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A FLUORESCENCE STUDY OF ISOFAGOMINE PROTONATION IN β-GLUCOSIDASE

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N-(10-chloro-9-anthracenemethyl)-isofagomine **5** and *N*-(10-chloro-9-anthracenemethyl)-1-deoxynojirimycin **6** were prepared, and their inhibition of almond β-glucosidase was measured. The isofagomine derivative **5** was found to be a potent inhibitor, while the 1-deoxynojirimycin derivative **6** displayed no inhibition at the concentrations investigated. Fluorescence spectroscopy of **5** with almond β-glucosidase at different pH values showed that the inhibitor nitrogen is not protonated when bound to the enzyme. Analysis of pH-inhibition data confirmed that **5** binds as the amine to the enzyme's unprotonated dicarboxylate form. This is a radically different binding mode than has been observed with isofagomine and other iminosugars in the literature.

Introduction

Sugar mimics containing a nitrogen atom in place of the ring-oxygen atom or anomeric carbon are some of the most potent inhibitors of glycosidases.[1] Many of these molecules are natural products most commonly being secondary metabolites in plants,[2] which suggest that they play a role protecting the plant from carbohydrate degrading pests. Examples of such molecules are 1-deoxynojirimycin (**1**), which is present in mulberries and obviously mimics D-glucose in the pyranose form and pyrrolidine **2** from tropical beans, which mimics D-fructose in the furanose form (Figure 1).[3] The nitrogen atom is very important for the inhibitory activity as the equivalent O-analogue is many orders of magnitude less potent.[4] The iminosugar does not necessarily have to be a strong base to be a glycosidase inhibitor, as pK_a's in the range from 3 to 10 are known among these compounds – yet basicity is normally a prerequisite for inhibition.[5] It is therefore obviously important to understand the function of the amine and especially whether it is actually the conjugate acid that is the inhibitor. Inhibition-pH data clearly show that iminosugars bind to β-glucosidase with one proton but whether that proton is bound at nitrogen or at one of the active site carboxylates is unknown.[6] In one case crystallography has shown that the inhibitor 4-*O*-glycopyranosyl isofagomine **3** (Figure 1) binds to cellulase as the conjugate acid[7] – yet it could be argued that **3** is a rather strong base (pK_a 8.4) making protonation particularly facile. Solid state NMR studies of labelled versions of azafagomine **4** (pK_a 5.3)[8] led to the conclusion that **4** bound to β-glucosidase as the conjugate acid and to isomaltase and glycoamylase as the base.[9] These studies suggest that iminosugar glycosidase inhibitors may or may not be protonated in the active site depending on the inhibitor's base-strength and the enzyme. However it could be interesting to observe the inhibitor protonation relative to pH because the inhibition of glycosidases by these compounds is heavily pH dependant.[4,9]

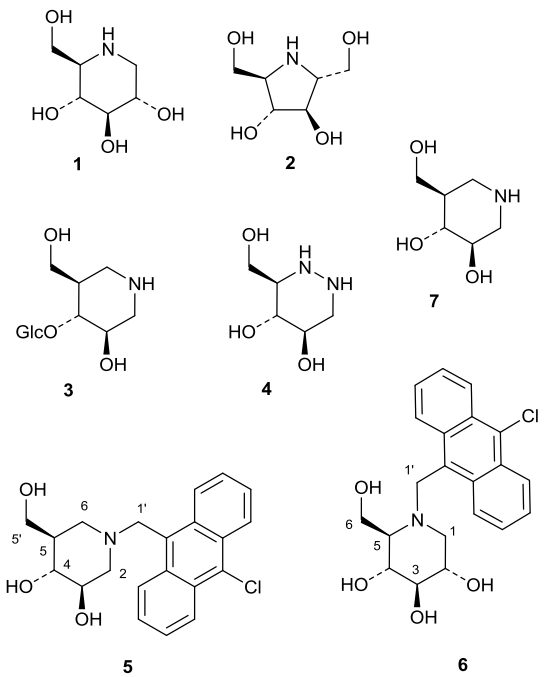


Figure 1. Iminosugar inhibitors

Photo-induced electrontransfer has been used as a principle in fluorescent pH indicators where an aminogroup attached to a fluorescent chloroanthracene quench the emission; however when it is protonated it does not.[11] The idea behind this study

was to use this principle to study iminosugar protonation whenbound to the enzymes. We have prepared compounds **5** and **6** (Figure 1), iminosugar analogues of the above mentioned pH indicators, and studied their inhibition of β-glucosidase.

