Substrates of the Human Neuraminidase and Sialic Acid Esterase Enzymes

by

Neha Khanna

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Department of Chemistry
University of Alberta

Abstract

Neuraminidase enzymes (NEU) catalyze the cleavage of sialic acid residues from sialylated oligosaccharides, glycoproteins, and glycolipids. The human neuraminidase enzymes (hNEU) are a family of four isoenzymes (NEU1, NEU2, NEU3, and NEU4), which cleave terminal sialic acid groups (exo-sialidase). Members of the hNEU family are proposed play important roles in health and disease by controlling the composition of cellular sialosides. The membrane-associated enzyme, NEU3, is responsible for cleaving glycolipid substrates and plays critical roles in cell signaling. Although gangliosides, such as GM3, are known as substrates for NEU3, there are several uncommon natural analogs of this substrate found in human cells, including Neu5Gc and 9-*O*-Ac-Neu5Ac derivatives.

This thesis presents the synthesis and characterization of a series of GM3 analogs {Neu5Acα(2→3)Gal*β*(1→4)Glc*β*(1→1)cer} with an octyl aglycone and containing either Neu5Ac, Neu5Gc, or 9-*O*-Ac-Neu5Ac terminal residues. Furthermore, we generated each of these compounds with either an α(2→3)- or α(2→6)-glycosidic linkage. Additionally, to examine the role of the sialic acid esterase (SIAE) enzyme, which is responsible for degredation of 9-*O*-Ac-Neu5Ac residues, we developed a synthesis of a chloro-acetate analog of the esterase substrate, which will be studied as an inhibitor or label of the SIAE.

Preface

This thesis is an original work by the author, Neha Khanna. The enzymes (CMP-Neu5Ac synthetase, Sialic acid aldolase, α(2→3)-sialyltransferase, α(2→6)-sialyltransferase) required for chemoenzymatic reactions in Chapter 2 and 3 were provided by Ruixiang Zheng (Blake).

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Last but not least, my special thanks to my parents and my sister for relying on my decisions and their support.

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List of Schemes

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List of Abbreviations

ABPP	Activity-based protein profile
BCR	B-cell receptor
BOC	Butyloxy carbonyl
BOC_2O	Di-*t*-butyl dicarbonate
BzCl	Benzoyl chloride
CAM	Ceric ammonium molybdate
CD22	Cluster of Differentiation-22
CMAH	Cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase
CMP	Cytidine monophosphate
CoA	Coenzyme A
CuAAC	Cu-catalyzed azide- alkyne cycloaddition
CSS	CMP-sialic acid synthetase
D50	Asparatic acid 50
DANA	2,3-Didehydro-2-deoxy-N-acetylneuraminic acid
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	Dichloromethane
DIPEA	*N,N*-Diisopropylethylaminie
DIC	*N,N'*-Diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	*N,N*-Dimethylformamide

DMSO	Dimethyl sulfoxide
E225	Glutamic acid 225
EtOAc	Ethyl acetate
Et_3N	Triethylamine
Fuc	Fucose
FP	Fluorophosphonates
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GD	Disialoganglioside
GT	Trisialoganglioside
GDP	Guanosine diphosphate
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
GlcUA	Glycosaminoglycan
GM	Monosialodihexosylganglioside
HMBC	Heteronuclear multiple-bond correlation spectroscopy
hNEU	Human Neuraminidases
HOBt	1-Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
HSQC	Heteronuclear single-quantum correlation spectroscopy
KDN	2-Keto-3-deoxy-D-glycero-D-galactonononic acid
MALDI	Mass-assisted laser desorption ionization

Man	Mannose
ManGc	*N*-glycolyl-D-mannosamine
MeOH	Methanol
NA	Neuraminidase
NaOH	Sodium hydroxide
NaOMe	Sodium methoxide
NaCl	Sodium chloride
Na_2SO_4	Sodium sulphate
$NaHCO_3$	Sodium bicarbonate
Neu	Neuraminic acid
Neu5Ac	5-Acetamido-2-keto-3,5-dideoxy-D-glycero-D-galactonononic acid
Neu5Gc	*N*-glycolylneuraminic acid
NHS	*N*-Hydroxysuccinimide
NMR	Nuclear magnetic resonance
PMe_3	Trimethyl phosphine
PPh_3	Triphenyl phosphine
PSA	Polysialic acid
SG	Sialoglycoconjugates
SIAE	Sialic acid esterase
SIAT	Sialic acid transferase
SOAT	Sialic acid *O*-acyltransferase
TCEP	Tris(carboxyethyl)phosphine

TFAA	Trifluoroacetic anhydride
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
TOF	Time of flight
p-TsOH	*p*-Toluenesulfonic acid
p-TsCl	*p*-Toluenesulfonyl chloride
UDP	Uridine diphosphate
UV	Ultraviolet
Xyl	Xylose
Y370	Tyrosine 370

Chapter 1

The human neuraminidase and sialic acid esterase enzymes

1.1 Introduction

Carbohydrates are an essential structural component and source of energy for living cells. Carbohydrates are polyhydoxylated carbonyl compounds, consisting of mainly aldehyde (aldoses) and ketones (ketoses). Carbohydrate antigens (also called glycans) are expressed on the cell surface as components of glycoproteins, glycolipids, and gangliosides. Glycans are also collectively referred to as glycoconjugates. Glycoconjugates contribute significantly to fundamental biological functions, such as cell differentiation, cell adhesion, cell-cell interaction, pathogen-host recognition, toxin-receptor interactions, cancer metastasis, immune responses, and regulation of signaling pathways.[1] Glycoconjugates with terminal sialic acids are recognized by an enzyme, Neuramindase.

Neuraminidases are enzymes that hydrolytically cleave the sialic acid residues linked to various glycoconjugates.[2] Neuraminidase enzymes are interchangeably referred to as sialidase enzymes due to their substrates, which are known as sialic or neuraminic acids. Neuraminidases are widely distributed in nature, and are found in virus, fungal, protozoal, bacterial, avian, and mammalian species. However, the enzymes are absent in plant, yeast, and insects.[3,4] There are two primary forms of neuraminidases: a) exo-α-sialidase (*N*-acylneuraminyl glycohydrolases) and b) endo-α-sialidase (endo-*N*-acylneuraminidase).[2,5] Exo-α-sialidases hydrolyze the glycosidically linked terminal sialic acids from glycoconjugates including glycoproteins, oligosaccharides, glycolipids, colominic acid, and synthetic substrates. Endo-α-sialidases, on the other hand, hydrolyze the internal glycosidic linkages in polysialyated compounds.[5]

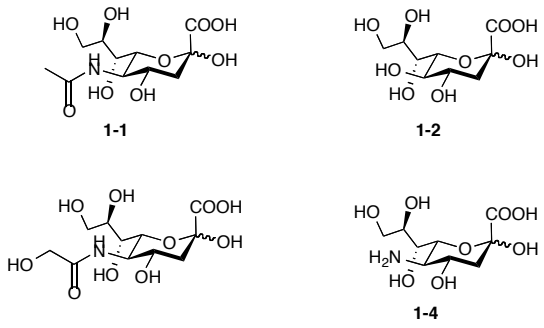
1.2 Sialic acids

Sialic acids are a family of α-keto acids with a nine-carbon backbone. These are also known as nonulsonic acids.[1] This nine-carbon chain of sialic acids is an exception to vertebrate glycoconjugates which are mainly composed of five and six-carbon sugars.[7] Sialic acids were first identified by Gunnar Blix and Ernst Klenk in the 1930s as a hydrolytic product of brain glycolipids or salivary mucins.[8] The word "sialic" is derived from the Greek word sialos meaning saliva (coined by Blix) while the term "neuraminic" is derived from neuro-, which refers to the neurological source of the glycolipids (coined by Klenk).[1] At physiological pH, sialic acids are negatively charged, which makes them ideal for ionic interactions with other charged species such as amino acids. In addition, sialic acids are easily accessible for intermolecular interactions as they are usually terminal sugars in glycoconjugates.[7]

The sialic acids are not a single compound but are a diverse family of more than 50 members of structurally distinct molecules.[9] Naturally occurring forms of sialic acids are *N*- or *O*-substituted derivatives of neuraminic acid (**1-1**, Neu) (**Scheme 1.1**), which itself is not found in nature. In fact, neuraminic acid is biosynthesized via condensation of neutral six-carbon unit *N*-acetyl-D-mannosamine with a three-carbon pyruvate in presence of sialic acid aldolase. The high-energy nucleotide sugar donor form of sialic acid is unusual in nature as compared to other vertebrate monosaccharides. Sialic acids are activated as cytidine mononucleotides, i.e., CMP-Sia, whereas other vertebrate monosaccharides are activated in the form of uridine or guanine dinucleotides, e.g., GDP-Man and -Fuc, UDP-Glc, -Gal, -GlcNAc, -GalNAc, -GlcUA, and –Xyl.[7]

The two most common representatives of sialic acids are 5-acetamido-2-keto-3,5-

dideoxy-D-glycero-D-galactonononic acid (**1-1**, *N*-acetylneuraminic acid, Neu5Ac or NANA) and 2-keto-3-deoxy-D-glycero-D-galactonononic acid (**1-2**, 2-keto-3-deoxynononic acid, KDN) (**Scheme 1.1**).[6] Neu5Ac is believed to be the biosynthetic precursor for all other members of sialic acid family.[10,11,12] The basic difference between Neu5Ac **1-1** and KDN **1-2** is the hydroxyl group at *C*5 position in KDN compared to *N*-acetyl group in Neu5Ac.[13] Hydroxylation of the 5-*N*-acetyl group of Neu5Ac gives another of the most commonly occurring member of sialic acids, *N*-glycolylneuraminic acid (**1-3**, Neu5Gc) (**Scheme 1.1**). Neuraminic acid (**1-4**, Neu), with an unsubstituted amino group, is considered to be derived from Neu5Ac via enzymatic deacetylation.[14]



Scheme 1.1 Structures of Neu5Ac (**1-1**), KDN (**1-2**), Neu5Gc (**1-3**) and Neu (**1-4**)

Some typical modifications of sialic acid include modification of the hydroxyl groups at *C*4, *C*7, *C*8, and *C*9 positions. Several other modifications such as acetylation, methylation, sulfonation, lactylation, methylation, and phosphorylation increase the diversity of sialic acids.[15,16] The linkage of sialic acid is another source of diversity. Sialic acids have three glycosidic linkages, α(2→3), α(2→6) and α(2→8), which are observed on several glycoproteins. The α(2→3) and α(2→6) is linked to β-D-galactopyranosyl (Gal) residues , α(2→6) to α-D-*N*-acetyl-galactosaminyl (GalNAc) and α(2→8) is linked

to other sialic acid residues in gangliosides. These linkages are generated by sialyltransferase enzymes such as ST3Gal, ST6Gal, ST6GalNAc, and ST8Sia. They are the four families that have been reported in humans,[17] and their specific activities are discussed in **Section 1.4.3**.

Several different characteristics of sialic acids make them a unique structural component of glycoconjugates. Sialic acids have been found to play critical roles in biological processes.[6] Due to the importance of sialic acids and their ubiquity in nature, there is great interest in the synthesis of Neu5Ac derivatives that can be either used as enzyme inhibitors or biological probes.[18,19,20]

1.3 Sialidases

Exo-sialidases hydrolyse the terminal silaic acid from glycoconjugates. In the hydrolytic mechanism the configuration of the starting material (sialoglycoside) is retained, which means if the starting glycoside has the α-configuration then the product that is formed will also have the α-configuration (**Figure 1.1**).[39]

The active site of sialidases contains a pair of carboxyl residues (D50, Asparatic acid 50 and E225, Glutamic acid 225), which play central roles in the enzyme's catalytic mechanism. The mechanism shown in **Figure 1.1**, involves the catalytic hydrolysis of a sialoside by NEU3 in four steps. In the first step, the dissocation of the reducing end of the saccharide occurs either via protonation of water or the D50 residue. Due to this dissosciation, an oxacarbenium intermediate is formed which is then attacked by the tyrosine 370 (Y370) nucleophile. This attack is assisted by neighbouring group participation of the E225 residue. Thus, the enzyme-sialic acid adduct formed is then

hydrolyzed through catalysis by D50 resulting the release the free α-sialic acid from the active site.[39]

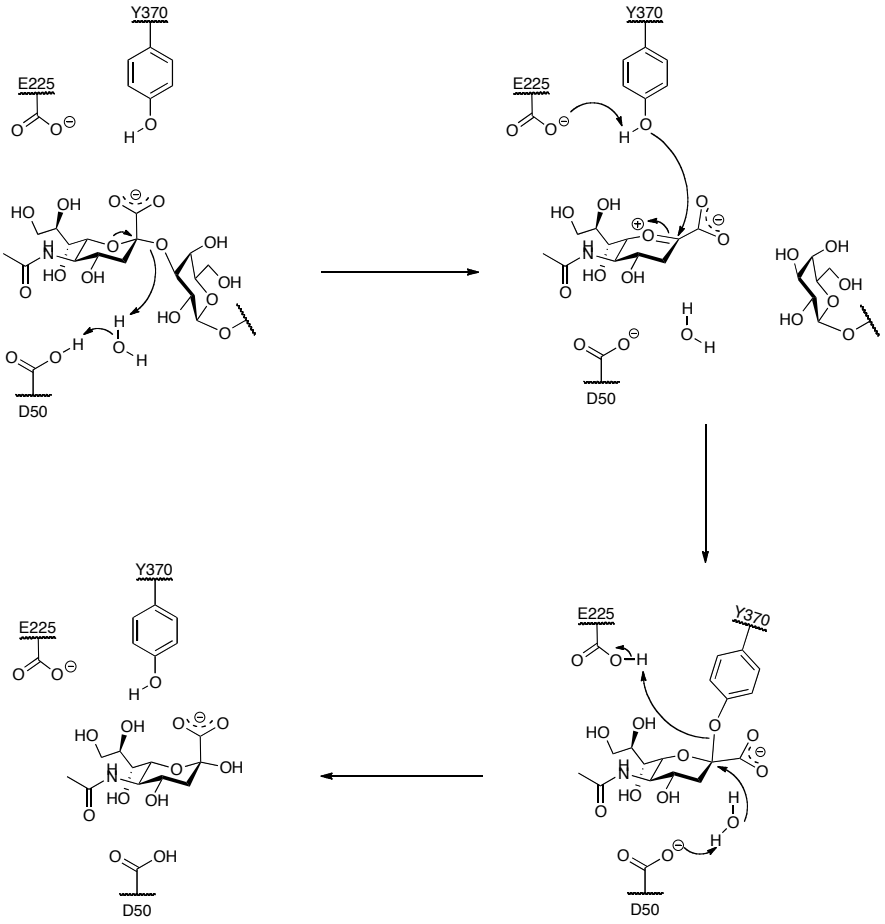


Figure 1.1 Proposed mechanism of substrate hydrolysis by NEU3

1.3.1 Viral sialidases

The surface of the influenza virus is composed of two main glycoproteins, hemagglutinin (HA) and neuraminidase (NA, also known as sialidase). There are 16 hemagglutinin (H1 to H16) and 9 neuraminidase subtypes (N1 to N9) that have been recognized in influenza.[21] These surface glycoproteins are also known as carbohydrate recognition proteins that target sialic acid. Viral hemagglutinin facilitates influenza

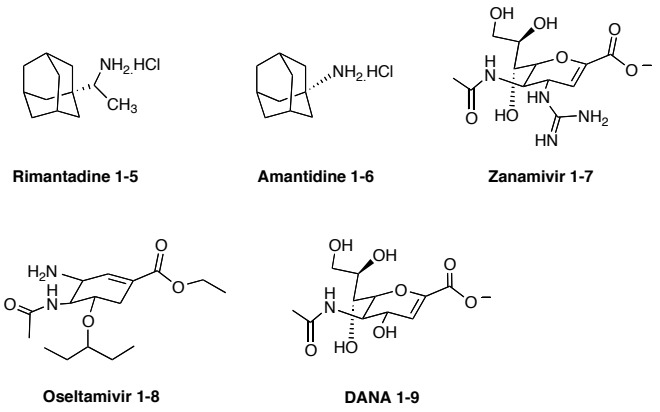
adhesion to target cell-surface glycoconjugates by recognizing terminal *N*-acetylneuraminic acid (Neu5Ac, **1-1**) residues on host cells.[22,23,24] HA facilitates the internalization process of the virus by fusing the viral envelope with the host cell membrane.[22,25,26] In comparison, the viral neuraminidase is an exoglycohydrolase, which contributes to the release of *N*-acetylneuraminic acid residues from both host cells and new viral glycoconjugates. This process allows the viral particles to migrate and invade new cells.[26] Thus, both viral hemagglutinin and neuraminidase are vital for the life cycle of the virus, and each of these surface glycoproteins are considered drug design targets.

1.3.2 Inhibitors of viral sialidases

The development of anti-viral drugs against influenza infection continues to be a major area of research in medicinal chemistry.[26] There are three classes of influenza viruses: influenza virus A, influenza virus B, and influenza virus C.[27] Out of these, influenza A and B are responsible for clinical influenza.[28] The most successful anti-influenza strategies have targeted the viral neuraminidase enzymes (vNEU), members of glycosyl hydrolase family 34, which cleave terminal *N*-5-acetyl-neuraminic acid (Neu5Ac) residues from host glycoproteins and glycolipids.[29]

The first generation anti-influenza neuraminidase inhibitors were Rimantadine (**1-5**) (**Scheme 1.2**) and its derivative Amantadine (**1-6**) (**Scheme 1.2**). These compounds specifically targeted influenza virus A.[30] The major drawbacks of these compounds were their lack of efficacy against influenza virus B strains, side effects, and rapid development of drug-resistant strains. These shortcomings have led to the design and discovery of a new generation of anti-influenza inhibitors.[26] Presently, two viral neuramindase inhibitors are used clinically, Zanamivir (**1-7**) and Oseltamivir (**1-8**)

(**Scheme 1.2**). The first example of structure-based designed inhibitor of neuraminidase was Zanamivir (**1-7**).[31] Later, a potent anti-influenza inhibitor with improved oral bioavailability Oseltamivir (**1-8**) was developed.[32,33] Both of these viral neuraminidase inhibitors were designed as transition-state mimics based on 2-deoxy-2,3-didehydro-*N*-5-acetylneuraminic acid (**1-9**) (DANA).[26]



Scheme 1.2 Structures of viral sialidase inhibitors

1.3.3 Human sialidases

Compared to the work done in developing the inhibitors of viral neuraminidase enzyme, very little work has been done to target their mammalian counterparts. The family of neuraminidase enzyme that are expressed in humans are responsible for cleavage and recycling of sialic acid residues of glycoproteins and glycolipids.[34] In 1933, mouse cytosolic sialidase was the first expressed and purified mammalian sialidase.[35] Based on the sequencing and order of cloning and expression in humans, these neuraminidase enzymes are classified as four different isoenzymes: NEU1, NEU2, NEU3 and NEU4.[4] Amongst these, only NEU2 has been crystallized.[36,37] The other enzymes have likely resisted structural analysis due to their hydrophobic content or, in the case of

NEU1, the requirement of a co-expression system. Due to this limitation, the design of specific inhibitors for these enzymes can be challenging. This problem can be partly overcome by using homology models for these enzymes based on the NEU2 crystal structure as a template. The first reported homology models for NEU1, NEU3 and NEU4 have been used for this purpose with limited success.[38] Our group has developed improved models of NEU3 and NEU4, which have been verified by more extensive modeling and site-directed mutagenesis.[39,40]

The human neuraminidase enzymes (hNEU) are present in different sub-cellular locations. NEU1 and NEU3 are found in plasma and lysosomal membranes of cells, NEU2 is found in the cytoplasm, and NEU4 is found in the lysosomal membrane and mitochondria.[3] In addition, hNEU enzymes also have tissue-specific expression. NEU1 is the most highly expressed sialidase with typically 10-20 times higher expression than NEU3 and NEU4. NEU2 is the lowest-expressed sialidase based on RT-PCR in human brain and lungs.[41] The human neuraminidases cleave $\alpha(2\rightarrow3)$, $\alpha(2\rightarrow6)$, and $\alpha(2\rightarrow8)$ linkage specificities. In general $\alpha(2\rightarrow6)$ linkages are found primarily in glycoproteins and $\alpha(2\rightarrow8)$ linkages are found primarily in glycolipids. The NEU1, NEU2, and NEU4 enzymes are known to hydrolyze glycoproteins, glycolipids and oligosaccharides, while NEU3 is specific to ganglioside substrates.[34,42,43] It has been reported that NEU2 cleaves both $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$ linkages, with preference of $\alpha(2\rightarrow6)$ over $\alpha(2\rightarrow3)$.[44] The hNEU enzymes also show some significant differences in optimal pH. NEU1, NEU3, and NEU4 are most active at approximately pH 4.5, whereas NEU2 is most active near neutral pH (5.5-6.5).[45]

Deficiency in the hNEU enzymes may lead to several diseases. For example,

deficiency of NEU1 results in lysosomal storage diseases.[46] Neuraminidase enzymes are also partly responsible for changes in metastasis and resistance to apoptosis of cancerous cells.[47,48,49] Due to the importance of hNEU enzymes in human health it is necessary to design inhibitors for these enzymes both as research tools, but also to explore potential therapeutic strategies where these enzymes may be overactive.

1.3.4 Inhibitors of human sialidases

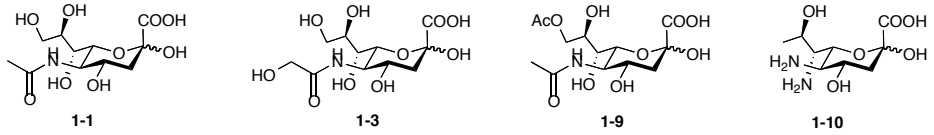
Structure-based drug design of inhibitors against hNEU enzymes has depended heavily on the crystal structure of NEU2.[37,44,51,52,53] The homology models have been used for the other three isoforms.[38,39]

Limited work has been done in testing the activity of inhibitors against the human sialidases as compared to viral sialidases. The known inhibitors of viral sialidases show weak potency against human enzymes.[41] DANA showed micromolar activity against NEU2, NEU3 and NEU4, while zanamivir was found to be a micromolar inhibitor of NEU2 and NEU3. On the other hand, oseltamivir was completely inactive against all of the hNEU enzymes. These results indicated that the *C*7– *C*9 binding pocket of the human enzymes are different from that of the viral enzymes. Some of the known anti-viral compounds have been tested against human neuraminidases.[37] For example, the derivatives of legionaminic acid (**1-10**, **Scheme 1.3**) have been tested against NEU2, which indicated limited inhibitory effects.[54] Synthetic derivatives of DANA with *C*9 modifications were active against both NEU1 and NEU3.[50,51] In 2012, Chen and coworkers reported that the modification at *C*9 and *N*5 positions of DANA with an azido group shows the best inhibitory effect with the highest potency against NEU2 and best selectivity over several other bacterial sialidases tested.[55]

Our group has reported highly selective inhibitors for NEU2 and NEU3 by modifying the *C*4 and *C*7 positions of DANA, respectively. These are the first reported compounds that have been confirmed to be selective for NEU2 and NEU3 over other hNEU.[56,131]

1.4 N-5 and O-9 modifications of Neu5Ac and their attachment with glycoconjugates

The family of sialic acids consist mainly of *N*- and *O*-substituted neuraminic acid (Neu5Ac).[12] Hydroxylation at *N*-5 position of Neu5Ac (**1-1**) gives Neu5Gc (**1-3**) whereas acetylation at *C*9 position gives 9-*O*-Ac-Neu5Ac (**1-9**) (**Scheme 1.3**). Neu5Gc is one of the less commonly expressed members of the sialic acid family in humans as is 9-*O*-Ac-Neu5Ac. The modifications of Neu5Gc and 9-*O*-Ac-Neu5Ac glycoconjugates and what is known of their effects on substrate activity are discussed in detail below.

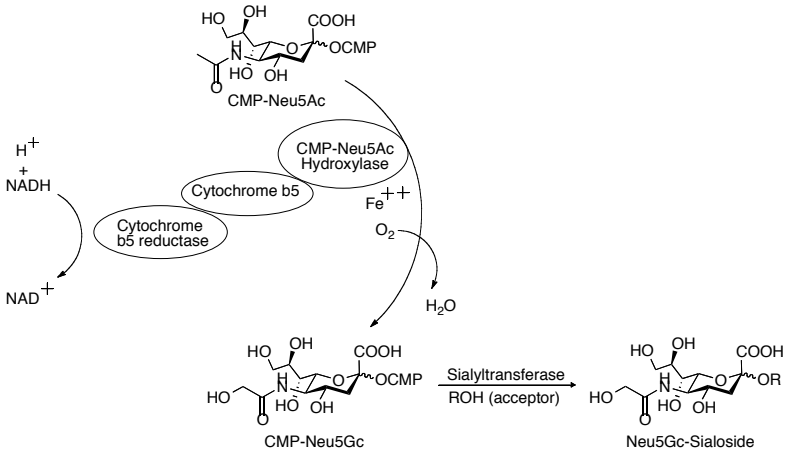


Scheme 1.3 Structures of Neu5Ac (**1-1**), Neu5Gc (**1-3**), 9-*O*-Ac-Neu5Ac (**1-9**) and Legionaminic acid (**1-10**)

1.4.1 N-Glycolylneuraminic acid (Neu5Gc)

The mammalian cell surface is covered with a complex range of sugars, which are often terminated by sialic acids. However, *N*-glycolylneuraminic acid (Neu5Gc) is not commonly found on the surface of human cells but is widely expressed in the tissues of many other mammals. As a result, this monosaccharide is sometimes referred to as non-human sialic acid.[57,58] Neu5Gc is different of Neu5Ac by only one additional oxygen

atom. The biosynthesis of Neu5Gc involves a modification from the CMP-sialic acid donor. The first step of biosynthetic incorporation of both sugars (Neu5Ac and Neu5Gc) involves conversion of free Neu5Ac to the corresponding cytidine-monophosphate-Neu5Ac-donor.[57] The biosynthesis of Neu5Gc results from modification of the Neu5Ac-nucleotide donor.[61-70] Schauer and coworkers discovered the hydroxylase/monooxygenase enzyme, CMP-Neu5Ac hydroxylase (CMAH) that catalyzes the transfer of one oxygen atom to CMP-Neu5Ac to generate CMP-Neu5Gc.[59,60] This conversion involves the usage of different cofactors such as cytochrome b5 and b5 reductase, iron (Fe), oxygen (O_2) and NADH (**Scheme 1.4**).[60-70]



Scheme 1.4 Biosynthetic pathway of Neu5Gc-sialoside from CMP-Neu5Ac

CMP-Neu5Gc acts as a donor to attach Neu5Gc to an acceptor (e.g. glycoproteins and glycolipids) through the action of a sialyltranferase enzyme. The product sialosides are involved in a broad range of biological processes such as intercellular adhesion, cell-signaling and microbial attachment. Investigating the roles of such sialosides in vertebrates has improved our understanding of normal physiology, disease and human evolution. Chen and coworkers have reported the activity of Neu5Gc galactosides as

substrates for human NEU2 and bacterial neuraminidases.[71,72] Substrate activity studies can be useful in designing selective sialidase inhibitors against hNEU2.[71] To date, the substrate activity of Neu5Gc sialosides has not been tested against any other human neuraminidase isoenzymes.

In comparison to other mammals, such as rodents and ungulates, human tissues are deficient in Neu5Gc.[73-75] In the 1970's, researchers investigated the role of Neu5Gc as a foreign antigen to humans when they observed immune responses against horse serum used for clinical treatments, which was found to be enriched with Neu5Gc.[76-78] The lack of Neu5Gc in human tissue is due to an inactivating mutation in the gene encoding CMP-*N*-acetylneuraminic acid hydroxylase (CMAH).

The consequences of CMAH gene inactivation in humans is the loss of Neu5Gc and accumulation of its metabolic precursor, Neu5Ac. Secondly, pathogens that bind to Neu5Ac would be more able to infect human cells, whereas pathogens that bind to Neu5Gc would not be able to infect humans. This change in the glycan structures of humans could also affect the function of sialic acid receptors in the immune system. Mutation of the gene encoding CMAH in humans has been proposed to play a role in human brain evolution from other mammals.[57,58]

Despite the lack of activity in CMAH in humans, Neu5Gc can be found in human tumors, fetal tissues, and in some cultured cell lines of human origin.[79-91] One reason for this observation may be the metabolic incorporation of Neu5Gc into human tissue from dietary sources of animal origin. Beef, pork, and lamb are rich sources of Neu5Gc. Cow's milk and fish products have lesser amounts of Neu5Gc whereas plants and poultry do not contain any Neu5Gc.[92] The non-human sialic acid, Neu5Gc, may play important roles in

human cancer. Recently, it has been reported that incorporation of Neu5Gc from food can lead to cancer initiation and progression as well as other inflammatory diseases. Thus, reducing the consumption of Neu5Gc containing food could prevent malignant diseases and cardiovascular disorders.[92]

1.4.2 9-O-Acetylneuraminic acid (9-O-Ac-Neu5Ac)

Among the sialic acid family, the most frequently occurring modification is *O*-acetylation. The *O*-acetylation can occur at the *C*7, 8, 9 positions of neuraminic acid to form *N*-acetyl-7,8, or 9-*O*-acetyl neuraminic acids. The family of *O*-acetylated sialoglycoconjugates are also abbreviated as O-AcSGs.[93,94] Under physiological conditions, *O*-acetyl esters at the *C*7 and *C*8 positions are not very stable and thus spontaneously migrate to the *C*9 position of neuraminic acid, which is the most common biologically occurring modification.[95,96] These *O*-AcSGs are involved in various physiological and pathological processes such as cell signaling, cell-cell adhesion, cell differentiation and metastasis.[95]

The *O*-acetylated sialoglycoconjugates are formed after activation of the sialic acid derivative, 9-*O*-Ac-Neu5Ac. The activated form of 9-*O*-Ac-Neu5Ac, cytidine monophosphate-9-*O*-Ac-Neu5Ac (CMP-9-*O*-Ac-Neu5Ac), acts as a donor to react with acceptor (e.g. glycoproteins and glycolipids) in the presence of a sialyltransferase. The linkage between the oligosaccharide chain of the glycoconjugate and sialic acid can be α(2→3), α(2→6), or α(2→8). Generally, the most common accepting subterminal sugar is galactose (Gal) or N-acetyl galactosamine (GalNAc), or less commonly N-acetyl glucosamine (GlcNAc).[97]

The *O*-acetylated sialoglycoconjugates can be formed by using *O*-acetyl

transferase enzymes. These enzymes catalyze the transfer of the acetyl group from acetyl-coenzyme A (CoA) onto sialoglycoconjugates at the *C*7, 8, or 9 positions.[96,98] However, the acetyl group is first inserted on the *C*7-OH group; from there it migrates to the *C*9 position non-enzymatically, presumably via an intermediate *C*8 *O*-Ac.[94,96] The *O*-acetyl esters can be removed from *O*-acetylated sialoglycoconjugates by using an esterase.[99,100]

The quantification of 9-*O*-acetylated sialoglycoconjugates (9-*O*-AcSGs) is often done by enzymatic or chemical hydrolytic cleavage. However, these methods may result in incomplete release of sialic acids, de-*O*-acetylation, and spontaneous migration of *O*-acetyl groups.[97] Earlier probes for detection of 9-*O*-AcSGs involved a variety of sialic acid-binding lectins or monoclonal antibodies.[91,101-106] But now these sialoglycoconjugates can be quantified by using MALDI-TOF mass spectrometry techniques.[107]

The accessibility of silaoglycoconjugates at the periphery of the cell surface makes them well suited to serve as molecular determinants of various biological processes.[97] The 9-*O*-AcSGs are involved as differentiation markers in developmental processes, inhibition of binding, invasion of malarial parasites, and also in protection against sialidases.[95,108,109,110] The acetylation of the hydroxyl group at *C*9 results in enhancing haemolysis.[97] The 9-*O*-AcSGs plays a regulatory role in preventing undesirable interactions of CD22 with targets in humans by masking the binding of the terminal sialic acid residues to human CD22.[111] As a consequence of this, 9-*O*-AcSGs prevents the initiation of CD22-dependent cellular responses, such as cell proliferation and differentiation, immune responses, and metastasis leading to a loss of host defense and immunosuppression.[97] Despite their importance in various biological processes, the

substrate activity of these 9-*O*-AcSGs against human neuraminidases enzyme has not been studied. If these variants of sialic acid play such important biological roles, any enzymes that modify them will act as regulators. The identification of differential activity among hNEU isoenzymes may help elucidate the roles of AcSGs and the enzymes that interact with them.

