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Progress Towards a Novel Synthesis of a Glycosylated Asparagine Residue
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Abstract: Glycoproteins are important compounds that are studied for their ability to regulate cellular behavior in several important biological processes. The function of these glycosylated proteins is still vastly unknown due to obstacles faced with synthesis of their highly specified structure. Prior literature has been unable to isolate these compounds in a stable form which would reveal information about their structure-to-function relationship. Therein we have developed a novel synthesis that furnishes a high yield of a hydrolytically stable, glycosylated asparagine residue that is suitable for solid-phase peptide synthesis of N-linked glycoproteins.

Introduction
Protein glycosylation is essential to post-translational modification (PTM) that modulates protein stability, folding, and function. The covalent attachment of carbohydrates onto an asparagine residue is characteristic of N-linked glycosylation, a phenomena involved in the signal regulation, protein-protein interaction, and immune response of mammalian cells. The understanding of glycosylation is key to decoding many complex biological processes including inflammatory responses, neuronal development, and cellular communication events. Our comprehension of the structure to function relationship of the glycoproteome is impeded by non-isolable glycoforms that constitute heterogeneous reaction mixtures.

Previous attempts at developing a glycosylated amino acid residue that is viable for further study proved to be a complicated and often unsuccessful journey. The desired N-linked product was often hydrolytically unstable, prone to mutarotation, or simply too inconvenient to generate. Most literature involving the study of glycoproteins reported the isolation of a protected glycoprotein derivative, but could not study the function of their achieved structure.

In response to this discontinuity in scientific understanding, we report our progress towards a novel route for the generation of a glycosylated amino acid residue that is suitable for solid-phase peptide synthesis of N-linked glycoproteins. To subvert issues found with the β-glycosylamine utilized by other groups, we employed a β-glucosylamine surrogate which can be cleaved off during a subsequent step in the synthesis. An
initial study was conducted to determine optimal conditions for coupling glycosyl hydrazide and a model acid. We included a representative coupling agent from each major class: uronium salt, phosphorous mixed anhydride, carbodiimide, mixed anhydride, phosphonium salt. In addition to examining coupling agents, we worked towards attaining higher yield of pure glycosyl hydrazide coupled to an asparagine residue. We explored a number of reaction variables including stoichiometry, solvent, bases, additives, concentrations, and even temperatures. Expansion of the project goal included the broadening the subject scope from N-Acetylglucosamine to include sugars such as glucose, galactose, xylose, and cellobiose.

**Experimental**

While the different sugar starting material will undergo the same synthetic transformation, they require different reaction conditions for optimization of product yield. Heating the mixture of unprotected saccharides with benzoyl hydrazide in methanol linked the hydrazine group to the sugar’s anomeric or 1’-carbon.

*Scheme 1. Attachment of the Benzoyl Hydrazide onto the Saccharide*

N-Acetylglucosamine (GlcNAc) was treated with benzoyl hydrazide to create a 1:2 mixture in ethanol and deionized water (3:1) with a catalytic amount of acetic acid. The reaction was stirred at 60°C for 48 hours and monitored for completion by thin-layer chromatography. White solid precipitate was isolated by gravity filtration, concentrated using DCM/EtOH and toluene, and purified by silica gel column chromatography (5:1 DCM/MeOH). The conversion of glucose to a glucosyl hydrazide follows a slightly different procedure to GlcNAc. The 1:1.5 mixture of glucose and benzoyl hydrazide was dissolved in EtOH and a catalytic amount of AcOH. The reaction stirred at 80°C for 3 hours before precipitate was purified with a wash of hot and then cold EtOH under vacuum filtration. Galactose was introduced to benzoyl hydrazide as a 1:1.5 mixture in EtOH and deionized H$_2$O (3:1) along with AcOH catalyst. The reaction was set to stir at 60°C for 24 hours until the precipitate was purified with a wash of hot and then cold EtOH under vacuum filtration. Xylose was treated with benzoyl hydrazide to form a 1:1.5 mixture in EtOH and AcOH catalyst. The reaction was stirred at 78°C for 3 hours before precipitate was purified with a wash of hot and then cold EtOH under vacuum filtration.

