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Anti-bacterial glycosyl triazoles – Identification of an N-acetylglucosamine derivative with bacteriostatic activity against Bacillus

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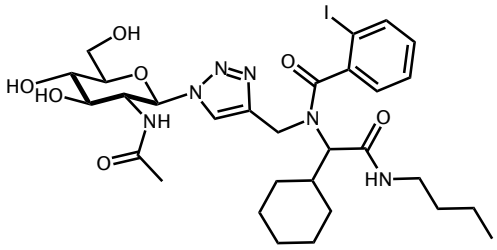
TOC Entry



bacteriostatic GlcNAcase inhibitor

Abstract

N-acetylglucosaminidases (GlcNAcases) play an important role in the remodeling and recycling of bacterial peptidoglycan. Inhibitors of bacterial GlcNAcases can serve as antibacterial agents and provide an opportunity for the development of new antibiotics. We report the synthesis of triazole derivatives of *N*-acetylglucosamine using a copper promoted azide-alkyne coupling reaction between 1-azido-*N*-acetylglucosamine and a small library of terminal alkynes prepared via the Ugi reaction. These compounds were evaluated for their ability to inhibit the growth of bacteria. Two compounds that show bacteriostatic activity against *Bacillus* were identified, with MIC values of approximately 60 μM in both cases. *Bacillus subtilis* cultured in the presence of sub-MIC amounts of the glycosyl triazole inhibitors exhibit an elongated phenotype characteristic of impaired cell division. This represents the first report of inhibitors of bacterial cell wall GlcNAcases that demonstrate inhibition of cell growth in whole cell assays.

The microbial glycome contains numerous attractive targets for antibiotic discovery.[1] Peptidoglycan (PG), the mesh-like heteropolymer that surrounds all bacterial cells (with the exception of mycoplasma), confers strength, support, and shape to bacteria, as well as providing resistance to internal turgor pressure.[2] Maintaining the integrity of PG is essential to bacterial viability, which is reflected by the number of different classes of clinically important antibiotics that target its biosynthesis. The PG sacculus is composed of the amino sugars *N*-acetylglucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc), linked *via* a β-(1,4)-glycosidic linkage to form an alternating copolymer.[3] Attached to the C-3 lactyl moiety of MurNAc is a highly variable pentapeptide composed of alternating *L*- and *D*- amino acids (Figure 1). Adjacent strands of the glycan copolymer are inter-connected through cross-links between these peptides. The biosynthesis of PG, particularly the cytoplasmic assembly and periplasmic cross-linking steps, is fairly well understood. Some of the most successful antibiotics to date, including the β-lactams and vancomycin, inhibit enzymes involved in PG biosynthesis. Conversely, remodeling of the invariant glycan backbone of PG by glycosidases and lyases is poorly understood despite the important roles of these enzymes in cell growth and division.[1,4] Many current cell wall acting antibiotics, which act on the highly variable stem peptide, are plagued by resistance issues. Because of the invariant glycan backbone, enzymes that act on it are particularly attractive antibiotic targets.

