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Efficient chemoenzymatic synthesis of globotriose and its derivatives with a recombinant α-(1→4)-galactosyltransferase

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Abstract

A truncated α-(1→4)-galactosyltransferase (LgtC) gene from *Neisseria meningitidis* was cloned. The recombinant glycosyltransferase was expressed in *Escherichia coli* BL21 (DE3) strain with high specific activity (5 units/mg protein). Its acceptor specificity was carefully characterized. Then the purified enzyme was utilized in highly efficient syntheses of globotriose and a variety of α-(1→4)-galactosylated derivatives as potential antibacterial agents. © 2002 Published by Elsevier Science Ltd.

Keywords: Globotriose; Chemoenzymatic synthesis; LgtC; α-(1→4)-Galactosyltransferase; Recombinant enzyme

1. Introduction

Carbohydrates are now recognized as being of major importance in a great variety of physiological and pathological processes.[1–3] In fact, glycans adorning the cells are involved in extensive recognition phenomena including development, differentiation, morphogenesis, fertilization, implantation, infection, cancer metastasis, etc.[4] For example, *Escherichia coli* O 157, the so-called "hamburger bug", is responsible for serious, and sometimes deadly, outbreaks of food poisoning.[5–7] As part of its infectivity strategy, *E. coli* O 157 produces a toxin (verotoxin) that binds to the carbohydrate globotriose [α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc, Fig. 1], which is found as a glycolipid, globotriaosylceramide [α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc-β-(1→Cer] in the membranes of many cells. Following binding of the toxin to globotriaosylceramide, it is transported through the membrane, brings protein synthesis to a halt, and kills the cell. Globotriaosylceramide is distributed in many tissues of the body and is particularly abundant on kidney cells. Kidney damage, as a side effect of *E. coli* O 157 infections, is especially a danger to children and the elderly. Additionally, globotriose found in the lipooligosaccharides (LOS) of the bacterial pathogens *Neisseria meningitidis* immunotype L1 and *N. gonorrhoeae*, participates in the invasion of these pathogens into mammalian cells.[8,9] Thus, globotriose and its synthetic derivatives could be used as effective inhibitors of pathogen invasion, thereby having important pharmaceutical potential in both experimental and clinical applications.[10–13]

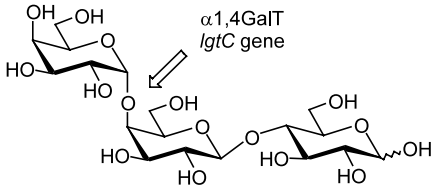

Fig. 1. Structure of globotriose [α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc] and the linkage installed by the α1,4-GalT.

Several reports exist of both chemical and enzymatic efforts on the synthesis of globotriose trisaccharide since its direct isolation from natural sources is impractical. Chemical syntheses of the free trisaccharide and globotriaosylceramide require lengthy protection and deprotection procedures.[14–22] The most difficult step in the chemical approach is the efficient construction of the α-(1→4)-linkage between two galactosyl residues.

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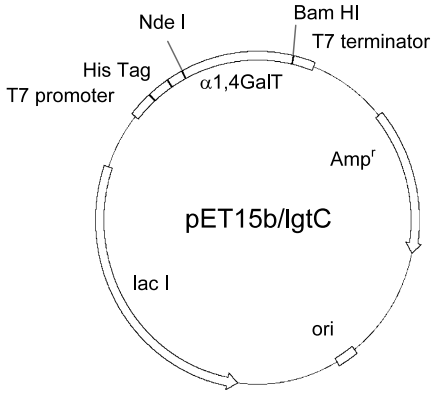


Fig. 2. Genetic map of α-(1→4)-galactosyltransferase expression plasmid.

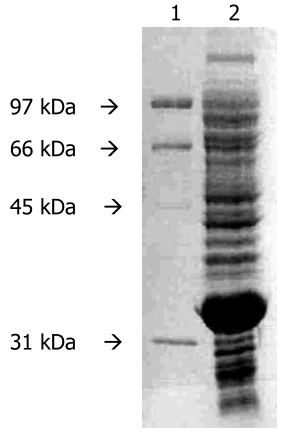


Fig. 3. SDS–PAGE of crude lysate from BL21 (DE3) cells expressing LgtC. Lane 1, molecular weight markers; lane 2, cell lysate.