1.4.3 Linkage between sialic acid derivatives and various glycoconjugates

Sialic acids have glycosidic linkages α(2→3) or α(2→6) to β-D-galactopyranosyl (Gal) residues, or α(2→6) to α-D-*N*-acetyl-galactosaminyl (GalNAc) or α-D-*N*-acetylglucosaminyl (GlcNAc) residues. Sialic acids are also found in α(2→8)-linkages to other sialic acid residues in gangliosides and in polysialic acid (PSA), which is a linear α(2→8)-homopolymer observed on several glycoproteins. This linkage is generated by sialyltransferase enzymes, and further expands the diversity of sialic acid residues on cell-surface glycoconjugates.[17,112,115] Sialyltransferases are a family of glycosyltransferases that catalyze the transfer of sialic acid from an activated sugar donar, cytidine mononucleotides-sialic acid (CMP-SA) onto the terminal non-reducing end of oligosaccharide chains of glycoproteins and glycolipids. The reaction takes place via nucleophilic displacement of CMP with inversion of configuration at the anomeric center.[113]

Twenty sialyltransferase enzymes are thought to be required for the synthesis of all known sialoglycoconjugates, which show high specificity for the anomeric linkage between sugar donar as well as oligosaccharide acceptor. Only 15 sialyltransferases have been cloned from animal sources to date.[112,113,114] Sialyltransferase genes may be differentially expressed in a tissue-, cell type-, and stage-specific manner. These enzymes

differ in their substrate specificity, tissue distribution and various biochemical parameters; however, one linkage can be synthesized by multiple enzymes.[114] In spite of their essential roles in the recognition and biosynthesis of specific sialylated oligosaccharide chains, so far there is only limited information available on their structures, substrate specificities, and recognition processes, and on the cellular mechanisms involved in the regulation of their transcriptional expression.[112,114]

1.5 Sialic acid acetyl esterase (SIAE) enzyme

The two enzymes that regulate the *O*-acetylation of sialic acids are Sialic acid transferase (SIAT) and Sialic acid acetylesterase (SIAE) (**Figure 1.2**). The role of SIAT is described above in section **1.4.3**. Sialic acid acetyl esterase was originally characterized by Varki and coworkers, and specifically removes *O*-acetyl esters from naturally occurring sialic acids.[116,117]

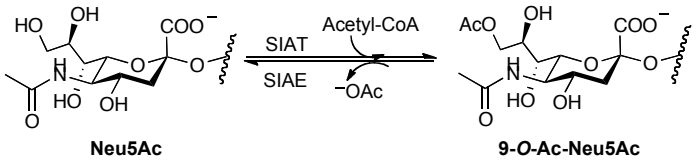


Figure 1.2 Regulation of *O*-acetylation of sialic acids

This *O*-acetylation and de-*O*-acetylation of sialic acid modification is an important factor in determining the peripheral structure of *N*-linked oligosaccharides.[99] These esters have been involved in cell adhesion, lectin recognition, tissue morphogenesis, and several other biological phenomena such as tumor antigenicity, and complement activation.[12,95,118] The activity of SIAE has been described in both viruses and vertebrates.[117]

Sialic acid acetylesterase is thought to be a key regulator of B cell tolerance. The B cell receptor (BCR) is negatively regulated by the CD22 co-receptor. The *cis* sialosides (found on the same cell surface) and *trans* sialosides (found on adjacent cell surface) are basically responsible for this activation process.[119] *O*-Acetylation of sialic acid antigen has been recognized as the mechanism that inhibits the interaction of sialosides with CD22 and can contribute to autoimmunity.[111,120,121] Thus, inhibition of SIAE could increase the concentration of acetylated sialic acid, which will not interact with CD22 and could result in activation of the BCR (**Figure 1.3)**. Studies in animal models have suggested that these enzymes are required for maintenance of immunological tolerance in mice.[121,122]

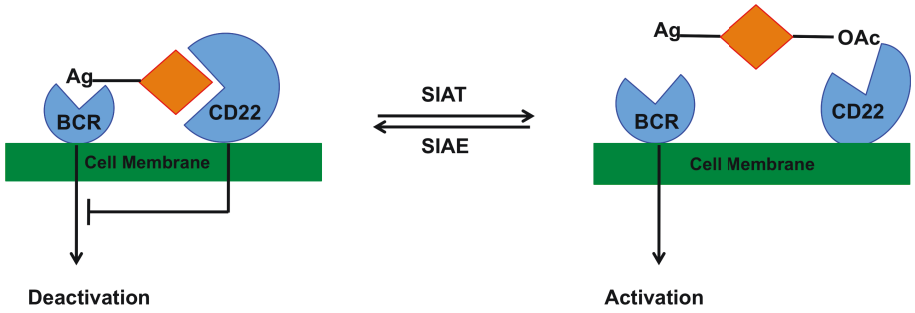


Figure 1.3 BCR negatively regulated by CD22 co-receptor. Deacetylated antigen sialosides binds with CD22 and hence deactivate BCR whereas acetylated antigen sialosides doesnot binds with CD22 and thus results in activation of BCR

Cravatt and coworkers have reported the non-specific labeling of SIAE using organophosphorus reagents.[123] Fluorophosphonates (FPs) are well-characterized affinity labels for serine hyrolases.[124,125] The SIAE was identified using irreversible FPs to label and sequence the protein as a novel member of the serine hydrolase superfamily.[123] The

Sialic acid acetylesterase – fluorophosphonates (SIAE-FP) interactions were characterized by using a gel-free version of ABPP (activity-based protein profiling). This ABPP enables probe-labeling sites for enzyme determination in complex proteomes.[126,127] These organophosphorus reagents are however non-specific, and are only suitable in vitro use and would not be useful for cell-based experiments due to their lack of specificity.

1.6 Project Objectives

Members of the human neuraminidase enzyme (hNEU) family are proposed to play important roles in health and disease by controlling the composition of cellular sialosides. Thus, there is a need to explore the substrate specificity of the human neuraminidase enzymes in order to design selective inhibitors and to understand their native activity. Although gangliosides, such as GM3, are known as substrates for human NEU3, there are uncommon natural analogs found in human cells with unknown substrate activity. The most notable of these include Neu5Gc and 9-*O*-Ac-Neu5Ac derivatives. We hypothesized that these modifications of the Neu5Ac residue could alter the substrate activity of glycolipids and glycoproteins for hNEU. In this thesis, we test this hypothesis by synthesizing GM3 analogs that contain these modifications.

In previous work, our group has reported that a hydrophobic aglycone is required for substrate recognition by the NEU3 enzyme.[128] More hydrophobic groups (logP > 4) were the best substrates for NEU3. In fact, octyl glycosides were of comparable activity as the native sphingolipids. Thus, GM3 analogs that contain an octyl chain are expected to be good substrates for NEU3 and are much simpler to generate. It was also found that large modifications at the *C*9 and *N*5 positions of Neu5Ac inhibited NEU3 activity.[128]

This finding suggests that naturally occurring modifications at *C*9 (9-*O*-AcNeu5Ac) and *N*5 (Neu5Gc) may also have reduced NEU3 activity.

There is limited information about the influence of different glycosidic linkages on the activity of hNEU. Chen and coworkers have studied the substrate activity of a Neu5Gc galactoside with several bacterial neuraminidases and the human cytosolic neuraminidase enzyme, NEU2.[71,72] In general, the α(2→3) analogs of Neu5Gc sialosides showed 2-4 fold higher activity as compared to the α(2→6) linked sialosides.[71] However, the substrate activity of Neu5Gc sialosides have not been tested against any other human neuraminidase enzymes.

Considering these results, we planned to synthesize a panel of trisaccharide analogs of GM3 containing modifications of the Neu5Ac residue, including Neu5Gc, 9-*O*-Ac-Neu5Ac, and both α(2→3) and α(2→6) glycosidic linkages (**Figure 1.4**). Enzymatic assays of these compounds with human neuraminidase isoenzymes (NEU1, NEU2, NEU3 and NEU4) would provide crucial insights into the role of these modifications in biological systems.

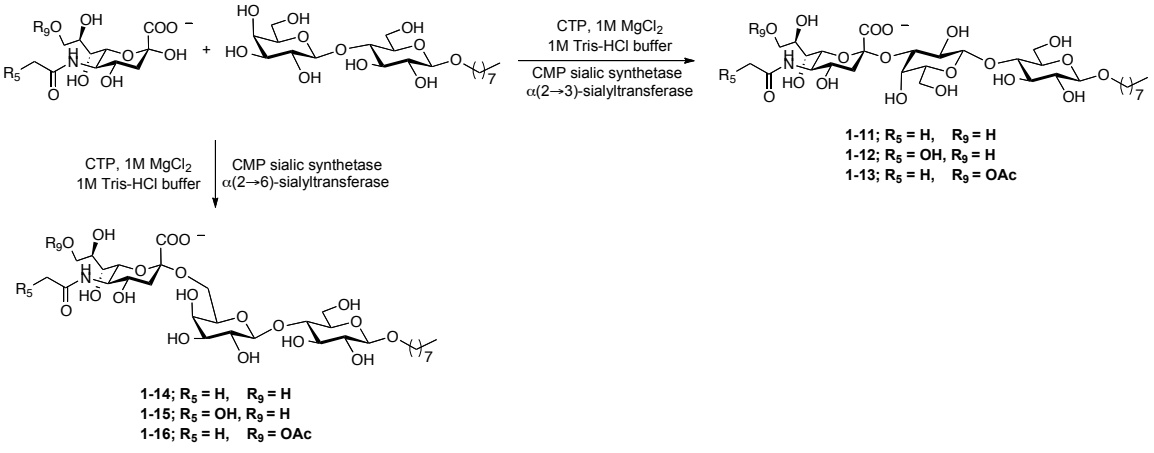


Figure 1.4 Structure of GM3 target analogs

The regulation of the 9-*O*-Ac modifications of sialic acid are a growing area of biological interest. The activity of the sialic acid esterase (SIAE) may be important for the study of autoimmune disease.[111,120,121] We designed candidate inhibitors of SIAE based on *C*-9-α-halo-esters of Neu5Ac. The α-halo ketones have been used as specific covalent inhibitors for other esterase enzymes.[129,130] We develop synthetic methods to obtain the chloro-acetate analog of the esterase substrate, which can also incorporate a tag to act as a probe to allow detection of SIAE (**Figure 1.5**).

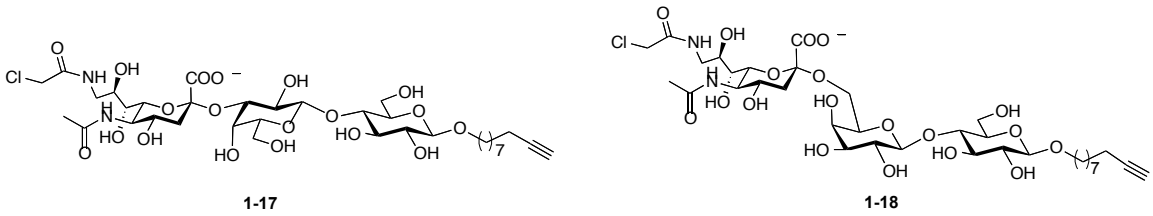


Figure 1.5 Proposed inhibitor of SIAE

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Chapter 2

GM3 Analogs for the exploration of substrate activity in the human neuraminidase (hNEU) enzymes

2.1 Introduction

The human neuraminidase enzymes are a family of glycosyl hydrolases that catalyze the hydrolytic cleavage of terminal sialic acid residues from sialylated oligosaccharides, glycoproteins, and glycolipids. These enzymes are widely distributed in nature, such as in viruses, fungi, protozoan, bacterial, and avian and mammalian species.[1] Dysfunction of these enzymes can lead to disorders of sialic acid storage, such as type I and type II sialidosis.[2,3] The human neuraminidase enzymes also play important roles in health and disease. Some of these roles are essential in cell function such as immune cell activation, cellular communication, signaling, adhesion, apoptosis, and metastasis of malignant cells.[4-15]

In humans, the known members of the human neuraminidase (hNEU; also known as sialidases) enzyme family include four isoenzymes (NEU1, NEU2, NEU3, and NEU4). All these enzymes are classified as exo-sialidases (EC 3.2.1.18) and are members of glycoside hydrolase family 33 in the CaZy database.[16] Out of these enzymes, NEU3 is known to be a peripheral membrane-associated enzyme with activity at the outer leaflet.[17-19] The NEU3 isoenzyme is selective for hydrolytic cleavage of glycoproteins and glycolipids over small soluble molecules. This suggests that its basic function is to process substrates that are bound to the membrane.[20] The NEU3 sialidase also shows a substantial preference for ganglioside substrates (such as glycolipids) over glycoproteins.[21]

Glycolipids are essential components of the plasma membrane and the specificity of NEU3 for these substrates could play an important role in mediating membrane signaling.[18] The specificity of NEU3 for commonly occurring natural ganglioside

substrates has been examined in previous studies. Many groups have observed that this enzyme hydrolyses α(2→3) sialosides (e.g., GM3, GD1a, and GT1b) and α(2→8) sialosides (e.g., GD3, GD1b, and GT1b). [6,11,21-25] Previous studies show that α(2→3) sialylated glycolipids with a branch point at the adjacent galactose residue e.g, Neu5Acα(2→3)[Gal*β*(1→3)GalNAc*β*(1→4)]Gal*β*(1→4)(Glc*β*(1→1)cer, as found in GM1 and GM2, are poor substrates for NEU3.[21-23]

The most common natural modifications of glycoconjugates are Neu5Gc and 9-*O*-acetyl-Neu5Ac. Neu5Gc is a modification of Neu5Ac with hydroxylation at the *N*-5 acetyl position, whereas 9-*O*-Ac-Neu5Ac is acetylated at the *C*-9 position of Neu5Ac (**Scheme 1.3**). In humans, both Neu5Gc and 9-*O*-Ac-Neu5Ac are one of the less commonly expressed members of the sialic acid family. The modifications of Neu5Gc and 9-*O*-Ac-Neu5Ac glycoconjugates and what is known of their effects on substrate activity are discussed in detail below.

The substrate activity of Neu5Gc galactosides against human NEU2 and bacterial neuramindases have been reported by Chen and coworkers.[27,28] These substrate activity studies can be useful in designing selective neuraminidase inhibitors against NEU2.[27] NEU2 is known to preferentially cleave α(2→3) linkages but also cleaves α(2→6) and α(2→8) sialosides.[26, 27]

The *O*-acetylation of Neu5Ac at *O*9 is the most commonly observed form of acetylation. The *O*-acetyl ester formation at the *C*-7 and *C*-8 positions of Neu5Ac are not very stable, and hence spontaneously migrate to *C*-9 position of Neu5Ac under physiological conditions.[29,30] Earlier strategies for detection of 9-*O*-Ac-sialoglycoconjugates have used a variety of sialic acid-binding lectins or monoclonal

antibodies.[31-37] Newer methods exploit MALDI-TOF mass spectrometry techniques for quantification of sialoglycoconjugates.[38] The 9-*O*-Ac-sialoglycoconjugates have been proposed to prevent the initiation of CD22-dependent cellular responses, such as cell proliferation and differentiation, immune response, and metastasis leading to a loss of host defense and immunosuppression.[39] Despite their importance in these biological processes, the substrate activity of these 9-*O*-Ac-sialoglycoconjugates with human neuraminidase enzymes has not been studied in detail.

Our group has developed a recombinant expression system for NEU2, NEU3, and NEU4.[40] This recombinant expression system has been used to explore the substrate tolerance for these enzymes.[40] Moreover, our group has examined the role of the lipid aglycone in the substrate recognition of NEU3. In these studies, it was observed that NEU3 required the presence of the hydrophobic aglycone for substrate activity. More hydrophobic groups (those having a logP > 4) were excellent substrates (k_{rel} > 0.9). Sandbhor *et al.* reported that modifications at the *C*9 or *N*5-Ac position of Neu5Ac with small groups in a trisaccharide analog of GM3 (*β*-octyl sialosides) were generally good substrates for NEU3.[41] However, larger modifications and charged groups did inhibit NEU3 activity. These results with synthetic glycans led us to consider whether native modifications of the glycolipid substrates of NEU3 could have significant effects on enzyme activity.

There are limited reports describing hydrolytic cleavage by hNEU of synthetic glycans. Chen and coworkers have reported that the α(2→3) analogs of Neu5Gc sialosides generally showed 2-4 fold higher activity as compared to the α(2→6) linked

sialosides.[27] However the activity of Neu5Gc as a substrate has not yet been tested against any other hNEU.

Considering these results, we hypothesized that Neu5Gc and 9-*O*-Ac-Neu5Ac analogs of glycolipids, such as GM3 (**Figure 2.1**), could have reduced substrate activity for NEU3. To explore this possibility, we set out to synthesize a panel of uncommon natural analogs of GM3, including Neu5Gc and 9-*O*-Ac-Neu5Ac analogs of GM3. These substrates were designed to include the required hydrophobic aglycone for substrate activity, and should be ideal compounds for testing with human neuraminidase isoenzymes (NEU1, NEU2, NEU3 and NEU4).

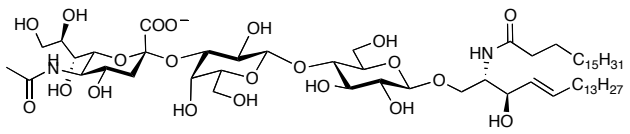


Figure 2.1. Structure of GM3

The following trisaccharide analogs of GM3 containing modifications of the Neu5Ac residue, including Neu5Gc, 9-*O*-Ac-Neu5Ac, with both α(2→3) and α(2→6) glycosidic linkages were synthesized (**Figure 2.2**).

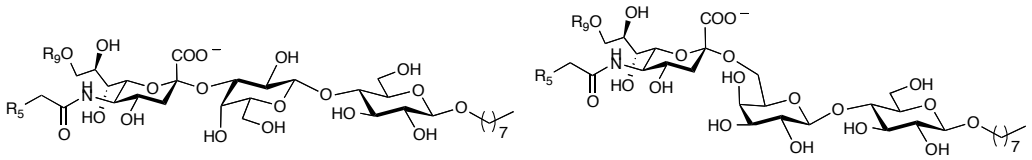
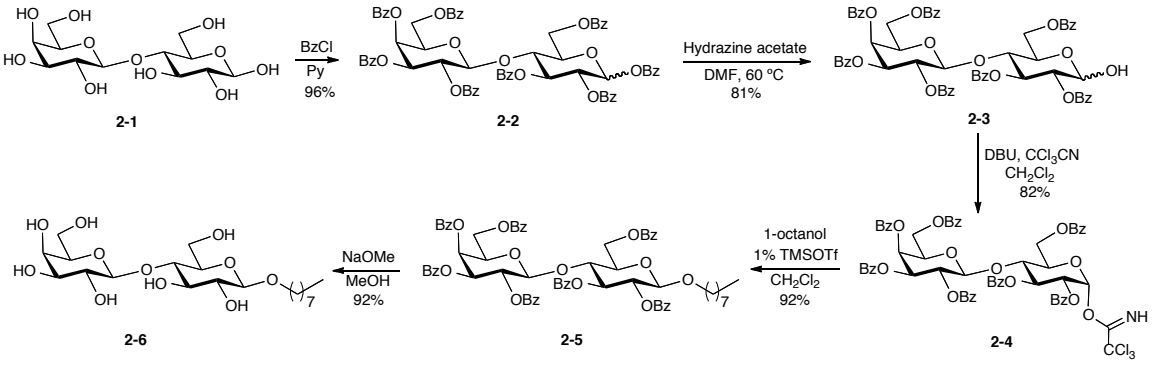


Figure 2.2. Structure of GM3 target analogs

2.2 Results and Discussion

2.2.1 Synthesis of β-octyl-lactoside

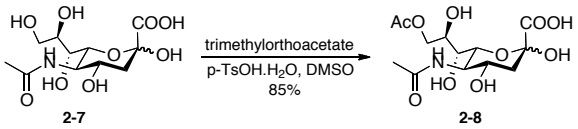
We generated glycolipid analogs of GM3 starting from *β*-octyl-lactoside following a previously reported strategy (**Scheme 2.1**).[41] Lactose is a commercially available naturally occurring disaccharide with a *β*(1→4) glycosidic linkage between galactose and glucose. As illustrated in **Scheme 2.1**, the 8-OH groups of *β*-lactose (**2-1**) were protected as benzoyl (OBz) esters by treatment with benzoyl chloride (BzCl) in pyridine to furnish compound **2-2** in 96% yield. The selective deprotection of the anomeric benzoyl group was achieved using hydrazine acetate at 60 ºC to give **2-3** in 81% yield. Compound **2-3** was then treated trichloroacetonitrile in the presence of 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) to give only the *β*-anomer of **2-4** in high yield (82%). The perbenzoylated lactosyl trichloroacetimidate glycosyl donor (**2-4**) underwent glycosylation with 1-octanol as an acceptor under acidic conditions to yield benzoyl protected *β*-octyl-lactoside (**2-5,** 92% yield). Finally, the benzoyl protecting groups were removed using freshly prepared sodium methoxide to provide the *β*-octyl-lactoside (**2-6,** 94% yield).[41]



Scheme 2.1 Synthesis of *β*-octyl-lactoside

2.2.2 Synthesis of 9-O-Ac-Neu5Ac

The 9-*O*-acetyl-sialic acid (**2-8**; 9-*O*-Ac-Neu5Ac) was synthesized in one step based on a known protocol (**Scheme 2.2**).[42] Sialic acid (**2-7**) was reacted with trimethylorthoacetate in the presence of a catalytic amount of *p*-TsOH in 85% yield. This is a very efficient method for substituting groups at 9-*O*-position of sialic acid in a single step without the use of protecting groups.

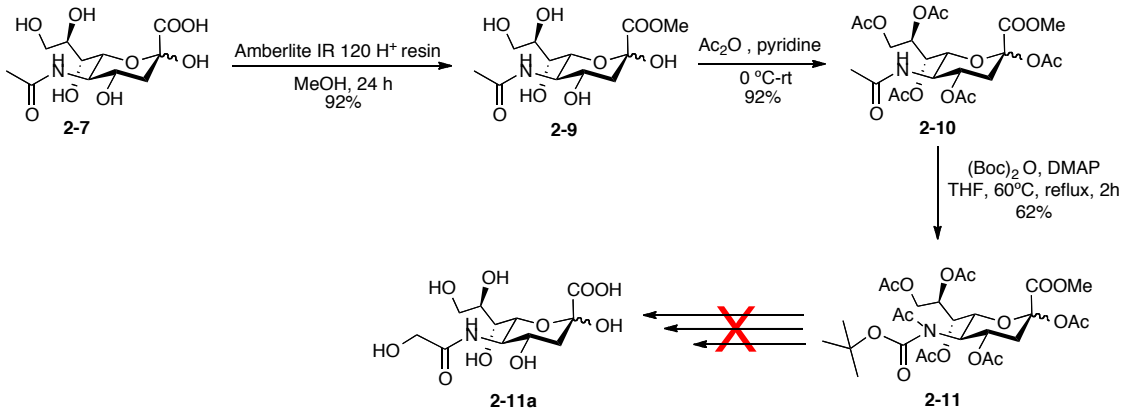


2-7 **2-8**

Scheme 2.2 Single step synthesis of 9-*O*-Neu5Ac

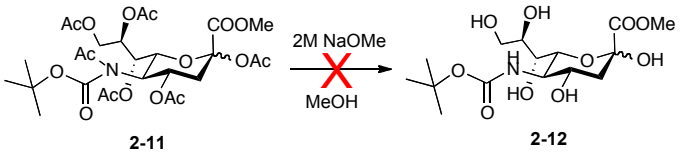
2.2.3 Synthesis of Neu5Gc

Earlier reported syntheses of Neu5Gc were based on ManGc and exploited a chemoenzymatic conversion to Neu5Gc.[43] In order to avoid the usage of enzymes, we first approached the synthesis of Neu5Gc from commercially available sialic acid (**2-7**). Sialic acid (**2-7**) was converted to its methyl ester (**2-9)** using Amberlite®-IR 120 H^+ resin in 92% yield and further acetylated using acetic anhydride with pyridine as a solvent to give **2-10** in 92% yield (**Scheme 2.3**)[44]. The NHAc group of **2-10** was then protected as t-butylcarbamate under refluxing conditions using Boc anhydride, 4-dimethylaminopyridine (DMAP) at 60 ºC to furnish **2-11** in 62% yield.



Scheme 2.3 Approach towards synthesis of Neu5Gc

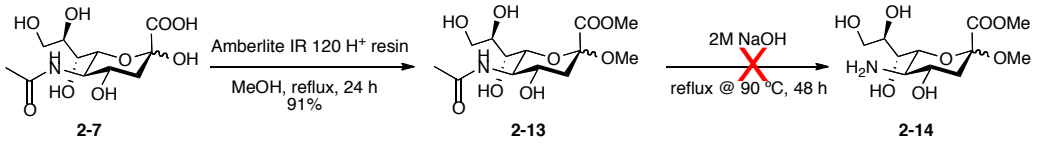
Global deprotection of the acetyl groups of **2-11** was attempted using freshly prepared NaOMe (2 M) in dry MeOH (**Scheme 2.4**). However, the target compound, **2-12**, was not obtained under these conditions; instead, we observed removal of all O-Ac groups while the N-Ac group was preserved.



Scheme 2.4 Deacetylation of *C*5-amino group of sialic acid

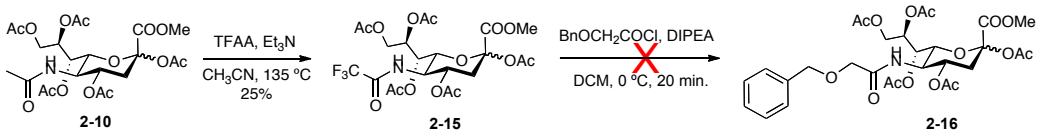
We also attempted deprotection of the N-Ac group using more harsh conditions (**Scheme 2.5**). For this, sialic acid (**2-7**) was converted to the *C*1-methyl ester (**2-13**) under refluxing conditions using Amberlite®-IR 120 H^+ resin in 91% yield. We then attempted *N*-deacetylation of compound **2-13** using 2M NaOH under reflux for 48 h at 90 ºC to afford **2-14**. Unfortunately, this method was unsuccessful and we could not detect

the formation of the expected product. We suspect that these conditions resulted primarily in decomposition of the starting material based on TLC observations.



Scheme 2.5 Attempted *N*-deacylation of sialic acid using harsh conditions

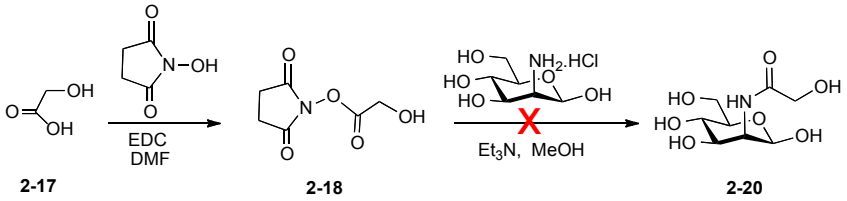
Thus, we changed our strategy and prepared a more electrophilic acetate derivative, **2-15** from **2-10**. We hoped that this group could be more easily displaced to allow formation of benzyloxyacetyl derivative **2-16**, which could then be used to obtain the desired Neu5Gc target. The required *N*-trifluoroacetamido derivative **2-15** was synthesized from **2-10** using trifluoroacetic anhydride (TFAA) in presence of Et_3N in low yield (25%) (**Scheme 2.6**).[45] Compound **2-15** was subjected to a nucleophillic displacement reaction by benzyloxyacetyl chloride in the presence of *N,N*-diisopropylethylamine (DIPEA). We obtained a mixture of inseparable compounds apparently derived from a rearrangement and polymerization of **2-15**, leading us to conclude that this strategy could not be used to obtain the desired product, **2-16**.



