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Large-scale synthesis of globotriose derivatives through recombinant *E. coli*†

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The carbohydrate chains decorating cell membranes and secreted proteins participate in a range of important biological processes. However, their ultimate significance and possible therapeutic potential have not been fully explored due to the lack of economical methods for their production. This study is an example of the use of a genetically engineered bacterial strain in the preparation of diverse oligosaccharides. Based on an *ex vivo* biosynthetic pathway, an artificial gene cluster was constructed by linking the genes of five associated enzymes on a plasmid vector. This plasmid was inserted into the *E. coli* NM522 strain to form globotriose-producing cells ('superbug' pLDR20-CKTUF). The specific strain was conveniently applied to the synthesis of globotriose trisaccharide and its derivatives, as potential neutralizers for Shiga toxin. This work demonstrates a novel and economical method for generating ligand diversity for carbohydrate drug development.

Introduction

Carbohydrates decorating cell surface molecules manifest important roles in cell adhesion, subcellular recognition, cancer metastasis and so on.[1–3] The science of glycobiology has experienced enormous expansion during the past twenty years but further advances are being hampered by the poor availability of glycoconjugates, owing to the inherent difficulties in the syntheses of these complex molecules.[4–6] Glycosyltransferase-catalyzed synthesis has been recognized as one of the most practical approaches, for its high efficiency and specificity under very mild conditions.[7,8] Furthermore, multienzyme *in situ* regeneration systems have been developed to alleviate the sugar nucleotide cost problem.[9–13] Because enzyme purifications are laborious processes and might cause decreases in enzymatic activity, whole-cell reactions without isolating enzymes have been developed for carbohydrate synthesis. Kyowa Hakko Kogyo Co. has demonstrated the viability of large-scale enzymatic synthesis of carbohydrates with coupled engineered bacteria.[14,15] Wang's group has also reported a novel approach for the synthesis of α-Gal epitopes using only one engineered *E. coli* strain (superbug) encoding all the enzymes in the biosynthetic pathway.[16] To demonstrate the versatility of this new strategy, herein we report the construction and application of a new superbug to the large-scale synthesis of carbohydrate derivatives with therapeutic prospects.

Globotriaosylceramide (Gb_3; CD77; Galα1,4Galβ1,4GlcβOCer, Fig. 1) is an important glycosphingolipid in the human body. Research on the invasion and toxicity mechanisms of Shiga toxin-producing *E. coli* (STEC) has revealed that Shiga toxin binds to globotriose (globotriaose, Galα1,4Galβ1,4GlcOH), the sugar part of Gb_3 in host cell membranes. The binding event facilitates the toxin's entry into the host cell ultimately causing various secondary complications such as septic shock, multiple organ failure and mortality.[17,18] A typical clinical example is hemolytic-uremic syndrome (HUS), which is a worldwide disease that kills thousands of children and seniors annually and is caused by the STEC infections.[19] Unfortunately, routine antibiotic therapies against HUS are not effective and might cause the release of cell-associated toxins into the bloodstream and further induce toxin gene expression.[20] Thus, considerable interest exists in developing agents to block the initial interaction between Shiga toxin and globotriose.[21,22] To this end, several groups have focused on the chemical and enzymatic synthesis of natural globotriose and globotriaosylceramide and their derivatives.[23–27] Some polymers, silica gel or even bacterium coated with globotriose have also been designed to absorb the toxin and were recently put into clinical trial.[28–32] Our contribution here is to provide means for the efficient synthesis of globotriose derivatives through a specific metabolically engineered bacterium.[33,34]

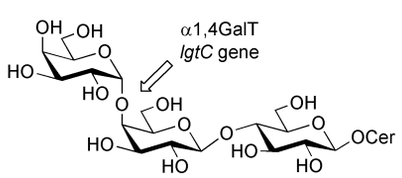
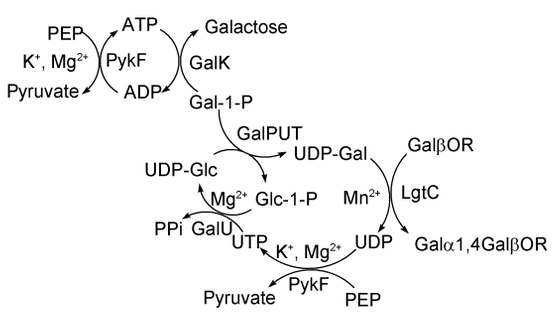


Fig. 1 Structure of *E. coli*, Shiga toxin receptor: Gb_3 (Galα1,4Galβ1,4GlcOCer).

