Chemical Tools for the Synthesis and Analysis of Glycans

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CHEMICAL TOOLS FOR THE SYNTHESIS AND ANALYSIS OF GLYCANS

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By Thamrongsak Cheewawisuttichai

Dissertation Advisor: Dr. Matthew Brichacek


Glycans can be found in every living organism from plants to bacteria and viruses to human. It has been known that glycans are involved in many biological processes such as structural roles, specific recognition with glycan-binding proteins, and host-pathogen recognitions. However, the understanding of glycan structure-function relationship is limited due to the structural complexity and heterogeneity. In this work, chemical approaches have been developed to facilitate the investigation of structures and functions of glycans. A tetrazine auxiliary was synthesized and its utility in a variety of glycomic applications including purification and fractionation of complex carbohydrates, compatibility in chemoenzymatic reaction with glycosyltransferases, and direct conjugation to chemical probes was demonstrated. A new chemoselective transformation of aldehydes to nitriles was discovered and found to be useful for a variety of substrates such as reducing sugars and simple aldehydes. Furthermore, the chemical tools were applied to the chemical investigation of poly(ADP-ribose) including the functionalization of ADP-ribose with the tetrazine auxiliary and the synthesis of a photoremovable β-NAD⁺ analogue.
DEDICATION

To my parents Bundhid and Samaporn, my brother Nutthapong and my aunt Jongrak Tunglamai for their everlasting love.

To my greatest supporters Margaret Kerr and Suttirak Chaiwongkarjohn for their limitless support.
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1.1. Introduction to Glycans and Their Biological Functions

Glycan is a generic term that refers to any type of carbohydrates from simple monosaccharides to complex oligo- and polysaccharides. Glycans can be found as glycoconjugates by connecting to different macromolecules such as proteins and lipids, and they are also found as free glycans.¹ Glycoproteins are polyamides that contain saccharides covalently attached via N- or O-linkages to the peptide backbone. It was reported that more than half of all proteins are glycosylated, of which 90% are observed to have N-glycosylation and around 10% are O-glycosylation.² Glycan in glycolipid is usually covalently linked to the terminal hydroxy group of the lipid moiety.¹

There are many monosaccharides available in nature; however, only 10 are commonly found in mammalian glycans including D-glucose, D-galactose, D-mannose, N-acetyl-D-glucosamine (GlcNAc), N-acetyl-D-galactosamine (GalNAc), L-fucose, D-xylose, D-sialic acid, D-glucuronic acid, and D-iduronic acid (Figure 1.1).³ To avoid the complication when drawing these glycan structures, the Consortium of Functional Glycomics (CFG) has agreed to use the glycan symbol system which uses shapes and colors to indicate monosaccharides.⁴ The symbols of 10 common monosaccharides are shown in Figure 1.2.
Figure 1.1: Monosaccharides commonly found in mammalian glycans.

Figure 1.2: Glycan symbols.
Each monosaccharide can form a glycosidic linkage from its anomeric carbon to any hydroxyl group of another monosaccharide to create disaccharide. The configuration at the anomeric center can be either an α or β form (Figure 1.3). Each different configuration plays a unique role in biological functions. The structures of glycan become more complicated when the number of monosaccharides is higher because they can form linear or branched oligosaccharides. The study of mammalian oligosaccharides revealed that 25% of oligosaccharides are in linear form, and the rest of them contain at least one branch.3 Also, the average number of monosaccharides in mammalian oligosaccharides is about 8 units.3

Glycans are involved in almost all biological processes. They are found in all living systems, including prokaryotic and eukaryotic cells. The roles of glycans can be divided into three major categories: (i) structural and modulatory roles, (ii) specific recognition with other biomolecules which can be divided into two subclasses including intrinsic...
(intraspecies) recognition and extrinsic (interspecies) recognition, and (iii) molecular mimicry of host glycans.\textsuperscript{1,5}

The most obvious structural role that glycans serve is a plant cell wall which contains cellulose and hemicelluloses.\textsuperscript{6} The glycocalyx (glycoproteins and glycolipids) covers the surface of endothelial cells in bacteria and other cells are involved in many processes such as cell adhesion, maintaining and providing humidity in cell, and providing protection of cell from other substances such as antibiotic compounds.\textsuperscript{7} Inside the cell, glycans are known to participate in the folding of proteins synthesized in the endoplasmic reticulum (ER) and Golgi by covalent modifications such as $O$-mannosylation.\textsuperscript{5,8} Furthermore, the glycosylated proteins can either prevent or induce the degradation of proteins from the proteasome.\textsuperscript{5,9} There are other roles of glycans related to physical properties such as increased water solubility of macromolecules, lubrication of mucins, protection from immune recognition, etc.\textsuperscript{5,9}

It is well known that glycans are involved in recognition and signaling processes. There are examples reported which signify the role of glycans in cellular trafficking such as the mannose-6-phosphate recognition in the ER-Golgi pathway.\textsuperscript{5} Figure 1.4 illustrates the interactions and recognitions of glycans with glycan-binding proteins (GBPs), bacteria, and viruses.\textsuperscript{10} The specific recognitions of glycans and GBPs (lectins, antibodies, and enzymes) mediate biological functions such as cell-cell adhesion, regulation of proteins and protein turnover, regulate phosphorylation.\textsuperscript{10} Furthermore, glycans can mediate the recognition between species such as host-pathogen recognition. For example, viruses recognize sialic acids on a host cell containing hemagglutinins.\textsuperscript{5}
Glycans are the most diverse biomacromolecules. The non-template formation and heterogeneity of glycan chains can participate in multiple ways in biological processes. Glycans are usually formed as mixtures, which made it difficult to predict their specific roles. Also, the different functions can be found from the same glycans depending on the cellular- and tissue-specific locations that glycans present. The study of glycan has advanced in the past decade; however, there are more glycans yet to be studied and discovered. Therefore, it is necessary to have robust and reliable methods to elucidate the structures and functions in glycobiology.
1.2. Methods to Elucidate Structures and Biological Functions of Glycans

There are four major classes of macromolecules in living systems, including proteins, nucleic acids, lipids, and glycans. Despite the advances in the study of proteins and nucleic acids (DNA and RNA), the development of tools in glycan studies is deficient. One of the major reasons that impedes developments in glycobiology is the diversity of glycans in nature. While proteins and nucleic acids are formed in a template-directed manner, glycans can be linear or branched at a variety of available hydroxy positions. Furthermore, the glycosidic linkages that connect two monosaccharides can be either an α- or β-linkage, and they have distinct biologically recognition. Therefore, it is a challenge for scientists to elucidate the structures of glycans and their biological functions.

1.2.1. Releasing of Glycans

In nature, glycans are in the form of glycoconjugates such as glycoproteins, glycolipids, glycosphingolipids, etc. There are two main strategies to release glycans: enzymatic release and chemical release. Peptide-N-glycosidase F (PNGase F) is an enzyme that cleaves all N-glycans that have a linkage between the core GlcNAc and asparagine residue.\(^\text{11}\) However, it cannot release glycans that contain fucose-linked GlcNAc attach to the protein.\(^\text{12}\) Other N-glycan releasing enzymes such as endoglycosidase H (endo H), which releases high-mannose glycans, endoglycosidase D (endo D) which cleaves all types of N-glycans, and endoglycosidase F3 (endo F3) which cleaves biantennary (two branches) and triantennary (three branches) glycans.\(^\text{11,13}\) Although there are many enzymes available for releasing N-glycans, the enzymatic release of O-glycans is challenging.
Currently, endo-\(\alpha\)-N-acetylgalactosaminidase (O-glycanase) is the only enzyme available which cleaves O-glycans between GalNAc and serine or threonine residues.\(^{14}\)

A chemical release is an option for releasing both N- and O-glycans from glycoconjugates. Reductive \(\beta\)-elimination under alkaline solution followed by sodium borohydride to reduce the terminal end of released glycans is commonly used for O-glycans.\(^{11,15}\) Hydrazinolysis is another approach to release N- and O-glycans using hydrazine to functionalize the reducing end of glycan.\(^{16,17}\) Recently, the oxidative release of glycans from their natural sources using sodium hypochlorite (NaClO) was reported to be an alternative approach.\(^{18}\) Although the chemical release of glycans is useful and accessible, there are concerns about the degradation of glycans during the chemical treatments.

### 1.2.2. Derivatization of Glycans

After releasing glycans from glycoconjugates, free glycans are usually derivatized with functional groups prior to further analysis. Due to the characteristics of native glycans which are very polar and lack a chromophore, derivatizing glycans with hydrophobic and fluorescent or UV active compounds would benefit the separation and detection of a glycan pool and would also enhance the ionization signal for mass spectrometry (MS).

Glycan is usually chemoselectively labeled at the reducing end because it contains an aldehyde group. A reductive amination is a popular approach for labeling glycans with chromophores or fluorophores. Details of the reactions on the reducing end of glycan will
be described in chapter 1, section 1.3. A variety of labeling compounds have been developed such as 2-aminobenzoic acid (2-AA), 2-aminobenzamide (2-AB), 2-aminopyridine (2-AP), and 8-aminopyrene-1,3,6-trisulfonic acid (APTS) (Figure 1.5).19

Permethylation is another approach to modify glycans by converting hydrogens on hydroxyl groups, amine groups, and carboxyl groups to a methyl group.15 This method is commonly used to derivatize glycans before analyzing with mass spectrometry (MS) because it enhances ion intensity and facilitates the spectral interpretation.20 Furthermore, the labile bond between sialic acid and other saccharides can be stabilized by permethylation.

![Figure 1.5. Structures of glycan labels.](image)

1.2.3. Mass Spectrometry

The structural complexities and variation in the physical properties of glycans have challenged scientists to investigate their structures relating to biological functions. There
are several approaches currently developed in an attempt to study and characterize the structures of glycans. At present, mass spectrometry (MS) is a powerful technique for obtaining structural information of glycans because it requires a small sample and provides high sensitivity. The limits of detection of MS techniques is in the range of femtomolar to attomolar. There are two general ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). The benefit of using the ESI technique is that it can be coupled with liquid chromatography. In addition, both neutral and anionic glycans (such as sialylated compounds) can be analyzed in positive mode via an ESI. In contrast, MALDI detects neutral glycans in a positive mode and anionic glycans in a negative mode. However, MALDI ionization is still widely used for the analysis of glycans obtained from biological samples because it has higher sensitivity and tolerance to contaminants compared to ESI. Also, MS spectra obtained from MALDI ionization is simple to determine compared to spectra from ESI.

The structural analysis of glycans can become more complicated when the sample contains mixtures of complex glycans. Therefore, the chromatographic separation of the mixtures prior to analysis by MS can be useful. Liquid chromatography is very common for the separation of complex glycans. There are several approaches for liquid chromatography depending on types of stationary phase. Reverse-phase liquid chromatography (RPLC) is the method that uses hydrophobic (C18 or C8) functionalized silica as the stationary phase. Hydrophilic interaction liquid chromatography (HILIC) is the technique that uses a highly polar stationary phase such as bare silica particles, amine-, amide-, or zwitterion-bonded silica. Glycans will interact with the stationary phase via
hydrogen bonding, dipole-dipole, or ion-dipole interactions.15 Porous graphitized carbon (PGC) is becoming more popular to use as a stationary phase. Although PGC is considered as a hydrophobic stationary phase, native glycans and reduced glycans tend to retain better than on a C18 column. Hydrophobic, polar, and ionic interactions between glycans and PGC column allow glycans to retain longer in the column.24 Figure 1.6 is an example demonstrating the separation and MS analysis of N-glycans released from immunoglobulin G (IgG).25 Figure 1.6a shows the ability to separate complex glycans through HILIC technique and Figure 1.6b shows MALDI-MS data obtained after permethylation of released glycans.

Figure 1.6: Glycans released from human IgG: (a) HILIC UHPLC chromatogram after 2-AB labeling and (b) MALDI-TOF-MS after permethylation. Reprinted with permission from ref 25. Copyright (2016) American Chemical Society.
1.2.4. Microarray

Inspired by the studies of DNAs and proteins through the array technologies, glycan microarrays were introduced to study the interaction of glycans with glycan-binding proteins (GBPs). The process of this technique includes (i) the immobilization of glycans on the solid surface, (ii) the introduction of GBPs, and (iii) detection, as demonstrated in Figure 1.7.26

![Illustration of processes in the study of glycan microarray.](Image)

Figure 1.7: Illustration of processes in the study of glycan microarray. Reprinted with permission from ref 26. Copyright 2016 Royal Society of Chemistry.

The approach to the immobilization of glycans on the surface is important because it can affect the interaction of GBP. There are two major strategies including noncovalent and covalent immobilizations.27 The most convenient strategy is to use polysaccharide arrays because glycans can be noncovalently immobilized on the nitrocellulose surface without chemical modification.28 However, this technique is limited to glycans with high molecular weights. The most commonly and extensively used technique to immobilize glycans on the surface is by chemically modifying the reducing end of glycans with a linker,
then covalently attaching on the surface. This strategy will allow for site-specific attachment. Furthermore, linkers that contain a chromophore or fluorophore provide the ability to determine the concentration of glycans printed on the surface array. Therefore, it is important to select the appropriate linker and type of reaction to attach the glycans and construct the glycan arrays. It is known that the nature of the linker, length, and functional group can affect the binding-proteins. There are several ligation reactions reported, and examples are shown in Table 1.1.\textsuperscript{27} The maleimide-thio reaction (entry 1) is one of the earliest methods for the immobilization of a glycan array.\textsuperscript{29,30} In this approach, carbohydrates were functionalized with a thiol-terminated linker and immobilized on the maleimide-coated surface. However, the major drawback of this type of reaction is that the thiol functional group can be readily oxidized with air, which requires caution while constructing the array. The selective reaction between hydrazide and epoxide was introduced in 2005.\textsuperscript{31} A hydrazide-based linker was functionalized onto a carbohydrate then immobilized on the surface that was coated with epoxide (entry 2). This reaction is highly selective even though there are other nucleophiles such as thiols and amines present in the reaction. The inverse-electron-demand Diels-Alder (IEDDA) reaction was introduced in 2012\textsuperscript{32} for the production of glycan arrays (entry 3). In this process, the electron-rich dienophile was attached to the carbohydrate, and the electron-poor diene (tetrazine) was coated on the surface. This reaction is fast and highly selective without the need of a catalyst. Copper-catalyzed cycloaddition between alkynes and azides were also developed for glycan array applications. In this approach, an alkyne-terminated linker was attached to a carbohydrate, then immobilized on the azide-coated
surface via a Cu(I)-catalyzed 1,3-dipolar cycloaddition. Similar to the IEDDA reaction, this reaction is highly chemoselective while there are other functional groups present.

Table 1.1 Example of the immobilization reactions on the surface array.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reaction</th>
<th>Representative scheme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Maleimide-thiol reaction</td>
<td><img src="image1" alt="Maleimide-thiol reaction" /></td>
</tr>
<tr>
<td>2</td>
<td>Epoxide-hydrazide reaction</td>
<td><img src="image2" alt="Epoxide-hydrazide reaction" /></td>
</tr>
<tr>
<td></td>
<td>Tetrazine-dienophile cycloaddition</td>
<td><img src="image3" alt="Tetrazine-dienophile cycloaddition" /></td>
</tr>
<tr>
<td>3</td>
<td>Tetrazine-dienophile cycloaddition</td>
<td><img src="image4" alt="Tetrazine-dienophile cycloaddition" /></td>
</tr>
<tr>
<td>4</td>
<td>Azide-alkyne cycloaddition</td>
<td><img src="image5" alt="Azide-alkyne cycloaddition" /></td>
</tr>
</tbody>
</table>
The interaction of glycans printed on the array and GBP\(\text{s}\) represents the interaction that happens in a cell (Figure 1.8a).\(^\text{33}\) Designing and constructing glycan arrays is important because the recognition of GBP\(\text{s}\) depends on the orientation of the glycans, the spacing between glycans, and the length of linker from the surface\(^\text{27,33,34}\) (Figure 1.8b). An array that contains a high density of glycans enables multivalent interaction between glycans and proteins, which is suitable for the study of GBP\(\text{s}\) that have weak interaction with glycans at the monovalent level.

![Figure 1.8: Comparing interaction between glycans and GBP\(\text{s}\): (a) in a cell, (b) on the microarray. Reprinted from ref 33 with permission from Elsevier.](image-url)
There are many detection methods for the study of glycan microarrays (Figure 1.9)\textsuperscript{27}. The most commonly used is fluorescence detection because the reagents are commercially available and the instruments that measure fluorescence are common for many laboratories.\textsuperscript{27} The general approach is to attach fluorophores on either GBPs or secondary reagents that bind the GBPs. Then, the binding interaction can be analyzed based on the fluorescence intensity (Figure 1.9a). Other glycan array detection approaches including mass spectrometry detection\textsuperscript{35} which have been used to monitor enzymatic reaction (Figure 1.9b) and surface plasmon resonance (SPR) imaging\textsuperscript{36,37} which is used for real-time imaging of binding interactions (Figure 1.9c).

![Detection Methods](image)

*Figure 1.9: Detection methods for the study of glycan microarrays: (a) fluorescence detection, (b) mass spectrometry detection, and (c) surface plasmon resonance detection. Reprinted with permission from ref 27. Copyright 2013 Royal Society of Chemistry.*
1.2.5. Imaging via Chemical Probes

For a complete understanding of the roles of glycans, it is necessary to be able to detect the changes of glycans and other events in biological systems. The development of imaging technology provides the ability to visualize and detect the changes of glycans in cellular processes by conjugating the chemical reporters onto glycans then visualizing by chemical probes that can specifically react with the reporters. This type of reaction is called bioorthogonal chemistry. Currently, there are many bioorthogonal reactions available depending on the types of applications. Examples of bioorthogonal reactions are presented in Table 1.2. Copper-catalyzed azide-alkyne cycloaddition (entry 1) is shown to be widely used for biomolecule labeling in vitro because the sizes of the functional groups are very small. However, it is not suitable for in vivo labeling because Cu(I) is cytotoxic.

Strain-promoted azide-alkyne cycloaddition (SPAAC) was later introduced for the chemoselective cycloaddition without using a cytotoxic metal catalyst (entry 2). This reaction was designed to use a ring-strained cyclooctyne which promptly reacts with azide under ambient condition. SPAAC has been widely used for biomolecule labeling for imaging in vivo. Another popular cycloaddition for biomolecule labeling is the reaction between an azide and triaryl phosphine, which is called Staudinger ligation (entry 3). This reaction is highly selective and suitable for a cellular environment. Although the rate of the Staudinger reaction is often slow, it is widely used for in vivo studies because this reaction is compatible with living cells. The IEDDA reaction between trans-cyclooctene and tetrazine (entry 4) is another popular choice for imaging in living
systems because the reaction rate is fast with a rate constant from $10^3$ to $10^6 \text{ M}^{-1}\text{s}^{-1}$.\textsuperscript{49,50}

Furthermore, only small amounts of reagents are required for the reaction without using an excess of chemical probes which improves the signal-to-noise ratios.\textsuperscript{38}

Table 1.2 Example of bioorthogonal reactions.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reaction</th>
<th>Reactant 1</th>
<th>Reactant 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Copper-catalyzed azide-alkyne cycloaddition (CuAAC)</td>
<td>$R-N_3$</td>
<td>$R\equiv\text{ }, \text{Cu(I)}$</td>
</tr>
<tr>
<td>2</td>
<td>Strain-promoted azide-alkyne cycloadditions (SPAAC)</td>
<td>$R-N_3$</td>
<td>$\text{ }$</td>
</tr>
<tr>
<td>3</td>
<td>Staudinger ligation 1,3-Cycloaddition</td>
<td>$R-N_3$</td>
<td>$\text{ }$</td>
</tr>
<tr>
<td>4</td>
<td>Inverse electron-demand Diels-Alder reaction</td>
<td>$\text{ }$</td>
<td>$\text{ }$</td>
</tr>
</tbody>
</table>
1.3. Chemoselective Reaction with Unprotected Carbohydrates

As introduced in section 1.2.2, the current methods to investigate biological functions of glycans can be accomplished by conjugating glycans with noncarbohydrate molecules (ex, auxiliaries, linkers, fluorophores), then studying the glycoconjugates through various techniques such as attachment on the microarrays, labeling of glycans for bioimaging,\textsuperscript{20} etc.

A number of chemoselective reactions have been developed over the past decade including \textit{O}-, \textit{N}-, \textit{S}-, and \textit{C}-linked glycoconjugates.\textsuperscript{51} In this thesis, only examples that are relevant to our projects will be described. The main idea of these reactions is to take advantage of the reducing end of carbohydrate as it contains an aldehyde in a ring-opened form (Scheme 1.1). This unique property provides the ability to conjugate directly at this position without disturbing other functional groups. Therefore, carbohydrates can be directly functionalized without a need for protecting groups.

\begin{center}
\includegraphics[width=\textwidth]{chemoselective_reactions.png}
\end{center}

\textbf{Scheme 1.1:} Schematic illustration of chemoselective reactions of unprotected carbohydrates.
1.3.1. Reductive Amination Reaction

Reductive amination is the reaction of an aldehyde and a primary amine resulting in the formation of an imine. An imine is then reduced with reducing agents such as NaBH₃CN to yield a secondary amine (Scheme 1.2). Reductive amination is widely used to label glycans for microarray applications because it is highly selective to aldehydes and tolerates other functional groups. Furthermore, it is a stoichiometric reaction which is useful for the quantitation of glycans based on the UV-absorbance or fluorescence intensity. Nevertheless, obtaining an unnatural ring-opened form as a product can be a drawback of this reaction because it can disturb the recognition of GBPs. There are proteins that only bind to ring-closed glycoconjugates such as anti-sialyl Lewisx (SLex) antibody 1 and 2, and intravenous immunoglobulin (IVIg). However, most GBPs demonstrate the ability to bind ring-opened glycoconjugates on an array. It is known that GBPs contain binding pockets that can access a few numbers of monosaccharide residues. Having a ring-opened form at the terminal monosaccharide may not disrupt their interactions. Cummings and coworkers have shown that ring-opened glycoconjugates on the array can have better interaction with several GBPs because the sterically restricted ring-closed form is reduced.
1.3.2. Formation of Oximes and Hydrazones

Oximes and hydrazones are products from the conjugation of carbohydrates with \(\alpha\)-nucleophiles (hydroxylamine and hydrazine). The reaction is similar to the reductive amination reaction as it occurs at the carbonyl carbon of an aldehyde, and the reaction is usually catalyzed by acid (Scheme 1.3). The attack of an \(\alpha\)-heteroatom on the carbonyl carbon results in the formation of a tetrahedral intermediate. After protonation of the hydroxyl group, the reaction undergoes dehydration resulting in oxime or hydrazone as the product (Scheme 1.3).\(^{51,53}\) The obtained product can be in both ring-opened (also both \(E\) and \(Z\)) and ring-closed (also both \(\alpha\) and \(\beta\)) forms (Scheme 1.4). These isomers are interconvertible. Therefore, the concentration of species are determined from their thermodynamic stability.\(^{51}\)
The hydrolytic stability is dependent on the inductive effect of the heteroatom.\textsuperscript{54} The hydrolysis of oxime and hydrazone can be obtained from the protonation of the imine nitrogen. In this case, oximes are more stable than hydrazones because the electronegativity of oxygen is higher than nitrogen, which prevents protonation of the imine nitrogen.\textsuperscript{51,53,54} The reversibility and the difference in hydrolytic stability of oximes and hydrazones are beneficial for various types of applications. Oximes are suitable for those applications that require a more stable linkage such as bioconjugation reactions, while hydrazones are better in applications that need labile linkage such as the controlled release of biologically active compounds.\textsuperscript{53,55}
1.3.3. Formation of \(N,O\)-Dialkyl Oxyamines

The substrates of \(N,O\)-dialkyl oxyamines are different from the oxyamines that form oximes in which they are dialkyl substituted on both nitrogen and oxygen on the molecules. The reaction is similar to the formation of the oximes that takes place on an aldehyde in its ring-opened form (Scheme 1.5). The advantage of \(N,O\)-dialkyl oxyamines compared to the oximes is that the conjugated products are in the thermodynamically most stable ring-closed forms, either \(\alpha\)- or \(\beta\)-form depending on the nature of the carbohydrate substrates.\(^{51}\) As demonstrated by Langenhan et al.\(^{56}\), neoglycoconjugated product from glucose presented in an only \(\beta\)-pyranose form while three isomers: \(\beta\)-pyranose, \(\alpha\)-pyranose, and \(\alpha\)-furanose were obtained as the products from mannose. Nevertheless, the equilibrium constant \((K_{eq})\) of this oxyamine glycoconjugation is low. It is usually required a high concentration of reagents (0.1 – 1 M) to increase the yield of the conjugated products.\(^{51}\)
The ring-closed form obtained as a product is a benefit for the study of glycan because it mimics native $O$- and $N$-glycosides. In the past decade, a variety of different $N,O$-dialkyl oxyamine linkers had been developed especially for the glycan microarray applications (Figure 1.10). In 2014, Boons and coworkers$^{57}$ developed a multifunctional linker 1-1 that contained a dialkyl oxyamine for the construction of the neoglycosides with reducing sugars and a hydrophobic chromophore for the UV detection and purification of the conjugated products via reverse-phase column. The advantage of this linker is that the terminal Fmoc (fluorenylmethyloxycarbonate) group on the linker can be removed yielding a free amine which can be used for the construction of the microarray. In 2017, the same group had modified their oxyamine linker to have hydrophobic chromophore and free amine on the same linker without the need of deprotection (1-2)$^{58}$ Another bifunctional dialkyl oxyamine linker (1-3) was reported by Timmer and coworkers$^{59}$ in

Scheme 1.5: Proposed mechanism for the formation of dialkyl-oxyamine.
2015. Linker 1-3 is capable of the formation neoglycosides with carbohydrates and the conjugation with chemical probes from the terminal azide group. The primary amine can also be obtained from this linker by reducing at the azide. Although linker 1-3 demonstrated to be highly stable toward hydrolysis, it is lack of a hydrophobic chromophore which might not be useful for the separation and purification purposes. The aminoxy-based fluorescent linker 1-4 was developed by Wilson et al.\textsuperscript{60} in 2016. This bifunctional linker contained dialkyl oxyamine for the neoglycoconjugation and the azide group for the conjugation with chemical probes or for the immobilization on the solid support after reduction of an azide to a primary amine. Unlike linker 1-3, linker 1-4 contains a fluorescent naphthalene group that aids in the separation and detection through reverse-phase chromatographic technique.

