Efficient Preparation of Natural and Synthetic Galactosides with a Recombinant β-1,4-Galactosyltransferase-/UDP-4′-Gal Epimerase Fusion Protein

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The numerous biological roles of LacNAc-based oligosaccharides have led to an increased demand for these structures for biological studies. In this report, an efficient route for the synthesis of β-galactosides using a bacterial β-4-galactosyltransferase/-UDP-4′-gal-epimerase fusion protein is described. The *lgtB* gene from *Neisseria meningitidis* and the *galE* gene from *Streptococcus thermophilus* were fused and cloned into an expression vector pCW. The fusion protein transfers galactose to a variety of different glucose- and glucosamine-containing acceptors, and utilizes either UDP-galactose or UDP-glucose as donor substrates. A crude lysate from *Escherichia coli* expressing the fusion protein is demonstrated to be sufficient for the efficient preparation of galactosylated oligosaccharides from inexpensive UDP-glucose in a multigram scale. Lysates containing the fusion protein are also found to be useful in the production of more complex oligosaccharides in coupled reaction mixtures , e.g., in the preparation of sialosides from *N*-acetylglucosamine. Thus, bacterially expressed fusion protein is well suited for the facile and economic preparation of natural oligosaccharides and synthetic derivatives based on the lactosamine core.

Introduction

N-Acetyllactosamine (LacNAc) is one of the most common core structures in oligosaccharides of glycoproteins, some of which are significantly elevated in various types of cancer patients.[1,2] LacNAc is also a precursor of sialyl Lewis^x, which play an important role in selectin-mediated adhesion of neutrophils to endothelial cells, the initial event in many inflammatory responses.[3] The widespread biological role of LacNAc-based oligosaccharides has led to an increased demand for these structures for biomedical studies, and therefore, simple and efficient synthetic routes to these compounds are desirable.

Numerous chemical approaches have been developed in order to prepare lactosamines by glycosylation between galactose and *N*-acetylglucosamine derivatives.[4−6] However, these procedures often involve tedious multiple protection and deprotection steps, where the total yields are low and the amounts prepared are seldom more than gram quantities. Therefore, to avoid the protection and deprotection steps entirely, much attention has been focused on enzymatic preparation of these structures using glycosyltransferases.[7−9] The high regio- and stereoselectivity and catalytic efficiency make these enzymes especially useful for the synthesis of complex carbohydrate structures. A few enzymes are commercially available, and the recent cloning of these enzymes, and advances in genetic engineering and recombinant DNA techniques, are increasing their availability. In most glycosyltransferase reactions, sugar nucleotides are used stoichiometrically, and the accessibility and cost of these compounds are also of major importance for the successful application of glycosyltransferases in synthesis.

Enzyme-catalyzed in vitro synthesis of LacNAc-containing saccharides with β-4-galactosyltransferases[10] and the corresponding nucleotide sugar, uridine-5′-diphosphogalactose (UDP-galactose), has been used to obtain galactose-containing oligosaccharides. The high cost of UDP-galactose and the inhibition of mammalian galactosyltransferases by UDP during the course of the reaction have led to the development of the in situ generation of UDP-galactose by the use of UDP-galactose-4′-epimerase.[11,12] In addition, an alternative enzymatic synthesis using glycosidases by transglycosylation has

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(1) Fukuda, M. *Biochim. Biophys. Acta* **1984**, *780* (*2*), 119−150.
(2) Montreuil, J. *Adv. Carbohydr. Chem. Biochem.* **1980**, *37*, 157−223.
(3) Lasky, L. A. *Annu. Rev. Biochem.* **1995**, *64*, 113−139.
(4) Kretzschmar, G.; Stahl, W. *Tetrahedron* **1998**, *54*, 6341−6358.
(5) Aly, M. R. E.; Ibrahim, E.-S. I.; El-Ashry, E.-S. H. E.; Schmidt, R. R. *Carbohydr. Res.* **1999**, *316*, 121−132.
(6) Ding, Y.; Fukuda, M.; Hindsgaul, O. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1903−1908.
(7) Wong, C. H.; Halcomb, R. L.; Ichikawa, Y.; Kajimoto, T. *Angew. Chem., Int. Ed.* **1995**, *34*, 412−432.
(8) Wong, C.-H.; Ichikawa, Y.; Krach, T.; Gautheron-Le Narvor, C.; Dumas, D.; Look, G. C. *J. Am. Chem. Soc.* **1991**, *113*, 8137−8145.
(9) Ichikawa, Y.; Look, G. C.; Wong, C. H. *Anal. Biochem.* **1992**, *202*, 215−238.
(10) Wong, C. H.; Wang, R.; Ichikawa, Y. *J. Org. Chem.* **1992**, *57*, 4343−4344.
(11) Ichikawa, Y.; Shen, G. J.; Wong, C. H. *J. Am. Chem. Soc.* **1991**, *113*, 4698−700.
(12) Zervosen, A.; Elling, L. *J. Am. Chem. Soc.* **1996**, *118*, 1836−1840.

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also been demonstrated.[13,14] In general, however, glycosidases exhibit lower regiospecificity and efficiency, resulting in low yields.

Until recently most glycosyltransferases used in enzymatic synthesis were of mammalian origin. However, many bacteria produce polysaccharide sequences that are identical or similar to those found on mammalian glycoproteins.[15,16] Using bacterial glycosyltransferases that catalyze reactions identical to mammalian glycosyltransferase reactions offers certain advantages. For example, facile production of large amounts of enzymes in bacteria instead of the dramatically reduced production in mammalian cells is typically required for mammalian enzymes.[17] Several published results on bacterial glycosyltransferases[18,19] have shown enzymatic activities similar to mammalian enzymes, but have also shown a wider range of substrate specificity. Recently, the development of bacterial-coupled glycosyltransferase reactions involving mixtures of an organism expressing a bacterial glycosyltransferase and organisms producing nucleotide sugars have also shown a promising route to the large-scale synthesis of oligosaccharides.[20,21]

Several reports have demonstrated the utility of coupling glycosyltransferase genes to other bacterial genes useful in the in situ synthesis of the nucleotide sugars used by the enzyme. For example, the *Escherichia coli* (*E. coli*) expressed fusion protein, consisting of a bacterial α-2,3-sialyltransferase fused to the *E. coli* cytidine-5′-monophospho-*N*-acetylneuraminic acid (CMP−NeuAc) synthetase, has been shown to be a very efficient tool for the preparation of sialosides with in situ formation of the expensive CMP−NeuAc.[22] Fang et al. reported a fusion of the mammalian α-1,3-galactosyltransferase (EC 2.4.1.151) with the *E. coli* (EC 5.1.3.2) UDP-glucose-4′-epimerase[23] and demonstrated that the enzyme produced in bacteria had utility in the synthesis of α-1,3 galactosides.[24] Since UDP-glucose is commercially available at a price nearly 100 times less than that of UDP-galactose, coupling the epimerase to galactosyltransferase represents a highly cost efficient way for preparing galactosides.

