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Efficient Chemoenzymatic Synthesis of O-Linked Sialyl Oligosaccharides

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Abstract: The tumor associated Tn (GalNAcα(1-1)-Thr/Ser)- and T (Galβ(1-3)-GalNAcα(1-1)Thr/Ser)-antigens and their sialylated derivatives are present on the surface of many cancer cells. Preparative synthesis of these sialylated T- and Tn-structures has been achieved mainly from a chemical synthetic approach due to the lack of the required glycosyltransferases. We demonstrate a flexible and efficient chemoenzymatic approach for using recombinant sialyltransferases including a chicken GalNAcα2,6-sialyltransferase (chST6GalNAc I) and a porcine Galβ(1-3)GalNAcα-2,3-sialyltransferase (pST3Gal I). Using these enzymes, the common O-linked sialosides Neu5Acα(2-6)GalNAcα(1-1)Thr, Galβ(1-3)[Neu5Acα(2-6)]GalNAcα(1-1)Thr, Neu5Acα(2-3)Galβ(1-3)GalNAcα(1-1)Thr, and Neu5Acα(2-3)Galβ(1-3)[Neu5Acα(2-6)]GalNAcα(1-1)Thr were readily prepared at preparative scale. The chST6GalNAc I was found to require at least one amino acid (Thr/Ser) for optimal activity, and is thus an ideal catalyst for synthesis of synthetic glycopeptides and glycoconjugates with O-linked glycans.

Introduction

Carbohydrate groups of glycoproteins exhibit tremendous structural diversity and are major components of the outer surface of animal cells. The two major classes are N-linked to asparagine and O-linked to threonine and serine. While the core regions of these carbohydrate groups have elements common to many cells, the terminal sequences vary in a tissue and cell-type specific manner in both developing and mature organisms.[1−3]

The most common O-linked carbohydrates are based on core structures represented by the Tn (GalNAcα(1-1)Thr/Ser)- and T (Galα(1-3)GalNAcα(1-1)Thr/Ser)-antigens (Scheme 1).[4] Sialylated versions of these O-antigens are expressed at low levels by many normal tissues, but become highly expressed in many types of human malignancies including colon, breast, pancreas, ovary, stomach, lung adenocarcinomas,[5,6] and myelogenous leukemias.[7] The abnormal expression distinguishes them as tumor associated antigens, and the specific biosynthetic pathway, based on the in vitro activities of recombinant glycosyltransferases, has been previously discussed.[8−10] These antigens are also targets for immunotherapy. Indeed, clinical trials have been reported with vaccine-induced antibody responses against the sialyl-Tn-antigen Neu5Acα(2-6)GalNAcα(1-1)Thr/Ser.[11−13]

In addition to their roles as antigens, the sialylated T- and Tn-antigens have been implicated in a variety of other biological functions. The sequence Neu5Acα(2-3)Galβ(1-3)[Neu5Acα(2-6)]GalNAc has been documented to be a cell surface receptor mediating attachment of several bacteria and virus pathogens.[14−16] Members of the Siglec family of cell adhesion molecules have been demonstrated to use them as carbohydrate ligands and to distinguish between glycans containing *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc).[17−19] Neu5Acα2-3 sialylated T-antigen has also recently been shown to be critical for normal development of $CD8^+$ T cells.[20]

Despite the importance of these sialylated O-linked glycans, methods for their efficient preparation have been limiting.

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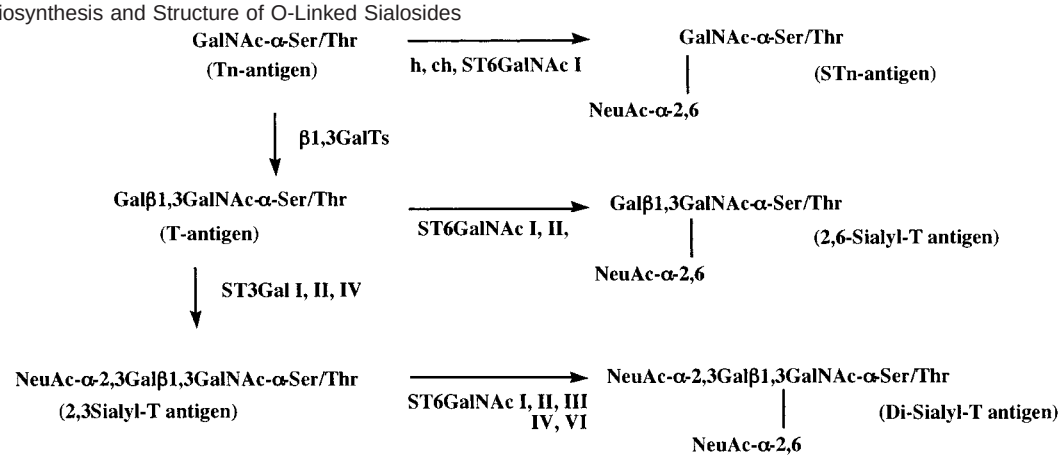
Scheme 1. Biosynthesis and Structure of O-Linked Sialosides



Strategies for pure chemical synthesis of O-linked glycans must address a host of issues, including appropriate blocking and deprotection steps, and the formation of the glycosidic bond of the 2-keto sugar *N*-acetylneuraminic acid, which is still considered one of the most difficult problems in synthetic carbohydrate chemistry.[21] Despite these obstacles, several groups have developed elegant solutions to the synthesis of the sialylated derivatives of the Tn- and T-antigens.[22−29] Nontheless, the complexity of the synthetic strategies restricts the ultimate scale and overall yields of the desired compounds, and consequently limits the availability of these compounds to the biomedical community.

Chemoenzymatic synthesis employing glycosyltransferases offers advantages for efficient production of sialosides, owing to the stereo- and regioselective catalysis and lack of blocking or deprotection steps for the addition of sialic acid.[30−33] While the scale of the reactions can be limited by the availability of the appropriate glycosyltransferase, use of recombinant glycosyltransferases to a large extent obviates this limitation.[31,34−38] A typical strategy for chemoenzymatic synthesis of oligosaccharides involves chemical synthesis of a simple glycoside that serves as a substrate for final steps involving the enzymatic addition of one or more sugar units. Sialosides are ideally suited for a chemoenzymatic synthesis strategy since the sialic acids are the most difficult sugars to handle in a chemical synthesis, and sialic acids are normally added as terminal sugars in the last steps of a biosynthetic pathway.

Several groups have reported the successful synthesis of α2-3 sialylated T-antigen, Neu5Acα(2-3)Gal*β*(1-3)GalNAcα(1-1)Thr, through a chemoenzymatic strategy employing sialyltransferase ST3Gal I[39−41] or by transglycosidation using a sialidase.[42] George et al.[43,44] recently reported the semipreparative chemoenzymatic synthesis of sialyl-Tn glycopeptide and a synthetic analogue of sialyl-Tn Neu5Acα(2-6)GalNAcα(1-spacer-1)Thr/Ser using a murine ST6GalNAc I.

In this report, we document the successful high yield expression of a chicken ST6GalNAc I, and present a detailed analysis of its substrate specificity, relevant for its use in the synthesis of all four possible sialyl-T- and sialyl-Tn-antigens in preparative scale.

Results and Discussion

Synthesis Strategy. As shown in Scheme 1, four sialylated O-linked glycans are derived from the Tn- and T-antigens. The primary decision in the synthesis strategy is which structures should be prepared chemically. Reliable procedures for the chemical synthesis of Tn- and T-antigens have been reported.[45−48] Thus, it was determined to prepare these compounds chemically, deprotect, and add the sialic acids enzymatically.