Scheme 2.6 Another approach for synthesizing Neu5Gc

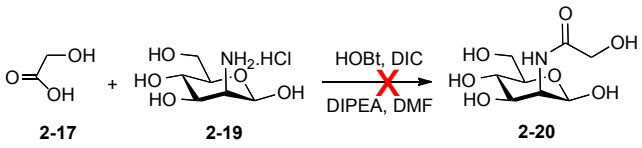
As we were unable to obtain our expected product, we moved to a chemoenzymatic strategy for making Neu5Gc.[43] These methods involve derivatized mannosamine hydrocloride, which can then be converted to Neu5Gc enzymatically. We

first prepared ManGc (**2-19**, *N*-glycolyl-D-mannosamine), from glycolic acid (**2-17**) and its corresponding NHS ester (**2-18**) (**Scheme 2.7**).[43] The NHS ester, **2-18**, was then treated with mannosamine hydrochloride in the presence of Et_3N as a base to give **2-20** as the desired product. Unfortunately, we obtained only starting material (observed by TLC). This result is likely due to the *in situ* decomposition of the NHS ester (**2-18**) to the free acid.



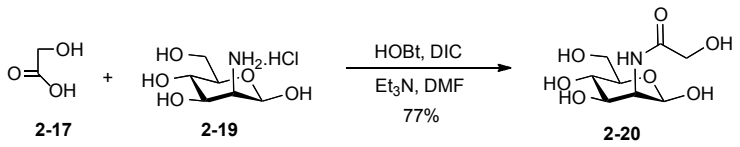
Scheme 2.7 Approach towards synthesis of ManGc

We optimized this strategy by incorporating coupling reagents, such as 1-hydroxybenzotriazole (HOBt) and *N,N′*-diisopropylcarbodiimide (DIC) (**Scheme 2.8).** The reaction mixture was initially stirred for 12 h, and was then refluxed at 150 ºC for 12 h. The coupling remained inefficient, and only starting material was obtained.



Scheme 2.8 Attempt of synthesizing of ManGc

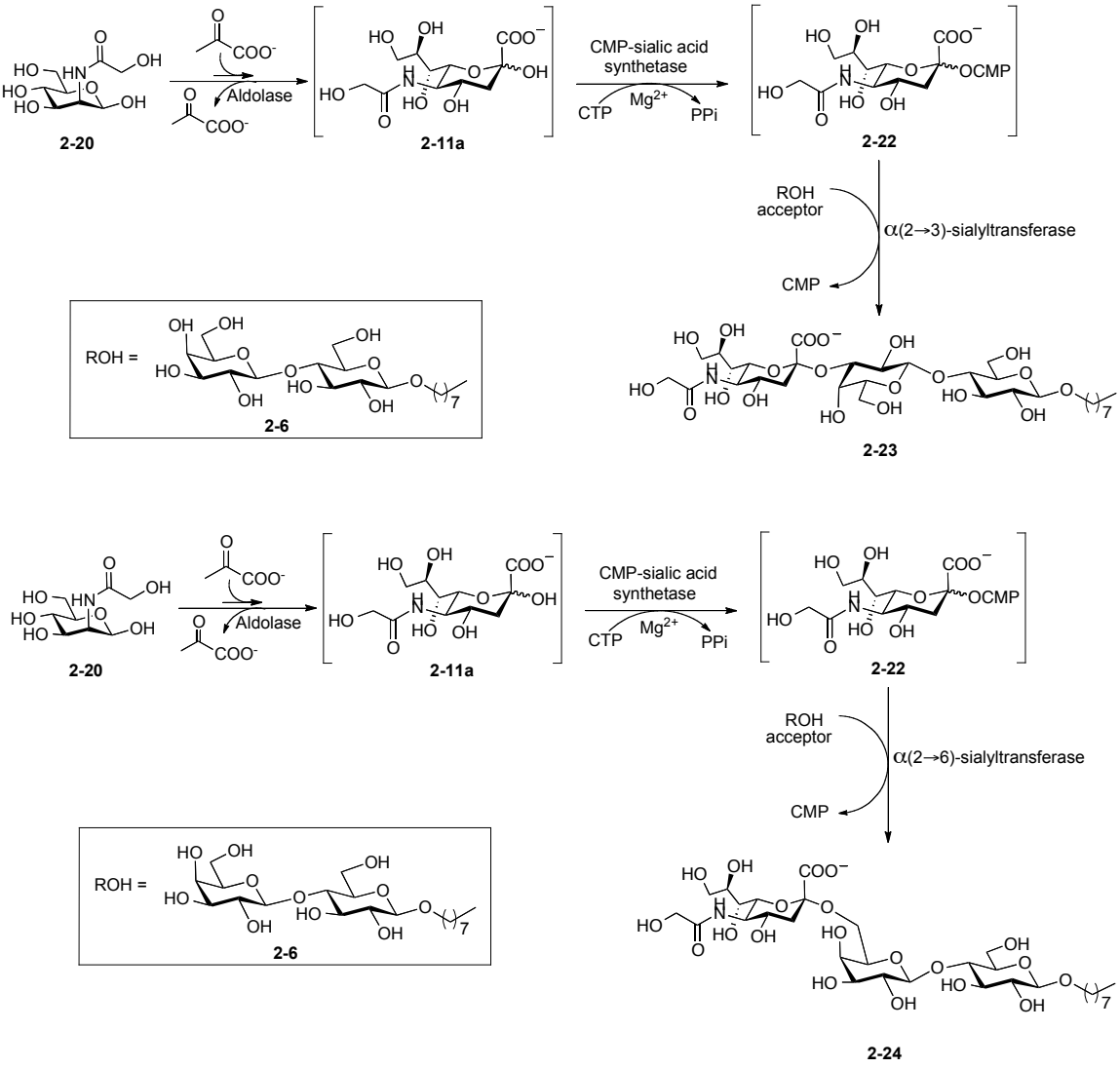
We successfully generated the target ManGc based on a reported modification of the strategy above (**Scheme 2.9**).[46] After replacement of the base in **Scheme 2.8** with Et_3N, we were able to obtain ManGc, **2-20**, in 87% yield.



Scheme 2.9 Synthesis of ManGc

2.2.4 Chemoenzymatic synthesis of GM3 analogs

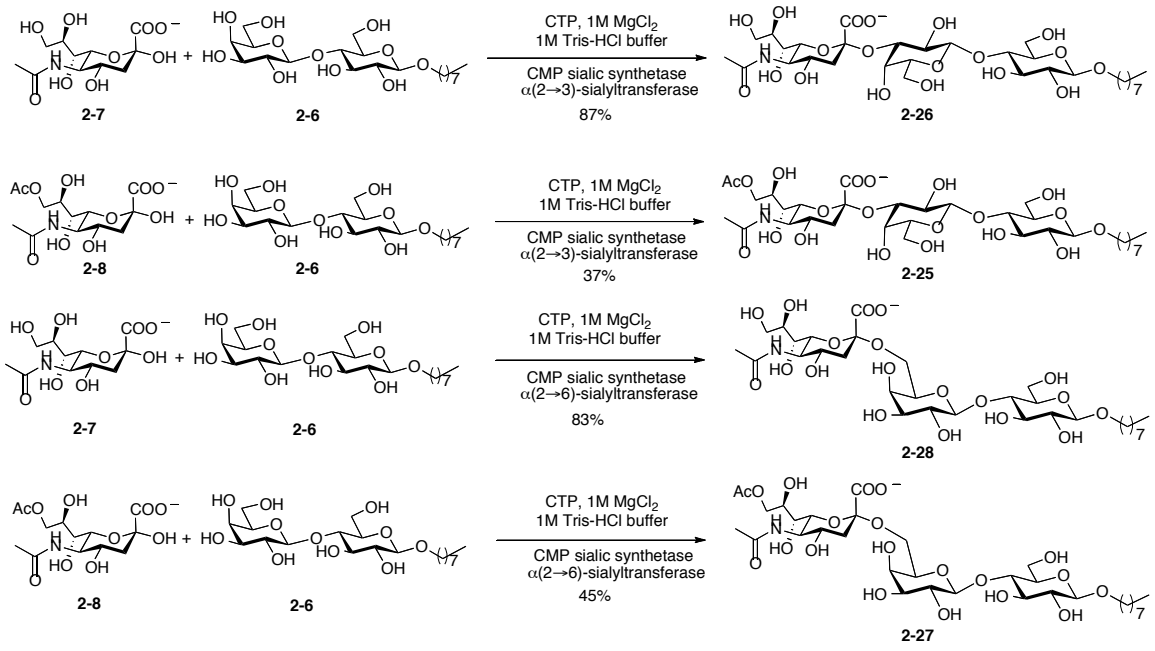
Chen and co-workers have reported a one pot three enzyme chemoenzymatic approach to the synthesis of sialosides, including Neu5Gc derivatives.[47,48] *N*-glycolyl-D-mannosamine (**2-20**) was converted *in situ* to the corresponding *N*-glycolyl-neuraminic acid (**2-7**) by the action of a recombinant sialic acid aldolase and sodium pyruvate (**Scheme 2.10**). The corresponding sugar-nucleotide donor, CMP-Neu5Gc (**2-22**) or CMP-sialic acid, could be formed by the *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS).[48] Finally, the desired trisaccharide (**2-23**) was generated by a *Pasteurella multocida* α(2→3)-sialyltransferase.[49] To generate sialosides with an α(2→6) linkage (**2-24**), we employed the *Photobacterium damsela* α(2→6)-sialyltransferase enzyme.[50]



Scheme 2.10 Synthesis of *N*-5 modified α(2→3) and α(2→6) trisaccharides

Synthesis of 9-*O*-acetyl-trisaccharides are more challenging due to the susceptibility of the *O*-Ac groups to hydrolysis. The chemoenzymatic conditions employed in **Schemes 2.9** and **2.10** use enzymes with an optimal pH of 8.8, and which require 12 h to reach completion. To avoid hydrolysis of the O-Ac groups, we optimized these conditions by reducing the pH and reaction time. Performing the transferase reaction with the 9-*O*-Ac-Neu5Ac at pH 7.2 for 3 h provided the desired product;

however, at reduced yields. We used this method for the generation of the *O*-9-Ac modified α(2→3) linked trisaccharide (**2-25**), pH 7.2 to yield **2-25** (37% yield). Even with these modified conditions, we observed partial hydrolysis of **2-25** to **2-26**, which had to be removed during purification. A similar strategy was applied for production of the α(2→3) linked Neu5Ac sialoside, **2-26**, at pH 8.8 (**Scheme 2.11**) at much higher yields. To produce the corresponding α(2→6) sialosides (**2-27**, **2-28**), we used identical methods with the replacement of the transferase enzyme to the α(2→6)-sialyltransferase at pH 7.2 and 8.8 respectively (**Scheme 2.11**).



Scheme 2.11 Synthesis of α(2→3) and α(2→6) sialosides

2.3 Conclusion

In this chapter we have successfully generated a set of analogs of GM3 sialosides that contain glycolyl and 9-*O*-acetylated modifications (**Figure 2.3**). We identified several challenges in the reported methods for generation of Neu5Gc, but were able to

obtain our desired target using a chemoenzymatic approach. The synthesis of 9-*O*-Ac sialosides was achieved through modifications of reported chemoenzymatic strategies to avoid hydrolysis of the desired *O*-acetyl groups. These targets will be of primary interest for the study of human neuraminidase enzyme activity. Although previous biological studies suggest that these modifications could result in reduced enzymatic activity for NEU3, this hypothesis remains to be tested directly.

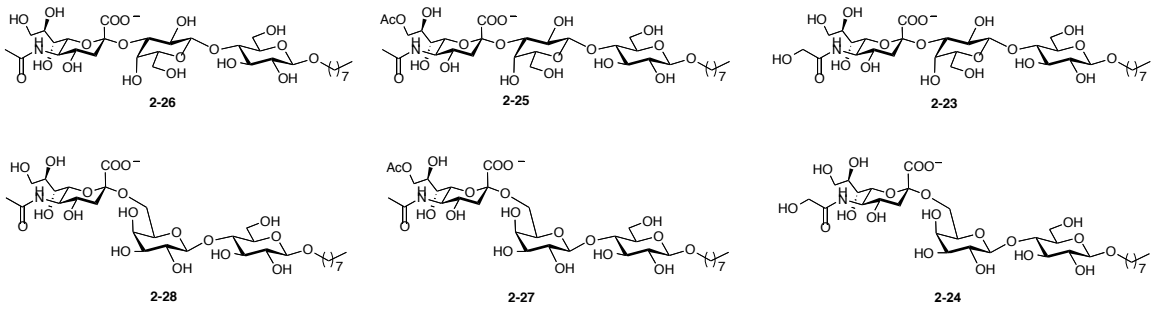


Figure 2.3 Target analogs of GM3 sialosides

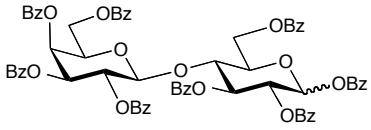
2.4 Experimental Details

2.4.1 General

All reagents were purchased from commercial sources and were used without further purification unless noted otherwise. Reaction solvents were purified by successive passage through columns of alumina and copper under an argon atmosphere using Innovative Technology, Inc. PURE SOLV (SPS-400-7). All reactions were performed under a positive pressure of argon at room temperature unless specified otherwise. The reactions were monitored by analytical TLC on silica gel 60-F_{254} (0.25 nm, Silicycle, Quebec, Canada) and visualization of the reaction components was achieved using UV fluorescence (254 nm) and/or by charring with acidified anisaldehyde solution in ethanol,

Ceric Ammonium Molybdate (CAM) or orcinol stain. Organic solvents were evaporated under reduced pressure at 40 ºC. Reaction products were purified by column chromatography on silica gel (230–400 mesh, Silicycle, Quebec, Canada), Iatrobeads 6RS-8060 (Shell-USA Inc.) if the eluent system contained greater than 10% methanol and by reversed phase C-18 silica with MeOH and H_2O as eluents. Flash column chromatography was performed using a Combiflash companion chromatography instrument (Teledyne Isco, Inc., Lincoln, NE) with Redisep and Silicycle flash silica gel columns (40-63 μm). The yields reported are after purification. NMR experiments were conducted on Varian 400, 500, 600, and 700 MHz instruments. Chemical shifts are reported relative to the deuterated solvent peaks in parts per million. Assignments of the NMR spectra were based on one-dimensional experiments (APT) and/or two-dimensional experiments (^{1}H–^{1}H COSY, ^{1}H–^{13}C HSQC and ^{1}H–^{13}C HMBC). Electrospray mass spectra (ES-MS) were recorded on Agilent Technologies 6220 TOF. For ES-MS spectra, samples were dissolved in $CHCl_3$ or CH_3OH and NaCl was added.

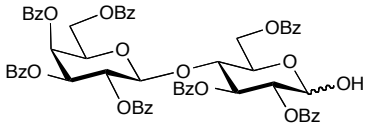
2.4.2 Synthetic methods



2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-benzoyl-α/β-D-glucopyranose (2-2):

To a suspension of β-lactose (514 mg, 1.5 mmol) in 10 mL pyridine, benzoyl chloride (1.67 mL, 14.4 mmol) was added slowly. The reaction mixture was stirred for 24 h. The excess of benzoyl chloride was quenched by the addition of MeOH at 0 ºC. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2

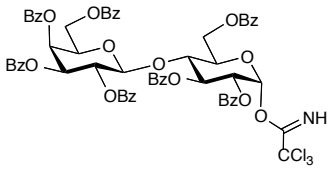
(25 mL) and washed with water (3x 30 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. Filtered the Na_2SO_4 and then organic layer was concentrated under reduced pressure. The product was purified by flash silica gel column chromatography using EtOAc–Hexane (1:2) to afford **2-2** (1.71 g, 96%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.13 (dd, *J* = 8.1, 1.2 Hz, 1H, ArH), 8.08 – 7.97 (m, 13H, ArH), 7.96 – 7.89 (m, 5H, ArH), 7.79 – 7.74 (m, 3H, ArH), 7.67 – 7.30 (m, 28H, ArH), 7.22 (ddd, *J* = 11.4, 10.0, 5.6 Hz, 6H, ArH), 6.17 (d, *J* = 8.1 Hz, 1H, H_1'), 5.98 (app. t, *J* = 9.3 Hz, 1H, H_3'), 5.84 – 5.75 (m, 3H, H_2', H_2'', H_4''), 5.41 (dd, *J* = 10.4, 3.4 Hz, 1H, H_3''), 4.93 (d, *J* = 7.9 Hz, 1H, H_1''), 4.64 – 4.54 (m, 2H, H_6'), 4.42 (m, 1H, H_4'), 4.10 (ddd, *J* = 9.9, 3.7, 1.9 Hz, 1H, H_5'), 3.92 (app. t, *J* = 6.6 Hz, 1H, H_5''), 3.84 – 3.72 (m, 2H, H_6''). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 165.8 (ArC), 165.6 (ArC), 165.4 (ArC), 165.3 (ArC), 165.2 (ArC), 164.8 (ArC), 164.5 (ArC), 133.8 (ArC), 133.6 (ArC), 133.5 (ArC), 133.42 (ArC), 133.41 (ArC), 133.39 (ArC), 133.30 (ArC), 133.2 (ArC), 130.2 (ArC), 130.1 (ArC), 130.0 (ArC), 129.9 (ArC), 129.81 (ArC), 129.8 (ArC), 129.73 (ArC), 129.71 (ArC), 129.68 (ArC), 129.60 (ArC), 129.5 (ArC), 129.45 (ArC), 128.88 (ArC), 128.84 (ArC), 128.79 (ArC), 128.75 (ArC), 128.63 (ArC), 128.61 (ArC), 128.60 (ArC), 128.5 (ArC), 128.4 (ArC), 128.43 (ArC), 128.3 (ArC), 128.32 (ArC), 101.1 (C_1''), 92.6 (C_1'), 75.6 (C_4'), 73.9 (C_3''), 72.8 (C_5'), 71.7 (C_5''), 71.4 (C_3'), 70.7 (C_2'), 69.8 (C_2''), 67.5 (C_4''), , 62.1 (C_6'), 61.0 (C_6''). HRMS (ESI) calculated for $C_{68}H_{54}O_{19}Na$ $[M+Na]^+$ 1197.3152, found: 1197.3144.



2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-α/β-D-glucopyranose (2-3):

To a solution of **2-2** (600 mg, 0.51 mmol) in DMF (10 mL) was added hydrazine acetate (220 mg, 2.44 mmol). The reaction mixture was stirred for 1.5 h at 60 ºC. Water (20 mL) was added to the reaction mixture, a white solid precipitate formed that was collected by filtration. The solid residue was dissolved in CH_2Cl_2 (10 mL) and washed with water (2x 15 mL). The organic layer was separated, dried over anhydrous Na_2SO_4. Filtered the Na_2SO_4 and evaporated the organic layer under vacuum. The crude residue was purified by flash silica gel column chromatography using EtOAc–Hexane (1:1) to furnish **2-3** (440 mg, 81%) as a white crystalline solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.07 – 7.93 (m, 13H, ArH), 7.89 (dd, *J* = 8.3, 1.2 Hz, 3H, ArH), 7.73 (dd, *J* = 8.3, 1.2 Hz, 3H, ArH), 7.66 – 7.25 (m, 28H, ArH), 7.25 – 7.13 (m, 6H, ArH), 6.13 (app. t, *J* = 9.7 Hz, 1H, H_3'), 5.77 – 5.62 (m, 3H, H_2', H_2'', H_4''), 5.42 – 5.37 (m, 1H, H_3''), 5.22 – 5.28 (m, 1H, H_1'), 4.94 (d, *J* = 7.8 Hz, 1H, H_1''), 4.59 (dd, *J* = 12.3, 1.9 Hz, 1H, H_6'), 4.51 (dt, *J* = 12.3, 3.5 Hz, 1H, H_6'), 4.38 (ddd, *J* = 11.7, 3.4, 2.0 Hz, 1H, H_4'), 4.28 – 4.22 (m, 1H, H_5'), 3.94-3.72 (m, 3H, H_5'', H_6''). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 166.0 (ArC), 165.9 (ArC), 165.64 (ArC), 165.62 (ArC), 165.5 (ArC), 165.43 (ArC), 165.40 (ArC), 165.32 (ArC), 165.29 (ArC), 165.20 (ArC), 164.84 (ArC), 164.82 (ArC), 133.5 (ArC), 133.47 (ArC), 133.46 (ArC), 133.3 (ArC), 133.2 (ArC), 133.1 (ArC), 130.0 (ArC), 129.9 (ArC), 129.8 (ArC), 129.78 (ArC), 129.77 (ArC), 129.76 (ArC), 129.70 (ArC), 129.64 (ArC), 129.62 (ArC), 129.5 (ArC), 129.4 (ArC), 129.0 (ArC), 128.83 (ArC), 128.81 (ArC), 128.76

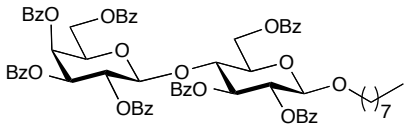
(ArC), 128.75 (ArC), 128.65 (ArC), 128.64 (ArC), 128.62 (ArC), 128.58 (ArC), 128.55 (ArC), 128.50 (ArC), 128.43 (ArC), 128.42 (ArC), 128.38 (ArC), 128.37 (ArC), 128.2 (ArC), 101.0 (C_1''), 90.4 (C_1'), 76.0 (C_4 '), 75.8 (C_3''), 74.3 (C_5'), 73.3 (C_5''), 71.9, 71.44, 71.42, 70.0 (C_3'), 67.53 (C_2'), 67.50 (C_2''), 62.3 (C_4''), 61.2 (C_6'), 61.1 (C_6''). HRMS (ESI) calculated for $C_{61}H_{50}O_{18}Na$ $[M+Na]^+$ 1093.2889, found: 1093.2887.



2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyltrichloroacetimidate (2-4):

Compound **2-3** (430 mg, 0.4 mmol), DBU (6 μL) and trichloroacetonitrile (481 μL, 4.8 mmol) were dissolved in CH_2Cl_2 (10 mL) and stirred for 2 h. The color of the reaction mixture changed to dark reddish brown. The solvent was evaporated and the residue was purified by column chromatography with EtOAc/Hexane (1:1) to yield 400 mg (82%) of **2-4** as an off-white crystalline solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.60 (s, 1H, -NH), 8.01 (dddd, *J* = 15.9, 12.8, 8.4, 1.3 Hz, 12H, ArH), 7.90 (dd, *J* = 8.3, 1.2 Hz, 2H, ArH), 7.75 (dd, *J* = 8.4, 1.3 Hz, 2H, ArH), 7.70 – 7.54 (m, 4H, ArH), 7.54 – 7.26 (m, 17H, ArH), 7.26 – 7.15 (m, 5H, ArH), 6.13 (d, *J* = 3.7 Hz, 1H, H_1'), 5.81 – 5.76 (m, 2H, H_2', H_2''), 5.57 (dd, *J* = 10.1, 3.7 Hz, 1H, H_3'), 5.43 (dd, *J* = 10.3, 3.3 Hz, 1H, H_3''), 4.98 (d, *J* = 8.1 Hz, 1H, H_1''), 4.63 – 4.54 (m, 2H, H_6'), 4.39 – 4.35 (m, 2H, H_4', H_5'), 3.93 (t, *J* = 7.0 Hz, 1H, H_5''), 3.85 (dd, *J* = 11.3, 6.2 Hz, 1H, H_6''), 3.77 (dd, *J* = 11.2, 7.2 Hz, 1H, H_6''). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 165.7 (ArC), 165.6 (ArC), 165.57 (ArC), 165.55 (ArC), 165.22 (ArC), 165.20 (ArC), 164.8 (ArC), 160.7 (ArC), 133.6 (ArC), 133.44 (ArC), 133.42 (ArC), 133.3 (ArC), 130.0 (ArC), 129.9 (ArC), 129.8 (ArC), 129.73 (ArC),

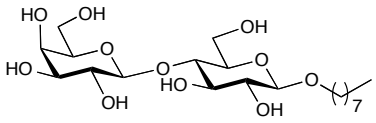
129.71 (ArC), 129.60 (ArC), 129.59 (ArC), 129.50 (ArC), 129.4 (ArC), 128.9 (ArC), 128.7 (ArC), 128.68 (ArC), 128.66 (ArC), 128.52 (ArC), 128.50 (ArC), 128.4 (ArC), 128.32 (ArC), 128.31 (ArC), 101.3 (C_1''), 93.1 (C_1'), 75.7 (C_4'), 71.9 (C_3''), 71.4 (C_5'), 70.5 (C_5''), 70.3 (C_3'), 70.0 (C_2'), 67.5 (C_2''), 61.9 (C_6'), 61.0 (C_6''). HRMS (ESI) calculated for $C_{63}H_{50}Cl_3NO_{18}Na$ $[M+Na]^+$ 1236.1986, found: 1236.1983.



***O*-2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl-octanol (2-5):**

1-Octanol (64.20 µL, 0.49 mmol) was added to a reaction mixture of compound **2-4** (400 mg, 0.33 mmol) and activated molecular sieves (2.5 g) in CH_2Cl_2 (10 mL). The reaction was charged with 1% TMSOTf (0.3 mL in CH_2Cl_2) at 0 ºC. The reaction was allowed to warm to room temperature and then stirred overnight. After completion, the reaction mixture was filtered on a pad of Celite. The product was purified by column chromatography (0 to 30% EtOAc in hexane) to yield 315 mg (92%) of **2-5**. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.05 – 7.95 (m, 13H, ArH), 7.91 (d, *J* = 7.7 Hz, 2H, ArH), 7.73 (d, *J* = 7.8 Hz, 2H, ArH), 7.59 (ddd, *J* = 22.3, 15.0, 7.4 Hz, 3H, ArH), 7.49 (q, *J* = 7.9 Hz, 5H, ArH), 7.45 – 7.28 (m, 9H, ArH), 7.21 (t, *J* = 7.6 Hz, 2H, ArH), 7.14 (t, *J* = 7.6 Hz, 2H, ArH), 5.83 (app. t, *J* = 9.4 Hz, 1H, H_3'), 5.77 – 5.72 (m, 2H, H_2', H_2''), 5.48 (dd, *J* = 9.9, 7.9 Hz, 1H, H_4''), 5.40 (dd, *J* = 10.3, 3.4 Hz, 1H, H_3''), 4.90 (d, *J* = 7.9 Hz, 1H, H_1''), 4.71 (d, *J* = 7.9 Hz, 1H, H_1'), 4.62 (dd, *J* = 12.1, 1.6 Hz, 1H, H_6'), 4.52 (dd, *J* = 12.2, 4.5 Hz, 1H, H_6'), 4.28 (app. t, *J* = 9.5 Hz, 1H, H_4'), 3.92 – 3.70 (m, 4H, H_5', H_5'', H_6''), 3.49 – 3.43 (m, 1H, H_6''), 1.53 – 1.38 (m, 2H, -O-CH_2), 1.23 – 0.95 (m, 10H,

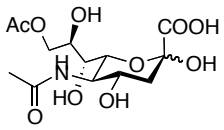
5xCH_2), 0.84 (t, *J* = 7.2 Hz, 3H, CH_3-octyl). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 165.9 (ArC), 165.6 (ArC), 165.5 (ArC), 165.4 (ArC), 165.3 (ArC), 165.2 (ArC), 164.8 (ArC), 133.5 (ArC), 133.47 (ArC), 133.45 (ArC), 133.33 (ArC), 133.32 (ArC), 133.2 (ArC), 133.1 (ArC), 130.0 (ArC), 129.8 (ArC), 129.78 (ArC), 129.77 (ArC), 129.6 (ArC), 129.53 (ArC), 129.51 (ArC), 128.9 (ArC), 128.72 (ArC), 128.71 (ArC), 128.6 (ArC), 128.54 (ArC), 128.53 (ArC), 128.37 (ArC), 128.36 (ArC), 128.2 (ArC), 101.2 (C_1''), 100.9 (C_1'), 76.1 (C_4'), 73.0 (C_3''), 72.9, 71.82 (C_5'), 71.81, 71.4 (C_5''), 70.3 (C_3'), 69.9 (C_2'), 67.6 (C_2''), 63.1 (C_6'), 62.5 (C_6''), 61.1, 32.8, 31.8, 31.7, 29.4, 29.33, 29.31, 29.2, 29.0, 25.7, 22.6, 14.1 (CH_3-octyl). HRMS (ESI) calculated for $C_{69}H_{66}O_{18}Na$ $[M+Na]^+$ 1205.4141, found: 1205.4139.



Octyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (2-6):

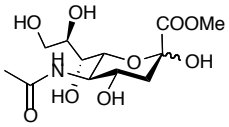
To a solution of compound **2-5** (300 mg, 0.25 mmol) in MeOH (5 mL) was added 1% freshly prepared NaOMe (1.9 mL). The reaction mixture was stirred for 24 h and monitored by TLC (1:5, MeOH: CH_2Cl_2; *Rf* = 0.2 to 0.3). After completion of the reaction, the reaction mixture was neutralized to pH 7 by adding Amberlite®-IR 120 H^+ resin. The reaction mixture was filtered and concentrated under reduced pressure. The crude product was purified by column chromatography by eluting with 0 to 40% of MeOH in CH_2Cl_2 in 92% yield as a white solid. ^{1}H NMR (500 MHz, D_2O): δ = 4.48 – 4.41 (d, *J* = 7.9 Hz, 2H, H_1', H_1''), 3.99 – 3.86 (m, 3H, H_3''), 3.82 – 3.50 (m, 11H), 3.29 (m, 1H, H_2'), 1.64 – 1.58 (m, 2H, -O-CH_2), 1.38-1.22 (m, 11H, 5xCH_2), 0.84 (t, *J* = 6.2 Hz, 3H, CH_3-octyl). ^{13}C NMR (126 MHz, D_2O): δ = 103.0 (C_1''), 102.3 (C_1'), 78.5 (C_4'),

75.4 (C_3''), 74.8 (C_5'), 74.5 (C_5''), 72.9 (C_3'), 72.6, 71.0, 70.8 (C_2'), 68.6 (C_2''), 61.3 (C_6'), 60.2 (C_6''), 31.1, 28.8, 28.5, 28.4, 25.1, 22.0, 13.4 (CH_3-octyl). HRMS (ESI) calculated for $C_{20}H_{38}O_{11}Na$ $[M+Na]^+$ 477.2306, found: 477.2301.



