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IRREGULARITY OF INTERACTION BETWEEN HOMOLOGOUS ω-AMINATED 1-METHOXYALKYL β-D-GLUCOPYRANOSIDES AND β-D-GLUCOSIDASE FROM SWEET ALMOND EMULSIN*

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ABSTRACT

A number of homologous (*R*)- and (*S*)-1-methoxyalkyl β-D-glucopyranosides, carrying at the ω-end of the C_{2-5} atom alkyl chain either an azido- or an amino group, were used as substrates or competitive inhibitors of β-D-glucosidase from sweet almond emulsin. All azides were cleaved at comparatively high rates, whereas the amines required extremely high enzyme concentrations and incubation times for only partial hydrolysis. Kinetic parameters were determined as far as possible. The influence of the amino group in the glycoside on K_i values or cleavage rates did not change in the expected regular way. Exceptional behavior was found with (*R*)-3-amino-1-methoxypropyl β-D-glucopyranoside (**4**). As compared to its diastereomer and to its smaller or larger homologues, compound **4** had a much higher affinity to the enzyme and was almost totally resistant to enzyme-catalyzed cleavage. Considering a likely three-dimensional structure of the enzyme-bound glycoside, a certain spatial orientation of interacting groups in the active site is discussed.

INTRODUCTION

In a previous publication[1] we described syntheses of homologous, ω-aminated 1-methoxyalkyl β-D-glucopyranosides (**2, 6, 8,** and **12**). Preliminary, qualitative experiments showed that such potentially ionic compounds, unlike their neutral analogues, such as bromo or azido derivatives, are resistant to hydrolysis by β-D-glucosidase from sweet almond emulsin. These findings are essentially substantiated in this paper by thorough kinetic investigations, although remarkable deviations from expected results were observed. We expected that effects on enzyme kinetics, for which the amino group is responsible, must change continuously with length of the alkyl chain separating it from the anomeric center of the glucoside,

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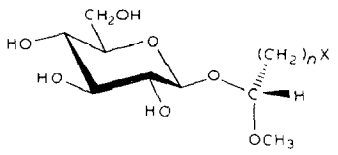
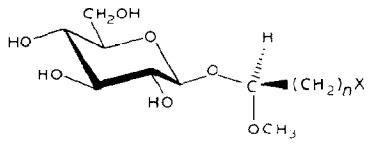


"R"



"S"

	n	X		n	X
1	1	N_3	**9**	1	N_3
2	1	NH_2	**10**	1	NH_2
3	2	N_3	**11**	2	N_3
4	2	NH_2	**12**	2	NH_2
5*	3	N_3			
6*	3	NH_2			
7*	4	N_3			
8*	4	NH_2			

* Stereoisomeric mixture at aglycon carbon atom

where enzyme-catalyzed cleavage takes place. This assumption can be verified only in part.

RESULTS

(*R*)-2-Azido-1-methoxyethyl β-D-glucopyranoside (**1**), (*S*)-2-azido-1-methoxyethyl β-D-glucopyranoside (**9**), (*R*)-3-azido-1-methoxypropyl β-D-glucopyranoside (**3**), (*S*)-3-azido-1-methoxypropyl β-D-glucopyranoside (**11**), (*R,S*)-4-azido-1-methoxybutyl β-D-glucopyranoside (**5**), (*R,S*)-5-azido-1-methoxypentyl β-D-glucopyranoside (**7**), (*R*)-2-amino-1-methoxyethyl β-D-glucopyranoside (**2**), (*S*)-2-amino-1-methoxyethyl β-D-glucopyranoside (**10**), (*R*)-3-amino-1-methoxypropyl β-D-glucopyranoside (**4**), (*S*)-3-amino-1-methoxypropyl β-D-glucopyranoside (**12**), (*R,S*)-4-amino-1-methoxybutyl β-D-glucopyranoside (**6**), and (*R,S*)-5-amino-1-methoxypentyl β-D-glucopyranoside (**8**) were submitted to hydrolysis by β-D-glucosidase from sweet almond emulsin. All azides (**1, 3, 5, 7, 9,** and **11**) were smoothly cleaved under standard conditions, at a rate comparable to that for 2-nitrophenyl β-D-glucopyranoside and considerably faster than methyl β-D-glucopyranoside. Kinetic parameters for enzyme-catalyzed hydrolysis are listed in Table I. In the following section the values are compared among themselves as well as with those obtained with an analogous series of amines **2, 4, 6, 8, 10,** and **12**. Amines **2, 6, 8,** and **12** were available[1]; amines **4** and **10** had to be prepared. All amines turned out to be resistant under conditions sufficient for complete hydrolysis of the corresponding azides. Such inertness has already been described for (*R*)- and (*S*)-2-amino-1-methoxyethyl α-D-glucopyranoside toward α-D-glucosidase from yeast[2].