![Figure 1.10: Existing N,O-dialkyl oxyamine linkers.](image)
1.3.4. Wohl Degradation

Wohl degradation is the conventional method to shorten aldose sugars which was first reported by Wohl in 1893. The reaction is involved in the formation of an oxime by hydroxylamine followed by the acetylation using acetic anhydride and sodium acetate to form an acetylated cyanohydrin. Then, the terminal cyanide group can be removed by the treatment with ammonia and silver hydroxide (Scheme 1.6). However, the original Wohl degradation method was modified by treating the cyanohydrin with sodium methoxide in methanol that led to the elimination of cyanide and deacetylation, also known as Zemplén degradation. The product obtained from the Wohl degradation is an aldose sugar that has one carbon shorter than the starting material.

Scheme 1.6: Reaction pathway of Wohl degradation.
As shown in Scheme 1.6, the cyanohydrin (nitrile containing hydroxyl group at the \( \alpha \)-carbon) is an intermediate from the Wohl degradation process, which can be useful in other applications. Several methods reported the transformation of aldehydes to nitriles, however, they focused on the transformation of aliphatic and aromatic aldehydes. Bandgar and Makone reported the transformation of aldehydes to nitriles using N-bromosuccinimide (NBS) and aqueous ammonia (Scheme 1.7).\(^6\) They demonstrated that the reaction was suitable for a variety of substrates from aromatic aldehydes to D-glucose and 2-deoxy-D-ribose.

![Scheme 1.7: Transformation of aldehydes to nitrile using NBS.](image)

Several research groups have shown that carbohydrate cyanohydrins could be used as the intermediates to form biologically active compounds. The formation of tetrazole-containing carbohydrate has been reported by forming cyanohydrin as an intermediate followed by the formation of a cyclic tetrazole (Scheme 1.8).\(^6\) It is known that tetrazole has biologically active properties such as antifungals\(^6\) and glycosidase inhibitor,\(^6\) however, the authors did not report the specific biological properties of the compound.\(^6\) Another example of the application of carbohydrate cyanohydrins was reported by Fernandes and coworkers\(^6\) in 2015. They synthesized ruthenium(II) and
iron(II) complexes containing carbohydrate cyanohydrins as the ligands (Scheme 1.9) and demonstrated that these complexes had the cytotoxic activity and could be used as the colorectal anticancer agents.\textsuperscript{67}

Scheme 1.8: Formation of tetrazole from carbohydrate cyanohydrin.

Scheme 1.9: Synthesis of ruthenium(II) and iron(II) complexes containing carbohydrate cyanohydrins as ligands.
1.3.5. Kiliani-Fischer Cyanohydrin Synthesis

Kiliani-Fischer synthesis is the reaction to elongate aldose sugars by adding one more carbon. The reaction involves the addition of the cyanide to the aldehyde forming cyanohydrin as an intermediate, which is subsequently hydrolyzed to form aldonic acid lactone. The treatment of the lactone with reducing agents such as NaBH₃ or with catalytic hydrogenation yields a new carbohydrate compound with one extra carbon (Scheme 1.10). However, the products obtained from this reaction are in the mixture of diastereomers.

Scheme 1.10: Reaction pathway for Kiliani-Fischer synthesis.
1.4. Thesis Overview

The structural complexity of glycan impedes the study and understanding of the roles of glycans in biological processes. The ultimate goal of this research is to develop chemical tools for a better understanding of the structures and functions of glycans. There are two chemical approaches presented herein including the development of a chemical auxiliary and the introduction of new synthetic methods, as shown in Figure 1.11.

![Figure 1.11: The overview of the thesis.](image)

The design and synthesis of a new chemical auxiliary are presented in Chapter 2 with the purpose of using the auxiliary in broad applications in glycomics research. A new $N,O$-dialkyl oxyamine auxiliary containing tetrazine moiety was synthesized in 3 steps with a 28% overall yield. The auxiliary is demonstrated to be useful in a variety of applications...
including purification and fractionation of complex glycans, compatibility with glycosyltransferases in chemoenzymatic reaction, and ability to directly conjugate to chemical probes via an inverse-electron-demand Diels-Alder reaction.

A new synthetic method for the transformation of aldehydes to nitriles is presented in Chapter 3. This new synthetic approach is demonstrated to be compatible with a variety of reducing sugars including monosaccharides, disaccharides, and silyl-protected saccharides. Furthermore, the reaction is also suitable for the formation of nitriles from aliphatic and aromatic aldehydes. This synthetic approach could be useful for the further transformation of the nitriles to other functional groups such as amines and heterocycles. Chapter 3 also presents a new efficient method to shorten reducing sugars by using weakly basic resins. This practical approach is applicable for the formation of carbohydrates through the shortening process.

Poly(ADP-ribose) (PAR) is a complex biopolymer that involved in many biological processes such as transcription and DNA damaged repair. The knowledge in the functions of PAR is limited due to the difficulty to obtain the defined length of PAR polymers at the workable scale. There are two approaches to obtain the defined length of PAR presents in Chapter 4. The first approach is using the chemical auxiliary to facilitate the fractionation and purification of PAR polymers. The second approach is the use of a novel β-NAD⁺ analogue containing a photoremovable protecting group as a substrate in the chemoenzymatic synthesis of ADP-ribose oligomers.
CHAPTER 2

DEVELOPMENT OF A MULTIFUNCTIONAL NEOGLYCOSIDE AUXILIARY FOR
APPLICATIONS IN GLYCOMIC RESEARCH

2.1. Introduction

Glycans, and more frequently the glycoconjugates of proteins and lipids, are implicated in virtually all important biological processes. These conjugated glycans can be recognized by glycan-binding proteins (GBPs). The interactions between glycans and GBPs play crucial roles in biological recognition processes such as cell signaling, protein folding, cell adhesion, host-pathogen interactions, etc. The structural complexities and diverse biological functions of glycans necessitates reliable and efficient techniques to conduct glycomic studies. The utilization of mass spectrometry allows the rapid determination of the connectivity of complex carbohydrates. However, mass spectrometric strategy requires separation techniques either by capillary electrophoresis or liquid chromatography for effectively analyzing glycan structures, especially their isomeric species. The study of glycan-protein interactions is often interrogated using microarray technologies in which glycans are modified for the attachment on a slide-type surface and visualized by fluorescently tagged proteins. Most of these analysis approaches usually require the derivatization of glycans with hydrophobic moiety for the separation, detection, and attachment purposes.

Chemoselective ligation reactions at the anomeric center of carbohydrates have been extensively developed during the past decade. The attachment of an auxiliary to a
reducing sugar facilitates examination of biological interactions through the introduction of fluorescent tags, immobilization on a surface microarray, conjugation to biomolecules, as well as increasing retention time for chromatographic separation.\textsuperscript{20,74} A neoglycoside auxiliary (secondary oxyamine) is preferred because it preserves a ring-closed conformation product simplifying recognition by GBPs. Several elegant auxiliaries have been reported exhibiting compatibility with chemoenzymatic extension and/or highly efficient conjugation to surfaces for the production of glycan microarrays.\textsuperscript{57,59,60,75–78} However, in order to visualize the biological processes of these glycoconjugates, they required further chemical modifications for conjugation to chemical probes. The purpose of this work is to develop a multifunctional auxiliary that can serve in a variety of glycomic applications and also enable direct bioorthogonal conjugation without further modification.

Inverse-electron-demand Diels-Alder (IEDDA) reactions between tetrazines and strained dienophiles such as trans-cyclooctene (TCO), cyclooctyne, and norbornene, have gained attention recently due to fast and highly chemoselective cycloadditions.\textsuperscript{79–81} The rate constants of the IEDDA reaction between tetrazine and TCO can be as high as $10^6$ M$^{-1}$s$^{-1}$.\textsuperscript{49,82,83} This exceptional kinetic reaction has been widely used to label small molecules for visualization under physiological condition.\textsuperscript{84,85} Previous studies demonstrated the use of glycans containing TCO for the cellular imaging via an IEDDA reaction with the tetrazine.\textsuperscript{85–87} Therefore, the auxiliary containing tetrazine moiety could be an alternative tool for labeling and studying the biological functions of glycans.
Herein, the synthesis of a multifunctional neoglycoside auxiliary and the applications of this auxiliary are reported. The hydrophobic UV active moiety on the auxiliary facilitates the isolation and purification of complex carbohydrate mixtures via a reverse-phase chromatographic technique. The auxiliary displayed compatibility with a common glycosyltransferase during chemoenzymatic synthesis. The stability of the neoglycoside conjugate was defined over a suitable pH range and the near-quantitative removal was achieved when desired. Moreover, the tetrazine moiety on the auxiliary enabled direct conjugation to chemical probes without further modification.

2.2. Experimental

2.2.1. General Information

$^1$H and $^{13}$C NMR spectra were recorded at 400 MHz and 101 MHz, respectively. For $^{13}$C NMR spectra of samples in the D$_2$O solvent, a small amount of methanol was added as the internal standard. Assignments were based on COSY, NOESY, and TOCSY experiments and comparison with published data.$^{88,89}$ HPLC analysis and preparation were performed on Gilson® Analytical to Semi-Preparative HPLC system using Kinetex® 5 μm EVO C18 100 Å 4.6x150 mm and Kinetex® 5u Biphenyl 100 Å 4.6x150 mm for analytical columns and Kinetex® 5u EVO C18 100 Å 21.1x150 mm for a preparative column. Mass spectrometry analysis was performed by the School of Chemical Sciences Mass Spectrometry Laboratory at the University of Illinois. LCMS was operated in an electrospray ionization technique (ESI) using Agilent 1100 series HPLC coupled with diode array detector (model G1315A) and mass selective detector (model G2445A). Thin-layer
chromatography (TLC) analysis was performed on silica gel 60 with fluorescent indicator F_{254} (EMD Millipore) and visualized with UV light (254 nm) where applicable. Silica gel column chromatography was performed on SiliaFlash® F60 (Silicycle, 40-64 µm). UV-vis absorption spectra were recorded from the SpectraMax i3x multi-mode detection platform (Molecular Devices).

All reagents were used directly without further purification. CMP-N-acetyl-neuraminic acid was purchased from CarboSynth. α-2,3-Sialyltransferase from Pasteurella multocida was purchased from Sigma. Cy5-TCO was purchased from Click Chemistry Tools. A mixture of xylooligosaccharides was a gift from TethyL research LLC.

Carbohydrate symbols: D-glucose (Glc, ), D-galactose (Gal, ), N-acetyl neuraminic acid (Neu5Ac, ), D-xylose (Xyl, ).

2.2.2. Synthetic Procedures

Synthesis of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanol (2-2)

The synthesis procedure of Yang and coworkers (2012) was followed with modification.\textsuperscript{90} Specifically, 4-(hydroxymethyl)-benzonitrile (2-1) 0.2 g (1.5 mmol, 0.1 eq) and Zn(OTf)\textsubscript{2} 0.272 g (0.75 mmol, 5 mol%) were added to a 20 mL vial under N\textsubscript{2}. Next, 0.78 mL of dried acetonitrile and 2.6 mL of hydrazine anhydrous were added to the reaction
vial. The reaction was heated to 60 °C for 48 h. The reaction was cooled to room temperature and the solution of sodium nitrite (2.07 g, 30 mmol, 2 eq) in 5 mL water was added to the reaction, followed by the addition of 1 M HCl. During the addition, the solution turned red and produced gas. Addition of 1 M HCl was continued until no gas formed. Then, the solution was transferred to a separatory funnel and extracted with EtOAc 3 times. The organic layer was dried with Na₂SO₄ and concentrated using a rotary evaporator. The crude product was purified by silica column chromatography (EtOAc:Hexanes = 40:60). Yield = 0.130 g (43%).

\(^1\)H NMR (400 MHz, CDCl₃) \(\delta\) (ppm): 8.51 (d, \(\text{J} = 8.5\ \text{Hz}, \ 2\text{H})\), 7.52 (d, \(\text{J} = 8.3, \ 2\text{H}\)), 4.77 (s, 2H), 3.03 (s, 3H)

\(^{13}\)C NMR (400 MHz, CDCl₃) \(\delta\) (ppm): 167.45, 164.15, 145.81, 131.19, 128.35, 127.61, 64.98, 21.40.

HR-MS (ESI) m/z calcd. for C₁₀H₁₁N₄O (M + H)\(^+\) 203.0927, found 203.0926.

**Synthesis of N-Boc-N'-methylhydroxylamine (2-4)**

\[
\begin{align*}
&\text{HO}_2^\cdot \text{N}^- \quad \text{Boc}_2 \\
\rightarrow \quad \text{EtN} \quad \text{CH}_2\text{Cl}_2 \\
&\text{HO}_2^\cdot \text{N}^- \text{Boc} \\
\end{align*}
\]

92%

To a 50 mL round-bottom flask was added Boc anhydride (Boc₂) (4.4251 g, 20 mmol, 1 eq), 10 mL dried methylene chloride, and N-methyl-hydroxylamine (2-3) (2.1248 g, 25.4 mmol, 1.25 eq). Next, triethylamine (2.85 mL, 39 mmol, 1.9 eq) was added dropwise to the solution. The reaction was stirred at room temperature overnight. The
reaction was then washed with water and saturated sodium chloride. The organic layer was dried with Na₂SO₄ and concentrated using a rotary evaporator. Yield = 2.64 g (92%).

¹H NMR (400 MHz, CDCl₃) δ (ppm): 3.13 (s, 3H), 1.43 (s, 9H).

¹³C NMR (101 MHz, CDCl₃) δ (ppm): 157.79, 81.97, 37.96, 28.42.

HR-MS (ESI) m/z calcd. for C₆H₁₃NO₃Na (M + Na)⁺ 170.0788, found 170.0795.

**Synthesis of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-t-butoxycarbamate-N'-methylhydroxylamine (2-5)**

![Chemical Structure](image)

To a 20 mL vial under N₂ was added compound 2-2 (0.1415 g, 0.65 mmol, 1 eq), triphenylphosphine (0.205 g, 0.78 mmol, 1.2 eq), and 5 ml of dried methylene chloride. The solution of N-Boc-N'-methylhydroxylamine (2-4) (0.115 g, 0.78 mmol, 1.2 eq) in 1 ml methylene chloride was subsequently added to the reaction. The reaction was cooled to 0 °C, followed by addition of diisopropyl azodicarboxylate (DIAD) (0.15 ml, 0.78 mmol, 1.2 eq) dropwise. The reaction was allowed to warm to room temperature and stirred overnight. The reaction was concentrated and then purified by silica column chromatography (EtOAc:Hexanes = 30:70). Yield = 0.1441 g (67%).

¹H NMR (400 MHz, CDCl₃) δ (ppm): 8.58 (d, J = 8.1 Hz, 2H), 7.62 (d, J = 8.2 Hz, 2H), 4.93 (s, 2H), 3.09 (s, 3H), 3.08 (s, 3H), 1.49 (s, 9H).
\[ ^{13}\text{C NMR (400 MHz, CDCl}_3 \text{)} \delta \text{ (ppm): 167.54, 164.10, 157.28, 140.64, 132.00, 130.14, 128.19, 81.78, 76.11, 37.30, 28.52, 21.41.} \]

HR-MS (ESI) m/z calcd. for C\textsubscript{16}H\textsubscript{21}N\textsubscript{5}O\textsubscript{3}Na (M + Na\textsuperscript{+}) \text{354.1537}, found 354.1547.

**Synthesis of (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamine (2-6)**

To a 20 mL vial under N\textsubscript{2} was added compound 2-5 (0.1109 g, 0.335 mmol, 1 eq) and 3 mL of dried methylene chloride. The solution was cooled to 0 °C. Trifluoroacetic acid (TFA) (0.5 mL, 3.5 mmol, 10 eq) was added dropwise. The reaction was kept at 0 °C for 30 min then allowed to warm to room temperature and stirred for 3 h. The reaction was quenched with water and pH was adjusted to basic with 2 M NaOH. The solution was extracted with methylene chloride 3 times. The organic layer was dried over Na\textsubscript{2}SO\textsubscript{4} and concentrated using a rotary evaporator. Yield = 0.0758 g (98%).

\[ ^{1}\text{H NMR (400 MHz, CDCl}_3 \text{)} \delta \text{ (ppm): 8.53 (d, } J = 8.3 \text{ Hz, 2H), 7.52 (d, } J = 8.4 \text{ Hz, 2H), 5.01 (s, 2H), 3.04 (s, 3H), 2.87 (s, 3H).} \]

\[ ^{13}\text{C NMR (101 MHz, CDCl}_3 \text{)} \delta \text{ (ppm): 167.44, 164.18, 143.31, 131.33, 129.00, 128.21, 75.27, 39.61, 21.39.} \]

HR-MS (ESI) m/z calcd. for C\textsubscript{11}H\textsubscript{14}N\textsubscript{5}O (M + H\textsuperscript{+}) \text{232.1193}, found 232.1192.
2.2.3. General Procedure for the Preparation of Neoglycoconjugates

The general procedure for neoglycoconjugation of auxiliary 2-6 and reducing sugars is described. To a 7 mL vial, reducing sugar (5 eq) was dissolved in a sodium phosphate buffer (0.25 M, pH 4.7). Compound 2-6 (1 eq) was dissolved in DMF (10% v/v) then added to the reaction mixture (final concentration 0.1 M). The reaction mixture was stirred at 40 °C for 24-48 h. Then, the solution was concentrated by rotary evaporator and purified by HPLC preparative C18 column (for compound 2-7, 2-8, 2-9, 2-10, 2-11, and 2-13, gradient of 80% water/ 20% acetonitrile to 50% water/ 50% acetonitrile in 10 minutes, for compound 2-12 gradient of 85% water/ 15% acetonitrile to 84% water/ 16% acetonitrile in 20 minutes). The collected fractions were concentrated to obtain the desired product.

\[(4-(6\text{-methyl}-1,2,4,5\text{-tetrazin-3-yl})\text{phenyl})\text{-N-methylhydroxyl-amino-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (2-7).}\]

Prepared according to the general procedure using D-lactose (0.133 g, 0.37 mmol) as reducing sugar to obtain compound 2-7 as a pink powder. Yield = 0.027 g (64%).
$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm):

<table>
<thead>
<tr>
<th></th>
<th>$^1$H NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc – H1</td>
<td>4.17 (d, $J = 8.3$ Hz)</td>
</tr>
<tr>
<td>Glc – H2</td>
<td>3.63 – 3.59</td>
</tr>
<tr>
<td>Glc – H3</td>
<td>3.63 – 3.59</td>
</tr>
<tr>
<td>Glc – H4</td>
<td>3.52 – 3.48</td>
</tr>
<tr>
<td>Glc – H5</td>
<td>3.80 – 3.77</td>
</tr>
<tr>
<td>Glc – H6a</td>
<td>3.93</td>
</tr>
<tr>
<td>Glc – H6b</td>
<td>3.80 – 3.73</td>
</tr>
<tr>
<td>Gal – H1</td>
<td>4.41 (d, $J = 7.8$ Hz)</td>
</tr>
<tr>
<td>Gal – H2</td>
<td>3.52 – 3.48</td>
</tr>
<tr>
<td>Gal – H3</td>
<td>3.63 – 3.59</td>
</tr>
<tr>
<td>Gal – H4</td>
<td>3.88</td>
</tr>
<tr>
<td>Gal – H5</td>
<td>3.63 – 3.59</td>
</tr>
<tr>
<td>Gal – H6a</td>
<td>3.72 – 3.66</td>
</tr>
<tr>
<td>Gal – H6b</td>
<td>3.72 – 3.66</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.28 (d, $J = 8.2$ Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.60 (d, $J = 8.2$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.86 (d, $J = 2.7$ Hz s, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.00 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.78 (s, 3H)</td>
</tr>
</tbody>
</table>

$^{13}$C (101 MHz, D$_2$O) $\delta$ (ppm): 167.86, 164.43, 141.38, 131.81, 130.71, 128.64, 103.56, 93.72, 78.74, 76.83, 76.28, 76.01, 75.24, 73.16, 71.61, 70.33, 69.22, 61.72, 60.77, 39.44, 20.74.

HR-MS (ESI) m/z calcd. for C$_{23}$H$_{34}$N$_5$O$_{11}$ (M + H)$^+$ 556.2249, found 556.2255.
**(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamo-α-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (2-8)**

Prepared according to the general procedure using D-maltose (0.155 g, 0.430 mmol) as reducing sugar to obtain compound 2-8 as a pink powder. Yield = 0.025 g (51%).

$^1$H NMR (400 MHz, D$_2$O) δ (ppm):

| Glc 1 – H1 | 4.17 (d, $J = 9.1$ Hz) |
| Glc 1 – H2 | 3.65 – 3.55 |
| Glc 1 – H3 | 3.78 – 3.76 |
| Glc 1 – H4 | 3.76 – 3.70 |
| Glc 1 – H5 | 3.52 – 3.48 |
| Glc 1 – H6a | 3.68 – 3.52 |
| Glc 1 – H6b | 3.68 – 3.52 |
| Glc 2 – H1 | 5.38 (d, $J = 3.9$ Hz) |
| Glc 2 – H2 | 3.58 – 3.55 |
| Glc 2 – H3 | 3.80 – 3.75 |
| Glc 2 – H4 | 3.39 |
| Glc 2 – H5 | 3.70 – 3.66 |
| Glc 2 – H6a | 3.91 – 3.88 |
| Glc 2 – H6b | 3.78 – 3.74 |
| CH aromatic | 8.24 (d, $J = 8.3$ Hz, 2H) |
| CH aromatic | 7.58 (d, $J = 8.3$ Hz, 2H) |
| CH$_2$-O-N | 4.86 (d, $J = 2.7$ Hz, 2H) |
| CH$_3$-tetrazine | 3.01 (s, 3H) |
| CH$_3$-N | 2.79 (s, 3H) |
$^{13}$C (101 MHz, D$_2$O) $\delta$ (ppm): 167.81, 164.28, 141.44, 131.64, 130.60, 128.54, 100.27, 93.73, 78.13, 77.29, 76.57, 75.20, 73.28, 72.34, 70.49, 69.93, 61.43, 61.08, 39.42, 20.73.

HR-MS (ESI) m/z calcd. for C$_{23}$H$_{34}$N$_5$O$_{11}$ (M + H)$^+$ 556.2249, found 556.2268.

