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Developing Anti-Adhesion Derivatives of N-acetylglucosamine for the Inhibition of Biofilm Formation

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Abstract

Antibiotic resistance has become a rising public health issue over the past several decades, with an urgent need to find a solution. Inhibiting the formation of biofilms would be an alternative anti-virulence strategy for treating infections while decreasing the spread of antibiotic resistance. Anti-virulence strategies utilize inhibitors that inhibit virulence instead of killing the bacterium, reducing the likelihood of bacteria developing resistance to the molecules. One bacterial virulence factor is adhesion; during colonization, bacterial pathogens adhere to host cells to avoid elimination by clearance mechanisms. Adhesion is mediated by bacterial surface lectins, in the form of elongated protein appendages, highly specific to carbohydrates on the host cell. We aim to mimic the carbohydrates found on the host cell to bind to the lectins on the bacterial pathogen and block adhesion. To achieve this, analogs of N-acetylglucosamine (GlcNAc) have been synthesized to target the F17G/GafD lectins found on strains of *Escherichia coli*. Due to carbohydrates exhibiting a relatively weak adhesion, focusing on increasing binding affinity is the main goal of this study. Modifications have been made to the anomeric position of GlcNAc to increase protein-carbohydrate contacts through hydrogen bonding and aromatic-aromatic interactions, in an effort to create higher affinity inhibitors. Three derivatives have been synthesized, achieved by the glycosidation of N-acetylglucosamine with 5-phenyl-1-pentanol, 2-phenylethyl alcohol, and 3-phenyl-1-propanol.

1. Introduction and Background

For decades, antibiotic resistance has persisted as a pressing concern, demanding an immediate resolution. With bacteria becoming resistant to antibiotics, there has been an increase in medical costs, hospital admissions, and mortality rates. In a 2019 report, antibiotic resistance has been the cause of 1.27 million deaths each year worldwide, with the United States having more than 2.8 million cases of antimicrobial-resistant infections annually.[1] Thirty-five thousand cases of antibiotic-resistant infections led to death in 2019 in the US and these numbers continue to increase.[1] One reason for these increases is the overuse of antibiotics. In 2014, the Center for Disease Control and Prevention (CDC) reported that 266.1 million courses of antibiotics were prescribed to outpatients in the United States, averaging about 5 prescriptions written annually for every 6 people. The CDC estimated that 28% of these antibiotics prescribed to outpatients were unnecessary. Inappropriate antibiotic use, including inappropriate selection, dosing, and duration, may soon approach nearly 50% of all antibiotic use in the upcoming years.[2]

Antibiotic resistance in bacteria can develop through several mechanisms, including the formation of biofilms of aggregated bacteria. This biofilm serves as a barrier that prevents antibiotics from penetrating and effectively killing the bacteria. Additionally, bacteria in biofilms often enter a dormant state, which lessens their susceptibility to antibiotics. Biofilms are common and are present in 60% to 80% of human microbial infections.[3,4] For bacteria to colonize a host and form biofilms, the bacterial pathogen often adheres to host cells to prevent them from being swept away during clearance mechanisms. Adhesion is mediated by bacterial surface lectins, presented on elongated protein appendages commonly referred to as fimbriae or pili, highly specific to carbohydrates on the host cell surfaces (Figure 1). The ability to inhibit the binding of the lectins by adding carbohydrates that are specific to the binding sites on the bacteria, and disrupting or preventing the formation of biofilms, could lead to the prevention of microbial diseases (Figure 2).[5]

