Azafagomine hydrazones: an argument against a "Flat" transition state in glycoside cleavage

Steen Uldall Hansen and Mikael Bols *

Department of Chemistry, Aarhus University, Langelandsgade 140, DK-8000 Aarhus, Denmark. E-mail: mb@kemi.aau.dk; Tel: +45 89 42 39 64; Fax: +45 86 19 61 99

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Two hydrazones, (3*R*,4*R*,5*R*)-4,5-dihydroxy-3-hydroxymethyl-2,3,4,5-tetrahydropyridazine (**2**) and (4*R*,5*R*)-4,5-dihydroxy-6-hydroxymethyl-2,3,4,5-tetrahydropyridazine (**3**), were obtained in good yield from oxidation of 1-azafagomine (**1**). Both **2** and **3** have the half-chair conformations commonly believed to be important in good transition state analogues and an almost identical molecular composition to the strong glucosidase inhibitor **1**. Yet **2** and **3** are very poor glucosidase inhibitors, which suggests that the half-chair geometry is far less important for a transition state analogue than its ability to accept protons.

Introduction

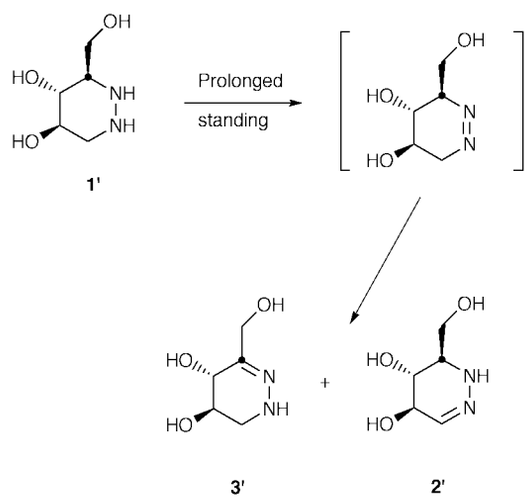
Glycosidase inhibitors and transition state analogues have been subject to intense research in the last decade.[1] One of the new inhibitors discovered in this period is the hydrazine 1-azafagomine (**1**), which is a potent inhibitor of both α- and β-glucosidases.[2] One of the problems that has been intensely debated is whether geometry or charge is the more important factor when designing a transition state analogue inhibitor. The half-chair geometry is considered to be the shape of the transition state of glycoside cleavage, with one important argument being that aldonolactones, lactams and related compounds are inhibitors of glycosidases.[1,3,4] This theory has been extended as far as the idea that the half-chair geometry is more important for a compound mimicking the transition state structure than having basic groups present,[3,4] and that compounds such as **1** that do not have this geometry are strong binding substrate analogues and not transition state analogues.

In the present work we have made two very close analogues of **1** that do have the half-chair geometry, in order to investigate their ability to inhibit glycosidases.

Results and discussion

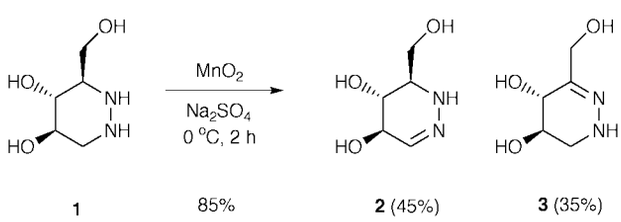
We recently carried out a synthesis of 3-(^{13}C) labelled **1**.[5] It was subsequently observed that in an originally pure sample of labelled **1** that had been left in an NMR tube for a long period of time, two new and less polar compounds had been formed. Chromatographic purification allowed small quantities of these labelled substances to be isolated and subjected to partial spectral characterisation. One of the compounds had a C-3 ^{13}C signal at 146.1 ppm, while the other had a C-3 signal at 57.3 ppm. Furthermore the ^{1}H NMR spectrum of the latter showed a proton at δ 6.42. It had previously been observed in our group that dilute solutions of **1**, upon prolonged standing at room temperature, tended to undergo decomposition, even though the exact nature of this chemistry was unclear at the time. Nevertheless both observations suggested that the hydrazine **1** was undergoing aerial oxidation.

It is known that hydrazines upon oxidation form the azo-compound (or diazene), which readily undergoes double bond isomerisation to the more stable hydrazone (Scheme 1).[6] Based on this information and the above NMR data, the structures **2′** and **3′** could be assigned to the byproducts. To confirm this it was decided to try to synthesise the two compounds in larger amounts.



