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Note

Regioselective synthesis of α-L-fucosyl-containing disaccharides by use of α-L-fucosidases of various origins

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The α-L-fucosyl group is found, in glycoproteins or glycolipids, (1→2)-linked to a D-galactosyl residue, or (1→3)-, (1→4)-, or (1→6)-linked to a 2-acetamido-2-deoxy-D-glucosyl residue. Recently, Svensson and Thiem[1] reported an enzymic synthesis of methyl α-L-fucopyranosyl-β-D-galactopyranosides by a transglycosylation of the L-fucosyl residue of 4-nitrophenyl α-L-fucopyranoside (**1**) to methyl β-D-galactopyranoside with the aid of an α-L-fucosidase from porcine liver. However, a mixture of disaccharides containing a (1→2)- and (1→6)-linked α-L-fucosyl group was obtained with a ratio of ~ 2:3. Moreover, an enzyme obtained from microorganisms is desirable for a preparative-scale synthesis of oligosaccharides, as a large amount of enzyme can be obtained easily. We report herein the regioselective synthesis of disaccharides containing a (1→2)-, (1→3)-, and (1→6)-linked α-L-fucosyl group by use of α-L-fucosidases from various origin. These α-L-fucosyl -containing disaccharides are important starting materials for the synthesis of higher mol. wt. oligosaccharides as components of glycoproteins or glycolipids.

On Q-Sepharose column chromatography of *Aspergillus niger* culture broth (Fig. 1), the α-L-Fucosidase activity was found in Peak A, together with α-D-glucosidase activity, but most of the other glycosidase activities appeared in Peaks B and C. Therefore, α-L-fucosidase from *Aspergillus niger* could be purified up to ~ 33 times by a one-step procedure. The collected fractions contained α-D-glucosidase activity (0.056 unit/mg), β-D-galactosidase activity (0.036 unit/mg), and β-D-mannosidase activity (0.031 unit/mg), together with α-L-fucosidase activity (0.033 unit/mg). This semipurified enzyme preparation was used in the following experiments without further purification.

Incubation of **1** and D-glucose in the presence of semipurified α-L-fucosidase was examined by l.c. (see Fig. 2). Peak B showed a molecular ion at *m/z* 327 for $[M + H]^+$ in SIMS mode mass spectrometry. After purification of Peak B on an activated carbon column chromatography, the ^1H- and ^{13}C-n.m.r. spectra of the disaccharide were recorded and analyzed on the basis of a relayed-COSY and a ^1H–^{13}C hetero-COSY spectra (Table I). The data were in agreement with those of methyl 3-O-α-L-fucopyranosyl-α- (**2**) and -β-D-glucopyranoside (**3**), as reported by Baumann *et al.*[2] In the ^1H-n.m.r.

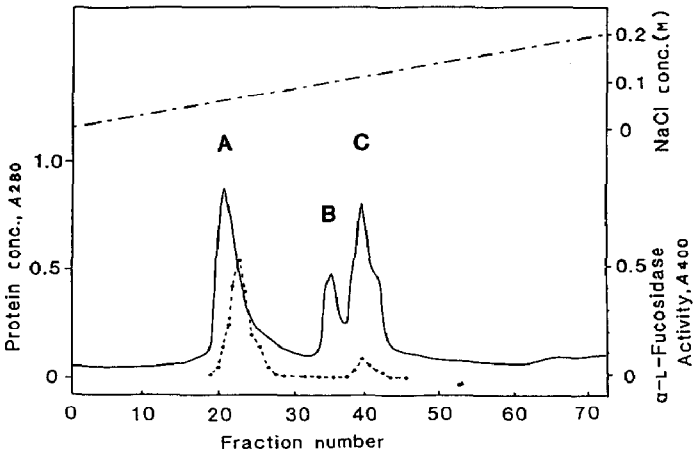
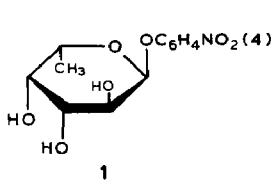


Fig. 1. Q-Sepharose column chromatography of Rhozyme: (—) Total protein content, (- -● - -● -) α-L-fucosidase activity, and (– · – · –) concentration of NaCl.