For synthesis of the critical sialic acid linkages, two recombinant sialyltransferases are required (Scheme 1), one that synthesizes the Neu5Acα(2-3)Gal*β*(1-3)GalNAcα(1-1)Thr/Ser sequence, and another that synthesizes the Neu5Acα(2-6)-GalNAcα(1-1)Thr/Ser sequence. The former sequence is efficiently produced by the sialyltransferase ST3Gal I, and its

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production in insect cells using a bacculovirus expression system has been previously reported.[39,49,50]

There were several options for production of the Neu5Acα-(2-6)GalNAcα(1-1)Thr/Ser linkage. An α2-6-sialyltransferase from *Photobacterium damsela* was demonstrated to be useful in the large scale synthesis of the oligosaccharide portion of sialyl-Tn, Neu5Acα(2-6)GalNAc. However, because it also transfers sialic acid to terminal galactose, and does not transfer to substituted GalNAc, it is not useful for the synthesis of α2-6 sialylated T-antigens Gal*β*(1-3)[Neu5Acα(2-6)]GalNAcα(1-1)-Thr.[30,51,52] Six members of the vertebrate GalNAc-α2-6-sialyl-transferase family have been cloned, which, in principle, can synthesize the Neu5Acα(2-6)GalNAc linkage.[9,53−63] Of these, ST6GalNAc I has the broadest substrate specificity, transferring sialic acid in α2-6 linkage to the GalNAc residue in the Tn-, T-, and α2-3 sialylated T-antigen structures. To date, ST6GalNAc I has been cloned and expressed from human, mice, and chicken.[9,54,63] The enzymatic feature of chST6GalNAc I from these three species differs significantly with respect to their use as a synthetic catalyst. Indeed, relative to a reference acceptor substrate, asialo-bovine submaxillary mucin (Gal*β*(1-3)GalNAc), a GalNAcαThr-glycopeptide, was a good acceptor (160%) for hST6GalNAc-I,[9] but was a poor acceptor (<1%) for mST6GalNAc I.[55] Preliminary analysis with the chST6GalNAc I showed that it also transferred well to GalNAcαThr substrates. For this reason, chST6GalNAc I was chosen as the target enzyme for synthesis of O-linked sialosides.

Preparation of Recombinant Sialyltransferase chST6GalNAc I. chST6GalNAc I plasmid DNA, pCEB-1800,[62] was used for production of recombinant protein in a Sf9 baculovirus system. To obtain expression of a secretable form of chST6GalNAc I, the expression vector was constructed by subcloning the dog proinsulin signal sequence (ins ss)[64] from the pGIR201Nhe3′ vector into the mammalian expression vector pcDNA3 generating pcDNAins. Thus, as described in the Experimental Section, the PCR-amplified chST6GalNAc I gene was cloned into this expression vector, and the resulting fusion construct was subcloned into the pVL1392 shuttle vector

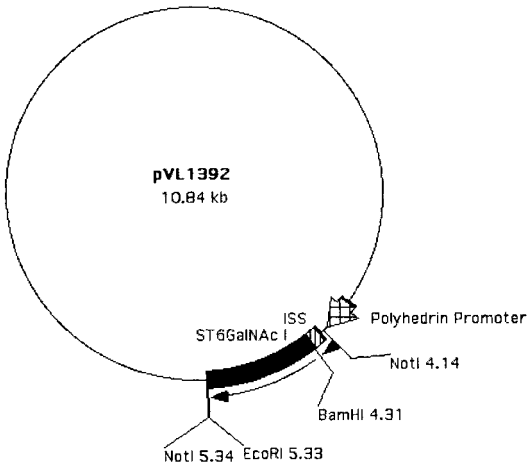

Figure 1. Plasmid construct for transfection.

(Pharmingen, San Diego, CA) which contained an ampicillin-resistant gene and a polyhedrin promoter (Figure 1). The plasmid DNA was used for recombinant baculovirus preparation using the BaculoGold transfection kit (Pharmingen, San Diego, CA). The enzyme was expressed at a level of approximately 10 units/L media. The soluble recombinant enzyme from 1 L culture supernatant was purified as described in the Experimental Section by affinity chromatography on CDP-hexanolamine-Sepharose. The typical yield was approximately 8 U of chST6GalNAc I.

Acceptor Specificity of ST6GalNAc I. A general concern about the use of mammalian glycosyltransferases for synthesis of oligosaccharides is that their stringent acceptor specificity may limit their utility as a synthetic tool. In the case of chST6GalNAc I, the natural substrates are oligosaccharides attached to glycoproteins, not the small molecule glycosides targeted for synthesis. To evaluate the utility of chST6GalNAc I for preparative synthesis of sialylated Tn- and T-antigens, and to determine to what extent the oligosaccharide substrate could be modified and still be recognized as an acceptor, a panel of different synthetic precursors of α-*N*-acetylgalactosamine (α-GalNAc) was prepared (Table 1).

2-Azidoethyl α-*N*-acetyl galactopyranoside **1** and its (2-*N*-acetylethyl)-derivative **2** were prepared from *N*-acetylgalactosmine and 2-azidoethanol[65] using a Lewis acid as a catalyst as reported previously.[66] Compounds **3c**−**7c** were prepared from the *N*-(9-fluorenylmethyloxycarbonyl)-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine phenacylester (**3a**) and *N*-(9-fluorenylmethyloxycarbonyl)-*O*-[4,6-di-*O*-2-azido-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine phenacylester (**7a**) glycopeptide building blocks.[47,48] Complete deprotection with Zemplen deacetylation followed by treatment with acetic anhydride gave the methylester of N^{α}-acetyl-L-threonine-glycosides **3c** and **7c**, respectively. *N*-Fluorenylmethyloxycarbonyl (Fmoc) protecting group could be preserved by carefully controlled deacetylation (pH < 9) followed by neutralization with Dowex (H^+) to give the amino acid glycoside **6** (72%).[67] Compound **3a** was deacetylated to give **4** (78%). The remaining azido functional

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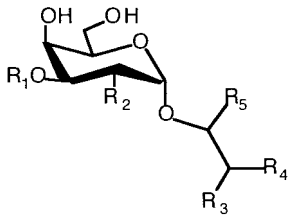
Table 1. chST6GalNAc I Activity of α-D-Galacto Derivatives



compound	R_1	R_2	R_3	R_4	R_5	cpm	nmol/mg min^{-1}
D-GalNAc	H	NHAc				0	0.00
1	H	NHAc	N_3	H	H	65	0.06
2	H	NHAc	NHAc	H	H	121	0.11
3c	H	NHAc	NHAc	$COOCH_3$	CH_3	9133	8.60
4	H	N_3	NHAc	$COOCH_3$	CH_3	3043	2.90
5	H	NH_2	NHAc	$COOCH_3$	CH_3	1421	1.30
6	H	NHAc	NHFmoc	$COOCH_3$	CH_3	13 277	12.50[a]
7	Galβ1,3	NHAc	NHAc	$COOCH_3$	CH_3	12 760	12.00

[a] Product was isolated by using Sep-Pak (C18) cartridges as described.[77]

group was further reduced by hydrogenolysis to the aminoglycoside **5** (82%).

