Carbohydrate Research, 200 (1990) 391–402
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Purification of α-L-fucosidase by *C*-glycosylic affinity chromatography, and the enzymic synthesis of α-L-fucosyl disaccharides

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(Received September 12th, 1989; accepted for publication, October 17th, 1989)

ABSTRACT

An α-L-fucosidase from porcine liver was purified using the new *C*-glycosylic affinity adsorbent, Sepharose-ε-aminocaproyl-3-(α-L-fucopyranosyl)propylamine. The *C*-fucosylic linkage was synthesized by a radical reaction of 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide with acrylonitrile. In transglycosylation reactions with *p*-nitrophenyl α-L-fucopyranoside or α-L-fucopyranosyl fluoride as donor and methyl β-D-galactopyranoside as acceptor, the enzyme mediated the formation of (1→2)- and (1→6)-linked α-L-fucosyl derivatives (6.5 and 10.0%, respectively).

INTRODUCTION

Many L-fucose-containing glycoconjugates have important biological functions in solution or on cell membranes, such as growth regulation, receptor function, cell–cell interactions, and antigenicity. Further, they may act as "chain-stoppers" in biosynthesis by controlling the extent of the chain elongation[1]. Generally, the L-fucose residues are α and at non-reducing positions. The most abundant linkages are (1→2) to galactose, and (1→3), (1→4), and (1→6) to 2-acetamido-2-deoxyglucose. Examples are glycosides found in human milk (lacto-*N*-fucopentanoses I–III) and in the blood-group substances (H, Le^a, and Le^b determinants). α-L-Fucose (1→2)- and (1→3)-linked to glucose and (1→4)-linked to fucose are found in Gram-negative bacteria.

Elegant chemical procedures have been developed[2] for the synthesis of complex oligosaccharides, but they are frequently cumbersome because of the many protection and deprotection steps, or difficult separations of mixtures of anomers, which is reflected in low overall yields. Therefore, enzymic syntheses are an attractive complement to chemical procedures. The main strategies for enzymic syntheses involve the glycosyl transfer potential of hydrolases with physiological or non-physiological substrates, or the scaling up of relevant biochemical processes. Hydrolases can be isolated easily and their use does not involve cofactors. On the other hand, hydrolases, although generally less expensive than glycosyl transferases, usually give lower yields and are not

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regioselective. The moderate yields of glycosylation products obtained with hydrolases reflect the competing hydrolysis reactions, and the relative concentrations of water and the glycosyl acceptor are important.

Hydrolases can show different acceptor specificy and, even though glycosylation may be unpredictable, several successful glycosylations have been reported, *e.g.*, by the use of α-D-glucosidase[3,4], β-D-galactosidase[5–10], and α,α-trehalase[11].

α-L-Fucosidases (α-L-fucoside fucohydrolase, EC 3.2.1.51) are important enzymes in the metabolism of biological substances that contain L-fucose, for example, glycoproteins, oligosaccharides, and glycolipids, and have been investigated extensively[12–17]. α-L-Fucosidases are classified broadly into three groups based on their aglycon specificity, namely, those from bacteria and moulds, mammals, and molluscs[18]. In humans, the genetically linked deficiency of this enzyme results in the neurovisceral storage disease fucosidosis[19].

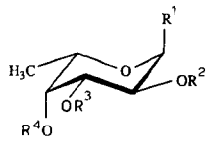
We now report on the glycosyl-transfer potential of porcine liver α-L-fucosidase for the synthesis of α-L-fucosyl disaccharides.

RESULTS AND DISCUSSION

Purification of α-L-fucosidase by affinity chromatography. — A solution of crude α-L-fucosidase was prepared by homogenization and ultracentrifugation of porcine liver. Standard methods for the semi-purification of enzymes, such as ammonium sulphate precipitation, gel filtration, and dialysis, were not used; instead, the crude enzyme was subjected to affinity chromatography. Several adsorbents have been used for the affinity chromatography of α-L-fucosidase from human, mammalian, and other sources[14], and the most successful purifications were obtained with agarose- or Sepharose-*N*-(ε-aminocaproyl)-β-L-fucopyranosylamine[20]. This and two other affinity adsorbents were synthesized and tested for isolation of pure α-L-fucosidase in a concentrated form.

p-Aminophenyl 1-thio-β-L-fucopyranoside[21] (**13**) was coupled to CH-Sepharose 4B. Despite results to the contrary[22], no α-L-fucosidase activity was retained by this material.

As a new approach to affinity adsorbents for glycosidases, an α-fucosyl *C*-glycosylic adsorbent was synthesized, following a method applied to glucose, galactose, and mannose[23]. Crystalline tri-*O*-acetyl-α-L-fucopyranosyl bromide[24] (**1**) was treated with tributyl tin hydride and acrylonitrile in refluxing toluene to give the *C*-glycosyl derivative, 3-(2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl)propiononitrile (**2**, 54%). The structure of **2** was evident from the upfield n.m.r. shift of the signal for H-1 to 4.21 p.p.m. (ddd, $J_{1',2'}$ 5.3 Hz). The acetyl groups were removed from **2** with methanolic sodium methoxide to give **3**, the nitrile group of which was reduced best by hydrogenation (Pd–C, PtO_2–C) in methanolic ammonia to give the amine **4**, together with ~ 15% of the di(fucosylpropyl)amine **5**. The amine **4** was characterized by the n.m.r. data of the fully acetylated compound **6** and the *N*-acetylated derivative **7**. Further, the reduction of **3**