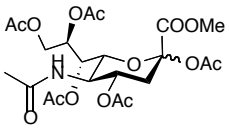
5-Acetamido-9-*O*-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonic acid (2-8):

Trimethylorthoacetate (0.64 mL, 5.0 mmol) and p-TsOH·H_2O (5 mg, catalytic amount) was added to a solution of *N*-Acetyl neuraminic acid (155 mg, 0.5 mmol) in DMSO (1 mL). After being stirred for 2 h, the reaction mixture was applied directly to a Dowex 1-X8 ($HCOO^-$) anion exchange resin. The column was washed with H_2O (100 mL) and then the compound was eluted with formic acid (1N, 50 mL). The eluent was lyophilized over 48 h to give **2-8** (85% yield) as a white solid. ^{1}H NMR (CD_3OD): δ = 4.37 (dd, *J* = 11.5, 2.5 Hz, 1H, H_{9b}), 4.14 (dd, *J* = 11.4, 6.3 Hz, 1H, H_{9a}), 4.08 – 4.00 (m, 2H, H_4, H_6), 3.94 – 3.81 (m, 2H, H_5, H_8), 3.51 (dd, *J* = 9.3, 1.0 Hz, 1H, H_7), 2.23 (dd, *J* = 12.8, 4.9 Hz, 1H, H_{3e}), 2.06 (s, 3H, -O-CO-C$\underline{H}_3$), 2.03 (s, 3H, -NH-CO-C$\underline{H}_3$), 1.85 (app.t, *J* = 12.0 Hz, 1H, H_{3a}). ^{13}C NMR (CD_3OD): δ = 173.6 (CO), 171.6 (C_1), 70.6, 69.0, 68.0, 66.5 (C_9), 66.3 (C_5), 52.8 (C_3), 39.6 (NH-CO-$\underline{C}H_3$), 21.2 (-O-CO-$\underline{C}H_3$), 19.4. HRMS (ESI) calculated for $C_{13}H_{20}NO_{10}$ $[M-H]^-$ 350.1093, found: 350.1089.



Methyl-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranosonate (2-9):

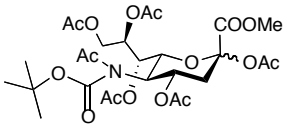
To a solution of *N*-Acetyl neuraminic acid (2.0 g, 6.46 mmol) in 75 mL MeOH added 10 g Amberlite®-IR 120 H^+ resin, and the mixture was stirred overnight. The reaction mixture turned from white cloudy to colorless. The reaction mixture was filtered, and then concentrated to give **2-9** (91.6% yield) as a white solid. The crude product was used for the next step without any further purification. ^{1}H NMR (500 MHz, D_2O): δ = 4.14 – 4.02 (m, 2H, H_4, H_6), 3.94 (t, *J* = 10.3 Hz, 1H, H_5), 3.90-3.82 (m, 4H, H_{9a}, -OCH_3), 3.75 (ddd, *J* = 9.0, 6.3, 2.6 Hz, 1H, H_{9b}), 3.68 – 3.60 (m, 1H, H_8), 3.57 (d, *J* = 9.2 Hz, 1H, H_7), 2.33 (dd, *J* = 13.1, 4.8 Hz, 1H, H_{3e}), 2.06 (s, 3H, NH-CO-C$\underline{H}_3$), 1.94 (dd, *J* = 12.9, 11.9 Hz, 1H, H_{3a}). ^{13}C NMR (125 MHz, D2O): δ = 175.7 (C$\underline{O}$OMe), 172.2 (C_1), 71.2 (C_2), 71.0, 69.1, 67.5 (C_9), 64.0 (C_5), 54.3, 52.9 (C_3), 39.5 (NH-CO-$\underline{C}H_3$), 22.9 (-COO-$\underline{C}H_3$). HRMS (ESI) calculated for $C_{12}H_{21}NO_9Na$ $[M+Na]^+$ 346.1109, found: 346.1111.



Methyl-5-acetamido-2,4,7,8,9-penta-*O*-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-α/β-nonulopyranosonate (2-10):

A solution of methyl-5-acetamido-3, 5-dideoxy-D-glycero-D-galactononulopyranosonate (1.9 g, 5.92 mmol) in pyridine (30 mL) was kept at 4 °C for 10 minutes. Acetic anhydride (27.98 mL, 296.1 mmol) was added dropwise for 1 h. The mixture was warmed to rt and stirred overnight. The solution was concentrated and the residue was purified by flash chromatography with 5:1 EA/hexane to give **2-10** as a white solid (92% yield, mixture of

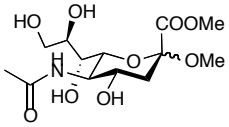
anomers in 2:1). ^{1}H NMR (500 MHz, D_2O): δ = 5.42 (dd, *J* = 7.7, 1.8 Hz, 1H, H_4), 5.38 (dd, *J* = 8.1, 1.9 Hz, 1H, H_7) 5.31 – 5.24 (m, 2H), 5.04 (ddd, *J* = 15.4, 10.3, 5.1 Hz, 1H, H_8), 4.64 (dd, *J* = 11.0, 2.0 Hz, 1H, H_{9a}), 4.41 (dd, *J* = 12.8, 2.7 Hz, 1H, H_6), 4.24 – 4.14 (m, 2H, H_{9b}), 3.80 (s, 3H, -OCH_3), 2.57 (dd, *J* = 13.7, 5.3 Hz, 1H, H_{3e}), 2.20 (s, 3H, -OCOC$\underline{H}_3$), 2.16 (s, 3H, -OCOC$\underline{H}_3$), 2.09 (s, 3H, -OCOC$\underline{H}_3$), 2.07 (s, 3H, -OCOC$\underline{H}_3$), 2.04 (s, 3H, -OCOC$\underline{H}_3$), 1.93 (s, 3H, -NHCOC$\underline{H}_3$). ^{13}C NMR (125 MHz, D_2O): δ = 174.4 (C$\underline{O}$OMe), 173.8 (-O$\underline{C}$OCH_3), 173.2 (-O$\underline{C}$OCH_3), 173.1 (-O$\underline{C}$OCH_3), 172.56 (-O$\underline{C}$OCH_3), 172.48 (-O$\underline{C}$OCH_3), 172.46 (C_1), 171.3, 169.5, 168.4, 97.0, 96.3, 72.7, 71.2 (C_2), 69.5, 69.1, 68.8, 68.7, 67.3, 67.2, 61.8, 53.9, 53.7, 48.7, 35.62 (NH-CO-$\underline{C}H_3$), 21.8 (-OCO$\underline{C}H_3$), 20.32 (-OCO$\underline{C}H_3$), 20.23 (-OCO$\underline{C}H_3$), 20.18 (-OCO$\underline{C}H_3$), 20.16 (-COO-$\underline{C}H_3$). HRMS (ESI) calculated for $C_{22}H_{31}NNaO_{14}$ $[M+Na]^+$, 556.1637, found 556.1629.



Methyl 5-(*N*-tert-butoxycarbonylacetamido)-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-α/β-nonulopyranosonate (2-11):

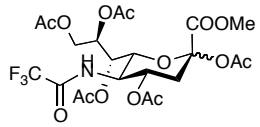
To a stirred solution of **2-10** (288 mg, 0.53 mmol) in THF (20 mL) was added di-tert-butyldicarbonate (Boc_2O) (371 mg, 1.7 mmol), and 4-dimethylaminopyridine (DMAP; 125 mg, 1.0 mmol). The reaction mixture was refluxed for 2 h. After completion, the mixture was cooled to room temperature, extracted with CH_2Cl_2, washed with 0.5 M aqueous HCl, water, and saturated $NaHCO_3$. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The crude produce was purified by flash chromatography (EA/hexane: 1:1) to give **2-11** as yellow oil (62% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.84 (ddd, *J* = 15.7, 10.6, 5.3 Hz, 1H, H_4), 4.54 (dd, *J* = 12.5, 2.6 Hz,

2H, H_7) 4.49 –4.38 (m, 3H, H_8, H_9), 3.83 (s, 3H, -OCH_3), 2.66 (dd, *J* = 13.6, 5.1 Hz, 1H, H_{3e}), 2.17 (s, 3H, -OCOC$\underline{H}_3$), 2.15 (s, 3H, -OCOC$\underline{H}_3$), 2.12 (s, 3H, -OCOC$\underline{H}_3$), 2.08 (s, 3H, -OCOC$\underline{H}_3$), 2.06 (s, 3H, -OCOC$\underline{H}_3$), 1.98 (s, 3H, -NHCOC$\underline{H}_3$), 1.66 (s, 9H, 3xC$\underline{H}_3$). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.8 (C$\underline{O}$OMe), 170.64 (-O$\underline{C}$OCH_3), 170.62 (-O$\underline{C}$OCH_3), 170.3 (-O$\underline{C}$OCH_3), 170.0 (-O$\underline{C}$OCH_3), 169.8 (-O$\underline{C}$OCH_3), 168.3, 166.5, 97.9, 84.9, 72.0 (C_2), 71.4, 69.5, 66.1, 62.0, 53.2, 52.3, 37.18, 37.17 (NH-CO-$\underline{C}H_3$), 27.92, 27.90, 26.8, 20.94 (-OCO$\underline{C}H_3$), 20.93 (-OCO$\underline{C}H_3$), 20.92 (-OCO$\underline{C}H_3$), 20.88 (-OCO$\underline{C}H_3$), 20.86 (-OCO$\underline{C}H_3$), 20.76 20.75 (-COO-$\underline{C}H_3$). HRMS (ESI) calculated for $C_{27}H_{39}NNaO_{16}$ $[M+Na]^+$, 656.2161, found 656.2153.



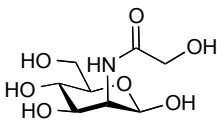
Methyl-5-acetamido-2-methoxy-3, 5-dideoxy-D-glycero-D-galacto-nonulopyranosonate (2-13):

To a solution of *N*-Acetyl neuraminic acid (100 mg; 0.32 mmol) in 10 mL MeOH at rt under N_2 was added 25 mg Amberlite®-IR 120 H^+ resin, and the mixture was stirred overnight. After completion, the mixture was filtered, and concentrated in vacuo to give **2-13** (90.7% yield) as a white solid. The crude product was used for the next step without any further purification.



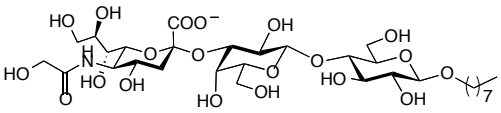
Methyl 5-(*N*-trifluoroacetamido)-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-D-glycero-D-galacto-2-α/β-nonulopyranosonate (2-15):

To a solution of **2-10** (100 mg, 0.31 mmol) in acetonitrile (1 mL) was added Et_3N (0.26 mL, 1.85 mmol) and trifloroacetic anhydride (0.13 mL, 0.93 mmol). The reaction was stirred at 135 ºC for 5 minutes. Methanol (20 mL) was added at 0 ºC, and the mixture was stirred for 5 minutes. The solvent was evaporated under reduced pressure. The crude residue was then purified by column chromatography, eluting with EA/ hexane (3:2) to give **2-15** as a yellow oil (25.4% yield). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.42 (d, *J* = 9.4 Hz, 1H, N<u>H</u>COCH$_3$), 5.45 (m, 1H, H_4), 5.34 (dd, *J* = 6.1, 1.8 Hz 1H, H_7), 5.21 (ddd, *J* = 8.8, 6.2, 2.6 Hz 1H, H_8), 4.52 (dd, *J* = 12.6, 2.6 Hz 1H, H_{9a}), 4.28 (dd, *J* = 10.7, 2.1 Hz 1H, H_6), 4.18 – 4.11 (m, 4H, H_{9b}), 4.09 – 4.02 (m, 1H, H_5), 3.82 (s, 3H, -OCH$_3$), 2.65 (dd, *J* = 13.5, 5.0 Hz 1H, H_{3e}), 2.21 (s, 3H, -OCOC<u>H</u>$_3$), 2.20 (s, 3H, -OCOC<u>H</u>$_3$), 2.12 (s, 3H, -OCOC<u>H</u>$_3$), 2.21 (s, 3H, -OCOC<u>H</u>$_3$) 2.08 – 2.07 (s, overlapping, 9H, -OCOC<u>H</u>$_3$, -NHCOC<u>H</u>$_3$). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 170.7 (<u>C</u>OOCF$_3$), 170.2 (<u>C</u>OOMe), 97.3, 71.6 (C_2), 70.4, 67.6, 67.2, 62.2, 62.1, 60.5, 53.5, 50.4, 35.9 (NHCO<u>C</u>H$_3$), 29.72, (-COO-<u>C</u>F$_3$), 29.70, 21.1 (-COO-<u>C</u>H$_3$), 20.77 (-COO-<u>C</u>H$_3$), 20.64 (-COO-<u>C</u>H$_3$), 20.63 (-COO-<u>C</u>H$_3$), 20.5 (-COO-<u>C</u>H$_3$). ^{19}F NMR (468.6 MHz, $CDCl_3$): δ = -75.9 (COC<u>F</u>$_3$). HRMS (ESI) calculated for $C_{22}H_{28}F_3NNaO_{14}$ $[M+Na]^+$, 610.1354, found 610.1342.



***N*-Glycolyl-D-mannosamine (2-20)**

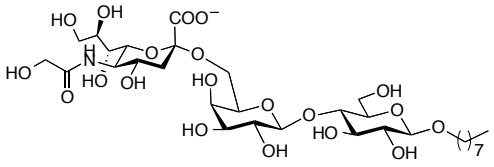
Glycolic acid (100 mg, 1.31 mmol) was added to a mixture of mannosamine hydrochloride (338.5 mg, 1.57 mmol) and triethylamine (0.22mL, 1.57 mmol) in anhydrous DMF (5 mL). After cooling the reaction mixture to 0 ºC, DIC (0.24 mL, 1.57 mmol) and HOBt (194.7 mg, 1.44 mmol) were added consecutively and the reaction mixture was stirred overnight. The reaction mixture was filtered. The solvent was concentrated using reduced pressure. The product was purified by flash chromatography ($CHCl_3$/ MeOH: 2:1) to give 238 mg (76.6%) of **2-20** as an off-white solid. The ^{1}H NMR and ^{13}C NMR data were consistent with previous reports.[46]



***O*-(5-glycolylamido-3,5,-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)- (2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (2-23):**

N-Glycolyl-D-mannosamine (2.25 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol), 1 M $MgCl_2$ (80 μL) and distilled H_2O (600 μL) were suspended in 1M Tris-HCl buffer (400 μL, pH 8.8). The reaction was then charged with *E. coli sialic acid aldolase*[50] (300 μL), *Neisseria meningitides* CMP-Neu5Ac synthetase (NmCSS)[48] (300 μL), octyl-β-lactoside, **2-6**, (2.5 mg, 6.2 μmol), *Pasteurella multocida* α(2→3)-sialyltransferase[49] (300 μL) and distilled H_2O (600 μL). The reaction mixture was stirred overnight. After completion, ethanol was added to the reaction mixture. The reaction

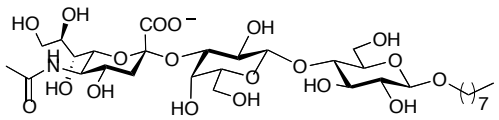
mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified by sep-pack C-18 reverse phase cartridge. The product was eluted with H_2O/MeOH (2:1). Pure product **2-23** (2.5 mg; 53%) was obtained as a white solid after concentration of the fractions on high vacuum. ^{1}H NMR (400 MHz, D_2O): δ = 4.53 (d, *J* = 7.7 Hz, 1H, H_1''), 4.47 (d, *J* = 7.9 Hz, 1H, H_1'), 4.12 (s, 2H, -NHCOC$\underline{H}_2$OH), 4.01-3.51 (m, 24H), 3.30 (t, *J* = 8.0 Hz, 1H, H_2'), 2.78 (dd, *J* = 12.5, 4.3 Hz, 1H, H_{3e}'''), 1.81 (t, *J* = 12.0 Hz, 1H, H_{3a}'''), 1.66 – 1.58 (m, 2H, -OCH_2), 1.38 – 1.22 (m, 10H, 5xCH_2), 0.85 (t, *J* = 6.6 Hz, 4H, CH_3-octyl). ^{13}C NMR (125 MHz, D_2O): δ = 175.2 (COO^-), 173.9 (-NH$\underline{C}$OCH_2OH), 102.7 (C_1''), 102.1 (C_1'), 99.9 (C_2'''), , 78.4, 75.5, 75.2, 74.8, 74.5, 72.9, 70.8, 69.4, 68.14, 68.08, 67.5, 62.6, 62.5, 61.1, 61.0, 60.9, 60.2, 52.2, 51.4, 31.1, 28.8, 28.5, 28.4, 25.1, 22.0, 13.4 (CH_3-octyl). HRMS (ESI) calculated for $C_{31}H_{54}NO_{20}^-$ $[M-H]^-$ 760.3244, found: 760.3345.



***O*-(5-glycolylamido-3,5,-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)- (2→6)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (2-24):**

N-Glycolyl-D-mannosamine (2.25 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol), 1 M $MgCl_2$ (80 μL) and distilled H_2O (600 μL) were suspended in 1M Tris-HCl buffer (400 μL, pH 8.8). Added *E. coli sialic acid aldolase*[50] (300 μL), *Neisseria meningitides* CMP-Neu5Ac synthetase (NmCSS)[48] (300 μL), octyl-β-lactoside, **2-6**, (2.5 mg, 6.2 μmol), *Photobacterium damsela* α(2→6)-sialyltransferase[50] (300 μL) and distilled H_2O (600 μL). The reaction mixture was stirred overnight. After completion,

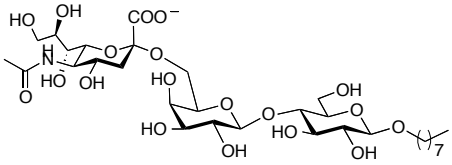
ethanol was added to the reaction mixture. The reaction mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified by sep-pack C-18 reverse phase cartridge. The product was eluted with H_2O/MeOH (2:1). Pure product **2-24** (3.2 mg, 68%) was obtained as a white solid after concentration of the fractions on high vacuum. ^{1}H NMR (400 MHz, D_2O): δ = 4.48 (d, *J* = 8.1 Hz, 1H, H_1''), 4.43 (d, *J* = 7.7 Hz, 1H, H_1'), 4.11 (s, 2H, -NHCOC$\underline{H}_2$OH), 4.01 –3.50 (m, 25H), 3.33 (t, *J* = 7.9 Hz, 1H, H_{3e}'''), 2.72 (dd, *J* = 12.4, 4.4 Hz, 1H, H_2'), 1.75 (t, *J* = 12.2 Hz, 1H, H_{3a}'''), 1.66 – 1.58 (m, 2H, -OCH_2), 1.38 – 1.24 (m, 11H, 5xCH_2), 0.85 (t, *J* = 6.6 Hz, 4H, CH_3-octyl). ^{13}C NMR (125 MHz, D_2O): δ = 175.7 (COO^-), 173.5 (-NH$\underline{C}$OCH_2OH), 103.3 (C_1''), 101.9 (C_1'), , 100.4 (C_2'''), , 79.7, 74.8, 74.7, 73.8, 72.8, 72.4, 72.3, 71.9, 70.83, 70.79, 68.6, 68.5, 68.4, 68.1, 63.6, 62.6, 61.0, 60.4, 51.5, 40.2, 31.1, 28.8, 28.5, 28.4, 25.1, 22.0, 20.1, 13.4 (CH_3-octyl). HRMS (ESI) calculated for $C_{31}H_{54}NO_{20}^-$ $[M-H]^-$ 760.3244, found: 760.3345.



***O*-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (2-26):**

N-Acetyl neuraminic acid (2.90 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol), 1M $MgCl_2$ (80 μL) and distilled H_2O (600 μL) were dissolved in 1M Tris-HCl buffer (400 μL pH 8.8). The reaction was then charged with *Neisseria meningitides CMP-Neu5Ac synthetase* (NmCSS)[48] (200 μL), octyl-β-lactoside, **2-6**, (2.5 mg, 6.2 μmol), *Pasteurella multocida* α(2→3)-sialyltransferase[49] (200 μL) and distilled H_2O (600 μL). The reaction mixture was stirred overnight. After completion, ethanol was added to

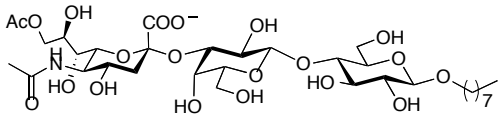
the reaction mixture. The reaction mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified over a sep-pack C-18 reverse phase cartridge. The product was eluted with MeOH/EA (1:1). The purified product **2-26** (4 mg; 87%) was obtained as a white solid after concentration of the fractions on high vacuum. ^{1}H NMR (500 MHz, D_2O): δ = 4.52 (d, *J* = 7.9 Hz, 1H, H_1''), 4.46 (d, *J* = 8.1 Hz, 1H, H_1'), 4.10 (dd, *J* = 9.9, 3.1 Hz, 1H, H_3''), 3.99 – 3.78 (m, 7H), 3.76-3.53 (m, 13H), 3.28 (t, *J* = 8.6 Hz, 1H, H_2'), 2.74 (dd, *J* = 12.4, 4.7 Hz, 1H, H_{3e}'''), 2.01 (s, 3H, -NHCOC$\underline{H}_3$), 1.77 (t, *J* = 12.1 Hz, 1H, H_{3a}'''), 1.64 – 1.57 (m, 2H, O-CH_2), 1.37 – 1.22 (m, 10H, 5xCH_2), 0.84 (t, *J* = 6.8 Hz, 3H, CH_3-octyl). ^{13}C NMR (125 MHz, D_2O): δ = 175.1 (COO^-) , 173.9 (NH$\underline{C}OCH_3$), 102.7 (C_1''), 102.1 (C_1'), 99.9 (C_2'''), 78.4, 75.5, 75.2, 74.8, 74.5, 72.92, 72.90, 71.8, 70.8, 69.4, 68.4, 68.2, 67.5, 62.6, 61.13, 61.11, 60.2, 51.7, 39.7 (C_3'''), 31.1, 28.8, 28.5, 28.4, 25.1, 22.1 (-NHCO$\underline{C}H_3$), 22.0 (-OCO$\underline{C}H_3$), 13.4 (CH_3-octyl). HRMS (ESI) calculated for $C_{31}H_{54}NO_{19}Na$ $[M+Na]^+$ 744.3295, found: 744.3279.



***O*-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→6)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (2-28):**

N-Acetyl neuraminic acid (2.90 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol), 1 M $MgCl_2$ (80 μL) and distilled H_2O (600 μL) were suspended in 1M Tris-HCl buffer (400 μL, pH 8.8). The reaction was then charged with *Neisseria meningitidis CMP-Neu5Ac* (NmCSS)[48] (200 μL), octyl-β-lactoside, **2-6**, (2.5 mg, 6.2 μmol), *Photobacterium damsela* α(2→6)-sialyltransferase[50] (200 μL) and distilled H_2O

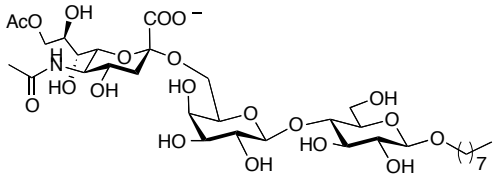
(600 μL). The reaction mixture was stirred overnight. After completion, ethanol was added to the reaction mixture. The reaction mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified by sep-pack C-18 reverse phase cartridge. The product was eluted with H_2O/ MeOH (2:1). Purified product **2-28** (3.82 mg; 83%) was obtained as a white solid after concentration under high vacuum. ^{1}H NMR (500 MHz, D_2O): δ = 4.44 (d, *J* = 8.1 Hz, 1H, H_1''), 4.39 (d, *J* = 7.6 Hz, 1H, H_1'), 3.97 – 3.46 (m, 20H), 3.28 (t, *J* = 8.4 Hz, 1H, H_2'), 2.67 (dd, *J* = 11.9, 5.0 Hz, 1H, H_{3e}'''), 2.01 (s, 3H, NHCOC$\underline{H}_3$), 1.70 (t, *J* = 12.2 Hz, 1H, H_{3a}'''), 1.63 – 1.54 (m, 2H, OCH_2), 1.35 – 1.19 (m, 10H, 5xCH_2), 0.82 (t, *J* = 6.6 Hz, 3H, CH_3-octyl). ^{13}C NMR (125 MHz, D_2O): δ = 174.9 (COO^-), 173.5 (NH$\underline{C}OCH_3$), 103.3 (C_1''), 101.9 (C_1'), 100.3 (C_2'''), 79.7, 74.8, 74.7, 73.7, 72.8, 72.6, 72.4, 71.8, 70.8, 70.8, 68.6, 68.5, 68.44, 68.42, 63.6, 62.7, 62.5, 60.3, 51.8, 40.2 (C_3'''), 31.1, 28.8, 28.5, 28.4, 25.1, 22.1, 22.0 (-NHCO$\underline{C}H_3$), 20.1 (-OCO$\underline{C}H_3$), 13.4 (CH_3-octyl). . HRMS (ESI) calculated for $C_{31}H_{54}NO_{19}Na$ $[M+Na]^+$ 744.3295, found: 744.3279.



***O*-(5-Acetamido-9-acetoxy-3,5,9-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)- (2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-octanol (2-25):**

5-Acetamido-9-*O*-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonic acid (2.75 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol), 1M $MgCl_2$ (80 μL) and distilled H_2O (600 μL) were suspended in 1M Tris-HCl buffer (400 μL, pH 7.2). The reaction was then charged with *Neisseria meningitides* CMP-Neu5Ac

synthetase (NmCSS)[48] (200 μL), octyl-β-lactoside, **2-6,** (2.5 mg, 6.2 μmol), *Pasteurella multocida* α(2→3)-sialyltransferase[49] (200 μL) and distilled H_2O (600 μL). The reaction mixture was stirred for 3 h. After completion, ethanol was added to the reaction mixture. The reaction mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified by sep-pack C-18 reverse phase cartridge. The product was eluted with H_2O/MeOH (1:2). Product **2-25** (1.8 mg; 37%) was obtained as a white solid after concentration of the fractions on high vacuum as a mixture of acetylated and non-acetylated product in 2:1 ratio. Low yield was observed due to formation of hydrolyzed product along with the acetylated one. ^{1}H NMR (400 MHz, D_2O): δ = 4.48 (d, *J* = 7.7 Hz, 1H, H_1''), 4.42 (d, *J* = 8.2 Hz, 1H, H_1'), 4.08 – 4.01 (m, 2H, H_3''), 3.97 – 3.46 (m, 25H), 3.24 (t, *J* = 8.3 Hz, 1H, H_2'), 2.74 (dd, *J* = 12.5, 4.6 Hz, 1H, H_{3e}'''), 2.08 (s, 2H, -O-CO-C<u>H</u>$_3$), 1.98 (s, 3H, -NH-CO-C<u>H</u>$_3$), 1.75 (t, *J* = 12.1 Hz, 1H, H_{3a}'''), 1.62 – 1.50 (m, 2H, -O-CH_2), 1.35 – 1.18 (m, 10H, 5xCH_2), 0.82 (t, *J* = 6.8 Hz, 3H, CH_3-octyl). ^{13}C NMR (125 MHz, D_2O): δ = 175.1 (COO^-), 173.9 (-NH<u>C</u>OCH_3), 102.7 (C_1''), 102.1 (C_1'), 99.9 (C_2'''), 78.4, 75.5, 75.2, 74.8, 74.5, 72.93, 72.89, 71.8, 70.8, 69.4, 68.4, 68.2, 67.5, 62.5, 61.1, 60.2, 51.7, 40.1 (C_3''') 31.2, 31.1, 28.8, 28.5, 28.4, 25.1, 23.3, 22.1 (-NHCO<u>C</u>H_3), 22.0 (-OCO<u>C</u>H_3), 13.4 (CH_3-octyl). HRMS (ESI) calculated for $C_{33}H_{56}NO_{20}^-$ $[M-H]^-$ 786.3401, found: 786.3406, 744.3289.



_O_-(5-Acetamido-9-acetoxy-3,5,9-trideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→6)-_O_-(β-D-galactopyranosyl)-(1→4)-_O_-(β-D-glucopyranosyl)-octanol (2-27):

5-Acetamido-9-*O*-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonic acid (2.75 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol), 1M $MgCl_2$ (80 μL) and distilled H_2O (600 μL) were suspended in 1M Tris-HCl buffer (400 μL, pH 7.2). The reaction was then charged with *Neisseria meningitides* CMP-Neu5Ac synthetase (NmCSS)[48] (200 μL), octyl-β-lactoside, **2-6,** (2.5 mg, 6.2 μmol), *Photobacterium damsela* α(2→6)-sialyltransferase[50] (200 μL) and distilled H_2O (600 μL). The reaction mixture was stirred for 3 h. After completion, ethanol was added to the reaction mixture. The reaction mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified by sep-pack C-18 reverse phase cartridge. The product was eluted with H_2O/MeOH (1:2). Purified product **2-27** (2.2 mg; 45%) was obtained as a white solid after concentration of the fractions on high vacuum. ^{1}H NMR (600 MHz, D_2O): δ = 4.44 (d, *J* = 8.0 Hz, 1H, H_1''), 4.39 (d, *J* = 7.7 Hz, 1H, H_1'), 4.17 (dd, *J* = 11.6, 5.6 Hz, 1H, H_3''), 4.09 – 4.04 (m, 1H), 3.96 – 3.47 (m, 25H), 3.28 (t, *J* = 8.5 Hz, 1H, H_2'), 2.67 (dd, *J* = 12.1, 4.0 Hz, 1H, H_{3e}'''), 2.08 (s, 3H, -O-CO-C$\underline{H}_3$), 1.98 (s, 3H, -NH-CO-C$\underline{H}_3$), 1.70 (t, *J* = 12.3 Hz, 1H, H_{3a}'''), 1.62 – 1.55 (m, 2H, -O-CH_2), 1.35-1.19 (m, 10H, 5xCH_2), 0.82 (t, *J* = 5.8 Hz, 3H, CH_3-octyl). ^{13}C NMR (125 MHz, D_2O): δ = 174.9 (COO^-), 174.4 (-O$\underline{C}OCH_3$), 173.5 (-NH$\underline{C}OCH_3$), 103.3 (C_1''), 101.9

(C_1'), 100.4 (C_2'''), 79.8, 74.8, 74.7, 73.7, 72.8, 72.6, 72.4, 72.1, 71.8, 70.8, 69.3, 68.6, 68.4, 65.7, 63.7, 62.7, 62.5, 60.4, 51.8, 40.2 (C_3'''), 31.2, 28.8, 28.6, 28.5, 28.4, 25.1, 22.1, 22.0 (-NHCO<u>C</u>H_3), 20.3 (-OCO<u>C</u>H_3), 13.4 (CH_3-octyl). HRMS (ESI) calculated for $C_{33}H_{56}NO_{20}^-$ $[M-H]^-$ 786.3401, found: 786.3396.

