[9] Synthesis of Galactose-Containing Oligosaccharides through Superbeads and Superbug Approaches: Substrate Recognition along Different Biosynthetic Pathways

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Introduction

Galactosides as carbohydrate receptors play critical roles in biological recognition events.[1] α-Galactosyl (α-Gal) epitopes and globotriose are two representative galactosyloligosaccharides with therapeutic significance. α-Gal epitopes (Galα1,3Galβ1,4GlcOR, Fig. 1) are oligosaccharides abundantly expressed on the surface of pig vascular endothelial cells (1×10^7 epitopes per cell).[2] Their interactions with preexisting natural antibodies (anti-Gal) in human are believed to be the main cause of the hyperacute rejection (HAR) in pig-to-human organ xenotransplantation.[3] Neutralization of anti-Gal with free α-Gal and its derivatives is a promising treatment to overcome HAR.[4] Globotriose (Galα1,4Galβ1,4Glc; Fig. 2) is the carbohydrate moiety of a functional cell surface receptor, globotriaosylceramide (Gb_3; Galα1,4Galβ1,4GlcβOCer), which can be recognized by Shiga toxin (Stx)-producing *Escherichia coli* (STEC), including

[1] (a) A. Kobata, *Acc. Chem Res*. **26,** 319 (1993); (b) C.-H. Wong, R. L. Halcomb, Y. Ichikawa, and T. Kajimoto, *Angew. Chem. Int. Ed. Engl*. **34,** 412 (1995); (c) R. A. Dwek, *Chem. Rev*. **96,** 683 (1996); (d) A. Varki, R. D. Cummings, J. Esko, H. Freeze, and G. Hart (Eds.), ''Essentials of Glycobiology.'' Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999.

[2] (a) D. K. C. Cooper, *Clin. Trans*. **6,** 178 (1992); (b) D. K. C. Cooper, A. H. Good, E. Koren, R. Oriol, A. J. Malcolm, R. M. Ippolito, F. A. Neethling, Y. Ye, E. Romano, and N. Zuhdi, *Transplant. Immunol*. **1,** 198 (1993); (c) U. Galili, *Sci. Med*. **5,** 28 (1998).

[3] (a) U. Galili, *Blood Cells* **14,** 205 (1988); (b) U. Galili, *Immunol. Ser*. **55,** 355 (1992); (c) U. Galili, *Immunol. Today* **14,** 480 (1993); (d) U. Galili, *Springer Semin. Immunopathol*. **15,** 155 (1993); (e) D. K. C. Cooper, E. Koren, and R. Oriol, *Immunol. Rev*. **141,** 31 (1994); (f) M. S. Sandrin, H. A. Vaughan and I. F. C. McKenzie, *Transplant. Rev*. **8,** 134 (1994); (g) B. E. Samuelsson, L. Rydberg, M. E. Breimer, A. Backer, M. Gustavsson, J. Holgersson, E. Karlsson, A.-E. Uyterwaal, T. Cairns, and K. Welsh, *Immunol. Rev*. **141,** 151 (1994); (h) R. Hamadeh, U. Galili, P. Zhou, and J. Griffiss, *Clin. Diagn. Lab. Immunol*. **2,** 125 (1995); (i) X. Chen, P. R. Andreana, and P. G. Wang, *Curr. Opin. Chem. Biol*. **3,** 650 (1999).

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FIG. 1. Structure of α-Gal (Galα1,3Galβ1,4Glc) epitope and the linkage created by α1,3-galactosyltransferase (α1,3GalT).

FIG. 2. Structure of globotriose (Galα1,4Galβ1,4Glc) and the linkage created by α1,4GalT.

O157:H7.[5] Research on Stx has revealed that Stx B subunit pentamer binds to the globotriose on human cells in the first stage of infection. This specific binding facilitates toxin entry into the host cell, resulting in cessation of protein synthesis, ultimately causing various secondary complications such as septic shock, multiple organ failure, and mortality.[6] In addition, globotriose found in the lipooligosaccharides of the bacterial pathogens *Neisseria meningitidis* immunotype L1 and *Neisseria gonorrhoeae* participates in the invasion of these pathogens into mammalian cells.[7]

[5] (a) C. A. Lingwood, *Biochim. Biophys. Acta* **1455,** 375 (1999); (b) J. C. Paton and A. W. Paton, *Clin. Microbiol. Rev*. **11,** 450 (1998); M. A. Karmali, M. Petric, C. Lim, P. C. Fleming, G. S. Arbus, and H. Lior, *J. Infect. Dis*. **151,** 775 (1985).

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[7] (a) R. J. P. M. Scholten, B. Kuipers, H. A. Valkenburg, J. Kankert, W. D. Zollinger, and J. T. Poolman, *J. Med. Microbiol*. **41,** 236 (1994); (b) J. Kihlberg and G. Magnusson, *Pure Appl. Chem*. **68,** 2119 (1996).

Therefore, globotriose and its derivatives have been used as effective Stx neutralizers[8] or inhibitors to prevent pathogen invasion.[9]

It is clear that further studies on preventing HAR and protecting humans from pathogen attack require easy access to substantial amounts of α-Gal and globotriose as well as their analogs.[10] Chemical synthesis of these compounds suffers from relatively low conversion due to multiple protection–deprotection steps and tedious purification at each stage of the synthesis.[11] Leloir glycosyltransferases, on the other hand, are highly regio- and stereoselective for the glycosidic linkage formation, thereby allowing the straightforward synthesis of glycoconjugates.[12] However, the scale-up application of glycosyltransferases is limited by the high cost of associated sugar nucleotide donors, especially the unnatural sugar nucleotides. One approach to overcome this drawback is to imitate the natural glycosylation pathway by which the sugar nucleotides are recycled during the reaction. Another advantage of sugar nucleotide recycling is that the low concentration of sugar nucleotide prevents its inhibitory effect on the glycosyltransferase-catalyzed reaction and, finally, increases the synthetic efficiency and yield.[13]

Sugar nucleotide regeneration is a complicated process involving interactions between multiple enzymes along the biosynthetic pathway. Besides their natural acceptor substrates, glycosyltransferases normally can recognize a variety of acceptor derivatives and produce glycoside derivatives in high yields.[14] Moreover, the enzymes involved in the generation of sugar nucleotides can also tolerate donor derivatives. Therefore, a wider range of

[8] K. Nishikawa, K. Matsuoka, E. Kita, N. Okabe, M. Mizuguchi, K. Kino, S. Miyazawa, C. Yamasaki, J. Aoki, S. Takashima, Y. Yamakawa, M. Nishijima, D. Terunuma, H. Kuzuhara, and Y. Natori, *Proc. Natl. Acad. Sci. USA* **99,** 7669 (2002).

