Regeneration of PAPS for the Enzymatic Synthesis of Sulfated Oligosaccharides

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This paper describes the study of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) regeneration from 3′-phosphoadenosine-5′-phosphate (PAP) for use in practical syntheses of carbohydrate sulfates which are catalyzed by sulfotransferases. Among the regeneration systems, the one with recombinant aryl sulfotransferase proved to be the most practical. This regeneration system was coupled with a sulfotransferase-catalyzed reaction, using a recombinant Nod factor sulfotransferase, for the synthesis of various oligosaccharide sulfates that were further glycosylated using glycosyltransferases.

Sulfated carbohydrates and glycopeptides have generated interest due to their roles in specific cell signaling and recognition events of both normal and disease processes,[1] such as chronic inflammation, cancer metastasis, cartilage formation, and hormone regulation. Recent studies have identified the 6-sulfo sialyl Lewis x (sLe^X) structure as the preferred ligand for L-selectin binding in lymphocyte homing to high-endothelial venules in peripheral lymph nodes.[2] Evidence for sulfation at the 6′-position of the sLe^X structure as the L-selectin ligand has also been reported.[3,4] Furthermore, the ligand for P-selectin adhesion has been identified as a tyrosine-sulfated sLe^X glycopeptide in which the sulfate group contributes significantly (∼8 kcal/mol) to the ligand binding.[5] The association of sulfation with selectin binding has, thus, elicited renewed efforts to synthesize sulfated carbohydrates and glycopeptides to study their biological activities.

Sulfotransferases are the family of enzymes which catalyze the transfer of a sulfuryl group (SO_3) from 3′-phosphoadenosine-5′-phosphosulfate (PAPS) (**1**) to an acceptor molecule. Sulfotransferases, present in most organisms and in all human tissues, mediate sulfation of different classes of acceptors for a variety of biological functions. To date, more than 30 sulfotransferase cDNAs have been isolated from animal, plant, and bacterial sources.[6,7] The varied and important roles sulfotransferases play in biological systems have only recently been uncovered, including detoxification, cell signaling, and modulation of receptor binding.[1,8] Drug design for the inhibition of these therapeutically interesting enzymes has quickly followed sulfotransferase discovery and will gain increasing importance as we have easy access to this class of molecules to study their biological roles more completely.[9−12]

In the course of biological sulfation, inorganic sulfate first becomes activated to a high-energy cofactor, which is followed by transfer of the sulfate group to the final acceptor (Scheme 1).[13] ATP is initially sulfated by ATP sulfurylase (EC 2.7.7.4), yielding adenosine-5′-phosphosulfate (APS) (**2**) and pyrophosphate. APS is then phosphorylated at the 3′ position by APS kinase (EC 2.7.1.25) to give PAPS, the universal donor of sulfate for most sulfotransferase enzymes.[14] Following sulfation, sulfotransferases release 3′-phosphoadenosine-5′-phosphate (PAP) (**3**), which is degraded in vivo through dephosphorylation at the 3′ position to form adenosine-5′-monophosphate (AMP) (**4**). Reactivation to ATP follows for the cycle to begin anew.[15] Several groups have reported the syntheses of PAPS,[16−19] which involved several difficult steps and gave very low yields.[20−22] The enzymatic preparation of PAPS on a nanomole-to-micromole scale using isolated enzymes has been described; however, it

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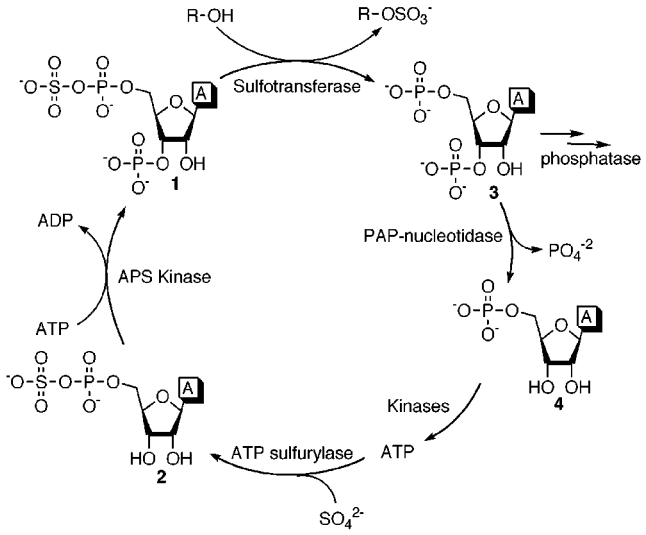
Scheme 1. PAPS Metabolism in Vivo



is not clear if this process is feasible for larger scale syntheses.[21] PAP has demonstrated micromolar product inhibition of sulfotransferases and, hence, must be removed from preparative reactions.[23] Also, the high cost and instability of PAPS ($45/mg; $t_{1/2}$ = 20 h at pH 8.0) have made previous preparative enzymatic sulfations untenable. In a preliminary study, we investigated the large-scale enzymatic synthesis of PAPS and its in situ regeneration for application to the enzymatic synthesis of sulfated molecules.[23]

The genes coding for ATP sulfurylase and APS kinase from *Escherichia coli* have been identified and are located in the cluster of *cys*CDHIJ.[24] ATP sulfurylase contains two subunits corresponding to genes *cys*E and *cys*N. APS kinase exists as one subunit from the *cys*C gene. Both ATP sulfurylase and APS kinase were expressed in *E. coli*.[23]

We used a representative enzymatic sulfation to study the practicality of PAPS regeneration; that is, the nod-factor sulfotransferase (NodST)-associated signal exchange between a nitrogen-fixing bacterium, *Rhizobium* sp., and its symbiont legume.[25] The bacterium excretes specific sulfated lipooligosaccharide markers, called Nod factors, that are required for root nodulation and bacterial infection. This sulfotransferase, found in the nodulation locus of *Rhizobium meiloti*, transfers a sulfuryl group from PAPS to the 6-position of a lipochitotetraose. This enzyme has also been found to accept bare chitoses as substrates for sulfation.[26]

In this paper we describe in detail the cloning and overproduction of ATP sulfurylase and APS kinase that are used for the enzymatic synthesis of PAPS on a millimole scale. PAPS regeneration for enzymatic synthesis is demonstrated by two recycling systems, using both of the multiple enzymes,[23] and by the aryl sulfotransferase IV with *p*-nitrophenyl sulfate as the sulfate donor. We also describe in detail the cloning and overproduction of NodST and its use for the enzymatic sulfation of three *N*-acetylchitoses, *N*,*N*′-diacetylchitobiose (**5a**), *N*,*N*′,*N*″-triacetylchitotriose (**5b**), and *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose (**5c**). These three sulfated chitoses are further enzymatically glycosylated with β(1,4)galactosyltransferase, α(2,3)sialyltransferase, and α(1,3)fucosyltransferase to yield sLe^X derivatives which are sulfated at variable distances from the Lewis x moiety. In the synthesis, it was found that the commercially available rat-liver sialyltransferase reactivity depends on the distance between the sulfate group and the acceptor sugar, while the bacterial sialyltransferase is not affected by the sulfate group in the substrate.

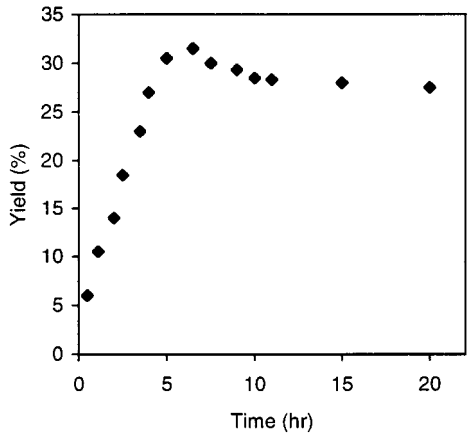


Figure 1. Synthesis of PAPS.

We believe the PAPS regeneration system described here is the most studied known to date and when coupled with a sulfotransferase and glycosyltransferases, the synthetic system will provide various sulfated carbohydrates in quantities large enough for the study of the effect of the sulfate group on carbohydrate-mediated biological recognitions.

Results and Discussion

Enzymatic Synthesis of PAPS. To enzymatically synthesize sulfated sLe^X analogues, we first cloned and overexpressed the enzymes necessary for PAPS synthesis and regeneration, ATP sulfurylase and APS kinase. ATP sulfurylase (*cys*D and *cys*N genes) and APS kinase (*cys*C genes) were cloned from *E. coli* K12 genomic DNA, ligated into the pRSET vector, and overexpressed in *E. coli* XL1-Blue. To avoid formation of inclusion bodies, the expression was carried out at 25 °C for 20 h following induction by IPTG. The enzymes were purified by ammonium sulfate precipitation and ion-exchange chromatography, and the expressed ATP sulfurylase and APS kinase were determined to yield 430 and 560 U/L, respectively.

For the synthesis of PAPS, the two recombinant enzymes above were used in a one-pot, coupled reaction. ATP was sulfated to form APS and pyrophosphate, which was hydrolyzed by the addition of inorganic pyrophosphatase (EC 3.6.1.1) to drive the reaction forward. APS was then phosphorylated by APS kinase with ATP, yielding PAPS and ADP. Due to the instability of PAPS, the coupled enzyme reaction needed to be optimized for the minimization reaction times. Figure 1 shows a general time course of PAPS synthesis in which peak

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Table 1. Reaction Conditions of the Coupled Enzymatic Synthesis of PAPS from ATP[a]

ATP (mM)	Na_2SO_4 (mM)	$MgCl_2$ (mM)	yield (%)
5	5	5	30.5
5	10	5	34.7
10	10	5	22.3
10	5	5	5.3
5	5	10	31.7
5	5	20	30.2
5	20	5	38.8
5	30	5	43.2
5	40	5	45.6

[a] Reaction conditions: 0.5 mL Tris−HCl (50 mM, pH 8.0), 20 mM KCl, 1 U ATP sulfurylase, 3.5 U APS kinase, 2 U inorganic pyrophosphatase, ATP, Na_2SO_4, and $MgCl_2$.

