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Anti-Adhesion Carbohydrates for Prevention of Biofilm Formation

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Abstract

Antibiotic resistance is a rapidly growing concern, making it increasingly important to develop new ways to treat bacterial infections. Anti-virulence strategies focus on disarming bacteria rather than killing them, putting less selective pressure on the bacteria and making it less likely bacteria will become resistant to the treatments. Virulence factors, the targets of anti-virulence, are tools that bacteria use to aid in the infection of the host. One virulence factor is the formation of biofilms, which occurs when lectins on the surface of bacteria bind to carbohydrates on the host cell. This research aims to synthesize carbohydrates with a strong binding affinity for bacterial lectins to inhibit the adhesion process. The GafD and F17G lectins are found on strains of *Escherichia coli* bacteria that cause diarrheal illness and bind to N-acetylglucosamine (GlcNAc). Derivatives of GlcNAc have been synthesized to use as inhibitors for bacterial adhesion. To synthesize derivatives, GlcNAc pentaacetate is used to allow for selective modification of the anomeric position by glycosidation with various alcohols. The acetate-protecting groups are then removed to yield the final product. Benzyl, phenyl, hexyl, butyl, and dodecyl glycosides have been successfully synthesized. Derivatives will be tested for inhibitory potential in competitive binding assays with GlcNAc and purified GafD protein.

1. Introduction

Antibiotic resistance is a growing problem within the medical world today, as drug-resistant pathogens currently account for ~700,000 deaths worldwide annually and it is estimated that the number may rise to ~10 million by the year 2050.[1] Martinez et al. claim that "the spread of resistant pathogens has been such that the possibility of returning to a pre-antibiotic era is real."[1] Antibiotic resistance arises as a result of antibiotic overuse and misuse. When antibiotics are administered, resistant cells are able to survive and then spread. The cycle then repeats itself with the use of stronger antibiotics and resistance increases.[1] This rapid rise in antibiotic resistance makes it increasingly important to discover new ways to treat bacterial infections. Given that this is a multi-faceted issue, it is unlikely that a single method will provide a solution to this problem, therefore it will be necessary to use multiple approaches that will work in tandem with each other to treat infections caused by drug-resistant bacteria.

One such approach is anti-virulence - combatting virulence factors which are the tools bacterial cells use to promote infection of the host. By minimizing or eliminating these virulence factors it would be possible to treat the infection without killing the pathogen, in turn putting less selective pressure on the bacteria and eliciting a slower evolution in the bacteria toward resistance than antibiotics.[2] There are several approaches to the anti-virulence strategy, such as interfering with the production of toxins, interfering with quorum sensing, and targeting the biofilm.

Biofilms are assemblages of microorganisms that are encased in a matrix and function as a group.[3] Biofilm formation consists of three main stages: attachment of cells to the surface, followed by the aggregation of microorganisms, and finally differentiation of biofilm into a mature structure.[4] This process is thought to be triggered by external stresses, such as the use of antibiotics.[3] Biofilms have cell

densities ranging from 10^8 to 10^{11} cells per gram of wet weight, and function as a barrier to protect bacteria from outside factors that may be detrimental to their survival,[5] including antibiotics.[6] Biofilms can attach themselves to a variety of surfaces including teeth and medical devices, allowing bacteria to more easily survive on these surfaces and eventually spread.[6] Preventing biofilm formation would make it more difficult for the bacteria to survive both within the body as well as on abiotic surfaces.

There are multiple ways to possibly stop the formation of biofilms, one method being the inhibition of adhesins. Adhesins are proteins on the surface of bacteria that bind to the host cell leading to aggregation and biofilm formations (Figure 1). In order to prevent adhesion and break up existing biofilms, competitive inhibitors could be synthesized for adhesins to bind to rather than onto the receptors on the host cell. An essential part of the adhesion process, and therefore biofilm formation, is the tiny wire-like structures on the surface of bacteria known as pili and, fimbriae. Carbohydrate-binding proteins, or lectins, on these fimbriae, adhere to glycan receptors on the host cell. This adherence is the first step in colonization.[5,7]