It was suggested that deprotonation by the amino substituent of a catalytically essential, proton-donating group is responsible for this protective effect. Ionic interaction between the conjugate base of this group and the ammonium group in

TABLE I

KINETIC DATA FOR THE AZIDO DERIVATIVES

Compound	K_M (*mM*)	V_{max} (*nmol/mL·min*)[a]	$\frac{V_{max}\ (oNPG)^b}{V_{max}}$
1	18.0	9.22	6.51
3	18.4	24.0	2.50
5	18.6	13.0	4.62
7	20.0	24.0	2.50
9	69.4	2.08	28.8
11	28.3	11.0	5.45

[a]ε(NADH, 366 nm) = 3.3 cm²/μmol. [b]V_{max}(oNPG) was determined to be 0.060 μmol/mL·min with ε(*o*-nitrophenol, 405 nm, pH = 6.8) = 1.85 m²/μmol.

the ligand is also the factor responsible for competitive glycosylase inhibition by numerous carbohydrate derivatives carrying amino functions[3]. Based on such assumptions, a gradually decreasing effect of an amino function is to be expected, the further it is removed from its proton-donating counterpart.

Preliminary, qualitative tests[1] already indicated that extremely high enzyme concentrations and very long incubation times can cause measurable hydrolysis of aminated glycosides. Therefore, we treated compounds **2, 4, 6, 8, 10,** and **12** with enzyme at ~100-fold the concentration normally used and extended the incubation times up to 48 h (see Table II). Under these conditions, significant differences in rates of hydrolysis could be detected (see Fig. 1). Concentrations of unchanged substrates and product (D-glucose) were determined by g.l.c. The expected decrease of protection by the amino group against enzyme-catalyzed hydrolysis with length of the alkyl chain in the aglycon was indeed observed when the extremes **2** and **10** (n = 1) and **8** (n = 4) were compared. Strangely, however, the rates for **4** and **12** (n = 2) were much lower when compared with that for **2** (n = 1) and measurably lower when compared with that for **10** (n = 1). The peculiar deviation of **4** and **12** (n = 2) from the expected steady change of properties is also found when inhibition constants of the amines were measured (see Table III). All com-

TABLE II

RESULTS OF G.L.C. ANALYSIS OF INCUBATION MIXTURES

Compound	*Remaining substrate (%) after an incubation time of (h)*				
	0	*2*	*5*	*21*	*48*
2	100	94	85	57	29
4	100	97	95	91	88
6	99	78	60	31	11
8	100	72	57	26	10
10	100	98	96	86	76
12	99	98	96	91	87

TABLE III

K_i-VALUES OF THE AMINO DERIVATIVES

Compound	**2**	**4**	**6**	**8**	**10**	**12**
K_i (mM)	53	3.5	46	63	47	80

pounds except **4** (n = 2) have unexceptional K_i-values, comparable to that for D-glucose, which indicates that here the amino groups have little or no influence on the affinity. Of the diastereomeric pair **4** and **12** (n = 2) only one, the (*R*)-isomer **4**, shows a significantly higher affinity (K_i = 3.5mM) for the active site of the enzyme.