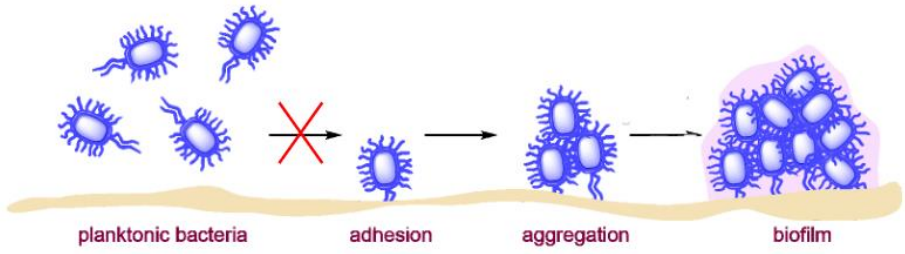


Figure 1. Biofilm formation process - preventing adhesion halts biofilm formation (McMahon)

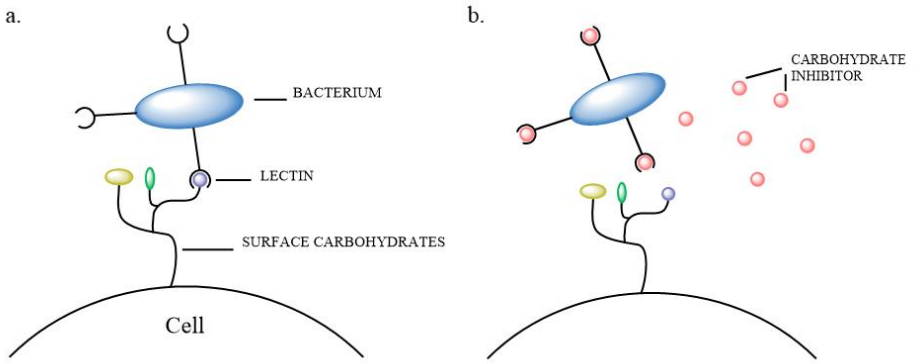


Figure 2. Inhibition of adhesion to the cell surface where (a.) represents normal adhesion between the bacterium and the lectin-binding site found on the cell surface. (b.) represents the adhesion of the carbohydrate-based inhibitor binding to the bacterium, thus preventing adhesion to the cell surface.

Carbohydrates exhibit relatively weak adhesion, with a low affinity for lectin-carrying bacteria. This implies that high doses or concentrations could be necessary for the successful prevention of bacterial adhesion.[6] This has presented issues for inhibitor design as the need for strong adhesion to the bacterial target site would ensure effective anti-virulence binding is successful. Some pathogens use several modes of adhesion, which may require a multi-target approach to find a carbohydrate that will successfully bind to all lectin sites. With this said, several hurdles must be addressed. First, there is an issue of the limited binding affinity of monovalent carbohydrates, which are essentially monosaccharides. This limitation arises because target proteins often exhibit multivalency, meaning they have multiple binding sites.[6] Hence, the interaction between monovalent carbohydrates and proteins tends to be low affinity. Additionally, there is the challenge posed by α- and β-anomers at the anomeric position, with β-anomers having the highest affinity, which further complicates the binding process.

In ongoing research, the ability to develop high-affinity monovalent ligand-carbohydrates is the main goal. Successful carbohydrate adhesion inhibitors would need to display at least some of the following characteristics: hydrophobic ridges, increased chain length, and/or aromatic rings.[7] It is also important that the carbohydrate synthesized has properties ensuring its stability in the body, including the ability to withstand pH changes while maintaining functionality. Higher affinity could also be accomplished by providing opportunities for additional hydrogen bonding, or by introducing covalent bonding in these binding pockets. This will enhance the inhibition potency, lowering the dosage needed overall. Increasing the binding affinity of monovalent carbohydrates could also be achieved by creating hydrophobic ridges to increase interactions with bacterial sites and improve the outcomes of interactions between the carbohydrates and the lectin protein's nonpolar regions. When looking at the ligand and the interaction between the

carbohydrate, extending the length of the spacer arms, or carbon chains, will help ensure that the carbohydrate has full access to the ligand binding site.[8] There would also be the need to account for the environment, such as the shape of binding pockets, and the orientation of the molecule where the binding will occur when deciding on a carbohydrate molecule that will best be suited for this task.

