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Selective trihydroxyazepane NagZ inhibitors increase sensitivity of *Pseudomonas aeruginosa* to β-lactams†

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AmpC β-lactamase confers resistance to β-lactam antibiotics in many Gram negative bacteria. Inducible expression of AmpC requires an *N*-acetylglucosaminidase termed NagZ. Here we describe the synthesis and characterization of hydroxyazepane inhibitors of NagZ. We find that these inhibitors enhance the susceptibility of clinically relevant *Pseudomonas aeruginosa* to β-lactams.

Resistance to frontline β-lactams in Gram negative bacteria is a growing clinical problem.[1] Given the prevalence of bacterial pathogens that harbour either inducible chromosomal AmpC or inducible plasmid-borne AmpC,[2–5] new strategies to suppress this resistance mechanism are needed.

Inducible AmpC expression is regulated by peptidoglycan metabolites produced within the bacterium.[6] The presence of β-lactams leads to increased cytosolic levels of the metabolite GlcNAc-1,6-anhydroMurNAcpeptide, which is cleaved by a conserved enzyme of the Gram-negative peptidoglycan cell wall recycling pathway termed NagZ (Fig. 1).[7] NagZ removes the non-reducing GlcNAc residue to liberate 1,6-anhydroMurNAc peptides, which induce transcription of AmpC β-lactamase to confer resistance to the β-lactam antibiotic.[7] β-Lactam therapy also frequently selects for mutations that cause hyperproduction of AmpC. One approach that is being explored to reverse resistance to β-lactams is to generate inhibitors of NagZ that can act to hinder the production of AmpC by disrupting its induction pathway.[8–10] One challenge facing this approach is that NagZ inhibitors can also inhibit functionally related human enzymes.

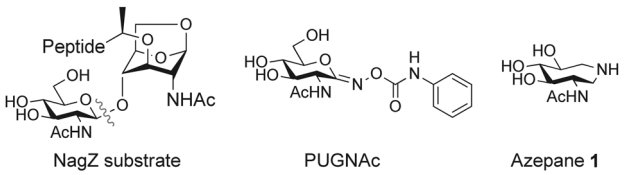

Fig. 1 NagZ substrate, product (GlcNAc), PUGNAc, and azepane **1**. The stem peptide is not shown for reasons of clarity.

NagZ enzymes belong to the GH3 family of the glycoside hydrolases.[11] Detailed kinetic[12,13] and X-ray structural studies[8,14,15] of NagZ enzymes have revealed that they use a two step mechanism involving the formation and breakdown of a covalent glycosyl-enzyme intermediate. The 2-acetamido group of the GlcNAc residue of the substrate plays no role in catalysis, which is in contrast to human β-acetylglucosaminidases from GH20[13,16] and GH84,[17,18] which use a catalytic mechanism in which the 2-acetamido group of the substrate acts as a nucleophile to yield a bicyclic oxazoline intermediate. β-Hexosaminidase A (HexA) and β-hexosaminidase B (HexB) are GH20 enzymes and their dysfunction gives rise to heritable lysosomal storage diseases. The GH84 enzyme known as O-GlcNAcase (OGA) removes O-linked β-*N*-acetylglucosamine residues (O-GlcNAc) from serine and threonine residues of nucleocytoplasmic proteins.[19,20] Examination of the active sites of the GH3, GH20, and GH84 enzymes reveals that the active site topology interacting with the 2-acetamido group of the substrate differs.[21] For GH20 and GH84 enzymes, this pocket envelops the acetamido group, whereas for the NagZ enzymes this substituent rests against the surface of the enzyme and is solvent exposed. Exploiting this discrepancy by incorporating *N*-acyl substituents of various volumes therefore offers a tractable route to generating selective inhibitors of NagZ.

Several different glycosidase inhibitors that can inhibit diverse *exo*-*N*-acetyl-β-glucosaminidases, including various enzymes from GH3, GH20, and GH84, have been reported. Among the most potent of these inhibitors are PUGNAc,[22] the iminosugar-based AcDNJ,[8,10] and the glycosylimidazoles.[23] An additional class that has gained attention over the last two decades is the family of polyhydroxylated azepanes.[24–28]

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Table 1 Inhibition of *Salmonella typhimurium* NagZ, OGA, and HexA by azepanes **1** and **9–16**

Compound	**1**	**9**	**10**	**11**	**12**	**15**	**16**
OGA K_i (μM)	0.7	2.2	47	190	NI	22	2.0
NagZ K_i (μM)	0.4	0.4	7.4	27	NI	NI	NI
HexA K_i (μM)	*3.6*	NI	NI	NI	NI	*51*	*14*
(K_i OGA/K_i NagZ)	1.8	5.5	6.7	7.1	NA	NA	NA
(K_i HexA/K_i NagZ)	2.1	+	+	+	NA	NA	NA

NI: no significant inhibition at 500 μM, NA: not applicable, italic numbers K_i by Dixon analysis, +: estimated to be at least >1000. For errors on these measurements see Table S2 (ESI).

