Expanding Glycomic Investigations Through Thiol-derivatized Glycans

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EXPANDING GLYCOMIC INVESTIGATIONS THROUGH THIOL-DERIVATIZED GLYCANS

By

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MSc in Chemistry, Bangor University, 2018

A DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy (in Chemistry)

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The University of Maine
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EXPANDING GLYCOMIC INVESTIGATIONS THROUGH THIOL-DERIVATIZED GLYCANS

By Robert Hurst

Dissertation Advisor: Dr. Matthew Brichacek


N-(2-thioethyl)-2-aminobenzamide (TEAB), a novel glycan auxiliary, was synthesized and its utility was evaluated. The auxiliary was conjugated to glycans by reductive amination with the water-stable reagent 2-picoline borane complex. Glycan products, which ranged from 1 to 7 linked hexoses, were all isolated in yields ranging from 60% to 90% after purification by reverse-phase chromatography. The novel conjugate introduces a convenient, shelf-stable thiol directly onto the desired free glycans with purification advantages and direct modification with efficient reactions through alkenes, halides, epoxides, disulfides, and carboxylates in yields of 49% to 93%. Subsequently, a thiol-selective modification of the BSA protein was used to generate a neoglycoprotein with a bifunctional PEG–maleimide linker. To further illustrate the utility of a thiol motif, 2-thiopyridine activation of a thiol-containing support facilitated the covalent chromatographic purification of labeled glycans in yields up to 63%. Additionally, initial proof of concept of implementation in a light printed microarray was explored and validated through FITC-labeled concanavalin A binding. The thiol-functionalized glycans produced greatly expand the diversity of bioconjugation tools that can be developed with glycans and enable a variety of biological investigations. Finally, some non-reductive amination techniques were preliminarily investigated which achieved labeling of a xylose species in non-anomeric positions in poor yields (<5%) alongside characterization of generated cyanohydrins and some select truncated product
DEDICATION

I dedicate my dissertation to my family, without their constant support and love I would not have been able to succeed or persevere through trying times. I thank my loving wife Kayla, who even on the hardest of days managed to cheer me up and love me even when I was not at my best. I hope that I can repay all the love, support, and cheer that she has given to me throughout my Ph.D. and during the move to the U.S., I am excited to begin the next chapter of our lives together and how we get to spend every day together no longer apart. I thank my parents for encouraging me to pursue my happiness and permitting me to form my own family here abroad despite their absence of them in my daily life, I miss you dearly and look forward to our next reunion. Words cannot express the gratitude and love that I feel for my in-laws, they have truly taken me in as one of their own and I could not imagine having my family here in the U.S. without them being a part of my daily life.

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CHAPTER 1
INTRODUCTION

1.1 Preface

Carbohydrates are ubiquitous throughout nature [1] and are likely one of the most abundant biomolecules on earth [2]. They can be found decorating proteins, as components of DNA [3], modifying RNA [4], and attached to a variety of lipids present on the cellular wall [5,6]. This umbrella term of carbohydrates is often disfavored by researchers who prefer the term “glycans”. Glycans can range from single monosaccharides such as glucose to long polymers like cellulose. Some of these complex structures are illustrated with the consortium of functional glycomics (CFG) notation in Figure 1.

Figure 1: Illustration of the glycome present on the cellular wall with glycans in CFG notation [4].

Glycan roles are often diverse but crucial to the functioning of all organisms [7]. Despite their importance understanding the exact biological mechanisms that they facilitate is challenging [8]. Unlike other classes of biomolecules, this field has historically lagged with most research focused on characterization, synthesis, and understanding of lipids, nucleic acids, and proteins,
while more complex structures such as carbohydrates or “glycans” are comparatively less understood due to synthetic challenges and characterization issues surrounding them [9–11].

Glycochemists utilize specialized terminology to describe the unique chemistry of hexoses in the study of the glycome. Much of this terminology is summarized in Figure 2, which illustrates the key process of "ring-chain tautomerism." Through this rapid equilibrium, the "reducing end" interconverts in stereochemistry rapidly, giving rise to a mixture of α and β isomers. This is just one example of the various complexities involved in the structures of glycans.

![Figure 2: Defining a glycan, dissection of glycan structure](image)

Study of the glycome and specifically glycans are particularly challenging compared to other biomolecules due to three main factors. The first is stereochemistry, as glycans typically have four stereocenters, resulting in 16 possible structures \(2^n\), where \(n\) is the number of anomeric carbons) for one hexose (or glycan) when freely reducing. This complexity is compounded as more glycans are added to a single chain, resulting in a vast array of potential structures [13]. Secondly, synthesis, glycans are difficult to synthesize due to the stereochemistry and the subsequent homogeneity of functional groups makes it particularly challenging to functionalize a specific hydroxyl, group for example C4 position vs the C3 position [14]. Lastly, Alcohols, these diverse structures are highly water soluble due to their ability to hydrogen bond and that they can form a wide variety of salts which significantly impacts their ability to be characterized [15] through mass
spectrometry (poor fragmentation) or their retention onto most chromatography media (highly hydrophilic) [16]. These fundamental challenges have resulted in the field of glycomics historically trailing the other three classes of biomolecules until recent decades.

1.2 Structure of glycans

Great strides have fortunately been occurring in the field of glycomics within recent decades giving rise to a classification of the glycome (Figure 3) and the systematic coding system created by the consortium of functional glycomics (CFG) [17].

![Figure 3: A chemical view of glycan classes with linkage types highlighted](image)

There are four major classes of glycans present in biology, a schematic displaying their generalized forms with their linkage to the associated biomolecule is drawn in Figure 3. Firstly, N-linked glycans bear an amide linkage at the reducing end to asparagine then to either a peptide or to decorate proteins [19]. The length of these glycans is at least 4 hexoses with complex branching occurring at the third glycan but can further branch as the chain progresses. Secondly, O-linked glycans are a more discrete sub-class of glycans where structures tend to be simpler in comparison to N-linked with an O-glycan ranging from 2 to 8 units [15]. However, it can branch like N-glycans but seldom more than 2 -3 branches are observed; these glycans are linked through an ether linkage at the reducing end to a serine or threonine residue. Thirdly, glycolipids are
glycans that are O-link to their lipid counterpart and can vary in size typically from 1 to 6 glycans. More complex species also exist bearing phosphate or sulfate species at a wide variety of positions [20]. Lastly, glycosylamino glycans (GAGs) are negatively charged polymers composed of a repeating disaccharide. There are four core disaccharide combinations that make up GAGs [21]. These GAG structures can either be linked via an N or O linkage at the reducing end. Overall, they are the heaviest but least complex class of glycans. In Figure 4, a more general “biological” overview is shown with the glycans labeled in the consortium of functional glycomics (CFG) notation.

![Figure 4: A biological representation of glycans on the cellular wall in CFG notation with minor subclasses of glycans noted [22] Copyright © 2009, The Consortium of Glycobiology Editors, La Jolla, California.](image)

Additional minor classes of glycans are drawn such as O-GlcNAc’s, which exist as 2-20 oligomers of N-acetyl-glucosamines that are linked to proteins [22]. Glycosylphosphatidylinositol (GPI) anchored glycoproteins, bear a unique phosphate group on certain terminal glycans. This complex class of glycans can vary greatly with branching akin to N-linked glycans in addition to GPIs bearing a wider variety of glycans. An example of this is the inositol linkage, a unique glycan referred to as a carbo cyclic sugar [23].
1.3 Function of glycans

With this biological overview in mind, it is crucial to discuss the importance of the biological functions of glycans within a cellular view. Figure 5 displays a generalized representation of the variety of roles glycans play within the cell environment specifically the importance of cell-cell signaling.

![Figure 5: Biological roles of glycosylation within the cell [24]](image)

Glycans are typically found on the outer layer of cell walls and allow cells to communicate with each other without the need for signaling molecules. These glycans play a vital role in cellular recognition and identity [25]. Surface glycans are crucial in identifying immune-privileged cells, which prevents T-cells from destroying healthy cells [26]. As a result, glycan-binding proteins or lectins that specifically recognize glycans become prime targets for viral and bacterial pathogens to exploit for cell entry or evasion from the immune system [27]. Various viruses use glycan-mediated pathways for cell entry, including SARS-CoV-2 and HIV-1. These viruses have highly glycosylated proteins that enable them to enter cells and avoid detection by the immune system,
also known as "immune privilege." [28,29]. As such, targeting and isolating glycans involved in cell entry is a crucial objective in therapeutic development. This is particularly important for diseases like HIV, where extensive research aims to develop a glycan-based vaccine [30].

The majority of glycans exist attached to the cellular structures but, there is still the significant presence of reducing (non-attached) glycans that exist in low concentrations expressed by cells, which are crucial for cellular signaling to permit cellular survival and proliferation [31]. An important example of this is the glycosaminoglycan (GAG) hyaluronan, also commonly referred to as hyaluronic acid (HA). It has been shown to be taken up by embryonic stem cells and can result in epithelial to mesenchymal transition shown in Figure 6 [32].

![Figure 6: Cellular uptake of hyaluronan into embryonic stem cells](image)

With this in mind, various studies have just begun to investigate the biological effects of HA with regards to its various molecular weights [33,34]. In addition to this free GAG, there are a wide variety of glycans (such as O-linked, GPI, lipid, etc.) that can also act as signaling
molecules. Now in hand with a fundamental understanding of the importance glycans play within biology, it is necessary to briefly discuss the complex biological machinery that eukaryotic life utilizes to decorate its cellular walls shown in Figure 7.

Figure 7: Enzymatic synthesis of glycans within the endoplasmic reticulum (ER) [35] with a list of enzymes that are involved in glycan synthesis on the right [36].

In summary, glycosyltransferases (GTs) are enzymes that catalyze the transfer of glycan molecules to a biological substrate, both inside and outside of the endoplasmic reticulum (ER). The 6 major classes of GTs are: N-acetylglucosaminyltransferases (GlcNAcTs), mannosyltransferases (ManTs), glucosyltransferases (GlcTs), galactosyltransferases (GalTs), fucosyltransferases (FucTs) and sialyltransferases (SiaTs). This process of glycosyltransferase occurs both outside and inside the ER where after the desired glycan has been synthesized utilizing a mixture of these enzymes it can then be either trimmed with a glycosidase (GH) or with a glycosynthase. The matured glycan is then transferred onto the biological substrate, such as a protein or peptide for N-glycans, and exported out of the ER [37]. While this process is technically challenging and currently not scalable, *E. Coli* can be engineered to express these enzymes for the efficient synthesis of complex glycans outside of biological conditions [36]. This approach is likely
to be the most scalable in the future of glycan synthesis, as it avoids the synthetic challenges behind chemical isolation of glycans or direct synthesis [38].

1.4 Chemical and enzymatic release of glycans

Previous research into glycan release is a relatively recent discovery, with most methods dating back to the 1970s [39]. The most prominent liberation methods since their discovery have been enzymatic tools. This has resulted in enzymatic tools being the main driver for the systematic identification and classification of the glycome allowing for the consistent cleavage of glycans at specific positions. A summary of the various tools utilized in the field of glycochemistry to release natural glycans is shown below in Figure 8.

![Figure 8: Chemical and enzymatic tools to release glycans from natural sources.](image)

Out of the many enzymatic digestion methods listed in Figure 8, the most popular and commercially available enzymatic tool is PNGaseF/A [40] which leaves the desired glycan entirely intact with the corresponding reducing glycan liberated. Unfortunately, there are significant limitations surrounding this enzyme: scaling this approach above single mg quantities is cost prohibitive, fucosylation at C3 prevents the enzyme from cleaving and the enzyme works only for N-glycans which limits it to one glycan type [41]. Therefore, this method is mainly suitable for
LC/MS studies and has limitations when it comes to studying \( O \)-linked glycans, which rely on endoglycosidases for partial liberation.

In contrast to enzymatic tools, the chemical release of glycans has been extremely challenging. The oldest chemical treatment for the release of glycans dates back to the 1980s and involves alkali digestion with \( \text{NaBH}_4 \). This treatment is effective at liberating \( O \)-glycans and \( N \)-glycans from proteins, with a yield of more than 75-80\% [42,43]. But there are significant drawbacks from this treatment with chemical peeling of glycans into their alditols subunits at various places, and the final product typically results in a non-homogenous mixture of alditols or de-acetylated chains. It is important to emphasize that the terminology ‘chemical peeling’ strictly relates to the degradation of the glycan but does not specific the exact mechanistic process.

Fortunately, a few decades later, hydrazinolysis became a well-established chemical process for glycans, dating back to 1993 [44], and it was the first significant chemical method to liberate milligram scales of complex glycans (I), intermediates (II and III) within the mechanism elucidated as shown in Figure 9 [45].

![Figure 9: Hydrazinolysis release of N-linked glycans proposed mechanism through acetohydrazine hydrolysis [44].](image-url)
Despite this ability to chemically release glycans, there were some major drawbacks to this process. The presence of water at ppm levels during the hydrazine removal of O-glycans can catalyze the process of β-elimination at the C3 position, resulting in the formation of an alkene and cleaving the glycan chain within O-glycans. A similar process can even occur in anhydrous hydrazine, which is why the optimized temperature for O-glycan release is 65 °C rather than 95 °C. In comparison to the generally more stable N-glycans, the yields are still significantly reduced when water becomes present. One study specifically investigated the effects of hydrazine monohydrate as a chemical release reagent to avoid the difficulties surrounding the drying process and isolation of glycoproteins; as well as maintaining anhydrous hydrazine. The authors reported some respectable yields of up to 32% using hydrazine monohydrate [46] vs the 80% of anhydrous hydrazine. However, they note that any time greater than >10 hrs results in chemical peeling of the glycans of interest. Despite the effectiveness of this chemical-releasing agent, which can be performed at a larger scale, many researchers still desired a water-compatible system since drying glycans to water levels below ppm is extremely energy-intensive and challenging, especially for crude samples.

In the past few decades, researchers have been investigating an aqueous-compatible system for releasing glycans. Cummings and Song have been the most successful in implementing an oxidative release of glycans [47]. Their initial attempts used the reagent N-bromosuccinimade [48] to halogenate the amide bond to then undergo β-elimination (a similar mechanism to Figure 10, VII) to remove the peptide chain [48].
This initial study gave poor yields and had significant chemical peeling (VIII and IX) but fortunately led to their innovation of bleach (NaClO) release. The authors claimed that they were able to release complex glycans on a scale of hundreds of milligrams from kilograms of crude biological materials by utilizing household bleach (Clorox 6.5 % w/v NaClO). The authors subsequently labeled their purified glycans with a fluorescent reporter, and isolated them using a combination of dialysis and hydrophilic interaction liquid chromatography (HILIC), without characterizing the intermediate crude liberated glycans.

Analysis of glycans isolated from various biological samples such as porcine brain, egg yolks, and egg whites, which would normally take months or years of glycoprotein isolation and challenging release, could now be completed within days using the bleach addition and dialysis method. Despite the success of this approach, there were still major questions regarding the study. For instance, the mechanism of the chlorination process and its potential effects on the integrity of the NHAc groups remain unclear. Additionally, the authors did not determine the yields on pure glycoproteins and simply relied on mass spectrometry of derivatized samples relative to an internal standard to estimate the purity of the resulting samples.
Additionally in the study of bleach oxidative release, the authors did not mention the effect of using a comparable halogenation reagent (NBS [49]) that had previously been shown to glycans truncate glycans in a 4:1 ratio (V: IX). However, this was not extensively discussed in their paper on bleach oxidative release, with only a few brief notes provided in the supplemental information. Although the tool demonstrated to be useful in terms of mass spectrometry characterization, it was unclear whether it can be scaled up to yield results comparable to those obtained using the hydrazine reagent. Further research was needed to address these questions and to compare the effectiveness of this process with other releasing tools.

1.5 Reductive amination of glycans

Moving on from the liberation techniques of glycans, it is necessary to purify and label the reducing glycans to enable biological investigations. Typically, glycans are typically subjected to the process of reductive amination, as illustrated in Figure 11 where the “open form” is trapped with a secondary amine linkage.

*Figure 11: Reductive amination of glycans exploiting tautomerization of glycans at the reducing end [50].*

Reductively aminated glycans are now trapped in this reduced state where the glycan is in the ring open form conjugated with an amine of interest. The amine is typically linked to an
aromatic functional group to enable detection, although it can also be done with aliphatic amines. The addition of the amine is a reversible process until the intermediate imine is reduced with a borane reagent. The borane reagent can be introduced in a variety of ways, most commonly in the form of sodium cyanoborohydride (Na BH₃CN) [50,51]. Acetic acid must be present to help facilitate the process of alcohol hydrolysis, however most borane reagents are not aqueous compatible. Therefore, this reaction is often run in dimethylsulfoxide (DMSO) to ensure reagent/starting material compatibility.

Overall, this process is highly efficient with labeling occurring up to 99% during the reaction, as determined via LC-MS studies of this reaction [52]. It is important to note that the cyclic ring is opened during this process to form a “ring open” product (XVII) which can affect the ability of the glycan to bind to certain substrates. Early studies of reductive amination utilized a variety of fluorophores such as ANTS, AA, and AB which were typically used to label glycans shown in Figure 12 [45].

![Chemical Structures](image)

**Figure 12:** Some important chemical reporters a) that are commonly attached to glycans through b) reductive amination [45].
The now labeled glycans possess a potent fluorophore which can either be a hydrophobic or hydrophilic residue, which can enable them to retain on chromatography media but, consistently allows for their detection using UV light. The most modern and popular aromatic reporter is 2-amino-N-(2-aminoethyl)-benzamide (AEAB). Due to the bifunctionality of this molecule the primary amine is left un-reacted as acidic conditions result in protonation of the more basic amine while the aromatic amine can label the glycan [53]. Once completed the resulting glycan now bears a flexible amine group that can be further derivatized to facilitate biological assays.

### 1.6 Purification of glycans

Glycochemists subject these fluorescently labeled glycans for purification with media (illustrated in Figure 13) such as porous graphitic carbon (PGC) [54], HILIC [55], weak anionic exchange (WAX) and in the case of ANTS retention to C18 media (reverse phase with ion-pairing reagents, femtomolar fM scale) [16].
However, due to the inconsistency in retention to PGC, WAX, and HILIC, many researchers have historically elected to avoid utilizing these chromatographic media as elution times are altered significantly by solvent /pH and sample composition [57]. Despite these challenges, HILIC chromatography has in recent years prevailed as the major chromatographic media to facilitate the isolation of large glycans allowing for discrete separation from natural pools of glycans [47]. In summary, HILIC chromatography is currently the only reliable method for separating glycans, but there are still challenges associated with this technique. The most critical of these is the existence of numerous HILIC stationary phases, each with its specific protocol, and
no consensus on the superior functional group for this chromatography. As a result, this technique is used inconsistently across the field of glycomics, unlike the more standardized C18 chromatography protocols, which are significantly more reproducible, despite changes in C18 media origin. However, it should be noted that C18 separation has been shown to be inappropriate for highly hydrophilic glycans, even when labeled with various auxiliaries [16].

1.7 Applications of derivatized glycans

There are several potential applications for derivatized glycans, with one of the most important techniques being their use in arrays. In this technique, multiple glycans are coated onto a specific area of a surface. This process can be performed on semi-purified crude pools of glycans (after size exclusion), or on isolated glycans, which are utilized for microarray analysis. The glycans are contact printed directly onto an epoxy (or NHS ester)-coated glass surface in microliter quantities, as shown in the image below in Figure 14.

Application of glycans (XVII) onto the surface must be done through manual micropipettes or an automated pipette system to give consistent spotting and uniformity onto the surface (the maximum precision is 0.1 µm) [59]. As a result, there is a variety of glycan microarrays now
commercially available which can range in price from smaller (100 glycans) arrays of $700 to more extensive libraries (300 glycans) for $2200. The cost of these arrays alone can be prohibitive for most biological assays as lectins in mg/µg quantities (100s of µM concentration is required) of fluorescently labeled proteins required for glycan binding assessment can range from hundreds to thousands of dollars.

Utilizing the fluorescent linker AEAB, the Cummings group explored the important effect of having the “open ring” (XVII) vs “closed ring” (Figure 15, XVIII) to investigate how the synthetic modification of reducing glycans may alter binding effects to a variety of lectins/antibodies.

![Figure 15: Comparisons of binding of “closed ring” analogs vs “open ring” to Aleuria aurantia lectin (AAL): glycans, 1-12 = N-linked and 13-26 = O-linked [60].](image)

Within this key study, a glycoarray of both "open ring" (XVII) and "closed ring" (XVIII) O-linked and N-linked complex glycans was created by depositing 26 glycans onto an NHS ester-coated slide using contact printing. They then applied 9 lectins modified with fluorescent probes to the surface of the glycoarray and measured the remaining fluorescence of the lectins after blocking, incubation, and sequential washing. Fluorescent measurements confirmed the theory that the majority of lectins bind to the end of the glycan rather than the entire molecule, as 7 out of the
9 lectins showed no statistically significant difference between binding to the "open ring" and "closed ring" forms of the glycans. The remaining 2 lectins (SLex1/2 antibodies) were notably binding to their binding partners’ human blood group A/B glycans which are only 4-5 glycan units long (O-linked glycans) where proximity to the terminal glycan vs the reducing end is considerably shorter compared to the larger branching N-linked glycans.

Overall, the study confirmed the importance of having reductively aminated “ring open” (XVII) glycans analogous to their natural “ring closed”(XVIII) counterparts with some exceptions relating to glycan size being too small to facilitate binding to certain lectins. In addition to this study, a great deal of other articles have also investigated the effects of “open ring” on a variety of glycosyltransferases (GTs) and found them to have broad acceptability across labeled glycans [61–63]. Recent studies have investigated metabolic labeling through azide-bearing monosaccharides (Figure 16, XIX) for biomimetic synthesis to generate complex branching glycans and glycoproteins.

![Figure 16: Azide chemistry as a tool for cellular metabolism [64].](image)

Labeled glycans can be isolated from crude samples through Staudinger ligation, which is referred to as a "pull-down" technique [64]. The term "catch and release" is also used
interchangeably with "pull-down". These are broad terms that describe the reversible addition of compounds to supports or the selective isolation of compounds [65].

To introduce the motif, the starting monosaccharide (V) is synthetically protected and then derivatized at a desired position with an azide then subsequently de-protected (XIX). The synthetic glycan is then fed into cells and incubated until the glycans are up taken and expressed by the ER to produce branching glycans on resulting glycoproteins/peptides. Additionally, they can be further reacted with potent fluorophores/solid supports [66,67] and digested via enzymes and released for LC-MS studies, which in turn can help facilitate a variety of experiments; for example, knock-out studies of genes can be used to determine gene relationship to glycan synthesis which can help determine cell viability to determine functions of the resulting glycans [68,69]. Therefore, the introduction of bio-orthogonality into a reactive auxiliary can serve as a potent tool for elucidating biological pathways.

1.8 Chemistry of thiols and applications

Inspired by both proteomics and previous glycan studies, a new motif for derivatizing glycans was proposed (Figure 17). Previous studies have mainly focused on nitrogen-containing species, offering limited orthogonality. In contrast, thiols have been found to be versatile and permit conjugation under a wide range of mild conditions with a broad range of species.
Briefly looking at thiol chemistry, a wide range of discrete chemical reactions have been shown, ranging from: thiolene/yne click [71,72], halide exchange, thioesterification, haloacetamide, and maleimide addition [70]. Additionally, the existence of thiols within nature is extremely low where sulfur containing amino acids only composes 3–6% of proteins alongside low natural abundance (0.5%) overall [73]. Moreover, thiols can form a wide variety of permanent (thioethers) or temporary bonds (disulfide/thioester), with diverse functional group tolerance (amides, carboxylic acids, alcohols, etc.) permitting for bio-orthogonal reactions. Overall, the reactivity of thiols can always outcompete their amine counterparts and reactions can occur in as little as 1-10 seconds making them superior nucleophiles [74].

Many proteomic investigations have sought to exploit this chemistry to further develop novel thiol-reactive motifs that can destabilize the thioether linkage allowing for delayed release. Such conjugation is displayed in Figure 18 with a modified maleimide (XXI to 5-MP) which
enabled pH cleavage of the thioether bond. This resulted in a reversible protein labeling (5-MP adduct) allowing for a “pull-down” approach of a protein of interest [75].

![Reversible thiol exchange](image)

**Figure 18:** Novel thiol-specific substrate (5-MP) designed for “pull-down” of reduced proteins or temporary chemical attachment [75].

Most thiol chemistry investigations concerning proteomics have previously focused on these “pull-down” approaches using thiol-bearing proteins alongside thiol reactive media, such as Sepharose-6B [76,77]. Implementation of a temporary disulfide bonds then enables the isolation of specific proteins without the need for chromatographic separations. Finally, self-assembled monolayers (SAMs) have also been of growing interest within the field of proteomics where research has focused on tailoring gold nanoparticles with various thiol peptides to enable delivery mechanisms for peptide-based therapeutics [78,79].

In terms of material chemistry, thiols have been of great importance due to the radically initiated thiol-ene reaction where initiators vary from thermal to photoinitiators. Consequently complex microarray generation (Figure 19 A) has been enabled as a result of these photoinitiators as they permit photolithography of thiol peptides in discrete patterns [80]. Synthetically modified peptides are terminally linked to a PEG spacer bearing an alkene or thiol. The analyte of interest is then reacted to the surface bearing the corresponding residue resulting in a linked surface. This
approach has several advantages over a traditional contact printed array, the most consequential being that the spacing of the analyte can be performed at nm levels. Finally, inclusion of a gradient spacer can also permit the variation in densities of multiple analytes to the surface which therefore permits the study of a multitude of combinations in a confined area.

![Figure 19: 2D (A) [80] vs 3D (B) [81] peptide arrays using gradient photomasks to control labeling density.](image)

Photolithography of peptides has even been expanded to three-dimensional systems like hydrogels [81] (Figure 19 B). The usage of hydrogels has been shown to be more analogous to mimicking in-vivo conditions due to their ability to alter their mechanical properties, such as degree of swelling and stiffness, as well as being three-dimensional structures [82,83]. This technique of photolithography has also seen some limited usage in glycan arrays, where a commercially available thiol modified glucose was investigated [84]. The major limitation in this study was that this technique could not be applied to natural glycans but did verify the importance of implementation of photolithography. Other early studies have also emphasized the significance of having defined spacing between glycans. This is because lectins have been shown to act in multivalent capacities, forming dimers or trimers and binding to multiple glycans on a surface [85].
1.9 Thesis overview

To address the outlined issues described within this chapter, this thesis seeks to expand glycomic investigations primarily through the introduction of a thiol-labeled fluorescent reporter based on the highly popularized AEAB. Alterations of the AEAB compound beyond the one amine functional group are viewed as unfavorable due to potentially adverse effects for binding to glycan specific species such as glycosidases. Utilizing this theorized novel glycan auxiliary (TEAB, 1) this thesis will explore a wide variety of topics ranging from; glycan library generation, applications of derivatized glycans, and chemoenzymatic synthesis and isolation of complex glycans. Additional investigation into non-reductive amination labeling of glycans will also be explored, to assess their utility and application in labeling at non-C1 positions. Outlined below are the stated objectives (numerically labeled) with their corresponding sub-objectives and strategies (alphabetically labeled) to achieve their overarching goal.

1) Synthesis of reporter: The compound referred to as TEAB (1) has been synthesized in previous literature [86] but has not been applied to the field of glycomics yet. Therefore, synthesis of this compound should be achieved in the stated two-step process following the referenced article with the potential for optimizations in yields and purifications. An outline of this proposed modification is shown in Figure 20.

![Figure 20: Proposed auxiliary, functional group interconversion form an amine to thiol forming TEAB, 1.](image-url)
2) **Glycan library:** Once synthesized (TEAB, 1), conjugation of the thiol-derivatized fluorophore to glycan species will be explored on a wide variety of commercially available glycans with varying lengths from 1 to 7 linked units to form a “glycan library. As a sub-objective, it will be determined if this novel glycan auxiliary (TEAB, 1) facilitates purification where previous auxiliaries have been shown to assist in glycan purification once labeled [16]. Few auxiliaries have enabled C18 retention, and modification of the amino group to the thiol group should in theory improve retention to reverse phase media (reducing hydrogen bonding) but to what extent would be unknown until applied.

![Figure 21: Proposed thiol bearing auxiliary TEAB (1) reductively amination of a variety of commercially available glycans to form a “glycan library.”](image)

3) **Applications:** A) Pending isolation of the variety of glycans, the TEAB glycoconjugates will then be applied to a wide range of chemical reactions (displayed in Figure 17) to validate the orthogonality of the thiol species (Figure 22). B) As these select thiol reactions are assessed for the resulting purity and efficiency of the transformations, the most favorable reactions will be selected for further applications. C) Applications will focus primarily on biological validation due to the importance of forming glycoarrays for screening biological targets or glycosylation of protein species to generate therapeutic targets. D) Additional applications surrounding covalent chromatography akin to Sepharose-6B will be explored as well. The ability to “catch and release”
glycans from crude mixtures would improve the overall workflow of glycochemistry to a 2-step process (reductive amination and “pull down”) which expedites glycan investigations.

**Objective 3: Applications**

**Figure 22:** Proposed applications of TEAB (1) labeled glycans utilizing various thiol-reactive species.

4) **Chemoenzymatic and complex glycan isolation:** Finally, to fully be a comparable auxiliary to AEAB the glycan auxiliary (TEAB, 1) must be able to facilitate in isolation of non-commercially available glycans (specifically N/O-linked glycans). Therefore, A) natural product isolation of a model N-linked glycan [87] must be attempted as well as B) utilization of the recently published bleach protocol [88]. C)Additional investigation into glycosidases [89,90] is also warranted as chemoenzymatic compatibility to the novel TEAB substrate should be validated alongside isolation of the resulting complex glycan.

5) **Investigations of non-reductive amination techniques:** Labeling of glycans at positions other than C1 may be necessary for enzymatic transformations of glycans shown in **Figure 23**. Therefore, it is essential to develop strategies for the labeling of glycans at positions such as C2 or C3 to enhance our understanding of glycans. Recently published protocols [91,92]
have utilized potent electrophiles, including iodonium reagents (A) and boronic acids (B), mediated by regioselective copper catalysts to facilitate these transformations.

Figure 23: Proposed site-selective labeling of glycans utilizing A) and B) which are copper mediated.

To expand the scope of this thesis, it is necessary to explore other chemical techniques for labeling glycans that include non-reductive amination methods utilizing these copper catalyzed systems.
CHAPTER 2

FLUORESCENT AUXILIARY DESIGN, AND DEVELOPMENT

2.1 Fluorescent auxiliary design

To improve upon previous generations of auxillaries, implementation of a novel glycan auxiliary which exploits the diverse chemical reactions of thiols was viewed as advantageous. Upon review of the literature, the most popular auxiliary appeared to be AEAB due to the bifunctionality and apparent advantages of a small, spaced arm off the amide bond. Therefore, it seemed prudent to change the primary amine into a reduced thiol (TEAB, Figure 24) 1 to permit a direct synthesis to the target molecule; as well as to avoid introducing further complexity by making the aliphatic arm longer which could affect chemoenzymatic modifications or binding to glycan specific proteins.

![Proposed auxillary](image)

**Figure 24:** Retrosynthesis of TEAB to precursors anthranilic acid and cysteamine.

Following similar protocols for the synthesis of AEAB, it seemed best to perform a similar disconnection at the amide linker (Figure 24) to start from the commercially available and highly economical Anthranilic acid (2, ~$50/250 g). This could then be amidated but required the thiol (3) to be converted into a disulfide bond to prevent thioesterification resulting in the cystamine hydrochloride salt (4, ~$50/25 g). Fortunately, synthesis of this compound [86] was previously published which utilized standard peptide coupling (no yield published) to the disulfide-linked arm (5).
2.2 Fluorescent auxiliary development

The protocol (Figure 25) utilized a standard sodium borohydride (NaBH₄) reduction of the subsequent compound followed by silica gel chromatography purification of the product. The initial attempts followed the protocol in the literature. This successfully resulted in the synthesis of the N,N’-(dithiodi-2,1-ethanediyl)bis[2-amino-benzamide] (DEAB, 5) however, the subsequent reduction appeared to suffer inconsistencies where the resulting thiol post purification was shown to be product through NMR and TLC analysis even though multiple equivalents of the reducing agent was used.

Figure 25: Synthesis of TEAB from AA using peptide coupling reagents and subsequent reduction [86].

A variety of other peptide coupling reagents such as HCTU (59%), DIC (22%), and DCC (25-40%) were investigated to improve yield and confirm DEAB (5) was being produced. Alternative reducing agents such as dithiothreitol (DTT), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), and 2-mercaptoethanol (βME) were also investigated but this process still
resulted in the isolation of the oxidized product (DEAB, 5) despite an observable change shown through TLC analysis over the course of the reaction.

2.2 Trial reductive amination

After several attempts to isolate the desired compound 1, it seemed prudent to abandon temporarily the isolation of the thiol compound and instead test the reductive amination process on a few select glycans to ensure this auxiliary did indeed react. Lactose monohydrate (6) was deemed a model substrate to test this process alongside a recently published reductive amination reagent 2-picoline borane (7) [52] (Figure 26).

![Figure 26: Reaction of lactose monohydrate with DEAB with the reducing agent 2-picoline borane.](image)

A recent study at the time showed that it was just as effective as the analog sodium cyanoborohydride but displayed water stability, meaning that the process of reductive amination could occur under aqueous conditions. The initial study involving this glycan of interest was first investigated with methanol and acetic acid as the solvent, as DEAB (5) is insoluble in water. The resulting labeling followed through successfully with HPLC chromatograms of the reaction showing the progression of the reaction and significant retention of the labeled compound (Figure 27, 6-7 minutes).
Figure 27: Reductive amination of lactose monohydrate with DEAB using analytical HPLC 1% to 100% AcN over 10 minutes.

The compound was then submitted to preparative C18 where the labeled glycan was isolated in 90% yield. This process was then repeated on 1,3-α-1,6-α-D-mannotriose and N-Acetyl-D-lactosamine as they are important binding substrates within biology (core components to N-glycans) and had important non-glucose residues. Both compounds were also isolated through preparative C18 chromatography with N-Acetyl-D-lactosamine-DEAB matching the same retention time as 8 in (38% yield) whilst 1,3-α-1,6-α-D-mannotriose-DEAB had slightly less retention at around 5.5 minutes (37% yield). Overall resulting in three labeled glycans with modest to excellent yields which all displayed significant C18 retention utilizing the novel glycan auxiliary DEAB (5).

To fully display the utility of this fluorophore, it was still necessary to obtain these compounds in the reduced thiol state (9) before they can be further reacted, therefore these compounds were subsequently reacted with TCEP as it is a water-soluble reagent with high selectivity for disulfides. The resulting chromatograms displayed interesting characteristics where the reduced thiol, 9 (Figure 28, peak at 6 minutes) was fully reacted.
Figure 28: HPLC traces of reduction of lactose DEAB (8) in water with TCEP.HCl (5 eq.) post reduction 5 to 30 minutes (partial oxidation) using a ramp of 1% to 100% AcN over 20 minutes.

However, after an additional 30 minutes, 50% of the compound had oxidized back to the disulfide 8, which was indicated by a peak at 7 minutes. This behavior was unusual as TCEP.HCl was used in excess (5 eq.) as well as the pH was acidic due to the HCl salt which should have slowed down any ambient air oxidation process.