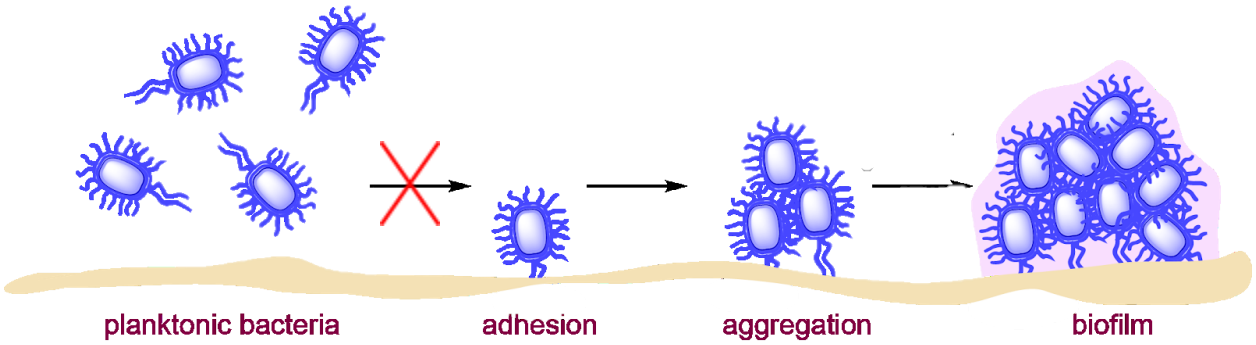


Figure 1. Biofilm formation process - preventing adhesion halts biofilm formation. (McMahon)

There has been prior research into the inhibition of biofilm formation through the inhibition of bacterial adhesins. For example, Sommer et al. have investigated LecB, an adhesin present on *Pseudomonas aeruginosa*. Derivatives of C-glycoside ligands of LecB were developed and tested for inhibitory potential. In general, it was found that C-glycosidic sulfonamides showed the highest potential for both LecB strain types.[8] These sulfonamides allow for a degree of modification that may allow for optimization by attaching cargo molecules or even targeted delivery of antibiotics.[8] This provides a positive outlook for the development of adhesin inhibitors for different bacterial strains and adhesin molecules.

This project is specifically focused on N-acetylglucosamine (GlcNAc) derivatives as inhibitors for F17G, a lectin on the F17 fimbriae present on *Escherichia coli (E. coli)* bacteria.[7] These bacteria are known to cause a variety of illnesses in humans including recurrent urinary tract infections, intestinal infections, neonatal sepsis, and meningitis.[9] The binding of F17-fimbriated *E. coli* is suspected to be density-dependent, requiring a high density of glycan receptors for adhesion, implying that charges may play a role in bacterial adhesion.[7] It has been determined that the F17G selectively binds glycan species with a terminal GlcNAc,[7] so the goal of this project is to develop similar structures to competitively inhibit F17G and stop biofilm formation.

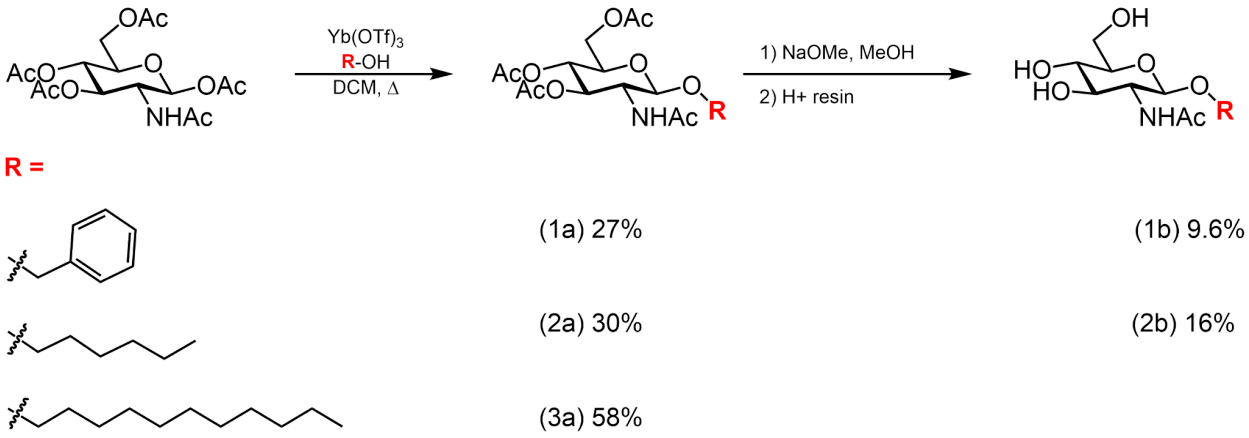
Olivia Pistor and Emma Founds in the McMahon group have already begun synthesizing GlcNAc derivatives with side chains at the amide and anomeric positions. These positions are ideal because they are the easiest to modify selectively, and their position allows for more bulky groups to be added without introducing too much steric hindrance that could prevent binding to the adhesin. Previously, butyl, hexyl, and benzyl amide derivatives have been synthesized. Other proposed derivatives include extending chain length, combining chain length and aromaticity, and changing the electronics and hydrogen-bonding ability of the aromatic group. The objective of this paper is to report the synthesis of GlcNAc derivatives modified at the anomeric position to then be used as F17G inhibitors.

