A New, Simple, High-Affinity Glycosidase Inhibitor: Analysis of Binding through X-ray Crystallography, Mutagenesis, and Kinetic Analysis

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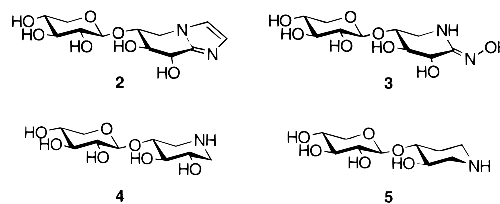
Retaining glycosidases catalyze the hydrolysis of glycosides with overall retention of anomeric stereochemistry, normally with the assistance of two carboxyl groups, one functioning as an acid/base and the other as a nucleophile.[1] The reaction usually proceeds through a covalent glycosyl enzyme, and the transition-states leading to and from this intermediate have been shown to possess considerable oxacarbenium-ion character (Figure 1a).[2] Many glycosidase inhibitors have been developed over the last 30 years, including a number of sugar-shaped heterocycles containing a basic nitrogen at positions that correspond to C1, O5, and the glycosidic oxygen, sites that develop partial charge at the transition state.[3,4] Of these, 1-*N*-iminosugars are particularly powerful inhibitors of retaining β-glycosidases. However, unlike the substrates for most glycosidases and most other iminosugar inhibitors, 1-*N*-iminosugars lack a hydroxyl at C2.[5,6] This is unfortunate since the interaction of active-site residues, particularly of the catalytic nucleophile, with OH2 has been suggested to provide upward of 30−40 kJ mol^{-1} to the stabilization of the transition state of enzyme-catalyzed glycoside hydrolysis.[7−10] The possibility therefore exists to develop much stronger inhibitors of this type if this interaction can be included. However, 1-*N*-iminosugars with a hydroxyl at C2 are not expected to be stable compounds, since they will presumably dehydrate in common with many hemiaminals.[11] An attractive way of incorporating this interaction into a 1-*N*-iminosugar is provided in compound **1**. By binding as the protonated iminol tautomer,[12] **1** could thereby provide a hydroxyl group at C2 and a positive charge at the anomeric position and in part reflect the planarity of the sugar ring at the transition state (Figure 2). Investigation of the mode of interaction of such a compound may cast light on the role of OH2 in the transition state of the enzyme-catalyzed reaction.

Xylobiose-derived inhibitors with sp^2-hybridized nitrogen atoms in place of the glycosidic oxygen, namely the imidazole **2** and the lactam oxime **3**, and two compounds with sp^3-hybridized nitrogen atoms in place of C1 or O5, the deoxynojirimycin **4** and the isofagomine **5**, were recently shown to be effective inhibitors of Cex, a retaining family 10 xylanase from *Cellulomonas fimi* (Table 1).[13] It was hoped that by installing a carbonyl into **5**, affording **1,** we could provide a simple, new class of nonbasic inhibitors of glycosidases, and of Cex in particular. Notably, there exist relatively few effective, nonbasic glycosidase inhibitors, the best of these being kifunensine,[14] the glyconotetrazoles[15] and various glyconolactones and their isosteric lactams.[16]



Compound **1** was prepared in a straightforward fashion from the known lactam **6**.[13] Benzoylation of **6** afforded the monobenzoate **7** that was subsequently xylosylated with tri-*O*-acetyl-α-D-xylopyranosyl trichloroacetimidate to afford the disaccharide **8**. Deacetylation was achieved under standard conditions to give **1** which was purified by flash chromatography and assayed as an inhibitor of Cex (Scheme 1). **1** was shown to act as a competitive inhibitor with a K_i value of 340 nM.[17,18] The K_i value determined is impressive considering that glyconolactams ordinarily bind rather poorly.[19] To probe whether this high affinity is due to its binding in the iminol tautomer proposed earlier, the X-ray crystal structure of the complex of **1** with Cex was determined (Figure 3).[20] This structure shows the inhibitor binding in the −1 and

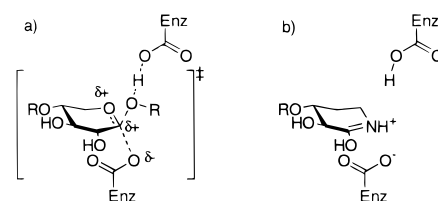


Figure 1. (a) Hypothetical transition-state of a retaining β-xylanase proceeding through an intermediate in a chair conformation. (b) Proposed mode of inhibition of an isofagomine lactam.

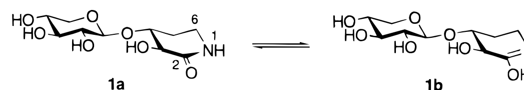


Figure 2. Potential tautomerisation of compound **1**.

