Substrates and Inhibitors of Enzymes Involved in Exopolysaccharide Dependent Biofilms

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Abstract

Many biofilm-forming bacteria produce a similar partially de-N-acetylated β-1,6-N-acetyl glucosamine homopolymer (dPNAG) to facilitate bacterial adhesion. In many medically important biofilm forming bacterial strains, including *Escherichia coli*, *Staphylococcus epidermidis* and *Staphylococcus aureus*, de-N-acetylation of the β-1,6-N-acetyl glucosamine homopolymer (PNAG) is catalyzed by a metal dependent de-N-acetylase. Sixty-five percent of all human persistent bacterial infections are considered to be biofilm related. *In vivo* studies have implicated the production and subsequent de-N-acetylation of PNAG is essential to bacterial virulence.

In this work, methods of monitoring and targeting PNAG-dependent biofilm processes are explored. A novel chromogenic glycosidase substrate based on a glycosyl carbamate was developed and used to effectively monitor the activity of Dispersin B (DspB), an enzyme capable of degrading PNAG/dPNAG. Additionally, an array of potential deacetylase inhibitors were synthesized with the goal of targeting the essential de-N-acetylation enzymes PgaB and IcaB, from *E. coli* and *S. epidermidis* respectively, for biofilm formation. These inhibitors were based on a carbohydrate scaffold containing either a metal chelating moiety or a transition state mimic.
Finally, a novel coumarin based substrate was developed to monitor *in vitro* de-N-acetylas activity of PgaB and IcaB. Using this substrate, the potency of the synthesized inhibitors was evaluated through a competitive fluorogenic assay. The most effective inhibitor, a chemoenzymatically-synthesized pentasaccharide derivative showed a $K_i$ value of 280 µM.
Acknowledgments

Ahhhh – Acknowledgments! The only place where you won’t find overwhelming amounts of science written in this wonderful document!

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<tr>
<td>9GlcNH₂</td>
<td>β-1,6-Glucosamine Nonasaccharide</td>
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<td>DCC</td>
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<td>Dichloromethane</td>
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<tr>
<td>DMAP</td>
<td>4-Dimethylamino Pyridine</td>
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<td>DNA</td>
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<td>Partially de-(N)-acetylated (\beta)-1,6-(N)-acetyl Glucosamine Homopolymer</td>
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<td>(N,N,N',N')-Tetramethyl-(O)-(1H-benzotriazol-1-yl)uronium Hexafluorophosphate</td>
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<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic Acid</td>
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<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>ICP-AES</td>
<td>Inductively Coupled Plasma-Atomic Emission Spectroscopy</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption Ionization</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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<td>min</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloprotease</td>
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<tr>
<td>MsCl</td>
<td>Mesyl Chloride</td>
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<tr>
<td>MSCRAMMs</td>
<td>Microbial Surface Components Recognizing Adhesive Matrix Molecules</td>
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<td>Mu-GlcNAc</td>
<td>Methylumbellifery-β-N-acetylglucosamine</td>
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<td>MWCO</td>
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<td>NOE</td>
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<td>PNAG</td>
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<td>pNP</td>
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1 General Introduction

1.1 Biofilm Structure and Life Cycle

Biofilms are complex communities of bacteria that have adhered to a surface and are held together by an extracellular matrix. This matrix is principally composed of biological polymers including DNA, proteins, and polysaccharides, as well as other small molecules. These colonies often form mushroom-like structures that allow the bacteria to be sheltered from hostile environments and host defenses. Biofilm life cycles can be divided into three major stages: attachment, growth, and release (Figure 1.1).

Figure 1.1 Model of the biofilm life cycle. 1-2) attachment of planktonic bacteria to a surface. 3-4) growth of the macroscopic biofilm structure. 5) release of planktonic to the environment. Image obtained from the Center of Biofilm Engineering, Montana State University.

1.1.1 Attachment

The development of a biofilm begins with planktonic bacteria adhering to a surface. The initial attachment is accomplished by weak van der Waals forces, electrostatic forces, and hydrophobic interactions between the bacteria and the surface. This initial attachment is
dynamic and reversible, during which hydrodynamic forces, repulsive forces, or nutrient availability may act as motives for bacteria to return to the planktonic population. Upon initial attachment to a surface, microbes may transition to a biofilm mode of existence. This involves the bacteria producing components necessary for permanent and irreversible attachment to the surface, known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). Well-studied Gram-positive biofilm producers \textit{Staphylococcus aureus} and \textit{Staphylococcus epidermidis} can produce and export to the cell-surface up to 20 different MSCRAMMs.\textsuperscript{5} These components have a common structure that includes an exposed binding domain, a cell-wall spanning domain, and a domain responsible for covalent or non-covalent attachment to a surface.\textsuperscript{6} For example, Biofilm Associated Protein (BAP) produced by \textit{S. aureus} and \textit{S. epidermidis} facilitates adherence through non-covalent hydrophobic interactions on inert surfaces.\textsuperscript{7}

\subsection*{1.1.2 Growth}

Upon permanent attachment to a surface, bacteria in the newly-forming biofilm begin replication and matrix formation. Significant up-regulation of the genes responsible for cell envelope and extracellular matrix formation, along with a concurrent down-regulation of genes responsible for motility, is observed.\textsuperscript{8} Growth persists until the colony reaches a mature size, with mushroom-like or tower-like profiles often being observed \textit{in vitro}, with its maximal size determined by the hostility of the environment. This stage of colony development and maturation is dependent on environmental conditions and can last for several days.\textsuperscript{4a,9}
1.1.3 Release

The final stage of a biofilm life cycle involves a partial breakdown of the extracellular matrix and release of planktonic cells from the mature biofilm. This stage of development allows for the initiation of new biofilm colonies distant from the initial attachment site. In *Actinobacillus actinomycetemcomitans*, Dispersin B (DspB), a β-hexosaminidase enzyme, is secreted into the extracellular environment to partially degrade the polysaccharide matrix and subsequently allow for planktonic release. Likewise, some bacterial strains including *Pseudomonas aeruginosa* produce and release rhamnolipid surfactants which can also result in biofilm dispersal.

1.2 Biofilm Resilience and Antibiotic Resistance

Biofilm-related infections typically occur from contact of a colonized surface with a host. Most commonly, these infectious surfaces include indwelling medical devices such as catheters, heart valves, and orthopedic devices, but biofilms can also be found on natural surfaces such as teeth, the lungs in the case of cystic fibrosis patients, and in the middle ear of patients with persistent otitis media infections. Common biofilm-forming bacteria that establish
colonies on indwelling medical devices include *S. epidermidis*, *S. aureus*, *E. coli*, *Acinetobacter baumannii*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Yersinia pestis*, *Acinetobacillus pleuropneumoniae*, and *P. aeruginosa*. Once such a device has been compromised by biofilm bacteria, chronic infection of the host can follow. In most cases, removal or replacement of the device is the only effective means of treatment.

Bacterial infections are regularly treated with antibiotics; however, this form of treatment has shown limited success when attempting to treat a bacterial biofilm infection. It is generally the case that biofilm-residing bacteria demonstrate increased antibiotic resistance in comparison to their planktonic counterparts. Some reports suggest this increase of antibiotic resistance to be up to 1000-fold. This resistance is likely a result of the multicellular nature of biofilms, rather than through the mechanisms of efflux pumps or acquired mutation. There are several hypotheses to explain this observation, all relating to a central theme: the communal existence of biofilm bacteria leads to their increased antibiotic resistance and environmental resilience. In fact, it has been shown that dispersion of a biofilm structure, for example through mechanical means, negates this antibiotic resistance improvement.

The extracellular matrix is thought to form a physical barrier, which sterically limits the penetration of some antibiotics including β-lactams, aminoglycosides and host antibodies to the biofilm core, leaving only peripheral microbes susceptible. The steric barrier of the biofilm matrix also limits the activity of the host’s phagocyte cells, which rely on an engulfing mechanism for bacterial removal, reserving their efficacy only towards planktonic bacteria.

Concurrently, the physiological heterogeneity of the bacteria in the biofilm can lead to antibiotic resistance. Planktonic bacteria exist in a uniform environment and thus live in similar physiological states. In a biofilm, gradients of nutrients, oxygen, waste, pH, etc exist which alter the physiological state of each bacterium in the biofilm depending on its specific local environment. It has been shown that biofilms consist of at least two subpopulations as a result of these chemical gradients: a growing aerobic subpopulation and a more dormant anaerobic subpopulation. Since many antibiotics such as aminoglycosides, β-lactams and fluoroquinolones target bacterial replication and metabolism pathways, the dormant subpopulation of bacteria is likely resistant to these antibiotic modes of action. *In vitro*
additions of compounds that stimulate anaerobic growth such as arginine and nitrates have shown to enhance the antimicrobial activity of these antibiotics.\textsuperscript{36}

Finally, biofilm bacteria have also demonstrated a higher adaptive tolerance. The mutation frequency of biofilm-growing bacteria has been observed to be significantly increased compared to isogenic, planktonic-growing bacteria, likely through a stress-induced mechanism.\textsuperscript{37} Additionally, an increased rate of horizontal gene transfer among proximal bacteria is also observed. This allows for easy imprint of antibiotic resistance amid the communal biofilm bacteria.\textsuperscript{38}

### 1.3 Extracellular Matrix

A commonality in all biofilm communities is the existence of an extracellular matrix.\textsuperscript{39} This matrix facilitates cohesion between the individual microbes in a biofilm, and it is composed of biomacromolecules including DNA, proteins, lipids, and long repeating polysaccharides, depending on the bacterial species/strain in question.\textsuperscript{40} Whereas DNA is thought to play an important role in the establishment of the biofilm structure, polysaccharides are considered to be the major structural component of the biofilm matrix.\textsuperscript{41} Bacterial biofilms that are devoid of polysaccharides are unable to form multilayer biofilms.\textsuperscript{42} The focus of this chapter will be on polysaccharide-based matrices.

#### 1.3.1 Psl and Pel Matrices

In \textit{P. aeruginosa}, an opportunistic pathogen, the exopolysaccharide matrix is composed of two main polysaccharides: the Pel exopolysaccharide, whose structure has not been fully characterized, and the Psl exopolysaccharide, a polysaccharide containing a pentasaccharide repeating unit consisting of D-mannose, L-rhamnose, and D-glucose (Figure 1.3).\textsuperscript{43} In general, \textit{P. aeruginosa} strains predominantly produce and secrete one of the two polysaccharides at any given time.\textsuperscript{44} The Psl exopolysaccharide is biosynthesized by the 12 gene operon \textit{pslABCDEFGHIJKL}. It has been predicted that the Psl exopolysaccharide pathway is initiated on a lipid carrier on the inner membrane and subsequent oligosaccharide subunit polymerization is then achieved in the periplasm. The final polysaccharide is then translocated across the periplasm and exported through the outer membrane. This process is similar to \textit{E. coli} capsule synthesis and export.\textsuperscript{45}
1.3.2 Alginate Matrix

Alginate is an acidic exopolysaccharide also found in *P. aeruginosa*, particularly strains involved in cystic fibrosis. Its importance in the pathology of cystic fibrosis has led to alginate being the first and best characterized biofilm matrix exopolysaccharide. The alginate polysaccharide consists of two monosaccharide uronic acid subunits: \( \beta-D \)-mannuronate residues and its C-5 epimer \( \alpha-L \)-guluronate residues (Figure 1.4). The biosynthesis of alginate is accomplished by the gene operon \( \text{algADEFGIJKL} \). Biosynthesis involves 1,4-polymerization of \( \beta-D \)-mannuronic acid residues in the cytoplasm and concurrent transport into the resulting polymannuronate polymer into the periplasm. The polysaccharide is then modified by epimerases to produce \( \alpha-L \)-guluronate residues within the mature polymer, and \( O \)-acetylated at various residues before final translocation across the periplasm and export into the environment. The final mature alginate matrix contains homopolymeric regions of poly-\( \beta-D \)-mannuronate and poly-\( \alpha-L \)-guluronate, as well as heteropolymeric regions. Alginate can facilitate the formation of gel like structures in the presence of chelating cations including sodium and calcium, with functional properties strongly correlated to the mannurate/guluronate ratio and sequence.
Figure 1.4 Structure of the alginate polysaccharide. It contains uronic acid residues β-D-mannuronate (M) and its C-5 epimer α-L-guluronate (G). R = H or Ac.

1.3.3 Cellulose Matrix

Cellulose is a polysaccharide comprised of β-1,4-glucose monomer subunits (Figure 1.5). It is the most abundant sugar polymer in the living world, existing as a structural component of cell walls of green plants. It has been identified as a biofilm matrix component of several bacterial species including Agrobacterium tumefaciens, E. coli, Pseudomonas fluorescens, and Gluconacetobacter xylinus. Cellulose consists of insoluble fibers that aggregate at the cell surface to form a gel-like substance. Hydrogen bonds between cellulose polymer strands lead to its fibrous structure. The gene operon responsible for cellulose polymer synthesis is the bcsABCD operon. It is polymerized by a multimeric protein complex located in the cytoplasmic membrane, followed by export into the extracellular environment, where multiple chains can begin to form higher order aggregates.

Figure 1.5 Structure of cellulose polysaccharide.

1.3.4 Poly N-Acetyl Glucosamine Matrix

In the biofilms of Gram-negative E. coli and Gram-positive S. epidermidis, which are both medicinally relevant bacteria, the extracellular matrix is composed of a β-1,6-N-acetyl
glucosamine homopolymer (PNAG, poly-N-acetylglucosamine), which is de-N-acetylated at variable positions along the scaffold (dPNAG, de-N-acetylated poly-N-acetylglucosamine) (Figure 1.6). De-N-acetylation widely varies among different bacterial species and strains, with isolated dPNAG exhibiting a range from 1% to 40% de-N-acetylation, with polymer strands on the scale of hundreds of monosaccharide units long. De-N-acetylation results in positive charges along the polymer at the de-N-acetylated sites, and is thus thought to lead to electrostatic interactions with negatively-charged functional groups including phosphates and carboxylates on a neighboring cell’s cell wall, leading to robust adhesion among bacterial cells. Strains of bacteria displaying only PNAG polysaccharide showed limited ability to form biofilms and demonstrated compromised antibiotic resistance. Additionally, these strains also demonstrated diminished virulence in mice. For the remainder of this thesis, only this polysaccharide matrix will be discussed in detail.
1.3.4.1 Biosynthesis of PNAG and dPNAG

In both Gram-positive and Gram-negative strains of bacteria, PNAG and dPNAG matrices are synthesized similarly, with differences accommodating for export of the polysaccharide through the extra membrane of Gram-negative bacteria. In *E. coli*, synthesis is accomplished by the *pgaABCD* operon,\(^{54}\) whereas in *S. epidermidis*, this is achieved by the *icaABCD* operon.\(^{19}\) It is not well understood whether dPNAG remains covalently attached to the cellular membrane or remains loosely associated to the bacterial cells by electrostatic or other non-covalent interactions in either system.
1.3.4.1.1 PNAG Biosynthesis in Gram-negative Bacteria

In Gram-negative E. coli, synthesis and extracellular display of dPNAG is accomplished by four proteins. In a model proposed by Itoh et al., initially, UDP-GlcNAc is polymerized into the β-1,6-N-acetyl glucosamine homopolymer PNAG and consecutively transported to the periplasmic space by the transmembrane proteins PgaC and PgaD, with PgaC predicted to be a glycosyl transferase (Figure 1.7). PgaD has recently been found to act as an allostERIC regulator of polysaccharide formation, and is dependent on the presence of cyclic di-GMP (c-di-GMP). Allosteric binding of c-di-GMP to PgaD allows for formation of a PgaCD complex, which in turn allows for the glycosyl transferase activity required for PNAG polymerization. Upon polymerization and transfer of the polysaccharide to the periplasmic space by PgaCD, PgaB is responsible for partial de-N-acetylation of the growing polysaccharide chain within the periplasm. PgaA is then thought to be responsible for the export of the developed polysaccharide into the extracellular space. Notably, ΔPgaB knockout strains of E. coli accumulate only PNAG into the periplasmic space; neither de-N-acetylation nor export is observed. The pgaABCD operon has also been implicated in dPNAG synthesis in other medically relevant Gram-negative bacterial strains including Aggregatibacter actinomycetemcomitans, A. baumannii, A. pleuropneumoniae, and Y. pestis.
1.3.4.1.2 PNAG Biosynthesis Gram-positive Bacteria

In Gram-positive *Staphylococcus* strains including *S. epidermidis*\(^60\) and *S. aureus*,\(^61\) the biosynthesis of dPNAG is similar to that of the Gram-negative strains, employing the *icaABC*D operon.\(^62\) In a model proposed by Götz *et al*, this system utilizes a complex of membrane-associated proteins IcaC, IcaA and IcaD to elongate and transport the growing PNAG into the extracellular space (Figure 1.8).\(^63\) IcaB, a secreted protein, is then subsequently responsible for the de-\(N\)-acetylation of the PNAG polysaccharide to form dPNAG. Akin to ΔPgaB *E. coli*, ΔIcaB strains of *S. epidermidis* result in bacteria with compromised biofilm-forming ability, and isolation of the polysaccharide reveals only the fully acetylated PNAG polymer.

Figure 1.7 Model of dPNAG biosynthesis and export in Gram-negative *E. coli*. PgaB is a periplasmic de-\(N\)-acetylase responsible for partial de-\(N\)-acetylation of PNAG. Image adapted and used with the permission of J. Poloczek.
1.3.4.2 PGA and IcaB

1.3.4.2.1 PGA

PGA, a lipoprotein localized in the periplasm, is a 672 amino acid enzyme with 2 domains: an N-terminal deacetylase domain and a C-terminal domain homologous to glycosyl hydrolases. Crystallographic studies of PGA have revealed the presence of a metal ion in the predicted deacetylase active site of the N-terminal domain, which shares sequence homology with other enzymes in the Carbohydrate Esterase Family 4 (CE4), a family of metalloenzymes. A number of crystal structures have been produced, containing different metal ions including Fe$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$, all bound to the same Asp115-His184-His189 triad of residues. In vitro studies of PGA expressed in the presence of different metals had varied rates of de-N-acetylase activity on synthetic β-1,6-N-acetyl glucosamine pentasaccharides, suggesting de-N-acetylation is metal-dependent, with Fe$^{2+}$, Co$^{2+}$, and Ni$^{2+}$ loaded enzyme demonstrating the highest activity. Additionally, PGA demonstrates size dependent de-N-acetylation activity when tested with oligomeric PNAG substrates, showing increased de-N-acetylation activity with increasing oligomer size ranging from tri- to pentasaccharide. Strong selectivity is also observed for de-N-acetylation of the third carbohydrate unit in a synthesized PNAG pentasaccharide.
suggesting a total of four flanking carbohydrate binding substitues in the active site, two on each side.

![Figure 1.9 Ribbon trace of the crystal structure of PgaB N-terminal deacetylase domain (residues 41-310). Trace is colored from blue at the N-terminus to red at the C-terminus. The active site nickel ion is shown in grey and is bound by Asp115-His184-His189 and an acetate molecule.](image)

1.3.4.2.2 IcaB

IcaB, a secreted protein found in Gram-positive *S. epidermidis* and *S. aureus*, is a 289 amino acid de-*N*-acetylase with sequence homology similar to that of the N-terminal domain of PgaB and other enzymes in the CE4 family. Similar to PgaB, it has been reported that deletion mutants of IcaB resulted in production of fully acetylated PNAG polysaccharide with limited cell surface retention, as well as a dramatically reduced pathogenicity in animal models of biofilm infections with *S. epidermidis* and *S. aureus*. Immunofluorescence spectroscopy has shown that IcaB does associate with the cellular membrane and is also present in the cellular medium. In *vitro* studies of IcaB in the presence of different metals also showed differential de-*N*-acetylase
activity with PNAG pentasaccharides, suggesting that de-N-acetylation is metal dependent. ICP-AES analysis on as-isolated IcaB showed zinc to be the predominant metal; however the addition of Co^{2+} to the as-isolated enzyme increased the de-N-acetylation activity four-fold. This sort of trend has been observed with many other Zn^{2+} metallohydrolases and other CE4 enzymes. In terms of substrate selectivity, IcaB displayed partial dependence on substrate length, showing negligible de-N-acetylation activity on monosaccharide and disaccharide PNAG substrates, and equivalent levels on trisaccharide to hexasaccharide PNAG substrates, suggesting a three unit binding site containing two occupancy sites and the active site. Tandem ESI-MS/MS analysis of the de-N-acetylated PNAG confirmed this assessment, and also showed a preference for de-N-acetylation to occur at the residue one sugar removed from the reducing terminus on pentasaccharides, signifying an extended binding motif for carbohydrate units on the non-reducing end of the PNAG polymer.