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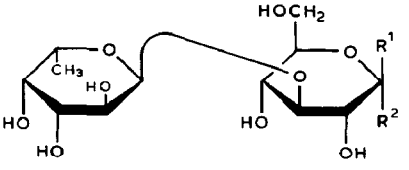
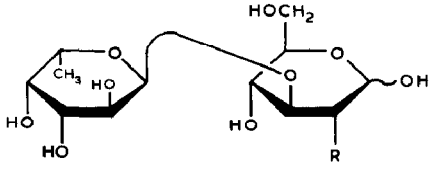


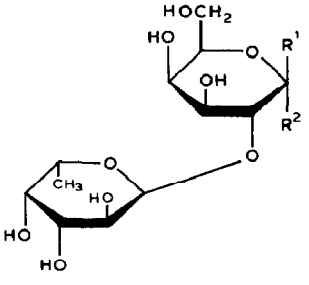
2 R^1 = H, R^2 = OMe

3 R^1 = OMe, R^2 = H



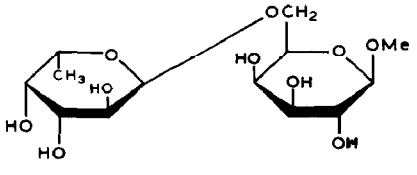
4 R = OH

5 R = NHAc



6 R^1 = OMe, R^2 = H

7 R^1, R^2 = H, OH



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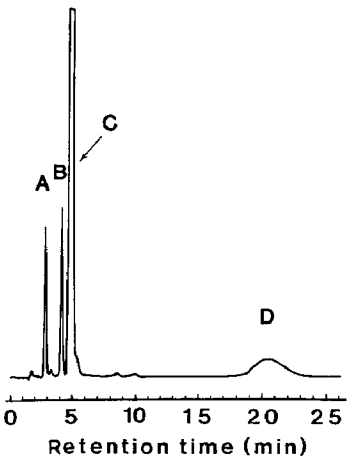

Fig. 2. L.c. pattern of the reaction between **1** and D-glucose in the presence of α-L-fucosidase from *Aspergillus niger* measured by a Bio-LC system: (A) L-fucose, (B) L-fucosyl-D-glucose, (C) D-glucose, and (D) 4-nitrophenyl α-L-fucopyranoside (**1**).

spectra of α-L-fucosyl-containing oligosaccharides, H-5 of the α-L-fucosyl residue is known to exhibit chemical shifts characteristic for the linkage of the L-fucosyl group[3]. The chemical shift of H-5 for the present disaccharide was at δ 4.38, and that for the corresponding methyl glycosides, **2** and **3**, were reported at δ 4.33 and 4.32, respectively. Therefore, the disaccharide was identified as 3-O-α-L-fucopyranosyl-D-glucose (**4**).

Similarly, when **1** was treated with 2-acetamido-2-deoxy-D-glucose in the presence of the same enzyme preparation, only one peak was observed in the disaccharide region of l.c. In the spectra of the purified disaccharide (see Fig. 3 and Table I), H-5 of

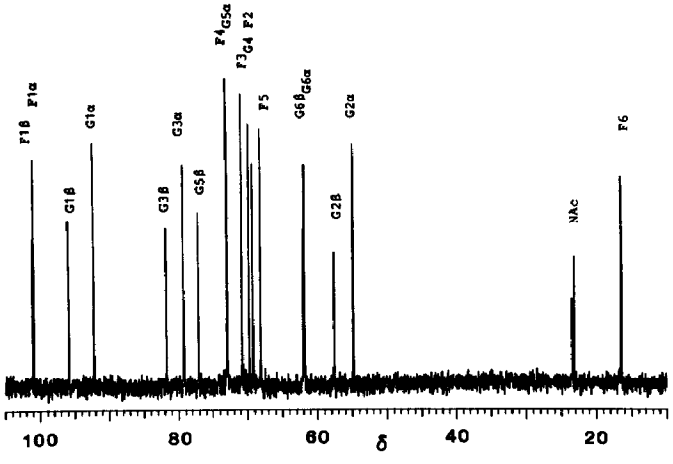

Fig. 3. ^{13}C-N.m.r. spectrum of **5**. Abbreviations: F = L-fucose; G = 2-acetamido-2-deoxy-D-glucose; α and β correspond to the anomeric forms of the reducing residue.

