Synthesis and Characterization of an Anomeric Sulfur Analogue of CMP-Sialic Acid

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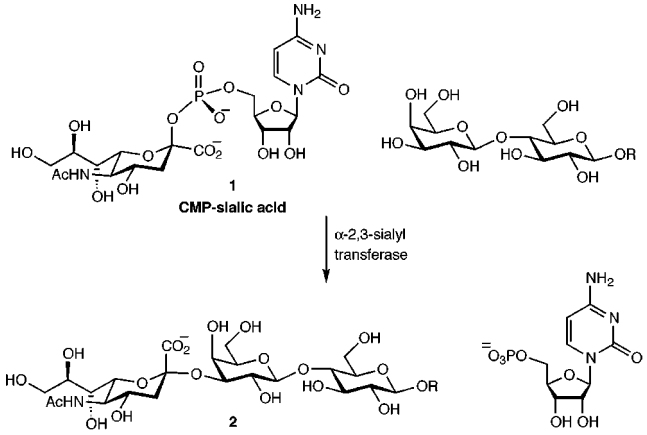
α-2,3-Sialyltransferase catalyzes the transfer of sialic acid from CMP-sialic acid (**1**) to a lactose acceptor. An analogue of **1** was synthesized in which the anomeric oxygen atom was replaced with a sulfur atom (**1*S***). The key step in the synthesis of **1*S*** was a tetrazole-promoted coupling of a cytidine-5′-phosphoramidite with a glycosyl thiol of a protected sialic acid. Compounds **1** and **1*S*** were characterized for their activity in a sialyl transfer assay. The rate of solvolysis in aqueous buffer of analogue **1*S*** was 50-fold slower than that of **1**. Analogue **1*S*** was found to be substrate for α-2,3-sialyltransferase. The K_m of **1*S*** was just 3-fold higher than that of **1**, while the k_{cat} of **1*S*** was 2 orders of magnitude lower compared to **1**.

Introduction

Sialic acid is a nine-carbon monosaccharide found at the termini of many complex oligosaccharides and glycoproteins in mammalian systems. Biological functions of sialic acid include cell−cell recognition, cellular adhesion, and receptor−ligand interactions.[1] Sialyltransferases catalyze the transfer of sialic acid from CMP-sialic acid (**1**) to a lactose acceptor to produce sialylated product **2** (Scheme 1). The reaction proceeds through a nucleophilic displacement with inversion of configuration at the anomeric center, with cytidine monophosphate (CMP) as the leaving group. Numerous sialyltransferases have been identified, each displaying unique substrate specificity.[2]

Our interest in sialyltransferases lies in their synthetic utility to sialylate oligosaccharides and glycoconjugates as an alternative to traditional chemical glycosylation. Enzymatic sialylation proceeds regio- and stereoselectively in aqueous solution without the need for tedious protecting group schemes. Chemical glycosylation with sialic acid is particularly difficult as a result of the presence of a carboxylate at the anomeric center. Consequently, the anomeric position is sterically hindered, and the electron-withdrawing nature of the carboxylate disfavors formation of an oxocarbenium ion at the anomeric carbon. Sialyltransferases have already seen some utility in the synthesis of oligosaccharides.[3] Unfortunately, the low catalytic constants (k_{cat}) for these enzymes, on the order of 10^2 min^{-1}, necessitate a time scale of days for preparative sialylations. A significant improvment in the synthetic utility of sialyltransferases would be achieved by understanding and potentially accelerating their reaction. We have therefore undertaken the synthesis and evaluation of an anomeric sulfur analogue (**1*S***, Figure 1) of CMP-sialic acid (**1**) as a potential substrate for α-2,3-sialyltransferase. Analogue **1*S*** appeared as a promising candidate for an active sialyltransferase substrate given that phosphorothioates in general are superior leaving groups compared to phosphates.[4] While our primary goal of accelerating the transferase reaction has not yet been realized, this report presents a convergent synthesis of this unique sialyl conjugate and its activity in a sialyltransfer assay.

Scheme 1



Results and Discussion

Synthesis of Sulfur Analogue 1*S*. A general route for the synthesis of CMP-sialic acid and CMP-conjugates containing modified sialic acids has been developed in our laboratory.[5] The synthesis of **1*S*** expands on this chemistry and demonstrates its application for the construction of phosphorothioate analogues of **1**. The key step in the synthesis is the coupling of a protected cytidine-5′-phosphoramidite with a glycosyl thiol of a

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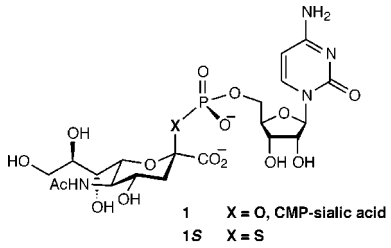
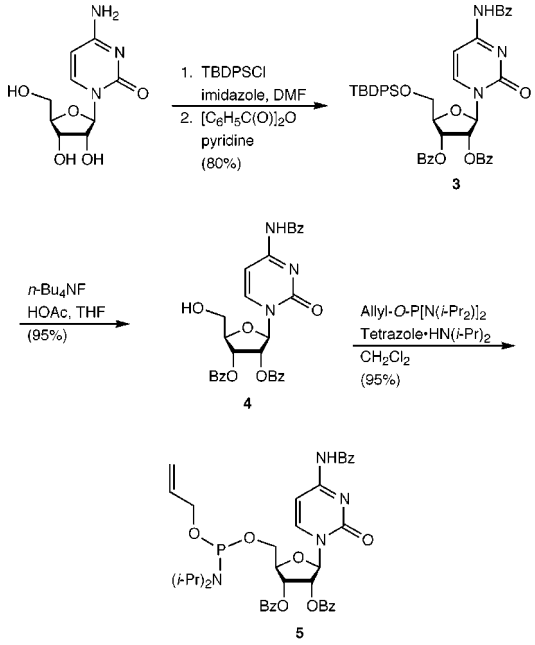


Figure 1. Structures of CMP-sialic acid (**1**) and the anomeric sulfur analogue (**1*S***) characterized in this work.

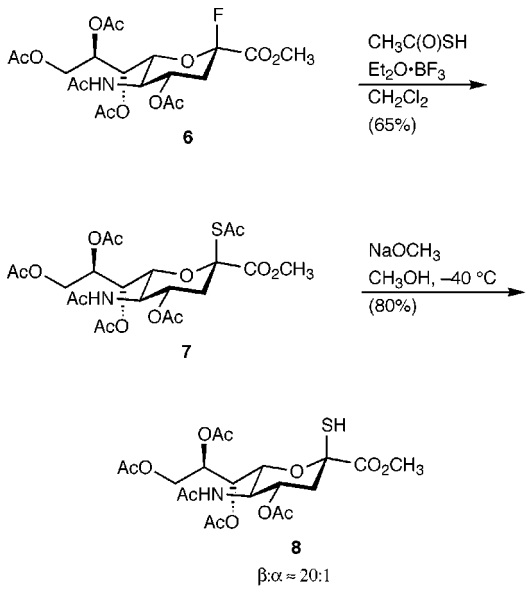
Scheme 2



protected sialic acid. The cytidine phosphoramidite was synthesized in four steps beginning with selective protection of the 5′-alcohol of cytidine as the TBDPS ether (Scheme 2). Benzoylation of the 2′- and 3′-alcohols and the C4-amine was followed by liberation of the 5′-alcohol with tetrabutylammonium fluoride to provide **4**. Phosphitylation with *O*-allyl tetraisopropylphosphordiamidite afforded the cytidine *O*-allyl phosphoramidite **5**.