2.3 Metal-catalyzed thiol oxidation

With this phenomenon shown through HPLC analysis, a review of surrounding thiol chemistry was needed to explain why thiol oxidation was catalyzed despite no other reagents being introduced. Fortunately, it was found that certain transition metals have been shown to coordinate with a variety of aminobenzamide analogs (Figure 29 A, from review [93]) like TEAB in a variety of ways.
It is suspected that similar to these analogs, TEAB likely underwent a similar coordination event where soft nucleophilicity of thiols coordinated a metal species and likely facilitated a process similar to the iron-catalyzed thiol oxidation from the literature [94] (Figure 29, B). With this working theory that TEAB (1) is a strong metal chelator (Figure 30) due to the abundance of lone pairs and electron density present on the aromatic ring.

It was suspected that the fluorescent reporter was likely contaminated either via reagents or through silica gel chromatography with transition metals resulting in re-occurrent oxidation of the thiol bond. To prove that this theory was indeed correct, it was decided that utilizing a stronger metal chelator should prevent the formation of these complexes which should permit reduction.
Chelex 10\textsuperscript{®} sodium resin, a polymeric form of sodium ethylenediaminetetraacetic acid (EDTA) was obtained to remove the impurities from the contaminated lactose-DEAB (8) and then reduced with TCEP as it was shown through HPLC to keep the desired thiol reduced the longest compared to DTT and βME. NMR spectroscopy data is shown below Figure 31 displaying the differences between 8 and 9.

\textbf{Figure 31:} NMR of reduced lactose-TEAB (9) and oxidized lactose-DEAB (8), notable shifts of the CH$_2$-SH at 3.04 ppm (DEAB) and 2.84 ppm (TEAB).

This approach resulted in the first reductive amination to form thiol-derivatized glycans that also possessed long-term stability. Once dissolved in solution these compounds remained stable as the thiol form for weeks (oxidation would occur due to ambient air) but as a solid, it could be stored indefinitely in the thiol form under refrigeration. Figure 31 shows the resulting differences between the oxidized and reduced form of the glycoconjugates, interestingly very little
NMR differences are observed aside from a slight broadening of most signals with the only major shift of the methylene (CH$_2$-SH) from 3.04 to 2.84 ppm and some slightly less discernible shifts in the aromatic region.

2.4 Optimization of TEAB synthesis

With this challenge surmounted and the hypothesis of metal contamination confirmed, it was deemed important to finally find an effective scalable synthetic approach to TEAB. To be able to outperform current fluorescent probes, a high-yielding synthesis had to be developed that was highly robust and required little to no chromatography in addition to avoiding all potential metal contaminants. A variety of potential protocols were reviewed but fundamentally it seemed highly advantageous to alter the starting material from anthranilic acid to isatoic anhydride (10) where literature [95] showed that a variety of different analogs could be prepared to utilize this ring-opening protocol. The initial protocol was taken and then altered, where the reaction (Figure 32) was run in water instead of organic solvent, but all other conditions remained except for the reactant being cysteamine hydrochloride (4).

Figure 32: Synthesis of TEAB fluorescent probe $^a$isolated yield.

The process followed successfully resulting in the desired product precipitating out from the reaction and all starting materials/reagents being washed away with excess nano pure water giving modest yields of around 30%. This protocol was then further optimized with equivalencies altered as well as time and temperature until the final process drawn in Figure 32 above was
obtained yielding the compound DEAB in an acceptable yield of 71%. The subsequent reduction step was viewed as problematic due to the resulting product, TEAB, being an oil with modest solubility in water. This resulted in consistently poor recovery despite changing the reducing reagents, such as TCEP, DTT, and NaBH₄. These reagents had to be extracted away from the compound in an aqueous solution to avoid the use of silica gel chromatography, which could potentially introduce metal impurities. With the highest recorded yield of 37% post-extraction, this alternate pathway was deemed undesirable. To find a solution, the chelating properties of TEAB were considered as it could potentially be exploited to create a shelf-stable compound, given that the auxiliary has the ability to form a transition metal complex. Zinc reduction (Figure 33) is an older protocol for the liberation of thiols from disulfides, historically discovered around the 19th century [96] but is seldom used in modern reductions of compounds as the conditions required are heavily reducing using zinc metal and strongly acidic environments making it intolerant to many functional groups.

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\text{Zn reduction (Figure 33) is an older protocol for the liberation of thiols from disulfides, historically discovered around the 19th century [96] but is seldom used in modern reductions of compounds as the conditions required are heavily reducing using zinc metal and strongly acidic environments making it intolerant to many functional groups.}
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\text{Therefore, a few modifications were made to an existing protocol [97] to develop a straightforward method. Heating zinc in a mixture of methanol, acetic acid, and water resulted in the desired Zn-TEAB 11. Once dissolved in a sufficiently acidic solution, it would reform TEAB 1. After a few hours of heating this mixture, the suspension was filtered to remove leftover zinc and the solvent was concentrated down until acetic acid remained. To transfer the compound, water was added to the flask, where it was found that upon the addition of water, precipitation would}
\]
occur resulting in an isolable off-white powder formed. The resulting precipitate was then filtered and washed with further excess water until no acetic acid remained and was then dried under vacuum. The resulting compound was then suspected to be a zinc complex (drawn in Figure 33) due to its physical properties, where a solid was recovered instead of the known oil that TEAB (1) had formed previously. In addition, this compound was only soluble in solvents that have been acidified with either 1M HCl or an organic acid (1% formic or acetic acid) indicating a pH-sensitive complex was present. NMR data of the complexes are shown below in Figure 34.

![NMR spectra](image)

**Figure 34:** TEAB (1) in D$_6$-Acetone (a) vs Zn-TEAB (II) in D$_2$O with formic acid (b) and in D$_6$-DMSO (c).

Mass spectrometry of the resulting compound showed the presence of this zinc ion complex (HRMS (ESI) m/z [M + H]$^+$: calcd for C$_{18}$H$_{23}$N$_4$O$_2$S$_2$Zn$^+$, 455.0554; found 455.0463) in the corresponding dimeric form where the hydrogen on the sulfur was missing with other smaller
fragments corresponding mass ions supporting a dimer structure. Interestingly NMR analysis of the compounds showed broad signals around the amide and CH$_2$ species (8.3, 3.4, and 2.7 ppm, Figure 34) suggesting coordination to the amide when in D$_6$-DMSO. HSQC phase edited spectrum of the Zn-TEAB, confirmed the broad signals at ~3.4 and 2.7 ppm to be the CH$_2$ groups on the aliphatic arm. Overall, it seemed highly advantageous for purification (1 day for the total synthesis of 11 from 10), yields (67% overall yield), and long-term stability (>2 months by HPLC) to utilize this Zn-TEAB (11) vs the unprotected TEAB (1). It is important to note that the structure of compound 11 was drawn as a simplified representation and may not accurately reflect the actual zinc complex that was formed.

2.5 Reductive amination optimization and generation of a glycan library

The resulting compound was submitted for reductive amination to lactose monohydrate and the overall protocol was optimized to ensure consistent removal of zinc salts Figure 35.

![Figure 35: Reductive amination using the novel auxiliary Zn-TEAB 11.](image)

Conditions such as temperature were increased to a more standard 65 °C (typical for sodium cyanoborohydride) and the solvent composition was altered to ensure the solubility of desired glycans (MeOH:Water: Acetic acid). The introduction of EDTA (non-sodium salt form) was deemed appropriate as it should have partial solubility in the solvent mixture and be more effective at removing contaminate metal ions compared to Chelex 100. It was observed that the addition of a reducing agent (TCEP) was optional. If the reaction was run for the specified duration with low
impurities, the formation of disulfide was found to be negligible, as indicated by HPLC analysis (<2%). When this overall process was applied to a variety of glycans as shown above, yields ranged from good to acceptable, (60-90%, Figure 36) where the lowest for acarbose was likely a result of the starting material not having a defined purity.

Figure 36: “Glycan library” of Model TEAB linked glycans with their corresponding yields “isolated yields.

The only exception to these conditions was maltoheptaose where the inclusion of TCEP significantly impacted preparative C18 chromatography of the 7 linked glycan. Recovery was found to be around 20% as the rest of the desired compound would co-elute in the dead volume with the TCEP reagent. The exclusion of the TCEP reagent would then increase the yield to the corresponding 83%, which was satisfactory. Ironically, smaller monosaccharides proved to be the most challenging to isolate from the reaction mixtures as they eluted a full minute closer to TEAB resulting in partial co-elution with unreacted TEAB giving poorer yields of around 70%.
2.6 Investigation of thiol modified AEAB

To further understand the C18 retention of the novel TEAB fluorophore as well as to understand the effect the aliphatic arm contributed, a direct modification to the existing AEAB was deemed necessary (Figure 37).

![Figure 37: Proposed thiol introduction to AEAB.](image)

It was decided that AEAB could be derivatized with 2-iminothiolane (24, Traut’s reagent) which is a cyclic thioimidate whose ring opens and forms a resulting thiol at the terminal alkyl chain and an amidine to the primary amine. AEAB was synthesized from methyl anthranilate and ethylene diamine through aminolysis which was then isolated with silica gel chromatography (26% yield) instead of utilizing the weeklong HCl precipitation of AEAB from the literature [53] with a reported 32% overall yield.

Instead of trying to directly modify AEAB in the way drawn in Figure 37, it was seen as more advantageous to utilize known reductive amination conditions to label the desired glycan with AEAB. Then subsequently modify it with 24 as it has been previously shown to be high yielding shown in Figure 38.
Figure 38: Traut's reagent modification of reductively aminated lactose with AEAB to generate a thiol-bearing species with an amidine linker (25).

The AEAB salt (23) was therefore reductively aminated utilizing our modified conditions Figure 38 without the presence of EDTA/TCEP in 1:1 stoichiometry of lactose monohydrate (6). Once reductive amination was completed, the resulting compound was then in-situ modified with 2-iminothiolane (24) by pH adjustment with NaHCO₃ (pH 8) and submitted onto C18 chromatography to assess subsequent retention and to see if these properties transferred over onto preparative HPLC like the TEAB probe.

As shown in Figure 39 above, the lactose-AEAB-thiol probe (25) did indeed have retention to C18 media under analytical conditions with a retention of 5 minutes. Unfortunately, once the reaction was transferred over to the preparative side, significant co-elution occurred where most of the compound eluted in the dead volume (fractions 1/2) alongside polar contaminants likely due to the amidine forming strong interactions with the salts present.
Figure 39: Analytical HPLC of crude reductive amination, lactose-AEAB-thiol conjugate (25) using a ramp of 1% to 100% AcN over 10 minutes.

However, isolation of a trace amount of impure compound was achieved on preparative HPLC (<1% yield) confirming the formation of the desired product the remainder of the product was found in the dead volume via NMR.

2.7 Summary

Overall, the synthesis of the first reductively aminated fluorescent thiol glycans was achieved in moderate to excellent yields (60% to 90% yields) with a one-day non-chromatographic preparation of TEAB as a stable zinc complex in 67% overall yield compared to the 32% yield of AEAB.HCl. Generation of these TEAB-linked glycans enabled C18 retention instead of the inconsistent and challenging HILIC separations resulting in a significant advancement over previous auxiliaries. This C18 retention has even been demonstrated with up to seven linked glycans on a mmol scale but investigation of more complex N-linked glycans should be evaluated to assess if this retention is transferred to longer-branching glycans. Investigation of thiol-modified AEAB showed that despite the increase in the length of the aliphatic arm, the resulting increase does not imbue the corresponding compound with significant C18 retention highlighting the effectiveness of the slight modification of one heteroatom affording TEAB. With hundreds of
milligram quantities of various TEAB-labeled glycans now available, further chemical modifications can be explored to validate the proposed utility of this new thiol motif.

2.8 Methods for the synthesis of compounds 1-25

2.9.1. Synthesis of N,N’-(dithiodi-2,1-ethanediyl)bis[2-amino-benzamide] (3)

Cysteamine dihydrochloride 1 (690 mg, 3.064 mmol, 1 eq.), isatoic anhydride 2 (1000 mg, 6.135 mmol, 2 eq.), DIPEA (2.69 mL, 9.192 mmol, 5 eq.) and DMAP (38 mg, 0.3110 mmol, 0.1 eq.) were dissolved in water (35 mL) in that order. The solution was then heated to 50 ºC and stirred overnight at which point the desired product had precipitated out from the crude mixture. The product was then filtered through P2 filter paper yielding the insoluble disulfide precipitate which was then washed with water until analytical HPLC of the product showed desired purity, yielding product 3 (851 mg, 71% yield).

\[ ^1H \text{NMR (500 MHz, D}_6\text{-Acetone with 0.25\% v/v TMS): } \delta 7.83 (\text{br, 1H}), 7.53 (\text{dd, } J = 7.9, 1.1 \text{ Hz, 1H}), 7.25 - 7.04 (\text{m, 1H}), 6.75 (\text{dd, } J = 8.2, 0.7 \text{ Hz, 1H}), 6.62 - 6.39 (\text{m, 1H}), 6.19 (\text{br, 1H}), 3.70 (\text{dd, } J = 12.9, 6.5 \text{ Hz, 3H}), 2.99 (\text{t, } J = 6.9 \text{ Hz, 3H}). \]

Compound previously published in literature [86]
2.9.2. Synthesis of \( N-(2\text{-ThioEthyl})-2\text{-AminoBenzamide} \) (1)

\[
\begin{align*}
\text{N,N'}-(\text{dithiodi-2,1-ethanediyl})\text{bis}[\text{2-amino-benzamide}] & \quad 3 \quad (100 \text{ mg, 0.256 mmol, 1 eq.}), \\
\text{TCEP} & \quad (367 \text{ mg, 1.28 mmol, 5 eq.}) \text{ and Chelex 100 sodium form (1 g) was suspended in MeOH (10 mL).}
\end{align*}
\]

The suspension was stirred for half an hour, Chelex was filtered off and the filtrate was then concentrated to dryness. The crude product was re-dissolved in water (10 mL) and the desired product was extracted with ethyl acetate (50 mL three times). The resulting organic layer was washed with a saturated NaCl solution (10 mL five times) and the resulting organic layer was concentrated yielding the colorless oil 1 (38 mg, 37\% yield).

\(^1\)H NMR (500 MHz, D\text{\textsubscript{6}}-Acetone): \(\delta 8.01\) (br, 1H), 7.73 (s, 1H), 7.51 (dd, \(J = 7.9, 1.4\) Hz, 1H), 7.14 (ddd, \(J = 8.4, 7.1, 1.5\) Hz, 1H), 6.74 (dd, \(J = 8.3, 1.0\) Hz, 1H), 6.52 (br, 2H), 3.61 – 3.46 (m, 2H), 2.80 – 2.69 (m, 2H); \(^{13}\)C\(^{\text{\textsubscript{1}}}\)H NMR (126 MHz, D\text{\textsubscript{6}}-Acetone): \(\delta 170.1, 150.5, 132.6, 128.4, 117.4, 116.0, 115.9, 43.5, 24.5\).

Compound previously published in literature [86]

2.9.3. Synthesis of \( N-(2\text{-ThioEthyl})-2\text{-AminoBenzamide zinc salt} \) (11)

\[
\begin{align*}
\text{N,N'}-(\text{dithiodi-2,1-ethanediyl})\text{bis}[\text{2-amino-benzamide}] & \quad 3 \quad (500 \text{ mg, 1.282 mmol, 1 eq.}) \text{ and zinc powder (420 mg, 6.462 mmol, 5 eq.) are suspended in MeOH, water and acetic acid (25 mL, 3.5:3.5:3). The suspension was then heated at 65 \text{ \degree C for 2.5 hrs and then left to cool. The excess}
\end{align*}
\]
undissolved metallic zinc was then filtered off from the solution using P2 filter paper. The crude mixture was then concentrated under vacuum to remove most of the methanol and water until a fine oil formed on the bottom of the flask. The product was then precipitated with an excess of water and the solid was filtered with P2 filter paper. The white precipitate was once again washed with water, finally yielding pure zinc salt 11 (547 mg, 94% yield).

\[ ^1H \text{NMR (500 MHz, DMSO): } \delta 8.27 \text{ (br, 1H), 7.46 (1H, d, } J = 7.6 \text{ Hz, H}_4, 7.10 \text{ (1H, t, } J = 7.3 \text{ Hz, H}_3), 6.66 \text{ (1H, d, } \text{= 8.2 Hz, H}_1), 6.45 \text{ (br, 1H), 6.35 (1H, s, H}_2), 3.47 \text{ (1H, br, H}_8), 2.72 \text{ (2H, br, H}_9) \]

\[ ^{13}C\{^1H\} \text{NMR (126 MHz, DMSO): } \delta 168.7 \text{ (C}_7), 149.6 \text{ (C}_6), 131.6 \text{ (C}_3), 128.1 \text{ (C}_4), 116.3 \text{ (C}_1), 114.8 \text{ (C}_5), 114.6 \text{ (C}_2), 43.6 \text{ (C}_8), 26.3 \text{ (C}_9) \]

HRMS (ESI) m/z [M + H]\(^+\): calcd for C\(_{18}\)H\(_{23}\)N\(_4\)O\(_2\)S\(_2\)Zn, 455.0554; found 455.0463.

2.9.4 General Synthesis of Glycoconjugates (6-17)

A glycan of interest (1.0 eq.), Zn-TEAB 11 (1.0 eq.), 2-Picoline borane (1.2 eq.), and EDTA (2.5 eq.) was dissolved in a MeOH, water, and acetic acid mixture (3.5:3.5:3, [glycan] = 0.1 M). The solution was then heated to 65 °C and stirred for 2.5 hrs. The reaction was then filtered through a cotton plug to remove the undissolved EDTA. All reactions were then concentrated under vacuum, filtered through a 0.45 µM filter, and purified by reverse phase chromatography on a semi-preparative C18 column. All desired glycans were eluted with a gradient of 99:1% to 50:50% (water: acetonitrile) over 15 minutes. If the resulting product contains trace amounts of
fluorophore due to partial co-elution, the starting material can be extracted away with a large excess of ethyl acetate with the product fully dissolved in water. All glycoconjugates should be stored as a solid with no solvent present in a -20 °C freezer to avoid oxidation if storing greater than a week.

2.9.5. Example synthesis of Lactose-TEAB (9)

Lactose (100 mg, 0.292 mmol, 1 eq.), 2-picoline borane complex (36 mg, 0.351 mmol, 1.2 eq.), Zn-TEAB 11 (133 mg, 0.292 mmol, 1 eq.) and EDTA (214 mg, 0.730 mmol, 2.5 eq.) were dissolved in MeOH, water and acetic acid mixture (3 mL, 3.5:3.5:3). The solution was heated at 65°C for 2.5 hrs, afterward the solution was filtered through a cotton plug removing un-dissolved EDTA and TCEP (209 mg, 0.730 mmol, 2.5 eq.) was added to ensure complete reduction of any disulfide. The reaction was then concentrated down to a crude oil (~ 2 mL) which was then filtered through a 0.45 µM filter and purified by reverse phase chromatography on a semi-preparative C18 column with a gradient of 99:1% to 50:50% (water: acetonitrile) over 15 minutes. The desired fraction was collected and concentrated to yield the off-white foaming solid, lactose-TEAB 9 (137 mg, 90% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v MeOH): δ 7.64 (d, J = 7.7 Hz, 1H), 7.56 (t, J = 7.8 Hz, 1H), 7.12 (d, J = 8.3 Hz, 1H), 7.03 (t, J = 7.5 Hz, 1H), 4.56 (d, J = 7.6 Hz, 1H), 4.29 –4.15 (m, J = 7.9, 3.7 Hz, 1H), 4.06 –3.87 (m, 5H), 3.85 –3.78 (m, 1H), 3.78 –3.51 (m, 9H), 3.33 (dd, J = 12.6, 8.6 Hz, 1H), 2.84 (t, J = 6.2 Hz, 2H). $^{13}$C{$^1$H} NMR (126 MHz, D$_2$O with 0.25% v/v
MeOH): δ 171.7, 148.0, 133.2, 128.7, 118.0, 117.1, 113.1, 103.2, 79.6, 75.1, 72.7, 71.3, 71.2, 70.8, 70.3, 68.6, 62.2, 60.8, 45.7, 38.5, 37.3. HRMS (ESI) m/z [M+H]^+: calcd for C_{21}H_{35}N_{2}O_{11}S^+, 523.1962; found 523.1957. [α]_{D}^{20} + 0.2 (c 0.37, MeOH).

2.9.6. Synthesis of D-glucose-TEAB (12)

Following the general synthesis procedure, the following reagents were reacted: D-glucose (100 mg, 0.555 mmol, 1 eq.), Zn-TEAB 11 (252 mg, 0.555 mmol, 1 eq.), 2-Picoline borane complex (70 mg, 0.666 mmol, 1.2 eq.) and EDTA (402 mg, 1.375 mmol, 2.5 eq.). Yielding compound 12 as a foaming white solid (154 mg, 77% yield).

^1H NMR (500 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 7.50 (d, J = 7.7 Hz, 1H), 7.44 (t, J = 11.4, 4.3 Hz, 1H), 6.93 (d, J = 8.3 Hz, 1H), 6.83 (t, J = 7.5 Hz, 1H), 4.02 (ddd, J = 8.3, 5.3, 4.2 Hz, 1H), 3.91 – 3.77 (m, 3H), 3.76 – 3.70 (m, 1H), 3.70 – 3.63 (m, 1H), 3.55 (t, J = 6.5 Hz, 2H), 3.45 (dd, J = 13.2, 4.0 Hz, 1H), 3.23 (dd, J = 13.2, 8.2 Hz, 1H), 2.77 (t, J = 6.5 Hz, 2H).$^{13}$C{[^1}H] NMR (126 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 172.2, 147.8, 133.5, 129.0, 117.7, 113.6, 71.8, 71.5, 71.2(2), 71.1(5), 63.3, 46.1, 42.8, 23.8. HRMS (ESI) m/z [M – H]^−: calcd for C$_{15}$H$_{23}$N$_{2}$O$_{6}$S$,^-$, 359.1227; found 359.1275. [α]_{D}^{20} - 5.9 (c 1.23, MeOH).
2.9.7. Synthesis of D-xylose-TEAB (13)

Following the general synthesis procedure, the following reagents were reacted: D-xylose (100 mg, 0.667 mmol, 1 eq.), Zn-TEAB 11 (302 mg, 0.667 mmol, 1 eq.), 2-Picoline borane complex (83 mg, 0.800 mmol, 1 eq.) and EDTA (487 mg, 1.668 mmol, 2.5 eq.). Yielding compound 13 as a foaming white solid (187 mg, 85% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v MeOH): δ 7.45 (d, J = 7.7 Hz, 1H), 7.41 (dd, J = 18.6, 11.0 Hz, 2H), 6.88 (d, J = 8.3 Hz, 1H), 6.77 (t, J = 7.5 Hz, 1H), 4.01 – 3.91 (m, J = 8.1, 4.1 Hz, 1H), 3.88 – 3.80 (m, J = 11.0, 4.5 Hz, 1H), 3.77 – 3.60 (m, 2H), 3.50 (t, J = 6.5 Hz, 2H), 3.43 – 3.32 (m, 2H), 3.23 (dd, J = 13.1, 8.1 Hz, 1H), 2.73 (t, J = 6.5 Hz, 2H). $^{13}$C{$^1$H} NMR (126 MHz, D$_2$O with 0.25% v/v MeOH): δ 171.7, 147.8, 133.2, 128.6, 117.0, 112.9, 72.2, 71.9, 69.9, 62.8, 45.8, 42.4, 23.4. HRMS (ESI) m/z [M – H]: calcd for C$_{14}$H$_{21}$N$_2$O$_5$S, 329.1171; found 329.1172. $\left[\alpha\right]_D^{20}$ - 4.8 (c 1.99, MeOH).

2.9.8. Synthesis of L-fucose-TEAB (14)

Following the general synthesis procedure, the following reagents were reacted: L-fucose (100 mg, 0.610 mmol, 1 eq.), Zn-TEAB 11 (277 mg, 0.610 mmol, 1 eq.), 2-Picoline borane complex (83 mg, 0.800 mmol, 1 eq.) and EDTA (487 mg, 1.668 mmol, 2.5 eq.). Yielding compound 14 as a foaming white solid (187 mg, 85% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v MeOH): δ 7.45 (d, J = 7.7 Hz, 1H), 7.41 (dd, J = 18.6, 11.0 Hz, 2H), 6.88 (d, J = 8.3 Hz, 1H), 6.77 (t, J = 7.5 Hz, 1H), 4.01 – 3.91 (m, J = 8.1, 4.1 Hz, 1H), 3.88 – 3.80 (m, J = 11.0, 4.5 Hz, 1H), 3.77 – 3.60 (m, 2H), 3.50 (t, J = 6.5 Hz, 2H), 3.43 – 3.32 (m, 2H), 3.23 (dd, J = 13.1, 8.1 Hz, 1H), 2.73 (t, J = 6.5 Hz, 2H). $^{13}$C{$^1$H} NMR (126 MHz, D$_2$O with 0.25% v/v MeOH): δ 171.7, 147.8, 133.2, 128.6, 117.0, 112.9, 72.2, 71.9, 69.9, 62.8, 45.8, 42.4, 23.4. HRMS (ESI) m/z [M – H]: calcd for C$_{14}$H$_{21}$N$_2$O$_5$S, 329.1171; found 329.1172. $\left[\alpha\right]_D^{20}$ - 4.8 (c 1.99, MeOH).
complex (76 mg, 0.732 mmol, 1.2 eq.) and EDTA (446 mg, 1.525 mmol, 2.5 eq.). Yielding compound 14 as a foaming white solid (136 mg, 65% yield).

\[ \text{1}^\text{H} \text{NMR (500 MHz, D}_2\text{O with 0.25\% v/v CH}_3\text{CN): \delta 7.52 (d, J = 7.7 Hz, 1H), 7.46 (t, J = 7.8 Hz, 1H), 6.96 (d, J = 8.4 Hz, 1H), 6.84 (t, J = 7.5 Hz, 1H), 4.26 – 4.06 (m, 2H), 3.67 (d, J = 9.0 Hz, 1H), 3.62 – 3.49 (m, 4H), 3.46 – 3.33 (m, J = 12.9, 11.9, 4.8 Hz, 3H), 2.80 (t, J = 6.5 Hz, 2H), 1.25 (d, J = 6.6 Hz, 4H).} \]

\[ \text{13C}\{^\text{1}^\text{H}\} \text{NMR (126 MHz, D}_2\text{O with 0.25\% v/v CH}_3\text{CN): \delta 172.3, 148.0, 133.5, 129.1, 117.6, 113.5, 73.6, 71.2, 68.7, 66.6, 46.6, 42.8, 23.8, 19.1. HRMS (ESI) m/z [M – H]: calcd for C}_15\text{H}_23\text{N}_2\text{O}_5\text{S}, 343.1328; found 343.1330. [\alpha]_{D}^{20} - 3.1 (c 0.25, MeOH).} \]

2.9.8. Synthesis of N-acetyl-D-glucoseamine-TEAB (15)

Following the general synthesis procedure, the following reagents were reacted: N-acetyl-D-glucoseamine (100 mg, 0.452 mmol, 1 eq.), Zn-TEAB 11 (205 mg, 0.452 mmol, 1 eq.), 2-Picoline borane complex (56 mg, 0.542 mmol, 1.2 eq.) and EDTA (330 mg, 1.130 mmol, 2.5 eq.). Yielding compound 15 as a foaming white solid (118 mg, 65% yield).

\[ \text{1}^\text{H} \text{NMR (500 MHz, D}_2\text{O): \delta 7.44 (d, J = 6.9 Hz, 1H), 7.39 (t, J = 13.7, 5.7 Hz, 1H), 6.91 (d, J = 8.4 Hz, 1H), 6.76 (t, J = 7.5 Hz, 1H), 4.20 (dt, J = 9.6, 5.0 Hz, 1H), 4.03 (d, J = 5.7 Hz, 1H), 3.90 – 3.84 (m, 1H), 3.71 (ddd, J = 17.0, 10.8, 3.0 Hz, 2H), 3.65 – 3.42 (m, 6H), 3.21 (dd, J = 13.9, 9.4 Hz, 1H), 2.72 (t, J = 6.5 Hz, 2H), 1.90 (s, 3H).} \]

\[ \text{13C}\{^\text{1}^\text{H}\} \text{NMR (126 MHz, D}_2\text{O with 0.25\% v/v CH}_3\text{CN): \delta 174.7, 172.1, 147.8, 133.4, 129.1, 117.7, 113.7, 71.9, 71.6, 70.1, 69.9, 63.1, 44.2, 42.8,} \]
23.8, 22.5. HRMS (ESI) m/z [M – H]−: calcd for C_{17}H_{26}N_{2}O_{6}S^{-}, 400.1542; found 400.1541. [α]_{D}^{20} + 28.1 (c 0.29, MeOH).

2.9.9. Synthesis of D-Allose-TEAB (16)

Following the general synthesis procedure, the following reagents were reacted: D-allose (100 mg, 0.667 mmol, 1 eq.), Zn-TEAB 11 (302 mg, 0.667 mmol, 1 eq.), 2-Picoline borane complex (83 mg, 0.800 mmol, 1.2 eq.) and EDTA (487 mg, 1.668 mmol, 2.5 eq.). Yielding compound 16 as a foaming white solid (183 mg, 76% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 7.58 (d, J = 7.8 Hz, 1H), 7.52 (t, J = 7.8 Hz, 1H), 7.03 (d, J = 8.4 Hz, 1H), 6.91 (t, J = 7.5 Hz, 1H), 4.19 – 4.12 (m, J = 5.1 Hz, 1H), 4.00 (s, 1H), 3.95 – 3.84 (m, 3H), 3.76 (dd, J = 11.9, 7.0 Hz, 1H), 3.69 – 3.58 (m, 4H), 3.32 (dd, J = 13.4, 8.6 Hz, 1H), 2.86 (t, J = 6.5 Hz, 2H). $^{13}$C($^1$H) NMR (126 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 172.2, 148.0, 133.5, 129.0, 117.6, 113.6, 113.6, 73.7, 72.9(0), 72.8(5), 70.4, 62.8, 45.5, 42.8, 23.8. HRMS (ESI) m/z [M – H]−: calcd for C_{15}H_{23}N_{2}O_{6}S^{-}, 359.1277; found 359.1274. [α]_{D}^{20} - 1.3 (c 0.75, MeOH).
2.9.10. Synthesis of D-ribose-TEAB (17)

![Chemical structure of D-ribose-TEAB](image)

Following the general synthesis procedure, the following reagents were reacted: D-ribose (100 mg, 0.667 mmol, 1 eq.), Zn-TEAB 11 (302 mg, 0.667 mmol, 1 eq.), 2-Picoline borane complex (83 mg, 0.800 mmol, 1.2 eq.) and EDTA (487 mg, 1.668 mmol, 2.5 eq.). Yielding compound 17 as a foaming white solid (170 mg, 77% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v MeOH): $\delta$ 7.49 (d, $J = 7.7$ Hz, 1H), 7.47 – 7.41 (m, 1H), 6.94 (d, $J = 8.4$ Hz, 1H), 6.82 (t, $J = 7.5$ Hz, 1H), 4.00 (ddd, $J = 8.7$, 5.9, 3.0 Hz, 1H), 3.92 – 3.79 (m, 3H), 3.75 (t, $J = 6.2$ Hz, 1H), 3.69 (dd, $J = 11.0$, 4.1 Hz, 2H), 3.54 (dd, $J = 12.1$, 4.9 Hz, 4H), 3.22 (dd, $J = 13.3$, 8.7 Hz, 1H), 2.77 (t, $J = 6.6$ Hz, 2H). $^{13}$C$[^1]$H NMR (126 MHz, D$_2$O with 0.25% v/v MeOH) $\delta$ 172.2, 148.0, 133.5, 129.0, 117.6, 113.6, 73.7, 72.6, 70.3, 63.0, 45.6, 42.8, 23.8. HRMS (ESI) m/z [M – H]$^-$: calcd for C$_{14}$H$_{21}$N$_2$O$_5$S$^-$, 329.1171; found 329.1176. [$\alpha]^D_{20}$ - 6.4 (c 0.62, MeOH).
2.9.11. Synthesis of D-maltose-TEAB (18)

Following the general synthesis procedure, the following reagents were reacted: D-maltose (100 mg, 0.292 mmol, 1 eq.), Zn-TEAB 11 (133 mg, 0.292 mmol, 1 eq.), 2-Picoline borane complex (36 mg, 0.350 mmol, 1.2 eq.) and EDTA (213 mg, 0.730 mmol, 2.5 eq.). Yielding compound 18 as a foaming white solid (104 mg, 68% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v MeOH): δ 7.52 (dd, J = 7.8, 1.2 Hz, 1H), 7.49 – 7.42 (m, 1H), 6.95 (d, J = 8.4 Hz, 1H), 6.84 (t, J = 7.5 Hz, 1H), 5.12 (d, J = 3.9 Hz, 1H), 4.05 (ddd, J = 7.7, 5.1, 2.8 Hz, 1H), 3.98 (dd, J = 7.3, 3.7 Hz, 1H), 3.96 – 3.71 (m, 8H), 3.65 (dd, J = 11.8, 7.3 Hz, 1H), 3.61 – 3.55 (m, 3H), 3.45 (dt, J = 8.5, 7.3 Hz, 2H), 3.35 (d, J = 5.8 Hz, 1H), 2.80 (t, J = 6.5 Hz, 2H). $^{13}$C{$^1$H} NMR (126 MHz, D$_2$O with 0.25% v/v MeOH): δ 171.8, 147.4, 133.1, 128.7, 117.3, 113.2, 100.6, 82.0, 73.0, 72.6(4), 72.5(8), 71.7, 71.4, 69.5, 69.2, 62.3, 60.5, 46.0, 42.4, 23.5. HRMS (ESI) m/z [M – H]: calcd for C$_{21}$H$_{33}$N$_2$O$_{11}$S, 521.1805; found 521.1808. $[\alpha]_D^{20} + 54.2$ (c 1.41, MeOH).
2.9.12. Synthesis of \(N\)-acetyl-D-lactoseamine-TEAB (19)

Following the general synthesis procedure, the following reagents were reacted: \(N\)-acetyl-D-lactoseamine (40 mg, 0.104 mmol, 1 eq.), Zn-TEAB 11 (48 mg, 0.104 mmol, 1 eq.), 2-Picoline borane complex (13 mg, 0.125 mmol, 1.2 eq.) and EDTA (76 mg, 0.260 mmol, 2.5 eq.). Yielding compound 19 as a foaming white solid (48 mg, 82% yield).

