Influence of diversity in sialic acid presentation on human neuraminidase enzyme activity

by

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Abstract

Sialic acids are an incredibly diverse family of carbohydrates that serve many roles in biological systems. Their presentation is influenced not only by structural modification of the sialic acid, but also by attachment through glycosidic linkages and their presence on different glycoconjugates. Many unusual sialic acids are unstable and difficult to study. Our group has an ongoing interest in sialic acid metabolism by the human neuraminidase enzymes (hNEU). The metabolism of unstable sialic acids such as 9-*O*-acetylated sialic acids (Neu5,9Ac$_2$) and polymers of sialic acid (polysialic acid, polysia) by hNEU has not been systematically defined. In this thesis we present studies towards understanding hNEU metabolism of sialic acids which vary in monosaccharide structure and glycosidic linkage.

Chapter 2 describes our initial study of hNEU activity towards Neu5,9Ac$_2$ by monitoring enzyme activity on simple fluorescent substrates based on 4MU-NANA (4-methylumbelliferyl α-D-*N*-acetylneuraminic acid). The substrate preference observed for NEU4 towards these simple substrates disagreed with those observed for octyl sialyllactoside mimics of the ganglioside GM3. We also observed an increase in the preference for Neu5Ac over Neu5,9Ac$_2$ for the α(2→6)-linked octyl sialyllactosides compared to the α(2→3)-linked substrates. These results confirmed the importance of considering the context of sialic acid presentation when studying its metabolism and inspired us to continue studying hNEU activity towards Neu5,9Ac$_2$ on different sialoside substrates.

We expanded our study of hNEU activity towards Neu5,9Ac$_2$ substrates in Chapter 3 to encompass more of the diversity of sialic acid presentation. We began by studying hNEU activity towards a Neu5,9Ac$_2$-enriched glycoprotein, which required the optimization of an assay used to detect bacterial neuraminidase activity on Neu5,9Ac$_2$ glycoproteins. For all four hNEU

isoenzymes, we observed a large preference for Neu5Ac over Neu5,9Ac$_2$ such that our assay could only detect the release of Neu5Ac. We then expanded the scope of our study to include α(2→8)-linked sialosides. We designed a panel of disialo substrates based on the ganglisoside GD3 with Neu5,9Ac$_2$ sialic acids and developed an HPLC assay to monitor hNEU kinetics on α(2→8)-linked sialic acids. The assay will be valuable for the study of hNEU activity on labeled gangliosides.

The challenge of studying metabolism of α(2→8)-linked sialic acids is apparent when considering polymers of α(2→8)-linked sialic acids, known as polysialic acid. The unique chemical and physical properties of polysia include enhanced susceptibility to acid-catalyzed hydrolysis. In Chapter 4 we detail the first systematic investigation of the activity of isoforms of hNEU towards polysialic acid. We found that NEU1, NEU3, and NEU4 hydrolyzed short polymers with degree of polymerization (DP) of 3-8. We did not detect hNEU activity on longer polymers (DP > 10), suggesting alternative mechanisms for polysialic acid regulation *in vivo*.

The work described in this thesis fills in longstanding questions in our understanding of sialic acid metabolism by hNEU enzymes. In doing this work, we have expanded chemical biology methods available for the investigation of sialic acid metabolism and the important roles of monosaccharide and glycosidic linkage diversity in substrate presentation. Overall, this work highlights the importance of considering the influence of sialic acid presentation on the role of sialosides in biological systems.

Preface

A version of **Chapter 1** is a manuscript in progress as a review article detailing methods for the study of sialoside metabolism.

A version of **Chapter 2** was been published as: Hunter, C.D.; Khanna, N.; Richards, M.R.; Darestani, R.R.; Zou, C.; Klassen, J.S.; Cairo, C.W., (2018) Human neuraminidase isoenzymes show variable activities for 9-*O*-acetyl-sialoside substrates. *ACS Chemical Biology,* 13, 4, 922-932. Methodology for synthesis of octyl sialyllactoside compounds was developed by Neha Khanna, molecular modelling was carried out by Michele R. Richards, ESI-MS assay was developed and implemented by Reza Rezaei Darestani and John Klassen, NEU1-NEU4 enzymes were expressed by Cecilia Zou. Carmanah Hunter executed the synthesis of compounds 2-2, 2-4, 2-5, 2-7, 2-8, 2-9; characterized compounds 2-2, 2-5 – 2-9, developed methods for the purification of 9-*O*-acetylated derivatives, optimized the solution-phase kinetics assay, implemented the solution-phase kinetics assay, and wrote the manuscript.

The experiments detailed in **Section 3.2.1** have been adapted into a manuscript in preparation Hunter, C.D., Porter, E., Cairo, C.W., Human neuraminidase activity towards modified sialic acids on glycoproteins. In **Section 3.2.1**, optimization of assay conditions and collection of preliminary data was done with Elizabeth Porter. NEU1 was produced by Hanh-Thuc Ton Tran, and NEU2-NEU4 were expressed and purified by Carmanah Hunter or other members of the Cairo Lab.

A version of **Chapter 4** has been adapted to a manuscript in preparation Hunter, C.D., Cairo, C.W., Hydrolysis of polysialic acids by human neuraminidase enzymes. For the neuraminidase experiments in **Chapter 4**, NEU1-NEU4 were expressed and purified as for

Chapter 3. Endo-N was a generous gift from Prof. Lisa Willis. Selective neuraminidase inhibitors in **Section 4.2.3** were synthesized by Dr. Tianlin Guo.

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I feel fortunate to have so many people to thank for their contributions, whether directly or indirectly, to the work in this thesis and my experience as a graduate student. First, I must thank my supervisor Dr. Christopher Cairo. It has been such a pleasure to work for Chris and I am so grateful for all the guidance, mentorship, and opportunites he has provided me throughout my graduate studies. I would also like to thank all the past and present members of the Cairo lab for both their contributions to the work in this thesis and for everything I have learned from them. Of them I would like to single out Dr. Michele Richards, whom I had the privilege of sharing an office with for 4 years. Mickey not only had seemingly endless patience for my questions and musings, but also helped me navigate the ups and downs of this experience.

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Throughout this experience my friends and family have been unwavering in their love and encouragement, and I wish I had the space to name them all individually. I was so lucky to have met a friend in Dr. Helen Clement in our first week of graduate school and to have shared so many experiences with her both in and out of academic life. All my friends both in and out of

the department have been a constant source of support and happiness. My family, particularly my parents and brother, have nurtured and empowered me to push my boundaries throughout my entire life. Lastly, thank you to my incredible partner Anushka Jayasuriya whose love and support I feel every day (and who read and edited this document) and our cats Greg and Zane who bring me so much joy (but occasionally physically prevented me from writing this document by placing themselves between me and my computer).

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List of Abbreviations

4MU	4-methylumbelliferyl
4MU-NANA	4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid
4MU-Neu5,9Ac$_2$	2′-(4-methylumbelliferyl)-α- D-9-*O*-acetyl-5-*N*-acetylneuraminic acid
9-*O*-Ac	9-*O*-acetyl
Achatinin-H	lectin from *Achatina fulica*
ALL	acute lymphoblastic leukemia
BDNF	brain-derived neurotrophic factor
BODIPY	boron dipyrromethene
BSM	bovine submaxillary mucin
BTP	benzothiazolylphenol
CD	cluster of differentiation
CD_3OD	deuterated methanol
CH_3OH	methanol
$CHCl_3$	chloroform
CMAH	cytidine monophosphate *N*-acetylneuraminic acid hydroxylase
CMC	critical micelle concentration
CMP	cytidine monophosphate
CSS	CMP-sialic acid synthetase
Cst-I	*Campylobacter jejuni* α(2→3) sialyltransferase
CstII	*Campylobacter jejuni* α(2→3/8) sialyltransferase (Cst-II)
CTP	cytidine triphosphate
D_2O	deuterated water

DANA	2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid
DMB	1,2-diamino-4,5-methylenedioxyenzene dihydrochloride
DMBA	4,5-dimethylbenzene-1,2-diamine
DMF	dimethylformamide
DNase I	deoxyribonuclease I
DP	degree of polymerization
E. Coli	*Escherichia coli*
ELS	evaporative light scattering
endo-N	endoneuraminidase-N
ESI	electrospray ionization
ESI-MS	electrospray-ionization mass spectrometry
EtOH	ethanol
FGF2	basic fibroblast growth factor
FRET	fluorescence/förster resonance energy transfer
GAL	galactose-6-oxidase and aniline-catalyzed oxime ligation
Gal	galactose
Glc	glucose
GSL	glycosphingolipid
H_2O	water
H-bond	hydrogen bond
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
His_6	polyhistidine tag
hNEU	human neuraminidase

HPLC	High pressure liquid chromatography
IPTG	isopropyl-β-$_D$-thiogalactopyranoside
KDN	2-keto-3-deoxy-D-glycero-D-galactononic acid
k_{rel}	relative rate
LacNAc	*N*-acetyllactosamine
LFA	lectin from *Limax flavus*
LOB	limit of blank
LOD	limit of detection
LOQ	limit of quantitation
LPS	lipopolysaccharide
MAH	lectin from *Maackia amurensis*
MAL	lectin from *Maackia amurensis*
MALDI	matrix-assisted laser desorption
ManGc	*N*-glycolyl-D-mannosamine
ManNAc	*N*-acetylmannosamine
MBP	maltose binding protein
MD	molecular dynamics
$MgCl_2$	magnesium chloride
MOPS	4-morpholinepropanesulfonic acid
MWCO	molecular weight cut-off
$Na_2S_2O_4$	sodium dithionite
NaCl	sodium chloride
NaOH	sodium hydroxide

NCAM	neural cell adhesion molecule
NEU	neuraminidase
NEU1	human neuraminidase 1
NEU2	human neuraminidase 2
NEU3	human neuraminidase 3
NEU4	human neuraminidase 4
Neu4,5Ac$_2$	4-*O*-acetylated sialic acid
Neu5,7Ac$_2$	7-*O*-acetylated sialic acid
Neu5,8Ac$_2$	8-*O*-acetylated sialic acid
Neu5,9Ac$_2$	9-*O*-acetylated sialic acid
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
Neu5Gc9Ac	N-glycolyl-9-*O*-acetylneuraminic acid
NH$_4$OAc	ammonium acetate
NH$_4$OH	ammonium hydroxide
Ni-NTA	nickel nitriloacetic acid
NMR	nuclear magnetic resonance
OD	optical density
OL	octyl lactoside
oligosia	oligosialic acid
OPD	*o*-phenylendiamine
OPME	one-pot multienzyme
PAL	periodate oxidation and aniline-catalyzed oxime ligation

Pd2,6ST	*Photobacterium damsela* α(2→6) sialyltransferase
PDA	photodiode array
***p*-NP**	*para*-nitrophenol
polysia	polysialic acid
PP_i	pyrophosphate
PSI	pound-force per square inch
RCF	relative centrifugal force
RIPA buffer	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
rpm	rotations per minute
Sia	sialic acid
SIAE	sialate-9-*O*-acetylesterase
SiaT	sialyltransferase
Siglec	sialic acid-binding immunoglobulin-type lectin
SNA	lectin from *Sambucus nigra*
SNFG	symbol nomenclature for glycans
SOAT	sialate-*O*-acetyltransferase
SubB	Subunit B of subtilase cytotoxin
StDev	standard deviation
synCAM	synaptic cell adhesion molecule
TBA	thiobarbituric acid
TLC	thin layer chromatography
Tris-HCl	2-amino-2-(hydroxylmethyl)-1,3-propanediol hydrochloride

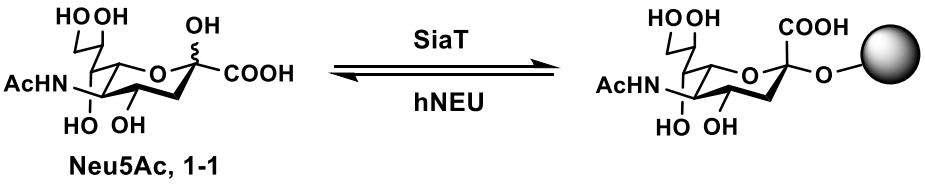
UV	ultraviolet
w/v	weight/volume

Chapter 1 : Introduction[a]

[a] A version of this Chapter is being adapted to a manuscript as a review article detailing methods for the study of sialoside metabolism

1.1 Sialosides in human health and disease

Sialic acids (Sia) are a family of α-keto acids with a 9-carbon backbone that cap the non-reducing end of glycoconjugates on glycoproteins and glycolipids. Featured at this outermost position on the glycan, sialic acids are poised to mediate intra- and intercellular communication and host-pathogen interactions.[1, 2] The roles of sialosides generally fall into two categories: formation of an epitope, or masking an epitope.[3] For instance, in cell migration, sialic acids on β1-integrins enhance binding to collagen-I,[4] but block the binding of galectin-3 to the underlying galactose.[5] The complement of cellular sialosides is crucial to human health and is maintained by opposing actions of sialyltransferase (SiaT) and sialidase (neuraminidase) enzymes (**Scheme 1.1**). To date, 20 human SiaT[6, 7] and 4 human neuraminidases (hNEU, NEU1-4) have been identified.[8] This broad assortment of enzymes that regulate addition and removal of sialic acids from the glycan hints at the remarkable heterogeneity of sialic acid presentation in human systems. Methods to study sialic acid metabolism that can account for heterogeneity are critical to elucidating the roles of sialic acids in normal and disease processes.



Scheme 1.1: Regulation of sialic acids by sialyltransferase and neuraminidase enzymes

1.2 Heterogeneity of sialic acid presentation

Sialic acids were first identified and named from salivary glycoproteins in 1936 by Blixt and were later independently discovered on neuronal glycolipids by Klenk in 1941 and referred to as "neuraminic acids".[9] Both terms have been adopted into scientific vernacular, and at the time it

was agreed that the term sialic acid should be used synonymously with *N*-acylated neuraminic acids.[10] In practice, the term "sialic acid" has most often been used synonymously with a particular *N*-acylated neuraminic acid: *N*-acetylneuraminic acid (5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid, Neu5Ac, **1-1**). The primary reason for this is that Neu5Ac is the most abundant form of sialic acid in humans. This seemingly innocuous shift in nomenclature belies the incredible diversity found in sialic acids and its impact on biological processes. To date over 50 different sialic acids have been identified, the most common of which is Neu5Ac, which is made biosynthetically from *N*-acetylmannosamine (ManNAc).[11] Most other sialic acids are derived biosynthetically from Neu5Ac through modification of one or more hydroxyl groups or at *C*-5 (**Figure 1.1A**).[12] The definition of sialic acids was expanded with the discovery of 2-keto-3-deoxy-D-glycero-D-galactononic acid (KDN, **1-3**), which has a hydroxyl group at C5 but the same stereochemistry as *N*-acylated neuraminic acids,[13] and is derived biosynthetically from mannose.[14, 15] KDN is primarily found in bacteria and lower vertebrates, but is present in trace amounts in mammalian systems.[16]

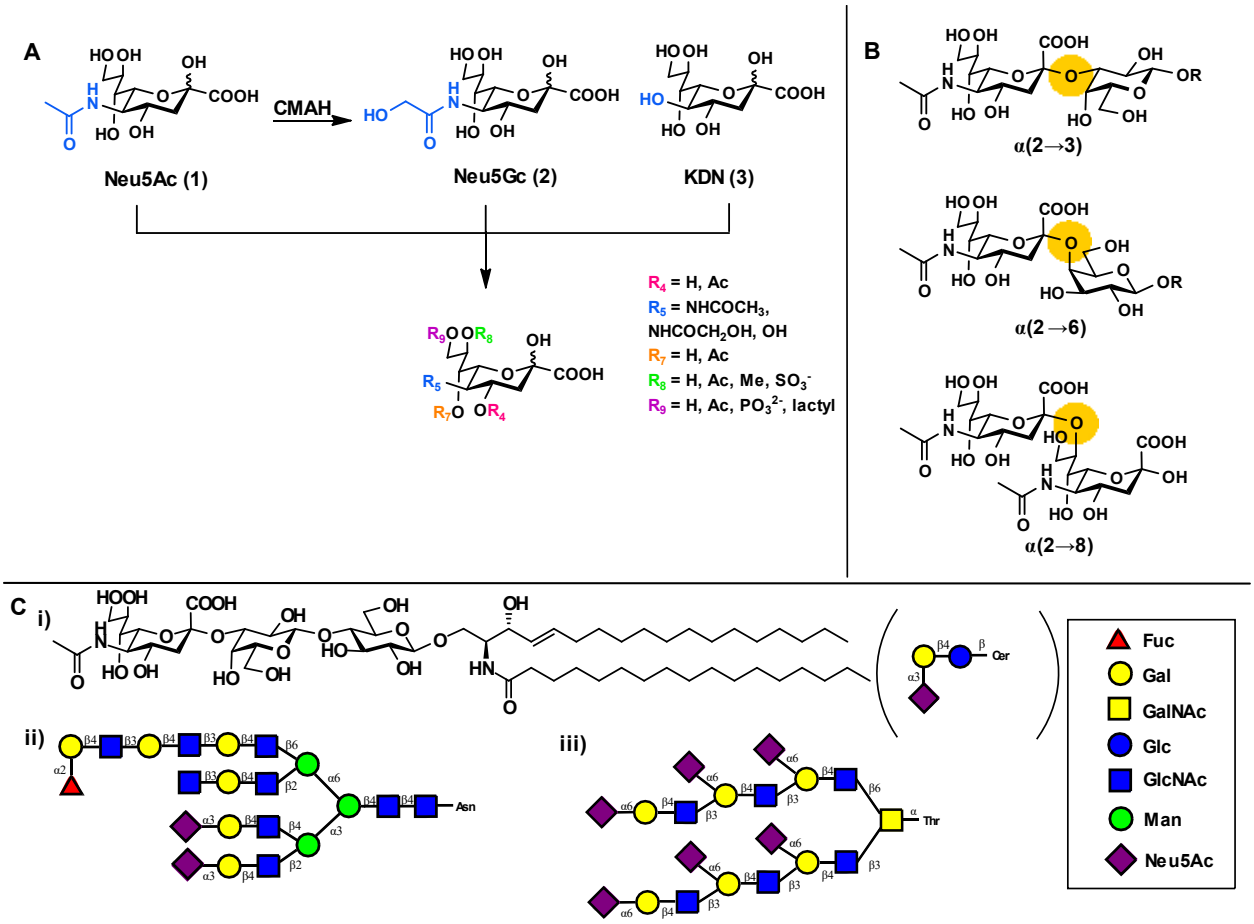


Figure 1.1: Diversity in sialic acid presentation A) diversity in sialic acid monosaccharide structure B) diversity in glycosidic linkage C) sialylglycoconjugate scaffolds i) ganglioside GM3 presented as both a line drawing and with SNFG (symbol nomenclature for glycans)[17] ii) representative example of a sialylated *N*-glycan[18] iii) representative example of a sialylated *O*-glycan.[19]

Sialic acid presentation throughout phylogeny is discussed extensively elsewhere.[12, 20, 21] Herein, we limit our discussion to sialic acids in mammalian systems. Hydroxylation of the *C*-5 amide of Neu5Ac (R_5, **Figure 1.1A**) gives *N*-glycolylneuraminic acid (Neu5Gc, **1-2**). This residue is uncommon in humans as exon deletion in the cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH) gene prevents humans from making Neu5Gc. However, Neu5Gc from meat

and dairy products can be incorporated into the human glycome.[22, 23]. The most common hydroxyl group modification is *O*-acetylation at *O*-4,7,8, or 9,[24] although acetylation at *O*-4 does not occur in humans.[25] Under physiological conditions acetyl groups at *O*-7 and *O*-8 migrate to the primary alcohol at *O*-9.[26, 27] 9-*O*-acetylated sialic acids (Neu5,9Ac_2) have numerous roles in both normal and disease processes (see **Chapter 2** and **Chapter 3**) and are regulated by the opposing action of sialate-*O*-acetyltransferase (SOAT)[28, 29] and sialate-9-*O*-acetylesterase (SIAE)[30, 31] enzymes. Less prevalent sialic acid modifications include 8-*O*-methyl or -sulfo, and 9-*O*-phosphoro or -lactyl groups.[12] Of these, only 9-*O*-phosphoro or -lactyl groups have been identified in humans, where 9-*O*-phosphoro-Neu5Ac is a biosynthetic precursor to sialylglycoconjugates,[32] and 9-*O*-lactyl-Neu5Ac has been identified on gastric mucins.[33]

The diversity of sialic acid presentation is further complicated by its attachment to the glycan. While the β-anomer of sialic acid is more thermodynamically favored, naturally occurring glycosidic linkages of sialic acid are in the α-conformation [34]; most commonly an α(2→3) or α(2→6) linkage to an underlying galactose (see **Chapter 2**), or an α(2→8) linkage to an underlying sialic acid (**Figure 1.1B**).[35] Different glycosidic linkage of the sialic acid can influence sialic acid recognition. For instance, Siglec 2 (CD22), a negative regulator of B-cell activation, only recognizes sialic acids with an α(2→6) linkage to galactose.[36, 37] Sialosides with α(2→8)-linkage are present as disialyl epitopes, as in the ganglioside GD3 (see **Chapter 3**),[38] but also as polymers of sialic acid (polysialic acid, polysia). The polyanionic nature of polysia contributes to its interesting chemical and biological properties which continue to be elucidated (see **Chapter 4**).[39]

Sialic acid-containing glycans are displayed on an array of glycoconjugates, providing an additional level of diversity in sialic acid presentation. Glycosphingolipids (GSLs) containing sialic acids are referred to as gangliosides, which almost exclusively have α(2→3) and α(2→8)

linked sialic acids (see **Chapter 2** and **Chapter 3**).[40] Gangliosides (**Figure 1.1C i)**) are amphiphilic and their physical properties influence their organization and biological function in cell membranes. Recently a study found that the lipid composition of liposomes influenced rates of ganglioside desialylation by viral and bacterial neuraminidases.[41] The properties of GSLs and GSL-based probes have been recently reviewed by our group,[40] and others.[42] Glycoproteins display sialosides (see **Chapter 3**) on both *N*- and *O*-linked glycans where the reducing end sugar is bound to the protein through an asparagine (*N*-glycan, **Figure 1.1C ii)**), and serine or threonine (*O*-glycan, **Figure 1.1C iii)**).[1, 43] Sialosides on *N*- and *O*-linked glycans generally have an α(2→3) or α(2→6) linkage to galactose, or occasionally an α(2→6)-linkage to *N*-acetylgalactosamine.[44] Certain proteins contain *N*- and *O*-glycans with polysialic acid.[43, 45] Thus, the heterogeneity of sialic acid presentation results from variations in the monosaccharide structure, glycosidic linkage, and the underlying glycoconjugate. The study of sialic acid metabolism requires consideration of all these levels of diversity.

1.3 Metabolism of sialosides by human neuraminidases

Neuraminidases, also referred to as sialidases, are glycosyl hydrolases that cleave the glycosidic bond between a sialic acid and its reducing end substituent. To date, four neuraminidase enzymes have been identified in the human system, denoted NEU1-NEU4.[46] All four hNEU isoenzymes are members of the GH33 family.[47] The hNEU are fairly ubiquitous across cell types, but have different primary subcellular localizations (see Table 1).[8, 46] The hNEU have many roles in normal and disease processes, including in cell migration,[48] inflammation,[49, 50] glucose homeostasis,[51, 52] and tumor malignancy.[53] Although it is apparent that the heterogeneity of sialic acid presentation influences the availability of sialic acids to hNEU,[54] the substrate specificities of

hNEU towards natural sialic acid presentations have not been fully elucidated (see **Table 1.1**). For instance, NEU3 is typically considered to be selective for glycolipid substrates; however, NEU3 activity on a glycoprotein substrate was recently reported.[50] Further, there is a lack of consensus in the literature on which hNEU modifies polysialic acid; some studies reported NEU1 to be exclusively active on polysia,[49] and others NEU4.[55] Reports of hNEU substrate specificities towards modified monosaccharides have been largely limited to unnatural sialic acid modifications.[56-58]

Table 1.1: Localization and reported substrate tolerance of hNEU [8, 46]

	NEU1	**NEU2**	**NEU3**	**NEU4***
Major subcellular localization	Lysosome	Cytosol	Plasma membrane	Lysosome, mitochondria, ER
Monosaccharide specificity	Neu5Gc<Neu5Ac[59]	Neu5Gc>Neu5Ac[58] Neu5,9Ac$_2$ <<<Neu5Ac[60]	ND	ND
Glycosidic linkage specificity[54]	α(2→3) > α(2→6), polysialic acid[49]	α(2→3) >>> α(2→6) α(2→3) > α(2→8)[61]	α(2→3) > α(2→6), α(2→3) > α(2→8)[48]	α(2→3) >> α(2→6), α(2→3) > α(2→8)[48], polysialic acid[55]
Glycoconjugate specificity	Oligosaccharides, glycoproteins	Oligosaccharides, glycoproteins, gangliosides	Gangliosides, some glycoproteins	Oligosaccharides, glycoproteins, gangliosides

* NEU4 has two isoforms, where one isoform has an additional 12 amino acids at the *N*-terminus

ND, not determined

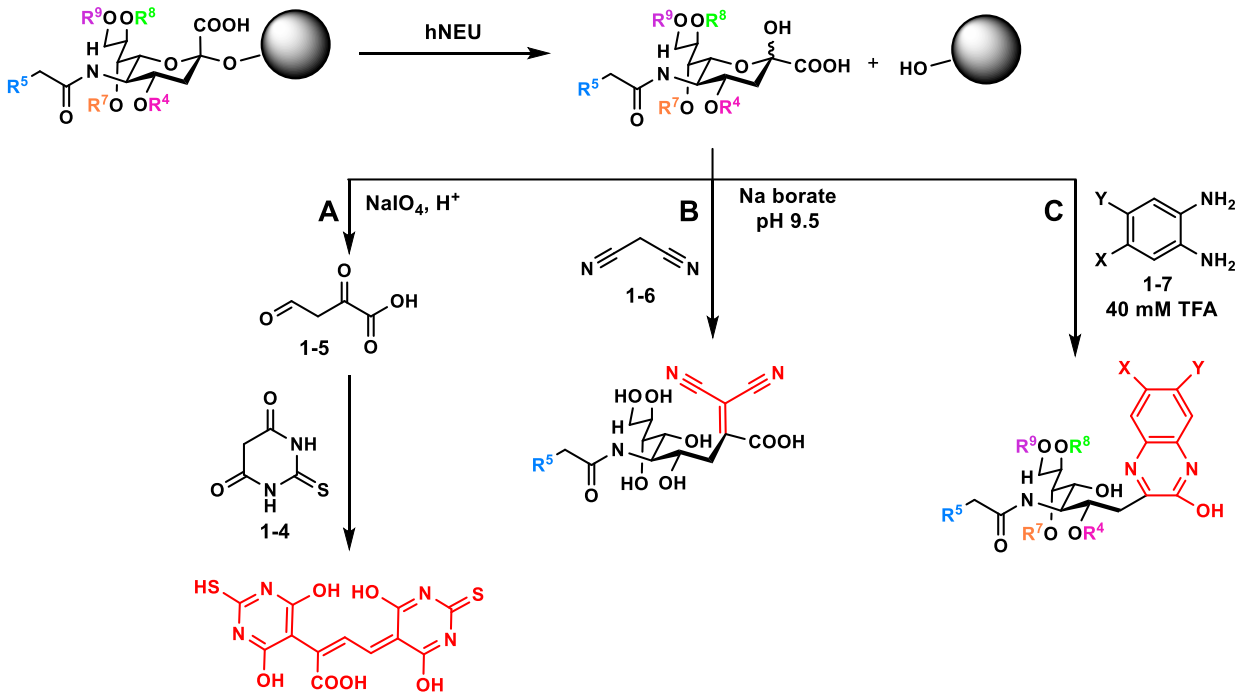
1.4 Chemical biology methods to detect sialic acids

There is no universal method to study sialic acid metabolism by NEU – rather, each has strengths and limitations. Methods to detect sialic acids allow for the study of sialic acid metabolism on natural substrates. The careful selection of a method to detect sialic acids, with consideration of which factors of sialic acid presentation the method accounts for, is critical to effectively studying sialoside metabolism. There is an assortment of chemical and biochemical tools available to detect sialic acids, both as a part of glycoconjugates and as free reducing monosaccharides.

1.4.1 Chemical methods to detect sialic acids

Chemical methods to detect sialic acid can be categorized as methods that detect free sialic acid or those that detect sialic acid on glycoconjugates. Methods to detect free sialic acids are common for studying sialic acid metabolism by neuraminidase enzymes and can detect neuraminidase activity on natural NEU substrates. Methods that detect free sialic acids do not discriminate sialic acid glycosidic linkage and many methods require harsh conditions that destroy labile modifications to sialic acid. A commonly used method for detecting free sialic acids is the thiobarbituric acid (TBA, **1-4**) method (**Scheme 1.2A**), simultaneously developed by Warren[62] and Aminoff[63, 64]. Free sialic acid is oxidized to generate an aldehyde at C6 and then further degraded to β-formylpyruvic acid (**1-5**) which reacts with TBA to generate a fluorophore.[65] The method suffers from harsh acidic conditions and cross-reactivity with other monosaccharides[66] and unsaturated lipids,[67] but it is still a popular method to detect sialic acid in hNEU activity assays[55, 68] and in complex samples.[69, 70] An alternative to the TBA assay is the detection of free sialic acid with malononitrile **(1-6, Scheme 1.2B**).[71] Although the assay conditions are relatively mild (pH

9.5), the basic conditions hydrolyze sialic acid hydroxyl group modifications (e.g 9-*O*-Ac) and malononitrile reacts with other electrophiles; particularly ketones and aldehydes[72]. Submitting the sample to reducing conditions prior to enzymatic cleavage of sialic acids has enabled quantitative sialic acid detection in cell culture supernatant.[73] Malononitrile detection of free sialic acid has been used to evaluate hNEU kinetics *in vitro*[74-76] and is particularly useful for studying NEU3[77-79] which prefers glycolipid substrates and does not tolerate the fluorogenic hNEU substrate 4MU-NANA well (*vide infra*).[80] Perhaps the most versatile method for detecting free sialic acids is through derivatization with 1,2-phenylenediamines (**1-7**, **Scheme 1.2C**), which react selectively with α-keto sugars to form a quinoxaline fluorophore.[81, 82] The mild acidic conditions preserve labile *O*-acetyl groups and different sialic acid species including Neu5Gc, Neu5Ac, and *O*-acetylated sialic acids can be resolved using reversed-phase HPLC.[83] The most popular 1,2-phenylenediamine for sialic acid labeling is 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) because it enables the most sensitive detection of sialic acids.[84] Other 1,2-phenylenediamines such as *o*-phenylendiamine (OPD)[85] and 4,5-dimethylbenzene-1,2-diamine (DMBA)[86] are more stable and cost-effective alternatives. 1,2-phenylenediamines have been used extensively to study sialic acid composition[87-91] and to monitor hNEU activity.[55, 92, 93]

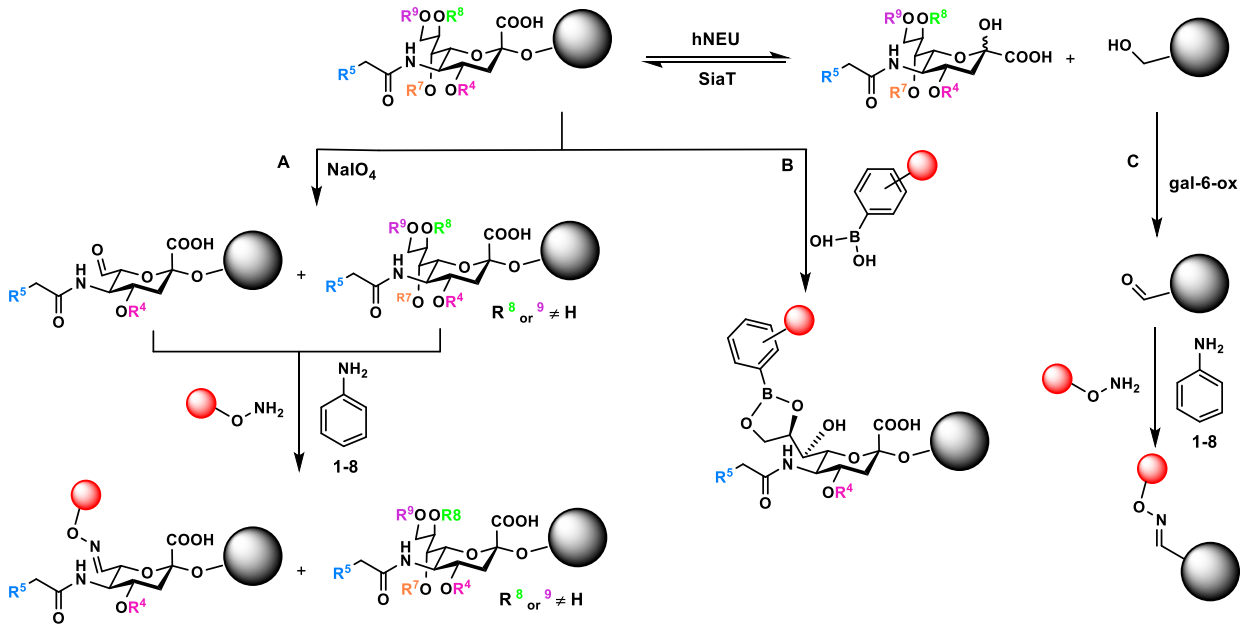


Scheme 1.2: Chemical methods to detect free sialic acids by A) thiobarbituric acid detection, B) malononitrile derivatization, and C) 1,2-phenylenediamine derivatization

Chemical methods to detect native sialic acids on glycoconjugates rely on the reactivity of the glycerol sidechain at *C*-7 – *C*-9 (**Scheme 1.3A**). Mild oxidation of the sidechain using sodium periodate generates an aldehyde at *C*-7 which can be labeled bioorthogonally.[34, 94] Historically resorcinol was used to label the aldehyde; [95] however, the reaction is not chemoselective.[96] More recently aniline (**1-8**) catalyzed - oxime ligation[97] after periodate oxidation was used to detect sialic acids on live cells[98, 99] and in zebrafish[100] (**Scheme 1.3A**). After isolation of protein fractions, this strategy was used to study the effect of ST3Gal4 knockout on glycoprotein sialylation in mice.[101] The method has also been adapted to study neuraminidase activity in a 96 well plate.[102] Periodate oxidation and aniline-catalyzed oxime ligation (PAL), coupled with a reciprocal method to detect galactose (galactose-6-oxidase and aniline-catalyzed oxime ligation, GAL; **Scheme 1.3C**),[103] was used to detect cell surface neuraminidase activity.[104] GAL has also been used to determine

neuraminidase linkage specificity on a glycan microarray.[105] The microarray was also used to determine neuraminidase monosaccharide specificity towards Neu5Gc and KDN,[105] however, labile modifications such as *O*-acetyl groups may not be stable to conditions for the generation and storage of glycan microarrays.[106] Modifications to the glycerol chain (such as *O*-acetylation) also block periodate oxidation,[95] making these modified sialic acids invisible to PAL.

Complexation of sialic acids by phenylboronic acids at the reducing end or the glycerol sidechain can be used as an alternative to chemical modification (**Scheme 1.3B**).[107] Phenylboronic acids complex 1,2- or 1,3-diols so also bind other carbohydrates,[108] but at physiological pH binding is selective for sialic acids.[109] The interactions between sialic acids and phenylboronic acids are low affinity,[109] and are weaker for sialosides than for free sialic acid,[110] but have still been used to detect of sialic acids on the cell surface.[111-114] Efforts to increase affinity and selectivity have included the addition of pendant amino[115] or urea[116, 117] groups for electrostatic interactions with the C1 carboxylic acid. Unlike other chemical methods to detect sialic acids, detection of sialic acids with phenylboronic acids does not chemically modify the sialic acid, making this interaction more analogous to biochemical methods to detect sialic acids than to chemical methods.



Scheme 1.3: Chemical methods to detect sialoside metabolism on glycoconjugates by A) PAL, B) phenylboronic acids, and C) GAL

1.4.2 Mass spectrometry methods to detect sialic acids

Mass spectrometry methods provide an alternative to solution-phase methods to detect sialosides. Mass spectrometry methods have high sensitivity compared to solution-phase methods and can provide unambiguous structural data; however, because the observable is molecular mass, differentiating isomers is non-trivial. In the context of sialosides, differentiating between α(2→3) and α(2→6) linkages or acetylation at different positions can be challenging.[118, 119] Further, different compounds may have varying response factors, making quantitation challenging.[120] Despite these issues, mass spectrometry provides an important and sensitive tool for the study of sialic acid metabolism. Glycan fingerprints of cellular *N*- and *O*-linked glycans have been determined by MALDI mass spectrometry after glycan purification and permethylation. The permethylated glycans have predictable fragmentation patterns which - in conjunction with

chemical and enzymatic degradation – can be used to elucidate glycan structure.[121] This approach was used to detect a reduction in α(2→6) sialylation on glycoproteins in gestational diabetes mellitus.[122] It was also used to investigate the effects of disrupting sialic acid biosynthesis on glycan composition and structure.[123, 124] The alkaline conditions used for permethylation would destroy labile sialic acid modifications, making the method incompatible for the study of modified sialic acids.

Mass spectrometry has been used to study neuraminidase specificity towards defined sialosides. Electrospray ionization mass spectrometry (ESI-MS) was used to study NEU3 substrate specificity on analogs of ganglioside GM3. Determination of relative rates was made possible by careful selection of internal standards that had comparable structural characteristics to both the sialic acid and lactoside products, which normalized the response factors.[56] Recently, a competitive universal proxy receptor assay (CUPRA) was developed.[125] The assay is indifferent to variance in response factor so does not need internal standards or calibration curves to determine hNEU substrate specificities, but does require conjugation of the glycan to an affinity tag. As such, the CUPRA assay does not account for the influence the aglycone may have on enzyme specificity. Conversely, ESI-MS has been used to study hNEU activity towards gangliosides embedded in picodiscs, which mimics ganglioside presentation and distribution in cell membranes.[126]

1.4.3 Biochemical tools to detect sialic acids

The unstable nature of many unusual sialosides[12, 106, 127] has historically resulted in these epitopes being overlooked using chemical methods for purification and analysis. Biochemical probes such as lectins, antibodies, and inactivated enzymes provide an alternative where unstable sialic acids can be studied *in situ* and under mild conditions. In general, lectins tend to be less

expensive than antibodies, while antibodies tend to have a higher affinity than lectins.[128] The substrate scope of biochemical probes can be broad and poorly defined, but glycan microarrays can help to alleviate this problem.[129, 130] The careful selection of a biochemical probe can generate data that compliments, or is an alternative to, chemical methods to detect sialic acids.

Sialic acid-binding lectins and their specificities have been reviewed thoroughly,[131] and we will only describe a few relevant examples herein. Some of the most widely used biochemical probes to detect sialic acids are the lectins from *Sambucus nigra* (SNA) and *Maackia amurensis* (MAL and MAH). These lectins discriminate sialic acid glycosidic linkages (SNA recognizes α(2→6)-linked sialosides and MAL and MAH recognize α(2→3)-linked sialosides) but do not distinguish between Neu5Ac, Neu5Gc, and Neu5,9Ac_2.[132, 133] There has historically been much confusion over MAH and MAL specificity.[134] These lectins recognize a charged group at *C*-3 of galactose rather than the core of the sialic acid residue, thus they can also recognize 3-*O*-sulfated galactose.[134, 135] The lectins from *Limax flavus* (LFA) and *Cancer antennarius* have no linkage preference, but LFA binds only Neu5Ac[136, 137] while the lectin from *Cancer antennarius* is selective for 4- and 9-*O*-acetylated sialic acids.[138, 139] The B subunit (SubB) of subtilase cytotoxin from *E. coli* has high specificity for α(2→3)-linked Neu5Gc.[140]. Engineering of SubB to decrease its specificity for α(2→3)-linked sialosides while maintaining its specificity towards Neu5Gc[141] enabled the detection of Neu5Gc in human serum, with potential applications as a tool for cancer diagnostics.[142, 143] The substrate specificities of lectins continue to be elucidated. Lectin Achatinin-H from *Achatina fulica* was reported as being selective for Neu5,9Ac_2-α(2→6)-GalNAc,[144] making it a probe for Neu5,9Ac_2 glycoproteins. However, the substrate specificity for Achatinin-H has not been systematically studied, and it has been shown to bind other substrates, including colominic acid.[145] Cholera toxin from *V. cholerae* is notable for its high affinity towards

ganglioside GM1,[146, 147] and research into *V. cholerae* infectivity was focused on this binding interaction.[148] Recently, fucosylated glycoproteins were also found to bind to cholera toxin[149] and Lewis X-displaying glycoproteins were identified as binders to cholera toxin, while infection was independent of GM1.[150] This example highlights the continued evolution of the understanding of lectin specificity.

There are too many antibodies available that recognize sialic acid epitopes for a thorough review of the topic in the scope of this Chapter. Instead, examples have been selected to provide an overview of the types of antibody probes available for studying sialic acid metabolism. Monoclonal antibodies tend to be specific for a sialic acid in the context of its reducing end substituents (for instance antibody MK2-34 is specific for Neu5Gc-GM2,[151] and antibody 14F7 is specific for Neu5Gc-GM3)[152] but polyclonal antibodies can be used to detect a specific sialic acid residue. Polyclonal antibodies from chicken are used to detect Neu5Gc independent of its glycosidic linkage or reducing end substituent.[153-155] There are many antibodies that are specific for ganglioside or *O*-acetylated ganglioside epitopes.[156, 157] Some antibodies that recognize gangliosides have well defined epitopes; for instance, an anti-GD2 antibody (Dinutuximab) is an approved drug for cancer immunotherapy.[158] Others suffer from the same undefined substrate scope as many lectin probes. Antibody D1.1 has been used extensively over the past 35 years as a probe to detect Neu5,9Ac_2 GD3[159, 160] but was recently found to cross-react with Neu5,9Ac_2 on glycoproteins.[161] There are reports of antibodies selective for sialylated complex-type *N*-glycans over asialo *N*-glycans and high-mannose-type *N*-glycans,[162] or that recognize portions of sialylated *N*-glycan epitopes.[163] A range of antibodies available to detect polysialic acid recognize small epitopes with degree of polymerization (DP) = as low as 2 and as large as DP > 11.[39] Antibody

selection for the detection of polysialic acid should be made carefully as DP impacts the conformation and biological activity of polysia.[164]

Biochemical probes can be engineered by inactivating enzymes that recognize carbohydrate epitopes, effectively converting the enzymes to lectins.[165] This approach is especially useful for poorly immunogenic epitopes such as polysialic acid, whose poor immunogenicity has been exploited as a potential polymer in drug delivery strategies.[166, 167] An inactive mutant of endosialidase from *Escherichia coli* (*E. coli*) K1 bacteriophage was generated that could bind, but not cleave, polysialic acid with DP >10.[168, 169] Expression of this modified endosialidase as a fusion protein with green fluorescent protein enabled one-step fluorescent labeling.[170, 171] The hemagglutinin esterase of influenza C virus removes the *O*-acetyl group from Neu5,9Ac_2.[172] After catalytic deactivation of the enzyme with diisopropyl fluorophosphate the protein can be used to detect Neu5,9Ac_2[173] without preference for glycosidic linkage or the reducing end moiety.[172, 173] Hemagglutinin esterases from other viruses have also been transformed into "virolectins" that label *O*-acetylated sialic acids.[174] The strategy of using inactivated enzymes as lectins could be expanded to other enzymes and that recognize other carbohydrate epitopes.[165]

Biochemical tools such as lectins, antibodies, and inactivated enzymes have been invaluable in the study of unstable sialic acids, however, they are limited by their substrate specificities which can be broad or not fully validated. The broad substrate specificities of many biochemical probes can lead to data outputs that overlook the heterogeneity of sialic acid presentation. Furthermore, the epitopes recognized by biochemical probes are often not fully defined, which can lead to misleading interpretations. As a result, defined sialoside substrates are still essential tools for studying the biological roles and metabolism of sialic acids.

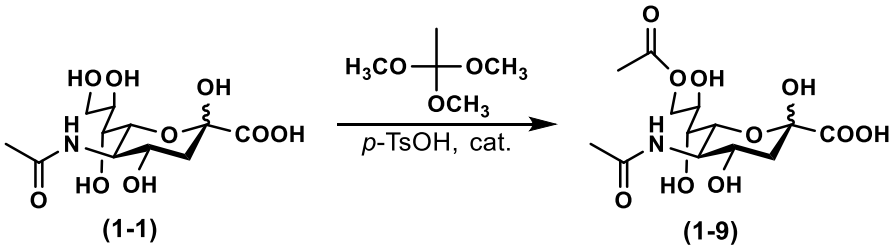
1.5 Defined substrates for the study of sialic acid metabolism.

Access to defined sialoside substrates enables a more controlled investigation into the impact of sialic acid presentation on their metabolism. *In vitro* studies with defined substrates can eliminate numerous variables present when analyzing complex biological mixtures, generating less ambiguous data. These studies, however, remove the sialic acid substrates from their biological context, where the nuances of sialic interactions and spatial distribution may influence their biological function.[175] Labelled sialic acid substrates can be useful for monitoring metabolism *in cellulo* or *in vivo*, but are not always good mimics of native substrates. Therefore, using a combination of defined sialoside substrates and the methods to detect sialic acids discussed in **Section 1.4** can enable a more complete understanding of sialic acid metabolism.

1.5.1 Methods for the synthesis of defined substrates

Chemical and chemoenzymatic methods have been used extensively to make sialoside probes encompassing the diversity in sialic acid presentation. Modified sialic acid monosaccharides have been generated through chemical modification of Neu5Ac or to *N*-acetylmannosamine (ManNAc), which is a biosynthetic precursor converted to a sialic acid through condensation with pyruvate by a sialic acid aldolase (sialic acid synthetase).[176, 177]. Following the biosynthetic pathway in mammals, Neu5Ac-9-phosphate was made from ManNAc-6-phosphate[178, 179] and was used to screen inhibitors of sialic acid phosphatase.[180] Both Neu5Gc[181] and Neu5,9Ac_2[182] have also been prepared by this strategy. Alternatively, selective acetylation of Neu5Ac using trimethyl orthoacetate generated Neu5,9Ac_2 (**1-9**) in a single step (**Scheme 1.4**), while protection of *O*-9 allowed the synthesis of 4-*O*-acetylated sialic acid (Neu4,5Ac_2).[183] Other partially acetylated sialic acids, including Neu5,7Ac_2 and Neu5,8Ac_2 have been made using carefully chosen protecting group strategies to avoid the hydrolysis or migration of the labile

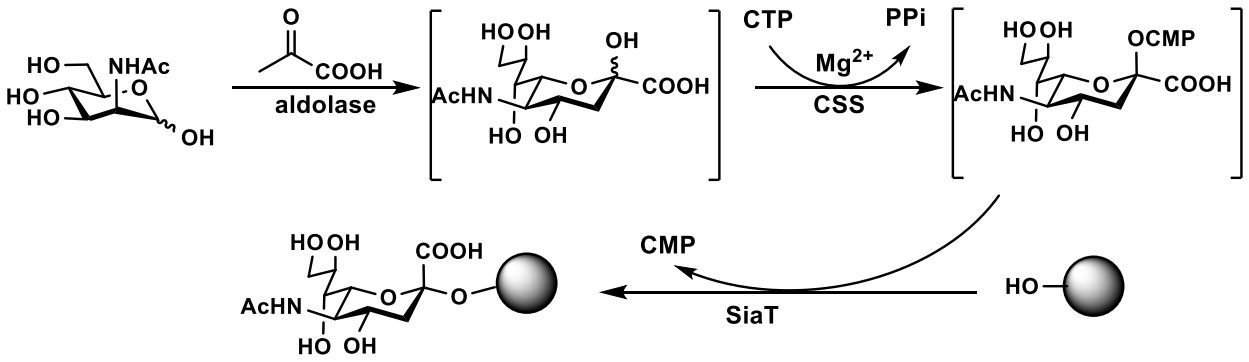
esters.[184, 185] Selective chemoenzymatic acetylation of Neu5Ac has also been used to generate Neu5,9Ac$_2$ sialosides.[186, 187] Direct conversion of Neu5Ac to Neu5Gc can be accomplished by de-*N*-acylation of the Neu5Ac methyl ester to neuraminic acid methyl ester, followed by *N*-acylation with glycolic acid,[188] or mannosamine-HCl can by acylated and converted to a sialic acid using a sialic acid synthetase (**Scheme 1.5**).[189, 190] These strategies can also be used to generate labelled *C*-5 derivatives. Numerous non-human and unnatural sialic acids have been made through both chemical and chemoenzymatic methods, and are reviewed elsewhere.[191, 192]



Scheme 1.4: One-step synthesis of Neu5,9Ac$_2$ from Neu5Ac using trimethyl orthoacetate.

Extensive effort has been applied to the development of sialic acid glycosylation strategies. Chemical α-sialylation is complicated by steric hindrance at the tertiary anomeric center, the absence of substituents at *C*-3 to guide stereoselectivity, and the thermodynamic stability of the β-sialoside.[192, 193] There are numerous strategies that have been developed for stereoselective glycosylation to make α(2→3)-, α(2→6)-, and α(2→8)-linked sialosides which have been reviewed elsewhere; [192-195] however, chemical sialylation remains an ongoing challenge. Chemoenzymatic sialylation exploits biosynthetic pathways for protecting group-free sialylation using sialyltransferases (SiaT). It provides a reliable stereoselective alternative to chemical methods but is limited by the substrate tolerance of the enzymes. One-pot multienzyme (OPME) chemoenzymatic approaches facilitate synthetic strategies by executing multiple chemical transformations without isolating intermediates. Consequently, sialosides can be generated from ManNAc, Neu5Ac, or variations thereof in a single step (**Scheme 1.5**).[196] Chemoenzymatic

syntheses of α(2→3)-, α(2→6)-, and α(2→8)-linked sialosides -including polysialic acid- have been thoroughly reviewed elsewhere.[40, 186, 197] An alternative to OPME chemoenzymatic synthesis is metabolic engineered microorganisms in which all the enzymes necessary for one or many synthetic steps are expressed. These cell factories circumvent the need for isolated enzymes and can be scalable.[198] Still, the development of a microbial strain that expresses all of the enzymes necessary for synthesis - without the enzymes that lead to side products or product degradation – is non-trivial.[199]



Scheme 1.5: General approach to one-pot multienzyme chemoenzymatic sialylation (CTP = cytidine triphosphate, PPi = pyrophosphate, CSS = CMP-sialic acid synthetase, CMP = cytidine monophosphate).