Results

Compounds **5** and **6** were prepared from the corresponding inhibitors isofagomine (**7**) and 1-deoxynojirimycin (**1**), respectively (Figure 1). Isofagomine (**7**) was prepared as previously described,[12] while **1** was obtained as the tetra-*O*-benzyl derivative according literature methods[13] and then debenzylated by hydrogenolysis with Pd/C catalyst in dilute

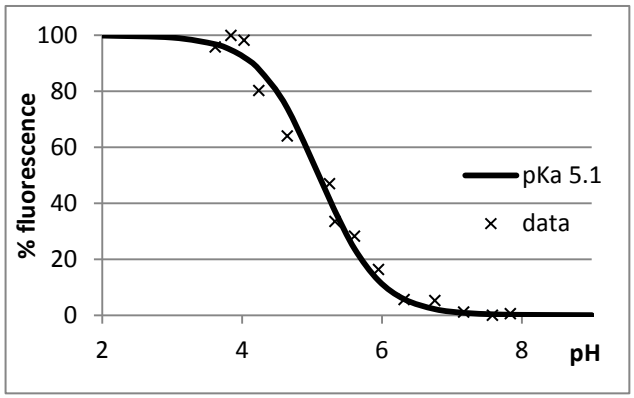


Figure 2. Fluorescence titration of **5**. Shown is the percentage of fluorescence emission as function of pH.

HCl solution. Attachment of the 10-chloroantracene group was performed by reductive amination of the amines **1** and **7** with 10-chloro-9-anthralaldehyde using sodium cyanoborohydride.

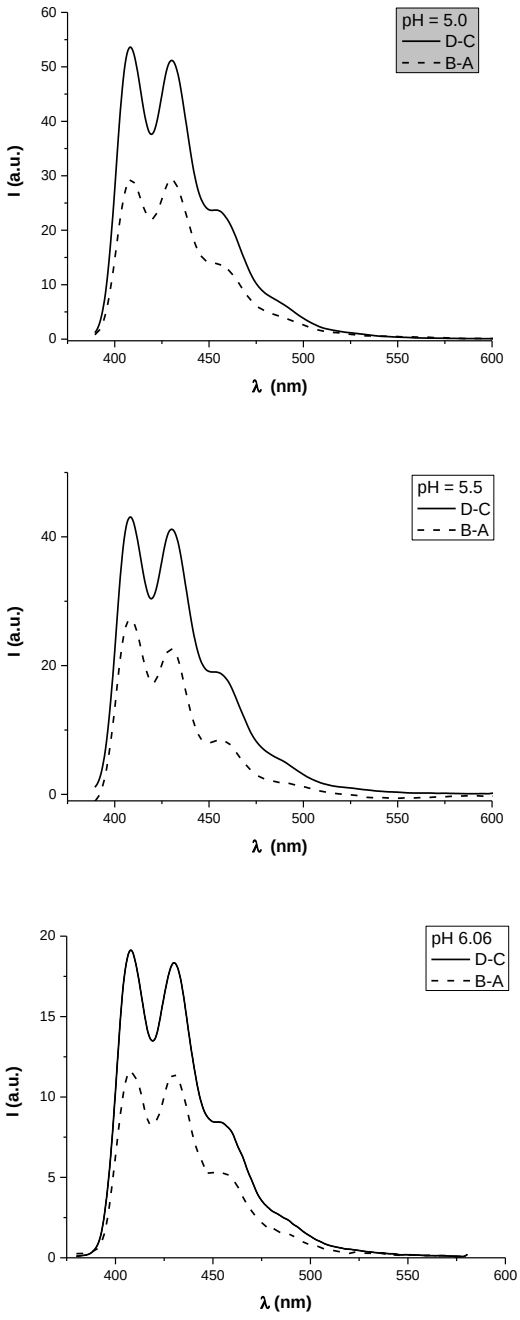
As predicted antracenyl compounds **5** and **6** displayed pH dependant fluorescence with a large fluorescence emission when protonated, and essentially no fluorescence in basic solution. The pK_a of **5** and **6** was measured by fluorescence titration (Figure 2), which gave a value 5.1 for both compounds. This may seem low compared to **1** (pK_a 6.7)[10] and **7** (pK_a 8.4)[8], however it is perfectly in line with previous chloroantracenemethyl derivatives, which also were 2-3 orders of magnitude less basic than the parent amine.[11] The reason is possibly partially electronic effects and steric hindrance towards protonation.