1.4 Biofilm Treatment Strategies

Sixty-five percent of all human persistent bacterial infections are considered to be biofilm related. Due to the antibiotic tolerance of biofilm-forming bacteria as previously discussed, novel strategies are required to combat growing biofilms. A number of anti-biofilm strategies have been proposed. These methods are based on several themes: prevention of initial formation, disruption or weakening of a maturing biofilm, or the targeted killing of the bacteria within a biofilm (Figure 1.10).
Figure 1.10 Outline of the stages in biofilm development and lists of strategies aimed at inhibiting/disrupting the biofilm at specific stages

1.4.1 Prevention of Biofilm Formation

1.4.1.1 Antibodies

The use of antibodies against specific components of the biofilm has been investigated; particularly, the development of antibodies towards PNAG and dPNAG in the hopes of creating an anti-biofilm vaccine. In one study, mice were immunized with goat antibodies towards chemically-modified native dPNAG (~85% de-N-acetylated) and subsequently infected with a lethal dose of *S. epidermidis*. Mice that were immunized against dPNAG showed significantly
decreased mortality rates. Interestingly, antibodies raised against native PNAG (~10% de-N-acetylation) showed low binding capability to dPNAG, whereas antibodies raised towards dPNAG showed equal binding capacity to both PNAG and dPNAG in ELISA.\textsuperscript{61} Attempts to conjugate dPNAG to a \textit{S. aureus}-specific immunogenic protein carrier (clumping factor A) failed to improve the opsonic killing activity, and the protective activity of raised antibodies towards it.\textsuperscript{71}

Future development in this field requires the availability of large amounts of well-defined dPNAG antigens. Work in our lab by Leung \textit{et al.} developed pentasaccharide-sized PNAG and dPNAG glycoconjugates.\textsuperscript{72} These oligosaccharides are available for use as antigens for the development of anti-PNAG/anti-dPNAG antibodies. Conversely, work by Ninfantiev \textit{et al.} has led to the synthesis of a PNAG nonasaccharide as well as several defined dPNAG nonasaccharide variants.\textsuperscript{73} One of these nonasaccharide variants, a $\beta$-1,6-glucosamine homopolymer (9GlcNH$_2$), representing fully de-N-acetylated PNAG was glycoconjugated to tetanus toxoid (9GlcNH$_2$-TT) and was used as a potential antigen candidate.\textsuperscript{74} \textit{In vitro} bactericidal killing of the following PNAG producing bacteria strains was accomplished using rabbit serum raised towards 9GlcNH$_2$-TT: \textit{Streptococcus pneumonia} (4/4 strains), \textit{Enterococcus faecalis} (3/3 strains), \textit{Streptococcus pyogenes} (3/3 strains), \textit{Neisseria gonorrhoeae} (7/7 strains) and \textit{Neisseria meningitidis} (5/5 strains).\textsuperscript{75} A control experiment using a \textit{P. aeruginosa} alginate strain showed no bactericidal activity, verifying PNAG-dependent activity. Mice immunized with rabbit antibodies towards 9GlcNH$_2$-TT demonstrated significantly reduced mortality when challenged with lethal doses of \textit{S. pyogenes}, \textit{S. pneumoniae}, \textit{Listeria monocytogenes}, and \textit{N. meningitidis}. Anti-malarial activity was also observed in mice.\textsuperscript{75} This antigen is currently undergoing clinical testing and has passed phase I clinical trials as a potential PNAG-based immunotherapy. Unfortunately none of the reported results targeted a pre-formed bacterial biofilm colony. Additionally, only limited utility of this antigen has been observed with \textit{S. epidermidis}, \textit{S. aureus} and \textit{E. coli}, due to interference of its opsonic killing activity by antibodies toward capsular polysaccharides.\textsuperscript{76}

1.4.1.2 Inhibition of Bacterial Attachment

One approach that has seen a significant undertaking is the modification of abiotic surfaces preceding biofilm exposure.\textsuperscript{77} A rough, irregular surface will increase the extent of
biofilm formation compared to a smooth surface, based on peak profile analysis of various polyurethane central venous catheters.\textsuperscript{78} The same can be said for a hydrophobic surface when compared to a charged surface, with the charged surface demonstrating a capability to inhibit \textit{S. epidermidis} adherence \textit{in vitro} and \textit{in vivo}.\textsuperscript{79} Thus, material scientists have grafted surfaces with a variety of different agents in the hope of preventing initial microbial colonization, including antibiotics, disinfectants, and antiseptics.\textsuperscript{77}

In one study, vancomycin-coated titanium has been shown to retain antibacterial activity for up to 11 months, even after multiple challenges \textit{in vitro}.\textsuperscript{80} Surfaces designed to retain non-covalently bound antibiotic surfaces have also been explored, but no system has been developed that retains antibiotic release over the therapeutically relevant time scale of weeks to months.\textsuperscript{81} Unfortunately, the \textit{in vivo} long-term efficacy of covalently bound antibiotics on the surface of implants has not been reported.\textsuperscript{82} This practice is most commonly employed with bone grafts, where grafts are impregnated with antibiotics prior to surgery.\textsuperscript{83} Long term efficacy is not an issue in this case since the graft is absorbed into the bone structure over the course of weeks.

Another strategy involves coating abiotic surfaces with silver nanoparticles. Historically, silver has been used to sterilize wound infections, and was used extensively during World War I.\textsuperscript{84} Though the potential toxicity to humans has diminished its use, the advent of nanotechnology has revived its popularity; the combination of small size and the large surface area-to-volume ratio of silver nanoparticles enables maximal proximate interactions with microbial membranes.\textsuperscript{85} Its bactericidal mode of action is owing to silver’s interaction with enzyme thiol-groups which can inactivate essential enzymes involved in DNA replication and electron transport.\textsuperscript{84, 86} Silver nanoparticles coated implants in rabbits showed > 95\% inhibition of \textit{P. aeruginosa} and \textit{S. epidermidis} biofilms. Moreover, silver accumulation in the host tissue was not observed 28 days after impregnation.\textsuperscript{87} Silver is now currently used as an exogenous wound dressing to combat biofilm formation.\textsuperscript{88}

Finally, recent evidence has demonstrated that exogenous bacterial biofilm exopolysaccharides can inhibit or destabilize biofilm formation from other species. For example, it was found that Psl and Pel solutions from \textit{P. aeruginosa} culture supernatants disrupted \textit{S. epidermidis} and \textit{S. aureus} biofilms without inhibiting bacterial growth.\textsuperscript{89} Furthermore, the presence of \textit{P. aeruginosa} inhibited \textit{S. epidermidis} biofilm formation in dual-species \textit{in vitro}
experiments. Conversely, *E. coli* that produced the Ec300p anti-adhesion polysaccharide were able to quickly disperse *S. aureus* from *E. coli/S. aureus* mixed biofilms. Additionally, this *E. coli* strain was significantly protected from colonization by invading planktonic *S. aureus*. It has been proposed that the disruption caused by these anti-biofilm polysaccharides is due to several reasons: the foreign polysaccharide competitively inhibit multivalent carbohydrate-protein interactions essential for adhesion, it acts as regulators for biofilm formation, and it acts as a signaling molecule that can impact gene expression patterns involved in biofilm formation and maintenance (Figure 1.1). A range of polysaccharides have demonstrated a broad spectrum of biofilm inhibition and disruption capacity. As such, polysaccharides have been considered for use as surface coatings for indwelling medical devices; however, this utility has yet to be explored.
1.4.2 Removal or Disruption of Existing Biofilms

1.4.2.1 Enzymatic Disruption of Biofilms

The treatment of biofilms with enzymes has been explored; in particular, enzymes capable of breaking down the extracellular matrix. Enzymes that have been explored in this way include DNases for *P. aeruginosa* biofilms, and glycosyl hydrolases for *E. coli* and *S. epidermidis*. Dispersin B, a 42 kDa exo-hexosaminidase, has been explored as an exogenous
treatment for PNAG biofilms. It has been shown to capably cleave $\beta$-1,6-$N$-acetyl glucosamine homopolymer into monosaccharide units.$^{94a}$ It has also demonstrated an ability to disperse mature biofilms, and in conjunction with the antimicrobial agent triclosan, nearly eliminate biofilm formation \textit{in vitro}.$^{29, 96}$ Recently, this synergistic combination has been marketed as topical wound treatment DispersinB®, and has been undergoing clinical trials since 2010. Unfortunately, as a protein, it is not hypothesized to be stable in blood, and therefore is not an ideal treatment for biofilms on implanted devices.

1.4.2.2 Chemical Disruption of Biofilms

Chemical treatment of biofilms has been explored in the context of quorum sensing (QS) inhibition.$^{97}$ Quorum sensing is a system of stimulus and response outputs correlated to population density among bacteria; a method of coordinating gene expression among proximal microbes.$^{97a, 98}$ In Gram-negative bacteria, such as \textit{E. coli} and \textit{P. aeruginosa}, crosstalk is accomplished by means of signal molecules such as \textit{N}-acyl homoserine lactones (AHL).$^{45}$ It has been proposed that this mechanism enables the arrest of virulence factors until enough bacteria have accumulated to conquer the host defense.$^{99}$ One goal for researchers has been to develop compounds to inhibit QS, and thus prohibit bacteria from producing virulence factors. An array of inhibitors have been developed and seen moderate success with \textit{P. aeruginosa} biofilms (Figure 1.12).$^{100}$ Unfortunately, the inhibition of QS may not be a broad spectrum treatment, as it has been shown to increase biofilm formation in certain \textit{Staphylococcus} strains.$^{56, 101}$
Another investigation into chemical biofilm treatment by Losick et al. involved the utility of D-amino acids to trigger biofilm disassembly.\textsuperscript{102} In particular, D-leucine, D-methionine, D-tryptophan, and D-tyrosine showed success against various bacterial strains, including PNAG dependent S. epidermidis and S. aureus. It was hypothesized that naturally-occurring biofilms incorporate D-amino acids other than D-alanine into their peptidoglycan as part of a signaling cascade to promote biofilm disassembly, and thus the accumulation of additional D-amino acid in the environment might enhance the rate of cascade.\textsuperscript{102-103} S. aureus cultures soaked in a medium containing either D-tyrosine (50 µM) or a D-amino acid cocktail containing the amino acids outlined above (15 nM each) inhibited biofilm formation \textit{in vitro} over 24 hours. Surface impregnation of D-amino acids also demonstrated effectiveness in preventing S. aureus biofilm growth on poly-urethane surfaces.\textsuperscript{104} Unfortunately, this concept has yet to be tested \textit{in vivo}, but does show great promise.\textsuperscript{102}

Finally, there have been several reports of the aquatic natural product bromoageliferin, a 2-aminimidazole compound disrupting mature biofilms of \textit{P aeruginosa} and S. aureus (Figure 1.13a).\textsuperscript{105} Mechanistic analysis of bromoageliferin led to the discovery that its inhibitory mode of action is based on its Zn$^{2+}$-chelating ability.\textsuperscript{106} Discovery of this natural product led to the synthesis and study of an array of 2-aminobenzimidazole derivatives based on the core structure of bromoageliferin (Figure 1.13b). One of the compounds, 2-ABI3, demonstrated IC$_{50}$ values of 890 nM, 1.4 µM, and 570 nM towards methicillin-resistant S. aureus, vanomycin-resistant \textit{Enterococcus faecium}, and S. epidermidis respectively, where IC$_{50}$ represents the concentration
of compound that inhibits biofilm development by 50% (Figure 1.13c). The addition of 200 µM of zinc suppressed the inhibitory ability of 2-ABI3, suggesting a zinc dependent mechanism. This chelative activity was also verified by $^1$H NMR analysis.106

![Chemical structures](A) Bromoageliferin  
![Chemical structures](B) 2-aminobenzimidazole  
![Chemical structures](C) 2-ABI3

Figure 1.13 Small molecule biofilm inhibitors

### 1.5 Purpose of the Current Study

As discussed previously, the dPNAG matrix is the defining component of biofilm formation and survival in medicinally relevant bacteria including *E. coli* and *S. epidermidis*. Studies with DspB, a glycosidase that specifically degrades the PNAG polysaccharide matrix, have demonstrated that depolymerization of the matrix in PNAG-based biofilms results in colony dispersal. Additional research on the *pga* and *ica* operons, specifically deletion studies, has shown that bacteria that is unable to de-$N$-acetylate the PNAG polymer is unable to effectively produce biofilm colonies.56, 101a This implies that PgaB and IcaB, the $\beta$-1,6-GlcNAc de-$N$-acetylase enzymes in Gram-negative and Gram-positive bacteria respectively are a crucial factor in the biofilm formation process, and thus represent attractive targets for potential antibiofilm compounds. To date, there are few reports of small molecules that specifically disrupt or target biofilms and none that target the extracellular matrix.

The purpose of this study was thus to develop and synthesize novel inhibitors to target PgaB and IcaB, in order to develop an agent that would ultimately inhibit biofilm formation and/or promote biofilm colony degradation. Additionally, we endeavored to develop assays to
study the effectiveness of current biofilm treatment methods as well as to determine the effectiveness of synthesized inhibitors.

Methods were also developed to evaluate the efficacy of DspB to treat biofilm-related infections. This was accomplished by developing a novel carbamate substrate to measure DspB activity in vitro via a continuous absorbance spectroscopy assay. This method improved on the existing method of detecting DspB activity in several ways, including utilizing a continuous assay as opposed to a discontinuous assay, and achieving a 30-fold greater analytical sensitivity resulting from improved enzyme recognition and improved catalytic consumption of the novel substrate. The scope of this study is presented in chapter 2.

Subsequently, an array of potential inhibitors was developed towards PgaB and IcaB. Previous work by Poloczek and Pokrovskaya in our lab has demonstrated both enzymes to be metal dependent with respect to their catalytic ability.\(^{64, 67}\) Thus the design of the inhibitors was based on a carbohydrate GlcNAc scaffold, mimicking the natural enzyme substrate and containing a metal binding moiety expected to bind to the enzymatic metal ion resulting in inhibition of its catalytic ability. Transition state mimic inhibitors based on trifluoromethyl ketone moieties were also explored. The scope of this study is presented in chapter 3.

Finally, a new method was developed to measure PgaB and IcaB activity. Earlier methods included a cumbersome discontinuous and indirect assay for measuring de-N-acetylation activity utilizing fluorescamine to detect the resulting free amines at specific time-points over a lengthy 24 hour period using PNAG oligomers (tri- through hexasaccharides) as substrates, which were both tedious to synthesize and purify. The new method involved the development of a new assay using a substrate that could be readily synthesized, and its activity monitored continuously over a short period (20 minutes). With this new approach, it was then possible to measure inhibitor effectiveness quickly and accurately. The scope of this work is presented in detail in chapter 4.
2 Substrate Development for Hexosaminidases

The synthesis of the acetal substrate was done by Dr. Somnath Dasgupta. This chapter has been reproduced in part with full permissions from:


2.1 Introduction

β-Hexosaminidases are a class of glycoside hydrolases that are responsible for the hydrolysis of hexosamine subunits from a carbohydrate polymer. Many hexosaminidases belong to the family 20 glycoside hydrolases class of enzymes. These exo-hexosaminidases use the 2-acetamido group as an anchimeric participating group to achieve glycoside hydrolysis and act on the non-reducing hexosamine residues (Figure 2.1).\(^{107}\) The mechanism of hydrolysis involves intramolecular nucleophilic attack of the glycosidic bond by the 2-acetamido carbonyl to form an oxazolinium ion intermediate, which is stabilized by enzymatic carboxylate residues. Upon expulsion of the polysaccharide chain, the oxazolinium intermediate is subsequently attacked by water to release the hexosamine monosaccharide.

Figure 2.1 Typical anchimeric participation mechanism for the hydrolysis of GlcNAc polymers by family 20 hexosaminidases.

β-Hexosaminidase activity has been implicated in a number of human genetic lysosomal disorders. As such, a considerable research effort has been committed to developing different assays for monitoring β-hexosaminidase activity.\(^{108}\) Assays using fluorogenic and chromogenic
substrates, such as p-nitrophenyl-β-N-acetylglucosamine (pNP-GlcNAc) or 4-methylumbelliferyl-β-N-acetylglucosamine (Mu-GlcNAc), are commonly used (Figure 2.2).\textsuperscript{109} Alternatively, using a synthesized radiolabeled ‘natural polysaccharide’, incorporating \textsuperscript{3}H onto a GlcNAc or N-acetylgalactosamine (GalNAc) residue, is also a viable method.\textsuperscript{110} Whereas chromogenic and fluorogenic substrates are ideal for patient diagnoses in hospital laboratories, radiolabeled substrates would be used for more specialized assays and diagnoses. The remainder of this chapter will focus on chromogenic substrates.

![Hexosaminidase activity](image.png)

Figure 2.2 Common chromogenic aryl glycoside substrates used to measure hexosaminidase activity.

Dispersin B, an exo-acting hexosaminidase from the oral pathogen \textit{A. actinomycetemcomitans}, is capable of cleaving PNAG-like β-1,6-linked \textit{N}-acetylglucosamine polymers from the non-reducing end.\textsuperscript{111} \textit{In vivo}, it is a secreted protein that mediates \textit{A. actinomycetemcomitans} biofilm attachment and detachment properties.\textsuperscript{58a} It has since been developed as a topical treatment for exogenous biofilm infections, and marketed as DispersinB\textsuperscript{®}.\textsuperscript{112}

Previous work on DspB has shown that it is capable of cleaving PNAG isolated from biofilm-forming \textit{E. coli}, and results in reaction products that include GlcNAc monomers and various uncharacterized GlcNAc oligomers.\textsuperscript{94b} Interestingly, no hydrolytic products were generated when tested with chitin, a β-1,4-linked \textit{N}-acetylglucosamine homopolymer. Analysis of DspB activity with the chromogenic carbohydrate substrates pNP-GlcNAc or Mu-GlcNAc
showed a heavy dependence on the identity of the aglycone (Figure 2.2).\textsuperscript{94b} DspB’s preference for the β-1,6-linkage has also been verified through molecular modeling.\textsuperscript{113} The kinetic parameters for DspB with pNP-GlcNAc and Mu-GlcNAc were poor in comparison to other family 20 hexosaminidases.\textsuperscript{94b} We proposed that due to the selectivity of DspB, a substrate that more closely mimics the natural β-1,6-linkage may show improved catalytic activity, in contrast to pNP-GlcNAc or Mu-GlcNAc, whose linkages directly to aryl rings may be sterically disfavored and better mimic linkages to secondary hydroxyls (Figure 2.3).

![Figure 2.3 Comparison of the β-1,4 and β-1,6 linkages in chitin and PNAG respectively.](image)

The work described in this chapter will discuss an ongoing effort to develop more suitable substrates for DspB that better mimic the β-1,6-linkage, by introducing less steric bulk around the glycosidic linkage.\textsuperscript{114} This was accomplished through the synthesis of glycosyl acetal and glycosyl carbamate substrates that release chromophores upon enzymatic hydrolysis.

Traditionally, β-glucuronic carbamates were synthesized to attach to organic drug molecules to form a prodrug to improve solubility and biological uptake and aid in site selective delivery to a cancer site via the ADEPT (Antibody Directed Enzyme Prodrug Therapy) principle.\textsuperscript{115} In vivo hydrolysis of the pro-moiety carbamate by native β-glucuronidase enzymes then reveals the active drug molecule. Alternatively, p-nitroaniline carbamates have been previously used to monitor protease activity through a cascade degradation mechanism involving the production of azaquinone methides, carbon dioxide, and p-nitroaniline. Prior to our work, neither glycosyl carbamates nor glycosyl acetals had been explored as chromogenic glycosidase substrates.\textsuperscript{116}
2.2 Results and Discussion

2.2.1 Synthesis of Acetal and Carbamate Substrates

The synthesized substrates are derived from N-acetyl glucosamine with either an acetal or carbamate linkage at the reducing end. These linkages were chosen for their flexibility proximal to the glycosidic oxygen in comparison to the traditionally-used aryl substrates and for their potential fragmentation upon enzymatic hydrolysis, which would lead to a signal from either p-nitrophenolate (pNP) or p-nitroanilide (pNA) moiety (Figure 2.4). A substrate with greater activity towards DspB would overcome the challenges inherent to the system, including dilute conditions or assays conducted in biological media.

![Chemical structures](image)

**Figure 2.4** Fragmentation pathways for the proposed acetal substrate 1 and carbamate substrate 2.

2.2.1.1 Synthesis of Acetal Substrate 1

The synthesis of the acetal substrate 1 began from the alkylation of the known compound 3,4,6-triacetyl-2-deoxy-2-phthalimido-D-glucose with 1-chloromethoxy-4-nitrobenzene, synthesized in two steps from pNP and chloromethylthioether, in basic conditions, yielding the β-glycoside product in 40% yield (Scheme 2.1). Removal of the phthalimido group with ethylenediamine in butanol, followed by acetylation of the resulting free amine in acetic
anhydride/pyridine yielded the per-acetylated substrate in 78% yield. Final global deprotection in Zemplén conditions afforded the final compound 1 in 90% yield.

Scheme 2.1 (a) ClCH₂SMe, NaI, NaH, DMF (b) SOCl₂, DCM. (c) NaI, K₂CO₃ (d) i. NH₂CH₂CH₂NH₂, Butanol, 120 °C. ii. Py, Ac₂O (e) MeOH, NaOMe.

2.2.1.2 Synthesis of Carbamate Substrate 2

The carbamate substrate 2 was synthesized in four steps from commercially available N-acetylglucosamine (Scheme 2.2). Following peracetylation, selective deacetylation of the anomeric hydroxyl with benzylamine gave the free hemiacetal. This was then treated with p-nitrophenyl isocyanate (pNP-NCO) in toluene with a catalytic base to afford the protected glycosyl carbamate 8, which precipitated out of the reaction mixture exclusively as the β-anomer in 91% yield. This result is consistent with the results found in the literature, where glucose derivatives were reacted with various aryl and alkyl isocyanates to yield only β-carbamates when conducted in the presence of weak bases. Final deprotection of the remaining acetyl groups proved challenging: Zemplén conditions resulted in the return of N-acetyl glucosamine and p-nitroisocyanate, whereas acidic HCl in methanol resulted in hydrolysis of the carbamate, yielding only N-acetyl glucosamine and pNA. The final substrate 2 was obtained by careful addition of 3.3 equivalents of hydrazine monohydrate in methanol, while monitoring the reaction by TLC.
over six hours. Crude NMR analysis of the reaction mixture suggested a 62% yield of the desired product by integration of the anomeric signals; however, 48% yield was obtained after flash column chromatography.

![Scheme 2.2](image_url)

Scheme 2.2 (a) pNP-NCO, cat TEA, Tol, rt, 12 h, 91%. (b) NH₂NH₂·H₂O, MeOH, rt, 6 h, 48%.

### 2.2.2 Substrate activity assay

Substrates 1 and 2 were submitted to enzymatic assays with either DspB, a β-1,6 hexosaminidase, or jack bean β-hexosaminidase, an enzyme with a preference for hydrolyzing β-1,4-linkages, while monitoring reaction progress by continuous absorbance spectroscopy analysis. Substrate 1 hydrolysis was monitored at A₄₀₅ over a range of concentrations (0.1- 5 mM 1, 0.1 µM DspB or 25 nM jack bean β-hexosaminidase, 50 mM phosphate, 100 mM NaCl, pH 6.0) resulting in the production of pNP, whereas substrate 2 was monitored by A₄₁₀ (same conditions as above), resulting in the subsequent production of pNA. These results were then compared to the enzymatic activity against the benchmark pNP-GlcNAc substrate under the same conditions, monitored at A₄₀₅. The major point of difference for these assays is that they were run continuously, whereas pNP-GlcNAc hydrolysis assays are typically measured discontinuously at specific time-points after alkaline spiking to improve the phenolate analytical signal.

