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A mechanism-based GlcNAc-inspired cyclophellitol inactivator of the peptidoglycan recycling enzyme NagZ reverses resistance to β-lactams in *Pseudomonas aeruginosa*

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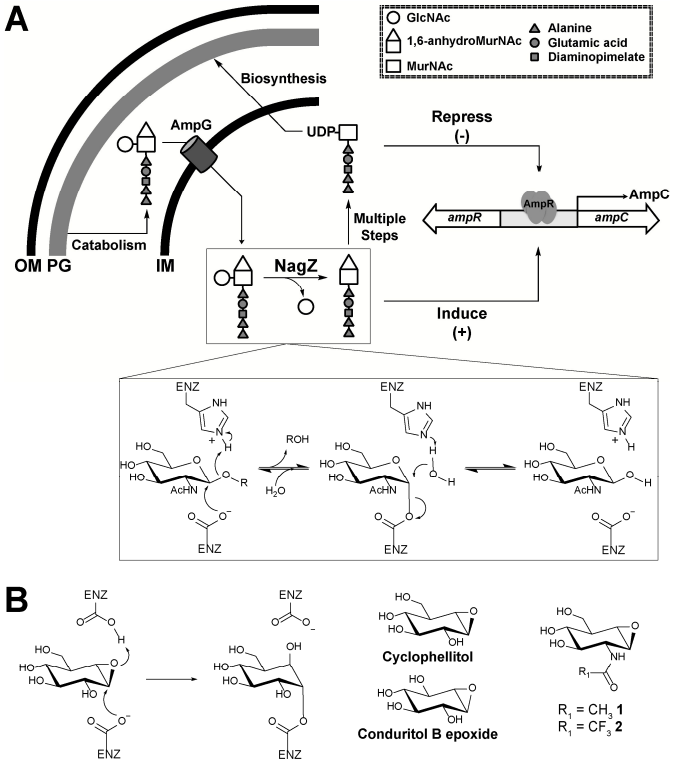
The development of a potent mechanism-based inactivator of NagZ, an enzyme critical to the production of inducible AmpC β-lactamase in Gram-negative bacteria, is presented. This inactivator significantly reduces MIC values for important β-lactams against a clinically relevant strain of *Pseudomonas aeruginosa*.

Medical organisations have highlighted the urgent need for new antibiotic strategies and have proposed a list of priority antimicrobial resistant pathogens, including several Gram-negative bacteria.[1,2] One particularly worrisome area is the increasing prevalence of resistance to β-lactam antibiotics, which are heavily relied upon to treat Gram-negative bacterial infections.[3] β-Lactamases, which are enzymes that deactivate β-lactams by cleaving the cyclic amide moiety, are the most problematic.[4] A class of these enzymes that pose a significant clinical problem are the inducible chromosomal AmpC β-lactamases,[5] which have a broad spectrum activity against β-lactams.[6] AmpC enzymes are generally resistant to classical β-lactam-based β-lactamase inhibitors such as clavulanic acid;[5] however, non-β-lactam based inhibitors effective against AmpC are now reaching the market.[7]

In contrast to the classical approach of directly blocking β-lactamase activity, one can also aim to block *ampC* gene expression. Expression of inducible AmpC depends on peptidoglycan (PG) metabolism and recycling.[8] During normal growth a large amount of PG is degraded and recycled (Scheme 1A).[8,9] PG degradation fragments, GlcNAc-1,6-anhydroMurNAc-peptides, are transported into the cytoplasm by AmpG, and further degraded to produce a series of 1,6-anhydroMurNAc-tri-, tetra- and pentapeptides (Scheme 1A).[10] One or more of these PG metabolites induces *ampC* by binding to its transcriptional regulator, AmpR.[11,12] Another molecule, that acts as a repressor of AmpR is UDP-MurNAc-pentapeptide; a PG building block that is formed by further metabolism of the 1,6-anhydroMurNAc peptides.[11,13,14] Overall, the regulation of AmpC expression, and the ability for bacteria to sense β-lactams is controlled by the relative concentrations of these PG ligands that modulate AmpR activity.[15,16]



Scheme 1. (A) The peptidoglycan recycling pathway shows the important biological activity of NagZ in the production of 1,6-anhydroMurNAc peptides (pentapeptide shown) to induce AmpC expression. Inset: The catalytic mechanism of NagZ (B) The inhibition by Cyclophellitol of retaining β-glucosidases that use two catalytic carboxylic acid residues. A GlcNAc-based cyclophellitol would allow for the formation of a covalent adduct with the enzymic nucleophile of NagZ, leading to its irreversible inhibition. Structures of the known epoxide-based inactivators Cyclophellitol and Conduritol B epoxide and the compounds of interest in this study.