DISCUSSION

a. Enzymic hydrolysis of ω-azido-1-methoxyalkyl β-D-glucopyranosides. — This is the first time that hydrolysis rates of homologous 1-methoxyalkyl β-D-glucosides carrying a polar, but not charged group, at the end of the alkyl chain have been compared. The differences are small yet significant. According to A. J. Kirby *et al.*[4] it is very likely that β-D-glucopyranosides, in order to be cleaved by β-D-glucosidase, have to adopt a conformation that allows overlap of a lone pair of the ring oxygen atom with the antibonding sigma orbital of the glycosidic bond ($\sigma^*_{C\text{-}1-O\text{-}1}$). Without this overlap, responsible also for the anomeric effect[5], the energy barrier would be too high for enzyme-catalyzed β-D-glycoside cleavage. In order to simplify the discussion of our results, we depict β-D-glucopyranosides bound to

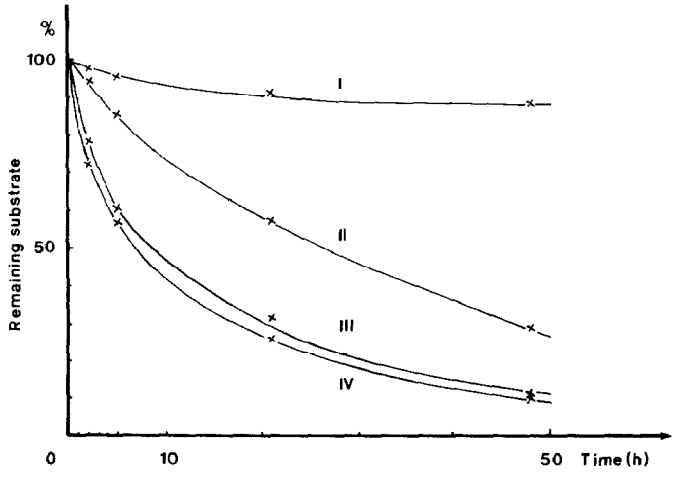


Fig. 1. Process of enzymic hydrolysis of ω-aminated acetal β-D-glucopyranosides (40 mM) with β-D-glucosidase from sweet almonds (2.7 U/mL) over a period of 48 h [Key: I, compound **4**; II, compound **2**; III, compound **6**; and IV, compound **8**].

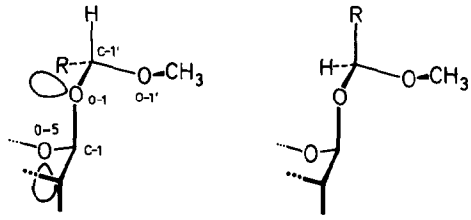


Fig. 2. Segment of (*R*)- and (*S*)-1-methoxyalkyl β-D-glucopyranoside arranged in a conformation, not necessarily with the ring as a chair, to allow for optimal lone pair–σ^* overlap.

β-D-glucosidase only as the relevant section O-5–C-1–C-2–O-1–aglycon, because it is unlikely that the extremely unfavourable, explicit $^1C_4$(D) conformation prevails in reality. A somewhat distorted conformation[6] might be sufficient to create the aforementioned stereoelectronic situation that would be required for glycoside cleavage. As the 1-methoxyalkyl aglycon groups in the substrates **1, 3, 5, 7, 9,** and **11** are not likely to be particularly fixed by the active site, they probably adopt a conformation also favoured by lone pair–σ^* overlap, which is only possible in a helical arrangement of the diacetal system (O-5–C-1–O-1–C-1′–O-1′–Me, see Fig. 2). This favoured helical conformation is also found in poly(oxymethylene)[7]. The assumptions thus made on the basis of stereoelectronic requirements would lead to the illustrated three-dimensional structures for the enzyme-bound 1-methoxyalkyl β-D-glucopyranosides of the *R*- and of the *S*-series (see Fig. 2).