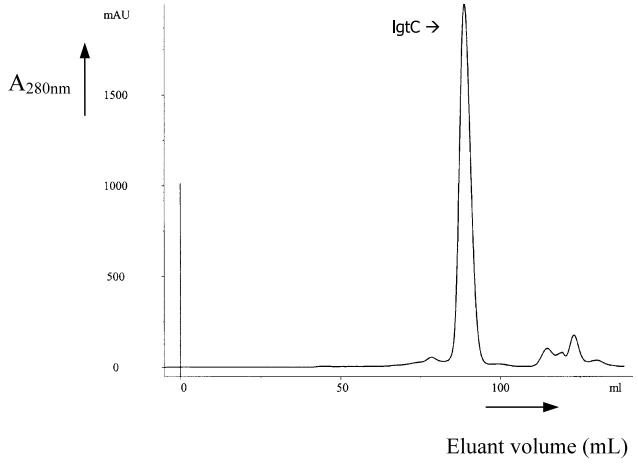


Fig. 4. FPLC elution trace for Ni–NTA purified LgtC.

The 4′-OH of the lactosyl residue is the most inert hydroxyl group in the molecule due to electrosteric effects, and the stereoselective construction of the 1,2-cis glycosyl linkage remains problematic.

Glycosyltransferases of the Leloir pathway are catalysts with high substrate specificity and absolute stereo- and regioselectivity for the glycosidic linkages. However, practical use of the Leloir pathway in oligosaccharide synthesis depends on the availability of the glycosyltransferases as well as the sugar nucleotide donor substrates. Although cloning and characterization of α-(1→4)-galactosyltransferase (α1,4-GalT) has been reported,[23,24] and globotriose has been synthesized with α1,4-GalT or engineered microorganisms,[12,25,26] no systematic synthesis of the derivatives of the trisaccharide utilizing a recombinant enzyme has been accomplished. Here we report a facile method for the synthesis of globotriose and its derivatives with LgtC, an α1,4-GalT.

2. Results and discussion

Cloning and expression of LgtC.—Full-length *N. meningitidis* LgtC is a 36-kDa protein. Previous studies have found that C-terminal proteolysis was encountered during purification and that the last 28 residues of LgtC are not essential for expression or activity of the enzyme.[23] A 25-residue C-terminal deletion variant has been shown to result in highest expression levels and was used in this study. The truncation was introduced at the genetic level. At first, the gene was amplified by polymerase chain reaction (PCR) from *N. meningitidis* MC58 chromosomal DNA. Then the appropriately digested PCR fragment was inserted in-frame with the hexahistidine tag-encoding region of the pET15b vector (Fig. 2).

The recombinant enzyme was expressed in *E. coli* BL21 (DE3) transformed with pET15b/LgtC. Expression of the LgtC was induced with 0.2 mM isopropyl 1-thio-β-D-galactospyranoside (IPTG), initially at 37 °C. Subsequent large-scale enzyme expression was carried out at 30 °C, as this temperature resulted in higher yields of soluble protein. SDS–PAGE indicates that LgtC-25 is expressed as 65% of all soluble protein after a 15 h induction (Fig. 3).

Following induction, the pelleted cells were suspended in 100 mL of 50 mM Tris–HCl, pH 8.5 buffer, lysed on ice by adding lysozyme to 100 μg/mL, and then centrifuged to remove unbroken cells and debris. The lysate was applied to an immobilized metal affinity column (IMAC), with Ni^{2+} as the cation. Single step Ni–NTA (nickel–nitrilotriacetic acid) affinity purification resulted in 92% purity as judged by FPLC analysis. In addition, the FPLC results indicated that LgtC is a monomer under our buffer conditions (Fig. 4).

About 300 units of LgtC activity could reproducibly be obtained from 1 L of bacterial culture (one unit of enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of galactose from

UDP-Gal to lactose per min at 37 °C). The purified enzyme has a molecular weight of 32 kDa by SDS–PAGE with a specific activity of 5 U/mg using the Lowry method[27] (data not shown). Thus, this expression system provides access to a large quantity of α-1,4GalT essential for the enzymatic synthesis of α-galactosyl oligosaccharides.

Substrate specificity of LgtC.—In nature, LgtC mediates the transfer of galactose from UDP-galactose to D-lactose terminated glycolipids. Wakarchuk et al. have reported that LgtC exhibits a fivefold higher reaction rate with lactose-terminated versus galactose-terminated FCHASE-AP.[23] Thus to facilitate the enzymatic synthesis of globotriose and, more importantly, their derivatives, we set out to study the acceptor specificity of LgtC.