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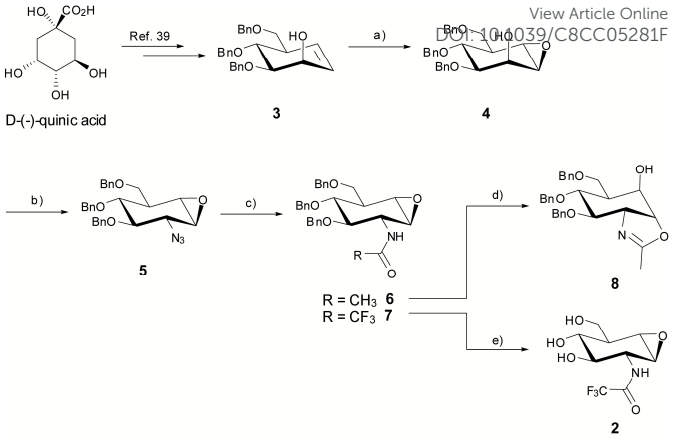
A key enzyme in PG recycling is the β-*N*-acetylglucosaminidase, NagZ, which cleaves the non-reducing GlcNAc residue from GlcNAc-1,6-anhydroMurNAc-peptides to produce the 1,6-anhydroMurNAc-peptides that activate AmpR (Scheme 1A).[17,18] Genetic studies have demonstrated that this enzyme is important for induction of AmpC β-lactamase.[19-21] Strategies to inhibit this enzyme using small molecules are therefore of interest since they could block formation of the AmpR inducer molecules. This approach to potentiate co-administered β-lactams has received some *in vitro* validation,[19,22-26] and structural insight into inhibition of NagZ has enabled the design of improved NagZ inhibitors.[27,28] Yet, none of the inhibitors have been able to recapitulate the effects seen when deleting the NagZ gene.[19-21]

NagZ is a member of glycoside hydrolase family 3 (GH3) and uses a two-step, double-displacement mechanism. Two residues play key catalytic roles. A histidine residue acts as a general catalytic acid/base[29,30] and an aspartate acts as a catalytic nucleophile to enable the formation and breakdown of a covalent glycosyl-enzyme intermediate (Scheme 1A).[10,30-33]† Generation of NagZ inhibitors has centred on mimicking the oxocarbenium ion-like transition states that are thought to flank the covalent glycosyl-enzyme intermediate to yield competitive inhibitors of the enzyme (ESI, Fig. S1).

One approach to NagZ inhibitors that has not been pursued, is to exploit the nucleophilicity of the catalytic nucleophile that acts to form the glycosyl-enzyme intermediate. Such mechanism-based inhibitors form covalent adducts with the catalytic nucleophile of the enzyme. One notable set of these mechanism-based inhibitors are the epoxide-containing cyclitols, of which Cyclophellitol[34], a natural product, and Conduritol B epoxide[35] are the best known (Scheme 1B).[36,37] These compounds and their derivatives have received attention for their ability to inactivate a wide range of glycoside hydrolases and have demonstrated utility.[38] We accordingly felt that the GlcNAc-based cyclophellitols **1** and **2**, would be excellent candidates for mechanism-based inactivation of NagZ. We noted studies of competitive NagZ inhibitors have shown that replacement of the acetamido group with a trifluoroacetamido moiety increases potency of inhibitors toward NagZ.[26]

Here, we present a significantly improved strategy for inhibiting NagZ-type enzymes as compared to previous reversible NagZ inhibitors that have had only limited success at reversing antibiotic resistance mediated by inducible AmpC. These epoxides in combination with β-lactams, increases the susceptibility of a clinically relevant Gram-negative bacterium possessing inducible AmpC to β- lactams, far beyond the effects seen previously with competitive inhibitors and provides the first clear validation that NagZ can be targeted with small molecules to yield effects matching those seen when deleting the NagZ gene.

Starting from D-(-)-quinic acid using established literature procedures[39] we obtained the allylic alcohol **3** in good overall yield (Scheme 2). At this point, we decided to first install the epoxide and later perform the required epimerization to give the correct stereochemistry at C2. Using the epoxidation



Scheme 2. a) MCPBA, CH_2Cl_2; b) PPh_3, DPPA, DEAD, toluene; c) i. PMe_3, THF:H_2O (20:1); ii. $(RC(O))_2O$, C_5H_5N, CH_2Cl_2; d) MeOH; e) $Pd(OH)_2/C$, H_2, MeOH.