Sialylation strategies have been applied to the synthesis of complex sialoglycoconjugates such as gangliosides, and *N*- and *O*-linked glycans. The synthesis of gangliosides is complicated by their amphiphilic nature. The glucosylceramide cassette strategy facilitates the synthesis of complex gangliosides.[200] while ganglioside analogs with simplified aglycones can be sufficient ganglioside mimics and are easier to synthesize.[56] A review detailing chemical and chemoenzymatic methods for the synthesis of modified gangliosides was recently published by our group.[40] The synthesis of *N*-glycans is challenging because of the incredible diversity of these often asymmetric branched oligosaccharides. Although convergent chemical synthetic strategies

have been used to make specific *N*-glycans,[201-203] the synthesis of sialylated *N*-glycans is typically accomplished through the chemoenzymatic extension of a core oligosaccharide isolated from natural sources or made through synthetic methods.[19, 204-207] Orthogonally protected core structures facilitate the synthesis of asymmetric *N*-glycans (**Figure 1.2**).[18, 208, 209] Comparatively, methods for the synthesis of *O*-glycans have lagged behind.[210] The continued development of general and orthogonal methods to access these complex *N*- and *O*-glycan structures will be valuable tools for generating defined sialoglycoconjugates.

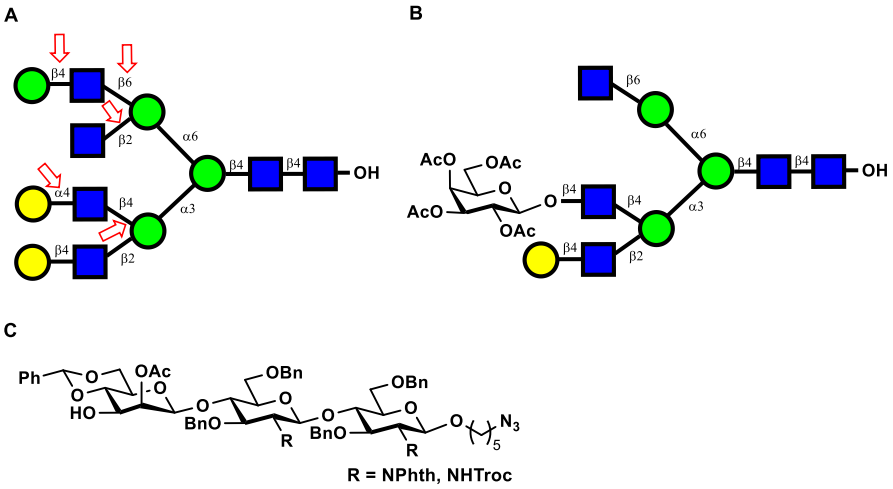


Figure 1.2: *N*-glycan oligosaccharide scaffolds with orthogonal reactivity to generate diverse *N*-glycan sialoglycoconjugates through chemoenzymatic synthesis A) tetraantennary scaffold that can be degraded with glycosidases at places marked with red arrows to generate asymmetric tetraantennary *N*-glycans,[18] B) tri-antennary scaffold with orthogonal reactivity. Per-acetylated galactose can be deprotected following the chemoenzymatic extension of the neighboring galactose to de-symmetrize the scaffold.[208] C) Bioorthogonally protected core structure for the modular synthesis of asymmetric high-mannose, hybrid, and complex-type *N*-glycans.[209].

The methods for the synthesis of sialoside substrates discussed herein can be used to generate defined natural sialoside substrates for the study of sialoside metabolism using the methods to

detect sialic acids discussed in **Section 1.4**. Alternatively, these methods can be applied to generate labelled substrates for the study of sialoside metabolism.

1.5.2 Labelled substrates to study sialic acid metabolism by neuraminidase

High-throughput fluorogenic substrates to study hNEU activity have been used extensively for over 40 years (**Figure 1.3A**). The most popular hNEU substrate is 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (4MU-NANA, **1-10**).[211, 212] Release of the sialic acid unmasks an alcohol, which upon deprotonation leads to increased fluorescence of the coumarin moiety. 4MU-NANA has been used extensively as a tool to normalize neuraminidase activity and to screen hNEU inhibitors.[74, 75, 213-215] Modifications to *C*-5[92, 216] and *C*-9[76] of the sialic acid have been used to probe the substrate tolerance of hNEU; however, 4MU-NANA is not a good mimic of natural substrates.[76] Recently, similar substrates have been developed to image neuraminidase activity in tissues.[217, 218] Neuraminidase activity released an insoluble, and highly fluorescent benzothiazolylphenol (BTP) aglycone from the soluble and minimally fluorescent, Neu5Ac-BTP (**1-11**).[219] These simple fluorogenic neuraminidase substrates have been useful tools to monitor neuraminidase activity, but do not take into account the influence of sialic acid glycosidic linkage or the reducing end substituent on enzyme activity.

A disaccharide substrate with a *p*-nitrophenol aglycone (Neu5Ac-α(2→3/6)-Gal-β-*p*-NP, **1-12**) enabled the differentiation of glycosidic linkages. The substrate was incubated with neuraminidase and an excess of β-galactosidase which would release *p*-NP upon removal of the sialic acid.[220] The coupled enzyme assay was adapted to be high-throughput[221] and was used to determine NEU2 specificity towards modified sialic acids.[58, 60] A Neu5Ac-α(2→8)-Neu5Ac-α(2→3)-Gal-β-*p* -NP substrate and the inclusion of an excess of neuraminidase specific for

α(2→3)-linked sialosides expanded the scope of the assay to α(2→8)-linked sialosides.[222] A 5-bromo-4-chloro indol has been used as an alternative to *p*-NP, and produces visible color upon sialic acid hydrolysis.[223] Monosialylated substrates with a BODIPY-tagged aglycone (**1-13**) have been used to determine Michaelis-Menten kinetics parameters for NEU1-NEU4 in a well-plate assay.[54] Prior to fluorescence detection, the negatively charged sialylated substrates were removed from the neutral product using anion exchange resin (**Figure 1.3B**).[224, 225] This assay could be applied to measure the kinetics of hNEU activity on any glycan containing one sialic acid. A ganglioside probe with a FRET donor at the *C*-9 of sialic acid and a FRET acceptor on the ceramide tail (**1-14**) was used to monitor neuraminidase activity in live cells where neuraminidase activity correlated with an increase in coumarin fluorescence and a decrease in BODIPY fluorescence, making it an "on-off" probe (**Figure 1.3C**).[226] Although the fluorophore at *C*-9 of sialic acid makes it a poor probe of hNEU specificity towards sialic acids, the probe is a better ganglioside mimic making it a probe of ganglioside processing by neuraminidases.

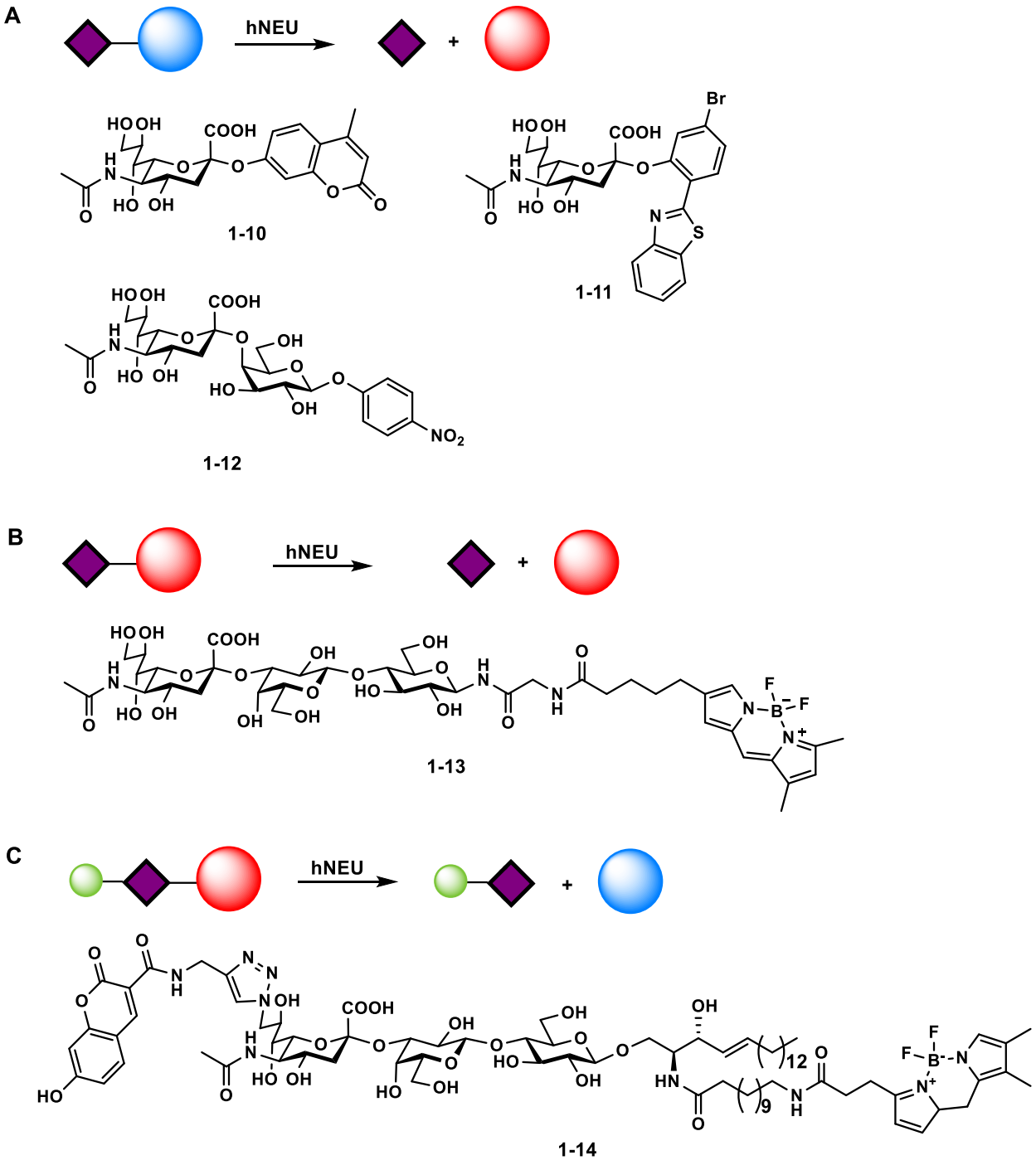


Figure 1.3: Selected labelled sialic acid substrates to study hNEU activity A) fluorogenic and "off-on" absorbance-based substrates to study hNEU activity, B) "on-on" fluorescent substrate, C) "on-off" FRET probe.

When studying the effects of sialic acid presentation on its metabolism, an informed evaluation of which factors influencing sialic acid presentation are most important to the study is imperative. Further, there is a trade-off between generating defined substrates that effectively represent sialic acid presentation and the synthetic effort required to produce these targets. A diverse chemical biology toolkit, providing access to an array of defined sialoside substrates and methods to detect sialic acids is essential to the study of sialoside metabolism in biological processes.

1.6 Project Objective

Our group has an ongoing interest in sialic acid metabolism by hNEU and its roles in biological processes. Previous work has demonstrated that hNEU-catalyzed hydrolysis of sialic acids is influenced by the diversity of sialic acid presentation at multiple levels – sialic acid structural modification, glycosidic linkage, and reducing end substituent. We identified gaps in the literature where hNEU activity towards various unstable sialosides, namely 9-*O*-acetylated sialic acids and polysialic acid, was undefined. These unusual sialic acids have been implicated in numerous normal and disease processes, but the instability of the substrates has hampered their study and there is a shortage of data on their metabolism by hNEU. *In this thesis, we will develop experimental methods to study the substrate tolerance of hNEU towards sialic acids with unstable presentations. We hypothesize that the hNEU isoenzymes will have different activities towards these sialic acids, which will provide insight into the roles of these enzymes and their substrates in human health and empower future study into the biological roles f these sialosides.*

In Chapter 2 we began our study of hNEU activity towards Neu5,9Ac_2 by monitoring hNEU activity on simple fluorescent substrates based on 4MU-NANA (**1-10**) and octyl sialyllactoside mimics of the ganglioside GM3 with α(2→3)- and α(2→6)-glycosidic linkages. Our results indicated that in order to fully understand hNEU activity towards Neu5,9Ac_2 we needed to expand

the scope of the study towards more Neu5,9Ac$_2$ substrates. In Chapter 3 we widened the scope of our study of Neu5,9Ac$_2$ as substrates for hNEU to include a sialoglycoprotein and α(2→8)-linked sialic acids. To study hNEU activity on these complex sialoside substrates we needed to adapt and expand the assays available to study hNEU activity. Polymers of α(2→8)-linked sialic acids (polysialic acid, polysia) have unique chemical and physical properties which make them challenging to study. Chapter 4 details the first systematic investigation of hNEU activity towards polysialic acid using purified enzymes. The work in this thesis expands our understanding of sialic acid metabolism by hNEU and highlights the importance of considering multiple factors of sialic acid presentation when studying its roles in biological systems.

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Chapter 2 : Human neuraminidase isoenzymes show variable activities for 9-O-acetyl-sialoside substrates.[a,b]

[a] A version of this chapter has been published as: Hunter, C.D.; Khanna, N.; Richards, M.R.; Darestani, R.R.; Zou, C.; Klassen, J.S.; Cairo, C.W., (2018) Human neuraminidase isoenzymes show variable activities for 9-*O*-acetyl-sialoside substrates. *ACS Chemical Biology*, 13, 4, 922-932.

[b] Methodology for synthesis of octyl sialyllactoside compounds was developed by Neha Khanna, molecular modelling was carried out by Michele R. Richards, ESI-MS assay was developed and implemented by Reza Rezaei Darestani and John Klassen, NEU1-NEU4 enzymes were expressed by Cecilia Zou.

2.1 Introduction

Sialic acids are a structurally diverse family of carbohydrates; however, the influence of subtle structural changes on their biological function is not well understood. These 9-carbon, α-keto acids are often the terminal (non-reducing) carbohydrate residue of human glycans.[1] Their location at the periphery of the glycan allows sialic acids to play roles in development, immune response, host-pathogen interactions, and tumor metastasis.[2, 3] The most common sialic acid in humans is the 5-*N*-acetylneuraminic acid (Neu5Ac), and it is considered to be the precursor for most sialic acids. Common modifications of Neu5Ac include a glycolyl group at *N*-5 (Neu5Gc), and *O*-acetate, -sulfate, -lactate, or -phosphate ester modifications of hydroxyl groups.[1] The 9-*O*-acetylated form of sialic acid (Neu5,9Ac_2) has been implicated in blocking lectin binding,[4] yet also enhances the affinity of influenza C hemagglutinin.[5] Ligands for immune cell lectins, such as Siglecs, can be masked by 9-*O*-acetylation of sialic acid.[6] The presence of Neu5,9Ac_2 has been associated with cancer cell survival through prevention of GD3-mediated apoptosis,[7] and protection of sialoside substrates from bacterial and viral neuraminidases.[8] Despite its recognized importance, the specific roles of Neu5,9Ac_2 residues have remained unclear. Routine study of Neu5,9Ac_2 has been hampered by the lability of the *O*-acetate, particularly under basic conditions;[9-11] limiting detection strategies to the use of antibodies, lectins, or influenza C hemagglutinin-esterase.[12] This fact prompted the recent development of a hydrolytically stable analog of Neu5,9Ac_2 (9-acetamido-9-deoxy-*N*-acetylneuraminic acid) for glycan microarrays.[11]

The 9-*O*-acetylation of sialosides is regulated *in vivo* by the action of two opposing enzyme activities: sialate-*O*-acetyltransferases (SOAT) and sialate-9-*O*-acetylesterases (SIAE). Human SIAE have been identified[13, 14] and are implicated in several diseases including rheumatoid arthritis and type I diabetes.[15] In childhood acute lymphoblastic leukemia, there is increased sialic acid *O*-

acetylation as a consequence of both decreased SIAE activity and increased SOAT activity.[16] Furthermore, increased 9-*O*-acetylation has been observed in melanoma, small cell lung carcinoma, glioblastoma, and breast carcinomas.[17] The removal of the 9-*O*-acetyl group by treatment with SIAE induced apoptosis in both leukemia[18] and glioblastoma cells.[7] While human SIAEs have been known for decades,[13, 14] human SOATs have been more challenging to isolate, due to the sensitivity of AcT activity to membrane solubilization.[19] The protein CASD1 has recently been found to be essential for sialic acid 9-*O*-acetylation in humans;[20] however, this enzyme may not be responsible for SOAT activity in gangliosides.[17]

While both acetylesterases and acetyltransferases are critical to the prevalence of Neu5,9Ac_2, the ability of these residues to modulate activity of sialic acid modifying enzymes is not well understood. Neuraminidase (also called sialidase) enzymes are glycosyl hydrolases which cleave the glycosidic linkage of sialiosides (EC 3.2.1.18). Four distinct human neuraminidase (hNEU) isoenzymes have been identified (NEU1, NEU2, NEU3, and NEU4). The hNEU isoenzymes differ in subcellular localization, tissue expression, and substrate preference.[21-25] Seyrantepe et al. demonstrated that both the sialic acid aglycone and reducing-end sugar have a large impact on the relative activity of hNEU.[23] While NEU2 and NEU4 can cleave glycoproteins, glycolipids, and oligosaccharides;[23, 26] NEU1 cleaved only glycoproteins and oligosaccharides, [23, 27] and NEU3 demonstrated a strong preference for glycolipid substrates.[23, 28] Sialic acid diversity also impacts hNEU activity. The hNEU are reported to have different activity for Neu5Gc substrates compared to Neu5Ac substrates.[29,30] Sialic acid oligomers containing Neu5Gc residues have reduced substrate activity with NEU1, NEU2, and NEU4.[31] The Neu5,9Ac_2 residue is known to impede the activity of bacterial and viral neuraminidases.[32] Reports differ on whether Neu5,9Ac_2 residues are substrates for mammalian NEU.[33-35]

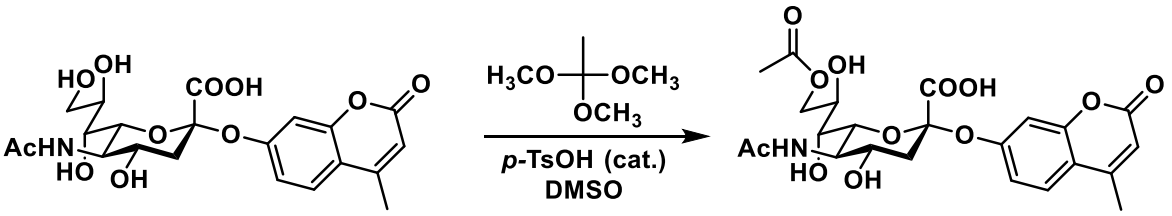
To the best of our knowledge, a comprehensive study probing the influence of sialic acid 9-*O*-acetylation on hNEU modulation has not been carried out. While recent work indicated that Neu5,9Ac$_2$, is a poor substrate for NEU2,[35] previous work with sialosides containing unnatural modifications at C-9 have suggested that variation among isoenzymes could result in disparate activity for Neu5,9Ac$_2$ substrates. Substitutions of the 9-OH of Neu5Ac with fluoride, methoxy, hydrogen, and azide groups almost completely inhibited NEU2 activity.[36] Sandbhor et al. reported that 9-azido, -amino, or -aryl groups reduced substrate activity of sialosides for NEU3.[37] Futhermore, the most selective inhibitors known for NEU1[38] and NEU4[39] involve modifications of 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) at the C-9 position.[25] These examples suggest that the C-9 pocket of hNEU enzymes is critical for recognition, and could result in differential activity for Neu5,9Ac$_2$ substrates among the isoenzymes.

Generation of defined sialosides containing Neu5,9Ac$_2$ is necessary for elucidation of enzyme specificity. While the generation of 9-*O*-acetyl sialiosides is challenging, there are examples using selective trimethyl orthoacetate chemistry.[40-46] Enzymatic strategies using *O*-acetyltransferases have been reported.[47] In order to test for effects of 9-*O*-acetylation on hydrolysis by hNEU, we generated a fluorogenic substrate containing Neu5,9Ac$_2$: 2′-(4-methylumbelliferyl)-α-D-9-*O*-acetyl-5-*N*-acetylneuraminic acid (4MU-Neu5,9Ac$_2$ **2-2**, **Scheme 2.1**). A similar strategy has been explored for testing the role of N-5 modifications of Neu5Ac on hNEU activity in cell lysates.[48] Using fluorogenic substrates in combination with purified isoenzymes, we observed that substitution of Neu5,9Ac$_2$ for Neu5Ac significantly blocked sialic acid cleavage by multiple isoenzymes of hNEU. Furthermore, we generated a panel of octyl sialyllactosides (**2-4 – 2-9**, **Figure 2.2**) containing Neu5Gc and Neu5,9Ac$_2$ residues with variation of glycosidic linkages to Gal (either α(2→3) or α(2→6)). We observed that trisaccharides containing Neu5,9Ac$_2$ were

generally poor substrates for NEU2, NEU3, and NEU4 isoenzymes, with NEU2 having the greatest sensitivity to this modified residue. These findings support a role for enzymes that regulate 9-*O*-acetylation of Neu5Ac (i.e. SIAE, SOAT) in altering hNEU activity on cellular sialosides.

2.2 Results and discussion

2.2.1 hNEU discriminate 4MU-Neu5,9Ac$_2$



2-1 **2-2**

Scheme 2.1: Synthesis of fluorogenic hNEU substrate 4MU-Neu5,9Ac2 (**2-2**) from 4MU-Neu5Ac (**2-1**)

To investigate the effect of 9-*O*-acetylation of sialic acid on hydrolysis by hNEU, we acetylated **2-1** at *O*-9 using the method of Furuhata and Ogura to generate 4MU-Neu5,9Ac$_2$ **2-2** (**Scheme 2.1**).[46] Incubation of **2-1** or the 9-*O*-acetylated derivative, **2-2**, with hNEU allowed us to monitor the relative rates of hydrolysis by fluorescence spectroscopy. The four hNEU isoenzymes exhibited dramatic differences in their ability to hydrolyze Neu5,9Ac$_2$ (**Figure 1.1**, **A2.1**). The hNEU isoenzymes discriminated between the unmodified and 9-*O*-modified substrates to different extents. NEU4 was the only isoenzyme to show a significant (2-fold) preference for the Neu5,9Ac$_2$ substrate. Of the other isoenzymes, NEU3 had a 5-fold preference for the Neu5Ac substrate. Remarkably, NEU2 activity was almost completely blocked by acetylation of the *O*-9 position.[35] Overall, these data clearly demonstrated that the 9-*O*-Ac modification could have a substantial,

isoenzyme-specific, influence on hNEU activity. However, we considered that the 4MU-based substrates may be poor mimics of the physiological substrates of the enzymes, and proceeded to investigate differences in hNEU activity in the context of trisaccharide substrates.

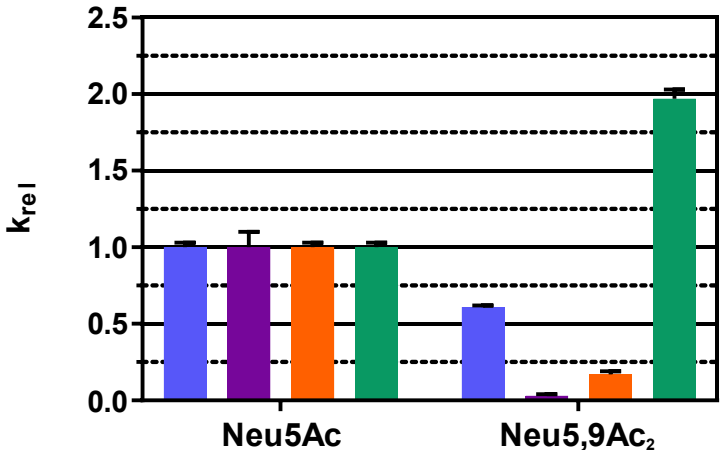


Figure 2.1: Effect of sialic acid 9-*O*-acetylation on hNEU hydrolysis of 4MU-substrates. Rates were obtained by linear regression of triplicate experiments. Data is presented as k_{rel} and normalized to 4MU-Neu5Ac **2-1**, and error bars correspond to standard error.

2.2.2 Synthesis of octyl sialyllactosides

Human neuraminidase substrate preference depends not only on sialic acid but also on the reducing end sugars and aglycone.[37] For instance, NEU1 is known to prefer 4MU-Neu5Ac **2-1** over 3'-sialyllactose (the carbohydrate moiety of **2-3**), which in turn was cleaved more efficiently than gangliosides. NEU3 demonstrated the opposite preferences, with gangliosides being preferred over 4MU-Neu5Ac (**2-1**).[23] The specificity of human NEU for glycolipid substrates containing LacNAc have been previously investigated.[49] To generate substrates for hNEU similar to physiological targets, we prepared analogs of the ganglioside GM3 containing modified sialic acid residues (Neu5Ac, Neu5Gc, and Neu5,9Ac$_2$) using a chemoenzymatic strategy (**Figure 2.2**, **A2.2**).

Previous work in our group had shown that an octyl chain is a sufficient mimic of the native ceramide in GM3 to maintain NEU3 activity.[37] This substitution simplified both the synthesis and purification of glycolipid substrates. Smutova et al. observed that the glycosidic linkage between sialic acid and galactose had a significant impact on hNEU activity.[49] With these data in mind, we designed a panel of octyl-sialyllactoside analogues with variable sialic acid residues to investigate substrate activity of hNEU. We varied the anomeric linkage of the sialic acid to the galactose residue, preparing both α(2→3) (**2-4**, **2-6**, **2-8**) and α(2→6) (**2-5**, **2-7**, **2-9**; **Figure 2.2**) linkages. We also synthesized substrates containing Neu5Gc, as the *N*-glycolyl group has been proposed to influence NEU2 activity.[36, 48]

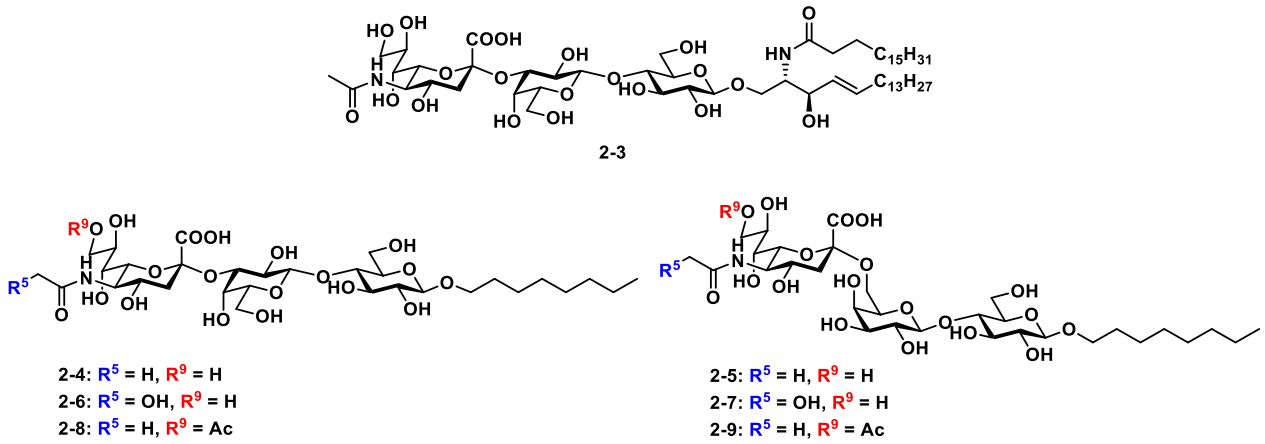


Figure 2.2: Structures of hNEU substrate GM3 **3-3** and octyl sialyllactoside targets containing α(2→3) (**2-4**, **2-6**, **2-8**) or α(2→6) (**2-5**, **2-7**, **2-9**) linkages.

The most facile route to modified sialic acid-containing substrates is through the one-pot, chemoenzymatic method developed by Chen and coworkers.[42, 50, 51] This approach circumvents the challenges faced by chemical methods.[52, 53] The acceptor for the chemoenzymatic sialylation was β-octyl lactoside, obtained in 5 steps from lactose.[37] The Neu5Ac residue was converted to Neu5,9Ac$_2$ in one step.[40] The starting materials Neu5Ac, Neu5Gc, Neu5,9Ac$_2$, and the acceptor

were subjected to a one-pot, two-enzyme reaction (CSS and SiaT) to yield compounds **2-4**, **2-5**, **2-7**, **2-8**, and **2-9** (**A2.2**).

The Neu5,9Ac_2 sialyllactoside targets **2-8** and **2-9** presented unique challenges for chemoenzymatic synthesis. Optimal conditions for the one-pot multienzyme reaction are alkaline (pH 8.8), which rapidly hydrolyzes the Neu5,9Ac_2 ester. Following Chen and coworkers protocol for chemoenzymatic synthesis of *O*-acetylated sialic acids, we lowered the pH of the reaction buffer to 7.2.[54] We observed partial hydrolysis at long reaction times, while shorter times (3 h) provided the best yields for Neu5,9Ac_2 α(2→6) product **2-9** (33%) after purification by C_{18} chromatography. Synthesis of the Neu5,9Ac_2 α(2→3) product **2-8** required purification by HPLC, and gave a low yield of the desired product (10%).

2.2.2 Solution phase kinetics of hNEU activity with octyl sialyllactosides

With octyl sialyllactosides **2-4** – **2-9** in hand as improved mimics of ganglioside targets, we sought to confirm changes to hNEU kinetics with these substrates. Substrates **2-4** – **2-9** lack any sensitive chromophore for detection of cleavage by fluorescence or UV spectroscopy. To monitor the reactions of **2-4** – **2-9** with hNEU we adapted a known assay for the detection of free sialic acid, in which malononitrile reacts with the ketone of open chain sialic acid under basic conditions to generate a fluorescent product with a limit of quantitation (LOQ) of 2 μM.[55] This fluorescent assay does not require modification of the substrates with a chromophore, and can therefore be used to enable the study of native substrates.[30, 49, 56] However, the low sensitivity of the assay in combination with the slow kinetics of NEU3 and NEU4 limited our analysis to the determination of relative rates (k_{rel}). In testing the activity of NEU1 with **2-4**, we found that the rate of cleavage was too slow for detection by this assay; therefore, we did not attempt to quantify

the kinetics of **2-4** – **2-9** against NEU1 with the malonitrile assay (**A2.3**). This observation is consistent with previous reports that gangliosides are poor substrates for NEU1.[23]

Among the remaining hNEU isoenzymes, we found that NEU2 was the least tolerant of modifications to the Neu5Ac residue or its glycosidic linkage. Due to this specificity, we had to alter the timescale of the experiments to obtain a full data set for NEU2. For the Neu5Ac and Neu5Gc α(2→3) substrates **2-4** and **2-6**, data was collected every 1 min over 4 min, while for substrates **2-5**, **2-7**, **2-8**, and **2-9** data was collected every 10 min over 40 min. Substrate **2-5**, which differs from **2-4** only in the α(2→6) glycosidic linkage, had more than a 30-fold reduction in activity. The Neu5Gc residue with an α(2→3)linkage **2-6**, showed a 2-fold loss of activity as compared to **2-4**; while the α(2→6)-linked Neu5Gc **2-7**, had very low activity (**Figure 2.3**). The observed preference of NEU2 for Neu5Ac over Neu5Gc contrasts previous reports using disaccharides containing a *p*-nitrophenyl-galactoside.[36] These structural differences in the substrates are likely responsible for disagreement as hNEU substrate preference is influenced by the aglycone. Furthermore, the glucose residue of 3'-sialyllactose participates in two hydrogen bonds with NEU2 outside of the active site.[57] Both Neu5,9Ac_2 substrates **2-8** and **2-9** had remarkably low rates of cleavage. Substrates **2-8** and **2-9** were hydrolyzed at least 100-fold slower than **2-4**, consistent with the data collected for the 4MU-NANA substrates with NEU2 (**Figure 2.3**, **A2.7**). Our results are consistent with a recent observation that Neu5,9Ac_2 sialosides resulted in nearly complete loss of NEU2 hydrolysis activity.[35, 36]

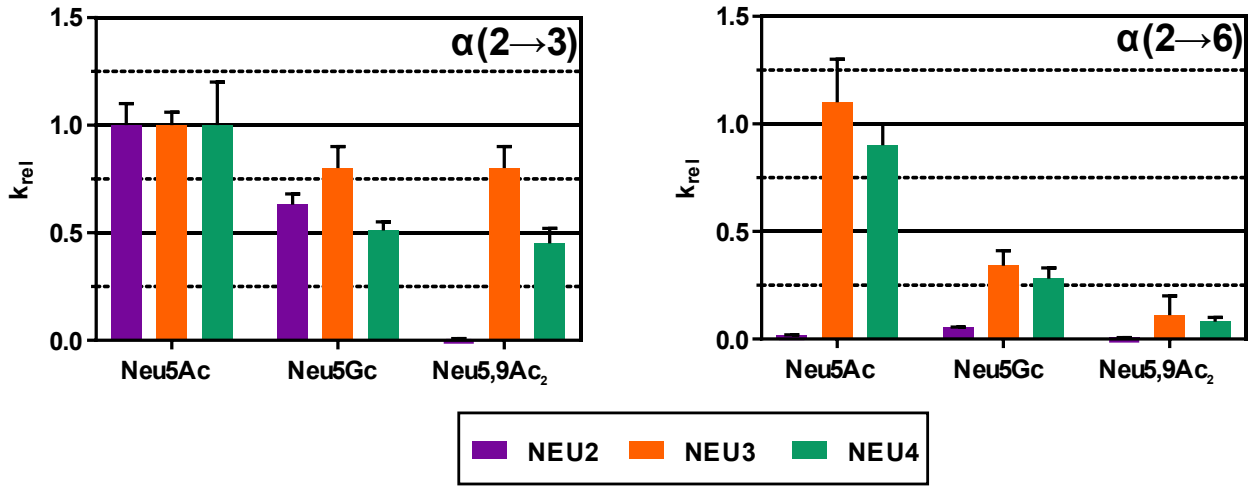


Figure 2.3: Substrate specificity of hNEU towards octyl sialyllactosides. Data are given as relative rates, normalized to α(2→3) Neu5Ac **2-4** substrate. Data for Neu5Ac and Neu5Gc α(2→3) octyl sialyllactosides **2-4** and **2-6** were taken every one minute for 4 minutes and rates for the remaining substrates were obtained by taking points every 10 min over 40 min. Rates were obtained by linear regression of triplicate experiments with error bars indicating standard error. NEU1 cleavage of octyl sialyllactosides **2-4** and **2-5** was too slow for detection and is not shown.

We observed that NEU3 was more tolerant of substrate modifications than NEU2. NEU3 had a preference for α(2→3) over α(2→6) linkages (**Figure 2.3**, **A2.7**) which was only significant with modified sialosides (Neu5Gc and $Neu5,9Ac_2$).[49] The incorporation of Neu5Gc for substrates **2-6** and **2-7** both showed reduction in activity to approximately half of that for Neu5Ac substrate **2-4**. NEU3 activity showed a similar reduction for $Neu5,9Ac_2$ (**2-8**, **2-9**) substrates relative to **2-4**. Interestingly, while the C-9 pocket of NEU3 has been exploited for the design of inhibitors of NEU3,[56] it did not accommodate the acetate group, nor a C-9 methylamide.[38] We concluded that NEU3 had a modest preference for sialosides with an α(2→3) linkage and only minor discrimination between that Neu5Gc and $Neu5,9Ac_2$ substrates (~2-fold reduction). The NEU3 kinetic data suffered from large standard errors which we attributed to slow kinetics for this

enzyme combined with the high LOQ of the malonitrile assay and limited quantities of the substrates. Therefore, we pursued alternative assays to confirm this finding (*vide infra*).

We found that NEU4 was also more tolerant than NEU2 with modified substrates. The NEU4 isoenzyme exhibited only a moderate preference for α(2→3) over α(2→6) linkages.[49] We observed NEU4 to have a preference for Neu5Ac over Neu5Gc residues. The crystal structure of NEU2 and homology models developed for NEU3 and NEU4 suggest that these three isoenzymes use similar active site residues (N86, Y178, E218 of NEU2; N88, Y179, E225 of NEU3; N86, Y177, E222 of NEU4) to recognize the N5 group.[58] Surprisingly, NEU4 exhibited a significant discrimination against Neu5,9Ac$_2$ substrates (**2-8**, **2-9**) compared to the Neu5Ac substrate **2-4** (**Figure 2.3**, **A2.7**). This result is the opposite of the substrate preference we observed with our 4MU-NANA substrates (**Figure 2.1**). The NEU4 isoenzyme has previously been shown to have a significant preference for 4-MU substrates over gangliosides, and the aglycone may have an influence on the observed activity.[23] This hypothesis is consistent with the observed differences between 4MU-Neu5Ac and GM3 interacting with the NEU2 active site.[57] This result emphasized the influence of the aglycone on substrate recognition by hNEU, and highlighted the limitations of 4MU substrates for the study of hNEU substrate specificity. Together, our results suggested a currently unrecognized role for 9-*O*-acetyl modifications in the regulation of sialic acid catabolism.

2.2.4 ESI-MS kinetics

Our kinetic studies of trisaccharide substrates for hNEU highlighted a need for more sensitive assays. In particular, while the 4MU-based substrates clearly indicated a NEU3 preference for Neu5Ac, the nature of the trisaccharide assays could not clearly resolve the effect of Neu5,9Ac$_2$ substrates on NEU3 hydrolysis. To address this issue, the Klassen lab designed an

electrospray ionization mass spectrometry (ESI-MS) assay to monitor the hydrolysis of octyl sialyllactosides by hNEU. Electrospray ionization is a sufficiently mild ionization technique to preserve the *O*-acetyl ester of **2-8** and **2-9** and is more sensitive than the malononitrile fluorescence assay. Additionally, the assay can simultaneously measure both substrate depletion and product formation by monitoring changes in substrate and product ion abundances, relative to an internal standard (leucine encephalin, and *N*-acetyl-9-azido-9-deoxy-neuraminic acid). The ESI-MS assay was implemented to monitor the kinetics of NEU3 with the α(2→3) substrates (**2-4**, **2-6**, and **2-8**; **Figure 2.3**). Control experiments were performed in the absence of enzyme to confirm that changes in abundance of the substrate and product were not due to in-source (gas phase) fragmentation of the substrate ions. With this assay, we confirmed the trends observed in the malononitrile assay. We ascertained a two-fold decrease in activity for Neu5Gc substrate **2-6** relative to **2-4**. We observed a 7-fold reduction in activity for Neu5,9Ac_2 substrate **2-8** relative to **2-4** – consistent with the 4MU assay for substrates **2-1** and **2-2**.

Table 2.1: Results of time-resolved ESI-MS data for NEU3 cleaving α(2→3) linked octyl sialyllactosides.

		Single Substrate[a]		**Mixture**[a]	
Substrate		Substrate depletion	Product formation	Substrate depletion	Product formation
2-4	Neu5Ac	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.1	1.00 ± 0.2
2-6	Neu5Gc	0.6 ± 0.1	0.42 ± 0.07	0.37 ± 0.07	0.44 ± 0.08
2-8	$Neu5,9Ac_2$	0.17 ± 0.06	0.06 ± 0.03	0.25 ± 0.02	0.11 ± 0.03

[a] Results are reported as relative rates (k_{rel}) using the mean of three runs, and errors corresponding to one standard deviation.

ESI-MS can differentiate between modified sialic acids based on the differences in molecular weights. This feature allowed us to test for competition between different sialic acid-containing substrates for the hNEU active site, which is likely to occur in physiological settings. Relative rates were determined for substrates **2-4**, **2-6**, and **2-8** measured both in isolation and in a mixture of all three substrates (**Table 2.1**, **Figure 2.4**). The differences in relative rates for substrates measured individually and in a mixture were not significant by one-way ANOVA. Therefore, we concluded that competition between substrates did not have a significant impact on sialic acid hydrolysis. These data confirmed the results of the malononitrile assay that NEU3 had substantially reduced activity Neu5Gc and $Neu5,9Ac_2$ substrates.

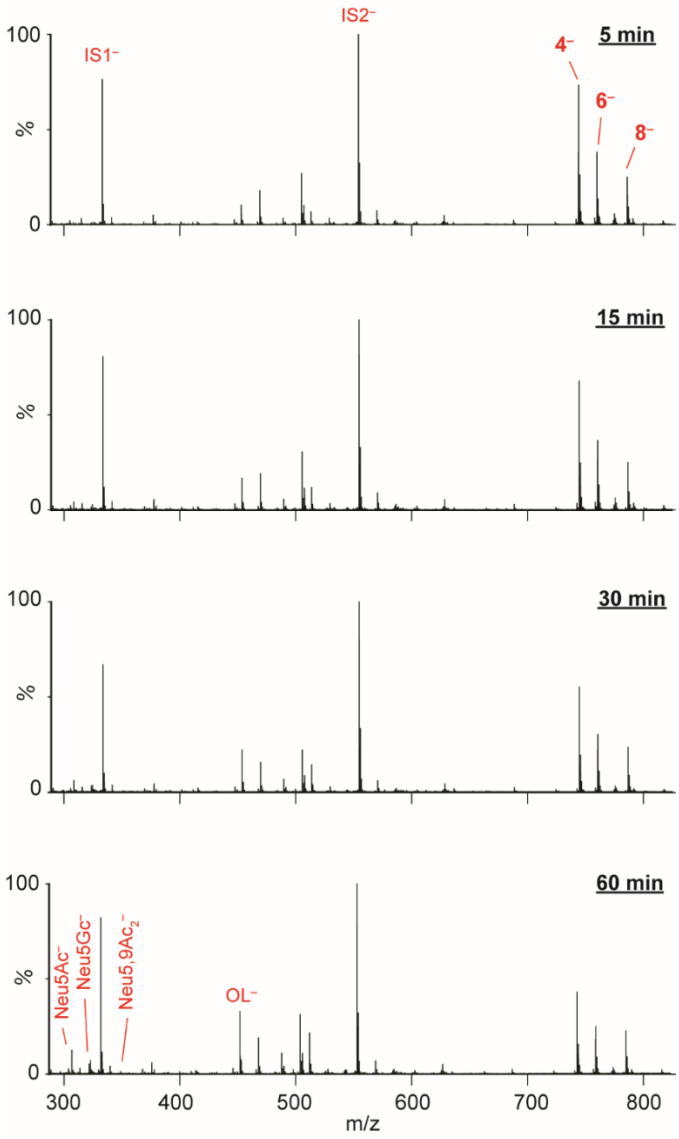


Figure 2.4: Time-resolved ESI-MS data acquired for NEU3 cleaving α(2→3) linked substrates. Mass spectra were measured in negative ion mode for 200 mM aqueous ammonium acetate solutions (pH 4.8, 22 °C) of **2-4**, **2-6**, and **2-8** (100 μM each) and NEU3 (0.0002 units). OL^- is the octyl lactoside anion; the internal standards are *N*-acetyl-9-azido-9-deoxy-neuraminic acid (IS1) and leucine enkephalin (IS2).

2.2.5 Molecular modeling of hNEU active sites with modified sialic acids

To provide insight into the surprising discrimination of hNEU for modified sialoside substrates, we performed molecular dynamics (MD) simulations of enzyme-substrate complexes. NEU2 is the only hNEU enzyme with an atomic resolution crystal structure.[59] Homology models for the other hNEU enzymes have been proposed based on the structure of NEU2.[58, 60] We used the crystal structure of NEU2 and our homology model of NEU3[60] to conduct MD simulations of NEU2 and NEU3 bound to the methyl sialyllactoside analogues of **2-4**, **2-6**, and **2-8**. The MD simulations provided structural insight into the observed relative rates of hydrolysis.

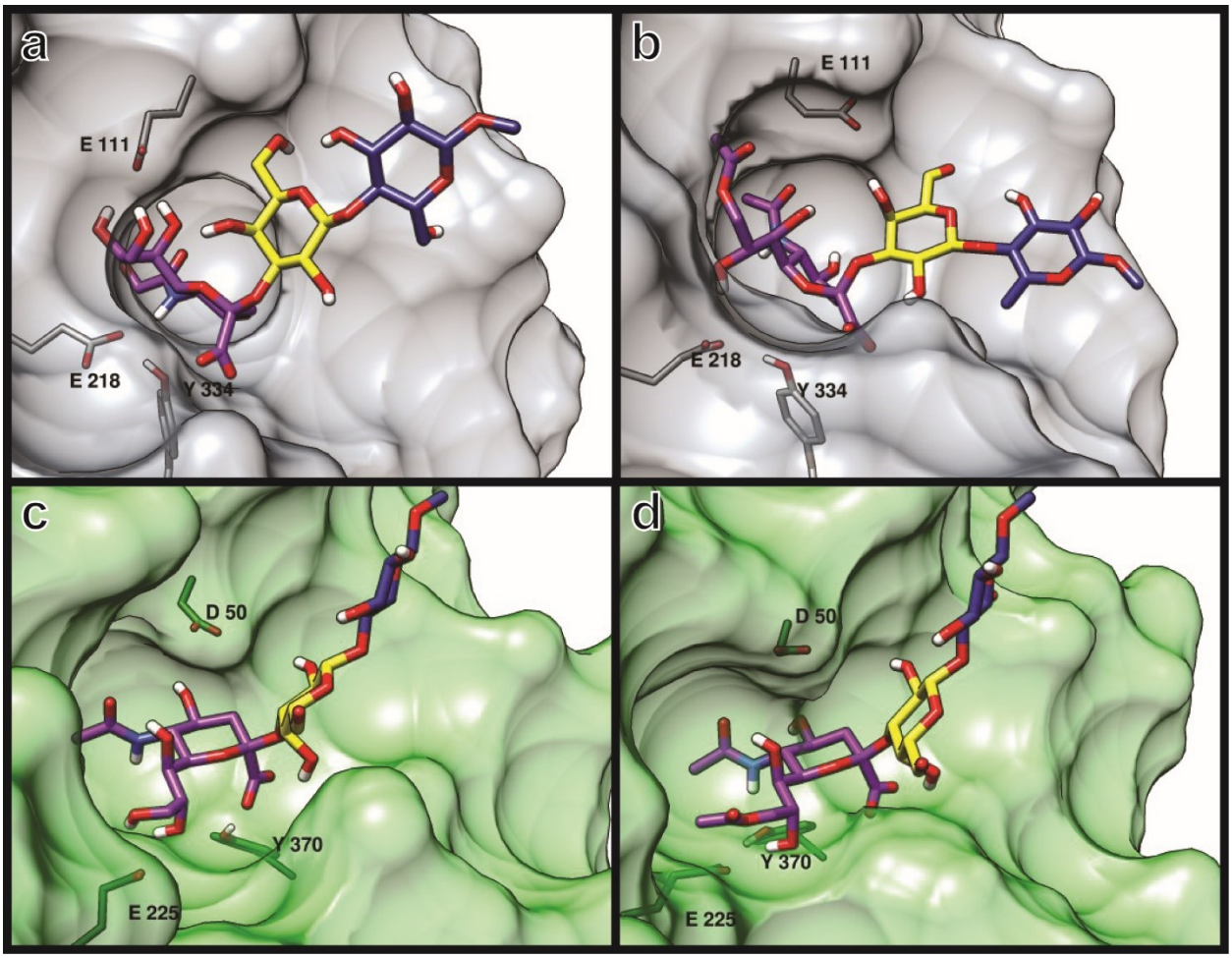


Figure 2.5: Models of substrate binding to NEU2 and NEU3. View of the active site of a) NEU2 with α(2→3) Neu5Ac-Lac-CH_3 (methyl sialyllactoside analogue of **2-4**) bound to the active site,

b) NEU2 with α(2→3) Neu5,9Ac$_2$-Lac-CH$_3$ (methyl sialyllactoside analogue of **2-8**) bound to the active site, c) NEU3 with methyl sialyllactoside analogue of **2-4** bound to the active site d) NEU3 with methyl sialyllactoside analogue of **2-8** bound to the active site

In NEU2, the preference for α(2→3)Neu5Ac over α(2→3)Neu5Gc substrates was supported by changes in the hydrogen bonding networks seen during MD simulations, particularly with the amino acid residues responsible for catalysis, the nucleophilic pair Y334–E218.[60] We found the H-bond between the carboxylate of E218 and H5N of **2-4** was occupied 24% of the time, but that same H-bond increased occupancy to 82% when **2-8** was in the active site (**Figure 2.5**). The increased occupancy of this E218 H-bond with H5N would make a key catalytic residue less available to deprotonate the nucleophile (Y334), reducing the rate of cleavage.

The C-9 pocket of NEU2 cannot accommodate large modifications of the glycerol side chain. In our MD simulations of **2-8** bound to NEU2, the 9-*O*-acetyl group forced the sialic acid ring into a boat conformation throughout the simulation (**Figure 2.5**, **A2.9**). This modification also led to further changes in the hydrogen bonding network in the NEU2 active site. When **2-4** was bound to NEU2, the O-7H of Neu5Ac formed a hydrogen bond to E111 96% of the time. This hydrogen bond decreased occupancy to 81% for Neu5Gc in **2-6**, and only 2% for Neu5,9Ac$_2$ in **2-8**, with a concomitant increase on a H-bond between O-4H in Gal and E111 (68% of the simulation time). Residue E111 has been implicated as necessary for correct positioning of the substrate in the NEU2 active site.[59] Thus, we propose that the 9-*O*-Ac modification cannot be sterically accommodated by the NEU2 active site.

Our MD simulations also supported that the C-9 pocket in NEU3 was larger than that of NEU2; allowing the sialoside rings of **2-4**, **2-6**, and **2-8** to remain in chair conformations throughout the simulations (**Figure 2.5**). Analysis of the hydrogen bonding networks between **2-**

4, **2-6**, and **2-8** with NEU3 active site residues responsible for catalysis (Y370, E225)[60] showed differences for modified sialosides (**Figure 2.5**). With **2-4**, a hydrogen bond between phenolic hydrogen of Y370 and the C-1 carboxylate of Neu5Ac was occupied for only 5% of the MD simulation. The same H-bond had higher occupancy when **2-6** or **2-8** was in the active site of NEU3 – for **2-6**, it was occupied for 64% of the simulation, and for **2-8**, it was occupied for 41% of the simulation. Increased H-bonding should reduce the nucleophilicity of Y370, and decrease the relative rate of catalysis for **2-6** and **2-8** when compared with **2-4**. The acetate group at C-9 also caused a shift of the trisaccharide in the active site for **2-8** relative to **2-4** (**Figure 2.5**). This shift was accompanied by changes in hydrogen bonding to D50, one of the catalytic residues for NEU3.[60] For **2-4** and **2-6**, D50 formed two key H-bonds to the glycan – one to O-4H of the sialic acid residues (51%–54% of the simulation) and another to O-2H of Gal (40–45% of the simulation). However, for **2-8**, the H-bond to Neu5,9Ac_2 decreased to only 2% occupancy and that to Gal decreased to 38%.

