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Enzymatic synthesis of α-L-fucosyl-*N*-acetyllactosamines and 3′-*O*-α-L-fucosyllactose utilizing α-L-fucosidases

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Abstract

An α-L-fucosidase from porcine liver produced α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-GlcNAc (2′-*O*-α-L-fucosyl-*N*-acetyllactosamine, **1**) together with its isomers α-L-Fuc-(1→3)-β-D-Gal-(1→4)-D-GlcNAc (**2**) and α-L-Fuc-(1→6)-β-D-Gal-(1→4)-D-GlcNAc (**3**) through a transglycosylation reaction from *p*-nitrophenyl α-L-fucopyranoside and β-D-Gal-(1→4)-D-GlcNAc. The enzyme formed the trisaccharides **1**–**3** in 13% overall yield based on the donor, and in the ratio of 40:37:23. In contrast, transglycosylation by *Alcaligenes* sp. α-L-fucosidase led to the regioselective synthesis of trisaccharides containing a (1→3)-linked α-L-fucosyl residue. When β-D-Gal-(1→4)-D-GlcNAc and lactose were acceptors, the enzyme formed regioselectively compound **2** and α-L-Fuc-(1→3)-β-D-Gal-(1→4)-D-Glc (3′-*O*-α-L-fucosyllactose, **4**), respectively, in 54 and 34% yields, based on the donor.

Keywords: α-L-Fucosidase; Transglycosylation; Blood group H substance; Fucosyl oligosaccharide

1. Introduction

α-L-Fucose is an important constituent of the carbohydrate chains of glycoconjugates involved in a variety of biological events, such as cell–cell recognition and antigenicity [1]. For example, immunological determinants of the ABH(O) type are known to be oligosaccharides having the common structure of α-L-Fuc-(1→2)-β-D-Gal-R. Furthermore, (1→2)-linked α-L-fucosyllactose is a major component of human milk oligosaccharides [2]. Great attention, therefore, has been paid to chemical and enzymatic syntheses of such oligosaccharides. Chemical procedures have been extensively developed [3–6], but they are frequently cumbersome because of many protection and deprotection steps. The enzymatic approach has utilized various glycosyltransferases and glycosidases. Fucosylated di- and trisaccharides of the type 2 ABO blood group have been obtained with porcine β-D-galactoside α-(1→2) fucosyltransferase [7]. From a practical viewpoint, the use of glycosidases is attractive for synthesizing oligosaccharides, because these enzymes are generally more available, and less expensive than glycosyltransferases, and do not require expensive sugar nucleotide donors [8]. Svensson and Thiem reported an enzymatic synthesis of

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methyl (1→2)- and (1→6)-linked α-L-fucopyranosyl-β-D-galactosides in the ratio of 2:3 with an α-L-fucosidase from porcine liver [9]. Ajisaka and Shirakabe developed the regioselective synthesis of disaccharides containing (1→2)-, (1→3)-, and (1→6)-linked α-L-fucosyl groups by use of α-L-fucosidases from various microorganisms [10]. The object of the present investigation was to develop a system for the enzymatic synthesis of fucosylated trisaccharide **1** of the determinant of type 2 blood group H substance, utilizing β-D-Gal-(1→4)-D-GlcNAc (LacNAc), which has been readily obtained on a gram-scale by our method [11,12]. The present paper reports a synthetic procedure for α-L-fucosyl-*N*-acetyllactosamine and 3′-*O*-α-L-fucosyllactose through α-L-fucosidase-catalyzed transglycosylation.

2. Materials and methods

Materials.—α-L-Fucosidase from *Alcaligenes* sp. was kindly supplied by Kumiai Chemical Industry Co. Ltd., (Shizuoka, Japan). Porcine liver extract (Catalase L 'Amano') was kindly supplied by Amano Pharmaceutical Co. Ltd., (Nagoya, Japan). LacNAc, β-D-Gal-(1→4)-β-D-Glc-OMe, β-D-Gal-(1→3)-β-D-Glc-OMe, and β-D-Gal-(1→4)-β-D-GlcNAc-OMe were prepared by our methods [11,12]. *p*-Nitrophenyl α-L-fucopyranoside (Fucα-*p*NP) and α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc (2′-*O*-α-L-fucosyllactose) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were obtained from commercial sources.

