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Facile enzymatic conversion of lactose into lacto-*N*-tetraose and lacto-*N*-neotetraose

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Lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc, LNT) and lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc, LNnT) were enzymatically synthesized by consecutive additions of GlcNAc and Gal residues to lactose. Lacto-*N*-triose II (GlcNAcβ1-3Galβ1-4Glc) was prepared first by the transfer of GlcNAc from UDP-GlcNAc to lactose by β-1,3-*N*-acetylglucosaminyltransferase from bovine serum. The resulting lacto-*N*-triose II was converted into LNT and LNnT utilizing two kinds of β-D-galactosidase-mediated transglycosylations. Thus, β-D-galactosidase from *Bacillus circulans* ATCC31382 induced regioselective galactosyl transfer from *o*-nitrophenyl β-D-galactoside to the OH-3″ position of lacto-*N*-triose II, and commercially available β-D-galactosidase from *B. circulans* to the OH-4″ position of lacto-*N*-triose II. These convenient processes are suitable for large-scale preparations of LNT and LNnT. As another method, LNT was directly synthesized from lactose as an initial substance, utilizing lacto-*N*-biosidase (*Aureobacterium* sp. L-101)-mediated transglycosylation with Galβ1-3GlcNAcβ-*p*NP donor.

Keywords: **Lacto-*N*-tetraose, lacto-*N*-neotetraose, β-D-galactosidase, β-1,3-*N*-acetylglucosaminyltransferase**

Abbreviations: **LNT, lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc); LNnT, lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc); UDP, uridine 5′-diphosphate; β-gal-3, β-D-galactosidase from *Bacillus circulans* ATCC 31382; β-1,3-GnT, β-1,3-*N*-acetylglucosaminyltransferase; HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detection; ATP, adenosine 5′-triphosphate; EtOH, ethanol; Galβ-*o*NP, *o*-nitrophenyl β-D-galactopyranoside.**

Introduction

There is high current interest in developing synthetic routes for obtaining oligosaccharides involved in glycolipids and glycoproteins. Our interest is directed toward an enzymatic approach to the synthesis of lacto-*N*-triose II, LNT and LNnT, because glycolipids containing these oligosaccharide units have been known to play an important role in biological recognition events. Generally, they are found as the common structural feature in the core region of glycolipids of the so-called lacto- and neolacto-series [1], and in the terminal position of lipooligosaccharide of *Neisseria meningitidis* [2,3]. They are also well-known as human milk oligosaccharides [4]. Accordingly, a sufficient supply of such oligosaccharides could be utilized as substrates for glycosidase and glycosyltransferase [5], as probes for lectin [6], and as carbohydrate antigens [7]. Conventional chemical methods for obtaining lacto-*N*-triose II, LNT, and LNnt have been developed [8–10], but they involve various elaborate procedures for protection, glycosylation, and deprotection. A process for synthesizing these oligosaccharides is clearly needed to enable practical use. A enzymatic method would facilitate large-scale synthesis of oligosaccharides in as short a path way as possible.

The present paper describes the regioselective synthesis of LNT and LNnT by a combination of glycosyltransferase-catalyzed reaction and glycosidase-catalyzed transglycosylation and the direct conversion of lactose into LNT through a lacto-*N*-biosidase-mediated transfer reaction.

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Materials and methods

Materials

A crude preparation of UDP-GlcNAc: Galβ1-4Glc (or GlcNAc) β-1,3-*N*-acetylglucosaminyltransferase (β-1,3-GnT) was prepared as a 25–50% saturated ammonium sulfate precipitation from bovine serum and used without further purification for the synthesis of lacto-*N*-triose II. β-D-Galactosidase (EC 3.2.1.23) from *Bacillus circulans* ATCC 31382 (β-gal-3) [11] which was kindly supplied by Meiji Milk Products Co. Ltd., Tokyo and commercially

available β-D-galactosidase from *B. circulans,* Biolacta (Daiwa Kasei, K. K., Osaka) were used as enzyme sources. Lacto-*N*-biosidase from *Aureobacterium* sp. L-101 culture broth was prepared by ammonium precipitation followed by two chromatographic procedures. The detailed purification of the enzyme will be reported elsewhere. UDP-GlcNAc was supplied by Yamasa Corporation, Chiba. Galβ1-3GlcNAcβ-*p*NP was synthesized by our method [12]. All other chemicals were obtained from commercial sources.

Enzyme assay

β-1,3-GnT activity was assayed as follows: Lactose (22 mg) and UDP-GlcNAc (3.6 mg) were dissolved in 1.0 ml of 150 mM sodium cacodylate buffer (pH 7.0) containing 4.0 mg of $MnCl_2$, 10 mg of ATP, 3.0 mg of NaCl, followed by an appropriate amount of β-1,3-GnT. The reaction was stopped by boiling for 5 min, and then the resulting lacto-*N*-triose II was measured by the high performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD) method. One unit of the enzyme was defined as the amount synthesizing 1 μmole of lacto-*N*-triose II per hour.

