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Synthesis of 1,5-dideoxy-1,5-imino-D-arabinitol (5-*nor*-L-fuco-1-deoxynojirimycin) and its application for the affinity purification and characterisation of α-L-fucosidase

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Abstract

The title, 1,5-dideoxy-1,5-imino-D-arabinitol (**2**), was synthesized in seven steps from D-arabinose with 5-azido-5-deoxy-D-arabinofuranose as key intermediate and 40% overall yield. An affinity procedure employing the *N*-carboxypentyl derivative of **2** linked to aminohexyl agarose permitted the isolation of pure α-L-fucosidase from bovine kidney homogenate in two steps. Inhibition constants of **2** with the purified enzyme ranged from 39 μM (pH 5.0) to 3 μM (pH 7.0) which was ~ 1,500-fold larger than K_I for L-fuco-1-deoxynojirimycin (**1**) at these pH values. A comparably large contribution of the methyl group of **1** to the inhibition was also indicated by the K_I values for D-*manno*- and L-*gulo*-1-deoxynojirimycin. The pH-dependence of K_I for **1**, **2**, and other basic analogues was very similar to that of K_I for L-fucose (205 μM at pH 5.0 to 25 μM at pH 7.0). On the other hand, K_M for *p*-nitrophenyl α-L-fucoside and K_I for methyl α-L-fucoside showed only small variations with pH. A higher affinity, of up to 100-fold, of the enzyme for L-fucose relative to methyl α-L-fucoside in conjunction with its different pH-dependence is tentatively explained by a hydrogen bond of the anomeric hydroxyl group as donor with an active site carboxylate with pK_a 6.1 as acceptor. The inhibitory potency of **2** was greatly lowered by *N,N*-dimethylation (K_I 3600 μM at pH 5.0 to 305 μM at pH 7.0).

Keywords: α-L-Fucosidase, affinity purification; α-L-Fucosidase, inhibition; α-L-Fucosidase, characterisation of active site; D-Arabinitol, 1,5-dideoxy,1,5-imino-, synthesis; D-Arabinitol, 1,5-dideoxy-1,5-imino-, inhibition of α-L-fucosidase

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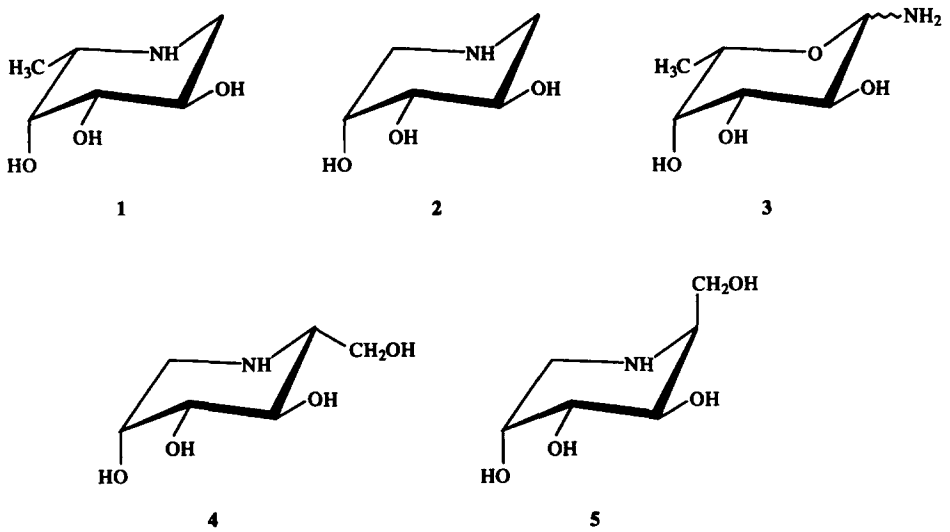
1. Introduction

Mammalian α-L-fucosidases (α-L-fucoside fucohydrolases, EC 3.2.1.51) are lysosomal enzymes involved in the degradative pathway of L-fucose containing glycoproteins and glycolipids. In humans, genetic defects in their expression cause the lysosomal storage disease fucosidosis, characterized by an accumulation of fucosylated glycolipids, glycopeptides and oligosaccharides in various tissues and severe effects on neural function [1]. The fucosidases resemble other glycosidases in being strongly inhibited by glycon analogues having a basic amino function at C-1, e.g. L-fucosylamine [2] and L-fuco-1-deoxynojirimycin [3,4]. A carboxylate group essential for catalysis was inferred for the enzyme from humans from the temperature dependence of pH-rate profiles [5] and from the reaction with the active-site directed inhibitor conduritol C *trans*-epoxide [6]. The mechanism of L-fucosidases is thus likely to be similar to that of most other glycosidases [7].

L-Fucosidases are not strictly specific for α-L-fucosides but they also hydrolyse *p*-nitrophenyl β-D-arabinoside at approximately a three-fold lower rate based on V_{max} or a 70-fold lower rate when V_{max}/K_M is compared [8]. This rather small effect of replacing the methyl group of L-fucose by hydrogen and the high affinity [3,4] for 1,5-dideoxy-1,5-imino-L-fucitol (fuco-deoxynojirimycin, fuco-dNM, **1**) prompted us to prepare the D-arabino-analogue of dNM, 1,5-dideoxy-1,5-imino-D-arabinitol (*nor*-fuco-dNM, **2**). From its inhibition data we expected to obtain information on that part of the glycon binding site complementary to L-fucose and on the binding energy contributed by the methyl group. In addition, **2** was thought to be a suitable affinity ligand for the purification of L-fucosidases. Compound **2** has already been synthesized [9] by a more elaborate route than the one described here but no inhibition data with α-L-fucosidases were reported (Scheme 1).

