Novel Methodologies for the Synthesis of Multivalent Glycoconjugates

By

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"A man can be as great as he wants to be. If you believe in yourself and have the courage, the determination, the dedication, the competitive drive, and if you are willing to sacrifice the little things in life and pay the price for the things that are worthwhile, it can be done."

~ Vince Lombardi

Abstract

Glycoconjugates, such as glycolipids and glycoproteins, play an important role in health and disease. The synthesis and biological evaluation of these glycoconjugates allows for the development of novel carbohydrate-based therapeutics and analytical tools. Traditionally, the conjugation of glycans to other substrates required the installment of an anomeric linker during the total synthesis of the glycan, however, this strategy does not allow for the conjugation of naturally isolated glycans. To address this concern, glycan conjugation methodologies without the need for protecting groups have been developed, including the use of oxyamine conjugation methodologies. In particular, the synthesis of a variety of novel bi-functional oxyamine linkers enabled the rapid assembly of various types of glycoconjugates, including fluorescent- and biotinylated-glycans, glycoproteins and multivalent glycodendrons.

The multivalent presentation of glycans to cell surface lectins is often required to induce a measurable biological response. This multivalent binding can be achieved by the presentation of glycans on dendrons as these 'glycodendrons' have increased affinity for their corresponding lectin compared to monovalent glycans. Moreover, these glycodendrons have several advantages, including high synthetic control, low cytotoxicity and in addition can be derivatised with molecular probes of choice, which can aid in the biological evaluation of these glycoconjugates. Accordingly, novel biotinylated and fluorescent dendrons were synthesised from a highly convergent second generation dendron core scaffold. These functionalised dendrons then allow for the nonavalent conjugation of carbohydrates, such as Lewis antigens, for their biological evaluation in the selective targeting of lectins.

Lewis antigens play an important role in host cell recognition, but these glycans are also involved in disease, such as in cancer metastasis and HIV-infection. The synthesis of Lewis antigens allows for the biological evaluation of these glycans, and moreover, could be employed in the development of novel glycan-based therapeutics and analytical tools. Accordingly, a novel high-yielding and efficient synthesis of a crystalline trisaccharide building block is presented, which can then be utilised in the synthesis of most Type-2 Lewis antigens. In particular, the global deprotection of the crystalline material gave the Lewis^X glycan antigen, a natural ligand for the C-Type lectin DC-SIGN on dendritic cells and macrophages.

Finally, the rapid assembling of complex multivalent glycodendrons is discussed by conjugating the glycan antigens to the functionalised multivalent dendrons through the use of the bi-functional oxyamine linker methodology. In particular, the synthesis of a fluorescent Lewis^X glycodendron is presented, and to demonstrate a potential biological application of this methodology, the fluorescent Lewis^X glycodendron is evaluated as a flow cytometry marker for the C-type lectin DC-SIGN on human macrophages.

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Preface

This doctoral thesis consists of six indivual chapters. Each chapter contains individual compound numbering and contains separate citation and reference lists. To further clarify; the compound numbering and citations are chapter bound, and the identical compounds and citations numbers may differ between the chapters.

The findings in this doctoral thesis has been/will also be presented in the following manuscripts:

- Munneke, S.; Prevost, J. R. C.; Painter, G. F.; Stocker, B. L.; Timmer, M. S. M. 'The Rapid and Facile Synthesis of Oxyamine Linkers for the Preparation of Hydrolytically Stable Glycoconjugates' *Org. Lett.* **2015**, *17*, 624–627.
- Munneke, S.; Painter, G. F.; Gainsford, G. J.; Stocker, B. L.; Timmer, M. S. M. 'Total Synthesis of Lewis^X Using a Late-Stage Crystalline Intermediate' *Carbohydr. Res.* **2015**, *414*, 1–7.
- Munneke, S.; Stocker, B. L.; Timmer, M. S. M.; Gainsford, G. J. '*N*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-*N*-(3-azidopropyl)-*O*-methyl-hydroxylamine' *Submitted to Acta Cryst. E*
- Munneke, S.; Hill, J. C.; Timmer, M. S. M.; Stocker, B. L. 'Synthesis and Hydrolytic Stability of *N*- and *O*-methyloxyamine linker Glycoconjugates' *Manuscipt in preparation*
- Munneke, S.; Stocker, B. L.; Timmer, M. S. M. 'The versatility of *N*-alkyl-methoxyamine bi-functional linkers for the preparation of various glycoconjugates' *Manuscipt in preparation*
- Munneke, S.; Kodar, K.; Painter, G. F.; Stocker, B. L.; Timmer, M. S. M. 'Synthesis of fluorescent multivalent glycodendrons for detection of DC-SIGN on macrophages' *Manuscipt in preparation*

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List of abbreviations

1G	First generation
2G	Second generation
3G	Third generation
Ac	Acetyl
AcOH	Acetic acid
AcSH	Thioacetic acid
AgOTf	Silver triflate
AIBN	Azobisisobutyronitrile
Alk	Alkyl
All	Allyl
APC	Antigen presenting cell
ATPase	Adenosinetriphosphatase
Bn	Benzyl
Boc	*tert*-Butyloxycarbonyl
BODIPY	Boron-dipyrromethene
BSA	Bovine serum albumin
Bu	Butyl
Bz	Benzoyl
BzCl	Benzoyl chloride
CAD	Charged aerosol detector
Calcd	Calculated
CD44	Cluster of differentiation
ClAc	Chloroacetyl
ClZ	2-Chlorobenzyloxycarbonyl
COSY	Homonuclear correlation spectroscopy
Cys	Cysteine
d	doublet
DC	Dendritic cell
DCC	*N*,*N*'-Dicyclohexylcarbodiimide
DCE	Dichloroethane
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
dd	Doublet of doublets
DDQ	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
dfBz	2,5-Difluorobenzoyl
DIC	*N*,*N*′-Diisopropylcarbodiimide
DiPEA	*N*,*N*-Diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DME	Dimethyl ether
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DMTST	Dimethyl(methylthio)sulfonium triflate
DNA	Deoxyribonucleic acid
EBP	Egg-binding protein
EGCase	Recombinant endoglycoceramidase
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ER	Endoplasmic reticulum
EtOAc	Ethyl acetate
FCS	Fetal calf serum
FI	Fluorescent intensity
FITC	Fluorescein isothiocyanate isomer I
Fmoc	Fluorenylmethyloxycarbonyl
Fuc	Fucose
FUT	Fucosyltransferase
Gal	Galactose
GalNAc	*N*-Acetylgalactosamine
G-CSF	Granulocyte-colony stimulating factor
GDP	Guanosine diphosphate
Glc	Glucose
GlcN	Glucosamine
GlcNAc	*N*-Acetylglucosamine
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMP	Good manufacturing practices
gp120	Envelope glycoprotein-120
HBTU	*N*,*N*,*N*′,*N*′-Tetramethyl-*O*-(*1H*-benzotriazol-1-yl)uronium hexafluorophosphate
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HRMS	High resolution mass spectometry
HSQC	Heteronuclear single quantum coherence
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
IFN	Interferon
IR	Infrared
Lac	Lactosyl
LacCer	Lactosyl ceramide
LCMS	Liquic chromatography mass spectrometer

Lev	Levulinoyl
Le^x	$Lewis^x$
LPS	Lipopolysaccharide
m/z	mass/charge
MALDI	Matrix-assisted laser desorption ionization
Man	Mannose
Me	Methyl
MeOH	Methanol
MFI	Median fluorescent intensity
MGL	Macrophage galactose-type lectin
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MsCl	Mesyl chloride
NaOAc	Sodium acetate
NHS	*N*-Hydroxysuccinimide
NIS	*N*-Iodosuccinimide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NP	Nanoparticle
NZD	New Zealand dollar
Obsd	Observed
OMe	Methoxy
OVA	Ovalbumin
PAMAM	Poly(amido amine)amine
PBS	Phosphate buffered saline
PE	Petroleum ether 50-60
p	Pentet
Pept	Peptide
PG	Protecting group
Ph	Phenyl
Phth	Phthaloyl
Piv	Pivaloyl
PMA	Phorbol-12-myristate-13-acetate
PMB	*para*-Methoxybenzyl
ppm	Parts per million
PRR	Pathogen recognising receptor
PSGL-1	P-Selectin glycoprotein ligand-1
Pyr	Pyridine
q	Quartet
RaNi	Raney-nickel
rt	Room temperature
s	Singlet
sat	Saturated
Siglec	Sialic-acid-binding immunoglobulin-like lectin
SMCC	Succinimidyl-4-[*N*-maleimido-methyl]cyclohexane-1-carboxylate
SPPS	Solid phase peptide synthesis
t	Triplet
TBABr	Tetrabutylammonium bromide
TBAI	Tetrabutylammonium iodide
TBDPS	*Tert*-butyl-diphenylsilyl
*t*Bu	*Tert*-butyl
TCA	Trichloroacetyl
TCEP	Tris(2-carboxyethyl)phosphine
TDS	*Tert*-butyldimethylsilyl
TES	Triethylsilyl
TFA	Trifluoroacetic acid
TfOH	Trifluorosulfonic acid
TGF	Transforming growth factor
THF	Tetrahydrofuran
THP-1	Leukemic human monocytic cell line
TIC	Total ion count
TLC	Thin layer chromatography
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TOCSY	Total correlation spectroscopy
TOF	Time-of-flight
Tol	Toluene
TRIS	Tris(hydroxymethyl)amino-methane
Troc	2,2,2-Trichloroethoxycarbonyl
TsOH	*para*-Toluenesulfonic acid
UDP	Uridine diphosphate
UV	Ultraviolet
WGA	Wheat germ agglutinin
Z	Benzyloxycarbonyl

Chapter 1.

Introduction.

1.1 General Introduction

The beginning of the 21st century has seen significant advances within the fields of chemistry and biology related to human health and disease. Nowadays, genomic- and proteomic-based treatments are readily available, and in addition carbohydrate-based treatments are emerging.[1] Glycoconjugates, such as glycolipids and glycoproteins, are essential to all forms of life. Cells are coated in glycans and these fundamental constituents are involved in a variety of biological processes such as host cell recognition, inflammation, cell signalling, proliferation and protein folding.[2,3] A better understanding of the role of glycans, and the successful use of glycan based treatments has pushed the field of glycobiology to the front of academic and pharmaceutical research.

The growing interest in glycobiology has led to the design of numerous novel agents such as glycan-based therapeutics,[4] immunotherapies,[5] small molecule drugs,[6] and analytical tools such glycan-arrays.[7,8] These agents often target lectins (carbohydrate binding proteins) which are expressed on the cell surface. Lectins generally have a weak affinity for their carbohydrate ligands and require multivalent binding in order to induce a measurable biological response. Accordingly, for therapeutic application the glycan epitope is often conjugated to a multivalent substrate such as proteins, liposomes or dendrimers. This conjugation, however, is not straightforward and often does not allow for the use of naturally isolated glycans and/or requires complicated multi-step synthetic protocols.

In this thesis the development of novel chemical tools for the synthesis of multivalent glycoconjugates is described. Several aspects of carbohydrate chemistry are discussed including the rapid assembly of glycoconjugates through the use of bi-functional oxyamine linkers, the

efficient total synthesis of the Lewis^X antigen and the multivalent presentation of these glycans through the use of functionalised dendrons. In addition, the synthesis and biological evaluation of a fluorescent glycodendron for the C-type lectin DC-SIGN will be presented.

1.1.1 Natural roles of glycans

Carbohydrates are essential for life in all organisms and are found either in, or on the surface of all cells. They are fundamental constituents of glycoproteins and glycolipids and are involved in a variety of biological processes.[2,3] Glycans, however, also play important roles in disease where defects in glycosylation processes or the overexpression of glycan antigens can lead to a wide variety of disorders, including cancers.[9] Moreover, pathogens, such as the human immunodeficiency virus (HIV), can evade and infect the host immune system by using a non-pathogenic glycan display.[10,11] Nevertheless, the immune system is quite efficient in protecting us against pathogens by identifying foreign glycan displays through the use of pathogen recognising receptors (PRRs), despite the extreme diversity of glycans found in nature.

1.1.2 Carbohydrate diversity

The structural diversity of glycoconjugates in nature is enormous due to the complexity of the carbohydrate constituents.[3,12] A hexose such as glucose contains five chiral centres, and can adopt four different configurations: α-pyranose, β-pyranose, α-furanose and β-furanose. Besides the stereochemistry of the individual carbohydrate moieties and the anomeric configuration of the hexose, the regiospecificity (*i.e.* 1′→1, 1′→2, 1′→3, *etc.*) of the glycosidic linkages in oligosaccharides gives rise to the vast number of glycans found in nature.[3] In addition, each glycan has specific functions, which may vary depending on the type of conjugate (*i.e.* glycolipid or glycoprotein). In contrast to proteins, the glycan structure is not encoded within the genome of an organism but it is post-translationally modified by glycosyltransferases and glycosidases.[13,14]

1.1.3 Glycoprotein synthesis and post-translational modification

Proteins are biosynthesised by ribosomes which translate messenger ribonucleic acid (mRNA) into polypeptide chains. The peptides and/or proteins then undergo post-translational modifications in order to change the phenotype of the protein, for example by folding, glycosylation, phosphorylation, methylation, lipidation, *N*-acetylation or proteolysis. This process can occur at any time during the life cycle of a protein. Despite the limited human genome the diversity of the post-translational modifications gives rise to the wide variety of proteins. Indeed, the glycosylation of proteins is thought to be one of the most important post-translational

modification processes due to the variety of functional modifications that the carbohydrates can impart on the protein.[12]

1.1.4 N-glycan and O-glycan biosynthesis

The biosynthesis of glycoproteins starts in either the endoplasmic reticulum (ER) or the Golgi apparatus depending on the type of glycan. N-glycans often terminate in *N*-acetylglucosamine (GlcNAc) and are bound through asparagine to proteins, whereas O-glycans are often initiated by *N*-acetylgalactosamine (GalNAc) and can either be linked to the hydroxyl of threonine, serine or tyrosine (Figure 1).

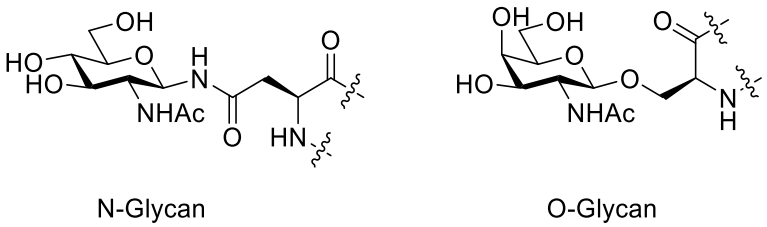


Figure 1. *N-glycans are often terminated in N-acetylglucosamine which is bound to the amide functionality of the amino acid asparagine, whereas O-glycans are often initiated by N-acetylgalactosamine and can either be bound to the hydroxyl of threonine, serine or tyrosine.*

The synthesis of N-glycans starts on the cytoplasmic surface of the endoplasmic reticulum (ER), where phosphorylated dolichol is glycosylated by a GlcNAc-1-phophotransferase, followed by other glycosylations to generate $Glc_3Man_9GlcNAc_2$ on the phosphorylated lipid carrier (Figure 2).[15,16] The glycosyltransferases require nucleotide-sugar donors, which are synthesised in the cytoplasm or nucleus. After dolichol glycosylation on the ER surface, the glycolipid flips to the inside of the ER, where glucosyl-transferases glycosylate the core saccharide. Next, the glycan is transferred to the asparagine of new proteins, which are biosynthesised by the ribosome, by an oligosaccharyltransferase to form the N-linkage, and the lipid carrier is recycled to the surface of the ER for subsequent glycosylation. The new glycoprotein is checked by calnexin, which is a chaperone that determines the protein folding and the quality of proteins. The correctly folded glycoprotein is then transferred to the Golgi apparatus where more than 200 glycosidases and glycosyltransferases further diversity the N-glycan structures. The newly formed glycoproteins can either be transported to the cell surface or excreted from the cell.

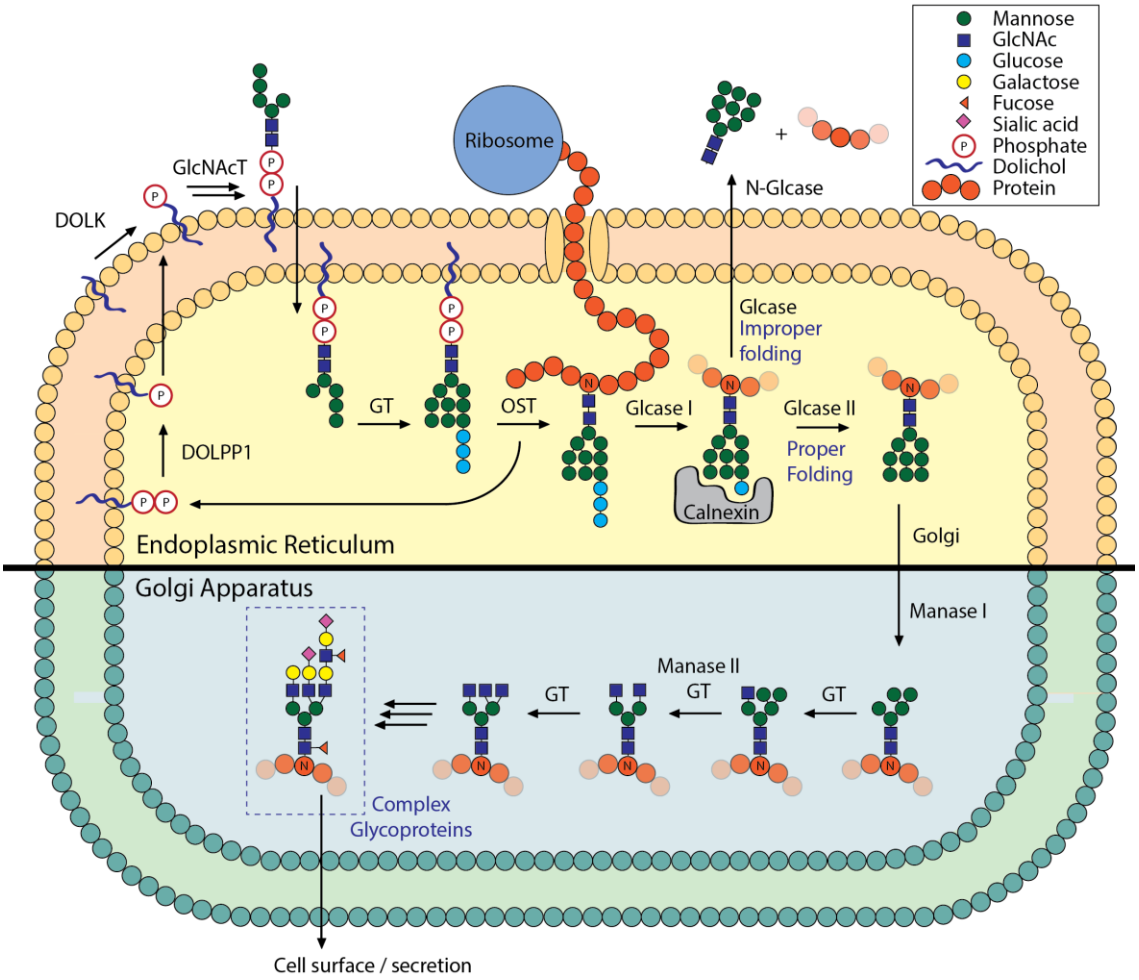


Figure 2. *Biosynthesis of protein bound N-glycans. Figure based on publications of Wolfert & Boons as well as Cantagrel & Lefeber.*[15,16]

The O-glycans are biosynthesised in the Golgi apparatus where the biosynthesis commences with the transfer of a GalNAc moiety onto the hydroxyl group of either serine or threonine by an *N*-acetylgalactosaminyltransferase.[17] From here, the core structure can be converted into complex O-glycoproteins in a similar fashion to N-glycans.

As mentioned above, post-translational modifications occur intracellularly, however, once glycoconjugates are expressed extracellularly, glycosidase and glycosyltransferase activity can still alter the glycan moieties as required, depending on the cellular environment.[13] This glycan alteration can tag proteins to target specific lectins or immune cells, which, for example, can affect cellular uptake, intracellular processing and antigen presentation.

It is important to note that glycosylation pathways differ between organisms, but also within classes, families and individuals. The presence or absence of glycosyltransferases and glycosidases, which are encoded within the DNA, greatly influences the glycan structure and diversity displayed on cells. The immune system can utilise these glycan displays for host cell

and pathogen recognition. Detection of foreign glycans expressed by pathogens, however, can result in pathogen digestion and antigen presentation, which ultimately can lead to immune activation against a particular pathogen.[15]

Unfortunately, many viruses can use (host) glycosylation mechanisms to not only avoid detection by immune cells but also aid infection of the host.[18] For example, the HIV envelope protein gp120 is highly glycosylated (with an average of 25 N-glycans per protein) in order to block antibody binding and proteolysis of the protein antigen, which is necessary for pathogen recognition by the immune system. In addition, these glycan antigens can bind to specific C-type lectins expressed on immune cells, which results in cellular internalisation and infection of the host cell.[19]

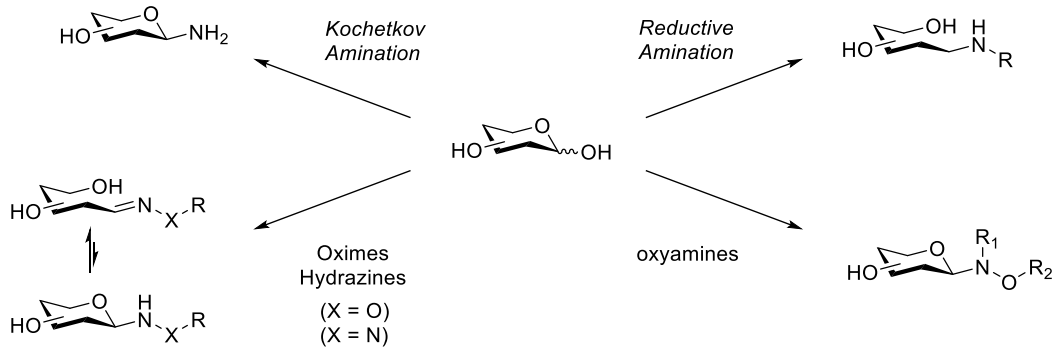
1.1.5 Carbohydrate-based therapeutics

While carbohydrates play an important role in both health and disease, it has taken a considerable amount of time for scientists to appreciate the role of carbohydrates in nature.[1] The structural elucidation and chemical conjugation of carbohydrates was pioneered in the 1890's when Emil Hermann Fischer, who later received a Nobel Prize for this work, reacted carbohydrates under acidic conditions with alcoholic solvents to form Fischer glycosides.[20] During the next 100 years, many different types of glycoconjugates were discovered and synthesised.[21] The field of glycomics, however, did not start to expand until the 1980s with significant breakthroughs such as the development of small molecule glycosidase and glycosyl transferase inhibitors,[6] and carbohydrate-based vaccines.[1,5]

Most of these small molecules target glycosyltransferases and glycosidases, whereas other glycan therapies often target lectins. For example, many iminosugars can be used as potent inhibitors for glycosidase and glycosyltransferases by mimicking the transition state of the natural substrate.[22,23] In contrast, glycans can be utilised in a therapeutic setting to target specific lectins to induce a biological response or for cell-selective targeting for immune activation or cell-depletion. This, however, requires the conjugation of the glycan antigen to a multivalent substrate, such as proteins or dendrimers, in order to obtain a significant biological response upon lectin binding.

1.2 Glycan Conjugation strategies

As described above, a number of biological functions have been attributed to glycoconjugates and, accordingly, there has been much interest in the construction of glycoconjugate mimetics,[21,24,25] glycopeptides,[26] and carbohydrate arrays,[7,27,28] and in the synthesis of fluorescent or biotinylated glycoconjugate probes.[29,30] The conjugation of carbohydrates to the substrate of choice often requires the use of a chemical linker at the non-reducing end of the glycan. Traditionally, the installation of such linkers requires the total synthesis of the glycan antigen, in which the linker is installed in an early stage of the synthesis. Such a strategy, however, is time-consuming and does not allow for the conjugation of carbohydrates from natural sources. Accordingly, much effort has been expended in the development of linker strategies that can be performed at the reducing end of free sugars without the need for protecting groups. These approaches include the use of Kochetkov amination,[31] reductive amination,[32] oximes/hydrazides[33–35] and the use of oxyamines (Scheme 1).[36]



Scheme 1. *Chemoselective chemical ligation methods for free sugars.*

1.2.1 Kochetkov Amination

The Kochetkov amination allows for the formation of a β-D-glycosylamine by incubating a carbohydrate with a free reducing end in an ammonium carbonate solution at 30–40 °C for more than 24 hours.[31] Although the scope and application of the Kochetkov amination is limited to amine conjugation chemistry, acylation reactions with the glycosylamine can then result in complex glycopeptide conjugates. This was nicely demonstrated by Danishefsky and co-workers who used the Kochetkov amination to conjugate complex sialylated tri-branched N-glycans to native peptide sequences so as to synthesise various glycoforms of erythropoietin (EPO) and study their role in prostate cancer.[37] Although the Kochetkov amination results in the native N-glycan linkage, often incomplete amination is observed.[38] Moreover, given the instability of the glycosylamine, the crude product is often used in amide ligation reactions. Here, removal of the large excess of ammonium formate is critical to prevent the reaction with activated peptides to form asparagine derivatives. The methodology was later adapted by Davies and co-workers,

whereby the glycoside in the presence of Lawesson's reagent was converted into a glycosylthiol, which then allowed for sulfur conjugation chemistry.[39]

1.2.2 Reductive Amination

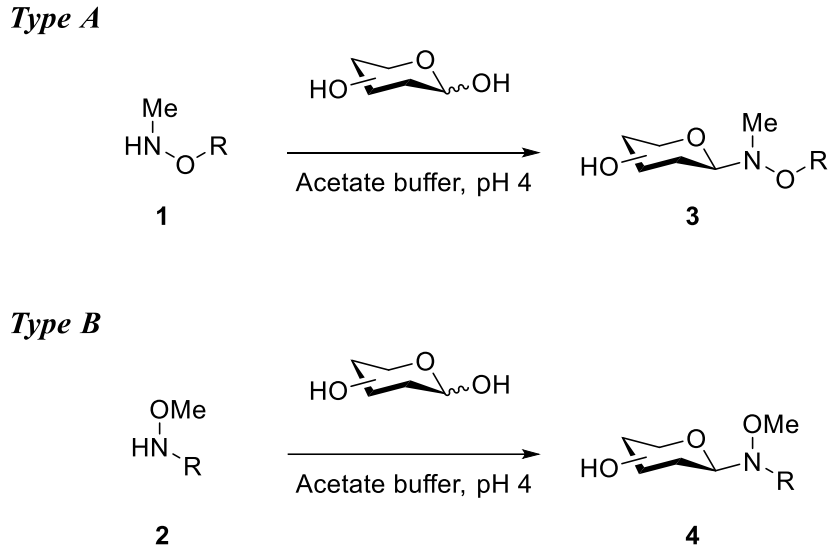
Reductive amination is widely used for the preparation of glycoconjugates. Here, the aldehyde functionality of the sugar is converted into the imine, which in turn is reduced by a reducing agent to give the 'ring-opened' glycoconjugate. There are many examples of the use of reductive amination for the synthesis of glycoconjugates, with a representative example being that of Van Kooyk and co-workers who synthesised $Lewis^X$ glycodendrons to target DC-SIGN on dendritic cells.[40] It can also be used to conjugate glycans directly to lysine moieties on proteins in order to obtain glycoproteins.[41] This technique, however, affects the structural integrity of the reducing end carbohydrate, which in turn could affect lectin binding.

1.2.3 Oxime/Hydrazine Ligation

Another widely studied conjugation method is the oxime and hydrazine glycan ligation. In 1895, Wolff discovered that hydrazines can react with carbohydrates to form stable glycoconjugates,[42] and since then, it has been determined that both oximes and hydrazines can be used in a condensation reaction under aqueous conditions to create glycoconjugates without the need for protecting groups.[43] Accordingly, both hydrazine and oximes have been used in various applications, which include the characterisation and isolation of glycan antigens,[44–46] and glycoconjugate synthesis.[47–50] Moreover, oximes and hydrazines can be used in analytical applications. For example, fluorescent hydrazines have been synthesised for use in HPLC analysis,[46] whereas biotinylated analogues were used to immobilise glycan antigens on plates for ELISA assays.[51] Although the use of oxime and hydrazine ligations is a robust technique for the conjugation of glycan antigens, in solution the obtained glycoconjugate excists in the ring-opened hydrazine and the closed pyranose and furanose forms,[52] which could affect lectin affinity and thus the biological activity of the conjugate.[48] In 2014, Unverzagt and co-workers reported the reaction of hydroxylamine glycosides with α-ketoacids, such as pyruvic acid, to form the native N-glycan acetamide linkage in various yields (23 – 80%). When these α-ketoacids were incorporated into more complex peptides, low yields were obtained.[53] This novel method, however, has great potential in the preparation of glycoconjugates. Nevertheless, in 1998 it was determined by Peri *et al.* that the use of *N*,*O*-dialkylated oxyamines in the condensation reaction resulted in the exclusive formation of the ring-closed glycoconjugate.[54] Thus, this latter strategy has particular merit as it does not affect the structural integrity of the reducing end sugar.

1.3 *N*,*O*-Dialkyl oxyamine glycoconjugation

The efficacy of oxyamines as a means to synthesise glycoconjugates was significantly enhanced with the introduction of *N*,*O*-dialkyl oxyamines. To this end, Peri *et al.* synthesised two types of oxyamine substrates for conjugation with carbohydrates (Scheme 2). These oxyamines are divided in two sub-types, the "Type A" *O*-alkyl-*N*-methyl oxyamines **1** and the "Type B" *N*-alkyl-*O*-methyl oxyamines **2**. Both types of oxyamines have been successfully conjugated to carbohydrates under mildly acidic aqueous conditions to form the ring-closed glycoconjugates **3** and **4**, respectively. The scope and application of the "Type A"oxyamine linker, however, has been well studied, whereas the "Type B" has been utilised less extensively.



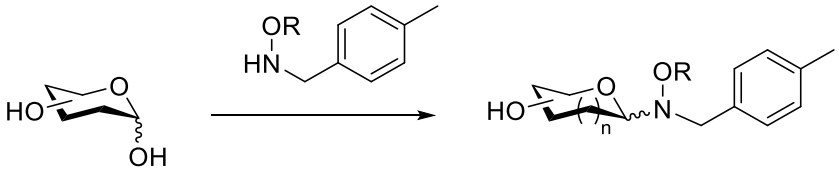
Scheme 2. *Oxyamine linkers for the conjugation of carbohydrates.*

1.3.1 Oxyamine glycan conjugation Scope and Limitations

The condensation reaction between reducing sugars and both "Type A" and "Type B" oxyamine derivatives can be performed under various conditions.[54,55] For example, solvent mixtures such as DMF:AcOH, Pyr:AcOH, MeOH:$CHCl_3$ or an aqueous acetate buffer at pH 4.5 can be used to give glycosides in good yields. It should be noted that these conjugation reactions are equilibrium reactions and accordingly require high concentrations of either (or both) the glycan or the oxyamine in order to obtain high yields.[43] Moreover, depending on the stereochemistry of the carbohydrate, it is possible to form mixtures of α-, β-, pyranosyl- and furanosyl-glycosides (Table 1).[55] For example, upon reaction of D-glucose with either *N*-methoxy-4-methylbenzylamine (entry 1) or *N*-benzyloxy-4-methylbenzylamine (entry 2), exclusive formation of β-pyranose glycoconjugates was observed, as the formation of α-pyranosides is disfavoured due to 1,3-diaxial interactions (Figure 3). Morover, the reaction of D-GlcNAc (entry 3) with oxyamines also resulted in the β-pyranose glycoconjugates. On the other hand,

reaction of the methoxyamine with *N*-acetyl-D-galactosamine (entry 4) and D-galactose (entry 5) gave a 41:59 and 94:6 mixture of β-pyranose and β-furanose, respectively. Here, it was suggested that the β-galactofuranosides do not experience the unfavourable steric interactions between the O-3 and C-5 groups which are experienced by glucofuranoside derivatives. Isolation of the individual structural isomers proved ineffective as the single isomers rapidly equilibrate to the pyranose/furanose mixtures. Moreover, it was found that the reaction with the more "Type A"-like linkers (R = Allyl, Bn; entry 6 and 7) results in different pyranose/furanose ratios, indicating that oxyamine substituents influence the outcome of these reactions. In the case of mannose (entry 8), the β-pyranose, α-pyranose and α-furanose isomers are formed, whereas the β-furanoside was not observed due to the unfavourable 1,2-*cis* and 4,5-*cis* configuration of this adduct. The formation of the α-pyranoside can be explained by the favourable 1,2-*trans* configuration of the hydroxyl substituents and the C-1/C-2 dipole effect.[55] It is also interesting to note that the α-furanose is formed as a minor product, despite the undesired O-4 and C-5-*cis* configuration of the hydroxyls. This indicates that the electronic effects of the 1,2-*trans* configuration may play a role in the formation of the α-pyranoside and α-furanoside of the mannose conjugates.

Table 1. The relative stereochemistry of the obtained glycosides.



Entry	Carbohydrate	R	Yield	β-Pyr	α-Pyr	β-Fur	α-Fur
1	Glc	Me	71	100	-	-	-
2	Glc	Bn	67	100	-	-	-
3	GlcNAc	Me	47	100	-	-	-
4	GalNAc	Me	78	41	-	59	-
5	Gal	Me	82	96	-	4	-
6	Gal	Allyl	47	89	-	11	-
7	Gal	Bn	56	59	-	41	-
8	Man	Me	91	37	41	-	21

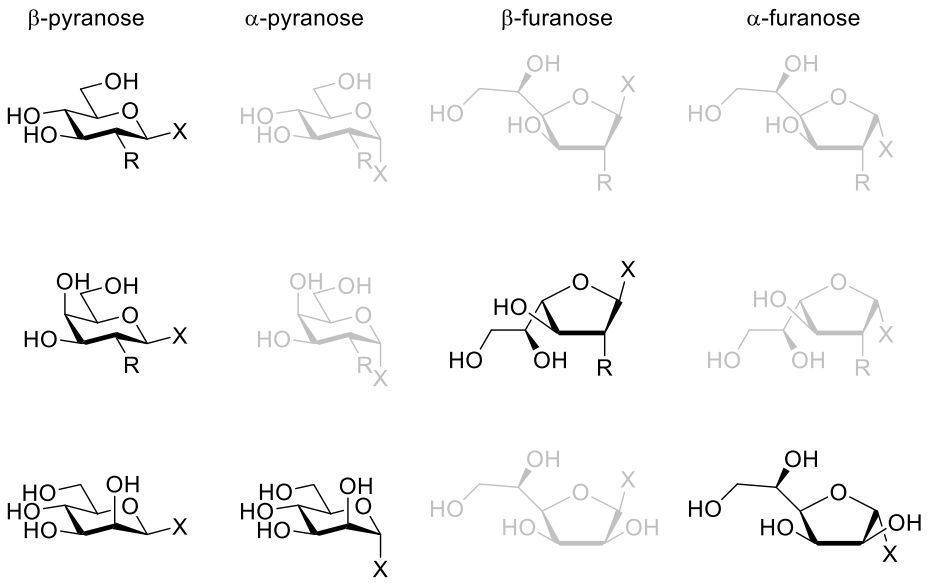


Figure 3. *Possible isomer formation in the glycosylation reaction of both "Type A" and "Type B" oxyamines with glucose (top row, R=OH), GlcNAc (top row R=NHAc), galactose (middle row, R=OH), N-acetylgalactosamine (middle row, R=NHAc) and mannose (bottom row). The isolated isomers are depicted in black whereas the grey isomers were not observed.*[55]

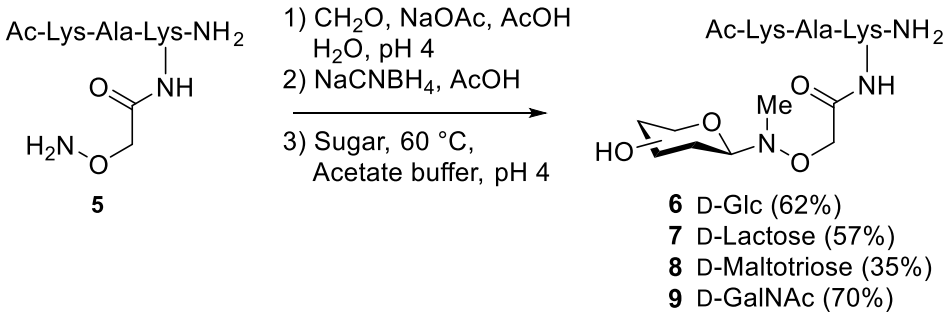
Although many carbohydrates are obtained as isomeric mixtures when using oxyamines, glucose and *N*-acetylglucosamine reducing end glycans result in the selective formation of the β-pyranoside. Given the importance of reducing end *N*-acetylglucosamine in N-glycans, these oxyamine glycoside conjugations nevertheless have much potential in the synthesis of biologically relevant glycoconjugates.

1.3.2 Oxyamine Stability

Since oxyamine glycosides are formed under acidic equilibrium conditions, the readily formed glycoconjugates can be hydrolysed to the hemiacetal glycan when subjected to aqueous conditions below pH 7. The hydrolytic stability of both "Type A" and "Type B" glycoconjugates has been investigated.[55,56] For both types, it was observed that the hydrolysis reaction is pseudo-first order, highly pH dependent, and that the half-life depends on the substituents on the nitrogen, the oxygen and the carbohydrate. When more electron withdrawing substituents are present, the half-life is increased. For example, benzyloxyamine glucosides ($t_{½}$ = 7d) are more stable than methoxyamine glucosides ($t_{½}$ = 1.4 d) when submitted to an aqueous buffer of pH 4.[56] Moreover, the hydrolysis rate of *N*-acetylglucosamine is much slower than glucose, which in turn hydrolyses slower than xylose oxyamines. Furthermore, *N*-acetylglucosamine derivatives show excellent hydrolytic stability at physiological pH.

1.3.3 Glycoprotein synthesis

Peri *et al.* synthesised novel glycopeptides employing the "Type A" oxyamine glycan conjugation (Scheme 3).[54] Here, the oxyamine functionalised peptide **5** was methylated via a two-step reductive amination with formaldehyde and $NaCNBH_3$. Next, the ligation with various glycans, including glucose, lactose and maltotriose, was performed under mildly acidic aqueous conditions to give the desired glycopeptides **6**-**9** in 35 – 70% yield.

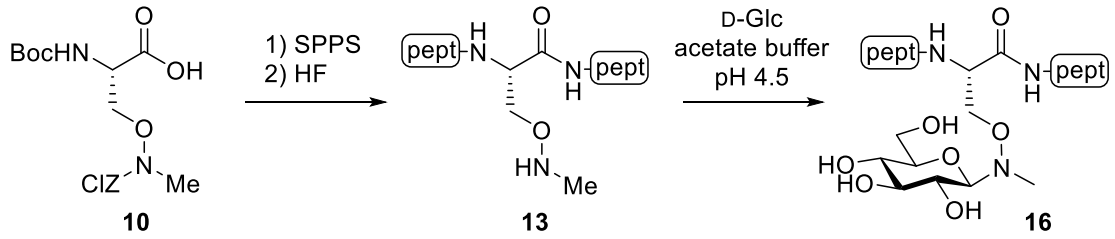


5

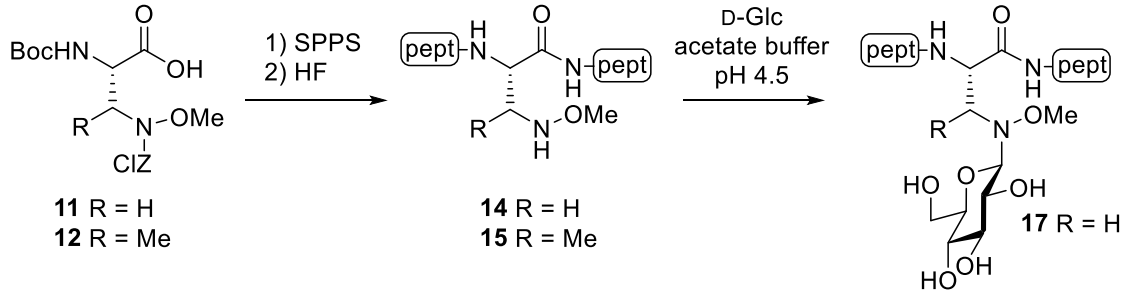
Scheme 3. *Synthesis of oxyamine functionalised glycopeptides* ***6-9****.*[54]

Following the initial work of Peri *et al.*, Carrasco *et al.* synthesised a novel series of both "Type A" and "Type B" oxyamine functionalised amino acids **10** – **12** (Scheme 4).[57,58] These amino acids were then utilised in solid phase peptide synthesis (SPPS) to form oxyamine peptides **13** – **15**, which in turn could be used in the conjugation with D-glucose to obtain glycosylated peptides **16** and **17** in good (75-80%) yields. Here, it should be noted, however, that the secondary oxyamine **15** could not be conjugated to D-glucose due to the steric hindrance of the methyl substituent. In a similar synthetic approach, oxyamine derived aspartate amino acids were synthesised by Filira *et al.* for the synthesis of small glycopeptides.[59]

Type A



Type B

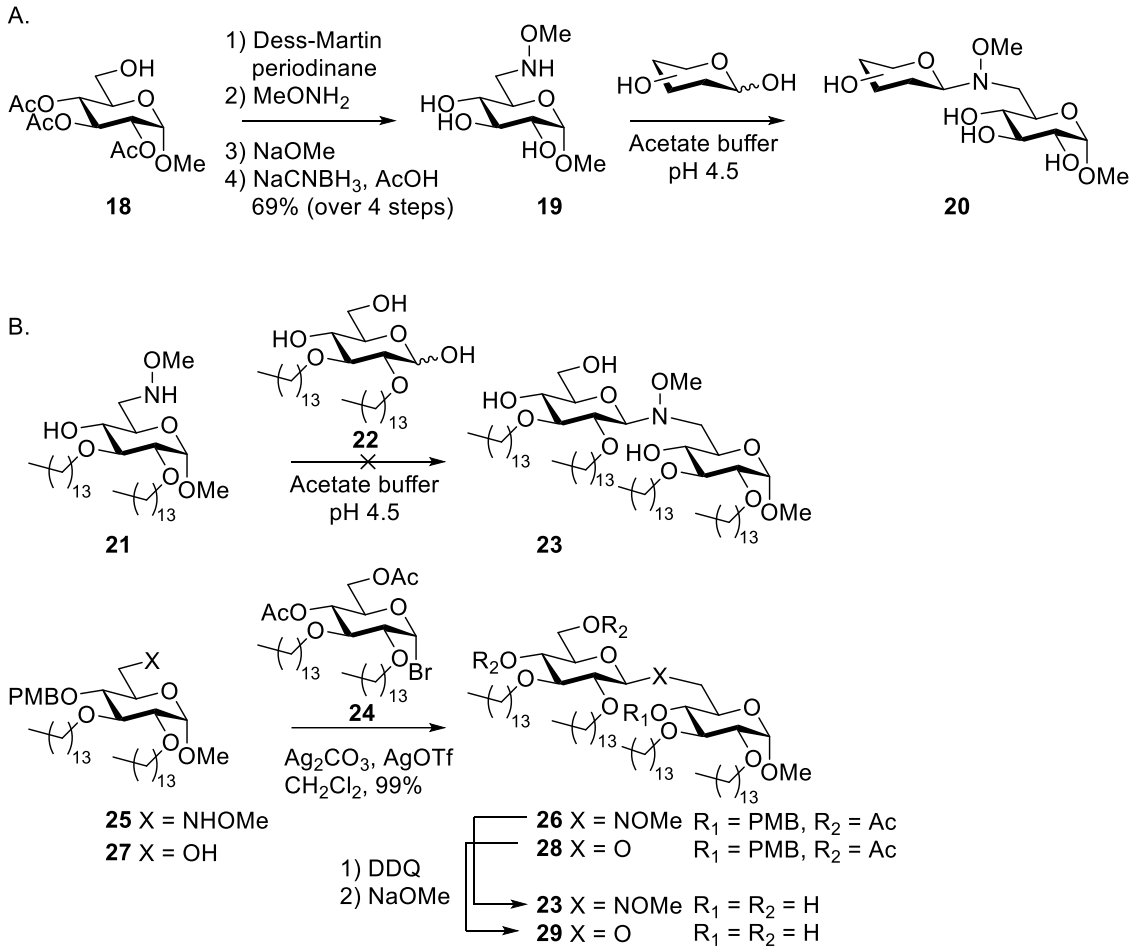


Scheme 4. *Oxyamine functionalised amino acids were used to synthesise glycopeptides.*[57]

1.3.4 Glycan-mimetics

Besides glycopeptide synthesis, Peri *et al.* also synthesised novel glycan mimetics by using the methoxyamine as an inter-glycosidic linkage to form oligosaccharides (A. Scheme 5).[60,61] These "Type B" oxyamine glycans could be obtained via a Dess-Martin oxidation of the 6-hydroxyl of methyl glucoside **18**, followed by imine formation with methoxyamine and subsequent deacetylation and imine reduction to give methoxyamine functionalised glucoside **19**. Next, the conjugation with a variety of reducing sugars, including Glc, Man, Gal and GlcNAc was performed to give disaccharide glycan mimetics **20**, however, as described above, the use of galactose and mannose resulted in a mixture of α/β- and furanose/pyranose-adducts.

This strategy was then further exploited towards the synthesis of Lipid A derivatives, however, in an attempt to conjugate glycolipid **21** and **22** via this methodology, disaccharide **23** was not observed despite varying the experimental conditions such as solvents and temperature (B. Scheme 5). Thus, a traditional glycosylation between donor **24** and acceptor **25** was performed to obtain the glycoside **26**. In addition, the use of the hydroxyl acceptor **27** gave the *O*-glycoside **28**. Both the oxyamine **26** and *O*-glycoside were deprotected to give glycolipids **23** and **29** and were tested for their ability to induce macrophages to release nitric oxide (NO), however, no response was observed. Instead, the glycolipids competitively bind Toll-like receptor-4 (TLR-4) compared to its natural ligand lipopolysaccharide (LPS), and thus can be used as antagonists for TLR-4. Both glycolipids **23** and **29** show very similar results, which indicates that the "Type B" methoxyamine glycosidic linkage does not interfere binding with the TLR-4 receptor.



Scheme 5. *The use of oxyamines towards glycan mimetics.*[61]

This mimetic concept was recently further explored by Ishida *et al.* who synthesised *N*-LacCer glycolipid **30** (Figure 4) to inhibit the hydrolytic activity of recombinant endoglycoceramidase-II (EGCase-II).[62] In this work, a "Type B" oxyamine derived ceramide lipid was synthesised and used in the conjugation with lactose to form *N*-LacCer **30**, which can inhibit EGCase II-mediated hydrolysis of *O*-LacCer **31**. The advantage of this route is that it is relatively easy to vary the carbohydrate moiety in order to alter the activity of the glycolipid, as the carbohydrate is introduced in the last step. In addition, the oxyamine glycosidic linkage shows potential for other glycosidase and glycosyl transferase inhibitors.

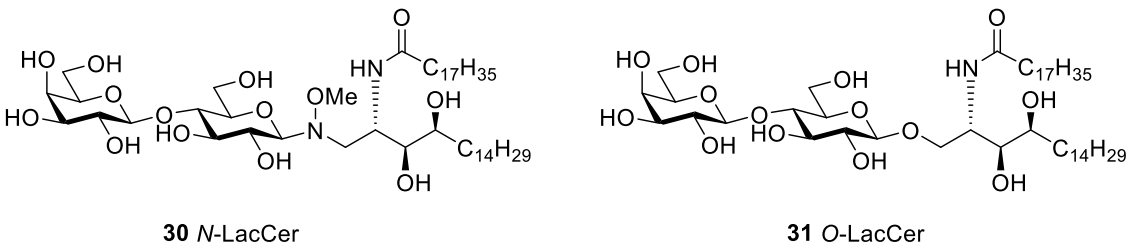
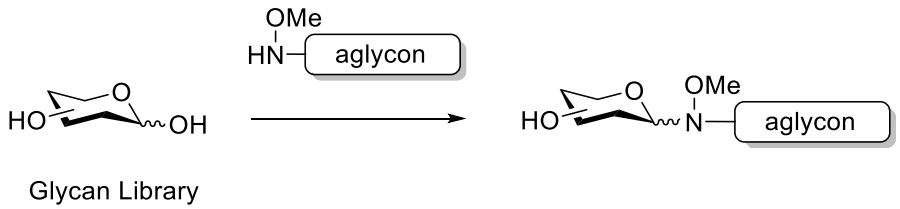


Figure 4. *N-LacCer **30** can inhibit the EGCase II-mediated hydrolysis of O-LacCer **31**.*[62]

1.3.5 Neoglycorandomisation

In an effort to further explore the use of oxyamine glycan conjugation, the research group of Thorson and co-workers conjugated glycan libraries of both natural and un-natural glycans to aglycon natural products via "Type B" oxyamine conjugation. (Scheme 6).[63] This process, also known as neoglycorandomisation, started with the conjugation of 78 glycans to oxyamine derivatised digitoxin.[64] Digitoxin is a glycosylated cardenolide (steroid) with potential anti-cancer activity that is attributed to its ability to mediate the inhibition of the plasma membrane Na^+/K^+-adenosinetriphosphatase (ATPase). By changing the glycan antigen, it was envisioned that the cancer cell specificity could be enhanced, and thus the library of oxyamine-glycan conjugates were tested in a high-throughput screening against nine human cancer cell lines. Although most glycoconjugates were obtained as furanose/pyranose mixtures, it was observed that some conjugates had a 20 fold increase in IC_{50} compared to the natural digitoxin. This nicely illustrated that the glycan moiety can alter the cell specificity of the drug and since then the concept of neoglycorandomisation has been employed for other drugs, such as colchicine,[65] vancomycin,[66] betulinic acid,[67] chlorambucil,[48] warfarin,[68] cyclopamine,[69] amphimedosides.[70] and perillyl glycosides.[71] This work has been recently summarised in an excellent review.[36]

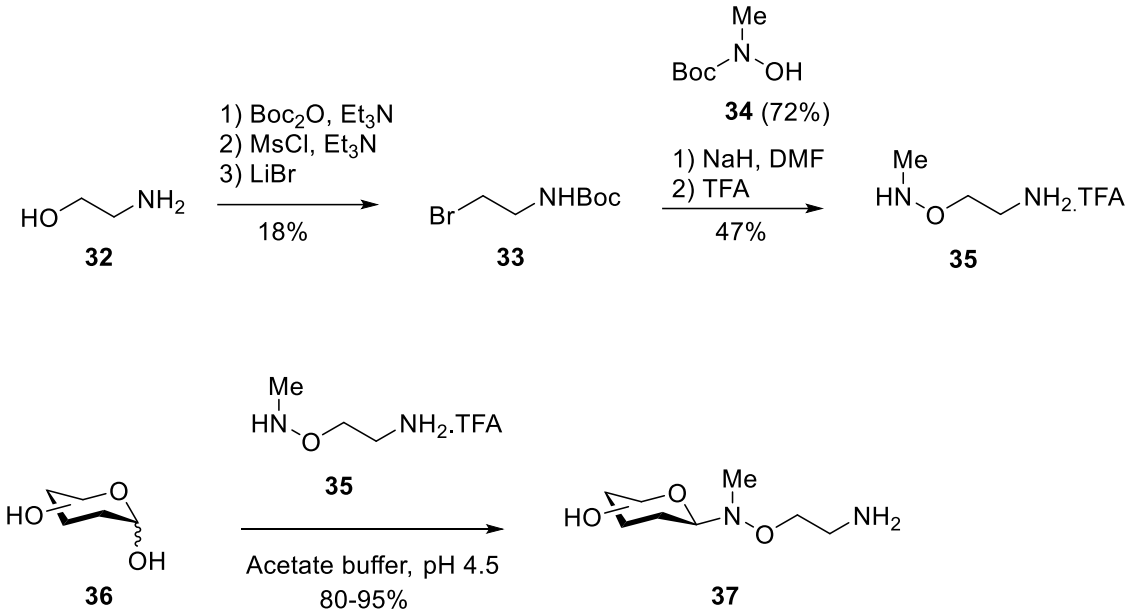


Scheme 6. *Neoglycorandomisation: reaction aglycons through oxyamines to glycan libraries allows for rapid screening of biological active glycoconjugates.*

Despite the potential of the neoglycorandomisation strategy and the fact that the glycoconjugates can be obtained in good yields, a large excess of the carbohydrate is typically required for the glycan conjugation step. This is a serious limitation of the methodolgy when complex and less readily available N-glycans are needed for conjugation. Accordingly, a high yielding glycan conversion was investigated through the use of bi-functional linkers.

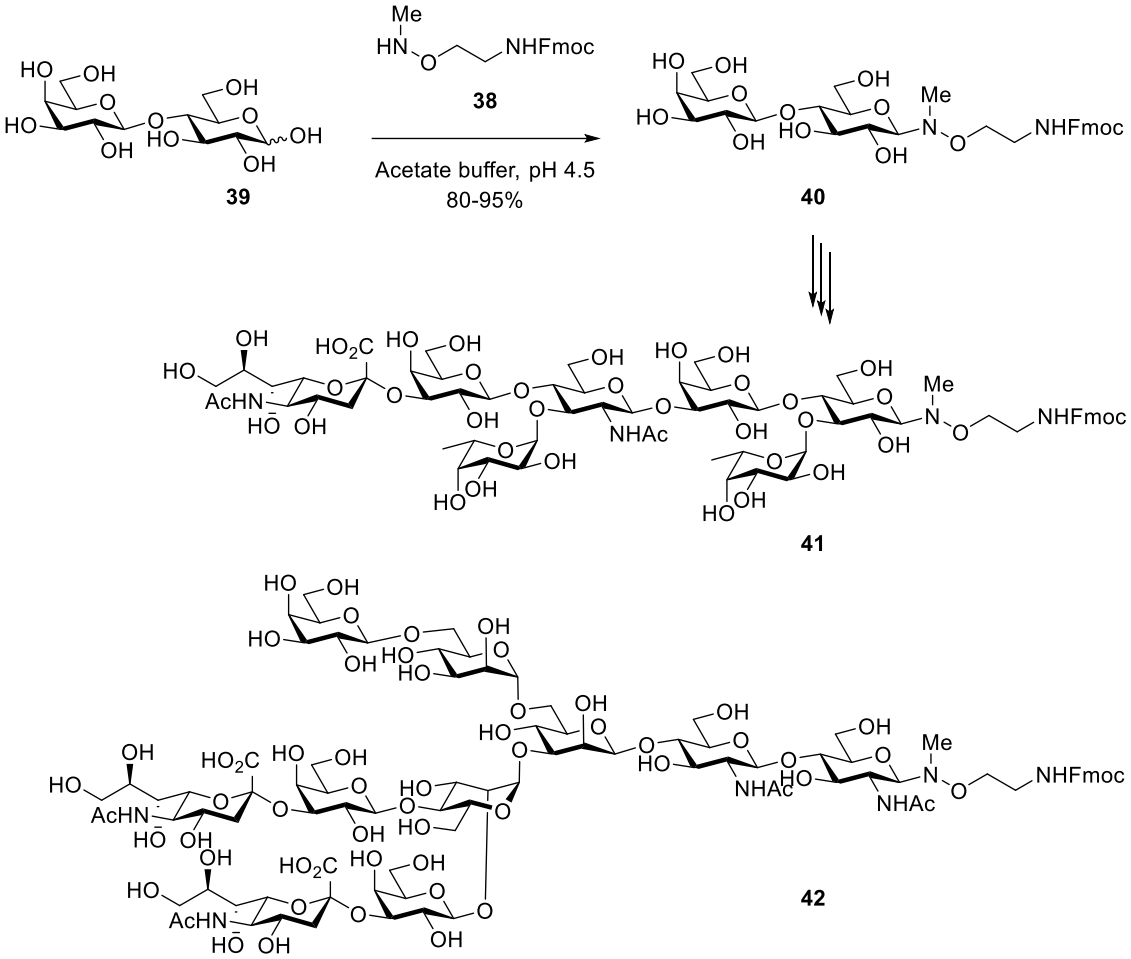
1.3.6 Bi-functional linkers

Bi-functional oxyamine linkers of "Type A" were first reported by Bohorov *et al.* for use in glycan arrays.[72] In this 5-step linker synthesis, hydroxylamine **32** was protected with an *N*-Boc protecting group. The alcohol was then mesylated and subjected to bromide substitution to give bromide **33** (Scheme 7). Next, *N*-methylhydroxylamine was *N*-Boc protected to give oxyamine **34**, which was used in the alkylation reaction with bromide **33**. After alkylation, the linker was treated with TFA to give the bi-functional linker **35** in 8% over 5-steps. With the "Type A" linker in hand, various glycans **36** were conjugated, including complex oligosaccharides, and the resulting conjugates **37** were printed on an NHS-ester activated glycan-array chip. This linker was later employed by Boons and co-workers to print complex asymmetrical *N*-glycans on micro-array slides.[73] Moreover, these linkers would allow for amide ligation reactions with the substrate of choice to form various glycoconjugates.



Scheme 7. *"Type A" bi-functional linker synthesis and subsequent glycan conjugation by Bohorov et al.*[72]

Recently, the group of Boons and co-workers reported the use of a *N*-Fmoc protected bi-functional "Type A" oxyamine linker in the chemoenzymatic synthesis of complex oligosaccharides (Scheme 8).[74] Instead of conjugating these linkers to readily synthesised glycoconjugates, linker **38** was condensed with lactose (**39**) to give disaccharide oxyamine **40**, which in turn was used throughout the chemoenzymatic synthesis of complex glycan ligands such as **41**. In this way, other complex glycans were synthesised, such as dodecasaccharide **42**.



Scheme 8. *Chemoenzymatic synthesis of oxyamine functionalised complex glycans.*[74]

In addition to the work of Bohorov and Boons, other "Type A" bi-functional linkers **43** – **46** have also been synthesised (Figure 5), as well as linker **35**. These linkers were conjugated to glucose so as to evaluate the relative stability of the constructs (**47** – **51**) as well as their lectin binding. Here, the latter was determined by immobilising *N*-acetyllactosamine oxyamine glycoconjugates to an array slide and employing a fluorescent Wheat Germ Agglutinin (WGA) binding assay. From these studies it was determined that the benzyl derivatives **49** and **51** were more stable glycoconjugates when compared to methyl derivatives **48** and **50**, however, the size of the benzyl group affects lectin binding. Moreover, oxime **47** is more stable than the methylated glycoconjugates **48** and **50**, but when the lectin binding of **47** is compared to the methyl- and benzyl-oxyamine conjugates **48** – **51**, oxime **47** showed significantly lower affinity for the lectin. This could be explained by the fact that oxime **47** exists in the ring-opened form in solution, which affects the structural integrity of the glycan.

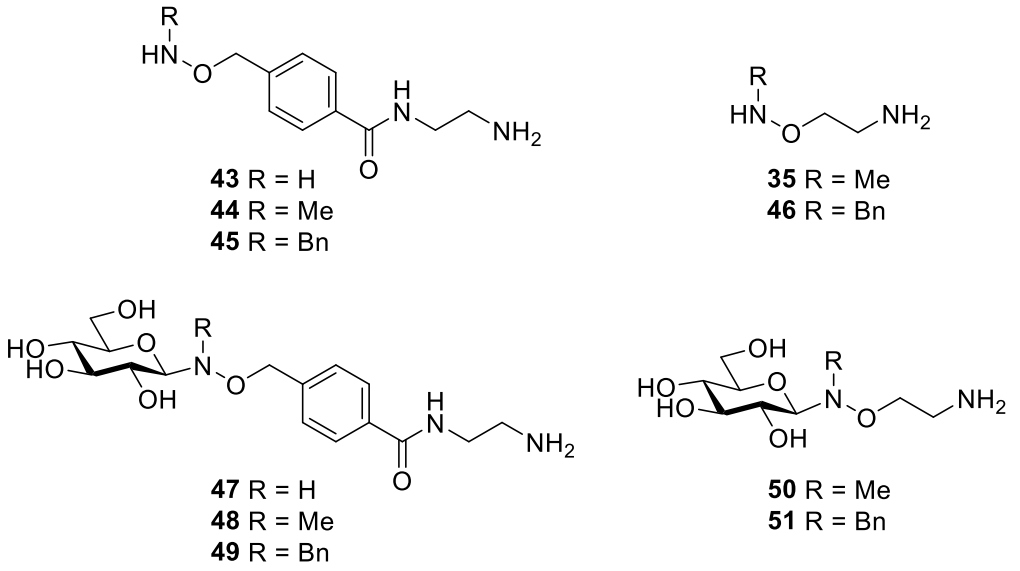
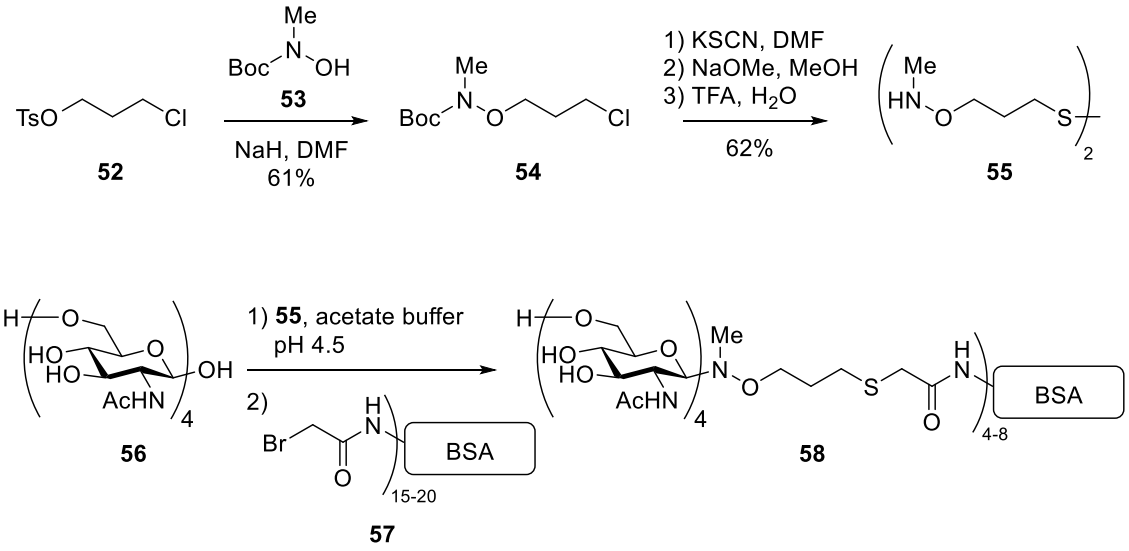


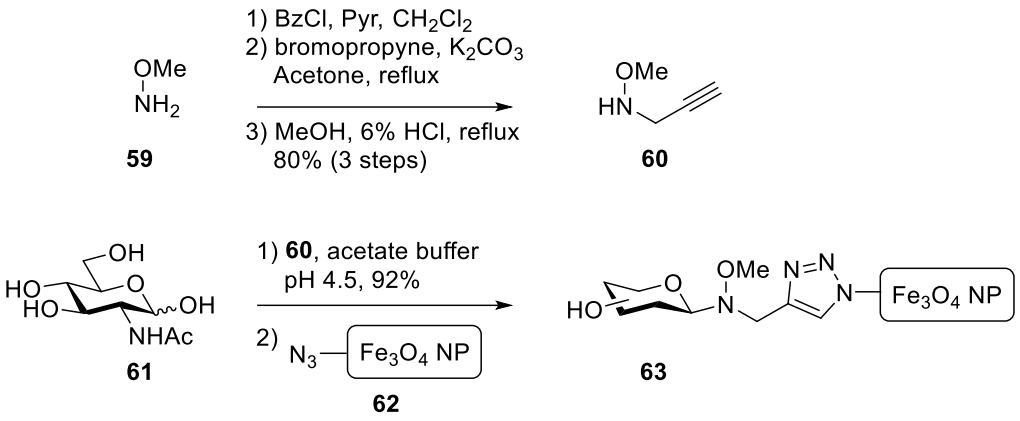
Figure 5. *"Type A" bi-functional oxyamine linkers and glycoconjugates were tested on hydrolytic stability and lectin binding.*[56]

In a similar fashion, a thiol bi-functional oxyamine linker of "Type A" was synthesised for the conjugation of glycans to bromoacetyl functionalised proteins (Scheme 9).[75] Here, 3-chloropropyl 4-methylbenzenesulfonate (**52**) was alkylated with *N*-Boc-*N*-methyl-hydroxylamine **53** to give chloride **54**. A thiol substitution of the chloride with KSCN, followed by thiocyanate hydrolysis and subsequent *N*-Boc cleavage gave bi-functional linker **55**. With the "Type A" linker in hand, *N*-acetylglucosamine oligosaccharide **56** was then reacted with bromoacetyl functionalised bovine serum albumin (BSA) **57** to give glycoprotein **58**, bearing four to eight oligosaccharides.[75]



Scheme 9. *Synthesis of "Type A" bi-functional linker* ***55*** *and conjugation of glucosamine antigens to BSA.*[75]

Beside the synthesis of oxyamine functionalised amino-acids, only one "Type B" bi-functional linker has been synthesised. El-Noubbou *et al.* demonstrated the use of oxyamines in the assembly of nanoparticles via the utilisation of the azide-alkyne Huisgen cycloaddition (Scheme 10). In this work, methoxyamine (**59**) was benzoylated, alkylated with bromopropyne, and de-benzoylated to give the "Type B" alkyne bi-functional linker **60** in three steps. Next, condensation of *N*-acetylglucosamine (**61**) and oxyamine **60** was performed, followed by the Huisgen cycloaddition with azide-functionalised iron oxide nanoparticle **62**, to form glycosylated nanoparticle **63**.



Scheme 10. *Synthesis of "Type B" alkyne oxyamine **60**, which allows for the synthesis of carbohydrate functionalised iron oxide nanoparticle **63** through the use of the copper-mediated Huisgen cycloaddition.*[76]

Given the ease of formation, and their potential use in the conjugation of naturally isolated reducing carbohydrate antigens without the need for protecting groups, oxyamine methodology has proven to be a useful strategy in the synthesis of glycoconjugates. In comparison to the Kochetkov amination, oxyamines have the advantage that they are more stable than the β-glycosylamine, and, in addition, are not restricted to amide ligations with other substrates. When compared to reductive amination and oxime/hydrazide conjugation, oxyamines have the advantage in that the ring-closed glycoconjugate is obtained which better mimics the native glycan.

As discussed above, both "Type A" *O*-linked oxyamine and "Type B" *N*-linked oxyamines have been utilised in the synthesis of various glycoconjugates, including glycopeptides, glycan mimetics and neoglycorandomised therapeutics. Moreover, the use of "Type A" bi-functional linkers has been extensively investigated for the conjugation of glycan antigens to the substrate of choice, whereas research towards "Type B" bi-functional linkers has been explored less. Although the use of oxyamine linkers allows for the synthesis of biologically relevant glycoconjugates, to induce a meaningful biological response, the multivalent presentation of glycan antigens is required, thus the synthesis of multivalent glycoconjugates will be discussed.

1.4 Multivalent Glycan presentation

1.4.1 Dendritic structures

In biological systems polyvalent interactions are collectively much stronger than their corresponding monovalent interactions.[77–79] Interactions between monomeric carbohydrate ligands and protein receptors are often of low affinity and, therefore, give weak or non-detectable biological response. Multivalent presentation of ligands, however, can enhance binding and downstream signalling. The cell wall is covered in carbohydrates and, as a result, interactions between cells are enhanced by multivalent presentation of glycan antigens to corresponding lectins. For example, the deadly chicken influenza virus H5N1 targets specific cells through multivalent binding to sialyl Lewis^{X} ligands.[80]

There are many structures which can be used for multivalent interactions with cell surfaces (Figure 6). For example, (branched) polymers decorated with glycan antigens have been used to enhance the biological response upon lectin binding.[81] More recently, glycan decorated liposomes have been used to transport toxins efficiently into B-cells.[82] Liposomes, however, have limited shelf lives compared to dendritic molecules. Unlike polymeric chains and liposomes, the synthesis of dendritic structures more readily allows for the control of the size and functionality of the target structures.[83] Moreover, it has been demonstrated that the multivalent presentation of glycan antigens on dendritic molecules significantly enhances lectin binding and results in a stronger biological response when compared to polymer-presented glycan antigens.[40]

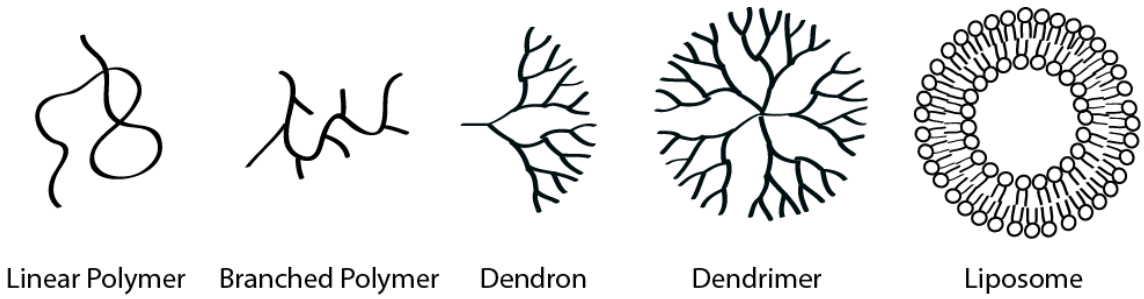


Figure 6. *Examples of multivalent structures.*

1.4.1 Dendritic features

The names dendrimer and dendron originate from the Greek word ‘dendros’, meaning branch. An obvious example of a branched structure in nature is a tree, which uses a dendritic structure above the ground to enhance its exposure to sunlight, whereas an underground dendritic root system allows efficient nutrient uptake. Unlike trees, synthetic dendrons are often symmetrical and are structurally well-defined branched molecules of a specific size and functionalised terminal surface area.[84] In contrast to other multivalent structures dendrons and dendrimers can be synthesised as single molecular species. Thus, dendron/dendrimer synthesis allows for the control

of physical and chemical properties of biomolecules that are presented in a multivalent fashion, which in turn allows for the close monitoring of their biological behaviour.[85] Accordingly, dendritic macromolecules have been used in various applications, including drug/gene delivery, cellular targeting, catalysis, and solubilisation.[86]

Dendrimers contain a core moiety and a layer-by-layer system that controls the size, branching and surface area of the dendritic structure. The central core of a dendrimer comprises either a single atom or a molecular moiety, such as a carbohydrate or amino acid.[87] The size of the dendritic structure can be regulated by the number of generations, which is determined by the number of focal points between the core and the periphery of the dendrimer. For example, a dendrimer with two focal points is a second generation (2G)-dendrimer. The outer shell can be decorated with the functional group of choice, for example glycan-decorated dendrimers are known as glycodendrimers. Dendrimer derivatisation with lectin binding specific carbohydrates can be used for dendrimer trafficking to specific cell types. Accordingly, this feature can be used to study biological processes or can be utilized in the development of therapeutic agents.[88]

Glycodendrons have similar properties to glycodendrimers, however, the former are bi-functional molecules. Glycodendrons also contain carbohydrates at the periphery of the dendron, however, instead of a symmetrical core, dendrons are synthesised from a bi-functional core. This allows for the additional functionalisation of dendrons with the substrate of choice, which can range from fluorescent molecules[89] to small molecule drug carriers,[90] as well as larger constructs such as peptide epitopes and proteins.[91]

1.4.2 Dendron functionalisation

1.4.2.1 Fluorescent dendrons

Fluorescent markers for the analysis of cellular processes have become a widely used tool in the field of biochemistry.[92,93] To this end, fluorescent molecules have been conjugated to a variety of molecules including glycans, proteins, lipids, nucleic acids and dendrons, and have found application in a number of fluorescent-based readout assays, including microscopy, flow cytometry and ELISA. For example, BODIPY-labelled glycodendrons have been synthesised for the detection of the DC-SIGN receptor on dendritic cells, and subsequently used to study the lysosomal uptake of DC-SIGN antigens.[89]

1.4.2.1 Biotinylated dendrons

The interaction between biotin and avidin is the strongest non-covalent binding known to date (K_d = 1.7 × 10^{-15} M), and accordingly, biotinylated dendrons have been conjugated to avidin or streptavidin proteins.[94] The rapid conjugation of biotinylated substrates with streptavidin probes containing, for example, fluorescent groups, enzyme reporters or antigens, has many applications including use in microscopy, flow cytometry as well as vaccine development.[95,96] Moreover, biotinylated constructs can be used in ELISA assays where the ELISA plate is coated with streptavidin, followed by the biotinylated dendron, which then results in the presentation of the glycodendrons on the surface of the plate.[97]

Biotinylated dendrimers have been used in various applications, including in the detection of influenza viruses,[98] radioimmunotherapy,[99] and cellular imaging.[100] For example, in studies by Xu *et al.* a biotinylated dendrimer-based MRI contrast agent was synthesised and subsequently conjugated to fluorescent avidin.[101] In this work, it was demonstrated that the supramolecular construct effectively targeted ovarian cancer tumours in mice, with efficient delivery of both the MRI contrast agent as well as the fluorophores to the cancer cells.

1.4.2.3 Dendron drug delivery vehicles

Dendrimers have been widely studied for their ability to deliver small molecule drugs to target cells.[102] These small molecule pharmaceuticals can either be covalently attached to the dendron or encapsulated in the void spaces within the dendrimer. The encapsulation of drugs within dendrimers requires void spaces of a particular size and moreover, it requires various degrees of either hydrogen bonding, ionic interaction or hydrophobic void space.[103] This encapsulation process has been used for many clinical drugs, such as diclofenac, doxorubicin and 10-hydroxycamptothecin. For the covalent attachment of drugs to dendrimers, key factors to consider are the stability of the conjugate in circulation and the cleavage of the drug within the target cell.[102] To this end, most strategies involve the acid-dependent release of the drug, which allows for drug release when the dendrimer gets internalised in endosomal vesicles. Covalently attached drugs are often used in vaccination strategies, whereby antigenic peptides are delivered to immune cells to induce an immune response. Here, dendrimers and functional dendrons can be used instead of carrier proteins. The advantage of dendrons over proteins is that it is often easier to specifically functionalise dendrimers, while the conjugation of proteins can lead to a mixture of products.

1.4.3 Size and functionalisation of dendrons

1.4.3.1 The effect of dendron size on the efficiency of lectin binding

The multivalent presentation of the glycan antigen is the most important property of glycodendrons when used for lectin binding studies. Although the synthesis of large dendrons seems desirable for the mimicry of biological events, several groups have reported on both the binding efficiency as well as the toxicity of large dendrons and have demonstrated that more highly branched dendrons are not necessarily better than their lesser branched counterparts. For example, Wang *et al.* showed that when comparing the half inhibition concentration (IC_{50}) of the 2G12 lectin between the mono-valent glycan and the tri-, nona- and 27-valent glycodendrons, all three dendrons showed significantly higher affinity for the lectin.[104] When comparing the IC_{50} per dendron (290, 4.6 and 4.1 nM, respectively), the second generation dendron is just as effective as the third generation dendron. However, when the efficiency per glycan is compared, the second generation has a lower IC_{50} than the third generation (10 and 41 nM, respectively). Moreover, it should be noted that the synthesis of second generation dendrons is more efficient and requires fewer glycan antigens.

The size of the dendron (without increasing the number of antigens), however, is important for lectin binding. This was nicely demonstrated by van Kooyk and co-workers, who used the reductive amination between the antigen $Lewis^X$ and different size (3G, 4G and 5G) poly(amido amine)amine (PAMAM) dendrimers to obtain $Lewis^X$-glycodendrons of various sizes.[40] Due to the coupling inefficiency and structural defects of the larger dendrimers, all dendrimers contained 16 ± 3 glycans, and thus the size of the dendrimers (23 ±2, 27 ±2 and 45 ±3 kDa) could be compared in lectin binding studies. It was observed that larger dendrons resulted in more efficient binding of the lectin. This supports the theory that the size of the dendron, and thus the spacing between glycans, might be more important for binding than the number of antigens when enough antigens are present.

1.4.3.2 Dendrimer cytotoxicity

Another important aspect of dendrimer chemistry is the biocompatibility and cytotoxicity of dendrimer constructs, particularly when these macromolecules are used in a pharmaceutical setting. Although each dendrimer/dendrons has a specific cytotoxicity, depending on the dendron functionalisation, several studies have considered dendron toxicity.[105] For example, it was found that the smaller generations (<G5) PAMAM-based dendrons have little or no harmful effect at concentration desired for biological applications, whereas larger (>5G) dendrimers are more cytotoxic.[106] In addition, no evidence of immunogenicity was observed for the smaller generations (<5G), although the functionalisation of dendrons and dendrimers will affect the biological response too, and thus the potential cytotoxicity of these macromolecules. Notwithstanding, preliminary research indicates that small dendritic structures have few *in vivo* side effects.[107]

1.4.4 Dendrimer design and synthetic approaches

1.4.4.1 Divergent vs convergent dendrimer synthesis

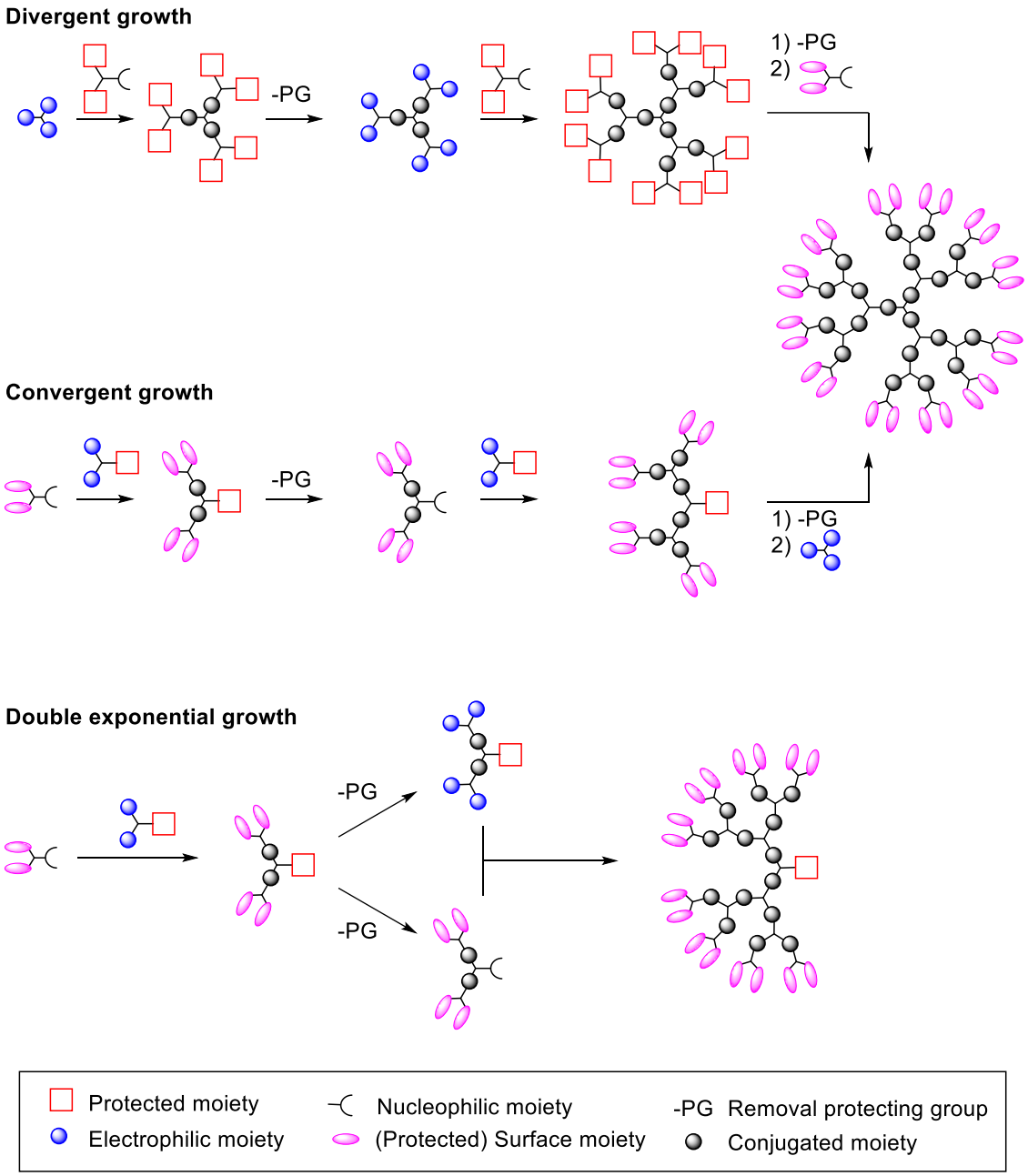
Traditionally, dendrimers and dendrons have been synthesised either via divergent or convergent approaches (Scheme 11).[108] In the divergent approach, the dendrimer is synthesised from the inside-out. Here a multifunctional core is activated and functionalised with a new generation. Repetition of these two steps increases the number of generations and thus the size of the dendrimer. It should be noted that structural defects developed during divergent dendrimer synthesis can be difficult to separate from the desired dendron. However, as divergent dendrimer synthesis allows for the use of cheap and readily available reagents, many commercially available dendrimers are synthesised via this approach.

The convergent synthetic route allows for the synthesis of dendron 'wedges' that are subsequently coupled to a dendron core structure to obtain a dendrimer. This allows for a more controlled synthesis, as structural defects are more easily removed. A drawback of the convergent synthesis is the requirement for excess dendron during the coupling with the core structure in order to obtain the dendrimer in sufficient yield.

More recently, double exponential growth methodology has been developed for the more efficient syntheses of dendrons.[83] Here, a protected first generation dendron is synthesised, which is then converted to a second generation dendron in a parallel synthesis. This route has particular merit since it minimises the number of steps to assemble larger dendrons, and in addition, it allows for the easy purification of the first generation dendron and accordingly, rapid assembly of second, third and fourth generation dendrons.

Using these methodologies, an overwhelming number of dendrons and dendrimers have been synthesised in the last three decades, each of them with unique structural features.[83,84,109] However, each of these dendrons and dendrimers have three things in common: a core structure, branching units and a functionalised periphery.

As mentioned above, the core structures can differentiate between dendrimers and dendrons. However, both dendrons and dendrimers can be synthesised from a large variety of core molecules, such as ammonia, diamine-alkanes, benzenes, amino acids, lipids, hydrocarbons and heteroatoms. In particular, the use of tris(hydroxymethyl)aminomethane (TRIS) has been useful in dendrimer chemistry as it allows for three fold symmetry. A more comprehensive review on the use of TRIS in dendron synthesis will be discussed in Chapter 3.



Scheme 11. *Synthetic approaches towards dendrons and dendrimers.*

1.5 Lewis Antigens

The Lewis antigens are a related set of glycans found in nature. They are divided in two subgroups, the Type-1 and Type-2 Lewis antigens, which are characterised by the presence of the α-Fuc-(1→4)-GlcNAc or α-Fuc-(1→3)-GlcNAc residues, respectively (Figure 7).[110] The structurally most basic Type-1 and Type-2 Lewis antigens are known as Lewis^A and Lewis^X. In the presence of a fucosyltransferase (FUT), these structures are converted into Lewis^B and Lewis^Y, whereas sialylation of the galactose moiety results in Sialyl Lewis^A and Sialyl Lewis^X derivatives. Moreover, sulfation of the 6-, 6′- or 3′-hydroxyl gives rise to sulfated derivatives.

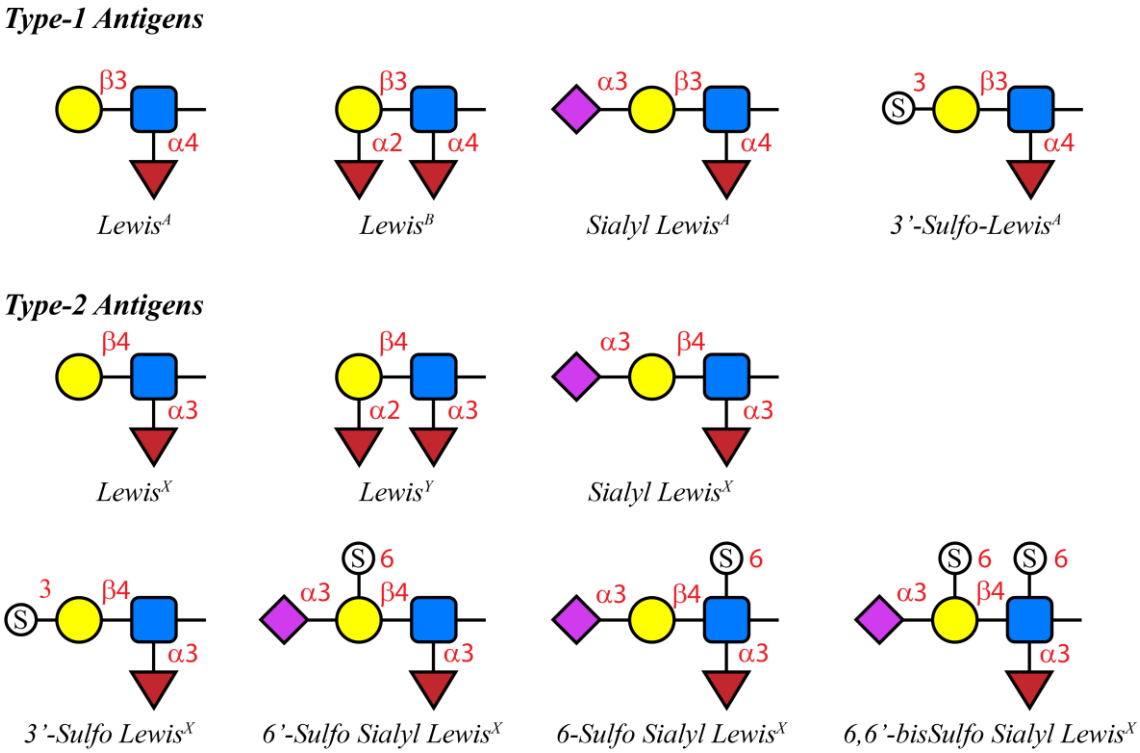


Figure 7. *Schematical presentation of Type-1 and Type-2 Lewis antigens; ● = Galactose; ■ = N-Acetylglucosamine; ▼ = Fucose; ◆ = Sialic acid; Ⓢ = sulfate.*

The biosynthesis of the Lewis antigens depends on the presence or absence of fucosyltransferases. The Type-1 Lewis antigens are encoded by genes on chromosomes *FUT3* and *FUT2*.[110] These genes have dominant alleles (*Le* and *Se*) which code for the fucosyltransferases, as well as non-functional recessive alleles (*le* and *se*). Individuals with the *Le* allele (*FUT3*) have the α-(1→3/4)-fucosyltransferase which produce Lewis^A and small amounts of Lewis^X antigens from the Type-1 and Type-2 precursor, respectively. The *Se* allele (*FUT2*) encodes for the α-(1→2)-fucosyltransferase, which in turn produces Lewis^B and Lewis^Y antigens. Individuals lacking both *Se* and *Le* genes are Lewis negative and do not produce Type-1 blood group antigens. The Type-2 Lewis antigens, however, can also be synthesised by α-(1→3)-fucosyltransferases

that are encoded within the *FUT6* and *FUT7* genes, of which the *FUT6* gene is expressed by 96% of the population.[111] In contrast to other erythrocyte antigens, Lewis glycans are synthesised in the tissue and distributed throughout the body via the excretion of glycolipids into the plasma.[112] Accordingly, Lewis antigens are widely distributed in body fluids and tissues and are mainly expressed on epithelia of glandular tissues.

1.5.1 Lewis Blood group antigens

Unlike Type-2 antigens, the Type-1 Lewis antigens (*e.g.* $Lewis^A$ and $Lewis^B$), are incorporated by erythrocytes with variable expression levels between individuals.[112] Moreover, the concentration of these Lewis blood group antigens may alter in case of disease, or during pregnancy. The incorporation of these antigens by immune cells might be involved with self-antigen detection, as anti-$Lewis^A$ and anti-$Lewis^B$ antibodies are found in Le(a-/b-) deficient individuals as well as pregnant women.[113] Although the function of $Lewis^A$ and $Lewis^B$ antigens remains somewhat uncertain, the sialylated blood group antigen, Sialyl $Lewis^A$, is involved in binding with E-selectins.

1.5.2 E-, L- and P- Selectin binding

One of the major features of Lewis antigens is the reversible binding of E-, L- and P-selectins expressed on the endothelium as well as on individual leukocytes.[114,115] The endothelium upregulates these cell adhesion selectins upon local inflammation or bacterial infection which, in turn, recruits leukocytes through the interaction of the glycan antigens expressed on immune cells. This causes the leukocyte to slow down through "rolling and tethering", and allows for the migration of the leukocyte into the tissue towards the site of inflammation.[115] Sialyl $Lewis^X$ is found on the glycoprotein P-Selectin Glycoprotein Ligand-1 (PSGL-1) expressed on immune cells, which mediates the interaction with the selectins.[116] Moreover, $Lewis^Y$ antigens are expressed on Cluster of Differentiation-44 (CD44), a transmembrane glycoprotein found on a large number of mammalian cell types.[117] The primary function of CD44 is binding of hyaluronan, but it can also mediate cell–cell interactions, cell adhesion, and tissue migration through the interaction between the Lewis antigen and the selectins.[114] The binding of glycan antigens to selectins, however, has also been linked to various disease, including viral infection, arthritis, thrombosis and tumour metastasis.[118–120]

1.5.3 Lewis antigens in disease

As mentioned above, the selectin mediated binding of leukocytes allows for the migration of these leukocytes into the tissue. Tumours, however, often upregulate Lewis antigens for migration of

cancer cells into new organs (metastasis). Sialyl Lewis^X overexpression is often found on cancer cells and is associated with cancer metastasis, and the overexpression of this biomarker can be used to detect these types of cancers through high-density antibody arrays.[120] In a similar fashion, Lewis^Y has been found to be expressed at various levels in human malignancies, and can be used as a biomarker for cancer-progenitors.[121,122] Besides tumour metastasis, Lewis antigens can be involved with other migratory complications. Sialyl Lewis^A, which is expressed on CD44v6 on human intestinal epithelial cells, mediates the transepithelial migration of granulocytes into the lumen. Patients suffering from inflammatory bowel disease showed highly upregulated Sialyl Lewis^A antigen levels, which could explain the excess of granulocytes present in the lumen of patients suffering from Crohn's disease and ulcerative colitis.[123] Moreover, sialylated glycoproteins are found on viruses such as the Herpes Simplex Virus Type-1 (HSV-1) and HIV, which allows for infection of the host through selectin-mediated binding.[118,124]

1.5.4 Lewis antigens and human fertilisation

Lewis^X, Lewis^Y and, in particular, Sialyl Lewis^X are involved in human fertilisation. For fertilisation to occur, the sperm cells initially need to bind to the highly specialised extracellular translucent matrix coating the egg known as the zona pellucida. This first interaction is mediated between the egg-binding protein (EBP) on sperm cells, and Lewis antigens on the zona pellucida.[125,126] Upon sperm cell binding to the zona pellucida, the acrosomal exocytosis is induced. Next, the acrosome-reacted sperm adheres to the plasma membrane of the egg cell and the fusion of the membranes results in the fusion of both nuclei.

1.5.5 Sulfated Lewis Antigens

Sulfated Lewis antigens have been found to have many important biological roles. For example, 3′-*O*-sulfo-Lewis^X is synthesised by Gal-3-*O*-sulfotransferase[127] and plays an important role in the regulation of integrin-associated cell adhesion,[128] selectin binding,[129] and is also over-expressed in particular tumour cells.[130] Moreover, sulfated sialyl Lewis^X ligands play an important role in the modulation of immune responses through the interaction with sialic-acid-binding immunoglobulin-like lectins (siglecs),[131] as well as the selectins.[132] The siglecs are a family of transmembrane cell-surface proteins which are selectively expressed on immune cells. Expression of the different siglecs ranges from single cell-types to a variety of immune cells.[131,133] Currently, fourteen different human siglecs and nine mouse variants are known. Each siglec can bind to specific carbohydrate ligands, and in particular, Siglec-8 and Siglec-9 are of interest as they bind to 6′-*O*-sulfo and 6-*O*-sulfo sialyl Lewis^X, respectively.[131] Engagement of siglecs can result in various immunological functions, such as cellular activation, adhesion, endocytosis, proliferation and apoptosis.[134,135]

1.5.6 Lectin binding Lewis antigens

Besides the binding of selectins, Lewis antigens have been shown to bind other lectins, including macrophage galactose-type lectin (MGL), Langerin, and Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN). The mouse MGL has been found to bind $Lewis^X$ antigens, however, the human MGL binds to α/β-GalNAc Tn-antigens (O-glycans) instead of Lewis antigens, and thus cannot be used to target human DCs through the use of Lewis antigens.[136] Langerin is expressed on Langerhans cells, which make up the outer cell layer of epithelial and mucosal tissue, and form the first line of defense against pathogens such as bacteria, fungi and viruses. Langerin is an extracellular C-type lectin with specific affinity for mannose and fucosylated glycans, including $Lewis^B$ and $Lewis^Y$. Upon binding of these pathogens, the pathogen-Langerin complex rapidly internalises into Birbeck granules for degradation of the pathogen, which results in antigen presentation and immune activation against the pathogens. This receptor has been the major focus of research against HIV. The binding of $Lewis^X$ to the C-type lectin DC-SIGN, however, results in internalisation of the complex in a similar fashion to Langerin, but instead of the formation of Birbank granules, internalisation results in lysosomal compartments for antigen degradation. This makes DC-SIGN an interesting target for vaccine development.

1.6 Targeting DC-SIGN with $Lewis^X$ antigens

1.6.1 C-type lectin DC-SIGN

Antigen-presenting cells (APCs), such as dendritic cells (DCs) and macrophages, are mostly located where pathogen invasion is most common, (*i.e.* on the skin, in the respiratory system as well as the digestive system). Both macrophages and DCs are capable of pathogen recognition and processing and play a crucial role in the early stages of pathogen elimination.[137] By phagocytosis and intracellular processing of pathogens, APCs are capable of presenting specific peptide antigens to effector T-cells in order to produce and stimulate specific immune responses against these pathogens.[138] They are efficient at detecting pathogens due to their display of pattern-recognition receptors (PRR), such as Toll-like receptors, Nod-like receptors, RIG-I-like receptors and C-type lectins, with each PRR recognising a different class of molecular patterns.[138] The molecular recognition of these PRRs is extremely diverse, ranging from nucleic acids to lipids and from carbohydrates to proteins, making these cells extremely efficient at pathogen recognition.

DC-SIGN is a C-type lectin and is expressed on the extracellular cell wall of myeloid DCs, human alveolar and lymph node macrophages, and endothelial cells.[19,139–143] Although DC-SIGN is highly expressed on most DCs, the expression on macrophages is dependent on the phenotype of

the macrophage.[141,144] DC-SIGN, also known as Cluster of Differentiation 209 (CD209), is encoded by the *CD209* gene and has a high affinity for the transmembrane glycoprotein ICAM3 on T-cells.[143] The C-type lectin comprises a Ca^{2+} mediated binding site to which a variety of carbohydrate ligands have been found to adhere, including high-mannose glycans and fucosylated oligosaccharides such as $Lewis^A$ and $Lewis^X$.[145–147] Moreover, glycan array screening of the DC-SIGN receptor revealed that at least 20 different glycans have a significant interaction with DC-SIGN.[148]

DC-SIGN is organised in tetramers,[146] and upon multivalent binding of the lectin, the complex is internalised by phagocytosis. This internalisation is independent of receptor cross-linking and the DC differentiation status.[149] After internalisation the natural ligand is released from the DC-SIGN receptor and the receptor is recycled to the cell surface. While inside the lysosome, the antigens get processed into smaller peptide antigen fragments. These antigens are then presented on MHC-II to activate $CD4^+$ T-cells. When using DC-SIGN antibodies, however, the recycling of the lectin is prevented which in turn inhibits the antigen uptake by $DC\text{-}SIGN^+$ cells. Moreover, the use of anti-DC-SIGN antibodies can result in an aberrant immune response with undesired immune activation. Accordingly, it could be desirable to utilise the DC-SIGN native glycan antigen to target these receptors in a therapeutic setting.

1.6.5 DC-SIGN mediated treatment

Given the importance of DC-SIGN in pathogen recognition and antigen presentation for immune activation, DC-SIGN has been studied for its potential use in therapeutic settings. In 2012, Van Kooyk and co-workers synthesised mannose and fucose functionalised fluorescent glycodendrons to target DC-SIGN on DCs (Figure 8).[89] These BODIPY labelled glycodendrons **64** were synthesised via the copper-mediated Huisgen cycloaddition and it was found that these glycodendrimers bind DC-SIGN and internalise without activation or maturation of the DC. Using a mannose glycodendron, Davies and co-workers synthesised a Virus-like glycodendrinanoparticle displaying up to 1620 glycan ligands to potentially block viral infection by Ebola via DC-SIGN.[91] Here, the second generation mannose dendrimer was conjugated to the $Q\beta\text{-}Hpg16_{180}$ nanoparticle through a copper-mediated Huisgen cycloaddition to give glycodendrinanoparticle **65**. It was observed that 1620 glycan ligands were required to block viral infection, whereas the nanoparticle containing 540 antigens insufficiently blocked infection. Furthermore, it was found that a 25 nM concentration of glycodendrinanoparticles was required to prevent HIV infection of DCs by more than 90% which was a similar result to that observed when using the anti-DC-SIGN antibody.[91]

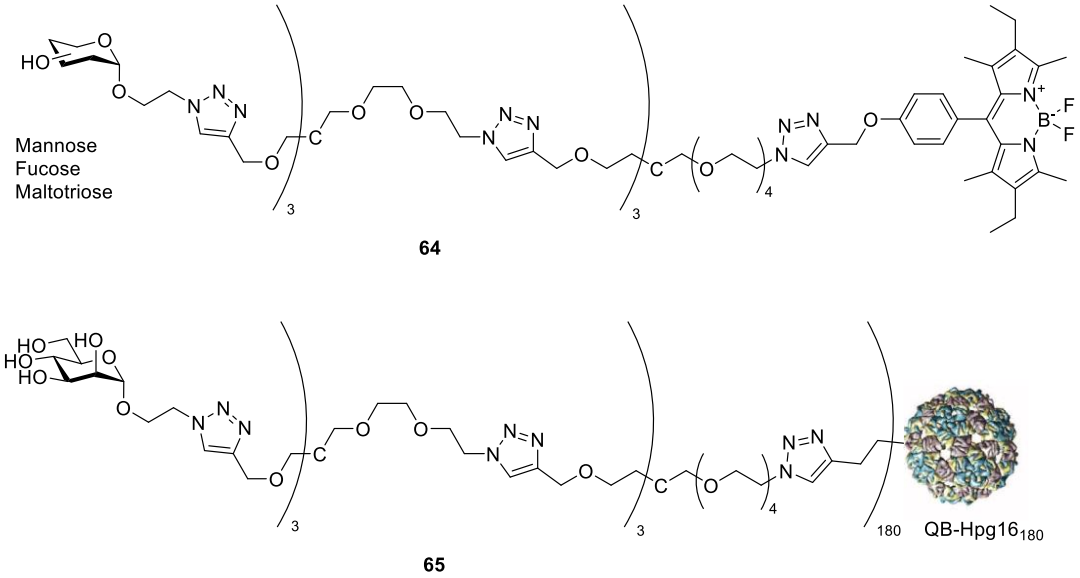
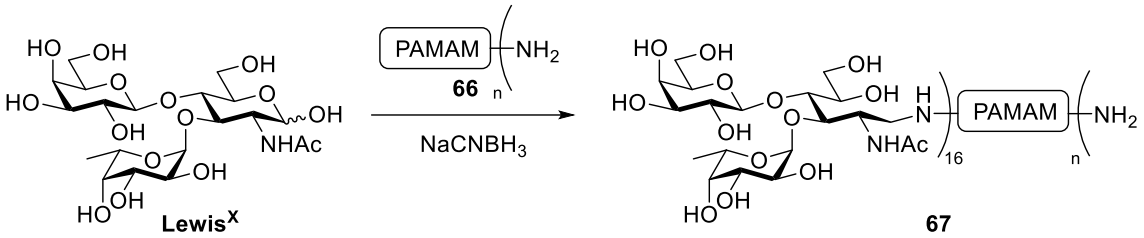


Figure 8. *BODIPY-labelled mannose- and fucose-dendrimers* ***64*** *were used to target DC-SIGN on DCs.*[89] *The mannose-glycodendrinanoparticle* ***65*** *can prevent DC-SIGN-mediated HIV-infection of DCs.* [91]

Although mannose (micromolar affinity) and fucose (high micromolar affinity) glycans can be used for DC-SIGN mediated treatments, it was shown that the mannose derivatives also bind to the receptor Langerin which leads to an undesired side effect in the treatment of HIV. Langerin also binds HIV, however, instead of infection of the host, Langerin binding results in HIV capturing and destruction through rapid Birbeck granule degradation. Thus, blocking the Langerin receptor in HIV treatment is undesirable. To prevent Langerin binding, Lewis^X glycan antigens can be used to target DC-SIGN. This approach was taken by Van Kooyk and co-workers who reported the synthesis of Lewis^X glycodendrimers and it was postulated that these glycodendrimers can prevent HIV-transmission through DC-SIGN on dendritic cells.[40] Using the reductive amination methodology, they conjugated the Lewis^X antigen to the commercially available PAMAM dendrimers **66** of various size to yield the 3G-, 4G- and 5G-dendrimers **67** each bearing 16 ± 2 Lewis^X antigens (Scheme 12). It was observed that the spacing between glycan ligands is important for biological activity, where the larger dendrons more efficiently bound to DC-SIGN. Moreover, they showed that these glycodendrimers had higher binding affinity for DC-SIGN binding when compared to Lewis^X containing polyacrylamide polymers. It was shown that over 95% of the DCs internalise these glycodendrons, and internalisation did not induce DC-activation. Notwithstanding, it was found that these glycodendrimers were able to completely inhibit HIV infection, even after two days in the presence of HIV.