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 sodium phosphate buffer (pH 6.0) and an appropriate amount of enzyme in a total volume of 0.1 ml was incubated for 10 min at 40°C. The reaction was stopped by adding 0.5 ml of 1.0 M Na_2CO_3, and then the liberated *o*-nitrophenol was measured spectrophotometrically at 420 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μmole of *o*-nitrophenyl β-D-galactopyranoside per min.

Lacto-*N*-biosidase activity was assayed as follows: A mixture containing 2 mM Galβ1-3GlcNAcβ-*p*NP in 0.2 ml of 40 mM acetate buffer (pH 5.5) and an appropriate amount of enzyme in a total volume of 50 μl was incubated for 10 min at 37°C. The reaction was stopped by adding 1 ml of 1.0 M Na_2CO_3 and 2 ml of H_2O, and then the liberated *p*-nitrophenol was measured spectrophotometrically at 405 nm. One unit of enzyme was defined as the amount hydrolyzing 1 μmole of Galβ1-3GlcNAcβ-*p*NP per min.

Analytical method

HPAEC-PAD analysis was conducted on a DX-300 BioLC system equipped with a pulsed amperometric detector (Dionex, Osaka, Japan). Oligosaccharides were separated on a CarboPac P-1 column (Dionex, Ø 4 × 250 mm) at a flow rate of 1 ml/min at room temperature. The elutions for lacto-*N*-triose II and LNnT were effected with 100 mM NaOH, and for LNT with 150 mM NaOH. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL JNM-LA 500 spectrometer at 25°C. Chemical shifts are expressed in δ relative to sodium 3-(trimethylsilyl)propionate as an external standard.

Preparation of lacto-N-triose II

Lactose (2.7 g) and UDP-GlcNAc (450 mg) were dissolved in 125 ml of 150 mM sodium cacodylate buffer (pH 7.0) containing 495 mg of $MnCl_2$, 183 mg of ATP and 365 mg of NaCl, followed by 850 mU of crude β-1,3-GnT preparation from bovine serum. The mixture was incubated for 90 h at 40°C and the reaction was terminated by boiling for 5 min. The precipitate was removed by centrifugation (12,000 rpm, 20 min), and the supernatant was loaded onto a charcoal-Celite column (Ø 4.4 × 87 cm) equilibrated with H_2O. The column was eluted with 0 (4 L)-40 (4 L) % EtOH of linear gradient, and the effluent solution was monitored by measuring the absorbances at 210 nm (*N*-acetyl group) and 485 nm (neutral sugar content, phenol-sulfuric acid method). As shown in Figure 1, the chromatogram showed three peaks (F-1, tubes 13–30; F-2, tubes 31–52; F-3, tubes 75–100). The third peak, which displayed coincident absorptions of 210 and 485 nm, was presumed to contain the transfer product. The F-3 fraction was combined, concentrated, and lyophilized to yield lacto-*N*-triose II (106.3 mg). F-1 and F-2 contained GlcNAc and lactose, respectively.

^{1}H-NMR data (D_2O) of lacto-*N*-triose II: δ 5.21 (d, *J* 4.0 Hz, H-1α), 4.68 (d, *J* 8.6 Hz, H-1″α), 4.67 (d, *J* 8.6 Hz, H-1″β, 4.65 (d, *J* 8.0 Hz, H-1β, 4.43 (d, 1H, *J* 7.9 Hz, H-1′), 4.14 (d, 1H, *J* 3.1 Hz, H-4′), and 2.03 (s, 3 H, NHCOCH_3). The ^{13}C-NMR signals of F-3 were assigned by HSQC and by comparing with the data for methyl and *p*-nitrophenyl β-lacto-*N*-triosides II [13]. ^{13}C-NMR data (D_2O) of lacto-*N*-triose II: δ 177.81 (C=O), 105.77 (C-1′β, 105.74 (C-1′α), 105.69 (C-1″), 98.55 (C-1β, 94.66 (C-1α), 84.80 (C-3′β, 84.77 (C-3′α), 81.22 (C-4α), 81.12 (C-4β, 78.51 (C-5″), 77.74 (C-5′), 77.64 (C-3β, 77.20 (C-2β, 76.64 (C-5β, 76.41 (C-3″), 74.24 (C-3α), 73.97

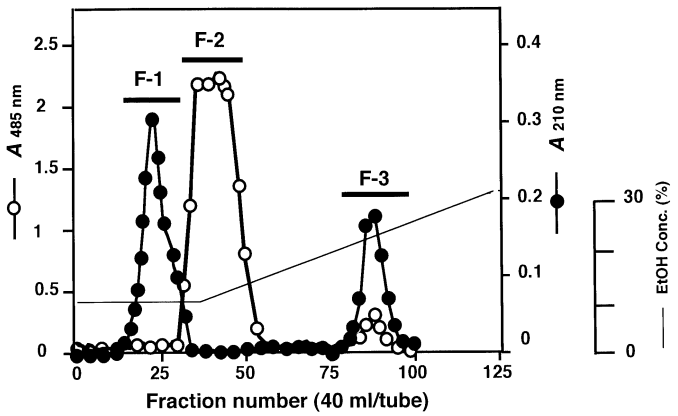


Figure 1. Charcoal-Celite chromatography of transglycosylation product formed from UDP-GlcNAc and lactose by β-1,3-GnT from bovine serum. ○, absorbance at 485 nm; ●, absorbance at 210 nm. F-1, lactose; F-2, GlcNAc; F-3; lacto-*N*-triose II.