Enzyme assay.—The activity of α-L-fucosidase from the porcine liver extract was assayed as follows: a mixture containing 0.4 mM Fucα-*p*NP in 0.9 mL of 50 mM NaOAc buffer (pH 4.8) and an appropriate amount of enzyme in total volume of 1 mL was incubated for 10 min at 37 °C. The reaction was stopped by adding 0.5 mL of 1 M Na_2CO_3 solution, and the liberated *p*-nitrophenol was determined spectrophotometrically at 410 nm. The activity of α-L-fucosidase from *Alcaligenes* sp. was assayed in a similar manner with 50 mM potassium phosphate buffer (pH 7) and incubation at 50 °C. One unit of activity was defined as the amount of enzyme that hydrolyzes 1 μmol of Fucα-*p*NP per min.

β-D-Galactosidase activity was assayed as follows: a mixture containing 2 mM *o*-nitrophenyl β-D-galactopyranoside (Galβ-*o*NP) in 0.9 mL of 50 mM NaOAc buffer (pH 4.8) and an appropriate amount of enzyme in a total volume of 1 mL was incubated for 10 min at 37 °C. The reaction was stopped by adding 0.5 mL of 1 M Na_2CO_3 solution, and liberated *o*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of enzyme activity was defined as the amount of the enzyme hydrolyzing 1 μmol of Galβ-*o*NP per min.

Analytical methods.—HPLC was performed with a Hitachi 6000 liquid chromatograph with a Shodex Asahipak NH2P-50 column (4.6 mm diameter × 250 mm, Showadenko Corp., Tokyo, Japan) developed with 1:3 or 9:41 water–MeCN at a flow rate of 0.8 mL/min with an L-4000 UV detector and an L-3350 RI monitor. ^1H and ^{13}C NMR spectra were recorded with a Jeol JNM-EX 270 FT-NMR spectrometer. Chemical shifts are expressed in δ values relative to sodium 4,4-dimethyl-4-silapentanoate as the external standard. FABMS analyses were carried out in the positive ion mode using a Jeol JMS DS303HF mass spectrometer, coupled to a Jeol DA-800 mass-data system. An accelerating voltage of 10 kV and mass resolution of 1000 were employed. A sample of 1 μL in distilled water was loaded onto a probe tip and mixed with 1 μL of glycerol as a matrix. Mass calibration was done with Ultramark.

Preparation of α-L-fucosidase from porcine liver.—The porcine liver extract (400 mL) was brought to 20% saturation with $(NH_4)_2SO_4$, stirred for 30 min, and kept for 9 h at 4 °C. The supernatant obtained by centrifugation was brought to 60% saturation with $(NH_4)_2SO_4$ and stirred for 1 h. The precipitate was recovered by centrifugation, dissolved in 200 mL of 50 mM NaOAc buffer (pH 6), and dialyzed overnight against the same buffer. The dialyzed enzyme solution was then charged onto a CM-Sepharose Fast Flow column (3.2 cm diameter × 24 cm) equilibrated with 50 mM NaOAc buffer (pH 6).

After applying the sample, the column was washed with 400 mL of the same buffer. The column was then eluted with a linear gradient of NaCl concentration from 0 (400 mL) to 1 M (400 mL) and the eluate was collected in 20-mL fractions. Fractions (F. nos. 21–27) containing α-L-fucosidase, which did not show β-D-galactosidase activity, were combined and concentrated to low volume with an Amicon Diaflo unit equipped with a PM-10 membrane operating at 2 kg/cm^2, giving a specific activity of 0.04 U/mg protein.

Preparation of α-L-*fucosidase from* Alcaligenes *sp.*—Strain KSF-9687, which has been deposited in the National Institute of Bioscience and Human Technology, Ministry of International Trade and Industry, Tsukuba, Japan, under accession no. P-16944, was grown in a medium of nutrient broth (NB). The culture was transferred from the NB to a 500-mL Erlenmeyer flask containing 100 mL of 0.25% yeast extract, 0.5% polypeptone, 0.1% KH_2PO_4, and 0.05% $MgSO_4 \cdot 7\ H_2O$ (adjusted to pH 7 with 0.2 N NaOH before the sterilization). The flask was shaken on a rotary shaker for 48 h at 37 °C at 200 rpm.