Scheme 12. *Reductive amination between Lewis^X and various sizes PAMAM-NH$_2$* ***66*** *gave 3G-, 4G- and 5G-glycodendrons* ***67*** *bearing 16 ± 2 Lewis antigens.*[40]

The ability of these Lewis antigens to bind DC-SIGN and internalise into DCs allows for cell-selective antigen delivery, and 'controlled' activation of the immune system against various diseases, and accordingly would allow for the development of novel vaccines. This requires the DC-SIGN mediated internalisation of glycodendrons containing antigenic peptides. Accordingly Garcia-Vallejo *et al.* prepared Lewis^B containing glycopeptide-PAMAM conjugates **68** (Figure 9).[150] Again, these glycodendrons showed sufficient binding to DC-SIGN, resulting in antigen delivery by internalization as was evidenced by flow cytometry. Most importantly, these glycodendrons were able to present these antigens to ovalbumin (OVA)-specific CD4$^+$ T-cells, which resulted in T-cell proliferation. Here it should be noted that the second generation was sufficient to induce a T-cell response, whereas larger dendrons did not induce stronger biological responses. Interestingly, these glycodendrons showed superior activity when compared to DCs activated with Lewis^B-OVA conjugates, which indicates that these glycodendrons are effective cell-targeting delivery vehicles.

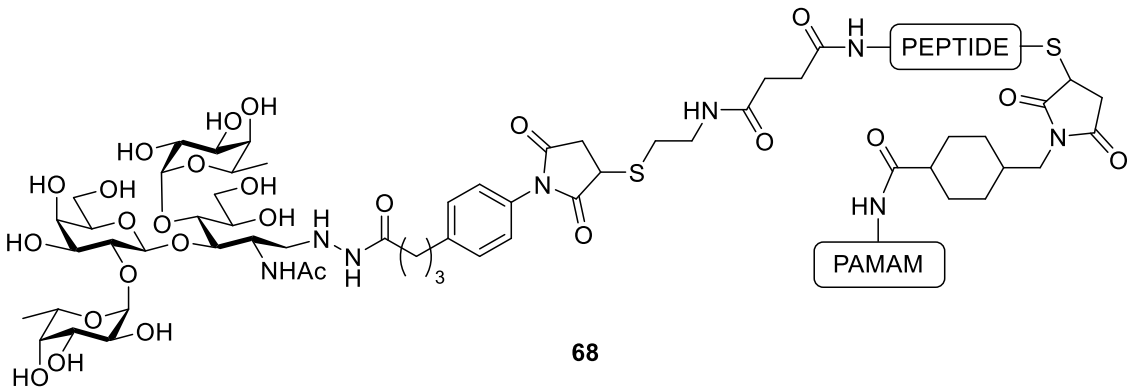


Figure 9. *Synthesis of PAMAM based glycodendrons bearing antigenic peptides such as SIINFEKL and ISQAVHAAHAEINEAGR.*[150]

Recently, targeting of DC-SIGN has also been achieved with the use of fucosylated gold nanoparticle **69** (Figure 10).[151] It was observed that the *N*-α-fucosyl-β-alanylamide bound with similar if not higher affinity for DC-SIGN compared to the Lewis^X antigen.[152,153] It was demonstrated that these nanoparticles were internalised by DCs through binding with DC-SIGN without triggering inflammatory cytokine responses of the DCs. Thus the synthesis of fucosylated gold nanoparticles allows for a less expensive and effective alternative to Lewis^X derived glycodendrons. However, many C-type lectins can bind fucosylated glycan antigens, and thus the selectivity for DC-SIGN C-type lectins remains questionable.

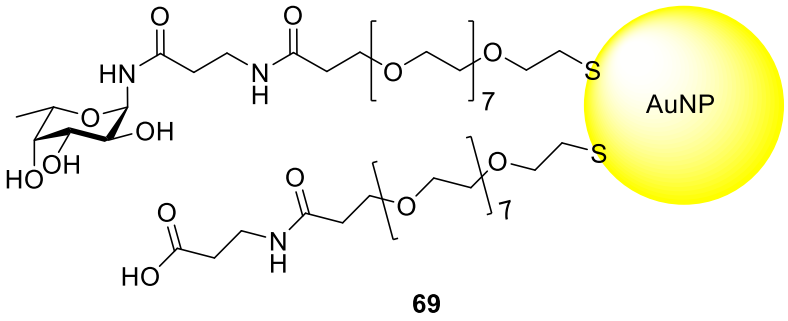


Figure 10. *Fucosylated gold nanoparticle **77** for targeting of DC-SIGN.*

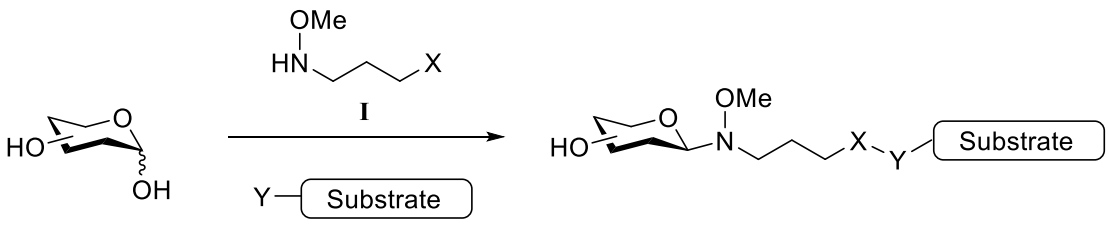
Besides Lewis antigens, high mannose glycodendrons were synthesised to target DC-SIGN on DCs. Wong and co-workers synthesised 1G – 3G glycodendrons bearing the $Man_9GlcNAc_2$ high mannose glycan antigen.[104] These glycodendrons were tested for DC-SIGN binding and it was observed that second generation glycodendrons were the most efficient for lectin binding. The third generation only showed a slight increase in activity, however, with lower activity per glycan antigen. Moreover, it was demonstrated that these glycodendrons are potential candidates for carbohydrate vaccines as well as antiviral agents related to HIV.

1.7 Thesis outline

The overall objective of the research described in this dissertation is to develop new and efficient strategies for the synthesis of multivalent glycodendrons. To achieve this, a novel bi-functional oxyamine linker strategy that allows for the conjugation of glycans to other complex biomolecules will first be discussed in Chapter 2. To enhance the binding affinity of these glycoconjugates, biotinylated and fluorescently labelled glycodendrons were then synthesised, as described in Chapter 3, while Chapter 4 concerns the synthesis of a representative carbohydrate antigen, Lewis^X. Finally, the combination of the bi-functional oxyamine linkers, the multivalent dendrons and the glycan antigen allows for the target goal of assembling a complex glycoconjugate, which is the focus of Chapter 5. To demonstrate a potential biological application of this methodology, the fluorescent Lewis^X glycodendron is evaluated as a flow cytometry marker for the C-type lectin DC-SIGN on human DCs and macrophages. A brief overview of the content of each chapter is provided below.

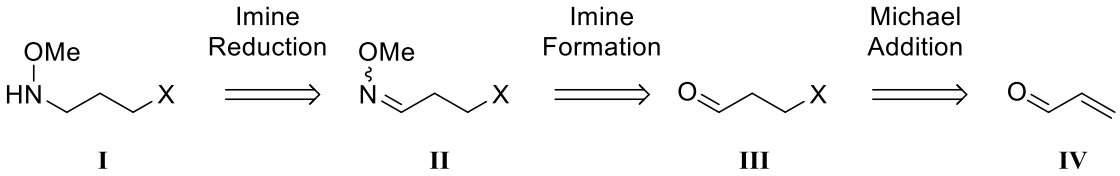
1.7.1 Proposed strategy for the rapid assembly of glycoconjugates through the use of bi-functional oxyamine linkers

The use of oxyamine glycoconjugation strategies has made a significant impact in the field of carbohydrate chemistry, as it allows for the conjugation of both naturally sourced and synthetic reducing-end carbohydrate antigens without the use of protecting groups. In particular, bi-functional linkers have been used to conjugate complex glycans to microarray slides, to test for their lectin binding ability. Accordingly, it is proposed to synthesise various glycoconjugates through the use of oxyamine bi-functional linkers (Scheme 13), which allow for the conjugation of glycan antigens to substrates of choice using two orthogonal reactions. As described in sections 1.2 and 1.3 above, the use of "Type A" bi-functional linkers has been explored by various groups, however, these strategies often involve the use of long and/or low-yielding synthetic routes. Herein, it was proposed that the "Type B" oxyamines could be more readily synthesised via reductive amination methodology and this work forms the focus of Chapter 2.



Scheme 13. *Rapid assembly of glycoconjugates through the use of bi-functional linker* ***I****.*

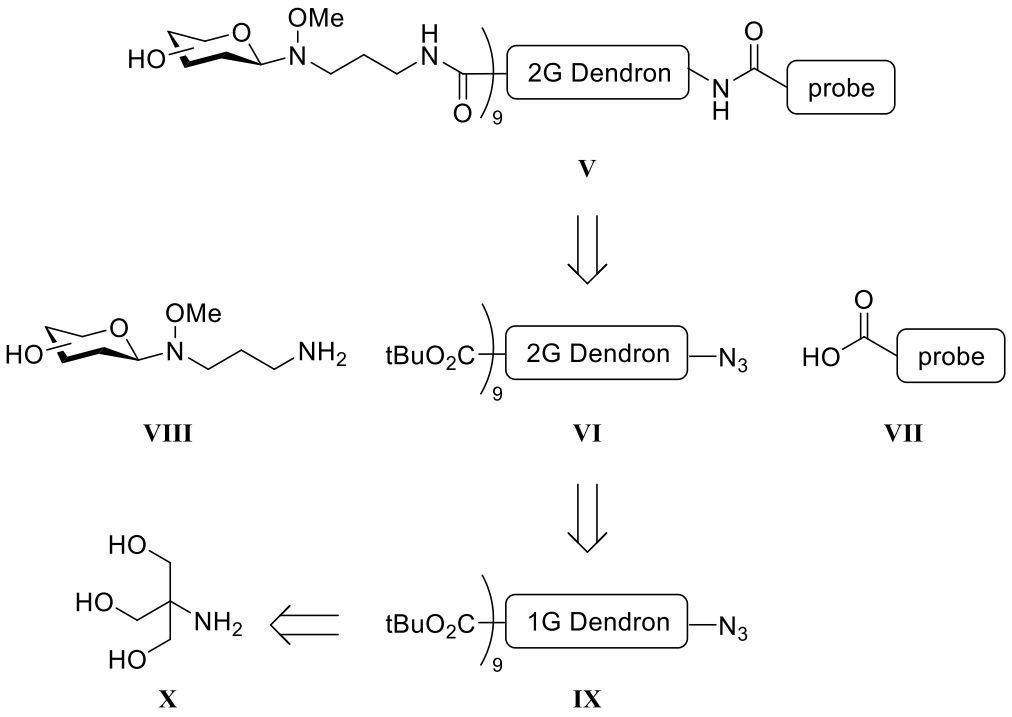
Retrosynthetically, it was proposed that the bi-functional linker **I** could be obtained via the reduction of the corresponding methoxyimine **II**, which in turn can be obtained from the reaction of aldehyde **III** with methoxyamine (Scheme 14). The second functional group that gives rise to the bi-functionality of the oxyamine linker can be introduced via a Michael addition of various nucleophiles with acrolein **IV**. For example, sodium azide can be used to synthesise the azide functionalised bi-functional linker, whereas thiols can be used to synthesise thiol-derived linkers. This strategy allows for the synthesis of various bi-functional linkers which all contain the oxyamine moiety for glycan conjugation, but which also include a second functional group for the required chemical ligation.



Scheme 14. *Retrosynthesis of bi-functional linkers.*

1.7.2 Proposed synthesis of novel glycodendrons

Glycosylated dendritic structures, such as glycodendrons, have been found to effectively bind cells with increased binding affinity for their corresponding lectin. Moreover, when compared to polymers and liposomes, dendritic structures can be prepared with high synthetic control, thereby allowing for more controlled biological studies to be undertaken. Accordingly, the synthesis of a multivalent second generation glycodendron was proposed in order to enhance glycan antigen activity (Scheme 15). The glycodendron **V** would contain various functionalities, including biotin and fluorescent groups, which would allow for the biological evaluation of these glycodendrons using various analytical techniques such as microscopy, ELISA, and flow cytometry. Thus, the synthesis of the target glycodendron **V** could be achieved using a highly convergent second generation dendron scaffold **VI**. Here, the functionalisation of dendron scaffold **VI** with the molecular probe **VII**, followed by glycosylation with oxyamine functionalised carbohydrate **VIII** gives the target glycoconjugate. This approach allows for the rapid modification of the dendron according to the required functionality. Specifically, in this thesis an oxyamine-functionalised Lewis^X glycan will be conjugated to a fluorescently labelled 2G dendron as a proof of concept.



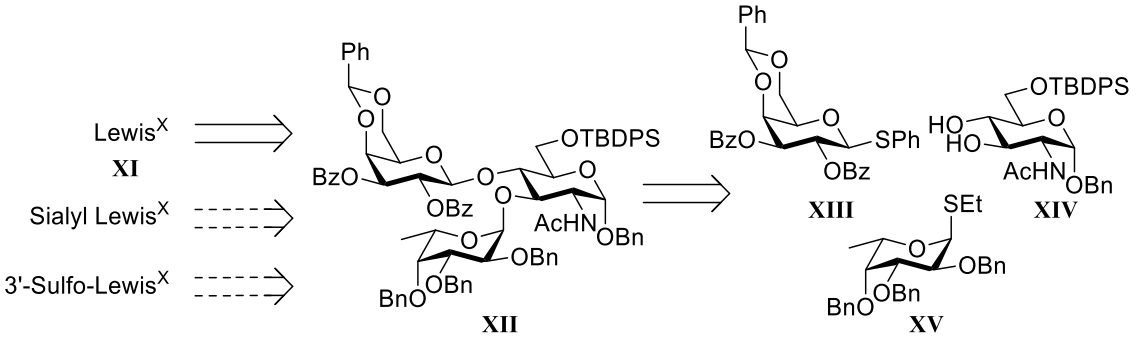
Scheme 15. *Retrosynthesis of TRIS-based second generation glycodendrons.*

There are several methodologies for dendron synthesis, including divergent, convergent and double exponential growth synthesis, of which the latter is the most efficient methodology. It was envisioned to use the double exponential growth synthetic route towards the highly convergent synthesis of the bi-functional second generation dendron scaffold **VI**. Accordingly, the second generation dendron core **VI** could be synthesised from the first generation dendron **IX** in three steps. Here, the synthesis of the first generation dendron **IX** can be achieved using tris(hydroxymethyl)aminomethane (TRIS) **X** as a core moiety. A more comprehensive review on the use of TRIS in dendron synthesis will be provided in the introduction of Chapter 3.

1.7.3 Synthesis of Lewis^X antigens

As described in section 1.5, Lewis antigens possess a variety of biological functions including selectin-mediated cell-adhesion process and Lectin-mediated immune activation against pathogens. Moreover, the over-expression of these Lewis antigens often is associated with various tumours and the targeting of highly glycosylated cells or the synthesis of these tumour-associated carbohydrate antigens for use in vaccines could allow for the development of better anti-cancer therapies. In addition, glycotherapies could be further developed whereby the glycan-lectin binding process is used to target specific cell types and to study lectin-mediated biological events.

Given the breath of biological activities attributed to the Lewis antigens in general, it was proposed that an efficient and rapid synthesis of the Lewis^X antigens was required via a route that would allow for the synthesis of other Type 2 Lewis antigens (*e.g.* Sialyl Lewis^X, and sulfated Lewis^X derivatives). Accordingly, it was proposed that these Lewis antigens, such as Lewis^X **XI**, could be prepared from an orthogonal protected "Type 2" Lewis antigen intermediate **XII**, which in turn could be synthesised from the corresponding monosaccharide building blocks **XIII-XV** via the regioselective glycosylation between galactose donor **XIII** and GlcNAc acceptor **XIV** followed by the [2+1] glycosylation with donor **XV** (Scheme 16). In this way, the synthetic strategy is not only highly efficient but also convergent. A thorough analysis of the previously reported syntheses of Lewis^X derivatives, as well as a novel synthesis of the Lewis^X antigen are presented in Chapter 4.

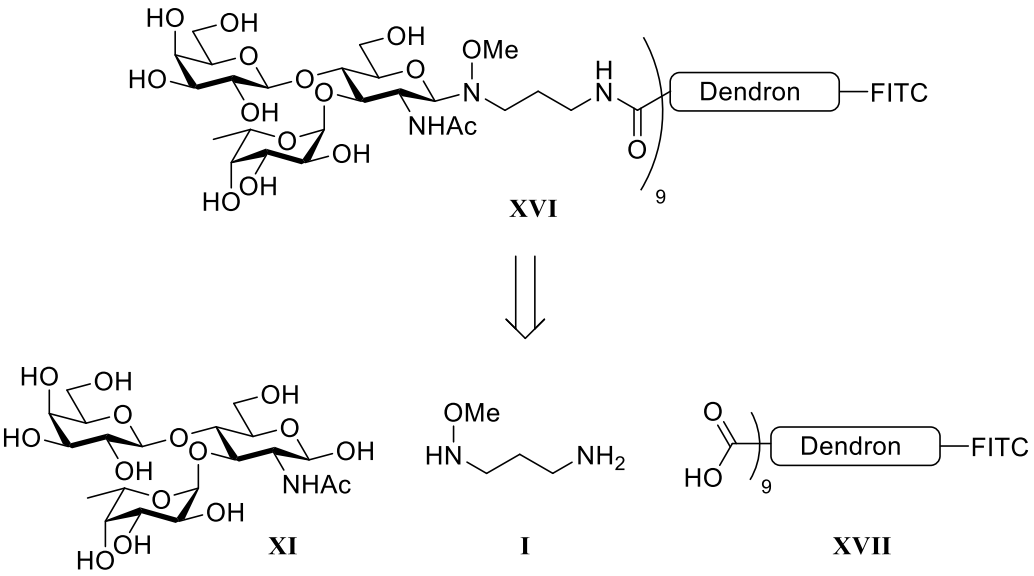


Scheme 16. *Retrosynthesis of "Type 2" Lewis antigens through the use of an orthogonally protected trisaccharide scaffold* ***XII****.*

1.7.4 Biological evaluation of Lewis^X-glycodendrons

The C-type lectin DC-SIGN is expressed on DCs and specific subsets of macrophages and binds to various Lewis antigens, including Lewis^X. The interaction of DC-SIGN with its ligands results in lysosomal uptake and degradation, followed by antigen presentation by the MHC-II complex to $CD4^+$ T-cells, which leads to immune activation. Here, it is important to note that the simultaneous multivalent binding of these ligands to the receptor is required to induce a biological response, and accordingly various multivalent scaffolds have been developed to target lectins efficiently. Chapter 5 concerns the combination of the bi-functional oxyamine linker strategy with the multivalent dendrons and the Lewis^X glycan antigen to prepare fluorescent glycodendrons **XVI** (Scheme 17). Here, the glycodendron will be prepared via the conjugation of Lewis^X **XI** to the bi-functional linker **I**, followed by the conjugation with the fluorescent dendron scaffold **XVII**. The glycodendron will then be used for the detection of DC-SIGN on human DCs as well as 'M1-like' and 'M2-like' macrophages via flow cytometry. The targeting of DC-SIGN by this glycodendron will provide proof-of-concept that the general targeting strategy works, and it is

envisioned that future work could include the specific delivery of antigenic peptides to these APCs. For example, peptide antigen functionalised dendrons could be used to selectively deliver peptide antigens to DC-SIGN^{+} cells, which could potentially activate the immune system, mediated by MHC-II antigen presentation to CD4^{+} T-cells.



Scheme 17. *Retrosynthesis of fluorescent multivalent Lewis^{X} glycodendron* ***XV****.*

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Chapter 2.

The rapid and facile synthesis of oxyamine linkers for the preparation of novel glycoconjugates.

2.1 Introduction

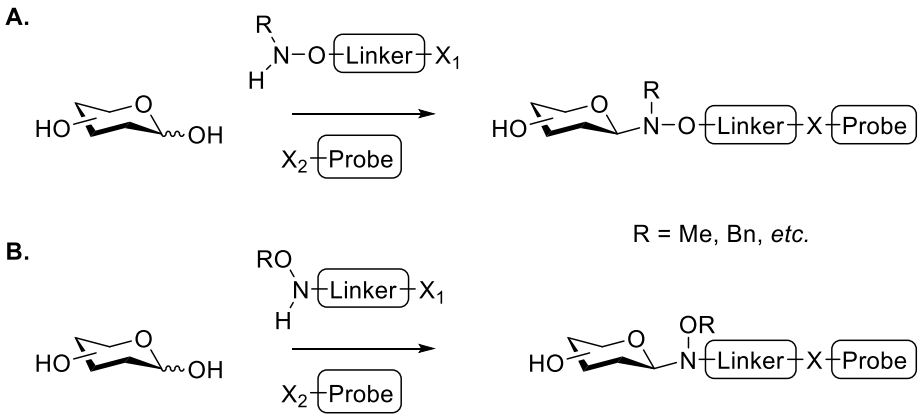
2.1.1 Conjugation of carbohydrates

Glycoconjugates are ubiquitous in nature and can be present in many different forms ranging from mono- to polysaccharides , and from glycolipids to glycoproteins.[1] A number of biological functions have been attributed to these glycoconjugates and, accordingly, there has been much interest in the construction of glycoconjugate mimetics,[2–4] glycopeptides,[5] and carbohydrate arrays,[6–8] and in the synthesis of fluorescent or biotinylated glycoconjugate probes.[9,10] To this end, synthetic strategies have been developed which allow for the conjugation of the carbohydrate of interest to a bifunctional linker that can then be further functionalised as required. These linkers can be introduced during the total synthesis of oligosaccharides, however, this is time-consuming and does not allow for the conjugation of carbohydrates from natural sources. Accordingly, much effort has been expended in the development of linker strategies at the reducing end of unprotected carbohydrates. These approaches include the use of Kochetkov amination,[11] reductive amination,[12] oximes/hydrazides[13–15] and oxyamines,[16] which were previously discussed in the introduction of this thesis.

2.1.2 Oxyamine Linkers

The focus of this thesis chapter concerns the synthesis of new oxyamine linkers that can be used for the construction of glycoconjugates. Here, it is important to note that the conjugation of carbohydrates with an oxyamine results in the formation of ring-closed glycoconjugates.[17] This has particular merit as it allows for the facile introduction of a variety of functionalised linkers,

without affecting the structural integrity of the reducing end sugar. *N*-Glycosyl oxyamines were first synthesised in the late 1970's to study *N*-aryl-*N*-hydroxy-glucuronylamine metabolites of anticancer drugs.[17–19] However, twenty years passed before oxyamines were realised in glycoconjugation when Peri *et al.* prepared neoglycopeptides and neoglycolipids.[17] In brief, other elegant applications of oxyamines include their use in the synthesis of glycan and glycolipid analogues,[20–22] in the neoglycorandomisation of methoxyamine-appended drug targets,[16] and to conjugate carbohydrates to amino acids.[23–29] Seminal examples include work by the groups of Blixt,[30] Carraso,[28] Nitz,[31] and Jensen,[32] and more recently, Boons and co-workers demonstrated the power of oxyamine-linkers by attaching complex N-glycans to microarrays[33] through the use of a 2-[(methylamino)oxy]ethylamine linker.[30] In all previously described studies, the oxyamine linkers were of 'Type A' (Scheme 1), and while the linkers clearly showed much potential in the synthesis of glycoconjugates, the linker synthesis was low yielding (10-25%) and required 5-6 synthetic steps. The strategies employed also required different approaches for different terminal functional groups. At the time these studies were initiated, there was an unmet need for the efficient, scalable, and preferably one-pot synthesis of a series of differentially functionalised oxyamine linkers.



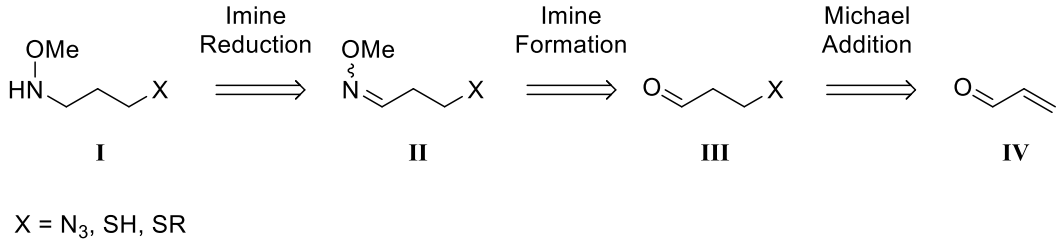
Scheme 1. *The two general strategies for the conjugation of carbohydrates to oxyamine linkers and further functionalisation with the probe of interest.*

2.1.4 Bifunctional Linker Design

2.1.4.1 Retrosynthesis of linkers

It was postulated that a variety of bifunctional linkers could be synthesised, including the azido-, amino-, thiol- and aminoacid linkers, by using a 3-step bifunctional linker synthesis (Scheme 2). Retrosynthetically, it was envisioned that the methoxyamine functionality in the linker could be obtained via a reductive amination of the corresponding aldehyde. Accordingly, the target oxyamine **I**, which can be equipped with different functional handles, was proposed to be prepared from the corresponding imine **II** via the reduction of the imine with $NaCNBH_3$. Imine

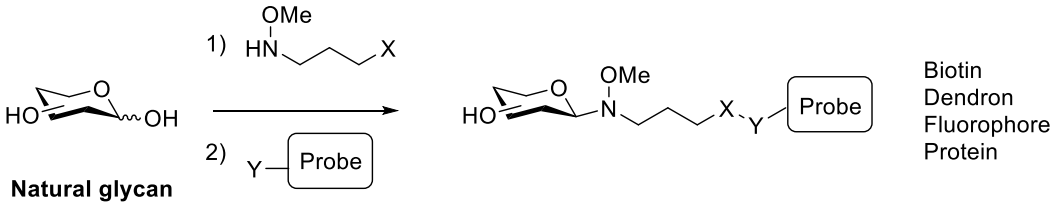
II could be synthesised from the condensation reaction of aldehyde **III** with methoxyamine. This reductive amination of aldehydes was first explored in 2002 by Peri *et al.* towards the synthesis of dimeric neoglycosides.[20] The aldehyde in turn could be obtained via the Michael addition of different nucleophiles with acrolein **IV**. Herein it should be noted that sodium azide, as well as thiols (*e.g.*, thiolacetic acid, cysteamine, *N*-Boc-cysteine and propane-1,3-dithiol), are known to undergo 1,4-addition to acrolein.[34] Accordingly, it was envisioned that these nucleophiles give rise to azide and thiol functionalised linkers, whereas the amine linker could be derived via the Staudinger reduction of the azide bifunctional linker.



Scheme 2. *Retrosynthesis of bifunctional methoxyamine linkers.*

Chemical ligation methods

Upon the successful synthesis of a variety of oxyamine linkers, it was proposed that the chemical ligation with the carbohydrate would be performed first, followed by the conjugation to the probe of choice (Scheme 3). For example, the azide derived bifunctional linker can be used in the conjugation with a glycan, followed by the copper mediated azide-alkyne Huisgen cycloaddition with alkynes,[35] or a copper-free strain-promoted alkyne-azide cycloaddition,[36] to obtain triazole containing glycoconjugates, while amine functionalised bifunctional linkers are suitable for classical peptide chemistry.[30] Thiol linkers are extremely useful for maleimide conjugate additions which are often used in protein conjugation[37,38] in thiol-ene addition reactions,[39] or the displacement of α-halo ketones. Accordingly, to illustrate the versatility of the oxyamine linker, it was proposed that a variety of glycoconjugates (*e.g.*, containing biotin, a fluorophore, a protein, or presented in a multivalent fashion on a dendrimer scaffold) would be synthesised.

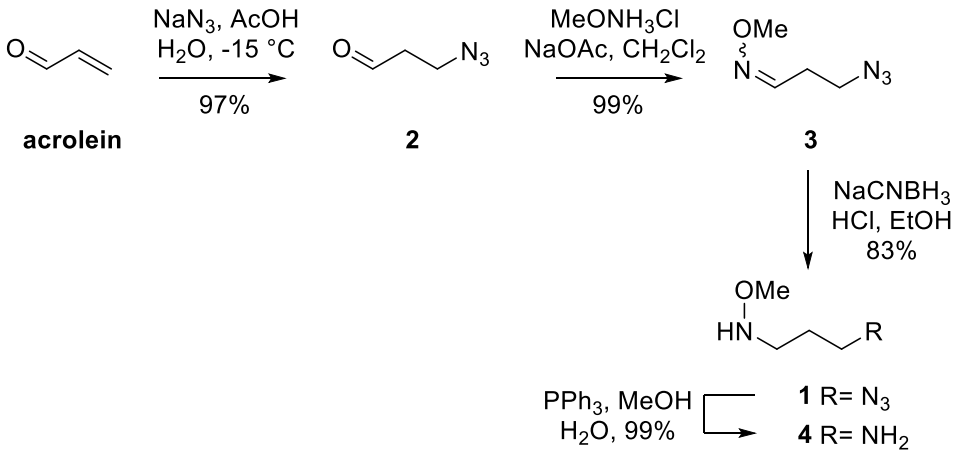


Scheme 3. *Synthesis of glycoconjugates utilising the chemoselective bifunctional linkers.*

2.2 Results and discussion

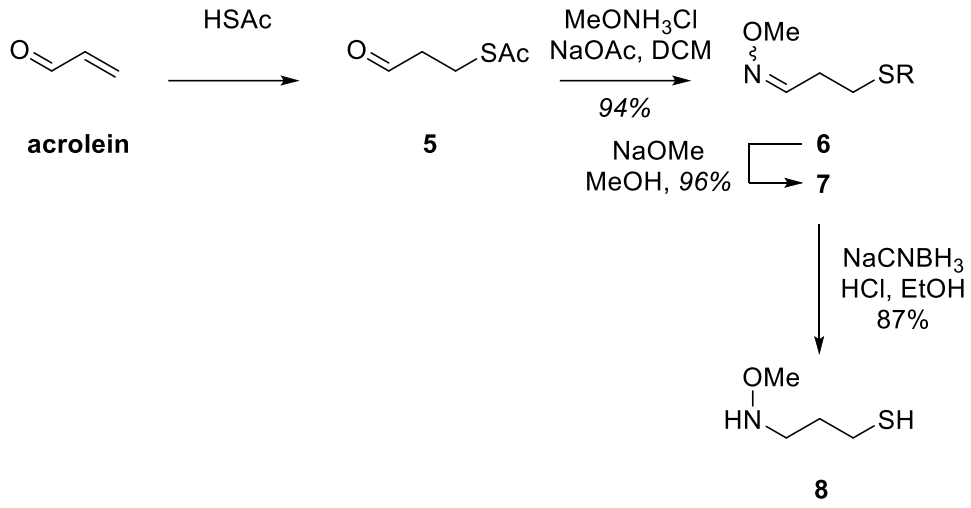
2.2.1 Linker synthesis

To explore the proposed synthetic strategy, the azide-functionalised oxyamine linker **1** was first prepared (Scheme 4). Here, acrolein was subjected to a Michael addition using sodium azide to generate 3-azidopropanal (**2**) in good yield on a gram scale.[34] Due to the instability and reactivity of the aldehyde, the reaction was performed at -15 °C and the mixture was directly used after basic aqueous work-up. Next, the condensation of propanal **2** with methoxyamine was performed under basic conditions to give imine **3**. At first, pyridine/ethanol was used as a solvent system,[20] however, the use of sodium acetate in CH_2Cl_2 was preferred due to practical advantages such as easier extraction.[40] Imine **3** could then be purified by silica gel column chromatography before being reduced with $NaCNBH_3$ in the presence of ethanolic HCl (freshly prepared from AcCl in ethanol) to give the desired bifunctional linker **1**. Purification of the methoxyamine was achieved by aqueous workup followed by column chromatography. In addition, it should be noted that amine **1** can either be purified by Kugelrohr distillation (bp. of ca. 150 °C at 10 mbar) or by silica gel column chromatography. Using this approach, azido methoxyamine **1** was synthesised in 80% yield over three steps. Purification of the intermediate products, however, is not required and indeed, the methoxyamine **1** can be prepared on a gram scale and in a higher (96%) yield if the intermediates are not isolated, but used directly after each extraction without concentration of the reaction mixture. Next, to prepare the amine functionalised oxyamine linker **4,** the azide **1** can be reduced using a Staudinger reaction in excellent yield. Oxyamine **4** can readily be purified by a reverse phase plug to remove the phosphine by-products. Infrared spectroscopy confirmed the reduction of the azide to the amine by the lack of the distinct azide stretch at 2100 cm^{-1}, which was previously observed for azide **1**, and all other characterization data, including ^{1}H and ^{13}C NMR, were in full accordance with the anticipated structures.



Scheme 4. *Synthesis of azide and amine functionalised oxyamine linkers.*

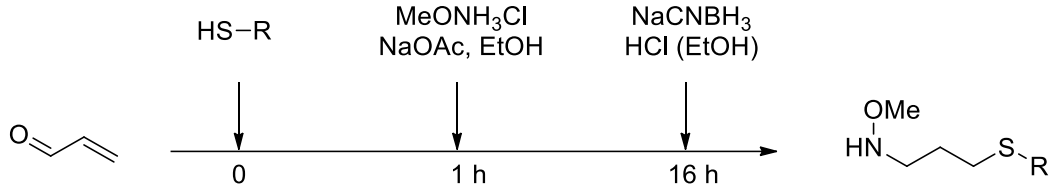
Having established the potential of the methodology, the repertoire of oxyamine functionalised bifunctional linkers was then extended by exploring the construction of a thiol-functionalised linker (Scheme 5). To this end, thioacetic acid was reacted with acrolein to provide the 1,4-adduct **5**, which was again condensed with methoxyamine to give the corresponding oxime **6**. While initial attempts to reduce the oxime in a one-pot approach led to the formation of the amine, S-to-N acyl migration from the thiol could not be prevented and the acetamide product was isolated in high yield. Therefore, the intermediate thioacetate was deacetylated under Zemplén conditions to give thiol **7**, in excellent yield. Finally, the reduction of imine **7** under the agency of $NaCNBH_3$ allowed for the successful synthesis of thiol-functionalised linker **8** in 79% overall yield (4 steps). Moreover, in 1965 Bauer and Ghosh prepared a similar thiol-functionalised methoxyamine, however, with an ethyl-, instead of propyl-linker length.[41]

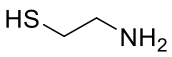
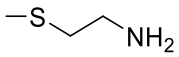
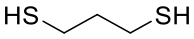
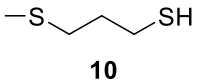
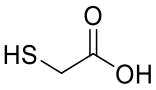
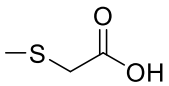
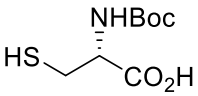
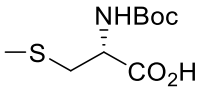


Scheme 5. *Synthesis of the thiol functionalised oxyamine linker **8**.*

With the goal of developing a one-pot procedure, the use of functionalised thiols for the Michael addition was explored (Table 1). To this end, the reaction of cysteamine with acrolein, followed by the addition of methoxyamine, led to the *in situ* formation of the oxime, which was subsequently reduced by the addition of $NaCNBH_3$, leading to the one-pot three-step synthesis of the amine-functionalised linker **9** (entry 1). The desired oxyamine **9** could be purified by silica gel column chromatography to yield the target linker in a good (66 %) yield. Given the success of this methodology, it was subsequently extended to the use of alternative thiol nucleophiles in order to synthesise an array of bifunctional linkers.

Table 1. *Three-step one-pot synthesis of oxyamine linkers.*



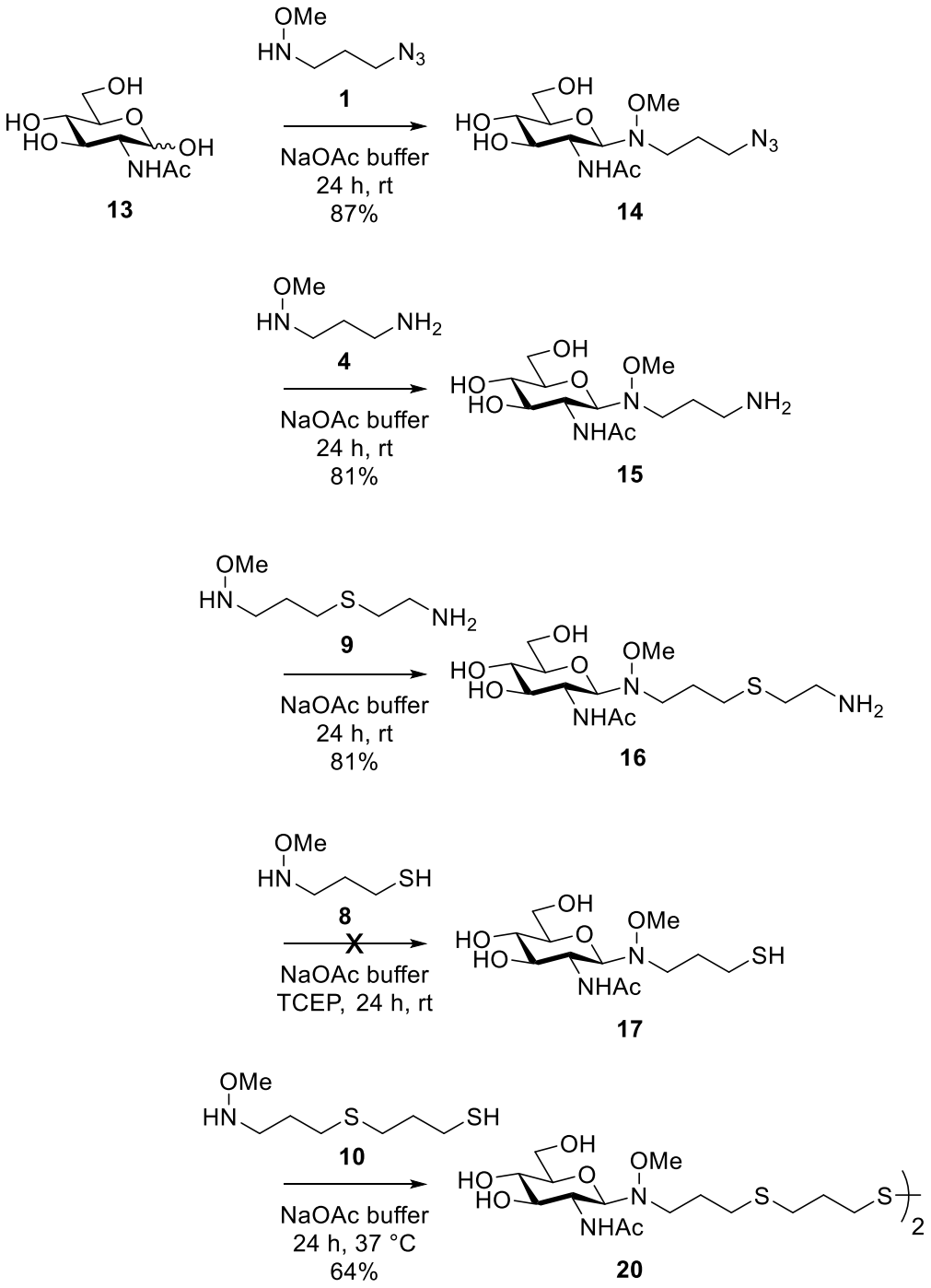
Entry	Thiol	Product	Yield
1		 **9**	66%
2		 **10**	76%
3		 **11**	79%
4		 **12**	51%

For the synthesis of a thiol-functionalised linker, neat propane-1,3-dithiol was then used to allow for the rapid synthesis of linker **10** in 76% yield (Table 1, entry 2). Here, 1,3-propanedithiol simultaneously acts as a reducing agent, which allows for the isolation of the sulfhydryl instead of the disulfide oxyamine after silica gel chromatography. In order to produce a carboxy-modified linker, 2-mercaptoacetic acid was used (entry 3) Again, the required linker **11** was readily prepared via the three-step one pot strategy and in 79% overall yield. It should be noted that concentration of the crude reaction mixture results in the formation of the ethyl ester derivative (^{1}H NMR δ 4.22 (q, 2H, $J_{CH2,CH3}$ = 7.1 Hz, CH_2 OEt), 3.53 (s, 3H, OCH_3), 2.97 (t, 2H, $J_{1,2}$ = 7.3 Hz, CH_2-1), 2.68 (t, 2H, $J_{2,3}$ = 7.3 Hz, CH_2-3), 1.81 (p, 2H, $J_{1,2}$ = $J_{2,3}$ = 7.3 Hz, CH_2-2), 1.27 (t, 3H, $J_{CH2,CH3}$ = 7.1 Hz, CH_3 OEt); *m/z* for $[C_8H_{18}NO_3S]^+$ calc. 208.1002, obs. 208.1013). Due to the high water solubility of linker **11**, an aqueous work-up procedure was not feasible and instead the reaction was quenched by the addition of 1M NaOH (aq.) before concentration and purification by silica gel flash column chromatography to give the target carboxy-modified linker. Finally, to

determine if the methodology was amenable to the use of more complex substrates, acrolein was subjected to a Michael addition with *N*-Boc-cysteine, followed by oxime formation and reduction (entry 4). Again, ethyl ester formation (*m/z* for $[C_{14}H_{29}N_2O_5S]^+$ calc. 337.1792, obs. 337.1820) was observed as well as some *N*-Boc cleavage (*m/z* for $[C_9H_{21}N_2O_3S]^+$ calc. 237.1267, obs. 237.1280), and thus the reaction was quenched with 1M NaOH followed by concentration and silica gel flash column chromatography to afford amino-acid derivative **12**. While the overall yield for the synthesis of cysteine linker **12** was modest (51% yield over three steps), this route nonetheless provides a rapid entry to an alkoxyamine functionalised amino acid.

2.2.2 Carbohydrate Ligation of methoxyamine linkers

With a series of oxyamine linkers in hand, the efficiency of glycan conjugation was then investigated. Given the importance of GlcNAc as an N-linked reducing end terminal sugar,[42] this substrate was initially used for linker conjugation. To this end, *N*-acetyl-glucosamine (**13**) and the appropriate linker (10 equiv.) were stirred in an aqueous 2M AcOH/NH_4OAc buffer at pH 4.5 (0.1 M glycan concentration) for 16 h at room temperature (Scheme 6). These solvent conditions were chosen as they should allow for the solubilisation of most glycans. The conjugation of azide linker **1** proceeded smoothly and in excellent yield to give N-glycan **14** in 87%. Here, it was found that direct concentration or lyophilisation of the reaction mixture resulted in neoglycoside hydrolysis, therefore reverse phase chromatography was used to purify glycoconjugate **14**. Alternatively, the neoglycosides was purified using a water based size exclusion column (BioGel P-2, 1200 x 18 mm, flow-rate 10 mL/hour), whereby the crude reaction mixture was directly loaded onto the size exclusion column, to give the neoglycoside in excellent yields. The conjugation of the sugar to the linker was confirmed by HMBCs between the CH_2-1 of the linker and the CH-1´ of the glycan (Figure 1). Moreover, the exclusive formation of the β-pyranoside was confirmed by the $J_{1',2'} = 9.8$ Hz. In addition, the N-glycan **14** was crystallised from MeOH/Et_2O and analysed by X-ray crystallography, which confirmed that the linker was in the β-configuration (Figure 2).



Scheme 6. *Conjugation of oxyamine linkers to GlcNAc.*

Next, *N*-acetylglucosamine (**13**) was successfully condensed with the amine functionalised linkers **4** and **9** to give both the corresponding N-glycans **15** and **16** in 81% yield. Interestingly, amine **15** had no retention using reverse phase chromatography and thus required purification by size exclusion chromatography. Size exclusion chromatography with a 0.1 M ammonium formate solution results in the formation of the formate salt of **15**. This, however, can be converted to the free amine or hydrochloride salt by filtering over a ion exchange resin, Dowex OH^- or Dowex Cl^- respectively (see SI for corresponding NMR spectra). The less polar glycoside **16**, however, was obtained after purification with reverse phase and silica gel column chromatography.

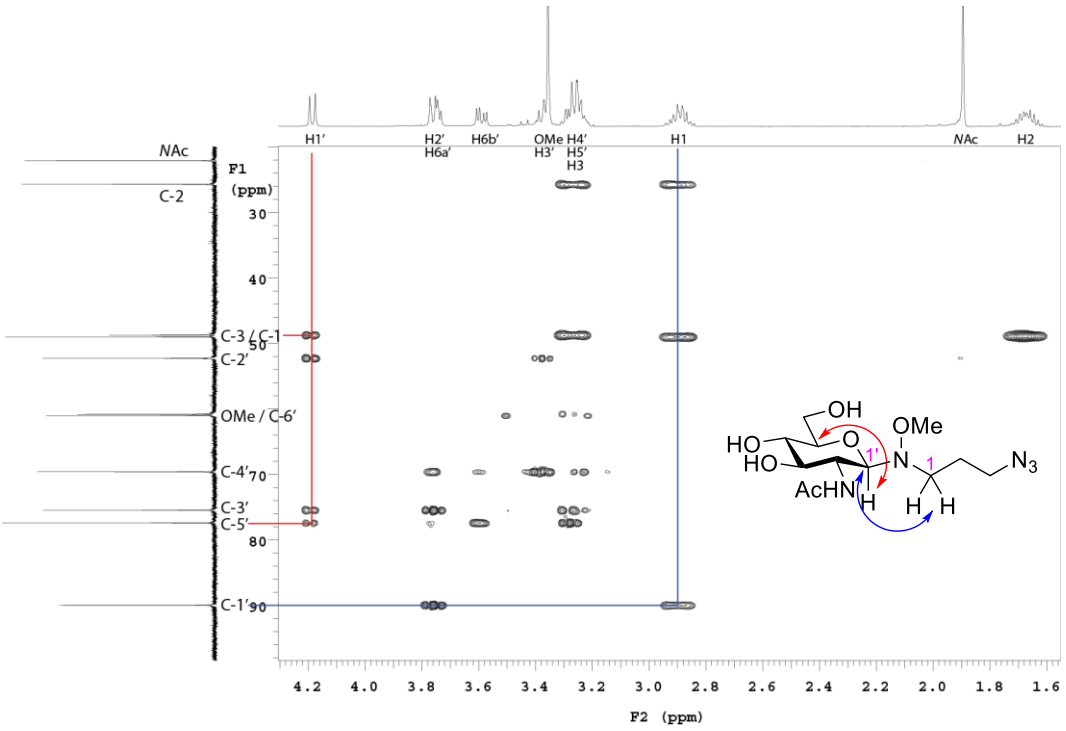


Figure 1. *HMBC of neoglycoside* ***14*** *(D_2O, 500MHz).*

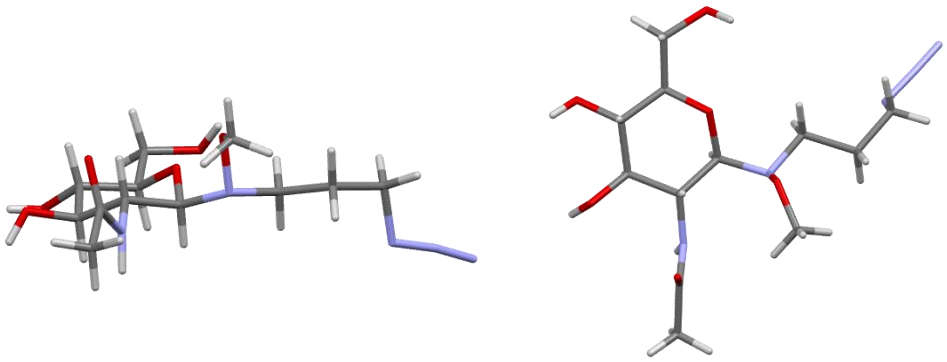
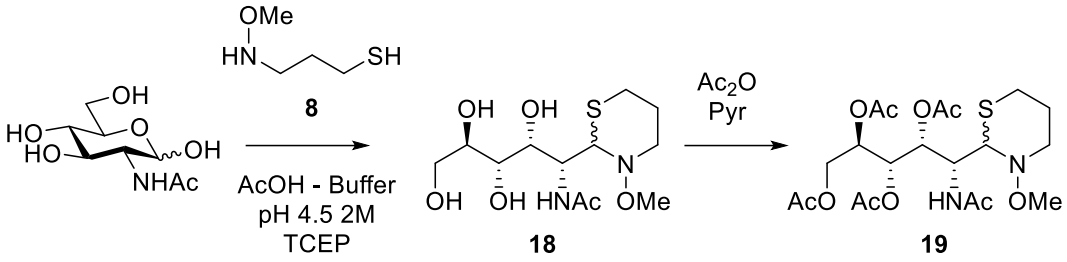


Figure 2. *X-ray crystallography structure for glycoside* ***14****. A) Sideways view B) Topview.*

Having succesfully prepared the azide-functionalised glycan **14**, *N*-acetylglucosamine (**13**) was then reacted with thiol functionalised linker **8** to obtain a condensation product in 81% yield, however, this did not turn out to be the desired glycosylamine **17**, but instead a diastereomeric mixture of *N*-methoxythiazinanes **18**, which are formed via the intramolecular 6-*endo*-*trig* ring-closure of the thiol and the imine. (Scheme 7). Here, TLC analysis indicated the formation of this newly formed glycoconjugate, and although this was supported by mass spectrometry (*m/z* for $[C_{12}H_{25}N_2O_6S]^+$ calc. 325.1428, obs. 325.1445), the structures could not be conclusively confirmed using NMR analysis. Surprisingly, NMR spectroscopy showed peak broadening in both the ^1H and the ^{13}C experiments, even when using different solvents [D_2O, CD_3OD, Pyr(D_5) and DMSO(D_6)] and varying temperatures (rt, 30, 40, 50 and 60 °C). Nevertheless, the structure

of the newly formed thiazinanes could be confirmed after acetylation of the crude condensation reaction products.



Scheme 7. *The formation of N-methoxy thiazinanes* ***18****, which after per-acetylation could be confirmed by NMR analysis.*

After acetylation of tetraols **18**, two products were observed by NMR-analysis, although peak broadening complicated the ^{1}H and ^{13}C NMR spectra. Fortunately NMR analysis at 50 °C proved sufficient for structural confirmation of the two diastereomeric thioaminals **19**. Although the relative stereochemistry of the two diastereoisomers could not be determined, a combination of COSY, HSQC (coupled and decoupled) and HMBC NMR-experiments enabled all resonances of each diastereoisomer to be assigned. To this end, the presence of the thiazinane was confirmed for both isomers by the HMBC between C-1' and $CH_{eq}N$ as well as $CH_{eq}S$. In addition, a coupled HSQC experiment showed a $^{1}J_{C1'\text{-}H1'}$ = 146 Hz for *isomer a* and $^{1}J_{C1'\text{-}H1'}$ = 145 Hz for *isomer b*, indicating they both contain the thioaminal functional group. Moreover, 1D-TOCSY experiments were used to analyse the 6-membered ring (Figure 3). Here, excitation of the *isomer a* equatorial CH_2NO (H_a) at 3.60 ppm gave the TOCSY resonances, throughout the spin system, at the relaxation delay times of 20, 40, 80 and 120 ms. Here, it was observed that for *isomer a* the coupling constants of the protons were indicative of the presence of a 6-membered ring. Unfortunately, selective excitation of CH_2NO (H_a) for *isomer b* could not be used for structure confirmation, due to overlapping TOCSY resonances.

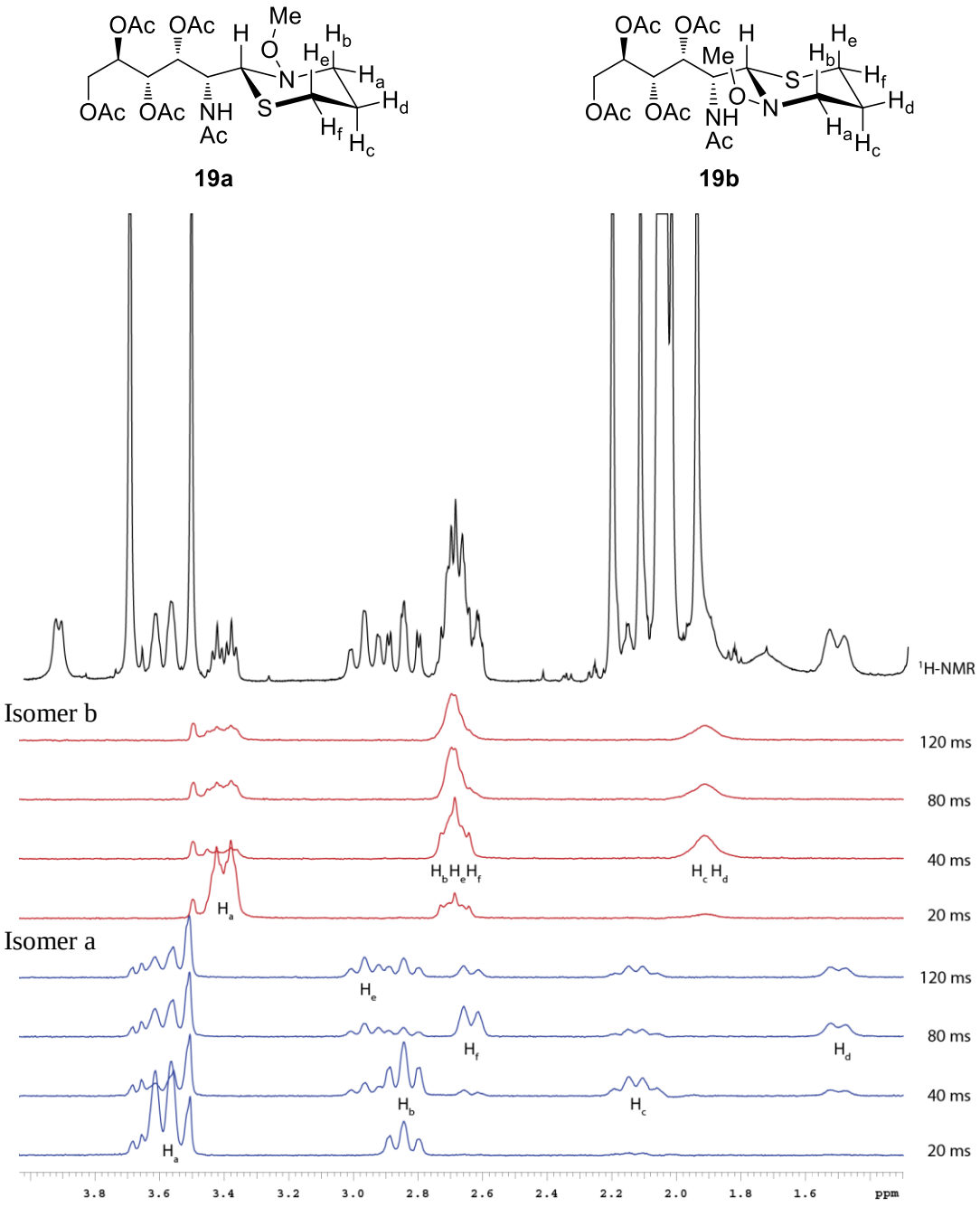


Figure 3. *TOCSY-NMR experiment with relaxation delay times of 20, 40, 80 and 120 ms. Selective excitation at 3.58 ppm (H_a) for isomer a (blue), and at 3.40 ppm (H_a) for isomer b (red) and the corresponding ^{1}H-NMR spectrum (black).*

In an attempt to prevent the 6-*endo-trig* cyclisation and formation of the thioaminals, the extended thiol linker chain **10** was used (Scheme 6). Gratifyingly, this led to the successful formation of the disulfide glycosylamine **20** in 64% yield. Here, it should be noted that the disulfide was obtained after exposure to air, however, the use of a reducing agent, such as *tris*(2-carboxyethyl)phosphine (TCEP), prevented the formation or reduced the already formed disulfide to the corresponding sulfhydryl.

2.2.2.1 Kinetic Stability Studies

As oxyamine-linkers are hydrolysed under acidic aqueous conditions, we investigated the rates of hydrolysis of glycoconjugate **14** at varying pH. Previous work in this field has shown that the substitution pattern of the nitrogen and oxygen in oxyamines affects their hydrolytic stability, with the electron-donating Bn group on either the oxygen or nitrogen of the oxime decreasing the rate of hydrolysis.[32] The disadvantage of the bulky benzyl group, however, is that conjugation to the glycan is not as efficient and lectin binding can be compromised.[32] Thus, glycoconjugate **14** was subjected to aqueous conditions at pH 4.75, 5, 6, 7 and 9 to evaluate linker stability (Figure 4). Using a ^{1}H-NMR experiment, the reactions were monitored over time and relative integration between CH_2-1 and H-1' gave the respective ratios between neoglycoside **14**, the hydrolysed glycan **13** and the linker **1**. As anticipated, the rates of hydrolysis were pH dependent, with the glycoconjugate **14** showing no appreciable cleavage at pH 7 and 9 over a 100 day period. At pH 6, only limited cleavage was observed with a $t_{1/2}$ = 2 years, which indicates that the linker is suited for use under physiological conditions. At pH 5 and 4.5, $t_{1/2}$ = 120 and 22 days, respectively, which was 2 to 5-fold better than the analogous benzylated oxyamine of 'Type A' (Figure 4).[32] Accordingly, the *N*-linked methoxyamine linker of 'Type B' can not only be readily prepared but, in addition, shows excellent hydrolytic stability.

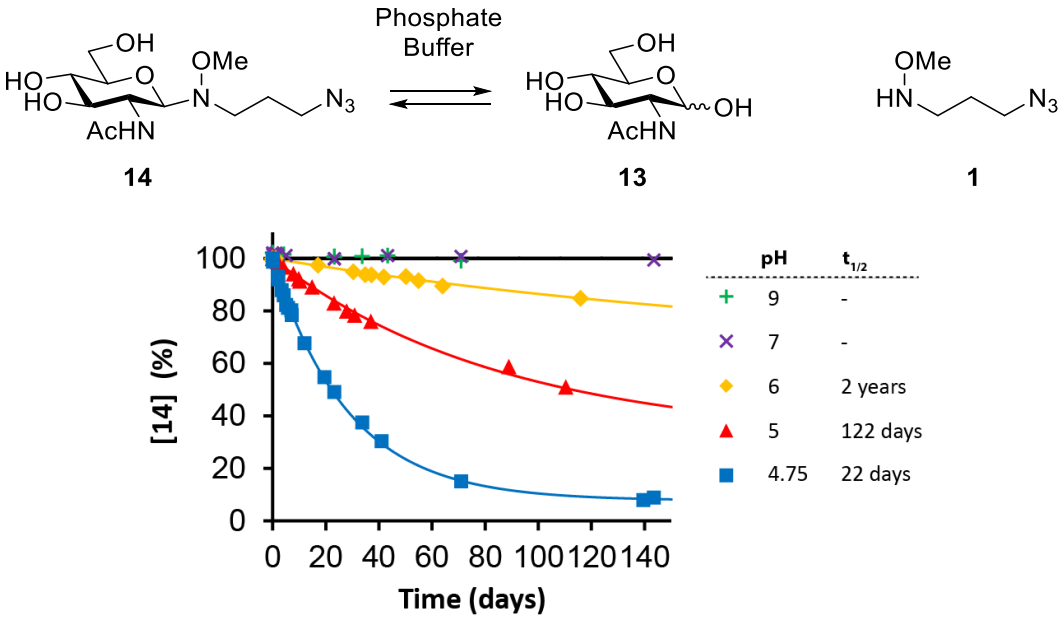


Figure 4. *Rates of hydrolysis of oxyamine linked GlcNAc **14** at pH 4.75, 5, 6, 7 and 9. As determined by ^{1}H-NMR analysis of 50 mM solutions of glycoside **4** in sodium phosphate buffered D_2O.*

To calculate the half-life for each reaction, the rate equation for chemical decay of the glycoside is described with:

$$N(t) = N_0\, e^{-\lambda t} \quad (1)$$

Where *N(t)* is the percentage of starting material left at time (*t*), N_0 is the amount of starting material at *t=0*, λ is the probability of one molecule to decay per time unit (decay constant) and *t* is the time. However, given the fact that the reaction reaches an equilibrium, the equation must be modified to describe that the reaction reaches the equilibrium N_{eq}:

$$N(t) = (N_0 - N_{eq})\, e^{-\lambda t} + N_{eq} \quad (2)$$

To determine the half-life a theoretical line is fitted to the data according to the least squares method. By fitting the line to the data, both the rate constant λ and the equilibrium N_{eq} were determined for each reaction. Next, the half-life can be determined by solving the equation (2) above for *N(t)=50*. For example, if N_{eq} = 7 and λ = 0.035 then a half-life of 22 days is calculated:

$$50 = (100 - 7)e^{-0.035t} + 7 \quad (3)$$

$$t_{(\frac{1}{2})} = \frac{ln\left(\frac{1}{43/93}\right)}{0.035} = 22\ days \quad (4)$$

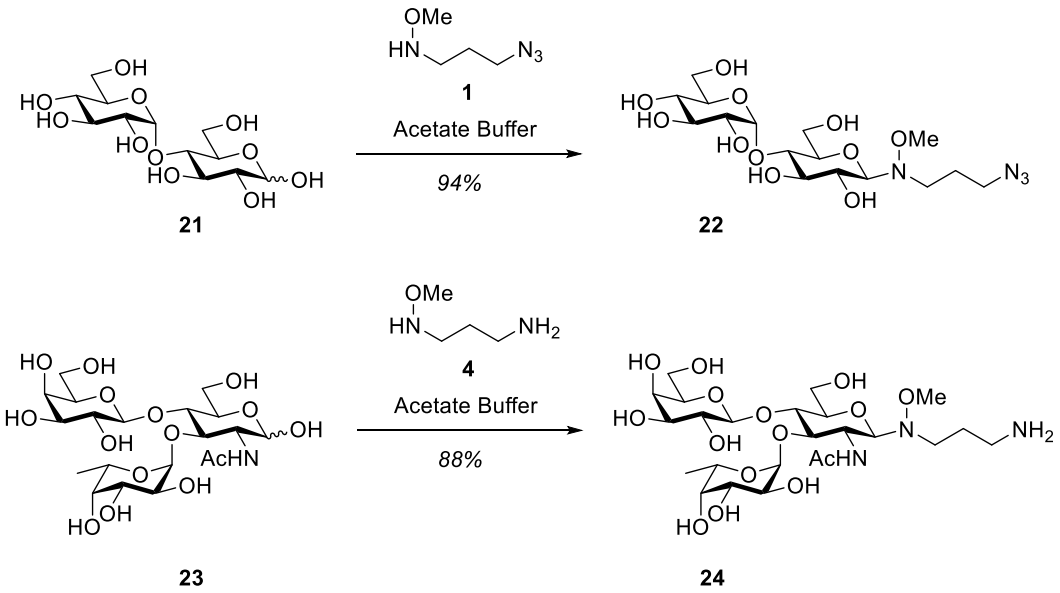
Currently, further investigations towards oxyamine stability are underway. In these experiments the glycoconjugates of 'Type A' and 'Type B' are directly compared under the same reaction conditions. Here, the effect of the glycan concentration, the pH and the buffer strength are examined. These results will be reported in due course.

2.2.2.2 Scope and limitation of carbohydrates ligation

Having demonstrated that a variety of oxyamine linkers can be conjugated to *N*-acetyl glucosamine (**13**), the chemoselective ligation of methoxyamine linkers to other carbohydrates was investigated. As nicely demonstrated in the neoglycorandomisation research field, a variety of carbohydrates can be used for the chemoselective ligation with methoxyamine linkers. However, only a few neoglycosides are selectively obtained in the β-pyranose form, where most glycans are obtained as α/β-mixtures as well as furanose/pyranose configurations.[43] To determine whether comparable α/β, pyranose/furanose configurations would be obtained when using methoxyamine linker **1**, a variety of reducing end sugars were conjugated to this linker and subsequently analyzed by ^{1}H-NMR. Accordingly, it was found that both GlcNAc and glucose were selectively obtained as the β-pyranoside. Moreover, D-galactose was successfully converted into the corresponding neoglycoside, however, with a 90:10 β-pyranose:β-furanose ratio.

Furthermore, mannose was obtained as an α-furanose and α/β-pyranose mixture, which is in line with Langenhans' study.[43] Accordingly, the selectivity of the ligation with all forms of oxyamine linkers appears to be similar and thus no further investigation was performed towards the nature of the reducing end sugar.

To further illustrate the versatility of the linker strategy, conjugation to more sterically hindered carbohydrates were undertaken. To this end, the disaccharide maltose (**21**) was successfully converted into glycoside **22** in excellent yield and with complete β-selectivity. Conjugation of the biologically relevant trisaccharide Lewis^X (**23**) was then performed, whereby the amine linker **4** and Lewis^X **23** were stirred in a 2 M NaOAc/NH_4OAc buffer at room temperature (Scheme 4). The reaction proved sluggish and only *ca.* 25% conversion was observed after 24 h by TLC-analysis. Increasing the temperature to 40 °C, however, led to a marked increase in reaction rate, with complete conversion to N-glycan **24** being observed after 36 h at this temperature. Purification by direct loading of the reaction mixture onto a Bio-Gel P-2 column for size exclusion chromatography then allowed for the isolation of conjugate **24** in 88% yield and as only the β-pyranosyl glycoside, as evidenced by $^1J_{CH\text{-}1'}$ = 154 Hz, and an HMBC between H-1´and C-5´ (Figure 5).



Scheme 8. *Neoglycosylation of the more bulky maltose **21** and the biological relevant Lewis^X **23**.*

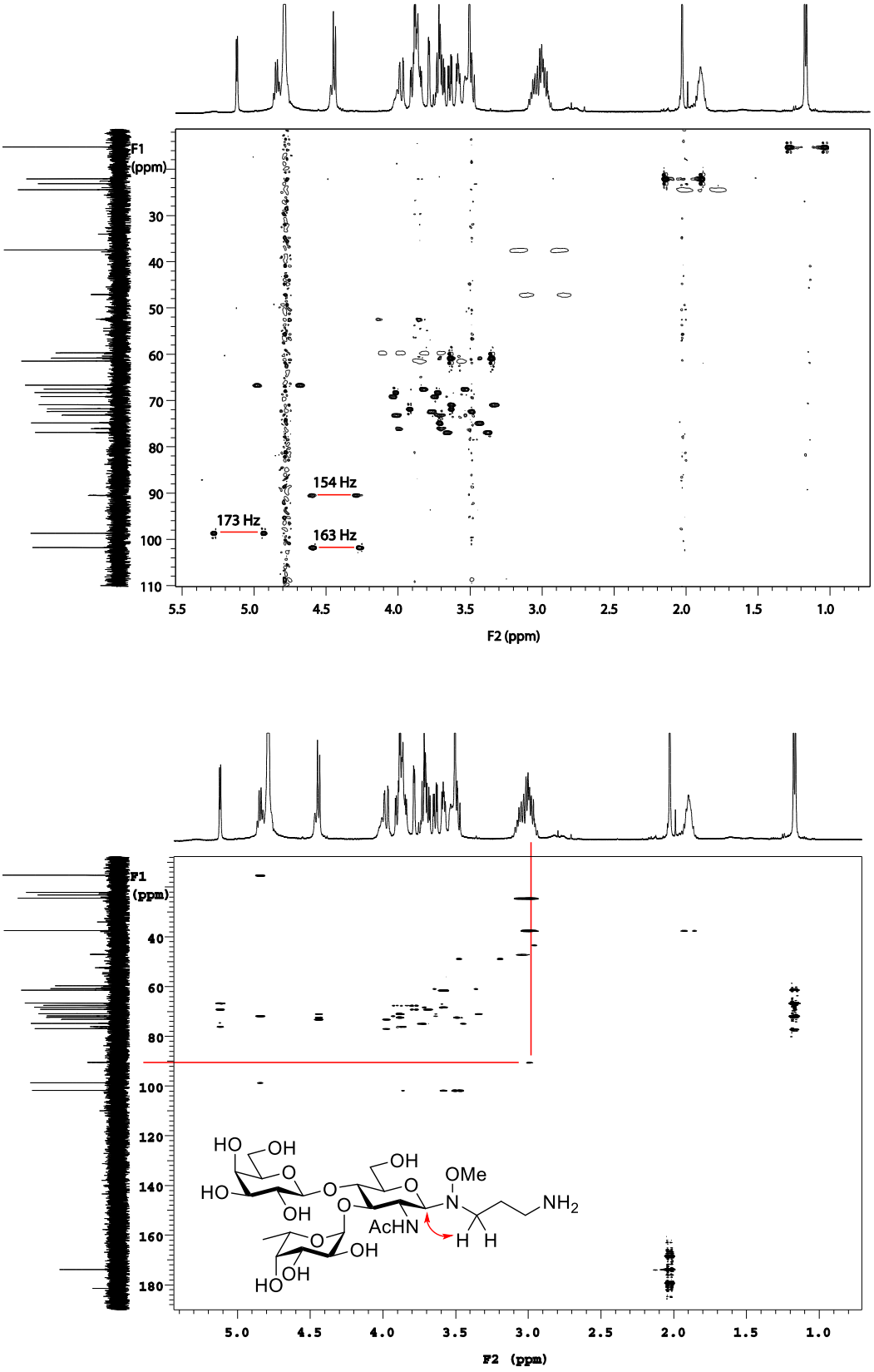
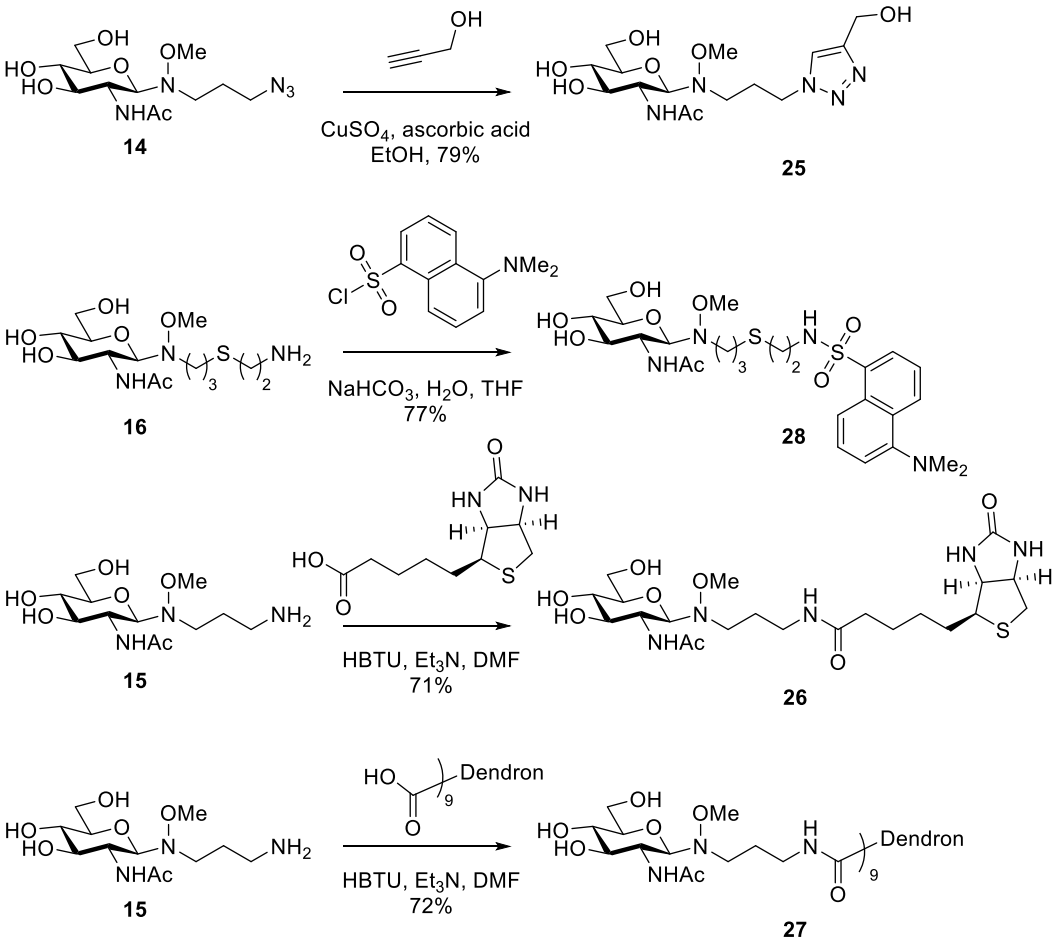


Figure 5. *Coupled HSQC (top) and HMBC (bottom) NMR experiments of Lewis^X-glycoconjugate* ***24****. (D_2O, 500MHz NMR)*

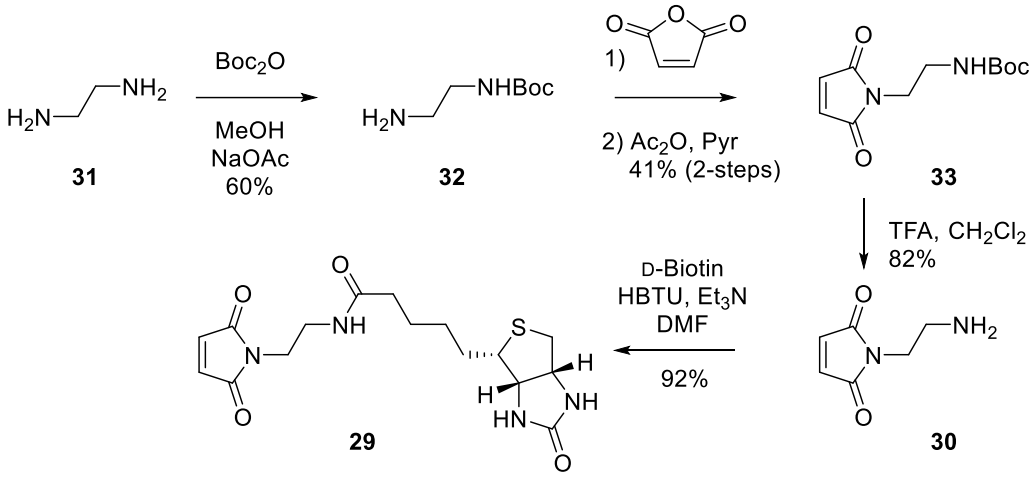
2.2.3 Conjugation of neoglycosides to probes

In order to determine whether the azide derived neoglycoside **14** could be used in the copper mediated azide-alkyne Huisgen cycloaddition, the azidoglycoconjugate **14** was successfully reacted with propargyl alcohol (Scheme 9). This led to the formation of triazole **25** in good yield (79%) and demonstrated that the azide-functionalised neoglycoside can indeed successfully be used in a cycloaddition reaction. The use of amine functionalised glycans was exemplified by the synthesis of a fluorescent glycoconjugate. Here, neoglycoside **16** was reacted with dansyl chloride under basic conditions to give sulfonamide **28** in 77% yield. In this way the potential to generate fluorescent glycans for the study of biological processes has been demonstrated.[10] Amine derivative **15** was also successfully conjugated with D-biotin using the *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) mediated peptide coupling, to give glycoconjugate **26** in 71% yield. Here, the biotinylated glycan was purified by a combination of silica gel and size exclusion chromatography. The conjugation of neoglycoside **15** with a nonavalent bifunctional second generation dendron allows for the multivalent presentation of the glycan in **27** which forms the subject of Chapter 3.

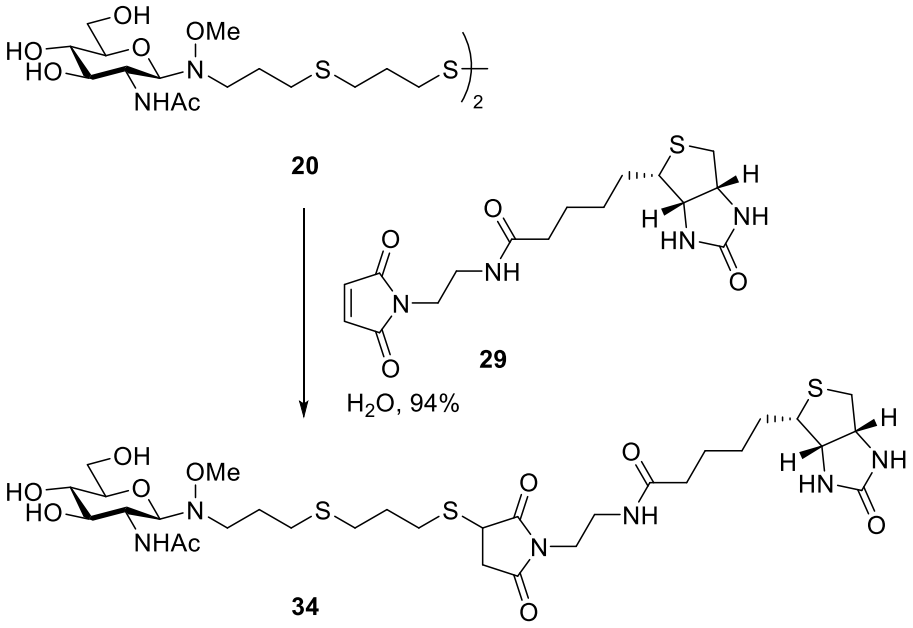


Scheme 9. *Conjugation of neoglycosides with various probes.*

To further demonstrate the scope and efficiency of the developed oxyamine linker methodology, it was proposed to conjugate thiol functionalised glycosides to maleimide **29**. This required the synthesis of biotinylated-maleimide **29** via the amide coupling of D-biotin with previously reported 2-aminoethyl-maleimide intermediate **30** (Scheme 10).[44] This intermediate was prepared from ethylene diamine (**31**) via mono *N*-Boc protection (→**32**), reaction with maleic anhydride and subsequent cyclisation to give maleimide **33**, and TFA treatment to give amine **30**. Subsequently, disulfide **20** and Michael acceptor **29** were successfully reacted in H_2O to give crude biotinylated adduct **34**, which was then purified by a combination of reverse phase (C_{18}) and size exclusion chromatography (Scheme 11). Both biotinylated adducts **26** and **34** can be used for the detection of carbohydrate binding antibodies in patients' blood following conjugation of the probes to streptavidin-coated plates, and subsequent incubation with patients' serum.[45,46]

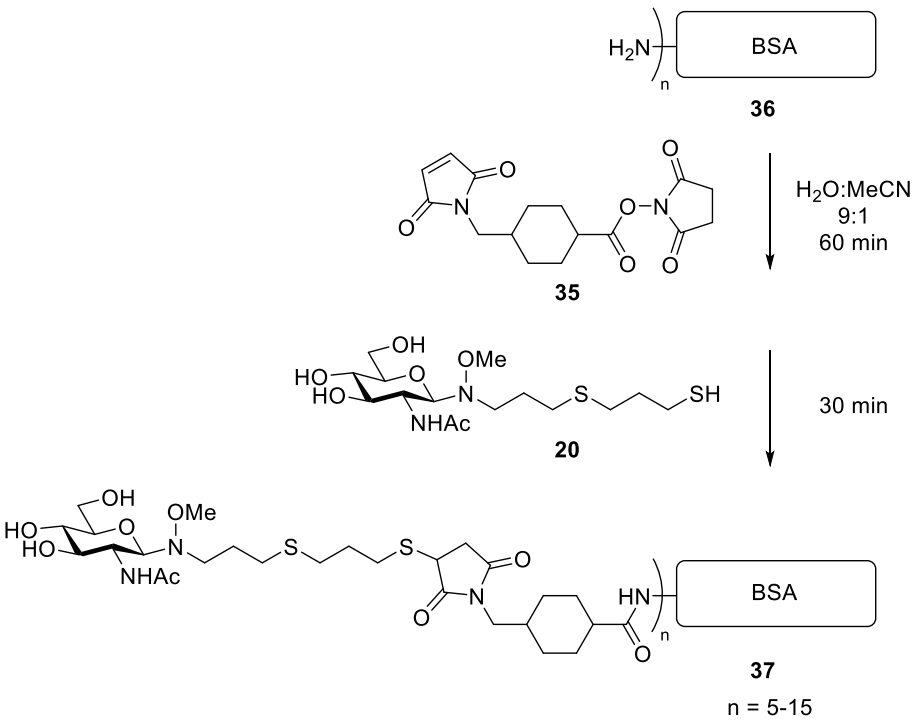


Scheme 10. *Synthesis of maleimide acceptor **29**.*



Scheme 11. *Synthesis of biotinylated neoglycoside* ***34****.*

The use of maleimide chemistry can also be extended to the formation of highly glycosylated proteins. Here, the commercially available bifunctional cross-linker succinimidyl-4-[*N*-maleimidomethyl]cyclohexane-1-carboxylate (SMCC) **35** was first used for the maleimide functionalisation of the serine residues in Bovine Serum Albumin (BSA) **36** (Scheme 12). The SMCC linker can then be used in the thiol-maleimide conjugation with the neoglycoside **20** to give glycoprotein **37**. The reaction progress was monitored using MALDI-TOF analysis, whereby the average molecular weight of the protein was measured after SMCC conjugation and subsequent glycosylation. Initially, a number of reaction conditions were explored to find the optimal conjugation conditions to obtain highly glycosylated proteins.



Scheme 12. *Glycosylation of BSA* ***36*** *using SMCC cross-linker* ***35*** *and neoglycoside* ***20*** *to give glycoprotein* ***37****.*

Given that SMCC **35** is hydrophobic, the cross-linker conjugation was first examined using different concentrations of DMSO in water. To this end, the protein was dissolved in water (1 mg/mL), followed by the addition of cross-linker (60 equiv.) in DMSO to give 10, 25, 50 and 75% DMSO in water. In addition, a duplicate experiment was performed using MeCN instead of DMSO. The reactions were incubated at rt for 30 min. and a MALDI-TOF sample was prepared. Next, 120 molar equivalents of thiol functionalised glycoside **20** in water was added and the reaction was incubated for a further 30 minutes, before another MALDI-TOF sample was prepared. From these experiments it was observed that both 10% DMSO and 10% MeCN were sufficient for conjugating the cross-linker to BSA, whereas higher concentrations of organic solvent did not result in much higher cross-linking. That said, low cross-linking (< 5 linkers) as well as GlcNAc binding was observed throughout all the experiments, which could be explained by low solubility of the protein. Accordingly, when the protein was dissolved in PBS an increased conjugation of both the cross-linker as well as the glycan was observed. Next, the amount of SMCC-linker was explored (60, 120, 180, 360, 720 equiv.) and it was observed that optimal conjugation was achieved using 180 equivalents of SMCC. Here it should be noted that a large excess of cross-linker requires an even larger excess of glycan. With these results in hand, the efficiency of the SMCC cross-linking step as well as the glycan conjugation was analysed over time (Figure 6). Again, BSA **36** (66.2 kDa) was dissolved in PBS, followed by the addition of SMCC **35** in MeCN (10% final concentration) and MALDI-TOF samples, as well as 10 μL

samples for glycoconjugation, were taken at 0, 15, 30, 60, 120, 240 and 480 min. To each of the 10 μL glycoconjugation samples, 10 equiv. of oxyamine **20** (10 mg/mL in water) was added, and for each of these reactions MALDI-TOF samples were taken after 0, 30 and 60 min. From these experiments, it was found that the SMCC conjugation reaction continued over time, however, the glycan conjugation was inhibited after 8-10 glycans were cross-linked. This decrease in glycan conjugation may be due to the SMCC-maleimide moiety cross-linking within the protein, or maleimide degradation without affecting the activated NHS-ester. Nevertheless, it was determined that optimal glycoconjugation can be achieved when the BSA protein **35** (10 mg/mL) was reacted with the SMCC cross linker **35** (180 equiv.) in 10% MeCN in PBS for 60 min before adding an excess of 360 equiv. of the neoglycoside **20** and incubating the glycoprotein for 60 min at rt. Although, glycoprotein **37** was never purified, numerous literature procedures describe this process using dialysis or size exclusion chromatography purification methods, thereby demonstrating yet another application of the oxyamine ligation strategy.[47]

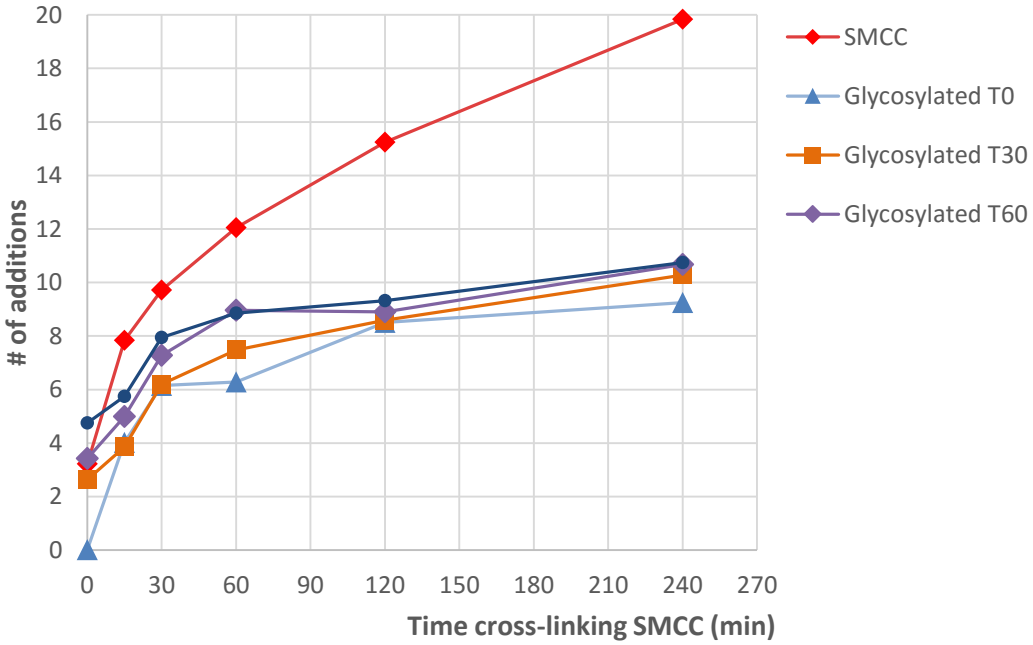


Figure 6. *Synthesis of BSA glycoprotein **37**. First conjugation of cross-linker SMCC **35** was monitored over time. In addition, glycoside **20** was added at each time point, and measured over a 4 h. time frame. Here, the average addition of cross-linker is calculated by measuring the molecular weight difference between BSA and cross-linked protein. In a similar fashion the glycosylation state is calculated, by subtracting the final molecular weight over the molecular weight increase after conjugation at each time point.*

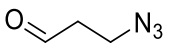
2.3 Conclusion

In conclusion, a variety of oxyamine linkers have been prepared in high yield and in few (3-4) steps. Key in the reaction sequence was the use of acrolein as a Michael acceptor which could then undergo reductive amination with methoxyamine. Many of the linkers can be synthesised using a one-pot protocol, and can be prepared on a gram scale. The linkers can be readily coupled to glycans in excellent yields, and hydrolytic stability studies revealed that the methoxyamine linker is stable over a large pH range, with exceptional stability at physiological pH. The use of the linker for conjugation to more complex glycans has been demonstrated by the highly efficient coupling to Lewis^{x}. In addition, the versatility of the differently functionalised oxyamine linkers was demonstrated via the synthesis of biotinylated, fluorescently-labelled, and protein-conjugated probes, which were prepared using different reaction conditions, incluing peptide ligation, thiol-maleimide Michael addition and sulfonylation. The ability of the oxyamine linker to participate in the copper-mediated azide-alkyne Huisgen cyclisation reaction was also demonstrated using a model substrate.

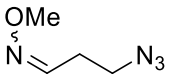
Compared to the existing methodology for oxyamine glycan linker synthesis the route presented here has a number of advantages. In particular, the linkers in this thesis are prepared in higher yields (50-96%) and fewer steps (3-4 steps) when compared to the the linkers of “Type A” (4-6 steps, 10-34%). In addition, these linkers can be prepared with the functional handle of choice, and thus can be used in the assembly of the glycoconjugate of choice.

2.4 Experimental

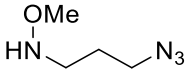
General Procedure. Unless otherwise stated all reactions were performed under atmospheric air. H_2O, methanol (MeOH, Pure Science), ethanol (EtOH, absolute, Pure Science), ethyl acetate (EtOAc, Pure Science), dichloromethane (LabServ) and petroleum ether (PE, Pure Science) were distilled prior to use. Acrolein (Merck), AcOH (Ajax Finechem), NaN_3 (BDH), 30 % aqueous NH_3 (J. T. Baker Chemical Co.), AcCl (Sigma Aldrich), PPh_3 (Acros), $NaCNBH_3$ (Aldrich), NaOH (Pure Science), NaOAc (Riedel-de Haën), $NaHCO_3$ (Pure Science), $MgSO_4$ (Pure Science), Na_2CO_3 (Pure Science), NH_4OAc (Sigma Aldrich), methoxyamine hydrochloride (Sigma Aldrich), thioacetic acid (Aldrich), sodium (Aldrich), 1,3-propanedithiol (Aldrich), cysteamine hydrochloride (Fluka), *N*-Boc-Cys-OH (Fluka), 2-mercaptoacetic acid (Aldrich) and *N*-Acetylglucosamine (Fluka) were used as received. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC-analysis on silica gel coated plastic sheets (0.20 mm, silicagel 60) with detection by UV-absorption (254 nm), by dipping in 10 % H_2SO_4 in EtOH followed by charring at ~150 °C, by dipping in I_2 in silica, or by dipping in a solution of ninhydrin in EtOH followed by charring at ~150 °C. Column chromatography was performed on Pure Science silica gel (40-63 micron). AccuBOND II ODS-C18 (Agilent) was used for reverse phase chromatography. Bio-Gel P-2 (600 × 10 mm) was used for size exclusion chromatography. Optical rotations were recorded using a Perkin-Elmer 241 polarimeter at the sodium D-line. Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm^{-1}). High-resolution mass spectra were recorded on an Agilent Technology 6530 Accurate Mass Q-TOF/LCMS spectrometer using positive electro-spray ionisation. Nuclear magnetic resonance spectra were recorded at 20 °C in D_2O, $CDCl_3$ or CD_3OD using either a Varian Unity-INOVA operating at 300 MHz or a Varian Unity operating at 500 MHz. Chemical shifts are given in ppm (δ) relative to tetramethylsilane. NMR peak assignments were made using COSY, HSQC and HMBC experiments.



3-Azido-propanal (2). Acetic acid (2.0 mL) was cooled to -20 °C and acrolein (0.92 mL, 13.7 mmol) was added, followed by dropwise addition of a solution of NaN_3 (1.34 g, 20.6 mmol) in water (5.2 mL) and the reaction mixture was stirred for 1 h at -20 °C. The reaction mixture was extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic extracts were washed with sat. aq. Na_2CO_3 (10 mL) and water (10 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo* to afford 3-azido-propanal (1.32 g, 97 %). Due to the instability of the aldehyde the crude material was directly used in the next step. R_f = 0.52 (PE/EtOAc, 80/20, v/v); IR (film) 2938, 2917, 2848, 2737, 2094, 1718, 1449, 1366, 1286, 1071 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 9.80 (bs, 1H, CH-1), 3.61 (t, 2H, $J_{2,3}$ = 6.3 Hz, CH_2-3), 2.72 (dt, 2H, $J_{1,2}$ = 0.9 Hz, $J_{2,3}$ = 6.3 Hz, CH_2-2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.4 (C-1), 44.4 (C-3), 42.6 (C-2); MS (EI): *m/z* 99 (M+, 1%), 71 (2%), 56 (5%), 55 (6%), 43 (97%), 28 (100%).



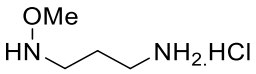
3-Azidopropanal *O*-methyl oxime (3). To a solution of crude 3-azido-propanal (847 mg, 8.55 mmol) in CH_2Cl_2 (10 mL), methoxyamine hydrochloride (852 mg, 10.3 mmol) and sodium acetate (1.40 g, 17.1 mmol) were added at rt and the reaction mixture was stirred for 16 h. The reaction mixture was quenched by the addition of sat. aq. Na_2CO_3 (10 mL) and extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic extracts were washed with brine (10 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by column chromatography (PE/EtOAc, 99/1 → 97/3, v/v) to give 3-azidopropanal *O*-methyl oxime (1.08 g, 99 %) as a colorless oil. R_f = 0.61 (PE/EtOAc, 90/10, v/v); IR (film) 2939, 2902, 2855, 2821, 2099, 1461, 1442, 1352, 1265, 1240, 1047, 910, 733 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.39 (t, 1H, $J_{1,2}$ = 5.5 Hz, H-1 *major*), 6.70 (t, 1H, $J_{1,2}$ = 5.3 Hz, H-1 *minor*), 3.89 (s, 3H, OCH_3 *minor*), 3.84 (s, 3H, OCH_3 *major*), 3.48 (t, 2H, $J_{2,3}$ = 6.8 Hz, CH_2-3 *major*), 3.45 (t, 2H, $J_{2,3}$ = 6.8 Hz, CH_2-3 *minor*), 2.60 (dt, 2H, $J_{1,2}$ = 5.8 Hz, $J_{2,3}$ = 6.6 Hz, CH_2-2 *minor*), 2.48 (dt, 2H, $J_{1,2}$ = 5.9 Hz, $J_{2,3}$ = 6.6 Hz, CH_2-2 *major*); ^{13}C NMR (125 MHz, $CDCl_3$) δ 147.3 (C-1 *minor*), 146.9 (C-1 *major*), 62.0 (OCH_3 *minor*), 61.7 (OCH_3 *major*), 48.5 (C-3 *major*), 48.1 (C-3 *minor*), 29.6 (C-2 *major*), 25.6 (C-2 *minor*); HRMS(ESI) *m/z* calcd. for $[C_4H_9N_4O]^+$: 129.0771, obsd.: 129.0779.



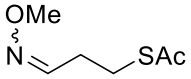
***N*-(3-Azidopropyl)-*O*-methylhydroxylamine (1)**. <u>*Procedure A (Stepwise):*</u> To a solution of 3-azidopropanal *O*-methyl oxime (934 mg, 7.29 mmol) in ethanol (15 mL), $NaCNBH_3$ (550 mg, 8.75 mmol) was added, followed by dropwise addition of 1M ethanolic HCl (8.75 mL, freshly prepared from AcCl and EtOH) and the reaction mixture was stirred at rt for 60 min. The crude mixture was concentrated and the white solid was suspended in sat. aq. Na_2CO_3 (30 mL) and extracted with CH_2Cl_2 (2 × 30 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*.

Purification of the residue by silica gel column chromatography (CH_2Cl_2/MeOH, 99/1 → 90/10, v/v) yielded oxyamine **1** as a colorless oil (787 mg, 83 %).

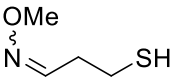
Procedure B (Without isolation of intermediates): Acetic acid (2.0 mL) was cooled to -20 °C and acrolein (0.92 mL, 13.7 mmol) was added, followed by dropwise addition of a solution of NaN_3 (1.34 g, 20.6 mmol) in water (5.2 mL) and the reaction mixture was stirred for 1 h at -20 °C. The crude mixture was quenched by addition of sat. aq. $NaHCO_3$ (10 mL) and the reaction mixture was extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic extracts were dried over $MgSO_4$ and filtered. To the solution in CH_2Cl_2, methoxyamine hydrochloride (1.37 g, 16.4 mmol) and sodium acetate (1.40 g, 17.1 mmol) were added at rt and the reaction mixture was stirred at rt for 16 h. The reaction mixture was then quenched by the addition of sat. aq. Na_2CO_3 (10 mL) and extracted with CH_2Cl_2 (2 × 10 mL). The combined organic extracts were dried over $MgSO_4$ and filtered. To the solution of crude product in CH_2Cl_2, $NaCNBH_3$ (1.03 g, 16.4 mmol) was added, followed by drop wise addition of 1M ethanolic HCl (18 mL, freshly prepared from AcCl and EtOH) and the reaction mixture was stirred at rt for 1 h. The crude mixture was concentrated and the white solid was suspended in sat. aq. Na_2CO_3 (30 mL) and extracted with CH_2Cl_2 (2 × 30 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification of the residue by silica gel column chromatography (CH_2Cl_2/MeOH, 99/1 → 90/10, v/v) yielded oxyamine **1** (1.71 g, 96 % over 3 steps) as a colorless oil. R_f = 0.15 (in EtOAc); IR (film) 2937, 2897, 2876, 2811, 2093, 1677, 1464, 1352, 1262, 1125, 1044 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 3.93 (s, 3H, OCH_3), 3.53 (t, 2H, $J_{2,3}$ = 6.6 Hz, CH_2-3), 3.45 (t, 2H, $J_{1,2}$ = 7.4 Hz, CH_2-1), 2.02 (tt, 2H, $J_{1,2}$ = $J_{2,3}$ = 6.8 Hz, CH_2-2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 61.6 (OCH_3), 48.2 (C-3), 46.6 (C-1), 22.8 (C-2); HRMS(ESI) *m/z* calcd. for $[C_4H_{11}N_4O]^+$: 131.0927, obsd.: 131.0929.



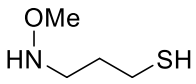
3-(Methoxyamino)propan-1-amine hydrochloride (4). To a solution of *N*-(3-Azidopropyl)-*O*-methylhydroxylamine **1** (95.6 mg, 0.74 mmol) in CH_2Cl_2:EtOH:H_2O (1:3:1), triphenylphosphine (385 mg, 1.47 mmol) was added and the reaction mixture was stirred at rt for 16 h. The crude mixture was concentrated *in vacuo*, 1M HCl (5 mL) was added and the mixture was filtered and the residue was purified by reverse phase chromatography (C_{18}, H_2O) to yield amine **4** as a white solid (102 mg, 0.73 mmol, 99 %). R_f = 0.24 (CH_2Cl_2:EtOH:MeOH:NH_3 (aq. 35%), 10:2:2:1, v/v/v/v); IR (film) 3400, 2950, 2766, 2695, 2492, 1611, 1475, 1272, 1163, 1016, 954, 758 cm^{-1}; ^1H NMR (300 MHz, D_2O) δ 3.85 (s, 3H, OCH_3), 3.40 (t, 2H, $J_{1,2}$ = 7.6 Hz, CH_2-1), 3.07 (t, 2H, $J_{2,3}$ = 7.6 Hz, CH_2-3), 2.08 (tt, 2H, $J_{1,2}$ =$J_{2,3}$ = 7.6 Hz, CH_2-2); ^{13}C NMR (125 MHz, D_2O) δ 61.5 (OCH_3), 45.9 (C-1), 36.8 (C-3), 21.7 (C-2); HRMS(ESI) *m/z* calcd. for $[C_4H_{13}N_2O]^+$: 105.1022, obsd.: 105.1023.



