A Unique Chemoenzymatic Synthesis of α-Galactosyl Epitope Derivatives Containing Free Amino Groups: Efficient Separation and Further Manipulation

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A novel chemoenzymatic approach for the synthesis of oligosacchrides containing free amino groups has been developed in which thermophilic glycosidases and a fusion enzyme containing catalytic domains of uridine-5′-diphospho-galactose 4-epimerase and α(1→3) galactosyltransferase were used. This methodology, in conjunction with a convenient purification procedure employing ion exchange chromatography, facilitates the lengthy and high-cost process of carbohydrate synthesis. The prepared oligosaccharides range from disaccharide lactosamine (**1a**), trisaccharide α-Gal-(1→3)-*β*-Gal-(1→4)-GlcNH$_2$ (**2**), tetrasaccharide *β*-Gal-(1→4)-*β*-GlcNH$_2$-(1→3)-*β*-Gal-(1→4)-*β*-Glc-N$_3$ (**7**), to pentasaccharide α-Gal-(1→3)-*β*-Gal-(1→4)-*β*-GlcNH$_2$-(1→3)-*β*-Gal-(1→4)-*β*-Glc-N$_3$ (**15**). Compounds **2** and **15** are derivatives of natural α-Gal epitopes. Both of them have shown comparable activities with their natural parent compounds toward human anti-Gal IgG. This method provides a practical approach for the preparation and purification of oligosaccharides containing free amino groups, which can be further derivatized for the enhancement of biological activities.

Introduction

Aminosugars, e.g. glucosamine, galactosamine, and mannosamine, are widely distributed in living organisms where they constitute building blocks of glycoconjugates such as glycopeptides and glycolipids.[1] They are found in milk, in blood group substances, and in lipopolysaccharide antigens, where they serve as part of the cell surface antigenic determinants (epitopes).[2] The chemical synthesis of oligosaccharides containing aminosugar moieties (amino oligosaccharides) relies on glycosylation employing the oxazoline method or phthalimido protecting scheme.[3] Recently, enzyme-catalyzed synthesis of oligosaccharides has evolved into a powerful shortcut for those chemical strategies.[4] Nevertheless, the advantages of enzymatic synthesis are sometimes overshadowed by the difficulty of isolating the product(s) from the reaction mixture normally containing very similar compounds.[5] Various separation methodologies, such as size exclusion chromatography (SEC),[4,5,6,7] ion exchange chromatography (IEC),[8] HPLC,[7] and charcoal adsorption,[8] have been evaluated for isolation efficiency. SEC, which is conceivably the most used method for separating enzyme-synthesized oligosaccharides, is limited to compounds which have significant molecular weight differences. Biomolecules such as nucleic acids and proteins are frequently isolated utilizing IEC. The use of IEC in carbohydrate chemistry was reported, exploiting the weakly acidic character of polyhydroxy groups of "neutral" sugars.[9] In principle, amino oligosaccharides should be easy to isolate with IEC since the unprotected amino group would have a strong affinity to the cation-exchange resin.[8] However, such amino/resin interactions will be lost if amino-protected monosaccharides or oligosaccharides are used in synthetic procedures as previously reported in the literature.[4]

α-Galactosyl epitopes (α-Gal),[2] which include trisaccharides [α-Gal-(1→3)-*β*-Gal-(1→4)-*β*-Glc-R] and [α-Gal-(1→3)-*β*-Gal-(1→4)-*β*-GlcNAc-R] and pentasaccharide [α-Gal-(1→3)-*β*-Gal-(1→4)-*β*-Glc-(1→3)-*β*-Gal-(1→4)-*β*-GlcNAc−R] (all structures bearing a α-Gal-(1→3)Gal*β* terminus), are abundantly expressed on the cell surface of most mammals with the exception of humans, apes, and other Old World primates. On the contrary, anti-Gal antibodies exist only in these primates. Studies concluded that the major hyperacute rejection during xenotransplantation was caused by α-Gal epitopes binding specifically to human anti-Gal antibodies (anti-Gal). This discovery has prompted experimental attempts to overcome hyperacute rejection by either depleting the recipient's anti-Gal through α-Gal immobilized affinity columns or antagonizing anti-Gal by infusing soluble synthetic α-Gal oligosaccharides.[2b,10] Our laboratory has developed an effective chemoenzymatic method to syn-

(1) (a) Varki, A. *Glycobiology* **1993**, *3*, 97. (b) Ferzi, T.; Solomon, J. C.; Yuen, C.-T.; Jeng, K. C. G.; Frigeri, L. G.; Hsu, D. K.; Liu, F.-T. *Biochemistry* **1994**, *33*, 6342.

(2) (a) Cooper, D. K. C.; Koren, E.; Oriol, R. *Immunol. Rev.* **1994**, *141*, 31. (b) Galili, U. *Immunol. Today* **1993**, *14*, 480. (c) Sandrin, M. S.; Vaughan, H. A.; McKenzie, I. F. C. *Transplant. Rev.* **1994**, *8*, 134. (d) Samuelsson, B. E.; Rydberg, L.; Breimer, M. E.; Backer, A.; Gustavsson, M.; Holgersson, J.; Karlsson, E.; Uyterwaal, A.-C.; Cairns, T.; Welsh, K. *Immunol. Rev.* **1994**, *141*, 151. (e) *Nature (Special Issue)* **1998**, January, *391*, 309. (f) Platt, J. L. *Nature* **1998**, *392*, 11 (Supp).

(3) Banoub, J.; Boullanger, P.; Lafont, D. *Chem. Rev.* **1992**, *92*, 1167.