#### 2.2.2.1 Acetal Substrate 1

Upon introduction of substrate 1 to the assay conditions outlined above, no increase in A₄₀₅ was detected over a 30 minute time period in the presence of either DspB or jack bean β-hexosaminidase. Further experiments were performed to determine whether this was a result of the glycosidic linkage not being cleaved or the resulting hydroxyl-pNP-acetal not fragmenting to formaldehyde and pNP. ¹H NMR analysis of the product from the enzyme incubation showed no glycosidic bond cleavage, suggesting that 1 was not a suitable substrate for either hexosaminidase. Further competition assays with pNP-GlcNAc (0.5 mM) in the presence and
absence of 1 (5 mM) showed no difference in the pNP-GlcNAc hydrolysis rate, signifying that 1 did not inhibit either enzyme, and the lack of activity could be a result of inadequate enzyme recognition towards 1.

Due to the similarity of 1 to pNP-GlcNAc, it was confounding to discover that no affinity for DspB or jack bean β-hexosaminidase was observed. From the ¹H NMR analysis of 1, one notable feature is the resonance of the acetyl protons at 1.46 ppm, which is significantly upfield from the typical 1.9 to 2.1 ppm region common to N-acetylglucosamine derivatives (Figure 2.5). We hypothesized that this was due to an anisotropic effect from the aryl ring, which may be in close proximity to the acetyl protons, leading to an enzymatically inactive conformation. Analysis of crystallized 2-phenylethyl glycosides have shown the aryl rings folded back towards the pyranoside ring, with similar ¹H NMR resonance shifts being observed in similar large, flexible aromatic glycosides; napththylmethyl-N-acetylglucosamine glycoside was reported to have acetyl proton resonance of 1.68 ppm. Unfortunately, ¹H NMR NOE experiments showed no through space contacts between the acetyl protons and aryl protons in 1, and thus our hypothesis could not be verified.
Figure 2.5 $^1$H NMR array of the acetal GlcNAc substrate in MeOD (bottom) and methyl-N-acetylglucosamine glycoside in D$_2$O (top). Note the difference in chemical shift for the acetyl protons (1.49 ppm vs 2.05 ppm).

2.2.2.2 Carbamate Substrate 2

Upon introduction of substrate 2 to the previously mentioned assay conditions, promising results were obtained. With DspB, a 20-fold improvement of $K_m$ was observed, combined with a 200-fold $k_{cat}/K_m$ when compared to the benchmark pNP-GlcNAc (Table 2.1). When 2 was assayed in the presence of jack bean $\beta$-hexosaminidase, there was only a small improvement in $K_m$ compared to pNP GlcNAc and a three-fold improvement in $k_{cat}$. Experimentally-obtained $K_m$ and $k_{cat}$ values for pNP-GlcNAc were consistent with literature values for both DspB and jack bean $\beta$-hexosaminidase. Due to the low solubility of the substrates, saturation could not be achieved. Michaelis-Menten kinetic parameters were obtained from double-reciprocal ($1/v$ vs $1/[2]$) plots using first order kinetics (Figure 2.6).
Table 2.1 Kinetic characterization of substrate 2 and comparison with pNP-GlcNAc.

<table>
<thead>
<tr>
<th>Enzyme/Substrate</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM/s$^{-1}$)</th>
<th>Literature $k_{cat}/K_m$ (mM/s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DspB/2</td>
<td>2.3 ± 0.2</td>
<td>14.0 ± 0.1</td>
<td>6.1</td>
<td>-</td>
</tr>
<tr>
<td>DspB/pNP-GlcNAc</td>
<td>46 ± 2</td>
<td>1.0 ± 0.1</td>
<td>0.021</td>
<td>0.02$^{91b}$</td>
</tr>
<tr>
<td>Jack bean/2</td>
<td>0.43 ± 0.02</td>
<td>22 ± 2</td>
<td>51</td>
<td>-</td>
</tr>
<tr>
<td>Jack bean/pNP GlcNAc</td>
<td>0.58 ± 0.02</td>
<td>7.9 ± 0.3</td>
<td>13.6</td>
<td>35.6$^{119}$</td>
</tr>
</tbody>
</table>

Figure 2.6 Double-reciprocal plot used to obtain Michaelis-Menten kinetic parameters for the carbamate substrate 2. This method was used to acquire the kinetic parameters in every case.

The improved $K_m$ for 2 with DspB supports the hypothesis that reduced steric congestion around the glycosidic linkage would improve recognition of the substrate as compared to pNP-GlcNAc. Consistent with previous work on 1,4-cleaving hexosaminidases, jack bean β-hexosaminidase showed no improved affinity for either aglycone, demonstrating a similar $K_m$ for
both 2 and pNP-GlcNAc. Fortuitously, both enzymes showed improved turnover of the glycosyl carbamate in comparison to pNP-GlcNAc, shown by the improved $k_{cat}$ values, demonstrating that glycosyl carbamates are ideal substrate candidates for other family 20 hexosaminidases. Overall, a 35-fold improvement in analytical sensitivity was observed with DspB and substrate 2 over pNP-GlcNAc (Figure 2.7). This is a result of improved kinetics and improved extinction coefficients of pNA over pNP in the reaction conditions at pH 6.0 (5450 M$^{-1}$ cm$^{-1}$ for pNA vs 7280 M$^{-1}$ cm$^{-1}$ for pNP).
2.2.3 2nd Generation Carbamate Substrate

Upon the successful application of carbamate substrate 2 with DspB, a new substrate was designed. The limitations of substrate 2 included limited solubility in aqueous media (5 mM) and the relatively low extinction coefficient of the pNA chromophore (5450 M$^{-1}$ cm$^{-1}$ at pH 6). To this end, the next generation carbamate contained a charged $N$-methyl-quinolinium moiety as the chromogenic species, which would be expected to expel 7-amino-1-$N$-methylquinolininium upon hexosaminidase cleavage.
2.2.3.1 Synthesis

The carbamate substrate 12 was synthesized in four steps from the commercially available N-acetylglucosamine (Scheme 2.3). Following peracetylation and selective deacetylation of the anomeric hydroxyl with benzylamine, the known free hemiacetal 7 was then treated with p-nitrobenzoic anhydride to afford the mixed anhydride 9. Nucleophilic attack of the anhydride center by 7-aminoquinoline yielded the carbohydrate carbamate 10, which could then be globally deprotected in the presence of hydrazine monohydrate in methanol. Methylation of the ring nitrogen with methyl iodide in DMF followed by precipitation upon the addition of ethanol led to the final product 12.

Scheme 2.3 (a) 3 eq (pNP)₂CO, TEA, DCM, 12 h, rt, 80%. (b) 2 eq 5-quinamine, TEA, DCM, rt, 6 h %. (c) NH₂NH₂·H₂O, MeOH, rt, 6 h 78%. (d) 1 eq MeI, DMF, rt, 3 h, 47%.

2.2.3.2 Substrate Activity

Compound 12 was found to be soluble in aqueous solution to at least 20 mM, compared to the maximal solubility of 5 mM for the carbamate substrate 2, which was expected due to its charged state. Unfortunately compound 12 also showed a limited stability in aqueous solution,
exhibiting a moderate background hydrolysis rate in the absence of enzyme (data not shown). As a result it was not tested in the presence of DspB.

2.3 Conclusions

In conclusion, novel acetal and carbamate substrates were synthesized and tested against two hexosaminidases. This is the first reported instance for both a glycosyl acetal and a glycosyl carbamate to be tested as an analytical substrate for glycosyl hydrolases. Traditionally, glycosyl carbamates have been used for development of prodrugs to improve solubility and site-specific delivery. Using the glycosyl carbamate structure 2, it was possible to monitor DspB activity in a continuous assay over a short time period with 35-fold greater sensitivity than the traditional pNP-GlcNAc under the same conditions.
3 Synthesis and Development of Inhibitors towards PgaB and IcaB

The synthesis of the phosphonamidate inhibitor was done by Dr. Vavara Pokrovskaya. This chapter has been reproduced in part with full permissions from:


3.1 Introduction

Based on sequence homology, IcaB and PgaB, proteins involved in the biofilm pathway for *S. epidermidis* and *E. coli* respectively, are predicted to have carbohydrate deacetylase activity. These proteins are homologous to other enzymes classified in the Carbohydrate Esterase Family 4 in the CAZy database (www.cazy.org). Well studied enzymes within this family have de-*N*- or de-*O*-acetylation activity on the carbohydrate polymers xylan or chitin, or peptidoglycan residues, and commonly exhibit metal-dependent activity. Based on crystallographic data acquired for PgaB, and comparison to other known CE4 enzymes, a catalytic mechanism for PgaB has been hypothesized utilizing a metal center to achieve de-*N*-acetylation of PNAG to produce dPNAG (Figure 3.1).
Due to the necessity of PNAG de-N-acetylation for efficient bacterial biofilm formation in *E. coli* and *S. epidermidis*, these enzymes make attractive drug targets. To date, no inhibitor has been identified for these enzymes. In this chapter, the synthesis of potential inhibitors against these proteins is presented. These inhibitors are based on a metal-chelating scaffold, and on transition state analogs. Finally, the development of a pentasaccharide inhibitor synthesized using a chemoenzymatic approach will be described.

### 3.1.1 Inhibitor Design

#### 3.1.1.1 Metal Chelating Inhibitors

Metalloenzymes catalyze a variety of hydrolytic reactions on many different substrates in important metabolic pathways. In particular, deacetylation is an example of a reaction commonly catalyzed by zinc-dependent enzymes. The biological importance of this metal center has made these enzymes an attractive pharmaceutical target for the development of inhibitors. Generally, these enzymes adopt a mechanism where metal-bound water acts as a nucleophile and stabilization of the resulting tetrahedral intermediate is also provided by the active site metal.
Increased understanding of these enzymes and their mechanistic pathways has led to the design of different types of inhibitors.

One class of metallohydrolases inhibitor includes compounds that specifically bind the metal center, with the most common subclass being coined “zinc binding groups” (ZBG). Notable examples include inhibitors targeting matrix metalloproteases (MMPs) a family of zinc dependent endopeptidases,\textsuperscript{122} inhibitors targeting histone deacetylases (HDACs),\textsuperscript{123} and glycosyl deacetylase inhibitors, specifically, LpxC inhibitors.\textsuperscript{124} A general principle for metalloenzyme drug design is that the metal binding group is responsible for binding energy, whereas the other functional groups on the small molecule provide binding selectivity.\textsuperscript{125}

### 3.1.1.1.1 Matrix Metalloprotease Inhibitors

Great efforts have gone into developing MMP inhibitors with anti-cancer activity.\textsuperscript{122a} A common feature found in the most effective inhibitors was a metal chelating moiety (Figure 3.2). These metal chelating moieties can be mono- or bi-dentate in character. Due to the sheer number of homologous MMP enzymes (>20), inhibitor discovery has been performed via broad spectrum library screening on all MMP enzymes to ensure target selectivity.\textsuperscript{122c} In one example, it was computationally determined that in a small molecule selective for MMP-9 binding the hydroxamic acid moiety accounted for 96% of the binding energy.\textsuperscript{126}

![MMP-9 inhibitor examples](image)

Figure 3.2 MMP-9 inhibitor examples that have undergone clinical trials for cancer treatment. Metal chelating hydroxamic acid group is denoted in red.

### 3.1.1.1.2 Histone Deacetylase Inhibitors

Histone deacetylases (HDACs) have been a popular target for scientists for many years as anti-cancer drug targets. Breslow \textit{et al.} were the first to discover the potent HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), marketed in the United States and Canada as
Vorinostat for the treatment of T cell lymphoma (Figure 3.3).\textsuperscript{123a} The mode of action of this small molecule entails binding of the HDAC Zn\textsuperscript{2+} metal center in a bidentate fashion through the hydroxamic acid and the aliphatic chain interacts with a hydrophobic pocket. Work by Miyata \textit{et al.} explored the binding of SAHA analogs to HDAC8, where each analog contained a substituted metal binding domain (Table 3.1).\textsuperscript{123b} This study provided great insight into the relative binding capacities of various common metal binding moieties for Zn\textsuperscript{2+}.

<table>
<thead>
<tr>
<th>Functional Group (R)</th>
<th>IC50 (µM)</th>
<th>Functional Group (R)</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CONHOH (SAHA)</td>
<td>0.28</td>
<td>-NHOCH\textsubscript{2}NH\textsubscript{2}</td>
<td>&gt;100</td>
</tr>
<tr>
<td>-NHCONHOH</td>
<td>80</td>
<td>-NHOCH\textsubscript{2}SH</td>
<td>0.39</td>
</tr>
<tr>
<td>-NHCONH\textsubscript{2}H</td>
<td>150</td>
<td>-NHSO\textsubscript{2}Me</td>
<td>7500</td>
</tr>
<tr>
<td>-NHOCH\textsubscript{2}OH</td>
<td>&gt;100</td>
<td>-SO\textsubscript{2}Me</td>
<td>230</td>
</tr>
</tbody>
</table>

Table 3.1 Relative inhibition activities of various SAHA analogs.
3.1.1.1.3 Glycosyl Deacetylase Inhibitors

LpxC, a zinc-dependent lipopolysaccharide (LPS) de-N-acetylasen, provides an attractive target for drug design as a potential antibiotic against Gram-negative bacteria.\textsuperscript{124a} LPS is a potent endotoxin found in the outer membrane of Gram-negative bacteria consisting of 3 parts: lipid A, the core oligosaccharide, and the O antigen, with the first two parts considered to be highly conserved among bacteria (Figure 3.4).\textsuperscript{126, 128} Early in the LPS biosynthesis, the core N-acetyl glucosamine residue is de-N-acetylated by LpxC, which in turn is re-acylated with a fatty chain before subsequent functionalization and polysaccharide elongation. Inhibition of LpxC leads to the formation of attenuated LPS, which subsequently lead to bacteria with compromised viability.\textsuperscript{129} Work by Hindsgaul et al. demonstrated the synthesis of an inhibitor with a carbohydrate scaffold and a metal-chelating hydroxamic acid moiety.\textsuperscript{124a} This compound demonstrated 50% inhibition of LpxC at 1 \textmu g/mL.\textsuperscript{124b} Although LpxC belongs to a different carbohydrate esterase family than PgaB and IcaB (CE11 vs CE4), LpxC shares some similarity with PgaB and IcaB in that it acts on the same GlcNAc scaffold and is metal dependant.
More recently, Urbanaik et al. have developed a library of carbohydrate-based metal binding inhibitors targeting glycosylphosphatidylinositol (GPI) de-N-acetylases, a causative agent in African sleeping sickness, based on the previously mentioned LpxC inhibitor (Figure 3.5). These compounds showed IC₅₀ values ranging from micromolar to molar.

Figure 3.4 Left – Biosynthesis of Lipid A and the role LpxC plays. Right – LpxC inhibitor developed by Hindsgaul et al.¹²⁴a, ¹²⁵

Figure 3.5 Left – GPI de-N-acetylase catalyzed reaction. Right – inhibitors synthesized and tested with GPI de-N-acetylase. Metal chelating regions in red. Values below inhibitors represent IC₅₀ values.¹³⁰a
3.1.1.2 Transition State Mimics

Transition state analogs are commonly employed to target enzymes. These analogs mimic a substrate’s transition state during the enzymatic reaction. Since the transition state is the highest energy state during the reaction course, enzymes stabilize this state in order to lower the energy barrier of the chemical reaction. As a result, the transition state is the tightest bound enzyme complex over the reaction course (Figure 3.6). Therefore, molecules that mimic the transition state of an enzyme-catalyzed reaction are ideal inhibitor candidates. A number of transition state analog inhibitors have also been developed for various metallodeacetylase enzymes and will be discussed in detail.

![Figure 3.6 Simplified reaction coordinate energy diagram of an enzyme-catalyzed reaction and an uncatalyzed reaction.](image)

3.1.1.2.1 Phosphonamidate Inhibitors

Phosphonamidates have long been employed in medicinal chemistry as transition state inhibitors for de-N-acetylase enzymes. Phosphorous-containing compounds are found to be amongst the most potent inhibitors of metalloproteases. The phosphorous center is tetrahedral with heteroatoms approximately positioned to mimic the tetrahedral transition state of amide
bond hydrolysis (Figure 3.7). Additionally, the phosphorous-heteroatom bonds are longer than ground state carbon-heteroatom bonds and are thus capable of mimicking the longer bonds in the transition state of the reaction with the native substrate.

NagA is a metal dependent enzyme that catalyzes the deacetylation of N-acetyl-D-glucosamine-6-phosphate to form D-glucosamine-6-phosphate (Figure 3.8), similar to the action of IcaB and PgaB. This enzyme is found in E. coli and homologs have also been identified in 300 different bacterial strains.\textsuperscript{132} Its overall function is involved in the process of catabolizing N-acetyl-D-glucosamine from chitobiose and recycling the peptidoglycan, an essential component of the bacterial cell wall, thus making it an attractive drug target.\textsuperscript{133} In E. coli, this enzyme contains a zinc ion in its active site coordinated by a glutamic acid residue and two histidine residues in a tetrahedral conformation.\textsuperscript{132}
Recently, work by Raushel et al involved development of a carbohydrate-based phosphonamidate to act as an inhibitor towards the metallo de-N-acetylase NagA (Figure 3.8). This inhibitor mimicked the substrate exactly with the exception of a methyl phosphonamidate moiety in place of the N-acetyl group. *In vitro* kinetic analysis of recombinant NagA showed it to be a moderately fast-acting enzyme when introduced to its natural N-acetyl-D-glucosamine-6-phosphate substrate ($k_{cat}/K_m$ of $5.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$). Introduction of the glycosyl phosphonamidate showed the molecule to be a remarkably strong competitive inhibitor, with a $K_i$ value of 34 nM. The authors also pointed out that this $K_i$ value is among the lowest ever measured for the inhibition of a deacetylase.
3.1.1.2.2 Fluoro Ketone Inhibitors

The incorporation of fluorine can result in profound changes in the physical and chemical properties of organic molecules. These changes can also affect the biological activity of the molecules. Trifluoromethyl groups in particular are exceptionally inductively electron withdrawing, boasting an inductive sigma constant (σI) of 0.43 (Table 3.2). When placed alpha to a ketone, it has the implication of drastically altering the equilibrium between the ketone and the corresponding geminal diol. In the case of acetone versus hexafluoroacetone, a $10^9$-fold shift in the equilibrium constant towards the geminal diol in aqueous media was observed (Figure 3.9). \(^{136}\)

<table>
<thead>
<tr>
<th>Substituent</th>
<th>Inductive Sigma Value (σI)</th>
<th>Substituent</th>
<th>Inductive Sigma Value (σI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>0</td>
<td>CF₃</td>
<td>0.43</td>
</tr>
<tr>
<td>CH₃</td>
<td>-0.04</td>
<td>Cl</td>
<td>0.47</td>
</tr>
<tr>
<td>C₆H₅</td>
<td>0.10</td>
<td>NO₂</td>
<td>0.76</td>
</tr>
<tr>
<td>OH</td>
<td>0.29</td>
<td>N(CH₃)₃⁺</td>
<td>0.93</td>
</tr>
</tbody>
</table>

Table 3.2 Inductive sigma values for various organic substituents. \(^{136}\) Positive values represent electron-withdrawing power relative to hydrogen.

![Equilibrium of ketones and geminal diols in aqueous solution.](image)

Figure 3.9 (A) Equilibrium of ketones and geminal diols in aqueous solution. (B) Comparison of equilibrium constants between acetone and hexafluoroacetone to their corresponding geminal diols. In this case, a $10^9$-fold difference was observed.
The ability of trifluoromethyl-ketone moieties to adopt the geminal diol conformation is an important measure of their role as potential metallohydrolase inhibitors. This conformation best resembles the tetrahedral intermediate of an acyl hydrolysis reaction. A crystal structure of carboxypeptidase A and a trimethylfluoro-ketone inhibitor shows the inhibitor bound to the active site in its geminal diol form. A SAHA mimic replacing the hydroxamadate group with a trifluoromethyl ketone moiety has also demonstrated comparable inhibition efficacies, exhibiting an IC<sub>50</sub> value of 6.7 µM (Figure 3.10).  

![Figure 3.10](image)

Figure 3.10 Left – Ketone binds to the hydrolase metal center in its geminal diol form. Right – SAHA analog containing a trifluoromethyl moiety, exhibiting a IC<sub>50</sub> value of 6.7 µM.

The effectiveness of fluoro ketone inhibitors is best observed when compared to similar non-fluorinated derivatives. A study using fluoro ketones as potential enzyme inhibitors was performed by Abeles et al. Working with angiotensin converting enzyme, a zinc metalloprotease, they demonstrated a 3000-fold improvement in inhibition capability of a trifluoro methyl ketone when compared to a simple methyl ketone (Figure 3.11). A similar 1000-fold improvement was observed when making similar modifications to a ketone inhibitor for pepsin, an aspartate protease demonstrating that fluoro ketone inhibitors are not metal dependent.

![Figure 3.11](image)

Figure 3.11 Inhibitors of angiotensin converting enzyme, a zinc protease.
3.2 Results and Discussion

3.2.1 Monosaccharide Inhibitors

3.2.1.1 Acyl and Sulfonyl Inhibitors

Many metal-chelating inhibitors have been designed for metal-dependent N-acetylamidases, such as histone de-N-acetylasases and other carbohydrate processing de-N-acetylasases such as Lipopolysaccharide de-N-acetylase LpxC. A library of inhibitors with ranging IC$_{50}$ values (100 – 500 uM) has also been identified for a Streptococcus peptidoglycan de-N-acetylase spPgdA from the CE4 family via a large in silico library screen. A common feature of these de-N-acetylamidase inhibitors has been a metal-coordinating functional group, capable of binding the active site metal. Common metal coordinating functional groups include free thiols and hydroxamic acid moieties, the latter being used as a successful inhibitor for LpxC, under the name TU-514 (Figure 3.12).$^{124a, b, 125}$ Due to these past results, part of the library of potential PgaB/IcaB inhibitors was based on a glucosamine scaffold with an installed metal coordinating group in the acetamide position.$^{139}$ Additionally, methylation of the amido nitrogen was investigated, as this would potentially allow for different orientations of the metal chelating groups, and has been shown to improve inhibitor efficacy for Zn metalloproteases, which have been proposed to act in a similar mechanism to PgaB.

