30. Inhibition of Emulsin by D-Gluconhydroximo-1,5-lactone and Related Compounds

by **Dieter Beer** and **Andrea Vasella***

Organisch-Chemisches Institut der Universität Zürich, Winterthurerstr. 190, CH–8057 Zürich

(28.X.85)

At pH 4.5 (citrate buffer), D-gluconhydroximo-lactone (**2**), the *N*-methylurethane **3** and the *N*-phenylurethane **4** inhibit competitively the hydrolysis of *p*-nitrophenyl *β*-D-glucopyranoside by emulsin. The IC_{50} values of **2**, **3**, and **4** were 1.6×10^{-4}, 1.0×10^{-4}, and 5.8×10^{-6} M, respectively. The K_i values of **2** and **4** were 9.8×10^{-5} and 2.3×10^{-6} M, respectively, while D-glucono-1,5-lactone (**1**) showed $IC_{50} = 1.1 \times 10^{-4}$ M and $K_i = 3.7 \times 10^{-5}$ M.

Introduction. – D-Glucono-1,5-lactone (**1**) is a well-known competitive inhibitor of *β*-glucosidases (EC 3.2.1.21) with K_i values of about 10^{-4}–10^{-5} M [1] [2]. According to X-ray analysis [3] [4] and ^{1}H-NMR spectra (see *Table 1*), **1** adopts both in the solid state and in aqueous solution (predominantly) a distorted half-chair conformation; thus, it has been considered a transition-state analogue for the enzymatic cleavage of *β*-D-glucopyranosides [5].

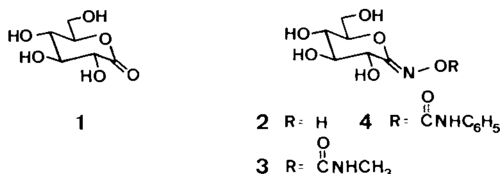

Table 1. *^{1}H-NMR Data of* **1**–**4**[a])

Chemical shifts [ppm] Compound	H–C(2)	H–C(3)	H–C(4)	H–C(5)	H–C(6)	H'–C(6)
1	4.18	3.87	3.85	4.22	3.84	3.92
2	4.19	3.73	3.72	4.02	3.87	3.99
3	4.35	3.86	3.79	4.20	3.86	4.04
4	4.35	3.85	3.78	4.20	3.87	4.03
Coupling constants [Hz]	J(2,3)	J(3,4)	J(4,5)	J(5,6)	J(5,6')	J(6,6')
1	9.0	9.0	9.0	4.0	2.5	12.5
2	8.5	8.3	9.9	4.3	1.6	12.2
3	8.0	8.5	9.5	4.1	2.0	12.5
4	7.9	8.0	9.2	4.7	2.2	12.8

[a]) The spectra were measured in D_2O (295 K; DHO = 4.80 ppm) at 400 MHz (**1** and **2**) or at 200 MHz (**3** and **4**).

The hydroximo-lactone **2** is expected to adopt a similar conformation. This is indeed the case for aqueous solution of **2**[1]) as evidenced by the coupling constants (*Table 1*). Similar conformations are also adopted by the urethanes **3** and **4** in aqueous solution (*Table 1*). The hydroximo-lactone **2** and the urethanes **3** and **4** are, therefore, expected also to inhibit β-D-glucopyranosides. Since the urethanes **3** and **4** are more lipophilic than the parent hydroximo-lactone, they might show a higher affinity for the enzyme and be stronger (competitive or non-competitive) inhibitors than **2**.

Experimental. – Emulsin (from sweet almonds; *Fluka*) with a standard activity under assay conditions of 2.8 µmol per min and per mg, *p*-nitrophenyl β-D-glucopyranoside (*Fluka, purum*) and D-glucono-1,5-lactone (**1**; *Merck, p.a.*) were used without any further purification.