pH	5.00	5.85	6.80	6.85	7.58	8.20
K_i (μM)	11.3	1.35	1.00	1.78	0.64	0.49

Table 1. Inhibition constants for the inhibition of β-glucosidase by **5** at different pH values. K_i values were measure in phosphate buffer containing 10% DMSO to ensure complete solubility of **5**.

In a nitrophenol assay the compounds were tested for inhibition of almond β-glucosidase. The isofagomine derivative **5** was found to be a competitive inhibitor with K_i values between 0.49 and 11 μM in the pH range 5 to 8.2 (Table 1), while the 1-deoxynojirimycin analogue **6** on other hand did not display appreciable competitive inhibition at a concentration of 200 μM and was therefore not further studied.

Fluorescence of **5** upon binding to almond β-glucosidase was studied by measuring fluorescence emission upon excitation at 358 nm at 5 different pH values of A) β-glucosidase alone, B) β-glucosidase and **5**, C) a blank solution and D) **5** alone. The substraction spectra B-A and D-C, which represents the net effect of the inhibitor **5** with or without the enzyme present, are shown in Figure 3. The enzyme used was chromatographically purified β-glucosidase that essentially showed one protein band in the SDS-page and its concentration was 40 μM on assumption it was completely active. Concentration of **5** was 0.8 μM. This means that, with the K_i values given in Table 1, most of the inhibitor is binding the enzyme active site even if 75% of the enzyme is an inactive form (denatured).



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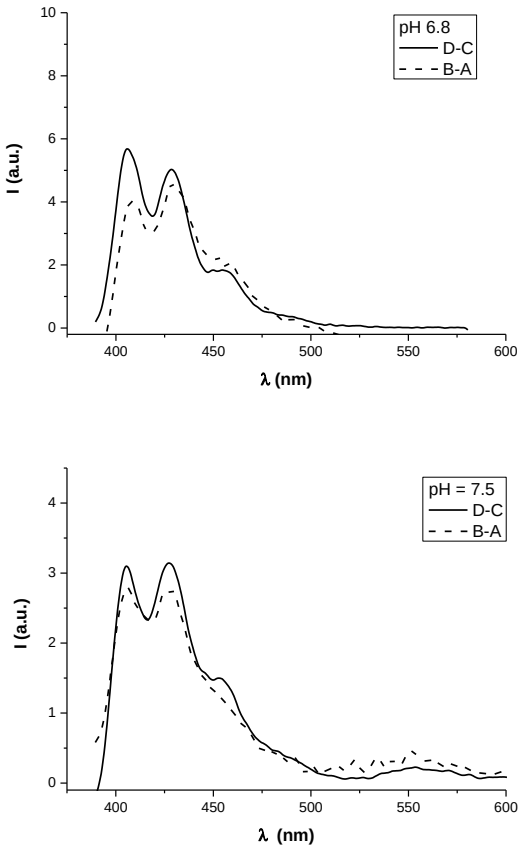

Figure 3. Subtracted fluorescence spectra of **5** with and without almond β-glucosidase at 5 different pH. (from top to bottom 5.0, 5.5, 6.1, 6.8, 7.5). Solid lines (D-C) and dashed lines (B-A) represents the net effect of **5** without or with enzyme present. All solutions contain 90% phosphate buffer and 10% DMSO.