2.3 Conclusion

Our substrate specificity studies of hNEU indicate that naturally occurring 9-*O*-acetyl (Neu5,9Ac_2) and 5-*N*-glycolyl (Neu5Gc) sialic acid modifications have a significant impact on hydrolysis by hNEU. Substrate specificity studies with 4MU substrates **2-1** and **2-2** indicated that the hNEU isoenzymes exhibited discrimination between Neu5Ac and Neu5,9Ac_2 substrates. While NEU4 preferred 4MU-Neu5,9Ac_2, NEU1, NEU2, and NEU3 were substantially less active against 9-*O*-Ac modified substrates. Notably, NEU2 was essentially inactive (100 times lower activity) on Neu5,9Ac_2 substrate **2-2**. These data confirm that 9-*O*-acetylation of sialic acid had a substantial and isoenzyme-specific impact on hNEU activity. By optimizing a known assay for the detection

of free sialic acid, we were able to study hNEU kinetics on trisaccharide substrates with a hydrophobic aglycone which acted as improved mimics of natural hNEU substrates. All three isoenzymes tested (NEU2, NEU3, and NEU4) had a 2-fold preference for Neu5Ac over Neu5Gc octyl sialyllactosides. Sialic acid hydrolysis by all hNEU isoenzymes discriminated against the 9-*O*-acetylation of sialic acid in the trisaccharide substrates. Consistent with the 4MU substrates, NEU2 was inactive (100-times lower activity) on Neu5,9Ac$_2$ substrates. Data for NEU3 was ambiguous by the malononitrile assay; however, an ESI-MS assay confirmed that NEU3 had a 7-fold preference for Neu5Ac over Neu5,9Ac$_2$ substrates. In contrast to the 4MU substrates, NEU4 demonstrated a 2-fold preference for Neu5Ac over Neu5,9Ac$_2$ octyl sialyllactoside substrates, indicating that the aglycone had an influence over observed activity. In general, we observed that substrate tolerance for NEU2, NEU3, and NEU4 followed a trend with Neu5Ac > Neu5Gc >> Neu5,9Ac$_2$. Furthermore, the presence of modified sialoside residues (Neu5Gc, Neu5,9Ac$_2$) accentuated hNEU preferences for the α(2→3) glycosidic linkage. We propose that a full understanding of the role of the 9-*O*-Ac modification of sialic acid will require additional study of hNEU activity on a variety of sialoglycoconjugates to account for the effects of reducing end sugars and aglycone on human neuraminidase specificity. Specifically, future study should include investigation of hNEU activity towards Neu5,9Ac$_2$ polysaccharides and glycoprotein substrates. Ultimately, the development of chemical tools to study the effects of 9-*O*-acetylation on hNEU activity towards complex glycoconjugates in cells will be essential for elucidating the role of this sialic acid modification in biological systems. Several examples suggest an important physiological role for 9-*O*-Ac metabolizing enzymes SOAT and SIAE, which have impacts in autoimmune diseases and cancers.[15, 17] Our findings strongly suggest that the 9-*O*-Ac modification

of sialic acids could be important in the regulation of hNEU activity and may provide a biochemical link between SIAE, SOAT, and hNEU enzymatic activity.

2.4 Materials and Methods

2.4.1 General Methods

All reagents were purchased from commercial sources and used without further purification unless otherwise noted. 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). Reactions were monitored by analytical TLC on silica gel 60-F254 (0.25 nm, Silicycle, QC, Canada). Visualization was achieved using UV fluorescence and/or by charring with 5% sulfuric acid in ethanol. Organic solvents were evaporated under reduced pressure at 40 °C. Reaction products were purified by column chromatography on silica gel (230-400 mesh, Silicycle, QC, Canada) unless othterwise noted. When the eluent system required greater than 10% methanol, Iatrobeads 6RS-8060 (Shell-USA Inc.) were used. HPLC was performed with a Waters Delta 600 pump, and a Waters 600 controller with Empower 2 software. Eluted peaks were detected with a Waters 2420 evaporative light scattering (ELS) detector or a Waters 2996 photodiode array (PDA) detector (Waters Ltd., Mississauga, ON, Canada). NMR experiments were conducted on Varian 400, 500, 600, and 700 MHz instruments. Chemical shifts are reported relative to deuterated solvent peaks or 3-(trimethylsilyl)-propionic-2,2,3,3,-d_4 acid sodium salt in D_2O as an internal standard. The ESI mass spectra were recorded on Agilent Technologies 6220 TOF after dissolving samples in $CHCl_3$ or CH_3OD and adding NaCl. Unless otherwise stated, relative rates reported are technical replicates, with preliminary independent replicates confirming the trends reported.

2.4.2 Enzyme Preparation

Enzymes for the one-pot sialylation reactions were prepared as described previously.[37, 42] The aldolase was *E. coli* sialic acid aldolase[51] expressed with a $(His)_6$ tag in *E. coli* strain BL21(DE3) and was purified using a Ni-NTA column then used at a concentration of 4.6 mg mL^{-1}. The CMP-Neu5Ac synthetase was *Neisseria meningitidis* CMP-Neu5Ac synthetase (NmCss)[61] expressed in *E. coli* strain AD202 grown to 2.4 x 10^8 cells mL^{-1} and used in a crude enzyme mixture. The α(2→3) sialyltransferase was *Campylobacter jejuni* α(2→3) sialyltransferase (CstI)[62] expressed in *E. coli* strain AD202 grown to 2.4 x 10^8 cells mL^{-1} and used in a crude enzyme mixture, and the α(2→6) sialyltransferase was *Photobacterium damsela* α(2→6) sialyltransferase (Pd2,6ST) expressed with a $(His)_6$ tag in *E. coli* strain Nova Blue (DE3). and was purified using a Ni-NTA column then used at a concentration of 3.0 mg mL^{-1}.[42, 63] Human neuraminidase enzymes NEU2 and NEU3 were expressed as fusion proteins with maltose-binding protein. Human neuraminidase enzyme NEU4 was expressed as a fusion protein with glutathione-S-transferase protein. Isoenzymes NEU2-4 were purified as described.[37, 60, 64] Human neuraminidase enzyme NEU1 was produced from HEK293E cells and was used as a crude cell lysate, in which the majority of the neuraminidase activity could be accounted for by NEU1 (**A2.3**). Specific activity of the neuraminidase enzymes was determined against 4-methylumbelliferyl α-$_D$-*N*-acetylneuraminic acid (4MU-NANA) in comparison to a standard curve of neuraminidase from *Clostridium perfringens*.

2.4.3 Solution-phase kinetics assay with 4MU substrates

Substrate (**2-1** or **2-2**), (30 μL, 0.5 mM in H_2O) was incubated at 37 °C for 15 min in 60 μL 0.1 M sodium acetate buffer at the enzyme's optimum pH (4.5 for NEU1, NEU3 and NEU4, 5.6 for

NEU2). Enzyme was added (30 µL, 3.33 x 10^{-5} U/µL) and the assay mixture was incubated at 37 °C. At timepoints of 0, 10, 20, 30, and 40 minutes, a sample of assay mixture (20 µL) was removed and quenched in 100 µL of 0.2 M Na glycine buffer (pH 10.2). Fluorescence was measured on a SpectraMax M2^e plate reader (Molecular Devices, Sunnyvale, CA, USA excitation 365 nm, emission 445 nm). Relative rates were determined, after background subtraction of fluorescence at time = 0, by linear regression forcing through time = 0 using Graphpad Prism and are an average of three runs. Relative rates are relative to a matched Neu5Ac control (4MU-Neu5Ac, **2-1**)

2.4.4 Kinetics assay employing malononitrile for the detection of free sialic acid

Enzymes were diluted to 15 µL in 20 mM MOPS with 0.2 M NaCl, pH 7.2. Substrates (**2-4** – **2-9**) (2.5 x 10^{-8} mol in 45 µL water) were incubated at 37 °C for 15 min in 120 µL 0.1 M sodium acetate buffer, followed by addition of the appropriate enzyme to a final pH at the enzyme's optimum (pH 5.6 for NEU2, pH 4.5 for NEU1, NEU3, and NEU4; 0.003 U for NEU1, 0.0013 U for NEU2, 0.00075 U for NEU3, 0.0029 U for NEU4). At each time point, 30 µL of the assay solution was removed and quenched into 50 µL 0.2 M sodium borate (pH 9.5), and 15 µL of 0.8% (w/v) malononitrile was added to the solution followed by heating to 100 °C for 20 min. Fluorescence was measured on a SpectraMax M2^e plate reader (Molecular Devices, Sunnyvale, CA, USA, excitation 357 nm, emission 434 nm). Relative rates were determined by linear regression of three average runs and are relative to a matched Neu5Ac control (α(2→3) Neu5Ac octyl sialyllactoside, **2-4**). Points were corrected for background and fitting was forced through the zero point using Graphpad Prism. Data were evaluated to exclude any outliers identified by applying Dixon's Q Test.[65] .

2.4.5 Synthetic methods

Chemoenzymatic synthesis of octyl sialyllactosides

Enzymatic reactions were performed with stirring at 37 °C. Reaction was monitored by TLC using 6:3:3:2 ethyl acetate: acetic acid: methanol: H_2O as an eluent system and charring with 5 % sulfuric acid in ethanol. Upon completion, ethanol was added and the reaction mixture was centrifuged at 17,000 rpm for 1 hour. The supernatant was collected and lyophilized. Crude product was purified with a Sep-pack C-18 reverse phase cartridge. The product was eluted with MeOH:H_2O (1:2).

*Method A: For Neu5Ac octyl sialyllactosides (**2-4**, **2-5**)*

Neu5Ac (2.90 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol, 1 M $MgCl_2$ (80 μL), and deionized H_2O (600 μL) were dissolved in 1 M Tris-HCl (400 μL, pH 8.8). The reaction was charged with CMP-Neu5Ac synthetase (200 μL), β-octyl lactoside (**SI4**) (2.5 mg, 6.2 μmol), sialyltransferase (200 μL), and deionized H_2O (600 μL). The reaction proceeded overnight.

*Method B: For Neu5Gc octyl sialyllactosides (**2-6**, **2-7**)*

N-glycolyl-D-mannosamine (ManGc, 2.25 mg, 9.4 μmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol, 1 M $MgCl_2$ (80 μL), and deionized H_2O (600 μL) were dissolved in 1 M Tris-HCl (400 μL, pH 8.8). The reaction was charged with sialic acid aldolase, CMP-Neu5Ac synthetase, β-octyl lactoside (**SI4**) (2.5 mg, 6.2 μmol), sialyltransferase (300 μL), and deionized H_2O (600 μL). The reaction proceeded overnight.

*Method C: For Neu5,9Ac$_2$ octyl sialyllactosides (**2-8**, **2-9**)*

Neu5,9Ac_2 (**SI6**) (2.5 mg, 8.5 mmol), cytidine triphosphate disodium salt (4.95 mg, 9.4 μmol, 1 M $MgCl_2$ (80 μL), and deionized H_2O (600 μL) were dissolved in 1 M Na HEPES (400 μL, pH 7.2). The reaction was charged with CMP-Neu5Ac synthetase (200 μL), β-octyl lactoside (**SI4**)

(2.0 mg, 5.0 μmol), sialyltransferase (200 μL), and deionized H_2O (600 μL). The reaction proceeded for 3 hours.

4-methylcoumarin-7-yl 5-acetamido-9-*O*-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid (2-2):

A solution of 4-MU-NANA (**2-1**, in 500 μL dry DMF), prepared as previously reported[66] (5 mg, 0.01 mmol), was charged with glacial acetic acid (5 μL) and trimethyl orthoacetate (20 μL, 0.157 mmol). The reaction was stirred for four hours and then dried under reduced pressure. The crude mixture was purified by HPLC on a C-18 reversed phase Waters (10 μm, 10 x 250 mm) column. Pure **2** was eluted with a linear gradient of 0-70% acetonitrile in water over 30 minutes, with a flow rate of 7 mL/min to give a 5 % yield. ^{1}H NMR data was consistent with previous reports.[46] ^{13}C NMR (125 MHz, D_2O) δ 177.9 (NH<u>C</u>OCH_3), 177.2 (O<u>C</u>OCH_3), 175.8 (C-1), 167.6 (C-2'), 160.1 (C-8'), 159.3 (C-10'), 156.3 (C-5'), 129.1 (C-6'), 120.3 (C-7'), 119.1 (C-4'), 114.6 (C-3'), 110.7 (C-9'), 105.2 (C-2), 76.6, 72.2, 71.5, 70.6, 68.9 (C-9), 54.6, 43.7 (C-3), 24.9 (NHCO<u>C</u>H_3), 23.1 (OCO<u>C</u>H_3), 20.9 (C-4'-<u>C</u>H_3). ESI-MS calculated for $C_{23}H_{27}NO_{12}$ $[M-H]^-$ 508.1460 found: 508.1462.

***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 (2-4):**

Compound **4** was prepared using Method A to yield 4 mg (87 %) as a white solid. ^{1}H and ^{13}C NMR data were consistent with previous reports.[37, 67]

***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 (2-5):**

Compound **5** was prepared using Method A to yield 3.8 mg (83 %) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.49 (d, *J* = 8.2 Hz, 1H, H-1'), 4.43 (d, *J* = 8.3 Hz, 1H, H-1''), 4.00-3.52 (H-5', H-6a', H-6b', H-5'', H-6a'', H-6b'', H-7''', H-8''', H-9a''', H-9b'''), 3.95 (1H, H-4''', from TOCSY), 3.93 (1H, OC<u>Ha</u>Hb$(CH_2)_6CH_3$, from TOCSY, 3.87 (1H, H-5''', from TOCSY), 3.70 (1H, OCHa<u>Hb</u>$(CH_2)_6CH_3$, from TOCSY), 3.69 (1H, H-3'', from TOCSY), 3.67 (1H, H-4''', from COSY), 3.65 (1H, H-3', from TOCSY), 3.54 (1H, H-2'', from COSY), 3.34 (t, *J* = 8.6 Hz, 1H, H-2'), 2.72 (dd, *J* = 12.6, 4.7 Hz, 1H, H-3eq'''), 2.04 (s, 3H, NHCOC<u>H</u>$_3$), 1.75 (t, *J* = 12.5 Hz, 1H, H-3ax'''), 1.66-1.61 (m, 2H, $OCH_2C\underline{H_2}(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C\underline{H_2})_5CH_3$), 0.87 (t, *J* = 6.6 Hz, 3H, $O(CH_2)_7C\underline{H_3}$). ^{13}C NMR (175 MHz, D_2O) δ 177.7 (NH<u>C</u>OCH_3, from HMBC), 175.8 (<u>C</u>OO-, from HMBC), 104.5 (C-1'', from HSQC), 103.0 (C-1', from HSQC), 102.9 (C-2''', from HMBC), 82.0 (C-2'', from HMBC), 78.5 (C-3''', from HMBC), 76.7, 74.7, 73.91, 73.86 (C-2', from HSQC), 73.1, 73.0 (C-4''', from HSQC), 72.7, 70.2 ($O\underline{C}H_2(CH_2)_6CH_3$, from HMBC), 69.3 (C-4'', from HSQC), 68.9, 64.5, 63.6, 62.8, 61.4, 53.2 (C-5''', from HSQC), 40.5 (C-3''', from HSQC), 29.8 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 29.5, 26.7, 26.5 ($OCH_2CH_2\underline{C}H_2(CH_2)_4CH_3$, from HMBC), 25.7 ($O(CH_2)_6\underline{C}H_2CH_3$, from HMBC), 23.4 (NHCO<u>C</u>H_3, from HSQC), 22.4, 14.5 ($O(CH_2)_7CH_3$, from HSQC). ESI-MS calculated for $C_{31}H_{55}NO_{19}$ $[M-H]^-$ 744.3296 found: 744.3300.

***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 (2-6):**

Compound **6** was prepared using Method B to yield 2.5 mg (53%) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.54 (d, *J* = 7.8 Hz, 1H, H-1''), 4.49 (d, *J* = 8.1 Hz, 1H, H-1'), 4.14 (d, *J* = 3.2 Hz,

1H, H-3’’), 4.13 (s, 2H, NHCOC$\underline{H_2}$OH), 4.01-3.53 (H-4’, H-5’, H-6a’, H-6b’,H-5’’, H-6a’’, H-6b’’, H-7’’’, H-8’’’, H-9a’’’, H-9b’’’), 3.97 (1H, H-4’’, from COSY), 3.95 (1H, H-5’’’, from TOCSY), 3.80 (1H, H-4’’’, from COSY), 3.78 (1H, H-6’’’, from TOCSY), 3.66 (1H, H-3’, from HSQC), 3.60 (1H, H-2’’, from COSY), 3.31 (t, J = 8.5 Hz), 1H, H-2’), 2.79 (dd, J = 12.4, 4.7 Hz, 1H, H-3e’’’), 1.83 (t, J = 12.2 Hz, 1H, H-3a’’’), 1.63 (m, 2H, $OCH_2C\underline{H_2}(CH_2)_5CH_3$), 1.39-1.26 (m, 10H, $OCH_2CH_2(C\underline{H_2})_5CH_3$), 0.87 (t, J = 7.0 Hz, 3H, $O(CH_2)_7C\underline{H_3}$. ^{13}C NMR (175 MHz, D_2O) δ 176.5 (NH-$\underline{CO}$-CH_2OH, from HMBC), 175.3 ($\underline{C}$OO-, from HMBC), 100.4 (C-2’’’, from HMBC), 103.4 (C-1’’, from HSQC), 102.7 (C-1’, from HSQC), 79.0, (C-2’’, from HMBC), 77.1, 76.8 (C-3’, from HMBC), 76.4 (C-3’’, from HSQC), 74.5, 74.2 (C-6’’’, from HSQC), 73.6, 72.0 (C-2’, from HMBC), 70.4, 69.1 (C-4’’’, from HMBC), 68.8, 68.6 (C-4’’, from HSQC), 63.0, 62.8, 61.7 (CH_2OH, from HSQC), 52.3 (C-5’’’, from HSQC), 52.3, 40.4 (C-3’’’, from HSQC), 32.6, 29.8 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 29.3, 28.5 ($OCH_2CH_2\underline{C}H_2(CH_2)_4CH_3$, from HMBC), 27.0, 24.4 ($O(CH_2)_6\underline{C}H_2CH_3$, from HMBC), 14.5 ($O(CH_2)_7\underline{C}H_3$, from HSQC). ESI-MS calculated for $C_{31}H_{55}NO_{20}$ $[M-H]^-$ 760.3245 found: 760.3245.

_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 (2-7):

Compound **7** was prepared using Method B to yield 3.2 mg (68%) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.49 (d, J = 8.3 Hz, 1H, H-1’), 4.44 (d, J = 8.1 Hz, 1H, H-1’’), 4.13 (s, 2H, CH_2OH), 4.01-3.53 (H-4’, H-5’, H-6a’, H-6b’, H-4’’, H-5’’, H-6a’’, H-6b’’, H-5’’’, H-6’’’, H-7’’’, H-8’’’, H-9a’’’, H-9b’’’) 3.81 (1H, H-4’’’, from COSY), 3.71 (2H, $OCH_2(CH_2)_6CH_3$, from COSY), 3.70 (1H, H-3’, from COSY), 3.57 (H-2’’, from COSY), 3.34 (t, J = 8.7 Hz, 1H, H-2’), 2.74 (dd, J = 12.4, 4.6 Hz, 1H, H-3eq’’’), 1.77 (t, J = 12.3 Hz, 1H, H-3ax’’’), 1.66-1.61 (m, 2H,

$OCH_2C\underline{H_2}(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C\underline{H_2})_5CH_3$), 0.87 (t, *J* = 7.3 Hz, 3H, $O(CH_2)_7C\underline{H_3}$. ^{13}C NMR (175 MHz, D_2O) δ 176.8 ($NH\underline{C}OCH_2OH$, from HMBC), 173.8 ($\underline{C}OO$-, from HMBC), 105.6 (C-1'', from HSQC), 104.3 (C-1', from HSQC), 101.6 (C-2''', from HMBC), 80.9 (C-2'', from HMBC), 79.8, 76.4, 75.6, 75.3, 73.2, 72.9, 71.5, 71.3, 70.6, 70.2, 69.9 (C-4''', from HMBC), 66.2, 64.9, 64.5, 63.5, 62.1 ($\underline{C}H_2OH$, from HSQC), 53.5, 40.9 (C-3''', from HSQC), 31.4, 29.7, 29.6 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 26.4, 25.3 ($O(CH_2)_6\underline{C}H_2CH_3$, from HMBC), 23.3, 14.8 ($O(CH_2)_7\underline{C}H_3$, from HSQC). ESI-MS calculated for $C_{31}H_{55}NO_{20}$ $[M-H]^-$ 760.3245 found: 760.3242.

***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 (2-8)**

Compound **8** was prepared using Method C to yield a mixture of **4**:**8** 1:2. To yield pure product **8**, the crude mixture was separated using HPLC on a C-18 reversed-phase Waters Xterra (3.5 μm, 4.8 x 150 mm) column. The trisaccharides were eluted with a linear gradient of 20–50% methanol in H_2O over 30 min, with a flow rate of 0.7 mL/min, to yield 0.4 mg (10%) of **8** as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.54 (d, *J* = 7.8 Hz, 1H, H-1''), 4.49 (d, *J* = 8.1 Hz, 1H, H-1'), 4.42 (dd, *J* = 11.8, 2.2 Hz, 1H, H-9a'''), 4.20 (dd, *J* = 11.8, 6.4 Hz, 1H, H-9b'''), 4.13-4.09 (m, 2H, H-8''', H-3'', from COSY, TOCSY), 4.02-3.54 ($OC\underline{H_2}(CH_2)_6CH_3$, H-3', H-4', H-5', H-6a', H-6b', H-5'', H-6a'', H-6b', H-6'''), 3.97 (1H, H-4'', from TOCSY), 3.87 (1H, H-5''', from TOCSY), 3.69 (1H, H-4''', from COSY), 3.66 (1H, H-7''', from TOCSY), 3.59 (1H, H-2'', from COSY), 3.31 (t, *J* = 8.3 Hz, 1H, H-2'), 2.77 (dd, *J* = 12.8, 4.8 Hz, 1H, H-3e'''), 2.15 (s, 3H, $OCOC\underline{H_3}$), 2.05 (s, 3H, $NHCOC\underline{H_3}$), 1.81 (t, *J* = 12.2 Hz, 1H, H-3a'''), 1.66-1.61 (m, 2H, $OCH_2C\underline{H_2}(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C\underline{H_2})_5CH_3$), 0.87 (t, *J* = 7.0 Hz, 3H, $O(CH_2)_7C\underline{H_3}$. ^{13}C NMR (175 MHz, D_2O) δ 176.1 ($NH\underline{C}OCH_3$, from HMBC), 175.4 ($O\underline{C}OCH_3$,

from HMBC), 175.0 (COO-, from HMBC), 103.3 (C-1'', from HSQC), 103.0 (C-1', from HSQC), 101.2 (C-2''', from HMBC), 79.6 (C-2'', from HMBC), 76.6, 76.3, 76.1, 76.1 (C-7''', from HMBC), 75.9, 74.8, 73.4 (C-2', from HMBC), 72.4, 68.9, 66.8 (C-9''', from HSQC), 63.4, 62.2, 62.2, 61.6 (C-4'', from HSQC), 60.7, 53.6, 40.6 (C-3''', from HSQC), 30.2 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 29.5-25.0 ($OCH_2CH_2(\underline{C}H_2)_4CH_2CH_3$ 23.9 ($OCH_2(CH_2)_5\underline{C}H_2CH_3$, from HMBC), 23.8 ($NHCO\underline{C}H_3$, from HSQC), 21.5 ($OCOCH_3$, from HSQC), 14.8 ($O(CH_2)_7\underline{C}H_3$, from HSQC). ESI-MS calculated for $C_{33}H_{57}NO_{20}$ $[M-H]^-$ 786.3401 found: 786.3403.

***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 (2-9):**

Compound **9** was prepared using Method C to yield 1.3 mg (33%) as a white solid. ^{1}H NMR (700 MHz, D_2O) δ 4.49 (d, *J* = 7.8 Hz, 1H, H-1'), 4.44 (d, *J* = 7.8 Hz, 1H, H-1''), 4.42 (dd, *J* = 12.0, 2.2 Hz, 1H, H-9a'''), 4.21 (dd, *J* = 12.0, 5.8 Hz, 1H, H-9b'''), 4.12 (ddd, *J* = 9.6, 5.8, 2.2 Hz, 1H, H-8'''), 4.01-3.52 ($OC\underline{H}_2(CH_2)_6CH_3$, H-6a', H-6b', H-5'', H-6a'', H-6b'', H-5''', H-6'''), 4.00 (1H, H-5', from TOCSY), 3.95 (1H, H-4'', from TOCSY), 3.81 (1H, H-4', from TOCSY), 3.68 (1H, H-3', from COSY), 3.68 (H-4''', from COSY), 3.67 (1H, H-3'', from COSY), 3.62 (1H, H-7''', from COSY), 3.55 (1H, H-2'', from COSY), 3.34 (t, *J* = 8.7 Hz, 1H, H-2'), 2.72 (dd, *J* = 12.6, 4.7 Hz, 1H, H-3eq'''), 2.14 (s, 1H, $OCOC\underline{H}_3$), 2.05 (s, 1H, $NHCOC\underline{H}_3$), 1.75 (t, *J* = 12.1 Hz, 1H, H-3ax'''), 1.67-1.61 (m, 2H, $OCH_2C\underline{H}_2(CH_2)_5CH_3$), 1.39-1.25 (m, 10H, $OCH_2CH_2(C\underline{H}_2)_5CH_3$), 0.87 (t, *J* = 6.8 Hz, 3H, $O(CH_2)_7C\underline{H}_3$). ^{13}C NMR (175 MHz, D_2O) δ 175.4 ($NH\underline{C}OCH_3$, from HMBC), 175.0 ($O\underline{C}OCH_3$, from HMBC), 174.4 (COO-, from HMBC), 103.9 (C-1'', from HSQC), 103.0 (C-1', from HSQC), 101.8 (C-2''', from HMBC), 76.7 (C-3', from HMBC), 75.1, 74.9, 74.26, 74.25, 73.7, 73.6 (C-2', from HSQC), 71.8, 71.7 (C-2'', from HSQC), 70.4 (C-8''', from HSQC), 69.3 (C-4''', from HMBC), 68.8 (C-4'', from HSQC), 67.4 (C-7''', from HMBC), 66.9

(C-9’’’, from HSQC), 63.5, 63.1, 52.8, 41.1 (C-3’’’, from HSQC), 32.6, 30.0, 29.7 ($OCH_2\underline{C}H_2(CH_2)_5CH_3$, from HSQC), 26.9, 26.3 ($O(CH_2)_6\underline{C}H_2CH_3$, from HMBC), 23.4, 23.3 ($NHCO\underline{C}H_3$, from HSQC), 21.5 ($OCO\underline{C}H_3$, from HSQC), 14.4 ($O(CH_2)_7\underline{C}H_3$, from HSQC). ESI-MS calculated for $C_{33}H_{57}NO_{20}$ $[M-H]^-$ 786.3401 found: 786.3396.

2.5: References

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Chapter 3 : Determination of hNEU activity on glycoproteins and glycolipid analogs.[a,b]

[a] Parts of this Chapter (Section 3.2.1) have been adapted to a manuscript Hunter, C.D., Porter, E., Cairo, C.W., Human neuraminidase activity towards modified sialic acids on glycoproteins.

[b] Optimization of assay conditions and collection of preliminary data was done with Elizabeth Porter. NEU1 was produced by Hanh-Thuc Ton Tran, and NEU2-NEU4 were expressed and purified by myself or other group members.

3.1 Introduction

Chemical modifications to sialic acids can influence their rates of hydrolysis by the human neuraminidase (hNEU) enzymes. Our study of 9-*O*-acetylated sialic acids on octyl sialyllactoside substrates (described in **Chapter 2**) suggested that this modification may generally impede the activity of the hNEU enzymes.[1] We noted, however, that the modification had different effects on hNEU-catalyzed hydrolysis on substrates with different sialic acid glycosidic linkages and reducing end sugars or aglycones. We concluded that in order to better understand the effect of 9-*O*-acetylation on hNEU-catalyzed hydrolysis of sialic acids we had to expand the scope of our study to include a broad range of well-defined substrates, including sialoglycoproteins and α(2→8)-linked sialosides.

Just as sialic acid glycosidic linkage and reducing end substituents may influence metabolism by hNEU, the Neu5,9Ac_2 residue plays unique roles in biological processes. Sialoglycoproteins containing 9-*O*-acetylated sialosides modulate B-cell immune response by blocking sialic acid recognition by CD22.[2, 3] Sialic acids on colonic mucins are heavily *O*-acetylated which protects them from neuraminidases of gut bacteria.[4, 5] In colon carcinomas this *O*-acetylation is reduced, unmasking sialyl Lewis^x - a contributing ligand in metastasis.[6-8] *In vitro*, Neu5,9Ac_2 levels on sialomucins were found to be tightly controlled throughout development and differentiation of murine erythroleukemia cells.[9] Sialomucins with Neu5,9Ac_2 have been identified as a differentiation marker on $CD4^+$ T-cells,[10] and sialic acid *O*-acetylesterase has been implicated in B-cell development.[11] Most studies of Neu5,9Ac_2 have monitored cell-surface Neu5,9Ac_2; however, in cells where cell surface Neu5,9Ac_2 is not detected Neu5,9Ac_2 sialoglycoproteins have been identified in the Golgi, suggesting an unexplored role for intracellular Neu5,9Ac_2.[12, 13] Sialic acid *O*-acetylation of glycoproteins also plays a role in pathological processes. In visceral

leishmaniasis, elevated levels of Neu5,9Ac$_2$ on erythrocyte glycoproteins were associated with shorter erythrocyte lifespan and anemia.[14, 15] In acute lymphoblastic leukemia (ALL) lower levels of Neu5,9Ac$_2$ glycoproteins was correlated with better prognosis,[16] and a role for Neu5,9Ac$_2$ on glycoproteins has been identified in the deployment of cancerous lymphoblasts from the bone marrow to circulating blood.[17] Increased levels of sialiate 9-*O*-acetyltransferase (SOAT) activity have been detected in ALL, which not only resulted in more acetylated sialoglycoproteins, but also disialo-ganglioside GD3 (Neu5Ac α(2→8) Neu5Ac α(2→3) Gal β(1→4) Glc β Ceramide, **3-2**).[18]

Likewise, acetylation of the α(2→8)-linked sialic acid on GD3 has been implicated in the pathogenesis of ALL and other cancers. Neu5,9Ac$_2$-GD3 promoted survival of cancerous lymphoblasts in ALL by blocking GD3-mediated apoptosis.[19] An increase in Neu5,9Ac$_2$-GD3 could be attributed to both an increase in SOAT activity and a decrease in sialate acetyl esterase (SIAE) activity.[20] The anti-apoptotic effect of Neu5,9Ac$_2$ GD3 was also observed in glioblastoma, where esterase activity could induce apoptosis.[21, 22] Elevated levels of Neu5,9Ac$_2$ GD3 have also been found in basal cell carcinoma,[23, 24] melanoma,[25, 26] medulloblastoma,[27] and breast cancer.[28, 29] Increased 9-*O*-acetylation of α(2→8)-linked sialic acids have been identified on other gangliosides in malignant cells. Elevated 9-*O*-acetylation of GD2 and GT3 was detected in breast cancer, [28 30] and Neu5,9Ac$_2$ GD2 was found in neuroblastoma,[31] and melanoma.[25] Neu5,9Ac$_2$-GD2 is emerging as a potential target in cancer immunotherapy.[32] These 9-*O*-acetylated α(2→8)-linked sialic acids also have roles in normal cellular processes. Neu5,9Ac$_2$ GD3 plays a role in development of the central nervous system[33, 34] and on the regulation of B- and T-lymphocytes.[35-37] In contrast to its anti-apoptotic effect in many cancers, Neu5,9Ac$_2$-GD3 is a pro-regulatory molecule for programmed cell death in erythrocytes.[38]

An understanding of hNEU activity towards Neu5,9Ac$_2$ on glycoproteins and α(2→8) sialosides would help to elucidate the specific roles of this small but impactful modification to sialic acid. To study hNEU activity on 9-*O*-acetylated glycoproteins and Neu5,9Ac$_2$ α(2→8)-linked sialosides we adapted and expanded assays available to monitor hNEU activity (see **Sections 1.4** and **1.5)**. We detected sialic acid released from a Neu5,9Ac$_2$ sialoglycoprotein with *o*-phenylenediamine (OPD). This assay was adapted from one originally used to study bacterial neuraminidase activity on a Neu5,9Ac$_2$ sialoglycoprotein[5] to quantify the activity of hNEU. Detection of free sialic acid is not a good strategy for studying neuraminidase kinetics on α(2→8)-linked sialosides because it is not possible to differentiate release of α(2→8) linked sialic acid from the underlying α(2→3/6)-linked sialoside. To study hNEU activity on α(2→8) Neu5,9Ac$_2$ we designed substrates with a chromophore in the aglycone and an HPLC method to monitor substrate degradation. These assays expand the toolkit of chemical biology methods available to study mammalian neuraminidases. Together, the results of these studies should provide a more complete picture of the effects of Neu5,9Ac$_2$ on hNEU-catalyzed hydrolysis and help to illuminate the roles of these modified sialic acids and the hNEU in biological systems.

3.2 Results and discussion

3.2.1 hNEU activity on 9-*O*-acetylated glycoproteins

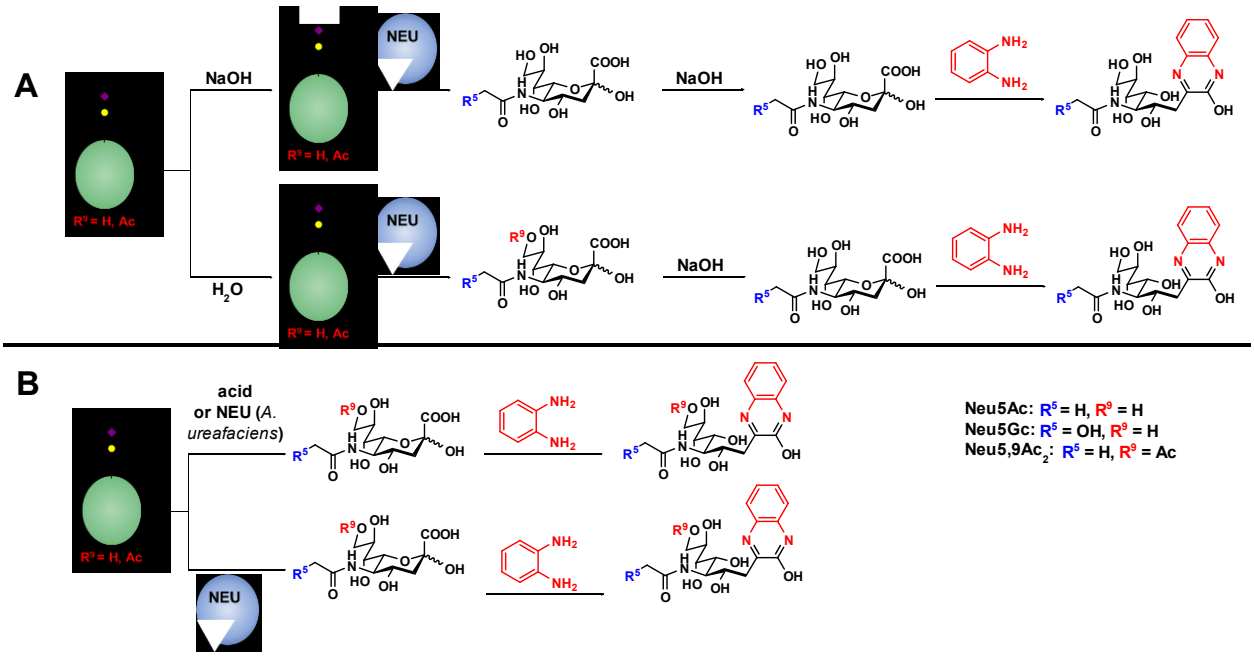


Figure 3.1: Assay workflow to detect modified sialic acid released from glycoproteins A) assay workflow implemented by Lewis and coworkers[5] B) assay workflow to detect sialic acid released by hNEU.

A strategy that has been used to probe the effects of *O*-acetylated sialic acids on glycoproteins is treatment of a sialoglycoprotein with base to hydrolyze the *O*-acetyl esters, followed by comparison of the treated protein to an untreated control. [4, 39-41] Bovine submaxillary mucin (BSM) is an inexpensive commercially available glycoprotein with α(2→6) linked sialosides enriched in *O*-acetylated sialic acids, particularly Neu5,9Ac$_2$, making it a popular model to study 9-*O*-acetylated glycoproteins. Recently, Lewis and coworkers used this strategy to study sialic acid released from BSM by sialidase and sialate-*O*-acetylesterase activity from *Bacteroidetes*.[5] The capacity for the sialidase to hydrolyze acetylated sialic acids versus its non-

acetylated variant was determined by comparing the amount of sialic acid it released from control BSM to BSM pre-treated with NaOH to hydrolyze any *O*-acetyl esters. After neuraminidase treatment, the samples were again submitted to basic conditions to remove any *O*-acetyl groups to standardize the analysis to detection of only Neu5Ac and Neu5Gc across all samples (**Figure 3.1A**). Free sialic acid release was labeled with a 1,2-phenylenediamine and detected after separation using HPLC.[5]

When we implemented this assay to study hNEU, we found that the assay had insufficient sensitivity for our purposes. We calculated a limit of detection (LOD) of 1.3 μM (**A3.1, Section 3.4.2**) for Neu5Ac labeled with *o*-phenylenediamine (OPD) and detection at 350 nm. Lewis and coworkers reported a small but significant signal with their enzyme-free base-treated negative control.[5] The background noise was inconsequential in studying bacterial neuraminidases where the signal was approximately 10-fold higher than the background; however, we noted that the hNEU enzymes were at least 8- and 15-fold less efficient at cleaving sialic acids from BSM than bacterial neuraminidases from *C. perfringens* and *A. ureafaciens*, respectively (**Figure 3.2**, **A3.2).** With the lower efficiency of hNEU towards BSM, the noise resulting from treatment of the protein with base was prohibitive to studying hNEU substrate tolerance.

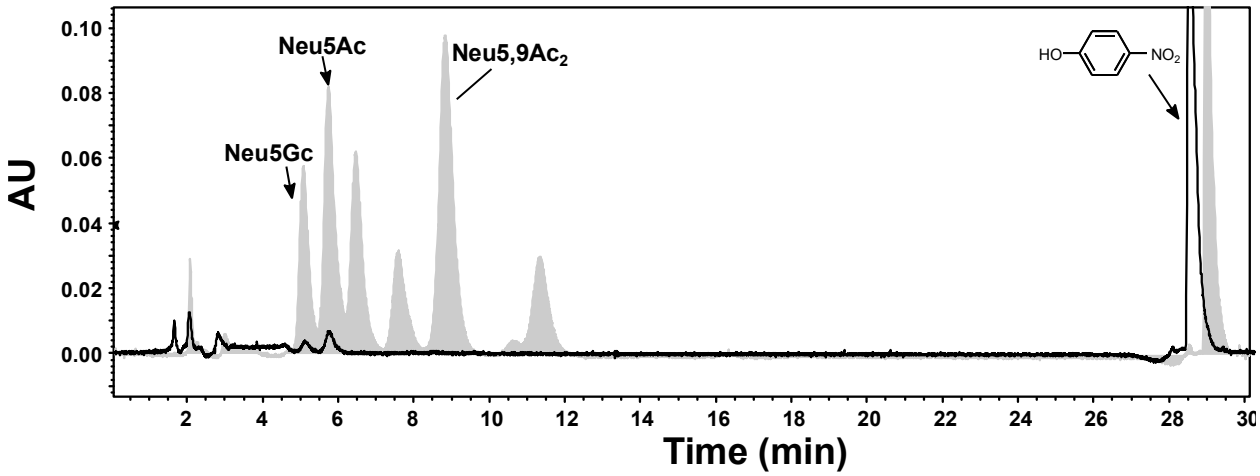


Figure 3.2: Representative run of sialic acids released from bovine submaxillary mucin by *A. ureafaciens* neuraminidase (grey) and by NEU2 (black). Enzyme activity was normalized to 1 mU based on 4MU-NANA.

To account for the lower efficiency of the hNEU towards BSM we modified the assay to avoid pre-treatment of BSM with base, meaning we could not compare the amount of sialic acid released from BSM with or without *O*-acetyl modifications. Instead, we compared the ratios of modified sialic acids released by hNEU to Neu5Ac (**Figure 3.1B**). This approach eliminated the noise from the negative control which allowed us to study hNEU activity; however, it complicated the analysis from detection of Neu5Gc and Neu5Ac to at least five different sialic acid species. We could definitively identify Neu5Gc, Neu5Ac, and Neu5,9Ac_2 using standards, and could tentatively assign Neu5,7Ac_2 and Neu5Gc9Ac based on the order of elution from the column.[5, 42]

To determine substrate preferences for the hNEU without pre-treatment of the samples with base, we needed to determine the sialic acid composition of BSM, which we report as ratios of Neu5Gc/Neu5Ac or Neu5,9Ac_2/Neu5Ac. Two methods for the total release of *O*-acetylated sialic acids from glycoconjugates are acid hydrolysis and hydrolysis with neuraminidase from *A.*

ureafaciens; however, both methods have limitations. The most common method is acid hydrolysis, which is widely used for total sialic acid release from glycoconjugates.[43] Conditions for acid hydrolysis of sialosides will also hydrolyze *O*-acetyl esters and may lead to migration of acetate groups from *O*-7 and *O*-8 to *O*-9.[44, 45] Treatment with neuraminidase from *A. ureafaciens* is an alternative control, but is not as efficient as acid treatment [44, 46] and discriminates against Neu5Gc and *O*-acetylated sialic acids.[5, 47, 48] We found the composition of sialic acid on BSM to be 1:0.7:1.1 of Neu5Ac:Neu5Gc:Neu5,9Ac_2 (acid hydrolysis) or 1:0.6:1.5 (*A. ureafaciens*) (**Figure 3.3**, **A3.2**). Conditions with a ratio of Neu5,9Ac_2/Neu5Ac below 1.1 or 1.5 (depending on the control) indicate a preference for Neu5Ac over Neu5,9Ac_2. In our significance test we used the acid hydrolysis condition (Neu5,9Ac_2/Neu5Ac = 1.1 ± 0.2) as our reference because it gave a more conservative evaluation of the preference for Neu5Ac over Neu5,9Ac_2. The lower ratio of Neu5,9Ac_2 to Neu5Ac from acid hydrolysis compared to *A. ureafaciens* neuraminidase is likely due to the loss of some *O*-acetyl groups.

Neuraminidase from *C. perfringens* (NanI) discriminated Neu5Gc to a greater extent than Neu5,9Ac_2 (**Figure 3.3**), consistent with previous reports.[47]

We next turned to the hNEU enzymes using the same assay. Due to the generally low activity of these enzymes, in some cases we quantified the minimum preference of hNEU for Neu5Ac by calculating a ratio of Neu5,9Ac_2 or Neu5Gc to Neu5Ac where the numerator was defined by the limit of detection (LOD) for the assay (indicated by an "x" in **Figure 3.3**). We observed that all hNEU isoenzymes had a moderate preference for Neu5Ac over Neu5Gc (**Figure 3.3A)**, consistent with our previous study of hNEU activity on Neu5Gc-containing glycolipids.[1] This preference was most pronounced for NEU3, and was shared by both bacterial enzymes tested. The discrimination of Neu5,9Ac_2 by hNEU was common to all isoenzymes. For NEU2-NEU4 we

could detect a greater than 10-fold preference for Neu5Ac over Neu5,9Ac_2 (**Figure 3.3B**). Comparison to the acid or the *A. ureafaciens* neuraminidase positive control indicates an 11- or 16-fold preference, respectively, of NEU2 for Neu5Ac over Neu5,9Ac_2. Previous work in our group and others found that NEU2 has very strict substrate tolerance which can be attributed to its constrained C9 active site pocket.[1, 48] For NEU3 we detected a 9- to 12-fold preference; and for NEU4 an 11- to 15-fold preference. NEU1 experiments found at least a 1.5- to 2-fold preference for Neu5Ac over Neu5,9Ac_2; however, we note that these experiments used a lower enzyme activity where the Neu5Ac peak was close to the LOD (see **Materials and Methods**) and the Neu5,9Ac_2 value was defined by the LOD. Previous results with an artificial substrate based on 4MU-NANA (**4**) found a 2-fold preference for Neu5Ac over Neu5,9Ac_2, and a selective NEU1 inhibitor developed by our lab contains a C9 acetamido group, suggesting that NEU1 may be more tolerant of an acetate at C9.[49] It is important to note that ratios defined by the limit of detection and not by a Neu5,9Ac_2 peak indicate a lower limit of substrate preference rather than absolute substrate preferences. Further study will be necessary to determine if NEU1 is more tolerant of Neu5,9Ac_2 than are the other hNEUs.

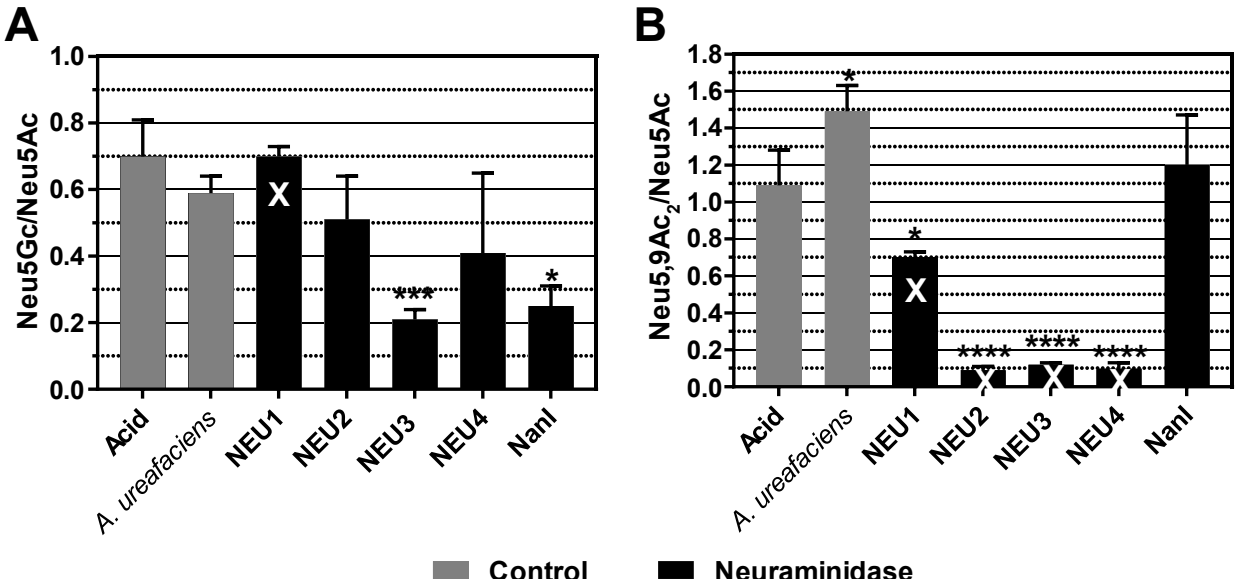


Figure 3.3: Release of modified sialic acids from bovine submaxillary mucin relative to Neu5Ac. Ratios smaller than those of the controls indicate preference for Neu5Ac over A) Neu5Gc, or B) $Neu5,9Ac_2$. Results are a mean of triplicate experiments, with error bars denoting one standard deviation. Unpaired t-tests comparing enzymatic conditions to release of sialic acid with the acid control were performed, and are indicated with * = $p > 0.05$, *** = $p > 0.001$, **** = $p > 0.0001$. Bars marked with an x indicate that the nominator was defined by the limit of detection of the assay rather than by a detected peak.

We have expanded our study of hNEU activity towards $Neu5,9Ac_2$ to include glycoprotein substrates. This work provides the first data point for NEU1 substrate tolerance towards $Neu5,9Ac_2$ on mimics of natural hNEU substrates. Considering that the sialic acid reducing end substituents and aglycone influence hNEU substrate tolerance, this data set on a sialoglycoprotein expands on the understanding of the substrate scope for NEU2-NEU4. Consistent with our previous study of $Neu5,9Ac_2$ – containing glycolipids, we observed the general trend of Neu5Ac > Neu5Gc > $Neu5,9Ac_2$. Further study on diverse sialoside substrates, particularly α(2→8)-linked sialosides,

will be important to generate a complete understanding of hNEU substrate specificity towards 9-*O*-acetylated sialic acids.

3.2.2 Aryl glycolipids for the study of hNEU activity on α(2→8)-linked Neu5,9Ac$_2$ substrates

Sialic acids are bound to glycans through a variety of different glycosidic linkages, most commonly an α(2→3) or α(2→6) linkage to a reducing end galactose or an α(2→8) linkage to a reducing end sialic acid. In our original study of hNEU activity on 9-*O*-acetylated sialic acids, we investigated the substrate activity of hNEU on mimics of ganglioside GM3 (**3-1**) where the sialic acid had an α(2→3) or α(2→6) glycosidic bond to the galactose. The data indicated that the prohibitive effect of the 9-*O*-acetyl group on hNEU activity may be enhanced with the presence of an α(2→6) versus α(2→3) linkage.[1] With this result in mind we sought to expand the scope of the study to include α(2→8)-linked Neu5,9Ac$_2$ sialosides. We decided on analogues of the glycolipid GD3 (**3-2**) as substrates because the carbohydrate moiety differs from GM3 by one sialic acid. This structural similarity would not only streamline the synthesis of these substrates, but also allow the direct comparison of the different glycosidic linkages. We designed a small panel of Neu5,9Ac$_2$ GD3 analogues and their GM3 precursors (**Figure 3.4**). These substrates contain non-reducing end α(2→8)-linked sialoside with an underlying α(2→3)-linked sialoside (**3-7**, **3-8**), or a non-canonical α(2→6)-linked sialoside (**3-9**, **3-10**).

Assays of hNEU activity on α(2→8)-linked sialosides are complicated by the presence of multiple sialic acids available to be cleaved by the hNEU enzymes. Methods to detect sialic acid hydrolysis through an increase in free sialic acids do not distinguish between glycosidic linkages. Therefore these methods would be unable to detect the release of α(2→8)-linked sialic acids exclusively over the α(2→3)- or α(2→6)-linked sialic acid exposed by their removal from the

glycan. As a result, we could not study hNEU kinetics on GD3 mimics using the malononitrile assay as we had for the GM3 mimics.[1] Instead we had to implement an assay in which we could detect changes to the substrate itself. We designed the glycolipid substrates with an aryl aglycone (**Figure 3.4**) which would afford for UV detection of the substrates after resolution using reversed phase HPLC.