Results and discussion

The biosynthetic pathway to Galα1,4Galβ1,4GlcOR with recycling of sugar nucleotide can be reconstituted with five enzymes derived from the Leloir pathway.[35] As shown in Scheme 1, the pathway consists of an α-1,4-galactosyltransferase from *Neisseria meningitidis* (LgtC)[36,37] and four enzymes (GalK, GalPUT, GalU and PykF)[38,39] for the regeneration of uridine 5′-diphosphogalactose (UDP-Gal). In this cycle, galactokinase (GalK, EC 2.7.1.6) first converts galactose into galactose-1-phosphate (Gal-1-P) with the consumption of one equivalent of phosphoenolpyruvate (PEP). Two enzymes,



Scheme 1 Biosynthetic pathway of globotriose Gb_3 derivatives.

† Electronic supplementary information (ESI) available: abbreviations; general methods and materials; cloning, overexpression and purification of LgtC; glycosyltransferase activity assays; and spectra of products **1**–**8**. See http://www.rsc.org/suppdata/ob/b3/b304911f/

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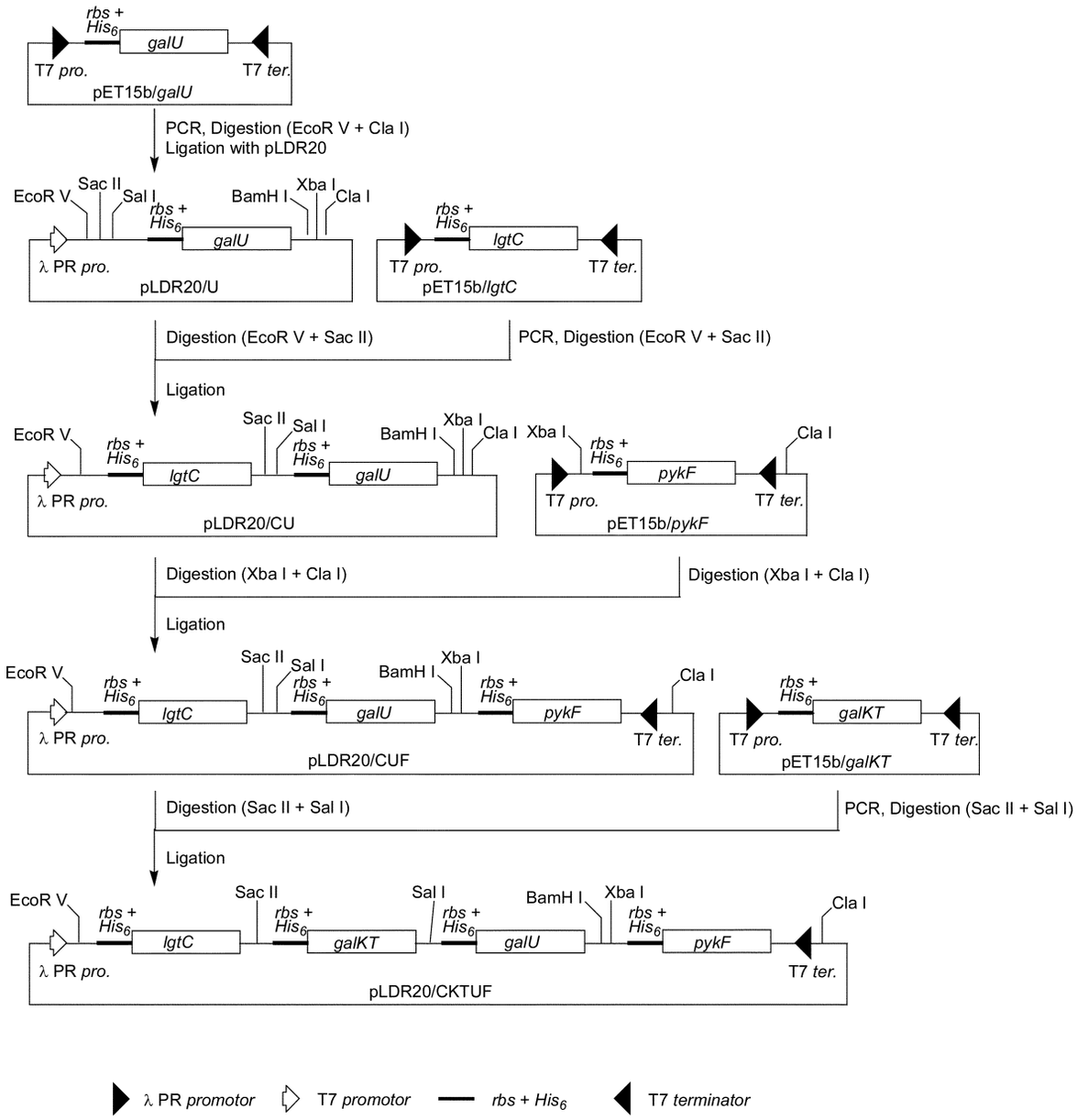


