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Enzymic synthesis of lacto-*N*-triose II and its positional analogues

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N-acetylhexosaminidase from *Nocardia orientalis* catalysed the synthesis of lacto-*N*-triose II glycoside (β-D-GlcNAc-(1-3)-β-D-Gal-(1-4)-β-D-Glc-OMe, **3**) with its isomers β-D-GlcNAc-(1-6)-β-D-Gal-(1-4)-β-D-Glc-OMe (**4**) and β-D-Gal-(1-4)-[β-D-GlcNAc-(1-6)]-β-D-Glc-OMe (**5**) through *N*-acetylglucosaminyl transfer from *N*,*N*′-diacetylchitobiose ($GlcNAc_2$) to methyl β-lactoside. The enzyme formed the mixture of trisaccharides **3**, **4** and **5** in 17% overall yield based on $GlcNAc_2$, in a ratio of 20:21:59. With *p*-nitrophenyl β-lactoside as an acceptor, the enzyme also produced *p*-nitrophenyl β-lacto-*N*-trioside II (β-D-GlcNAc-(1-3)-β-D-Gal-(1-4)-β-D-Glc-$OC_6H_4NO_2$-*p*, **6**) with its isomers β-D-GlcNAc-(1-6)-β-D-Gal-(1-4)-β-D-Glc-$OC_6H_4NO_2$-*p* (**7**) and β-D-Gal-(1-4)-[β-D-GlcNAc-(1-6)]-β-D-Glc-$OC_6H_4NO_2$-*p* (**8**). In this case, when an inclusion complex of *p*-nitrophenyl lactoside acceptor with β-cyclodextrin was used, the regioselectivity of glycosidase-catalysed formation of trisaccharide glycoside was substantially changed. It resulted not only in a significant increase of the overall yield of transfer products, but also in the proportion of the desired compound **6**.

Keywords: lacto-*N*-triose II, enzymic synthesis, *N*-acetylhexosaminidase, transglycosylation

Abbreviations: $GlcNAc_2$, 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1-4)-2-acetamido-2-deoxy-D-glucose; NAHase, *N*-acetylhexosaminidase; β-CD, β-cyclodextrin

Introduction

The oligosaccharide components of glycosphingolipids are of interest because of their numerous roles in biological events such as cell-cell recognition. The glycosphingolipids of the lacto series are called lacto- and lactoneo-tetraglycosylceramides and the corresponding lipid with one less sugar is lactotriglycosylceramide [1]. They have the common trisaccharide lacto-*N*-triose II (β-D-GlcNAc-(1-3)-β-D-Gal-(1-4)-D-Glc) unit. There is at present a great interest in developing synthetic routes to such an oligosaccharide unit which is normally located on the cell surface as a component of glycolipid. An organic chemical method for obtaining the lacto-*N*-triose II unit has been developed [2–4]; it requires multistep manipulations including protection, glycosylation and deprotection. From a practical viewpoint, the use of glycosidase is an attractive alternative in the synthesis of such trisaccharide structures. Several GlcNAc-containing disaccharides have been prepared conveniently by an *N*-acetylglucosaminyl transfer reaction utilizing NAHase (EC 3.2.1.52) and lysozyme (EC 3.2.1.17) [5–7]. Our interest was therefore directed to an enzymic approach to the synthesis of lacto-*N*-triose II. This compound would be useful as an exogenous substrate for NAHase, a probe for lectins, a common synthetic intermediate of blood group I antigen [8] and a starting material for synthesis of tetrasaccharides, lacto-*N*-tetraose (β-D-Gal-(1-3)-β-D-GlcNAc-(1-3)-β-D-Gal-(1-4)-D-Glc) and lacto-*N*-neotetraose (β-D-Gal-(1-4)-β-D-GlcNAc-(1-3)-β-D-Gal-(1-4)-D-Glc). Thus, the objective of the present investigation is to develop a system for selective transfer of *N*-acetylglucosaminyl residues onto C-3 of the Gal residue of lactoside acceptor, based on this approach.

The present paper describes a preparative synthetic

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method for lacto-*N*-triose II glycoside with its positional analogues, utilizing the transglycosylation reaction catalysed by NAHase from *N. orientalis* and the manipulation of the regioselectivity of the glycosidase by the use of an inclusion complex of *p*-nitrophenyl lactoside acceptor with β-CD.

Materials and methods

Materials

Commercially available β-D-galactosidase preparations (Biolacta, Daiwa Kasei Co., Ltd, Osaka, Japan) prepared from the culture filtrates of *Bacillus circulans* were used as an enzyme source. Chitinase prepared from the culture filtrates of *N. orientalis* was directly used for the enzymic synthesis without further purification [9]. The charcoal Celite column for the separation of transglycosylation products was prepared as follows: equal parts by weight of dry charcoal and Celite were slurried in water and packed into a glass column. All other chemicals were obtained from commercial sources.

Enzyme assays

β-D-Galactosidase activity was assayed as follows. A mixture containing 2 mM *o*-nitrophenyl β-D-galactopyranoside in 0.9 ml of 50 mM sodium phosphate buffer (pH 6.0) and an appropriate amount of enzyme in a total volume of 1.0 ml was incubated for 10 min at 30 °C. The reaction was stopped by adding 0.1 M Na_2CO_3 (2 ml), and then the liberated *o*-nitrophenol was determined spectrophotometrically at 420 nm. One unit of activity was defined as the amount of the enzyme releasing 1 μmol of *o*-nitrophenol per min. NAHase activity was assayed as follows. The enzyme (20 μl) was incubated with 0.98 ml of 2 mM *p*-nitrophenyl *N*-acetyl-β-glucosaminide in 0.1 M sodium citrate buffer (pH 5.0) at 40 °C for 15 min. After incubation, the enzymic reaction was stopped by adding 2 ml of 1.0 M sodium carbonate solution and the *p*-nitrophenol liberated was measured by the absorbance at 405 nm. One unit of enzyme activity was defined as the amount of enzyme which released 1 μmol of *p*-nitrophenol per min.

