Synthesis of Anti-Adhesion Carbohydrates Against Bacterial Biofilms

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Abstract

Antibiotic resistance is a growing problem as misuse of these drugs has increased and more bacterial infections become resistant. Bacterial adhesion to host cells is a virulence factor leading to colonization and biofilm formation, making infections harder to treat. Anti-virulence methods block bacterial virulence factors, like adhesion, providing an alternative to antibiotic treatment. Creating anti-adhesion molecules to bind to bacteria, making them incapable of adhering to a host cell, is a known anti-virulence method. F17G and GafD are *E. coli* lectins that facilitate adhesion by binding to N-acetyl-ß-D-glucosamine (GlcNAc) found in the microvilli of intestinal epithelial cells. The goal of this project is to make derivatives of GlcNAc to serve as inhibitors of F17G/GafD-mediated *E. coli* adhesion. Multiple modifications have been made at the amide position to increase the potency of inhibitors by varying sterics, electronics, and binding groups. Synthesized inhibitors have been purified to be used for future testing of their effectiveness at halting adhesion.

1. Introduction and Background

Antibiotic resistance is rapidly growing as a public health crisis due to the overuse of antibiotics. Development of antibiotics is currently a challenge as bacteria quickly develop resistance before the new antibiotics can be useful. According to the Center for Disease Control (CDC), approximately 2.8 million people become infected with antibiotic resistant bacteria and 35,000 people die from these infections every year.[1] The evolutionary trait of antibiotic resistance develops quickly and bacteria have developed many mechanisms to evolve with antibiotics. One factor, known as a biofilm, occurs when the bacteria adhere together and to the host cell, creating a larger mass of bacteria that is difficult to break through. In biofilms, bacteria produce polymeric molecules that add another barrier of separation between them and antibiotics, known commonly as an extracellular matrix.[2] When bacteria are in biofilms, the bacteria's growth and metabolism slow which makes them further resistant to antibiotics as antibiotics target growth and metabolic processes.[4] As bacteria evolve and find new ways to resist antibiotics, a different method to treat bacterial infections has emerged, known as anti-virulence strategies.

Anti-virulence aims to treat bacterial infections by targeting virulence factors, such as biofilm formation, that bacteria use during colonization and infection, while not killing the bacteria. Other examples of anti-virulence strategies include, but are not limited to, the inactivation of bacterial toxins and the neutralization of antitoxin antibodies after a patient has been exposed, blocking adhesion, and inhibiting quorum sensing.[9] This project aims to block the virulence factor of adhesion, which is the first step in biofilm formation. As anti-virulence strategies do not kill the bacteria, there is less evolutionary pressure for the bacteria to develop resistance.[3] This research project aims to utilize anti-virulence strategies to stop the formation of biofilms.

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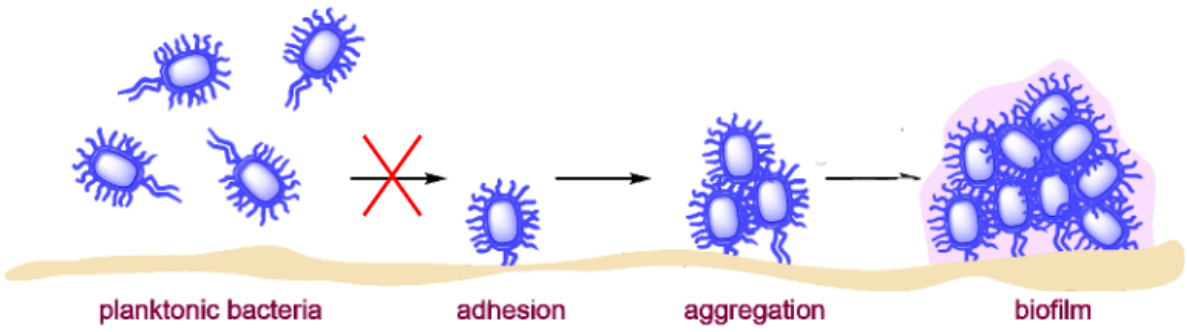


Figure 1. Biofilm formation step, red X indicates where inhibitors halt the biofilm process (McMahon)