To determine the optimal reaction conditions and achieve high-yielding production, the effects of buffer, metal ion, and thiol were investigated. Although the activity assay for LgtC was previously performed in HEPES–NaOH buffer at pH 7.5,[23] our assays were conducted in Tris–HCl buffer at pH 7.0 for the compatibility with recombinant UDP-Gal 4-epimerase (GalE, EC 5.1.3.2).[28] Our results indicate that there is no significant difference for LgtC activities in these buffers, and Tris salt is much easier removed from the reaction mixture with gel-permeation chromatography. We confirmed a previous report that Mn^{2+} increases LgtC activity due to stabilizing the UDP part during the bond cleavage.[24] Moreover, LgtC needed to be activated by incubation with DTT prior to use in synthesis.[23,29]

Twenty-six compounds, including derivatives of lactose, galactose, and glucose were assayed as potential unnatural acceptors for our α1,4-GalT. Unlike the recombinant α1,3-GalT, which can accept a wide range of substrates,[30] LgtC is active towards a narrower spectrum of acceptors (Table 1). β-D-Gal-(1→4)-D-Glc disaccharide derivatives (entries 2–4) were good acceptors for LgtC. Compared to lactose (100%), the enzyme activities for these compounds ranged from 19% to 104%. Apparently the activity was affected by the nature of the aglycons (OMe > OH > SPh > OBn) linked to the lactosyl residue.

Synthesis of globotriose and its derivatives.—Enzyme purified on a Ni–NTA affinity column has been employed in the synthesis of galactose-terminated saccharides. However, the high cost of UDP-galactose donor has significantly limited the application of this transferase in large-scale synthesis of galactosyl oligosaccharides. A cheaper alternative to UDP-galactose is UDP-glucose, which can be converted to UDP-galactose using GalE. Recently, we have successfully cloned and overexpressed GalE,[28] and used it to generate UDP-galactose. We have previously demonstrated that the optimal pH for GalE activity was 7.0 in Tris–HCl buffer, the same as for LgtC in this study. Therefore, the reactions catalyzed by two recombinant enzymes were carried out at pH 7.0 in 10 mM Tris–HCl buffer (Scheme 1).

Using the recombinant enzyme, globotriose and a variety of derivatives were synthesized on preparative scale. The acceptors that were used in this study include lactose **1a**, methyl β-lactoside (**1b**), β-thiophenyl lactoside (**1c**), β-D-Gal-(1→3)-D-AraOH (**1d**), benzyl β-lactoside (**1e**), lactulose (**1f**), lactitol (**1g**), and methyl β-D-galactoside (**1h**) (Table 2). Acceptors bearing a β-D-Gal terminus afforded good reaction results, consistent with the acceptor specificity studies. Methyl β-D-galactoside is not a good substrate ($< 5\%$ conversion). The reaction products were characterized by NMR spectroscopy and mass spectrometry (MS).

Spectrometric analysis of enzymatic products.—Large-scale incubations yielded sufficient amounts of galactosylated products to allow structural analysis by ^{1}H and ^{13}C NMR spectroscopy. In the spectra of the product obtained in the incubation of lactose and UDP-Gal, the chemical shift of the new anomeric hydrogen at 4.78 ppm, as well as the chemical shift of 100.46 ppm for anomeric carbon, indicated clearly that a Gal residue was attached to the acceptor molecule. A coupling constant of 4.1 Hz for the H-1 resonance and the chemical shift for the C-1 resonance of Gal showed that this residue was in the α-anomeric configuration. The significant downfield shift of the C-4′ resonance of Gal ($\Delta\delta = 8.5$ ppm), but not in the other resonances, indicates that the Gal residue had been introduced to C-4 of Gal. The α-anomeric configuration of the Gal residue was also evident from the crosspeaks between the anomeric proton and the anomeric carbon of Gal in the HMQC spectrum (data not shown). Hence, the structure of the product was found to be α-D-Gal-(1→4)-β-D-Gal-(1→4)-D-Glc. By similar reasoning, it was concluded that the products of the reaction with lactose derivatives contained the newly formed α-D-Gal-(1→4)-β-D-Gal structure. The product structures were also analyzed by low and high-resolution FABMS showing that one Gal residue was attached to each lactose derivative.

3. Summary

We describe an effective expression system that enables the production of highly active and soluble α1,4-GalT. The recombinant galactosyltransferase was characterized with respect to its acceptor specificity and then applied in the efficient chemoenzymatic syntheses of globotriose and its derivatives. In addition, this work provides the demonstration of the enzymatic formation of novel, unnatural galactosyl oligosaccharides containing the galabiose moiety.