Incorporation of the sulfur at the anomeric position of sialic acid employed Lewis acid catalyzed glycosylation with a sulfur nucleophile (Scheme 3). Peracetylated sialic acid methyl ester was treated with anhydrous HF−pyridine as described by Olah and co-workers[6] to afford the β-fluoride **6**. Activation of **6** with boron trifluoride in the presence of excess thiolacetic acid for 12 h afforded the thiolacetate **7** with good β-selectivity (β:$\alpha \approx$ 20:1, determined by ^{1}H NMR).[7] The initial addition of thiolacetic acid occurs within minutes to afford a kinetic distribution of anomers (β:$\alpha \approx$ 1:1); the products equilibrate under the reaction conditions over several hours to give the thermodynamically more stable β product, as expected by the anomeric effect. Selective *S*-deacylation was achieved with sodium methoxide at −40 °C to provide **8** (Scheme 3).

Scheme 3



Tetrazole-promoted activation of phosphoramidite **5**[8] in the presence of glycosyl thiol **8** at −40 °C cleanly afforded the corresponding thiophosphite product (Scheme 4). In early experiments, this P(III) product was isolated but was prone to oxidation by ambient molecular oxygen during workup and purification (ca. 25% of product oxidized). Therefore, the thiophosphite was not isolated but rather oxidized in situ at −40 °C with dimethyldioxirane (Scheme 4).[9] Purification over lipophilic sephadex (Sephadex LH-20, CH_3CN as mobile phase) afforded the phosphorothioate triester **9** in 80% yield as a 2:1 mixture of diastereomers. The use of the *O*-allyl phosphate protecting group allowed deallylation of **9** under neutral conditions by palladium(0)-catalyzed allyl ester exchange with potassium 2-ethylhexanoate (Scheme 4).[10] Sialyl conjugates such as **9** are not compatible with the more common base-labile phosphate protecting groups such as β-cyanoethyl because conditions for removing these groups can lead to elimination across the C2−C3 bond of the sialic acid. In contrast, anionic phosphate diesters such as **10** are relatively robust, allowing for smooth deacylation with sodium methoxide and final methyl ester saponification with sodium hydroxide to afford sulfur analogue **1*S***. The ^{1}H NMR spectrum of **1*S*** bears marked similarity to that of **1**, including phosphorus coupling to the axial proton of the C3 methylene. The ^{31}P NMR spectrum displays a sharp singlet at δ 14.73 ppm (H_3PO_4 = 0 ppm), consistent with data from bridging phosphorothioate diesters reported in RNA systems.[11]

Sialyl Transfer Assayed by HPLC. Activity assays for sialyltransferases typically monitor the consumption of a radiolabeled CMP-sialic acid substrate. Consumption of **1** does not accurately reflect the formation of sialylated product, but rather is a composite of four different reactions: (1) solvolysis of **1** free in solution to produce sialic acid and CMP, (2) enzyme-catalyzed solvolysis to produce sialic acid and CMP, (3) enzyme-catalyzed solvolysis to produce sialyl phosphate and cytidine, (4) the

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(7) Preparation of **7** from the α-fluoride has been reported: Kanie, O.; Nakamura, J.; Kiso, M.; Hasegawa, A. *J. Carbohydr. Chem.* **1987**, *6*, 105. A synthesis of the α anomer of **1*S*** has been reported: Smalec, B.; von Itzstein, M. *Carbohydr. Res.* **1995**, 266, 269.
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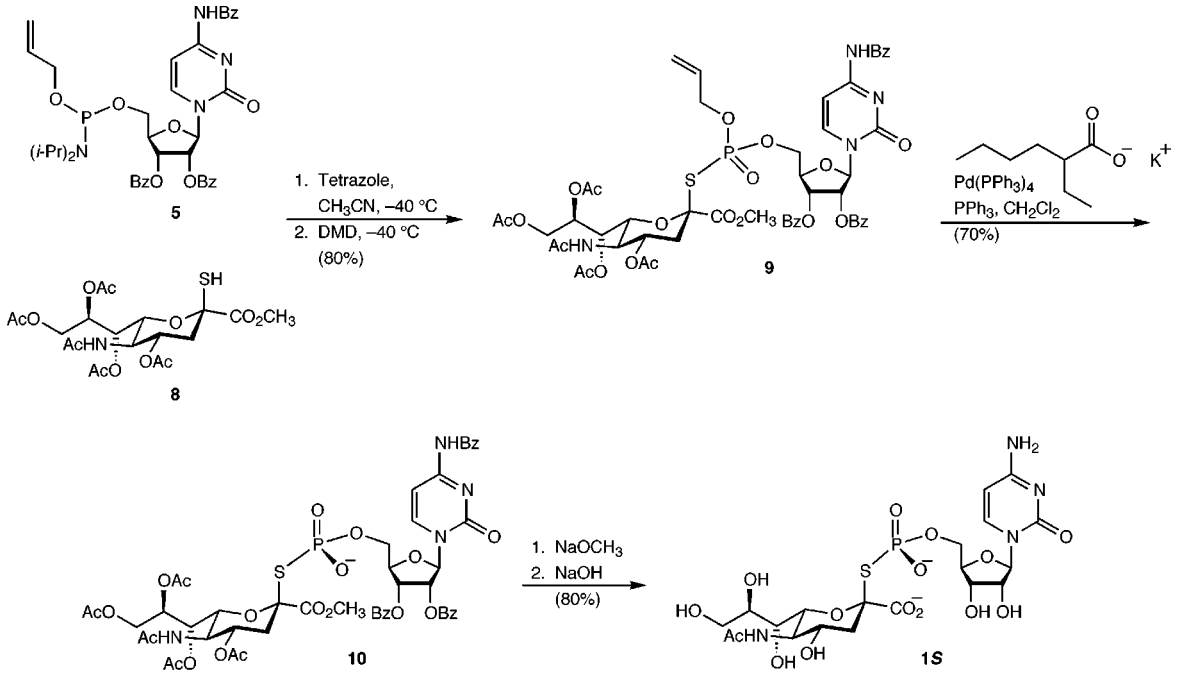
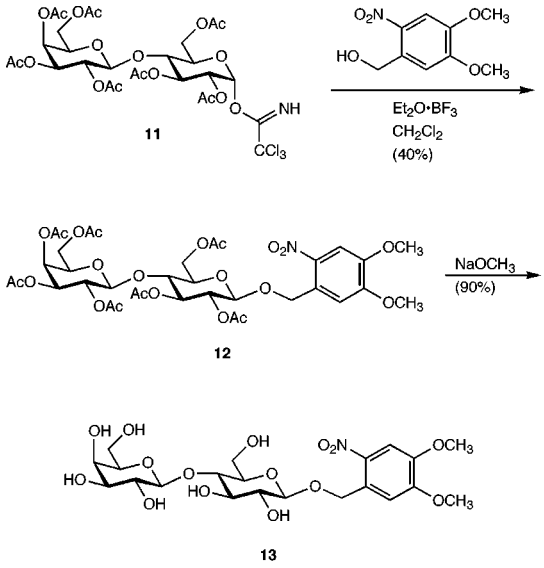
Scheme 4



Scheme 5



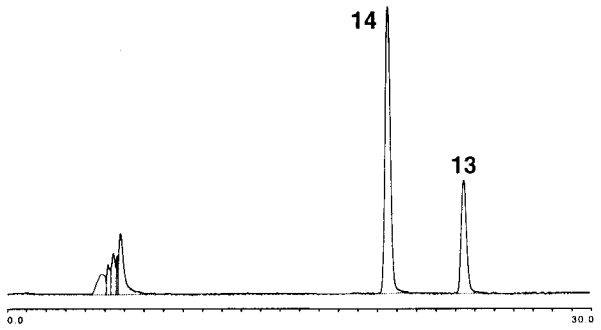


Figure 2. A rp-HPLC trace of the reaction of **1** (0.5 mM) and **13** (20 μM) with α-2,3-ST (20 mU/mL) monitored by measuring absorption at 348 nm. The reaction was performed at 37 °C in MES buffer (200 mM, pH 6.0) with NaCl (100 mM), disodium-EDTA (0.5 mM), and Triton X-100 (0.01%). The reaction was injected directly onto the HPLC after 2 h reaction time to afford 68% **14**.

sialyl transfer reaction of interest. The alternative to monitoring consumption of **1** is to quantitate the radiolabeled sialylated product after its isolation by size-exclusion chromatography.[12] For this work, a HPLC assay was developed to allow rapid quantitation specifically of the transfer of sialic acid without the requirement of a radiolabeled substrate. A lactose acceptor bearing a UV-chromophore was synthesized in two steps from the lactose trichloroacetimidate **11** (Scheme 5).[13] Standard borontrifluoride-catalyzed glycosylation[14] of **11** with 4,5-dimethoxy-2-nitrobenzyl alcohol afforded **12** exclusively as the β-anomer. Deacylation of **12** with sodium methoxide gave the water-soluble lactose acceptor **13**, which displayed a λ_{max} at 348 nm ($\epsilon_{348} = 6000$ M^{-1} cm^{-1}). Such a UV absorption is far removed from the cytosine chromophore (λ_{max} at 272 nm). The transfer of sialic acid from **1** to **13** catalyzed by α-2,3-sialyltransferase (α-2,3-ST) is cleanly resolved with reversed-phase HPLC (rp-HPLC) down to 20 μM **13** (Figure 2). The identity of the sialylated product as **14** was confirmed by ^{1}H NMR and high-resolution mass spectrometry of a preparative-scale reaction product.

