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The rational design of an iminosugar inhibitor able to mimic substrate distortion occurring during retaining-cellulase hydrolysis†

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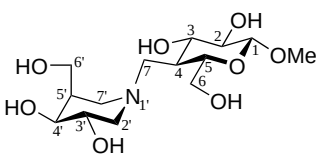
Abstract—This paper reports the rational design, synthesis and biochemical evaluation of a new type of cellulase inhibitor. This isofagomine isomer analogous to the β-L-idopyranose linked to a glucosidic unit by a methylenic bond is able to mimic the pseudo-axial orientation of the 'aglycon' observed in the X-ray structure of a non-hydrolyzable thiooligosaccharide/cellulase complex.

Cellulases are *endo*-glycoside hydrolases which specifically catalyze hydrolysis of the β-1,4-glycosidic linkages in cellulose, the most abundant biopolymer on earth.[1] Cellulases have found widespread application in detergent, textile and paper industries,[2] and much effort has been directed to further understand the structure/function relationships of these enzymes or to improve their effectiveness as well.

There is some doubt about the exact conformation and stereoelectronic nature of the transition state adopted by the sugar moiety during enzyme-mediated glycoside bond cleavage. However, significant explanations about the catalytic mechanism of *endo*-β-glycoside hydrolases have been recently obtained both from the kinetic evaluation of specific inhibitors and information derived from crystallographic techniques. The growing number of 3D structures of cellulases complexed with inhibitors (thiooligosaccharides,[3] 2-deoxy-2-fluoro-D-glycosides,[4] tetrazole-glycosides[5]) provided important architectural features to the enzyme's active site such as the location and the identification of the catalytic residues and the number of subsites involved in the substrate recognition. To date, four crystal structures of *endo*-β-glycoside hydrolases have been solved,[6–9] each disclose significant ring distortion into either a skew-boat ($^{1}S_{3}$) or boat ($^{2,5}B$) conformation of the glucosyl unit situated in the −1 subsite of these enzymes. This phenomenon, occurring during the formation of the Michaelis complex with the still unhydrolyzed substrate,[7] would assist the glycosidic hydrolysis of β-D-pyranosides according to the antiperiplanar lone pair principle[10] by inducing a pseudo-axial orientation of the aglycon and facilitate the attack of a water molecule or a nucleophilic amino acid at the anomeric center.



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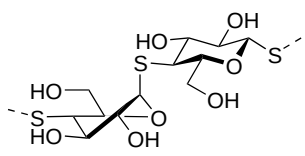

Figure 1. Target inhibitor **1** and two internal residues of thio-cellopentaoside complexed in the −1 and +1 subsites of Cel7B.[6]

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† This paper is dedicated to Professor Joachim Thiem on the occasion of his 60th birthday.

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Non-hydrolyzable substrates able to mimic the pseudo-axial orientation of the aglycon, but without presenting ring puckering constraints may generate a new potent class of glycoside hydrolase inhibitors. Few examples of skew–boat or boat-mimicking compounds have been reported in the literature and of these only one polyhydroxylated isoquinuclidine[11] exhibited potent activity against a β-D-mannosidase. No *endo*-glycoside hydrolase inhibitors of this type have been reported and we decided to accept the challenge to design, synthesize and evaluate a putative retaining-cellulase inhibitor.

The target disaccharide **1** consists of an isofagomine isomer analogous to the inverted $^1C_4$ chair of an β-L-idopyranose linked to a glucosidic unit by a methylenic bond (Fig. 1). Its structure was studied by molecular modeling in order to mimic the pseudo-axial orientation of the 'aglycon' observed in the cellulase distorded substrate and using the methyl thio-cellopentaoside in complex with Cel7B from *Fusarium oxysporum* as a model.[6]