The molar concentration of the tested substances effecting a 50% inhibition of emulsin was in each case determined by preincubating the enzyme (10 mU/0.5 ml H_2O; blank: 0.5 ml H_2O) and the inhibitor (the concentrations were 2×10^{-3}, 2×10^{-4}, 2×10^{-5}, and 2×10^{-6} M; 0.9 ml 0.05M citrate buffer; pH 4.5) for 10 min at 37°, then adding the substrate (10 µmol/0.5 ml; final substrate concentration: 5.3×10^{-3} M) and incubating for 5, 10, 15, and 20 min[2]). The reaction was stopped by addition of borate buffer soln. (0.2 M; pH 9.2; 1.8 ml).

Michaelis and inhibition constants (K_m and K_i, resp.) were determined by preincubating the enzyme (5 mU/0.1 ml H_2O; blank: 0.1 ml H_2O) without or with the inhibitor (the concentrations for **1** and **2** were 1.6×10^{-5} and 4.7×10^{-5} M; the concentrations for **4** were 9×10^{-7}, 3.4×10^{-6}, 6.9×10^{-6}, and 1.0×10^{-5} M; 0.9 ml citrate buffer; pH 4.5) for 10 min at 37° and then adding the substrate (the concentrations were 3.9×10^{-4}, 4.9×10^{-4}, 6.6×10^{-4}, 9.7×10^{-4}, and 2.0×10^{-3} M; 0.9 ml H_2O). After the incubation (2 to 8 min), borate buffer (pH 9.2; 1.8 ml) was added. In all the tests, the amount of *p*-nitrophenolate liberated was determined by reading the absorption at 400 nm (ε = 15500)[3]).

Results. – The molar concentrations for 50% inhibition of emulsin at pH 4.5 by the hydroximo-1,5-lactone **2**, the *N*-methylurethane **3**, and the *N*-phenylurethane **4** are indicated in *Table 2*.

The D-gluconhydroximo-1,4-lactone showed no inhibition up to concentrations of 5 mM. In this concentration range, a weak inhibition by D-glucono-1,4-lactone had been noted [8], which could be due to a lactone isomerization. The hydroximo-lactones appear to be more stable under assay conditions. While the inhibition by the glucono-1,5-lactone **1** decreased after prolonged preincubation, such a treatment did not affect the inhibition by the hydroximo-lactone **2** or by the urethanes **3** and **4**.

Table 2. *Enzyme Kinetic Data*

Inhibitor	Inhibitor concentration (M) required for 50% inhibition[a])	K_i (M)	K_i/K_m
1	1.1×10^{-4}	3.7×10^{-5}	1.3×10^{-2}
2	1.6×10^{-4}	9.8×10^{-5}	3.5×10^{-2}
3	1.0×10^{-4}	[b])	–[b])
4	5.8×10^{-6}	2.3×10^{-6}	8.2×10^{-4}

[a]) The substrate concentration was 5.3×10^{-3} M.
[b]) Value not determined.

---

[1]) The ^{1}H-NMR spectrum of a (D_6)DMSO solution of **1** shows the coupling constants $J(2,3) = 8.5$, $J(3,4) = 7.5$, and $J(4,5) = 8.1$ Hz [7], which have been interpreted as characterizing an equilibrium between distorted half-chair and boat conformers [7]. In the same solvent, **2** adopts a different conformation ($J(2,3) = 3.9$, $J(3,4) = 5.0$, and $J(4,5) = 10.0$ Hz). We thank Prof. Dr. *K. Bock*, The Technical University of Denmark, DK-2800 Lingby, for his contribution to the conformational analysis of **2**.

[2]) The reaction rates were found to be constant during the period of observation.

[3]) The concentrations of *p*-nitrophenolate were such that the optical density never exceeded 0.8.

