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PAPER

Synthesis and evaluation of inhibitors of *E. coli* PgaB, a polysaccharide de-*N*-acetylase involved in biofilm formation†

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Many medically important biofilm forming bacteria produce similar polysaccharide intercellular adhesins (PIA) consisting of partially de-*N*-acetylated β-(1 → 6)-*N*-acetylglucosamine polymers (dPNAG). In *Escherichia coli*, de-*N*-acetylation of the β-(1 → 6)-*N*-acetylglucosamine polymer (PNAG) is catalysed by the carbohydrate esterase family 4 deacetylase PgaB. The de-*N*-acetylation of PNAG is essential for productive PNAG-dependent biofilm formation. Here, we describe the development of a fluorogenic assay to monitor PgaB activity *in vitro* and the synthesis of a series of PgaB inhibitors. The synthesized inhibitors consist of a metal chelating functional group on a glucosamine scaffold to target the active site metal ion of PgaB. Optimal inhibition was observed with *N*-thioglycolyl amide (K_i = 480 μM) and *N*-methyl-*N*-glycolyl amide (K_i = 320 μM) glucosamine derivatives. A chemoenzymatic synthesis of an *N*-thioglycolyl amide PNAG pentasaccharide led to an inhibitor with an improved K_i of 280 μM.

Introduction

Surface associated, matrix embedded colonies of bacteria, known as biofilms, are responsible for approximately 65% of all chronic human bacterial infections.[1] Bacteria residing in biofilms have a high tolerance to antibiotics and are sheltered from the host's immune system making biofilm associated infections challenging to eradicate.[2,3] During biofilm formation bacteria excrete an extracellular matrix to facilitate the adherence between bacteria and the colonized surface. Exopolysaccharides form an essential component of these biofilm matrices.[4,5] Many medically important biofilm forming bacterial strains, including *Staphylococcus epidermidis*[6] and *aureus*,[7] *Escherichia coli*,[8] *Acinetobacter baumannii*,[9] *Bordetella* species,[10,11] *Actinobacillus pleuropneumoniae*,[12] *Burkoholderia* species[13] and *Yersinia pestis*,[14] generate similar partially de-*N*-acetylated β-(1 → 6)-D-*N*-acetylglucosamine homopolymers (dPNAG), also known as the polysaccharide intercellular adhesin (PIA), as a key biofilm matrix exopolysaccharide.

In gram negative and gram positive bacteria, partial de-*N*-acetylation of the β-(1 → 6)-D-*N*-acetylglucosamine homopolymer (PNAG) is essential for the function of the polysaccharide in biofilm formation (Fig. 1). Deletion of the PNAG de-*N*-acetylase, IcaB, in *S. epidermidis* results in secretion of the PNAG exopolysaccharide into the growth media rather than localization of the exopolysaccharide at the bacterial surface.[15] The *ΔicaB S. epidermidis* strain was less virulent and displayed an attenuated biofilm formation phenotype.[16] In *E. coli* disruption of the PNAG de-*N*-acetylase, PgaB, results in a PNAG polysaccharide that fails to be exported from the periplasm and abolished bacterial biofilm formation.[8,17] These studies suggest that PNAG de-*N*-acetylases are good targets for therapeutic intervention in biofilm related infections.

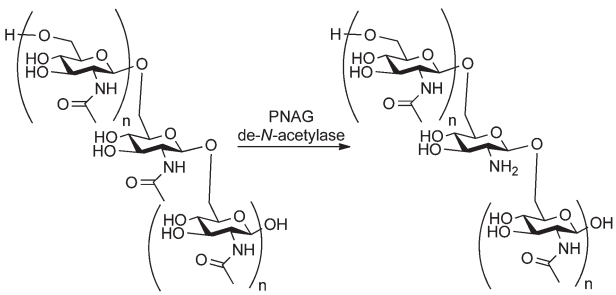

Fig. 1 PgaB catalyzed de-*N*-acetylation. Polymeric PNAG is tens to hundreds of monosaccharides in length and the degree of de-*N*-acetylation varies between bacterial species (10–40%).[8]

From sequence homology PNAG de-*N*-acetylases have been classified as members of the family 4 carbohydrate esterases (CE4) in the CAZY database[18] (http://www.cazy.org). CE4 family enzymes are metal dependent hydrolases having a His-His-Asp metal coordinating triad as well as conserved catalytic residues. Recently, we have solved the first structure of a PNAG

