The Inhibition of Bovine Kidney Hexosaminidase by *N*-Acetylglucosamine-Related 1,2,3- and 1,2,4-Triazoles Is in Agreement with an '*anti*'-Protonation

by **Narendra Panday** and **Andrea Vasella***

Laboratorium für Organische Chemie, ETH-Zentrum, Universitätstrasse 16, CH-8092 Zürich

The *N*-acetylglucosamine-related 1,2,4-triazole **14** and 1,2,3-triazole **16** have been prepared by *N*-acetylation of the known amines **19** and **20**, and their K_i values determined against bovine kidney β-*N*-acetylglucosaminidase, a mammalian hexosaminidase. The 1,2,3-triazole **16** ($K_i = 4$ μM) is a markedly weaker inhibitor than the isosteric azoles **13**–**15**. The K_i value of the 1,2,4-triazole **14** (0.034 μM) is smaller than that of the tetrazole **13** (0.2 μM), but larger than that of the imidazole **15** (0.0035 μM), confirming the correlation between inhibitory strength and basicity of the azole, as expected on the basis of an *anti*-protonation mechanism of mammalian hexosaminidases.

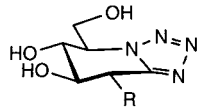
Introduction. – Retaining β-glycosidases are inhibited by the triazole **2**, but hardly by its isomer **4** (*Fig.*) [1–3]. This has been taken as strong evidence that inhibition by these inhibitors depends on (partial) protonation of the heteroatom corresponding to the glycosidic O-atom by the catalytic acid of the enzyme, that protonation occurs laterally, and that protonation of the substrate must occur in more or less the same way as of these transition-state-analog inhibitors [4], *i.e.*, laterally and not from above [1], as had been deduced earlier [5][6]. This contention was confirmed by a correlation of basic and inhibitory properties for the azoles **1**–**3** and their *manno*- and *galacto*-analogues against β-glycosidases from families 1 and 2[1]) [2][7]. It was further evidenced by crystal-structure analyses [8] of β-glycosidases in complex with substrates or substrate analogues and by modeling studies [9]. These led to the conclusion that lateral protonation is a general feature of β-glycosidases, and that these enzymes make use of one of two protonation trajectories that serve as basis to classify β-glycosidases into *syn*- and *anti*-protonating enzymes [9].

The K_i values of the *N*-acetylglucosamine-related tetrazole **7**, the imidazole **9**, and the pyrroles **11** and **12** have been determined against bovine kidney β-*N*-acetylglucosaminidase [11][12][14] and a mammalian hexosaminidase[2]) [16] (a *bona fide* member of the family 20 β-*N*-acetylglucosaminidases [17][18]). These β-*N*-acetylglucosaminidases appear to be '*anti*'-protonators, as indicated by a crystal-structure analysis and by

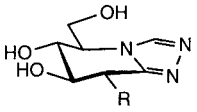
---

[1]) Families 1 and 2 refer to the β-glucosidase from *C. saccharolyticum* and the β-galactosidase from *E. coli*, respectively. The β-mannosidase from snail (used for the determination of the inhibitory properties of the *manno*-azoles) has not yet been assigned to any family.

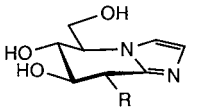
[2]) Mammalian hexosaminidases are lysosomal enzymes (pH optimum $\approx$ 4.4) with both β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities ([15][16] and refs. cit. therein). According to the amino-acid sequence determined for some of these enzymes or their genes [17], they are members of family 20 glycosyl hydrolases.