Discussion

The fluorescence spectra show that at pH 6.8 and 7.5 the fluorescence of **5** is essentially unaffected by the presence of the enzyme (Figure 3). Due to the low K_i of the inhibitor at these pH (Table 1) most if not all of the inhibitor must be bound to the active site but since there is no fluorescence the nitrogen of **5** is not protonated or the fluorescence is quenched by another functional group in the enzyme. At pH values 6.06, 5.5 and 5.0 we see an increasing fluorescence from **5** in the presence (B-A) and absence of enzyme (D-C) with latter consistently being twice as high. This means that binding of the inhibitor to the enzyme decrease it fluorescence if not completely then partially. The simplest interpretation of these data is that **5** is not protonated in the active site of β-glucosidase. The fact that significant fluorescence is seen in the enzyme containing samples (B-A, Figure 3) at low pH could simply be because of the presence of some unbound inhibitor, as we see the K_i tends to rise at low pH to a value of 11.3 μM at pH 5.

Inhibitors such as **1** and **7** are known to give a bell-shaped curve when $1/K_i$ is plotted versus pH, which means that inhibition is highest at a certain pH which depend on the inhibitor's and the enzyme's pK_a values.[4,14] This behaviour appears widespread if not the norm among iminosugar inhibitors[14] and detailed analysis of the data show that the inhibitor and glycosidase in essentially all cases binds as an EHI complex i.e. with just one proton.[6] The K_i data of Figure 1 does however not appear to conform to this norm. A plot of $1/K_i$ versus pH (Figure 4) shows that the data does not conform to the curve one would expect from an inhibitor of pK_a 5.1 binding to β-glucosidase as the EHI-complex. However surprisingly the data fit well with the inhibitor binding as an EI-complex i.e. without any protons. In other words this means that binding follow the deionisation of the least acidic carboxylate in the enzyme (pK_a 6.7) and that unprotonated **5** binds exclusively to dicarboxylate form of the enzyme.

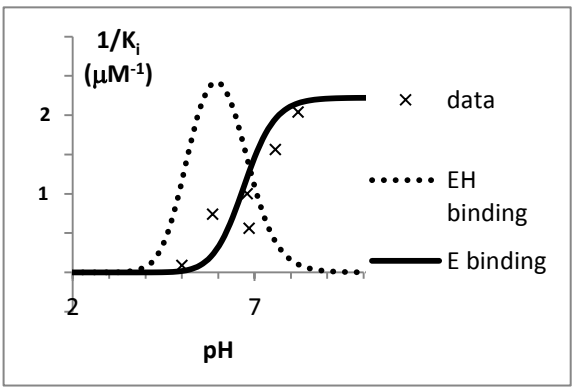

Figure 4. 1/*K*i versus pH for inhibition of almond pH glucosidase by **5** in phosphate buffer containing 10% DMSO. The data is compared with predicted curves for inhibition if **5** binds to the enzyme with one proton (EH binding) or none (E binding). It is seen that the E binding curve can fit the data, while the EH curve cannot.

The pH independent binding constant is $K_i(4)$ = [I][E]/[EI] = 0.45 μM. This is in accordance with the fluorescence data discussed above as no fluorescence is seen when **5** binds to the enzyme and none would be seen from an EI complex.

These results show that in compound **5**, unlike its parent isofagomine and other iminosugars, the nitrogen atom has no importance for binding as it is can neither be involved in salt bridge or hydrogen bonding interactions with the active site carboxylate groups (Figure 5). The attachment of the anthracyl group has apparently made protonation of any of the basic groups in the active site unfeasible presumably due to steric reasons. Perhaps the negatively charged carboxylate groups repel each other and thus provide enough space for **5** to enter the active site. Compound **5** is still a strong and competitive inhibitor and this must be due to lipophilic and/or aromatic interactions of the anthracyl group as the nitrogen can play no role and simple monosaccharides are poor inhibitors. To our

knowledge compound **5** is the first glycosidase inhibitor where EI complex formation has been observed.