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de-*N*-acetylase, PgaB from *E. coli*.[19,20] The N-terminal de-*N*-acetylase domain of PgaB is structurally homologous to other well characterized CE4 family members such as the *Colletotrichum lindemuthianum* chitin de-*N*-acetylase, *Cl*CDA, and the *Streptococcal* peptidoglycan de-*N*-acetylase, *Sp*PgdA.[21,22] Despite the structural similarities, PgaB is specific for PNAG, and shows no activity on chitin (β-(1 → 4)-*N*-acetylglucosamine) substrates. PgaB also has unique metal dependence, showing optimal activity with Fe^{2+}, Ni^{2+}, and Co^{2+} in contrast to the Zn^{2+} dependency observed for other de-*N*-acetylases in the CE4 family.

To probe the active site of PgaB and to develop an inhibitor of PNAG de-*N*-acetylation, we have developed a chromogenic assay for PgaB activity, synthesized a series of potential inhibitors and assayed these against PgaB. The inhibitors are based on a methyl 2-deoxy-2-amino-β-D-glucopyranoside scaffold in which the amino group displays a range of potential metal chelating functional groups. One of the optimal metal chelating functional groups, a thioglycolyl amide, was chemoenzymatically installed on a PNAG pentasaccharide scaffold yielding the highest affinity inhibitor (K_i = 280 μM).

Results and discussion

Assay development

The catalytic efficiency for de-*N*-acetylation by PgaB on synthetic PNAG oligomers is low (k_{cat}/K_m for pentasaccharide = 0.25 M^{-1} s^{-1}), and saturation of enzymatic activity cannot be achieved due to the limited solubility of the substrate.[19] Furthermore, the previous assay used to monitor the de-*N*-acetylation reaction of the natural substrate required a cumbersome discontinuous fluorescamine assay. A simpler continuous assay was developed to allow for more rapid analysis of PgaB inhibition and to allow full kinetic characterization of the enzymatic activity.

A series of chromogenic acetyl esters were evaluated as PgaB substrates (Fig. 2). The compounds were dissolved in DMSO and added (0.10–10 mM) to PgaB (10 μM) in HEPES buffer (100 mM, pH 7.5). Acetate hydrolysis was monitored by absorbance or fluorescence spectroscopy. *p*-Nitrophenyl acetate (pNPA), 5-acetoxymethylquinolinium (5-AMQ)[23] and 7-acetoxymethylquinolinium (7-AMQ)[23] were substrates for PgaB but these substrates suffered from high rates of background hydrolysis. 4-Methylumbelliferyl acetate (AMC) and 3-carboxyumbelliferyl acetate (ACC)[24] showed good stability to hydrolysis and also served as substrates for PgaB. The limiting solubility of AMC (~1 mM) prevented saturation of the enzymatic activity; however, ACC was soluble up to 10 mM concentrations allowing for the determination of the kinetic parameters of PgaB (k_{cat} = 0.013 ± 0.002 s^{-1}, K_m = 1.2 ± 0.2 mM).

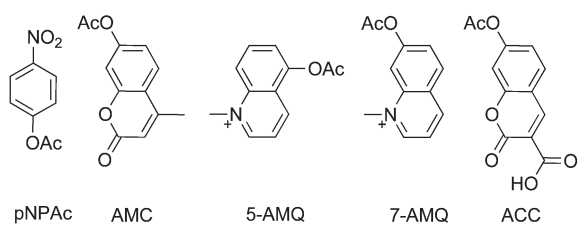


Fig. 2 Acetyl esters evaluated as substrates for PgaB.

Inhibitor design

Many inhibitors have been developed for metal dependent *N*-acetylamidases, such as the histone de-*N*-acetylases, and other carbohydrate processing de-*N*-acetylases, such as lipopolysaccharide de-*N*-acetylase LpxC.[25,26] A number of potential inhibitors (IC_{50} = 100–500 μM) have also been identified for the CE4 *Streptococcal* peptidoglycan de-*N*-acetylase through a large *in silico* library screen.[27] Recently, Urbaniak *et al.* have explored the inhibition of *N*-acetylglucosamine-phosphatidylinositol de-*N*-acetylase from *Trypanosoma brucei* using a series of GlcNAc derivatives.[28] The common feature of the *N*-acetylamidase inhibitors identified is a metal coordinating functional group, which ligates the de-*N*-acetylase active site metal ion. Based on the success of the glucose based hydroxamic acid inhibitor (TU-514) against lipopolysaccharide de-*N*-acetylases, we installed a range of metal coordinating groups on a glucosamine scaffold with the aim of identifying a PgaB inhibitor (Fig. 3).[29] In addition, methylation of the amide nitrogen was evaluated, as similar modifications of metal chelating groups led to higher affinity inhibitors for Zn metalloproteases, which are proposed to act by a similar mechanism to PgaB.[30] Although analysis has shown that monosaccharides are not substrates of PgaB,[19] it was envisaged that once an optimal metal chelating functional group was identified it could be installed on a larger PNAG oligosaccharide *via* a chemoenzymatic synthesis to yield an improved inhibitor.