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[10] P. Sears and C.-H. Wong, *Angew. Chem. Int. Ed. Engl.* **38,** 2300 (1999).

[11] (a) K. Koike, M. Sugimoto, S. Sato, Y. Ito, Y. Nakahara, T. Ogawa, *Carbohydr. Res.* **163,** 189–208 (1987); (b) K. C. Nicolaou, T. Caufield, H. Kataoka, T. Kumazawa, *J. Am. Chem. Soc.* **110,** 7910–7912 (1998); (c) D. Qui, R. R. Schmidt, *Liebigs Ann.* 217–224 (1992); (d) W. Zhang, J.-Q. Wang, J. Li, L.-B. Yu, P. G. Wang, *J. Carb. Chem.* **18,** 1009–1017; 1999.

[12] (a) M. M. Palcic, *Curr. Opin. Biotechnol.* **10,** 616 (1999); (b) P. Sears and C.-H. Wong, *Science* **291,** 2344 (2001); (c) X. Chen, P. Kowal, and P. G. Wang, *Curr. Opin Drug Discov. Dev.* **3,** 756 (2000); (d) J. Zhang, B. Wu, Z. Liu, P. Kowal, X. Chen, J. Shao, and P. G. Wang, *Curr. Org. Chem.* **5,** 1169 (2001).

[13] (a) C.-H. Wong, S. L. Haynie, and G. M. Whitesides, *J. Org. Chem.* **47,** 5418 (1982); (b) M. Gilbert, R. Bayer, A.-M. Cunningham, S. DeFrees, Y. Gao, D. C. Watson, N. M. Young, and W. W. Wakarchuk, *Nat. Biotechnol.* **16,** 769 (1998).

[14] (a) J. Fang, J. Li, X. Chen, Y. Zhang, J. Wang, Z. Guo, K. Brew, and P. G. Wang, *J. Am. Chem. Soc.* **120,** 6635 (1998); (b) J. Zhang, P. Kowal, J. Fang, P. Andreana, and P. G. Wang, *Carbohydr. Res.* **337,** 969 (2002).

glycosides would be generated, starting from donor derivatives and acceptor derivatives. To provide convenient tools for glycobiologists and carbohydrate chemists, we have developed ''superbeads'' and ''superbug'' technologies and successfully applied them as independent toolkits for the synthesis of galactose-containing oligosaccharides.[15]

UDP-Gal Superbeads

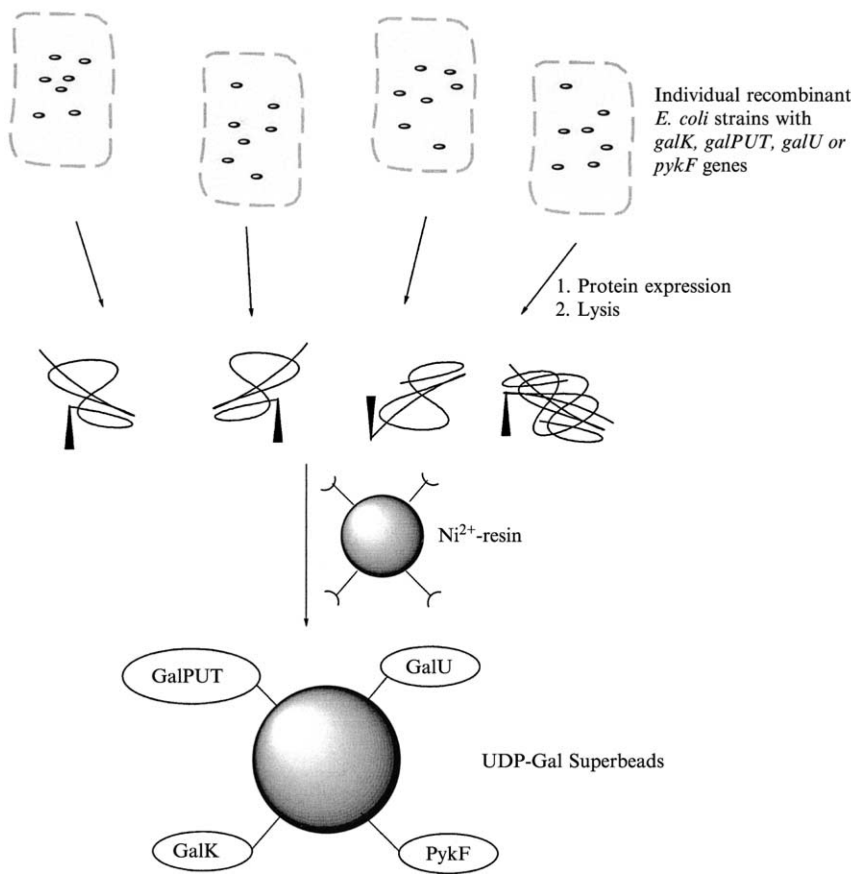
An immobilized enzyme system has the advantages of ease of product separation, increased stability, reusability, and improved kinetics.[16] Superbeads, which are essentially agarose resin-immobilized enzymes along the biosynthetic pathway for glycoconjugate synthesis, inherit these advantages. This technology for sugar nucleotide regeneration involves the following steps: (1) cloning and overexpression of individual N-terminal hexahistidine (His_6)-tagged enzymes along the sugar nucleotide biosynthetic pathway, (2) stepwise testing of the activity of the recombinant enzymes, (3) coimmobilizing onto nickel-nitrilotriacetate (Ni^{2+}-NTA) beads, and (4) combining the superbeads with glycosyltransferases (either on the beads or in solution) for glycoside synthesis (Scheme 1).