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Chapter 3

Studies towards inhibitors of the 9-*O*-acetyl sialic acid esterase (SIAE)

3.1 Introduction

The 9-*O*-acetyl sialic acid esterase (SIAE) is the enzyme that catalyzes the removal of the *O*-acetyl ester from the 9-position of naturally occurring sialic acids.[1] These enzymes are found in vertebrates and higher invertebrates, but not in plants or lower invertebrates.[1,2] The removal of acetyl groups from the 9-position of sialic acids by SIAE has been described in certain mammalian viruses, in human erythrocytes, and in murine and equine liver.[1,3-6] All of these organisms either contain sialic acid itself or modify residues from a host organism. Studies of SIAE enzymes have suggested that while they can cleave small synthetic esters, they are otherwise specific for sialic acid substrates.[3,4,6] However, the basis of specificity of these 9-*O*-acetylsialic acid esterases (SIAE) has not been explored in detail.[1] The *O*-acetylation and de-*O*-acetylation of sialic acids, which is regulated by two enzymes, namely, sialic acid transferase (SIAT; also known as the sialic acid *O*-acyltransferase, SOAT) and sialic acid acetylesterase (SIAE), are thought to be involved in a variety of biological processes. These include cell adhesion, endogenous lectin recognition, tumor antigenicity, virus binding, and complement activation.[2,5,6]

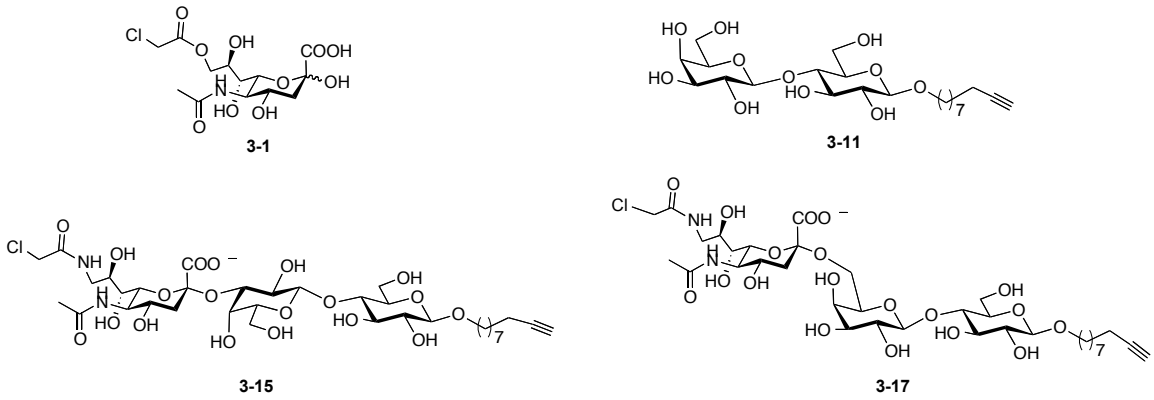
The sialic acid acetylesterase enzyme also plays essential roles in maintaining immunological tolerance by negatively regulating the B lymphocyte antigen receptor.[7] The BCR co-receptor, CD22, regulates the activation of B cells and hence involved in autoimmune diseases. The interaction of *cis* and *trans* sialosides with CD22 are implicated in the activation process.[8] Deacetylated sialosides act as ligands for CD22, which in turn suppress the activation of the BCR. The consequence of deactivation of the BCR can be immunodeficiency, autoimmunity, and B-cell malignancy.[9,10,11,12] In contrast,

the modification of sialic acid with acetylation by SIAT results in the loss of CD22 binding, thus the enzyme acts as an indirect activator of the BCR. Specific inhibitors of SIAE could be used to maintain the activation of the BCR, and could be used in immunotherapy or immunological research. Increased concentrations of acetylated sialic acid, which cannot interact with CD22, should result in activation of the BCR (**Figure 1.2, Chapter 1)**. Thus, SIAE inhibitors could be used as an essential tool for understanding how sialoside ligands balance CD22-BCR activation.

SIAE and its sequence homologues are novel members of the serine hydrolase superfamily.[13] Diisopropyl fluorophosphate (DFP), which is a covalent modifier of the active site serine nucleophiles of esterases, is known to inhibit the SIAE enzymes.[14] Previous studies have found that fluorophosphonates (FPs) are excellent affinity labels for many different serine hyrolases.[16,17] These organophosphorus reagents are however non-specific. As a result, the lack of specificity for organophosphorus reagents limits their utility for specific labeling of SIAE.[13] The active site of the SIAE enzymes is different from most serine hydrolases.[15] The amino acid sequence in the active site of SIAE contains arginine residues, which are not present in serine hydrolases.[1,15] Varki and coworkers have postulated that SIAE enzymes have serine active site nucleophiles with an essential arginine residue.[1]

In this chapter, we aimed to design and synthesize sialosides for labeling and inhibition of SIAE. Reagents that can covalently inhibit SIAE could be used to identify isoforms of the enzyme or detect its activity in specific tissues. We aimed to generate *C*-9-α-halo-ketones as candidate inhibitors of SIAE enzymes (**3-15** and **3-17**). We also proposed the synthesis of 9-*O*-chloroacetyl-Neu5Ac (**3-1**) so as to confirm that the 9-

position of Neu5Ac could be substituted in a single step by chloroacetate. Ketones with an α-halo group are well known as covalent inhibitors for esterase enzymes.[18,19] We describe the synthesis of a chloro-acetate analog of a known SIAE substrate, GM3. We also include an alkyne label (**3-11**) for the incorporation of additional probes that could be used for visualization and detection of SIAE by fluorescence spectroscopy or mass spectrometry (**Scheme 3.1**).

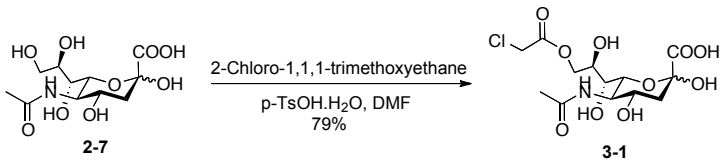


Scheme 3.1 Proposed inhibitors of SIAE

3.2 Results and Discussion

3.2.1 Synthesis of 9-O-chloroacetyl-Neu5Ac

The synthesis of 9-*O*-chloroacetyl-Neu5Ac was carried out as a model reaction to confirm that the 9-position of Neu5Ac could be substituted in a single step by chloroacetate analogous to the reactivity shown in **Scheme 2.2**.[25] We were pleased to find that preparation of the 9-*O*-chloroacetyl-Neu5Ac (**3-1**, **Scheme 3.2**) was achieved in good yield (79%) using 2-chloro-1,1,1-trimethoxyethane with catalytic amount of *p*-tosyl monohydtrate.



Scheme 3.2 One step synthesis of 9-*O*-chloroacetyl-Neu5Ac

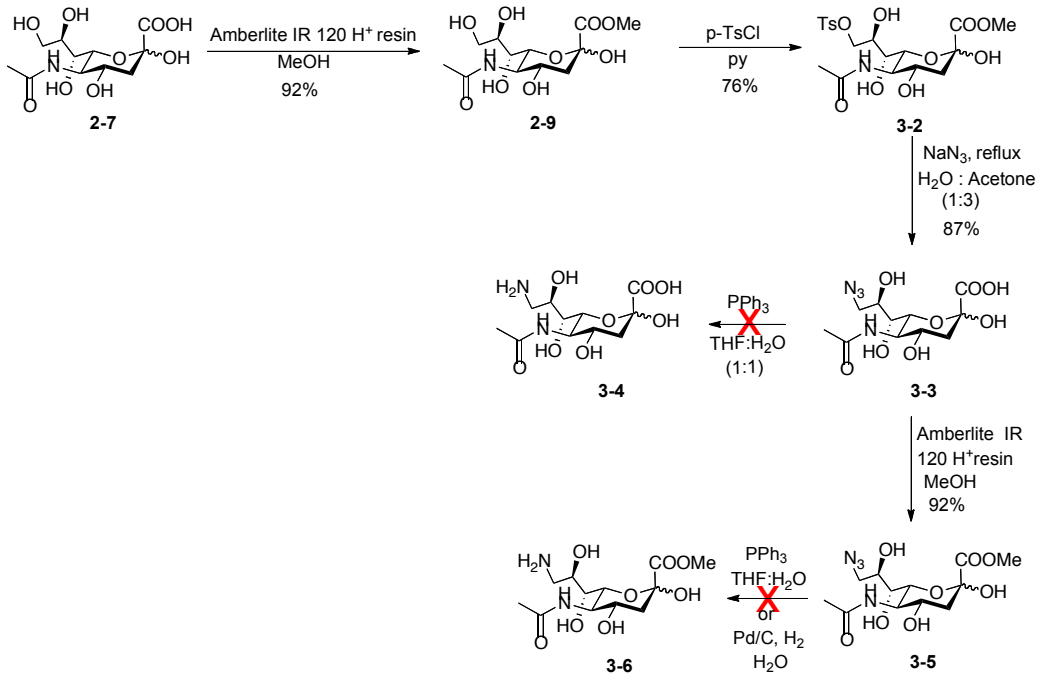
After achieving the one step synthesis of 9-*O*-chloroacetyl-Neu5Ac (**3-1**), we considered that the ester linkage of **3-3** could be unstable in biological systems. We then focused our attention on the preparation of the 9-amino-Neu5Ac to prepare the analogous amide derivative.

3.2.2 Synthesis of 9-amino-Neu5Ac

We started the synthesis of 9-amino-Neu5Ac from commercially available sialic acid (**2-7**). The sialic acid (**2-7**) was first converted to its methyl ester derivative (**2-9**) using Amberlite®-IR 120 H^+ resin in 92% yield. The methyl ester, **2-9**, was treated with *p*-tolulenesulfonyl chloride (TsCl) in presence of pyridine to furnish 9-*O*-Ts-Neu5Ac (**3-4**) in 76% yield (**Scheme 3.3**).[20] The substitution of an azido group at the C-9 of the tosyl derivative **3-2** was achieved by using sodium azide in a mixture of water–acetone (1:3) to afford 9-azido-Neu5Ac (**3-3**) in 87% yield. We then attempted to reduce the azide functionality of **3-3** using triphenyl phosphine (PPh_3) with a THF–H_2O mixture to give 9-amino-Neu5Ac (**3-4**). Unfortunately, this reaction did not provide expected product but only starting material was detected.

To test if the free carboxylic acid at *C*1 of the Neu5Ac residue was interfering with the reduction we examined methyester analogs of 9-azido-Neu5Ac. Compound **3-5** was generated in good yield using standard conditions (80% yield). The methylester, **3-5**, was also unreactive with triphenyl phosphine (PPh_3) in a THF–H_2O solution. Alternative

reduction conditions, including Pd/C under a hydrogen atmosphere, were unable to generate the reduced 9-amino-methyester-Neu5Ac (**3-6**).



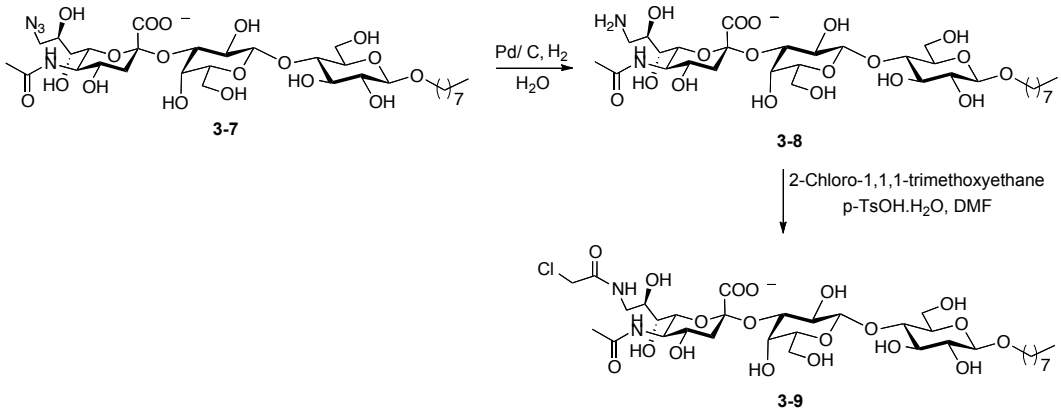
Scheme 3.3 Planned synthetic route to 9-amino-Neu5Ac

Based on these results, we decided to first synthesize the sialoside using 9-azido-Neu5Ac and 9-decynelactose and then develop a strategy for reduction of the azide group followed by chloro-acetate substitution to give the desired product.

3.2.3 Synthesis of 9-azido-sialyl octyl lactoside

We based our synthetic route for synthesis of the 9-chloroacetamido-sialyl-9-decyne-lactose (**3-15** and **3-17**) on the test reactions between 9-amino-sialyl-octyllactose (**3-8**)[20] and 2-chloro-1,1,1-trimethoxyethane in the presence of a catalytic amount of *p*-toluenesulphonyl monohydrate in DMF to give 9-chloroacetamido-sialyl octyl-lactoside

(**3-9**) by TLC. The 9-amino-sialyl-octyllactose (**3-8**) was formed by the reduction of 9-azido-sialyl-octyllactose (**3-7**) using Pd/C (**Scheme 3.4**).[20]

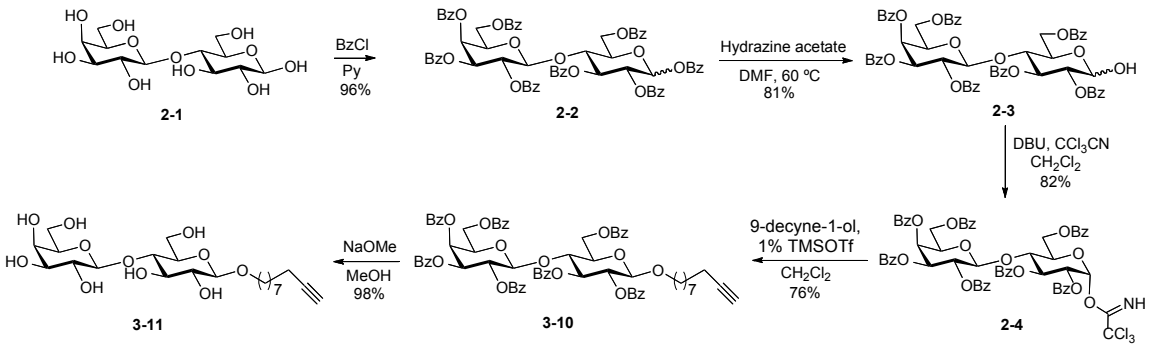


3-7 3-8 3-9

Scheme 3.4 Synthesis of 9-chloroacetamido-sialyl-octyllactoside

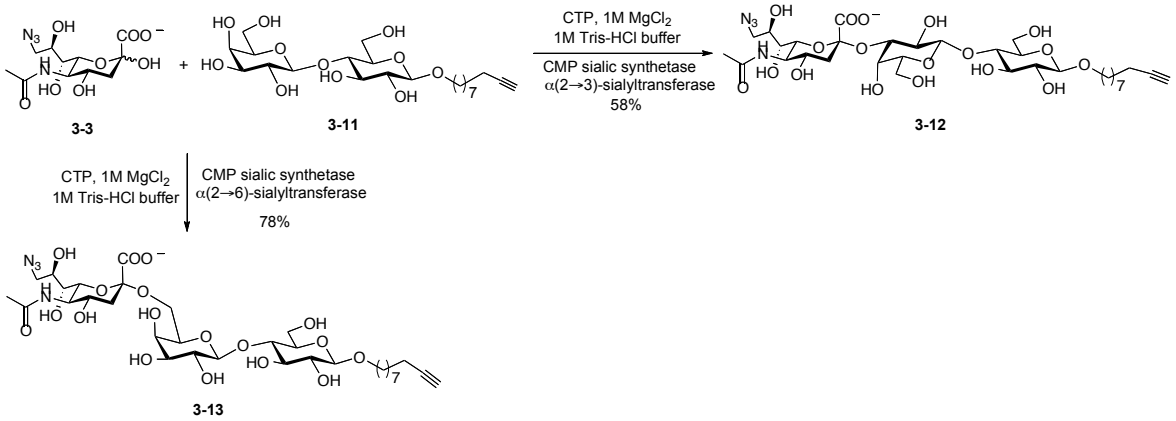
3.2.4 Synthesis of 9-decyne-lactoside

We next synthesized 9-decyne-lactoside (compound **3-11**) as a starting material for the target glycolipid analog of GM3 (compound **3-15** and **3-17**). The terminal alkyne was intended for use in future reactions by Cu-catalyzed azide-alkyne cycloaddition (CuAAC). The strategy for synthesizing 9-decyne-lactoside was based on the synthesis *β*-octyl-lactoside (**Scheme 2.1**, **Chapter 2**). The strategy is summarized in **Scheme 3.5**, with the first three steps identical to those of **Scheme 2.1**. The benzoyl-protected 9-decynelactoside (**3-10**, 73% yield) was synthesized after the completion of the glycosylation reaction between the perbenzoylated lactosyl trichloroacetimidate glycosyl donor (**2-4**) and 9-decyne-1-ol under acidic conditions. The benzoyl-protected 9-decynelactose (**3-10**) was then subjected to debenzoylation in the presence of freshly prepared sodium methoxide to provide 9-decyne-lactoside (**3-11**, 98% yield).



Scheme 3.5 Synthesis of 9-decyne-lactoside

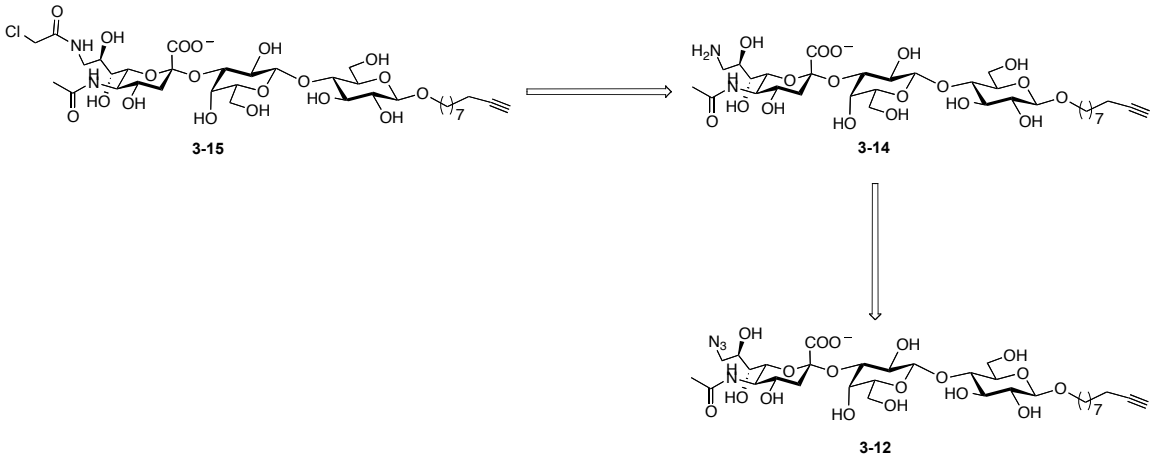
Following chemoenzymatic methods developed by Chen and coworkers, we prepared the 9-azido trisaccharides using the monosaccharides **3-3** and substrate **3-11**.[21,22] Both α(2→3) and α(2→6) linked sialosides were prepared in moderate to excellent yield (**Scheme 3.6**).



Scheme 3.6 Synthesis of 9-azido-sialyl-dec-9-yne-lactoside

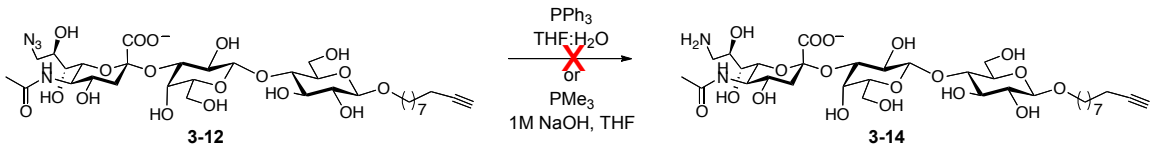
3.2.5 Synthesis of 9-chloroacetamido-sialyl-dec-9-yne-lactoside

For the formation of targets **3-15** and **3-17**, the reduction of 9-azido trisaccharides (**3-12** and **3-13**), followed by substitution with 2-chloro-1,1,1-trimethoxyethane was envisioned (**Scheme 3.7**).



Scheme 3.7 Steps involved in synthesis of target substrate of SIAE

The reduction of 9-azido-sialyl-dec-9-yne-lactoside (**3-12**) was attempted using triphenylphosphine in THF:H_2O. Unfortunately, the Staudinger reduction of **3-12** was not successful under these conditions. Attempts with more reactive conditions, i.e., trimethylphosphine and NaOH in THF were also unsuccessful, with isolation of the starting material (**Scheme 3.8**).



Scheme 3.8 Attempted reduction of 9-azido-sialyldec-9-yne-lactoside

3.3 Conclusion

The azide derivatives (**3-12** and **3-13**) were successfully synthesized via chemoenzymatic route. However, the target molecules 9-chloroacetamido-sialyl-9-decyne-lactoside (**3-15** and **3-17**) could not be synthesized even after trying different reaction conditions. We were successful in synthesizing the 9-*O*-chloroacetyl-Neu5Ac (**3-**

1) in a single step. Also, the synthesis of trisaccharide **3-9** shows that the chloroacetyl group can be specifically incorporated at 9-position of sialic acid in a trisaccharide. Future work will need to develop reduction conditions suitable for conversion of **3-12** to **3-14** to allow access to targets **3-15** and **3-17**.

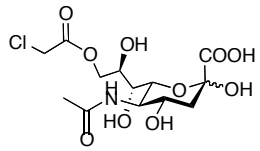
3.4 Experimental methods

3.4.1 General

All reagents were purchased from commercial sources and were used without further purification unless noted otherwise. Reaction solvents were purified by successive passage through columns of alumina and copper under an argon atmosphere using Innovative Technology, Inc. PURE SOLV (SPS-400-7). All reactions were performed under a positive pressure of argon at room temperature unless specified otherwise. The reactions were monitored by analytical TLC on silica gel 60-F_{254} (0.25 nm, Silicycle, Quebec, Canada) and visualization of the reaction components was achieved using UV fluorescence (254 nm) and/or by charring with acidified anisaldehyde solution in ethanol, Ceric Ammonium Molybdate (CAM) or orcinol stain. Organic solvents were evaporated under reduced pressure at 40 ºC. Reaction products were purified by column chromatography on silica gel (230–400 mesh, Silicycle, Quebec, Canada), Iatrobeads 6RS-8060 (Shell-USA Inc.) if the eluent system contained greater than 10% methanol and by reversed phase C-18 silica with MeOH and H_2O as eluents. Flash column chromatography was performed using a Combiflash companion chromatography instrument (Teledyne Isco, Inc., Lincoln, NE) with Redisep and Silicycle flash silica gel columns (40-63 μ m). The yields reported are after purification. NMR experiments were conducted on Varian 400, 500, 600, and 700 MHz instruments. Chemical shifts are

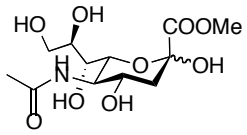
reported relative to the deuterated solvent peaks in parts per million. Assignments of the NMR spectra were based on one-dimensional experiments (APT) and/or two-dimensional experiments ($^{1}H–^{1}H$ COSY, $^{1}H–^{13}C$ HSQC and $^{1}H–^{13}C$ HMBC). Electrospray mass spectra (ES-MS) were recorded on Agilent Technologies 6220 TOF. For ES-MS spectra, samples were dissolved in $CHCl_3$ or CH_3OH and NaCl was added.

3.4.2 Synthetic methods



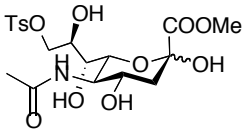
5-Acetamido-9-*O*-(2-cholroacetyl)-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonic acid (3-1):

2-chloro-1,1,1-trimethyoxyethane (0.23 mL, 1.62 mmol) and p-TsOH.H_2O (2 mg) was added to a solution of *N*-Acetyl neuraminic acid (50 mg, 0.16 mmol) in DMF (0.5 mL). After being stirred for 2 h under argon, the reaction mixture was applied directly to a Dowex 1-X 8 ($HCOO^-$) anion exchange resin. The column was washed with H_2O (50 mL) and then the compound was eluted with formic acid (1N, 25 mL). The eluent was lyophilized over 48 h to yield 48.5 mg (78.7%) of **3-3** as a white solid. ^{1}H NMR (400 MHz, D_2O): δ = 4.44 (d, *J* = 11.5, Hz, 1H, H_9), 4.30 (dd, *J* = 11.4, 6.3 Hz, 1H, H_9), 4.27 (s, 2H, -COC$\underline{H}_2$Cl), 4.03 – 3.93 (m, 3H), 3.91 – 3.85 (m, 2H), 3.56 (d, *J* = 8.9, 1H), 2.18 (dd, *J* = 12.8, 4.7 Hz, 1H, H_{3e}), 2.02 (s, 3H, -NHCOC$\underline{H}_3$), 1.79 (app.t, *J* = 12.2 Hz, 1H, H_{3a}). ^{13}C NMR (126 MHz, D_2O): δ = 171.0 (CO), 170.0 (C_1), 71.0, 69.2, 68.8 (C_9,) 68.7, 68.2 (C_5), 53.2 (C_3), 42.1 (-CO$\underline{C}H_2$Cl) 40.3 (NH-CO-$\underline{C}H_3$), 23.1 (-O-CO-$\underline{C}H_3$). HRMS (ESI) calculated for $C_{13}H_{20}ClNO_{10}$ $[M-H]^-$ 384.0776, found: 384.0698.



_N_-acetyl neuraminic acid methlyester (2-9)[20]:

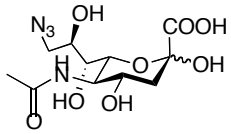
To a solution of *N*-Acetyl neuraminic acid (2.0 g; 6.46 mmol) in 75 ml MeOH was added 10 g Amberlite IR-120 (H^+ resin) and the mixture was stirred overnight. The reaction changed from cloudy white to colorless. The reaction mixture was filtered and concentrated, to give **2-9** as a white solid (91.6% yield). ^{1}H NMR (500 MHz, D_2O): δ = 4.14 – 4.02 (m, 2H, H_4, H_6), 3.94 (t, *J* = 10.3 Hz, 1H, H_5), 3.90 – 3.82 (m, 4H, H_{9a}, -OCH_3), 3.75 (ddd, *J* = 9.0, 6.3, 2.6 Hz, 1H, H_{9b}), 3.68 – 3.60 (m, 1H, H_8), 3.57 (d, *J* = 9.2 Hz, 1H, H_7), 2.33 (dd, *J* = 13.1, 4.8 Hz, 1H, H_{3e}), 2.06 (s, 3H, NH-CO-C$\underline{H}_3$), 1.94 (dd, *J* = 12.9, 11.9 Hz, 1H, H_{3a}). ^{13}C NMR (125 MHz, D_2O): δ = 175.7 (C$\underline{O}$OOMe), 172.2 (C_1), 71.2 (C_2), 71.0, 69.1, 67.5 (C_9), 64.0 (C_5), 54.3, 52.9 (C_3), 39.5 (NH-CO-C$\underline{H}_3$), 22.9 (-COO-C$\underline{H}_3$). HRMS (ESI) calculated for $C_{12}H_{21}NO_9Na$ $[M+Na]^+$ 346.1109, found: 346.1111.



5-Acetamido-9-*O*-tosyl-3,5-dideoxy-D-*glycero*-D-*galacto*-2-nonulosonic methyl ester (3-2)[20]:

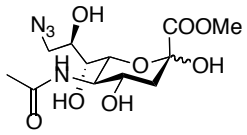
To a solution of Neu5Ac methyl ester **2-9** (483 mg; 1.5 mmol) in pyridine (15 mL) was added *p*-toluensulfonyl chloride (429 mg, 2.25 mmol) which was cooled to -10 °C. After 1 hour at -10 °C, the mixture was brought to 0-5 °C overnight with stirring. After completion of the reaction, pyridine was removed. The residue was purified by flash chromatography using 0 to 5% methanol/ethyl acetate. The product was obtained as a

white solid (538 mg, 76%). ^{1}H NMR (500 MHz, D_2O): δ = 7.78 (d, *J* = 8.4 Hz, 2H, ArH), 7.45 (d, *J* = 8.4 Hz, 2H, ArH), 4.25 (dd, *J* = 10.5, 2.3 Hz, 1H, H_{9b}), 4.15 (dd, *J* = 10.5, 5.0 Hz, 1H, H_{9a}), 4.08 (q, *J* = 7.2 Hz, 1H, H_4), 4.03 – 3.92 (m, 2H, H_6) 3.90 – 3.70 (m, 5H, H_5, H_8, OCH_3), 3.50 (d, *J* = 9.2 Hz, 1H, H_7), 2.48 (s, 3H, -ArC$\underline{H}_3$), 2.24 (dd, *J* = 13.0, 4.9 Hz, 1H, H_{3e}), 2.07 (s, 3H, -NH-CO-C$\underline{H}_3$), 1.86 (dd, *J* = 13.0, 11.6 Hz, 1H, H_{3a}). ^{13}C NMR (125 MHz, D_2O): δ = 175.0 (CO), 171.3 (C_1), 146.7 (ArC), 130.6 (ArC), 130.3 (ArC), 127.9 (ArC), 95.3, 72.6, 70.2, 67.7, 67.6 (C_9), 66.6 (C_5), 61.8, 53.5 (C_3), 52.1, 38.7 (NH-CO-$\underline{C}H_3$), , 22.1, 20.9 (-O-CO-$\underline{C}H_3$), 13.3. HRMS (ESI) calculated for $C_{19}H_{27}NO_{11}SNa$ $[M+Na]^+$ 500.1203, found: 500.1201.



