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2,5-Dideoxy-2,5-imino-D-mannitol and -D-glucitol. Two-step bio-organic syntheses from 5-azido-5-deoxy-D-glucofuranose and -L-idofuranose; evaluation as glucosidase inhibitors and application in affinity purification and characterisation of invertase from yeast

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ABSTRACT

Glucose isomerase (EC 5.3.1.5) catalyzes the quantitative isomerisation of 5-azido-5-deoxy-D-gluco- (**7**) and -L-idofuranose (**9**), respectively, into the corresponding ketoses, 5-azido-5-deoxy-D-fructopyranose (**8**) and -L-sorbopyranose (**10**), respectively. Upon catalytic hydrogenation over palladium-on-charcoal, the fructose derivative **8** gives the natural product and the efficient glycosidase inhibitor 2,5-dideoxy-2,5-imino-D-mannitol (**4**), while the sorbose derivative **10** affords 2,5-dideoxy-2,5-imino-D-glucitol (**5**). This represents a preparatively very simple and efficient two-step synthesis of these biologically active compounds. Both are strong inhibitors of α- and β-glucosidases from various sources, the D-*manno*-isomer **4** being distinctly more active. Because of its structural relationship with β-D-fructofuranose, compound **4** is also a very good inhibitor of invertase from yeast and, as such, was for the first time employed, after immobilization on aminohexyl-sepharose, for the purification of this enzyme.

INTRODUCTION

Several sugar analogues with a nitrogen atom replacing oxygen in the carbohydrate ring have been found as natural products and were shown to behave as highly selective reversible inhibitors of glycosidases with inhibition constants in the micromolar and, in quite a few cases, even submicromolar range[1]. Some of the first examples of such compounds were nojirimycin (5-amino-5-deoxy-D-glucose[2,3], **1**)

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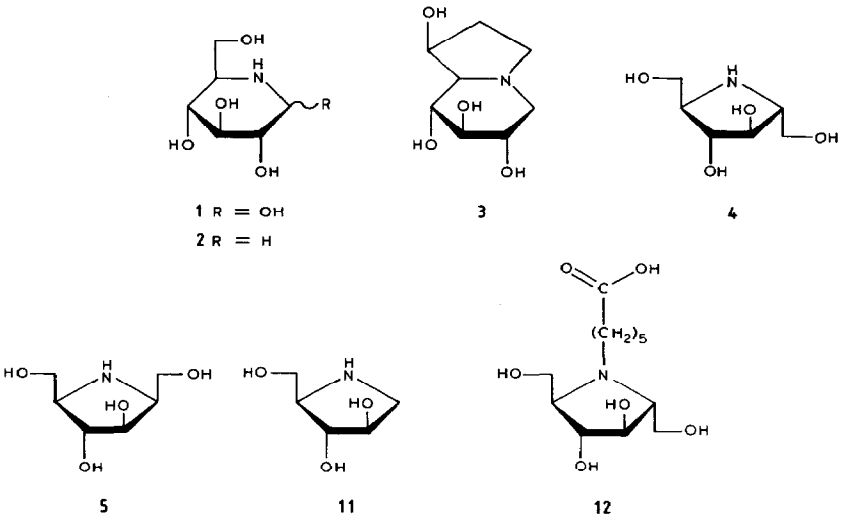


and 1-deoxynojirimycin (1,5-dideoxy-1,5-imino-D-glucitol[3–5], **2**). Many others have been subsequently discovered or synthesized in the past, such as the D-*manno*-[6], the D-*galacto*-[7,8], the L-*fuco*-[9], and the 2-acetamido-2-deoxy-D-*gluco*-isomers[10]. This variety of products is extended by bicyclic analogues, such as the highly active mannosidase inhibitor swainsonine[11] and the powerful glucosidase inhibitor castanospermine[12] (**3**), as well as a range of interesting derivatives thereof[13]. Bearing in mind that the five-membered ring analogue of 1-deoxynojirimycin (**2**), namely 2,5-dideoxy-2,5-imino-D-mannitol (**4**), had been shown[1] to exhibit a higher activity than the six-membered ring counterpart **2** against α- as well as β-glucosidases, we became interested in other modified analogues of this molecule for comparison with the parent compound.