Construction of in Vitro *Biosynthetic Pathway of UDP-Gal*

The biosynthetic pathway for galactoside synthesis involves at least one galactosyltransferase and four sugar nucleotide regeneration enzymes (Scheme 2): Galactokinase (GalK, EC 2.7.1.6) phosphorylates galactose to galactose 1-phosphate (Gal-1-P) and consumes one molecule of phosphoenolpyruvate (PEP). Galactose-1-phosphate uridylyltransferase (GalPUT, EC 2.7.7.10) and glucose-1-phosphate uridylyltransferase (GalU, EC 2.7.7.9) then work together to produce UDP-Gal from Gal-1-P and UTP through intermediates glucose 1-phosphate (Glc-1-P) and UDP-Glc. Pyruvate kinase (PykF, EC 2.7.1.40) is responsible for the formation of UTP from UDP, the by-product of the galactosylation, with the consumption of the second molecule of PEP.

[15] (a) X. Chen, J. Fang, J. Zhang, Z. Liu, J. Shao, P. Kowal, P. Andreana, and P. G. Wang, *J. Am. Chem. Soc*. **123,** 2081 (2001); (b) X. Chen, J. Zhang, P. Kowal, Z. Liu, P. R. Andreana, Y. Lu, and P. G. Wang, *J. Am Chem. Soc*. **123,** 8866 (2001); (c) X. Chen, Z. Liu, J. Zhang, W. Zheng, P. Kowal, and P. G. Wang, *ChemBioChem* **3,** 47 (2002).

[16] (a) N. L. S. Clair and M. A. Navia, *J. Am. Chem. Soc*. **114,** 7314 (1992); (b) J. Thiem and T. Wiemann, *Synthesis-Stuttgart* 141 (1992); (c) L. Bulow and K. Mosbach, *Trends Biotechnol*. **9,** 226 (1991); (d) L. Bulow, *Eur. J. Biochem*. **163,** 443 (1987); (e) C. Suge and C. Merienne, *Carbohydr. Res*. **151,** 147 (1986).



SCHEME 1. Preparation of UDP-Gal regeneration superbeads.

To construct the UDP-Gal regeneration cycle, genes for these four enzymes are individually amplified from the *E. coli* K-12 genome, cloned into the pET15b vector, and expressed in *E. coli* BL21 (DE3) with N-terminal His_6 tags.[15a,c,17]

The feasibility of UDP-Gal regeneration was tested by the combined activity of these biosynthetic enzymes and a recombinant α-1,3-galactosyltransferase (α1,3GalT). In the radioactivity assays, ^{3}H-labeled galactose (*Gal) is transferred to the acceptor substrate, $LacO(CH_2)_7CH_3$ (Lac-grease), via a stepwise combination of the action of several enzymes

[17] Z. Liu, J. Zhang, X. Chen, and P. G. Wang, *ChemBioChem* **3,** 348 (2002).

$$2\ PEP + Gal + ROH \rightleftharpoons 2\ Pyruvate + PPi + Gal\text{-}OR$$

SCHEME 2. Biosynthetic pathway for galactosides with regeneration of UDP-Gal.

along the pathway. After the reactions, *Gal was removed from the reaction mixture and the radio-labeled product *Galα1, 3LacO$(CH_2)_7CH_3$ having hydrophobic moieties would bind to the Sep-Pak C_{18} cartridge and be eluted with methanol for radioactive counting. The second method of testing the regeneration cycle is to quantify the formation of α-Gal product by high-performance liquid chromatography (HPLC) with lactose as an acceptor. The high efficiency of each enzyme in the UDP-Gal regeneration cycle is demonstrated in stepwise experiments (steps 1–5 in Table I). Higher yields in this assay might represent the preference of the transferase for lactose (Lac) over Lac-grease.

Construction of UDP-Gal Superbeads

UDP-Gal regeneration beads (superbeads) are obtained by incubating Ni^{2+}-NTA resin with a mixture of the cell lysate consisting of the same number of activity units of His_6-tagged recombinant GalK, GalPUT, GalU, and PykF (Scheme 1). When combined with one or more galactosyltransferase(s), either on the beads or in solution, these UDP-Gal regeneration beads can catalyze the synthesis of a wider range of galactosides.

Synthesis of Galactose-Containing Oligosaccharides with UDP-Gal Superbeads

By using UDP-Gal regeneration beads in combination with β1,4-galactosyltransferases, LacNAc is synthesized from N-acetylglucosamine (GlcNAc) and galactose (Table II, entry 1). Furthermore, α-Gal and

TABLE I
HPLC ANALYSIS FOR PRODUCTION OF GALα1,3LACOH WITH PURIFIED RECOMBINANT ENZYMES OF BIOSYNTHETIC PATHWAY[a]

Step	Enzyme(s)	Starting material	Product (%)
1	α1,3GalT	UDP-Gal + Lac	95
2	GalPUT + α1,3GalT	Gal-1-P + UDP-Glc + Lac	95
3	GalK + GalPUT + α1,3GalT	ATP + Gal + UDP-Glc + Lac	95
4	GalK + GalPUT + α1,3GalT + GalU	ATP + Gal + UTP + Glc-1-P (cat.) + Lac	90
5	GalK + GalPUT + α1,3GalT + GalU + PykF	ATP + Gal + PEP + UDP (cat.) + Glc-1-P (cat.) + Lac	90

[a] See text for abbreviations.