\(^1\)H NMR (500 MHz, D\(_2\)O with 0.25% v/v CH\(_3\)CN): \(\delta\) 7.47 (d, \(J = 7.8 \text{ Hz}, 1\text{H}\)), 7.43 (t, \(J = 7.8 \text{ Hz}, 1\text{H}\)), 6.94 (d, \(J = 8.4 \text{ Hz}, 1\text{H}\)), 6.80 (t, \(J = 7.5 \text{ Hz}, 1\text{H}\)), 4.49 (d, \(J = 7.6 \text{ Hz}, 1\text{H}\)), 4.47 – 4.34 (m, 1H), 4.00 – 3.83 (m, 5H), 3.83 – 3.75 (m, 1H), 3.75 – 3.47 (m, 11H), 3.22 (dd, \(J = 13.1, 9.2 \text{ Hz}, 1\text{H}\)), 2.76 (t, \(J = 6.5 \text{ Hz}, 2\text{H}\)), 1.96 (s, 3H). \(^{13}\)C{\(^1\)H} NMR (126 MHz, D\(_2\)O with 0.25% v/v CH\(_3\)CN): \(\delta\) 174.8, 172.1, 147.9, 133.4, 129.0, 118.7, 117.6, 113.6, 103.5, 79.4, 75.5, 73.1, 71.6, 70.1, 69.0, 62.3, 61.3, 50.7, 44.3, 42.8, 23.8, 22.5. HRMS (ESI) m/z [M + H]: calcd for C\(_{23}\)H\(_{38}\)N\(_3\)O\(_{11}\)S, 564.2225; found 564.2227. [\(\alpha\)]\(_D\)\(^{20}\) - 3.5 (c 0.27, MeOH).
2.9.13 Synthesis of 1,3-α-1,6-α-D-mannotriose-TEAB (20)

Following the general synthesis procedure, the following reagents were reacted: 1,3-α-1,6-α-D-mannotriose (40 mg, 0.079 mmol, 1 eq.), Zn-TEAB 11 (36 mg, 0.079 mmol, 1 eq.), 2-Picoline borane complex (10 mg, 0.0952 mmol, 1.2 eq.) and EDTA (58 mg, 0.198 mmol, 2.5 eq.). Yielding compound 20 as a foaming white solid (46 mg, 85% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 7.49 (dd, J = 7.8, 1.2 Hz, 1H), 7.47 – 7.40 (m, 1H), 6.93 (d, J = 8.4 Hz, 1H), 6.82 (t, J = 7.5 Hz, 1H), 5.06 (d, J = 1.1 Hz, 1H), 4.89 (d, J = 1.3 Hz, 2H), 4.15 – 4.07 (m, 1H), 4.07 – 4.02 (m, 2H), 3.99 (dd, J = 3.3, 1.7 Hz, 1H), 3.98 – 3.82 (m, 6H), 3.82 – 3.58 (m, 10H), 3.58 – 3.51 (m, 3H), 3.27 (dd, J = 13.4, 8.0 Hz, 1H), 2.76 (t, J = 6.5 Hz, 2H). $^{13}$C{$_1$H} NMR (126 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 172.2, 147.8, 133.5, 129.1, 119.0, 117.7, 113.7, 102.9, 100.4, 79.9, 74.0, 73.2, 71.0, 70.8(2), 70.8(0), 70.6, 70.5, 70.4, 69.7, 69.0, 67.2, 66.9, 61.4, 61.1, 46.5, 42.8, 23.8. HRMS (ESI) m/z [M + H]$^+$: calcd for C$_{27}$H$_{45}$N$_2$O$_{16}$S, 684.2412; found 685.2490. [α]$_D^{20}$ + 43.8 (c 0.44, MeOH).
2.9.14. Synthesis of acarbose-TEAB (21)

Following the general synthesis procedure, the following reagents were reacted: acarbose (100 mg, 0.155 mmol, 1 eq.), Zn-TEAB 11 (71 mg, 0.155 mmol, 1 eq.), 2-Picoline borane complex (20 mg, 0.186 mmol, 1.2 eq.) and EDTA (113 mg, 0.388 mmol, 2.5 eq.). Yielding compound 21 as a foaming white solid (77 mg, 60% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v MeOH): $\delta$ 7.75 (t, J = 7.7 Hz, 1H), 7.61 (dd, J = 18.5, 11.0 Hz, 1H), 7.47 (dd, J = 13.0, 6.5 Hz, 2H), 5.78 (d, J = 3.2 Hz, 1H), 5.29 (dd, J = 16.9, 3.9 Hz, 1H), 4.96 (d, J = 3.8 Hz, 1H), 4.19 – 3.42 (m, 29H), 3.15 (t, J = 10.3 Hz, 1H), 2.68 (t, J = 6.6 Hz, 2H), 1.29 (d, J = 6.2 Hz, 3H). $^{13}$C{$^1$H} NMR (126 MHz, D$_2$O with 0.25% v/v MeOH): $\delta$ 170.8, 147.3, 133.8, 129.1, 121.2, 120.2, 118.0, 116.7, 115.7, 114.7, 100.8, 100.1, 82.5, 78.1, 73.6, 72.9, 72.7, 72.0, 71.8, 71.3, 70.8, 68.9, 68.6, 66.9, 64.8, 63.3, 62.6(1), 61.5(8), 60.9, 56.9, 49.4, 42.8, 23.7, 17.8. HRMS (ESI) m/z [M – H]: calcd for C$_{34}$H$_{54}$N$_5$O$_{18}$S$^-$, 824.3122; found 824.3122. $[\alpha]_D^{20} + 100.2$ (cm 0.30, MeOH).

2.9.15. Synthesis of maltoheptaose -TEAB (22)

Following the general synthesis procedure, the following reagents were reacted: maltoheptaose (20 mg, 0.017 mmol, 60 % purity, 1 eq.), Zn-TEAB 11 (8 mg, 0.017 mmol, 1 eq.),
2-Picoline borane complex (2 mg, 0.021 mmol, 1.2 eq.) and EDTA (12 mg, 0.043 mmol, 2.5 eq.). Yielding compound 17 as a foaming white solid (11 mg, 83% yield).

\[^{1}\text{H NMR (500 MHz, D}_2\text{O with 0.25% v/v CH}_3\text{CN): }\delta 7.50 (d, J = 7.6 \text{ Hz, 1H}), 7.44 (t, J = 7.9 \text{ Hz, 1H}), 6.94 (d, J = 8.4 \text{ Hz, 1H}), 6.82 (t, J = 7.5 \text{ Hz, 1H}), 5.39 (s, 5H), 5.11 (d, J = 3.6 \text{ Hz, 1H}), 4.10 – 3.75 (m, 28H), 3.75 – 3.52 (m, 20H), 3.42 (t, J = 8.0 \text{ Hz, 2H}), 3.34 (d, J = 5.8 \text{ Hz, 1H}), 2.78 (t, J = 6.3 \text{ Hz, 2H}).\]^\[^{13}\text{C}[^{1}\text{H}] \text{ NMR (126 MHz, D}_2\text{O with 0.25% v/v CH}_3\text{CN): }\delta 172.3, 147.8, 133.5, 129.1, 119.6, 118.8, 117.6, 113.5, 100.7, 100.2, 100.1, 82.5, 77.4(2), 77.3(7), 77.2, 73.8(1), 73.7(8), 73.3, 73.2, 72.9, 72.2, 72.0, 71.9, 71.8, 71.7, 71.4, 69.8, 69.4, 62.6, 60.9(4), 60.8(8), 60.8, 46.2, 42.8, 23.9, 23.7. HRMS (ESI) m/z [M + H]^+: calcd for C\text{\textsubscript{51}}H\text{\textsubscript{85}}N\text{\textsubscript{2}}O\text{\textsubscript{36}}S\text{\textsuperscript{+}}, 1333.4603; found 1333.4603. [\alpha\textsubscript{D}\textsuperscript{20} + 70.5 (c 0.14, H\textsubscript{2}O).]

2.9.16. Synthesis of 2-amino-N-(2-aminoethyl)-benzamide (AEAB, 23) modified from literature [53]

![Structural diagram of AEAB](image)

Methyl anthranilate (100 mg, 0.662 mmol, 1 eq.) was added to ethylenediamine (1 mL, 18.5 mmol, 30 eq.) where the mixture was then heated at 100 °C for 12hrs. This was then followed by evaporation to dryness where the residue was then loaded onto silica gel (Ø 5 cm, h\textsubscript{C} 15 cm, V\textsubscript{Fr} 12 mL) and eluted with MeOH: DCM (10:90) with R\textsubscript{f} 0.5 (in place of the weeklong precipitation method described in the literature) which then yielded AEAB 23 (31 mg, 26 % yield).
$^{1}$H NMR (500 MHz, D$_6$-Acetone): δ 8.01 (s, 1H), 7.52 (dd, J = 7.9, 1.5 Hz, 1H), 7.12 (t, J = 8.5, 7.1, 1.5 Hz, 1H), 6.73 (dd, J = 8.2, 1.0 Hz, 1H), 6.51 (t, J = 8.2, 7.1, 1.2 Hz, 2H), 3.64 – 3.48 (m, 4H). Compound previously published in literature [53]

2.9.17. Synthesis of lactose-AEAB-2- iminothiolane (25)

![Chemical Structure](image)

AEAB (30 mg, 0.168 mmol, 1eq.), lactose monohydrate (60 mg, 0.168 mmol, 1eq.), and 2-picoline borane (28 mg, 0.202 mmol, 1.2 eq.) were dissolved in MeOH:water: acetic acid (2 mL, 3.5:3.5:3) and heated to 65 °C for 2.5 hrs. The reaction was then cooled, and pH was adjusted with sat. NaHCO$_3$ (1 mL) where 2-iminothiolane (28 mg, 0.202 mmol, 1.2 eq.) was added to the solution. The reaction was then left to stir for 30 minutes until the lactose-AEAB conjugate fully reacted which was determined by HPLC analysis of the reaction. The crude reaction was then submitted to prep-C18 for purification with a ramp of 1% to 50% AcN similar to TEAB conjugates resulting in the recovery of the corresponding lactose-AEAB-2- iminothiolane 25 (<1 mg, <1% yield) with the majority of the reaction eluting in the dead volume alongside polar contaminates.

$^{1}$H NMR (500 MHz, D$_2$O) δ 7.45 (d, J = 7.6 Hz, 1H), 7.37 (s, 1H), 6.89 (dd, J = 22.8, 8.0 Hz, 2H), 5.09 (s, 1H), 4.14 (dd, J = 15.4, 8.5 Hz, 2H), 4.04 – 3.61 (m, 9H), 3.51 (dd, J = 27.7, 6.9 Hz, 2H), 3.29 (d, J = 6.4 Hz, 1H), 3.16 (d, J = 14.7 Hz, 1H), 2.72 (dd, J = 18.8, 11.6 Hz, 1H), 2.63 – 2.53 (m, 1H), 2.50 – 2.39 (m, 1H), 2.14 (dd, J = 22.6, 15.6 Hz, 2H), 1.92 (d, J = 22.9 Hz, 2H).
CHAPTER 3
APPLICATIONS OF THIOL-DERIVATIZED GLYCANS

3.1 Chemical modifications of TEAB glycoconjugates

With a large library of TEAB glycan conjugate probes ready, the chemoselective conjugation of the thiol-derivatized glycans was investigated inspired by the wide range of thiol chemistry in Figure 17. Lactose-TEAB (9) was selected to identify optimal conditions to react with a diverse range of functional groups (Figure 40). To display the utility of a thiol group over an amino derivative a wide variety of coupling partners were selected to highlight the selective chemistry of thiols.

Figure 40: Synthesis of TEAB-modified lactose targets “isolated yield. (a) (NH₄)₂CO₃ (1 eq.), water, 30 mins.; (b) LAP (0.05% w/v), water 30 mins irradiation at 365 nm, 10 mW/cm²; (c) i) Dipyridyl disulfide (25 eq.), DMF, 10 mins; ii) Hexane thiol (2 eq.), DMF, 30 mins.; (d) (NH₄)₂CO₃ (1 eq.), water, 2 hrs.; (e) DCC (2 eq.), DMAP (0.1 eq.), DMF, 2 hrs.; (f) TCEP (0.7 eq.), (NH₄)₂CO₃ (1 eq.), MeOH, 65 °C, 8 hrs.; (g) (NH₄)₂CO₃ (1 eq.), water, 1.5 hrs.
First, conjugate addition to the maleimide functional group (33) was performed in the presence of a volatile base (NH₄)₂CO₃ to promote the generation of the thiolate ion to attack the maleimide present. The addition was achieved in 30 minutes yielding the desired product 26, which was then purified from trace salts via prep-C18 in 76% yield. Halo acetamide (35) addition to the lactose-TEAB (9) also proceeded in a similar fashion to maleimide addition except with a longer time period of 2 hrs resulting in 26 in 91% yield post purification. Simple alkylation with an alkyl halide (37) produces thioether 29 slowly over 8 hours. Oxidation of the thiol (9) could be observed and the inclusion of TCEP in the reaction conditions provides the desired product in 64% yield after purification.

Next, thiol-ene click addition to the endo-norbornene-cis-5,6-dicarboxylic acid (34) was performed with the photo-initiator lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, 0.05% w/v) or with 1-[4-(2-hydroxyethoxy)-phenyl]-2-hydroxy-2-methyl-1-propane-1-one (I2959, 0.05% w/v) where both proceeded in an exo-selective manner resulting in product 27. Interestingly I2959 was consistently slower to form the desired thiol-ene product in comparison to LAP which is likely a result of high molar absorptivity at the wavelength of irradiation (365 nm) vs the TEAB fluorophore which is highly absorbing at that wavelength.

The desired product was purified with prep-C18 in 93% yield and stereochemistry was indicated by 2D NOESY and HSQC NMR supported by the literature [98]. In Figure 41, a timelapse NMR of the reaction (with I2959) was recorded where the reaction progressed with the corresponding peaks to the thiol-ene “clicked” product slowly growing in.
Figure 41: Thiol-ene click of lactose-TEAB (9, 1 eq.) to 34 (1 eq.) with I2959 (0.05% w/v) utilizing NMR monitoring of the reaction.

As additional UV light was provided, integral values also corroborate this as the noroborene alkene (~6.25 ppm) reduce relative to the aromatic group (~6.8 ppm). This figure is an excellent example of how controllable the thiol-ene “click” process is, where once the UV light is removed the resulting chemical reaction slows down significantly to the point where the reaction can be observed at different time intervals.

An uncommon transformation known for thiols is the process of thiol-yne “click” chemistry [71] where similar to thiol-ene chemistry, thiols are added to the alkyne (40) except this process is repeated twice shown in Figure 42.
The resulting product is usually a mixture of enantiomers with mono (42) and bis (41) derivatized species. Therefore, an investigation into preparing these thiol-yne products was warranted to try to assess if the formation was more competitive against the thiol-ene reaction. Subsequently, the excess ratio of lactose-TEAB (9, 1 eq.) to the alkyne and alkene (4 eq. each) reactants were dissolved in CD$_3$OD and reacted together in the presence of photoinitiator (LAP, 0.5% w/v). The corresponding mixture was then analyzed via NMR with integration taken of the known alkene (~6.4 ppm) and alkyne peaks (~2.7 ppm) (Figure 42) and the ratio between the two was monitored from pre-irradiation to an hour post-irradiation. This resulted in the integrals

**Figure 42:** Competition reaction of thiol-yne (40, 4 eq.) vs thiol-ene (34, 4 eq.) reaction with the integration of the corresponding starting materials pre-irradiation and post-irradiation.
remaining close to similar values of the starting material alkyne relative to this alkene indicating that the reaction proceeded in an approximate 1:1 ratio despite the alkyne being able to react twice with the Lactose-TEAB product. Exact quantification would be challenging with NMR analysis due to the mixture of products and the overlap of NMR signals. It is important to note that the most reactive species for thiol-ene addition (norbornene diacid) was utilized therefore this ratio of products may be significantly impacted if the electronics on the alkene or alkyne species are altered.

The preparation of asymmetric disulfide (28) is a reversible conjugation to the lactose-TEAB where an unstable disulfide bridge is formed by an excess of dipyridyl disulfide (DPDS) reagent Figure 43. The desired intermediate (43, which is a thiol reactive species due to by-product 2-thiopyrdine being thermodynamically favorable) could be isolated and purified by prep-C18 but only in a modest yield (30%). But overall yield is improved if in-situ reacted with the desired thiol, which helps promote C18 retention and reduces co-elution with the unreacted reagent.

Figure 43: Disulfide exchange of hexane thiol to activated TEAB to form 35, followed by the exchange to 28.

Alternatively, the crude product can be used in situ to further react with 1-hexane thiol (HT) providing disulfide 28 in 49% yield once again after reverse phase chromatography, interestingly retention of the alkylated product isn’t significantly better than the precursor thiol despite the significant increase in the aliphatic arm. Pre-activation of hexane thiol with dipyridyl
disulfide was also investigated to improve overall yield. However, the stoichiometric excess of the reagent (DPDS) prevented the purification of the desired compound with silica gel chromatography. Unfortunately, the electronics present on the 2-thiopyridine-based reagent (which gives it unique thiol exchanging properties) result in a rapid equilibrium between forming a thione or thiol (behaving like a charged species). This was found to significantly complicate the purification of many reagents bearing this species which often resulted in co-elution with either prep-C18 or silica gel despite the derivative bearing a significantly different residue. Due to the utility of this exchange reaction and the potential ability to vary C18 retention for more complex glycans, a pilot study into a pre-activated derivative was conducted Figure 44.

![Figure 44: Pre-activation of TEAB (1) with DPDS for subsequent reductive amination and HPLC chromatogram (1% to 100% AcN over 10 minutes) of reductive amination yielding 43.](image)

Where the TEAB reporter was derivatized to DPDS and purified with the 2-thiopyridine conjugate (44) in 87% yield instead of modifying the glycan post reductive amination shown in.
Once isolated, this activated intermediate 44 was then reductively aminated with lactose monohydrate to determine if activated TEAB would survive reductive amination conditions. Unfortunately, HPLC analysis showed that a significant portion of the activated compound 44 was undergoing unfavorable side reactions resulting in the sub-optimal generation of the product 43 (60%, 5.6 minutes), 8 (25 %, 5.0 minutes), and 45 (15%, 5.7 minutes). Utilizing this method to generate an activated TEAB glycoconjugate would be more efficient (~30%) than the previous approach in Figure 43 but still would have significant limitations in yield and would prove challenging to isolate due to the resulting byproducts present. Overall, exploration of this chemistry provided insight into the limitations of 2-thiopyrdine-based chemistry and served as a useful study to help elucidate different pathways to form asymmetric disulfides.

The amino acid thioesterification with the BOC-protected leucine (36) was an important example of glycosylation of an amino acid, which proved to produce a stable thioester 30 in a moderate yield (50%, unoptimized) using N,N'-dicyclohexylcarbodiimide (DCC, 2 eq.) and DMAP (0.1 eq.).

Native chemical ligation (NCL) [99] of the thioester produced was then investigated (shown in Figure 45), as the resulting product would have been a close analog to N-glycans as the peptide linkage. Where the thioester group undergoes a nucleophilic substitution via the secondary amine resulting in a shift from the thiol group to the amine present on the aromatic ring 47/49.
Figure 45: Native chemical ligation (NCL) process and desired NCL of Lactose TEAB thioester (47/49).

These NCL products would have been unique products (with respect to thiol derivatives) that could have emphasized the importance of thiol chemistry and created close structural analogs to glycopeptides. Generation of the thioester to the BOC-Leucine (30) and the subsequent hexenoic acid was performed to see if a sterically less hindered species (48) would help promote the chemical reaction. Unfortunately, both derivatives did not result in NCL (49) occurring even if conditions were altered to aid in the nucleophilic substitution such as; change in buffer (PBS, ascorbic), change in temperatures (80, 60, and 40 °C), time (3, 6 and 14 hrs) as well as the addition of species that catalyze this reaction (imidazole, thiophenol, selenophenol). Unfortunately, the thioester consistently underwent a hydrolysis event (or simply stayed unreacted) and either formed the disulfide or thiol if a reducing agent was present, and typically the addition of catalysts promoted this formation faster as they are more nucleophilic which typically out-competed the secondary amine. The hypothesis surrounding the lack of formation of the desired NCL products is likely due to either the steric hindrance present around the amine due to the lactose or it could be attributed to the poor reactivity of the secondary amine resulting from the ortho amide withdrawing electron density from the aromatic ring.
To conclude direct chemical modifications of TEAB, an epoxide ring opening (32) proceeded similar to maleimide addition (26) and acetamide alkylation (29), with a slightly shorter time of 1.5 hrs giving product 24 in 75% yield post purification. It is important to note that products 32, 31, 29, and 26 can be obtained with minimal impurities or salts without C18 purification if a 1:1 stoichiometry, concerning substrate and TEAB, is utilized which would significantly improve overall yields. Overall, out of the seven reactions displayed in Figure 40, amines can only participate in two of the reactions and cannot form a reversible covalent bond, displaying a significant advantage thiol chemistry has over amines.

3.2 Neoglycoprotein synthesis utilizing TEAB glycans

After demonstrating chemoselective conjugation with the thiol to a variety of coupling partners, the model lactose-TEAB probe (9) was applied to a more complex system to demonstrate the selectivity of a combined thiol-fluorophore-glycan. Upon reviewing the literature[76] bovine serum albumin (BSA) was chosen as a model protein partner to conjugate a glycan with since it possesses a singular reduced cysteine residue. A variety of linkers were assessed with three potential model coupling partners shown in Figure 46.

![Figure 46: Bifunctional linkers investigated for attachment to BSA.](image)

Therefore, the need to develop a thiol-specific bifunctional linker utilizing the demonstrated conjugation techniques was required resulting in three model linkers investigated. A traditional bismaleimide linker (50) was seen as the ideal substrate as it was previously employed in literature [100] to link BSA at the lone cysteine residue alongside other halo acetamide linkers. It was
envisioned that investigation of thiol-reactive species which were reversible, or light controlled could be highly utile for bioconjugation purposes thusly di(ethylene glycol)divinyl ether (53) and thiol activation with a 2-thiopyridine derivative (52, MNA) was warranted.

Figure 47: Thiol-ene addition of 53 to generate mono addition spacer 54 resulting in a mixture of products with starting material.

Firstly 53, was a commercially available bis-allylated ethylene glycol species which was seen as an acceptable linker due to the flexibility of the spacing group present. Preliminary attempts of reacting Lactose TEAB (9) with the desired alkene to form a mono-substituted product which would have resulted in a terminating alkene to further react with the desired protein thiol. Three methods of initiating the reaction were investigated; chemically (TEMED), heat (AIBN), and light (LAP) mediated to produce the desired product. Unfortunately, all three methods with the compound (53) in 10 eq. resulted in significant byproduct formation where the Lactose TEAB (9) was added to the linker twice instead of the mono-substituted product. The elution of the compound was close to the bis product, and its formation was rather significant, increasing the stoichiometric excess to reduce this by-product formation was concerning as significant co-elution would start to occur with the presence of more ethylene glycol reagent (53). Therefore, this method of generating a BSA reactive species was regarded as disadvantageous and other methods were further studied instead. Generation of a directly thiol-reactive species seemed advantageous as installation of a reactive bis linker proved to be problematic with double addition to both ends. Therefore, further expansion on previous work with thiopyridine appeared to be a valid route. Instead of utilizing the
2-thiopyrdine motif, a more water-soluble motif appeared superior due to the poor solubility properties of the previous compound (shown in Figure 48).

![Figure 48: Activation of thiol with 5-mercaptopyrdine-2-carboxylic acid (MNA) with "isolated yield."

Therefore 6,6'-dithiodinicotinic acid (55), which is a more water-soluble derivative of the 2,2’-dithiodipyr dine (DPDS) due to the carboxylic acids present was utilized to ensure complete solvation of the adduct. The transformation was run in pH 10 water, to dissolve 55 where the Lactose-TEAB (9) probe was added dropwise to the reaction. After 30 minutes the reaction was acidified, and the solution was filtered to remove the excess reagent to allow for prep-C18 purification resulting in lactose-TEAB-MNA (56) 92 % yield of the desired compound ready for conjugation with protein. Synthesis of the bismaleimide linker 60 was unfortunately required where a sequential two-step reaction occurred shown in Figure 49.

![Figure 49: Synthesis of Bismaleimide spacer 60 with "isolated yields."

Ring opening of the maleic anhydride occurs in the first step where the amine (57) adds through nucleophilic addition of maleic anhydride (58), the resulting intermediate (59) can then
be precipitated or purified with silica gel chromatography to result in the intermediate excellent yield (90 %). The second step then required the intermediate amide to attack the ester product generated from acetic anhydride addition resulting in ring closure and formation of the desired 60. The solvent for this step was modified from the published acetone to dimethylformamide (DMF) to enable higher temperatures and solvation of all compounds.

To generate the activated maleimide (62) beforehand to eliminate uncertainty in reaction with the protein of interest, synthesis was attempted with excess stoichiometry to the maleimide linker (60) with the addition of Lactose-TEAB (9) dropwise (shown in Figure 50). Purification of the reaction was attempted via prep-C18 chromatography where unfortunately co-elution occurred between the two compounds as they exhibited extremely close retentions <10-second shift between the two.

![Figure 50: Synthesis of mono-addition to Lactose-TEAB (62) with "isolated yield."](image)

Size exclusion chromatography was employed to purify the products in a 33% yield of 61+62 was obtained. Following a similar trend to compound 44, the ratio of products proved to be: 7:3; 61:62 where a majority of the bis-labeled species (61) co-eluted with the desired mono species (62) making this route highly unfavorable. Therefore, an in-situ approach (shown in Figure
was developed to install the bifunctional linker onto the BSA protein instead of solving challenging purifications of bis vs mono substituted compounds.

Figure 51: Attachment of bifunctional maleimide linker (60) and lactose TEAB (9) to BSA forming 63.

Similar to the formation of compound 26, the thiolate ion present in the BSA was added to the PEG-bismaleimide 60 [100]. After 1 hour the now linked BSA-PEG-maleimide was then submitted to dialysis in a 10-13K MWCO tube for 24 hrs. to remove any small molecule reagents. The modified protein in the solution was then reacted with a slight excess of Lactose-TEAB (9) for an additional hour and finally purified using dialysis once again. After freeze-drying, the sample, the identity of the glycoprotein 63 was confirmed using MALDI mass-spectrometry (theoretical: ~ 67,307 Da, observed mass: 67,309 Da) indicating a significant shift of ~ 846 Da from the original BSA protein (66,463 Da). Analytical HPLC was used to track reaction progress as well as a confirmation of the removal of starting materials from the reaction.

Concurrently with the maleimide linker, the activated Lactose-TEAB-MNA (66) compound was also added to the BSA protein and allowed to stir for an hour Figure 52.
Post modification, the protein underwent dialysis with a 10-13K MWCO tube, and purification of the protein was achieved with fluorescence remaining of the resulting product (64) despite multiple days of dialysis. HPLC monitoring of the reaction suggested that the protein was labeled (removal of starting material observed) in a similar manner to the bis maleimide linker however, due to the costs of MALDI analysis it was decided that the more stable neoglycoprotein was preferred for such analysis and that the more significant shift of ~846 Da would be easier to determine as glycan labeled (vs a ~523 Da shift). Therefore, this experiment was considered complete, and the results indicated attachment of the desired glycan was achieved but MALDI analysis would be required to confirm the desired product was synthesized.

3.3 Catch and release of glycans

To illustrate further the importance of the soft nucleophilic nature of the thiol present on a fluorescent auxiliary, inspiration was taken from a 2-thiopyridine motif installed onto a Sepharose support [77,101] which was previously utilized as a method to purify proteins through covalent chromatography in low µmol/g loading relative to the support utilized [76]. Preliminary studies into the modification of derivatized glycans with the 2-thiopyridine motif decidedly ruled out a
small molecule-based approach to develop a “Catch and release” or “pull down” procedure (Figure 53). Therefore, an investigation into a similarly activated support seemed prudent where a thiol-linked stationary phase was needed and then could be subsequently reacted in mmol/g levels.

Figure 53: Activation of Sepharose-6B from literature and modified chitosan preparation from literature.

Initial thoughts for derivatizing a thiol-modified support were taken from the process of preparing Sepharose-6B [101] where post-amine functionalization, a subsequent thiol addition could be performed then activation of the thiol could be achieved which also facilitated quantification. Therefore, following a similar route it was suspected that from the published literature [76,77] of functionalizing [102] the amine-abundant chitosan (65) with thiol-glycolic acid utilizing 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), a small modification of the thiol-glycolic acid with dipyridyl disulfide giving 66 could be performed in situ to avoid large scale synthesis. Unfortunately, characterization of the resulting support (67) was found to
be challenging and determination of the degree of attachment was proving problematic resulting in sub mmol/g functionalization with inconsistent results after some initial trials therefore this approach was discarded for the more direct route.

Fortunately, further research into the market availability of a thiol functionalized support resulted in finding one product that could meet the required high degree of functionality for a pull-down approach (mmol/g of resin loading) which was the SiliMetSH silica gel (68) acquired from Silicycle designed for metal removal from crude reactions. Importantly, the thiol content of the silica gel was pre-quantified at 1.54 mmol/g, making it easy to quantify the degree of functionalization and the remaining thiol content in our experiments.

In order to functionalize a large quantity of thiol support (68) on a gram scale and to avoid the issues observed with previous dipyridyl disulfide activations, which involved a significant excess of reagent, an in-situ activation approach was chosen. This involved dissolving the water-soluble reagent in pH 10 water with a molar excess (eq.) and then adding the thiol silica with hydrogen peroxide to facilitate the oxidation of 5-mercaptopyrdine-2-carboxylic acid (MNA) onto the support (68) [103].

![Figure 54: Theorized attachment of 5-mercaptopyrdine-2-carboxylic acid (MNA) to thiol silica gel (68) thiol via hydrogen peroxide oxidation forming 69.](image)

However, this activation method was found to be inconsistent, with significant amounts of MNA washing off from the support despite excess washing with water (~pH 10) and other organic
solvents like DMF. This was likely due to the dissolution of the silica gel (which occurs when the pH is above ~10) and some affinity of MNA via hydrogen bonding from the carboxylic acid of MNA to the silica gel. As a result, a new approach using DPDS as the reagent was developed, as shown in Figure 55.

![Figure 55: Attachment of 2,2-dipyridyl disulfide (DPDS) to silica gel thiol releasing 2-thiopyridine (TP).](image)

After some modifications to the procedure, the reagent MNA was replaced with 2-thiopyridine (TP), which is less soluble and required a change in solvent. The method reverted back to the non-oxidative reaction, using a stoichiometric excess of dipyridyl disulfide (DPDS) relative to the silica gel (68). Despite using a molar excess of the reagent, functionalization of the silica gel remained low, with a value of 0.094 mmol/g determined through UV-Vis absorption at 375 nm of diluted aliquots of the reaction relative to a calibration curve. To increase the reactivity of the solid support, a new method using sulfuryl chloride (SO₂Cl₂) was considered Figure 56.

![Figure 56: Chlorination of thiol support (58) with SO₂Cl₂ followed then by treatment with 2-thiopyrdine (TP).](image)

Sulfuryl chloride was previously studied as a method of preparing symmetric disulfides [104] through chlorination of the thiol (71) and then the formation of the disulfide bond (70). This process was repeated through chlorination of the support with SO₂Cl₂ (10 or 25 eq. in DCM or
neat) and done in excess of reagent to prevent symmetric disulfide formation allowing for the chlorinated thiol to be treated with 2-thiopyridine (2 eq.) in dry DCM which resulted in the desired thiol selective support (70). Rapid chlorination of the silica gel (71) was achieved by performing the reaction neat (slow addition of silica to SO$_2$Cl$_2$) and was proven to be the most effective way to generate the reactive support (70, 1.99 mmol/g vs ~0.6 mmol/g, Table 1) where excess SO$_2$Cl$_2$ could be removed via evaporation.

**Table 1: Optimizing activation of SiliMetSH (70) with various protocols.**

<table>
<thead>
<tr>
<th>Conditions (500 mg SiliMet-SH, 68)</th>
<th>Raw absorbance diluted (a.u.)</th>
<th>Mercaptopyridine adjusted dilution (nmol)</th>
<th>Activated resin (mmol/g)</th>
<th>SH unactivated (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPDS (2 eq.) DMF (10 mL)</td>
<td>2.8393</td>
<td>1.499</td>
<td>0.094</td>
<td>1.446</td>
</tr>
<tr>
<td>DPDS (10 eq.) DMF (10 mL)</td>
<td>1.113</td>
<td>1.395</td>
<td>0.094</td>
<td>1.446</td>
</tr>
<tr>
<td>SO$_2$Cl$_2$ (10 eq.) TP (2 eq.)</td>
<td>2.2265</td>
<td>1.165</td>
<td>0.710</td>
<td>0.830</td>
</tr>
<tr>
<td>DCM (10 mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SO$_2$Cl$_2$ (25 eq.) TP (2 eq.)</td>
<td>2.37</td>
<td>1.243</td>
<td>0.454</td>
<td>0.986</td>
</tr>
<tr>
<td>DCM (10 mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SO$_2$Cl$_2$ (10 eq.) TP (2 eq.)</td>
<td>1.048</td>
<td>0.523</td>
<td>1.994</td>
<td>-0.454</td>
</tr>
<tr>
<td>Neat then DCM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*DPDS = 2,2-dipyridyl disulfide, TP = 2-Thiopyridine.

The resulting chlorinated silica was then treated with TP (2 eq.) and after 10 minutes, the reaction mixture was concentrated and redissolved in DMF for quantification. Preliminary quantification of the support suggested that higher activation of thiols was achieved, where the SH content was determined to be 1.54 mmol/g by the manufacturer compared to the expected 1.99 mmol/g. With this promising result, the overall process was scaled up to 15 g and repeated to produce a large quantity of activated support for testing the "catch and release" of select model glycans. The activated Silimet-SH (70) was scaled up and treated with aqueous washings (sodium sulfite, sodium bicarbonate, and 1M HCl to re-acidify) to remove any remaining chlorine or SO$_2$Cl$_2$ present. The complete removal of chlorine was confirmed by suspending a portion of the
support in water and measuring its pH. The pH was found to become acidic before washing the support, but after washing, it was no longer acidic, which was indicative of the absence of chlorine. Additionally, chlorine test strips were used to verify the absence of chlorine. The support was also washed with an excess of organic solvents (DCM, methanol, and acetone) to remove any unreacted TP. The proposed functionalized silica was then validated by testing lactose-TEAB (9) alongside a direct TCEP reduction. Both tests confirmed an approximate loading of ~0.31 mmol SH/g silica, which was lower than the supposed manufacturer value (1.54 mmol/g) and measured value (1.99 mmol/g) quantified. The lower loading could be attributed to poor swelling of the non-chlorinated support, which prevented access to all activated motifs, or chlorine present on non-thiol functional groups, such as silanols, which promoted oxidation of TP to the dipyridyl disulfide form, indicating higher activation than achieved. Despite the lower loading, the process was deemed successful and feasible for purifying standard reductive amination reactions, requiring less than 1 g of silica. The process can now be applied to crude and pure TEAB glycoconjugates.