4. Experimental

Materials.—LacSPh, LacOBn, and galactoside derivatives (Compound **7**–**11**, **17**, **22**, **23**, **26** in Table 1) were synthesized previously [Refs. 31–32 and unpublished data]. Ni–NTA agarose resin and DNA miniprep spin kit were from Qiagen (Santa Clarita, CA). Taq DNA polymerase, BamHI, and Nde I were from Fisher Scientific Co. (Pittsburgh, PA). UDP-[galactose-6-^{3}H] was purchased from Amersham. UDP-glucose, UDP-galactose and Dowex 1×8 resin were obtained from Sigma Chemical Co. (St. Louis, MO). Deionized water was obtained with a Water Pro PS system (Labconco, Kansas, MO). All other chemicals are from commercial vendors.

Bacterial strains and plasmids.—T7 promoter and T7 terminator primers, and *E. coli* BL21 (DE3) competent cells [F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm (DE3)*] were from Novagen, Inc. (Madison, WI). *E. coli* DH5α competent cells (*lacZΔM15 hsdR recA*) was from Gibco-BRL Life Technology (Rockville, MD). Plasmid vector pET15b was from Promega Corp. (Madison, WI). Chromosomal DNA of *N. meningitidis* MC58 (L1) was a kind gift from Dr Michel Gilbert (Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada).

Cloning.—The truncated *LgtC* gene was amplified directly from the chromosomal DNA of *N. meningitidis* MC58 (L1) (primers 5′-CGGAATTCATATGGA-CATCGTATTTGCG-3′ and 5′-GCCGGATCCT-

Table 1
Acceptor specificity of LgtC

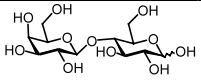
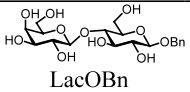
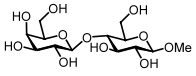
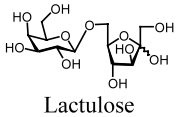
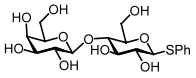
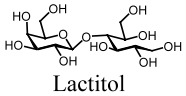
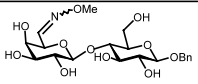
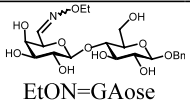
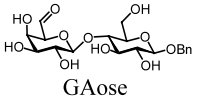
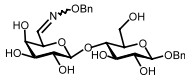
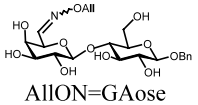
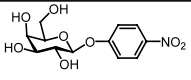
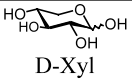
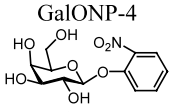
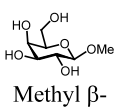
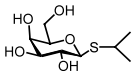
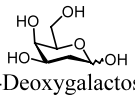
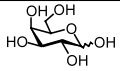
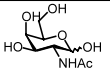
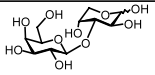
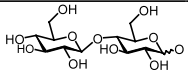
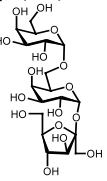
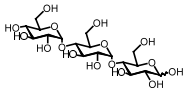
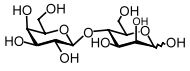
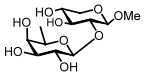
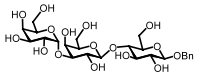
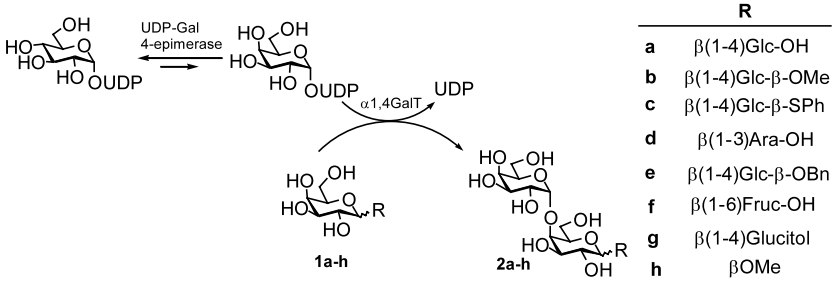
Entry	Acceptor Compound	Relative activity (%)	Entry	Acceptor Compound	Relative activity (%)
		Lactose derivatives			
1	 Lactose	100	**4**	 LacOBn	19.0
2	 LacOMe	104	**5**	 Lactulose	15.1
3	 LacSPh	87.9	**6**	 Lactitol	1.1
		6"-Lactose derivatives			
7	 MeON=GAose	1.86	**10**	 EtON=GAose	<0.1
8	 GAose	<0.1	**11**	 BnON=GAose	<0.1
9	 AllON=GAose	<0.1			
		Monosaccharides and their derivatives			
12	 GalONP-4	1.3	**15**	 D-Xyl	0.75
13	 GalONP-2	1.3	**16**	 Methyl β-galactoside	0.20

Table 1 (*Continued*)

