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Inhibitors for Bacterial Cell-Wall Recycling

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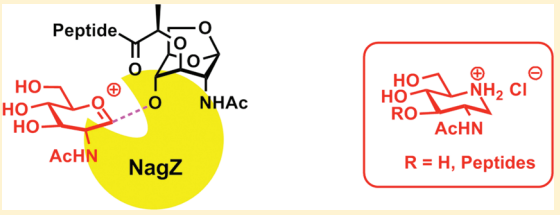
Supporting Information

ABSTRACT: Gram-negative bacteria have evolved an elaborate process for the recycling of their cell wall, which is initiated in the periplasmic space by the action of lytic transglycosylases. The product of this reaction, β-D-*N*-acetylglucosamine-(1→4)-1,6-anhydro-β-D-*N*-acetylmuramyl-L-Ala-γ-D-Glu-*meso*-DAP-D-Ala-D-Ala (compound **1**), is internalized to begin the recycling events within the cytoplasm. The first step in the cytoplasmic recycling is catalyzed by the NagZ glycosylase, which cleaves in a hydrolytic reaction the *N*-acetylglucosamine glycosidic bond of metabolite **1**. The reactions catalyzed by both the lytic glycosylases and NagZ are believed to involve oxocarbenium transition species. We describe herein the synthesis and evaluation of four iminosaccharides as possible mimetics of the oxocarbenium species, and we disclose one as a potent (compound **3**, K_i = 300 ± 15 nM) competitive inhibitor of NagZ.



KEYWORDS: *Iminosaccharide, NagZ, MltB, peptidoglycan, β-lactam*

The bacterial cell wall is recycled in the normal course of growth of many Gram-negative bacteria. The recycled peptidoglycan components are processed during the growth and maturation of the cell wall and in response to damage by antibiotics.[1] Peptidoglycan recycling in the periplasm commences with the catalytic action of the lytic transglycosylases (Figure 1). These enzymes catalyze the nonhydrolytic fragmentation of the glycosidic bond between the *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) residues of the peptidoglycan-derived muropeptides. This unusual fragmentation gives the NAG-1,6-anhydromuramyl derivative **1** as a product. Following internalization through the AmpG permease, compound **1** is transformed via a series of enzymatic reactions to key intermediates, enabling merger of the recycling process with the biosynthetic events that lead to the formation of Lipid II. An early cytoplasmic event of muropeptide recycling is the hydrolytic action of the NagZ glycosylase, which removes NAG from **1** to produce compound **2**. Subsequent reaction of the protease AmpD removes the peptide segment from compound **1** or **2**.[2,3] Once formed, Lipid II is transported to the surface of the plasma membrane, where it is the building unit for the *de novo* synthesis of the peptidoglycan.

The distinct reactions of lytic transglycosylase(s) and NagZ—one is a nonhydrolytic transglycosylase and the other a hydrolytic glycosidase—are nonetheless proposed to go through conformationally distinct oxocarbenium species in the key step of their respective reactions. The oxocarbenium species in the former entraps the C6 hydroxyl group as a nucleophile, whereas a different oxocarbenium species in the latter is captured by a water molecule. The subjects of iminosaccharide properties, conformations, and bioactivities have been reviewed.[4,5] Here we evaluate the piperidine iminosaccharides **3**–**6**, inspired by the structures of the NAG (**3**) and the NAM (**4**–**6**) moieties found in the bacterial peptidoglycan, as possible mimics of oxocarbenium species generated in the course of the reactions by these two enzymes.

We report herein their syntheses and their inhibitory properties against purified recombinant NagZ of *Pseudomonas aeruginosa* and the lytic transglycosylase MltB of *Escherichia coli*. As indicated above, compound **3** potentially mimics the oxocarbenium species encountered in the turnover of **1** by NagZ and compounds **3**–**6** are potential mimics for the species in reaction of lytic tranglycosylases.

The synthetic plan to iminosaccharide derivatives **3**–**6** was envisioned to proceed via a protected 2-acetamido-1,2-dideoxynojirimycin (**7**) as the common intermediate. Several syntheses of piperidine iminosaccharides have been reported.[6−20] Our approach to intermediate **7**, as depicted in the retrosynthetic route of Figure 2, is based on the synthesis of lactam **8a**, followed by its reduction to the piperidine. While this route is conceptually based on the prior syntheses of GlcNAc iminosaccharide mimetics,[17−20] it incorporates a protecting group strategy that is advantageous to structure−activity development toward incorporation of the lactate moiety at C3. This orthogonal protective strategy also allows facile incorporation of a second saccharide at C4.