Solvolysis of 1*S*. The first characterization of **1*S*** with respect to **1** was the solvolysis in aqueous solution. Substrate **1** (0.5 mM) was incubated at 37 °C in HEPES buffer (100 mM, pH 7.5) or MES buffer (100 mM, pH 6.0) with sodium chloride (100 mM) and disodium-EDTA (0.5 mM), and its transformation to CMP and sialic acid was monitored by rp-HPLC. The solvolysis of **1** displayed first-order behavior and increased in rate upon lowering the pH from 7.5 to 6.0 (Figure 3). The same experiment with **1*S*** conducted in parallel revealed an unexpectedly slow rate of solvolysis. Substrate **1*S*** displayed almost 2 orders of magnitude slower solvolysis than **1** (Figure 3). As observed for **1**, solvolysis of **1*S*** was also accelerated by lowering the pH of the reaction. The stability of **1*S*** was

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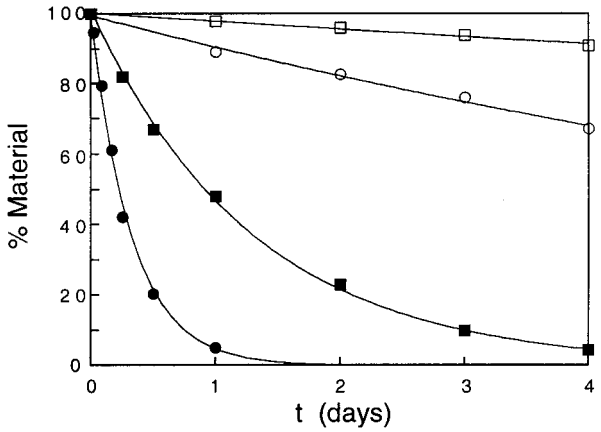
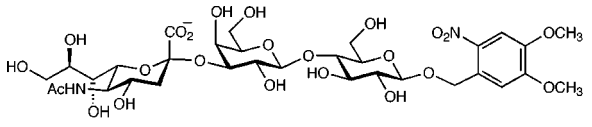


Figure 3. Reaction profiles for the solvolysis of **1** and **1*S*** in aqueous solution. Reactions were performed at 37 °C with either **1** (0.5 mM) or **1*S*** (0.5 mM) in HEPES buffer (100 mM, pH 7.5) or MES buffer (100 mM, pH 6.0) with NaCl (100 mM) and disodium-EDTA (0.5 mM): (■) **1**, pH = 7.5, $k = 0.032$ h^{-1}; (●) **1**, pH = 6.0, $k = 0.13$ h^{-1}; (□) **1*S***, pH = 7.5, $k = 0.0006$ h^{-1}; (○) **1*S***, pH = 6.0, $k = 0.003$ h^{-1}.

surprising, considering that replacing the anomeric oxygen atom with sulfur should result in a better leaving group in the form of a phosphorothioate, with the sulfur bearing a negative charge.[4]

Sialyl Transfer Activity of 1*S*. Sialyl transfer assays were conducted at 37 °C in aqueous MES buffer (200 mM, pH 6.0) with sodium chloride (100 mM), disodium-EDTA (0.5 mM, the STs do not require divalent metal cofactors),[15] and lactose acceptor **13** (20 μM). With a saturating excess of **1** (0.5 mM), transformation of **13** to **14**, as monitored by rp-HPLC (Figure 2), displayed first-order behavior and afforded a final end point of 95% **14**. Sulfur analogue **1*S*** was also observed to be a substrate for α-2,3-ST, affording the same product **14**. This structural assignment was confirmed by comparison of the product with the authentic sample of **14** prepared from **1**; the two compounds were found to be identical in terms of HPLC retention time (co-injection), UV-absorption spectra, and mass spectra of the product collected directly from the HPLC [MALDIMS calculated for $(M + Na)^+$ 851; found 851].



14

Michaelis−Menton (K_m) and catalytic constants (k_{cat}) for substrates **1** and **1*S*** were determined using initial rate velocities as a function of substrate concentration (Table 1).[16] The apparent binding affinity of **1*S*** for α-2,3-ST was just 3-fold lower than that of **1**. This modest decrease may be attributed to the slightly longer bond lengths and smaller bond angles of sulfur compared to oxygen (e.g., for methanol, C−O = 1.43 Å, C−O−H = 109°; for methanethiol, C−S = 1.82 Å, C−S−H = 100°).[17] A much larger effect, nearly 2 orders of magnitude, was observed for the catalytic constants. Replacement of oxygen with sulfur greatly decreased the rate of transfer. Thus, although **1*S*** is a substrate for α-2,3-ST, it appears to be significantly less reactive than the natural substrate (**1**).

Table 1. Kinetic and Thermodynamic Parameters for Transferase Substrates[a]

substrate	K_m (mM)	k_{cat} (min^{-1})
1	0.3 ± 0.1	170 ± 20
1*S*	0.9 ± 0.2	2.0 ± 0.2

[a] Reactions conducted at 37 °C in MES buffer (200 mM, pH 6.0) with NaCl (100 mM), disodium-EDTA (0.5 mM), Triton X-100 (0.01%), **13** (20 μM), and α-2,3-ST (20 mU/mL for **1**, 100 mU/mL for **1*S***). The concentration of transferase substrate was varied from 20 to 1000 μM. Each value is the mean ± range of three independent determinations.

Summary

In summary, this work expands on a general synthetic route to CMP-sialic acid conjugates[5] to prepare an anomeric sulfur analogue (**1*S***) of CMP-sialic acid (**1**). Although **1*S*** is a substrate for α-2,3-ST, it is 2 orders of magnitude less reactive toward sialyl transfer than **1**. One possible implication from these results is that the reaction of **1** with α-2,3-ST requires initial protonation of the anomeric oxygen atom by the enzyme. Sulfur, carrying less basic lone electron pairs than oxygen, would be slower to protonate. While this hypothesis is consistent with the apparent reactivity of **1*S***, it cannot be concluded unequivocally from the limited data presented here. Given the remarkable stability of **1*S*** in conjuction with its competent binding, it could possibly find use as a relatively inert substrate analogue in ongoing efforts to obtain X-ray structures of sialyl transferases.

Experimental Section

General Procedures and Materials. Authentic CMP-sialic acid (**1**, disodium salt) was obtained from CalBiochem at a purity of 91%, the impurities being CMP (7%) and cytidine (2%). Aliquots of **1** were prepared in aqueous HEPES buffer (10 mM, pH 7.5) at a concentration of 5 mM and stored frozen at −20 °C; aliquots were consumed within 2 weeks. Rat liver α-2,3-ST was obtained from CalBiochem at a concentration of 1 mU/μL. The enzyme was stored at −20 °C and consumed within 2 weeks.