Following the process illustrated (Figure 57), it was found that crude reactions could be purified through this method of washing and subsequent release with excess PBu₃ (no change past 30 eq.) although other reducing agents were trialed (TCEP, PPh₃) they were found to be challenging to remove from the glycoconjugates.
The glycans that were released were then precipitated and any remaining organic compounds were removed by washing with dichloromethane. Other solvents such as acetone and methanol were also investigated, but they consistently resulted in poorer yields (~30\%) for the smaller glycans. Most of the product loss was found to occur in the initial immobilization step, where the filtered solvent from the initial "catch" was found to contain the functionalized glycan in the disulfide form (37-40\% of original thiol by mass) due to the presence of 2-thiopyridine. No significant amount of product in the reduced state thiol, CH$_2$ at ~2.75 ppm, was observed in NMR analysis of the initial filtrate, indicating complete capture of the desired thiol. To confirm that activation of the support was the cause of oxidation, lactose-TEAB (9) was suspended with the starting SiliMetSH (68), and the solution was stirred for an hour with no significant oxidation observed. Therefore, some impurity introduced from the generation of the activation (likely SO$_2$Cl$_2$ related) was determined to be the cause. Saturated solutions of EDTA sodium form and maltol described in the literature [105] were applied to small portions of the activated support.

<table>
<thead>
<tr>
<th>Glycan</th>
<th>Yield from isolated glycan</th>
<th>Yield from crude reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-lactose-TEAB (9)</td>
<td>60%$^a$</td>
<td>52%$^a$</td>
</tr>
<tr>
<td>acarbose-TEAB (21)</td>
<td>63%$^a$</td>
<td>58%$^a$</td>
</tr>
</tbody>
</table>

Figure 57: Finalized “Catch and release” of TEAB labeled glycans, $^a$isolated yield.
as a method to potentially remove redox catalyzing species but the effect of both chelators on overall yield was found to be negligible.

Overall, this study presented a novel and highly scalable "catch and release" method for glycan isolation, which resulted in tens of milligrams of purified product. Further exploration of other thiol-containing supports may improve activation or reduce the number of redox-catalyzing species present, leading to further optimization of the process. Although there is room for improvement to increase yields, the efficiency of this process was acceptable as it eliminates a complex chromatography step. Based on the results obtained, this method was deemed satisfactory, and the study was concluded. The process of “catch and release” through a reversible modification to a glycan is highly novel, with no knowledge of this process being achieved with other auxiliaries to the author’s knowledge. Previous Staudinger or “click” based approaches have typically resulted in the desired substrate being permanently attached to the media utilized which have inherent limitations in further studies. Depending on the favorability in the field of glycomics for auxillaries this process of “catch and release” may incentivize other glycochemists to utilize this auxiliary.

3.4. Preparation of thiol-reactive surface

To further demonstrate the utility of the novel TEAB auxiliary, it was necessary to utilize it in the creation of glycoarrays using a thiol-coated surface, showing its superiority over an amino derivative. While previous studies have demonstrated the use of commercially available amine-reactive surfaces that can also react with thiols, simply replicating this method and validating the arrays with glycan-binding enzymes would not fully illustrate the significance of this work. It is important to note that there are limitations to labeling glycans onto coated surfaces, as discussed in the introduction. Specifically, the precision of glycoarray printing is constrained by the ability
to dispense only microliter quantities of substrate onto a surface, which restricts resolutions to below µM levels. Additionally, the cost of conventional automated pipetting instruments can be prohibitively expensive, impacting the overall cost of producing commercial glycoarrays.

In order to improve on previous research and develop a more cost-effective and precise method of generating glycoarrays, a review of previous literature was necessary [84]. This led to the discovery of a small study that also investigated thiol-functionalized small monosaccharides (not through reductive amination). In this study, commercially available monosaccharide derivatives (glucose) were printed onto the surface (73, Figure 58) using UV light to directly generate radicals onto the sulfur present, followed by de-protection of the monosaccharide (72) with base hydrolysis.

![Diagram](image)

**Figure 58:** Published crude glycosylation of glass surface with 1,2-polybutene spacer and subsequent de-acetylation [84].

The article presented a few ways to generate a thiol reactive surface (methyl acrylic acid and 1,2-polybutene, 71) and had various methods to create different thicknesses of coatings with atomic force microscopy defining the morphologies. The work presented what appeared to be a straightforward way to obtain a surface that would facilitate light-mediated printing of glycans.
The procedure was replicated as drawn in Figure 59 with some modifications on the sulfur coating and the final step of introducing the alkene.

**Figure 59: Modified preparation of alkene-coated slides (71) for thiol-ene reaction [84].**

The thiol-coated microscope slides (75) were prepared through the dip coating protocol (described in the supplemental) instead of the spin coating method (described in the main body of the article) with 3-(Trimethoxysilyl)-1-propanethiol (74) and HCl for 24 hrs. This was implemented due to inconsistencies in replicating the spin coating method. When the spin coating of the slides (500 rpm, 30 seconds) was performed with the stated method of “baking” at 150 °C, the uniformity of the coating was visibly irregular with mm differences on the surface (71). This was likely attributed to some erroneous notation in either rpm or time in the spin coating procedure, therefore the “dip coating” method (for 24 hrs) was utilized which resulted in a visually more uniform surface. Iodine starch colorimetric titration was then used to confirm the presence of thiols on the glass slide resulting in similar levels stated by literature (~3.2 μmol/mm²).

The subsequent alkylation of the surface had to also be modified with a radical initiator azobisisobutyronitrile (AIBN) in place of the UV irradiation of the surface due to difficulties in obtaining a similar lamp with the same wattage and UV cut-off. Variations of AIBN (5, 10, 20 % w/v), heat (60, 70, and 80 °C), and solvent (toluene and THF) were investigated to alkylate the
surface. However, inconsistent functionalization would usually occur where half the slide would alkylate which was determined by staining the surface with Lactose-TEAB (9, 0.1 M) and LAP (0.5% w/v) shown in Figure 60.

![Figure 60: Staining of polybutene coated microscope slides (71) a) UV curing of glycans to surface, b) resulting wetting properties of the surface where glycosylated c) UV fluorescence of glycosylated regions.](image)

Inconsistent functionalization was attributed to the density of the 1,2-polybutene (76, ~1.2 kg/mol) where poor stirring would result in a higher concentration in the lower part of the reaction vessel (slides were placed vertically) resulting in partial functionalization of the slide. Another explanation could be partial polymerization of the alkene was also occurring due to large concentrations of initiator being employed. As a side note, photobleaching of the slides would occur if irradiation of the slides (with UV curing torch) lasted longer than 15 minutes which would result in loss of resulting wetting properties of the slide lack alongside the lack of observable fluorescent spots on the surface.

Therefore, it was evaluated that the 1,2-polybutene (76) was a poor choice for the alkene coating of the surface and instead a more biocompatible polymer would be better suited for biological assays to avoid non-specific binding to the surface Figure 61.
Thusly, the polymer was substituted with a common polyethylene glycol (PEG) spacer (1 kg/mol) that was bis allylated by a Williamson ether synthesis with allyl bromide and sodium hydride. The method of generating radicals onto the thiols was also optimized from the dip coating of slides for 24 hrs with UV irradiation to a drop casting approach with AIBN in an air oven at 100 °C. Iodine starch titration was once again used to estimate the consumption of thiols to confirm thiol content had decreased by a half (~1.6 mmol/mm²) which indicated the presence of allyl-containing PEG (77) on the drop casted side of the slide.

3.5 Light-mediated printing for glycoarray generation

The now pegylated slides (77) then underwent glycosylation shown in Figure 62, where fluorescence quantification of the TEAB fluorophore was measured, allowing for the optimization of various parameters: LAP (0.1, 0.5, 1% w/v), time of irradiation (1, 5, 10 minutes), irradiation power (10, 20, 40 mW/cm²) and concentration of glycans 78 (1, 5, 10 mM) which were varied until optimized to the values given in Figure 62.
Utilizing the optimal glycosylation conditions, the quantifiable fluorescence of TEAB was then used to further optimize the PEGylation conditions where the concentration of PEG (76) and AIBN (1, 5, 10, 20% w/v) and time (10, 15, 60, 120 minutes) were varied. It is important to note that no significant fluorescence was observed when PEG and AIBN concentrations were dropped below 5% w/v supporting the fact that thiol-ene addition is occurring on the alkene-PEG (77) rather than disulfide formation to the SH-coated glass.

3.6 Enzymatic validation of glycoarray

To fully establish that the surface was glycosylated, FITC labeled Concanavalin A from Canavalia ensiformis (Jack beans, 1 mg/mL) was then used as a probe as described in the literature [106] to investigate the binding of various glycans present on the surface. A slide of 9 glycans was generated in triplicate spots and a control region of PEG allyl-capped was used to ensure sufficient washing of the surface. The variability in spotting, consistency of the surface, and washing of the FITC Concanavalin A were optimized to minimize the variability of each well on the slide. The slides were pre-blocked with the non-binding protein (BSA) before the addition of the glycan
specific Concanavalin A to ensure non-specific binding to the slide. Fluorescence was then measured of the bound Concanavalin A resulting in the graph below Figure 63.

Figure 63: FITC Concanavalin A binding assay of Glycosylated PEG-coated slides in CFG notation with error bars denoted by 95% confidence intervals (n = 3). Blank is defined as a non-glycosylated region of the slide measured at the same time.

The measurements taken at the blank region contributed an average of 51% (with an instrument error of +/- 273 a.u.) to the error of the samples measured, as assessed by two standard deviations. Despite this, the binding assay showed a significant signal for the primary binding target of Concanavalin A, which is 1,3-α-1,6-α-D-mannotriose (20). The error was low enough to determine that there was a significant, replicable binding event to 20 in comparison to a non-binding monosaccharide such as D-ribose-TEAB (17). Furthermore, partial binding was observed to a variety of glucose-bearing glycans, which have also been reported to display partial binding to Concanavalin A in other literature [84].
In summary, the protocols developed in this study offer an effective method for creating a thiol-reactive surface suitable for photolithography of glycans. This approach improves upon the previous alkene modification method by utilizing a bio-compatible PEG spacer, which provides greater flexibility and distance from the surface. Assessment of the morphology of the surface, consistency, and further characterization of the surface should be performed before implementation of commercial availability to ensure consistency. An additional study could also be performed to demonstrate the precision of the photolithography approach described here as this study elected to avoid using any photomasks to generate the glycoarrays. Indeed, utilizing such an approach would permit gradient functionalization of the surface where multiple glycans could be printed in a defined way allowing a matrix characterization of various glycans to a biological target (either a cellular or an enzymatic study).

3.7 Summary

This chapter discusses several important applications of thiol-derivatized glycans. These glycans were chemically modified at the thiol end, and their characterization was carried out using traditional small molecule synthesis methods. The chapter explores complex methods of neoglycoprotein synthesis, which involve a two-step process of glycan modification to a non-glycosylated protein, followed by MALDI mass spectrometry. Additionally, the chapter investigates the expansion of thiol conjugations into a non-chromatographic purification of glycoconjugates, resulting in a novel methodology of activation of thiol-bearing supports. This protocol enables the purification of crude TEAB-glycans in up to 60% yield without the need for traditional chromatography, using a 5-step procedure.

Finally, the chapter discusses the refurbishment of existing protocols for an alkene-generated surface, using more recent heat/photoinitiators and a more bio-compatible spacer. This
methodology culminates in a diagnostic biological assay, validating the presence of the glycans on the surface of a microscope slide, which implies significant improvements in precision and subsequent cost for the future of glycoarray generation through light-mediated printing.

3.8 Methods for compounds 26-79

3.7.1. Synthesis of lactose-TEAB-maleimidoundecanoic acid (26)

Lactose TEAB (50 mg, 0.0958 mmol, 1 eq.), 11-maleimidoundecanoic acid (27 mg, 0.0958 mmol, 1 eq.), and ammonium carbonate (9 mg, 0.0929 mmol, 1 eq.) were dissolved in water (1 mL) the mixture was then left to stir for half an hour. The reacted product was then concentrated giving the crude product with trace salt impurities; therefore, it was further purified with prep-C18 with a gradient of 99:1 % to 0:100 % (water: acetonitrile), resulting in an overall yield of 26 (58 mg, 76% yield).

$^1$H NMR (500 MHz, CD$_3$OD): $\delta$ 7.33 (dd, $J = 7.8$, 1.4 Hz, 1H), 7.19 – 7.12 (m, 1H), 6.70 – 6.64 (m, 1H), 6.50 – 6.42 (m, 1H), 4.34 (d, $J = 7.6$ Hz, 1H), 3.96 – 3.91 (m, 1H), 3.86 (dd, $J = 8.8$, 3.3 Hz, 1H), 3.81 – 3.68 (m, 4H), 3.65 – 3.49 (m, 5H), 3.46 – 3.38 (m, 3H), 3.38 – 3.31 (m, 3H), 3.27 (dd, $J = 12.9$, 4.6 Hz, 1H), 3.21 – 2.98 (m, 4H), 2.78 (ddd, $J = 13.3$, 7.2, 6.1 Hz, 1H), 2.38 (ddd, $J = 18.6$, 3.5, 2.3 Hz, 1H), 2.09 – 1.98 (m, 2H), 1.43 (dt, $J = 14.6$, 7.2 Hz, 4H), 1.15 (s, 13H). $^{13}$C\{$^1$H\} NMR (126 MHz, CD$_3$OD): $\delta$ 182.1, 179.1, 177.2, 172.2, 150.5, 133.8, 129.3, 117.5, 116.2, 113.0, 105.4, 82.8, 76.9, 74.8, 73.1, 72.9, 72.6, 71.3, 70.2, 63.7, 62.4, 47.1, 39.7(8), 39.7(5), 39.7(2), 38.5,
37.0, 32.3, 30.7, 30.5, 30.2, 28.5, 27.7, 27.4. HRMS (ESI) m/z [M – H]⁻: calcd for C₃₆H₅₆N₅O₁₅S⁻, 802.3432; found 802.3430. [α]D²⁰ - 3.0 (c 0.33, MeOH).

3.7.2. Synthesis of lactose-TEAB-noroborene dicarboxylic acid (27)

Lactose TEAB (50 mg, 0.0958 mmol, 1 eq.), endo-norbornene-cis-5,6-dicarboxylic acid (17 mg, 0.0958 mmol, 1 eq.), and LAP (0.05 w/v %) were dissolved in water (10 mL). The solution was then irradiated at 365 nm light, 10 mW/cm² for half an hour. The crude product was submitted onto prep-C18 with a gradient of 99:1 % to 50:50 % (water: acetonitrile with 0.1 % TFA) yielding product 27 (63 mg, 93% yield).

¹H NMR (400 MHz, D₂O with 0.25% v/v acetone): δ 7.46 (1H, d, J = 7.5 Hz, H15), 7.39 (1H, t, J = 7.9 Hz, H16), 6.87 (1H, d, J = 8.4 Hz, H18), 6.78 (1H, t, J = 7.6 Hz, H17), 4.44 (1H, d, J = 7.7 Hz, H4), 4.14 – 4.04 (1H, m), 3.93 – 3.75 (5H, m), 3.72 – 3.63 (1H, m, H12b), 3.63 – 3.53 (6H, m), 3.53 – 3.32 (3 H, m, H9, 10a, 22), 3.14 (1H, dd, J = 12.8, 8.5 Hz, H10b), 2.91 – 2.75 (4H, m, H21, 25, 26), 2.39 (2H, d, J = 13.5 Hz, H24, 27), 2.08 – 2.07 (1H, m, H23a), 1.62 (1H, d, J = 10.3 Hz, H28a), 1.34 (1H, d, J = 8.8 Hz, H28b), 1.23 – 1.08 (1H, m, H23b). ¹³C{¹H} NMR (126 MHz, D₂O with 0.25% v/v acetone): δ 180.9 9 (C, C29), 175.4 (C, C30), 171.2 (C, C19), 147.3 (C, C13), 132.5 (CH, C16), 128.2 (CH, C15), 118.0 (C, C14), 116.7 (CH, C18), 112.6 (CH, C17), 102.7 (CH, C4), 79.0 (CH), 74.6 (CH), 72.2 (CH), 70.8 (CH), 70.7 (CH), 70.2 (CH), 69.7 (CH, C7), 68.0 (CH), 61.7 (CH₂, C12), 60.3 (CH₂, C6), 50.2 (CH, C26), 48.9 (CH, C25), 46.3 (CH, C27), 45.3 (CH₂, 10), 40.7 (CH, 22), 40.6 (CH, C24), 38.4 (CH₂, C20), 35.9 (CH₂, C28), 34.2 (CH₂,
C23), 30.6 (CH2, C21). HRMS (ESI) m/z [M + H]+: calcd for C30H45N2O15S+, 705.2541; found 705.2527. \([\alpha]_D^{20} + 2.6\) (c 0.28, MeOH).

3.7.3. Synthesis of lactose-TEAB-disulfide hexane (28)

Dipyriddyl disulfide (264 mg, 1.20 mmol, 25 eq.) was dissolved in DMF (1 mL). Lactose TEAB (25 mg, 0.0479 mmol, 1.0 eq.) was dissolved in a separate portion of DMF (1 mL) and the latter mixture was then added to the stirring thiol activating reagent dropwise over a period of 5 minutes. The reaction mixture was then stirred for a further 5 minutes which was then diluted with water (10 mL). DCM (5 mL, five times) was then used to extract any remaining dipyriddyl disulfide reagent. At this point, the activated thiopyridine intermediate can be isolated with prep-C18 gradient of 1 % to 50 % acetonitrile yielding the solid in poor yield (8 mg, 30 % yield). The aqueous layer was then concentrated under air and re-dissolved in DMF (1 mL). Hexane thiol (7 \(\mu\)L, 0.0479 mmol, 1 eq.) was added and left to stir for an hour to generate the disulfide of interest. The crude mixture was concentrated with air and re-dissolved in MeOH (2 mL) and submitted onto prep-C18 with a gradient of 99:1 % to 0:100 % (water: acetonitrile) yielding 28 (15 mg, 49% yield).

\(^1\)H NMR (500 MHz, CD3OD): \(\delta\) 7.46 (dd, J = 7.9, 1.2 Hz, 1H), 7.32 – 7.25 (m, 1H), 6.81 (d, J = 8.1 Hz, 1H), 6.63 – 6.57 (m, 1H), 4.47 (d, J = 7.7 Hz, 1H), 4.10 – 4.01 (m, 1H), 3.95 – 3.81 (m, 2H), 3.79 – 3.69 (m, 1H), 3.62 (t, J = 6.9 Hz, 1H), 3.60 – 3.52 (m, 1H), 3.49 (dd, J = 9.7, 3.3 Hz, 1H), 3.40 (dd, J = 12.9, 4.9 Hz, 1H), 3.28 (dd, J = 12.9, 7.6 Hz, 1H), 2.91 (t, J = 6.9 Hz, 1H), 2.80 – 2.67 (m, 1H), 1.68 (dt, J = 14.9, 7.4 Hz, 1H), 1.39 (dd, J = 14.8, 7.4 Hz, 1H), 1.35 – 1.25 (m,
2H), 0.90 (t, J = 7.0 Hz, 1H). $^{13}$C{$^{1}$H} NMR (126 MHz, CD$_3$CN): δ 170.8, 150.7, 133.7, 128.9, 116.4, 115.6, 112.7, 105.5, 84.6, 76.4, 74.4, 72.8, 72.6, 72.0, 71.4, 70.0, 63.5, 62.5, 46.9, 39.5, 38.5, 32.1, 29.8, 28.8, 23.3, 14.3. HRMS (ESI) m/z [M + H]$^+$: calcd for C$_{27}$H$_{47}$N$_2$O$_{11}$S$_2$$^+$, 639.2621; found 639.2618. $[\alpha]_{D}^{20}$ - 8.2 (c 0.11, MeOH).

3.7.4. Synthesis of lactose-TEAB-acetamide (29)

![Lactose-TEAB-acetamide](image)

Lactose TEAB (50 mg, 0.0958 mmol, 1 eq.), bromo acetamide (13 mg, 0.0958 mmol, 1 eq.), and ammonium carbonate (9 mg, 0.0958 mmol, 1 eq.) were dissolved in water (1 mL). The solution was stirred for 2 hours and submitted onto prep-C18 99:1 % to 50:50 % (water: acetonitrile) yielding 29 (50 mg, 91% yield).

$^1$H NMR (500 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 7.46 (d, J = 7.7 Hz, 1H), 7.42 (t, J = 7.8 Hz, 1H), 6.89 (d, J = 8.4 Hz, 1H), 6.79 (t, J = 7.5 Hz, 1H), 4.47 (d, J = 7.7 Hz, 1H), 4.17 – 4.06 (m, J = 7.4, 4.4 Hz, 1H), 3.99 – 3.80 (m, 5H), 3.72 (dd, J = 11.8, 5.8 Hz, 1H), 3.67 – 3.47 (m, 8H), 3.47 – 3.37 (m, 1H), 3.32 (s, 2H), 3.18 (dd, J = 12.4, 8.5 Hz, 1H), 2.84 (t, J = 6.4 Hz, 2H). $^{13}$C{$^{1}$H} NMR (126 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 176.0, 172.1, 148.3, 133.6, 129.0, 118.5, 117.5, 113.5, 103.5, 79.8, 75.4, 73.0, 71.6, 71.5, 71.1, 70.6, 68.9, 62.5, 61.2, 46.0, 38.8, 34.9, 32.0. HRMS (ESI) m/z [M + H]$^+$: calcd for C$_{23}$H$_{38}$N$_3$O$_{12}$S$^+$, 580.2176; found 580.2179. $[\alpha]_{D}^{20}$ + 1.6 ° (c 0.42, MeOH).
3.7.5. Synthesis of lactose-TEAB-BOC-L-Leucine thioester (30)

Lactose TEAB (50 mg, 0.0958 mmol, 1 eq.), DCC (39 mg, 0.192 mmol, 2 eq.), DMAP (1 mg, 0.00958 mmol, 0.1 eq.), BOC-L-Leucine (44 mg, 0.192 mmol, 2 eq.) was dissolved in DMF (1 mL). The solution was stirred for 12 hrs and then concentrated under air to be re-dissolved in MeOH (2 mL) and submitted onto prep-C18 with a gradient of 99:1 % to 0:100 % (water: acetonitrile) yielding 30 (35 mg, 50% yield).

\(^1\)H NMR (500 MHz, D\(_6\)-DMSO): \(\delta \) 8.39 (t, \(J = 5.4 \text{ Hz}, 1\text{H}\)), 7.88 (dd, \(J = 13.0, 7.9 \text{ Hz}, 1\text{H}\)), 7.59 (d, \(J = 7.8 \text{ Hz}, 1\text{H}\)), 7.49 (t, \(J = 7.9 \text{ Hz}, 1\text{H}\)), 7.24 (t, \(J = 7.6 \text{ Hz}, 1\text{H}\)), 6.69 (d, \(J = 8.4 \text{ Hz}, 1\text{H}\)), 6.51 (dd, \(J = 15.4, 7.9 \text{ Hz}, 1\text{H}\)), 5.57 (d, \(J = 7.9 \text{ Hz}, 1\text{H}\)), 5.12 (d, \(J = 3.5 \text{ Hz}, 1\text{H}\)), 4.79 – 4.65 (m, 3H), 4.65 – 4.51 (m, 3H), 4.51 – 4.39 (m, 2H), 4.28 (d, \(J = 7.4 \text{ Hz}, 2\text{H}\)), 4.10 (d, \(J = 4.5 \text{ Hz}, 1\text{H}\)), 4.09 – 3.99 (m, 1H), 3.83 (s, 2H), 3.79 – 3.61 (m, 6H), 3.61 – 3.42 (m, 8H), 3.17 (ddd, \(J = 17.1, 11.4, 4.9 \text{ Hz}, 3\text{H}\)), 2.98 (dt, \(J = 11.4, 8.0 \text{ Hz}, 2\text{H}\)), 1.65 (ddd, \(J = 12.9, 10.5, 6.6 \text{ Hz}, 6\text{H}\)), 1.50 (ddd, \(J = 28.3, 19.0, 4.4 \text{ Hz}, 5\text{H}\)), 1.33 – 1.19 (m, 5H), 1.19 – 0.97 (m, 3H), 0.84 (dd, \(J = 20.4, 6.5 \text{ Hz}, 6\text{H}\)).

\(^{13}\)C\(^{1}\)H NMR (126 MHz, D\(_6\)-DMSO): \(\delta \) 203.0, 169.1, 156.6, 155.5, 149.6, 132.4, 128.3, 114.6, 113.7, 111.2, 104.3, 82.4, 78.6, 75.4, 73.3, 71.3, 71.2, 71.1, 68.9, 68.0, 62.2, 60.2, 59.3, 47.5, 45.5, 33.3, 28.2, 25.3, 24.5, 24.2, 22.9, 21.0. HRMS (ESI) m/z [M + H]: calcd for C\(_{32}\)H\(_{54}\)N\(_3\)O\(_{14}\)S\(^+\), 736.3326; found 736.3327. \([\alpha]_{D}^{20}\) - 14.6 (c 0.16, MeOH).
3.7.6. Synthesis of lactose-TEAB-butyne (31)

![Chemical structure of lactose-TEAB-butyne (31)]

1-Bromo-butyne (18 μL, 0.192 mmol, 5 eq.), lactose TEAB (20 mg, 0.0383 mmol, 1 eq.), and triethylamine (27 μL, 0.192 mmol, 5 eq.) were dissolved in methanol (1 mL). The sample was heated at 65 °C in a sealed vial and stirred for 6 hrs where TCEP (8 mg, 0.0268 mmol, 0.7 eq.) was added to the mixture to reduce any disulfide formed. The mixture was heated and stirred for a further 2 hrs then concentrated under vacuum and treated with NaOH (1 M, 0.2 mL) and re-concentrated to remove any triethylamine. The crude product was then submitted onto prep-C18 with a gradient of 99:1 % to 0:100 % (water: acetonitrile) yielding 31 (14 mg, 64% yield).

$^1$H NMR (500 MHz, D$_2$O): $\delta$ 7.49 (dd, $J$ = 7.8, 1.4 Hz, 1H), 7.46 – 7.41 (m, 1H), 6.92 (d, $J$ = 8.3 Hz, 1H), 6.81 (t, $J$ = 7.5 Hz, 1H), 4.49 (d, $J$ = 7.7 Hz, 1H), 4.17 – 4.08 (m, 1H), 3.90 (qdd, $J$ = 15.1, 8.5, 3.1 Hz, 6H), 3.73 (dd, $J$ = 11.8, 5.9 Hz, 1H), 3.69 – 3.51 (m, 8H), 3.45 (dd, $J$ = 12.8, 3.9 Hz, 1H), 3.19 (dd, $J$ = 12.8, 8.3 Hz, 1H), 2.87 (t, $J$ = 6.5 Hz, 2H), 2.78 (t, $J$ = 6.8 Hz, 2H), 2.55 (td, $J$ = 6.8, 2.6 Hz, 2H), 2.41 (t, $J$ = 2.6 Hz, 1H). $^{13}$C{$^1$H} NMR (126 MHz, D$_2$O with 0.25% v/v CH$_3$CN): $\delta$ 172.1, 148.2, 133.5, 129.0, 118.7, 117.6, 113.5, 103.6, 84.3, 79.8, 75.4, 73.0, 71.6, 71.5, 71.1, 70.9, 70.6, 68.9, 62.5, 61.2, 46.1, 39.2, 31.1, 30.3, 19.3. HRMS (ESI) m/z [M + H]$^+$: calcd for C$_{25}$H$_{39}$N$_2$O$_{11}$S$^+$, 575.2275; found 575.2276. $[\alpha]^{20}_D$ - 4.4 (c 0.24, MeOH).

3.7.7. Synthesis of lactose-TEAB-propanediol (32)

![Chemical structure of lactose-TEAB-propanediol (32)]
Lactose TEAB (50 mg, 0.0958 mmol, 1 eq.), glycidol (7 μL, 0.0958 mmol, 1 eq.), and ammonium carbonate (9 mg, 0.0958 mmol, 1 eq.) were dissolved in water (1 mL). The solution was stirred for 1.5 hrs, concentrated under vacuum, and submitted onto prep-C18 with a gradient of 99:1 % to 50:50 % (water: acetonitrile) yielding 32 (43 mg, 75% yield).

$^1$H NMR (500 MHz, D$_2$O): δ 7.49 (d, J = 7.7 Hz, 1H), 7.43 (t, J = 7.7 Hz, 1H), 6.92 (d, J = 8.4 Hz, 1H), 6.81 (t, J = 7.5 Hz, 1H), 4.49 (d, J = 7.7 Hz, 1H), 4.21 – 4.10 (m, 1H), 4.01 – 3.80 (m, 6H), 3.80 – 3.53 (m, 12H), 3.45 (dd, J = 12.8, 3.6 Hz, 2H), 3.19 (dd, J = 12.7, 8.3 Hz, 1H), 2.95 – 2.75 (m, 3H), 2.66 (dd, J = 12.7, 3.7 Hz, 1H). $^{13}$C{$^1$H} NMR (126 MHz, D$_2$O with 0.25% v/v CH$_3$CN): δ 172.0, 148.2, 133.5, 129.0, 118.5, 117.5, 113.5, 103.5, 79.8, 75.4, 73.0, 71.6, 71.5, 71.2, 71.1, 70.6, 68.9, 64.8, 62.5, 61.2, 46.0, 39.3, 34.7, 31.8. HRMS (ESI) m/z [M + H]$^+$: calcd for C$_{24}$H$_{41}$N$_2$O$_{13}$S$, 597.2329$; found 597.2324. $[^\alpha]_{D}^{20}$ - 5.2 (c 0.16, MeOH).

3.7.8. Synthesis of thiol-yne bis reaction excess Lactose-TEAB (41)

Lactose TEAB (50 mg, 0.0956 mmol, 10 eq.), 4-pentyonic acid (1 mg, 0.0102 mmol, 1 eq.), and LAP (6 mg, 0.0205 mmol, 2 eq.) were dissolved in water (2 mL). The reaction was irradiated at 365 nm light utilizing a hand torch for 15 minutes, where the crude reaction was purified with prep-C18 ramp 1 to 100 % AcN over 15 minutes yielding a mixture of isomers 41 (42 % yield, 5 mg)
$^1$H NMR (500 MHz, D$_2$O) $\delta$: 7.79 (dd, $J = 21.4, 7.8$ Hz, 1H), 7.75 – 7.61 (m, 1H), 7.57 – 7.36 (m, 2H), 4.52 (d, $J = 7.7$ Hz, 1H), 4.23 (s, 1H), 3.91 (s, 3H), 3.86 (d, $J = 11.7$ Hz, 2H), 3.81 – 3.59 (m, 8H), 3.59 – 3.43 (m, 2H), 3.04 (t, $J = 6.0$ Hz, 2H).

3.7.9. Synthesis of thiol-yne mono reaction excess alkyne reagent (42)

![Mixture of isomers](image)

Lactose TEAB (10 mg, 0.0192 mmol, 1 eq.), 4-pentyonic acid (20 mg, 0.191 mmol, 10 eq.), and LAP (6 mg, 0.0205 mmol, 2 eq.) were dissolved in water (2 mL). The reaction was irradiated at 365 nm light utilizing a hand torch for 15 minutes, where the crude reaction was purified with prep-C18 ramp 1 to 100 % AcN over 15 minutes yielding a mixture of isomers 42 (92 % yield, 11 mg)

$^1$H NMR (500 MHz, D$_2$O) $\delta$: 7.84 – 7.56 (m, 2H), 7.50 (dd, $J = 18.2, 8.1$ Hz, 6H), 6.11 – 5.93 (m, 1H), 5.64 (ddd, $J = 49.1, 15.1, 8.1$ Hz, 1H), 4.44 (d, $J = 7.7$ Hz, 2H), 4.16 (s, 2H), 3.93 – 3.71 (m, 11H), 3.71 – 3.35 (m, 5H), 2.92 (dt, $J = 21.5, 6.4$ Hz, 3H), 2.76 (d, $J = 34.3$ Hz, 2H), 2.47 (d, $J = 4.9$ Hz, 2H), 2.39 – 2.13 (m, 7H).

3.7.10. Thiol-yne vs thiol-ene competition reaction

Lactose-TEAB (10 mg, 0.0192 mmol, 1 eq.), 4-pentyonic acid (7.5 mg, 0.0768 mmol, 4 eq.), endo-norbornene-cis-5,6-dicarboxylic acid (14 mg, 0.0768 mmol, 4 eq.), and LAP (0.05 % w/v) were dissolved in water (2 mL). The reaction was irradiated at 365 nm light utilizing a hand torch and NMR analysis was taken of the crude and the reaction pre-addition of LAP.

3.5.6. Synthesis of 2-thiopyrdine-activated-TEAB- (44)
TEAB-SH (50 mg, 0.255 mmol, eq.) was dissolved in DCM (4 mL) where separately 2,2'-dipyridyldisulfide (281 mg, 1.274 mmol, 5 eq.) was also dissolved in another portion of DCM (1 mL). Dipyridyl disulfide solution was added drop wise to the TEAB solution over 10 minutes and after 30 minutes of stirring the solution were concentrated. The sample was re-dissolved in DCM and loaded onto silica gel (Ø 5 cm, hC 15 cm, VFr 12 mL) the product was eluted with 4 column volumes of isocratic EtOAc: Hexanes (40:60) yielding compound 44 (Rf = 0.3) as a colorless oil (78 mg, 87 % yield).

1H NMR (500 MHz, D6-Acetone) δ: 8.51 – 8.36 (m, 1H), 8.07 – 7.88 (m, 1H), 7.88 – 7.67 (m, 2H), 7.55 (dd, J = 7.9, 1.4 Hz, 1H), 7.21 (ddd, J = 6.2, 4.8, 2.3 Hz, 1H), 7.15 (ddd, J = 8.5, 7.1, 1.5 Hz, 1H), 6.75 (dd, J = 8.3, 0.9 Hz, 1H), 6.55 (ddd, J = 8.2, 7.2, 1.2 Hz, 1H), 6.23 (s, 2H), 3.79 – 3.53 (m, 2H), 3.09 (t, J = 6.6 Hz, 2H).

3.7.11. Reductive amination to 2-thiopyrdine-activated lactose-TEAB (43)

TEAB-modified linker (24 mg, 0.0766 mmol, 1.5 eq.), Lactose monohydrate (18 mg, 0.051 mmol, 1 eq.), and 2-Picoline-borane (7 mg, 0.0612 mmol, 1.2 eq.) was dissolved in the acetic acid: MeOH (1:9) mixture. The crude reaction was heated to 50 °C and left to stir for 2 hrs and the reaction mixture was analyzed with HPLC.

1H NMR (500 MHz, CD6OD) δ: 8.37 (d, J = 4.5 Hz, 1H), 7.83 (d, J = 8.1 Hz, 1H), 7.76 (t, J = 7.7 Hz, 1H), 7.48 (d, J = 7.8 Hz, 1H), 7.30 (t, J = 7.3 Hz, 1H), 7.25 – 7.13 (m, 1H), 6.81 (d, J = 8.4 Hz, 1H), 6.60 (t, J = 7.5 Hz, 1H), 4.59 (s, 1H), 4.47 (d, J = 7.7 Hz, 1H), 4.13 – 3.98 (m, 1H), 3.96
– 3.21 (m, 6H), 3.05 (t, J = 6.6 Hz, 2H). \(^{13}\)C \(_{\text{\`H}}\) NMR (126 MHz, D\(_2\)O) \(\delta\): 171.60, 158.65, 149.15, 148.05, 138.66, 133.24, 128.63, 122.03, 121.45, 117.61, 116.98, 113.06, 103.21, 79.56, 75.09, 72.69, 71.32, 71.16, 70.75, 70.29, 68.59, 62.17, 60.84, 49.00, 45.68, 38.42, 37.88, 0.97.