![Figure 3.12 A) TU-514, a proposed metal-chelating lipopolysaccharide de-N-acetylase inhibitor for LpxC. B) Proposed metal-chelating inhibitor structures for IcaB and PgaB, where R = H or Me, Y = CH$_2$NH$_2$, CH$_2$OH, CH$_2$SH, NHOH.](image-url)

The acyl and sulfonyl inhibitor array was initiated from the starting scaffold molecule methyl β-2-deoxy-2-aminoglucopyranoside, which could be obtained in three steps from the
commercially available \( N \)-acetylglucosamine (Scheme 3.1). Using protection-group-free chemistry, condensation of \( N \)-acetylglucosamine with a small excess of tosyl hydrazide in DMF, followed by precipitation with diethyl ether resulted in a multigram scale production of 13.\(^{140}\) Oxidation of the glycosyl hydrazide donor with NBS in the presence of methanol yielded the methyl glycoside 14, which could then be subsequently de-\( N \)-acetylated in the presence of hydrazine monohydrate to quantitatively yield the divergent starting material 15 upon acidic workup.\(^{141}\) Conversely, 15 could also be obtained in 3 steps from glucosamine hydrochloride; initial acetylation and bromination of the carbohydrate by acetyl bromide yielded the 3,4,6-triacetyl-2-deoxy-2-aminoglucosyl bromide 16.\(^{142}\) Direct methanolysis of 16 in the presence of pyridine, followed by subsequent de-\( O \)-acetylation in acidic conditions also yielded the divergent starting material 15, in large scale.

Upon synthesis of the divergent starting material, the free amine of 15 could then be acetylated to yield a variety of potential inhibitors (Scheme 3.2). HBTU-assisted condensation of 15 with benzyloxyacetic acid or Cbz-Gly-OH followed by hydrogenation over Pd/C yielded the hydroxyl and amino inhibitors 19 and 21 respectively. Conversely, condensation with pentafluorophenyl \( S \)-acetylthioglycolate (SAMA-OPFP) followed by alkaline deprotection under Zemplén conditions yielded the thiol inhibitor 23. The hydroxylurea inhibitor 24 and the sulfonylate inhibitor 25 could also be accessed by acetylation of 15 with 1-(4-nitrophenol)-\( N \)-hyxroxyxycarbamate or with mesyl chloride respectively.\(^{143}\)
Synthesis of inhibitors with a methylated amide required the production of a divergent monosaccharide containing a secondary methylated amine. To this end, benzyl protected methyl 2-aminomethyl-2-deoxy-β-D-glucoside was synthesized in four steps from 14 (Scheme 3.3).\textsuperscript{144} Initial per-$O$-benzylation of 15 with benzyl bromide followed by $N$-carbamylation of the amide with Boc$_2$O in the presence of catalytic DMAP yielded the mixed imide 27. De-$N$-acylation of the mixed imide with NaOMe resulted in the expulsion of acetate, yielding the carbamate monosaccharide 28. Reduction with LiAlH$_4$ yielded the secondary methylamine 29 in good yield, which could then be divergently functionalized to produce the secondary methyl amide inhibitors.
Synthesis of the methylated inhibitors proceeded from compound 29 in a similar fashion to the previously described non-methylated inhibitors (Scheme 3.4). DCC-assisted condensation of 29 with benzyloxyacetic acid or Cbz-Gly-OH followed by global deprotection employing hydrogenation over Pd/C yielded the hydroxyl and amino inhibitors 35 and 36, respectively. Similarly, the hydroxylurea and sulfamate inhibitors could be accessed by condensation of the amine with 1-(4-nitrophenol)-N-hydroxycarbamate or mesyl chloride followed by global deprotection employing hydrogenation over Pd/C to yield compounds 37 and 38, respectively. Finally, condensation of the secondary amine with the sulfamylating agent benzyl N-(chlorosulfonyl)carbamate (BNCC) yielded the sulfamate inhibitor 39 after global deprotection of the benzyl groups via hydrogenation over Pd/C.145
Scheme 3.4 (a) 1 eq DCC, 1 eq benzyloxyacetic acid, TEA, EtOAc:DCM (1:1), rt, 12 h, leading to 30, 89%. (b) 1 eq DCC, 1 eq Cbz-Gly-OH, TEA, EtOAc:DCM (1:1), rt, 12 h, leading to 31, 92%. (c) 1 eq 1-(4-Nitrophenol)-N-hydroxycarbamate, TEA, DMF, rt, 12 h, leading to 32, 72%. (d) 1 eq MsCl, TEA, EtOAc, rt, 12 h, leading to 33, 88%. (e) 1.2 eq BNCC, 1.2 eq TEA, DCM, rt, 3 h, leading to 34, 88%. (f) 10% Pd/C, 1 atm H₂, MeOH, rt, 12 h, 75–82%.

Lastly, modified chemical schemes were required for the synthesis of the sulfamate derivative and the methylthioglycolyl amide derivative, due to their inherent incompatibility with the previously described schemes (Scheme 3.5). Owing to the reactivity of the sulfamylating agent BNCC, hydroxyl protecting groups were required. To this end, 28 was treated with TFA to afford the benzyl-protected glucosamine derivative 40, which could then be condensed with BNCC followed by subsequent global deprotection by hydrogenation over Pd/C to yield the sulfamate derivative 42. Conversely, due to the incompatibility of sulfur with hydrogenation conditions, an alternative synthesis for the methylthioglycolyl amide derivative was as follows; the methyl glucosamine derivative 29 was initially debenzylated by hydrogenation over Pd/C to yield the unprotected glucosamine 43, which could then be acylated using SAMA-OPFP after which subsequent base-catalyzed hydrolysis of the thioester would yield the methylthioglycolyl amide inhibitor 45 as a symmetric disulfide.
Scheme 3.5 A) (a) 1:1 TFA:DCM, rt, 30 min, 81%. (b) 1.5 eq BNCC, 1.2 eq TEA, DCM, rt, 3 h, 88%. (c) 10% Pd/C, 1 atm H₂, MeOH, 12 h, 75%. B) (a) 10% Pd/C, 1 atm H₂, MeOH, 12 h, 78%. (b) 1 eq SAMA-OPFP, 1.2 eq TEA, DMF, rt, 12 h, 69%. (c) 0.1 eq NaOMe, MeOH, rt, 10 min, 64%.

3.2.1.1.1 Crystal Structure of 35

Compound 19 was dissolved in a minimum of methanol, whereupon ethyl acetate was slowly added to that solution, resulting in a transition from clear and colourless solution to slightly cloudy. Sonication of the mixture for 10 minutes yielded a separation of the sugar from the solution as a slightly yellow oil. The reaction mixture was then begrudgingly left open to air for 60 hours, after which colourless, needle-shaped crystals (2 – 4 cm in length) grew in place of the oil. The crystals were then filtered, dried, and submitted to X-ray crystallography analysis (Figure 3.13).
3.2.1.2 N-Alkyl Inhibitor Synthesis

Starting from the glucosamine scaffold 15, reductive amination with glyoxylic acid in the presence of refluxing formic acid through a transfer hydrogenation mechanism led to the formation of the N-alkyl-N-formyl carbohydrate 46 (Scheme 3.6). Several methods were attempted for the deformylation of compound 46, including basic Zemplén conditions (MeONa/MeOH) and methanolic HCl, however these methods proved unsuccessful. The reported route of aqueous 2M HCl was unavailable to us due to the potential hydrolysis of the glycosidic linkage. It was finally determined that hydrazine hydrate at 50 °C afforded the final inhibitor 47 in modest yield after recrystallization.

Scheme 3.6 a) 4 eq glyoxylic acid, formic acid, reflux, 4 h, 62%. B) NH₂NH₂·xH₂O, 50 °C, 12 h, 52%.
3.2.1.3 Phosphonamidate Inhibitor Synthesis

Synthesis of the glycosyl phosphonamidate inhibitor was adapted from a procedure reported by Xu et al.\textsuperscript{134} Starting from glucosamine hydrochloride, initial per-\textit{O}-acetylation of the carbohydrate by acetyl bromide yielded the 2-deoxy-2-amino-1,3,4,6-tetra-\textit{O}-acetyl-\textbeta-D-glucopyranosyl hydrochloride 49 (Scheme 3.7).\textsuperscript{147} Phosphonylation of the amine was then accomplished by using methyl methylchlorophosphonate in chloroform in the presence of TEA under reflux conditions to obtain 50 in moderate yield. Introduction of the compound to Lewis base-activated glycosidation conditions resulted in the thiobenzyl glycoside 51. Finally, global hydrolysis of the acetyl groups and the methyl phosphonyl ester with aqueous ammonia in methanol resulted in the final phosphonamidate inhibitor 52.

![Scheme 3.7](image.png)

Scheme 3.7 (a) Methyl methylchlorophosphonate, CHCl\textsubscript{3}, TEA, reflux, 5 h, 48%. (b) BnSH, BF\textsubscript{3}·OEt\textsubscript{2}, DCM, rt, 63%. (c) NH\textsubscript{3}(aq), MeOH/H\textsubscript{2}O, rt, 4 h, 98%.

3.2.1.4 2-C Carbohydrate Inhibitors

Synthesis of the 2-C inhibitor array was accomplished from the scaffold molecule 52a, which could be synthesized in two steps from the commercially available D-Gluca.\textsuperscript{148} Initially, per-\textit{O}-benzylaion of D-Gluca with benzyl bromide and sodium hydride resulted in the benzylated gluca in good yield. This was then followed by the radical addition of dimethyl malonate to the double bond using cerium ammonium nitrate as a radical initiator in the presence of methanol and sodium bicarbonate to yield a diastereomeric mixture of the gluco product 52a and the manno product 52b in 65% and 10% yield respectively, according to crude \textsuperscript{1}H NMR. Fortunately, the gluco diastereomer was easily isolable by flash column chromatography.
Scheme 3.8 (a) 4.5 eq BnBr, 3.3 eq NaH, DMF, 0°C – rt, 12 h, 74%. (b) 2 eq cerium ammonium nitrate, 10 eq dimethyl malonate, NaHCO₃, MeOH, rt, 3 h, 65% for the gluco-product, 10% for the manno-product (relative yields obtained by crude ¹H NMR).

With compound 52a in hand, synthesis of 2-C inhibitors containing a bis-binding moiety could be synthesized (Scheme 3.9). Initial base catalyzed hydrolysis of the methyl esters with 2 M sodium hydroxide in dioxane yielded the bis-carboxylic acid glycoside 53 in excellent yield. This was followed by a global debenzylation by hydrogenation over Pd/C to yield the final inhibitor 54 in moderate yield.

Scheme 3.9 (a) 1:1 2M NaOH/Dioxane, 12 h, rt, 86%. (b) 10% Pd/C, 1 atm H₂, MeOH, 12 h, rt, 77% for top, 72% for bottom. (c) 5 eq TMS-CF₃, cat CsF, DME, rt, 12 h, 88%.

Transformation of the methyl esters to a trifluoromethyl ketone was accomplished using a method developed by Shreeve et al. Briefly, the glycosyl ester was dissolved in ethylene glycol dimethyl ether along with the trifluoromethylating agent TMS-CF₃ and the catalytic initiator CsF, resulting in excellent yield of the bis-trifluoromethyl ketone product 55. Following
this step, global debenzylation of the carbohydrate by hydrogenation over Pd/C yielded the final inhibitor 56 in moderate yield.

Finally, in order to attain a mono-functionalized inhibitor, an additional step was necessary, involving microwave-assisted decarboxylation of one of the methyl esters (Scheme 3.10). This was accomplished by dissolving the bis-methyl ester 52a in DMSO in the presence of an excess of lithium iodide, and submitted to microwave irradiation (200 W, 10 bar, 100 °C, 20 min) to yield the mono ester product 57 in decent yield. This step was also accomplished by using the same reagents, but refluxing at 180 °C for 5 hours. The reflux method, however, resulted in a lower yield. This product could then be subjected to similar reaction conditions as described above to obtain the monocarboxylic acid inhibitor 59 and the mono-trifluoromethyl inhibitor 61.

Scheme 3.10 (a) 3 eq LiI, microwave irradiation (200 watts, 10 bar, 100 °C, 20 min), DMSO, 62% (b) 1:1 2M NaOH/Dioxane, 12 h, rt, 82%. (c) 5 eq TMS-CF₃, cat CsF, DME, rt, 12 h, 84%. (d) 10% Pd/C, 1 atm H₂, MeOH, 12 h, rt., 72% for top, 81% for bottom
3.2.2 Chemoenzymatic Synthesis of a Polysaccharide Inhibitor

Previous work has shown PgaB to have no measurable de-\textit{N}-acytlaition activity on GlcNAc or any monosaccharide derivative. However, increased catalytic activity has been observed against larger PNAG substrates up to pentasaccharide in length.\textsuperscript{64} Thus, the synthesis of a larger oligosaccharide inhibitor was envisioned. An added advantage of a chemo-enzymatic approach is that PgaB de-\textit{N}-acetylation of the pentasaccharide provides optimal regiochemistry of the metal binding group (Scheme 3.11).
Scheme 3.11 (a) HF-Pyridine (neat), rt, 5 days. 40 mg per gram of GlcNAc used after HPLC size exclusion purification. (b) PgaB, 50 mM HEPES, pH 7.5, 37 °C, 24 h. (c) 10 eq N-hydroxysuccinyl 2-(octanoylthio)acetate, 1:1 H$_2$O:MeOH, rt, 2 h. (d) 0.1 eq NaOMe, MeOH, rt, 1 h.

PNAG pentasaccharide was obtained through a one-step procedure involving the dissolution of GlcNAc in a HF-pyridine solution (a procedure modified from work done by Defaye et al) to produce a mixture of oligomers from length two to eight. Size exclusion
chromatography was then used to isolate substrates of uniform lengths. Purified PNAG pentasaccharide was subjected to Pgb-catalyzed de-\textit{N}-acetylation, resulting in an inseparable mixture of PNAG and mono-de-\textit{N}-acetylated dPNAG (Pgb expression and methodology is presented in Chapter 4). The mixture was then carried forward to an amine acylation step with activated S-octanoylthioglycolate-\textit{N}-hydroxysuccinamide ester to provide a product that could be separated from the starting material PNAG pentasaccharide using RP-HPLC. Base-catalyzed hydrolysis of the octanoyl thioester resulted in the pentasaccharide inhibitor 64 with a thioglycolamide metal binding moiety (Figure 3.14).
Figure 3.14 MALDI Mass spectra of compounds 63 (top) and 64 (bottom)

3.3 Conclusions

In this chapter, the synthesis of an array of inhibitors was presented. These inhibitors include compounds designed to be metal-chelating in their mode of inhibition, as well as inhibitors representing transition state analogs. This array represents a diverse library of functional groups and inhibitor styles, improving the chances of a successful hit. The development of a pentasaccharide inhibitor through a chemoenzymatic approach was also
described. Since both IcaB and PgaB have shown size dependence for PNAG substrate length in terms of de-N-acetylation activity, it is hypothesized that a pentasaccharide inhibitor would demonstrate an increased binding affinity.
4 Assay Development and Inhibitor Evaluation with PgaB and IcaB

Development of the PgaB expression methodology was done by Dr. Joanna Poloczek. Expression and testing of inhibitors with IcaB was performed by Dr. Varvara Pokrovskaya. This chapter has been reproduced in part with full permissions from:


4.1 Introduction

Based on sequence homology, the N-terminal domain of PgaB and IcaB have predicted carbohydrate deacetylase functionality similar to enzymes classified in the Carbohydrate Esterase Family 4 (CE4) family of enzymes in the CAZy database. This class of enzymes consists of metal dependant hydrolases containing a His-His-Asp metal binding triad, along with other conserved catalytic residues. The recently solved structure of the N-terminal domain of PgaB has confirmed that the enzyme is structurally homologous to other well-characterized CE4 family enzymes like the chitin de-\textit{N}-acetylase \textit{CICDA} from \textit{Colletotrichum lindemuthianum} and the peptidoglycan de-\textit{N}-acetylase \textit{SpPgdA} from \textit{Streptococcus pneumoniae}.

Previous work in our lab was done to characterize these enzymes and their substrate activity \textit{in vitro}. Several factors have been shown to play a role in de-\textit{N}-acetylase activity for PgaB and IcaB (Figure 4.1). The activity of both of these enzymes was shown to be dependent on the length of the PNAG substrate length, with PgaB activity peaking with pentasaccharide substrate, whereas IcaB showed maximal activity with trisaccharide. Carbohydrate polymer linkage also affected activity, with both enzymes showing no de-\textit{N}-acetylase activity on short polymers of chitin, which is comprised of $\beta$-1,4-linked \textit{N}-acetylg glucosamine units, despite their similarity to the chitin de-\textit{N}-acetylase \textit{CICDA}. Finally, both enzymes demonstrated varied metal dependence. PgaB showed optimal activity with, Co$^{2+}$, Fe$^{2+}$ and Ni$^{2+}$, in contrast to Zn$^{2+}$ dependency observed in other CE4 deacetylases. IcaB, on the other hand demonstrated optimal activity with Co$^{2+}$ and Zn$^{2+}$, as has been observed with other CE4 enzymes.
A significant challenge in monitoring IcaB and PgaB activity is the low activity of the enzymes necessitating long incubations. Using PNAG pentasaccharides, activity could be measured using a discontinuous fluorescamine-based assay to probe for the production of free amines. Despite the optimization of reaction conditions for both enzymes, they have still demonstrated extremely slow kinetic parameters compared to other well-studied CE4 enzymes (Table 4.1). As a result, meaningful assay results required large amounts of substrate, and had to be monitored discontinuously over a timescale of hours to days. However, an assay capable of
evaluating deacetylase activity quickly and continuously is required for efficient inhibitor evaluation.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IcaB</td>
<td>PNAG pentasaccharide</td>
<td>0.03$^{67}$</td>
</tr>
<tr>
<td>PgaB</td>
<td>PNAG pentasaccharide</td>
<td>0.26$^{64}$</td>
</tr>
<tr>
<td>spPgdA</td>
<td>Chitin trisaccharide</td>
<td>150$^{124c}$</td>
</tr>
<tr>
<td>C/CDA</td>
<td>Chitin pentasaccharide</td>
<td>89000$^{151}$</td>
</tr>
</tbody>
</table>

Table 4.1 Kinetic parameters for a selection of CE4 enzymes.

To this end, new methodology was explored for monitoring PgaB and IcaB activity using synthetic substrates. Once a suitable method was established, the efficacy of potential PgaB and IcaB inhibitors was explored.

4.2 Results and Discussion

4.2.1 Enzyme Purification

4.2.1.1 PgaB

An MBP-PgaB(22-672) construct in a pIADL plasmid (prepared by Joanna Poloczek) was expressed in E. coli BL21(DE3) cells. A 1 L culture was grown at 37 °C in the presence of kanamycin (100 mg/L) to an OD$_{600}$ of 0.3. Nickel (II) sulfate (0.26 g, 100 mmol) was then added to the culture and the temperature was lowered to 10 °C. At this point, the cells were induced with isopropyl 1-thio-β-D-galactopyranoside (0.123 g, 0.5 mmol) for 16 hours. After centrifugal pelleting (3750 x g, 70 min) of the culture, the cells were then resuspended in 10 mL of buffer containing HEPES (50 mM, pH 7.5), NaCl (300 mM), and a Complete Mini protease inhibitor cocktail tablet (Roche) at a final concentration of 2 ml per gram of pellet weight. Lysing of the cells was achieved by sonicating the suspension on ice, followed by centrifugal pelleting of the cellular debris (16 000 x g, 1 hour). The resulting soluble fraction was then passed through an amylose resin (Sigma-Aldrich) column. MBP-PgaB was then eluted with buffer containing
HEPES (50 mM, pH 7.5). The fractions containing MBP-PgaB were concentrated using centrifugal filter units (Millepore, 30K MWCO) and stored at 4°C. The enzyme was stable and active for approximately 1 month under these conditions. Protein concentrations were measured using a predicted molar extinction coefficient of 218 070 M⁻¹. Purity of the fractions could also be assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).⁶⁴

### 4.2.1.2 IcaB

An IcaB<sub>His10</sub> construct in a UT032 plasmid was expressed in <i>E.coli</i> BL21(DE3) cells. A 1 L culture was grown at 37 °C in LB media with 100 mg/L ampicillin to an OD<sub>600</sub> of 0.5. The cells were then cooled to 10 °C, and allowed to grow until an OD<sub>600</sub> of 0.7–0.9 was reached. At this point, the cells were induced with isopropyl β-D-thiogalactoside (0.185 g, 0.75 mmol). After 24 h at 10 °C, the cells were centrifuged (3430 x g, 40 min) and the cell pellet was suspended in 20 mL of buffer containing sodium phosphate (20 mM, pH 7) NaCl (1 M) and glycerol (5% (v/v)) (buffer A). Lysing of the cells was accomplished by sonication of the resulting suspension on ice. The resulting crude extract was centrifuged to pellet and remove cell debris (16260 x g, 40 min). The soluble fraction was then gently mixed with 0.5 mL of Ni(II)-NTA resin (QIAGEN) for 1 hr before being loaded into a column (1 cm diameter). Unbound proteins were washed from the resin with 10 mL of buffer A containing 20 mM imidazole. IcaB<sub>His10</sub> was then eluted with buffer A containing 150 mM imidazole. All purification steps were performed at 4 °C. The purified protein was dialyzed overnight against buffer A and subsequently washed with buffer containing sodium phosphate (50 mM, pH 8) and NaCl (0.5 M) (buffer B) by centrifugation in an Amicon Ultra-15 10 000 Da MWCO centrifugal filter (Millipore), and concentrated to 3 mg/mL and stored at 4 °C in buffer B. The purity of the protein as analyzed by 15% SDS-PAGE was >95%. Protein mass was confirmed by electrospray ionization mass spectrometry (ESI-MS). The protein concentrations were determined using a molar extinction coefficient of 39 400 M⁻¹ cm⁻¹ calculated from a BCA assay on purified protein. The purified IcaB<sub>His10</sub> was digested with Factor Xa protease (New England Biolabs, Ipswich, MA) according to the recommended protocol to remove the His10 tag. Briefly, 1 mg of IcaB<sub>His10</sub> was incubated with 9 μg of Factor Xa at room temperature for 4 h in Factor Xa reaction buffer (75 mM HEPES, pH 6.5, 75 mM NaCl, 100 mM Na<sub>2</sub>SO<sub>4</sub>, 2 mM CaCl<sub>2</sub>). After protease digestion, Factor Xa protease was removed from the reaction mixture by affinity chromatography using Xarrest Resin. Xarrest resin slurry (100 μL, pre-equilibrated with Factor Xa reaction buffer) was added to the
cleavage reaction. The resin was resuspended by gentle mixing and incubated for 10 min at room temperature. The mixture was then centrifuged (1000 x g, 5 min) and the soluble fraction collected. In order to remove cleaved His-tag peptide, the sample was washed 4 times on a 10 000 Da MWCO centrifugal filter with buffer B, resulting in purified IcaB enzyme. The effectiveness of the digestion could be analysed by SDS-PAGE.67

4.2.2 Assay Development

Previous work on measuring PgaB and IcaB activity involved using PNAG oligomers as substrates and indirectly measuring free amine production with the aid of fluorescamine (Figure 4.2).64, 67, 152. Several unresolved challenges with this method included the low catalytic efficiency of de-N-acetylation on PNAG oligomers ($k_{cat}/K_m = 0.25$ M$^{-1}$s$^{-1}$ for PNAG pentasaccharide with PgaB), the inefficient methods of obtaining purified PNAG pentasaccharide, which featured a 5 day reaction time and 4 size exclusion chromatography steps for purification, and the low solubility of PNAG pentasaccharides (1 mM in aqueous solution), which, in turn, prevented determination of isolated $K_m$ or $k_{cat}$ values. In addition, monitoring the de-N-acetylation reaction of the pentasaccharide substrate required a cumbersome discontinuous fluorescamine assay.
Figure 4.2 A) Reaction of PgaB with PNAG oligomers. B) Reaction of fluorescamine with an amine to yield a highly fluorescent product. R in this case represents dPNAG. C) Competing hydrolysis reaction of fluorescamine yielding a non-fluorescent by-product.