(4) (a) Wong, C.-H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 412 and 521. (b) Toone, E. J.; Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *Tetrahedron* **1989**, *45*, 5365. (c) Takayama, S.; Wong, C.-H. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 3. (d). Wong, C.-H.; Haynie, S. L.; Whitesides, G. M. *J. Org. Chem.* **1982**, *47*, 6.

(5) Kimura, T.; Takayama, S.; Wong, C.-H. *Angew. Chem., Int. Engl.* **1996**, *35*, 2348.

(6) Nilsson, K. G.; Eliasson, A.; Lorek, U. *Biotechnol. Lett.* **1995**, *17*, 717.

(7) Iwanowitsch, A.; Friebolin, H.; Carganico, R.; Errenst M.; Weissharr, G.; Brunner, H.; Mann, H. *Biol. Chem.* **1998**, *379*, 737.

(8) Samain, E.; Drouillard, S.; Heyraud, A.; Driguez, H.; Geremia, R. A. *Carbohydr. Res.* **1997**, *302*, 35.

(9) Rochlin, R. D.; Pohl, C. A. *J. Liq. Chromatogr.* **1983**, *6*, 1577.

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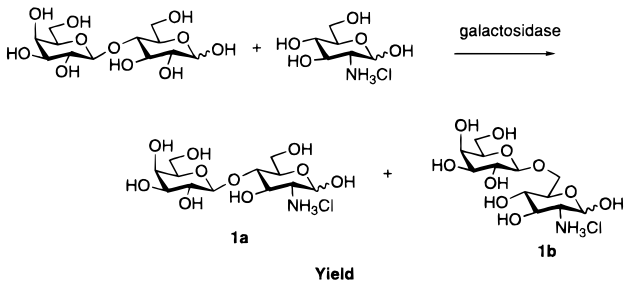
Scheme 1



Yield

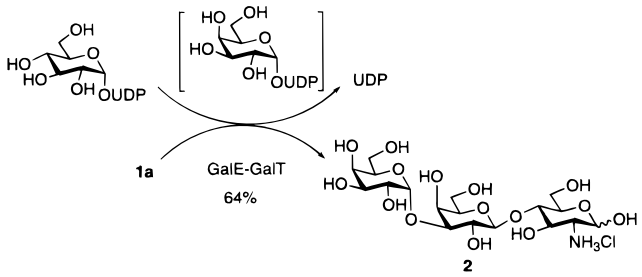
Enzyme	1a	1b
Gly01-006	19	0
Gly01-008	3	10
Gly01-009	23	0
Galactosidase from *Bacillus circulans*	10	6

thesize α-Gal epitopes utilizing α(1→3)-galactosyltransferase.[11] More recently we have focused our attention on chemoenzymatic synthesis of α-Gal analogues containing versatile handles, such as a free NH_2 group,[12] which can be used for further synthetic manipulation to generate diversity. These derivatives would present the possibility of identifying unnatural ligands with enhanced binding affinity toward anti-Gal antibodies. This paper reports a unique chemoenzymatic preparation of oligosaccharides with free amino groups ranging from disaccharides to pentasaccharides. In this synthetic process, saccharides bearing free amino groups were used as acceptors of enzyme-catalyzed glycosylations, involving thermophilic glycosidases and fusion enzymes, to produce more complex glycoconjugates. This novel approach, coupled with IEC methodology, allows for a convenient way of synthesizing and purifying amino saccharides that could be used as intermediates for further derivatization.[3,13]

Results and Discussion

Disaccharide **1a**, lactosamine, which can be found not only in α-Gal epitopes but also in the core structure of many glycoproteins and glycolipids, was chosen as the primary target. β(1→4) Galactosyltransferase-catalyzed glycosylation, which is convenient and gives definite regio- and stereoselectivity, was first employed using UDP-galactose (UDP-Gal) as donor and glucosamine as acceptor. Unfortunately no significant preferred product was obtained. A library of thermophilic glycosidases from Diversa Corporation,[12] which included 10 enzymes, as well as a common galactosidase from *Bacillus circulans*, was then screened. The results indicated that two thermophilic enzymes, Gly001-06 and Gly001-09, had excellent regioselectivity toward glucosamine (Scheme 1). Both enzymes gave the desired product **1a** with a β(1→4)-linkage. Compound **1a** was isolated and purified on a cation-exchange resin column using 0.15 M HCl as eluent, followed by anion-exchange resin to neutralize the acid with yields of 19% and 23%, respectively. Glucosamine was recovered by eluting with 0.6 M HCl while the resin was simultaneously regenerated. The reaction with Gly001-09 was scaled up to a multigram level and afforded a similar yield. Thermophilic enzyme Gly001-08 rendered a β(1→6)-linked isomer **1b** in 10% yield and **1a** as a minor product (3%). It is noteworthy that the reactions with thermophilic enzymes as catalysts were conducted at 85 °C or even 90 °C. The conventional galactosidase from *B. circulans* also gave the identical isomers in decreased regioselectivity (**1a**, 10% yield; **1b**, 6% yield) at room temperature. Since these regioisomers have the same molecular weight, it is almost impossible to separate them using SEC. Fortunately, individual isomers were successfully isolated with IEC using a dilute concentration of HCl (0.05 M).