Table 2. Effect of ATP Concentration and Regeneration on the Enzymatic Synthesis of PAPS

ATP (mM)	PEP (mM)	yield (%)
5	0[a]	45.6
5	5[b]	63.2
2	0[a]	47.1
2	2[b]	81.4

[a] Reaction conditions: 0.5 mL Tris−HCl (50 mM, pH 8.0), 20 mM KCl, 40 mM Na_2SO_4, 5 mM $MgCl_2$, 1 U ATP sulfurylase, 3.5 U APS kinase, 2 U inorganic pyrophosphatase, ATP. [b] Conditions same as in footnote *a*, with 20 U pyruvate kinase and PEP included.

production occurs at 6 h. PAPS hydrolysis became a problem thereafter. Further optimization was also carried out with regard to ATP, sulfate, and magnesium concentration (Table 1). Greater yields were realized as sulfate concentration was increased. The highest yields were accomplished when the sulfate concentration surpassed that of ATP, indicating that the equilibrium of ATP sulfurylase lies far to the left.[27] Varying magnesium concentration, on the other hand, did not affect reaction yields. Finally, we found that an accumulation of ADP significantly inhibits product formation, and its removal by pyruvate kinase (EC 2.7.1.40), which regenerates ATP in situ from ADP with phospho(enol)pyruvate (PEP), increased yields dramatically (Table 2). We rationalized this outcome to be product inhibition of APS kinase by ADP; however, substrate inhibition by ATP upon ATP sulfurylase could also account for increased yields at lower ATP concentrations.[27] With the optimized procedures scaled up to produce 100 mg of product, PAPS was obtained in 82.3% yield when 2 mM ATP with ATP regeneration was used. PAPS was further purified from the reaction mixture by ion-exchange chromatography.

Cloning of NodST. Nod factors from *Rhizobium* sp. have been found to be β(1,4)-*N*-acetylglucosamine oligosaccharides having various *N*-linked fatty acyl groups on the nonreducing sugar in place of the *N*-acetyl group. Preliminary studies indicate that various *N*-acetylchitoses serve as substrates for the enzyme NodST, including di-, tri-, and tetra-β(1,4)-*N*-acetylglucosamine.[26] Another interesting feature is the apparently exclusive sulfation of the reducing terminal sugar at the 6-hydroxy group.[28] We, therefore, found this enzyme to be a desirable catalyst for the syntheses of sulfated oligosaccharides for the study of selectin−ligand binding.

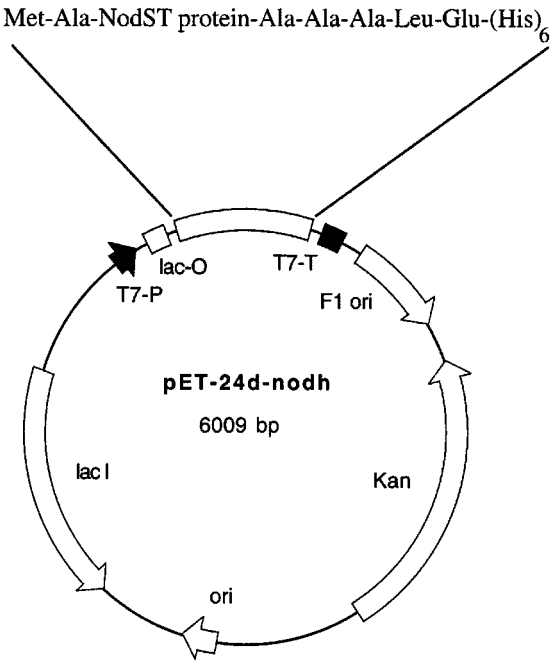

Figure 2. Plasmid map of Nod-factor sulfotransferase (NodST).

The gene encoding NodST was amplified by PCR from whole *R. meiloti* cells, cloned into the pET24 vector (Figure 2) and expressed in *E. coli* BL21(DE3). At high induction levels, >95% of the enzyme was found in inclusion bodies, and careful expression conditions were required. Optimized expression conditions employed induction at low IPTG concentrations and expression at 15 °C. Purification by nickel agarose yielded expression levels of 440 U/L. To determine NodST activity, we developed a new radioactive assay for sulfotransferase activity by using ion-exchange chromatography with selective elution. This assay uses [^{35}S]PAPS followed by loading onto a small anion-exchange resin column and elution with a NaCl solution that selectively elutes sulfated carbohydrate product and retains unreacted [^{35}S]-PAPS. In this manner, we were able to quantify NodST activity.

PAPS Regeneration for Sulfotransferase-Catalyzed Enzymatic Synthesis. Despite the high cost of PAPS, the major obstacle to enzymatic synthesis with sulfotransferases is the complication of product inhibition by PAP, even in low micromolar concentrations.[23,29] PAPS regeneration is, therefore, essential to synthetic production of sulfotransferase products on even sub-milligram scales. In planning a PAPS regeneration strategy, we deduced that the in vivo metabolism of PAPS could provide the necessary machinery for successful PAPS regeneration in vitro (Scheme 1). We found that the commercially available 3′-nucleotidase (EC 3.1.3.6) catalyzes the 3′-phosphate cleavage of PAP to 5′-AMP in addition to its known activity of hydrolyzing 3′-AMP. Hydrolysis of the 3′-phosphate of PAPS was also observed, although at a lower rate than PAP. We concluded that addition of commercially available myokinase (EC 2.7.4.3) and pyruvate kinase (EC 2.7.1.40) can catalyze the phosphorylation of 5′-AMP to ATP in the presence of ATP and PEP. Finally, in situ synthesis of PAPS from ATP, as described above with ATP sulfurylase, APS

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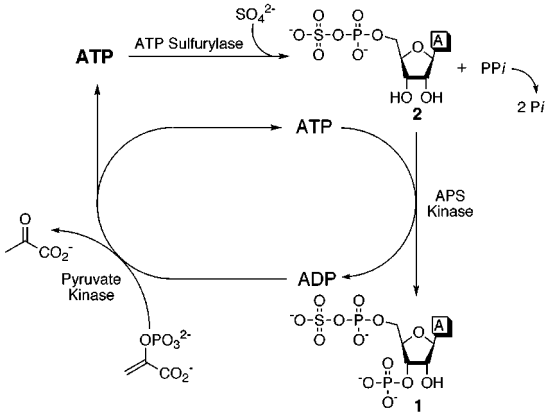
Scheme 2. Synthesis of PAPS



kinase, and inorganic pyrophosphatase, completes the regeneration cycle shown in Scheme 3.

We were able to couple the above six enzymes to the enzymatic sulfation of *N*,*N*′-diacetylchitobiose by recombinant NodST and a catalytic amount of ATP. The reaction was conducted at 25 °C for 2 days, and the resultant *N*,*N*′-diacetylchitobiose 6-sulfate was purified by size-exclusion chromatography, yielding 20 mg with an 84% yield.

PAPS Regeneration with Aryl Sulfotransferase IV (AST-IV) for Sulfotransferase-Catalyzed Enzymatic Synthesis of Sulfates. In an effort to simplify the above regeneration system, it was necessary to explore alternative approaches to PAPS synthesis. As Scheme 1 indicates, there is not a metabolic pathway for sulfation of PAP to form PAPS other than the reverse activity of sulfotransferases. We, therefore, explored known sulfotransferase literature and found aryl sulfotransferase to be a feasible means of sulfating PAP from simple available chemicals. AST-IV (EC 2.8.2.1) is a mammalian detoxification enzyme that has been studied for several years, and work has been published concerning its expression and purification,[23,30,31] acceptor specificity and inhibition,[32−35] and mechanism.[36−38] Recently Lin et al. demonstrated that the resolved physiologically relevant form can be utilized to assay picomole quantities of PAPS and PAP.[39] Recombinant AST-IV displays very good expression in *E. coli* and has been found to exist in two oxidative forms which can be easily interconverted under the appropriate conditions.[38] Although the physiological sulfation of phenols by AST-IV has been carefully studied for two decades, the reverse-physiological catalysis of PAP to PAPS has not been the focus of AST-IV kinetic studies. This catalysis is driven by a high concentration of *p*-nitrophenyl sulfate (**7**) to form the high-energy PAPS product.[40] We, therefore, reasoned that this reverse synthesis could be easily coupled with a sulfotransferase such as NodST for the regeneration of PAPS through the addition of *p*-nitrophenyl sulfate.