	R^1	R^2–R^4		R^1	R^2	R^3	R^4
1	Br	Ac	8	$CH_2CH_2CH_2NHAc$	H	$C(CH_3)_2$	
2	CH_2CH_2CN	Ac	9	OAc	Ac	Ac	Ac
3	CH_2CH_2CN	H	10	F	Ac	Ac	Ac
4	$CH_2CH_2CH_2NH_2$	H	11	F	H	H	H
5	$CH_2CH_2CH_2]_2NH$	H	12	p-NO_2PhO	H	H	H
6	$CH_2CH_2CH_2NHAc$	Ac	13	p-NH_2PhS	H	H	H
7	$CH_2CH_2CH_2NHAc$	H					

was performed after isopropylidenation to give the derivative **8**, the structure of which was proved by n.m.r. spectroscopy.

The *C*-fucosyl compound **4** was coupled, without purification, to CH-Sepharose 4B via an ε-aminocaproyl linkage. The resulting adsorbent bound the above α-L-fucosidase, which could be eluted with L-fucose. The capacity of the column was ~0.16 activity unit of enzyme per mL of wet gel [*cf.* the value of 0.19 for Sepharose *N*-(ε-aminocaproyl)-β-L-fucopyranosylamine]. The efficiency of the new adsorbent was not reduced after repeated use, as reported for a fucopyranosylamine column[25]. The fact that β-L-fucopyranosylamine is a good competitive inhibitor for α-L-fucosidase[12,14] has been explained by the fact that N-1, whatever the configuration, can form hydrogen bonds to the polar hydrolytic groups in the active site of the enzyme, *e.g.*, carboxyl groups[26]. This explanation cannot apply to the new *C*-fucosylic affinity adsorbent, but the interaction of the enzyme with the groups at C-2 to C-5 in the fucosyl unit may be of importance for the affinity. Further, in addition to the L-fucose recognition site, the enzyme may have a hydrophobic site that binds to substituents. This view is supported by the observation that the enzyme readily accepts both *p*-nitrophenyl and 4-methylumbelliferyl fucosides in spite of considerable differences in their structure[15].

Figure 1 and the data in Table I illustrate the results for a typical run on the *C*-fucosylic affinity adsorbent. The recovery of α-L-fucosidase activity was 65–80%, the purification in one step from the solution of crude enzyme was ~3700-fold, and the specific activity was 15.0 units.mg^{-1} of protein. Reference data for porcine liver fucosidase are not available in the literature, but the value accords, for example, with a specific activity of 19.4 for purified human-liver α-L-fucosidase[3]. The results for the β-L-fucopyranosylamine affinity adsorbent were almost identical (Table I).

Polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulphate, on the purified enzyme revealed a single major unit with a mol. wt of ~50 000 preceded by a faint contaminant protein band. α-L-Fucosidase forms a tetramer[13–15,27], which can

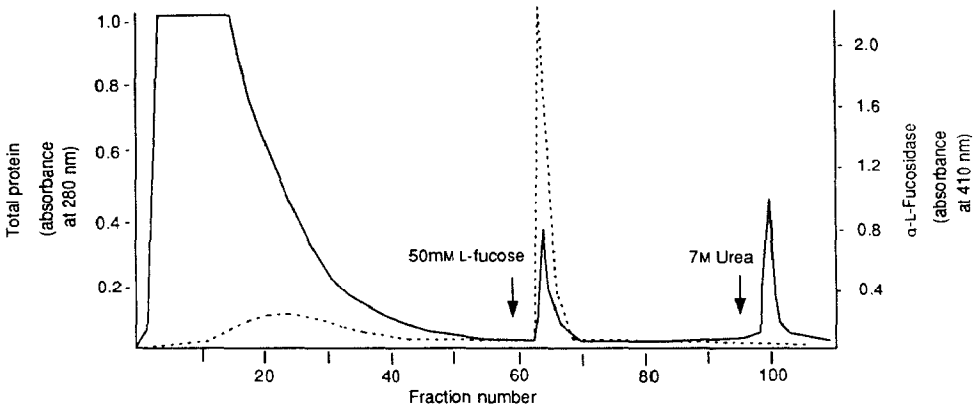


Fig. 1. *C*-Fucosylic affinity chromatography of the crude α-L-fucosidase from porcine liver: total protein, ——; enzyme activity, -----.

TABLE I

Purification of porcine α-L-fucosidase (EC 3.2.1.51)

Fraction	*Protein* ($mg.mL^{-1}$)	*Activity*[a] ($U.mL^{-1}$)	*Specific activity* ($U.mg^{-1}$)	*Purification factor*	*Yield* (%)
Supernatant solution	39.0	0.16	0.004	1	100
After affinity chromatography					
I α-*C*-Fucosylpropylamine	0.18	2.7	15.0	3770	78
II β-Fucosylamine[b]	0.20	2.5	13.6	3400	75

[a]A unit of activity relates to the hydrolysis of 1 μmol of *p*-nitrophenyl α-L-fucopyranoside per min at 37°.
[b]Values are not optimized.

dissociate into monomers each with one active site and with a mol.wt. 47 000–60 000 depending on the source of the enzyme. The tetramer is believed to be much more active than the monomer and to be bound more tightly to the affinity adsorbent than the monomer, which is only retarded[14]. Therefore, the small fraction of slightly retarded α-L-fucosidase in the affinity chromatography experiments now reported may be the monomer, and the bound form the tetramer. The pH curve for the enzyme activity showed a broad optimum with a maximum of 4.8, and >85% of the activity was found in the range 4.4–5.5, which accords with results for enzymes from other mammalian sources[13–16].