TABLE I

^{1}H- and ^{13}C-N.m.r. data (δ values) of (1→8)-linked α-L-fucosyl disaccharides

Residue or group		*Compound*					
		2[a]	**3**[a]	**4**		**5**	
				α anom.[b]	*β anom.*[b]	*α anom.*[b]	*β anom.*[b]
^{1}H-n.m.r.							
Fuc	H-1	5.20	5.24	5.27	5.27	5.07	5.10
	H-2	3.81	3.81	3.79	3.79	3.79	3.79
	H-3	3.88	3.88	3.89	3.89	3.90	3.90
	H-4	3.83	3.80	3.82	3.82	[c]	[c]
	H-5	4.33	4.32	4.38	4.38	4.40	4.40
	H-6	1.20	1.21	1.15	1.15	1.26	1.27
Glc	H-1	4.78	4.38	5.22	4.69	5.24	4.62
or	H-2	3.73	3.45	3.71	3.43	4.11	3.70
GlcNAc	H-3	3.73	3.60	3.76	3.59	3.89	3.81
	H-4	3.49	3.48	3.48	3.49	3.64	3.54
	H-5	3.66	3.47	[c]	[c]	3.98	[c]
	H-6a	3.76	3.73	[c]	[c]	[c]	[c]
	H-6b	3.87	3.92	[c]	[c]	[c]	[c]
NH*C*OCH$_3$						2.19	2.19
^{13}C-n.m.r.							
Fuc	C-1	100.4	100.4	100.4	100.4	100.9	101.1
	C-2	69.3	69.3	69.2	69.2	69.2	69.3
	C-3	70.6	70.6	70.4	70.4	70.8	70.8
	C-4	72.8	72.8	72.8	72.8	73.0	73.0
	C-5	67.7	67.8	67.7	67.7	68.1	68.1
	C-6	16.1	16.1	16.1	16.1	23.5	23.2
Glc	C-1	100.1	103.9	93.0	96.6	92.3	95.9
or	C-2	72.6	74.3	72.5	75.5	54.9	57.6
GlcNAc	C-3	81.7	84.0	80.8	83.8	79.3	81.8
	C-4	69.2	69.3	69.0	69.0	69.8	69.9
	C-5	72.5	76.8	72.8	76.7	73.1	77.2
	C-6	61.7	61.8	61.5	61.7	62.0	61.9
NHCO*C*H$_3$						16.7	16.4

[a] Values reported by Baumann *et al.* [b] α or β Anomer of D-glucose or 2-acetamido-2-deoxy-D-glucose residue. [c] Not determined.

the α-L-fucosyl group appeared at δ 4.40, and C-3 of the α and β anomer of the 2-acetamido-2-deoxy-D-glucose residue at δ 79.3 and 81.8, respectively. The lower-field shifts of the C-3 signals indicated that the L-fucosyl group is bound to O-3 of the 2-acetamido-2-deoxy-D-glucose residue and, therefore, the structure of this disaccharide is 2-acetamido-2-deoxy-3-O-α-L-fucopyranosyl-D-glucose (**5**).

Subsequently, **1** was treated with methyl β-D-galactopyranoside in the presence of α-L-fucosidases from various origin. Among the enzymes studied, α-L-fucosidases from *Corynebacterium* sp., and from ampullaria afforded transglycosylated products. Fig. 4

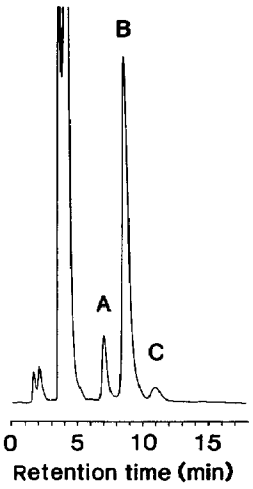

Fig. 4. L.c. pattern of the reaction between **1** and methyl β-D-galactopyranoside in the presence of α-L-fucosidase from *Corynebacterium* sp., measured by use of LiChrospher-NH_2 column: (A) α-L-fucose, (B) methyl β-D-galactopyranoside, and (C) methyl 2-*O*-α-L-fucopyranosyl-β-D-galactopyranoside (**6**).