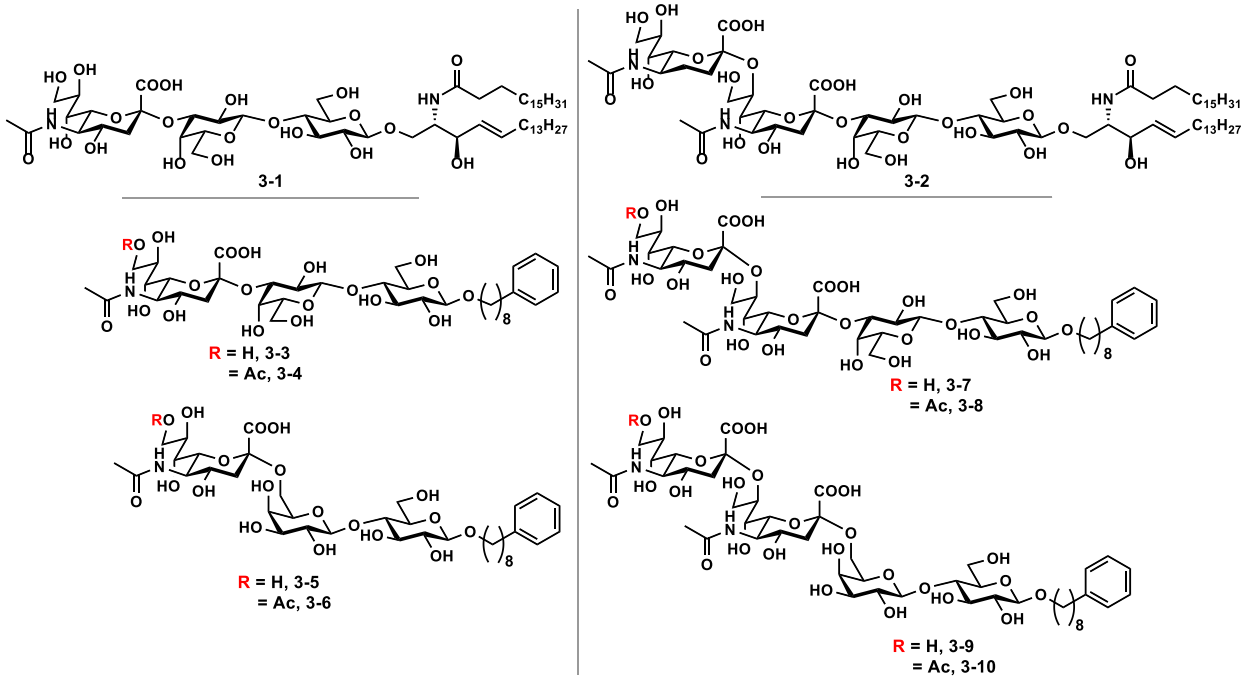
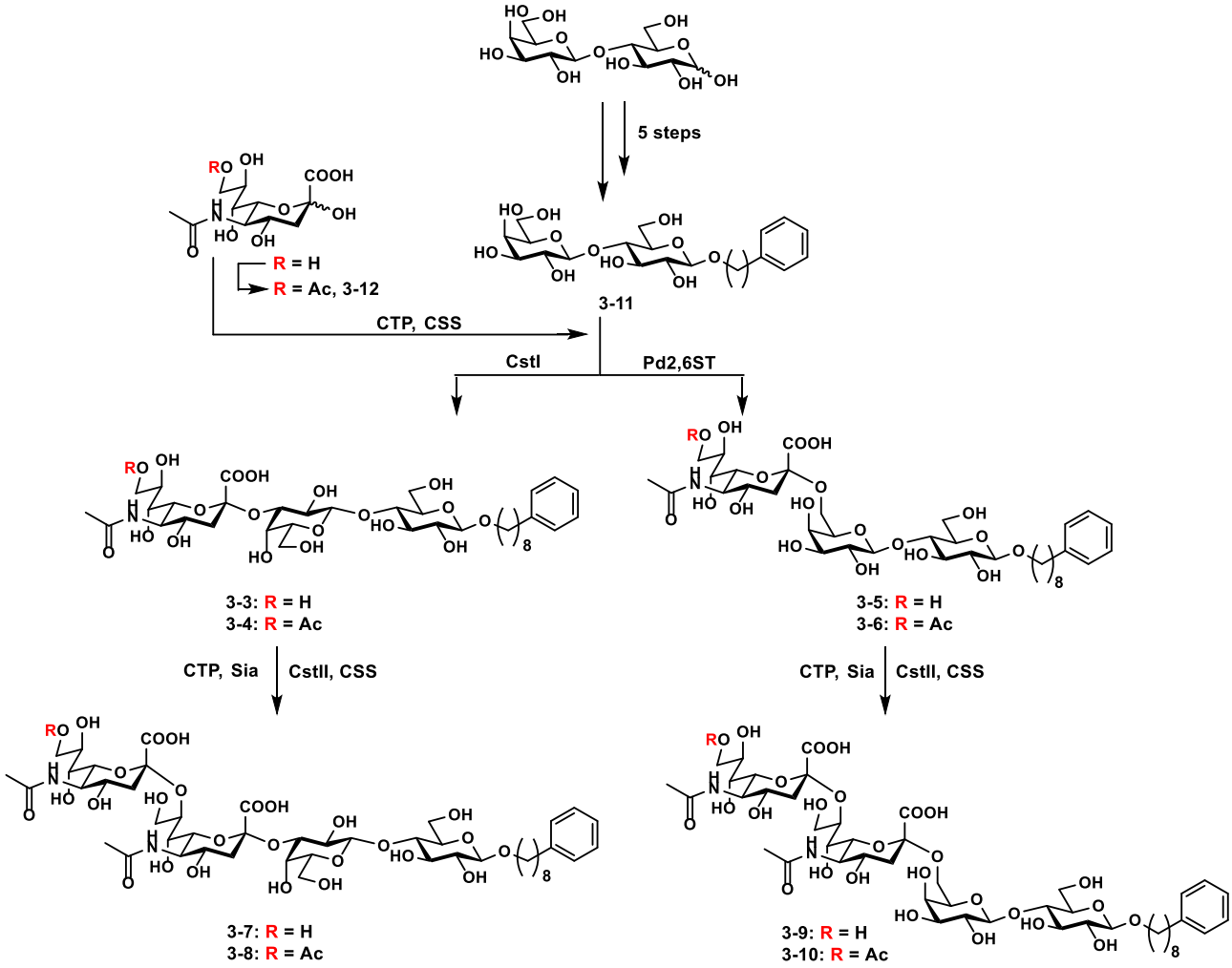


Figure 3.4: Aryl glycolipid target based on GM3 and GD3 targets.

3.2.3 Synthesis of aryl glycolipids

The aryl glycolipids **3-3** –**3-7** and **3-9** were made through a combination of synthetic and chemoenzymatic methods. Starting from lactose, lactoside **3-11** was synthesized in 5 steps as reported.[1, 50] Consecutive sialylations of **3-11** using the one-pot multienzyme chemoenzymatic approach developed by Chen and coworkers generated the GM3 and GD3 analogues (**Scheme 3.1**). From lactoside **3-11**, CMP-sialic acid synthetase (CSS) and the α(2→3) SiaT Cst-I[51] generated **3-3** and **3-4**, while **3-5** and **3-6** were made through the action of CSS and the α(2→6)

SiaT Pd2,6ST. The GD3 analogues were made through sialylation of **3-3** and **3-5** with CSS and the bifunctional α(2→3/8) SiaT Cst-II.[52] The α(2→8) sialyltransferase activity of Cst-II can polymerize sialic acid to generate polymers of α(2→8)-linked sialic acids (polysialic acid), but stoichiometric control of Neu5Ac in the one-pot chemoenzymatic reaction prevented further sialylation of the GD3 analogues.[53]



Scheme 3.1: Chemoenzymatic synthesis of GD3 targets

Synthesis of the Neu5,9Ac$_2$ sialosides was complicated by esterase activity present in the crude enzyme mixtures. As with our parallel efforts into the synthesis of the Neu5,9Ac$_2$ GM3 analogues detailed in **Chapter 2**, Neu5,9Ac$_2$ GM3 analogue **3-4** was made as a mixture with its

ester hydrolysis product **3-3**, and would require further purification. Because compound **3-6** was made successfully without de-*O*-acetylation to **3-5**, we believe that the de-*O*-acetylation of **3-4** could be attributed to esterase activity in the crude mixture of the α(2→3) SiaT Cst-I. The synthesis of Neu5,9Ac_2 GD3 was also hampered by esterase activity. Despite extensive optimization of the chemoenzymatic reaction, we never detected the formation of compound **3-8**, only its ester hydrolysis product **3-7**.

To identify and separate the esterase activity present in the crude enzymatic mixtures we applied an esterase assay using *p*-nitrophenol acetate as the esterase substrate (**Figure 3.5A**).[54] We could detect the hydrolysis of the ester through the increase in absorbance at 405 nm.[55] After unsuccessful attempts to separate esterase activity from sialyltransferase activity using various molecular weight cut-off filters, we tried a different preparation of Cst-II purified using ion exchange chromatography (personal communication from Warren Wakarchuk, University of Alberta). Despite decreasing the esterase activity in the CstII enzyme mixture (**Figure 3.5B**), our attempts to synthesize compound **3-8** were unsuccessful.

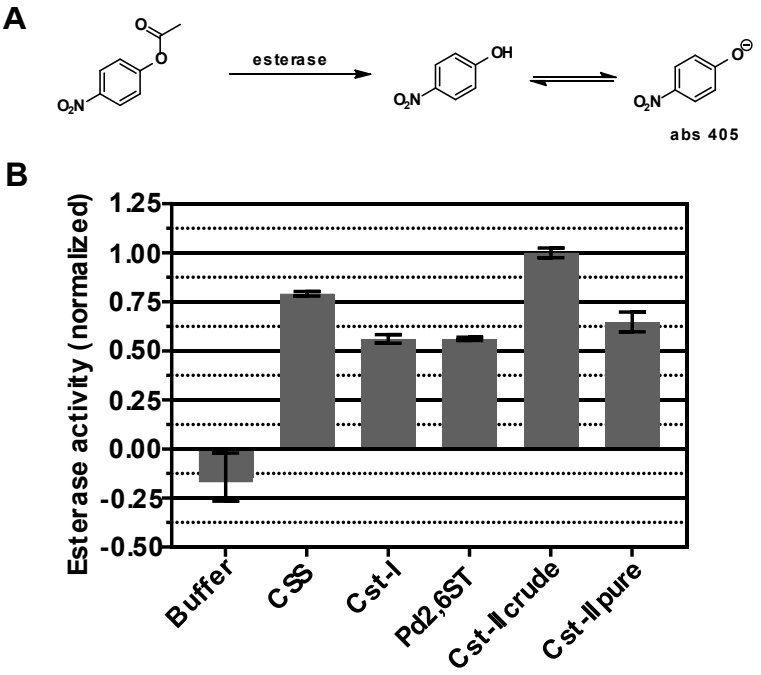


Figure 3.5: Efforts to eliminate esterase activity from one-pot multienzyme synthesis A) mechanism of the esterase assay B) results of the esterase assay with esterase activity normalized to crude Cst-II.

3.2.4 HPLC assay for hNEU activity on aryl glycolipids

The GD3 (**3-7**), GM3 (**3-3**), and lactoside (**3-11**) analogues were resolved using reversed-phase HPLC with an isocratic elution of 30 % acetonitrile in water and detection at 220 nm (**Figure 3.6**). Benzoic acid was used as an internal standard to normalize peak areas. The limit of detection (LOD) was 24 μM for GD3 analogue **3-7**, 18 μM for GM3 analogue **3-3**, and 45 μM for lactoside **3-11**. The higher LOD of compound **3-11** is likely due to the reduced solubility of the substrate observed in water, which suggests that detection of **3-11** should not be used for quantitation.

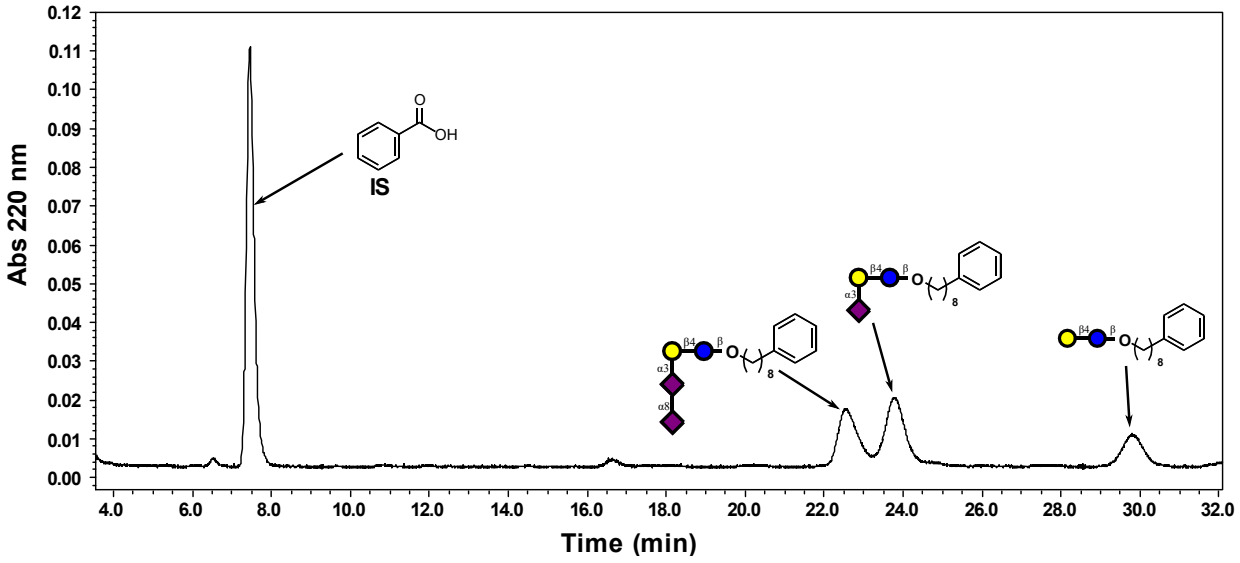


Figure 3.6: Separation of GD3 (**3-7**), GM3 **(3-3**), and lactoside (**3-11**) analogues using HPLC, with benzoic acid as an internal standard

To validate the HPLC assay to study hNEU kinetics, we obtained Michaelis-Menten parameters for NEU2 and NEU3 cleaving the GM3 analogue **3-3** (data not shown). The kinetic parameters determined by the HPLC assay agree with values obtained by other methods, although there was significant noise in the assay which may limit its application. We calculated a K_M of 300 μM for NEU2 (data not shown), which agrees well with the K_M of 325 μM from the malononitrile assay (**A3.2**), and with literature reports.[56] For NEU3 we calculated a K_M of 200 μM (data not shown), consistent with previous reports.[57] Attempts to measure a K_M for NEU4 cleaving **3-3** were unsuccessful even in the presence of Triton X-100, suggesting that GM3 is a poor substrate for NEU4. These data are consistent with reports that NEU4 prefers glycoproteins and oligosaccharides over ganglioside substrates.[58] The agreement of the Michaelis-Menten parameters measured using this HPLC assay to other methods and literature values confirmed its utility for measuring hNEU kinetics. The assay is limited due to noise, contributing to large standard errors; however, as compared to typical measures of NEU kinetics which rely on thin

layer chromatography (TLC)[56, 57] the assay is easier to standardize and provides similar measurements. This assay should be particularly useful for studying the kinetics of hNEU on ganglioside mimics with multiple sialic acids, because it can detect multiple species simultaneously during the successive removal of each sialic acid.

3.3 Conclusions

There is no universal method for studying hNEU substrate specificity and to do so requires the selection and implementation of appropriate assays. We report two methods which expand the chemical biology toolbox to study hNEU substrate specificity on natural unstable sialosides. We adapted an assay to study bacterial neuraminidase activity on Neu5,9Ac_2 glycoproteins for the lower activity of hNEU. All four hNEU were more efficient at cleaving Neu5Ac over Neu5,9Ac_2 to such an extent we could not detect the release of Neu5,9Ac_2 by hNEU. Sialic acid release was detected after derivatization with OPD which is selective for α-keto acids. As such, the assay should be compatible with complex biological samples. To study hNEU activity on α(2→8)-linked Neu5,9Ac_2 we developed an HPLC assay to monitor the degradation of aryl glycolipids. We validated the assay for studying hNEU kinetics by using it to determine Michaelis-Menten parameters for NEU2 and NEU3 cleaving GM3 analogue **3-3**. Esterase activity in our enzyme mixture prohibited the synthesis of Neu5,9Ac_2 GD3 analogues **3-8** and **3-10**. Purified enzymes should eliminate this barrier, after which relative rates of sialic acid release from substrates **3-3** to **3-10** can be measured using the HPLC assay for aryl glycolipids. This assay would be valuable for studying hNEU kinetics on any glycolipid with more than one sialic acid residue.

3.4 Materials and Methods

3.4.1 General methods:

All reagents were purchased from commercial sources and used as received, unless other wise noted. Enzymes for the one-pot sialylation to make GM3 analogues **3-3, 3-5**, and **3-6** were prepared as described previously.[50, 59] The CMP-Neu5Ac synthetase (CSS) was *Neisseria meningitidis* CMP-Neu5Ac synthetase (NmCss)[60] and the α(2→3) sialyltransferase (SiaT) was *Campylobacter jejuni* α(2→3) sialyltransferase (CstI),[51] both expressed in *E. coli* strain AD202 and used as crude enzyme mixtures. The α(2→6) sialyltransferase was *Photobacterium damsela* α(2→6) sialyltransferase (Pd2,6ST) was expressed with a $(His)_6$ tag in *E. coli* strain Nova Blue (DE3) and purified using a Ni-NTA column.[59, 61] The α(2→8) sialyltransferase (SiaT) used for the synthesis of **3-7** and **3-9** was *Campylobacter jejuni* α(2→3/8) sialyltransferase (Cst-II),[52] expressed in *E. coli* strain AD202 and used as a crude enzyme mixture. Reactions were monitored by thin layer chromatography on silica gel 60-F254 (0.25 nm, Silicycle, QC, Canada) with visualization by charring with 5 % sulfuric acid in ethanol. Organic solvents were evaporated under reduced pressure, and water was removed by lyophilization. Chemoenzymatic reactions were purified using sep-pak C_{18} plus short cartridges, 360 mg sorbent per cartridge, 55-105 μM particle size (Waters Ltd., Mississauga, ON, Canada). NMR experiments were run on Varian 500, 600, and 700 MHz instruments with chemical shifts reported relative to deuterated solvent peaks. ESI mass spectra were measured on Agilent Technologies 6220 TOF. HPLC was performed with a Waters Delta 600 pump, a Waters 600 controller, and a Waters 2996 photodiode array (PDA) detector with Empower 2 software (Waters Ltd., Mississauga, ON, Canada). Bovine submaxillary mucin Type I-S, 9-24 % bound sialic acids was purchased from Sigma Aldrich. Neuraminidases from *Clostridium perfringens* and *Arthrobacter ureafaciens* were purchased from Sigma Aldrich. The human neuraminidase enzymes NEU2-NEU4 were expressed as fusion with maltose binding protein, as previously reported. [50, 62, 63] NEU1 was overexpressed in HEK293E cells and used as

crude cell lysate.[1] Neuraminidase activity was determined in comparison to a standard curve of neuraminidase from *Clostridium perfringens* using 4-methylumbelliferyl α-$_D$-*N*-acetylneuraminic acid (4MU-NANA) as a substrate. Unless otherwise stated, results reported are technical replicates, with preliminary independent replicates confirming the trends reported.

3.4.2 LOB and LOD of the assay to detect sialic acid release from 9-*O*-acetylated glycoproteins:

To determine the limit of detection (LOD) for the quinoxaline derivatized sialic acids, 30 µL of 5 µM Neu5Ac (or H_2O for limit of blank, LOB) was mixed with 30 µL of 40 mM TFA, 30 µL derivatization mixture (25 mM *o*-phenyldiamine (OPD), 18 mM $Na_2S_2O_4$, 0.5 M ß-mercaptoethanol), and 10 µL 2 mM *p*-nitrophenol (internal standard). The mixture was heated for 3 hours at 50 ℃ after which it was analyzed by HPLC on a C-18 reverse phase column with isocratic elution with 84:9:7 H_2O: acetonitrile: methanol and detection at 350 nm. The LOB and LOD were calculating using peak areas normalized to the internal standard with $LOB = mean_{blank} + 1.645(StDev_{blank})$ and $LOD = LOB + 1.645(StDev_{sample})$.[64] The peak areas for the LOB were obtained by integrating the baseline with the average retention times and peak widths detected for the LOD.

3.4.3 Acid hydrolysis of sialic acids from bovine submaxillary mucin:

Bovine submaxillary mucin (250 µg) was dissolved in 40 µL of water and 30 µL 2 M acetic acid. The mixture was heated for 3 hrs at 80 ℃ to release all sialic acids.[46] Derivitization mixture ((25 mM σ-phenyldiamine (OPD), 18 mM $Na_2S_2O_4$, 0.5 M ß-mercaptoethanol) and 10 µL of 2 mM ρ-nitrophenol were added. Protein was removed using centrifugal filters (30 kDa molecular weight

cut-off) for 30 minutes at 14 000 rpm. The filtrate was heated for 3 hrs at 50 ℃ and the labeled sialic acids were analyzed after separation by HPLC on a C-18 reversed phase column with an isocratic solvent composition of H_2O:acetonitrile:methanol 84:9:7. Elution peaks were detected at 350 nm.

3.4.4 Neuraminidase release of sialic acids from bovine submaxillary mucin:

Bovine submaxillary mucin (250 µg) was dissolved in 20 µL 20 mM sodium acetate buffer pH 4.5 (5.5 for NEU2). 20 µL (1 mU) of enzyme was added (or water as a negative control). After 5 hrs incubation at 37 ℃, 30 µL 40 mM TFA, 30 µL derivatization mixture (25 mM σ-phenyldiamine (OPD), 18 mM $Na_2S_2O_4$, 0.5 M ß-mercaptoethanol), and 10 µL of 2 mM ρ-nitrophenol were added. Protein was removed using centrifugal filter units (30 kDa molecular weight cut-off) for 30 minutes at 14 000 rpm after which the filtrate was heated for 3 hrs at 50 ℃. The labeled sialic acids were analyzed after separation by HPLC on a C-18 reversed phase column with an isocratic solvent composition of H_2O:acetonitrile:methanol 84:9:7. Peaks were detected at 350 nm. For NEU1 which had lower activity, 500 µg of bovine submaxillary mucin was used. The volumes of all reagents were also doubled, and 1 mU enzyme activity was used.

3.4.5 LOB and LOD of HPLC assay for aryl glycolipids:

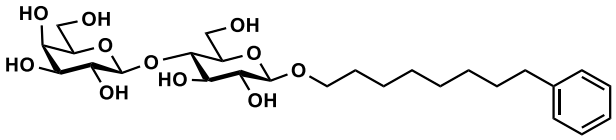
To determine the limit of detection of aryl glycolipids separated by HPLC on a C-18 reversed phase column, 20 µL of water (LOB) or of 60 µM each compounds **3-1**, **3-2**, and **3-6** (lactoside, GM3 analogue, and GD3 analogue, respectively), 20 µL of 0.2 M sodium acetate pH 4.5, 30 µL 0.2 M sodium borate pH 9.5, and 10 µL 0.5 mM phenol were mixed and separated using HPLC on a reversed phase C-18 column. The glycolipids were eluted with 30 % acetonitrile in water (both

with 0.1 % trifluoroacetic acid) over 30 minutes with detection at 220 nm. The LOB and LOD were calculated as described in section **3.4.2**.

3.4.6 Michaelis-Menten kinetics of hNEU cleaving compound 3-2:

Compound **3-2** (x·2 µM) in 100 µL H_2O was mixed with 80 µL 0.2 M sodium acetate pH 4.5 (NEU3, NEU4) or pH 5.5 (NEU2) and 20 µL (1 mU) hNEU. The assay mixture was incubated at 37 °C. At timepoints of 0, 5, 10, 15 minutes (NEU2) or 0, 10, 20, 30 minutes (NEU3, NEU4) 40 µL of the assay mixture was quenched in 30 µL 0.2 M sodium borate pH 9.5 and 10 µL 0.5 mM benzoic acid. Protein was removed using centrifugal filters (10 kDa molecular weight cut-off) for 45 minutes at 14 000 rpm. The mixture was analyzed by HPLC on a reversed-phase C-18 column with an isocratic elution of 30 % acetonitrile in water (both with 0.1 % trifluoroacetic acid) over 30 minutes. Peaks were detected at 220 nm and peak areas were normalized to benzoic acid. Michaelis-Menten parameters were calculated using GraphPad Prism.

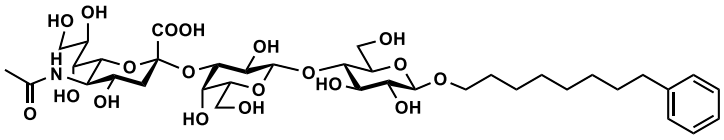
3.4.7 Synthetic methods



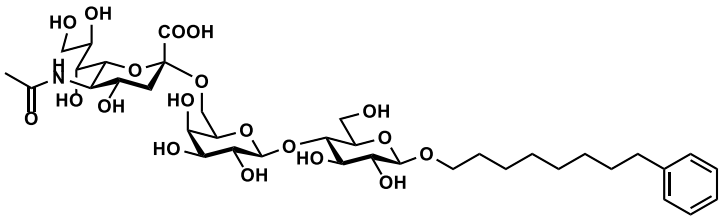
O-(β-D-galactopyranosyl)-(1 → 4)-O- (β-D-glucopyranosyl)-8-phenyl-octanol (3-11)

Compound **3-11** was made in 5 steps as previously described.[1, 50] ^{1}H NMR (700 MHz, CD_3OD) δ 7.23 (t, J = 7.7, 2H, H_c), 7.15 (d, J = 7.7 Hz, 2H, H_b), 7.13 (t, J = 7.7 Hz, 1H, H_d), 4.36 (d, J = 7.7 Hz, 1H, H-1''), 4.28 (d, J = 7.7 Hz, 1H, H-1'), 3.91-3.68 (m, 6H), 3.60-3.47 (m, 6H), 3.39 (ddd, J = 9.7,4.2, 2.6 Hz, 1H, H-4'), 3.23 (dd, J = 9.7, 7.9 Hz, 1H, H-2'), 2.59 (t, J = 7.7 Hz, 2H,

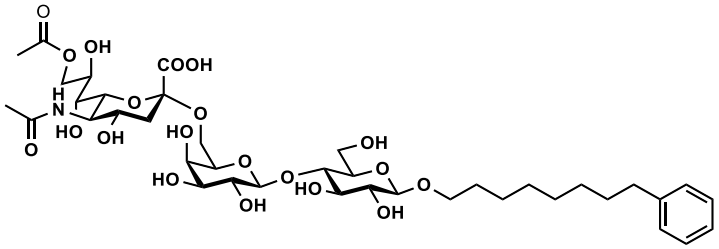
$O(CH_2)_7C\underline{H_2}Ar$), 1.64-1.58 (m, 4H, $OCH_2C\underline{H_2}(CH_2)_4C\underline{H_2}CH_2Ar$), 1.40-1.29 (m, 8H, $O(CH_2)_2(C\underline{H_2})_4(CH_2)_2$ Ar). ^{13}C (175 MHz, CD_3OD) δ 144.3 (C_a), 129.7 (C_b), 129.5 (C_c), 126.9 (C_d), 105.4 (H-1''), 104.5 (H-1'), 80.99, 77.4, 76.8, 76.3, 75.13, 75.07 (C-2'), 72.9, 71.2, 70.6, 62.8, 62.3, 37.2 ($O(CH_2)_7\underline{C}H_2Ar$), 33.1, 31.1, 30.8, 30.6, 27.4.



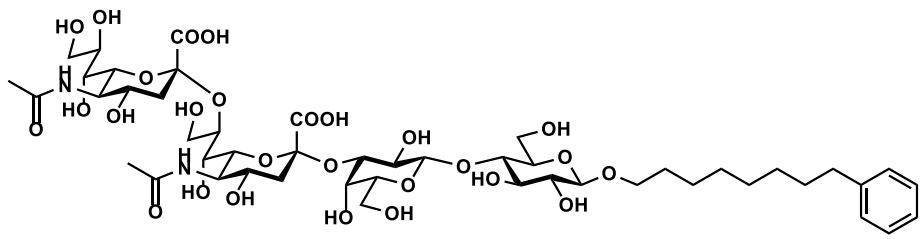
3-3: 5 mg (0.016 mmol) Neu5Ac, 10 mg CTP (0.021 mmol), 1.8 mL H_2O, 800 µL 1 M Tris HCl pH 8.8 and 160 µL 1 M $MgCl_2$ were added to 5 mg (0.0094 mmol) of lactoside **3-11**. The reaction was charged with 200 µL CSS and 200 µL Cst-I and the reaction was stirred at 37 °C overnight. Upon completion the reaction was quenched with 1 mL EtOH and the reaction was spun down at 17 000 rpm for 1 hr. The solution was concentrated under vacuum and the product was purified using a C_{18} sep pak cartridge with a gradient of 0 - 50 % MeOH in H_2O. Yield: 5.2 mg (70 %) ^1H NMR (700 MHz, D_2O) δ 7.37 (t, *J* = 7.7 Hz, 2H, H_c), 7.31 (d, *J*= 7.7 Hz, 2H, H_b), 7.26 (t, *J* = 7.7 Hz, 1H, H_d), 4.54 (d, *J* = 7.8 Hz, 1H, H-1''), 4.48 (d, *J* = 8.0 Hz, 1H, H-1'), 4.12 (dd, *J* = 10.5, 2.8 Hz, 1H, H-3''), 4.01-3.96 (m, 2H, H-4''), 3.93-3.55 (m, 17H), 3.30 (t, *J* = 8.4 Hz, 1H, H-2''), 2.77 (dd, *J* = 12.3, 4.9 Hz, 1H, H-3_{ax}'''), 2.64 (t, *J* = 7.6 Hz, 2H, $O(CH_2)_7C\underline{H_2}Ar$), 2.04 (s, 3H, $NHCOC\underline{H_3}$), 1.81 (t, *J* = 12.3 Hz, 1H, H-3_{eq}'''), 1.65-1.59 (m, 4H, $OCH_2C\underline{H_2}(CH_2)_4C\underline{H_2}CH_2Ar$), 1.38-1.28 (m, 8H, $O(CH_2)_2(C\underline{H_2})_4(CH_2)_2$ Ar) ^{13}C (175 MHz, D_2O) δ 176.0 ($NH\underline{C}OCH_3$), 174.9 ($\underline{C}OO^-$), 144.7 (C_a), 129.6 (C_b), 129.5 (C_c), 126.7 (C_d), 103.6 (H-1''), 103.0 (H-1'), 100.8 (C-2'''), 79.3 (C-2''), 76.5 (C-3'') , 76.2 , 75.7, 75.4, 73.8 (C-2'), 72.8, 71.7, 70.4, 69.3 (H-4'''), 69.1, 68.5 (C-4''), 63.6, 62.0, 61.1, 60.6 (C-5'''), 52.7, 40.7, 36.0 ($O(CH_2)_7\underline{C}H_2Ar$), 31.7, 29.7, 29.4, 29.3, 29.1, 25.9, 23.0. ESI-MS calculated for $C_{37}H_{58}NO_{19}$ $[M-H]^-$ 820.3609 found: 820.3604.



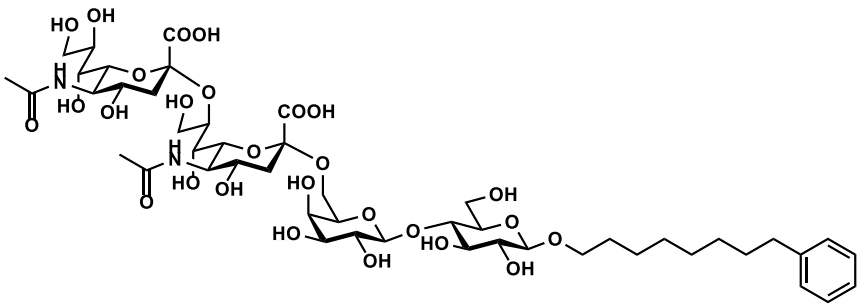
3-5: 5 mg (0.016 mmol) Neu5Ac, 10 mg (0.021 mmol) CTP, 1.8 mL H_2O, 800 µL 1 M Tris HCl pH 8.8 and 160 µL 1 M $MgCl_2$ were added to 5 mg (0.0094 mmol) of lactoside **3-11**. The reaction was charged with 200 µL CSS and 200 µL Pd2,6ST and the reaction was stirred at 37 °C overnight. Upon completion the reaction was quenched with 1 mL EtOH and the reaction was spun down at 17 000 rpm for 1 hr. The solution was concentrated under vacuum and the product was purified using a C_{18} sep pak cartridge with a gradient of 0 - 50 % MeOH in H_2O. Yield: 3.9 mg (51 %) ^{1}H NMR (700 MHz, D_2O) 7.37 (t, *J* = 7.7 Hz, 2H, H_c), 7.31 (d, *J*= 7.7 Hz, 2H, H_b), 7.26 (t, *J* = 7.7 Hz, 1H, H_d) 4.48 (d, *J* = 8.1 Hz, 1H, H-1'), 4.43 (d, *J* = 7.8 Hz, 1H, H-1''), 4.00-3.77 (m, 9H), 3.70-3.52 (m, 11H), 3.33 (t, *J* = 8.4 Hz, 1H, H-2'), 2.72 (dd, *J* = 12.3, 4.9 Hz, 1H, H-3_{ax}'''), 2.65 (t, *J* = 7.6 Hz, 2H, $O(CH_2)_7C\underline{H_2}Ar$), 2.04 (s, 3H, $NHCOC\underline{H_3}$), 1.75 (t, *J* = 12.3 Hz, 1H, H-3_{eq}'''), 1.66-1.59 (m, 4H, $OCH_2C\underline{H_2}(CH_2)_4C\underline{H_2}CH_2Ar$), 1.38-1.29 (m, 8H, $O(CH_2)_2(C\underline{H_2})_4(CH_2)_2$ Ar) δ ^{13}C (175 MHz, D_2O) δ 175.8 ($NH\underline{C}OCH_3$), 174.3($\underline{C}OO^-$), 144.5 (C_a), 129.5 (C_b), 129.4 (C_c), 126.6 (C_d), 104.1 (C-1'), 102.8 (C-1''), 101.2 (C-2'''), 80.5, 75.6, 75.5, 74.5, 73.6, 73.4, 73.2, 72.6, 71.7, 71.6, 69.3, 69.2, 64.4, 63.5, 62.1, 61.7, 60.3, 52.3, 41.0, 35.8, 31.6, 29.5, 29.24. 29.18, 29.0, 25.8, 22.9. ESI-MS calculated for $C_{37}H_{58}NO_{19}$ $[M-H]^-$ 820.3609 found: 820.3606.



3-6: 5 mg (0.016 mmol) Neu5,9Ac$_2$, 10 mg (0.021 mmol) CTP, 1.8 mL H_2O, 800 µL 1 M HEPES pH 7.2 and 160 µL 1 M $MgCl_2$ were added to 5 mg (0.0094 mmol) of lactoside **3-11**. The reaction was charged with 200 µL CSS and 200 µL Pd2,6ST and the reaction was stirred at 37 °C for 3 hrs. The reaction was quenched with 1 mL EtOH and the reaction was spun down at 17 000 rpm for 1 hr. The solution was concentrated under vacuum and the product was purified using a C_{18} sep pak cartridge with a gradient of 0 - 50 % MeOH in H_2O. Yield: 0.9 mg (11 %) ^{1}H NMR (700 mHz, D_2O) δ 7.37 (t, *J* = 7.7 Hz, 2H, H_c), 7.31 (d, *J* = 7.7 Hz, 2H, H_b), 7.26 (t, *J* = 7.7 Hz, 1H, H_d), 4.49 (d, *J* = 8.2 Hz, 1H, H-1'), 4.44 (d, *J* = 7.7 Hz, 1H, H-1''), 4.42 (dd, *J* = 11.9, 2.2 Hz, 1H, H-9a'''), 4.21 (dd. *J* = 11.9, 5.7 Hz, 1H, H-9b'''), 4.12 (ddd, 9.2, 5.7, 2.2 Hz, 1H), 4.00-3.53 (m, 17H), 3.33 (t, *J* = 8.6 Hz, 1H, H-2'), 2.72 (dd, *J* = 12.7, 4.9 Hz, 1H, H-3$_{ax}$'''), 2.65 (t, *J* = 7.6 Hz, 2H, O(CH$_2$)$_7$C<u>H$_2$</u>Ar), 2.14 (s, 3H, CH$_2$COC<u>H$_3$</u>), 2.05 (s, 3H, NHCOC<u>H$_3$</u>), 1.75 (t, *J* = 12.7 Hz, 1H, H-3$_{eq}$'''), 1.66-1.60 (m, 4H, OCH$_2$C<u>H$_2$</u>(CH$_2$)$_4$C<u>H$_2$</u>CH$_2$Ar), 1.38-1.29 (m, 8H, O(CH$_2$)$_2$(C<u>H$_2$</u>)$_4$(CH$_2$)$_2$ Ar). δ ^{13}C (175 MHz, D_2O) 175.7 (NH<u>C</u>OCH$_3$, from HMBC), 175.2 (CH$_2$<u>C</u>OCH$_3$, from HMBC), 174.3 (COO$^-$, from HMBC), 144.5 (C_a), 129.5 (C_b), 129.4 (C_c), 126.6 (C_d), 104.1 (C-1'), 102.8 (C-1''), 101.4 (C-2'''), 80.6 (C-2''), 75.5 (C-3'), 74.6, 73.6 (C-2'), 73.2, 71.6, 71.5, 71.2, 70.1 (HSQC), 69.5 (HSQC),69.4 (HSQC), 69.2 (HSQC), 69.1 (HSQC) 66.5, 64.5 (HSQC), 61.4 (HSQC) 61.1, 52.6, 41.1, 35.8 (O(CH$_2$)$_7$<u>C</u>H$_2$Ar), 31.6, 29.5, 29.22, 29.16, 29.0, 25.8, 21.1. ESI-MS calculated for $C_{39}H_{61}NO_{20}$ [M-H]$^-$ 862.3714 found: 862.373.



3-7: 2 mg (0.004 mmol) CTP, 0.75 mg (0.002 mmol) Neu5Ac, 1.2 mL H_2O, 400 µL 1 M Tris HCl pH 8.8 and 80 µL 1 M $MgCl_2$ were added to 2 mg (0.002 mmol) of **3-3**. The reaction was charged with 200 µL CSS and 200 µL Cst-II and the reaction was stirred at 37 °C for 2 hrs. The reaction was quenched with 500 µL EtOH and the reaction was spun down at 17 000 rpm for 1 hr. The solution was concentrated under vacuum and the product was purified using a C_{18} sep pak cartridge with a gradient of 0 - 50 % MeOH in H_2O. Yield: 0.8 mg (30 %) ^{1}H NMR (700 mHz, D_2O) δ 7.37 (t, *J* = 7.7 Hz, 2H, H_c), 7.31 (d, *J* = 7.7 Hz, 2H, H_b), 7.26 (t, *J* = 7.7 Hz, 1H, H_d), 4.53 (d, *J* = 7.8 Hz, 1H, H-1''), 4.48 (d, *J* = 8.2 Hz, 1H, H-1'), 4.19 (dd, *J* = 12.2, 3.5 Hz, 1H), 4.16-4.14 (m, 1H), 4.1 (dd, *J* = 10.0, 3.0 Hz, 1H, H-3''), 4.03-3.96 (m, 2H, H-4'', H-X), 3.95-3.53 (m, 23 H), 3.31 (t, *J* = 9.1 Hz, 1H, H-2'), 2.79 (dd, *J* = 12.4, 4.4 Hz, 1H, H-3_{ax}''''), 2.69 (dd, *J* = 12.4, 4.4 Hz, 1H, H-3_{ax}'''), 2.65 (t, *J* = 7.6 Hz, 2H, $O(CH_2)_7C\underline{H_2}Ar$), 2.08 (s, 3H, $NHCOC\underline{H_3}$''''), 2.04 (s, 3H, $NHCOC\underline{H_3}$'''), 1.75 (t, *J* = 12.2 Hz, 2H, H-3_{eq}''', H-3_{eq}''''), 1.66-1.60 (m, 4H, $OCH_2C\underline{H_2}(CH_2)_4C\underline{H_2}CH_2Ar$), 1.38-1.29 (m, 8H, $O(CH_2)_2(C\underline{H_2})_4(CH_2)_2$ Ar). ^{13}C (125 MHz, D_2O) δ 176.0 ($NH\underline{C}OCH_3$''', $NH\underline{C}OCH_3$''''), 174.5 (COO^-), 174.3 (COO^-), 144.7 (C_a), 129.6 (C_b), 129.5 (C_c), 126.7 (C_d), 103.7 (C-1''), 103.0 (C-1'), 101.5 (C-2''''), 101.2 (C-2'''), 79.2, 79.1, 76.5, 76.2 (C-3''), 75.8, 75.4, 75.0, 73.9, 73.6 (C-2'), 72.7, 71.7, 70.34, 70.28, 69.5, 69.1, 68.9, 68.5 (C-4'''), 63.6, 62.6, 62.1, 61.0, 53.3, 52.7, 41.5, 40.7, 36.0 ($O(CH_2)_7\underline{C}H_2Ar$), 31.7, 29.7, 29.4, 29.3, 29.1, 25.9, 23.3, 23.0. ESI-MS calculated for $C_{48}H_{74}N_2O_{27}$ $[M-2H]^{-2}$ 555.2245 found: 555.2253.



3-9: 5 mg (0.010 mmol) CTP, 2.5 mg (0.008 mmol) Neu5Ac, 1.2 mL H_2O, 400 µL 1 M Tris HCl pH 8.8 and 80 µL 1 M $MgCl_2$ were added to 4.5 mg (0.005 mmol) of **3-5**. The reaction was charged with 200 µL CSS and 200 µL Cst-II and the reaction was stirred at 37 ℃ for 2 hrs. The reaction was quenched with 500 µL EtOH and the reaction was spun down at 17 000 rpm for 1 hr. The solution was concentrated under vacuum and the product was purified using a C_{18} sep pak cartridge with a gradient of 0 - 50 % MeOH in H_2O. Yield: 2.4 mg (39 %) ^{1}H NMR (700 mHz, D_2O) δ 7.37 (t, *J* = 7.7 Hz, 2H, H_c), 7.31 (d, *J* = 7.7 Hz, 2H, H_b), 7.26 (t, *J* = 7.7 Hz, 1H, H_d), 4.49 (d, *J* = 8.2 Hz, 1H, H-1'), 4.44 (d *J* = 7.8 Hz, 1H, H-1''), 4.22-4.19 (m, 1H, H-6a''), 4.14 (dd, *J* = 12.1,3.9 Hz, 1H, H-x), 4.00-3.78 (m, 12H), 3.74-3.53 (m, 13H), 3.32 (t, *J* = 8.6 Hz, 1H, H-2'), 2.79 (dd, *J* = 12.4, 4.5 Hz, 1H, H-3_{ax}'''), 2.66-2.61 (m, 3H, H-3_{ax}'''', $O(CH_2)_7C\underline{H_2}Ar$), 2.08 (s, 3H, $NHCOC\underline{H_3}$''''), 2.04 (s, 3H, $NHCOC\underline{H_3}$'''),1.75 (t, *J* = 12.2 Hz, 1H, H-3_{eq}'''), 1.70 (t, *J* = 12.2 Hz, 1H, H-3_{eq}''''), 1.66-1.60 (m, 4H, $OCH_2C\underline{H_2}(CH_2)_4C\underline{H_2}CH_2Ar$), 1.38-1.29 (m, 8H, $O(CH_2)_2(C\underline{H_2})_4(CH_2)_2$ Ar). ^{13}C (125 MHz, D_2O) δ 175.85 ($NH\underline{C}OCH_3$'''), 175.78 ($NH\underline{C}OCH_3$''''), 174.22 (COO^-'''), 174.20 (COO^-''''), 144.5 (C_a), 129.45 (C_b), 129.36 (C_c), 126.6 (C_d), 104.1 (C-1''), 102.7 (C-1'), 101.8 (C-2''''), 101.3 (C-2'''), 95.8, 80.6, 79.4, 75.6, 75.5, 75.0, 74.6, 73.6, 73.5, 73.2, 72.6, 71.7, 71.6, 70.5, 69.4, 69.0, 68.7, 64.6, 63.5, 62.5, 61.2, 53.2, 52.6, 41.4, 41.0, 35.8 ($O(CH_2)_7\underline{C}H_2Ar$), 31.6, 29.5, 29.23, 29.17, 29.0, 25.8, 23.2, 22.9. . ESI-MS calculated for $C_{48}H_{74}N_2O_{27}$ $[M-2H]^{-2}$ 555.2245 found: 555.2248.

3.4.8 Esterase assay

ρ-nitrophenyl acetate (**3-12**) was made in one step from ρ-nitrophenol. Briefly, 0.69 g (5 mmol) of ρ-nitrophenol (**3-13**) was dissolved in 10 mL CH_2Cl_2 and 0.69 mL (10 mmol) triethylamine as added. The mixture was put over ice and 0.57 mL (6 mmol) acetic anhydride was added. The reaction was removed from ice and stirred for 1 hr at room temperature. The reaction was quenched with 50 mL H_2O and 100 mL ethyl acetate. The water was removed and the reaction was washed successively with 30 mL saturated ammonium chloride, 25 mL saturated sodium bicarbonate, and 40 mL brine. The reaction was dried with magnesium sulfate before evaporation under reduced pressure. Yield 844 mg (94 %) ^{1}H NMR (700 mHz, $CDCl_3$) δ 8.26 (d, J = 9.1 Hz, 2H, H_b), 7.27 (d, J = 9.1 Hz, 2H, H_c), 2.34 (s, 3H, $COCH_3$) ^{13}C NMR (125 mHz, $CDCl_3$) δ 168.4 ($COCH_3$), 155.4(C_d), 145.4 (C_a), 125.2 (C_b), 122.3 (C_c), 21.1 ($COCH_3$) For the esterase assay, the proportions of the reagents used for the chemoenzymatic reactions were kept to scale. 48 µL 0.5 M HEPES pH 7.2 was added to 60 µL H_2O and 12 µL of enzyme mixture or enzyme buffer (20 mM Tris pH 8.3 with 1 M NaCl). After addition of 10 µL 20 mM **3-12** in iso-propanol the assay was monitored at 405 nm for 30 minutes, with measurements taken every 5 minutes. Esterase activity was evaluated by relative rate of release of **3-**13, determined by linear regression using GraphPad Prism.

3.4.9 Partial purification of Cst-II

Cst-II was expressed in *E. coli* strain AD202. An overnight starter culture (5 mL) was used to inoculate 200 mL LB broth with 150 µg/mL ampicillin. The cells were grown at 37 ℃ with shaking at 180 rpm until OD_{600} = 0.4. Protein production was induced with 0.5 mM IPTG and the cells were shaken overnight 180 rpm at 37℃. The cells were harvested by centrifugation at 8000 RCF for 15 min. Cells were resuspended in 10 mL 20 mM Tris pH 8.3 per gram of cells. Dnase I and

protease inhibitor cocktail were added, and the cells were disrupted at 20 000 PSI. The lysate was spun down 20 000 RCF for 30 minutes, then the supernatant was spun at 100 000 RCF for 1 hour. After filtration through a 0.2 μm nylon filter the supernatant was loaded onto HiPrep Q ion exchange resin. The column was washed with 20 mM Tris pH 8.3 and the enzyme was eluted with a gradient of 0 - 0.5 M NaCl in 20 mM Tris pH 8.3. The enzyme eluted at 0.15 M NaCl. (personal communication from Warren Wakarchuk)

3.5 References

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Chapter 4 : Hydrolysis of polysialic acids by human neuraminidase enzymes[a,b]

[a] A version of this chapter has been converted to a manuscript in preparation Hunter, C.D., Cairo, C.W., Hydrolysis of polysialic acids by human neuraminidase enzymes.

[b] NEU1 was produced by Hanh-Thuc Ton Tran, and NEU2-NEU4 were expressed and purified by C.D.H as well as other members of the Cairo Lab. Endo-N was a generous gift from Prof. Lisa Willis. Selective neuraminidase inhibitors in **Section 4.2.3** were synthesized by Dr. Tianlin Guo.

4.1 Introduction

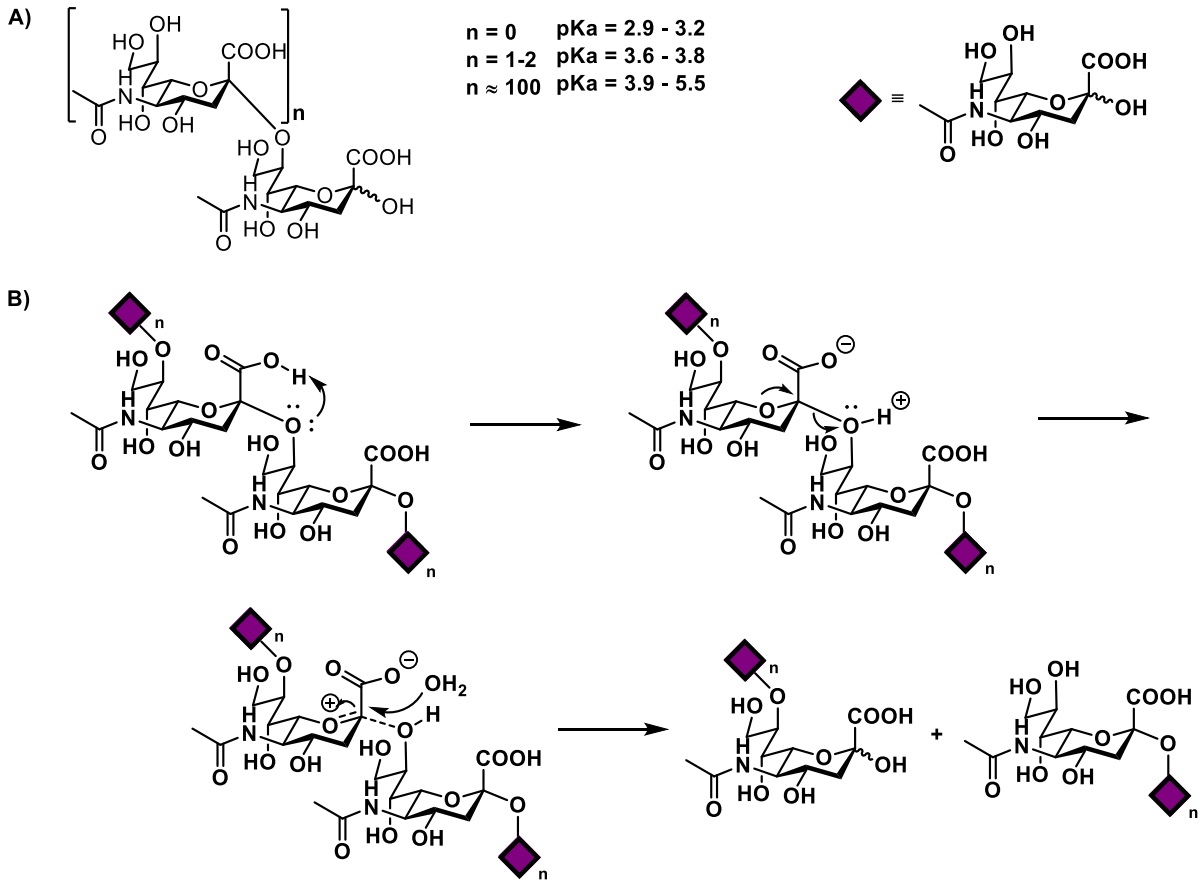
Hydrolysis of the glycosidic bonds of sialic acid by sialidase (neuraminidase) enzymes modulates cell signaling by removing sialic acid epitopes or by exposing epitopes masked by sialic acids. The regulation of sialic acid by neuraminidase enzymes plays important roles in many biological processes including cell-cell interaction, host-pathogen interactions, and tumor malignancy.[1, 2] To date, four human neuraminidase (hNEU) isoenzymes have been identified which differ in subcellular localization and substrate tolerance.[1] Small molecule inhibitors[3] and unnatural sialic acid substrates[4-6] have been valuable tools to further the understanding of hNEU structure and substrate tolerance; however, many natural sialic acid substrates are unstable and difficult to isolate which has impeded their study.[7, 8]

Diversity in sialic acid presentation is generated at three levels: modification to one or more hydroxyl groups or at *C*-5, the attachment of glycosidic bonds (most commonly α(2→3, 6, or 8)), and different reducing end glycans or aglycones.[9] Previous work in our group and others indicated that all three levels of sialic acid diversity influence hNEU catalyzed hydrolysis, both independently and in synergy.[4, 10, 11] These results suggest that in order to understand the interaction of uncommon sialic acids and hNEU it is important to study a broad range of well-defined substrates.