Proton NMR (^{1}H NMR) spectra were recorded at 500 MHz. Chemical shifts are expressed in parts per million (δ) and are referenced to residual protium in the NMR solvent: $CD_3S(O)$-CD_2H, δ 2.49; CD_2HOD, δ 3.31; DOH, δ 4.80; C_6D_5H, δ 7.16. Carbon NMR (^{13}C NMR) spectra were recorded at 125 MHz. Chemical shifts (δ ppm) are referenced to the carbon signal for the solvent: DMSO-d_6, 39.51; C_6D_6, 128.39; carbon spectra recorded in D_2O are referenced to an external standard of DMSO-d_6. Fluorine NMR (^{19}F NMR) spectra were recorded at 470 MHz and are referenced to an internal standard of C_6H_5F (δ 0.00). Phosphorus NMR (^{31}P NMR) spectra were recorded at 202 MHz and are referenced to an external standard of 1% aqueous H_3PO_4 (δ 0.00).

2′,3′-*O*,*N*^{4}-Tribenzoyl-5′-*O*-*tert*-butyldiphenylsilyl Cytidine (3). Cytidine (6.0 g, 25 mmol, 1.0 equiv) and imidazole (4.2 g, 63 mmol, 2.5 equiv) were dissolved in dimethylformamide (45 mL). *tert*-Butyldiphenylsilyl chloride (7.0 mL, 27 mmol, 1.1 equiv) was added dropwise over a period of 10 min. The reaction was stirred at room temperature for 1 h and then quenched by the addition of methanol (10 mL). The solvents were removed by rotary evaporation at 40 °C under reduced pressure. The resulting syrup was partitioned between water and dichloromethane. The aqueous layer was further extracted

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with dichloromethane. The organic extracts were combined, and upon standing, the silylated product crystallized from solution within ca. 10 min. The white crystals were filtered and dried under vacuum. The crystalline product was dissolved in pyridine (40 mL, 500 mmol, 20 equiv). Benzoic anhydride (56 g, 250 mmol, 10 equiv) was added, and the reaction was stirred at room temperature for ca. 2 d until TLC (SiO_2, 1/1 hexane/ethyl acetate) indicated a single product ($R_f = 0.30$). The reaction was quenched with water (20 mL), and the solvents were removed by rotary evaporation at 40 °C under reduced pressure. The resulting syrup was partitioned between water and dichloromethane. The aqueous layer was further extracted with dichloromethane, and the combined organic extracts were washed with 5% aqueous HCl, saturated aqueous sodium bicarbonate, and saturated aqueous sodium chloride. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The product was purified over silica gel (300 mL), eluting with 6/4 hexane/ethyl acetate to 1/1 hexane/ethyl acetate. The product was obtained as a white foam (15.8 g, 80%). ^{1}H NMR (DMSO-d_6): δ 11.38 (s, 1 H), 8.31 (d, $J = 7.4$ Hz, 1 H), 8.02 (m, 2 H), 7.87 (m, 3 H), 7.68−7.60 (m, 7 H), 7.54−7.30 (m, 14 H), 6.28 (d, $J = 2.9$ Hz, 1 H), 5.91 (m, 2 H), 4.61 (q, $J = 3.9$, 5.2 Hz, 1 H), 4.11 (dd, $J = 3.3$, 11.5 Hz, 1 H), 3.97 (dd, $J = 4.1$, 11.7 Hz, 1 H), 1.01 (s, 9 H). IR (cm^{-1}): 3068, 2918, 1731. HRFABMS: calcd for $(M + H)^+$, 794.2898; found, 794.2930.

2′,3′-*O*,*N*^{4}-Tribenzoyl Cytidine (4). The protected cytidine derivative **3** (15.5 g, 19.5 mmol, 1.0 equiv) was dissolved in tetrahydrofuran (30 mL). Acetic acid (1.7 mL, 29 mmol, 1.5 equiv) was added, followed by tetrabutylammonium fluoride solution (1.0 M in THF, 59 mL, 59 mmol, 3.0 equiv). The reaction was stirred at room temperature for ca. 1 h until TLC (SiO_2, 1/1 hexane/ethly acetate) indicated complete conversion to a polar product ($R_f = 0.10$). The solvent was removed by rotary evaporation, and the resulting syrup was partitioned between saturated aqueous sodium bicarbonate and dichloromethane. The aqueous layer was further extracted with dichloromethane, and the combined organic extracts were washed with saturated aqueous sodium chloride. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The 5′-alcohol was purified over silica gel (300 mL), eluting first with 1/1 hexane/ethyl acetate to remove the *tert*-butyldiphenylsilyl fluoride, followed by 100% ethyl acetate to elute the product. To the product solution was slowly added approximately equal volume hexane to afford a white crystalline product. The white crystals were filtered and dried under vacuum (10.5 g, 95%). Mp = 180−182 °C. ^{1}H NMR (DMSO-d_6): δ 11.31 (s, 1 H), 8.52 (d, $J = 7.0$ Hz, 1 H), 8.02 (d, $J = 7.5$ Hz, 2 H), 7.93 (d, $J = 7.6$ Hz, 2 H), 7.83 (d, $J = 7.4$ Hz, 2 H), 7.68−7.58 (m, 3 H), 7.53−7.39 (m, 7 H), 6.38 (d, $J = 5.2$ Hz, 1 H), 5.85 (t, $J = 5.3$ Hz, 1 H), 5.79 (t, $J = 5.6$ Hz, 1 H), 5.50 (t, $J = 5.1$ Hz, 1 H), 4.53 (q, $J = 3.2$, 4.0 Hz, 1 H), 3.88 (m, 1 H), 3.81 (m, 1 H). ^{13}C NMR (DMSO-d_6): δ 167.39, 164.73, 164.49, 163.62, 154.55, 145.73, 133.84, 132.81, 129.29, 128.82, 128.45, 96.87, 88.46, 83.19, 74.43, 71.49, 60.52. IR (cm^{-1}): 3313, 3063, 2930, 1728. HRFABMS: calcd for $(M + H)^+$, 556.1720; found, 556.1694.

3-Propenyl-(2′,3′-*O*,*N*^{4}-tribenzoyl Cytidine-5′)-yl-*N*,*N*′-diisopropylphosphoramidite (5). The 5′-alcohol **4** (1.8 g, 3.2 mmol, 1.0 equiv) and tetrazole-diisopropylammonium salt (0.28 g, 1.6 mmol, 0.5 equiv) were dissolved in dichloromethane (10 mL). Allyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphordiamidite (1.75 mL, 5.4 mmol, 1.7 equiv) was added, and the solution stirred at room temperature for ca. 4 h until TLC (SiO_2, 1/1 hexane/ethyl acetate) indicated complete conversion of the 5′-alcohol to a less polar product ($R_f = 0.30$). The reaction solution was concentrated to a volume of ca. 5 mL and then purified directly over silica gel (250 mL), eluting with 1/1 hexane/ethyl acetate. The 5′-phosphoramidite was obtained as a clear, colorless oil (2.2 g, 95%). ^{1}H NMR (DMSO-d_6): δ 11.38 (s, 1 H), 8.37 (d, $J = 7.7$ Hz, 1 H), 8.02 (dd, $J = 1.8$, 7.4 Hz, 2 H), 7.92 (dd, $J = 1.8$, 7.6 Hz, 2 H), 7.84 (dt, $J = 1.4$, 8.1 Hz, 2 H), 7.68−7.63 (m, 3 H), 7.54−7.40 (m, 7 H), 6.35 (d, $J = 4.4$ Hz, 1 H), 5.92 (m, 1 H), 5.82 (m, 2 H), 5.30 (m, 1 H), 5.10 (m, 1 H), 4.68 (m, 1 H), 4.20−3.80 (m, 4 H), 3.58 (m, 2 H), 1.13 (d, $J = 6.8$ Hz, 12 H). ^{13}C NMR (DMSO-d_6): δ 167.44, 164.61, 164.46, 163.69, 154.39, 145.59, 135.61, 133.87, 132.83, 129.32, 128.75, 128.45, 123.51, 115.53, 115.24, 96.69, 89.19, 81.70, 74.32, 71.42, 63.91, 42.46, 24.35. ^{31}P NMR (DMSO-d_6, H_3PO_4 reference): δ 148.34, 148.24. IR (cm^{-1}): 3069, 2968, 1733. HRFABMS: calcd for $(M + H)^+$, 743.2846; found, 743.2869.