The specificity of the chST6GalNAc I was evaluated using the purified recombinant enzyme and the prepared α-galactopyranoside-epitopes in Table 1. The assays were performed with CMP-[C^{14}]Neu5Ac, and the relative transfer of [C^{14}]Neu5Ac to the corresponding *N*-acetylgalactoside acceptor was measured as described in the Experimental Section. Neither free *N*-acetylgalactosamine (GalNAc), nor the α-glycosides **1** and **2**, served as suitable acceptor substrates for the chST6GalNAc I enzyme (Table 1), indicating that a α-glycosidic linkage alone is not sufficient. Kurosawa et al.[63] previously reported low activity toward α-glycosides of GalNAc-benzyl and GalNAc-(1-1)SerNAc for chST6GalNAc I. However, as shown in Table 1, GalNAc- or (Galβ(1-3)GalNAc- α-linked to the hydroxyl group of the carboxymethylester derivative of L-ThrNAc (**3c**, **7c**) gave a rate of transfer 80−200-fold higher than to compound **2**. For this reason, the carboxymethylester-ThrNAc was used as the standard aglycon in O-linked oligosaccharide synthesis. Moreover, replacement of the *N*-acetyl group on GalNAc with N_3 or NH_2 reduced transfer, but not to the extent of precluding preparative synthesis. Additionally, replacement of ThrNAc with ThrFmoc enhanced activity adding an important synthetic dimension for preparation of glycosylated amino acids for peptide synthesis.[68]

Synthesis of Sialylated Tn- and T-antigens. With the substrate specificity of the target glycosides in hand, chST6GalNAc I was used in preparative chemoenzymatic synthesis. Five different O-linked sialoside epitopes containing the amino acid aglycon, threonine, were synthesized in high yield and high purity (Figure 2). The highly expensive nucleotide donor, CMP-Neu5Ac, required for the enzymatic transfer of *N*-acetyl neuraminic acid (Neu5Ac), was prepared crude from CTP and Neu5Ac and was catalyzed by the bacterial fusion protein ST3-/CMP-Neu5Ac-synthetase.[31] The crude nucleotide sugar was used in the sialylation reactions after removal of fusion protein by 10 000 MW cutoff membrane filter.[34] CMP-Neu5Gc was synthesized from Neu5Gc[69] and CTP as described for CMP-Neu5Ac above.

Thus, enzymatic sialylation of **3c** and **7c** with chST6GalNAc I enzyme (0.6−2 U) and CMP-Neu5Ac (1.5 equiv) was efficiently carried out to give, after purification, the STn (**8**, 349 mg, 97%)- and sialyl(2,6)-T-epitopes (**10**, 74 mg, 93%), respectively. The enzyme showed a strict specificity for the GalNAc residue on the T-epitope. A 2.5-fold excess of donor, increased amount of enzyme, together with longer reaction times did not incorporate any additional sialic acid to the terminal galactose residue on compound **10** (data not shown). Sialyl-(2,3)-T-epitope (**9**, 78 mg, 95%) was prepared from **3c** with the porcine ST3Gal I enzyme. Compound **9** was further elongated with chST6GalNAc I to generate the di-sialyl-T-epitope (**11**, 77 mg, 73%). CMP-Neu5Gc donor could also be effectively utilized by the chST6GalNAc I, and Neu5Gc-Tn-epitope (**12**, 49 mg, 87%) was isolated. The enzymatic products were subject to ion-exchange and size-exclusion chromatography for purification. All enzymatic reactions could easily be scaled up.

Conclusion

Enzyme-catalyzed sialylation to produce sialylated oligosaccharides has been established as a powerful tool in the field of carbohydrate chemistry. Overexpression and purification of the chST6GalNAc I has enabled a detailed acceptor specificity study and evaluation of its potential for preparative synthesis of sialylated T- and Tn-structures. The specificity study shows that by stripping down the natural glycoprotein acceptor to a single amino acid residue α-O-linked to the glycosidic acceptor, the chST6GalNAc I enzyme could still effectively be used for synthesis of O-linked sialoside epitopes with different functionalities. The purified recombinant chST6GalNAc I together with pST3Gal I are shown to be important tools for simple and efficient preparation of O-linked sialoside epitopes.

Experimental Section

General Method. Solvent concentration 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), acetone 29.7 ppm (^{13}C in D_2O). MALDI-FTMS spectra were recorded with an IonSpec Ultima FTMS (IonSpec Corp., Irvine, CA) using dihydroxybenzoic acid as matrix. Thin-layer chromatography was performed on Kieselgel 60 F_{254} Fertigplatten (Merck, Darmstadt, Germany). After development with 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

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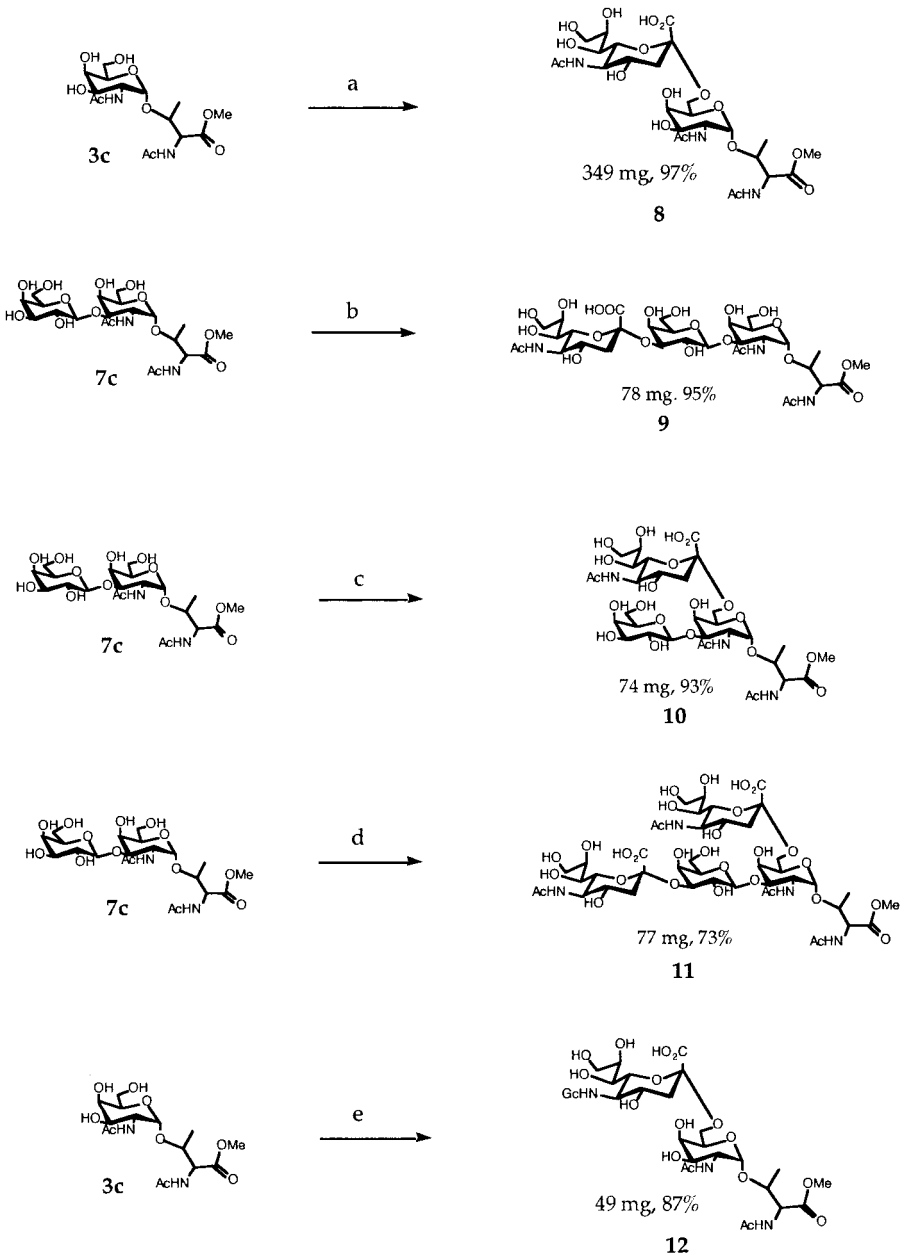

Figure 2. Enzymatic preparation of O-linked sialosides. Reagents and conditions: (a) chST6GalNAc I (2 U), CMP-Neu5Ac (1.5 equiv); (b) pST3Gal I (0.6 U), CMP-Neu5Ac (1.4 equiv); (c) chST6GalNAc I (0.6 U), CMP-Neu5Ac (1.5 equiv); (d) (i) pST3Gal I (1.2 U), CMP-Neu5Ac (1.5 equiv), (ii) chST6GalNAc I (1 U), CMP-Neu5Ac (1.5 equiv); (e) chST6GalNAc I (1 U), CMP-Neu5Gc (1.5 equiv).