An example of this strategy being used in the past is with the treatment of uropathogenic *Escherichia coli*, which are the cause of urinary tract infections. Urinary tract infections (UTIs) pose a serious threat when it comes to antibiotic resistance. Patients who tend to get recurring UTIs are commonly treated with antibiotics, causing the development of antibiotic-resistant forms of uropathogenic *E. coli* (UPEC). FimH is a well-investigated lectin that is present on the type 1 pili in *E. coli.* Cusumano et al. studied UTIs and were able to confirm that these infections were caused by the adhesion of FimH to mannosylated receptors.[9] They were then able to synthesize a low-molecular weight mannoside that inhibits the adhesion of FimH to the urinary tract. Humans cannot completely metabolize D-mannose. Of the dose of D-mannose, 20-30% enters the urine from the blood circulation within 60 minutes, where it has the potential to interact with mannose-sensitive structures of UPEC, thereby decreasing the pathogenic effect of the bacterium. Approximately a third of supplemental D-mannose is excreted in the urine, where it has the potential to prevent pathogenic *E. coli* from adhering to uroepithelial cells.[10]

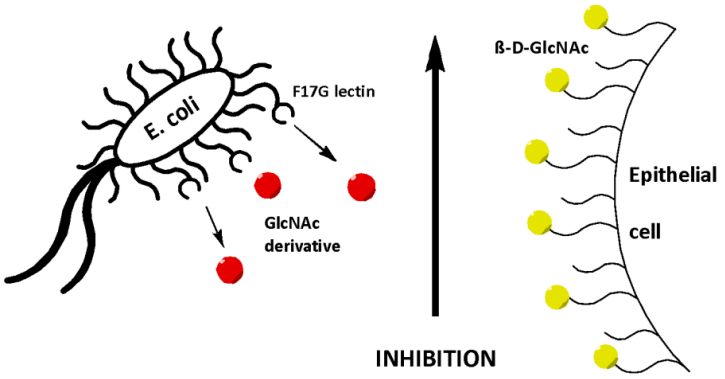
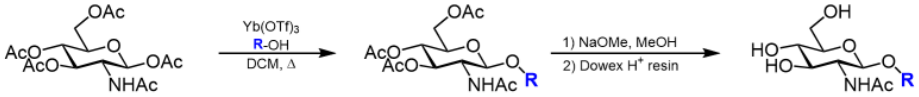


Figure 3. GlcNac derivatives to bind to the F17G lectin.[3]

We are studying another *E. coli* lectin, the F17G/GafD lectin, which binds to GlcNAc on epithelial cells. (Figure 3). F17G and GafD are very similar lectins, but F17G is present in *E. coli* bacteria in livestock and animals, while GafD is found in human pathogen strains. Recently, research has been conducted by previous and current members of the McMahon research group, to synthesize derivatives of N-acetylglucosamine (GlcNAc), a monosaccharide derivative of glucose, creating high-affinity inhibitors of the F17G/GafD lectins. Researchers focused primarily on both the anomeric and amide groups located on C1 and C2 of the GlcNAc structure, which included the synthesis of butyl, hexyl, and benzoyl amide derivatives. An enzyme-linked

immunosorbent assay (ELISA) will be conducted to test the binding affinity of their inhibitors in comparison to the natural GlcNAc ligand.

In the McMahon research lab, current studies use N-acetylglucosamine to create derivatives with the modification to the anomeric position (Scheme 1), attempting to increase the binding affinity of these inhibitors, preventing the adhesion of F17G/GafD lectins to the natural GlcNAc. Alcohols with longer carbon chains, aromatic groups, and potential hydrogen bonding interactions were chosen for the modifications because they are more likely to have a high binding affinity. This approach will help in identifying the most effective derivatives for the target protein. The results obtained will further aid in the development of future drugs, improving their efficiency and specificity.



Scheme 1. General synthesis scheme of N-acetylglucosamine (GlcNAc) derivatives at the anomeric position.