This report describes the construction, expression, and characterization of a bifunctional fusion protein combining the β-1,4-galactosyltransferase from *Neisseria meningitidis* (lgtB)[25] with an UDP-glucose-4′-epimerase, the *galE* gene, from *Streptococcus thermophilus*.[26] In a preliminary report, this enzyme was shown to be useful in the synthesis of lacto-*N*-neotetraose.[27] As reported here, detailed characterization of the enzymatic properties reveals the potential of this fusion protein for synthesis of diverse variety of galactosides. Because the *E. coli* strain used for expression was deficient in endogenous proteases and β-galactosidase, it was found that crude lysates could be used to efficiently produce galactosides in multigram scale using UDP-glucose as a donor substrate.

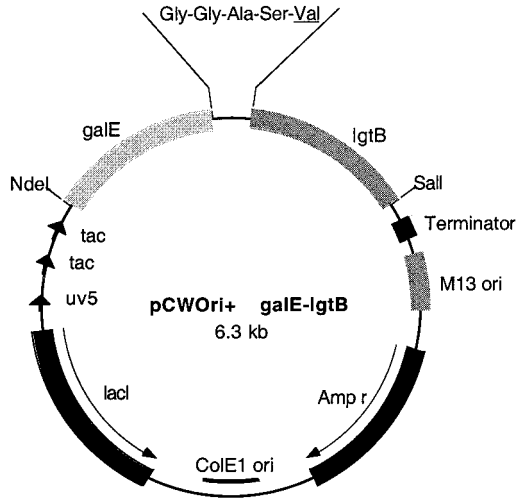


Figure 1. Construction of the β4galT−galE fusion plasmid.

Results and Discussion

Recombinant DNA methods were used to produce a fusion of the two enzymes used in glycosylation reactions, β-1,4-galactosyltransferase (lgtB) and UDP-glucose-4′-epimerase (galE). The linking of these enzymes was achieved by joining the genes in an arrangement where a minimal linker region is produced between the two proteins, and the methionine at the beginning of the transferase was changed to valine (Figure 1). The two genes were then combined in a three-fragment ligation into the vector pCWori$^+$ and the resulting DNA was introduced into *E. coli* AD202 cells. The fusion protein is produced at high levels in *E. coli* and is mostly soluble in the cytoplasm of the cells (data not shown). The choice of *E. coli* strain AD202 for expression was critical as the LgtB protein has been shown to be rapidly inactivated by the endogenous OmpT protease. This strain is also deficient in the *E. coli* enzyme β-galactosidase, which could otherwise degrade the products of the galactosyltransferase.[25]

Bacterial lysates containing the recombinant enzymes, β4galT−galE fusion and the native β4galT,[25] were produced as described in experimental procedure. The activities of the β4galT−galE and the native β4galT in the lysates was approximately 25 and 160 U/L, respectively, using *N*-acetylglucosamine as the acceptor. To investigate the effectiveness of the UDP-glucose-4′-epimerase in the fusion protein, lysates containing the native β4galT and the β4galT−galE were compared for their

(13) Li, J.; Wang, P. G. *Tetrahedron Lett.* **1997**, *38*, 7967−7970.
(14) Vetere, A.; Paoletti, S. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 6−13.
(15) Krivan, H. C.; Roberts, D. D.; Ginsburg, V. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6157−6161.
(16) Verheul, A. F. M.; Snippe, H.; Poolman, J. T. *Microbiol. Rev.* **1993**, *57*, 34−49.
(17) Johnson, K. F. *Glycoconjugate J.* **1999**, *16*, 141−146.
(18) Gilbert, M.; Cunningham, A. M.; Watson, D. C.; Martin, A.; Richards, J. C.; Wakarchuk, W. W. *Eur. J. Biochem.* **1997**, *249*, 187−194.
(19) Blixt, O.; van Die, I.; Norberg, T.; van den Eijnden, D. H. *Glycobiology* **1999**, *9*, 1061−1071.
(20) Endo, T.; Satoshi, K.; Kazuhiko, T.; Shingo, K.; Akio, O. *Carbohydr. Res.* **1999**, *316*, 179−183.
(21) Endo, T.; Koizumi, S.; Tabata, K.; Ozaki, A. *Appl. Microbiol. Biotechnol.* **1999**.
(22) Gilbert, M.; Bayer, R.; Cunningham, A. M.; DeFrees, S.; Gao, Y.; Watson, D. C.; Young, M. N.; Wakarchuk, W. W. *Nat. Biotechnol.* **1998**, *16*, 769−772.
(23) Fang, J.; Chen, X.; Zhang, W.; Janczuk, A.; Wang, P. G. *Carbohydr. Res.* **2000**, *329*, 873−878.
(24) Fang, J.; Chen, X.; Zhang, W.; Wang, J.; Andreana, P. R.; Wang, P. G. *J. Org. Chem.* **1999**, *64*, 4089−4094.
(25) Wakarchuk, W. W.; Cunningham, A.; Watson, D. C.; Young, M. *Protein Eng.* **1998**, *11*, 295−302.
(26) Poolman, B.; Royer, T. J.; Mainzer, S. E.; Schmidt, B. F. *J. Bacteriol.* **1990**, *172*, 4037−4047.
(27) Yan, F.; Wakarchuk, W. W.; Gilbert, M.; Richards, C. J.; Whitfield, M. D. *Carbohydr. Res.* **2000**, *328*, 3−16.

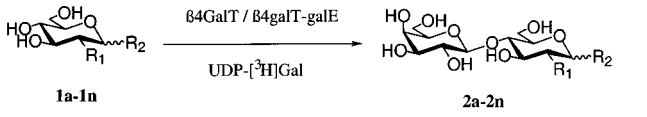
Enzyme	Donor Substrate		
	UDPgal	UDPglc	UDPglcNAc
β4galT	100%	13%	0%
β4galT-galE	100%	104%	0%

Figure 2. Relative product transfer of UDP donor substrates (2 mM) to glcNAc (10 mM) acceptor. Results are expressed as the percent of completion of the reaction based on the amount of the UDP sugar substrate.

ability to synthesize LacNAc using UDP-galactose and UDP-glucose as donor substrates (Figure 2). Under conditions where the native β4galT quantitatively converted UDP-galactose to UDP and LacNAc, only 13% of the UDP-glucose was consumed, with a corresponding production of a neutral disaccharide (see below). In contrast to the native β4galT, the β4galT−galE fusion protein efficiently utilized both UDP-galactose and UDP-glucose as donor substrates for the synthesis of LacNAc.

Because the native β4galT utilized UDP-glucose to some extent, albeit at a lower rate, we were concerned about the potential for the galactosyltransferase to transfer glucose, in addition to galactose, and thereby produce a mixture of glucosylated and galactosylated products when UDP-glucose was used as a donor substrate. Accordingly, large scale reactions were performed with the native β4galT and UDP-glucose in order to characterize the resulting disaccharide product. The disaccharide produced was found to be exclusively LacNAc (Galβ1,4GlcNAc) by NMR (not shown). Thus, it appears that the galactosyltransferase does not utilize UDP-glucose as a substrate, and that the LacNAc product formed with UDP-glucose results from a partial conversion by the endogenous *E. coli* UDP-glucose-4′-epimerase to UDP-galactose .