Inhibitor synthesis

Methyl β-D-2-deoxy-2-aminoglucopyranoside (**2**) served as the starting scaffold for synthesis of the monosaccharide based inhibitors. Using the protecting-group-free chemistry based on glycosyl tosylhydrazides, the methyl glycoside **1** could be synthesized in two steps from *N*-acetylglucosamine. De-*N*-acetylation of **1** with hydrazine hydrate gave **2** in excellent yield.[31] Alternatively, *via* direct methanolysis of 3,4,6-triacetyl-2-deoxy-2-aminoglucosyl bromide, the methyl glycoside **2** could be generated and subsequently deacetylated.[32] The free amine of **2** was then condensed with benzyloxyacetic acid and Cbz-Gly-OH using HBTU, or *via* direct acylation with pentafluorophenyl *S*-acetylthioglycolate (SAMA-OPFP) to provide compounds **4**, **6** and **8** after deprotection. The hydroxylurea **9** could be accessed by acylation of **2** with 1-(4-nitrophenol)-*N*-hydroxycarbamate.[33] Compound **2** was also sulfonylated with mesylchloride to provide compound **10** (Scheme 1).

Benzyl protected methyl 2-aminomethyl-2-deoxy-β-D-glucoside (**14**) was synthesized in four steps from **1**. Initial

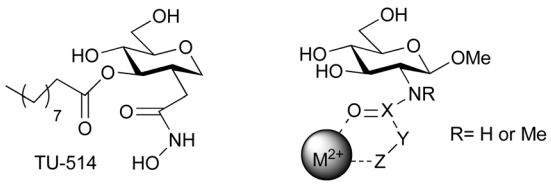
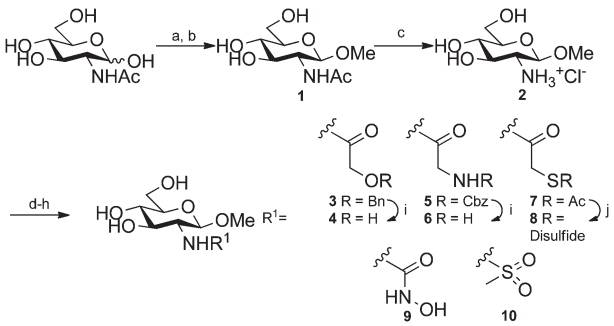


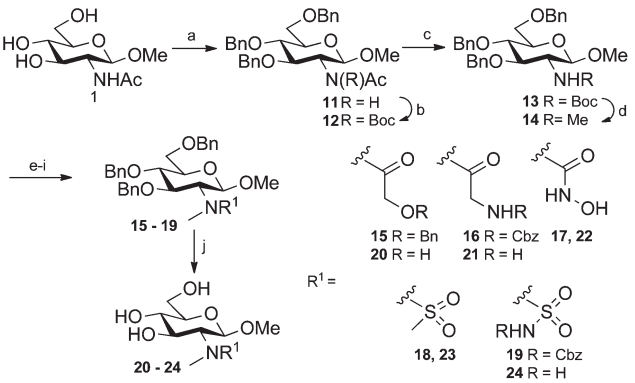
Fig. 3 Structure of TU-514 a potent lipopolysaccharide de-*N*-acetylase (LpxC) inhibitor (left) and the proposed inhibitor structures (right) where X, Y, Z represent atoms forming a bidentate metal chelate.

per-*O*-benzylation followed by *N*-carbamylation with Boc_2O and catalytic DMAP gave the protected mixed imide **12** as previously reported.[34] De-*N*-acylation of **12** with catalytic NaOMe followed by reduction with $LiAlH_4$ gave the protected methylamine **14** in good yield. The secondary amine was then functionalized as has been described above (Scheme 1). After final deprotection potential inhibitors **20–24** were isolated in good yield (Scheme 2). Rotamers were observed in the cases of the per-*O*-benzylated species in both ^{13}C NMR and ^{1}H NMR spectra at room temperature, as expected by literature precedent.[35] In most cases only a single rotamer was observed for the final *N*-Me derivatives by ^{1}H NMR in D_2O at room temperature; however, some signal broadening was observed, and distinct rotamers could be seen with compound **21**.