2. Experimental

2.1 General Methods

All reagents were purchased from commercially available resources. Anhydrous solvents were dried under molecular sieves in a solvent system. Reaction progress was monitored using thin-layer chromatography (TLC) and visualized under ultraviolet light (UV) or in potassium permanganate stain. Reactions were concentrated using Heidolph rotary evaporator. Products were characterized using proton nuclear magnetic resonance spectroscopy (^{1}H NMR) on a 400 MHz Varian spectrometer. ^{1}H NMR data are reported as follows: chemical shift, multiplicity (s= singlet, d= doublet, t=triplet, dd= doublet of doublets, m= multiplet, br. s= broad singlet), coupling constants (Hz), integration, proton identifier.

Synthesis was done according to methods from Shultz et al. (Scheme 1) for all derivatives synthesized (Scheme 1).



Scheme 1. Synthesis of GlcNAc derivatives with modified anomeric position.

2.2 Glycosylation of per-acetylated GlcNAc with benzyl alcohol

Acetylated benzyl glycoside (1a) was synthesized by adding benzyl alcohol (3.2 equiv, 4.09 mmol, 0.425 ml) to GlcNAc pentaacetate (1 equiv, 1.284 mmol, 0.5 g) dissolved in dry dichloromethane (122 equiv, 156.6 mmol, 10 mL) along with ytterbium triflate (0.13 equiv, 0.16123 mmol, 0.1 g) at 60 °C. The reaction was run overnight under a reflux condenser in an inert argon atmosphere. The reaction was then allowed to cool to room temperature and removed from the condenser. The product was washed three times with deionized water, dried with $MgSO_4$ and concentrated by rotary evaporation. Product was purified using column chromatography with a mobile phase consisting of 80% Ethyl acetate and 20% hexanes to yield a white solid (28%). The structure was then confirmed using ^{1}H NMR spectroscopy. H NMR (CDCl3): δ 7.33-7.38 (m, 5H, Ph), 5.30 (d, J = 9.2 Hz, 1H, NH), 5.20, (dd, J = 9.6, 9.6 Hz, 1H, H-3), 5.08 (t, J = 9.2 Hz, 1H, H-4), 4.89 (d, J = 12 Hz, 1H, CH2Ph), 4.63 (d, J = 8 Hz, 1H, H-1) 4.62 (d, J = 12.4 Hz, 1H, CH2Ph), 4.31 (dd, J = 4.8, 4.8 Hz, 1H, H-6a), 4.19 (dd, J = 2.8, 2.4 Hz, 1H, H-6b), 3.99 (m, 1H, H-2), 3.68 (m, 1H, H-5), 2.12 (s, 3H, NHAc), 2.03 (s, 6H, 2COCH3), 1.92 (s, 3H, COCH3).