Enzymic glycosylation using α-L-fucosidase. — The use of *p*-nitrophenyl α-L-fucopyranoside[28] (**12**) as glycosyl donor presented problems because of its low solubility in aqueous solutions (<10 $mg.mL^{-1}$). Therefore, the use of organic co-solvents was investigated since some enzymes can function as well under these conditions[6,9,29] as in aqueous buffers; in the presence of co-solvents, although competing hydrolysis would be minimized, the enzymic activity may also be decreased.

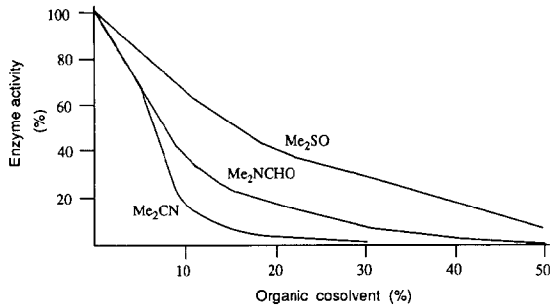


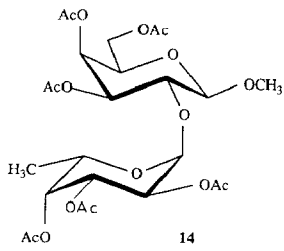
Fig. 2. Effect of the co-solvent on the activity of α-L-fucosidase with *p*-nitrophenyl α-L-fucopyranoside as substrate (see Experimental).

The activity of the purified α-L-fucosidase was decreased dramatically in the presence of *N,N*-dimethylformamide, dimethyl sulfoxide, and acetonitrile, up to 50% (Fig. 2). The best results were obtained with dimethyl sulfoxide which gave, for example, 35% of residual activity in a 30% mixture with the buffer solution. Other solvents caused considerable loss of activity, even in concentrations down to 10%. As expected, the solubility of **12** in aqueous 30% dimethyl sulfoxide was at least twice that in the buffer solution.

Because of the low solubility of **12** in aqueous solutions, other glycosyl donors were considered. Since glycosyl fluorides are useful substrates for hydrolases[4,7,8,30–32], α-L-fucopyranosyl fluoride (**11**) was synthesized. Reaction of L-fucose tetra-acetate[33] (**9**) with 70% hydrogen fluoride in pyridine–dichloromethane at room temperature gave crystalline 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl fluoride (**10**, 67%). Compound **10**, which was identified on the basis of ^{1}H-n.m.r. data ($J_{1,2}$ 2.7, $J_{1,F}$ 53.7 Hz), was deacylated using methanolic sodium methoxide to give crystalline α-L-fucopyranosyl fluoride (**11**, 94%), which was a substrate for the purified enzyme; however, the pH was crucial because hydrolysis occurred at <4.5.

For glycosylation on a mmol scale, the glycosyl donors **11** and **12** were mixed with the acceptors and purified enzyme in concentrated solutions of buffer with or without organic co-solvents at 37°. The reactions were monitored by t.l.c. and stopped when the glycosyl donor was almost consumed. The products were then acetylated and fractionated by column chromatography on silica gel, and the disaccharide fractions were subjected to preparative h.p.l.c..

The results of the enzymic fucosylation of methyl β-D-galactopyranoside are presented in Table II. Depending on the conditions, methyl 2-*O*- and 6-*O*-(α-L-fucopyranosyl)-β-D-galactopyranoside were formed in various amounts, and their structures were elucidated by ^{1}H-n.m.r. spectroscopy of the respective hexa-acetates **14** and **15**. The best result was obtained with the *p*-nitrophenyl glycoside **12** as the donor in phosphate buffer with 30% dimethyl sulfoxide for 5 days, which yielded 16.5% of a 2:3 mixture of **14** and **15**. When the glycosyl fluoride **11** was the donor, the ratio was shifted to 1:1. The CH-Sepharose-immobilized enzyme gave a yield comparable to that of the reaction in solution.



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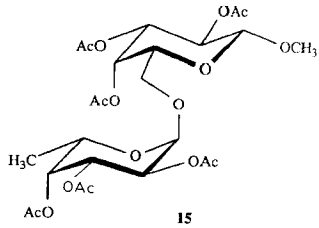

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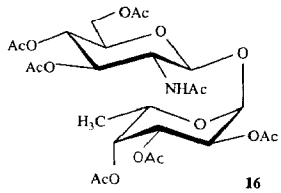

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TABLE II

Enzymic fucosylation of methyl β-D-galactopyranoside[a]

Donor	*Conditions (0.1M NaH_2PO_4 buffer, pH 4.8)*	*Reaction time*	*Yield (%)*[b]		
			14	**15**	*Total*
11	Pure buffer	7h	2.9	6.1	9.0
11	30% Me_2SO	24h	5.0	4.7	9.7
12	30% Me_2SO	5d	6.5	10.0	16.5
12	Immobil. enzyme[c]	24h	2.5	6.5	8.5

[a]The reaction mixture contained 1 mmol of glycosyl donor, 2.5 mmol of acceptor, and 2 U of α-L-fucosidase in 2.1 mL of buffer, and was stirred at 37°. [b]Yields of acetylated compounds. [c]Immobilized enzyme (4 U) in 5 mL of buffer was stirred at room temperature.