For the acceptor-specificity studies, the variety of different acceptor substrates that were used in this study includes D-glucose (**1a**), *p*-nitrophenyl β-D-glucopyranoside (**1b**), isopropyl β-D-thio-glucopyranoside (**1c**), D-glucosamine (**1d**), glucosamine derivatives **1e**−**i**,[28,29] **1j** and **k**,[19] **1l** and **m**,[30] and **1n**.[31,32] All of these monosaccharides were good to excellent acceptor substrates (Figure 3). Compounds **1e**,**h**,**i**, and **n** were used in the large-scale preparation of LacNAc derivatives in good yields. The β4gal-transferase used in this study showed a broad specificity to the anomeric aglycon and to the amino group at the 2-deoxy position on the gluco-derivative. Spacer arms with different functionalities were tolerated by the enzyme, an important consideration for the synthesis of glycoconjugates that must be coupled with biopolymers (proteins, lipids) or synthetic polymers. It is also worth noting that glucosamine (**1d**), which has an unprotected amino group, served as an acceptor for the enzyme. Surprisingly, compounds **1l** and **m**, with the allyloxycarbonylamino protecting group, act as similar or better acceptor substrates than the lacto-*N*-triose derivative **1j**, which is closer to the natural substrate in the lipooligosaccharide biosynthesis in *N. meningitidis*.[33]



1a-1n **2a-2n**

			Glycosyltransferase activity (%)		
			β4galT	β4galT-galE	
	R1	R2	10mM	10mM	1mM
1a	OH	OH	60	80	80
1b	OH	β*O*pNP	60	60	60
1c	OH	β*S*iPr	210	210	330
1d	NH_2	OH	30	30	50
1e	NHAc	OH	**100**	**100**	**100**
1f	NHAc	β*O*pNP	120	120	140
1g	NHAc	βOMe	200	220	350
1h	NHAc	$\beta OCH_2CH_2N_3$	340	360	410
1i	NHAc	$\beta OCH_2CH_2OCH_2CH_2N_3$	330	300	480
1j	NHAc	β3Galβ4GlcβO$CH_2CH_2N_3$	600	440	530
1k	NHAc	βNH-squarate[a]	60	60	70
1l	NHAlloc	βSCH_2CH_2COOMe	520	520	1000
1m	NHAlloc	$\beta OCH_2CH_2OCH_2CH_2N_3$	460	550	2470
1n	NPht	βSCH_2CH_3	220	160	190

Figure 3. Relative acceptor substrate specificity of compounds **1a**−**n**. For each enzyme, the transfer of galactose to the various acceptors is expressed relative to the transfer observed using *N*-acetylglucosamine as the acceptor substrate. [a]βNH-squarate is a derivative of 3,4-Diethoxy-3-cyclobutene-1,2-dione (diethyl squarate).

The synthetic 2-amino-2-deoxy-*N*-phthalimido-β-D-thio-glucoside (**1n**) was also used as an excellent acceptor substrate and demonstrates that the enzyme could be used in combination with chemical protection group strategies in the preparation of synthetic building blocks.[32,34] The amino group can, in those cases, be further modified by synthetic manipulations.[35]

It is significant that the crude lysate was adequate for efficient production of galactosides, obviating the need to utilize purified enzymes, as previously described by others.[19,36−38] This was possible due to the use of the *E. coli* strain AD202 that is deficient in the *ompT* protease, previously shown to rapidly inactivate the β-1,4-galactosyltransferase.[25] In addition, the AD202 is deficient in the *E. coli* β-galactosidase, which reduces product formation due to hydrolysis of the product formed unless it is removed by purification (not shown).

As examples for use of the fusion protein for larger scale reactions, galactosides **2e**,**h**,**i** and **n** were produced on a preparative scale (e.g., 4−6 g acceptor substrate) using crude lysate and a minimal amount of the donor substrate UDP-glucose (1.1 equiv). Isolated yields of the disaccharide were up to 90%, after ion-exchange and size exclusion chromatography purifications, as compared to the chemical preparations of LacNAc derivatives where total yields after deprotections and repeated purifications

(28) Eklind, K.; Gustafsson, R.; Tiden, A. K.; Norberg, T.; Aberg, P. M. *J. Carbohydr. Chem.* **1996**, *15* (9), 1161−1178.
(29) Nilsson, S.; Bengtsson, M.; Norberg, T. *J. Carbohydr. Chem.* **1992**, *11*, 265−85.
(30) Boullanger, P.; Jouineau, M.; Bouammali, B.; Lafont, D.; Descotes, G. *Carbohydr. Res.* **1990**, *202*, 151−64.
(31) Fügedi, P.; Garegg, P. *Carbohydr. Res.* **1986**, *149*, C9−C12.
(32) Nilsson, M.; Norberg, T. *J. Carbohydr. Chem.* **1989**, *8*, 613−627.
(33) Wakarchuk, W.; Martin, A.; Jennings, M. P.; Moxon, E. R.; Richards, J. C. *J. Biol. Chem.* **1996**, *271*, 19166−19173.
(34) Arnarp, J. H., M.; Lonngren, J. *J. Chem. Soc., Perkin Trans. 1* **1982**, 1841−1844.
(35) Banoub, J.; Boullanger, P.; Lafont, D. *Chem. Rev.* **1992**, *92*, 1167.
(36) Kleene, R.; Krezdorn, C. H.; Watzele, G.; Meyhack, B.; Herrmann, G. F.; Wandrey, C.; Berger, E. G. *Biochem. Biophys. Res. Commun.* **1994**, *201*, 160−167.
(37) Seto, N. O. L.; Palcic, M. M.; Hindsgaul, O.; Bundle, D. R.; Narang, S. A. *Eur. J. Biochem.* **1995**, *234*, 323−328.
(38) Williams, M. A.; Kitagawa, H.; Datta, A. K.; Paulson, J. C.; Jamieson, J. C. *Glycoconjugate J.* **1995**, *12*, 755−761.

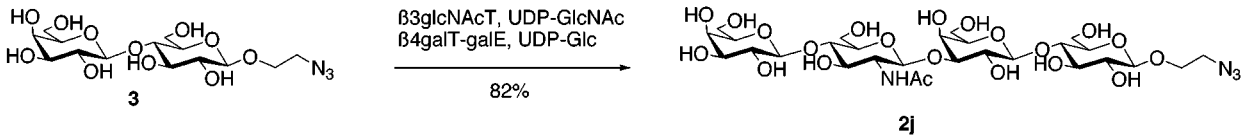


Figure 4. One-pot synthesis of lacto-N-neotetraose derivative **2j** using β3glcNAcT/UDPglcNAc and β4galT−galE fusion/UDPglc.

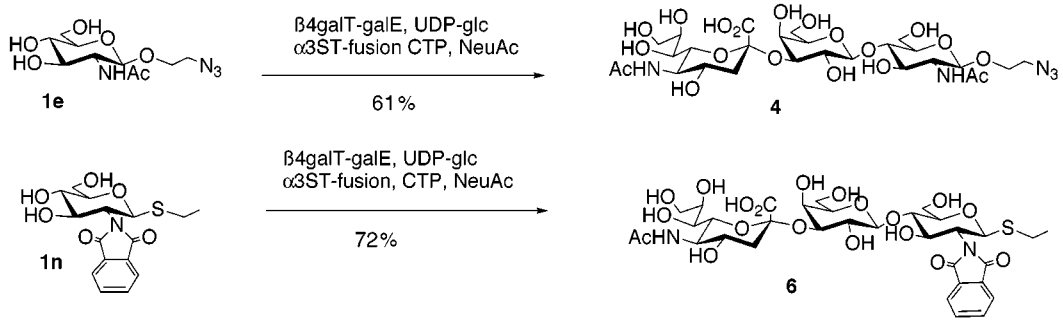


Figure 5. One-pot, one-step synthesis of α-2,3-sialosides.