As demonstrated in Figure 1, adhesion is a virulence factor that is the beginning step in biofilm formation. The adhesion step is mediated by lectin-carbohydrate interactions in which bacterial lectins bind to the host cell's carbohydrate outer coating.[4] As lectins are the driving force of bacterial adhesion and in turn, bacterial biofilms, it is a priority to halt these interactions to combat antibiotic resistance. *Escherichia coli* (*E. coli)* in particular has several different lectins dependent on the strain, expressed on long extensions from the surface of a bacterial cell called fimbriae. In naturally occurring settings, lectins have a low binding affinity to carbohydrate ligands.[6] Research on an *E coli.* lectin, FimH, which plays a role in urinary tract infections, has helped showcase how synthetic inhibitors can stop adhesion.[7] FimH naturally binds to the carbohydrate mannose, and several research groups have developed potent mannoside FimH inhibitors. Han et al. found that they were able to block adhesion with the addition of biaryl groups with hydrogen bond acceptors such as an amide.[15] Janetka et. al used their mannose derivatives that have a higher binding affinity with FimH in clinical trials for *E. coli* urinary tract infections.[17]

This project is focused on two of *E. coli's* lectins, F17G and GafD. F17G is the primary lectin of interest as it has been identified in human infections and GafD is the lectin found in livestock infections.[5] The F17G and GafD lectins found in enterotoxigenic *E. coli* are almost identical due to their conserved sequence and structure.[5] The F17G lectin is an adhesive protein that binds to N-acetyl-ß-D-glucosamine (GlcNAc)-presenting receptors on the microvilli of intestinal epithelial cells.[7] As seen in Figure 2, the crystal structure of F17G is known and the binding site for GlcNAc has been identified.[16] The structure of F17G allows for more information to create an inhibitor that could have higher binding affinity than the natural ligand. This project will develop carbohydrate-based inhibitors that prevent lectin binding based on the F17G crystal structure to host ligands.

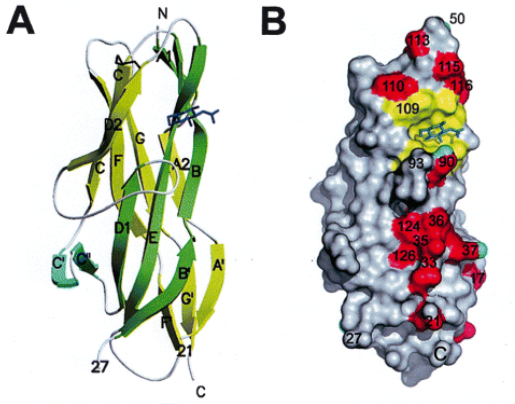


Figure 2. A. Ribbon structure of F17G B. Crystal structure of F17G lectin with GlcNAc binding site shown[16]

For this project, a successful inhibitor will serve as an anti-adhesion molecule that helps to disrupt biofilms and treat bacterial infections while minimizing potential development of antibiotic resistance using anti-virulence strategy. Modified derivatives of GlcNAc are designed to bind to the lectin with an increased binding affinity. Previous researchers in the McMahon group have begun to work on the synthesis of these derivatives. The McMahon group has been able to optimize key steps in the synthetic scheme and making various modifications at the anomeric and amide position on GlcNAc. Many of these derivatives have varying chain lengths and aromatic groups due to interest in sterics and π-stacking capabilities, further considerations for derivatives involving electronics is of interest. These inhibitors will be implemented in various binding assays to determine their ability to inhibit biofilm formation.