Chemical approaches to the natural product[14] have started from D-glucose[15], L-sorbose[16], and D-mannitol[17]. Cyclisation of 5-keto-D-fructose with benzylamine led to compound **4** as a side product, the corresponding D-*gluco*-isomer **5** being the main product of the reaction[18]. A bio-organic method[19] employing rabbit muscle aldolase has also been used for the synthesis of **4**. In addition, the nonnatural isomer at C-2 of **4**, 2,5-dideoxy-2,5-imino-D-glucitol (**5**), was also synthesised by an aldolase catalysed carbon–carbon bond formation and introduced as a glycosidase inhibitor by Wong and co-workers[8].

In order to compare the effect of structural modifications in the 1-deoxynojirimycin and the 2,5-dideoxy-2,5-imino-D-mannitol system, we needed access to both structural types and looked for a simple means of synthesising both ring systems from the same precursor. Attempts to isomerize chemically 5-azido-5-deoxy-D-glucose[20] (**6**) to the corresponding D-fructose derivative, employing the Lobry de Bruyn–Alberda van Ekenstein rearrangement under a variety of condi-

tions[21], was not successful. Turning our attention towards a possible biochemical approach, we found glucose isomerase (EC 5.3.1.5) to be a suitable candidate to catalyze the required aldose–ketose transformation.

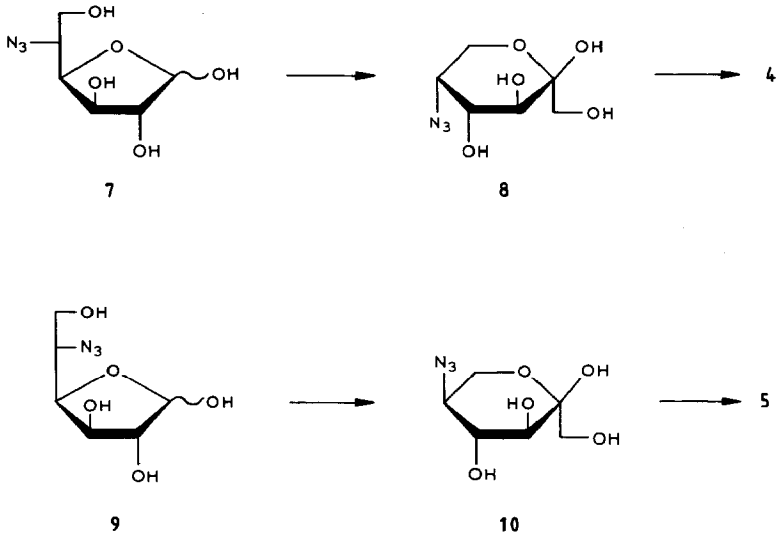
Despite its well-known industrial use, there is only little information available on the use of glucose isomerase in organic synthesis, apart from the isomerization of isotopically labelled D-fructose[22]. Pioneering work on the substrate specificity of soluble as well as immobilized glucose isomerase was carried out by Bock and co-workers[23]. It was demonstrated that this enzyme also converts glucose derivatives modified at C-6 or C-3, such as 6-deoxy, 6-*O*-methyl-, and 3-*O*-methyl-D-glucose, as well as 3-deoxy-D-*ribo*-hexose into the corresponding D-*fructo*-isomer (conversion rates between 10 to 38%). Other hexoses and glucose derivatives modified at C-4 were not accepted as substrates. More recent results by Wong and co-workers[24] essentially confirm the findings of Bock et al.

Both groups found that 5-deoxy-D-*xylo*-hexofuranose ("5-deoxy-D-glucose") was converted quantitatively into the corresponding keto-pyranose, despite the fact that this substrate was unstable to the reaction conditions in the absence of enzyme. This remarkable observation which, to our knowledge has not been further investigated, led us to try to isomerize other glucofuranose derivatives lacking a hydroxyl group at C-5, such as 5-azido-5-deoxy- (**6**), 5-*O*-benzyl-[25], and 5-deoxy-5-fluoro-D-glucofuranose[26], as well as derivatives chain-extended at C-6. Quantitative conversion to the corresponding D-fructopyranose derivative was observed in all cases investigated, with isolated yields ranging between 70 and 80% employing C-6-unsubstituted substrates, and 50–60% for C-alkylated aldoses. Interestingly the conversion is equally efficient for substrates with an L-*ido* configuration. Both stereoisomers of derivatives chain-extended at C-6 could also be converted[27].