Scheme 2

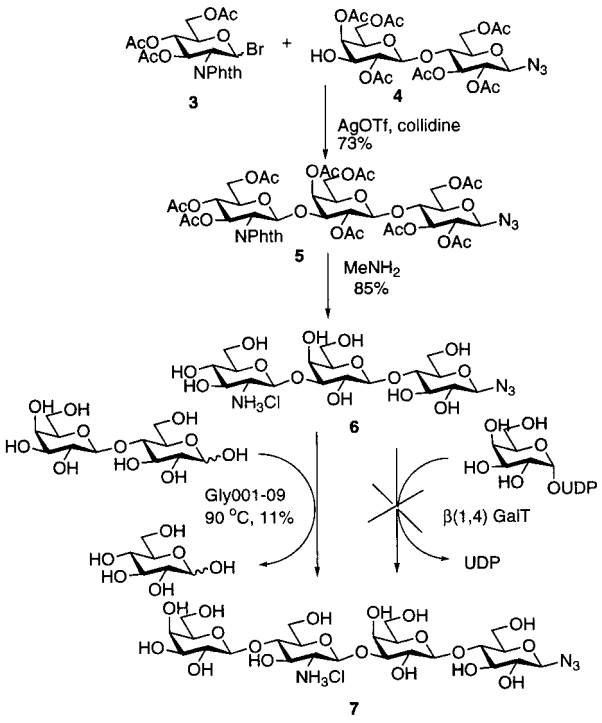


To further demonstrate the potential of IEC, the trisaccharide α-Gal-(1→3)-β-Gal-(1→4)-GlcNH_3Cl **2** was prepared from **1a**. A fusion enzyme[14] (GalE-GalT) containing both uridine-5′-diphospho-galactose 4-epimerase (GalE) and α(1→3) galactosyltransferase (GalT) was used as a catalyst with UDP-glucose (UDP-Glc) as a glycosylation donor. This enzyme showed excellent regio- and stereoselectivity (Scheme 2). The desired product **2** was isolated in 64% yield using IEC (0.05 M HCl as eluent), and the starting material was recovered in 22% yield (0.15 M HCl). GalE-GalT was constructed by in-frame fusion of the *Escherichia coli* gene GalE to the 3′-terminus of a truncated bovine GalT gene within a high-expression plasmid. It is particularly noteworthy that this enzyme has two functions: one as an epimerase and the other as a galactosyltransferase. It uses relatively inexpensive UDP-glucose (UDP-Glc) instead of high-cost UDP-Gal as a donor. Three galactosidases, α-galactosidase from *Aspergillus niger*, α-galactosidase from Green Coffee Bean, and Gly001-10, were evaluated to catalyze a transgalactosylation between lactose and **1a**. Although all three galactosidases exhibited catalytic activity after the reactions were conducted over long periods, the yields were quite low and a mixture of **2** and its regioisomer was recovered.

The preparation of tetrasaccharide [β-Gal-(1→4)-β-GlcNH_2-(1→3)-β-Gal-(1→4)-β-Glc-N_3 HCl] **7** began with the glycosylation of 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide **3**[15] and *O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)−(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl azide **4**[16] to afford trisaccharide **5** in a

(10) Rieben, R.; Von Allmen, E.; Korchagina, E.; Nydegger, U.; Neethling, F. A.; Kujundzic, M.; Koren, E.; Bovin, N.; Cooper, D. K. C. *Xenotransplantation* **1995**, *2*, 98.
(11) Fang, J.; Li, J.; Chen, X.; Zhang, Y.; Wang, J.; Guo, Z.; Zhang, W.; Yu, L.; Wang, P. G. *J. Am. Chem. Soc.* **1998**, *120*, 6636.
(12) Fang, J.; Xie, W.; Li, J.; Wang, P. G. *Tetrahetraon Lett.* **1998**, *329*, 919.
(13) Liang, R.; Yan, L.; Loebach, J.; Ge, M.; Uozumi, Y.; Sekanina, K.; Horan, N.; Gildersleeve, J.; Thonpson, C.; Smith, A.; Biswas, K.; Still, W. C.; Kahne, D. *Science* **1996**, *274*, 1520.
(14) Chen, X.; Fan, H.; Zhang, Y.; Brew, K.; Wang, P. G. Manuscript in preparation.
(15) Lemieux, R. U.; Takeda, T.; Chung, B. Y. *ACS Symp. Ser.* **1976**, *39*, 90.
(16) Zhang, W.; Wang, J.; Li, J.; Yu, L.; Wang, P. G. *J. Carbohydr. Chem.* **1999**, submitted.

Scheme 3



yield of 73% (Scheme 3). The azido group was purposefully introduced for its synthetic flexibility in the solid-phase synthesis of glycopeptides,[17] glycopolymers, and glycodendrimers.[18] It can also be transformed to other useful glycosylation functionalities such as glycosyl fluorides for orthogonal oligosaccharide synthesis.[19] Deprotection of **5** using a 40% methylamine aqueous solution afforded **6** in 85% yield. Compound **6** did not undergo glycosylation with UDP-Gal and β(1→4)galactosyltransferase; however, it still served as a substrate for the thermophilic enzyme Gly001-09 to yield an important intermediate amino tetrasaccharide **7** in 11% yield after purification with IEC.

In comparison, tetrasaccharide **7** was also prepared on the basis of chemical transformation (Scheme 4). In a subsequent synthetic scheme, peracetyllactose **8** was chemically transformed to its azido derivative **9**.[20] Via an efficient and simple isopropylidenation using acetone and trimethylsilyl chloride (TMSCl),[21] **9** was converted to the desired 3′,4′-*O*-isopropylidene derivative **10**. Benzoylation of the free hydroxyl groups in **10**, followed by deisopropylidenation in a 60% acetic acid solution, rendered compound **12**. Compound **1a** was modified to Schmidt type donor **13**.[22] Compounds **12** and **13** were then coupled through a regioselective Schmidt glycosylation to give **14** in 86% yield. Deprotection of **14** with methylamine afforded **7** in 92% yield.