4.2.2.1 Synthesis of Chromogenic Enzyme Substrates

A series of chromogenic acetyl esters were synthesized or purchased, and evaluated as PgaB substrates (Figure 4.3). Such esters have been used to study other de-O- and de-N-acetylase enzymes. 153
Figure 4.3 Acetyl esters evaluated as substrates for PgaB under continuous assay conditions.

5-Acetoxyethylquinolinium (5-AMQ) and 7-acetoxyethylquinolinium (7-AMQ) were easily and efficiently synthesized in 2 steps from 5-quinolinol and 7-quinolinol respectively (Scheme 4.1). 3-Carboxyumbelliferyl acetate (ACC) was synthesized in two steps: initial condensation of Meldrum’s acid and 2,4-dihydroxybenzaldehyde in ethanol to give 3-carboxyumbelliferyl, followed by acetylation of the phenol group with acetic anhydride in DMF.\textsuperscript{154} p-Nitrophenyl acetate (pNPAc) and 4-methylumbelliferyl acetate (AMC) were commercially available.

Scheme 4.1 A) (a) Ac\textsubscript{2}O, Py, 3 h, 97% for 5-quinolinol, 96% for 7-quinolinol. (b) 3eq MeI, THF, rt 2 h. 82% for 5-quinolinol, 78% for 7-quinolinol 82%. B) (a) EtOH, cat AcOH, cat pip, 6 h, 74%. (b) DMF, 60 °C, 20 eq Ac\textsubscript{2}O, cat TEA, 3 h, 83%.
4.2.2.2 Evaluation of Proposed Chromogenic Enzyme Substrates

Enzyme reactions were carried out in 50 µL solution volumes containing HEPES (100 mM, pH 7.5), PgaB (10 µM), and varying concentrations of pNPAc, AMC, 5-AMQ, 7-AMQ, and ACC. (0.05–1 mM for pNPAc, AMC, 0.05-10m M for 5-AMQ, 7-AMQ and ACC) at 25 °C in a 96 well plate. Substrates were all predissolved in DMSO and added to the reaction mixtures directly as such. The final DMSO concentration was 10% (v/v) in all cases. Due to substrate solubility constraints and background absorbance levels, 1-10 mM was used as a maximum concentration of substrate. Reactions were monitored in real time using the increase of either absorbance signal or fluorescence signal at the wavelengths found in Table 4.2, resulting from the hydrolysis and release of acetate over 10 min. Background hydrolysis rate were also monitored and subtracted from the values measured for the enzyme-catalyzed reactions.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Absorbance/Excitation (nm)</th>
<th>Fluorescence Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcO-(\text{pNPAc})</td>
<td>405</td>
<td>-</td>
</tr>
<tr>
<td>O(\text{Ac})</td>
<td>354</td>
<td>445</td>
</tr>
<tr>
<td>5-AMQ</td>
<td>460</td>
<td>-</td>
</tr>
<tr>
<td>7-AMQ</td>
<td>454</td>
<td>-</td>
</tr>
<tr>
<td>ACC</td>
<td>386</td>
<td>446</td>
</tr>
</tbody>
</table>

Table 4.2 Absorbance / excitation and fluorescence emission wavelengths for the chromogenic substrates tested with PgaB.

When tested under the conditions described above, all of the tested compounds were found to be substrates for PgaB; however, the pNPAc, 5-AMQ, and 7-AMQ substrates suffered from high rates of background hydrolysis, making them unsuitable for further studies. AMC and ACC were more stable to hydrolysis, so experiments were continued with these two substrates only. The limiting solubility of AMC (~1 mM) prevented saturation of enzymatic activity, thus this substrate was not evaluated further. Fortunately, ACC was soluble up to 10 mM, which allowed for the determination of the kinetic parameters of PgaB and subsequently IcaB.
4.2.2.3 Determination of Enzyme Kinetics

4.2.2.3.1 PgaB

Enzyme reactions were carried out in 50 µL solutions containing HEPES (100 mM, pH 7.5), PgaB (10 µM), and varying concentrations of ACC (0.05 –10 mM) at 25 ºC in a Corning 3686 half-area 96 well black plate. A TECAN Safire2 plate reader was used to quantify fluorescence. ACC was predissolved in DMSO and added directly to the reaction mixtures as such. The final DMSO concentration was 10% (v/v) in all cases. Reactions were monitored in real time in a continuous assay measuring the increase of a fluorescence signal at 446 nm, resulting from the hydrolysis of the acetyl moiety with excitation at 386 nm over a 20 min period to determine steady-state initial velocity kinetics. Michaelis-Menten kinetic parameters were obtained using non-linear regression analysis of the data (Figure 4.4). A slow but measurable background hydrolysis rate was also monitored and subtracted from the enzyme-catalyzed reaction before data analysis. A control experiment replacing PgaB with bovine serum albumin (BSA, 10 µM) showed no increase in reaction rate above background. All assays were performed in triplicate. A calibration curve for ACC was obtained under the reaction conditions using 7-hydroxycoumarin-4-carboxylic acid as a standard to calculate reaction rate. A $k_{cat}$ value of 0.013s$^{-1}$ and a $K_m$ value of 1.2 mM were determined for the activity of PgaB on ACC (Table 4.3).
Figure 4.4 Rates of PgaB-catalyzed ACC deacetylation rates at varying ACC concentrations. Conditions were as follows: HEPES buffer (100 mM, pH 7.5) containing 10% (v/v) DMSO, 10 µM PgaB, ACC (0.5 mM – 10 mM). Assays were done in triplicate. Rates were obtained by measuring the increase in fluorescence emission at 446 nm resulting from the acetylosis of ACC. \( V_{max} \), \( K_m \) and \( k_{cat} \) were determined from non-linear regression analysis of the data to obtain a curve.

4.2.2.3.2 IcaB

For the continuous fluorogenic assay, purified IcaB\textsubscript{His10} (30 uM) in buffer B and CoCl\textsubscript{2} (30uM) were incubated at room temperature for 30 min. ACC was predissolved in DMSO and added directly to the reaction mixtures as such. The final DMSO concentration was 10% (v/v) in all cases. To determine the steady-state initial velocity kinetics the substrate concentrations were varied from 0.01 to 5 mM. The sample was excited at 386 nm and continuously monitored for the increase in fluorescence emission at 446 nm. The production of phenol was quantified using 7-hydroxycoumarin-4-carboxylic acid as a standard. The fluorescence intensity data were
analyzed as above, using nonlinear regression analysis. All assays were performed in triplicate. A $k_{\text{cat}}$ value of 0.0013 s$^{-1}$ and a $K_m$ value of 0.5 mM were determined for the activity on IcaB on ACC (Table 4.3).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (s$^{-1}$ mM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgaB</td>
<td>0.013 ± 0.002</td>
<td>1.2 ± 0.2</td>
<td>0.01</td>
</tr>
<tr>
<td>IcaB</td>
<td>0.0013 ± 0.0001</td>
<td>0.5 ± 0.1</td>
<td>0.0025</td>
</tr>
</tbody>
</table>

Table 4.3 Kinetic parameters for PgaB and IcaB with ACC.

### 4.2.3 Evaluation of Inhibitors towards IcaB and PgaB

The potential synthesized de-N-acetylase inhibitors presented in chapter 3 were tested with Ni$^{2+}$-loaded PgaB and IcaB in a competitive assay in the presence of the fluorogenic substrate ACC. Previous work in our lab has shown that PgaB loaded with a selection of divalent ions was active *in vitro*, particularly with Fe$^{2+}$; however this variant was unstable in ambient atmospheric conditions and therefore the Ni$^{2+}$ loaded enzyme was used. Inhibitors stored as disulphides were pre-incubated with DTT for 10 minutes prior to assay onset to reduce the disulphide and bring the inhibitor into its active form. Overnight incubation of the inhibitors at 10 mM with the enzymes under assay conditions followed by $^1$H NMR analysis showed no observable hydrolysis of the amide bonds, indicating that none of the inhibitors acted as substrates for the enzymes. Inhibitors demonstrating greater than 10% inhibition of enzymatic ACC deacetylation at 10 mM were carried forward to full kinetic analysis. Methyl 2-amino-2-deoxy glucopyranoside 4 showed no inhibition of the enzyme at 1 mM.

#### 4.2.3.1 Obtaining Inhibitor Activity

Enzyme reactions were carried out in 50 µL solution volumes in a Corning 3686 half-area 96-well black plate. The solution contained HEPES (100 mM, pH 7.5), PgaB (10 µM), and ACC (5 mM) for PgaB, or IcaB$_{\text{His}10}$ (30 uM), sodium phosphate (50mM, pH 8), and NaCl (0.5M). In the IcaB case only, CoCl$_2$ (30uM) was also added to the reaction mixture. ACC was predissolved in DMSO in both cases and added directly to the reaction mixtures as such. The final DMSO
concentration was 10% (v/v) in all assays. Reactions were carried out in the presence of each inhibitor (all at 10 mM, except for 1 mM in the case of 21, 36, and 64). Reactions containing a free thiol (23, 45, 64) were pre-incubated with 1.1 equivalents of DTT for 5 min before assay onset. Reactions containing an inhibitor candidate with a primary amine were run at a maximal concentration of 1 mM due to its high background signal. Reactions were monitored in real time using the increase of fluorescence signal at 446 nm resulting from the hydrolysis of the acetyl moiety, over a 20 min period. Percent activity was measured as the relative difference of ACC acetolysis rate in the presence and absence of each inhibitor. Samples were corrected for background hydrolysis by measuring fluorescence signals of reactions lacking only the PgaB or IcaB enzyme. All assays were performed in triplicate.

4.2.3.2 Determining Kinetic Parameters of Inhibitors

Enzyme reactions were carried out in 50 µL solution volumes in a Corning 3686 half-area 96-well black plate. The solution contained HEPES (100 mM, pH 7.5), PgaB (10 µM), and varying concentrations of ACC (0.05 – 10 mM) for PgaB, or IcaB_{His10} (30 µM) sodium phosphate (50 mM, pH 8) and NaCl (0.5 M), CoCl$_2$ (30 µM), and varying concentrations of ACC (0.05 – 10 mM) for IcaB. Reactions were carried out in the presence of 3 different inhibitor concentrations (1 mM, 5 mM, 10 mM or 0.1 mM, 0.5 mM 1 mM for 64 only). Final DMSO concentration was 10% in every assay reaction mixture. Reactions containing a free thiol (23, 45) were pre-incubated with 1.1 equivalents of DTT for 5 min before assay onset. Reactions were monitored in real time over a 20 min period using the increase of fluorescence signal at 446 nm resulting from the hydrolysis of the acetyl moiety. A slow but measurable background hydrolysis rate was also monitored and subtracted from the enzyme-catalyzed reaction. All assays were performed in duplicate.

$K_i$ values were obtained for each inhibitor by line-fitting the double reciprocal (1/rate vs 1/[ACC]) of each curve to obtain an $\alpha$ coefficient at each concentration, representing the change in slope (slope = $\alpha K_m/V_{max}$) . From the derived $\alpha$ values, $K_i$ values could be obtained from the following formula ($\alpha = 1 + [I]/K_i$). The average $K_i$ value (n = 3) is reported. Obtained Lineweaver-Burk plots revealed that all the inhibitors were competitive in nature, as they had similar y-intercepts. Obtained Lineweaver-Burk plots revealed that all the inhibitors were competitive in nature, as they had similar y-intercepts.
4.2.3.3 N-Acyl Inhibitors

Of the primary amide inhibitors, the thioglycolyl amide derivative 23 showed significant inhibitory activity towards PgaB ($K_i = 480 \, \mu M$), whereas the hydroxyglycolyl and aminoglycolyl amide derivatives 21 and 23 showed only minimal inhibitory capability (Table 4.4). This is consistent with previously reported inhibitors designed for other de-N-acetylases, as thioglycolyl amides have shown greater inhibitory capacity than their hydroxy and amino counterparts. The secondary amide inhibitors as a whole displayed much better inhibitory capacity than their primary amide counterparts. In particular, the hydroxyglycolyl inhibitor 35 was found to be the best inhibitor for PgaB activity ($K_i = 320 \, \mu M$). The amino counterparts 21 and 36 demonstrated no measurable inhibitory capacity (Table 4.4). Interestingly, the amino derivatives were the best inhibitors towards IcaB, in contrast to the PgaB results.
Table 4.4 Inhibition of PgaB and IcaB by N-acyl inhibitors. \(^a\) Remaining PgaB or IcaB enzyme activity in the presence of 10 mM inhibitor. \(^b\) Remaining PgaB or IcaB enzyme activity in the presence of 1 mM inhibitor. \(^c\) Inhibitor preincubated with 1.1 eq DTT prior to assay onset.
Figure 4.5 Lineweaver-Burk inhibitor plots for compounds 23 and 35 with PgaB.
One factor to take into consideration is the conformation of the amide bond. Crystal structures of inhibitors bound to other metallodeacetylas show the metal-binding heteroatom and the carbonyl oxygen in an eclipsed gauche conformation (Figure 4.6a).\textsuperscript{155} A crystal structure of compound 19 shows the hydroxyl moiety hydrogen bonded with the amido nitrogen, locking it in an anti-conformation relative to the carbonyl oxygen, and thus unsuitable for bivalent metal binding (Figure 4.6b,c).\textsuperscript{156} This factor could partly account for the limited inhibitory activity from the primary amide series of inhibitors, particularly the glycolyl inhibitors 19, 21, and reduced 23, where this hydrogen bond network would limit rotation around the C(O)-C\textsubscript{α} bond.\textsuperscript{157} Methylation of the amido nitrogen, as in the secondary amide series of inhibitors would effectively prevent this hydrogen bonding from occurring. As a result, adopting the anti-conformation relative to the carbonyl oxygen would be less energetically favourable, in turn promoting the eclipsed gauche conformation (Figure 4.6d).
Another conformational factor to take into account is the rotation around the C2-N bond and the N-C(O) bond. There are 4 plausible states in which the acetamido functionality could exist (Figure 4.7). NMR-based conformational analysis of methyl 2-deoxy-2-acetamido-β-D-glucopyranoside has shown that a significant population exists in the (E)-anti conformation, whereas methyl 2-deoxy-2-N-methyl-N-acetamido-β-D-glucopyranoside adopts primarily the (Z)-anti conformation. The improved inhibitory activity of the N-methyl inhibitors suggests that the preferred bound conformation of the acyl metal chelates may be the (E)-anti conformation. Oddly, the best inhibitor of the N-methyl series, 35, contains a metal chelating group that has not been previously reported as an effective inhibitor of amidases.
Figure 4.7 Possible conformations of the amide bond in GlcNAc derivatives. (Z)-Anti was found to be the principle conformation when R = H. (E)-Anti was found to be the principle conformation when R = Methyl.

4.2.3.4 N-Sulfonyl and N-Phosphonyl Inhibitors

Of the sulfonyl inhibitors, the N-methylated inhibitors 38 and 39 exhibited greater inhibitory potential than their non-sulfonyl counterparts towards PgaB in line with the acyl results; the sulfonamide inhibitor 39 was shown to have a $K_i$ value of 680 µM (Table 4.5). The increased inhibitory capability of the sulfamide inhibitors 42 and 39 over the sulfonamide derivatives 25 and 38 could be due to additional hydrogen bonding. Unfortunately a conclusive study exploring the comparative inhibitory effects of sulfamides versus sulphonamides has not been explored. The phosphonamidate inhibitor 51 showed a similar $K_i$ value of 620 µM similar to the $K_i$ value calculated for the sulfamide inhibitor 39. This similarity is not surprising due to the conformational and electronic similarity of phosphonamidates and sulfonamides/sulfamides. Unfortunately, these compounds only showed limited inhibition of IcaB.
<table>
<thead>
<tr>
<th>Structure</th>
<th><img src="image" alt="Structure 25" /></th>
<th><img src="image" alt="Structure 42" /></th>
<th><img src="image" alt="Structure 51" /></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Residual Activity (%)</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PgaB</td>
<td>93 ± 3</td>
<td>91 ± 2</td>
</tr>
<tr>
<td>IcaB</td>
<td>-</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td><strong>$K_i$ (µM)</strong></td>
<td>PgaB</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IcaB</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Structure</th>
<th><img src="image" alt="Structure 38" /></th>
<th><img src="image" alt="Structure 39" /></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Residual Activity (%)</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>PgaB</td>
<td>82 ± 3</td>
</tr>
<tr>
<td>IcaB</td>
<td>90</td>
<td>93</td>
</tr>
<tr>
<td><strong>$K_i$ (µM)</strong></td>
<td>PgaB</td>
<td>5800 ± 100</td>
</tr>
<tr>
<td>IcaB</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.5 Inhibition of PgaB and IcaB by $N$-acyl inhibitors. <sup>a</sup> Remaining PgaB or IcaB enzyme activity in the presence of 10 mM inhibitor.
4.2.3.5  \( N \)-Alkyl and \( C\)-2 Inhibitors

The \( N \)-alkyl inhibitor 47 was tested against PgaB and IcaB activity and was found to show no inhibition towards both enzymes (Table 4.6). It was hoped that the carboxylate group would mimic the substrate tetrahedral intermediate chemically and proximally. It is possible that the poor inhibitory capacity of 47 could be a result of suboptimal placement of the carboxylate to effectively mimic the tetrahedral intermediate.

| Structure |  
| --- | --- |
| ![Structure of 47](image) |  

<table>
<thead>
<tr>
<th>Residual Activity (( % ))^a</th>
<th>PgaB</th>
<th>98 ± 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>IcaB</td>
<td>104</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>( K_i (\mu M) )</th>
<th>PgaB</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>IcaB</td>
<td>-</td>
<td></td>
</tr>
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</table>

Table 4.6 Inhibition of PgaB and IcaB by \( N \)-alkyl inhibitors. ^a Remaining PgaB or IcaB enzyme activity in the presence of 10 mM inhibitor.

Carbon-branched carbohydrates have long been used as important subunits in many antibiotics. C-2 carbohydrates have demonstrated activity against a number of bacterial carbohydrate deacetylase enzymes including LpxC and other GPI de-\( N \)-acyetylase inhibitors.\(^{124a,130a}\) These C-2 derivatives provide a number of advantages. A methylene connection provides a greater degree of rotational freedom over an amide, allowing for a greater diversity of potential binding modes to an enzyme. Additionally, these derivatives are non-hydrolyzable, and as such, there is no risk of potential inhibitors acting as a substrate for the targeted enzyme.
Of the C-2 inhibitors tested, none of them showed significant inhibitory capacity towards PgaB relative to the other acyl and sulfonyl inhibitors tested (Table 4.7). Fortunately, compound 59 was shown to be an adequate inhibitor towards IcaB, with a $K_i$ value of 460 µM.

<table>
<thead>
<tr>
<th>Structure</th>
<th>(\text{Residual Activity (%)}^a)</th>
<th>(K_i (\mu M))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PgaB 98 ± 5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IcaB 104</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PgaB 77 ± 5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IcaB 102</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PgaB 84 ± 3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IcaB 22</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PgaB 86 ± 5</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>IcaB 82</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4.7 Inhibition of PgaB and IcaB by C-2 inhibitors. \(^a\) Remaining PgaB or IcaB enzyme activity in the presence of 10 mM inhibitor.

This result was unexpected, given the success of trifluoromethyl ketones with other metalloenzymes. Their lack of efficacy could be due to the molecules not hydrating to the geminal diol. \(^{19}\)F NMR studies of these compounds in D\(_2\)O showed primarily the presence of the ketone, and minuscule amounts of the hydrate (26:1 for 61) (Figure 4.8). No evidence of the hydrate was observed in the \(^1\)H or \(^{13}\)C NMR. Comparatively, in the case of (S)-2-amino-8,8,8-trifluoro-7-oxo-octanoic acid, a potential arginase inhibitor, a 15:1 ketone/hydrate ratio was observed in D\(_2\)O.\(^{160}\)
Figure 4.8 $^{19}$F NMR of compound 61 in D$_2$O. Peak at -74.2 ppm represents the ketone. Peak at -84.4 ppm represents the hydrate.

It has been shown that an electron withdrawing group opposite of the trifluoro group is required for significant hydration of the ketone (Figure 4.9). In the cases described here, the carbohydrate ring may not be inductively electron-withdrawing to shift the equilibrium towards the hydrate.