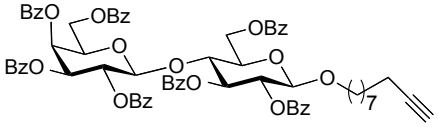
5-Acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-D-*galacto*-2-nonulosonic acid (3-3)[20]:

Compound **3-4** (365 mg; 0.77 mmol) and sodium azide (248 mg; 3.82 mmol) were refluxed at 75 ºC in water:acetone (12 mL; 1:3) for 12 h. The solvent was removed under reduced pressure, and the product was purified by flash chromatography (80% isopropanol in water/ethyl acetate; 0 to 50% linear gradient) to obtain **3-5** (226 mg; 88.6%). ^{1}H NMR (500 MHz, D_2O): δ = 4.10 – 3.99 (m, 2H, H_9), 3.92 – 3.86 (m, 2H, H_4, H_6), 3.59 (dd, *J* = 13.2, 2.8 Hz, 1H, $H_{5/8}$), 3.50 (dd, *J* = 9.2, 1.0 Hz, 1H, $H_{5/8}$), 3.46 (dd, *J* = 13.0, 6.1 Hz, 1H, H_7), 2.19 (dd, *J* = 13.0, 4.8 Hz, 1H, H_{3e}), 2.08 (s, 3H, NHCOC$\underline{H}_3$), 1.87 (t, *J* = 12.6 Hz, 1H, H_{3a}). ^{13}C NMR (125 MHz, D_2O): δ = 176.7 (COO^-), 174.8 (NH$\underline{C}OCH_3$), 171.2 (C_1), 96.4, 70.1, 69.1, 69.0 (C_9), 67.3 (C_5), 67.28, 53.9 (C_3), 52.3, 39.4 (NH-CO-$\underline{C}H_3$), 22.1. HRMS (ESI) calculated for $C_{11}H_{17}N_4O_8$ $[M-H]^-$ 333.1052, found: 333.1054.



9-azido *N*-acetyl neuraminic acid methlyester (3-5)[20]:

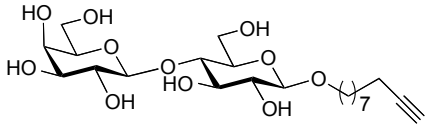
To a solution of 9-azido *N*-Acetyl neuraminic acid **3-5** (30 mg; 0.09 mmol) in 3 ml MeOH was added 210 mg Amberlite IR-120 (H^+ resin). The mixture was stirred overnight. The reaction mixture turned from cloudy white to colorless. The mixture was filtered and the filtrate was concentrated to give 25 mg of **3-7** (79.8% yield) as a white solid. ^{1}H NMR (500 MHz, D2O): δ = 4.17 – 4.01 (m, 2H, H_9), 3.92 – 3.84 (m, 2H, H_4, H_6), 3.78 (s, 3H, OCH_3), 3.59 (dd, *J* = 13.2, 2.8 Hz, 1H, $H_{5/8}$), 3.54 (dd, *J* = 9.2, 1.0 Hz, 1H, $H_{5/8}$), 3.47 (dd, *J* = 13.0, 6.1 Hz, 1H, H_7), 2.30 (dd, *J* = 13.0, 4.8 Hz, 1H, H_{3e}), 2.04 (s, 3H, NHCO$\underline{C}H_3$), 1.89 (t, *J* = 12.6 Hz, 1H, H_{3a}). ^{13}C NMR (125 MHz, D2O): δ = 174.8 (CO), 171.4 (C_1), 95.4, 70.3 (C_2), 69.0 (C_9), 68.8, 68.7 (C_9), 66.8, 66.7, 53.9 (C_3), 53.5, 38.7 (NH-CO-$\underline{C}H_3$), 22.1 (COO$\underline{C}H_3$). HRMS (ESI) calculated for $C_{12}H_{20}N_4O_8Na$ $[M+Na]^+$ 371.1179, found: 371.1173.



***O*-2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl-dec-9-yne (3-10):**

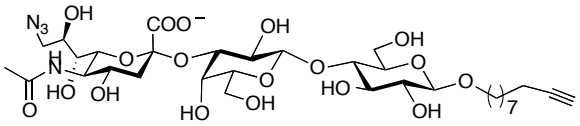
9-Decyne-1-ol (51.57 µL, 0.30 mmol) was added to a reaction mixture of compound **2-4** (235 mg, 0.20 mmol) and activated molecular sieves (2 g) in dry CH_2Cl_2 (12 mL). The reaction mixture was charged with 1% TMSOTf (trimethylsilyl trifluoromethanesulfonate) (0.3 mL in CH_2Cl_2) at 0 °C. The reaction was allowed to warm to room temperature and then stirred overnight. After completion, the reaction

mixture was filtered on a pad of Celite. The product was purified by column chromatography (0 to 30% EtOAc in hexane) to yield 175 mg (72.5%) of **3-1**. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.07 – 7.97 (m, 13H, ArH), 7.92 (d, *J* = 7.3 Hz, 2H, ArH), 7.76 (d, *J* = 7.3 Hz, 2H, ArH), 7.63 (ddd, *J* = 22.2, 14.9, 7.5 Hz, 3H, ArH), 7.52 (q, *J* = 7.9 Hz, 5H, ArH), 7.47 – 7.31 (m, 9H, ArH), 7.25 (t, *J* = 7.8 Hz, 2H, ArH), 7.17 (t, *J* = 7.8 Hz, 2H, ArH), 5.83 (t, *J* = 9.5 Hz, 1H, H_3'), 5.77 – 5.72 (m, 2H, H_2', H_2''), 5.48 (dd, *J* = 9.7, 7.9 Hz, 1H, H_4''), 5.40 (dd, *J* = 10.4, 3.4 Hz, 1H, H_3''), 4.90 (d, *J* = 7.9 Hz, 1H, H_1''), 4.71 (d, *J* = 7.9 Hz, 1H, H_1'), 4.63 (dd, *J* = 12.1, 1.8 Hz, 1H, H_6'), 4.52 (dd, *J* = 12.2, 4.5 Hz, 1H, H_6'), 4.28 (t, *J* = 9.5 Hz, 1H, H_4'), 3.93 – 3.70 (m, 4H, H_5', H_5'', H_6''), 3.49-3.43 (m, 1H, H_6''), 2.13 (td, *J* = 7.5, 2.6 Hz, 2H, ≡CH-C$\underline{H}_2$), 1.98 (t, *J* = 9.6 Hz, 2H, O-CH_2), 1.60 – 1.00 (m, 12H, ≡CH-$CH_2C\underline{H}_2$, 5xCH_2). ^{13}C NMR (126 MHz, $CDCl_3$): δ = 165.9 (ArC), 165.6 (ArC), 165.5 (ArC), 165.4 (ArC), 165.3 (ArC), 165.2 (ArC), 164.8 (ArC), 133.5 (ArC), 133.4 (ArC), 133.37 (ArC), 133.35 (ArC), 133.3 (ArC), 133.2 (ArC), 133.1 (ArC), 130.0 (ArC), 129.8 (ArC), 129.7 (ArC), 129.6 (ArC), 129.61 (ArC), 129.5 (ArC), 128.9 (ArC), 128.7 (ArC), 128.68 (ArC), 128.6 (ArC), 128.5 (ArC), 128.52 (ArC), 128.3 (ArC), 128.26 (ArC), 128.2 (ArC), 101.2 (C_1''), 100.9 (C_1'), 84.8 (C_4'), 73.0 (C_3''), 72.9, 71.8, 71.83 (C_5'), 71.4 (C_5''), 70.3 (C_3'), 69.9 (C_2'), 68.0 (C≡C), 67.5 (C_2''), 62.5 (C_6''), 61.1, 29.3, 29.0, 28.9, 28.6, 28.4, 25.7, 18.3. HRMS (ESI) calculated for $C_{71}H_{66}O_{18}Na$ $[M+Na]^+$ 1229.4147, found: 1229.4135.



***O*-(β-D-galactopyranosyl-(1→4)-*O*-(β-D-glucopyranosyl)-dec-9-yne (3-11):**

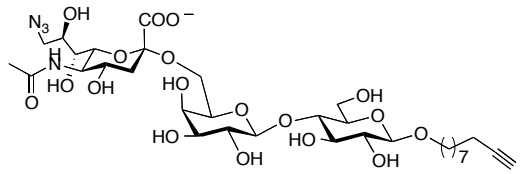
To a solution of compound **3-1** (175 mg, 0.25 mmol) in dry MeOH (5 mL) was added 1% freshly prepared NaOMe (1.9 mL). The reaction mixture was stirred for 24 h and monitored by TLC (1:5, MeOH:CH_2Cl_2; *Rf* = 0.2 to 0.3). After completion of the reaction, the mixture was neutralized to pH 7 by adding Amberlite IR 120 acidic resin. The reaction mixture was filtered from the resin and concentrated under reduced pressure. The crude product was purified by column chromatography by eluting with 0 to 40% of MeOH in CH_2Cl_2 to yield 68 mg (98%) as a white solid. ^{1}H NMR (400 MHz, D_2O): δ = 4.42 (d, *J* = 8.0 Hz, 2H, H_1', H_1''), 3.75 – 3.46 (m, 15H), 3.25 (m, 1H, H_2'), 2.13 (td, *J* = 7.5, 2.6 Hz, 2H, ≡CH-C<u>H</u>$_2$), 1.58 (m, 2H, O-CH_2), 1.47 (m, 2H, ≡CH-CH_2C<u>H</u>$_2$), 1.38 – 1.24 (m, 10H, 5xCH_2). ^{13}C NMR (126 MHz, D_2O): δ = 103.0 (C_1''), 102.1 (C_1'), 78.5 (C_4'), 75.4 (C_3''), 74.8 (C_5'), 74.5 (C_5''), 72.9 (C_3'), 72.6, 71.0 (C_2'), 68.9 (C≡C), 68.6 (C_2''), 61.1 (C_6'), 54.3 (C_6''), 28.7, 28.3, 28.1, 27.9, 27.7, 25.0, 17.5. HRMS (ESI) calculated for $C_{22}H_{38}O_{11}Na$ $[M+Na]^+$ 501.2312, found: 501.2306.



***O*-(5-Acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-dec-9-yne (3-12):**

9-azido sialic acid, **3-5**, (5.24 mg; 0.02 mmol), cytidine triphosphate disodium salt (8.22 mg; 0.02 mmol), $MgCl_2$ (1M, 160 µL) and distilled H_2O (1200 µL) were dissolved in 1M

Tris-HCl buffer (800 μL; pH 8.8). The reaction was then charged with *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)[22] (200 μL), dec-9-yne-β-lactoside, **3-2**, (5.0 mg, 0.10 mmol), *Pasteurella multocida* α(2→3)-sialyltransferase[23] (200 μL), and distilled H_2O (1200 μL). The reaction was stirred overnight at 37 ºC. After completion, ethanol was added to the reaction mixture. The reaction mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified over a sep-pack C-18 reverse phase cartridge. The product was eluted with H_2O/MeOH (2:1). Pure product **3-9** (4.8 mg; 58%) was obtained as a white solid after concentrated the fractions on high vacuum. ^{1}H NMR (400 MHz, D_2O): δ = 4.46 (d, *J* = 7.9 Hz, 1H, H_1''), 4.41 (d, *J* = 7.9 Hz, 1H, H_1'), 4.14 (dd, *J* = 9.9, 2.8 Hz, 1H, H_1''), 4.00-3.00 (m, 22H), 3.24 (t, *J* = 8.5 Hz, 1H, H_2'), 2.71 (dd, *J* = 12.4, 4.6 Hz, 1H, H_{3e}'''), 2.15 (t, *J* = 7.1 Hz, 2H, ≡CH-C<u>H</u>$_2$) 1.98 (s, 3H, -NH-CO-C<u>H</u>$_3$), 1.74 (t, *J* = 12.2 Hz, 1H, H_{3a}'''), 1.62 – 1.53 (m, 2H, O-CH_2), 1.50 – 1.42 (m, 2H, ≡CH-CH_2-C<u>H</u>$_2$), 1.38 – 1.22(m, 10 H, 5xCH_2). ^{13}C NMR (125 MHz, D_2O): δ = 176.0 (COO^-), 174.8 (-NH<u>C</u>OCH_3), 103.6 (C_1''), 103.0 (C_1'), 100.8 (C_2'''), 79.3, 76.6, 76.2, 75.8, 75.7, 75.4, 73.8, 73.7, 71.7, 71.4, 70.4, 69.8, 69.3, 68.4, 62.0, 61.2, 54.1, 52.7, 43.5, 40.7 (C_3'''), 31.6, 29.7, 29.2, 29.0, 28.8, 28.6, 25.9, 23.0 (-NHCO<u>C</u>H_3), 20.1, 18.4 (≡CH-<u>C</u>H_2), 13.7. HRMS (ESI) calculated for $C_{33}H_{53}N_4O_{18}$ [M-H]$^-$ 793.3360, found: 793.3352.



***O*-(5-Acetamido-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2→6)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyl)-dec-9-yne (3-13):**

9-azido sialic acid, **3-5**, (5.24 mg; 0.02 mmol), cytidine triphosphate disodium salt (8.22 mg; 0.02 mmol), $MgCl_2$ (1M, 160 μL) and distilled H_2O (1200 μL) were dissolved in 1M Tris-HCl buffer (800 μL; pH 8.8). The reaction was charged with *Neisseria meningitidis* CMP-sialic acid synthetase (NmCSS)[22] enzyme preparation (200 μL), dec-9-yne-β-lactoside, **3-2**, (5.0 mg, 0.10 mmol), *Photobacterium damsela* α(2→6)-sialyltransferase[24] (200 μL), and distilled H_2O (1200 μL). The reaction was stirred overnight at 37 ºC. After completion, ethanol was added to the reaction mixture. The reaction mixture was centrifuged and the supernatant was then lyophilized. The crude product was purified over a sep-pack C-18 reverse phase cartridge. The product was eluted with H_2O/MeOH (2:1). Pure product **3-10** (6.2 mg; 78%) was obtained as a white solid after concentration of the fractions on high vacuum. ^{1}H NMR (400 MHz, D_2O): δ = 4.43 (d, *J* = 8.1 Hz, 1H, H_1''), 4.37 (d, *J* = 7.6 Hz, 1H, H_1'), 4.00 – 3.41 (m, 22H), 3.27 (t, *J* = 8.4 Hz, 1H, H_2'), 2.65 (dd, *J* = 12.4, 4.6 Hz, 1H, H_{3e}'''), 2.15 (t, *J* = 7.0 Hz, 2H, ≡CH-C$\underline{H}_2$) 1.98 (s, 3H, -NH-CO-C$\underline{H}_3$), 1.68 (t, *J* = 12.2 Hz, 1H, H_{3a}'''), 1.62 – 1.53 (m, 2H, O-CH_2), 1.50-1.42 (m, 2H, ≡CH-CH_2 -C$\underline{H}_2$), 1.38-1.22(m, 10 H, 5xCH_2). ^{13}C NMR (125 MHz, D_2O): δ = 175.8 (COO^-), 174.4 (-NH$\underline{C}OCH_3$), 104.2 (C_1''), 102.9 (C_1'), 101.3 (C_2''), 80.7, 75.7, 75.6, 74.7, 73.7, 73.4, 73.3, 71.8, 71.7, 71.3, 70.0, 69.5, 69.3, 64.6, 61.3, 54.1, 52.8, 41.1, 29.7, 29.3, 29.0, 28.8, 28.6, 25.9, 23.1 (NHCO$\underline{C}H_3$), 18.4 (≡CH-$\underline{C}H_2$). HRMS (ESI)

calculated for $C_{33}H_{53}N_4O_{18}$ $[M-H]^-$ 793.3360, found: 793.3364.

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Chapter 4

Summary and future work

4.1 Summary

In this thesis, we describe the use of chemoenzymatic methods to generate a panel of novel sialic acid-containing trisaccharides for use in enzymatic studies. We were successful in synthesizing the target analogs of GM3 sialosides, which included variation of the glycosidic linkage α(2→3) and α(2→6) as well as the uncommon sialosides Neu5Gc and 9-*O*-Ac-Neu5Ac (**Figure 2.3**, **Chapter 2**). We employed a chemoenzymatic strategy based on established methods.[1,2] The most challenging aspects were purification and synthesis of 9-*O*-acetate trisaccharides due to susceptibility of the *O*-Ac groups to hydrolysis at pH 8.8 (optimal for chemoenzymatic reactions). We were able to overcome these issues by optimizing the chemoenzymatic reaction conditions by reducing the pH and reaction time. Purifications were carried out using C-18 sep pak columns by slowly decreasing the polarityof eluent in order to avoid the mixture of acetylated and non-acetylated trisaccharides.

The primary focus of this work was the generation of modified sialosides for enzymatic substrate studies. However, we also considered that the 9-*O*-Ac-sialosides are regulated by the action of sialic acid esterase (SIAE) and acetyltransferase (SIAT) enzymes.[3,4] Although esterase enzymes have been studied using a variety of methods, the SIAE has not been extensively studied.[3] We developed a method for the synthesis of 9-chloroacetate and 9-chloroacetamide sialosides. Future work will be required to complete the synthesis of the target substrate 9-chloroacetamide trisaccharides (**Scheme 3.7**, **Chapter 3**).

4.2 Future Work

With the acetyl and glycolyl analogs of the GM3 oligosaccharide prepared, several lines of investigation are now possible. The first of these would be to test the substrate activity of these compounds against each of the human neuramindase isoenzymes (NEU1, NEU2, NEU3 and NEU4). Previous work from our group has used a mass spectrometry-based assay to examine the substrate activity of GM3 analogs that varied in their lipid aglycone.[5] Substrates shown in **Figure 2.3**, **Chapter 2** could be screened by using this mass spectrometry-based assay.

Other high-throughput screening methods using chromophore, fluorophore, or chemilluminescent labeled sialic acids have several limitations.[6-10] First is the difficulty in chemically synthesizing naturally occurring sialic acid residues directly linked to the label. Due to this difficulty, most of these studies are limited to a few naturally occurring sialic acid forms.[11] Secondly; these compounds do not study the effect of sialyl linkages and the structure of respective monosaccharide on sialidase substrate specificity.

To overcome these limitations, Chen and coworkers have reported a coupled enzyme colorimetric assay for substrate specificity studies of sialidases.[14] This assay allows for the systematic study of the effect of the sialic acid structures, sialyl linkages, and the structures of the respective monosaccharide residues on the sialidase substrate specificity using a convenient and commonly accessible 96-well plate based high-throughput screening format.[14] However, this method requires the use of para-nitrophenol attached to the anomeric position of di- or trisaccharide substrates.

Another high-throughput method for quantifying glycoprotein sialylation is by using malonitrile as a fluorophore, which is added during the assay.[12] This method

requires three steps: chemical reduction, enzymatic release of sialic acid, and chemical derivatization of the sialic acid using malononitrile (fluorophore).[12,13] This method is accurate, rapid (15 min), and specific.

The naturally occurring modifications at C-9 (9-*O*-AcNeu5Ac) and N-5 (Neu5Gc) are hypothesized to show reduced activity based on the results reported by Sandbhor and coworkers.[5] They found that GM3 analogs that contain an octyl chain are expected to be good substrates for NEU3 and are much simpler to generate the large modifications at the *C*-9 and *N*-5 positions of Neu5Ac inhibited the NEU3 activity. Also, the 9-*O*-acetyl-GD3 have been reported with strongly reduced cleavage by membrane-bound ganglioside sialidase with only 12% relative activity compared to GM3.[15] For the sialoside linkages, the α(2→3) linked sialosides generally shows 2-4 fold higher activity as compared to the α(2→6) linked sialosides.[5,16] Mass spectrometry based kinetic assays of these compounds (**Figure 4.1**) with human neuraminidase isoenzymes (NEU1, NEU2, NEU3 and NEU4) would provide an essential understanding of the role of these modifications in biological systems.

We designed the synthesis of the chloroacetate analog (**3-15** and **3-17**), which are expected to be the substrates for the SIAE enzyme. Although, we were able to synthesize an intermediate, 9-azido-sialyl-9-decyne lactose (**3-12** and **3-13**) chemoenzymatically but unfortunately we were not able to reduce the azide to amine. However our test reaction for the substitution of chloroacetate specifically at 9-position of sialic acid was successful. Also, the synthesis of a trisaccharide with an octyl chain (**3-9**) shows that the chloroacetyl group can be specifically incorporated at 9-position of sialic acid in a trisaccharide. We propose the reduction of azide in presence of alkyne by using TCEP

(tris(carboxyethyl)phosphine)), Ascorbate in presence of Ru, modified staudinger reduction conditions (PPh_3, diethyl ether/ H_2O).[17,18]

The alkyne-modified version of this compound should be capable of labeling the SIAE isoform that reacts with lipid analogs. A "tag-free'' ABPP (activity-based protein profile) strategies will be used for the analysis of SIAE enzyme activity. This strategy is based on the Cu-catalyzed azide-alkyne cycloaddition reaction. Proteins are first labeled by an azido-sulfonate ester probe and then treated with an alkyne-tag under click-chemistry conditions. These types of compounds can be used to label components of the cell and be visualized using fluorescence.[19,20]

Future work will be required to confirm that this compound does covalently modify the enzyme (**Figure 1.**3, **Chapter 1**). If the compound is specific for SIAE, then it should be a useful probe to determine the number and prevalence of the SIAE within cell or tissue samples.

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Appendix

Immobilization strategy for synthesis of sialosides

In addition to solution-phase strategies for chemoenzymatic synthesis of sialosides, we developed an improved protocol for use of SiaT enzymes. Solution phase application of enzymes has several drawbacks. First, the enzyme is generally difficult to recover, and must be separated from the product. Additionally, the enzymes require fresh preparation before synthesis and can degrade over time requiring repeated preparations.

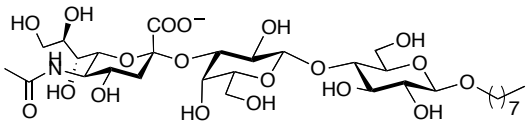
We considered that immobilization of the enzymes could be used to improve the protocol. The enzymes used were immobilized in calcium alginate as described below.[1] Immobilized enzymes were then used in the reaction mixture. The advantages of using immobilized enzymes over the enzymes in solution are easy purification (just filter the immobilized enzyme), reuse of the enzyme, and extended stability at 4 ºC. The reaction is somewhat slower than the solution phase equivalent, with typical times extended from one to two days for reaction completion with immobilized enzymes with similar yields. If purification is the concern, then immobilized enzymes strategy will be better over the enzymes in solution.

Preparation of immobilized enzymes with cross-linking reagents

Sodium Alginate (20 mg) was added to 0.5 mL of a solution containing *Neisseria meningitides CMP-NEU5Ac synthetase* and 0.5 ml of *Pasteurella multocida* α*-(2,3)-sialyltransferase*. Gluteraldehyde (40 mL) was added, and the mixture was stirred for 2 hours. The alginate solution was then added dropwise into 5 mL of 1% calcium chloride and was allowed to stand for 30 min, generating alginate gel beads. The gel beads were washed twice with 5 mL of 1M Tris-HCl buffer (pH 8.8) containing 0.1% of calcium chloride solution, followed by 1M Tris-HCl reaction buffer (pH 8.8) three times (the

same buffer which is used for chemoenzymatic reaction) and was then used for chemoenzymatic reaction.

Chemoenzymatic synthesis using immobilized enzymes



_O_-(5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2→3)-_O_-(β-D-galactopyranosyl)-(1→4)-_O_-(β-D-glucopyranosyl)-octanol (2-26):

N-Acetyl neuraminic acid (2.90 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol), 1M $MgCl_2$ (80 μL) and distilled H_2O (600 μL) were dissolved in 1M Tris-HCl buffer (400 μL pH 8.8). The reaction was then charged with resin - bound enzymes, octyl-β-lactoside, **2-6**, (2.5 mg, 6.2 μmol), (200 μL) and distilled H_2O (600 μL). The reaction mixture was stirred for 48 h. After completion, the resin was filtered and the reaction mixture was lyophilized. The crude product was purified over a sep-pack C-18 reverse phase cartridge. The product was eluted with MeOH/EA (1:1). The purified product **2-26** (4 mg; 87%) was obtained as a white solid after concentration of the fractions on high vacuum.

Stability of resin – bound enzymes

These resin - bound enzymes after filtration from the reaction mixture were stored at 4 ºC for two weeks. To check the stability of these enzymes a test reaction on the same substrate was carried out. The test reaction shows the formation of product on TLC. This demonstrates that the enzymes are still active and can be reused again.

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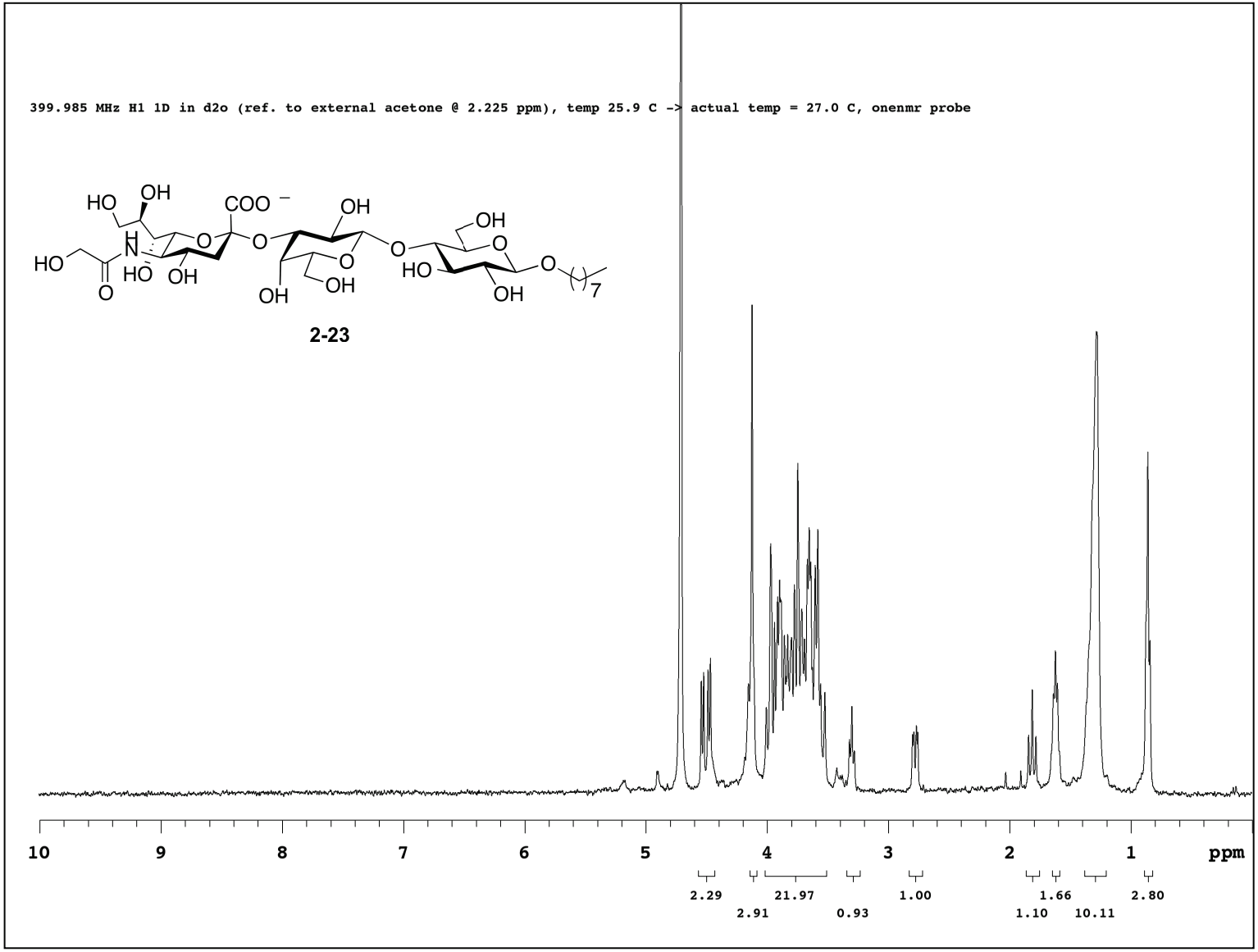
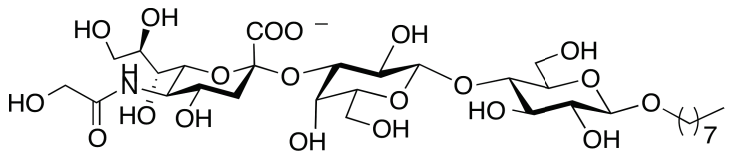
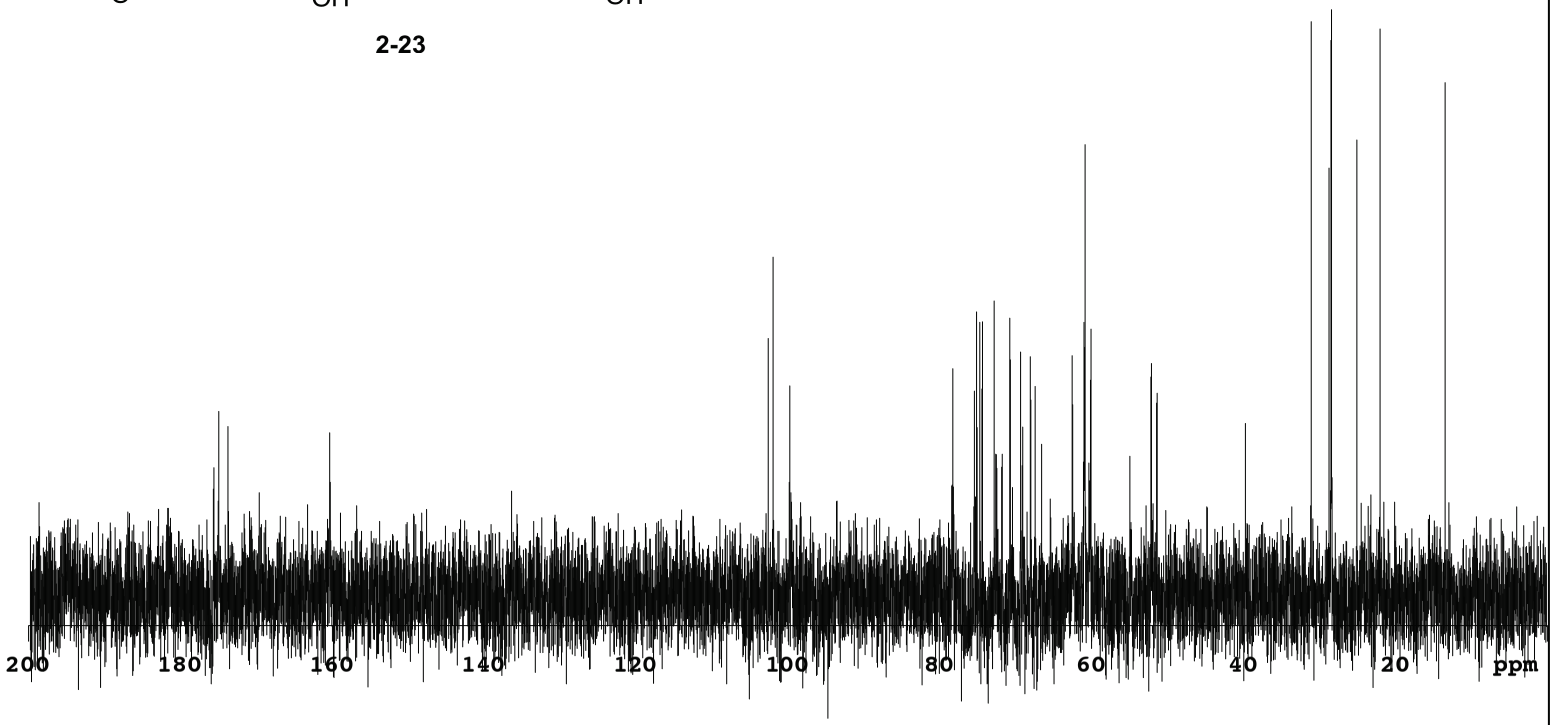
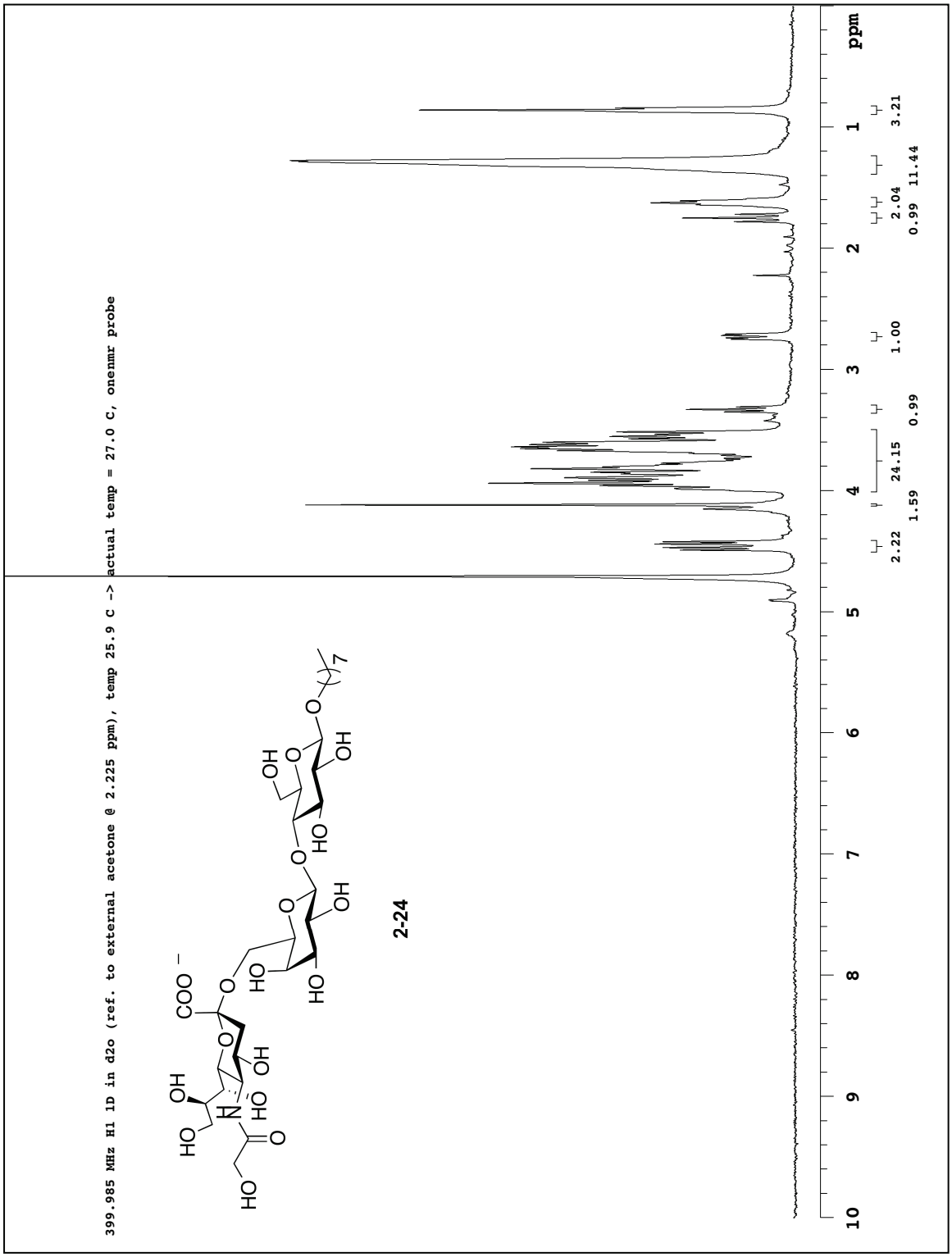