2. Results and discussion

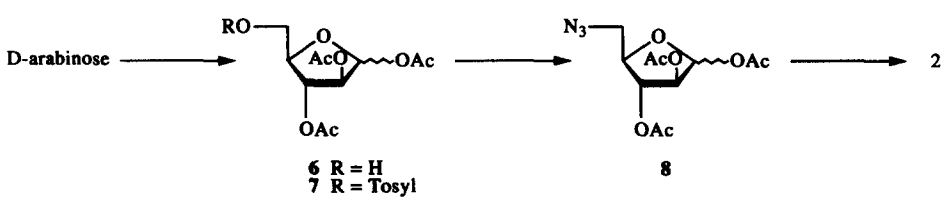
Compound **2** was synthesized in the following straightforward manner employing only standard procedures and relatively cheap and easily available chemicals: D-arabinose was treated with 1.5 equiv of chlorotriphenylmethane (trityl chloride) in pyridine for 24 h to give the 5-*O*-trityl derivative which was *O*-acetylated in situ by addition of 5 equiv of acetic anhydride to the reaction mixture. After removal of the excess acetic anhydride by addition of methanol and concentration under reduced pressure the syrupy residue was dissolved in dichloromethane and washed with 5% aqueous HCl. The organic layer was dried and boron fluoride–ethyl ether and methanol were added to cleave the trityl ether in a standard procedure [10]. By this three-step sequence, 1,2,3-tri-*O*-acetyl-D-arabinofuranose (**6**) was obtained after chromatography in 60% overall yield. ^{1}H NMR spectra revealed that the α/β-ratio of the product usually was between 2:1 and 4:1. Compound **6** was converted into the 5-*p*-toluenesulfonate (**7**, 85%) which was reacted with sodium azide in *N*,*N*-dimethylformamide at 90°C to give 1,2,3-tri-*O*-acetyl-5-azido-5-deoxy-D-arabinofuranose (**8**) in 94% yield. Subjecting compound **5** to Zemplén conditions gave free 5-azido-5-deoxy-D-arabinofuranose in practically quantitative yield.



Scheme 1. The structures of **4** and **5** were drawn in an orientation which brings the hydroxyl and imino groups in the same position as in **1** and **2**.

Catalytic hydrogenation over palladium-on-charcoal (5%) under ambient pressure for 16 h led to the desired inhibitor **2** which, after crystallization from methanol–ether could be isolated as the free base in 85% yield. This sequence of reactions represents a considerable improvement over the previously described procedure [9] and allows a convenient access to multi-gram quantities of the product. Attempts to gain access to compound **2**, by a sequence involving methyl-D-arabinofuranoside, turned out to be not as efficient in term of simplicity of procedures and overall yield (Scheme 2).

Isolation of α-L-fucosidase.—"Aza-sugars" of the deoxynojirimycin type and their five-membered analogues are well suited as affinity ligands for glycosidases (see ref. [11] for examples) because of their high affinity (K_I values in the μM range) and the ease of attaching them to the gel support by means of an "arm" linked to the ring nitrogen. Previously described ligands for the affinity purification of α-L-fucosidases are 6-aminocaproyl-β-L-fucosylamine [12,13], *p*-aminophenyl 1-thio-β-L-fucopyranoside [14], 3-(α-L-fucopyranosyl)propylamine [15], and the *N*-5-carboxypentyl derivative of **1** [4,16]. The first three have rather low affinities for the enzyme (e.g. K_I 2.6 mM [13]), resulting in low capacities of the affinity gel [12,15] or even failure to bind (cited from



Scheme 2.

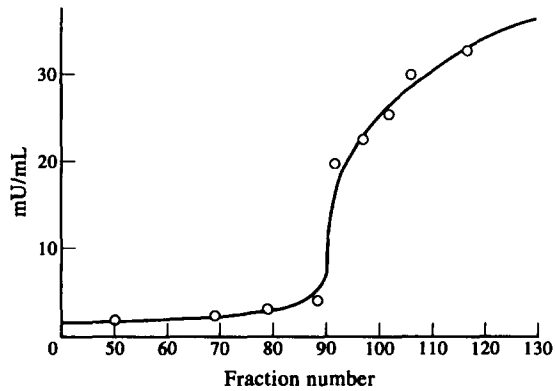


Fig. 1. Affinity binding of α-L-fucosidase from a heat denatured extract from bovine kidney [425 mL (Table 1) with 56 mU/mL α-L-fucosidase activity] on *N*-(5-carboxypentyl)-2 linked to EAH-Sepharose. Bed volume 5.5 mL, flow rate 3.0 mL/h, fraction size 3.0 mL. Ordinate gives enzyme activity in the effluent.

ref. [15] for the 1-thiofucopyranoside [14]). The *N*-5-carboxypentyl derivative of **1** has been applied to the isolation of α-L-fucosidases from almonds [16] but the enzyme from bovine kidney bound too tightly to be eluted effectively [17].

As preliminary measurements have shown compound **2** to have K_I values much larger than **1**, but still in the range suitable for affinity chromatography (40 μM at pH 5 and 2.5 μM at pH 7), we converted it into the *N*-(5-carboxypentyl) derivative by reductive alkylation with methyl 6-oxocaproate and linked it to aminohexyl agarose. Reaction with ninhydrin before and after ligand coupling gave an intra-column concentration of 4 μmol ligand per mL settled gel. As K_I value for **2** was much lower at pH 7 than at pH 5, the former pH was selected for the binding and washing steps whereas elution of the enzyme with 50 mM L-fucose was carried out at pH 5. The results of passing a partially purified homogenate from bovine kidney over the affinity gel are

Table 1
Purification of α-L-fucosidase from bovine kidney

Purification step	Volume (mL)	Protein (mg)	Activity (U)	Spec. activ. (U per mg prot.)	Purification-fold	Yield (%)
Crude extract						
Supernatant (20000 g) from 500 g fresh tissue	550	21000	28.4	0.00135	1	100
Heat denaturation with 450 mL of crude extract	425	8100	23.7	0.0029	2.2	93
Affinity chromatography (Fig. 1)						
Fraction 1–90	270	n.d.	0.5	—	—	2.1
Fraction 91–140	150	n.d.	5.0	—	—	21
Eluate with L-fucose (50 mM)	18.5	2.54	15.0	5.9	7400	63

n.d. not determined.