The synthesis of the sulfamate derivative **27** and the *N*-methylthioglycolyl amide derivative **30** required slightly modified schemes. Due to the reactivity of the sulfamylating agent (benzyl *N*-(chlorosulfonyl)carbamate) (BNCC)[36] hydroxyl protecting groups were required. Thus, **14** was treated with TFA to liberate the amine which reacted smoothly to form the Cbz-protected sulfamate **26**. Global deprotection of this compound by hydrogenation yielded **27**. The incompatibility of the thioglycolyl amide with hydrogenation conditions required deprotection of **14** to give **28** prior to acylation. After final acetolysis compound **30** was isolated as the symmetrical disulfide (Scheme 3).



Scheme 1 Synthesis of inhibitors **4**, **6**, **8–10**. (a) Tosyl hydrazide (1.1 equiv.), DMF : H_2O : AcOH (5 : 1 : 0.1), rt, 24 h, 92%. (b) NBS (2.4 equiv.), MeOH (20 equiv.), DMF, rt, 1 h, 78%. (c) $NH_2NH_2{\cdot}H_2O$, 110 °C, 24 h, 96%. (d) HBTU (1 equiv.), benzyloxyacetic acid (1 equiv.), TEA (1.2 equiv.), DMF, rt, 12 h, leading to **3**, 84%. (e) HBTU (1 equiv.), Cbz-Gly-OH (1 equiv.), TEA (1.2 equiv.), DMF, rt, 12 h, leading to **5**, 76%. (f) SAMA-OPFP (1 equiv.), TEA (1.2 equiv.), DMF, rt, 12 h, leading to **7**, 78%. (g) 1-(4-Nitrophenol)-*N*-hydroxycarbamate (1 equiv.), TEA (1.2 equiv.), DMF, rt, 12 h, leading to **9**, 54%. (h) MsCl (1 equiv.), TEA (2 equiv.), DMF, rt, 12 h, leading to **10**, 58%. (i) 10% Pd/C, 1 atm H_2, MeOH, rt, 12 h, 81–89%. (j) NaOMe (0.1 equiv.), MeOH, rt, 10 min, 92%.

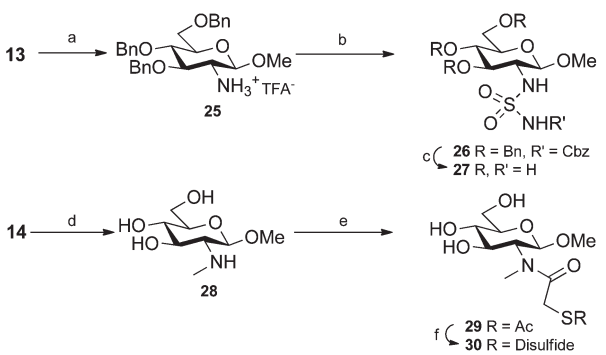


Scheme 2 Synthesis of inhibitors **20–24**. (a) BnBr (3.5 equiv.), NaH (3.5 equiv.), DMF, 0 °C, 16 h, 76%. (b) Boc_2O (2 equiv.), DMAP (0.25 equiv.), THF, 60 °C, 12 h. (c) NaOMe (0.2 equiv.), MeOH, rt, 1 h, 72% over 2 steps. (d) $LiAlH_4$ (3 equiv.), THF, 60 °C, 6 h, 68%. (e) DCC (1 equiv.), benzyloxyacetic acid (1 equiv.), TEA (0.2 equiv.), EtOAc : DCM (1 : 1), rt, 12 h, leading to **15**, 89%. (f) DCC (1 equiv.), Cbz-Gly-OH (1 equiv.), TEA (0.2 equiv.), EtOAc : DCM (1 : 1), rt, 12 h, leading to **16**, 92%. (g) 1-(4-Nitrophenol)-*N*-hydroxycarbamate (1 equiv.), TEA (0.2 equiv.), DMF, rt, 12 h, leading to **17**, 72%. (h) MsCl (1 equiv.), TEA (1.2 equiv.), EtOAc, rt, 12 h, leading to **18**, 88%. (i) TEA (1.2 equiv.), DCM, rt, 3 h, 88%. (j) 10% Pd/C, 1 atm H_2, MeOH, 12 h, 75–82%.