399.985 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe
HO
OH
COO −
OH
OH
HO
N
H
HO
OH
O
O
O
O
O
HO
OH
OH
OH
O
7
2-23
10
9
8
7
6
5
4
3
2
1
ppm
2.29
2.91
21.97
0.93
1.00
1.10
1.66
10.11
2.80

125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe

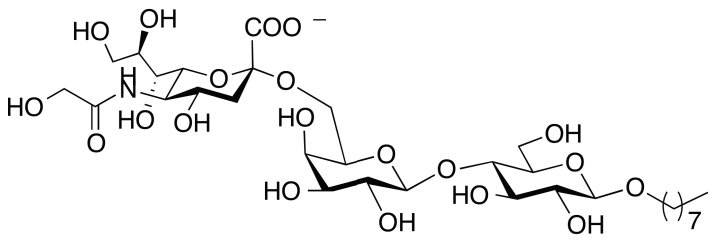


2-23

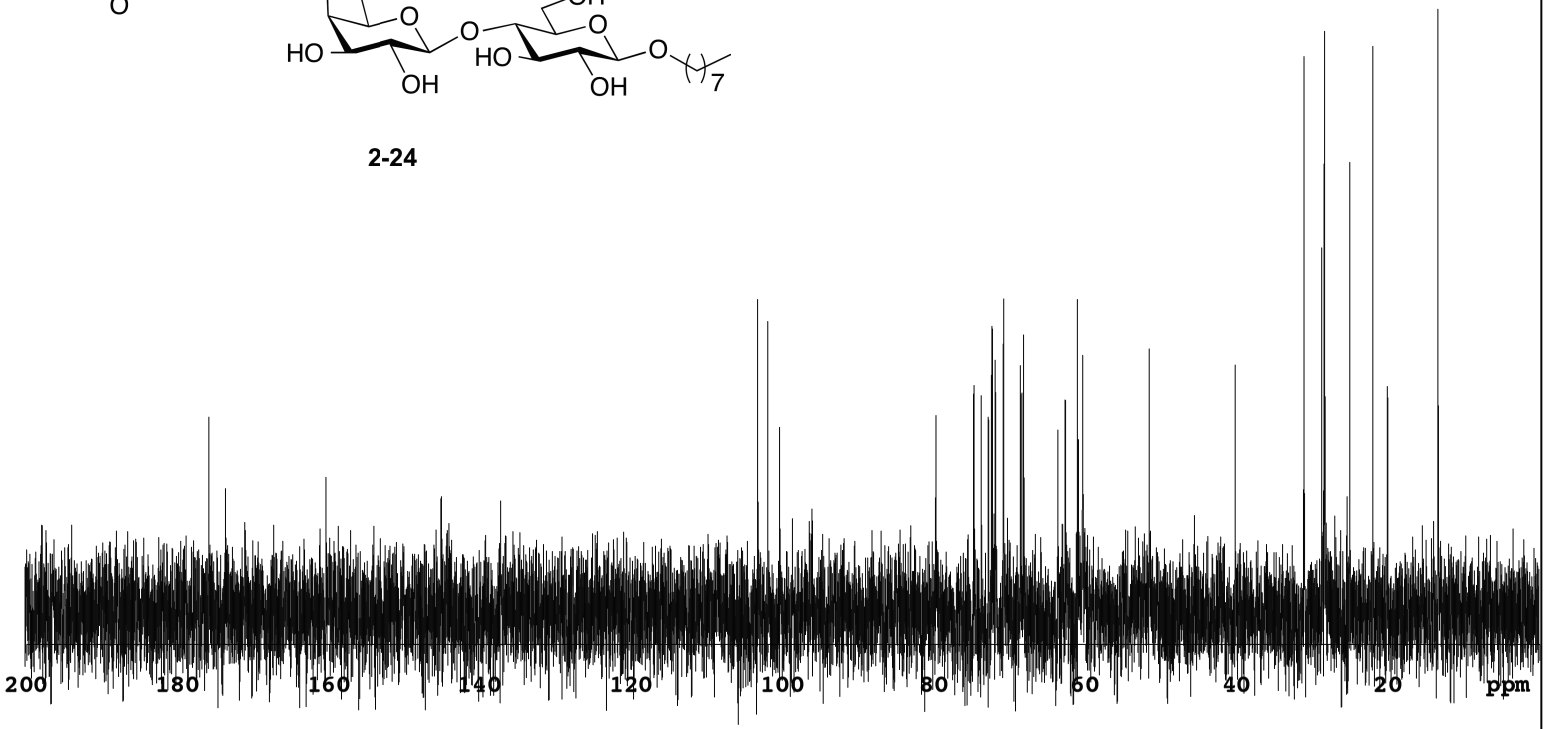


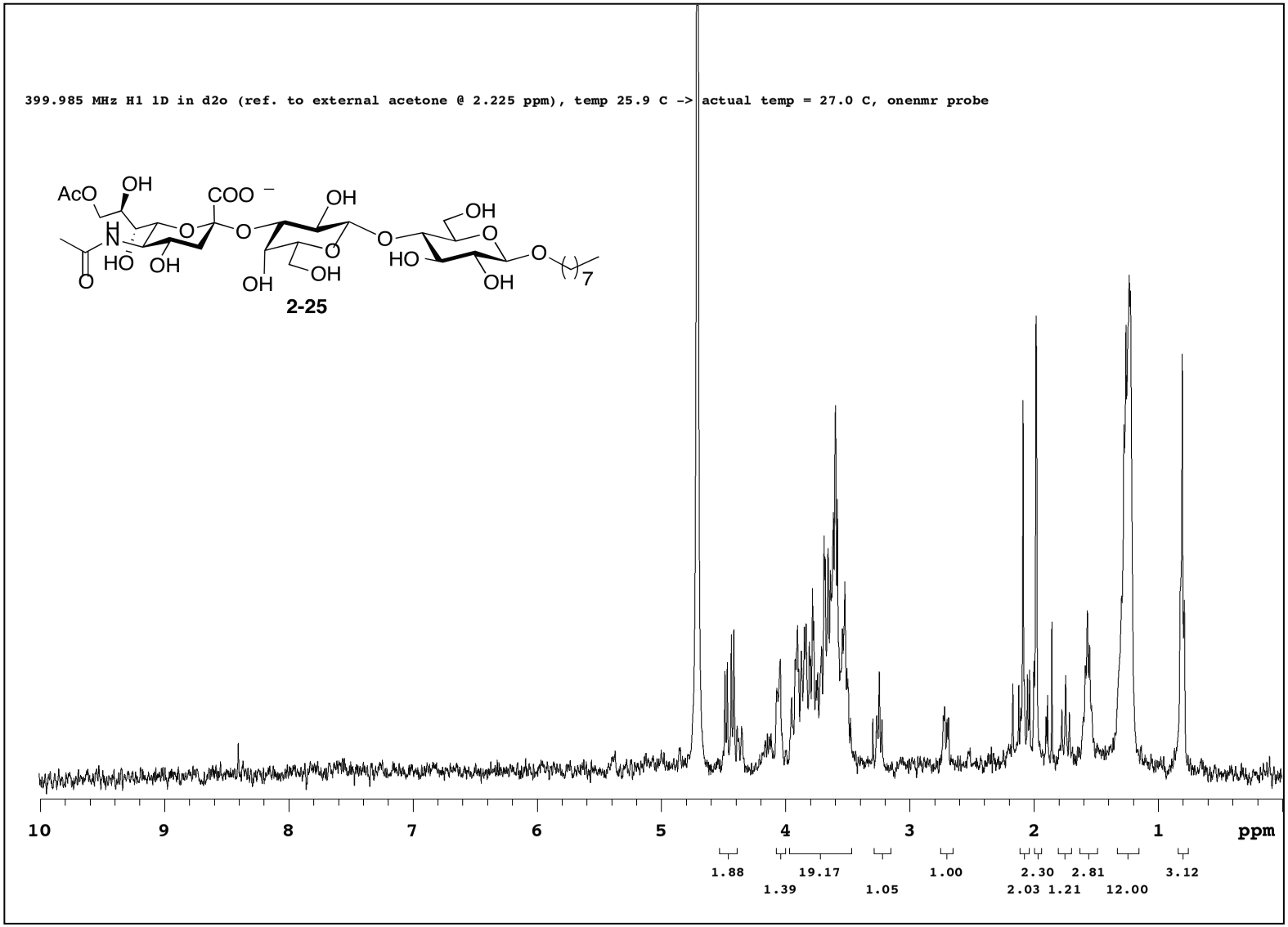

399.985 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe
2-24
ppm
2.22
24.15
0.99
1.59
1.00
2.04
0.99
11.44
3.21

125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe

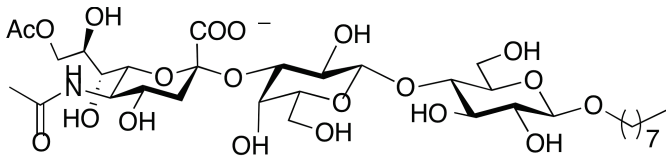


2-24

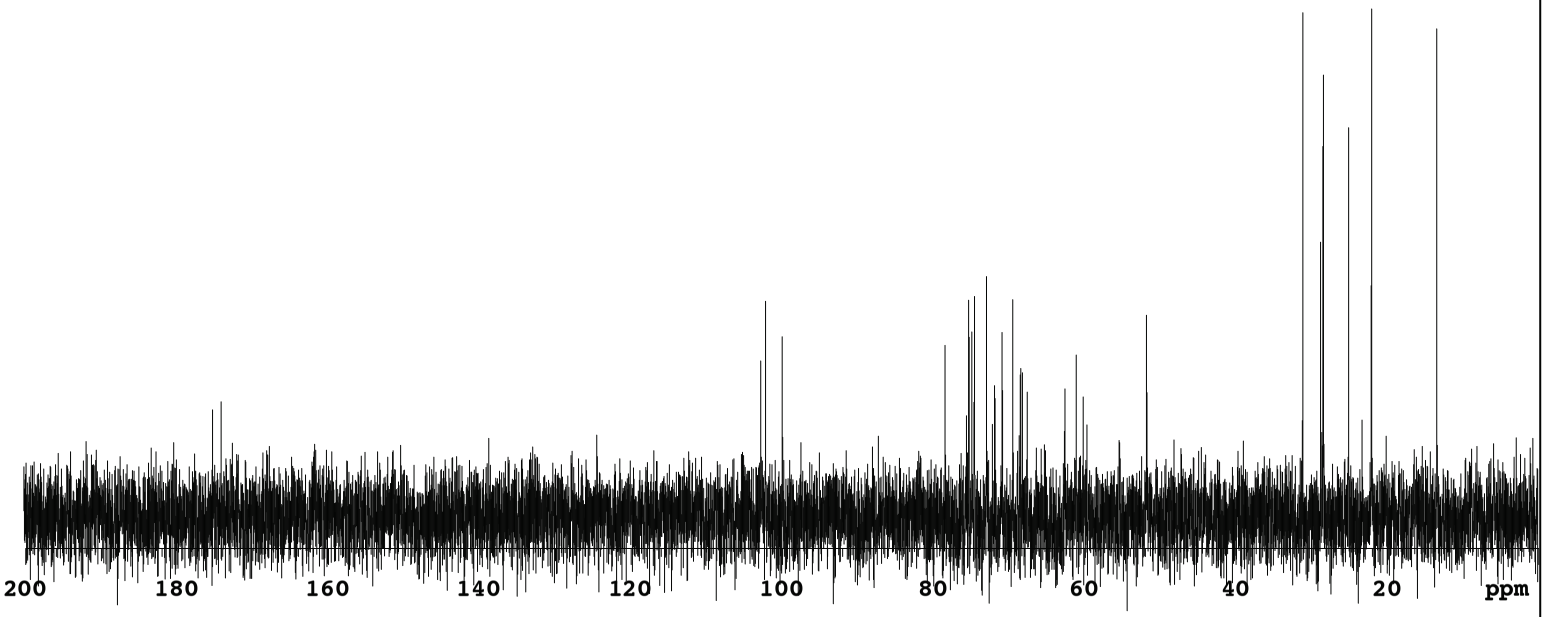


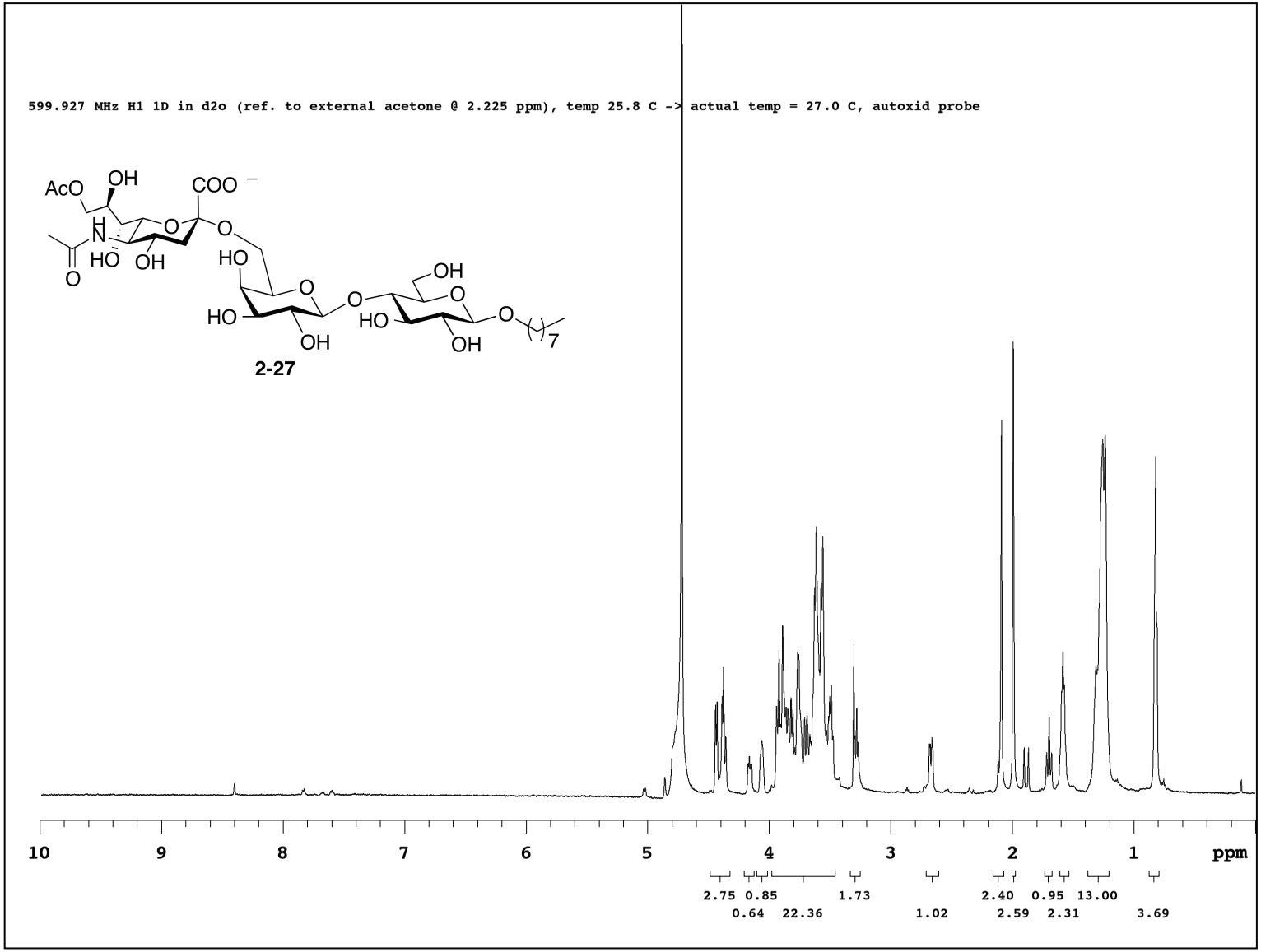

399.985 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe
AcO
OH
COO −
OH
OH
N
H
HO
OH
O
O
O
O
O
HO
OH
OH
OH
O
7
2-25
10
9
8
7
6
5
4
3
2
1
ppm
1.88
19.17
1.00
2.30
2.81
3.12
1.39
1.05
2.03
1.21
12.00

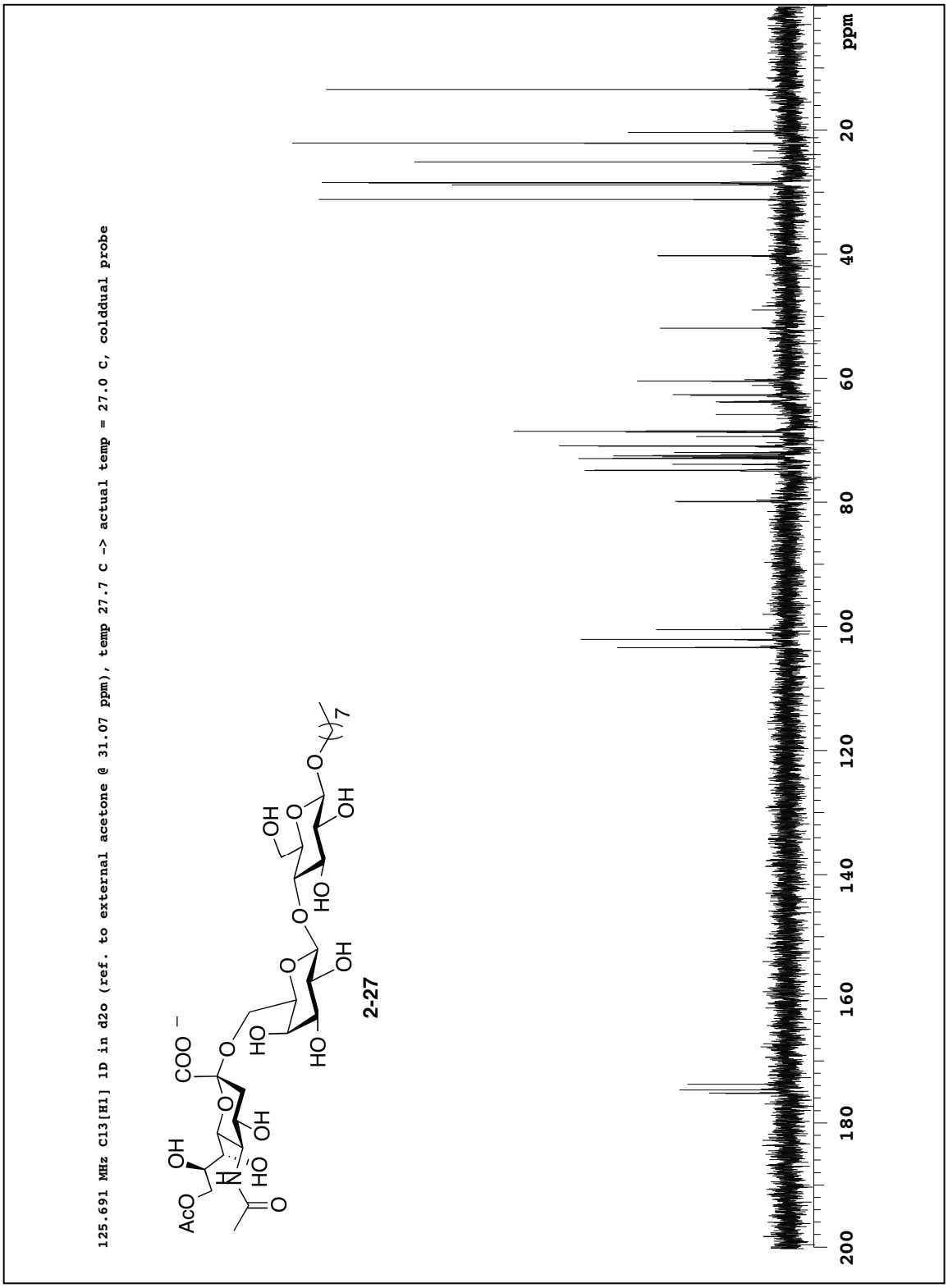
125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe



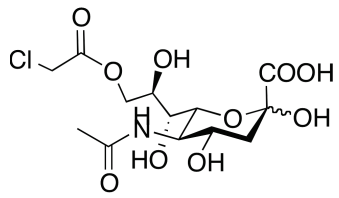
2-25



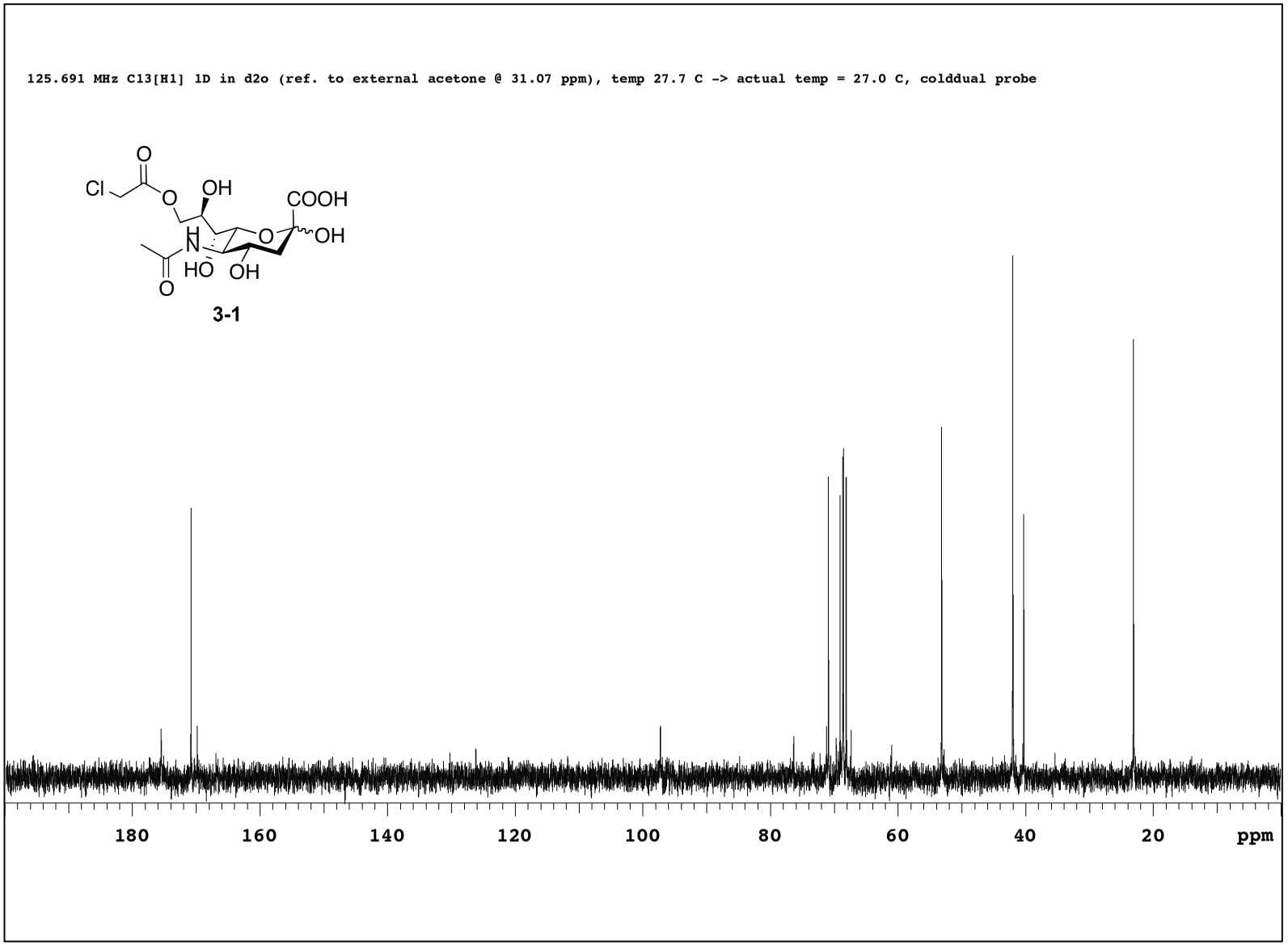

599.927 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.8 C -> actual temp = 27.0 C, autoxid probe
AcO
OH
COO −
N
H
HO
OH
O
HO
O
O
HO
OH
O
HO
OH
O
7
2-27
10
9
8
7
6
5
4
3
2
1
ppm
2.75 0.85
0.64 22.36
1.73
1.02
2.40 0.95 13.00
2.59 2.31
3.69