strategy of Shing[39] allowed for the hydroxy-directed MCPBA epoxidation of **3** which gave us exclusively the epoxide **4** in good yield. This material was then exposed to Mitsunobu conditions which afforded us the azide **5**. We then treated the azide **5** to Staudinger conditions and trapped the intermediate amine with either acetic anhydride to give the acetamide **6** or trifluoroacetic anhydride to give **7**. With these compounds in hand all that was required was removal of the protecting groups to give the desired epoxides **1** and **2** respectively. Unfortunately, dissolution of the acetamide **6** in methanol resulted in its spontaneous conversion to the oxazoline **8** in almost quantitative yield. We found this also occurred in other protic solvents (ethanol, isopropanol) as well as aprotic solvents including tetrahydrofuran and acetonitrile. These results were disappointing, but we thought that the trifluoroacetamide **7** would be far more resistant to this conversion due to the reduced nucleophilicity of the carbonyl group of the amide. Gratifyingly, the trifluoroacetamide **7** was stable in methanol and thus, after palladium-catalysed hydrogenolysis, we obtained the desired polyol **2**.

We then tested the ability of compound **2** to inactivate NagZ enzymes using *Bc*NagZ, a model NagZ from *Burkholderia cenocepacia* that has been used in other NagZ inhibitor studies.[26,28] Incubation of *Bc*NagZ with **2** results in rapid time-dependent inactivation of the enzyme (Fig. 1A). We determined the kinetic parameters governing inactivation (k_i and K_i) by plotting k_{obs} versus [I] (Fig. 1B). In all cases the inactivation data fitted well to a single exponential decay equation. Compound **2** shows a K_i value of 8.0 ± 1.0 μM and a k_i value of 0.17 ± 0.01 min^{-1}. We also confirmed that the inactivation of *Bc*NagZ by **2** was irreversible (ESI, Fig S2).

One important caveat in the development of NagZ inhibitors for eventual clinical use is the potential concomitant inhibition of functionally related human enzymes. These enzymes include the GH84 β-glucosaminidase human *O*-GlcNAcase,[40] the GH20 human β-hexosaminidases[41] and GH89 α-*N*-acetylglucosaminidase NAGLU.[42] Early competitive inhibitors suffered from a lack of selectivity and thus derivatives were required to obtain selectivity for NagZ (ESI, Fig. S1). An important feature of these enzymes is that they

use different catalytic mechanisms as compared to NagZ-like enzymes. The GH20[32] and GH84[43] enzymes use substrate-assisted catalysis involving the 2-acetamido group as a nucleophile and the GH89 enzymes act on α-GlcNAc linked moieties[44]. None of these enzymes form a NagZ-like covalent glycosyl-enzyme intermediate and therefore they should not be inactivated using the approach described here. Moreover, inspection of the active sites of these enzymes suggest that they are less likely to accommodate the *N*-trifluoroacetamido group of compound **2**.[27] To confirm that epoxide **2** did not act as a time-dependent inhibitor of these enzymes we incubated them with a large inhibitor concentration for extensive time periods. No significant time dependent inactivation was observed for all enzymes tested (ESI, Fig. S3) suggesting that the compound likely acts as a poor reversible inhibitor of these enzymes. Moreover, analysis of the data shows that epoxide **2** inactivates NagZ on a useful timescale Given the high conservation of GH3 NagZ among Gram-negative bacteria,[28] this approach could potentiate β-lactams against numerous pathogens carrying an NagZ-mediated inducible *ampC* gene.

To gain insight into the molecular basis for inhibition of NagZ by **2**, we determined the X-ray structure of *Bc*NagZ in complex with **2** by molecular replacement to 1.9 Å resolution (ESI, Table S1, PDB 6DTE). Electron density for **2** was observed within the enzyme active site, where a covalent bond between C1 of **2** and the enzymic nucleophile of the *Bc*NagZ (Asp253) is clearly observed, confirming the basis for the mechanism-based inactivation of NagZ enzymes by **2** (Fig. 2A). The adduct causes the hexose ring of **2** to sit lower in the active site, closer to Asp 253 as compared to the product of the normal reaction, GlcNAc (PDB 4GNV, Fig. 2B). Aside from this difference, **2** and GlcNAc bind similarly. Further, the catalytic loop containing the general acid/base His183, which is typically disordered in the absence of substrate or product[28,30] adopts a catalytically competent conformation in the presence of **2**. In summary, NagZ binds **2** very similarly, in overall geometry, to GlcNAc.

