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A xylobiose-derived isofagomine lactam glycosidase inhibitor binds as its amide tautomer†

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The atomic-resolution structure of a xylobiose-derived isofagomine lactam in complex with the xylanase Xyn10A from *Streptomyces lividans* reveals that the lactam is bound to the enzyme as the amide tautomer, with "reversed" protonation-states for nucleophile and acid–base.

Substantial efforts have been expended in the design and study of glycosidase inhibitors. Whilst these studies have been partially successful, with inhibitors known for many glycosidases, our understanding of the mechanisms by which many of these inhibitors function remains relatively limited. Furthermore, given the extremely tight-binding (10^{-22} M) of the transition-state by this class of enzyme,[1] even the nanomolar inhibition constants for the best mimics are comparatively poor. Dissection of the binding mode of known inhibitors is particularly important if we are to improve the potency of inhibitors for their development into therapeutic agents. Additionally, such compounds are of great utility as mechanistic probes in studies of the nature of the transition state of the enzyme-catalyzed reaction. Inhibitors are assessed for their ability to mimic the transition state of the enzyme-catalyzed reaction through studies with enzyme mutants.[2,3] Those inhibitors for which good mimicry exists may then be studied by X-ray crystallography in complex with the enzyme in question, providing a structural view of the interactions that are likely to be of importance at the transition state of the enzyme-catalyzed reaction. Ideally such complexes should be determined at "atomic" resolution[4] allowing accurate placement of all atoms involved in the enzyme–inhibitor interactions as well as determination of protonation and tautomeric-states for protein and ligand.[5]

Retaining glycosidases function through a two-step double displacement mechanism.[6] In the first step an enzymic carboxylate performs a nucleophilic attack on the anomeric carbon while a second carboxyl group functions as a general acid assisting the departure of the aglycon. In the second step the intermediate glycosyl enzyme is hydrolyzed through general base assistance by the second carboxyl group, affording a sugar hemiacetal with net retention of anomeric configuration. We recently described a novel lactam derivative (**1**) related to isofagomine that is a powerful inhibitor (K_i 0.34 μM) of the retaining family 10 endoxylanase Cex from *Cellulomonas fimi* (Table 1).[7] Asn126 (Cex numbering; this residue is equivalent to Asn127 of the *Streptomyces lividans* Xyn10A described here), a residue known to interact with the 2-hydroxyl group of the substrate, was used as a probe for inhibitor–protein interactions about the 2-position of the ligand. Studies of inhibitor binding to the Asn126Ala mutant of Cex, supported binding of **1** as its iminol tautomer **1b** (Fig. 1), but the 2.0 Å resolution structure of **1** bound to Cex did not allow determination of which tautomeric form was bound. The potent inhibition shown by isofagomine-derived lactams appears to be relatively general, with a closely related galactoisofagomine lactam (**6**) being an excellent inhibitor (18 nM) of the β-galactosidase from *Aspergillus oryzae*.[8] Thus, the question as to the tautomeric-state in which isofagomine lactams such as **1** and **6** bind to glycosidases is crucial.

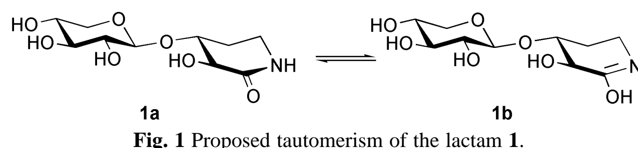

Fig. 1 Proposed tautomerism of the lactam **1**.

In order to clarify the binding mode of **1** we have determined the atomic-resolution structure of the inhibitor **1** bound to a closely related homologue of Cex, the xylanase Xyn10A from *S. lividans*. **1** is a competitive inhibitor of Xyn10A with a K_i of 8 μM, Table 1.‡ Xyn10A crystals, grown at the pH optimum for catalysis, diffract to atomic resolution and allow precise, unbiased determination of the relevant bond lengths and hence assignment of the bound tautomer.

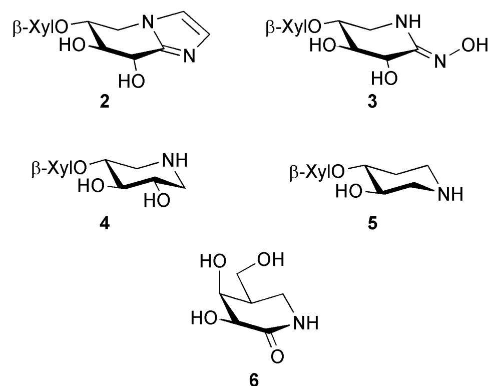

Table 1 Inhibition constants of xylobiose-derived inhibitors against Xyn10A from *S. lividans* (this work) and Cex from *C. fimi*[a]

	K_i/μM	
Compound	Xyn10A	Cex
1	8	0.34
2	1.1	0.15
3	4.7	0.37
4	8	5.8
5	0.028	0.13

[a] Data from ref. 7.

† Electronic supplementary information (ESI) available: details of data and structure quality for complex of **1** with Xyn10A. See http://www.rsc.org/suppdata/cc/b3/b301829f/

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The crystal structure, at 1.05 Å resolution reveals two molecules of **1** binding in the −2/−1 and +2/+3 subsites (Fig. 2). A single imidazole from the crystallisation buffer lies in the +1 subsite. In the "catalytic" −1 subsite the isofagomine lactam is found in a $^4H_5$ (half-chair) conformation with C6, N1, C2, O2 and C3 co-planar, as observed for the single crystal structure of the lactam moiety of **1** and consistent with partial delocalisation across the C–N bond.