3.7.12. Synthesis of lactose-TEAB-thioester (48)

\[ \text{Lactose-TEAB} \]

Lactose-TEAB 9 (50 mg, 0.092 mmol, 1 eq.) hexenoic acid (22 \(\mu\)L, 0.188 mmol, 2 eq.), DCC (39 mg, 0.186 mmol, 2 eq.) and DMAP (1 mg, 0.0092 mmol, 0.1 eq.) were dissolved in DMF. The solution was stirred for 7 hrs and then heated at 80 °C for 3 hrs to promote conversion. The crude mixture was concentrated and purified with prep-C18 yielding desired compound 38 (30 mg, 53 % yield).

\(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\): 7.46 – 7.29 (m, 2H), 6.89 (d, J = 8.3 Hz, 1H), 6.77 (t, J = 7.5 Hz, 1H), 5.75 (d, J = 6.6 Hz, 1H), 5.04 – 4.89 (m, 1H), 4.48 (d, J = 7.7 Hz, 1H), 4.10 (d, J = 3.7 Hz, 1H), 3.86 (ddd, J = 19.1, 16.0, 6.0 Hz, 3H), 3.77 – 3.47 (m, 4H), 3.47 – 3.34 (m, 1H), 3.23 – 3.09 (m, 1H), 2.61 (dd, J = 17.5, 10.3 Hz, 1H), 1.98 (dd, J = 13.8, 6.8 Hz, 1H), 1.76 – 1.54 (m, 1H).

\(^{13}\)C \(_{\text{\`H}}\) NMR (126 MHz, D\(_2\)O) \(\delta\): 199.31, 166.36, 142.72, 133.06, 127.86, 123.19, 112.31, 111.64, 109.91, 107.76, 97.80, 74.10, 69.67, 67.25, 65.88, 65.74, 65.34, 64.85, 63.18, 56.74, 55.44, 40.28, 37.53, 33.47, 26.79, 22.86, 19.11.

3.7.13. Attempted native chemical ligation of lactose-TEAB-thioester (51/52)

\[ \text{Lactose-TEAB} \]

\[ \text{H} \]
Compound **48/30** (0.0345 mmol, 1 eq.) was dissolved in phosphate buffer (1 mL, 0.1 M, pH 7.5) and stirred for 2 hrs, after no sign of conversion the sample was heated at 75 °C for an additional 2 hrs. Thiophenol (1% v/v) and TCEP (20 mg, 0.0698 mmol, 2 eq.) and stirred overnight at 75 °C. Starting material underwent hydrolysis when left overnight and no desired product **51/52** was isolated or observed with NMR.

3.7.14. Attempted synthesis of lactose-TEAB-bis allyl PEG (54)

![Lactose-TEAB-bis allyl PEG](image)

Lactose-TEAB (30 mg, 0.0577 mmol, 1 eq.) and di(ethylene glycol) divinyl ether (90 µL, 0.557 mmol, 10 eq.) was dissolved in water. The chosen initiator was then added in 1 eq. and the crude reaction was analyzed with HPLC which consistently yielded various products that could not be separated with prep-C18 chromatography.

3.7.15. Synthesis of MNA-activated lactose-TEAB- (56)

![MNA-activated lactose-TEAB](image)

6,6-dithionicotinic acid (900 mg, 2.78 mmol, 50 eq.) was dissolved in 1M NaOH and pH adjusted to ~10 resulting in a final volume of 20 mL. Lactose-TEAB (20 mg, 0.0372 mmol, 1 eq.) was separately dissolved in water (1 mL) and then added dropwise to the stirring solution of 6,6-dithionicotinic acid. After half an hour, pH was acidified and filtered through a cotton plug to remove the precipitated excess reagent. The filtrated was concentrated and submitted to prep-C18 with a ramp of 1 to 100 % AcN over 15 minutes to yield desired product **56** (92 % yield, 24 mg).
\textsuperscript{1}H NMR (500 MHz, D$_2$O) $\delta$: 8.55 (dd, J = 7.4, 2.0 Hz, 1H), 7.99 – 7.89 (m, 2H), 7.78 (t, J = 7.2 Hz, 1H), 7.64 (t, J = 7.8 Hz, 1H), 6.96 – 6.78 (m, 1H), 4.56 (d, J = 7.8 Hz, 1H), 4.37 – 4.20 (m, 1H), 4.12 – 3.50 (m, 18H), 3.46 – 3.36 (m, 2H), 3.06 (d, J = 26.6 Hz, 1H), 2.90 (d, J = 27.8 Hz, 1H), 2.15 (t, J = 22.6 Hz, 1H), 1.98 – 1.83 (m, 1H), 1.16 (dt, J = 44.2, 7.2 Hz, 1H). \textsuperscript{13}C\{\textsuperscript{1}H\} NMR (126 MHz, D$_2$O) $\delta$: 168.41, 164.67, 147.78, 141.49, 135.45, 134.46, 133.69, 129.50, 129.00, 125.08, 123.84, 116.20, 109.98, 102.82, 78.82, 75.27, 72.47, 71.13, 71.03, 70.42, 68.59, 68.01, 62.00, 60.99, 53.78, 39.49, 36.93, 33.82.

3.7.16. Synthesis of N,N’-[1,2-ethanediylbis(oxy-2,1-ethanediyl)]bismaleimide (60)

![Chemical structure of compound 60.]

Compound 60 was synthesized according to the protocols described in literature, [100] utilizing the following amounts: triethylene glycol diamine 57 (0.96 mL, 6.4 mmol, 1 eq.), maleic anhydride (1255 mg, 12.8 mmol, 2 eq.), triethylamine (0.90 mL, 6.4 mmol, 1 eq.), sodium acetate trihydrate (524 mg, 6.4 mmol, 1 eq.) and acetic anhydride (6.04 mL, 64.0 mmol, 10 eq.). The procedure was modified, where DMF (20 mL) was utilized over the reported acetone solvent for the condensation reaction. The crude product was concentrated and purified with silica gel chromatography over the reported precipitations. The sample was re-dissolved in MeOH: DCM (5:95) and loaded onto silica gel (Ø 5 cm, h 15 cm, V$_{FR}$ 12 mL) the product eluted with isocratic MeOH: DCM (5:95) yielding compound 60 ($R_f$ = 0.3).

3.7.17. Attempted synthesis of Lactose-TEAB-N,N’-[1,2-ethanediylbis(oxy-2,1-ethanediyl)]monomaleimide (62)
Lactose-TEAB (20 mg, 0.0372 mmol, 1 eq.) and separately 60 (600 mg, 1.858 mmol, 50 eq.) were dissolved in DMF (0.5 mL) each. Lactose-TEAB solution was added dropwise to the reaction over 10 minutes and lifted to stir for half an hour, where it was then submitted to size exclusion chromatography (Bio-Rad P-2, fine particle size 45–90 μm; Ø 2.5 cm, h_c 25 cm, V_Fr 20 mL) and eluted with water yielding a colorless solid compound 62 (10 mg, 33 % yield in 30% purity).

^1^H NMR (500 MHz, D_2O) δ 7.41 (ddd, J = 26.3, 15.3, 8.1 Hz, 1H), 6.85 (dt, J = 13.2, 6.6 Hz, 1H), 6.79 – 6.61 (m, 1H), 6.27 (d, J = 12.4 Hz, 1H), 5.90 (t, J = 11.6 Hz, 1H), 4.45 (dd, J = 7.4, 4.3 Hz, 1H), 4.16 – 3.94 (m, 1H), 3.96 – 3.71 (m, 3H), 3.59 (dddd, J = 43.0, 31.1, 16.7, 9.6 Hz, 9H), 3.43 – 3.08 (m, 2H), 3.08 – 2.85 (m, 1H), 2.69 (t, J = 19.7 Hz, 1H), 2.07 (d, J = 6.2 Hz, 1H).

3.7.18. Synthesis of lactose-TEAB-PEG-bismaleimide BSA (63)

BSA (10 mg, 0.000150 mmol, 1 eq.) and N,N’-[1,2-ethanediylbis(oxy-2,1-ethanediyl)]bismaleimide 60 (2.3 mg, 0.00750 mmol, 50 eq.) was dissolved in water (1 mL) the
solution was left to stir for 1 hr. The solution then underwent dialysis with 10-13K MWCO tubing against water (3.5 L) with the dialysate being changed every 12 hrs for 24 hours. Lactose TEAB 9 (0.4 mg, 0.000766 mmol, 5.1 eq.) was then added to the solution and left to stir for an additional hour. The solution underwent dialysis again, placed inside of a 10-13K MWCO tubing, and left to undergo dialysis against water (3.5 L) for a further 3 days with the dialysate being changed every 12 hrs. The product was then lyophilized yielding the synthetic BSA glycoprotein 63 (10 mg) in excellent yield. The final product was analyzed via MALDI to determine connectivity to the desired lactose-TEAB-maleimide connector.

3.7.19. Synthesis of lactose-TEAB-PEG-bismaleimide BSA (64)

BSA (10 mg, 0.000150 mmol, 1 eq.) and activated Lactose-TEAB-MNA (37 mg, 0.0548 mmol, 400 eq.) were dissolved in water (1 mL) the solution was left to stir for 1 hr. The solution underwent dialysis again, placed inside of a 10-13K MWCO tubing, and left to undergo dialysis against water (3.5 L) for a further 3 days with the dialysate being changed every 12 hrs. The final product was not analyzed via MALDI and HPLC analysis confirmed significant removal of any remaining Lactose-TEAB-MNA (56) conjugate, but fluorescence of the solution was maintained suggesting 64 was present.
3.7.20 Attempted activation of Chitosan modified (67) from the literature [102,107]

To the stirring solution of DMF: water (1:1, 10 mL), 2,2-dipyridyl disulfide (300 mg, 1.328 mmol, 1 eq.), and thioglycolic acid (0.08 mL, 1.124 mmol, 1 eq.) was added in rapid succession then allowed to stir at room temperature for an hour. The polymer support was then added in 100 mg quantities along with EDCI (0.5 mL, 4.916 mmol, 3.75 eq.) and further stirred for 3 hours. The polymer support (65) was then washed with the aqueous DMF until the runoff displayed no more fluorescence with a saturated thiol solution. The degree of conjugation was measured by applying a saturated solution of cysteine to the resin support and absorbance was measured with a UV detector.

3.7.21 Attempted activation of SiliMet-SH with MNA (69)

2-Mercaptonicotinic acid (196 mg, 1.26 mmol, 10 eq.) was dissolved within water (20 mL, ~ pH 8-9.0), and SiliMetSH (100 mg, 0.126 mmol, 1 eq.) was added to the stirring yellow solution. H2O2 (0.53 mL) was then slowly added dropwise to the solution until color change was observed. The silica was then filtered off and washed with water (100 mL), DMF (100 mL), methanol (100 mL), and acetone (100 mL). Continuous further washings of the silica still resulted in a visible run of the 2-thiopyrdine, and full removal of the reagent could not be achieved.
3.7.22 Preparation of activated SiliMet-SH (70)

SO₂Cl₂ (25 g, 185.23 mmol, 10 eq.) was poured into a round bottom flask to which SiliMetSH (12.500 g, 19.25 mmol SH content from supplier, 1 eq.) was slowly added to the fuming mixture over a period of 5 minutes. Once completed the silica gel was placed onto a rotary evaporator and heated to 45 °C, reduced pressure was applied until complete removal of any excess SO₂Cl₂. Separately 2-thiopyridine (4.280 g, 38.5 mmol, 2 eq.) was dissolved in dry dichloromethane (150 mL) and slowly added to the dried chlorinated silica. The sample was stirred for 10 minutes and then filtered through a glass sinter, the resin was then washed with dichloromethane, methanol, and acetone. The silica was then further washed with sodium bicarbonate (20 mL) to quench any unreacted chlorine present and re-acidified with 1M HCl (40 mL) and further washed with water (200 mL) until runoff was between pH 6-7. Finally, the activated silica was dried with acetone (200 mL) and dried further under vacuum overnight, yielding the purified activated SiliMet-SH (70, 14.678 g).

3.7.23. Optimized catch and release process

To the solution of recently reductively aminated glycans, activated resin (~ 0.31 mmol of SH/ g of resin) was added to the solution and stirred for 2 hrs, the solution was diluted with water
and then centrifuged for 3000 rpm at 5 minutes which was then repeated with methanol and then acetone. The resin was then recovered and suspended in PBu₃ (10 eq.) with ethanol: water (1:1), where it was stirred for 30 minutes, and the solution separated from the resin via syringe filter (1 µM), the process was repeated an additional two times. The resin was further washed with additional water and methanol then the filtrates combined to be concentrated under vacuum. The yellow crude oil was then precipitated with dichloromethane and centrifuged at 3000 rpm for 15 minutes, the supernatant was filtered through a syringe filter (1 µM) which was then discarded, and additional dichloromethane was added to the pellet and the process repeated a further two times. Finally, the precipitate was dissolved in a minimal amount of water and syringe filtered (1 µM) into a scintillation vial to which the solution was concentrated, yielding the desired product.

3.7.24. Example catch and release of acarbose TEAB

Following the optimized procedure, a crude reaction of the reductively aminated acarbose TEAB (21, theoretical yield: 64 mg, 0.0776 mmol, 1 eq.) was added to the activated SiliMet-SH (70, 250 mg). The suspension was left to stir for 2 hrs which was then placed into a centrifuge tube, the suspension was diluted up to 15 mL with water and centrifuged at 3000 rpm for 5 minutes. The supernatant was decanted, and the process was repeated with the same volume of methanol and acetone. The resin was recovered back into a scintillation vial then PBu3 (157 mg, 0.775 mmol, 10 eq.) was added and diluted with water: ethanol (1:1, 5 mL). The suspension was stirred vigorously for 30 minutes, and the solution was back-filtered with a PTFE syringe filter (1 µM), this process was repeated 3 times during which the resin was washed with water (10 mL) and methanol (10 mL) then filtrates pooled together and concentrated until dry. The resulting yellow oil was then diluted with dichloromethane (50 mL) and once again centrifuged at 3000 rpm for 15 minutes. The supernatant was then discarded, additional dichloromethane (30 mL) was added to
the pellet and the process was repeated a further two times. The pellet was dissolved in water (5 mL x 3) and filtered through a PTFE syringe filter (1 μM). The filtered solution was dispensed into a scintillation vial which was then concentrated under rotary evaporation to yield the desired acarbose TEAB (21, 37 mg, 0.0450 mmol) in 58 % yield.

3.7.25. Preparation of bis-allylated PEG 1000 (76)

Following literature [108] PEG 1000 MW (10.000 g, 10 mmol, 1 eq.) was added to a 3-necked round bottom flask and flushed with nitrogen, to which THF (100 mL) was added and stirred until dissolved. To the stirring solution NaH (60% in oil, 1.3 g, 55 mmol, 5 eq.) was slowly added to the solution and then allyl bromide (8.64 mL, 100 mmol, 10 eq.) was added dropwise. The reaction was then heated at 40 °C with additional portions of NaH (5 eq.) being added every 30 minutes until a total of 15 eq. was reached. After 12 hrs the solution was left to cool and then concentrated to obtain the crude product, which was then re-dissolved in DCM (30 mL) and filtered. Again, the sample was concentrated to dryness and the crude solid was then washed with hexanes (200 mL) three times to remove mineral oil leftover from the NaH yielding the diallyl PEG 1000 MW 76 (8.734 g, 7.74 mmol, ~95% purity) 77% yield with trace mineral oil by NMR.

$^1$H NMR (500 MHz, CDCl$_3$): δ 5.94 – 5.82 (m, $J = 10.4$, 7.1, 4.1, 1.5 Hz, 2H), 5.25 (d, $J = 17.2$, 1.5 Hz, 2H), 5.15 (d, $J = 10.4$ Hz, 2H), 4.08 – 3.90 (m, 5H), 3.80 – 3.42 (m, 76H). $^{13}$C {$^1$H} NMR (126 MHz, CDCl$_3$): δ 134.87, 117.18, 72.32, 70.70, 70.61, 69.50.
3.7.26. Preparation of allyl PEG microscope slides (77)

Microscope slides were acquired from Fischer scientific, and the surface was prepared according to the literature [84] where fresh piranha solution was applied to the glass surface for 1 hr and then the surface was washed and dried overnight under vacuum. The slides were then taken and left in a solution of MPTMS (5% v/v) in THF with HCl (0.4% v/v, 10 mL/slide) which was then left for 24 hrs under nitrogen. The dip-coated slides were then removed from the solution and sequentially washed with DCM, toluene, hexanes, acetone, and dichloromethane again. The slides were then dried under a vacuum to which a portion was taken, and the thiol content was estimated utilizing the iodine and starch colorimetric indicator which approximated around ~ 3.6 µmol/mm². A saturated solution of divinyl PEG 1000 MW (10% w/v), AIBN (10% w/v) in toluene (9.23 mL) was then prepared and a portion of the solution (1 mL) was deposited onto the thiol glass slide in a culture dish. The slide was then ‘baked’ in an air oven at 100 °C for 1 hr, the slide was then removed and sequentially washed with an excess of toluene, dichloromethane, methanol, water, and acetone. The now pegylated slides (77) were then utilized for glycosylation and free thiol content was estimated to have decreased to ~1.6 µmol/mm² with the iodine starch colorimetric assay.
3.7.27. Glycosylation of PEG-coated microscope slides (79)

To the pegylated slides, a solution of the glycan of interest 78 (10 mM) and LAP (1 % w/v) in water was dispensed (2 μL) onto the surface. The solution was exposed to UV light (365 nm, 10 mW/cm²) for a period of 1 minute which was then washed away with excess water, methanol, and acetone leaving fluorescent spots when viewed with a UV flashlight (365 nm). The slide was then dried under a vacuum resulting in the prepared glycosylated surface (79)
CHAPTER 4

ISOLATION OF GLYCANS FROM NATURAL SOURCES

4.1 Chemical release of TEAB glycoconjugates

As described in the introduction, there are several ways to chemically release glycans from natural sources, but only a select few can achieve this in a non-destructive way to yield complex-reducing glycans. To condense everything displayed in Figure 8, there are only three common methods still utilized in modern literature PNGase F, hydrazine, and at the time the recent reagent bleach (NaClO, [47]). PNGase F is commercially available however it is cost-inefficient to prepare mg quantities of reducing glycans utilizing this method which also would not work on fucosylated glycans at the reducing end. This thus leaves two practical options to chemically release glycans from complex mixtures: hydrazine, which will not work if molar equivalencies of water are present, and bleach, which has been suggested to be extremely flexible and efficient at chemically releasing glycans, requiring ~0.6% v/v of NaClO in reactions.

This realistically left only one option to chemically prepare complex-reducing glycans from biological mixtures, as obtaining glycoproteins (in lyophilized form) in milligram quantities is cost-prohibitive. To reliably utilize the bleach protocol described by Cumming et al, it was decided that a better understanding of the limitations of the chemical release was necessary. The mechanism presented within the initial article (Figure 10) was a best guess based on previous work so it seemed prudent to determine if this reagent did indeed work and if it could be performed on other glycoconjugates. Therefore, the described bleach protocol was implemented onto the lactose-TEAB (9) glycoconjugate to ascertain if this protocol did work and to address the discrepancies between the NaClO and NBS release as (NBS) presented truncation (3:1 ratio) of glycans while the other (NaClO) did not.
The glycoconjugate (9) was released according to the bleach protocol where HPLC indicated complete removal of the starting material. The crude reaction was quenched with formic acid (described by literature) and was concentrated to remove any volatile by-products present. The reaction was then re-reductively aminated with TEAB-SH (1, 5 eq.) utilizing the optimized conditions and purified to result in a ~12.5% yield recovery of the truncated glycan with no significant observation of the original glycan Figure 64.

Figure 64: NaClO release of lactose-TEAB (9) followed by subsequent reductive amination with TEAB-SH in excess.

This preliminary release and subsequent reattachment looked promising and indicated acceptable recovery even though the linkage was no longer an amide bond to a peptide and resulted in truncation (80). Interestingly, this release directly disputed the mechanism put forward in the bleach protocol release. Suggesting that the removal of the amine/amide is on the glycan side reforming the reducing sugar rather than a beta elimination with the peptide previously discussed. This implies that the amine is being halogenated as shown but is then suitably unstable leaving group which permits ring formation. Therefore, the reaction was repeated once again, this time using a structurally closer analog to N-glycans, namely N-acetyl-lactose amine-TEAB (19), as shown in Figure 65.
In agreement with 9, the presence of the NHAc group on the C2 position introduced less truncation in the final product isolated where a mixture of products (19+80) was observed shown in Figure 66.

This corroborates the previous reaction as the NHAc group is a more stable group and is seldom reactive unless under basic conditions which would presumably result in less truncation and higher isolation of the desired product. Unfortunately, as the mixture of products was at 4.7 minutes with less than 10-20 second shifts between all three peaks, it proved too challenging to determine the exact ratio of products forming even with co-injection of starting material did not
assist in peak identification. To disentangle the mixture of products (shown in Figure 67), it was necessary to implement HILIC chromatography.

![Figure 67: NaClO release of N-acetyl-lactose amine-TEAB (19) followed by subsequent reductive amination. HPLC HILIC chromatogram of crude reaction (90% to 60% AcN in 20 minutes).](image)

A linear ramp decreasing from 90% to 60% (can) was adequate to separate the two major isomers (peaks at 7 and 8 minutes), with excess TEAB eluting rapidly (peak at 3 minutes). The mixture was then separated into three fractions through repeated analytical injections (100 µL) until the entire sample (2 mL) was separated (shown in Figure 68).

![Figure 68: NaClO release of N-acetyl-lactose amine-TEAB (19) followed by subsequent reductive amination, “analytical preparative” HILIC chromatogram of crude reaction (90% to 60% AcN in 20 minutes).](image)

Fractions ranged from; 5-7 minutes, 7-10 minutes, and 10-12 minutes with the initial and post-filtrate discarded (C18 chromatography was used to confirm no product present). The samples were concentrated and then submitted for analysis with mass spectrometry alongside NMR
analysis ($^1$H, $^{13}$C($^1$H), DEPT, HSQC, HMBC, TOCSY, DOSY, and COSY) to assign their respective identities.

Overall, it was found that there were only two products in a significant ratio between starting materials 19 and 80 (75:25 respectively by HPLC) that had formed with a worse overall yield (<10 % yield). Due to less than a milligram quantity (including both products) recovered an exact yield could not be determined due to the error in weighing. Truncation was likely a preferred pathway for this mechanism with a better-leaving group when not acetylated (9) which would explain the comparatively worse yield (19). Despite the poor yields and significant degree of truncation (which was not mentioned in the original literature) being observed, it was tentatively decided that this could simply be attributed to the fact that a more stable secondary aromatic amine bond was being broken vs the less stable amide bond to a peptide. Which could explain the inconsistencies that were being observed in the literature. Therefore, to fairly evaluate the NaClO release of glycoproteins, an exact substrate that could match the literature had to be obtained.

4.2 Natural product isolation of Sialylglycopeptide (SGP) from egg yolk

To render an accurate assessment of the NaClO-mediated release of glycans from biological mixtures, an unequivocal substrate had to be studied. Although there are various commercially available glycoproteins, they are not cost-effective to procure, and quantifying yields would not be accurate. The only glycopeptide that is relatively easy to obtain is the “poster child” of glycomics: the Sialylglycopeptide (SGP). This glycopeptide is highly abundant in hen eggs, specifically in the egg yolk. Numerous studies have previously investigated various natural product isolation methods to obtain hundreds of milligrams of SGP however one protocol stood out as superior in the literature [87]. It was the most recent protocol in the isolation of SGP and appeared to be the most effective due to the high yields given (0.8 mg/g of powder) and the ability
to utilize egg yolk powder (dried, in kg quantities) where other protocols had to lyophilize egg yolks (in sub-kg quantities). The paper stated that spray-dried egg yolk powder was suitable for the isolation of SGP and that despite heating between 120 – 150 °C SGP (81) glycans remained intact from this process. Overall, this process (shown in Figure 69) would save time and energy intensive drying which would therefore expedite the whole process.

**Figure 69:** Previously published [87] the isolation of SGP (81) from spray-dried egg yolks in a 4-step process.

The protocol for SGP (81) isolation was relatively straightforward with much of the isolation process focused on washing with ethanol for the removal of lipids and fats (composes the majority of egg yolks by weight). This was then followed by an aqueous ethanol extraction (40 %) of the SGP compound from most organics and the dried cake that would be discarded. This process was developed through LC-MS analysis of filtrates which determined when SGP was eluting out in the washings. Once completed the ethanol (aq.) filtrate was dried and submitted for a non-traditional chromatography step required which involved celite packed with activated carbon (1:1) where the carbon acts like a filler reducing the back pressure generated from pushing solvent through the column. The celite (diatomaceous earth) present is a sedimentary rock that originates from fossilized remains of hard-shelled algae, diatoms where an SEM image of the porosity is shown in Figure 70.
Figure 70: Scanning electron microscopy (SEM) image of celite [109] alongside an image of the celite column used.

The theory of how celite works as a filtering aid is that the porous silica particles (ranging from 3 µm to millimeter size) can swell when solvated, which allows for the entrapment of the analyte of interest (in this case, SGP 81). The column was run with trifluoracetic acid (TFA) acidified aqueous acetonitrile (0, 5, 10, and 25% v/v with 0.1% v/v TFA) where fractions (40 mL) were collected of the final runoff. In the protocol described, the procedure utilized LC-MS to determine which fractions contained the glycan of interest, unfortunately, due to lack of access to expensive instrumentation a more innovative way had to be utilized to determine when the glycan of interest was eluting. Fortunately, it was found that the phenol-sulfuric acid assay was an effective colorimetric assay that had been previously established and specifically utilized in the quantification of carbohydrates Figure 71.

Figure 71: Proposed dehydration of carbohydrates with H₂SO₄ followed by phenol addition to generate 485 nm absorbing species by literature [110].
According to literature [110], carbohydrates can be quantified using the protocol described above Figure 71. First, \( \text{H}_2\text{SO}_4 \) is used to dehydrate carbohydrates, resulting in the formation of a furan species (82). This furan species can then undergo nucleophilic addition at the generated aldehyde. The resulting species (83) has a high absorbance of 485 nm, enabling the quantification of carbohydrates down to the nanomolar level. This method was used to visualize glycan-containing fractions, and was even applied to the initial washings of the column to ensure that no desired glycans were being removed. After selecting the desired fractions, the final step involved size exclusion with P2 media. The phenol sulfurous acid assay was then utilized again on the subsequent size exclusion of the glycaN-containing fractions shown in Figure 72 and the fractions of interest was recovered.

![Relative absorbance at 485 nm vs fraction number](image)

**Figure 72:** *Phenol sulfurous acid assay of size excluded fractions after celite column to isolate SGP.*

The product was characterized by NMR and was found to have an overall yield of \( \sim 1 \) mg, far short of the predicted yield of 364 mg from using 0.5 kg of egg yolk powder. The resulting NMR of the obtained product was highly disappointing where no distinct peaks were observed and
were essentially noise. To ensure that no mistakes were made during the process, the procedure was repeated (2 more times) which took multiple months to dry and isolate filtrates as well as multiple days to run the activated carbon:celite column consistently resulting in no significant amount of material. LC-MS analysis was performed on the isolated extracts which did suggest the presence of the SGP (81, peak observed with 1903 g/mol) just not in the stated yields or purity.

Troubleshooting of the published protocol was then undertaken, where the unconventional celite:carbon column was scrapped and replaced with a Trypsin digestion step. Trypsin is an effective lysine/arginine digestion enzyme that is commonly used in biological studies and is widely commercially available (liters of enzyme can be bought). It is an extremely robust enzyme and has been well characterized in other studies with high catalytic turnover for the hydrolysis of peptides.

![Figure 73: Phenol sulfuric acid assay of size excluded fractions after Trypsin digestion to isolate SGP 81.](image)

The concentrated aqueous ethanol filtrates were treated in an excess of the enzyme and then concentrated again which were submitted to size exclusion with a P2 and G25 column due to
the amount of crude material present. This attempt did successfully result in some product isolated but was interestingly not the desired 81 but rather one of the smaller pre-coursers which is known to be in egg yolk but not in as high of quantity relative to 81. This was determined through NMR data of the resulting compound shown below in Figure 74.

![Figure 74: HSQC NMR with suspected G1 glycan (84) isolated utilizing size exclusion of Trypsin digestion of crude aqueous ethanol extracts from egg yolk powder. Highlighted in red circles are anomeric carbons.](image)

Despite being structurally similar to 81, the G1 glycan (84) is smaller and missing the important sialic acids that contribute to some key glycan binding interactions. The tentative identity was assigned by examining NMR data from the bleach paper [47] which resembled the NMR that was assigned to the G1 glycan (84). ESI mass spectrometry was also taken of the compound isolated and two large peaks with the corresponding m/z of 1547.9 and 1569.9. The proposed structure was not far off with an m/z of 1505 g/mol but still had a significant shift of 42-54 g/mol which could be accounted by salt impurities present in the sample or perhaps degradation.
as acetyl groups did not appear to be present (~2.0 ppm). Either way, the isolated compound did not corroborate the literature [87] but it did confirm that the celite column was not the issue as SGP was not present. Instead, the data suggested that a significant change to the structure of the glycan had occurred. The most likely explanation for de-sialylation/de-glycosylation occurring was attributed to the spray drying process that was used to prepare the egg yolk powder which contradicted the paper’s claim that egg yolk powder is a viable source for SGP isolation (no specification on the exact brand).

To verify the theory that spray drying was causing the issue, the entire protocol had to be repeated one final time using fresh hen egg yolks instead of the spray-dried powder. Therefore, 72 medium eggs (Eggland brand, not free range) were purchased from the local store. The egg yolks were separated from the whites and then dried using rotary evaporation at 65 °C, with the anti-foaming agent 1-octanol used to prevent foaming and bumping. After complete removal of water from the foaming solid, the SGP isolation procedure was attempted again on the dried egg yolks, though significant amounts of 1-octanol remained.

Gratifyingly, the protocol did finally work which yielded SGP in a 318 mg yield (81, 4.4 mg/ egg yolk) out of the theoretical 364 mg, the 48 mg difference in yield could be attributed to variation in eggs and the number of yolks per gram of spray dried powder which again can vary (~36 yolks/336 g of powder). The NMR spectrum in Figure 75 of the resulting product was satisfactorily identical to the published NMR of the SGP isolated in the literature [87].
Three distinct features of this NMR show that it is indeed a complex glycan near the D$_2$O peak (~ 5.0 ppm), at least 5 distinct anomic protons (protons at the ether linkage between glycans) can be shown in this region alongside characteristic peptide peaks between 1.7 ppm – 0.8 ppm. Another key characteristic is the asparagine $\beta$ proton at ~2.5 ppm (doublet of multiplets), these NMR signals (determined by literature [87]) must be removed to be able to determine liberation of the N-glycan as these are the protons adjacent to the amide connection. Finally, the important acetyl methyls can be observed (~2.0 ppm) which integrate to approximately 19 hydrogens just over the 18 hydrogens present in SGP which can be accounted for by some slight over-integration. It is important to note that the material is not entirely SGP (81) and there is the presence (~10%) of other similarly structured glycans which is characterized in Figure 69.
In conclusion, this study reaffirms previous findings that spray dried egg yolk powder is not a suitable source for obtaining large quantities of SGP (81). However, the published protocol was found to work as described by the authors, with a substituted method for glycan detection using a colorimetric assay to identify the desired glycans. Moreover, it is likely that substituting spray dried egg yolks with freeze-dried egg yolks would resolve the inconsistency of this process and provide a shelf-stable alternative.

4.3 Bleach oxidative release of biological samples and SGP

Now in hand with a model example of an N-glycan, testing of chemical release was required. Previous testing of the bleach protocol had indicated some significant issues surrounding the chemical release of glycans that were not described in the paper. Where truncation was significantly observed on non-acetylated glycans along with partial truncation of N-acetyl-bearing glycans (3:1 ratio). Additionally, overall yields were concerning where 15% or less of the sample was recovered when treated with bleach, it was unknown if the glycans are being degraded but it was known that the fluorescent signal for the TEAB fluorophore was no longer present. This implies that either a significant portion of glycans was undergoing chemical peeling (pathway is indicated in bleach release Figure 10) or that the fluorophore is undergoing an elimination where it remains attached to the glycan but loses the electronics that result in its observability by UV-Vis (possible halogenation of amide bond followed by cleavage).
The isolated SGP was now released with NaClO, utilizing the described protocol for bleach release of proteins as described by literature[47]. A crude sample of the reaction was taken and analyzed by NMR. Below in Figure 77, is the crude quenched reaction of SGP (81) treatment with NaClO utilizing the described protocol for glycoprotein release.

Figure 76: Theorized bleach release of SGP (81) followed by reductive amination of glycan (85).

Figure 77: $^1$H NMR of NaClO treatment (~6 eq.) of SGP (81).
Established earlier as the peptide region (1.7-0.8 ppm) NaClO treatment resulted in shifts occurring where the doublets previously observed have now broadened and are no longer clear signals. Diagnostically the peak within this NMR was the asparagine amide β proton at ~2.6 ppm, the removal of this peak is crucial to determine the liberation of the N-glycan to allow for reductive amination. Although broadened, it is challenging to say if the peaks have been “removed” due to the overlap of adjacent peaks therefore the sample was recovered, and the next step of the protocol was carried out.

Subsequently, the sample was treated with the optimized reductive amination conditions using TEAB (1). However, only partial reductive amination of the glycan was observed, resulting in the formation of a broad peak at approximately 3 minutes when a standard 1% to 100% AcN ramp was used. Due to the high levels of salts and surfactants present, the compound was not retained in prep-C18 and eluted into the dead volume.

To purify the compound and evaluate the degree of reductive amination, G25 media was used with water as the eluent for size exclusion chromatography. This resulted in the NMR spectra shown below Figure 78 with trace amounts of reductive occurring.
Once again, the bleach oxidative release method appeared to have failed, as less than 5% of the reaction was undergoing reductive amination, while the majority of the sample was not. However, some significant changes were observed in the NMR, including the movement of anomeric peaks (~5.0 ppm) and degradation of most peptide peaks (1.7-0.8 ppm).

The experiment was repeated several times, with variations in the quantities of bleach used (6, 9, and 20 eq.), type of bleach (ACS grade or Clorox), the quenching agent used (formic acid or sodium thiosulfate), and increased ratios of reagents for reductive amination. Despite these modifications, the NMR results remained largely the same. There was little reductive amination of the SGP, despite utilizing a molar excess of reagents, and higher degradation was observed when significant excess (20 eq.) was used. An additional experiment was performed on N-acetyl-glucosamine (86) to help determine if chemical peeling could be caused by the NaClO present.
N-acetyl-glucosamine was treated as if it were a glycoprotein and treated with bleach followed by quenching and NMR analysis (shown in Figure 79) of the mixture.

Figure 79: \(^1\)H NMR of NaClO treatment of N-acetyl-glucosamine (86).

This yielded significant chemical peeling of the starting material which can be observed in the NMR above, notably shifts in the acetyl region (~2.0 ppm) displayed at least 5 side products being generated from peeling generating uncertainties in the bleach release process.