TABLE II
SYNTHESES OF GALACTOSIDE ANALOGS WITH UDP-GAL REGENERATION SUPERBEADS

Entry	Gal derivative	GalT	Acceptor	Product	Yield (%)
1	Gal	α1,3GalT		**1**	92
2	Gal	α1,3GalT		**2**	85
3	Gal	α1,4GalT		**3**	86
4	Gal[a]	α1,3GalT β1,4GalT		**4**	76
5	2-Deoxy-Gal	α1,3GalT		**5**	69
6	[1-^{13}C]Gal	α1,3GalT		**6**	83
7	[1-^{13}C]Gal[a]	α1,3GalT β1,4GalT		**7**	95

[a] Two equivalents; others are 1 equivalent.

globotriose derivatives are synthesized from other oligosaccharide acceptors such as β-benzyllactoside (LacβOBn) and β-methyllactoside (LacβOMe) (Table II, entries 2, 3, 5, and 6). In addition, the UDP-Gal regeneration beads can be used in combination with multiple galactosyltransferases. For example, the combination of α1,3GalT and β1,4GalT with the superbeads can generate Galα1,3GAlβ1,4GlcNAc sequence starting from

GlcNAc and two equivalents of galactose (Table II, entries 4 and 7). Another powerful synthetic potential of the superbeads is that they can also synthesize unnatural sugar nucleotides and oligosaccharide derivatives with modifications at the nonreducing end of the sugar residues. When 2-deoxygalactose or 1-^{13}C-labeled galactose is used as starting monosaccharide instead of galactose, a novel 2″-deoxy-α-Gal epitope or a 1-^{13}C-labeled α-Gal is generated (Table II, entries 5–7). These labeled α-Gal derivatives are important tools to probe the interaction of α-Gal epitopes with anti-Gal antibodies. From these results, we conclude that the enzymes in the UDP-Gal regeneration cycle can accept different galactose derivatives as well as natural galactose.

Acceptor specificity studies on β1,4GalT, α1,3GalT, and LgtC have shown that these galactosyltransferases can utilize a broad spectrum of acceptors.[14,18] However, few data are available on the donor specificity of these glycosyltransferases because of the limited availability of the sugar nucleotide derivatives. Our superbeads technology is a tool to analyze the donor substrate specificity for a certain glycosyltransferase provided that a sugar nucleotide can be processed by the regeneration enzymes. Superbeads, therefore, can be used for the study of both the acceptor and substrate specificities.

UDP-Gal Superbug

Because the expression and purification of multiple enzymes individually are laborious and the purification process may result in decreases in enzymatic activities, whole cell reactions without isolating enzymes have been developed for carbohydrate synthesis.[19] After demonstration of the UDP-Gal synthetic pathway and the successful syntheses with recombinant enzymes on superbeads, we focused on using whole *E. coli* cells as biocatalysts. In this approach, genes encoding all related proteins in the biosynthetic pathway of galactoside are cloned in tandem into a single vector and transformed into the *E. coli* expression host. In contrast to

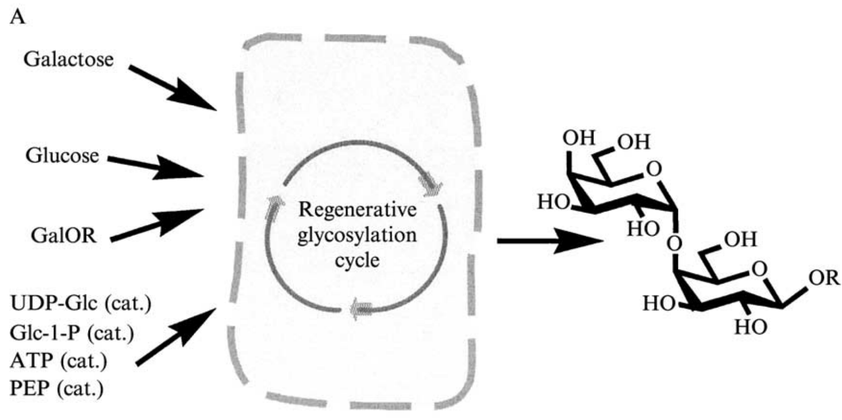
[18] (a) C. H. Hokke, A. Zervosen, L. Elling, D. H. Joziasse, and D. H. van Den Eihnden, *Glycoconj. J.* **13,** 687 (1996); (b) J. W. Fang, X. Chen, W. Zhang, A. Janczuk, and P. G. Wang, *Carbohydr. Res.* **329,** 873 (2000); (c) X. Chen, W. Zhang, J.-Q. Wang, J. W. Fang, and P. G. Wang, *Biotech. Prog.* **16,** 595 (2000); (d) K. Sujino, T. Uchiyama, O. Hindsgaul, N. O. L. Seto, W. Wakarchuk, and M. M. Palcic, *J. Am. Chem. Soc.* **122,** 1261 (2000).

[19] (a) S. Koizumi, T. Endo, K. Tabata, and A. Ozaki, *Nat. Biotechnol.* **16,** 847 (1998); (b) T. Endo, S. Koizumi, K. Tabata, S. Kakita, and A. Ozaki, *Carbohydr. Res.* **316,** 179 (1999); (c) K. Tabata, S. Koizumi, T. Endo, and A. Ozaki, *Biotech. Lett.* **22,** 479 (2000); (d) T. Endo, S. Koizumi, K. Tabata, and A. Ozaki, *Appl. Microbiol. Biotechnol.* **53,** 257 (2000); (e) T. Endo, S. Koizumi, K. Tabata, S. Kakita, and A. Ozaki, *Carbohydr. Res.* **330,** 439 (2001).

superbeads, superbug contains the necessary galactosyltransferases to accomplish the full biosynthetic cycle. Such a bacterial strain capable of simultaneous galactoside production and UDP-Gal regeneration has been named "UDP-Gal superbug" in our laboratory.[15b,c]