Cloning of AST-IV from rat-liver cDNA library was conducted as previously reported within a pET19 vector (Figure 3) and expressed in *E. coli* BL21 cells. Following expression at 25 °C for 20 h, the enzyme was purified and resolved to a single oxidative form (βAST-IV)[38] on a nickel agarose column. The enzyme was bound to the nickel column and washed following routine procedures. Prior to elution, the resin-bound enzyme was incubated with alkaline phosphatase and β-mercaptoethanol. The resin was washed a second time, and the resolved, purified enzyme was eluted and stored as a 50% glycerol stock. Apparent K_M and V_{max} of βAST-IV were determined as functions of PAP concentration. A double-reciprocal Lineweaver−Burk plot gave $K_M = 26.4 \pm 3.4\ \mu$M and $V_{max} = 6.04 \pm 0.24\ \mu$mol/min for the reverse-physiological reaction.[41]

Recombinant NodST was used with PAPS regeneration by βAST-IV and *p*-nitrophenyl sulfate[41] for the synthesis of *N*,*N*′-diacetylchitobiose 6-sulfate (**6a**), *N*,*N*′,*N*″-triacetylchitotriose 6-sulfate (**6b**), and *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose 6-sulfate (**6c**) (Scheme 4). These reactions were incubated at 25 °C for 4 days, and the products were purified by size-exclusion chromatography to yield 49%, 65%, and 95% yields, respectively. The position of the sulfation of each chitooligosaccharide was determined by ^{1}H and ^{13}C NMR and electrospray mass spectrometry (ESI-MS). The ^{1}H NMR spectrum of compound **6b** showed a downfield shift of two sets of H6 protons (δ 4.20, 4.16 and 4.25, 4.12) according to reducing-end α and β anomers. ^{13}C NMR also showed a downfield shift of two C6 carbons (δ 67.04, 66.97). These facts indicated that sulfation occurred at the 6 position. ESI-MS revealed an $[M - H]^-$ ion of $m/z = 706$ in the negative mode. Positive mode gave an $[M + Na]^+$ ion of $m/z = 730$, as well as degradation peaks $m/z = 628$, 407, and 204 corresponding to the removal of the sulfate group ($m/z = 628$) and successive losses of *N*-acetylglucosamine (GlcNAc) units, respectively. This fragmentation pattern was similar to those of Nod factors,[28,42] indicating that the sulfate group is on the reducing-end sugar. The positions of the sulfations of compounds **6a**,**c** were also determined to be the 6 position of the reducing-end GlcNAc by the examination of ^{1}H and ^{13}C NMR and ESI-MS.

The continuous assay for the kinetic analysis of NodST using PAPS regeneration with βAST-IV[41] was carried out. Five chitooligosaccharides, *N*-acetyllactosamine (LacNAc), and an *N*-acetyllactosamine dimer (LacNAc dimer) were examined as substrates, and their kinetic parameters (K_M and k_{cat}) are summarized in Table 3. *N*,*N*′*N*″,*N*‴-Tetraacetylchitotetraose (**5c**) was the most preferred substrate among the chitooligosaccharides tested. Of particular interest is the 60-fold increase in acceptor specificity from LacNAc to LacNAc dimer, although we do not know which GlcNAc residue was sulfated.

Enzymatic Synthesis of Sulfated Sialyl Lewis X Analogues. The study of differentially sulfated sLe^X analogues will help to reveal the nature of ligand−selectin binding and to inform the design of future

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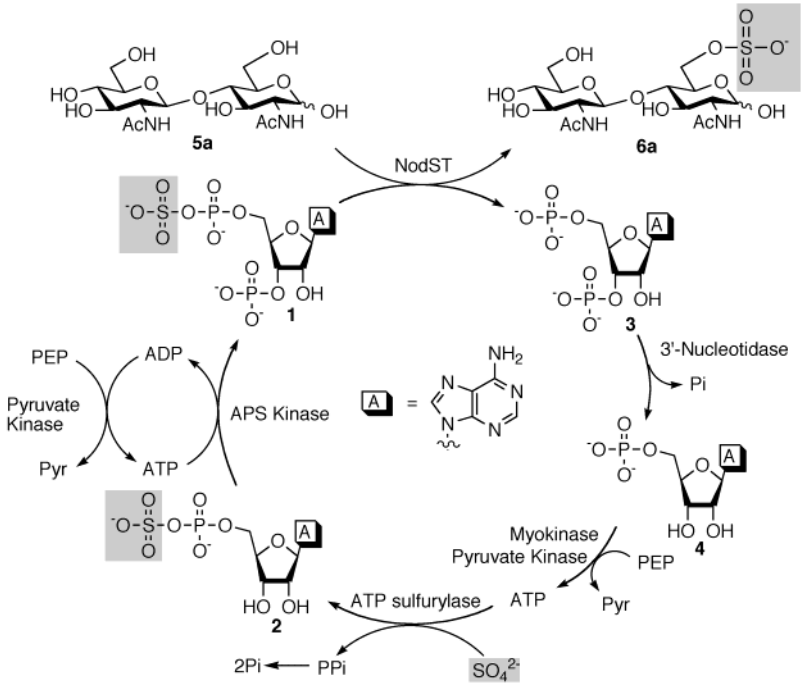
Scheme 3. NodST-Catalyzed Synthesis of *N,N′*-Diacetylchitobiose 6-Sulfate Using Multienzymatic PAPS Regeneration



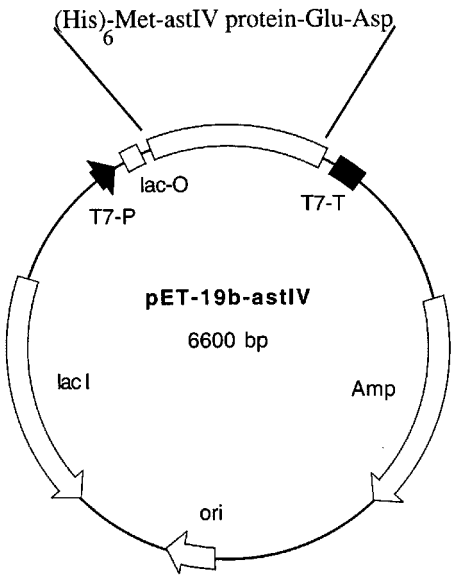


Figure 3. Plasmid map of aryl sulfotransferase IV (AST-IV).

inhibitors of leukocyte recruitment. 6-Sulfo sLe^X ganglioside has been recently chemically synthesized,[43] and a chemo-enzymatic preparation of a 6′-sulfo sLe^X was reported several years ago.[44] With sulfated chitoses in hand, we were able to enzymatically complete saccharide decoration to append the sLe^X moiety to the nonreducing end of **5a**−**c**. The complete enzymatic synthesis of sLe^X is now readily practicable with commercially available glycosyltransferases.[45] Of interest is the ability of glycosyltransferases to accept sulfated substrates, because the timing of the biosynthesis of sulfated molecules is unclear. Studies that probe the substrate specificity of glycosyltransferases for sulfated molecules have been reported,[46] and information about biosynthetic timing will benefit glycosyltransferase inhibitor design.

The sulfated oligosaccharides were quantitatively galactosylated by bovine β(1,4)galactosyltransferase (βGalT), and the products were purified via ion-exchange chromatography followed by size-exclusion chromatography. Sialylation of galactosyl *N,N′,N″,N‴*-tetraacetylchitotetraose 6-sulfate (**9c**) was achieved by the commercially available recombinant rat α(2,3)sialyltransferase (ST3). Because this enzyme does not accept **9a**,**b** efficiently, recombinant *Neisseria* ST3 was employed for the sialylation of chitotriose derivative (**9b**) and chitobiose derivative (**9a**). Each reaction gave sialylated oligosaccharides (**10a**−**c**) in excellent yield after purification by ion exchange and size-exclusion chromatography. Fucosylation of sialyl galactosyl *N,N′,N″*-triacetylchitotriose 6-sulfate (**10b**) and chitotetraose derivative (**10c**), with either recombinant human α(1,3)fucosyltransferase V or VI (FucT V, VI), proceeded smoothly to give monofucosylated products **11b**,**c**, respectively, in excellent yields. The SLe^x structure inside **11b** was determined by the observation of ROESY cross-peaks between Fuc H5 and Gal H2, Fuc H6 and Gal H2, and NeuAc H3ax and Gal H3.[45] ^{1}H NMR chemical shifts of **11c** were essentially the same as those of **11b**, which suggests that **11c** also has an sLe^x structure. On the other hand, fucosylation of chitobiose derivative (**10a**) with FucT V under the same conditions did not proceed at all. However, fucosylation with FucT VI proceeded smoothly to give **11a** in excellent yield. The effects of sulfation on the kinetic parameters of glycosyltransferases are under investigation. In any event, the enzymatic strategy described here provides a practical