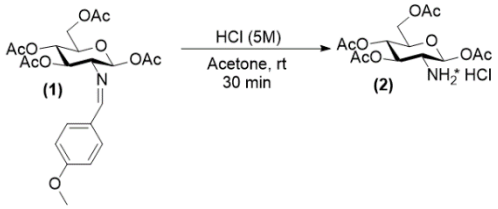
2. Experimental Methods

2.1 General Methodology

Chemical reagents were acquired commercially. All reaction flasks were first oven dried and then cooled and kept under an inert atmosphere using argon gas, unless stated otherwise. Reactions were monitored using thin layer chromatography (TLC) on SiliaPlates (TLG-R10014B-323) and visualized with ultraviolet light and potassium permanganate stain ($KMnO_4$). All concentrations were done under reduced pressure using a Heidolph rotary

evaporator. Analysis of crude and purified products was performed on a 400 MHz Varian nuclear magnetic resonance (NMR) spectrometer. NMR solvent resonance was used as the internal standard - ^{1}H spectra were referenced to 7.27 ppm ($CDCl_3$), 4.80 ppm (DMSO) and 4.78 (1) ppm (CD_3OD). ^{1}H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, dd = doublet of doublets, ddd = doublet of doublet of doublets, td = triplet of doublets, qd = quartet of doublets, m = multiplet, br. s.= broad singlet), coupling constants (Hz), and integration.

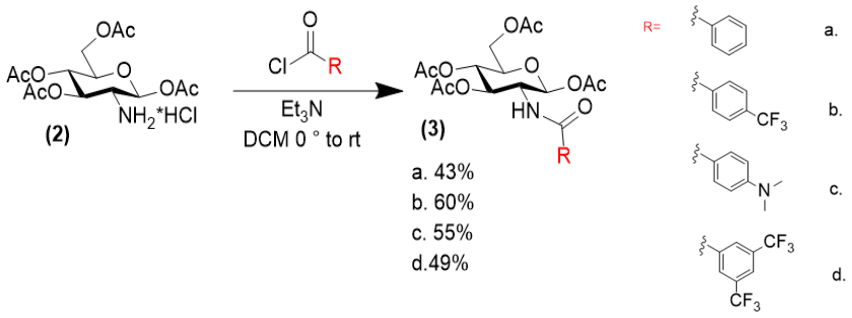
2.2 General Procedure for Amide Formation



Scheme 1: Deprotection of acetoxy imine to form acetoxyl Glucosamine hydrochloride

Synthesis of **2** was adapted from the literature.[11] **1 (1.83 mmol, 850 mg, 1 equiv)** was stirred in acetone **(**6.9 mL**)**. Hydrochloric acid **(HCl - 5M, 0.43 mL)** was added and the reaction was stirred for 30 minutes at room temperature. An additional portion of acetone (4 mL) was added to the flask as the reaction solidified. After 30 minutes, diethyl ether (7 mL) was added to the reaction and stirred for another hour at room temperature. The crude product was then vacuum filtered and washed with diethyl ether to yield a white, flaky solid (0.635g, 91% yield). Product NMR data matched previous literature and was then immediately used for the following reactions.

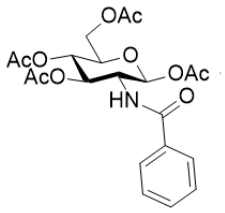
2.3 Procedure for Derivatization at Amide Position of Per-acetylated Glucosamine Hydrochloride



Scheme 2: Addition of benzoyl to Glucosamine hydrochloride

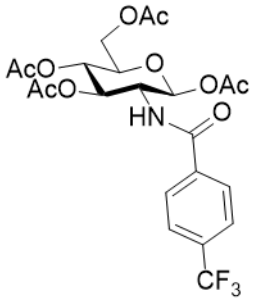
Synthesis of 3 was adapted according to the literature.[12] **2 (1 equiv)** was mixed with triethylamine **(2 equiv)** and benzoyl chloride **(1.2 equiv)** and stirred in 0. 2M dichloromethane (DCM) at 0 °C then warmed to room temperature and stirred for 2 hours. The reaction was monitored by using thin layer chromatography (TLC). The crude products were diluted with ethyl acetate and the organic layer was washed successively with deionized water, 1 M NaOH and saturated NaCl brine. Purification utilized column chromatography with gradient mobile phases. Fractions containing the product (as confirmed by TLC) were combined and concentrated by rotary evaporation at 40 °C, resulting in a white solid product confirmed via spectroscopy.

