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Molecular Basis for Inhibition of GH84 Glycoside Hydrolases by Substituted Azepanes: Conformational Flexibility Enables Probing of Substrate Distortion

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Glycosidase inhibitors are enjoying much interest, as they are finding applications in the treatment of diabetes and influenza and as chemical chaperones in lysosomal storage disorders.[1] Iminoalditols rank among the most powerful glycosidase inhibitors and have accordingly been the topic of extensive study.[2,3] Two major classes of six-membered-ring iminoalditols are the nojirimycin class,[4] in which the endocyclic ring oxygen of a pyranose is replaced by nitrogen, and the noeuromycin class,[5] in which the anomeric center is replaced by nitrogen and the endocyclic oxygen by a methylene unit. The reliable potency of these inhibitors toward glycoside hydrolases derives from beneficial charge−charge interactions with conserved enzymatic residues found at the active center. Their potency is often attributed to mimicry of electrostatic features of the oxocarbenium ion-like transition states proposed for most glycosidases.[6,7] Five-membered-ring iminoalditols have also been found to be potent inhibitors of glycoside furanosidases[8] and nucleoside hydrolases.[9] Interestingly, such five-membered-ring iminoalditols have also been found to potently inhibit a wide range of glycoside pyranosidases.[2,10−14]

More recently, unnatural seven-membered-ring iminoalditols (perhaps more appropriately referred to as azepanes) have been found to inhibit glycosidases.[15] This observation has invigorated efforts directed toward their efficient synthesis and probing of their biological activities.[15−20] Although recent efforts have highlighted the basis for the binding of five-membered-ring iminoalditols to glycosidases,[14] the molecular details mediating binding by azepanes to glycosidases remains unknown.

One glycosidase hydrolase that has generated recent interest is the cytoplasmic β-*N*-acetylglucosaminidase (*O*-GlcNAcase)[21] that processes nucleocytoplasmic proteins post-translationally modified with 2-acetamido-2-deoxy-D-glucopyranose residues [Figure 1, GlcNAc (**1**)] β-O-linked to serine or threonine residues (*O*-GlcNAc).[22] This family 84 glycoside hydrolase (GH84) plays a role in multiple cellular processes.[23] Its catalytic mechanism has been shown to involve anchimeric assistance from the acetamido group of the substrate.[24] Notably, the structure of a β-*N*-acetylglucosaminidase from *Bacteroides thetaiotaomicron* (*Bt*GH84) has revealed an active site in which all but one amino acid residue is conserved with respect to that of human *O*-GlcNAcase,[25] which makes *Bt*GH84, along with the Clostridial GH84 enzyme,[26] valuable models for studying inhibition of *O*-GlcNAcase.[27,28] Human *O*-GlcNAcase inhibitors offer insights into the biological roles of *O*-GlcNAc without suffering the loss of protein−protein interactions that may complicate interpretation of gene knockouts and knockdowns.[29]

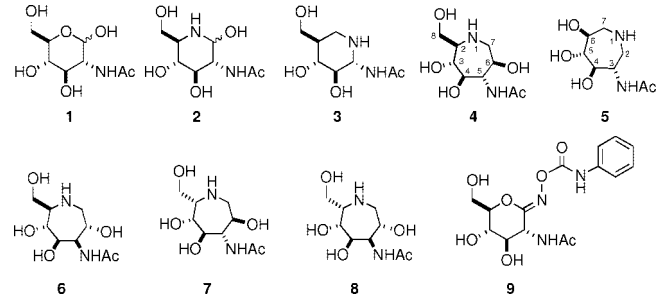

Figure 1. Structures of GlcNAc (**1**), nojirimycin-like **2**, noeuromycin-like **3**, β-D-gluco-like configured target azepane **4**, target azepane **5**, diastereomers of the target azepanes with α-D-manno- (**6**), α-L-ido- (**7**), and β-L-gulo-like (**8**) configurations, and PUGNAc (**9**).