(C-2α), 72.97 (C-5α), 72.88 (C-2′α), 72.85 (C-2′β, 72.53 (C-4″), 71.22 (C-4′α), 71.19 (C-4′β, 63.81 (C-6′), 63.33 (C-6″), 62.93 (C-6β, 62.80 (C-6α), 58.51 (C-2″), 25.00 ($NHCOCH_3$). Other physiological data of lacto-*N*-triose II were almost identical to the data reported previously [14].

Preparation of LNT

o-Nitrophenyl β-D-galactoside (60 mg) and lacto-*N*-triose II (36 mg) were dissolved in 3 ml of 40 mM sodium acetate buffer (pH 5.5), followed by 250 mU of β-gal-3. The mixture was incubated for 11 h at 50°C and the reaction was terminated by boiling for 5 min. The resulting insoluble material was removed by centrifugation (5,000 rpm, 15 min), and the supernatant was loaded onto a Chromatorex-ODS DM 1020T column (Ø 1.0 × 57 cm) equilibrated with 5% MeOH in order to remove the *o*-nitrophenol liberated during the reaction. The column was eluted with the same solution. The eluate fractions (26–55 ml) showing absorbance at 485 nm were combined and concentrated to a small volume (15 ml). The concentrate was loaded onto a charcoal-Celite column (Ø 2.2 × 28 cm) equilibrated with H_2O. The column was washed with 150 ml of H_2O, 300 ml of 7.5% EtOH, and then eluted with 7.5 (1.0 L)-35 (1.0 L) % EtOH of linear gradient and the eluate was collected in 10-ml fractions. The effluent solution was monitored by measuring the absorbance at 210 nm. As shown in Figure 2, the chromatogram contained two peaks (F-1, tubes 43–65; F-2, tubes 84–102). The F-2 fraction was combined, concentrated, and lyophilized to give 16.3 mg. It was further treated with *B. circulans* β-D-galactosidase (Biolacta) in order to selectively hydrolyze undesired LNT isomer in the product mixture. Thus, the F-2 was dissolved in 16.3 ml of 50 mM sodium phosphate buffer (pH 7.0) containing the β-D-galactosidase (316 U) and incubated for 21 h at 40°C. The reaction was terminated by boiling for 5 min. Then the solution was rechromatographed on a charcoal-Celite column (Ø 2.2 × 10 cm) equilibrated with H_2O. The column was eluted with 100 ml of H_2O and 60 ml of 7.5% EtOH, and then eluted with 7.5 (300 ml)-35 (300 ml) % EtOH of linear gradient. The F-2′ fraction (276–290 ml) showing absorption at 210 nm was combined, concentrated, and lyophilized to yield 7.1 mg. The F-1 fraction contained lacto-*N*-triose II (16 mg) used as an acceptor substrate. The ^{1}H- and ^{13}C-NMR data of F-2′ were identical to those of LNT reported previously [15]. Other physiological data of LNT were almost identical to the data of F-2′ reported previously [16].

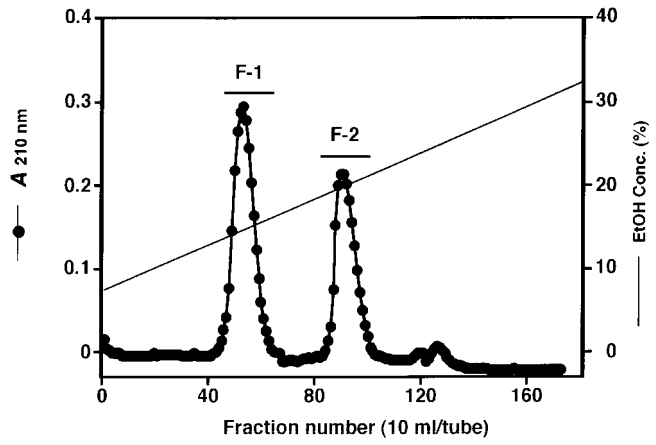


Figure 2. Charcoal-Celite chromatography of transglycosylation products formed from Galβ-*o*NP and lacto-*N*-triose II by β-gal-3. ●, absorbance at 210 nm. F-1, lacto-*N*-triose II; F-2, LNT.