2.3 Deprotection of per-acetylated benzyl glycoside

Benzyl glycoside (1b) was synthesized by dissolving (1a) (1 equiv, 0.36 mmol, 0.1565 g) in anhydrous methanol (4.38 mL) and adding sodium methoxide (3 equiv, 1.07 mmol, 2.15 mL). The reaction was run overnight at room temperature under an inert argon atmosphere. Amberlite resin (washed using 1M HCl and rinsed with water until neutral pH) was then added to the reaction and left to stir until the pH of the reaction reached ~5. The resin was then filtered out using cotton and the product was

concentrated using a rotary evaporator. The product was purified using a C18 Sep-pak to yield a white solid (9.6%). The structure was confirmed using ^{1}H NMR spectroscopy. H NMR (CD_3OD): δ 7.18-7.25 (m, 5h, Ph), 4.8 (d, J= 13 Hz, 1H, CH_2Ph), 4.6 (d, J= 12 Hz, 1H, CH_2Ph), 4.4 (d, J= 8 1H, H-1), 3.8 (dd, J= 2, 12, 1H, H-6a), 3.7-3.6 (m, 2H, H-6b, H2), 3.4-3.5 (m, 3H, H-3, H-4, H-5), 1.9 (s, 3H, NHCOCH3).

2.4 Glycosylation of per-acetylated GlcNAc with hexanol

Acetylated hexyl glycoside (2a) was synthesized by adding GlcNAc pentaacetate (1 equiv, 0.26 mmol, 0.10 g) to dry dichloromethane (31.5 mmol, 2 mL). Hexanol (2.58 equiv, 0.67 mmol, 0.084 mL) was then added along with ytterbium triflate (0.12 equiv, 0.03 mmol, 0.02g). The reaction was then left to stir at 60 °C overnight under a reflux condenser in an inert argon atmosphere. The reaction was then allowed to cool to room temperature before being rinsed thrice with deionized water, dried with magnesium sulfate, and filtered using cotton. The product was then purified using column chromatography with a mobile phase of 20% hexanes and 80% ethyl acetate to yield a white solid (30%). The structure of the product was confirmed using ^{1}H NMR spectroscopy. ^{1}H NMR (CDCl3): δ 5.46 (d, J = 8.4 Hz, 1H, NH), 5.3 (dd, J = 8.8, 9.2 Hz, 1H, H-3), 5.10 (t, J = 9.6, 9.6 Hz, 1H, H-4), 4.70 (d, J = 8.8 Hz, 1H, H-1), 4.29 (dd, J = 5.2, 4.4 Hz, 1H, H-6a), 4.5 (dd, J = 2.4, 2.4 Hz, 1H, H-6b), 3.88 (m, 2H, H-2, OCH2), 3.7 (m, 1H, H-5), 3.45 (m, 1H, OCH2), 2.1, 2.05, 1.9 (s, 12H, 3COCH3), 1.30 (br s, 8H, OCH2(CH2)4), 0.9 (t, J = 6.8, 7.2 Hz, 3H, OCH2(CH2)4CH3).

2.5 Deprotection of per-acetylated hexyl glycoside

Hexyl glycoside (2b) was synthesized by dissolving (2a) (1 equiv, 0.078 mmol, 0.034 g) anhydrous methanol (306 equiv, 23.9 mmol, 0.97 mL) and sodium methoxide (3 equiv, 8.105 mmol, 0.46 mL) and stirring at room temperature overnight in an inert argon atmosphere. Amberlite resin was then added to the reaction and left to stir until the pH of the reaction reached ~5. The resin was then filtered out using cotton and the product was concentrated using a rotary evaporator. The product was then purified using C18 Sep-pak to yield a white solid (16%). The structure was then confirmed using ^{1}H NMR spectroscopy. H NMR (CD3OD): δ 4.32 (d, J = 8.4 Hz, 1H, H-1), 3.91-3.22 (m, 8H, H-2, H-3, H-4, H-5, H-6, OCH2), 1.9 (s, 3H, NHCOCH3), 1.5, 1.3 (br m, 8H, OCH2(CH2)4), 0.8 (t, J = 7.2, 6.8, 3H, O(CH2)5CH3).