Scheme 2 Plasmid construction for the globotriose-producing superbug (pLDR20/CKTUF). Introduced restriction sites: EcoR I, Sac II, Sal I, Xba I, Cla I. Abbreviation: rbs, ribosomal binding site.

galactose-1-phosphate uridylyltransferase (GalPUT, EC 2.7.1.10) and glucose-1-phosphate uridylyltransferase (GalU, EC 2.7.1.9), condense galactose-1-phosphate and uridine 5′-triphosphate (UTP) to form UDP-Gal and pyrophosphate (PPi). This process involves glucose-1-phosphate and uridine 5′-diphosphoglucose (UDP-Glc) as intermediates. LgtC will then transfer the galactosyl residue from UDP-Gal to the acceptor to form the α-1,4-galactosylated product. The resulting uridine 5′-diphosphate (UDP) can be phosphorylated to UTP by pyruvate kinase (PykF, EC 2.7.1.40) with the consumption of another equivalent of PEP. Overall, production of one equivalent of globotriose requires one equivalent each of galactose and lactose, and two equivalents of PEP. In the *in vitro* system, PEP and/or adenosine 5′-triphosphate (ATP) have to be added stoichiometrically to provide the energy for the glycosylation. However, in living bacterial cells, the high-energy phosphates may come from the normal cellular metabolism, as will be discussed later.

To construct the globotriose generating superbug, LgtC, GalK, GalPUT, GalU and PykF have been individually cloned into the pET15b vector. In order to eliminate the need for isopropyl-1-thio-β-D-galactosylpyranoside (IPTG), an expensive inducer, and to prevent the degradation of acceptor in host cells, we need to turn away from the pET15b vector system and the DE3 lysogen hosts. The pLDR20 vector was employed here allowing the use of β-galactosidase negative hosts and temperature-induced expression. The multi-enzyme plasmid construction was accomplished with the subsequent insertion of *galU*, *lgtC*, *pykF* and *galT* + *galK* genes, respectively, with the corresponding ribosomal binding sites (rbs) and N-terminal His_6-tags into the pLDR20 plasmid as shown in Scheme 2. Each gene (except for the *galK* gene, which has a natural *rbs* in the coding sequence of upstream *galT* gene in the *gal* operon) was preceded by a Shine–Dalgarno sequence for ribosome binding to assure adequate translation. A λ P_R promoter, a cI857 repressor gene, an ampicillin resistance gene, and a T7 terminator were also added in the plasmid for expression control. A *lacZ- E. coli* strain such as NM522 was used as host to eliminate the hydrolysis of substrate by the internal β-galactosidase (Fig. 2). Plasmid pLDR20-CKTUF was transformed into the NM522 strain to form globotriose-producing cells.[16] The recombinant cells were cultured at 30 °C until the optical density (OD_{600}) of the cell culture reached ~1.0, at which point the enzyme expression was switched on by increasing the

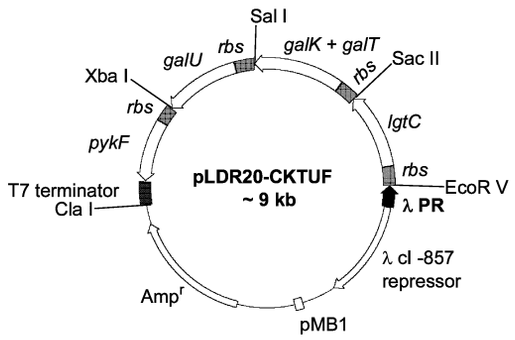
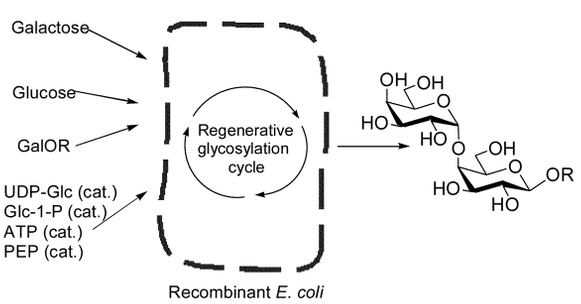


Fig. 2 Plasmid map of superbug CKTUF harboring five genes (*lgtC*, *galK*, *galPUT*, *galU*, and *pykF*).

culture temperature from 30 °C to 40 °C. SDS-PAGE results revealed (data not shown) that the co-expression of the proteins had no adverse effects on cell growth and that all proteins were expressed in approximately equal amounts.