Methyl(5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-fluoro-β-D-glycero-D-galacto-2-nonulopyranosid)onate (6). Sialic acid (3.0 g, 9.7 mmol, 1.0 equiv) was dissolved in pyridine (16 mL, 195 mmol, 20 equiv). Acetic anhydride (14 mL, 145 mmol, 15 equiv) was added, and the reaction was stirred at room temperature for 2 h. The solvents were removed by rotary evaporation at 40 °C under reduced pressure. The resulting syrup was dissolved in methanol (100 mL), and cesium carbonate (4.1 g, 12.6 mmol, 1.3 equiv) was added. After dissolution and CO_2 evolution had ceased, the solution was concentrated under vacuum. The syrup was dissolved in dimethylformamide (15 mL), and iodomethane (12 mL, 195 mmol, 20 equiv) was added. The reaction was stirred at room temperature for 24 h and then concentrated by rotary evaporation at 40 °C under reduced pressure. The crude mixture was partitioned between water and dichloromethane. The aqueous layer was further extracted with dichloromethane, and the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated to a volume of ca. 20 mL. The solution was transferred to a 50-mL polypropylene centrifuge tube and concentrated to a minimum volume (ca. 8 mL) under a stream of nitrogen. The tube was placed in an ice−water bath, and hydrogen fluoride−pyridine (10 mL, 70% HF) was added. After stirring for 15 min at 0 °C, the icebath was removed, and the reaction was stirred at room temperature for 4 h. The reaction solution was then transferred carefully with a syringe to a 1-L flask containing cold saturated aqueous sodium bicarbonate (600 mL) and dichloromethane (200 mL) with vigorous stirring. After neutralization, the mixture was transferred to a 1-L separatory funnel, and the aqueous layer was further extracted with dichloromethane. The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The glycosyl fluoride was purified over silica gel (200 mL), eluting with 6/4 ethyl acetate/hexane to 8/2 ethyl acetate/hexane. The product was obtained as a clear, colorless oil (3.4 g, 70%). TLC (SiO_2, 100% ethyl acetate): $R_f = 0.35$. ^{1}H NMR (C_6D_6): δ 5.64 (d, $J = 10.1$ Hz, 1 H), 5.62 (dd, $J = 2.4$, 6.3, 1 H), 5.54 (td, $J = 2.6$, 6.3 Hz, 1 H), 5.19 (td, $J = 4.5$, 11.3, 1 H), 4.75 (dd, $J = 2.7$, 12.5 Hz, 1 H), 4.55 (q, $J = 10.5$ Hz, 1 H), 4.26 (dd, $J = 2.6$, 8.4 Hz, 1 H), 4.24 (dd, $J = 6.3$, 12.5 Hz, 1 H), 3.34 (s, 3 H), 2.40 (dt, $J = 4.5$, 13.9 Hz, 1 H), 2.05 (dq, $J = 13.8$, 36.5 Hz, 1 H), 1.99 (s, 3 H), 1.94 (s, 3 H), 1.79 (s, 3 H), 1.74 (s, 3 H), 1.71 (s, 3 H). ^{13}C NMR (C_6D_6): δ 170.94, 170.74, 170.61, 170.44, 170.04, 165.24 (d, $J = 29.1$ Hz), 108.77 (d, $J = 228$ Hz), 73.97, 71.11, 68.95, 67.92, 63.04, 53.95, 53.24, 48.81, 36.18 (d, $J = 27.4$ Hz), 23.35, 21.15, 20.95, 20.79, 20.76. ^{19}F NMR (C_6D_6, C_6H_5F reference): δ 2.62 (dd, $J = 4.6$, 36.6 Hz). IR (cm^{-1}): 2959, 1748. HRFABMS: calcd for $(M + H)^+$, 494.1674; found, 494.1694. Anal. Calcd for $C_{20}H_{28}NO_{12}F$: C, 48.68; H, 5.72; N, 2.84. Found: C, 48.64; H, 5.73; N, 2.87.

Methyl (5-Acetamido-4,7,8,9-tetra-*O*-acetyl-2-*S*-acetyl-3,5-dideoxy-2-thio-β-D-glycero-D-galacto-2-nonulopyranosid)onate (7). The glycosyl fluoride **6** (970 mg, 2.0 mmol, 1.0 equiv) was dissolved in dichloromethane (28 mL). Thiolacetic acid (0.63 mL, 10 mmol, 5.0 equiv, freshly distilled under reduced pressure at 2 °C) was added, followed by borontrifluoride-ethyl etherate (1.22 mL, 10 mmol, 5.0 equiv, freshly distilled), thus affording the following concentrations: glycosyl fluoride, 70 mM; thiolacetic acid, 350 mM; borontrifluoride-etherate, 350 mM. The reaction was stirred at room temperature for 12 h and then was quenched by the addition of saturated aqueous sodium bicarbonate (100 mL). The mixture was transferred to a separatory funnel, and the aqueous layer was further extracted with dichloromethane. The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The product was purified over silica gel (150 mL), eluting with 6/4 ethyl acetate/hexane to 8/2 ethyl acetate/hexane. The thiolacetate was obtained as a clear, colorless oil (700 mg, 65%). TLC (SiO_2, 100% ethyl acetate): $R_f = 0.30$. ^{1}H NMR (C_6D_6): δ 5.68 (t, $J = 5.5$ Hz, 1 H), 5.40 (dt, $J = 2.4$, 8.1

Hz, 1 H), 5.21 (dd, J = 2.2, 12.4 Hz, 1 H), 5.08 (td, J = 4.5, 11.1 Hz, 1 H), 4.84 (d, J = 10.3 Hz, 1 H), 4.49 (dd, J = 7.9, 12.3 Hz, 1 H), 4.45 (q, J = 10.5 Hz, 1 H), 4.26 (dd, J = 2.6, 10.5 Hz, 1 H), 3.58 (s, 3 H), 2.50 (dd, J = 4.8, 13.7 Hz, 1 H), 1.98 (dd, J = 11.7, 13.7 Hz, 1 H), 1.93 (s, 3 H), 1.86 (s, 3 H), 1.85 (s, 3 H), 1.77 (s, 3 H), 1.65 (s, 3 H), 1.62 (s, 3 H). IR (cm^{-1}): 2956, 1745. HRFABMS: calcd for $(M + H)^+$, 550.1594; found, 550.1594.

Methyl (5-Acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-*β*-D-glycero-D-galacto-2-nonulopyranosid)onate (8). The thiolacetate **7** (570 mg, 1.04 mmol, 1.0 equiv) was dissolved in methanol (21 mL), and the solution was cooled to −40 °C. Sodium methoxide (500 mM in methanol, 2.1 mL, 1.04 mmol, 1.0 equiv, 50 mM final) was added dropwise over a period of 5 min and the reaction stirred at −40 °C for 30 min. The reaction was quenched at −40 °C by addition of ammonium chloride crystals (165 mg, 3.1 mmol, 3.0 equiv) and the solution was allowed to warm to room temperature. Upon dissolution of all salts, the solution was concentrated. The crude product was partitioned between water and dichloromethane. The aqueous layer was further extracted with dichloromethane, and the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The product was purified over silica gel (20 mL), eluting with 8/2 ethyl acetate/hexane to afford the glycosyl thiol as a clear, colorless oil (420 mg, 80%). TLC (SiO_2, 100% ethyl acetate): R_f = 0.30. ^{1}H NMR (C_6D_6): δ 5.67 (dd, J = 2.1, 4.9 Hz, 1 H), 5.60 (m, 1 H), 5.24 (d, J = 10.3 Hz, 1 H), 5.18 (td, J = 4.0, 6.2 Hz, 1 H), 5.05 (dd, J = 2.1, 12.3 Hz, 1 H), 4.52 (dd, J = 2.2, 10.7 Hz, 1 H), 4.43 (q, J = 10.4 Hz, 1 H), 4.30 (dd, J = 7.8, 12.5 Hz, 1H), 3.36 (s, 3 H), 2.75 (br s, 1 H), 2.49 (dd, J = 4.9, 13.9 Hz, 1 H), 2.16 (dd, J = 11.7, 13.9 Hz, 1 H), 1.97 (s, 3 H), 1.91 (s, 3 H), 1.74 (s, 3 H), 1.72 (s, 3 H), 1.66 (s, 3 H). ^{13}C NMR (C_6D_6): δ 170.66, 170.65, 170.62, 170.49, 170.10, 84.79, 73.13, 72.58, 69.83, 68.54, 63.50, 53.21, 49.93, 39.05, 23.37, 21.25, 21.05, 20.84. IR (cm^{-1}): 2961, 1748. HRFABMS: calcd for $(M + H)^+$, 508.1489; found, 508.1493. Anal. Calcd for $C_{20}H_{29}NO_{12}S$: C, 47.33; H, 5.76; N, 2.76; S, 6.32. Found: C, 47.53; H, 5.78; N, 2.78; S, 6.51.