2.6 Glycosylation of per-acetylated GlcNAc with Dodecanol

Acetylated dodecyl glycoside (3a) was synthesized by adding GlcNAc pentaacetate (1 equiv, 0.64 mmol, 0.25g) to dry dichloromethane (78.3 mmol, 5 mL) along with dodecanol (2.6 equiv, 1.67 mmol, 0.26 mL) and ytterbium triflate (0.1 equiv, 0.08 mmol, 0.05 g) and stirring at 60 °C overnight in a high pressure sealed flask. The reaction was then removed from heat and allowed to cool to room temperature before being washed three times with deionized water, dried using $MgSO_4$ and filtered using a funnel and cotton. The product was purified using column chromatography with a mobile phase consisting of 20% hexanes and 80% ethyl acetate to yield a white solid (58%). The structure was then confirmed using ^{1}H NMR spectroscopy. ^{1}H NMR (CDCl3): δ 5.5 (d, J = 8.4 Hz, 1H, NH), 5.3 (dd, J = 8.8, 14.4 Hz, 1H, H-3), 5.10 (t, J = 9.6 Hz, 1H, H-4), 4.70 (d, J = 8.4 Hz, 1H, H-1), 4.29 (dd, J= 4.8, 4.4 Hz, 1H, H-6a), 4.2 (dd, J= 2.4, 4.8 Hz, 1H, H-6b), 3.88 (m, 2H, H-2, OCH2), 3.7 (m, 1H, H-5), 3.45 (m, 1H, OCH2), 2.1, 2.05, 1.9 (s, 12H, 3COCH3), 1.30 (br s, 20H, OCH2(CH2)10), 0.9 (t, J= 6.8, 7.2 Hz, 3H, OCH2(CH2)4CH3).

2.7 Deprotection of per-acetylated dodecyl glycoside

Dodecyl glycoside (3b) was synthesized by dissolving (3a) (1 equiv, .306 mmol, .145 mg) in anhydrous methanol (308 equiv, 94.30 mmol, 3.82 mL) and adding sodium methoxide (3 equiv, 0.91 mmol, 0.052 mL). The reaction was then left to stir overnight at room temperature in an inert argon atmosphere. Dowex resin was then added and left to stir for ~15 minutes, pH reached ~2. The resin was then removed using cotton and the product was concentrated using rotary evaporator. The structure of the crude product was then confirmed using ^{1}H NMR spectroscopy. H NMR (CD3OD): δ 4.32 (d, J = 8.4 Hz, 1H, H-1), 3.84-3.56 (m, 8H, H-2, H-3, H-4, H-5, H-6, OCH2), 1.9 (s, 3H, NHCOCH3), 1.4, 1.2 (br m, 20H, OCH2(CH2)10), 0.8 (t, J = 6.8, 3H, O(CH2)5CH3). Purification was attempted but the product was contaminated.

3. Results and Discussion

GlcNAc derivatives with hexyl, benzyl, and dodecyl chains added at the anomeric position were successfully synthesized using a method developed by Shultz et al. An issue that arose with the glycosidation reaction was the evaporation of solvent when using a reflux condenser. For the synthesis of (3a) a high-pressure sealed flask was used, which appeared to result in a better yield. For future experiments, high-pressure sealed flasks will be used in replacement of the reflux condenser.

Yields of final derivatives were low, with much of the product lost during purification of the deprotected compounds. In an attempt to combat this, the use of C18 Sep-paks was attempted, however, because of the polarity of the sugar compared with the non-polar side chains solubility issues arose. C18 Sep-paks typically use one fraction of methanol and one fraction of water, however, derivatives were not fully soluble in water. Due to this solubility issue, product was present in all fractions. An attempt was made to take multiple water fractions and multiple methanol fractions but the product was still present in both solvents. It was then decided that column chromatography was more efficient. Additionally, it is possible that the Amberlite resin is destroying the product during the workup of the deprotection reaction, due to its acidity. An alternative resin, Dowex, was used for the workup of (3b). The crude product appeared mostly pure on ^{1}H NMR spectra, and purification by normal phase column chromatography was attempted. Unfortunately, (3b) was contaminated by an outside source during the process of purification through column chromatography. Dowex resin will continue to be used in the future for the workup of glycosidation reactions.

4. Conclusions

The goal of this project is to develop GlcNAc derivatives to use as inhibitors for the F17G/GafD lectins present on enteropathogenic *E. coli*. Several GlcNAc derivatives modified at the anomeric position were successfully synthesized in low to moderate yield. Modifications have been made to previously established synthetic pathways to further optimize yield of GlcNAc derivatives, such as the use of high-pressure sealed flasks and the use of Dowex resin rather than Amberlite. These derivatives, once fully purified and characterized, will be tested for their inhibitory ability of F17G binding via an ELISA-like assay.