The superbug production of oligosaccharides is a two-step procedure, distinct from the commonly employed fermentative processes. The first step involves the growth of the recombinant *E. coli* NM522 cells and the subsequent expression of the enzymes. In the second step, the cells are harvested from the culture media, permeabilized and employed as biocatalysts in the reaction (Scheme 3). This two-step process avoids the possible inhibition of cell growth by substrates and product and allows the use of high cell concentrations in the reaction (*i.e.* high catalyst concentrations) and facile manipulation of substrate concentrations. The permeabilization by repeated freezing and thawing and using detergent (Triton X-100) allows for better transfer of substrates and products into and out of the cells.[40]



Scheme 3 Synthesis of globotriose derivatives with superbug CKTUF.

Small-scale (1 mL) reactions were carried out at room temperature for 36 h to search for the optimum condition. The formation of the globotriose was monitored by high performance liquid chromatography (HPLC) using a refractive index (RI) detector (Fig. 3).[37] The results revealed that the optimal condition for the CKTUF superbug catalyzed reaction was as follows: acceptor (25 mM), Gal (50 mM), Glc (50 mM), $MnCl_2$ (10 mM), $MgCl_2$ (10 mM), KCl (100 mM), catalytic amounts (2 mM) of UDP-Glc, ATP, glucose-1-phosphate (Glc-1-P) and catalytic amount (5 mM) of PEP in 50 mM of HEPES buffer (pH 7.5). Reaction time course studies (data not shown) indicated that the appearance of product reached a plateau after 20 h at room temperature as monitored by HPLC.

As we mentioned before, in an *in vitro* setting, two equivalents of high-energy phosphate in the form of PEP are required for the formation of every glycosyl bond (Scheme 1). However, in our *ex vivo* setting, only a catalytic amount of PEP is added to optimize the reaction yield. It is clear that majority of the energy necessary to account for the product concentrations observed is being generated by normal cellular metabolism. The remaining metabolites, namely ATP, Glc-1-P and UDP-Glc are not consumed but re-circulated. That means the permeabilized cells continue to carry out at least some of the normal metabolic functions. Moreover, the superbug technology does not require reducing agents such as dithiothreitol (DTT), which is normally needed to activate LgtC for *in vitro* reactions.[39,41] This may be due to the increased stability of enzymes in the *in vivo* reductive environment compared to conditions *in vitro*.[37]

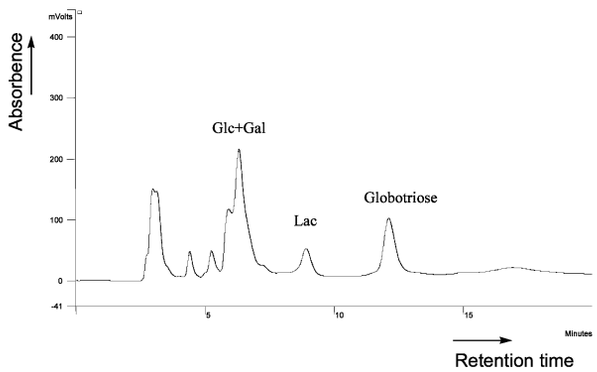


Fig. 3 HPLC Profile of reaction mixture with superbug CKTUF. Column: MICROSORB™-100 Å NH_2 5U. Detector: RI (Varian Star 9040).

The capacity of the superbug technology was explored in the gram-scale synthesis of globotriose derivatives. Table 1 presents a comparison of derivative syntheses carried out with purified recombinant enzymes and whole cells.[37] It is apparent that good acceptors for purified LgtC are accepted well by the CKTUF superbug. The structures of the products were confirmed with NMR and MS. For most substrates shown, the purified enzyme reactions give slightly higher yields, probably due to the much higher concentration of sugar nucleotide (48 mM) in the reaction mixtures. The most notable exception to this is benzyl β-lactoside (LacOBn) (Entry 2). This acceptor proved better in the whole cell catalyzed reaction. In this case, the cell-confined LgtC transferase may prefer benzyl β-lactoside, as it is a better mimic of the natural lactosylceramide substrate.[36]