If these are the "ground states" of the enzyme-bound substrates, it becomes clear, judging from models, that the (*R*) isomers are more crowded than the (*S*) isomers. This higher ground-state energy could account for higher rates of enzymic hydrolysis of the (*R*) isomers. In all β-pairs thus far tested[1], this is the case. The same consideration can be applied to α-pairs[2,8,9] (see Fig. 3). Here the (*S*) isomers are more sensitive to enzyme-catalyzed hydrolysis. Looking at the structures shown in Figs. 2 and 3, one may well consider initiation of cleavage by enzymic protonation not of O-1 but of O-1′, a mechanism that has been discussed for the cleavage of acetal glycosides in aqueous acid[9]. This mechanism could explain the comparatively high enzymic cleavage rates. We consider irrelevant the fact that C-1′ in all

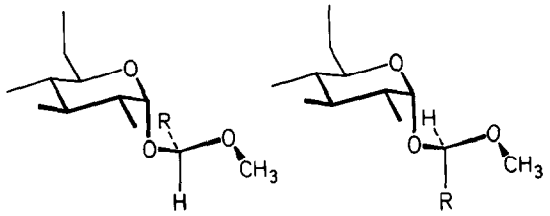


Fig. 3. Segment of (*S*)- and (*R*)-1-methoxyalkyl α-D-glucopyranosides arranged in a conformation to allow for optimal lone pair–σ^* overlap.

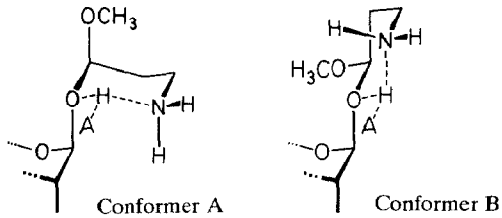


Fig. 4. Two conformational arrangements of the aglycon in (*R*)-3-amino-1-methoxypropyl β-D-glucopyranoside (**4**) showing optimal H-bonding interactions between the polar groups in the aglycon and a proton-donating group (A–H) of emulsin β-D-glucosidase.

1-methoxyalkyl β-D-glucosides is a secondary center, as the rates of hydrolysis of propyl and isopropyl β-D-glucopyranosides are approximately equal[10].

A significant difference in hydrolysis rates between homologous pairs of the same configuration, such as **1** and **3**, and **9** and **11** (see Table I), is in agreement with the general idea that the influence of a polar group, here an azido group, which stabilizes a substrate against hydrolytic cleavage[9], falls off rapidly with distance. In fact compound **3** is hydrolyzed 2.6 times, and compound **11** almost five times, faster than their respective shorter-chain homologues **1** and **9**.

b. Enzymic hydrolysis of ω-amino-1-methoxyalkyl β-D-glucopyranosides. — Under extreme conditions, glycosides bearing amino groups in the aglycon can be enzymically hydrolyzed. The fact that, here again, (*R*) isomers are generally more susceptible to enzymic hydrolysis may be explained by their higher ground-state energy (see section *a*). Puzzling is the almost total stability of compounds **4** and **12** ($n = 2$) in comparison with the lower homologues **2** and **10** ($n = 1$) or the higher ones **6** ($n = 3$) and **8** ($n = 4$) (see Fig. 1 and Table II). The generally very high stability of these glycosides cannot be explained solely by the inductive effect (-I) of an ω-amino group or the corresponding ammonium group. This effect ought to decrease with lengthening of the alkyl chain. It rather points to a folding back of the alkyl chain to the glycosidic oxygen atom and the catalytically active proton-donor group, thus leading to an optimal protection against general acid catalysis by the enzyme (see Figs. 4 and 5). In such a complex, the proton would be ideally placed to hydrogen-bond with the amino nitrogen and with the glycosidic oxygen (O-1) in a 6-membered system that is only possible with the 3-aminopropyl chain

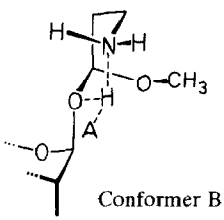


Fig. 5. Conformational arrangement of the aglycon in (*S*)-3-amino-1-methoxypropyl β-D-glucopyranoside (**12**) showing optimal H-bonding interactions between the polar groups in the aglycon and a proton-donating group (A–H) of emulsin β-D-glucosidase.