Analytical methods

HPLC was performed with an Asahipak packed column NH2P-50 (4.6 × 250 mm) and a YMC-packed column Type AQ-312 (ODS) (6 × 150 mm) in a Hitachi L-4000 ultraviolet detector. Elution of the former column was effected with 1:4 H_2O-MeCN and the latter with 1:9 H_2O-MeOH. The flow rate was 1.0 ml min^{-1} at a pressure of 60 kg cm^{-2}. ^{13}C- and ^{1}H-NMR spectra were determined with a JEOL JNM-EX 270 spectrometer operating at 68 MHz in the pulsed Fourier-transform mode with complete proton decoupling and at 270 MHz, respectively. Chemical shifts are expressed in ppm relative to sodium 3-(trimethylsilyl)-propionate (TPS) as an internal standard. FAB-MS analyses were carried out in the positive- and negative-ion mode using a JEOL JMS SX-102 mass spectrometer, coupled to a JEOL DA-800 mass data system. An accelerating voltage of 10 kV and a mass resolution of 1000 was employed. The sample (3 μl) in distilled water was added to the magic bullet. CID FABMS/MS experiments on $[M + H]^+$ and $[M-H]^-$ ions were performed by linked scanning using He as the collision gas. Linked scans at B/E were generated by the JEOL Compliment data system. The spectrum of oligosaccharide was recorded with JEOL JMS HX/HX 110A mass spectrometer, operating at the full accelerating potential (10 kV) and coupled to a JEOL DA-800 mass data system. The molecular weight of the sample was estimated from *m/z* value of quasi molecular-ion $[M + H]^+$ peak. Specific rotation was determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken Corp., Ltd).

Preparation of methyl β-lactoside(1) and p-nitrophenyl β-lactoside (2)

(**1**) To a solution of lactose (9.4 g) and methyl β-D-glucoside (10.6 g) in 50 mM phosphate buffer (26.5 ml, pH 6.0) was added β-D-galactosidase (20 U) from *B. circulans*. The mixture was incubated at 40 °C for 20 h and terminated by heating at 95 °C for 10 min. The resulting insoluble material was centrifuged off. The supernatant was directly applied to a charcoal-Celite column (5.5 × 58 cm). The column was first eluted with water (1 l) and then with a linear gradient of 0 (4 l)-27% (4 l) ethanol. The elution was monitored by measuring the absorbance at 490 nm (carbohydrate content, determined by the phenol-sulfuric acid method). The eluate (60 ml fractions) showed four main peaks (F-1: tubes 35–60, F-2: tubes 65–80, F-3: tubes 92–110 and F-4: tubes 121–135). Fractions F-1 and F-2 contained the unreacted methyl β-D-glucoside and lactose, respectively. Fractions F-3 and F-4, which were presumed to contain transfer products, were each combined and concentrated to afford yields of 1.2 g (crystallized from ethanol) and 1.1 g (crystallized from ethanol), respectively. All physical data for F-3 was identical to those of β-D-Gal-(1-4)-β-D-Glc-OMe reported previously [10].

F-4 had:$[\alpha]_D^{25}$ −3.4° (*c* 1, H_2O); mp 163 °C; and *m/z* 357 $(M + H)^+$. NMR data (D_2O): ^{1}H, 4.65 (d, 1H, *J* 7.7 Hz, H-1′), 4.42 (d, 1H, *J* 7.9 Hz, H-1) and 3.58 (s, 3H, OMe); ^{13}C, 106.18 (C-1′), 105.79 (C-1), 87.56 (C-3), 78.33 (C-5), 78.20 (C-5′), 75.52 (C-2), 75.40 (C-3′), 74.07 (C-2′), 71.41 (C-4′), 71.16 (C-4), 63.90 (C-6′), 63.58 (C-6) and 60.04 (OMe). When F-4 was hydrolysed with *B. circulans* β-D-galactosidase, it gave D-galactose and methyl β-D-glucoside in the molar ratio of 1:1 by HPLC.

From these data, F-4 was identified as β-D-Gal-(1-3)-β-D-Glc-OMe.

(**2**) To a solution (20 ml) of lactose (4 g) and *p*-nitrophenyl β-D-glucoside (3.33 g) in 20 mM phosphate buffer (pH 7.0) containing 20% MeCN (4 ml) was added β-D-galactosidase from *B. circulans* (34 U). After being incubated for 6 h at 40 °C, the mixture was centrifuged. The supernatant was directly loaded on a Toyopearl HW-40S column (5 × 100 cm). The eluate was monitored by measuring the absorbance at 300 nm (*p*-nitrophenyl group) and at 490 nm (phenol-sulfuric acid method). The eluate (20 ml fractions) showed three main peaks (F-1: tubes 152–161, F-2: tubes 163–174 and F-3: tubes 199–222) for which the absorbance at 300 nm coincides with that at 490 nm. F-1 and F-2 were each combined and concentrated to afford **2** (509 mg, crystallized from methanol) and β-D-Gal-(1-3)-β-Glc-D-$OC_6H_4NO_2$-*p* (216 mg, crystallized from methanol), respectively. F-3 contained *p*-nitrophenyl β-glucoside used as the acceptor. This procedure was repeated an additional three times.