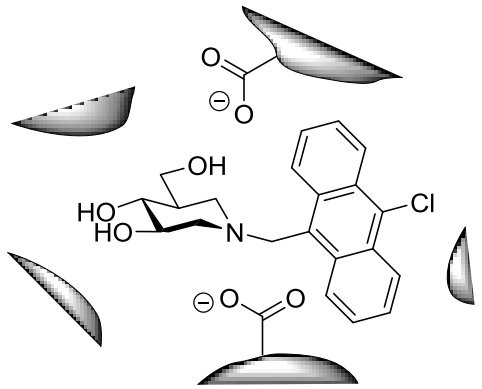

Figure 5. Depiction of the nonfluorescent form of **5** binding to dicarboxylate form of the β-glucosidase active site. Binding is presumably primarily caused by lipophilic interaction with the aromatic system and hydrogen bonding to the 3 hydroxyl groups.

Conclusions

This work has shown that 10-chloro-9-anthracyl-isofagomine (**5**) is a competitive inhibitor of β-glucosidase and that it binds as the unprotonated amine form. This is perhaps not so surprising as **5** has a comparatively low basicity (pK_a = 5.1). It is more remarkable that **5** exclusively bind to the uncatalytic dicarboxylate form of β-glucosidase. This is in contrast to isofagomine (**7**) and other iminosugars that have so far been analysed.[6,7,10,12] The fact that a relatively simple alkylation of **7** changed its binding behaviour profoundly, suggest that similar behaviour could be widespread among iminosugar analogues.

Experimental

Procedure for synthesis of 5 or 6. A mixture of iminosugar (0.15 mmol) and 10-chloro-9-anthralaldehyde (36 mg, 0.15 mmol) in methanol (15 ml) and $CHCl_3$ (1 ml) was heated to reflux until a homogeneous solution was formed. Then $NaCNBH_3$ (18 mg, 0.29 mmol) was added. After 10 min HOAc (0.1 ml) was added, and the mixture heated to about 60 ^oC for 4 h and furthermore stirred at room temperature for 24 h. The solution was evaporated, and the residue chromatographed with $CHCl_3$ until the lipophilic compounds were eluted (10-chloro-9-anthralaldehyde and 10-chloro-9-anthracenemethanol, ~65%) and then changed to $CHCl_3$/MeOH (8:1) giving the product in about 30% yield.

***N*-(10-chloro-9-anthracenemethyl) isofagomine (5).** yellow crystals, ^{1}H-NMR (500 MHz, D_2O): δ 8.08 (d, 2H, J 8.8 Hz), 7.90 (d, 2H, J 8.8 Hz), 7.46 (m, 4H), 4.95 (s, 2H, H-1'), 3.33-3.71 (m, 6H, H-2eq, H-6eq, H-3, H-4, H-5'a, H-5'b), 3.07 (m, 2H, H-2ax, H-6ax), 1.74 (m, 1H, H-5). ^{13}C-NMR (500 MHz, d6-DMSO): 134.5 (1C), 132.0, 131.3 (4C), 127.8, 127.6, 125.6, 124.8 (8C), 121.6 (1C), 71.0, 68.0 (C-3 & C-4), 58.8 (C-5'), 55.1, 54.0, 51.2 (C-1', C-2 & C-6), 40.8 (C-5) ppm. UV (nm): 399 (3.93), 378 (3.96), 360 (3.76), 344 (3.46), 327 (3.14), 251 (>4.32), 220 (4.13). HRMS(ES) calc. for M+H$^+$ 372.1366, found 372.1364.

***N*-(10-chloro-9-anthracenemethyl) 1-deoxynojirimycin (6).** yellow crystals, ^{1}H-NMR (500 MHz, d6-DMSO): δ 8.6 (d, 1H), 8.5 (d, 1H,), 8.2 (dd, 1H), 7.9 (dd, 2H), 7.7 (m, 4H), 5.5 (m, 1H, H-1'a), 5.4 (m, 1H, H-1'b), 3.8 (bd, 1H, H-6a), 3.7 (dd, 1H, H-6b), 3.6 (m, 1H, H-2), 3.4 (m, 5H, H-4, 4OH), 3.2 (t, 1H, H-3), 3.1 (dd, 1H, H-1eq), 2.8 (bs, 1H, H-5), 2.7 (dd, 1H, H-1ax). ^{13}C-NMR (500 MHz, d6-DMSO): 132.9, 130.2 (2C), 127.8, 127.2, 126.7, 126.2 (8C), 125.5 (2C), 124.3 (2C), 76.7 (C-3), 67.7, 66.8 (C-2 & C-4), 60.1 (C-5), 57.6 (C-6), 55.2 (C-1'), 46.3 (C-1) ppm. HRMS(ES) calc. for M+H$^+$ 388.1310, found 388.1391.