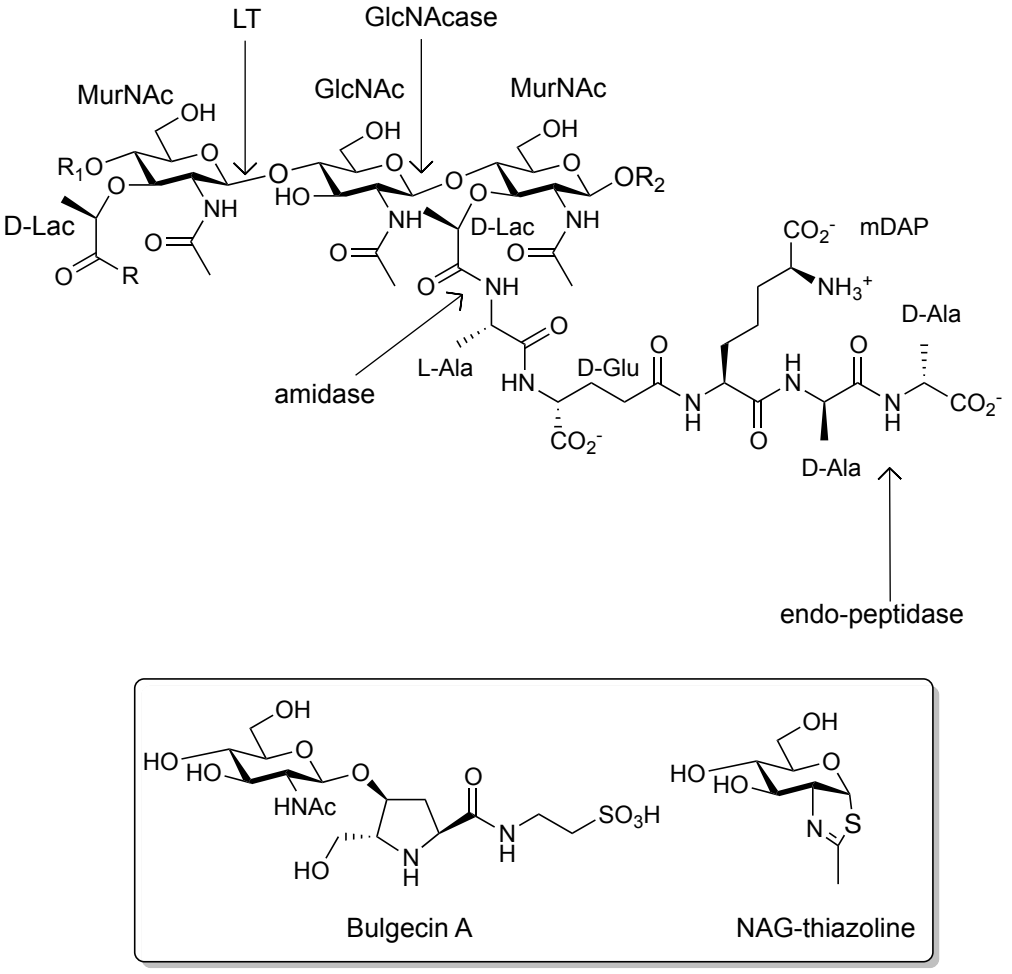


Figure 1. Repeat structure of peptidoglycan (A1-γ chemotype) and cleavage sites of major bacterial autolysins; Box - structures of Bulgecin A and NAG-thiazoline, inhibitors of cell wall glycosyl hydrolases.

Autolysins, also referred to as peptidoglycan hydrolases, are a family of enzymes that include glycosidases and peptidases and are important for cell wall remodeling (Figure 1).[2,4,5] These enzymes play particularly important roles in cell division, motility and macromolecular assembly (e.g. pili). There are two classes of autolysins that act on the glycan backbone of PG - lytic transglycosylases (LT) and *N*-acetylglucosaminidases (GlcNAcase). GlcNAcases can be further categorized as *endo*-glycosidases, such as LytD from *Bacillus subtilis*, or *exo*-glycosidases like LytG.[3,6,7] In Gram-positive bacteria,

genetic and phenotypic analysis has identified an important role for GlcNAcases in vegetative cell growth and division.[8,9] Additionally, bacteria possess a cytosolic *exo*-acting GlcNAcase (e.g. NagZ) that acts on a disaccharide glycopeptide intermediate generated during PG recycling.[10,11] Attempts to inhibit cleavage of the PG glycan backbone have focused on LTs from Gram-negative bacteria, which can be inhibited by the substrate analog NAG thiazoline and the natural product bulgecin A (Figure 1).[12-15] While these inhibitors show activity *in vitro*, they do not demonstrate a measurable minimum inhibitory concentration in whole cell assays. NAG thiazoline treatment of *E. coli* resulted in the formation of shorter cells without affecting cell viability.[15] NAG thiazoline has also been shown to inhibit a number of Gram-positive enzymes that are involved in *N*-glycan modification.[16,17] Treatment of *Pseudomonas aeruginosa* with a NagZ inhibitor attenuated β-lactam resistance, demonstrating the potential for modulating antibiotic activity by inhibiting PG recycling.[18] An iminosugar based inhibitor of NagZ has recently been reported.[19] To date, no inhibitors targeting GlcNAcases that act on the cell wall have demonstrated antimicrobial activity in susceptibility tests. We have previously reported the synthesis of galactosyl and glucosyl triazoles as inhibitors for galactosidases and glucosidases.[20] Numerous subsequent studies have demonstrated the generality of the glycosyl triazole pharmacophore for glycosidase inhibition.[21-26] Based on this precedent, we sought to examine whether glycosyl triazole derivatives of GlcNAc would inhibit bacterial GlcNAcase activity. GlcNAc triazoles (GNTs) have previously been shown to inhibit O-GlcNAcase and have been examined as inhibitors for human hexosaminidase, but to the best of our knowledge have not been examined for anti-bacterial effects.[27,28]