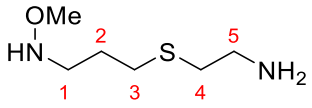
***S*-3-(Methoxyimino)propyl thioacetate (6)**. Acrolein (0.90 mL, 13.5 mmol) was added dropwise to thioacetic acid (1.04 mL, 14.8 mmol) and the reaction mixture was stirred at rt for 2 h. The crude *S*-3-oxopropyl thioacetate **5** was used in the next step. To a solution of crude *S*-3-oxopropyl thioacetate **5** (13.5 mmol) in CH_2Cl_2 (13.5 mL), methoxyamine hydrochloride (1.35 g, 16.2 mmol) and sodium acetate (2.21 g, 26.9 mmol) were added and the reaction mixture was stirred 16 h at rt. The crude mixture was then quenched with sat. aq. Na_2CO_3 (25 mL) and extracted with CH_2Cl_2 (2 × 10 mL), and the combined organic extracts were washed with brine (10 mL), dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (PE/EtOAc, 95/5 → 80/20, v/v) to give a mixture of *S*-3-(methoxyimino)propyl thioacetate **6** (2.05 g, 94 % over 2 steps) as a colorless oil. R_f = 0.69 (PE/EtOAc, 75/25, v/v); IR (film) 2991, 2961, 2939, 2901, 2820, 1689, 1634, 1466, 1428, 1355, 1282, 1132, 1104, 1085, 1038, 952, 912, 892, 858, 734, 623 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.29 (t, 1H, $J_{1,2}$ = 5.7 Hz, H-1 *major*), 6.58 (t, 1H, $J_{1,2}$ = 5.4 Hz, H-1 *minor*), 3.80 (s, 3H, OCH_3 *minor*), 3.75 (s, 3H, OCH_3 *major*), 2.97 (t, 2H, $J_{2,3}$ = 7.1 Hz, CH_2-3 *major*), 2.95 (t, 2H, $J_{2,3}$ = 7.2 Hz, CH_2-3 *minor*), 2.54 (dt, 2H, $J_{1,2}$ = 5.4 Hz, $J_{2,3}$ = 7.2 Hz, CH_2-2 *minor*), 2.41 (dt, 2H, $J_{1,2}$ = 5.7 Hz, $J_{2,3}$ = 7.1 Hz, CH_2-2 *major*), 2.28 (s, 3H, SAc *minor*), 2.27 (s, 3H, SAc *major*); ^{13}C NMR (125 MHz, $CDCl_3$) δ 195.22 (C=O *minor*), 195.19 (C=O *major*), 148.6 (C-1, C=N *minor*), 148.1 (C-1, C=N *major*), 61.7 (OCH_3 *minor*), 61.4 (OCH_3 *major*), 30.6 (SAc major + *minor*), 29.8 (C-2 *major*), 25.9 (C-3 *major*), 25.8 (C-2 *minor*), 25.6 (C-3 *minor*); HRMS(ESI) *m/z* calcd. for $[C_6H_{12}NO_2S]^+$: 162.0583, obsd.: 162.0585.



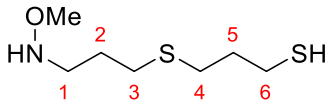
3-Mercaptopropanal *O*-methyl oxime (7). *To prevent disulfide formation all solvents used during the reaction and purification were pre-purged with argon gas.* To imine **6** (151 mg, 0.93 mmol), 1M methanolic sodium methoxide (2 mL, freshly prepared from sodium and methanol) was added at rt and the reaction mixture was stirred under argon for 30 min. The reaction mixture was then washed with sat. aq. Na_2CO_3 (10 mL) and extracted with CH_2Cl_2 (2 × 10 mL), and the combined CH_2Cl_2 solutions were dried over $MgSO_4$, filtered and concentrated *in vacuo* to give crude 3-mercaptopropanal *O*-methyl oxime **7** (107 mg, 96 %) as a colorless oil. R_f = 0.63 (PE/EtOAc, 75/25, v/v); IR (film) 3002, 2990, 2937, 2923, 2819, 1465, 1437, 1279, 1080, 1036, 916, 857, 743 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.39 (t, 1H, $J_{1\text{-}2}$ = 5.6 Hz, H-1 *major*), 6.71 (t, 1H, $J_{1\text{-}2}$ = 4.7 Hz, H-1 *minor*), 3.87 (s, 3H, OCH_3 *minor*), 3.84 (s, 3H, OCH_3 *major*), 2.88-2.46 (m, 4H, CH_2-2 *minor*, CH_2-2 *major*, CH_2-3 *minor*, CH_2-3 *major*), 1.52 (t, 1H, $J_{3\text{-}SH}$ = 8.0 Hz, SH *major*), 1.44 (t, 1H, $J_{3\text{-}SH}$ = *7.8* Hz, SH *minor*); ^{13}C NMR (125 MHz, $CDCl_3$) δ 149.0 (C-1 *minor*) 148.4 (C-1 *major*), 61.9 (OCH_3 *major*), 61.6 (OCH_3 *minor*), 33.9 (C-2 *major*), 30.0 (C-2 *major*), 21.8 (C-3 *major*), 21.4 (C-3 *minor*); HRMS(ESI) *m/z* calcd. for $[C_4H_{10}NOS]^+$: 120.0478, obsd.: 120.0483.



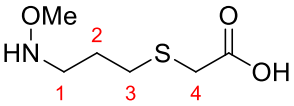
3-(Methoxyamino)propane-1-thiol (8). *To prevent disulfide formation all solvents used during the reaction and purification were pre-purged with argon gas.* To a solution of 3-mercaptopropanal *O*-methyl oxime (95 mg, 0.80 mmol) and $NaCNBH_3$ (60 mg, 0.96 mmol) in ethanol, 1M ethanolic HCl (2 mL, freshly prepared from AcCl and EtOH) was added dropwise and the reaction mixture was stirred at rt for 1 h. The crude mixture was concentrated *in vacuo*, suspended in sat. aq. Na_2CO_3 (30 mL) and extracted with CH_2Cl_2 (2 × 30 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*. 1,3-propanedithiol was added to reduce disulfides. The residue was purified by silica gel flash column chromatography (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 30/2/2/1 → 20/2/2/1, v/v/v/v) yielded 3-(methoxyamino)propane-1-thiol **8** (84 mg, 87 %) as a colorless oil. R_f = 0.65 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 25/2/2/1, v/v/v/v); IR (film) 3246, 3237, 3064, 2939, 2842, 2411, 2366, 2360, 2343, 1739, 1676, 1440, 1365, 1217, 1121, 1023 cm^{-1}; ^1H NMR (300 MHz, D_2O) δ 4.04 (s, 3H, OCH_3), 3.59 (t, 2H, $J_{1\text{-}2}$ = 7.5 Hz, CH_2-1), 2.79 (t, 2H, $J_{2\text{-}3}$ = 6.8 Hz, CH_2-3), 2.16 (p, 2H, $J_{1\text{-}2}$ = $J_{2\text{-}3}$ = 7.4 Hz, CH_2-2); ^{13}C NMR (125 MHz, D_2O) δ 64.3 (OCH_3), 50.4 (C-1), 30.2 (C-2), 23.7 (C-3); HRMS(ESI) *m/z* calcd. for $[C_4H_{12}NOS]^+$: 122.0634, obsd.: 122.0635.



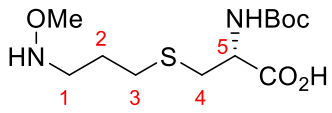
2-(3-(Methoxyamino)propylthio)ethanamine (9). To cysteamine hydrochloride (186 mg, 1.64 mmol) in ethanol (2 mL), acrolein (0.10 mL, 1.50 mmol) was added dropwise and the reaction mixture was stirred at rt for 2 h. The crude mixture was dissolved in EtOH (5 mL) and methoxyamine hydrochloride (150 mg, 1.80 mmol) and sodium acetate (246 mg, 3.00 mmol) were added and the reaction mixture was stirred at rt for 16 h. To the mixture, $NaCNBH_3$ (141 mg, 2.24 mmol) was added, followed by dropwise addition of 1M ethanolic HCl (5 mL, freshly prepared from AcCl and EtOH), and the reaction mixture was stirred at rt for 1 h. The crude mixture was concentrated and the white solid was suspended in sat. aq. Na_2CO_3 (30 mL) and extracted with CH_2Cl_2 (2 × 30 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated. Purification of the residue by silica gel column chromatography (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 20/2/2/1 → 10/2/2/1, v/v/v/v) yielded methoxyamine **9** (163 mg, 66 %) as a colorless oil. R_f = 0.56 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 25/2/2/1, v/v/v/v); IR (film) 3381, 3247, 3015, 2939, 2933, 1738, 1698, 1635, 1508, 1464, 1376, 1230, 1217, 1030 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 3.55 (s, 3H, OCH_3), 3.21 (t, 2H, $J_{4,5}$ = 6.6 Hz, CH_2-5), 3.00 (t, 2H, $J_{1,2}$ = 7.2 Hz, CH_2-1), 2.86 (t, 2H, $J_{4,5}$ = 6.6 Hz, CH_2-4), 2.65 (t, 2H, $J_{2,3}$ = 7.4 Hz, CH_2-3), 1.86 (p, 2H, $J_{1,2}$ = $J_{2,3}$ = 7.2 Hz, CH_2-2); ^{13}C NMR (125 MHz, D_2O) δ 60.5 (OCH_3), 49.1 (C-1), 39.5 (C-5), 33.3(C-3), 28.3(C-4), 26.0 (C-2); HRMS(ESI) *m/z* calcd. for $[C_6H_{12}NO_2S]^+$: 165.1056, obsd.: 165.1058.



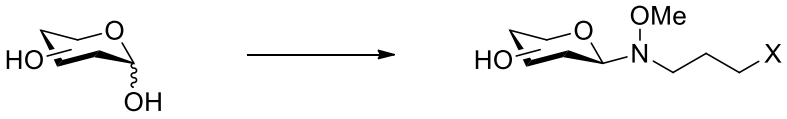
3-(3-(Methoxyamino)propylthio)propane-1-thiol (10). To neat 1,3-propanedithiol (1.50 mL, 15.0 mmol), acrolein (0.20 mL, 2.99 mmol) was added dropwise and the reaction mixture was stirred at rt for 2 h. The crude reaction mixture was diluted with EtOH (5 mL) and methoxyamine hydrochloride (300 mg, 3.59 mmol) and sodium acetate (492 mg, 6.00 mmol) were added and the reaction mixture was stirred at rt for 16 h. To the crude mixture, $NaCNBH_3$ (282 mg, 4.49 mmol) was added, followed by dropwise addition of 1M ethanolic HCl (10 mL, freshly prepared from AcCl and EtOH), and the reaction mixture was stirred at rt for 1 h. The crude mixture was concentrated *in vacuo*, suspended in sat. aq. Na_2CO_3 (30 mL) and extracted with CH_2Cl_2 (2 × 10 mL). The combined organic extracts were dried over $MgSO_4$, filtered and concentrated *in vacuo*. Purification of the residue by silica gel column chromatography (CH_2Cl_2/MeOH, 100/0 → 98/2, v/v) yielded methoxyamine **10** (442 mg, 76 %) as a colorless oil. R_f = 0.60 (PE/EtOAc, 50/50, v/v); IR (film) 3246, 3933, 2851, 2807, 1692, 1464, 1436, 1366, 1295, 1261, 1190, 1075, 1026, 958, 914, 834, 732, 647, 626 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 5.59 (bs, 1H, NH), 3.52 (s, 3H, OCH_3), 3.00 (dt, 2H, $J_{1,NH}$ = 1.0 Hz, $J_{1,2}$ = 6.9 Hz, CH_2-1), 2.66 – 2.61 (m, 4H, CH_2-4, CH_2-6), 2.59 (t, 2H, $J_{6,SH} = J_{5,6}$ = 7.1 Hz, CH_2-3), 1.88 (p, 2H, $J_{4,5} = J_{5,5}$ = 7.1 Hz, CH_2-5), 1.81 (p, 2H, $J_{1,2} = J_{2,3}$ = 7.0 Hz, CH_2-2), 1.36 (t, 1H, $J_{6,SH}$ = 8.0 Hz, SH); ^{13}C NMR (125 MHz, D_2O) δ 62.0 (OCH_3), 50.8 (C-1), 33.4 (C-5), 30.4 (C-4), 29.9 (C-3), 27.3 (C-2), 23.6 (C-6); HRMS(ESI) *m/z* calcd. for $[C_7H_{18}NOS_2]^+$: 196.0824 , obsd.: 196.0819.



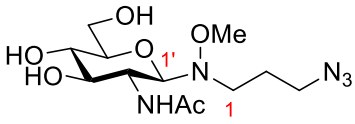
2-(3-(Methoxyamino)propylthio)acetic acid (11). To neat 2-mercaptoacetic acid (0.16 mL, 2.25 mmol), acrolein (0.10 mL, 1.50 mmol) was added drop wise at 0 °C and the reaction mixture was stirred at rt for 30 min. The crude was dissolved in ethanol (1.0 mL) and sodium acetate (246 mg, 3.00 mmol) and methoxyamine hydrochloride (250 mg, 2.99 mmol) were added and the reaction mixture was stirred at rt for 16 h. To the crude mixture, $NaCNBH_3$ (141 mg, 2.24 mmol) and 1M ethanolic HCl (7.5 mL, freshly prepared from ethanol and AcCl) were added and the reaction mixture was stirred for 1 h. The crude reaction mixture was quenched by the addition of 1M NaOH to pH 9 and the crude was concentrated *in vacuo*. Purification of the residue by silica gel column chromatography (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%, 10/2/2/1 → 5/2/2/1, v/v/v/v)) afforded amino acid **11** (214 mg, 79 %); R_f = 0.31 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 10/2/2/1, v/v/v/v); IR (film) 3128, 3039, 2939, 2807, 1664, 1562, 1440, 1401, 1298, 1223, 1139, 1043, 903, 785, 687, 587 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.04 (s, 3H, OCH_3), 3.59 (t, 2H, $J_{1,2}$ = 7.6 Hz, CH_2-1), 3.56 (s, 2H, CH_2-4), 2.88 (t, 2H, $J_{2,3}$ = 7.1 Hz, CH_2-3), 2.17 (t, 2H, $J_{1,2} = J_{2,3}$ = 7.3 Hz, CH_2-2); ^{13}C NMR (125 MHz, D_2O) δ 174.9 (C=O), 61.9 (OCH_3), 47.7 (C-1), 33.4 (C-3), 28.9 (C-4), 22.7 (C-2); HRMS(ESI) *m/z* calcd. for $[C_6H_{12}NO_3S]^-$: 178.0543, obsd.: 178.0547.



***S*-(3-(Methoxyamino)propyl)-*N*-Boc-L-cysteine (12)**. To neat *N*-Boc-Cys-OH (332 mg, 1.50 mmol), acrolein (0.1 mL, 1.50 mmol) was added dropwise and the reaction mixture was stirred at rt for 2 h. The crude reaction mixture was dissolved in a mixture of CH_2Cl_2 (1 mL) and EtOH (1 mL), methoxyamine hydrochloride (250 mg, 1.8 mmol) and sodium acetate (246 mg, 3.0 mmol) were added and the reaction mixture was stirred at rt for 16 h. To the crude mixture, $NaCNBH_3$ (141 mg, 2.24 mmol) was added, followed by dropwise addition of 1M ethanolic HCl (7.5 mL, freshly prepared from AcCl and EtOH), and the reaction mixture was stirred at rt for 60 min. The crude reaction mixture was quenched by the addition of 1M NaOH to pH 9 and the crude was concentrated *in vacuo*. Purification of the residue by silica gel column chromatography (CH_2Cl_2/MeOH, 95/5 → 80/20, v/v) yielded methoxyamine **12** (236 mg, 51 %) as a colorless oil. R_f = 0.15 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 10/2/2/1, v/v/v/v); $\alpha_D^{20.1}$ = -7.5 (c = 1, MeOH); IR (film) 3335, 2976, 2934, 1687, 1584, 1509, 1416, 1394, 1367, 1249, 1167, 1051, 1025, 868, 777, 659, 578 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 4.22 – 4.17 (m, 1H, H-5), 3.50 (s, 3H, OCH_3), 3.01 (dd, 1H, $J_{4a,5}$ = 4.4 Hz, $J_{4a,4b}$ = 13.7 Hz, H-4a), 2.95 (t, 2H, $J_{1,2}$ = 7.0 Hz, CH_2-1), 2.84 (dd, 1H, $J_{4b,5}$ = 7.3 Hz, $J_{4a,4b}$ = 13.7 Hz, H-4b), 2.64 (t, 2H, $J_{2,3}$ = 7.2 Hz, CH_2-3), 1.79 (p, 2H, $J_{1,2}$ = $J_{2,3}$ = 7.1 Hz, CH_2-2), 1.45 (s, 9H, CH_3 *t*Bu); ^{13}C NMR (125 MHz, CD_3OD) δ 177.7 (COOH), 157.7 (C=ONH), 80.5 (Cq), 61.6 (OCH_3), 56.1 (C-5 αCH), 50.8 (C-1), 35.5 (C-4), 30.9 (C-3), 28.8 (CH_3 *t*Bu), 27.7 (C-2); HRMS(ESI) *m/z* calcd. for $[C_{12}H_{25}N_2O_5S]^+$: 309.1502, obsd.: 309.1479.



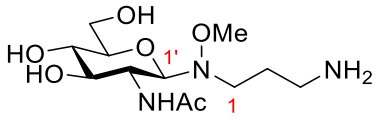
General procedure for the chemical ligation of methoxyamine linkers with carbohydrates. To a solution of carbohydrate (0.15 mmol) in an AcOH/NH_4OAc buffer (1.5 mL, 2M, freshly prepared, pH 4.5), methoxyamine linker (1.50 mmol) was added and the reaction mixture was stirred at rt for 24 h at the indicated temperature. The crude mixture was then directly loaded onto a size exclusion column (Bio-Gel P-2, 1200 × 18 mm) and eluted with a 0.1 M aq. NH_4HCO_3 solution. Lyophilisation of the product fractions afforded the neoglycoside. Alternatively, the neoglycoside can be purified by directly loading the crude reaction mixture onto a reverse phase column (C_{18}) and/or by purification by silica gel column chromatography to afford the neoglycoside**.**



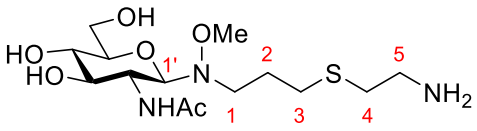
***N*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-*N*-(3-azidopropyl)-*O*-methylhydroxylamine (14).** *N*-Acetyl-glucosamine (34.0 mg, 0.15 mmol) and 3-azido-1-

methoxyaminopropane **1** (195 mg, 1.50 mmol) were reacted in an AcOH/NH_4OAc buffer (1.5 mL, 2M, freshly prepared, pH 4.5) at rt for 24 h. Purification by reverse phase chromatography (C_{18}, H_2O/MeOH, 100/0 → 60/40, v/v) afforded neoglycoside **14** (44.6 mg, 87 %) as a white solid. R_f = 0.64 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 35%), 10/2/2/1, v/v/v/v); $\alpha_D^{20.3}$ = -19 (c = 0.1, MeOH); IR (film) 3369, 3296, 3253, 3003, 2953, 2943, 2889, 2099, 2067, 1735, 1647, 1605, 1575, 1567, 1487, 1446, 1419, 1377, 1349, 1308, 1253, 1079, 1020, 828 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.33 (d,1H, $J_{1',2'}$ = 9.8 Hz, H-1′), 3.89 (dd, 1H, $J_{1',2'} = J_{2',3'}$ = 9.6 Hz, H-2′), 3.88 (dd, 1H, $J_{5',6'}$ = 1.5 Hz, $J_{6a',6b'}$ = 12.3 Hz, H-6a′), 3.73 (dd, 1H, $J_{5',6'}$ = 5.3 Hz, $J_{6a',6b'}$ = 12.3 Hz, H-6b′), 3.51 (dd, 1H, $J_{2',3'} = J_{3',4'}$ = 8.7 Hz, H-3′), 3.50 (s, 3H, OCH_3) 3.46 – 3.34 (m, 4H, H-4′, H-5′, CH_2-3), 3.09 – 2.98 (m, 2H, CH_2-1), 2.04 (s, 3H, CH_3 Ac), 1.88 – 1.74 (m, 2H, CH_2-2); ^{13}C NMR (125 MHz, D_2O) 173.9 (C=O), 90.0 (C-1′), 77.4 (C-5′), 75.5 (C-3′), 69.6 (C-4′), 61.0 (OCH_3), 60.8 (C-6′), 52.3 (C-2′), 49.0 (C-3), 48.7 (C-1), 25.7 (C-2), 22.1 (CH_3 Ac); HRMS(ESI) *m/z* for $[C_{12}H_{24}N_5O_6]^+$ calcd.: 334.1721, obsd.: 334.1719.

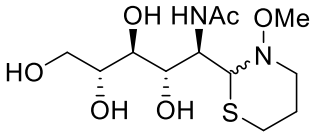
Hydrolytic stability experiment. To a solution of neoglycoside **14** (8.0 mg) in D_2O (0.5 mL), NaH_2PO_4 and Na_2HPO_4 were added, resulting in a 100 mM solution with the pH of 4.75, 5, 6, 7 or 9. The decay was measured over time, using ^1H-NMR analysis, in which the intergral of CH_2-1 was measured against the integral of CH-1'. As described in section 2.2.2.1, a line was fitted to the data according to the least-square method, and the half-lives were calculated based on the fitted line.



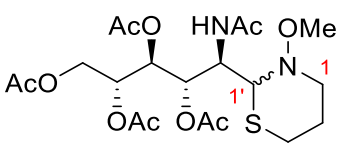
***N*-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-*N*-(3-aminopropyl)-*O*-methylhydroxylamine (15)**. *N*-Acetyl-glucosamine (34.0 mg, 0.15 mmol) and 3-(methoxyamino)propan-1-amine hydrochloride **4** (211 mg, 1.50 mmol) were reacted in an AcOH/NH_4OAc buffer (1.5 mL, 2M, freshly prepared, pH 4.5) at rt for 24 h. Purification by size exclusion chromatography (Bio-Gel P-2) afforded neoglycoside **15** (28.3 mg, 0.092 mmol, 81 %). R_f = 0.16 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 35%), 5/2/2/1, v/v/v/v); $\alpha_D^{20.3}$ = -21 (c = 0.1, MeOH); IR (film) 3401, 3375, 3322, 3311, 3291, 3278, 2938, 2885, 2727, 2058, 1651, 1567, 1490, 1459, 1437, 1376, 1315, 1257, 1108, 1023, 633 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.41 (d, 1H, $J_{1',2'}$ = 9.9 Hz, H-1′), 3.92 (dd, 1H, $J_{5',6'}$ = 0.9 Hz, $J_{6a',6b'}$ = 12.5 Hz, H-6a′), 3.85 (dd, 1H, $J_{1',2'} = J_{2',3'}$ = 9.8 Hz, H -2′), 3.75 (dd, 1H, $J_{5',6'}$ = 4.9 Hz, $J_{6a',6b'}$ = 12.5 Hz, H-6b′), 3.55 (dd, 1H, $J_{2',3'} = J_{3',4'}$ = 9.1 Hz, H-3′), 3.52 (s, 3H, OCH_3), 3.45 – 3.40 (m, 2H, H-4′, H-5′), 3.12 – 3.01 (m, 4H, CH_2-1, CH_2-3), 2.06 (s, 3H, CH_3 Ac), 1.93 (p, 2H, $J_{1,2} = J_{2,3}$ = 7.1 Hz, CH_2-2); ^{13}C NMR (125 MHz, D_2O) δ 174.1 (C=O Ac), 90.5 (C-1′), 77.4 (C-5′), 75.2 (C-3′), 69.6 (C-4′), 61.1 (OCH_3), 60.8 (C-6′), 52.3 (C-2′), 47.9 (C-1), 37.6 (C-3), 24.5 (C-2), 22.1 (CH_3 Ac); HRMS(ESI) *m/z* calcd. for $[C_{12}H_{26}N_3O_6]^+$: 308.1816, obsd.: 308.1820.



2-(3-[Methoxy(2-acetamido-2-deoxy-β-D-glucopyranosyl)amino] propylthio)ethan-1-amine (16). *N*-Acetylglucosamine (12.0 mg, 54.2 µmol) and oxyamine **8** (89.1 mg, 0.54 mmol) were reacted in an AcOH/NH_4OAc buffer (1.5 mL, 2M, freshly prepared, pH 4.5) at rt for 72 h. Purification with reverse phase chromatography (C_{18}, H_2O/MeOH, 100/0 → 90/10, v/v), followed by silica gel column flash chromatography (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 10/2/2/1 → 5/2/2/1, v/v/v/v) afforded neoglycoside **16** (16.1 mg, 81 %). R_f = 0.53 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33 %), 5/2/2/1, v/v/v/v); $\alpha_D^{20.3}$ = -25 (c = 0.1, MeOH); IR (film) 3287, 2938, 2056, 1690, 1650, 1559, 1434, 1375, 1316, 1131, 1080, 1034, 947, 632 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.34 (d, 1H, $J_{1',2'}$ = 9.7 Hz, H-1′), 3.93 – 3.85 (m, 2H, H-2′, H-6a′), 3.89 (t, 1H, $J_{1',2'} = J_{2',3'}$ = 9.7 Hz, H-2′), 3.73 (dd, 1H, $J_{5',6'}$ = 5.3 Hz, $J_{6a',6b'}$ = 12.4 Hz, H-6b′), 3.56 – 3.47 (m, 4H, H-2′, OCH_3), 3.46 – 3.37 (m, 2H, H-4′, H-5′), 3.07 – 3.00 (m, 2H, CH_2-1), 2.85 (t, 2H, $J_{4,5}$ = 6.6 Hz, CH_2-5), 2.71 – 2.54 (m, 4H, CH_2-3, CH_2-4), 2.04 (s, 3H, CH_3 Ac), 1.81 (m, 2H, CH_2-2). ^{13}C NMR (125 MHz, D_2O) δ 173.9 (C=O), 90.1 (C-1′), 77.5 (C-5′), 75.5 (C-3′), 69.7 (C-4′), 61.1 (OCH_3), 60.9 (C-6′), 52.3 (C-2′), 50.3 (C-1), 39.4 (C-5), 32.9 (C-3), 28.4 (C-4), 26.4 (C-2), 22.2 (CH_3 Ac); HRMS(ESI) *m/z* calcd. for $[C_{14}H_{30}N_3O_6S]^+$: 368.1850, obsd.: 368.1864.

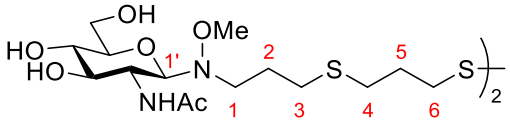


D-gluco-2-(1-Acetamido-2,3,4,5-tetrahydroxy-pentyl)-3-methoxy-1,3-thiazinane (18). *N*-Acetylglucosamine (34 mg, 0.15 mmol) and 3-(methoxyamino)propane-1-thiol **8** (182 mg, 1.50 mmol) were reacted in an AcOH/NH_4OAc buffer (1.5 mL, 2M, freshly prepared, pH 4.5) containing 5% TCEP at rt for 72 h. Purification with reverse phase chromatography (C_{18}, H_2O/MeOH, 100/0 → 60/40, v/v) afforded thiazinane **18** (44 mg, 89 %) as an colourless oil. R_f = 0.32 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 10/2/2/1, v/v/v/v); IR (film) 3298, 2940, 1646, 1545, 1428, 1374, 1318, 1284, 1190, 1079, 1037, 951, 867, 844, 694, 632 cm^{-1}; ^1H NMR (300 MHz, D_2O, 50 °C) δ 4.85 – 4.62 (m, 2H), 4.63 – 4.38 (m, 2H), 4.12 – 3.72 (m, 10H), 3.35 – 2.91 (m, 4H), 2.40 – 2.13 (m, 5H), 2.11 – 1.77 (m, 1H); ^{13}C NMR (150 MHz, D_2O) δ 174.1, 173.8, 72.2, 71.3, 69.3, 68.4, 68.0, 62.6, 62.3, 58.6, 58.0 53.5, 50.6, 27.2, 22.1, 22.0, 17.8; HRMS(ESI) *m/z* calcd. for $[C_{12}H_{25}N_2O_6S]^+$: 325.1428, obsd.: 325.1426.



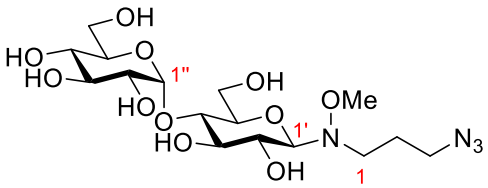
D-gluco-2-(1-Acetamido-2,3,4,5-tetra-acetoxy-pentyl)-3-methoxy-1,3-thiazinane (19). The mixture of thiazinanes **18** (12.0 mg, 37.0 µmol) was co-evaporated with pyridine:acetic

anhydride (3 mL, 2/1, v/v) and dissolved in acetic anhydride (2 mL) and pyridine (1 mL) and stirred overnight. The crude reaction mixture was concentrated and purified by silica gel column chromatography (PE/EtOAc, 95/5 → 80/20, v/v) to give a diastereomeric thioaminals **19** (17.8 mg, 98%) as a colorless oil. R_f = 0.38 (EtOAc); IR (film) 2951, 2847, 2816, 1742, 1668, 1514, 1429, 1370, 1313, 1211, 1033, 953, 916, 859, 845, 795, 729, 646, 633 cm^{-1}; ^1H NMR (300 MHz, D_2O, 50 °C) δ 6.06 (d, 1H, $J_{NH,2'}$ = 9.8 Hz, NH *isomer A*), 5.64 (d, 1H, $J_{NH,2'}$ = 10.1 Hz, NH *isomer B*), 5.50 (dd, 1H, $J_{3',4'}$ = 2.0 Hz, $J_{2',3'}$ = 9.4 Hz, H-3′ *isomer A*), 5.43 (dd, 1H, $J_{3',4'}$ = 2.0 Hz, $J_{2',3'}$ = 8.6 Hz, H-3′ *isomer B*), 5.43 – 5.37 (m, 2H, H-4′ *isomer A+B*), 5.23 – 5.13 (m, 2H, H-5′ *isomer A+B*), 4.84 (dt, 1H, $J_{1',2'}$ = $J_{2',3'}$ = 5.5 Hz, $J_{2',NH}$ = 10.0 Hz, H-2′ *isomer B*), 4.53 (bd, 1H, $J_{1',2'}$ = 4.5 Hz, $^1J_{CH}$ = *147 Hz*, H-1′ *isomer A*), 4.40 (dt, 1H, $J_{1',2'}$ = $J_{2',3'}$ = 4.9 Hz, $J_{2',NH}$ = 9.7 Hz, H-2′ *isomer 7A*), 4.28 (dd, 1H, $J_{5',6a'}$ = 3.2 Hz, $J_{6a',6b'}$ = 12.4 Hz, H-6a′ *isomer B*), 4.22 (dd, 1H, $J_{5',6a'}$ = 2.9 Hz, $J_{6a',6b'}$ = 12.5 Hz, H-6b′ *isomer A*), 4.15 – 4.07 (m, 2H, H-6b′ *isomer A+B*), 3.90 (bd, 1H, $J_{1',2'}$ = 5.2 Hz, $^1J_{CH}$ = *149 Hz*, H-1′ *isomer B*), 3.68 (s, 3H, OCH_3 *isomer A*), 3.63 – 3.52 (m, 1H, H-1_{eq} *isomer A*), 3.49 (s, 3H, OCH_3 *isomer B*), 3.39 (dt, $J_{1eq,2eq}$ = $J_{1eq,2ax}$ = 4.3 Hz, $J_{1eq,1ax}$ = 13.3 Hz, H-1_{eq} *isomer B*), 2.95 (dt, 1H, $J_{2eq,3ax}$ = 1.7 Hz, $J_{2ax,3ax}$ = $J_{3eq,3ax}$ = 13.0 Hz, H-3_{ax} *isomer B*), 2.83 (ddd, 1H, $J_{1ax,2eq}$ = 2.9 Hz, $J_{1ax,2ax}$ = 13.0 Hz, $J_{1ax,-1eq}$ = 15.5 Hz, H-1_{ax} *isomer B*), 2.75 – 2.57 (m, 3H, H-3_{eq} *isomer B*, H-1_{ax} *isomer A*, H-3_{ax} *isomer A*, H-3_{eq} *isomer A)*, 2.18 (s, 3H, CH_3 Ac), 2.10 (s, 3H, CH_3 Ac), 2.04 (s, 3H, CH_3 Ac), 2.03 (s, 3H, CH_3 Ac), 2.02 (s, 3H, CH_3 Ac), 2.00 (s, 3H, CH_3 Ac), 1.97 – 1.84 (m, 5H, CH_3 Ac, H-2_{eq}, H-2_{ax} *isomer A*), 1.49 (bd, 1H, H-2_{eq} *isomer B*); ^{13}C NMR (150 MHz, D_2O) δ 171.2 (C=O), 170.8 (C=O), 170.53 (C=O), 170.46 (C=O), 170.3 (C=O), 170.2 (C=O), 169.9 (C=O), 169.8 (2 × C=O), 169.6 (C=O), 70.9, 70.5, 69.9, 69.4 (C-3′, C-4′ *isomer A+B*) 69.0 (C-5′ *isomer A+B*), 68.6 (C-1′ *isomer B*), 67.6 (C-1′ *isomer A*), 62.3 (C-6′ *isomer A/B*), 62.1 (C-6′ *isomer A/B*), 59.8 (OCH_3 *isomer B*), 58.2 (OCH_3 *isomer A*), 52.9 (CH_2NO *isomer B*), 51.6 (CH_2NO *isomer A*), 51.5 (C-2′ *isomer A*), 49.5 (C-2′ *isomer B*), 28.1 (CH_2S *isomer B*), 26.8 (CH_2S *isomer A*), 23.4 (C-CH_2-C *isomer A/b)*, 23.2 (C-CH_2-C *isomer A/b*), 20.9, 20.8, 20.7 (CH_3 Ac); TOCSY NMR (300 MHz, D_2O, 50 °C) *Isomer a* δ 3.59 (excited, app. bd, $J_{1ax,1eq}$ = 14.6 Hz, H-1eq), 2.84 (app. bt, $J_{1ax,1eq}$ = $J_{1ax,2ax}$ = 13.1 Hz, H-1ax), 2.13 (app. bq, $J_{1ax,2ax}$ = $J_{2ax,2eq}$ = $J_{2ax\text{-}3ax}$ = 13.0 Hz, H-2 ax), 1.50 (app. bd, $J_{2eq,2ax}$ = 14.3 Hz, H-2eq), 2.97 (app. bt, $J_{2ax,3ax}$ = $J_{3ax,3eq}$ = 13.0 Hz, H-3ax), 2.64 (app. bd, $J_{3eq,3ax}$ = 13.3 Hz, H-3eq); HRMS(ESI) *m/z* calcd. for $[C_{20}H_{33}N_2O_{10}S]^+$: 493.1850, obsd.: 493.1856.

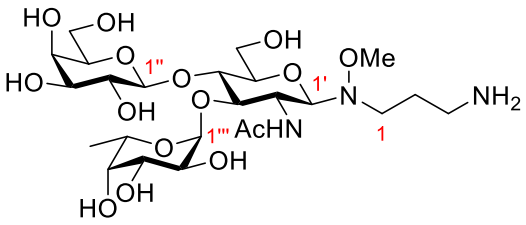


1,2-Bis(3-[3-(methoxy[2-acetamido-2-deoxy-β-D-glucopyranosyl]amino) propylthio]propyl)-disulfane (20). *N*-Acetylglucosamine (7.8 mg, 35.3 µmol) and 3-(3-(methoxyamino)propylthio)propane-1-thiol **10** (69.0 mg, 0.35 mmol) were reacted a mixture of AcOH/NH_4OAc buffer (0.2 mL, 2M, freshly prepared, pH 4.5), ethanol (0.2 mL) and CH_2Cl_2 (0.1 mL) at 40 °C for 72 h. The crude reaction mixture was directly loaded on a

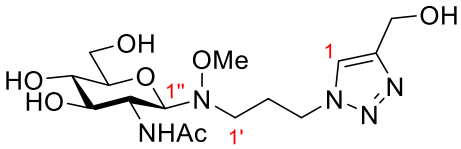
size exclusion column (Bio-Gel P-2, 600 × 10 mm) and eluted with 0.1 M aq. NH_4HCO_3. Lyophilisation of the product fractions afforded disulfide **20** (9.0 mg, 64%) as a colorless oil. R_f (Sulfhydryl) = 0.34 (10/2/2/10 CH_2Cl_2/EtOH/MeOH/NH_3(aq, 33 %)), R_f = (disulfide) = 0.17 (10/2/2/1 CH_2Cl_2/EtOH/MeOH/NH_3(aq, 33 %)); $\alpha_D^{19.8}$ = +3.5 (c = 0.1, MeOH); IR (film) 3313, 3273, 3094, 3070, 2656, 2909, 1632, 1557, 1487, 1439, 1355, 1215, 1195, 1111, 1038, 1000, 986, 903, 755, 721, 691, 620, 604 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.34 (d, 2H, $J_{1',2'}$ = 9.8 Hz, H-1′), 3.92 – 3.86 (m, 4H, H-6a′, H-2′), 3.74 (dd, 2H, $J_{5',6b'}$ = 5.4 Hz, $J_{6a',6b'}$ = 12.4 Hz, H-6b′), 3.55 – 3.46 (m, 8H, OCH_3, H-3′), 3.44 – 3.36 (m, 4H, H-4′, H-5′), 3.10 – 3.00 (m, 4H, CH_2-1), 2.85 (t, 4H, $J_{5,6}$ = 7.1 Hz, CH_2-6), 2.70 (t, 4H, $J_{4,5}$ = 7.1 Hz, CH_2-4), 2.68 – 2.57 (m, 4H, CH_2-3), 2.04 (s, 6H, CH_3 *N*Ac), 2.00 (p, 4H, $J_{4,5}$ = $J_{5,6}$ = 7.0 Hz, CH_2-5), 1.86 – 1.78 (m, 4H, CH_2-2); ^{13}C NMR (125 MHz, D_2O) δ 173.8 (C=O), 90.0 (C-1′), 77.4 (C-5′), 75.5 (C-3′), 69.6 (C-4′), 61.1 (OCH_3), 60.8 (C-6′), 52.3 (C-2′), 50.3 (C-1), 36.5 (C-6), 29.4 (C-4), 28.6 (C-3), 27.9 (C-5), 26.3 (C-2), 22.1 (CH_3 *N*Ac); HRMS(ESI) *m/z* calcd. for $[C_{30}H_{59}N_4O_{12}S_4]^+$: 795.3007 , obsd.: 795.3018; HRMS(ESI) *m/z* calcd. for $[C_{15}H_{31}N_2O_6S_2]^+$: 399.1618, obsd.: 399.1609.



***N*-(4-*O*-(α-D-glucopyranosyl)-β-D-glucopyranosyl)-*N*-(3-azidopropyl)-*O*-methylhydroxylamine (22)**. To a solution of maltose **21** (23 mg, 67 µmol) in a AcOH/$AcONH_4$ buffer (0.5 mL, 2M, freshly prepared, pH 4.5), 3-azido-propan-1-methoxyamine **1** (88 mg, 0.067 mmol) was added and the reaction mixture was stirred at room temperature for 24h. The rude mixture was directly loaded on a size exclusion column (BioRad, P2) and eluted with 0.1 M aq. NH_4HCO_3. Lyophilisation of the product fraction afforded neoglycoside **22** (28.8 mg, 94%) as a white foam. R_f = 0.54 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 35%), 10/2/2/1, v/v/v/v); $\alpha_D^{17.6}$ = +24 (c = 1, MeOH); IR (film) 3349, 2931, 2098, 1568, 1439, 1355, 1262, 1145, 1075, 1025, 906, 858, 783, 592, 528 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 5.41 (d, 1H, $J_{1'',2''}$ = 3.9 Hz, H-1″), 4.19 (d, 1H, $J_{1',2'}$ = 9.2 Hz, H-1′), 3.91 (dd, 1H, $J_{5',6'}$ = 1.8 Hz, $J_{6a',6b'}$ = 12.1 Hz, H-6a′), 3.85 (dd, 1H, $J_{5'',6a''}$ = 1.9 Hz, $J_{6a'',6b''}$ = 12.2 Hz, H-6a″), 3.81 – 3.64 (m, 5H, H-5′, H-6b′, H-6b″, H-3″, H-5″), 3.62 (s, 3H, OCH_3), 3.61 – 3.38 (m, 7H, H-4′, H-2′, H-2″, H-3′, H-3a, H-3b, H-4″), 3.19 – 3.11 (m, 1H, H-1a), 3.00 – 2.94 (m, 1H, H-1b), 1.94 – 1.85 (m, 2H, H-2); ^{13}C NMR (125 MHz, D_2O) δ 99.5 (C-1″), 91.9 (C-1′), 77.6 (C-5′), 76.5 (C-4′), 76.0 (C-3′), 72.8, 72.6 (C-3″, C-5″), 71.6 (C-2″), 69.6 (C-2′), 69.3 (C-4″), 62.0 (OCH_3), 60.8 (C-6′), 60.4 (C-6″), 49.9 (C-1), 49.1 (C-3), 25.9 (C-2); HRMS(ESI) *m/z* calcd. for $[C_{16}H_{31}N_4O_{11}]^+$: 455.1984, obsd.: 455.1986.

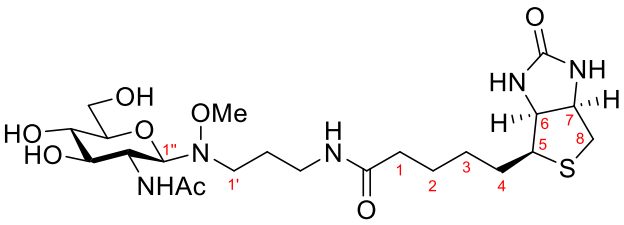


***N*-(2-Acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-4-*O*-(β-D-galacto-pyranosyl)-β-D-glucopyranosyl)-*N*-(3-aminopropyl)-*O*-methylhydroxylamine (24)**. To a solution of Lewis^X **23** (5.6 mg, 10.6 µmol) in a AcOH/NH_4OAc buffer (0.5 mL, 2M, freshly prepared, pH 4.5), 3-(methoxyamino)propan-1-amine hydrochloride **4** (15.9 mg, 113.2 µmol) was added and the reaction mixture was stirred at 40 °C for 35 h. The crude mixture was directly loaded on a size exclusion column (Bio-Gel P-2, 1200 × 18 mm) and eluted with 0.1 M aq. NH_4HCO_3. Lyophilisation of the product fractions afforded neoglycoside **24** (5.7 mg, 88%). R_f = 0.10 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 35%), 5/2/2/1, v/v/v/v); $\alpha_D^{19.5}$ = -9.3 (c = 0.2, MeOH); IR (film) 3341, 2925, 2852, 17117, 1647, 1586, 1466, 1451, 1415, 1380, 1350, 1302, 1233, 1193, 1085, 1026, 968, 917 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ 5.12 (d, 1H, $J_{1''',2'''}$ = 3.9 Hz, H-1'''), 4.84 (q, 1H, $J_{5''',6'''}$ = 6.7 Hz, H-5'''), 4.49 – 4.42 (m, 1H, H-1′, H-1″), 4.05 (m, 2H, H-2′, H-6a′), 3.93 – 3.81 (m, 5H, H-3‴, H-4″, H-4′, H-3′, H-6b′), 3.80 (d, 1H, $J_{3''',4'''}$ = $J_{4''',5'''}$ = 2.9 Hz, H-4‴), 3.76 (m, 3H, H-6a″, H-6b″, H-2‴), 3.64 (dd, 1H, $J_{3'',4''}$ = 3.2 Hz, $J_{2'',3''}$ = 9.9 Hz, H-3″), 3.60 – 3.55 (m, 1H, H-5″), 3.54 – 3.46 (m, 1H, H-5″), 3.54 – 3.46 (m, 5H, OCH_3, H-5′, H-2″), 3.11 – 2.92 (m, 4H, CH_2-1, CH_2-3), 2.02 (s, 3H, CH_3 Ac), 1.95 (m, 2H, CH_2-2), 1.17 (d, 3H, $J_{5''',6'''}$ = 6.7 Hz, H-6‴); ^{13}C NMR (125 MHz, D_2O) 170.5 (C=O), 101.8 (C-1″), 98.7 (C-1‴), 90.5 (C-1′), 76.9 (C-5′), 76.1 (C-3′), 74.8 (C-5″), 73.2 (C-4′), 72.4 (C-3″), 71.8 (C-4‴), 70.9 (C-2″), 69.1 (C-3‴), 68.3 (C-4″), 67.6 (C-2‴), 66.7 (C-5‴), 61.4 (C-6″), 60.9 (OCH_3), 59.7 (C-6′), 52.5 (C-2′), 47.2 (C-1), 37.5 (C-3), 24.5 (C-2), 22.1 (CH_3 Ac), 15.2 (C-6‴); HRMS(ESI) *m/z* calcd. for $[C_{24}H_{46}N_3O_{15}]^+$: 616.2923, obsd.: 616.2938.

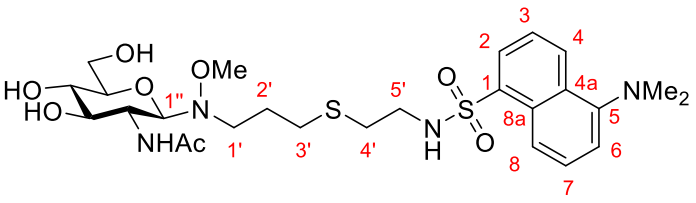


(1-(3-(Methoxy(2-acetamido-2-deoxy-b-D-glucosyl)amino)propyl)-1H-1,2,3-triazol-4-yl)methanol (25). To a solution of azide **14** (11.8 mg, 35 µmol) and propagyl alcohol (0.1 mL) in ethanol (1 mL), a solution of L-ascorbic acid (6.2 mg, 35 µmol) and $CuSO_4$ (1 mg) were added and the reaction mixture was stirred for 3 d at rt. The crude mixture was filtered and washed with H_2O (2 mL) and concentrated *in vacuo*. Purification with reverse phase chromatography (C_{18}, H_2O/MeOH, 100/0 → 90/10, v/v) yielded triazole **25** (10.7 mg, 79%) as a colourless oil. R_f = 0.53 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 5/2/2/1, v/v/v/v); α_D^{19} = -0.82 (c = 0.1, MeOH); IR (film) 3350, 2934, 2874, 1643, 1555, 1442, 1378, 1319, 1227, 1104, 1054, 1033, 1021 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD) δ 7.99 (s, 1H, triazole), 4.73 (s, 2H, C=C-CH_2), 4.58 – 4.45 (m, 2H, CH_2 H-3), 4.33 (d, 1H, $J_{1',2'}$ = 9.8 Hz, H-1′), 3.91 – 3.85 (m, 2H, H-2′, H-6a′), 3.73 (dd, 1H, $J_{5',6b'}$ = 5.4 Hz, $J_{6a',6b'}$ = 12.4 Hz, H-6b′), 3.53 (dd, 1H, $J_{2',3'}$ = 8.7 Hz, $J_{3',4'}$ = 9.7 Hz, H-3′), 3.50 (s, 3H, OCH_3), 3.42 (t, 1H, $J_{2',3'}$ = $J_{4',5'}$ = 8.7 Hz, H-4′), 3.40 – 3.35 (m, 1H, H-5′), 3.03-2.89 (m, 2H, H-1), 2.24 –

2.10 (m, 2H, H-2), 2.06 (s, 3H, CH_3 Ac); ^{13}C NMR (125 MHz, CD_3OD) δ 173.9 (C=O Ac), 146.7 (Cq C=C), 124.0 (CH=C), 90.1 (C-1″), 77.4 (C-5″), 75.4 (C-3″), 69.6 (C-4″), 61.2 (OCH_3), 60.8 (C-6″), 54.5 (CH_2 allylic), 52.3 (C-2″), 48.4 (C-1′), 48.2 (C-3′), 27.0 (C-2′), 22.1 (CH_3 Ac); HRMS(ESI) *m/z* calcd. for $[C_{15}H_{28}N_5O_7]^+$: 390.1983, obsd.: 390.1986.

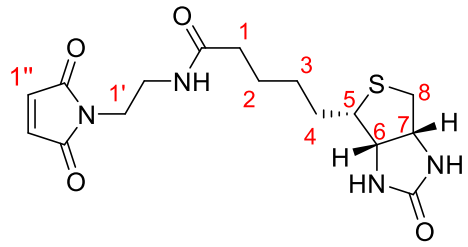


***N*-(3-(methoxy[2-acetamido-2-deoxy-β-D-glucopyranosyl]-amino)propyl)-D-biotinamide (26).** To a solution of amine **15** (12.0 mg, 39.1 µmol) in DMF (0.2 mL), D-biotin (13.0 mg, 53.7 µmol), Et_3N (12 µL) and HBTU (30.0 mg, 79 µmol) were added and the reaction mixture was stirred for 2 h. at rt. To the reaction mixture, H_2O (2 mL) was added and the white participate was filtered and washed with water (2 × 1 mL). The liquid residue was then purified by reverse phase chromatography (elute in H_2O), concentrated *in vacuo*, followed by silica gel flash chromatograpy (CH_2Cl_2/EtOH/MeOH/NH_3(aq. 33%), 15/2/2/1 → 10/2/2/1, v/v/v/v) to afford glyconjugate **26** (17.6 mg, 84 %) as a colourless oil. R_f = 0.58 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 5/2/2/1, v/v/v/v); $\alpha_D^{20.0}$ = +3.1 (c = 0.2, MeOH); IR (film) 3343, 2927, 2857, 1671, 1652, 1637, 1559, 1542, 1457, 1437, 1395, 1375, 1317, 1266, 1110, 1078, 1053, 1033 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ 4.58 (dd, 1H, $J_{7,8}$ = 4.7 Hz, $J_{6,7}$ = 7.9 Hz, H-7), 4.39 (dd, 1H, $J_{5,6}$ = 4.5 Hz, $J_{6,7}$ = 7.9 Hz, H-7), 4.30 (d, 1H, $J_{1'',2''}$ = 9.8 Hz, H-1″), 3.90 – 3.84 (m, 2H, H-6a″, H-2″), 3.71 (dd, 1H, $J_{5'',6''}$ = 5.4 Hz, $J_{6a'',6b''}$ = 12.5 Hz, H-6b″), 3.49 (t, 1H, $J_{3'',4''}$ = $J_{4'',5''}$ = 8.9 Hz, H-3″), 3.47 (s, 3H, OCH_3), 3.42 – 3.34 (m, 2H, H-4″, H-5″), 3.34 – 3.28 (m, 1H, H-5), 3.28 – 3.22 (m, 1H, H-3a′), 3.22 – 3.15 (m, 1H, H-3b′), 3.03 (m, 3H, CH_2-1′, H-8a), 2.75 (d, 1H, $J_{8a,8b}$ = 13.1 Hz, H-8b), 2.23 (t, 2H, $J_{1,2}$ = 7.2 Hz, CH_2-1), 2.02 (s, 3H, CH_3 *NAc*), 1.78 – 1.50 (m, 6H, CH_2-2′, CH_2-2, CH_2-4), 1.44 – 1.34 (m, 2H, CH_2-3); ^{13}C NMR (125 MHz, D_2O) δ 176.6 (C=O amide), 174.0 (C=O acetamide), 165.3 (C=O carbamide), 90.0 (C-1″), 77.3 (C-5″), 75.5 (C-3″), 69.6 (C-4″), 62.0 (C-6), 61.0 (OCH_3), 60.8 (C-6″), 60.1 (C-7), 55.3 (C-5), 52.2 (C-2″), 48.7 (C-1′), 39.6 (C-8), 37.0 (C-3′), 35.4 (C-1), 27.7 (C-3), 27.6 (C-4), 25.9 (C-2′), 25.0 (C-2), 22.1 (CH_3 Ac); HRMS(ESI) *m/z* calcd. for $[C_{22}H_{39}N_5O_8SNa]^+$: 556.2414, obsd.: 556.2416.



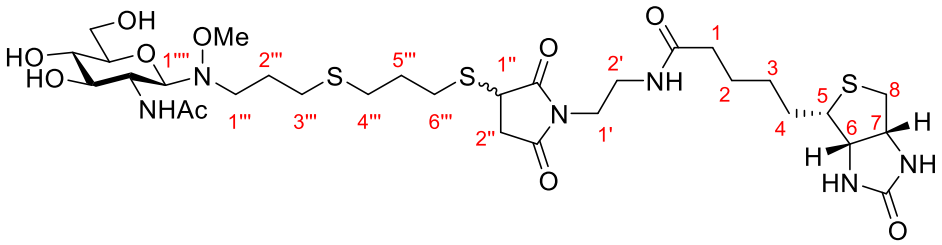
5-(dimethylamino)-*N*-(2-((3-(methoxy(2-acetamido-2-deoxy-β-D-gluco-pyranosyl)amino)-propyl)-thio)ethyl)naphthalene-1-sulfonamide (28). To a solution of amine **16** (10.9 mg, 29.7 µmol) in H_2O (2 mL), $NaHCO_3$ (15 mg, 179 µmol) was added. Dansylchloride (12.0 mg, 44.5 µmol) was dissolved in distilled THF (1 mL) and added drop wise to the reaction mixture. After 2 hours the THF was concentrated

in vacuo and the remaining aqueous reaction mixture was purified by reverse phase chromatography (C_{18}, H_2O/MeOH, 100/0 → 25/75, v/v) to give fluorescent glycoside **28** (13.8 mg, 77%); R_f = 0.50 (CH_2Cl_2/MeOH, 8/1, v/v); $\alpha_D^{17.8}$ = -14.4 (c = 0.5, MeOH); IR (film) 3304, 3283, 3088, 2939, 2795, 1650, 1572, 1503, 1456, 1409, 1374, 1356, 1316, 1232, 1201, 1142, 1076, 1037, 945, 793, 686, 625, 573, 554, 536 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 8.57 (d, 1H, $J_{2,3}$ = 8.6 Hz, H-2), 8.34 (d, 1H, $J_{3,4}$ = 8.7 Hz, H-4), 8.21 (d, 1H, $J_{7,8}$ = 7.3 Hz, H-8), 7.62 – 7.56 (m, 2H, H-3, H-7), 7.28 (d, 1H, $J_{6,7}$ = 7.6 Hz, H-6), 4.22 (d, 1H, $J_{1'',2''}$ = 9.8 Hz, H-1″), 3.85 (dd, 1H, $J_{5'',6a''}$ = 1.8 Hz, $J_{6a'',6b''}$ = 12.0 Hz, H-6a″), 3.78 (dd, 1H, $J_{1'',2''} = J_{2'',3''}$ = 9.9 Hz, H-2″), 3.68 (dd, 1H, $J_{5'',6b''}$ = 5.6 Hz, $J_{6a'',6b''}$ = 12.0 Hz, H-6b″), 3.47 (s, 3H, OCH_3), 3.41 (dd, 1H, $J_{2'',3''} = J_{3'',4''}$ = 9.0 Hz, H-3″), 3.36 – 3.27 (m, 1H, H-4″), 3.21 (ddd, 1H, $J_{5'',6a''}$ = 1.9 Hz, $J_{5'',6b''}$ = 5.4 Hz, $J_{4'',5''}$ = 9.6 Hz, H-5″), 3.02 (t, 1H, $J_{4',5'}$ = 7.2 Hz, H-5′), 2.98 – 2.85 (m, 8H, H-1′, 2 × *N*-CH_3), 2.49 – 2.32 (m, 4H, H-3′, H-4′), 1.95 (s, 3H, CH_3 Ac), 1.69 – 1.59 (m, 1H, H-2′); ^{13}C NMR (125 MHz, CD_3OD) δ 173.3 (C=O Ac), 153.2, 137.1, 131.32, 131.30 (C-1/4a/5/8a), 130.9 (C-2), 130.1 (C-8), 129.2, 124.3 (C-3/C-7), 120.6 (C-4), 116.5 (C-6), 92.4 (C-1″), 79.8 (C-5″), 77.5 (C-3″), 71.8 (C-4″), 62.9 (C-6″), 62.2 (OCH_3), 54.1 (C-2″), 51.4 (C-1′), 45.8 ($N(CH_3)_2$), 43.9 (C-5′), 32.4 (C-4′), 30.2 (C-3′), 28.5 (C-2′), 23.0 (CH_3 Ac); HRMS(ESI) *m/z* calcd. for $[C_{26}H_{41}N_4O_8S_2]^+$: 601.2360, obsd.: 601.2362.

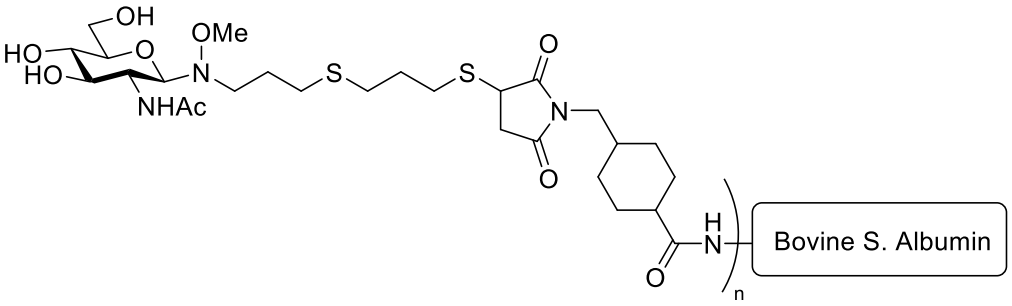


1-(2-D-biotinamidoethyl)-1*H*-pyrrole-2,5-dione (29). To a solution of 2-aminoethyl maleimide[44] (159 mg, 1.13 mmol) and D-biotin (363.4 mg, 1.31 mmol) in DMF (10 mL), HBTU (516 mg, 1.36 mmol) and triethylamine (0.1 mL) was added and the reaction mixture was stirred at rt for 2 h. The crude reaction mixture was concentrated and purified by reverse phase column chromatography (C_{18}, H_2O/MeOH, 100/0 → 70/30, v/v) and size exclusion chromatography (Sephadex LH-20, CH_2Cl_2/MeOH, 50/50, v/v) to give biotin functionalised maleimide **29** as a white solid (380 mg, 1.04 mmol, 92 %). R_f = 0.59 (CH_2Cl_2/MeOH, 80/20, v/v); $\alpha_D^{20.6}$ = + 26.5 (c = 0.2, MeOH); IR (film) 3283, 3087, 2936, 2864, 1701, 1552, 1461, 1439, 1408, 1360, 1332, 1266, 1169, 1101, 1055, 1033, 828, 763, 696 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 6.82 (s, 2H, HC=CH), 4.60 (s, 1H, NH), 4.49 (dd, $J_{6,7}$ = 4.9 Hz, $J_{7,8}$ = 7.7 Hz, 1H, H-7), 4.67 (dd, 1H, $J_{6,7}$ = 4.9 Hz, $J_{5,6}$ = 7.8, Hz H-6), 3.61 (dd, 2H, $J_{1',2'}$ = 5.5 Hz, CH_2-1′), 3.36 (dd, 2H, $J_{1',2'}$ = 5.5 Hz, CH_2-2′), 3.24 – 3.17 (m, 1H, H-5), 2.93 (dd, 1H, $J_{7\text{-}8a}$ = 5.0 Hz, $J_{8a\text{-}8b}$ = 12.8 Hz, H-8a), 2.70 (d, 1H, $J_{8a\text{-}8b}$ = 12.8 Hz, H-8b), 2.12 (dt, 2H, $J_{1a,2}$ = 3.4 Hz, $J_{1b,2}$ = 7.4 Hz, CH_2-1), 1.79 – 1.66 (m, 1H, CH_2-4a), 1.66 – 1.52 (m, 3H, CH_2-2, CH_2-4b), 1.50 – 1.35 (m, 2H, CH_2-3); ^{13}C NMR (125 MHz, CD_3OD) δ 176.3 (C=O amide), 172.6 (2 × C=O *maleimide*), 166.1 (C=O *carbamate*), 135.5 (C=C), 63.3 (C-6), 61.6 (C-7), 56.9 (C-5), 41.0 (C-8), 38.8 (C-2′), 38.4 (C-1′), 36.7 (C-1),

29.7 (C-3), 29.4 (C-4), 26.6 (C-2); HRMS(ESI) *m/z* calcd. for $[C_{16}H_{23}N_4O_4S]^+$: 367.1435, obsd.: 367.1429.



1-(2-D-biotinamidoethyl)-3-(3-(3-(methoxy[2-acetamido-2-deoxy-β-D-glucopyranosyl]-amino)propylthio)propylthio)pyrrolidine-2,5-dione (34). To a solution of neoglycoside **20** (7.0 mg, 17.6 μmol) in H_2O (1 mL), maleimide **29** (9.0 mg, 24.6 mmol) was added, and the reaction mixture was stirred at rt for 1 h. The crude reaction mixture was purified using size exclusion chromatography (Bio Gel P-2) to yield glycoconjugate **34** (12.6 mg, 16.5 μmol, 94%) as colourless oil. R_f = 0.54 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 5/2/2/1, v/v/v/v); IR (film) 3338, 3286, 2933, 2871, 2859, 1700, 1648, 1559, 1452, 1439, 1332, 1265, 1188, 1107, 1080, 1026, 969, 901, 847, 760 699 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ 4.60 (dd, 1H, $J_{7,8}$ = 5.0 Hz, $J_{6,7}$ = 7.8 Hz, H-7), 4.42 (dd, 1H, J_{5-6} = 4.5 Hz, J_{6-7} = 7.8 Hz, H-6), 4.32 (d, 1H, $J_{1'''',2''''}$ = 9.8 Hz, H-1′′′′), 4.02 – 3.98 (m, 1H, H-1′′), 3.91 – 3.85 (m, 2H, H-2′′′′, H-6a′′′′), 3.72 (dd, 1H, $J_{5'''',6''''}$ = 5.2 Hz, $J_{6a'''',6b''''}$ = 12.3 Hz, H-6b′′′′), 3.71 – 3.58 (m, 2H, CH_2-1′), 3.53 – 3.46 (m, 4H, H-3′′′′, OCH_3), 3.45 – 3.35 (m, 4H, CH_2-2′, H-5′′′′, H-4′′′′), 3.35 – 3.24 (m, 2H, CH_2-5, H-2a′′), 3.06 – 2.95 (m, 3H, CH_2-1′′′, H-8a), 2.93 – 2.79 (m, 2H, CH_2-6′′′), 2.77 (d, 1H, J_{8a-8b} = 13.0 Hz, H-8b), 2.72 (m, 5H, H-2b′′, CH_2-4′′′, CH_2-3′′′), 2.19 (t, 1H, $J_{1,2}$ = 7.3 Hz, CH_2-1), 2.03 (s, 3H, CH_3 Ac), 1.96 – 1.87 (m, 2H, CH_2-5′′′), 1.84 – 1.76 (m, 2H, CH_2-2′′′), 1.76 – 1.66 (m, 1H, H-4a), 1.62 – 1.50 (m, 3H, H-4b, CH_2-2), 1.42 – 1.33 (m, 2H, CH_2-3); ^{13}C NMR (125 MHz, D_2O) δ 182.0, 180.8 (2 × C=O *pyrrolidine*), 179.5 (C=O *biotin*), 176.3 (C=O *NAc*), 167.8 (*C*=O *carbamate-biotin*), 92.6 (C-1′′′′), 80.0 (C-4′′′′), 78.1 (C-3′′′′), 72.2 (C-5′′′′), 64.6 (C-6), 63.7 (OCH_3), 63.4 (C-6′′′′), 62.8 (C-7), 57.8 (C-5), 54.9 (C-2′′′′), 52.9 (C-1′′′), 42.5 (C-1′′), 42.3 (C-8), 41.2 (C-1′), 39.1 (C-2′), 38.5 (C-2′′), 38.0 (C-1), 32.4 (C-6′′′), 32.3 (C-4′′′), 31.2 (C-3′′′), 30.6, 30.5 (C-5′′′, C-3), 30.2 (C-4), 28.9 (C-2′′′), 27.5 (C-2), 24.8 (CH_3 Ac); HRMS(ESI) *m/z* calcd. for $[C_{31}H_{53}N_6O_{10}S_3]^+$: 765.2980, obsd.: 765.2975.



Glycoprotein 37. To a solution of Bovine Serum Albumin **36** (1 mg, 0.015 μmol) in PBS (1 mL), SMCC cross-linker **35** (0.9 mg, 2.73 μmol) in MeCN (0.1 mL) was added and the reaction mixture was incubated at rt. To 10 μL of the crude reaction mixture, neoglycoside **20** (2.2 mg, 5.45 μmol) in water (10 μL) was added and the reaction mixture was allowed to stir at rt to obtain crude glycoprotein **37**.

Preparation of MALDI-TOF Matrix solution.

Matrix solution. To a solution of cinnapinic acid (15 mg) in MeCN:H_2O (1 mL, 1/1, v/v), TFA (1 μL) was added and the mixture was vortexed for 3 min, and centrifuged for 10 minutes, to obtain the matrix solution.

Sample preparation. 1-2 uL of the desired sample (~1.0 mg/mL) was added to 20 μL of the matrix solution and a pipette was used to mix the sample. *NOTE, do not use vortex since this will precipitate the protein.* Next, 2 μL of the sample/matrix was spotted on the MALDI-plate and allowed to dry for 1 h. before measuring the MALDI-TOF.

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Chapter 3.

Design and synthesis of novel fluorescent and biotinylated multivalent glycodendrons.

3.1 Introduction

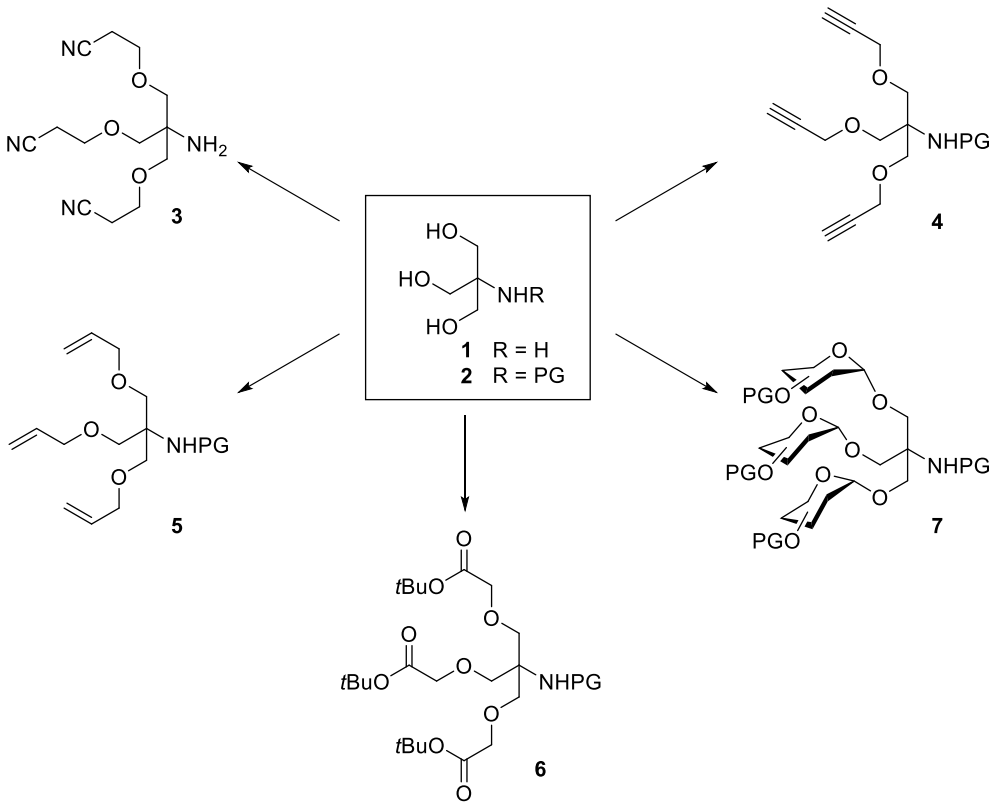
Dendrimers and dendrons are commonly used to enhance or decrease a specific activity, and accordingly have been investigated for their use in a large variety of applications[1] including light harvesting and fluorescent sensors,[2,3] catalysis,[4] as well as for biomedical purposes.[5] In particular, the use of glycodendrimers in biomedical applications has significantly increased over the last few decades and glycodendrons are now being used as magnetic resonance imaging contrast agents,[6,7] antigen carriers and adjuvants for vaccines,[8,9] cell-specific delivery vehicles for the treatment and diagnosis of disease,[10–12] or to simply solubilise active pharmaceuticals.[13]

On a molecular level, inter-cellular interactions are often mediated by multivalent interactions between carbohydrate antigens and their corresponding lectins. In fact, the low binding affinity between carbohydrate antigens and lectins requires multivalent interactions in order to induce a biological response.[14] The key feature of glycodendrons is their multivalent display of carbohydrate antigens and accordingly such glycodendrons are able to mimic cellular interactions. This allows glycodendrons to be used in biomedical applications, as described above.[9] As discussed in Chapter 1, several synthetic methods have been developed for the assembly of multivalent dendrons,[15] whereas this chapter provides an overview of the TRIS-based dendrimer/dendron syntheses. Moreover, the synthesis of a novel second generation dendron scaffold is discussed, which allows for the rapid

functionalisation and oxyamine glycan conjugation towards the assembly of complex glycoconjugates for the biological purpose of choice.

3.1.1 TRIS-based dendrons

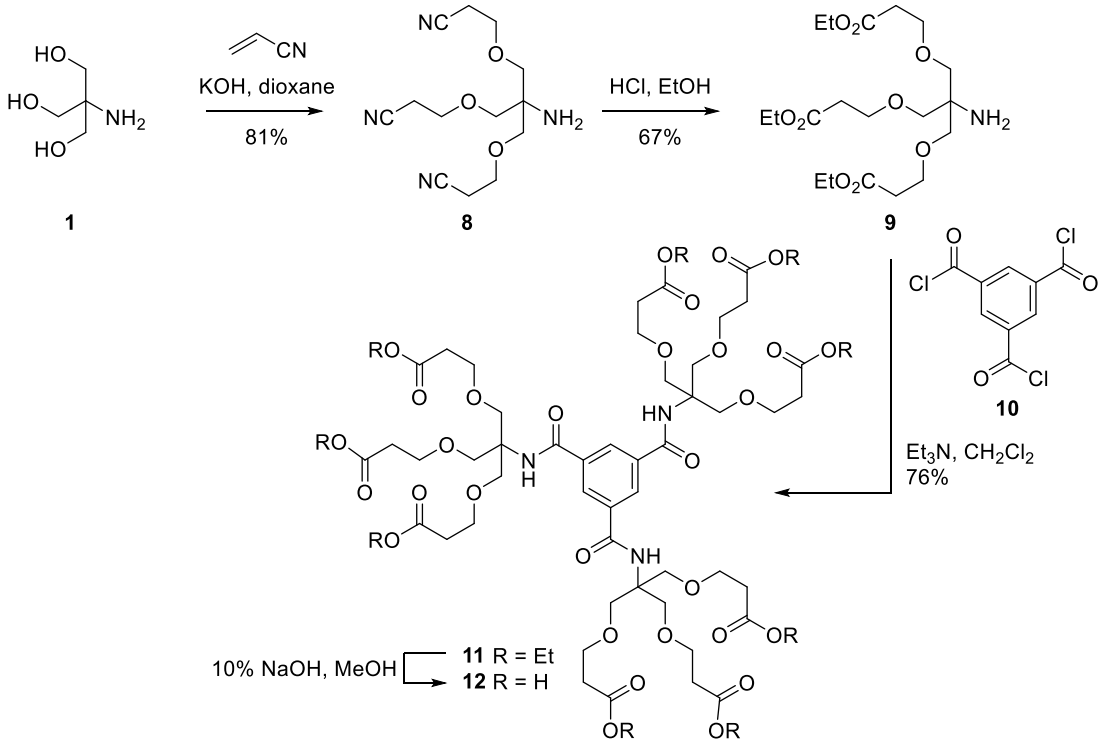
Tris(hydroxymethyl)aminomethane (TRIS) (**1**) as a branching unit in dendrons and dendrimers has been utilised in various synthetic methodologies.[16–19] Key in the synthesis of TRIS-based dendrons is the tri-*O*-alkylation reaction, and accordingly, several alkylation reactions have been reported (Scheme 1). For example, reaction of TRIS (**1**) with acrylonitrile gives rise to trinitrile **3**, whereas the conjugate addition of the *N*-protected TRIS **2** with either propargyl bromide, allyl bromide or *tert*-butyl bromoacetate gives rise to dendrons **4**, **5** and **6**, respectively. Alternatively, the *N*-protected TRIS **2** can be used as an acceptor in glycosylation reactions to give the trivalent glycodendron **7**.



Scheme 1. *Different approaches for the tri-O-alkylation of TRIS.*

3.1.1.1 TRIS alkylation with acrylonitrile

In 1991, Newkome *et al.* first reported the use of TRIS (**1**) as a core scaffold for dendrimer synthesis.[16] Their synthesis commenced with a Michael addition of TRIS to acrylonitrile to give 1,4-adduct **8** in 81% yield (Scheme 2). Next, the nitrile functional groups were converted to the ethyl esters under acidic conditions to give first generation dendron **9**. The amine was then reacted with 1,3,5-benzenetricarbonyl trichloride (**10**) to obtain nonaester **11** which after ester hydrolysis gave the nona-valent acid **12**.



Scheme 2. *First dendrimer synthesis featuring the TRIS moiety.*[16,20]

Following the initial work by Newkome *et al.*, many TRIS-based dendrons have been synthesised utilising the initial alkylation with acrylonitrile. For example, in 1999 Sliedregt *et al.* synthesised glycodendron glycolipid **13** in 9 steps from the afore mentioned common intermediate **9** in order to associate glycosides with liposomes via the incorporation of the acylated cholesterol derivative (Figure 1).[21,22] The liposomes were then used to target liver cells via the selective binding of asialoglycoprotein receptors to the galactose residues of the glycodendron.

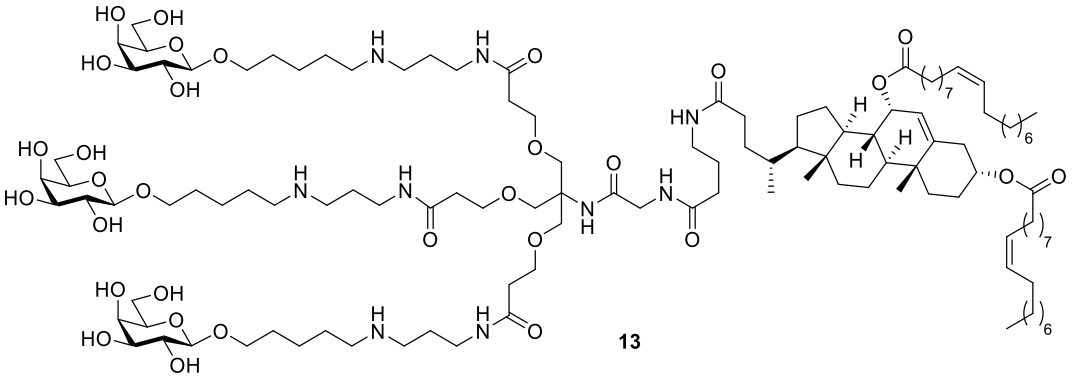


Figure 1. *Galactose glycodendron lipids allow for liposome association to target liver cells.*[21]

More recently, this synthetic route has been used by Seeberger and co-workers for the synthesis of second generation glycodendron **14** functionalised with a cyclodextrin for the selective delivery of doxorubicin to liver cells (Figure 2).[11] Here, the doxorubicin was trapped within the cyclodextrin moiety, which allowed the release of the active drug in the target cells. It was found that doxorubicin-containing glycodendrons were able to selectively induce apoptosis in liver cells, whereas the empty cyclodextrin glycodendrons did not result in liver cell apoptosis. This nicely demonstrates the ability of multivalent glycodendrons to target cell-specific receptors and deliver cytotoxic drugs in order to deplete these cell types, and, therefore, validates the potential use of dendrimers as drug-delivery vehicles.

Although acrylonitrile has been successfully employed in glycodendrimer synthesis, an extra step is required for the conversion to the ester derivative, which ultimately lowers the overall efficiency of the synthesis.

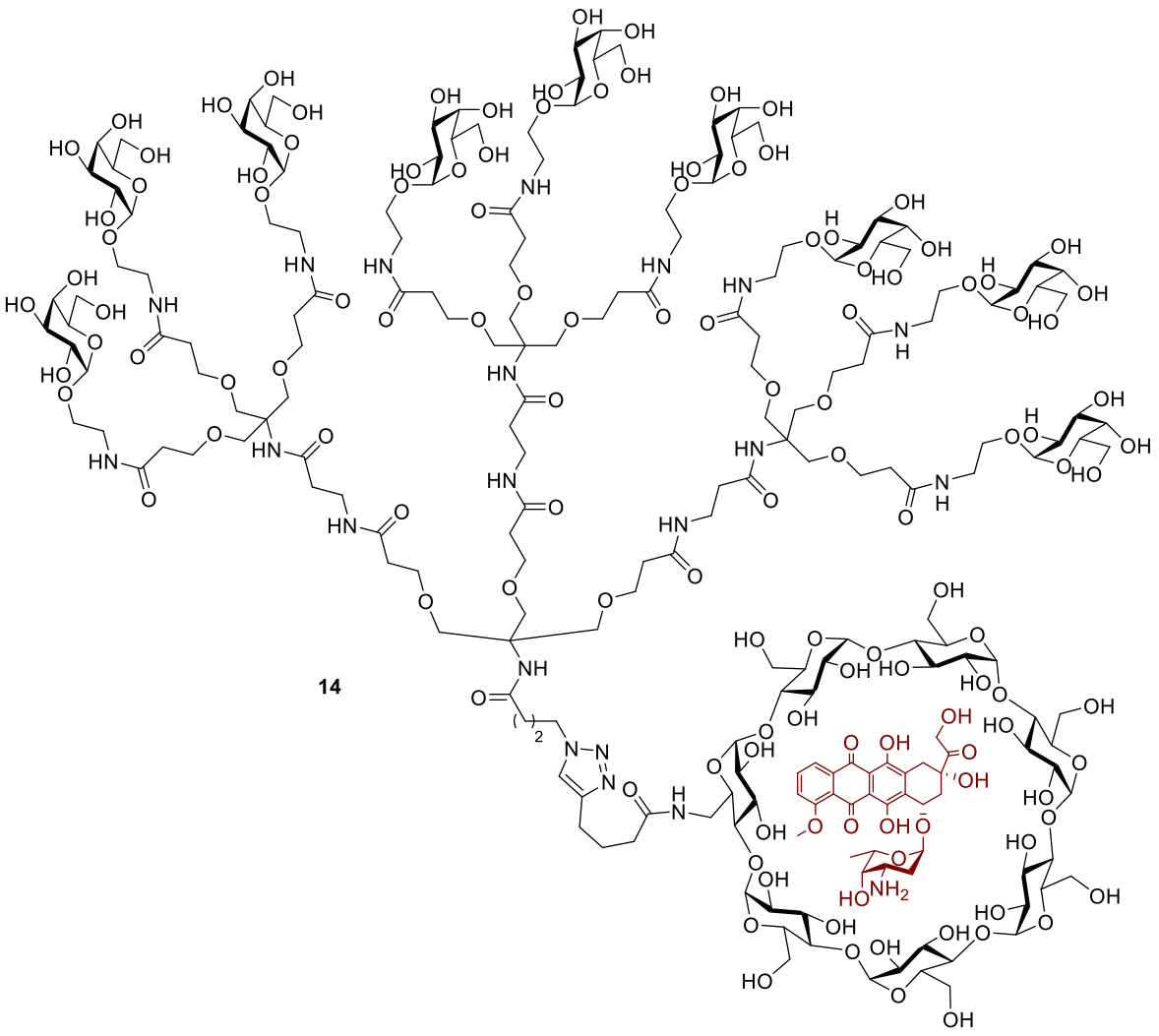
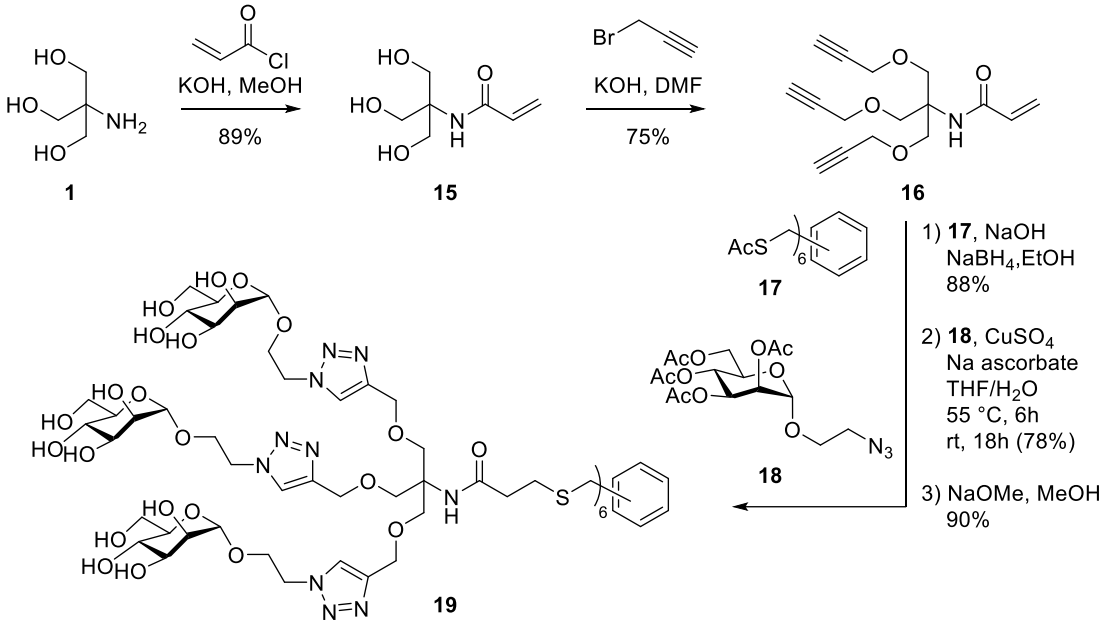


Figure 2. *A galactose-glycodendron functionalised β-cyclodextrin for the liver cell-specific delivery of doxorubicin.*

3.1.1.2 Propargylation of TRIS

The tri-*O*-propargylation of *N*-protected TRIS with propargyl bromide can be performed to give the first generation dendron, which in turn is suitable for the Huisgen cycloaddition reaction with azide derivatives.[17,23–25] Although the use of copper in late stage dendrimer chemistry is not ideal due to the cytotoxicity of copper and the ability of dendrons to host metals in its void spaces,[26,27] the Huisgen cycloaddition has been used extensively throughout the field of dendrimer chemistry.[28,29] For example, in 2008 Chabre *et al.* presented the synthesis of triazole based dendrons and dendrimers, whereby TRIS (**1**) was reacted with acryloyl chloride to give triol **15**, which subsequently was reacted with propargyl bromide to give dendron **16** (Scheme 3).[17] Next, the dendron was reacted with thiol **17** to give the octadecapropargylated dendritic scaffold, which, was then used in the copper-mediated

azide-alkyne Huisgen cycloaddition with azido-glycan **18** to give octadeca-valent glycodendrimer **19** after deacetylation.



Scheme 3. *Glycodendrimer **19** synthesis utilising an azide-alkyne Huisgen cycloaddition reaction.*[17]

In a similar fashion, Rojo and co-workers synthesised fluorescently labelled glycodendrons **20-22** utilising the copper catalysed azide-alkyne cycloaddition reaction (Figure 3).[25]Although, instead of TRIS, pentaerythritol was used as the branching moiety towards the synthesis of several second generation glycodendrons, which were then used to analyse the DC-SIGN-mediated internalization by dendritic cells. A synthetic intermediate was then used in the synthesis of virus-like glycodendrinanoparticles bearing up to 1620 glycans, to study its ability to block DC-SIGN and prevent infection by the Ebola virus.[30]

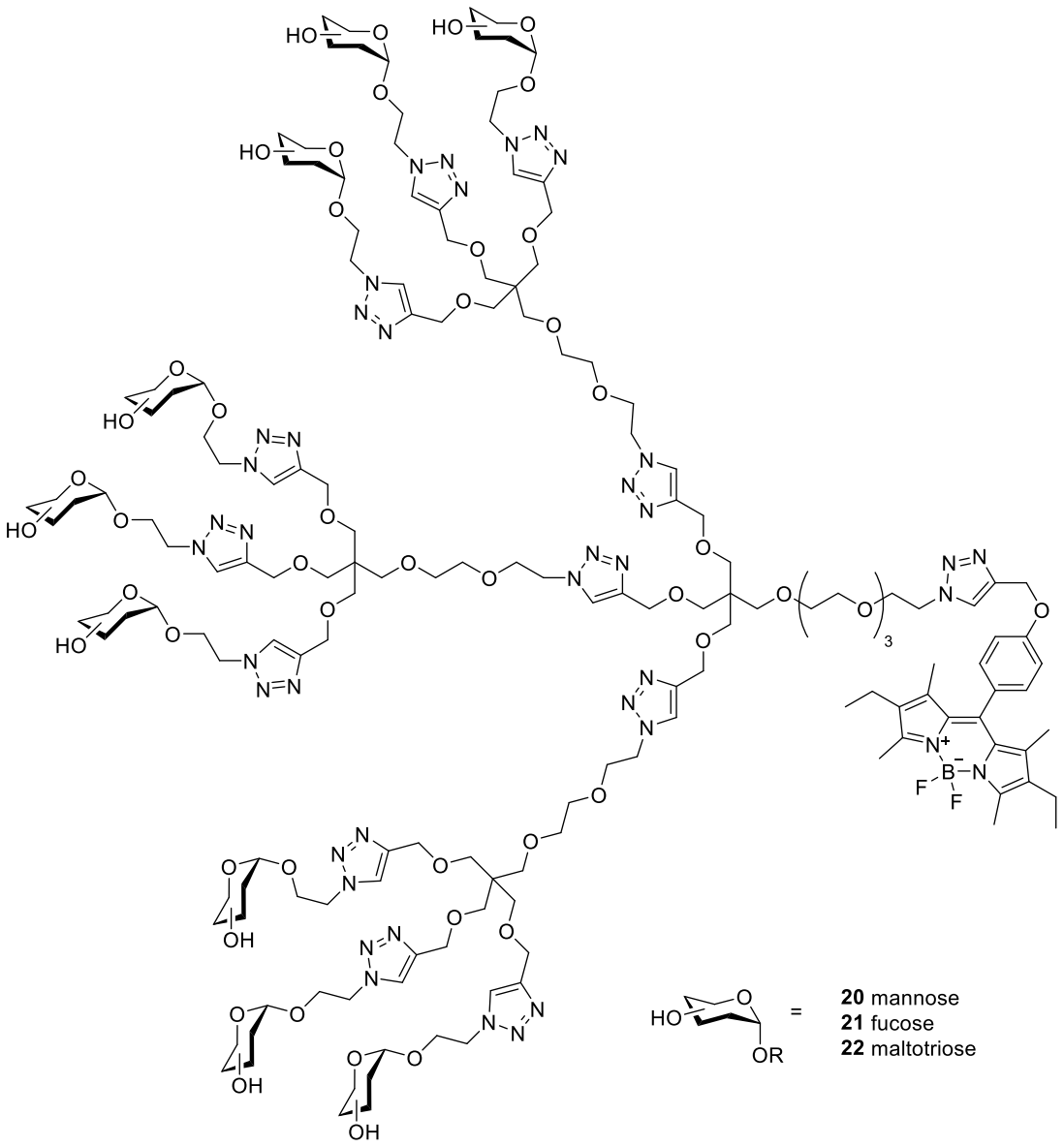
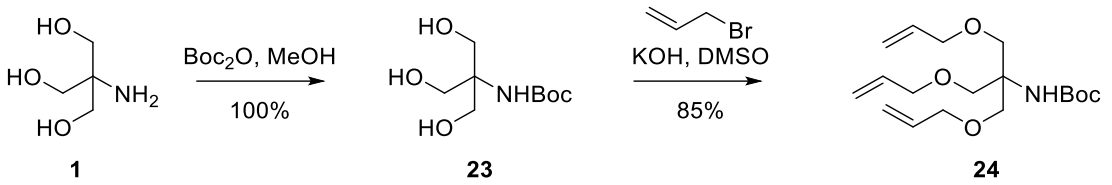


Figure 3. *BODIPY-labelled glycodendrons were synthesised to study DC-SIGN-mediated internalization by human dendritic cells.*[25]

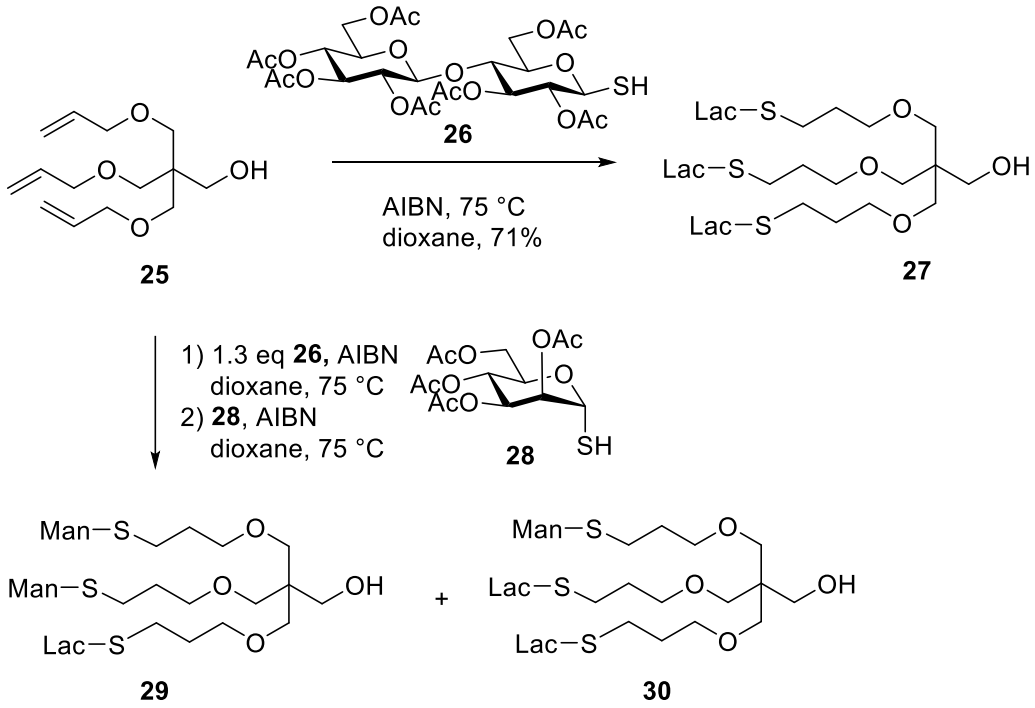
3.1.1.3 Allylation of TRIS

Kaplánek *et al.* demonstrated the use of allyl bromide in the tri-*O*-alkylation of TRIS derivatives, where TRIS (**1**) was *N*-Boc protected (→**23**) and subsequently allylated with allyl bromide using KOH in DMSO to give trialkene **24** in good yields (Scheme 4).[18] Although these dendrons were not used for conjugation of biomolecules, the tri-*O*-allylation would allow for the synthesis of sulfur derived dendrimers, through the use of thiol-ene conjugation reactions.[31] The use of sulfur chemistry in the synthesis of glycodendrons has been discussed in an excellent review.[32]



Scheme 4. *Synthesis of triallyl dendron* ***24****.*[18]

Similar trialkenyl dendrons such as **25**, were used in thiol-ene conjugations, as was demonstrated by Gómes-Garcia *et al.* who used thioglycosides such as lactose derivative **26** to form glycodendron **27** using catalytic azobisisobutyronitrile (AIBN) (Scheme 5).[33] On the other hand, more complex, asymmetrical glycodendrons could be obtained when the lactose glycan was coupled first followed by the thiol-ene conjugation with mannoside **28**. This, however, resulted in the formation of a 3:1 mixture of **29** and **30** respectively. These glycodendron were then used in the convergent synthesis of a β-cyclodextrin glycodendron.

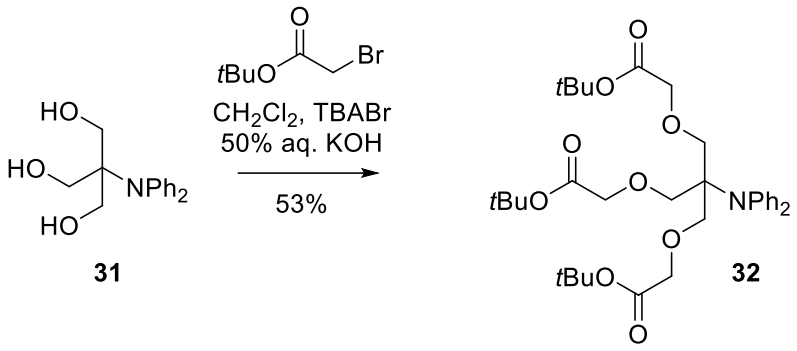


Scheme 5. *Thiol-ene conjugation in the synthesis of glycodendrons.*[33]

These thiol-ene conjugations, however, require radical initiation and conjugation at high temperatures which can complicate the conjugation of oligosaccharides. In addition, protected glycoconjugates were used which requires a late stage deprotection step on large glycoconjugates complicating the assembly of these macromolecules.

3.1.1.4 TRIS alkylation with tert-butyl bromoacetate

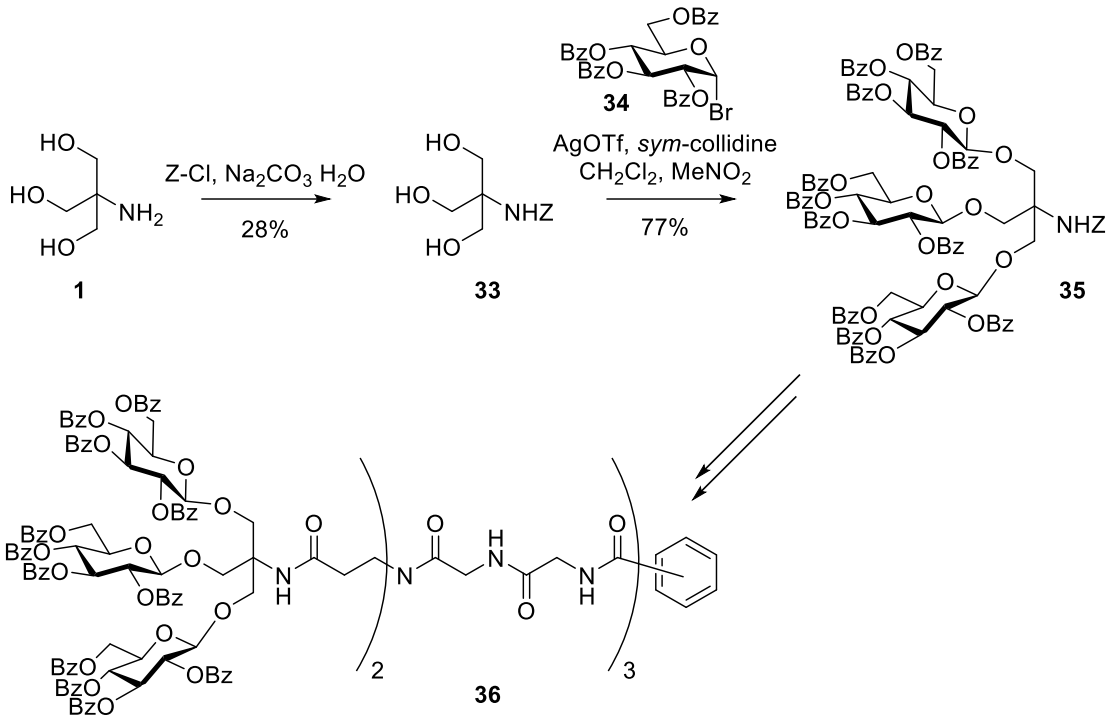
In 2001, Dupuy *et al.* reported the tri-*O*-alkylation of *N,N*-diphenyl TRIS **31** with *tert*-butyl bromoacetate.[34] The alkylation was performed in a two-phase system (CH_2Cl_2 : 50% aq. NaOH) with a phase transfer catalyst to give dendron **32** in 53% (Scheme 6). The resulting dendron, however, was synthesised for phase transfer methodology purposes and was not further utilised. In contrast to the use of acrylonitrile for the tri-*O*-alkylation of TRIS derivatives, the advantage of using *tert*-butyl bromoacetate as an alkylation agent is that it directly results in ester formation and thus shortens the synthetic route by one step. In 1999, a similar alkylation was included in patented methodology by Martinez, whereby a similar triol was reacted with *tert*-butyl bromoacetate and KOtBu in *t*BuOH. The yield of this reaction, however, was not reported.[35]



Scheme 6. *Tri-O-alkylation of TRIS derivative **31** with tert-butyl bromoacetate.*[34]

3.1.1.5 Direct tri-O-glycosylation of TRIS

As mentioned above, the use of TRIS derivatives as acceptors in glycosylation reactions gives rise to trivalent glycodendrons, which in turn can be used in a convergent route to make larger generation glycodendrons. In 1996, Stoddart and co-workers reported the synthesis of an octadeca-valent glycodendron via this convergent synthetic route (Scheme 7).[19,36] Here, TRIS (**1**) was benzyloxycarboxyl (Z)-protected and the resulting carbamate **33** was glycosylated with glycosyl bromide **34** to give glycoside **35**. With the dendron in hand, further derivatisation gave octadeca-valent glycodendron **36**.



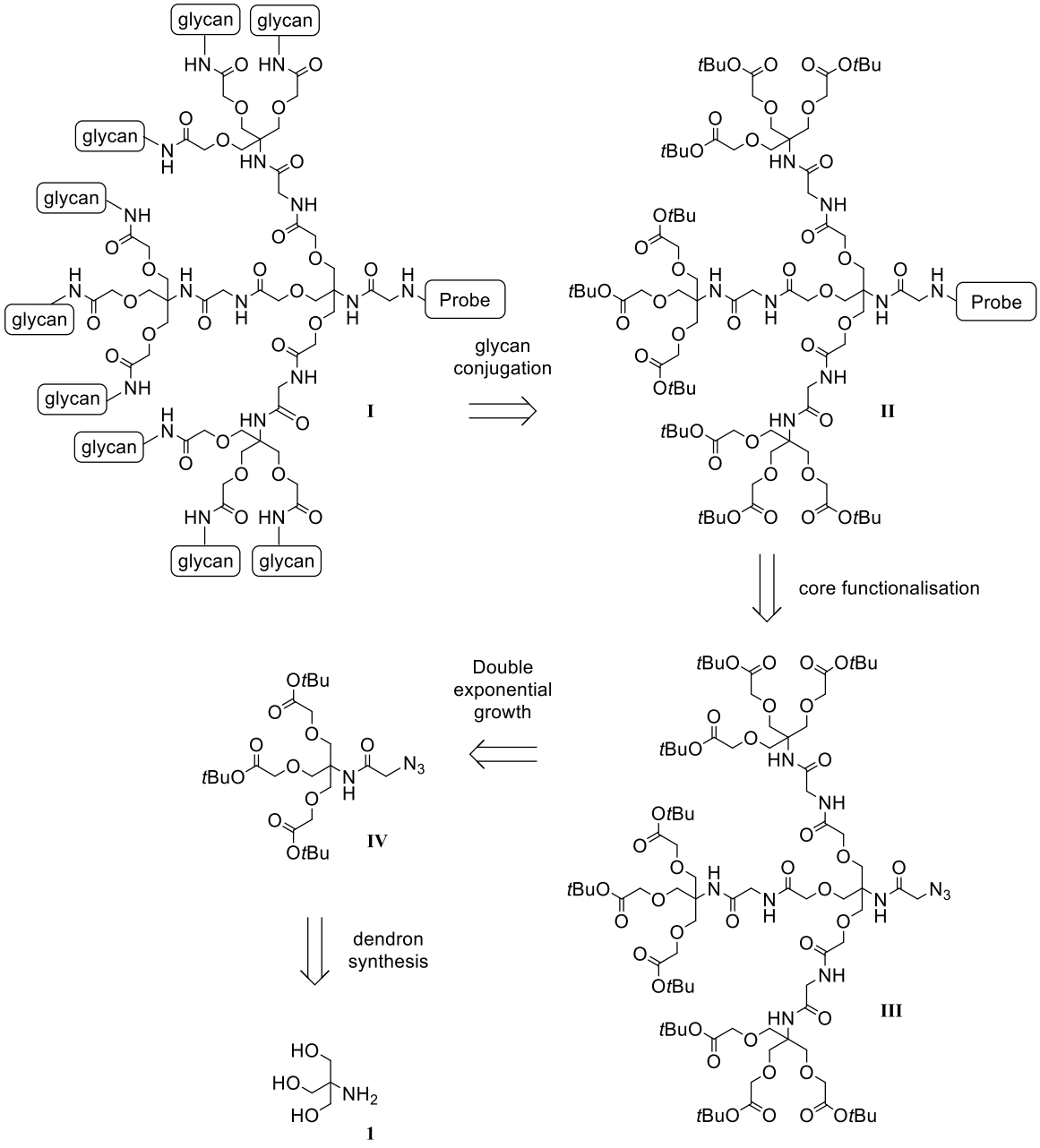
Scheme 7. *Convergent synthesis of TRIS-glycodendrons by Stoddart and co-workers.*[19]

Using this glycosylation method, Furneaux *et al.* synthesised trivalent α-mannose-dendrons for the liposome-mediated antigen delivery to dendritic cells.[37] In 2006, this type of glycodendron was functionalised with paramagnetic gadolinium (Gd) metal for MRI purpose.[6] More recently, glycodendrons were conjugated to photosensitisers to exclusively target cancer cell lines, but these glycodendrons did not show cell specificity in vitro, when compared between healthy cells and two tumour cell lines.