Figure 4.9 Hydration equilibrium of trifluoromethyl ketones in aqueous media.
4.2.3.6 Chemoenzymatic Approach: Pentasaccharide Inhibitor

The chemoenzymatically-synthesized pentasaccharide inhibitor 64 was tested against PgaB and found to have a $K_i$ of 280 $\mu$M (Table 4.8). This value represents the best inhibitor against PgaB found to date. Compared to its chemically-similar monosaccharide thiol- inhibitor 23, 64 was found to have a two-fold increase in inhibitory efficacy. A larger gain in affinity was expected however, given the length dependence of PgaB, which demonstrates higher activity with pentasaccharide PNAG over monosaccharide GlcNAc. Further work is required to determine with confidence the reason behind this limited improvement. Unfortunately, only enough inhibitor was synthesized to test it against PgaB only, and no experiments were performed with IcaB.

<table>
<thead>
<tr>
<th>Structure</th>
<th><img src="image.png" alt="Pentasaccharide Inhibitor" /></th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual Activity (%)$^a$</td>
<td>PgaB</td>
</tr>
<tr>
<td></td>
<td>IcaB</td>
</tr>
<tr>
<td>$K_i$ (µM)</td>
<td>PgaB</td>
</tr>
<tr>
<td></td>
<td>IcaB</td>
</tr>
</tbody>
</table>

Table 4.8 Inhibition of PgaB and IcaB by polysaccharide inhibitor 64. $^a$Remaining PgaB enzyme activity in the presence of 1 mM inhibitor. $^b$Inhibitor preincubated with 1.1 eq DTT prior to assay onset.
4.3 Conclusions

In this chapter, a new method of monitoring PgaB and IcaB activity was explored using chromogenic acetyl substrates. With this method, it was possible to test the inhibitory efficacy of the array of synthesized inhibitors outlined in chapter 3. The thiol-inhibitor 23 was found to be the best monosaccharide inhibitor of PgaB, whereas the C-2 inhibitor 59 was found to be the best inhibitor towards IcaB. Finally, the chemoenzymatically-synthesized pentasaccharide inhibitor 64 was found to be the best inhibitor of PgaB, with a \( K_i \) value of 280 µM.
5 Future Perspectives

5.1 Substrate development for hexosaminidases

The availability of substrates to monitor hexosaminidase and glycosidase activity is of great interest to the scientific community. Novel glycosyl substrates could be of great value in helping determine the substrate specificity of glycosidase enzymes. As demonstrated with DspB and the carbamate substrate 2, this compound could be used to further elucidate whether other enzymes can act on a 1,6 linkage without consuming potentially-valuable native polysaccharide substrates. Among family 20 glycosyl hydrolases in the CAZy database, only DspB from *A. actinomycetemcomitans* is classified as a β-1,6-N-acetylglcosaminidase. Considering the prevalence of the β-1,6 linkage of GlcNAc in nature, especially among PNAG-producing microbes, it is unlikely that DspB is the only β-1,6 specific glycosidase. Thus, assays with substrate 2 in conjunction with the commonly-used pNP-GlcNAc, could shed light on the function of other newly-isolated β-hexosaminidases.

Alternatively, despite the inactivity of the acetal substrate 1, its conceptual utility has yet to be disproven. If our hypothesis is correct that its ineffectiveness is due to a non-reactive conformation, exchange of the p-nitrophenol group for another chromophoric analyte may resolve the issue. Particularly, substitution of the 5- or 7-hydroxyquinolinium chromophores appears promising. Due to the charged quinolinium nitrogen, these compounds would have a vastly different electrostatic profile than the pNP acetal substrate counterpart, and such a change may also induce a change in conformation (Figure 5.1). Additionally, their chromogenic properties make them ideal analytes, as a drastic increase in absorbance at 460 nm and 464 nm is observed upon hydrolysis to 5- and 7-hydroxyquinolinium, resulting from the newly possible hydroxyl conjugation. This shift in absorbance wavelength could be used to monitor reaction progress.\[162\]
Finally, β-Hexosaminidases have been implicated in a number of disease states. Human lysosomal β-hexosaminidases catalyze the hydrolysis of GlcNAc or GalNAc residues from the reducing end of a number of biologically-significant glycoconjugates. Three isoforms are known to exist: β-hexosaminidase A (HexA), β-hexosaminidase B (HexB), and β-hexosaminidase S (HexS). These isoforms play an important role in the lysosomal catabolism of glycosphingolipids (GSLs) (Figure 5.2). Defects in any of the three β-hexosaminidase genes can result in the accumulation of non-degraded glycolipids within the lysosomal compartments and leading to severe neurodegenerative storage diseases including Tay-Sachs disease (HexA mutation) and Sandhoff disease (HexB mutation), which are devastating neurological disorders that usually lead to death before the age of four.
Positive diagnoses of these diseases are dependent on the reliability of hexosaminidase assays of patient blood samples. Serum or leukocytes are most commonly used as HexA/B/S sources, however dried blood spots on filter paper has also been evaluated, with the latter being effective only with homozygote carriers.\textsuperscript{165} Unfortunately, with the current methodology, false positives can range from 2\% to 35\% depending on the ethnicity of the individual.\textsuperscript{166} This is a result of certain variations of HexA having reduced activity towards artificial substrates, but no impairment in its ability to degrade the physiological substrate GM2. Although false negative results are rare in comparison, 10\% of the cases result in an inconclusive result among the general population, resulting in the need for more expensive genetic mutation analysis.\textsuperscript{167}

Therefore, novel glycoside substrates hold great potential for improving the reliability of measuring HexA/B/S activity in human patient samples, ultimately improving the clinical standard of care. Increased sensitivity and specificity could reduce the number of false positives, providing greater respite to the affected patients and decreasing the burden on the medical
system. With the goal of improving this pharmological assay, novel $N$-acetylgalacto- carbamate or acetal substrates could be tested with human HexA/B/S for glycosidic activity.

5.2 Inhibitor development for PgaB and IcaB

Strains of bacteria lacking the $pgaB$ or $icaB$ genes produce only the PNAG polysaccharide show limited ability to form biofilms and demonstrate compromised antibiotic resistance. Successful development of an effective inhibitor for PgaB and/or IcaB could lead to the development of a treatment strategy towards the dispersal of bacterial biofilms. Unfortunately, when compounds 23, 35, and 38 were tested against $S.\ epidermidis$ 1457 in biofilm inhibition assays at a concentration of 1 mM, no inhibition was observed. This is likely due to the weak activity of these compounds. Based on these findings, tighter-binding inhibitors are likely required for future utility of this strategy.

One approach that may aid in inhibitor development is further crystallographic analysis of the target enzyme. Although the structure of PgaB has been obtained through X-ray crystallography, attempts to co-crystallize PgaB with either an inhibitor or a PNAG polysaccharide substrate have been unsuccessful. Such data would offer valuable information regarding the binding mode of potential inhibitors and could aid in structure-based drug design. This includes important points of contact made between the inhibitors and the enzyme, and the alignment of available binding pockets surrounding the active site which can be taken advantage of with further functionalization of the inhibitor candidates.

Another experimental method that could offer information about inhibitor binding would be isothermal titration calorimetry (ITC). As mentioned previously, it was observed that in the case of an MMP-9 inhibitor, the metal-chelating hydroxamic acid moiety accounted for 96% of the binding energy. To test the assumption that the inhibitors are acting through a metal-chelating mechanism in the active sites of PgaB and IcaB, utilizing an array of similar metal-chelating inhibitors and obtaining the binding energy of each could offer insight. For example, using an array of $N$-(methyl)thioglycolamide, $N$-(cyclohexyl)thioglycolamide, and the successful thiol inhibitors 23 and 64, it may be possible to differentiate the binding energies resulting from the metal-chelating thioglycolamide moieties (Figure 5.3).
Figure 5.3 Array of compounds to test using isothermal calorimetry

Another area to be explored could be the use of non-carbohydrate base inhibitors. As observed with lipopolysaccharide deacetylase LpxC, despite the tight binding of the carbohydrate inhibitor Tu-514, with a $K_i$ of 640 nM, a series of small molecule aryl compounds were found to be over 100-fold more effective, with the best one, CHIR-090, demonstrating a $K_i$ of 2 nM (Figure 5.4a).\textsuperscript{125, 169} Additionally, utilizing a computational molecule library screen, a small molecule aryl inhibitor of the CE4 family pneumococcal peptidoglycan deacetylase PgdA was discovered, exhibiting an IC\textsubscript{50} value of 130 µM under the reported reaction conditions (Figure 5.4b). Computational docking studies suggested that it bound to the metal site in a bidentate fashion, utilizing the carboxylic acid moiety and the ketone oxygen.\textsuperscript{153} Common features of these non-carbohydrate inhibitors include metal-binding moieties to bind the metal centers and aryl groups which can mimic the carbohydrate rings of native substrates.
A) LpxC inhibitors

![TU-514](image1)

\[ K_i = 640 \text{ nM} \]

![CHIR-090](image2)

\[ K_i = 2 \text{ nM} \]

B) PgdA inhibitor

![IC50](image3)

\[ IC_{50} = 130 \text{ mM} \]

Figure 5.4 A) Inhibitors developed against LpxC. B) Small molecule inhibitor developed against pneumococcal peptidoglycan deacetylase PgdA.

Finally, due to the low affinity of PgaB and IcaB to oligosaccharides of their native substrate (refer to table 4.1), it may ultimately prove perplexing to find effective carbohydrate-based inhibitors. Additionally, due to their high polarity, carbohydrate-based drugs have limited capability to cross the enterocyte layer of the small intestine when administered orally, and suffer from fast metabolic renal extraction. In principle, given our access to the structures of PgaB and IcaB, using a computational structure-based virtual screening followed by a biochemical evaluation of a small molecule library may unveil novel small molecule inhibitors to these enzymes. This virtual screening method has seen success with several enzyme targets, including the previously-mentioned PgdA.
6 Experimental

6.1 General Methods

Reagents were obtained from Sigma-Aldrich or Acros Organics and were used without further purification. If appropriate, reactions were carried out under an inert atmosphere of nitrogen. Standard syringe techniques were applied for the transfer of dry solvents and air- or moisture-sensitive reagents. Reactions were monitored by TLC using Silica Gel 60 F254 (EMD Science) with detection by quenching of fluorescence and/or by visualization with phosphomolybdic acid in ethanol (0.5% w/v), methanolic H₂SO₄ (10% v/v), or ninhydrin in ethanol (0.2% w/v). ¹H and ¹³C NMR spectra were recorded at 25 °C with a Mercury 300 MHz or a Varian 400 MHz (75 MHz or 100 MHz for ¹³C, respectively) spectrometer. ¹H-NMR chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard, or a residual proton peak of the solvent: δ = 7.26 ppm for CDCl₃, δ = 4.81 ppm for D₂O, δ = 3.31 ppm for CD₃OD. Multiplicities are reported as: s (singlet), d (doublet), t (triplet), dd (doublet of doublets), or m (multiplet). Broad peaks are indicated by br. Coupling constants are reported as J-values in Hz. The number of protons (n) for a given resonance is indicated as nH, and is based on spectral integration values. ¹³C-NMR chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS) as an internal standard, or a carbon peak of the solvent: δ = 77.0 for CDCl₃, δ = 49.0 for CD₃OD. Multiplicities in carbon represent different rotamers and are reported as: d (doublet). Flash chromatography was performed on Silica-P Flash Silica Gel 60 (40–63 μm particle size, Silicycle). High-resolution mass spectra were obtained from an ABI/Sciex QStar mass spectrometer (ESI). Absorption spectra were measured on a Schimadzu UV-2401PC spectrophotometer. Fluorescence measurements were obtained on a TECAN Safire2 plate reader. HPLC was performed on a Waters 1525 binary HPLC pump with a Waters 2487 dual k absorbance detector or a Gilson 321 HPLC pump with a Gilson UV–Vis 156 dual k absorbance detector.
6.2 Procedures

4-nitrophenoxymethoxy 2-pthalimido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (5)

3,4,6-triacetyl-2-deoxy-2-pthalimido-α-glucose (500 mg, 1.15 mmol) was dissolved in acetonitrile (5 ml) and to it K₂CO₃ (238 mg, 1.72 mmol), was added followed by the addition of 4-nitrophenoxymethoxychloride (430 mg, 2.3 mmol), and NaI (258 mg, 1.72 mmol). The reaction was allowed to proceed overnight at room temperature. TLC analysis showed generation of a new spot (EtOAC:n-pentane, 1:2, Rf 0.5). Solvent was evaporated and the crude mixture was diluted with dichloromethane (25 ml), and washed with water (50 ml), the organic phase was separated, dried over MgSO₄, filtered and evaporated to syrup in vacuo. The crude product thus obtained was purified by flash chromatography eluting with (EtOAC:n-pentane, 1:3) to furnish 4-nitrophenoxymethoxy 2-pthalimido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside 4 (269 mg, 40%), as colourless paste. ¹H NMR (CDCl₃, 300 MHz) δ: 7.68-7.53 (m, 6H), 6.79-6.72 (m, 2H), 5.82 (t, 1H, J = 9.2 Hz.), 5.71 (d, 1H, J = 8.6 Hz), 5.44-5.37 (m, 2H), 5.18 (t, 1H, J = 9.1 Hz), 4.39-4.28 (m, 2H), 4.22 (dd, 1H, J = 2.1 Hz, 10.2 Hz), 3.97-3.92 (m, 2H), 2.13 (s, 3H), 2.02 (s, 3H), 1.81 (s, 3H).¹³C NMR (75 MHz, CDCl₃) δ: 170.6(2), 170 (2), 169.4, 160.1, 141.6, 134 (2), 125.3 (2), 123.3 (4), 115.2 (2), 94.1, 88.1, 72.2, 70.1, 68.5, 61.7, 54, 20.7, 20.6, 20.4. HRMS cald for C₂₇H₂₆N₂O₁₃Na(M+Na)⁺: 609.1333; found 609.1337.

4-nitrophenoxymethoxy 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (6)

Compound 5 (200 mg, 0.34 mmol) was dissolved in butanol (5 ml) under Nitrogen and to it ethylene diamine (1 ml) was added. The solution was heated to 120 °C for 20 h, after which the solvent was evaporated in a rotavapour and the crude was dried under pump. To the crude pyridine (3 ml) and Acetic anhydride (2 ml) was added and the solution was stirred at room temperature overnight. The solvent was evaporated and the crude mixture was diluted with dichloromethane (25 ml), and washed with 1N HCl (25 ml), water (50 ml), and sodium bicarbonate (25 ml), the organic phase was separated, dried over MgSO₄, filtered and evaporated to syrup in vacuo. The crude product thus obtained was purified by flash chromatography eluting with (EtOAC:n-pentane, 1:1) to furnish 4-nitrophenoxymethoxy 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside 5 (132 mg, 78%), as colourless
paste. $^1$H NMR (CDCl$_3$, 300 MHz) $\delta$: 8.16-8.11 (m, 2H), 7.07-7.02 (m, 2H), 5.44 (d, 1H, $J = 7.6$ Hz), 5.34 (d, 1H, $J = 7.6$ Hz), 5.23 (t, 1H, $J = 8.9$ Hz), 5.13-5.05 (m, 2H), 4.85 (d, 1H, $J = 8.5$ Hz), 4.23 (dd, 1H, $J = 4.5$ Hz, 12 Hz), 4.12 (dd, 1H, $J = 2.3$ Hz, 10 Hz), 3.97-3.93 (m, 1H), 3.70-3.66 (m, 1H), 2.04 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H), 1.46 (s, 3H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$: 171, 170.7, 169.8, 161.6, 142.5, 125.7 (2), 116.1 (2), 97.3, 89.1, 72.2, 72, 68, 61.7, 53.9, 22.8, 20.7, 20.6 (2). HRMS cald for C$_{21}$H$_{26}$N$_2$O$_2$Na(M+Na)$^+$: 521.1383; found 521.1386.

4-nitrophenoxymethoxy 2-acetamido-2-deoxy-$\beta$-D-glucopyranoside (1).

Compound 6 (100 mg, 0.2 mmol) was dissolved in MeOH (5mL) followed by addition of NaOMe (0.5 M in MeOH, 0.1 mL) PH Paper alkaline and the solution was allowed to stir at room temperature for 6 hours. The solution was then neutralized with Amberlite IR 120 H$^+$ resin and filtered. The filtrate was evaporated to afford pure compound 4-nitrophenoxymethoxy 2-acetamido-2-deoxy-$\beta$-D-glucopyranoside 1 (67 mg, 90%) as amorphous white solid. $^1$H NMR (MeOD, 300 MHz) $\delta$: 8.24-8.19 (m, 2H), 7.21-7.15 (m, 2H), 5.55 (d, 1H, $J = 7.6$ Hz), 5.46 (d, 1H, $J = 7.6$ Hz), 4.75 (d, 1H, $J = 8.5$ Hz), 3.89 (dd, 1H, $J = 8.5$ Hz, 1.7 Hz, 10.4 Hz), 3.72-3.63 (m, 2H), 3.47-3.41 (ddd, 1H, $J = 8.5$ Hz, 1.5 Hz, 8.7 Hz), 3.36-3.27 (m, 3H), 1.49 (s, 3H).$^{13}$C NMR (75 MHz, MeOD) $\delta$: 173.5, 163.6, 143.7, 126.6 (2), 117.6 (2), 99.3, 90.5, 78.4, 75.5, 72, 62.8, 57, 22.7. HRMS cald for C$_{15}$H$_{20}$N$_2$O$_9$Na(M+Na)$^+$: 395.1067; found 395.1064.

4-Nitrophenyl carbamoyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-$\beta$-D-glucopyranoside (8)

Compound 7 (200 mg, 0.58mmol) was dissolved in toluene (10 mL) followed by the addition of a catalytic amount of triethylamine (0.05 mL) and p-nitrophenyl isocyanate (190mg, 1.16mmol). The solution was stirred at room temperature for 6 hours, at which point a yellow solid had precipitated. The solution was chilled in an ice bath and pure 4-nitrophenyl carbamoyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-$\beta$-D-glucopyranoside was isolated by filtration (253 mg, 86% yield) $^1$H NMR (CDCl$_3$, 400 MHz) $\delta$: 8.22 (d, 2H, $J = 9.2$ Hz), 7.51 (d, 2H, $J = 9.2$ Hz), 5.61 (d, 1H, $J = 8.8$ Hz.), 5.58 (bd, 1H, $J = 5.2$) 5.13-5.05 (m, 2H), 4.45 (d, 1H, $J = 8.5$ Hz), 4.35 (dd, 1H, $J = 4.5$ Hz, 12 Hz), 4.15 (dd, 1H, $J = 2.3$ Hz, 12 Hz), 3.86-3.72 (m, 1H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H).$^{13}$C
NMR (75 MHz, CDCl₃) δ: 171.4, 170.6, 169.8, 161.2, 148.5, 141.2, 130.7 (2), 116.1 (2), 102.3, 89.2, 73.1, 71.9, 68, 61.4, 53.7, 22.4, 20.8, 20.6 (2). HRMS cald for C₂₁H₂₆N₂O₁₂Na(M+Na)⁺: 534.1374; found 534.1379.

4-Nitrophenyl carbamoyl 2-acetamido-2-deoxy-β-D-glucopyranoside (2)

Compound 8 (200 mg, 0.4mmol) was stirred as a slurry in MeOH (10 mL) and to it was added hydrazine monohydrate (0.05ml 0.13 mmol). After 4 hours, the reaction mixture was then evaporated under reduced pressure, and the remaining residue was purified by flash chromatography eluting with MeOH : DCM (1:20) to afford 4-nitrophenyl carbamoyl 2-acetamido-2-deoxy-β-D-glucopyranoside (80 mg, 48% yield) as a faint yellow powder.¹H NMR (MeOD, 400 MHz) δ: 8.22 (d, 2H, J = 9.2Hz), 7.51 (d, 2H, J = 9.2Hz), 5.61 (d, 1H, J = 8.8 Hz), 3.95 (m, 2H), 3.75 (dd, 1H, J = 1.7 Hz, 10.4 Hz), 3.62-3.31 (m, 3H), 2.04 (s, 3H).¹³C NMR (75 MHz, MeOD) δ: 173.5, 143.6, 141.7, 129.2 (2), 113.6 (2), 105.2, 90.5, 78.1, 75.2, 72.3, 62.4, 57.3, 22.4. HRMS cald for C₁₅H₁₉N₃O₉Na(M+Na)⁺: 408.1062; found 408.1068.

Methyl 2-ammonium-2-deoxy-β-D-glucopyranoside acetate¹⁴⁰,¹⁴² (15)

N-Acetylglucosamine (5 g, 25 mmol) and tosylhydrazide (5.5 g, 25 mmol) in a mixture of DMF (20 mL), water (4.5 mL) and acetic acid (0.5 mL) was stirred at 37 °C for 24 h. The solution was then poured into diethyl ether (1 L) and stirred vigorously to promote formation of a white precipitate, 13. Vacuum filtration of the suspension yielded the glycosyltosylhydrazide donor (10.2 g, 92%). The glycosyl donor (5 g, 12 mmol) was then dissolved in DMF (150 mL) containing MeOH (10 mL, 240 mmol). N-Bromosuccinimide (2.2 g, 26 mmol) was then added to the solution portion-wise over 5 min 0 °C. Upon completion of the addition, the solution was allowed to stir for 30 min at room temperature. The solution was then evaporated under reduced pressure, and the resulting residue was dissolved in hot EtOH (100 mL), and chilled to -20 °C to allow crystallization of the succinimide. Filtration and subsequent evaporation of the EtOH filtrate followed by flash chromatography (10% MeOH in DCM) of the resulting residue yielded 14 (1.8 g 62%) as a white solid. 1 (1.8 g, 8 mmol) was then dissolved in hydrazine monohydrate (10 mL) and allowed to
stir at 110 °C for 48 h. The solution was then evaporate under reduced pressure, followed by the addition of 1M AcOH (5 mL) and a subsequent evaporation under reduced pressure to form the acetate salt. Recrystallization of the resulting residue with MeOH/EtOAc yielded 15 (1.85 g, 96%).