14	 IPTG	1.1	**17**	 2-Deoxygalactose	<0.1
18	 Galactose	0.15	**19**	 GalNAc	<0.1
	Miscellaneous				
20	 Gal-β-(1-3)-AraOH	37.4	**24**	 Cellobiose	0.83
21	 Raffinose	6.5	**25**	 Maltotriose	0.40
22	 Gal-β-(1-4)-ManOH	6.3	**26**	 Methyl β-D-Fuc-β-(1-2)-Xyloside	<0.1
23	 Gal-α-(1-3)LacOBn	1.4			



Scheme 1. General procedure for galactosylation of lactose acceptors (**1a**–**h**) using the two-enzyme system.

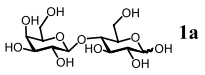
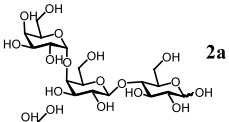
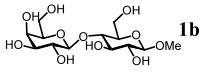
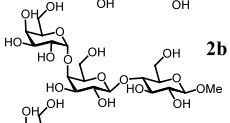
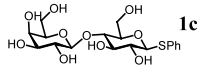
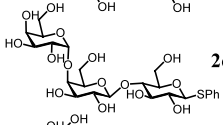
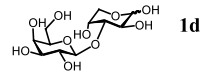
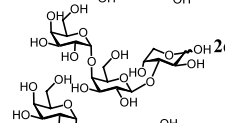
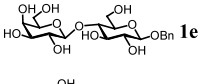
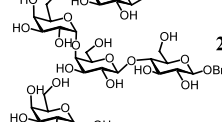
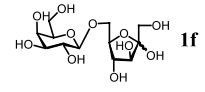
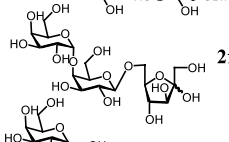
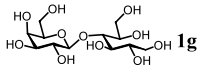
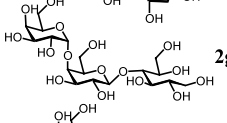
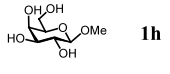
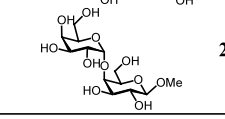
CATCAGTGCGGGACGGCAAGTTTGCC-3′). The truncation involved 25 N-terminal residues. PCR was done in Thermocycler I (Thermolyne, Dubuque, Iowa) using Taq polymerase (30 cycles; 94 °C, 1 min; 50 °C, 1 min; 72 °C, 1.5 min). The PCR product was purified by a QIAquick PCR Purification Kit and a QIAEX II Gel Extraction Kit (Qiagen) and digested with Nde I and BamH I restriction enzymes for insertion into the cloning vector. The digested products were ligated into the multiple cloning site of pET15b plasmid. The resulting plasmid pET15b/LgtC-25 was transformed into *E. coli* DH5α cloning host for amplification. The cells were grown on LB agar plates containing 100 μg/mL ampicillin. Positive clones were identified by restriction mapping and sequencing of the isolated plasmid. Recombinant pET15b/LgtC plasmid with the correct sequence was inserted into the expression host, *E. coli* BL21 (DE3). The cells were then grown in 3 mL LB broth for 8 h and stored at −80 °C as glycerol stocks.

Expression.—A 50-mL pre-culture was grown overnight and then transferred to 1 L of LB broth. The cells were cultured in medium containing 100 μg/mL ampicillin with rapid shaking (250 rpm) at 30 °C in a C25 incubator shaker (New Brunswick Scientific,

Edison, NJ). Growth was monitored by measuring optical density at 600 nm using a Beckman DU-600 spectrometer. When the A_{600} of the culture reached 0.3, IPTG (isopropyl 1-thio-β-D-galactopyranoside) was added to a concentration o0f 200 μM to induce the expression of α-1,4GalT. After 15 h, the cells were harvested by centrifugation at 5000 rpm for 15 min and washed with washing buffer (pH 8.5, 20mM Tris–HCl, 20% sucrose). Lysis buffer (pH 8.5, 20 mM Tris–HCl, 1% Triton X-100, 200 μg/mL lysozyme) was added, and the mixture was stirred vigorously for 10 min at rt. The mixture was then lysed on ice for 1 h and then sonicated to shear the released nucleic acids. The lysate and inclusion bodies were separated by centrifugation at 31,000*g* for 30 min.