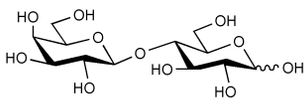
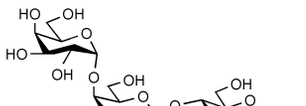
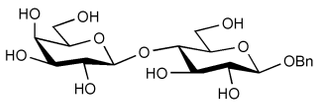
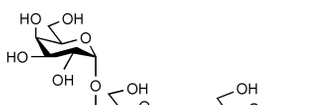
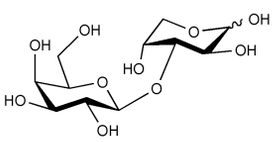
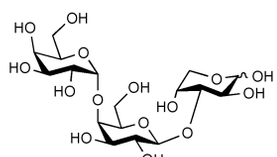
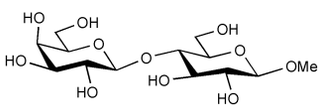
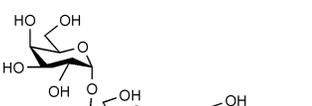
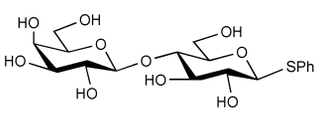
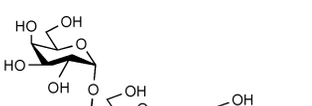
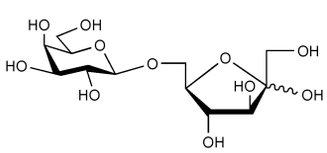
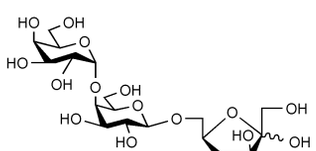
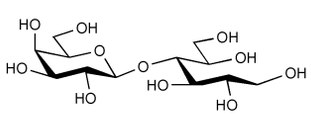
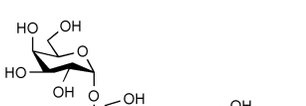
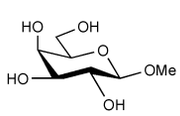
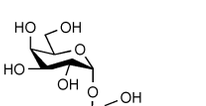
Despite the diminished yields in whole cell reactions, the cost per gram of product obtained is substantially reduced in comparison to synthesis using purified recombinant enzymes. Most of the products can be synthesized at a price range from \$30–\$50 g^{-1} (based on the most pricy components: PEP and UDP-Glc). The lower cost is due to the absence of purification of individual enzymes and omission of the reductive agent, but most significantly due to the non-stoichiometric use of expensive intermediates. This is attributable to the cells' ability to both recycle these metabolites as well as to provide the energy required for the formation of the glycosidic bonds.

Conclusion

In summary, the superbug synthesis is a practical method towards large-scale production of carbohydrates with defined regio- and stereospecificities under mild conditions. An *ex vivo* biosynthetic pathway was constructed as a single artificial gene cluster. The plasmid was then transplanted into an *E. coli* expression system and the recombinant cells were used to synthesize globotriose and a series of derivatives. The use of whole cells alleviates the source of energy problem in large-scale enzymatic synthesis.

Given the efficiency of our system, and the increasing availability of recombinant glycosyltransferases, various *E. coli* strains can be constructed for specific glycosyl linkages. Then, glycochemists can use the superbug cell as a convenient tool in the constructions of diverse glycoconjugates and unnatural derivatives to meet the increasing demands.

Table 1 Comparison of syntheses of globotriose derivatives with recombinant enzymes and whole cells

Entry	Acceptor	Product	Yield (%)	
			Whole cells	Enzyme
1		 **1**	75	92
2		 **2**	85	66
3		 **3**	60	77
4		 **4**	50	84
5		 **5**	50	81
6		 **6**	45	45
7		 **7**	20	10
8		 **8**	10	5

Experimental

Construction of superbug CKTUF (Scheme 2)

The plasmid for the CKTUF superbug was constructed as described in ref. 16 into the pLDR20 plasmid vector. Primers lgtC-N (5′-GGATCCATATGACTAGTGATATCAATAATTT-TGTTTAACTTTAAGAAGG-3′) and lgtC-C (5′-TCCCCGC-GGTCATCAGTGCGGGACGGCAAGTTTGCC-3′) were used to amplify the *lgtC* gene with ribosomal binding site and codons for His_6-tag from pre-constructed plasmid pET15b-lgtC-25aa. The PCR product was digested and inserted into EcoR V and Sac II two restriction sites of the plasmid

pLDR20-U (constructed in ref. 16) to form plasmid pLDR20-CU. Then, *pykF* gene and *galT* + *galK* genes were inserted to form plasmid pLDR20-CKTUF (Fig. 2). This final plasmid harboring five genes was transformed into NM522 competent cells.

General procedure for one-pot synthesis of globotriose and its derivatives with superbug

NM522 (pLDR20-CKTUF) cells were grown in 4 L shake flasks or a 10 L fermentor. The expression of the target genes in the superbug was initiated by increasing the temperature from 30 °C to 40 °C. After expression at 40 °C for 3–3.5 h, the cells were separated from the medium by centrifugation (5000*g* × 30 min) and suspended in 100 mL of Tris-HCl buffer (20 mM, pH 8.5) containing 1% Triton X-100. For better results, the cell suspension was stored at −20 °C and freeze–thawed twice before being applied in the reaction.

For small-scale analysis, the reactions were performed with 0.14 g (wet weight) of cells in 1 mL reaction volume containing Gal (50 mM), Lac (25 mM), Glc (50 mM), PEP (5 mM), Glc-1-P (2 mM), UDP-Glc (2 mM), ATP (2 mM), $MgCl_2$ (10 mM), KCl (100 mM), $MnCl_2$ (10 mM), HEPES (50 mM, pH 7.4). The reaction was carried out at room temperature and the formation of the trisaccharide product was monitored by HPLC (Fig. 3). To optimize the conditions, multiple 1 mL reactions were set up with different starting material compositions.