Methyl 2-(2-benzylxy)acetamido-2-deoxy-β-D-glucopyranoside (18)

The title compound was prepared by dissolving compound 15 (200 mg, 0.93 mmol), benzylxyacetic acid (220 mg, 0.93 mmol) and triethylamine (55 µL, 1 mmol) in DMF. HBTU (355 mg, 0.93 mmol) was then added portion wise to the mixture at 0 °C over 30 min and allowed to stir overnight at room temperature. The solution was then evaporated under reduced pressure and the resulting residue was purified by flash chromatography (8% MeOH in DCM) to afford 18 (198 mg, 84%) as a white solid. $^1$H NMR (400 MHz, D$_2$O) δ 7.56 – 7.41 (m, 5H, Ar-H), 4.69 (s, 2H, CH$_2$), 4.54 (d, $J$ = 8.5 Hz, 1H, H-1), 4.14 (d, $J$ = 5.3 Hz, 2H, CH$_2$Ar), 3.97 (dd, $J$ = 12.1 Hz, 1.8 Hz, 1H, H-6a), 3.84 – 3.73 (m, 2H, H-6b, H-5), 3.64 (dd, $J$ = 10.3 Hz, 8.7 Hz, 1H, H-2), 3.54 (s, 3H, CH$_3$), 3.52 - 3.47 (m, 2H, H-3, H-4). $^{13}$C NMR (75 MHz, D$_2$O) δ 173.37, 136.72, 128.99, 128.79, 128.76, 101.93, 76.07, 73.80, 73.37, 70.18, 68.60, 60.90, 57.28, 55.32. HRMS m/z calcd. for C$_{16}$H$_{24}$NO$_7$ (M+H$^+$) 342.1536, found 342.1547.

Methyl 2-(2-hydroxy)acetamido-2-deoxy-β-D-glucopyranoside (19)

The title compound was prepared by dissolving compound 18 (100 mg, 0.41 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H$_2$ gas (1 atm) for 12 h. The suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 19 (82 mg, 89%) as a colorless oil. $^1$H NMR (400 MHz, D$_2$O) δ 4.57 (d, $J$ = 8.4 Hz, 1H, H-1), 4.16 (s, 2H, CH$_2$), 3.98 (dd, $J$ = 12.3 Hz, 1.9 Hz, 1H, H-6a), 3.84 – 3.76 (m, 2H, H-6b, H-5), 3.67 (dd, $J$ = 10.3 Hz, 8.6 Hz, 1H, H-2), 3.55 (s, 3H, CH$_3$), 3.53 – 3.45 (m, 2H, H-3, H-4). $^{13}$C NMR (75 MHz, D$_2$O) δ 175.77, 101.93, 76.08, 73.86, 70.15, 61.17, 60.91, 57.24, 55.30. HRMS m/z calcd. for C$_9$H$_{16}$NO$_7$Na (M+Na$^+$) 274.0906, found 274.0921
Methyl 2-(2-benzylcarboxyamino)acetamido-2-deoxy-β-D-glucopyranoside (20)

The title compound was prepared by dissolving compound 15 (200 mg, 0.93 mmol), Cbz-Gly-OH (180 mg, 0.93 mmol) and triethylamine (55 µL, 1 mmol) in DMF. HBTU (355 mg, 0.93 mmol) was then added portion wise to the mixture at 0 °C over 30 min and allowed to stir overnight at room temperature. The solution was then evaporated under reduced pressure and the resulting residue was purified by flash chromatography (8% MeOH in DCM) to afford 20 (178 mg, 76%) as a white solid. ¹H NMR (400 MHz, D₂O) δ 7.58 – 7.39 (m, 5H, Ar-H), 4.52 (d, J = 8.4 Hz, 1H, H-1), 4.22 (d, J = 5.3 Hz, 2H, CH₂Ar), 3.97 (dd, J = 12.0 Hz, 1.9 Hz, 1H, H-6a), 3.90 – 3.70 (m, 4H, H-6b, H-5, CH₂), 3.66 – 3.58 (m, 1H, H-2), 3.58 – 3.42 (m, 5H, H-3, H-4, CH₃). ¹³C NMR (75 MHz, D₂O) δ 175.74, 160.93, 137.29, 130.29, 129.38, 129.17, 104.55, 84.41, 78.61, 76.49, 72.70, 70.1, 63.48, 59.92, 58.30. HRMS m/z calcd for C₁₇H₂₅N₂O₈ (M+H⁺) 385.1554, found 385.1561

Methyl 2-(2-amino)acetamido-2-deoxy-β-D-glucopyranoside (21)

The title compound was prepared by dissolving compound 20 (100 mg, 0.40 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. The suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 21 (72 mg, 81%) as a colourless oil. ¹H NMR (400 MHz, D₂O) δ 4.57 (d, J = 8.4 Hz, 1H, H-1), 3.98 (dd, J = 12.3 Hz, 1.9 Hz, 1H, H-6a), 3.91 (d, J = 2.8 Hz, 2H, CH₂), 3.84 – 3.76 (m, 2H, H-6b, H-5), 3.67 (dd, J = 10.3 Hz, 8.6 Hz, 1H, H-3), 3.55 (s, 3H, CH₃), 3.53 – 3.45 (m, 2H, H-4, H-2). ¹³C NMR (75 MHz, D₂O) δ 175.77, 101.93, 76.08, 73.86, 70.15, 61.17, 60.91, 57.24, 55.30. HRMS m/z calcd. For C₉H₁₈N₂O₆Na (M+Na⁺) 273.0900, found 273.0918
Methyl 2-(2-thioacetyl)acetamido-2-deoxy-\(\beta\)-D-glucopyranoside (22)

The title compound was prepared by dissolving compound 15 (200 mg, 0.93 mmol) and triethylamine (55 \(\mu\)L, 1 mmol) in DMF. Pentafluorophenyl 2-(acetyllthio)acetate (310 mg, 0.93 mmol) was then added to the mixture and allowed to stir overnight at room temperature. The solution was evaporated under reduced pressure and the resulting residue was purified by flash chromatography (8% MeOH in DCM) to afford 22 (160 mg, 78%) as a white solid. 

\(^1\)H NMR (400 MHz, D\(_2\)O) \(\delta\) 4.49 (d, \(J = 8.3\) Hz, 1H, H-1), 3.96 (dd, \(J = 12.2\) Hz, 1.6 Hz, 1H, H-6a), 3.74 - 3.64 (m, 4H, H-6b, H-2, CH\(_2\)), 3.60 (dd, \(J = 9.8\) Hz, \(J = 8.6\) Hz, 1H, H-3), 3.52 (s, 3H, OCH\(_3\)), 3.51 – 3.44 (m, 2H, H-3, H-4), 2.45 (s, 3H, CH\(_3\)). 

\(^{13}\)C NMR (100 MHz, D\(_2\)O) \(\delta\) 199.48, 171.71, 102.14, 76.04, 73.77, 70.16, 60.90, 57.40, 56.12, 33.24, 29.67. HRMS m/z calcd. for C\(_{11}\)H\(_{19}\)NO\(_7\)SNa (M+Na\(^+\)) 332.0778, found 332.0774

2,2'-disulfanediylbis(N-(Methyl-2-deoxy-\(\beta\)-D-glucopyranos-2-yl)acetamide) (23)

The title compound was prepared by dissolving compound 22 (80 mg, 0.3 mmol) in MeOH (5 mL) containing a catalytic amount of NaOMe and the solution was stirred for 10 min. Amberlite resin (H\(^+\)) was added to the mixture until the solution was neutral. Filtration and evaporation under reduced pressure yielded the symmetrical disulfide 23 (65 mg, 92%) as a white powder. 

\(^1\)H NMR (400 MHz, D\(_2\)O) \(\delta\) 4.54 (d, \(J = 8.4\) Hz, 1H, H-1), 3.98 (d, \(J = 11.9\) Hz, 1H, H-6a), 3.78 (d, \(J = 9.1\) Hz, 2H, CH\(_2\)), 3.68 – 3.43 (m, 8H, H-6b, H-5, H-3, H-4, OCH\(_3\), H-2). 

\(^{13}\)C NMR (100 MHz, D\(_2\)O) \(\delta\) 175.23, 100.27, 76.34, 72.38, 69.93, 60.60, 57.58, 55.94, 23.11. C\(_{18}\)H\(_{33}\)N\(_2\)O\(_4\)S\(_2\) (M+H\(^+\)) 533.1484, found 533.1469
Methyl 2-(3-hydroxy)ureayl-2-deoxy-β-D-glucopyranoside (24)

The title compound was prepared by dissolving compound 15 (200 mg, 0.93 mmol) in MeOH with triethylamine (55 µL, 1 mmol). 4-nitrophenyl carboxamate (385 mg, 1.5 mmol),\(^4\) was subsequently added portion wise over 30 min at 0 °C and the mixture was allowed to stir overnight. Following complete consumption of the glycosyl starting material, as judged by TLC, the reaction mixture was evaporated under reduced pressure, and the resulting residue was dissolved in 1M AcOH (25 mL) and washed with 3x50 mL EtOAc. Collection and evaporation of the aqueous layer under reduced pressure, followed by flash chromatography of the resulting residue (8% MeOH in DCM) yielded 24 (110 mg, 54%) as a white solid. \(^1\)H NMR (400 MHz, D\(_2\)O) δ 4.54 (d, \(J = 8.1\) Hz, 1H, H-1), 3.98 (dd, \(J = 12.3\) Hz, 1.8 Hz, 1H, H-6a), 3.79 (dd, \(J = 12.3\) Hz, 5.6 Hz, 1H, H-6b), 3.62 – 3.42 (m, 7H, H-2, H-3, CH\(_3\), H-4, H-5). \(^13\)C NMR (75 MHz, D\(_2\)O) δ 163.57, 100.43, 76.05, 74.14, 70.20, 60.95, 57.30, 56.00. HRMS m/z calcd. for C\(_8\)H\(_{16}\)N\(_2\)O\(_7\)Na (M+Na\(^+\)) 275.0855, found 275.0849

Methyl 2-methylsulfonamido-2-deoxy-β-D-glucopyranoside (25)

The title compound was prepared by dissolving compound 15 (200 mg, 0.93 mmol) and triethylamine (110 µL, 2 mmol) in DMF. Mesyl chloride (65 µL, 1 mmol) was then added to the mixture slowly and allowed to stir overnight at room temperature. The mixture was evaporated under reduced pressure and the resulting residue was purified by flash chromatography (8% MeOH in DCM) to afford 25 (112 mg, 58%) as a white solid. \(^1\)H NMR (400 MHz, CD\(_3\)OD) δ 4.37 (d, \(J = 8.4\) Hz, 1H, H-1), 3.89 (dd, \(J = 12.3\) Hz, 1.9 Hz, 1H, H-6a), 3.69 (dd, \(J = 12.4\) Hz, 5.8 Hz, 1H, H-6b), 3.54 (s, 3H, OCH\(_3\)), 3.46 – 3.34 (m, 4H, H-2, H-3, H-4, H-5), 3.11 (s, 3H, S-CH\(_3\)). \(^13\)C NMR (100 MHz, D\(_2\)O) δ 100.83, 77.01, 73.21, 70.50, 60.41, 57.38, 56.02, 39.12 HRMS m/z calcd. for C\(_8\)H\(_{17}\)NO\(_3\)SNa (M+Na\(^+\)) 294.0711, found 294.0713
Methyl 2-methylamino-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside (29)

The title compound was synthesized in 3 steps following a literature procedure. Methyl 2-acetamido-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside 26 (5 g, 10 mmol) was dissolved in THF (100 mL). Boc Anhydride (4.4 g, 20 mmol) and DMAP (0.5 g, 0.5 mmol) were then added and the solution was then refluxed for 16 h. The solution was then evaporated to dryness under reduced pressure. The resulting residue was then dissolved in MeOH (100 mL) containing a catalytic amount of NaOMe for 4 h, at which point the Boc protected compound precipitated. Vacuum filtration of resulting solid yielded methyl 2-tert-butylicalboxamido-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside 28 (3.8 g, 7.2 mmol). The resulting sugar (28) was then dissolved in THF (100 mL) and to the solution, on ice, was slowly added LiAlH₄ (21.6 mmol) a 1M solution in THF. Following complete addition of LiAlH₄, the solution was refluxed for 12 h. The solution was then neutralized with water, washed consecutively with a 200 mL of a 0.1 M solution of sodium potassium tartrate, water, brine, and the resulting organics were dried over magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography (1:1 EtOAc/Pentane) afforded 29 (2.1 g, 4.8 mmol) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.56 – 7.12 (m, 15H, ArH), 4.95 (d, J = 11.5 Hz, 1H, Ar-CH₃Hb), 4.79 (d, J = 10.8 Hz, 1H, Ar-CH₃Hb), 4.74 – 4.55 (m, 4H, Ar-CH₂, Ar-CH₂), 4.16 (d, J = 7.9 Hz, 1H, H-1), 3.81 – 3.65 (m, 3H, H-6a, H-6b, H5), 3.57 – 3.44 (m, 5H, H-4, H-3, OCH₃), 2.51 – 2.43 (m, 4H, H-2, NCH₃), 1.26 (s, 1H, N-H). ¹³C NMR (75 MHz, CDCl₃) δ 138.53, 138.26, 137.86, 128.64, 128.64, 128.59, 128.56, 128.49, 128.36, 128.08, 127.93, 127.75, 100.22, 100.08, 79.63, 79.39, 79.26, 78.54, 75.17, 74.96, 73.63, 68.82, 61.49, 57.01, 42.73, 29.84, 28.56, 28.51. HRMS m/z calcd. for C₂₉H₃₆NO₅ (M+H⁺) 478.2596, found 478.2588.
Methyl 2-N-methyl-(2-benzyloxy)acetamido-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside (30)

The title compound was prepared by dissolving 29 (250 mg, 0.55 mmol), benzyloxyacetic acid (120 mg, 0.6 mmol) and triethylamine (30 µL, 0.58 mmol) in a 50:50 EtOAc/DCM solution. DCC (140 mg, 0.66 mmol) was then added portion wise to the mixture at 0°C over 30 min and allowed to stir overnight at room temperature. The mixture was then filtered through celite and evaporated under reduced pressure. The resulting residue was then dissolved in EtOAc, washed consecutively with 1M NaOH\(_{\text{(aq)}}\), water, and brine, and dried with magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography (1:1 EtOAc/Pentane) afforded 30 (260 mg 89%) as a colourless oil. \(^1\)H NMR (400 MHz, CDCl\(_3\); mixture of rotamers) \(\delta\) 7.40 – 7.04 (m, 20H, Ar-H), 4.81 – 4.63 (m, 2H, Ar-CH\(_2\)), 4.60 – 4.37 (m, 7H, Ar-CH\(_2\), H-1), 4.32 – 4.15 (m, 1.4H, COCH\(_2\)), 3.97 (d, \(J = 13.8\) Hz, 0.6H, COCH\(_2\)), 3.73 – 3.55 (m, 5H, H-6a, H-6b, H-5, H-2, H-4), 3.48 – 3.30 (m, 4H, H-3, OCH\(_3\)), 2.98 - 2.79 (br s, 2.2H, NCH\(_3\)), 2.68 (s, 1.8H NCH\(_3\)). \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 171.07, 138.65, 138.30, 137.98, 137.96, 137.85, 137.57, 128.66, 128.61, 128.56, 128.52, 128.49, 128.44, 128.39, 128.33, 128.17, 128.15, 128.08, 128.04, 128.00, 127.96, 127.88, 127.78, 127.68, 100.43 (d), 79.05, 78.98, 75.14, 75.07, 74.96, 74.82, 73.72, 73.56, 73.10, 69.67, 68.89, 68.53, 61.31, 57.13, 34.05, 27.83, 25.07. HRMS \(m/z\) calcd. for C\(_{38}\)H\(_{43}\)NO\(_7\)Na (M+Na\(^+\)) 648.2938, found 648.2931.

Methyl 2-N-methyl-(2-benzylcarboxyamino)acetamido-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside (31)

The title compound was prepared by dissolving 29 (250 mg, 0.55 mmol), Cbz-Gly-OH (120 mg, 0.6 mmol) and triethylamine (30 µL, 0.58 mmol) in a 50:50 EtOAc/DCM solution. DCC (140 mg, 0.66 mmol) was then added portion wise to the mixture at 0°C over 30 min and allowed to stir overnight at room temperature. The mixture was then filtered through celite and evaporated under reduced pressure. The resulting residue was then dissolved in EtOAc, washed consecutively with 1M NaOH\(_{\text{(aq)}}\), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography (1:1 EtOAc/Pentane) afforded 31 (268 mg, 92%) as a colourless oil. \(^1\)H NMR (400 MHz, CDCl\(_3\); mixture of rotamers) \(\delta\) 7.45 –
Methyl 2-N-methyl-(3-hydroxy)ureayl-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside (32)

The title compound was prepared by dissolving 29 (250 mg, 0.55 mmol) and triethylamine (30 µL, 0.58 mmol) in DCM. 4-nitrophenyl carboxamate (210 mg, 0.66 mmol) was then added slowly to the mixture at 0 °C over 30 min and allowed to stir overnight at room temperature. The mixture was washed consecutively with 1M NaOH, water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography (2:1 EtOAc/Pentane) afforded 32 (204 mg, 72%) as a colourless oil. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 11.01 (br s, 1H, OH) 7.62 – 6.91 (m, 15H, Ar-H), 4.95 (d, $J = 11.3$ Hz, 1H, Ar-CH$_a$H$_b$), 4.84 – 4.73 (m, 2H, H-1, CH$_a$H$_b$), 4.69 – 4.52 (m, 4H, Ar-CH$_2$, Ar-CH$_2$), 4.31 (d, $J = 7.7$ Hz, 1H, N-H), 3.79 – 3.42 (m, 10H, H-6a, H-6b, H-5, H-4, H-2, H-3, OCH$_3$), 2.54 (s, 3H, N-CH$_3$). $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 161.03, 138.65, 138.40, 135.86, 129.55, 129.49, 129.42, 129.34, 129.33, 129.27, 129.19, 129.01, 128.84, 128.80, 128.63, 128.59, 99.88, 79.82, 77.38, 77.11, 73.51, 71.60, 67.66, 58.40, 54.76, 53.46, 26.10. HRMS $m/z$ calcd. for C$_{36}$H$_{36}$N$_2$O$_7$Na (M+Na$^+$) 559.6523, found 559.6531.
Methyl 2-N-methyl-(methylsulfonamido)-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside (33)

The title compound was prepared by dissolving 29 (250 mg, 0.55 mmol) and triethylamine (60 µL, 1.15 mmol) in DCM. MsCl (50 µL, 0.82 mmol) was then added slowly to the mixture at 0 °C over 30 min and allowed to stir overnight at room temperature. The mixture was then washed consecutively with 1M NaOH(aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography (1:1 EtOAc/Pentane) afforded 33 (232 mg, 88%) as a colourless oil. 1H NMR (400 MHz, CDCl3) δ 7.40 – 7.10 (m, 15H, ArH), 4.87 – 4.65 (m, 4H, ArCH2, ArCH2), 4.50 – 4.35 (m, 3H, H-1, ArCH2), 3.65 – 3.53 (m, 6H, H-6a, H-6b, H-5, H-3, H-4, H-2), 3.32 (s, 3H, OCH3), 2.98 (s, 3H, SCH3), 2.58 (s, 3H, NCH3). 13C NMR (75 MHz, CDCl3) δ 137.61, 137.51, 134.72, 128.41, 128.35, 128.28, 128.27, 128.23, 128.18, 128.13, 128.05, 127.94, 127.86, 127.69, 127.66, 127.62, 127.49, 127.44, 99.78, 78.48, 74.86, 74.75, 74.62, 73.21, 68.16, 67.77, 66.67, 56.41, 31.06, 21.72. HRMS m/z calcd. for C30H37NO7S Na (M+Na+) 578.1120, found 578.1131.