Dimethyldioxirane.[18] In a 1-L flask equipped with short-path distilling head and powder addition funnel was vigorously stirred water (160 mL), acetone (104 mL, spectrophotometric grade), and sodium bicarbonate (96 g). Oxone (100 g) was added over a period of 5 min. The system was placed under vacuum (water aspirator), and the receiving flask (100 mL) was placed in an acetone/solid CO_2 bath. Oxone (100 g) was added though the addition funnel over a period of 10 min. Collection continued for another 10 min to afford ca. 30 mL of a light yellow solution. Calcium sulfate (5 g, finely crushed) was added as a drying agent. The flask was covered with a rubber septum and stored at −20 °C vented with a needle to allow the condensed CO_2 to escape. Typically, dimethyldioxirane was obtained at a concentration of ca. 100 mM. Titration was performed on the day of use by measuring the oxidation of thioanisole by ^{1}H NMR integration of the aromatic resonances.

3-Propenyl (2′,3′-*O*,*N*^{4}-Tribenzoyl Cytidine-5′)-yl (Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-*β*-D-glycero-D-galacto-2-nonulopyranosid-2))-yl Phosphorothioate (9). The glycosyl thiol **8** (240 mg, 0.48 mmol, 1.0 equiv) and the 5′-phosphoramidite **5** (605 mg, 0.82 mmol, 1.7 equiv) together in a flask were rendered anhydrous by concentration from toluene (2 × 5 mL). Activated 3-Å molecular sieves (1 g, finely ground) were added, followed by acetonitrile (6 mL, distilled first from CaH_2, then from P_2O_5). The suspension was stirred at room temperature for 1 h and then cooled to −40 °C. In a second flask were stirred tetrazole (102 mg, 1.4 mmol, 3 equiv) and activated 3-Å molecular sieves (0.2 g) in acetonitrile (2 mL) at room temperature for 1 h. The reaction was initiated at −40 °C by transfer of the tetrazole solution to the thiol/amidite flask. The reaction was stirred at −40 °C for 2 h. To oxidize the phosphite, dimethyldioxirane (100 mM in acetone, 12 mL, 1.2 mmol, 2.5 equiv) was added at −40 °C, and the reaction was stirred at −40 °C for 1 h. Excess dimethyldioxirane was quenched at −40 °C by addition of dimethyl sulfide (0.09 mL, 1.2 mmol, 2.5 equiv). The suspension was warmed to room temperature and filtered over Celite. The clear solution was concentrated by rotary evaporation at 30 °C to a volume of ca. 3 mL and immediately purified over a column of LH-20 sephadex (Sigma) in acetonitrile (column dimensions, 3 cm × 35 cm; flow rate, 1 drop per 5 s). The phosphorothioate **9** eluted first. The product, a white oil, was obtained as a 2:1 mixture of diastereomers (450 mg, 80%). TLC (SiO_2, 8/2 ethyl acetate/hexane): R_f = 0.10. ^{1}H NMR (C_6D_6, major diastereomer): δ 10.10 (br s, 1 H), 8.15−7.95 (m, 7 H), 7.16−6.92 (m, 10 H), 6.30−4.55 (m, 19 H), 3.76 (s, 3 H), 2.72 (br dd, J = 4.7, 13.5 Hz, 1 H), 2.06 (m, 1 H), 2.03 (s, 6 H), 1.85 (s, 3 H), 1.74 (s, 3 H), 1.61 (s, 3 H). ^{31}P NMR (C_6D_6, H_3PO_4 reference): δ 21.17 (minor) and 20.79 (major). IR (cm^{-1}): 3069, 1741. EIMS: calcd for $(M + H)^+$, 1165; found, 1165.

Triethylammonium 2′,3′-*O*,*N*^{4}-Tribenzoyl Cytidine-5′-yl (Methyl (5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-2-thio-*β*-D-glycero-D-galacto-2-nonulopyranosid-2))-yl Phosphorothioate (10). The phosphorothioate triester **9** (325 mg, 0.28 mmol, 1.0 equiv), triphenylphosphine (30 mg, 0.12 mmol, 0.4 equiv), and tetrakis(triphenylphosphine)-palladium(0) (30 mg, 0.03 mmol, 0.1 equiv) were dissolved in dichloromethane (10 mL). Deallylation was initiated by addition of potassium 2-ethylhexanoate (100 mM in ethyl acetate, 3.1 mL, 0.31 mmol, 1.1 equiv). The reaction was stirred for ca. 30 min until TLC (SiO_2, 9/1 dichloromethane/methanol) indicated complete conversion to a polar product (R_f = 0.15). The reaction solution was partitioned between aqueous triethylammonium bicarbonate (0.1 M) and dichloromethane. The aqueous layer was further extracted with dichloromethane, and the combined organic extracts were dried (Na_2SO_4), filtered, and concentrated. The product was purified over silica gel (50 mL), eluting with 96/2/2 dichloromethane/methanol/triethylamine to afford the triethylammonium salt of **10** as a white oil (240 mg, 70%). ^{1}H NMR (CD_3OD): δ 8.65 (d, J = 7.5 Hz, 1 H), 8.01 (m, 4 H), 7.88 (dd, J = 1.2, 8.4 Hz, 2 H), 7.77 (d, J = 7.5 Hz, 1 H), 7.63 (m, 2 H), 7.55 (m, 3 H), 7.45 (t, J = 7.9 Hz, 2 H), 7.37 (t, J = 7.9 Hz, 2 H), 6.52 (d, J = 5.4 Hz, 1 H), 5.93 (t, J = 10.0 Hz, 1 H), 5.87 (t, J = 11.1 Hz, 1 H), 5.56 (td, J = 4.7, 11.1 Hz, 1 H), 5.50 (dd, J = 2.4, 6.0 Hz, 1 H), 5.29 (td, J = 2.5, 6.2 Hz, 1 H), 4.73 (m, 1 H), 4.60 (d, J = 2.4 Hz, 1 H), 4.58 (t, J = 2.2 Hz, 1 H), 4.42 (m, 1 H), 4.30 (m, 1 H), 4.22 (dd, J = 6.4, 12.3 Hz, 1 H), 3.98 (t, J = 10.4 Hz, 1 H), 3.81 (s, 3 H), 3.13 (q, J = 7.4 Hz), 2.96 (dd, J = 4.7, 13.5 Hz, 1 H), 2.10 (m, 1 H), 2.08 (s, 3 H), 2.04 (s, 3 H), 1.99 (s, 3 H), 1.93 (s, 3 H), 1.83 (s, 3 H), 1.28 (t, J = 7.4 Hz). ^{31}P NMR (CD_3OD, H_3PO_4 reference): δ 11.30. IR (cm^{-1}): 3420, 2982, 1735. EIMS: calcd for $(M - H)^-$, 1123; found, 1123.