This method, however, requires the use of protected glycosyl donors, which then requires global deprotection of macromolecules, complicating the synthesis of large glycodendrons. Moreover, the short distance between the three antigens makes these dendrimers extremely compact. This might affect the lectin binding as these glycodendrons are relatively bulky when compared to the other TRIS-based glycodendrons.

3.1.2 Project aim and Retrosynthesis

The aim of the research described in this chapter is to synthesise novel glycodendrons for use in biomedical applications. To this end, it was envisioned that an orthogonal dendron core, which allows for the rapid functionalisation with the antigen and probes of choice, should be prepared (Scheme 8). The carbohydrate antigen of choice can then be coupled to the periphery of the dendron. Here, it should be noted that the use of bifunctional methoxyamine linkers, as described in Chapter 2, will be utilised to conjugate the desired carbohydrate to the multivalent dendron scaffold. Thus, to obtain glycodendrons **I**, a peptide conjugation protocol was envisioned for the glycan conjugation, and accordingly a carboxylic acid functionalised periphery of the dendron was desired, which in turn could be readily obtained from ester derivatives **II**. Orthogonality at the core of the dendron allows for functionalisation with the probe (*e.g.* biotin and fluorophores), and again it was envisioned to use a peptide conjugation protocol to functionalise the amine core with carboxylic acid derivatised probes to obtain the dendrons **II**. Accordingly, the amine core dendron could be obtained from azide **III** via the Staudinger reaction. As described above, sufficient lectin binding can be achieved with nonavalent second generation glycodendrons, and accordingly, the synthesis of a bifunctional second generation dendron **III** was proposed. Here, it was anticipated that the efficient double exponential growth methodology could be used, thereby allowing for the synthesis of the second generation dendron **III** from the first generation dendron **IV**. Finally, dendron **IV** can be synthesised in two steps from the branching unit TRIS (**1**), via amine conjugation and tri-*O*-alkylation.

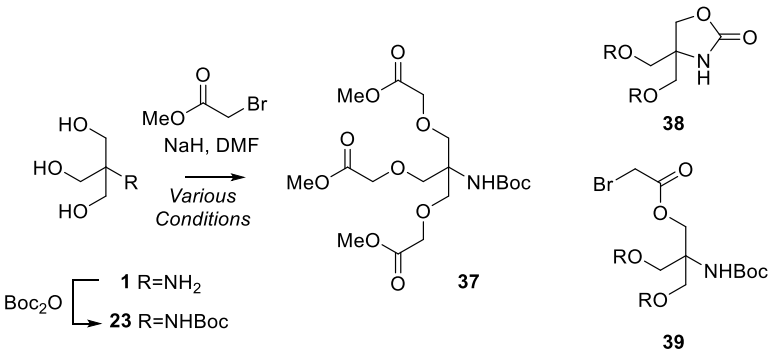


Scheme 8. *Retrosynthesis of bi-functional glycodendrons.*

3.2 Results and discussion

3.2.1 Initial strategy

In an initial attempt to synthesise multivalent dendrons, it was anticipated that the synthesis of a first generation dendron could be achieved via the amine-protection and *O*-alkylation of the hydroxyls in TRIS (**1**). Thus the synthesis commenced with the smooth *N*-Boc protection of TRIS (**1**) to give triol **23** (Scheme 9).[18] Next, the alkylation reaction was investigated and sodium hydride was slowly added to triol **23** in the presence of methyl bromoacetate in DMF. Here, a large number of products were observed using TLC-analysis. Although some of the desired tri-alkylated product **37** was observed using LC-MS analysis, a variety of byproducts were also observed in the LC-MS chromatogram (Figure 4). Here, intramolecular reaction of the alcohol with the carbamate of the *N*-Boc protecting group gave cyclic carbamate **38**, which is a reaction that has previously been used in the synthesis of spiropiperidines.[38,39] Moreover, the formation of α-bromoacetate esters (*i.e* **39**) indicates that esterification occurs instead of the desired alkylation reaction. Mono- and di-alkylated products were also observed in the LC-MS chromatogram.



Scheme 9. *Alkylation attempt of triol **23** to yield trimethyl ester **37**. During the alkylation reaction numerous byproducts were formed, which could be separated and analysed using LCMS (Figure 4).*

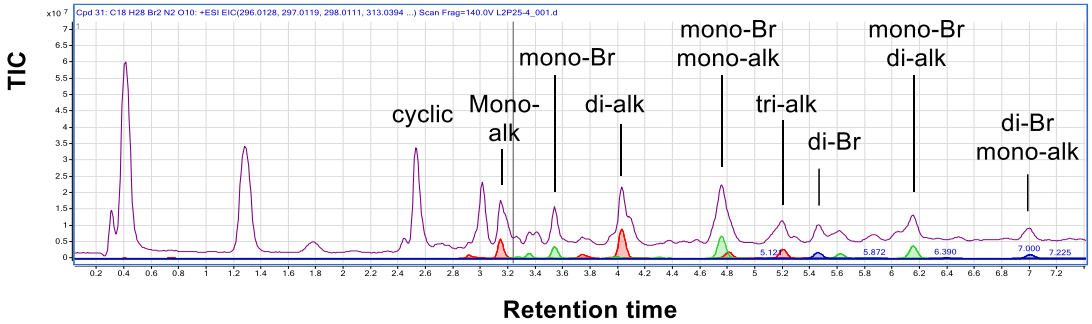
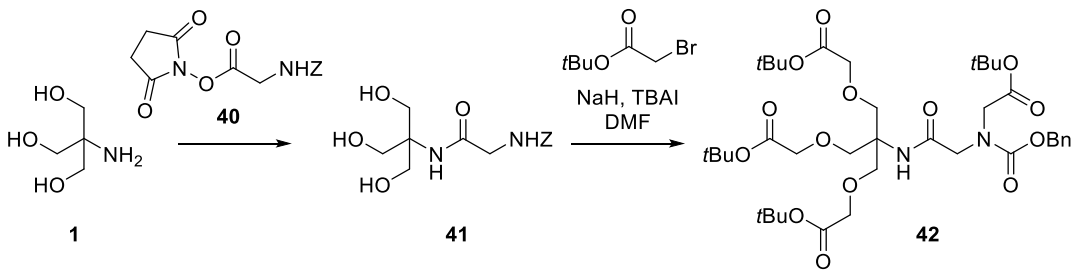


Figure 4. *LCMS (TIC) trace of alkylation reaction with some of the indicated byproducts.*

Accordingly, it was thought that conversion of the amine in TRIS to the glycinamide would prevent the cyclisation reaction and use of a bulky alkylating agent *tert*-butyl bromoacetate would prevent esterification byproducts. Thus, TRIS **1** was reacted with NHS-activated *N*-Z protected glycine **40** to give the desired amide **41** (Scheme 10). Next, alkylation of the triol with *tert*-butyl bromoacetate was explored. First, the tri-*O*-alkylation reaction was examined using the previously reported conditions.[34] To this end, the triol was reacted with *tert*-butyl bromoacetate in a bi-phasic system comprising 50% aq. KOH, CH_2Cl_2 and TBABr as a phase transfer catalyst. Using these conditions only small amounts of alkylation products were observed, therefore alternative alkylation conditions were explored. Initially, triol **41** was submitted to alkylation conditions utilizing *tert*-butyl bromoacetate, NaH and TBAI in DMF. While cyclisation with concomitant removal of the Z-group was not observed, overalkylation of the carbamate gave rise to tetraester **42**, which was confirmed by HMBC NMR analysis. Even though the formation of the over-alkylated byproducts was undesired, the basis of successful dendron chemistry was unveiled: by changing the Z-protecting group to an azide derivative, formation of the over-alkylated product could be prevented.

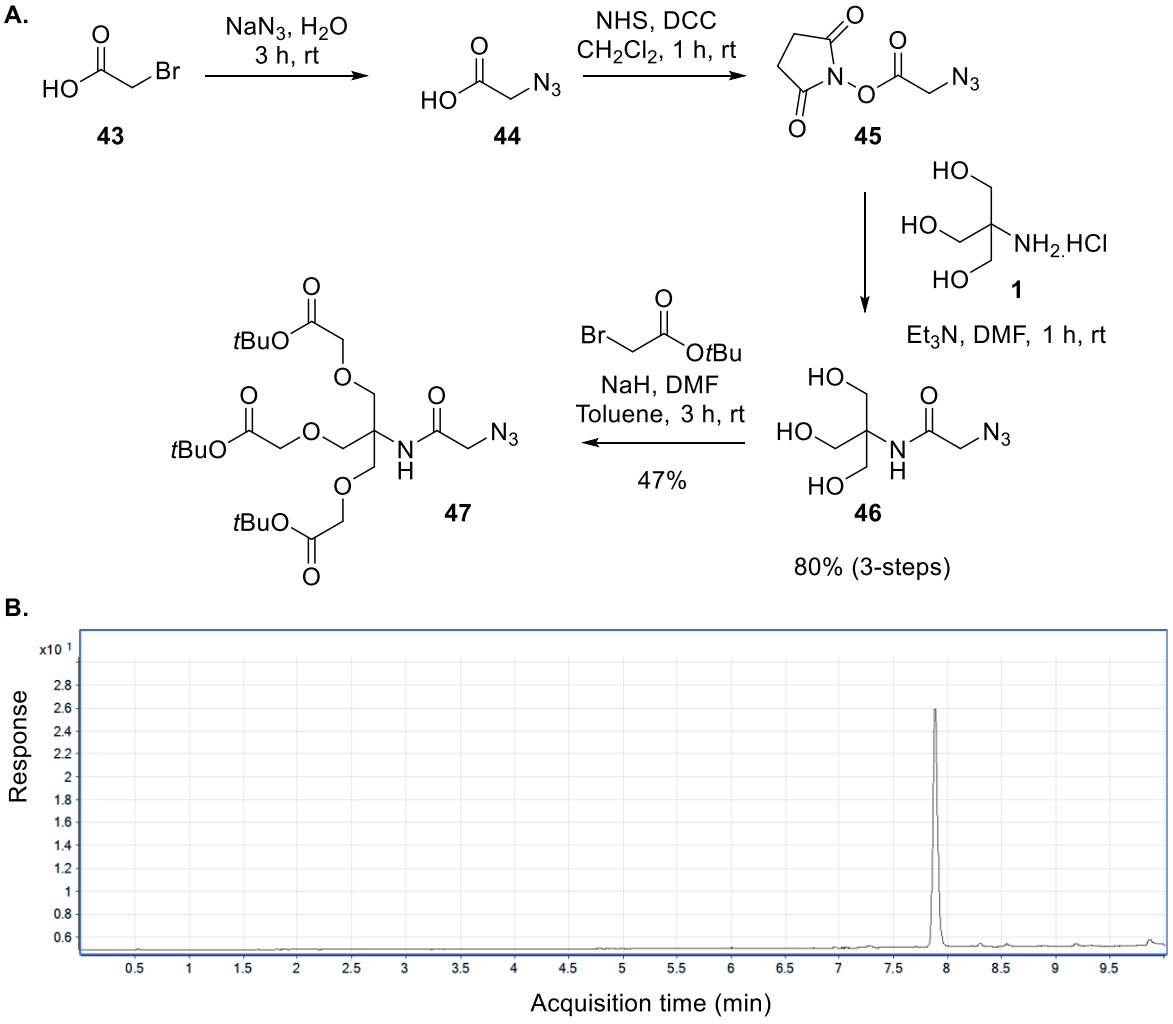


Scheme 10. *Alterations in the dendron design led to the formation of the over-alkylated dendron **42**.*

3.2.2 First generation dendron synthesis

Thus, in the alternative approach, the synthesis of first generation dendron commenced with the quantitative substitution reaction of 2-bromoacetic acid (**43**) with sodium azide to give azidoacetic acid (**44**) (A, Scheme 11). Next, activation of the carboxylic acid by a DCC-mediated coupling with *N*-hydroxysuccinimide gave the activated ester **45**, which was selectively conjugated with TRIS **1** to afford triol **46** in 80% yield over three steps. Here, it should be noted that an excess of TRIS prevents the formation of *O*-acylated byproducts. With the triol in hand, alkylation with NaH and *tert*-butyl bromoacetate in DMF yielded the first generation dendron in moderate yields (ca 30%), however by LCMS there was no evidence of the previously found type of byproducts. Small-scale optimisation of the alkylation reaction (temperature, speed of addition and choice of solvent) revealed that a reasonably quick addition of NaH to the mixture of triol **46** and *tert*-butyl bromoacetate in a 1:1

DMF:toluene mixture at room temperature gave dendron **47** in 47% yield. Similar yields could also be achieved on a gram scale, which allowed for the large scale synthesis of the novel dendron core structure. In order to prevent future purification problems of larger dendrons, the first generation dendron was purified using silica gel flash chromatography as well as reverse phase (C_{18}) chromatography to obtain the dendron in excellent (94%) purity (B, Scheme 11).



Scheme 11. *Synthesis of first generation dendron* ***47****. After purification, the LC-CAD trace shows a 94% purity of the first generation dendron. C18 LC conditions: 50/50 → 100/0 MeCN/H_2O, v/v (10 min gradient); Retention time (min): 7.9 (****47****).*

Structural elucidation of the dendron with both ^{1}H and ^{13}C-NMR spectra revealed the trilateral symmetry of the first-generation dendron (Figure 5). This symmetry simplifies the ^{1}H and ^{13}C NMR spectra of these dendritic molecules. Incomplete coupling, however, results in less symmetrical products and almost certainly more complex NMR-spectra.

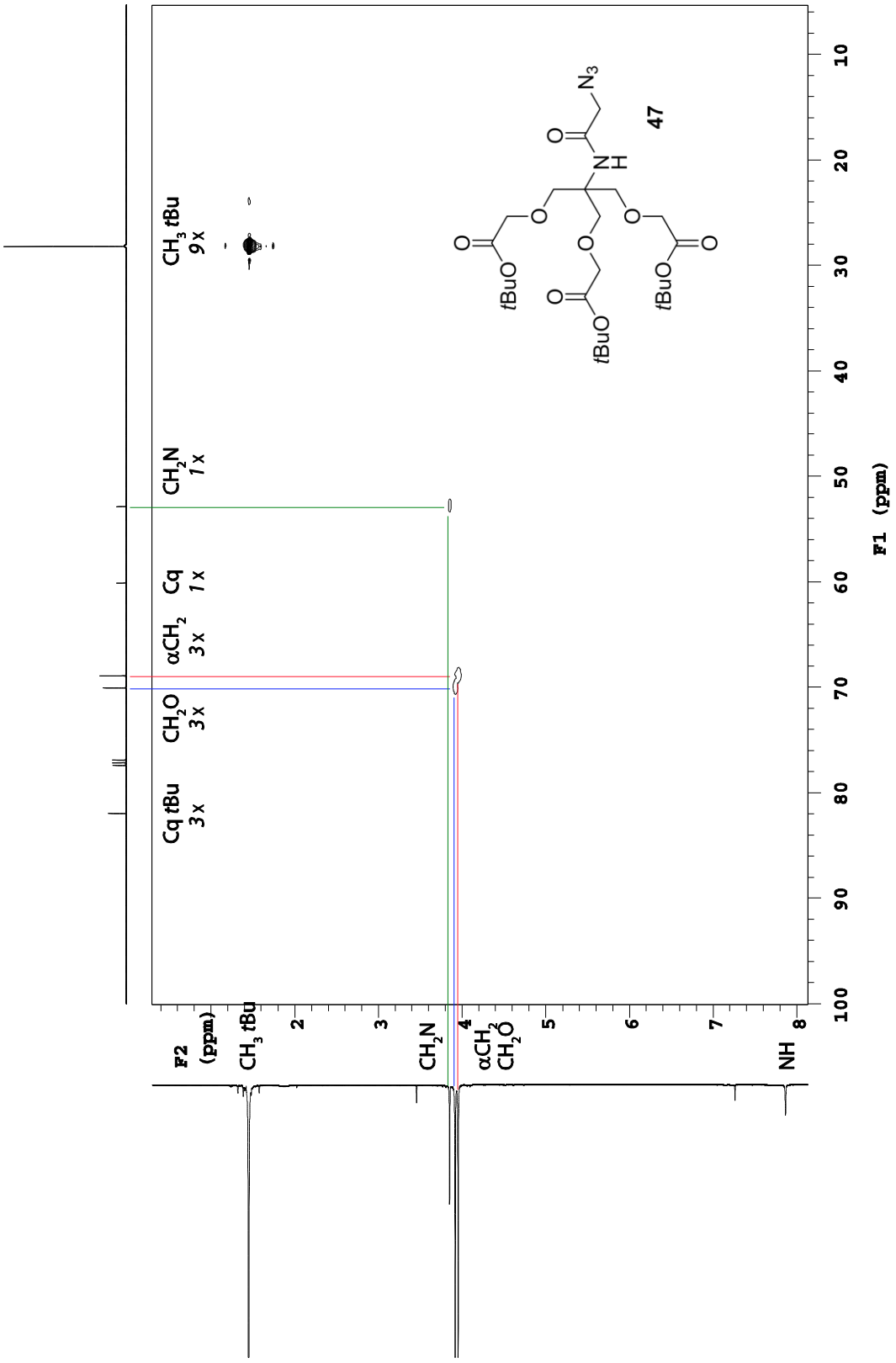
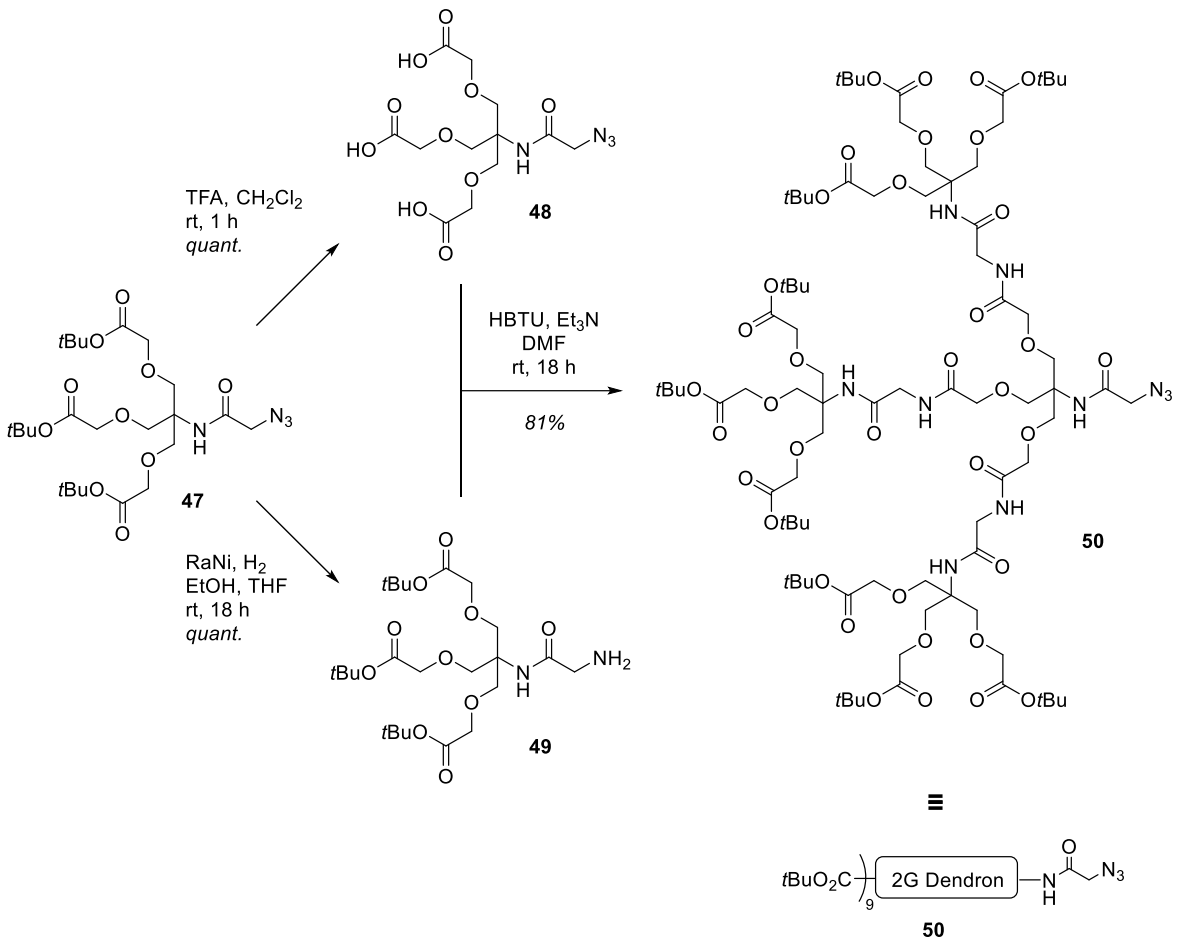


Figure 5. *HSQC experiment of the first generation dendron* ***47*** *showing the C_3-symmetry in both the proton as well as the carbon NMR spectra.*

3.2.3 Second generation dendron synthesis

With the first generation in hand, the synthesis of the second generation dendron was explored using the double exponential growth approach. Here, the first generation dendron **47** was divided into two batches for the conversion into two different reactive substrates, followed by the conjugation of both compounds to afford the second generation dendron. Accordingly, for the *tert*-butyl deprotection of **47**, aqueous 6M HCl was initially used, but this reaction proved slow and sluggish due to the insolubility of the ester-protected dendron in water. When the hydrolysis was performed in a mixture of aqueous 6M HCl and *tert*-butanol, the reaction showed full conversion of the starting material, however, cleavage of the glycinamide was also observed as evidenced by MS analysis. Accordingly, other deprotection strategies were explored and gratifyingly, the use of TFA/CH_2Cl_2 (1/1, v/v) at room temperature for 1 hour led to the formation of the tri-valent acid **48** in excellent yield. Here, it is important to note that the use of alcoholic solvents should be avoided, in order to prevent ester formation, until the TFA is eluted with water on the reverse phase chromatography column.

Next, azide reduction of the first generation dendron **47** was performed using a Staudinger reaction in CH_2Cl_2/MeOH/H_2O (2/9/1, v/v/v) to give primary amine **49** which was purified using reverse phase chromatography and isolated in a quantitative yield. IR-spectroscopy was used to confirm the absence of the azide peak (~2100 cm^{-1}). Alternatively, a Raney-Nickel hydrogenation reaction was used to give the primary amine **49** in quantitative yield, which has the advantage that it is readily purified via a silica gel plug.



Scheme 12. *Synthesis of second generation dendron **50** using a double exponential growth coupling strategy.*

With both triacid **48** and amine **49** in hand, the coupling of triacid **48** and 4.5 equivalents of amine **49** using HBTU and Et_3N was then undertaken to give second generation dendron **50** in 81% yield and in 95% purity after chromatography on both silica gel and C_{18} beads, respectively (Figure 6). The combination of both purification techniques was required in order to obtain a sufficiently pure sample of the dendron. Again, these reactions could be performed on a gram scale in order to produce the second generation dendron **50** on a multi-gram scale.

In terms of characterisation, both ^{1}H and ^{13}C-NMR spectra showed the C_3-symmetry of the second generation dendron **50**. In addition, two-dimensional data (HMBC and HSQC) were used to confirm the coupling between building blocks. With the desired bifunctional dendron core in hand, further functionalisation of the dendron, as well as the glycan conjugation were examined.

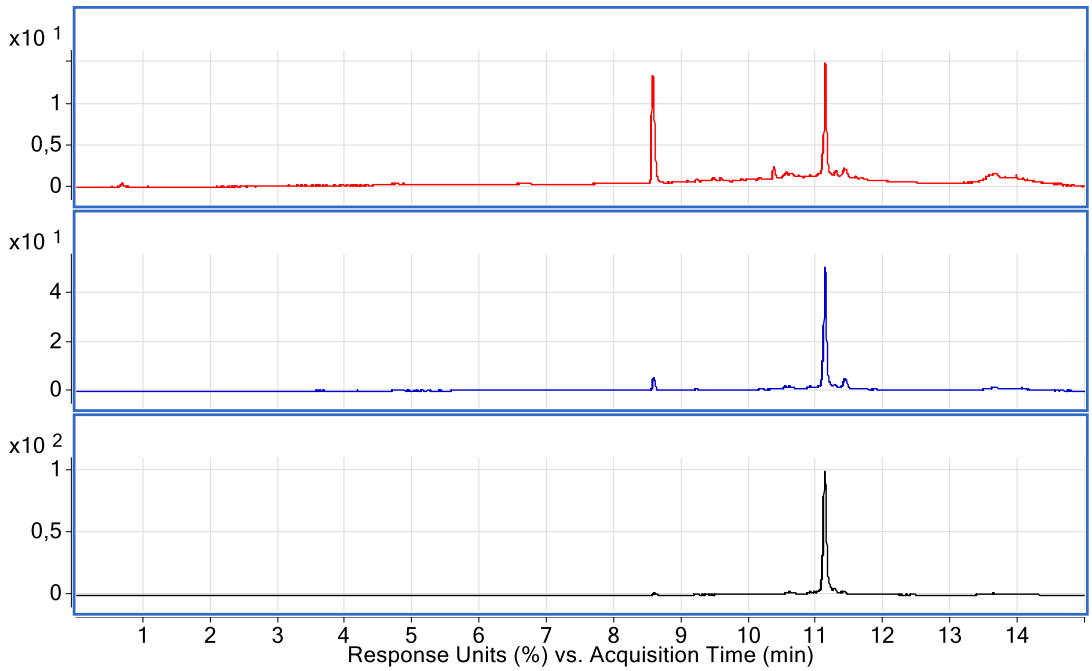
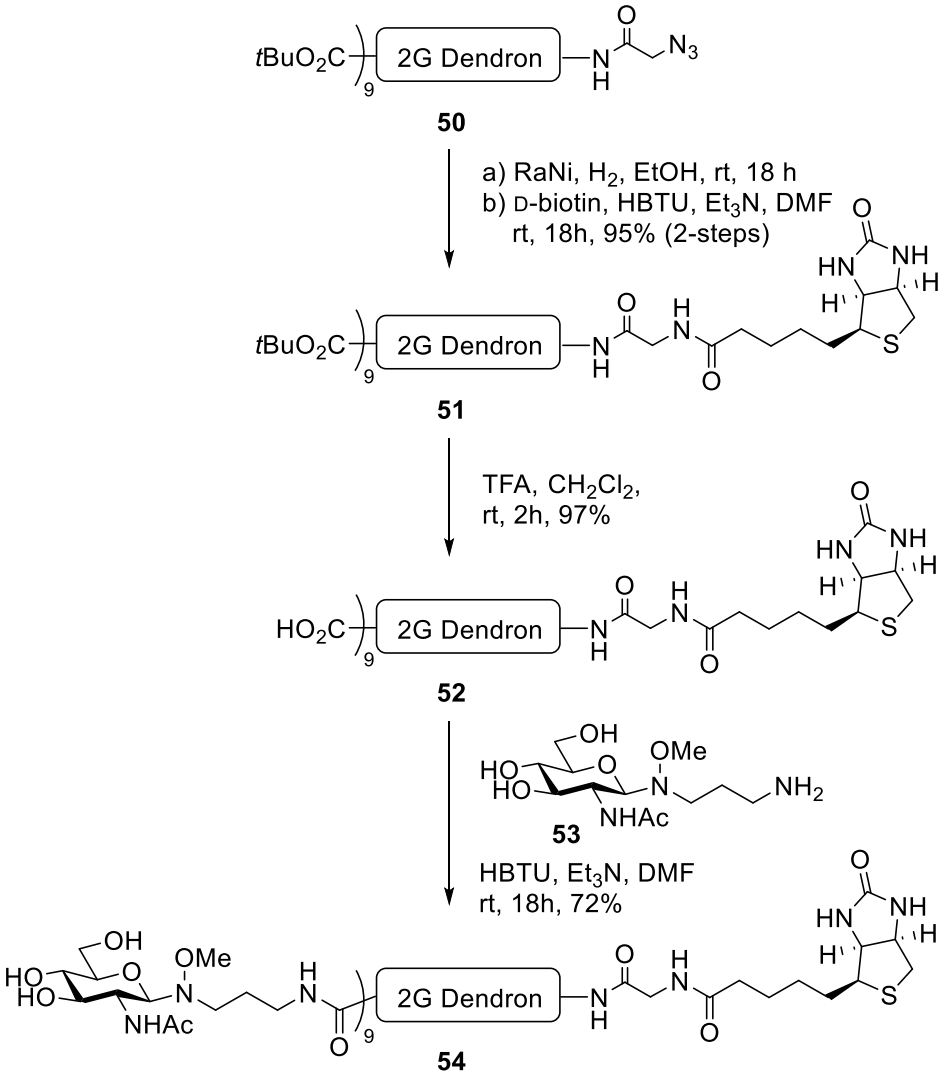


Figure 6. *LC-CAD trace for dendron **50** after extraction (red, top), after silica gel column chromatography (blue, middle) and ODS-reverse phase chromatography (black, bottom). C_{18} LC conditions: 5/95 → 100/0 MeCN/H_2O, v/v (10 min gradient), 2 min 100/0 MeCN:H_2O, v/v; Retention times (min): 8.6 (**49**), 11.1 (**50**);*

3.2.4 Dendron functionalisation

3.2.4.1 Biotinylated glycodendrons

After the successful preparation of the orthogonally protected dendron **50**, the synthesis of the second generation biotin-functionalised glycodendron was undertaken. To this end, the azide was first converted to the corresponding amine via a Raney-Nickel hydrogenation reaction and the crude product was then coupled to D-biotin using an HBTU-mediated peptide ligation (Scheme 13). This gave the biotin functionalised dendron **51** in 96% yield over two steps. Again, glycodendron **51** was purified by both silica gel as well as reverse phase chromatography.



Scheme 13. *Functionalisation of the second generation dendron* ***54****.*

Removal of the *tert*-butyl esters in dendron **51** using the previously optimised TFA/CH_2Cl_2 methodology yielded **52** in 2 hours. The crude reaction mixture was then concentrated, co-evaporated with H_2O and subsequently subjected to a coupling reaction with amine-functionalised carbohydrates. To establish that the bifunctionalised methoxyamine linker (see Chapter 2) was suitable for multivalent conjugation strategies, an amide coupling between dendron **52** and oxyamine glycoside **53** was attempted whereby an excess of 18 molar equivalents of amine **53** was used in order to facilitate the conjugation. Using HRMS-analysis of the crude reaction mixture, it was determined that the previous conjugation conditions (HBTU and Et_3N in DMF) gave rise to the desired glycodendron

54 in about an hour, however, the reaction mixture was stirred overnight to ensure optimal conjugation. Purification of the glycodendron **54** was then achieved by membrane dialysis (cellulose ester dialysis membrane, molecular weight cut-off 500D). Here, the crude reaction mixture was diluted with water and dialysed for 48 hours with a 0.1 M Na_2HPO_4 buffer at pH 7.5 to prevent oxyamine linker hydrolysis (see Chapter 2). After dialysis, the glycodendron was lyophilised and the product was purified by reverse phase chromatography (C_8) to give glycodendron **54** in a good (72%) yield. The glycodendron **54** was analysed using ^{1}H NMR analysis, and in addition, HSQC analysis (Figure 7) showed the presence of all CH resonances, which could not be observed in the ^{13}C spectrum. Taken as a whole, the synthesis of the biotin functionalised glycodendron demonstrated the use of the dendron scaffold to present carbohydrates in a multivalent fashion and moreover, the effectiveness of the oxyamine linker for glycan conjugation.

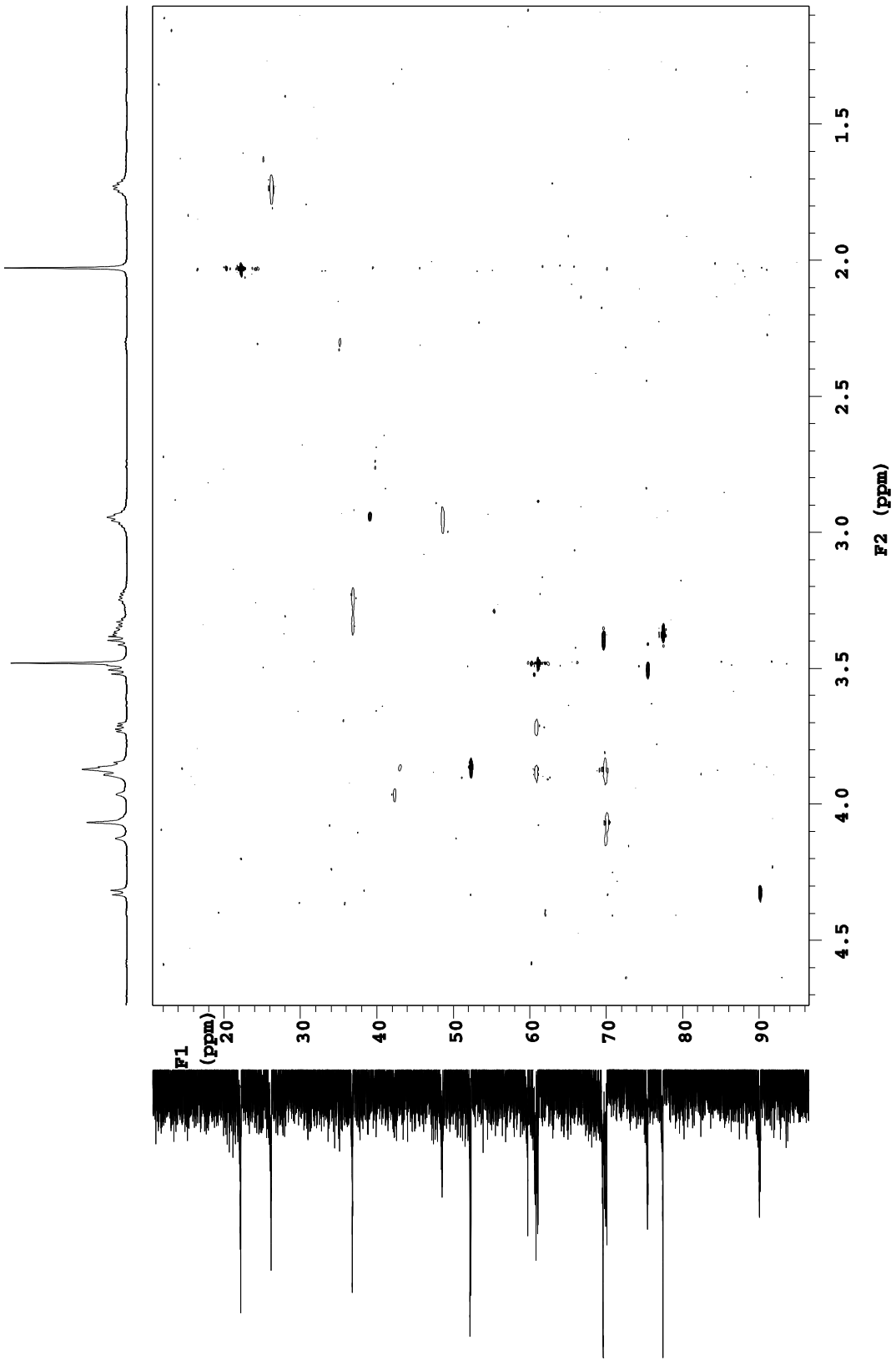
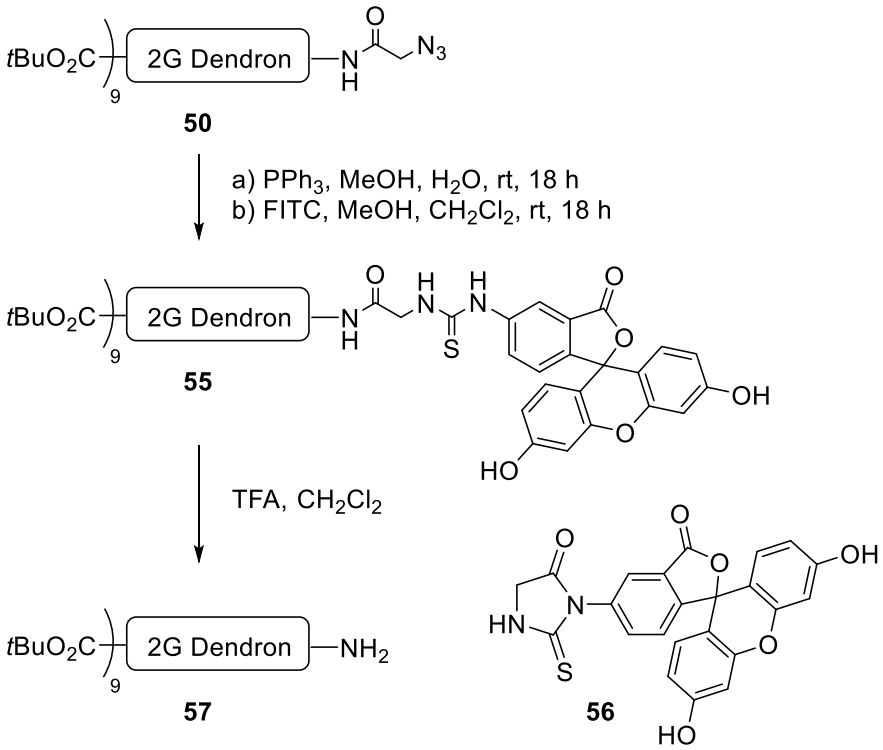


Figure 7. *HSQC of glycodendron* ***54****. (D_2O, 500MHz)*

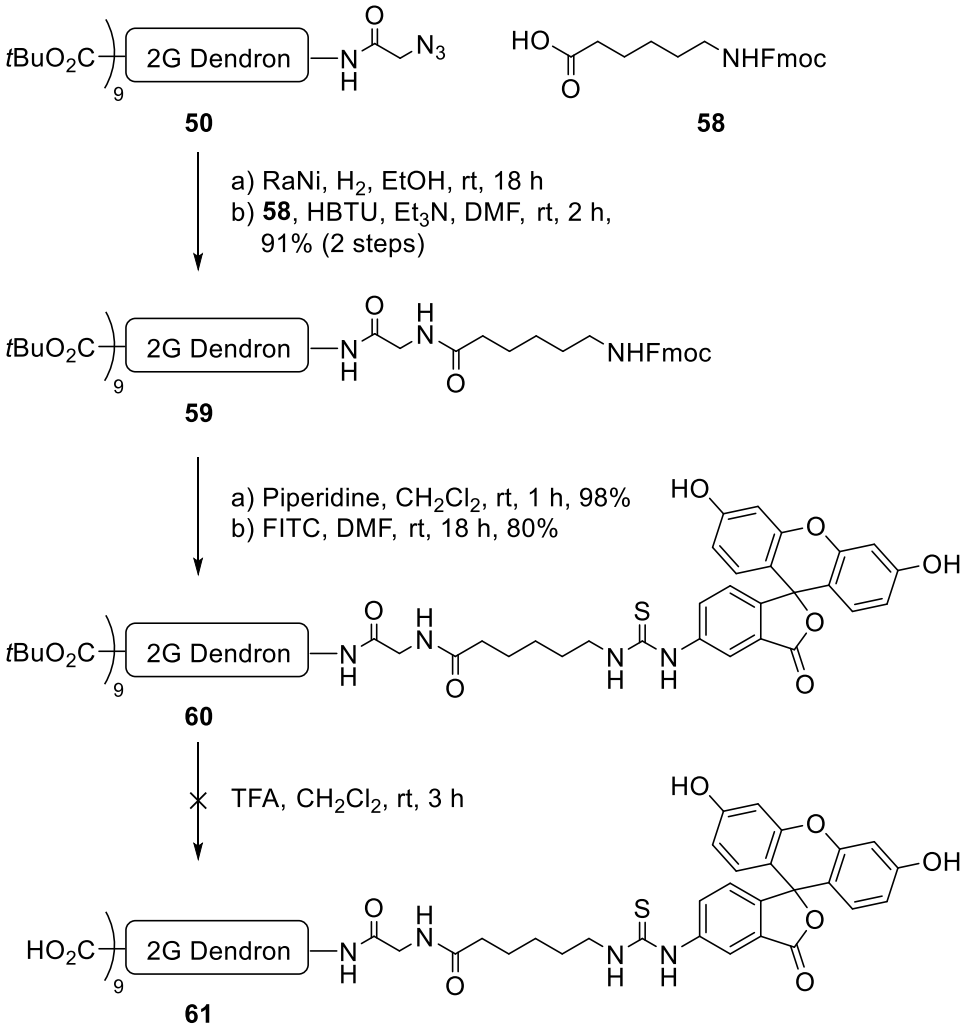
3.2.4.2 Fluorescent glycodendrons

In addition to biotin-functionalised glycodendrons, a strategy for the synthesis of fluorescent glycodendrons was explored as these latter constructs have wide application in microscopy and in flow cytometry. To this end, the azide moiety of the second generation dendron **50** was reduced to the corresponding amine, followed by the smooth ligation with fluorescein isothiocyanate isomer I (FITC) to give the fluorescent dendron **55** (Scheme 14). Next, TFA treatment of **55** in CH_2Cl_2 effectively removed the *tert*-butyl esters, however, in addition the Edman degradation reaction, which can be used to determine amino acid sequences,[40] was observed. Here, the glycinamide moiety was eliminated to give the cyclic byproduct **56** [HRMS(ESI) *m/z* calcd. for $[C_{23}H_{15}N_2O_6S]^+$: 447.0645, obsd.: 447.0660][41] and the corresponding dendron without glycine moiety **57** [^{1}H NMR (500MHz, D_2O) δ 4.16 (s, 18H, 9 × CH_2O), 4.14 (s, 6H, 3 × CH_2O), 3.93 (s, 6H, 3 × CH_2C=O, 3.84 (s, 18H, 9 × CH_2C=O), 3.81 (s, 6H, 3 × CH_2N); HRMS(ESI) *m/z* calcd. for $[C_{46}H_{73}N_7O_{36}]^{2+}$: 649.7043, obsd.: 649.7051]. Optimisation of this reaction was thought to be difficult due to the fact that nine esters need to be cleaved without the undesired Edman degradation reaction, which occurs below pH 3. Thus, an alternative method for the synthesis of the fluorescent glycodendrons was investigated.



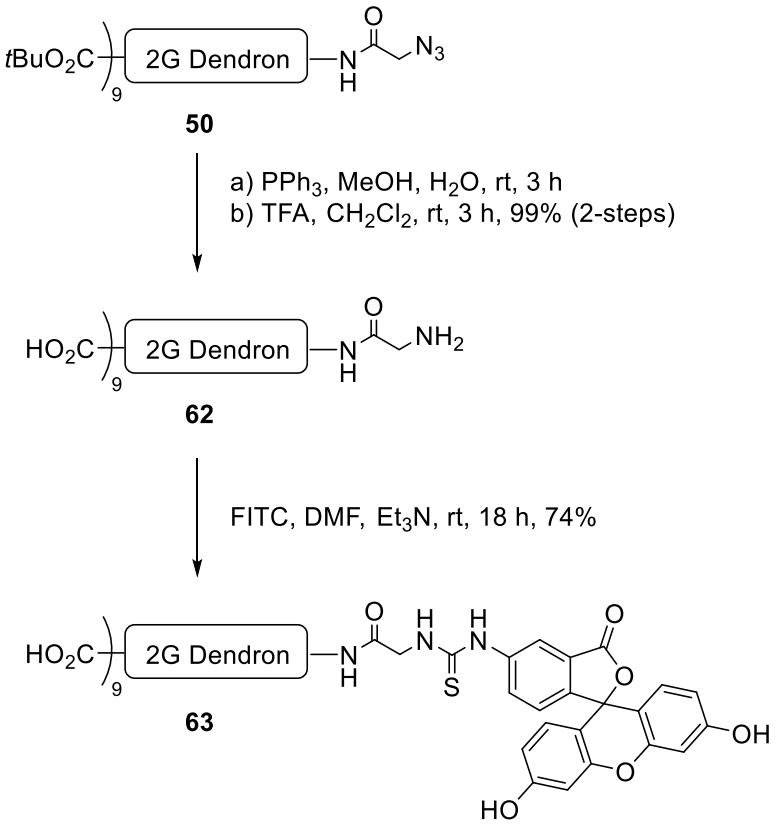
Scheme 14. *Attempted synthesis of fluorescein-based dendrons.*

To prevent the Edman degradation byproduct, the azide of the second generation dendron **50** was converted to the corresponding amine and conjugated to Fmoc-6-aminocaproic acid **58**[42] to give dendron **59** in an excellent yield (91% over 2-steps) (Scheme 15). Fmoc deprotection with piperidine then gave the hexanamide amine dendron, which was readily purified using size exclusion chromatography to remove the piperidine. Next, FITC conjugation in DMF with Et_3N as a base gave the fluorescent dendron **60** in good (80%) yield. In an attempt to synthesise dendron **61**, dendron **60** was treated with TFA, however, degradation of the fluorophore resulted in the isolation of an unknown dendron. Thus, another synthetic approach was required.



Scheme 15. *An alternative attempt to synthesise a fluorescent dendron.*

In the next approach, dendron **50** was deprotected to the amino acid, followed by conjugation with FITC (Scheme 16). Here, the second generation dendron was deprotected using the Staudinger azide reduction and subsequent TFA treatment to give the amino-acid **62** in an excellent yield (99%), which could be readily purified by filtration to remove the triphenylphosphine salts. Next, conjugation with FITC in DMF and Et_3N gave the desired fluorescent dendron **63** in an excellent yield (74%). Here it should be noted that the dendron was obtained as the triethylamine salt after size exclusion chromatography, however, the dendron could be obtained as the nona-valent acid after an ion exchange plug (Dowex H^+). With the fluorescent dendron in hand, chemical ligation with amine functionalised glycans gives rise to the desired fluorescent dendrons. In Chapter 5, the conjugation of the Lewis^X glycan with dendron **63**, as well as the biological evaluation of these glycodendrons is discussed.



Scheme 16. *Synthesis of fluorescent dendron* ***63****.*

3.3 Conclusion

In summary, the design and synthesis of a novel glycodendron has been presented. Even though the field for dendrimer chemistry has rapidly expanded in the last decades, with many dendron core-structures now being commercially available, the presented strategy allows for the synthesis of a novel dendron core structure that can be functionalised as required. In the presented synthetic route, a first generation dendron was synthesised from the TRIS core moiety in 2-steps. Here, optimisation of the amine protection, as well as the alkylation reaction, resulted in the formation of the first generation dendron **47** on a multi-gram scale. Next, the double exponential growth approach was used, which allows for the rapid 3-step assembly of a second generation dendron **50** in 81% yield on a gram scale. Here, the combination of silica gel column chromatography as well as reverse phase (C_{18}) column chromatography proved to be very successful in the purification of these macromolecules.

With the bi-functional second generation dendron in hand, rapid functionalisation allows for use of these dendrons in various applications. Accordingly, a core-biotin derivatised dendron was then synthesised in two steps, and after ester deprotection, the dendron was subjected to glycosylation through the use of the oxyamine amine functionalised *N*-acetylglucosamine **53** to give the biotinylated glycodendron **54.**

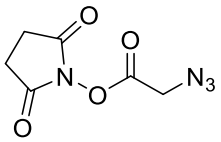
In addition, a multivalent fluorescent glycodendron was synthesised for use in microscopy and flow cytometry. Here, it was required to globally deprotect the second generation dendron **50** to the amino acid before coupling with FITC to give the desired fluorescent dendron **63** in a good yield. This fluorescent dendron was later utilised for conjugation with the Lewis^X glycan, as will be discussed in Chapter 5.

Taken as a whole, the novel and efficient synthesis of second generation dendrons allows for the rapid functionalisation of the dendron with the substrate of choice. Here, the conjugation with D-biotin or a fluorescent moiety allows for the use of these dendrons in various biological assays, including microscopy, ELISA and flow cytometry. Moreover, it was demonstrated that the effective use of the oxyamine linker strategy for glycan conjugation allows for the efficient assembly of complex multivalent bio-molecules.

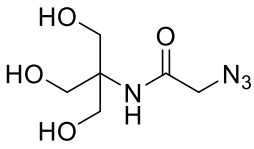
3.4 Experimental

General procedure. Prior to use, THF (Pancreac) was distilled from sodium and benzophenone, CH_2Cl_2 (Fisher) was distilled from P_2O_5, DMF was distilled from BaO, Et_3N (Sigma) was distilled from KOH, and H_2O was distilled. Bromoacetic acid (Sigma), NaN_3 (BDH), conc HCl (Panreac), *N*-hydroxysuccinamide (Aldrich), *N,N'*-Dicyclohexylcarbodiimide (Aldrich), TRIS(hydroxymethyl)aminomethane (Sigma), NaH (Aldrich, 60% dispersed in mineral oil), *tert*-butyl bromoacetate (Apollo Scientific), trifluoroacetic acid (Sigma), Raney-Nickel (Sigma-Aldrich), HBTU (Apollo Scientific), D-biotin (Acros), Ph_3P (Acros), fluorescein isothiocyanate isomer I (Sigma), anhydrous Et_2O (Pancreac), EtOAc (Pancreac), petroleum ether (Pure Science), toluene (Panreac), MeOH (Pure Science), $CHCl_3$ (Pancreac), EtOH (absolute, Pure Science), $NaHCO_3$ (Pure Science), $Na_2HPO_4.7H_2O$ (J.T.Baker), NaOH (Pure Science), $MgSO_4$ (Pure Science) and NaCl (Pancreac) were used as received.

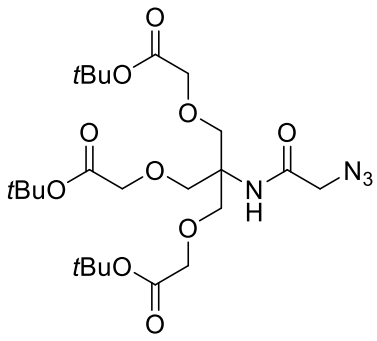
All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC-analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, with fluorescent indicator UV_{254}) with detection by UV-absorption (short wave UV – 254 nm; long wave UV – 366 nm), by dipping in 10% H_2SO_4 in EtOH followed by charring at ~150 °C, by dipping in I_2 in silica, or by dipping into a solution of ninhydrin in EtOH followed by charring at ~150 °C. Column chromatography was performed on Pure Science silica gel (40 – 63 micron). AccuBOND II ODS-C_{18} (Agilent) was used for reverse phase chromatography. Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 20 °C in D_2O, CD_3OD, $CDCl_3$, or pyridine-d_5 using either a Varian INOVA operating at 500 MHz or Varian VNMRS operating at 600 MHz. Chemical shifts are given in ppm (δ) relative to solvent residues. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.



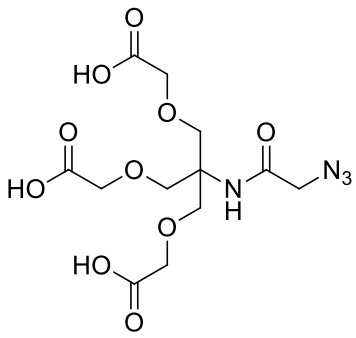
***N*-Hydroxysuccinimidyl azidoacetate (45).** To a solution of bromoacetic acid **43** (15.1 g, 0.11 mol) in water (30 mL), NaN_3 (30.0 g, 0.43 mmol) was added and the reaction mixture was stirred at r.t. for 3 h. The reaction mixture was carefully diluted with 6M aq. HCl (50 mL) (*CAUTION: formation of hydrazoic acid*) and extracted with Et_2O (2 × 100 mL). The organic layers were dried with $MgSO_4$, filtered and concentrated *in vacuo*, to give azide **44** as a colourless oil (10.7 g, 0.11 mmol, 99%). *The crude reaction product was used in the esterification reaction without further purification.* To a solution of crude 2-azido acetic acid **44** (9.70 g, 96.0 mmol) in dry CH_2Cl_2 (100 mL), *N*-hydroxysuccinamide (13.2 g, 115 mmol) and *N*,*N'*-dicyclohexylcarbodiimide (17.8 mL, 115 mmol) were added at 0 °C and the reaction mixture was stirred at r.t. for 18 h. The reaction mixture was filtered and concentrated *in vacuo*. To the crude residue, EtOAc (150 mL) was added and the solids were removed by filtration and the mixture was concentrated *in vacuo*. The residue was purified by silica gel column chromatography (PE/EtOAc, 75/25 → 25/75, v/v) and crystallised from CH_2Cl_2/PE to yield title compound **45** as a white solid (17.9 g, 90.2 mmol, 94%). R_f = 0.25 (PE/EtOAc, 1/1, v/v); Mp 110-115 °C; IR (film) 2995, 2935, 2110, 1818, 1781, 1727, 1428, 1416, 1369, 1278, 1199, 1087 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 4.24 (s, 2H, CH_2-N_3), 2.88 (s, 4H, 2 × CH_2C=O); ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.6 (2 × *N*-C=O), 164.3 (*O*-C=O), 48.1 (CH_2-N_3), 25.7 (2 × αCH_2).



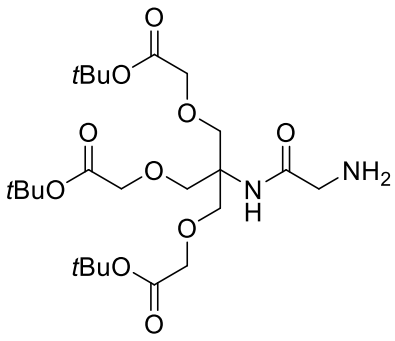
***N*-Azidoacetyl-1,1,1-tris(hydroxymethyl)aminomethane (46).** To a solution of succinimidyl ester **45** (8.83 g, 44.6 mmol) in freshly distilled DMF (100 mL), TRIS(hydroxymethyl)aminomethane **1** (26.9 g, 222 mmol) and Et_3N (0.6 mL, 4.5 mmol) were added and the reaction mixture was stirred at r.t. for 18 h. The reaction mixture was concentrated *in vacuo*, then co-evaporated with H_2O to remove traces of DMF. The residue was purified by silica gel column chromatography (dry-loading, PE:EtOAc, 50/50 → 0/100, v/v) yielded the title compound **46** as a white foam (7.84 g, 38.4 mmol, 86%). R_f = 0.50 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 5/2/2/1, v/v/v/v); IR (film) 3359, 2979, 2946, 2890, 2113, 1739, 1650, 1540, 1454, 1367, 1282, 1229, 1217, 1053 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.00 (s, 2H, CH_2-N_3), 3.76 (s, 6H, 3 × CH_2-*O*); ^{13}C NMR (125 MHz, D_2O) δ 170.4 (C=O Amide), 62.1 (Cq tris), 60.1 (3 × CH_2-*O*), 51.9 (CH_2-N_3); HRMS(ESI) *m/z* calcd. for $[C_6H_{13}N_4O_4]^+$: 205.0931, obsd.: 205.0930.



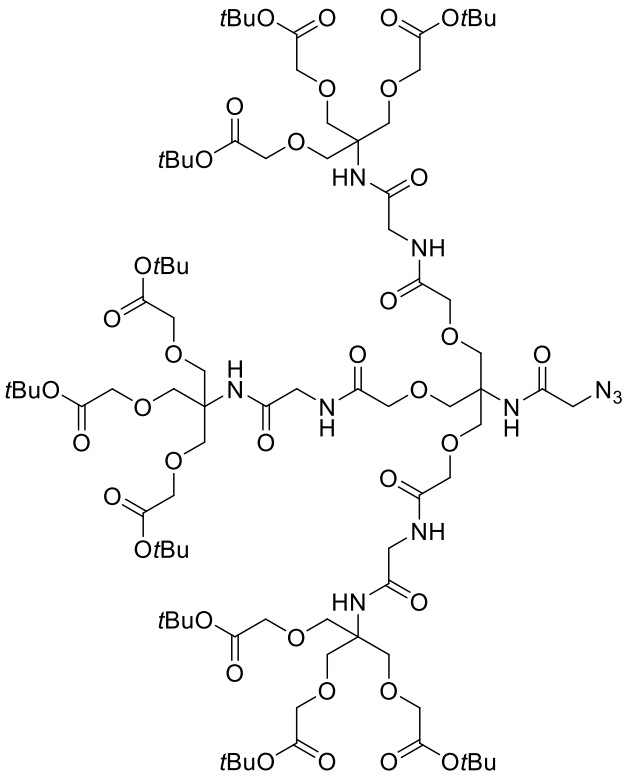
***N*-Azidoacetyl-1,1,1-tris(*tert*-butyloxycarbonylmethyloxymethyl)aminomethane (47).** To a solution of triol **46** (366 mg, 1.79 mmol) in DMF (9 mL) and toluene (9 mL), *tert*-butyl bromoacetate (1.06 mL, 7.16 mmol) and NaH (286 mg, 7.16 mmol) were added and the reaction mixture was stirred at r.t. for 4 h. The reaction mixture was quenched by the addition of ice water (20 mL) and was then extracted with Et_2O (2 × 100 mL). The organic layers were dried with $MgSO_4$, filtered and concentrated *in vacuo*. Purification by silica gel column chromatography (PE:EtOAc, 85/15 → 75/25, v/v) and reverse phase column chromatography (C_{18}, H_2O/MeOH, 50/50 → 20/80, v/v) yielded tri-alkylated **47** as a white foam (464 mg, 0.85 mmol, 47%). R_f = 0.44 (PE/EtOAc, 70/30, v/v); IR (film) 3316, 2979, 2935, 2107, 1745, 1684, 1536, 1473, 1428, 1393, 1368, 1229, 1158, 1119, 1037, 971, 915, 845, 731, 647 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.86 (s,1H, NH), 3.95 (s, 6H, 3 × CH_2C=O), 3.92 (s, 6H, 3 × CH_2-*O*), 3.85 (s, 2H, CH_2-N_3), 1.45 (s, 27H, CH_3 *t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2 (3 × C=O ester), 167.5 (1 × C=O amide), 82.0 (3 × Cq *t*Bu), 70.1 (3 × CH_2-*O*), 68.9 (3 × CH_2C=O), 60.1 (Cq tris), 52.9 (CH_2-N_3), 28.2 (9 × CH_3 *t*Bu); HRMS(ESI) *m/z* calcd. for $[C_{24}H_{43}N_4O_{10}]^+$: 547.2974, obsd.: 547.2981.



***N*-Azidoacetyl-1,1,1-tris(carboxymethyloxymethyl)aminomethane (48).** To a solution of dendron **47** (508 mg, 0.93 mmol) in CH_2Cl_2 (5 mL), freshly distilled trifluoroacetic acid (5 mL) was added and the reaction mixture was stirred at r.t. for 2 h. The reaction mixture was then concentrated *in vacuo* and coevaporated with CH_2Cl_2 (10 mL). The residue was purified by reverse phase column chromatography (C_{18}, H_2O/MeOH, 100/0 → 70/30, v/v) yielded acid **48** as a colourless oil. R_f = 0.20 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 33%), 5/2/2/1, v/v/v/v); IR (film) 3418, 2930, 2114, 1727, 1661, 1547, 1427, 1249, 1121 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.20 (s, 6H, 3 × CH_2C=O), 3.98 (s, 6H, 3 × CH_2-*O*), 3.90 (s, 2H, CH_2-N_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.3 (3 × C=O), 170.2 (1 × C=O amide), 69.6 (3 × CH_2-*O*), 68.1 (3 × CH_2C=O), 59.9 (Cq tris), 52.0 (CH_2-N_3); HRMS(ESI) *m/z* calcd. for $[C_{12}H_{19}N_4O_{10}Na]^+$: 401.0915, obsd.: 401.0920.

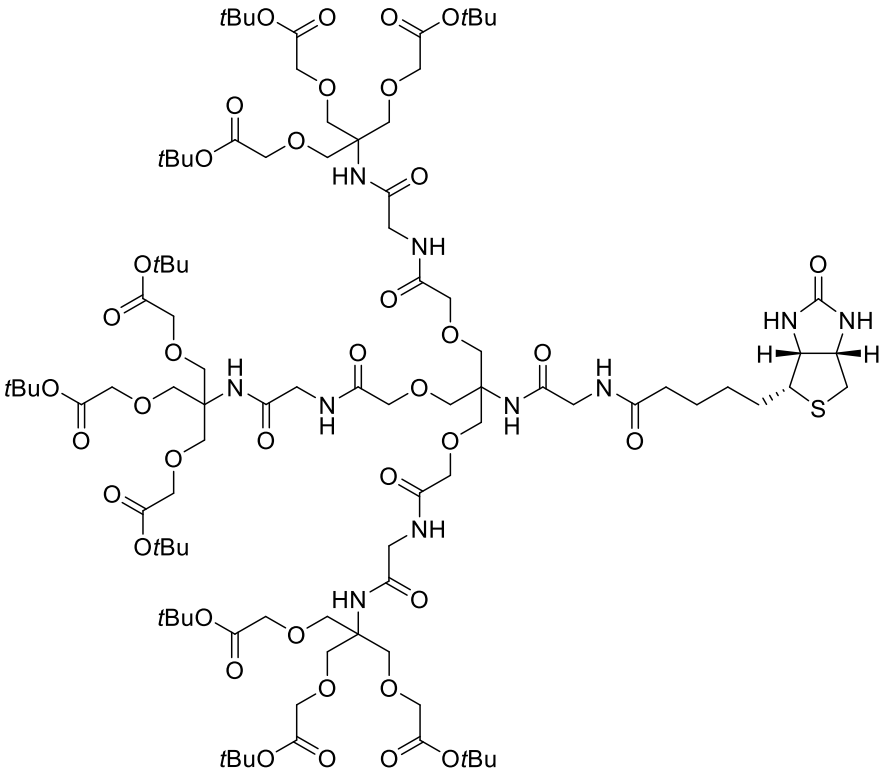


***N*-Glycyl-1,1,1-tris(*tert*-butyloxycarbonylmethyloxymethyl)-aminomethane (49).** To a solution of dendron **47** (220 mg, 0.40 mmol) in ethanol (1 mL) and THF (1 mL), activated Raney-Nickel (120 mg) was added and hydrogen gas was then bubbled through the reaction mixture at r.t. for 18 h. The reaction mixture was filtered, washed with sat. NaCl (aq.) (50 mL) containing 1M NaOH (1 mL), extracted with CH_2Cl_2 (2 × 50 mL) and the combined organic layers were dried, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc/MeOH, 100/0 → 85/15, v/v) yielded amine **49** as a colourless oil (207 mg, 0.40 mmol, 99%). R_f = 0.09 (EtOAc/MeOH, 90/10, v/v); IR (film) 3336, 2978, 3933, 1745, 1674, 1520, 1368, 1300, 1229, 1159, 1121, 1044, 847 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.82 (s, 1H, NH), 3.92 (s, 6H, 3 × CH_2C=O), 3.88 (s, 6H, 3 × CH_2-*O*), 3.30 (s, 2H, CH_2-NH_2), 2.07 (bs, 2H, NH_2) 1.42 (s, 27H, 9 × CH_3 *t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.2 (1 × C=O amide), 170.3 (3 × C=O ester), 81.8 (3 × Cq *t*Bu), 70.2 (3 × CH_2-*O*), 69.0 (3 × CH_2C=O), 59.5 (Cq tris), 45.6 (CH_2-NH_2), 28.2 (9 × CH_3 *t*Bu); HRMS(ESI) *m/z* calcd. for $[C_{24}H_{45}N_2O_{10}Na]^+$: 543.2888, obsd.: 543.2891.



***N*-Azidoacetyl-1,1,1-tris(1,1,1-tris[*tert*-butyloxycarbonylmethyloxymethyl]methylamidocarbonylmethylamidocarbonylmethyloxy-methyl)aminomethane (50).** First generation tri-acid **48** (118 mg, 0.31 mmol) and primary amine **49** (728 mg, 1.40 mmol) were co-evaporated with DMF (2 × 5 mL). To the mixture DMF (3.1 mL),

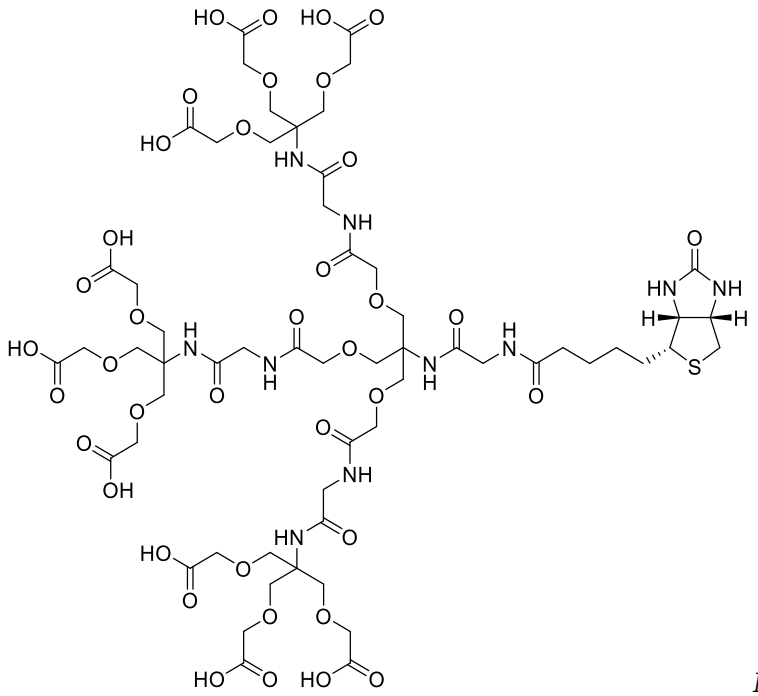
HBTU (590 mg, 1.56 mmol) and NEt_3 (0.65 mL, 4.67 mmol) were added and the reaction mixture was stirred at r.t. for 14 h. The reaction mixture was diluted with Et_2O (50 mL) and washed with 0.1M HCl (aq) (2 × 50 mL). The combined water layers were extracted with Et_2O (2 × 50 mL) and the combined organic layers were dried with $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (PE/EtOAc, 75/25 → 0/100, v/v; followed by EtOAc/MeOH 100/0 → 95/5, v/v) and reverse phase column chromatography (C_{18}, H_2O/MeOH, 50/50 → 0/100, v/v) yielded second generation dendron **50** as a colourless oil (475 mg, 0.25 mmol, 81%). R_f = 0.48 (EtOAc/MeOH, 95/5, v/v); IR (film) 3316, 3970, 2934, 2107, 1745, 1670, 1526, 1368, 1231, 1159, 1120, 917, 846, 731 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 7.62 (s, 3H, 3 × NH-TRIS), 7.44 (s, 1H, 1 × NH-TRIS), 7.24 (t, 3H, $J_{NH,CH2}$ = 5.0 Hz, 3 × NH gly), 4.06 (s, 6H, 3 × CH_2C=O), 4.01 (d, 6H, $J_{CH2,NH}$ = 4.5 Hz, 3 × CH_2-N), 3.98 (s, 2H, CH_2-N_3), 3.95 (s, 18H, 9 × CH_2C=O), 3.93 (s, 6H, 3 × CH_2-O), 3.89 (s, 18H, 9 × CH_2-O), 1.45 (s, 54H, 18 × CH_3 *t*-Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3 (9 × C=O ester), 169.6 (3 × C=O amide) , 168.5 (3 × C=O amide) , 168.2 (1 × C=O amide), 82.0 (Cq *t*Bu), 70.7 (3 × CH_2-*O*), 70.6 (3 × CH_2C=O), 70.2 (9 × CH_2-*O*), 68.9 (9 × CH_2C=O), 60.0 (3 × Cq tris), 59.8 (1 × Cq tris) 52.5 (CH_2-N_3), 42.2 (3 × CH_2-NH), 28.3 (27 × CH_3 *t*Bu); HRMS(ESI) *m/z* calcd. for $[C_{84}H_{146}N_{10}O_{37}]^{2+}$: 943.4920, obsd.: 943.4929.



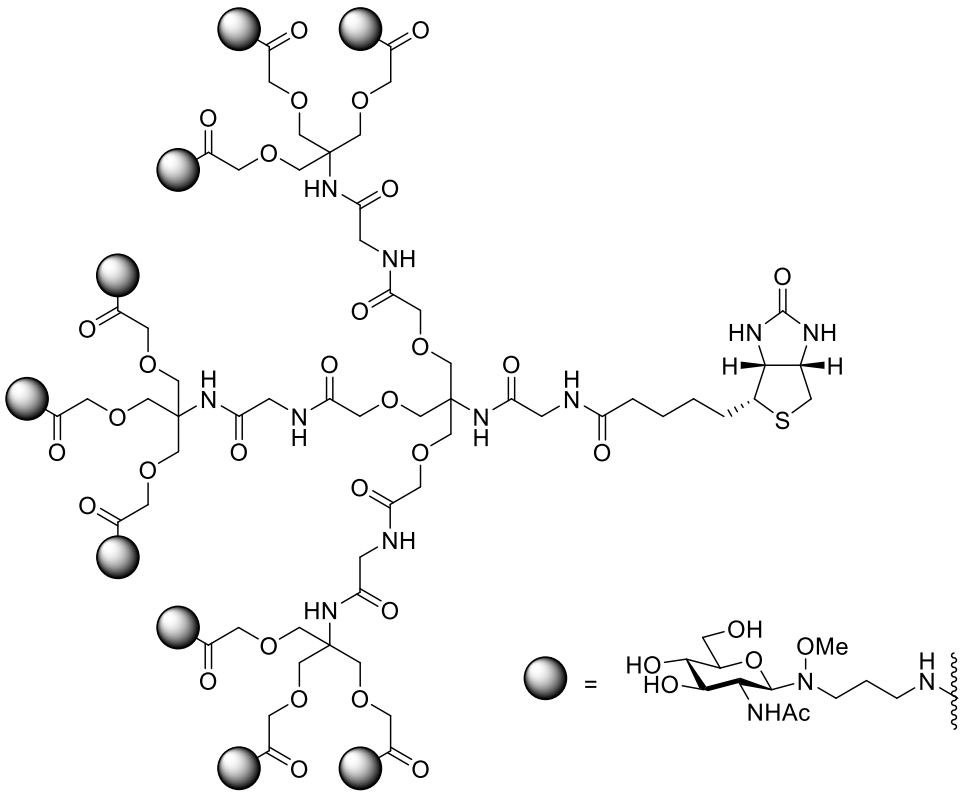
***N*-(2-D-Biotinylamido-acetyl)-1,1,1-tris(1,1,1-tris[*tert*-butyl-oxycarbonylmethyloxymethyl]methylamidocarbonyl-methylamidocarbonylmethyloxymethyl)aminomethane (51).** To a solution of second generation dendron **50** (139 mg, 73.7 µmol) in ethanol (1 mL) and THF (1 mL), activated Raney-Nickel (100

mg) was added and hydrogen gas was bubbled through the reaction mixture at r.t. for 20 h. The reaction mixture was filtered, washed with sat. aq. NaCl (50 mL) containing 1M NaOH (1 mL), extracted with CH_2Cl_2 (2 × 50 mL) and concentrated *in vacuo*. The resulting oil was then passed through a silica gel plug (EtOAc/MeOH, 100/0 → 85/15, v/v) to yield crude amine as a colourless oil, which was then used without further purification. HRMS(ESI) *m/z* calcd. for $[C_{84}H_{148}N_8O_{37}]^{2+}$: 930.4967, obsd.: 930.4960.

To crude second generation dendron amine in DMF (0.73 mL), D-biotin (27.0 mg, 110 µmol), HBTU (55.9 mg, 147 µmol) and NEt_3 (25 µL) were added and the reaction mixture was stirred at r.t. for 18 h. The reaction was diluted with Et_2O (50 mL) and washed with sat. aq. NaCl (2 × 50 mL). The combined water layers were extracted with Et_2O (2 × 50 mL) and the combined organic layers were dried with $MgSO_4$, filtered and concentrated *in vacuo*. Purification by column chromatography (CH_2Cl_2/MeOH, 100/0 → 90/10, v/v) and reverse phase column chromatography (C_{18}, H_2O/MeOH, 50/50 → 0/100, v/v) yielded 2nd generation dendron **51** as a colourless oil (146 mg, 70.1 µmol, 95%). R_f = 0.50 (CH_2Cl_2/MeOH, 90/10, v/v); IR (film) 3309, 3064, 2979, 2933, 1744, 1668, 1527, 1368, 1230, 1158, 1118, 1035, 846, 733 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.66 (s, 3H, 3 × NH-TRIS), 7.64 (t, 1H, $J_{CH2,NH}$ = 5.7 Hz, NH-gly), 7.57 (s, 1H, NH-TRIS), 7.54 (t, 3H, $J_{CH2,NH}$ = 5.1 Hz, 3 × NH-gly), 6.30 (s, 1H, NH biotin), 5.65 (s, 1H, NH biotin), 4.45 (dd, 1H, $J_{7,8a}$ = 5.3 Hz, $J_{6,7}$ = 7.0 Hz, H7-biotin), 4.27 (dd, 1H, $J_{5,6}$ = 5.1 Hz, $J_{6,7}$ = 7.0 Hz, H6-biotin), 4.00 (s, 6H, 3 × CH_2O), 3.97 (t, 6H, $J_{CH2,NH}$ = 4.3 Hz, 3 × CH_2N), 3.92 (s, 18H, 9 × CH_2O), 3.88 – 3.80 (m, 26H, 9 × CH_2C=O, 3 × CH_2C=O, CH_2N), 3.08 (dt, 1H, $J_{5,6}$ = 4.8 Hz, $J_{4,5}$ = 7.0 Hz, H5-biotin), 2.85 (dd, 1H, $J_{7,8a}$ = 5.3 Hz, $J_{8a,8b}$ =13.0 Hz, H8a-biotin), 2.69 (d, 1H, $J_{8a,8b}$ = 12.8 Hz, H8b-biotin), 2.28 – 2.16 (m, 2H, CH_2-1-biotin), 1.71 – 1.50 (m, 6H, CH_2-2, CH_2-3, CH_2-4-biotin), 1.41 (s, 81H, 27 × CH_3 *t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.4 (C=O biotin), 170.6 (C=O dendron), 170.1 (9 × C=O), 170.0 (3 × C=O), 169.0 (3 × C=O), 163.9 (C=O carbamide biotin), 81.9 (9 × Cq *t*Bu), 70.6 (3 × CH_2C=O), 70.4 (3 × CH_2-O), 70.0 (9 × CH_2-O), 68.8 (9 × CH_2C=O), 61.8 (C6-biotin), 60.1 (C7-biotin), 59.9 (3 × Cq tris), 59.5 (1 × Cq tris), 55.5 (C5-biotin), 43.7 (1 × CH_2-N), 42.2 (3 × CH_2-N), 40.3 (C8-biotin), 35.2 (C1-biotin), 28.2 (C2-biotin), 28.0 (27 × CH_3), 27.9 (C4-biotin), 25.3 (C3-biotin); HRMS(ESI) *m/z* calcd. for $[C_{94}H_{162}N_{10}O_{39}S]^{2+}$: 1043.5355, obsd.: 1043.5351.

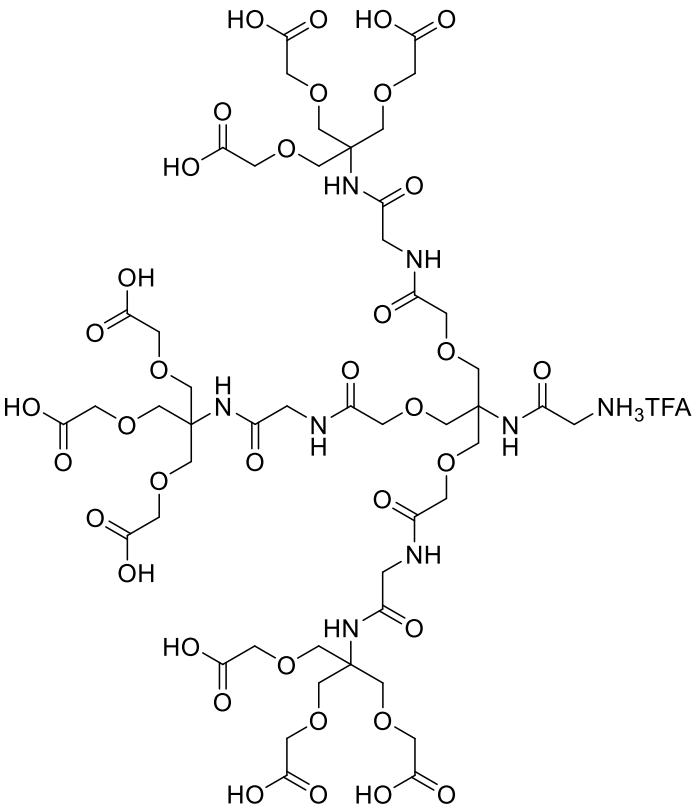


***N*-(2-D-Biotinylamidoacetyl)-1,1,1-tris(1,1,1-tris[carboxymethyloxymethyl]methylamidocarbonylmethylamidocarbonyl-methyloxymethyl)aminomethane (52).** To a solution of second generation dendron **51** (12.0 mg, 5.8 µmol) in CH_2Cl_2 (1 mL), freshly distilled trifluoroacetic acid (1 mL) was added and the reaction mixture was stirred at r.t. for 3 h. The crude reaction mixture was concentrated *in vacuo* and coevaporated with water (10 × 1 mL). Purification by reverse phase column chromatography (C_{18}, H_2O/MeOH, 100/0 → 70/30, v/v) yielded nona-acid **52** (8.8 mg, 5.6 mmol, 97%). R_f = 0.1 (EtOAc/MeOH, 80/20, v/v); IR (film) 3360, 3005, 2990, 2978, 2948, 1721, 1656, 1552, 1472, 1431, 1343, 1245, 1200, 1123, 1032, 1018, 979 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.57 (dd, 1H, $J_{7,8a}$ = 5.1 Hz, $J_{6,7}$ = 7.5 Hz, H7-biotin), 4.39 (dd, 1H, $J_{5,6}$ = 4.6, $J_{6,7}$ = 7.5 Hz, H6-biotin), 4.18 (s, 18H, 9 × CH_2CO), 4.12 (s, 6H, 3 × CH_2CO), 3.95 (s, 6H, 3 × CH_2NH), 3.88 (s, 18H, 9 × CH_2O), 3.86 (s, 6H, 3 × CH2O), 3.28 (dt, 1H, $J_{5,6}$ = 5.3 Hz, $J_{4,5}$ = 8.0 Hz, H5-biotin), 2.96 (dd, 1H, $J_{7,8a}$ = 5.0 Hz, $J_{8a,8b}$ = 13.0 Hz, H8a-biotin), 2.74 (d, 1H, $J_{8a,8b}$ = 13.0 Hz, H8b-biotin), 2.30 (t, 2H, $J_{1,2}$ = 7.2 Hz, CH_2-1-biotin), 1.74 – 1.50 (m, 4H, CH_2-4-biotin, CH_2-2-biotin), 1.39 (p, 2H, $J_{2,3}$ = $J_{3,4}$ = 7.4 Hz, CH_2-3-biotin); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.1 (C=O biotin), 174.2 (9 × C=O), 172.8 (3 × C=O), 171.3 (1 × C=O), 170.7 (3 × C=O), 165.2 (C=O carbamide biotin), 70.0 (3 × CH_2C=O), 69.8 (3 × CH_2-O), 69.7 (9 × CH_2-O), 68.0 (9 × CH_2C=O), 62.0 (C6-biotin), 60.2 (C7-biotin), 59.8 (3 × Cq tris), 59.6 (1 × Cq tris), 55.2 (C5-biotin), 43.0 (1 × CH_2-N), 42.4 (3 × CH_2-N), 39.6 (C8-biotin), 35.1 (C1-biotin), 27.9 (C2-biotin), 27.6 (C4-biotin), 25.0 (C3-biotin); HRMS(ESI) *m/z* calcd. for $[C_{58}H_{90}N_{10}O_{39}S]^{2+}$: 791.2538, obsd.: 791.2536.



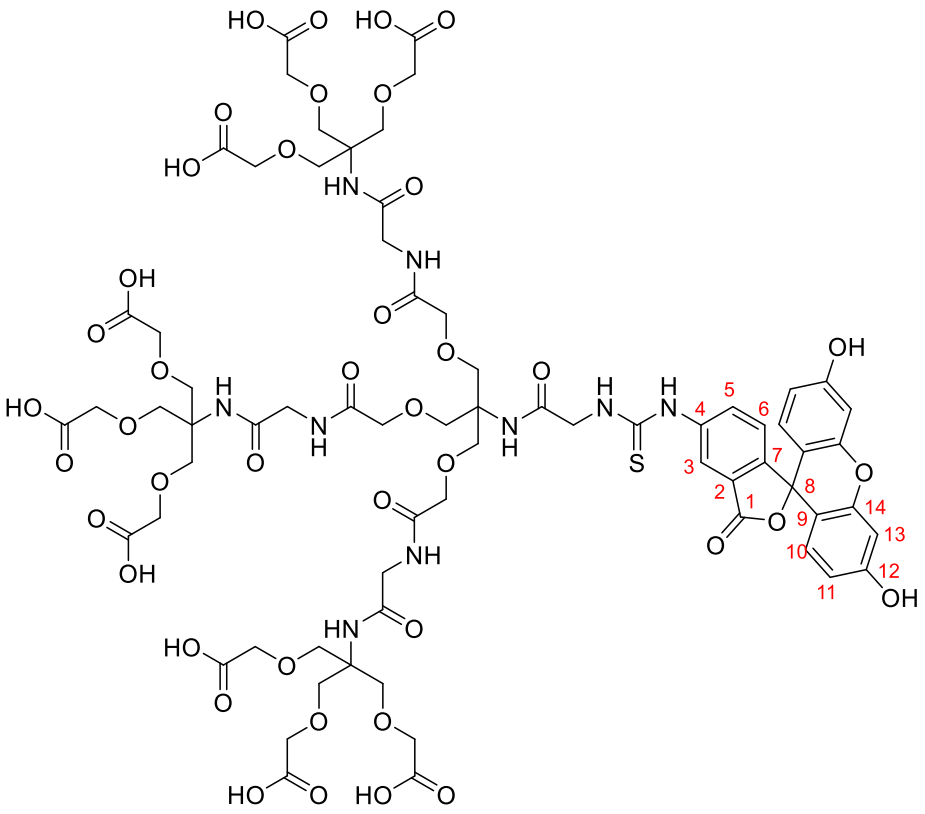
***N*-(2-D-Biotinylamido-acetyl)-1,1,1-tris(1,1,1-tris[*3-(N*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-*O*-methyl-hydroxylamine)propylamidocarbonylmethyloxymethyl]methyl-amidocarbonylmethylamidocarbonylmethyloxy-methyl)aminomethane (54).** Second generation dendron nona-acid **52** (1.5 mg, 0.95 µmol) and GlcNAc-amine **53** (5.3 mg, 17.1 µmol) were co-evaporated with freshly distilled DMF (2 × 2 mL). To the mixture DMF (0.2 mL) was added and the reaction mixture was half concentrated under reduced pressure to remove traces of Et_2NH. To the reaction mixture, HBTU (13.7 mg, 36 µmol) and distilled NEt_3 (25 µL) were added and the reaction mixture was stirred at r.t. for 14 h. The reaction mixture was diluted with water (2 mL) and purified using a dialysis in a Na_2HPO_4 solution (1 g/L), to remove the conjugation byproducts. The water was replaced twice a day for 4 days. The dendron was then purified using reverse phase column chromatography (C_8, H_2O/MeOH, 100/0 → 70/30, v/v) to give glycodendron **54** as a colourless oil (2.8 mg, 0.68 µmol, 72%). ^{1}H NMR (600 MHz, D_2O) δ 4.58 (dd, 1H, $J_{7,8a}$ = 5.1 Hz, $J_{6,7}$ = 7.7 Hz, H7-biotin), 4.40 (dd, 1H, $J_{5,6}$ = 4.7 Hz, $J_{6,7}$ = 7.7 Hz, H6-biotin), 4.32 (d, 9H, $J_{1,2}$ = 9.7 Hz, 9 × H-1 GlcNAc), 4.12 (s, 6H, 3 × CH_2C=O dendron), 4.07 (s, 18H, 9 × CH_2C=O dendron), 3.96 (s, 6H, 3 × CH_2-N dendron), 3.92 – 3.83 (m, 37H, 1 × CH_2N dendron, 9 × H-2 GlcNAc, 9 × H-6a GlcNAc, 9 × CH_2-dendron), 3.72 (dd, 9H, $J_{5,6a}$ = 5.5 Hz, $J_{6a,6b}$ = 12.4 Hz, 9 × H-6b GlcNAc), 3.51 (dd, 9H, $J_{2,3}$ = 8.8 Hz, $J_{3,4}$ = 9.8 Hz, 9 × H-3 GlcNAc), 3.48 (s, 27H, 9 × $NOCH_3$), 3.43 – 3.28 (m, 28H, 9 × CH_2a-3 linker, 9× H-4 GlcNAc, 9 × H-5 GlcNAc, H5-biotin), 3.24 (dt, 9H, $J_{2,3}$ = 7.2 Hz, $J_{3a,3b}$ = 13.4 Hz, 9 × CH_2b-3 linker), 3.01 – 2.87 (m, 19H, 9 × CH_2-1 linker, H8a-biotin), 2.75 (d, 1H, $J_{8a,8b}$ = 12.9 Hz,

H8b-biotin), 2.30 (t, 2H, $J_{1,2}$ = 7.3 Hz, CH_2-1-biotin), 2.03 (s, 27H, 9 × *N*-Ac), 1.88 – 1.78 (m, 1H, H4a-biotin), 1.77 – 1.69 (m, 18H, 9 × CH2-2 linker), 1.69 – 1.58 (m, 1H, H4a-biotin) 1.58 – 1.50 (m, 2H, CH_2-2-biotin), 1.44 – 1.36 (m, 2H, CH_2-3-biotin); HRMS(ESI) *m/z* calcd. for $[C_{166}H_{298}N_{37}O_{84}S]^{3+}$: 1395.6641, obsd.: 1395.6648.



***N*-Glycyl-1,1,1-tris(1,1,1-tris[carboxymethyloxymethyl]methylamidocarbonylmethylamidocarbonylmethyloxymethyl)aminomethane trifluoroacetic acid (62).** To a solution dendron **50** (60.5 mg, 31.8 μmol) in MeOH/H_2O/CH_2Cl_2 (3 mL, 3/1/1, v/v/v), triphenylphosphine (25.0 mg, 95.4 μmol) was added and the reaction mixture was stirred at rt for 18 h. The crude reaction mixture was concentrated *in vacuo* and co-evaporated with CH_2Cl_2 (2 × 10 mL). The residue was dissolved in CH_2Cl_2 (3 mL), TFA (3 mL) was added and the reaction mixture was stirred at rt for 3 h. The solvent was evaporated by an argon stream, H_2O (10 mL) was added, and the phosphine salts were removed via filtration, and the resulting residue was concentrated *in vacuo* to give crude amino acid **62**. (45.9 mg, 31.6 μmol, 99%). R_f = 0.05 (*t*BuOH/AcOH/H_2O, 4/1/1, v/v/v); IR (film) 3344, 3003, 2981, 2950, 2937, 2930, 1718, 1651, 1543, 1473, 1458, 1434, 1241, 1199, 1120, 1032, 952 cm^{-1}; ^1H NMR (500 MHz, D_2O) δ 4.06 (s, 18H, 9 × CH_2C=O), 4.00 (s, 6H, 3 × CH_2C=O), 3.83 (s, 6H, 3 × CH_2N), 3.77 (s, 6H, 3 × CH_2O), 3.75 (s, 18H, 9 × CH_2O), 3.71 (s, 2H, 1 × CH_2NH_2); ^{13}C NMR (125 MHz, D_2O) δ 174.1 (9 × C=O CO_2H), 172.6 (3 × C=ONH), 170.6 (3 × C=O), 166.7 (1 × C=O), 162.8, 162.5, 162.2, 161.9 (C=O

TFA), 119.4, 117.1, 114.8, 112.5 (CF_3 TFA), 69.83, 69.77 (3 × CH_2O, 3 × $CH_2C{=}O$), 69.6 (9 × CH_2O), 67.9 (9 × $CH_2C{=}O$), 59.9 (1 × Cq tris), 59.7 (3 × Cq tris), 42.3 (3 × CH_2NH), 40.6 (1 × CH_2NH_2); HRMS(ESI) *m/z* calcd. for $[C_{48}H_{76}N_8O_{37}]^{2+}$: 678.2150, obsd.: 678.2136.



***N*-[*N*-([(3',6'-Dihydroxy-3-oxospiro[isobenzofuran-1(*3H*),9'-(*9H*)xanthen]-5-yl)amino]-thioxomethyl)glycyl]-1,1,1-tris(1,1,1-tris[carboxymethyloxymethyl]methylamido-carbonylmethylamidocarbonylmethyloxymethyl)aminomethane (63).** To a solution of dendron **62** (25.1 mg, 17.3 μmol) in DMF (3 mL), fluorescein isothiocyanate isomer I (7.4 mg, 19.0 μmol) and Et_3N (1 mL) were added and the reaction mixture was stirred at rt for 18 h. The crude reaction mixture was concentrated *in vacuo*. The residue was purified with size exclusion chromatography (LH-20, CH_2Cl_2/MeOH, 50/50, v/v) and concentrated *in vacuo* to give the trimethylamine salt of dendron **63**. The dendron was co-evaporated with H_2O and purified using ion exchange chromatography (Dowex H^+, elute with H_2O) to give dendron **63** (22.3 mg, 12.8 mmol, 74%) as a yellow oil. R_f = 0.05 (*t*BuOH/AcOH/H_2O, 4/1/1, v/v/v); IR (film) 3463, 3366, 3074, 3015, 2970, 2946, 1738, 1656, 1548, 1436, 1366, 1229, 1216, 1206, 1119, 1051, 1032, 897 cm^{-1}; ^1H NMR (500 MHz, CD_3OD) δ 8.22 (s, 1H, H-3), 8.13 – 7.98 (m, NH), 7.94 (s, 3 × NH), 7.88 (s, 1 × NH), 7.84 (d, 1H, $J_{5,6}$ = 8.8 Hz, H-5), 7.17 (d, 1H, $J_{5,6}$ = 8.8 Hz, H-6), 6.78 – 6.63 (m, 4H, 2 × H-13, 2 × H-10),

6.58 (d, 2H, $J_{9,10}$ = 8.2 Hz, 2 × H-11), 4.35 (s, 2H, 1 × CH_2NH), 4.20 – 4.02 (bs, 24H, 3 × $CH_2C{=}O$, 9 × $CH_2C{=}O$), 4.02 – 3.92 (m, 12H, 3 × CH_2O, 3 × CH_2NH), 3.92 – 3.86 (m, 18H, 9 × CH_2O); ^{13}C NMR (125 MHz, CD_3OD) δ 183.5 (C=S), 174.5 (9 × C=O), 173.0 (3 × C=O), 172.0 (1 × C=O glyc), 171.4 (3 × C=O glyc), 171.2 (C=O C-1), 161.5, 154.2 (C-12, C14), 149.7 (C-7), 142.2 (C-2/4/8), 132.1 (C-5), 130.4 (C-13), 130.3 (C-2/4/8), 128.9 (C-2/4/8), 125.8 (C-6), 120.3 (C-3), 113.8 (C-11), 111.5 (C-9), 103.6 (C-10), 71.4 (3 × CH_2-O), 71.0 (9 × CH_2O), 69.3 (3 × $CH_2C{=}O$), 69.2 (9 × $CH_2C{=}O$), 61.3 (3 × C_q tris), 61.2 (1 × C_q tris), 48.9 (1 × CH_2NH), 43.5 (3 × CH_2NH); HRMS(ESI) *m/z* calcd. for $[C_{69}H_{87}N_9O_{42}S]^{2+}$: 872.7329, obsd.: 872.7335.

3.5 References

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Chapter 4.

Highly efficient synthesis of Lewis^X via a versatile late-stage crystalline trisaccharide intermediate.

4.1 Introduction

The Lewis antigens are a related set of glycans and are part of glycoconjugates (*i.e.*glycolipids and/or glycoproteins) found in nature. They are divided into two subgroups, the Type-1 and Type-2 Lewis antigens, which are characterised by the presence of the Fuc-α-(1→4)-GlcNAc or Fuc-α-(1→3)-GlcNAc residues, respectively.[1] In addition, several of the Lewis glycans may also be sialyated and/or sulfated (e.g. Sialyl Lewis^X). These antigens are synthesised in the tissue and subsequently distributed throughout the body via excretion of glycolipids into the plasma.[2] The Type-1 antigens, also known as the Lewis blood group antigens (e.g. Lewis^a and Lewis^b, Figure 1), are incorporated into erythrocytes and various tissues.[2,3] The other sub-class, Type-2 antigens (*i.e.* Lewis^X, Lewis^Y, Sialyl Lewis^X, 3'-Sulfo-Lewis^X, 6-Sulfo-Sialyl Lewis^X, 6'-Sulfo-Sialyl Lewis^X and 6,6'-Sialyl Lewis^X) are serological characters widely distributed in human and animal organisms.[2,3] These Lewis glycans have important roles in biology and members of both subsets of Lewis antigens have been implicated in favourable and unfavourable selectin-dependent leukocyte and cell adhesion processes[2,3] with, for example, Sialyl Lewis^A and Sialyl Lewis^X being used as serological markers for cancer prognosis and progression,[4–6] while Sialyl Lewis^X also has an essential role in human fertilisation.[7] Targeting the dendritic cell (DC) C-type lectin, DC-SIGN, which binds Lewis glycans (*i.e.* Lewis^X, Lewis^Y, Lewis^b and Lewis^a), has been shown to increase antigen cross-presentation and enhance vaccine efficacy for cancer and intracellular pathogens.[8–12] Accordingly, there has been much interest in targeting this lectin using antigen-conjugated antibodies or antigen-conjugated Lewis glycans (*e.g.* Lewis^X), whereby the latter approach is thought to be advantageous due to reduced side-effects and the ease of synthesis.[13] In

this chapter the synthesis of the Lewis^X ligand will be discussed, with the aim to synthesise a versatile trisaccharide intermediate, which can be used towards the synthesis of other Lewis glycans.

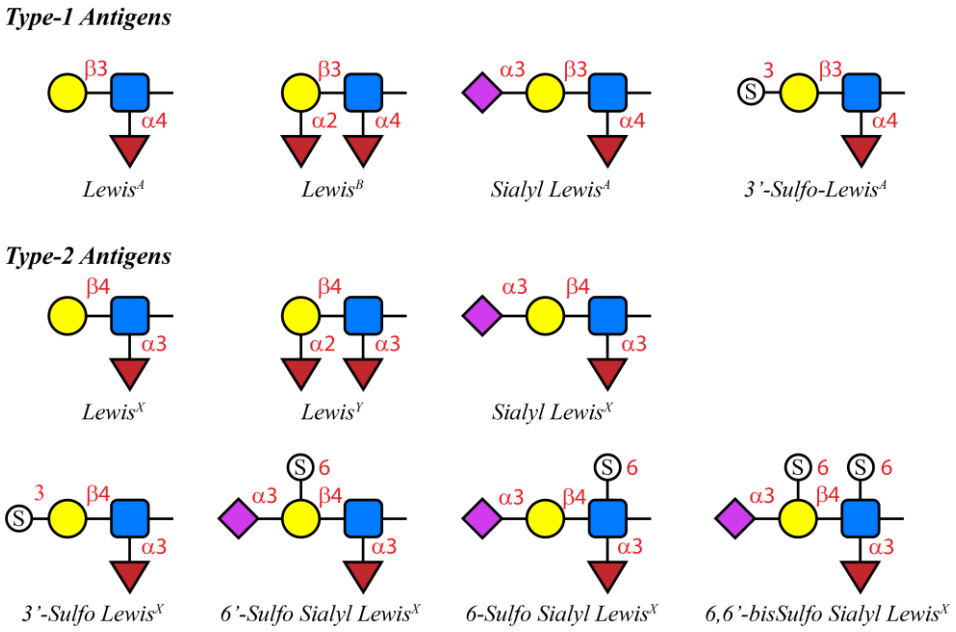


Figure 1. *Schematical presentation of Type-1 and Type-2 Lewis antigens; ● = Galactose; ■ = N-Acetylglucosamine; ▼ = Fucose; ◆ = Sialic acid; Ⓢ = sulfate.*

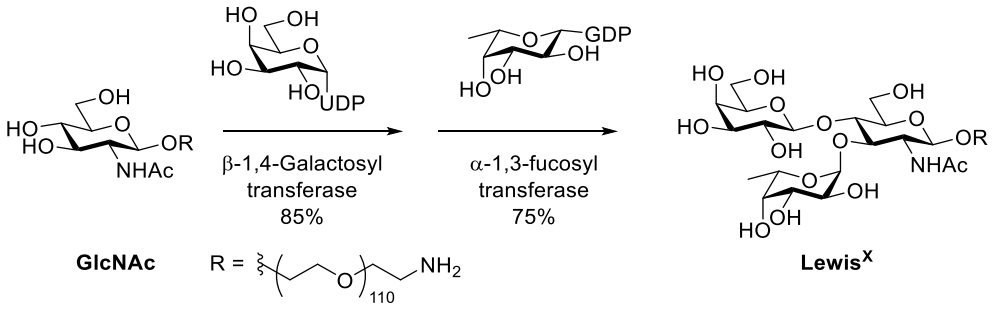
4.1.1 Lewis^X Synthesis

Although many of the Lewis structures are commercially available, they are rather expensive, with for example, 10 mg of Lewis^X costing ~$1000 NZD.[14] The availability of Lewis^X at a lower cost would allow for further investigation into the biological functions of Lewis^X, particularly as more complex Lewis^X constructs (e.g. fluorescent derivatives) can be more readily prepared. Moreover, the efficient synthesis of Lewis^X would not only provide greater amounts of material, but if the synthetic route is developed with an appropriate protecting group strategy other more complex glycan ligands, such as Sialyl Lewis^X, Lewis^Y and sulfated derivatives can also be synthesised.

The goal of this section of research is to develop an efficient and scalable route towards the synthesis of Lewis^X. Here, several aspects come to play, including using building blocks that can be readily prepared (*i.e.* preferably in a low number of synthetic steps and in a highly convergent route). To begin, however, an overview of the key strategies for the synthesis of Lewis^X will be described.

4.1.1.1 Chemoenzymatic synthesis

Lewis^X has been previously synthesised chemoenzymatically using two glycosyltransferases: *β*-1,4-galaclosyltransferase and *α*-1,3-fucosyltansferase (Scheme 1).[15] Using these transferases, Lewis^X glycans can be synthesised in two steps and good yield (ca 80%). In addition, the glycan could be synthesised or elongated on the surface of the desired complex, for example, on glycodendrons,[16] glyco-nanoparticles[17] or glycolipids.[15] Other enzymes, such as α-2,3-sialyl transferases, can also be used to prepare more complex glycans from the Lewis^X core. On the other hand, chemoenzymatic synthesis can be costly. The required building blocks, UDP-galactose and GDP-fucose, as well as the glycosyltransferases themselves, are rather expensive and only available in small quantities. In addition, glycan complexity is restricted to the enzymes available thus limiting the diversity of the enzymatically produced antigens. Increasing the scale of the enzymatic reactions may also be problematic as enzymes can be affected by temperature, pressure, chemical environment and the concentration of substrate. Even though the use of chemoenzymatic routes is becoming more popular, the above mentioned limitations allow for more efficient chemical syntheses of complex glycans.