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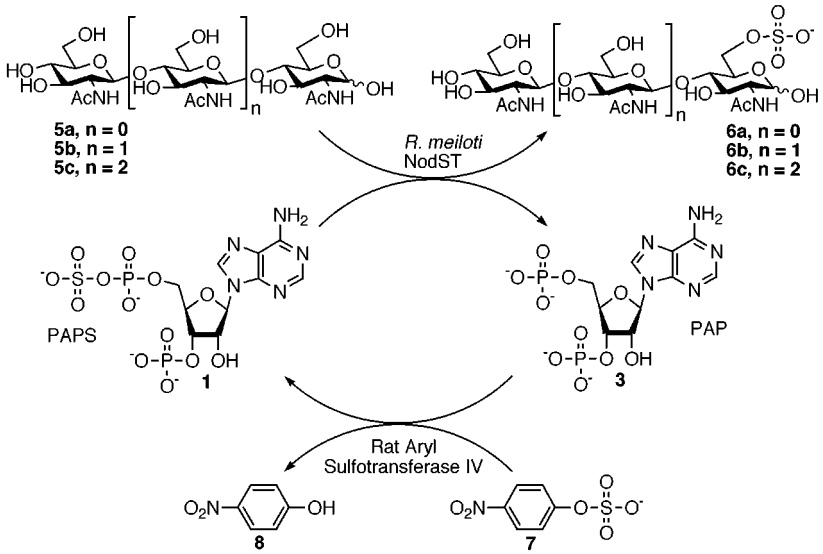
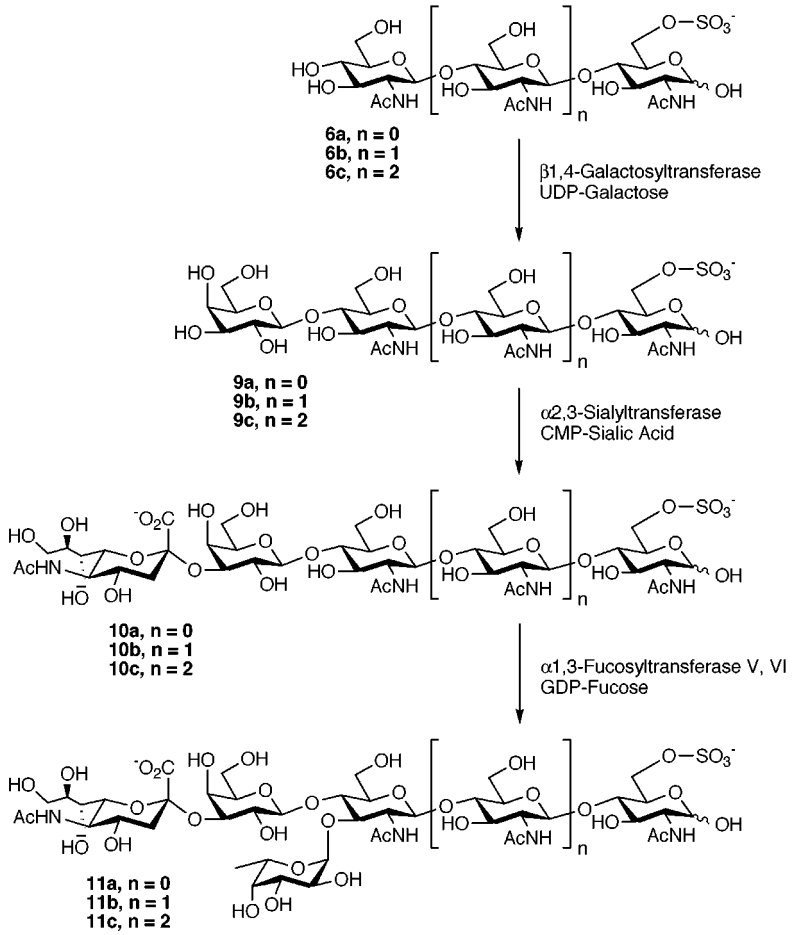
Scheme 4. Synthesis of Sulfated Chitooligosaccharides with AST-IV-Catalyzed PAPS Regeneration



Scheme 5. Synthesis of Sialyl Le^x 6-Sulfate Analogues



entry to various carbohydrate sulfates which may find use in the study of their biological function.

Experimental Section

General Methods. The vector pTrcHis was obtained from Invitrogen Co. (San Diego, CA). *E. coli* strain XL1-Blue was obtained from Stratagene Co. (San Diego, CA). Vectors pET19 and pET24 and *E. coli* strain BL21(DE3) were obtained from Novagen, Inc. (Milwaukee, WI). *E. coli* strain K12 (ATCC 10798) was obtained from American Type Culture Collection. Rat-liver cDNA library was obtained from Clonetech (Palo Alto, CA). The microorganisms were maintained on Luria-Bertani (LB) medium and agarose plates. β(1,4)Galactosyltransferase, α(2,3)sialyltransferase, α-(1,3)-fucosyltransferase V and VI, UDP-galactose, CMP-sialic acid, and GDP-fucose were purchased from Calbiochem (San Diego, CA). All other molecular biology reagents were purchased from New England Biolabs (Beverly, MA). Myokinase, pyruvate kinase, inorganic pyrophosphorylase, ATP, *p*-nitrophenyl sulfate, and all other

Table 3. Acceptor Specificity for NodST[a]

acceptor substrates	$K_{M,acceptor}$[b] (μM)	k_{cat}[b] (min^{-1})
GlcNAcβ(1,4)GlcNAc	243 ± 40	23.6 ± 1.7
GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAc	103 ± 12	27.5 ± 2.9
GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAc	20.4 ± 3.6	19.7 ± 0.8
GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAc	98.1 ± 12.4	25.9 ± 1.2
GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAcβ(1,4)GlcNAc	45.7 ± 6.0	22.7 ± 1.0
Galβ(1,4)GlcNAc	5575 ± 1057	8.0 ± 1.3
Galβ(1,4)GlcNAcβ(1,3)Galβ(1,4)GlcNAc	87.0 ± 6.3	3.4 ± 0.1

[a] The acceptor sugar concentrations were chosen around their respective K_M values. The PAPS concentration was held constant at 20 μM. Kinetic constants were derived from the nonlinear, least-squares fit of the Michaelis−Menten equation with the LineWeaver program. [b] Apparent kinetic values.

reagents were obtained from Sigma Co. (St. Louis, MO). 3′-Phosphoadenosine-5′-[S^{35}]-phosphosulfate was purchased from Amersham Life Science. ScintiVerseI scintillation cocktail and $MnCl_2$·4 H_2O were purchased from Fisher Scientific Co. ^{1}H NMR spectra were recorded at 500 or 600 MHz. ^{13}C NMR spectra were recorded at 125 or 150 MHz. ^{31}P NMR spectra were recorded at 162 MHz. GlcNAc residues were assigned as I, II, ... from the reducing end. High-resolution mass spectra (HRMS) were recorded using either the fast atom bombardment (FAB) method in a *m*-nitrobenzyl alcohol matrix or the matrix-assisted laser desorption ionization (MALDI) method in a DHB matrix doped with NaI. Analytical TLC was performed using silica gel 60 F_{254} precoated glass plates (Merck) and visualized by quenching of fluorescence and/or by charring after treatment with cerium molybdophosphate or orcinol in 10% H_2SO_4−EtOH. Size-exclusion chromatography was performed on Bio-Gel P2, P4, or P10 gel, fine (Bio-Rad Laboratories) with 0.03 M NH_4HCO_3 unless otherwise noted. Protein concentrations were determined using the Coomassie protein staining reagent with albumin standards as purchased from Pierce. The scintillation counter used was the Beckman LS 3801. *N*,*N*′-Diacetylchitobiose, *N*,*N*′,*N*″-triacetylchitotriose, and *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose were prepared according to published procedures.[47]

Cloning, Overexpression, and Purification of ATP Sulfurylase. The ATP sulfurylase gene was obtained using a polymerase chain reaction from *E. coli* K12 genomic DNA. The 5′ oligonucleotide contained the sequence ATATTGAGCTC-GATCAAATACGACTTACTCACCTG with a *Sac*I restriction site preceding the 5′ start codon. The 3′ oligonucleotide contained the sequence GCGCAAGCTTTTATTATTTATC-CCCCAGCAAATC with a *Hin*dIII site following the 3′ stop codon. The PCR product (2.3 kb) was purified and digested with *Hin*dIII and *Sac*I and ligated with a similarly digested pTrcHis vector. The ligated plasmid was transferred into *E. coli* XL1-Blue, and a single colony containing the desired plasmid was chosen to inoculate 5 mL of LB broth containing 25 μg/mL of ampicillin for overnight growth. The culture was used to inoculate four 1-L volumes of LB/ampicillin and grown to an $OD_{600} = 0.4$. The culture was induced with 0.5 mM IPTG and incubated at 25 °C for 20 h. The culture cell pellet was resuspended in 50 mL Tris−HCl (50 mM, pH 7.6), disrupted by French pressure, and centrifuged at 18000×*g* for 50 min. The supernatant was fractionated with $(NH_4)_2SO_4$, and the 40−75% fraction was collected and resuspended in Tris−HCl (50 mM, pH 7.6). Dialysis overnight at 4 °C against the same buffer was followed by DEAE Sepharose 6B-Cl (3 × 40 cm) chromatography with a linear gradient from 0 to 1 M KCl (pH 7.6, 0.5 L total). Enzyme production yielded 430 U/L.

Cloning, Overexpression, and Purification of APS Kinase. The ATP sulfurylase gene was cloned and overexpressed exactly as ATP sulfurylase with the exception of the PCR primer sequence. The 5′ oligonucleotide contained the sequence ATATTGAGCTCGCGCTGCATGACGAAAAC with a *Sac*I restriction site preceding the 5′ start codon. The 3′ oligonucleotide contained the sequence GCGCAAGCTTTTATT-AGGATCTGATAATATCGTT with a *Hin*dIII site following the 3′ stop codon. The PCR product (800 bp) was treated as above. For the $(NH_4)_2SO_4$ fractionation, the 45% fraction was isolated and subjected to dialysis and DEAE-Sepharose purification as above. Enzyme production yielded 560 U/L.

APS Kinase Activity Assay. The activity of the forward reaction (APS consumption) was measured using a modified pyruvate kinase−lactate dehydrogenase-coupled assay of Burnell and Whatley.[48] To 1 mL of Tris−HCl buffer (50 mM, pH 8.0) containing 5 mM ATP, 1 mM APS, 5 mM $MgCl_2$, 5 mM Na_2SO_4, 20 mM KCl, 0.3 mM NADH, and 0.4 mM PEP were added 50 U of pyruvate kinase, 50 U of lactate dehydrogenase, and 30 μL of APS kinase enzyme solution. The reaction was initiated by adding APS after the NADH absorbance (340 nm) stabilized. The rate of NADH dehydrogenation was measured to determine activity.