CKTUF Superbug: Synthesis of Globotriose and Its Derivatives

The superbug for globotrioside synthesis is constructed according to the biosynthetic pathway shown in Scheme 3B. To prevent the degradation of acceptor by β-galactosidase activity in the host strain of the pET system,



SCHEME 3. Synthesis of globotriose derivatives with superbug CKTUF. (A) Schematic presentation of the synthesis and (B) biosynthetic pathway of globotriose synthesis.

and to eliminate the need of isopropyl-1-thio-β-D-galactopyranoside (IPTG), β-galactosidase negative strain *E. coli* NM522 or DH5α and the heat-inducible pLDR20 vector are best suited for superbug application. The multienzyme plasmid construction is accomplished with the subsequent insertion of *galU*, *lgtC*, *pykF*, and *galPUT* + *galK* genes, respectively, with the corresponding ribosomal binding sites (rbs) and N-terminal His_6 tags into the pLDR20 plasmid. Each gene (except the *galK* gene, which has a natural rbs in the coding sequence of the upstream *galPUT* gene in the *gal* operon) is preceded by a Shine–Dalgarno sequence for ribosome binding to assure adequate translation. Because *galK* and *galPUT* exist in the same *gal* operon and close to each other, they are cloned together into the pET15b vector and then into the pLDR20 vector (Scheme 4). The plasmid pLDR20-CKTUF was then transformed into the NM522 strain to form globotriose-producing cells. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of this superbug indicates that all five enzymes are expressed. The activities of these enzymes are further confirmed by the synthesis of globotriose and its derivatives.

It should be mentioned that our whole cell synthesis of globotriose oligosaccharides is actually a two-step procedure, which is distinct from the commonly employed fermentative processes.[20] 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 harvested cells are permeabilized by surfactant treatment and a multiple freeze–thaw procedure. They are then employed as catalysts in the reaction mixture that contains glucose, galactose, acceptor (lactose or its derivative), and catalytic amounts of PEP, ATP, Glc-1-P, and UDP-Glc. This two-step process avoids the possible inhibition of cell growth by substrates and product; moreover, it allows the use of high cell concentrations (i.e., high catalyst concentrations) and facile manipulation of substrate concentrations. The permeabilization allows for better transfer of substrates and sugar products into and out of the cells.[18c]

The CKTUF superbug has been compared with purified recombinant enzymes for capacity to synthesize globotriose and its derivatives[14b] (Table III). Good acceptors for purified LgtC are accepted well by the CKTUF superbug. For most substrates shown, the purified enzyme reactions give higher yields, probably because of the higher concentration of sugar nucleotide in the reaction mixtures. The most notable exception to this

[20] (a) R. Patnaik and J. Liao, *Appl. Environ. Microbiol.* **60,** 3903 (1994); (b) J. T. Kealey, L. Liu, D. V. Santi, M. C. Betlack, and P. J. Barr, *Proc. Natl. Acad. Sci. USA* **95,** 505 (1998); (c) K. Li and J. W. Frost, *J. Am. Chem. Soc.* **120,** 10545 (1998).

TABLE III
SYNTHESES OF GLOBOTRIOSE DERIVATIVES WITH RECOMBINANT ENZYMES AND WHOLE CELLS[a]

Entry	Acceptor	Product		Yield (%) Whole cells	Yield (%) Enzyme
1	[structure]	[structure]	**8**	75	92
2	[structure]	[structure]	**3**	85	66
3	[structure]	[structure]	**9**	60	77
4	[structure]	[structure]	**10**	50	84
5	[structure]	[structure]	**11**	50	81
6	[structure]	[structure]	**12**	45	45
7	[structure]	[structure]	**13**	20	10
8	[structure]	[structure]	**14**	10	5

[a] See Ref. 14b for detailed reaction conditions for globotriose synthesis with purified enzymes. The whole cell-catalyzed reactions are described in the experimental section.

is benzyllactoside (LacOBn) (Table III, entry 2). This acceptor proved better in the whole cell-catalyzed reaction. In this case, the cell-confined LgtC transferase may prefer benzyllactose, as it is a better mimic of the natural lactosylceramide substrate.[21]

[21] W. W. Wakarchuk, A. Cunningham, D. C. Watson, and N. M. Young, *Protein Eng.* **11,** 295 (1998).

αKTUF Superbug: Synthesis of α-Gal Epitope

Given the efficiency of the superbug system, and the increasing availability of recombinant glycosyltransferases, various superbug strains can be conveniently constructed for specific glycosyl linkages. For example, once the *lgtC* gene is replaced by the gene encoding α1,3Gal T, the new superbug pLDR20-αKTUF is generated to synthesize α-Gal epitope. Similar to the globotriose superbug, plasmid pLDR20-αKTUF is obtained by sequential insertion of the genes encoding the five enzymes involved in the synthesis of α-Gal epitope into the pLDR20 vector. The resulting plasmid is transformed into NM522 to obtain the α-Gal superbug.[15c]

Another feature of this superbug reaction is that deoxygalactose can be utilized by superbug as a substitute for galactose to generate deoxy-α-Gal. The substrate tolerance of the superbug is being investigated further.

Conclusion

The superbeads and superbug are versatile tools that have shown utility in synthesis of galactoside analogs. At present, we are constructing other sugar–nucleotide regeneration systems to synthesize diverse glycoconjugates and unnatural derivatives. The success of superbeads and the superbug technology offers the promise of easily synthesizing diversified oligosaccharides and glycoconjugates with uncommon or even unnatural sugar residues to meet increasing biochemical demands.

On the other hand, the substrate recognition between substrates and enzymes has a dual function. With the superbeads and superbug approaches, it holds promise for the screening of possible inhibitors in the biosynthetic pathway, if the substrates cannot be adopted as suitable donor or acceptors.