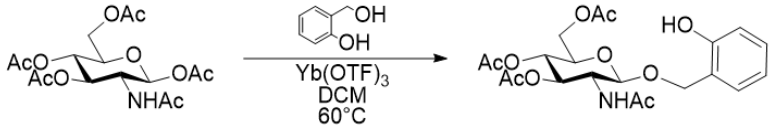
2. Experimental Methods

General Methodology

Glycosidation of N-acetylglucosamine with 2-hydroxybenzyl alcohol, 5-phenyl-1-pentanol, 5-benzylamino-1-pentanol, 2-phenylethyl alcohol, and 3-phenyl-1-propanol followed the general methodology from Schultz, V. et al.[11] All chemical reagents were purchased from commercially available sources. Anhydrous solvents were extracted from a solvent system and dried over molecular sieves. Thin layer chromatography (TLC) on SiliaPlates was used to track the progress and purity of the reactions, and UV light or potassium permanganate stain ($KMnO_4$) was used to visualize the spots. A Heidolph rotary evaporator was used to concentrate reaction samples. Column chromatography using silica (SiO_2 40-63 µm, 230-400 mesh) was used for purification. Proton nuclear magnetic resonance (^{1}H-NMR 400 MHz Varian spectrometer) spectroscopy was used to characterize the products using standards of $CDCl_3$ at 7.27 ppm.

2.1. Synthesis of (2R,3S,4R,5R,6R)-5-acetamido-2-(acetoxymethyl)-6-((2-hydroxybenzyl)oxy)tetrahydro-2H-pyran-3,4-diyl diacetate[11]

2.1.1. Glycosidation of per-acetylated N-acetylglucosamine with 2-hydroxybenzyl alcohol

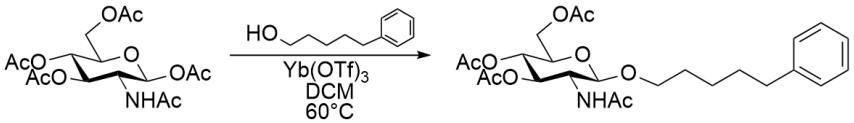


Scheme 2. Synthesis of acetylated 2-hydroxybenzyl glycoside.

Commercially available per-acetylated N-acetylglucosamine (0.25 g, 0.64 mmol, 1 equiv.) was refluxed with 2-hydroxybenzyl alcohol (0.25 g, 2.02 mmol, 3.15 equiv.), $Yb(OTf)_3$ (0.0397 g, 0.064 mmol, 0.1 equiv.) in dichloromethane (DCM) (5.0 mL), and left to stir at 60 °C overnight. The solution was allowed to cool to room temperature and then added to a separatory funnel with DCM, then washed three times with deionized water. The organic layer was then dried over magnesium sulfate and filtered. Column chromatography with a mobile phase of 1:5 hexane:ethyl acetate was used for purification. Samples were concentrated using rotary evaporation, resulting in a white crystalline product. Characterization of the structure was analyzed using ^{1}H-NMR spectroscopy, but due to unclear results, continuation of characterization for this product was not taken.

2.2. Synthesis of N-((2R,3R,4R,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-((5-phenyl-pentyl)oxy)tetrahydro-2H-pyran-3-yl)acetamide[11]

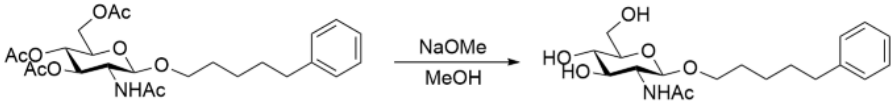
2.2.1. Glycosidation of per-acetylated N-acetylglucosamine with 5-phenyl-1-pentanol



Scheme 3. Synthesis of acetylated 5-phenylpentyl glycoside.