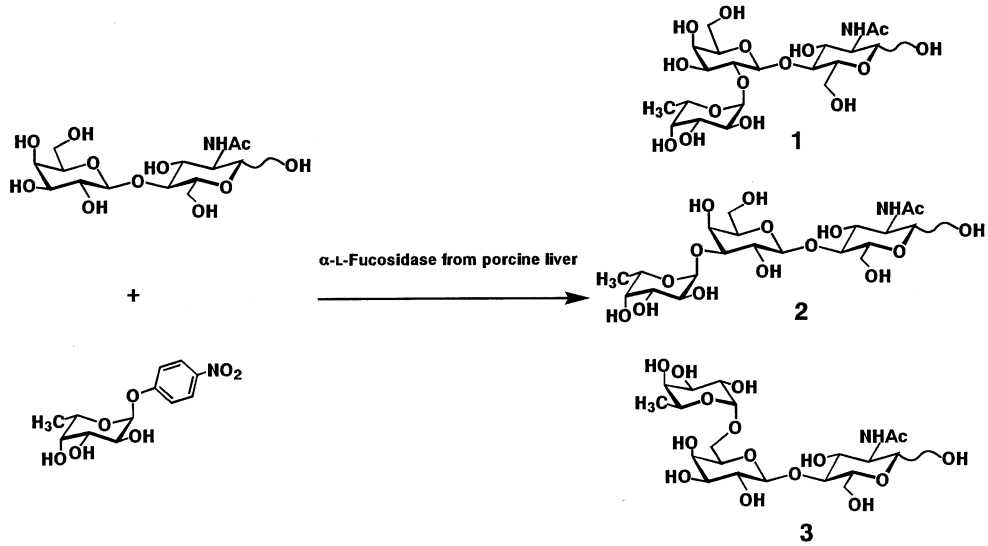
Step 1: Streamline-DEAE. A 4-L broth containing the cells was adjusted to pH 7 with 0.5 M potassium phosphate buffer (pH 7). The broth was applied on a Streamline-DEAE (Amersham Pharmacia Biotech) column (5.0 cm diameter × 20 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7) at room temperature. After the column had been washed with the same buffer, the α-L-fucosidase activity was eluted with the same buffer containing 0.2 M NaCl.

Step 2: Column chromatography on Butyl-Toyopearl 650M. Solid $(NH_4)_2SO_4$ was added to make 1.5 L of the active fraction from Step 1 up to 1 M in final concentration. The enzyme solution was put onto a Butyl-Toyopearl 650M column (2.6 cm diameter × 30 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7) containing 1 M $(NH_4)_2SO_4$, and the column was washed with the same buffer at 4 °C. The column was eluted with a linear gradient of $(NH_4)_2SO_4$ from 1 M (300 mL) to 0 M (300 mL). The active fractions were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7).

Step 3: Fucose–Cellulofine column chromatography. The dialyzed enzyme solution was charged onto a Fucose–Cellulofine [13] column (1.6 cm diameter × 10 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7); the column was washed with the same buffer, and the enzyme activity was then eluted with 50 mM potassium phosphate buffer (pH 7) containing 50 mM L-fucose at 4 °C. The active fractions were dialyzed against 10 mM potassium phosphate buffer (total activity, 56 U; specific activity, 19.7 U/mg).

Preparation of α-L-*Fuc-(1→2)-β-*D-*Gal-(1→4)-*D-*GlcNAc* (**1**) *and its isomers* α-L-*Fuc-(1→3)-β-*D-*Gal-(1→4)-*D-*GlcNAc* (**2**) *and* α-L-*Fuc-(1→6)-β-*D-*Gal-(1→4)-*D-*GlcNAc* (**3**), *utilizing porcine liver* α-L-*fucosidase (Scheme 1).*—To a solution of Fucα-*p*NP (70 mg) and LacNAc (1.2 g) in 9 mL of 50 mM potassium phosphate buffer (pH 4.8) containing 0.5 mL of MeOH was added partially purified α-L-fucosidase from porcine liver (0.45 U). The mixture was incubated for 30 h at 40 °C, and the reaction was terminated by boiling for 5 min. The resulting insoluble material was filtered and the filtrate was placed directly onto an activated charcoal–Celite column (2.6 cm diameter × 25 cm). The column was eluted successively with 500 mL of water and 500 mL of 12% EtOH, and then eluted with a linear gradient of 12% (500 mL) to 40% (500 mL) EtOH. The elution was monitored by measuring the absorbance at 210 nm for the characteristic absorption of the *N*-acetyl group and at 485 nm (carbohydrate content, determined by the phenol–H_2SO_4 method). The chromatogram (Fig. 1) showed four peaks displaying coincident absorbance at 210 and 485 nm. Fraction F-1 contained LacNAc used as the acceptor. F-2, F-3, and F-4 were collected, concentrated, and lyophilized to afford **1** (6.8 mg), **2** (6.4 mg), and **3** (3.9 mg), respectively.