Inhibitor evaluation

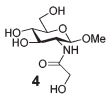
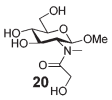
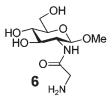
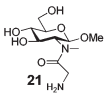
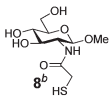
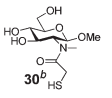
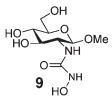
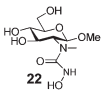
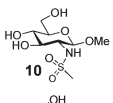
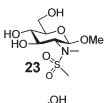
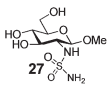
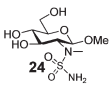
The potential de-*N*-acetylase inhibitors (**4**, **6**, **8**, **9**, **10**, **20–24**, **27**, **30**) were tested for their inhibitory potency against Ni^{2+} loaded PgaB in a competitive assay with the coumarin substrate ACC. We have previously shown PgaB to also be active in its Fe^{2+} form but this enzyme is unstable under ambient conditions and was not evaluated.[19] None of the inhibitors were substrates of PgaB as determined by ^{1}H NMR after overnight incubation of the compounds at 10 mM under the assay conditions. Inhibitors containing a disulfide (**8**, **30**) were preincubated with DTT prior to assay onset to reduce the disulfide. Compounds showing greater than 10% inhibition of coumarin deacetylation in the presence of 10 mM inhibitor were subjected to full kinetic analysis. In all fully characterized inhibitors, competitive inhibition was observed with the coumarin substrate. Methyl 2-deoxy-2-amino-glucopyranoside (**2**) showed no inhibition of PgaB at 1 mM, whereas at higher concentrations the free amine caused a significant increase in background signal during the assay. The results of the inhibitor assay are summarized in Table 1.

Of the primary amide derivatives (**4**, **6**, **8**, **9**, **10**, and **27**) only the reduced thioglycolyl amide, **8**, showed significant inhibitory activity against PgaB (K_i = 480 μM). Thioglycolyl amides are known to be good inhibitors for many metalloamidases.[37] Surprisingly compounds **9** and **27** showed little activity, as inhibitors



Scheme 3 Synthesis of inhibitors **27** and **30**. (a) 1 : 1 TFA : DCM, rt, 30 min, 81%. (b) BNCC (1.5 equiv.), TEA (1.2 equiv.), DCM, rt, 3 h, 88%. (c) 10% Pd/C, 1 atm H_2, MeOH, 12 h, 75%. (d) 10% Pd/C, 1 atm H_2, MeOH, 12 h, 78%. (e) SAMA-OPFP (1 equiv.), TEA (1.2 equiv.), DMF, rt, 12 h, 69%. (f) NaOMe (0.1 equiv.), MeOH, rt, 10 min, 64%.

Table 1 Inhibition of PgaB

Inhibitor	Residual activity[a] (%)	K_i (μM)	Inhibitor	Residual activity[a] (%)	K_i (μM)
 **4**	92 ± 3	—	 **20**	17 ± 2	320 ± 10
 **6**	98 ± 5[c]	—	 **21**	96 ± 4[c]	—
 **8**[b]	24 ± 2	480 ± 10	 **30**[b]	52 ± 3	920 ± 20
 **9**	106 ± 4	—	 **22**	103 ± 3	—
 **10**	93 ± 3	—	 **23**	82 ± 3	5800 ± 100
 **27**	91 ± 2	—	 **24**	33 ± 4	680 ± 20

[a] Remaining PgaB deacetylase activity with 10 mM inhibitor. [b] Inhibitor incubated with DTT (1 equiv.) prior to assay. [c] Remaining PgaB deacetylase activity with 1 mM inhibitor.