Commercially available per-acetylated N-acetylglucosamine (0.25 g, 0.64 mmol, 1 equiv.) was refluxed with 5-phenyl-1-pentanol (0.340 mL, 2.02 mmol, 3.15 equiv.) $Yb(OTf)_3$ (0.0397 g, 0.064 mmol, 0.1 equiv.), and in dichloromethane (5.0 mL), left to stir at 60 °C overnight. The solution was allowed to cool to room temperature and then added to a separatory funnel with DCM, then washed three times with deionized water. The organic layer was then dried over magnesium sulfate and filtered. Column chromatography with a mobile phase of 1:5 hexane:ethyl acetate was used for purification. Samples were placed in the rotary evaporator to remove any unwanted solvents, leaving behind a white solid (27.4%). The structure was confirmed using ^{1}H-NMR spectroscopy. ^{1}H NMR ($CDCl_3$): δ 7.1-7.3 (m, 5H, Ph), 5.46 (d, *J* = 8.4 Hz, 1H, NH), 5.3 (t, *J* = 9.6 Hz, 1H), 5.05 (t, *J* = 9.6 Hz, 1H, CHO_2), 4.65 (d, *J* = 8.4 Hz, 1H), 4.25 (dd, *J* = 2.4, 2.4 Hz, 1H), 4.15 (dd, *J* = 4.4, 4.8 Hz, 1H), 3.88 (m, 2H, OCH_2), 3.65 (m, 1H), 3.45 (m, 1H, OCH_2), 2.08, 2.05, 1.9 (s, 12H, 3 $COCH_3$), 1.30 (br s, 8H, OCH_2-$(CH_2)_4$-Ph).

2.2.1.1 Deacetylation of per-acetylated N-acetylglucosamine with 5-phenyl- 1-pentanol



Scheme 4. Zemplen deacetylation of acetylated 5-phenylpentyl glycoside.

Deacetylation of acetylated 5-phenylpentyl glycoside was achieved by dissolving the compound (0.06 g, 0.122 mmol, 1 equiv.) in anhydrous methanol (0.08M, 0.73 mL) and sodium methoxide (0.5M, 0.73 mL, 3 equiv.) and stirring at room temperature, overnight, in an inert argon atmosphere using a balloon. Dowex resin was added to the mixture and stirred for 10 minutes until a final pH of 4 was obtained. The resin was then removed by filtration, and the mixture was concentrated by rotary evaporation. The product was then purified using C18 Sep-pak, using 5:1 dichloromethane:methanol, to yield a white solid (88%). Characterization of the structure was confirmed using ^{1}H-NMR spectroscopy. ^{1}H NMR (CD_3OD): δ 7.1-7.3 (m, 5H, Ph), 4.4 (d, 1H, *J* = 8.4 Hz, 1H, anomeric), 3.25-3.8 (m, 8H, CH_2), 2.62 (t, *J* = 3.8 Hz, OCH_2), 1.95 (d, *J* = 10 Hz, 3H, $NHCOCH_3$), 1.3-1.7 (br m, 8H, OCH_2-$(CH_2)_4$-Ph).

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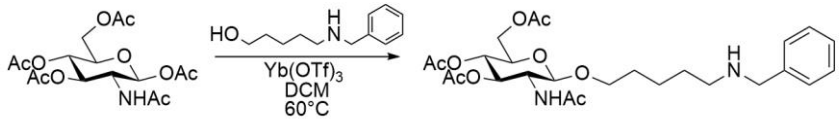
2.3. Synthesis of N-((2R,3R,4R,5S,6R)-2-((5-(benzylamino)pentyl)oxy)-4,5-dihydroxy- 6-(hydroxymethyl)tetrahydro-2H-pyran-3-yl)acetamide[11]

2.3.1. Glycosidation of per-acetylated N-acetylglucosamine with 5-benzylamino-1-pentanol

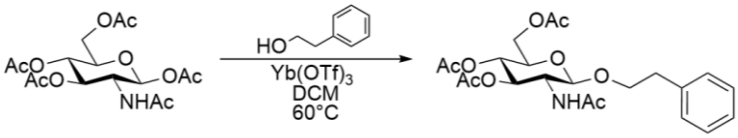


Scheme 5. Synthesis of acetylated 5-benzylamino pentanol glycoside.