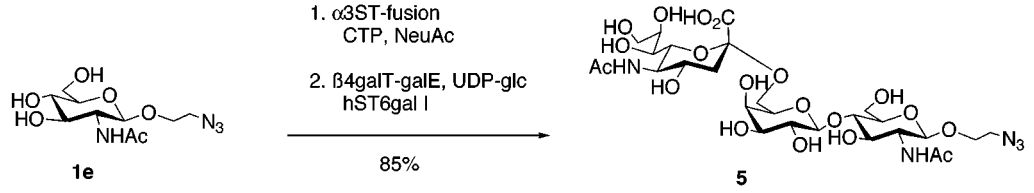


Figure 6. One-pot, two-step synthesis of α-2,6-sialoside.

seldom exceed 50%.[4,6,39] The reaction scale is limited only by the method used for purification, and larger reactions could easily be considered.

The fusion protein was also investigated for its ability to form larger oligosaccharides in coupled glycosyltransferase reactions. A lacto-*N*-neotetraose (LNnT) tetrasaccharide derivative **2j** was synthesized from the corresponding lactoside **3** in a single reaction step (Figure 4). For this reaction, the β4galT−galE fusion protein and a bacterial β-3-*N*-acetylglucosaminyl transferase (lgtA) from *N. meningitidis*[19,25] were used, together with the donor substrates, UDP-glucose and UDP-*N*-acetylglucosamine, respectively. Because the β3glcNAc transferase was also expressed in the strain AD202, bacterial lysates were employed. From this coupled reaction, the LNnT tetrasaccharide was obtained in 82% overall yield relative to the β-lactoside acceptor substrate.

To investigate the synthesis of sialosides, one-pot coupled reactions were evaluated with two sialyltransferases, ST3gal−CMPNeuAc synthetase fusion protein[22] and the ST6gal I transferase,[40] which form the NeuAcα2,-3Gal and NeuAcα2,6Gal sequences, respectively. The important roles in biological recognition that sialosides with these sequences play has been well documented.[3,41] As illustrated in Figure 5, the ST3gal-CMPNeuAc synthetase and β4galT−galE fusion proteins were used to convert the *N*-acetylglucosamine glycosides **1e** and **n** to the corresponding α-2,3-sialylated trisaccharides **4** and **6** in a single step. The donor substrates were UDP-glucose for the galactosyltransferase. For the sialyltransferase, NeuAc and CTP were used as stoichiometric substrates for the synthesis of CMP−NeuAc; the CMP−NeuAc synthetase of the fusion protein was also used.

To use the sialyltransferase ST6gal I, CMPNeuAc was first synthesized in the absence of acceptor substrates using the ST3gal-CMPNeuAc synthetase fusion protein (Figure 6). Once the enzyme was removed by ultrafiltration, **1e** and the other enzymes were added to produce the corresponding α-2,6-sialylated trisaccharide. In all cases the products were isolated in good yields (61−85%). It is noteworthy that compound **6** is well suited for further derivatization by protecting groups for use as synthetic building blocks for more complex sialoside derivatives.[42,43]

Conclusion

There have been rapid advances in the development of enzymatic methods of glycosylation which have each enhanced the use of enzymes in a chemoenzymatic approach to the synthesis of both natural and unnatural oligosaccharides. The β4galT−galE fusion protein described here carries out two sequential steps of an important biosynthetic galactosylation pathway. Because the synthesis can be done with crude bacterial lysates and inexpensive substrates, preparative scale synthesis of β-4-galactosides can be done efficiently and at low cost. Due to the broad specificity of the bacterial glycosyltransferase and the compatibility of reaction conditions in coupled one-pot mixtures (e.g., for preparation of sialosides), the β4galT−galE fusion protein should be of

(39) Toepfer, A.; Schmidt, R. R. *Carbohydr. Res.* **1993**, *247*, 159−164.
(40) Weinstein, J.; de Souza-e-Silva, U.; Paulson, J. *J. Biol. Chem.* **1982**, *257*, 13835−13844.
(41) Sharon, N.; Lis, H. *Sci. Am.* **1993**, *268*, 82−89.
(42) Mehta, S.; Gilbert, M.; Wakarchuk, W. W.; Whitfield, D. M. *Org. Lett.* **2000**, *2*, 751−753.
(43) Ito, Y.; Gaudino, J. J.; Paulson, J. C. *Pure Appl. Chem.* **1993**, *65*, 753−762.

significant value in the preparation of diverse galactosides with the Galβ1,4Glc(NAc) sequence.

Experimental Section

General Methods. Concentration of solvents was performed under reduced pressure at <40 °C bath temperature. NMR spectra were recorded at 30 °C using a Varian Unity Inova 500 spectrometer. The following reference signals were used: acetone 2.225 ppm (^{1}H in D_2O) and acetone 29.7 ppm (^{13}C in D_2O). MALDI-FTMS spectra was recorded with an IonSpec Ultima FTMS (IonSpec Corp., Irvine, CA, using dihydroxybenzoic acid as the matrix. Thin-layer chromatography was performed on Kieselgel 60 F_{254} Fertigplatten (Merck, Darmstadt, Germany). After development with the appropriate eluants, spots were visualized by UV light and/or by dipping in 5% sulfuric acid in ethanol followed by charring. Water was produced from a NanoPure Infinity Ultrapure water system (Barnstead/Thermolyne, Dubuque, IA) and degassed by vacuum treatment before use. Compounds **1l** and **m** were kindly provided by Dr. T. Norberg (Swedish University of Agricultural Sciences, Uppsala, Sweden); compound **1j** was synthesized by enzymatic 3-*N*-acetylglucosaminylation of 2-azidoethyl β-D-lactopyranoside[44] using crude lysate β-1,3-*N*-acetylglucosaminyl transferase.[19,25] [^{3}H]-UDPglcNAc (0.17 Ci/mol), [^{3}H]-UDPgal (0.58 Ci/mol), and [^{3}H]-UDPglc (0.56 Ci/mol) were from NEN Life Science Products, Inc. (Boston, MA). The radioactive nucleotide-sugars were diluted with unlabeled UDP-sugars (Sigma) to the desired specific radioactivity. All other oligosaccharide substrates were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of the Galactosyltransferase/Epimerase Fusion Protein (β4galT−galE). The *S. thermophilus* UDP-glucose-4′-epimerase (galE) gene was amplified from a plasmid (pTGK-EP1) provided by Cytel Corp. using primers derived from the nucleotide sequence of galE from *S. thermophilus* (GenBank accession M38175). The 5′ primer (galE−5p) was a 58-mer that included a *Bam*H I site (shown in bold italics) and the ATG start codon (shown in bold) (5′-GGGACA***GGATCC***ATCG**ATG**CTTAGGAGGTCATATGGCAATTTTAGTATTAGGTGGAGC-3′). The 3′ primer (GalE−3p) was a 42-mer that contained the *Nhe* I site (shown in bold italics; 5′-GGGGGG***GCTAGC***GCCGCCTCCTCGATCATCGTACCCTTTTGG-3′).