Compound **1** had m/z 530 $[M+H]^+$; ^{1}H NMR (D_2O, 30 °C): δ 5.31 (H-1α), 5.21 (d, $J_{1,2}$ 2.97 Hz, H-1″), 4.72 (d, $J_{1,2}$ 8.1 Hz, H-1β), 4.55 (d, $J_{1,2}$ 7.56 Hz, H-1′α), 4.48 (d, $J_{1,2}$ 8.1 Hz, H-1′β), 2.05 (s, 3 H, NAc), and 1.23 (d, $J_{5,6}$ 6.7 Hz, H-6″).



Scheme 1.

Compound **2** had m/z 530 $[M+H]^+$; ^1H NMR (D_2O, 30 °C): δ 5.23 (d, $J_{1,2}$ 2.43 Hz, H-1α), 5.20 (d, $J_{1,2}$ 4.05 Hz, H-1″), 4.56 (t, H-1′), 2.07 (s, 3 H, NAc), and 1.23 (d, $J_{5,6}$ 6.48 Hz, H-6″).

Compound **3** had m/z 530 $[M+H]^+$; ^1H NMR (D_2O, 30 °C): δ 5.16 (d, $J_{1,2}$ 2.45 Hz, H-1α), 4.89 (d, $J_{1,2}$ 3.65 Hz, H-1α″), 4.88 (d, $J_{1,2}$ 3.95 Hz, H-1β″), 4.44 (d, $J_{1,2}$ 7.6 Hz, H-1′), 2.03 (s, 3 H, NAc), and 1.27 (d, $J_{5,6}$ 6.7 Hz, H-6″).

Preparation of compound **2** *and* α-L-*Fuc-(1→3)-β-*D-*Gal-(1→4)-Glc* (**4**) *and other related compounds utilizing* Alcaligenes *sp.* α-L-*fucosidase (Scheme 2)*

Compound **2**. Fucα-*p*NP (60 mg) and LacNAc (2 g) were dissolved in 20 mL of 20 mM potassium phosphate buffer (pH 7), and α-L-fucosidase from *Alcaligenes* sp. (0.6 U) was added. The mixture was incubated for 24 h at 50 °C and the reaction was terminated by boiling for 5 min. The resulting insoluble material was centrifuged and the filtrate was put directly onto an activated charcoal–Celite column (2.2 cm diameter × 95 cm). The column was eluted successively with 1 L of water and 1 L of 10% EtOH, and then with a linear gradient of 10% (1 L) to 40% (1 L) EtOH. The eluate was collected in 20-mL fractions and showed one main peak (3100–

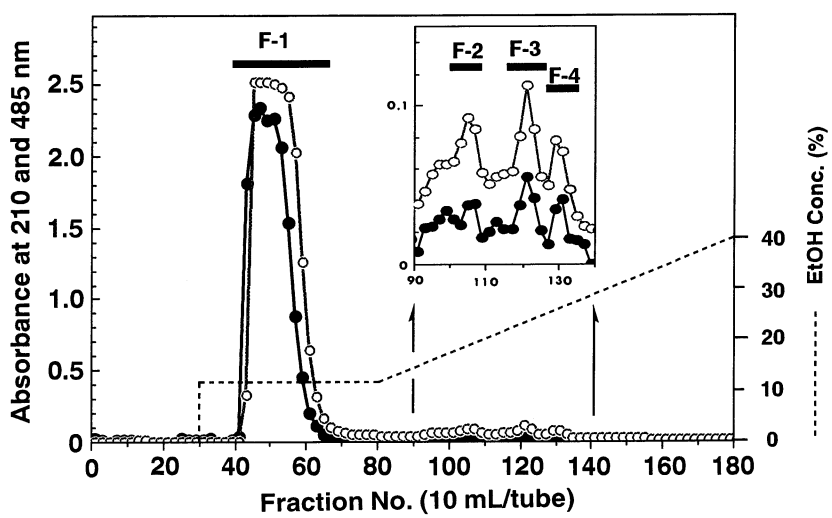
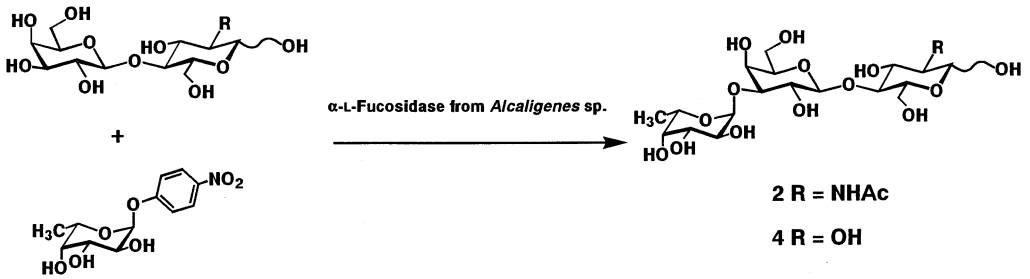


Fig. 1. Chromatographic separation of transglycosylation products with LacNAc and Fucα-*p*NP as substrates by the use of α-L-fucosidase from porcine liver. Absorbance at 210 nm (○) and 485 nm (●), and concentration of ethanol (dashed line).