RESULTS AND DISCUSSION

When 5-azido-5-deoxy-D-glucofuranose **6** (easily available in six steps from D-glucofuranurono-6,3-lactone[20]) was shaken at 60°C with a four- to five-fold excess (w/w) of immobilized glucose isomerase in water containing trace amounts of magnesium sulfate, a smooth conversion to 5-azido-5-deoxy-D-fructopyranose[16,19] (**7**) took place within 3 h. After removal of the immobilized enzyme by filtration, the mixture was concentrated under reduced pressure, the residue dissolved in a minimal amount of ethyl acetate, and the solution was passed over a short column of silica gel. The rapidly crystallizing material obtained after removal of the solvent was conventionally hydrogenated on a Parr apparatus over palladium-on-charcoal to give the desired product **4** in 70% overall yield. The same procedure, applied to 5-azido-5-deoxy-L-idofuranose **8**, easily accessible in 5 steps and 50% yield from D-glucofuranurono-6,3-lactone[20,28], led, via 5-azido-5-deoxy-L-sorbopyranose[8,19] (**10**) to 2,5-dideoxy-2,5-imino-D-glucitol[8] (**5**) in 64% yield for both steps.

Inhibition constants (K_i) of pyrrolidines **4** and **5** are summarized in Table I and



compared to those of 1-deoxynojirimycin (**2**) and castanospermine (**3**). For all enzymes tested, the *manno* configured product **4** was a much better inhibitor than the *gluco* compound **5**. Superposition of molecular models of **4** and **2** showed all the hydroxyl groups of **4** to be located in almost the same position as those of **2** when the NH groups were in the same place. A similar alignment could not be achieved with the *gluco* isomer **5**.

TABLE I

Inhibition [a] of α- and β-glucosidases and of β-fructofuranosidase by 1,5- and 2,5-dideoxy-1,5- and 2,5-imino-D-hexitols and by castanospermine

Enzyme	Inhibitor			
	2	**4**	**5**	**3**
α-Glucosidase (Yeast, pH 6.5)	25	0.73	80	> 1500
β-Glucosidase (Almonds, pH 5.0)	300	1.7	52	1.5
β-Glucosidase (A. wentii, pH 5.0)	2.0	57	1150	0.9
β-Glucosidase (Bovine kidney, pH 5.0)	250	44	500	25
β-Fructofuranosidase				
(Yeast) pH 5.0	> 5000	6.8	450	n.d.
pH 6.0	n.d.	3.5	330	n.d.
pH 7.0	n.d.	1.1	74	n.d.

[a] K_i Values in μM; n.d., not determined

The positional equivalence of the hydroxyl groups at C-1 of **4** and at C-2 of **2**, in conjunction with the inhibition data, demonstrate the importance of the C-2 hydroxyl group of D-glucose for biological activity. This is also born out by the observation[29] that fagomine (1,2-dideoxynojirimycin), at 1 mM, showed no inhibition with α-glucosidase from yeast and β-glucosidase from apricot emulsin, (i.e., $K_i > 5000$ μM). On the other hand, 1,4-dideoxy-1,4-imino-D-arabinitol (**12**) inhibited yeast α-glucosidase 100-fold better than **2** and ≥ 7-fold better than **4** (ref 30). This could point to steric hindrance with the hydroxymethyl group on C-2 of **4**, partly compensated by hydrogen bond interactions. With β-glucosidase from almonds, the inhibitory potency of **4** was similar to that of **2** (ref 30).