The *N. meningitidis* β-1,4-galactosyltransferase was amplified from pCW-lgtB(MC58)[25] using primers derived from the nucleotide sequence of lgtB from *N. meningitidis* (GenBank accession NMU25839). The 5′ primer (LgtB−NheI) was a 38-mer (5′-GGGGGG***GCTAGC***GTGCAAAACCACGTTATCAGCTTAGC-3′) containing the *Nhe* I (shown in bold italics) and the 3′ primer (LgtB−SalI) was a 45-mer (5′-GGGGGG***GTCGAC***CTATTATTGGAAAGGCACAATGAACTGTTCGCG-3′) containing the *Sal* I site (shown in bold italics). The thermocycler parameters were 94 °C 3 min and 30 cycles of 55 °C for 30 s, 72 °C for 30 s, and 94 °C for 30 s. PCR was performed with Pwo polymerase as described by the manufacturer (Roche Molecular Systems). The plasmid containing the fused gene products, pCWgalE-LgtB, was constructed as follows (Figure 1). The UDP-glucose-4′-epimerase PCR product was digested with *Bam*H I and *Nhe* I, and the β-1,4-galactosyltransferase PCR product was digested with *Nhe* I and *Sal* I and then recovered from the reaction mixtures using Prep-a-Gene resin according to the manufacturer's instructions (BioRad). The two genes were then combined in a three-fragment ligation under standard conditions with the vector pCWori$^+$ [25] that had been digested with *Bam*H I and *Sal* I. DNA was introduced into *E. coli* AD202 using electroporation with 1 μL of the ligation reaction. Transformants were screened using colony PCR with primers specific for vector sequences flanking the cloning site. Colonies with inserts of the correct size were then grown in liquid culture and tested for enzyme activity. The strain containing the functional fusion protein is designated pCW:galE-lgtB/AD202.

Preparation of Bacterial Extracts Containing the Galactosyl- and *N*-Acetylglucosaminyl-Transferases. A single colony from an agar plate containing either the lgtB β-4-galactosyltransferase (pCW:lgtB/AD202),[25] the β4galT−galE fusion protein (pCW:galE-lgtB/AD202), or the lgtA β-3-*N*-acetylglucosaminyl transferase (pWC:lgtA/AD202)[33] was inoculated into a 100 mL culture containing 2YT medium (10 g Tryptone, 5 g Yeast extract, 7.5 g NaCl per liter) supplemented with ampicillin (150 mg/L). The culture was grown at 37 °C until a $OD_{600} = 0.4-0.6$, and was then added to the 2YT medium (1 L) supplemented with ampicillin (150 mg/L), and the culture was grown at 37 °C until a $OD_{600} = 0.4-0.6$. Expression was induced with *iso*-propyl thiogalactopyranoside (IPTG) at a final concentration of 1 mM, and the cells were collected 6 h after induction by centrifugation for 15 min at 5 000*g*. The pellets were then resuspended to 1/10 of the culture volume and washed in buffer containing Tris−HCl (25 mM, pH 7.5), $MnCl_2$ (10 mM), and Triton X (0.25%(v/v)). After an additional 15 min centrifugation at 5 000*g* the pellets were resuspended in the same buffer to 1/20 of the culture volume. The cells were lysed by a microfluidizer (1108, Microfluids Corp., Newton, MA), and were immediately cooled on ice. Lysates were centrifuged for 30 min at 20 500*g*. For the three glycosyltransferases, the activity in the clarified lysates was approximately 160, 25, and 25 U/L, respectively. Extracts were stored at −20 or −80 °C and were typically used within two months.

Preparation of Sialyltransferases. *ST3gal-CMPNeuAc synthetase fusion protein*. This was prepared and assayed as described previously.[22] *hST6gal I transferase.* The human homologue of ST6gal I was obtained by PCR using the reverse transcript product of human liver as follows: Two oligonucleotides, a forward primer CG***GGATCC***ATGATTCACACCAACCTGAAG (5′−3′; nt 1−21) with an internal *Bam*H I site (shown in bold italics), and a reverse primer, G***CTCTAG***ATTAGCAGTGAATGGTCCGGAAGCC (5′−3′; nt 1221−1198) with an internal *Xba* I site (shown in bold italics) were used for amplification, using the first strand cDNA from human liver as a template (Clontech). The conditions for PCR using pfu DNA polymerase were 94 °C for 30 s, 60 °C for 30 s, and 73 °C for 1 min, for 25 cycles. Agarose gel analysis showed the generation of a single band with the expected size (1.2 kb). This PCR generated fragment was digested with *Bam*H I and *Xba* I and subcloned into a similarly digested pBSKS$^+$ vector (Stratagene) following standard molecular biology techniques.[45] Dideoxy double-stranded sequencing of the full-length clone confirmed hST6gal I when compared with that of a previously published sequence.[40,46] For expression as a soluble form, two restriction sites, *Xba* I and *Bam*H I, were introduced into its cDNA by polymerase chain reaction (PCR) as follows: a forward primer CG***TCTAGA***CCACAGGGGCCGCCAGACCCTC (5′−3′; nt 214−236) with an internal *Xba* I site (shown in bold italics), and a reverse primer CG***GGATCC***TTAGCAGTGAATGGTCCGGAAG (5′−3′; nt1222−1200), with an internal *Bam*H I site (shown in bold italics) were used for amplification, using as a template cDNA for the hST6gal I subcloned in pBluescript above. The conditions for PCR were 94 °C for 30 s, 56 °C for 1 min, and 73 °C for 2 min, for 25 cycles. Gel analysis showed the generation of a single band of the desired size (1.05 kb). This band was purified using Genclean II (Bio 101, San Diego, CA), digested with *Xba* I and *Bam*H I, and then subcloned into a similarly digested mammalian expression vector $pcDNA_{ins}$, described previously.[47] The sequence of the resulting expression vector, termed pcDNAins:

(44) Chernyak, A. Y.; Sharma, G. V. M.; Kononov, L. O.; Krishna, P. R.; Levinsky, A. B.; Kochetkov, N. K.; Rao, A. V. R. *Carbohydr. Res.* **1992**, *223*, 303−309.

(45) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1989.

(46) Grundmann, U.; Nerlich, C.; Rein, T.; Zettlmeissl, G. *Nucleic Acids Res.* **1990**, *18*, 667.