Compound **2** had: $[\alpha]_D^{25}$ −70.4° (*c* 1, H_2O); mp 256 °C (from methanol); and *m/z* 464 $(M + H)^+$. NMR data (D_2O): ^1H, 8.31 (d, 2H, *J* 8.6 Hz, *m*-Ph), 7.29 (d, 2H, *J* 8.6 Hz, *o*-Ph), 5.34 (d, 1H, *J* 7.9 Hz, H-1), 4.52 (d, 1H, *J* 7.9 Hz, H-1′); ^{13}C, 164.49 (Ph carbon attached to the phenolic oxygen), 145.50 (*p*-Ph), 128.93 (*m*-Ph), 119.32 (o-Ph), 105.77 (C-1′), 102.08 (C-1), 80.74 (C-4), 78.20 (C-5′), 77.93 (C-5), 76.89 (C-3), 75.36 (C-3′), 75.29 (C-2), 73.78 (C-2′), 71.37 (C-4′), 63.86 (C-6′), and 62.62 (C-6). β-D-Gal-(1-3)-β-D-Glc-$OC_6H_4NO_2$-*p* had: $[\alpha]_D^{25}$ −46.0° (*c* 1, H_2O); mp 208 °C (from methanol); and *m/z* 464 $(M + H)^+$. NMR data (D_2O): ^1H, 8.29 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.28 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.31 (d, 1H, *J* 7.3 Hz, H-1), 4.72 (d, 1H, *J* 7.3 Hz, H-1′); ^{13}C, 164.51 (Ph carbon attached to the phenolic oxygen), 145.50 (*p*-Ph), 128.95 (*m*-Ph), 119.33 (*o*-Ph), 106.14 (C-1′), 102.05 (C-1), 86.72 (C-3), 78.69 (C-5′), 78.18 (C-5), 75.40 (C-3′), 75.29 (C-2), 74.07 (C-2′), 71.43 (C-4′), 70.76 (C-4), 63.92 (C-6′) and 63.25 (C-6).

*Preparation of methyl β-lacto-N-trioside II (β-D-GlcNAc-(1-3)-β-D-Gal-(1-4)-β-D-Glc-OMe (**3**)), β-D-GlcNAc-(1-6)-β-D-Gal-(1-4)-β-D-Glc-OMe(**4**) and β-D-Gal-(1-4)-[β-D-GlcNAc-(1-6)]-β-D-Glc-OMe (**5**)*

To a solution of $GlcNAc_2$ (0.55 g) and **1** (1.05 g) in 20 mM acetate buffer (pH 5.0, 4 ml) was added NAHase from *N. orientalis* (15 U). The molar ratio of the former and latter compounds was 1:2 and the total substrate concentration was 40%. The mixture was incubated for 20 h at 40 °C and terminated by heating at 95 °C for 10 min. The resulting insoluble material was centrifuged off. The supernatant was directly applied to a charcoal-Celite column as in Fig. 1a. The column was first eluted with water (3.5 l) and then with a linear gradient of 0 (3 l)–25% (3 l) ethanol. The solution was monitored by measuring the absorbance at 210 nm (characteristic absorption of the *N*-acetyl group) and at 490 nm. The chromatogram showed three main peaks (F-1: tubes 110–144, F-2: tubes 182–196, and F-3: tubes 202–225) for which the absorbance at 210 nm coincides with that at 490 nm. Peak F-1 contained mainly **1** used as the

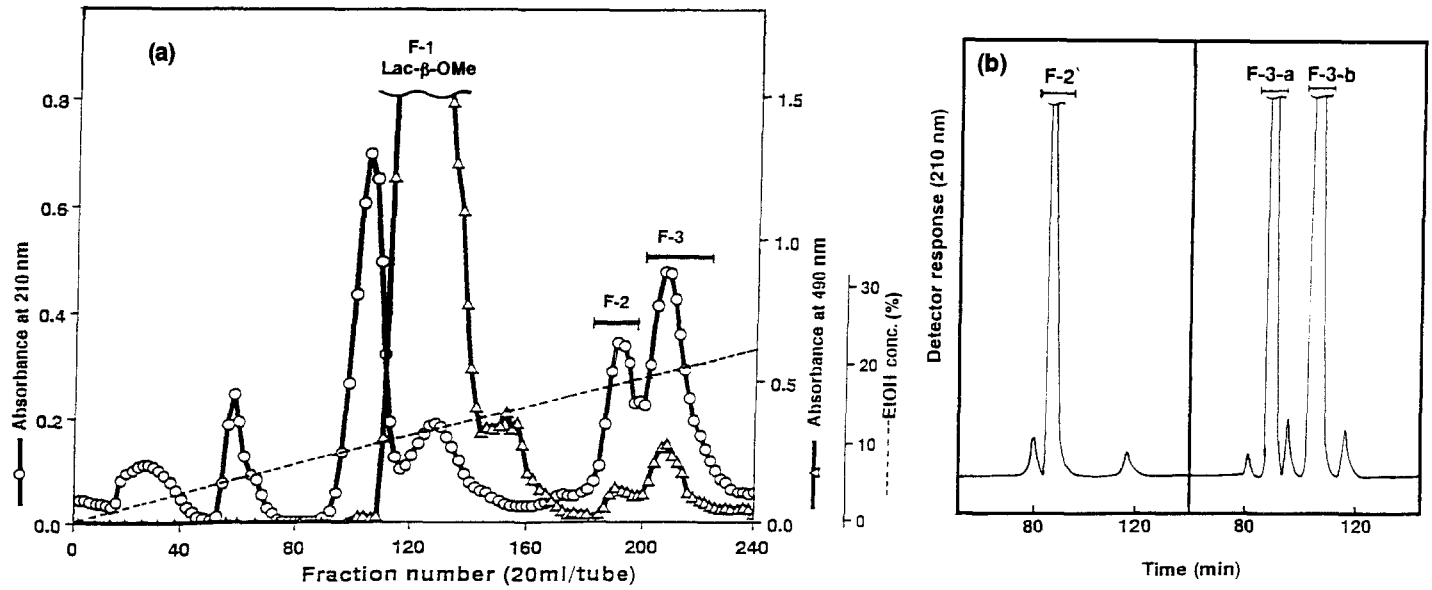

Figure 1. Chromatographic separation of transglycosylation products formed by the action of NAHase on $GlcNAc_2$ and **1**. (a) Chromatography of carbohydrates was carried out on a column (5.5 × 59 cm) of charcoal-Celite at room temperature. (b) HPLC was performed with a Asahipak NH2P-50 column (2.15 × 30 cm). Elution of the column was effected with acetonitrile/water (3:1, v/v).