General Methods and Procedures

Materials and Chemical Reagents

The polymerase chain reaction (PCR) purification kit, QIAEX II gel extraction kit, QIAamp tissue kit, and DNA miniprep spin kit are from Qiagen (Santa Clarita, CA). All restriction enzymes, *Taq* DNA polymerase, 1-kb DNA ladder, and T4 DNA ligase are obtained from Promega (Madison, WI). Sodium chloride and ScintiVerse BD are from Fisher Scientific (Pittsburgh, PA). IPTG, ampicillin, [6-^{3}H]galactose, Dowex 1X8 anion-exchange resin, PEP, ATP, UDP-Glc, Glc-1-P, HEPES hemisodium salt, $MgCl_2$, $MnCl_2$, KCl, β-D-lactose, β-methyllactoside, lactulose, lactitol, β-methyllactoside, *3-O*-β-D-galactopyranosyl-D-arabinose, and bovine

β1,4-GalT are obtained from Sigma (St. Louis, MO). β-Benzyllactoside and phenyl-1-thio-β-D-lactoside have been synthesized previously.[22] All other chemicals are obtained in reagent grade from commercially available sources.

Bacterial Strains and Plasmids

Plasmid vector pLDR20 (ATCC 87205) is purchased from American Tissue Culture Collection (ATCC, manassas, VA). Plasmid vector pET15b and *E. coli* competent strain BL21 (DE3) [F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)] are from Novagen (Madison, WI). Plasmids pET15b-*α1,3GalT*, pET15b-*lgtC*, pET15b-*galK*, pET15b-*galPUT*, pET15b-*galU*, and pET15b-*pykF* are constructed as described previously.[14,15c,17] *Escherichia coli* competent strain DH5α (*lacZ*Δ*M15 hsdR recA*) is from Gibco–BRL Life Technology (Rockville, MD). *Escherichia coli* competent strain NM522 {*supE thi-1* Δ(*lac-proAB*) Δ(*mcrB-hsdSM*)5(r_K^- m_K^+) [*F proAB lacI*^{q}*Z*Δ*M15*]} is from Stratagene (La Jolla, CA). Chromosomal DNA of *Neisseria meningitidis* MC58(L3) is a kind gift from M. Gilbert (Institute for Biological Sciences, National Research Council of Canada, Ottawa, ON, Canada).

HPLC Analysis for α-Gal Production

(See Table I.) The assays are carried out at room temperature (24°) for 3 days in a final volume of 250 ml in 2-[4-(2-hydroxyethyl)-l-piperazinyl] ethanesulfonic acid (HEPES) buffer (100 m*M*, pH 7.0) containing starting materials and enzyme solutions (20 mU for each enzyme). The reaction is stopped by heating in boiling water for 10 min to precipitate the enzymes. After centrifugation for 20 min, the supernatant is analyzed by HPLC [Microsorb, 100-Å pore size amino column with CH_3CN–H_2O (65:35, v/v) as the eluent] with a refractive index (RI) detector.

Starting materials: step 1, UDP-Gal (50 m*M*), lactose (50 m*M*), $MnCl_2$ (10 m*M*); step 2, Gal-1-P (50 m*M*), UDP-Glc (50 m*M*), lactose (50 m*M*), $MnCl_2$ (10 m*M*); step 3, ATP (50 m*M*), Gal (50 m*M*), UDP-Glc (50 m*M*), lactose (50 m*M*), $MnCl_2$ (10 m*M*); step 4, ATP (5 m*M*), Gal (5 m*M*), UTP (5 m*M*), Glc-1-P (1 m*M*), lactose (5 m*M*), $MgCl_2$ (10 m*M*), $MnCl_2$ (10 m*M*); step 5, ATP (5 m*M*), Gal (5 m*M*), PEP (5 m*M*), Glc-1-P (1 m*M*), UDP (1 m*M*), lactose (5 m*M*), $MgCl_2$ (10 m*M*), KCl (100 m*M*), $MnCl_2$ (10 m*M*).

[22] (a) W. Zhang, J. Wang, J. Li, L. Yu, and P. G. Wang, *J. Carbohydr. Chem*. **18,** 1009 (1999); (b) J. Fang, Ph.D. thesis. Wayne State University, Detroit, MI, 2000.

Synthesis of Galactose-Containing Oligosaccharides with UDP-Gal Superbeads

(See Table II; and the supplementary materials in Ref. 15a for detailed reaction conditions.) Superbeads are prepared by incubation of Ni^{2+}-NTA agarose with cell lysate of α1,3GalT, β1,4GalT, or LgtC (5 ml, 5 U), washed with Tris-HCl buffer (20 m*M*, pH 8.0) containing 0.5 *M* NaCl, and added to a mixture of starting materials in HEPES buffer (100 m*M*, pH 7.5). The reaction is carried out in a rotor mixer for 4 days at room temperature (24), when thin-layer chromatographic analysis [2-propanol–NH_4OH–H_2O, 7:3:2 (v/v/v)] indicates that the reaction is complete. After the reaction, the superbeads are separated from the reaction mixture by centrifugation and washed for another batch of reaction. The mixture is passed through Dowex 1-X8 (Cl^-) anion-exchange resin and purified by gel-permeation chromatography (Sephadex G-15; Sigma) with water as the mobile phase. The product-containing fractions are pooled and lyophilized.