Starting material **9** was prepared from D-glucosamine in three steps with an overall yield of 77% by small variation of known procedures[21,22] (Scheme 1). Briefly, D-glucosamine was selectively *N*-acetylated, and an α-OBn group was introduced at C1 with thermodynamic control. Finally, 4,6-*O*-benzylidene was introduced in the presence of *p*-TsOH to give compound

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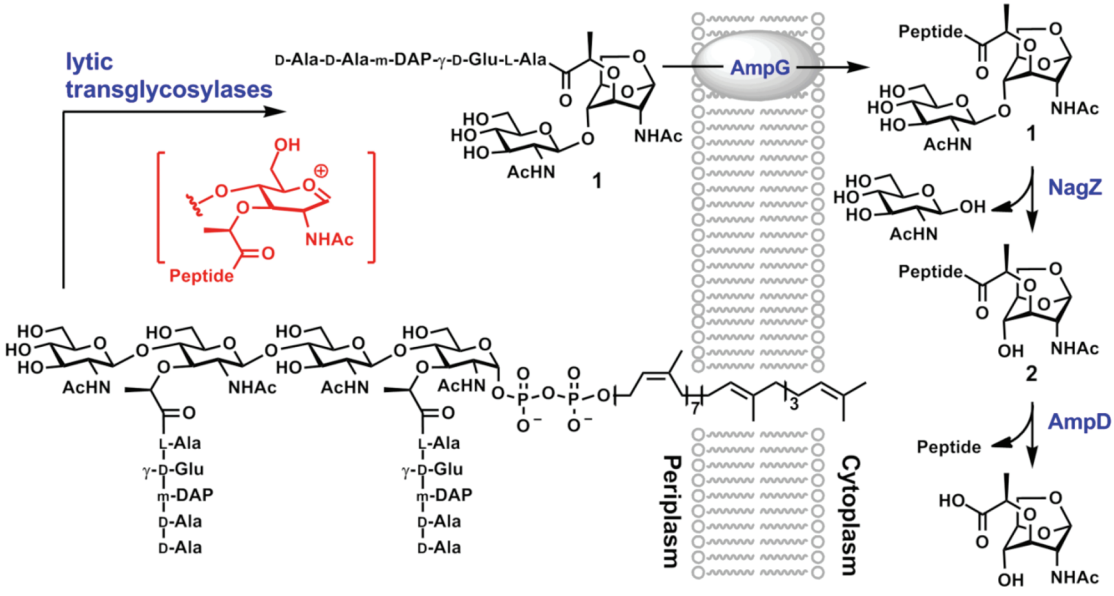


Figure 1. Schematic of the early events of cell-wall recycling.

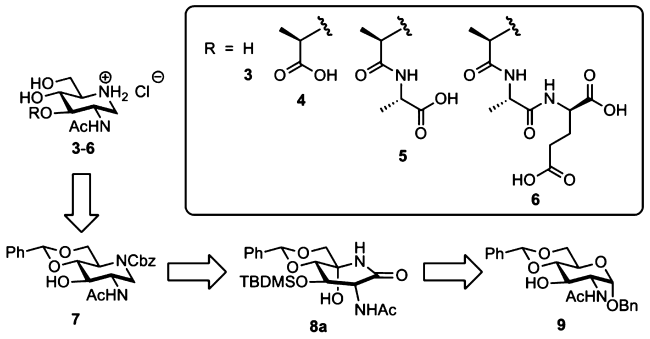
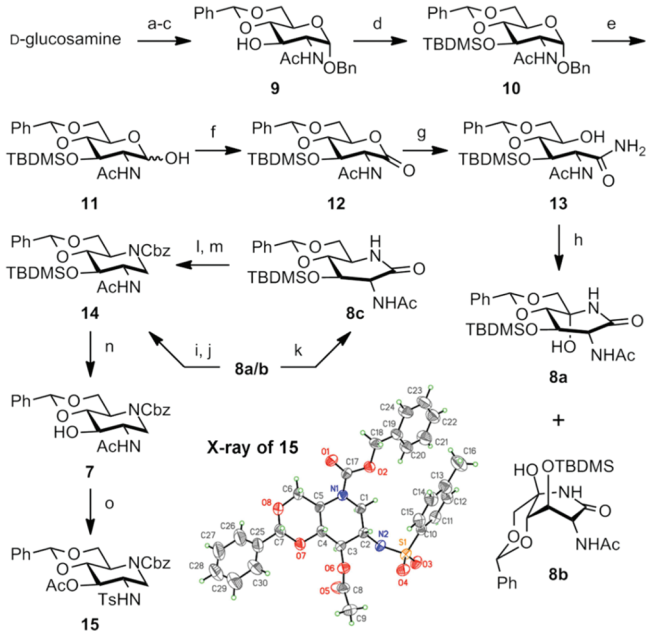


Figure 2. Iminosaccharides (**3**–**6**) based on the structural templates of NAG and NAM and their retrosynthetic analysis.