Scheme 2.

3400 mL) as the transfer product. The fraction was collected, concentrated, and lyophilized to afford **2** (60.8 mg) in 54% yield, based on the donor added.

Compound **4**. Fucα-*p*NP (60 mg) and lactose (1.4 g) were dissolved in 20 mL of 20 mM potassium phosphate buffer (pH 7), and the same enzyme (0.6 U) was then added. The mixture was then applied to an activated charcoal–Celite column as already described, with the same elution conditions. The eluate, collected in 20-mL fractions, showed one main peak (2020–2220 mL) as the transfer product. The fraction was collected, concentrated, and lyophilized, giving **4** (35.2 mg) in 34% yield, based on the donor.

Compound **4** had m/z 489 $[M+H]^+$; ^{1}H NMR (D_2O, 26 °C): δ 5.23 (d, $J_{1,2}$ 3.96 Hz, H-1α), 5.18 (d, $J_{1,2}$ 3 96 Hz, H-1″), 4.68 (d, $J_{1,2}$ 8.25 Hz, H-1β), 4.52 (t, H-1′), and 1.26 (H-6″); ^{13}C NMR (D_2O): δ 105.33 (C-1′), 103.74 (C-1″), 98.60 (C-1α), 94.64 (C-1β), 83.16 (C-3′), 81.31 (C-4α), 8l.19 (C-4β), 78.09 (C-5′), 77.61 (C-5β), 77.23 (C-3β), 76.64 (C-2β), 74.59 (C-4″), 74.29 (C-3α), 73.98 (C-2α), 73.24 (C-2′), 72.92 (C-5α), 72.24 (C-3″), 71.46 (C-4′), 71.27 (C-2″), 69.99 (C-5″), 63.77 (C-6′), 62.93 (C-6β), 62.80 (C-6α), and 18.17 (C-6″).

Other related compounds, α-L-Fuc-(1→3)-β-D-Gal-(1→4)-β-D-Glc-OMe, α-L-Fuc-(1→3)-β-D-Gal-(1→3)-β-D-Glc-OMe, and α-L-Fuc-(1→3)-β-D-Gal-(1→4)-β-D-GlcNAc-OMe, were prepared according to the same method as already described. The yields are summarized in Table 2.

α-L-Fuc-(1→3)-β-D-Gal-(1→4)-β-D-Glc-OMe had m/z 503 $[M+H]^+$; ^{1}H NMR (D_2O, 26 °C): δ 5.18 (d, $J_{1,2}$ 3.96 Hz, H-1″), 4.51 (t, H-1′), 4.41 (d, $J_{1,2}$ 7.92 Hz, H-1), and 1.22 (H-6″); ^{13}C NMR (D_2O): δ 105.91 (C-1′), 105.55 (C-1), 103.74 (C-1″), 83.14 (C-3′), 81.22 (C-4), 78.09 (C-5′), 77.57 (C-5), 77.25 (C-3), 75.61 (C-2), 74.59 (C-4″), 73.22 (C-2′), 72.22 (C-3″), 71.45 (C-4′), 71.25 (C-2″), 69.99 (C-5″), 63.76 (C-6′), 62.89 (C-6), 60.04 (OCH_3), and 18.15 (C-6″).

α-L-Fuc-(1→3)-β-D-Gal-(1→3)-β-D-Glc-OMe had m/z 503 $[M+H]^+$; ^{1}H NMR (D_2O, 26 °C): δ 5.19 (d, $J_{1,2}$ 3.96 Hz, H-1″), 4.74 (d, $J_{1,2}$ 7.26 Hz, H-1), 4.43 (d, $J_{1,2}$ 8.25 Hz, H-1′), and 1.22 (H-6″); ^{13}C NMR (D_2O): δ 105.87 (C-1′), 105.77 (C-1), 103.74 (C-1″), 87.31 (C-3), 83.38 (C-3′), 78.31 (C-5), 78.08 (C-5′), 75.58 (C-2), 74.57 (C-4″), 73.44 (C-2′), 72.22 (C-3″), 71.45 (C-2″), 71.25 (C-4′), 71.09 (C-4), 70.01 (C-5″), 63.76 (C-6′), 62.89 (C-6), 60.04 (OCH_3), and 18.15 (C-6″).