Methyl 2-N-methyl-N-benzylcarboxyaminosulfonamido-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside (34)

The title compound was prepared by first dissolving chlorosulfonyl isocyanate (0.80 µL, 0.75 mmol) and benzyl alcohol (95 µL, 0.75 mmol) in DCM, and allowed to stir for 30 min at 0 °C. Following this, the DCM solution was combined with a DCM solution containing 29 (250 mg, 0.55 mmol) and triethylamine (60 µL, 1.2 mmol), and allowed to stir for 3 h. The mixture was then washed consecutively with 1M NaOH(aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography (1:1 EtOAc/Pentane) afforded 34 (232 mg, 88%) as a colourless oil. 1H NMR (400 MHz, CDCl3) δ 7.42 – 6.99 (m, 20H, ArH), 5.07 (s, 2H, ArCH2), 4.77 – 4.68 (m, 4H, ArCH2, ArCH2), 4.55 – 4.41 (m, 3H, H-1, ArCH2), 4.26 (brs, 1H, NH), 3.69 – 3.56 (m, 5H, H-6a, H-6b, H-5, H-3, H-4), 3.32 (m, 4H, OCH3, H-2), 2.74 (s, 3H, NCH3). 13C NMR (100 MHz, CDCl3) δ 151.55, 137.98, 137.88, 137.63, 136.32, 135.09, 128.78, 128.72, 128.65, 128.64, 128.60, 128.56, 128.50, 128.42,
Methyl 2-N-methyl-(2-hydroxy)acetamido-2-deoxy-β-D-glucopyranoside (35)

The title compound was prepared by dissolving 30 (200 mg, 0.41 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate was evaporated under reduced pressure to afford 35 (68 mg, 82%) as a white powder. ¹H NMR (400 MHz, D₂O; mixture of rotamers) δ 4.89 (d, J = 7.9 Hz, 1H, H-1) 4.39 (br d, J = 5.2 Hz, 2H, CH₂), 4.03 – 3.71 (m, 3H, H-6a, H-6b, H-5), 3.58 – 3.48 (br m, 5H, H-4, H-3, CH₃), 3.34 – 3.23 (br m, 1H, H-2), 2.95 (br s, 3H, NCH₃). ¹³C NMR (100 MHz, D₂O; mixture of rotamers) δ 175.00, 99.86, 76.03 - 75.98 (d), 70.69 - 70.58 (d), 70.36, 61.74, 60.93-60.87 (d), 60.24 - 60.14 (d) 57.48 – 57.36 (d), 27.97. HRMS m/z calcd. For C₁₀H₁₉NO₇Na (M+Na⁺) 288.1223, found 288.1222

Methyl 2-N-methyl-(2-amino)acetamido-2-deoxy-β-D-glucopyranoside (36)

The title compound was prepared by dissolving 31 (200 mg, 0.41 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 36 (64 mg, 78%) as a white powder. ¹H NMR (400 MHz, CD₃OD; mixture of rotamers) δ 4.50 (br d, J = 7.5 Hz, 1H, H-1), 4.00 – 3.76 (br m, 3H, H-6a, H-6b, H-5), 3.63 (br m, 2H, CH₂), 3.52 – 3.18 (br m, 6H, OCH₃, H-4, H-2, H-3), 2.95 (br s, 1.2H, NCH₃), 2.80 (br s, 1.8H, NCH₃). ¹³C NMR (100 MHz, CD₃OD; mixture of rotamers) δ 172.47, 101.07 - 100.99 (d), 77.69 – 77.56 (d), 72.13, 63.37, 62.50 – 62.42 (d), 57.04, 43.10, 28.09. HRMS m/z calcd. For C₁₀H₂₁N₂O₆(M+H⁺) 265.1393, found 265.1394
Methyl 2-N-methyl-(3-hydroxy)ureayl-2-deoxy-β-D-glucopyranoside (37)

The title compound was prepared by dissolving 32 (200 mg, 0.38 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 37 (54 mg, 75%) as a white powder. ¹H NMR (400 MHz, D₂O) δ 4.75 (d, J = 7.3 Hz, 1H, H-1), 3.93 (dd, J = 11.5 Hz, 4.3 Hz, 1H, H-6a), 3.86 (m, 1H, H-5), 3.74 (dd, J = 11.1 Hz, 7.1 Hz, 1H, H-6b), 3.60 (s, 3H, OCH₃), 3.50 – 3.40 (m, 3H, H-2, H-3), 2.62 (s, 3H, NCH₃). ¹³C NMR (75 MHz, D₂O) δ 161.43, 100.33, 73.97, 72.05, 68.12, 58.86, 55.21, 53.91, 25.26. HRMS m/z calcd. for C₈H₁₆N₂O₇Na (M+Na⁺) 289.0735, found 289.0747

Methyl 2-N-methyl-(methylsulfonamido)-2-deoxy-β-D-glucopyranoside (38)

The title compound was prepared by dissolving 33 (200 mg, 0.38 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 38 (71 mg, 75%) as a colourless paste. ¹H NMR (400 MHz, D₂O) δ 4.67 (d, J = 8.4 Hz, 1H, H-1), 3.92 (dd, J = 11.3 Hz, 1.9 Hz, 1H, H-6a), 3.81 (m, 1H, H-5) 3.69 (dd, J = 11.4, 5.8 Hz, 1H, H-6b), 3.59 (s, 3H, OCH₃), 3.46 – 3.34 (m, 3H, H-2, H-3, H-4), 3.11 (s, 3H, SCH₃), 2.74 (s, 3H, NCH₃). ¹³C NMR (100 MHz, D₂O) δ 101.98, 76.34, 72.58, 69.72, 60.24, 57.58, 55.12, 40.21, 23.31. HRMS m/z calcd. for C₈H₁₇NO₇SNa (M+Na⁺) 289.0859, found 289.0851
Methyl 2-N-methyl-N-aminosulfonamido-2-deoxy-β-D-glucopyranoside (39)

The title compound was prepared by dissolving 34 (200 mg, 0.42 mmol) in MeOH (5 mL), followed by the addition of a catylitic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 39 (74 mg, 75%) as a colourless oil. 

\[ \text{1H NMR (400 MHz, D}_2\text{O)} \delta 4.61 (d, J = 8.1 Hz, 1H, H-1) 3.93 (dd, J = 7.8 Hz, 4.6 Hz, 1H, H-6a), 3.88 – 3.84 (m, 1H, H-5), 3.78 – 3.71 (m, 1H, H-6b), 3.60 (s, 3H, OCH₃), 3.48 – 3.44 (m, 3H, H-4, H-2, H-3), 2.80 (s, 3H, NCH₃).} \]

\[ \text{13C NMR (100 MHz, D}_2\text{O)} \delta 99.69, 75.60, 70.51, 70.32, 63.12, 60.63, 56.76, 21.46.} \]

HRMS \text{m/z calcd. for C}_{8}H_{18}N_{2}O_{7}NaS (M+Na⁺) 309.0726, found 309.0726

Methyl 2-benzylcarboxyaminosulfonamido-2-deoxy-3,4,6-tri-O-benzyl-β-D-glucopyranoside (41)

The title compound was prepared by first dissolving chlorosufonylisocyanate (0.80 µL, 0.75 mmol) and benzyl alcohol (95 µL, 0.75 mmol) in DCM, and allowed to stir for 30 min at 0 °C. Following this, the reaction mixture was combined with a DCM solution containing 40 (240 mg, 0.55 mmol) and triethylamine (60 µL, 1.2 mmol), and allowed to stir for 3 h. The mixture was then washed consecutively with 1M NaOH (aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography (1:1 EtOAc/Pentane) afforded 41 (232 mg, 88%) as a colourless oil. 

\[ \text{1H NMR (400 MHz, CDCl₃)} \delta 7.40 – 7.04 (m, 20H), 4.88 – 4.61 (m, 3H), 4.60 – 4.37 (m, 6H), 4.35 – 4.15 (m, 2H), 3.73 – 3.55 (m, 3H), 3.48 – 3.30 (m, 4H),} \]

\[ \text{13C NMR (100 MHz, CDCl₃)} \delta 156.78, 151.43, 137.87, 137.77, 137.52, 136.20, 134.97, 128.67, 128.61, 128.54, 128.53, 128.48, 128.44, 128.38, 128.30, 128.20, 128.12, 127.95, 127.91, 127.88, 127.74, 127.70, 100.04, 78.74, 75.12, 75.01, 74.88, 73.46, 68.42, 68.02, 66.92, 56.66.} \]

HRMS \text{m/z calcd. for C}_{37}H_{42}N_{2}O_{9}S (M+Na⁺) 699.2385, found 694.2388.
Methyl 2-aminosulfonamido-2-deoxy-β-D-glucopyranoside (42)

The title compound was prepared by dissolving 41 (200 mg, 0.42 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 42 (74 mg, 75%) as a colourless oil. ¹H NMR (400 MHz, D₂O) δ 4.53 (d, J = 8.4 Hz, 1H, H-1), 3.78 (dd, J = 12.3 Hz, 2.0 Hz, 1H H-6a ), 3.59 - 3.54 (m, 2H H-6b, H-5), 3.47 – 3.25 (m, 6H, H-4, H-2, H-3, CH₃) ¹³C NMR (100 MHz, D₂O) δ 102.65, 76.60, 74.51, 70.32, 60.12, 57.63, 56.76. HRMS m/z calcd. for C₈H₁₈N₂O₇NaS (M+Na⁺) 294.0825, found 294.0820

Methyl 2-methylamino 2-deoxy-β-D-glucopyranoside (43)

The title compound was prepared by dissolving 29 (200 mg, 0.41 mmol) in aldehyde free MeOH (5 mL) followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate evaporated under reduced pressure to afford 43 (64 mg, 78%) as a colorless film. ¹H NMR (400 MHz, D₂O) δ 4.47 (d, J = 8.5 Hz, 1H, H-1), 3.77 (dd, J = 12.6 Hz, 4.3 Hz, 1H, H-6a), 3.60 (dd, J = 12.4 Hz, 6.4 Hz, 1H, H-6b), 3.50 (dd, J = 10.6, 8.4 Hz, 1H, H-3), 3.43 (s, 3H, OCH₃), 3.37 – 3.27 (m, 2H, H-5, H-4), 2.84 (dd, J = 10.6 Hz, 8.5 Hz, 1H, H-2), 2.51 (s, 3H, NCH₃). ¹³C NMR (100 MHz, D₂O) δ 100.27, 76.34, 72.38, 69.93, 60.60, 57.58, 55.94, 23.34. HRMS m/z calcd. for C₈H₁₉NO₅ (M+H⁺) 206.1014, found 206.1022
Methyl 2-N-methyl-(2-thioacetyl)acetamido-2-deoxy-β-D-glucopyranoside (44)

The title compound was prepared by dissolving compound 43 (50 mg, 0.23 mmol) and triethylamine (12 µL, 0.25 mmol) in DMF. Pentafluorophenyl 2-(acetylthio)acetate (75 mg, 0.23 mmol) was then added to the mixture and allowed to stir overnight at room temperature. The mixture was evaporated under reduced pressure. The resulting residue was then purified by flash chromatography (8% MeOH in DCM) to afford 44 (35 mg, 69%) as a white solid. 

\(^1\)H NMR (400 MHz, D₂O) δ 4.72 (d, J = 8.3 Hz, 1H, H-1), 3.94 (dd, J = 12.2 Hz, 1.6 Hz, 1H, H-6a), 3.74 – 3.44 (m, 9H, H-6b, H-5, H-2, CH₂, H-4, H-3, OCH₃), 2.78 (s, 3H, N-CH₃), 2.32 (s, 3H, CH₃). 

\(^{13}\)C NMR (100 MHz, D₂O) δ 197.04, 169.27, 99.70, 73.61, 71.34, 67.72, 58.47, 54.97, 53.68, 30.80, 29.09, 27.23. HRMS m/z calcd. for C₁₁H₁₉NO₇SNa (M+Na⁺) 323.0978, found 323.0981

Methyl 3,4,6-tri-O-benzyl-2-deoxy-2-C-[bis-(methoxycarbonyl)methyl]-β-D-glucopyranoside (52a)

The title compound was synthesized in two steps following a literature procedure. D-glucal (1 g, 7.5 mmol) was dissolved in DMF (150 mL) in the presence of BnBr (5.8 g, 0.0340 mol) and cooled to 0 ºC. Sodium hydride (0.55 g, 0.024 mol) was then slowly added and the solution allowed to rise to room temperature and stirred overnight. The mixture was then diluted with EtoAc and subsequently washed with 1 M NaOH(aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure, yielded per-O-benzylated D-glucal which was then dissolved in MeOH (200 mL) containing NaHCO₃. Dimethyl malonate (9.5 mL, 0.075 mol) and cerium ammonium nitrate (8 g, 0.015 mol) and stirred for 3 hours. The resulting solution was neutralized with water and evaporated to dryness. The resulting suspension was dissolved in EtOAc and washed with 1 M HCl(aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure, followed by flash chromatography yielded 52a (48% over 2 steps, 1.89 g) as a clear oil. All acquired spectral data were in agreement with literature values.
Methyl 3,4,6-tri-O-benzyl-2-deoxy-2-C-[malonic acid]-β-D-glucopyranoside (53)
The title compound was prepared by dissolving 52a (0.2 g, 0.35 mmol) in a 1:1 solution of 2M NaOH/dioxane and stirred overnight at room temperature. The solution was then diluted with DCM and washed with 1M HCl(aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure yielded 53 (86%, 0.16 g) as a clear oil. All acquired spectral data were in agreement with literature values.\(^\text{148}\)

Methyl 2-deoxy-2-C-[malonic acid]-β-D-glucopyranoside (54)
The title compound was prepared by dissolving 53 (0.1 g, 0.18 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H\(_2\) gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate was evaporated under reduced pressure to afford 54 (35 mg, 77%) as a white powder. All acquired spectral data were in agreement with literature values.\(^\text{148}\)

Methyl \(\text{3,4,6-tri-O-benzyl-2-deoxy-2-C-[1,1,1,5,5,5-hexafluoropentane-2,4-dione]-β-D-glucopyranoside (55)}\)
The title compound was prepared by dissolving 52a (0.2 g, 0.35 mmol) in ethylene glycol dimethyl ether (5 mL) in the presence of TMS-CF\(_3\) (0.55 mL, 3.5 mmol) and a catalytic amount of cesium fluoride and allowed to stir overnight at room temperature. The solution was then diluted with DCM, and washed consecutively with HCl(aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure followed by flash chromatography (8:1 pentane/EtOAc) yielded 55 (71%, 0.14 g) as a colorless oil. \(^1\)H NMR (400 MHz, D\(_2\)O) \(\delta\) 4.61 (d, \(J = 8.2\) Hz, 1H, H-1), 3.96 – 3.40 (m, 5H, H-6b, H-6a, H-5, H-4, H-3), 3.31 (s, 3H, OCH\(_3\)), 3.13 (m, 1H, H-7), 2.34 (m, 1H, H-2). HRMS \(m/z\) calcd. for C\(_{33}\)H\(_{32}\)F\(_6\)O\(_7\)Na (M+Na\(^+\)) 677.2093, found 677.2101.
Methyl 2-deoxy-2-C-[1,1,1,5,5,5-hexafluoropentane-2,4-dione]-β-D-glucopyranoside (56)
The title compound was prepared by dissolving 53 (0.12 g, 0.18 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate was evaporated under reduced pressure to afford 54 (42 mg, 72%) as a white powder. 

1H NMR (400 MHz, CDCl₃)  δ 7.42 – 7.20 (m, 15H, ArH), 4.95 (m, 2H, ArCH₂), 4.70 – 4.52 (m, 5H, ArCH₂, ArCH₂, H-1), 4.21 (m, 1H, H6a) 4.09 (m, 1H, H-6b), 3.90-3.70 (m, 3H, OCH₃), 3.35 (m, 1H, H-7), 2.35 (m, 1H, H-2). 

13C NMR (100 MHz, D₂O)  δ 160.70, 160.41, 119.20, 118.75 103.42, 76.31, 74.92, 70.18, 61.14, 57.87, 56.12, 42.21. 

19F NMR (376 MHz, D₂O)  δ -75.2 (2) (COCF₃), -84.9 (2) (C(OH)₂CF₃) 

HRMS m/z calcd. for C₁₂H₁₄F₆O₇Na (M+Na⁺) 407.0601, found 407.0605.

Methyl 3,4,6-tri-O-benzyl-2-deoxy-2-C-[(methoxycarbonyl)methyl]-β-D-glucopyranoside (57)
The title compound was prepared following a procedure developed by Yin et al. 148 Briefly, 52a (1 g, 1.7 mmol) was dissolved in DMSO (10 mL) in the presence of excess lithium iodide (2.2 g, 17 mmol) and submitted to microwave irradiation (200 W, 10 bar, 100 °C, 20 min). The solution was then diluted with EtOAc and washed 5 times with water, followed by brine and drying with magnesium sulphate, and evaporated to dryness. Flash chromatography of the resulting mixture (10:1 pentane/EtOAc) yielded 57 (62%, 0.51 g) as a colorless oil. All acquired spectral data were in agreement with literature values. 

Methyl 3,4,6-tri-O-benzyl-2-deoxy-2-C-[acetic acid]-β-D-glucopyranoside (58)
The title compound was prepared by dissolving 57 (0.15 g, 0.28 mmol) in a 1:1 solution of 2M NaOH/dioxane and stirred overnight at room temperature. The solution was then diluted with DCM and washed with 1M HClₐq, water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure yielded 53 (82%, 0.11 g) as a clear oil. All acquired spectral data were in agreement with literature values. 

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Methyl 2-deoxy-2-C-[acetic acid]-β-D-glucopyranoside (59)
The title compound was prepared by dissolving 58 (0.1 g, 0.2 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate was evaporated under reduced pressure to afford 54 (72%, 30 mg) as a white powder. All acquired spectral data were in agreement with literature values. 148

Methyl 3,4,6-tri-O-benzyl-2-deoxy-2-C-[1,1,1-trifluoropropan-2-one]-β-D-glucopyranoside (60)
The title compound was prepared by dissolving 57 (0.15 g, 0.28 mmol) in ethylene glycol dimethyl ether (5 mL) in the presence of TMS-CF₃ (0.26 ml, 0.175 mmol) and a catalytic amount of cesium fluoride and allowed to stir overnight at room temperature. The solution was then diluted with DCM, and washed consecutively with HCl(aq), water, and brine, dried with magnesium sulphate. Evaporation under reduced pressure followed by flash chromatography (8:1 pentane/EtOAc) yielded 55 (64%, 96 mg) as a colorless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.42 – 7.10 (m, 15H, ArH), 4.95 – 4.60 (m, 6H, ArCH₂), 4.4 (d, 1H, H-1, J = 8.1 Hz), 3.75 – 3.40 (m, 8H, H-6a, H-6b, H-5, H-3, H-4, OCH₃), 2.50 (m, 2H, CH₂) 2.18 (m, 1H, H-2) HRMS m/z calcd. for C₃₁H₃₃F₃O₆Na M+Na⁺ 581.2219, found 581.2213.

Methyl 2-deoxy-2-C-[1,1,1-trifluoropropan-2-one]-β-D-glucopyranoside (61)
The title compound was prepared by dissolving 53 (75 mg, 0.13 mmol) in MeOH (5 mL), followed by the addition of a catalytic amount of 10% Pd/C. The resulting suspension was stirred vigorously under H₂ gas (1 atm) for 12 h. Upon reaction completion, the suspension was filtered through celite, and the resulting filtrate was evaporated under reduced pressure to afford 54 (81%, 30 mg) as a white powder (ketone/hydrate 26:1). ¹H NMR (400 MHz, D₂O) δ 4.40 (d, J = 8.3 Hz, 1H, H-1), 3.94 (dd, J = 12.2 Hz, 1.6 Hz, 1H, H-6a), 3.71 (dd, J = 12.2 Hz, 6.6 Hz, 1H, H-6b), 3.61 (dd,
$J = 9.4 \text{ Hz}, 8.7 \text{ Hz}, 1\text{H}, 
3.52 \text{ (s, 3H, OCH}_3\text{)}, 3.42 \text{ (m, 2H, H-4, H-5), 2.55 \text{ (m, 2H, CH}_2\text{)},} 
2.19 \text{ (m, 1H, H-2).}^{13}\text{C NMR (100 MHz, D}_2\text{O) }\delta 162.33, 119.4, 100.42, 75.3, 71.24, 68.80, 
57.40, 54.81, 49.68, 35.02. \text{^{19}F NMR (376 MHz, D}_2\text{O) }\delta -74.2 \text{ (COCF}_3\text{)}, -84.4 \text{ (C(OH)_2CF}_3\text{)}$

HRMS $m/z$ calcd. for C$_{10}$H$_{15}$F$_3$O$_6$Na (M+Na$^+$) 311.0713, found 311.0720.

**GlcNAc-GlcNAc-GlcNAcSOc-GlcNAc-GleNAc (63)**

The title compound was prepared by dissolving GlcNAc pentasaccharide (50 mg, 0.045 mmol) in HEPES buffer (50 mM, pH 7.5) to a final concentration of 10 mM, followed by the addition of MBP-PgaB to a final concentration of 50 µM. The solution was then incubated at 37 °C for 48 h. Desalting of the de-N-acetylated pentasaccharide on HPLC p2 size exclusion column yielded a 1:1 mixture of product and starting material (38 mg), which was used without further purification. The obtained powder (38 mg) was then dissolved in water (0.5 mL), and N-hydroxysuccinidyl 2-(octoylthio)acetate (100 mg, 0.55 mmol), dissolved in MeOH (0.5 mL), was then added to the solution and allowed to mix for 2 h. Upon complete consumption of the starting material, as judged by MALDI mass spectrometry, the reaction mixture was then evaporated under reduced pressure, redissolved in water, washed with EtOAc, and the aqueous layer was then subjected to reverse phase HPLC to isolate 63 (7.2 mg, 0.007 mmol) as a white foam. $^1$H NMR (400 MHz, D$_2$O) $\delta$ 5.17 (d, $J = 3.5 \text{ Hz, 0.4H, H-1}\alpha$), 4.62 – 4.51 (m, 4H, H-1’, H-1’’, H-1’’, H-1’’’’), 4.25 – 3.37 (m, 34H), 2.17 – 1.99 (m, 12H), 1.58 - 1.52 (m, 2H), 1.30 - 1.22 (m, 8H), 0.86 – 0.78 (m, 3H) CH$_3$C$_{18}$H$_{33}$N$_2$O$_{14}$S$_2$ (M+Na$^+$) 1214.8043, found 1214.8051
**GlcNAc-GlcNAc-GlcNAcSH-GlcNAc-GlcNAc (64)**

The title compound was prepared by dissolving 63 (7.2 mg, 0.007 mmol) in MeOH (0.5 mL) containing NaOMe (1 µM) for 1 hr. Upon complete consumption of the starting material, as judged by MALDI mass spectrometry, ambersite resin (H⁺) was added to the mixture until the pH was neutral, and the solution was evaporated under reduced pressure. The residue was then redissolved in AcOH (0.5 M, 0.5 mL), and washed 3 times with EtOAc (0.5 mL). The aqueous phase was then freeze-dried, yielding 64 (4.6 mg, 0.005 mmol) as a white foam. ¹H NMR (500 MHz, D₂O) δ 5.19 (d, J = 3.5 Hz, 0.4H, H-1α), 4.70 (d, J = 8.6 Hz, 0.6H, H-1β), 4.60 – 4.53 (m, 4H, H-1’, H-1”, H-1”’, H-1””’), 4.25 – 3.37 (m, 32H), 2.17 – 1.99 (m, 12H, CH₃). C₁₈H₃₃N₂O₁₄S₂ (M+Na⁺) 1088.1461, found 1088.1462.
References


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Staphylococcus aureus


Appendix A

Selected NMR spectra
Appendix B

Lineweaver-Burk and Michaelis-Menten plots
PgaB ACC deacetylation rates at various ACC concentrations. Conditions were as follows: HEPES buffer containing 10% (v/v) DMSO (100 mM, pH 7.5), 10 µM PgaB, ACC (0.5 mM – 10 mM). Assays were done in triplicate. Rates were obtained by measuring the increase of fluorescence emission at 446 nM resulting from the acetylosis of ACC. $V_{\text{max}}$, $K_M$ and $k_{\text{cat}}$ were determined from direct curve fitting of the data.
Top – Absorbance spectra of 1 (ACC) and 2 (Deacetylated ACC) at 5mM in 100mM HEPES containing 10% (v/v) DMSO. Bottom – Fluorescence spectra of 2 when excited at either 346 or 388.
Lineweaver Burke plots – All inhibitors showed competitive enzyme inhibition. The value of $\alpha$ was assessed as the factor increase of the slope in the presence of different inhibitor concentrations ($\text{slope} = \alpha K_m/V_{\text{max}}$). Using each obtained $\alpha$ coefficient, a mean $K_i$ value was derived from the following formula ($\alpha = 1 + [I]/K_i$).