Results

We prepared a 21-member GNT library by coupling 1-azido-*N*-acetylglucosamine (**1**) to a series of terminal alkynes that were prepared using a multicomponent Ugi reaction (Figure 2). Each Ugi reaction was carried out using either propargyl amine or propiolic acid as the alkyne component to provide either propionamides or *N*-propargyl amides as the diamide products. While Ugi-reactions can sometimes require prolonged reaction times, we identified conditions that reduced the reaction time to 4-5 hours at elevated temperature and in many cases proceeded to completion, enabling use of the Ugi product directly in the next reaction.[29-31] The Ugi-derived terminal alkynes were then subjected to a copper accelerated azide-alkyne coupling reaction using a redox couple of copper powder and copper (II) sulfate. To further facilitate synthesis of the library we simplified the purification of the compounds by using the alkyne-building blocks in excess for the copper accelerated azide-alkyne coupling reaction. This ensured complete consumption of the azido sugar **1**, and the unreacted alkyne residue could be easily removed using a silica plug after the reaction, eliminating the need for column chromatography. Removal of trace copper salts from the reaction was carried out by incubating the reaction solution with a commercially available copper chelating resin prior to purification using the silica plug.

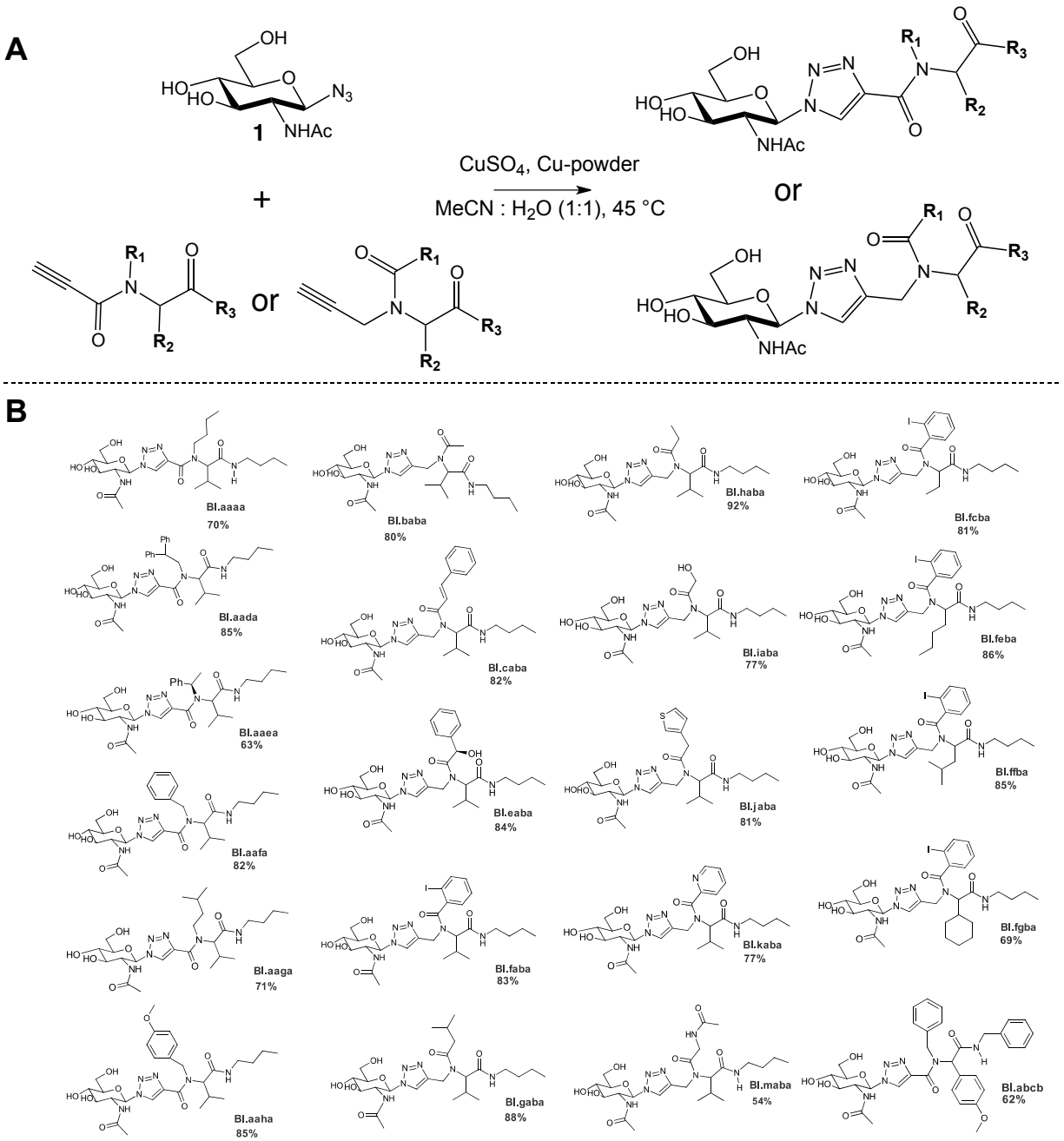
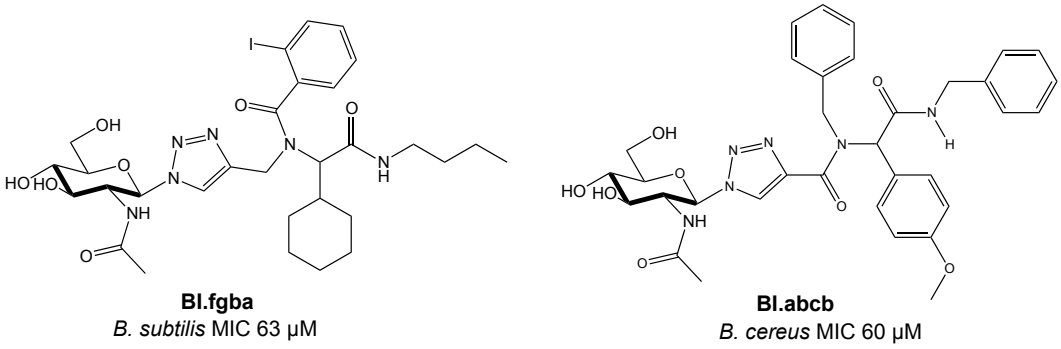