acceptor. The latter two peaks (F-2 and F-3) were presumed to contain transfer products. The F-2 and F-3 fractions were each collected, concentrated and lyophilized to give residues of 50 and 120 mg, respectively. The former fraction was further treated with 2-aminopyridine, which was established for pyridylamination of reducing sugar [11], in order to remove the undesirable reducing sugar *N*, *N'*, *N''*-triacetylchitotriose ($GlcNAc_3$) produced. Thus, F-2 (50 mg) was dissolved in a solution of 2-aminopyridine (296 mg) in glacial acetic acid (192 μl) and methanol (230 μl). The solution was heated at 90 °C for 20 min. After volatiles were removed by evaporation under a stream of nitrogen at 60 °C, reducing reagent (prepared by 23 mg of borane-dimethylamine complex being dissolved in 384 μl of acetic acid) is added, and the solution was mixed well and heated at 90 °C for 30 min. The reaction mixture was dried under a stream of nitrogen gas with methanol and toluene. The residue was dissolved in 1 ml of water and the solution was applied to a column of Dowex 50W × 12 (H^+ form, 1.2 × 5 cm) equilibrated with water. After the column was washed with 100 ml of water, the nonadsorbed portion was concentrated to low volume (2 ml) and 1/4 vol of the solution was applied to HPLC. The fraction was separated into one main peak (F-2') as shown in Fig. 1b. The remaining solution was similarly treated. The eluates corresponding to the F-2' peak were combined, concentrated, and lyophilized to give compound **4** (26 mg). The F-3 fraction was dissolved in 4 ml of water and 1/8 vol of the solution was resolved by HPLC. The fraction was separated into two main peaks (F-3-a and F-3-b). The remainder of F-3 was similarly treated. The eluates corresponding to F-3-a and F-3-b peaks were each combined, concentrated, and lyophilized to afford compounds **3** (25 mg) and **5** (71 mg), respectively.

Compound **3** had: $[\alpha]_D^{25}$ + 3.7° (*c* 1, H_2O) and *m/z* 560 $(M + H)^+$. ^1H NMR data (D_2O): 4.66 (d, 1H, *J* 8.3 Hz, H-1''), 4.41 (d, 1H, *J* 7.7 Hz, H-1), 4.38 (d, 1H, *J* 7.9 Hz, H-1'), 3.55 (s, 3H, OMe) and 2.01 (s, 3H, Ac). Compound **4** had: $[\alpha]_D^{25}$ −18.0 ° (*c* 1, H_2O) and *m/z* 560 $(M + H)^+$. ^1H NMR data (D_2O): 4.59 (d, 1H, *J* 8.6 Hz, H-1''), 4.42 (d, 1H, *J* 7.6 Hz, H-1'), 4.39 (d, 1H, *J* 7.9 Hz), 3.55 (s, 3H, OMe) and 2.03 (s, 3H, Ac). Compound **5** had: $[\alpha]_D^{25}$ −6.9° (*c* 1, H_2O) *m/z* 560 $(M + H)^+$. ^1H NMR data (D_2O); 4.53 (d, 1H, *J* 8.2 Hz, H-1''), 4.37 (d, 1H, *J* 7.9 Hz, H-1'), 4.31 (d, 1H, *J* 7.6 Hz, H-1), 3.53 (s, 3H, OMe) and 2.04 (s, 3H, Ac).

Preparation of p-*nitrophenyl β-lacto-N-trioside (6) (β-D-GlcNAc-(1-3)-β-D-Gal-(1-4)-β-D-Glc-$OC_6H_4NO_2$*-p), *β-D-GlcNAc-(1-6)-β-D-Gal-(1-4)-β-D-Glc-$OC_6H_4NO_2$*-p(*7) and β-D-Gal-(1-4)-[β-D-GlcNAc-(1-6)]-β-D-Glc-$OC_6H_4NO_2$*-p *(8)*

To a solution of previously dissolved **2** (1.6 g) and β-CD (3.9 g) in 20 ml of 20 mM acetate buffer (pH 5.0) was added $GlcNAc_2$ (1.4 g) followed by NAHase (40 U). The molar ratio of the donor and acceptor was 1:1 and the total substrate concentration was about 15%. The mixture was incubated for 12 h at 40 °C and terminated by heating at 95 °C for 10 min. To the reaction mixture was added 80 ml of 40% methanol. When allowed to stand overnight at 5 °C, β-CD partially was crystallized out. The crystalline material was filtered off by a glass filter. The filtrate was concentrated to a low volume (20 ml) and directly loaded onto Toyopearl HW-40S column (4.5 × 90 cm). The elution was monitored by measuring