(47) Datta, A. K.; Chammas, R.; Paulson, J. C. *J. Biol. Chem.* **2001** (in press).

hST6gal I, was confirmed by dideoxy double-stranded sequencing of the entire subcloned fragment, including the restriction sites used. For expression in the baculoviral system, the cDNA with the insulin signal sequence was released by digestion with *Not* I and *Bam*H I, and it was subcloned into similarly digested pVL1392 following standard molecular biological techniques. The plasmid DNA was used for transfection of Sf9 using the BaculoGold Technique following the protocol of the supplier (Pharmingen). After four days of post-transfection, the supernatant was used for subcloning using blue agar, as suggested by the supplier of the reagents (Gibco-BRL). A single clone was selected and amplified using the standard procedure. The supernatant containing the viral particles was then used for expression of the protein using a multiple of infection (MOI) of five. After 72 h of infection, the supernatant was collected and used for enzyme assays as follows: the clarified cell supernatant (1 L) was diluted with sodium cacodylate (50 mL, 500 mM, pH 6.5) and loaded onto a CDP−hexanolamine column (5 mL) equilibrated with sodium cacodylate buffer (50 mM, pH 6.5) containing NaCl (100 mM). The column was then washed with the same buffer until OD_{280} reached the background level and eluted with sodium cacodylate buffer (50 mM, pH 4.8) containing NaCl (1.5 M). Fractions containing enzymatic activity were collected, pooled, and dialyzed against sodium cacodylate buffer (50 mM, pH 6.5) containing NaCl (100 mM) and glycerol (50%) for 48 h at 4 °C prior to assaying the activity. Enzymatic activity was determined, as previously described,[48] and approximately 10 U/L cell culture was isolated and determined with lactose as an acceptor substrate. The enzyme could be stored over a 3−4 month period at −20 °C without a loss of activity.

Standard Glycosyltransferase Assays for the Galactosyl- and *N*-Acetylglucosaminyl-Transferases. The assays were conducted in a final volume of 100 μL at 37 °C for 30 min, containing the acceptor *N*-acetylglucosamine (10 mM) or lactose (10 mM) and the donor [H^3]-UDPgal (2 mM 0.58 Ci/mol) or [H^3]-UDPglcNAc (2 mM 0.17 Ci/mol), respectively, in sodium cacodylate buffer (50 mM, pH 7.5), $MnCl_2$ (10 mM), and bovine serum albumin (1%). Reactions were initiated by addition of the appropriate crude glycosyltransferase (10 μL ≈ 0.5 mU) diluted in buffer containing bovine serum albumin (1%). In all cases, reactions were limited to a transfer of less than 20% of the nucleotide donor substrate. Reactions were terminated by the addition of water (0.5 mL). The reaction mixtures were then passed through pasteur pipet columns of Dowex resin (1-X8, 200−400 mesh, Cl^- form, 0.5 × 5 cm, 1 mL). The columns were washed with water (1.5 mL), and the effluents were directly collected in scintillation vials and the radioactivity was assayed by liquid scintillation counting. Background activity was obtained using identical assay mixtures lacking acceptor and was subtracted. A unit of activity is defined as that amount of enzyme which converts 1 μmol of acceptor to product per minute under the conditions described above.

Substrate Specificity Assays for β4galT−galE Fusion and β4galT. *Acceptor substrate*. The assays were conducted in a final volume of 100 μL at 37 °C for 30−60 min, containing the acceptor (1 mM or 10 mM) and [H^3]-UDPgal (2 mM, 0.58 Ci/mol) in sodium cacodylate buffer (50 mM, pH 7.5), $MnCl_2$ (10 mM), and bovine serum albumin (1%). Reactions were initiated by addition of the appropriate crude glycosyltransferase (10 μL ≈ 0.5 mU) diluted in buffer containing bovine serum albumin (1%). In all cases, reactions were limited to a transfer of less than 20% of the UDPgal. Transfer of galactose to the various acceptors is expressed relative to the transfer observed using *N*-acetylglucosamine as the acceptor substrate (100%). The reactions were analyzed as described above. *Donor substrate*. The assays were conducted in a final volume of 100 μL at 37 °C for 30 min, containing the acceptor *N*-acetylglucosamine (10 mM), one of the donors [H^3]-UDPgal (2 mM, 0.58 Ci/mol), [H^3]-UDPglc (2 mM, 0.17 Ci/mol), or [H^3]-UDPglcNAc (2 mM, 0.17 Ci/mol) in sodium cacodylate buffer (50 mM, pH 7.5), $MnCl_2$ (10 mM), and bovine serum albumin (1%). Reactions were initiated by addition of the appropriate crude glycosyltransferase β4galT or β4galT−galE fusion (10 μL ≈ 0.5 mU) diluted in buffer containing bovine serum albumin (1%). In all cases, reactions were limited to a transfer of less than 20% of the nucleotide donor substrate. The reactions were analyzed as described above.

General Procedure for Large-Scale Synthesis of Galactosides. Reactions contained the acceptor substrate (20 mmol, 40 mM) and UDP-glucose (22 mmol, 44 mM) in sodium cacodylate buffer (500 mL 100 mM, pH 7.5) with $MnCl_2$ (20 mM) and bovine serum albumin (0.1%). Reactions were initiated by addition of the fusion protein β4galT−galE (15 U based on the galactosyltransferase activity). The reaction was slowly stirred at room temperature (25 °C) for 3 days, and the pH was periodically checked and kept at 7.5 by dropwise addition of NaOH (2 M). The mixture was then ultrafiltered through a 10 000 MWCO membrane to remove proteins and passed through a Dowex anion resin (1-X8, 200−400 mesh, HCO_3^- form, 2.5 × 20 cm). The eluent and washings were collected and passed through a Dowex cation resin (200−400 mesh, H^+ form, 2.5 × 20 cm) and further purified by gel permeation chromatography (Sephadex G15, 5 × 140 cm). At all steps, the product was monitored by thin-layer chromatography using the eluent system ethyl acetate/methanol/acetic acid/water (*X*: 3:3:2 by volume, where *X* varies between 4 and 12). The following galactosides were prepared according to this method unless otherwise specified.

β-D-Galactopyranosyl-(1→4)-*O*-2-acetamido-2-deoxy-D-glucopyranose (2e). (3.6 g, 47%) Selected ^{1}H NMR (D_2O) δ (α-anomer = 2.04 (s, 3H, CH_3CONH), 3.56 (dd, 1H, H-2′), 3.67 (m, 1H, H-3′), 3.77 (m, 2H, H-4, H-5′), 3.91 (m, 2H, H-2, H-3), 3.93 (d, 1H, H-4′), 4.48 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 5.24 (d, 1H, $J_{1,2}$ 2.5 Hz, H-1)); (β-anomer = 2.04 (s, 3H, CH_3CONH), 3.55 (dd, 1H, H-2′), 3.67 (m, 1H, H-3′), 3.72 (m, 1H, H-3), 3.74 (m, 2H, H-2, H-4), 3.93 (d, 1H, H-4′), 4.48 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 4.73 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1)). ^{13}C NMR (D_2O) δ 21.4, 21.6, 53.2, 55.7, 59.4, 59.6, 60.5, 68.0, 68.7, 69.7, 70.4, 71.9, 72.0, 74.3, 74.8, 77.9, 78.3, 90.0, 94.3, 102.4, 173.9. MALDI-FT-MS: *m*/*z* calcd for (M + Na), 406.1320 (M + Na); found, 406.1321.