Purification of recombinant α-(1→4)-galactosyltransferase.—LgtC was purified from the crude lysate at 4 °C using a Ni–NTA agarose affinity column which binds to the N-terminal His_6-tag sequence in the recombinant enzyme. The Ni^{2+} column was equilibrated with 3 column volumes (CV) of binding buffer (5 mM imidazole, 20 mM Tris–HCl, pH 7.9, 0.5 M NaCl) before loading the cell lysate. The column was then washed with 6 CV of binding buffer, followed by 6 CV of washing buffer (60 mM imidazole, 20 mM Tris–HCl, pH 7.9, 0.5 M NaCl). The protein was eluted with 6 CV of elution buffer (200 mM imidazole, 20 mM Tris–HCl, pH 7.9, 0.5 M NaCl). After elution, the fractions containing the purified enzyme (detected by a UV spectrophotometer) were combined, and the purified enzyme was dialyzed at 4 °C against 10% glycerol, 20 mM Tris–HCl, pH 7.9 buffer for enzyme activity assays and enzymatic reactions. Polymerization status and purity of the sample were determined by fast-performance liquid chromatography FPLC (ÄKTA FPLC, Amersham Pharmacia Biotech, Piscataway, NJ) carried out using a Superdex 200 prep column pre-equilibrated with 50 mM ammonium acetate buffer (pH 7.0).

Table 2
Galactoside yields in practical synthesis with recombinant LgtC

Entry	Acceptor	Product	Yield (%)
1	 **1a**	 **2a**	92
2	 **1b**	 **2b**	84
3	 **1c**	 **2c**	81
4	 **1d**	 **2d**	77
5	 **1e**	 **2e**	66
6	 **1f**	 **2f**	45
7	 **1g**	 **2g**	10
8	 **1h**	 **2h**	5

Enzyme activity assay.—Enzymatic assays for LgtC were performed at 37 °C for 15 min in a final volume of 100 μL containing Tris–HCl (10 mM, pH 7.0), $MnCl_2$ (10 mM), DTT (5 mM), bovine serum albumin (0.1%), UDP-D-[6-^{3}H]galactose (0.3 mM) (final specific activity of 1000 cpm/nmol), lactose (50 mM), and varying amounts of activated enzyme. Lactose was omitted for blank. Enzyme activation consisted of incubating the Ni–NTA column purified enzyme in buffer made up of 62.5 mM HEPES, pH 7.0, and 6.25 mM DTT for 45 min at enzyme to buffer ratio of 1:4. The assay was stopped by adding equal volume (100 μL) of ice-cold 100 mM EDTA. Unreacted radioactive donor was removed by binding to Dowex 1 × 8-200 [Cl^-] anion-exchange resin in a water suspension [0.8 mL, 1:1 (v/v)]. After centrifugation (10,000 rpm, 5 min), supernatant (0.5 mL) was collected in a 20-mL plastic vial, and ScintiVerse BD (10 mL) was added. The vial was vortexed thoroughly before the radioactivity of the mixture was counted in a liquid scintillation counter (Beckmann LS-3801 counter). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of galactose from UDP-Gal to lactose per min at 37 °C.

Acceptor specificity assay for α1,4-GalT.—This assay was performed as described above, except that the acceptor for α1,4-GalT was varied, and the reaction was carried out in 10 mM Tris–HCl buffer at pH 7.0. The acceptor has been excluded for the background count.

General procedure for galactosylation of lactose acceptors (**1a**–**h**) *using the two-enzyme system.*—To a mixture of the acceptor (480 μmol, 40 mM), UDP-glucose (576 μmol, 48 mM), $MnCl_2$ (10 mM), and bovine serum albumin (BSA, 0.1%) in Tris–HCl buffer (10 mM, pH 7.0, 12 mL) were added the enzymes UDP-galactose-4-epimerase (10 U) and LgtC (7 U). The reaction was shaken under an argon atmosphere at rt (ca. 25 °C) for 2 days. The mixture was passed through Dowex-1 × 8 [Cl^-] anion-exchange resin and purified with gel-perme-

ation chromatography (Sephadex G-15, 120 × 4 cm) with double-distilled water as the mobile phase. The desired fractions were pooled and lyophilized to give the derivatives of globotriose.

Analysis of oligosaccharide products.—^{1}H and ^{13}C NMR spectra were obtained using a 400-MHz Varian Mercury-400 or a 500-MHz Varian Unity-500 NMR spectrometer with D_2O as solvent. Low-resolution and high-resolution mass spectra (FAB) were run at the mass spectrometry facility at Wayne State University and University of California at Riverside, respectively. Thin-layer chromatography was conducted on Baker Si250F Silica Gel TLC plates with a fluorescent indicator. The following data were obtained.