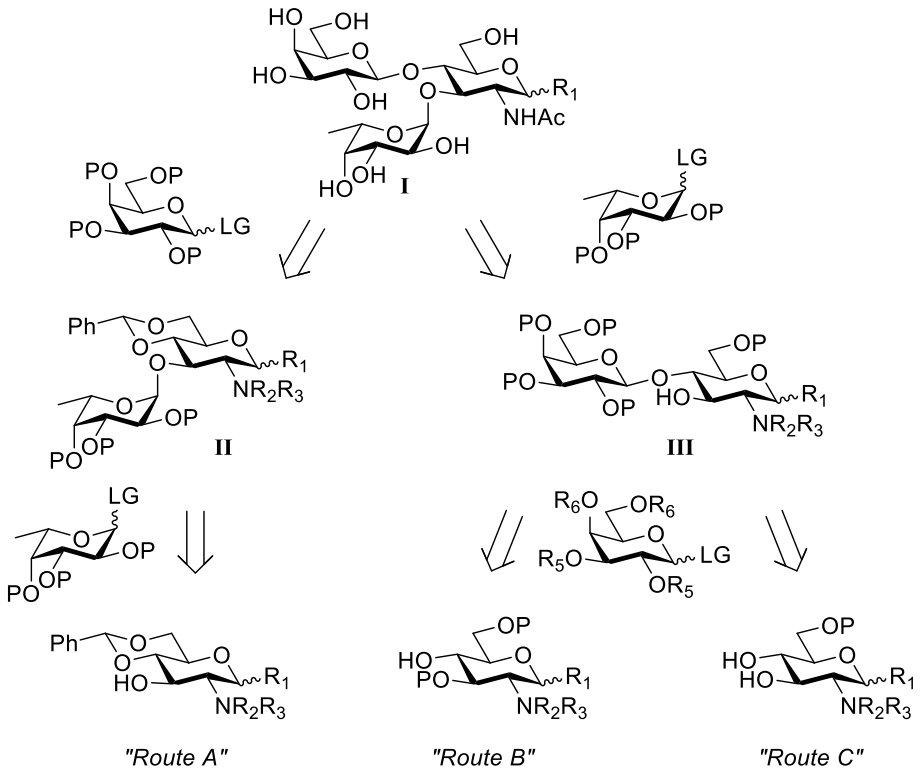


Scheme 1. *The use of glycosyltransferases to synthesise Lewis^X derivative.*[18]

4.1.1.2 Chemical synthesis

Retrosynthetically, there are three main strategies that have been used for the synthesis of Lewis^X **I** (Scheme 2). The key differences in the routes are whether the Fuc-α-(1→3)-GlcN **II** or Gal-β-(1→4)-GlcN **III** backbone is synthesised first, and whether backbone **II** can be prepared via a regioselective glycosylation. In the first approach, *"Route A"*, the first glycosylation reaction is between a glucosamine acceptor and a fucosyl donor to obtain the Fucα(1→3)GlcN backbone, which upon further modification can be used as an acceptor for the glycosylation with a galactose donor to obtain the desired Lewis^X structure. In all previously reported *"Route A"* syntheses, benzylidene protected glucosamine acceptors were used for the selective 3-*O*-glycosylation with a variety of fucosyl donors. The benzylidene acetal was then converted to the 6-*O*-benzyl protecting group, followed by glycosylation of the glucosamine 4-OH with a galactose donor. In the *"Route B"* strategy, the galactose donor is coupled to the 4-OH of a glucosamine acceptor, followed by protecting group manipulation and glycosylation with the fucose donor. Key in this

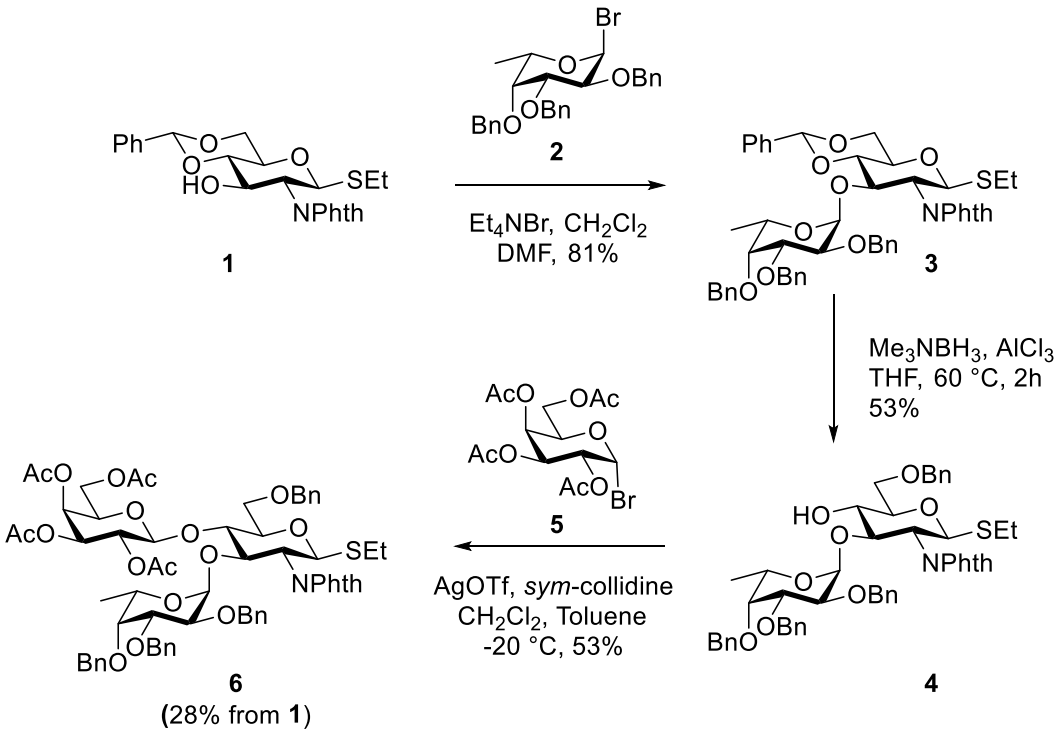
approach is to temporarily protect the glucosamine 3-OH with a specific protecting group, which after galactosylation allows for selective deprotection. This route can be improved when a regioselective glycosylation is used thereby allowing for two consecutive couplings in *"Route C"*. This section will give a brief overview on each of these different approaches towards the synthesis of Lewis^X.



Scheme 2. *Retrosynthetic approaches towards the synthesis of Lewis^X.*

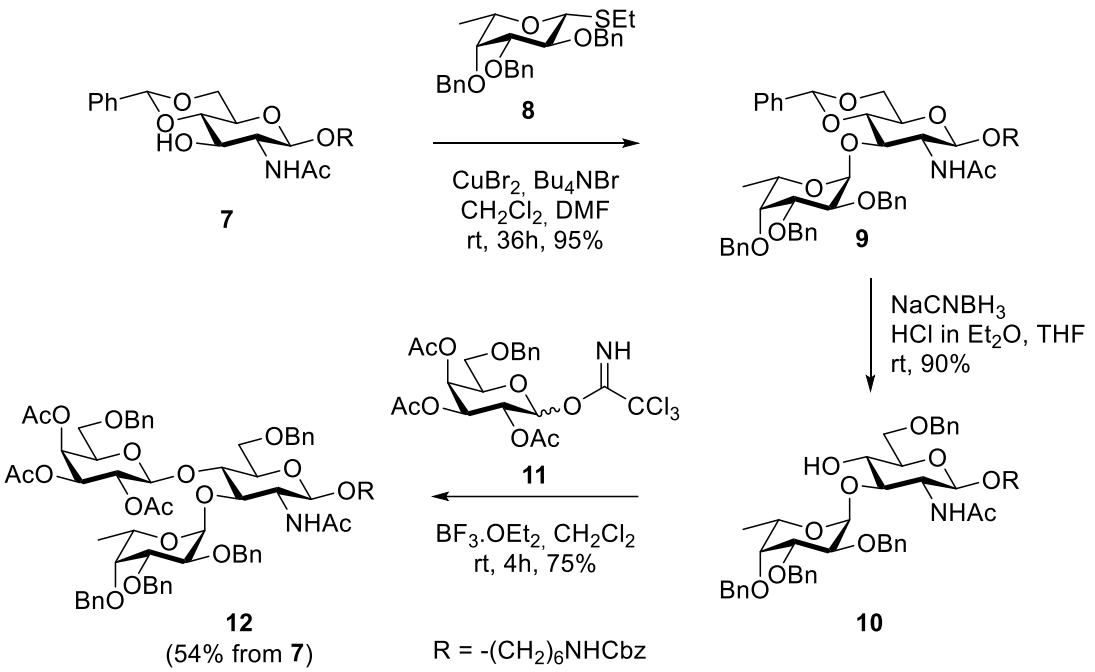
Route A. Lewis X synthesis via the Fucα-1,3-GlcNAc disaccharide

In the *"Route A"* approach, the glucosamine acceptor is coupled with a fucoside donor before glycosylation with the galactose donor *en route* to the synthesis Lewis^X. This route was first employed by Hans Lönn in 1984, whereby acceptor **1** was reacted with glycosyl bromide **2** to give disaccharide **3** in 81% with the α-fucoside linkage (Scheme 3).[19,20] Reductive ring-opening of the benzylidene acetal with trimethylamine-borane complex and aluminum trichloride then gave acceptor **4** in 66% yield. Glycosylation of the acceptor proved sluggish and, according to the authors, could only be achieved with galactosyl bromide **5** to give protected Lewis^X **6** in 53% yield. Here, the participating 2-*O*-acetate gives rise to the β-linked galactose moiety. The combined yield over these three steps was 28% from glucosamine acceptor **1**.[20]



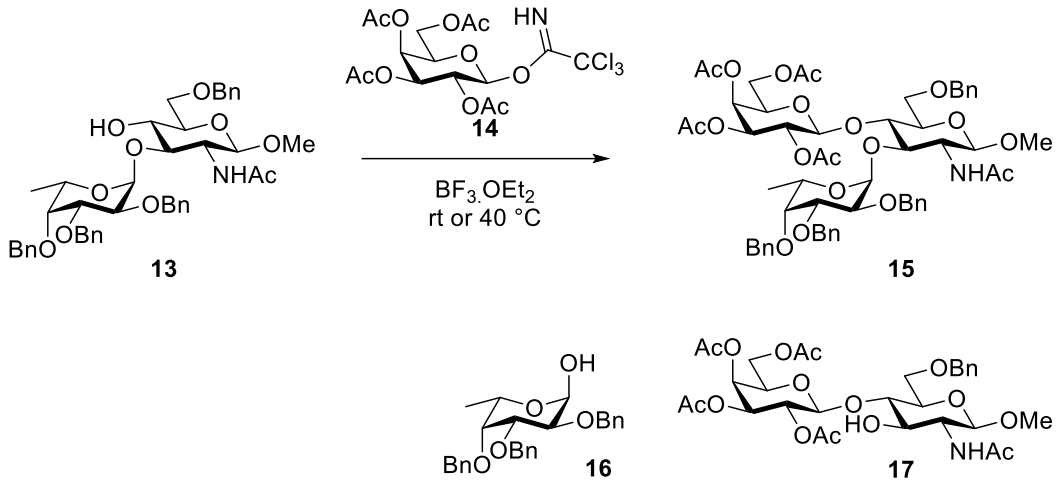
Scheme 3. *First synthesis of Lewis^X derivative using the "Route A" approach.*[19]

Following the seminal work of Lönn, several other groups have also used the *"Route A"* approach for the synthesis of Lewis^X and related glycans. In 1995, Stahl *et al.* successfully reacted acetamide acceptor **7** with fucosyl donor **8** using mild activation conditions to give disaccharide **9** in a good yield and α-selectivity (Scheme 4). A subsequent regioselective reductive ring-opening of the benzylidene acetal with $NaCNBH_3$/HCl then gave acceptor **10** in 90%. Next, the acceptor was used in a glycosylation reaction with galactose donor **11** to give trisaccharide **12** in 75% yield.[21] Here, it is interesting to note that a relatively large amount of donor (1.7 equivalents) was required to achieve sufficient coupling yields. This is probably due to the unreactive nature of the 4-OH acceptor. Nevertheless, the overall yield of 54% over these three steps is the highest reported yield to date for the assembly of the Lewis^X backbone, with the use of an *N*-acetylglucosamine moiety. The use of the *N*-acetyl glucosamine moiety throughout the synthesis has the obvious advantage in that it does not require late stage deprotection or conversion steps, when compared to N-protected glycans.



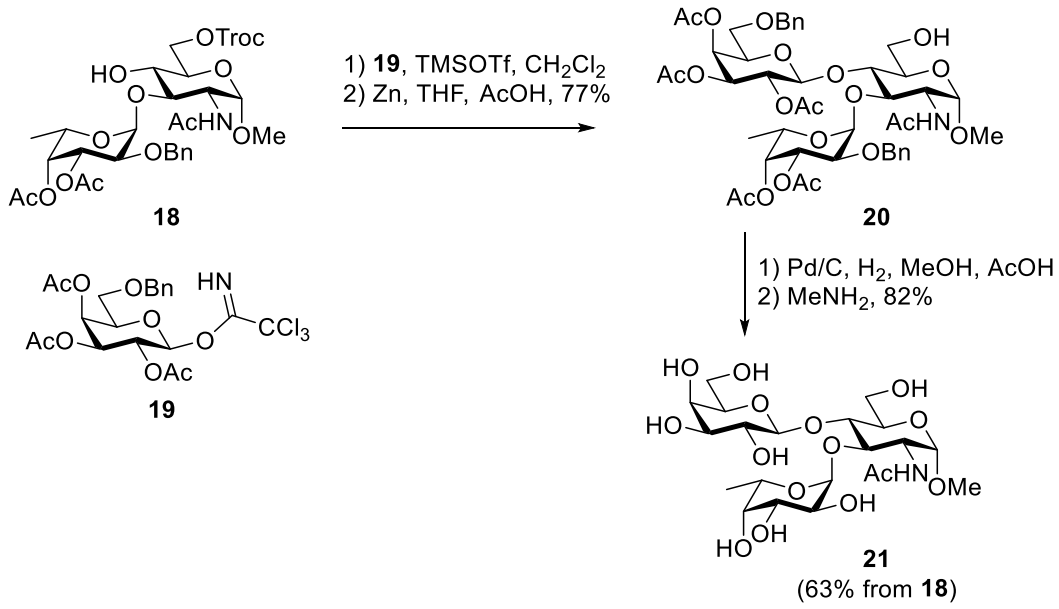
Scheme 4. *The use of N-acetyl glucosamine acceptor* ***7****. This synthesis of Lewis*^{X} ***12*** *has the advantage that it does not require a deprotection or conversion step in the global deprotection sequence.*[21]

The difficulty of 4-OH glycosylation with 3-O-fucosylated glucosamine acceptors, such as **10** above, has also been reported by others in the synthesis of Lewis^X and related structures.[20,22–25] This lack of reactivity has been attributed to the steric hindrance around the glucosamine 4-OH.[23] For example, while reacting highly benzylated acceptor **13** with donor **14**, Hendel *et al.* observed that the resulting trisaccharide **15** could undergo degradation under acidic conditions, due to the acid lability of the armed fucosyl group, to give fucose **16** and LacNAc **17**, thus resulting in low yielding glycosylation reactions (Scheme 5).[23] These results have also been supported by Wang *et al.* who found similar degradation products in their glycosylation reactions.[25]



Scheme 5. *Poor reactivity of disaccharide acceptor **13** and degradation of trisaccharide **15** results in low yielding glycosylation reactions.*[23]

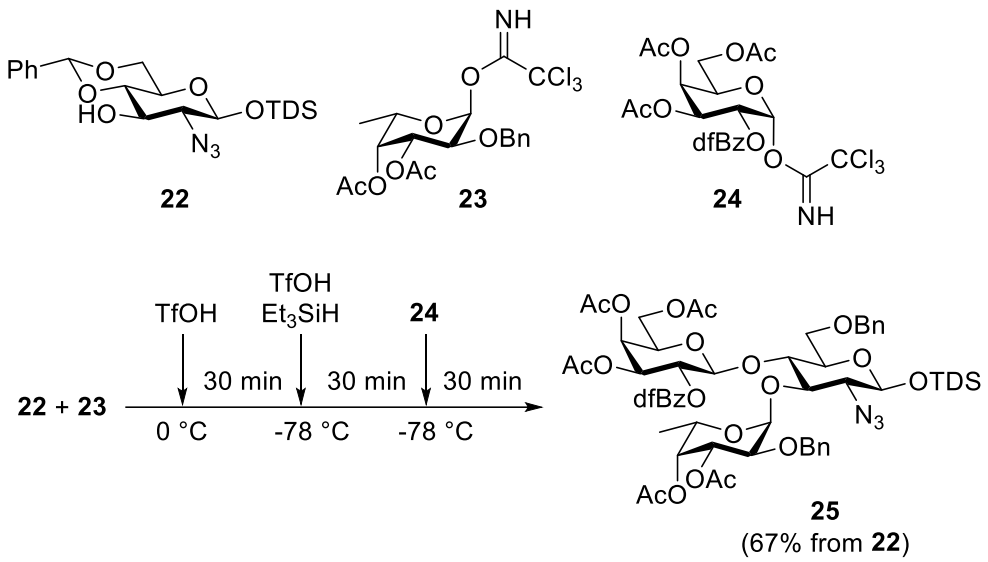
In order to solve this problem, Schmidt and co-workers reacted the less bulky and more stable, 2-*O*-benzyl-3,4-di-*O*-acetyl fucose protected acceptor **18** with donor **19** to obtain Lewis^X derivative **20** in a good yield of 77% (Scheme 6).[26,27] This, however, requires more steps for the fucose building block synthesis and results in a less versatile trisaccharide **20**, which complicates the synthesis of larger oligosaccharides. Global deprotection, however, resulted in the formation of methyl glycoside **21** in good yield.



Scheme 6. *Utilisation of the more electron withdrawing 2-O-benzyl-3,4-di-O-acetyl protected fucose residue in acceptor **18** results in higher glycosylation yields.*[26]

Finally, it should be noted that *N*-protection increases the reactivity of the glucosamine acceptor. In 2001, it was revealed by Crich and Dudkin that 4-OH of *N*-acetyl glucosamine acceptors are less reactive, compared to azide and *N*-phthalimide protected amines.[28] Thus, in order to increase the reactivity of the acceptor, different nitrogen protection group strategies have been employed. For example, *N*-Troc[29–32] as well as *N*-Phth[20,24,33,34] protecting groups have been successfully used to slightly increase glycosylation yields *en route* towards glycans containing the core Lewis^X motif. These protecting groups, however, are introduced at an early stage of the synthesis and require an additional deprotection step in the late stage synthesis of oligosaccharides, which can sometimes be problematic.

Interestingly, the combination of the *N*-protection and the 2-*O*-benzyl-3,4-di-*O*-acetyl fucose protecting group strategy was used by Boons *et al.* in a remarkable one-pot synthesis of a variety of oligosaccharides, including a protected Lewis^X.[35] Here, the glycosylation reaction of acceptor **22** and donor **23** was combined with the reductive opening of the benzylidene acetal and the glycosylation with galactose donor **24** to form protected Lewis^X **25** in 67% yield from acceptor **22**. Indeed, this is the highest yield to date for Lewis^X compounds, synthesised via *"Route A"*.



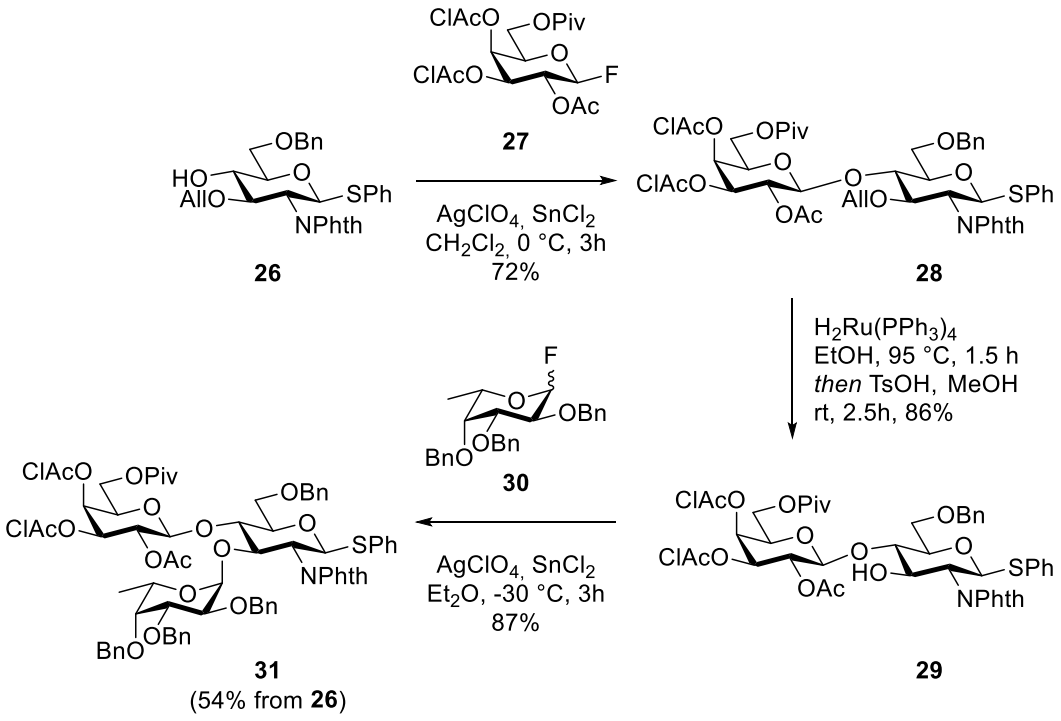
Scheme 7. *One-pot synthesis of Lewis^X oligosaccharide **25**, combining the reductive benzylidene opening with two glycosylation reactions.*[35]

In summary, several groups have reported the synthesis of Lewis^X derivatives via *"Route A"*. Here, the use of benzylidene protected glucosamine derivatives is used to introduce the fucose moiety on the 3-OH. Next, selective ring-opening results in the disaccharide acceptor for glycosylation with galactose acceptors, while different protection strategies result in different reactivities of the acceptor and/or donor. A major challenge of this synthetic route, however, is the poor reactivity of the Fuc-α-(1→3)-GlcN acceptor and the potential lability of the fucosyl group during the glycosylation reaction. On the contrary, other synthetic strategies such as in

"Route B" and *"Route C"* avoid this problem by synthesising the Gal-β-(1→4)-GlcNAc disaccharide first.

Route B. Lewis^X synthesis via the Gal-β-1,4-GlcNAc disaccharide

The *"Route B"* approach towards the synthesis of Lewis^X was first employed by Nicolaou and co-workers, who glycosylated a 3-*O*-allyl protected acceptor **26** with galactosyl fluoride donor **27**, which is compatible with the thioglycoside acceptor, to obtain disaccharide **28** in 72% (Scheme 8).[36] Selective removal of the allyl protecting group with a ruthenium hydride catalyst then gave acceptor **29**. Again, a fluoride donor **30** was required to obtain the protected Lewis^X derivative **31**, which was then used in the synthesis of an impressive trimeric Le^X glycolipid. Here, the *N*-phthalamide protecting group was converted into the acetamide at a late stage in the synthesis. To avoid this late stage conversion, Nicolaou reported a slightly improved method, in which a 3-*O*-allyl protected *N*-acetylglucosamine acceptor was used for the synthesis of Sialyl Lewis^X.[37]



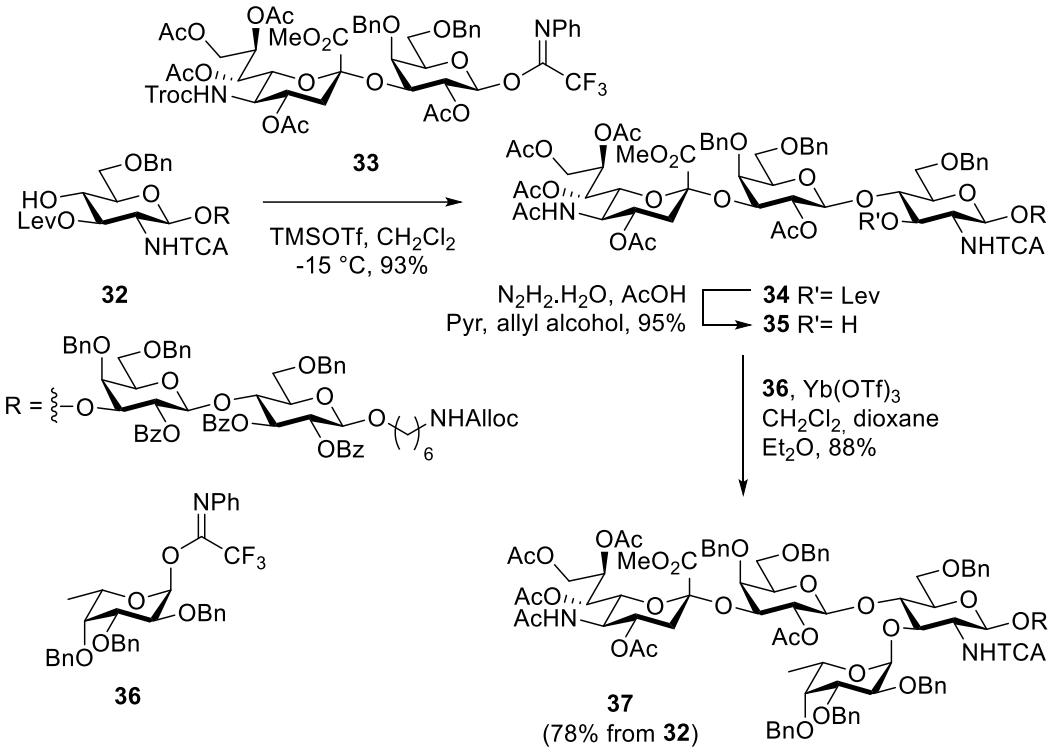
Scheme 8. *First Lewis^X derivative synthesis employing the "Route B" strategy.*[36]

Following Nicolaou's publications, several groups have employed the *"Route B"* strategy using different protecting groups for the 3-hydroxyl of glucosamine, with a number of groups using a 3-*O*-chloroacetyl.[38–43] Alternatively, carbonates[44] and para-methoxybenzyl[45,46] protecting groups have been used as temporary protecting groups. In addition, some syntheses of Lewis^X derivatives

via *"Route B"* involve the use of the *N*-acylated amine, while others have employed *N*-TCA[47,48] or *N*-phthalimido protecting groups.[36]

Via these approaches, a number of different Lewis^X derivatives have been prepared with overall yields ranging from 16 to 70% for the assembly of the core glycan structure (i.e. excluding the preparation of building blocks).

To date, however, the highest yield of 78% for the synthesis of Sialyl Lewis^X, which contains the Lewis^X motif, over three steps, was reported by Seeberger and co-workers (Scheme 9). Here, trisaccharide acceptor **32**, containing the 3-*O*-levulinoyl protecting group was coupled with donor **33** to give the pentasaccharide **34**. Subsequent selective removal of the 3-*O*-levulinoyl protecting group with hydrazine gave acceptor **35**, which was glycosylated with imidate donor **36** to give the desired hexasaccharide **37**.[47,48]

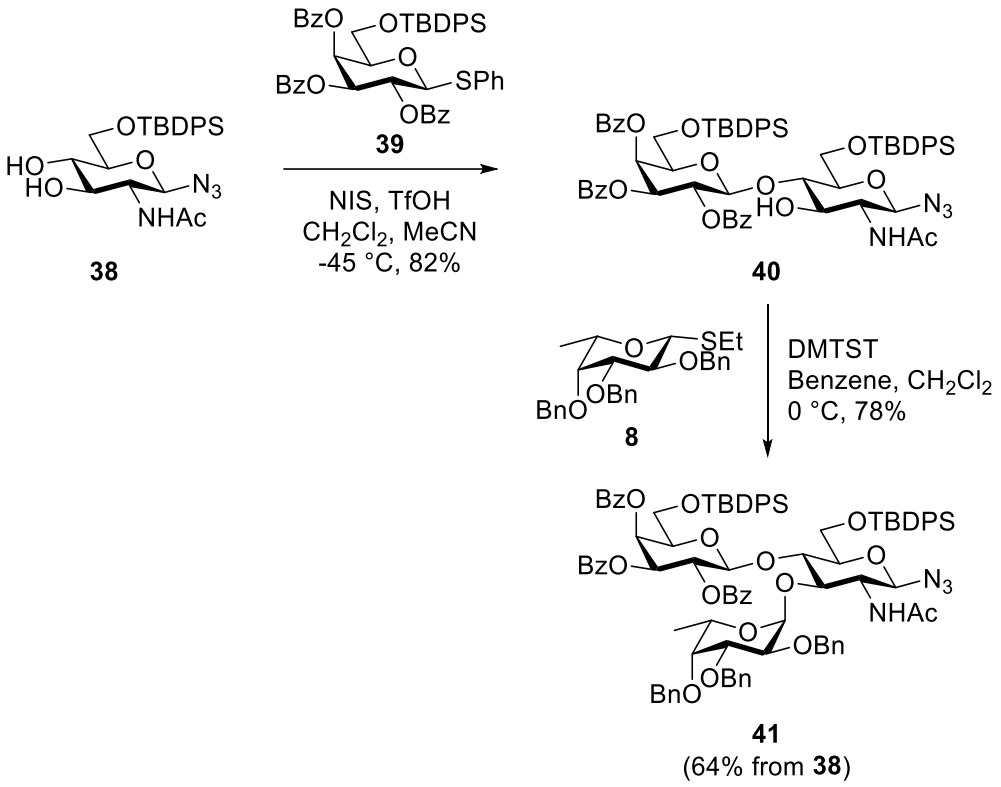


Scheme 9. *Most efficient synthesis of a Sialyl Lewis^X derivative by Seeberger and co-workers, employing the "Route B" approach with a 3-O-Lev protecting group.*[47]

In contrast to *"Route A"*, this synthetic approach allows for the synthesis of the linear saccharides, followed by fucosylation to obtain Lewis antigens. In comparison *"Route B"* has been shown to be slightly higher yielding, which could be explaned by the use of less reactive disaccharide acceptors employed in *"Route A"*. Although both routes have successfully been employed in Lewis^X syntheses, *"Route C"* has particular merit since it allows for more straightforward glucosamine building block syntheses. In addition, two consecutive glycosylation reactions are used to obtain Lewis^X derivatives, which thus allows for a rapid assembly of the Lewis^X backbone.

Route C. Regioselective glycosylation towards LacNAc derivatives.

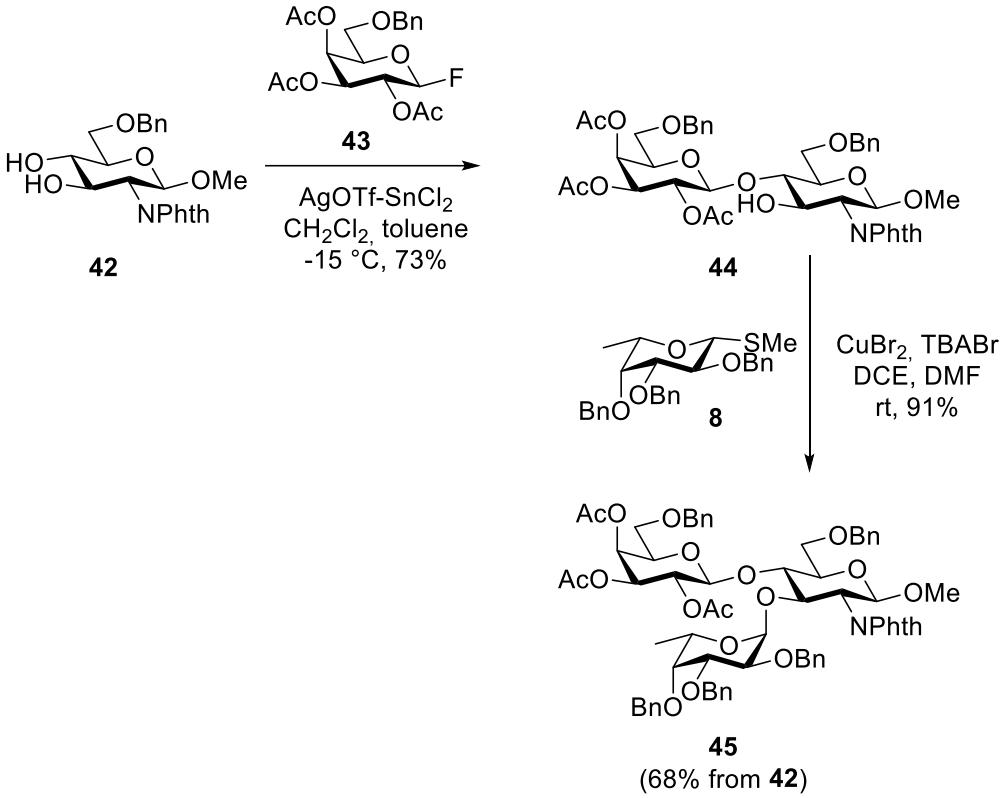
The first regioselective glycosylation *en route* to Lewis^X was reported in 1996, by Roy and co-workers (Scheme 10).[49] Here, the 6-*O*-(*tert*-butyl diphenylsilyl) protected azido-GlcNAc acceptor **38** was successfully used in glycosylation with thioglycoside donor **39** to give the target disaccharide in 82% yield. It was suggested that the bulky 6-*O*-silane protecting group blocked the top side of the GlcNAc, thereby favouring the regioselective glycosylation of the 4-hydroxyl. The resulting disaccharide **40** was then used in a glycosylation reaction with fucosyl donor **8** to give the protected Lewis^X trisaccharide **41** in 64 % yield over two steps. To date, this is the highest reported yield over the two glycosylations, while using an *N*-acetylglucosamine acceptor.



Scheme 10. *Regioselective glycosylation of 3,4-dihydroxyl glucosamine acceptor **38** with glycosyl donor **39**. The resulting disaccharide acceptor **40** is then reacted with fucose donor **8** to obtain Lewis^X derivative **41**.*[49]

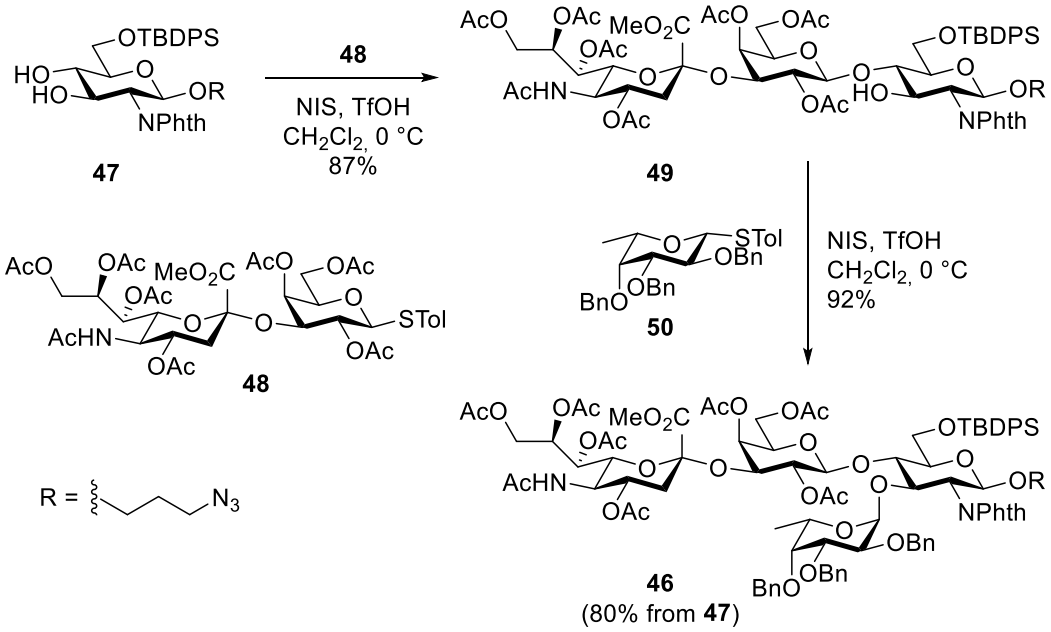
Since then, *"Route C"* has been used by numerous research groups who made slight modifications to either the donor or the acceptor *en route* to the synthesis of Lewis^X and related structures.[49–65] For example, the use of the *N*-phthalimide protecting group has been used to increase the yields in glycosylation reactions. Here, *N*-phthalimide acceptor **42** was glycosylated with fluoride donor **43** to give disaccharide **44**, which in turn was glycosylated with fucoside donor **8** to give protected Lewis^X **45** in 68% yield over the two steps (Scheme 11).[50] Other examples include the use of

"Route C" for the automated one-pot synthesis of a Lewis^X thioglycoside by Huang and co-workers in 68-71% yield.[51] This work builds on earlier work by Tanaka *et al.* for the synthesis of dimeric and trimeric Lewis^X.[52,53] Although the use of automated one-pot strategies allows for rapid assembly of the Lewis^X derivatives, relatively large amounts (5 equivalents) of donors are required to obtain high yields. In addition, the building blocks used require extensive protecting group manipulation.



Scheme 11. *The use of the N-phthalimide protecting group in the synthesis of Lewis^X derivative **45**.* [50]

To date, the highest yield (80%) in the regioselective glycosylation reactions, was reported by Cao *et al.* for the assembly of Sialyl Lewis^X **46** (Scheme 12).[54] Here, diol acceptor **47** was glycosylated with Sia-α(2→3)Gal donor **48** to give trisaccharide **49** in 87%, which in turn was reacted with thiofucoside **50** to form Sialyl Lewis^X **46** in 92% yield. Even though this route allows for the construction of protected Lewis^X derivatives in high yields, a late stage conversion of the *N*-phthalimide into the acetamide is required, which ultimately increases the number of steps in the final stages of the synthesis of these complex antigens. Moreover, this route also does not allow for the synthesis of other Lewis glycans.



Scheme 12. *Highest yielding glycosylation sequence for the synthesis of Sialyl Lewis^X* ***46*** *by Cao et al.*[54]

4.1.1.3 Building block syntheses and glycosylation strategies

As described above, many different glycosyl donors and acceptors can be used for the synthesis of Lewis^X and related structures. Here, the protecting group strategy of the glucosamine acceptor dictates the route by which Lewis^X can be synthesised. For example, when *N*-acetylglucosamine is protected at the anomeric position, and a 4,6-*O*-benzylidene acetal is installed, the acceptor is suitable for the *"Route A"* approach. Instead of a glycosylation reaction, a 3-O-protecting group could be installed, followed by the reductive opening of the benzylidene acetal to give an acceptor suitable for *"Route B"*. The acceptor for *"Route C"*, however, can be obtained more efficiently, by protecting the anomeric position, as well as the primary 6-OH of *N*-acetylglucosamine in two steps. The glycosyl donors, however, do not differ between the different synthetic strategies, and thus similar donors were used throughout all routes.

In order to obtain the α-fucoside, a non-participating protecting group at the 2-hydroxyl is required, and thus per-benzylated donors are often used. However, these armed fucosides can be more prone to hydrolysis, and thus other protecting groups (e.g. acetates) for the 3- and 4-hydroxyls of the carbohydrate have been used.[35,55,56] Alternatively, a fucose donor with TBDMS protecting groups at the 2-, 3- and 4-position was used *en route* to protected Lewis^X glycans, however, these bulky protecting groups resulted in a low-yielding (24%) glycosylation reaction.[57] Alternatively, the less-bulky triethylsilane (TES) protected fucose donor was employed by Kopitzki *et al*., however, a 1:1 α:β-ratio was observed in the glycosylation reaction.[58] The activation method of the fucoside donor depends on the type of glycoside, for example thioethyl fucoside donors can be activated with DMTST,[49] NIS/TfOH,[59] or more mildly with

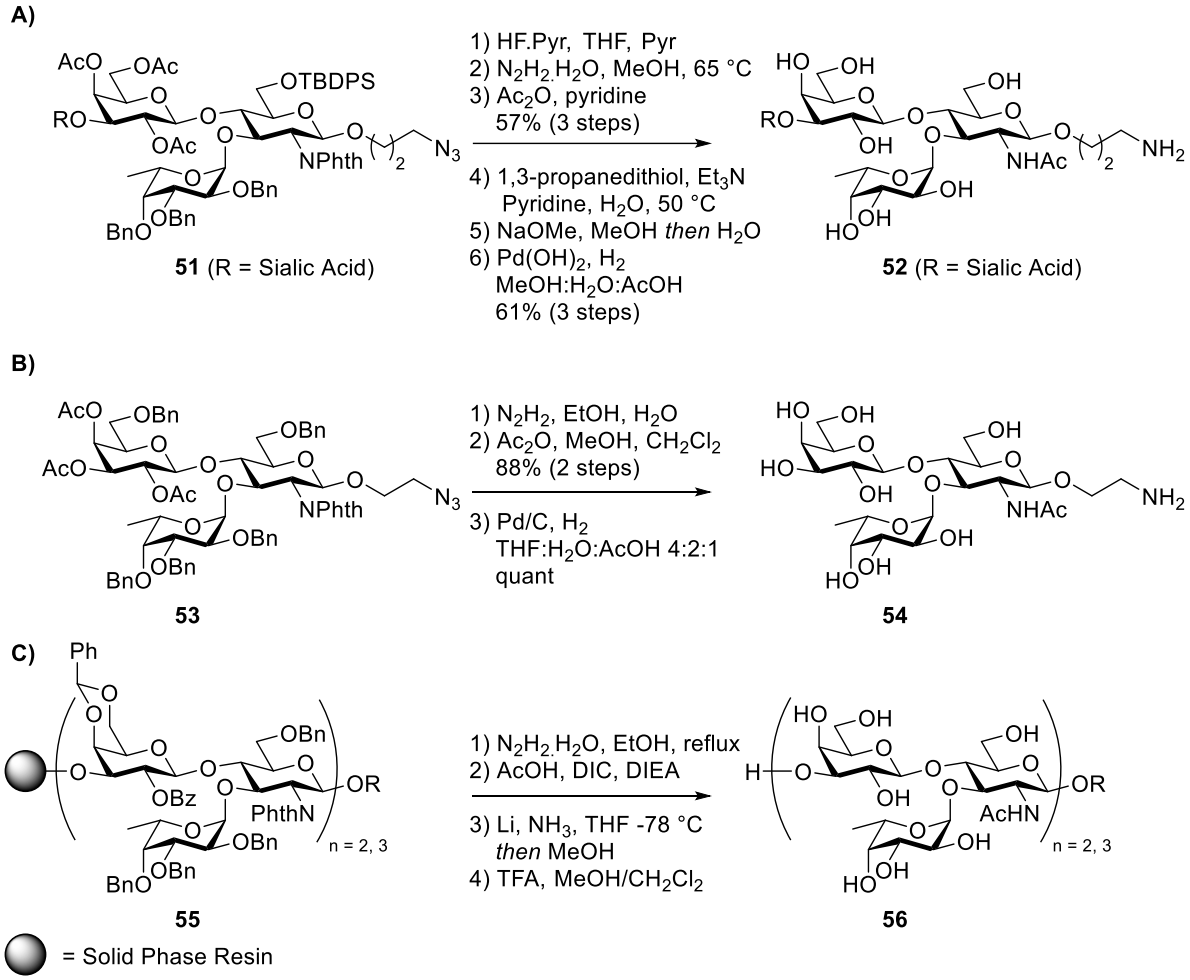
$Cu(Br)_2$/TPABr.[66] Alternatively, imidate donors,[47,55] or bromide/fluoride donors[60] can be activated in the presence of a thioglycoside acceptor, thereby allowing for the use of glucosamine donors.[20,34,36,51,61] Here, however, it is interesting to note that the halide and imidate donors are often obtained from a fucosyl thioglycoside donor, which increases the number of steps to synthesise the donor, and thus lowers the overall efficiency of the route.

To introduce the β-galactose moiety in Lewis^X, a participating 2-*O*-protecting group on the galactose donor is required. Accordingly, benzoyl and acetate protecting groups at this position have been employed. The protecting group strategy for the 3-, 4- and 6-OH differs between the Lewis^X glycan syntheses and depends on the desired functionality. For example, per-acetylated thioglycoside donors can be obtained in 2 steps from galactose, and thus allow for efficient donor synthesis. Alternatively, 4,6-*O*-benzylidene acetal donors have been used to obtain an orthogonally protected product that more readily allows for the synthesis of other Lewis^X glycans. In addition, Siaα(2→3)Gal disaccharide donors have been used to obtain Sialyl Lewis^X structures,[27,29,42,43,45,47,54,56,62] though again, this restricts the type of Lewis glycan that can be prepared.

4.1.1.4 Deprotection strategies

Many synthetic routes appear to have focussed on the assembly of the backbone of desired Lewis^X glycans and have omitted their global deprotection strategies, even though these late stage reactions can be tedious and difficult to optimise. Although each protected oligosaccharide requires a unique deprotection sequence, depending on the protecting groups used, a few relevant examples are given.

In 2008, Cao *et al.* reported the global deprotection of Sialyl Lewis^X **51** where the silyl protecting group was removed with HF.pyridine, followed by the conversion of the *N*-phthalimide to the acetamide (Scheme 13, A).[54] Here, the phthalimide was reacted with hydrazine.hydrate in methanol under reflux conditions to give the deacetylated glycosylamine, which in turn was per-acetylated. Next, azide reduction with 1,3-propanedithiol, deacetylation under Zemplén conditions and a $Pd(OH)_2$ mediated hydrogenation gave tetrasaccharide **52** in 35% over 6 steps. More recently, this deprotection sequence was improved by Lu *et al.* who reported a two-step deacetylation and phthalimide conversion in 88% yield, followed by the quantitative conversion of trisaccharide **53** into Lewis^X **54** (Scheme 13, B).[63] This deprotection sequence features a hydrazine deacetylation/phthalimide removal, followed by selective *N*-acetylation and a hydrogenation with Pd/C in THF:H_2O:AcOH under 1 atm. hydrogen.



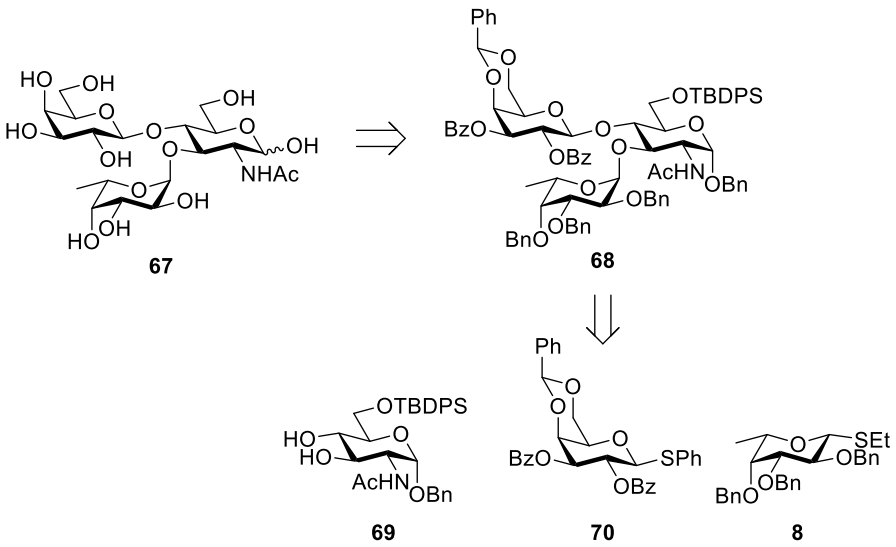
Scheme 13. *Global deprotection strategies to obtain Lewis^X derivatives.*[53,54,63]

Alternatively, a Birch reaction can be employed for the global deprotection of oligosaccharides. For example, dimeric and trimeric Lewis^X structures **55** were fully deprotected in a 4-step sequence (Scheme 13, C).[53] First, the phthalimide was converted to the acetamide in two steps. Next, a Birch reaction removed all benzyl and benzylidene acetal protecting groups. When the Birch reaction was quenched with methanol, the formation of NaOMe resulted in benzoate deprotection. Finally, cleavage of the resin with TFA, MeOH/CH_2Cl_2 resulted in the formation of dimeric and trimeric Lewis^X **56**.

4.1.2 Retrosynthesis of Lewis^X

The main goal in this chapter is to develop an efficient synthesis for Lewis^X and other Lewis antigens. As described above, three general synthetic routes have been reported to synthesise the Lewis^X antigen. Here, *"Route C"* is the most efficient strategy, employing a regioselective glycosylation reaction, followed by fucosylation to obtain Lewis^X derivatives. This route allows for more efficient acceptor synthesis compared to other routes. In addition, the two consecutive glycosylations are high yielding and do not require an extra deprotection step. Thus, it was envisioned that the Lewis^X trisaccharide **67** could be synthesised via global deprotection of the protected trisaccharide **68** (Scheme 14). Trisaccharide **68** can in turn be obtained via an initial glycosylation of *N*-acetylglucosamine diol acceptor **69** with galactosyl donor **70**, followed by the coupling with fucose donor **71**. Here, the thioglycoside donors were chosen as they are more stable (*i.e.* increase shelf life) and easily prepared, compared to glycosyl imidates or halides. All three monosaccharide building blocks were previously synthesised from the corresponding monosaccharides *N*-acetyl-D-glucosamine,[67] D-galactose[68,69] and L-fucose.[70]

Herein, it should be noted that the protected Lewis^X **68** is a versatile building block for the synthesis of the other Lewis glycans. For example, removal of the benzoates, followed by a regioselective glycosylation with a sialic acid building block, allows for rapid access to a protected Sialyl Lewis^X.[37,71] Alternatively, sulfonation of the 3'-hydroxyl results in the 3'-sulfo Lewis^X derivative.[21] On the other hand, removal of the benzylidene acetal, followed by sulfonation gives 6'-sulfo Lewis^X motifs.[72] Protecting group manipulation to obtain the galactose 2-OH acceptor allows for the synthesis of Lewis^Y glycans.[34] Derivatisation of the glucosamine 6-position, can be achieved after removal of the silane protecting group, and allows for the assembly of 6-sulfo Lewis structures.[72] Thus, all seven type-2 Lewis structures can be obtained using trisaccharide **68**.

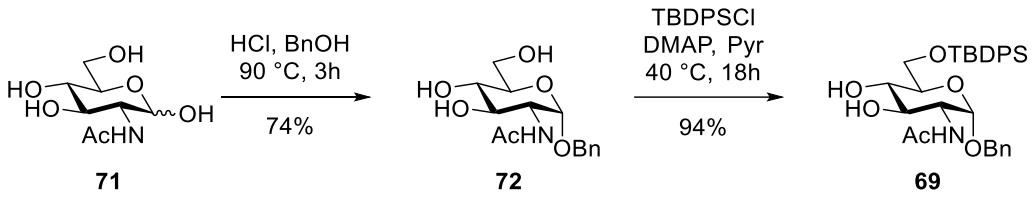


Scheme 14. *Retrosynthesis of Lewis^X* ***67****.*

4.2 Results and discussion

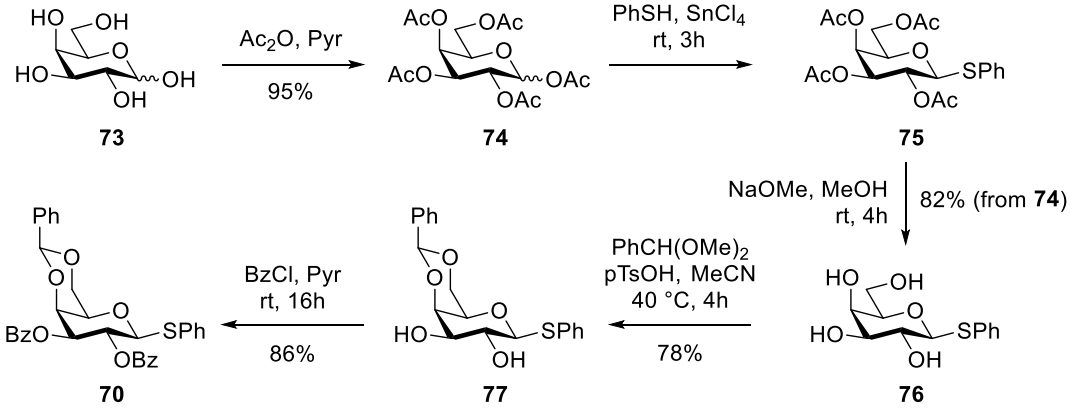
4.2.1 Synthesis of Lewis^X building blocks

The glucosamine acceptor **69** was synthesised in two steps, starting with a Fischer glycosylation of benzyl alcohol with *N*-acetyl glucosamine (**71**) (Scheme 15). This reaction was performed on a 6.5 gram scale to obtain the α-benzyl glycoside **72**, which was isolated in 74% yield.[73,74] The benzyl glycoside was then used in the selective silylation reaction with TBDPS-Cl and DMAP in pyridine to obtain acceptor **69**.[67] In our hands, the reaction was sluggish at room temperature due to the insolubility of the sugar moiety, but by increasing the temperature of the reaction to 40 °C, a smooth conversion of the triol into diol-acceptor **69** was achieved.



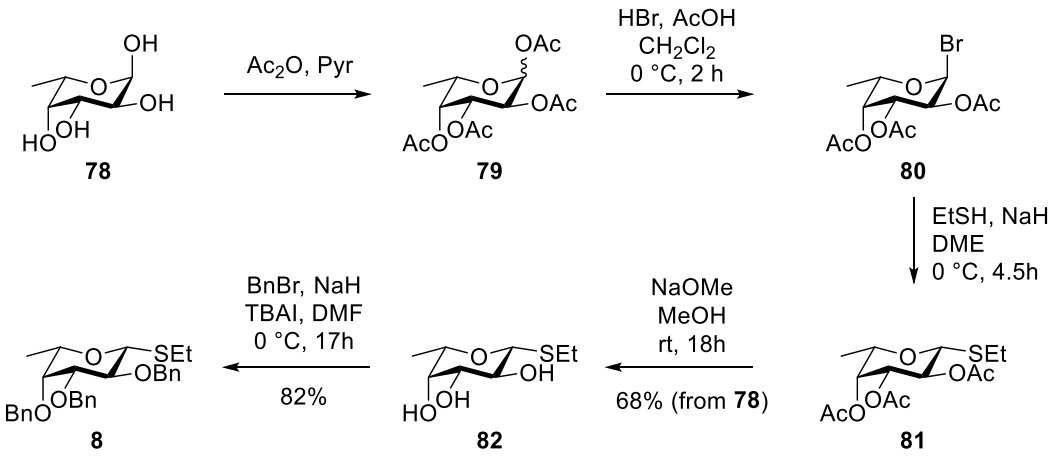
Scheme 15. *Two step synthesis of N-acetyl glucosamine acceptor **69**.*

The galactose donor **70** was synthesised in 5 steps, on a multi-gram scale, starting from D-galactose (**73**).[68,75] Here, peracetylation of galactose gave pentaacetate **74** that was used without purification. Activation of the anomeric acetate in **74** with $SnCl_4$ in the presence of thiophenol gave thioglycoside **75**, which was treated with NaOMe to give the tetraol **76**.[68] Next, the 4,6-*O*-benzylidene protecting group was installed using benzaldehyde dimethyl acetal and pTsOH in acetonitrile at 40 °C to give diol **77**.[68] The diol was then treated with benzoyl chloride in pyridine to give the desired crystalline donor **70** in good yield.[69] Here, the ability of the benzoyl protecting group to participate is required for β-selective glycosylation with acceptor **69**.



Scheme 16. *Synthesis of galactose building block **70**.*

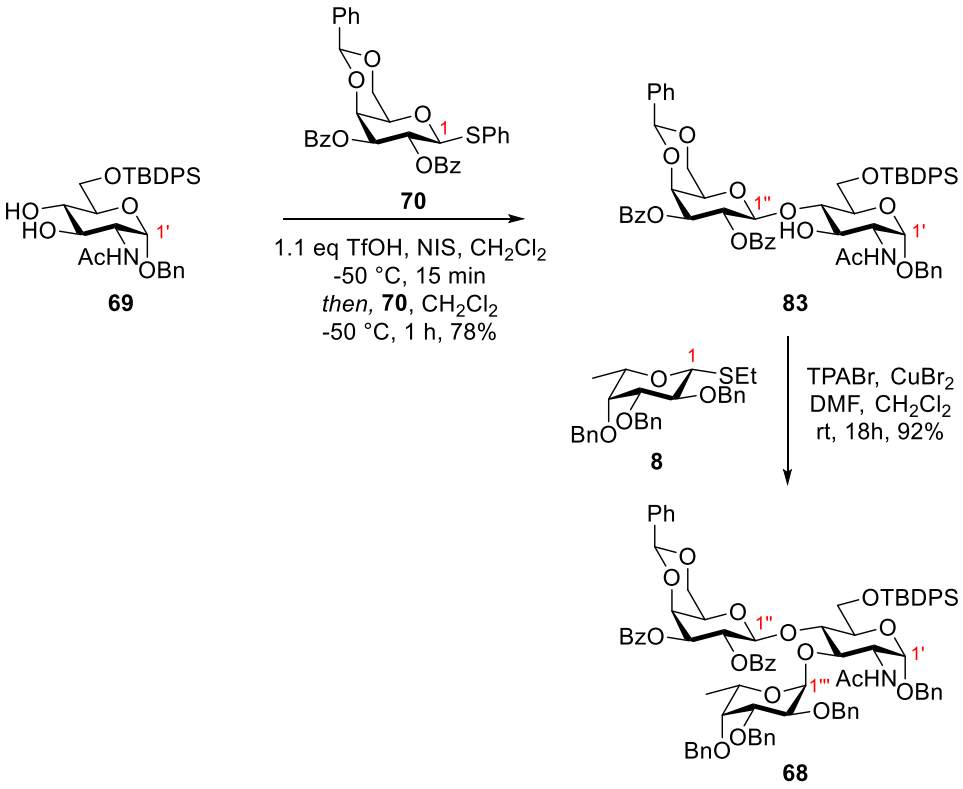
The third building block, fucose donor **8**, was synthesised in five steps from L-fucose (**78**) (Scheme 17). Here, acetylation gave fucoside **79**, which was reacted with HBr to give bromide **80**. Next, the bromide was treated with ethanethiol and sodium hydride to give thioglycoside **81**, which was deacetylated to give triol **82** in 68% over four steps. In order to obtain the α-fucopyranoside during glycosylation, a non-participating protecting group was required at the 2-position. To this end, triol **82** was perbenzylated using benzyl bromide, TBAI and sodium hydride to give thioglycoside **8** in 82% yield.[76] This allowed for a highly efficient synthesis of the fucose donor, although it should be noted that others have found such highly armed fucose donors to be labile during glycosylation reaction, as previously described.[23]



Scheme 17. *Building block synthesis fucose donor **8**.*

4.2.2 Glycosylation reactions

After the synthesis of the necessary building blocks, the assembly of the Lewis^X trisaccharide backbone started with the regioselective glycosylation of glucosamine acceptor **69** with thioglycoside donor **70** to give disaccharide **83** (Scheme 18). In trial reactions on small scale, it was found that slow addition of the donor (1.2 equivalents) to a mixture of the acceptor, NIS and TfOH at -50 °C resulted in a high yielding (74%) regioselective glycosylation. It was also observed that when the reaction was performed at higher temperatures (or allowed to warm to 0 °C) small amounts of the over-glycosylated trimer were formed. Given that the acceptor is synthesised in fewer steps than the donor, in subsequent gram scale couplings, the acceptor was used in slight excess.



Scheme 18. *Glycosylation reactions to obtain protected Lewis^X* ***68****.*

The best yield (78%) on a gram scale was obtained when TfOH was added to a mixture of NIS and a slight excess of GlcNAc acceptor **69** (1.1 equiv.) at -50 °C, prior to the addition of galactosyl donor **70** (1 equiv.). The resulting disaccharide **83** was purified by silica gel flash column chromatography and was obtained as the desired β-pyranoside, whereas the α-anomer was not observed.

The configuration at the anomeric centre was confirmed by NMR analysis, which revealed a $J_{1'',2''}$ coupling of 8.2 Hz while an HMBC between H-1´´ and C-5´´ confirmed the pyranose configuration. A combination of 1D- and 2D-NMR analysis was used to assign all proton and carbon resonances, and for example, the regioselectivity of this method was confirmed using HMBC NMR analysis, in which correlations between H-1´´ and C-4´, as well between H-4´ and C-1´´ were observed (Figure 2).

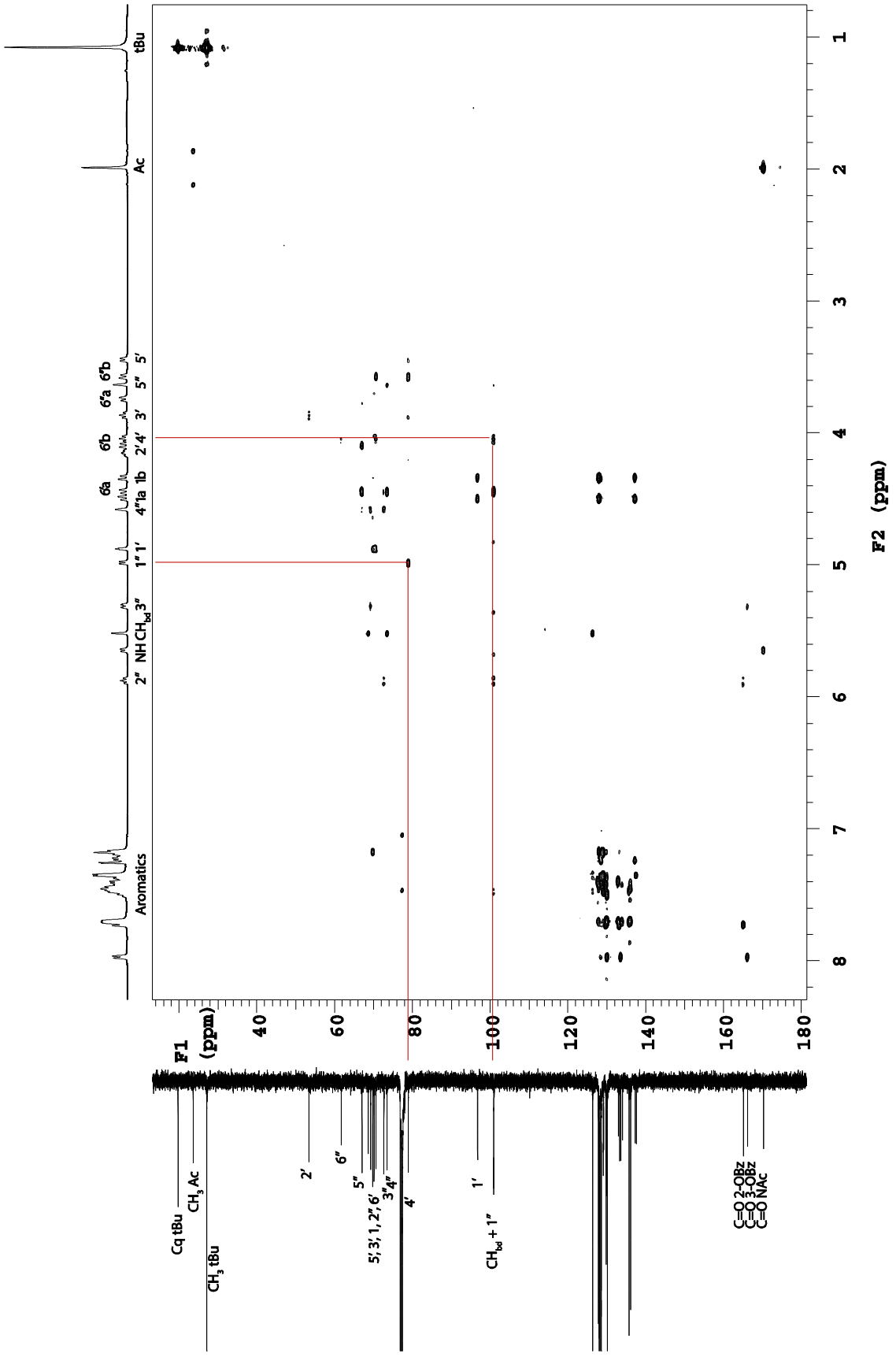
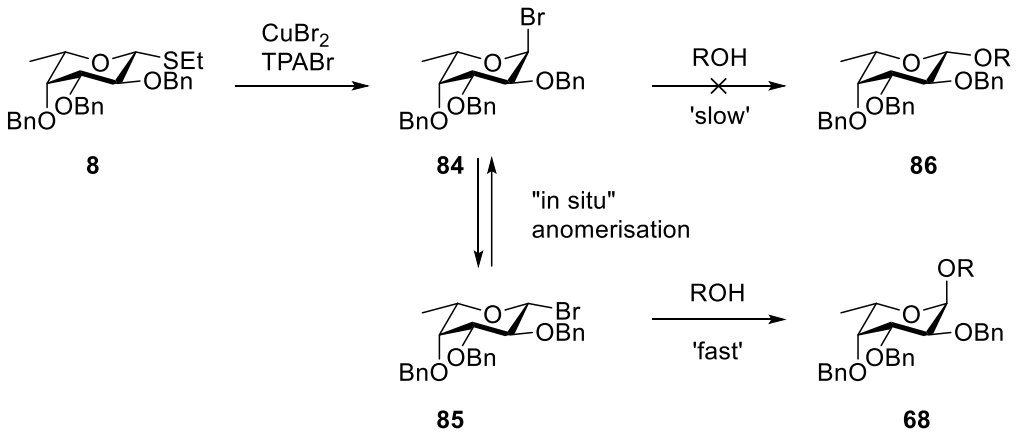


Figure 2. *HMBC analysis of disaccharide* ***83*** *confirms the regioselectivity of the glycosylation reaction.*

With the disaccharide acceptor in hand, the glycosylation between disaccharide **83** and L-fucose donor **8** was attempted. Here, the thioethyl donor **8** was activated with copper(II)bromide and tetrapropylammonium bromide in DMF and CH_2Cl_2 at room temperature, which resulted in the smooth formation of the desired trisaccharide **68**. Although the $J_{1''',2'''}$ coupling constant could not be observed, the $^{1}J_{H\text{-}1''',C\text{-}1'''}$ coupling constant of 170 Hz confirmed the formation of the α-anomer, whereas the β-anomer was not observed.[77] To explain the α-selectivity, the reaction mechanism needs to be considered (Scheme 19).[78] Here, the S_N2-like activation of the thioglycoside **8** results in the formation of the relatively stable α-bromide **84**, which in turn anomerises 'in situ' to the more reactive β-bromoglycoside **85** in the presence of the 'Br^-' nucleophile, that originates from tetrapropylammonium bromide (TPABr). The relative reaction rate between the α- and β-bromides, whereby the α-bromide reacts slowly while the β-bromide reacts more quickly, then results in the selective formation of the α-glycoside **68** via an S_N2 like substitution with the acceptor, whereas β-glycoside **86** was not observed.



Scheme 19. *In situ anomerisation with TPABr results in the selective formation of the α-L-fucopyranoside **68**.*

Finally, the trisaccharide **68** was purified using silica gel column chromatography (R_f = 0.23, 2/1 PE/EtOAc, v/v), however, the trisaccharide can also be crystallised from methanol. This allows for crystallisation of the trisaccharide from the crude reaction mixture, thus significantly simplifying the purification process, especially during large-scale synthesis. Recrystallisation of the initial crystalline product, resulted in large (1–2 mm) crystals that were suitable for X-ray crystallography (Figure 3). The structure of trisaccharide **68** was confirmed and, in particular, the confirmation of the glycosidic linkages were clearly as required.

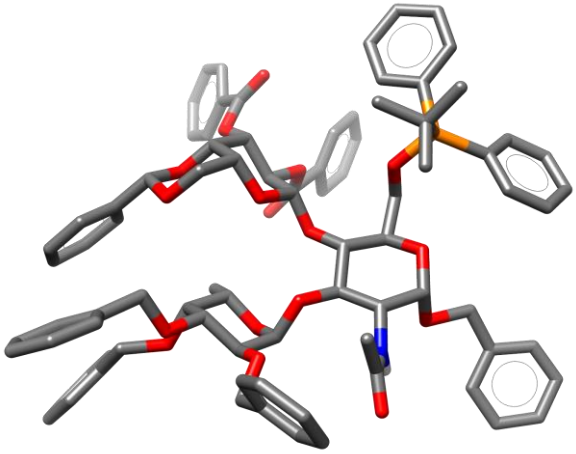
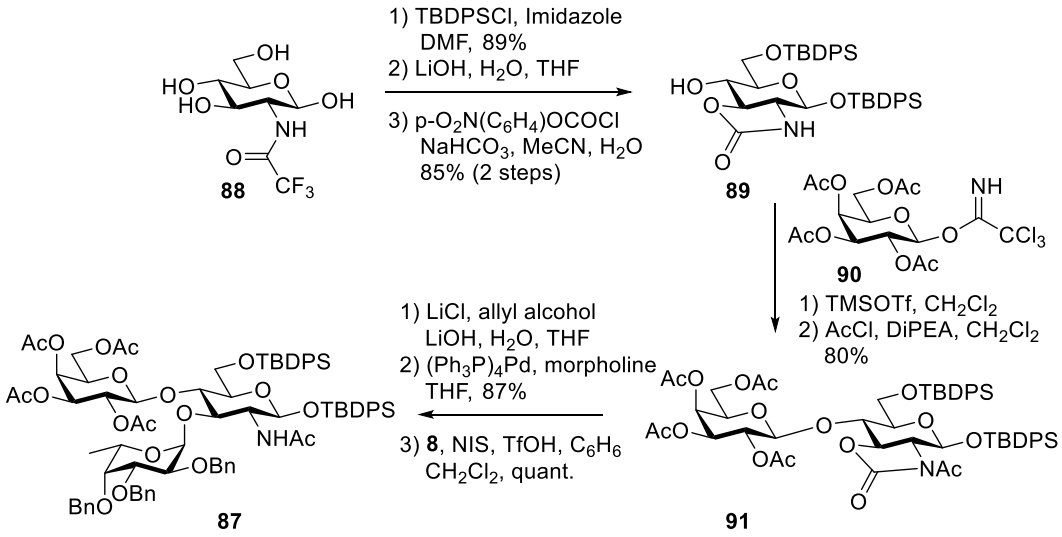


Figure 3. *XRD Crystal structure of the protected Lewis^X trisaccharide* ***68****.*

Interestingly, only one protected Lewis^X derivative has previously been reported as a crystalline compound, however no crystal structure of this material was obtained.[79] Here, Lewis^X **87** was synthesised in 8 steps from trifluoroacetyl glucosamine **88** (Scheme 20). The 3-OH of glucosamine **88** was temporarily protected with a carbonate to give acceptor **89**, which underwent glycosylation with imidate **90**, followed by acetylation to yield disaccharide **91**. Removal of the carbonate via hydrogenation of the *O*-alloc intermediate, followed by fucosylation gave trisaccharide **87**. Unfortunately, the authors did not report the full global deprotection, and instead only removed the benzyl and acetate protecting groups. In contrast to their extensive synthetic route, the synthetic route employed in this thesis only requires four steps to obtain Lewis^X **68** in good yields from *N*-acetyl glucosamine. In addition, trisaccharide **68** contains a benzylidene acetal protecting group, which allows for further functionalisation of Lewis^X into more complex ligands, such as Sialyl Lewis^X derivatives and/or sulfated glycans.



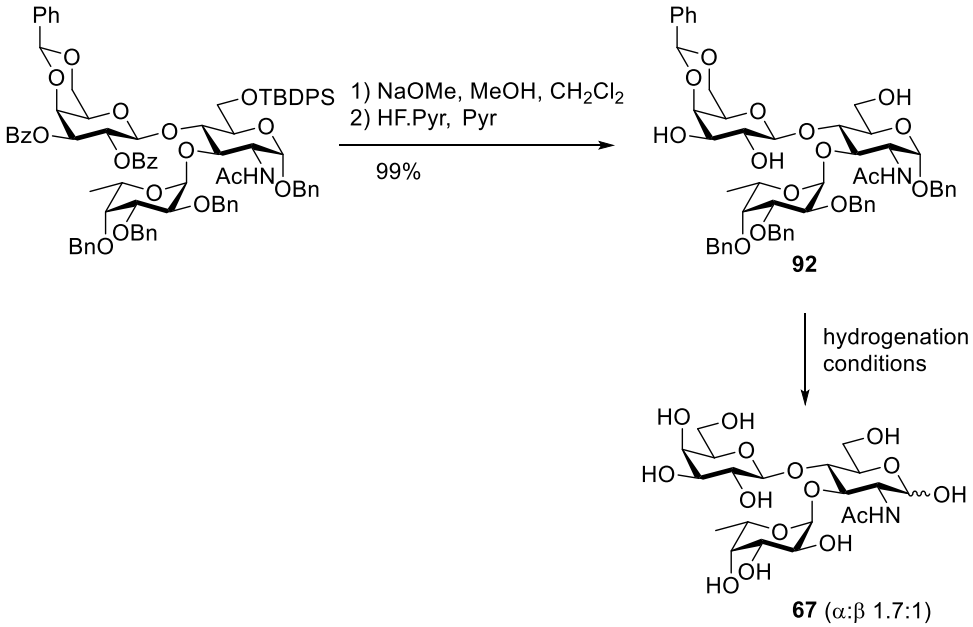
Scheme 20. *Synthesis by Nagai et al. towards crystalline Lewis^X* ***91****.*[79]

Another advantage of the isolation of a late stage crystalline compounds is that it could allow for shorter and thus cheaper Good Manufacturing Practices (GMP)-controlled syntheses of Lewis^X containing pharmaceutical products. GMP-synthesis is an expensive process due to many regulations, guidelines, extensive documentation such as good laboratory and clinical practices, hygiene and safety regulations. A late stage crystalline material is therefore advantageous as it reduces the number of steps required in GMP-synthesis, as crystalline material can conveniently be used as feedstock, without needing to analyse prior intermediates using GMP-guidelines.

Taken as a whole, the new synthetic strategy reported in this thesis has resulted in the highest yield of a protected Lewis^X derivative (72% from **69**) utilising an *N*-acetylglucosamine acceptor. While other groups have reported higher yielding syntheses using *N*-phtalimido protected glucosamines,[52,54,59] two extra steps are required at a late-stage synthesis to obtain the desired *N*-acetyl derivative. In addition, the versatile protecting group strategy of crystalline trisaccharide **68** allows for further derivatisation of Lewis^X, for example in sulfated Lewis^X derivatives, as well as Sialyl Lewis^X. To complete the synthesis of Lewis^X, global deprotection was required.

4.2.3 Deprotection strategy

Deprotection of trisaccharide **68** started with debenzoylation under Zemplén conditions in MeOH:CH_2Cl_2 (1/1, v/v) (Scheme 21). The reaction was quenched with Dowex H^+ and concentrated *in vacuo* and the crude mixture was then submitted to HF.pyridine in pyridine to give triol **92** in an excellent (99%) yield after purification by silica column chromatography. With the triol in hand, several hydrogenolysis methods were examined in order to obtain the Lewis^X trisaccharide (**67**).



Scheme 21. *Three step deprotection sequence to synthesise Lewis^X.*

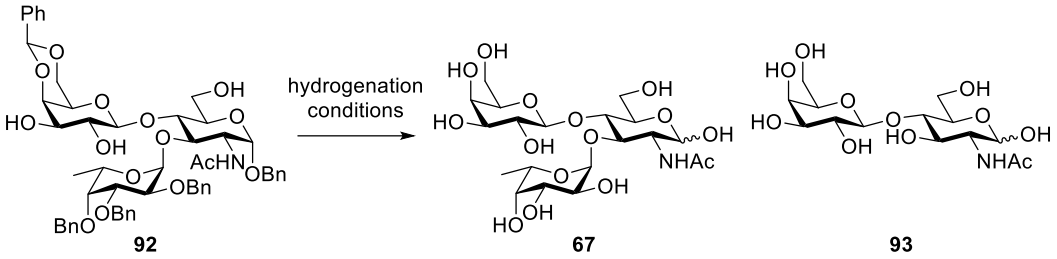
Although a few groups have reported high yielding global deprotection strategies for the synthesis of Lewis^X and related glycans, in our hands, it took much effort to optimise the final deprotection step (Table 1). When methanol was used as a solvent, the reaction was slow and after 5 days, debenzylation was complete, yet a significant amount of the defucosylated disaccharide **93** (ca 80%) was also formed (entry 1). Here, it should be noted that a large excess of palladium, or filtration followed by resubmission of the reaction did not increase the yield of the desired trisaccharide. By using 3.5 bar of hydrogen gas in a Fisher Porter bottle, the hydrogenation occurred faster, however, again the fucose moiety was cleaved (entry 2). When Pd/C, rather than $Pd(OH)_2$, was used similar results were observed (entry 3). To speed up the reaction, a mixture of $CHCl_3$: MeOH was used, which is known to be slightly acidic and thus favor the hydrogenolysis reaction (entry 4). Although these conditions resulted in a rapid reaction, they also favoured cleavage of the fucose moiety. With the addition of formic acid, the reaction was complete overnight, but again the disaccharide was the only product observed (entry 5). Thus, given the known acid sensitivity of the fucosyl linkage, it was envisioned to keep the reaction mixture basic.

To this end, the addition of NH_4HCO_2, which forms NH_3, H_2 and CO_2 during the reaction,[80] resulted in a sluggish low yielding reaction accompanied by undesired disaccharide **93** formation (entry 6). When the hydrogenolysis reaction was performed in the presence of pyridine (residue from the HF.pyridine silane cleavage), a smooth but slow (5 days) conversion to Lewis^X (**67**) was observed (entry 7). Unfortunately, pyridine was hydrogenated to form piperidine, which proved difficult to remove with either reverse phase or size exclusion chromatography. Notwithstanding, this result suggested that the addition of a base might aid in the synthesis of trisaccharide (**67**) and

thus other bases were therefore investigated. The use of ammonia in methanol resulted in disaccharide formation as well as the formation of what was thought to be a methylated amine, as evidenced by mass spectrometry (entry 8). This by-product may have been formed by a reductive amination of the acetamide, with traces of formaldehyde present in methanol. The use of triethylamine resulted in the isolation of the product in moderate yield, however, again disaccharide formation was observed (entry 9). Here it should also be noted that a large excess of Et_3N most likely poisoned the catalyst and resulted in the long reaction time. Nevertheless, after 5 days, 34% of the desired product was obtained on a small scale reaction.

During the course of these studies, the hydrogenation reactions were monitored using HRMS, and it was observed that the benzyl protecting groups were removed within 24 hours, whereas the benzylidene acetal removal proved sluggish. And upon further literature research, it was found that the use of AcOH greatly increases the reaction speed and, in particular, seems essential for the deprotection of benzylidene acetals. Accordingly, triol **92** was submitted to the hydrogenation conditions reported by Lu *et al.*[63] and gratifyingly it was found that the hydrogenation with Pd/C in THF/H_2O/AcOH (4/2/1, v/v/v) and atmospheric hydrogen gas was completed within three hours, with clean conversion to the desired Lewis^X antigen **67**, which was isolated in a high (98%) yield as an α/β-mixture.

Table 1. *Hydrogenation conditions for the formation of Lewis^X.*



Entry	Cat.	Solvent	Additives	Time	Isolated Yield 67	Observed
1[a]	$Pd(OH)_2$	MeOH	-	5 d	~10%	**67** + **93**
2[b]	$Pd(OH)_2$	MeOH	-	5 d	~10%	**67** + **93**
3[b]	Pd/C	MeOH	-	5 d	~10%	**67** + **93**
4[a]	$Pd(OH)_2$	MeOH:$CHCl_3$	-	18 h	-	**93**
5[a]	$Pd(OH)_2$	MeOH	HCO_2H	18 h	-	**93**
6[a,b]	$Pd(OH)_2$	MeOH	NH_4HCO_2	5 d	15%	**67** + **93**
7[a]	$Pd(OH)_2$	MeOH	Pyridine	5 d	~40%	**67**[c]
8[a]	$Pd(OH)_2$	MeOH	NH_3 (MeOH)	1 d	-	**93**[d]
10[a]	$Pd(OH)_2$	EtOH	Et_3N	5 d	34%	**67**[e]
11[a]	Pd/C	4:2:1 THF: H_2O:AcOH		3 h	98%	**67**

a) Reaction performed at 1.0 bar

b) Reaction performed at 3.5 bar

c) Contaminated with piperidine

d) Methylated byproduct formed

e) ca. 5% of dimer **93** was observed

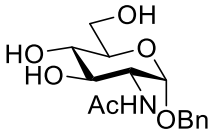
4.3 Conclusion

In summary, a number of different strategies can be used for the synthesis of Lewis^X derivatives. In the *"Route A"* approach, a benzylidene acetal glucosamine acceptor is used in the glycosylation with fucosyl donors. Next, reductive opening of the benzylidene acetal gives the disaccharide acceptor for glycosylations with galactose donors. Even though several groups reported the successful assembly of Lewis^X using this route, others noted the unreactivity of this type of acceptors. To avoid this problem, *"Route B"* can be used which involves the glycosylation of a 3-*O*-protected glucosamine acceptor with a galactosyl donor to give a lactosamine derivative. Selective deprotection of the 3-hydroxy protecting group then gave the disaccharide acceptor, which in turn was fucosylated. This route was further improved by employing a regioselective glycosylation of a diol acceptor with a galactose donor, followed by the consecutive glycosylation with a fucosyl donor (*"Route C"*). This route allows for a more efficient acceptor synthesis, and more importantly, results in the high yielding assembly of Lewis^X derivatives.

Thus, in this thesis, the *"Route C"* strategy was employed to synthesise Lewis^X. First, the required monosaccharide building blocks were synthesised in good yields (2-5 step, 54-70% overall yield). The regioselective glycosylation of the glucosamine acceptor with the galactose donor resulted in the required disaccharide acceptor in good yield. Next, the α-selective fucosylation was performed with the fucose donor, and the resulting trisaccharide was obtained as a crystalline material. Compared to previous reported synthetic routes, Lewis^X was obtained in an excellent overall yield of 72% from the glucosamine acceptor. With the versatile trisaccharide in hand, global deprotection afforded DC-SIGN antigen Lewis^X. Although the final hydrogenation step proved troublesome, an overall deprotection yield of 97% could be achieved if the benzoyl and TBDPS groups were removed first, and the hydrogenolysis was performed using the conditions previously reported by Lu *et al.* It is envisioned that this highly efficient route (36% over 10-steps) will find further application, not only in the synthesis of Lewis^X probes for studying the function of DC-SIGN (see Chapter 5), but also for the synthesis of other members of the Type-2 family of Lewis antigens.

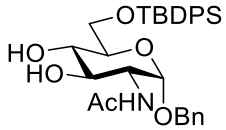
4.4 Experimental

General procedure. Unless otherwise stated all reactions were performed under argon. Prior to use, THF (Pancreac) was distilled from sodium and benzophenone, pyridine was distilled and dried over 4Å molecular sieves (4Å MS), CH_2Cl_2 (Fisher) was distilled from P_2O_5, DME (Sigma) was distilled from $LiAlH_4$, MeCN (Panreac) was distilled from CaH, DMF was distilled from BaO, and H_2O was distilled. N-Acetylglucosamine (Fluka), D-galactose (J. T. Baker), L-fucose (Pfanstiehl Inc.), BnOH (Ajax Chemicals), conc HCl (Panreac), TBDPS-Cl (Aldrich), DMAP (Pierce), $SnCl_4$ (Aldrich), PhSH (Koch-Light Laboratories), KF (Riedel-de Haën), NaOMe (freshly prepared from sodium [Aldrich] and MeOH), benzaldehyde dimethyl acetal (Aldrich), pTsOH (Aldrich), BzCl (Aldrich), Ac_2O (Peking Reagent), HBr (33% in AcOH, Acros), $LiAlH_4$ (Aldrich), EtSH (Sigma-Aldrich), BnBr (Fluka), TBAI (Riedel-de Haën), NaH (Aldrich, 60% dispersed in mineral oil), N-iodosuccinimide (Aldrich), TfOH (Aldrich), TPABr (Aldrich), $CuBr_2$ (Chempur), HF.Pyridine (Aldrich), CaOAc (Sigma), AcOH (Sigma-Aldrich), Pd/C (Aldrich), Et3N (Sigma), Dowex H^+ (Sigma-Aldrich 50WX8), 4Å molecular sieves (ROTH), Toluene (Panreac), anhydrous Et_2O (Pancreac), EtOAc (Pancreac), petroleum ether (Pure Science), MeOH (Pure Science), $CHCl_3$ (Pancreac), EtOH (absolute, Pure Science), $NaHCO_3$ (Pure Science), $Na_2S_2O_3$ (Merck) and NaCl (Pancreac) were used as received. All solvents were removed by evaporation under reduced pressure. Reactions were monitored by TLC-analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, with fluorescent indicator UV_{254}) with detection by UV-absorption (short wave UV – 254 nm; long wave UV – 366 nm), by dipping in 10% H_2SO_4 in EtOH followed by charring at ~150 °C, by dipping in I_2 in silica, or by dipping into a solution of ninhydrin in EtOH followed by charring at ~150 °C. Column chromatography was performed on Pure Science silica gel (40 – 63 micron). AccuBOND II ODS-C_{18} (Agilent) was used for reverse phase chromatography. Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 20 °C in D_2O, CD_3OD, $CDCl_3$, or pyridine-d_5 using either a Varian INOVA operating at 500 MHz or Varian VNMRS operating at 600 MHz. Chemical shifts are given in ppm (δ) relative to solvent residues. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.

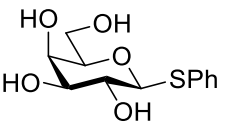


Benzyl 2-acetamido-2-deoxy-α-D-glucopyranoside (2).[73,74] To a solution of *N*-acetylglucosamine (6.40 g, 28.9 mmol) in benzyl alcohol (50 mL, 480 mmol), conc. aq. HCl (3.0 mL) was added and the reaction mixture was stirred at 90 °C for 3 h. The crude mixture was cooled down to rt, poured into Et_2O (500 mL) and

left to crystallise at -4 °C for 18 h. The crystalline product was filtered and washed with petroleum ether. Purification by silica gel column chromatography (CH_2Cl_2/MeOH, 95/5 → 88/12, v/v) gave benzyl glycoside **2** (6.67 gr, 21.4 mmol, 74%) as a white foam. R_f = 0.25 (CH_2Cl_2/MeOH, 90/10, v/v); Mp 182 – 184 °C; $\alpha_D^{17.8}$ = +222 (c = 0.1, MeOH); IR (film) 3298, 3092, 2938, 2901, 2844, 1648, 1552, 1497, 1455, 1375, 1309, 1230, 1156, 1093, 1047, 778, 732, 695 cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD) δ 7.40 – 7.25 (m, 5H, CH_{arom}), 4.85 (d, 1H, $J_{1´,2´}$ = 3.6 Hz, H-1´), 4.74 (d, 1H, $J_{1a,1b}$ = 12.0 Hz, H-1a), 4.49 (d, 1H, $J_{1a,1b}$ = 12.0 Hz, H-1b), 3.89 (dd, 1H, $J_{1´,2´}$ = 3.6 Hz, $J_{2´,3´}$ = 10.8 Hz H-2´), 3.83 (dd, 1H, $J_{5´,6a´}$ = 1.6 Hz, $J_{6a´,6b´}$ = 11.4 Hz, H-6a´), 3.73 – 3.64 (m, 3H, H-6b´, H-3´, H-4´), 3.36 (dd, 1H, $J_{4´,5´}$ = 9.6 Hz, H-5´), 1.95 (s, 3H, CH_3 Ac); ^{13}C NMR (125 MHz, CD_3OD) δ 173.6 (C=O Ac), 139.0 (Cq_{arom}), 129.4 (C-o_{arom}), 129.3 (C-m_{arom}), 128.8 (C-p_{arom}), 97.5 (C-1´), 74.1 (C-4´), 72.7 (C-3´), 72.5 (C-5´), 70.1 (C-1), 62.7 (C-6´), 55.4 (C-2´), 22.5 (CH_3 Ac); HRMS(ESI) *m/z* calcd. for $[C_{15}H_{22}NO_6]^+$: 312.1442, obsd.: 312.1446.



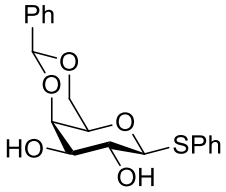
Benzyl 2-acetamido-6-*O*-*tert*-butyldiphenylsilyl-2-deoxy-α-D-glucopyranoside (3). To a solution of benzyl glycoside **2** (837 mg, 8.74 mmol) in pyridine (10 mL), TBDPS-Cl (2.75 mL, 33.9 mmol) and DMAP (200 mg, 1.64 mmol) were added and the reaction mixture was stirred at 40 °C for 18 h. The crude mixture was concentrated *in vacuo* and purified by silica gel column chromatography (CH_2Cl_2/MeOH, 100/0 → 95/5, v/v) to give diol **3** (1.39 gram, 94%) as a white solid. R_f = 0.50 (CH_2Cl_2/MeOH, 95/5, v/v); $\alpha_D^{17.8}$ = +58.4 (c = 0.5, $CHCl_3$); IR (film) 3324, 3071, 2930, 2857, 1739, 1655, 1544, 1472, 1456, 1428, 1376, 1217, 1112, 1048, 1025, 823, 775, 739, 701 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73 – 7.68 (m, 4H, CH_{arom}), 7.46 – 7.27 (m, 11H, CH_{arom}), 5.84 (d, 1H, $J_{2´,NH}$ = 8.5 Hz, NH), 4.88 (d, 1H, $J_{1´,2´}$ = 3.0 Hz, H-1´), 4.72 (d, 1H, $J_{1a,1b}$ = 11.8 Hz, H-1a), 4.44 (d, 1H, $J_{1a,1b}$ = 11.8 Hz, H-1b), 4.14 – 4.06 (m, 1H, H-2´), 3.94 – 3.85 (m, 2H, H-6a´, H-6b´), 3.76 (m, 2H, H-3´, H-4´), 3.63 (dd, 1H, $J_{4´,5´}$ = $J_{5´,6a´}$ = $J_{5,6b´}$ = 9.1 Hz, H-5´), 3.24 (bs, 1H, OH), 2.86 (bs, 1H, OH), 2.00 (s, 3H, CH_3 Ac), 1.07 (s, 9H, CH_3 *t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.0 (C=O), 137.1, 135.81, 135.78, 133.3, 133.2, 130.0, 128.8, 128.3, 128.2, 127.9 (C_{arom}), 96.5 (C-1´), 74.5 (C-3´), 72.9 (C-5´), 71.5 (C-4´), 69.4 (C-1), 64.3 (C-6´), 53.8 (C-2´), 27.0 (CH_3 *t*Bu), 23.4 (CH_3 Ac), 19.4 (Cq *t*Bu); HRMS(ESI) *m/z* calcd. for $[C_{31}H_{40}NO_6Si]^+$: 550.2619, obsd.: 550.2620.



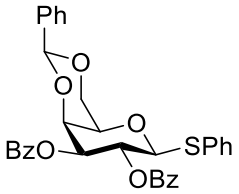
Phenyl 1-thio-β-D-galactopyranoside (76). 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose **74** (20.0 g, 51.2 mmol) was co-evaporated with toluene (2×), dissolved in CH_2Cl_2 (150 mL) and cooled to 0 °C. Thiophenol (6.3 mL, 61.4 mmol) and $SnCl_4$ (0.6 mL, 5.12 mmol) were added and the reaction mixture and stirred at 0 °C for 3 h. under argon. The reaction was quenched with a 3M solution of potassium fluoride

(100 mL) and the organic layer was washed with water, saturated sodium bicarbonate solution, brine and dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was dissolved in EtOAc and passed through a plug of silica gel to remove residual tin by-products, to give the crude thioglycoside **75** as a yellow oil (23.9 g). The data obtained for this compound matched literature values.[68]

To a suspension of the crude mixture (23.9 g) in methanol (200 mL), sodium methoxide was slowly added until the solution reached pH 13. The reaction mixture was stirred for 4 h and neutralised with Dowex-H^+. The resin was removed by filtration and the methanolic solution was concentrated *in vacuo* to give crude tetraol **76** as a white solid, which was used without further purification. The data obtained for this compound matched literature values.[68]

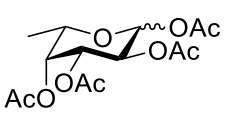


Phenyl 4,6-*O*-benzylidene-1-thio-β-D-galactopyranoside (77). To a solution of phenyl 1-thio-β-D-galactopyranoside **76** (4.80 g, 17.6 mmol) in acetonitrile (100 mL), benzaldehyde dimethyl acetal (2.9 mL, 19.4 mmol) and pTsOH (200 mg, 1.64 mmol) were added and the reaction mixture was stirred at 40 °C for 4 h. The reaction mixture was neutralised with triethylamine (0.4 mL, 3 mmol), concentrated *in vacuo* and purified by silica gel column chromatography (CH2Cl2/MeOH, 100/0 → 95/5, v/v). Crystallisation from PE/EtOAc (2/1, v/v) afforded diol **77** (4.96 gram, 13.7 mmol, 78%) as white crystals. The data obtained for this compound matched literature values.[68]

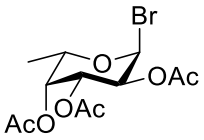


Phenyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-1-thio-β-D-galacto-pyranoside (70). To a solution of diol **77** (4.90 g, 13.6 mmol) in pyridine (68 mL), benzoyl chloride (4.7 mL, 40.8 mmol) was added dropwise and the reaction mixture was stirred at r.t. for 16 h. The crude mixture was diluted with CH_2Cl_2 (100 mL) and washed with aq. 1 M HCl (100 mL), and the water layer was extracted with CH_2Cl_2 (50 mL). The combined organics were washed with sat. $NaHCO_3$ (100 mL) and brine (100 mL), dried with $MgSO_4$, filtered and concentrated *in vacuo*. The product was crystallised from CH_2Cl_2/PE to give donor **70** (6.65 g, 86%) as white crystalls. R_f = 0.55 (PE/EtOAc, 1/1, v/v); Mp 210 °C; $\alpha_D^{17.8}$ = +52 (c = 0.1, $CHCl_3$); IR (film) 3062, 2937, 2855, 1726, 1601, 1584, 1480, 1451, 1403, 1368, 1316, 1275, 1219, 1176, 1130, 1093, 1070, 1027, 944, 817, 772, 709 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.02 - 7.91 (m, 4H, CH_{arom}), 7.65 – 7.60 (m, 2H, CH_{arom}), 7.56 – 7.22 (m, 14H, CH_{arom}), 5.82 (t, 1H, $J_{2,3}$ = 10.0 Hz, H-2), 5.52 (s, 1H, CHPh), 5.37 (dd, 1H, $J_{2,3}$ = 10.0 Hz, $J_{3,4}$ = 3.5 Hz, H-3), 4.97 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-1), 4.61 (dd, 1H, $J_{3,4}$ = 3.3 Hz, $J_{4,5}$ = 0.6 Hz, H-4), 4.46 (dd, 1H, $J_{5,6a}$ = 1.6 Hz, $J_{6a,6b}$ = 12.5 Hz, H-6a), 4.10 (d, 1H, $J_{5,6b}$ = 1.6 Hz, $J_{6a,6b}$ = 12.4 Hz, H-6b), 3.77 (bs, 1H, H-5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.3 (C=O

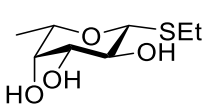
Bz), 165.1 (C=O Bz), 137.7, 134.1, 133.5, 133.3, 131.2, 130.1, 129.9, 129.8, 129.2, 129.2, 128.9, 128.5, 128.4, 128.3, 126.6 (C_{arom}), 101.1 (CHPh), 85.5 (C-1), 74.2 (C-3), 73.8 (C-4), 70.1 (C-5), 69.3 (C-6), 67.2 (C-2); HRMS(ESI) *m/z* calcd. for $[C_{33}H_{32}NO_7S]^+$: 586.1894, obsd.: 586.1920.



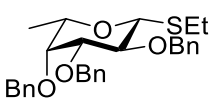
1,2,3,4-Tetra-*O*-acetyl-α/β-L-fucopyranose (79). L-Fucose (**78**) (4.00 g, 24.4 mmol) was dissolved in acetic anhydride (25 ml) and pyridine (50 mL) and the reaction mixture was stirred at r.t. for 18 h. The reaction mixture was concentrated *in vacuo*, co-evaporated three times with toluene, redissolved in EtOAc, washed with water and brine, dried with $MgSO_4$, filtered and concentrated *in vacuo*. The crude per-acetylated product **79** (8.4 g) was used without further purification. The data obtained for this compound matched literature values.[70]



2,3,4-Tri-*O*-acetyl-α-L-fucopyranosyl bromide (80). To a solution of crude **79** (8.4 g, 24.4 mmol) in CH_2Cl_2 (16 mL) at 0 °C, HBr (16 mL, 33% in AcOH) was slowly added and the reaction mixture was stirred for 2.5 h, where it was allowed to warm to r.t. The reaction mixture was co-evaporated with toluene (4 × 25 mL) and the resulting crude bromide **80** (9.05 g) was used without further purification. The data obtained for this compound matched literature values.[70]

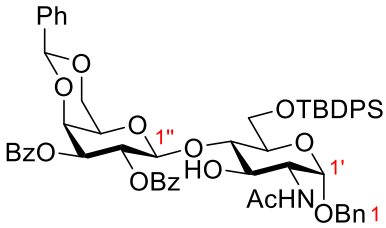


Ethyl α-L-thiofucopyranoside (82). To a solution of sodium hydride (2.40 g, 60.0 mmol) in DME (53 mL, distilled from $LiAlH_4$) at 0°C, ethanethiol (5.40 mL, 101 mmol) was slowly added while vigorously stirring the reaction mixture. After 30 min, a solution of crude bromide **80** (9.01 g, 24.4 mmol) in DME (27 mL, distilled from $LiAlH_4$) was added and the reaction mixture stirred at r.t. for 3.5 h. After TLC-analysis (PE/EtOAc, 50/50, v/v) showed complete conversion of the starting material, MeOH (80 mL) and NaOMe were added until the reaction mixture reached pH 10, and the reaction mixture was stirred at r.t. for 18 h. The reaction mixture was neutralised with Dowex H^+, filtered, washed with MeOH, and concentrated *in vacuo*. Purification by column chromatography (CH_2Cl_2/MeOH, 100/0 → 90/10, v/v) yielded **82** as a colourless oil (3.43 g, 68% over four steps) which was crystallised from Et_2O to yield white crystals. The data obtained for this compound matched literature values.[70]



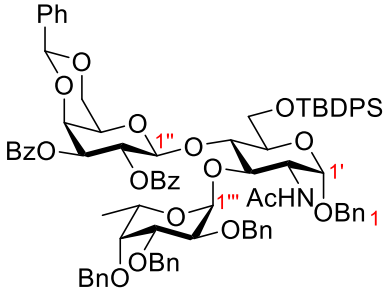
Ethyl 2,3,4-tri-*O*-benzyl-β- L-fucopyranoside (8). Thiofucoside **82** (1.13 g, 5.44 mmol) was co-evaporated with DMF (3 × 50 mL), and for the final co-evaporation the solution was partially concentrated leaving ca. 27 mL DMF.

The reaction was cooled to 0 °C under inert atmosphere, benzyl bromide (2.33 mL, 19.6 mmol), sodium hydride (0.849 g, 21.2 mmol) and TBAI (100 mg) were added at 0 °C and the reaction mixture was stirred for 17 h. and allowed to warm to r.t. The reaction mixture was quenched with methanol (100 mL), concentrated *in vacuo*, dissolved in EtOAc, washed with water and brine, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (PE/EtOAc, 98/2 → 90/10, v/v) yielded **8** as a colourless oil which crystallised upon refrigeration (2.14 g, 82%). R_f = 0.36 (PE/EtOAc, 5/1, v/v); Mp 51.0 – 52.1 °C; $[\alpha]_D^{24}$ = +6.0 (c= 1.0, $CHCl_3$); IR (film) 3088, 3063, 3030, 2977, 2929, 2868, 1497, 1454, 1357, 1208, 1165, 1125, 1089, 1067, 1048, 912, 876, 697, 667 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.42 – 7.28 (m, 15H, CH_{arom}), 5.00 (d, 1H, $J_{CH2a,CH2b}$ = 11.7 Hz, CH_2a 4-*O*-Bn), 4.91 (d, 1H, $J_{CH2a,CH2b}$ = 10.0 Hz, CH_2a 2-*O*-Bn), 4.81 (d, 1H, $J_{CH2a,CH2b}$ = 10.3 Hz, CH_2b 2-*O*-Bn), 4.78 (d, 1H, $J_{CH2a,CH2b}$ = 11.7 Hz, CH_2a 3-*O*-Bn), 4.75 (d, 1H, $J_{CH2a,CH2b}$ = 12.0 Hz, CH_2b 3-*O*-Bn), 4.71 (d, 1H, $J_{CH2a,CH2b}$ = 11.7 Hz, CH_2b 4-*O*-Bn), 4.40 (d, 1H, $J_{1,2}$ = 9.8 Hz, H-1), 3.83 (t, 1H, $J_{1,2}$ = $J_{2,3}$ = 9.5 Hz, H-2), 3.62 (d, 1H, $J_{3,4}$ = 2.9 Hz, H-4), 3.57 (dd, 1H, $J_{2,3}$ = 9.3 Hz, $J_{3,4}$ = 2.7 Hz, H-3), 3.49 (q, 1H, $J_{5,6}$ = 6.3 Hz, H-5), 2.79 (dq, 1H, $J_{CH2a,CH2b}$ = 12.5 Hz, $J_{CH2a,CH3}$ = 7.6 Hz, CH_2a SEt), 2.72 (dq, 1H, $J_{CH2a,CH2b}$ = 12.5 Hz, $J_{CH2b,CH3}$ = 7.6 Hz, CH_2b SEt), 1.31 (t, 3H, $J_{CH2,CH3}$ = 7.6 Hz, CH_3 SEt), 1.21 (d, 3H, $J_{5,6}$ = 6.3 Hz, CH_3 H-6); ^{13}C NMR (125 MHz, $CDCl_3$) δ138.8, 138.6, 138.5, 128.6, 128.4, 128.3, 128.3, 127.8, 127.8, 127.7, 127.6 (C_{arom}), 85.1 (C-1), 84.6 (C-3), 78.5 (C-2), 76.6 (C-4), 75.8 (CH_2 2-*O*-Bn), 74.7 (C-5), 74.6 (CH_2 4-*O*-Bn), 73.0 (CH_2 3-*O*-Bn), 24.8 (CH_2 SEt), 17.4 (C-6), 15.1 (CH_3 SEt); HRMS(ESI) *m/z* calcld. for $[C_{29}H_{34}O_4SNa]^+$: 501.2076, obsd.: 501.2079.