Although successful experiments had been conducted on a model glycopeptide and N-acetyl sugar, it remained challenging to determine whether the failure of the bleach oxidative release method was due to the samples' characteristics. The samples used in the study were considerably more diluted, contained a higher number of amide linkages, and other biological species that could have hindered or slowed down the chemical cleavage of the attached glycan. As a result, a final series of experiments was necessary to evaluate the bleach protocol accurately.
To accomplish this, select biological samples were chosen, including egg yolks, whites, and yeast. The bleach protocol had already been used to characterize two of the samples, egg yolks and whites, and defined NMR of the isolated glycans was available. The third sample, yeast, had its complex glycan profile previously assessed in other studies, revealing it to be high mannose bearing with two N-acetyl bearing glycans. Consequently, the study had three well-characterized samples that, if NMR of the glycans could be obtained, would enable straightforward assessment of whether the correct complex glycans had been isolated. The process of bleach release of glycans from biological samples is a direct method that is outlined in Figure 80.

![Figure 80: Bleach oxidative release of glycans from biological materials in CFG notation followed by reductive amination [47].](image)

The samples are subjected to a process that involves treating them with dilute bleach, quenching with formic acid, dialysis (1K MWCO) for 12 hours, and concentration. This process is repeated twice, yielding crude glycans that can be reductively aminated.

After applying the bleach release protocol, three samples of glycans were obtained from egg yolks, whites, and yeast, with yields ranging from 0.8 to 5 g. The NMR spectra of these samples appeared (shown in Figure 81) to be closely correlated to their respective sources, indicating successful chemical release and purification of the glycans. However, it is important to note that potential impurities may not have been accounted for in the reported yields.
Figure 81: $^1$H NMR of bleach released complex glycans isolated from egg whites (87), egg yolks (88), and yeast (89).

Three crude glycans were isolated from the bleach release method which seemed to be adequately pure for reductive amination however there was a notable presence of peptides around 2.5 ppm and 1.25 ppm with the yeast-released glycans looking the purest out of the three samples (87-89). The glycans were then submitted to reductive amination once more, but unsurprisingly once size excluded resulted in similar spectra as shown in Figure 78. Where TEAB fluorophore present in the samples was not consistent (extremely low signal or in excess/broad) and could be significantly removed (<5 % functionalization) once submitted to dialysis. This resulted in a problematic conclusion, where NaClO did facilitate the isolation of crude glycans, but they would not undergo reductive amination even if they were submitted for further NaClO oxidation. Attempts to utilize the "catch and release" approach with the activated SiliMetSH support to isolate trace amounts of product yielded some glycans, but the yields were low (<5%) and only resulted
in single milligram quantities. The glycans obtained were typically impure, with varying NMR signals that did not fully correlate with the respective starting materials.

Overall, it was finally concluded that bleach oxidative release was not an appropriate method for breaking the amide bond present in N-glycans with several experiments on glycoconjugates indicating discrepancies with the proposed mechanism. It is presumed that the isolation of the complex glycans shown in

**Figure 81** could be achieved, as the glycans were not as reactive to the bleach oxidation compared to proteins and other biological species present. This would likely leave carbohydrates greater than 1 kg/mol (from dialysis purification), but they may have suffered some chemical peeling from the process as shown with TEAB glycoconjugates and N-acetyl-glucosamine. During the time of this study, another article was published[111], which also displayed similar results to the conclusions drawn here. The observation of NaClO-related degradation of the acetyl (86) residue and overall poor release (20-fold less than PNGaseF) corroborating the findings presented here.

### 4.4 Hydrazinolysis of complex glycans

Ungratifyingly there was now only one approach left, hydrazinolysis [112] which is highly sensitive to moisture where the presence of molar quantities of water can degrade the glycans of interest. Hydrazinolysis is applied to dilute samples (10 mg glycoprotein/mL of hydrazine) with the hydrazine mixture heated between 6-24 hrs at 95 °C (under nitrogen) and then evaporated. Due to the explosive risk with hydrazine, many researchers elect to avoid utilizing significant quantities (greater than single mL’s) to mitigate compression and combustion of the vacuum pump. To remove this risk, inspiration was taken from the previous SGP protocol where precipitation with
ethanol was applied to the sample which was diluted (ethanol: N\textsubscript{2}H\textsubscript{4}, 9:1) to precipitate liberated glycan. After centrifugation, and the pellet recovered, the now de-acetylated glycans were re-dissolved in water with sodium bicarbonate (sat.) and acetic anhydride (10 eq.) was added. Re-acetylation was important to re-introduce the acetyl groups on the now de-acetylated glycans which yield the conserved reducing sugars. The sample was then dialyzed with 1K MWCO tubing over multiple days (with water as the dialysate) to ensure complete recovery of the glycan which was then submitted for reductive amination with TEAB. Dialysis was repeated with TCEP and disodium EDTA in the initial dialysate to ensure the presence of thiols as well as the removal of any metal species present, the subsequent dialysates had no additives afterward.

Hydrazinolysis was applied to SGP (81), and the bleach released samples (87-89) where out of the four samples, only one sample with significant TEAB labeling had reacted, which was the more carefully isolated SGP glycan. The TEAB peaks appeared to be present at 7.5 and 7 ppm (correlating with previous TEAB glycoconjugates) within Figure 82.
Figure 82: $^1$H NMR of SGP (CFG notation included) that has undergone hydrazinolysis and subsequent reductive amination (85) with internal standard tBuOH.

However, there is some broadening across all peaks in the sample due to either molecular levels of water or salt present. Notably, the asparagine peak (at 2.5 ppm) appeared to have been removed with some slight increase in integration of the acetyl region (~26H) relative to the aromatic region. The acetyl region should integrate at around 18 hydrogens (like Figure 75) suggesting that complete reductive amination of the sample may not have been obtained which could have been caused by the incomplete release of the SGP (81, hydrazine release is typically between 75-90 % efficient). Recovery of the reaction appeared to be high with the isolation of 12 mg of sample (20 mg of starting SGP) which translated to an acceptable 70% yield (85).

Unfortunately, the bleach samples (87-89) did not proceed as cleanly and did not have consistent TEAB peaks although they were more significant than utilizing NaClO (functionalization varied from sample to sample). Bleach-released egg yolk (88) sample did match closely to the NMR in Figure 82 but the integration of TEAB signals was lower (1:40 aromatic: acetyl group). Overall, it is again indicative that the bleach protocol may have resulted in chemical peeling on most of the samples which would track with the previous experiments where truncation and peeling were found.

Now tentatively assigned TEAB SGP (85) glycan should still be further investigated, where purification away from any non-labeled glycan can be achieved utilizing the “catch and release” method developed previously or potentially chromatographically (HILIC/C18). Mass spectrometry of the glycan must also be performed to confirm the assigned identity to enable its future use in biological assays. Usage of the bleaching protocol to remove proteins may still yet be a viable method but alternative approaches to more glycaN-sensitive isolation should be considered. Precipitations utilized by the protocol [87] appear to apply to a wide variety of glycans
(if bearing small peptide fragments) however digestion of larger protein-linked glycans would be necessary to permit the aqueous ethanol separation. In conclusion, hydrazine appears to still be the most efficacious chemical-releasing reagent for glycans but its sensitivity towards the presence of water and subsequent removal still requires improvement.

4.5 **Hydrolysis of Sodium hyaluronate**

Isolation of additional complex glycans that poses utility in future biological experiments such as glycoarrays and investigation of commercially available GAGs was considered. Research surrounding GAGs is promising with significant findings around the presence of these glycans and their effects on cellular identity. Previous studies have investigated acid hydrolysis on GAGs, but the focus was shifted towards this specific study involving sodium hyaluronate (HA) to prepare smaller oligomers **Figure 83**[113], the investigation had an exceptional performance in studying the rate kinetics of the acid digestion with NMR and SEC analysis of corresponding oligomers.

![Sodium hyaluronate](image)

**Figure 83:** *Hydrolysis study on sodium hyaluronate (HA) to prepare various-sized oligomers.*

Utilizing the generated kinetics plots of their obtained samples, estimation of the degree of hydrolysis could be predicted permitting the preparation of various oligomers of defined sizes. Conservation of the reducing end is maintained throughout as the GAG polymer will generate new reducing ends as the ether bonds are hydrolyzed at the C1 position, multiple studies have exploited this approach before and applied it to a variety of GAGs [114,115]. As **HA** is an extremely
abundant GAG and stable to hydrolysis conditions where the N-acetyl groups are conserved it was selected over other sulfated GAGs as they can undergo removal of the sulfate functional groups. As proof of concept, this approach was replicated and followed by reductive amination with the generalized conditions. The NMR analysis of the resulting products is shown below in Figure 84.

![Figure 84: 1H NMR of hydrolysis of HA from 5-230 hrs followed by reductive amination to TEAB.](image)

Purification was achieved with dialysis or size exclusion chromatography on the labeled samples which resulted in a variety of molecular-weighted GAG oligomers of interest. Preliminary analysis was conducted with 1H NMR without optimization, where relaxation time (d1) and 90-degree pulse width (pw90) were at instrument defaults as well as lacking an internal standard. Variations in Mₘ based on the integration of the acetyl group (2.10-1.92 ppm) relative to aromatic TEAB peaks (7.60-6.60 ppm) indicated isolation of varying-sized oligomers Figure 84.
Deviations in target $M_w$ can be attributed to power outages during the initial setup of the reaction alongside accuracy in the preparation of the HCl (0.1 M) solution, but achieving a variety of oligomers was the desired goal with a variety of $M_w$. To enable the usage of these GAGs for biological studies, size exclusion chromatography (SEC) should be done to determine the exact $M_w$ with perhaps some more quantitative NMR performed. The identity of the impurity at 1.88 ppm is likely ammonium acetate (likely a result of the G25 size exclusion column) but based on literature [113] it is known that all acetyl groups for the oligomers should be located at 2.00 ppm.

In conclusion, while the method of preparing labeled GAG oligomers is not a novel approach, it has allowed for the expansion of the TEAB labeled glycan library, which could be utilized in future complex biological studies that focus on GAG-mediated processes. However, it is important to note that this pilot study has limitations, as there is an assumption that all TEAB peaks are attached to the HA and that all HA present is functionalized. Despite this limitation, the acquired data suggests that smaller TEAB functionalized oligomers are present in the isolated samples with a molecular weight close to the predicted values.

4.6 Periodate oxidation for release of non-reducing glycans

Preliminary exploration of other chemical approaches to prepare non-reducing sugars to permit reductive amination with TEAB was also considered. Sodium metaperiodate (NaIO$_4$) oxidation of diols to generate dialdehydes is selective where cis 1,2 diols have higher reactivity compared to trans 1,2 diols [116]. This approach of the introduction of aldehydes could be effective at labeling non-reducing sugars where the reducing end has formed an ether linkage to an additional glycan or can be linked to other biologically active residues such as terpenes/peptides. A prime example of this is the widely known sucrose (90, “table sugar”) which in theory should be unreactive towards reductive amination as the terminal fructose residue has its reducing end
linked to a glucose residue. Therefore, to enable the reactivity of this simple glycan, an aldehyde would have to be introduced to facilitate reductive amination. This approach of periodate oxidation has been applied before to carbohydrates such as cellulose [117,118] to introduce chemically reactive species, but the investigation Figure 85 to determine the exact selectivity of reaction for non-cis diol species was deemed relevant to potentially expand the glycolibrary.

**Figure 85:** Periodate oxidation of sucrose followed by subsequent TEAB labeling to form 91.

Following this analysis, sucrose was treated with sodium meta periodate where the selectivity of the oxidation of sucrose (90) was unknown as it possessed multiple trans 1,2 diols Figure 85. After an hour of oxidation with periodate, NMR was taken off the crude sample and aldehydes were determined to be present by the formation of peaks at ~8.2 ppm. Once complete, the addition of excess 2-picoline borane was performed as it was a mild reducing agent which should quench any remaining periodate. Reductive amination of the sample was then followed where it was found that oxidation was primarily occurring on the glucose residue through NMR and ESI mass spectrometry of the sample. Observation of primarily glucose-cleaved species made sense as the glucose offered two 1,2 diols that could be oxidatively opened, interestingly it was found that the aldehydes were being reduced back to alcohols and reductive amination was occurring primarily on the glucose side instead of the fructose side. Overall, the reaction proceeded surprisingly cleaner than expected however separation of all isomers in significant yields (greater
than single mg quantities) could not be achieved with standard C18 chromatography, but the isolation of the mixture was yielding the major compound (91). TOCSY was performed on the mixed isolated species to determine TEAB connectivity to the sucrose which indicated the elucidated structure above through spin coupling analysis. Additionally, the simpler sialic acid glycan was chosen to react with the periodate oxidation to permit reductive amination Figure 86.

Figure 86: Periodate oxidation of sialic acid (92) followed by subsequent TEAB labeling (93-94).

Sialic acid (66) is an important glycan species that is present in a variety of mammalian-related N-linked glycans such as SGP (57). The presence of sialic acid (66) is important for cell-cell signaling pathways[119] which therefore meant it was important to isolate a TEAB-labeled sample for future biological assays. Attempts were made previously to reductively aminate the glycan as it is a reducing sugar, but no significant reaction was observed, this was attributed to an unfavorable ring opening due to the carboxylic acid present at the anomeric center. Thusly, the glycan was treated with periodate allowing for oxidation of the two available sets of 1,2 diols on the aliphatic arm. Unsurprisingly the oxidation of the freer terminal diol was the major product (94, 72%) with the minor product (93 alcohol removed, 28%) in a sub-optimal 26 % yield (based on the highest MW product) but did result in a previously non-reactive glycan being labeled. Finally, with this moderately successful release in hand, an additional experiment was conducted
on raffinose (95), which is similar to sucrose in structure but bears an additional galactose unit at the end of Figure 87.

![Diagram of Periodate oxidation of raffinose followed by subsequent TEAB labeling with tentatively assigned products in a 3:1 ratio based on the integration of anomeric centers and $^{13}$C{$^1$H} data.]

**Figure 87:** Periodate oxidation of raffinose followed by subsequent TEAB labeling with tentatively assigned products in a 3:1 ratio based on the integration of anomeric centers and $^{13}$C{$^1$H} data.

The experiment was repeated and the same trend in the sucrose experiment was observed where the 1,2 diols present on the glucose were the preferred oxidizing species which resulted in significant removal of the galactose on the isolated glycan. Interestingly, despite the cis 1,2 diol present, the truncated product (96 + 97) was forming in a significant 3:1 ratio based on anomeric in $^1$H and $^{13}$C{$^1$H} NMR. The exact identity was not confirmed with ESI mass spectrometry, but the tentative identities based on the sucrose experiment were assigned above, further analysis would still be required to determine the exact connectivity to the glycans.

Despite being non-reducing sugars, when the reductive amination was run without the periodate oxidation and left overnight it was found that a significant amount of sucrose would undergo hydrolysis (~3 % yield). The purified product was unsurprisingly Glucose-TEAB (12), the hydrolysis event was even observed with raffinose (95) with a more prominent cleavage (~57 % yield) occurring with the resulting species being Lactose-TEAB (9) which was confirmed through HSQC analysis. Overall, it seemed as though periodate oxidation was more complex than
it initially appeared with increasing complexity in the assignment of identity when glycans were extended greater than disaccharides. Thusly, utilization of this reagent may not be appropriate for natural glycan isolation especially when glycans are greater than a trisaccharide, due to the vast number of isomers formed.

4.7 Natural product synthesis utilizing glycosylation enzymes

To conclude this chapter and to permit the isolation of some more advanced glycans with more defined identities where samples were not isolated from biological mixtures. To achieve this goal, a preliminary study of select glycosylation enzymes was conducted to ensure their compatibility with TEAB (1) and to emphasize its effectiveness as an auxiliary. Two model enzymes were chosen for investigation, namely the simpler α-2,3-sialyltransferase enzyme that sialylates lactose, and the highly complex Poly ADP-ribose polymerase (PARP) enzyme that creates varying length branches of ADP-Ribose (ADPr). The fractionation of a few oligomers from the PARP enzyme would be a significant breakthrough in the field of glycomics, highlighting the effectiveness of the TEAB motif if achieved.

To start the investigation, work performed by predecessors in Figure 88 was followed, using the α-2,3-sialyltransferase enzyme. This enzyme transfers a sialic acid residue from CMP-\(N\)-acetyl-neuraminic acid onto a lactose residue (98), generating a trisaccharide (99) of unique biological importance [120]. Previous attempts of using this enzyme have been successful, and it has been found to have generalized specificity for labeled substrates with bulky tetrazine groups present.
Thusly, the above work was repeated on lactose-TEAB (9) to demonstrate that TEAB-labeled substrates did indeed permit compatibility with glycosyltransferase enzymes. The protocol was followed (results shown in Figure 89) utilizing the same $\alpha$-2,3-sialyltransferase enzyme with identical equivalencies; it is important to note that the sialyltransferase enzyme was stored incorrectly under refrigeration instead of being frozen therefore the efficiency of the enzyme may have degraded over the 5 years of storage in solution.

Figure 89: Comparison of $^1$H NMR of Lactose-TEAB (9) to suspected Sialyated Lactose-TEAB (100).
Once the sialylation was performed under the previously shown conditions, the product was submitted to prep-C18 chromatography which resulted in the isolation of the suspected sialylated product (100). NMR comparison of the two compounds proved promising with several new peaks formed throughout the spectra, most importantly the new anomeric position present at 4.87 ppm. Unfortunately, it appears to be a mixture of products with the compound being in a mixture of disulfide and thiol form (peaks at 2.69 and ~3ppm), it is challenging to determine if starting material is still present but cannot entirely be ruled out.

Overall, it can be stated that partial sialylation did occur from the distinct differences between the two NMRs, but this process should be repeated in the future with a new sialyltransferase enzyme to definitively confirm the identity of the product alongside mass spectrometry for characterization of the compound.

Focus now shifted toward the PARP enzyme where work could be expanded further through the implementation of the novel glycan modification tools developed in previous chapters. The role of PARP is to generate a variety of oligomers of ADPr which act as key signaling molecules on DNA to identify damaged sections that can facilitate repair in breaks Figure 90 [121]. Isolation of large amounts of oligomers from PARP is significant discovery and a reproducible methodology to achieve this process would be a significant finding within the field of glycochemistry.
Essentially poly ADP-Ribose (pADPr) will be considered a complex mixture of glycans of varying defined length and branching that is desired as fractionated oligomers. A great deal of research has still been focused on characterizing this complex biological pathway which means isolation and fractionation of these complex oligomers is still highly valued but only achieved once previously [90]. To improve on that previous work, it was suspected that reductive amination could be employed to label and assist in the fractionation of the various oligomers generated by the PARP enzyme.

Previous attempts at the enzymatic synthesis of pADPr have been promising with some controlled PARP extension of NAD$^+$ photocaged at the 2$^\prime$OH position performed by predecessors [122]. It was decided that a different approach fractionate out oligomers of ADPr followed closely to the original published literature which achieved fractionation Figure 91.
Figure 91: Published PARP extension of ADP-Ribose to form poly ADP-Ribose followed by hydrolysis and fractionation [90].

Upon review of the literature, a protocol for PARP extension of ADPr was found where HIII histones (from calf thalamus) were linked to ADPr by various PARP enzymes forming branching oligomers on the histones. The polymerized ADP-Ribose was detached with base hydrolysis (KOH) of the phosphate groups followed by ion exchange chromatography to fractionate the desired oligomers from 2-17mers with medium and high molecular weight fractions isolated as well.

This approach was replicated where ADPr was attached to HIII histones via the PARP1 enzyme (~30µg scale of NAD+) utilizing activated DNA as a scaffold with nicotinamide cleaved as a byproduct. Utilizing ion exchange chromatography (shown in Figure 92), consumption of the NAD+ precursor was observed over 24 hours which indicated successful polymerization, and the sample was concentrated.
Figure 92: Ion pairing HPLC of NAD⁺ consumption by PARP1 presumably attached to Histones as pADPr: 1% to 5% AcN over 20 minutes in 100 mM Et₃NHOAc buffer at pH~5.

Before a detachment of the pADPr was performed, confirmation that PARylation had indeed occurred to the HIII substrate was required. Therefore a ¹H NMR (Figure 93) was taken of the crude reaction with 10,000 scans over 48 hours to confirm if the 30 µg of the sample was observable.

Figure 93: ¹H NMR of crude PARP extension of ADPr onto HIII histones with labeled nicotinamide peaks (101).

Successfully the crude product was observed through ¹H NMR with the specific observation of the cleaved nicotinamide 101 (~8-7.5 ppm) present in the crude reaction indicating
significant polymerization had occurred. The product was then recovered and submitted for KOH hydrolysis to cleave the pADPr from the HII histones which would permit fluorescent labeling and fractionation. Once hydrolyzed, the sample was then reductively aminated with TEAB in excess to fluorescently label the desired oligomers. The resulting sample was purified with G-25 size exclusion to remove excess reagents, unfortunately, subsequent ion-pairing chromatography indicated a significant presence of TEAB fluorophore which prevented further HPLC analysis.

Therefore, to obtain some insight into the reaction of the presumably pADPr-TEAB (102) the entire sample was size excluded again and submitted for ESI mass spectrometry (shown in Figure 94) to see if there was any presence of the desired molecular ions.

![ESI mass spectrometry of crudely labeled TEAB-pADPr (102).](image)

Interestingly, there was some presence of a re-occurring ~ +/- 178 m/z spacing between ions in the mass spectrum obtained. Unfortunately, fragment identification of the peaks could not be determined suggesting some significant un-desired reaction was occurring during the reductive amination step or some unknown salt (Na⁺, K⁺ or organic). It was identified that the spacing
between the mass ions did not match peptide-based fragmentation (not a broad distribution) which would indicate the observed ions are related products to the polymerization of ADPr. Overall, this more direct approach did result in the polymerization of pADPr but could not be utilized to fractionate the desired oligomers. Elimination of the reductive amination step seemed to be the most prudent way to avoid alteration of the product therefore a different approach was envisioned to give a selective release shown in Figure 95.

![Diagram](image)

**Figure 95:** An envisioned approach for reversible linked HIII histones (105) and pADPr-TEAB (102) through labeling of amine residues present (blue colored residues). HIII histone shown is from chicken, not calf thymus.

Pre-labeling of ADP-ribose with TEAB (103) would be chemically less harsh but would require the introduction of a reversible species onto the histones; allowing for TEAB to form a reversible bond to a motif introduced onto histones which could then be cleaved through either a reduction or hydrolysis. Implementation of a 5-MP residue appeared to be the best candidate as a coupling partner as it was designed to be a temporary linkage to proteins where slightly basic (pH 8.5) conditions would result in cleavage of the 5-MP residue releasing free thiol.
Overall, this envisioned approach enabled multiple redundant pathways to permit ligation of the 5-MP species to either ADPr-TEAB followed by addition to histones (approach B) or directly to the histones followed by ADPr-TEAB addition (approach A). Approach A (synthetic pathway drawn in Figure 95) was the favored approach for the introduction of the reversible residue due to the poor long-term stability of 5-MP (releases thiols in 2-3 days at neutral pH).

Figure 96: Attempted synthesis of NHS ester activated 5-MP linker (112) with isolated yields.

With this envisioned plan in hand, synthesis of precursor 5-MP and modification of ADPr with TEAB was begun. Both steps were run concurrently to expedite synthesis, preparation of 108 was followed by literature [75] and proceeded efficiently yielding the precursor 108 in acceptable yield (40 %). Once isolated generation of the reactive species had to be achieved where initial plans were to prepare an NHS-activated species (112) that could be applied to the histones. Interestingly the addition of glycine (110) ran smoothly but subsequent activation with NHS (111) would consistently not form due to a suspected self-elimination where the alkene would substitute the NHS ester (112) generated. Analysis of the isolated products consistently resulted in the lack of presence of both the alkene peaks in the NMR (~5 ppm) or any NHS ester-related peaks from the crude reaction and isolated fractions.
Therefore, an alternative approach was devised with the reaction of the precursor 108 with glucosamine (shown in Figure 97) which would allow the glycoconjugate 5-MP (113) to undergo a reductive amination to the amine-rich histones following established protocols [123].

![Figure 97: Attempted synthesis of glucosamine linked 5-MP from 113](image)

Again, the reaction of the precursor to the glucosamine substrate was observed to facilitate poorly (10-20% formation) despite multiple attempts with various free base preparation of the glucosamine. Isolation of the unprotected glucosamine-5-MP (113) also proved challenging due to the inability to utilize standard silica gel chromatography. The poor reactivity was attributed to the steric hindrance of the tertiary amine product, unfortunately, at this point time constraints prohibited further exploration of this pathway and further attempts were stopped.

Generation of the TEAB-linked ADPr (drawn in Figure 98) proceeded rather cleanly following the generalized protocol. The ADPr-TEAB product (103) was generated in a modest yield (22 %) with some unique by-product (~25%) co-eluting alongside it which appeared to other phosphorous species with peaks at ~0 and 18 ppm in the $^{31}$P{$^1$H}.
Figure 98: \(^1\)H and \(^{31}\)P\(^1\)H NMR of crude ADPr-TEAB (103) with unknown byproducts

Once again due to time constraints, the product was ungratifyingly left in this crude state and attempts at further modification of the histones were abandoned. Overall, this strategy promises to be a novel approach to synthetically generating pADPr species without significant byproduct formation, unlike the previous hydrolysis release. Further investigation of 5-MP species should be considered as a wide variety of functional groups can be installed allowing for linkage to the histones. Finally, the presence of other phosphorous-reduced species may explain why the labeling of hydrolyzed pADPr did not yield any matching mass ions to the theorized products and should be further identity elucidated.

4.8 Summary

To summarize, the study aimed to investigate the effectiveness of bleach oxidative release of glycans. The results showed significant deviations from the published literature [47] and suggested that the protocol was not effective for large scale glycan isolation, with yields lower
than 5%. While bleach oxidative release may be useful for LC-MS studies, it may not be appropriate for isolating complex glycans. The proposed mechanisms in the literature could not account for these deviations, and the study highlights the need for further research in this area. In addition, it was found that spray-dried egg yolk powder is not a viable source for SGP which contradicts claims made by literature [87] where no observable SGP was isolated utilizing their protocols. SGP was isolated using protocols from previous literature [87] with some improvements, using a colorimetric assay instead of complex mass spectrometry. The resulting glycan was further analyzed using hydrazinolysis and reductive amination to obtain $^1$H NMR spectra, but ESI mass spectrometry is still required for validation. Hydrolysis of GAGs was also successful, allowing for future studies on chain length effects. Sodium periodate oxidation was used to isolate non-reducing glycans in low yield but does not work for more complex glycans. Enzymatic extension of Lactose-TEAB showed partial sialylation, but validation using mass spectrometry/$^1$H NMR is still needed. Finally, the PARP enzymatic extension of ADP-ribose showed promising results, but more research is needed for labeling and fractionation of desired oligomers.

4.9 Methods for compounds 80-113

4.9.1. Bleach Truncation of Lactose-TEAB (80)

\[
\text{Lactose-TEAB (9, 20 mg, mmol, 1 eq.) was dissolved in water (1 mL) which was then reacted with Clorox bleach (0.2 mL). The solution was then stirred for 15 minutes and then quenched with formic acid (10 µL) where the crude mixture was concentrated, and the remaining}
\]

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precipitate was then re-conjugated to TEAB utilizing the generalized protocol. After the sample was concentrated, it was then submitted to prep-C18 where truncated lactose-TEAB 80 was recovered (3 mg, 15% yield).

4.9.2. Bleach Truncation of TEAB-N-Acetyl-lactoseamine (80 and 19)

\[
\text{N-Acetyl-D-lactosamine-TEAB (19, 10 mg, mmol, 1 eq.) was dissolved in water (0.5 mL) which was then reacted with Clorox bleach (0.1 mL). After stirring for 15 minutes followed by quenching with formic acid (5 µL), the crude mixture was concentrated, and the remaining precipitate was then re-conjugated to TEAB utilizing the generalized protocol. Isolation of the desired compounds was attempted with prep-C18 purification, which resulted in elution in the dead volume. Where the sample was recovered and analyzed with HILIC chromatography which was then purified through analytical run-off of the collection. Resulting in two fractions of interest containing the truncated (80) and non-truncated product (19) in a 25:75 ratio respectively, yield was not quantifiable due to an error in weighing (<10% yield).}
\]

4.9.3. Sulfuric Acid-Phenol assay detection of crude glycans[110]
Concentrated H$_2$SO$_4$ (150 μL, 37 % v/v), glycan dissolved sample (50 μL), and phenol aqueous (30 μL, 5 % w/v) were pipetted into a vial and then heated at 80 °C for 5 minutes. An aliquot (100 μL) of the sample was taken and placed into a microwell plate and absorbance at 485 nm was measured.

4.9.4. Attempted extraction of SGP (81) from egg yolk powder based on literature[87]

Egg yolk powder (454 g) was suspended in ethanol (400 mL of 95%) and stirred for 1 h, (five times) to extract lipids from the bulk starting material. The suspension was filtered and dried via suction filtration to yield an off-white, dry powder; the filtrate was discarded. To extract the SGP, the off-white powder was suspended in 300 mL of 40% ethanol and mechanically stirred for 1 hr (5 times). During each filtration, the solid was washed thoroughly with plenty of extraction solvent (300 mL wash each extraction) to ensure high recovery since the long filtration time from the large scale would lead to evaporation of the solvent in the solid. Filtrates were then pooled and concentrated under reduced pressure at 65 °C alongside air drying overnight (while this is near completion, last 400 mL of solvent. Start set-up of Celite-Carbon column). Cold aqueous ethanol (40%, 50 mL) was added to the concentrated solution to precipitate proteins which were removed by centrifugation (3000 rpm, 10 min). The resulting solution was concentrated and purified by an active carbon/Celite column chromatography (60 g of active carbon and 60 g of Celite). Elution of the column was started with water (750 mL, 0.1 % TFA), 5 % acetonitrile in water (750 mL, 0.1 % TFA v/v), and 10 % acetonitrile in water (750 mL, 0.1% TFA v/v). SGP was released from the column by eluting with 25% acetonitrile in water (0.1% TFA v/v). Fractions of 40 mL were collected and analyzed with the phenol-sulfuric acid assay, fractions of approximately 9-22 contained glycan of interest. Fractions containing the desired product were concentrated under rotary evaporation and subjected to size chromatography (Bio-Rad P-2, fine particle size 45–
90 μm; Ø 2.5 cm, h 25 cm, V_Fr 20 mL) and eluted with a 0.1 M ammonium bicarbonate where glycan containing fractions were detected with the phenol-sulfuric acid assay. SGP-containing fractions were lyophilized to yield no SGP 81 (theorized 364 mg, or 0.8 mg SGP/g of egg yolk powder).
4.9.5. Trypsin digestion of ethanol (aq.) extracts from dried yolk powder yielding G1 (84)

Following the above protocol, the procedure was stopped post centrifuging the samples isolated. Recovered samples were then added to Trypsin solution from porcine pancreas (10 mL, 25 g/L of trypsin) and incubated at 37 °C for 48 hrs. The resulting sample was concentrated and submitted for P2 (Bio-Rad P-2, fine particle size 45–90 μm; Ø 2.5 cm, h 25 cm) and G25 (Sephadex G25, fine particles; Ø 2.5 cm, h 25 cm, VFr 20 mL) size exclusion where fractions of interest (8-14) were determined by phenol-sulfuric acid assay and subsequently concentrated yielding the tentatively assigned G1 glycan (84).
4.9.6. Extraction of SGP from fresh egg yolks (81)

Fresh hen eggs (72 eggs) equating to (~0.5kg) of egg yolk powder were separated from yolk/whites which resulted in ~1.5 liters of yolk where shells and whites were discarded. This solution was evaporated at 65 °C portion-wise in a round bottom flask (1 L) or under air if left overnight with the addition of 1-octanol (200 mL) as an anti-foaming agent. Once completed the dried yolk was utilized under the same conditions described in the attempted isolation of SGP from egg yolk powder with SGP-containing fractions ranging from 10-13. Overall, this resulted in an off-white solid SGP (81) of 318 mg yield with an approximate purity of 90% (4.4 mg/egg yolk) with $^1$H NMR data matching literature [87].

$^1$H NMR (500 MHz, D$_2$O_salt) δ: 4.99 (s, 1H), 4.90 (d, $J = 9.8$ Hz, 2H), 4.63 (s, 1H), 4.53 (t, $J = 6.6$ Hz, 1H), 4.44 (d, $J = 27.8$ Hz, 2H), 4.36 – 4.22 (m, 4H), 4.21 – 4.13 (m, 1H), 4.08 (d, $J = 29.1$ Hz, 2H), 4.03 – 3.93 (m, 3H), 3.93 – 3.26 (m, 37H), 2.86 (dd, $J = 14.0$, 6.7 Hz, 2H), 2.71 (dd, $J = 16.0$, 5.9 Hz, 1H), 2.59 (dd, $J = 16.6$, 7.3 Hz, 1H), 2.54 – 2.37 (m, 2H), 2.37 – 2.25 (m, 2H), 2.17 (s, 1H), 2.08 (dd, $J = 13.7$, 5.4 Hz, 1H), 2.02 – 1.82 (m, 18H), 1.82 – 1.51 (m, 6H), 1.31 (s, 1H), 1.28 (d, $J = 6.4$ Hz, 1H), 1.24 (d, $J = 6.9$ Hz, 2H), 1.06 (d, $J = 6.2$ Hz, 2H), 0.83 (d, $J = 6.5$ Hz, 3H).
4.9.7. Bleach oxidative release of N-linked glycans from biological samples (87-89)

Following the published literature[47], yeast (250 g), egg whites (30 eggs), and egg yolks (30 eggs) were diluted with water (~4.5 L) and treated with Clorox bleach (~1L) for 15 minutes. Samples were then quenched with formic acid (75 mL) and centrifuged at 3000 rpm for 15 minutes (separation of precipitated proteins) for the supernatant to be collected. Supernatants were concentrated under rotary evaporation (65 °C) where they were then re-diluted (~200 mL each) and dialyzed for 12 hrs with 1K MWCO tubing against water. The process of bleach oxidative release was repeated with water (2 L), Clorox bleach (100 mL), and formic acid (17 mL). After concentration of the final dialyzed samples, yielded crude glycans in the following amounts:

**Yeast 89** 5.345 g; $^1$H NMR (500 MHz, D$_2$O) δ: 5.44 (d, J = 8.1 Hz, 1H), 5.31 (s, 3H), 5.24 – 4.98 (m, 14H), 4.24 (s, 4H), 4.19 – 3.56 (m, 100H), 3.45 (t, J = 9.4 Hz, 2H), 3.36 (s, 5H), 2.24 (s, 7H).

**Egg yolk 88** 1.835 g; $^1$H NMR (500 MHz, D$_2$O_salt) δ: 5.39 (s, 1H), 5.32 (d, J = 15.9 Hz, 2H), 5.25 (s, 1H), 5.11 (d, J = 11.6 Hz, 5H), 5.03 (s, 4H), 4.64 (s, 40H), 4.46 (d, J = 11.2 Hz, 28H), 4.12 (d, J = 63.5 Hz, 130H), 4.04 – 3.42 (m, 215H), 3.34 (s, 16H), 2.51 (d, J = 28.8 Hz, 19H), 2.35 (d, J = 7.6 Hz, 22H), 2.21 (s, 53H), 2.04 (d, J = 20.0 Hz, 43H), 1.83 – 1.64 (m, 36H), 1.53 (s, 55H), 1.30 (s, 317H), 0.89 (s, 124H).