9. Transformation of **9** to **11** was straightforward, with introduction of the silyl protective group at C3 and removal of the benzyl protective group at C1. This orthogonal protective scheme is critical for selective functional group manipulation toward the target molecules **3**–**6**.

The PCC oxidation of **11** provided lactone **12**, which was converted to **13** by ammonolysis. We observed transient formation of the methyl ester of C1 during the reaction; however, it was slowly converted to carboxamide **13**, which was oxidized with a catalytic amount of tetrapropylammonium perruthenate (TPAP) in the presence of *N*-methylmorpholine *N*-oxide (NMO) to the diastereomeric mixture of hydroxyl lactams **8a** (D-gluco) and **8b** (L-ido) in a 2:3 ratio. Stereoselective 2-fold reduction of the lactam mixture (**8a**/**b**) using borane dimethylsulfide, followed by amine protection with CbzCl, gave the protected 2-acetamido-1,2-dideoxynojirimycin **14** in 29% yield from the mixture of **8a**/**b**. In an attempt at improving the low yield, we pursued a stepwise approach[17−20] for stereoselective dehydroxylation of the mixture **8a**/**b** using $NaBH_3CN$/trifuloroacetic acid (35%), followed by reduction of lactam (**8c**) with $BH_3{\cdot}SMe_2$ (64%). We observed no transformation of **8b** during the first reaction, which is the main reason for the low yield. Overall, the two-step approach was not advantageous over the one-pot treatment with borane. In preparation of similar molecules, Vasella et al.

Scheme 1. Synthesis of the Key Intermediate 7[a]



[a]Reagents and conditions: (a) NaOMe, MeOH, 40 °C; Ac_2O, 40 °C; (b) BnOH, AcCl, 50 °C; (c) $PhCH(OMe)_2$, TsOH, DMF, 70 °C, 88% (3 steps); (d) TBDMSCl, imidazole, DMF, 70 °C, quant.; (e) H_2, Pd/C, THF, rt, quant.; (f) PCC, CH_2Cl_2, rt; (g) NH_3/MeOH, CH_2Cl_2, rt, 77% (2 steps); (h) TPAP, NMO, CH_2Cl_2, rt, 61%; (i) $BH_3{\cdot}SMe_2$/THF, CH_2Cl_2, rt; (j) CbzCl, Pyr, CH_2Cl_2, rt, 29% (2 steps); (k) $NaBH_3CN$, TFA, MeCN, rt, 35%; (l) $BH_3{\cdot}SMe_2$/THF, CH_2Cl_2, rt; (m) CbzCl, Pyr, CH_2Cl_2, rt, 64% (2 steps); (n) TBAF, THF, rt, quant.; (o) TsCl, Py, reflux, 76%.

used Jones oxidation or Dess−Martin periodinane, followed by acid treatment (acetic acid or $BF_3{\cdot}OEt_2$) to improve the proportion of the tri-*O*-benzyl protected derivative of **8a** over **8b**.[18] This strategy was not applicable to our chemistry, as some of the protective groups for the required orthogonal protection would not survive the treatment. Despite the low yield, we were

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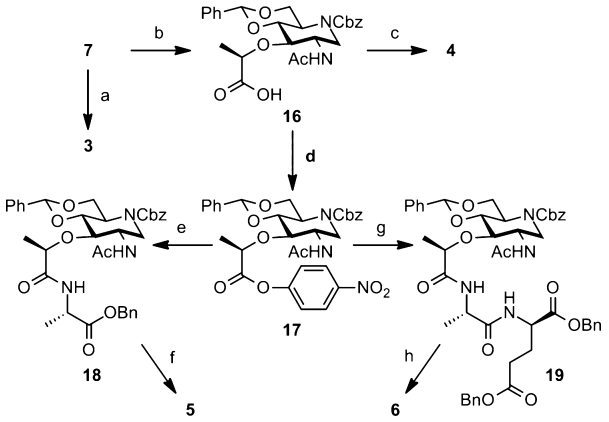
able to isolate compound **14** in pure form in quantity, and we carried it over to the next stage of the synthesis.