Table 2
Michaelis constant K_M for *p*-nitrophenyl α-L-fucopyranoside and inhibition constants K_I for L-fucose and its nitrogen containing analogues (K_M and K_I in μM)

	pH 5.0	pH 6.0	pH 7.0
p-Nitrophenyl α-L-fucopyranoside	200	250	380
L-Fucose	205	30	25
Methyl α-L-fucopyranoside	4500	2100	3600
L-Fucopyranosylamine (**3**)	4.9	0.75	1.2
L-fuco-deoxynojirimycin (**1**)	0.017	0.0027	0.0013
nor-fuco-deoxynojirimycin (**2**)	24	4.3	2.2
D-*manno*-Deoxynojirimycin (**4**)	64	9.5	6.6
L-*gulo*-Deoxynojirimycin (**5**)	n.d.	16	9.0
N-Carboxypentyl-*nor*-fuco-deoxynojirimycin (**9**)	130	n.d.	18
N,*N*-Dimethyl-*nor*-fuco-deoxynojirimycin (**10**)	3600	700	305

n.d. not determined.

presented in Fig. 1 and Table 1. The amount of L-fucosidase bound in the saturation experiment (2.8 U/mL gel, Fig. 1) was ⩾ 33-fold higher than reported for gels with *N*-caproyl-β-L-fucosylamine [12,15] and 3-(α-L-fucopyranosyl)propylamine [15] as ligands when the different temperatures during the assay (25 vs. 37°C) are taken into account. The enzyme eluted with L-fucose was shown to be pure by SDS-PAGE (M_r 50 kDa); its specific activity (6.8 U/mg assayed at 25°C, 14 U/mg at 37°C) was in good agreement with the data for the enzymes from human [5] and porcine [15] liver.

The enzyme activity eluting from the affinity columns prior to saturation (e.g. 1.5 mU/mL at 50% saturation (Fraction 50); 3.8 mU/mL at 90% saturation (Fraction 90) was distinctly lower than expected from the ligand concentration in the column (4 mM) and the K_I determined for the *N*-5-carboxypentyl derivative of **2** (18 μM; Table 2).

Apparent K_I values calculated from the activity eluted at fraction 50 and 90 and from amount of enzyme retained in the column were 6 μM and 9.9 μM, respectively, i.e. much lower than expected because steric effects with the gel-matrix and diffusion problems tend to give values for K_I (apparent) which are higher than K_I in solution. However, if the tetrameric structure reported for the human L-fucosidase [18,6] does also hold for the bovine enzyme, the calculated values should be corrected by a factor of four because in the solution experiments each subunit can bind an inhibitor molecule whereas a tetrameric enzyme molecule will be retained by a single immobilized inhibitor molecule, thus giving a four-fold larger equivalent weight. The differences between the corrected K_I (apparent) values (24 and 40 μM, respectively) and the solution value of 18 μM demonstrate that the formation of the enzyme ligand complex is close to equilibrium and that practically all of the immobilized ligand is accessible to the enzyme.

Inhibition studies.—The inhibition constants K_I (Table 2) were calculated from experiments with a five-fold variation of substrate concentrations; the competitive character of the inhibition followed from the absence of systematic deviations over this concentration range. L-Fucose was included in this study for comparison with its basic (cationic) analogues. At pH 5.0, its K_I value (0.2 mM) was similar to the K_I values

reported for other mammalian α-L-fucosidases at this pH [18]. At pH 6.0 and 7.0, where no data from the literature were available, K_I values were 6.8- and 8.2-fold lower, respectively. This finding was unexpected as the basic (cationic) inhibitors showed a similar pH-dependence of K_I whereas K_M for the substrate varied little with pH (Table 2) or is reported to be pH-independent with the enzyme from humans [5]. The pH-rate profile, on the other hand, resembled that of a crude preparation from bovine liver [19] with a sharp maximum at pH 5.5, half-maximal activities at pH 3.3 and 6.7 and a shoulder at pH 4.0 (not shown). A similar asymmetric pH-profile but with maximal activity at pH 4.5 was reported for the enzymes from rat epididymis [19] and human liver [20].

Experiments to evaluate the individual contributions of α- and β-L-fucose to the inhibition seemed to indicate that both anomers were of similar inhibitory potency. Stock solutions of α-L-fucose, measured within 4 min after dissolution, gave about the same apparent K_I value as solutions with the anomers at equilibrium. It should be noted, however, that mutarotation under the assay conditions (e.g. 50 mM phosphate/citrate at pH 6.0 and 25°C) proceeded with a half-life of ~ 6 min.

When the potential substrate methyl α-L-fucopyranoside was tested as inhibitor for the hydrolysis of *p*-nitrophenyl α-L-fucoside, we found a pH-dependence of K_I which differed markedly from that for L-fucose (Table 2). Furthermore, the affinity of the enzyme for the fucoside was up to 100-fold lower than for L-fucose. A tentative explanation for the high affinity for L-fucose and its pH-dependence could be the formation of a hydrogen bond with the anomeric hydroxyl group as donor and a carboxylate group of $pK_a \sim 6.1$ (see below) as acceptor.