Interestingly, all glucosidases (except that from *Aspergillus wentii*) were better inhibited by **4** than by **2**, a point already noted by Fleet et al.[30] for the yeast and almond enzymes. It may well be that hydrogen bond interactions with **4** bear a better resemblance to a transition state flattened at the anomeric carbon of substrates than comparable interactions with **2**. A reason for the different response of the β-glucosidases from almonds and from *A. wentii*, to the structural differences of **4** and **2**, could be their different susceptibility to a replacement of the C-2 hydroxyl group by hydrogen. This resulted in a 10^3-fold decrease of k_{cat} and only a small effect on substrate binding with the almond enzyme[31], but a 2.5 10^5-fold decrease of k_{cat} and a 30-fold decrease of substrate affinity with the fungal enzyme[32]. Another point to be considered in the discussion of the inhibitory potency of polyhydroxy piperidines vs. pyrrolidines is the greater basicity of the latter; e.g., **2** has a pK_a of 6.3 and **4** has a pK_a of 7.2; **11** is expected to have a $pK_a > 8$.

The inability of castanospermine (**3**) to inhibit the α-glucosidase from yeast has been discussed[33] in terms of an active site evolved towards a transition state, with a boat-like conformation to which the rigid structure of **3** could not adapt. The conformation would put the C-2 hydroxyl group in a more axial position than in the usually accepted half-chair conformation flattened at C-1. The larger difference between the K_i values of **4** and **6** for yeast α-glucosidase, relative to the other enzymes, appears to support the hypothesis of Hosie and Sinnott[33].

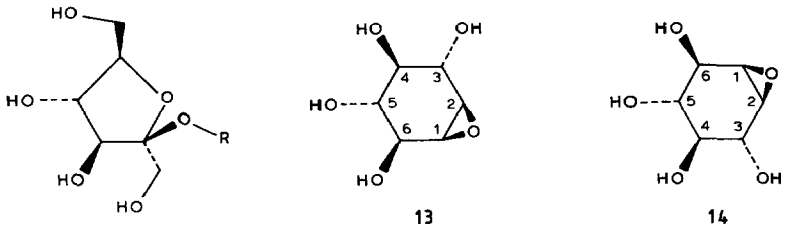
β-Fructofuranosidase (invertase) from yeast is inhibited by the basic glycon analogue **4** up to 10^5-fold better than by β-D-fructofuranose (K_i 140 mM for D-fructose[34] measured with the α:β equilibrium mixture of the furanose and pyranose forms with 31% β-D-fructofuranose[35]). The strong inhibition by **4**, which had already been noted by Card and Hitz[16], points to a mechanism similar to that of other glycosidases, i.e., a mechanism involving a transition state with a (partial) positive charge at the anomeric carbon atom stabilized by a carboxylate group in close proximity. The presence of the latter had been inferred from the irreversible inhibition of yeast invertase by conduritol B epoxide (1,2-D,L-anhydro-*myo*-inositol, CBE)[36], of which the D-enantiomer reacts with α- and β-glucosidases with the formation of an ester bond between an aspartate group and *scyllo*- and *chiro*-inositol, respectively[1]. The pH-dependence of the inactivation of invertase had

indicated a base with pK_a 3.05 (the carboxylate) and an acidic group with pK_a 6.8. The corresponding values for substrate hydrolysis were pK_a 2.9 for the base and pK_a 6.5 for the acid. From the pH-dependence of the inhibition of invertase by **4** (see Table I), we conclude that the protonated inhibitor is bound with its $-NH_2^+$ group (pK_a 7.2) in close proximity to the carboxylate with pK_a 3, because there is little change in the state of ionisation of both species on going from pH 5 to pH 7. Binding of unprotonated **4**, or association with the group with pK_a 6.5, would have caused a much larger shift of the K_i values with increasing pH.

Additional information about the active site of invertase was obtained by studying the *N*-methyl and *N,N*-dimethyl derivatives of **4** (**4a** and **4b**, respectively). The inhibition by **4a** (K_i 20 μM) and **4b** (K_i 130 μM, both at pH 6.0) indicated steric clashes of the *N*-substituents within the active site. The pH dependence of the K_i values, including that of the permanently cationic **4b**, was closely parallel to that of the parent compound (data not shown). This, and the moderate impairment of inhibitory potency, places yeast invertase among the small group of glycosidases which are strongly inhibited by cationic rather than basic glycon analogues[1]. The increase of inhibitory potency with increasing pH is probably caused by the ionisation of the acidic group with pK_a 6.5 to 6.6.