Compound **2a** *(223 mg, 92%) α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-D-glucopyranose.* ^{1}H NMR (500 MHz, D_2O): δ 5.06 (d, 0.4 H, $J_{1,2}$ 3.6 Hz, H-1α), 4.78 (d, 1 H, $J_{1'',2''}$ 4.1 Hz, H-1″), 4.50 (d, 0.6 H, $J_{1,2}$ 8.1 Hz, H-1β), 4.34 (d, 1 H, $J_{1',2'}$ 7.6 Hz, H-1′), 4.19 (t, 1 H, *J* 6.6 Hz), 3.87 (m, 2 H,), 3.39–3.82 (m, 14.4 H), 3.11 (t, 0.6 H, $J_{1,2}$ 8.6 Hz, H-2β); ^{13}C NMR (125 MHz, D_2O): δ 103.41, 103.37, 100.46, 95.86, 91.94, 78.82, 78.71, 77.51, 75.58, 74.99, 74.56, 74.04, 72.30, 71.59, 71.35, 71.06, 70.96, 70.30, 69.28, 69.08, 68.71, 60.65, 60.53, 60.18, 60.06; FABMS: 527 [M + Na$^+$]; HRFABMS: Anal. Calcd for $C_{18}H_{32}O_{16}Na$ [M + Na$^+$] 527.1588; Found 527.1581.

Compound **2b** *(209 mg, 84%) methyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside.* ^{1}H NMR (500 MHz, D_2O): δ 4.79 (d, 1 H, $J_{1'',2''}$ 4.1 Hz, H-1″), 4.35 (d, 1 H, $J_{1',2'}$ 8.1 Hz, H-1′), 4.25 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 4.20 (t, 1 H, *J* 6.6 Hz), 3.40–3.89 (m, 16 H), 3.42 (s, 3 H, OMe), 3.14 (t, 1 H, $J_{1,2}$ 8.6 Hz, H-2). ^{13}C NMR (125 MHz, D_2O): δ 103.42, 103.18, 100.46, 78.78, 77.49, 75.58, 74.96, 74.60, 73.02, 72.30, 71.05, 70.96, 69.27, 69.08, 68.71, 60.64, 60.52, 60.15, 57.35. FABMS: 541 [M + Na$^+$]; HRFABMS: Anal. Calcd for $C_{19}H_{34}O_{16}Na$ [M + Na$^+$] 541.1745; Found 541.1736.

Compound **2c** *(232 mg, 81%) phenyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-1-thio-β-D-glucopyranoside.* ^{1}H NMR (500 MHz, D_2O): δ 7.24–7.41 (m, 5 H, SPh), 4.88 (d, 1 H, $J_{1'',2''}$ 3.0 Hz, H-1″), 4.76 (d, 1 H, $J_{1,2}$ 7.6 Hz, H-1), 4.32 (d, 1 H, $J_{1',2'}$ 7.6 Hz, H-1′), 4.17 (t, 1 H, *J* 6.1 Hz), 3.85 (m, 2 H), 3.40–3.81 (m, 15 H), 3.22 (t, 1 H, $J_{1,2}$ 9.1 Hz, H-2). ^{13}C NMR (125 MHz, D_2O): δ 132.00, 131.77, 129.45, 128.26, 103.33, 100.42, 87.24, 78.88, 78.29, 77.45, 75.95, 75.55, 72.25, 71.66, 71.00, 70.92, 69.25, 69.04, 68.68, 62.58, 60.60, 60.50, 60.17. FABMS: 619 [M + Na$^+$]; HRFABMS: Anal. Calcd for $C_{24}H_{36}O_{15}SNa$ [M + Na$^+$] 619.1673; Found 619.1698.

Compound **2d** *(175 mg, 77%) α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→3)-β-D-arabinopyranose.* ^{1}H NMR (500 MHz, D_2O): δ 5.11 (d, 0.4 H, $J_{1,2}$ 3.6 Hz, H-1β), 4.79 (d, 1 H, $J_{1'',2''}$ 3.0 Hz, H-1″), 4.45 (d, 0.4 H, $J_{1',2'}$ 8.1 Hz, H-1′β), 4.43 (d, 0.6 H, $J_{1',2'}$ 6.1 Hz, H-1′α), 4.38 (d, 0.6 H, $J_{1,2}$ 7.6 Hz, H-1α), 4.21 (t, 1 H, *J* 6.6 Hz), 3.46–4.06 (m, 16 H). ^{13}C NMR (125 MHz, D_2O): δ 101.34, 100.99, 100.40, 96.87, 92.61, 79.70, 77.48, 76.69, 75.55, 72.37, 70.94, 70.85, 70.37, 69.27, 69.09, 68.73, 67.12, 66.38, 66.02, 60.64. FABMS: 497 [M + Na$^+$]; HRFABMS: Anal. Calcd for $C_{17}H_{30}O_{15}Na$ [M + Na$^+$] 497.1482; Found 497.1480.