The starting materials are acceptor (0.24 mmol), ATP (13.2 mg, 24 μmol), PEP (91.2 mg, 0.48 mmol), UDP (10 mg, 24 μmol), Glc-1-P (7.3 mg, 24 μmol), Gal (54 mg, 0.3 mmol), $MgCl_2$ (10 m*M*), $MnCl_2$ (10 m*M*), and KCl (100 m*M*); the total volume is 30 ml. For entry 1 (Table II), the superbeads are added directly to a reaction mixture containing 5 U of commercial available β1,4GalT. For entries 2, 3, 5, and 6 (Table II), superbeads (5 ml, containing 7.5 U of individual enzymes) are obtained by incubation with 16 ml of cell lysate mixture of GalK, GalPUT, GalU, and PykF with a volume ratio of 4:1:1:2. For entries 4 and 7 (Table II), the reaction mixture contains 2 equivalents of Gal or its derivatives and 4 equivalents of PEP. For the gram-scale synthesis of Galα1,3LacOBn: superbeads (40 ml) are obtained by incubation with 120 ml of cell lysate mixture (GalK, GalPUT, GalU, and PykF at a volume ratio of 4:1:1:2) and subsequent incubation with cell lysate of α1,3GalT (40 ml, 40 U). Starting materials are LacOBn (1 g, 2.4 mmol), ATP (132 mg, 240 μmol), PEP (912 mg, 4.8 mmol), UDP (100 mg, 240 μmol), Glc-1-P (73 mg, 240 μmol), μmol), Gal (540 mg, 3 mmol), $MgCl_2$ (10 m*M*), $MnCl_2$ (10 m*M*), and KCl (100 m*M*), and the total volume is 250 ml.

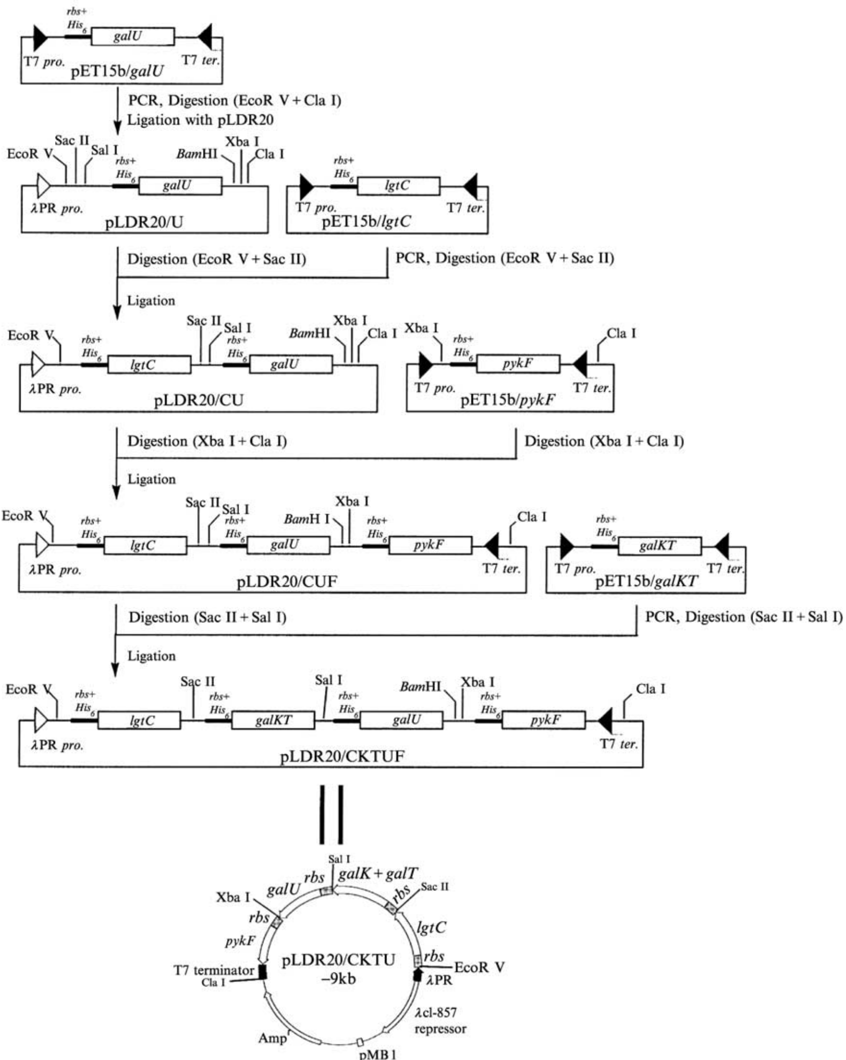
Construction of Superbug CKTUF-NM522

By using the preconstructed plasmids PET15b-*galK*, PET15b-*galPUT*, pET15b-*galU*, pET15b-*lgtC*, and pET15b-*pykF* as the PCR templates, the genes of the enzymes involved in the biosynthetic pathway of globotriose are subcloned one by one into the pLDR20 vector with the ribosomal binding site and the His_6 tag-encoding sequence preceding

each gene (Scheme 4) to form the final plasmid pLDR20-CKTUF. This pLDR20 vector contains an ampicillin resistance gene, a p_R promoter, and a *c*I857 repressor gene. Because *galK* and *galPUT* exist in the same *gal* operon and close to each other, they are cloned together into the pET15b vector and then into the pLDR20 vector. Briefly, the stepwise construction of plasmid pLDR20-CKTUF is as follows: first, the *galU* gene is amplified by PCR from pET15b-*galU* with primers *galU*-F′ (5′-CCGGATATCCCGCGGGTCGACAATAATTTTGTTTAACTTTAAGAAGG-3′) and *galU*-R′ (5′-GCATCGATGGTCTAGAGGATCCTTACCITAATGCCCATCTC-3′), which introduces the *Eco*RV, *Sac*II, and *Sal*I, or *Xba*I and *Cla*I restriction sites, respectively. The PCR product is digested with *Eco*RV and *Cla*I and inserted into the multiple-cloning site of the pLDR20 vector previously cut with the same enzymes. Successful cloning is verified by restriction mapping and the expression of GalU is confirmed by SDS–PAGE. Second, primers *lgtC*-F′ (5′-GGATCCATATGACTAGT GATATCAATAATTTTGTTTAACTTTAAGAAGG-3′) and *lgtC*-R′ (5′-TCCCCGCGGTCATCAGTGCGGGACGGCAAGTTTGCC-3′) are used to amplify the *lgtC* gene from plasmid PET15b-*lgtC*. The PCR product is digested and inserted into the *Eco*RV and *Sac*II restriction sites of the plasmid pLDR20-U to form plasmid pLDR20-CU. Third, the pET15b-*pykF* plasmid is digested with *Xba*I and *Cla*I, and the smaller fragment containing the *pykF* gene, ribosomal binding site, sequence for the N-terminal His_6 tag, and T7 terminator is purified and inserted into the *Xba*I and *Cla*I restriction sites of the plasmid pLDR20-CU to form plasmid pLDR20-CUF. Finally, the *galK* and *galPUT* genes are amplified from plasmid pET15b-*galKT* (constructed by inserting the gene sequence encoding both *galK* and *galPUT* into a pET15b vector between the *Nde*I and *Bam*HI restriction sites) by using the primers *galKT*-F′ (5′-TCCCCGCGGCCCGGGAATAATTTTGTTTAACTTTAAGAAGG-3′) and *galKt*-R′ (5′-CGCGTCGACTCAGCACTGTCCTGCTCCTTG-3′). The PCR product is digested and inserted into the *Sac*II and *Sal*I restriction sites of plasmid pLDR20-CUF to form plasmid pLDR20-CKTUF. This final plasmid pLDR20-CKTUF, harboring five genes, is subsequently transformed into competent NM522 cells to make NM522 (pLDR20-CKTUF) globotriose-producing cells (Scheme 4).