To prepare pentasaccharide [α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNH$_2$-(1→3)-β-Gal-(1→4)-β-Glc-N$_3$ HCl] **15**, compound **7** from either Scheme 3 or Scheme 4 was galactosylated with GalE-GalT. Compound **15** was isolated with IEC in an 87% yield (Scheme 5).

To evaluate the consequence of the substitution of acetamido group in α-Gal epitopes with free amino group, compounds **2** and **15** along with their parent α-Gal-(1→3)-β-Gal-(1→4)-GlcNHAc **16** and α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNHAc-(1→3)-β-Gal-(1→4)-β-Glc-N$_3$ **17**[11] were subjected to an ELISA assay.[23] The results demonstrated compounds **2** and **15** had comparable inhibition activities toward human anti-Gal IgG with their parent compounds **16** and **17** (Scheme 6). This indicated the free amino groups did not significantly change the binding ability of α-Gal epitopes to the antibody. Work is in progress to attach a variety of functional groups on the amino group and generate a large number of α-Gal derivatives to screen for the tight-binding structures.

Conclusion

Enzyme-catalyzed glycosylation to produce amino sugar moieties is a powerful tool in the field of carbohydrate chemistry. This efficient stereo- and regioselective enzymatic method is being exploited to prepare oligosaccharides with unprotected amino groups, producing products ranging from disaccharide to pentasaccharide. This novel approach, combined with IEC methodology, allows for a facile way to synthesize and purify amino saccharides. Upon isolation of these amino oligosaccharides, further chemical or enzymatic manipulation will lead to more complex molecules for identification of ligands with enhanced biological activities.

Experimental Section

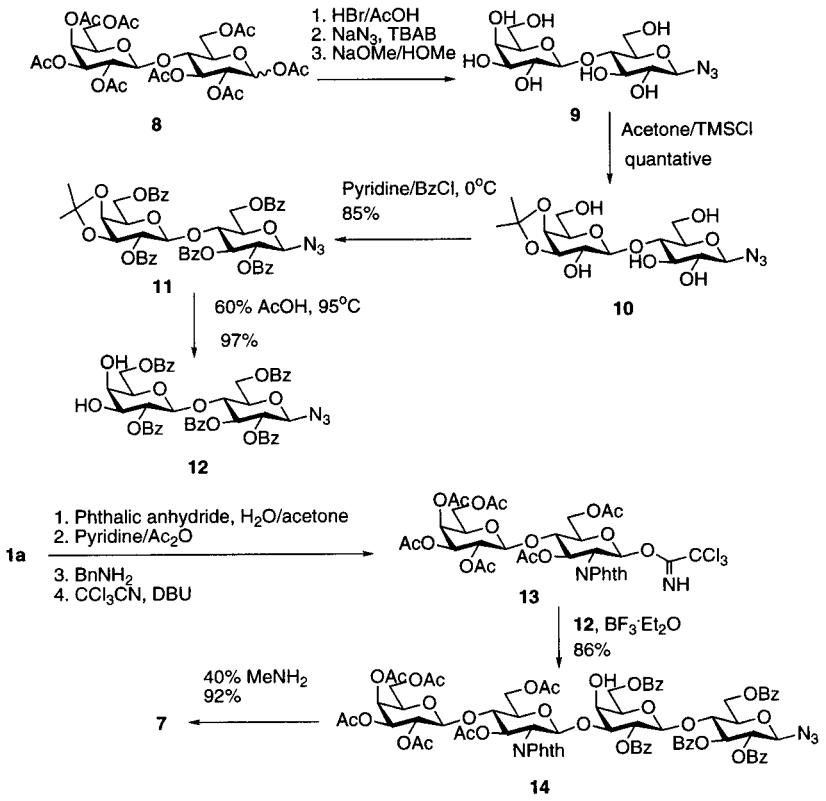
General. ^{1}H and ^{13}C NMR spectra were recorded on a 400 or 500 MHz spectrometer. Mass spectra (FAB or ESI) were run at the mass spectrometry facility at the University of California, Riverside. Thin-layer chromatography was conducted on Baker Si$_{250F}$ silica gel TLC plates with a fluorescent indicator. The library of thermophilic glycosidases was a gift from Diversa Corporation (San Diego, CA). Bovine serum albumin (BSA), Type AB human serum, peroxidase conjugated-goat anti-human IgG antibody, and mouse laminin (a basement membrane glycoprotein containing 50−70 α-Gal epitopes per molecule) were purchased from Sigma. Other reagents are all from commercial available sources.

General Procedure of Ion Exchange Chromatography. The reaction mixture was loaded onto a cation-exchange column after the reaction was conducted for a period of time. Unreacted starting materials and byproducts, which did not contain amino groups, were removed with distilled water, and the column was washed with a dilute HCl solution. The concentration of HCl was dictated by the type of product: monosaccharide, 0.6 M; disaccharide, 0.15 M; 0.05 M HCl was required for the isolation of regioisomers **1a** and **1b**; trisaccharide, 0.06 M; tetrasaccharide, 0.025 M; pentasaccharide, 0.01 M. The elution was monitored by TLC. The fractions containing the desired products were collected and neutralized with anion-exchange resin (Dowex, Marathon WBA, OH$^-$ form, 20−50 mesh). After filtration, the anion-exchange resin was washed with distilled water. The combined solution was concentrated under reduced pressure and then lyophilized to give the pure product as a hydrochloride salt.