Preparation of LNnT

Lactose (30 mg) and lacto-*N*-triose II (50 mg) were dissolved in 1.8 ml of 20 mM sodium acetate buffer (pH 6.0) followed by 250 m U of β-D-galactosidase (Biolacta). The mixture was incubated for 36 h at 40°C and the reaction was terminated by boiling for 5 min. The resulting insoluble material was removed by centrifugation (12,000 rpm, 15 min), and the supernatant was loaded onto a charcoal-Celite column (Ø 4.5 × 50 cm) equilibrated with H_2O. The column was washed with 800 ml of H_2O followed by 800 ml of 20% EtOH, and then eluted with 20 (4.0 L)-40 (4.0 L) % EtOH of linear gradient. The effluent solution was monitored by measuring the absorbances at 210 and 485 nm. The chromatogram showed two peaks (F-1, 330–1040 ml; F-2, 1080–2880 ml). The F-2 fraction was combined and concentrated to small volume (1.5 ml). The concentrate was loaded onto a Bio-Gel P-2 column (Ø 2.5 × 90 cm) and the eluate was collected in 4-ml fractions. The chromatogram showed three peaks (F-2a, 256–288 ml; F-2b, 328–360 ml; and F-2c, 376–392 ml). The F-2b fraction, which displayed coincident absorptions of 210 and 485 nm, was combined, concentrated, and lyophilized to yield 12 mg. F-2c contained lacto-*N*-triose II (19 mg) used as the acceptor substrate. The ^{1}H- and ^{13}C-NMR data of F-2b were identical to those of LNnT reported previously [15]. Other physiological data of F-2b were almost identical to the LNnT data reported previously [16].

Direct conversion of lactose into LNT

Lactose (3425 mg) and Galβ1-3GlcNAcβ-*p*NP (500 mg) were dissolved in 55 ml of 40 mM sodium acetate buffer (pH 5.5), followed by 20 U of lacto-*N*-biosidase from *Aureobacterium* sp. L-101. The mixture was incubated for 5 h at 40°C and the reaction was terminated by boiling for 5 min. The resulting insoluble material was removed by centrifugation (12,000 rpm, 15 min), and the supernatant was loaded onto a Chromatorex-ODS DM 1020T column (Ø 3.0 × 60 cm) equilibrated with 20% MeOH in order to remove the *o*-nitrophenol liberated during the reaction. The column was eluted with the same solution. The eluate fractions (181–510 ml) showing absorbance at 210 nm were

combined and concentrated to a small volume (30 ml), and the solution was loaded onto a charcoal-Celite column (Ø 5.5 × 58 cm) equilibrated with H_2O. The column was washed with 500 ml of H_2O followed by 3 L of 7.5% EtOH, and then eluted with 7.5 (3.0 L)-35 (3.0 L) % EtOH of linear gradient. The effluent solution was monitored by measuring the absorbance at 210 nm. The chromatogram showed two peaks (F-1, 3160–3480 ml; F-2, 3643–3800 ml). The F-2 fraction was combined and concentrated to small volume (1.5 ml). The concentrate was loaded onto a CarboPac P-1 column (Dionex, Ø 4 × 250 mm) as mentioned above. The main peak (18–21 min) was neutralized with 1 N acetic acid, desalted by a Micro Acilyzer G1 with an AC-220-10 cartridge (Asahikasei Co. Ltd., Kawasaki, Japan), concentrated, and lyophilized to yield LNT (4 mg). The ^{1}H- and ^{13}C-NMR data were identical to those of LNT reported previously [15]. Other physiological data of LNT were almost identical to the data reported previously [16].

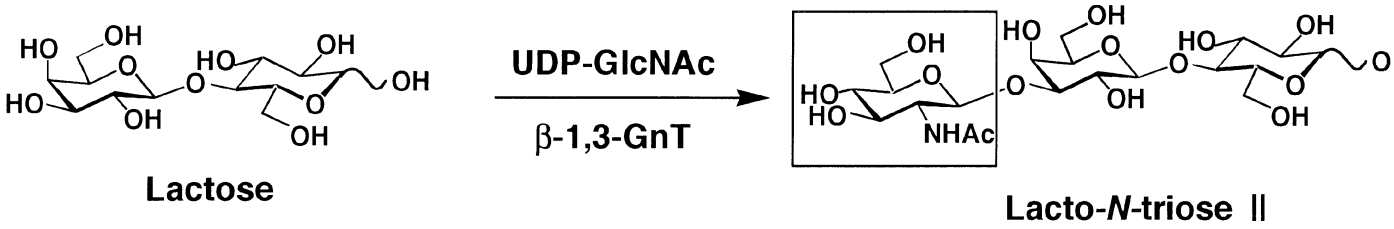
Results and discussion

Facile enzymatic conversions of lactose into LNT and LNnT were developed with the use of two sequential enzymatic reactions, respectively. Direct conversion of lactose into LNT was then performed enzymatically.