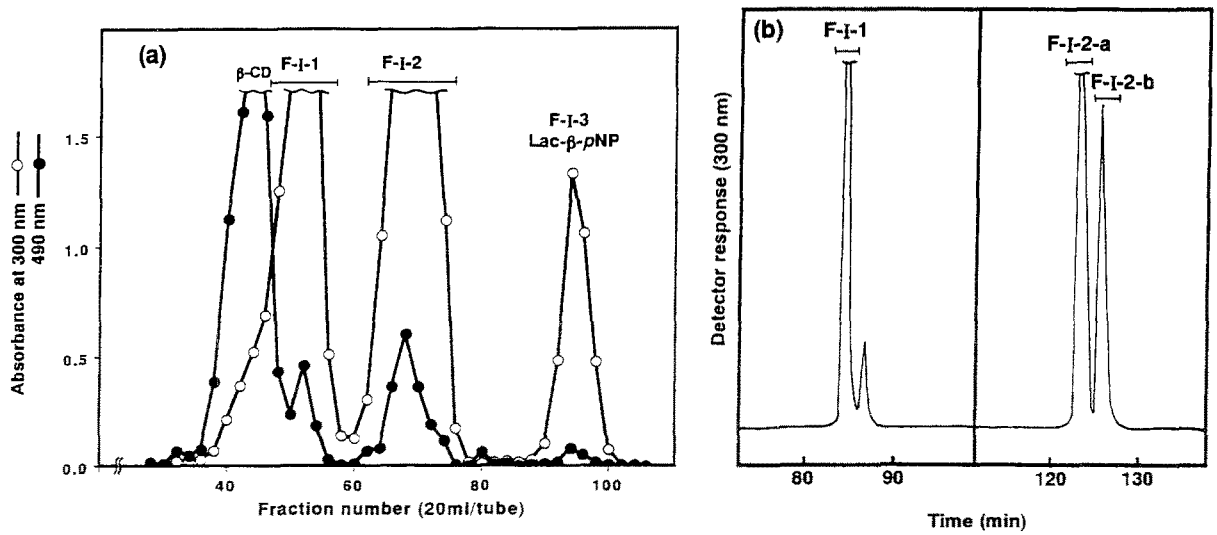

Figure 2. Chromatographic separation of transglycosylation products formed by the action of NAHase on $GlcNAc_2$ and **2**. (a) Chromatography of carbohydrates was carried out on a column (4.5 × 90 cm) of Toyopearl HW-40S eluted with 3:1 H_2O-MeOH. (b) HPLC was performed with a YMC-packed AQ-323 S-5 120A (ODS) column (2 × 50 cm). Elution of the column was effected with water/methanol (5:1, v/v).

the absorbance at 300 nm (*p*-nitrophenyl group) and at 490 nm. The eluate was collected in 20 ml fractions. It was fractionated into two fractions (F-I: tubes 32–81, and F-II: tubes 82–98). Peak F-II contained mainly **2** used as the acceptor. F-I was concentrated to a small volume (20 ml), which was then subjected to rechromatography as above. The chromatogram showed three main peaks (F-I-1: tubes 47–58, F-I-2: tubes 63–76, and F-I-3: tubes 92–100) for which the absorbance at 300 nm coincides with that at 490 nm as in Fig. 2a. The first two peaks were presumed to contain transfer products. The eluates corresponding to F-I-1, which still contained β-CD judging from overlapping of the absorption at 490 nm, were concentrated to a small volume (2 ml) and 1/4 was resolved by HPLC as in Fig. 2b. The remaining aliquots were similarly processed. The eluates corresponding to F-I-1′ were combined, concentrated and lyophilized to afford compound **8** (30.3 mg). Fraction F-I-2 was concentrated to a small volume (2 ml) and 1/4 vol was also applied to HPLC as above (Fig. 2b). The fraction was separated into two main peaks (F-I-2-a and F-I-2-b). The remaining aliquots were similarly treated. The eluates corresponding to F-I-2-a and F-I-2-b peaks were each combined, concentrated and lyophilized to afford compounds **6** (28.0 mg) and **7** (45.2 mg), respectively. Peak F-I-3 contained **2** used as the acceptor.

Compound **6** had: $[\alpha]_D^{25}$ −55.3° (*c* 0.2, H_2O/CH_3CN = 3/7) and *m/z* 667 $(M + H)^+$. ^1H NMR data (D_2O): 8.28 (d, 2H, *J* 9.2 Hz, *m*-Ph), 7.25 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.31 (d, 1H, *J* 7.9 Hz, H-1), 4.69 (d, 1H, *J* 8.2 Hz, H-1″), 4.48 (d, 1H, *J* 7.9 Hz, H-1′) and 2.04 (s, 3H,Ac). Compound **7** had: $[\alpha]_D^{25}$ −46.0° (*c* 1, H_2O/CH_3CN = 3/7) and *m/z* 667 $(M + H)^+$. ^1H NMR data (D_2O): 8.26 (d, 2H, J 9.2 Hz, *m*-Ph), 7.24 (d, 2H, *J* 9.2 Hz, *o*-Ph), 5.30 (d, 1H, *J* 7.6 Hz, H-1), 4.61 (d, 1H, *J* 8.2 Hz, H-1″), 4.46 (d, 1H, *J* 7.6 Hz, H-1′) and 2.06 (s, 3H, Ac). Compound **8** had: $[\alpha]_D^{25}$ −55.5° (*c* 0.4, H_2O/CH_3CN = 3/7) and *m/z* 667 $(M + H)^+$. ^1H NMR data (D_2O): 8.28 (d, 2H, *J* 9.2 Hz, *m*=Ph), 7.24 (d, *J* 9.2 Hz, *o*-Ph), 5.30 (d, 1H, *J* 7.6 Hz, H-1), 4.52 (d, 1H, *J* 8.2 Hz, H-1″), 4.37 (d, 1H, *J* 7.6 Hz, H-1′), and 1.84 (s, 3H, Ac).