(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-$\beta$-d-glucopyranoside (2-9)

Prepared according to the general procedure using d-glucose (0.084 g, 0.467 mmol) as reducing sugar to obtain compound 2-9 as a pink powder. Yield = 0.0613 g (66%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm):

<table>
<thead>
<tr>
<th>Glc 1</th>
<th>4.16 (d, $J = 8.8$ Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc 2</td>
<td>3.58 (t, $J = 8.9$ Hz)</td>
</tr>
<tr>
<td>Glc 3</td>
<td>3.50 (t, $J = 8.5$ Hz)</td>
</tr>
<tr>
<td>Glc 4</td>
<td>3.36</td>
</tr>
<tr>
<td>Glc 5</td>
<td>3.36</td>
</tr>
<tr>
<td>Glc 6a</td>
<td>3.88 (d, $J = 12.5$ Hz)</td>
</tr>
<tr>
<td>Glc 6b</td>
<td>3.71 (dd, $J = 12.3$ Hz, $J = 4.7$ Hz)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.17 (d, $J = 8.1$ Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.53 (d, $J = 8.1$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.84 (s, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>2.98 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.79 (s, 3H)</td>
</tr>
</tbody>
</table>
\(^{13}\)C (101 MHz, D\(_2\)O) \(\delta\) (ppm): 167.76, 164.16, 141.51, 131.49, 130.51, 128.44, 93.88, 77.97, 77.73, 75.14, 70.65, 70.05, 61.45, 39.41, 20.73.

HR-MS (ESI) m/z calcd. for C\(_{17}\)H\(_{24}\)N\(_5\)O\(_6\) (M + H)+ 394.1721, found 394.1736.

\((4-(6\text{-methyl}-1,2,4,5\text{-tetrazin-3-yl})\text{phenyl})-N\text{-methylhydroxylamino-\(\beta\text{-2-acetamido-2-deoxy-\(\beta\text{-}D\text{-glucopyranoside (2-10)\)}}\)

Prepared according to the general procedure using \(N\text{-acetyl-\(\beta\text{-D-glucosamine (0.112 g, 0.506 mmol) as reducing sugar to obtain compound 2-10 as a pink powder. Yield = 0.0205 g (46%).}\)

\(^1\)H NMR (400 MHz, D\(_2\)O) \(\delta\) (ppm):

| GlcNAc 1 | 4.24 (d, \(J = 9.7\) Hz) |
| GlcNAc 2 | 4.00 (t, \(J = 9.8\) Hz) |
| GlcNAc 3 | 3.51 (t, \(J = 9.8\) Hz) |
| GlcNAc 4 | 3.46-3.34 (m) |
| GlcNAc 5 | 3.46-3.34 (m) |
| GlcNAc 6a | 3.89 (dd, \(J = 12.4, J = 1.9\) Hz) |
| GlcNAc 6b | 3.74 (dd, \(J = 12.4\) Hz, \(J = 4.9\) Hz) |
| NH-acetyl | 2.03 (s, 3H) |
| CH aromatic | 8.23 (d, \(J = 8.4\) Hz, 2H) |
| CH aromatic | 7.56 (d, \(J = 8.3\) Hz, 2H) |
| CH\(_2\)-O-N | 4.70 (d, \(J = 6.1\) Hz, 2H) |
| CH\(_3\)-tetrazine | 3.00 (s, 3H) |
| CH\(_3\)-N | 2.75 (s, 3H) |
$^{13}\text{C}$ (101 Hz, D$_2$O) δ (ppm): 174.65, 167.79, 164.27, 141.51, 131.57, 130.71, 128.45, 92.40, 77.90, 76.02, 74.42, 70.20, 61.39, 53.01, 39.13, 23.00, 20.73.

HR-MS (ESI) m/z calcd. for C$_{19}$H$_{27}$N$_6$O$_6$ (M + H)$^+$ 435.1987, found 435.2012.

(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-β-D-galactofuranoside (2-11a)

Prepared according to the general procedure using β-D-galactose (0.074 g, 0.411 mmol) as reducing sugar. Compound 2-11a was obtained as a pink powder. Yield = 0.0032 g (10%).

$^1$H NMR (400 MHz, D$_2$O) δ (ppm):

<table>
<thead>
<tr>
<th>Gal 1</th>
<th>4.49 (d, J = 6.2 Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal 2</td>
<td>4.27 (dd, J = 6.2 Hz, J = 0.5 Hz)</td>
</tr>
<tr>
<td>Gal 3</td>
<td>4.14 (dd, J = 8.6 Hz, J = 7.4 Hz)</td>
</tr>
<tr>
<td>Gal 4</td>
<td>3.85 (dd, J = 8.7 Hz, J = 2.7 Hz)</td>
</tr>
<tr>
<td>Gal 5</td>
<td>3.76 (m)</td>
</tr>
<tr>
<td>Gal 6a</td>
<td>3.64 (d, J = 2.8 Hz)</td>
</tr>
<tr>
<td>Gal 6b</td>
<td>3.65 (s)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.38 (d, J = 8.3 Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.68 (d, J = 8.3 Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.91 (d, J = 2.3 Hz, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.05 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.75 (s, 3H)</td>
</tr>
</tbody>
</table>
$^{13}\text{C}$ (101 MHz, D$_2$O) $\delta$ (ppm): 167.93, 164.57, 141.44, 131.97, 130.67, 128.73, 98.45, 81.64, 77.16, 75.72, 75.36, 70.75, 63.42, 40.05, 20.72.

HR-MS (ESI) m/z calcd. for C$_{17}$H$_{24}$N$_{5}$O$_{6}$ (M + H)$^+$ 394.1721, found 394.1725.

(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-$\beta$-D-galactopyranoside (2-11b)

Prepared according to the general procedure using D-galactose (0.074 g, 0.411 mmol) as reducing sugar. Compound 2-11b was obtained as a pink powder. Yield = 0.0183 g (57%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm):

<table>
<thead>
<tr>
<th></th>
<th>$^1$H NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal 1</td>
<td>4.16 (d, $J$ = 9.1 Hz)</td>
</tr>
<tr>
<td>Gal 2</td>
<td>3.77 (t, $J$ = 9.2 Hz)</td>
</tr>
<tr>
<td>Gal 3</td>
<td>3.65 (dd, $J$ = 9.4 Hz, $J$ = 3.4 Hz)</td>
</tr>
<tr>
<td>Gal 4</td>
<td>3.92 (d, $J$ = 3.2 Hz)</td>
</tr>
<tr>
<td>Gal 5</td>
<td>3.66-3.64</td>
</tr>
<tr>
<td>Gal 6a</td>
<td>3.81-3.77</td>
</tr>
<tr>
<td>Gal 6b</td>
<td>3.73-3.69</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.27 (d, $J$ = 8.2 Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.61 (d, $J$ = 8.2 Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.88 (s, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.02 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.79 (s, 3H)</td>
</tr>
</tbody>
</table>
$^{13}$C (101 MHz, D$_2$O) $\delta$ (ppm): 167.83, 164.35, 141.68, 131.62, 130.56, 128.54, 94.46, 77.26, 74.98, 74.64, 69.59, 68.20, 61.84, 38.72, 20.72.

HR-MS (ESI) m/z calcd. for C$_{17}$H$_{24}$N$_5$O$_6$ (M + H)$^+$ 394.1721, found 394.1734.

(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-$\alpha$-D-mannopyranoside (2-12a), (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-$\beta$-D-mannopyranoside (2-12b), and (4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-$\alpha$-D-mannofuranoside (2-12c)

Prepared according to the general procedure using D-mannose (0.099 g, 0.55 mmol) as reducing sugar. The ratio of the anomic configurations of the products could not be certainly determined due to the difficulty to separate individual isomers. Therefore, the collected fractions contained all products were combined and determined the ratio of the anomic configurations by $^1$H NMR. The yield was reported as the overall yield. Yield = 0.02878 g (66%). All three products were obtained as a pink powder.
For the NMR analysis of each isomer, the collected fractions from HPLC preparative chromatogram that contained mixtures of isomer were rejected and the fractions that contained only one isomer were concentrated and analyzed by NMR.

\textbf{(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamo-no-\alpha-d-mannopyranoside (2-12a)}

\(^1\)H NMR (400 MHz, D\textsubscript{2}O) \(\delta\) (ppm):

<table>
<thead>
<tr>
<th></th>
<th>(^1)H NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man 1</td>
<td>4.16 (d, (J = 0.6) Hz)</td>
</tr>
<tr>
<td>Man 2</td>
<td>4.10 (d, (J = 2.0) Hz)</td>
</tr>
<tr>
<td>Man 3</td>
<td>3.61 – 3.60</td>
</tr>
<tr>
<td>Man 4</td>
<td>3.75 (dd, (J = 12.3) Hz, (J = 6.1) Hz)</td>
</tr>
<tr>
<td>Man 5</td>
<td>3.41 – 3.37</td>
</tr>
<tr>
<td>Man 6a</td>
<td>3.91 (dd, (J = 12.3) Hz, (J = 2.2) Hz)</td>
</tr>
<tr>
<td>Man 6b</td>
<td>3.61 – 3.60</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.34 (d, (J = 8.4) Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.64 (d, (J = 8.4) Hz, 2H)</td>
</tr>
<tr>
<td>CH\textsubscript{2}-O-N</td>
<td>4.93 (d, (J = 6.0) Hz, 2H)</td>
</tr>
<tr>
<td>CH\textsubscript{3}-tetrazine</td>
<td>3.03 (s, 3H)</td>
</tr>
<tr>
<td>CH\textsubscript{3}-N</td>
<td>2.79 (s, 3H)</td>
</tr>
</tbody>
</table>

\(^{13}\)C (101 MHz, D\textsubscript{2}O) \(\delta\) (ppm): 167.91, 164.55, 141.53, 131.89, 130.60, 128.70, 94.00, 79.02, 75.27, 74.61, 70.18, 67.52, 61.72, 40.94, 20.70.

HR-MS (ESI) m/z calcd. for C\textsubscript{17}H\textsubscript{24}N\textsubscript{6}O\textsubscript{6} (M + H)\(^+\) 394.1721, found 394.1725.
(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-β-D-
mannopyranoside (12b)

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm):

<table>
<thead>
<tr>
<th></th>
<th>$^1$H NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man 1</td>
<td>4.17 (d, $J = 3.3$ Hz)</td>
</tr>
<tr>
<td>Man 2</td>
<td>4.18 – 4.16</td>
</tr>
<tr>
<td>Man 3</td>
<td>3.66 – 3.64</td>
</tr>
<tr>
<td>Man 4</td>
<td>3.69 – 3.66</td>
</tr>
<tr>
<td>Man 5</td>
<td>3.65 – 3.63</td>
</tr>
<tr>
<td>Man 6a</td>
<td>3.80 – 3.77</td>
</tr>
<tr>
<td>Man 6b</td>
<td>3.74 – 3.70</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.38 (d, $J = 8.4$ Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.67 (d, $J = 8.3$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.87 (d, $J = 2.9$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.04 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.67 (s, 3H)</td>
</tr>
</tbody>
</table>

$^{13}$C (101 MHz, D$_2$O) $\delta$ (ppm): 167.95, 164.62, 141.53, 132.05, 131.12, 128.77, 94.80, 75.96, 74.70, 71.51, 69.13, 67.58, 61.74, 41.03, 20.76.

HR-MS (ESI) m/z calcd. for C$_{17}$H$_{24}$N$_5$O$_6$ (M + H)$^+$ 394.1721, found 394.1715.
(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-α-D-mannofuranoside (2-12c)

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm):

<table>
<thead>
<tr>
<th></th>
<th>$^1$H NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man 1</td>
<td>4.54 (d, $J = 7.3$ Hz)</td>
</tr>
<tr>
<td>Man 2</td>
<td>4.38 (dd, $J = 6.9$ Hz, $J = 4.5$ Hz)</td>
</tr>
<tr>
<td>Man 3</td>
<td>4.25 (dd, $J = 4.4$ Hz, $J = 2.2$ Hz)</td>
</tr>
<tr>
<td>Man 4</td>
<td>3.58 (dd, $J = 12.2$ Hz, $J = 5.7$ Hz)</td>
</tr>
<tr>
<td>Man 5</td>
<td>3.88 (m)</td>
</tr>
<tr>
<td>Man 6a</td>
<td>3.94 (dd, $J = 9.2$ Hz, $J = 2.3$ Hz)</td>
</tr>
<tr>
<td>Man 6b</td>
<td>3.74 (dd, $J = 11.7$ Hz, $J = 3.0$ Hz)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.39 (d, $J = 7.6$ Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.66 (d, $J = 7.7$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.89 (d, $J = 5.1$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.03 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.72 (s, 3H)</td>
</tr>
</tbody>
</table>

$^{13}$C (101 MHz, D$_2$O) $\delta$ (ppm): 167.97, 164.69, 141.35, 132.13, 130.70, 128.82, 98.83, 80.77, 75.40, 73.27, 72.11, 69.64, 63.74, 40.33, 20.76.

HR-MS (ESI) m/z calcd. for C$_{17}$H$_{24}$N$_5$O$_6$ (M + H)$^+$ 394.1721, found 394.1725.
(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-β-D-xylopyranoside

(2-13a)

Prepared according to the general procedure using D-xylose (0.063 g, 0.42 mmol) as reducing sugar to obtain compound 2-13a as a pink powder. Yield = 0.0206 g (67%).

$^1$H NMR (400 MHz, D$_2$O) δ (ppm):

<table>
<thead>
<tr>
<th></th>
<th>$^1$H NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xyl 1</td>
<td>4.09 (d, $J = 9.1$ Hz)</td>
</tr>
<tr>
<td>Xyl 2</td>
<td>3.62-3.48</td>
</tr>
<tr>
<td>Xyl 3</td>
<td>3.42 (t, $J = 8.9$ Hz)</td>
</tr>
<tr>
<td>Xyl 4</td>
<td>3.25 (t, $J = 11$ Hz)</td>
</tr>
<tr>
<td>Xyl 5a</td>
<td>3.94 (dd, $J = 11.3$ Hz, $J = 5.4$ Hz)</td>
</tr>
<tr>
<td>Xyl 5b</td>
<td>3.62-3.48</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.36 (d, $J = 8.0$ Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.64 (d, $J = 8.1$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$O-N</td>
<td>4.88 (d, $J = 3.0$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.04 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.74 (s, 3H)</td>
</tr>
</tbody>
</table>

$^{13}$C (101 Hz, D$_2$O) δ (ppm): 167.91, 164.59, 141.61, 131.87, 130.58, 128.68, 94.81, 77.77, 75.14, 70.55, 69.82, 67.49, 39.39, 20.72

HR-MS (ESI) m/z calcd. for C$_{16}$H$_{22}$N$_5$O$_5$ (M + H)$^+$ 364.1616, found 364.1616.
2.2.4. Hydrolytic Stability Study

The hydrolysis method of neoglycosides developed by Nitz and coworkers (2009) was followed with several modifications. Briefly, glycoconjugate 2-7 was prepared at 2.2 mM in 20 mM of sodium phosphate buffer pH 5.0, 7.0, and 9.0, then incubated at 40 °C. The reaction was monitored at a given time by taking 200 μL of samples and diluting with 400 μL of 200 mM sodium phosphate buffer pH 7.0 and subsequently analyzed by HPLC reverse-phase column with a gradient of 80% water/20% acetonitrile to 50% water/50% acetonitrile in 10 minutes. The peak areas of the remaining glycoconjugate at given times were converted to percent remaining and plotted as a linear decay of ln[%remaining glycoconjugate] vs time. The pseudo-first-order rate constants of each pH were determined by the equation: ln[A] = ln[A]₀ − kt, where [A] = concentration of glycoconjugate at a given time, [A]₀ = initial concentration of glycoconjugate, t = time, and k = rate constant.

The degradation products from the reaction of glycoconjugate 2-7 under sodium phosphate pH 9 could be identified by LCMS using the ESI technique.
2.2.5. Hydrolysis of Conjugated Product

To a 20 mL vial was added glycoconjugate 2-6 0.0166 g (0.0289 mmol) and 6 mL of 0.25% of TFA in water. The reaction was stirred at room temperature for 3 h. Then, DOWEX 1X8 (OH form) 0.9 g was added to the solution and stirred for 10 minutes. DOWEX resins were then removed from the solution by filtration. The auxiliary was removed by extracting with methylene chloride 3 times. The aqueous layer was concentrated using a rotary evaporator to obtain unconjugated D-lactose (yield = 96%).

2.2.6. Synthesis of N-Acetyl-Neuraminic Acid-α-(2→3)-Lactopyranoside

To a 7 mL vial, glycoconjugate 2-6 (1 mM, 1 eq) and CMP-N-acetyl-neuraminic acid (1 mM, 1 eq) were added to 3 mL of Tris.HCl buffer (0.1 M, pH 8.0). Next, 50 µL of α-2,3-sialyltransferase (1 mg/mL) was added to the reaction mixture then incubated at 37 °C. The reaction was monitored by HPLC (gradient of 99% water/ 1% acetonitrile to 60%
water/ 40% acetonitrile in 20 minutes). The reaction was left overnight (18 h) to reach to the completion. Upon completion, the reaction was concentrated by rotary evaporator then purified by HPLC preparative column (gradient of 99% water/ 1% acetonitrile to 60% water/ 40% acetonitrile in 20 minutes). The collected fractions were concentrated to obtain the desired product (2-17) as pink powder. Yield = 0.0024 g (91%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm):

<table>
<thead>
<tr>
<th></th>
<th>$^1$H NMR (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc 1</td>
<td>4.17 (d, $J = 8.3$ Hz)</td>
</tr>
<tr>
<td>Glc 2</td>
<td>3.62 – 3.60</td>
</tr>
<tr>
<td>Glc 3</td>
<td>3.62 – 3.60</td>
</tr>
<tr>
<td>Glc 4</td>
<td>3.54 – 3.50</td>
</tr>
<tr>
<td>Glc 5</td>
<td>3.57 – 3.55</td>
</tr>
<tr>
<td>Glc 6a</td>
<td>3.94</td>
</tr>
<tr>
<td>Glc 6b</td>
<td>3.86 – 3.82</td>
</tr>
<tr>
<td>Gal – H1</td>
<td>4.49 (d, $J = 7.9$ Hz)</td>
</tr>
<tr>
<td>Gal – H2</td>
<td>3.54 – 3.50</td>
</tr>
<tr>
<td>Gal – H3</td>
<td>N/A</td>
</tr>
<tr>
<td>Gal – H4</td>
<td>N/A</td>
</tr>
<tr>
<td>Gal – H5</td>
<td>N/A</td>
</tr>
<tr>
<td>Gal – H6a</td>
<td>3.74 – 3.66</td>
</tr>
<tr>
<td>Gal – H6b</td>
<td>3.74 – 3.66</td>
</tr>
<tr>
<td>Neu5Ac – H3a</td>
<td>2.71 (dd, $J = 12.6$ Hz, $J = 4.7$ Hz)</td>
</tr>
<tr>
<td>Neu5Ac – H3b</td>
<td>1.76 (t, $J = 12.3$ Hz)</td>
</tr>
<tr>
<td>Neu5Ac – H4</td>
<td>3.62 – 3.60</td>
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<tr>
<td>Neu5Ac – H5</td>
<td>3.87 – 3.78</td>
</tr>
<tr>
<td>Neu5Ac – H6</td>
<td>3.91</td>
</tr>
<tr>
<td>Neu5Ac – H7</td>
<td>4.07</td>
</tr>
<tr>
<td>Neu5Ac – H8</td>
<td>N/A</td>
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<tr>
<td>Neu5Ac – H9</td>
<td>N/A</td>
</tr>
<tr>
<td>Neu5Ac – NH-acetyl</td>
<td>1.99 (s, 3H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.40 (d, $J = 8.2$ Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.67 (d, $J = 8.3$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.89 (d, $J = 3.9$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.04 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.78 (s, 3H)</td>
</tr>
</tbody>
</table>

N/A = not assigned
$^{13}$C (101 Hz, D$_2$O) δ (ppm): 175.64, 174.57, 167.95, 164.71, 141.30, 132.11, 130.82, 128.81, 103.25, 100.43, 93.75, 78.59, 76.84, 76.25, 76.12, 75.84, 75.29, 73.52, 72.42, 70.39, 70.03, 69.04, 68.73, 68.13, 63.20, 60.75, 52.32, 40.28, 39.46, 22.68, 20.73.

HR-MS (ESI) m/z calcd. for C$_{34}$H$_{51}$N$_{6}$O$_{19}$ (M + H)$^+$ 847.3204, found 847.3245.

### 2.2.7. Conjugation of Neoglycoside Auxiliary to Mixture of Xylooligosaccharides

![Reaction Scheme]

To a 7 mL vial was added a mixture of xylooligosaccharides 0.373 g (~0.9 mmol, 5 eq) and 1.5 mL of 0.25 M sodium phosphate buffer pH 4.7. Compound 2-6 (0.0415 g, 0.18 mmol, 1 eq) was dissolved in 0.5 mL of DMF, then added to the xylooligosaccharides solution. The reaction was stirred at 40 °C for 48 h. The solution was concentrated and then purified by reverse-phase preparative HPLC (gradient of 90% water/ 10% acetonitrile to 80% water/ 20% acetonitrile in 30 minutes). The fractions containing products were concentrated separately using a rotary evaporator. The collected fractions were analyzed by $^1$H NMR showing the pure product of n = 2 to n =4 as pink powder. Yield n = 2 (2-18, 0.0096 g, 11%), n = 3 (2-19, 0.0118 g, 11%), n = 4 (2-20, 0.0062 g, 3%). The collected fractions for longer glycans (n = 5 – 7) were analyzed by LCMS.
$n = 2 \ (2\cdot18)$

$^1\text{H NMR (400 MHz, D}_2\text{O) } \delta (\text{ppm})$:

<table>
<thead>
<tr>
<th></th>
<th>$^1\text{H NMR (ppm)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xyl 1 – H1</td>
<td>4.09 (d, $J = 8.5$ Hz)</td>
</tr>
<tr>
<td>Xyl 1 – H2</td>
<td>3.55</td>
</tr>
<tr>
<td>Xyl 1 – H3</td>
<td>3.38</td>
</tr>
<tr>
<td>Xyl 1 – H4</td>
<td>3.23</td>
</tr>
<tr>
<td>Xyl 1 – H5a</td>
<td>3.92</td>
</tr>
<tr>
<td>Xyl 1 – H5b</td>
<td>3.58</td>
</tr>
<tr>
<td>Xyl 2 – H1</td>
<td>4.42</td>
</tr>
<tr>
<td>Xyl 2 – H2</td>
<td>3.21</td>
</tr>
<tr>
<td>Xyl 2 – H3</td>
<td>3.53</td>
</tr>
<tr>
<td>Xyl 2 – H4</td>
<td>3.29</td>
</tr>
<tr>
<td>Xyl 2 – H5a</td>
<td>4.06</td>
</tr>
<tr>
<td>Xyl 2 – H5b</td>
<td>3.69</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.30 (d, $J = 8.3$ Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.61 (d, $J = 8.3$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_2$-O-N</td>
<td>4.86 (d, $J = 1.7$ Hz, 2H)</td>
</tr>
<tr>
<td>CH$_3$-tetrazine</td>
<td>3.02 (s, 3H)</td>
</tr>
<tr>
<td>CH$_3$-N</td>
<td>2.74 (s, 3H)</td>
</tr>
</tbody>
</table>

$^{13}\text{C (101 Hz, D}_2\text{O) } \delta (\text{ppm})$: 167.90, 164.55, 141.59, 131.83, 130.59, 128.67, 102.47, 94.67, 77.06, 76.24, 75.84, 75.17, 73.41, 70.51, 69.82, 65.85, 65.33, 39.41, 20.73.