Sequential conversion of lactose into LNT and LNnT

*Synthesis of lacto-*N*-triose II*

In our previous study, *p*-nitrophenyl lacto-*N*-trioside II was synthesized from *N, N′*-diacetylchitobiose and Galβ1-4Glcβ-*p*NP using trans-*N*-acetylglucosaminylation catalyzed by β-*N*-acetyl-D-hexosaminidase from *Nocardia orientalis*. It resulted in only a low yield of the desired compound, because of the low regioselectivity of the hydroxyl linkages to the acceptor [13]. On the other hand, β-1,3-GnT, which catalyzes the transfer of the GlcNAc residue of UDP-GlcNAc to the OH-3′ position of lactose and LacNAc, has been found in Novikoff ascites tumor cells [17], human serum [18,19] and colostrum [20], bovine serum [20], and rat rete testis [21]. Recently, cDNA encoding a human β-1,3-GnT, which is essential for poly-*N*-acetyllactosamine synthesis, has been isolated by expression cloning [22]. The enzymatic method for obtaining lacto-*N*-triose II was first reported on the μmolar scale using β-1,3-GnT from human serum [23]. The object of the present investigation is to develop a convenient enzymatic procedure suitable for large-scale preparation of lacto-*N*-triose II. Sufficient amounts of β-1,3-GnT and UDP-GlcNAc are needed for the practical synthesis. In this case, β-1,3-GnT is easily obtained from bovine serum and UDP-GlcNAc has become commercially available in large amounts in recent years. The enzyme used in the present study was a crude product prepared as a 25–50% saturated ammonium sulfate precipitation. The enzyme catalyzed the transfer of a GlcNAc moiety to the OH-3′ position of the lactose unit as mentioned above (Scheme 1), in a 26% yield based on the donor substrate. Lacto-*N*-triose II was readily synthesized on a mmolar scale and conveniently isolated by charcoal-Celite chromatography (Fig. 1). The time course of lacto-*N*-triose II production was examined by the HPAEC-PAD method. Maximum production was observed after 50 h and the concentration varied little during the subsequent reaction. The efficiency of transglycosylation was very dependent on pH, because the enzyme activity was considerably weakened in the acidic region of pH 5. As a result, the supply of a sufficient amount of lacto-*N*-triose II led to the practical syntheses of LNT and LNnT as mentioned below.

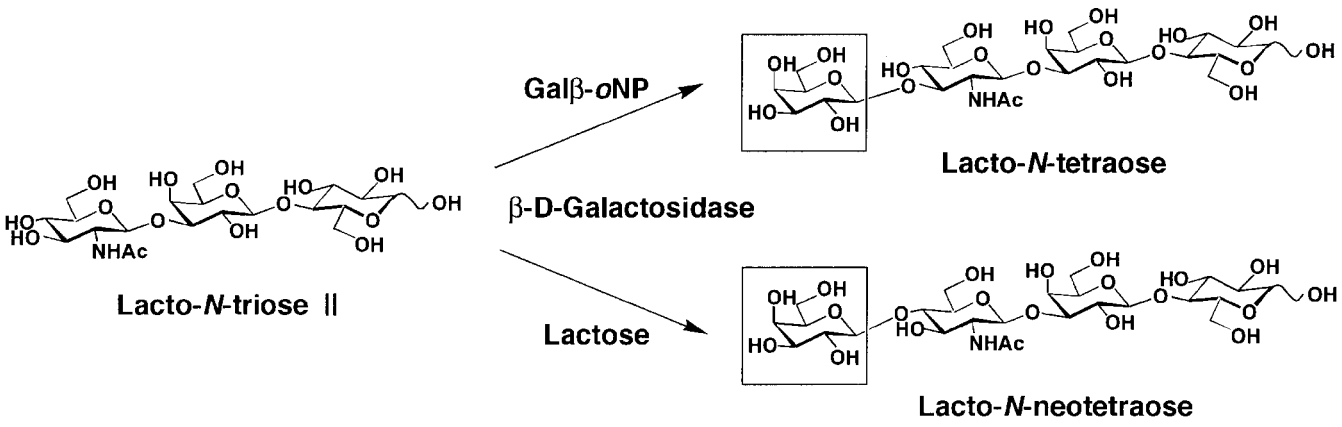


Synthesis of LNT and LNnT

Fujimoto *et al.* have reported that β-gal-3 induces a preponderant β-D-Gal transfer from *p*-nitrophenyl β-D-galactopyranoside to the OH-3 position of GlcNAc, GalNAc, and their glycosides [24]. The methodology was extended to the regioselective synthesis of LNT. When lacto-*N*-triose II was used as an acceptor and Galβ-*o*NP as a donor, the enzyme catalyzed the formation of LNT in a yield of 20% based on the acceptor added (Scheme 2). LNT was separated by successive chromatography on ODS and charcoal-Celite columns as in Figure 2. Unreacted lacto-*N*-triose II acceptor, which is very valuable substance, was recovered by straightforward chromatography and reutilized for the synthesis. The resulting LNT fraction was still contaminated by its β-(1 → 6) isomer (Fig. 3a). This isomer was therefore selectively removed by hydrolytic treatment with β-D-galactosidase from *B. circulans* (Biolacta), which does not hydrolyze β-(1 → 3) linked galactosyl-oligosaccharide.

When the lyophilized fraction was incubated in a 0.1% concentration with the enzyme, the isomer was hydrolyzed completely after 21 h. The hydrolyzate was then rechromatographed on a charcoal-Celite column, enabling the selective removal of the isomer from the LNT fraction (Fig. 3b).

The formation of LNT and the degradation of Galβ-*o*NP donor were directly proportional to time within 8 h (Fig. 4). The maximum LNT production was reached at 8 h and its concentration then varied little during the subsequent reaction.