Commercially available per-acetylated N-acetylglucosamine (0.25 g, 0.64 mmol, 1 equiv.) was refluxed with 5-benzylamino-1-pentanol (0.387 mL, 2.016 mmol, 3.15 equiv.) and $Yb(OTf)_3$ (0.0397 g, 0.064 mmol, 0.1 equiv.), and in dichloromethane (5.0 mL), left to stir at 60 °C for 5 days total. Initially, it was planned to run this reaction for 48 hours. The extra time was due to a power outage occurring, as well as the hot plate malfunctioning, creating an unknown period outside the desired temperature. The solution was allowed to cool to room temperature and then added to a separatory funnel with DCM, then washed three times with deionized water. The organic layer was then dried over magnesium sulfate and filtered. Column chromatography with a mobile phase of 1:5 hexane:ethyl acetate was used for purification. Samples were placed in the rotary evaporator to remove any unwanted solvents, leaving behind a red oil-like residue. Characterization of the structure was analyzed using ^{1}H-NMR spectroscopy and determined that this reaction was unsuccessful.

2.4 Synthesis of N-((2R,3R,4R,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-phenethoxy- tetrahydro-2H-pyran-3-yl)acetamide[11]

2.4.1. Glycosidation of per-acetylated N-acetylglucosamine with 2-phenylethyl alcohol

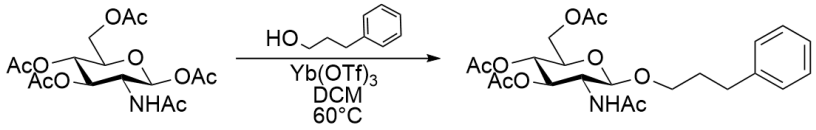


Scheme 6. Synthesis of acetylated 2-phenylethyl alcohol glycoside.

Commercially available per-acetylated N-acetylglucosamine (0.25 g, 0.64 mmol, 1 eq.) was refluxed with 2-phenylethyl alcohol (0.242 mL, 2.016 mmol, 3.15 eq), $Yb(OTf)_3$ (0.0397 g, 0.064 mmol, 0.1 eq.), and in dichloromethane (5.0 mL), left to stir at 60 °C for 48 hours. The solution was allowed to cool to room temperature and then added to a separatory funnel with DCM, then washed three times with deionized water. The organic layer was then dried over magnesium sulfate and filtered. Column chromatography with a mobile phase of 1:5 hexane:ethyl acetate was used for purification. Samples were placed in the rotary evaporator to remove any unwanted solvents, leaving behind a white solid. The crude structure was confirmed using ^{1}H-NMR spectroscopy, accounting for impurities that are seen in the spectra. ^{1}H NMR ($CDCl_3$): δ 7.20-7.33 (m, 5H, Ph), 5.27 (d, J = 8.6 Hz, 1H, NH), 5.25 (d, J = 9.3 Hz, 1H, CH), 5.07 (t, J = 19.2 Hz, 1H, CH), 4.61 (d, J = 8.3 Hz, 1H, anomeric), 4.27 (m, 1H, CH), 4.14 (m, 1H, CH), 3.88 (m, 2H, OCH_2), 2.93 (d, J = 10.3 Hz, 1H, CH_2), 2.88 (t, J = 6.5 Hz, 1H, CH_2), 2.11, 2.09, 2.08, 1.9 (s, 12H, $4COCH_3$).

2.5. Synthesis of N-((2R,3R,4R,5S,6R)-4,5-dihydroxy-6-(hydroxymethyl)-2-(3-phenyl-propoxy)tetrahydro-2H-pyran-3-yl)acetamide[11]

2.5.1. Glycosidation of per-acetylated N-acetylglucosamine with 3-phenyl-1-propanol



Scheme 7. Synthesis of acetylated 3-phenyl-1-propanol glycoside.