We next set out to test if epoxide **2** could increase the susceptibility of bacteria harbouring inducible AmpC β-lactamase to β-lactams. *Pseudomonas aeruginosa* is a nosocomial Gram-negative pathogen that harbours an inducible *ampC*.[45] Previous work has shown that genetic inactivation of *nagZ* in clinically problematic AmpC-derepressed mutants of *P. aeruginosa* markedly increases susceptibility to β-lactams supporting the validity of this target.[19,46]

To determine if **2** potentiates β-lactams, clinically important β-lactams, ceftazidime, aztreonam and imipenem, were assayed against the most clinically relevant AmpC derepressed mutant of *P. aeruginosa* (Δ*dacB*)[46] (Table 1). Minimal inhibitory concentration (MIC) assays demonstrated that treating cultures with **2** (100 μM) restored the efficacy of ceftazidime and aztreonam, reducing MICs to below their resistance breakpoints.[47] Susceptibility to imipenem also increased slightly; however, imipenem is already effective against this strain since it is a poor AmpC substrate. As expected, **2** does not exhibit any inherent antibacterial or bacterostatic properties on its own, even at concentrations

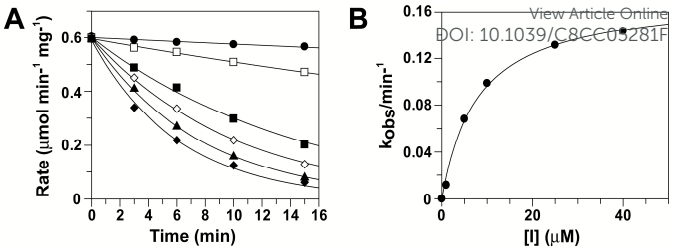


Figure 1. Time-dependent inactivation of *Bc*NagZ by **2**: A) Experimental data represent *Bc*NagZ activity of control samples (without **2**) over time. [**2**] used with *Bc*NagZ: 0 (●), 1 (□), 5 (■), 10 (◊), 25 (▲), 40 (♦) μM. B) Curves are the nonlinear fits of data to a single exponential decay equation; B) Plot of the inactivation rate constants (k_{obs}) as a function of the concentration of **2** for *Bc*NagZ.

as high as 1 mM. Notably, MICs obtained in the presence of **2** are similar to MICs obtained for the Δ*nagZ* knockout of this strain,[20,46] which is a marked improvement over competitive NagZ inhibitors that have been evaluated as β-lactam potentiators. We speculate that the improved activity of epoxide **2** represents stems from it being an irreversible inactivator of NagZ, which makes it reasonable that the effects seen here reflect genetic inactivation of NagZ. Moreover, it may also be that the structural similarity of **2** to GlcNAc, which can be scavenged by many bacteria, may enable its active transport into the bacterial cytosol. Accordingly, we believe that **2** and potential analogues, or other covalent inhibitors, represent an exciting new strategy to advance this adjunct antimicrobial approach.

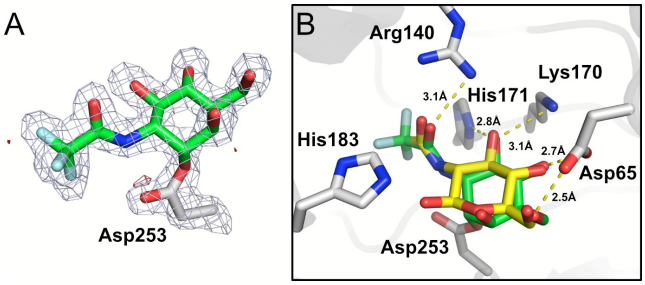


Figure 2. Crystal structure of *Bc*NagZ bound to **2**. A) Electron density (sigma-A weighted difference map (mF_o-DF_c) contoured at 4σ) for **2** (green carbon atoms) covalently bound to Asp253 in the *Bc*NagZ active site. B) *Bc*NagZ active site bound to **2** (green carbon atoms) with the *Bc*NagZ product complex PDB 4GNV superposed. The GlcNAc product is shown with yellow carbon atoms.

In summary, there is a pressing need for new strategies to overcome inducible chromosomal AmpC β-lactamase resistance to β-lactams. Here we describe a new class of NagZ inhibitor that provides chemical potentiation of β-lactams against clinically problematic *P. aeruginosa* that is equivalent to genetic inactivation of the enzyme. This strategy accordingly opens a new chemical approach to suppressing β-lactam resistance in clinical pathogens that carry NagZ-mediated inducible AmpC β-lactamase.

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Table 1. Susceptibility of *P. aeruginosa* Δ*dacb* to various β-lactams. Values were determined by the serial dilution according to CLSI guidelines. Measurements were performed in triplicate.

	MIC [μg ml^{-1}]		
Inhibitor	Ceftazidime	Aztreonam	Imipenem
-**2**	32	16	2
+**2**	4	4	1

Conflicts of Interest

There are no conflicts to declare.

Notes and references

†NagZ-like enzymes have in some cases been reported to proceed by phosphorolysis rather than hydrolysis.[48]

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