Synthesis of Globotriose and Its Derivatives with Superbug CKTUF-NM522

(See Table III.) Globotriose-producing superbug NM522 (pLDR20-CKTUF) is grown in 4-liter shake flasks. The expression of the target genes in the superbug is initiated by increasing the temperature from 30 to 40°.



SCHEME 4. Construction of plasmid pLDR20-CKTUF for CKTUF superbug.

After being shaken at 40° for 3–3.5 h, the cells are separated from the medium by centrifugation (5000 *g* for 20 min at 4°) and suspended in 100 ml of Tris-HCl buffer (20 m*M*, pH 8.5) containing 1% Triton X-100. For better results, the cell suspension is freeze–thawed twice before being applied in the reaction. For small-scale analysis, the reaction is performed with 0.14 g (wet weight) of cells in a 1-ml reaction volume containing Gal (50 m*M*), Lac (25 m*M*), Glc (50 m*M*), PEP (5 m*M*), Glc-1-P (2 m*M*), UDP-Glc (2 m*M*), ATP (2 m*M*), $MgCl_2$ (10 m*M*), KCl (100 m*M*), $MnCl_2$ (10 m*M*), and HEPES (50 m*M*, pH 7.4). The reaction is carried out at room temperature and formation of the trisaccharide product is monitored by HPLC with a Microsorb 100-A pore size amino column using CH_3CN–H_2O (65:35, v/v) as eluent. To optimize the conditions, multiple 1-ml reactions are set up with different starting material compositions.

Large-scale production consists of two steps: culturing the superbug cells and producing trisaccharide as catalyzed by the cells. NM522 (pLDR20-CKTUF) cells are first grown at 30° in a 10-liter fermentor, and then enzyme expression is induced by increasing the temperature to 40° for 3 h. The cells are separated from the medium by centrifugation. The cell pellet (65 g, wet weight) is stored at −20° and freeze–thawed twice before use in the reaction. Gram-scale synthesis is performed with a variety of galactose or lactose derivatives as acceptors for the LgtC. For a typical synthesis reaction, to a 250-ml flask is 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*). Superbug cells [12 g in 72 ml of Tris-HCl buffer (20 m*M*, pH 8.5) containing 1% Triton X-100, obtained from a 2-liter bacterial culture] are then added to bring the total reaction mixture volume to 120 ml. The reaction is agitated with a magnetic stirrer at room temperature (24°) for 36 h. The reaction is monitored by thin-layer chromatograpy (TLC) [2-proponal–H_2O–NH_4OH (7:3:2, v/v/v)] and HPLC. After 36 h, the reaction is stopped by heating the flask to 100° for 10 min. Insoluble components are sedimented by centrifugation at 5000 *g* for 20 min and the pellet is washed twice with 50 ml of deionized water. The combined supernatants are subsequently passed through an anion-exchange column and a cation-exchange column. The concentrated eluate is loaded onto a Sephadex G-15 gel-filtration column (120 × 4 cm) with water as the mobile phase. The desired fractions are pooled and lyophilized to give the derivatives of globotriose. The following compounds have been prepared according to this method.

*α-D-Galactopyranosyl-(1 → 4)-β-D-galactopyranosyl-(1 → 4)-D-glucopyranose (**8**).* (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; mass spectrometry (MS) [fast atom bombardment (FAB)] 527 ($M + Na^+$); high-resolution MS (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 (**3**).* (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 ($M + Na^+$); 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 (**9**).* (756 mg, 50%) ^{1}H NMR (500 MHz, D_2O): δ4.79 (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 M Hz, 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) 541 ($M + Na^+$); 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-thioglucopyranoside (**10**).* (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) 619 ($M + Na^+$); 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 (**11**).* (830 mg, 60%) ^{1}H NMR (500 MHz, D_2O): δ5.11 (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): δ101.34, 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) 497 ($M + Na^+$); 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)-β-D-fructofuranose (**12**).* (662 mg, 45%) ^1H NMR (500 MHz, D_2O): δ4.77 (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): δ103.16, 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) 527 ($M + Na^+$); 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-gludititol (**13**).* (296 mg, 20%) ^1H NMR (400 MHz, D_2O): δ4.91 (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): δ103.72, 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 ($M + Na^+$).

*Methyl-α-D-galactopyranosyl-(1 → 4)-β-D-galactopyranoside (**14**).* (104 mg, 10%) ^1H NMR (500 MHz, D_2O): δ4.81 (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) 379 ($M + Na^+$); HRMS: calculated for $C_{13}H_{24}O_{11}Na$ ($M + Na^+$) 379.1216, found 379.1218.

Acknowledgments

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