Polymers of α(2→8) linked sialic acids (polysialic acid, polysia) are sialylglycoconjugates with unique chemical properties. In human systems polysia is predominantly found on NCAM, which accounts for approximately 80 % of total polysia,[12] and where chains of degrees of polymerization (DP) up to 90 residues long have been detected.[13] Polysialic acid has also been found on synCAM,[14] neuropilin,[15] E-selectin,[16] CD36 in human milk,[17] the α-subunit of the voltage-sensitive sodium channel[18], and on polysialyltransferases ST8SiaII and ST8SiaIV.[19] The

biological role of polysialic acid has generally been considered to be as a non-specific anti-adhesive or pro-migratory molecule for cells.[20, 21] In recent years specific binders to polysialic acid have emerged, including BDNF and other neurotrophins,[22] and FGF2.[23] Polysialyltransferase activity regulates the activation of CD4+ T-cells[24] and knock-out of the polysialyltransferase ST8SiaIV resulted in T-cell defects in mice.[25] In some cancers dysregulation of polysia promoted malignancy.[26-29] Polysialic acid regulates neuronal development and plasticity,[30] and it has recently been gaining traction as a molecule associated with mental disorders.[31] In various parts of the brain polysialic acid is increased in bipolar disorder,[32] but decreased in depression,[32] schizophrenia,[33] and under acute stress.[34] Understanding the mechanisms of polysialic acid regulation would help to elucidate its specific roles in human health and disease.

The unique chemical properties of polysia can largely be attributed to the *C*-1 carboxylic acid on the sialic acid monomers, making the polymer polyanionic at neutral pH. This polyanionic nature causes polysia to organize large quantities of surrounding water, resulting in a large hydration volume,[35] which is influenced by charge screening from ions in the surrounding solution.[36] The carboxylic acids also influence the stability of the polymer. Like other polymers containing carboxylic acids,[37] the pKa of the carboxylic acids of polysia increase with increasing chain length (**Scheme 4.1A**).[38] An intramolecular self-cleavage mechanism, where a protonated *C*1 carboxylic acid acts as a proton donor to the glycosidic O2, is proposed to explain the susceptibility of polysia to acid-catalyzed hydrolysis (**Scheme 4.1B**).[38] Notably, polysialic acid is known to hydrolyze even under the mild acidic conditions present in cellular compartments such as endosomes and lysosomes.[38] Protonation of the C1 carboxylate also leads to the formation of lactones between the carboxylic acid and C9 of the neighboring reducing end residue.[39, 40] The C1 carboxylate contributes to the formation of polysia tertiary structure.[41]



Scheme 4.1: Polysialic acid hydrolysis under acidic conditions A) the pKa of internal carboxylic acids is higher than that of external carboxylic acids B) proposed intramolecular self-cleavage mechanism.[38]

The chemical properties of polysia make it challenging to study. Polysialic acid studies *in vitro* have relied heavily on reducing end reactivity for the thiobarbituric acid assay,[42, 43] or 1,2-phenylenediamines for analysis of chain length using anion exchange HPLC.[43] Both reducing end chemistries require acidic conditions which would further degrade the polymer, making accurate analysis of polysia difficult.[44, 45] *In cellulo* analysis of polysia has relied almost exclusively on anti-polysia antibodies that recognize epitopes that are not always well defined.[45] A polysia-

specific probe has been developed from an inactive mutant of endosialidase that binds but does not cleave polysia,[46-48] however, it has yet to gain traction as a common tool for polysia analysis.

Literature reports of hNEU cleaving polysialic acid are limited and often contradictory. *In vitro* and *in cellulo* studies have reported that NEU4 is the only isoenzyme to cleave polysialic acid,[43, 49] while another reported NEU1 to be the isoenzyme that regulates polysia.[12] An *in vivo* study in rats detected an increase in NEU1 in brain tissues upon LPS stimulation, which corresponded to a decrease in polysia,[50] and NEU1 knockdown experiments led to an increase in polysia on hippocampal granule cells.[51] Although these results provide strong evidence for NEU1 regulation of polysia, they do not provide direct evidence for NEU1 cleaving polysia. The indirect data, contradictions in the literature, and heterogeneity of polysia substrates highlight the need for a systematic study of polysialic acid degradation by the hNEU enzymes. Such a systematic study should consider the chemical properties of polysialic acid and its susceptibility to non-enzymatic hydrolysis. Our access to purified, recombinant hNEU makes us uniquely poised to carry out such a systematic study *in vitro.*

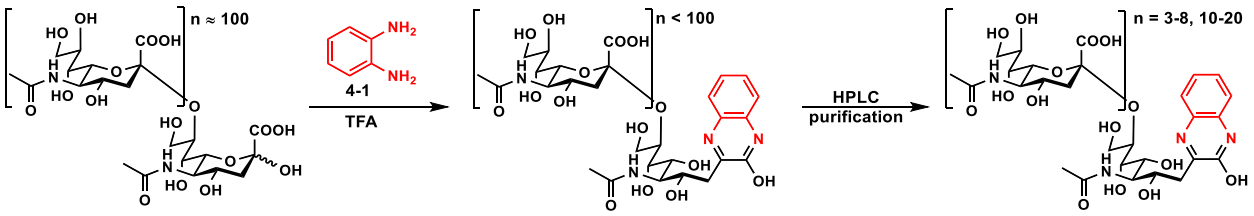
4.2 Results and Discussion

4.2.1 Preparation of substrates for degradation assays.

To systematically study polysialic acid degradation we required defined polysia substrates and a reliable method to detect polysia chain length. Reducing-end labeling of sialic acids with 1,2-phenylenediamines followed by HPLC separation is a well-established method for the analysis of sialic acid monosaccharides[52, 53] and polymers.[54] Sialic acid labeling with 1,2-phenylenediamines requires acidic conditions which in turn promote the intramolecular self-cleavage of polysialic acid.[38] The labeling reaction has been optimized to minimize, but does not

completely eliminate, polysia hydrolysis.[44, 54] Further, under acidic conditions polysia of higher DP hydrolyzed faster than that of lower DP, so background hydrolysis from 1,2-phenyldiamine derivatization may be inconsistent across samples.[38, 44] The drawbacks to determining polysia chain length through labeling with 1,2-phenyldiamines can be circumvented by labeling the polysia substrates prior to being submitted to their test conditions. This early labeling not only eliminated background hydrolysis due to labeling from the polysia degradation assay, but also allowed us to separate and isolate pools of polysialic acid of known DP using preparative HPLC to provide defined substrates.[44]

The most common diamine used for sialic acid labeling is 1,2-diamino-4,5-methylenedioxyenzene dihydrochloride (DMB) because it enables sensitive fluorescent detection. However, DMB is unstable to both light and oxygen while being relatively expensive.[55] A more stable and inexpensive alternative,[56] *o*-phenylenediamine (OPD, **4-1**) (**Scheme 4.2**), provided sufficient sensitivity for our purposes. Starting from commercially available and inexpensive colominic acid (average DP ≈ 100), shorter fragments of labeled polysia were produced by reducing end labeling with OPD over 2 hours at 50 °C to promote hydrolysis to smaller chain lengths (**Scheme 4.2**).[54] After labeling, anion exchange chromatography with preparative HPLC was used to isolate polymers of defined length. For our degradation assays we generated pools of short chain (DP 3-8) and long chain (DP 10-20) sialic acids, which we henceforth refer to as oligo- and poly-sialic acid, respectively.[45] These lengths were selected based on conformational studies that suggested polysialic acid can adopt helical structures where one helical turn is 8-9 sialic acid residues. [41, 57, 58] This model indicates that oligosia (DP < 8) may have a different tertiary structure relative to longer chain polysia (DP 10+), which we reasoned could influence its availability for hNEU-catalyzed hydrolysis.



Scheme 4.2: Preparation of sialic acid polymers for degradation assays

4.2.2 Degradation assays

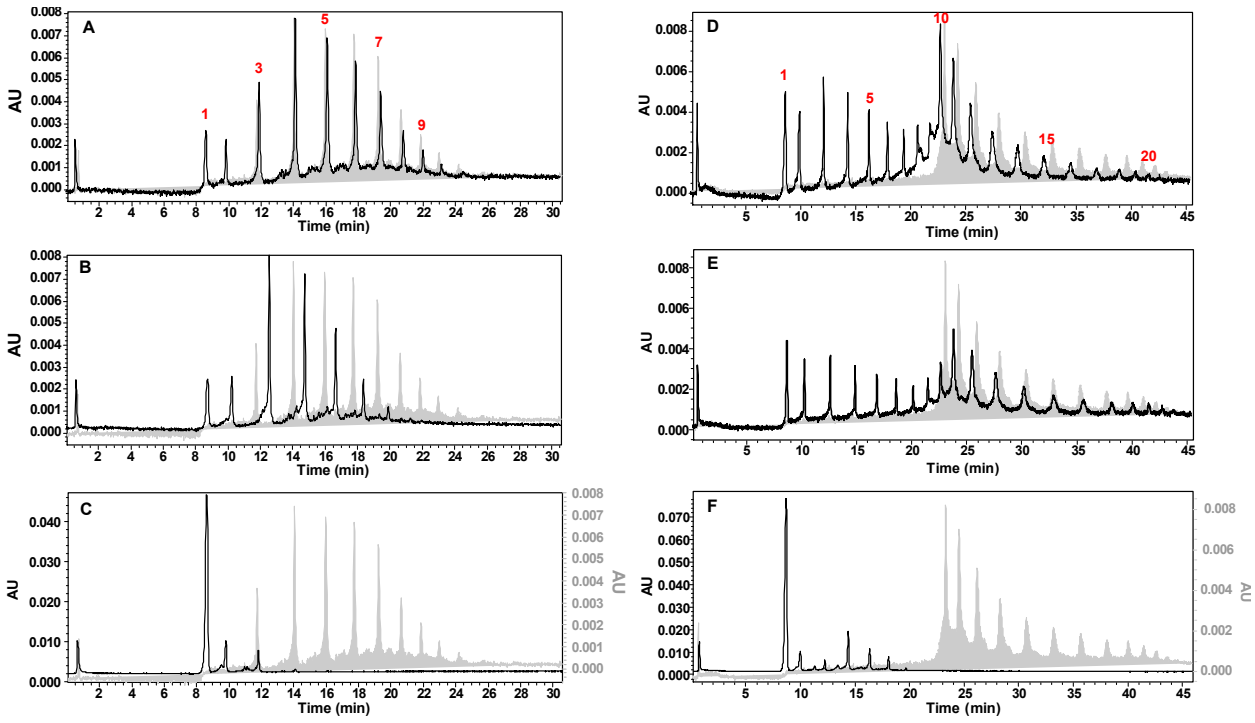


Figure 4.1: Representative runs of oligosia (A-C) and polysia (D-F) degradation assays. Samples were measured after 5 hrs at 37 °C where the grey chromatogram indicates time = 0, and the black chromatogram indicates treatment with A,D) pH 4.5 control B,E) 1 mU NEU3 C,F) 65 µg/mL Endo-N.

To study the degradation of oligo- and polysia, we implemented an endpoint assay where the labeled substrates were incubated with neuraminidase for 5 hrs at 37 °C before quenching. The samples were analyzed by HPLC using anion exchange chromatography (DNAPac PA-100) to

separate polymers of different DP. To generate a succinct data output we calculated a weighted average of the peak areas from the raw data (**Figure 4.1**) to produce an average degree of polymerization. Because polymers of sialic acid undergo intramolecular self-cleavage under mildly acidic conditions, and the pH optimum of hNEU is also acidic (4.5 for NEU1, NEU3, and NEU4 and 5.5 for NEU2); enzyme-free pH controls were implemented in addition to a time = 0 control (**Figure 4.2**). The enzyme-free controls used hNEU storage buffer to ensure consistency in pH and salt concentration (see **Section 4.4**). Pooled oligosia (DP 3-8) and polysia (DP 10-20) were stable at pH 7 over the 5 hour experiment, in agreement with previous results,[59] and only underwent minor hydrolysis at pH 5.5. At pH 4.5 oligosia had a ΔDP of -0.82 ± 0.03 over 5 hours (a decrease in molecular weight of 50.7 g mol^{-1}/hr)(**Figure 4.1A**), and hydrolysis occurred more rapidly for polysia (**Figure 4.1D, Figure 4.2**) (ΔDP -4.2 ± 0.1, -247.6 g mol^{-1}/hr), consistent with previous reports.[38, 44] Taken together, these data agree with the postulated intramolecular self cleavage introduced by Varki and coworkers where a protonated carboxylic acid with unusually high pKa (3.91-5.53 for DP ≈ 100) provides an intramolecular proton transfer to catalyze the hydrolysis reaction (**Scheme 4.1**).[38]

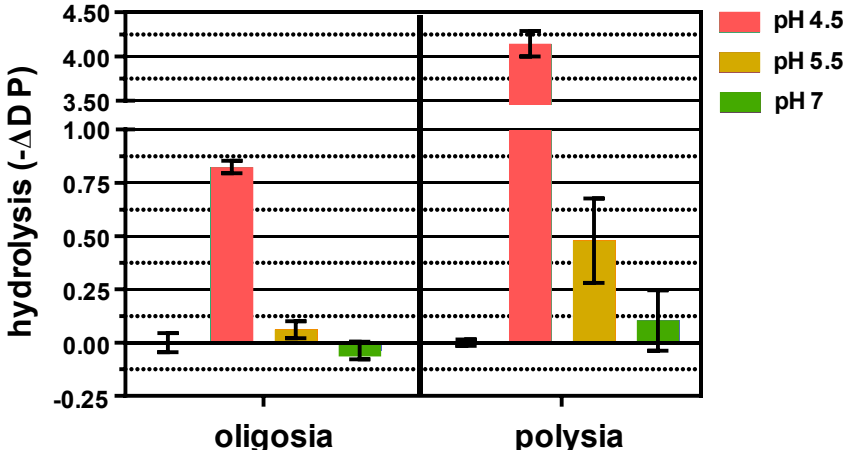


Figure 4.2: Enzyme-free hydrolysis of oligosia and polysia over 5 hours at pH 4.5, 5.5 and 7. Hydrolysis is presented as -ΔDP (over 5 hrs), the change in the average degree of polymerization of the sample, with data normalized to time = 0. Results are presented as means of triplicate experiments with error bars denoting standard deviation.

Bearing in mind that pH has a strong influence on enzyme-independent hydrolysis of oligosia and polysia, we tested oligosia degradation by neuraminidases both at the enzyme optimum pH (4.5 for NEU1, NEU3, NEU4 and 5.5 for NEU2) as well as at pH 7. Endoneuraminidase-N (Endo-N, from *E. coli* K1 bacteriophage) was used as a positive control and has endoneuraminidase activity which could not be normalized using our exosialidase enzyme activity assay based on 4MU-NANA. Endo-N treatment (65 µg/mL)[60] of oligosia converted most of the sample to monosaccharide (**Figure 4.1C**) at both pH 4.5 and pH 7 (**Figure 4.3A**), confirming that the reducing-end labeling conditions did not disrupt the structure of oligosia. We note that a previous study found that Endo-N has low activity at pH 7;[60] however, since our assay was not optimized to study Endo-N specificity we may not expect to observe Endo-N preferences under these conditions. We were surprised to observe that neuraminidase from *A. ureafaciens,* reported

to be a universal neuraminidase for α(2→3,6,8,9) linked sialic acids,[42, 61] did not hydrolyze oligosia compared to the pH controls at both pH 4.5 and pH 7 (**Figure 4.3B**).

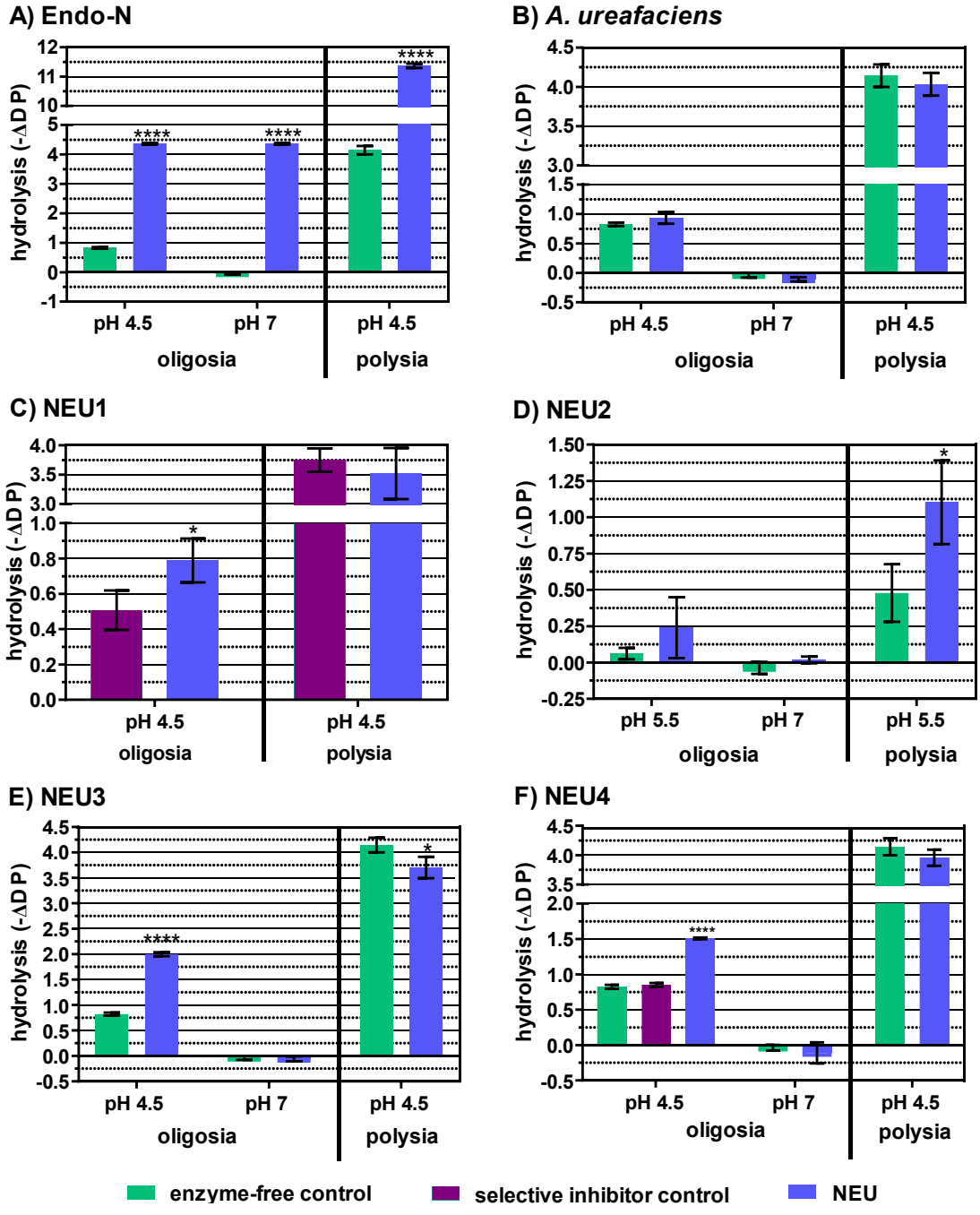


Figure 4.3: Neuraminidase-catalyzed degradation assays. Enzyme activity was normalized to 1 mU enzyme activity apart from endo N, for which enzyme activity could not be normalized and

so was used at 65 μg/mL, and NEU1 which was normalized to 0.5 mU. For NEU3 at pH 7, 0.75 mU of enzyme activity was used due to its low activity away from the enzyme optimum pH. Results are presented as means of triplicate experiments with error bars denoting standard deviation. Unpaired t-tests were performed comparing the enzymatic conditions to their respective pH controls, where * = $p < 0.05$ and **** = $p<0.0001$.

The hNEU isoenzymes exhibited different activity towards oligosia depending on pH. The enzyme activity for all neuraminidase enzymes was normalized to 0.5-1 mU at the pH used for the degradation assay. At the enzyme optimum pH, NEU3 hydrolyzed oligosia fastest with a ΔDP of -2.00 ± 0.04 compared to -0.82 ± 0.03 for the pH 4.5 control (**Figure 4.3E**). The NEU4 isoenzyme also had significant activity on oligosia (ΔDP of -1.53 ± 0.01, **Figure 4.3F**). At its optimum pH (5.5) NEU2 did not show detectable hydrolysis of oligosia relative to the pH control (**Figure 4.3D**), nor did it have activity at a more acidic pH (4.5, data not shown). These results agree with reports that NEU2 has a strong preference for α(2→3) over α(2→6) and α(2→8) linked sialic acids and that it does not to cleave colominic acid.[62] Previous studies in our group and others suggest that the NEU2 active site pocket that accommodates the glycerol chain is very constrained, limiting its substrate tolerance.[10, 63] We observed moderate, but detectable, enzyme-catalyzed hydrolysis by NEU1 at pH 4.5 (**Figure 4.3C**). Our NEU1 enzyme preparation was not active at pH 7, preventing measurement under this condition. In general, hNEU isoenzymes showed activity at acidic enzyme optimum pH (except for NEU2); however, none of the hNEU cleaved oligosia at pH 7. This observation suggests that oligosia is not degraded by hNEU at the cell surface *in vivo*.

We proceeded to test hNEU activity on polysia (DP 10-20) at the enzyme optimum pH (**Figure 4.3**). Our results showed that with the exception the Endo-N positive control, none of the neuraminidases tested had appreciable activity on polysia. NEU2 treatment showed a small but

significantly different ΔDP compared to the pH 5.5 control ($p < 0.05$), suggesting that it may be cleaving polysia. We note that NEU3 treatment had a similarly significant ($p < 0.05$) ΔDP compared to its control which suggested that the DP was increasing slightly with treatment. Taken together, we ascribe both of these observations to noise in the assay rather than authentic differences in enzyme activity. We conclude that all four hNEU isoenzymes have minimal or no activity towards polysia. We note that previous reports found that NEU2 did not cleave colominic acid.[62]

4.2.3 Inhibition of oligosia degradation by selective hNEU inhibitors

Our group and others have developed isoenzyme specific inhibitors of hNEU.[64-68] We have previously demonstrated that our NEU3[64] and NEU4[65] inhibitors can block glycolipid processing *in vitro*. To confirm that oligosia degradation under hNEU treatment conditions was a result of neuraminidase activity, we used a NEU4 selective inhibitor (**Figure 4.4, 4-2**) to block hydrolysis of oligosia by NEU4.[65] Addition of the inhibitor to the assay mixture at 1.6 μM (10x the IC_{50} concentration measured on a fluorescent substrate)[65] gave a ΔDP identical to the pH control (**Figure 4.3F**), indicating that NEU4-catalyzed hydrolysis of oligosia was completely blocked by the NEU4-selective inhibitor. This result confirmed that the additional hydrolysis compared to the pH controls under hNEU treatment conditions could be attributed to hNEU activity.

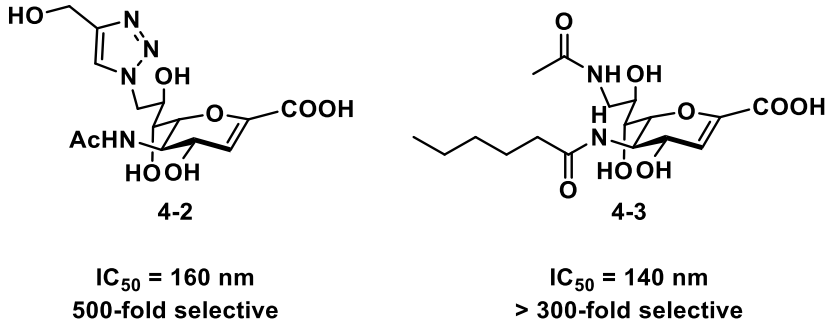


Figure 4.4: Structures of selective hNEU inhibitors. NEU4 inhibitor **4-2** was is 500-fold selective for NEU4 and NEU1 inhibitor **4-3** is 300-fold selective for NEU1.

A selective hNEU inhibitor was also used to confirm NEU1 activity. While NEU2-NEU4 were expressed in *E. coli* as recombinant MBP fusion proteins, NEU1 was overexpressed in HEK293 cells from a crude cell lysate.[10] We previously confirmed that all neuraminidase activity present in the sample was from NEU1 [10] (see **Chapter 2**) but considering the sensitivity of oligosia/polysia to small changes in pH and salt concentration (*vide infra*) we could not use the enzyme-free conditions as controls for NEU1. Instead, we used selective NEU1 inhibitor **4-3**[66] at 10x the IC_{50} concentration (**Figure 4.3C**) which confirmed that the differences observed could be attributed to NEU1 enzyme activity on oligosia. As with the other isoenzymes, we did not observe NEU1 activity towards polysia compared to the control.

4.2.4 Influence of salt concentration on oligosia degradation.

Our systematic investigation of hNEU activity on oligosia/polysia provides evidence that hNEU may only cleave short polymers of sialic acid and only at the acidic enzyme optimum pH. We hypothesize that these results could be explained through two factors 1) the relative ionic strength of polysia to the surrounding solution influencing association kinetics of the substrate to hNEU, or 2) different conformations of the polymer. These two factors would be difficult to isolate

experimentally because conformational studies on polysia suggest that the carboxyl group is integral to its conformation.[41, 57] The relative ionic strength of the solution to the polymer can be changed either by altering the charge on the polymer, as we did by changing the pH of the solution and the polymer length, or by varying the salt concentration of the solution. To test whether the relative ionic strength of the polymer influenced its accessibility to hNEU, we monitored oligosia hydrolysis at three different salt concentrations: 260 mM (used in our assays, 150 mM NaOAc/HEPES from assay buffer, 100 mM NaCl, 10 mM MOPs from hNEU buffer), 160 mM (150 mM NaOAc HEPES, 10 mM MOPS), and 80 mM (75 mM NaOAc HEPES, 5 mM MOPS).

Similar to changing the charge on polysialic acid, changing the salt concentration of the buffer also had an effect on oligosia hydrolysis (**Figure 4.5**). Increasing the salt concentration had a small protective effect against hNEU-independent hydrolysis, which was consistent with an observation reported by Manzi *et al*.[38] Analogous to our results from altering the charge on polysia, in our system varying the salt concentration of the buffer did not change the ΔDP after Endo-N treatment. Conversely, higher salt concentration correlated with an increase in NEU4-catalyzed hydrolysis of oligosia. These data make sense when considering the polyanionic nature of oligosia/polysia. A previous study demonstrated that decreasing salt concentrations increased the anti-adhesive properties of polysialic acid by reducing charge screening and changing the hydration volume of polysialic acid.[36] In this context, our experiments suggest that the relative ionic strength of oligosia/polysia may influence the association kinetics of the polymer to the enzyme. Although our experiments with varying salt concentrations support ionic strength of the polymer as a barrier to hNEU catalyzed hydrolysis of polysia, they do not rule out conformational differences between oligosia and polysia which may act as a barrier for hNEU catalysis. Considering the influence of the C1 carboxyl group on polysia conformation,[41] we propose that

the results of this study are likely best explained by changes in both polysia charge and conformation.

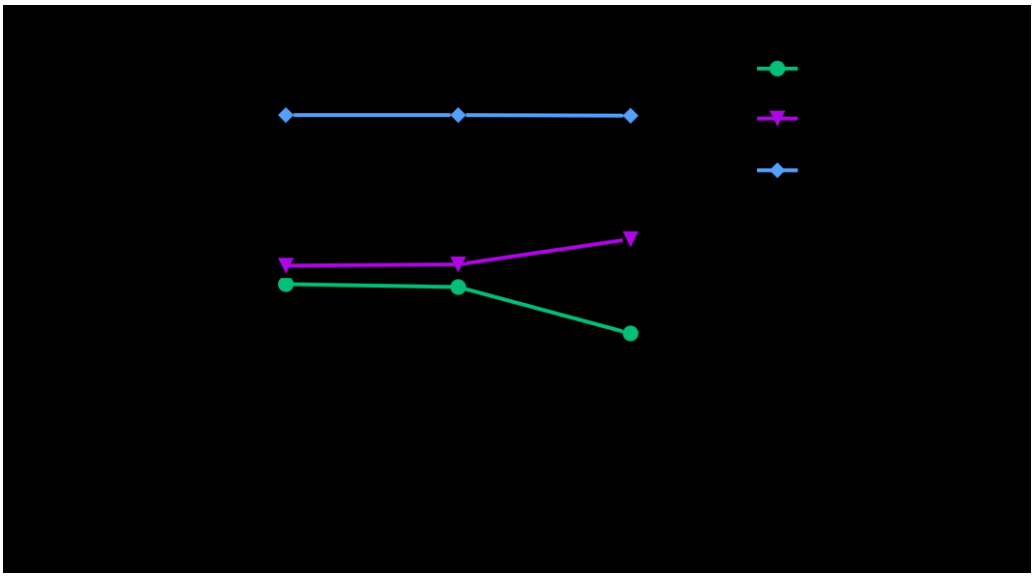


Figure 4.5: Effect of buffer salt concentrations on oligosia hydrolysis. Each point represents a mean of triplicate experiments with error bars denoting one standard deviation. Enzyme activity was normalized to 1 mU with the exception of Endo-N, which was used at 65 μg/mL. Unpaired t-tests were performed comparing the enzymatic conditions to the pH control at each buffer concentration, where * = $p < 0.05$ ** = $p < 0.01$, **** = $p < 0.0001$.

4.2.5 The ongoing challenge of studying polysia.

The sensitivity of our degradation assays to different salt concentrations highlights the challenge of studying polysialic acid in a controlled and reliable manner. The unique chemistry of polysia makes it a challenging target. Factors which complicate the study of polysialic acid include the DP, sample consistency, pH, salt concentration, and methods of detection. This challenge becomes apparent when reviewing the literature for examples of neuraminidase activity towards polysialic acid.

Neuraminidase from *Arthrobacter ureafaciens* is reported to be an α(2→3,6,8,9) neuraminidase[42, 61] and has been used to degrade polysia to smaller DP for both preparatory purposes and in the analysis of polysialic acid degradation. In fact, colominic acid has been used as a substrate to determine enzymatic activity of *A. ureafaciens* neuraminidase; however, the released sialic acid was detected using the thiobarbituric acid assay which requires the sample to be subjected to both strong acid and boiling.[42, 69] Many reports using *A. ureafaciens* neuraminidase for preparatory purposes employed large amounts of enzyme for small amounts of polysia at acidic pH, which would promote hydrolysis of polysia independent of enzyme activity.[13, 39, 70] In analytical studies of polysia degradation, *A. ureafaciens* neuraminidase released approximately 10 % of available monosaccharides from oligosia over 80 minutes, although the enzyme activity used was not reported.[71] High concentrations of *A. ureafaciens* neuraminidase (10-fold higher enzyme activity than in this study) was reported to hydrolyze polysia qualitatively faster than a pH control.[72] In this context, our observation that *A. ureafaciens* neuraminidase did not cleave oligosia/polysia indicates that activity of the enzyme towards polysialic acid could be much lower than is generally considered; and may be attributed to acid-catalyzed hydrolysis.

Apart from NEU3, all hNEU have been reported to hydrolyze oligosia at their enzyme optimum pH *in vitro*. In one study, murine NEU2 and NEU4 exhibited similar activity towards oligosia while NEU1 was approximately twice as active on the substrate. Monosaccharide release was detected after DMB derivatization[71] Conversely, a study of murine sialidases determined that only NEU4 cleaved oligosia where the initial DP was either 2 or 6. Monosaccharide release was measured using the thiobarbituric acid assay[43] The study also used anti-polysia antibodies to show that NEU4b could cleave polysialic acid *in vitro* and *in cellulo*. The antibodies used (12E3 and 12F8) recognize epitopes of DP > 5 and an undefined epitope, respectively.[45] In contrast to our

observation that no hNEU were active on oligosia at pH 7, they detected murine NEU4b-catalyzed removal of polysia from NCAM *in vitro* at near-neutral pH.[43] The results of the study were in disagreement with a previous study by the same group that used anti-polysialylated NCAM antibodies to determine that murine NEU4a was the active isoenzyme on polysialylated NCAM.[49] Data in the literature further deviate from consensus when considering more recent studies that point to NEU1, not NEU4, as the isoenzyme involved in polysia regulation in mouse microglial cells after an inflammatory stimulus. Polysialic acid was detected with the 12E3 anti-polysia antibody, and polysia disappearance could be blocked by a general neuraminidase inhibitor.[12] An *in vivo* study using anti-polysia mAb 735 detected a decrease in polysia upon LPS stimulation that corresponded to an increase in NEU1.[50]

The data presented in this study suggest that polysialic acid regulation and turnover may be largely independent of neuraminidase-catalyzed hydrolysis of polysialic acid, although follow-up studies will be necessary to confirm this result. This idea is not baseless. *In vitro* studies showing enzyme-free polysialic acid hydrolysis under mild acidic conditions prompted speculation that enzyme-free hydrolysis of polysia may occur in the acidic environments of endosomes and lysosomes.[38, 73, 74] Evidence for this mechanism of polysia turnover is emerging. Desialylation of polysialylated NCAM was found to be dependent on endocytosis.[75] More recently, an antibody specific for polysialic acid (DP >3) was internalized by cells – likely through antibody-induced receptor internalization - and co-localized with endosomal and lysosomal markers.[76] Further investigation of polysia trafficking and degradation, as well as follow-up studies of hNEU activity on polysia will be essential to elucidate the mechanisms of polysia regulation.

4.3 Conclusion

The lack of agreement throughout the literature on polysialic acid degradation emphasizes the unique chemistry of this substrate and the challenges impeding its study. Herein, we have reported the first systematic study of polysialic acid degradation with all four hNEU enzymes. Our assay design has enabled us to directly compare several factors that influence polysia degradation kinetics, including degree of polymerization, pH, and relative ionic strength of the surrounding media. Our results suggest that hNEU can only hydrolyze polymers of sialic acid with short degree of polymerization at acidic pH, and we provide evidence to suggest that the relative ionic strength of polysia influences its susceptibility to hNEU-catalyzed hydrolysis. We hypothesize that hNEU activity towards polysia depends both on the relative ionic strength to the solution and the conformation of the substrate because these factors are not likely independent of one another. Further study of polysia conformation would be required to confirm this hypothesis. Our findings provide a base that can be used as a foundation for future work into the interesting chemistry and biology of polysialic acid.

4.4 Materials and Methods

4.4.1 General methods

All reagents were purchased from commercial sources and used without further purification unless otherwise noted. HPLC was performed with a Waters Delta 600 pump, and a Waters 600 controller with Empower 2 software. Eluted peaks were detected with a Waters 2996 photodiode array (PDA) detector (Waters Ltd.). Neuraminidase from *A. ureafaciens* was purchased from Millipore Sigma. Endo-N was a generous gift from Dr. Lisa Willis. Human neuraminidase enzymes NEU2-NEU4 were expressed as fusion proteins with maltose binding protein and were purified as described.[4, 77, 78] Human neuraminidase enzyme NEU1 was produced from HEK293E cells and was used as a

crude cell lysate.[10] Specific activity of the exosialidase enzymes was determined in comparison to a standard curve of neuraminidase from *Clostridium perfringens* against 4-methylumbelliferyl α-D-*N*-acetylneuraminic acid (4MU-NANA). Neuraminidase activity was normalized to 1 mU, with exceptions. Our NEU1 expression systems has lower activity[10] than the other isoenzymes so 0.5 mU NEU1 was used at pH 4.5. At pH 7, NEU1 had no activity and NEU3 had lower activity, so was used at 0.75 mU. Endo-N could not be normalized using the exosialidase assay so was used at 65 μg/mL. Unless otherwise stated, results reported are technical replicates, with preliminary independent replicates confirming the trends reported.

4.4.2 Oligo- and polysialic acid sample preparation

Colominic acid (average MW 30 000 kDa, 45 mg) was dissolved in 4.5 mL of 0.25 M β-mercaptoethanol, 9 mM $Na_2S_2O_4$, and 20 mM trifluoroacetic acid. 8 mg (0.07 mmol) of 1,2-phenylenediamine was added and the mixture was heated at 50 °C for 2 hrs. 10 μL of 28 % NH_4OH was added and the mixture was incubated at 37 °C for 2 hrs.[52, 54]

Labelled polysialic acid was separated on a DNA Pac PA-100 anion exchange column (9 x 250 mm) with a gradient of 2 M NH_4OAc (pH 8) in water 3 mL/min: 0%, 0%, 20%, 25%, 32.5%, 100%, 100%, at 0, 5, 20, 35, 45, 48, 55 minutes, respectively.[43, 44] Peaks were detected at 350 nm. Fractions were collected and pooled to obtain oligosialic acid (DP 3-8) and polysialic acid (DP 10-20). For oligosialic acid, solvent was removed under vacuum and remaining salt was removed by repeated washes through a centrifugal filter unit (3 kDa MWCO) before lyophilization. For polysialic acid solvent was concentrated under vacuum and salt was removed by dialysis (3 kDa MWCO) followed by repeated washes through a centrifugal filter unit (3kDa MWCO), before lyophilization.

4.4.3 Oligo- and polysialic acid hydrolysis assays

To aliquots of oligo- or polysialic acid (0.1 mg of oligo- or 0.8 mg of poly-) 20 μL of assay buffer was added. Unless otherwise stated, the assay buffer was 0.3 M sodium acetate HEPES at pH 4.5, 5.5, or 7. For controls, 20 μL of neuraminidase buffer (0.2 M NaCl, 20 mM MOPS, 10 mM maltose and 10% glycerol, pH 7.2) was added and for enzymatic assays 20 μL of 1 mU of enzyme in neuraminidase buffer was added (for NEU1, 0.5 mU of enzyme in 1X RIPA buffer was used). Mixtures were incubated at 37 °C for 5 hrs. 10 μL of ethanol and 30 μL of 0.2 M NH_4OAc pH 8 with 1 mM benzoic acid were added. The mixture was washed through a centrifugal filter unit (30 kDa MWCO) at 14 000 rpm for 30 min and the filtrate was analyzed after separation on a DNA Pac PA-100 anion exchange column (4 x 150 mm), with monitoring at 350 nm. The gradient was 2 M NH_4OAc (pH 8) in water 2 mL/min: : 0%, 0%, 20%, 25%, 100%, 100%, at 0, 5, 20, 35, 38, 43 minutes, respectively (oligosia) or : 0%, 0%, 20%, 25%, 32.5%, 100%, 100%, at 0, 5, 20, 35, 45, 48, 55 minutes, respectively (polysia).

For inhibitor assays NEU1 or NEU4 was incubated with their respective inhibitors for 10 minutes at room temperature prior to addition to the oligosialic acid. Final concentrations of each inhibitor was 10x their Ic_{50} values. For the NEU1 inhibitor (C5-hexanamido-C9-acetamido-DANA) the concentration was 1.4 μM,[66] and for the NEU4 inhibitor (9-[4-hydroxymethyl-[1,2,3]triazol-1-yl]-2,3-didehydro-*N*-acetylneuraminic acid, C9-4HMT-DANA) the concentration was 1.6 μM.[79]

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Chapter 5 : Conclusions and future outlooks

5.1 General Conclusions

Sialic acids have exceptionally heterogeneous presentations in biological systems; however, many methods used to study sialic acids do not account for that heterogeneity (as discussed in Chapter 1). As a result, many roles of sialic acids remain unclear. The metabolism of sialic acids by human neuraminidase enzymes is influenced by the heterogeneity of sialic acid presentation, but the substrate tolerance of these enzymes towards various unstable sialic acids is poorly understood. This thesis has investigated the role of sialic acid modifications in hNEU metabolism of sialosides using model and glycoprotein substrates. In Chapters 2 and 3, we investigated hNEU tolerance of 9-*O*-acetylated sialic acids (Neu5,9Ac$_2$). We observed a general preference for Neu5Ac over Neu5,9Ac$_2$, and found that the degree of this preference was dependent on sialic acid glycosidic linkage and aglycone,[1] indicating that an understanding of the context of sialic acid presentation is important to understanding its metabolism by hNEU. In Chapter 4 we implemented the first systematic study of polysialic acid metabolism by hNEU. We found that shorter polymers with degrees of polymerization (DP) of 3-8 were hydrolyzed by hNEU; however, we did not observe hNEU-catalyzed hydrolysis of longer polymers (DP 10-20). We provide evidence for relative ionic strenght of polysialic acid as a factor changing its availability to hNEU. Overall, we note the importance of considering the larger context of sialic acid presentation when studying its metabolism by hNEU - whether it be the glycosidic linkage and reducing end substituents influencing hNEU tolerance of Neu5,9Ac$_2$, or DP influencing hNEU-catalyzed hydrolysis of polysia.

5.2 Methods to study sialic acid metabolism by hNEU

Our studies of sialic acid metabolism by hNEU required adaptation and expansion of the known methods to monitor sialic acid metabolism to account for increasingly complex sialoside

substrates. Our initial studies in hNEU specificity for Neu5,9Ac_2 were with the simple fluorogenic substrate 4MU-NANA (4-methyllumbelliferyl-*N*-acetylneuraminic acid)[1] which does not have a glycosidic bond to a reducing end sugar and contains an aromatic aglycone (see **Section 1.5.2**). We expanded the substrate scope to octyl sialyllactoside mimics (with α(2→3) or α(2→6) linked sialosides) of the monosialo-ganglioside GM3.[1] hNEU specificity studies with these octyl sialyllactoside substrates required adaptation of sialic acid detection using malononitrile for enzyme kinetics[2] (see **Section 1.4.1**).

More complex substrates such as glycoprotein and disialo substrates were not amenable to hNEU assays using malononitrile. To study hNEU specificity towards a Neu5,9Ac_2-enriched glycoprotein we needed a method that could distinguish between modified sialic acids, so we used reducing end derivatization with a 1,2-phenylenediamine (see **Section 1.4.1**). This strategy has been used to study bacterial neuraminidase specificity[3, 4] but was optimized to study the hNEU which have significantly lower activity. To study hNEU specificity on disialo substrates with α(2→8)-linked sialic acids we could not use a method to detect free sialic acids because they do not discriminate between glycosidic linkages. Instead we designed substrates with a chromophore in the aglycone that could be resolved using HPLC. This approach should be useful for studying hNEU kinetics on any glycolipid substrate with multiple sialic acids.

The problem of accounting for multiple sialic acids is also apparent in the study of polysialic acid (polysia) metabolism. Reducing end labeling with 1,2-phenylenediamines followed by analysis using anion exchange HPLC is a popular method for DP analysis of polysia,[5] but the conditions required for derivatization cause acid-catalyzed hydrolysis of the polymer.[6] By labeling polysia prior to enzymatic conditions, we could directly monitor enzymatic degradation of the polymer. The informed selection of methods to study sialic acid metabolism that accounts for the

chemical properties and diversity of sialic acids is essential to provide interpretable data in these systems.

5.3 Metabolism of modified sialic acids by hNEU on complex biological samples.

The workflow we developed to study hNEU activity on bovine submaxillary mucin is amenable to studying hNEU activity on more complex biological samples. Not only could the method be used to study other sialoglycoproteins, but potentially cell and tissue samples – provided potential contamination by pyruvate was accounted for. Reported methods for glycoprotein and ganglioside extraction from tissue samples do not require harsh acidic or basic conditions[7] and should be compatible with particularly unstable sialosides such as *O*-acetylated sialic acids.

Mouse erythrocytes almost exclusively have *O*-acetylated sialic acids.[8, 9] Considering the importance of sialic acid spatial distribution on their function,[10] mouse erythrocytes would be a good model for future studies of hNEU activity towards Neu5,9Ac_2 on complex biological samples and could provide insight into their physiological roles

5.4 hNEU metabolism of aryl glycolipids

In the work presented herein we were not able to complete the synthesis of Neu5,9Ac_2-GD3 aryl glycolipids **3-8** and **3-10**. Future studies should look towards a sialic acid 9-*O*-acetyltransferase (SOAT) enzyme to acetylate Neu5Ac-GD3 substrate **3-7**.[11] This approach has the potential to have higher yields than our original approach because the yield of the one-pot multienzyme sialylations are low when run at neutral pH to preserve the ester of Neu5,9Ac_2.

The HPLC assay for aryl glycolipids could be applied to other disialo glycolipid substrates. The hNEU are reported to have a particular intolerance for α(2→8)-Neu5Gc.[12] The enzyme activity of hNEU towards Neu5Gc-GD3 analogues could be studied by this method. Kinetic data

for hNEU metabolism of ganglioside substrates is limited and has relied on TLC methods.[13, 14] The kinetics of any glycolipid – particularly those with more than one sialic acid – can be monitored effectively using this approach.

An alternate approach to studying hNEU substrate tolerance on complex glycolipid glycans with HPLC is to use mass spectrometry, which is generally faster and more sensitive. In Chapter 2, we detailed our collaboration with the Klassen group where they used ESI-MS to monitor hNEU activity on our octyl sialyllactoside substrates. The relative rates observed with ESI-MS agreed with those obtained by solution-phase methods, however, the method required internal standards to normalize the response factor.[1] Recently, an ESI-MS approach to studying protein-glycan binding interactions without internal standards was developed.[15] The competitive universal proxy receptor assay (CUPRA) would not be amenable to studying glycolipid analogs, but could be used to study hNEU kinetics on the carbohydrate moiety of glycolipids.[15]

5.5 Polysialic acid

Our systematic study of hNEU activity towards polysialic acid identified new questions surrounding polysia metabolism. The data presented herein should be followed up by studies with better mimics of natural polysia substrates. A follow-up with hNEU activity on polysialylated glycoproteins would help to confirm the results of this study. The controlled polysialylation of glycoproteins has been reported[16] and could be used to generate short and long polysialic acid on a glycoprotein. *In cellulo* data would be necessary to confirm the results obtained in *in vitro* studies. Polysialic acid immobilized on beads could be an effective probe of extracellular neuraminidase activity.

Changes in polysialic acid hydration and conformation may both be contributing factors to the resistance of polysia against hNEU-catalyzed hydrolysis. We found evidence that suggests that

the relative ionic strength of the polymer may influence the association of polysia to the hNEU. Experiments with divalent cations such as calcium and magnesium at their physiologically relevant concentrations would also help to illuminate the influence of salts on polysia regulation. Relative ionic strength and conformation are not likely to be independent factors. Conformational studies using NMR[17, 18] would help to elucidate the role polysia conformation may have in blocking hNEU-catalyzed hydrolysis of polysia.

There are still significant barriers to accurate DP analysis of polysia – both in polysia release from glycoconjugates and in polysia labeling. Current labelling chemistries require acidic conditions that result in hydrolysis of the polymer.[5, 6] Release of the polysia from a glycoprotein is typically accomplished using acidic conditions that not only hydrolyze the glycosidic bond between galactose and sialic acid, but also the bonds between sialic acid monomers in polysia.[19] An alternative to polysia release from the glycan using acidic conditions is to use an endo-ß-galactosidase to cleave the bond between the galactose and *N*-acetylglucosamine.[6] This method releases polysia from glycoconjugates without hydrolysis of the polymer but the substrate specificity of the endo-ß-galactosidase is limited.[20] A mild labeling chemistry that does not result in polysia hydrolysis would also be an invaluable tool for polysia analysis.

5.6 Outlook

Many sialic acid presentations are unstable and difficult to isolate or synthesize, which has resulted in a shortage of molecular data to describe their regulation. In this thesis I have developed methods to study the substrate tolerance of the human neuraminidase isoenzymes towards some of these unstable sialic acids: namely, 9-*O*-acetylated sialic acids and polysialic acids. This work should inform future study into the roles of these sialosides and hNEU in human health and disease. Discrimination of 9-*O*-acetylated sialosides by hNEU may provide a biochemical link between

sialic acid *O*-acetyltransferase and *O*-acetylesterase activities and hNEU regulation of sialosides. Our observation that hNEU did not hydrolyze polysia with DP 10+ suggests the potential for an alternate mechanisms of polysialic acid turnover. Finally, the work presented in this thesis highlights the intricacies of sialic acid presentation and emphasizes the importance of diverse chemical biology methods to study the roles of sialic acids and their presentation in biological systems.