When protonated, the basic nitrogen of azepanes is thought to confer resemblance to the oxocarbenium ion-like transition state proposed for NagZ and other glycoside hydrolases. The azepanes also display increased conformational flexibility compared to the six-membered iminosugar inhibitors.[29] This increased plasticity enables these molecules to be accommodated within the active sites of glycoside hydrolases, as illustrated by the (3*S*,4*R*,5*R*,6*S*)-3-acetamido-4,5,6-trihydroxyazepane **1**, that mimics GlcNAc and inhibits eukaryotic GH20 and GH84 enzymes with K_i values ranging from 0.4 μM to 11 μM.[26] The versatility of the azepane scaffold prompted us to explore the potential of various (3*S*,4*R*,5*R*,6*S*)-3-*N*-acyl-4,5,6-trihydroxyazepanes as inhibitors of NagZ.

We find that azepane **1** is a good inhibitor of NagZ but shows poor selectivity (Table 1). To gain insight into the molecular basis of NagZ inhibition by azepane **1** we determined the X-ray crystal structure of this molecule bound to NagZ from the opportunistic human pathogen *Burkholderia cenocepacia* (*Bc*NagZ). The complex was solved by molecular replacement using a model of *Bc*NagZ (PDB 4GNV) from which ligands and solvents were removed. Like other NagZ enzymes *Bc*NagZ is a single-domain enzyme containing a $(\beta/\alpha)_8$ barrel fold (TIM barrel) (Fig. S1, ESI†). The structure of this enzyme in complex with **1** reveals several hydrogen-bonding interactions with the enzyme that contribute to the potency of this molecule as an inhibitor of GH3 NagZ enzymes (Fig. 2A).

In particular, the catalytic nucleophile of *Bc*NagZ, Asp253, forms a 2.7 Å hydrogen-bond with the endocyclic nitrogen (N1) of **1**—an interaction that cannot occur between the nucleophile and GlcNAc, (Fig. 2B) suggesting this interaction of **1** underlies its potency against NagZ. The structures also reveal significant space

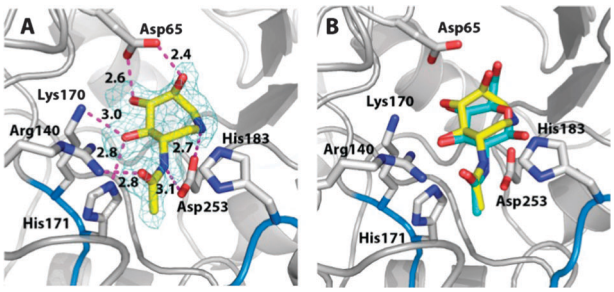
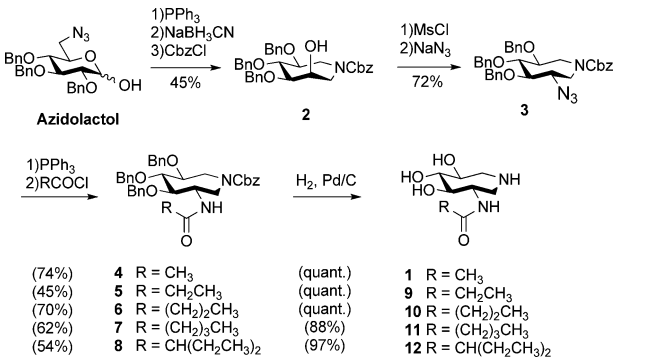


Fig. 2 Crystal structure of *Bc*NagZ bound to azepane **1**. (A) Active site residues (grey) and **1** (yellow) are drawn as sticks. The NagZ consensus sequence (blue) has the putative histidine general acid–base in sticks. Hydrogen bonding interactions are represented by dashed magenta lines. Electron density is an F_o–F_c map (3.0 rmsd). (B) Superimposition of the *Bc*NagZ–**1** complex with that of *Bc*NagZ bound to GlcNAc (cyan). See Table S1 (ESI†) for crystal and refinement statistics.



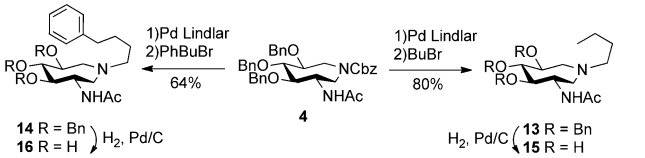
Scheme 1 Synthesis of 3-*N*-acyl azepanes **1** and **9–12**.

surrounding the endocyclic nitrogen as well as the acetyl group. Based on these structures, and the structures of GH84 OGA homologues as well as GH20 human hexosaminidase, we envisioned that selectivity toward NagZ could be obtained by exploring structural variations at the acetamido group and through alkylation of the endocyclic nitrogen. We therefore generated a panel of 3-*N*-acyl derivatives of (3*S*,4*R*,5*R*,6*S*)-3-acetamido-4,5,6-trihydroxyazepane **1** and evaluated these against NagZ, OGA, and HexA.