α-L-Fuc-(1→3)-β-D-Gal-(1→4)-β-D-Glc-NAc-OMe had m/z 544 $[M+H]^+$; ^{1}H NMR (D_2O, 26 °C): δ 5.15 (d, $J_{1,2}$ 3 96 Hz, H-1″), 4.51 (t, H-1′), 4.44 (d, $J_{1,2}$ 7.26 Hz, H-1), and 1.22 (H-6″); ^{13}C NMR (D_2O): δ 177.55 (C=O), 105.55 (C-1′), 104.76 (C-1), 103.79 (C-1″), 83.22 (C-3′), 81.38 (C-4), 78.15 (C-5′), 77.65 (C-5), 75.45 (C-3), 74.64 (C-4″), 73.31 (C-2′), 72.29 (C-3″), 71.50 (C-4′), 71.30 (C-2″), 70.06 (C-5″), 63.81 (C-6′), 62.95 (C-6), 59.98 (OCH_3), 57.86 (C-2), 25.05 ($COCH_3$) and 18.22 (C-6″).

Hydrolysis reaction of Alcaligenes *sp.* α-L-*fucosidase on* O-α-L-*fucosyllactose*.—To a 200 μL solution containing 5 mM of **4** or 2′-*O*-α-L-fucosyllactose in 0.1 M sodium phosphate buffer (pH 8), 44 mU of α-L-fucosidase from *Alcaligenes* sp. was added. The solution was incubated at 50 °C and samples (10 μL) were taken out at appropriate time intervals (0, 3, 6, 9, 12, and 15 min) during the incubation. After boiling for 5 min, the mono- and

oligosaccharides were derivatized with ethyl *p*-aminobenzoate according to the published methods [14,15]. The derivatized sugars were analyzed on a Hitachi F-1050 fluorescence spectrophotometer by HPLC with a Wakosil-II 5C18 HG column (4.6 cm diameter × 150 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan) eluted with 1:9 MeCN–0.02% trifluoroacetic acid at a flow rate of 1 mL/min.

3. Results and discussion

Transglycosylation reaction using α-L-fucosidase from porcine liver.—A crude α-L-fucosidase preparation from porcine liver, prepared by precipitation with 20–65% saturated ammonium sulfate, was applied to a CM-Sepharose Fast Flow column. Most of the β-D-galactosidase, which hydrolyzes the LacNAc used as acceptor, was not absorbed by the gel and eluted out with 50 mM NaOAc buffer (pH 6), and the adsorbed part of α-L-fucosidase was eluted with a linear gradient of NaCl from 0 to 1 M. The partially purified enzyme preparation, almost devoid of β-D-galactosidase activity, was used for the fucosylation reaction without further purification. Transglycosylation of α-L-fucosidase from porcine liver was performed with Fucα-*p*NP and LacNAc for 30 h at 40 °C. Compounds **1**–**3** were isolated from the reaction mixture by an activated charcoal–Celite column (Fig. 1) in the ratio of 40:37:23. These products were obtained in 13% total yield, based on the donor added. Compounds **1**–**3** all showed molecular ions at m/z 530 for $[M+H]^+$ by FABMS, together with fragment ions at m/z 204, 222, 366, 384, and 512 (HexNAc–water, HexNAc, deoxyHex-HexNAc, Hex-HexNAc, and deoxyHex-Hex-HexNAc–water, respectively). These data show the trisaccharides to consist of deoxyHex-Hex-HexNAc. Further, the structures of **1**–**3** were elucidated by ^{1}H and ^{13}C NMR. Table 1 summarizes ^{13}C NMR spectral data of these compounds. Compounds **1**–**3** were thus identified as α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-GlcNAc, α-L-Fuc-(1→3)-β-D-Gal-(1→4)-D-GlcNAc, and α-L-Fuc-(1→6)-β-D-Gal-(1→4)-D-GlcNAc, respectively. Fig. 2 shows the transglycosylation profile induced by the α-L-fucosidase in a reaction system containing LacNAc acceptor. The transfer products formed were analyzed by HPLC. The time at which maximal production of the total amount of **1**–**3** was 24 h, and the proportions of the three compounds varied little during reaction. The fucosylation shows only low regioselectivity, because it occurs without discrimination at the O-2, O-3, and O-6 positions of the Gal moiety, as shown in Fig. 3. As a result, the desired compound **1** was obtained in a low yield of 5% based on the donor. No transfer product was observed when lactose instead

Table 1
^{13}C chemical shifts of α-L-fucosyl-disaccharides in D_2O solution.