Procedure for fluorescence measurements. The inhibitor **5** was dissolved at 0.012 mg/mL in DMSO and diluted 4 times with DMSO to create a stock solution of concentration 8.05 μM. β-glucosidase from almonds was obtained from Sigma (G4511, 10-30 units/mg).
Four solutions were made:

A. 3 mg β-glucosidase G4511 in 0.45 mL phosphate buffer and 0.05 mL DMSO. Protein-concentration was 40 μM.
B. 3 mg β-glucosidase G4511 in 0.45 mL phosphat buffer and 0.05 mL inhibitor stock solution. Protein-concentration was 40 μM and [**5**] was 0.8 μM
C. 0.45 mL phosphat buffer and 0.05 mL DMSO
D. 0.45 mL phosphat buffer, and 0.05 mL inhibitor stock solution. [**5**] was 0.8 μM

Fluorescence spectra was taken of samples A-D irradiating with light of wavelength 358 nm on a Perkin Elmer LS50 instrument. The subtracted spectra (B-A) and (D-C) are given in Figure 3.

Procedure for measuring β-glucosidase inhibition. These experiments were performed at 25 ^oC in an aqueous phosphate buffer (0.1M) containing 10% DMSO to make sure the inhibitor was completely dissolved. As substrate 4-nitrophenyl β-D-glucopyranoside was used, while the enzyme was almond β-glucosidase of the quality described above. In a multisample spectrophotometer 5-8 reactions with variant substrate concentration (1-20 mM) was simultaneous started by addition of enzyme (0.3 nM) and the formation of 4-nitrophenol followed by measuring absorbance at 400 nM in the first 2 minutes. This was performed with and without the presence of the inhibitor in a concentration close to the expected K_i. From these data the K_m could be determined with and without inhibition and from those values K_i was calculated (using K_i = [I]/(K_m'/K_m - 1).

Analysis of inhibition-pH data. The method from reference 6 was used. 1/Ki have the following dependency of H concentration (for details see ref 6):

$$\frac{1}{K_{i(obs)}} = \frac{1}{K_{i(1)}*\left(\frac{K_{AE1}*K_{AE2}*K_{AI}}{H^3}+\frac{K_{AE1}*K_{AE2}}{H^2}+\frac{K_{AE1}*K_{AI}}{H^2}+\frac{K_{AE1}}{H}+\frac{K_{AI}}{H}+1\right)} + \frac{1}{K_{i(2)}*\left(\frac{K_{AE2}*K_{AI}}{H^2}+\frac{K_{AE2}}{H}+\frac{K_{AI}}{H}+1+\frac{K_{AI}}{K_{AE1}}+\frac{H}{K_{AE1}}\right)} + \frac{1}{K_{i(3)}*\left(\frac{K_{AE2}}{H}+\frac{K_{AE2}}{K_{AI}}+1+\frac{H}{K_{AI}}+\frac{H}{K_{AE1}}+\frac{H^2}{K_{AI}*K_{AE1}}\right)} + \frac{1}{K_{i(4)}*\left(1+\frac{H}{K_{AI}}+\frac{H}{K_{AE2}}+\frac{H^2}{K_{AI}*K_{AE2}}+\frac{H^2}{K_{AE1}*K_{AE2}}+\frac{H^3}{K_{AI}*K_{AE1}*K_{AE2}}\right)}$$

The EHI and EI binding curves shown in Figure 4 was made in a spreadsheet by entering the relevant acid constants (**5**: $10^{-5.1}$, β-glucosidase: $10^{-4.4}$ and $10^{-6.7}$ and reasonable values for the

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constants K_i(1-4). For the EHI binding curve $K_i(3) = 3 \times 10^{-7}$ while K_i(1,2 and 4) was set to 1. For the EI binding curve $K_i(4) = 4.5 \times 10^{-7}$ while K_i(1-3) was set to 1.

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Supporting information
NMR spectra of compounds **5** and **6** (6 pages) are available.

Notes and references
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