LNnT was similarly synthesized from lacto-*N*-triose II as an acceptor and lactose as a donor substrate by means of *B. circulans* β-D-galactosidase (Biolacta)-mediated transgalactosylation. We have already reported that the enzyme is quite useful for β-(1 → 4) galactosyl transfer to some acceptor sugars [25–27]. The enzyme also catalyzed the regioselective transgalactosylation from lactose onto OH-4″ of lacto-*N*-triose II as mentioned above (Scheme 2). The molar ratio of the donor and the acceptor was about 1 : 1, and the total substrate concentration was about 4.4%. No significant amounts of positional isomers were detected during the reaction. LNnT was easily separated by successive chromatography on charcoal-Celite and Bio-Gel P-2 columns. As a result, LNnT was prepared with a 19% yield based on the acceptor. The same highly regioselectivity of the enzyme has been observed also in some acceptors: *N*, *N*′-diacetylchitobiose, *N*, *N*′, N″-triacetylchitotriose, lactose [28] and *N*-acetyllactosamine derivatives [29]. In contrast, when monosaccharide (Gal and Glc) glycosides were used as acceptor substrates, the regioselectivity of the enzyme was substantially decreased. The galactosylation occurred not only preferentially to the OH-4 position of acceptors but also to the OH-6 and -3 positions [27,30]. The change in

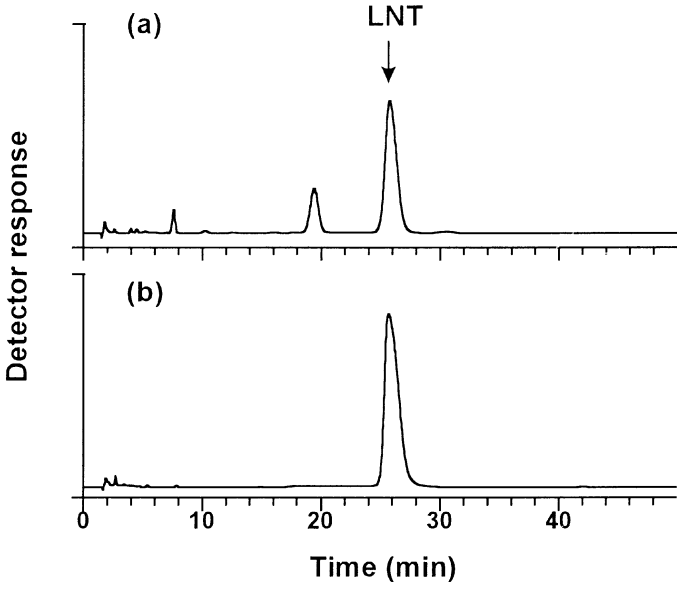


Figure 3. HPAEC-PAD analyses of transglycosylation products formed from Galβ-*o*NP and lacto-*N*-triose II by β-gal-3. The HPAEC-PAD profiles of the LNT fraction (F-2) obtained by charcoal-Celite chromatography (a), and the LNT fraction (F-2′) obtained by hydrolytic treatment with β-D-galactosidase (Biolacta) and charcoal-Celite chromatography (b).

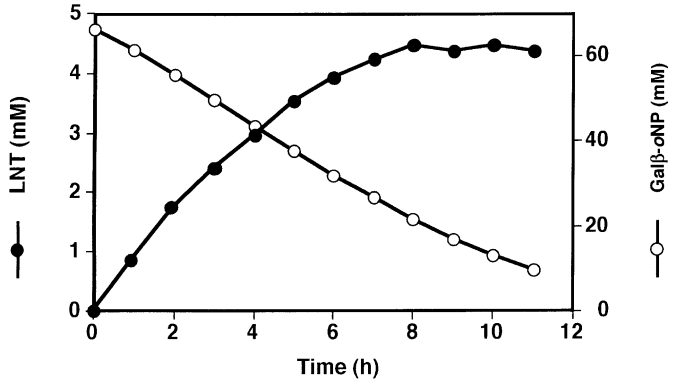


Figure 4. Time course of the formation of LNT and the degradation of Galβ-*o*NP. Sixty milligrams of Galβ-*o*NP and 36 mg of lacto-*N*-triose II were incubated at 50°C in 3 ml of 40 mM Na-Ac. buf. (pH 5.5) with 250 mU of β-gal-3. ●, concentration of LNT; ○, concentration of Galβ-*o*NP.

this regioselectivity seems to be due to differences in the size of the acceptor substrate.