2-Azidoethyl (β-D-Galactopyranosyl)−(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside (2h). (6.2 g, 69%) Selected ^{1}H NMR (D_2O) δ 2.04 (s, 3H, CH_3CONH), 3.41−3.52 (m, 2H, $OCH_2CH_2N_3$), 3.55 (dd, 1H, H-2′), 3.68 (dd, 1H, H-3′), 3.72 (dd, 1H, H-4), 3.73 (dd, 1H, H-3), 3.78 (dd, 1H, H-2), 3.78, 4.06 (2 m, 2H, $OCH_2CH_2N_3$), 3.93 (d, 1H, H-4′), 4.48 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 4.61 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1). ^{13}C NMR (D_2O) δ 21.3, 49.4, 54.1, 59.1, 60.1, 67.6, 67.8, 70.0, 71.5, 71.6, 73.9, 74.4, 77.5, 100.0, 102.0, 173.7. MALDI-FT-MS *m*/*z* calcd for (M + Na), 475.1647 ; found, 475.1631.

5-Azido-3-oxapentyl (β-D-Galactopyranosyl)−(1→4)-*O*-2-acetamido-2-deoxy-β-D-glucopyranoside (2i). (8.9 g, 90%) Selected ^{1}H NMR (D_2O) δ 2.04 (s, 3H, CH_3CONH), 3.46−3.72 (m, 2H, $OCH_2CH_2N_3$), 3.55 (dd, 1H, H-2′), 3.68 (dd, 1H, H-3′), 3.72 (dd, 1H, H-4), 3.73 (dd, 1H, H-3), 3.78 (dd, 1H, H-2), 3.78−4.06 (2 m, 6H, $OCH_2CH_2OCH_2CH_2N_3$), 3.93 (d, 1H, H-4′), 4.48 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 4.61 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1). ^{13}C NMR (D_2O) δ 21.3, 49.4, 54.2, 59.2, 60.1, 67.7, 68.1, 68.4, 68.8, 70.1, 71.5, 71.6, 73.9, 74.5, 77.6, 100.0, 102.0, 173.7. MALDI-FT-MS *m*/*z* calcd for (M + Na), 519.1909; found, 519.1893.

Ethyl (β-D-Galactopyranosyl)−(1→4)-*O*-2-deoxy-2-*N*-phthalimido-β-D-thio-glucopyranoside (2n). (7.5 g, 73%) The crude enzymatic mixture was ultrafiltered through a 10 000 MWCO membrane and then purified in two parts on a silica C18 column (5 × 15 cm), washed with water 10 column volumes, and eluted with 60% methanol. The combined eluates were concentrated to dryness by evaporation to give the disaccharide. Selected ^{1}H NMR (D_2O) δ 1.15 (t, 3H, CH_3CH_2S−), 2.70 (m, 2H, CH_3CH_2S−), 3.56 (dd, 1H, H-2′), 3.67 (dd, 1H, H-3′), 3.92 (d, 1H, H-4′), 4.15 (t, 1H, H-2), 4.50 (dd, 1H, H-3), 4.51 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1′), 5.45 (d, 1H, $J_{1,2}$ 11.0 Hz, H-1), 3.81 (dd, 1H, H-4), 7.87−7.93 (m, 4H, Ar−). ^{13}C NMR (D_2O) δ 13.9, 23.8, 55.1, 59.9, 60.6, 68.1, 70.0, 70.5, 72.0, 74.8, 78.0,

(48) Paulson, J. C.; Rearick, J. I.; Hill, R. L. *J. Biol. Chem.* **1977**, *252*, 2363−2371.

78.5, 80.5, 102.3, 123.0, 123.4, 130.3, 134.7, 169.2. MALDI-FT-MS *m*/*z* calcd for (M + Na), 538.1354; found, 538.1359.

2-Azidoethyl (*β*-D-Galactopyranosyl)−(1→4)-*O*-(2-acetamido-2-deoxy-*β*-D-glucopyranosyl)−(1→3)-*O*-(*β*-D-galactopyranosyl)−(1→4)-*O*-*β*-D-glucopyranoside (2j). To reactions containing the lactoside (**3**) (288 mg, 0.70 mmol), UDP-*N*-acetylglucosamine, (592 mg, 0.91 mmol), UDP-glucose (555 mg, 0.91 mmol), $MnCl_2$ (20 mM), and bovine serum albumin (BSA) 0.1%, in sodium cacodylate buffer (40 mL, 500 mM, pH 7.5) were added the enzyme *β*3glcNAcT (1 U) and *β*4galT−galE fusion (1 U). The reaction was slowly stirred at room temperature (25 °C) for 3 days and the pH was periodically checked and kept at 7.5 by the addition of NaOH (2 M). The mixture was then ultrafiltered through a 10 000 MWCO membrane and passed through a Dowex anion resin (1-X8, 200−400 mesh, HCO_3^- form, 1.5 × 10 cm). The eluent and washings were collected and passed through a Dowex cation resin (200−400 mesh, H^+ form, 1.5 × 10 cm) and further purified by gel permeation chromatography (Sephadex G15, 2.5 × 140 cm). Appropriate fractions were collected and lyophilized to give the tetrasaccharide **2j** (444 mg, 0.57 mmol, 82%). Selected ^{1}H NMR (D_2O) *δ* 2.04 (s, 3H, C*H*$_3$CONH), 3.35, 3.52 (m, 2H, OCH$_2$C*H*$_2$N$_3$), 3.78, 4.06 (2 m, 2H, OC*H*$_2$CH$_2$N$_3$), 4.16 (d, 1H, H-4′), 4.45 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1′), 4.49 (d, 1H, $J_{1,2}$ 7.5 Hz, H-1′), 4.54 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.72 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1″). ^{13}C NMR (D_2O) *δ* 21.0, 49.4, 54.0, 58.7, 58.9, 59.8, 59.8, 67.1, 67.4, 69.8, 71.0, 71.3, 71.5, 71.6, 73.1, 73.4, 73.6, 73.7, 74.2, 77.0, 77.2, 80.9, 101.0, 101.6, 101.7, 101.8, 173.7. MALDI-FT-MS *m*/*z* calcd for (M + Na), 799.2703; found, 799.2708.