HR-MS (ESI) m/z calcd. for C$_{21}$H$_{30}$N$_5$O$_9$ (M + H)$^+$ 496.2038, found 496.2025.
\( n = 3 \ (2-19) \)

\( \text{H NMR (400 MHz, D}_2\text{O)} \ \delta \ \text{(ppm):} \)

<table>
<thead>
<tr>
<th></th>
<th>( ^1\text{H NMR (ppm)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xyl 1 – H1</td>
<td>4.09 (d, ( J = 8.5 ) Hz)</td>
</tr>
<tr>
<td>Xyl 1 – H2</td>
<td>3.57</td>
</tr>
<tr>
<td>Xyl 1 – H3</td>
<td>3.38</td>
</tr>
<tr>
<td>Xyl 1 – H4</td>
<td>3.27</td>
</tr>
<tr>
<td>Xyl 1 – H5a</td>
<td>3.92</td>
</tr>
<tr>
<td>Xyl 1 – H5b</td>
<td>3.60</td>
</tr>
<tr>
<td>Xyl 2 – H1</td>
<td>4.42</td>
</tr>
<tr>
<td>Xyl 2 – H2</td>
<td>3.21</td>
</tr>
<tr>
<td>Xyl 2 – H3</td>
<td>3.50</td>
</tr>
<tr>
<td>Xyl 2 – H4</td>
<td>3.29-3.35</td>
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<tr>
<td>Xyl 2 – H5a</td>
<td>4.06</td>
</tr>
<tr>
<td>Xyl 2 – H5b</td>
<td>3.77-3.66</td>
</tr>
<tr>
<td>Xyl 3 – H1</td>
<td>4.43</td>
</tr>
<tr>
<td>Xyl 3 – H2</td>
<td>3.23</td>
</tr>
<tr>
<td>Xyl 3 – H3</td>
<td>3.53</td>
</tr>
<tr>
<td>Xyl 3 – H4</td>
<td>3.29-3.35</td>
</tr>
<tr>
<td>Xyl 3 – H5a</td>
<td>4.06</td>
</tr>
<tr>
<td>Xyl 3 – H5b</td>
<td>3.77-3.66</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>8.27 (d, ( J = 8.1 ) Hz, 2H)</td>
</tr>
<tr>
<td>CH aromatic</td>
<td>7.59 (d, ( J = 8.2 ) Hz, 2H)</td>
</tr>
<tr>
<td>CH(_2)-O-N</td>
<td>4.85 (d, ( J = 1.5 ) Hz, 2H)</td>
</tr>
<tr>
<td>CH(_3)-tetrazine</td>
<td>3.01 (s, 3H)</td>
</tr>
<tr>
<td>CH(_3)-N</td>
<td>2.73 (s, 3H)</td>
</tr>
</tbody>
</table>

\( ^{13}\text{C (101 Hz, D}_2\text{O)} \ \delta \ \text{(ppm):} \ 167.87, 164.47, 141.61, 131.75, 130.55, 128.62, 102.47, 102.28, 94.67, 77.00, 76.98, 76.22, 75.82, 75.15, 74.29, 73.39, 73.32, 70.51, 69.81, 65.84, 65.30, 63.60, 39.40, 20.73. \)

HR-MS (ESI) \( m/z \) calcd. for \( \text{C}_{26}\text{H}_{38}\text{N}_5\text{O}_{13} \ (M + H)^+ \ 628.2461, \) found 628.2482.
\[ n = 4 \ (2-20) \]

\[ \begin{align*}
\text{Xyl 1} - \text{H1} &: 4.10 \ (d, J = 8.1 \text{ Hz}) \\
\text{Xyl 1} - \text{H2} &: 3.57 \\
\text{Xyl 1} - \text{H3} &: 3.39 \\
\text{Xyl 1} - \text{H4} &: 3.3.27-3.37 \\
\text{Xyl 1} - \text{H5a} &: 3.94 \\
\text{Xyl 1} - \text{H5b} &: 3.61 \\
\text{Xyl 2} - \text{H1} &: 4.42-4.46 \\
\text{Xyl 2} - \text{H2} &: 3.20-3.25 \\
\text{Xyl 2} - \text{H3} &: 3.49-3.56 \\
\text{Xyl 2} - \text{H4} &: 3.3.27-3.37 \\
\text{Xyl 2} - \text{H5a} &: 4.05-4.09 \\
\text{Xyl 2} - \text{H5b} &: 3.68-3.78 \\
\text{Xyl 3} - \text{H1} &: 4.42-4.46 \\
\text{Xyl 3} - \text{H2} &: 3.20-3.25 \\
\text{Xyl 3} - \text{H3} &: 3.49-3.56 \\
\text{Xyl 3} - \text{H4} &: 3.27-3.37 \\
\text{Xyl 3} - \text{H5a} &: 4.05-4.09 \\
\text{Xyl 3} - \text{H5b} &: 3.68-3.78 \\
\text{Xyl 4} - \text{H1} &: 4.42-4.46 \\
\text{Xyl 4} - \text{H2} &: 3.20-3.25 \\
\text{Xyl 4} - \text{H3} &: 3.49-3.56 \\
\text{Xyl 4} - \text{H4} &: 3.27-3.37 \\
\text{Xyl 4} - \text{H5a} &: 4.05-4.09 \\
\text{Xyl 4} - \text{H5b} &: 3.68-3.78 \\
\text{CH aromatic} &: 8.37 \ (d, J = 8.2 \text{ Hz, 2H}) \\
\text{CH aromatic} &: 7.65 \ (d, J = 8.2 \text{ Hz, 2H}) \\
\text{CH}_2\text{-O-N} &: 4.88 \ (d, J = 2.6 \text{ Hz, 2H}) \\
\text{CH}_3\text{-tetrazine} &: 3.04 \ (s, 3H) \\
\text{CH}_3\text{-N} &: 2.74 \ (s, 3H)
\end{align*} \]
$^{13}$C (101 Hz, D$_2$O) δ (ppm): 167.92, 164.62, 141.56, 131.92, 130.62, 128.71, 102.48, 102.29, 102.29, 94.67, 77.01, 76.99, 76.95, 76.23, 75.83, 75.19, 74.28, 74.28, 73.40, 73.33, 73.33, 70.51, 69.82, 65.85, 65.31, 63.60, 63.60, 39.41, 20.73.

HR-MS (ESI) m/z calcd. for C$_{31}$H$_{46}$N$_{5}$O$_{17}$ (M + H)$^+$ 760.2883, found 760.2870.

\( n = 5 \) (2-24)

m/z calcd. (M+H)$^+$ 892.3, found 892.0.

\( n = 6 \) (2-25)

m/z calcd. (M+H)$^+$ 1024.4, found 1024.1.

\( n = 7 \) (2-26)

m/z calcd. (M+H)$^+$ 1156.4, found 1156.2.
Glycoconjugate 2-7 (55 μM, 1.08 eq) and Cy5-TCO (2-21) (51 μM, 1 eq) were dissolved in Tris.HCl buffer (50 mM, pH 7.4) to a final volume of 510 μL. The reaction mixture was incubated at room temperature. The reaction was monitored by HPLC biphenyl column (gradient of 80% water/ 20% methanol to 30% water/ 70% methanol in...
30 minutes) using UV detector at 647 nm and 254 nm to monitored Cy5-TCO and 2-7, respectively. The HPLC chromatograms are shown in Figure 2.5. Then, the solution was concentrated and desalted using Sephadex G-10 eluting with water. The compound 2-23 was collected and analyzed by mass spectrometry. Interestingly, the aromatic pyrazine product from the oxidation of 1,4-dihydropyrazine 2-23 could also be observed from the mass spectrometric results. Presumably, the 1,4-dihydropyrazine 2-23 was oxidized by air during the desalting step.\textsuperscript{92,93}

HR-MS (ESI) m/z calcd. for C\textsubscript{69}H\textsubscript{92}N\textsubscript{7}O\textsubscript{23}S\textsubscript{3} (M-H-2H)\textsuperscript{-} 1482.5412, found 1482.5398, and m/z calcd. for C\textsubscript{69}H\textsubscript{91}N\textsubscript{7}O\textsubscript{23}S\textsubscript{3} (M-2H-2H)\textsuperscript{-} 740.7668, found 740.7646.

2.3. Results and Discussion

2.3.1. Design and Synthesis of Auxiliary

The multifunctional auxiliary was designed to fit in broad applications in glycomic research. The secondary oxyamine is preferred because it preserved a ring-closed conformation of the conjugated product. The hydrophobic UV active moiety on the auxiliary facilitates the isolation and purification of complex carbohydrate mixtures via a reverse-phase chromatographic technique. This auxiliary displayed stability during the chemoenzymatic synthesis. Moreover, the tetrazine moiety on the auxiliary enabled direct conjugation to chemical probes without further modification.
The synthesis route of auxiliary 2-6 is shown in Scheme 2.1. Commercially available 4-(hydroxymethyl)-benzonitrile (2-1) reacted with acetonitrile, hydrazine, and the presence of zinc triflate in a one-pot manner to form hydroxybenzyl tetrazine (2-2) in moderate yield.\(^{90}\) Extended reaction time to 48 h was necessary to improve the reported 27% yield to 43% yield.\(^{90}\) Compound 2-2 was subsequently reacted with \(N\text{-Boc-}N'\text{-methylhydroxylamine 2-4 via Mitsunobu condition and purified by silica column to obtain compound 2-5. Finally, the Boc protecting group could be removed with TFA in dichloromethane and extracted with basic aqueous to give product 2-6 in 98% yield.}

![Scheme 2.1: Synthesis of auxiliary 2-6.](image)

The UV-vis absorption spectrum of auxiliary 2-6 revealed that there are two characteristic maxima absorption wavelengths (Figure 2.1). At the UV region, there is the strong absorption at 270 nm (\(\varepsilon_{270\text{ nm}} = 22,127 \text{ Lmol}^{-1}\text{cm}^{-1}\)). There is a weaker absorption maximum at the visible region at 540 nm (\(\varepsilon_{540\text{ nm}} = 439 \text{ Lmol}^{-1}\text{cm}^{-1}\)) which can be seen at
high concentration. This UV-vis absorption spectrum demonstrated that auxiliary 2-6 provides the ability to monitor the reaction at either UV or visible regions to avoid interference from other compounds in the reaction.

![Figure 2.1: UV-vis absorption spectra of 2-6 at 1 mM and 0.1 mM.](image)

Partition coefficient (logP) is a ratio that determines the distribution of the compound between octanol and water as shown in Eq 1 where \( c_{\text{oct}} \) = concentration of a compound in octanol and \( c_{\text{wat}} \) = concentration of a compound in water.\(^{94}\) In drug discovery, logP value is one of the important parameters to be considered. It can be used to predict the ability of the compound to permeate through the membrane at the target site.\(^{94-96}\) A compound with high logP value can permeate through the membrane easily. The logP value of the auxiliary 2-6 was predicted on the ChemDraw program (Chemdraw
Prime 17.1) to be 0.96 indicating that compound 2-6 has a lipophilic property that can provide the ability to interact with a non-polar stationary for conjugated glycans. The predicted logP value reveals that the auxiliary 2-6 can potentially be useful for pharmaceutical applications, such as increasing the ability of highly polar biologically active compounds to permeate through the cell membrane.

\[ \log P = \log \left( \frac{C_{\text{oct}}}{C_{\text{wat}}} \right) \]  

Eq 1

### 2.3.2. Evaluation of Auxiliary with Reducing Sugars

With the neoglycoside auxiliary 2-6 in hand, the glycoconjugate library was created by reaction with different reducing sugars (Table 2.1). It is known that the rate and efficiency of the oxyamine ligation is heavily dependent on the concentration of the saccharide and oxyamine, as well as the solvent and acid catalyst.\(^{97}\) The most efficient and general conditions utilized a sodium phosphate buffer (pH = 4.7), although, other buffers (NH\(_4\)OAc and NaOAc) were found to be viable. It should be noted here that approximately 10% of DMF improves the rate of ligation by improving the solubility of the auxiliary in the reaction medium. The effect from the optimal stoichiometry of the saccharide and auxiliary was evaluated. Two complementary reactions (Table 2.1, entries 1 and 2) demonstrate that the utilization of a five-fold molar excess of either the sugar or the auxiliary results in an acceptable yield of the conjugate. All subsequent entries in Table 2.1 utilized an excess of reducing sugars due to the substrates being readily available and inexpensive. The conjugation of seven reducing sugars was carried out under the
established conditions and purified by reverse-phase chromatography on C18 functionalized silica gel. Hexoses, pentoses, disaccharides, and N-acetyl containing glycans were examined and provided the expected products with yields ranging between 46% and 68%.

Table 2.1. Conjugation of auxiliary 2-6 with reducing sugars.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reducing sugar</th>
<th>Equivalent</th>
<th>Time (d)</th>
<th>Product</th>
<th>Yielda (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>d-Lactose (Lac)</td>
<td>5</td>
<td>1</td>
<td>2-7</td>
<td>64</td>
</tr>
<tr>
<td>2</td>
<td>d-Lactose (Lac)</td>
<td>1</td>
<td>5</td>
<td>2-7</td>
<td>51</td>
</tr>
<tr>
<td>3</td>
<td>d-Maltose (Mal)</td>
<td>5</td>
<td>1</td>
<td>2-8</td>
<td>51</td>
</tr>
<tr>
<td>4</td>
<td>d-Glucose (Glc)</td>
<td>5</td>
<td>1</td>
<td>2-9</td>
<td>66</td>
</tr>
<tr>
<td>5</td>
<td>N-Acetyl-d-glucosamine</td>
<td>5</td>
<td>1</td>
<td>2-10</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>(GlcNAc)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>d-Galactose (Gal)</td>
<td>5</td>
<td>1</td>
<td>2-11</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>d-Mannose (Man)</td>
<td>5</td>
<td>1</td>
<td>2-12</td>
<td>66</td>
</tr>
<tr>
<td>8</td>
<td>d-Xylose (Xyl)</td>
<td>5</td>
<td>1</td>
<td>2-13</td>
<td>68</td>
</tr>
</tbody>
</table>

a Isolated yield of all isomers
The conjugated products were characterized by NMR and mass spectrometry. The stereochemistry at the anomeric carbon was determined by $J_{H1-H2}$ coupling constant and compared with published results\textsuperscript{56,88} (Table 2.2). As expected, glucose-based conjugated products (Lac, Mal, Glc, and GlcNAc) were exclusively formed in the $\beta$-pyranose configuration. The xylose product was determined to also predominately be the $\beta$-pyranoside along with only trace amounts of $\alpha$-pyranoside. The conjugation of galactose gave only $\beta$ configured products but was observed in both the pyranose and furanose forms in a ratio of 85:15.

The exclusive formation of $\beta$-pyranoside can be rationalized by the favorable 6-exo-trig ring-closing on the $Re$ face\textsuperscript{88} to avoid a 1,3-axial interaction.\textsuperscript{60,88,89} The reaction of mannose with the neoglycoside auxillary resulted in the most complex mixture due to the presence of axial alcohol on C2. Three different isomers including $\alpha$-pyranoside, $\beta$-pyranoside, and $\alpha$-furanoside were observed and were difficult to distinguish. In contrast to glucose and galactose, the 6-exo-trig ring-closing of mannoside occurs preferentially on the $Si$ face and due to favourability of the opposing dipole-dipole interactions at C1 and C2 leading to the $\alpha$-pyranoside as a major product.\textsuperscript{60,88,89}
Table 2.2 Configuration ratio of conjugated products.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Compound</th>
<th>Configuration ratio</th>
<th>( \beta )-Pyranose</th>
<th>( \alpha )-Pyranose</th>
<th>( \beta )-Furanose</th>
<th>( \alpha )-Furanose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2-7</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2-8</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>2-9</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>2-10</td>
<td></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>2-11</td>
<td></td>
<td>85</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(2-11b)</td>
<td></td>
<td></td>
<td></td>
<td>(2-11a)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>2-12</td>
<td></td>
<td>40</td>
<td>45</td>
<td>0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>(2-12b)</td>
<td></td>
<td></td>
<td>(2-12a)</td>
<td></td>
<td>(2-12c)</td>
</tr>
<tr>
<td>7</td>
<td>2-13</td>
<td></td>
<td>97</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(2-13a)</td>
<td></td>
<td></td>
<td>(2-13b)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.3.3. Stability Study

The hydrolytic stability of glycoconjugate 2-7 was studied under aqueous sodium phosphate buffer at pH values of 5, 7, and 9 at 40 °C. The reactions were monitored by HPLC with UV detection at 254 nm. The peak area of the remaining glycoconjugate 2-7 at the given times was recorded and converted to the remaining concentration (Figure 2.2). The hydrolysis rate constants were calculated from the pseudo-first-order condition (See appendix A). As expected, the hydrolysis rate of glycoconjugate 2-7 is rapid at pH 5 with the half-life at 44 h. At pH 7, glycoconjugate 2-7 was very stable with half-life estimated to be 990 h providing a useful tool for the in vitro and in vivo investigation of glycan functions. Under basic condition at pH 9, the half-life was determined to be 158 h. Interestingly, the neoglycoside hydrolysis product was not observed. Instead, it was found that at pH 9 hydrolysis of the tetrazine moiety occurs with hydrazide (2-14), oxadiazole (2-15), and benzonitrile (2-16) derivatives observed as the degradation products (Scheme 2.2).
The degradation mechanisms could be rationalized based on the previous study that investigated the degradation of tetrazine under basic alkali. As shown in Scheme 2.3a, the degradation product 2-14 was produced from the attacking of hydroxide at the C-6 position on the tetrazine ring followed by proton transfer resulting in the formation of tetrahedron intermediate (2-28). The cheletropic exclusion was then taken place which led to the opening of the tetrazine ring and releasing of nitrogen gas to form 2-29. The proton transfer (tautomerism) on the compound 2-29 led to the formation of degraded product hydrazide 2-14. The nucleophilic elimination at the C-3 position of 2-14
caused further degradation to yield degraded product benzonitrile 2-16 (Scheme 2.3b). The proton transfer on 2-14 led to the formation of 2-30 followed by the nucleophilic addition at C-3 position resulting in the formation of 2,3-dihydrooxadiazole 2-31 (Scheme 2.3c). However, this compound was not stable and could be oxidized under air resulting in the formation of aromatic oxadiazole 2-15.98

Scheme 2.2: Degradation of glycoconjugate 2-7 in sodium phosphate pH 9.

Scheme 2.3: Proposed degradation mechanism of 2-7 under basic condition.
2.3.4. Removal of Auxiliary from a Conjugated Product

The advantage of oxyamine auxiliaries is that they can be easily removed from the conjugated carbohydrate with the presence of acid. A previous study demonstrated that the treatment of oxyamine-linked glycoconjugate with 0.25% v/v TFA would not affect to the acid-labile fucoside and sialoside.\textsuperscript{57} Thus, the glycoconjugate 2-7 was treated with 0.25% of TFA in water and incubated at room temperature for 3 h. Then, the reaction was neutralized by DOWEX 1X8 (OH\textsuperscript{-} form) resins which were easily removed by filtration (Scheme 2.4). The auxiliary was conveniently removed by extraction with dichloromethane and the unconjugated carbohydrate could be recovered from the aqueous layer. After evaporation, unconjugated lactose was obtained in 96% yield.

Scheme 2.4: Removal of auxiliary from glycoconjugate 2-7.
2.3.5. Applications

With the intention of using the multifunctional auxiliary in applications beyond glycomics research, the compatibility and stability of 2-6 were evaluated in the chemoenzymatic synthesis of complex oligosaccharides (Scheme 2.5). The glycoconjugate 2-7 was the prudent choice to illustrate chemoenzymatic extension due to lactose being a common motif in human milk oligosaccharides.\textsuperscript{78,99} The glycoconjugate 2-7 and CMP-N-acetyl-neuraminic acid were treated with \(\alpha\)-(2,3)-sialyltransferase resulted in the formation of glycoconjugate trisaccharide 2-17 with the addition of \(\alpha\)-(2,3)-linked Neu5Ac moiety (Scheme 2.5). This auxiliary demonstrated that it increased the affinity of the trisaccharide product to the hydrophobic stationary phase during reverse-phase chromatography relative to the unconjugated and could be monitored by the UV detector. After completion, the product was conveniently purified on a preparative HPLC column to obtain product in excellent yield.

Scheme 2.5: Chemoenzymatic extension of glycoconjugate 2-7.
The structural complexity and high polarity of oligosaccharides in nature impede glycomic investigations due to the difficulty in purification and isolation. To demonstrate the utility of auxiliary 2-6, the fractionation of a complex oligosaccharide mixture was attempted. A mixture of xylooligosaccharides (XOs) was chosen as it contained xylose oligomers of several chain lengths. The conjugation condition was carried out as previously described above (Figure 2.3a). After conjugation at the reducing end of xylose oligomers, the formation of conjugated xylosides could be analyzed by HPLC. After 48 h of reaction time, the conjugated products were purified by preparative reverse-phase chromatography. The HPLC chromatogram showed the ability to fractionate conjugated XOs up to 7 xylose units (Figure 2.3b). The conjugated xylodioside (2-18), xylotrioside (2-19), and xylotetraside (2-20) were collected and characterized by NMR and mass spectrometry and the longer conjugated glycans were identified by low-resolution ESI mass spectrometry. The results demonstrated that this auxiliary can aid in the fractionation and purification of carbohydrate mixtures.
Finally, the ability to visualize oligosaccharides at the cellular level is necessary for the study of biological functions of glycans. Towards this goal, auxiliary 2-6 containing the tetrazine moiety can be readily joined with the strained alkene of a chemical probe through the IEDDA reaction. The bioorthogonal labeling of glycoconjugate 2-7 with Cy5-TCO was conducted by incubating the substrates in Tris buffer pH 7.4 at room
temperature (Scheme 2.6). The reaction was monitored by HPLC using a biphenyl-functionalized silica column using UV detection at 647 nm which was specific for the Cy5 and 254 nm to indicate 2-7 (Scheme 2.4). As expected, the starting materials were rapidly consumed to form an initial adduct (2-23), and then slowly rearranged to form a 1,4-dihydropyrazine 2-24. This isomeric rearrangement could be observed from HPLC chromatograms which proceeded to the completion within 18 h (Figure 2.4). The cycloaddition product 2-24 was then desalted by Sephadex G10 and identified by mass spectrometry.

Scheme 2.6: IEDDA cycloaddition of glycoconjugate 2-7 and Cy5-TCO (2-21).
Figure 2.4: HPLC chromatograms of the IEDDA cycloaddition of glycoconjugate 2-7 and Cy5-TCO.
2.4. Conclusions

A novel multifunctional neoglycoside auxiliary containing tetrazine moiety (2-6) was successfully synthesized with the overall yield of 28%. The auxiliary was conjugated with a variety of reducing sugars to create a conjugated glycan library. The yields of all conjugated products were in the range of 46 – 64%. All conjugated products from glucose-based saccharides provided the only β configuration. Conjugated products from galactose and mannose gave α and β configurations in both pyranose and furanose forms. The hydrolytic stability of glycoconjugate 2-7 was studied at different pH values which revealed the high stability near physiological conditions. However, the glycoconjugate 2-7 was less stable under acidic conditions due to the high rate of hydrolysis and relative stable under basic condition. The degradation of the tetrazine moiety was observed in basic solution which could be detected by mass spectrometry. The conjugated auxiliary could be efficiently cleaved under mildly acidic conditions and conveniently removed by simple liquid-liquid extraction. The auxiliary demonstrated the capability and efficiency in different glycomic applications. During the chemoenzymatic reaction, the auxiliary appeared to be suitable for the enzyme to incorporate sialic acid onto the glycoconjugate 2-7. The hydrophobic property of the auxiliary facilitated fractionation and purification of complex oligosaccharides mixtures by a reverse-phase HPLC technique. Furthermore, the tetrazine moiety allowed the conjugated product to be bioorthogonally labeled via IEDDA reaction.
CHAPTER 3
TRANSFORMATION OF ALDEHYDES TO NITRILES IN AQUEOUS SOLUTION

3.1. Introduction

Nitrile is an important functional group in organic synthesis because it can be transformed into several functional groups such as amine, carboxylic acid, ester, ketone, and heterocycles. Furthermore, nitriles are involved in broad applications, including pharmaceutical and materials. In the past decades, several methods reported the formation of nitriles from different functional groups such as amine, amide, and alcohol. However, these methods require harsh conditions or metal complex catalysts. The transformation of aldehydes to nitriles has become more attractive because the reaction conditions are mild and do not require toxic reagents.

Formation of nitriles through dehydration of oximes is one of the most classical methods. In general, an aldehyde is reacted with hydroxylamine to form aldoxime followed by dehydration with acetic anhydride, thionyl chloride, or lead oxide to form a desirable nitrile (Scheme 3.1). This dehydration condition is considerably harsh; it requires an excess of dehydrating agents, high temperature, and has low functional group tolerance.
To avoid using harsh conditions and increase the compatibility of the reaction with a variety of functional groups, several approaches have been reported to convert aldehydes to nitriles in a one-pot manner. In 2012, Nantz et al.\textsuperscript{117} reported a method to transform aldehydes to nitriles using $O$-(diphenylphosphinyl)hydroxylamine (DPPH, 3-1) as a nitrogen source (Scheme 3.2a) in toluene at 85 °C. In 2015, An and Yu reported an alternative method to transform aldehydes to nitriles using $O$-(4-CF$_3$-benzoyl)-hydroxylamine (CF$_3$-BHA, 3-2) as a nitrogen source.\textsuperscript{118} This transformation could be done at room temperature using a Brønsted acid as a catalyst (Scheme 3.2b). A method to convert aldehydes to nitriles in aqueous medium was reported in 2016 by Moura-Letts and coworkers.\textsuperscript{119} In this method, hydroxylamine-$O$-sulfonic acid (3-3) was used as a nitrogen source (Scheme 3.2c). The main advantage of this method is that the product can be easily isolated by extraction with a suitable organic solvent.
Reducing sugar is a sugar containing an aldehyde group when it is in a ring-opened form. Although many publications reported the formation of nitriles from aldehydes, only a few examples studied cyanohydrin formations from reducing sugars. A novel method for the formation of cyanohydrins from reducing sugars in one step is described herein. O-Phenylhydroxylamine hydrochloride (3-4) was used as a nitrogen source, and the reaction proceeded under the aqueous condition at room temperature. This reaction is demonstrated to be suitable for a variety of carbohydrates including mono- and disaccharides, silyl-protected saccharide, as well as aliphatic and aromatic aldehydes.