2.3a Benzoyl Derivatization at Amide Position of Per-acetylated Glucosamine Hydrochloride



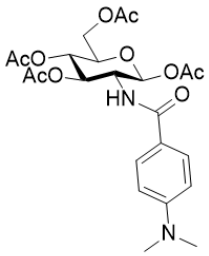
The product was made according to the general procedure. The product was purified via column chromatography with a gradient mobile phase of 100% DCM to 20:1 DCM:MeOH. Product yielded a white solid (43%). Product NMR data matched previous literature[12]. ^{1}H NMR (400 MHz,$CDCl_3$) δ = 7.7 (4H, d, J= 7.2 Hz), 7.5 (2H, d, J= 7.6 Hz), 7.4 (4H, t, J= 7.2), 6.4 (2H, d, J=7 Hz), 5.8 (2H, d, J= 8.8 Hz), 5.26 (4H, t, J=4 Hz), 4.6 (2H, s), 4.4 (2H, dd, J_1=4.4 Hz, J_2=12.6 Hz), 3.9 (4H, s), 2.19 (1H, s), 2.13 (2H, s), 2.08 (4H ,d, J=6.8 Hz), 2.01 (2H, s), 1.26 (2H, s).

2.3b 4-trifluoromethylbenzoyl Derivatization at Amide Position of Per-acetylated Glucosamine Hydrochloride



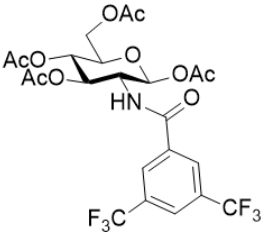
The product was made according to the general procedure. The product was purified with column chromatography with a gradient mobile phase of 100% DCM to 10:1 DCM:MeOH. Product yielded a white solid (60%). Product NMR data matched previous literature[12]. ^{1}H NMR (400 MHz,$CDCl_3$) δ =8.2 (5H, dd, J_1=8.4, J_2=12.8), 7.83 (5H, d, J= 8 Hz), 7.73 (10H, dd, J_1=8.4 Hz, J_2=18.8 Hz), 6.49 (2H, d, J=9.2 Hz), 5.84 (2H, d, J=9.2 Hz), 5.29-5.25 (5H, m), 4.57 (2H, d, J=9.6 Hz), 4.43 (1H, d, J= 7.2 Hz), 4.32 (3H, dd, J_1=4.8 Hz, J_2=12.4 Hz), 4.19-4.11 (18H, m), 3.68 (1H, s), 3.51 (1H, s), 2.15 (2H, s), 2.11 (2H, s), 2.08 (4H, s), 2.03 (1H, s), 1.43 (2H, t, J= 7.2 Hz).

2.3c 4-N,N-dimethylbenzoyl Derivatization at Amide Position of Per-acetylated Glucosamine Hydrochloride



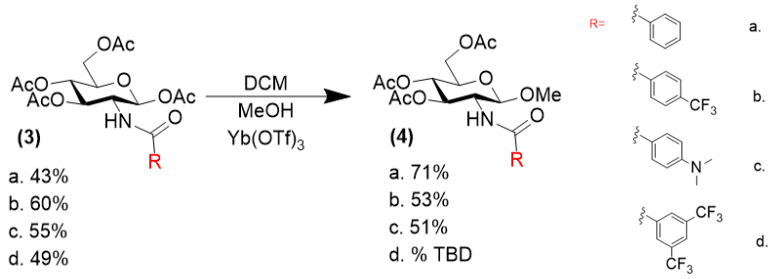
The product was made according to the general procedure. The product was purified with column chromatography in 10:1 DCM:MeOH. Product yielded a white solid (55%). Product NMR data matched previous literature[12]. ^{1}H NMR (400 MHz,$CDCl_3$) δ = 7.6 (5H, d, J=8.4 Hz), 6.65 (5H, d, J=8 Hz), 6.01 (2H, d, J=9.2 Hz), 5.79 (2H, d, J=9.2 Hz), 5.25-5.21 (4H, m), 4.56 (2H, s), 4.3 (2H, d, J= 12.8 Hz), 4.16 (2H, d, J= 12 Hz), 3.85 (2H, s), 2.18 (4H, quint., J= 1.6 Hz), 2.12 (7H, d, J= 4 Hz), 2.06 (14H, s).