Ultrapure water system (Barnstead/Thermolyne, Dubuque, IA). The cDNA of chST6GalNAc I was kindly provided by Dr. S. Tsuji, RIKEN, Wako, Japan. Plasmid DNA for pGIR201Nhe3′ containing proinsulin signal sequence was kindly provided by Dr. K. Drickamer, University of Oxford, Oxford, U.K. All other chemicals substrates were purchased from Sigma Chemical Co. (St. Louis, MO). Ovine submaxillary mucin was prepared as described.[70] CMP[^{14}C]NeuAc were purchased from Du Pont−New England Nuclear (Boston, MA). The radioactive nucleotide-sugar was diluted with unlabeled CMP-Neu5Ac-sugar (Sigma) to the desired specific radioactivity.

Production of Recombinant chST6GalNAc I. Construction of Recombinant Secretable chST6GalNAc I. For expression of a secretable form of chST6GalNAc I, the mammalian expression vector pcDNA3 (Invitrogen, San Diego, CA) was used after modifications as described earlier.[71] In brief, plasmid DNA for the vector pcDNA3 was digested with *Kpn*I and *Eco*RV and then ligated using T4 DNA ligase in the presence of T7 DNA polymerase (unmodified with 3′-5′ exonuclease activity). This treatment deleted the unique restriction sites *Bam*HI and *Eco*RI besides *Kpn*I and *Eco*RV from the modified pcDNA3, herefrom denoted as pcDNA3$_{mod}$. A dog proinsulin signal sequence was then subcloned into this modified vector to obtain pcDNAins which was finally confirmed by double-stranded sequencing.

For subcloning the secretable form of chST6GalNAc-I into pcDNA$_{ins}$ vector, two restriction sites *Bam*HI and *Eco*RI were introduced into its cDNA by polymerase chain reaction (PCR) as follows: a forward primer, CGGGATCCCAAGACTGAGCCACAGTGGGAT (5′-3′; nt. 725−745), with an internal *Bam*HI site (underlined) and a reverse primer, CGGAATTCTCAGGATCTCTGGTAGAGCTTC (5′-3′; nt 1711−1732), with an internal *Eco*RI site (underlined) were used for amplification using the cDNA for the chST6GalNAc I previously subcloned in pBluescript[72] as template. The conditions for PCR were

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94 °C, 30 s, 56 °C, 1 min, and 73 °C, 2 min for 20 cycles. The gel analysis showed the generation of a single band of about 1 kb. This band was purified using Geneclean II (Bio 101, San Diego, CA), digested with *Bam*HI and *Eco*RI, and then subcloned into similarly digested mammalian expression vector $pcDNA_{ins}$ described above. The sequence of the resulting expression vector, termed sST6GalNAc I, was confirmed by dideoxy double-stranded sequencing[73] of the entire subcloned fragment, including the restriction sites used.

Expression of chST6GalNAc I Using Baculoviral System. For expression, the cDNA for the chST6GalNAc I was released from the above constructed sST6GalNAc I by digestion with *Eco*RI and *Not*I. The fragment was purified by gel electrophoresis and ligated into similarly digested pVL1392 (Pharmingen, San Diego, CA). Transformation, plasmid DNA isolation, and other molecular biological techniques were done following the standard protocols (Pharmingen). The sequence of the resulting expression vector, termed pVL-sST6GalNAc I, was confirmed by dideoxy double-stranded sequencing[73] of the entire subcloned fragment, including the restriction sites used. To produce the Baculo viral stock, the plasmid DNA was used for transfection of Sf9 using the BaculoGold Technique (Pharmingen) and following the protocol of the supplier (Pharmingen). For protein expression, High Five cells (Invitrogen) were kept up to 6×10^6 cells/mL density at 27 °C in Insect Select media (BioWhitticker) using a refrigerated shaker incubator. Cell density was brought to 2×10^6 with fresh media (350 mL) using a 1 L fernback baffled shaker flask, and Bacula virus stock was added at a MOI of 5. After 96 h or 70% viability, the cells were harvest by centrifugation at 5000*g*, and the recombinant protein from the supernatant was purified as follows.

Affinity Purification of chST6GalNAc I. The clarified supernatant was diluted by 1/10 vol cacodylic acid (500 mM, pH 6.5) and loaded onto a cytidine-5′-diphospho-(CDP)-hexanolamine column (5 mL) (Calbiochem Inc., San Diego, CA) equilibrated with cacodylic acid buffer (50 mM, pH 6.5) containing sodium chloride (100 mM). The column was washed with cacodylic acid buffer (50 mM, pH 6.5) until the absorbance (A_{280}) reached background level, and the recombinant protein was eluted with cacodylic acid buffer (50 mM, pH 6.5) containing sodium chloride (1.5 M). Fractions (1 mL) containing chST6GalNAc I activity were collected and pooled and finally diluted with glycerol (1 vol). The enzyme was stored at least 6 months at −20 °C without loss of any activity. The typical yield of enzyme from 1 L of culture fluid was 8 U with a specific activity of 1 U/mg protein.

Assay for chST6GalNAc I. Enzyme activity was performed as described previously[74] in 2-(*N*-morpholino)ethanesulfonic acid buffer (MES) (100 μL, 50 mM, pH 6.5) containing asialo ovine submaxillary mucin (80 μg), sodium chloride (100 mM), diluted purified enzyme (5 μL), bovine serum albumin (1 mg/mL), and CMP[^{14}C]Neu5Ac (90 μM, final specific activity 2.7 Ci/mol) at 37 °C for 30 min. The reaction was quenched by freezing, and the thawed sample was loaded onto a gel filtration column (Sephadex G50 0.7 × 13 cm) and eluted with 0.1 M sodium chloride. The flow-through was directly collected in scintillation vials, and the radioactivity was assayed by liquid scintillation counting (Beckman LS counter). Values (μmol transferred/min incubation) obtained in this standard assay were multiplied by the factor 5.5, derived from kinetic analysis[75] to give units of enzyme. One unit (U) of enzyme activity is defined as the amount of enzyme that catalyzes the transfer of 1 μmol of Neu5Ac from CMP-Neu5Ac to asialo ovine submaxillary mucin per min at saturating substrate concentrations.

Enzyme activity for different acceptors was assayed at 37 °C for 30 min in a final volume of 100 μL containing cacodylic acid (50 mM, pH 6.5), CMP[^{14}C]Neu5Ac (0.5 mM, final specific activity 0.70 Ci/mol), acceptor (1 mM), and diluted enzyme (3.8 mU). Reactions were terminated by the addition of water (0.7 mL). The reaction mixtures were then passed through Pasteur pipet columns of Dowex resin (1-X8, 200−400 mesh, PO_4^{2-} form, 0.5 × 5 cm, 1 mL). The columns were washed with water (1.3 mL), the effluents were directly collected in scintillation vials, and the radioactivity was assayed as described above.

General Procedure for Preparation of Crude CMP-Neu5Ac and CMP-Neu5Gc. Cytidine 5′triphosphate (CTP) (980 mg, 1.72 mmol) and *N*-acetyl neuraminic acid or *N*-glycolyl neuraminic acid[69] (500 mg, 1.62 mmol) were dissolved in Tris-HCl (100 mL, 10 mM, pH 8.5), containing $MgCl_2$ (20 mM). ST3-CMPNeu5Ac-synthetase fusion protein (300 U) was added, and the reaction proceeded at room temperature for 1 h. The pH was kept constant (8.5) with NaOH (2 M). When all CTP was consumed according to TLC (^{i}PrOH/H_2O/NH_3, 6:3:2 by vol), the reaction mixture was filtered through a 10 000 MW cutoff membrane to remove the fusion protein, and the filtrate was lyophilized. Prior to use, the crude residue was dissolved in water, and the pH was set to 7.0 by HCl (2 M). The approximate concentration of nucleotide-sugar was determined by absorbance (A_{271}).