Scheme 3.2: Transformation of aldehydes to nitriles using different hydroxylamine sources.
Furthermore, a new convenient method to shorten reducing sugars using weakly basic resins which can be easily removed by filtration is presented.

3.2. Experimental

3.2.1. General Information

All chemicals and reagents were purchased and used without further purification. Deuterium oxide buffer solution was prepared by dissolving 0.227 g of NaH$_2$PO$_4$ and 0.221 g of NaH$_2$PO$_4$ in 16 mL of deuterium oxide and measured the pD value of the buffer solution by pH meter. It is worth noting here that the pH meter was calibrated with aqueous (H$_2$O) calibration buffer. Therefore, pD was calculated based on Krezel and Bal$^{120}$ (pD = pH + 0.45). Concentrated phosphoric acid and 1 M NaOH in deuterium oxide were used to adjust the pD values. Lewatit MP 62 resin was purchased from Sigma and washed with ethanol through the vacuum filtration before used. $^1$H and $^{13}$C NMR spectra were recorded at 400 MHz, 101 MHz, respectively. For $^{13}$C NMR spectra of samples in the D$_2$O solvent, a small amount of methanol was added as the internal standard. Thin-layer chromatography (TLC) analysis was performed on silica gel 60 with fluorescent indicator F$_{254}$ (EMD Millipore) and visualized with UV light (254 nm) where applicable. Silica gel column chromatography was performed on SiliaFlash® F60 (Silicycle, 40-64 μm). Mass spectrometry analysis was performed by the School of Chemical Sciences Mass Spectrometry Laboratory at the University of Illinois.
3.2.2. General Procedure for the Formation of Cyanohydrins

To a 7 mL vial was added reducing sugar (1 equiv), O-phenylhydroxylamine hydrochloride (3-4) (1.2 equiv), and 0.2 M sodium phosphate pD 7.25 to the final concentration of 0.1 M. The reaction was stirred at room temperature until the oxime intermediates were disappeared (monitored by $^1$H NMR). Then, the reaction mixture was transferred into a separatory funnel and washed with diethyl ether for 10 times. The remaining aqueous solution was evaporated to dryness. Ethanol was added to the solid residue to extract the product from the phosphate salt. The phosphate salt residues in the ethanol solution were removed by centrifugation. The solvent was evaporated to obtain the desired product.

\[ \text{Reducing sugar} \quad \text{O-H} \quad + \quad \text{H}_2\text{N-O} \quad \text{HCl} \quad \xrightarrow{0.2 \text{ M Sodium phosphate pD 7.25, r.t.}} \quad \text{R}=\text{N} \]

\[ \text{D-Ribonitrile (3-5)} \]

Prepared according to the general procedure using d-ribose (0.0226 g, 0.150 mmol) as reducing sugar. Yield = 0.0208 g (94%).
\( ^1\)H NMR (400 MHz, D\(_2\)O) \( \delta \) (ppm): 4.88 (d, \( J = 3.5 \) Hz, 1H), 3.88 – 3.75 (m, 2H), 3.72 – 3.60 (m, 2H).

\( ^{13}\)C NMR (101 MHz, D\(_2\)O) \( \delta \) (ppm): 119.22, 72.46, 72.14, 63.65, 63.02.

HR-MS (ESI) m/z calcd. for C\(_5\)H\(_9\)NO\(_4\)Na (M + Na)\(^+\) 170.0429, found 170.0431.

\textit{d-}Arabinonitrile (3-6)

Prepared according to the general procedure using \textit{d}-arabinose (0.0086 g, 0.0577 mmol) as reducing sugar. Yield = 0.0071 g (83%).

\( ^1\)H NMR (400 MHz, D\(_2\)O) \( \delta \) (ppm): 4.90 (d, \( J = 2.3 \) Hz, 1H), 3.85 – 3.76 (m, 2H), 3.76 – 3.70 (m, 1H), 3.66 (dd, \( J = 11.6, 5.4 \) Hz, 1H).

\( ^{13}\)C NMR (101 MHz, D\(_2\)O) \( \delta \) (ppm): 120.42, 72.02, 70.47, 63.15, 62.29.

HR-MS (ESI) m/z calcd. for C\(_5\)H\(_9\)NO\(_4\)Na (M + Na)\(^+\) 170.0429, found 170.0434.
**d-Xylonitrile (3-7)**

Prepared according to the general procedure using d-xylose (0.0489 g, 0.326 mmol) as reducing sugar. Yield = 0.0471 g (98%).

$^1$H NMR (400 MHz, D$_2$O) δ (ppm): 4.71 (dd, $J = 5.8$, 0.9 Hz, 1H), 3.91 – 3.80 (m, 2H), 3.69 – 3.54 (m, 2H).

$^{13}$C NMR (101 MHz, D$_2$O) δ (ppm): 119.75, 72.02, 71.21, 62.94, 62.86.

HR-MS (ESI) m/z calcd. for C$_5$H$_9$NO$_4$Na (M + Na)$^+$ 170.0429, found 170.0434.

**L-Arabinonitrile (3-8)**

Prepared according to the general procedure using L-arabinose (0.046 g, 0.307 mmol) as reducing sugar. Yield = 0.0455 g (100%).

$^1$H NMR (400 MHz, D$_2$O) δ (ppm): 4.89 (d, $J = 2.2$ Hz, 1H), 3.84 – 3.75 (m, 2H), 3.74 – 3.68 (m, 1H), 3.65 (dd, $J = 11.6$, 5.5 Hz, 1H).


\[ ^{13}C \text{ NMR (101 MHz, D}_2\text{O)} \delta (\text{ppm}): 120.44, 72.09, 70.49, 63.17, 62.27. \]

HR-MS (ESI) m/z calcd. for C\textsubscript{5}H\textsubscript{9}NO\textsubscript{4}Na (M + Na)\textsuperscript{+} 170.0429, found 170.0433.

**2-Deoxy-\textit{d}-ribonitrile (3-9)**

Prepared according to the general procedure using 2-deoxy-\textit{d}-ribose (0.0114 g, 0.085 mmol) as reducing sugar. Yield = 0.0107 g (96%).

\[ ^{1}H \text{ NMR (400 MHz, D}_2\text{O)} \delta (\text{ppm}): 3.99 - 3.91 (m, 1H), 3.84 - 3.71 (m, 1H), 3.70 - 3.56 (m, 2H), 2.87 (ddd, } J = 17.2, 4.1, 0.5 \text{ Hz, 1H}), 2.77 (ddd, } J = 17.2, 6.8, 0.5 \text{ Hz, 1H}). \]

\[ ^{13}C \text{ NMR (101 MHz, D}_2\text{O)} \delta (\text{ppm}): 120.04, 73.97, 67.67, 62.89, 22.64. \]

HR-MS (ESI) m/z calcd. for C\textsubscript{5}H\textsubscript{9}NO\textsubscript{3}Na (M + Na)\textsuperscript{+} 154.0480, found 154.0484.

**\textit{d}-Gluconitrile (3-10)**

Prepared according to the general procedure using \textit{d}-glucose (0.0082 g, 0.045 mmol) as reducing sugar. Yield = 0.0072 g (90%).
$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 4.72 (d, $J$ = 7.3 Hz, 1H), 4.06 (dd, $J$ = 7.4, 1.6 Hz, 1H), 3.82 (dd, $J$ = 11.8, 2.4 Hz, 1H), 3.79 – 3.70 (m, 2H), 3.64 (dd, $J$ = 11.5, 5.3 Hz, 1H).

$^{13}$C NMR (101 MHz, D$_2$O) $\delta$ (ppm): 119.75, 71.22, 71.14, 70.09, 63.47, 63.16.

HR-MS (ESI) m/z calcd. for C$_6$H$_{11}$NO$_5$Na (M + Na)$^+$ 200.0535, found 200.0528.

**d-Galactonitrile (3-11)**

Prepared according to the general procedure using d-galactose (0.0162 g, 0.090 mmol) as reducing sugar. Yield = 0.0155 g (97%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 4.91 (d, $J$ = 1.9 Hz, 1H), 3.96 – 3.86 (m, 2H), 3.68 – 3.62 (m, 3H).

$^{13}$C NMR (101 MHz, D$_2$O) $\delta$ (ppm): 120.61, 71.46, 70.40, 69.16, 63.63, 62.43.

HR-MS (ESI) m/z calcd. for C$_6$H$_{11}$NO$_5$Na (M + Na)$^+$ 200.0535, found 200.0540.
**d-Allonitrile (3-12)**

Prepared according to the general procedure using d-ahloose (0.0139 g, 0.077 mmol) as reducing sugar. Yield = 0.0086 g (63%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 4.87 (dd, $J = 3.9$, 1.1 Hz, 1H), 3.96 (ddd, $J = 7.6$, 3.9, 1.1 Hz, 1H), 3.91 – 3.84 (m, 1H), 3.78 – 3.70 (m, 2H), 3.63 (ddd, $J = 11.9$, 7.3, 1.1 Hz, 1H).

$^{13}$C NMR (101 MHz, D$_2$O) $\delta$ (ppm): 119.47, 73.04, 72.70, 72.38, 63.71, 62.73.

HR-MS (ESI) m/z calcd. for C$_6$H$_{11}$NO$_5$Na (M + Na)$^+$ 200.0535, found 200.0540.

**d-Mannonitrile (3-13)**

Prepared according to the general procedure using d-mannose (0.0335 g, 0.186 mmol) as reducing sugar. Yield = 0.0317 g (96%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 4.62 (d, $J = 8.4$ Hz, 1H), 4.03 (dd, $J = 8.3$, 1.5 Hz, 1H), 3.81 (dd, $J = 11.7$, 2.6 Hz, 1H), 3.75 – 3.69 (m, 1H), 3.68 – 3.58 (m, 2H).
$^{13}$C NMR (101 MHz, D$_2$O) δ (ppm): 120.94, 70.98, 70.82, 69.07, 63.67, 62.71.

HR-MS (ESI) m/z calcd. for C$_6$H$_{13}$NO$_5$Na (M + Na)$^+$ 200.0535, found 200.0537.

**$\textit{d-Maltonitrile (3-14)}$**

Prepared according to the general procedure using $\textit{d}$-maltose (0.0355 g, 0.098 mmol) as reducing sugar. Yield = 0.0186 g (53%).

$^1$H NMR (400 MHz, D$_2$O) δ (ppm): 5.08 (d, $J = 3.7$ Hz, 1H), 4.91 (d, $J = 5.4$ Hz, 1H), 4.12 (ddd, $J = 5.3$, 3.9, 1.1 Hz, 1H), 3.92 (pd, $J = 5.7$, 4.8, 3.0 Hz, 2H), 3.86 – 3.61 (m, 6H), 3.55 (dd, $J = 10.0$, 3.9 Hz, 1H), 3.40 (t, $J = 9.6$ Hz, 1H).

$^{13}$C NMR (101 MHz, D$_2$O) δ (ppm): 119.75, 101.47, 101.33, 80.40, 73.30, 72.90, 72.70, 72.26, 69.99, 63.03, 62.92, 60.96.

HR-MS (ESI) m/z calcd. for C$_{12}$H$_{21}$NO$_{10}$Na (M + Na)$^+$ 362.1063, found 362.1060.
**L-Fuconitrile (3-15)**

Prepared according to the general procedure using L-fucose (0.0133 g, 0.0812 mmol) as reducing sugar. CD3OD (20% v/v) was added to the reaction to increase the solubility. Yield = 0.0078 g (59%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 4.92 (d, $J$ = 2.0 Hz, 1H), 4.11 – 4.03 (m, 1H), 3.85 (dd, $J$ = 9.5, 2.1 Hz, 1H), 3.46 (dd, $J$ = 9.5, 1.6 Hz, 1H), 1.23 (d, $J$ = 6.5 Hz, 3H).

$^{13}$C NMR (101 MHz, D$_2$O) $\delta$ (ppm): 120.65, 72.57, 71.86, 66.11, 62.55, 19.23.

HR-MS (ESI) m/z calcd. for C$_6$H$_{11}$NO$_4$Na (M + Na)$^+$ 184.0586, found 184.0591.

**N-Acetyl-d-glucosaminonitrile (3-16)**

Prepared according to the general procedure using N-acetyl-d-glucosamine (0.0250 g, 0.114 mmol) as reducing sugar. Yield = 0.0174 g (70%).
$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 4.93 (d, $J = 8.2$ Hz, 1H), 4.25 (dt, $J = 8.2$, 0.7 Hz, 1H), 3.89 – 3.82 (m, 1H), 3.77 – 3.73 (m, 2H), 3.70 – 3.59 (m, 1H), 2.06 (s, 3H).

$^{13}$C NMR (101 MHz, D$_2$O) $\delta$ (ppm): 174.88, 117.83, 71.22, 70.44, 69.37, 63.47, 57.21, 22.36.

HR-MS (ESI) m/z calcd. for C$_8$H$_{14}$N$_2$O$_5$Na (M + Na)$^+$ 241.0800, found 241.0803.

3.2.3. Synthesis of 5-O-Tert-Butyl-Diphenylsilyl-D-Ribonitrile

To a 7 mL vial was added 5-O-tet-butyldiphenylsilyl-D-ribose (0.131 g, 0.337 mmol, 1 equiv), O-phenylhydroxylamine hydrochloride (0.059 g, 0.404 mmol, 1.2 equiv), 2 mL of 0.2 M sodium phosphate pH 7.25 and 2 mL of methanol. The reaction was stirred at room temperature until the change of starting materials could not be observed (monitored by TLC). The reaction mixture was evaporated to dryness. The crude mixture was purified by silica column ($R_f = 0.3$, 40:60 EtOAc:Hexanes). Yield = 0.0895 g (69%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 7.76 – 7.56 (m, 4H), 7.55 – 7.34 (m, 6H), 4.77 (d, $J = 3.9$ Hz, 1H), 3.93 – 3.81 (m, 3H), 3.78 (dd, $J = 8.0$, 4.3 Hz, 1H), 1.08 (s, 9H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ (ppm): 135.66, 132.50, 130.38, 128.15, 117.98, 72.83, 71.72, 64.65, 64.28, 27.00, 19.36.

HR-MS (ESI) m/z calcd. for C$_{21}$H$_{28}$NO$_4$Si (M + H)$^+$ 386.1788, found 386.1789.
3.2.4. General Procedure for the Formation of Nitriles

To a 7 mL vial, aldehyde (1 equiv) and O-phenylhydroxylamine hydrochloride (3-4) (1.2 equiv) were dissolved in 4:1 of methanol:0.5 M sodium phosphate pH 6.5 to the final concentration of 0.1 M. The reaction was stirred at 60 °C until the changes of starting materials could not be observed (monitored by TLC). Then, the reaction mixture was cooled to room temperature and extracted with 3 mL CH₂Cl₂ a total of 5 times. The combined organic layer was dried over Na₂SO₄ and concentrated by rotary evaporator. The crude product was purified by silica column chromatography.

4-Hydroxy-3-methoxybenzonitrile (3-18)

Prepared according to the general procedure using vanillin (3-28) (0.0254 g, 0.167 mmol) as an aldehyde. The reaction time was 8 h. The crude product was purified by silica column chromatography (EtOAc:Hexanes 40:60). Yield = 0.0248 g (99%).
$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 7.22 (dd, $J = 8.2, 1.8$ Hz, 1H), 7.08 (d, $J = 1.7$ Hz, 1H), 6.96 (d, $J = 8.2$ Hz, 1H), 3.92 (s, 3H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ (ppm): 150.01, 146.74, 127.10, 119.39, 115.33, 113.82, 103.35, 56.39

HR-MS (ESI) m/z calcd. for C$_8$H$_8$NO$_2$ (M + H)$^+$ 150.0555, found 150.0557

**Trans-Cinnamonic Acid (3-19)**

Prepared according to the general procedure using trans-cinnamaldehyde (3-27) (0.025 g, 0.189 mmol) as an aldehyde. The reaction time was 72 h. The crude product was purified by silica column chromatography (EtOAc:Hexanes 10:90). Yield = 0.0155 g (63%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 7.49 – 7.36 (m, 6H), 5.88 (d, $J = 16.7$ Hz, 1H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ (ppm): 150.74, 133.62, 131.35, 129.28, 127.52, 118.27, 96.42

HR-MS (ESI) m/z calcd. for C$_9$H$_7$N (M)$^+$ 129.0578, found 129.0581.
**N-Boc-4-(cyanomethyl)piperidine (3-20)**

Prepared according to the general procedure using N-Boc-4-piperidine-acetaldehyde (3-29) (0.0308 g, 0.136 mmol) as an aldehyde. The reaction time was 72 h. The crude product was purified by silica column chromatography (EtOAc:Hexanes 40:60). Yield = 0.0183 g (60%).

$^1$H NMR (101 MHz, CDCl$_3$) $\delta$ (ppm): 4.14 (s, 2H), 2.70 (t, $J$ = 12.2 Hz, 2H), 2.30 (d, $J$ = 6.4 Hz, 2H), 1.93 – 1.73 (m, 3H), 1.44 (s, 9H), 1.32 – 1.17 (m, 2H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ (ppm): 154.74, 118.24, 79.82, 43.48, 33.47, 31.40, 28.55, 24.21.

HR-MS (ESI) m/z calcd. for C$_{12}$H$_{20}$N$_2$O$_2$Na (M + Na)$^+$ 247.1422, found 247.1430
(3S)-Citronellylnitrile (3-21)

Prepared according to the general procedure using (-)-citronellal (3-30) (0.0244 g, 0.158 mmol) as an aldehyde. The reaction time was 24 h. The crude product was purified by silica column chromatography (EtOAc:Hexanes 5:95). Yield = 0.0139 g (58%).

\[ ^1H\text{ NMR (400 MHz, CDCl}_3\text{) } \delta \text{ (ppm): 5.07 (tp, } J = 7.1, 1.5 \text{ Hz, } 1H), 2.42 - 2.16 \text{ (m, } 2H), 2.10 - 1.95 \text{ (m, } 2H), 1.86 \text{ (dq, } J = 13.1, 6.5 \text{ Hz, } 1H), 1.68 \text{ (s, } 3H), 1.61 \text{ (s, } 3H), 1.46 \text{ (ddt, } J = 13.3, 8.5, 6.5 \text{ Hz, } 1H), 1.40 - 1.22 \text{ (m, } 1H), 1.07 \text{ (d, } J = 6.7 \text{ Hz, } 3H). \]

\[ ^{13}C\text{ NMR (101 MHz, CDCl}_3\text{) } \delta \text{ (ppm): 132.43, 123.62, 119.05, 35.99, 30.08, 25.84, 25.39, 24.58, 19.53, 19.50.} \]

HR-MS (ESI) m/z calcd. for C_{10}H_{17}N (M)^{+} 151.1361, found 151.1368.

3.2.5. General Procedure for the Elimination of Cyanohydrins

To a 20 mL vial was added sugar cyanohydrin and ethanol to a final concentration of 0.01 M. Lewatit MP 62 resins (approximately 10 times by mass compared to the mass of cyanohydrin) were added to the mixture then the vial was connected to a gas bubbler with a 5% NaOH trap by way of a Tygon® tube and host adapter. The purpose of this apparatus was to trap cyanide gas produced during the reaction. The reaction was stirred (300 rpm) at 70 °C for 7 h. The resins were removed by vacuum filtration, and the solution was evaporated to obtain the desired product.
3.2.5.1. Preparation of D-Lyxose (3-22)

Prepared according to the general procedure using D-galactonitrile (3-11) (0.0058 g, 0.033 mmol) as sugar cyanohydrin. Yield = 0.0039 g (79%).

$^1$H NMR spectrum of product matched existing $^1$H NMR of commercially available D-lyxose.

3.2.5.2. Preparation of D-Arabinose (3-23)

Prepared according to the general procedure using D-mannonitrile (3-13) (0.0065 g, 0.0365 mmol) as sugar cyanohydrin. Yield = 0.0041 g (75%).

$^1$H NMR spectrum of product matched existing $^1$H NMR of commercially available D-arabinose.
3.2.5.3. Preparation of α-D-Glucopyranosyl-(1→3)-D-Arabinopyranose

Prepared according to the general procedure using D-maltonitrile (3-14) (0.0037 g, 0.01 mmol) as sugar cyanohydrin. Yield = 0.0018 g (58%).

\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) (ppm): 5.41 (d, \(J = 3.8\) Hz, 1H), 5.22 (d, \(J = 3.3\) Hz, 0.5H), 4.65 (d, \(J = 7.9\) Hz, 0.5H), 4.05 – 3.58 (m, 9H), 3.47 (t, \(J = 9.5\) Hz, 1.5H), 3.35 – 3.30 (m, 0.5H).

HR-MS (ESI) m/z calcd. for C\(_{11}\)H\(_{19}\)O\(_{10}\) (M-H)\(^-\) 311.0978, found 311.0977.

3.3. Results and Discussion

3.3.1. Optimization Study with D-Ribose

In a preliminary study, the reaction of D-ribose and O-phenylhydroxylamine hydrochloride (3-4) in an aqueous solution yielded cyanohydrin. This observation led to an evaluation of the reaction conditions under various aqueous environments at room temperature (Table 3.1). All aqueous solutions in the optimization studies were prepared from deuterium oxide, which allowed for monitoring the reaction by \(^1\)H NMR spectroscopy. The NMR spectra demonstrated that the oxime was formed as an intermediate, then the phenyl group was subsequently eliminated, and the terminal nitrile (3-5) was obtained as a product (Scheme 3.3). The incompletion from entries 1 and
indicated that the presence of acid in solution prevented the reaction to proceed to the completion because the hydrolysis rate of the oxime is high under a low pH environment. Compound 3-4 was used as a hydrochloride salt, which increased the acidity of the solution. The cyanohydrin formation under phosphate buffer conditions at pD 5.45 and pD 7.45 could proceed to the completion within 7 h (entries 3 and 4). It was reasoned that under the buffer conditions, the hydrochloric acid produced from a starting material was neutralized. The pD values of the reaction mixtures were recorded, and the change of the pD values was not observed. Therefore, the reaction condition of 0.2 M of sodium phosphate buffer at pD 7.45 was used as a general procedure for further investigations.

Scheme 3.3: Proposed reaction mechanism.
Next, various types of hydroxylamine sources were evaluated for the formation of cyanohydrin from reducing sugar under the standard condition of 0.2 M sodium phosphate pD 7.45 (Table 3.2). The reactions were monitored by $^1$H NMR spectroscopy for 24 h at room temperature. As expected, the cyanohydrin product was not obtained from the reaction of D-ribose with O-benzylhydroxylamine hydrochloride (3-25) (entry 2); however, only an oxime was observed. The cyanohydrin was obtained from the reaction of D-ribose with hydroxylamine-O-sulfonic acid (3-3) in 90% yield after 24 h (entry 3). The incomplete reaction after 24 h could be rationalized from the fact that sulfuric acid was obtained as a byproduct, which increased the hydrolysis rate of an oxime intermediate. For the reaction of D-ribose with O-(diphenylphosphinyl)-hydroxylamine (3-1) and O-
(mesitylsulfonyl)hydroxylamine (3-26) (entries 4 and 5, respectively), only a trace of cyanohydrin product was observed. The poor solubility of substrate 3-1 and 3-26 in water was believed to be a major reason. Furthermore, a previous study demonstrated that oxime intermediates from the reaction of 3-1 with aldehydes were stable at room temperature, but the nitrile products could be observed after heating the reaction at 85 °C for several hours. The quantitative yield from entry 1 demonstrated that 3-4 was a suitable nitrogen source for the formation of cyanohydrins from a variety of carbohydrates.

Table 3.2 Evaluation of cyanohydrin formation with hydroxylamine sources.