Compound	Residue	Chemical shifts (δ)							
		C-1	C-2	C-3	C-4	C-5	C-6	NH*C*OCH$_3$	NHCO*C*H$_3$
1	GlcNAc (α)	93.49	56.66	72.58	79.32	73.48	63.02	177.39	24.85
	GlcNAc (β)	97.86	56.82	75.31	79.01	77.77	63.05	177.39	25.14
	Gal	103.23	78.18	75.47	71.50	76.51	64.02		
	Fuc	102.32	71.14	72.09	74.63	69.88	18.19		
2	GlcNAc (α)	93.48	56.66	72.36	81.81	73.19	62.93	177.39	24.83
	GlcNAc (β)	97.81	59.19	75.44	81.40	77.77	63.05	177.66	25.12
	Gal (α)	105.66	73.37	83.25	71.55	78.20	63.84		
	Gal (β)	105.61	73.37	83.25	71.55	78.20	63.84		
	Fuc	103.83	71.37	72.25	74.72	70.10	18.26		
3	GlcNAc (α)	93.55	56.48	72.88	83.22	73.60	62.89	177.57	24.94
	GlcNAc (β)	97.52	58.94	75.49	83.22	77.41	63.07	177.82	25.21
	Gal (α)	106.34	73.60	75.60	71.45	76.85	70.48		
	Gal (β)	106.41	73.60	75.60	71.45	76.85	70.37		
	Fuc	102.30	71.25	72.40	74.73	69.77	18.28		

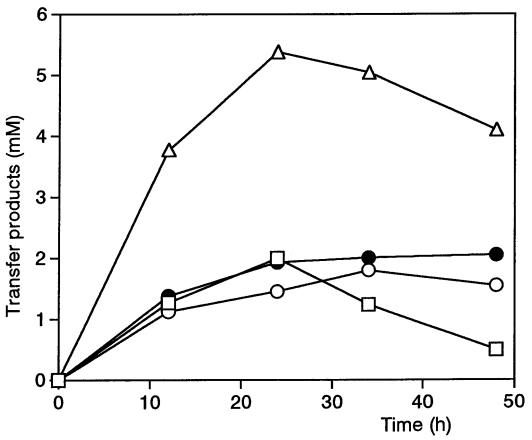


Fig. 2. Time-course of formation of 2′-fucosyl-*N*-acetyllactosamine and isomers by transglycosylation mediated by an α-L-fucosidase from porcine liver. The enzyme reaction was performed with Fucα-*p*NP (4.5 mg), LacNAc (75 mg), and enzyme (28 mU) in 0.4 mL of 0.1 M NaOAc buffer (pH 4.8) containing 5% MeOH. The amounts of compounds **1** (●), **2** (○), and **3** (□) were analyzed by HPLC. (△) shows the total amount of three compounds.

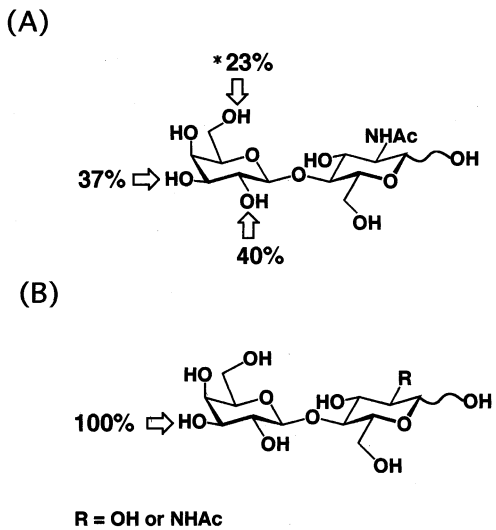


Fig. 3. Percentages of α-L-fucosyl-disaccharides formed by α-L-fucosidase from (A) porcine liver and (B) *Alcaligenes* sp. * The numbers show the individual transglycosylation as compared with the total.

of LacNAc was used as an acceptor, which was consistent with the result reported by Svensson and Thiem [9]. The porcine liver α-L-fucosidase-mediated fucosylation seems to recognize subtle differences in the acceptor structure.

Transglycosylation reaction utilizing α-L-fucosidase from Alcaligenes *sp.*—An α-L-fucosidase from the culture of *Alcaligenes* sp. was partially purified by successive chromatography on Streamline-DEAE and Butyl-Toyopearl 650M columns, and by affinity chromatography on a Fucose–Cellulofine column. The partially purified enzyme, completely devoid of β-D-galactosidase activity, was used for the enzyme synthesis.