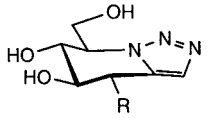
1 R = OH, K_i = 5 μM [10]
7 R = NHAc, K_i = 0.2 μM [11]



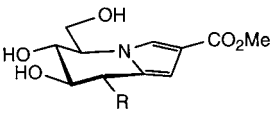
2 R = OH, K_i = 0.17 μM [2]
8 R = NHAc, K_i = 0.034 μM [this work]
13 R = NH_2



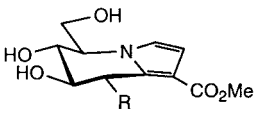
3 R = OH, K_i = 0.02 μM [2]
9 R = NHAc, K_i = 0.0035 μM [a]) [12]



4 R = OH, K_i = 1000 μM [a]) [1]
10 R = NHAc, K_i = 4 μM [this work]
14 R = NH_2



5 R = OH, K_i = 1150 μM [a]) [13]
11 R = NHAc, K_i = 19 μM [14]



6 R = OH, K_i = 410 μM [a]) [13]
12 R = NHAc, K_i = 20 μM [14]

[a]) K_i Value estimated on the basis of IC_{50} ($K_i \approx 0.5\ IC_{50}$).

Figure. *Inhibition constants of tetrahydroazolopyridine-type inhibitors against β-glucosidases from* Caldocellum saccharolyticum (**1**–**6**) *at pH 6.8 and 37° and β*-N*-acetylglucosaminidase from bovine kidney* (**7**–**12**) *at pH 4.2 and 37°*

the K_i values for **7**, **9**, **11**, and **12**[3]) [9][18]. Since the mechanism of action of hexosaminidases of family 20 differs markedly from that of glycosidases of families 1 and 2 with regard to the nature of the catalytic nucleophile [18][20][21], we considered it useful to determine also the inhibition by the 1,2,4-triazoles **8** and **10**, considering that substituents on the azole moiety (as, *e.g.*, in **5**, **6**, **11**, and **12**) may strongly influence the inhibitory properties of azolopyridine-type inhibitors [13][22][23].

Results and Discussion. – The *N*-acetylglucosamine-derived 1,2,4-triazole **8** and 1,2,3-triazole **10** were prepared by acetylation of the amines **13** and **14** [13] with Ac_2O in THF/MeO 1:1, followed by *O*-deacetylation with NH_3 in MeOH. Purification by FC gave **8** in 66% and **10** in 87% yield. The J(H,H) values confirm the $^7H_6$ conformation as it has been observed for the related tetrahydroazolopyridines **7** and **9** [11][12].

The 1,2,4-triazole **8** and the 1,2,3-triazole **10** inhibit *β*-*N*-acetylglucosaminidase from bovine kidney competitively with K_i values of 0.034 and 4 μM, respectively, at pH 4.2 and 37°. The 1,2,3-triazole **10** is a slightly stronger inhibitor than the pyrrolopyridine inhibitors **11** and **12**, but a much weaker inhibitor than the tetrazole **7**, the 1,2,4-triazole **8**, and the imidazole **9**. This is in keeping with the requirement of a lateral '*anti*'-protonation of the 'glycosidic heteroatom'. In agreement with the extrapolated correlation between the basicity of the isosteric inhibitors **7**–**9** and their inhibition of mammalian hexosaminidases, the 1,2,4-triazole **8** is a stronger inhibitor than the tetrazole **7**, but weaker than the imidazole **9**, the difference between the inhibitory strength of **7** and **8** ($\Delta\Delta G_{K_i} = 1.1$ kcal/mol) and of **8** and **9** ($\Delta\Delta G_{K_i} = 1.4$ kcal/mol) being of similar magnitude as the corresponding values for the glucose analogues **1**–**3** ($\Delta\Delta G_{K_i} = 2.1$ and 1.3 kcal/mol).

[3]) And, similarly, by the inhibition by 2-acetamido-2-deoxyglucono-1,5-lactone (0.16 μM), the corresponding hydroximo derivative (0.45 μM) and the corresponding lactam (1.8 μM) [19].

We thank the *Japanese Human Science Fund* and *Oxford Glycosciences Ltd.*, Abingdon, UK, for generous support.

Experimental Part

General. Solvents were distilled. TLC: *Merck* silica gel *60F-254* plates; detection by heating with mostain (400 ml of 10% H_2SO_4 soln., 20 g of $(NH_4)_6Mo_7O_{24}\cdot 6H_2O$, 0.4 g of $Ce(SO_4)_2$). Flash chromatography (FC): silica gel *Fluka 60* (0.04–0.063 mm). ^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz): chemical shifts δ in ppm and coupling constants J in Hz. FAB-MS: 3-Nitrobenzyl-alcohol matrix.