and not with the 2-aminoethyl nor any longer chain. This assumption requires the ideal helical arrangement of the diacetal (see section *a* and Fig. 2) to become at least partly disturbed. Two conformations (A and B) seem feasible, with the bulky propyl group being turned away from the hydroxymethyl group of the (*R*) isomer (compound **4**). For the (*S*) isomer (compound **12**), the ideal stereoelectronic requirements can prevail in conformer B (see Fig. 5) and this may account for the lower ground-state energy and higher stability of the (*S*) isomers. The longer the alkyl chain — as in compound **6** and **8**, which are diastereomeric mixtures — the more difficult the "folding back" process (and thereby the protective influence of the amino group) becomes. That "folding back" is possible, albeit not optimal, even with the longer alkyl chain in **6** and **8** explains why the ionizable amino group still exerts a protective influence, in contrast to the nonionizable azido group in compounds **5** and **7**.

c. ω-Amino-1-methoxyalkyl β-D-glucopyranosides as competitive inhibitors. — It is not surprising that the amines **2, 4, 6, 8, 10,** and **12** are competitive inhibitors of β-D-glucosidase activity. Striking, however, is the irregularity of the inhibitory effect. All of the amines, except one, have K_i values around 60mM, which is about three times the affinity of free D-glucose. It is not clear why (*R*)-3-amino-1-methoxypropyl β-D-glucopyranoside (**4**), which also happens to be one of the most resistant amines against enzyme hydrolysis, should have an affinity 20 times larger than its diastereomer **12** and all the other amines (**2, 6, 8,** and **10**). Considering again the conformers A and B of the (*R*) series (see Fig. 4), the latter may be visualized as having additional polar binding interaction between O-1′ of the methoxy group and a partner group -X at the active site, which could also bind to the ring oxygen atom O-5. Drawing the segment shown in Fig. 6 reveals a possible arrangement having eight atoms of the inhibitor involved in a *trans*-decalin system held together by polar groups (-X and -AH) of the enzyme.

In this respect it is noteworthy that the natural substrates for β-D-glucosidase from emulsin, the cyanogenic glycosides amygdalin and prunasin, also have the (*R*) configuration[11] at C-1′ (cyanohydrin carbon, see Fig. 6) like the competitive inhibitor **4**. With the benzene ring in prunasin taking the part of the alkyl chain, the polar cyano group of the former could, just as the methoxy group of the latter, interact with its natural partner -X at the active site.

The reason why all ω-amino-1-methoxyalkyl glycosides thus far tested, in-

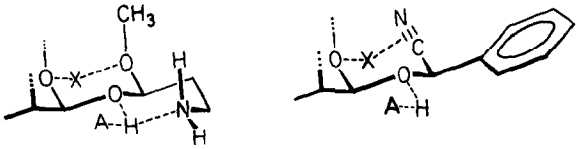


Fig. 6. Conformational arrangement of the aglycon in (*R*)-3-amino-1-methoxypropyl β-D-glucopyranoside (**4**) allowing not only for optimal H-bonding but also for interactions of two oxygen atoms with a hypothetical polar group X of emulsin β-D-glucosidase. For comparison, the same arrangement is shown for prunasin, the natural substrate of emulsin β-D-glucosidase.

cluding those in former experiments[2,9], never reach the affinities found with such other basic competitive inhibitors as nojirimycin, swainsonin, and several glycosylamines[3] may be attributable to flexibility of the aglyconic sidearm that leads to an unfavourable binding entropy. Another reason may be the presence of the glycosidic oxygen O-1, competing with nitrogen for the proton of the -AH group.

EXPERIMENTAL

Methods. — All reactions were monitored by t.l.c. on silica gel 60 F_{254} (Merck) using the solvents indicated. Preparative column chromatography was carried out on silica gel 60 (0.04–0.063 mm, Merck). Optical rotations were measured with a Perkin–Elmer 141 polarimeter. ^{1}H-N.m.r. spectra were recorded with a Bruker WM-250 spectrometer at 250 MHz in D_2O (internal sodium 4,4-dimethyl-4-silapentane-1-sulphonate). G.l.c. was performed with a Pye Unicam GCD chromatograph with glass columns and SE 52 (3%) on Chromosorb G, AW-DMCS, and recorded with a Shimadzu Chromatopack C-R2AX apparatus.