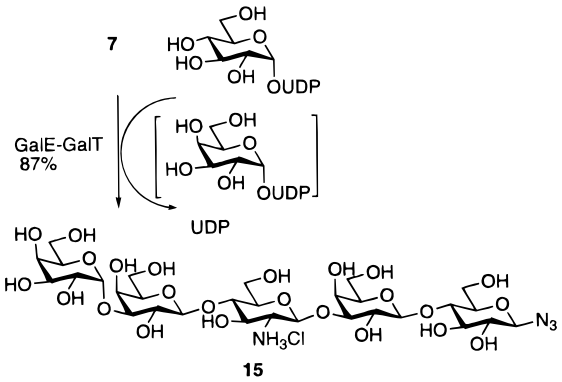
General Procedure of Glycosidase-Catalyzed Glycosidation. To a mixture of lactose (20 mmol) and glucosamine hydrochloride (2 mmol) was added the following quantity of enzyme [Gly001-06, 2 mL (1.1 mg/mL); Gly001-08, 2 mL (1.9 mg/mL); Gly001-09, 2 mL (1.8 mg/mL); or galactosidase from

(17) Kunz, H.; Dombo, B. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 711.
(18) Roy, R. *Curr. Opin. Struct. Biol.* **1996**, *6*, 692.
(19) Broder, W.; Kunz, H. *Bioorg. Med. Chem.* **1997**, *5*, 1.
(20) Tropper, F. D.; Andersson, F. O.; Braun, S.; Roy, R. *Synthesis* **1992**, 618.
(21) Nishida, Y.; Thiem, J. *Carbohydr. Res.* **1994**, *263*, 295.
(22) Sadozai, K. K.; Nukada, T.; Ito, Y.; Nakahara, Y.; Ogawa, T. *Carbohydr. Res.* **1986**, *157*, 101.
(23) Galili, U.; Ron, M.; Sharon, R. *J. Immunol. Methods* **1992**, *151*, 117.

Scheme 4



Scheme 5



Scheme 6

Compound	IC_{50}
α-Gal-(1→3)-β-Gal-(1→4)-GlcNH$_2$ (**2**)	9.4 μM
α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNH$_2$-(1→3)-β-Gal-(1→4)-β-Glc-N$_3$ (**15**)	24.2 μM
α-Gal-(1→3)-β-Gal-(1→4)-GlcNHAc (**16**)	9.0 μM
α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNHAc-(1→3)-β-Gal-(1→4)-β-Glc-N$_3$ (**17**)	8.2 μM

B. circulans, 10 mg]. The volume of the solution was adjusted to 10 mL using a phosphate buffer (50 mM, pH 6.0). The reactions were conducted at 90 °C for Gly001-06, 85 °C for Gly001-08 and Gly001-09, and at room temperature for galactosidase from *B. circulans*. After the mixtures were stirred for 3 days (Gly001-06 and Gly001-09), 3 h (Gly001-08), or 2 h (galactosidase from *B. circulans*), the product(s) was isolated with IEC as previously described. The ratios of regioisomers were calculated from ^{1}HNMR of the isomeric mixtures and the weights of isolated individual pure compounds. ***O*-β-D-Galactopyranosyl-(1→4)-2-amino-2-deoxy-D-glucopyranose (1a):** ^{1}H NMR (D_2O, selected data) $\delta = 5.31$ (d, $J = 3.6$ Hz, 1-H α), 4.85 (d, $J = 8.4$ Hz, 1-H, β), 4.34 (d, $J = 7.6$ Hz, 1′-H, β); ^{13}C NMR (D_2O, selected data) $\delta = 103.30$ (1′-C, β), 92.80 (1-C β), 89.11 (1-C α); HRFABMS calcd for $C_{12}H_{24}NO_{10}$ (M^+) 342.1400, found 342.1399. ***O*-β-D-Galactopyranosyl-(1→6)-2-amino-2-deoxy-D-glucopyranose (1b)**: ^{1}H NMR (D_2O, selected data) $\delta = 5.30$ (d, $J = 2.8$ Hz, 1-H α), 4.85 (d, $J = 8.0$ Hz, 1-H β), 4.36 (d, $J = 7.2$ Hz, 1′-H, β); ^{13}C NMR (D_2O, selected data) $\delta = 103.64$ (1′-C, β), 93.11 (1-C, β), 89.46 (1-C, α).

General Procedure of the Fusion Enzyme GalE-GalT-Catalyzed Glycosylation. GalE-GalT was added to a Tris-HCl buffer (100 mM, pH = 7.0) solution of substrate (40 mM), UDP-glucose (44 mM), $MnCl_2$ (10 mM), and bovine serum albumin (BSA) (0.1%). After the mixture was agitated under argon at room temperature for 3 days, the product was separated in a cation-exchange column as previously described. It is important to note that the substrate was also recovered.

***O*-α-D-Galactopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-*O*-D-2-amino-2-deoxyglucopyranose Hydrochloride (2).** Compound **2** (605 mg, 64% yield) was synthesized via a GalE-GalT-catalyzed reaction with **1a** as the substrate and then was isolated with IEC as previously described. **1a** (22%) was recovered. **2**: ^{1}H NMR (D_2O, selected data) δ 4.35 (d, $J = 7.5$ Hz, 1H), 4.79 (d, $J = 8.1$ Hz), 4.96 (d, $J = 3.6$ Hz, 1H), 5.27 (d, $J = 3.6$ Hz); ^{13}C NMR (D_2O, selected data) δ 88.68, 92.35, 95.21, 102.70; HRFABMS calcd for $C_{18}H_{34}NO_{15}$ (M^+) 504.1928, found 504.1955.