<table>
<thead>
<tr>
<th>Entry</th>
<th>R-ONH₂</th>
<th>Time (h)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>H₂N</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>3-4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>C₆H₄OH₂</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3-25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>H₂N</td>
<td>24</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>3-3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>H₂N</td>
<td>24</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>3-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>H₂N</td>
<td>24</td>
<td>trace</td>
</tr>
<tr>
<td></td>
<td>3-26</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Determined by ³¹H NMR
3.3.2. Transformation of Reducing Sugars to Cyanohydrins

The transformation of 13 reducing sugars to cyanohydrins is reported in Table 3.3. All carbohydrate substrates were reacted with 3-4 in 0.2 M sodium phosphate at pD 7.25 and the reactions were monitored by $^1$H NMR spectroscopy. Phenols produced as byproduct could be easily removed from the reaction mixtures by extraction with diethyl ether. In most cases, after evaporation of the aqueous solution to dryness, the cyanohydrin products were obtained by dissolving the dried mixture with ethanol. The exception to this purification protocol was the case of silyl-protected ribose (entry 13); the reaction mixture was purified by silica column. The reactions of 3-4 with pentoses (entries 1 to 4) was highly efficient; they proceeded to the completion within 12 h. Interestingly, it required longer reaction time for the substrate 2-deoxy-D-ribose (entry 5) to proceed to completion. The reactions of hexoses (entries 6 to 8) and disaccharides (entry 10) were slower and often required 48 h to reach completion. However, the reaction of D-mannose (entry 9) required only 24 h for the full conversion. It could be reasoned that the axial OH group on the C-2 position did not interfere during the nucleophilic attack on the carbonyl carbon. L-Fucose, GlcNAc, and silyl-protected ribose (entries 11, 12, and 13, respectively) required longer reaction time than typical likely due to the steric hindrance on the substrates. The isolated yield from the reaction of maltose was lower than expected because the maltonitrile was poorly soluble in ethanol. The reaction of L-fucose and N-GlcNAc showed lower of isolated yields than expected because both cyanohydrin products were partially soluble in diethyl ether, which were removed during the purification process.
Table 3.3 Transformation of reducing sugars to cyanohydrins.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Reducing sugar</th>
<th>Time (h)</th>
<th>Yield (^a) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D-Ribose</td>
<td>12</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>D-Arabinose</td>
<td>12</td>
<td>81</td>
</tr>
<tr>
<td>3</td>
<td>D-Xylose</td>
<td>12</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>L-Arabinose</td>
<td>12</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>2-Deoxy-D-ribose</td>
<td>16</td>
<td>96</td>
</tr>
<tr>
<td>6</td>
<td>D-Glucose</td>
<td>48</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>D-Galactose</td>
<td>48</td>
<td>97</td>
</tr>
<tr>
<td>8</td>
<td>D-Allose</td>
<td>48</td>
<td>63</td>
</tr>
<tr>
<td>9</td>
<td>D-Mannose</td>
<td>24</td>
<td>96</td>
</tr>
<tr>
<td>10</td>
<td>D-Maltose</td>
<td>48</td>
<td>53</td>
</tr>
<tr>
<td>11</td>
<td>L-Fucose</td>
<td>72</td>
<td>59</td>
</tr>
<tr>
<td>12</td>
<td>N-Acetyl-D-glucosamine (GlcNAc)</td>
<td>72</td>
<td>70</td>
</tr>
<tr>
<td>13</td>
<td>5-O-TBDPS-D-ribose(^b,c)</td>
<td>72</td>
<td>69</td>
</tr>
</tbody>
</table>

\(^a\)Isolated yield; \(^b\)MeOH was added (50% v/v) to increase solubility; \(^c\)TBDPS = tert-butyldiphenylsilyl
3.3.3. Optimization Study with Cinnamaldehyde

The scope of the aldehyde substrates was extended to aromatic, aliphatic, and \( \alpha,\beta \)-unsaturated aldehydes. Reaction conditions used for carbohydrate compounds needed to be modified to be used for these hydrophobic substrates since the organic are poorly soluble in water. Cinnamaldehyde (3-27) was chosen to optimize reaction conditions. A 4:1 ratio of methanol to aqueous sodium phosphate pH 6.5 was found to be the best solvent for dissolving 3-27. Since the reactions could be monitored by TLC, the protonated solutions were used for the reaction instead of using the deuterated condition. The concentration of sodium phosphate affects the yields of the products. As shown in Table 3.4, a 0.5 M of sodium phosphate increased the yields of the product as compared to a 0.2 M (entries 1 and 2). However, the yield decreased when a 1 M sodium phosphate (entry 3) was used because it caused the reaction mixture to become heterogeneous. The effect of biphasic conditions prepared from n-butanol and sodium phosphate buffer was also studied (Table 3.4, entries 4 and 5). The concentration of sodium phosphate did not affect the yield of the product. Nevertheless, these biphasic conditions were less efficient than monophasic conditions. Therefore, the condition in entry 2 was chosen as a general procedure to be used with organic compounds.
3.3.4. Transformation of Hydrophobic Aldehydes to Nitriles

To study the formation of nitriles from hydrophobic compounds, reactions using a 4:1 ratio of methanol to 0.5 M sodium phosphate pH 6.5 was used as a solvent, and conducted at 60 °C. The results in Table 3.5 suggests that this condition is efficient for the selective transformation of aldehydes to nitriles with a variety of substrates, especially, for the transformation of vanillin (3-28) which could be completed within 8 h with exceptional yield (entry 1). This reaction is also suitable for an acid-labile functional group, such as tert-butyloxycarbonyl protecting group (Boc) (3-29), as shown in entry 3.

Table 3.4 Optimization study with cinnamaldehyde.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Condition</th>
<th>Time (h)</th>
<th>Isolated yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4:1 of MeOH:0.2 M sodium phosphate pH 6.5</td>
<td>72</td>
<td>32</td>
</tr>
<tr>
<td>2</td>
<td>4:1 of MeOH:0.5 M sodium phosphate pH 6.5</td>
<td>72</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>4:1 of MeOH:1 M sodium phosphate pH 6.5</td>
<td>72</td>
<td>38</td>
</tr>
<tr>
<td>4</td>
<td>1:1 of n-BuOH:0.5 M sodium phosphate pH 6.5</td>
<td>72</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>1:1 of n-BuOH:1.0 M sodium phosphate pH 6.5</td>
<td>72</td>
<td>51</td>
</tr>
</tbody>
</table>
Furthermore, the unsaturated aliphatic aldehyde (3-30) could also be transformed to a nitrile under the same condition in an acceptable yield (58%).

Table 3.5 Transformation of aldehydes to nitriles.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Aldehyde</th>
<th>Time (h)</th>
<th>Isolated Yield (%)</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1.png" alt="image" /></td>
<td>8</td>
<td>99</td>
<td><img src="image2.png" alt="image" /></td>
</tr>
<tr>
<td>2</td>
<td><img src="image3.png" alt="image" /></td>
<td>72</td>
<td>63</td>
<td><img src="image4.png" alt="image" /></td>
</tr>
<tr>
<td>3</td>
<td><img src="image5.png" alt="image" /></td>
<td>72</td>
<td>60</td>
<td><img src="image6.png" alt="image" /></td>
</tr>
<tr>
<td>4</td>
<td><img src="image7.png" alt="image" /></td>
<td>24</td>
<td>58</td>
<td><img src="image8.png" alt="image" /></td>
</tr>
</tbody>
</table>
3.3.5. Application

Wohl degradation was selected to demonstrate the application of the carbohydrate cyanohydrins. It is well known that the Wohl degradation is one of the most common methods for shortening aldose sugars through the formation of acetylated cyanohydrin and elimination of the terminal cyanide by using ammonia or silver hydroxide (Scheme 3.4). The goal of this study is to find an alternative and robust approach to shortening sugars by using the weakly basic resins, which allow for convenient purification through filtration.

![Scheme 3.4: Conventional Wohl degradation.](image)

Previously, weakly basic resins (WBS) were used to remove palladium and platinum ions from aqueous solution.\textsuperscript{121,122} It was hypothesized that these types of resin would act similarly to ammonia to eliminate the terminal cyanide. Therefore, three cyanohydrins were chosen to study in a new degradation approach using Lewatit MP62 resin as a base (Table 3.6). The resin is very efficient for the reaction. By using the resin, d-Galactonitrile (3-11) could be degraded to d-lyxose (3-22), and d-mannonitrile (3-14) could be degraded to d-arabinose (3-23) (entries 1 and 2, respectively). The same reaction condition was also applied for removing terminal cyanide from a disaccharide cyanohydrin. Entry 3 shows that cyanide was eliminated from a d-malonitrile (3-14) to
form D-glucopyranose-(1→3)-D-arabinopyranose (3-24), which is a new type of reducing sugar. This practical approach provides an alternative way to produce rare reducing sugars from the commercially available ones.

Table 3.6 Degradation of cyanohydrins.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Time (h)</th>
<th>Product (Yield)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>3-11 (79%)</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>3-13 (75%)</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>3-14 (60%)</td>
</tr>
</tbody>
</table>
3.4 Conclusions

A new method for transforming aldehydes to nitriles using commercially available O-phenylhydroxylamine hydrochloride as a nitrogen source is described. The method works exceptionally well under aqueous solution with the presence of sodium phosphate buffer. Several reducing sugars were transformed to cyanohydrins in high yields. The lower yields obtained from D-maltose was due to the solubility issue. This method was modified to be compatible with hydrophobic compounds by using a mixture of an organic solvent and sodium phosphate buffer. Several functionalized aliphatic and aromatic aldehydes were transformed to nitriles in acceptable yields. The reaction conditions were also suitable for a compound containing an acid-labile functional group.

A new method for shortening reducing sugars by using weakly basic resins was demonstrated. The cyanohydrin compounds (3-11, 3-13, 3-14) were used as reactants for the demonstration. The reducing sugars obtained from the degradation of D-galactonitrile, D-mannonitrile, and D-maltonitrile were D-lyxose (3-22), D-arabinose (3-23), and D-glucose-α-(1→3)-D-arabinose (3-24), respectively.
CHAPTER 4
SYNTHESIS OF PHOTOCAGED β-NAD⁺ ANALOGUE AND CHEMOENZYMATIC SYNTHESIS
OF POLY(ADP-RIBOSE)

4.1. Introduction

Poly(ADP-ribose) (poly(adenosine diphosphate ribose)) or PAR is a natural polymer that was discovered more than 50 years ago as a product from the enzymatic reaction of β-NAD⁺ with poly(ADP-ribose) polymerase (PARP) (Scheme 4.1). To date, there are at least 18 enzymes in the PARP family based on the similarity at the catalytic domain as shown in Figure 4.1. However, there are only 4 enzymes that have been known to process the poly(ADP-ribosyl)ation (PARylation) including PARP1, PARP2, tankyrase1 (TANK1), and tankyrase2 (TANK2). PARylation is involved in many biological processes such as DNA-damage repair, transcription, mitosis, and apoptosis. During DNA strand breaks (either single- or double-strand breaks), PARP1 is activated, and it starts catalyzing PAR formation onto itself (automodification) or associated proteins such as histones (heteromodification).

Scheme 4.1: Incorporation of PAR on target protein by PARP enzyme.
PAR polymers involve in many biological processes through the noncovalent interactions with a variety of associated proteins.\textsuperscript{133,134} For example, PAR that is formed during the activation of PARP1 at the DNA-damage site can act as a scaffold to recruit PAR-binding proteins involving in DNA repairs, such as XRCC1, ALC1, APLF, and CHFR.\textsuperscript{135} Furthermore, the interaction between PAR and associated proteins depends on the chain length of PAR. For example, during the DNA-damaged repair, long PAR polymers (>40
units) are preferred for associated DNA repair proteins XPA, DEK, and ChK1 but short PAR polymers are preferred for DNA repair proteins p53 and WRN.¹³⁶

PAR composes of ADP-ribose between 2 to 200 units connecting at the 2’-OH of adenosine via an α-glycosidic linkage. In every 20 to 50 repeating units, the branching of the polymer can be observed at the 2’’-OH of the ribose¹³⁶ (Figure 4.2). PAR structure can be considered as a combination of polynucleotide and polysaccharide. PAR contains ribose (reducing sugar) at the terminal end and can branch at the hydroxyl group via a glycosidic linkage, which makes its properties similar to the carbohydrates (glycans).

![Figure 4.2: Structure of poly(ADP-ribose).](image-url)
The structural complexity and heterogeneity of PARs impede scientists to study their biological functions related to their structures. One of the reasons is the lack of tools to access PAR in homogeneous forms. This work is intended to use the chemical tools to obtain PAR in homogeneous forms by two approaches. The first approach uses chemical auxiliary to facilitate fractionation and purification of the PAR polymers. Another approach uses a new β-NAD⁺ analogue substrate for the chemoenzymatic synthesis of PAR in a controlled manner.

Recently, Mitchison and coworkers reported the in vitro enzymatic synthesis of PAR obtaining the polymers in hundreds of milligram scales with a variety of polymer sizes. They also demonstrated the fractionation of PAR through an anion exchange chromatogram to obtain the defined length polymers. However, this method is still not feasible to obtain a specifically defined length of PAR polymer at a workable yield. This Chapter focused on applying the auxiliary 2-6 from Chapter 2 to PAR envisioning that auxiliary 2-6 can facilitate fractionation and purification to obtain the defined length of PAR polymers.

Although PAR polymers with specifically defined lengths can be obtained via chemical synthesis, there are many labile functional groups and specific stereochemistry in PAR polymer that challenge synthetic chemists. Filippov and coworkers reported the synthesis of PAR oligomers through the solid-phase synthetic approach. Although this method demonstrated a robust approach to obtain a specifically defined length of PAR, it required multiple synthetic steps for preparing starting materials that were used as building blocks.
Recently, Marx and coworkers\textsuperscript{139} reported modified β-NAD\textsuperscript{+} analogues that could be used as substrates for wild-type PARP1 for labeling target proteins. The report shows that by changing hydroxyl group at 2’ position of β-NAD\textsuperscript{+} to hydrogen (compound 4-1 and 4-2, Figure 4.3), PARP1 still recognized these compounds as substrates and they were used as chain-terminators during PARylation. Inspired by this modification, it is envisioned that the formation of PAR can also be controlled selectively through the chemoenzymatic synthesis by modifying the substrate at the 2’ position. With this approach, it could be able to terminate a PAR formation by adding a protecting group at 2’-OH (compound 4-20). After selective deprotection, a 2’-OH will be available for further elongation.

![Figure 4.3: Structure of β-NAD\textsuperscript{+} analogue substrates for PARP1 as chain terminators.](image)

**4.2. Experimental**

**4.2.1. General Information**

All chemicals and reagents were purchased and used without further purification.

All the reactions were performed in flame dried glassware under a nitrogen atmosphere unless stated. Dimethylformamide (DMF), pyridine, methylene chloride, and methanol were dried over 4 Å molecular sieves. Thin-layer chromatography (TLC) analysis was
performed on silica gel 60 with fluorescent indicator F₂₅₄ (EMD Millipore) and visualized with UV light (254 nm) where applicable. Silica gel column chromatography was performed on SiliaFlash® F60 (Silicycle, 40-64 µm). ¹H, ¹³C, and ³¹P NMR spectra were recorded at 400 MHz, 101 MHz, and 162 MHz, respectively. HPLC analysis and preparation were performed on Gilson® Analytical to Semi-Preparative HPLC system using Kinetex® 5 µm EVO C18 100 Å 4.6x150 mm and SiliaChrom C18 5 µm 100 Å 4.6x150 mm for analytical columns and Kinetex® 5u EVO C18 100 Å 21.1x150 mm for a preparative column. Mass spectrometry analysis was performed by the School of Chemical Sciences Mass Spectrometry Laboratory at the University of Illinois.

Human PARP1 enzyme (high specific activity) was purchased from Trevigen. ADP-ribose and deoxyribonucleic acid from calf thymus were purchased from Sigma. Adenosine 5’-monophosphate was purchased from Acros. Beta-nicotinamide adenine dinucleotide was purchased from Alfa Aesar. Nicotinamide mononucleotide (NMN) was purchased from Accela Chembio.

List of solvents used on HPLC:

Solvent A = water

Solvent B = acetonitrile

Solvent C = 8 mM Et₃NHOAc in water

Solvent D = 8 mM Et₃NHOAc in acetonitrile
4.2.2. Synthetic Procedures

*Synthesis of compound 4-3a and 4-3b*

The synthetic procedure was modified from Szczepankiewicz *et al.*\textsuperscript{140} To a 7 mL vial was added Na$_2$CO$_3$ (0.032 g, 0.3 mmol, 2 equiv) and 0.8 mL of glacial acetic acid. After Na$_2$CO$_3$ dissolved, β-NAD$^+$ (0.1 g, 0.15 mmol, 1 equiv) was added to the solution. The reaction mixture was heated at 90 °C for 2 h. The reaction was removed from the heat then 2 mL of cold denatured alcohol (90:5:5 of ethanol:methanol:2-propanol) was added dropwise resulting in the formation of precipitates. The precipitates were filtered via vacuum filtration and washed with 6 mL of cold denatured alcohol. The solid was dried under high vacuum to obtain the product as light brown solid. Yield = 0.0663 g (73%).

$^1$H NMR spectrum of product matched $^1$H NMR of reference 140.
**Synthesis of compound 4-4**

To a 7 mL vial was added compound 4-3a and 4-3b (0.025 g, 0.0415 mmol, 1 equiv) and 0.42 mL of 1 M NaOH. The reaction mixture was stirred at room temperature for 50 min. The mixture was neutralized with glacial acetic acid (pH of the solution was checked by pH paper). Then, 2 mL of cold denatured alcohol (90:5:5 of ethanol:methanol:2-propanol) was added dropwise resulting in the formation of precipitates. The precipitates were filtered through cotton-packed glass pipet. The precipitates that remained in the glass pipet were removed by dissolving with water. After removing water by a rotary evaporator, a brown solid was obtained as the product. It should be noted here that hydrolysis at the pyrophosphate was also observed, but we chose to do conjugation in the next step without further purification. Yield = 0.0192 g (85%).

The $^1$H NMR was determined based on $^1$H NMR of purchased ADPr.

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 8.59 (s, 1H), 8.49 (s, 1H), 6.12 (d, $J = 6.0$, 1H), 5.29 (d, $J = 3.9$ Hz, 0.4H), 5.18 (d, $J = 2.4$ Hz, 0.6H), 4.50 (m, 2H), 4.42 – 4.31 (m, 1H), 4.31 – 3.90 (m, 7H).
**Synthesis of compound 4-5**

To a 7 mL vial, compound 4-4 (0.068 g, 0.11 mmol, 1 equiv) was dissolved in 0.8 mL sodium phosphate buffer (0.25 M, pH 4.7). Another 7 mL vial, compound 2-6 (0.076 g, 0.33 mmol, 3 equiv) was dissolved in 0.2 mL DMSO then added to the 4-4 solution. The reaction mixture was stirred at 40 °C for 72 h. The conjugated product was purified by HPLC preparative C18 column (gradient of 99% solvent A/ 1% solvent B to 60% solvent A/ 40% solvent B in 10 minutes). Yield = 0.0137 g (16%).

$^1$H NMR (400 MHz, D$_2$O) δ (ppm): 8.29 (s, 1H), 8.20 (d, J = 8.3 Hz, 2H), 7.95 (s, 1H), 7.50 (d, J = 7.9 Hz, 2H), 5.91 (d, J = 5.6 Hz, 1H), 4.85 – 4.83 (m, 2H), 4.65 – 4.52 (m, 2H), 4.44 (t, J = 4.6 Hz, 1H), 4.32 (s, 1H), 4.28 – 4.18 (m, 4H), 4.19 – 4.04 (m, 3H), 3.07 (s, 3H), 2.67 (s, 3H).

$^{31}$P NMR (162 MHz, D$_2$O) δ (ppm): -10.10 (d, J = 10.1 Hz).
Synthesis of compound 4-8

The synthetic procedure of Chaulk and MacMillan was followed with modification. To a 40 mL vial was added adenosine (4-6) (0.5 g, 1.87 mmol, 1 equiv) and dry DMF 17 mL under N₂. The solution was heated to 100 °C until adenosine dissolved. The solution was cooled in the iced-water bath. To a 7 mL vial, sodium hydride (0.112 g, 2.8 mmol, 1.5 equiv) was washed with hexanes 3 times then suspended in 2 mL of dry DMF under N₂. The suspension was transferred to adenosine solution and the reaction mixture was stirred in the iced-water bath for 45 min. To a 7 mL vial was added 2-Nitrobenzylbromide (4-7) (0.6 g, 2.8 mmol, 1.5 equiv) and dry DMF 2 mL, then transferred to the reaction mixture. The reaction was stirred at room temperature for 5 h. The reaction was added dropwise to ice-cold water and left stirring at room temperature for overnight. Light yellow precipitates were collected by vacuum filtration and dried under high vacuum. The product was used for the next reaction with further purification. Yield = 0.519 g (69%).

The product could be further purified by washing the precipitates with acetonitrile through vacuum filtration.
\(^1\)H NMR (400 MHz, DMSO-d\(_6\)) \(\delta\) (ppm): 8.35 (s, 1H), 8.10 (s, 1H), 7.97 (d, \(J = 8.1\) Hz, 1H), 7.59 (q, \(J = 8.3\), 7.8 Hz, 2H), 7.49 (t, \(J = 7.5\) Hz, 1H), 7.36 (s, 2H), 6.11 (d, \(J = 6.1\) Hz, 1H), 5.47 – 5.39 (m, 2H), 5.04 (d, \(J = 14.4\) Hz, 1H), 4.87 (d, \(J = 14.8\) Hz, 1H), 4.60 (t, \(J = 5.5\) Hz, 1H), 4.42 (q, \(J = 5.1\) Hz, 1H), 4.05 (q, \(J = 3.6\) Hz, 1H), 3.70 (dt, \(J = 12.8, 4.6\) Hz, 1H), 3.58 (ddd, \(J = 11.6, 7.1, 3.7\) Hz, 1H).

\(^{13}\)C NMR (101 MHz, DMSO-d\(_6\)) \(\delta\) (ppm): 156.16, 152.40, 148.96, 147.04, 139.64, 133.96, 133.61, 133.57, 128.51, 124.37, 119.35, 86.47, 86.09, 81.13, 68.93, 67.80, 61.43.

HR-MS (ESI) m/z calcd. for C\(_{17}\)H\(_{19}\)N\(_6\)O\(_6\) (M + H)* 403.1366, found 403.1360.

**Synthesis of compound 4-9**

The synthesis procedure of Chaulk and MacMillan was followed with modification.\(^{141}\) To a 20 mL vial, precipitates of 4-8 (0.358 g, 0.889 mmol, 1 equiv) was evaporated from dry acetonitrile 2 times then added 8.0 mL of dry pyridine and trimethylsilyl chloride (0.56 mL, 4.45 mmol, 5 equiv). The reaction was stirred at room temperature for 45 min. Benzoyl chloride (0.52 mL, 4.45 mmol, 5 equiv) was added to the reaction mixture and stirred at room temperature for 3.5 h. To the reaction was added 2.5 mL of water then cooled in the iced-water bath for 5 min. Cold NH\(_4\)OH (29%, 12 mL)
was added to the reaction and stirred in the iced-water bath for 50 min. The reaction mixture was concentrated by a rotary evaporator. During evaporation, a small amount of silica gel was added to the concentrating mixture. The product was purified by silica column chromatography (MeOH:CH$_2$Cl$_2$ = 5:95). Yield = 0.269 g (60%).

$^1$H NMR (400 MHz, CDCl$_3$) δ (ppm): 9.31 (s, 1H), 8.71 (s, 1H), 8.05 (s, 1H), 8.04 (d, $J$ = 9.4 Hz, 2H), 7.83 (dd, $J$ = 7.9, 1.5 Hz, 1H), 7.66 – 7.58 (m, 1H), 7.53 (dd, $J$ = 8.3, 6.8 Hz, 2H), 7.39 (td, $J$ = 7.4, 1.5 Hz, 1H), 7.36 – 7.28 (m, 2H), 5.95 (d, $J$ = 7.4 Hz, 1H), 5.04 (d, $J$ = 12.9 Hz, 1H), 4.94 (dd, $J$ = 7.4, 4.5 Hz, 1H), 4.68 (d, $J$ = 13.0 Hz, 1H), 4.66 (d, $J$ = 4.4 Hz, 1H), 4.35 (s, 1H), 3.96 (d, $J$ = 13.0 Hz, 1H), 3.77 (d, $J$ = 12.6 Hz, 1H).

$^{13}$C NMR (101 MHz, CDCl$_3$) δ (ppm): 164.78, 152.26, 150.39, 147.94, 143.33, 133.53, 133.42, 133.14, 131.84, 129.80, 129.40, 129.10, 129.00, 128.10, 124.98, 124.45, 89.19, 88.15, 81.13, 70.76, 70.13, 63.20.

HR-MS (ESI) m/z calcd. for C$_{24}$H$_{23}$N$_6$O$_7$ (M + H)$^+$ 507.1628, found 507.1624.
The synthesis procedure of Hergenrother et al. was followed with modification. To a 20 mL vial was added compound 4-9 (0.267 g, 0.528 mmol, 1 equiv), imidazole (0.180 g, 2.64 mmol, 5 equiv), dry DMF 3 mL. Tert-butyldimethylsilyl chloride (0.398 g, 2.64 mmol, 5 equiv) was dissolved in 2 mL of dry DMF then added to the reaction mixture dropwise. The reaction was stirred at room temperature for 18 h. The reaction was concentrated and purified by silica column chromatography (50:50 EtOAc:Hexanes). Yield = 0.279 g (72%).