2.3d 3,5-bis(trifluoromethyl) benzoyl Derivatization at Amide Position of Per-acetylated Glucosamine Hydrochloride



The product was made according to the general procedure. The product was purified via column chromatography using a mobile phase of 20:1 DCM:MeOH. Product yielded a white solid (49%). Product NMR data matched previous literature[12]. ^{1}H NMR (400 MHz,$CDCl_3$) δ =8.49 (1H, s), 8.2 (4H, s), 8.1 (1H, d, J=14 Hz), 7.99 (2H, s), 6.89 (2H, d, J=9.6 Hz), 5.82 (2H, d, J= 9.2 Hz), 5.38 (2H, t, J=9.6 Hz), 5.27 (3H, q, J= 8.8 Hz), 4.63 (2H, q, J= 7.2 Hz), 4.47 (2H, q, J= 6.8 Hz), 4.42 (1 H, q, J=7.6 Hz), 4.32 (2H, dd, J_1=4.4 Hz J_2=10.8 Hz), 4.2-4.12 (2H, m), 3.96-3.91 (2H, m), 2.11 (3H, d, J= 10.4 Hz), 2.07 (1H, d, J=12.4 Hz), 1.47 (3H, t, J=6.8 Hz).

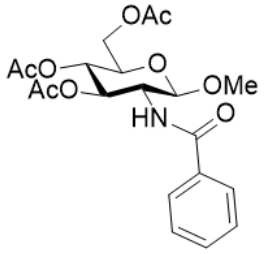
2.4 General Procedure for Methyl Glycosylation of Per-acetylated Derivatives



Scheme 3: Glycosylation at the anomeric position of benzoyl Glucosamine derivatives

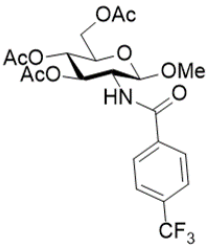
Synthesis of **4** was adapted according to the literature.[13] **3 (1 equiv)** was mixed with anhydrous DCM **(1 mL)** and ytterbium(III) trifluoromethanesulfonate ($Yb(OTf)_3$) **(0.12 equiv)** were combined and stirred in anhydrous methanol **(3.2 equiv)** for 1 hour at 60 °C in a high-pressure flask with a blast shield. After 1 hour, additional anhydrous DCM and methanol were added to the round bottom flask. The reaction was left to run for another 24 hours before being removed from the heat to cool to room temperature. The reaction flask was rinsed with DCM to transfer into a vial to wash the organic layer. The organic layer was washed with deionized (DI) water three times before being dried over $MgSO_4$ and filtered. The condensed product resulted in a white solid. The reaction was monitored by using thin layer chromatography (TLC) and purified with column chromatography phase. Confirmed products via spectroscopy.

2.4a Benzoyl Derivative of Methyl Glycosylation of Per-acetylated Derivatives



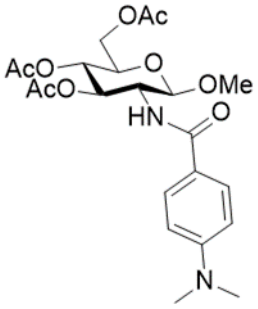
The product was made according to the general procedure. The product was not purified and was immediately used in the next step. Product yielded a white solid (71%). NMR spectra matched according to the literature.[13] ^{1}H NMR (400 MHz,$CDCl_3$) δ =7.72 (5H, d, J=6.8 Hz), 7.51 (2H, t, J=7.2 Hz), 7.43 (4H, t, J=6,8 Hz), 6.13 (2H, d, J=8 Hz), 5.39 (1H, t, J=9.6 Hz), 5.29 (3H, s), 5.14 (1H, t, J=9.6 Hz) 4.63 (2H, d, J=10 Hz), 4.3 (3H, dd, J_1=4.8 Hz, J_2=12,4 Hz), 4.19-4.14 (5H, m), 3.5 (6H, s), 2.1 (8H, s), 2.07 (8H, s), 2.04 (7H, s).