Applications of such other acceptors as D-glucose, L-fucose, and lactose did not result in any identified fucosylation, and 2-acetamido-2-deoxy-D-glucose yielded <2% of the disaccharide **16** with a trehalose-type interglycosidic linkage. Although no enzymic glycosylation using α-L-fucosidase has been reported hitherto, the specificity for galactose can be assumed to be due to the porcine liver enzyme.

Thus, porcine liver α-L-fucosidase has potential for the mild, stereo- and regioselective fucosylation of galactose derivatives. No attempts were made to optimize the yields reported.

EXPERIMENTAL

General. — Melting points were recorded with a Reichert melting-point microscope and are uncorrected. Optical rotations were determined with a Perkin–Elmer 241 polarimeter. ^{1}H-N.m.r. spectra were recorded with a Bruker WH-300 (300 MHz) instrument. Photometric measurements were recorded with a Perkin–Elmer 551 spec-

trophotometer. All reactions were monitored by t.l.c. on Silica Gel FG_{254} (Merck), with detection by u.v. light or by charring with sulphuric acid. Column chromatography was performed on Silica Gel 60 (230–400 mesh, Merck). Preparative h.p.l.c. was performed with a Waters 1145 pump (20 mL.min^{-1}), a Knauer type 98.00 refractometer detector, a column (16 × 250 mm) of Nucleosil 100 (5 μm), and elution with toluene–ethyl acetate (3:1). CH-Sepharose 4B was obtained from Pharmacia. Solutions of enzymes were concentrated by ultrafiltration using Amicon concentrators with PM-10 Diaflo membranes at 50 to 70 p.s.i. Protein was determined by the method of Bradford[34], using bovine serum albumin as the standard.

Enzyme assay. — The enzyme solution (10–50 μL) was added to 200mM Na_2HPO_4 buffer (pH 4.8, 200 μL) and 2mM *p*-nitrophenyl α-L-fucopyranoside (300 μL), then incubated for 5–30 min at 37°. The reaction was stopped by the addition of 100mM sodium tetraborate buffer (pH 9.0, 600 μL), and the *p*-nitrophenol liberated was determined spectrophotometricially (absorbance at 410 nm). One unit of α-L-fucosidase activity is defined as the amount of the enzyme which hydrolyses 1 μmol of *p*-nitrophenyl α-L-fucopyranoside per min.

3-(2,3,4-Tri-O-acetyl-α-L-fucopyranosyl)propiononitrile (**2**). — A solution of tributyl tin hydride (2.6 g, 9 mmol) and α,α′-azoisobutyronitrile (200 mg, 1.2 mmol) in dry toluene (40 mL) was added dropwise during 1 h to a stirred and refluxing solution of 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl bromide[24] (**1**; 2.1 g, 6 mmol) and acrylonitrile (4.8 g, 90 mmol) in anhydrous toluene (150 mL). After cooling, concentration, and extraction with hexane (to separate tin compounds), the residue was purified by column chromatography (toluene–ethyl acetate, 5:1) to yield **2** (1.1 g, 57%), m.p. 105–107° (from hexane–ether), $[\alpha]_D^{20}$ −92.5° (*c* 1, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.21 (d, 3 H, H-6′,6′,6′), 1.83 (ddt, 1 H, CH_2-3a), 2.00–2.19 (m, 1 H, CH_2-3b), 2.02, 2.08, and 2.15 (3 s, 9 H, 3 Ac), 2.45 (t, 2 H, CH_2-2), 3.98 (dq, 1 H, H-5′), 4.24 (ddd, 1 H, H-1′), 5.16 (dd, 1 H, H-3′), 5.28 (dd, 1 H, H-4′), 5.32 (dd, 1 H, H-2′); $J_{2,3}$ 7.2, $J_{3a,3b}$ 14.5, $J_{1',3a}$ 3.1, $J_{1',3b}$ 11.7, $J_{1',2'}$ 5.3, $J_{2',3'}$ 9.5, $J_{3',4'}$ 3.2 $J_{4',5'}$ 2.4, $J_{5',6'}$ 6.5 Hz.

Anal. Calc. for $C_{15}H_{21}NO_7$ (327.3): C, 55.04; H, 6.47. Found: C, 55.15; H, 6.66.

3-(α-L-Fucopyranosyl)propiononitrile (**3**). — A solution of **2** (0.70 g) in methanolic M sodium methoxide (10 mL) was stirred for 3 h, then filtered through a short column of Amberlite IR-120 (H^+) resin, and concentrated. Column chromatography ($CHCl_3$–EtOAc–MeOH, 3:2:1) of the residue and recrystallization from methanol–ether gave **3** (0.38 g, 88%), m.p. 177–178°, $[\alpha]_D^{20}$ −124° (*c* 1, methanol). ^{1}H-N.m.r. data (CD_3OD): δ 1.23 (d, 3 H, H-6′,6′,6′), 1.86–2.11 (m, 2 H, CH_2-3), 2.49 (dt, 2 H, CH_2-2), 3.62 (dd, 1 H, H-3′), 3.69 (dd, 1 H, H-4′), 3.79 (dq, 1 H, H-5′), 3.91 (dd, 1 H, H-2′), 3.96 (ddd, 1 H, H-1′); $J_{1',3a}$ 3.7, $J_{1',3b}$ 10.9, $J_{1',2'}$ 5.6, $J_{2',3'}$ 8.7, $J_{3',4'}$ 3.4, $J_{4',5'}$ 2.0, $J_{5',6'}$ 6.5 Hz.