*Scheme 2. Coupling of the Glycosyl Hydrazide and the Asparagine Residue*

The GlcNAc-hydrazide compound was
then coupled to the acyl chloride group of an Fmoc-protected asparagine residue at room temperature. The glycosyl hydrazide was dissolved in anhydrous dioxane supplemented with 4Å molecular sieves and sodium bicarbonate. The mixture was treated with duplicate equivalents of amino acid chloride wherein the reaction stirred for 1.5 hours before the addition of the second equivalent and was quenched with EtOH 1.5 hours after the addition of the second equivalent. A white precipitate was isolated by gravity filtration, concentrated, and purified by silica gel column chromatography (15:1 DCM/MeOH). The glycosyl hydrazide product developed using the glucose glycosyl hydrazide was also coupled to the Fmoc-protected amino acid residue at room temperature. Glucose coupling was conducted in a similar manner to the GlcNAc coupling, including the use of dioxane as a solvent and two equivalents of acyl chloride as the coupling agent. Purification of the coupled glycosyl hydrazide material was done by column chromatography (15:1 DCM/MeOH). The glycosyl hydrazide with the galactose attachment was also coupled to the Fmoc-protected amino acid residue at room temperature. The reacted mixture was purified by gravity filtration, concentrated, and purified by column chromatography (15:1 DCM/MeOH) to yield the coupled galactose and Fmoc-protected asparagine. The glycosyl hydrazide developed using the xylose saccharide was also dissolved in anhydrous dioxane and treated with two equivalents of amino acid chloride to yield the coupled saccharide and Fmoc-protected asparagine under an inert atmosphere. The completed reaction product was purified by column chromatography (15:1 DCM/MeOH). All purification reactions were analyzed by UV-vis and product characterization was done by NMR spectroscopy.

Scheme 3. Hydrolysis of Pfp Ester from Coupled Glycosyl Hydrazide

The highly reactive pentafluorophenyl (Pfp) ester which promotes the coupling reaction of the amino acid chloride with the GlcNAc glycosyl hydrazide of was later hydrolyzed to yield a carboxylic acid terminus. Hydrolysis was conducted at 60˚C for 24 hours using a 4:2:1 mixture of AcOH, THF, and deionized H2O. The product was co-evaporated with toluene and purified by column chromatography (5:1 DCM/MeOH, 0.5% AcOH). The Pfp ester of the glucose-coupled glycosyl hydrazide was hydrolyzed at 60˚C for 48 hours and purified by column chromatography (6:1 DCM/MeOH, 0.5% AcOH). The galactose-based coupled product hydrolyzed the pentafluorophenyl ester group at 60˚C up to 48 hours and the product was purified by column chromatography (6 DCM/MeOH, 0.5% AcOH). The xylose-based coupled product hydrolyzed Pfp at 60˚C for 24 hours and product was purified by column chromatography (7:1 DCM/MeOH, 0.5% AcOH). Hydrolysis of the coupled glycosyl hydrazides of all sugar derivatives succeeded in generating the terminal carboxylic acid on the asparagine residue. Characterization of hydrolyzed product was obtained by NMR spectroscopy and mass spectroscopy.
Scheme 4. Hydrazine Bond Cleavage from the Coupled Amino Acid

A samarium iodide cleaving agent was created by combining samarium metal and iodine dissolved in anhydrous THF in a 2:1 mixture, under an inert atmosphere for 60ºC for 18 hrs. The hydrazine bond depicted in the reaction scheme was then cleaved in the hydrolyzed product using SmI₂ in a solution of MeOH and THF. The cleavage produced the N-linked asparagine residue with a GlcNAc attachment. The hydrazine bond of the hydrolyzed and glucose-glycosylated amino acid was also cleaved by SmI₂ in a solution of MeOH and THF to produce the N-linked asparagine residue with a glucose attachment. The same reaction conditions were used to cleave the hydrazine bond of the galactose-glycosylated and xylose-glycosylated asparagine residues to produce an N-linked asparagine residue with a galactose and xylose attachment, respectively.

Results and Discussion

The various saccharides utilized in our experiment were chosen based on relevance to our understanding of glycoprotein function and based on previous literature. After optimization of reaction conditions to proceed with the glycosylating the asparagine residue, we were able to generate significant amounts of products from each step of the synthesis. Cellobiose was excluded from further study after low product yield and inconclusive characterization data that was obtained most likely due to its disaccharide structure that lowers thermodynamic stability of the product. The remaining saccharides had structures compatible with the chemistry being performed (Table 1) and thus underwent the mechanistic process in moderate to high yield, which allowed us to generate large amounts of hydrolyzed product ready for hydrazine bond cleavage to produce the glycosylated protein.

Table 1. Substituents on the Saccharides used for Protein Glycosylation

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>X₁</th>
<th>X₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylglucosamine</td>
<td>CH₃OH</td>
<td>Ph</td>
</tr>
<tr>
<td>Glucose</td>
<td>CH₃OH</td>
<td>OH</td>
</tr>
<tr>
<td>Galactose</td>
<td>CH₃OH</td>
<td>OH</td>
</tr>
<tr>
<td>Xylose</td>
<td>H</td>
<td>OH</td>
</tr>
</tbody>
</table>

*Hydroxyl group on 4'-carbon is axial whereas same region on all other sugars is equatorial.