***O*-(3,4,6-Tri-*O*-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl Azide (5).** A suspension of **4** (1.1 g, 1.75 mmol), 4A molecular sieves (2 g), AgOTf (1.37 g, 5.2 mmol), and collidine (0.7 mL, 5.2 mmol) in anhydrous CH_2Cl_2 (10 mL) was stirred for 2 h at room temperature and then cooled to −78 °C. A solution of compound **3** (2.6 g, 5.2 mmol) in CH_2Cl_2 (10 mL) was added dropwise to the cooled suspension. The reaction mixture was gradually warmed to room temperature and stirred for an additional 12 h. The reaction mixture was filtered through a pad of Celite, and the solvent was removed under reduced

pressure. Chromatography of the residue over SiO_2 with 5:4 ethyl acetate−hexane afforded **5** (1.24 g, 73%): ^{1}H NMR ($CDCl_3$) δ 7.81−7.72 (m, 4H), 5.74 (dd, J = 9.0, 11.0 Hz, 1H), 5.34 (m, 2H), 5.19 (t, J = 10.0 Hz, 1H), 5.06 (t, J = 9.0 Hz, 1H), 4.82−4.78 (m, 2H), 4.55 (d, J = 9.0 Hz, 1H), 4.50 (dd, J = 2.0, 12.0 Hz, 1H), 4.37 (dd, J = 2.0, 12.0 Hz, 1H), 4.26 (d, J = 8.0 Hz, 1H), 4.16−4.02 (m, 5H), 3.81−3.73 (m, 3H), 3.69 (t, J = 9.0 Hz, 1H), 3.56 (ddd, J = 2.0, 5.0, 10.0 Hz, 1H), 2.15 (s, 3H), 2.11 (s, 3H), 2.10 (s, 3H), 2.09 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.96 (s, 3H), 1.83 (s, 3H), 1.78 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 170.82, 170.58, 170.43, 170.11, 169.69, 169.60, 169.43, 169.38, 168.60, 134.26, 123.70, 109.85, 100.65, 97.61, 87.66, 75.47, 75.06, 74.83, 72.17, 71.84, 71.13, 70.78, 70.60, 70.04, 68.74, 68.65, 61.75, 61.57, 60.76, 54.49, 20.81, 20.72, 20.66, 20.59, 20.44, 20.37; HRFABMS calcd for $C_{44}H_{52}NO_{25}Na$ (M + Na) 1059.2818, found 1059.2869.

***O*-(2-Amino-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl Azide Hydrochloride (6).** A suspension of **5** (920 mg, 0.88 mmol) in 10 mL of a 40% $MeNH_2$ aqueous solution was stirred at room temperature for 12 h. The mixture was then concentrated under reduced pressure to give the residue. The residue was purified with IEC to afford **6** (420 mg, 85%): ^{1}H NMR (D_2O) δ 4.81 (d, J = 8.4 Hz, 1H), 4.77 (d, J = 8.8 Hz, 1H), 4.50 (d, J = 8.0 Hz, 1H), 4.18 (d, J = 3.2 Hz, 1H), 4.00−3.66 (m, 12H), 3.55−3.42 (m, 3H), 3.31 (t, J = 8.4 Hz, 1H), 2.90 (t, J = 9.2 Hz, 1H); ^{13}C NMR (D_2O) δ 103.21, 103.06, 90.59, 82.53, 78.23, 77.33, 76.66, 75.65. 75.00, 74.43, 73.19, 70.76, 70.16, 68.98, 61.60, 61.03, 60.53, 57.01; HRFABMS calcd for $C_{18}H_{33}N_4O_{14}$ (M^+) 529.1993, found 529.2002.

***O*-(3,4-*O*-Isopropylidene-β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl Azide (10).** A mixture of **9** (600 mg, 1.63 mmol), acetone (10 mL), and trimethylsilyl chloride (4 mL) was stirred at room temperature under N_2. After 3 h, the mixture was suspended with hexane (10 mL) and evaporated under reduced pressure to give **10** (660 mg, 100%) without further purification: ^{1}H NMR (CD_3OD) δ 4.56 (d, J = 8.5 Hz, 1H), 4.37 (d, J = 8.5 Hz, 1H), 4.20 (d, J = 3.5 Hz, 1H), 4.06 (t, J = 6.0 Hz, 1H), 3.95−3.75 (m, 4H), 3.61−3.53 (m, 3H), 3.45 (t, J = 7.5 Hz, 1H), 3.31 (s, 1H), 3.32 (t, J = 7.5 Hz, 1H), 1.48 (s, 3H), 1.33 (s, 3H); ^{13}C NMR (CD_3OD) δ 110.43, 103.41, 91.15, 80.15, 79.60, 77.84, 75.70, 74.66, 74.36, 73.84, 73.74, 61,72, 60.92, 27.72, 25.82; HRFABMS calcd for $C_{15}H_{25}N_3O_{10}Na$ (M + Na) 430.1438, found 430.1420.

***O*-(3,4-*O*-Isopropylidene-2,6-di-*O*-benzoyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl Azide (11).** After a mixture of **10** (650 mg, 1.59 mmol), pyridine (6 mL), and BzCl (3 mL) was stirred at 0 °C for 12 h, it was concentrated under reduced pressure. The residue was purified on silica gel (CH_2Cl_2) to give **11** (1.25 g, 85%): ^{1}H NMR ($CDCl_3$) δ 8.14−7.97 (m, 10H), 7.66−7.31 (m, 15H), 5.77 (t, J = 9.2 Hz, 1H), 5.39 (dd, J = 8.8, 9.6 Hz, 1H), 5.17 (t, J = 7.6 Hz, 1H), 4.81 (d, J = 8.4 Hz, 1H), 4.66 (dd, J = 1.6, 12.0 Hz, 1H), 4.64 (d, J = 8.0 Hz, 1H), 4.53 (dd, J = 4.4, 12.0 Hz, 1H), 4.31−4.23 (m, 3H), 4.12 (dd, J = 2.0, 5.6 Hz, 1H), 3.98−3.94 (m, 1H), 3.88−3.84 (m, 1H), 3.75 (dd, J = 7.6, 11.6 Hz, 1H), 1.55 (s, 3H), 1.29 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 167.08, 166.98, 166.63, 166.25, 166.05, 134.63, 134.49, 134.37, 134.27, 131.04, 130.87, 130.74, 130.65, 130.53, 130.36, 129.84, 129.64, 129.57, 129.57, 129.33, 112.04, 101.23, 89.27, 78.17, 76.27, 75.94, 74.68, 74.22, 73.44, 72.49, 72.34, 63.91, 63.42, 28.56, 27.27; HRFABMS calcd for $C_{50}H_{45}N_4O_{3215}Na$ (M + Na) 950.2748, found 950.2770.