Scheme 1 Oxidation of 1-azafagomine (**1**). ● means ^{13}C.

MnO_2 has been reported to be a good reagent for oxidation of hydrazines.[6] Treatment of (−)-**1**[7] with MnO_2 and Na_2SO_4 in MeOH according to this procedure led to an excellent yield, 84%, of the two hydrazones **2** and **3** (Scheme 2). They were separated and obtained in 45 and 35% yield, respectively.



Scheme 2 Synthesis of hydrazones **2** and **3**.

The two hydrazones **2** and **3** are interesting molecules, because they have almost identical structures to the potent glucosidase inhibitor **1** except for the presence of a double bond. The effect of the double bond is twofold. It decreases the basicity of the nitrogen atoms and flattens the ring from a chair

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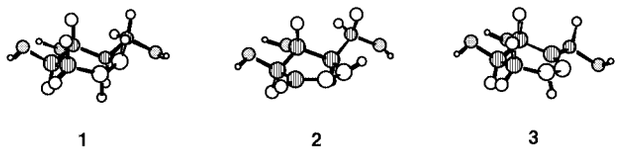


Fig. 1 Molecular models of **1**–**3** showing the $^4C_1$ conformation of **1**, the $^4H_3$ conformation of **2** and $^6H_5$ conformation of **3**.

to a half-chair. Molecular modelling of **2** and **3** clearly shows that both molecules prefer a half-chair conformation, where either the atoms N2–N1–C6–C5 or C4–C3–N2–N1 are almost perfectly in one plane (Fig. 1). ^{1}H NMR of **2** confirms this, since the vicinal couplings J_{34} and J_{45} are large (8–10 Hz), showing a $^4H_3$ conformation. The ^{1}H NMR of **3** is rather poorly resolved, but modelling shows the $^6H_5$ conformation (Fig. 1). A comparison of the modelled structures with that of **1** showed that the hydroxy groups are superimposable within 0.15 Å, so the OH groups are expected to be able to interact with an enzyme as do those of **1**. On the other hand hydrazones are weaker bases than hydrazines.[8] So **2** and **3** are weaker bases than **1**, which has a pK_a of 3.9. Therefore compounds **2** and **3** are ideally suited to test the hypothesis of geometry *contra* charge in transition state analogue design. If geometry is more important, in a transition state analogue, than the ability to accept charge, **2** and **3** should be expected to be at least fairly strong glucosidase inhibitors.

Therefore the inhibition of glucosidases by **2** and **3** was investigated (Table 1). Both **2** and **3** were very poor inhibitors of three glucosidases that **1** inhibits strongly. In the most extreme case, almond β-glucosidase, the hydrazones did not inhibit the enzyme even at 1 mM, meaning that the difference between **2/3** and **1** in binding to the enzyme is over 3000. Towards α-glucosidase and isomaltase some inhibition was seen, particularly by **2**.

The obvious explanation for the poor inhibition of **2** and **3** is that thay lack sufficient basicity to become charged and form an electrostatic bond to the enzyme. The fact that they have half-chair geometry does not appear to compensate the loss in binding from reduced base strength. The results suggest that geometry is less important, or at least overrated, when designing glycosidase transition state analogues.

Finally, one may ask why is it that aldonolactones, such as D-glucono-1,5-lactone, are, in some cases, quite potent glycosidase inhibitors, if geometry is not a crucial factor? The explanation may be, as suggested by Heightman and Vasella,[1] that the lactone carbonyl group can hydrogen bond to the catalytic carboxylate of the enzyme. The hydrazones **2** and **3** lack this protruding group and would lack this contribution to binding.

Experimental

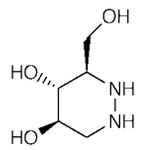
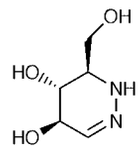
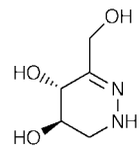
General

^{13}C NMR and ^{1}H NMR spectra were recorded on a Varian Gemini 200 instrument. Mass spectra were obtained on a Micromass LCT-QTOF instrument. Concentrations were performed on a rotary evaporator at temperatures below 40 °C. Molecular modelling was carried out using CS Chem 3D pro ver. 3.5.1 from CambridgeSoft on MacIntosh Powerbook 3400c. Structures were minimised using MOPAC with the potential function AM1.