Table 2. Product Yield Following Each Reaction Scheme

<table>
<thead>
<tr>
<th>Saccharide</th>
<th>Scheme 1</th>
<th>Scheme 2</th>
<th>Scheme 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Acetylglucosamine</td>
<td>84</td>
<td>38</td>
<td>88</td>
</tr>
<tr>
<td>Glucose</td>
<td>93</td>
<td>26</td>
<td>69</td>
</tr>
<tr>
<td>Galactose</td>
<td>64</td>
<td>62</td>
<td>-</td>
</tr>
<tr>
<td>Xylose</td>
<td>83</td>
<td>52</td>
<td>94</td>
</tr>
</tbody>
</table>

The initial step of the reaction links benzoyl hydrazide to the anomeric (1')-carbon of the saccharide. Due to the racemic nature of the 1'-hydroxy group on the sugar, we obtain a mixture of products included our desired β-glycosyl hydrazide. All four sugars were determined to have the beta configuration.
in high yield (Table 2), where purity was confirmed by TLC analysis. NMR spectroscopy was conducted to observe the protons on the sugar. The defining characteristic of a successful hydrazide addition is a singlet peak corresponding to a single anomeric proton adjacent to the proton on the beta-nitrogen of the hydrazide. Glucose was attached to the benzoyl hydrazide in greatest yield out of all the sugars at 93%. Galactose, a close relative to glucose differing only by the stereochemistry of one hydroxyl group, had the lowest product yield at 64%. Purification of the GlcNAc-glycosyl hydrazide required the use of silica gel chromatography and had a yield of 84% while all other sugar products were isolated using an ethanol wash.

The glycosyl hydrazide was coupled to the amino acid chloride in the following step. The reaction vessel was nitrogenated before and after addition of both the dioxane solvent as well as each equivalent of amino acid chloride. Sodium bicarbonate and molecular sieves were filtered from the mixture following the ethanol quench and the reaction was concentrated before purification. We obtained low to moderate yields for all sugars used in the starting material. Lower yields may be attributed to degraded quality of anhydrous solvent or incomplete dissolving of glycosyl hydrazide prior to addition of the chloride reagent. Galactose was coupled with highest yield at 62% and isolated as a pale yellow-white crystalline solid after purification. Glucose was coupled to the amino acid chloride with lowest yield at 26%.

We were able to convert glucose, GlcNAc, galactose, and xylose into the hydrolyzed and coupled glycosyl hydrazide observed after the termination of Scheme 3. Hydrolysis conditions were unchanging for each saccharide derivative. Acetic acid and deionized water aided in the cleavage of the Pfp ester and oxidation into a carboxylic acid. Duration of hydrolysis varied for each derivative and ranged from 24-48 hours. Reaction progresses was monitored by TLC analysis and reaction was terminated when there was sufficient purity of end product and lack of starting material. Purification involved the use of silica gel chromatography but column conditions varied for each coupled glycosyl hydrazide. Each sugar material required a small amount of acetic acid to aid in the elution of desired product from the column. Xylose required a more nonpolar environment to elute off the column and had the highest hydrolysis yield at 94%. The hydrolysis yield for galactose is not listed due to impurity in student-collected data.

Hydrazine bond cleavage was conducted by SmI₂ as per protocol from previous literature. Samarium iodide was generated in an anhydrous environment and the hydrolyzed material treated with cleaving agent was handled as a light-sensitive reaction. The round bottom flask was sealed to prevent oxidation and covered with aluminum foil to minimize light exposure. Only the hydrolyzed GlcNAc material was able to be cleaved and purified, as the other sugars underwent cyclization. The identity of this undesired material residing among the mixed products was determined from mass spectrometry analysis. Although the conversion to product was seen by TLC for the other glycosylated asparagine residues, the coelution of byproducts with the product material inhibited purification. Derivitizing these substrates with acetylation also proved unsuccessful due to cyclization of asparagine to the amine on C1.
Conclusion

Protein glycosylation is a critical PTM aids in cellular stability, folding, and function in all lifeforms, which makes it an important biological phenomenon to understand. In our research, glycosylation was conducted using GlcNAc, glucose, galactose, and xylose as the viable saccharide attachments. Optimal reaction conditions varied for each sugar, but all products exhibited the same structural characteristics. Hydrazide formation was effective in high yield and purity for all sugars. Coupling of the glycosyl hydrazide to the amino acid chloride yielded low amounts of products even after variation of coupling agent, temperature, solvent, and other reaction components conducted by previous members in the Langenhan laboratory. Hydrolysis was high in yield for all coupled glycosyl hydrazide products. Hydrazine bond cleavage only succeeded for the Glc-NAc starting material and was characterized by NMR and mass spectrometry. This project has found an efficient, novel synthesis for a glycosylated asparagine residue using Glc-NAc that does not experience the same instability and toxicity as its precursors from previous literature. A future goal for the group will be to obtain highly specific characterization data for all obtained products.

References