Our finding that the D-glucose analogue **2** showed no inhibition on invertase, at concentrations up to 1 mM, i.e., $K_i > 5000$ μM, indicates that, in any possibly complex with the α-glucosyl binding site, the NH group of **2** is too far removed from the carboxylate (and the acidic group) to give a contribution to the binding energy by ion-pair formation as with **4** in the fructofuranosyl site. This requires the stereochemistry of the reaction with CBE to be reevaluated.

Braun[37] had shown that the enzyme reacts with the L-enantiomer of CBE (**13**) and forms an ester of *chiro*-inositol. To account for these findings, he proposed a model involving binding of CBE at the glucosyl site where it is attacked by the carboxylate in a position to stabilize the positive charge of the transition state of substrate hydrolysis. This carboxylate was assumed to have sufficient flexibility to fulfill both functions which require different orientations in the enzyme–inhibitor and enzyme–substrate complex. Our results with **2** show that this is unlikely. Therefore, we propose that **13** is bound at the fructose binding site with maximal hydrogen bond interactions as shown in Scheme 1. The acid, donating a proton to the glycosidic oxygen of bound substrates, will then be near the oxirane oxygen of the inhibitor to act in the same way. For the D-enantiomer **14** the distance to the oxirane will be too large. The carboxylate, to stabilize the positive charge developing at the anomeric carbon of substrates, will be in a position to react with the epoxide from 'below'. If this would occur at C-1, it would result in the highly unfavorable *trans*-diequatorial mode of ring opening which would give *scyllo*-inositol. We therefore assume that the reaction occurs at C-2 to give L-*chiro*-inositol as shown by Braun[37]. The rather slow inactivation of invertase by **13** (3.9 $M^{-1}min^{-1}$ compared with 75 to 3700 $M^{-1}min^{-1}$ for the reaction with other β-glycosidases with glycon related epoxides[1]) might be due to the less favorable interaction with



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Scheme 1. A β-D-fructofuranoside drawn with L- (**13**) and D-1,2-anhydro-*myo*-inositol (**14**) in an orientation which will give maximal hydrogen bond interactions when the epoxides are bound at the fructosyl binding site of invertase. The carboxylate, assumed to stabilize a positive charge at the anomeric carbon atom during bond cleavage, should be visualized 'below' the furanose ring, and the acid donating a proton to the glycosidic oxygen, 'above'.

the glycon binding site, and to stereochemical problems with the orientation of the carboxylate which probably favors the unproductive attack at C-1, leaving only a small proportion of productive contacts with C-2.

Affinity purification of Invertase from yeast.—1-Deoxynojirimycin (**2**) and its D-*manno*-, D-*galacto*-, and 2-acetamido analogues have successfully been applied to the affinity purification of various mammalian enzymes (α-glucosidases and α-mannosidase from the endoplasmatic reticulum, lysosomal and cytosolic β-glucosidase, β-galactoceramidase from the brain, and *N*-acetyl-β-glucosaminidase from kidney)[38–43]. The basic glycon analogues are well suited for this purpose because their K_i values are in the μM range and their conversion into *N*-(ω-carboxyalkyl)-derivatives has, in general, only moderate effects on their binding properties with the respective glycosidases. By coupling the carboxyl group with an aminoalkyl derivative of agarose, the inhibitor is provided with an 'arm' of sufficient length to permit formation of the enzyme–inhibitor complex when the inhibitor is bound to an insoluble, macroporous gel.

We have applied this principle to the purification of invertase from bakers yeast by converting **4** into its *N*-(5-carboxypentyl)-derivative (**12**) by reductive alkylation with adipic acid methyl ester aldehyde and H_2–$Pd(OH)_2$. The carboxylic derivative obtained on hydrolysis of the ester was coupled to aminohexyl-Sepharose with a water soluble carbodiimide and *N*-hydroxysuccinimide for activation. As a result, conversion of **4** into **12** gave a more than 10-fold decrease of the inhibitory potency [K_i (pH 6.0) 50 μM vs. 3.5 μM for **4**]. Nevertheless, it was possible to bind 80% of the invertase activity from a yeast autolysate partially purified by precipitation with ammonium sulfate and chromatography on DEAE-cellulose. Ammonium sulfate and DEAE-cellulose completely removed the α-glucosidase(s) present in the crude extract[44], which otherwise would have interfered with the affinity purification because of their high affinity for **4** (K_i 0.73 μM). The bound enzyme was recovered by eluting the gel with a 10 mM solution of **4**; (yield 85%, purification 250-fold).