Table 1. Kinetic Parameters for Xylobiose-Derived Iminosugars and *p*-Nitrophenyl β-Cellobioside with Wild-Type Cex and the N126A Mutant[a]

K_i (μM)	1	2	3	4	5	*p*-NP β-cellobioside
wild-type	0.34	0.15[a]	0.37[a]	5.8[a]	0.13[a]	k_{cat}/K_m = 26.3 s^{-1} mM^{-1}
N126A	2000	200	1300	4800	1.6	k_{cat}/K_m = 0.0044 s^{-1} mM^{-1}
$\Delta\Delta G°$ (kcal mol^{-1})	−5.3	−4.4	−5.0	−4.1	−1.5	$\Delta\Delta G^{\ddagger}$ = 5.4 kcal/mol^{-1}

[a] Data taken from ref 13.

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(17) Compound **1** was a poorer inhibitor of Bcx (K_i = 9 mM), a retaining family 11 xylanase from *Bacillus circulans*.

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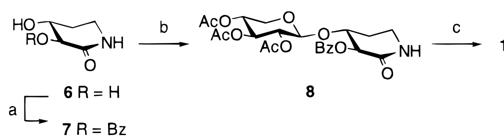
Scheme 1



[a] (a) BzCl, Pyr, −40°, 68%; (b) tri-*O*-acetyl-α-D-xylopyranosyl trichloroacetimidate, $BF_3.Et_2O$, $(CH_2Cl)_2$, 71%; (c) NaOMe, MeOH, 76%.

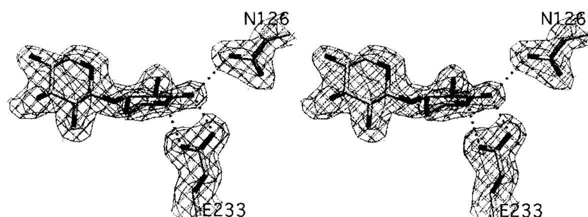


Figure 3. Stereodiagram of the $2|F_o| - |F_o|$ electron density from the protein model, contoured at 1σ. The stick model of the isofagomine is superimposed on the density in an E_5 conformation but was not used in the phase calculation.

−2 subsites in an E_5 conformation, consistent with its binding as the iminol tautomer, with C6, N1, C2, C3 and O2 lying very nearly in a plane.[21,22] While the amide tautomer of **1** necessarily requires atoms N1, C2, C3 and O2 to lie in a plane, upon tautomerisation to the iminol there is an additional requirement that C6 lies in this same plane, as observed. The catalytic nucleophile, Glu233 lies directly below N1 at a distance of 2.98 Å. The observation of significant interactions between the nucleophile and both N1 and O2 is most consistent with **1** binding in the protonated iminol form. Indeed, if the amide tautomer was bound to Cex, these interactions with the nucleophile would be strongly destabilizing. If **1** is in fact bound as the protonated iminol, the proton on nitrogen is directed in the plane of the ring and is not able to form a strong hydrogen bond with the nucleophile. Nonetheless, a strong electrostatic interaction between the nucleophile and the iminium ion is still possible. Also observed in the crystal structure is a relatively short contact of 2.59 Å between OE2 of the nucleophile and the lactam oxygen, reminiscent of that (2.37 Å) observed in the X-ray crystal structure of the cellobiosyl enzyme formed with the H205N/E127A Cex double mutant.[8] This strong interaction is suggestive of a hydrogen bond between OE2 of the nucleophile and O2 of the inhibitor. A significant H-bonding interaction (2.97 Å) is also seen between the amide nitrogen of N126 and O2, again consistent with a hydrogen bond from a 2-hydroxyl. N126 is a highly conserved residue in family 10 and in clan GH-A and, on the basis of crystal structures of the 2-deoxy-2-fluoro-cellobiosyl[7] and 2-deoxy-2-fluoro-xylobiosyl enzyme,[23] has been suggested as a residue that hydrogen bonds directly with OH2.