Motivated by interest in the function of this enzyme and the need for useful inhibitors, coupled with the lack of understanding of the molecular basis upon which azepanes inhibit glycosidases more generally,[15,30] we undertook detailed studies of the inhibition of *O*-GlcNAcase by several new azepanes. Here we report the synthesis of new azepanes such as **4** and **5**, which, unlike their six-membered-ring counterparts that bear relatively unstable hemiaminal (Figure 1, **2**)[31] or *gem*-diamine (Figure 1, **3**)[32] functionalities, should be chemically stable in biological media by virtue of the inserted methylene unit (Figure 1, **4** and **5**). We then investigate the potency of these inhibitors toward GH84 enzymes, including human *O*-GlcNAcase, and evaluate the detailed molecular basis for their potency using X-ray structural studies and molecular simulations.

As a first step, we synthesized azepanes **4** and **5**, since these molecules are analogous to the nojirimycin and noeuromycin classes of inhibitors, respectively, except that a methylene unit is inserted adjacent to the endocyclic nitrogen. Otherwise, both molecules have a relative stereochemical disposition of substituents that mimics that of the natural D-gluco-configured substrates. We therefore anticipated that these molecules would inhibit *O*-GlcNAcase. These two targets of interest were conveniently obtained from known azepanes[33,34] using two concise routes (Supplemental Scheme 1 in the Supporting Information).

To generate additional molecules that would allow us to verify or reject our hypothesis that the relative configuration of substituents is essential for potent inhibition of *O*-GlcNAcase, we prepared several conveniently accessible diastereomers having *incorrect* configurations. Azepanes **6**, **7**, and **8**, displaying α-D-manno-, α-L-

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Table 1. Inhibition Constants (K_i) for the Inhibitors and Enzymes Studied[a]

inhibitor	K_i (μM)	
	O-GlcNAcase	*Bt*GH84
GlcNAc (**1**)[b]	1500[c]	not determined
PUGNAc (**9**)	0.046[c,d]	0.010[e]
4	51 ± 5	89 ± 3
5	11 ± 1	8 ± 1
6	280 ± 40	>1000
7	480 ± 90	490 ± 130
8	1600	660 ± 50

[a] Assays for human *O*-GlcNAcase were carried out at 37 °C using *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (pNP-GlcNAc) as the substrate in 50 mM NaP_i, 100 mM NaCl (pH 7.4). For *Bt*GH84, assays were carried out at 25 °C using pNP-GlcNAc as the substrate in 50 mM MES, 200 mM NaCl (pH 6.5). See the Supporting Information for further details. [b] Product of the *O*-GlcNAcase-catalyzed reaction. [c] Reference 24. [d] Reference 21. [e] Reference 25.

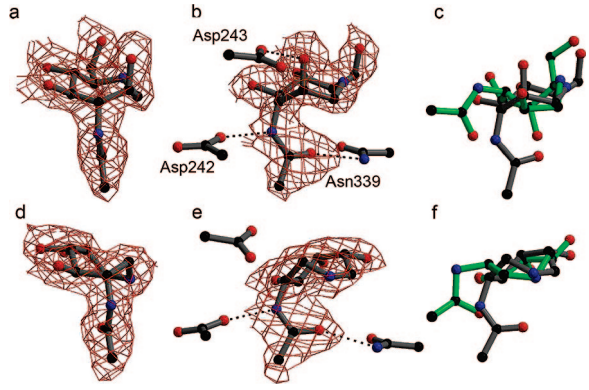

Figure 2. Observed electron density ($2F_{obs} - F_{calc}$ at 1σ) for azepanes **4** and **5** bound to *Bt*GH84 and major conformations of **4** and **5** in solution. (a) Mono "side-on" view of **4**. (b) Mono "front-on" view of **4**, with some residues mentioned in the text shown in ball-and-stick representation. (c) Major conformer of **4** as established by MM (green) overlaid with the bound structure (gray). (d−f) Same as (a−c) but with **5** rather than **4**.

ido- and β-L-gulo-like configurations respectively, were obtained from the corresponding seven-membered-ring azido alcohols.[33] We expected compounds **6**−**8** to be poor inhibitors of *O*-GlcNAcase because of the absence of the sequential trans relationship of the substituents seen in azepanes **4** and **5** as well as in the nojirimycin (**2**) and noeuromycin (**3**) classes of inhibitors.