Results and discussion

Enzymic synthesis of methyl β-lactoside (1) and p-nitrophenyl β-lactoside (2)

1 and **2**, which were used as acceptor substrates for the present enzymic synthesis of lacto-*N*-triose II and its positional analogues, were enzymically prepared utilizing transgalactosylation from lactose donor to, respectively, methyl β-glucopyranoside and *p*-nitrophenyl glucopyranoside acceptors using the β-D-galactosidase from *B. circulans* according to our previously established method [12]. The desired compounds **1** and **2** were readily synthesized on a gram scale and conveniently isolated by chromatography on a column of charcoal-Celite and on a column of Toyopearl HW 40S, respectively. These enzyme reactions were efficient enough to allow the one-pot preparation of the desired disaccharide glycosides. In each case, the β-(1-3)-linked isomer was formed in appreciable amounts along with the desired β-(1-4)-linked compound. We have recently reported that, when GlcNAc, GalNAc, or their *p*-nitrophenyl β-glycosides was acceptor, β-D-galactosyl transfer with the enzyme occurred preferentially at O-4 of the sugar, but did not at O-3 [12, 13]. An interesting result from these studies was the capacity of the β-D-galactosidase to catalyze formation of the β-(1-3)-linked disaccharide, when β-D-glucoside was the acceptor instead of the GlcNAc β-glycoside.

Enzymic synthesis of methyl β-lacto-N-trioside II (3) and its positional analogues (4 and 5)

The enzyme used in the present study was the crude NAHase from *N. orientalis*, which was prepared as an 20–70% ammonium sulfate fraction from the culture filtrates. This material contains chitinase activity in addition to NAHase, but is completely devoid of β-D-galactosidase, and was used without further purification. When **1** was used as acceptor and $GlcNAc_2$ as *N*-acetylglucosaminyl donor, three *N*-acetylglucosaminyltrisaccharide products **3**, **4**, and **5** were observed by HPLC, in 17% overall yield based on the donor added and in a ratio of 20:21:59. This indicates that about three fifth of the *N*-acetylglucosaminylation occurs at the C-6 position on the reducing side and one-fifth each at C-6′ and C-3′ on the nonreducing side. Fig. 3 is a transglycosylation profile of the reaction of NAHase with **1** and $GlcNAc_2$. Maximum production of the desired compound **3** was observed after 20 h, although its production

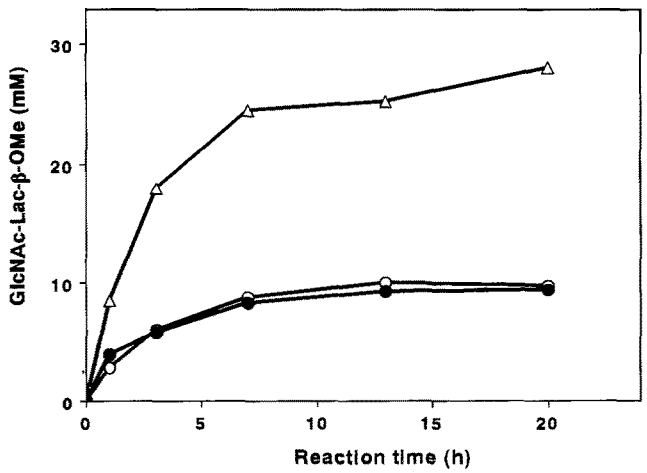

Figure 3. Time course of NAHase-mediated isomer formation of **3**, **4** and **5**. The amounts of **3** (○), **4** (●) and **5** (△) products as a function of time were examined on the 0.5 ml scale, as described in the Materials and methods section, and samples were analysed by HPLC during incubation.

was about one third that of **5**. Thus, the enzyme induced *N*-acetylglucosaminyl transfer to the primary hydroxyl (OH-6) of the acceptor rather than the secondary hydroxyl group (OH-4). These were first separated into three fractions (F-1, F-2 and F-3) by chromatography on a carbon-Celite column (Fig. 1a). F-1 recovered **1** used as the acceptor. The purification of F-2 into compound **4** was somewhat cumbersome, because this fraction was greatly contaminated by $GlcNAc_3$. Therefore, for the sake of operational simplicity, F-2 was further treated with 2-aminopyridine, which led to pyridylamination of the unwanted $GlcNAc_3$. The unreacted **4** was easily separated from the pyridylaminated $GlcNAc_3$ on a column of cation-exchange resin. **4** was finally purified by HPLC. F-3 was easily resolved into two components **3** and **5** by HPLC (Fig. 1b). FAB-MSMS indicated that **3** and **4** are a trisaccharide consisting of HexNAc-Hex-Hex-OMe. Thus, the sugar sequence was confirmed, in the positive-ion mode, by the presence of *m/z* 204 (fragment from the nonreducing end of HexNAc), 366 (fragment from the nonreducing end of HexNAc-Hex) and 560 ($[M + H]^+$). In the negative-ion mode, **5** gave a $[M-H]^-$ ion at *m/z* 558 together with a fragment ion at *m/z* 396 (fragment from the reducing end of HexNAc-Hex-OMe) and 355 (fragment from the reducing end of HexNAc-Hex-OMe). It indicates that **5** is a branched trisaccharide (Hex-(HexNAc)Hex-OMe). Based on their sugar sequences, the structures of the transfer products were elucidated by their ^1H- and ^{13}C-NMR spectra. NMR signal assignments were made using carbon-proton shift correlation spectroscopy (CH-COSY) as shown in Table 1.