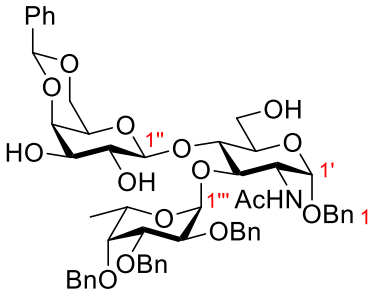
Benzyl 2-acetamido-4-*O*-(2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranosyl)-2-deoxy-6-*O*-*tert*-butyldiphenylsilyl-α-D-glucopyranoside (10). Glycosyl acceptor **3** (680 mg, 1.24 mmol) was co-evaporated with toluene (3 × 3 mL) and dissolved in dry CH_2Cl_2 (5 mL). In a separate flask, glycosyl donor **6** (798 mg, 1.40 mmol) was co-evaporated with toluene (3 × 3 mL) and dissolved in dry CH_2Cl_2 (10 mL). Activated molsieves (4Å) were added to both the donor and the acceptor solutions, and both solutions were stirred at rt for 30 min. *N*-iodosuccinamide (631 mg, 2.81 mmol) was added to the mixture with acceptor **3** and the reaction mixture was cooled to -50 °C. Freshly distilled TfOH (124 µL) was added at -50 °C and the reaction mixture was stirred for 15 min before adding the solution of donor **6**. The crude reaction mixture was stirred for 1 h at -50 °C and when TLC analysis showed full conversion of the glycosyl donor, the reaction mixture was quenched by the addition of $NaHCO_3$ (830 mg). The crude reaction mixture was diluted with CH_2Cl_2 (50 mL), washed with sat. aq. $Na_2S_2O_3$ (30 mL) and the water layer was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layers were dried with $MgSO_4$, filtered and concentrated *in vacuo*. Purification by silica gel flash column chromatography (PE/EtOAc

75/25 → 100/0, v/v) gave disaccharide **10** (909 mg, 73%) as a white foam. R_f = 0.56 (EtOAc); $\alpha_D^{17.8}$ = +91.1 (c = 1, $CHCl_3$); IR (film) 3422, 3382, 3069, 2931, 2889, 2857, 1722, 1668, 1602, 1531, 1452, 1428, 1369, 1315, 1273, 1219, 1177, 1112, 1070, 1040, 1027, 821, 773, 741, 708 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.97 (d, 2H, $J_{o\text{-}m}$ = 7.3 Hz, CH-*o* Bz), 7.76 – 7.66 (m, 6H, CH_{arom}), 7.55 – 7.30 (m, 16H, CH_{arom}), 7.28 – 7.14 (m, 8H, CH_{arom}), 5.88 (t, 1H, $J_{1'',2''} = J_{2'',3''} = J_{2'',NH}$ = 8.8 Hz, H-2´´), 5.64 (d, 1H, $J_{2',NH}$ = 8.8 Hz, NH) 5.52 (s, 1H, CHPh), 5.31 (dd, 1H, $J_{3'',4''}$ = 3.0 Hz, $J_{2'',3''}$ = 10.7 Hz, H-3´´), 4.98 (d, 1H, $J_{1'',2''}$ = 8.2 Hz, H-1´´), 4.88 (d, 1H, $J_{1',2'}$ = 3.2 Hz, H-1´), 4.58 (d, 1H, $J_{3'',4''} = J_{4'',5''}$ = 3.0 Hz, H-4´´), 4.50 (d, 1H, $J_{1a,1b}$ = 12.0 Hz, H-1a), 4.45 (d, 1H, $J_{6a',6b'}$ = 12.0 Hz, H-6a´), 4.34 (d, 1H, $J_{1a,1b}$ = 12.0 Hz, H-1b), 4.20 – 4.00 (m, 3H, H-2´, H-6b´, H-4´), 3.87 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.8 Hz, H-3´), 3.75 (d, 1H, $J_{6a'',6b''}$ = 11.6 Hz, H-6a´´), 3.64 (bs, 1H, H-5´´), 3.57 (d, 1H, $J_{6a'',6b''}$ = 11.6 Hz, H-6b´´), 3.45 (d, 1H, $J_{4',5'}$ = 9.9 Hz, H-5´), 1.99 (s, 3H, CH_3 Ac), 1.08 (s, 9H, CH_3 *t*Bu); ^{13}C NMR (125 MHz, D_2O) δ 170.3 (C=O Ac), 166.2 (C=O 3-*O*-Bz), 165.1 (C=O 2-*O*-Bz), 137. 6, 137.3, 136.1, 135.7, 134.0, 133.6, 133.3, 133.0, 130.1, 130.0, 129.8, 129.2, 129.1, 128.6, 128.5, 128.3, 128.1, 128.0, 127.8, 126.4 (C_{arom}), 100.93 (CPh), 100.91 (C-1´´), 96.8 (C-1´), 79.0 (C-4´), 73.5 (C-4´´), 72.7 (C-3´´), 70.7 (C-5´), 70.2 (C-3´), 69. 8 (C-1), 69.3 (C-2´´), 68.6 (C-6´), 67.0 (C-5´´), 61.7 (C-6´´), 53.4 (C-2´), 27.1 (CH_3 *t*Bu), 23.6 (CH_3 Ac), 19.7 (Cq *t*Bu); HRMS(ESI) *m/z* calcd. for $[C_{58}H_{62}NO_{13}Si]^+$: 1008.3985, obsd.: 1008.4009.



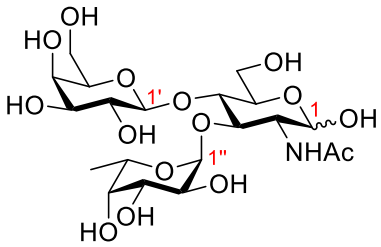
Benzyl 2-acetamido-4-*O*-(2,3-di-*O*-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranoside)-2-deoxy-6-*O*-*tert*-butyl-diphenylsilyl-α-D-glucopyranoside (11). A mixture of glycosyl acceptor **10** (0.90 g, 0.89 mmol) and glycosyl donor **8** (512 mg, 1.07 mmol)[23,76] were co-evaporated with dry DMF (3 × 3 mL) and dissolved in dry DMF (5 mL) and dry CH_2Cl_2 (10 mL). Activated molsieves (4Å) were added and the reaction mixture was stirred at rt for 30 min. TPABr (570 mg, 2.14 mmol) and $CuBr_2$ (480 mg, 2.14 mmol) were added and the reaction mixture was stirred ar rt for 15 h. When TLC analysis showed full conversion of the glycosyl acceptor, the crude reaction mixture was quenched by the addition of sat. aq. $Na_2S_2O_3$ (30 mL). The water layer was extracted with CH_2Cl_2 (3 × 10 mL), and the combined organic layers were washed with brine, dried with $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified by silica gel flash column chromatography (PE/EtOAc, 9/1 → 2/1, v/v) and crystallised from methanol to afford trisaccharide **11** (1.05 g, 0.74 mmol, 92%) as white crystals. R_f = 0.23 (PE/EtOAc, 2/1, v/v); $\alpha_D^{24.4}$ = +110 (c = 0.5, $CHCl_3$); IR (film) 3088, 3064, 3032, 3007, 2932, 2894, 2859, 1735, 1671, 1497, 1453, 1428, 1367, 1315, 1273, 1250, 1250, 1219, 1165, 1143, 1098, 1061, 1046, 1027, 1002, 772, 708, 699 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (d, 2H, $J_{o,m}$ = 7.7 Hz, CH-*o* Bz), 7.87 – 7.81 (m, 4H, CH_{arom}), 7.64 – 7.07 (m, 39H, CH_{arom}), 5.88 (t, 1H, $J_{2'',3''}$ = 9.0 Hz, C-2´´), 5.60 (s, 1H,

CHPh), 5.49 (d, 1H, $J_{2',NH}$ = 9.9 Hz, NH), 5.28 – 5.20 (m, 3H, H-1´´, H-1´´´, H-3´´), 4.95 (q, 1H, $J_{5''',6'''}$ = 6.7 Hz, H-5´´´), 4.84 – 4.77 (m, 2H, H-1´, CH_2-a 2´´´-*O*-Bn), 4.73 (d, 1H, $J_{a,b}$ = 11.7 Hz, CH_2-a 3´´´-*O*-Bn), 4.68 (d, 1H, $J_{a,b}$ = 11.7 Hz, CH_2-b 3´´´-*O*-Bn), 4.58 – 4.48 (m, 4H, H-4´´, CH_2-b 3´´´-*O*-Bn, H-2´, H-6a´), 4.41 – 4.31 (m, 3H, H-1a, H-1b, H-4´), 4.20 (d, 1H, $J_{a,b}$ = 11.4 Hz, CH_2-a 4´´´-*O*-Bn), 4.12 (d, 1H, $J_{6a',6b'}$ = 12.1 Hz, H-6b´), 4.06 – 4.01 (m, 1H, H-3´´´), 3.99 (t, 1H, $J_{2',3'} = J_{3',4'}$ = 9.7 Hz, H-3´), 3.96 – 3.89 (m, 2H, H-2´´´, H-6a´´), 3.54 – 3.47 (m, 3H, H-6b´´, H-5´´, CH_2-b 4´´´-*O*-Bn), 3.29 (d, 1H, $J_{4',5'}$ = 9.8 Hz, C-5´), 3.25 (bs, 1H, H-4´´´), 1.81 (s, 3H, CH_3 Ac), 1.33 (d, 3H, $J_{5''',6'''}$ = 6.2 Hz C-6´´´), 1.14 (s, 9H, CH_3 *t*Bu); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.7 (C=O Ac), 166.0 (C=O 3-*O*Bz), 164.8 (C=O 2-*O*Bz), 139.9, 139.8, 138.6, 137.8, 137.1, 136.2, 135.6, 134.0, 133.6, 133.2, 132.4, 130.3, 130.1, 130.0, 129.7, 129.2, 129.1, 129.0, 128.7, 128.64, 128.55, 128.52, 128.48, 128.45, 128.35, 128.3, 128.23, 128.20, 128.1, 128.0, 127.9, 127.7, 127.6, 127.5, 127.4, 127.1, 126.9, 125.9 (C_{arom}), 99.9 (C-1´´), 99.8 (CHPh), 97.9 (C-1´´´), 96.8 (C-1´), 79.4 (C-4´´´), 78.8 (C-3´´´), 75.3 (C-2´´´), 75.0 (CH_2 4´´´-*O*-Bn), 73.9 (C-4´), 73.6 (C-4´´), 72.9 (C-3´´, CH_2 2´´´-*O*-Bn), 72.7 (C-3´), 72.0 (CH_2 3´´´-*O*-Bn), 71.3 (C-5´), 70.1 (CH_2 1-*O*-Bn), 69.2 (C-2´´), 69.1 (C-6´), 66.6 (C-5´´, C-5´´´), 61.3 (C-6´´), 54.1 (C-2´), 27.1 (CH_3 *t*Bu), 23.7 (CH_3 Ac), 19.7 (Cq *t*Bu), 16.4 (C-6´´´); HRMS(ESI) *m/z* calcd. for $[C_{85}H_{89}NO_{17}SiNa]^+$: 1446.5792, obsd.: 1446.5797.



Benzyl 2-acetamido-4-*O*-(4,6-O-benzylidene-β-D-galacto-pyranosyl)-3-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-α-D-glucopyranoside (12). To a solution of trisaccharide **11** (201 mg, 141 µmol) in methanol (3.0 mL) and CH_2Cl_2 (3.0 mL), 1M methanolic NaOMe (2.0 mL) was added and the reaction mixture was stirred at rt for 4 h. The reaction mixture was quenched by the addition of Dowex H^+ and the reaction mixture was filtered, washed with MeOH and concentrated *in vacuo*. The crude product was used in the next step without further purification. R_f = 0.57 (PE/EtOAc, 1/1, v/v); HRMS(ESI) *m/z* calcd. for $[C_{71}H_{81}NO_{15}SiNa]^+$: 1238.5268, obsd.: 1238.5276.; The crude trisaccharide was dissolved in pyridine (5 mL), HF.pyridine (0.5 mL) was added at 0 °C and the reaction mixture was stirred ar rt for 18 h. After TLC analysis showed complete conversion, the mixture was diluted with CH_2Cl_2 (25 mL) and washed with 1M aq. $Ca(OAc)_2$ (50 mL). The aqeaous layer was extracted with CH_2Cl_2 (2 × 25 mL) and the combined organic extracts were washed with brine, dried with $MgSO_4$, filtered and concentrated *in vacuo*. The residue was co-evaporated with toluene (3 × 5 mL) in order to remove traces of pyridine, and purified by silica gel flash column chromatography (EtOAc/MeOH, 100/0 → 95/5, v/v) to afford triol **12** (137 mg, 140 µmol, 99%) as a white foam. R_f = 0.45 (EtOAc/MeOH, 8/1, v/v), $\alpha_D^{24.6}$ = -24.2 (c = 0.5, $CHCl_3$); IR (film) 3428, 3334, 3063, 3031, 2974, 2929, 2906, 2872, 1658, 1543, 1497, 1454, 1397, 1363, 1338, 1246, 1213, 1165,

1136, 1093, 1047, 966, 909, 858, 734, 697 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 7.48 – 7.42 (m, 2H, CH_{arom}), 7.31 – 7.14 (m, 22H, CH_{arom}), 7.11 (t, 1H, *J* = 7.6 Hz, CH_{arom}), 7.00 (t, 2H, *J*= 7.6 Hz, CH_{arom}), 6.79 (d, 1H, $J_{NH,2'}$ = 7.2 Hz, NH), 5.47 (s, 1H, CHPh), 5.38 (d, 1H, $J_{1'',2''}$ = 3.2 Hz, H-1´´), 4.98 (d, 1H, $J_{1''',2'''}$ = 3.4 Hz, H-1´´´), 4.80 (d, 1H, $J_{1',2'}$ = 7.6 Hz, H-1´), 4.70 – 4.62 (m, 3H, CH_2a 2-*O*-Bn, CH_2a 3-*O*-Bn, CH_2a 4-*O*-Bn), 4.60 (d, 1H, $J_{CH2a,CH2b}$ = 10.4 Hz, CH_2b 2-*O*-Bn), 4.55 (d, 1H, $J_{1a,1b}$ = 12.0 Hz, CH_2a-1-*O*-Bn), 4.52 (d, 1H, $J_{CH2a,CH2b}$ = CH_2b 3-*O*-Bn), 4.35 (d, 1H, $J_{1a,1b}$ = 12.0 Hz, CH_2b-1-*O*-Bn), 4.31 – 4.20 (m, 2H, H-6a´´, H-5´´´), 4.16 (dd, $J_{2',3'}$ = 9.1 Hz, $J_{3',4'}$ = 10.2 Hz, H-3´), 4.10 – 4.03 (m, 2H, H-4´, H-4´´), 4.00 (bd, $J_{6a',6b'}$ = 12.2 Hz, H-6a´), 3.98 – 3.90 (m, 3H, H-2´, H-2´´, H-6b´´), 3.84 (dd, 1H, $J_{3''',4'''}$ = 2.5 Hz, $J_{2''',3'''}$ = 10.0 Hz, H-3´´´), 3.74 – 3.64 (m, 3H, H-2´´, H-5´, H-6b´), 3.59 – 3.51 (m, 2H, H-4´´´, H-5´´), 3.51 – 3.44 (m, 2H, H-3´´, OH), 2.85 (bs, 1H, OH), 1.70 (bs, 2H, 2 × OH), 1.45 (s, 3H, CH_3 Ac), 1.04 (d, 3H, $J_{5''',6'''}$ = 6.6 Hz, H-6´´´); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.3 (C=O, Ac), 138.9, 137.8, 137.5, 137.5 (CH_{arom}), 129.2, 128.8, 128.6, 128.51, 128.50, 128.33, 128.28, 128.10, 128.06, 128.0, 127.7, 127.3, 126.5 (CH_{arom}), 102.3 (C-1´´), 101.1 (CHPh), 98.6 (C-1´´´), 96.3 (C-1´), 79.4 (C-3´´´), 77.7 (C-4´´´), 77.5 (C-2´´´), 76. 7 (C-3´), 75.6 (C-4´), 75.3 (C-4´´), 75.1 (CH_2 4-OBn), 74.7 (CH_2 2-OBn), 73.2 (C-2´´), 72.8 (C-5´´), 72.2 (CH_2 3-OBn), 71.1 (C-5´), 69.9 (CH_2-1), 69.5 (C-6´´), 67.5 (C-5´´´), 67.0 (C-3´´), 61.0 (C-6´), 54.1 (C-2´), 22.8 (CH_3 Ac), 17.1 (C-6´´´); HRMS(ESI) *m/z* calcd. for $[C_{55}H_{64}NO_{15}]^+$: 978.4270, obsd.: 978.4274.



2-Acetamido-2-deoxy-3-*O*-(α-L-fucopyranosyl)-4-*O*-(β-D-galactopyranosyl))-α/β-D-glucopyranoside (Le^X). To trisaccharide **12** (137 mg, 140 µmol) in distilled THF (4 mL), H_2O (2 mL), AcOH (1.0 mL) and Pd/C (100 mg) were added and hydrogen gas was bubbled though the reaction mixture at rt for 3 h. The crude mixture was diluted with water (20 mL), filtered over paper (Whatman 42) and lyophilised. Purification by size exclusion chromatography (BioGel P-2, 1200 × 10 mm) gave Le^X (72.5 mg 137 µmol, 98%) as a white foam. R_f = 0.12 (*n*-butanol/AcOH/H_2O, 4/1/1, v/v/v); IR (film) 3313, 2971, 2938, 1640, 1555, 1427, 1378, 1162, 1118, 1071, 1034, 1021, 968, 811 cm^{-1}; ^1H NMR (300 MHz, D_2O) *α-anomer:* δ 5.11 – 5.08 (m, 2H, H-1´, H-1), 4.84 (q, 1H, $J_{5'',6''}$ = 6.7 Hz, H-5´´), 4.46 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1´ α), 4.15 (dd, 1H, $J_{1,2}$ = 3.5 Hz, $J_{2,3}$ = 10.4 Hz, H-2 α), 4.03 – 3.92 (m, 4H, H-3, H-4, H-5, H-6a), 3.92 – 3.81 (m, 8H, H-6b, H-3´´, H-4´), 3.79 (d, 1H, $J_{3'',4''}$ = $J_{4'',5''}$ = 3.0 Hz, H-4´´), 3.77 – 3.62 (m, 4H, H-6´a, H-6´b, H-2´´, H-3´), 3.62 – 3.57 (m, 1H, H-5´), 3.53 – 3.46 (m, 1H, H-2´), 2.03 (s, 3H, CH_3 Ac), 1.19 – 1.15 (m, 3H, H-6´´); *β-anomer:* δ 5.11 – 5.08 (m, 1H, H-1´), 4.84 (q, 1H, $J_{5'',6''}$ = 6.7 Hz, H-5´´), 4.72 (d, 1H, $J_{1,2}$ = 8.1 Hz, H-1 β), 4.45 (d, 1H, $J_{1',2'}$ = 7.8 Hz, H-1´ β), 4.03 – 3.92 (m,

2H, H-4, H-6a), 3.92 – 3.81 (m, 5H, H-6b, H-3´´, H-4´, H-2, H-3), 3.79 (d, 1H, $J_{3'',4''} = J_{4'',5''}$ = 3.0 Hz, H-4´´), 3.77 – 3.62 (m, 4H, H-6´a, H-6´b, H-2´´, H-3´), 3.62 – 3.57 (m, 2H, H-5, H-5´), 3.53 – 3.46 (m, 1H, H-2´), 2.03 (s, 6H, CH_3 Ac), 1.19 – 1.15 (m, 6H, H-6´´); ^{13}C NMR (125 MHz, D_2O) δ 174.4 (C=O β), 174.2 (C=O α), 101.76 (C-1´ β), 101.74 (C-1´ α), 98.6 (C-1´´ β), 98.5 (C-1´´ α), 94.7 (C-1 β), 91.0 (C-1 α), 75.4 (C-5 β), 74.91 (C-3 β), 74.85 (C-5´ α + β), 73.2 (C-4 α+β), 72.8 (C-3 α), 72.4 (C-3´ α+β), 71.8 (C-4´´ α+β), 71.3 (C-5 α), 71.0 (C-2´ α+β), 69.20 (C-3´´ α), 69.15 (C-3´´ β), 68.3 (C-4´ α+β), 67.6 (C-2´´ α+β), 66.6 (C-5´´ α+β), 61.5 (C-6´ α+β), 59.7 (C-6 β), 59.6 (C-6 α), 56.9 (C-2 β), 54.0 (C-2 α), 22.2 (CH_3 Ac β), 21.9 (CH_3 Ac α), 15.2 (C-6´´ α+β); HRMS(ESI) *m/z* calcd. for $[C_{20}H_{36}NO_{15}]^+$: 530.2079, obsd.: 530.2093.

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Chapter 5.

The synthesis and biological evaluation of fluorescent Lewis^X glycodendrons to target DC-SIGN lectins on macrophages.

5.1 Introduction

When monocytes migrate into the tissue in response to pathogenic infection or inflammation, they differentiate into macrophages (Mϕs) and dendritic cells (DCs). Here, the upregulation of pathogen recognising receptors (PRRs), such as Toll-like receptors (TLRs) and C-type Lectin receptor (CLRs), is essential in modulating the immune responses.[1,2] In particular, signaling through the C-type lectin Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) on DCs and Mϕs, can result in the internalisation of the pathogen via endocytosis and antigen processing, which can lead to immune activation against the pathogen through major histocompatibility complex class II (MHC-II)-mediated antigen presentation to $CD4^+$ T-cells.[3] DC-SIGN binds several carbohydrate ligands, including high mannose and Lewis glycans such as the trisaccharide Lewis^X.[4]

Viruses, such as human immunodeficiency virus (HIV), Ebola and Hepatitis C, however, can also use the DC-SIGN-mediated internalisation to invade and infect the host. In this context, the virus can use the glycosylation mechanism of the host cell to glycosylate their viral proteins in order to prevent immune detection. The highly glycosylated proteins form a multivalent display of native ligands which can then efficiently bind to host cells, and allow for the subsequent internalisation of the virus by the host.[5]

In a similar fashion, glycan-based multivalent substances can be utilised to target lectins on specific cells. These agents have the potential to be developed into novel therapeutics and may be employed in vaccination strategies. Accordingly, the objective of this research is to selectively target DCs and Mϕs through DC-SIGN by using highly glycosylated Lewis antigen glycodendrons. In particular, the use of fluorescent glycodendrons would allow for the biological evaluation of DC-SIGN^{+} cells as a flow cytometry marker and demonstrate the potential of the Lewis^{X} glycodendron for other biological applications.

5.1.1 DC-SIGN expression on subsets of macrophages

When monocytes migrate from the blood into the tissue, they differentiate into either DCs or Mϕs depending on the presence or absence of specific cytokines.[6] Mϕs can differentiate into either 'M1-like' or 'M2-like' macrophages, whereby these descriptors represent the two general subsets of Mϕs.[7,8] Herein, it should also be noted that Mϕs exhibit great plasticity and can change phenotype depending on their local environment.

'M1-like' macrophages are characterised as pro-inflammatory macrophages as they produce antimicrobial cytotoxic cytokines, and can activate T-cells in a TH1 mediated pro-inflammatory response.[9] Polarisation into 'M1-like' Mϕs is induced by lipopolysaccharide (LPS), interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and granulocyte macrophage-colony stimulating factor (GM-CSF).[7,10] On the other hand, 'M2-like' Mϕs are immunosuppressive cells and are distinguished by the abundant expression of scavenger receptors, such as the mannose receptor.[7,9] 'M2-like' Mϕs can participate in tissue repair, and promote a TH2 immune response.[9] Mϕs can be polarised into 'M2-like' Mϕs by stimulation with Interleukin-4 (IL-4), IL-10, IL-13, IL-33, transforming growth factor-β (TGF-β) and macrophage-colony stimulating factor (M-CSF).[11]

DC-SIGN expression is highly upregulated upon stimulation with M-CSF, and as a result, the receptor can be found in high concentrations on 'M2-like' macrophages, whereas the expression on the 'M1-like' phenotype is minimal.[12] Moreover, DC-SIGN is expressed on tumour associated macrophages (TAM), which contributes to tumour growth, survival and metastasis, as well as immune suppression against the tumour.[12] In tumour environments, M-CSF is highly upregulated which causes 'M2-like' polarisation and Mϕ recruitment that stimulates pro-tumourigenic activities such as blood vessel preparation. This makes DC-SIGN an attractive target for anti-tumour therapeutics.

5.1.1.1 THP-1 derived DCs and Macrophages

Puig-Kröger *et al.* reported on the DC-SIGN expression of the human THP-1 leukemic cells, monocytes and Mϕs.[13] These THP-1 leukemic cells are widely used in monocyte-macrophage differentiation and serve as a model to study macrophages. Stimulation of these THP-1 cells with phorbol-12-myristate-13-acetate (PMA) results in the differentiation of these monocytes into Mϕs. Stimulation with PMA+IL-4 results in 'M2-like' Mϕs, whereas PMA+IFN-γ+LPS stimulation gives rise to 'M1-like' Mϕs. Thus, by changing the stimuli that THP-1 cells are exposed to in solution, different Mϕ-phenotypes can be generated.

Puig-Kröger *et al.* observed that THP-1 cells, as well as PMA-stimulated cells, have low expression levels of DC-SIGN, however, when THP-1 cells were differentiated with PMA and IL-4, these leukocytes produced high levels of DC-SIGN, comparable to the DC-SIGN transfected K562-cells. In these studies, the pro-inflammatory 'M1-like' Mϕs were not examined for DC-SIGN expression.

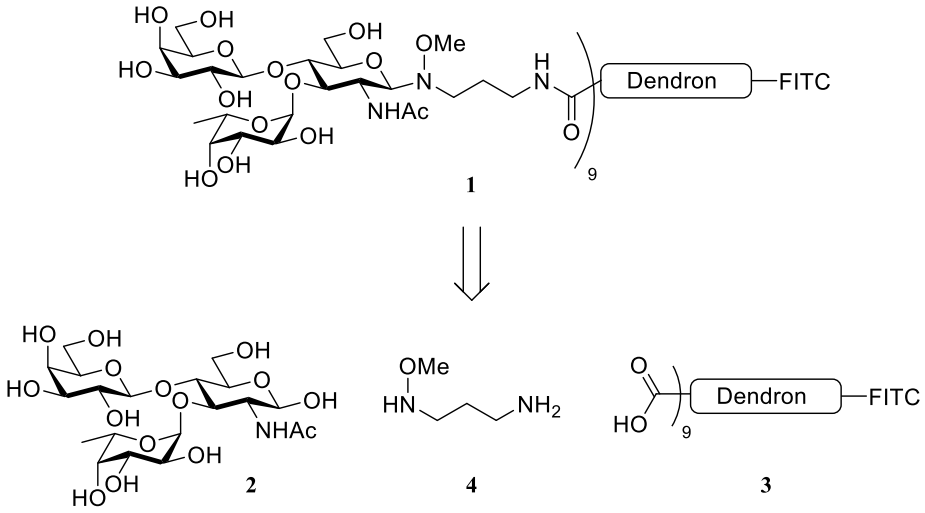
5.1.2 Development of DC-SIGN-mediated therapeutics

As was discussed in section 1.6.5, several glycoconjugates have been synthesised to target the DC-SIGN lectin on DCs and Mϕs, including glycodendrons and glycoproteins. For example, Wong and co-workers synthesised high-mannose glycodendrons in the development of a novel DC-SIGN-mediated vaccine.[14] They showed that these glycodendrons could bind DC-SIGN and inhibit the interaction between gp120 on HIV and DC-SIGN on DCs, and thus possibly prevent HIV infection. Moreover, Davies and co-workers synthesised a glycodendrinanoparticle containing 1620 mannose antigens to prevent HIV infection by blocking the DC-SIGN receptor and demonstrated that these nanoparticles could completely prevent HIV infection.[15]

Van Kooyk and co-workers have also reported the synthesis of several PAMAM-based glycodendrons through the use of reductive amination methodology.[16] In particular, glycodendrons bearing the Lewis^X antigens have been prepared and shown to have high affinity for DC-SIGN.[16] These glycodendrons were used to target DC-SIGN selectively and, in addition, can be used to prevent HIV infection through DC-SIGN. Moreover, DC-SIGN could be targeted using glycodendrons bearing immunogenic peptide antigens for the development of novel vaccines.[17] Taken as a whole, Lewis antigen glycodendrons show great promise in the development of novel therapeutics, and accordingly there is a need for the rapid assembly of these glycoconjugates.

5.1.3 Assembly of Lewis^X-glycodendrons

As described above, the DC-SIGN lectin is an interesting target in the development of novel therapeutics. Several synthetic strategies have been reported for the synthesis of DC-SIGN binding glycodendrons, however, further development of these strategies can result in better substrates that target DC-SIGN. Besides the development of DC-SIGN mediated vaccines, the selective targeting of DC-SIGN on tumour associated macrophages has great potential in the treatment of many types of cancers.[18] With Lewis glycans being excellent substrates for the DC-SIGN lectin, and by increasing binding affinity for these glycans through the use of multivalent glycodendron scaffolds, it was envisioned that multivalent Lewis^X glycodendrons could bind DC-SIGN$^+$ cells with high selectivity. As discussed in Chapter 3, the oxyamine ligation strategy was utilised to conjugate *N*-acetylglucosamine to multivalent substrates such as a biotinylated dendron scaffold, to obtain highly glycosylated dendrons. Accordingly, it was proposed to synthesise a fluorescent Lewis^X glycodendron **1** by conjugating the Lewis^X glycan **2** to a fluorescent second generation dendron **3** via the use of a bi-functional oxyamine linker **4** (Scheme 1). The synthesis of these three individual components was discussed in Chapters 2, 3 and 4.



Scheme 1. *Retrosynthesis of fluorescent multivalent Lewis^X glycodendron **1**.*

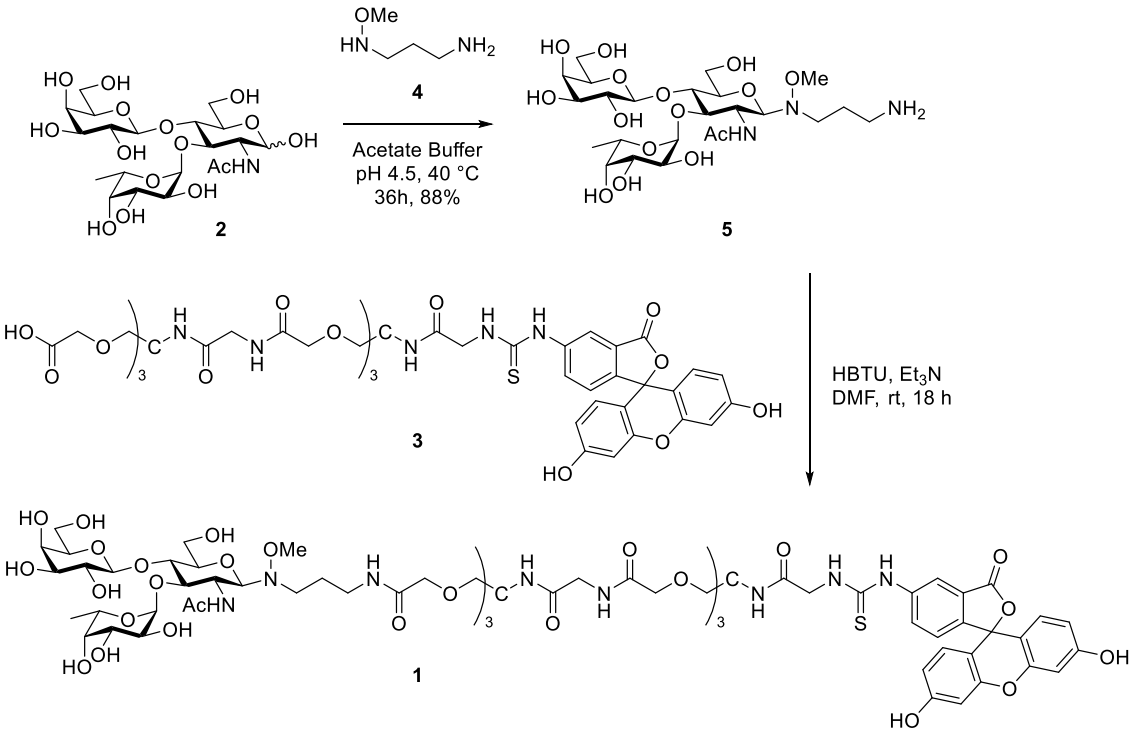
The fluorescent glycodendron **1** can then be used in the detection of DC-SIGN$^+$ cells via flow cytometry assays, while using the anti-DC-SIGN antibody as a positive control and unglycosylated dendron **3** as a negative control. First, the glycodendron will be tested against various types of human THP-1 derived Mϕs ('M0-like', 'M1-like' and 'M2-like'), where the 'M2-like' Mϕs should show high DC-SIGN expression, and the other subsets have lower DC-SIGN levels. The specificity for DC-SIGN-mediated binding will be examined via the co-staining with the fluorescent anti-DC-SIGN-antibody. These results will indicate if these fluorescent

glycodendrons are suitable as flow cytometry reagents for DC-SIGN^{+} cells. Moreover, these fluorescent glycodendrons can be utilised to study DC-SIGN-mediated internalisation, which may give a better insight into the biological functions of this lectin, but could also lead to the further development of DC-SIGN-mediated therapeutics, such as vaccines and anti-tumourigenic agents. To achieve this, alteration of the Lewis^{X} glycodendron with antigenic peptides instead of fluorophores may result in the DC-SIGN-mediated internalisation by DCs and Mϕs followed by the antigen presentation to CD4^{+} T-cells, a desired effect in vaccination strategies. In a similar fashion, the synthesis of glycodendrons bearing toxins may find its use in the depletion of tumour associated Mϕs.

5.2 Results and discussion

5.2.1 Glycodendron synthesis

As described in Chapter 2, the conjugation of natural glycan antigens with the substrate of choice can be achieved using bi-functional oxyamine linkers. These reactions can be performed under buffered conditions without the use of protecting groups and, depending on the type of linker used, allow for the rapid assembly of a variety of glycoconjugates. For the assembly of the target glycodendron, Lewis^X (**2**) was condensed with amine-functionalised bi-functional linker **4** (Scheme 2). Initially, the reaction was performed at room temperature, however, only a small amount of glycoconjugate was observed. When the reaction was heated to 40 °C for 36 hours, Lewis^X was converted into the desired trisaccharide and direct purification by size exclusion chromatography (BioGel P2) then allowed for the isolation of the desired trisaccharide as the ammonium formate salt. The ammonium formate salt, however, could not be used in peptide ligation strategies due to the conjugation of formic acid to form the formamide derivative. Accordingly, ion exchange chromatography with Dowex OH$^-$ resulted in the formation of the free amine **5** in a good yield (88%) and the resulting trisaccharide was suitable for conjugation with the fluorescent dendron.



Scheme 2. *Synthesis of glycodendron **1** by conjugating Lewis^X **2** to the fluorescein derived second generation dendron **3** by utilising the bi-functional methoxyamine linker **4**.*

Next, conjugation with the fluorescein isothiocyanate (FITC)-labelled second generation dendron **3** was performed using the HBTU-mediated peptide ligation methodology. Here, the fluorescent dendron **3** (1 equiv.) was dissolved in freshly distilled DMF and the volume of the solvent was then reduced by half, so as to remove traces of dimethylamine which could react with the activated ester. This solution was then added to the Lewis^X glycan **5** (18 equiv.), and reacted by the addition of HBTU (27 equiv.) and Et_3N (36 equiv.). Here, the order of addition is important as the Lewis^X glycan **5** has poor solubility in DMF, however, completely dissolves after the addition of HBTU and Et_3N. After one hour, the reaction mixture was analysed using HRMS and it was observed that a mixture of the hepta-, octa- and nona-valent glycodendron was formed (*m/z* for $[C_{285}H_{476}N_{36}O_{168}S]^{4+}$ calcd.: 1781.4903, obsd.: 1781.4906). When the reaction mixture was stirred overnight, the MS showed a similar product ratio, and thus the glycodendron was purified using size exclusion chromatography (Sephadex CM C-25, 0.1 M aq. NH_4HCO_2) to obtain the fluorescent glycodendron product. The glycodendron was lyophilised twice and dissolved in H_2O (1 mL). HRMS analysis of the glycodendrons, showed the presence of the hexa-, hepta-, octa- and nona-glycosylated dendrons (Figure 1). To determine the final concentration, the solution was analysed using a colorimetric assay (UV-VIS) by diluting 50, 100, 150 and 200 µL with 1500 µL water (Figure 2). Here the amount of stock solution is related to the absorbance (Figure 2 **A**), and this can then be correlated to the amount of glycodendron present in the stock solution (Figure 2 **B**) based on an average molecular weight of 6.5 kDa and the extinction coefficient of 85000. The concentration of the stock solution was thus determined to be 0.66 mg/mL (0.1 µM). The stock solution was used in the biological evaluation of the C-type lectin DC-SIGN.

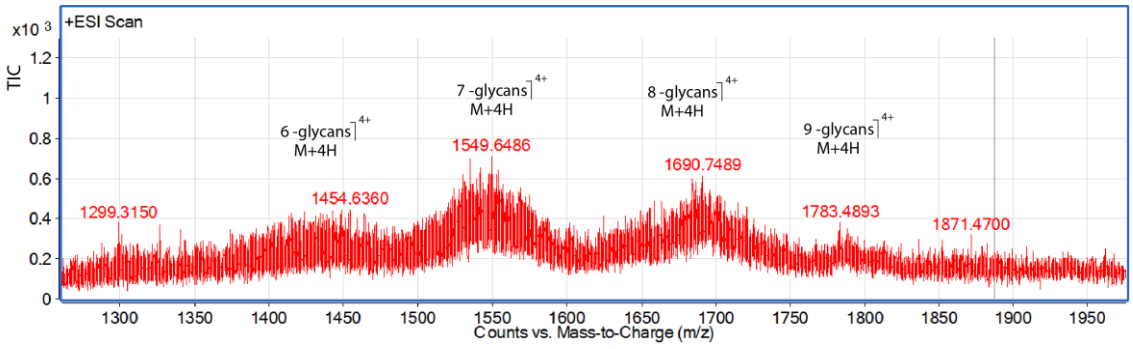


Figure 1. *HRMS of the fluorescent Lewis^X glycodendron stock solution.*

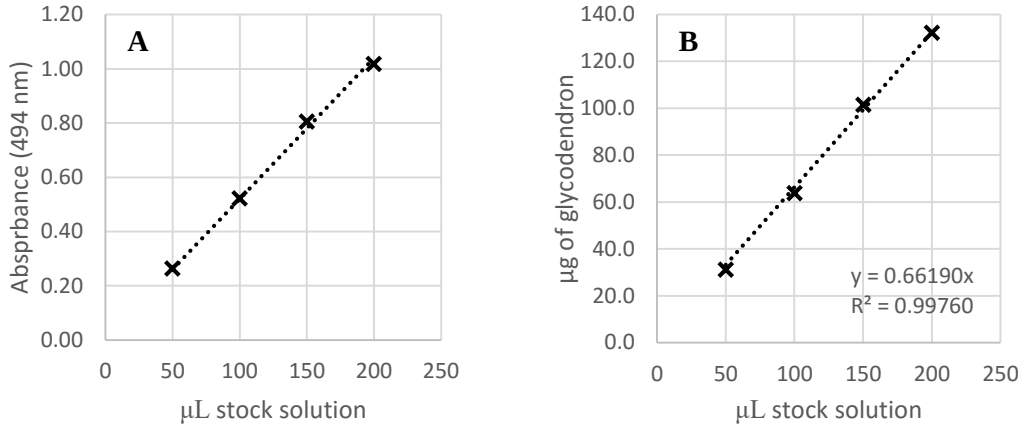


Figure 2. *Colorimetric assay showed a concentration of 0.66 mg/mL (100 µM) of glycodendron* ***1*** *stock solution.*

5.2.2 Biological evaluation of fluorescent glycodendron

The THP-1 acute monocytic lymphoma cell line was cultured in Fetal Calf Serum (FCS), Glutamax and Penstrep to obtain enough cells for differentiation and subsequent flow cytometry analysis. Next, the cells were differentiated into 'M0-like' Mϕs (PMA), 'M1-like' Mϕs (PMA + IFN-γ + LPS), or 'M2-like' Mϕs (PMA + IL-4). For the flow cytometry assays, the different types of cells were incubated for 1 hour at 4 °C with the various substrates (FITC glycodendron, positive and negative control), washed twice, and analysed accordingly.

5.2.2.1 Preliminary Flow cytometry results

First, the THP-1 cells as well as the different types of Mϕs were compared by CD14, CD86 and DC-SIGN expression (Figure 3). Interestingly, it was observed that undifferentiated THP-1 cells express high levels of DC-SIGN which is in contrast with previously reported experiments.[13] When these THP-1 cells were stimulated with PMA, the expression of CD14 and DC-SIGN was reduced. Stimulation of these THP-1+PMA derived Mϕs with LPS and IFN-γ upregulates CD14, CD86 as well as DC-SIGN. Stimulation with IL-4, however, did not result in the upregulation of DC-SIGN and, in addition, did not affect CD14 and CD86 expression.

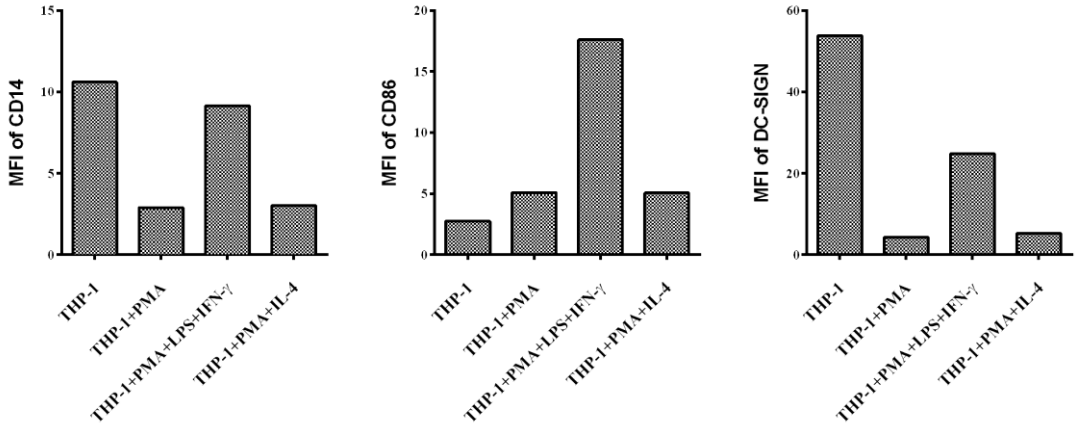


Figure 3. *Cellular expression of CD14, CD86 and DC-SIGN on different subsets of cells; MFI (median fluorescent intensity).*

The THP-1 cells stimulated with PMA and IL-4 were used to study the DC-SIGN binding of the anti-DC-SIGN antibody (positive control), the unglycosylated dendron **3** (negative control) and the Lewis^X glycodendron **1** (Figure 4). Here, it was observed that the anti-DC-SIGN antibody did bind to the Mϕs in a concentration-dependent manner. The negative control did not show binding to the Mϕs in low µM concentration, whereas the highest concentration tested (100 µM) gave a small increase in mean fluorescence. The Lewis^X glycodendron also bound to the cells in a concentration-dependent manner, with observed binding above 0.1 µM concentrations, whereas binding was not observed in the low nanomolar concentration range.

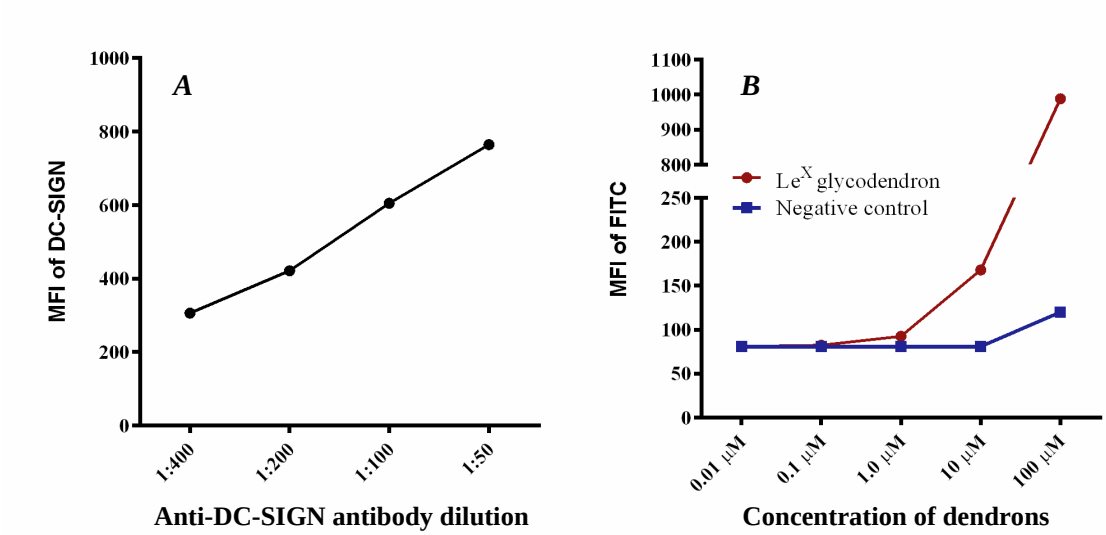


Figure 4. *The expression of DC-SIGN on THP-1+PMA+IL-4 cells measured with A) anti-DC-SIGN antibody and B) unglycosylated dendron **3** (negative control) and the fluorescent Lewis^X glycodendron **1**.*

To investigate the DC-SIGN mediated binding of Lewis^X glycodendrons, co-staining of the glycodendron with the anti-DC-SIGN antibody was then conducted on the 'M2-like' Mϕs (Figure 5). Here, it is important to note that the co-staining with the antibody did not interfere with the glycan-mediated binding, as the co-stain resulted in similar binding of the fluorescent glycodendron when compared to the glycodendron single staining (Figure 5A).

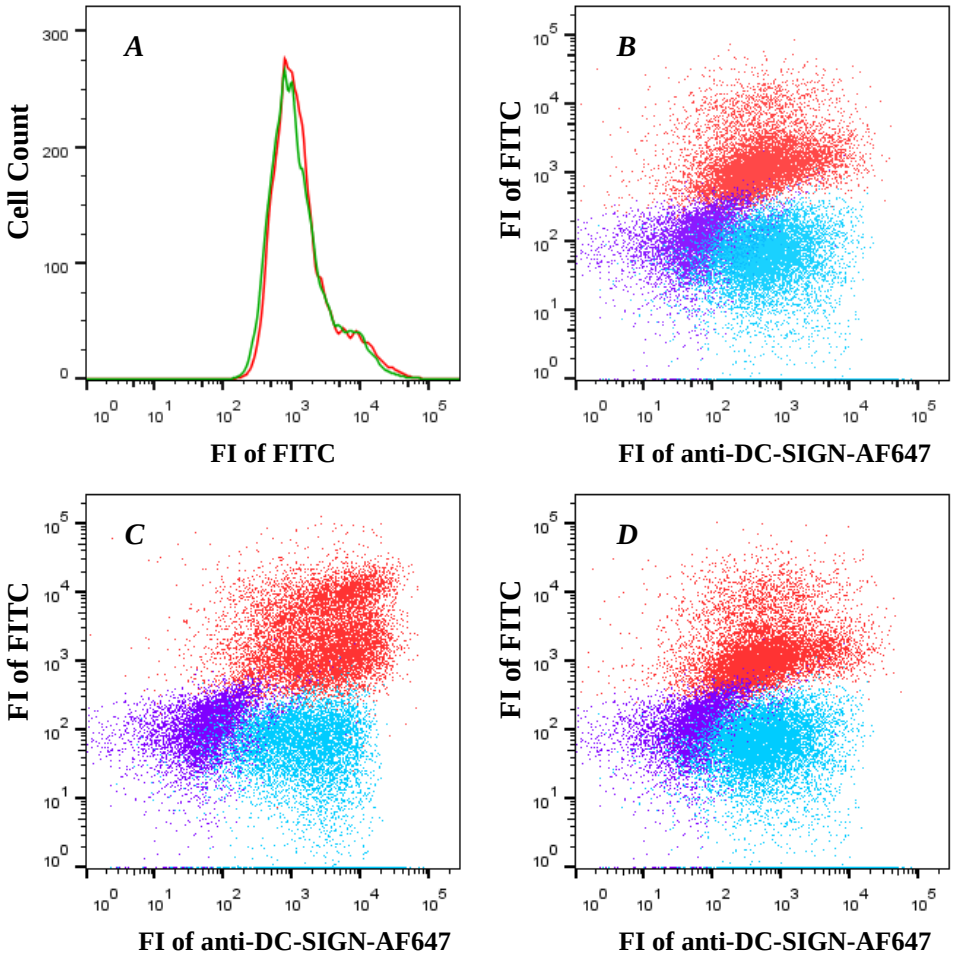


Figure 5. *(A) The costaining with the antibody does not affect glycodendron binding to THP-1+PMA+IL-4 cells, single staining with 100μM glycodendron (green), co-staining of the anti-DC-SIGN antibody with 100μM glycodendron (red); (B-D) The expression of DC-SIGN measured with the glycodendron and the anti-DC-SIGN antibody on B) THP-1+PMA cells; (C) THP-1+PMA+LPS+IFN-γ cells; (D) THP-1+PMA+IL-4 cells, using single staining with the anti-DC-SIGN antibody (blue), co-staining of the antibody with 100μM glycodendron (red) and unstained cells (purple).*

Next, the co-staining of the different type of Mϕs was conducted using the anti-DC-SIGN antibody and the glycodendron **1** (100 μM concentration) (Figure 5B, 5C and 5D). Here it was observed that all DC-SIGN$^+$ cells are FITC$^+$. Interestingly, in the 'M1-like' Mϕs there are two FITC$^+$ populations which are both DC-SIGN$^+$, but show different glycodendron binding, which

might either indicate non-specific binding or binding to other lectins. However, as the negative control (non-glycosylated dendron **3**) showed low levels of unspecific binding, it appears that the glycan-probe is binding to receptors other than DC-SIGN. Lewis^X, however, is only known to bind to Langerin (on Langerhans cells) and MGL-1 (on mouse Mϕs), and accordingly, this requires further investigation.

Taken as a whole, these data demonstrate that the Lewis^X glycodendron can be used to target Mϕs. This binding appears to occur predominantly via the DC-SIGN lectin, however, there also seems to be a small sub-population of Mϕs which bind the glycodendron independent of DC-SIGN. This will require further investigation using additional antibodies which are known to bind other lectins (*i.e.* Langerin or E-, P- and L-selectins), or biotinylated constructs for the purification of these lectins via streptavidin-columns. Moreover, to explore whether the glycodendron can be used to distinguish between 'M1-like' Mϕs and 'M2-like' Mϕs, different types of macrophages need to be produced, as the THP-1 cells generated in these assays contain low levels of DC-SIGN, regardless of their differentiation conditions.

5.3 Conclusion

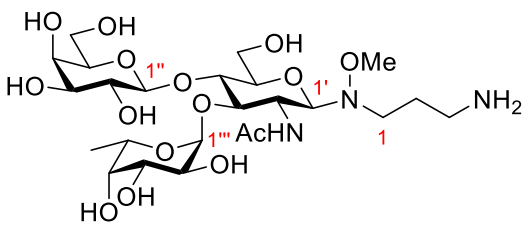
In summary, the synthesis of a complex fluorescent glycodendron has been achieved by using a bi-functional oxyamine linker to conjugate the Lewis^X antigen to a multivalent dendron scaffold. Here, condensation of Lewis^X with the bi-functional oxyamine linker gave the amine-functionalised glycoconjugate in a good 88% yield and the amide ligation with acid-functionalised dendrons also proved to be extremely efficient with eight to nine conjugations per dendron scaffold. The glycodendron was purified by size exclusion chromatography and, to determine the glycodendron concentration of the stock solution, a UV-VIS colorimetric assay was performed.

As proof-of-concept, the fluorescent glycodendron was used as a fluorescent marker in flow cytometry for the C-type lectin DC-SIGN on human THP-1 derived Mϕs. It was observed that the glycodendron binds to THP-1 derived Mϕs in a concentration-dependent manner, with binding being observed at micromolar concentrations. Although preliminary results indicate that these fluorescent glycodendrons are suitable as flow cytometry markers, further investigations are required.

Overall, the combination of glycan antigens, bi-functional oxyamine linkers and multivalent dendron scaffolds has proven to be an effective strategy towards the rapid assembly of complex glycodendrons which then allows for the biological evaluation of these macromolecules. Ideally, future work will demonstrate that these and related Lewis antigen glycodendrons have great potential as DC-SIGN-mediated therapeutics.

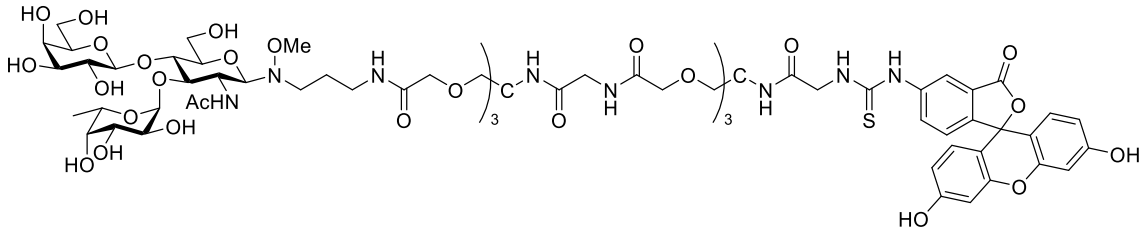
5.4 Experimental

General procedure. Prior to use, DMF was distilled from BaO, Et_3N (Sigma) was distilled from KOH, and H_2O was purified by millipore. AcOH (Ajax Finechem), NH_4OAc (Sigma Aldrich), NH_4HCO_2 (Sigma Aldrich) and HBTU (Apollo Scientific) were used as received. Reactions were monitored by TLC-analysis on Macherey-Nagel silica gel coated plastic sheets (0.20 mm, with fluorescent indicator UV_{254}) with detection by UV-absorption (short wave UV – 254 nm; long wave UV – 366 nm), by dipping in 10% H_2SO_4 in EtOH followed by charring at ~150 °C, or by dipping into a solution of ninhydrin in EtOH followed by charring at ~150 °C. Infrared spectra were recorded as thin films using a Bruker Tensor 27 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory and are reported in wave numbers (cm^{-1}). Nuclear magnetic resonance spectra were recorded at 20 °C in D_2O using either a Varian INOVA operating at 500 MHz or Varian VNMRS operating at 600 MHz. Chemical shifts are given in ppm (δ) relative to solvent residues. NMR peak assignments were made using COSY, HSQC and HMBC 2D experiments.



***N*-(2-Acetamido-2-deoxy-3-*O*-(α-L-fuco-pyranosyl)-4-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl)-*N*-(3-aminopropyl)-*O*-methylhydroxylamine (5)**. To a solution of Lewis^X **23** (5.6 mg, 10.6 µmol) in a AcOH/NH_4OAc buffer (0.5 mL, 2M, freshly prepared, pH 4.5), 3-(methoxyamino)propanyl-amine hydrochloride **4** (15.9 mg, 113.2 µmol) was added and the reaction mixture was stirred at 40 °C for 35 h. The crude mixture was directly loaded onto a size exclusion column (Bio-Gel P-2, 1200 × 18 mm) and eluted with 0.1 M aq. NH_4HCO_2. Lyophilisation of the product fractions afforded neoglycoside **24** (5.7 mg, 88%). R_f = 0.10 (CH_2Cl_2/EtOH/MeOH/NH_3 (aq. 35%), 5/2/2/1, v/v/v/v); $\alpha_D^{19.5}$ = -9.3 (c = 0.2, MeOH); IR (film) 3341, 2925, 2852, 17117, 1647, 1586, 1466, 1451, 1415, 1380, 1350, 1302, 1233, 1193, 1085, 1026, 968, 917 cm^{-1}; ^{1}H NMR (500 MHz, D_2O) δ 5.12 (d, 1H, $J_{1''',2'''}$ = 3.9 Hz, H-1‴), 4.84 (q, 1H, $J_{5''',6'''}$ = 6.7 Hz, H-5‴), 4.49 – 4.42 (m, 2H, H-1′, H-1″), 4.05 (m, 2H, H-2′, H-6a′), 3.93 – 3.81 (m, 5H, H-3‴, H-4″, H-4′, H-3′, H-6b′), 3.80 (d, 1H, $J_{3''',4'''}$ = $J_{4''',5'''}$ = 2.9 Hz, H-4‴), 3.80 – 3.68 (m, 3H, H-6a″, H-6b″, H-2‴), 3.64 (dd, 1H, $J_{3'',4''}$ = 3.2 Hz, $J_{2'',3''}$ = 9.9 Hz, H-3″), 3.60 – 3.55 (m, 1H, H-5″), 3.54 – 3.46 (m, 1H, H-5″), 3.54 – 3.46 (m, 5H, OCH_3, H-5′, H-2″), 3.11 – 2.92 (m, 4H, CH_2-1, CH_2-3), 2.02 (s, 3H, CH_3 Ac), 1.95 (m, 2H, CH_2-2), 1.17 (d, 3H, $J_{5''',6'''}$ = 6.7 Hz, H-6‴); ^{13}C NMR (125 MHz, D_2O) 170.5 (C=O), 101.8 (C-1″), 98.7 (C-1‴), 90.5 (C-1′), 76.9 (C-5′), 76.1 (C-3′), 74.8 (C-5″), 73.2 (C-4′), 72.4 (C-3″), 71.8 (C-4‴), 70.9 (C-2″), 69.1 (C-3‴), 68.3 (C-4″), 67.6 (C-2‴), 66.7 (C-5‴), 61.4 (C-6″),

60.9 (OCH_3), 59.7 (C-6′), 52.5 (C-2′), 47.2 (C-1), 37.5 (C-3), 24.5 (C-2), 22.1 (CH_3 Ac), 15.2 (C-6′′); HRMS(ESI) *m/z* calcd. for $[C_{24}H_{46}N_3O_{15}]^+$: 616.2923, obsd.: 616.2938.



***N*-[*N*-([(3',6'-Dihydroxy-3-oxospiro[isobenzofuran-1(*3H*),9'-(*9H*)xanthen]-5-yl)amino]-thioxomethyl)glycyl]-1,1,1-tris(1,1,1-tris[3-*(N*-(2-acetamido-2-deoxy-3-*O*-(α-L-fuco-pyranosyl)-4-*O*-(β-D-galactopyranosyl)-β-D-gluco-pyranosyl)-*N*-(3-aminopropyl)-*O*-)-*O*-methyl-hydroxylamine)propylamidocarbonylmethyloxymethyl]methylamidocarbonyl-methylamidocarbonylmethyloxy-methyl)aminomethane (1).** To Lewis^X trisaccharide **5** (6 mg, 9.8 µmol), a solution of FITC labeled second generation dendron **3** (0.94 mg, 0.54 µmol) in freshly distilled DMF (250 µL) was added followed by the addition of HBTU (5.6 mg, 15 µmol) and Et_3N (10 µL, 72 µmol) and the reaction mixture was stirred at rt for 18h. The crude mixture was diluted with H_2O (1 mL) and purified by size exclusion chromatography (Sephadex CM C-25, 0.1M aq. NH_4HCO_2). Lyophilisation of the product afforded fluorescent glycodendron **1** as a yellow foam. HRMS(ESI) *m/z* calcd. for $[C_{285}H_{476}N_{36}O_{168}S]^{4+}$: 1781.4903, obsd.: 1781.4906.

THP-1 derived Mϕs differentiation. The following experiments were performed by Kristel Kodar. The THP-1 acute monocytic lymphoma cell line were cultured in RPMI 1640 medium supplemented with 10% Fetal Calf Serum (FCS), 1% Glutamax and 1% Penstrep, and seeded 2.5×10^5 cells/mL. Differentiation of the THP-1 cells was induced via the treatment with PMA (50 ng/mL, 48 h.) to obtain 'M0-like' Mϕs, followed by addition of IL-4 (20 ng/mL, 24 h.) for 'M2-like' Mϕs, or IFN-γ (10 ng/mL) and LPS (10 ng/mL, 24h) for 'M1-like' Mϕs. For subsequent analysis, the differentiated cells were detached from the tissue culture plates by incubating the cells in PBS on ice.

Flow cytometry. The various types of Mϕs were stained by incubating for 1 hour at 4 °C with either the FITC-labelled glycodendron **1**, negative control **3** or the anti-DC-SIGN antibody, washed twice with PBS, and DC-SIGN expression was measured by flow cytometry. The fluorescent Lewis^X glycodendron and the negative control (non-glycosylated dendron **3**) were used in various concentrations (10^{-1}, 10^1, 10^2, 10^3, 10^4 and 10^5 nM, diluted in 10% FCS), while the human anti-DC-SIGN antibody (Phycoerythrin-labelled) positive control was diluted by a factor of 2 (1/50 → 1/1600, v/v).

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Chapter 6.

Conclusion and future prospects.

6.1 Conclusion

The overall objective of this dissertation was to develop new and efficient strategies for the synthesis of multivalent glycoconjugates. The research was divided into three separate projects: the oxyamine glycan conjugation methodology, the synthesis of functionalised multivalent dendrons and the total synthesis of a Lewis antigen. The elements of these research projects were then combined to complete the synthesis of fluorescent Lewis^X glycodendrons for the detection of the C-type lectin DC-SIGN on macrophages.

In **Chapter 2**, novel methodology was presented for the synthesis of a variety of bi-functional oxyamine linkers in high yield and few (3-4) steps. The oxyamine linkers were employed for the conjugation of representative glycan antigens to the substrate of choice, without the need for protecting groups. Here the oxyamine was used in the glycan conjugation, whereas the second functional group on the linker allowed for a variety of ligation reactions, including peptide ligation, thiol-maleimide Michael addition and sulfonylation. The substrates conjugated to the oxyamine-functionalised glycans via these ligation reactions included molecular probes, such as biotin and fluorescent molecules, as well as multivalent substrates, such as proteins and dendrons. Moreover, the hydrolytic stability of the oxyamine linkage was investigated, and it was determined that these glycoconjugates have excellent half-lives under physiological conditions.

Multivalent ligation is required to induce strong biological responses, and accordingly, the synthesis of multivalent dendrons was the focus of **Chapter 3**. Here, an efficient double exponential growth strategy allowed for the rapid assembly of a novel second generation dendron core. Functionalisation of this dendron scaffold with biotin, followed by glycan conjugation, allowed for the synthesis of biotinylated glycodendrons which can be used in a number of

applications. In addition, the synthesis of fluorescently labelled glycodendrons allowed for the use of these macromolecules in fluorescent microscopy as well as in flow cytometry, as described in Chapter 5.

En route to the formation of complex glycoconjugates, the synthesis of a novel trisaccharide crystalline intermediate which can be used in the synthesis of most Type-2 Lewis antigens was described in **Chapter 4**. In the synthetic route, all monosaccharide building blocks were readily synthesised on a large scale, in few steps, and in good yields. The regioselective glycosylation between the galactose donor and the 3,4-di-hydroxyl GlcNAc acceptor yielded the Gal-β-(1,4)-GlcNAc disaccharide, which was then 3-*O*-fucosylated to give the Lewis^X trisaccharide intermediate as a crystalline product. In summary, the trisaccharide was obtained in good yields and in few steps (38% over 7 steps, longest linear route). For the purpose of this thesis, the trisaccharide was globally deprotected to obtain the Lewis^X antigen in excellent yield.

Finally, **Chapter 5** concerned the synthesis of the complex Lewis^X glycodendrons, by conjugating the glycan antigen and multivalent dendron scaffold through the use of the bi-functional oxyamine linker. As a proof-of-concept, a fluorescent Lewis^X glycodendron was synthesised and tested as a flow cytometry marker for the C-type lectin DC-SIGN on human THP-1 derived macrophages. Preliminary results indicated that these Lewis^X glycodendrons can be used in the selective targeting of DC-SIGN$^+$ cells, however, further investigations are required to confirm these studies, and will be reported in due course.

6.2 Future prospects

The work in this doctoral thesis discussed the rapid assembly of complex glycoconjugates via the use of novel bi-functional linker methodology. In particular, the synthesis of a "Type B" oxyamine is discussed, however, in order to investigate the relative reactivity of both "Type A" and "Type B" oxyamines, future work requires the direct comparison between these two types of linkers. To this end, both types of glycoconjugates could be directly compared in various hydrolysis studies whereby the effect of glycan concentration, buffer strength and pH on hydrolytic stability could be compared.

Further insight into the formation and hydrolysis of oxyamine-linked glycoconjugates may also result in the development of more stable glycoconjugates, and may result in higher glycan conversions and thus higher yielding glycoconjugate syntheses. Moreover, the oxyamine-mediated conjugation of other glycan epitopes, such as tumour associated glycan antigens, to multivalent substrates such as dendrons, proteins and microarray slides, would allow for the development of further glycoconjugates that can be studied for their biological function, and in addition, may allow for the development of novel therapeutics such as anti-cancer vaccines.

As described in Chapter 3, dendritic structures are required for the multivalent presentation of glycan antigens. It was shown by Van Kooyk and co-workers that the larger size dendrimers have lower IC_{50} values, compared to more compact glycodendrimers. With this in mind, bi-functional linkers of various lengths could be synthesised in order to investigate the effect of dendron size on biological activity. Alternatively, the second generation dendron scaffold could be elongated with polyethylene glycol linkers to increase the size of the glycodendrons.

In Chapter 4, the synthesis of Lewis^X, a glycan antigen that plays an important role in targetting of the C-type lectin DC-SIGN, was described. Although Chapter 4 concerns the synthesis of Lewis^X, other Type-2 Lewis antigens (*i.e.* Lewis^Y, Sialyl Lewis^X and sulfated derivatives) could be synthesised from the orthogonally protected trisaccharide intermediate. Moreover, the common intermediate may find use in the synthesis of tumour associated glycan antigens such as dimeric Lewis^X, which may aid in the development of anti-tumour therapeutics.

As described above, bi-functional oxyamine linkers are powerful tools in the synthesis of glycodendrons. Accordingly, Lewis^X glycodendrons were synthesised for the targeting of DC-SIGN. Further experiments are required to demonstrate that the Lewis^X glycodendrons can be used to selectively target DC-SIGN, which will be undertaken in due course. If, however, these macromolecules can be used to selectively target this lectin, leading to endocytosis by dendritic cells and macrophages, these glycodendrons can be utilised in vaccines. Derivatisation of the glycodendron with antigenic peptides for the DC-SIGN-mediated peptide delivery to antigen

presenting cell may lead to immune activation against the peptide antigen through CD-4^{+} T-cells (Figure 1). Moreover, toxic molecules could be added to the dendron so as to facilitate cell-selective depletion of DC-SIGN^{+} cells. This concept could be employed as anti-cancer therapeutics, whereby specific lectins on cancer cells are targeted for toxin delivery. Other glycodendrons could also be prepared for the use in other diseases. For example, the synthesis of 6′-O-sulfo-sialyl Lewis^{X} would allow for the selective targeting of Siglec-8, which is expressed on inflammatory cells such as eosinophils and mast cells. Upon ligation of the siglec, eosinophil apoptosis is induced. Accordingly, 6′-O-sulfo-sialyl Lewis^{X} glycodendrons could be used to decrease eosinophil levels in eosinophilia patients and, in addition, the glycodendron might find use for the relief of symptoms in patients with asthma.

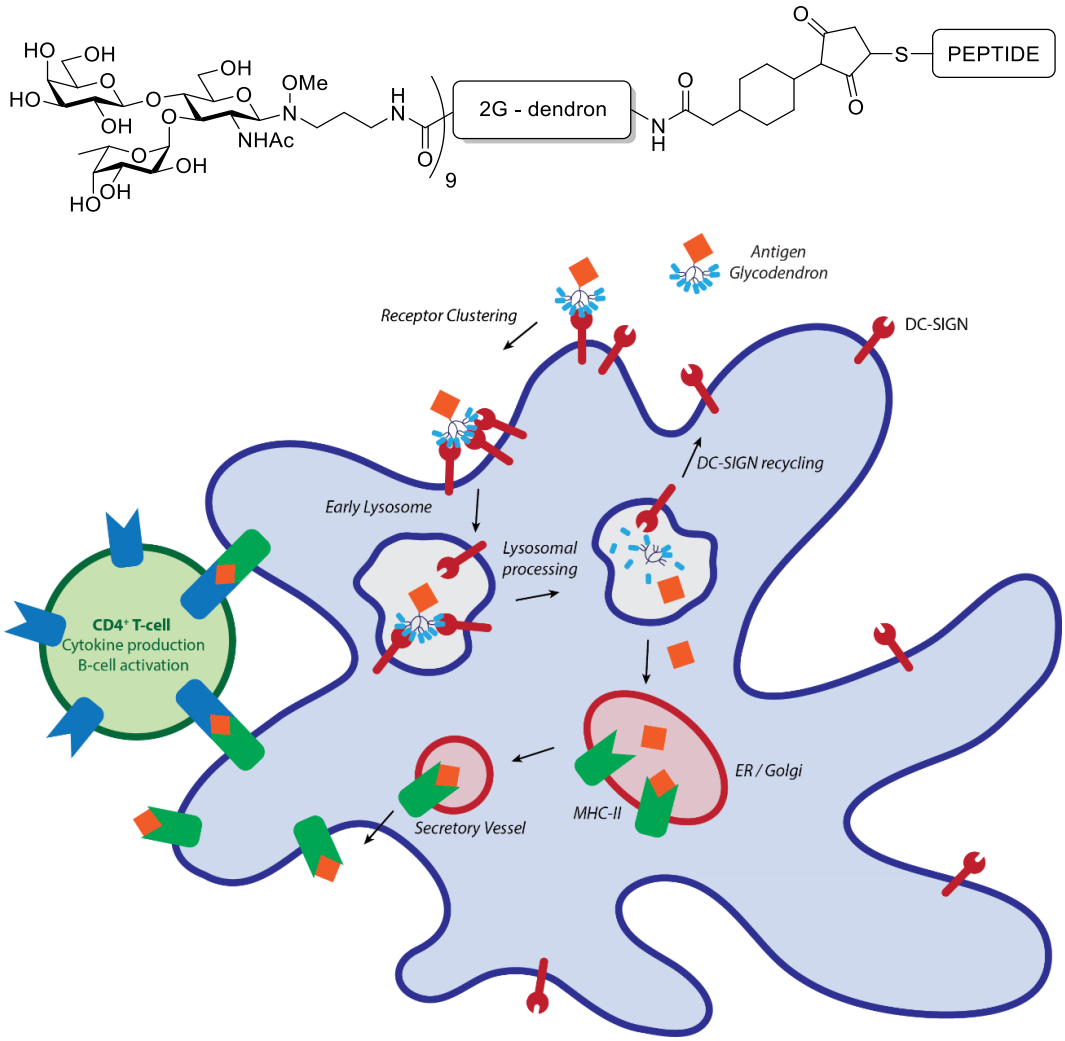

Figure 1. *Antigen-functionalised Lewis^{X} glycodendrons can selectively target DCs and macrophages through the lectin DC-SIGN. Multivalent binding with Lewis antigens results in internalisation in lysosomes, where the antigens get processed, and DC-SIGN recycles to the cell-surface. The antigen then gets transferred to the endoplasmic reticulum (ER) and Golgi apparatus where it is bound to MHC-II, and the complex is secreted to the cell surface. This allows for activation of CD4^{+} T-cells against specific antigens, through the MHC-II mediated antigen presentation to T-cell receptors.*

Appendix Chapter 2.

The Rapid and Facile Synthesis of Oxyamine Linkers for the Preparation of Novel Glycoconjugates.

Table of contents

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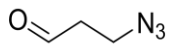

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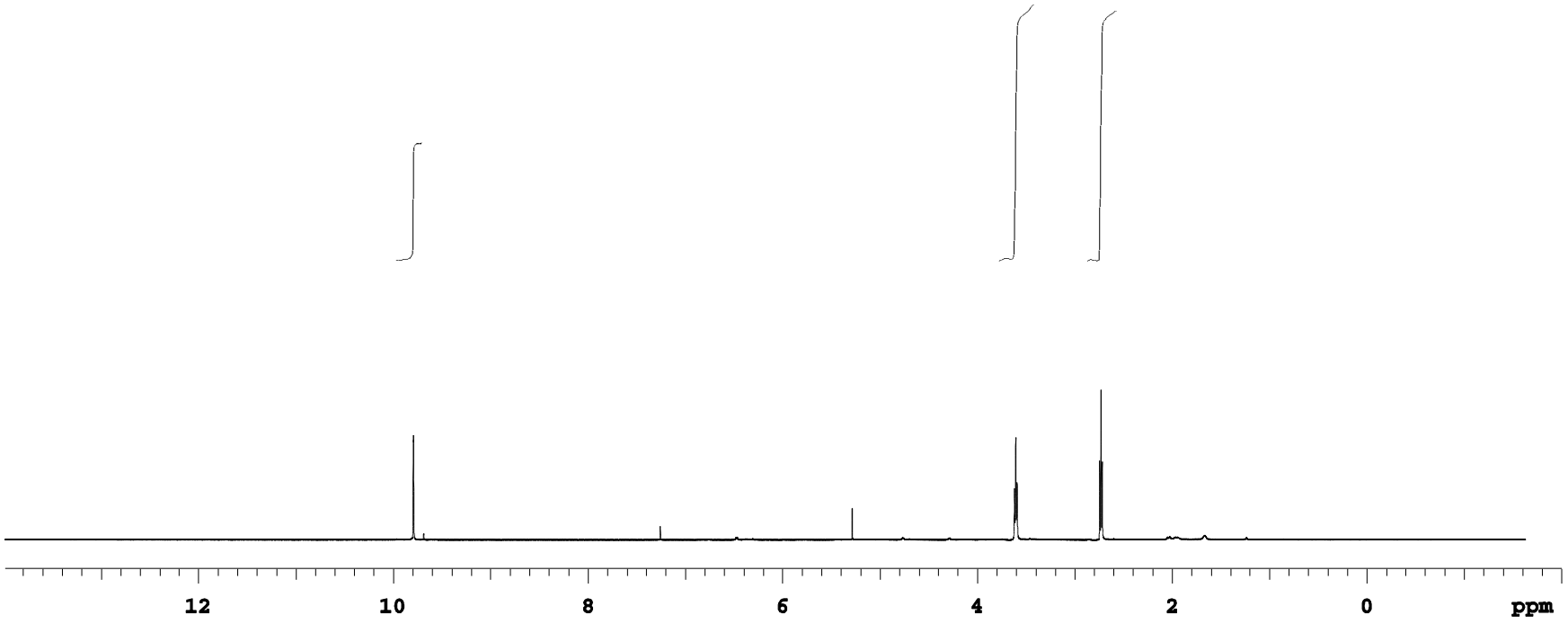

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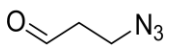

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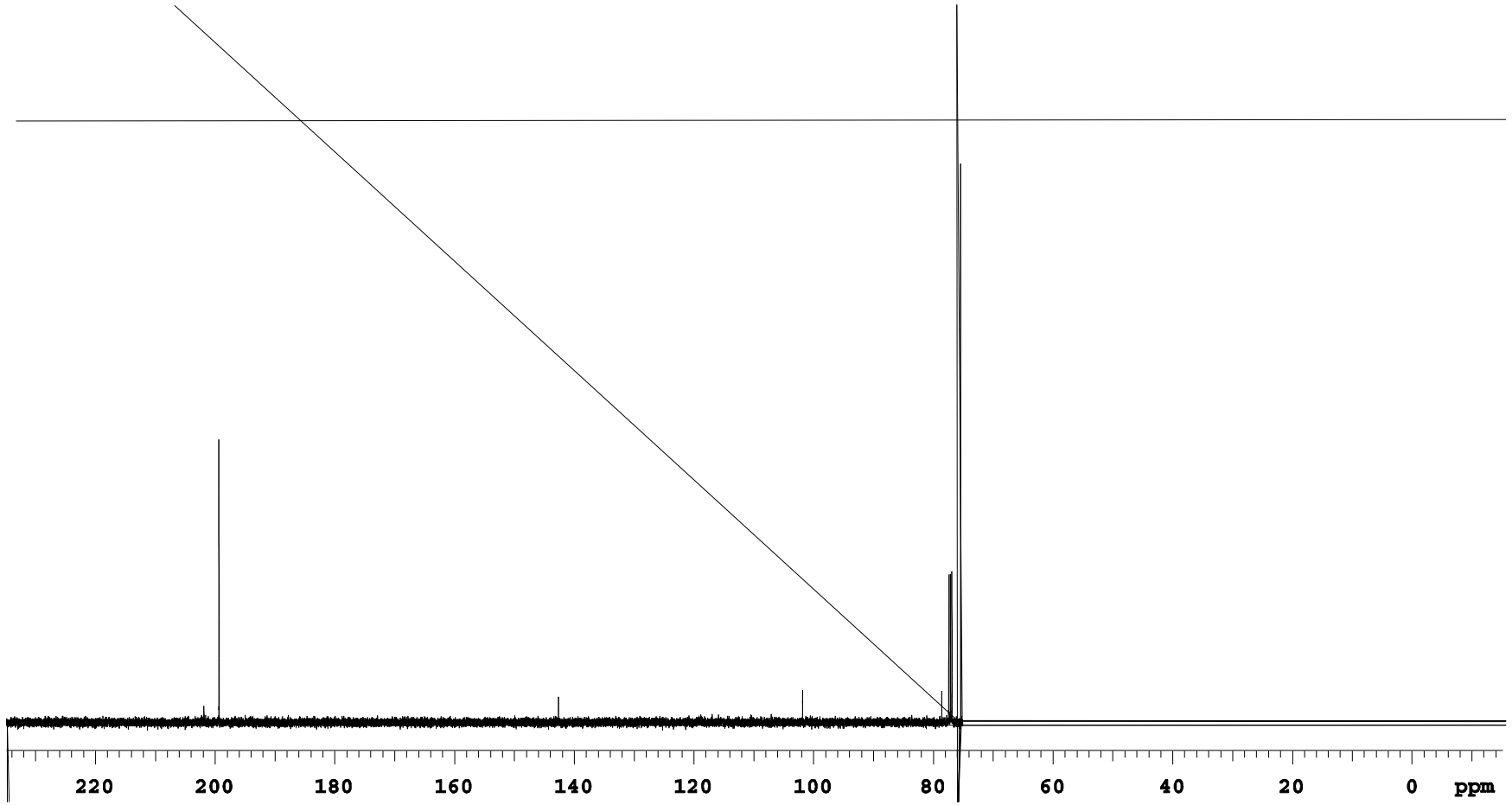

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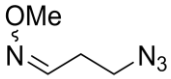

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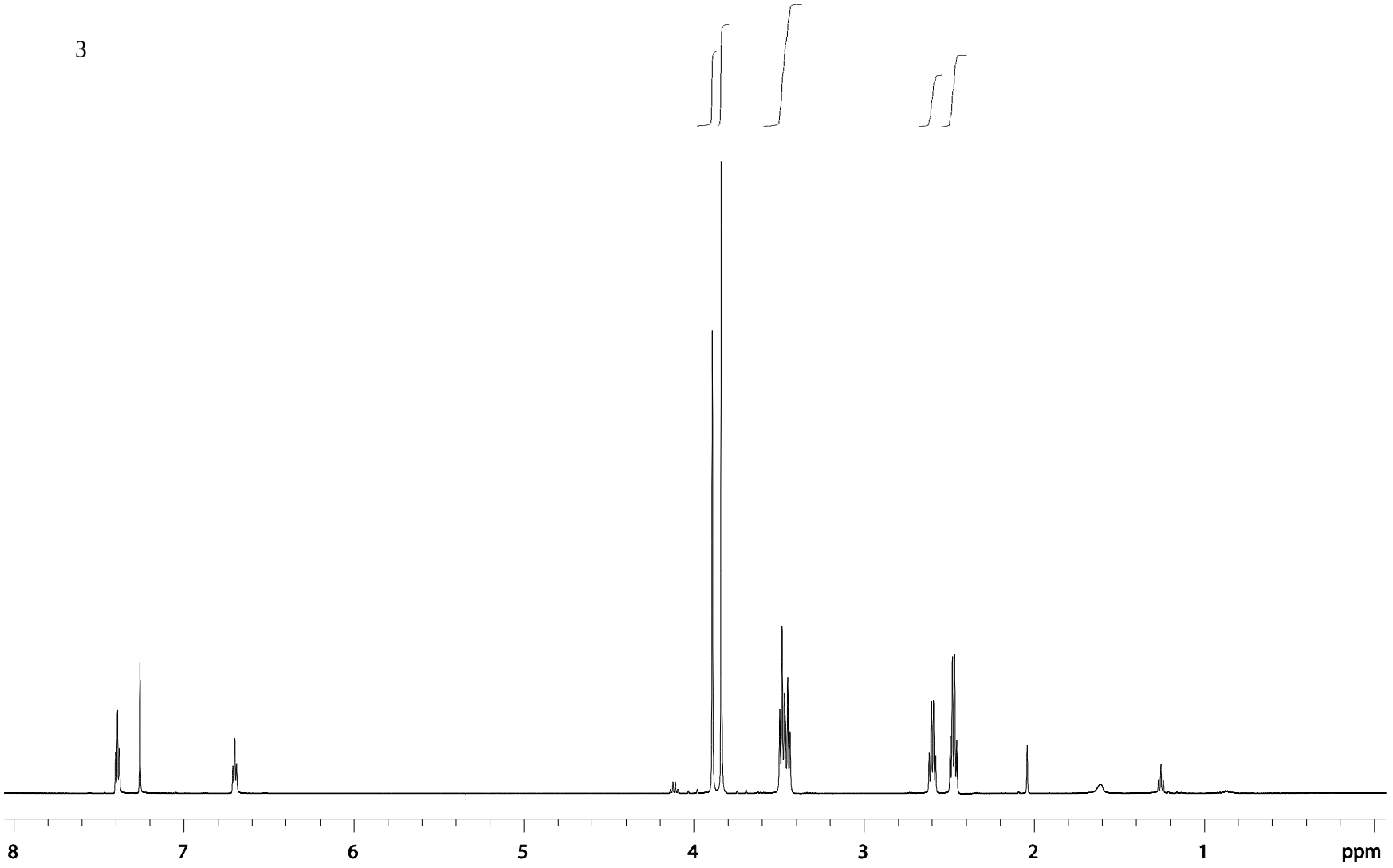

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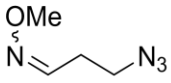

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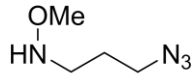

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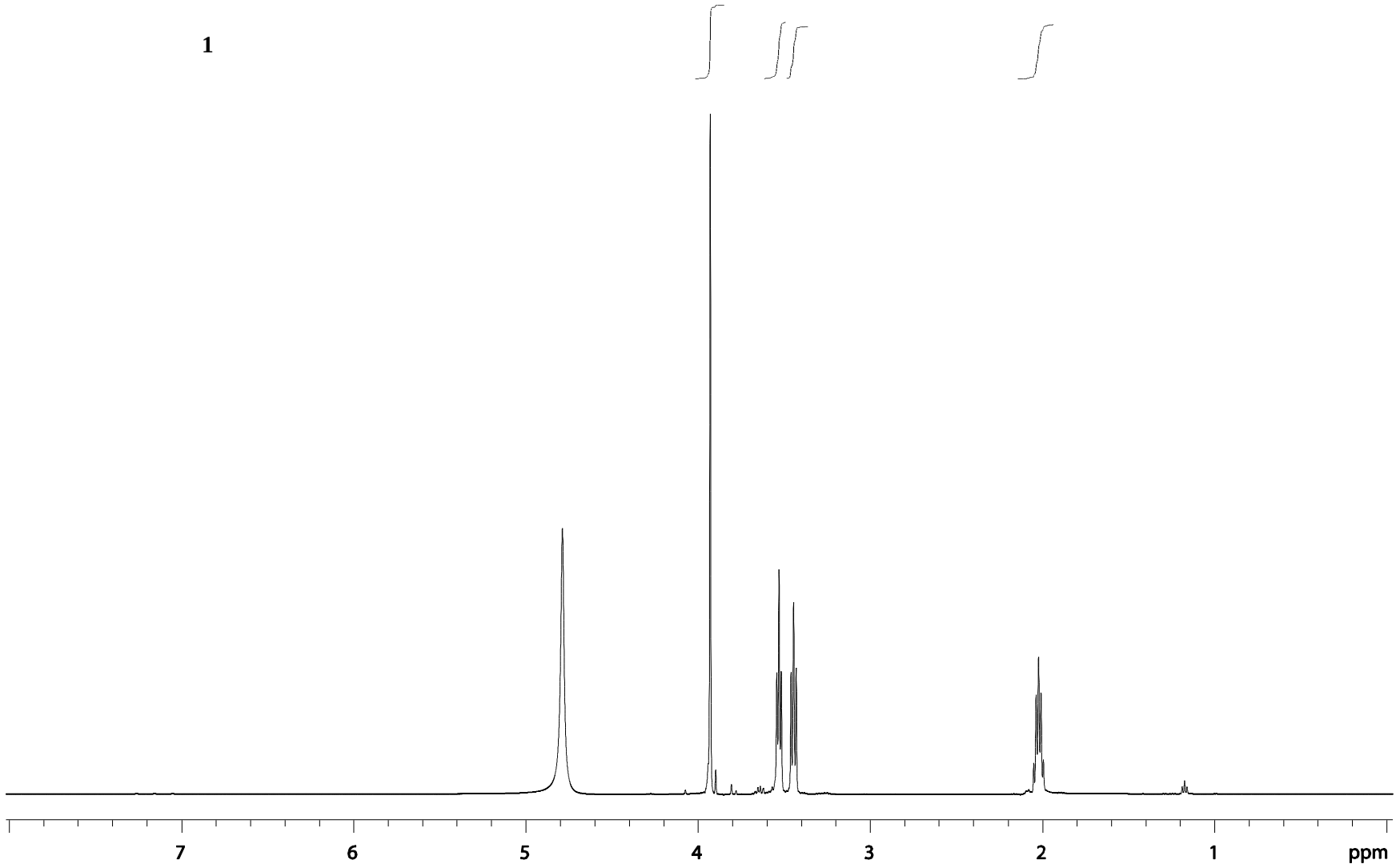

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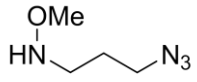

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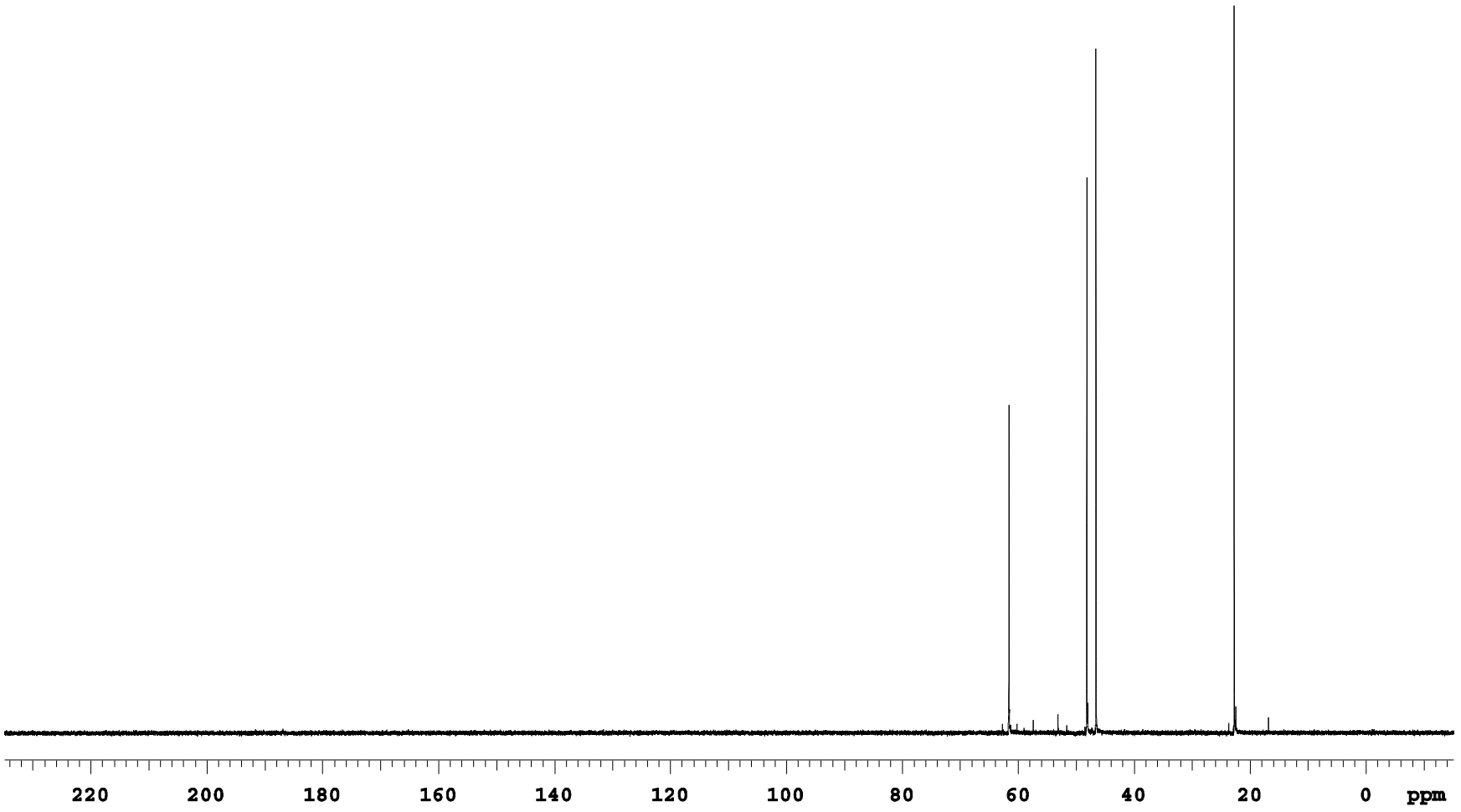

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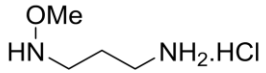

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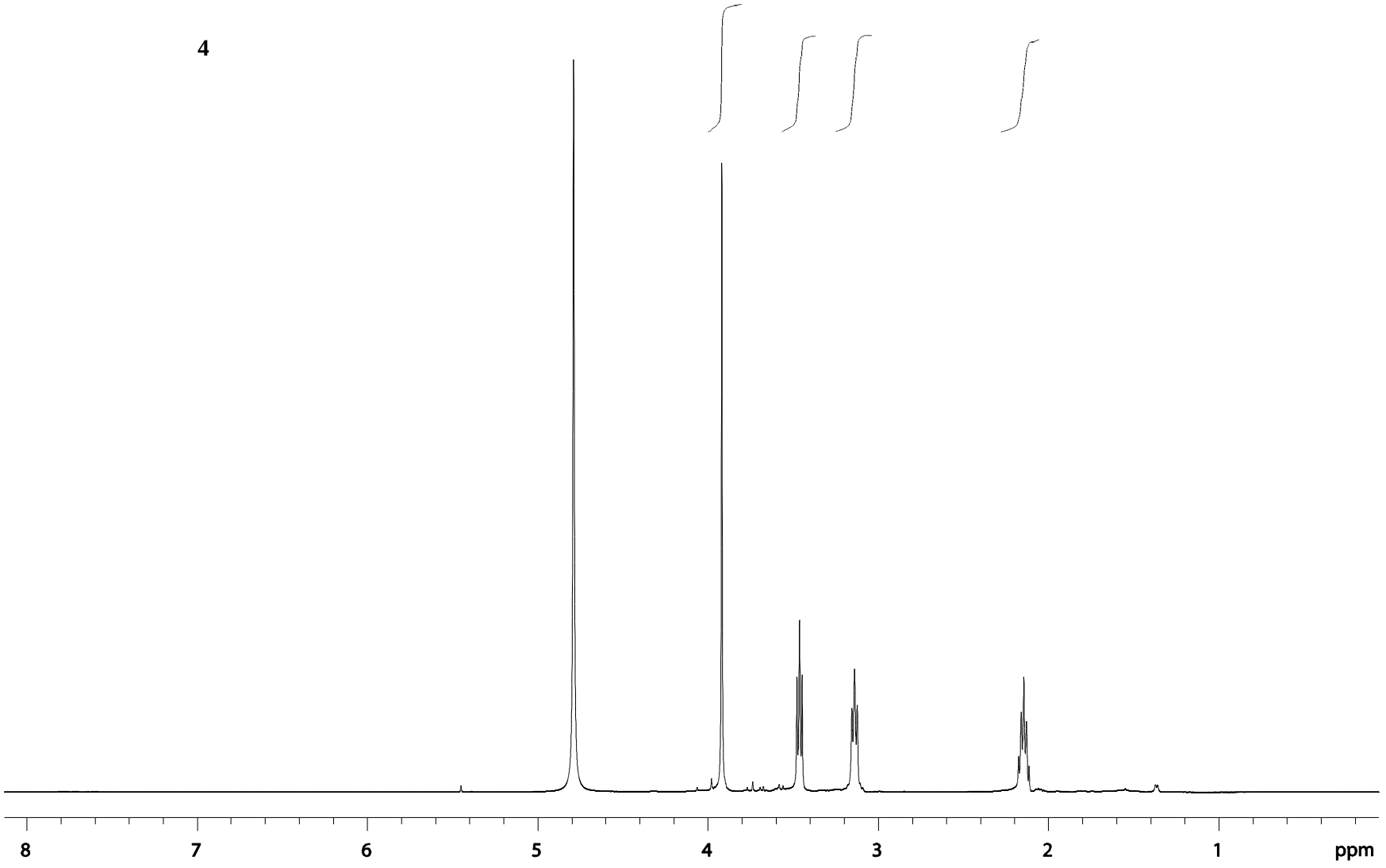

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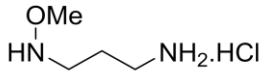

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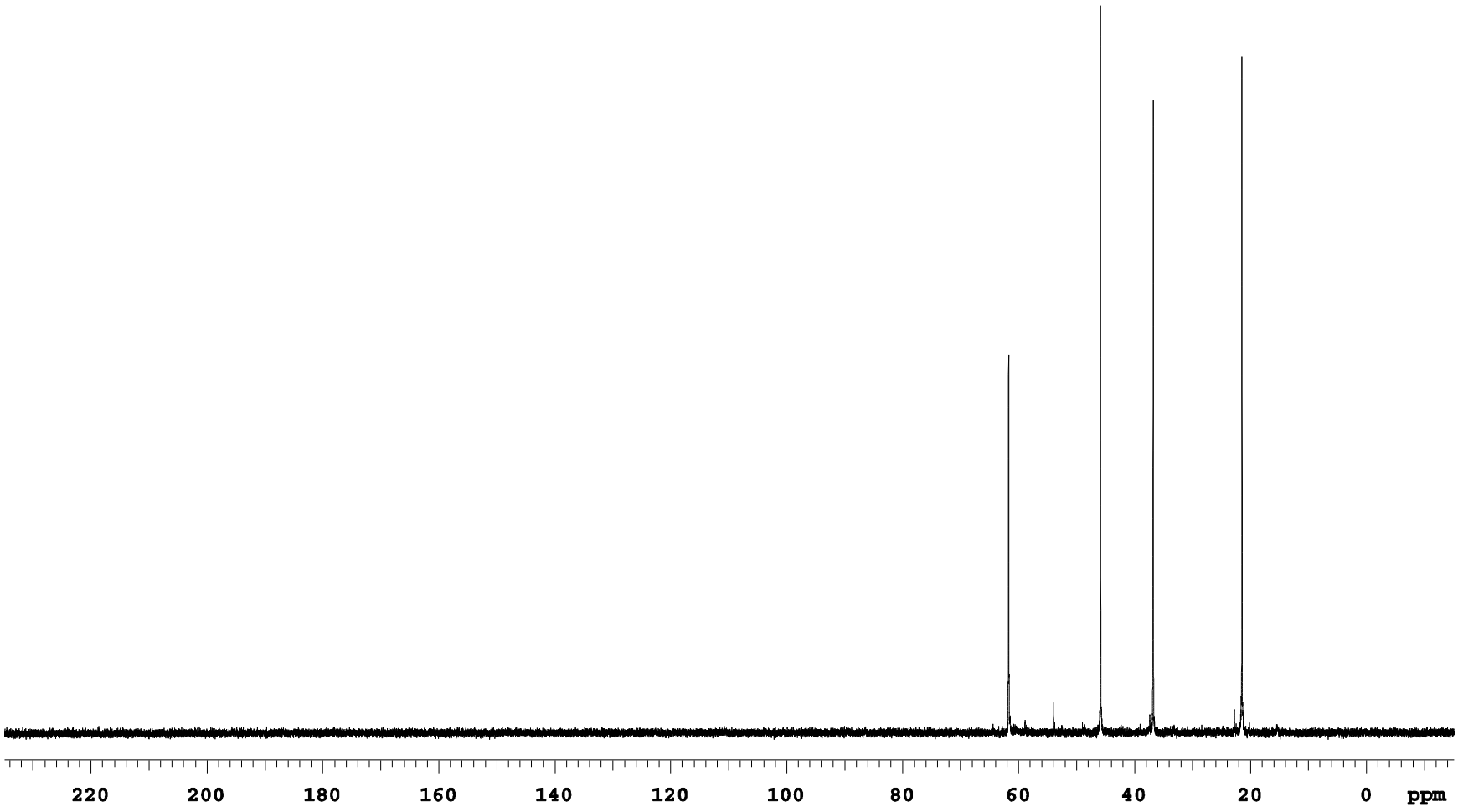

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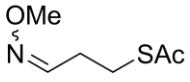

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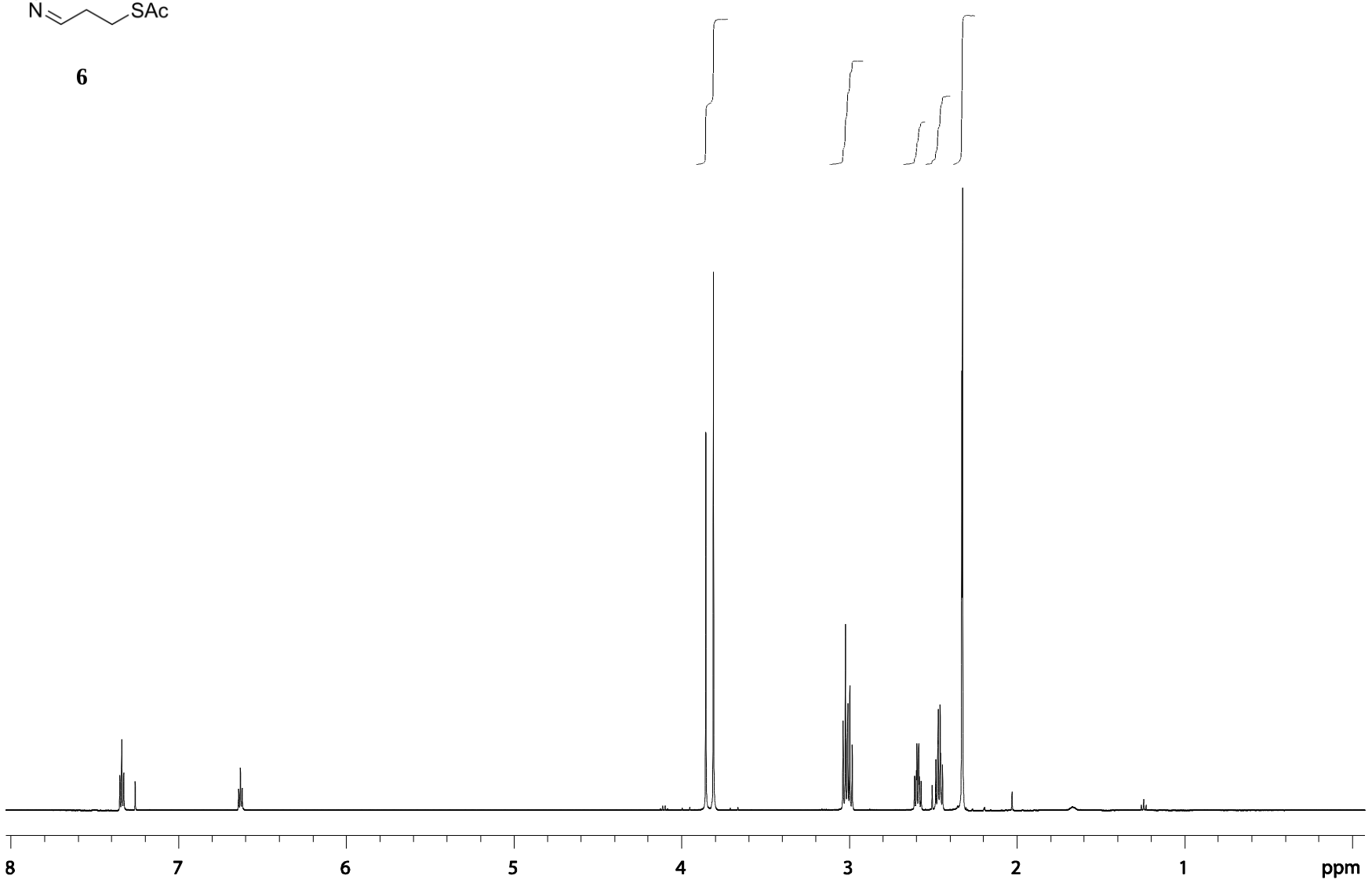