Compound **2e** *(188 mg, 66%) benzyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside.* ^{1}H NMR (500 MHz, D_2O): δ 7.36–7.43 (m, 5 H, Ph), 4.89 (d, 1 H, $J_{1'',2''}$ 4.1 Hz, H-1″; d, 1 H, $J_{a,b}$ 11.4 Hz, OC*Ha*HbPh), 4.71 (d, 1 H, $J_{a,b}$ 11.4 Hz, OCHa*Hb*PH), 4.51 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 4.46 (d, 1 H, $J_{1',2'}$ 8.1 Hz, H-1′), 4.31 (t, 1 H, *J* 6.5 Hz), 3.48–3.98 (m, 16 H), 3.29 (t, 1 H, $J_{1,2}$ 8.9 Hz, H-2); ^{13}C NMR (125 MHz, D_2O): δ 136.66, 128.93, 128.88, 128.65, 103.41, 101.12, 100.46, 78.79, 77.49, 75.57, 74.99, 74.63, 73.07, 72.30, 72.20, 71.66, 71.05, 70.96, 69.27, 69.08, 68.71, 62.62, 60.65, 60.20, 59.70; FABMS: 617 [M + Na$^+$]; HRFABMS: Anal. Calcd for $C_{25}H_{38}O_{16}Na$ [M + Na$^+$] 617.2058; Found 617.2042.

Compound **2f** *(109 mg, 45%) α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→6)-D-fructofuranose.* ^{1}H NMR (500 MHz, D_2O): δ 4.77, 4.74 (d, 1 H, $J_{1'',2''}$ 4.1 Hz, H-1″), 4.43 (d, 0.6 H, $J_{1',2'}$ 8.1 Hz, H-1′), 4.35 (d, 0.4 H, $J_{1',2'}$ 7.6 Hz, H-1′), 4.18 (t, 1 H, *J* 6.6 Hz), 3.37–4.09 (m, 18 H). ^{13}C NMR (125 MHz, D_2O): δ 103.16, 102.48, 101.04, 100.41, 100.33, 98.26, 85.24, 84.36, 80.89, 80.18, 77.63, 77.52, 77.02, 75.54, 74.77, 72.38, 72.23, 70.95, 70.84, 69.27, 69.08, 68.81, 68.72, 66.76, 66.14, 63.98, 63.08, 62.72, 62.52, 60.65, 60.58, 60.37. FABMS: 527 [M + Na$^+$]; HRFABMS: Anal. Calcd for $C_{18}H_{32}O_{16}Na$ [M + Na$^+$] 527.1588; Found 527.1601.

Compound **2g** *(24 mg, 10%) α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-D-gluctitol.* ^{1}H NMR (400M Hz, D_2O): δ 4.91 (d, 1 H, $J_{1'',2''}$ 3.2 Hz, H-1″), 4.52 (d, 1 H, $J_{1',2'}$ 7.3 Hz, H-1′), 4.29 (t, *J* 6.5 Hz, 1 H), 3.50–4.00 (m, 19 H). ^{13}C NMR (100 MHz, D_2O): δ 103.72, 100.51, 80.15, 77.59, 75.46, 72.46, 72.19, 71.52, 71.28, 71.12, 69.87, 69.33, 69.19, 68.86, 62.76, 62.24, 60.76, 60.51. FABMS: 529 [M + Na$^+$].

Compound **2h** *(9 mg, 5%) methyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranoside.* ^{1}H NMR (500 MHz, D_2O): δ 4.81 (d, 1 H, $J_{1',2'}$ 3.6Hz, H-1′), 4.23 (d, 1 H, $J_{1,2}$ 8.1 Hz, H-1), 4.20 (t, 1 H, *J* 6.1 Hz), 3.88 (m, 2 H), 3.76 (m, 2 H), 3.69 (m, 2 H), 3.54–3.64 (m, 3 H), 3.43 (s, 3 H, OMe), 3.38 (m, 2 H). ^{13}C NMR (125 MHz, D_2O): δ 103.98, 100.38, 77.47, 75.17, 72.51, 71.09, 70.94, 69.22, 69.06, 68.79, 60.61, 60.29, 57.30. FABMS: 379 [M + Na$^+$]; HRFABMS: Anal. Calcd for $C_{13}H_{24}O_{11}Na$ [M + Na$^+$] 379.1216; Found 379.1218.

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