ATP Sulfurylase Activity Assay. The activity assay was run in the reverse direction, quantifying desulfation of APS. To 1 mL of Tris−HCl buffer (50 mM, pH 8.0) containing 1 mM APS, 5 mM $MgCl_2$, 0.6 mM NADH, 1 mM glucose-6-phosphate, and 1 mM $Na_4P_2O_7$ were added 5 U of glucose-6-phosphate dehydrogenase, 10 U of hexokinase, and 30 μL of ATP sulfurylase enzyme solution. The reaction was initiated by adding APS after the NADH absorbance (340 nm) stabilized. The rate of NADH dehydrogenation was measured to determine activity.

Synthesis of APS by ATP Sulfurylase. A solution of Tris−HCl buffer (50 mM, pH 8.0, 5 mL) containing 20 mM KCl, 40 mM Na_2SO_4, 5 mM $MgCl_2$, 5 mM ATP (13.8 mg), 2 U ATP sulfurylase, and 20 U inorganic pyrophosphatase was incubated at room temperature under N_2. After 10 h, the reaction solution was chromatographed through a Mono-Q anion-exchange column with a linear gradient of NH_4HCO_3 from 20 mM to 0.8 M. The desired fractions, monitored by a UV detector (254 nm), were collected, and the existing NH_4HCO_3 salt was removed by adding Dowex 50W-X8 (H^+). The resulting solution was lyophilized and then applied to a column of Dowex 50W-X8 (Na^+) to yield the product as white powder (8.9 mg, 76% yield). The TLC, FPLC, ^{1}H NMR, and ^{31}P NMR data were identical to those of an authentic sample (Sigma Co.): ^{31}P NMR (D_2O) δ −10.51. ESI-MS (*m*/*z*): negative 448 $[M - Na]^-$; positive 494 $[M + Na]^+$.

One-Pot Synthesis of PAPS by ATP Sulfurylase and APS Kinase. The quantitative analysis of PAPS was first carried out by an FPLC system (Pharmacia Co.). Varied concentrations of authentic PAPS (Sigma Co.) were prepared (5, 2.5, 1.25, 0.5, 0.25 mM) and applied to a 0.5- × 5-cm Mono-Q anion-exchange column with UV detection (254 nm). After loading, the column was washed with 4 mL of H_2O, a 55 mL of a gradient of NH_4HCO_3 (50 mM to 1 M), and, finally, 5 mL of 1 M NH_4HCO_3. A linear relationship was established between the PAPS concentration and the area of the signal which was detected.

A solution of Tris−HCl buffer (50 mM, pH 8.0, 100 mL) containing 20 mM KCl, 40 mM Na_2SO_4, 5 mM $MgCl_2$, 2 mM ATP, 2 mM PEP, 20 U ATP sulfurylase, 560 U APS kinase, 400 U inorganic pyrophosphorylase, and 2000 U pyruvate kinase was incubated at room temperature under N_2. After 8 h, the reaction solution was filtered with a 10,000 NMWL regenerated-cellulose membrane (Millipore Co., Bedford, MA)

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to remove proteins and insoluble precipitates. The filtrate was chromatographed by using the same method as described above, and the reaction yield was determined to be 80.5% at this stage. The desired fractions were collected, and the existing NH_4HCO_3 salt was then removed using Dowex 50W-X8 (H^+). The resin was added until CO_2 evolution ceased. To prevent the acid decomposition of PAPS, the pH of the solution was kept above 7.0. The resulting neutral solution was then lyophilized to yield 76.8 mg of product (72.7%) as a white powder. To compare this with an authentic sample, the product was passed through a column with Dowex 50-X8 (Li^+) to yield the lithium salt. TLC, FPLC, ^{1}H NMR and ^{31}P NMR data were identical to those of an authentic sample: ^{31}P NMR (D_2O) δ 3.12, −10.51. ESI-MS (*m*/*z*): negative 518 $[M + H - 2Li]^-$; positive 532 $[M + H]^+$.

Cloning, Overexpression and Purification of NodST. The *Nod* H gene was obtained using PCR from *R. meiloti* genomic DNA. The 5′ oligonucleotide contained the sequence ATATTGAATTCATTTTCATGACCCATTCCA with an *Eco*RI site preceding the 5′ start codon. The 3′ oligonucleotide contained the sequence GGCGCGGATCCTTAGTCGTTAG-CAAGCTC containing a *Bam*HI site following the 3′ stop codon. The PCR product was purified and digested using *Bam*HI and *Eco*RI and ligated using a similarly digested pET 24 vector. The ligated plasmid was transferred into *E. coli* XL1-Blue for amplification and subsequently transferred into *E. coli* BL21(DE3). A single colony containing the desired plasmid was chosen to inoculate 5 mL of LB broth containing 50 μg/mL of kanamycin for overnight growth. The culture was used to inoculate two 1-L volumes of LB/kanamycin and was grown at 37 °C to an OD = 0.52. The culture was induced with 50 μM IPTG and incubated at 15 °C for 16 h. The cell pellet was resuspended in 100 mM Tris, pH 7.6, with 5 mM β-mercaptoethanol, disrupted by French press, and the soluble enzyme was purified via nickel agarose chromatography (1.5 × 10 cm, 250 mM imidazole elution). Production totaled 440 U/L.

Radioisotopic Assay for Sulfotransferase Activity. The assay mixture contained 2 mM PAPS, [^{35}S]PAPS, 2 mM *N*,*N*′,*N*″-triacetylchitotriose, 20 mM $MgCl_2$, and the enzyme in 100 mM Tris−HCl, pH 7.5. The mixture was incubated at 25 °C for 15, 30, and 60 min. The reaction was terminated by loading the mixture onto a QAE Sephadex A25 column (0.5 × 4 cm) and immediately washing it with 3 mL of H_2O. The column was then washed with 3 mL of 300 mM NaCl and subsequently with 3 mL of 2 M NaCl. Aliquots from the three washing steps were collected separately, and the radioactivity for each was counted by liquid scintillation. The sulfated sugar eluted in the 300 mM NaCl fraction and the unreacted PAPS and [^{35}S]PAPS eluted in the 2 M NaCl fraction. The amount of sulfated sugar was calculated from the second fraction and compared with residual [^{35}S]PAPS. Two negative controls were run for each time point, one omitting the sugar substrate and one omitting the enzyme. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of sulfated sugar/min.

***N*,*N*′-Diacetylchitobiose 6-Sulfate (6a) by Multienzymatic PAPS Regeneration.** A solution of 5 mM *N*,*N*′-diacetylchitobiose (20 mg, 47 μmol) in 9.4 mL of Tris−HCl buffer (100 mM, pH 7.5) with 1.5 mM ATP (7.8 mg, 14 μmol), 15 mM PEP (36.4 mg, 155 μmol), 40 mM Na_2SO_4, 20 mM $MgCl_2$, 3 U ATP sulfurylase, 2.5 U APS kinase, 12 U inorganic pyrophosphatase, 600 U pyruvate kinase, 300 U myokinase, 2.4 mL of NodST solution, and 0.36 U of 3′-nucleotidase was incubated for 2 days at 25 °C. The product was detected by TLC with $CHCl_3$/MeOH/AcOH/H_2O (25:15:2:1) and *p*-anisaldehyde stain. The reaction was centrifuged to remove the precipitate and the supernatant was concentrated and passed through a Sephadex-G25 column twice with water as the eluent to yield **6a** (19.9 mg, 84%): ^{1}H NMR (600 MHz, D_2O) δ 5.17 (0.6H, d, J = 3.1 Hz), 4.70 (0.4H, d, J = 8.3 Hz), 4.62 (0.6H, d, J = 8.3 Hz), 4.61 (0.4H, d, J = 8.8 Hz), 4.24 (0.4H, brd, J = 10.1 Hz), 4.19 (0.6H, dd, J = 2.2, 11.0 Hz), 4.16 (0.6H, dd, J = 3.5, 11.0 Hz), 4.12 (0.4H, dd, J = 2.6, 9.2 Hz), 4.07 (0.6H, m), 2.08, 2.07, 2.02 (6H, each s); ^{13}C NMR (125 MHz, D_2O) 175.64, 175.58, 175.3, 102.14, 102.11, 95.8, 91.4, 80.0, 79.5, 76.7, 74.5, 73.2, 70.5, 70.1, 68.8, 67.14, 67.05, 61.3, 56.9, 56.3, 54.4, 24.1, 23.1, 22.8. ESI-MS (*m*/*z*): negative 503 $[M - H]^-$.

***N*,*N*′-Diacetylchitobiose 6-Sulfate (6a) by βAST-IV PAPS Regeneration.** *N*,*N*′-Diacetylchitobiose (50 mg, 0.118 mmol) in a 23.5-mL solution of bis-tris propane−HCl (100 mM, pH 7.0) with *p*-nitrophenyl sulfate (60.5 mg, 0.235 mmol), PAP (1 mg, 84 μM), 5 mM DTT, 2.0 U NodST, and 4.3 U βAST-IV was incubated at room temperature for 3 days. The solution was applied to a DEAE-Sephadex A-25 column (AcO^- form), and the column was washed with water; then the product was eluted with 0.2 M NaOAc. The fractions containing the product were pooled and passed through a Dowex HCR-W8 column (H^+), and the void fraction was concentrated in vacuo. The residue was passed through a Dowex HCR-W8 (Na^+) column and lyophilized to give **6a** (30.4 mg, 49%) as a sodium salt: HR−MALDI−FTMS calcd for $C_{16}H_{27}N_2O_{14}SNa_2$ $[M - H + 2Na]^+$, 549.0978; found, 549.0980.