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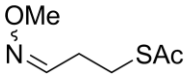

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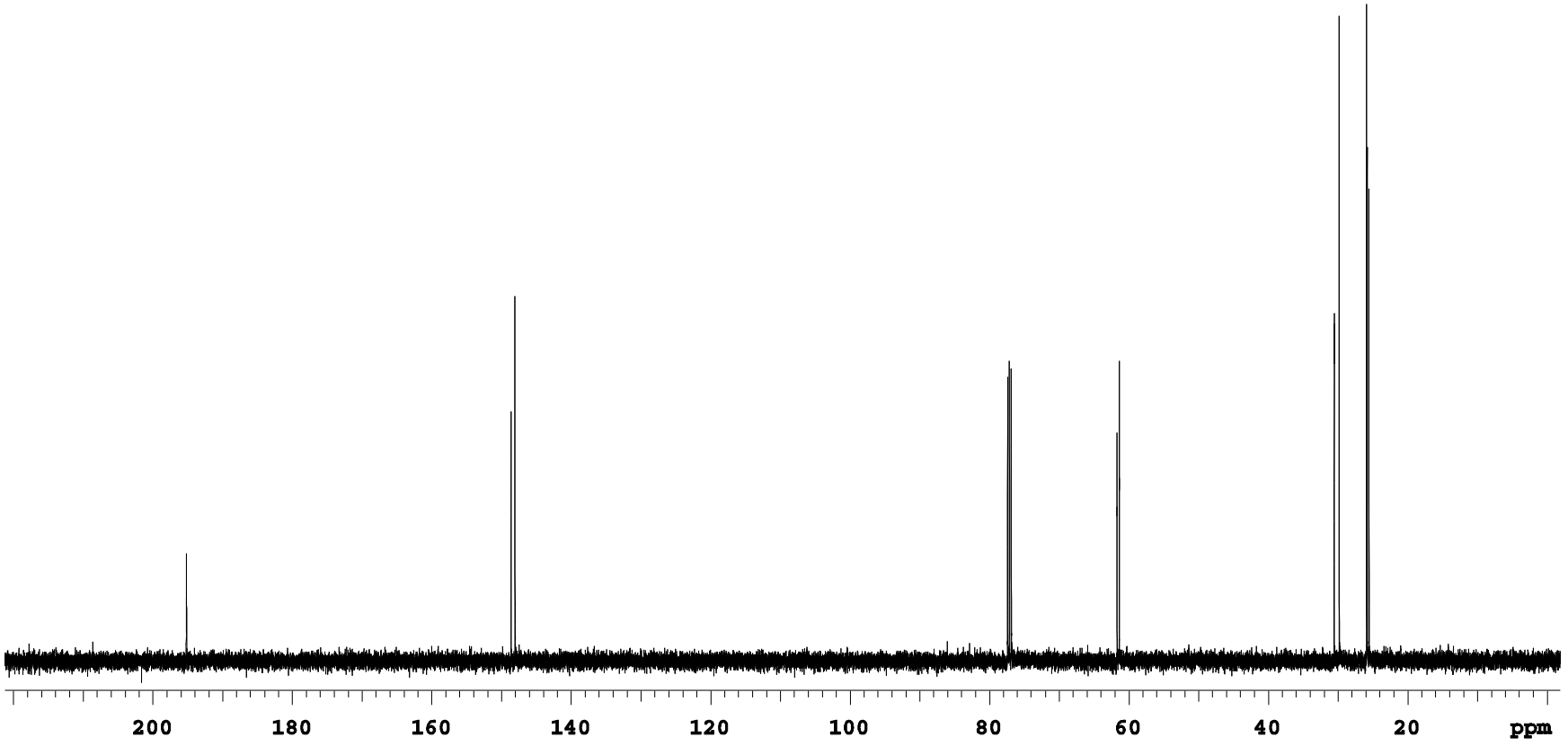

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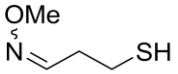

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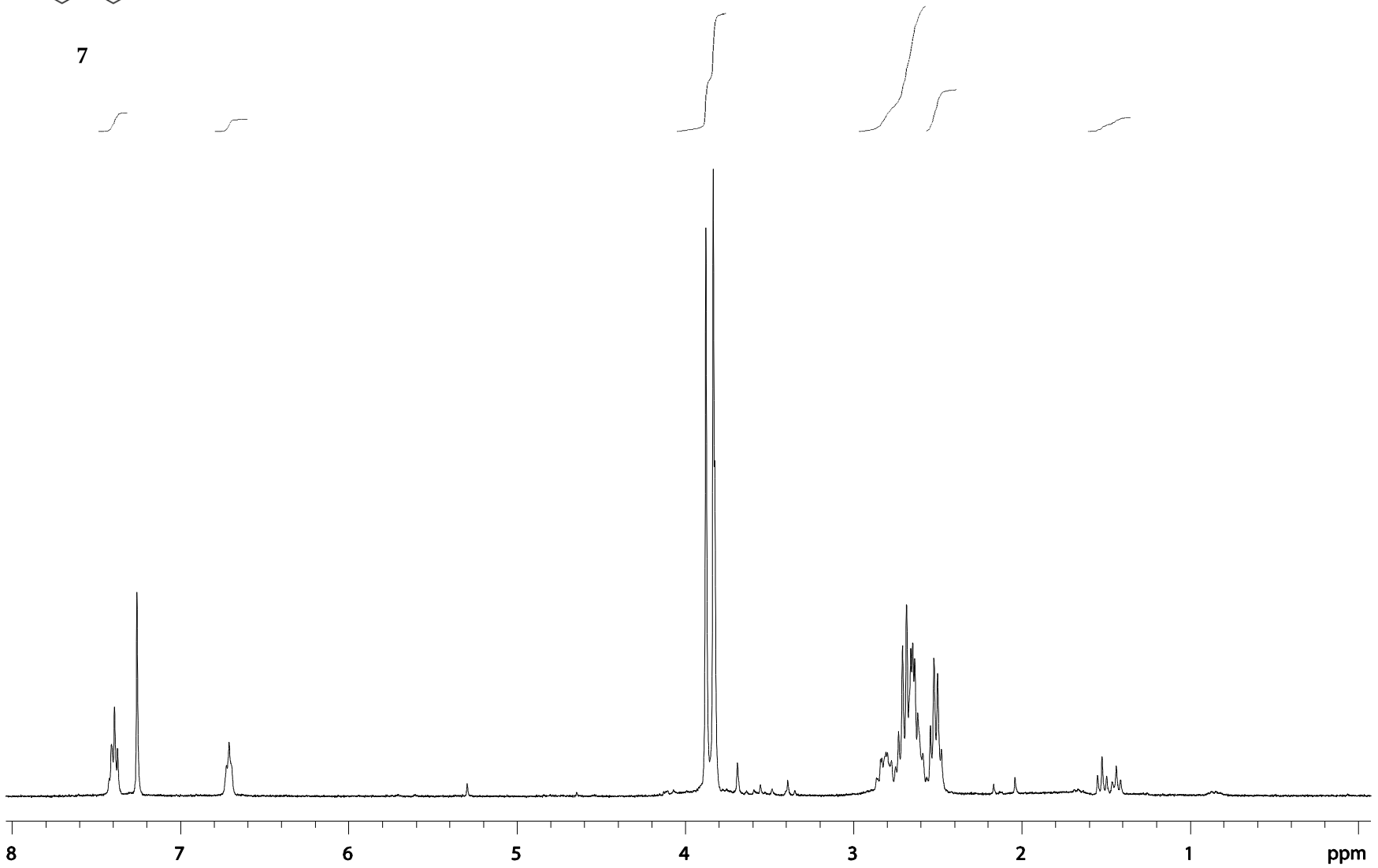

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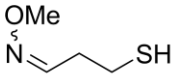

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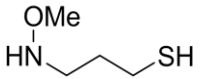

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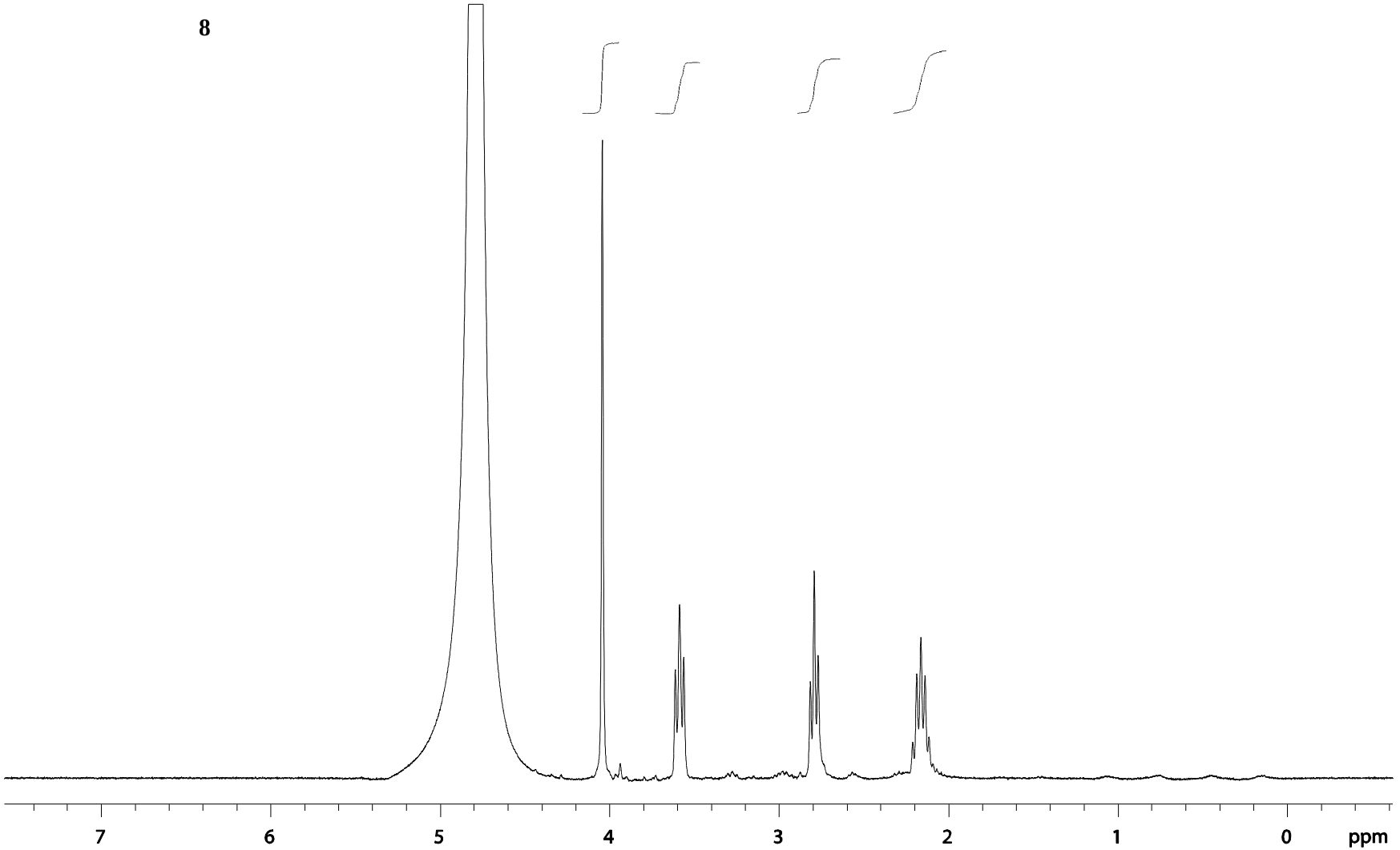

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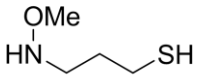

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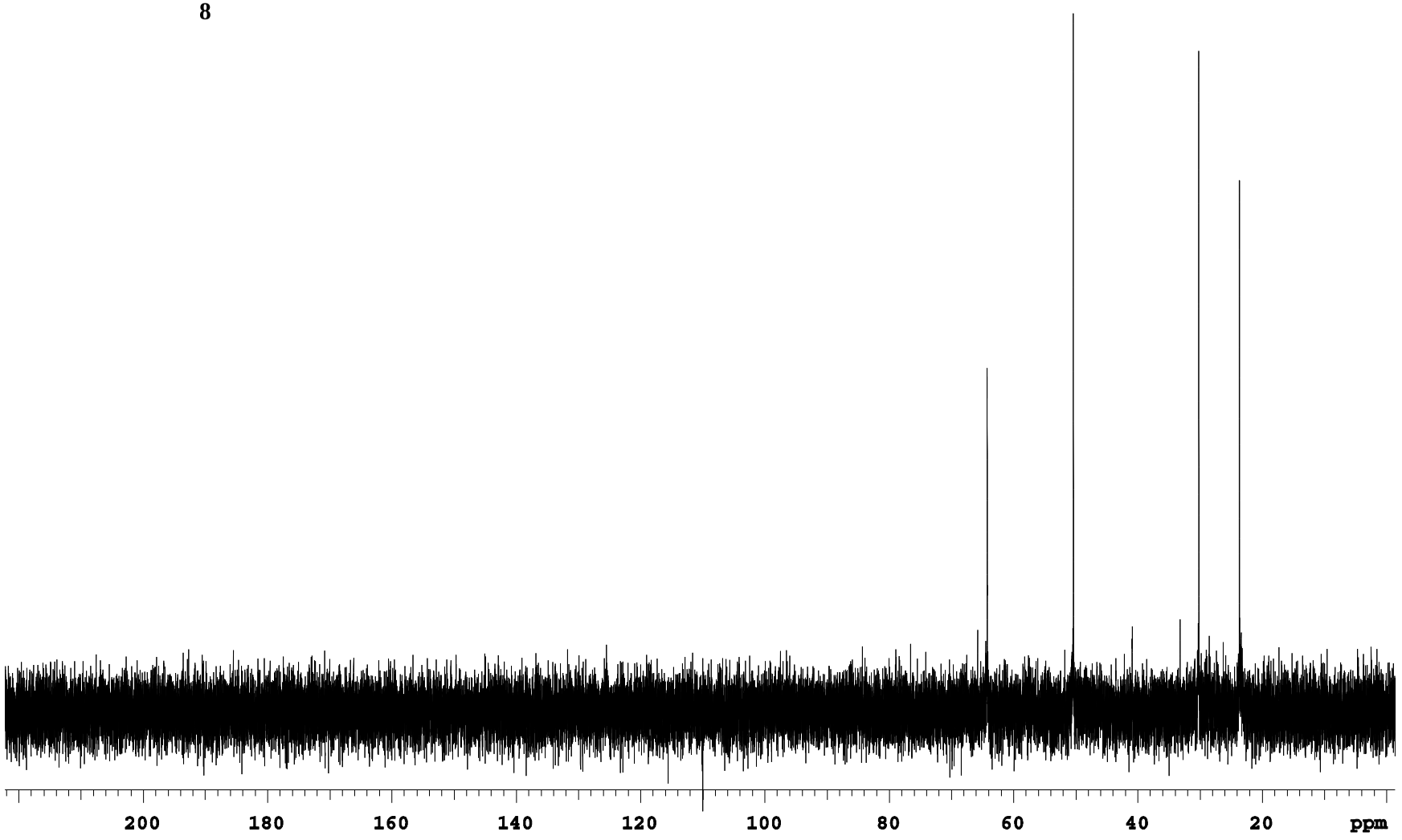
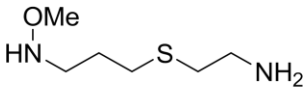


^{1}H NMR (D_2O, 500 MHz)



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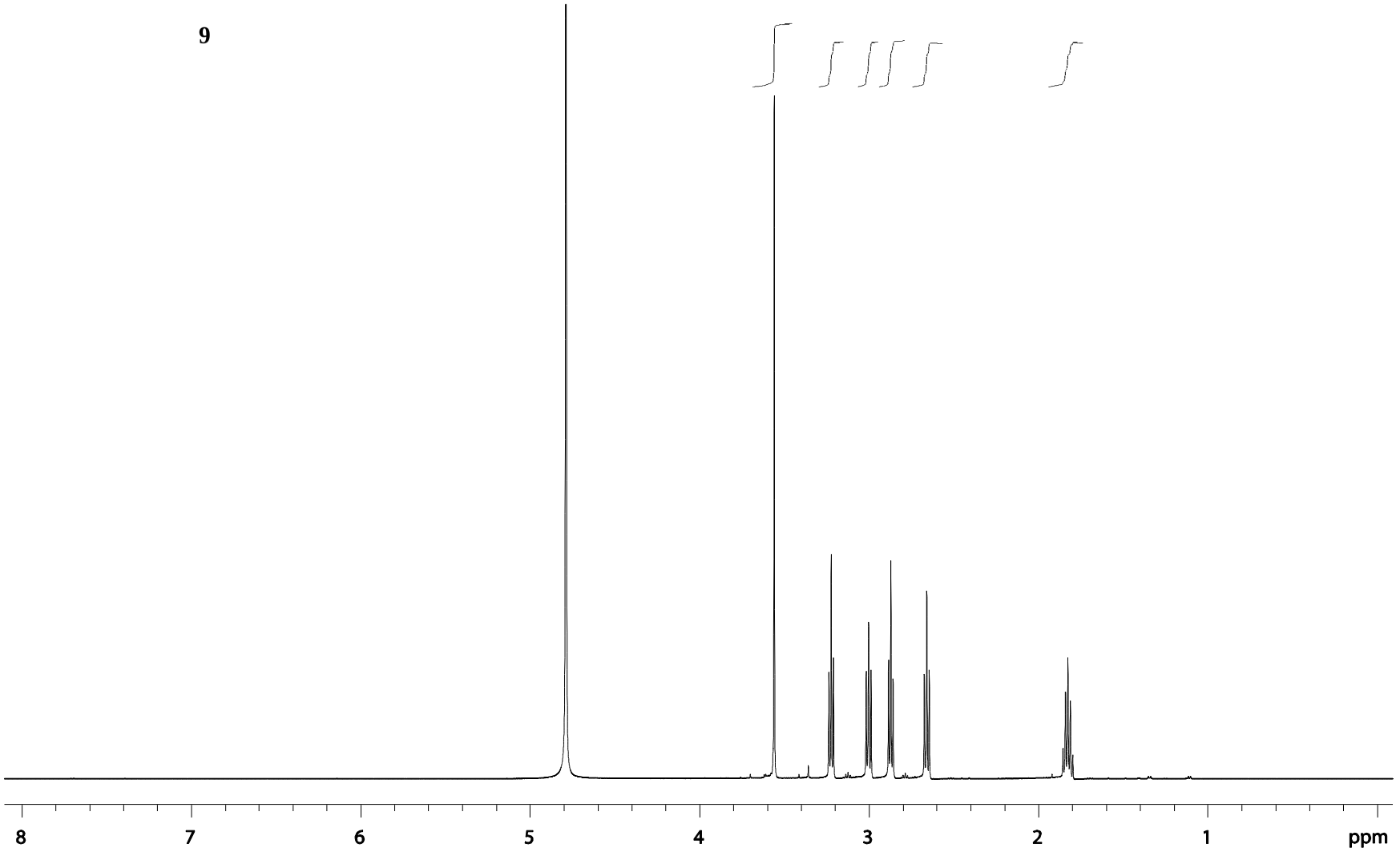
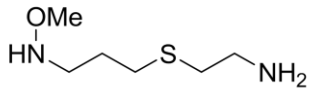


^{13}C NMR (D_2O, 125 MHz)



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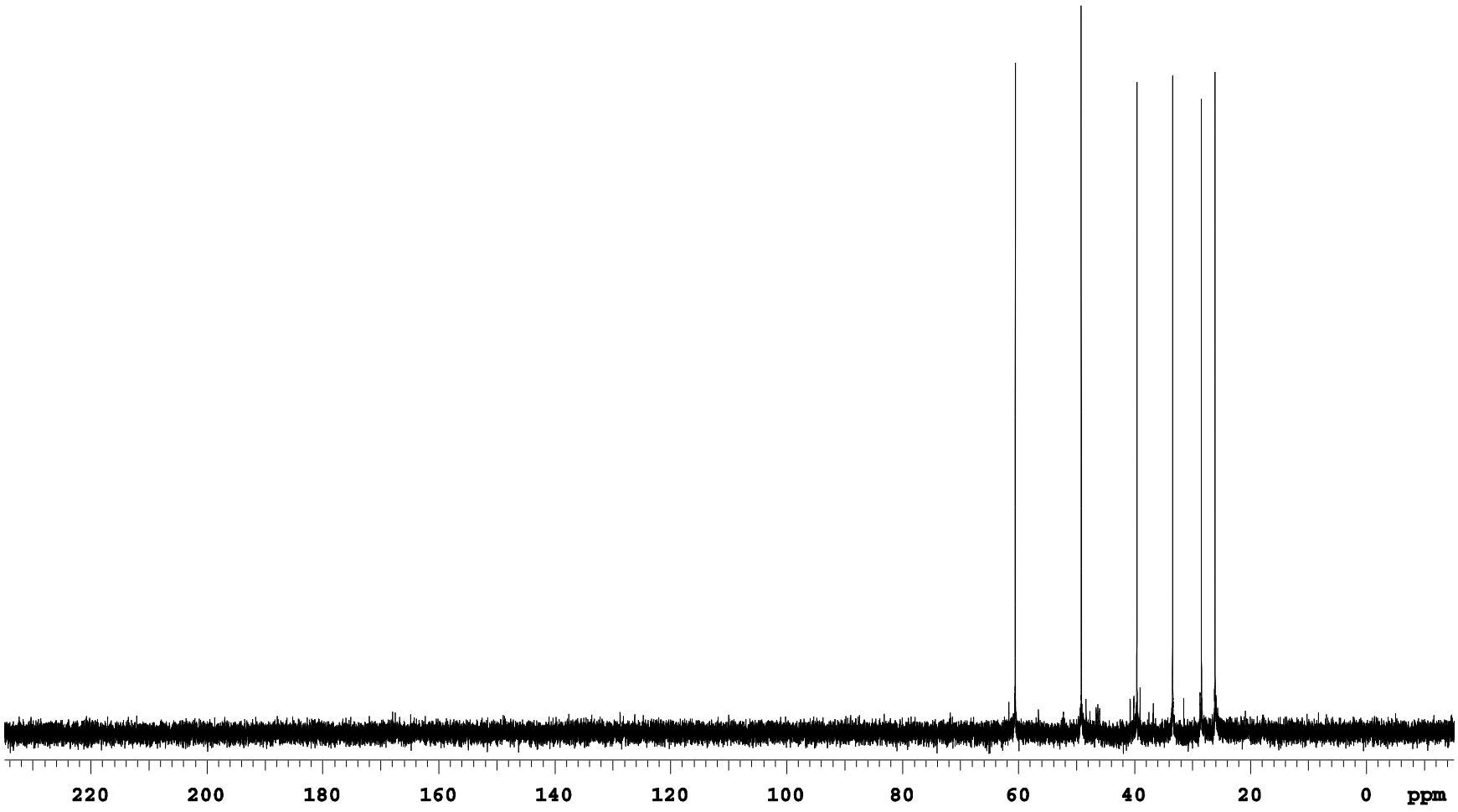
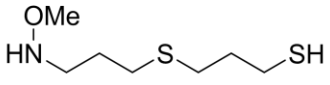


^{1}H NMR (D_2O, 500 MHz)



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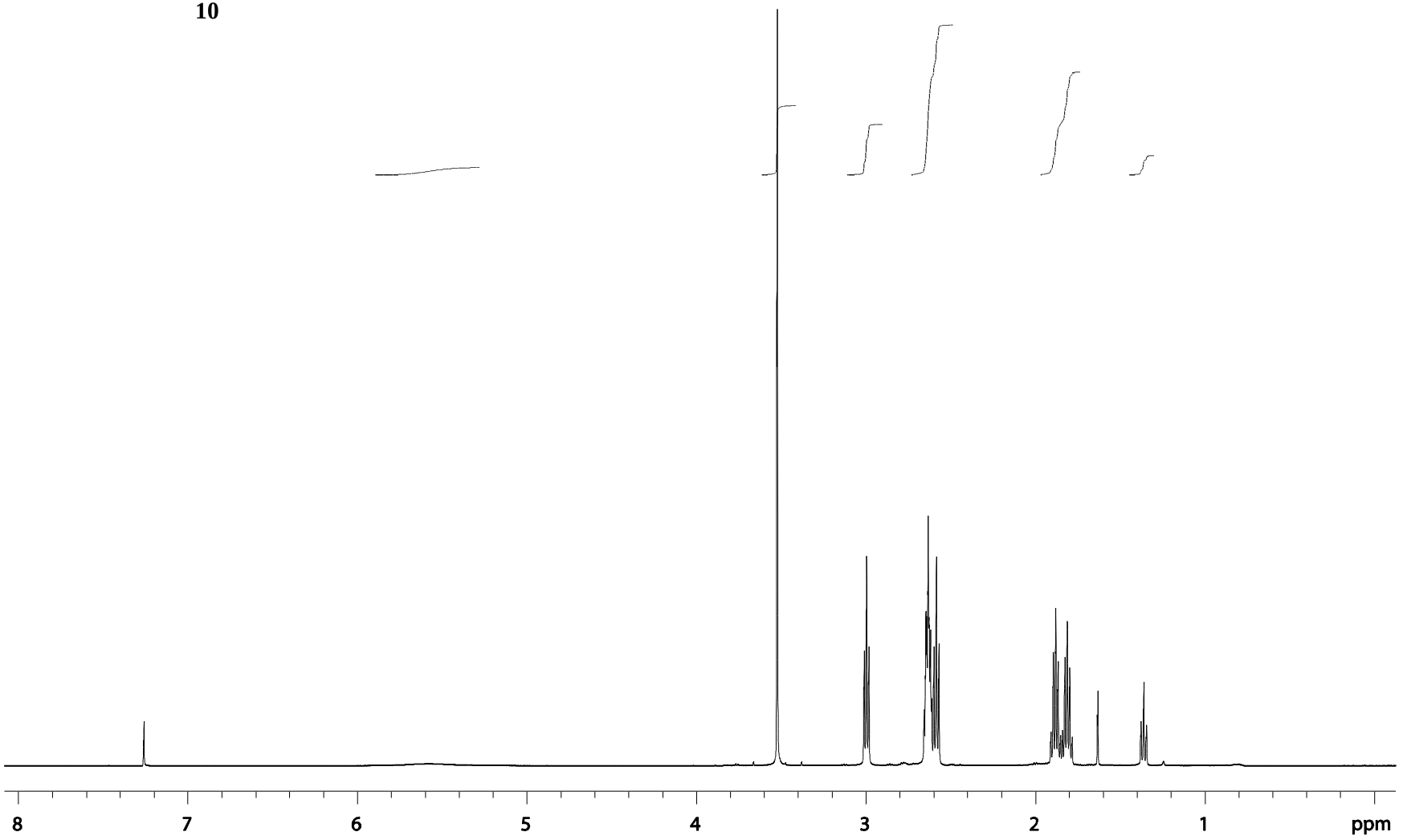
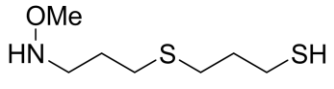


^{13}C NMR (D_2O, 125 MHz)



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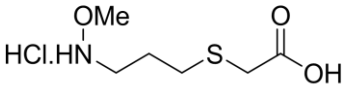
^{1}H NMR (D_2O, 500 MHz)



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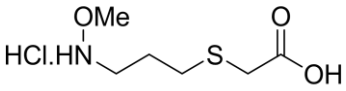
^{13}C NMR (D_2O, 125 MHz)



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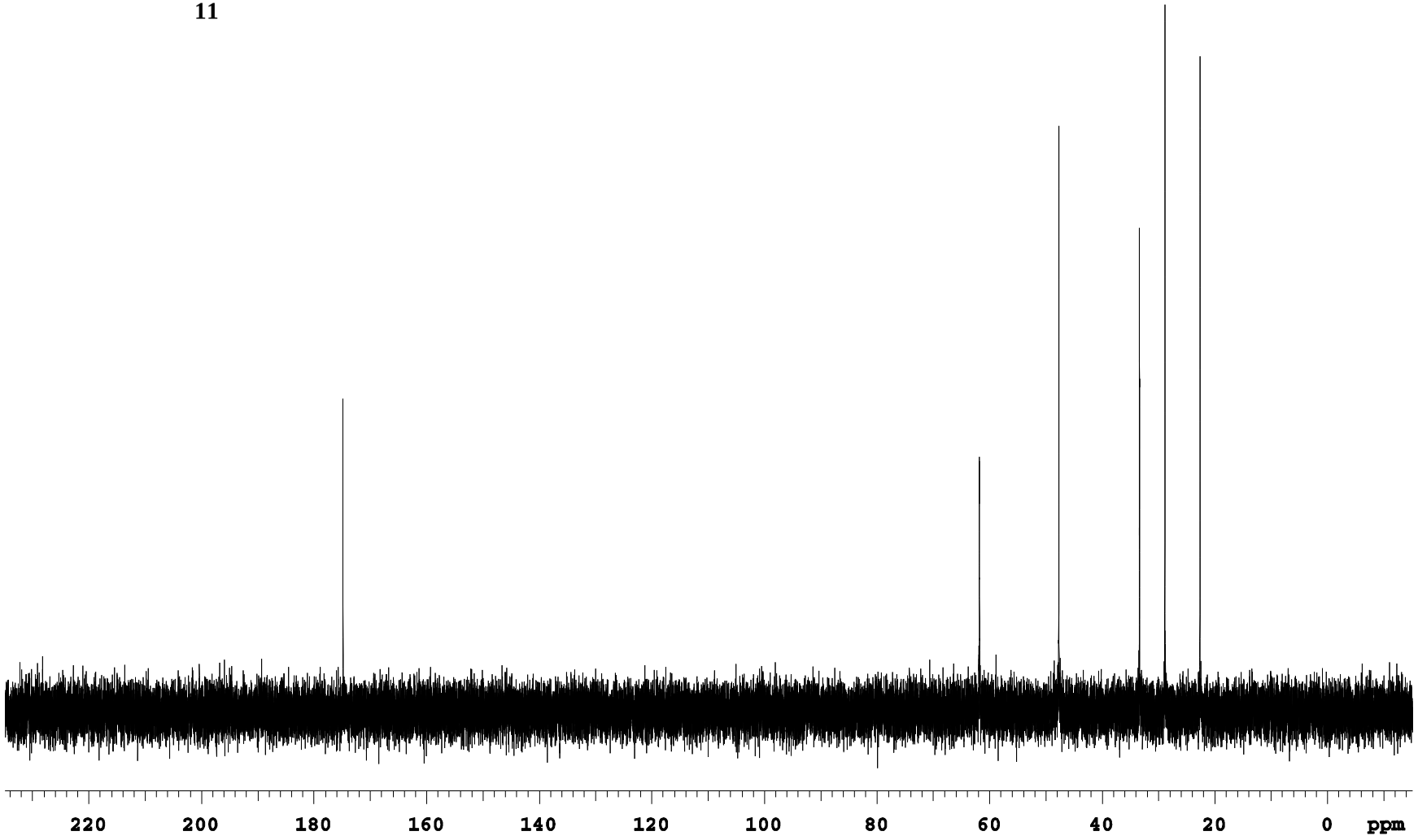
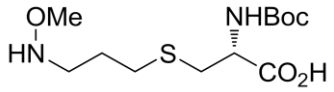


^{1}H NMR (CD_3OD, 500 MHz)



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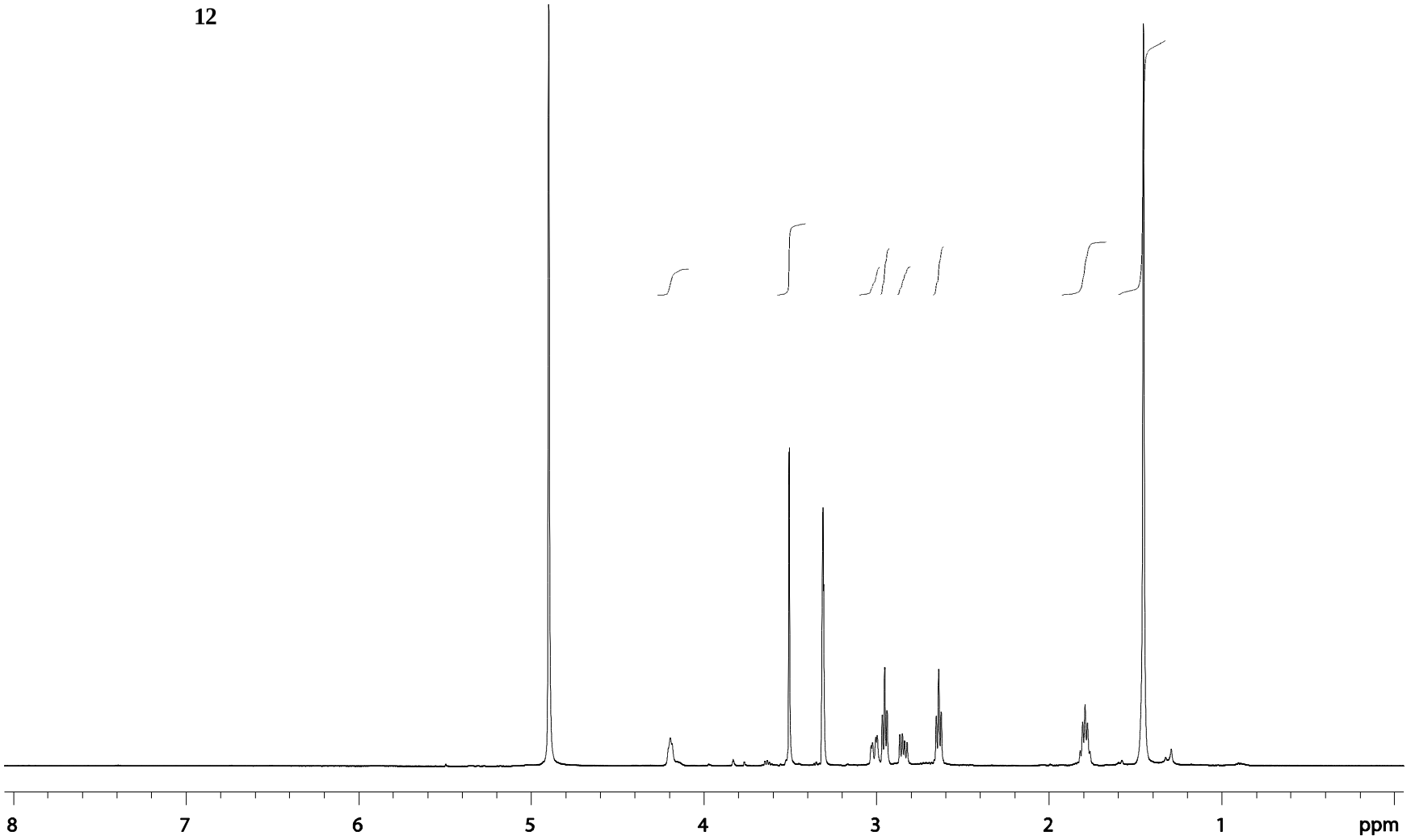
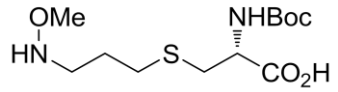


^{13}C NMR (CD_3OD, 125 MHz)



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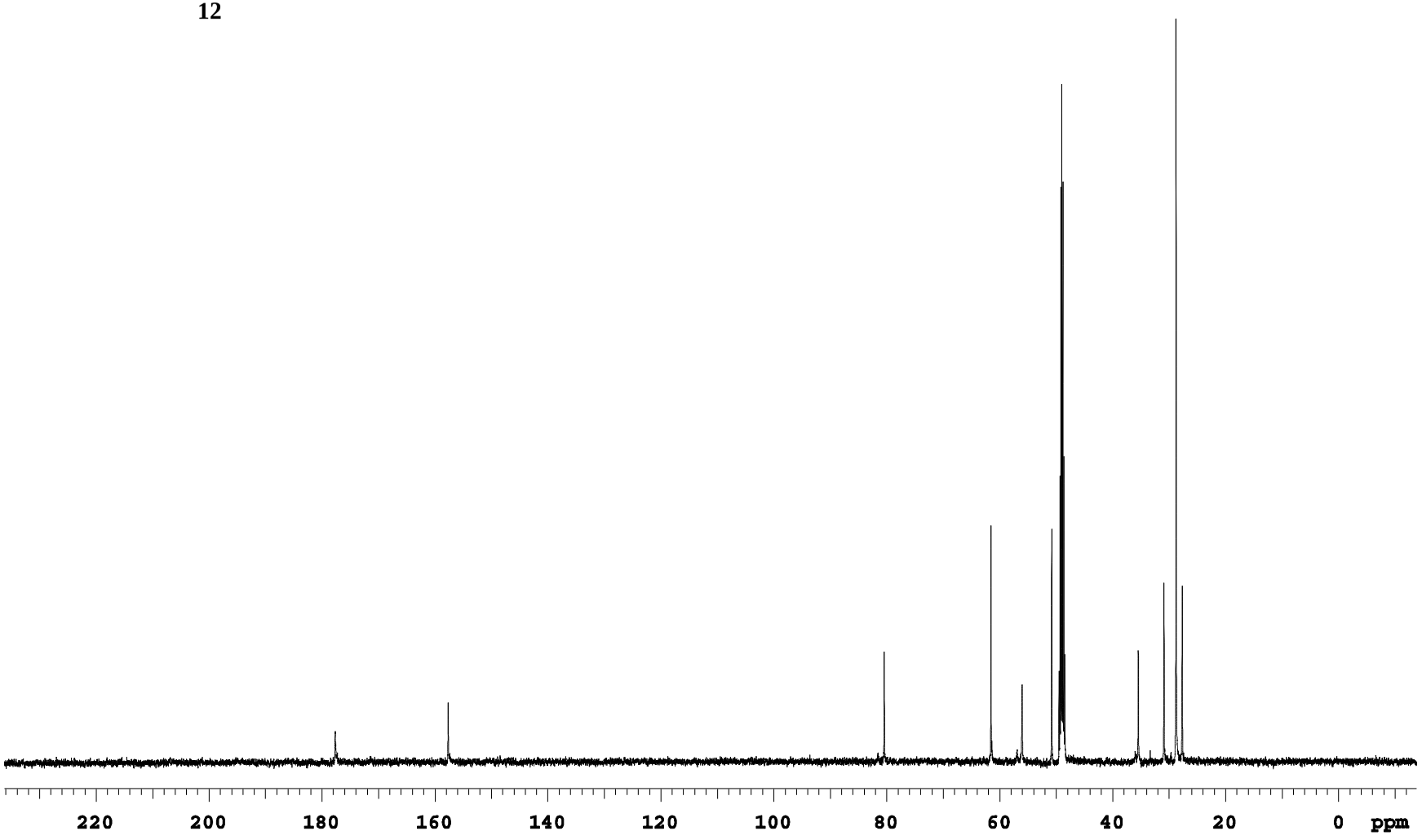
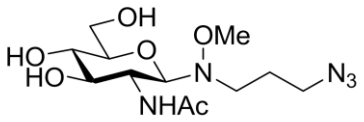
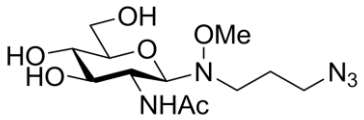


^{1}H NMR (D_2O, 500 MHz)



14

^{13}C NMR (D_2O, 125 MHz)



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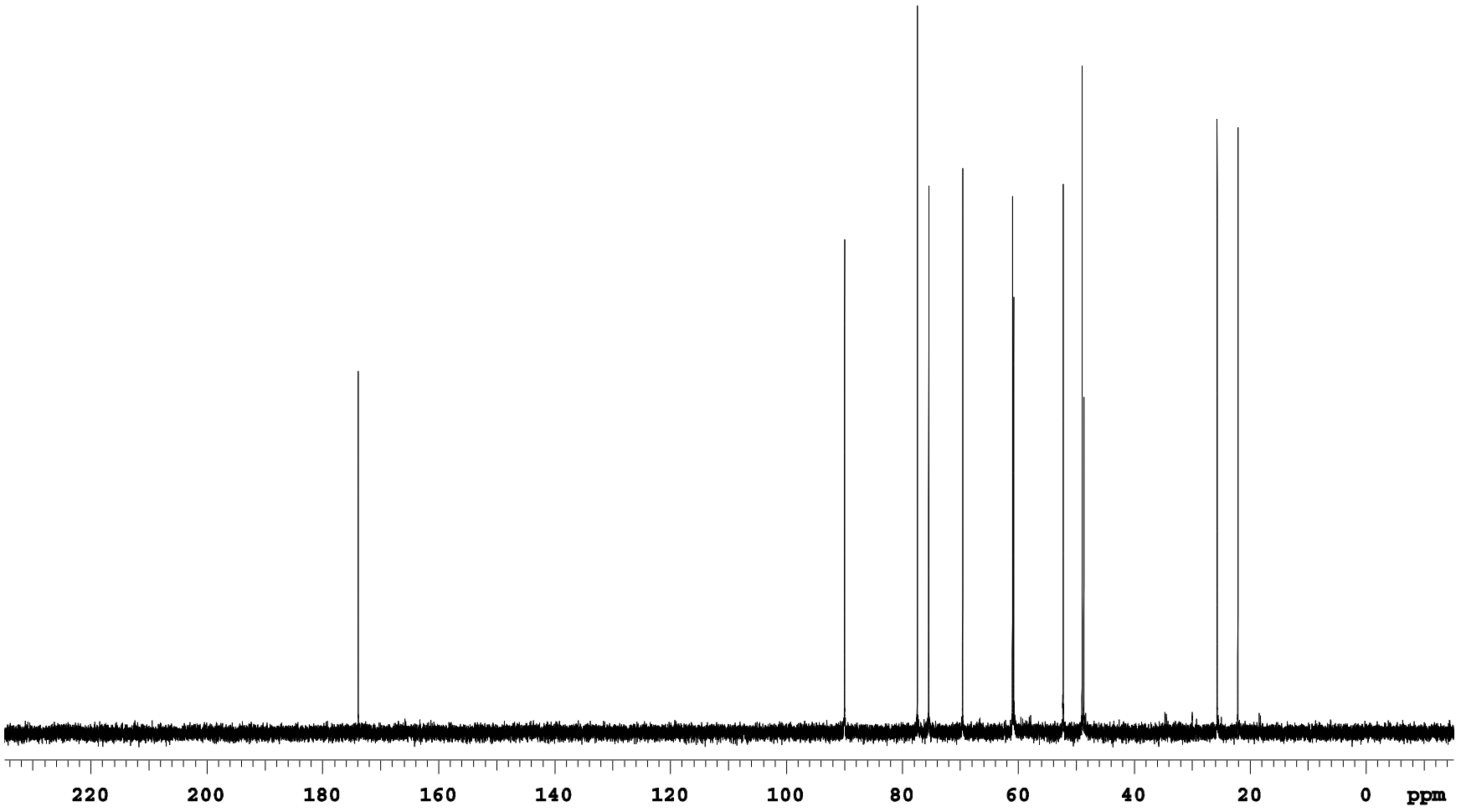
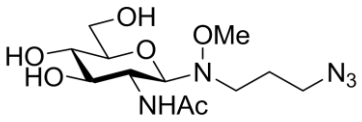
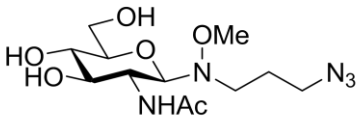


COSY (D_2O, 500 MHz)



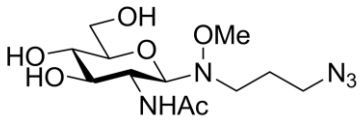
14

HSQC (D_2O, 500 MHz)



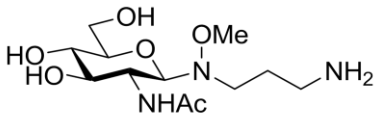
14

HMBC (D_2O, 500 MHz)



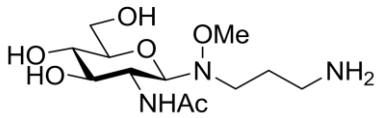
14

^{1}H NMR (D_2O, 500 MHz)



15

^{13}C NMR (D_2O, 125 MHz)



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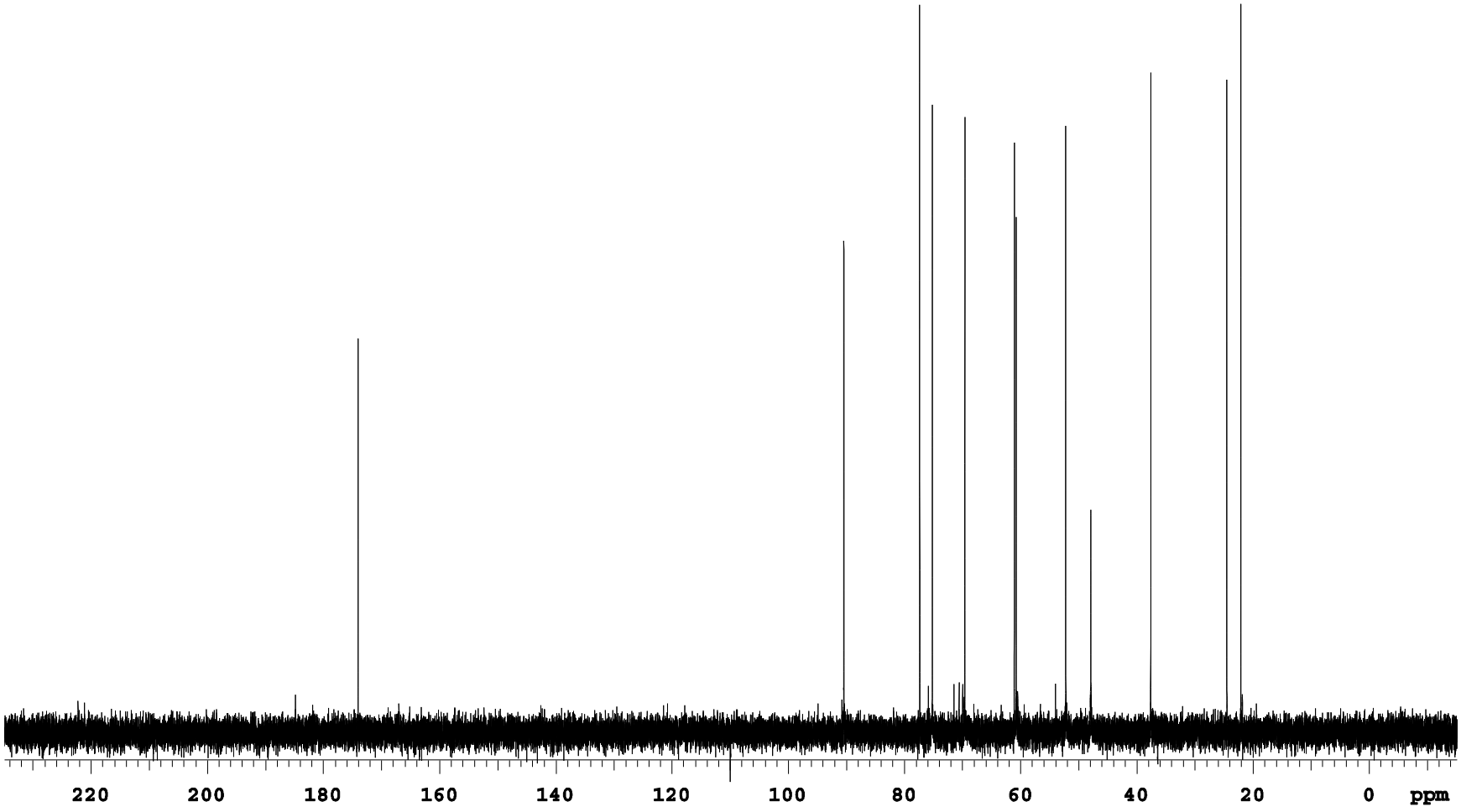
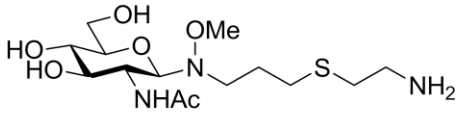
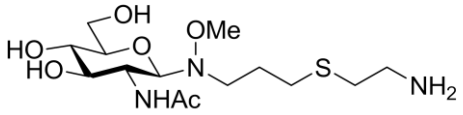


^{1}H NMR (D_2O, 500 MHz)



16

^{13}C NMR (D_2O, 125 MHz)



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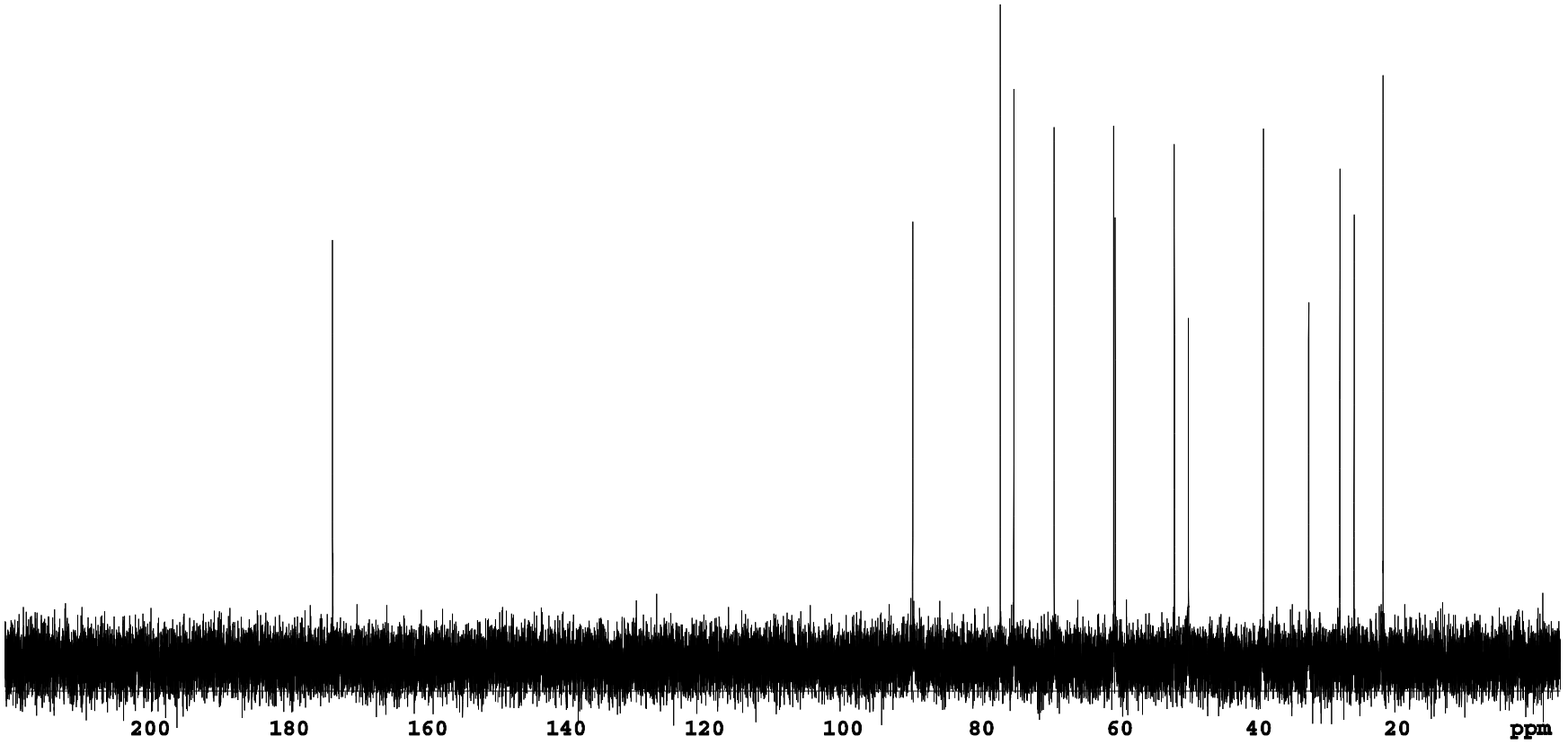
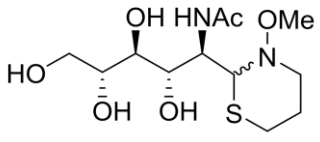


^{1}H NMR (D_2O, 50 °C, 300 MHz)



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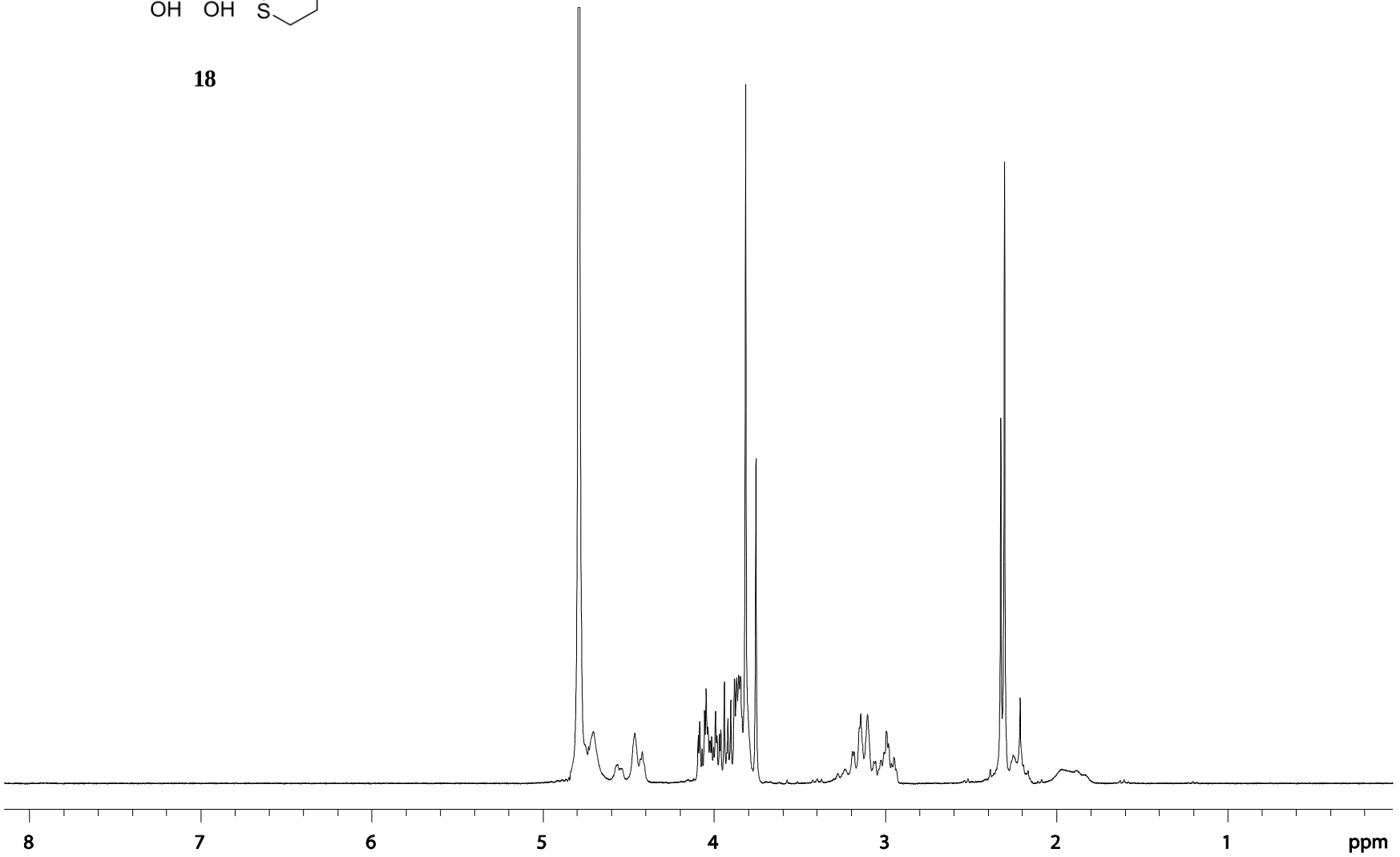
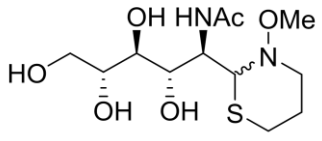


^{13}C NMR (D_2O, 150 MHz)



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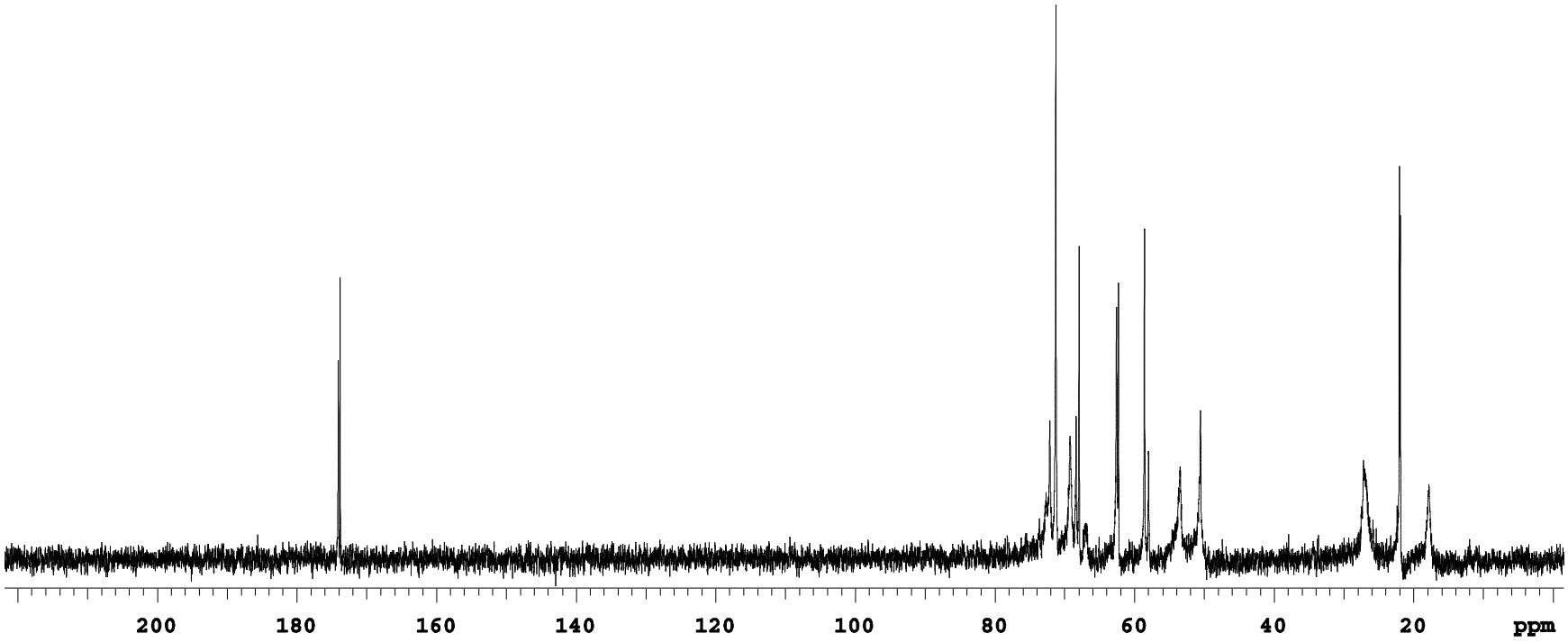
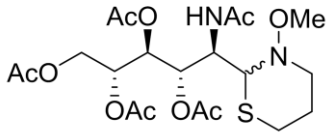


^{1}H NMR ($CDCl_3$, 50 °C, 300 MHz)



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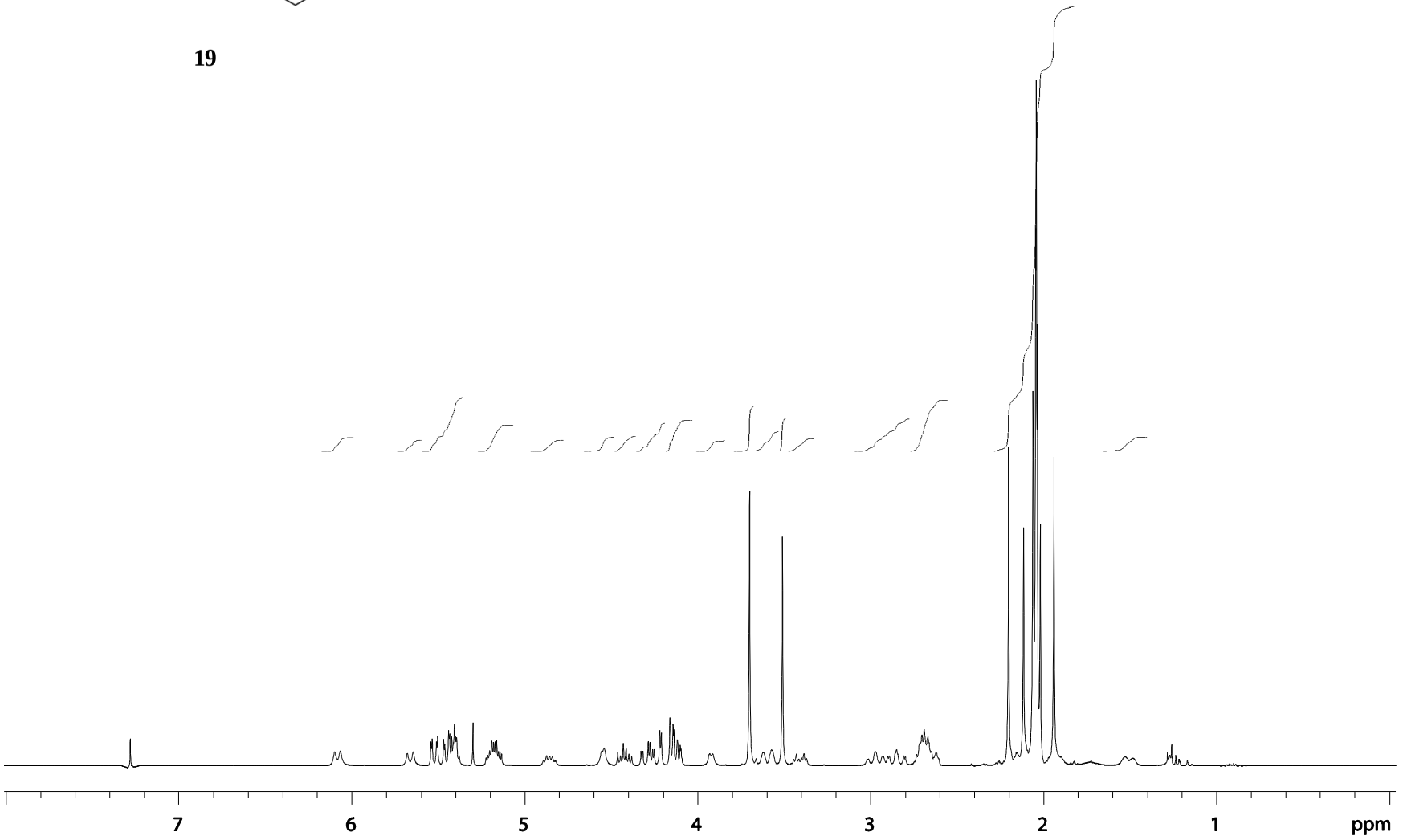
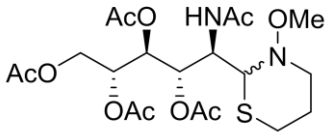


^{13}C NMR ($CDCl_3$, 50 °C, 75 MHz)



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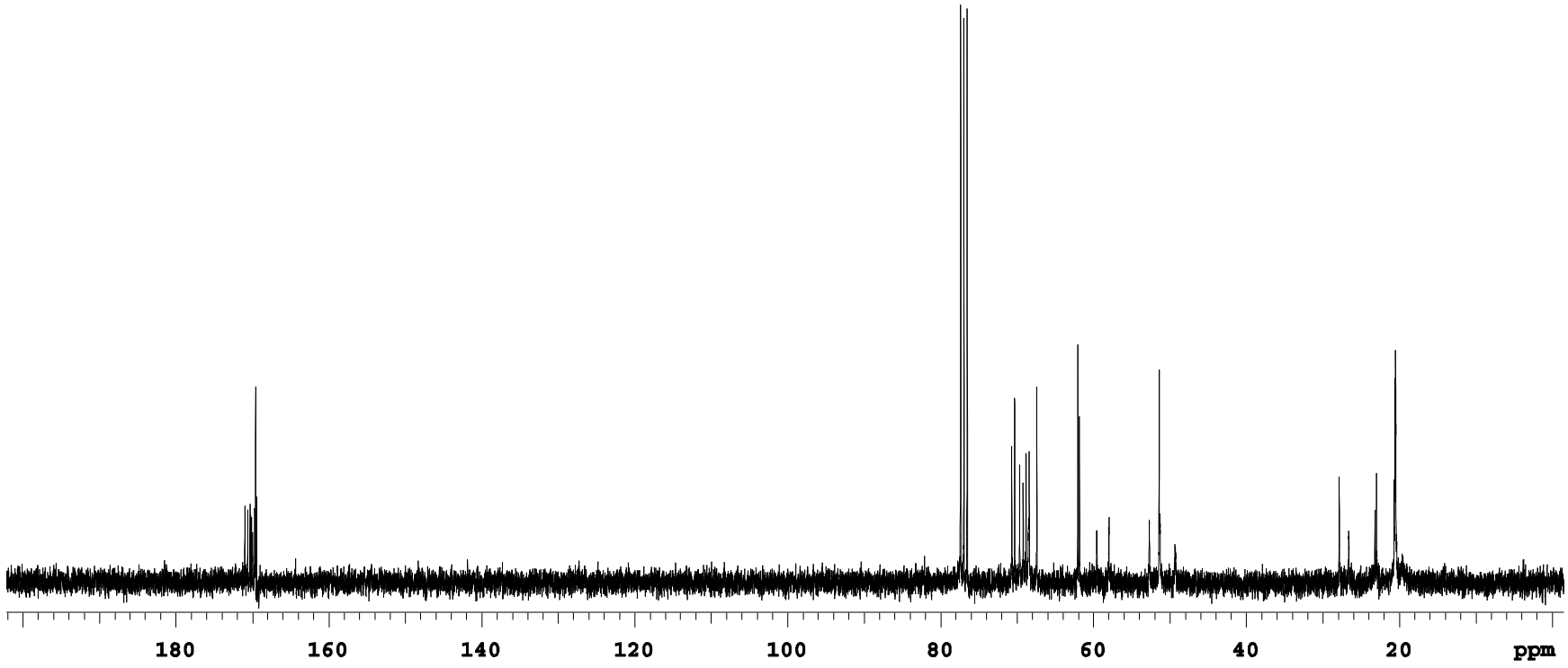
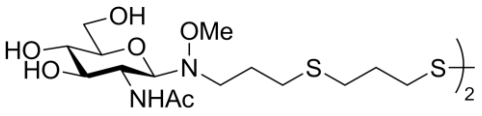
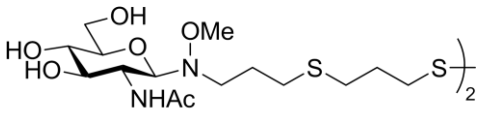


^{1}H NMR (D_2O, 125 MHz)



20

^{13}C NMR (D_2O, 125 MHz)



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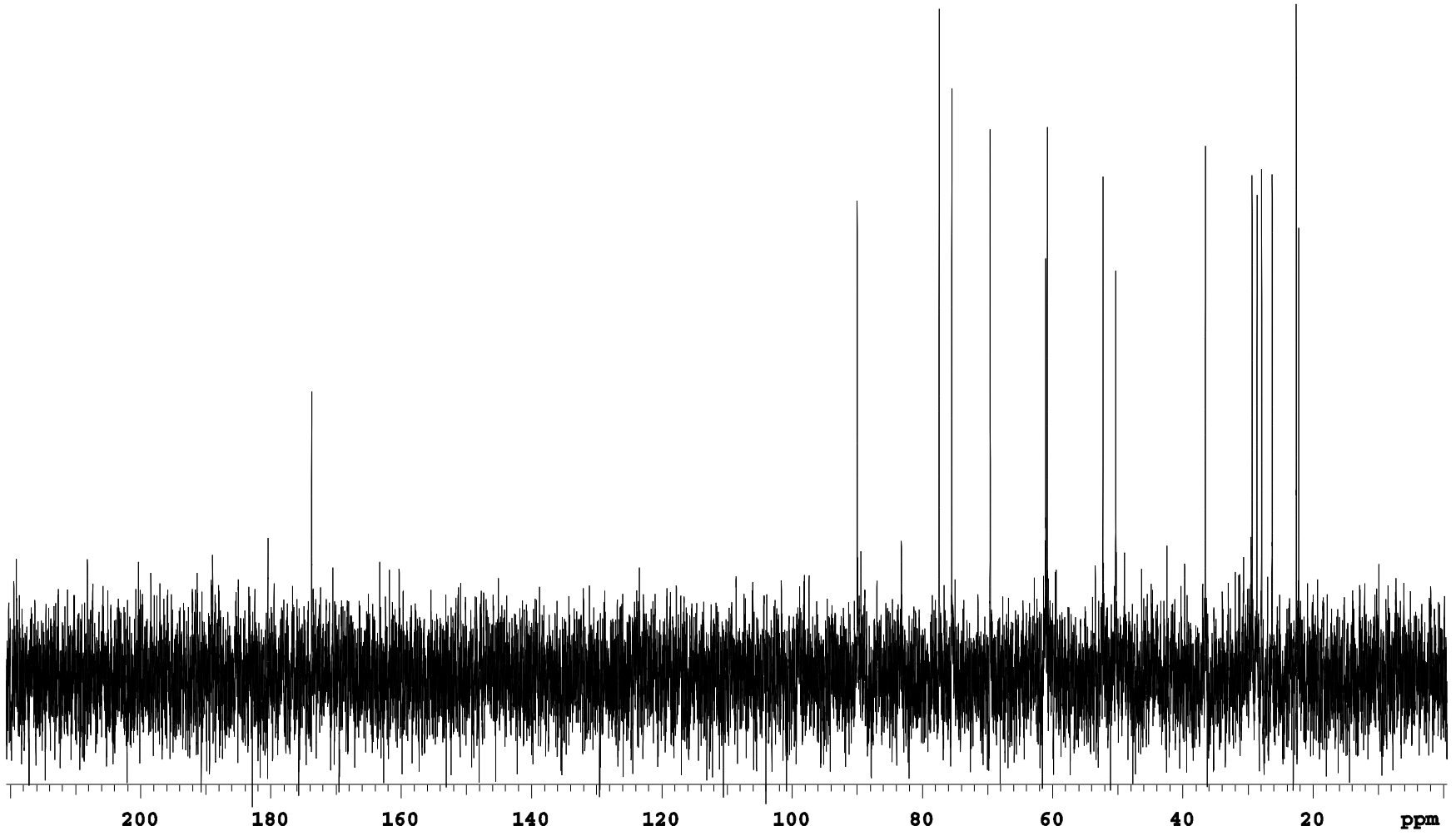
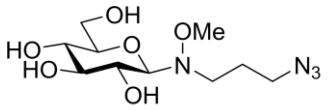
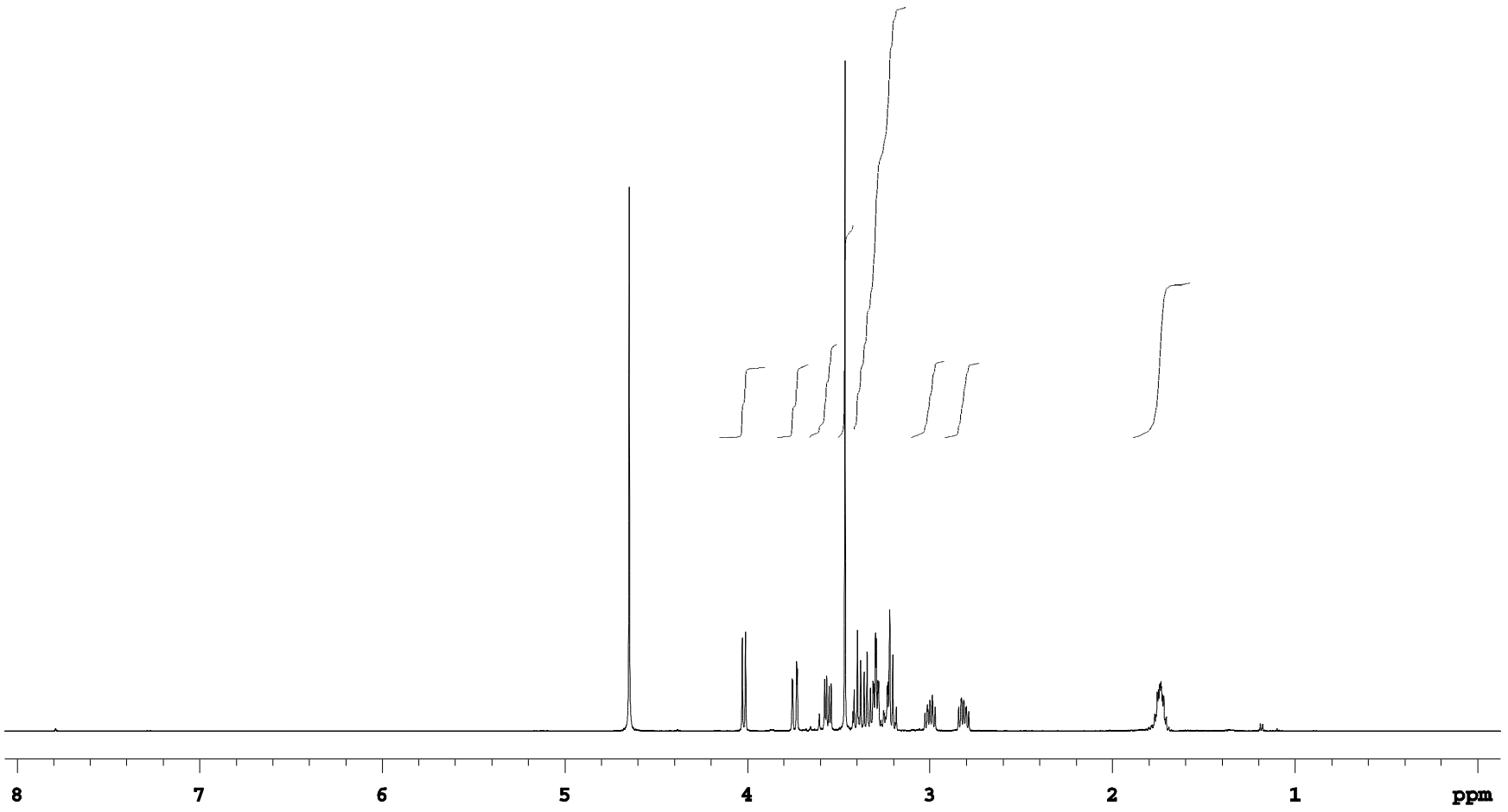
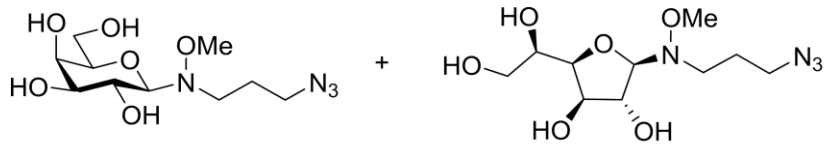


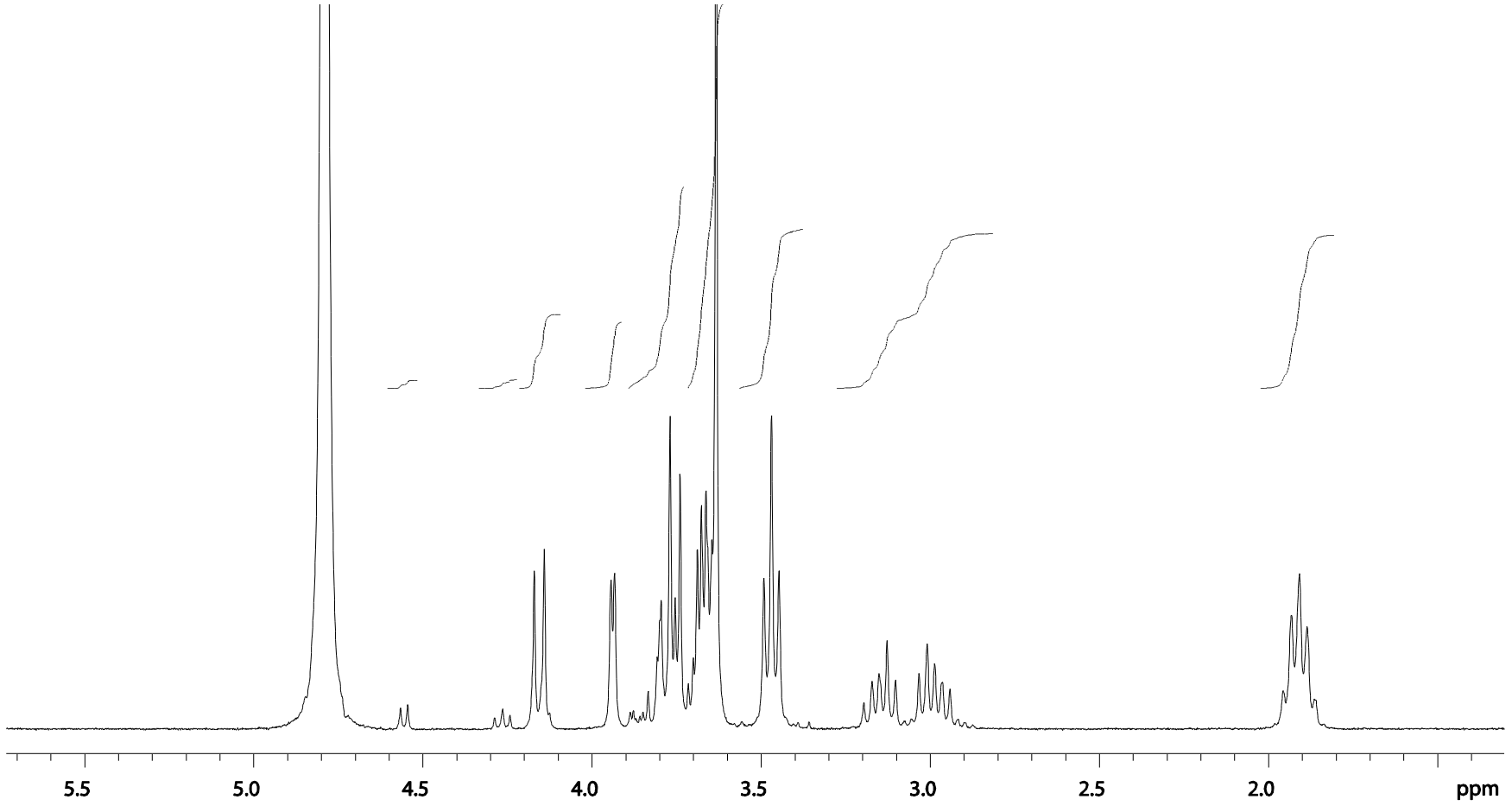
^{1}H NMR (D_2O, 500 MHz)



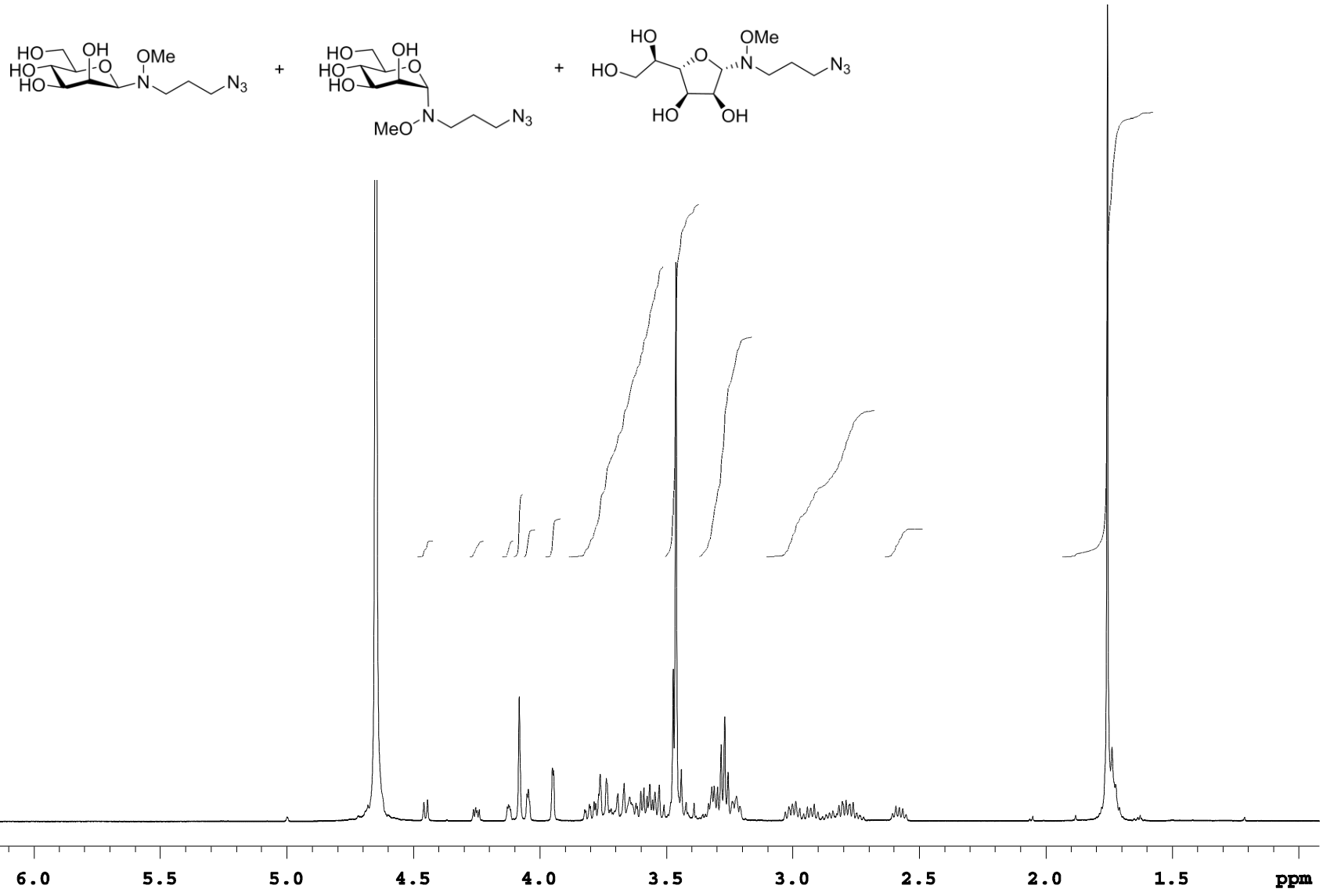


^{1}H NMR (D_2O, 500 MHz)

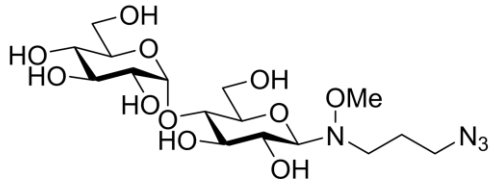




^{1}H NMR (D_2O, 500 MHz)

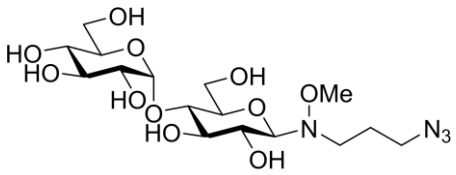


^{1}H NMR (D_2O, 500 MHz)



22

^{13}C NMR (D_2O, 125 MHz)



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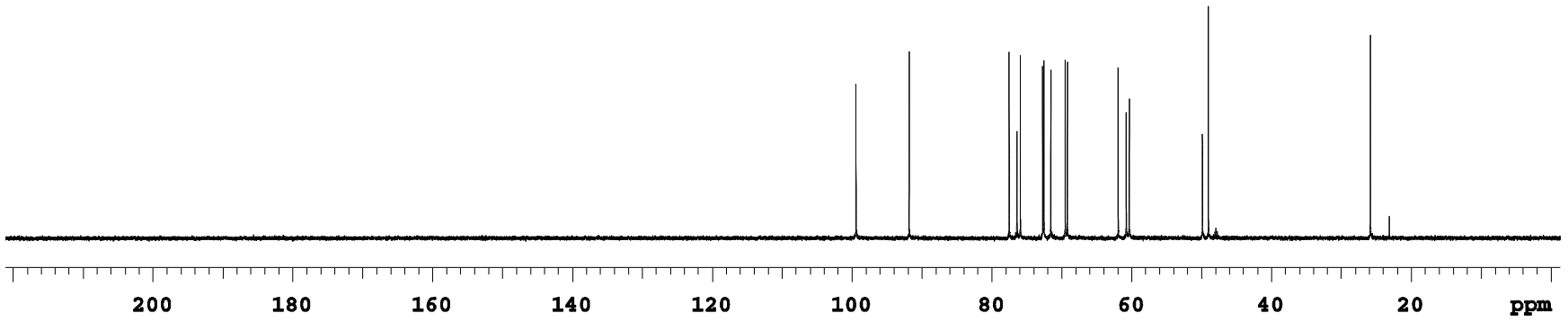
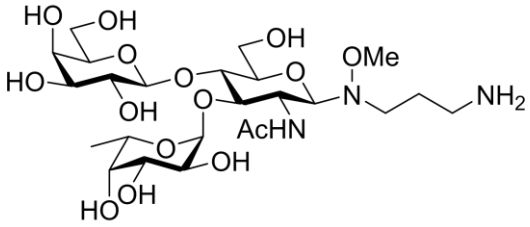
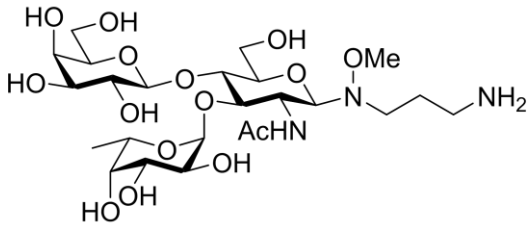


^{1}H NMR (D_2O, 500 MHz)

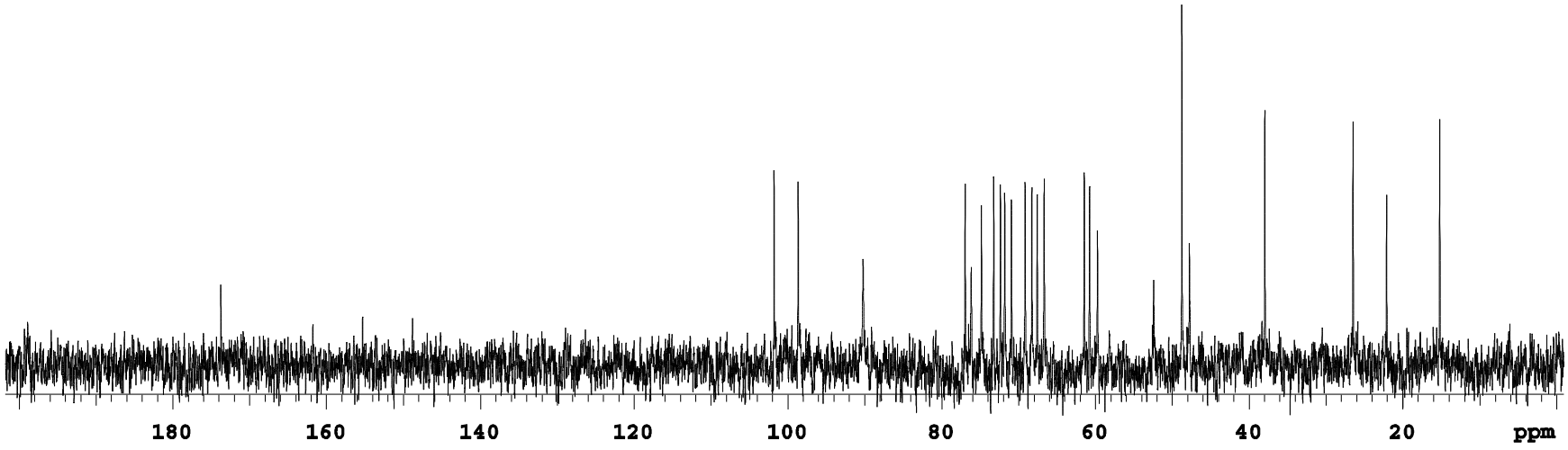


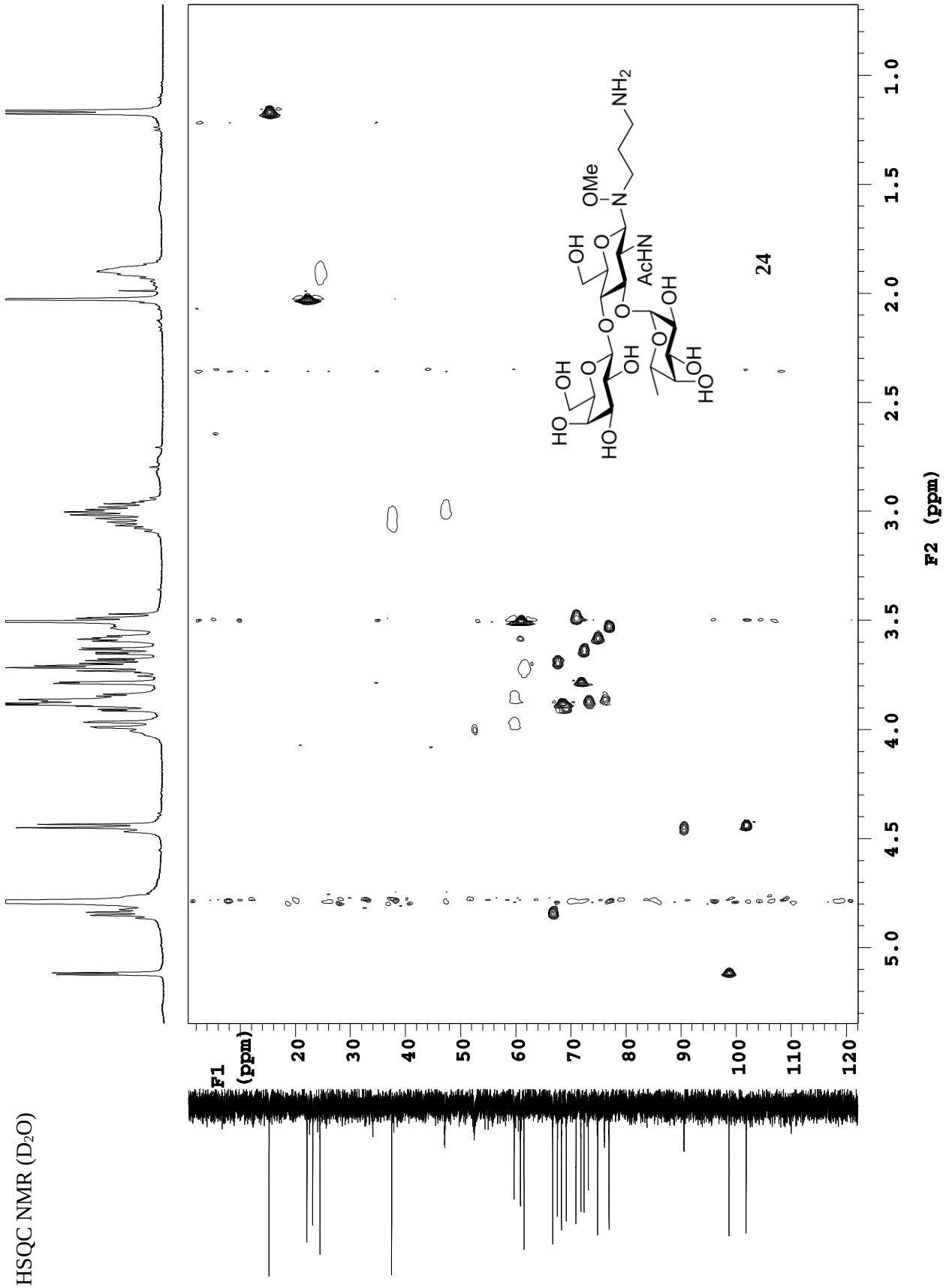
24

^{13}C NMR (D_2O, 125 MHz)

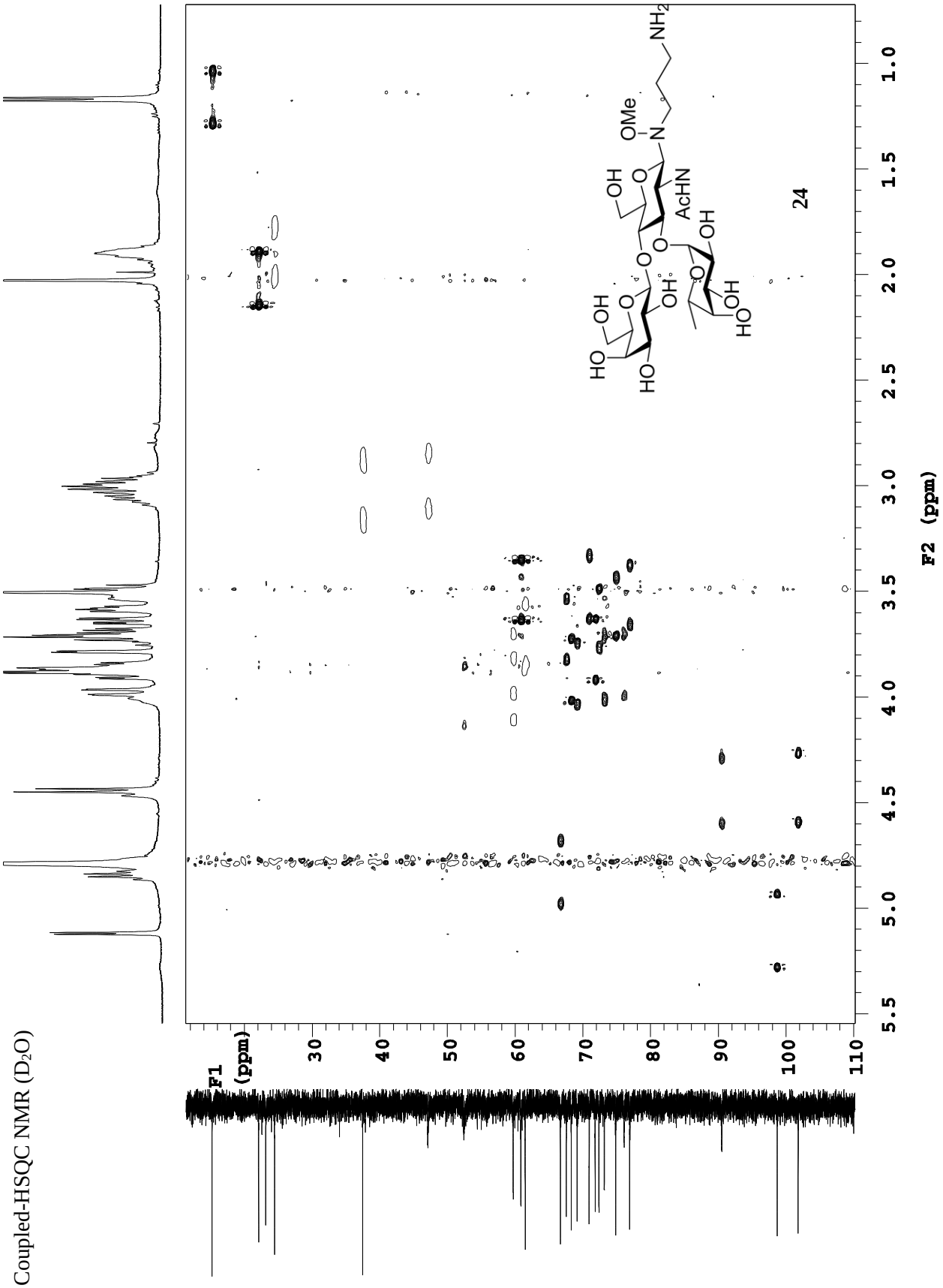


24

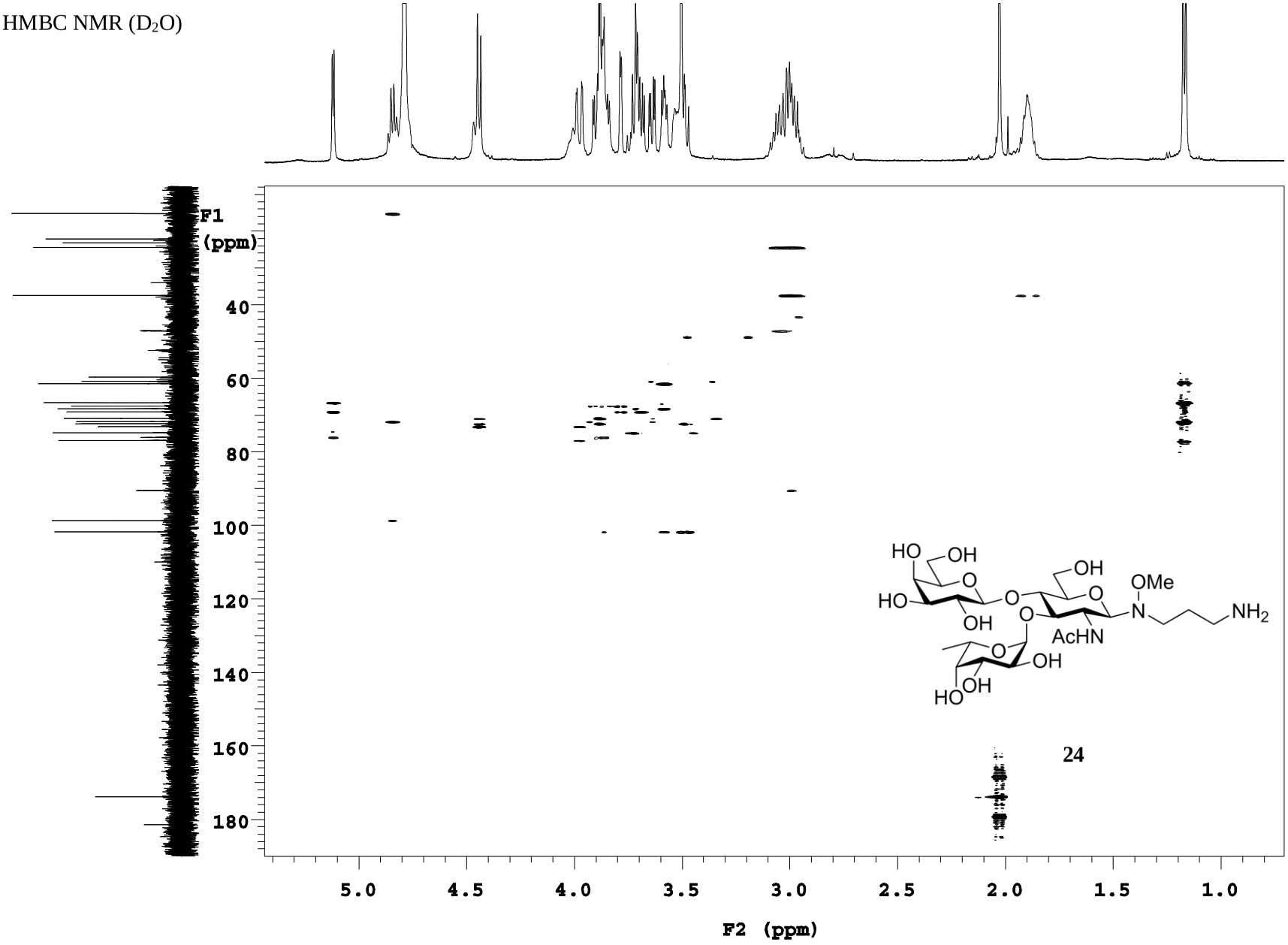



HSQC NMR (D_2O)
F1 (ppm)
F2 (ppm)
24

Coupled-HSQC NMR (D_2O)