2-Azidoethyl *N*-Acetyl-2-deoxy-α-D-galactopyranoside (1). *N*-Acetyl galactosamine (300 mg, 1.36 mmol) was dissolved in 2-azidoethanol (2 mL), and boron trifluoridetherate (1 mL, 7.0 mmol) was added. The mixture was stirred at room temperature for 2 h. The reaction was neutralized with sodium hydroxide (10 M) and loaded onto a Sephadex G15 (5 × 140 cm) column and eluted with 5% ^{n}BuOH. Fractions containing **1** were collected, lyophilized, and a second round of purification was performed. Isolated fractions gave **1** (309.7 mg, 78%). ^{1}H NMR (D_2O): δ 2.05 (s, 3H, C*H*$_3$CONH), 3.49−3.54 (m, 2H, OCH$_2$C*H*2N$_3$), 3.75−3.77 (m, 2H, 6a, 6b), 3.67, 3.91 (2 m, 2H, OC*H*$_2$CH$_2$N$_3$), 3.94 (dd, 1H, H-3), 3.99 (t, 1H, H-5), 4.00 (d, 1H, H-4), 4.18 (dd, 1H, H-2), 4.97 (dd, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 21.5, 49.3, 49.8, 60.7, 66.0, 67.0, 67.3, 70.6, 96.5, 174.0. Maldi-FT-MS: expected, *m*/*z* = 291.1299 (M + H); found, *m*/*z* = 291.1295.

2-*N*-Acetylethyl *N*-Acetyl-2-deoxy-α-D-galactopyranoside (2). 1 (100.0 mg, 345 μmol) and Pd/C (10%, 20 mg) were taken up in methanol (10 mL) and hydrogenated with H_2 at room temperature and atmospheric pressure. The mixture was filtrated, washed with methanol (5 mL), and acetic anhydride (0.3 mL) was added. After 10 min at room temperature, the mixture was evaporated to dryness, dissolved in H_2O (2 mL), and further purified by gel filtration chromatography (Sephadex G15 1.6 × 140 cm) to give **2** (93.2 mg, 87%). ^{1}H NMR (D_2O): δ 2.01 (s, 3H, C*H*$_3$CONH), 2.04 (s, 3H, C*H*$_3$CONH), 3.43−3.50 (m, 2H, OCH$_2$C*H*$_2$N$_3$), 3.74−3.78 (m, 2H, 6a, 6b), 3.54, 3.78 (2 m, 2H, OC*H*$_2$CH$_2$N$_3$), 3.87 (dd, 1H, H-3), 3.92 (t, 1H, H-5), 3.98 (d, 1H, H-4), 4.16 (dd, 1H, H-2), 4.89 (dd, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): 21.3, 21.4, 49.2, 60.7, 65.8, 67.3, 68.0, 70.0, 70.5, 96.6, 173.8, 174.0. MALDI-FT-MS *m*/*z* calcd for (M + Na), 329.1319; found, 329.1322. ***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-(3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine Phenacylester (3a).** This was prepared as described previously.[47,48,76] **3a**: 8.0 g (75%). ^{1}H NMR ($CDCl_3$): δ 1.44 (d, 3H, H-γ), 1.63, 2.05, 2.07 (s, 3H, C*H*$_3$-CO), 2.18 (s, 3H, C*H*$_3$CONH), 3.80 (dd, 1H, H-2), 4.13 (d, 1H, CH$_2$ (Fmoc)), 4.29 (t, 1H, H-5), 4.32 (m, 1H, CH (Fmoc)), 4.35, 4.42 (m, 2H, 6a, 6b), 5.34 (t, 1H, H-3), 5.48 (d, 1H, H-4), 4.57 (d, 1H, H-α, $J_{\alpha,\beta}$ 2.0 Hz), 4.64 (m, 1H, H-β), 5.54 (d, 1H, H-1, $J_{1,2}$ 3.5 Hz), 5.93 (d, 1H, NH (thr)), 5.32, 5.60 (d, 2H, CH$_{a/b}$ (phenacyl)), 7.2−8.0 (m, Ar). ^{13}C NMR ($CDCl_3$): δ 19.1, 20.6, 47.0, 58.2, 58.4, 61.8, 66.7, 66.9, 67.5, 67.6, 68.4, 76.1, 98.1, 120−144, 156.8, 169.8, 169.9, 170.0, 170.3, 191.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 795.2484; found, 795.2479.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-galactopyranosyl)-L-threonine Phenacylester (3b).**

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This was prepared as described previously.[47,48] **3b**: 3.5 g (88%). ^{1}H NMR ($CDCl_3$): δ 1.44 (d, 3H, H-γ), 1.63, 2.01, 2.04 (s, 9H, CH_3CO), 2.18 (s, 3H, CH_3CONH), 4.10, 4.15 (m, 2H, 6a, 6b), 4.30 (t, 1H, H-5), 4.30 (m, 1H, CH (Fmoc)), 4.47 (d, 2H, CH_2 (Fmoc)), 4.54 (m, 1H, H-β), 4.57 (d, 1H, H-α, $J_{\alpha,\beta}$ 2.0 Hz), 4.68 (dd, 1H, H-2), 5.18 (dd, 1H, H-3), 5.28, 5.60 (d, 2H, $CH_{a/b}$ (phenacyl)), 5.42 (d, 1H, H-1, $J_{1,2}$ 3.5 Hz), 5.44 (d, 1H, H-4), 5.74 (d, 1H, NH (thr)), 6.01 (d, 1H, NH (Ac), 7.2−8.0 (m, Ar). ^{13}C NMR ($CDCl_3$): δ 18.5, 20.6, 20.7, 22.8, 47.1, 47.4, 58.4, 62.2, 66.6, 67.2, 67.5, 67.5, 68.6, 76.7, 99.9, 120−144, 156.6, 170.3, 170.3, 170.4, 170.8, 171.0, 191.1. MALDI-FT-MS *m*/*z* calcd for (M + Na), 811.2685; found, 811.2687.

***N*-(Acetyl)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine Methylester (3c). 3b** (3.5 g, 4.4 mmol) was dissolved in MeOH (70 mL) and NaOMe (0.5 M, 15 mL). The mixture was stirred at room temperature for 30 min. The reaction was treated with Ac_2O (10 mL) and stirred for 10 min. The mixture was neutralized with sodium methoxide (0.5 M) and concentrated by evaporation. The residue was loaded onto a Sephadex G15 (5 × 140 cm), and the compound was eluted with 5% ^{n}BuOH. Fractions containing the product were collected and lyophilized to give **3c** (798 mg, 47%) as a white powder. ^{1}H NMR (D_2O): δ 1.27 (d, 3H, γ-CH_3), 2.06 (s, 3H, CH_3CONH), 2.15 (s, 3H, CH_3CONH), 3.74 (s, 3H, COOMe), 3.74−3.76 (m, 2H, 6a, 6b), 3.87 (dd, 1H, H-3), 3.94 (d, 1H, H-4), 4.01 (t, 1H, H-5), 4.08 (dd, 1H, H-2), 4.48 (q, 1H, β-CH), 4.64 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.89 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.5, 21.1, 21.6, 49.3, 52.4, 56.7, 60.7, 66.9, 67.9, 70.7, 74.7, 98.1, 171.6, 173.8, 174.3. MALDI-FT-MS *m*/*z* calcd for (M + Na), 401.1530; found, 401.1524.