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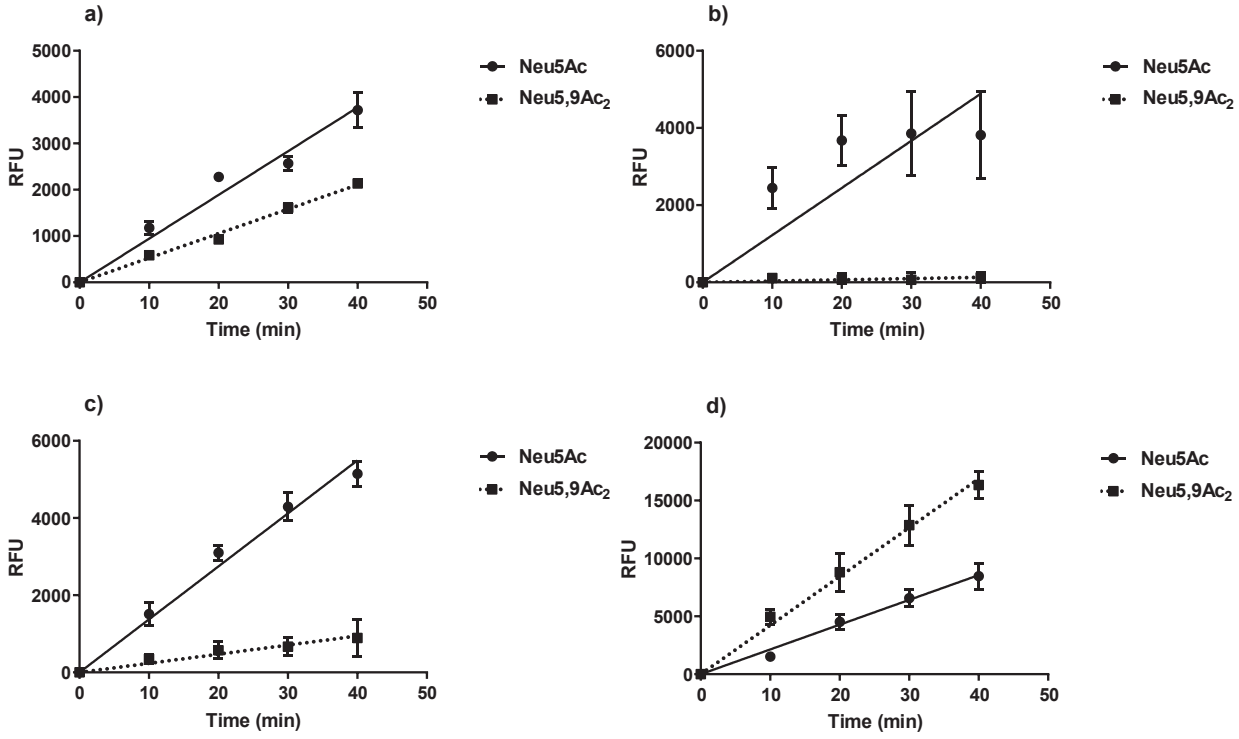
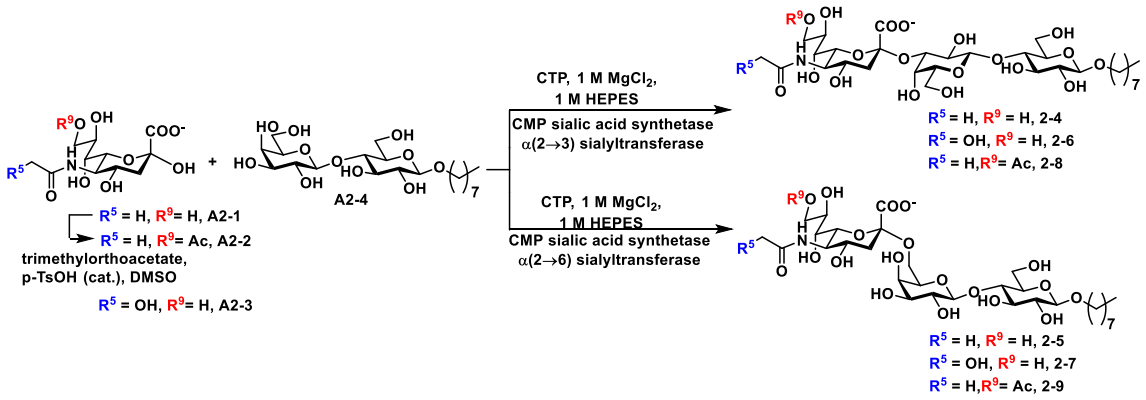
A.2 Supporting Information for Chapter 2

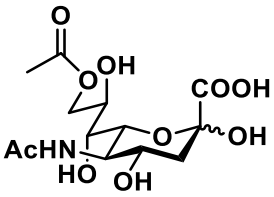
A2.1: Kinetics of hNEU cleaving 4MU-Neu5Ac **2-1** (solid line) and 4MU-Neu5,9Ac$_2$ **2-2** (dashed line) for a) NEU1, b) NEU2, c) NEU3, d) NEU4. Slopes were fit by linear regression forced through zero with triplicate experiments. Error bars denote standard error.



A2.2: One-pot-two-enzyme synthesis of octyl GM3 derivatives.

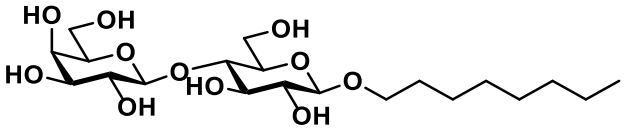


Experimental procedures for the synthesis of A2-2 and A2-4.



5-Acetamido-9-*O*-acetyl-3,5-dideoxy-D-glycero-β-D-galacto-2-nonulopyranosonic acid (A2-2):

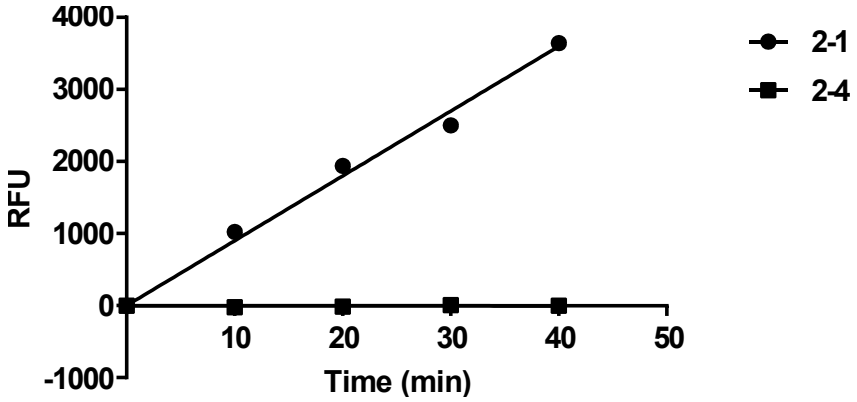
Starting from *N*-acetylneuraminic acid, **(SI6)** was prepared in one step, as previously reported.[1] ^{1}H and ^{13}C NMR data were consistent with previous reports.[1, 2]



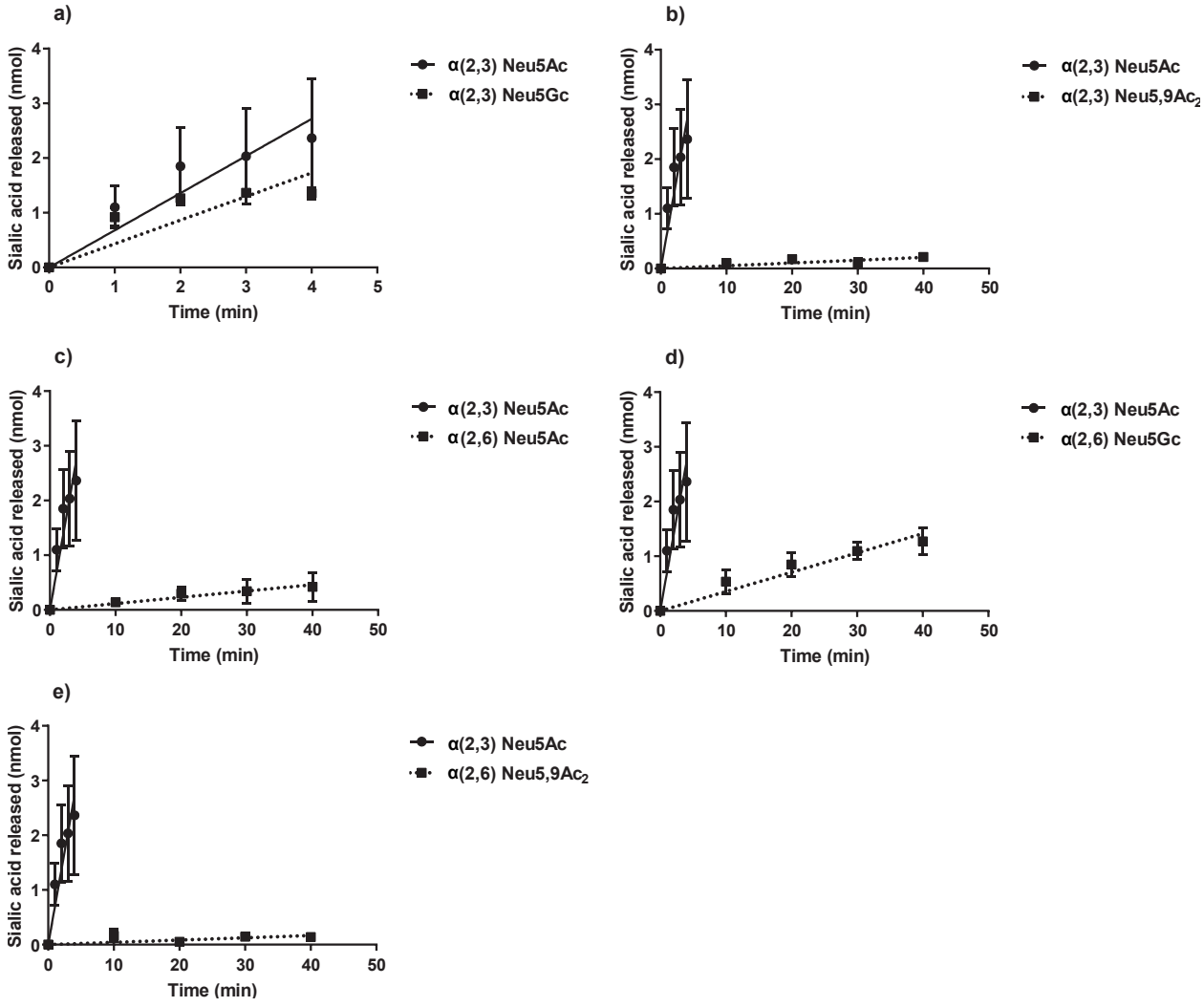
Octyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (A2-4):

Starting from β-lactose, octyl β-D-galactopyranosyl-(1→4)-β-D-glycopyranoside was prepared as previously reported.[3] ^{1}H and ^{13}C NMR data were consistent with previous reports.[3]

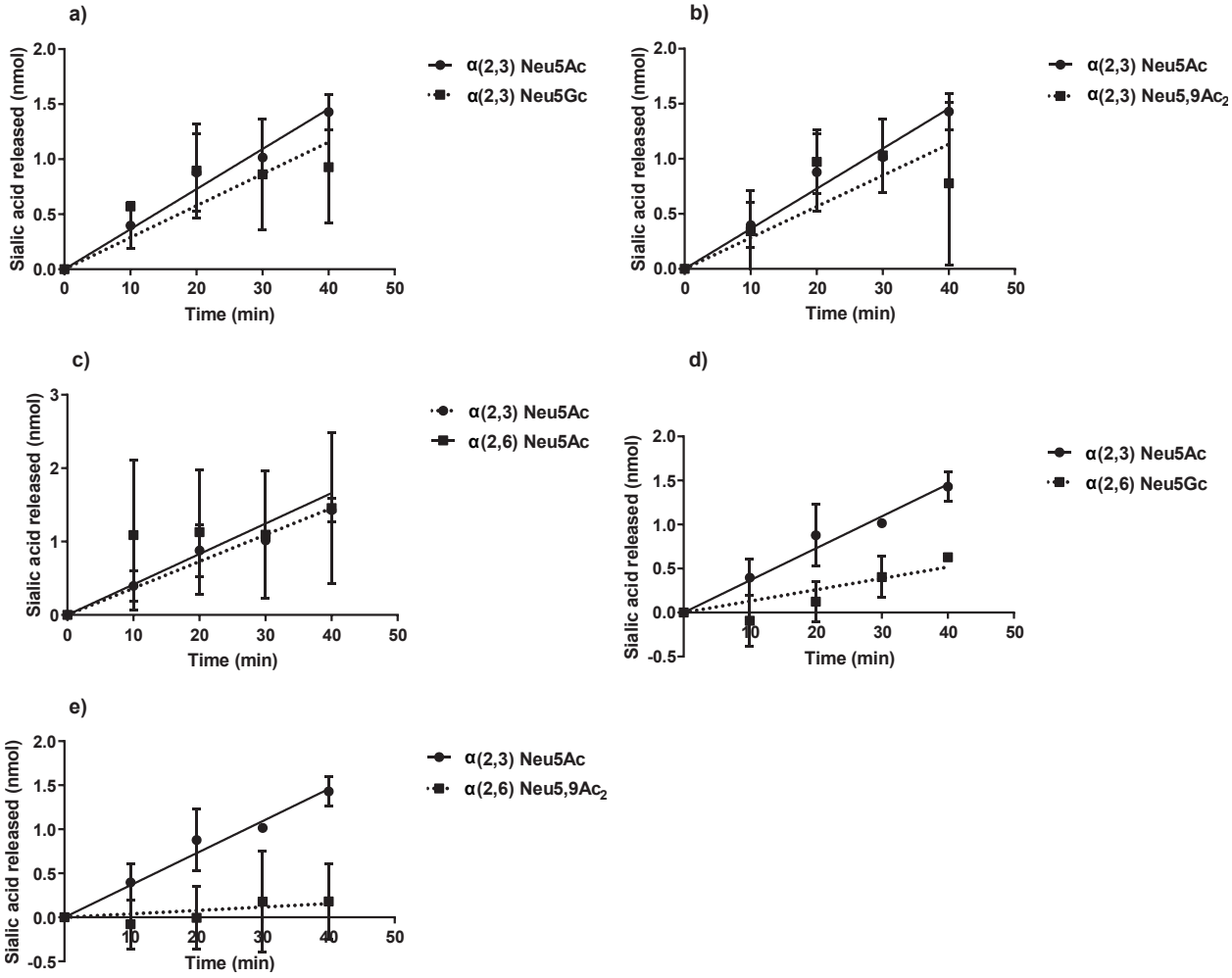
A2.3: Control experiment showing that NEU1 does not cleave octyl sialyllactosides 4. 4MU-Neu5Ac is a positive control. The result is consistent with previous reports that NEU1 does not cleave glycolipid substrates,[4] and supports that the majority of the neuraminidase activity in the crude HEK293E cell lysate is NEU1.



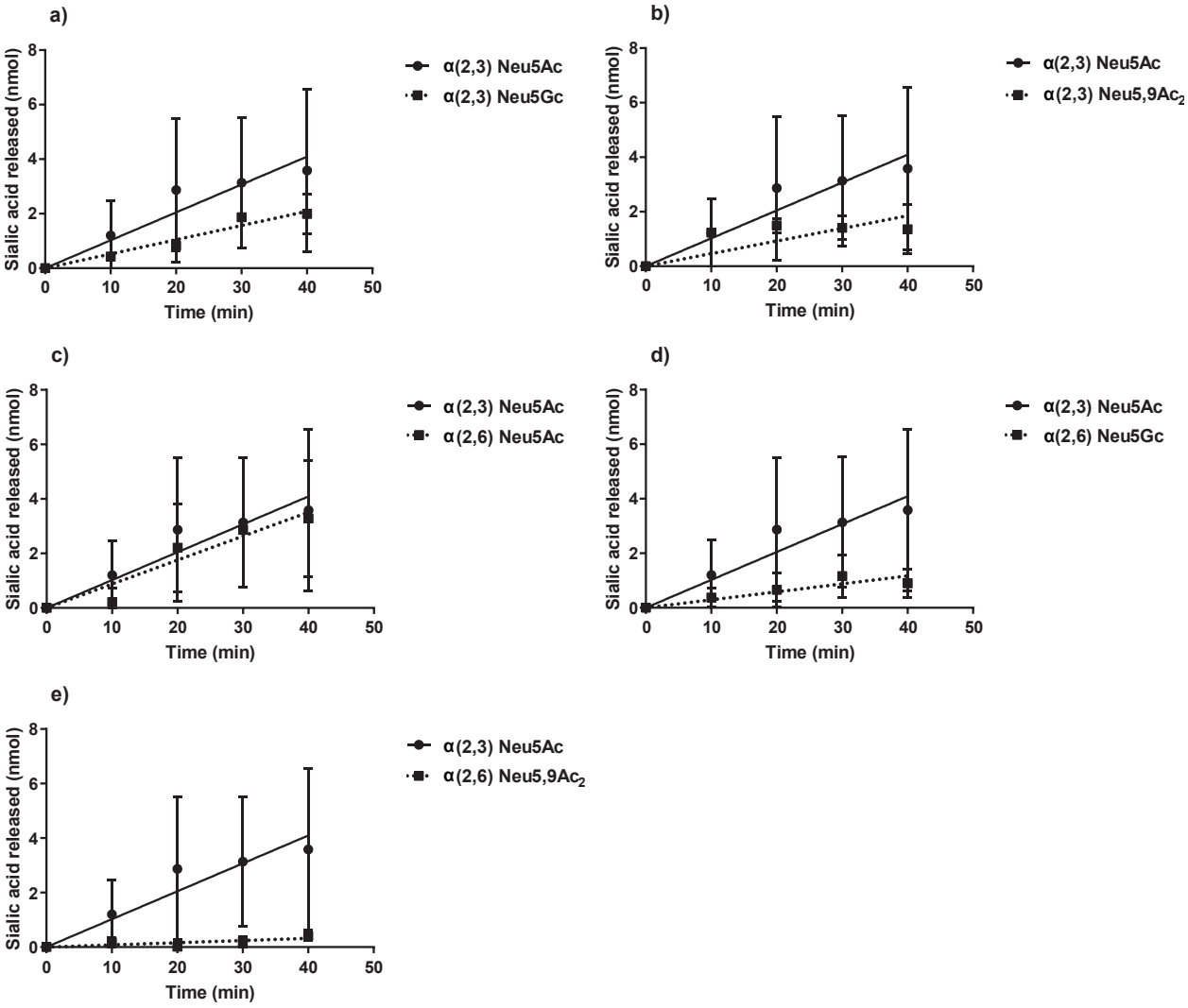
A2.4: Kinetics of NEU2 cleaving octyl sialyllactosides (**2-5** – **2-9** – dashed line) in reference to α(2→3) Neu5Ac octyl sialyllactoside (**2-4** – solid line, matched control replotted on every panel). Slopes were fit by linear regression forced through zero of triplicate experiments, error bars denote standard error.



A2.5: Kinetics of NEU3 cleaving octyl sialyllactosides (**2-5** – **2-9** – dashed line) in reference to α(2→3) Neu5Ac octyl sialyllactoside (**2-4** – solid line, matched control replotted on every panel). Slopes were obtained by linear regression forced through zero of triplicate experiments, error bars denote standard error.



A2.6: Kinetics of NEU4 cleaving octyl sialyllactosides (**2-5** – **2-9** – dashed line) in reference to α(2→3) Neu5Ac octyl sialyllactoside (**2-4** – solid line, matched control replotted on every panel). Slopes were obtained by linear regression forced through zero of triplicate experiments, error bars denote standard error.



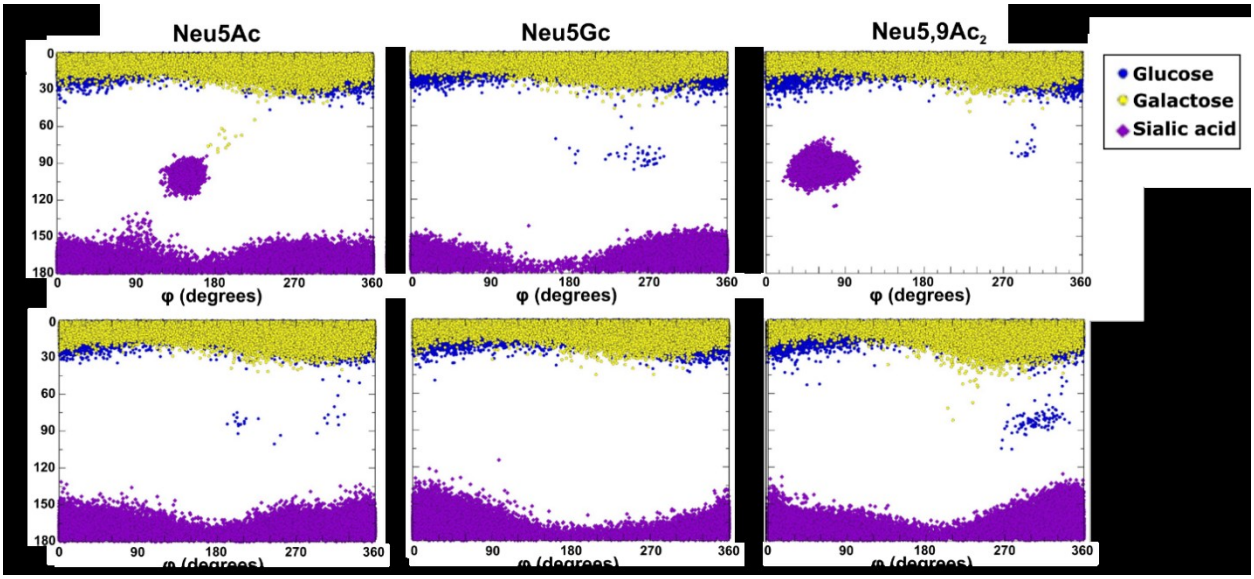
A2.7. Summary of kinetics assays showing the effect of sialic acid modification on hydrolysis with hNEU. Rates were obtained by taking points every 10 min for 40 min (or every 1 min for 4 min compounds **2-4** and **2-6** only); followed by a linear regression of triplicate experiments. Data is in the form of relative rates, ± standard error, normalized to α(2→3) Neu5Ac.

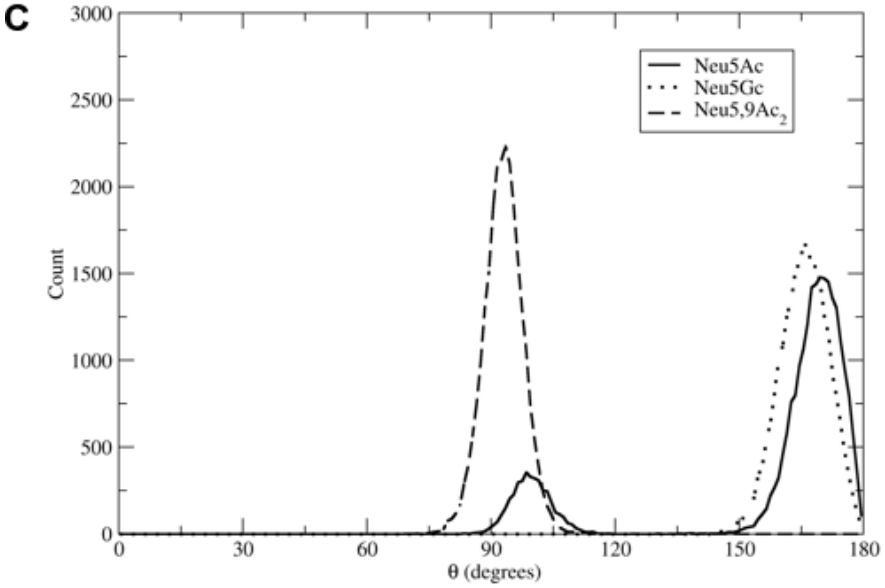
Assay	*4MU*		*Octyl sialyllactosides*					
Compound	**2-1**	**2-2**	**2-4**	**2-5**	**2-6**	**2-7**	**2-8**	**2-9**
NEU1	1.00 ± 0.03	0.61 ± 0.01						
NEU2	1.0 ± 0.1	0.02 ± 0.01	1.0 ± 0.1	0.017 ± 0.002	0.64 ± 0.05	0.052 ± 0.003	0.007 ± 0.001	0.006 ± 0.001
NEU3	1.00 ± 0.03	0.17 ± 0.02	1.00 ± 0.05	1.1 ± 0.2	0.8 ± 0.1	0.34 ± 0.07	0.8 ± 0.1	0.11 ± 0.09
NEU4	1.00 ± 0.03	1.97 ± 0.06	1.00 ± 0.2	0.9 ± 0.1	0.51 ± 0.04	0.29 ± 0.05	0.45 ± 0.07	0.08 ± 0.02

A2.8 Results from extra sum of squares F-test comparing the linear regression of the rates of hNEU-catalyzed hydrolysis of 4MU sialoside substrate **2-2** to **2-1** or octyl sialyllactoside substrates **2-5 - 2-9** to **2-4.** Results are reported as p values with $p<0.05$ indicating slopes are significantly different.

Assay	*4MU*		*Octyl sialyllactosides*					
Compound	**2-1**	**2-2**	**2-4**	**2-5**	**2-6**	**2-7**	**2-8**	**2-9**
NEU1		<0.0001						
NEU2		<0.0001		<0.0001	0.0056	<0.0001	<0.0001	<0.0001
NEU3		<0.0001		0.6	0.098	<0.0001	0.12	<0.0001
NEU4		<0.0001		0.5	0.022	0.0013	0.012	<0.0001

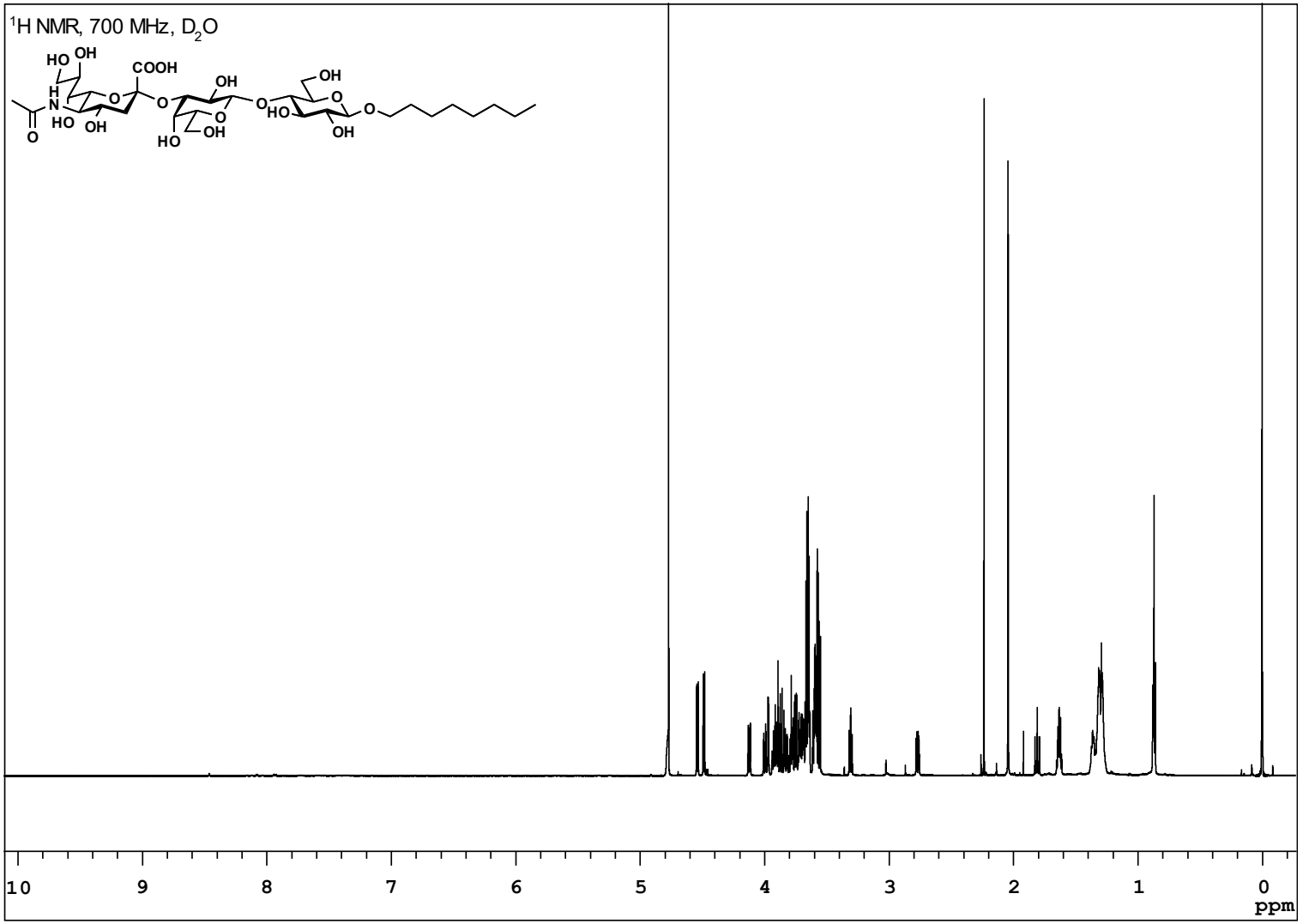
A2.9: Ring conformations from 25-ns MD simulations for GM3 trisaccharides of **2-4**, **2-6**, and **2-8** bound to a) NEU2 and b) NEU3 c) Histogram of ring conformations (along θ) from a 25-ns MD simulation for the GM3-analogs **2-4**, **2-6**, and **2-8** bound to NEU2. Neu5Ac in 4 is shown as a solid line and has two populations, one at θ = 99° (OS3, 18%) and a second at θ = 170° (2C5, 82%). Neu5Gc of 6 is shown as a dotted line and has one population at θ = 166° (2C5). Neu5,9Ac2 in 8 is shown as a line with long dashes and has one population at θ = 93° (B2,5).



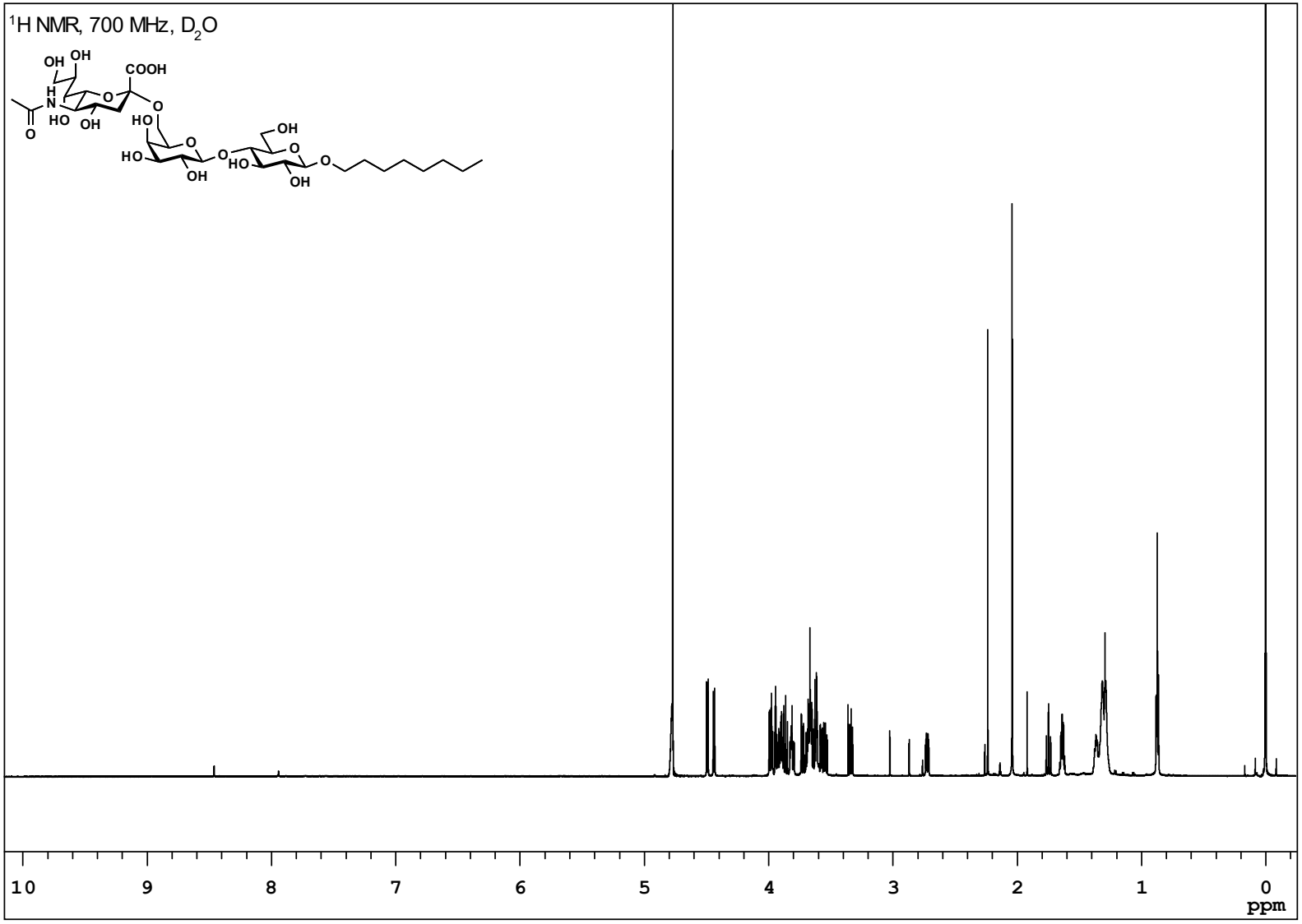

C
3000
2500
2000
1500
1000
500
0
Count
0
30
60
90
120
150
180
θ (degrees)
Neu5Ac
Neu5Gc
Neu5,9Ac2

A2.10 NMR data

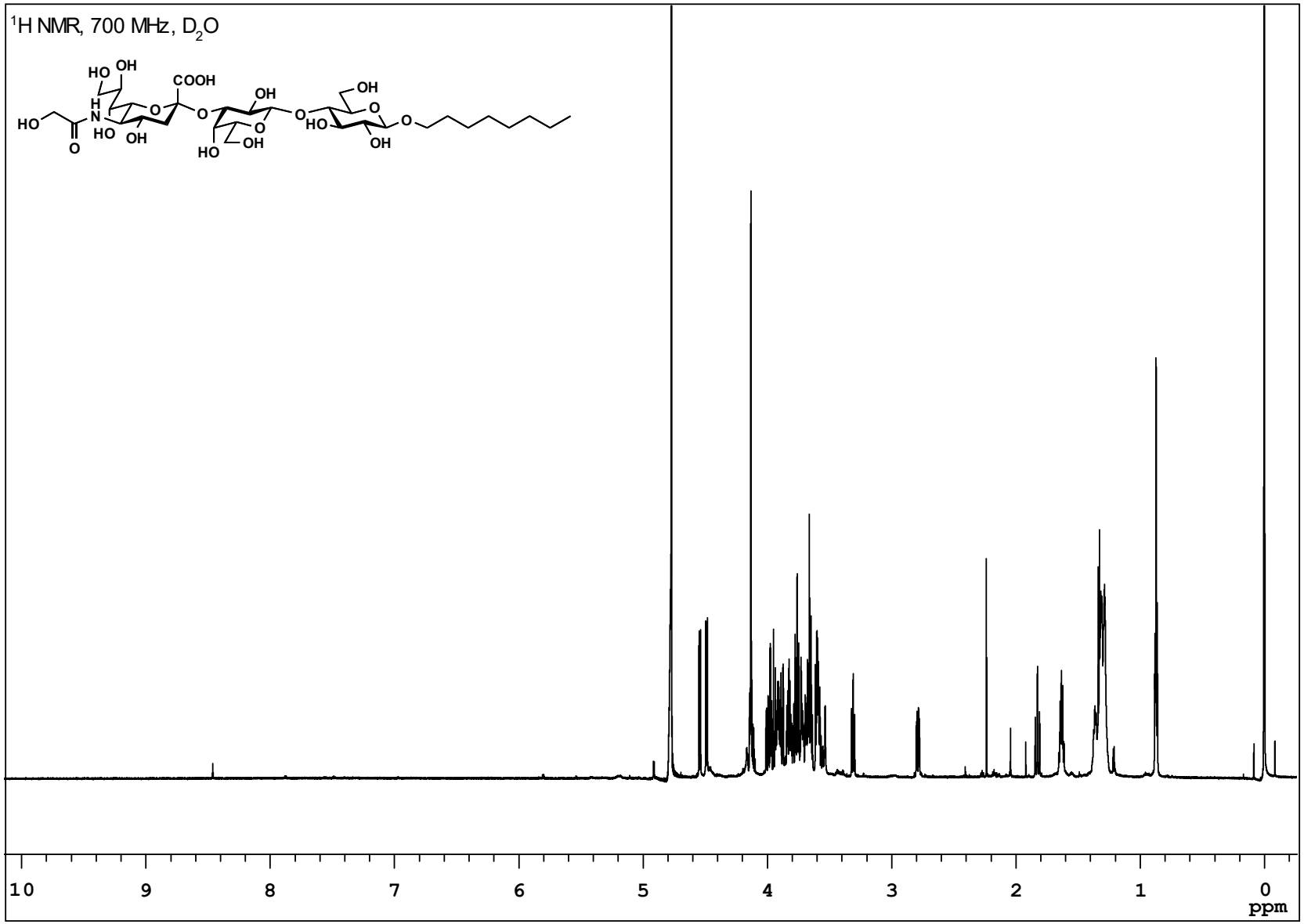
Compound 2-4



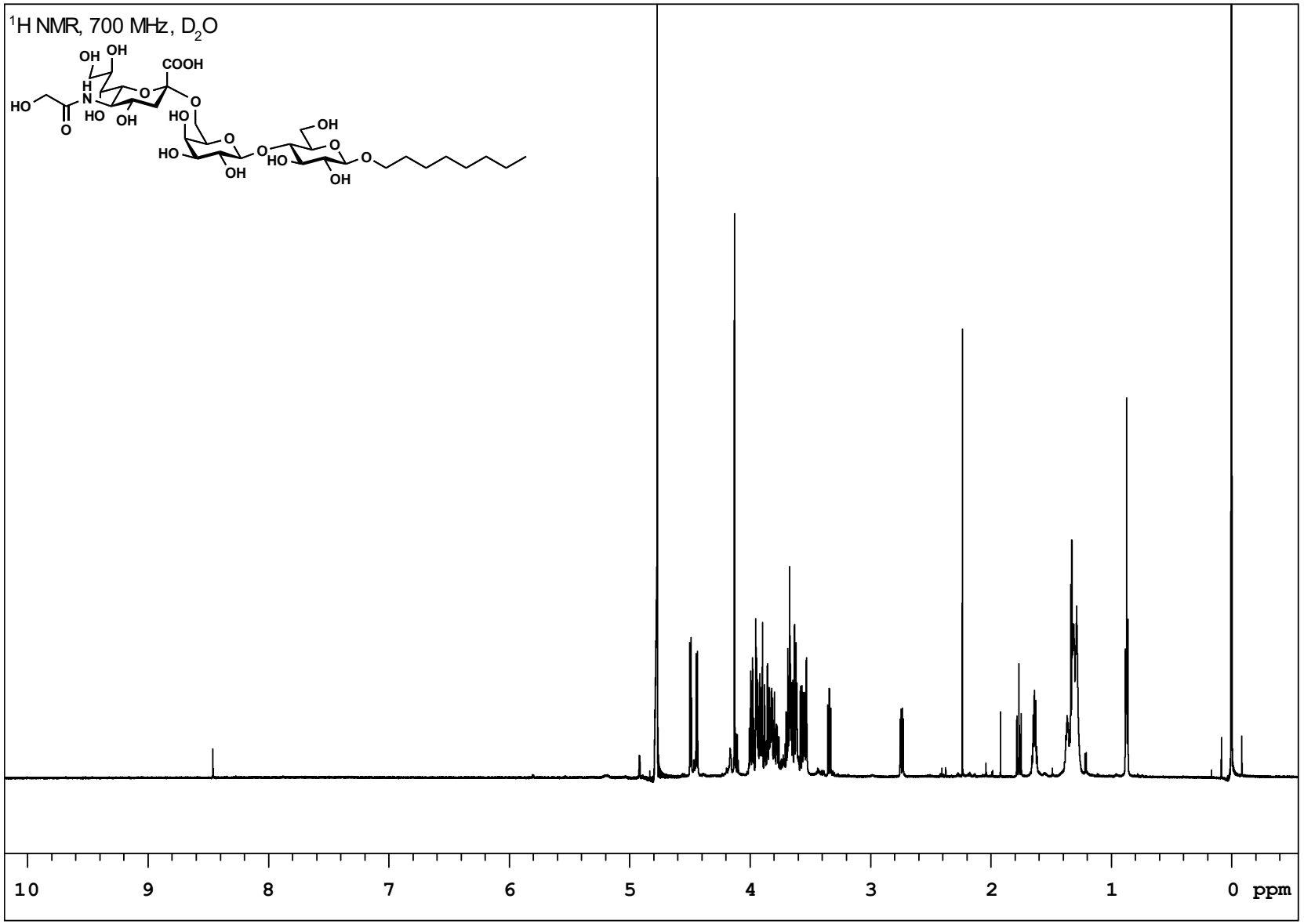
Compound 2-5



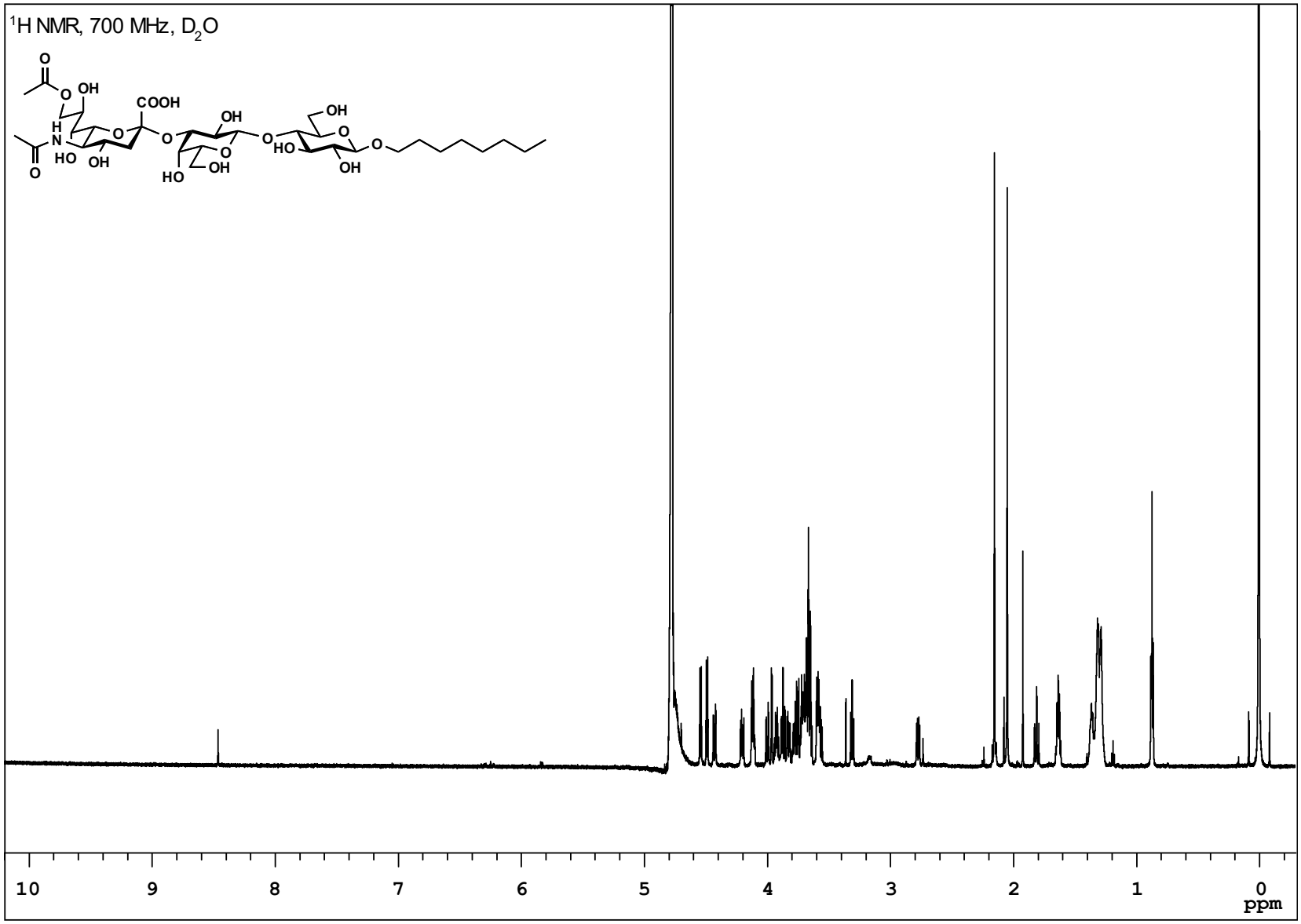
Compound 2-6



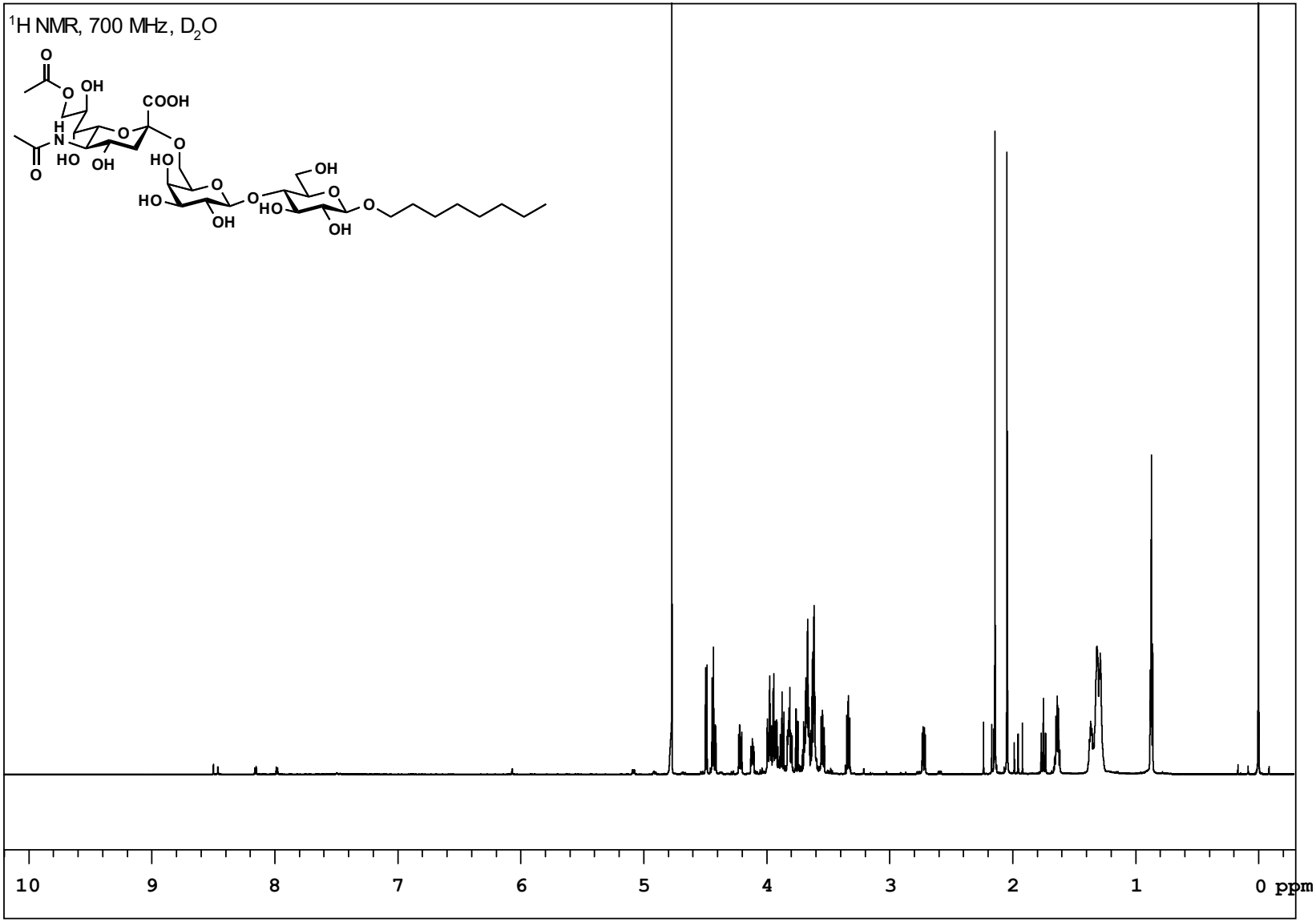
Compound 2-7



Compound 2-8



Compound 2-9



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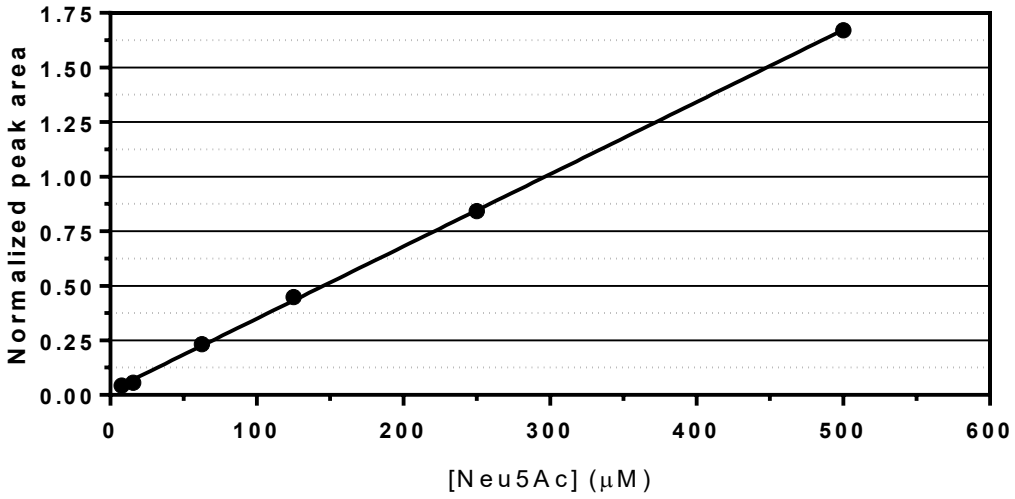
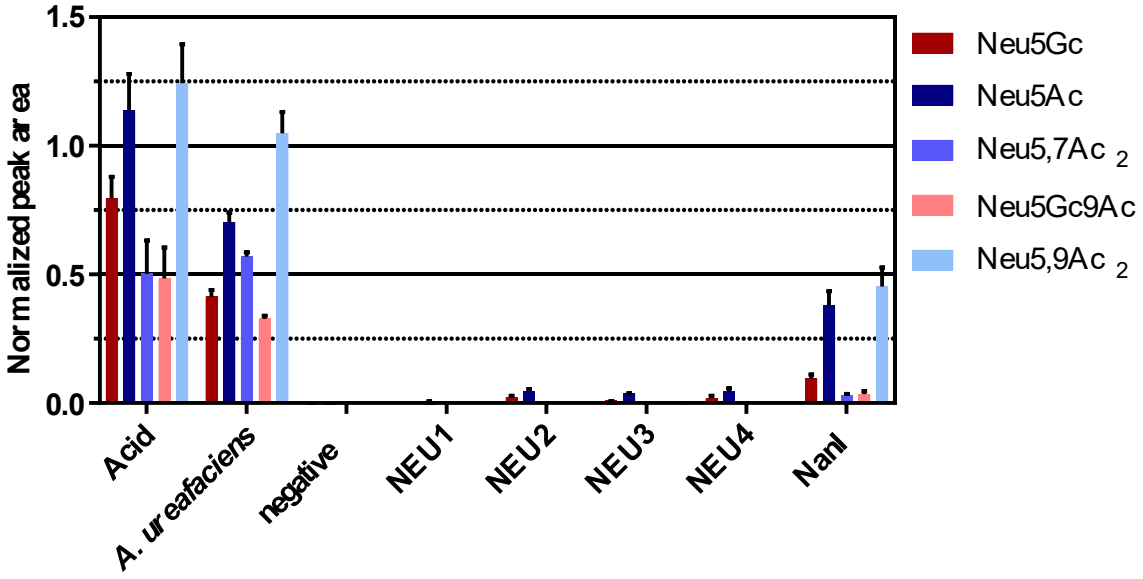
A.3. Supporting information for Chapter 3

A3.1: Calibration curve of Neu5Ac derivatized with 1,2-phenyldiamine σ-amino aniline

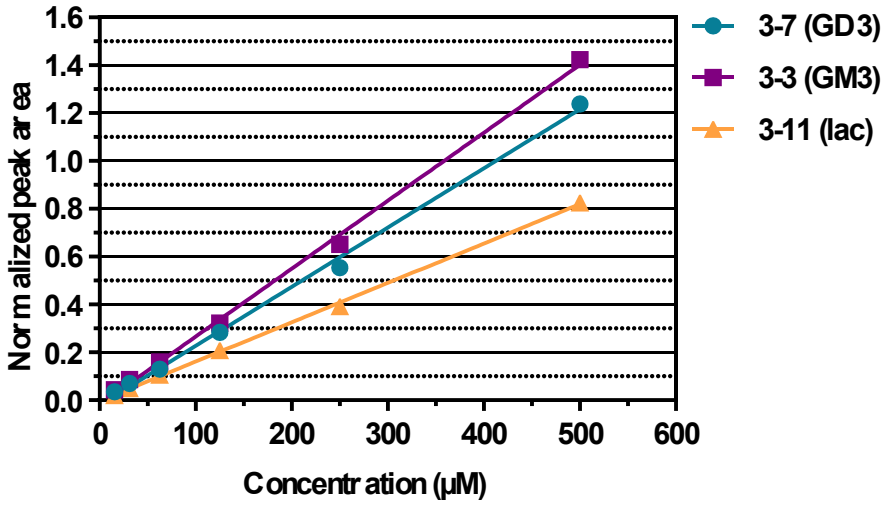


A3.2: Sialic acids released from bovine submaxillary mucin BSM. Peak areas were normalized using ρ-nitrophenol as an internal standard.