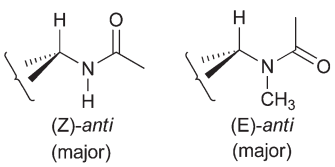
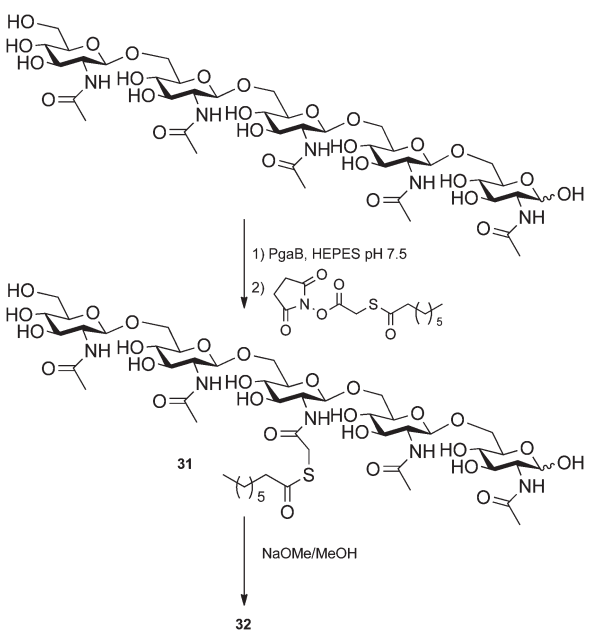


Fig. 4 Preferred conformers of *N*-acylglucosamine amides.

containing these metal chelating groups have shown promising activity with the histone de-*N*-acetylases.[37,38]

The *N*-methyl derivatives displayed dramatically improved inhibitory activity over the N–H derivatives. Conformational analysis of methyl 2-*N*-methyl-*N*-acetamido-2-deoxy-β-D-glucopyranoside has shown a significant population of the acetyl group in the (*E*)-*anti* conformation in comparison with the primary amide of methyl 2-acetamido-2-deoxy-β-D-glucopyranoside which adopts primarily a (*Z*)-*anti* conformation (Fig. 4).[35,39] The improved affinity observed with the *N*-methyl derivatives suggests that the preferred bound conformation of the acyl metal chelates may be (*E*)-*anti*. Interestingly the best inhibitor of the *N*-Me derivatives, **20**, is a metal chelating group that has not been previously reported as an inhibitor of amidases.



Scheme 4 Synthesis of thioglycolyl amide pentasaccharide **32**.

Chemoenzymatic synthesis

PgaB shows no measurable de-*N*-acetylation activity towards methyl β-D-2-acetamido-2-deoxyglucopyranoside.[19] However, PgaB shows increased catalytic activity against larger PNAG substrates with measurable de-*N*-acetylation of trisaccharide and increasing activity on oligomers up to pentasaccharide in length. Thus, it was hypothesized that introduction of one of the productive metal chelating groups identified above into a larger oligosaccharide would yield a higher affinity inhibitor.

Based on the thioglycolyl amide inhibitor **8** an improved inhibitor was synthesized using a chemoenzymatic approach (Scheme 4). PgaB was used to de-*N*-acetylate a PNAG pentasaccharide.[40] Previous analysis of the de-*N*-acetylation products produced by PgaB showed that the predominant product of a pentasaccharide substrate was mono-de-*N*-acetylation at the

central glucosamine residue.[19] Due to the low catalytic activity of the enzyme complete de-*N*-acetylation was not achieved and a mixture of the starting material and the mono-de-*N*-acetylated pentasaccharide was carried forward to the acylation step. Acylation with *S*-octanoylthioglycolate *N*-hydroxysuccinamide ester provided a product which could be readily separated from the fully acetylated pentasaccharide by RP-HPLC. The octanoyl thioester could be cleaved under basic conditions and the free octanoic acid removed by acidic extraction (Scheme 4).

Analysis of the inhibition of compound **32**, after reduction of the disulfide bond, against PgaB was determined using the same protocol as described for the monosaccharide inhibitors. The pentasaccharide **32** showed a two-fold increase in inhibitory (K_i = 280 μM) activity over compound **8**. A greater gain in affinity was expected given the observed improvement in substrate turnover by PgaB on larger PNAG oligomers. Further work is required to discern the binding mode of compounds **8** and **32** to the enzyme to explain this result with confidence. Likely the optimal binding orientation of the thioglycolyl amide **8** is different from the orientation of a monosaccharide within a larger oligomer.

Conclusion

In conclusion, we have synthesized a series of glucosamine derivatives bearing potential metal chelating groups as inhibitors against PgaB. *N*-Methylated chelators showed consistently stronger inhibition than their primary amide counterparts, yielding high micromolar inhibitors. Using a chemoenzymatic synthesis it was possible to generate a thioglycolyl amide functionalized pentasaccharide inhibitor with a K_i value of 280 μM against PgaB. Not surprisingly preliminary biofilm inhibition assays with *S. epidermidis 1457* using compounds **8**, **20**, **24**, at a concentration of 1 mM, showed no inhibition likely due to the weak activity of these compounds.

Acknowledgements

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