EXPERIMENTAL

General methods.—Melting points were recorded on a Tottoli apparatus and are uncorrected. Optical rotations were measured at 20°C from 0.5–5% solutions on a JASCO Digital Polarimeter with a path length of 10 cm. ^{1}H NMR spectra (at 300 MHz) and ^{13}C NMR spectra (at 75.47 MHz) were recorded on a BRUKER MSL 300 spectrometer. TLC was performed on precoated aluminium sheets (E. Merck, 5554). For preparative chromatography, Silica Gel 60 (E. Merck) was used. Immobilized glucose-isomerase (EC 5.3.1.5) was from Sweetzyme T (Novo Industries, Denmark).

*5-Azido-5-deoxy-*D*-fructopyranose* (**8**).—To a 10% aqueous solution of 5-azido-5-deoxy-D-glucofuranose[20] (**7**; 4.00 g, 19.50 mmol) containing magnesium sulphate (50 mg), immobilized glucose-isomerase (12 g) was added and the mixture was spun on a rotary evaporator at 60°C until starting material could no longer be detected by TLC (EtOAc). The immobilized enzyme was removed by filtration and carefully washed with water. The filtrate and washings were combined and filtered over a plug of activated charcoal, and the resulting colourless solution was concentrated and applied onto a short column of silica gel employing 4:1 Et OAc–petroleum ether as the eluent. Evaporation of the solvent from the product containing fractions gave the known[16,19] fructose derivative **7** (3.25 g. 81%); mp 115–18°C (lit.[16] mp 117–19°C); ^{1}H and ^{13}C NMR spectroscopic data were essentially identical with values given in ref 19.

*5-Azido-5-deoxy-*L*-sorbopyranose* (**10**).—The same procedure, used for **7**, was applied to 5-azido-5-deoxy-L-idofuranose[20] (**8**, 2.00 g, 9.75 mmol) resulting in the known [8,19] sorbose derivative **9** (1.52 g, 76%); $[\alpha]_D^{20}$ − 52.8° (*c* 1.2, MeOH); lit.[8] −54.12° (*c* 3.64, MeOH); ^{13}C NMR spectroscopic data are virtually identical with values given in ref. 19.

*2,5-Dideoxy-2,5-imino-*D*-mannitol* (**4**).—A 5% solution of 5-azido-5-deoxy-D-fructopyranose (**7**, 3.00 g, 14.6 mmol) in 1:1 MeOH–water was hydrogenated at ambient temperature on a Parr apparatus under 0.4 MPa H_2 pressure in the presence of Pd–C (5%, 350 mg) for 48 h. The catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. To the colourless residue, dry MeOH and Et_2O were added to obtain the crystalline product **4**, which was isolated as the free base by filtration (slightly yellow powder, 2.20 g, 88%); mp 114–115°C (lit.[16] 115–117°C); $[\alpha]_D^{20}$ +55.0° (*c* 1.1, H_2O); lit.[16] +55.8° (*c* 1.0, H_2O); pK_a 7.2; ^{1}H and ^{13}C NMR spectroscopic data were in agreement with values given in ref 19.

The *N*-methyl derivative of **4** was prepared by reductive alkylation with formaldehyde[46] and the iodide salt of the *N,N*-dimethyl derivative by methylation with methyl iodide[38]. Both compounds were obtained as glassy solids and were pure by TLC (3:2:0.4 $CHCl_3$–MeOH–concd aq NH_3) and gave the expected ^{1}H NMR spectra.

*2,5-Dideoxy-2,5-imino-*D*-glucitol* (**5**).—Following the procedure for the hydro-

genation of the D-*fructo*-isomer **8**, 5-azido-5-deoxy-L-sorbopyranose (**10**, 1.00 g, 4.87 mmol) was converted to yield the pyrrolidine derivative **5** (670 mg, 84%) as a slightly yellow powder; mp 132–136°C (lit.[18] 139–142.5°C, corr.); $[\alpha]_D^{20}$ +26.1° (*c* 1.2, H_2O); lit.[8] +25.75° (*c* 4, H_2O); lit.[18] +27.6° (MeOH); ^1H and ^{13}C NMR spectra are superimposable with spectra published in ref 8.