Glycosidases are inhibited by glycosylamines 100- to greater than 1000-fold better than by the corresponding hexoses, where the response of the α-specific enzymes is mainly in the lower part of this range [7]. α-L-Fucosidase appeared to be an exception because the reported K_I for L-fucosylamine (**3**, 0.07 mM [2]) was only four-fold lower than K_I for L-fucose. Inclusion of **3** in our study showed, however, that K_I(L-fucose)/K_I(L-fucosylamine) ≈ 40 (Table 2), thus showing the response of α-L-fucosidase to be close to the normal range. At pH 6 and, to a lesser extent at pH 5 and 7, the decomposition of **3** caused the enzyme activity to increase; e.g. from 23 to 36% of the uninhibited rate within 8 min after the addition of 20 μM **3** at pH 6.0. Freshly prepared aqueous solutions of **3** showed complex changes of optical rotation and pH (see Experimental section), indicating anomerization and concomitant hydrolysis. A quantitative interpretation of the apparent K_I values calculated from the "initial" rates in terms of individual contributions from the α- and β-anomers of **3** met with difficulties because of the overlap of anomerization and (two) rates of hydrolysis. The value for K_I at pH 7.0 differed from that of the other inhibitors in being consistently higher than at pH 6 (Table 2). Our tentative explanation is that α-L-fucosidase is strongly inhibited only by the cationic form of basic glycon derivatives whereas **3** ($pK_a \leqslant 7.0$) is less than 50% protonated at pH 7.0.

The inhibition constants for **3** given in Table 2 are considered to be approximate values for the β-anomer for the following reasons:

(i) **3** crystallizes as β-anomer as shown by the formation of *N*-caproyl-β-L-fucosylamine under conditions where mutarotation is very slow [21];

(ii) when **3** was dissolved in half the equivalent of dilute hydrochloric acid for the determination of pK_a, the pH dropped from 7.0 to 5.66 within the first 3 min and then started to rise slowly until the pH 8.6 was reached after 24 h (see Experimental section). Similar observations have been made with β-glucosylamine and some *N*-alkyl derivatives where the initial fall in pH was ascribed to the formation of the less basic α-anomer [22];

(iii) the K_I values given in Table 2 were calculated from rates measured directly after the addition of **3** from fresh stock solutions in dimethyl sulfoxide where no mutarotation was observed for at least 2 h. Interestingly, the rates measured at pH 6.0 increased to a constant value within 8–10 min which resulted in values for K_I (apparent) that were two-fold higher than those calculated from the initial rates. As the rate increase was much faster than the hydrolysis of **3** and L-fucose inhibited about 40-fold less potently than **3**, we conclude that β-L-fucosylamine is a better inhibitor than its α-anomer.

Glycon analogues which have the ring oxygen atom replaced by the NH-group are among the most powerful non-covalent inhibitors of glycosidases [7]. As shown by published data [3,4] and the K_I values in Table 2, L-fuco-dNM (**1**) is a prominent example for the large enhancement of affinity ($\geqslant$ 10,000-fold in terms of K_I or 5.5 kcal/mol as expressed by $\Delta\Delta G$) caused by converting the neutral sugar into this type of basic analogue. The strong inhibition is usually explained by the electrostatic interactions of the protonated imino group with a closely positioned carboxylate of the active site. The pH-dependence of the inhibition should, therefore, depend on the ionization state of both the inhibitor and the carboxylate. As the former is predominantly cationic over the pH-range studied here (pK_a 8.4 [20]), the observed variation of K_I with pH can be used to calculate pK_a of the putative carboxylate. Assuming that other ionizations of the enzyme have no effect and interactions of the inhibitor with the undissociated carboxyl group can be neglected, we get from the combination of pK_I values at pH 5–7 (see Experimental section) pK_a 6.1 $\pm$ 0.1. A very similar result was obtained when the same procedure was applied to the K_I values of L-fucose. Thus, it appears likely that the ionizing groups responsible for the pH-dependence of K_I for the neutral sugar and its cationic analogues are identical. [1]

The conversion of **1** into its 5-*nor*-derivative **2** gave a compound with a 1500-fold larger K_I, i.e. replacement of the methyl group of **1** by hydrogen caused a decrease in the standard free energy of binding amounting to 4.3 kcal/mol. This is about five-fold larger than expected from the hydrophobic interaction energy of a methyl group as estimated from partition experiments [23]. An explanation for this large energy contribution by the methyl group could be a glycon binding site with a pocket closely adapted to this part of the substrate. This could allow short range van der Waals interactions which

[1] Winchester et al. [20] have studied the inhibition by **1** with the α-L-fucosidase activity of an extract from human liver. From a plot of %-inhibition vs. pH with half-maximal inhibition at pH 3.6 and approach to a plateau of maximal inhibition at pH > 5.5, they concluded that a carboxyl group with pK_a 3.5 to 4 is responsible for the pH-dependence. In our opinion, this conclusion is not correct because the effect of the inhibitor and substrate concentrations on the pH curve had not been taken into account. High inhibitor concentrations will shift the curve to lower pH values.

are much less efficient in liquid partition systems because of looser packing and continuous fluctuations. Also, the absence of the methyl group might give **2** a greater flexibility within the binding site, thus making hydrogen bonds less effective.

D-*manno*-l-Deoxynojirimycin (*manno*-dNM, **4**) can be considered as an analogue of **1** which lacks the C-6 methyl group and has a β-configured hydroxymethyl group at C-1 [2]. Comparison of its inhibition constant ($K_I \sim 11$ μM at pH 6.5 for bovine α-L-fucosidase [24]; K_I 5.5 μM for the human enzyme [20]) with K_I for **1** (0.0027 μM at pH 6.0, Table 2) also demonstrates the importance of the methyl group on C-5 for efficient binding. A comparison with K_I for **2** (K_I 4.3 μM at pH 6.0) demonstrates that the "β-configurated" hydroxymethyl group has only a marginally detrimental effect on the enzyme inhibitor interactions. The inhibitory potency of the "α-configured" L-*gulo*-l-deoxynojirimycin (**5**) was very similar to that of **4**.