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References:

(1) Martínez, O. F.; Cardoso, M. H.; Ribeiro, S. M.; Franco, O. L. Recent Advances in Anti-Virulence Therapeutic Strategies with a Focus on Dismantling Bacterial Membrane Microdomains, Toxin Neutralization, Quorum-Sensing Interference and Biofilm Inhibition. *Frontiers in Cellular and Infection Microbiology* **2019**, *9* (APR). https://doi.org/10.3389/fcimb.2019.00074.

(2) Dufrêne, Y. F.; Viljoen, A. Binding Strength of Gram-Positive Bacterial Adhesins. *Frontiers in Microbiology*. 2020. https://doi.org/10.3389/fmicb.2020.01457.

(3) Qi, L.; Li, H.; Zhang, C.; Liang, B.; Li, J.; Wang, L.; Du, X.; Liu, X.; Qiu, S.; Song, H. Relationship between Antibiotic Resistance, Biofilm Formation, and Biofilm-Specific Resistance in Acinetobacter Baumannii. *Frontiers in Microbiology* **2016**, *7* (APR). https://doi.org/10.3389/fmicb.2016.00483.

(4) Roy, R.; Tiwari, M.; Donelli, G.; Tiwari, V. Strategies for Combating Bacterial Biofilms: A Focus on Anti-Biofilm Agents and Their Mechanisms of Action. *Virulence*. 2018. https://doi.org/10.1080/21505594.2017.1313372.

(5) Vengadesan, K.; Narayana, S. V. L. Structural Biology of Gram-Positive Bacterial Adhesins. *Protein Science*. 2011. https://doi.org/10.1002/pro.613.

(6) Gebreyohannes, G.; Nyerere, A.; Bii, C.; Sbhatu, D. B. Challenges of Intervention, Treatment, and Antibiotic Resistance of Biofilm-Forming Microorganisms. *Heliyon*. 2019. https://doi.org/10.1016/j.heliyon.2019.e02192.

(7) Lonardi, E.; Moonens, K.; Buts, L.; de Boer, A. R.; Olsson, J. D. M.; Weiss, M. S.; Fabre, E.; Guérardel, Y.; Deelder, A. M.; Oscarson, S.; Wuhrer, M.; Bouckaert, J. Structural Sampling of Glycan Interaction Profiles Reveals Mucosal Receptors for Fimbrial Adhesins of Enterotoxigenic Escherichia Coli. *Biology (Basel)* **2013**, *2* (3). https://doi.org/10.3390/biology2030894.

(8) Sommer, R.; Rox, K.; Wagner, S.; Hauck, D.; Henrikus, S. S.; Newsad, S.; Arnold, T.; Ryckmans, T.; Brönstrup, M.; Imberty, A.; Varrot, A.; Hartmann, R. W.; Titz, A. Anti-Biofilm Agents against Pseudomonas Aeruginosa: A Structure-Activity Relationship Study of C-Glycosidic LecB Inhibitors. *Journal of Medicinal Chemistry* **2019**, *62* (20). https://doi.org/10.1021/acs.jmedchem.9b01120.

(9) Sharma, G.; Sharma, S.; Sharma, P.; Chandola, D.; Dang, S.; Gupta, S.; Gabrani, R. Escherichia Coli Biofilm: Development and Therapeutic Strategies. *Journal of Applied Microbiology*. 2016. https://doi.org/10.1111/jam.13078.

(10) Biswas, N. N.; Yu, T. T.; Kimyon, Ö.; Nizalapur, S.; Gardner, C. R.; Manefield, M.; Griffith, R.; Black, D. S. C.; Kumar, N. Synthesis of Antimicrobial Glucosamides as Bacterial Quorum Sensing Mechanism Inhibitors. *Bioorganic and Medicinal Chemistry* **2017**, *25* (3). https://doi.org/10.1016/j.bmc.2016.12.024.

(11) Shultz, et. al. Chemoenzymatic Synthesis of 4-Fluoro-N-Acetylhexosamine Uridine Diphosphate Donors: Chain Terminators in Glycosaminoglycan Synthesis J. Org. Chem. 2017, 82, 2243−2248