The TBDMS group of **14** was removed in quantitative yield to give key intermediate **7**. The structure of **7** was confirmed by X-ray crystallographic analysis of its crystalline tosyl derivative **15**. We have discussed separately the mechanism of the type of *N,O*-acetyl migration shown in the formation of **15**.[23] This solid-state structure of **15** is the first of 2-acetamido-1,2-dideoxynojirimycin derivatives (the solid-state structure of the *O*-deprotected 1,5-piperidinone lactam is known[18]). The structure of **15** confirms both the regiochemistry and stereochemistry of all the transformations shown in Scheme 1.

Introduction of the lactate moiety into **7** gave **16**, which was then converted to the activated ester **17** (Scheme 2). Amides

Scheme 2. Synthesis of Iminosaccharides 3−6[a]



[a]Reagents and conditions: (a) H_2, Pd/C, HCl, ^{i}PrOH, rt, quant.; (b) (*S*)-2-chloropropionic acid, NaH, THF, 60 °C, 91%; (c) H_2, Pd/C, HCl, ^{i}PrOH, rt, quant.; (d) *p*-nitrophenyl trifluoroacetate, Py, TEA, CH_2Cl_2, rt, 72%; (e) L-alanine benzyl ester, TEA, THF, CH_2Cl_2, reflux, 85%; (f) H_2, Pd/C, HCl, ^{i}PrOH, rt, quant.; (g) dibenzyl *N*-(L-alaninyl)-D-glutamate, TEA, THF, CH_2Cl_2, reflux, 90%; (h) H_2, Pd/C, HCl, ^{i}PrOH, rt, quant.

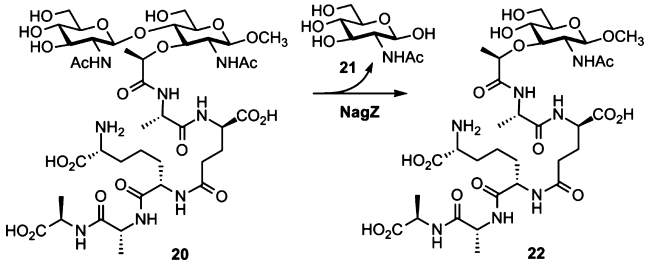
18 and **19** were conveniently formed by aminolysis. Deprotection of **7**, **16**, **18**, and **19** by catalytic hydrogenolysis gave iminosaccharides **3**, **4**, **5**, and **6**, respectively. The ^{1}H NMR data for **3** coincided with literature values.[7] Structures related to **4**−**6** were prepared previously for evaluation as immunoadjuvants.[9,10]

The four compounds were evaluated for binding to the *E. coli* MltB lytic transglycosylase. We reported previously the purification of a His-tag version of this protein that introduced several amino acids to its C-terminus.[24] Here, we used a new His6x-tagged construct having only two additional C-terminal amino acids, and also purified to homogeneity (Supporting Information). Compound **3** is missing the lactate moiety that is found in NAM (no substituent at C3). Compounds **4**, **5**, and **6** each show progressively larger portions of the peptide stem appended to the lactate. The reaction of lytic tranglycosylase MltB from *E. coli* (used in the present work) was characterized earlier,[24−26] including a quantitative analysis of the turnover chemistry of MltB using a synthetic peptidoglycan sample.[24]

The ability of the MltB lytic transglycosylase to bind the iminosaccharides was assessed using the intrinsic fluorescence of the tryptophan and/or tyrosine residues (of which there are eight and sixteen in the protein, respectively) of this enzyme. On the basis of the MltB X-ray structure, Trp165 is on a loop close to the active site, and Trp247, though more distal, makes interactions with the loop (amino acids Ser216−Met227) that comprises a portion of the binding site. In addition, several tyrosine residues (Tyr117, Tyr191, Tyr259, Tyr338, Tyr344) line the active-site groove. Upon excitation at 280 nm, the emission spectrum showed a maximum at 334 nm, consistent with the existence of a Tyr-to-Trp energy transfer. Compounds **3** and **4** showed saturable binding to a single site (K_d = 174 ± 9 μM and 1000 ± 200 μM, respectively), accompanied by a nonsaturable and presumably nonspecific binding mode (k_{ns} = 0.128 ± 0.004 μM^{-1} and 0.060 ± 0.006 μM^{-1}, respectively). This nonspecific binding may occur along the extended active site of MltB. The interactions of compounds **5** and **6** with MltB were simpler: their data fit to a hyperbolic one-site saturation equation (K_d = 189 ± 8 μM and 1010 ± 20 μM, respectively). In retrospect, the unexceptional K_d values for these piperidine iminosaccharides could be rationalized on the basis of a key mechanistic insight by Dijkstra et al.[27] These authors argued that, subsequent to the formation of the oxocarbenium species, the glucosamine ring should adopt a boat conformation to predispose spatially the C6 hydroxyl group for entrapment by the oxocarbenium ion in the intramolecular ring formation. The formation of the higher-energy boat conformation for the reaction intermediate is facilitated by the enzyme, which might not take place with our synthetic inhibitors, hence their relatively poor ability in inhibiting this enzyme.