***N*,*N*′,*N*″-Triacetylchitotriose 6-Sulfate (6b) by βAST-IV PAPS Regeneration.** *N*,*N*′,*N*″-Triacetylchitotriose (251 mg, 400 μmol) in an 80-mL solution of bis-tris propane−HCl (100 mM, pH 7.0) with 10 mM *p*-nitrophenyl sulfate (205.8 mg, 0.0.8 mmol), PAP (10 mg, 20 μmol), 5 mM DTT, 6.7 U NodST, and 14.4 U βAST-IV was incubated at room temperature. Formation of *N*,*N*′,*N*″-triacetylchitotriose 6-sulfate was monitored by TLC (7:1:2 *n*-propanol/30% NH_4OH/H_2O) and liberation of *p*-nitrophenol (**8**) at 400 nm. After 3 days, the solution was passed through a column of LiChroPrep 18 (water). The void fraction was absorbed to a BioRad AG1-X8 column (HCO_2^- form) and eluted with a linear gradient (0−0.1 M) of ammonium benzenesulfonate. Fractions containing the product were concentrated and purified twice by BioGel P-2 chromatography (water) and lyophilized to give **6b** (180 mg, 64%) as a white foam: ^{1}H NMR (500 MHz, D_2O) δ 5.19 (0.6H, d, J = 5.2 Hz), 4.72 (0.4H, d, J = 7.7 Hz), 4.63 (0.6H, d, J = 8.1 Hz), 4.62 (0.4H, d, J = 8.1 Hz), 4.595 (0.6H, d, J = 8.5 Hz), 4.591 (0.4H, d, J = 8.5 Hz), 4.25 (0.4H, brd, J = 10.3 Hz), 4.20 (0.6H, dd, J = 1.8, 11.0 Hz), 4.16 (0.6H, dd, J = 3.7, 11.0 Hz), 4.12 (0.4H, dd, J = 3.7, 9.6 Hz), 4.08 (0.6H, ddd, J = 1.8, 3.7, 9.9 Hz), 2.084, 2.080, 2.06, 2.03 (9H, each s); ^{13}C NMR (125 MHz, D_2O) δ 175.5, 175.4, 175.2, 171.7, 102.1, 101.9, 95.7, 91.3, 79.8, 79.7, 79.3, 76.7, 75.2, 74.2, 73.1, 73.0, 73.0, 70.5, 70.0, 68.7, 67.04, 66.97, 61.3, 60.7, 56.8, 56.4, 55.7, 54.3, 22.98, 22.95, 22.89, 22.7. ESI-MS (*m*/*z*): negative 706 $[M - H]^-$; positive 730 $[M + Na]^+$, 628 $[730 - SO_3]^+$, 407 $[730 - GlcNAcSO_3]^+$, 204 $[730 - (GlcNAc)_2SO_3]^+$. HR−MALDI−FTMS calcd for $C_{24}H_{40}N_3O_{19}S$ $[M - H]^-$, 706.1977; found, 706.2004.

***N*,*N*″,*N*‴-Tetraacetylchitotetraose 6-Sulfate (6c) by βAST-IV PAPS Regeneration.** Procedure exactly as above, with *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose (160 mg, 193 μmol) as starting material. Product purified twice by BioGel P-2 chromatography (water) to yield **6c** (170 mg, 95%): ^{1}H NMR (500 MHz, D_2O) δ 5.18 (0.6H, d, J = 3.3 Hz), 4.71 (0.4H, d, J = 8.1 Hz), 4.62−4.56 (3H, m), 4.25 (0.4H, brd, J = 10.6 Hz), 4.19 (0.6H, dd, J = 2.2, 11.0 Hz), 4.15 (0.6H, dd, J = 3.7, 11.0 Hz), 4.11 (0.4H, dd, J = 3.3, 11.0 Hz), 4.07 (0.6H, ddd, J = 2.2, 3.7, 9.9 Hz), 2.08, 2.07, 2.06, 2.05, 2.03 (12H, each s); ^{13}C NMR (125 MHz, D_2O) δ 67.07, 66.67. ESI-MS (*m*/*z*): positive 955 $[M - H + 2Na]^+$, 828 $[955 - SO_3Na_2]^+$, 610 $[955 - GlcNAcSO_3Na_2]^+$, 407 $[955 - (GlcNAc)_2SO_3Na_2]^+$, 204 $[955 - (GlcNAc)_3SO_3Na_2]^+$. HR−FAB−MS calcd for $C_{32}H_{53}N_4Na_2O_{24}S$ $[M - H + 2Na]^+$, 955.2566; found, 955.2563.

Galβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (9a). A solution of **6a** (12.9 mg, 24.5 μmol) in 4.5 mL of HEPES buffer (100 mM, pH 7.4) with UDP−galactose (18.0 mg, 29.5 μmol), 0.25% Triton X-100, 20 mM $MnCl_2$, 56 mU β(1,4)galactosyltransferase, and 2.2 U alkaline phosphatase was incubated for 2 days at room temperature. The reaction solution was applied to a DEAE-Sephadex A-25 column (AcO^- form), and the column was washed with water; then the product was eluted with 0.2 M NH_4OAc. The fractions containing the product were pooled and passed through a Dowex HCR-W8 column (H^+), and the void fraction was concentrated. The syrup was purified by BioGel P-2 chromatography and lyophilized to give **9a** (16.4 mg, 98%)

as a white foam: ^{1}H NMR (600 MHz, D_2O) δ 5.18 (0.6H, d, $J_{1,2}$ = 3.5 Hz, H1$^{I}\alpha$), 4.71 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{I}\beta$), 4.65 (0.6H, d, $J_{1,2}$ = 8.3 Hz, H1$^{II}\alpha$), 4.64 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{II}\beta$), 4.46 (1H, d, $J_{1,2}$ = 7.9 Hz, H1^{Gal}), 4.25 (0.4H, brd, J = 10.1 Hz, H6a$^{I}\beta$), 4.20 (0.6H, dd, $J_{5,6a}$ = 2.2, $J_{6a,6b}$ = 11.0 Hz, H6a$^{I}\alpha$), 4.17 (0.6H, dd, $J_{5,6b}$ = 3.5, $J_{6a,6b}$ = 11.0 Hz, H6b$^{I}\alpha$), 4.13 (0.4H, dd, $J_{5,6b}$ = 2.9, $J_{6a,6b}$ = 10.8 Hz, H6b$^{I}\beta$), 4.07 (0.6H, ddd, $J_{4,5}$ = 10.1, $J_{5,6a}$ = 2.2, $J_{5,6b}$ = 3.5 Hz, H5$^{I}\alpha$), 3.97 (1H, dd, $J_{5,6a}$ = 2.2, $J_{6a,6b}$ = 12.3 Hz, H6a^{II}), 3.91 (1H, d, $J_{3,4}$ = 3.1 Hz, H4^{Gal}), 3.90 (0.6H, dd, $J_{1,2}$ = 3.5, $J_{2,3}$ = 11.0 Hz, H2$^{I}\alpha$), 3.68−3.64 (1H, m, H5^{II}), 3.65 (1H, dd, $J_{2,3}$ = 10.1, $J_{3,4}$ = 3.1 Hz, H3^{Gal}), 3.63 (1H, dd, $J_{5,6a}$ = 4.4, $J_{6a,6b}$ = 11.8 Hz, H6a^{Gal}), 3.54 (1H, dd, $J_{5,6b}$ = 6.6, $J_{6a,6b}$ = 11.8 Hz, H6b^{Gal}), 3.53 (1H, dd, $J_{1,2}$ = 7.9, $J_{2,3}$ 10.1 Hz, H2^{Gal}), 2.079, 2.076, 2.03 (6H, each s, Ac × 2); ^{13}C NMR (150 MHz, D_2O) δ 175.6, 175.3, 103.7, 102.0, 101.9, 95.8, 91.4, 79.8, 79.3, 78.8, 76.2, 75.6, 73.3, 73.2, 73.1, 73.0, 72.9, 71.8, 70.0, 69.4, 68.8, 67.1, 67.0, 63.3, 61.9, 60.6, 56.9, 55.9, 54.4, 23.1, 23.0, 22.7. HR−MALDI−FTMS calcd for $C_{22}H_{37}N_2O_{19}SNa_2$ [M − H + 2Na]$^+$, 711.1507; found, 711.1519.

Galβ1−4GlcNAcβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (9b). Galactosylation of **6b** (14.2 mg, 19.6 μmol) was carried out under the same conditions as described for **6a**. The reaction solution was applied to a DEAE-Sephadex A-25 column (AcO$^-$ form), and the column was washed with water; then the product was eluted with 0.2 M NH_4OAc. The fractions containing the product were pooled and concentrated. The syrup was purified by BioGel P-4 chromatography and lyophilized to give **9b** (16.6 mg, 95%) as a white foam: ^{1}H NMR (600 MHz, D_2O) δ 5.18 (0.6H, d, J = 3.1 Hz), 4.70 (0.4H, d, J = 8.3 Hz), 4.63−4.60 (2H, m), 4.45 (1H, d, J = 7.5 Hz), 4.24 (0.4H, brd, J = 10.1 Hz), 4.19 (0.6H, dd, J = 2.2, 11.0 Hz), 4.15 (0.6H, dd, J = 4.0, 11.0 Hz), 4.11 (0.4H, dd, J = 3.5, 11.0 Hz), 4.07 (0.6H, ddd, J = 2.2, 4.0, 10.1 Hz), 3.97 (1H, dd, J = 1.8, 12.3 Hz), 3.91 (1H, d, J = 3.1 Hz), 3.52 (1H, dd, J = 7.9, 9.6 Hz), 2.075, 2.071, 2.05, 2.02 (9H, each s); ^{13}C NMR (150 MHz, D_2O) δ 179.6, 175.6, 175.4, 103.8, 102.1, 101.9, 95.8, 91.4, 79.9, 79.7, 79.4, 79.0, 76.2, 75.7, 75.3, 73.4, 73.1, 73.0, 71.8, 70.1, 69.4, 68.8, 67.2, 61.9, 60.8, 56.0, 55.9, 54.4, 23.1, 23.0, 22.7, 22.5. HR−MALDI−FTMS calcd for $C_{30}H_{50}N_3O_{24}S$ [M − H]$^-$, 868.2505; found, 868.2625.