TABLE II

^{13}C-N.m.r. data (δ values) of α-L-fucosyl-D-galactose derivatives

Residue or group		*Compound or group*			
		6	**8**	**7**	
				α anom.[a]	*β anom.*[a]
Fuc	C-1	101.2	100.5	102.4	101.0
	C-2	69.5	69.1	69.5	69.5
	C-3	70.7	70.5	70.5	70.6
	C-4	73.1	72.8	72.8	73.0
	C-5	68.0	67.9	68.4	67.9
	C-6	15.3	15.5	16.3	16.2
Gal	C-1	103.9	105.1	93.0	96.3
	C-2	79.3	71.8	79.2	80.3
	C-3	74.3	74.6	70.4	74.4
	C-4	69.9	69.8	69.2	69.9
	C-5	76.0	73.9	71.2	76.1
	C-6	62.1	68.5	62.2	62.0
	OCH_3	58.2	58.3		

[a] α or β Anomer of D-galactose residue.

shows a representative l.c. pattern of the reaction mixture incubated in the presence of α-L-fucosidase from *Corynebacterium* sp., Peak C indicating a newly produced disaccharide. The disaccharide was purified by chromatography on an activated-carbon column and was identified as methyl 2-O-α-L-fucopyranosyl-β-D-galactopyranoside (**6**) by its ^{1}H- and ^{13}C-n.m.r. spectra (Table II). On the other hand, the reaction mixture that

was incubated in the presence of α-L-fucosidase from ampullaria afforded methyl 6-O-α-L-fucopyranosyl-β-D-galactopyranoside (**8**) (Table II). In the ^{13}C-n.m.r. spectrum of **6**, C-2 of the D-galactopyranosyl residue resonated at δ 79.3, which was 7.5 p.p.m. down-field from the C-2 signal of methyl β-D-galactopyranoside. In the ^{13}C-n.m.r. spectrum of **7**, C-6 of the D-galactopyranosyl residue also showed a lower-field shift of 5.9 p.p.m. These lower-field shifts indicated a binding of the L-fucosyl group to O-2 and O-6 of the D-galactopyranosyl residue, respectively. Although Svensson and Thiem[1] had synthesized a mixture of **6** and **7** by use of an α-L-fucosidase from porcine liver, we could synthesize **6** and **7** separately by use of α-L-fucosidases from *Corynebacterium* sp. or ampullaria. In the presence of an α-L-fucosidase from *Corynebacterium* sp., **1** and D-galactose gave 2-O-α-L-fucopyranosyl-D-galactose (**7**) by a reaction similar to that giving **6** (see Fig. 5).

In the transglycosylation reaction, the accumulation of disaccharides depends on a delicate balance between formation and hydrolysis reaction. Even in the reaction using α-L-fucosidase from *Fussarium oxysporum* or bovine liver, which did not give any disaccharide (data not shown), a disaccharide might have been formed but an hydrolysis reaction occurred as soon as it was formed. In the reaction using an α-L-fucosidase from *Aspergillus niger*, no reaction occurred when methyl β-D-galactopyranoside was the acceptor. Similarly, α-L-fucosidases from *Corynebacterium* sp. and ampullaria did not show transglycosylation activity when D-glucose or 2-acetamido-2-deoxy-D-glucose was used as an acceptor, which indicated that each α-L-fucosidase has an unexpectedly strict substrate specificity. *Aspergillus niger* has been reported to produce two different α-L-fucosidases, one that cleaves selectively the α-L-(1→2) linkage[4], and another that hydrolyzes only the α-L-(1→6) linkage[5]. The present semipurified α-L-fucosidase preparation hydrolyzed the α-L-(1→2), but failed to cleave the α-L-(1→6) linkage. Moreover,