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ (ppm): 9.04 (s, 1H), 8.80 (s, 1H), 8.35 (s, 1H), 8.07 – 7.98 (m, 3H), 7.72 (d, $J = 7.8$ Hz, 1H), 7.61 (t, $J = 7.4$ Hz, 1H), 7.58 – 7.49 (m, 3H), 7.40 (t, $J = 7.2$ Hz, 1H), 6.33 (d, $J = 4.4$ Hz, 1H), 5.18 – 5.01 (m, 2H), 4.59 (dt, $J = 14.2$, 4.4 Hz, 2H), 4.22 (q, $J = 3.2$ Hz, 1H), 4.02 (dd, $J = 11.6$, 3.6 Hz, 1H), 3.81 (dd, $J = 11.4$, 2.9 Hz, 1H), 0.93 (s, 9H), 0.91 (s, 9H), 0.12 (s, 3H), 0.11 (s, 6H), 0.09 (s, 3H).

$^{13}$C NMR (101 MHz, CDCl$_3$) $\delta$ (ppm): 164.79, 153.02, 152.93, 151.70, 149.67, 141.90, 134.34, 133.95, 133.02, 129.15, 129.05, 128.83, 128.39, 128.08, 124.91, 123.60, 87.16, 85.87, 82.57, 70.34, 69.29, 62.25, 26.24, 25.90, 18.68, 18.26, -4.47, -5.15.
HR-MS (ESI) m/z calcd. for C_{36}H_{51}N_{6}O_{7}Si_{2} (M + H)^{+} 735.3358, found 735.3364.

Synthesis of compound 4-11

The synthesis procedure of Hergenrother et al. was followed with modification. To a 20 mL vial was added compound 4-10 (0.285 g, 0.373 mmol, 1 equiv) and THF 5.2 mL, stirred in iced-water bath. Trichloroacetic acid (2.8 g, 17.16 mmol, 46 equiv) was dissolved in 1.3 mL of water, then added to the 4-10 solution dropwise. The reaction was stirred in iced-water bath for 4.5 h. The reaction was quenched by adding saturated NaHCO_{3} solution. The reaction was extracted with CH_{2}Cl_{2} 3 times and dried with Na_{2}SO_{4}. The solvent was removed by rotary evaporator to obtain product as a light-yellow foam.

Yield = 0.219 g (95%).

{\textsuperscript{1}}H NMR (400 MHz, CDCl_{3}) \(\delta\) (ppm): 8.98 (d, \(J = 14.7\) Hz, 1H), 8.74 (s, 1H), 8.10 (s, 1H), 8.07 – 8.03 (m, 2H), 7.93 – 7.87 (m, 1H), 7.69 – 7.60 (m, 1H), 7.59 – 7.52 (m, 2H), 7.43 – 7.38 (m, 2H), 7.35 – 7.29 (m, 1H), 6.03 (d, \(J = 7.9\) Hz, 1H), 4.97 (d, \(J = 14.1\) Hz, 1H), 4.90 (dd, \(J = 7.8, 4.4\) Hz, 1H), 4.70 (d, \(J = 14.1\) Hz, 1H), 4.67 (d, \(J = 4.9\) Hz, 1H), 4.26 (s, 1H), 3.97 (dd, \(J = 13.1, 1.7\) Hz, 1H), 3.82 – 3.70 (m, 1H), 0.92 (s, 9H), 0.14 (s, 3H), 0.10 (s, 3H).
A modified procedure of Filippov et al.\textsuperscript{138} was proceeded. To a 7 mL vial was added compound \textbf{4-11} (0.027 g, 0.0434 mmol, 1 equiv) and DMF 0.2 mL. Another 7 mL vial was added 1-methylimidazolium chloride (0.0154 g, 0.1302 mmol, 3 equiv), 1-methylimidazole (0.0071 g, 0.0868 mmol, 2 equiv), and DMF 0.3 mL. The mixture was transferred to the \textbf{4-11} solution dropwise. Then, di-tert-butyl-\(N,N'\)-diisopropylphosphoramidite (\textbf{4-12}) (0.021 mL, 0.0615 mmol, 1.5 equiv) was added to the mixture dropwise. The reaction mixture was stirred at room temperature for 40 min. Tert-butyl-peroxide in decane (5-6 M) (0.24 mL, 0.238 mmol, 5.5 equiv) was added to the reaction mixture and the reaction was stirred at room temperature for 1 h. The reaction was quenched with 1 mL of saturated NaHCO\textsubscript{3} solution. The reaction was extracted with EtOAc (5 mL, 3 times). The organic was washed with water (5 mL, 1 time) then dried over
Na₂SO₄. The solution was concentrated and purified by silica column chromatography (5:95 MeOH:CH₂Cl₂). Yield 0.0247 g (70%).

¹H NMR (400 MHz, CDCl₃) δ (ppm): 9.28 (s, 1H), 8.78 (s, 1H), 8.35 (s, 1H), 8.07 – 7.96 (m, 3H), 7.69 (dt, J = 7.9, 1.3 Hz, 1H), 7.64 – 7.57 (m, 1H), 7.52 (tdd, J = 8.1, 6.5, 1.1 Hz, 3H), 7.38 (ddd, J = 8.2, 7.4, 1.6 Hz, 1H), 6.30 (d, J = 4.9 Hz, 1H), 5.10 (d, J = 14.7 Hz, 1H), 5.02 (d, J = 14.8 Hz, 1H), 4.69 (t, J = 4.8 Hz, 1H), 4.61 (t, J = 4.2 Hz, 1H), 4.39 – 4.26 (m, 2H), 4.13 (dt, J = 12.6, 4.8 Hz, 1H), 1.49 (s, 18H), 0.89 (s, 9H), 0.14 (s, 3H), 0.08 (s, 3H).

³¹P NMR (162 MHz, CDCl₃) δ (ppm): -8.79.

HR-MS (ESI) m/z calcd. for C₃₈H₅₄N₆O₁₀SiP (M + H)⁺ 813.3408, found 813.3417.

**Synthesis of compound 4-14**

To a 7 mL vial was added compound 4-13 (0.269 g, 0.331 mmol, 1 equiv) and CH₂Cl₂ 3 mL, stirred in the iced-water bath. Trifluoroacetic acid (TFA) (0.26 mL, 3.31 mmol, 10 equiv) was added to the 4-3 solution dropwise. The reaction was stirred in the iced-water bath for 2 h. The reaction was then warmed to room temperature and stirred for another 5 h. The solvent was removed by rotary evaporator and purified by HPLC.
preparative C18 column (gradient of 80% solvent A/ 20% solvent B to 50% solvent A/ 50% solvent B in 10 minutes). Yield = 0.083 g (43%).

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ (ppm): 8.83 (s, 1H), 8.72 (s, 1H), 8.14 – 8.08 (m, 2H), 7.96 – 7.89 (m, 1H), 7.80 – 7.72 (m, 1H), 7.71 – 7.65 (m, 1H), 7.64 – 7.54 (m, 3H), 7.45 (t, $J$ = 7.6 Hz, 1H), 6.40 (d, $J$ = 4.4 Hz, 1H), 5.23 (d, $J$ = 14.5 Hz, 1H), 5.08 (d, $J$ = 14.4 Hz, 1H), 4.66 (t, $J$ = 4.8 Hz, 1H), 4.59 (t, $J$ = 4.4 Hz, 1H), 4.35 (q, $J$ = 4.5, 3.7 Hz, 1H), 4.33 – 4.28 (m, 1H), 4.27 – 4.18 (m, 1H).

$^{13}$C NMR (101 MHz, CD$_3$OD) $\delta$ (ppm): 168.51, 153.09, 153.02, 152.76, 150.15, 149.14, 143.71, 134.73, 134.57, 134.43, 134.14, 130.55, 129.77, 129.52, 125.54, 122.66, 88.61, 88.49, 85.62, 83.66, 70.28, 65.88.

$^{31}$P NMR (162 MHz, CD$_3$OD) $\delta$ (ppm): 1.26.

HR-MS (ESI) m/z calcd. for C$_{24}$H$_{24}$N$_{6}$O$_{10}$P (M + H)$^+$ 587.1292, found 587.1288.

**Synthesis of compound 4-15**

![Synthesis Scheme](image)

To a 7 mL vial was added compound **4-14** (0.016 g, 0.027 mmol, 1 equiv) and MeOH 1 mL, stirred in the iced-water bath. The solution of 7 M NH$_3$ in MeOH was added to the **4-14** solution dropwise. The reaction was stirred in the iced-water bath for 2 h. The
reaction was then warmed to room temperature and stirred for another 42 h. The solvent was removed by a rotary evaporator and purified by HPLC preparative C18 column (gradient of 99% solvent A/ 1% solvent B to 90% solvent A/ 10% solvent B in 10 minutes).

Yield = 0.011 g (84%).

$^1$H NMR (400 MHz, CD$_3$OD ) $\delta$ (ppm): 8.51 (s, 1H), 8.16 (s, 1H), 7.90 (dd, $J = 8.2, 1.3$ Hz, 1H), 7.72 – 7.64 (m, 1H), 7.52 (td, $J = 7.6, 1.4$ Hz, 1H), 7.40 (ddd, $J = 8.1, 7.4, 1.5$ Hz, 1H), 6.25 (d, $J = 6.1$ Hz, 1H), 5.15 (d, $J = 14.5$ Hz, 1H), 4.96 (d, $J = 14.6$ Hz, 1H), 4.66 (dd, $J = 6.1$, 4.9 Hz, 1H), 4.60 (dd, $J = 4.9$, 3.0 Hz, 1H), 4.32 – 4.26 (m, 1H), 4.13 (m, 2H).

$^{13}$C NMR (101 MHz, CD$_3$OD) $\delta$ (ppm): 157.21, 153.84, 150.71, 148.82, 141.04, 135.29, 134.43, 130.22, 129.40, 125.44, 120.07, 87.29, 86.68, 84.39, 71.21, 70.17, 65.70.

$^{31}$P NMR (162 MHz, CD$_3$OD) $\delta$ (ppm): 2.27.

HR-MS (ESI) m/z calcd. for C$_{17}$H$_{20}$N$_{6}$O$_9$P (M + H)$^+$ 483.1029, found 483.1021.

**Synthesis of compound 4-17**

The synthesis was performed under a normal atmosphere. To a 20 mL vial was added 1.56 mL of acetic anhydride and 1.54 mL of pyridine, stirred in the iced-water bath.
Nicotinamide mononucleotide (NMN) (4-16) (0.05 g, 0.149 mmol, 1 equic) was dissolved in 0.4 mL of water, then added to the acetic anhydride/pyridine solution dropwise. The reaction was stirred in the iced-water bath for 1 h, then warmed to room temperature and stirred for another 5 h. The solvent was removed by a rotary evaporator. The residue was evaporated repeatedly with 2 mL of water 3 times, then left on high-vacuum overnight. To the residue was added 2 mL of 1:1 water:pyridine and stirred at room temperature for 3 h. The solvent was removed by rotary evaporator. The residue was evaporated repeatedly with 1 mL of 1:1 water:pyridine for 5 times, then left it on high-vacuum overnight. The product was used for the next step without further purification. Yield = 0.0554 g (89%).

$^1$H NMR (400 MHz, D$_2$O) $\delta$ (ppm): 9.50 (p, $J = 0.7$ Hz, 1H), 9.32 (d, $J = 6.3$ Hz, 1H), 8.99 (dt, $J = 8.2$, 1.7 Hz, 1H), 8.32 – 8.26 (m, 1H), 6.58 (d, $J = 5.1$ Hz, 1H), 5.64 – 5.59 (m, 1H), 5.56 (dd, $J = 5.4$, 3.7 Hz, 1H), 4.35 (ddd, $J = 11.9$, 4.4, 2.1 Hz, 1H), 4.17 (ddd, $J = 11.9$, 4.7, 1.9 Hz, 1H), 2.17 (s, 3H), 2.15 (s, 3H).

$^{13}$C NMR (400 MHz, D$_2$O) $\delta$ (ppm): 172.67, 172.48, 146.80, 143.23, 140.69, 134.28, 128.84, 97.87, 84.98, 76.73, 70.91, 63.56, 19.88.

$^{31}$P NMR (162 MHz, D$_2$O) $\delta$ (ppm): 1.14.

HR-MS (ESI) m/z calcd. for C$_{15}$H$_{20}$N$_2$O$_{10}$P (M + H)$^+$ 419.0856, found 419.0851.
**Synthesis of compound 4-19**

To a 7 mL vial was added compound 4-17 (0.0202 g, 0.0483 mmol, 2.8 equiv), 1,1’-carbonyldiimidazole (CDI) (0.078 g, 0.483 mmol, 28 equiv) and DMF 1 mL. The mixture stirred at room temperature for 4 h. To the reaction was added 5 mL of dry MeOH and stirred at room temperature for 30 min. The solvent was removed by a rotary evaporator to afford 4-18. The compound 4-18 was left on high-vacuum overnight.

A 0.3 mL of DMF was added to compound 4-18. In another vial, compound 4-15 was added 0.35 mL of 0.5 M MgCl₂ in DMF then transferred to 4-18 solution dropwise. The reaction was stirred at room temperature for 24 h. The solvent was removed by rotary evaporator and purified by HPLC preparative C18 column (gradient of 99% solvent A/ 1% solvent B to 20% solvent A/ 10% solvent B in 10 minutes). Yield = 0.0068 g (43%).

³¹H NMR (400 MHz, D₂O) δ (ppm): 9.30 (s, 1H), 9.18 – 9.11 (m, 1H), 8.86 – 8.77 (m, 1H), 8.22 – 8.14 (m, 1H), 8.13 (s, 1H), 8.01 (s, 1H), 7.61 – 7.57 (m, 1H), 7.38 – 7.31 (m, 2H), 7.24 (ddd, J = 8.8, 6.7, 2.3 Hz, 1H), 6.36 (d, J = 4.3 Hz, 1H), 5.82 (d, J = 7.2 Hz, 1H), 5.52 – 5.43 (m, 2H), 5.22 (d, J = 13.9 Hz, 1H), 4.72 – 4.63 (m, 3H), 4.61 (dd, J = 5.5, 2.0 Hz, 1H), 4.43 (m, 1H), 4.34 (m, 1H), 4.21 (m, 3H), 2.15 (s, 3H), 2.12 (s, 3H).

³¹P NMR (162 MHz, D₂O) δ (ppm): -10.30 (d, J = 18.9 Hz), -10.63 (d, J = 20.1 Hz).

HR-MS (ESI) m/z calcd. for C₃₂H₃₇N₈O₁₈P₂ (M+H)⁺ 883.1701, found 883.1682.
Synthesis of compound 4-20

To a 7 mL vial was added compound 4-19 (0.0017 g, 1.88 µmol, 1 equiv) and dry MeOH 0.36 mL, stirred in iced-water bath. Then, 0.14 mL of cold 7 M NH₃ in MeOH was added to the 4-19 solution dropwise (final concentration of NH₃ was 2 M). The reaction was stirred in the iced-water bath for 5 h. The solvent was removed by a rotary evaporator. The residue was evaporated repeatedly with 1 mL of water 3 times to afford product 4-20. Yield = 0.0013 g (85%).

¹H NMR (400 MHz, D₂O) δ (ppm): 9.25 (d, J = 1.6 Hz, 1H), 9.09 – 9.05 (m, 1H), 8.75 (dt, J = 8.2, 1.3 Hz, 1H), 8.15 – 8.12 (m, 1H), 8.11 (s, 1H), 7.98 (s, 1H), 7.57 (dd, J = 7.7, 1.1 Hz, 1H), 7.34 – 7.31 (m, 2H), 7.21 (ddd, J = 8.9, 6.6, 2.4 Hz, 1H), 6.01 (d, J = 5.5 Hz, 1H), 5.81 (d, J = 7.5 Hz, 1H), 5.21 (d, J = 13.7 Hz, 1H), 4.70 – 4.64 (m, 2H), 4.59 (m, 2H), 4.49 (t, J = 2.4 Hz, 1H), 4.42 (t, J = 5.3 Hz, 1H), 4.39 (dd, J = 5.0, 2.8 Hz, 1H), 4.37 – 4.30 (m, 2H), 4.24 – 4.11 (m, 3H).

³¹P NMR (162 MHz, D₂O) δ (ppm): -10.35 (d, J = 22.0 Hz), -10.62 (d, J = 21.4 Hz).

HR-MS (ESI) m/z calcd. for C₂₈H₃₃N₈O₁₆P₂ (M+H)+ 799.1490, found 799.1476.
4.2.3. Chemoenzymatic Study

4.2.3.1. Reaction of PARP1 with Natural β-NAD⁺

The reaction condition of Lin et al. was followed with modification. To a 2 mL vial was added natural β-NAD⁺ (100 µM), activated DNA (0.25 µg/µL), and PARP1 (0.15 µM) in 100 µL reaction buffer (containing 100 mM Tris.HCl, 10 mM MgCl₂, 1 mM DTT, pH 8.0). The mixture was incubated in a water-bath at 37 °C. The reaction was monitored with the UV detection at 254 nm at the given times by taking 10 µL of sample and diluting with 90 µL of water and subsequently analyzed by HPLC reverse-phase column with gradient of 99% solvent C/ 1% solvent D to 95% solvent C/ 5% solvent D in 10 min.

4.2.3.2. Reaction of PARP1 with β-NAD⁺ Analogue

To a 2 mL vial was added β-NAD⁺ analogue 4-20 (100 µM), ADPr (100 µM), activated DNA (0.25 µg/µL), and PARP1 (0.15 µM) in 100 µL reaction buffer (containing 100 mM Tris.HCl, 10 mM MgCl₂, 1 mM DTT, pH 8.0). The mixture was incubated in a water-bath at 37 °C. The reaction was monitored with the UV detection at 254 nm at the given times by taking 10 µL of sample and diluting with 90 µL of water and subsequently analyzed by HPLC reverse-phase column with gradient of 99% solvent C/ 1% solvent D to 90% solvent C/ 10% solvent D in 15 min, then gradient of 90% solvent C/ 10% solvent D to 50% solvent C/ 50% solvent D in 30 sec, then hold 50% solvent C/ 50% solvent D for 10 min.
4.3 Results and Discussion

4.3.1. Synthesis of ADP-Ribose from β-NAD⁺

The first approach in this chapter is the use of auxiliary 2-6 to facilitate fractionation and purification of PAR. The efficiency of the conjugation method between auxiliary 2-6 and ADP-ribose (ADPr), which is a monomer of PAR, and the stability of the conjugated product were determined prior to the study with PAR polymers. Although ADPr is commercially available, it is highly expensive ($219/100 mg, Sigma-Aldrich). Therefore, ADPr was synthesized from β-NAD⁺ ($78.50/1 g, Sigma-Aldrich) through the two step sequences (Scheme 4.2). The first step was the synthesis of 2’- and 3’-O-acetyl-ADP-ribose (4-3a and 4-3b) from β-NAD⁺ which reported by Szczepankiewicz et al. The initial step of this transformation was the substitution of acetate at the C-1 position (4-3c). However, this acetate ester underwent ester migration to the hydroxyl group at C-2 and C-3 positions, which were more thermodynamically stable (Scheme 4.3), resulting in the formation of compound 4-3a and 4-3b as products. The reaction of 4-3a and 4-3b in diluted NaOH in 50 min gave product 4-4 in 85% yield. However, it should be noted here that by mixing 4-3a and 4-3b with NaOH for a long period (more than 1.5 h) could result in the undesired adenosine monophosphate (AMP) as a product.
Scheme 4.2: Synthesis of ADPr from β-NAD⁺.

Scheme 4.3: Ester migration.
4.3.2. Neoglycoconjugation of ADP-Ribose with Auxiliary

With the auxiliary 2-6 and ADPr 4-4 in hand, the glycoconjugation between 2-6 and 4-4 was conducted following the procedure explained in Chapter 2 but a three-fold molar excess of auxiliary 2-6 was used instead (Scheme 4.4). Also, due to the solubility issue of ADPr in DMF, DMSO was used as a cosolvent. The reaction was monitored by HPLC reverse-phase column. It is known that ADPr is highly polar and generally unable to retain in the reverse-phase column when using water:acetonitrile as a mobile phase. Surprisingly, the hydrophobicity from 2-6 allows the conjugated product 4-5 to retain in the C18 column when using water:acetonitrile as a mobile phase, and the product 4-5 can be purified by HPLC preparative C18 column. Unfortunately, the stability issue of the conjugated product and the low yield discouraged for further investigations. It was shown that the conjugated product could be hydrolyzed during the removal of solvent from the rotary evaporator. The removal of the solvent after the purification by HPLC could be achieved via a freeze-dry technique. Furthermore, there was only 16% yield obtained from the conjugation reaction, which is not feasible for the conjugation with PAR polymers that are more complex than ADPr. Therefore, the reaction condition requires further optimization, such as increasing the concentration of the substrates and increase the solubility of the substrate by changing the solvents that used in the reaction.

Scheme 4.4: Glycoconjugation of ADPr with 2-6.
4.3.3. Design of the Photocaged β-NAD⁺ Analogue and Strategy Toward Synthesis of ADP-Ribose Oligomers in a Controlled Manner

The photocaged β-NAD⁺ analogue was designed based on the knowledge that PARP1 elongates the PAR polymer at the 2’-OH position. It is believed that by modifying this position with protecting group, it can prevent the undesired elongation. There are 2 requirements for a suitable protecting group. First, after putting the protecting group on the β-NAD⁺, the PARP1 enzyme should still recognize it as a substrate. Second, the protecting group should be removed under the mild condition, and the removal process should not disturb other functional groups on the polymer. The photoremovable 2-nitrobenzyl group is chosen in this aspect because it can be selectively removed by the UV light¹⁴⁴ (Scheme 4.5).

![Scheme 4.5: Deprotection of photoremovable 2-nitrobenzyl with UV light.](image)

A previous study reported that PARP1 could catalyze the formation of the PAR chain onto a functionalized-ADPr.¹⁴⁵ In this aspect, it is envisioned that with the presence of ADPr (free ADPr) in the solution containing PARP1 and β-NAD⁺, PARP1 can catalyze the formation of PAR from β-NAD⁺ onto a free ADPr. With the similar events but using a β-NAD⁺ analogue (4-20) as a substrate, the PARP1 enzyme would catalyze the formation of
a dimer of ADPr analogue (Scheme 4.6a). After selective removal of the protecting group by UV light, the 2'-OH of an ADPr dimer will be available for the addition of the next monomer analogue. By repeating this process for several rounds, the desired length of the ADPr oligomers will be obtained (Scheme 4.6b).

Scheme 4.6: Proposed a new approach for chemoenzymatic synthesis of PAR: (a) synthesis of ADP-ribose dimer and (b) synthetic sequence for the synthesis of ADPr oligomers.
4.3.4. Synthesis of Photocaged β-NAD⁺ Analogue

The β-NAD⁺ analogue is designed to contain a photoremoveable 2-nitrobenzyl group at the 2'-OH (4-20). In general, the introduction of the benzyl group from benzyl halide onto the hydroxyl group can be done by using a strong base. However, those reaction conditions would not be practical in this aspect because β-NAD⁺ contains a variety of labile functional groups. According to the retrosynthetic analysis (Scheme 4.7), the formation of pyrophosphate between nicotinamide mononucleotide (NMN, 4-16) and the photocaged AMP (4-15) can be achieved through the imidazolide activating process. The photocaged AMP can be obtained from the introduction of a phosphate group at the 5’-OH of the adenosine 4-8.