The reaction of NagZ of *E. coli* has been investigated by analogues of compound **1**, isolated from the bacterial culture and which showed complex kinetic behavior with NagZ.[28] As the complex kinetics did not allow steady-state measurements at saturation, the authors estimated a K_m for substrates in the range 30−35 μM for this enzyme.[28] Using a homogeneous synthetic sample of **1**,[2,3] we characterized **1** as a substrate for NagZ of *P. aeruginosa*. Compound **2** was confirmed as the product of this NagZ reaction both by mass spectrometry and by comparison with an authentic synthetic sample (Supporting Information). NagZ hydrolysis of **1** showed saturation, and the following kinetic parameters for its turnover were evaluated: k_{cat} = 6.6 ± 0.2 s^{-1}, K_m = 104 ± 2 μM, and k_{cat}/K_m = 6.3 ± 0.2 × 10^4 M^{-1} s^{-1}).

Park et al. reported that an isolated radioactive cell wall is degraded in the presence of NagZ of *E. coli*. Hence, NagZ might have other cell wall processing activities.[29] In our hands, NagZ of *P. aeruginosa* recognized a cognate synthetic compound, the NAG-NAM-pentapeptide **20** (see Supporting Information), as a substrate (k_{cat} = 0.77 ± 0.04 s^{-1}, K_m = 165 ± 36 μM, and k_{cat}/K_m = (0.5 ± 0.1) × 10^4 M^{-1} s^{-1}). NagZ ordinarily would not encounter **20**, as only **1** is internalized in the course of recycling of the peptidoglycan. However, this experiment reveals that NagZ might not have evolved for specific turnover of compound **1**, with its unusual bicyclic 1,6-anhydromuramyl moiety as the focus of evolution of function.



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We used a chromogenic substrate for NagZ, *p*-nitrophenyl β-D-*N*-acetylglucosamine, for evaluation of our iminosaccharides as potential NagZ inhibitors. Kinetic evaluation of compounds **3**−**6** with NagZ identified **3** as a potent competitive inhibitor (K_i = 300 ± 15 nM). The magnitude of this K_i is comparable to the K_i values reported previously for **3** against several β-*N*-acetylglucosaminidases.[6,20] Compounds **4**, **5**, and **6** were much less effective competitive inhibitors (respective K_i values of 51 ± 4 μM, 35 ± 3 μM, and 33 ± 2 μM). The poorer inhibition by **4**, **5**, and **6** is understandable in terms of their C3 substitution, compared to the unsubstituted NAG moiety of the endogenous substrate for NagZ.

The significance of NagZ-dependent exoglycosidase catalysis has increased following the discovery of its central role[28,29] in peptidoglycan recycling by *E. coli*.[1,30] Peptidoglycan recycling is monitored by many of the Gram-negative bacteria for the purpose of controlling induction of the expression of β-lactamase enzyme, in response to cell wall damage by β-lactam antibiotics.[31] Small molecule inhibition of NagZ attenuates β-lactamase expression with concomitant improvement in *in vitro* β-lactam efficacy, in both *E. coli* and *P. aeruginosa*.[32−34] Given the successful application of structure-based design toward potent and selective GlcNAc pyranosylidene aminocarbamate inhibitors of NagZ[35,36] and the proven synthetic strategies toward improved iminosaccharide inhibitor efficacy,[6−16,20,37,38] our observation of potent NagZ inhibition by a piperidine-based GlcNAc iminosaccharide augurs well for future application of the iminosaccharide for small molecule interrogation of these pathways.[36,39] Moreover, as it has now been proven that peptidoglycan recycling occurs in at least one Gram-positive bacterium (*Bacillus subtilis*)[40] and engages a mechanistically distinct NagZ ortholog,[41] this inhibitor class may have broad-spectrum implications toward efforts to preserve clinical relevance for the β-lactam antibiotics.[42]

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data for all new compounds, including the 1D (^{1}H, ^{13}C NMR, DEPT) and 2D NMR spectra (H−H COSY and C−H HETCOR), and the crystallographic information file (CIF) of compound **15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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