Maximum-likelihood refinement, in which geometry for the isofagomine lactam moiety (and the catalytic nucleophile and acid–base) were not restrained to assumed target values, revealed C2–O and C–N bond lengths of 1.24 and 1.32 Å, respectively. For comparison, we searched the Cambridge Structural Database (Version 5.23) for high resolution structures. As iminols are usually unstable molecules we also examined small molecule structures of imidates. There are several hundred such structures in the database, but only those molecules that satisfied the following were included. First, the substituent on nitrogen should be either hydrogen or sp^3-hybridized carbon to prevent conjugation of the nitrogen lone pair with an adjacent π-system. Second, the substituent on carbon should be either hydrogen or sp^3-hybridized carbon, again to avoid conjugation with the iminol double bond. The most common values of the C=N bond length of iminols and imidates are 1.26–1.28 Å, and for C–O bonds, 1.34–1.36 Å (Fig. 3). In contrast for amides, a recent study of the CSD revealed that the most commonly represented bond lengths for the amido C=O bond is 1.22–1.24 Å and for the C–N bond almost all values lie between 1.30–1.40 Å.[9] Thus our data are consistent only with the inhibitor binding as the amide tautomer, **1a**.

Also evident from the X-ray structure is the protonation state of the enzyme. As seen on other systems[5] both electron density (H-atom omit density not shown), Fig. 2, and refined bond lengths indicate that the nucleophile is protonated with C–O bond lengths of 1.32 and 1.22 Å (σ 0.010Å). Conversely, both bond lengths of the acid–base refine to 1.26 Å consistent with delocalisation. Binding of the lactam tautomer to the enzyme with deprotonated nucleophile would be expected to be destabilizing as a negatively charged carboxylate would be opposed to the lactam carbonyl. In the protonated form this interaction becomes favorable (Fig. 4).

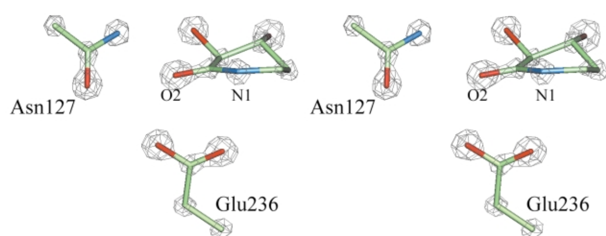

Fig. 2 1.05 Å maximum-likelihood weighted $2F_{obs} - F_{calc}$ electron density at 2.8 e Å^{-3} (divergent stereo) of the lactam **1** bound to Xyn10A. Only the −1 subsite is shown.

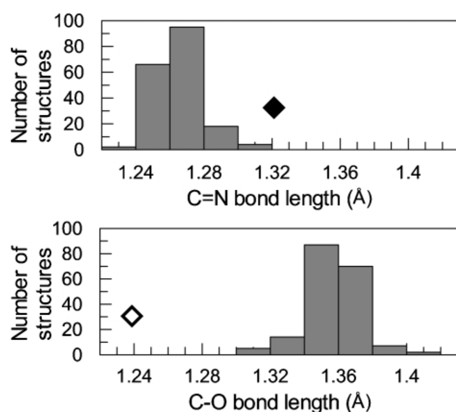

Fig. 3 Histogram of C=O and C–N bond lengths for 185 iminols and imidates from the CSD. Plotted for the same histogram are the bond lengths for the lactam **1** observed here. The closed diamond (◆) indicates the observed C2–N1 bond length and the open diamond (◇) indicates the corresponding C2–O2 bond length.

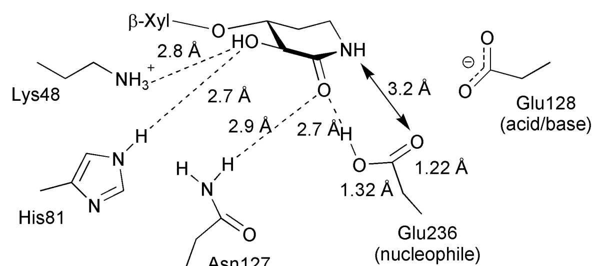

Fig. 4 Interactions of **1** bound to Xyn10A. Only the −1 subsite is shown. Distances between the non-hydrogen atoms are shown.

Atomic resolution analysis has allowed dissection of both the tautomeric form of **1**, and the protonation state of the enzyme. **1** binds Xyn10A as the amide tautomer and the enzyme has a protonated nucleophile and deprotonated acid–base residue. These data illustrate that binding of effective glycosidase inhibitors may occur to glycosidases in atypical protonation states, in this case with the usual protonation state of the nucleophile and acid–base residue reversed. Further insight into the binding mode of these inhibitors could be gained through studies of the pH dependence of inhibition and transition state mimicry, and should lead to the development of more powerful glycosidase inhibitors.

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Notes and references

‡ *Experimental*: kinetics and K_i determinations were performed at pH 7.5 in 50 mM NaPi, 0.1% BSA at 37 °C, essentially as described previously.[10] Crystals of Xyn10A were grown as in ref. 10 from 20 mg ml^{-1} protein with 16–18% polyethylene glycol monomethyl ether 5000, 5% isopropanol, 0.1 M imidazole pH 7.5. A small quantity of powdered **1** was added to the liquor surrounding a single crystal which was soaked for 3 min. Data, to 1.05 Å resolution (R_{merge} 0.06, 98% complete), were collected from a single crystal at 100 K in a stream of N_2 gas on European Synchrotron Radiation Facility beamline ID29 and were processed with the HKL suite.[11] The structure refined with REFMAC[12] (final R_{cryst} 0.10, R_{free} 0.13) and programs from the CCP4 suite.[13] Coordinate accession code 1od8.

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