Compound **2** has been synthesized by a chemical method [16]. However, it involved elaborate procedures for protection, glycosylation, and deprotection [16]. In the present study, compound **2** was efficiently synthesized from LacNAc and Fucα-*p*NP on a 100-mmol scale by transglycosylation utilizing the α-L-fucosidase. It was conveniently isolated by activated charcoal–Celite chromatography in high yield (54%), based on the donor added. In this case, the molar ratio of the donor to acceptor was 1:25 and the total substrate concentration was ~10%. The production of compound **2** as a function of time was monitored by HPLC with an Asahipak NH2P-50 column. The chromatogram showed that only one transfer product was formed during the entire course of reaction (50 h). This is also the case for the formation of **4** in the reaction with lactose as the acceptor. Compound **4** was obtained as a single transfer-product in high yield (34%), based on the donor. This trisaccharide has been synthesized by Baer and Abbas [17]. Use of other acceptors, β-D-Gal-(1→4)-β-D-GlcNAc–OMe, β-D-Gal-(1→4)-β-D-Glc–OMe, and β-D-Gal-(1→3)-β-D-Glc–OMe, also resulted in highly regioselective transglycosylation from Fucα-*p*NP to the 3-position of the Gal moiety by use of the α-L-fucosidase (Table 2). This result indicates that introduction of the methyl group at the β anomeric position of the acceptors enhanced the yield of transfer products. These reactions were efficient enough to permit one-pot preparations of compound **2** and its analogues. In this way, the enzyme fucosylated the O-3′ position of the galactose-containing disaccharide acceptors regioselectively, in contrast with the porcine liver enzyme, and generated an unusual carbohydrate linkage. The α-L-Fuc-(1→3)-D-Gal linkage is not found in glycoconjugates isolated from diverse sources, but its presence has been reported as part of higher homologues of oligosaccharides from human milk [18]. Miyauchi et al. have reported that antiserum against 3′-*O*-α-L-fuco-syllactose–*p*-isothiocyanato-phenethylamine–BSA

Table 2
Regioselective synthesis of α-L-fucosyl-disaccharides, utilizing *Alcaligenes* sp. α-L-fucosidase

Acceptor	Substrate [a] (%, w/v)	Product	Yield [b] (%)
Galβ-(1→4)-Glc	7.3	Fucα-(1→3)-Galβ-(1→4)-Glc (**4**)	34
Galβ-(1→4)-Glcβ–OMe	7.3	Fucα-(1→3)-Galβ-(1→4)-Glcβ–OMe	42
Galβ-(1→3)-Glcβ–OMe	7.4	Fucα-(1→3)-Galβ-(1→3)-Glcβ–OMe	67
Galβ-(1→4)-GlcNAc	10.3	Fucα-(1→3)-Galβ-(1→4)-GlcNAc (**2**)	54
Galβ-(1→4)-GlcNAcβ–OMe	10.3	Fucα-(1→3)-Galβ-(1→4)-GlcNAcβ–OMe	67

[a] Total substrate concentration.
[b] Based on the donor added.

preferentially reacts with adenocarcinoma and embryonal carcinoma cells [19]. It was shown that the unusual oligosaccharide **4** is useful as a probe for investigating biological functions.

In a separate experiment, the relative rates of hydrolysis of 2′-*O*-α-L-fucosyllactose and **4** were examined using the α-L-fucosidase from *Alcaligenes* sp. The reactions were linear from 3 to 15 min, and the rate of attack on **4** was arbitrarily set at 100. The relative hydrolysis rate of 2′-fucosyllactose was 77.5 as compared with that of **4**, a difference of only one-fourth. Thus, 2′-*O*-α-L-fucosyllactose seems to be a good substrate for hydrolysis. In general, glycosidases are known to be responsible for both hydrolytic and transfer activities. Therefore, the α-L-fucosidase was also expected to produce a trisaccharide substituted by an α-L-fucosyl group at the O-2′ of lactose, in addition to the O-3′ substituted product. However, the enzyme induced highly regioselective transglycosylation to the 3-position of the Gal moiety from Fucα-*p*NP and no 2′-*O*-α-L-fucosyllactose was detected during the entire reaction, as already mentioned. There may be other factors affecting the regioselectivity of this transglycosylation reaction.

In conclusion, a fucosylated trisaccharide **1** of the type 2 blood group H substance was prepared by using porcine liver α-L-fucosidase-mediated transglycosylation. We have also developed a practical synthetic method for obtaining fucosylated trisaccharides **2** and **4**, which contain the unusual α-(1→3) linkage.

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