**Egg whites 87** 810 mg; $^1$H NMR (500 MHz, D$_2$O_salt) δ: 5.40 (s, 1H), 5.33 (s, 1H), 5.26 (s, 1H), 5.19 (d, J = 19.7 Hz, 3H), 5.12 – 4.92 (m, 11H), 4.69 (s, 8H), 4.66 – 4.31 (m, 32H), 4.31 – 3.29 (m, 218H), 3.22 (d, J = 8.5 Hz, 5H), 3.11 (s, 3H), 2.94 (d, J = 43.7 Hz, 4H), 2.89 – 2.64 (m, 9H), 2.55 (d, J = 14.8 Hz, 7H), 2.34 (d, J = 11.8 Hz, 34H), 2.24 – 1.78 (m, 64H), 1.59 (s, 14H), 1.48 – 1.11 (m, 20H), 0.92 (s, 37H).
4.9.8. Reductive amination of complex glycans

Complex glycan (50 mg), Zn-TEAB 11 (50 mg, 0.110 mmol, 1 eq.), 2-Picoline borane 7 (14 mg, 0.132 mmol, 1.2 eq.), and EDTA (80 mg, 0.275 mmol, 2.5 eq.) were dissolved in MeOH:Water: Acetic acid (3.5:3.5:3, 0.5 mL). The reaction mixture was heated at 65 °C for 2.5 hrs, the crude reaction was diluted and filtered where purification was performed with either dialysis 1K MWCO or with exclusion media (Sephadex G25, fine particles; Ø 1 cm, h_c 5 cm, V_{Fr} 100 µL) and eluted with water and UV-Vis measured at 326 nm to determine glycan location.

4.9.9. Attempted pull-down of complex glycans post reductive amination.

Following the previous generalized procedure, a crude reaction of complex labeled glycan (50 mg, SM), and activated SiliMet-SH (200 mg) was combined and then followed the generalized pull-down procedure and characterized with ^1H NMR.

4.9.10. Bleach Chemical Peeling of N-acetyl-glucosamine (86)

GluNAc (60, 40 mg) was dissolved in water (2 mL) and NaClO (5 %, reagent grade, 0.4 mL) was added and left to stir for 15 minutes. Quenching was then performed with formic acid (0.04 mL) where the reaction was then concentrated through rotary evaporation and NMR was taken to determine the bleach effect of pure glycans.

4.9.11. Hydrazinolysis of complex glycans yielding 85 [112]

Complex glycan (20 mg) was placed inside a vial (7 mL) alongside a stir bar and sealed under nitrogen. Hydrazine anhydrous (2 mL, 10 mg/mL of glycoprotein) was then added to the complex glycan of interest, a blast shield was placed in front of the reaction and the solution was heated to 95 °C for 18 hrs. After completion of the hydrazinolysis, the samples were cooled to room temperature and ethanol (18 mL) was added to the solution where precipitation occurred. The suspension was transferred to two centrifuge tubes (15 mL) and centrifuged at 3000 rpm for
15 minutes where the desired pellet was recovered in a separate vial and the supernatant discarded. Following re-acetylation protocols, the solid was dissolved in sat. NaHCO₃ (1 mL) and then stirred with acetic anhydride (10 µL, ~0.0698 mmol, ~10 eq.) for 20 minutes. Another portion of acetic anhydride was added to ensure re-acetylation of complex glycans, the now liberated complex glycans were then placed inside a dialysis tube (1K MWCO) and underwent dialysis for 24 hrs against water. This process was then followed with 4.9.8 to prepare the reductively aminated glycans of interest.

4.9.12. Acid hydrolysis of hyaluronic acid from the literature [113]

Sodium hyraulanate (250 mg, 1M Da) was suspended in HCl (0.1 M, 10 mg/mL, 25 mL) and heated at 60 °C. After the desired time had been reached, an aliquot (5 mL) was withdrawn from the reaction and quenched with a sat. bicarbonate solution until pH was ~8. Samples were then added to a dialysis tube 1K MWCO with water as a dialysate for 12 hrs yielding the prepared oligomer.

4.9.13. Reductive amination of various oligomers of hyaluronic acid

Following the general synthesis procedure, the following reagents were reacted: Prepared oligomer (50 mg), Zn-TEAB 11 (50 mg, 0.110 mmol, 1 eq.), 2-Picoline borane complex 7 (14 mg, 0.132 mmol, 1.2 eq.) and EDTA (80 mg, 0.275 mmol, 2.5 eq.). Post reductive amination, samples were purified with a size exclusion column (G25, fine particles; Ø 1 cm, h 5 cm, VFr 100 µL) and eluted with ammonium acetate (0.1 M) to yield purified labeled HA samples.
4.9.14. Periodate oxidation and reductive amination of sucrose (90)

Sucrose (90, 50 mg, 0.140 mmol, 1 eq.) and sodium periodate (30 mg, 0.140 mmol, 1 eq.) were dissolved in water (1 mL) and stirred for an hour. To quench any remaining sodium periodate 2-picoline borane complex 7 (80 mg, 0.350 mmol, 5 eq.) was left to stir for 10 minutes. Zn-TEAB 11 (40 mg, 0.084 mmol, 0.6 eq.) and MeOH: Acetic acid mixture was added where the reaction was heated at 65 °C for 2.5 hrs. The reaction was purified with C18 purification resulting in three fractions of interest. The composition was a majority of sucrose-linked products at the glucose side with a poor overall yield of the combined products 91 (16 mg, ~10 % yield).

4.9.15. Reductive amination of sucrose forming Glucose-TEAB (12)

Following the general synthesis procedure, the following reagents were reacted: Sucrose (100 mg, 0.667 mmol, 1 eq.), Zn-TEAB 11 (302 mg, 0.667 mmol, 1 eq.), 2-Picoline borane complex 7 (83 mg, 0.800 mmol, 1.2 eq.) and EDTA (487 mg, 1.668 mmol, 2.5 eq.). The reaction was run overnight instead of for 2.5 hrs, which yielded Glucose-TEAB (12) as a white solid (10 mg, 3 % yield).
4.9.16. Periodate oxidation and reductive amination of Sialic acid (93+94)

Sialic acid 92 (100 mg, 0.326 mmol, 1 eq.) (0.7 mL) and sodium periodate (70 mg, 0.326 mmol, 1 eq.) were dissolved in water which was stirred for 1 hr. To quench, 2-picoline borane complex 7 (170 mg, 1.630 mmol, 5 eq.) was then added to the reaction mixture and stirred for 10 minutes. Zn-TEAB 11 (89 mg, 0.196 mmol, 0.6 eq.) was then added to the quenched solution along with MeOH (0.7 mL) and acetic acid (0.6 mL), heated at 65 °C for 2.5 hrs. Prep-C18 resulted in two fractions of interest (F8 – 25 mg and F9 – 15 mg) with an estimated ratio of 28 %: 72 % between the two products (93+94) formed with a suboptimal yield (~26 % yield based on the highest molecular weight product).

4.9.17. Periodate oxidation and reductive amination of raffinose (96+97)

Raffinose pentahydrate 95 (100 mg, 0.168 mmol, 1 eq.) was dissolved in water (0.7 mL) and sodium periodate (36 mg, 0.168 mmol, 1 eq.) was added and stirred for 1 hr. To quench the reaction 2-picoline borane complex 7 (88 mg, 0.840 mmol, 5 eq.) was added to the reaction mixture and stirred for 10 minutes. Zn-TEAB 11 (46 mg, 0.101 mmol, 0.6 eq.) was then added along with
MeOH (0.7 mL) and acetic acid (0.6 mL), and then at 65 °C for 2.5 hrs. Preparative C18 chromatography was then applied to the mixture (96+97) resulting in two fractions of interest F29 - 9 mg (Mixture) and F31 – 5 mg (Truncated).

4.9.18. Reductive amination of raffinose forming Lactose-TEAB (9)

Following the general synthesis procedure, the following reagents were reacted: Raffinose pentahydrate 95 (100 mg, 0.168 mmol, 1 eq.), Zn-TEAB 11 (76 mg, 0.168 mmol, 1 eq.), 2-Picoline borane complex (22 mg, 0.201 mmol, 1.2 eq.) and EDTA (123 mg, 0.420 mmol, 2.5 eq.). Yielding Lactose-TEAB (9) as a white solid (57 % yield, 50 mg).

4.9.19. Synthesis of Sialic acid-lactose-TEAB (100)

Lactose TEAB (9, 1 mM, 1 eq.), CMP-N-acetyl-neuraminic acid (from New England Biolabs, 1 mM, 1 eq.), and α-2,3-sialyltransferase (from New England Biolabs, 20 µL, 1 mM) were dissolved in Tris.HCl (0.1 M, pH 8.0, 1 mL) where the sample was incubated for 24 hrs. The resulting crude reaction was submitted for prep-C18 1-100% AcN over 20 minutes yielding partially (20%) Sialyated Lactose-TEAB 100 (~1.4 mg).

4.9.20. PARP extension of ADPr modified from the literature [90]

NAD+ (100 µM, 0.25 mg/mL, 33 µL, 1000 eq.), DNA (25 µg/mL, 1.25 µL in Tris.HCl buffer), PARP1 (from New England Biolabs, 0.583 µg/mL or 10 units/mL, 5 µL, 1 eq.) and HIII Histones from calf thymus (3 µg, 1.5 µM, 10 eq., in Tris.HCl buffer) was added to the prepared buffer (120 µL) of Tris.HCl (100 mM), MgCl₂ (10 mM), DTT (1.0 mM) of pH 8.0. The overall concentration of NAD+ became 0.15 µM after addition to the solution, however, literature [90] ran the reaction higher at ~5 µM for NAD+, and PARP1 was diluted to 0.5 µM post addition of solutions. After the reaction mixture was prepared, the sample was incubated for 48 hours (even though HPLC indicated competition at 24 hrs) at 37 °C with gentle agitation. The rate of
consumption of NAD+ proceeded linearly (1.5 µg/hr) where: ~3 µg/2hrs, 15 µg at 10 hrs, and 36 µg at 24 hrs for PARP1 (10 µL, 0.5 µM). The reaction was then concentrated, and NMR analysis was taken along with HPLC to confirm the consumption of starting materials.

4.9.21. Detachment of pADPr modified followed by reductive amination from the literature [90]

Following the literature, the crude solution of pADPr (~30 µg) was dissolved in KOH (1 M, 10 µL) with EDTA (50 mM) and heated at 60 °C for 1 hr. After hydrolysis was complete the sample was recovered and acidified with acetic acid where the sample was then concentrated with rotary evaporation. Once concentrated the sample was then reductively aminated with Zn-TEAB 11 (1 mg, 0.00220 mmol, 1 eq.), and 2-picoline borane complex (0.28 mg, 0.00264 mmol, 1.2 eq.) for 2.5 hrs at 50 °C. Excess reagents were precipitated with water (1 mL) and syringe filtered away, where the filtrate was collected followed by size exclusion purification (Sephadex G25, fine particles; Ø 1 cm, h c 5 cm, V Fr 100 µL) with ammonium bicarbonate (0.1 M) as the eluent. UV-Vis was taken off the fractions to determine the location of the glycans of interest, this process was repeated twice to remove excess TEAB, and the sample was submitted for ESI mass spectrometry analysis.

4.9.22. Synthesis of 5-MP precursor (108) from the literature[75]

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\begin{align*}
\text{Furfuryl alcohol (106, 0.87 mL, 7.848 mmol, 1 eq.) and NaHCO}_3 (1.7 g, 20.238 mmol, 2.6 eq.) were dissolved in dry methanol (10 mL) and cooled between -45-35°C under nitrogen. To the stirring solution, liquid bromine (0.561 mL, 8.2404 mmol, 1.05 eq.) dissolved in dry methanol (5 mL) was added dropwise and then left for 15 minutes. After 2 hrs of stirring the solution was then left to warm to room temperature where methanol was removed by rotary evaporation. The crude solid was then extracted with ethyl acetate (20 mL x 3) with saturated brine (20 mL) and the}
\end{align*}
\]
organic layer was concentrated yielding a crude intermediate (107, 620 mg). The crude intermediate was then acetylated with pyridine (20 mL) and acetic anhydride (1.5 mL, 11.772 mmol, 1.5 eq.). The solution was left for a further 30 minutes, the solution was quenched with water (1 mL) and then concentrated under rotary evaporation. The sample was re-dissolved in MeOH: DCM (5:95) and loaded onto silica gel (Ø 2.5 cm, hC 15 cm, VFr 6 mL) the product eluted with isocratic MeOH: DCM (5:95) yielding compound 108 (Rf = 0.7 and 0.5 of the two isomers). This resulted in a colorless oil of acceptable yield (108, 800 mg, 40 % yield, ~3:1 isomers) where the two isomers could be separated by silica gel chromatography but was combined as reactivity is the same between the two. Compound previously characterized by literature[75].

1H NMR (500 MHz, CDCl3) δ: 6.19 – 6.05 (m, 1H), 5.97 (ddd, J = 7.0, 5.5, 2.4 Hz, 1H), 5.92 (d, J = 5.9 Hz, 1H), 5.76 (d, J = 12.2 Hz, 1H), 5.51 (s, 1H), 4.33 (d, J = 11.3 Hz, 1H), 4.24 (d, J = 2.9 Hz, 1H), 4.06 (d, J = 11.4 Hz, 1H), 3.52 (t, J = 3.8 Hz, 2H), 3.44 (s, 1H), 3.25 (d, J = 3.4 Hz, 1H), 3.17 (s, 1H), 2.05 (dd, J = 9.6, 4.9 Hz, 2H).

4.9.23. Synthesis of Glycine 5-MP (110)

Mixed compound 106 (50 mg, 0.25 mmol, 1 eq.) was dissolved in HCl (0.1 M, 0.5 mL) to cleave the protecting ester for 1 hr. The solution was neutralized with NaHCO3 (sat.) followed by glycine (38 mg, 0.5 mmol, 2 eq.) addition along with HEPES buffer (0.5 M, 0.1 mL, pH 7.5). The reaction was stirred overnight and then concentrated under rotary evaporation. The sample was then re-dissolved in MeOH: DCM (5:95) and loaded onto silica gel (Ø 2.5 cm, hC 15 cm, VFr 6 mL) the product eluted with isocratic MeOH: DCM (1:99) yielding compound x (Rf = 0.6) yielding a yellow oil of 110 (28 mg, 74% yield).
$^1$H NMR (500 MHz, CDCl$_3$) $\delta$: 7.07 (d, $J = 5.8$ Hz, 1H), 6.28 (d, $J = 5.2$ Hz, 1H), 5.29 (s, 1H), 4.92 (d, $J = 1.8$ Hz, 1H), 4.42 (s, 2H), 3.21 (s, 1H).


![Image of Glycine 5-MP](image)

Glycine 5-MP 110 (15 mg, 0.0999 mmol, 1 eq.), DCC (21 mg, 0.199 mmol, 2 eq.) or EDCI (38 mg, 0.199 mmol, 2 eq.), N-hydroxy succinimide 111 (23 mg, 0.199 mmol, 2 eq.), and triethylamine (70 $\mu$L, 0.4995 mmol, 5 eq.) were dissolved in DCM (1 mL). The solution was stirred overnight and then submitted for silica gel chromatography ($Ø$ 2.5 cm, $h$C 15 cm, V$_{Fr}$ 6 mL) where the crude compound was eluted with MeOH: DCM (5:95) in fractions 3-12.

4.9.25. Attempted glycosylation of 5-MP (113)

![Image of Glucosamine](image)

Mixed compound 108 (50 mg, 0.25 mmol, 1 eq.) was dissolved in HCl (0.1 M, 0.5 mL) to cleave the protecting ester for 1 hr. The solution was neutralized with NaHCO$_3$ (sat.) followed by glucoseamine (89 mg, 0.493 mmol, 2 eq.) addition along with HEPES buffer (0.5 M, 0.1 mL, pH 7.5). The reaction was stirred overnight and then concentrated under rotary evaporation. Extraction of the compound was attempted with HCl (1M, 5 mL) with ethyl acetate (10 mL x 3 times) where the organic layer was concentrated and the solid triturated with methanol (10 mL). Resulting in the isolation of a red solid, NMR was taken off the corresponding product, but low functionalization (113) was observed in the crude NMR with the majority of the compound was still as the non-reacted glucoseamine.
4.9.26. Reductive amination of ADPr-TEAB (103)

Following the general synthesis procedure, the following reagents were reacted: ADP-Ribose (30 mg, 0.0536 mmol, 1 eq.), Zn-TEAB 11 (24 mg, 0.0536 mmol, 1 eq.), and 2-Picoline borane complex 7 (83 mg, 0.800 mmol, 1.2 eq.). The reaction mixture was heated at a lower temperature of 50 °C to avoid hydrolysis of phosphates and yielded compound ADPr-TEAB (103) as a white solid (9 mg, 22 % yield with 75% purity).

$^1$H NMR (500 MHz, D$_2$O) $\delta$: 8.64 – 8.57 (m, 1H), 8.55 (s, 1H), 8.46 (d, $J = 7.9$ Hz, 1H), 8.33 (s, 1H), 8.27 (s, 1H), 8.04 (s, 1H), 7.94 (s, 1H), 7.92 – 7.84 (m, 1H), 7.42 (d, $J = 7.6$ Hz, 2H), 7.31 (t, $J = 7.6$ Hz, 1H), 6.94 (s, 1H), 6.90 – 6.83 (m, 2H), 6.07 (d, $J = 5.2$ Hz, 1H), 4.67 (t, $J = 5.0$ Hz, 1H), 4.53 (dd, $J = 9.8$, 5.7 Hz, 1H), 4.40 (s, 1H), 4.34 – 4.15 (m, 4H), 4.15 – 4.06 (m, 1H), 4.04 (s, 1H), 3.96 (s, 1H), 3.81 (t, $J = 5.9$ Hz, 1H), 3.74 – 3.63 (m, 2H), 3.56 (t, $J = 6.4$ Hz, 1H), 3.49 – 3.31 (m, 1H), 3.21 (t, $J = 11.7$ Hz, 1H), 3.06 – 2.96 (m, 6H), 2.94 – 2.85 (m, 4H), 2.78 (s, 2H).

$^{31}$P$^{1}$H NMR (202 MHz, D$_2$O) $\delta$: 0.16, -10.51, -10.61, -11.11, -11.21.
CHAPTER 5
NON-REDUCTIVE AMINATION LABELING

5.1 Non-reductive labeling of glycans

Despite the significant advancements shown in chapters 1 through 4 regarding the reductive amination of glycans to the fluorophore TEAB and the multitude of applications demonstrated, a major limitation remains. Attachment can only occur at the reducing end (C1), while linkages formed at other alcohols (C2-C6) can facilitate metabolic studies of glycans (attachment to proteins) and the assessment of enzymes that promote ether cleavage between glycans. Consequently, much research is still focused on conserving this biologically important end and labeling other alcohols to facilitate studies surrounding these pathways. To address this limitation, we collaborated with another research group at UMaine whose research focus is on xylanase enzymes, which are enzymes that hydrolyze polymeric xylose (xylans) at various positions.

To enable a mechanistic study of the novel xylodase enzyme recently discovered by our collaborator, fluorescently labeled xylose and xylans were needed to determine at which position was undergoing hydrolysis at C2 or C3 ether. Two promising methodologies Figure 99 A) iodonium reagents (114) [92] and B) boronic acid reagents (118) [92] were recently explored where copper catalysts permitted the labeling of monosaccharides forming ether bonds, at non-anomeric positions.
Figure 99: Recently published labeling strategies of monosaccharides at the non-anomeric position utilizing copper catalysts [91,92].

Route A) [91] was previously performed on protected monosaccharides but was viewed as the most robust route due to the general ease of iodonium (84) preparation as well as a stereoselective catalyst that was commercially available. Route B) [92] was also an effective backup where boronic acid (85) preparation could be prepared similarly to iodination (Sandmeyer approach, one step [124]) but required extensive drying of all reagents to enable the addition to the xyloside due to water sensitivity of the reaction. Coumarins are well-studied species with high fluorescent activity once cleaved (ether to alcohol) and are extremely popular with significant commercial availability [125,126] therefore they were the desired motif installed onto the glycan Figure 100.
Iodonium reagents (114, hypervalent iodine) are an oxidized form of iodine where the halide is attached to two aryl rings and then precipitated out to form a corresponding iodonium salt. The species generated is a unique and excellent electrophile, withdrawing electron density from the aromatic rings attached and permitting substitution of the iodonium species present. Boronic acids (118) are traditional motifs that have been utilized in a wide variety of palladium-mediated couplings (Suzuki), mechanistically this reaction involves some proposed coordination of the boronic acid to the glycan of interest permitting the remaining alcohol to substitute the boronic acid in a stereoselective manner. Overall, both C2 (125) and C3 (124) products are desired in either ideally pure forms or if necessary mixed, the cleavage performed by the enzyme should preferentially prefer one isomer to the other and fluorescence can be measured alongside NMR analysis to quantify. However, this analysis is the responsibility of the collaborator, and preparation of the auxiliary linked xylosides is performed by this work described here.

5.2 Iodonium reagent preparation and attempted labeling of TBS-methyl-xyloside

Preparation of the iodine reagent has been previously described, where a Sandmeyer reaction from the corresponding amine (126) can be performed resulting in the now iodine-bearing species. Unfortunately, significant ring opening (ester hydrolysis) of the coumarin species
typically occurs during the formation of the iodine product (127) resulting in sub-optimal yields of 18% which could not be avoided despite changes in reagents (H₂SO₄ vs HCl) and order of addition alongside pH neutralization of the reaction mixture when diazonium salt had formed. Oxidation of the resulting iodine species with mCPBA proceeds efficiently where mesitylene and triflic acid (OTf) are present during the reaction and affords the hypervalent iodonium species (122) through precipitation with diethyl ether as stated through the generalized protocol [92] resulting in the novel iodonium reagent 122 shown in Figure 101.

![Figure 101: Synthesis of mesitylene iodonium coumarin reagent as a trifluoroacetic acid salt (122) with isolated yields.](image)

After obtaining the reactive iodonium coumarin reagent, attempts were made to conjugate it with β-methyl-xyloside (β-MX) in toluene, but solubility issues led to a switch to DMSO as a solvent (Figure 102).

![Figure 102: Attempted conjugation of iodonium reagent (122) to xyloside derivative (124/125).](image)

However, modifying the substrate and solvent to a mixed protected TBS-β-methyl-xyloside (128) did not yield the desired product, suggesting poor solubility of the iodonium reagent.
was the problem. Replication of a previously published protected monosaccharide using the iodonium reagent also failed, and the recovery of starting materials confirmed the issue was solvation. Unfortunately, any solvent that could solvate all compounds in the reaction would likely interfere with the copper catalyst or iodonium reagent, preventing the reaction from proceeding. As a result, the team decided to explore the boronic acid approach, which had been successful with unprotected species in more polar solvents.

5.3 Boronic acid reagent preparation and labeling of β-MX

Preparation of the boronic acid reagent had also been elucidated previously, where again another Sandmeyer reaction could be used to generate the boronic acid species in one step. Other contemporary methods (boronic ester formation followed by hydrolysis [127]) to synthesize the desired compound were assessed but this route proved to be the most direct way. Utilizing a sorbitol extraction of the product, the boronic acid (123) could be manipulated into the aqueous phase to then be released via acidification where it could be extracted back into the organic phase for isolation giving an overall 20% yield Figure 104.

![Figure 103: Preparation of boronic acid coumarin (129) via Sandmeyer reaction [124] “isolated yield."

 Desired species 123 was afforded, and implementation of this reagent for conjugation of the β-MX was then attempted utilizing the stated conditions. Initial attempts at generating the products of interest seemed to be partially successful despite xylose not being one of the dozen
substrates implemented for this reaction [91]. The reaction described is shown below in Figure 104.

![Fig 104: Copper-mediated Chan-Lam coupling of β-MX to boronic acid coumarin (123).](image)

The product was then acetylated to facilitate purification and determination of the degree of selectivity which was found to be in a 4:1 ratio (C3: bis; 129:130) which was unsurprising as both C6 and C2 should coordinate to the boronic acid followed by a copper-mediated Chan-Lam coupling. Analysis of crude reaction through NMR indicated the formation of the desired product was between 5-10% therefore attempts at re-optimization of this reaction seemed prudent. Variations of time, temperature (60 °C, 80 °C), base (eq.), solvent (AcN, DMSO, Methanol), substrate (mixed TBS protected 128), and other catalysts (various copper and nickel catalysts) were tried but consistently resulted in poor product formation especially if the temperature was increased past 40 °C. Variations in the substrate to TBS were attempts at modulating specificity for the substrate to generate other labeled species which did not proceed. Ion exchange to a BF4 salt (131) [128] (from a B(OH)2) of 123 was attempted but still did not assist in the reactivity of the compound. Regrettably, the reaction could not be further optimized, fortunately, the reaction had been run enough times in this process of optimization permitting isolation of the desired
product in low yield (6 mg) but it was a sufficient quantity for collaborators to enable testing of the substrate. To further improve the yield of this conjugation, an attempt at utilizing a smaller substrate (same boronic acid 134 in the original paper) was tried with an in-situ Von-Pechmann condensation to form the desired coumarin species (129/130) shown in Figure 105.

**Figure 105:** Conjugation of 3-hydroxyphenyl boronic acid (132) with subsequent Von-Pechmann condensation.

The condensation was run in-situ as detection of the corresponding intermediate product (135) with HPLC was difficult, NMR analysis did confirm that there was labeling occurring to the 3-hydroxyphenyl boronic acid (134), but determining efficiency was challenging to determine due to paramagnetic effects in NMR. In-situ Von-Pechmann condensation to ethyl acetoacetate was attempted but proved to be unfavorable (~5 % for non-protected and ~20% for TBS protected 128) with no significant signals forming of the corresponding coumarin (129/130) species in HPLC. The resulting NMR analysis of the crude reaction suggested the degradation of desired compounds from the strong acids (InCl₃ [129] or MeSO₃H [130]) which were employed to promote condensation. Unfortunately, it was determined that the desired β-MX substrate was indeed the issue which explains why it was not a listed substrate in the original paper and further attempts at optimizing this process were halted.
5.4 Transformation of aldehydes into nitriles

As a part of the broader scope of this thesis to explore other glycan labeling techniques, nitrile conversion of aldehydes was viewed as a potentially useful transformation that also permitted their conversion back into truncated carbohydrate products allowing for the generation of uncommon glycan species. Utilizing the reagent O-phenylhydroxylamine hydrochloride (136) reduction of the aldehydes into nitriles could be achieved through the formation of aldoximes (not drawn). Once the cyanohydrin (137) has been isolated it can then undergo a suspected Wohl-type degradation to form the truncated glycan (138). The generalized process of reduction followed by degradation is outlined in Figure 106, where sodium phosphate buffered solution facilitated the transformation due to the neutralization of the HCl salt (136).

Once the cyanohydrin (137) has been isolated heating with a weakly basic resin (Lewatit MP62) permitted the removal of the cyano group through a suspected Wohl-type degradation. The conceptualization and optimization of this work were performed primarily by a predecessor, but further characterization of the cyanohydrins and degraded products was still necessary for the publication of this work. Thusly the process described in Figure 106 was repeated on a wide variety of substrates and characterization through $^1$H/$^1$C NMR was obtained on all products shown in Figure 107.
Most importantly optical rotation was taken of all compounds due to the stereocenters present, once completed the results were published in the literature [131]. Overall, this process of cyanohydrin generation and subsequent truncation proved efficient with yields ranging from good to excellent where the major limitation in yields resulted from the process of purification through precipitations. Perhaps future studies could seek to exploit this route of glycan labeling further to generate specific glycans of interest for biological studies.

5.5 Summary

In summary, the new methods developed for labeling glycans have some limitations in terms of substrate specificity, and they are not tolerant of water. Although partial success was achieved in isolating the C3 and bis-labeled coumarin xyloside, which will enable further analysis
of the xylosidase enzyme by collaborators, it is unlikely that this process can be repeated on mixed xylan species due to the extremely low yields obtained and poor substrate specificity. Unfortunately, there are currently no other viable options for a more effective route of synthesis for these coumarin-labeled xylans. However, the copper-mediated labeling strategies have shown high selectivity in protected glycan labeling without the need for extensive stereoselective protecting groups, which makes further research on this approach worthwhile. In addition, further characterization of the predecessor’s work was performed, which will allow for the publication of cyanohydrin preparation and the subsequent truncation of glycans into potentially useful products of interest.

5.6 Methods for compounds 114-154

5.5.1. Synthesis of 7-iodo-4-methyl-coumarin (127)

![Chemical structure of 7-iodo-4-methyl-coumarin](image)

7-Amino-4-methyl-coumarin 126 (100 mg, 0.571 mmol, 1 eq.) was dissolved in HCl (2 mL, 37 %). Sodium nitrite (44 mg, 0.628 mmol, 1.1 eq.) was added in water (3 mL) to the reaction mixture at -10 °C. The reaction was left to stir for 1 hr and then KI (284 mg, 1.713 mmol, 3 eq.) was added to the mixture and left for a further 24 hrs. The final mixture was extracted with ethyl acetate (60 mL) and water (40 mL). The organic layer was washed with sat. sodium bicarbonate (40 mL), sodium thiosulfate (40 mL), and brine (40 mL) which was then concentrated. The mixture was purified with silica gel chromatography (Ø 2.5 cm, hC 15 cm, VFr 6 mL) with ethyl acetate: hexanes (40:60) yielding an off-white solid 127 from F10-20 (15 % yield, 24 mg). Compound previously synthesized in literature [132].

5.5.2. Synthesis of 7-iodonium-4-methyl-coumarin mesitylene TfOH salt (122)
7-iodo-4-methylcoumarin 127 (15 mg, 0.0525 mmol, 1 eq.), mesitylene (8.2 µL, 0.0789 mmol, 1.1 eq.), and mCPBA (10.7 mg, 0.0789 mmol, 1.1 eq.) was dissolved in AcN (1 mL) and cooled to 0 °C. TfOH (8 µL, 0.0862, 1.7 eq.) was dissolved in an additional portion of DCM (1 mL) and slowly added to the reaction mixture. The mixture was refluxed at 77 °C for 30 min. The product was triturated with diethyl ether (20 mL) and dried to yield compound 122 (65 % yield, 19 mg).

$^1$H NMR (500 MHz, D$_6$-DMSO) δ: 8.06 (s, 1H), 7.83 (s, 2H), 7.24 (s, 2H), 6.55 (d, J = 1.3 Hz, 1H), 2.61 (s, 7H), 2.41 (d, J = 1.2 Hz, 4H), 2.31 (s, 3H). $^{13}$C($^1$H) NMR (126 MHz, D$_6$-DMSO) δ: 158.61, 153.22, 152.27, 143.39, 141.74, 129.87, 129.28, 128.26, 122.83, 122.22, 116.57, 115.85, 39.52, 26.31, 20.50, 17.94.

5.5.3. Attempted synthesis of coumarin labeled β-MX with iodonium reagent (122)

Cu(OTf)$_2$ (0.6 mg, 0.018 mmol, 0.05 eq.), S,S box ligand (1.2 mg, 0.036 mmol, 0.1 eq.) and triethylamine (16 µL, 0.0543 mmol, 3 eq.) was added to a dried vial sealed with nitrogen. Toluene (1 mL) was then added, and the copper catalyst was pre-activated at 80 °C for 15 minutes. In a separate vial of mesitylene iodonium, reagent 122 (19 mg, 0.036 mmol, 1.2 eq.) and 2,3,4,6-tetra-benzyl-D-glucopyranose (18 mg, 0.033 mmol, 1 eq.) was sealed and evacuated with nitrogen. The pre-activated catalyst was allowed to cool and then added to the second vial, the mixture was heated at 50 °C for 12 hrs.

5.5.4. Synthesis of 7-boronic acid-4-methyl-coumarin (123)
7-Amino-4-methyl-coumarin 126 (1000 mg, 5.710 mmol, 2.5 eq.), tetrahydroxydiboron (1600 mg, 1.142 mmol, 2 eq.) and HCl (1.4 mL, 14.280 mmol, 2.5 eq.) were dissolved in water (1 mL) left to stir for 15 minutes. NaNO₂ (480 mg, 6.85 mmol, 1.2 eq.) and NaOAc (940 mg, 11.420 mmol, 2 eq.) were dissolved in water (40 mL) and added to the azido coumarin mixture. The reaction was then left to warm to room temperature for 30 minutes. Then pH was adjusted to ~ 8 with K₂CO₃ sat. solution and EtOAc (10 mL) added. Sorbitol/ Na₂CO₃ (1 M) was added to the aqueous layer, the biphasic solution was agitated and extracted three times with EtOAc (20 mL). The pH was acidified and then further extracted three more times with EtOAc (20 mL), the organic phase washed with brine solution (20 mL) and dried of Na₂SO₄. Then concentrated to yield a brown solid 123 (20 % yield, 241 mg). Previously characterized in literature [127]

5.5.5. Preparation of 7-iodonium-4-methylcoumarin mesitylene BF₄ salt (131)

Following protocol [128], 7-boronic acid-4-methylcoumarin 123 (20 mg, 0.0980 mmol, 1 eq.) was dissolved in CH₃OH by adding dropwise an aqueous solution of KHF₂ (25 mg, 0.323 mmol, 3.3 eq.) and stirring for 10 min; the solvent was then evaporated, and the residue was extracted with acetone and evaporated to obtain a crude solid (131).

¹H NMR (500 MHz, D₆-DMSO) δ: 7.96 (d, J = 8.3 Hz, 1H), 7.86 (dd, J = 19.6, 8.2 Hz, 3H), 7.78 (d, J = 8.4 Hz, 1H), 7.64 (t, J = 8.2 Hz, 2H), 7.54 – 7.40 (m, 6H), 7.43 – 7.33 (m, 6H), 7.26 (d, J = 6.6 Hz, 2H), 6.50 – 6.42 (m, 2H), 2.47 (d, J = 27.9 Hz, 20H), 2.43 (dd, J = 8.9, 5.2 Hz, 4H), 2.40 – 2.36 (m, 3H), 2.35 (d, J = 5.0 Hz, 3H), 2.33 – 2.29 (m, 3H), 2.32 – 2.24 (m, 6H). ¹³C{¹H} NMR
(126 MHz, D$_6$-DMSO) $\delta$: 160.22, 159.79, 152.64, 139.11, 126.62, 125.06, 123.61, 123.08, 118.98, 118.30, 114.51, 59.76, 39.52, 20.77, 18.09, 16.33, 14.09.

5.5.6. Synthesis of mixed protected TBS-β-methyl-xyloside (128)

β-methyl-xyloside (200 mg, 1.218 mmol, 1 eq.), TBSCl (220 mg, 1.462 mmol, 1.2 eq.), and imidazole (207 mg, 3.045 mmol, 2.5 eq.) were dissolved in DMF (0.3 M). The solution was stirred at room temperature for 24 hrs and then the sample was submitted for silica gel chromatography (Ø 5 cm, h$_C$ 15 cm, V$_F$ 12 mL) yielding the mixed species of TBS protected β-methyl-xyloside 128 as a colorless oil (230 mg, 67 % yield).