2.4b 4-trifluoromethylbenzoyl Derivative of Methyl Glycosylation of Per-acetylated Derivatives



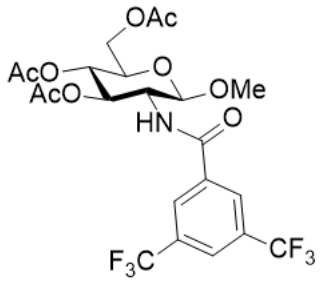
The product was made according to the general procedure. The product was not purified and was immediately used in the next step. Product yielded a white solid (51%). NMR spectra matched according to the literature[13]. ^{1}H NMR (400 MHz,$CDCl_3$) δ = 8.13 (2H, d, J= 8 Hz), 7.85 (1H, d, J=8.4 Hz), 7.73 (3H, d, J= 8.4 Hz), 5.3 (2H, q, J= 4.4Hz), 5.01 (2H, t, J= 10 Hz), 4.61 (2H, d, J=8.4 Hz), 4.37-4.224 (7H, m), 4.11-3.95 (6H, m), 3.78 (2H, t, J=5.6 Hz), 3.56 (2H, d, J= 6.8 Hz), 3.42 (9H, q, J= 3.6 Hz), 2.02 (3H, s), 1.96 (2H, s), 1.87 (2H, s), 1.34 (5H, t, J= 7.2 Hz).

2.4c 4-N, N-dimethyl benzoyl Derivative of Methyl Glycosylation of Per-acetylated Derivatives



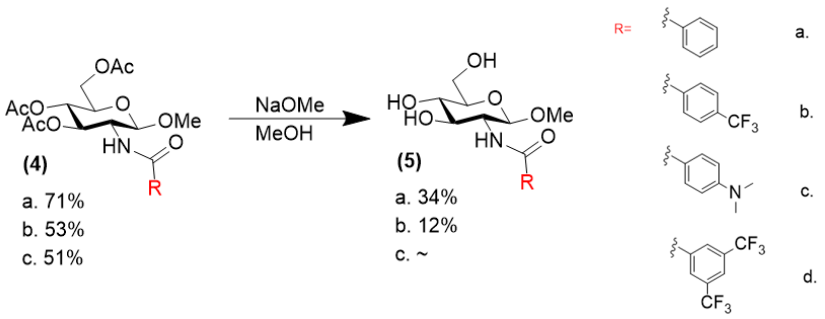
The product was made according to the general procedure. The product was not purified and was immediately used in the next step. Product yielded a white solid (53%). NMR spectra matched according to the literature[13]. ^{1}H NMR (400 MHz,$CDCl_3$) δ =7.64 (6H, d, J= 8.8 Hz), 6.66 (7H, d, J=8.8 Hz), 5.98 (2H, d, J= 8.8 Hz), 5.4 (3H, t, J= 9.6 Hz), 5.15 (3H, t, J= 10 Hz), 4.65 (2H, d, J=8 Hz), 4.33-4.13 (9H, m), 3.48 (6H, s), 2.02 (3H, s), 1.96 (2H, s), 1.87 (2H, s).

2.4d 3,5-bis(trifluoromethyl) benzoyl Derivative of Methyl Glycosylation of Per-acetylated Derivatives



The product was made according to the general procedure. The product was purified via automated column with a gradient mobile phase from 100% DCM to 20:1 DCM:MeOH. Product yielded a white solid (%TBD: Confirmed product via spectroscopy). NMR spectra matched according to the literature[13]. ^{1}H NMR (400 MHz,$CDCl_3$) δ =8.2 (6H, s), 8.02 (3H, s), 6.48 (2H, d, J=8.8 Hz), 5.43 (2H, t, J= 10.8 Hz), 5.21 (2H, t, J=9.6 Hz), 4.69 (2H, d, J= 8 Hz), 4.35 (3H, dd, J_1=4.8 Hz, J_2=4.4 Hz), 4.2 (5H, t, J=2.4 Hz), 3.73 (2H, d, J=2.4 Hz), 3.53 (9H, s),), 2.02 (3H, s), 1.96 (2H, s), 1.87 (2H, s).