Commercially available per-acetylated N-acetylglucosamine (0.25 g, 0.64 mmol, 1 eq.) was refluxed with 3-phenyl-1-propanol (0.274 mL, 2.016 mmol, 3.15 eq), $Yb(OTf)_3$ (0.0397 g, 0.064 mmol, 0.1 eq.) and in dichloromethane (5.0 mL), left to stir at 60 °C for 48 hours. The solution was allowed to cool to room temperature and then added to a separatory funnel with DCM, then washed three times with deionized water. The organic layer was then dried over magnesium sulfate and filtered. Column chromatography with a mobile phase of 1:5 hexane:ethyl acetate was used for purification. Samples were placed in the rotary evaporator to remove any unwanted solvents, leaving behind a white solid. The crude structure was confirmed using ^{1}H-NMR spectroscopy, accounting for impurities that are seen in the spectra. ^{1}H NMR ($CDCl_3$): δ 7.17-7.32 (m, 5H, Ph), 5.47 (d, J = 8.5 Hz, 1H, NH), 5.29 (t, J = 9.4, 1H, CH), 5.08 (t, J = 9.6 Hz, 1H, CH), 4.67 (d, J = 8.3 Hz, 1H, anomeric), 4.26 (dd, J = 4.8, 4.8 Hz, 2H, CH_2), 4.14 (d, J = 2.4 Hz, 1H, CH),

3.88 (m, 2H, OCH_2), 3.50 (m, 2H, OCH_2), 2.5 (m, 1H, CH), 2.06, 1.91 (m, 12H, $3COCH_3$), 1.28 (dd, J = 3.6, 5.9 Hz, 2H, CH2).

3. Results and Discussion

The glycosidation product of per-acetylated N-acetylglucosamine with 2-hydroxybenzyl alcohol was not successfully synthesized. Multiple compounds were detected by ^{1}H NMR, making it challenging to identify them. This might have happened due to ytterbium triflate reacting with the 2-hydroxybenzyl alcohol, leaving the starting GlcNAc material unreacted. Another possible outcome was that two different products could have been synthesized due to the multiple hydroxyl groups located on the molecule. Further optimization of the reaction conditions and purification methods may be necessary to obtain the desired product. A different glycosylation strategy, such as variations in the time the initial reaction is left to stir at 60 °C, could be explored to achieve the desired product. Another strategy could include adding a protection group to one of the hydroxyl groups.

The analogous reaction with 5-phenyl-1-pentanol was successful in producing the desired product. After fractions were separated by column chromatography, they were concentrated using a rotary evaporator, yielding a white solid, and an oil-like substance which was determined to be our starting material. A yield of 27.4% was obtained of pure product. This low yield could have been a result of unreacted starting material during synthesis and/or product loss during the transfer steps, but referring to the Schultz, V. et al. literature, a lower yield is to be expected. Product structures were confirmed using ^{1}H-NMR. Peaks used to determine if our alcohol had reacted with peracetylated GlcNAc and added in at the anomeric position include the new aromatic signals found at 7.25 ppm, as well as the anomeric proton peak at 4.4 ppm, shifted upfield from 7.3 ppm in going from an acetate ester to an alkyl acetal. Once the structure had been confirmed, the final deprotection of the compound was also successful, with a yield of 88% for the deprotected product. Final key ^{1}H NMR characterization peaks include the aromatic protons found at 7.25 ppm, and an anomeric proton at 4.40 ppm. The peaks around 2 ppm in the original NMR were from the acetate groups; with a successful reaction being indicated by those peaks no longer being there. Also, the CH/CH_2 peaks next to the hydroxyl groups on the ring have shifted from 4 ppm to the 3 ppm range.