(3*R*,4*R*,5*R*)-4,5-Dihydroxy-3-hydroxymethyl-2,3,4,5-tetrahydropyridazine (2) and (4*R*,5*R*)-4,5-dihydroxy-6-hydroxymethyl-2,3,4,5-tetrahydropyridazine (3)

To 70 mg (0.47 mmol) of D-azafagomine (**1**) in 15 mL MeOH at 0 °C was added 105 mg (0.74 mmol) Na_2SO_4 and 315 mg (3.62 mmol) of activated MnO_2. The solution was stirred for 2 h at 0 °C and then filtered through a thin layer of Celite. After evaporation the two hydrazones could be separated by flash chromatography in EtOAc–EtOH (5:1). Total yield: 58 mg (84%). Yield of **2**: 31 mg (45%). $[\alpha]_D^{22}$ +9.6° (H_2O). ^{1}H NMR (D_2O): δ 6.42 (d, 1H, H6, $J_{6,5}$ 1.8 Hz), 3.96 (dd, 1H, H5, $J_{5,4}$ 8.4 Hz), 3.64 (dd, 1H, H3′a, $J_{3'a,3'b}$ 12.1 Hz, $J_{3'a,3}$ 3.3 Hz), 3.45 (m, 2H, H3′b, H4), 2.94 (ddd, 1H, H3, $J_{3,4}$ 10.3 Hz, $J_{3,3'b}$ 6.6 Hz). ^{13}C NMR (D_2O): 141.7 (C5), 68.7, 66.8 (C4, C5), 58.1, 57.3 (C3, C3′). HRMS (ES): *m*/*z* 169.0595 (M + Na^+), calcd for $C_5H_{10}N_2O_3$ + Na^+: 169.0589. Yield of **3**: 24 mg (35%). $[\alpha]_D^{22}$ −131.5° (H_2O). ^{1}H NMR (D_2O): δ 4.06 (d, 1H, H3′a, $J_{3'a,3'b}$ 13.6 Hz), 3.85 (d, 1H, H3′b), 3.72 (m, 2H, H4, H5), 2.87 (m, 2H, H6). ^{13}C NMR (D_2O): δ 146.1 (C3), 65.3, 63.2 (C4, C5), 60.4 (C3′), 43.2 (C6). HRMS (ES): *m*/*z* 169.0586 (M + Na^+), calcd for $C_5H_{10}N_2O_3$ + Na^+: 169.0589.

Table 1 The inhibition constants (K_i) in μM for the competitive inhibition of glycosides by the inhibitors **1**–**3**

	K_i/μM		
Structure			
Compound	**1**	**2**	**3**
β-Glucosidase	0.32	>1000	>1000
α-Glucosidase	6.9	182	244
Isomaltase	0.27	237	758

Enzymatic assays

The substrates 4-nitrophenyl α- and β-D-glucopyranoside, and the enzymes α-glucosidase (yeast, Sigma G 5003), isomaltase (yeast, Sigma I 1256) and β-glucosidase (sweet almonds, Sigma G 4511) were purchased from Sigma Chemical Co. All assays were carried out in a sodium phosphate buffer (0.05 M, pH 6.8) at 25 °C. Formation of the product, 4-nitrophenol, was measured continually at 400 nm using a Milton Roy Genesys 5 spectrometer. In all kinetic runs less than 1% of the initial substrate was consumed, assuring the constancy of the substrate concentration.

Inhibition constant (K_i) determinations were performed as follows: Two thermostatted solutions of (1) 1 ml of 0.1 M buffer, 800 μl substrate in varied concentration ([S] = 0.25K_M to 4K_M, where K_M is the Michaelis constant for the substrate) and 100 μl water and (2) 100 μl enzyme were mixed, and the reaction was immediately monitored. From 5 experiments with varied substrate concentration initial reaction rates were calculated from the slope of the 1st order plot of product absorption *vs.* reaction time. K_M and V_{max} (maximum rate of substrate turnover) were calculated from a Hanes plot. From 5 experiments the Michaelis constant in the presence of the inhibitor (K_M') was calculated from the Hanes plot and from K_M and K_M', K_i was calculated.

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