With azepanes **4**−**8** in hand, we evaluated their inhibitory properties against both human *O*-GlcNAcase and *Bt*GH84 (Table 1). As we anticipated, azepanes **4** and **5** are potent competitive inhibitors [see Supplemental Figure (SF) 3], suggesting that these molecules adopt a conformation within the enzyme active site that places these groups in positions that mimic those of the substrate. Support for this view is gained from the observation that azepanes **6**−**8** are poor inhibitors of these GH84 enzymes, likely because of the inappropriate configuration of substituents. Azepanes **4** and **5** therefore bind some 30- and 140-fold more tightly than the product (GlcNAc, **1**, K_i = 1500 μM,[24] Figure 1) of the *O*-GlcNAcase-catalyzed reaction itself.

In order to gain insight into the molecular basis of inhibition of *O*-GlcNAcase, crystals of *Bt*GH84 were soaked in solutions containing azepanes **4**−**8**. In keeping with their respective K_i values, ordered binding to the enzyme was only observed for **4** and **5**. These 3D structures, determined to ~2.3 Å resolution, reveal clear, conformationally unambiguous electron density for both **4** and **5** bound in the active site of *Bt*GH84 (Figure 2). These 3D structures elegantly offer a molecular rationale for the stereochemical requirements for binding to GH84 enzymes. As we had anticipated, the enzyme-bound dispositions of the substituents of compounds **4** and **5** closely match those of the glucopyranose-configured inhibitors of GH84 enzymes such as PUGNAc (**9**)[21,35] (Figure 1). Conversely, compounds **6**−**8**, which display configurations equating to α-D-manno, α-L-ido and β-L-gulo configurations, have incorrect stereochemistry at centers C5/C6 (**6**), C2 (**7**), and C2/C5/C6 (**8**), respectively (see **4** in Figure 1 for numbering).

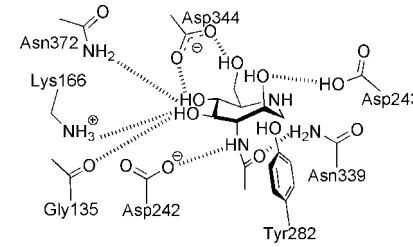

Figure 3. Representation of close (<3.1 Å) contacts of **4** with BtGH84.

Briefly, this configurationally selective binding is promoted by hydrogen bonding of the hydroxymethyl group and O3 to Asp^{344} and of O3 to Asn^{372}, with O4 receiving a hydrogen bond from Lys^{166} while donating one to the main-chain carbonyl of Gly^{135} (Figure 3). At the reaction center, the amide nitrogen forms a hydrogen bond with catalytic residue Asp^{242}. Most notably, we also observe an unusual conformation for the ring of azepane **4** in which both the acetamido group and the pseudoanomeric hydroxyl groups are oriented transdiaxially. This conformation is likely made possible by favorable interactions of these two substituents with the conserved residues Asp^{242} and the acid/base Asp^{243} (Figures 2 and 3). This arrangement, in which the "anomeric" hydroxyl group hydrogen-bonds to the general acid is reminiscent of the rather rarely observed enzyme−substrate complexes in which the general acid H-bonds to the glycosidic oxygen.[6] Azepane **5** adopts a conformation placing the "pseudoanomeric" carbon (C2) in the same position as that observed for C6 of azepane **4**, despite the lack of the "anomeric" hydroxyl group. It is also notable that consistent with the requirements for neighboring-group participation in catalysis, this transaxial arrangement places the *N*-acetyl carbonyl group 3.4 Å from the "anomeric" center with, in the case of **4**, a C═O···C6···O6 angle of ~150°, corresponding to an appropriate geometry for in-line nucleophilic substitution at the anomeric carbon. Correct attack orientation for the carbonyl is likely also enhanced by the hydrogen bond to Asn^{339}.