![Scheme 4.7: Retrosynthetic analysis.](image-url)
The synthetic route of the photocaged AMP is shown in Scheme 4.8. The synthesis of 2-nitrobenzyl adenosines (4-8 and 4-9) was achieved following a published procedure. The treatment of adenosine (4-6) with sodium hydride in DMF followed by the addition of 2-nitrobenzyl bromide (4-7) led to the selective alkylation at the 2'-OH to form 4-8. After the precipitation, compound 4-8 can be used for the next step without further purification. There was an attempt to introduce a phosphate group onto a 5'-OH of the 4-8 by using phosphoryl chloride. However, the multiple phosphorylated products were observed, and they were difficult to determine which hydroxyl group was phosphorylated. Therefore, the amine and the 3'-OH were protected (Compound 4-11) before the introduction of the phosphate. The primary amine on the adenine was protected with the benzoyl group in pyridine to obtain 4-9 in 60% yield. Subsequently, the two hydroxyl groups were silylated with TBSCI in DMF to afford 4-10. Then, a silyl protecting group at the 5'-OH was selectively deprotected using trichloroacetic acid in THF/H₂O to afford 4-11 in 95% yield. Di-tert-butyl phosphoramidite (4-12) and the combination of 1-methylimidazole and 1-methylimidazolium chloride were chosen for the phosphorylation of 4-11 to afford 4-13 because this condition reduced the amount of H-phosphonate byproduct. The deprotection of di-tert-butyl on the phosphate and the TBS group on the 3'-OH could be done simultaneously using TFA in CH₂Cl₂. After purification with reverse-phase HPLC, compound 4-14 was obtained in an acceptable yield. Finally, the benzoyl protecting group was removed by methanolic ammonia to afford photocaged AMP 4-15.
Nicotinamide mononucleotide (NMN) (4-16) is commercially available and was used without further purification. The attempt to use an unprotected NMN for a pyrophosphate formation was not practical because multiple pyrophosphate products were observed. One of the reasons is that NMN is poorly soluble in an organic solvent, even in DMF. Furthermore, the hydroxyl groups on the NMN could also act as a nucleophile to react at the activated phosphate during the activation process. Therefore, 2’- and 3’-OH on NMN were protected with acetate group prior to the activation step by using acetic anhydride in pyridine/water to afford 4-17 (Scheme 4.9).
The pyrophosphate synthesis of compound 4-17 and 4-15 was achieved through the activation of the phosphate group on 4-17 with CDI in dry DMF (Scheme 4.10). Ten equivalents of CDI were used to form the monophosphate imidazolide 4-18. The excess CDI was quenched with dry methanol. Next, compound 4-15 was dissolved in a DMF solution containing 0.5 M MgCl₂ then transferred to the solution of 4-18 dropwise to afford the protected β-NAD⁺ analogue 4-19. There was an attempt to remove the acetate protecting group by Et₃N in MeOH containing EDTA upon purification. However, the product was difficult to purify by reverse-phase HPLC because its polarity is similar to byproducts. Therefore, compound 4-19 was purified before the deprotection of the acetate group. The acetate deprotection was done in 2 M NH₃ in MeOH at 0 °C. It was shown that by using a high concentration of ammonia (7 M NH₃ in MeOH), the nicotinamide group was cleaved to form an undesired photocaged ADP-ribose. Finally, photocaged β-NAD⁺ 4-20 was obtained through the multiple evaporations with water to remove excess ammonia and ammonium acetate.
4.3.5. Chemoenzymatic Synthesis of ADP-Ribose Dimer Using Photocaged $\beta$-NAD$^+$ Analogue

Since the concentrations of substrate ($\beta$-NAD$^+$) and the amount of enzyme could affect the activity of the enzyme. The reaction conditions were investigated to find the suitable condition for the PARylation of PARP1. Natural $\beta$-NAD$^+$ was used in this study and the reaction was monitored by HPLC with the UV detection at 254 nm. It was found that the reaction contained 100 µM of natural $\beta$-NAD$^+$ and 0.15 µM of PARP1 was suitable for the PARylation, as indicated by the HPLC chromatograms that $\beta$-NAD$^+$ was consumed by the enzyme within 30 min (Figure 4.4). However, the formation of AMP could also be observed from the reaction mixture after 24 h.

Scheme 4.10: Synthesis of $\beta$-NAD$^+$ analogue 4-20.
With the β-NAD⁺ analogue 4-20 in hand, the formation of the ADPr dimer analogue (as shown in Scheme 4.6a) was attempted in vitro with PARP1 using the similar condition as describes for a natural β-NAD⁺. If the ADPr dimer analogue is formed, the HPLC chromatogram should show the decrease of 4-20 and ADPr peaks and the increase of a new peak between two starting material peaks. However, the HPLC chromatograms (Figure 4.5) showed that β-NAD⁺ analogue 4-20 was not consumed by the enzyme after 24 h indicating that the desired ADPr dimer analogue was not formed. The decrease of ADPr peak did not indicate the formation of the expected dimer; however, ADPr was hydrolyzed to AMP after 24 h, as confirmed with the standard AMP.
To determine whether compound \textbf{4-20} was a suitable substrate for PARP1, the study of PARylation of PARP1 using both natural \(\beta\)-NAD\(^+\) and \(\beta\)-NAD\(^+\) analogue \textbf{4-20} as substrates was conducted. The HPLC chromatograms (Figure 4.6) demonstrated that only natural \(\beta\)-NAD\(^+\) was consumed by the enzyme as indicated by the decrease of \(\beta\)-NAD\(^+\) peak and the increase of NMN peak, which was compared with the NMN standard.
Unfortunately, the decrease in β-NAD\(^+\) analogue 4-20 could not be observed. This information was therefore led to the conclusion that β-NAD\(^+\) analogue 4-20 was not a suitable substrate for the PARP1 enzyme.

![Chemical structures and HPLC chromatograms]

Figure 4.6: HPLC chromatogram from the reaction of PARP 1 with natural β-NAD\(^+\) and β-NAD\(^+\) analogue 4-20.

For further investigation, ADPr and natural β-NAD\(^+\) were used to evaluate the desired approach whether PARP1 could be able to catalyze the elongation of PAR from β-NAD\(^+\) onto ADPr in solution. The HPLC chromatograms (Figure 4.7) showed that the peak of β-NAD\(^+\) only decreased slightly indicating that the activity of PARP1 was decreased compared to the experiment that used only natural β-NAD\(^+\) (Figure 4.4), which was
consumed by the enzyme within 30 min. The ADPr peak decreased after 24 h with the increase of AMP peak. It was reasoned that the low concentration of the enzyme affected the rate of the reaction while the presence of ADPr in the solution decreased the enzymatic activity. The increase of AMP peak with the decrease of ADPr peak indicated that the reaction condition was not suitable for ADPr for a long period. Therefore, the reaction conditions need to be further evaluated, such as increasing the amount of enzyme and decreasing the concentration of ADPr to maintain the enzymatic activity.

Figure 4.7: HPLC chromatogram from the reaction of PARP1 with natural β-NAD$^+$ and ADPr.
4.4 Conclusions and Future Directions

The neoglycoside auxiliary 2-6 was successfully conjugated with ADPr under sodium phosphate buffer pH 4.7. The conjugated product was obtained in 16% yield. Although this conjugation reaction provided enough efficiency to reasonably obtain the conjugated product, it might not be sufficient for the conjugation with PAR. The structures of PAR are more complicated than ADPr and will result in the lower efficiency of the conjugation. As mentioned in Chapter 1, section 1.3.3 that the equilibrium constant of neoglycoconjugation is low, and the reaction is usually required high concentration of the substrates. The reaction condition for the conjugation of auxiliary 2-6 with ADPr is needed to be further optimized by increasing the concentration of the substrates. Furthermore, the solubility of both substrates can be enhanced by changing the solvents used in the reaction.

A photocaged β-NAD⁺ analogue (4-20) was successfully synthesized from the pyrophosphate formation between the photocaged AMP (4-15) and the acetylated NMN (4-17). The photoremovable 2-nitrobenzyl was installed at the 2’-OH of adenosine before the addition of a phosphate at the 5’-OH to obtain the photocaged AMP. The two hydroxyl groups (2’’- and 3’’-OH) on NMN needed to be protected with acetyl group before the activation of the phosphate to avoid the formation of byproducts during the pyrophosphate coupling with 4-15.

Chemoenzymatic synthesis of ADP-ribose dimer using β-NAD⁺ analogue 4-20 and ADP-ribose as substrates was not successful due to the inability of PARP1 to recognize compound 4-20 as a substrate. The protecting group on the β-NAD⁺ analogue is needed
to be modified to be recognized by the enzyme, such as using a smaller protecting group.

The results from the chemoenzymatic reaction of natural β-NAD$^+$ and ADP-ribose suggested that the reaction conditions need to be further evaluated by increasing the amount of enzyme and decreasing the concentration of ADPr. However, the reason that precluded from a full investigation of the chemoenzymatic reaction is a lack of ability to access a high amount of the enzyme.
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APPENDIX A: PSEUDO FIRST-ORDER HYDROLYSIS GRAPHS OF COMPOUND 2-7

Figure A.1. Pseudo first-order hydrolysis graph of compound 2-7 at pH 5.

Figure A.2. Pseudo first-order hydrolysis graph of compound 2-7 at pH 7.
159

$y = -0.0044x + 0.669$

$R^2 = 0.9634$

$t_{1/2} = 158\ h$

Figure A.3. Pseudo first-order hydrolysis graph of compound 2-7 at pH 9.
APPENDIX B: NMR SPECTRA FOR CHAPTER 2

Figure B.1. $^1$H NMR spectrum of compound 2-2.

Figure B.2. $^{13}$C NMR spectrum of compound 2-2.
Figure B.3. $^1$H NMR spectrum of compound 2-4.

Figure B.4. $^{13}$C NMR spectrum of compound 2-4.
Figure B.5. $^1$H NMR spectrum of compound 2-5.

Figure B.6. $^1$H NMR spectrum of compound 2-5.
Figure B.7. $^1$H NMR spectrum of compound 2-6.

Figure B.8. $^{13}$C NMR spectrum of compound 2-6.
Figure B.9. $^1$H NMR spectrum of compound 2-7.

Figure B.10. $^1$H NMR spectrum of compound 2-7.
HR-MS (ESI) m/z calcd. for C_{23}H_{34}N_{5}O_{11} (M + H)^+ 556.2249, found 556.2255.

Prepared according to the general procedure A using D-maltose (0.155 g, 0.430 mmol) as a reducing sugar to obtain compound 2-8 as a pink powder. Yield = 0.025 g (51%).

**1H NMR (400 MHz, D$_2$O)**

- Glc 1–H$_1$: 4.17 (d, J = 9.1 Hz)
- Glc 1–H$_2$: 3.65–3.55
- Glc 1–H$_3$: 3.78–3.76
- Glc 1–H$_4$: 3.76–3.70
- Glc 1–H$_5$: 3.52–3.48
- Glc 1–H$_6a$: 3.68–3.52

Figure B.11. $^1$H NMR spectrum of compound 2-8.

Figure B.12. $^{13}$C NMR spectrum of compound 2-8.
Prepared according to the general procedure A using D-glucose (0.084 g, 0.467 mmol) as a reducing sugar to obtain compound 2-9 as a pink powder. Yield = 0.0613 g (66%).

$^1$H NMR (400 MHz, D$_2$O) $d$ (ppm):

- Glc 1: 4.16 (d, $J$ = 8.8 Hz)
- Glc 2: 3.58 (t, $J$ = 8.9 Hz)
- Glc 3: 3.50 (t, $J$ = 8.5 Hz)
- Glc 4: 3.36
- Glc 5: 3.36
- Glc 6a: 3.88 (d, $J$ = 12.5 Hz)
- Glc 6b: 3.71 (dd, $J$ = 12.3 Hz, $J$ = 4.7 Hz)

CH aromatic: 8.17 (d, $J$ = 8.1 Hz, 2H)

Figure B.13. $^1$H NMR spectrum of compound 2-9.

Figure B.14. $^{13}$C NMR spectrum of compound 2-9.
$\text{H NMR (400 MHz, D}_2\text{O)}$

- 13C (101 MHz, D$_2$O) 167.76, 164.16, 141.51, 131.49, 130.51, 128.44, 93.88, 77.97, 77.73, 75.14, 70.65, 70.05, 61.45, 39.41, 20.73.

$\text{HR-MS (ESI) m/z calcd. for C}_{17}\text{H}_{24}\text{N}_5\text{O}_6 (M + H)}^+ 394.1721, \text{found 394.1736.}$

(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-2-acetamido-2-deoxy-D-glucopyranoside (2-10).

Prepared according to the general procedure A using N-acetyl-D-glucosamine (0.112 g, 0.506 mmol) as a reducing sugar to obtain compound 2-10 as a pink powder.

Yield = 0.0205 g (46%).

$\text{H NMR (ppm)}$

- GlcNAc 1 $4.24 \text{ (d, } J = 9.7 \text{ Hz)}$
- GlcNAc 2 $4.00 \text{ (t, } J = 9.8 \text{ Hz)}$
- GlcNAc 3 $3.51 \text{ (t, } J = 9.8 \text{ Hz)}$
- GlcNAc 4 $3.46 - 3.34 \text{ (m)}$

Figure B.15. $^1\text{H NMR spectrum of compound 2-10.}$

Figure B.16. $^{13}\text{C NMR spectrum of compound 2-10.}$
C (101 Hz, D$_2$O) ppm: 174.65, 167.79, 164.27, 141.51, 131.57, 130.71, 128.45, 92.40, 77.90, 76.02, 74.42, 70.20, 61.39, 53.01, 39.13, 23.00, 20.73.

HR-MS (ESI) m/z calcd. for C$_{19}$H$_{27}$N$_6$O$_6$ (M + H)$^+$ 435.1987, found 435.2012.

(4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino-D-galactofuranoside (2-11a) prepared according to the general procedure A using D-galactose (0.074 g, 0.411 mmol) as a reducing sugar. Compound 2-11a was obtained as a pink powder. Yield = 0.0032 g (10%).

GlcNAc 5.46 - 3.34 (m)
GlcNAc 6a 3.89 (dd, J = 12.4, J = 1.9 Hz)
GlcNAc 6b 3.74 (dd, J = 12.4 Hz, J = 4.9 Hz)
NH-acetyl 2.03 (s, 3H)
CH aromatic 8.23 (d, J = 8.4 Hz, 2H)
CH aromatic 7.56 (d, J = 8.3 Hz, 2H)
CH$_2$-O-N 4.70 (d, J = 6.1 Hz, 2H)
CH$_3$-tetrazine 3.00 (s, 3H)
CH$_3$-N 2.75 (s, 3H)

Figure B.17. $^1$H NMR spectrum of compound 2-11a.

Figure B.18. $^{13}$C NMR spectrum of compound 2-11a.
Prepared according to the general procedure A using D-galactose (0.074 g, 0.411 mmol) as a reducing sugar. Compound 2-11b was obtained as a pink powder. Yield = 0.0183 g (57%).

**1H NMR (400 MHz, D2O)**

- Gal 1: 4.16 (d, J = 9.1 Hz)
- Gal 2: 3.77 (t, J = 9.2 Hz)
- Gal 3: 3.65 (dd, J = 9.4 Hz, J = 3.4 Hz)
- Gal 4: 3.92 (d, J = 3.2 Hz)
- Gal 5: 3.66 - 3.64
- Gal 6a: 3.81 - 3.77
- Gal 6b: 3.73 - 3.69

**CH aromatic**

- 8.27 (d, J = 8.2 Hz, 2H)
- 7.61 (d, J = 8.2 Hz, 2H)

**CH**

- 4.88 (s, 2H)

Figure B.19. 1H NMR spectrum of compound 2-11b.

Figure B.20. 13C NMR spectrum of compound 2-11b.
Figure B.21. $^1$H NMR spectrum of compound 2-12a.

Figure B.22. $^{13}$C NMR spectrum of compound 2-12a.
**Figure B.23.** $^1$H NMR spectrum of compound **2-12b**.

**Figure B.24.** $^{13}$C NMR spectrum of compound **2-12b**.
HR-MS (ESI) m/z calcd. for C17H24N5O6 (M + H)+ 394.1721, found 394.1715.

(4-(6-(methyl-1,2,4,5-tetrazin-3-yl)phenyl)-N-methylhydroxylamino)a-D-mannofuranoside (2-12c)

Figure B.25. $^1$H NMR spectrum of compound 2-12c.

Figure B.26. $^{13}$C NMR spectrum of compound 2-12c.
1H NMR spectrum of compound 2-13a.

13C NMR spectrum of compound 2-13a.
2.2.7. Hydrolysis of Conjugated Auxiliary

To a 20 mL vial was added glycoconjugate 2-6 0.0166 g (0.0289 mmol) and 6 mL of 0.25% of TFA in water. The reaction was stirred at room temperature for 3 h. Then, DOWEX 1X8 (OH form) 0.9 g was added to the solution and stirred for 10 minutes. DOWEX resins were then filtered out of the solution. The auxiliary was removed by extracting with CH2Cl2 3 times. The aqueous layer was concentrated using rotary evaporator to obtain unconjugated D-lactose (yield = 96%).

2.2.4. Synthesis of N-Acetyleneuraminic acid 2\textregistered3-Lactopyranoside (2-17)

To a 7 mL vial, glycoconjugated 2-6 (1 mM, 1 eq) and CMP-N-acetyl-neuraminic acid (1 mM, 1 eq) were added to 3 mL of Tris.HCl buffer (0.1 M, pH 8.0). Next, 50 µL of α₂,₃-sialyltransferase (1 mg/mL) was added to the reaction mixture then incubated at 37°C. The reaction was monitor by HPLC (gradient of 99% water/ 1% acetonitrile to 60% water/ 40% acetonitrile in 20 minutes). The reaction was left overnight to reach to completion. Upon completion, the reaction was concentrated by rotary evaporator then

Figure B.29. ¹H NMR spectrum of compound 2-17.

Figure B.30. ¹³C NMR spectrum of compound 2-17.
$n = 2$ ($2^{-18}$)

H NMR (400 MHz, D$_2$O) $\delta$ (ppm):

- **Xyl 1–H1**: 4.09 (d, $J = 8.5$ Hz)
- **Xyl 1–H2**: 3.55
- **Xyl 1–H3**: 3.38
- **Xyl 1–H4**: 3.23
- **Xyl 1–H5a**: 3.92
- **Xyl 1–H5b**: 3.58

- **Xyl 2–H1**: 4.42
- **Xyl 2–H2**: 3.21
- **Xyl 2–H3**: 3.53
- **Xyl 2–H4**: 3.29
- **Xyl 2–H5a**: 4.06
- **Xyl 2–H5b**: 3.69

- **CH aromatic**: 8.30 (d, $J = 8.3$ Hz, 2H)
- **CH aromatic**: 7.61 (d, $J = 8.3$ Hz, 2H)

- **CH$_2$-O-N**: 4.86 (d, $J = 1.7$ Hz, 2H)
- **CH$_3$-tetrazine**: 3.02 (s, 3H)
- **CH$_3$-N**: 2.74 (s, 3H)

**Figure B.31.** $^1$H NMR spectrum of compound 2-18.

**Figure B.32.** $^{13}$C NMR spectrum of compound 2-18.
$^1$H NMR (ppm): 167.90, 164.55, 141.59, 131.83, 130.59, 128.67, 102.47, 94.67, 77.06, 76.24, 75.84, 75.17, 73.41, 70.51, 69.82, 65.85, 65.33, 39.41, 20.73.

HR-MS (ESI) m/z calcd. for C$_{21}$H$_{30}$N$_5$O$_9$ (M + H)$^+$ 496.2038, found 496.2025.

$n = 3 (2-19)$

Figure B.33. $^1$H NMR spectrum of compound 2-19.

Figure B.34. $^1$H NMR spectrum of compound 2-19.
Figure B.35. $^1$H NMR spectrum of compound 2-20.

Figure B.36. $^{13}$C NMR spectrum of compound 2-20.
APPENDIX C: NMR SPECTRA FOR CHAPTER 3

Figure C.1. $^1$H NMR spectrum of compound 3-5.

Figure C.2. $^{13}$C NMR spectrum of compound 3-5.
Figure C.3. $^1$H NMR spectrum of compound 3-6.

Figure C.4. $^{13}$C NMR spectrum of compound 3-6.
Figure C.5. $^1$H NMR spectrum of compound 3-7.

Figure C.6. $^1$H NMR spectrum of compound 3-7.
Figure C.7. $^1$H NMR spectrum of compound 3-8.

Figure C.8. $^{13}$C NMR spectrum of compound 3-8.
Figure C.9. $^1$H NMR spectrum of compound 3-9.

Figure C.10. $^{13}$C NMR spectrum of compound 3-9.
Figure C.11. $^1$H NMR spectrum of compound 3-10.

Figure C.12. $^{13}$C NMR spectrum of compound 3-10.
Figure C.13. $^1$H NMR spectrum of compound 3-11.

Figure C.14. $^{13}$C NMR spectrum of compound 3-11.
Figure C.15. $^1$H NMR spectrum of compound 3-12.

Figure C.16. $^{13}$C NMR spectrum of compound 3-12.
Figure C.17. $^1$H NMR spectrum of compound 3-13.

Figure C.18. $^{13}$C NMR spectrum of compound 3-13.
Figure C.19. $^1$H NMR spectrum of compound 3-14.

Figure C.20. $^{13}$C NMR spectrum of compound 3-14.
Figure C.21. $^1$H NMR spectrum of compound 3-15.

Figure C.22. $^{13}$C NMR spectrum of compound 3-15.
Figure C.23. $^1$H NMR spectrum of compound 3-16.

Figure C.24. $^{13}$C NMR spectrum of compound 3-16.
Figure C.25. $^1$H NMR spectrum of compound 3-17.

Figure C.26. $^{13}$C NMR spectrum of compound 3-17.
Figure C.27. $^1$H NMR spectrum of compound 3-18.

Figure C.28. $^{13}$C NMR spectrum of compound 3-18.
Figure C.29. $^1$H NMR spectrum of compound 3-19.

Figure C.30. $^{13}$C NMR spectrum of compound 3-19.
Figure C.31. $^1$H NMR spectrum of compound 3-20.

Figure C.32. $^{13}$C NMR spectrum of compound 3-20.
**Figure C.33.** $^1$H NMR spectrum of compound 3-21.

**Figure C.34.** $^{13}$C NMR spectrum of compound 3-21.
Figure C.35. $^1$H NMR spectrum of compound 3-22.
Figure C.36. $^1$H NMR spectrum of compound 3-23.
Figure C.37. $^1$H NMR spectrum of compound 3-24.
Figure D.1. $^1$H NMR spectrum of compound 4-3a and 4-3b.
Figure D.2. $^1$H NMR spectrum of compound 4-4.
Figure D.3. $^1$H NMR spectrum of compound 4-5.

Figure D.4. $^{31}$P NMR spectrum of compound 4-5.
Figure D.5. $^1$H NMR spectrum of compound 4-8.

Figure D.6. $^{13}$C NMR spectrum of compound 4-8.
Figure D.7. $^1$H NMR spectrum of compound 4-9.

Figure D.8. $^{13}$C NMR spectrum of compound 4-9.
Figure D.9. $^1$H NMR spectrum of compound 4-10.

Figure D.10. $^{13}$C NMR spectrum of compound 4-10.
Figure D.11. $^1$H NMR spectrum of compound 4-11.

Figure D.12. $^{13}$C NMR spectrum of compound 4-11.
Figure D.13. $^1$H NMR spectrum of compound 4-13.

Figure D.14. $^{31}$P NMR spectrum of compound 4-13.
Figure D.15. $^1$H NMR spectrum of compound 4-14.

Figure D.16. $^{13}$C NMR spectrum of compound 4-14.
Figure D.17. $^{31}$P NMR spectrum of compound 4-14.
Figure D.18. $^1$H NMR spectrum of compound 4-15.

Figure D.19. $^{13}$C NMR spectrum of compound 4-15.
Figure D.20. $^{31}$P NMR spectrum of compound 4-15.
Figure D.21. $^1$H NMR spectrum of compound 4-17.

Figure D.22. $^{13}$C NMR spectrum of compound 4-17.
Figure D.23. $^{31}$P NMR spectrum of compound 4-17.
Figure D.24. $^1$H NMR spectrum of compound 4-19.

Figure D.25. $^{31}$P NMR spectrum of compound 4-19.
Figure D.26. \( ^1H \) NMR spectrum of compound 4-20.

Figure D.27. \( ^31P \) NMR spectrum of compound 4-20.
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BIOGRAPHY OF THE AUTHOR

Thamrongsak Cheewawisuttichai was born on February 6, 1990 in Petchabun, Thailand. When he was 15 years old, he moved to Bangkok, Thailand. He graduated from Suankularb Wittayalai School in 2008. He attended Chulalongkorn University and graduated in 2012 with a Bachelor’s degree in Chemistry. After graduation, he spent 4 months working in a quality control laboratory at Mead Johnson nutrition, Thailand and spent 1 year working as a Sales representative at Thai Unique Ltd., Bangkok. He came to the United stated in 2013 to study English as a second language (ESL) program at College of the Canyon, Valencia, CA. He attended the University of Maine in September 2014 to pursue a doctoral degree in organic chemistry. He knew that he loves doing synthesis, so he decided to join Dr. Brichacek group where he can have fully experience in organic synthesis. After graduation, Thamrongsak will join the Chemistry Department at Worcester State University as a visiting postdoctoral associate. Thamrongsak is a candidate for the Doctor of Philosophy degree in Chemistry from the University of Maine in August 2019.