Gram-scale synthesis was performed with a variety of galactose or lactose derivatives as acceptors for the LgtC. For a typical synthesis reaction, in a 250 mL flask was added acceptor (2.92 mmol), Gal (1.05 g, 5.84 mmol), Glc (1.05 g, 5.84 mmol), PEP (111 mg, 0.584 mmol), ATP (129 mg, 0.234 mmol), UDP-Glc (143 mg, 0.234 mmol), Glc-1-P (72 mg, 0.234 mmol), and 12 mL of each of the following stock solutions: HEPES buffer (0.5 M, pH 7.4), $MnCl_2$ (0.1 M), $MgCl_2$ (0.1 M), and KCl (1 M). Then superbug cells [12 g in 72 mL Tris-HCl buffer (20 mM, pH 8.5) containing 1% Triton X-100, obtained from 2 L bacterial culture] were added to bring the total reaction mixture volume to 117 mL. The reaction was agitated with a magnetic stirrer at room temperature (22 °C) for 36 h. The reaction was monitored by thin-layer chromatography (TLC) [*i*-PrOH : H_2O : NH_4OH = 7 : 3 : 2 (vol/vol/vol)] and HPLC. After 36 hours, the reaction was stopped by heating the flask to 100 °C for 10 min. Insoluble components were sedimented by centrifugation at 5000*g* for 20 min and the pellet washed twice with 50 mL deionized water. The combined supernatants were passed through an anion exchange column and then a cation exchange column. The concentrated elute was loaded on a Sephadex G-15 gel filtration column (120 cm × 4 cm) with water as the mobile phase. The desired fractions were pooled and lyophilized to give the derivatives of globotriose. The following compounds were prepared.

α-D-galactopyranosyl-(1⟶4)-β-D-galactopyranosyl-(1⟶4)-D-glucopyranose (1)

(1.10 g, 75%) ^{1}H-NMR (500 MHz, D_2O): δ 5.06 (d, J = 3.6 Hz, 0.4 H), 4.78 (d, J = 4.1 Hz, 1 H), 4.50 (d, J = 8.1 Hz, 0.6 H), 4.34 (d, J = 7.6 Hz, 1 H), 4.19 (t, J = 6.6 Hz, 1 H), 3.87 (m, 2 H), 3.39–3.82 (m, 14.4 H), 3.11 (t, J = 8.6 Hz, 0.6 H); ^{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; MS (FAB) *m*/*z* 526.94 (M + Na^+, 100%); HRMS: calculated for $C_{18}H_{32}O_{16}Na$ (M + Na^+) 527.1588, found 527.1581.

Benzyl α-D-galactopyranosyl-(1⟶4)-β-D-galactopyranosyl-(1⟶4)-β-D-glucopyranoside (2)

(1.47 g, 85%) ^{1}H-NMR (500 MHz, D_2O): δ 7.36–7.43 (m, 5 H), 4.89 (d, J = 4.1 Hz, 1 H; d, J = 11.4 Hz, 1 H), 4.71 (d, J = 11.4 Hz, 1 H), 4.51 (d, J = 8.1 Hz, 1 H), 4.46 (d, J = 8.1 Hz, 1 H), 4.31 (t, J = 6.5 Hz, 1 H), 3.48–3.98 (m, 16 H), 3.29 (t, J = 8.9 Hz, 1 H); ^{13}C NMR (125 MHz, D_2O): δ 136.7, 129.02, 128.96, 128.7, 103.5, 101.2, 100.5, 78.8, 77.6, 75.7, 75.1, 74.7, 73.2, 72.4, 72.3, 71.7, 71.1, 71.0, 69.4, 69.1, 68.8, 62.7, 60.7, 60.6, 60.3; MS (FAB) 617.19 (M + Na^+, 41%); HRMS: calculated for $C_{25}H_{38}O_{16}Na$ (M + Na^+) 617.2058, found 617.2042.

Methyl α-D-galactopyranosyl-(1⟶4)-β-D-galactopyranosyl-(1⟶4)-β-D-glucopyranoside (3)

(756 mg, 50%) ^{1}H-NMR (500 MHz, D_2O): δ 4.79 (d, J = 4.1 Hz, 1 H), 4.35 (d, J = 8.1 Hz, 1 H), 4.25 (d, J = 8.1 Hz, 1 H), 4.20 (t, J = 6.6 Hz, 1 H), 3.40–3.89 (m, 16 H), 3.42 (s, 3 H), 3.14 (t, J = 8.6 Hz, 1 H); ^{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; MS (FAB) 540.99 (M + Na^+, 100%); HRMS: calculated for $C_{19}H_{34}O_{16}Na$ (M + Na^+) 541.1745, found 541.1736.