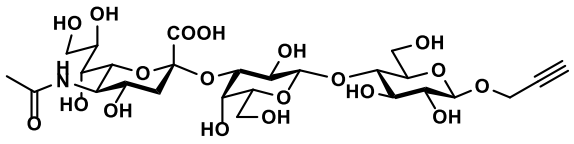


Condition	Normalized Peak Area		
	Neu5Ac	**Neu5Gc**	**Neu5,9Ac$_2$**
Acid	1.1 ± 0.1	0.80 ± 0.08	1.3 ± 0.2
A. ureafaciens	0.70 ± 0.04	0.41 ± 0.03	1.05 ± 0.08
NanI	0.38 ± 0.06	0.09 ± 0.02	0.45 ± 0.07
NEU1	0.0062 ± 0.0002	N/A	N/A
NEU2	0.049 ± 0.008	0.018 ± 0.004	N/A
NEU3	0.036 ± 0.001	0.008 ± 0.001	N/A
NEU4	0.04 ± 0.01	0.019 ± 0.009	N/A

A3.3: Calibration curve of aryl glycolipids separated using reversed phase HPLC with detection at 220 nm

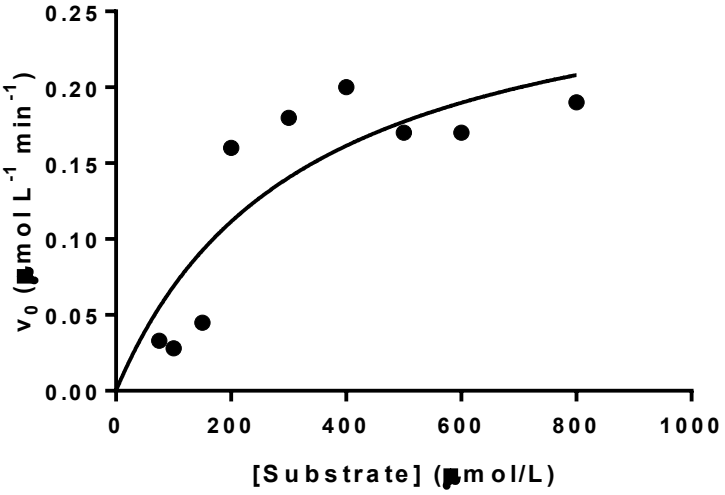


A3.4: Michaelis-Menten kinetics for NEU2 cleaving GM3 mimic octyne α(2→3) sialyllactose



Octyne sialyllactose was made using a previously reported synthetic route. ^{1}H NMR (700 MHz, D_2O) δ 4.54 (d, *J* = 8.0 Hz, 1H, H-1''), 4.49 (d, *J* = 8.1 Hz, 1H, H-1'), 4.12 (dd, *J* = 9.8, 3.2 Hz, 1H, H-3''), 4.02-3.56 (m, 22H), 3.31 (appt, 1H, H-2'), 2.71 (dd, *J* = 12.4, 4.7 Hz, 1H, H-3_{eq}'''), 2.35 (t, *J* = 2.7 Hz, 1H, $O(CH_2)_6CC\underline{H}$), 2.22 (td, *J* = 7.1, 2.7, 2H, $O(CH_2)_5C\underline{H_2}CCH$), 2.04 (s, 3H, $NHCOC\underline{H_3}$), 1.81 (t, *J* = 12.3 Hz, 1H, H-3_{eq}'''), 1.65 (appq, *J* = Hz, 2H, $OCH_2C\underline{H_2}(CH_2)_4CCH$) 1.56-1.51 (appq, 2H, $O(CH_2)_4C\underline{H_2}CH_2CCH$), 1.47-1.36 (m, 4H, $O(CH_2)_2C\underline{H_2}C\underline{H_2}(CH_2)_2CCH$). ^{13}C NMR (175 MHz, D_2O) δ 175.8 ($NH\underline{C}OCH_3$), 174.7 (C-1'''), 103.5 (C-1''), 102.9 (C-1'), 100.7 (C-2'''), 87.4, 79.2, 76.4 (C-3''), 76.0, 75.6, 75.3, 73.7, 72.6, 71.5, 70.2, 69.8, 69.2, 69.0, 68.3, 63.4, 61.9, 61.0, 52.5, 40.5 (C-3'''), 29.4 ($OCH_2\underline{C}H_2(CH_2)_4CCH$), 28.5, 28.4 ($O(CH_2)_4\underline{C}H_2CH_2CCH$), 25.4, 22.9 ($NHCO\underline{C}H_3$), 18.3 ($(CH_2)_5\underline{C}H_2CCH$). ESI-MS calcd for $C_{31}H_{51}NO_{19}$ $[M-H]^-$, 740.2983; found, 740.2975.

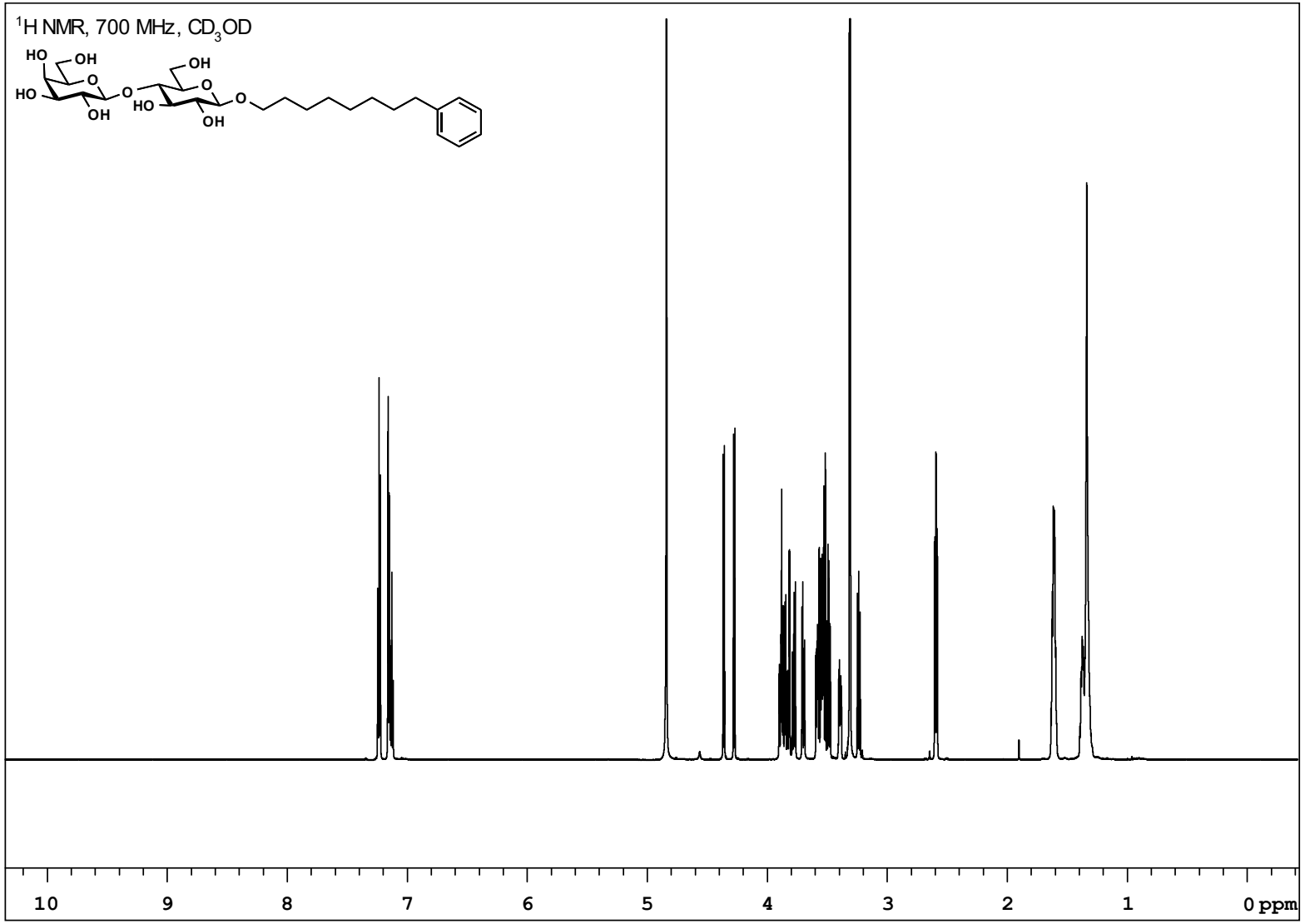
Solution phase K_M and V_{max} measurements of NEU2 cleaving the GM3 oligosaccharide were determined with slight modifications to a previously reported assay.[1] In brief, octyne sialyllactose in 45 µL water (75 – 800 µM) was mixed with 120 uL 0.3 M ammonium acetate (pH 6) and 15 uL (1 mU) NEU2. The assay mixture was incubated at 30 ºC. At timepoints of 0, 10, 20, 30, and 40 minutes, 30 µL aliquots were quenched in 50 µL sodium borate buffer (0.2 M, pH 9.5). Malononitrile (15 µL, 0.8 % w/v in H_2O) was added to each aliquot and heated for 20 minutes at 100 ºC, after which 28 µL was transferred to a 384 well plate. Fluorescence was measured on a SpectraMax M2^e plate reader (Molecular Devices), excitation 357 nm emission 434 nm.

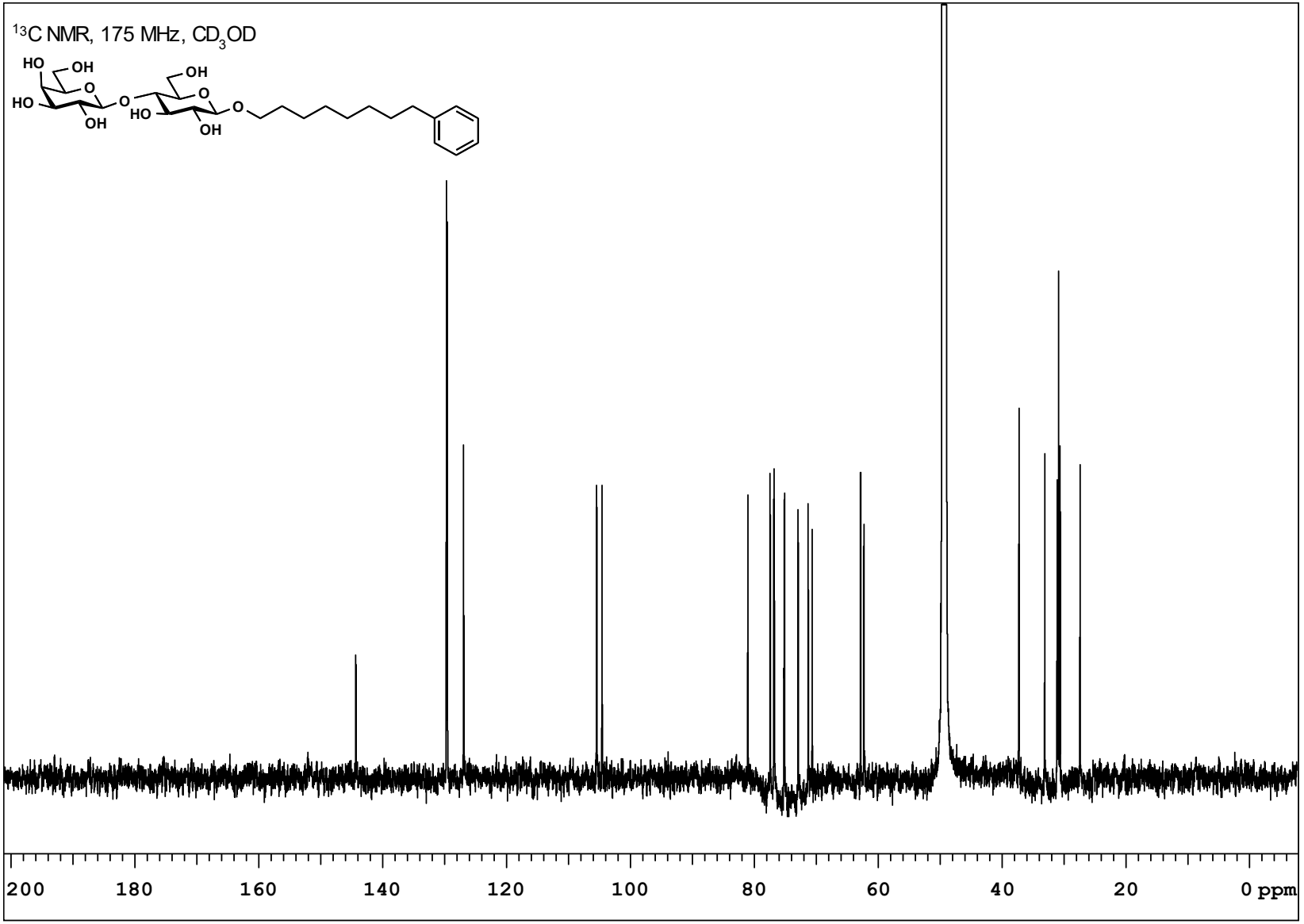


K_M and V_{max} determination for NEU2 cleaving octyne GM3. Curve fitting was done using GraphPad Prism to obtain a K_M of 325 ± 221 μM and V_{max} of 0.29 ± 0.09 μmol L^{-1} min^{-1}.

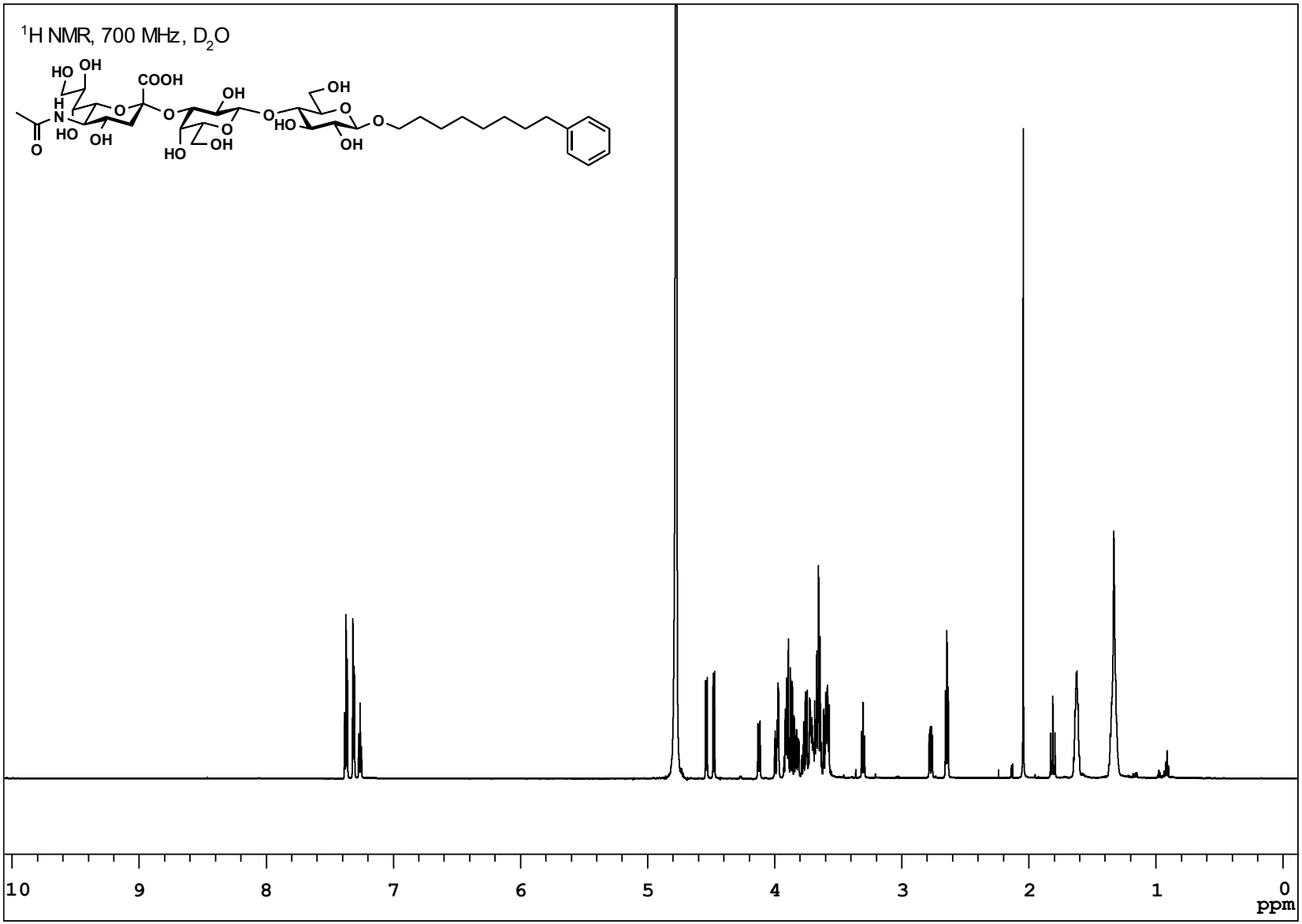
A3.5 NMR of aryl glycolipids

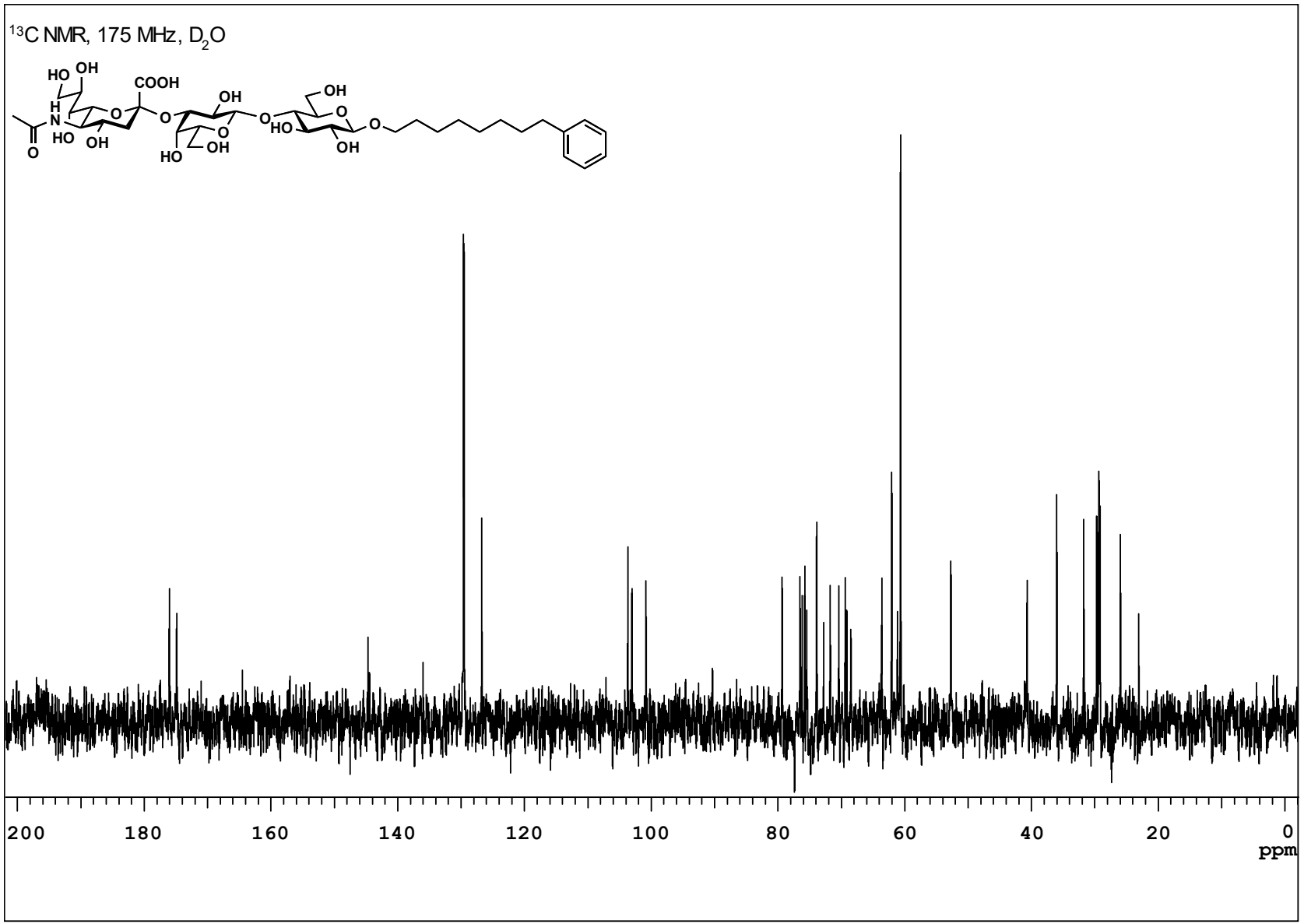
Compound **3-11**



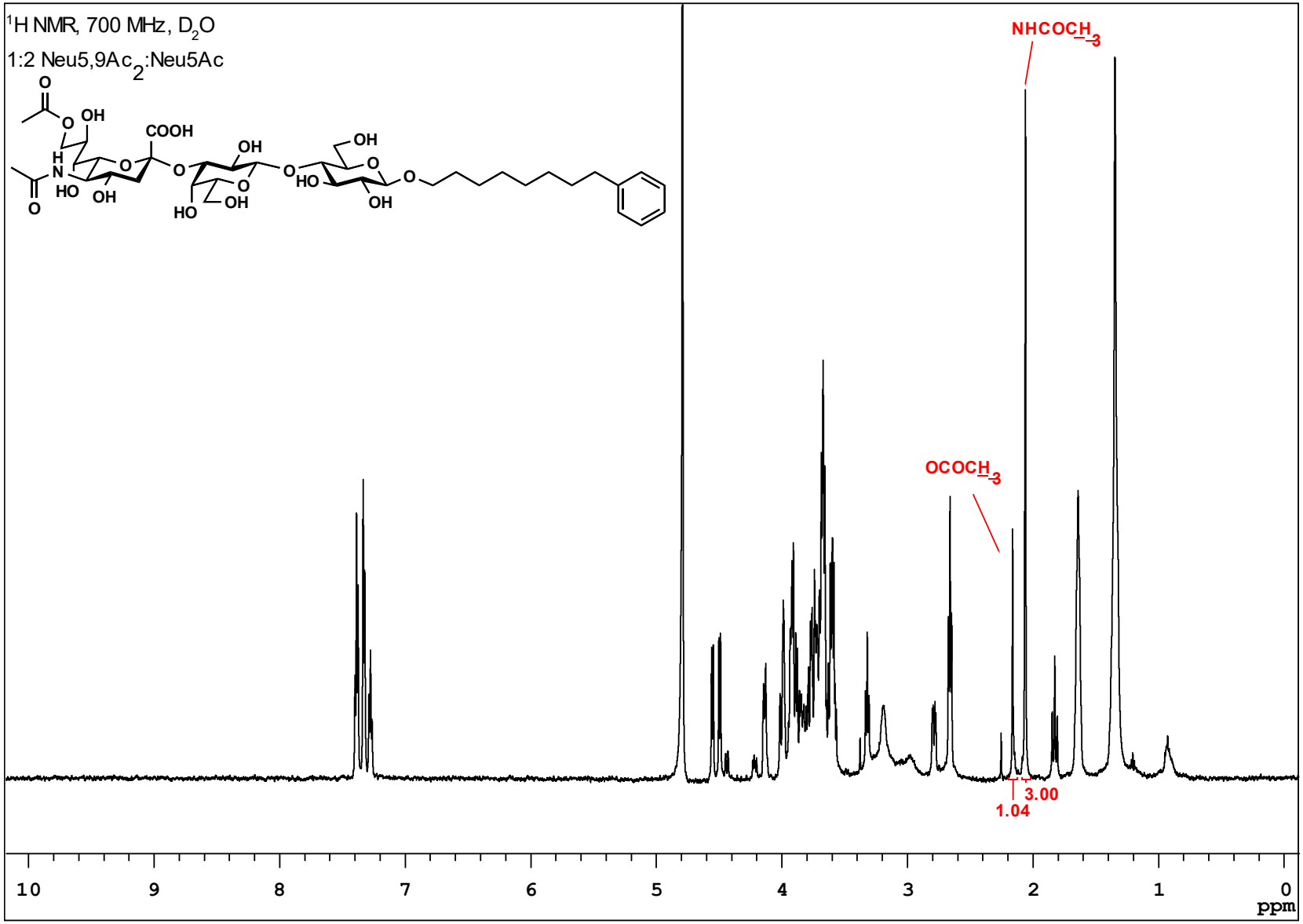

^{13}C NMR, 175 MHz, CD_3OD
HO
OH
O
HO
O
OH
HO
OH
O
HO
O
OH
200
180
160
140
120
100
80
60
40
20
0 ppm

Compound **3-3**

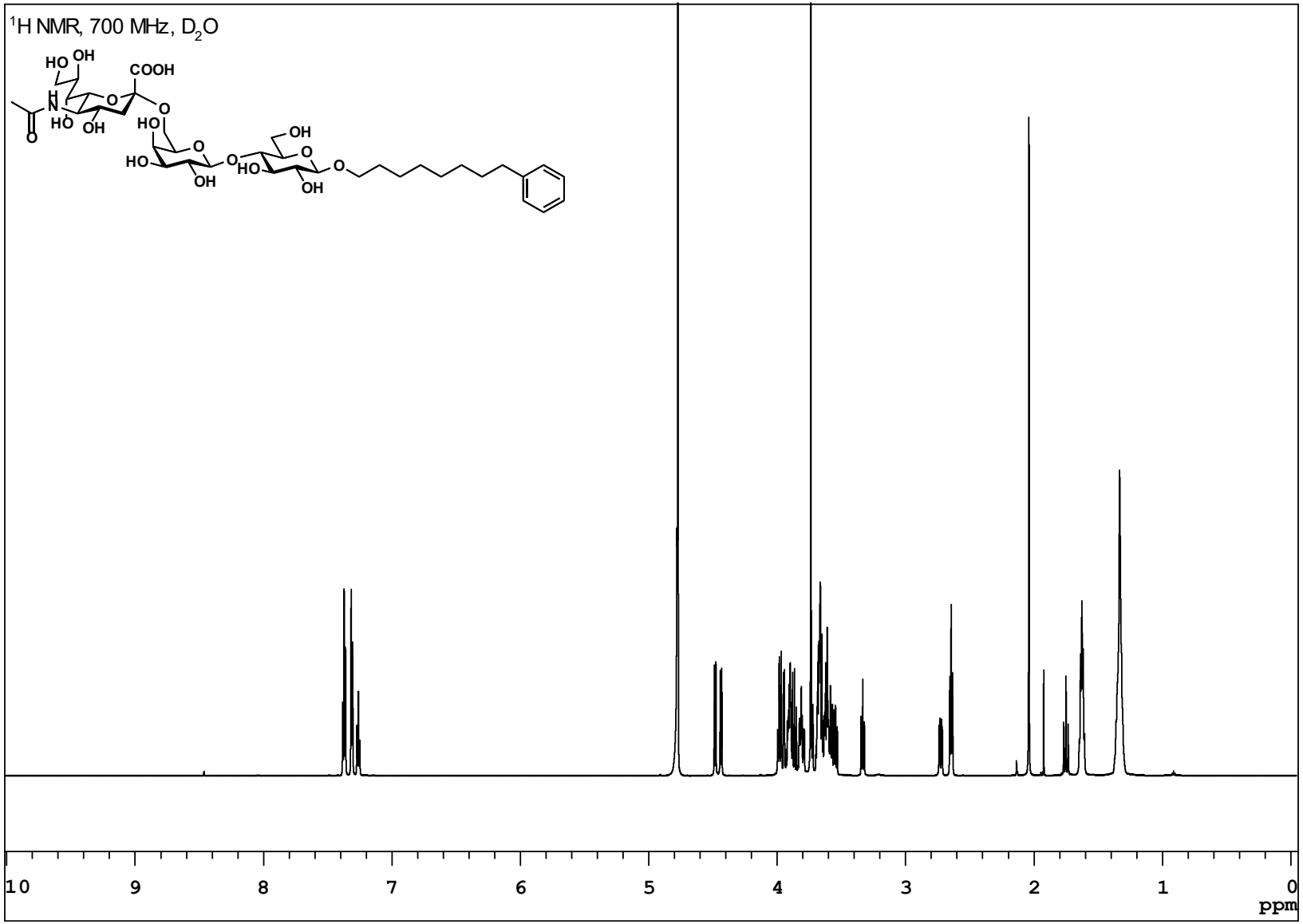


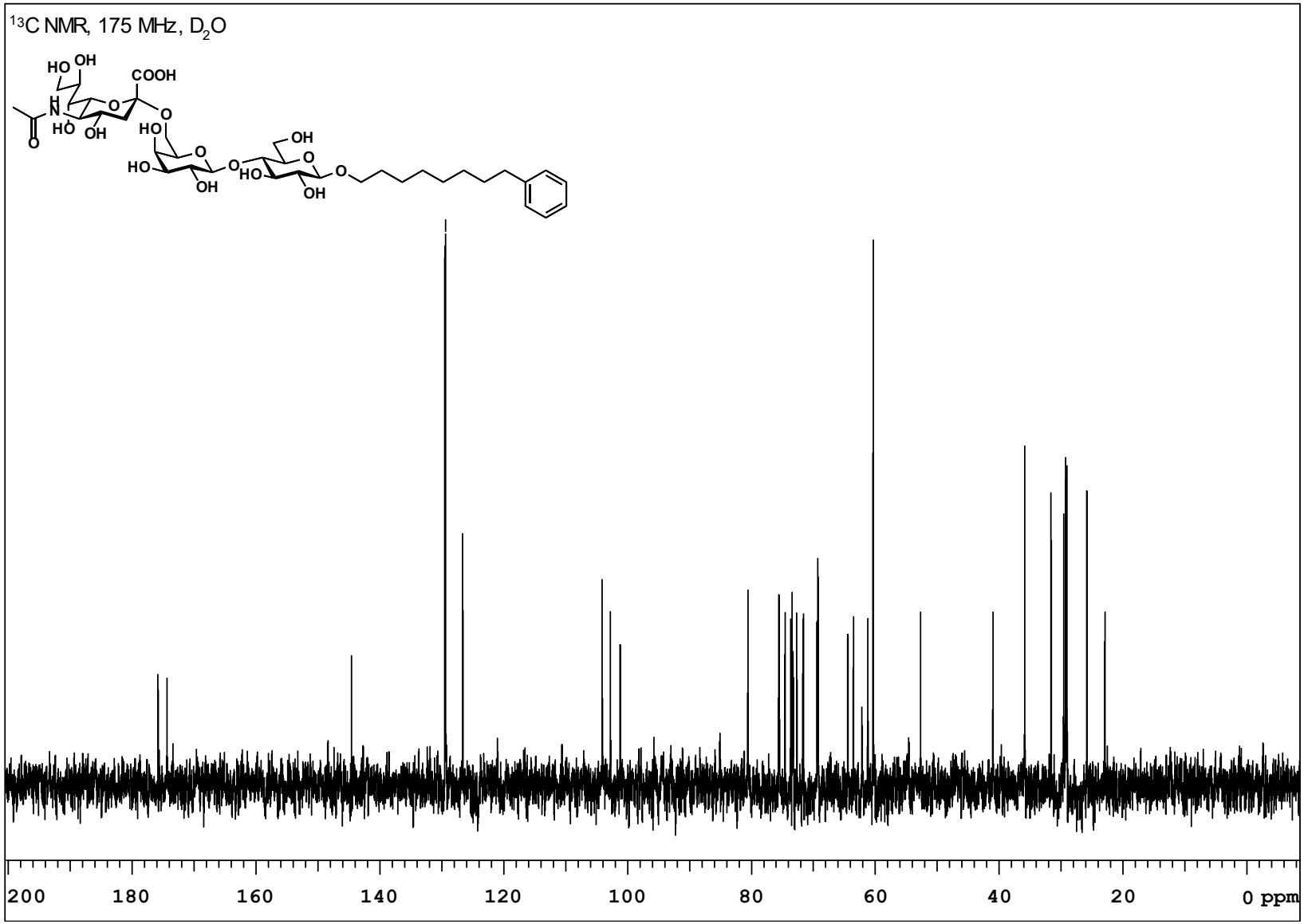

13C NMR, 175 MHz, D2O
200
180
160
140
120
100
80
60
40
20
0
ppm

Compound 3-4

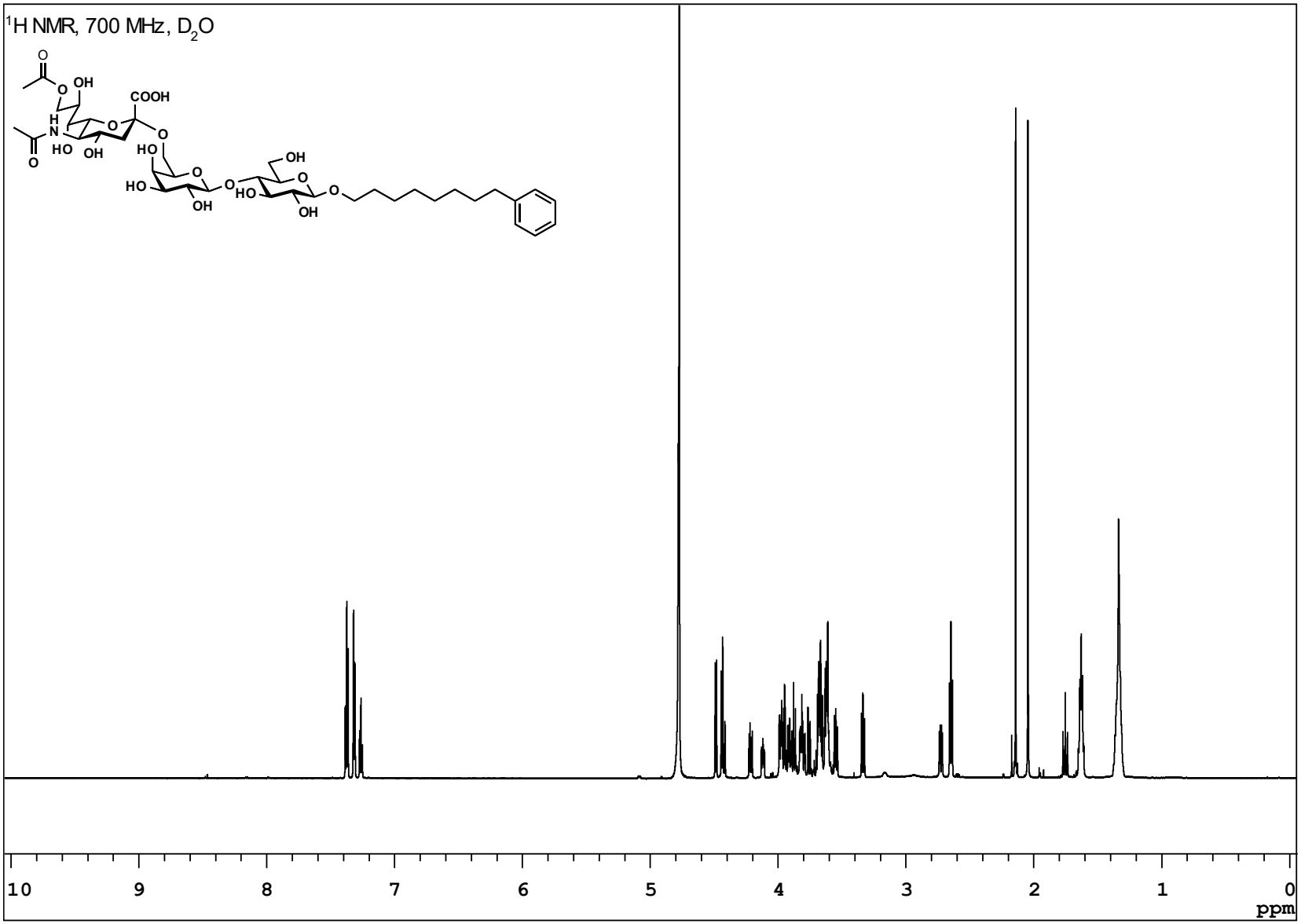

1H NMR, 700 MHz, D2O
1:2 Neu5,9Ac2:Neu5Ac
NHCOCH3
OCOCH3
3.00
1.04
10
9
8
7
6
5
4
3
2
1
0
ppm

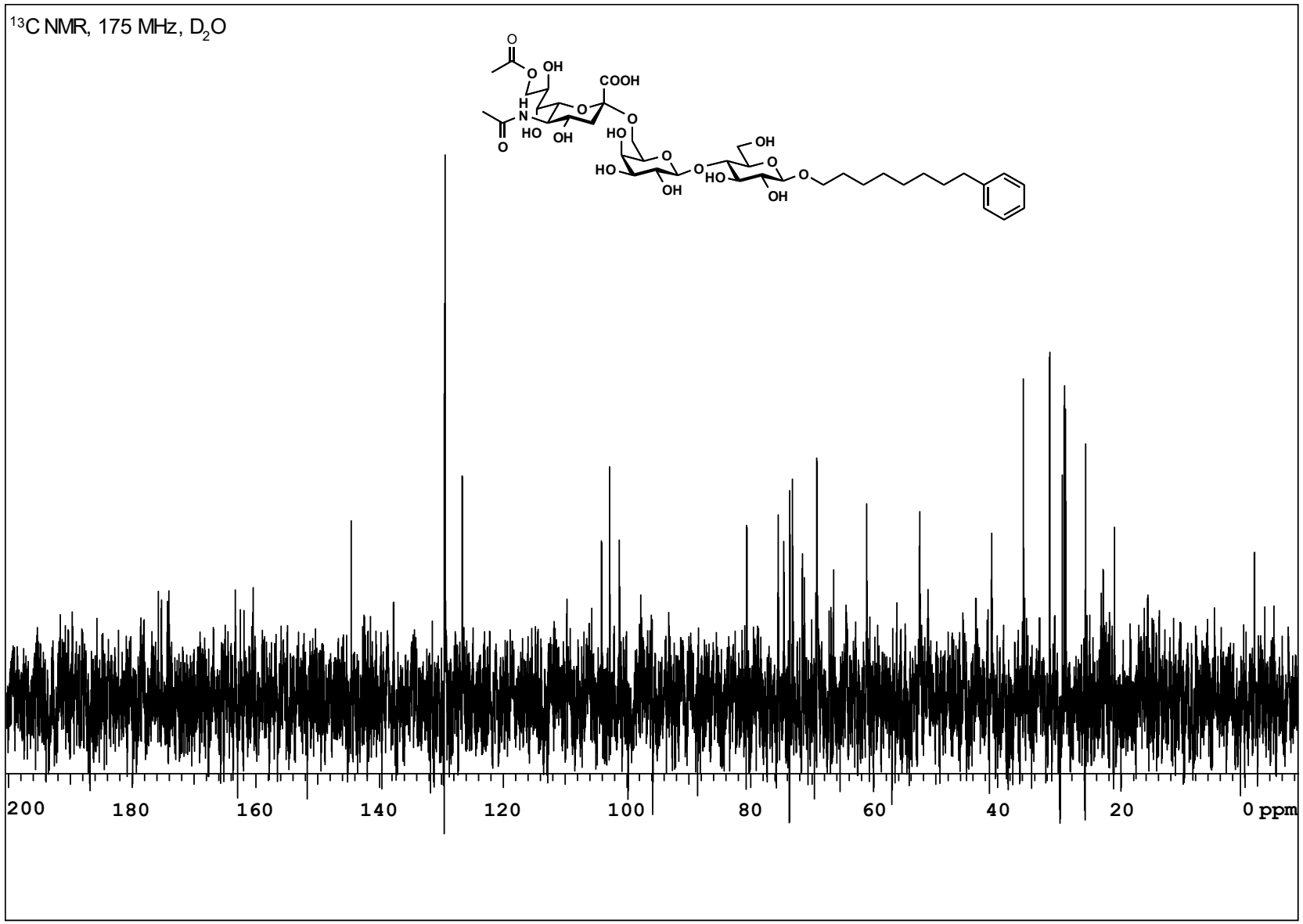
Compound **3-5**



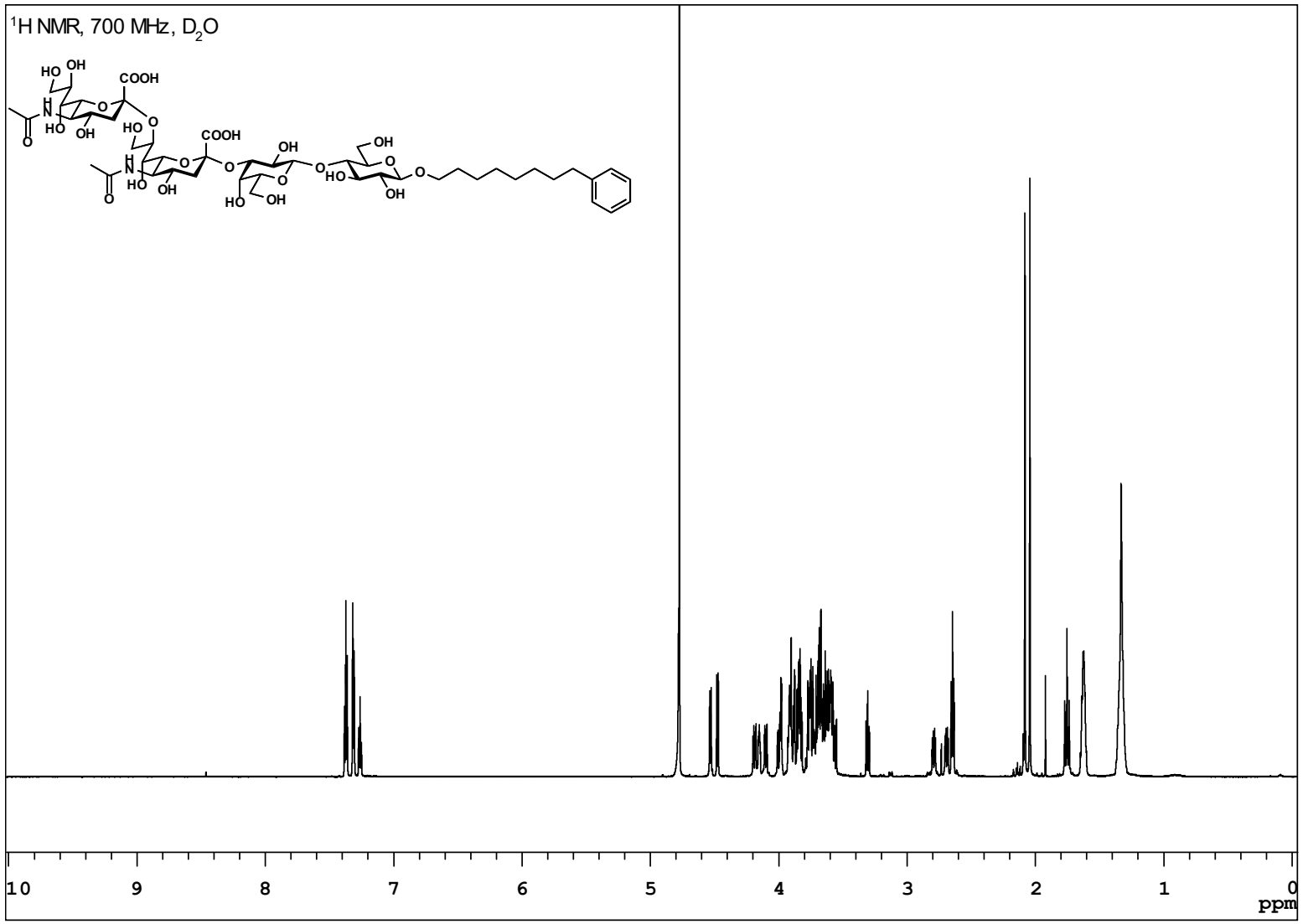

13C NMR, 175 MHz, D2O
HO OH
COOH
O
N
H
HO OH
HO
O
HO
O
HO
OH
O
HO
OH
OH
O
O
200
180
160
140
120
100
80
60
40
20
0 ppm

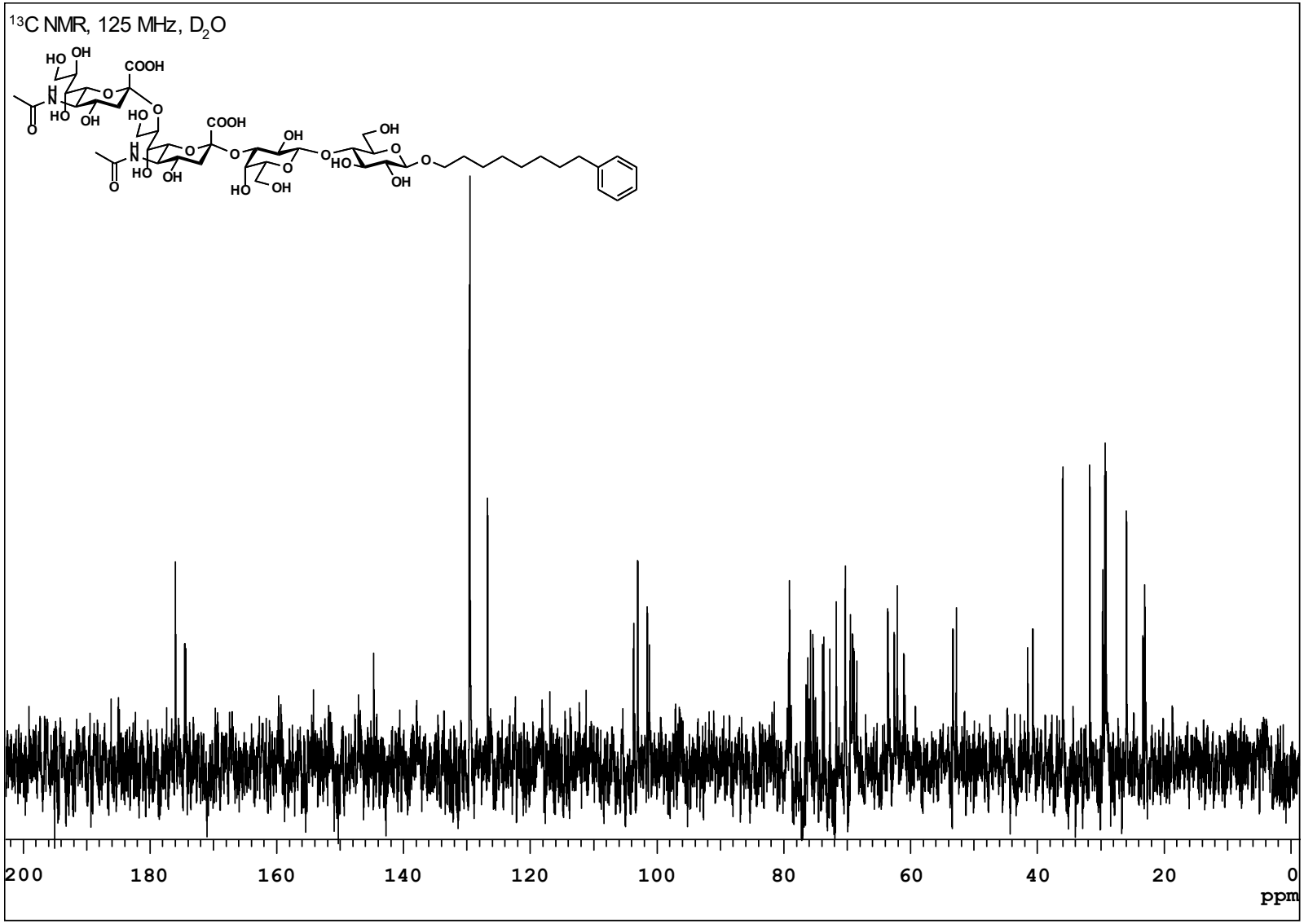
Compound **3-6**



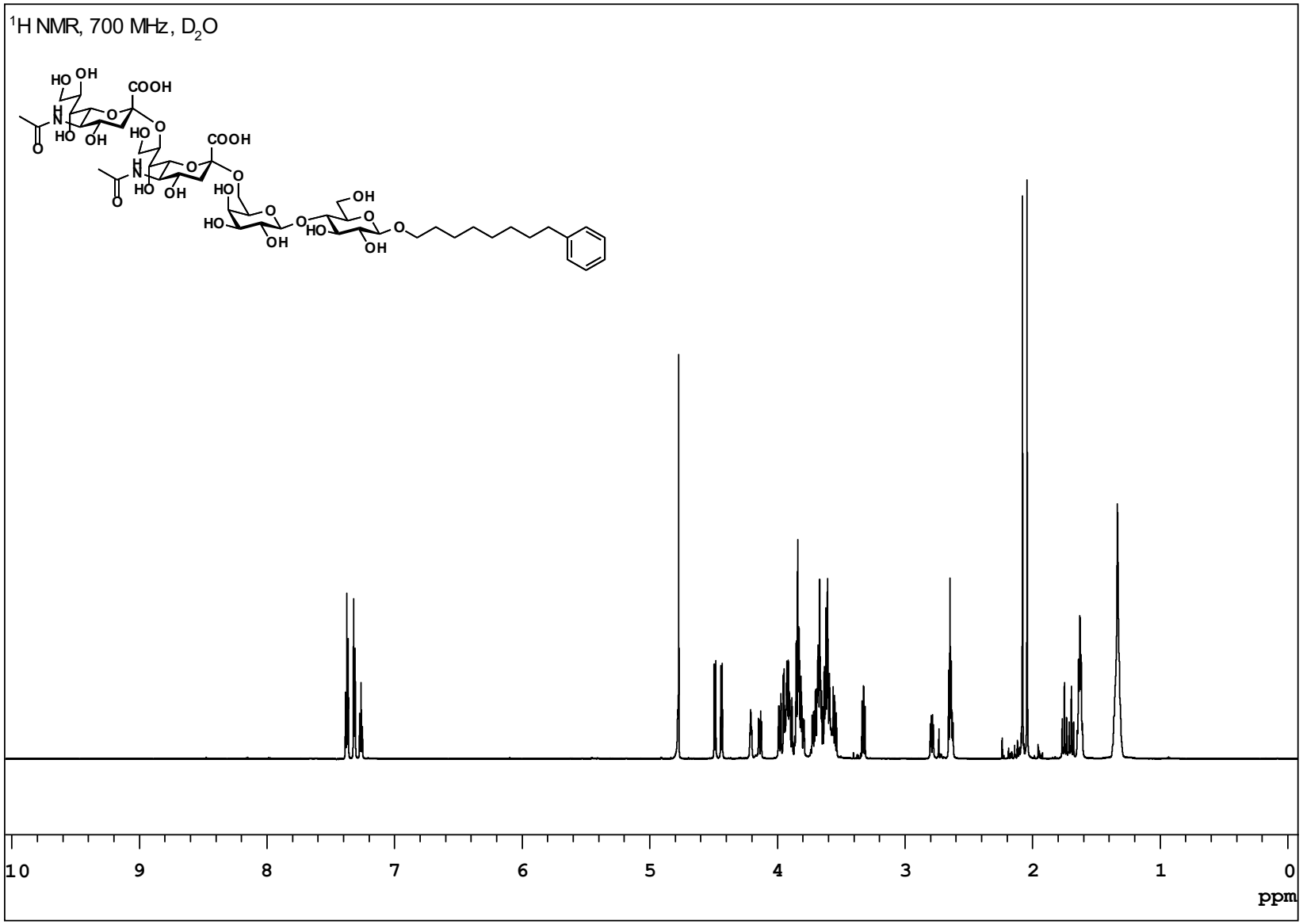

13C NMR, 175 MHz, D2O
200
180
160
140
120
100
80
60
40
20
0 ppm

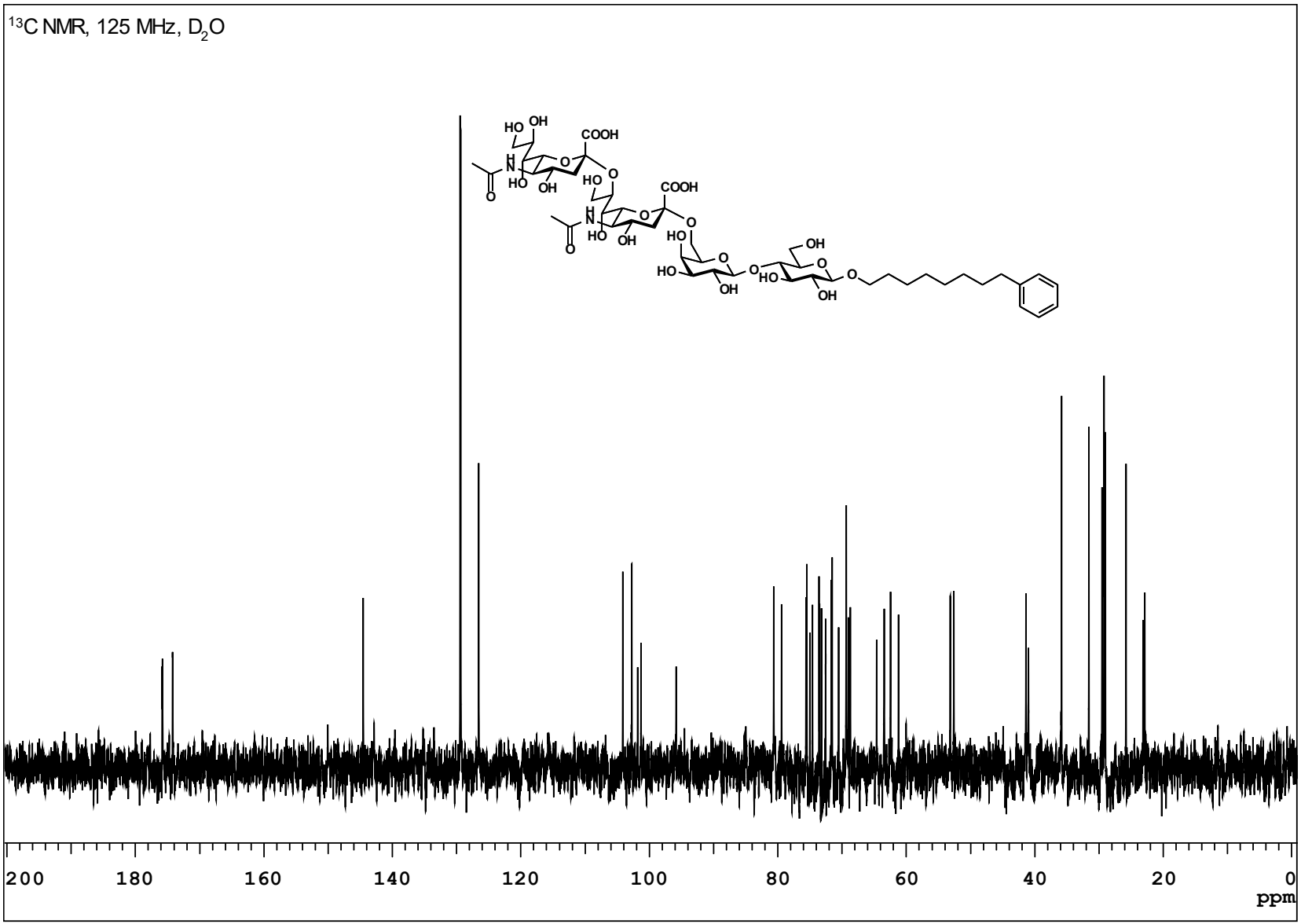
Compound **3-7**




13C NMR, 125 MHz, D2O
200
180
160
140
120
100
80
60
40
20
0
ppm

Compound **3-9**




13C NMR, 125 MHz, D2O
200
180
160
140
120
100
80
60
40
20
0
ppm

References

1. Sandbhor, M.S., Soya, N., Albohy, A., Zheng, R.B., Cartmell, J., Bundle, D.R., Klassen, J.S., and Cairo, C.W. (2011) Substrate recognition of the membrane-associated sialidase NEU3 requires a hydrophobic aglycone. *Biochemistry*. 50, 32, 6753-6762.
2. Hunter, C.D., Khanna, N., Richards, M.R., Rezaei Darestani, R., Zou, C., Klassen, J.S., and Cairo, C.W. (2018) Human neuraminidase isoenzymes show variable activities for 9-O-acetyl-sialoside substrates. *ACS Chemical Biology*, 13, 4, 922-932.