Galβ1−4GlcNAcβ1−4GlcNAcβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (9c). Galactosylation of **6c** (20.0 mg, 21.4 μmol) was carried out under the same conditions as described for **6a**. Purification twice by BioGel P-2 chromatography gave **9c** (23.2 mg, 99%) as a white foam after lyophilization: ^{1}H NMR (500 MHz, D_2O) δ 5.18 (0.6H, d, J = 2.9 Hz), 4.70 (0.4H, d, J = 8.1 Hz), 4.62−4.57 (3H, m), 4.45 (1H, d, J = 7.7 Hz), 4.24 (0.4H, brd, J = 11.0 Hz), 4.19 (0.6H, dd, J = 2.2, 11.0 Hz), 4.15 (0.6H, dd, J = 3.7, 11.0 Hz), 4.12−4.10 (0.4H, m), 4.08−4.05 (0.6H, m), 3.97 (1H, brd, J = 10.3 Hz), 3.91 (1H, d, J = 3.3 Hz), 3.53 (1H, dd, J = 7.7, 9.9 Hz), 2.075, 2.071, 2.051, 2.046, 2.02 (12H, each s). HR−MALDI−FTMS calcd for $C_{38}H_{63}N_4O_{29}S$ [M − H]$^-$, 1071.3299; found, 1071.3252.

NeuAcα2−3Galβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (10a). A solution of **9a** (10.4 mg, 15.2 μmol) in 3.0 mL of HEPES buffer (100 mM, pH 7.5) with CMP-sialic acid (12 mg, 18.2 μmol), 20 mM $MgCl_2$, 0.2 mM DTT, *Neisseria* α(2,3)sialyltransferase, and 10 U alkaline phosphatase was incubated at room temperature for 1 day. The reaction mixture was applied to a DEAE-Sephadex A-25 column (AcO$^-$ form) and eluted with a linear gradient of NH_4OAc (0−0.3 M). The fractions containing the product were pooled and passed through a Dowex HCR-W8 column (H$^+$), and the void fraction was concentrated. The residue was purified twice by BioGel P-2 chromatography and lyophilized to give **10a** (15.1 mg, quant) as a white foam: ^{1}H NMR (600 MHz, D_2O) δ 5.18 (0.6H, d, $J_{1,2}$ = 3.1 Hz, H1$^{I}\alpha$), 4.71 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{I}\beta$), 4.64 (0.6H, d, $J_{1,2}$ = 8.3 Hz, H1$^{II}\alpha$), 4.63 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{II}\beta$), 4.54 (1H, d, $J_{1,2}$ = 7.7 Hz, H1^{Gal}), 4.25 (0.4H, brd, J = 11.0 Hz, H6a$^{I}\beta$), 4.19 (0.6H, dd, $J_{5,6a}$ = 1.8, $J_{6a,6b}$ = 11.0 Hz, H6a$^{I}\alpha$), 4.16 (0.6H, dd, $J_{5,6b}$ = 3.9, $J_{6a,6b}$ = 11.0 Hz, H6b$^{I}\alpha$), 4.12−4.10 (0.4H, m, H6b$^{I}\beta$), 4.11 (1H, dd, $J_{1,3}$ = 9.9, $J_{3,4}$ = 3.1 Hz, H3^{Gal}), 4.07 (0.6H, m, H5$^{I}\alpha$), 3.99−3.97 (1H, m, H6a^{II}), 3.94 (1H, d, $J_{3,4}$ = 3.1 Hz, H4^{Gal}), 3.56 (1H, dd, $J_{1,2}$ = 7.7, $J_{2,3}$ = 9.9 Hz, H2^{Gal}), 2.75 (1H, dd, $J_{3e,3a}$ = 12.3, $J_{3e,4}$ = 4.8 Hz, H3e^{Neu}), 2.078, 2.075, 2.03, 2.02 (9H, each s, Ac × 3), 1.79 (1H, t, $J_{3e,3a}$ = $J_{3a,4}$ = 12.3 Hz, H3a^{Neu}). ESI-MS (*m*/*z*): negative 978 [M − 2H + Na]$^-$, 956 [M − H]$^-$, 665 [956-NeuAc]$^-$.

NeuAcα2−3Galβ1−4GlcNAcβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (10b). Sialylation of **9b** (11.1 mg, 12.5 μmol) was carried out under the same conditions as mentioned for **9a**. The reaction mixture was applied to a DEAE-Sephadex A-25 column (AcO$^-$ form) and washed with water and 0.1 M NH_4OAc; then it was eluted with 0.2 M NH_4OAc and concentrated. The residue was purified by BioGel P-2 chromatography and lyophilized to yield **10b** (14.9 mg, quant) as a white foam: ^{1}H NMR (600 MHz, D_2O) δ 5.17 (0.6H, d, $J_{1,2}$ = 3.1 Hz, H1$^{I}\alpha$), 4.70 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{I}\beta$), 4.62−4.59 (2H, m, H1^{II}, 1^{III}), 4.53 (1H, d, $J_{1,2}$ = 7.7 Hz, H1^{Gal}), 4.24 (0.4H, brd, J = 10.5 Hz, H6a$^{I}\beta$), 4.19 (0.6H, dd, $J_{5,6a}$ = 1.8, $J_{6a,6b}$ = 11.0 Hz, H6a$^{I}\alpha$), 4.15 (0.6H, dd, $J_{5,6b}$ = 3.5, $J_{6a,6b}$ = 11.0 Hz, H6b$^{I}\alpha$), 4.12−4.10 (0.4H, m, H6b$^{I}\beta$), 4.11 (1H, dd, $J_{2,3}$ = 9.7, $J_{3,4}$ = 3.1 Hz, H3^{Gal}), 4.06 (0.6H, m, H5$^{I}\alpha$), 3.99−3.97 (1H, m, H6a^{III}), 3.94 (1H, d, $J_{3,4}$ = 3.1 Hz, H4^{Gal}), 3.55 (1H, dd, $J_{1,2}$ = 7.7, $J_{2,3}$ = 9.7 Hz, H2^{Gal}), 2.74 (1H, dd, $J_{3e,3a}$ = 12.3, $J_{3e,4}$ = 4.4 Hz, H3e^{Neu}), 2.072, 2.068, 2.04, 2.021, 2.018 (12H, each s, Ac × 4), 1.81 (1H, t, $J_{3e,3a}$ = $J_{3a,4}$ = 12.3 Hz, H3a^{Neu}). ESI-MS (*m*/*z*): negative 1181 [M + Na − 2H]$^-$, 1159 [M − H]$^-$, 868 [1159 − NeuAc]$^-$; positive 1227 [M − 2H + 3Na]$^+$, 1205 [M − H + 2Na]$^+$.

NeuAcα2−3Galβ1−4GlcNAcβ1−4GlcNAcβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (10c). A solution of **9c** (12.4 mg, 11.3 μmol) in 2.0 mL of HEPES buffer (100 mM, pH 7.4) with CMP-sialic acid (8.2 mg, 12.5 μmol), 20 mM $MnCl_2$, 0.25% Triton X-100, 20 mU recombinant rat α(2,3)sialyltransferase, and 40 U alkaline phosphatase was incubated at room temperature for 1 day. Purification twice by BioGel P-2 chromatography gave **10c** (15 mg, 95%) as a white foam after lyophilization: ^{1}H NMR (500 MHz, D_2O) δ 5.18 (0.6H, d, $J_{1,2}$ = 3.3 Hz, H1$^{I}\alpha$), 4.70 (0.4H, d, $J_{1,2}$ = 8.4 Hz, H1$^{I}\beta$), 4.62−4.57 (3H, m, H1^{II}, 1^{III}, 1^{IV}), 4.53 (1H, d, $J_{1,2}$ = 7.7 Hz, H1^{Gal}), 4.24 (0.4H, brd, J = 11.0 Hz, H6a$^{I}\beta$), 4.19 (0.6H, brd, J = 9.2 Hz, H6a$^{I}\alpha$), 4.15 (0.6H, dd, $J_{5,6b}$ = 3.3, $J_{6a,6b}$ = 11.4 Hz, H6b$^{I}\alpha$), 4.12−4.09 (0.4H, m, H6b$^{I}\beta$), 4.10 (1H, dd, $J_{2,3}$ = 9.9, $J_{3,4}$ = 3.3 Hz, H3^{Gal}), 4.08−4.05 (0.6H, m, H5$^{I}\alpha$), 3.94 (1H, d, $J_{3,4}$ = 3.3 Hz, H4^{Gal}), 2.75 (1H, dd, $J_{3e,3a}$ = 12.5, $J_{3e,4}$ = 4.4 Hz, H3e^{Neu}), 2.07, 2.05, 2.03, 2.02 (15H, each s, Ac × 5), 1.79 (1H, t, $J_{3e,3a}$ = $J_{3a,4}$ = 12.5 Hz, H3a^{Neu}). ESI-MS (*m*/*z*): negative 1362 [M − H]$^-$; positive 1408 [M − H + 2Na]$^+$, 1386 [M + Na]$^+$, 1364 [M + H]$^+$, 1307 [1386 − SO_3]$^+$, 1285 [1364 − SO_3]$^+$.

NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAcβ1−4GlcNAc6OSO$_3$ (11a). A solution of **10a** (2.0 mg, 2.0 μmol) in 0.66 mL of MES buffer (50 mM, pH 6.0) with GDP−fucose (1.4 mg, 2.2 μmol), 15 mM $MnCl_2$, 10 mU α(1,3)fucosyltransferase VI, and 10 U alkaline phosphatase was incubated at room temperature for 2 days. The reaction mixture was applied to a DEAE-Sephadex A-25 column (AcO$^-$ form) and washed with water; then it was eluted with 0.2 M NH_4OAc and concentrated. The residue was purified by BioGel P-2 chromatography twice and lyophilized to yield a mixture of **11a** (2.4 mg, quant): ^{1}H NMR (600 MHz, D_2O) δ 5.18 (0.6H, d, $J_{1,2}$ = 3.1 Hz, H1$^{I}\alpha$), 5.10 (1H, d, $J_{1,2}$ = 3.5 Hz, H1^{Fuc}), 4.81 (1H, m, H5^{Fuc}), 4.70 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{I}\beta$), 4.65 (0.6H, d, $J_{1,2}$ = 8.8 Hz, H1$^{II}\alpha$), 4.64 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{II}\beta$), 4.51 (1H, d, $J_{1,2}$ = 7.9 Hz, H1^{Gal}), 4.25 (0.4H, brd, J = 11.0 Hz, H6a$^{I}\beta$), 4.20 (0.6H, dd, $J_{5,6a}$ = 1.8, $J_{6a,6b}$ = 11.0 Hz, H6a$^{I}\alpha$), 4.13 (0.6H, dd, $J_{5,6b}$ = 4.0, $J_{6a,6b}$ = 11.0 Hz, H6b$^{I}\alpha$), 4.10−4.06 (1H, m, H6b$^{I}\beta$, 5$^{I}\alpha$), 4.08 (1H, dd, $J_{2,3}$ = 9.7, $J_{3,4}$ = 3.1 Hz, H3^{Gal}), 3.92 (1H, d, $J_{3,4}$ = 3.1 Hz, H4^{Gal}), 3.77 (1H, d, $J_{3,4}$ = 3.1 Hz, H4^{Fuc}), 3.52 (1H, dd, $J_{1,2}$ = 7.9, $J_{2,3}$ = 9.7 Hz, H2^{Gal}), 2.75 (1H, dd, $J_{3e,3a}$ = 12.3, $J_{3e,4}$ = 4.4 Hz, H3e^{Neu}), 2.071, 2.068, 2.03, 2.02 (9H, each s, Ac × 3), 1.79 (1H, t, $J_{3e,3a}$ = $J_{3a,4}$ = 12.3 Hz, H3a^{Neu}), 1.16 (3H, d, $J_{5,6}$ = 6.6 Hz, H6^{Fuc}). ESI-MS (*m*/*z*): negative 1124 [M + Na − 2H]$^-$, 1102 [M − H]$^-$; positive 1148 [M − H + 2Na]$^+$, 1126 [M + Na]$^+$, 1104 [M + H]$^+$, 1046 [1126 − SO_3]$^+$, 1025 [1104 − SO_3]$^+$, 980 [1126 − Fuc]$^+$, 958 [1104 − Fuc]$^+$.

NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAcβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (11b). A solution of **10b** (4.7 mg, 4.0 μmol) in 1.2 mL of cacodylate buffer (25 mM, pH 6.2) with GDP−

fucose (3.0 mg, 4.7 μmol), 10 mM $MnCl_2$, 3.0 mU recombinant human α(1,3)fucosyltransferase V, and 12 U alkaline phosphatase was incubated at room temperature for 3 days. The reaction mixture was lyophilized and purified 3 times by BioGel P-2 and P-4 chromatography and lyophilized to give **11b** (5.5 mg, quant) as a white foam: ^{1}H NMR (600 MHz, D_2O) δ 5.21 (0.6H, d, $J_{1,2}$ = 3.1 Hz, H1$^{I}\alpha$), 5.12 (1H, d, $J_{1,2}$ = 3.9 Hz, H1^{Fuc}), 4.81 (1H, m, H5^{Fuc}), 4.73 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{I}\beta$), 4.65−4.63 (2H, m, H1^{II}, 1^{III}), 4.53 (1H, d, $J_{1,2}$ = 7.9 Hz, H1^{Gal}), 4.27 (0.4H, brd, J = 11.0 Hz, H6a$^{I}\beta$), 4.22 (0.6H, brd, J = 9.2 Hz, H6a$^{I}\alpha$), 4.18 (0.6H, dd, $J_{5,6b}$ = 3.7, $J_{6a,6b}$ = 10.7 Hz, H6b$^{I}\alpha$), 4.14 (0.4H, m, H6b$^{I}\beta$), 4.11−4.09 (0.6H, m, H5$^{I}\alpha$), 4.11 (1H, dd, $J_{2,3}$ = 9.6, $J_{3,4}$ = 3.5 Hz, H3^{Gal}), 3.95 (1H, d, $J_{3,4}$ = 3.5 Hz, H4^{Gal}), 3.60 (1H, dd, $J_{1,2}$ = 7.9, $J_{2,3}$ = 9.6 Hz, H2^{Gal}), 2.78 (1H, dd, $J_{3e,3a}$ = 12.3, $J_{3e,4}$ = 4.4 Hz, H3e^{Neu}), 2.11, 2.10, 2.07, 2.06, 2.05 (12H, each s, Ac × 4), 1.82 (1H, t, $J_{3e,3a}$ = $J_{3a,4}$ = 12.3 Hz, H3a^{Neu}), 1.19 (3H, d, $J_{5,6}$ = 6.6 Hz, H6^{Fuc}). ESI-MS (*m*/*z*): negative 1327 $[M + Na - 2H]^-$, 1305 $[M - H]^-$, 1014 [1305 − NeuAc]$^-$; positive 1351 $[M - H + 2Na]^+$, 1329 $[M + Na]^+$, 1307 $[M + H]^+$, 1249 $[1329 - SO_3]^+$, 1205 $[1351 - Fuc]^+$, 1060 [1351 − NeuAc]$^+$, 1028 $[1329 - GlcNAcSO_3]^+$, 1006 $[1307 - GlcNAcSO_3]^+$.

NeuAcα2−3Galβ1−4(Fucα1−3)GlcNAcβ1−4GlcNAcβ1−4GlcNAcβ1−4GlcNAc6OSO$_3$ (11c). Fucosylation of **10c** (5.7 mg, 4.1 μmol) with GDP-fucose (2.9 mg, 4.6 μmol) was carried out under the same conditions as described for **10b**. The reaction mixture was lyophilized and the residue was purified by BioGel P-2 and P-10 chromatography and lyophilized to give **11c** (5.7 mg, 90%) as a white foam: ^{1}H NMR (600 MHz, D_2O) δ 5.21 (0.6H, d, $J_{1,2}$ = 3.5 Hz, H1$^{I}\alpha$), 5.12 (1H, d, $J_{1,2}$ = 4.0 Hz, H1^{Fuc}), 4.81 (1H, m, H5^{Fuc}), 4.73 (0.4H, d, $J_{1,2}$ = 8.3 Hz, H1$^{I}\beta$), 4.65−4.60 (3H, m, H1^{II}, 1^{III}, 1^{IV}), 4.53 (1H, d, $J_{1,2}$ = 7.9 Hz, H1^{Gal}), 4.27 (0.4H, brd, J = 10.5 Hz, H6a$^{I}\beta$), 4.22 (0.6H, brd, J = 9.2 Hz, H6a$^{I}\alpha$), 4.18 (0.6H, dd, $J_{5,6b}$ = 3.5, $J_{6a,6b}$ = 10.5 Hz, H6b$^{I}\alpha$), 4.15−4.09 (1H, m, H5$^{I}\alpha$, H6b$^{I}\beta$), 4.11 (1H, dd, $J_{2,3}$ = 10.1, $J_{3,4}$ = 2.6 Hz, H3^{Gal}), 3.95 (1H, d, $J_{3,4}$ = 2.6 Hz, H4^{Gal}), 3.55 (1H, dd, $J_{1,2}$ = 7.9, $J_{2,3}$ = 10.1 Hz, H2^{Gal}), 2.78 (1H, dd, $J_{3e,3a}$ = 12.3, $J_{3e,4}$ = 4.8 Hz, H3e^{Neu}), 2.11, 2.10, 2.08, 2.07, 2.05 (15H, each s, Ac × 5), 1.82 (1H, t, $J_{3e,3a}$ = $J_{3a,4}$ = 12.3 Hz, H3a^{Neu}), 1.19 (3H, d, $J_{5,6}$ = 6.6 Hz, H6^{Fuc}); ESI-MS (*m*/*z*): negative 1531 $[M + Na - 2H]^-$, 1509 $[M - H]^-$, 1218 [1509 − NeuAc]$^-$; positive 1555 $[M - H + 2Na]^+$, 1533 $[M + Na]^+$, 1511 $[M + H]^+$, 1454 $[1533 - SO_3]^+$, 1431 $[1511 - SO_3]^+$, 1408 [1555 − Fuc]$^+$, 1386 [1533 − Fuc]$^+$, 1366 [1511 − Fuc]$^+$, 1263 [1555 − NeuAc]$^+$, 1231 [1533 − $GlcNAcSO_3$]$^+$.

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Supporting Information Available: ^{1}H NMR spectra of **6a**−**c**, **9a**−**c**, **10a**−**c**, **11a**−**c**, ^{13}C NMR spectra of **6a**,**b**, **9a**,**b**, and 2D-ROESY spectrum of **11b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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