*(5*R*,6*R*,7*R*,8*S*)*-N-*[6,7-Dihydroxy-5-(hydroxymethyl)-5,6,7,8-tetrahydro[1,2,4]triazolo[1,2-*a*]pyridin-8-yl]-acetamide* (**8**). A soln. of **13** (5 mg, 0.025 mmol) in MeOH/THF 1:1 (0.5 ml) was treated with Ac_2O (0.05 ml) and stirred for 4 h at 23°. The solvent was evaporated at 40°, the residue was dissolved in 2M NH_3 in MeOH (0.5 ml) and stirred for 1 h at 40°. Evaporation of the solvent and FC (AcOEt/MeOH/H_2O 20:2:1) gave **8** (4 mg, 66%). Colourless solid. R_f (AcOEt/MeOH 5:1) 0.05. ^{1}H-NMR (D_2O): 2.08 (*s*, AcN); 3.82–3.89 (*m*, H–C(6), H–C(7)); 4.12 (*dd*, J = 12.1, 5.3, CH–C(5)); 4.02 (br. *ddd*, $J \approx$ 8.1, 5.4, 2.6, H–C(5)); 4.22 (*dd*, J = 11.8, 2.2, CH–C(5)); 4.97 (*d*, J = 9.1, H–C(8)); 8.65 (*s*, H–C(3)). ^{13}C-NMR (D_2O): 24.04 (*q*, Me); 51.08 (*d*, C(8)); 61.98 (*t*, CH_2C(5)); 65.39, 70.97, 74.45 (3*d*, C(6), C(7), C(8)); 144.01 (*d*, C(3)); 154.37 (*s*, C(8a)); 177.72 (*s*, C=O). CI-MS: 243 (100, $[M+1]^+$).

*(4*S*,5*R*,6*S*,7*R*)*-N-*[5,6-Dihydroxy-7-(hydroxymethyl)-4,5,6,7-tetrahydro[1,2,3]triazolo[1,5-*a*]pyridin-4-yl]-acetamide* (**10**). As for the conversion of **13** to **8**, but with **14** (20 mg, 0.1 mmol), MeOH/THF 1:1 (1 ml), and Ac_2O (0.1 ml): **10** (21 mg, 87%). Colourless solid. R_f (MeOH/AcOEt 1:9) 0.25. ^{1}H-NMR (CD_3OD): 2.03 (*s*, AcN); 3.75 (*t*, $J \approx$ 9.0, irrad. at 4.97 → *d*, J = 9.0, H–C(5)); 4.10 (*dd*, J = 9.0, 8.5, H–C(6)); 4.13 (*dd*, J = 11.5, 2.5, CH–C(7)); 4.21 (*dt*, J = 8.4, 2.5, H–C(7)); 4.52 (*dd*, J = 11.8, 2.8, CH–C(7)); 4.97 (*d*, J = 9.0, H–C(4)). 7.51 (*d*, H–C(3)). ^{13}C-NMR (CD_3OD): 22.81 (*q*, Me); 48.68 (*d*, C(4)); 60.02 (*t*, CH_2–C(7)); 65.38, 69.73, 73.90 (3*d*, C(5), C(6), C(7)); 132.19 (*d*, C(3)); 137.95 (*s*, C(3a)); 174.13 (*s*, C=O). FAB-MS: 243 (100, $[M+1]^+$).

Enzyme Inhibition. The inhibition constants (K_i) were determined in the presence of 4 inhibitor concentrations which bracket the K_i value. 4-Nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (GlcNAc-Np) from *Sigma* (No. N-9376) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) from bovine kidney from *Sigma* (No. A-2415) were used. The suspension of the enzyme in 3.2M $(NH_4)_2SO_4$ (0.1 ml, 5 U) was dissolved in 10 ml of citrate buffer (0.5M, pH 4.2). Citrate buffer (0.5M, pH 4.2, 100 µl), inhibitor soln., or H_2O (300 µl), resp., and enzyme soln. (5 mU in citrate buffer, 100 µl) were incubated at 37° for 10 min, and GlcNAc-Np (5.0, 2.5, 1.6, 1.0, or 0.5 mM in H_2O, 500 µl) was added. The rate of substrate hydrolysis was determined by quenching the reaction after 5 min using 0.02M borate buffer (pH 9.2) and measuring the absorption at 400 nm. K_i Values were determined by taking the slopes from the *Lineweaver-Burk* plots [24] and plotting them against the inhibitor concentrations [25]. After fitting the data to a straight line, the negative [I] intercept of this plot gave the appropriate K_i value.