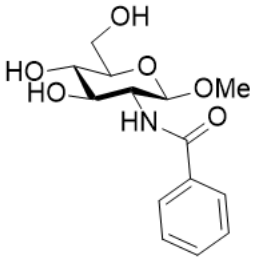
2.5 General Deacetylation Procedure of Methylated Derivatives



Scheme 4: Deprotection of Glucosamine derivative.

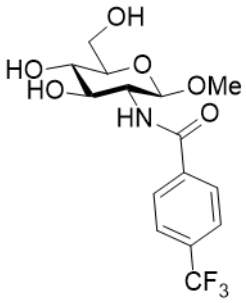
Synthesis of **5** was adapted according to the literature.[13] **4 (1 equiv)** was mixed with 0.08 M dry methanol and 0.5M sodium methoxide in anhydrous methanol **(3 equiv)** was added to **4**. The reaction was left to run overnight. Amberlite was initially washed with 1M HCl, then washed with DI water until the drips were neutral. The washed amberlite was added to the reaction flask and left to run for 30 minutes. The crude product was separated from the Amberlite by filtration. Purification for all products was carried out by passing the mixture through a C18 reverse-phase Sep-Pak cartridge by loading with MeOH and DI water, washed with DI water and eluted with MeOH resulting in a white solid. This reaction resulted in a fine, white solid product confirmed via spectroscopy.

2.5a Benzoyl Derivative of Deacetylated Methylated Derivatives



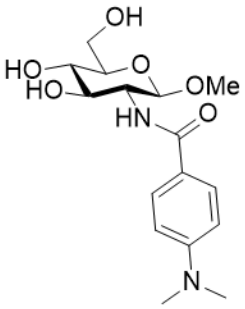
The product was made according to the general procedure. Product yielded a white solid (34%). NMR Spectra matched according to the literature.[13] ^{1}H NMR (400 MHz,$CDCl_3$) δ = 7.78 (5H, quint, J= 6.8 Hz), 7.47 (3H, quint, J= 2.4 Hz), 7.4 (5H, t, J= 6.8 Hz), 4.42 (2H, d, J=8.4 Hz), 3.88-3.83 (5H, m), 3.66 (3H, q, J=6.4 Hz), 3.65 (2H, t, J=5.6 Hz), 3.42-3.24 (10H, m), 2.09 (1H, s).

2.5b 4-trifluoromethylbenzoyl Derivative of Deacetylated Methylated Derivatives



The product was made according to the general procedure. Product yielded a white solid (12%). NMR Spectra matched according to the literature.[13] ^{1}H NMR (400 MHz,$CDCl_3$) δ =8.11 (8H, d, J=5.6 Hz), 7.92 (7H, d, J=7.6 Hz), 7.7 (9H, d, J=8 Hz), 4.39 (2H, d, J=8.8 Hz), 3.85-3.79 (5H, m), 3.64 (3H, t, J=6.4 Hz), 3.56-3.50 (5H, m), 3.4 (7H, s), 2.2-2.18 (3H, m), 2.1 (1H, s).

2.5c 4-N, N-dimethylbenzoyl Derivative of Deacetylated Methylated Derivatives



The product was made according to the general procedure. The product was unable to be purified as all the product was lost in the workup. No ^{1}H NMR collected due to loss of product.