A.4 Supporting information for Chapter 4

A4.1 Average degree of polymerization (average DP) for oligosialic acid degradation assays. Data presented is an average of triplicate experiments with error denoting one standard deviation

Condition	**Average DP**		
	pH 4.5	pH 5.5	pH 7
t = 0			5.58 ± 0.04
pH control	4.76 ± 0.03	5.52 ± 0.04	5.62 ± 0.04
NEU1	4.8 ± 0.2		
NEU2		5.3 ± 0.2	5.56 ±0.02
NEU3	3.58 ± 0.04		5.63 ± 0.05
NEU4	4.05 ± 0.01		5.7 ± 0.2
ureafaciens	4.64 ± 0.01		5.69 ± 0.04
endo-N	1.22 ± 0.03		1.22 ± 0.03
NEU1 + inh.	5.1 ± 0.1		
NEU4 + inh.	4.74 ± 0.03		

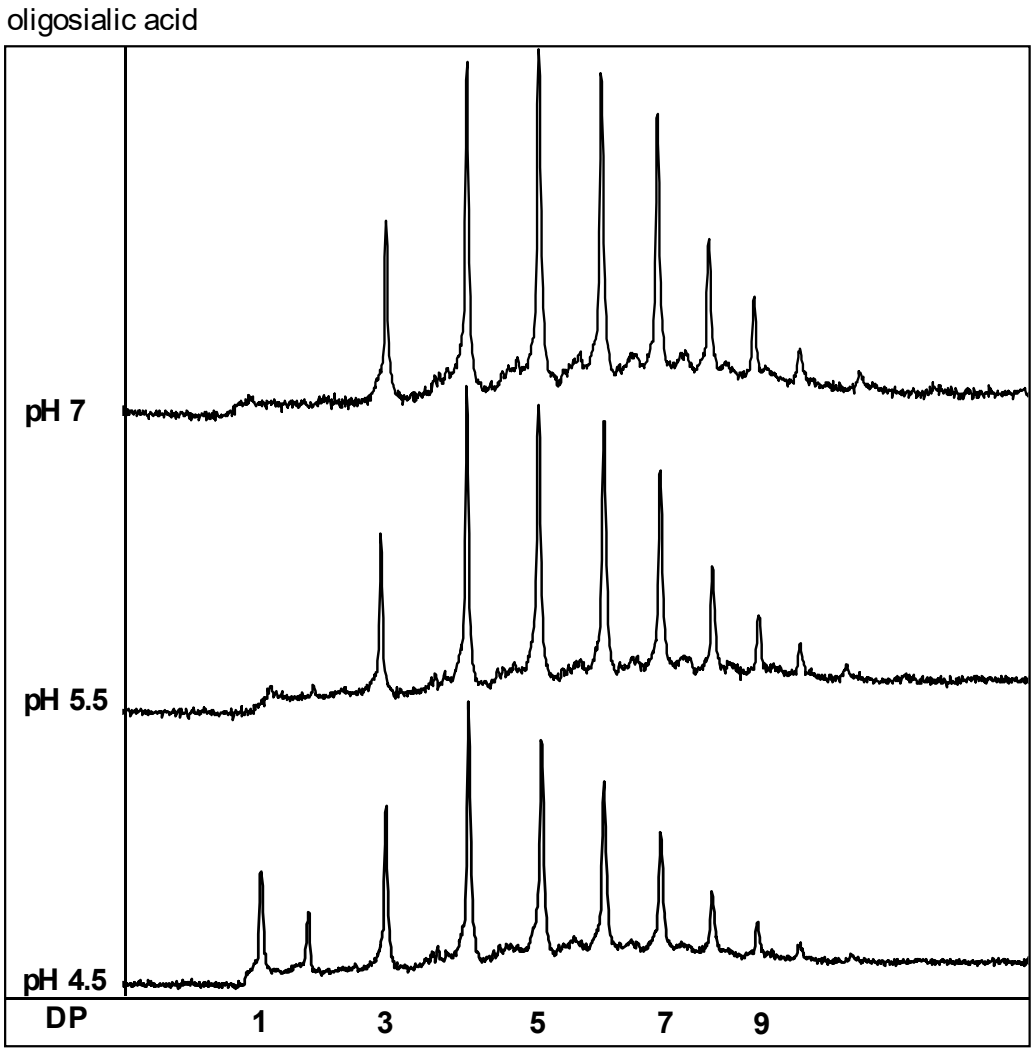
A4.2 Average degree of polymerization (average DP) for polysialic acid degradation assays. Data presented is an average of triplicate experiments with error denoting one standard deviation

Condition	Average DP
t = 0	13.15 ± 0.01
pH 4.5 control	9.0 ± 0.1
pH 5.5 control	12.7 ± 0.2
pH 7 control	13.0 ± 0.1
NEU1 + inh.	9.4 ± 0.2
NEU1	9.6 ± 0.4
NEU2	12.0 ± 0.3
NEU3	9.4 ±0.2
NEU4	9.2 ± 0.1
ureafaciens	9.1 ± 0.1
endo-N	1.77 ± 0.07

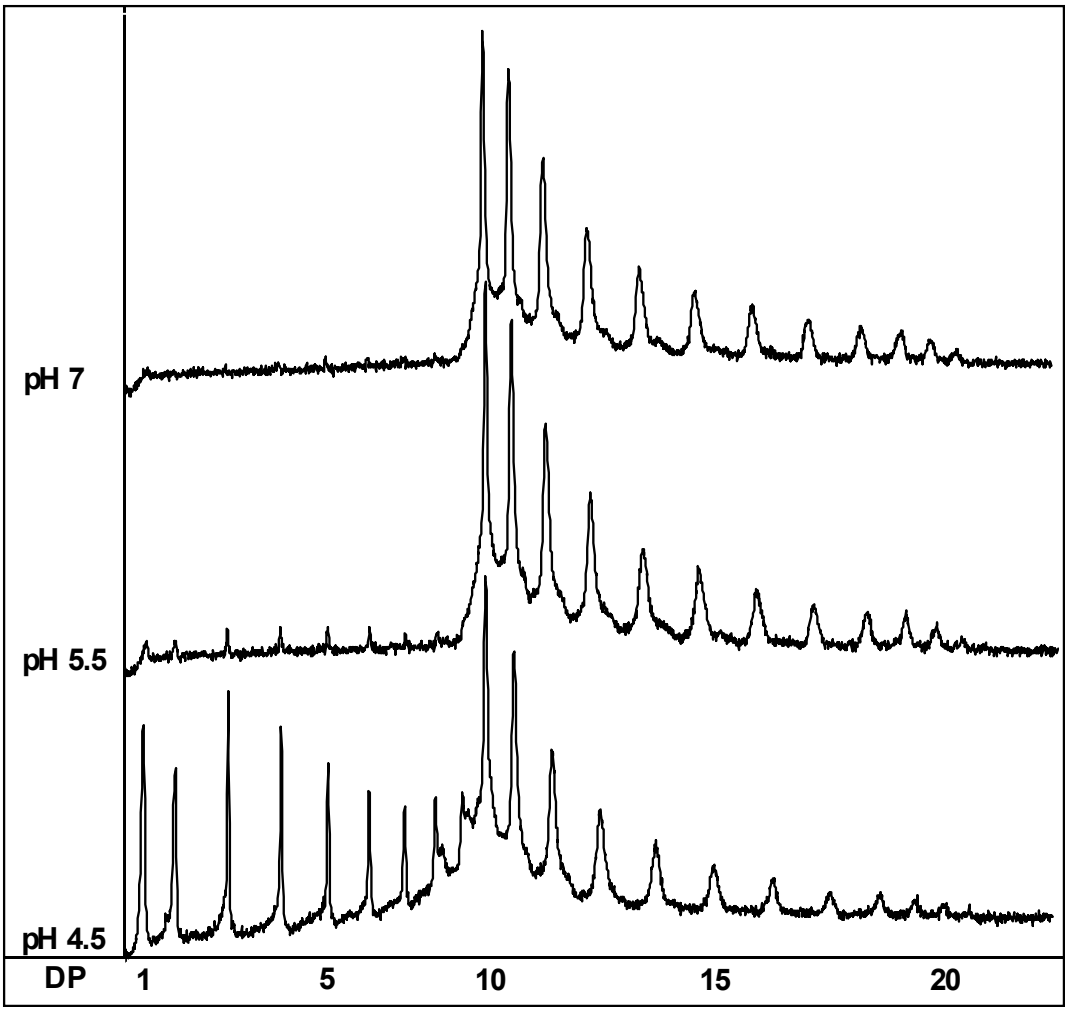
A4.3 Average degree of polymerization (average DP) for oligosialic acid degradation assays examining the effect of changing salt concentration. Data presented is an average of triplicate experiments with error denoting one standard deviation

Condition	**Average DP**		
	260 mM	160 mM	80 mM
pH 4.5 control	4.76 ± 0.03	4.41 ± 0.05	4.39 ± 0.06
NEU4	4.05 ± 0.01	4.24 ± 0.03	4.26 ± 0.08
endo-N	1.22 ± 0.03	1.21 ± 0.01	1.22 ± 0.02

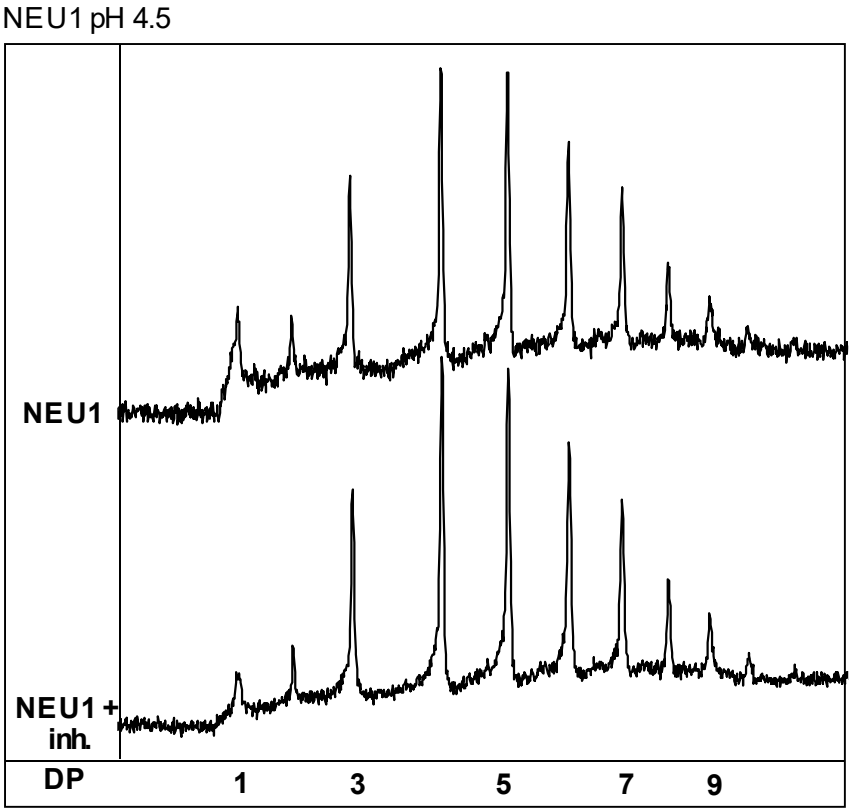
A4.4 Representative chromatograms for pH controls



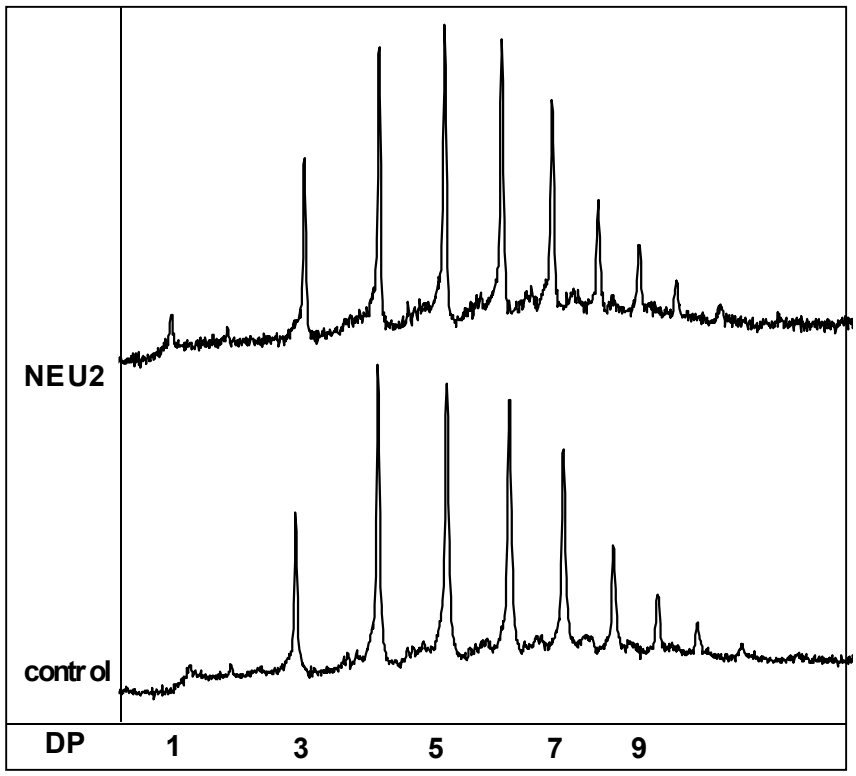
polysialic acid



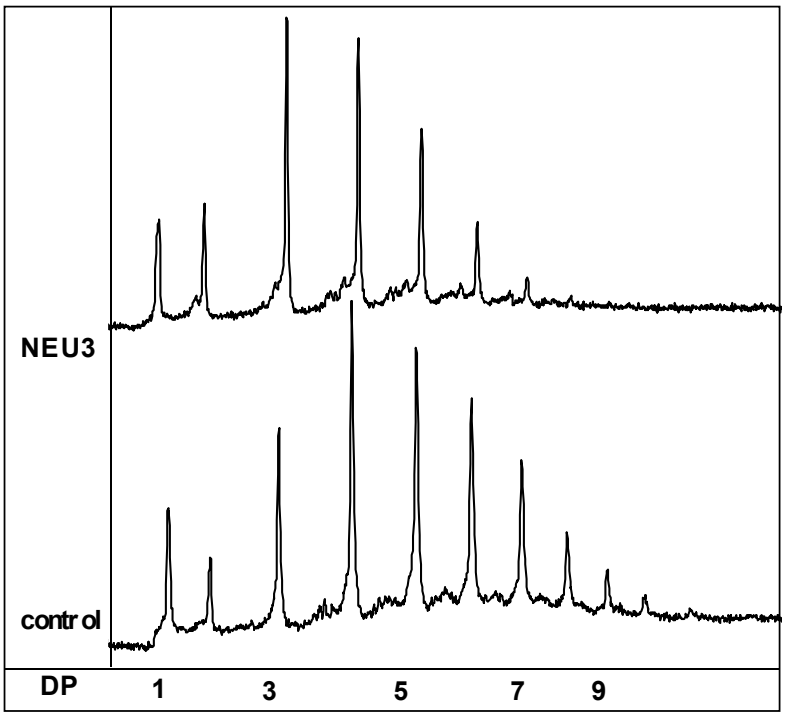
A4.5 Representative chromatograms from oligosialic acid degradation assays.



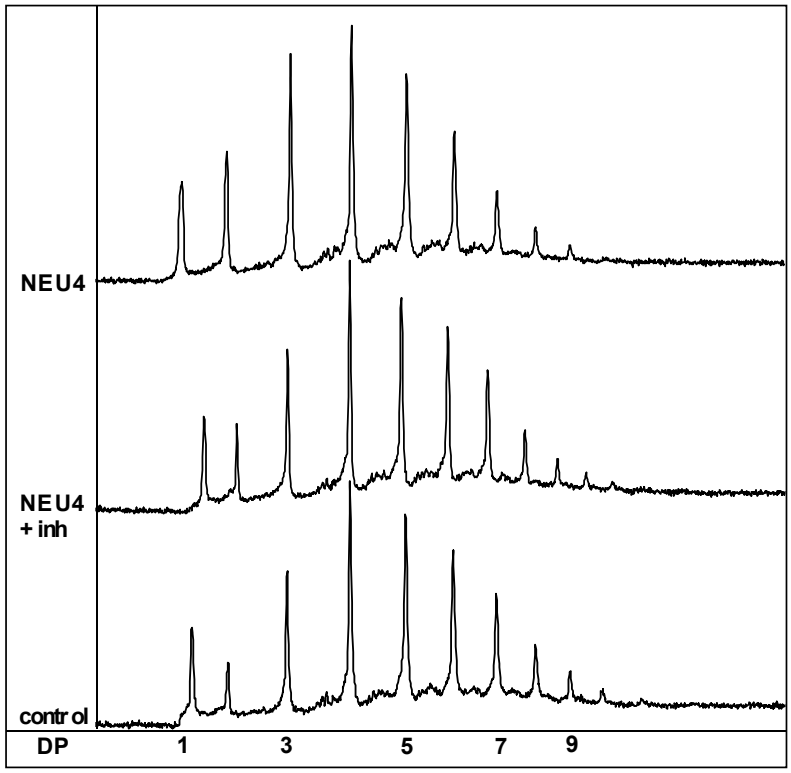
NEU2 pH 5.5



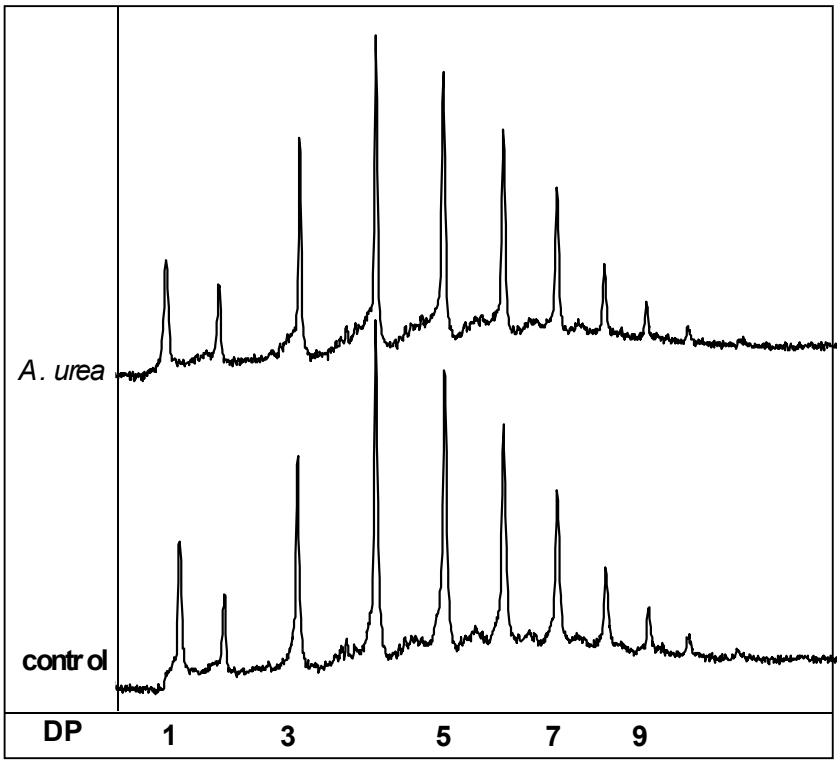
NEU3 pH 4.5



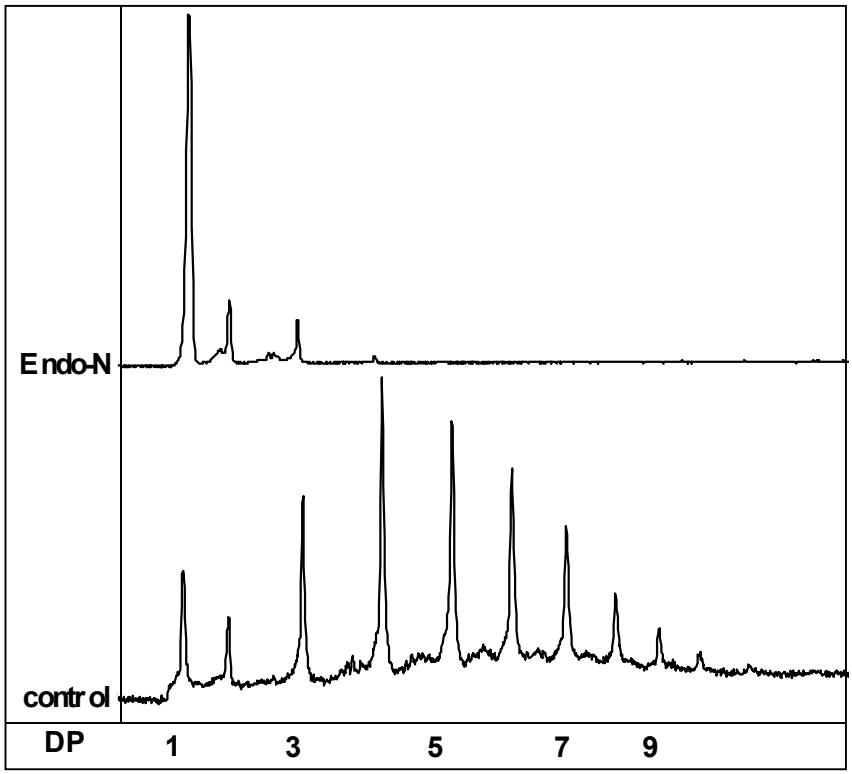
NEU4 pH 4.5



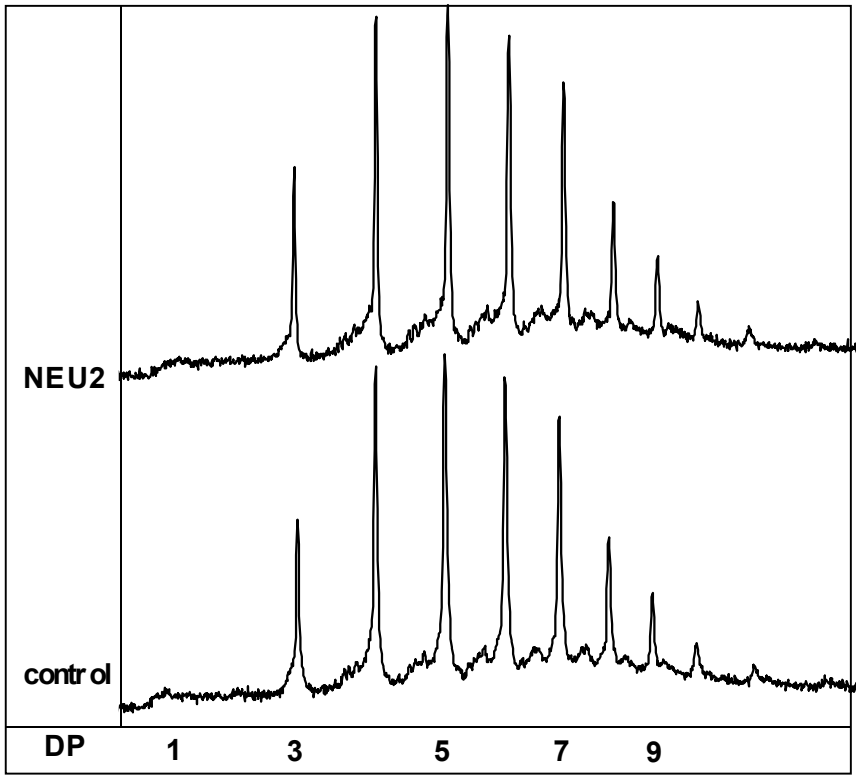
A. ureafaciens pH 4.5



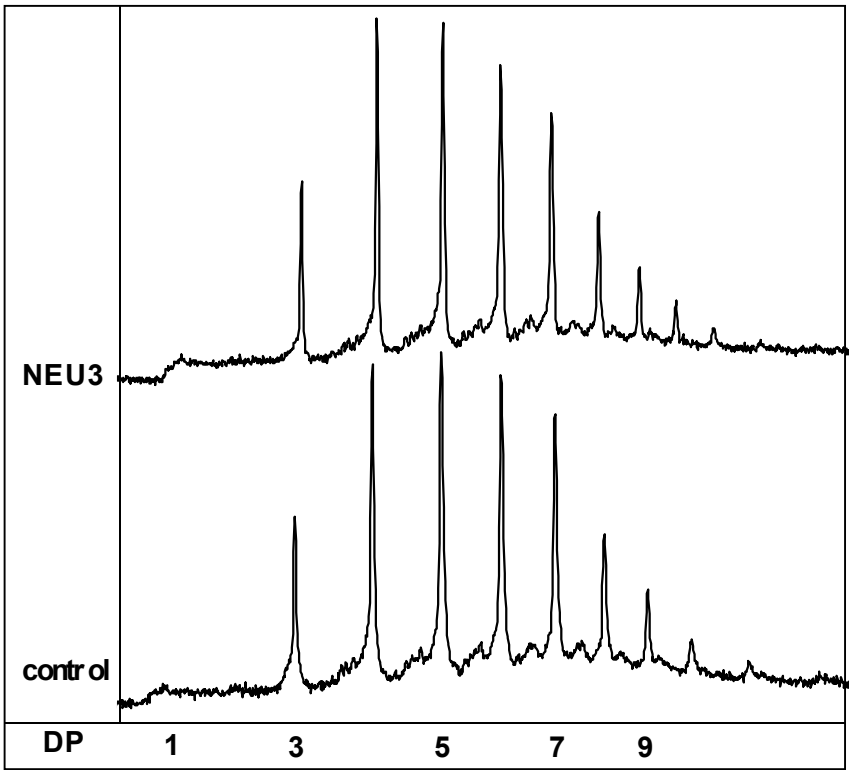
Endo-N pH 4.5



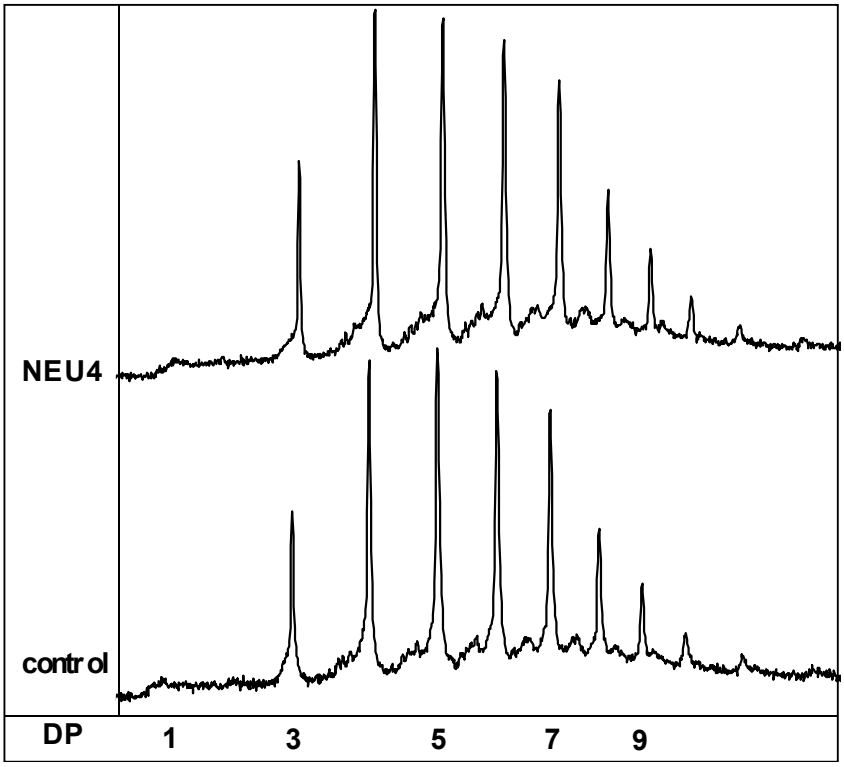
NEU2 pH 7



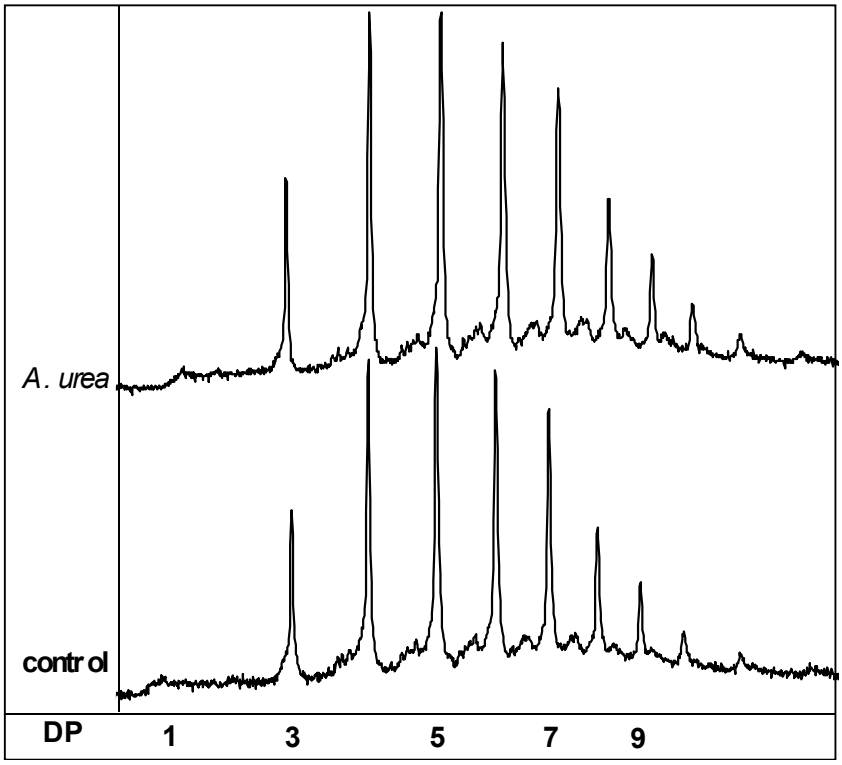
NEU3 pH 7



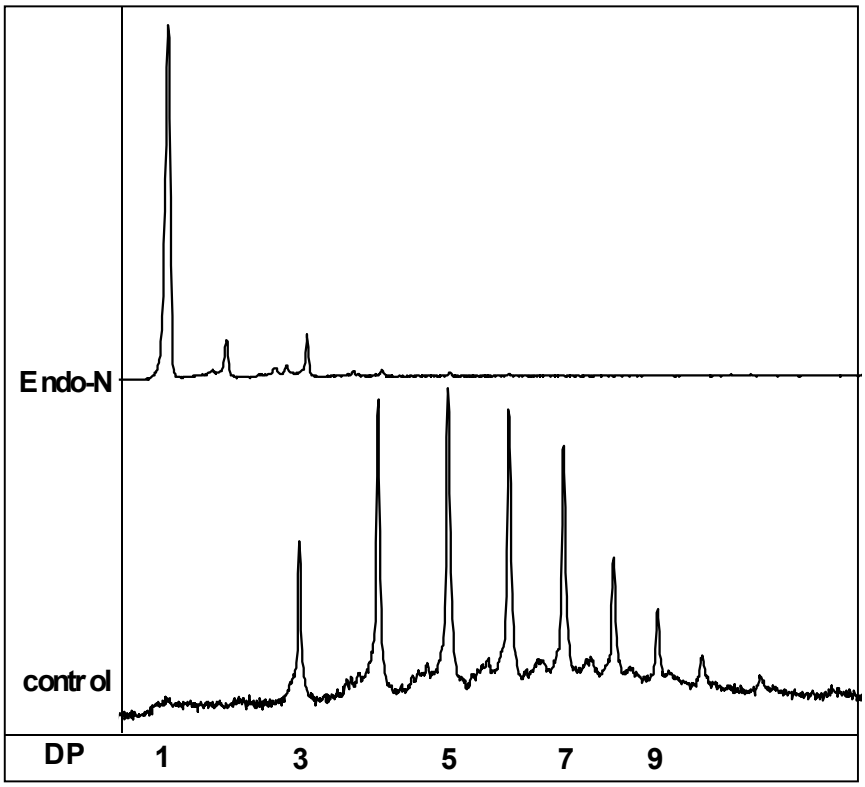
NEU4 pH 7



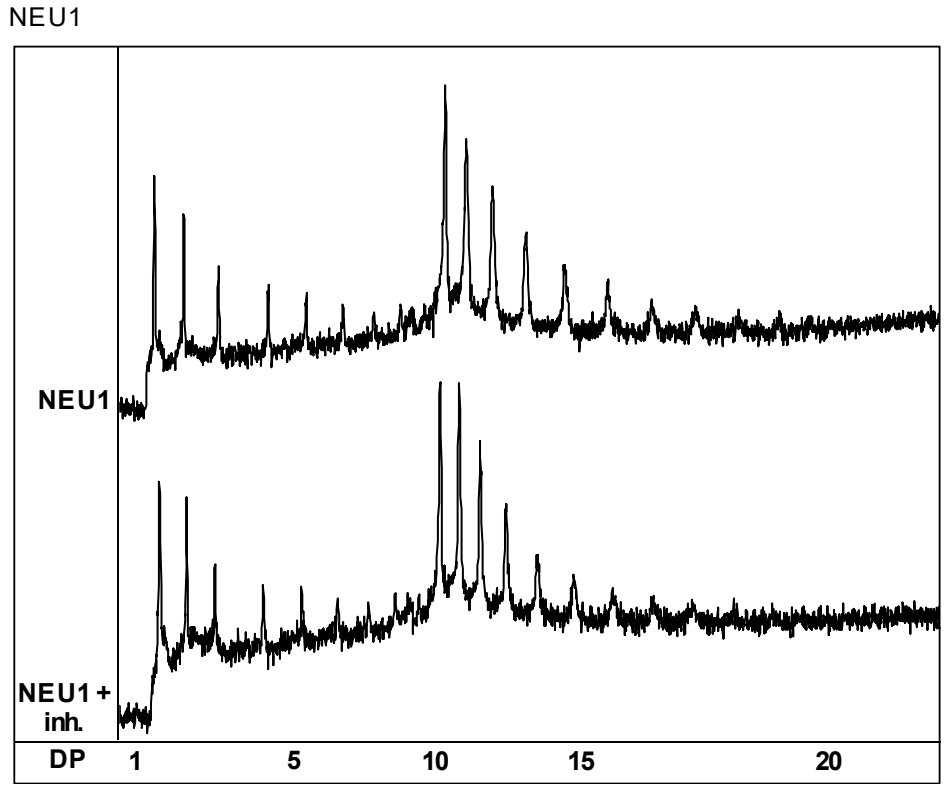
A. ureafaciens pH 7

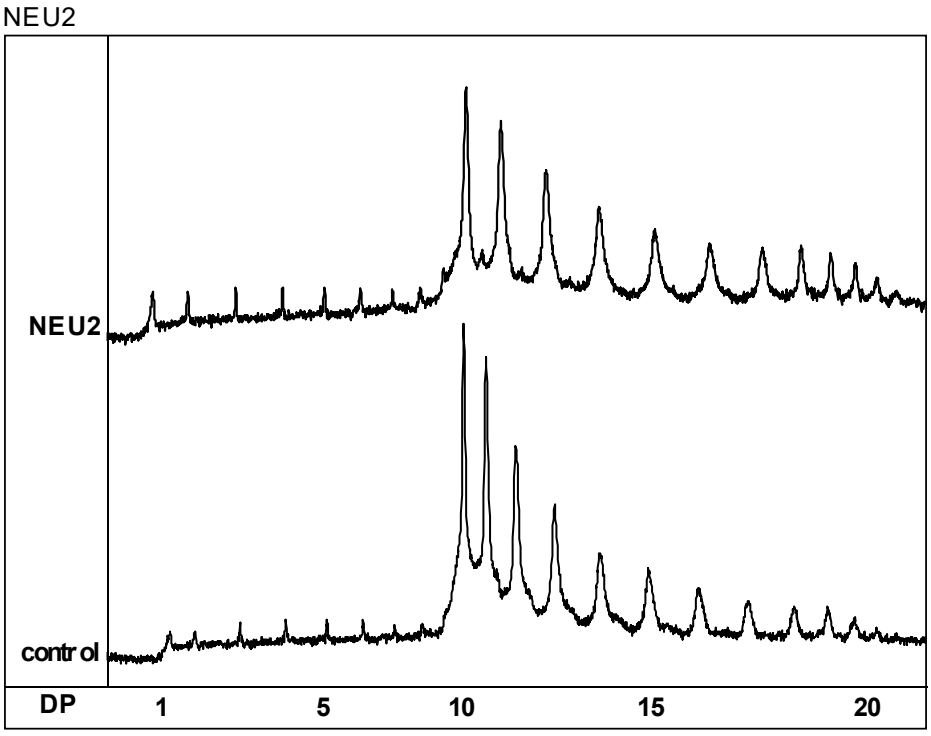


Endo-N pH 7

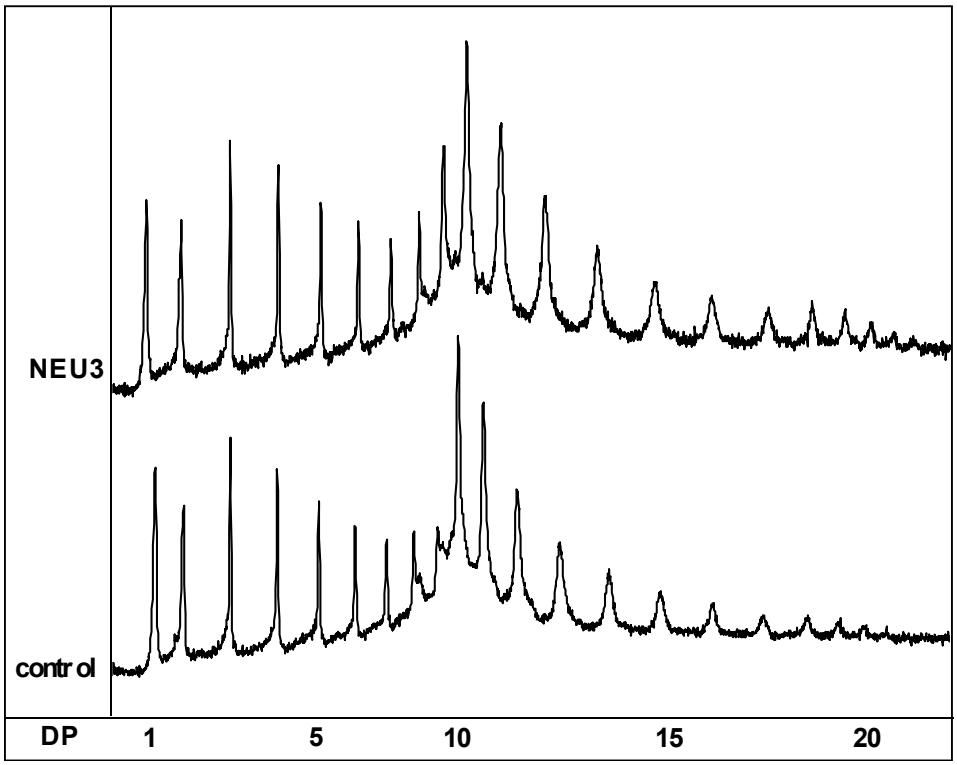


A4.6 Representative chromatograms from polysialic acid degradation assays.

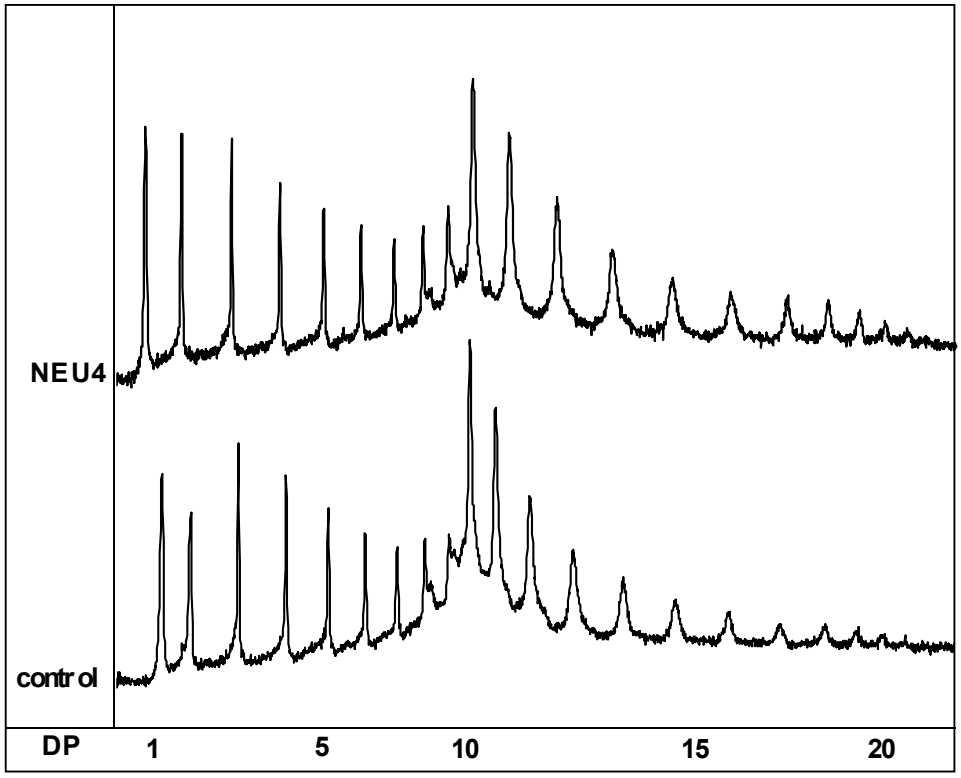




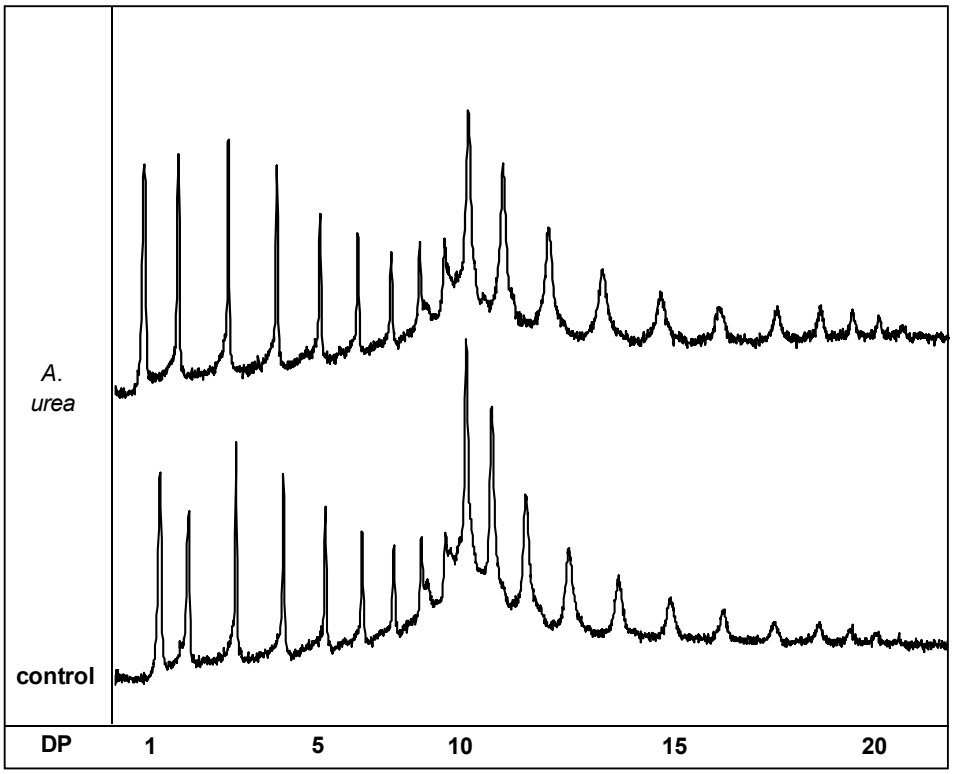
NEU3



NEU4



A. ureafaciens



Endo-N