N-Alkyl substitutents on **1** cause an approximate five-fold increase of its K_I as shown with the *N*-methyl derivative and human α-L-fucosidase by Winchester et al. [20] and, in addition, with the *N*-carboxypentyl derivative and the bovine enzyme by Paulsen et al. [4]. A similar effect of *N*-alkylation on K_I was seen in this study with **2** and its *N*-carboxypentyl derivative (Table 2). *N*,*N*-Dimethylation of **2**, on the other hand, caused K_I to increase 150-fold. This large decrease of inhibitory potency is probably not due to the conversion of a basic glycon analogue into a permanently cationic one as discussed for other glycosidases [7] because **2** (pK_a 8.3) is > 95% cationic even at pH 7.0. Instead, we propose that one of the methyl groups is in a position where it causes steric repulsions within the active site. With the cationic form of the monoalkyl derivatives, this position can be occupied by the proton.

3. Conclusion

In conjunction with previous studies on mammalian α-L-fucosidase, the following conclusions may be drawn from our results:

(i) The glycon site provides a close fit for the methyl group of L-fucose as its absence in **2**, **4**, and **5** caused K_I to increase 1500-fold over K_I for **1**. This corresponds to a contribution by the methyl group of 4.3 kcal mol^{-1} to the free energy of binding.

(ii) The basic analogues of L-fucose **1** to **5** bind as cations up to 10,000-fold better than L-fucose, presumably by the formation of an ion-pair with a carboxylate group having pK_a 6.1. The additional binding energy (5.5 kcal mol^{-1}) provided by electrostatic interactions may even be larger because L-fucose binds exceptionally strong by the formation of a hydrogen bond with the same carboxylate. An ion-pair as well as hydrogen bond formation of this strength indicate that water access to this part of the enzyme inhibitor complex is strongly impaired. Whereas steric effects of *N*-monoalkylation were small, a large effect on K_I was seen with the *N*,*N*-dimethyl derivative of **2**, pointing to close contacts with one side of the pyranose ring. α-L-Fucosidase resembled most other glycosidases [7] in being more weakly inhibited by glycosylamines than by the corresponding glycon analogues having an endocyclic nitrogen at C-1.

(iii) The close contacts of the enzyme with the methyl group at C-5 and the endocyclic nitrogen atom on C-1 is in marked contrast with seemingly loose interactions

with exocyclic substituents at C-1 as indicated by only small detrimental effects of the hydroxymethyl groups of **4** and **5**, even though its orientation in **4** is opposite to the anomeric configuration of the substrate. Flexible enzyme ligand contacts around C-1 are, in addition, indicated by the close correspondence of K_I values for methyl α-L-fucopyranoside and *N*-caproyl-β-L-fucopyranosylamine (2600 μM [13]) and by the small and possibly inverse effects of the anomeric configuration on the inhibition by L-fucose and L-fucosylamine.

(iv) Studies with α-L-fucosidase from humans [5] have shown that two carboxyl groups participate in the hydrolysis of *p*-nitrophenyl α-L-fucoside, one with pK_a 3.5 as general base or nucleophile and the other one with pK_a 7.3 as general acid. As these pK_a values were derived from the pH-dependence of V_{max}, they refer to ionizations of the enzyme substrate complex. Furthermore, it has been shown [6] that the reaction proceeds by a two-step mechanism where the deglycosylation of a β-L-fucosyl enzyme intermediate is rate limiting. As acid catalysis appears unlikely for this step, the group ionizing with pK_a 7.3 is probably not directly involved in catalysis; the group with pK_a 3.5 presumably acts a general base in the hydrolysis of the fucosyl enzyme intermediate. Carboxyl groups with pK_a 3.7 (nucleophile) and 6.1 (general acid) were inferred [6] from inactivation studies with conduritol C *trans*-epoxide where the pH-dependence is largely governed by ionizations of the free enzyme. From the stereochemistry of the reaction (no reaction with conduritol C *cis*-epoxide), it was concluded that the nucleophile binding the epoxide is identical with the carboxyl group which forms the fucosyl enzyme during substrate hydrolysis. From the similarity of the pK_a values calculated from the pH-dependence of K_I for L-fucose and its cationic analogues (pK_a 6.1 ± 0.1) with the pK_a of the general acid in the epoxide reaction, which is supposed to have the same function in the first step of substrate hydrolysis [6], we conclude that the inhibitors studied here are bound with their cationic centres close to this group.

(v) In view of the great importance of the C-5 methyl group of L-fucose with respect to enzyme inhibitor interactions, as demonstrated by the K_I values of **2**, **4**, and **5** vs. K_I of **1**, a reinvestigation of the activity of α-L-fucosidase with *p*-nitrophenyl β-D-arabinopyranoside [8] referred to in the Introduction seems justified.

4. Experimental

General methods.—Melting points were recorded on a Tottoli apparatus and are uncorrected. Optical rotations were measured at 22°C 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 with residual non-deuterated solvent as the internal standards. TlC was performed on precoated aluminium sheets (Merck 5554). For preparative chromatography, Silica Gel 60 (Merck) was used.

*1,2,3-Tri-*O*-acetyl-*D*-arabinofuranose* (**6**).—To a 5% soln of D-arabinose (15.1 g, 100 mmol) in dry pyridine, chlorotriphenylmethane (28 g, 150 mmol) was added and the mixture was stirred at 50°C. After 24 h, the soln was allowed to reach ambient