$^1$H NMR (500 MHz, CDCl$_3$) $\delta$: 4.51 (d, J = 3.8 Hz, 1H), 4.43 (d, J = 3.2 Hz, 1H), 4.15 (d, J = 7.1 Hz, 1H), 4.10 – 3.94 (m, 3H), 3.81 (dd, J = 11.5, 5.3 Hz, 1H), 3.73 (dd, J = 8.0, 5.1 Hz, 1H), 3.70 – 3.58 (m, 4H), 3.55 – 3.50 (m, 4H), 3.50 – 3.43 (m, 7H), 3.44 – 3.34 (m, 3H), 3.32 – 3.22 (m, 3H), 3.13 (t, J = 8.3 Hz, 1H), 3.05 (d, J = 7.3 Hz, 1H), 2.27 (t, J = 7.0 Hz, 1H), 2.23 (t, J = 8.1 Hz, 1H), 2.17 (d, J = 0.4 Hz, 2H), 2.11 (t, J = 9.7 Hz, 1H), 1.56 (s, 2H), 0.96 – 0.85 (m, 30H), 0.19 – 0.07 (m, 20H).

5.5.7. Synthesis of C3 and bis labeled coumarin β-methyl-xyloside (129+130)

7-boronicacid-4-methylcoumarin 123 (10 mg, 0.049 mmol, 2.5 eq.), Methyl-β-Xylopyranoside (3 mg, 0.020 mmol, 1 eq.), Cu(OAc)$_2$ (4 mg, 0.038 mmol, 2 eq.), DIPEA (13 µL,
0.076 mmol, 4 eq.), and activated molecular sieves 4Å (50 mg/mL) were dissolved in dry acetonitrile (1 mL, 15 ppm water content). The reaction was then sealed and flushed under nitrogen and heated at 40 °C for 16 hrs. NMR analysis of crude reactions post alumina filtration consistently displayed less than 10% labeling occurring (129+130).

This process was repeated with variations in equivalencies of reagents, catalyst, concentration, heat, and time. All attempts were pooled except for variations in copper catalysts, sorbitol/Na₂CO₃ (1M) was used to extract excess boronic acid reagent and finally purified with a prep-C18 yielding the 6 mg of the labeled substrates.

^1H NMR (500 MHz, D₆-Acetone) δ: 7.93 – 7.76 (m, 1H), 7.75 – 7.58 (m, 3H), 7.41 – 7.29 (m, 1H), 7.13 – 6.96 (m, 4H), 6.92 (dd, J = 8.7, 2.4 Hz, 1H), 6.81 (dd, J = 7.0, 2.5 Hz, 1H), 6.35 (t, J = 4.9 Hz, 1H), 6.19 – 6.07 (m, 2H), 4.50 – 4.39 (m, 1H), 4.37 – 4.20 (m, 2H), 4.16 – 4.04 (m, 1H), 4.00 – 3.87 (m, 2H), 3.84 (ddd, J = 10.0, 8.6, 5.4 Hz, 1H), 3.72 (dd, J = 18.8, 10.1 Hz, 1H), 3.69 – 3.63 (m, 2H), 3.48 – 3.43 (m, 4H), 3.43 – 3.39 (m, 3H), 2.58 – 2.50 (m, 2H), 2.48 – 2.38 (m, 7H), 2.37 – 2.32 (m, 1H), 2.15 – 2.08 (m, 3H).
5.5.8. Characterization of coumarin-xyloside products through acetylation (131+132)

To the crude mixture from reaction 5.5.7., acetic anhydride (14 µL, 0.147 mmol, 3 eq.), triethyl amine (36 µL, 0.245 mmol, 5 eq.), and DMAP (0.4 mg, 0.0049 mmol, 0.1 eq.) were added. The crude mixture was stirred for 1 hr and then separated with prep-C18 with ramp 1% to 100% yielding the resulting products 131+132 (F11 0.1 mg and F12-1 mg).

F12- 1H NMR (500 MHz, CDCl₃) δ: 7.55 – 7.42 (m, 1H), 7.14 – 6.88 (m, 3H), 6.17 (d, J = 3.5 Hz, 1H), 5.40 – 5.24 (m, 1H), 5.08 (dd, J = 14.4, 6.8 Hz, 1H), 5.01 – 4.88 (m, 1H), 4.56 – 4.42 (m, 2H), 4.23 (dd, J = 12.3, 4.5 Hz, 1H), 4.16 (dd, J = 11.9, 5.2 Hz, 1H), 3.63 – 3.35 (m, 4H), 2.40 (d, J = 1.3 Hz, 3H), 2.15 – 1.88 (m, 6H).

F11- 1H NMR (500 MHz, CDCl₃) δ: 7.59 – 7.43 (m, 1H), 7.02 (t, J = 11.6 Hz, 1H), 6.98 – 6.91 (m, 1H), 6.17 (d, J = 1.0 Hz, 1H), 5.18 – 4.94 (m, 2H), 4.58 – 4.40 (m, 2H), 4.29 – 4.14 (m, 1H), 3.56 – 3.37 (m, 4H), 2.40 (d, J = 1.0 Hz, 3H), 2.15 – 1.92 (m, 7H).

5.5.9. Attempted synthesis of labeled 3-hydroxyphenyl boronic acid β-methyl-xyloside (135)

![Diagram](image)
β-methyl-xyloside (200 mg, 1.218 mmol, 1 eq.), 3-hydroxyphenyl boronic acid 133 (420 mg, 3.046 mmol, 2.5 eq.), copper acetate (442 mg, 2.436 mmol, 2 eq.), and activated molecular sieves (50 mg/mL) were dissolved in acetonitrile (10 mL) with DIPEA (0.85 mL, 4.872 mmol, 4 eq.). The solution was sealed under nitrogen and heated at 65 °C for 16 hrs. For the following based on NMR analysis: β-methyl-xyloside resulted in ~5% conversion, TBS protected-β-methyl-xyloside resulted in ~ 30% conversion.

5.5.10. Attempted Pechmann condensation of crude labeled coumarin β-methyl-xyloside 129/130 [129,130]

To a portion of 5.5.9 (135), either; InCl₃ (2 mg, 0.0106 mmol, 0.1 eq.) or MeSO₃H (0.69 µL, 0.0106 mmol, 0.1 eq.) was added to the solution. Followed by the addition of ethyl acetoacetate (13.5 µL, 0.106 mmol, 1 eq.) and either heated at 65 °C (InCl₃) or stayed at room temperature for 30 minutes where the corresponding reaction was then analyzed with ¹H NMR and HPLC.
CONCLUSIONS AND FUTURE DIRECTIONS

Throughout this thesis, it has been demonstrated multiple times that this novel fluorescent reporter developed has vast chemical potential in facilitating studies surrounding the glycome. Numerous methodologies and applications have been shown throughout, which have displayed the flexibility of thiol chemistry where multiple reagents have facilitated a variety of functional group formations where the more traditional amine chemistry cannot. There is much room left to investigate surrounding this reporter and a great deal of future biological studies that could be conducted involving modified glycans.

From a chemical perspective, significant issues involving recently published methodologies in isolating complex glycans have been discovered throughout the course of this thesis. These recent methodologies signify the immense difficulty in complex glycan isolations in tangible scales as well as the necessity for further innovation into the chemical release of glycans from biological mixtures.

A major utility of the TEAB fluorophore is its ability to facilitate in “catch and release” of the thiol end, investigations into an improvement of the first generation of “catch and release” is of major importance. Further innovation should be taken from the sulfur gold chemistry where thiols have been shown to form self-assembled molecules readily, it is envisioned that this could be exploited alongside electrochemistry to release glycans bound to the surface. This approach would be reagentless and could be utilized repeatedly without the need for chemical activation of the surface. Gold nanoparticles can be generated in discrete sizes and shapes which should permit for manipulation of the surface areas allowing for the generation of large porous structures enabling significantly orders of magnitude higher attachment of glycans to the surface compared to the traditional chemical resin developed. In addition to that, gold nanoparticles are prime targets for
synthesis where numerous studies have focused on the introduction of various biologically (glycans, peptides) active species to target specific biological diseases, now that there is a significant development in the installation of thiol groups onto glycans this could be applied to the wide variety of glycans have been functionalized.

From a biological perspective, numerous assays are now permitted with this novel glycan fluorophore mixture. The lowest hanging fruit is to further exploit the UV printing of TEAB glycans similar to the more recent peptide arrays where complex mixtures of defined ratios can be printed. Additionally, the implementation of the TEAB glycans into hydrogels is crucial to replicating physiological environments and can expand and surpass the amino derivatives in a fashion that they cannot facilitate. Finally, chemoenzymatic synthesis of pADPr can be now attempted, despite its challenges in synthesis there are numerous routes to obtain 5-MP linked histones which would permit discrete cleavage of TEAB-pADPr without byproduct formation and facilitate ion-exchange purification.

In conclusion, there are many exciting directions for this system to be applied, with numerous natural product analogs that can be now synthesized utilizing the techniques described within this thesis.
BIBLIOGRAPHY


APPENDIX

General information
All materials and reagents were obtained from either Sigma-Aldrich (St. Louis, MO, USA), Oakwood Scientific (Estill, SC, USA), BioSynth (Gardner, MA, USA), or Fischer Scientific (Waltham, MA, USA). Unless stated otherwise all reagents were of purity $\geq 95\%$. All water used throughout procedures was filtered through a Barnstead NANOpure system (Van Nuys, CA USA) with a resistance of 18.1 M$\Omega$ cm. NMR was obtained on a Bruker NEO Avance 500 MHz (Billerica, MA, USA) and a Varian INOVA 400 MHz (Palo Alto, CA, USA). The coupling constant ($J$) values are given in hertz (Hz) and $\delta$ values are given in ppm. The chemical multiplicities have been abbreviated as follows: $s =$ singlet, $d =$ doublet, $t =$ triplet, $q =$ quartet, $br =$ broad signal, quint $=$ quintet, $m =$ multiplet (denotes complex pattern), and their combinations as well. Analytical HPLC was performed on an Agilent 1260 infinity II system (Santa Clara, CA, USA) equipped with a UV-Vis detector and a Kinetex 5 μm EVO C18 100 Å column (Torrance, CA, USA), 150 $\times$ 4.6 mm. Prep-HPLC was performed on a Gilson 322 system (Madison, WI, USA) equipped with a UV-Vis-155 detector and a Kinetex 5 μm EVO C18 100 Å column (Torrance, CA, USA), 150 $\times$ 21.2 mm. HILIC was performed with a Comosil 5 μm 120 Å column (San Diego, CA, USA). Silica gel column chromatography was performed on SiliaFlash® F60 gel (Silicycle 40–64 μm, Quebec City, Quebec, Canada). Mass spectrometry data were collected through the University of Illinois School of Chemical Sciences’ mass spectrometry service. ESI was collected on a Waters Quattro Ultima mass spectrometer, and MALDI analysis was performed on a Bruker Autoflex Speed LRF MALDI. UV-Vis and fluorescence data were acquired on a SpectraMax i3x reader (San Jose, CA, USA), and UV irradiation was performed with an OmniCure S2000 Spot UV curing system (Saint Louis, MO, USA). Optical rotation was measured on a Jasco DIP-370 digital polarimeter (Easton, MD, USA).
$^1$H NMR data of N,N'-(dithiodi-2,1-ethanediyl)bis[2-aminO-benzamide] (3)
$^1$H and $^{13}$C[1H] NMR data of N-(2-ThioEthyl)-2-AminoBenzamide (1)
$^1$H, $^{13}$C$^1$H and HSQC NMR data of $N$-(2-ThioEthyl)-2-AminoBenzamide zinc salt (11)
Phase-sensitive ge-2D HSQC using PEP and matched sweep adiabatic pulses for inversion and refocusing with gradients in the back-inept of compound 5 – (HSQCEDETGPSISP2.3)
Analytical C18 of oxidation analysis of TEAB 1 (1 mg / mL water)

Analytical HPLC of TEAB T=0, gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes

Analytical HPLC of TEAB T = 1 month, gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes

Analytical HPLC of TEAB T = 2 months, gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes
Analytical C18 of oxidation analysis of Zn-TEAB 11 (1 mg / mL water)

Analytical HPLC of Zn-TEAB T=0, gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes

Analytical HPLC of Zn-TEAB T = 1 month, gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes

Analytical HPLC of Zn-TEAB T = 2 months, gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes
Analytical C18 of standard reductive amination of a glycan – maltoheptaose -TEAB 22

Analytical HPLC of crude reaction, gradient 1:99% to 0:100% (water: acetonitrile) over 10 minutes

Analytical HPLC of purified product, gradient 1:99% to 0:100% (water: acetonitrile) over 10 minutes

Analytical HPLC of partially oxidized product, gradient 1:99% to 0:100% (water: acetonitrile) over 10 minutes
$^1$H and $^{13}$C($^1$H) NMR data of D-glucose-TEAB (12)
$^{1}\text{H}$ and $^{13}\text{C}[^{1}\text{H}]$ NMR data of D-xylose-TEAB (13)
$^{1}H$ and $^{13}C\{^1H\}$ NMR data of L-fucose-TEAB (14)
$^1$H and $^{13}$C$^1$H NMR data of N-acetyl-D-glucoseamine-TEAB (15)
$^1$H and $^{13}$C$[^1]$H NMR data of D-allose-TEAB (16)
$^1\text{H}$ and $^{13}\text{C}[^1\text{H}]$ NMR data of D-ribose-TEAB (17)
$^1$H and $^{13}$C($^1$H) NMR data of D-maltose-TEAB (18)
$^1$H and $^{13}$C$[^1]$H NMR data of N-Acetyl-D-lactosamine TEAB conjugate (19)
$^1$H and $^{13}$C$[^1]$H NMR data of β-lactose-TEAB conjugate (9)
UV-Vis absorption and fluorescence data of lactose-TEAB conjugate (9)

Acquired in water; $\lambda_{\text{max}_1}$ 278 nm, $\lambda_{\text{max}_2}$ 326 nm $\lambda_{\text{ex}_2}$ 440 nm – 4700 M$^{-1}$ cm$^{-1}$ at 326 nm
$^1$H and $^{13}$C[$^1$H] NMR data of 1,3-$\alpha$-1,6-$\alpha$-D-mannotriose-TEAB conjugate (20)
$^{1}H$ and $^{13}C\{^1H\}$ NMR data of acarbose-TEAB (21)
$^1$H and $^{13}$C{$^1$H} NMR data of maltoheptaose-TEAB (22)
**1H NMR of 2-aminO-N-(2-aminoethyl)-benzamide (AEAB, 23)**

![NMR spectrum of 2-aminO-N-(2-aminoethyl)-benzamide](image1)

**1H NMR of Lactose-AEAB-2-imunothiolane conjugate (25)**

![NMR spectrum of Lactose-AEAB-2-imunothiolane conjugate](image2)
$^1$H and $^{13}$C[$^1$H] NMR data of lactose-TEAB-maleimidoundecanoic acid (26)
$^{1}H$, $^{13}C$($^{1}H$), NOESY, HSQC NMR data of lactose-TEAB-noroborene dicarboxylic acid (27)
Phase-sensitive ge-2D NOESY with z-spoil of compound 27 – (NOESYGGPPPP)
Phase-sensitive ge-2D HSQC using PEP and matched sweep adiabatic pulses for inversion and refocusing with gradients in back-inet of compound 27 – (HSQCEDETGPSISP2.3)
$^{1}\text{H}$ and $^{13}\text{C}[^{1}\text{H}]$ NMR data of activated lactose-TEAB-disulfide pyridine (43)
$^1$H and $^{13}$C$[^1]$H NMR data of lactose-TEAB-difulfidehexane (29)
$^1$H and $^{13}$C$[^1$H$]$ NMR data of lactose-TEAB-acetamide (30)
\(^1\)H and \(^{13}\)C\(^{1\text{H}}\) NMR data of lactose-TEAB-BOC-L-Leucine thioester (31)

![NMR spectrum and structure diagram](image-url)
$^{1}H$ and $^{13}C\{^{1}H\}$ NMR data of lactose-TEAB-butyne (32)
$^{1}$H and $^{13}$C$[^{1}$H$]$ NMR data of lactose-TEAB-propanediol (33)
$^1$H and $^{13}$C{$_1^1$H} NMR of hexenoic thioester (44)
$^1$H and $^{13}$C[$^1$H] NMR of bis-Lactose-TEAB-Alkyne (42)
$^1$H NMR of mono-Lactose-TEAB-Alkyne (41)

$^1$H NMR of competition experiment between Lactose-TEAB: Alkyne vs Alkene
$^1$H and COSY NMR of TEAB-pyridyl disulfide (48)
NOESY NMR of TEAB-pyridyl disulfide (48)

\[ \text{^1H NMR of mixed maleimide linker to Lactose-TEAB (62/61)} \]
$^1$H and $^{13}$C$^1$H NMR of Lactose-TEAB-MNA disulfide (56)
Spectroscopy and HPLC data of lactose-TEAB-bismaleimide-BSA (64)

Maleimide reaction - 1 hr

Gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes

Dialysis after maleimide - 24 hr

gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes
1 hr with 5 eq. Lactose TEAB (9)

Dialysis 12 hr

gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes
Dialysis 24 hr

gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes

Dialysis 36 hr

gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes

Dialysis 60 hr

gradient 1:99% to 0:100% (water : acetonitrile) over 10 minutes
Calculated mass of 63: 66,429 Da + 522 Da + 308 Da = 67,259 Da ~ 67.26 KDa
(BSA Protein) + Lactose-TEAB (9) + bismaleimide (60)

MALDI (Base Peak = 67,245 Da ~ 67.25 KDa)
UV-Vis quantification of activated Sili-MetSH (70)

Calibration curve of 2-mercaptopyridine in DMF

![Graph showing calibration curve]

y = 1.8355x + 0.0482
R² = 0.9997

Modification of compound 59 based on 2-thiopyridine produced

**Protocol 1:** SO₂Cl₂ (x eq.) and 2-thiopyridine (2 eq.) were dissolved in dry DCM (3 mL) was poured into a vial to which SiliMetSH (500 mg, 0.77 mmol SH content from supplier, 1 eq.) was slowly added to the fuming mixture over a period of 5 minutes. Once completed the suspension was stirred for 10 minutes and then placed onto a rotary evaporator at 45 °C, reduced pressure was applied until the complete removal of solvent. The dried solid was then re-dissolved in dry DMF and UV-Vis absorption was taken of the diluted sample.

**Protocol 2:** SO₂Cl₂ (10 eq.) was dispensed into a vial to which SiliMetSH (500 mg, 0.77 mmol SH content from supplier, 1 eq.) was slowly added to the fuming mixture over a period of 1 minute. Once completed the silica gel was placed onto a rotary evaporator and heated to 45 °C, reduced pressure was applied until complete removal of any excess SO₂Cl₂. Separately 2-thiopyridine (2 eq.) was dissolved in dry DCM (3 mL) and slowly added to the chlorinated silica. After stirring the suspension for 10 minutes, the suspension was then concentrated and re-dissolved in dry DMF, and UV-Vis absorption was taken of a diluted sample.
<table>
<thead>
<tr>
<th>Reaction conditions varied</th>
<th>Activated SH (mmol)</th>
<th>Remaining SH (mmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 1 - SO₂Cl₂ (10 eq.) TP (2 eq.)</td>
<td>0.355</td>
<td>0.415</td>
</tr>
<tr>
<td>Protocol 1 - SO₂Cl₂ (25 eq.) TP (2 eq.)</td>
<td>0.277</td>
<td>0.493</td>
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<tr>
<td>Protocol 2</td>
<td>0.997</td>
<td>-0.227</td>
</tr>
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</table>

TP = 2-Thiopyridine

**Column format of compound (70) for thiol catchment**

Application of compound 70 in a column format was investigated where the initial filtration step was applied with similar loadings and concentrations relative to the described optimized stirring format, a flow rate of ~1 mL per 3 minutes was used with 70 (250 mg) equilibrated in water within a cotton plug pipette. Purified glycan (9, 40 mg) was loaded into the column dissolved in water (2 mL), the column was then further washed with water (5 mL), methanol (5 mL), and acetone (5 mL) where the resulting filtrate was then concentrated under reduced pressure. It was found that the initial filtration had consumed 19 mg, 41% (approximated by mass loss of dried filtrate 21 mg) of the derivatized glycan with the remaining filtrate containing 43:57 (S-H: S-S) determined by NMR quantification of the CH₂ triplet at 3.00 ppm (S-S) and ~2.75 ppm (S-H) not accounting for the 2-thiopyridine in the resulting mass.
$^{1}$H, $^{13}$C$^{1}$H NMR data of compound (76)
UV-Vis optimization of allylated PEG microscope slides (77)

Adjusting the time of PEGylation – 10 w/v% PEG, 5 w/v% AIBN in toluene at varying times.

Adjusting concentrations of initiator and PEG - All samples reacted for 1 hr with the stated solution in toluene

All samples were run in triplicate with 3 sample wells on a 10 mm x 25 mm cut from the same prepared slide batch, 10 mL aliquots were dispensed onto each well. Fluorescence was measured with 50 flashes per read of the wells analyzed with 385 nm excitation and 490 nm emission, reading from underneath the plate. Blank is defined as a non-reacted (non-pegylated) region on a thiol-coated glass slide measured at the same time as the other readings with the same settings defined, all samples have been made relative to the blank region to determine significance. All regions were thiol-ene clicked with the optimized conditions: lamp 22.41 mW/cm² for 1 minute with glycan 9 (10 mM), LAP (1 w/v%). The slide (25 mm x 75 mm) was drop cast with 1 mL of the stated solution for the stated time at 100 °C in an air oven. The error was measured as one standard deviation between all triplicate sample spots.
UV-Vis optimization of glycosylated PEG microscope slides

Adjusting irradiation time - Lamp 22.41 mW/cm² for x minutes with glycan 9 (10 mM) and LAP (1 w/v%)

Adjusting lamp power - Lamp x mW/cm² for 5 minutes with glycan 9 (10 mM) and LAP (1 w/v%)

Additionally, 2-Hydroxy-4’-(2-hydroxyethoxy)-2-methylpropiophenone (I2959) was investigated as a photoinitiator however efficiency of initiation was found to be significantly lower compared to LAP, requiring orders of magnitude higher concentrations to achieve observable fluorescent spots.
**Adjusting LAP** - Lamp 22.41 mW/cm² for 5 minutes with glycan 9 (10 mM), LAP (x w/v%)  

- 1 w/v%  
- 0.5 w/v%  
- 0.1 w/v%  

![Bar graph showing relative fluorescence intensity for different LAP concentrations.]

**Adjusting glycan concentration** - Lamp 22.41 mW/cm² for 5 minutes with glycan 9 (x mM), LAP (1 w/v%)  

- 10 mM  
- 5 mM  
- 1 mM  

![Bar graph showing relative fluorescence intensity for different glycan concentrations.]

All samples were run in triplicate with 3 sample wells on a 10 mm x 25 mm cut from the same prepared slide batch. 10 mL aliquots were dispensed onto each well. Fluorescence was measured with 50 flashes per read of the wells analyzed with 385 nm excitation and 490 nm emission, reading from underneath the plate. Blank is defined as a non-reacted (non-glycosylated) region on the glass slide measured at the same time as the other readings with the same settings defined, all samples have been made relative to the blank region to determine significance. All samples reacted with 10 w/v% PEG, and 5 w/v% AIBN in toluene for 1 hr at 100 °C with 1 mL deposited onto a slide (25 mm x 75 mm). The error was measured as one standard deviation between triplicate sample spots on the same slide.
## Fluorescence data of Concanavalin A assay

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<th>Sample</th>
<th>Well</th>
<th>Average</th>
<th>Relative fluorescence</th>
<th>σ</th>
<th>σ²</th>
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<td>1</td>
<td>2</td>
<td>3</td>
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<td>Blank (non-glycosylated)</td>
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<td>D-maltose (18)</td>
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<td>β-lactose (9)</td>
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<td>Maltoheptaose (22)</td>
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</tbody>
</table>

Fluorescence was measured with 50 flashes per read of the wells analyzed with 485 nm excitation and 525 nm emission, reading from underneath the plate. Blank is defined as a non-reacted (non-glycosylated) region on the glass slide measured at the same time as the other readings with the same settings defined, all samples have been made relative to the blank region to determine significance.
Truncation of \(N\)-acetyl-lactoseamine-TEAB (19) to products 80 and 9

Sample – Post prep fraction after TCEP reduction. Products @ 4.763 minutes

CO-injection truncated lactose. Peak @ 4.629-minute increase

CO-injection NeuAc-Lactose. Peak @ 4.667-minute increase

10-minute, Gradient used 1->100 AcN, on Phenomenex 4
Analytical HILIC, sample – Post prep fraction after TCEP reduction

Preparatory HILIC – 100 uL injections and minute collections.

Sample - Concentrated fractions 7-10 minutes

10-minute, Gradient used 1-100 %AcN, on Phenomenex 4
UV-Vis of phenol sulfuric acid assay of SGP (81) from egg yolk powder attempt

Phenol sulfuric acid assay: Celite: Carbon (1:1) column fractions

Phenol sulfuric acid assay: P2 media fractions
$^1$H and $^{13}$C($^1$H) NMR of tentatively assigned G1 glycan isolated from trypsin digestion of spray-dried egg yolks (84)
HSQC NMR of tentatively assigned G1 glycan isolated from trypsin digestion of spray-dried egg yolks (84)
UV-Vis of Phenol-sulfuric acid assay of celite column from fresh egg yolk (SGP 81 yielding)

Samples 1-3 are the wash extracts (0.75 L, 0%, 5%, and 10% can with 0.1 % v/v TFA), and 4-23 are fractions (40 mL) of the column at 25:75 (can: Water) 0.1% v/v TFA.

SGP isolated in fractions 10-13.

$^1$H of SGP (81) isolated from egg yolks utilizing [87]
$^{1}H,^{13}C^{(1}H \}$ of complex glycans isolated from yeast (89) utilizing NaClO treatment
HSQC, HMBC of complex glycans isolated from yeast (89) utilizing NaClO treatment
DEPT of complex glycans isolated from yeast (89) utilizing NaClO treatment

$^1$H NMR of complex glycans isolated from egg yolks (88) utilizing NaClO treatment
$^1$H NMR of complex glycans isolated from egg whites (87) utilizing NaClO treatment

$^1$H NMR of complex glycans isolated from yeast (89, 3 mg) utilizing NaClO treatment and ‘pull down’ methodology
$^1$H NMR of complex glycans isolated from egg whites (87, 5 mg) utilizing NaClO treatment and ‘pull down’ methodology

$^1$H NMR of complex glycans isolated from egg yolks (88, 4 mg) utilizing NaClO treatment and ‘pull down’ methodology
Hydrazine treatment of complex glycans followed by reductive amination and dialysis purification.
$^1$H NMR of TEAB-SGP (85) isolated from egg yolks utilizing hydrazine treatment w/tBuOH standard
$^1$H NMR of TEAB-complex glycans isolated from yeast utilizing NaClO then hydrazine treatment w/tBuOH standard
$^1$H NMR of TEAB-complex glycans isolated from egg whites utilizing NaClO then hydrazine treatment w/tBuOH standard
$^1$H NMR of TEAB-complex glycans isolated from egg yolks utilizing NaClO then hydrazine treatment w/tBuOH standard
$^1$H NMR of Sucrose periodate oxidation intermediate
$^1$H NMR of Hyaluronic acid-TEAB after 230 hrs

$^1$H NMR of Hyaluronic acid-TEAB after 87 hrs
$^1$H NMR of Hyaluronic acid-TEAB after 50 hrs

$^1$H NMR of Hyaluronic acid-TEAB after 23 hrs
$^1$H NMR of Hyaluronic acid-TEAB after 5 hrs

96(3-93 Acetyle
$\rightarrow$186 mer
$\approx$70,534 g/mol

$\delta$ (ppm)

$\delta$ (ppm)
$^{1}\text{H, 2D COSY, NMR of Sucrose periodate oxidation post reductive amination (91)}$
2D TOCSY, $^{13}C\{^1H\}$ NMR of Sucrose periodate oxidation post reductive amination (91)
1D TOCSY, HSQC, NMR of Sucrose periodate oxidation post reductive amination (91)
HMBC NMR of Sucrose periodate oxidation post reductive amination (91)

ESI of major product from sucrose periodate oxidation (91) post reductive amination
$^1$H NMR of Sialic-acid-TEAB mixture of products (75:25; 94:93)

$^{13}$C($^1$H) NMR of Sialic-acid-TEAB mixture of products (75:25; 94:93)

COSY NMR of Sialic-acid-TEAB mixture of products (75:25; 94:93)
NOESY NMR of Sialic-acid-TEAB mixture of products (75:25; 94:93)
TOCSY NMR of Sialic-acid-TEAB mixture of products (75:25; 94:93)

$^1$H, $^{13}$C($^1$H), 2D COSY, 2D TOCSY and 2D NOSY NMR of major raffinose periodate oxidation post reductive amination (96/97)
$^{13}$C($^1$H), 2D COSY NMR of major raffinose periodate oxidation post reductive amination (96/97)
2D TOCSY and 2D NOSY NMR of major raffinose periodate oxidation post reductive amination (96/97)
$^1$H, $^{13}$C($^1$H) NMR of minor raffinose periodate oxidation post reductive amination (96/97)
2D COSY, 2D TOCSY NMR of minor raffinose periodate oxidation post reductive amination (96/97)
2D NOSY NMR of minor raffinose periodate oxidation post reductive amination (96/97)
H NMR of Isolated Sialylated Lactose-TEAB (100)

H NMR of pADPr (30 µg) attached to HIII histones, crude reaction
ADP-Ribose standard

NAD+ standard

PARP enzymatic extension at 18 hrs

100 mM Et$_3$NOAc buffer at pH ~5 from 1% to 5% can with a Vydac C18 column (2.5 mm x 30 mm)
$^1$H NMR of isolated precursor (~2:1 isomers) 5-MP (108)

$^1$H NMR of isolated Glycine ester 5-MP (110)
$^1$H NMR of attempted crude NHS ester activated 5-MP (112)

$^1$H NMR of attempted crude glucoseamine-5MP reaction precipitation from methanol (113)
$^1$H and $^{31}$P($^1$H) NMR of ADPr-TEAB crude product (103)

$^1$H NMR of C4 protected TBS-$\beta$-methyl-xyloside (128)
$^1$H NMR of 7-IodO-4-methylcoumarin (127)

Variations in synthesis of 7-IodO-4-methylcoumarin (89) no greater than 15% yield

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<tr>
<th>Conditions modified following described protocol</th>
<th>Yield of compound</th>
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<tr>
<td>$\text{H}_2\text{SO}_4$, NaNO$_2$ (1.1 eq.), KI (1.1 eq.), 24 hrs</td>
<td>15 mg (semi-pure)</td>
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<td>$\text{H}_2\text{SO}_4$, NaNO$_2$ (1.1 eq.), KI (10 eq.), 24 hrs</td>
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<td>$\text{H}_2\text{SO}_4$, NaNO$_2$ (1.1 eq.), KI (1.1 eq.), CuI (1.1 eq.), 24 hrs</td>
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<td>$\text{H}_2\text{SO}_4$, NaNO$_2$ (1.1 eq.), KI (1.1 eq.), CuI (1.1 eq.), reflux, 12 hrs</td>
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<td>$\text{H}_2\text{SO}_4$, NaNO$_2$ (1.1 eq.), KI (1.1 eq.), Cu (1.1 eq.), 24 hrs</td>
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<td>$\text{H}_2\text{SO}_4$, NaNO$_2$ (1.1 eq.), KI (3 eq.), 24 hrs</td>
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$^1$H and $^{13}$C{$^1$H} NMR of iodonium reagent (122)

$^{13}$C{$^1$H} NMR of iodonium reagent (122)
$^1$H NMR of attempted conjugation of iodonium to TBS-β-methyl-xyloside, no observable reaction occurring
Crude $^1$H and $^{13}$C($^1$H) NMR of boronic acid prepared as a BF$_4$ salt (131)
$^1$H NMR of 7-boronic acid-4-methylcoumarin (123)

$^{13}$C NMR of 7-boronic acid-4-methylcoumarin (123)
$^1$H NMR of sorbitol extracted crude reaction filtered through alumina.

$^1$H NMR of isolated acetylated-β-methyl-xyloside major product (131)
$^{13}$C NMR of isolated acetylated-β-methyl-xyloside major product (131)

$^1$H NMR of isolated acetylated-β-methyl-xyloside minor product (132)
$^1$H NMR of mixed β-methyl-xyloside isolated product (129+130)

$^{13}$C NMR of mixed β-methyl-xyloside isolated product (129+130)
COSY NMR of mixed β-methyl-xyloside isolated product (129+130)
HSQC NMR of mixed β-methyl-xyloside isolated product (129+130)
HMBC NMR of mixed β-methyl-xyloside isolated product (129+130)
$^{1}H$ NMR of various copper and nickel catalysts to promote conjugation to $\beta$-methyl-xyloside filtered through alumina.
Mixed-mode chromatography in pharmaceutical and biopharmaceutical applications
Author: Kelly Zhang Xiaodong Lu
Publication: Journal of Pharmaceutical and Biomedical Analysis
Publisher: Elsevier
Date: 5 September 2016
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Transformation of aldehydes into nitriles in an aqueous medium using O-phenylhydroxylamine as the nitrogen source
Author: Thongvannak Cheangvichitcha, Robert D. Hunt, Matthew Bickmore
Publication: Carbohydrate Research
Publisher: Elsevier
Date: April 2021
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BIOGRAPHY OF AUTHOR

Robert ‘Robin’ Daniel Hurst was born in his home country of England in Horsham, where he spent his childhood in the sleepy town. Robin has fond memories involving chemistry from his teenage years, where he insisted on proving wrong his apathetic chemistry teacher. He will never forget the day when he was told in a parent-teacher meeting that ‘Robin will be lucky if he achieves a D in chemistry’, fortunately for Robin, he figured out that he was taking the multiple-choice tests wrong as he was not reading the instructions and ended up with an A*. After succeeding in secondary school with high A’s across all classes, Robin stumbled his way through his college years taking A-levels and becoming an anguished teenager. Luckily an inspiring college teacher Dr. Rob Hussey took Robin under his tutelage and helped him realize what the color ‘pink’ was after failing his 3rd acid-base titration. Post stumbling through his awkward teenage years, Robin recovered academically and attended Bangor University, Wales. Where he discovered his love for the wilderness, attending 12-mile hikes around the Welsh countryside and mountains with his close-knit band of friends. Having spent 4 years within the Bangor walking club (UMWC) alternating between guide and social secretary, he met his future wife during his final year of his masters and proposed soon after she returned to the United States. Robin followed her to the University of Maine where he entered a Ph.D. program to finally prove that chemistry teacher wrong as well as to make Dr. Hussey proud of the knowledge that he imparted upon him. Spending 5 years under Prof. Brichacek, Robin has learned a great deal from his tutelage and has been grateful to have contributed to the field of glycochemistry; as well as having had the fantastic teaching opportunity as the senior department TA learning from the best of the chemistry department of UMaine. Robin is a candidate for the Degree of Doctor of Philosophy in Chemistry from the University of Maine in August 2023.