Anal. Calc. for $C_9H_{15}NO_4$ (201.2): C, 53.72; H, 7.51; N, 6.96. Found: C, 53.67; H, 7.54; N, 6.83.

3-(α-L-Fucopyranosyl)propylamine (**4**). — A mixture of **3** (230 mg), 10% PtO_2–C (60 mg), and 10% Pd–C (70 mg) in methanol (10 mL) containing conc. ammonia (1 mL) was hydrogenated at atmospheric pressure for 4 h, then filtered, and concentrated to give syrupy **4**, $[\alpha]_D^{20}$ −70° (*c* 0.6, methanol). ^{1}H-N.m.r. data (D_2O; internal acetone, δ

2.23): δ 1.19 (d, 3 H, H-6′,6′,6′), 1.58–1.82 (m, 4 H, CH_2-2,3), 2.95 (m, 2 H, CH_2-1), 3.74–3.79 (q, 1 H, H-5′) and 3.93–4.03 (m, 4 H, H-1′,2′,3′,4′), 3.81. The elemental analysis of the syrup was not satisfactory, probably because of the formation of the di(fucosylpropyl)amine **5**.

N-*Acetyl*-3-*(2,3,4-tri*-O-*acetyl*-α-L-*fucopyranosyl)propylamine* (**6**). — Syrupy **4** (100 mg) was treated with acetic anhydride in pyridine (2 mL, 1:1) for 2 h at 90°. The mixture was concentrated and the residue was eluted from a short column of silica gel with CH_2Cl_2–EtOAc–MeOH (6:2:1) to give **6**. ^{1}H-N.m.r. data ($CDCl_3$): δ 1.13 (d, 3 H, H-6′,6′,6′), 1.41–1.78 (m, 4 H, CH_2-2,3), 1.95, 1.98, 2.03, and 2.12 (4 s, 12 H, Ac), 3.25 (m, 2 H, CH_2-1), 3.86 (dq, 1 H, H-5′), 4.15 (ddd, 1 H, H-1′), 5.15 (dd, 1 H, H-3′), 5.24 (dd, 1 H, H-4′), 5.27 (dd, 1 H, H-2′); $J_{1',3a}$ 3.5, $J_{1',3b}$ 11.1, $J_{1',2'}$ 5.6, $J_{2',3'}$ 10.0, $J_{3',4'}$ 3.4, $J_{4',5'}$ 1.9, $J_{5',6'}$ 6.4 Hz.

N-*Acetyl*-3-*(*α-L-*fucopyranosyl)propylamine* (**7**). — Compound **6** (50 mg) was *O*-deacetylated with methanolic M sodium methoxide (5 mL) for 3 h at room temperature. The mixture was neutralized with Amberlite IR-120 (H^+) resin, filtered, and concentrated to give **7**, $[\alpha]_D^{20}$ −76° (*c* 0.4, methanol). ^{1}H-N.m.r. data (CD_3OD): δ 1.12 (d, 3 H, H-6′,6′,6′), 1.36–1.64 (m, 4 H, CH_2-2,3), 1.84 (s, 3 H, AcN), 3.20 (t, 2 H, CH_2-1), 3.64 (dd, 1 H, H-3′), 3.67 (dd, 1 H, H-4′), 3.78 (dq, 1 H, H-5′), 3.83–3.90 (m, 2 H, H-1′,2′); $J_{2',3'}$ 8.8 $J_{3',4'}$ 3.5, $J_{4',5'}$ 1.9, $J_{5',6'}$ 6.4 Hz.

N-*Acetyl*-3-*(3,4*-O-*isopropylidene*-α-L-*fucopyranosyl)propylamine* (**8**). — A solution of **3** (300 mg, 1.49 mmol) in 2,2-dimethoxypropane (3 mL) and anhydrous acetone (1.5 mL) was stirred with a catalytic amount of *p*-toluenesulphonic acid for 3 h at room temperature, then neutralized ($NaHCO_3$), and concentrated. A solution of the residue in toluene was filtered and concentrated. The resulting syrup was dissolved in anhydrous tetrahydrofuran (20 mL), lithium aluminium hydride (1.5 g, 39.5 mmol) was added, and the suspension was stirred and heated for 6 h under reflux. The cold solution was treated successively with methanol and water, stirred with ethyl acetate, filtered, and concentrated, and a solution of the residue in acetone was dried ($MgSO_4$) and concentrated. Preparative t.l.c. (hexane–ethyl acetate, 1:3) of part of the residue (270 mg) gave **8** (9 mg). N.m.r. data ($CDCl_3$): ^{1}H, δ 1.21 (d, 3 H, H-6′,6′,6′), 1.28 and 1.43 (s, 6 H, CMe_2), 1.40–1.70 (m, 4 H, CH_2-2,3), 1.90 (s, 3 H, AcNH), 2.50 (s, 1 H, HO-2), 3.21 (m, 2 H, CH_2-1), 3.66 (t, 1 H, H-4′), 3.85 (m, 1 H, H-1′), 4.02 (dt, 1 H, H-5′), 4.06 (dd, 1 H, H-3′), 4.18 (dd, 1 H, H-2′); $J_{2',3'}$ 7.1, $J_{3',4'}$ 3.0, $J_{4',5'}$ 1.8, $J_{5',6'}$ 6.5 Hz; ^{13}C, δ 17.8 (C-6′), 23.3 (CH_3CONH), 24.6 and 25.7 [$C(CH_3)_2$], 27.0 and 27.7 (CH_2-1,2), 39.6 (CH_2-3), 65.1, 69.5, 70.9, 75.0, and 75.3 (C-1′–C-5′), 109.1 [$C(CH_3)_2$], 170.3 (CH_3CONH). Mass spectrum: *m/z* 288 (0.7%) ($M^+ + 1$).