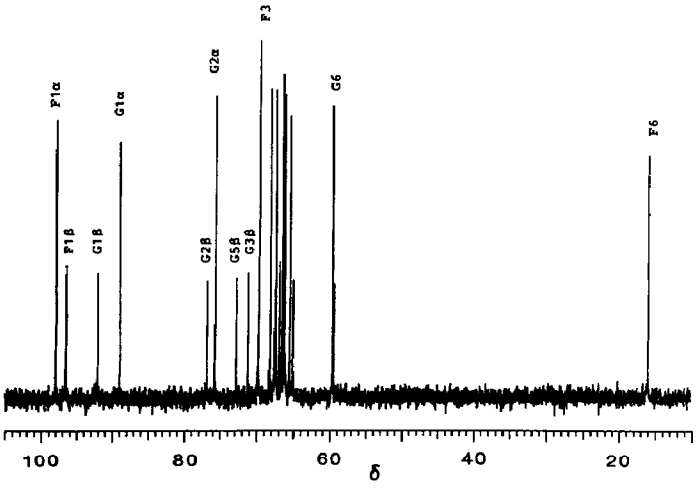

Fig. 5. ^{13}C-N.m.r. spectrum of **7**. Abbreviations: G = D-galactose; for other abbreviations, see legend to Fig. 3.

TABLE III

Synthesis of α-L-fucosyl-containing disaccharides with various α-L-fucosidases

Acceptor	*Origin of enzyme*	*Linkage*	*Yield (%)*
β-D-GalOMe	*Corynebacterium* sp.	α-L-(1→2)	25
D-Gal	*Corynebacterium* sp.	α-L-(1→2)	18
D-Glc	*Aspergillus niger*	α-L-(1→3)	61
D-GlcNAc	*Aspergillus niger*	α-L-(1→3)	58
β-D-GalOMe	Ampullaria	α-L-(1→6)	14

the present enzyme preparation did not hydrolyze 3-O-α-L-fucosyllactose, as had been reported[4]. However, it hydrolyzed **4** and **5**, which were not examined by Bahl[4]. The α-L-fucosidase from *Aspergillus niger* used in the present study may be the same one as that used by Bahl[4].

α-L-Fucosyl-containing disaccharides, components of glycoconjugate, were synthesized easily and regioselectively by selection of the appropriate enzyme (see Table III). These syntheses could be extended to a large scale. For example, about 1 g of **5** could be obtained by use of the α-L-fucosidase from *A. niger*; in that case a large-bore, activated-carbon column (7 × 80 cm) was used for the purification of the product.

EXPERIMENTAL

Materials. — α-L-Fucosidases (EC 3.2.1.51) from *Corynebacterium* sp. and ampullaria were purchased from Takara Shuzou Co., Ltd. (Kyoto, Japan) and Funakoshi Pharmaceuticals Co. Ltd. (Tokyo, Japan), respectively. Both enzymes were used without further purification. As a source of *Aspergillus niger* culture broth, "Rhozyme" was purchased from Genecor Inc. (CA, USA). Q-Sepharose and MONO-Q column were obtained from Pharmacia–LKB, (Uppsala, Sweden).

Analytical procedures. — ^{1}H- and ^{13}C-N.m.r. spectra were recorded with a Varian XL-400 n.m.r. spectrometer operating at 400 and 100 MHz, respectively. In ^{13}C-n.m.r. spectra, chemical shifts were referred to the methyl signal of internal acetonitrile (δ 1.27). The mass spectrum was recorded with an Hitachi M-2000 mass spectrometer operating in the SIMS mode. Two types of l.c. were employed for the analysis of transglycosylation reaction. One was a Bio-LC system (Dionex Co., CA, U.S.A.), equipped with a Carbopac PA1 column and a pulsed amperometric detector, and a 100mM NaOH solution was used as an eluent. The other was a reversed-phase l.c. using LiChrospher-NH_2 column (Merck) and RI detector, and 4:1 acetonitrile–water as eluent. The yields in the transglycosylation reaction were calculated on the basis of **1**.

Semipurification of α-L-fucosidase. — Rhozyme (2.8 g) was dissolved in 20mM potassium phosphate buffer (280 mL; pH 7.0) and dialyzed extensively against the same buffer (pH 7.0). The Rhozyme solution was applied onto a Q-Sepharose column (2.6 × 30 cm) which was eluted with a salt gradient from 0 to 0.5M NaCl in 20mM potassium phosphate buffer (pH 7.0, 2 L in total). Fractions containing α-L-fucosidase activity

were pooled and concentrated to 2mL by a membrane filtration with Amicon PM-30. α-L-Fucosidase activity, measured with **1** as a substrate, was 0.37 unit/mL.