***N*-(Acetyl)-*O*-(2-azido-2-deoxy-α-D-galactopyranosyl)-L-threonine Methylester (4). 3a** (150 mg, 0.19 mmol) was dissolved in MeOH (2 mL) and NaOMe (0.5 M, 0.5 mL). The mixture was stirred at room temperature for 30 min. The reaction was treated with Ac_2O (0.3 mL) and stirred for 10 min. The mixture was neutralized with Dowex resin (1 × 8, 200−400 mesh, H^+), filtered, and concentrated. The residue was redissolved in water and loaded onto a SepPak C18 column (0.5 g). The column was washed with water (2 vol), and the compound was eluted with 10−60% MeOH. Appropriate fractions were collected and evaporated to give **4** (55 mg, 78.8%). ^{1}H NMR (D_2O): δ 1.29 (d, 3H, γ-CH_3), 2.12 (s, 3H, CH_3CONH), 3.58 (dd, 1H, H-2), 3.74−3.76 (m, 2H, 6a, 6b), 3.84 (s, 3H, COOMe), 3.99 (dd, 1H, H-3), 4.00 (d, 1H, H-4), 4.02 (t, 1H, H-5), 4.48 (q, 1H, β-CH), 4.67 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 5.10 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.1, 21.1, 52.6, 56.9, 59.7, 60.6, 67.0, 68.3, 70.8, 75.7, 98.8, 171.6, 174.2. MALDI-FT-MS *m*/*z* calcd for (M + Na), 581.2106; found, 581.2129.

***N*-(Acetyl)-*O*-(2-amino-2-deoxy-α-D-galactopyranosyl)-L-threonine Methylester (5). 4** (25 mg, 74 μmol) was treated with PdC (10%) in methanol (5 mL) and hydrogenated with H_2 at room temperature and atmospheric pressure. The mixture was filtrated and concentrated to give the amino glycoside **5** (21.0 mg, 82%). ^{1}H NMR (D_2O): δ 1.30 (d, 3H, γ-CH_3), 2.11 (s, 3H, CH_3CONH), 3.07 (dd, 1H, H-2), 3.74−3.76 (m, 2H, 6a, 6b), 3.84 (s, 3H, COOMe), 3.95 (d, 1H, H-4), 3.97 (dd, 1H, H-3), 4.03 (t, 1H, H-5), 4.40 (q, 1H, β-CH), 4.64 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 5.04 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.1, 21.1, 50.4, 52.6, 56.4, 60.7, 67.9, 70.9, 75.3, 76.4, 98.6, 171.8, 174.1. MALDI-FT-MS *m*/*z* calcd for (M + H), 337.1605; found, 337.1605.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-L-threonine Methylester (6). 3b** (100 mg, 126.8 μmol) was dissolved in MeOH (10 mL), and a few drops of NaOMe (0.5 M) were added until pH indicated not more than 9. The mixture was stirred at room temperature for 30 min, and the reaction was neutralized with Dowex (1 × 8, 200−400 mesh, H^+), filtered, and concentrated. The residue was redissolved in EtOAc and MeOH (1:1, 10 mL) and loaded onto a Silica column (1.5 × 10 cm) packed in EtOAc and eluted with EtOAc (2 vol), EtOAc/MeOH (5/1, 2 vol), and EtOAc/MeOH (1/1). Fractions containing the product were pooled and concentrated to give **6** (50 mg, 72%). ^{1}H NMR (D_2O): δ 1.27 (d, 3H, γ-CH_3), 2.06 (s, 3H, CH_3CONH), 2.15 (s, 3H, CH_3CONH), 3.74 (s, 3H, COOMe), 3.74−3.76 (m, 2H, 6a, 6b), 3.87 (dd, 1H, H-3), 3.94 (d, 1H, H-4), 4.01 (t, 1H, H-5), 4.08 (dd, 1H, H-2), 4.28 (m, 1H, CH (Fmoc)), 4.48 (q, 1H, β-CH), 4.54 (d, 2H, CH_2 (Fmoc)), 4.64 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.89 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz), 7.2−8.0 (m, Ar). ^{13}C NMR (D_2O): δ 19.3, 23.2, 49.3, 52.8, 51.2, 60.1, 62.8, 67.7, 69.6, 70.3, 72.9, 76.5, 100.6, 120−145, 159.2, 172.4, 173.9. MALDI-FT-MS *m*/*z* calcd for (M + Na), 581.2106; found, 581.2129.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[4,6-di-*O*-acetyl-2-azido-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine Phenacylester (7a).** This was prepared as described previously.[47,48,76] **7a**: 0.80 g (39%). ^{1}H NMR ($CDCl_3$): δ 1.42 (d, 3H, H-γ), 1.59, 1.97, 2.03, 2.04, 2.06, 2.14 (s, 18H, CH_3CO), 2.15 (s, 3H, CH_3CONH), 3.92 (t, 1H, H-5′), 4.02 (dd, 1H, H-2), 4.13 (d, 1H, CH_2 (Fmoc)), 4.10, 4.17 (m, 2H, 6a′, 6b′), 4.29 (t, 1H, H-5), 4.32 (m, 1H, CH (Fmoc)), 4.00, 4.20 (m, 2H, 6a, 6b), 5.01 (dd, 1H, H-3′), 5.36 (d, 1H, H-4′), 5.34 (t, 1H, H-3), 5.49 (d, 1H, H-4), 4.56 (d, 1H, H-α, $J_{\alpha,\beta}$ 2.0 Hz), 4.61 (m, 1H, H-β), 4.74 (d, 1H, H-1′, $J_{1,2}$ 7.5 Hz), 5.18 (dd, 1H, H-2′), 5.50 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz), 5.91 (d, 1H, NH (thr)), 5.35, 5.57 (d, 2H, $CH_{a/b}$ (phenacyl)), 7.2−8.0 (m, Ar). ^{13}C NMR ($CDCl_3$): δ 19.1, 20.5, 20.6, 20.6, 20.7, 47.1, 58.5, 60.3, 60.9, 62.9, 66.7, 67.3, 67.7, 68.8, 69.3, 70.7, 74.8, 76.0, 98.9, 101.4, 120−144, 156.7, 169.4−170.4, 191.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 1083.3329; found, 1083.3353.

***N*-(9-Fluorenylmethyloxycarbonyl)-*O*-[2-acetamido-4,6-di-*O*-acetyl-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine Phenacylester (7b).** This was prepared as described previously.[47,48] **7b**: 0.62 g (88%). ^{1}H NMR ($CDCl_3$): δ 1.42 (d, 3H, H-γ), 1.59, 1.97, 2.03, 2.04, 2.06, 2.14 (s, 18H, CH_3CO), 2.15 (s, 3H, CH_3CONH), 3.76 (t, 1H, H-5′), 4.58 (dd, 1H, H-2), 4.13 (d, 1H, CH_2 (Fmoc)), 4.02, 4.17 (m, 2H, 6a′, 6b′), 4.27 (t, 1H, H-5), 4.32 (m, 1H, CH (Fmoc)), 4.00, 4.20 (m, 2H, 6a, 6b), 5.01 (dd, 1H, H-3′), 5.27 (d, 1H, H-4′), 4.04 (t, 1H, H-3), 5.14 (d, 1H, H-4), 4.56 (d, 1H, H-α, $J_{\alpha,\beta}$ 2.0 Hz), 4.61 (m, 1H, H-β), 4.89 (d, 1H, H-1′, $J_{1,2}$ 7.5 Hz), 5.01 (dd, 1H, H-2′), 5.49 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz), 6.26 (d, 1H, NH (Ac), 5.59 (d, 1H, NH (thr)), 5.28, 5.60 (d, 2H, $CH_{a/b}$ (phenacyl)), 7.2−8.0 (m, Ar). ^{13}C NMR ($CDCl_3$): δ 17.5, 20.5, 20.5, 20.6, 20.7, 22.9, 47.1, 49.0, 58.6, 60.8, 62.9, 66.8, 66.9, 67.2, 68.0, 68.6, 69.3, 70.4, 70.7, 72.8, 76.9, 99.4, 101.2, 120−144, 156.5, 169.4−170.4, 192.6. MALDI-FT-MS *m*/*z* calcd for (M + Na), 1099.353; found, 1099.3530.