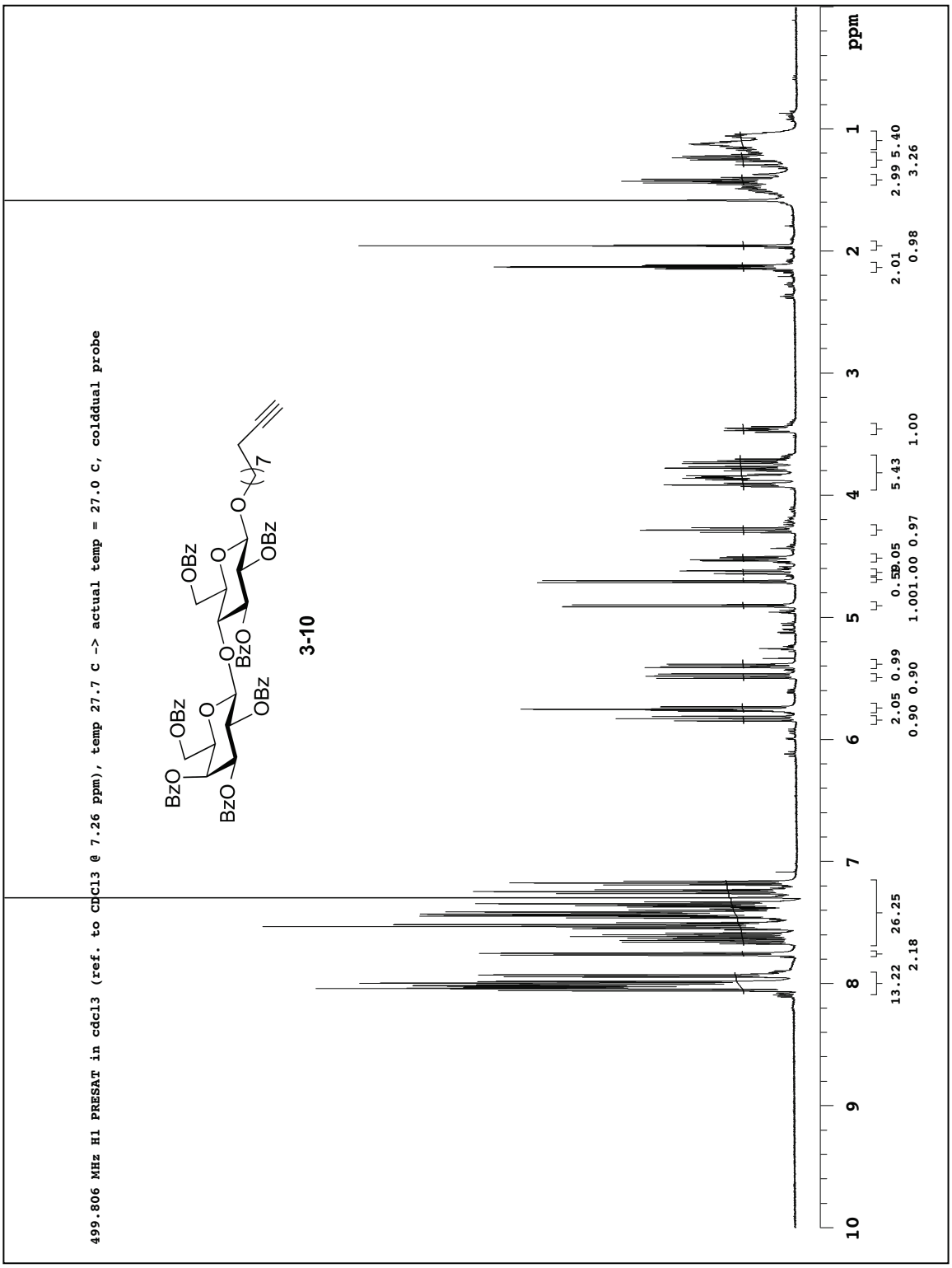

125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe
2-27
ppm
200
180
160
140
120
100
80
60
40
20

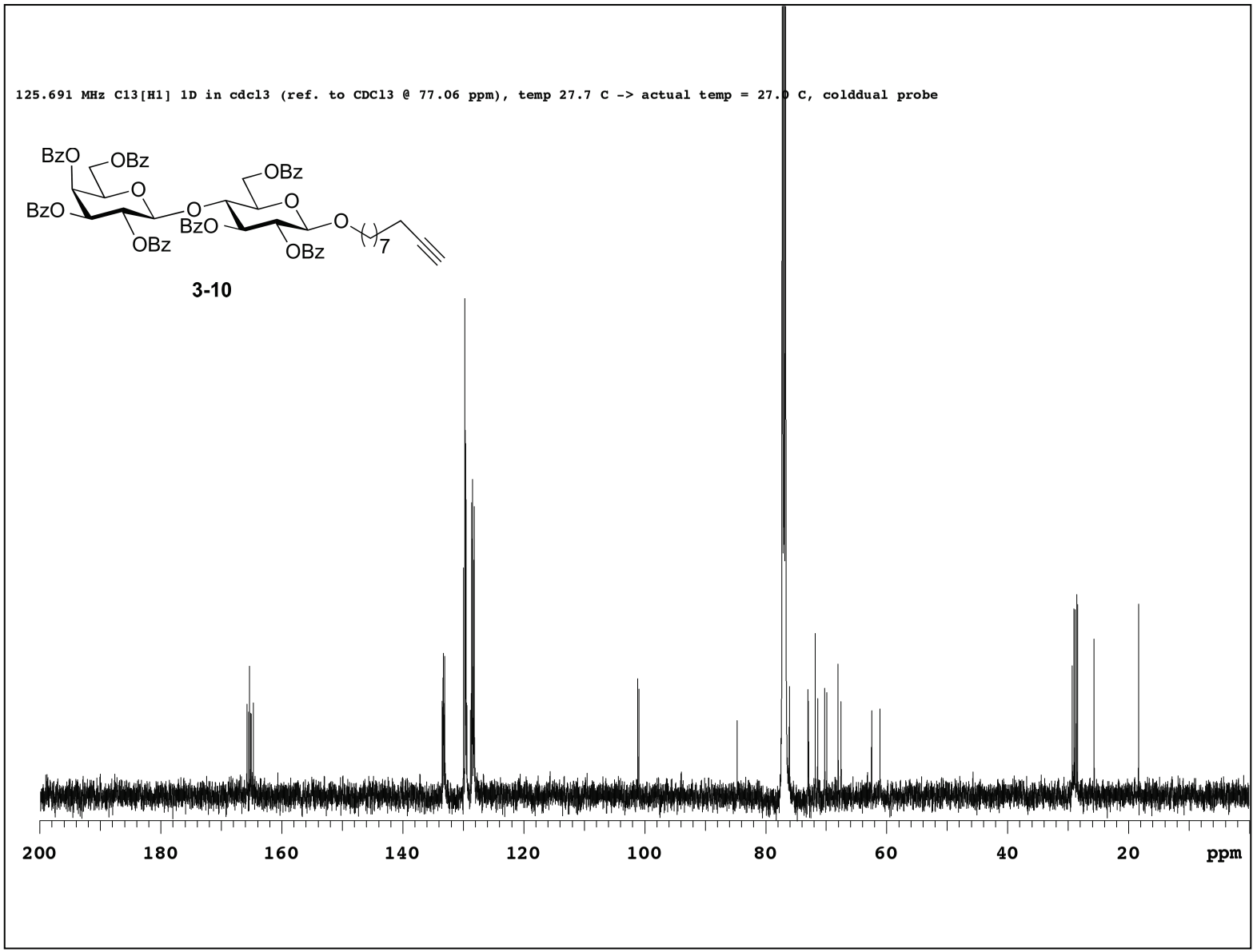
399.985 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe

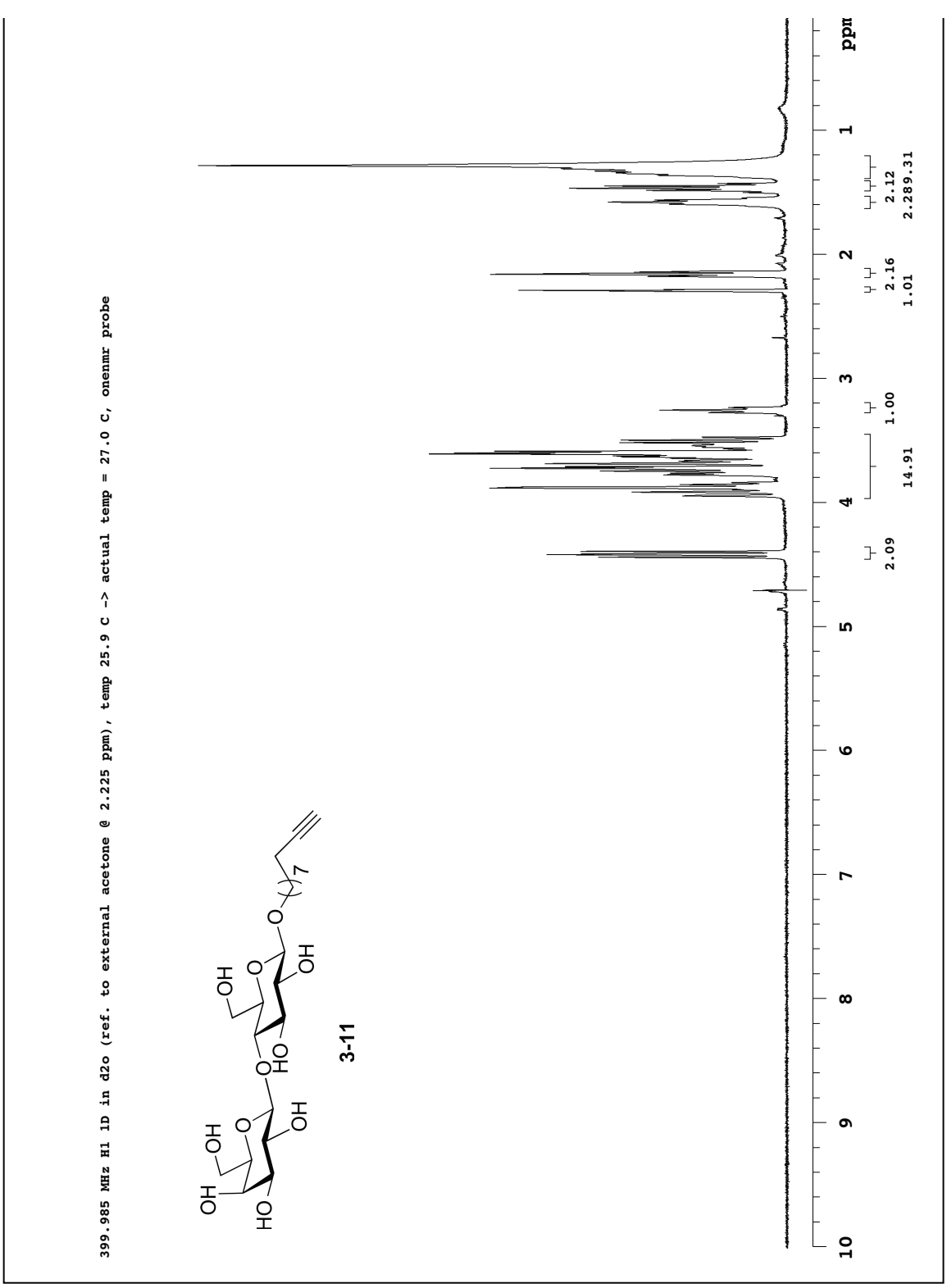


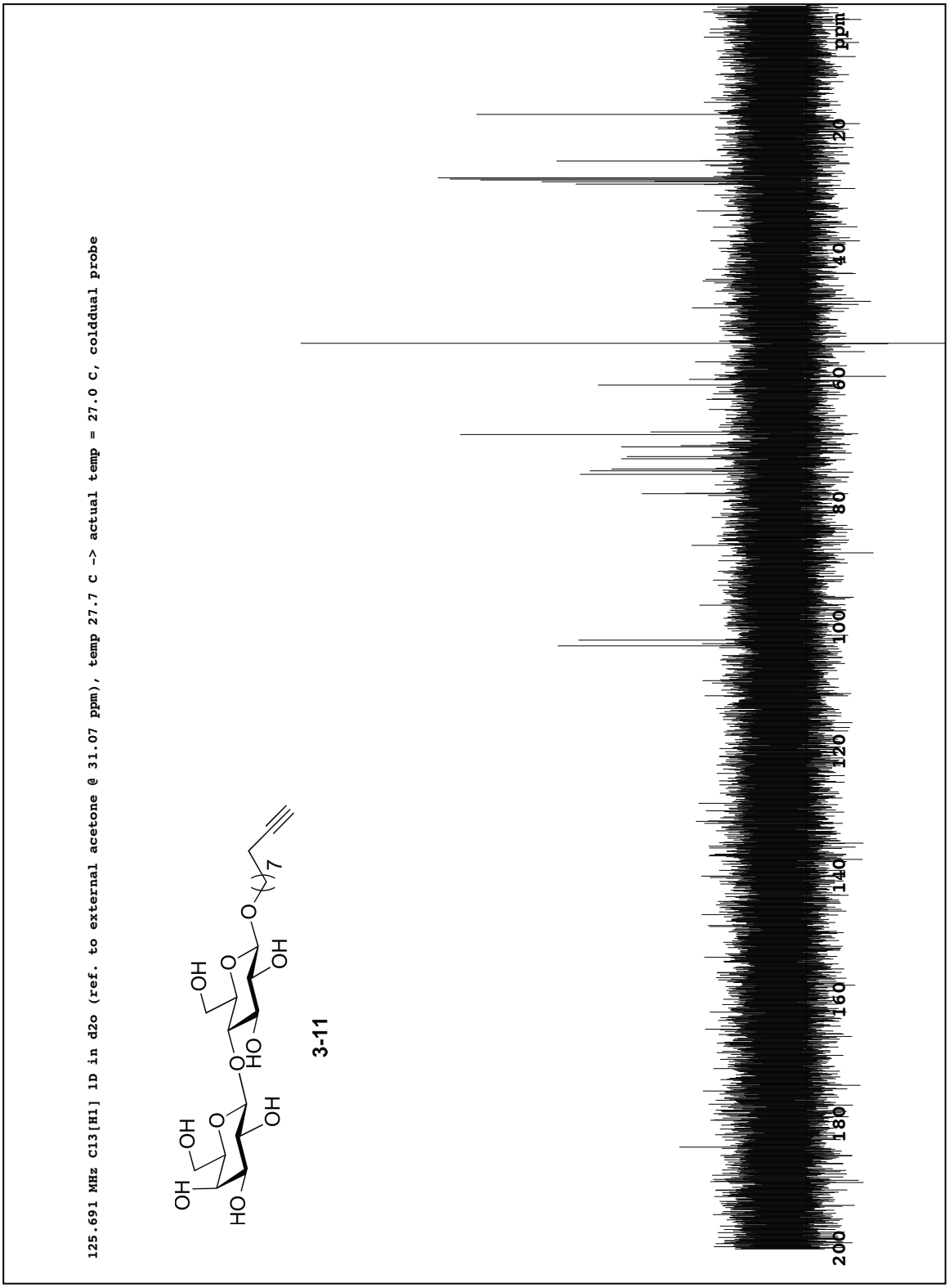
3-1

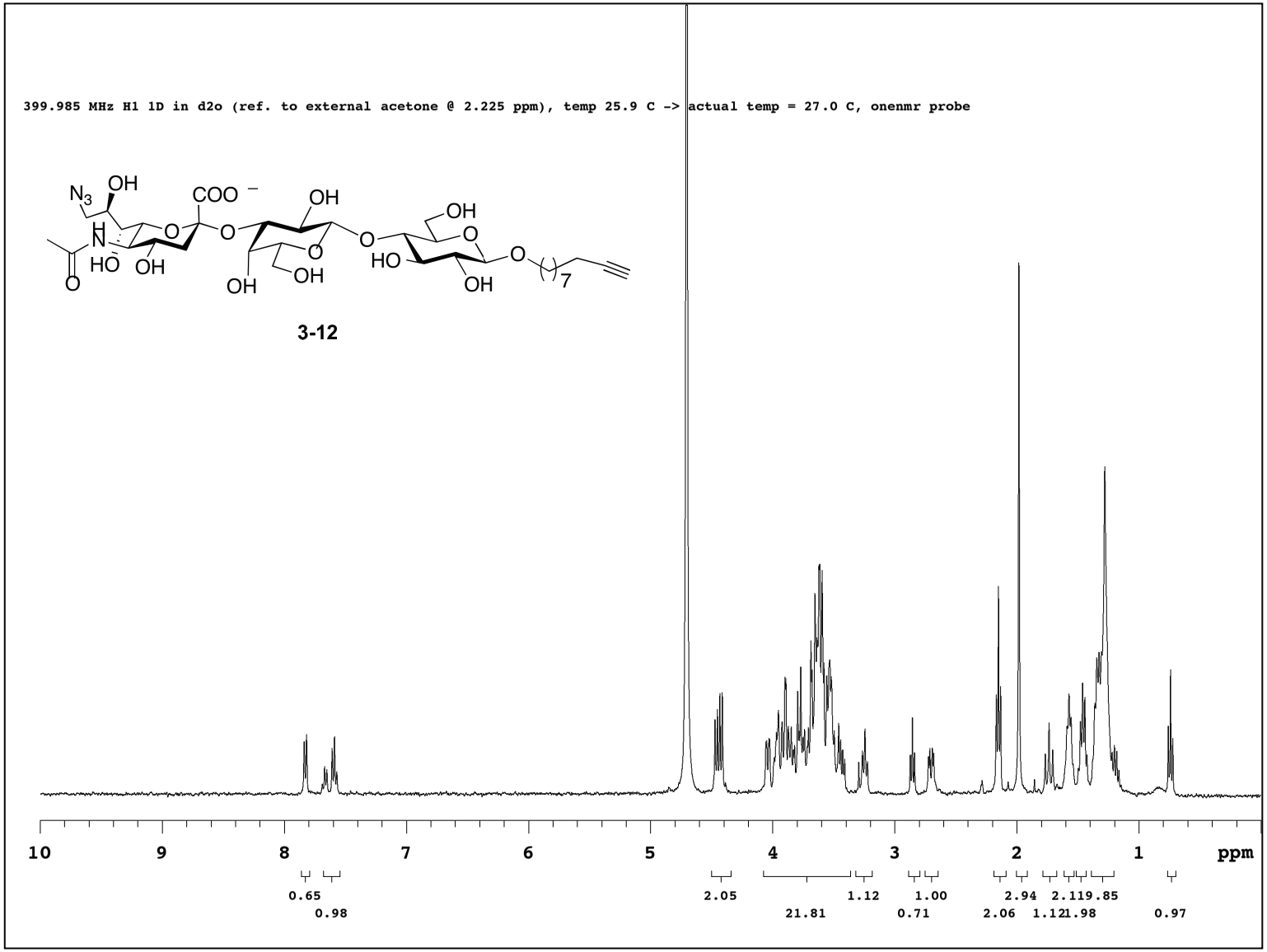

125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe
Cl
O
O
OH
COOH
H
N
O
OH
HO
OH
O
3-1
180
160
140
120
100
80
60
40
20
ppm

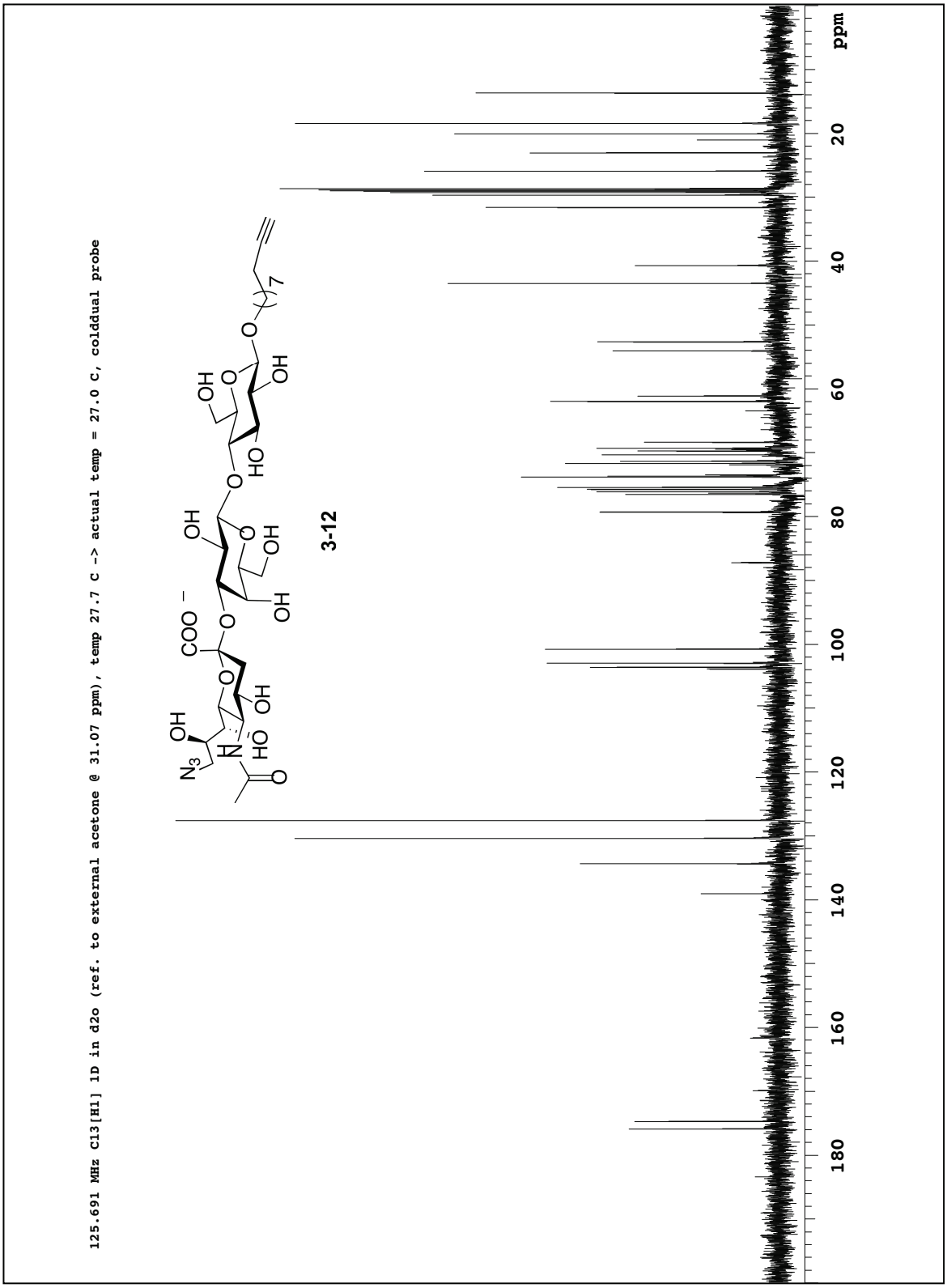

499.806 MHz H1 PRESAT in cdcl3 (ref. to CDCl3 @ 7.26 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe
BzO
OBz
O
BzO
OBz
O
BzO
OBz
O
7
3-10
ppm

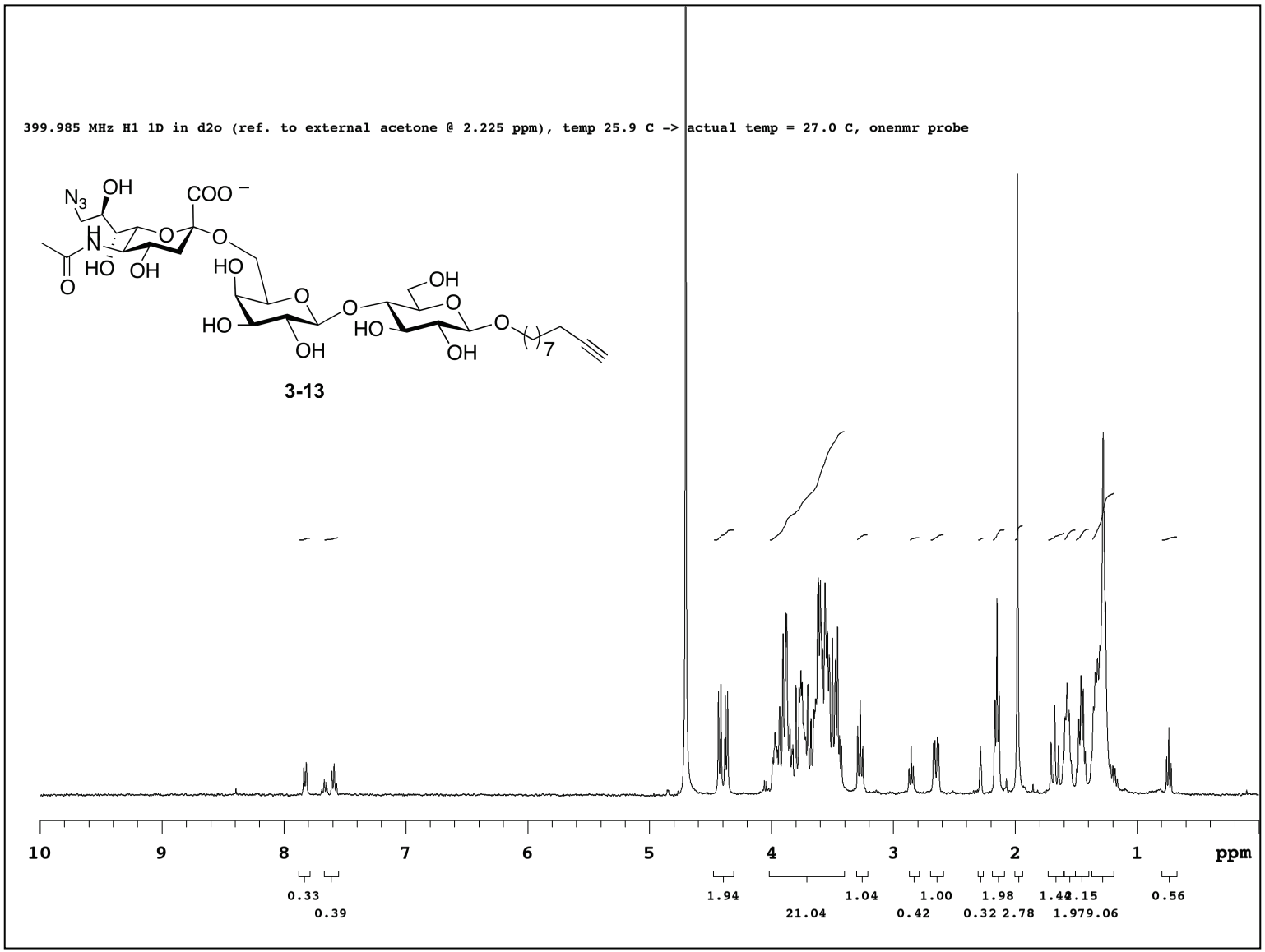

125.691 MHz C13[H1] 1D in cdcl3 (ref. to CDCl3 @ 77.06 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe
BzO
OBz
O
BzO
O
BzO
OBz
OBz
O
O
7
OBz
3-10
200
180
160
140
120
100
80
60
40
20
ppm

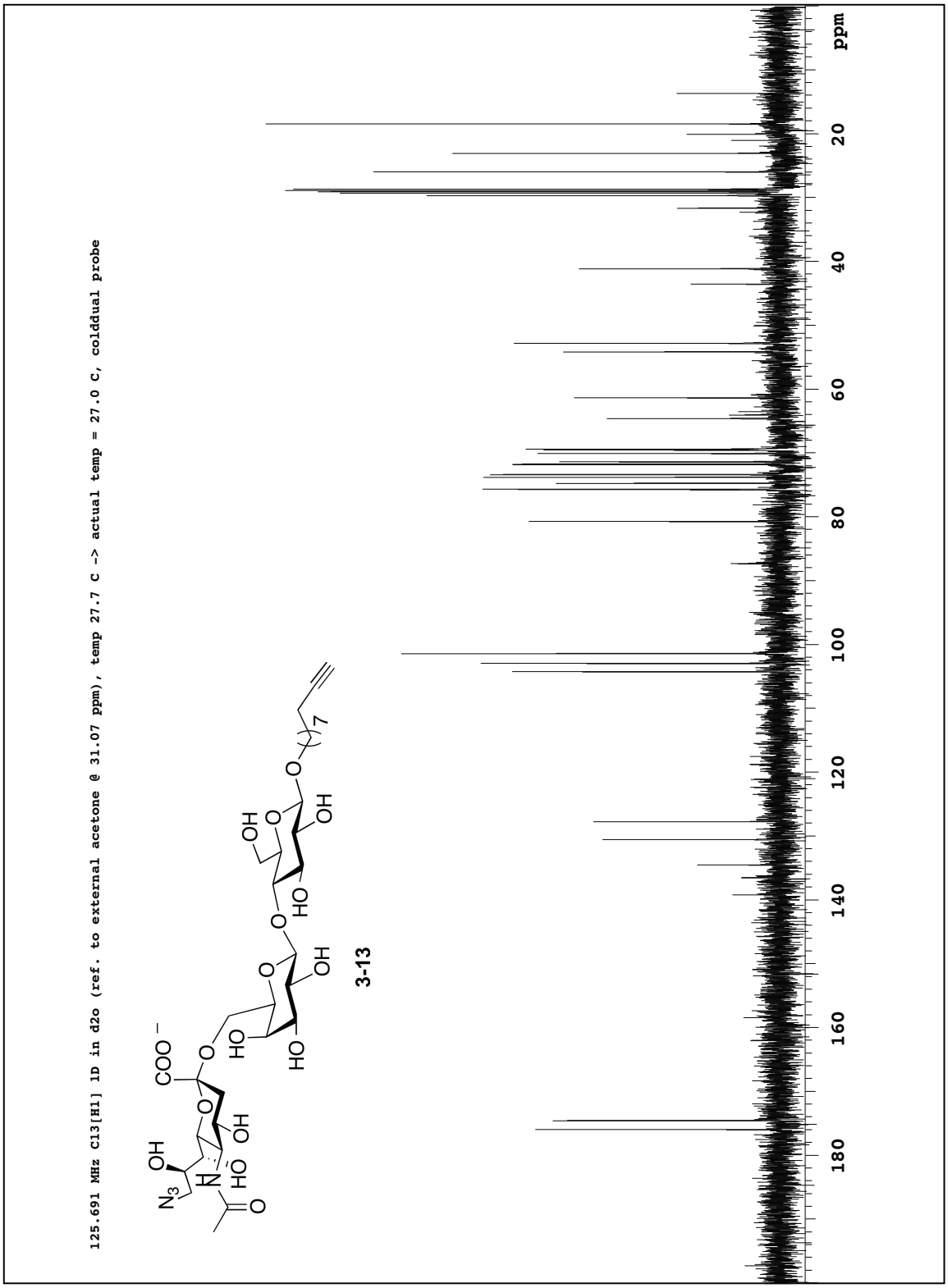

399.985 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe
3-11
ppm
2.09
14.91
1.00
2.16
1.01
2.12
2.289.31


125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe
3-11
ppm
200
180
160
140
120
100
80
60
40
20


399.985 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe
3-12
10
9
8
7
6
5
4
3
2
1
ppm
0.65
0.98
2.05
21.81
1.12
0.71
1.00
2.06
2.94
1.12
2.11
1.98
9.85
0.97


125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe
3-12
ppm
180
160
140
120
100
80
60
40
20


399.985 MHz H1 1D in d2o (ref. to external acetone @ 2.225 ppm), temp 25.9 C -> actual temp = 27.0 C, onenmr probe
3-13
10
9
8
7
6
5
4
3
2
1
ppm
0.33
0.39
1.94
21.04
1.04
0.42
1.00
0.32
1.98
2.78
1.44
1.15
1.97
9.06
0.56


125.691 MHz C13[H1] 1D in d2o (ref. to external acetone @ 31.07 ppm), temp 27.7 C -> actual temp = 27.0 C, colddual probe
3-13
ppm
180
160
140
120
100
80
60
40
20