3. Results and Discussion

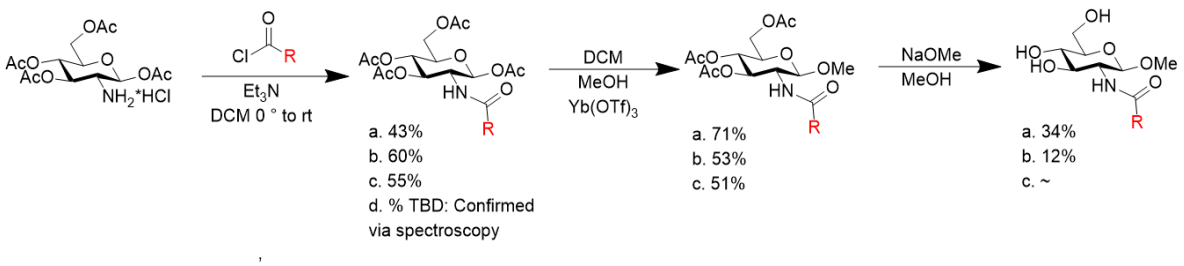


Figure 3 : Overall synthetic scheme for inhibitor synthesis

The procedure for the addition of the benzoyl group **(3a)** was successful in forming the product but difficult to purify, resulting in 43% yield after column chromatography. The product proved to have solubility issues, as it did not dissolve in methanol and had somewhat low solubility in ethyl acetate, dichloromethane, and acetone. These solubility issues made it difficult to purify properly using column chromatography. Similar solubility issues were seen with **(3b,c,d)**. The potential impurity is excess benzoyl chloride that has not reacted. The derivation of GlcNAc at the amide position with aromatic groups has proven to be tricky as it is difficult to remove the excess chloride from the product. Use of an automated column may be helpful as it can more accurately separate compounds that are very similar in polarity, rather than a normal column.

Synthesis of **(4)** from the O-methyl glycosylation is important to ensure that the sugar ring is locked in a closed conformation and has better selectivity for the ß form of GlcNAc. This reaction initially had issues with the use of the reflux condenser, and consistently needed more solvent added due to evaporation and leakage of solvent. This issue was remedied by use of high-pressure rated glassware with a blast shield so that there was less solvent evaporation during the heating process. The methyl glycosylation of the per-acetylated GlcNAc has been optimized but has a lower yield when R contains more electron withdrawing groups. The deprotection of GlcNAc needs further work for optimization. The workup for the reaction uses Amberlite resin that is acidic to neutralize the reaction, however it appears to be too acidic as much of the di-methyl amine product is lost on the resin. Switching this resin to one that is less acidic may be helpful for ensuring that more product is conserved throughout the course of the reaction. NMR spectroscopy suggests that there are mixes of fully deprotected sugars and partially deprotected sugars. It has been difficult to fully separate these accurately, resulting in low yields.

Several derivatives of GlcNAc have been synthesized at the amide position, and others in the McMahon group have made various derivatives at the anomeric position on the molecule that are in the process of characterization. However, before expanding the derivative library, the purification of the inhibitor molecules needed to be optimized for use in assays. Deprotection and purification of the current derivatives have had some issues, but once optimized, more potential inhibitors can be synthesized by modifying factors such as hydrophobicity, steric bulk and chain length, electronics, and hydrogen-bonding capability. By using electron-withdrawing and donating groups, it will affect how the molecule interacts with lectins, allowing for the assessment of the structure-activity relationships and ultimately design inhibitors that will increase affinity. Extending the chain length will also allow for the molecule to increase the surface area that has the potential to interact with the lectins. The synthesized derivatives will be tested for binding to the F17G lectin in an enzyme-linked immunosorbent assay (ELISA) competition experiment. In these assays, we will use different concentrations of the inhibitors against F17G-GlcNAc binding to evaluate effectiveness. This assay will be performed with the use of immobilized Lamin that is linked to GlcNAc in its natural form. The lectin and synthesized inhibitors will be introduced at the same time to test for binding affinity.

4. Conclusion

Ultimately, the goal of this project was to create a variety of inhibitors that can be used to block biofilm formation, which in turn will inhibit bacterial infections. To date, four GlcNAc derivatives designed as *E. coli* F17G lectin inhibitors have been synthesized with modifications at the amide position. the benzoyl, 4-trifluoromethylbenzoyl, 4-*N,N*-dimethylbenzoyl, and 3,5-bis(trifluoromethyl) benzoyl amides. The purification of the initial amide derivatives, synthesized from the amine and corresponding benzoyl chlorides, was optimized through use of gradient mobile phases for column chromatography. Additionally, the O-methyl glycosylation reaction was optimized by using a sealed high-pressure flask in place of a reflux condenser. Further optimizations will continue to be made to these procedures to produce a library of potential adhesion inhibitors. These molecules will then be tested for binding to *E. coli* adhesins, and ultimately as biofilm inhibitors. The absence of biofilms will significantly decrease the bacteria's antibiotic resistance, thus making infections easier to treat. Research on adhesion inhibitors allow for the use of anti-virulence intervention to help aid in reducing the negative effects of the ever-growing issue of antibiotic resistance.

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