Phenyl α-D-galactopyranosyl-(1⟶4)-β-D-galactopyranosyl-(1⟶4)-β-D-1-thio-glucopyranoside (4)

(870 mg, 50%) ^{1}H-NMR (500 MHz, D_2O): δ 7.24–7.41 (m, 5 H), 4.76 (d, J = 3.0 Hz, 1 H), 4.32 (d, J = 7.6 Hz, 1 H), 4.17 (t, J = 6.1 Hz, 1 H), 3.85 (m, 2 H), 3.40–3.81 (m, 15 H), 3.22 (t, J = 9.1 Hz, 1 H); ^{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; MS (FAB) 634.96 (M + K^+, 25%), 618.99 (M + Na^+, 100), 597.08 (M + H^+, 3); HRMS: calculated for $C_{24}H_{36}O_{15}SNa$ (M + Na^+) 619.1673, found 619.1698.

α-D-Galactopyranosyl-(1⟶4)-β-D-galactopyranosyl-(1⟶3)-β-D-arabinofuranose (5)

(830 mg, 60%) ^{1}H-NMR (500 MHz, D_2O): δ 5.11 (d, J = 3.6 Hz, 0.4 H), 4.79 (d, J =3.0 Hz, 1 H), 4.44 (t, J = 8.1 Hz, 1 H), 4.38 (d, J = 7.6 Hz, 0.6 H), 4.21 (t, J = 6.6 Hz, 1 H), 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; MS (FAB) 496.94 (M + Na^+, 100%); HRMS: calculated for $C_{17}H_{30}O_{15}Na$ (M + Na^+) 497.1482, found 497.1480.

α-D-Galactopyranosyl-(1⟶4)-β-D-galactopyranosyl-(1⟶6)-D-fructofuranose (6)

(662 mg, 45%) ^{1}H-NMR (500 MHz, D_2O): δ 4.77 (d, J = 4.1 Hz, 0.6 H), 4.74 (d, J = 3.6 Hz, 0.4 Hz), 4.43 (d, J = 8.1 Hz, 0.6 H), 4.35 (d, J = 7.6 Hz, 0.4 H), 4.18 (t, J = 6.6 Hz, 1 H), 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; MS (FAB) 526.86 (M + Na^+, 100%); HRMS: calculated for $C_{18}H_{32}O_{16}Na$ (M + Na^+) 527.1588, found 527.1601.

α-D-Galactopyranosyl-(1⟶4)-β-D-galactopyranosyl-(1⟶4)-D-gluctitol (7)

(296 mg, 20%) ^{1}H-NMR (400 MHz, D_2O): δ 4.91 (d, J = 3.2 Hz, 1 H), 4.52 (d, J = 7.3 Hz, 1 H), 4.29 (t, J = 6.5 Hz, 1 H), 3.50–4.00 (m, 19 H), 1.28 (d, J = 6.5 Hz, 1 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; MS (FAB) 529.00 (M + Na^+, 67%); MS (ESI): 505.00 (M − H^+, 100%).

Methyl α-D-galactopyranosyl-(1⟶4)-β-D-galactopyranoside (8)

(104 mg, 10%) ^{1}H-NMR (500 MHz, D_2O): δ 4.81 (d, J = 3.6 Hz, 1 H), 4.23 (d, J = 8.1 Hz, 1 H), 4.20 (t, J = 6.1 Hz, 1 H), 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), 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; MS (FAB) 378.88 (M + Na^+, 100%); HRMS: calculated for $C_{13}H_{24}O_{11}Na$ (M + Na^+) 379.1216, found 379.1218.