REFERENCES

[1] T. D. Heightman, M. Locatelli, A. Vasella, *Helv. Chim. Acta* **1996**, *79*, 2190.
[2] T. Granier, N. Panday, A. Vasella, *Helv. Chim. Acta* **1997**, *80*, 979.
[3] T. Granier, F. Gaiser, L. Hintermann, A. Vasella, *Helv. Chim. Acta* **1997**, *80*, 1443.
[4] P. Ermert, A. Vasella, M. Weber, K. Rupitz, S. G. Withers, *Carbohydr. Res.* **1993**, *250*, 113.
[5] C. A. Vernon, *Proc. R. Soc. London B* **1967**, *167*, 389.
[6] D. C. Phillips, *Proc. Natl. Acad. Sci. U.S.A.* **1967**, *57*, 484.
[7] T. D. Heightman, A. Vasella, K. E. Tsitsanou, S. E. Zographos, V. T. Skamnaki, N. G. Oikonomakos, *Helv. Chim. Acta* **1998**, *81*, 853.
[8] J. Sakon, W. S. Adney, M. E. Himmel, S. R. Thomas, P. A. Karplus, *Biochemistry* **1996**, *35*, 10648.
[9] T. D. Heightman, A. T. Vasella, *Angew. Chem., Int. Ed.* **1999**, *38*, 750.
[10] P. Ermert, A. Vasella, *Helv. Chim. Acta* **1991**, *74*, 2043.
[11] T. D. Heightman, P. Ermert, D. Klein, A. Vasella, *Helv. Chim. Acta* **1995**, *78*, 514.
[12] K. Tatsuta, S. Miura, S. Ohta, H. Gunji, *J. Antibiot.* **1995**, *48*, 286.
[13] N. Panday, M. Meyyappan, A. Vasella, *Helv. Chim. Acta* **2000**, *83*, in press.
[14] N. Panday, T. Granier, A. Vasella, *Helv. Chem. Acta* **1998**, *81*, 475.
[15] E. Conzelmann, H. Sandhoff, *Adv. Enzymol. Relat. Areas Mol. Biol.* **1987**, *60*, 89.

[16] G. Legler, E. Lüllau, E. Kappes, F. Kastenholz, *Biochim. Biophys. Acta* **1991**, *1080*, 89.
[17] B. Henrissat, P. Coutinho, 'Internet address: http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html', last update: 29.02.2000.
[18] I. Tews, A. Perrakis, A. Oppenheim, Z. Dauter, K. S. Wilson, C. E. Vorgias, *Nat. Struct. Biol*. **1996**, *3*, 638.
[19] M. Horsch, L. Hoesch, G. W. J. Fleet, D. M. Rast, *J. Enzyme Inhibit*. **1993**, *7*, 47.
[20] S. Drouillard, S. Armand, G. J. Davies, C. E. Vorgias, B. Henrissat, *Biochem. J.* **1997**, *328*, 945.
[21] D. J. Vocadlo, C. Mayer, S. He, S. G. Withers, *Biochemistry* **2000**, *39*, 117.
[22] N. Panday, Y. Canac, A. Vasella, *Helv. Chim. Acta* **2000**, *83*, 58.
[23] N. Panday, A. Vasella, *Synthesis* **1999**, *Special Issue*, 1459.
[24] H. Lineweaver, D. Burk, *J. Am. Chem. Soc*. **1934**, *56*, 658.
[25] I. H. Segel, 'Enzyme Kinetics', John Wiley & Sons, New York, 1975.

Received April 14, 2000