temperature and acetic anhydride (50 mL, 530 mmol) was added. After 6 h, MeOH (35 mL) was added and the mixture was concentrated under reduced pressure. The resulting syrup was dissolved in CH_2Cl_2 (300 mL), the solution was washed with 5% aq HCl and dried over Na_2SO_4. After filtration, $Et_2O \cdot BF_3$ (15 mL, 1 M in ether) and MeOH (50 mL) were added to the filtrate. As soon as TLC (1 : 1, petroleum ether–EtOAc) indicated that the trityl ether had been converted to slower moving material, the reaction mixture was extracted with great care with 5% aq $NaHCO_3$ and dried over Na_2SO_4. Removal of the solvent under reduced pressure and chromatography (4 : 1, petroleum ether–EtOAc) of the oily residue gave syrupy compound **6** as an inseparable mixture of anomers (16.6 g, 60%). ^{1}H NMR data ($CDCl_3$): δ 6.30 (d, $J_{1,2}$ 3.7 Hz, H-1β), 6.10 (s, H-1α), 5.31 (m, H-2β, H-3β), 5.16 (d, $J_{2,3}$ 1.5 Hz, H-2α), 5.03 (dd, $J_{3,4}$ 5 Hz, H-3α), 4.15 (dd, $J_{4,5}$ 8.5 Hz, H-4α), 4.00 (m, H-4β), 3.81–3.61 (m, H-5α, H-5′α, H-5β, H-5′β), 2.85 (bs, 5-OH), α/β-ratio approx. 3 : 1 (by integration over selected parts of the spectrum); ^{13}C NMR data ($CDCl_3$): δ 99.4 (C-1α), 93.6 (C-1β), 85.1 (C-4α), 82.6 (C-4β), 81.1 (C-2α), 76.7 (C-3α), 75.6 (C-2β), 74.6 (C-3β), 63.4 (C-5β), 61.7 (C-5α); the signals of the *O*-acetyl groups appear in the expected regions. Anal. Calcd for $C_{11}H_{16}O_8$: C, 47.83; H, 5.84. Found: C, 47.61; H, 5.94.

*1,2,3-Tri-*O*-acetyl-5-*O*-p-toluenesulfonyl-*D*-arabinofuranose* (**7**).—To a 5% solution of compound **6** (16.0 g, 58 mmol) in CH_2Cl_2, pyridine (25 mL) and *p*-toluenesulfonyl chloride (15 g, 79 mmol) were added and the mixture was kept at ambient temperature until TLC (2 : 1 petroleum ether–EtOAc) indicated quantitative conversion into a less polar UV-active product. Methanol (25 mL) was added and the mixture was concentrated under reduced pressure. Chromatography (6 : 1, petroleum ether–EtOAc) gave compound **7** as a colourless syrup (20.8 g, 83%). ^{1}H NMR data ($CDCl_3$): δ 6.29 (d, $J_{1,2}$ 2.5 Hz, H-1β), 6.07 (s, H-1α), 5.26 (m, H-2β, H-3β), 5.11 (s, H-2α), 4.95 (d, $J_{3,4}$ 4.0 Hz, H-3α), 4.30–4.25 (m, H-4α), 4.23–4.21 (m, H-5α, H-5′α), 4.17–4.04 (m, H-4β, H-5β, H-5′β); ^{13}C NMR data ($CDCl_3$): δ 99.4 (C-1α), 93.5 (C-1β), 82.7 (C-4α), 80.4 C-2α), 79.5 (C-4β), 76.8 (C-3α), 75.3 (C-2β), 74.5 (C-3β), 69.7 (C-5β), 68.4 (C-5α); the signals of the *O*-acetyl and the *O*-sulfonyl groups are located in the expected positions. Anal. Calcd for $C_{18}H_{22}O_{10}S$: C, 50.23; H, 5.15. Found: C, 49.98; H, 5.24.

*1,2,3-Tri-*O*-acetyl-5-azido-5-deoxy-*D*-arabinofuranose* (**8**).—To a 10% solution of sulfonate **7** (20.0 g, 46.5 mmol) in DMF, NaN_3 (13 g, 200 mmol) was added and the mixture was stirred at 90°C until TLC indicated quantitative conversion of the starting material into a single faster moving product. The reaction mixture was allowed to reach ambient temperature and CH_2Cl_2 (350 mL) was added to precipitate most of the inorganic salts. After filtration, the filtrate was concentrated under reduced pressure and the remaining residue was partitioned between CH_2Cl_2 (300 mL) and water (300 mL). The organic layer was dried over Na_2SO_4 and the filtrate concentrated under reduced pressure. The oily residue was purified on silica gel (6 : 1, petroleum ether–EtOAc) to give azidodeoxysugar **8** as a colourless syrup (13.2 g, 94%). ^{1}H NMR data ($CDCl_3$): δ 6.31 (d, $J_{1,2}$ 3.7 Hz, H-1β), 6.14 (s, H-1α), 5.29 (m, H-2β, H-3β), 5.13 (s, H-2α), 4.96 (d, $J_{3,4}$ 4.8 Hz, H-3α), 4.23 (dd, H-4α), 4.06 (m, H-4β), 3.61 (dd, $J_{4,5}$ 3.3, $J_{5,5}$ 13.4 Hz, H-5α), 3.53 (dd, $J_{4,5}$ 3.6, $J_{5,5}$ 13.3 Hz, H-5β), 3.37 (dd, $J_{4,5'}$ 4.8 Hz, H-5′α), 3.37 (m, H-5′β); ^{13}C NMR data ($CDCl_3$): δ 99.2 (C-1α), 93.4 (C-1β), 84.0 (C-4α),

80.8 (C-4β), 80.6 (C-2α), 77.3 (C-3α), 75.1 (C-2β), 74.9 (C-3β), 53.0 (C-5β), 51.3 (C-5α); the resonances of the *O*-acetyl groups are located in the expected regions. Anal. Calcd for $C_{11}H_{15}O_7N_3$: C, 43.86; H, 5.02. Found: C, 44.05; H, 5.11.