2-Azidoethyl (Sodium[5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosyl]-onate-(2→3)−(*β*-D-galactopyranosyl)−(1→4)-*O*-2-acetamido-2-deoxy-*β*-D-glucopyranoside (4). 1e (0.24 g, 0.83 mmol), NeuAc (0.55 g, 1.70 mmol), CTP (1.0 g, 1.70 mmol), and UDP-glucose (0.80 g, 1.3 mmol) were dissolved in sodium cacodylate (100 mL, 200 mM, pH 7.5) containing $MgCl_2$ (20 mM) and $MnCl_2$ (20 mM). *β*4galT−galE fusion (5 U) and ST3gal-CMPNeuAc synthetase fusion (300 U) were added and the reaction was slowly stirred at room temperature for 48 h. When all the starting material was converted to product (TLC: EtOAc/HOAc/MeOH/H_2O; 6:3:3:2 by volume) the mixture was ultrafiltered through a 10 000 MWCO membrane. The filtrate was passed through Dowex anion resin (1-X8, 200−400 mesh, phosphate- form, 4 × 10 cm) and lyophilized. The residue was loaded onto a column of Sephadex G15 (5 × 140 cm), equilibrated, and eluted with 5% *n*-BuOH. Appropriate fractions were collected and lyophilized to give **4** (0.39 g, 0.51 mmol, 61%). Selected ^{1}H NMR (D_2O) *δ* 1.80 (t, 1H, H-3eq″), 2.03 (s, 3H, C*H*$_3$CONH), 2.04 (s, 3H, C*H*$_3$-CONH), 2.76 (dd, 1H, H-3ax″), 3.40−3.52 (m, 2H, OCH$_2$C*H*$_2$N$_3$), 3.57 (dd, 1H, H-2′), 3.72 (dd, 1H, H-4), 3.74 (dd, 1H, H-3), 3.78 (dd, 1H, H-2), 3.78, 3.96 (d, 1H, H-4′), 4.05 (2 m, 2H, OC*H*$_2$-CH$_2$N$_3$), 4.11 (dd, 1H, H-3′), 4.55 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 4.60 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1). ^{13}C NMR (D_2O) *δ* 21.5, 21.7, 39.1, 49.8, 51.2, 54.5, 59.5, 60.5, 62.1, 66.9, 67.6, 67.8, 68.2, 68.9, 69.0, 71.2, 71.9, 72.3, 74.3, 74.6, 75.0, 77.8, 100.5, 102.0, 173.3, 174.1, 174.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 788.2420; found, 788.2418.

2-Azidoethyl (Sodium[5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosyl]-onate-(2→6)−(*β*-D-galactopyranosyl)−(1→4)-*O*-2-acetamido-2-deoxy-*β*-D-glucopyranoside (5). This reaction was carried out in two steps. The first step was to synthesize the CMP−NeuAc in the absence of any acceptor substrate using the ST3gal-CMP-NeuAc synthetase fusion protein. NeuAc (0.52 g, 1.7 mmol) was dissolved in Tris-HCl (200 mL, 100 mM, pH 9.0) containing $MgCl_2$ (20 mM). CTP (1.0 g, 1.7 mmol) and ST3gal-CMPNeuAc synthetase fusion protein (300 U) were added, and the reaction pH was kept constant at 8.5 by the addition of NaOH (2 M). When all CTP was converted to CMP−NeuAc (1 h), as confirmed by TLC (*i*-PrOH/H_2O/NH_3; 6:2:1 by volume), the mixture was filtered through a 10 000 MWCO membrane. The filtrate was then used to prepare the desired sialoside, essentially as described for compound **4**. To the filtrate compound **1e** (0.22 g, 0.76 mmol), UDP-glucose (0.70 g, 1.1 mmol), *β*4galT−galE fusion protein (5 U), and ST6gal I (5 U) were added. The pH was adjusted to 7.0 with HCl (2 M) and the reaction was slowly stirred at room temperature for 48 h. When all starting material had been converted to product (TLC: EtOAc/HOAc/MeOH/H_2O; 6:3:3:2 by volume), the mixture was filtered through a 10 000 MWCO membrane. The filtrate was passed through a Dowex anion resin (1-X8, 200−400 mesh, phosphate form, 4 × 10 cm) and lyophilized. The residue was loaded onto Sephadex G15 column (5 × 140 cm) and the compound was eluted with 5% *n*-BuOH. Appropriate fractions were collected and lyophilized to give **5** (0.50 g, 0.65 mmol, 85%). Selected ^{1}H NMR (D_2O) *δ* 1.71 (t, 1H, H-3eq″), 2.03 (s, 3H, C*H*$_3$CONH), 2.07 (s, 3H, C*H*$_3$CONH), 2.67 (dd, 1H, H-3ax″), 3.41−3.52 (m, 2H, OCH$_2$C*H*$_2$N$_3$), 3.54 (dd, 1H, H-2′), 3.68 (dd, 1H, H-3′), 3.72 (dd, 1H, H-4), 3.74 (dd, 1H, H-3), 3.78 (dd, 1H, H-2), 3.78, 4.06 (2 m, 2H, OC*H*$_2$CH$_2$N$_3$), 3.93 (d, 1H, H-4′), 4.45 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 4.63 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1). ^{13}C NMR (D_2O) *δ* 21.4, 21.8, 39.6, 49.8, 51.4, 54.3, 59.9, 62.2, 62.8, 67.7, 67.8, 68.2, 67.9, 70.2, 71.2, 71.9, 72.0, 72.1, 73.2, 74.0, 80.3, 100.3, 103.0, 161.1, 173.0, 174.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 766.2601; found, 766.2604.

Ethyl (Sodium[5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosyl]onate)−(2→3)−(*β*-D-galactopyranosyl)−(1→4)-*O*-2-deoxy-2-*N*-phthalimido-*β*-D-thioglucopyranoside (6). Compound **1n** (0.30 g, 0.85 mmol), NeuAc (0.55 g, 1.70 mmol), CTP (1.0 g, 1.70 mmol), and UDP-glucose (0.80 g, 1.31 mmol) were suspended in sodium cacodylate (100 mL, 200 mM, pH 7.5), containing $MgCl_2$ (20 mM) and $MnCl_2$ (20 mM). *β*4galT−galE fusion (15 U) and ST3gal-CMPNeuAc synthetase fusion (300 U) were added, and the reaction was slowly stirred at room temperature for 18 h. When all starting material was converted to product (TLC: EtOAc/HOAc/MeOH/H_2O; 6:3:3:2 by volume), the mixture was filtered through a 10 000 MWCO membrane. The filtrate was slowly loaded onto silica C18 column (5 × 10 cm) and washed with five volumes of water. The compound was eluted with 60% MeOH to give the trisaccharide **6** (0.50 g, 0.61 mmol, 72%). Selected ^{1}H NMR (D_2O) *δ* 1.15 (t, 3H, C*H*$_3$CH$_2$S−), 1.81 (t, 1H, H-3eq″), 2.04 (s, 3H, C*H*$_3$CONH), 2.70 (m, 2H, CH$_3$C*H*$_2$S−), 2.77 (dd, 1H, H-3ax″), 3.59 (dd, 1H, H-2′), 3.83 (t, 1H, H-4), 3.96 (d, 1H, H-4′), 4.13 (dd, 1H, H-2), 4.15 (t, 1H, H-3′), 4.50 (dd, 1H, H-3), 4.61 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 5.45 (d, 1H, $J_{1,2}$ 11.0 Hz, H-1), 7.87−7.93 (m, 4H, Ar-). ^{13}C NMR (D_2O) *δ* 13.9, 21.5, 23.8, 39.1, 51.2, 54.6, 59.7, 60.6, 62.1, 67.0, 67.6, 67.8, 68.9, 70.0, 71.3, 72.4, 74.7, 75.0, 78.0, 78.6, 80.5, 102.1, 123.4, 123.0, 130.3, 134.7, 169.2, 173.5, 174.4. MALDI-FT-MS *m*/*z* calcd for (M + Na), 851.2127; found, 851.2145.

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Supporting Information Available: Copies of ^{1}H, ^{13}C NMR spectra for compounds **2e**,**h**−**j**, and **n**, **4**, **5,** and **6**. This material is avilable free of charge via the Internet at http://pubs.acs.org.

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