Conversion of lactose into LNT

Many exoglycosidases have been shown to be potential tools for the synthesis of oligosaccharides [31–33]. In contrast, only a limited number of reports are available on the transglycosylation activities of endoglycosidases [34–38]. Commercially available lacto-*N*-biosidase as an endoglycosidase, that specifically hydrolyzes lacto-*N*-biosidic linkages in internal oligosaccharide chains [39,40] was first isolated from *Streptomyces* sp. 142. We also found a high activity of the enzyme in the culture fluid of *Aureobacterium* sp. L-101. Both lacto-*N*-biosidases acted on synthetic Galβ1-3GlcNAcβ-*p*NP to hydrolyze Galβ1-3GlcNAc and *p*-nitrophenol, that is, it can bypass a block of the disaccharide. Therefore, our interest was directed toward an enzymatic conversion of lactose into LNT. As a result, when Galβ1-3GlcNAcβ-*p*NP was used as a donor and lactose was used as an acceptor, the enzyme from *Aureobacterium* sp. L-101 catalyzed the transfer of a β-lacto-*N*-biosyl unit to the OH-3′ position of lactose as mentioned above (Scheme 3). The resulting LNT was roughly separated by successive chromatography on ODS Chromatorex DM1020T and charcoal-Celite columns and complete separation was finally effected by HPAEC-PAD. The actual yield was very low (0.6% based on the donor substrate). The time course of LNT production and the degradation of donor substrate could be observed by HPAEC-PAD, as shown in Figure 5. The maximum LNT production was reached at 3 h and its concentration rapidly decreased during the subsequent reaction. The yield of LNT at 3 h was observed by analysis to be 3.7% based on the donor. The large difference in the yields between the actual and analytical data should be accompanied by a loss of LNT through the process of three chromatographic separation procedures. This is the first report that lacto-*N*-biosidase-mediated transglycosylation enabled the direct synthesis of LNT from lactose as an initial substrate, although it was rather inefficient.

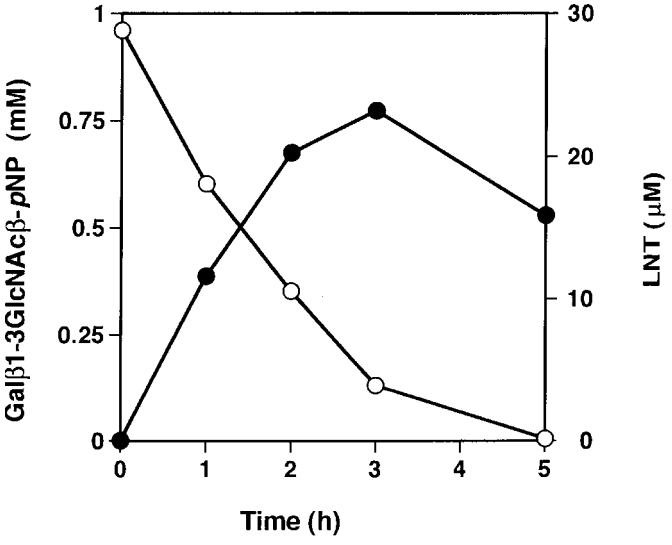


Figure 5. Time course of the formation of LNT and the degradation of Galβ1-3GlcNAcβ-*p*NP. Four milligrams of Galβ1-3GlcNAcβ-*p*NP and 27.4 mg of lactose were incubated at 40°C in 300 μl of 40 mM Na-Ac. buf. (pH 5.5) with 200 mU of lacto-*N*-biosidase. ●, concentration of LNT; ○, concentration of Galβ1-3GlcNAcβ-*p*NP.

Conclusion

We developed facile conversion of lactose into LNT and LNnT by consecutive use of β-1,3-GnT and β-D-galactosidase-mediated transglycosylations, respectively (Fig. 6). The yields (19–26 %) were considered to be sufficiently high for a practical method, because of the simplicity of enzymatic syntheses. Excess of unreacted substrates are also recovered by straightforward column chromagtogra-

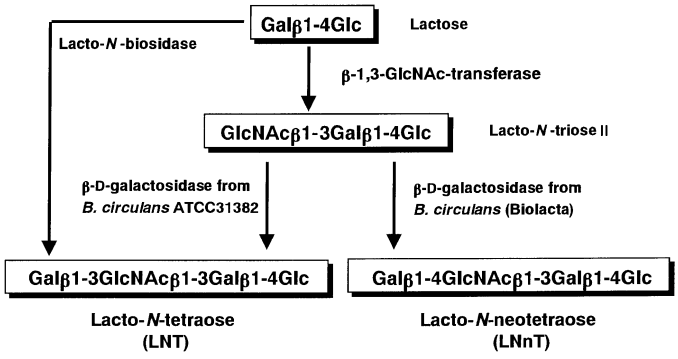


Figure 6. Enzymatic conversion of lactose into LNT and LNnT.

phy and reutilized for the synthesis. Furthermore, we found a system for transfer of the lacto-*N*-biosyl unit to the OH-3′ position of lactose, utilizing lacto-*N*-biosidase from *Aureobacterium* sp. L-101, but its efficiency was very low. Such well defined oligosaccharides would be useful as substrates for glycosidases and glycosyltransferases involved in biosynthetic routes for forming glycoconjugates, as probes for lectin, and as common synthetic intermediates of carbohydrate antigens, glycoconjugates, and milk oligosaccharides. The synthetic oligosaccharides are also useful as starting substances for glycopolymers [41–43], which are valuable tools for investigating biological recognition phenomena.