Disodium Cytidine-5′-yl 5-Acetamido-3,5-dideoxy-2-thio-*β*-D-glycero-D-galacto-2-nonulopyranosid-2-yl Phosphorothioate (1*S*). The protected phosphorothioate diester **10** (triethylammonium salt, 150 mg, 0.12 mmol, 1.0 equiv) was dissolved in methanol (5 mL). Sodium methoxide (0.5 M in methanol, 0.70 mL, 0.36 mmol, 3.0 equiv) was added, and the reaction was stirred at room temperature for 3 h. The reaction was quenched by addition of ammonium bicarbonate crystals (47 mg, 0.60 mmol, 5.0 equiv). Upon dissolution of all salts, the solution was concentrated. The crude product was partitioned between water and ethyl acetate. The aqueous layer was washed with ethyl acetate and then passed though a Waters C_{18} SepPak. The solution was concentrated to a dry powder by lyophilization, and the resulting methyl ester was dissolved in aqueous sodium hydroxide solution (0.1 M, 12 mL, 1.2 mmol, 10 equiv). The saponification was allowed to proceed at room temperature for 30 min and then was quenched by addition of ammonium bicarbonate crystals (95 mg, 1.2 mmol). The resulting solution was transferred to a dialysis membrane (SpectraPor, MWCO = 100) and dialyzed against aqueous sodium bicarbonate (10 mM, 4 L) for 48 h. The solution was concentrated by lyophilization. The product, a white powder, was obtained as the disodium salt. The yield was determined

(18) Adam, W.; Chan, Y. Y.; Cremer, D.; Gauss, J.; Scheutzow, D.; Schindler, M. *J. Org. Chem.* **1987**, *52*, 2800.

by UV ($\epsilon = 9200\ M^{-1}\ cm^{-1}$ at 272 nm) (65 mg, 80%). For sialic acid transfer assays, a solution of **1*S*** in aqueous HEPES buffer (10 mM, pH 7.5) was prepared at a concentration of 5 mM and stored until use at −20 °C. ^{1}H NMR (D_2O): δ 7.94 (d, J = 7.5 Hz, 1 H), 6.10 (d, J = 7.5 Hz, 1 H), 5.98 (d, J = 4.1 Hz, 1 H), 4.32−4.24 (m, 5 H), 4.20−4.10 (m, 2 H), 3.96−3.86 (m, 3 H), 3.61 (dd, J = 6.9, 11.9 Hz, 1 H), 3.44 (d, J = 10.0 Hz, 1 H), 2.50 (dd, J = 4.6, 13.7 Hz, 1 H), 2.04 (s, 3 H), 1.82 (ddd, J = 2.0, 6.4, 13.9 Hz, 1 H). ^{13}C NMR (D_2O): δ 176.12, 175.41, 166.86, 161.49, 158.47, 142.41, 97.25, 90.10 (d, J = 4.4 Hz), 89.63, 83.37 (d, J = 8.8 Hz), 74.90, 72.69, 70.21, 69.55, 68.21, 65.63 (d, J = 6.2 Hz), 64.04, 52.76, 43.44 (d, J = 9.8 Hz), 22.82. ^{31}P NMR (H_3PO_4 reference): δ 14.73. HRFABMS: calcd for $(M - H_2 + Na_3)^+$, 697.0781; found, 697.0810.

(4,5-Dimethoxy-2-nitrophenyl)methyl *O*-(2,4,6-Tri-*O*-acetyl-*β*-D-galactopyranosyl)-(1→4)-*O*-2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranoside (12). The lactose trichloroacetimidate **11**[13] (270 mg, 0.35 mmol, 1.0 equiv) and 4,5-dimethoxy-2-nitrobenzyl alcohol (370 mg, 1.8 mmol, 5 equiv) were dissolved in dichloromethane (20 mL). Borontrifluoride-ethyl etherate (0.025 mL, 0.18 mmol, 0.5 equiv) was added, and the reaction was stirred at room temperature for 5 min. The reaction solution was partitioned between aqueous saturated sodium bicarbonate and dichloromethane. The organic layer was dried (Na_2SO_4), filtered, and concentrated. The product was purified over silica gel (40 mL), eluting first with 6/4 hexane/ethyl acetate to remove the excess benzyl alcohol followed by 1/1 hexane/ethyl acetate to elute the product. The lactose product was obtained as a clear, pale-yellow oil (120 mg, 40%). TLC (SiO_2, 1/1 hexane/ethyl acetate): $R_f = 0.10$. ^{1}H NMR (C_6D_6): δ 7.50 (s, 1 H), 7.17 (s, 1 H), 5.53 (dd, J = 7.9, 10.6 Hz, 1 H), 5.49 (d, J = 3 Hz, 1 H), 5.43 (t, J = 9.3 Hz, 1 H), 5.35 (m, 2 H), 5.13 (dd, J = 3.4, 10.4 Hz, 1 H), 4.98 (d, J = 15.0 Hz, 1 H), 4.46 (dd, J = 1.9, 12.0 Hz, 1 H), 4.38 (d, J = 7.9 Hz, 1 H), 4.34 (d, J = 7.8 Hz, 1 H), 4.21−4.08 (m, 3 H), 3.71 (t, J = 9.5 Hz, 1 H), 3.55 (t, J = 7.0 Hz, 1 H), 3.54 (s, 3 H), 3.29 (m, 1 H), 3.21 (s, 3 H), 1.97 (s, 3 H), 1.96 (s, 3 H), 1.80 (s, 3 H), 1.76 (s, 3 H), 1.75 (s, 3 H), 1.68 (s, 3 H), 1.58 (s, 3 H). ^{13}C NMR (C_6D_6): δ 170.51, 170.48, 170.29, 170.21, 170.04, 169.65, 169.37, 154.87, 148.82, 139.80, 129.62, 110.37, 108.60, 102.03, 101.01, 77.51, 73.54, 73.46, 72.73, 71.92, 71.28, 70.06, 69.05, 67.40, 62.86, 61.45, 56.27, 55.84, 21.13, 20.87, 20.85, 20.69, 20.59, 20.51. IR (cm^{-1}): 2942, 1752, 1523, 1223. HRFABMS: calcd for $(M + Na)^+$, 854.2331; found, 854.2328. Anal. Calcd. for $C_{35}H_{45}NO_{22}$: C, 50.54; H, 5.45; N, 1.68. Found: C, 50.60; H, 5.54; N, 1.67.

(4,5-Dimethoxy-2-nitrophenyl)methyl *O*-(*β*-D-Galactopyranosyl)-(1→4)-*β*-D-Glucopyranoside (13). The lactose derivative **12** (50 mg, 0.06 mmol, 1.0 equiv) was dissolved in methanol (5 mL). Sodium methoxide (0.5 M in methanol, 0.24 mL, 0.12 mmol, 2.0 equiv) was added, and the reaction was stirred at room temperature for 2 h. The reaction was quenched by addition of ammonium chloride crystals (16 mg, 0.3 mmol, 5 equiv). After dissolution of all salts, the solution was concentrated. The crude product was dissolved in a minimum volume of water (ca. 10 mL) and transferred to a dialysis membrane (SpectraPor, MWCO = 100). The product was desalted by dialysis against water (4 L) for 48 h in the dark. The product was concentrated by lyophilization and obtained as a pale-yellow powder (29 mg, 90%). For sialic acid transfer assays, a solution of **13** in water was prepared at a concentration of 0.2 mM and stored until use at −20 °C ($\epsilon = 6000\ M^{-1}\ cm^{-1}$ at 348 nm). ^{1}H NMR (D_2O): δ 7.84 (s, 1 H), 7.44 (s, 1 H), 5.24 (d, J = 15.0 Hz, 1 H), 5.20 (d, J = 15.0 Hz, 1 H), 4.59 (d, J = 8.0 Hz, 1 H), 4.46 (d, J = 7.9 Hz, 1 H), 4.00 (s, 3 H), 3.98 (m, 1H), 3.96 (s, 3 H), 3.93 (d, J = 3.0 Hz, 1 H), 3.83−3.65 (m, 6 H), 3.60−3.52 (m, 3 H), 3.44 (t, J = 8.5 Hz, 1 H). MALDIMS: calcd for $(M + Na)^+$, 560.1591; found, 560.1601.