Enzymic reactions. — β-D-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) from sweet almonds, and alcohol dehydrogenase (ADH, alcohol NADH oxidoreductase, EC 1.1.1.1) from yeast were purchased from Boehringer Mannheim. NADH (reduced β-nicotinamide adenine dinucleotide, Na_2 salt) was obtained from Serva. The enzymic reactions were performed at 25° in 0.1M sodium phosphate buffer (pH 6.8). Kinetic parameters of the azides **1, 3, 5, 7, 9,** and **11** (Table I) were obtained by determining the initial rate of liberation of the corresponding ω-azidoaldehyde by measuring[12] the decrease of NADH-extinction at 366 nm. The assays were carried out with 2.5 to 250mM of glucoside and 0.69mM of NADH, containing 143 μg/mL of ADH and 12.5 μg/mL of β-D-glucosidase. Inhibition constants of the amines **2, 4, 6, 8, 10,** and **12** were determined using an assay with 0.76 to 20.4mM *o*-nitrophenyl β-D-glucopyranoside, 0 to 50mM inhibitor (0 to 9.2mM in the case of **4**), and 8 μg/mL of β-D-glucosidase. Enzymic hydrolysis of the amines was carried out in buffer solution containing amine (40mM) and β-D-glucosidase (1 mg/mL). Aliquots were taken after 0, 2, 5, 21, and 48 h, monitored by t.l.c. (7:3:3:2:3:2 1-propanol–EtOH–EtOAc–C_5H_5N–H_2O–AcOH) and, after drying and trimethylsilylation[13], submitted to quantitative g.l.c. analysis. Samples without enzyme showed no detectable hydrolysis of glucosides within two days.

(S)-*2-Amino-1-methoxyethyl* β-D-*glucopyranoside* (**10**). — A solution of (*S*)-2-azido-1-methoxyethyl β-D-glucopyranoside[1] (**9**) (381 mg, 1.36 mmol) in EtOH (30 mL) was hydrogenated in the presence of Adams' catalyst (18 mg PtO_2) for 7 h. Platinum was filtered off and the filtrate evaporated to give **10** as a colourless syrup (340 mg); $[\alpha]_D^{22}$ −17.5° (*c* 1.0, EtOH); R_F 0.41 (7:3:3:2:3:2 1-propanol–EtOH–EtOAc–C_5H_5N–H_2O–AcOH); ^{1}H-n.m.r. data: 4.70 (d, $J_{1,2}$ 8.0 Hz, H-1), 3.28–3.56 (m, $J_{5,6a}$ 5.3, $J_{5,6b}$ 1.8 Hz, H-2–H-5), 3.71 (dd, $J_{6a,6b}$ 12.3 Hz, H-6a), 3.89 (dd, H-6b), 4.86 (t, $J_{1',2'}$ 5.3 Hz, H-1'), 2.84 (d, H-2'), and 3.49 (s, OMe).

(R)-*3-Amino-1-methoxypropyl* β-D-*glucopyranoside* (**4**). — (*R*)-3-Azido-1-

methoxypropyl β-D-glucopyranoside[1] (**3**) (320 mg, 1.09 mmol) in EtOH (30 mL) was hydrogenated (28 mg PtO_2) as described for compound **10**. The product was purified by column chromatography (6:3:1 $CHCl_3$–MeOH–$NH_3 \cdot H_2O$), yielding syrupy **4** (280 mg, 96%); $[\alpha]_D^{22}$ −29.5° (*c* 1.0, EtOH); R_F 0.39 (7:3:3:2:3:2 1-propanol–EtOH–EtOAc–C_5H_5N–H_2O–AcOH); ^{1}H-n.m.r. data: 4.61 (d, $J_{1,2}$ 7.8 Hz, H-1), 3.29 (t, $J_{2,3}$ 7.8 Hz, H-2), 3.35–3.55 (m, $J_{5,6a}$ 5.3, $J_{5,6b}$ 1.2 Hz, H-3–H-5), 3.73 (dd, $J_{6a,6b}$ 12.0 Hz, H-6a), 3.91 (dd, H-6b), 4.89 (t, $J_{1',2'}$ 5.6 Hz, H-1'), 1.98 (q, $J_{2',3'}$ 6.9 Hz, H-2'), 2.96 (t, H-3'), and 3.50 (s, OMe).

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