-*O*-acetyl-6-*O*-Ac-Glu(OMe)-thio-pentanoate-β-D-glucopyranosyl azide (182)