Enzymic synthesis of p-*nitrophenyl β-lacto-*N-*trioside II (6) and its positional analogues (7 and 8)*

When **2** was the acceptor instead of **1**, one β-(1-3)- (**6**) and two β-(1-6)- (**7**, **8**) *N*-acetylglucosaminyl-trisaccharides were observed by HPLC, in total yield of 2.9% (based on the donor) and in a ratio of 14:21:65. In this case, the regioselectivity with respect to C-3′ vs. C-6′ was a little different from that of **1** acceptor. The minor product (14%) was shown to be the desired β-(1-3) compound **6**. This problem was partially solved by using β-CD, which should form an inclusion complex with a β-nitrophenyl group (*vide infra*). When **2** was dissolved in amounts equimolar with β-CD, the transfer products **6**, **7** and **8** were observed by HPLC, in 7.0% total yield and in a ratio of 27:43:30. It not only resulted in a significant improvement of the yield, but also in the higher proportion of **6** and **7** to **8**. These were separated by chromatography on a Toyopearl HW 40S column and purified by HPLC with an ODS column (Fig. 2). Structural assignments were made as indicated above. FAB-MSMS indicated that **6** and **7** are a trisaccharide consisting of HexNAc-Hex-Hex-$OPhNO_2$. The sugar sequence was confirmed in the positive ion mode by the presence of 667 ($[M + H]^+$) together with the fragment ions at *m/z* 204, 366 and 528 (from HexNAc, HexNAc-Hex and HexNAc-Hex-Hex, respectively). In the negative ion mode, **8** gave a $[M-H]^-$ ion at *m/z* 665 with a fragment ion at *m/z* 503 (fragment from the reducing end of HexNAc-Hex-$OPhNO_2$). The sugar sequence is shown to be a branched structure. Structure assignments were further made by ^1H- and ^{13}C-NMR data as in Table 2. By the use of an inclusion complex of the acceptor molecule with β-CD in this reaction system, strikingly different results were obtained with respect both to yield and to regioselectivity. The reaction of **1** in the presence of β-CD was used as a control to ascertain the efficiency of transglycosylation, but CD did not exert its effect in this reaction system. Fig. 4 is a transglycosylation profile of the reaction of the enzyme with $GlcNAc_2$ and **2** in the presence and absence of β-CD. The time for maximum formation of the three transfer products was ~10 h at 40 °C and the concentration then varied little during the subsequent reaction. The maximum production in the

Table 1. ^{13}C chemical shifts of compounds **3**, **4** and **5** in D_2O solution.

β-GlcNac-(1-3)-β-Gal-(1-4)-β-Glc-OMe (3)		*β-GlcNAc-(1-6)-β-Gal-(1-4)-β-Glc-OMe (4)*						*β-Gal-(1-4)-[β-GlcNAc-(1-6)-]β-Glc-OMe(5)*			
III	*II*	*I*		*III*		*II*	*I*	*III*		*II*	*I*
Compound		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*CH_3*	*C=O*	*-OCH_3*	
	I	105.89	75.60	77.22	81.22	77.59	62.91			60.04	
3	II	105.79	72.83	84.78	71.18	77.72	63.34				
	III	105.66	58.51	76.41	72.70	78.51	63.77	25.00	177.79		
	I	105.87	75.18[a]	77.21	81.71	77.50	62.84			60.04	
4	II	105.87	73.64	75.67[a]	71.27	76.62	71.09				
	III	103.88	58.33	76.37	72.69	78.67	63.50	25.14	177.41		
	I	105.80	75.29	77.21	82.05	76.24	70.26			60.02	
5	II	103.93	58.29	76.51	72.72	78.60	63.50	25.18	177.12		
	III	106.16	73.67	75.44	71.25	78.24	63.74				

[a]These assignments may be changed.

Table 2. ^{13}C chemical shifts of compounds **6**, **7**, and **8** in D_2O solution.

β-GlcNAc-(1-3)-β-Gal-(1-4)-β-Glc-pNP (6) *III* *II* *I*		*β-GlcNAc-(1-6)-β-Gal-(1-4)-β-Glc-pNP (7)* *III* *II* *I*							*β-Gal-(1-4)-[β-GlcNAc-(1-6)-]β-Glc-pNP(8)* *III* *II* *I*			
Compound		*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*CH_3*	*C=O*	*o-Ar*	*m-Ar*	*p-Ar*	*c-Ar*
	I	102.07	75.27	77.93	80.72	76.89	62.62			119.32	128.93	145.50	164.49
6	II	105.79	72.85	84.80	71.18	77.75	63.79[a]						
	III	105.68	58.51	76.41	72.54	78.51	63.32[a]	25.00	177.79				
	I	102.05	75.33	76.87	81.17	77.84	62.55			119.30	128.93	145.46	164.47
7	II	105.86	73.64	75.17	71.30	76.64	71.16						
	III	103.88	58.33	76.42	72.69	78.69	63.50	25.16	177.43				
	I	101.89	75.09	76.80	81.62	76.73[a]	70.03			119.24	129.04	145.48	164.45
8	II	103.70	58.18	76.53[a]	72.70	78.63	63.54	24.98	176.96				
	III	100.18	73.69	75.31	71.28	78.27	63.77						

[a]These assignments may be changed.

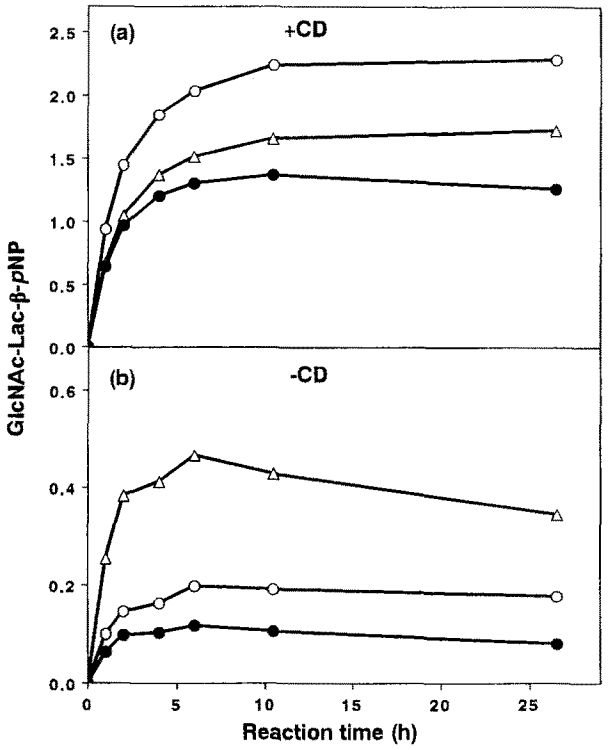

Figure 4. Time course of NAHase-mediated isomer formation of **6**, **7** and **8** in the presence (a) and absence (b) of β-CD. The amounts of **6** (○), **7** (●) and **8** (△) products as a function of time were examined on the 0.5 ml scale as in Fig. 3.