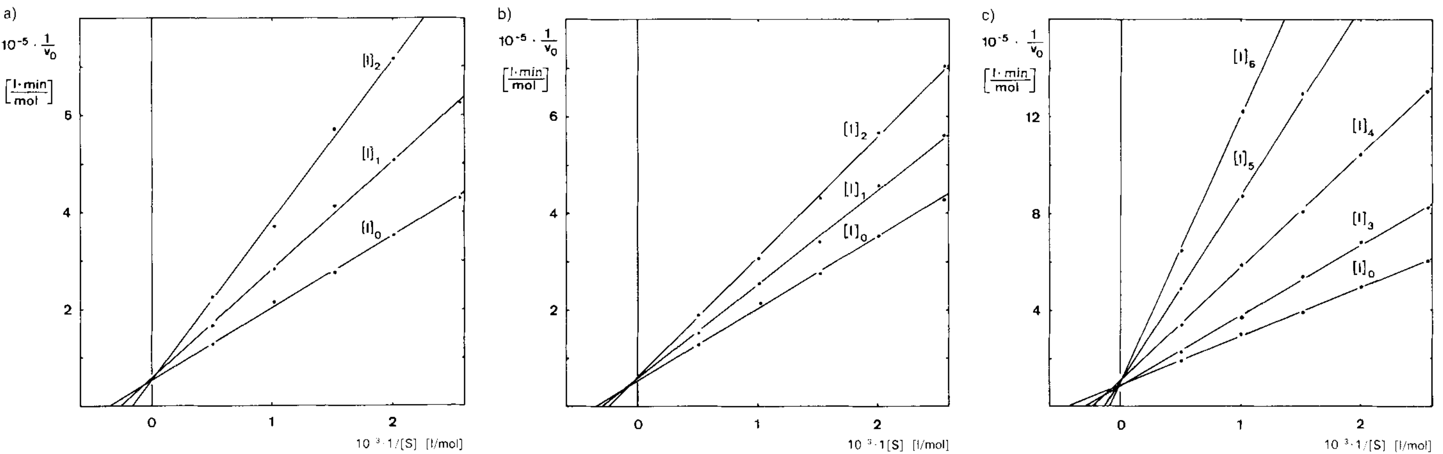

Fig. 1. Lineweaver-Burk *Plots. a)* Inhibition by **1**; *b)* inhibition by **2**; *c)* inhibition by **4**. Inhibitor concentrations: $[I]_0 = 0$; $[I]_1 = 1.6 \times 10^{-5}$ M; $[I]_2 = 4.7 \times 10^{-5}$ M; $[I]_3 = 9 \times 10^{-7}$ M; $[I]_4 = 3.4 \times 10^{-6}$ M; $[I]_5 = 6.9 \times 10^{-6}$ M; $[I]_6 = 1.0 \times 10^{-5}$ M.