***O*-(2,6-Di-*O*-benzoyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl Azide (12).** After a suspension of **11** (780 mg, 0.84 mmol) in 60% AcOH (20 mL) was stirred at 95 °C for 5 h, it was concentrated under reduced pressure. The residue was purified on silica gel (20:1 CH_2Cl_2−MeOH) to give **12** (730 mg, 97%): ^{1}H NMR ($CDCl_3$) δ 8.09−7.93 (m, 10H), 7.63−7.33 (m, 15H), 5.71 (t, J = 9.2 Hz, 1H), 5.42−5.33 (m, 2H), 4.80 (d, J = 8.8 Hz, 1H), 4.63 (d, J = 7.6 Hz, 1H), 4.60−4.55 (m, 2H), 4.17 (t, J = 9.6 Hz, 1H), 4.05 (dd, J = 6.4, 10.8 Hz, 1H), 3.95 (m, 1H), 3.85 (m, 1H), 3.73 (d, 7.2 Hz, 1H), 3. 62 (dd, J = 6.4, 10.2 Hz, 1H), 3.54 (t, J = 6.0 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 167.56, 167.20, 167.06, 166.95, 166.26, 134.67, 134.63, 134.58, 131.02, 130.84, 130.75, 130.53, 129.78, 129.74, 129.66, 129.57, 101.98, 89.11, 76.80, 76.26, 74.72, 73.85, 73.73, 73.66, 72.14, 69.61, 63.45, 62.92; HRFABMS calcd for $C_{47}H_{41}N_4O_{15}Na$ (M + Na) 910.2435, found 910.2462.

***O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→4)-*O*-(3,6-di-acetyl-2-phthalimido-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-(2,6-di-*O*-benzoyl-β-D-galactopyranosyl)-(1→4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranosyl Azide Hydrochloride (14).** A suspension of compound **13** (40 mg, 0.052 mmol), compound **12** (46 mg, 0.058 mmol), and 4A molecular sieves (200 mg) in anhydrous CH_2Cl_2 (3 mL) was stirred for 2 h at room temperature and then cooled to −78 °C. A 0.5 M solution of BF_3·Et_2O in CH_2Cl_2 (0.2 mL) was added dropwise to the cooled suspension, and the reaction mixture was gradually warmed to 0 °C over a period of 3 h and stirred for an additional 2 h at 0 °C. The reaction was quenched with 2 drops of Et_3N, and the mixture was evaporated under reduced pressure. Chromatography of the residue over SiO_2 in 55:1 toluene−EtOH afforded **14** (72 mg, 86%): ^{1}H NMR ($CDCl_3$) δ 8.10−7.88 (m, 5H), 7.64−7.16 (m, 17H), 7.04 (t, J = 7.5 Hz, 2H), 5.63 (t, J = 9.0 Hz, 1H), 5.56 (dd, J = 7.5, 9.5 Hz, 1H), 5.48 (d, J = 8.5 Hz, 1H), 5.31−5.23 (m, 3H), 5.09 (dd, J = 7.0, 10.0 Hz, 1H), 4.95 (dd, J = 3.0, 10.5 Hz, 1H), 4.68 (d, J = 9.0 Hz, 1H), 4.51 (d, J = 8.0 Hz, 1H), 4.48 (d, J = 8.0 Hz, 1H), 4.38 (d, J = 11.5 Hz, 1H), 4.28 (dd, J = 4.5, 12.0 Hz, 1H), 4.22 (dd, J = 4.5, 11.5 Hz, 1H), 4.16 (dd, J = 8.5, 1.0 Hz, 1H), 4.08 (t, J = 8.5 Hz, 1H), 4.04−3.99 (m, 3H), 3.82 (t, J = 6.5 Hz, 1H), 3.76−3.65 (m, 3H), 3.52 (t, J = 5.5 Hz, 1H), 2.11 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 2.00 (s, 3H), 1.95 (s, 3H), 1.79 (s, 3H); ^{13}C NMR ($CDCl_3$) δ 171.04, 170.99, 170.76, 170.22, 169.83, 166.72, 166.46, 166.05, 165.82, 164.71, 134.56, 134.14, 133.98, 133.74, 133.44, 130.58, 130.49, 130.33, 130.24, 130.20, 130.11, 129.94, 129.37, 129.17, 129.11, 128.94, 128.86, 123.82, 101.77, 101.12, 98.90, 88.68, 82.08, 77.42, 75.74, 75.54, 73.50, 73.04, 73.00, 71.87, 71.65, 71.56, 71.27, 71.04, 69.76, 68.27, 67.17, 30.40, 21.39, 21.31, 21.22, 21.07; HRFABMS calcd for $C_{79}H_{76}N_4O_{32}Na$ (M + Na) 1615.4340, found 1615.4372.