Preparation of Sepharose-linked affinity adsorbent. — The procedure recommended by Pharmacia was followed. CH-Sepharose 4B (10 g) was allowed to swell and washed with mM HCl (1 L) for 15 min at 4° on a sintered-glass filter. A solution of 3-(α-L-fucopyranosyl)propylamine (**4**, 300 mg), in 0.1M $NaHCO_3$ (pH 8.0) containing 0.5M NaCl (5mL per g of powder) was shaken with the gel for 2 h at room temperature. Excess of ligand was removed by washing, and the remaining active groups were blocked with 0.1M Tris-HCl buffer (pH 8) for 1 h. The product was washed first with

0.1M sodium acetate buffer (pH 4) containing 0.5M NaCl, then with 0.1M Tris–HCl buffer (pH 8) containing 0.5M NaCl, and, finally, with 50mM Na_2HPO_4 buffer (pH 5.2), and stored at 4–8°.

Likewise, β-L-fucopyranosylamine[20] (300 mg) was attached to CH-Sepharose 4B.

Purification of α-L-fucosidase. — Porcine liver (300 g) was cut into pieces and, together with 0.1M sodium acetate buffer (pH 5.5, 600 mL) containing 0.1M NaCl and NaN_3 (0.02%), was homogenized in a Waring Blendor (high speed, 4 × 20 s). The crude homogenate was heated for 30 min at 60°, cooled to 10°, and centrifuged for 30 min at 30000*g* at 4°. The supernatant fluid was filtered through cotton and stored in a refrigerator. The supernatant solution showed an enzymic activity of 0.16 $U.mL^{-1}$, with a specific activity of 0.004 $U.mg^{-1}$ of protein.

Affinity chromatography of α-L-fucosidase. — A column of 3-(α-L-fucopyranosyl) propylamine coupled to CH-Sepharose 4B (*column I*, 15 mL of wet gel) was pre-equilibrated with 50mM phosphate buffer (pH 5.2) containing 20mM NaCl and NaN_3 (0.02%). The column was loaded with the solution of the enzyme (60 mL, 15 $mL.h^{-1}$) and washed with the above-mentioned phospate buffer (30 mL.h; 15 $mL.fraction^{-1}$). When the u.v. absorbance at 280 nm was ~0, the column was eluted with 50mM L-fucose in the phosphate buffer (30 mL; 15 $mL.h^{-1}$; 5 $mL.fraction^{-1}$) and then with phosphate buffer. After assaying for α-L-fucosidase activity, fractions containing high activity were combined and concentrated by ultrafiltration. Further elution with M NaCl or 7M urea did not release α-L-fucosidase activity. A small fraction of enzyme activity was not retained fully and was eluted at the end of the major unretained protein fraction (see Fig. 1).

Affinity chromatography on Sepharose-ε-aminocaproyl-β-L-fucopyranosylamine (*column II*) under the above conditions gave almost identical results (see Table I). The capacities of the columns (units per mL of wet gel) were 0.16 for *I* and 0.19 for *II*. The typical recovery of activity from *columns I* and *II* was in the range 65–80%. There appeared to be no appreciable loss in the capacity for binding α-L-fucosidase by *columns I* and *II* after several cycles of this procedure. Before the columns were reused, they were washed on sintered-glass filters with three cycles of alternating pH, as described above.

The concentrated high-activity fractions of α-L-fucosidase were diluted with 0.1M phosphate buffer (pH 4.8) and concentrated (4 × 20 mL) to remove L-fucose, which inhibits α-L-fucosidase. The activity measured was 2.7 $U.mL^{-1}$, with a specific activity of 15.0 $U.mg^{-1}$ of protein after purification on *column I*.

pH Optimum. — The pH optimum of purified α-L-fucosidase was determined as described under enzyme assay conditions, with 5 μL of enzyme sample (2×10^{-2} U) in KH_2PO_4–Na_2HPO_4 buffer in the pH range 3.0–7.0.

Enzyme activity for mixtures that contained organic solvents. — The mixtures contained up to 50% of Me_2SO, Me_2NCHO, and MeCN. The enzyme fraction (10 μL, 5×10^{-3} U) was mixed with 0.2M NaH_2PO_4 buffer (pH 4.8, 160 μL), 2mM *p*-nitrophenyl α-L-fucopyranoside (220 μL), organic solvent (0, 40, 80, 120, 160, or 240 μL), and distilled water up to a total volume of 800 μL. After incubation for 30 min at 37° and addition of 0.2M sodium tetraborate buffer (400 μL, pH 9.0), the absorbance of the

solution was measured at 410 nm against unincubated samples. The results are presented in Fig. 2.