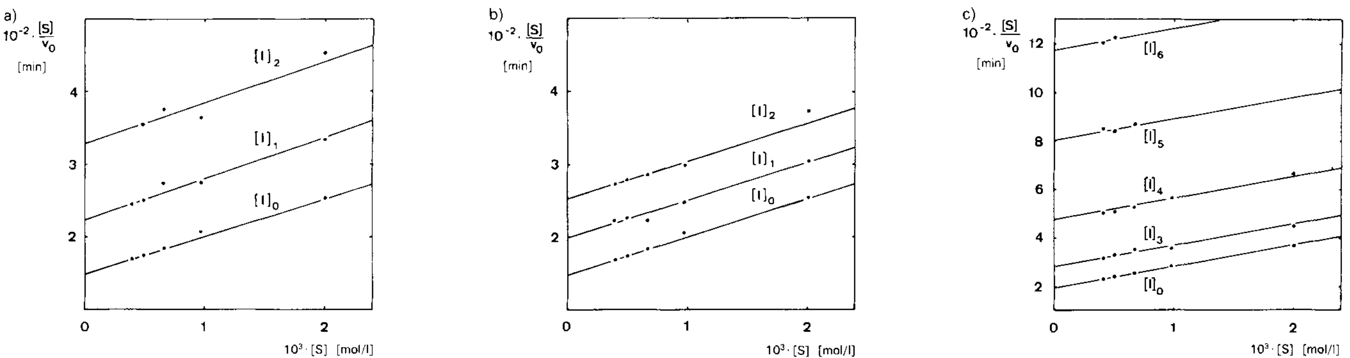

Fig. 2. Hanes-Woolf *Plots. a)* Inhibition by **1**; *b)* inhibition by **2**; *c)* inhibition by **4**. Inhibitor concentrations: $[I]_0 = 0$; $[I]_1 = 1.6 \times 10^{-5}$ M; $[I]_2 = 4.7 \times 10^{-5}$ M; $[I]_3 = 9 \times 10^{-7}$ M; $[I]_4 = 3.4 \times 10^{-6}$ M; $[I]_5 = 6.9 \times 10^{-6}$ M; $[I]_6 = 1.0 \times 10^{-5}$ M.

The *Michaelis* constant (K_m) of emulsin at pH 4.5 with *p*-nitrophenyl β-D-glucopyranoside as substrate was determined to be 2.8 mM. The K_i values of the lactone **1**, the hydroximo-lactone **2** and the *N*-phenylurethane **4**, calculated from *Lineweaver-Burk* plots *(Fig. 1)*, are collected in *Table 2*.

The K_i/K_m ratios of the lactone **1** and the hydroximo-lactone **2** are in the same range, the value for the *N*-phenylurethane **4** being 15 times smaller than that of **1**. *Hanes-Woolf* plots *(Fig. 2)* of the kinetic data showed competitive inhibition for all substances tested.

Discussion. – As expected, both the hydroximo-lactone **2** and the urethanes **3** and **4** inhibited the β-glucosidase activity of emulsin. The similarity of the enzyme inhibition by the lactone **1** and the hydroximo-lactone **2**[4]) shows that the additional OH group of **2** has little or no influence on the interaction of the inhibitor with the enzyme. It may, however, serve as a handle for the introduction of other substituents which should prove useful for the purpose of affinity chromatography [9] or for the preparation of inhibitors of aglycon-specific enzymes [10].

We thank the *Swiss National Science Foundation* and *Sandoz AG*, Basel, for generous support.

REFERENCES

[1] J. Conchie, A. L. Gelman, G. A. Levvy, *Biochem. J.* **1967,** *103,* 609.
[2] G. A. Levvy, S. M. Snaith, *Adv. Enzymol. Relat. Areas Mol. Biol.* **1972,** *36,* 151.
[3] M. L. Hackert, R. A. Jacobson, *J. Chem. Soc., Chem. Commun.* **1969,** 1179.
[4] M. L. Hackert, R. A. Jacobson, *Acta Crystallogr., Ser. B* **1971,** *27,* 203.
[5] D. H. Leaback, *Biochem. Biophys. Res. Commun.* **1968,** *32,* 1025.
[6] D. Beer, A. Vasella, *Helv. Chim. Acta* **1985,** *68,* 2254.
[7] Z. Walaszek, D. Horton, I. Ekiel, *Carbohydr. Res.* **1982,** *106,* 193.
[8] E. T. Reese, F. W. Parrish, M. Ettlinger, *Carbohydr. Res.* **1971,** *18,* 381.
[9] J. N. Kanfer, G. Petrovich, R. A. Mumford, *Anal. Biochem.* **1973,** *55,* 301.
[10] W. Hoesel, E. E. Conn, *Trends Biochem. Sci.* **1982,** *7,* 219.

[4]) In a similar way as **1**, which has a weak action on α-glucosidases (K_i range *ca.* 10^{-3} M [8]), the hydroximo-lactone **2** inhibits intestinal maltase weakly. We thank Dr. *W. Pirson, F. Hoffmann-La Roche & Co. AG,* Basel, for the determination of the inhibition.