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References

1 Macher BA, Sweeley CC (1978) *Meth Enzymol* **50:** 236–50.
2 Tsai CM, Civin CI (1991) *Infect Immun* **59:** 3604–9.
3 Wakarchuk W, Martin A, Jennings M, Moxon ER, Richards CR (1996) *J Biol Chem* **271:** 19166–73.
4 Kobata A (1978) *Meth Enzymol* **50:** 263–71.
5 Koenderman AHL, Koppen PL, Van den Eijnden DH (1987) *Eur J Biochem* **166:** 199–208.
6 Feizi T, Solomon JC, Yuen CT, Jeng KCG, Frigeri LG, Hsu DK, Liu FT (1994) *Biochemistry* **33:** 6342–49.
7 Yu H, Sipes JM, Cashel J, Bakos MA, Goldblum RM, Roberts DD (1995) *Arch Biochem Biophys* **322:** 299–305.
8 Takamura T, Chiba T, Ishihara H, Tejima S (1980) *Chem Pharm Bull* **28:** 1804–9.
9 Ito Y, Sato M, Mori M, Ogawa T (1988) *J Carbohydr Chem* **7:** 359–76.
10 Kameyama A, Ishida H, Kiso M, Hasegawa A (1990) *Carbohydr Res* **200:** 269–85.
11 Ito Y, Sasaki T (1997) *Biosci Biotech Biochem* **61:** 1270–6.
12 Murata T, Itoh T, Hayakawa Y, Usui T (1996) *J Biochem* **119:** 575–82.
13 Matahira Y, Tashiro A, Sato T, Kawagishi H, Usui T (1995) *Glycoconj J* **12:** 664–71
14 Aspinall GO, Percival E, Rees DA and Rennie M (1967) In *Rodd's Chemistry of Carbon Compounds,* vol. I part F, p. 657.
15 Strecker G, Wieruszeski J-M, Michalski J-C and Montreuil J (1988) *Glycoconj J* **6:** 67–83.
16 Aspinall GO, Percival E, Rees DA, Rennie M (1967)In *Rodd's Chemistry of Carbon Compounds,* vol. I, part F, p. 663.
17 Van den Eijnden DH, Winterwerp H, Smeeman P and Schiphorst WECM (1983) *J Biol Chem* **258:** 3435–7
18 Piller F, Cartron J-P (1983) *J Biol Chem* **258:** 12293–9
19 Hosomi, O., Takeya, A. and Kogure, T. (1984) *J Biochem* **95:** 1655–9
20 Hosomi O, Takeya A (1989) *Jpn J Vet Sci* **51:** 1–6.
21 Tsuji Y, Urashima T, Matsuzawa T (1996) *Biochim Biophys Acta* **1289:** 115–21.
22 Sasaki K, Kurata-Miura K, Ujita M, Angata K, Nakagawa S, Sekine S, Nishi T, Fukuda M (1997) *Proc Natl Acad Sci USA* **94:** 14294–9.
23 Yates AD, Watkins WM (1983) *Carbohydr Res* **120:** 251–68.
24 Fujimoto H, Miyasato M, Ito Y, Sasaki T, Ajisaka K (1998) *Glycoconj J* **15:** 155–60.
25 Usui T, Kubota S, Ohio H (1993) *Carbohydr Res* **244:** 315–23.
26 Usui T, Ogawa K, Nagai H, Matsui H (1992) *Anal Biochem* **202:** 61–7.
27 Usui T, Morimoto S, Hayakawa Y, Kawaguchi M, Murata T, Matahira Y, Nishida Y (1996) *Carbohydr Res* **285:** 29–39.
28 Singh S, Scigelova M, Vic G, Crout DHG (1996) *J Chem Sci Perkin Trans* **1:** 1921–6.
29 Takayama S, Shimazaki M, Qiao L, Wong CH (1996) *Bioorg Med Chem Lett* **6:** 1123–6.
30 Murata T, Itoh T, Usui T (1998) *Glycoconj J* **15:** 575–82.
31 Murata T, Usui T (1997) *Biosci Biotech Biochem* **61:** 1059–66.
32 Ichikawa Y, Look GC, Wong CH (1992) *Anal Biochem* **20:** 215–38.
33 Ichikawa Y (1997) *Trends Glycosci Glycotech* **9:** S47–S59.
34 Trimble R, Atkinson P, Tarentino A, Plummer TJ, Maley F, Tomer K (1986) *J Biol Chem* **261:** 12000–5.
35 Bardales R, Bhavanandan V (1989) *J Biol Chem* **264:** 19893–7.
36 Takegawa K, Yamaguchi S, Kondo A., Kato I, Iwahara S (1991) *Biochem Int* **25:** 829–35.
37 Yamamoto K, Kadowaki S, Watanabe J, Kumagai H (1994) *Biochem Biophys Res Commun* **203:** 244–52.
38 Saitoh H, Takagaki K, Majima M, Nakamura T, Matsuki A, Kasai M, Narita H, Endo M (1995) *J Biol Chem* **270:** 3741–7.
39 Falk P, Hoskins L, Larson G (1990) *J Biochem* **108:** 466–74.
40 Sano M, Hayakawa K, Kato I (1993) *J Biol Chem* **268:** 18560–6.
41 Tsuchida A, Akimoto S, Usui T Kobayashi K (1998) *J Biochem* **123:** 715–21.
42 Zeng X, Murata T, Kawagishi H, Usui T Kobayashi K (1998) *Biosci Biotech Biochem* **62:** 1171–8.
43 Kobayashi K, Kamiya S, Matsuyama M, Murata T Usui T (1998) *Polymer J* **30:** 653–8.

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