Figure 2. A) Synthetic route for preparation of of GNTs using copper accelerated azide-alkyne coupling; B) Structures of GNTs synthesized and evaluated for antibacterial activity. Yields provided correspond those for the azide-alkyne coupling after purification using a silica plug.

The library of GNTs was screened for its ability to inhibit the growth of a panel of different Gram-positive bacteria. Glycosyl triazole compounds were screened in a whole

cell assay against a number of Gram positive organisms using a resazurin microtiter assay.[32] The library was initially screened against all test organisms at a single concentration (250 μM) (Supplementary data). This high initial concentration was chosen as many soluble derivatives of PG bind to bacterial autolysins with K_D values in the high micromolar range.[33] Compounds showing at least 40% inhibition in growth were selected for further investigation. Follow up studies with 6 compounds that inhibited growth by more than 40% indicated that not all of these compounds reduced bacterial growth in a concentration dependent manner. We identified two compounds that inhibited the growth of *Bacillus cereus* and *Bacillus subtilis* in the micromolar range (Figure 3). GNT ***B1.abcb*** inhibited *B. cereus* with an MIC value of 39 μg/mL (60μM) , and ***B1.fgba*** inhibited *B. subtilis* with an MIC value of 45 μg/mL (63 μM). Both ***B1.abcb*** and ***B1.fgba*** were screened in a standard bactericidal assay.[34] Briefly, *B. subtilis* and *B. cereus* were grown in either the absence or presence of inhibitor (at the MIC) for 4 hours. The cells were harvested, washed and subject to serial dilution prior to plating on nutrient broth agar. Colony counts for growth in the presence of ***Bl.fgba*** or ***Bl.abcb*** ($8.50 \pm 3.00 \times 10^7$ cfu/mL and $7.00 \pm 1.70 \times 10^8$ cfu/mL respectively) were similar to controls in the absence of the compounds ($9.40 \pm 2.88 \times 10^7$ cfu/mL for *B. subtilis*, and $2.67 \pm 1.53 \times 10^8$ cfu/mL for *B. cereus*) indicating that these compounds are bacteriostatic in nature. In order to assess the role of the glycone and aglycone components in inhibition, the galactose derivatives of ***Bl.fgba*** and ***Bl.abcb*** were synthesized and tested, and exhibited no antimicrobial activity.