Immobilization of α-L-fucosidase. — CH-Sepharose (0.5 g) was swollen and washed with M HCl (200 mL). The gel was transferred to a solution of enzyme fraction (3 mL, 4 U) and 0.1M $NaHCO_3$ buffer (5 mL, pH 8.3). The pH of the solution was adjusted to 8.3 and the mixture was shaken at room temperature for 4 h. The gel was filtered and washed, as described above, and stored at 4°.

2,3,4-Tri-O-acetyl-α-L-fucopyranosyl fluoride (**10**). — To a stirred solution of 1,2,3,4-tetra-*O*-acetyl-α-L-fucopyranose[34] (**9**; 2.8 g, 8.4 mmol) in dichloromethane (1 mL) at −15° was added 70% hydrogen fluoride in pyridine. The mixture was allowed to attain room temperature within 2 h, stirred for 4 h, poured into ice–water, and extracted immediately with chloroform (100 mL). The extract was washed with saturated aqueous sodium hydrogen carbonate (100 mL) and water (100 mL), dried ($MgSO_4$), and concentrated. Flash-column chromatography (toluene–ethyl acetate, 8:1, containing 0.5% of triethylamine) of the residue and crystallization from hexane gave **10** (1.65 g, 67%), m.p. 80–81°, $[\alpha]_D^{20}$ −113° (*c* 1, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.20 (d, 3 H, H-6,6,6), 2.01, 2.12, and 2.18 (3 s, 9 H, 3 Ac), 4.35 (q, 1 H, H-5), 5.17 (ddd, 1 H, H-2), 5.34–5.39 (m, 2 H, H-3,4), 5.76 (dd, 1 H, H-1); $J_{1,F}$ 53.7, $J_{1,2}$ 2.8, $J_{2,F}$ 23.7, $J_{2,3}$ 11.8, $J_{5,6}$ 6.5 Hz.

Anal. Calc. for $C_{12}H_{17}FO_7$ (292.3): C, 49.31; H, 5.86. Found: C, 49.29; H, 5.75.

α-L-Fucopyranosyl fluoride (**11**). — Conc. ammonia (2 mL) was added to a solution of **10** (1.5 g) in methanol (10 mL), the solution was stirred for 4 h and then concentrated, and the residue was recrystallized from methanol–ether to yield **11** (0.80 g, 94%), m.p. 237–239°, $[\alpha]_D^{20}$ −138° (*c* 0.7, methanol). ^{1}H-N.m.r. data (CD_3OD, Me_4Si): δ 1.24 (d, 3 H, H-6,6,6), 3.67–3.82 (m, 3 H, H-2,3,4), 4.07 (q, 1 H, H-5), 5.49 (dd, 1 H, H-1); $J_{1,F}$ 54.3, $J_{1,2}$ 2.64, $J_{5,6}$ 6.6 Hz.

Anal. Calc. for $C_6H_{11}FO_4$ (166.2): C, 43.37; H, 6.67. Found: C, 43.19; H, 6.87.

Enzymic glycosylations with α-L-fucosidase. — In the reactions with α-L-fucopyranosyl fluoride, solid sodium hydrogen carbonate was added to neutralize liberated hydrogen fluoride.

The following conditions were used: *A*, enzyme fraction (2 U), 0.1M NaH_2PO_4 buffer (pH 4.8, 1.4 mL), and Me_2SO (0.7 mL) were stirred with donor and acceptor at 37°; *B*, enzyme fraction (2 U) in 0.1M NaH_2PO_4 buffer (pH 4.8, 2.1 mL) was stirred with donor and acceptor at 37°; *C*, immobilized enzyme (4 U) in 0.1M NaH_2PO_4 buffer (pH 4.8, 5 mL) was shaken at room temperature with donor and acceptor.

After ~95% of the donor had reacted, the reaction was stopped by heating (5 min, 90°), the mixture was concentrated, and toluene (3 × 10 mL) was evaporated from the residue, which was taken up in pyridine (5 mL), and acetic anhydride (4 mL) was added dropwise. After initial reaction at room temperature for 30 min, the mixture was heated for 4 h to 80° and then concentrated (t.l.c.; toluene–EtOAc, 1:1). The acetylated products were separated from polar components by elution from a short column of silica gel (100 g) with chloroform–ethyl acetate (3:1). Preparative h.p.l.c. (toluene–ethyl acetate, 3:1) then gave the crystalline disaccharide derivatives described below.

Methyl 2-O- (**14**) *and 6-O-(α-L-fucopyranosyl)-β-D-galactopyranoside hexa-acetate* (**15**). — *p*-Nitrophenyl α-L-fucopyranoside (**12**; 200 mg, 0.70 mmol) and methyl β-D-galactopyranoside (540 mg, 2.8 mmol) were reacted under conditions *A* (30% Me_2SO) for 5 d; acetylation and purification then gave **14** (27 mg, 6.5%) and **15** (41 mg, 10.0%).