Given the unusual conformation of these azepanes bound to BtGH84 and the considerable conformational flexibility of these molecules, we aimed to ascertain whether these inhibitors adopt a conformation in solution that might resemble the one observed within the enzyme active site or, more likely, whether they adopt a different conformation that may then either be "selected" from solution or, alternatively, bind and then "mold" to the active site to optimize the interactions. To gain insight into this question, we examined the NMR data and carried out molecular mechanics (MM) and molecular dynamics (MD) simulations. For azepane **4**, the observed vicinal coupling constants ($J_{5,6}$ = 6.5 Hz; $J_{4,5}$ = 8.6 Hz; $J_{3,4}$ = 8.6 Hz; $J_{2,3}$ = 7.3 Hz; $J_{2,8a}$ = 3.5 Hz; $J_{2,8b}$ = 9.3 Hz; $J_{6,7a}$ = 2.7 Hz; $J_{6,7b}$ = 6.5 Hz) resemble those experimentally observed by Peczuh and co-workers[36] for methyl α-D-glycero-D-idoseptanoside **(10)** (see SF 2). Compound **10** has been described as adopting primarily a $^{3,4}TC_{5,6}$ conformation in solution, with substituents at positions 1, 2, 3, 4, and 5 in equatorial-like orientations (numbering as in SF 2). The relatively small values we observe for $J_{5,6}$ and $J_{2,3}$ of **4**, however, indicate that other minor conformers are also present

in solution. MM3* calculations also indicate that other conformations are present (see SF 4), although the contribution of the $^{4,5}TC_{2,3}$ conformer likely is greater than 80%. On the basis of these results (Figure 2c), the predominant conformers in solution do not match that of the enzyme-bound inhibitor, and therefore, the enzyme either selects for a minor solution conformer or optimizes the conformation of the inhibitor within the active site.

For azepane **5**, the experimental couplings ($J_{2a,3} = 2.4$ Hz; $J_{2b,3} = 6.6$ Hz; $J_{3,4} = 6.6$ Hz; $J_{4,5} = 6.6$ Hz; $J_{5,6} = 9.7$ Hz; $J_{6,7a} = 9.7$ Hz; $J_{6,7b} = 2.7$ Hz) also suggest one major conformation, especially around the region defined by couplings between H5 and H6 and H6 and H7a,b, which, in view of the magnitude of the couplings, likely have an antitype relationship. The value of 6.6 Hz for $J_{3,4}$ and $J_{4,5}$, however, suggests that conformers having different torsion angles in this region also contribute. MD simulations using the MM3* force field offer more precise insight into the actual conformational equilibrium: an almost 1:1 ratio between the $^{4,5}C_{N,2}$ and $^{5,6}T_{2,3}$ conformers is consistent with the experimental coupling constants (see SF 5).[36] Here, too, it is clear that the conformation in solution differs significantly from that seen when the inhibitor is bound to *Bt*GH84 (Figure 2f).

The use of chemically stable but conformationally flexible polyhydroxylated azepanes thus allows the ring to adopt alternate conformations within the enzyme active site. These conformations offer insight into the preferred distorted conformation of bound substrates. The 1,2 transdiaxial arrangement of the pseudoanomeric β-hydroxyl and acetamido groups in azepane **4** occurs by virtue of its adoption of a $^{N}C_{4,5}$ chair conformation, whereas **5** adopts a slightly distorted $^{2}B_{5,6}$ conformation (Figures 2 and 3). These seven-membered-ring conformations, both of which place the "anomeric" carbon above the ring, are analogous to the ^{4}E and $^{1,4}B$boat conformations adopted by the relatively rarely observed Michaelis complexes of GlcNAc-hydrolyzing enzymes, including mutant chitinases[37] and β-*N*-acetylglucosaminidases,[38] and are further supported by the strong inhibition of these enzymes by polyhydroxylated isoquinuclidines.[39]

In summary, complexation of these azepane inhibitors with a glycoside hydrolase reveals the detailed basis for inhibition by this growing class of compounds. The structures of these GH84 complexes further suggest how substrates may bind and thereby enable hydrolysis with retention of anomeric configuration through neighboring-group participation. The conformational flexibility of the azepane class of inhibitors therefore may offer a generally useful approach for continued study of glycosidase conformational pathways.

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Supporting Information Available: Synthetic procedures, X-ray structural figures and data, figures of MD simulations, kinetic data, and details of experimental protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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