presence of β-CD was ~6 fold that in its absence. The increased solubility of the acceptor facilitated the production of transfer products. Thus, **2** in the presence of β-CD shows much higher solubility (8%) than that (2%) in the absence of β-CD. Furthermore, a change of regioselectivity could be achieved by using an inclusion complex of the acceptor with β-CD. In this way, the efficiency of the transglycosylation process is collectively reinforced by the presence of a minimal amount of water, an excess of substrate [13] and by the presence of β-CD. Furthermore, two thirds of the *N*-acetylglucosaminidation occurs at the C-3′ and C-6′ of the galactose moiety and 30% at C-6 of glucose, whereas, in the absence of β-CD, the most reactive OH is in the C-6 position as in Fig 5. The existence of the bulky β-CD region in an inclusion complex with the lactoside acceptor was clearly effective for diminishing the *N*-acetylglucosaminyl transfer onto C-6 at the reducing end glucose residue of the acceptor, which could be due to steric hindrance of the CD. As a result, the desired compound **6** was obtained in improved yield by using β-CD. Some researchers have demonstrated that the regioselectivity of glycosidase-catalysed formation of disaccharides can be changed by using α- or β-glycosyl acceptors with various aglycons [14, 15]. In the present case, the observed regioselectivity of the NAHase-catalysed reactions is also changed by manipulating the nature of hydrophobic *p*-nitrophenyl group in the glycosyl acceptor. In general, glycosidases do exhibit some regioselectivity, but this selectivity is less predictable and lower than that of such polysaccharide hydrolases as amylase [16], cellulase [17], β-D-mannanase [18] and lysozyme [19, 20].

Conclusions

It was found that the yield of methyl trisaccharide glycoside was higher than that of the corresponding phenyl glycoside. The chemical change of the acceptor can influence the regioselectivity of glycosidase-catalysed transglycosylation. The yields (7–17% of the donor) obtained in this study are comparable with those reported for enzymic synthesis of similar HexNAc-containing disaccharide glycosides [5, 6]. Lacto-*N*-triose II glycoside and its positional analogues might be useful for inhibition studies in biological experiments. Nitrophenyl

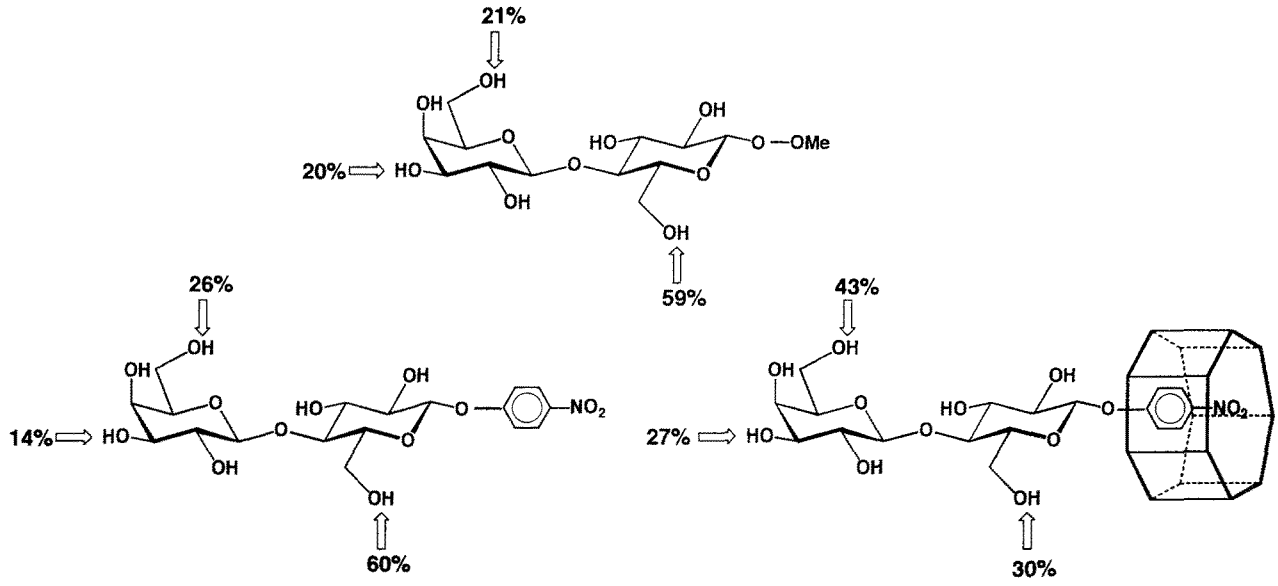

Figure 5. Structures of carbohydrates used as targets for NAHase-catalysed *N*-acetylglucosaminidation. Arrows indicate the position of *N*-acetylglucosaminidation. Percentages above the arrows are the fraction of a given transglycosylation compared with the total.

glycosides can be used as enzyme substrates or can be reduced to aminated glycosides. The amino function may be derivatised for reactions with electrophiles [21, 22]. The approach described above can be further extended to the synthesis of lacto-*N*-tetraose and lacto-*N*-neotetraose.

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