***N*-(Acetyl)-[2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranosyl]-L-threonine Methylester (7c).** Acetylated disaccharide **7b** (580 mg, 538 μmol) was dissolved in MeOH (10 mL) and NaOMe (2 mL, 0.5 M). After reaction at room temperature for 12 h, Ac_2O (1.5 mL) was then added to the mixture. After 10 min of reaction, the mixture was neutralized with sodium methoxide (0.5 M) and concentrated by evaporation. The residue was loaded onto a Sephadex G15 (5 × 140 cm), and the compound was eluted with 5% ^{n}BuOH. Appropriate fractions were collected and lyophilized to give **7c** (200 mg, 71%) as a white powder. ^{1}H NMR (D_2O): δ 1.27 (d, 3H, γ-CH_3), 2.04 (s, 3H, CH_3CONH), 2.15 (s, 3H, CH_3CONH), 3.52 (dd, 1H, H-2′), 3.62 (dd, 1H, H-3′), 3.65 (dd, 1H, H-5′), 3.74 (s, 3H, COOMe), 3.74−3.76 (m, 2H, 6a, 6b), 3.74−3.76 (m, 2H, H-6a′, H-6b′), 3.91 (d, 1H, H-4′), 4.01 (dd, 1H, H-3), 4.05 (t, 1H, H-5), 4.22 (d, 1H, H-4), 4.24 (dd, 1H, H-2), 4.47 (dd, 1H, H-1′, $J_{1,2}$ 7.5 Hz), 4.49 (q, 1H, β-CH), 4.65 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.88 (dd, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.5, 21.1, 21.6, 48.1, 52.4, 56.7, 60.6, 60.7, 68.1, 68.2, 70.0, 70.4, 72.0, 74.5, 74.7, 76.0, 98.3, 104.1, 171.7, 173.7, 174.3. MALDI-FT-MS *m*/*z* calcd for (M + Na), 563.2059; found, 563.2073.

***N*-(Acetyl)-(sodium[5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosyl]-onate-6-*O*-(2-acetamido-2-deoxy-α-D-galacto-pyranosyl))-L-threonine Methylester (8).** Tn-antigen **3c** (201 mg, 0.55 mmol) was mixed with crude CMP-Neu5Ac (0.82 mmol, pH 7.0). Water (10 mL) and chST6GalNAc I (2 U) were added, and the reaction

was kept at 37 °C overnight. The reaction was completed after adding 10% extra CMP-Neu5Ac and an additional 6 h of incubation. The mixture was filtered through Dowex 1 × 8−200 (1.5 × 7 cm, phosphate) and washed with 4 vol of water. The filtrate was lyophilized, and the residue was loaded onto Sephadex G15 (5 × 160 cm) eluted with 5% ^{n}BuOH. Appropriate fractions were collected and lyophilized to give the disaccharide **8** (349 mg, 97%). ^{1}H NMR (D_2O): δ 1.30 (d, 3H, γ- CH_3), 1.68 (t, 1H, H-3′$_{ax}$), 2.03 (s, 3H, CH_3CONH′), 2.05 (s, 3H, CH_3CONH), 2.15 (s, 3H, CH_3CONH), 2.71 (dd, 1H, H-3′$_{eq}$), 3.58 (dd, 1H, H7′), 3.67 (d, 1H, H-4′), 3.70 (dd, 1H, H-6′), 3.74 (s, 3H, COOMe), 3.64−4.10 (m, 2H, 6a, 6b), 3.65−3.90 (m, 2H, H-9′), 3.82 (dd, 1H, H-5′), 3.83 (t, 1H, H-5), 3.86 (dd, 1H, H-3), 3.88 (m, 1H, H-8′), 3.99 (d, 1H, H-4), 4.07 (dd, 1H, H-2), 4.45 (q, 1H, β-CH), 4.64 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.86 (dd, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.5, 21.1, 21.5, 21.6, 39.5, 49.3, 51.3, 52.3, 56.7, 62.1, 63.3, 66.8, 67.6, 67.7, 68.0, 72.0, 75.2, 98.3, 99.7, 172.7, 173.7, 174.3, 174.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 692.2485; found, 692.2506.

Sodium *N*-(Acetyl)-[5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosyl]-onate-3-*O*-(2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranosyl)-L-threonine Methylester (9). T-antigen **7c** (53.0 mg, 0.10 mmol) was mixed with crude CMP-Neu5Ac (0.14 mmol, pH 7.0). Water (10 mL) and pST3Gal-I (0.6 U) were added, and the mixture was reacted at 37 °C for 14 h. When TLC (6:3:3:2, EtOAc, MeOH, HOAc, H_2O by vol) showed complete consumption of the disaccharide, the mixture was passed through a Dowex 1 × 8−200 (1.5 × 4 cm, phosphate) column, and the filtrate was lyophilized. The residue was purified on a gel filtration column (Sephadex 2.5 × 160 cm) eluted with 5% ^{n}BuOH. Appropriate fractions containing the trisaccharide were collected and lyophilized to give sialyl (2,3)-T-epitope **9** (78.0 mg, 95%). ^{1}H NMR (D_2O): δ 1.26 (d, 3H, γ-CH_3), 1.78 (t, 1H, H-3″$_{ax}$), 2.03 (s, 3H, CH_3CONH″), 2.04 (s, 3H, CH_3-CONH), 2.15 (s, 3H, CH_3CONH), 2.75 (dd, 1H, H-3″$_{eq}$), 3.54 (d, 1H, H-2′), 3.59 (dd, 1H, H7″), 3.64 (dd, 1H, H-5′), 3.66 (d, 1H, H-4″), 3.68 (dd, 1H, H-6″), 3.74 (s, 3H, COOMe), 3.74−3.76 (m, 2H, 6a, 6b), 3.74−3.76 (m, 2H, 6a′, 6b′), 3.65−3.90 (m, 2H, H-9″), 3.85 (dd, 1H, H-5″), 3.89 (m, 1H, H-8″), 3.92 (d, 1H, H-4′), 4.01 (dd, 1H, H-3), 4.04 (t, 1H, H-5), 4.07 (dd, 1H, H-2), 4.07 (dd, 1H, H-3′), 4.22 (d, 1H, H-4), 4.50 (q, 1H, β-CH), 4.53 (d, 1H, H-1′, $J_{1,2}$ 7.5 Hz), 4.65 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.89 (dd, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.5, 21.1, 21.5, 21.7, 39.3, 48.1, 51.1, 52.4, 56.6, 60.5, 60.7, 62.1, 66.8, 67.6, 67.8, 68.0, 68.3, 70.5, 71.4, 72.2, 74.4, 74.8, 75.1, 76.3, 98.3, 99.0, 104.1, 171.6, 173.3, 173.9, 174.3, 174.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 854.3013; found, 854.2991.