1,5-Dideoxy-1,5-imino-D-*arabinitol* (**2**).—To a 5% solution of fully protected azido-deoxysugar **8** (5.0 g, 16.6 mmol) in dry MeOH, NaOMe (1 mL, 1 M soln) was added at ambient temperature and the reaction was monitored by TLC. When all starting material was converted into a single slower moving product, the clear solution was neutralized by addition of Amberlite IR 120, H^+ ion exchange resin. The resin was removed by filtration to give a soln of chromatographically pure 5-azido-5-deoxy-D-arabinofuranose. Palladium-on-charcoal (5%, 800 mg) was added and the mixture was stirred under an atmosphere of hydrogen at ambient temperature and pressure for 16 h. The catalyst was removed by filtration and the clear, colourless solution was concentrated under reduced pressure. The resulting glassy residue slowly crystallized at 5°C. Trituration with MeOH–ether gave pure free base **2** as colourless crystals (1.88 g, 85%); mp 182–185°C (dec); $[\alpha]_D$ −71° (*c* 0.6, MeOH). ^{1}H NMR data (D_2O) δ 3.97 (m, 1 H, H-4), 3.75 (ddd, 1 H, $J_{1a,2}$ 9.5, $J_{1e,2}$ 5, $J_{2,3}$ 8.7 Hz, H-2), 3.55 (dd, 1 H, $J_{3,4}$ 3.0 Hz, H-3), 3.04 (dd, 1 H, $J_{1a,1e}$ 13.2 Hz, H-1e), 2.90 (dd, 1 H, $J_{4,5e}$ 3.4, $J_{5a,5e}$ 14.1 Hz, H-5e), 2.67 (dd, 1 H, $J_{4,5a}$ 1.8 Hz, H-5a), 2.35 (dd, 1 H, H-1a); ^{13}C NMR data (D_2O): δ 74.8 (C-3), 69.7, 69.3 (C-2, C-4), 49.6, 49.1 (C-1, C-5). For comparison with published data, a small sample of the free base was converted by treatment with an excess of HCl in MeOH into the corresponding hydrochloride, which was obtained as a colourless glass; lit. [9]: mp 191–192°C; $[\alpha]_D$ −18° (*c* 0.7, MeOH); lit. [9] −16° (*c* 0.9, MeOH). ^{1}H NMR (D_2O): δ 3.91 (ddd, 1 H, $J_{3,4}$ 3.0, $J_{4,5a}$ 2.4, $J_{4,5e}$ 5.5 Hz, H-4), 3.75 (ddd, 1 H, $J_{1a,2}$ 8.5, $J_{1e,2}$ 4.3, $J_{2,3}$ 7.9 Hz, H-2), 3.44 (dd, 1 H, H-3), 3.09 (dd, 1 H, $J_{1a,1e}$ 12.8 Hz, H-1e), 2.97 (dd, 1 H, $J_{5a,5e}$ 12.8 Hz, H-5e), 2.88 (dd, H-5a), 2.62 (dd, 1 H, H-1a); ^{13}C NMR (D_2O): δ 71.6 (C-3), 65.9, 65.5 (C-2, C-4), 47.0, 46.4 (C-1, C-5), pK_a 8.3 (potentiometric titration).

N-*(5-Carboxypentyl)-1,5-dideoxy-1,5-imino*-D-*arabinitol* (**9**).—Reductive alkylation of **2** with adipic acid semialdehyde methyl ester and $H_2/Pd(OH)_2$ on charcoal was carried out as described for *N*-alkyl derivatives of 1-deoxynojirimycin [26]. The 5-carbomethoxyxypentyl derivative of **2** was hydrolysed by keeping its aqueous solution at pH 12 for 6 h, filtration over a small column with Dowex 50 × 4 (H^+-form), and elution of **9** with 5% aq ammonia. Compound **9** could not be crystallized but was pure by chromatography (4 : 2 : 0.4, $CHCl_3$–MeOH–20% aq ammonia). ^{1}H NMR (80 MHz, D_2O): δ 1.2–1.9 (m, 6 H, *N*-pentyl H-2 to H-4), 2.33 (2 H, t, *N*-pentyl H-5), 2.72–2.30 (m, 4 H, *N*-pentyl H-1, H-1a, H-5a), 2.95–3.10 (1 H, m, H-5e), 3.10–3.25 (m, 1 H, H-1e), 3.67 (1 H, d,d, $J_{2,3}$ 8.2, $J_{3,4}$ 3.0 Hz, H-3), 3.95 (1 H, d,d,d, $J_{1e,2}$ 4, $J_{2,3}$ 8.2, $J_{1a,2}$ 9 Hz, H-2), 4.1 −4.2 (1 H, m, H-4).

N,N-*Dimethyl-1,5-dideoxy-1,5-imino*-D-*arabinitol iodide salt* (**10**).—Compound **2** was converted into its *N*-methyl derivative by reductive alkylation with formaldehyde [26]. The *N*-methyl derivative of **2** (64 mg) was stirred overnight at 45°C with CH_3I (0.3 mL), the mixture taken to dryness, and the residue crystallized from EtOH ether to give **10** (70.2 mg); mp 169–169°C. ^{1}H NMR (D_2O): δ 3.42 and 3.50 (6 H, 2 s, *N*-CH_3), 3.5–3.75 (3 H, m, H-1a, H-5a, H-5e), 3.92–4.10 (2 H, m, H-1e, H-3), 4.30–4.6 (2 H, m, H-2, H-4).

Other inhibitors.—Methyl α-L-fucopyranoside [6], D-*manno*-(**4**), and L-*gulo*-l-deoxynojirimycin (**5**) [27] were prepared as described. L-*fuco*-l-Deoxynojirimycin (**1**) was provided by Professor Hans Paulsen (Hamburg).

L-Fucosylamine (**3**) was synthesized according to Blumberg et al. [21]. As the lability of glycosylamines in aq soln precludes accurate potentiometric titrations pK_a of **3** was estimated from the pH of solutions containing **3** (50 mM) and HCl (25 mM). The readings fell from the initial (5 s) pH 7.0 to 5.83 within 90 s, remained constant for ~ 5 min and then rose slowly to reach pH 6.5 after 30 min and pH 7.1 after 1 h. After 24 h at room temperature and short heating to 95°C the pH had risen to 8.9 instead of pH 9.2 as calculated for complete hydrolysis, indicating that additional reactions [28] had taken place.