HMBC NMR (D_2O)



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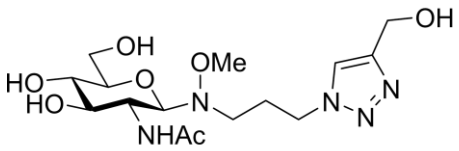
^{1}H NMR (D_2O, 500 MHz)



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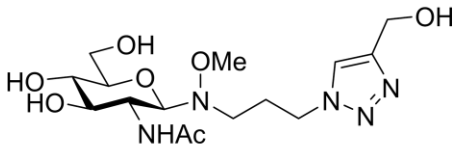
^{13}C NMR (D_2O, 125 MHz)



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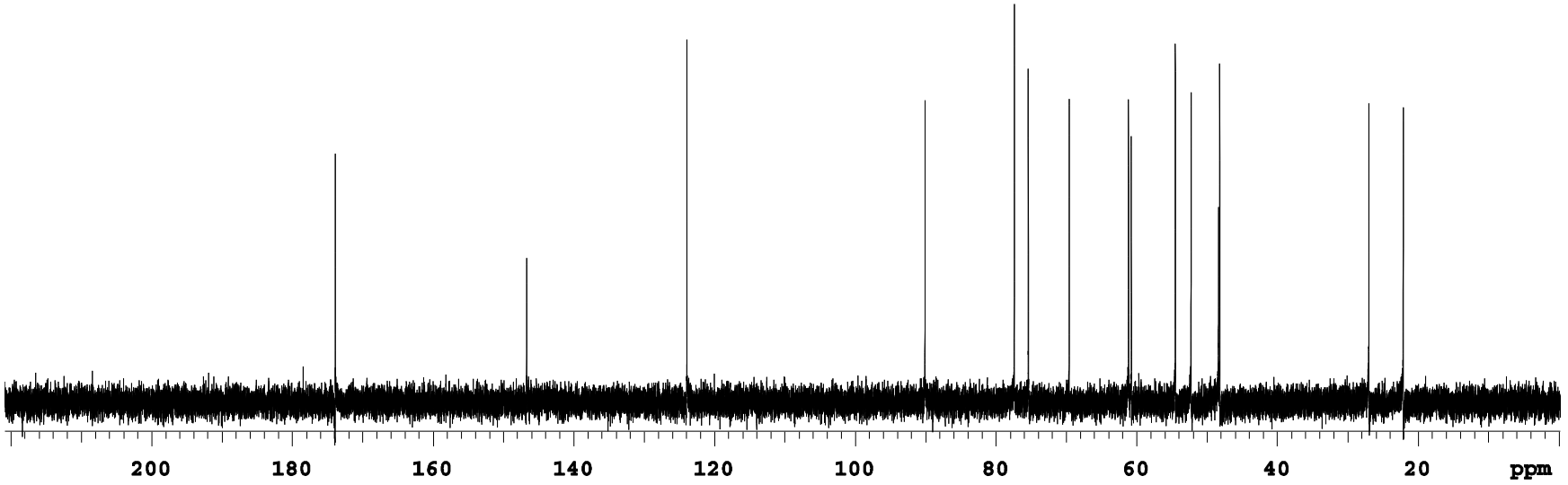
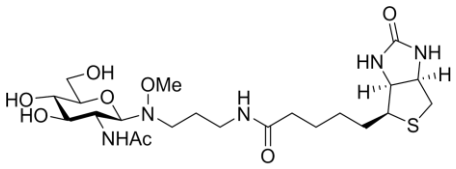


^{1}H NMR (D_2O, 500 MHz)



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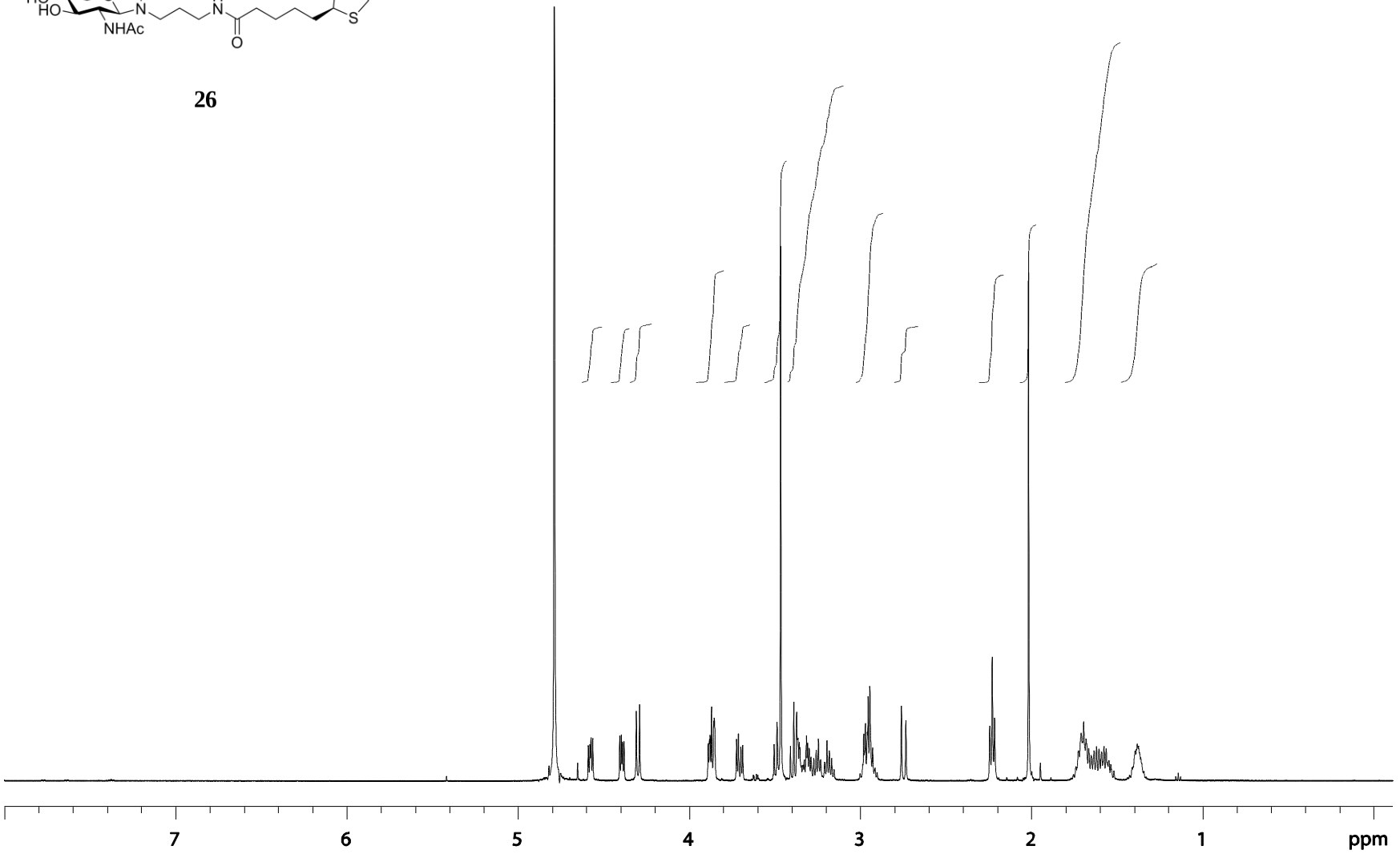
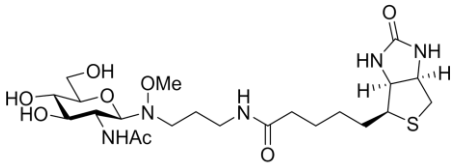


^{13}C NMR (D_2O, 125 MHz)



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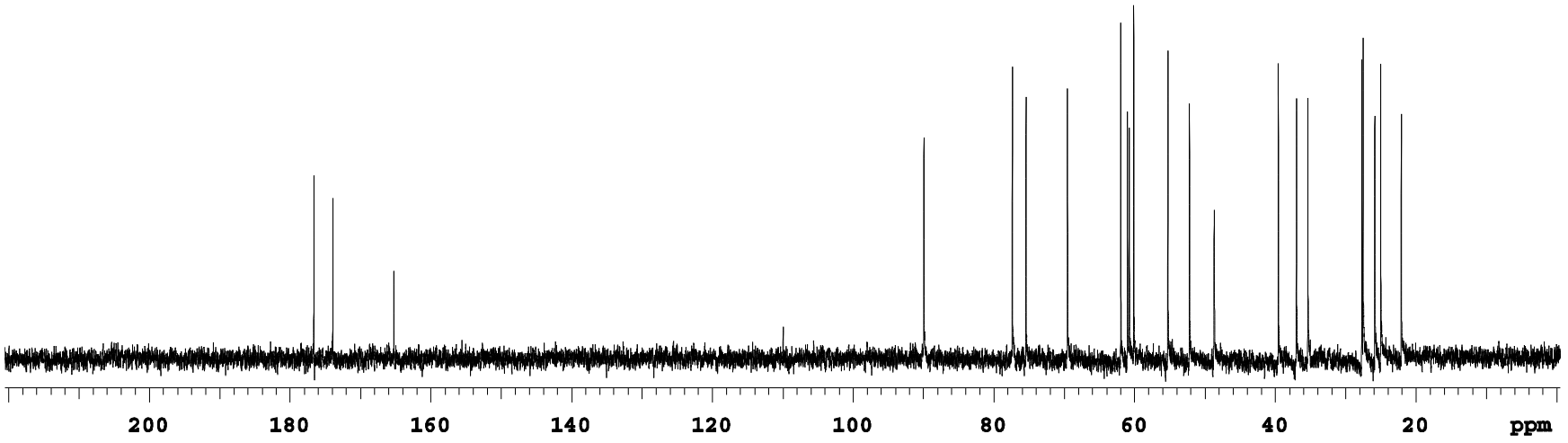
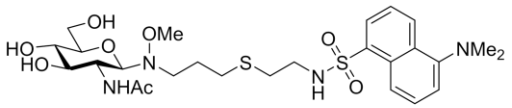
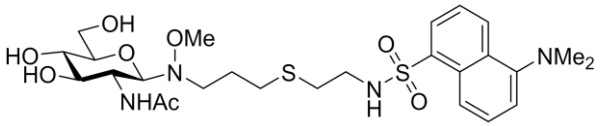


^{1}H NMR (D_2O, 500 MHz)



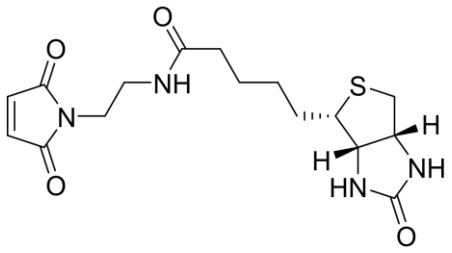
28

^{13}C NMR (D_2O, 125 MHz)



28

^{1}H NMR (CD_3OD, 500 MHz)



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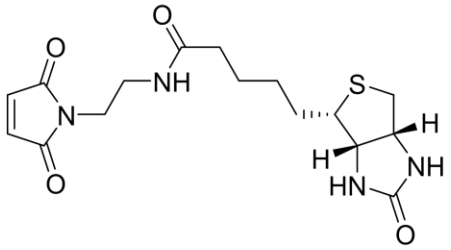
^{13}C NMR (CD_3OD, 125 MHz)



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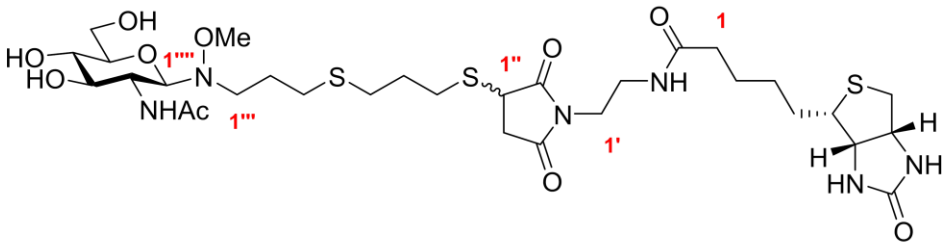
^{1}H NMR (D_2O, 500 MHz)



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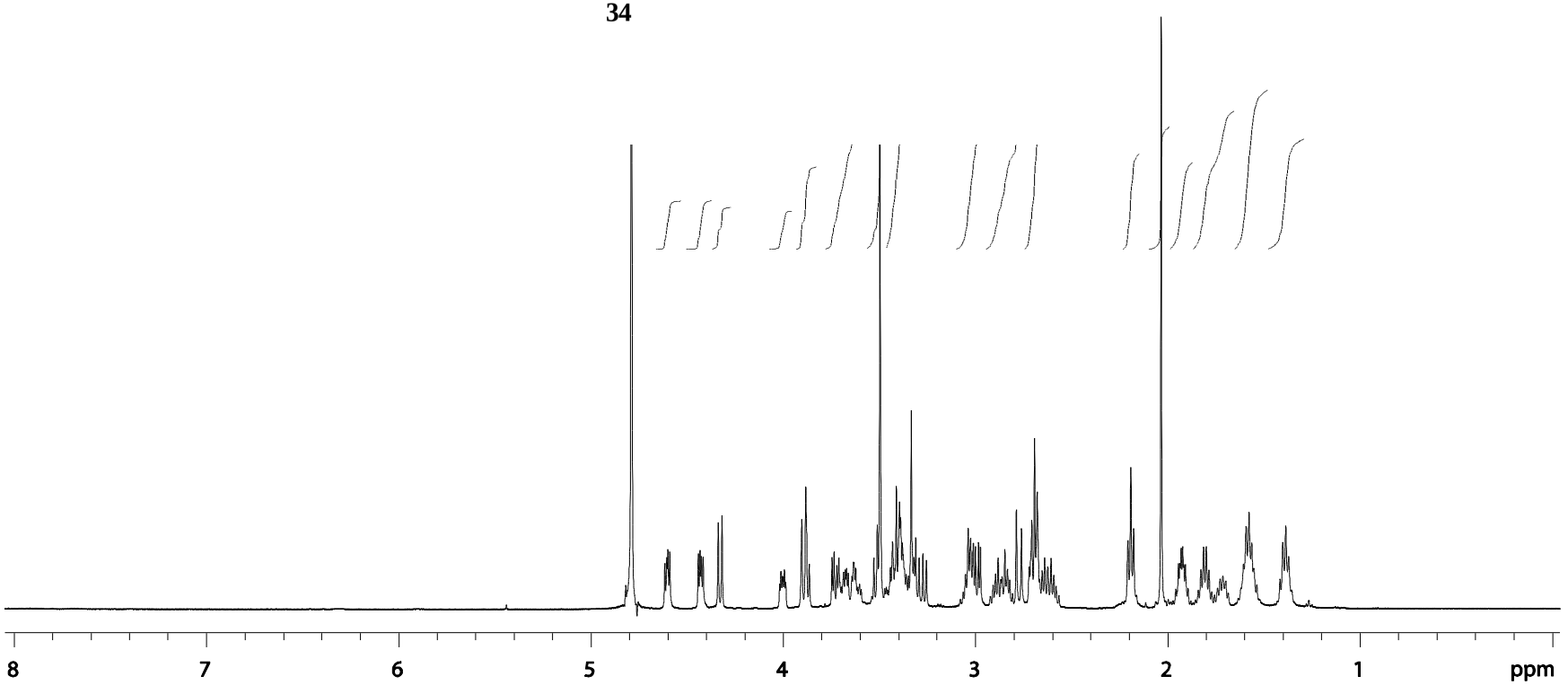
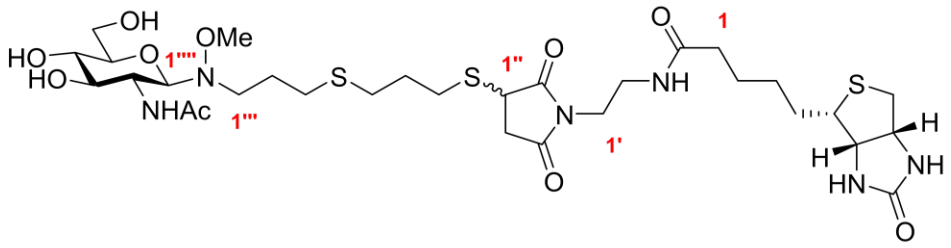


^{13}C NMR (D_2O, 125 MHz)



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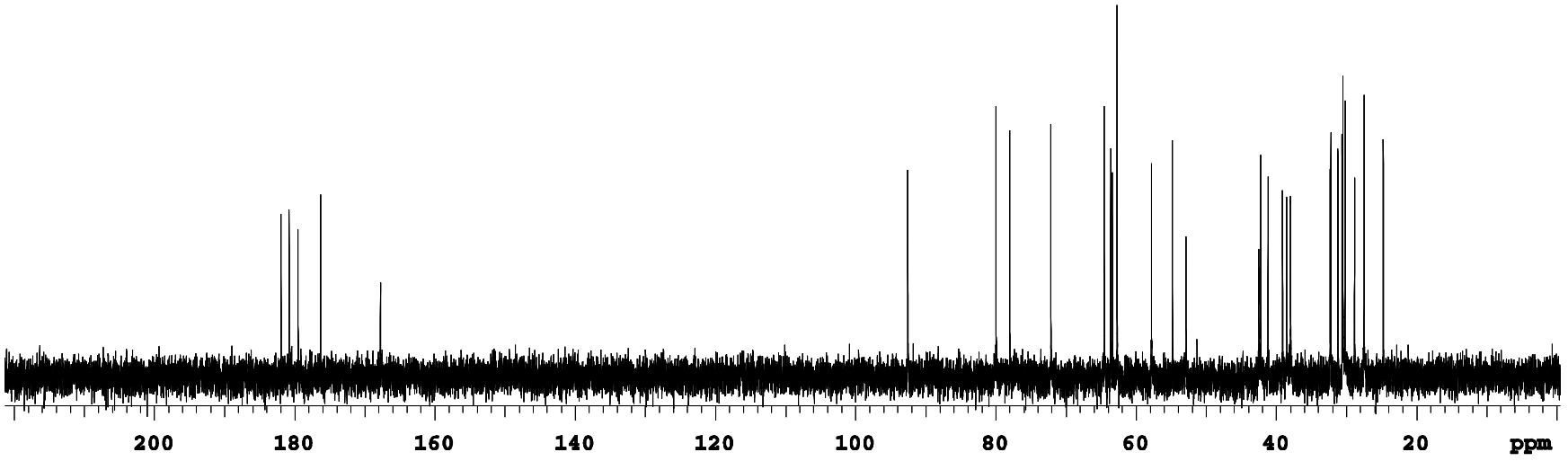
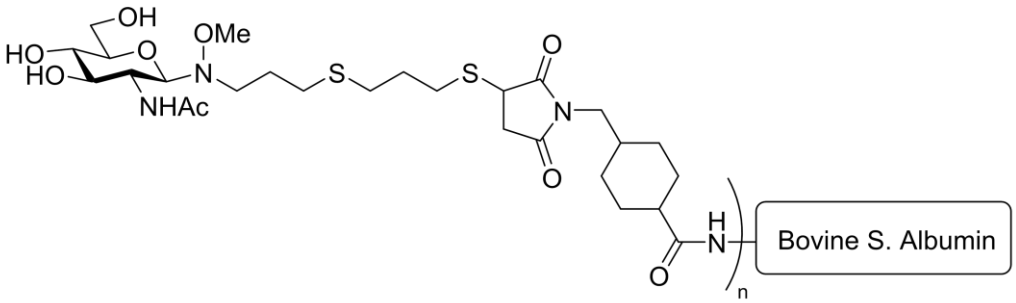


Maldi-TOF (Cinnapinic acid matrix)



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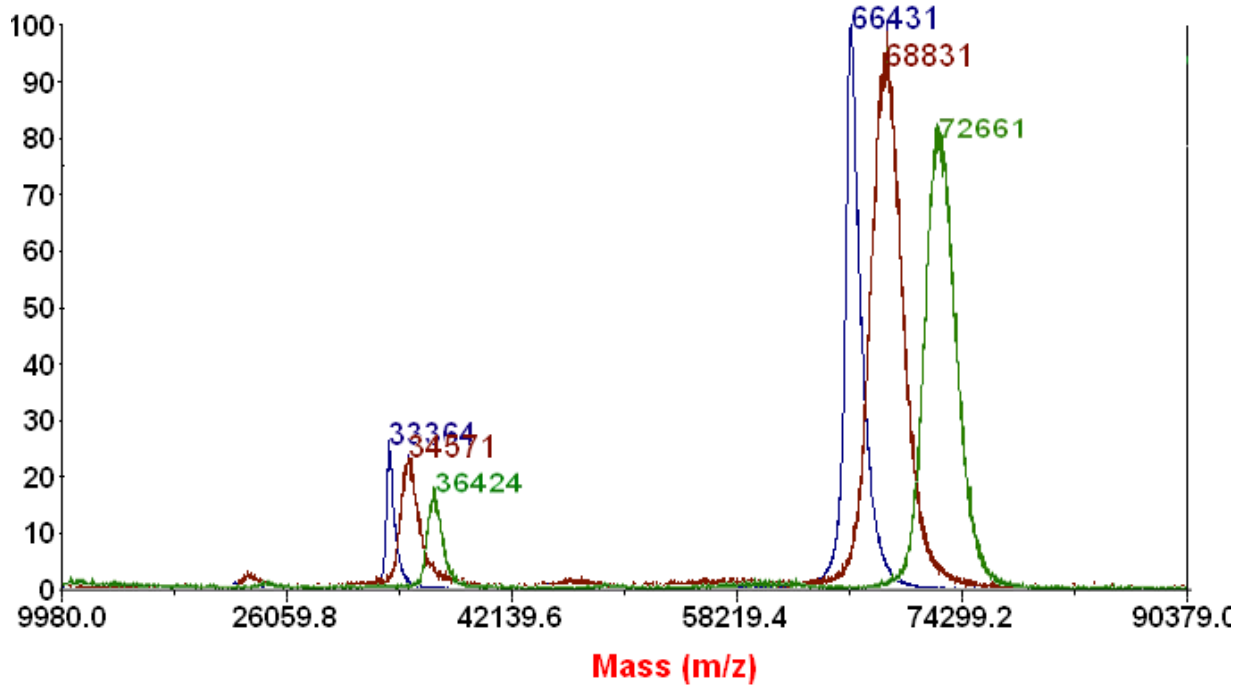
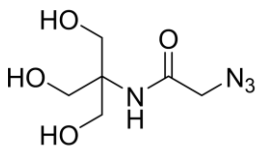


Supporting Information Chapter 3.

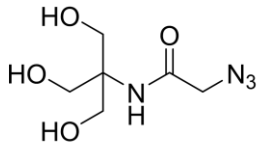
Design and synthesis of novel fluorescent and biotinylated multivalent glycodendrons.

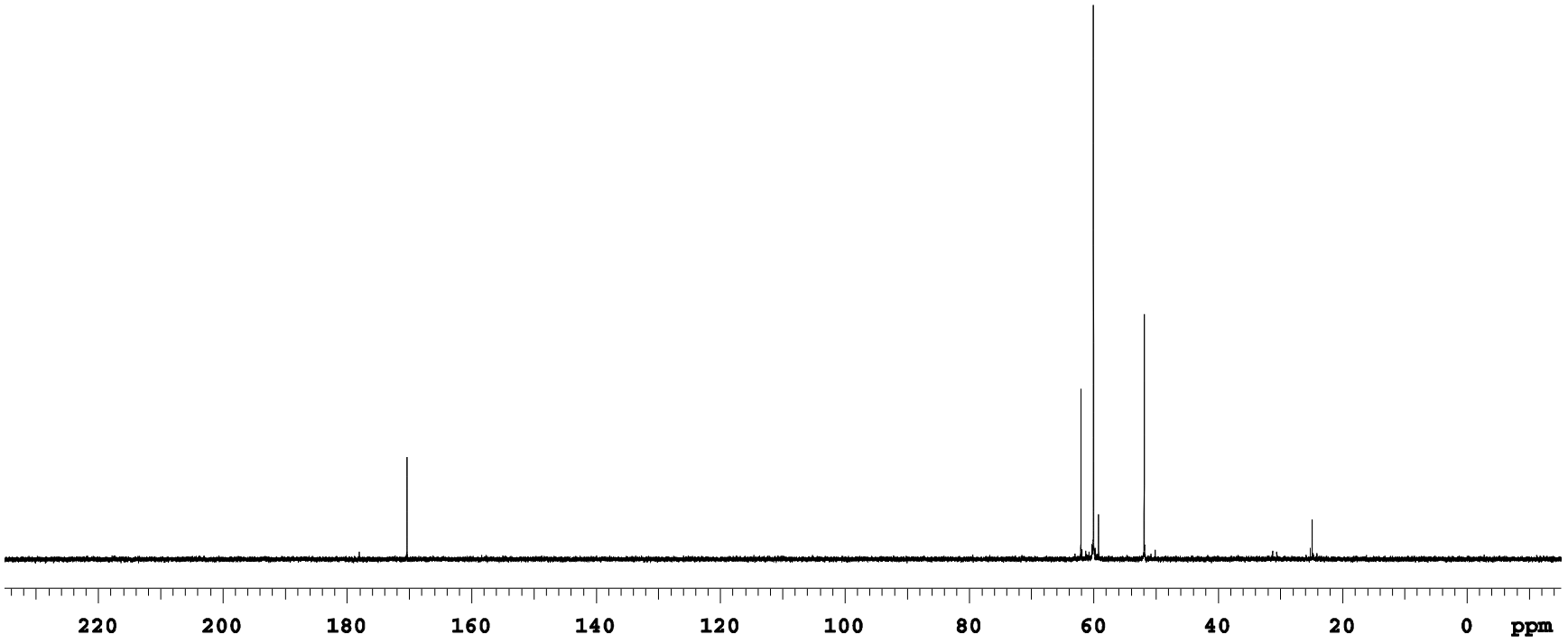
Table of content

^{1}H NMR (D_2O, 500 MHz)

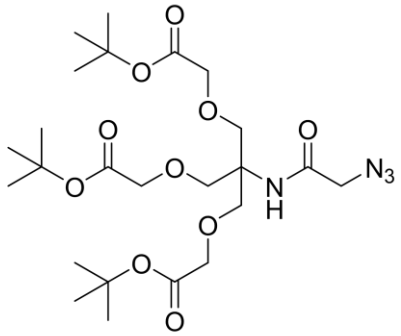


^{13}C NMR (D_2O, 125 MHz)

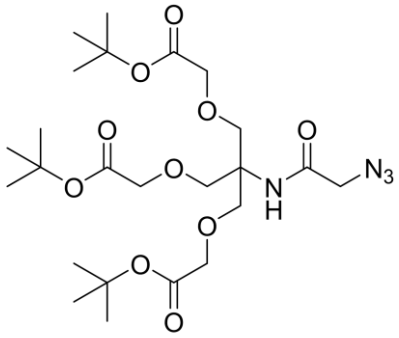




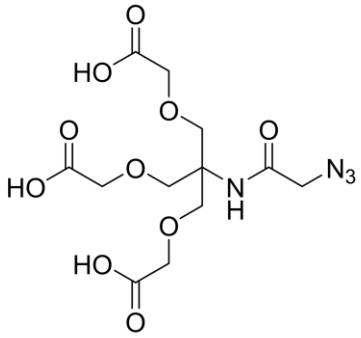
^{1}H NMR ($CDCl_3$, 500 MHz)

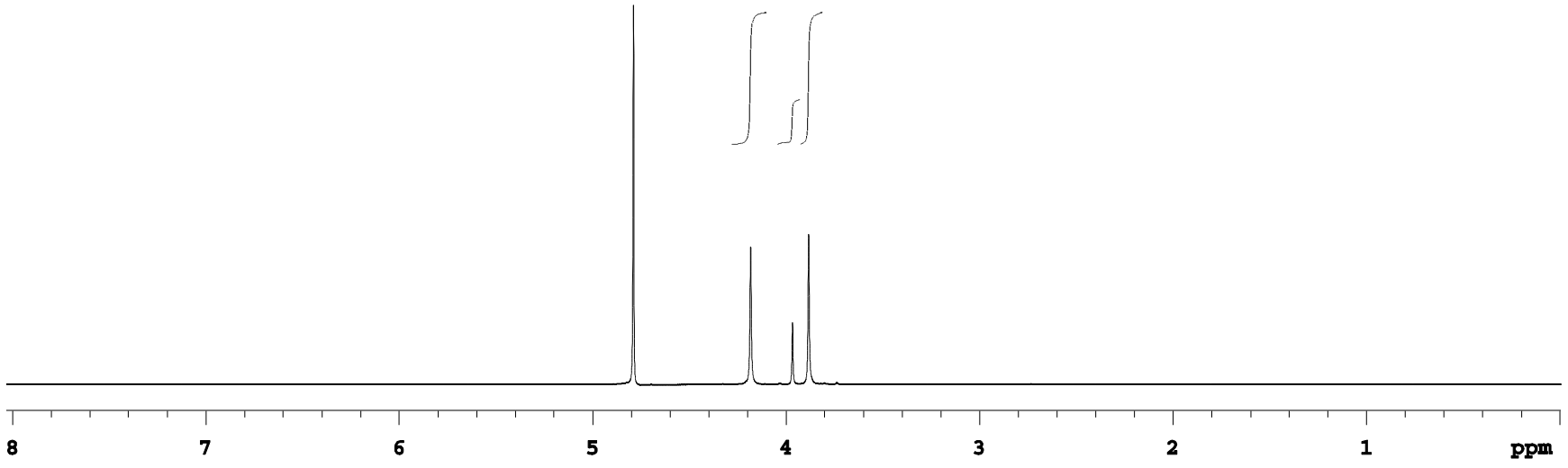


^{13}C NMR ($CDCl_3$, 125 MHz)

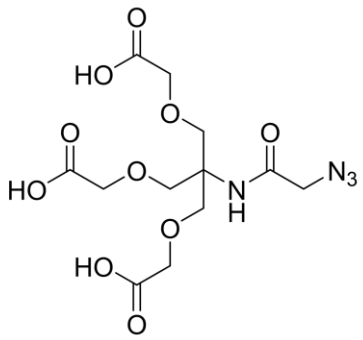


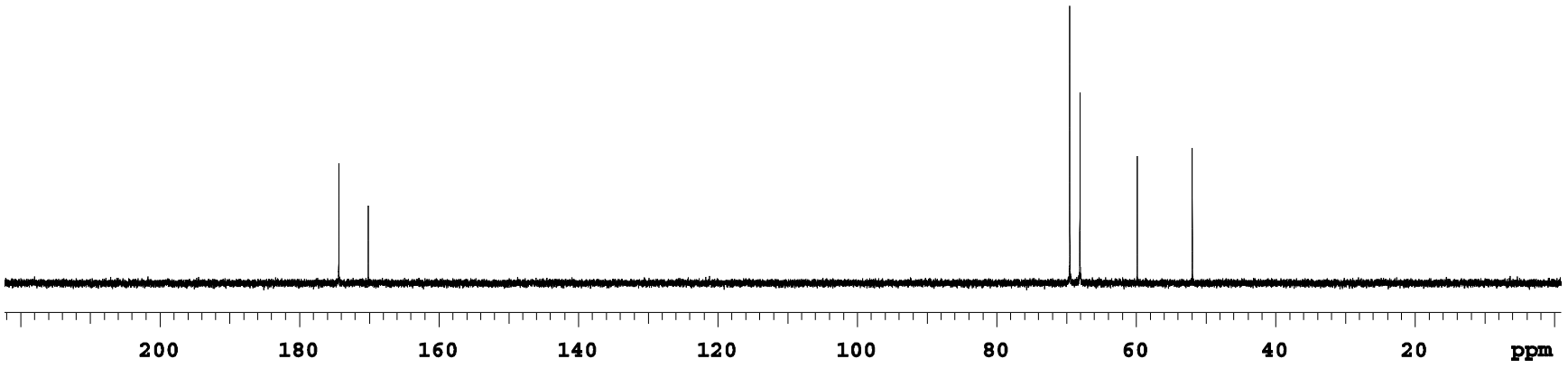
^{1}H NMR (D_2O, 500 MHz)



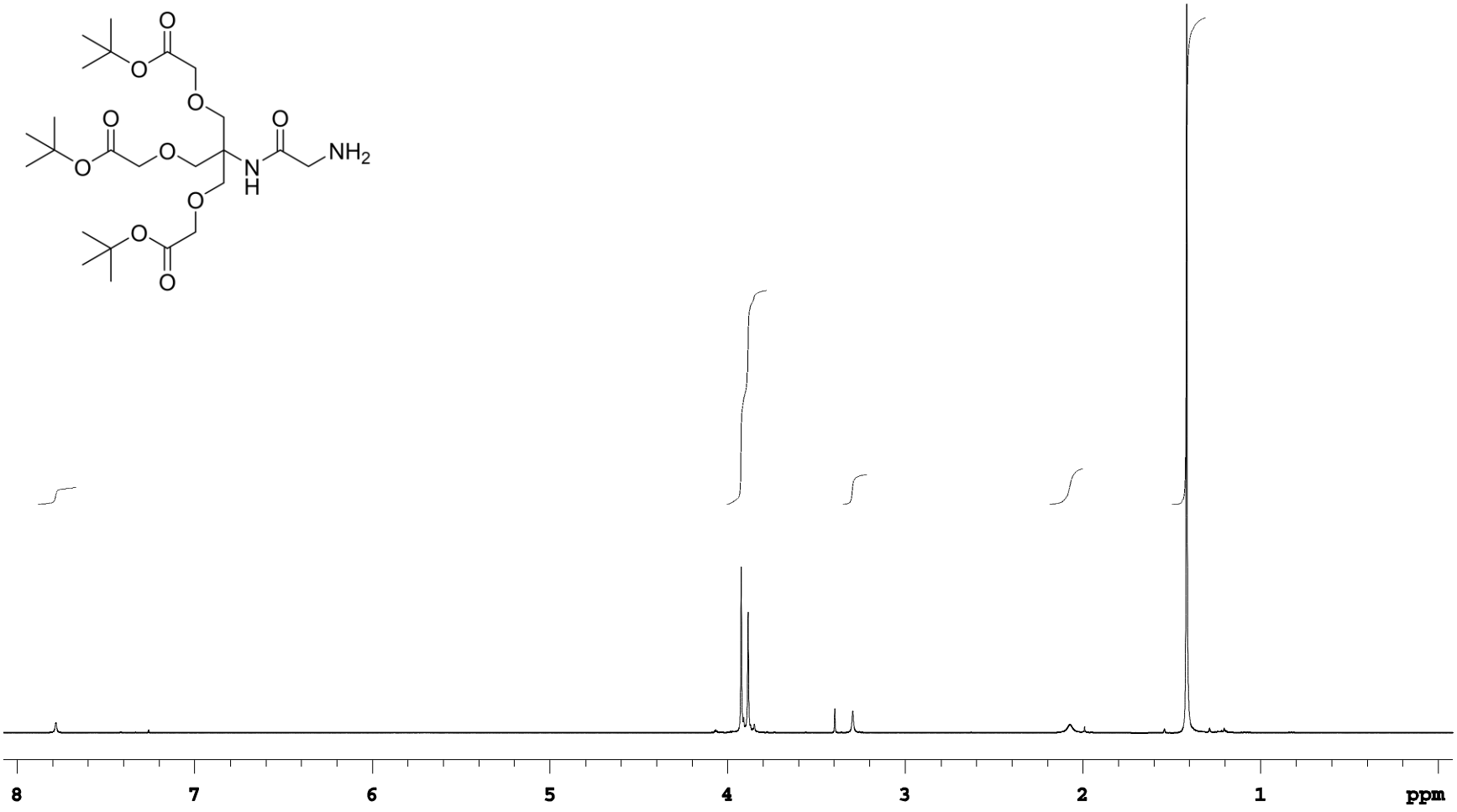


^{13}C NMR (D_2O, 125 MHz)

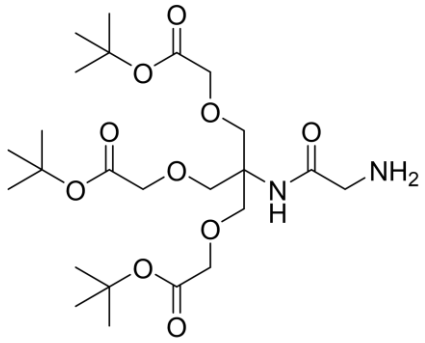




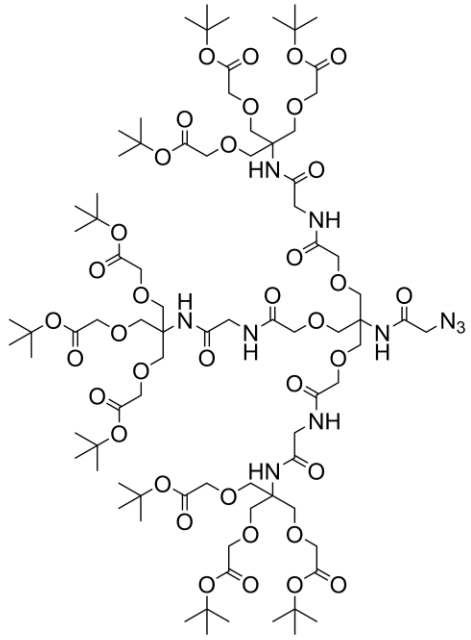
^{1}H NMR ($CDCl_3$, 500 MHz)



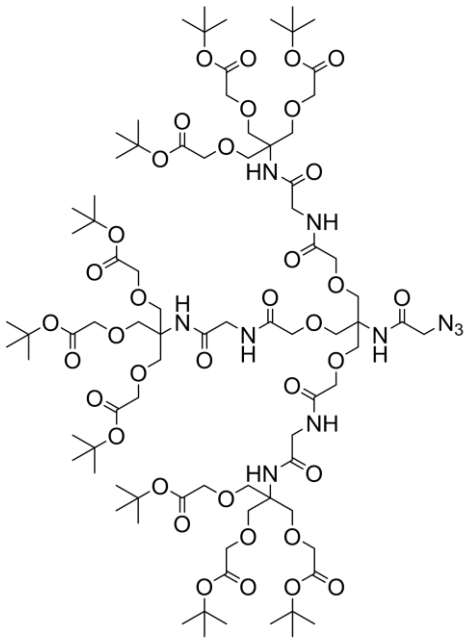
^{13}C NMR ($CDCl_3$, 125 MHz)



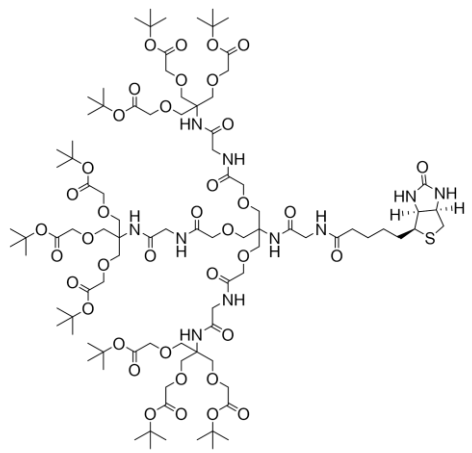
^{1}H NMR ($CDCl_3$, 500 MHz)



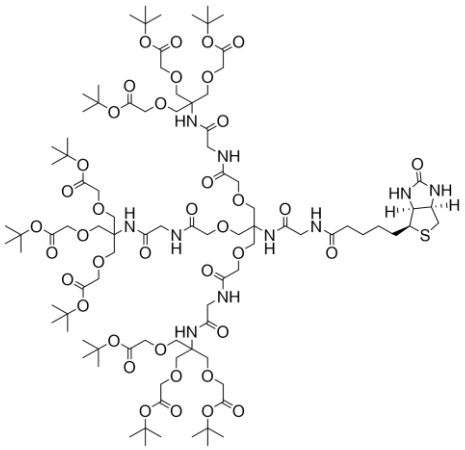
^{13}C NMR ($CDCl_3$, 125 MHz)



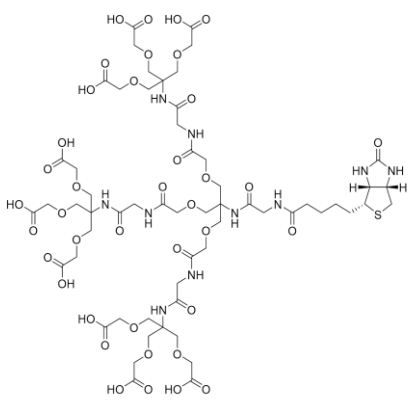
^{1}H NMR ($CDCl_3$, 500 MHz)



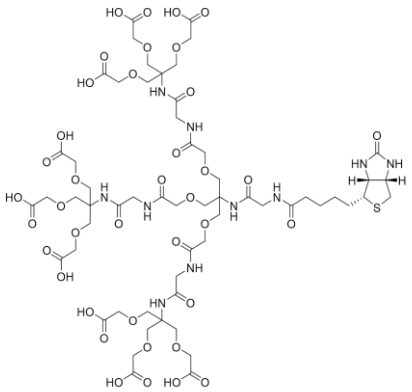
^{13}C NMR ($CDCl_3$, 125 MHz)

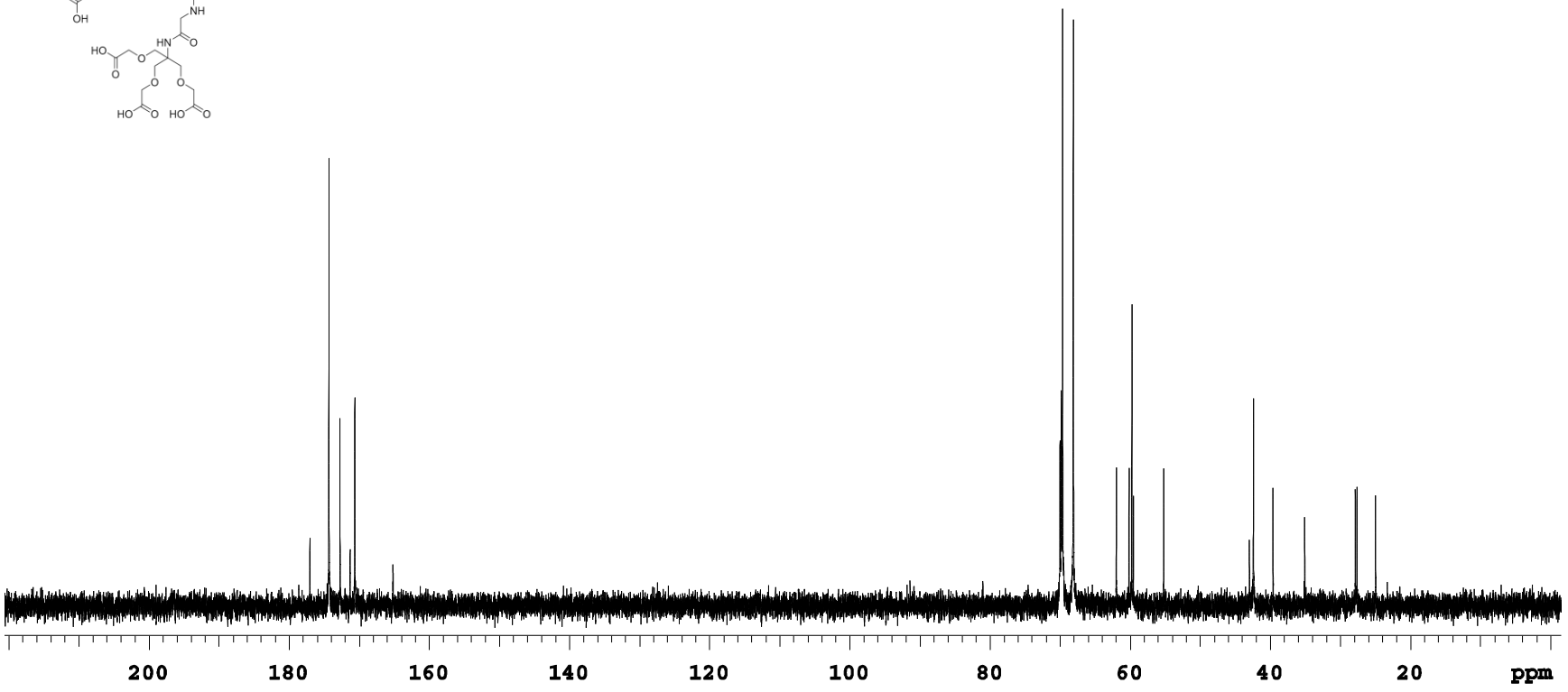


^{1}H NMR (D_2O, 500 MHz)

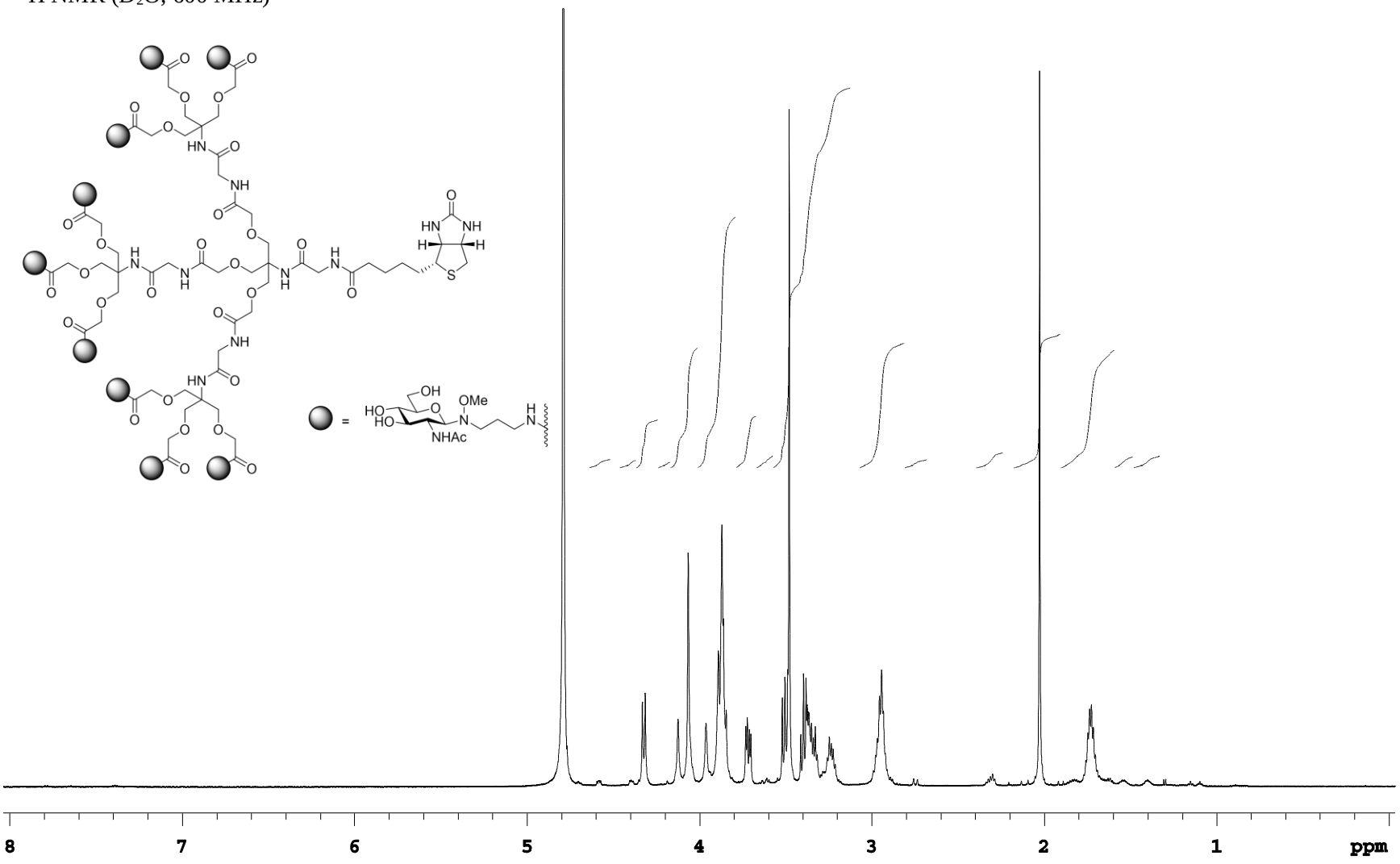


^{13}C NMR (D_2O, 125 MHz)

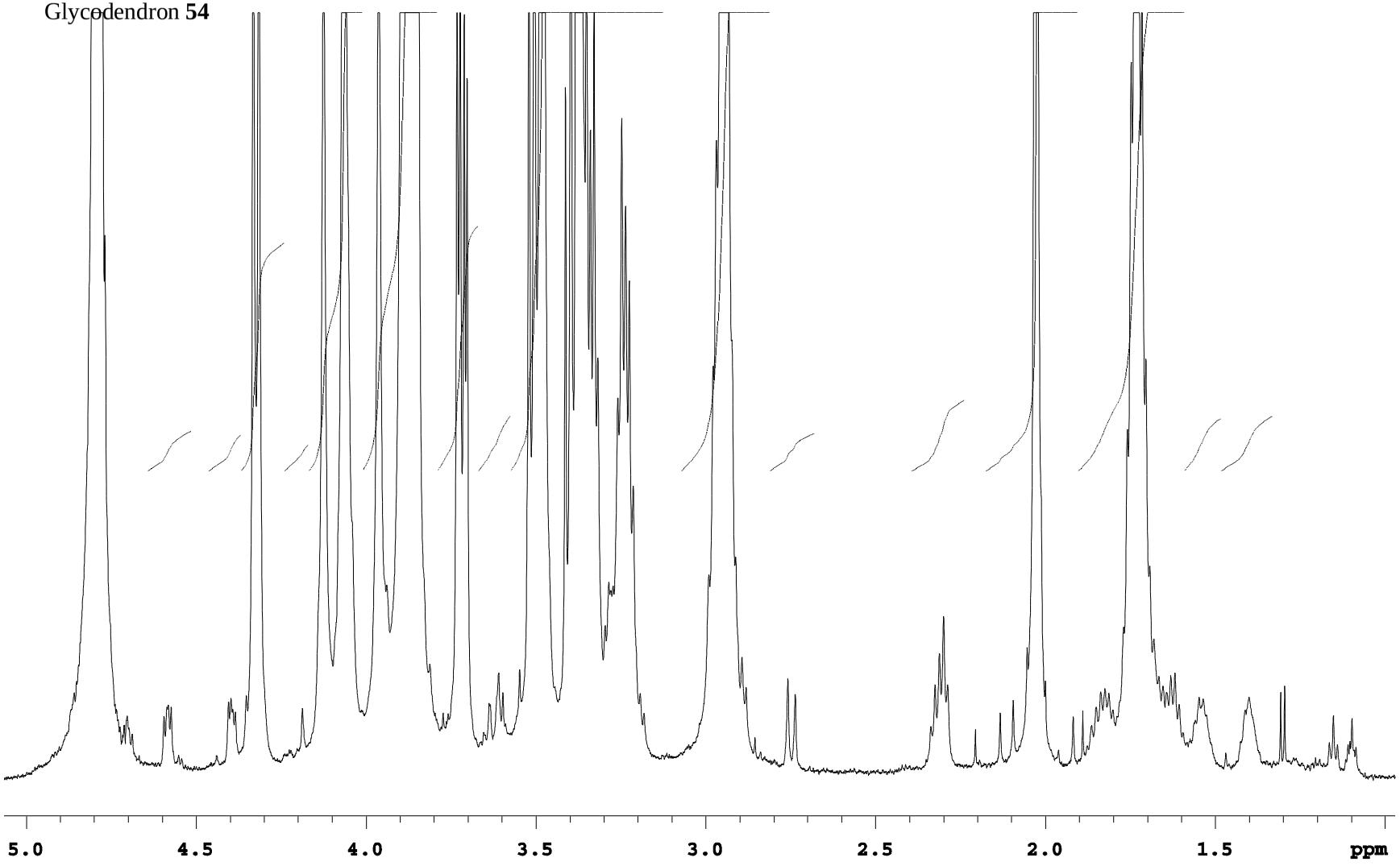


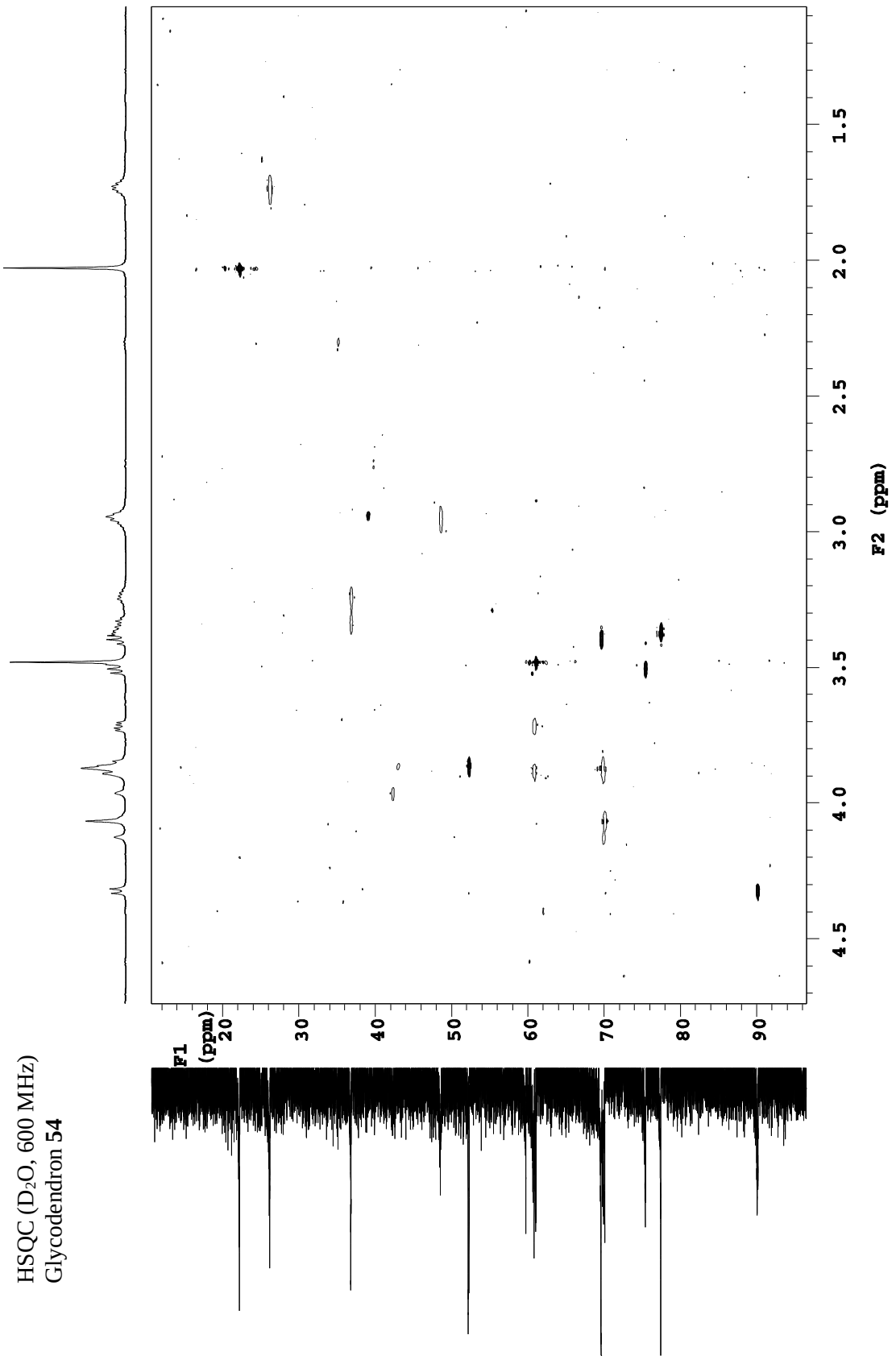


^{1}H NMR (D_2O, 600 MHz)



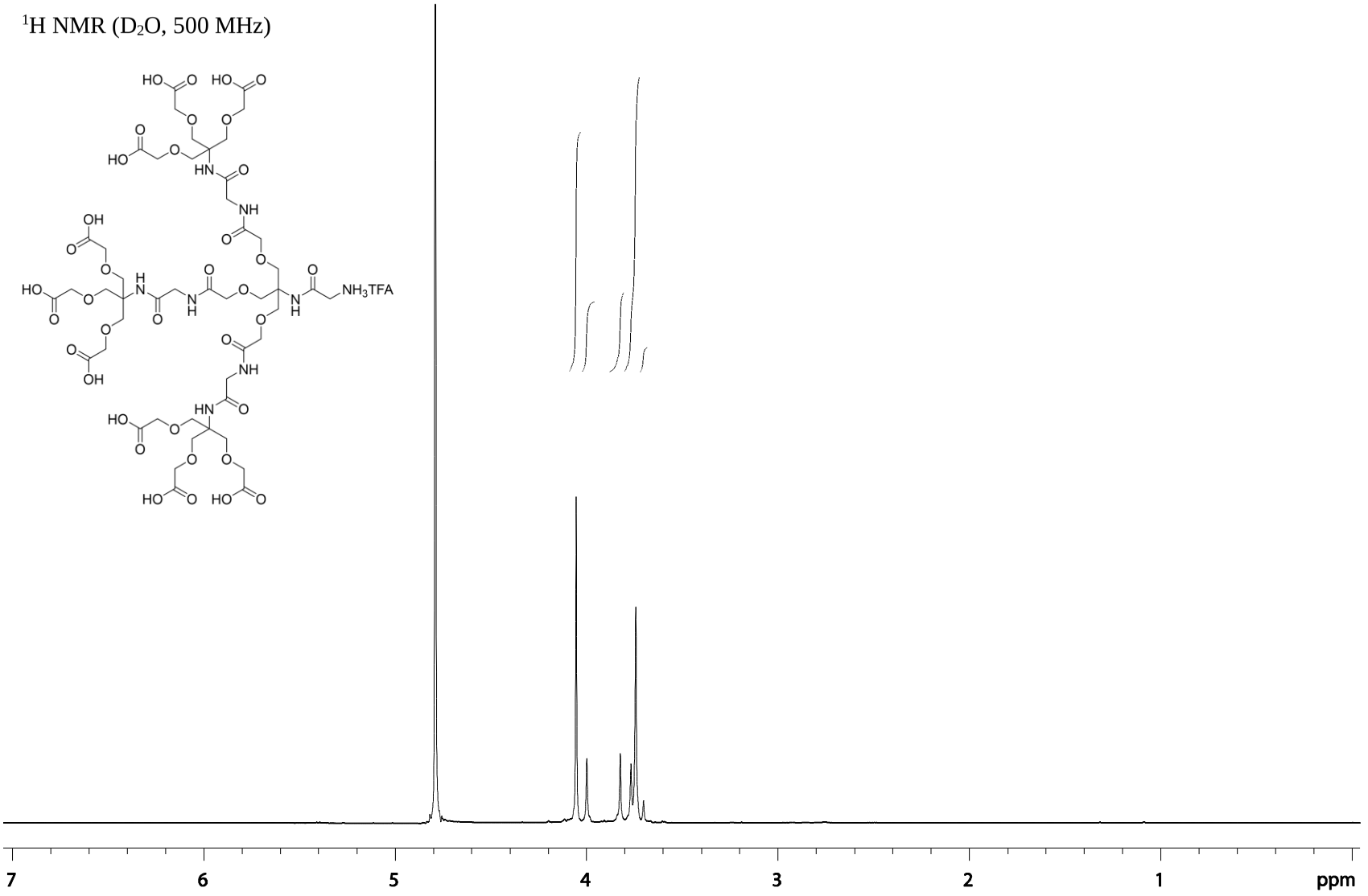
^{1}H NMR (D_2O, 600 MHz)
Expansion of Appendix-76
Glycodendron **54**



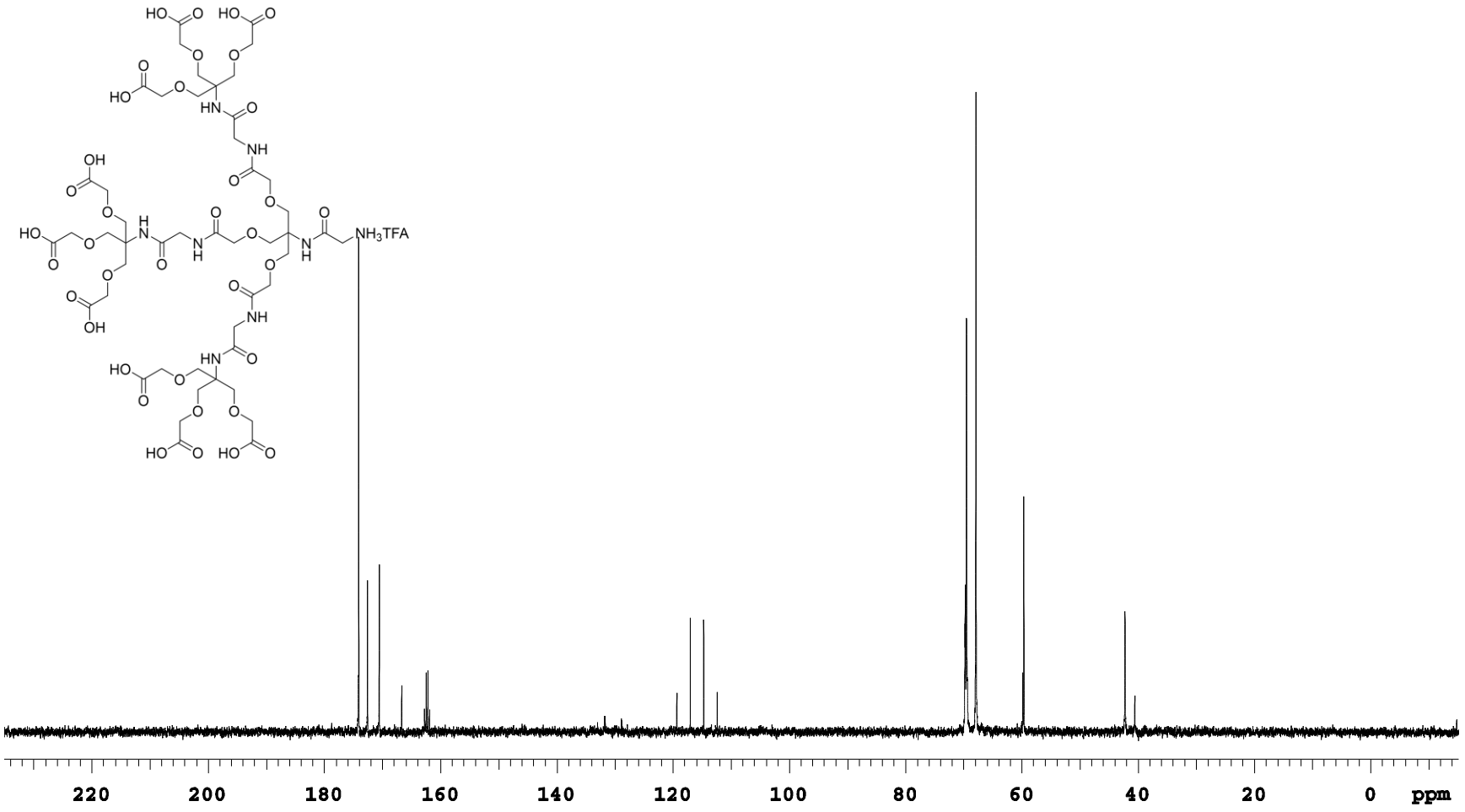


HSQC (D_2O, 600 MHz)
Glycodendron **54**

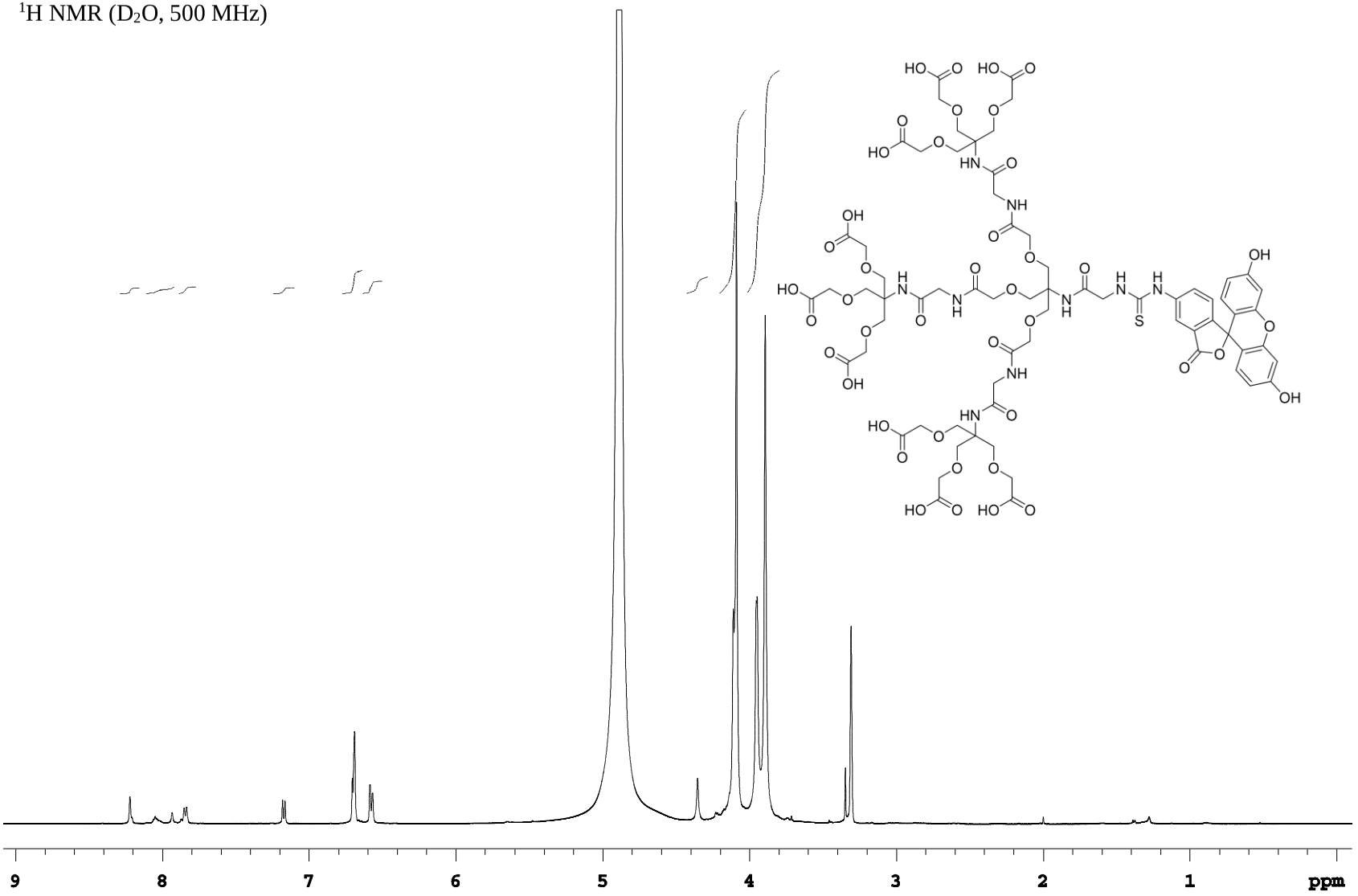
^{1}H NMR (D_2O, 500 MHz)



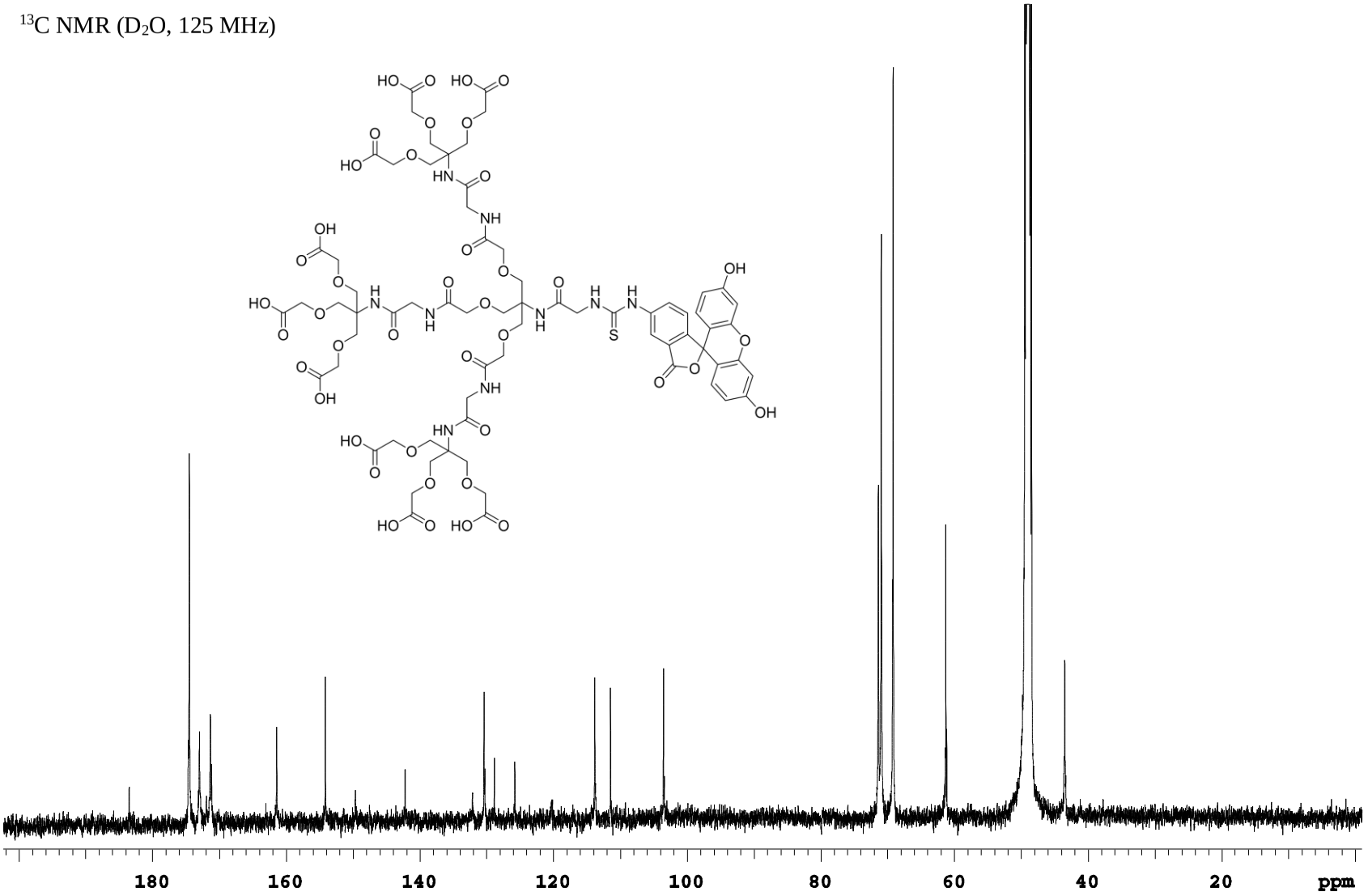
^{13}C NMR (D_2O, 125 MHz)



^{1}H NMR (D_2O, 500 MHz)



^{13}C NMR (D_2O, 125 MHz)

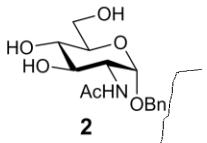


Appendix Chapter 4.

The Highly Efficient Synthesis of a crystalline trisaccharide for the construction of Lewis^X and related antigens.

Table of content

^{1}H-NMR (500 MHz, CD_3OD)



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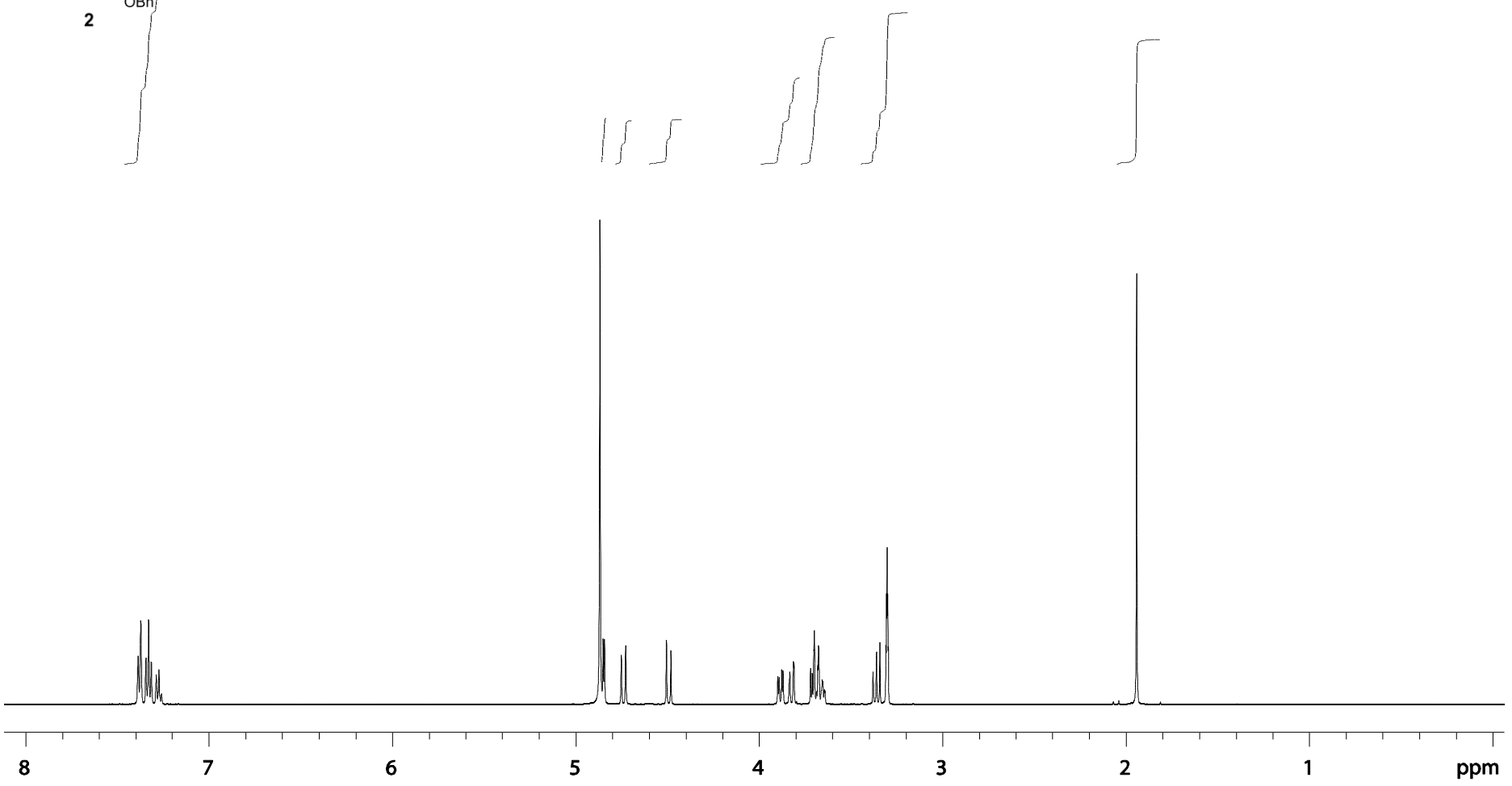
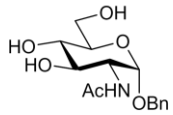


^{13}C-NMR (125 MHz, CD_3OD)



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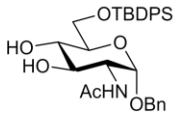
^{1}H-NMR (500 MHz, $CDCl_3$)



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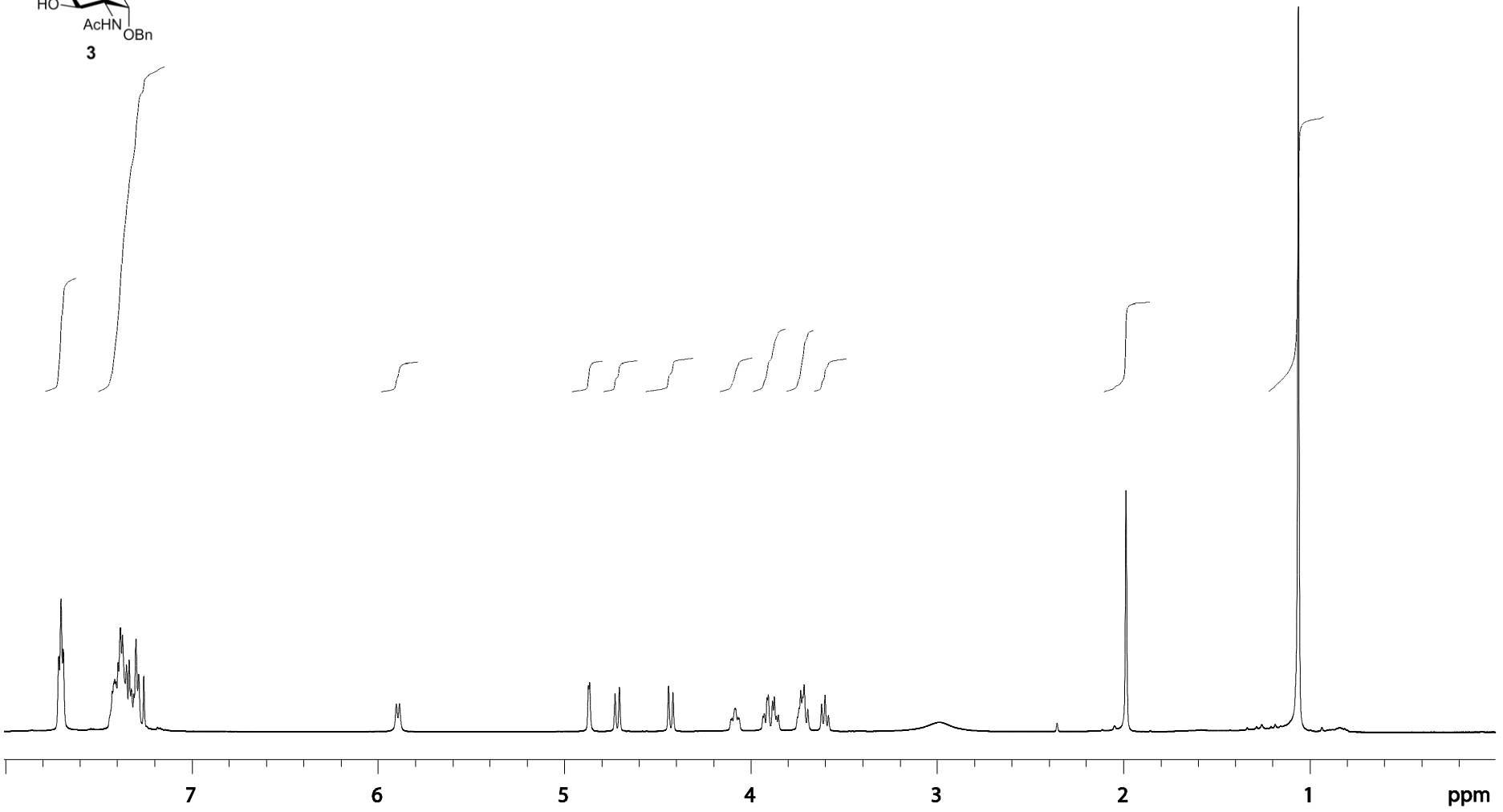
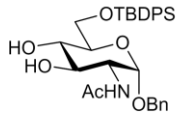


^{13}C-NMR (125 MHz, $CDCl_3$)



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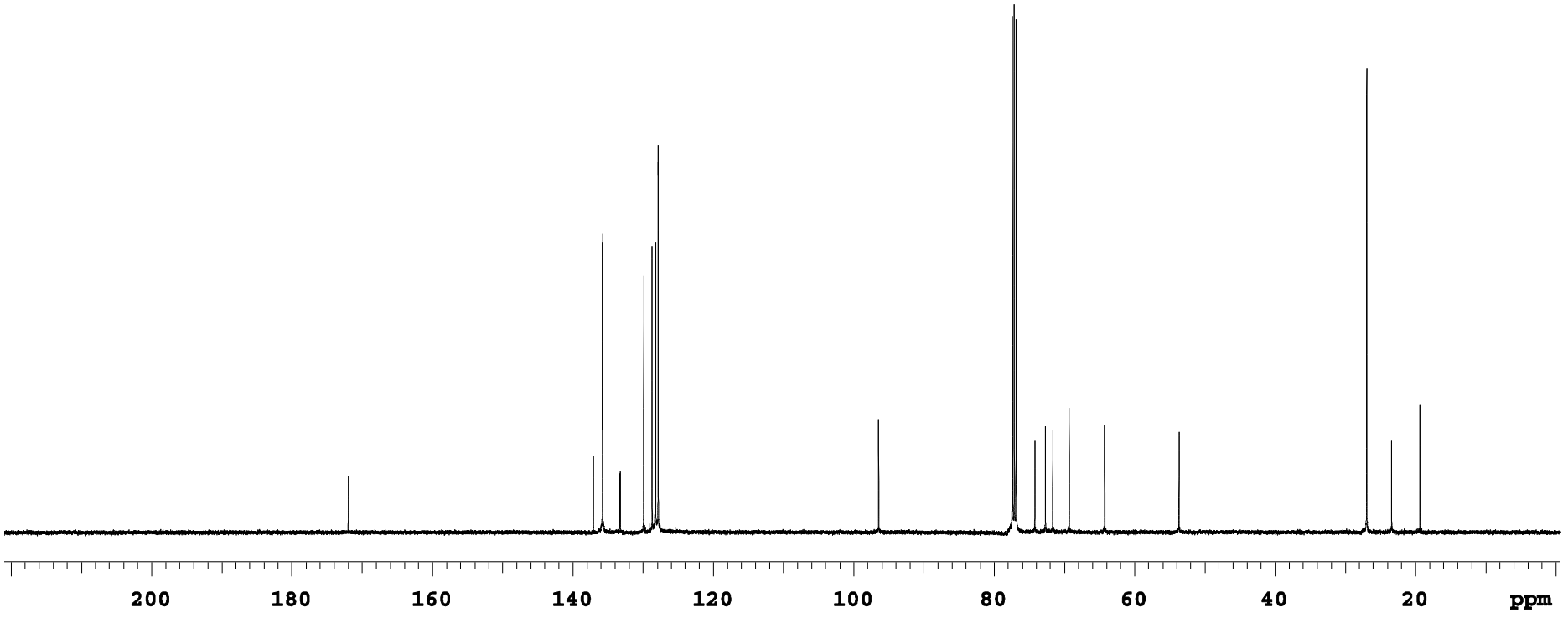

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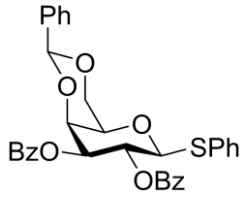

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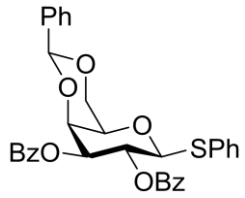

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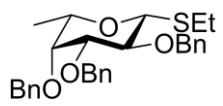

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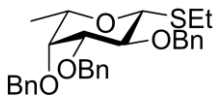

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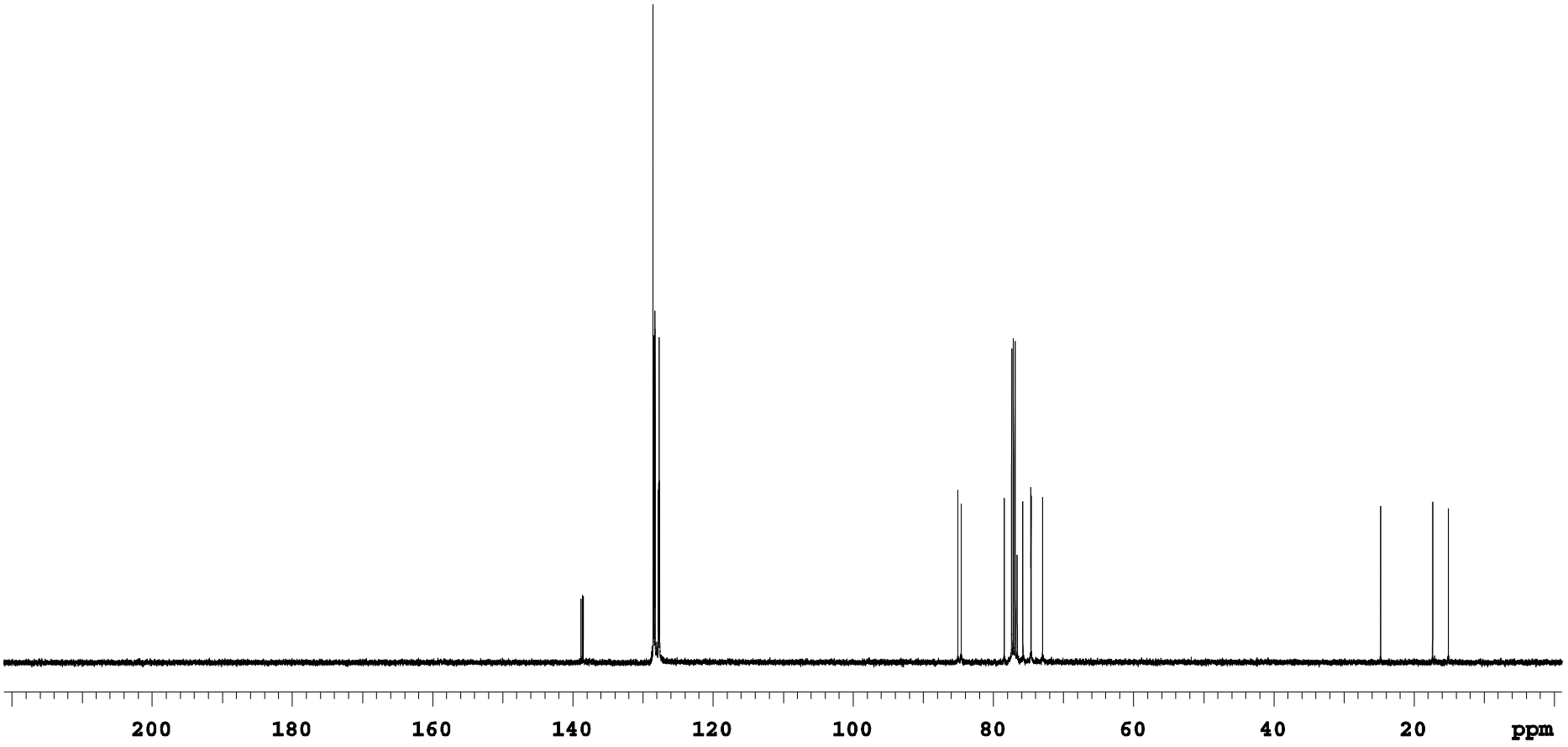

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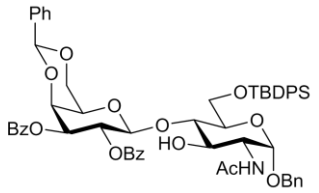

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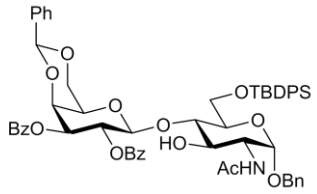

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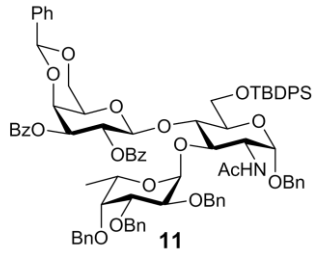

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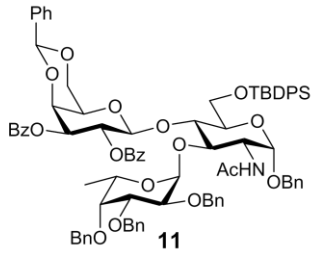

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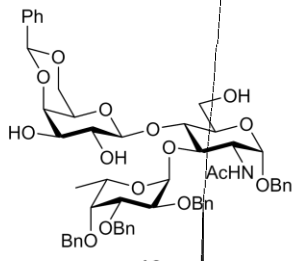

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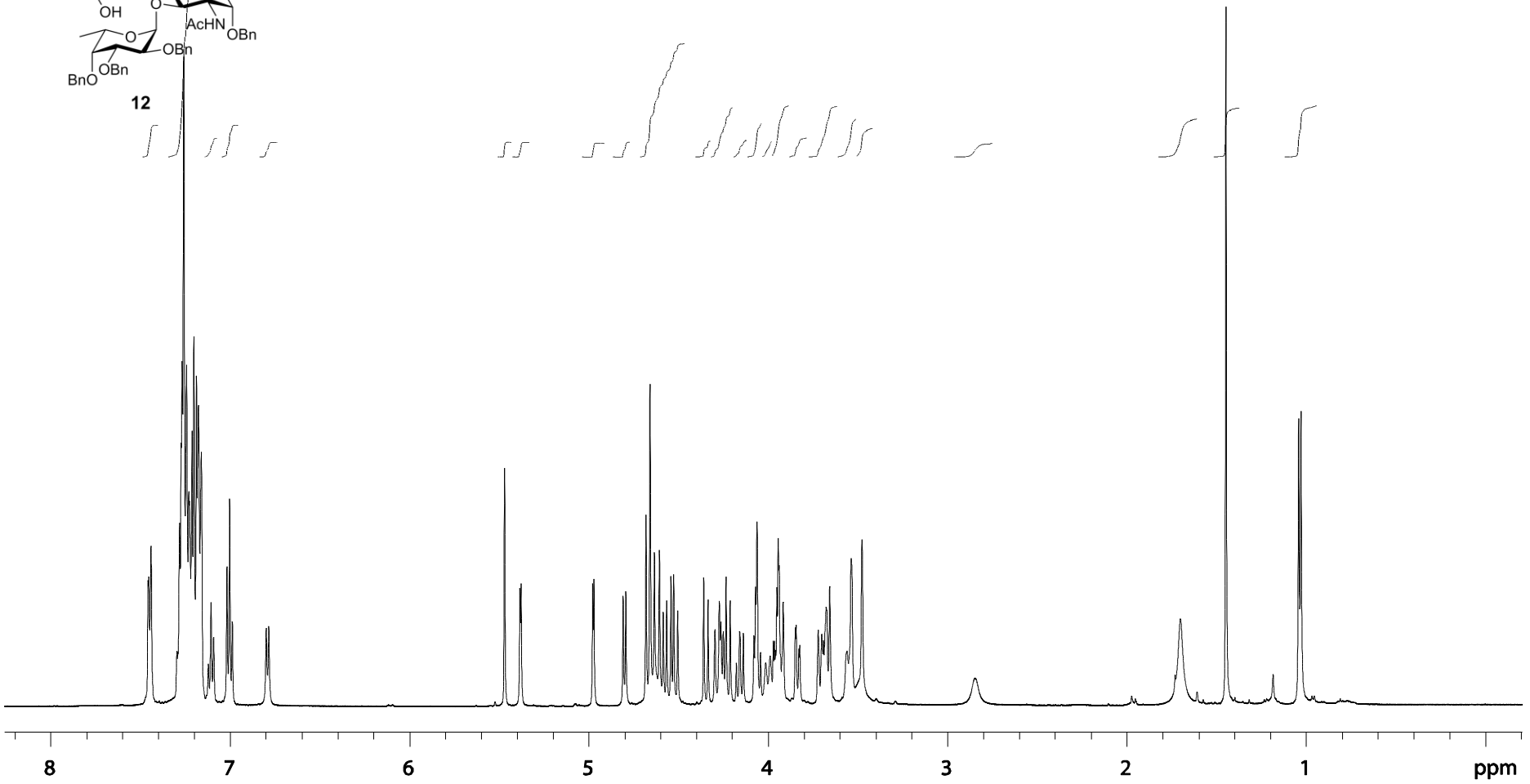

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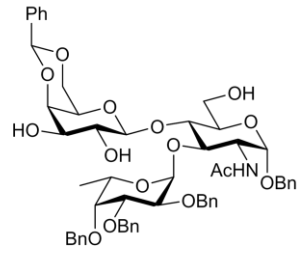

12

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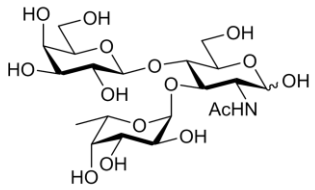
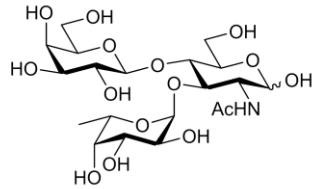
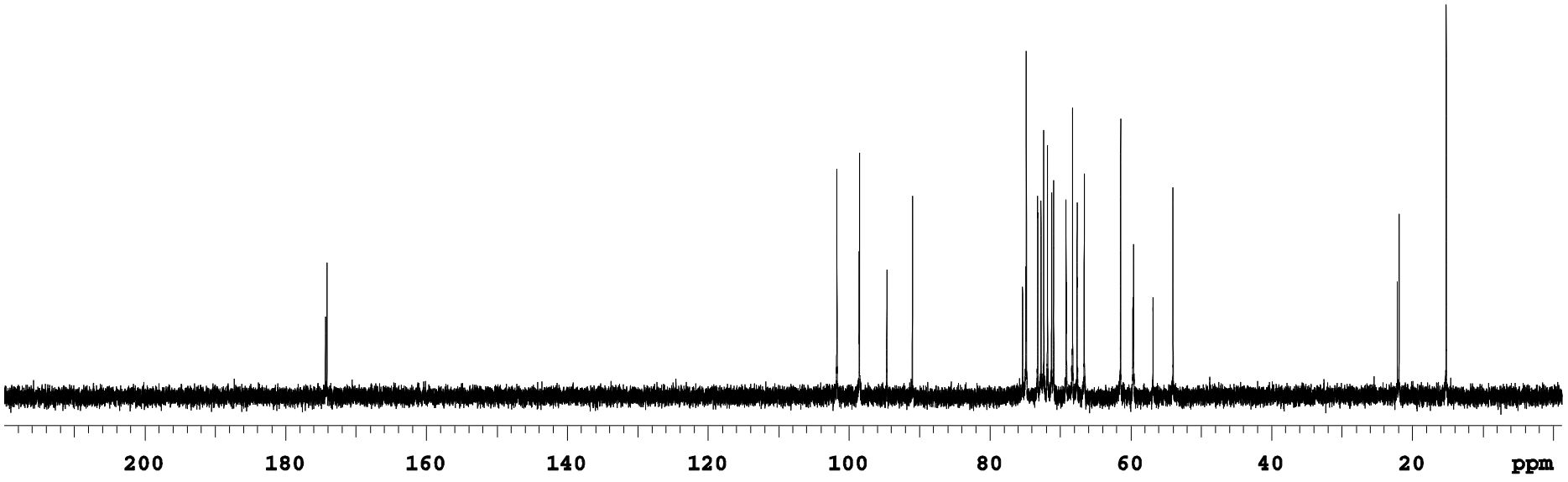


Lewis^{X}

^{13}C-NMR (125 MHz, D_2O)



Lewis^X

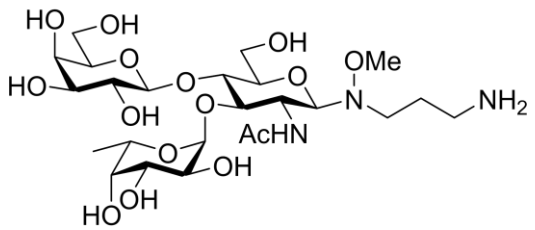


Appendix Chapter 5.

The synthesis and biological evaluation of fluorescent Lewis^X glycodendrons to target DC-SIGN lectins on Macrophages and Dendritic cells.

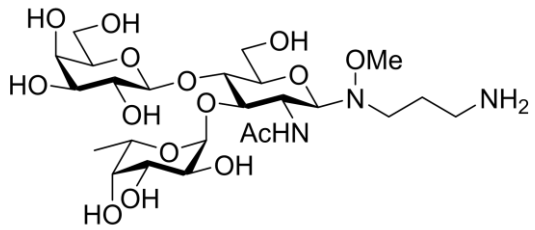
Table of content

^{1}H NMR (D_2O, 500 MHz)



24

^{13}C NMR (D_2O, 125 MHz)



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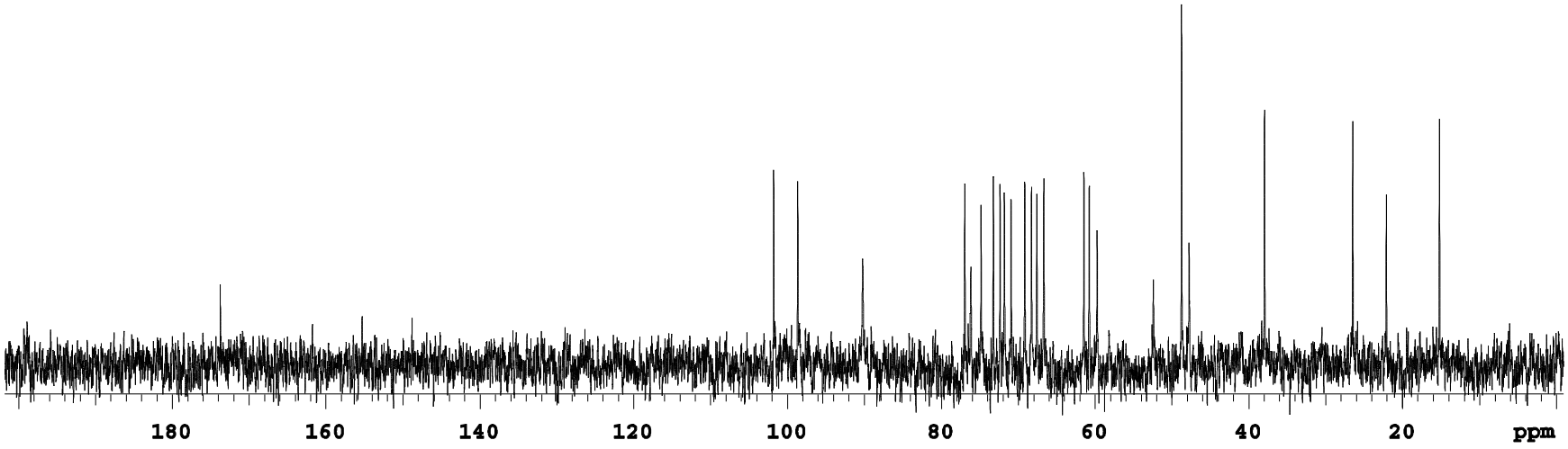

UV-VIS spectra of fluorescent Lewis^X glycodendron

Spectrum/Peak Report Date 5/26/2015 Time 18:00:44 Page 1 of 1

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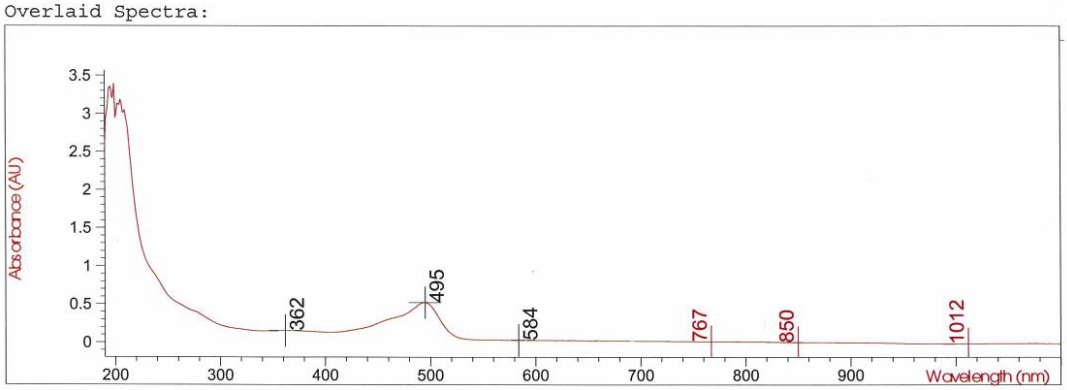

#	Name	Peaks(nm)	Abs(AU)	Valleys(nm)	Abs(AU)
1		495.0	0.52230	1012.0	-2.0363E-2
1		362.0	0.15248	850.0	1.0519E-3
1		584.0	2.7593E-2	767.0	8.9579E-3
2		495.0	0.52230	1012.0	-2.0363E-2
2		362.0	0.15248	850.0	1.0519E-3
2		584.0	2.7593E-2	767.0	8.9579E-3

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