We modified the route to **1**[30a] by using PPh_3 on a solid support. It enabled smooth conversion of the starting azidolactol into the corresponding N,O acetal. Its reduction with $NaBH_3CN$ followed by protection of the endocyclic nitrogen as its benzyl carbamate afforded 3-hydroxyazepane **2** in 45% yield. Activation of the free OH as its mesylate followed by azide displacement furnished azidoazepane **3** in 75% yield. Reduction with PPh_3 followed by acylation of the resulting crude amine with acyl anhydrides or acyl chlorides furnished the corresponding 3-*N*-acyl azepanes **4–8** in 45–74% yield. Hydrogenolysis yielded target azepanes **1** and **9–12** (Scheme 1).

The azepane inhibitor scaffold was also elaborated by endocyclic N-alkylation. Such modified polyhydroxylated piperidines can be selective and potent inhibitors of various glycosidases, including family GH20 β-glucosaminidases.[30b] We therefore examined such modifications on the selectivity and potency of azepanes for these enzymes. Such N-alkylation of azasugars mimics the aglycon moiety of substrates of these enzymes.[31] Here we investigated the commonly seen *n*-butyl group and a phenylbutyl group that we intended to mimic the aglycon phenylcarbamate substituent of PUGNAc. Azepane **4** was hydrogenolyzed under mild conditions to selectively remove the Cbz group and N-alkylated using butyl bromide or phenylbutyl bromide in the presence of K_2CO_3 to yield azepanes **13** (80% yield) and **14** (64% yield) respectively. Hydrogenolysis under acidic conditions furnished the corresponding trihydroxyazepanes **15** and **16** (Scheme 2).

With this set of compounds in hand we evaluated their inhibition of NagZ, OGA, and HexA. We find that increasing the



Scheme 2 Synthesis of *N*-alkyl azepanes **15** and **16**.

size of the *N*-acyl group in the series including **1**, **9–11** has a modest effect with regard to inhibition of NagZ and OGA in that the K_i values increase with the bulk of this *N*-acyl group by approximately 30-fold for NagZ and 200-fold for OGA. Interestingly, branching of the aliphatic group, as in compound **12**, leads to a loss of inhibition against all these enzymes. As previously observed for other inhibitors, increasing the bulk of the *N*-acyl group beyond acetyl leads to a dramatic loss of potency against HexA as seen for compound **9**, which differs in structure from **1** by only one methylene unit.

Because N-alkylation of iminosugars has often resulted in significantly enhanced binding to glycoside hydrolases, including to OGA, we speculated that N-alkylated azepanes **15** and **16** would be more potent than **1** against these enzymes. Surprisingly, however, we find that both these compounds abolished inhibition against NagZ and impaired binding to OGA by a modest 2–30 fold and to HexA by 4–15 fold.

Since some of these azepanes are good inhibitors of NagZ, in particular **10**, which retained fair potency and now showed some (7-fold) selectivity for NagZ over both OGA and Hex, we decided to explore their potential to block NagZ in bacteria and attenuate inducible AmpC-mediated β-lactam resistance within a relevant bacterial model representative of clinically occurring *P. aeruginosa*. Spontaneous inactivation of *dacB*, which encodes the non-essential penicillin-binding protein 4 (PBP4), is the most common cause of AmpC hyper-production and high-level β-lactam antibiotic resistance in *P. aeruginosa*.[32] We therefore tested whether **1** or **10** could potentiate the efficacy of the β-lactam ceftazidime against a *dacB* null mutant of *P. aeruginosa* (Table S3, ESI†). Neither **1** nor **10** had any effect on bacterial growth on their own. However, use of 1 mM **1** halved the minimum inhibitory concentration (MIC) of *P. aeruginosa* Δ*dacB* from 24 μg ml^{-1} ceftazidime in the absence of the inhibitor to 12 μg ml^{-1}. Azepane **10** led to a greater 3-fold reduction in MIC to 8 μg ml^{-1}, despite this derivative being 18-fold less potent *in vitro* relative to azepane **1**, suggesting that its uptake or diffusion into the cells must occur more easily. Notably, this value meets the susceptibility breakpoint of the Clinical and Laboratory Standards Institute (CLSI) for ceftazidime.

The synthetic accessibility of polyhydroxy azepanes, their stability and their ability to suppress β-lactam resistance in the clinically relevant *P. aeruginosa dacB* null mutant suggest that these compounds could prove useful. The availability of a NagZ–azepane complex will facilitate the design of improved NagZ azepane inhibitors. Finally, the recent observation that inactivation of NagZ also blocks the emergence of mutations giving rise to β-lactam resistance[33] makes NagZ an enzyme of high interest; its inhibition should not only block antibiotic resistance but could also hinder development of mutations leading to β-lactam resistance.

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