Enzymic studies.—Enzymes: α-Glucosidase was from yeast (Boehringer, Mannheim), K_m 0.25 mM; β-Glucosidase from almonds (Sigma G-4511), K_m 1.4 mM; β-glucosidase from *A. wentii*, isolated from spray-dried culture filtrate (Röhm GmbH, Darmstadt) as described[32], K_m 0.03 mM; the lysosomal β-glucosidase from bovine kidney was isolated as described for the enzyme from calf spleen[46].

α-Glucosidase activity was determined with 4-nitrophenyl-α-D-glucopyranoside in sodium phosphate pH 6.5, 50 mM as described[44]. The activity of β-glucosidases from almonds and *A. wentii* were determined with 4-methylumbelliferyl-β-D-glucopyranoside in sodium citrate–phosphate buffer, pH 5.0, 50 mM[45].

The β-glucosidase from bovine kidney was studied with 4-nonylumbelliferyl-β-D-glucopyranoside, in the same buffer, supplemented with 8 mM sodium taurocholate[46]. For the determination of invertase activity, sucrose (50 mM) was incubated at 30°C, in 50 mM sodium acetate pH 5.0, with an appropriate amount of enzyme solution, samples taken at 2 min intervals, and the reaction stopped with 10% Na_2CO_3 (5 vol). Glucose plus fructose were determined by the benzamidine method of Kai et al.[47]

Inhibition constants.—K_i Values were calculated from the reaction rates in the presence (v_i) and absence (v_0) of inhibitor with the equation

$$K_i = [I]/(v_i/v_0 - 1) \cdot (1 + [S]/K_m)$$

where [S] is the concentration of substrate with Michaelis constant K_m. At least three different concentrations of inhibitor were used, selected to give 20 to 80% inhibition. Values for K_i were reproducible to within ±10%.

Preparation of the affinity gel for invertase.—Reductive alkylation of **4** with adipic acid semialdehyde methyl ester was essentially carried out as described[47] for the *N*-alkylation of **2**. The product, the *N*-5-carboxypentyl derivative of **4**, (**12**) could not be crystallized, but was pure by TLC (3:2:0.4 $CHCl_3$–MeOH–1 M aq NH_3); **4**, R_f 0.10; methyl ester of **12**, R_f 0.81; *12*, R_f 0.45. The ^1H NMR spectrum (300 MHz, D_2O) showed all signals required by the structure. Coupling of **12** to EAH-Sepharose 4B (Pharmacia) was done as described[38].

Isolation of invertase.—A thick paste, prepared from bakers yeast (20 g) and EDTA buffer pH 7.0 (10 mL), was subjected to autolysis by the addition of toluene (3 mL) and incubation at 35°C for 18 h. The thin suspension was centrifuged (20 min, 10 000*g*), and the supernatant brought to 80% saturation by the addition of solid ammonium sulfate, centrifuged after 3 h at 4°C and the supernatant dialyzed overnight against 10 mM sodium phosphate, pH 7.0. This solution was passed over DEAE-cellulose (20 g) equilibrated in the same buffer, and the effluent slowly

agitated with the affinity gel (0.12 vol) for 18 h at 4°C. The gel, transferred to a column, was washed with 30 vol of buffer, pH 7.0, and the enzyme eluted with 10 mM **4** in sodium acetate pH 5.0 (50 mM). The inhibitor was removed by repeated (3 times) ultrafiltration and dilution with a 1:10 concentration–dilution ratio.

Electrophoresis of the purified enzyme, in the presence of sodium dodecyl sulfate, revealed a broad band corresponding to M_r 210 to 270 kDa. The molecular mass data and the diffuse appearance show that it is the heterogeneously glycosylated external enzyme[4]. The maximal specific activity was 5400 U/mg protein, and the K_m 25 mM (lit.[48] 3300 U/mg, K_m 20 mM)).

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