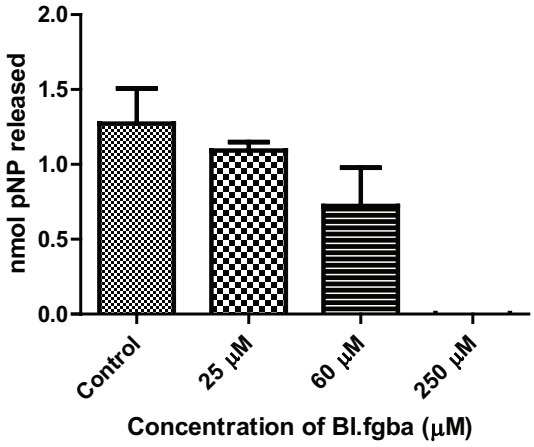
Bl.fgba
B. subtilis MIC 63 µM

Bl.abcb
B. cereus MIC 60 µM

Figure 3. GNTs with antibacterial activity against *Bacillus*

Incubation of *B. subtilis* cells with the synthetic substrate β-*p*-nitrophenyl GlcNAc (*p*NP-GlcNAc) in the presence of ***B1.fgba*** resulted in a concentration dependent inhibition of nitrophenol release (Figure 4A), with complete inhibition observed at 250 µM inhibitor. This result confirms that the likely bacterial target is indeed a GlcNAcase. The differences in potency between the in vitro assay and the anti-bacterial assay may reflect poor access to the target in the whole cell assay. Additionally, it should be noted that *p*NP-GlcNAc is not the natural substrate for a bacterial GlcNAcase, and lacks many protein-substrate contacts such as the stem peptide as well as an extended glycan chain. Thus, the ability of ***Bl.fgba*** to inhibit *p*NP-GlcNAc hydrolysis does not necessarily reflect its ability to inhibit peptidoglycan hydrolysis. Treatment of *B. subtilis* with ***B1.fgba*** at a concentration below its MIC (0.8 x MIC), resulted in a phenotype with highly elongated cells and the appearance of chains of cells (Figure 4B). This is suggestive of a disruption in the cell division/septation machinery, processes that are known to require GlcNAcase activity. This phenotype is reminiscent of that observed in *Lactococcus lactis* that lacks AcmA or AcmD, which are orthologs of LytG in *B. subtilis* and involved in cleavage of the septum during cell division.[35,36]

A)



B)

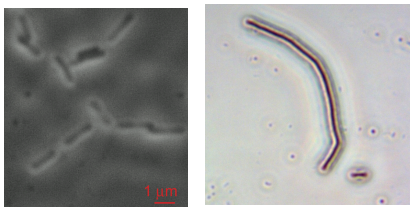


Figure 4. (A) Dose dependent inhibition of *p*NP-GlcNAc hydrolysis by intact *B. subtilis* cells in the presence of ***Bl.fgba*** confirming that the target is a GlcNAcase. (B) Morphological changes to *B. subtilis* upon exposure to ***Bl.fgba***. Control cells (left) were grown in the presence of 1 % DMSO and show the typical rod shaped cells. Cells on the right were treated with 0.8 x MIC (48 μM) ***Bl.fgba*** and exhibit an elongated phenotype.

In summary we have identified two inhibitors of bacterial GlcNAcases based on a glycosyl triazole scaffold. The bacteriostatic activity of both of these GNTs, in conjunction with biochemical evidence for inhibition for *p*NP-GlcNAc hydrolysis and the impaired cell division phenotype of cells treated with ***Bl.fgba***, is strongly suggestive of disrupted autolysin activity. There have been prior reports of compounds that inhibit purified bacterial GlcNAcases in vitro, as well as compounds that are known GlcNAcase inhibitors that exhibit antibacterial activity indirectly by sensitizing the bacteria to β-lactam antibiotics. However, to the best of our knowledge, this is the first report of

compounds that exhibit inhibition of GlcNAcase activity that also directly reduce bacterial growth. While the MIC values exhibited by ***Bl.fgba*** and ***Bl.abcb*** are modest, our results demonstrate an important proof of concept and validate the glycosyl triazole scaffold as a viable one for further optimization and development. Our current efforts are directed at identifying the molecular target(s) of these compounds and improving the potency of the GNTs.

Acknowledgements

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