Molecular modeling and conformational analyses of different target compounds were undertaken and the disaccharide **1** was expected to be a good mimic of the distorded substrate when the azasugar moiety adopts a $^1C_4$ chair conformation. The iminocyclitol ring was set in a $^1C_4$ chair so that the C-3′ and C-4′ secondary hydroxyl groups remained in an equatorial position. In the axial position were set the C-6′ hydroxymethyl group and, more importantly, the *N*-1′-linked methylenic bond. A total of 36 conformers was prepared under SYBYL 6.5 (Tripos Inc. St. Louis, MO), corresponding to the combination of the clockwise (c) and reverse-clockwise (r) orientations of the secondary hydroxyl groups for each ring, and of the three possible orientations of the hydroxymethyl groups in each ring, analogous to the *gg*, *gt* and *tg* conformers of cellobiose.[12] With a protonated nitrogen present in the imino bond, the torsional angles representing the orientations of the rings about the imino/methylenic bond linkage were defined as $\phi = \Theta$(H1′–N1′–C7–C4) and $\varphi = \Theta$(N1′–C7–C4–H4). Using an approach identical to that used for protonated methyl α-acarviosinide,[13] the conformational space for the two linkage torsional angles was evaluated at $\varepsilon = 4$ with MM3(92)[14–16] at 20° intervals, leaving the remaining geometric features relaxed. At each grid point, the lowest energy value from the different sets of conformers were used to construct a contour map using Xfarbe 2.3 (Fritz–Haber Institut der MPG, Berlin).[17] Local minima were found by a multistep procedure, where all structures that could contribute to local minima were rotated and optimized at local low-energy grid points, then re-optimized with no torsional angle restrictions. The global minimum was the lowest energy structure obtained and is found at $\phi/\varphi = -45°/-55°$. Only two local minima with relative energies of 2.8 and 5.5 kcal/mol above the global minimum are observed at $\phi/\varphi = 62°/48°$ and $\phi/\varphi = 58°/156°$, respectively. All minima appear to be contained in sharp energy wells, suggesting that interring flexibility is unlikely (Fig. 2). The global minimum of **1** has a conformation close to the −35°/−49° observed for the thioglycosidic linkage between subsites −1 and +1 in the complex studies with Cel7B.[6] The axially-linked conformer of **1**, is not however subject to the significant ring puckering constraints observed in the thioanalogue, a feature that validates the objectives of the modeling approach.

The pseudo-disaccharide **1** was synthesized from the triflate **2**[18] and the glycoside **5**[19] in a seven-step sequence (Scheme 1). Displacement of the triflate of **2** by tetrabutylammonium cyanide[20] led, in a 43% yield, to the methyl 4-cyano-β-D-glucoside **3**. 'Super hydride' reduction afforded the aminomethyl derivative **4** with 43% yield. Acetolysis of **5** catalyzed by sulfuric acid[21] followed by de-*O*-acetylation led in 71% yield to the triol **6**. The dialdehyde intermediate **7**, obtained by a periodate oxidative cleavage[22] of **6** between C-5 and C-6 was directly subjected to a double reductive amination[23] on **4**. Following coupling and de-*O*-benzylation, the disaccharide **1**,[24] obtained in 32% yield, was fully characterized.

The conformational study of **1** in solution was investigated by NMR to determine if the iminocyclitol adopts the $^1C_4$ conformation as assumed in the modeling studies. At 25°C, the title compound does not present a strained conformation, as indicated by broad signals of the methylenic protons next to *N*-1′. Complete attribution of ^{1}H and ^{13}C spectra could only be solved at 75°C in pyridine-d_5, unfortunately under these conditions a 5.2 Hz coupling constant between H-3′ and H-4′ prevented the assignment of the average conformation to either $^1C_4$ or $^4C_1$.

Inhibition studies of the *endo*-cellulase Cel7B from *Humicola insolens* were performed at pH 8.5 using cellotriose as substrate[25] and **1** appeared to act as a

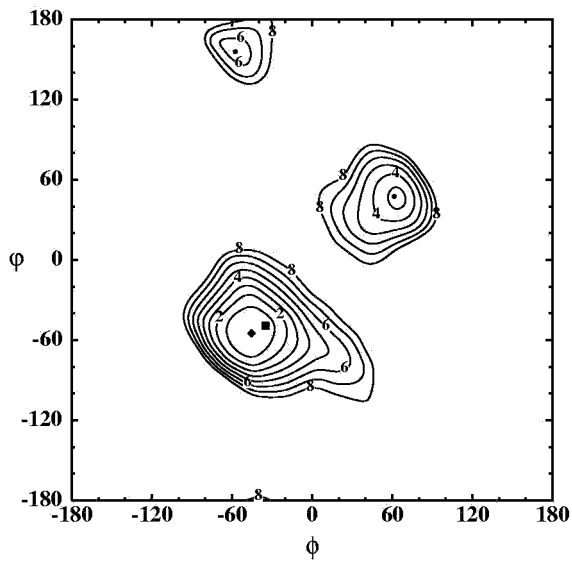
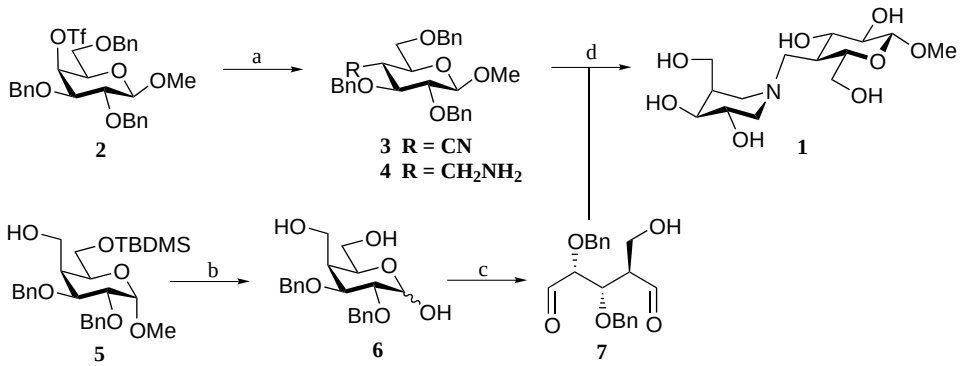