To determine the importance of interactions of O2 of **1** with the N126 residue, we measured the K_i values of the inhibitor with the Cex N126A mutant and compared this with K_i values for the inhibitors **2**-**5**. Table 1 shows K_i values for compounds **1**−**5** with wild-type Cex and the N126A mutant, as well as values for the contributions of the interactions with N126 to inhibitor binding. As can be seen for compounds **2**−**4** (all of which possess a 2-hydroxyl) binding to the N126A mutant is considerably weaker than to wild-type enzyme, as expected due to the loss of important hydrogen bonding in the N126A mutant.[24] By contrast, binding of **5** to the mutant is compromised to a much lesser extent, consistent with the absence of an interaction at that position. Significantly, the consequence of the mutation of N126 upon binding of **1** is very similar to that seen for inhibitors **2**−**4**, which contain a 2-hydroxyl group. This strongly implies that there are similar interactions in the two cases and thus that **1** binds in its iminol form. It is interesting to compare these results with the effect upon catalysis of mutating N126, as reflected in k_{cat}/K_m values. The loss in transition state stabilization observed ($\Delta\Delta G^{\ddagger} = 5.4$ kcal mol^{-1}) is very similar to that seen for binding of these inhibitors, implying that these inhibitors are, at least in part, mimicking the reaction transition state. However, a more detailed analysis will be required to properly probe this behavior. The tautomerization energy for the amide-iminol conversion is likely to be of the order of 11 kcal mol^{-1},[25] indicating that the concentration of the iminol form in solution is very low. Thus, if the K_i value observed results from the small amount of the iminol present, then the true K_i value for this tautomer must be several orders of magnitude lower. An important approach to tighter binding inhibitors may therefore involve inclusion of structural elements that stabilize this tautomer. Compound **1** therefore represents an example of a possible new class of potent glycosidase inhibitors and work is continuing to explore their generality as glycosidase inhibitors.

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Supporting Information Available: Experimental details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(18) Inhibition constants were determined at 37 °C using a 0.05 M NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0) and 2,4-dinitrophenyl β-cellobioside as a substrate. Measurements were started by addition of Cex. Measurements of the increase of absorption at 400 nm per min in a continuous assay yielded reaction rates. Michaelis parameters (V_{max} and K_m) were extracted from these data by best fit to the Michaelis−Menten equation. Estimates of K_i values (for N126A Cex) were obtained by measuring rates at a fixed substrate concentration with a range of inhibitor concentrations (6−10) which encompassed the K_i value ultimately determined, generally from 0.3 to 3 K_i. The observed rates were plotted in the form of a Dixon plot and the K_i value was determined by an intersection of this line with a horizontal line drawn through $1/V_{max}$. Full K_i determinations were performed by measurement of rates at a series of seven substrate concentrations (generally from 0.3 to 3 K_m) in the presence of a range of inhibitor concentrations (typically 5 concentrations) which bracket the K_i value.

(19) Although low K_i values for glyconolactams have been observed in many cases, the values observed have been similar to those of the corresponding deoxynojirimycin. In this case the lactam **1** binds more than 10-fold more tightly than the xylobiose-derived deoxynojirimycin **4**. See ref 16.

(20) Cex crystals were grown in a 0.1 M NaOAc buffer (pH 4.6) containing 16% PEG 4000. Cex crystals were soaked in artificial mother liquor containing inhibitor for several hours prior to data collection. Diffraction data were recorded in-house to 2.0 Å resolution at 100 K on a Mar345 image plate using Osmic mirror focused Cu Kα X-rays, generated from a rotating anode operating at 100 mA and 50 kV. Oscillations of 1° were collected in 120 s exposures. Recorded reflections were indexed, integrated, and scaled in the Denzo/Scalepack MarHKL suite (a) Otwinowski, Z.; Minor, W. Processing of X-ray diffraction data collected in oscillation mode. In *Methods in Enzymology*; Carter, C. W., Jr., Sweet, R. M., Eds.; Academic Press: New York, 1997; Vol 276, pp 307−326. The data set was 99% complete with R_{sym} of 5.7% and an average I/s of 13.3, using 258 208 observations for 21 259 unique reflections. The crystals belonged to tetragonal space group $P4_12_12$ with cell dimensions $a = b = 86.99$ Å and $c = 80.36$ Å and $\alpha = \beta = \gamma = 90°$. The structure was refined using wild-type Cex as a starting model (PDB code 2EXO) with CNS. (b) Brünger, A. T; Adams, P. D.; Clore, G. M.; DeLano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. *Acta Crystallogr.* **1998**, *D54*, 905−921; to an *R*-factor of 20.9% (*R*-free 25.0%). In order not to bias ligand geometry, dihedral angles were left unimposed. The rmsd for the Cα backbone atoms between this complex and that of native Cex was 0.315 Å.

(21) The torsional angles measured about the C6−N1−C2−C3 and C6−N1−C2−O2 systems of **1** were 21° and 175°, respectively.

(22) The single-crystal X-ray structure of the lactam **6** showed it to be in a $^5H_4$ conformation, Williams, S. J. and Withers, S. G. Unpublished data.

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(24) The X-ray structure of the N126A Cex mutant is essentially identical to that of the wild-type other than a slight movement of Trp84. This residue, which lies behind N126 in wild-type Cex, is observed to have moved towards the void left by the Asp to Ala mutation (a 30° rotation about χ-1) and may slightly obstruct the active site.

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