Synthesis of 3-O-α-L-fucopyranosyl-D-glucose (**4**). — D-Glucose (125 mg) and **1** (25 mg) were dissolved in 0.1M acetate buffer (pH 5.0, 2.5 mL) and *N,N*-dimethylformamide (250 μL). The semipurified α-L-fucosidase solution (200 μL, 0.074 unit) was added to the substrate solution, and the mixture was incubated for 12 h at 37°. The reaction mixture was heated in a boiling water bath for 10 min, the denatured enzyme was filtered off, and the filtrate was applied onto an activated carbon column (2 × 45 cm). The monosaccharides were washed with water (500 mL), and the disaccharide was eluted with a gradient of 0–20% ethanol (v/v) in water (1 L in total). The carbohydrate content of each fraction was measured with the phenol–H_2SO_4 reagent[6]. Fractions containing the disaccharide with a purity > 95% were pooled and concentrated to dryness to give **4** as an amorphous solid (17.5 mg, 61% yield).

Anal. Calc. for $C_{12}H_{22}O_{10}$:C, 44.17; H, 6.80. Found: C, 43.99; H, 6.77.

2-Acetamido-2-deoxy-3-O-α-L-fucopyranosyl-D-glucose (**5**). — 2-Acetamido-2-deoxy-D-glucose (125 mg) and **1** (25 mg) were condensed, as described for the synthesis of **4** to give **5**, an amorphous solid (17,3 mg, 58% yield).

Anal. Calc. for $C_{14}H_{25}O_{10}$: C, 45.77; H, 6.86; N, 3.81. Found: C, 45.54; H, 6.66; N, 3.95.

Methyl 2-O-α-L-fucopyranosyl-β-D-galactopyranoside (**6**). — Methyl β-D-galactopyranoside (194.2 mg) and **1** (28.5 mg) were dissolved in 0.1M potassium phosphate buffer (pH 8.0, 900 μL) and dimethyl sulfoxide (300 μL). α-L-Fucosidase from *Corynebacterium* sp. (5 μL, 0.1 unit) was added and the solution was incubated for 16 h at 37°. The processing was the same as described above for the synthesis of **4**, except that the activated carbon column was eluted with a gradient of 0–40% ethanol (v/v) in water (1.2 L in total) to give **6** as an amorphous solid (8.5 mg, 25% yield).

Anal. Calc. for $C_{13}H_{24}O_{10}$: C, 45.88; H, 7.11. Found: C, 45.66; H, 7.36.

2-O-α-L-Fucopyranosyl-D-galactose (**7**). — D-Galactose (360 mg) and **1** (57 mg) were dissolved in 0.1M potassium phosphate buffer (pH 8.0, 3.8 mL) and dimethyl sulfoxide (1.2 mL), and α-L-fucosidase from *Corynebacterium sp.* (20 μL, 0.4 unit) was added. The mixture was incubated for 16 h at 37°, and **7** was obtained as an amorphous solid (11.4 mg, 18% yield) as described for the synthesis of **4**.

Anal. Calc. for $C_{12}H_{22}O_{10}$: C, 44.17; H, 6.80. Found: C, 44.46; H, 6.47.

Methyl 6-O-α-L-fucopyranosyl-β-D-galactopyranoside (**8**). — Methyl β-D-galactopyranoside (194.2 mg) and **1** (28.5 mg) were dissolved in 0.1M acetate buffer (pH 5.0, 900 μL) and dimethyl sulfoxide (300 μL). α-L-Fucosidase from ampullaria (50 μL, 0.2 unit) was added and the mixture was incubated for 16 h at 37° to give **8** as an amorphous solid (4.9 mg, 14% yield), as described for the synthesis of **6**.

Anal. Calc. for $C_{13}H_{24}O_{10}$: C, 45.88; H, 7.11. Found: C, 45.72; H, 7.22.

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