***O*-(β-D-Galactopyranosyl)-(1→4)-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl Azide Hydrochloride (7).** A suspension of **14** (60 mg, 0.038 mmol) in 3 mL of 40% $MeNH_2$ was stirred at room temperature for 12 h. The mixture was then concentrated to give the residue. The residue was purified with IEC to afford **7** (25 mg, 92%). The spectra data were identical with that of the product prepared with a Gly001-09-catalyzed transglycosylation between lactose and **6**. **7**: ^{1}H NMR (D_2O) δ 5.01 (d, J = 8.0 Hz, 1H), 4.76 (d, J = 8.8 Hz, 1H), 4.50 (d, J = 7.6 Hz, 1H), 4.45 (d, J = 8.0 Hz, 1H) 4.18 (d, J = 2.8 Hz, 1H), 3.98−3.63 (m, 20 Hz), 3.52 (t, J = 8.0 Hz, 1H), 3.30 (t, J = 8.4 Hz, 1H), 3.16 (t, J = 9.2 Hz, 1H); ^{13}C NMR (D_2O) δ 103.70, 103.14, 100.68, 90.59, 82.36, 78.58, 78.22, 77.33, 76.10, 75.68, 75.64, 75.00, 73.22, 73.13, 71.61, 71.29, 70.74, 69.20, 69.04, 61.77, 61.57, 60.56, 60.25, 56.35; HRFABMS calcd for $C_{24}H_{43}N_4O_{19}$ (M^+) 691.2522, found 691.2557.

***O*-(α-D-Galactopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-*O*-(2-amino-2-deoxy-β-D-glucopyranosyl)-(1→3)-*O*-(β-D-galactopyranosyl)-(1→4)-β-D-glucopyranosyl Azide Hydrochloride (15).** Compound **15** (30 mg, 0.034 mmol) was synthesized in 87% yield from **7** (29 mg, 0.039 mmol) in the manner as previously described. **15**: ^{1}H NMR (D_2O) δ 4.98 (d, J = 3.5 Hz, 1H), 4.87 (d, J = 8.5 Hz, 1H), 4.60 (d, J = 8.0 Hz, 1H), 4.37 (d, J = 8.0 Hz, 1H), 4.35 (d, J = 8.0 Hz, 1H), 4.04−4.02 (m, 3H), 3.85−3.48 (m, 25H), 3.15 (t, J = 8.0 Hz, 1H), 3.02 (dd, J = 8.5, 10.5 Hz, 1H); ^{13}C NMR (D_2O) δ 103.60, 103.11, 100.66, 96.10, 90.57, 82.33, 78.75, 78.17, 77.82, 77.31, 75.76, 75.62, 74.97, 73.19, 71.49, 71.34, 70.71, 70.21, 69.92, 69.77, 69.01, 68.82, 65.45, 61.72, 61.57, 60.51, 60.27, 56.29; HRFABMS calcd for $C_{30}H_{53}N_4O_{24}$ (M^+) 853.3050, found 853.3016.

Isolation of Polyclonal Anti-Gal Antibody from Human Serum. Polyclonal anti-Gal antibody was isolated from commercially available human serum with an α-Gal (immobilized trisaccharide[α-Gal-(1→3)-β-Gal-(1→4)-β-GlcNAc]) affinity chromatography column. Type AB human serum (25 mL) was kept in a 56 °C water bath for 30 min to inactivate

the complement. The inactivated serum was then passed through the column to allow for the complexation between the anti-Gal antibody and the anti-Gal epitope. After an extensive wash with phosphate buffered saline (PBS, pH 7.4), the bound anti-Gal antibody remaining on the column was then eluted with a glycine−HCl buffer (pH 2.8). The pH value of the elute was adjusted to 7.2 using 0.1 M NaOH, and the resulting antibody solution was stored as frozen aliquots (about 200 μg/mL) in PBS.

ELISA Inhibition Assay. An ELISA assay was conducted using mouse laminin as the solid-phase antigen. The purified human polyclonal anti-Gal antibody was first incubated with varying concentrations of compounds **2**, **15**, **16**, and **17** for 2 h at 37 °C with gentle shaking. The mixture (50 μL) was then added to each microtiter plate well (Immulon 4) containing mouse laminin (10 μg/mL) and incubated for 1.5 h at room temperature. After removing any unbound anti-Gal antibody with PBS (pH 7.4), a secondary antibody (1/1000 peroxidase conjugated-goat anti-human IgG; 50 μL/well) was introduced and the newly conjugated antibody complex was incubated for 1 h at room temperature. The substrate (TMB (3,3′,5,5′-tetramethylbenzidine):H_2O_2, 9:1; BioRad) specific for the peroxidase was added at 100 μL/well, and the enzymatic reaction was quenched by adding 1 N H_2SO_4 (100 μL/well). A UV detector (BioRad Microplate Reader, model 3550-UV, at 450 nm) was implemented to measure the varying concentration levels. PBS, in addition with the secondary antibody, was used as a background marker, and anti-Gal antibody with secondary antibody as maximum staining (0% inhibition). The % inhibition was calculated with eq 1:

$$(M - S)/(M - B) = \%\ \text{inhibition} \qquad (1)$$

S refers to the OD_{450} reading of the sample with different concentrations of compounds **2**, **15**, **16**, or **17**, B the OD_{450} reading of the background staining, and M the OD_{405} reading of the maximum staining. IC_{50} was calculated from the curve of inhibition−concentration.

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Supporting Information Available: ^1H and ^{13}C NMR spectra for compounds **1a**, **1b**, **2**, **6**, **7**, and **15**. This material is available free of charge via the Internet at http://pubs.acs.org.

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