The glycosidation reaction with per-acetylated N-acetylglucosamine with 2-phenylethyl alcohol and 3-phenyl-1-propanol was successful in producing the desired product, based on crude ^{1}H-NMR. Modifications were made for these reactions, extending the reaction time to 48hrs to compensate for unreacted starting material observed in the former reaction. Characterization of the crude products involved similar peak analysis in ^{1}H-NMR, focusing on aromatic protons, acetate groups, and varying alkane groups located upfield around 1-2 ppm. Future steps include column chromatography to separate any byproducts and concentration via a rotary evaporator, along with confirmation of product structures after final deprotection steps are completed.

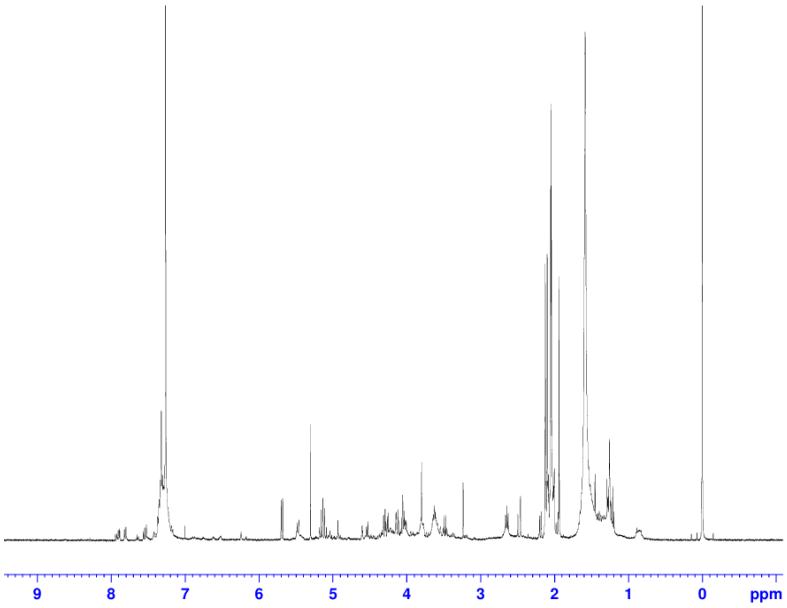


Figure 4. ^{1}H NMR results of the glycosidation product of per-acetylated N-acetylglucosamine with 5-benzylamino pentanol. The peak found at 5.7ppm helps determine that synthesis of our desired compound was unsuccessful. This is due to the anomeric proton with an acetate group still bound at the anomeric position. This peak is found more downfield than expected, which would be expected if our alcohol molecule did not bind to GlcNAc.

The glycosidation product of per-acetylated N-acetylglucosamine with 5-benzylamino pentanol was not successfully synthesized. While analyzing the ^{1}H NMR results, it appeared to be mostly starting material, with no clear indication of our compound being synthesized. In determining this, the doublet peak located at 5.7ppm is likely the anomeric position that is bound to an acetate group, found on the starting material, meaning our alcohol did not react (Figure 4). The acetate group is more electron withdrawing, which would explain why our peak is located more downfield. Synthesis could have been impacted by a power outage occurring twice for an unknown length of time, as well as hot plate errors, thus, creating fluctuations in temperature. Due to these circumstances, the initial reaction was left over the course of 5 days.

4. Conclusion

The focus of this research is to develop GlcNAc derivatives for the inhibition of the F17G/GafD lectins present on enteropathogenic *E. coli*. Multiple GlcNAc derivatives modified at the anomeric position were successfully synthesized in low to moderate yield.

Modifications have been made to previously establish synthetic pathways to further optimize the yield of GlcNAc derivatives, such as increased reaction time, the use of high-pressure sealed flasks, and the use of Dowex resin rather than Amberlite. Future and ongoing studies are being attempted to synthesize more derivatives. Once fully purified and characterized, this library of derivatives will be tested for their inhibitory ability of F17G binding via an ELISA-like assay.

Acknowledgment

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