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References

1 A. Kobata, *Acc. Chem. Res.*, 1993, **26**, 319.
2 K. M. Koeller and C.-H. Wong, *Nat. Biotechnol.*, 2000, **18**, 835.
3 S. Hurtley, R. Service and P. Szuromi, *Science*, 2001, **291**, 2337.
4 X.-S. Ye and C.-H. Wong, *J. Org. Chem.*, 2000, **65**, 2410.
5 O. J. Plante, E. R. Palmacci and P. H. Seeberger, *Science*, 2001, **291**, 1523.
6 P. Sears and C.-H. Wong, *Science*, 2001, **291**, 2344.
7 M. M. Palcic, *Curr. Opin. Biotechnol.*, 1999, **10**, 616.
8 K. M. Koeller and C.-H. Wong, *Nature*, 2001, **409**, 232.
9 C.-H. Wong, S. L. Haynie and G. M. Whitesides, *J. Org. Chem.*, 1982, **47**, 5416.
10 P. Wang, G.-J. Shen, Y.-F. Wang, Y. Ichikawa and C.-H. Wong, *J. Org. Chem.*, 1993, **58**, 3985.
11 Y. Ichikawa, R. Wang and C.-H. Wong, *Methods Enzymol.*, 1994, **247**, 107.
12 A. Zervosen and L. Elling, *J. Am. Chem. Soc.*, 1996, **118**, 1836.
13 M. Gilbert, R. Bayer, A. M. Cunningham, S. DeFrees, Y. Gao, D. C. Watson, N. M. Young and W. W. Wakarchuk, *Nat. Biotechnol.*, 1998, **16**, 769.
14 S. Koizumi, T. Endo, K. Tabata and A. Ozaki, *Nat. Biotechnol.*, 1998, **16**, 847.
15 T. Endo and S. Koizumi, *Curr. Opin. Struct. Biol.*, 2000, **10**, 536.
16 X. Chen, Z.-Y. Liu, J. Zhang, W. Zhang and P. G. Wang, *ChemBioChem*, 2002, **3**, 47.
17 J. P. Nataro and J. B. Kaper, *Clin. Microbiol. Rev.*, 1998, **11**, 142.
18 C. A. Lingwood, *Biochim. Biophys. Acta*, 1999, **1455**, 375.
19 M. P. Jackson, *Microb. Pathog.*, 1990, **8**, 235.
20 J. C. Paton and A. W. Paton, *Clin. Microbiol. Rev.*, 1998, **11**, 450.
21 J. J. Donnelly and R. Rappuoli, *Nat. Med. (N Y.)*, 2000, **6**, 257.
22 G. Mulvey, P. I. Kitov, P. Marcato, D. R. Bundle and G. D. Armstrong, *Biochimie*, 2001, **83**, 841.
23 K. Koike, M. Sugimoto, S. Sato, Y. Ito, Y. Nakahara and T. Ogawa, *Carbohydr. Res.*, 1987, **163**, 189.
24 K. C. Nicolaou, T. Caulfield, H. Kataoka and T. Kumazawa, *J. Am. Chem. Soc.*, 1988, **110**, 7910.
25 D. X. Qui and R. R. Schmidt, *Liebigs Ann. Chem.*, 1992, 217.
26 M. Z. Liu, H. N. Fan and Y. C. Lee, *Biochimie*, 2001, **83**, 693.
27 P. I. Kitov and D. R. Bundle, *J. Chem. Soc., Perkin Trans. 1*, 2001, 838.
28 G. D. Armstrong, P. C. Rowe, P. Goodyer, E. Orrbine, T. P. Klassen, G. Wells, A. Mackenzie, H. Lior, C. Blanchard, F. Auclair, B. Thompson, D. J. Rafter and P. N. McLaine, *J. Infect. Dis.*, 1995, **171**, 1042.
29 T. Takeda, K. Yoshino, E. Adachi, Y. Sato and K. Yamagata, *Microbiol. Immunol.*, 1999, **43**, 331–337.
30 P. I. Kitov, J. M. Sadowska, G. Mulvey, G. D. Armstrong, H. Ling, N. S. Pannu, R. J. Read and D. R. Bundle, *Nature*, 2000, **403**, 669.
31 A. W. Paton, R. Morona and J. C. Paton, *Nat. Med. (N. Y.)*, 2000, **6**, 265.
32 F. Yan, M. Gilbert, W. W. Wakarchuk, J.-R. Brisson and D. M. Whitfield, *Org. Lett.*, 2001, **3**, 3265.
33 M. D. Burkart, *Org. Biomol. Chem.*, 2003, **1**, 1.
34 E. Rodriguez and R. McDaniel, *Curr. Opin. Microbiol.*, 2001, **4**, 526.
35 H. H. Freeze, in *Essentials of Glycobiology*, ed. A. Varki, *et al.*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999, p. 69.
36 W. W. Wakarchuk, A. Cunningham, D. C. Watson and N. M. Young, *Protein Eng.*, 1998, **11**, 295.
37 J. Zhang, P. Kowal, J. Fang, P. Andreana and P. G. Wang, *Carbohydr. Res.*, 2002, **337**, 969.
38 X. Chen, J. Fang, J. Zhang, Z. Liu, J. Shao, P. Kowal, P. Andreana and P. G. Wang, *J. Am. Chem. Soc.*, 2001, **123**, 2081.
39 Z. Liu, J. Zhang, X. Chen and P. G. Wang, *ChemBioChem*, 2002, **3**, 348.
40 X. Chen, W. Zhang, J.-Q. Wang, J. W. Fang and P. G. Wang, *Biotechnol. Prog.*, 2000, **16**, 595.
41 M. Dieckelmann, S. Levesque, D. C. Watson and W. W. Wakarchuk, *Glycobiology*, 1999, **9**, 147.