Figure 2. MM3(92) steric energy map of the axially-linked conformer of **1**. Isoenergy contour lines are found in 1 kcal/mol increments above the global minimum. Global minimum (◆) and local minima (●) are shown. The conformation of the thioanalogue of Cel7B is shown (■).



Scheme 1. Synthesis of the imino-disaccharide **1**. Conditions: (a) (i) Bu_4NCN, toluene (43%), (ii) $LiHBEt_3$ (15 equiv.), THF (43%); (b) (i) Ac_2O, H_2SO_4 (cat.) (ii) MeOH/MeONa (71% overall); (c) $NaIO_4$, H_2O/MeOH; (d) (i) $NaBH_3CN$, AcOH, MeOH (62%), (ii) H_2, Pd/C, MeOH, HCl (51%).

competitive inhibitor (K_i 200 μM). In comparison, cellobiose, the major reaction product of cellulose hydrolysis by Cel7B, inactivates Cel7B to a lesser extent (K_i 900 μM).[25] The K_i observed for **1** is similar to the one observed for a thio-trisaccharide substrate analogue.[26]

In conclusion, we have reported the rational design and the synthesis of the first *C*-linked imino-disaccharide cellulase inhibitor **1**. This inhibitor exhibits two features expected to contribute to binding to cellulase active sites at the time of formation of the Michaelis complex: (1) a 1-*N*-imino linkage that when protonated should interact with the nucleophile via an ion-pair, and (2) an axial glycosidic-like bond to an 'aglycon' to mimic substrate distortion. An extension of this methodology to the synthesis of an iminocyclitol motif into higher oligomers may be beneficial for glycoside hydrolase inactivation by increasing the affinity of the ligand in cellulases.

Acknowledgements

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24. Data for **1**: $[\alpha]_D$ −12.9 (*c* 0.49, H_2O); ^{13}C NMR (100 MHz, pyridine-d_5; 348 K): δ 105.54 (C-1), 78.45 (C-5), 76.65 (C-2), 76.22 (C-3), 72.20 (C-4′), 70.26 (C-3′), 64.50 (C-6), 63.07 (C-6′), 59.34 (C-7), 57.80 (C-2′), 53.62 (C-7′), 42.64 (C-4), 41.05 (C-5′); ^1H NMR (400 MHz, pyridine-

d_5; 348 K): δ 4.53 (1H, $J_{1,2}$ 7.37 Hz, H-1), 4.30 (1H, $J_{3',4'}$ 5.2 Hz, H-4′), 4.26 (1H, $J_{6'b,5'}$ 5.9 Hz, H-6′b), 4.21 (1H, $J_{3',2'a}$ 2.8 Hz, $J_{3',2'b}$ 2.8 Hz, H-3′), 4.14 (1H, $J_{6a,6b}$ −12 Hz, H-6a), 4.06 (1H, H-6b), 4.11 (1H, $J_{6'a,5'}$ 6.5 Hz, H-6′a), 3.86 (1H, H-2), 3.83 (1H, H-3), 3.52 (1H, $J_{5,6a}$ 4 Hz, $J_{5,6b}$ 4 Hz, H-5), 3.12 (1H, $J_{2'b,3'}$ 2.8 Hz, $J_{2'a,2'b}$ −11.5 Hz, H-2′b), 2.97 (3H, $J_{7'a,5'}$ 6.5 Hz, $J_{7'b,5'}$ 6.5 Hz, $J_{7'a,7'b}$ 0 Hz, $J_{7b,4}$ 5.7 Hz, H-7′a, H-7′b, H-7b), 2.91 (1H, $J_{2'a,3'}$ 5 Hz, H-2′a), 2.727 (1H, $J_{7a,4}$ 7 Hz, H-7a), 2.726 (1H, $J_{5',4'}$ 3.65 Hz, H-5′), 2.42 (1H, $J_{4,5}$ 9.95 Hz, H-4); HRMS (FAB) calcd for $C_{14}H_{27}NO_8$ $(M+H)^+$ 338.1815, found 338.1815.

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