Sodium *N*-(Acetyl)-[5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosyl]-onate-6-*O*-(2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-α-D-galactopyranosyl)-L-threonine Methylester (10). T-antigen **7c** (50.0 mg, 95.0 μmol) was mixed with crude CMP-NeuAc (143 μmol, pH 7.0). Water (10 mL) and chST6GalNAc-I (0.6 U) were added, and the mixture was reacted at 37 °C for 14 h. When TLC (6:3:3:2, EtOAc, MeOH, HOAc, H_2O by vol) showed complete consumption of the disaccharide, the mixture was passed through a Dowex (1 × 8−200 mesh, 1.5 × 4 cm, phosphate) column, lyophilized, and loaded onto a gel filtration column (Sephadex 2.5 × 160 cm) eluted with 5% ^{n}BuOH. Appropriate fractions containing the trisaccharide were concentrated to dryness to give sialyl (2,6)-T-epitope **10** (75.0 mg, 96%). ^{1}H NMR (D_2O): δ 1.30 (d, 3H, γ- CH_3), 1.66 (t, 1H, H-3″$_{ax}$), 2.03 (s, 3H, CH_3CONH″), 2.03 (s, 3H, CH_3CONH), 2.15 (s, 3H, CH_3CONH), 2.70 (dd, 1H, H-3″$_{eq}$), 3.50 (d, 1H, H-2′), 3.58 (dd, 1H, H7″), 3.63 (dd, 1H, H-5′), 3.70 (dd, 1H, H-4″), 3.68 (dd, 1H, H-6″), 3.74 (s, 3H, COOMe), 3.70, 4.13 (m, 2H, 6a, 6b), 3.74−3.76 (m, 2H, 6a′, 6b′), 3.65−3.90 (m, 2H, H-9″), 3.86 (dd, 1H, H-5″), 3.89 (m, 1H, H-8″), 3.90 (d, 1H, H-4′), 3.99 (dd, 1H, H-3), 3.90 (t, 1H, H-5), 4.23 (dd, 1H, H-2), 3.61 (dd, 1H, H-3′), 4.24 (d, 1H, H-4), 4.48 (q, 1H, β-CH), 4.46 (d, 1H, H-1′, $J_{1,2}$ 8.0 Hz), 4.65 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.86 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.5, 21.1, 21.5, 21.7, 39.6, 48.1, 51.3, 52.3, 56.7, 60.5, 62.1, 63.3, 67.7, 68.1, 68.1, 69.1, 70.0, 71.2, 72.0, 72.0, 74.5, 75.1, 76.1, 98.3, 99.8, 104.2, 171.6, 173.1, 173.7, 174.3, 174.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 854.3013; found, 854.3000.

Di-sialyl-T-epitope (11). T-antigen **7c** (50.0 mg, 95 μmol) was mixed with crude CMP-Neu5Ac (143 μmol, pH 7.0), pST3Gal-I (1.2 U) was added, and the mixture was reacted at 37 °C for 4 h. When TLC (6:3:3:2, EtOAc, MeOH, HOAc, H_2O by vol) showed complete consumption of the disaccharide, the mixture was loaded onto a gel filtration column (Sephadex 1.5 × 160 cm) eluted with 5% ^{n}BuOH. Appropriate fractions were concentrated to dryness. The residue (142 mg) was dissolved in crude CMP-NeuAc pH 7.0 as described above, and chST6GalNAc-I (1 U) was added. The mixture was reacted at 37 °C for 24 h. When completed, the mixture was passed through a Dowex 1 × 8−200 (phosphate, 1 × 4 cm) column and lyophilized. The residue was then loaded onto a Sephadex G15 (2.5 × 160 cm) eluted with 5% ^{n}BuOH. Appropriate fractions was collected and lyophilized to give **11** (76.7 mg, 73%). Selected ^{1}H NMR (D_2O): δ 1.30 (d, 3H, γ-CH_3), 1.66 (t, 1H, H-3‴$_{ax}$), 1.78 (t, 1H, H-3‴$_{ax}$), 2.03 (s, 3H, CH_3CONH), 2.03 (s, 3H, CH_3CONH″), 2.03 (s, 3H, CH_3CONH‴), 2.16 (s, 3H, CH_3-CONH), 2.72 (dd, 1H, H-3″$_{eq}$), 2.73 (dd, 1H, H-3‴$_{eq}$), 3.53 (d, 1H, H-2′), 3.65 (dd, 1H, H-5′), 3.66 (d, 1H, H-4″), 3.69 (d, 1H, H-4‴), 3.73 (s, 3H, COOMe), 3.70−4.12 (m, 2H, 6a, 6b), 3.75−3.80 (m, 2H, 6a′, 6b′), 3.98 (d, 1H, H-4′), 3.99 (dd, 1H, H-3), 3.82 (t, 1H, H-5), 4.22 (dd, 1H, H-2), 4.07 (dd, 1H, H-3′), 4.22 (d, 1H, H-4), 4.48 (q, 1H, β-CH), 4.52 (d, 1H, H-1′, $J_{1,2}$ 8.0 Hz), 4.66 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.87 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz). Selected ^{13}C NMR (D_2O): δ 17.5, 21.1, 21.5, 21.6, 21.7, 39.6, 48.0, 51.1, 51.3, 52.3, 56.7, 60.4, 62.1, 63.3, 66.8, 67.6, 67.7, 67.8, 68.0, 68.3, 69.0, 69.1, 71.0, 71.2, 71.4, 72.0, 72.2, 74.3, 75.2, 75.3, 76.4, 98.4, 99.8, 104.1, 171.6, 173.3, 173.9, 174.2, 174.3, 174.5. MALDI-FT-MS *m*/*z* calcd for (M + Na), 1145.3967; found, 1145.3991.

Sodium *N*-(Acetyl)-[5-glycolylamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulo-pyranosyl]-onate-6-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl))-L-threonine Methylester (12). Tn-antigen **3c** (30.0 mg, 79.3 μmol) was mixed with crude CMP-Neu5Gc (119 μmol, pH 7.0). Water (10 mL) and chST6GalNAc-I (1 U) were added, and the reaction was kept at 37 °C for 24 h. The reaction was completed after adding 10% extra CMP-Neu5Gc and an additional 6 h of incubation. The mixture was filtered through Dowex 1 × 8−200 (1.5 × 7 cm, phosphate) and washed with 4 vol of water. The filtrate was lyophilized, and the residue was loaded onto Sephadex G15 (5 × 160 cm) eluted with 5% ^{n}BuOH. Appropriate fractions were collected and lyophilized to give the disaccharide **12** (49.0 mg, 87%). ^{1}H NMR (D_2O): δ 1.30 (d, 3H, γ-CH_3), 1.69 (t, 1H, H-3′$_{ax}$), 2.05 (s, 3H, CH_3CONH), 2.15 (s, 3H, CH_3CONH), 2.73 (dd, 1H, H-3′$_{eq}$), 3.58 (dd, 1H, H7′), 3.77 (dd, 1H, H-4′), 3.65 (dd, 1H, H-6′), 3.75 (s, 3H, COOMe), 3.64−4.10 (m, 2H, 6a, 6b), 3.65−3.90 (m, 2H, H-9′), 3.90 (dd, 1H, H-5′), 3.83 (dd, 1H, H-5), 3.86 (dd, 1H, H-3), 3.88 (m, 1H, H-8′), 4.00 (d, 1H, H-4), 4.07 (dd, 1H, H-2), 4.13 (s, 2H, HOCH_2CONH), 4.49 (q, 1H, β-CH), 4.64 (d, 1H, α-CH, $J_{\alpha,\beta}$ 2.0 Hz), 4.87 (d, 1H, H-1, $J_{1,2}$ 4.0 Hz). ^{13}C NMR (D_2O): δ 17.5, 21.0, 21.6, 39.6, 49.3, 51.0, 52.3, 56.7, 60.4, 62.0, 63.3, 66.8, 67.4, 67.6, 68.0, 69.3, 71.3, 71.8, 75.1, 98.2, 99.8, 171.6, 172.8, 173.7, 174.3, 175.2. MALDI-FT-MS *m*/*z* calcd for (M + Na), 708.2434; found, 708.2460.

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Supporting Information Available: Copies of ^{1}H NMR, spectra for compounds **8**, **9**, **10**, **11**, and **12** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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