The hexa-acetate **14** had m.p. 148–150° (from hexane–ether), $[\alpha]_D^{20}$ −113° (*c* 1.2, chloroform). ^{1}H-N.m.r. data (benzene-d_6): δ 1.12 (d, 3 H, H-6′,6′,6′), 1.51, 1.66, 1.66, 1.76, 1.87, and 1.93 (6 s, 18 H, 6 Ac), 3.17 (s, 3 H, OMe), 3.41 (ddd, 1 H, H-5), 3.83 (dd, 1 H, H-1), 4.06 (dd, 1 H, H-6b), 4.12 (dd, 1 H, H-2), 4.13 (dd, 1 H, H-6a), 4.62 (dq, 1 H, H-5′), 5.19 (dd, 1 H, H-3), 5.42 (dd, 1 H, H-4), 5.44 (dd, 1 H, H-2′), 5.60 (dd, 1 H, H-4′), 5.74 (d, 1 H, H-1′), 5.83 (dd, 1 H, H-3′); $J_{1,2}$ 7.7, $J_{2,3}$ 10.0, $J_{3,4}$ 3.5, $J_{4,5}$ 0.8, $J_{5,6a}$ 7.0, $J_{5,6b}$ 7.3, $J_{6a,6b}$ 10.2, $J_{1',2'}$ 3.8, $J_{2',3'}$ 11.0, $J_{3',4'}$ 3.4, $J_{4',5'}$ 1.1, $J_{5',6'}$ 6.5 Hz.

Anal. Calc. for $C_{25}H_{36}O_{16}$ (592.6): C, 50.67: H, 6.12. Found: C, 50.50; H, 6.03.

The hexa-acetate **15** had m.p. 164–166° (from hexane–ether), $[\alpha]_D^{20}$ −102° (*c* 1, chloroform). ^{1}H-N.m.r. data (benzene-d_6): δ 1.00 (d, 3 H, H-6′,6′,6′), 1.58, 1.67, 1.74, 1.75 (×2), and 1.90 (5 s, 18 H, 6 Ac), 3.29 (s, 3 H, OMe), 3.39 (dd, 1 H, H-5), 3.55 (dd, 1 H, H-6b), 3.61 (dd, 1 H, H-6a), 3.91 (dq, 1 H, H-5′), 4.18 (d, 1 H, H-1), 5.14 (dd, 1 H, H-3), 5.30 (d, 1 H, H-1′), 5.48 (dd, 1 H, H-4′), 5.51 (dd, 1 H, H-4), 5.52 (dd, 1 H, H-2′), 5.66 (dd, 1 H, H-2), 5.68 (dd, 1 H, H-3′); $J_{1,2}$ 8.0, $J_{2,3}$ 10.5, $J_{3,4}$ 3.4, $J_{4,5}$ 0.6, $J_{5,6a}$ 6.8, $J_{5,6b}$ 6.3, $J_{6a,6b}$ 11.0, $J_{1',2'}$ 3.6, $J_{2',3'}$ 11.0, $J_{3',4'}$ 3.5, $J_{4',5'}$ 1.1, $J_{5',6'}$ 6.5 Hz.

Anal. Found: C, 50.05; H, 6.01.

Similarly, under conditions *A* (30% Me_2SO), α-L-fucopyranosyl fluoride (**11**; 165 mg, 1.00 mmol) and methyl β-D-galactopyranoside (540 mg, 2.8 mmol) were reacted for 24 h, to give **14** (29 mg, 5.0%) and **15** (28 mg, 4.7%).

Under conditions *B*, **11** (165 mg, 1.00 mmol) and methyl β-D-galactopyranoside (540 mg, 2.8 mmol) were reacted for 7 h, to give **14** (17 mg, 2.9%) and **15** (36 mg, 6.1%).

Under conditions *C*, **12** (100 mg, 0.35 mmol) and methyl β-D-galactopyranoside (300 mg, 1.56 mmol) were reacted for 24 h, to give **14** (6 mg, 2.5%) and **15** (13.5 mg, 6.0%).

Reactions with other glycosyl acceptors. — Under conditions *A* and *B* with D-glucose, lactose, and L-fucose as acceptors, no glycosylation was observed. The reaction of **11** with 2-acetamido-2-deoxy-D-glucose under conditions *B* gave 2,3,4-tri-*O*-acetyl-α-L-fucopyranosyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (**16**, ~2%). ^{1}H-N.m.r. data ($CDCl_3$): δ 1.27 (d, 3 H, H-6′,6′,6′), 1.95, 1.99, 2.03, 2.05, 2.05, 2.08, and 2.16 (7 s, 21 H, 7 Ac), 3.70 (ddd, 1 H, H-5), 3.90 (ddd, 1 H, H-2), 4.04 (dd, 1 H, H-6b), 4.10 (q, 1 H, H-5′), 4.25 (dd, 1 H, H-6a), 4.88 (d, 1 H, H-1), 5.01–5.08 (m, 2 H, H-3,3′), 5.25–5.37 (m, 3 H, H-2′,4,4′), 5.38 (d, 1 H, H-1′), 5.70 (d, 1 H, NHAc); $J_{1,2}$ 8.3, $J_{2,3}$ 10.5, $J_{2,NH}$ 8.5, $J_{5,6b}$ 2.3, $J_{5,6a}$ 5.4, $J_{6a,6b}$ 12.3, $J_{1',2'}$ 3.8, $J_{5',6'}$ 6.6 Hz.

ACKNOWLEDGMENTS

Support of this work by the Fonds der Chemischen Industrie, the Deutsche Forschungsgemeinschaft, the Bundesministerium für Forschung und Technologie, and the Swedish Board for Technical Development (to S.C.T.S.) is gratefully acknowledged.

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