Optical rotation was measured on a Perkin–Elmer 141 polarimeter with solutions of **3** (10 mg/mL) in water (pH 9.6) and readjusted buffer solutions at pH 7.0, 6.0 and 1.0. Mutarotation was most rapid at pH 6.0 from the initial (extrapolated) $[\alpha]^{20}_{579}$ −44,2° to $[\alpha]^{20}_{579}$ −56.0° after 10 min, −71.2° after 30 min and −76.7° after 24 h. L-Fucose (equilibrium) had $[\alpha]^{20}_{579}$ −78.9°. At pH 7.0, the initial mutarotation rate was similar but became much slower after 10 min, reaching $[\alpha]^{20}_{579}$ −56.3° after 30 min. The optical rotation remained constant for at least 120 min in solns of **3** in Me_2SO ($[\alpha]^{20}_{579}$ − 36.2°).

Preparation and analysis of the affinity gel.—Compound **9** was coupled to EAH-Sepharose 4B (Pharmacia) as described [29]. Samples of the moist gel (10–20 mg, freed from interstitial buffer by suction) were heated 15 min to 100°C with ninhydrin reagent [1 mL of a soln of ninhydrin (0.8 g), hydrindantin (0.12 g) in 2-methoxy-ethanol (30 mL) and 4 M sodium acetate buffer pH 5.5 (10 mL)]. After cooling on ice, the samples were diluted with 50% aq EtOH (1.5 mL) and the gel particles allowed to settle for 10 min at room temperature. The content of amino groups was calculated from A_{540} and a standard curve prepared with 1-aminobutane. The concentration of bound ligand in the gel particles was calculated from the content of amino groups before and after coupling. This value was corrected for the amount of interstitial buffer (~ 16%) determined from the weight loss on suction of a known amount of settled gel to give the ligand concentration in the column.

Isolation of α-L-fucosidase.—Calf kidneys, fresh from the slaughterhouse (500 g), were homogenized at 4°C in 50 mM sodium citrate–phosphate buffer pH 5.5 (500 mL) and centrifuged at 20,000 × g for 30 min. The supernatant (550 mL) was heated to 60°C in portions of ~ 140 mL, rapidly cooled in ice to 10°C and centrifuged as above. The supernatant was adjusted to pH 7.0 by addition of 5% aq Na_2CO_3 and NaCl added to give a final concentration of 0.1 M.

A part of this solution (425 mL) was pumped at 4°C over the affinity gel (bed volume 5.5 mL) with a flow rate of 3.0 mL/h, the effluent collected in fractions of 3.0 mL and assayed for L-fucosidase activity (see below). The column was then washed with 50 mM phosphate citrate buffer pH 5.5 (~ 50 mL) until protein could no longer be detected in the effluent. Elution of α-L-fucosidase was done with 50 mM L-fucose in the same buffer (32 mL). The concentration of L-fucose was reduced to < 0.1 mM by repeated ultrafiltration (Diaflow YM 30 membrane (Amicon)) and 1 : 10 dilutions of the concentrate.

Protein concentrations were determined according to Lowry et al. [30]; the purity was

checked and the molecular weight of the subunits determined by the method of Weber and Osborne [31] with ovalbumin and bovine serum albumin as reference. If required, protein concentration was increased by precipitation according to Wessel and Flügge [32]. In order to increase the storage stability (4°C) solutions of the purified enzyme were supplemented with bovine serum albumin (1% w/v) and NaN_3 (0.05% w/v).

Enzymic studies.—Unless stated otherwise, the activity of α-L-fucosidase was determined with 1 mM *p*-nitrophenyl α-L-fucopyranoside (Sigma) in 50 mM phosphate buffer pH 5.5 at 25°C. Samples were taken at appropriate time intervals, quenched by the addition of 5% aq Na_2CO_3 and *p*-nitrophenol determined at 410 nm (ϵ_{410} 18,400 M^{-1} cm^{-1}) with a Zeiss PM 2K spectrophotometer. Measurements at pH 6.0 and 7.0 were usually done by continously recording A_{410}. If required, the concentration of *p*-nitrophenol was calculated from the photometer readings multiplied by a correction factor from the ratio of A_{410} of a standard solution of *p*-nitrophenol added to the assay buffers and Na_2CO_3, respectively.

Michaelis constants K_M were taken from Lineweaver–Burk plots with five substrate concentrations from 1 to 0.1 mM. Inhibition constants K_I were calculated from the slopes of Lineweaver–Burk plots in the presence [slope (*I*)] and absence of inhibitor [slope (0)]:

$$K_I = [I] \Big/ \left(\frac{\text{slope } (I)}{\text{slope } (0)} - 1 \right)$$

Purely competitive inhibition was observed with all inhibitors tested.

Estimation of pK_a *of the anionic group interacting with cationic inhibitors.*—Assuming that K_I is determined solely by the cationic form of the inhibitors and by the degree of ionization α of an anionic group at the active site, the pH-dependence of K_I should follow the Henderson–Hasselbalch equation, provided other ionizations of the enzyme do not affect the pK_a of this group. With the (hypothetical) inhibition constant K_I' at full ionization of the anionic group, we get for $1/K_I$ at an arbitrary pH value $1/K_I = \alpha/K_I'$. We thus get from the Henderson–Hasselbalch equation:

$$pK_a = \text{pH} + \log\left(\frac{\alpha}{1-\alpha}\right) = \text{pH} + \log\left(\frac{K_I'/K_I}{1 - K_I'/K_I}\right) = \text{pH} + \log\frac{K_I'}{K_I - K_I'}$$

The two unknowns pK_a and K_I' were calculated for L-fucose and compounds **1**, **2**, **4**, and **10** from the three possible combinations of two K_I values at different pH (Table 2). Except for L-fucose pK_a was 6.11 ± 0.1 (SD). For L-fucose the K_I values at pH 5 and 6 gave pK_a 6.2; K_I at pH 6 and 7 gave pK_a 4.8 and at pH 5 and pH 7 pK_a 5.9.

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