High-Performance Liquid Chomatography (rp-HPLC). All solutions in this work were analyzed with a Varian HPLC solvent delivery system and Varian UV-1 absorbance detector. All samples were eluted though a Beckman Ultrasphere C_{18} reversed-phase analytical HPLC column (particle size, 5 μm; column dimensions, 4.6 mm × 250 mm) at a flow rate of 0.50 mL/min. Solvolysis reactions employed a 10-μL injection loop with a mobile phase of 100% aqueous triethylammonium bicarbonate buffer (20 mM, pH 8) for 20 min. Sialic acid transfer assays employed a 50-μL injection loop with a linear gradient of 80/20 aqueous triethylammonium bicarbonate buffer (20 mM, pH 8)/methanol at $t = 0$ min to 50/50 aqueous triethylammonium bicarbonate buffer (20 mM, pH 8)/methanol over a period of 20 min; elution continued for another 10 min at 50/50 aqueous buffer/methanol. Peaks were detected at 272 nm for solvolysis reactions and 348 nm for transfer assays. Retention times for reaction components are as follows: CMP, 9 min; **1**, 12 min; **14**, 20 min; **13**, 24 min.

4,5-Dimethoxy-2-nitrobenzyl *O*-5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic Acid-(2→3)-*O*-*β*-D-Galactopyranosyl-(1→4)-*β*-D-glucopyranoside (sodium salt) (14). In a 15-mL polypropylene centrifuge tube was combined **1** (12 mg), **13** (5 mg), aqueous HEPES buffer (0.9 mL, 1 M, pH 7.5, sodium salt), aqueous sodium chloride solution (0.9 mL, 1 M), aqueous disodium-EDTA solution (0.9 mL, 5 mM, pH 8), aqueous Triton X-100 solution (0.9 mL, 0.1%), and water (5.0 mL). The reaction was initiated by addition of α-2,3-ST (0.45 mL, 1 mU/μL), thus affording the following concentrations of reactants at the onset of the reaction: **1**, 2 mM; **13**, 1 mM; HEPES buffer, 100 mM; sodium chloride, 100 mM; disodium-EDTA, 0.5 mM; Triton X-100, 0.01%; α-2,3-ST, 50 mU/mL. The reaction was incubated at 37 °C for 24 h in the dark. HPLC analysis after 24 h indicated 75% conversion to **14**. CMP-sialic acid (6 mg) and α-2,3-ST (0.45 mL, 1 mU/μL) were added, and the reaction was incubated for another 24 h in the dark. HPLC analysis after this time indicated complete conversion of **13** to **14**. The reaction solution was loaded directly onto a column of C_{18}-modified reversed-phase silica (Fluka, 400 mesh, column dimensions 10 mm × 90 mm). Buffers, salts, and cytidine byproducts were eluted with 30 mL 9/1 aqueous sodium bicarbonate solution (10 mM)/methanol. Product **14** (yellow) was then eluted with 7/3 aqueous sodium bicarbonate solution (10 mM)/methanol. The methanol was removed by rotary evaporation and the resulting aqueous solution was concentrated by lyophilization to afford **14** as a pale-yelleow powder (6 mg, 70%). $\epsilon = 6000\ M^{-1}\ cm^{-1}$ at 348 nm. ^{1}H NMR (D_2O): δ 7.71 (s, 1 H), 7.40 (s, 1 H), 5.20 (d, J = 15.0 Hz, 1 H), 5.16 (d, J = 15.0 Hz, 1 H), 4.57 (d, J = 8.0 Hz, 1 H), 4.53 (d, J = 7.9 Hz, 1 H), 4.12 (dd, J = 3.2, 9.9 Hz, 1 H), 4.00−3.80 (m, 6H), 3.97 (s, 3 H), 3.92 (s, 3 H), 3.78−3.54 (m, 12 H), 3.43 (t, J = 8.7 Hz, 1 H), 2.75 (dd, J = 4.6, 12.5 Hz, 1 H), 2.03 (s, 3 H), 1.79 (t, J = 12.2 Hz, 1 H). MALDIMS: calcd for $(M + Na)^+$, 851.2546; found, 851.2543.

Solvolysis of 1 and Analogue 1*S*. Solvolysis reactions were performed in a volume of 1.0 mL at 37 °C in 1.5-mL Eppendorf tubes. CMP-sialic acid (0.10 mL, 5.0 mM) was added to a solution composed of water (0.60 mL), HEPES buffer (0.10 mL, 1 M, pH 7.5, sodium salt), aqueous sodium chloride solution (0.10 mL, 1 M), and aqueous disodium-EDTA solution (0.10 mL, 5 mM, pH 8), thus affording the following concentrations of reactants at the onset of the solvolysis reaction: **1**, 0.5 mM; HEPES buffer, 100 mM; sodium chloride, 100 mM; disodium-EDTA, 0.5 mM. The reaction was incubated at 37 °C. An aliqout of the solvolysis reaction was injected onto the HPLC at $t = 0$. Aliqouts of 50 μL were removed at appropriate time intervals (see Figure 2), frozen in liquid nitrogen, and stored frozen until analysis by HPLC. The percent **1** remaining was calculated relative to $t = 0$. The **1** used in this work was obtained at a purity of 91%, containing CMP (7%) and cytidine (2%). The data were fit to the equation $y = e^{-kt}$, where y is the percent **1** remaining, t is time, and k is the first-order rate constant. For solvolysis at pH 6.0, aqueous MES buffer (0.10 mL, 1 M, pH 6.0, sodium salt) was used in place of HEPES. Solvolysis of the synthetic analogue **1*S*** was performed as described for **1**.

K_m Determination for 1. To each of seven 0.65-mL Eppendorf tubes was added 200 μL of a solution composed of water (400 μL), aqueous MES buffer (400 μL, 1 M, pH 6.0, sodium salt), aqueous sodium chloride solution (200 μL, 1 M), aqueous disodium-EDTA solution (200 μL, 5 mM, pH 8), aqueous Triton X-100 solution (200 μL, 0.1%), and an aqueous

solution of **13** (200 μL, 0.2 mM). To each reaction solution was added a 50-, 25-, 10-, 5-, 3-, 2- or 1-μL aliquot of aqueous **1** (5 mM). Water was then added to afford a final volume of 250 μL for all seven solutions. The reactions were initiated at 37 °C by the addition of α-2,3-ST (5 μL, 1 mU/μL), thus affording the following concentrations of reactants at the onset of the reaction: MES buffer, 200 mM; sodium chloride, 100 mM; disodium-EDTA, 0.5 mM; Triton X-100, 0.01%; **13**, 20 μM; **1**, 1000, 500, 200, 100, 60, 40, or 20 μM; α-2,3-ST, 20 mU/mL. Aliquots of 70 μL were removed at 10 and 20 min, frozen in liquid nitrogen, and stored frozen until analysis by HPLC. Formation of **14** was less than 15% at 1000 μM **1** and ca. 1% at 20 μM **1**. Initial reaction velocities were obtained by fitting the data to $y = mx + b$, where y is the percent **14**, x is time, and m is the velocity. Velocities were then plotted against [**1**] and fitted to the equation $y = m1x/(m2 + x)$, where y is the velocity, x is [**1**], $m1$ is $[\alpha\text{-2,3-transferase}]_0$ (k_{cat}), and $m2$ is K_m.

K*$_m$ Determination for Sulfur Analogue 1*S*.** To each of seven 0.65-mL Eppendorf tubes was added 175 μL of a solution composed of: water (200 μL), aqueous MES buffer (400 μL, 1 M, pH 6.0, sodium salt), aqueous sodium chloride solution (200 μL, 1 M), aqueous disodium-EDTA solution (200 μL, 5 mM, pH 8), aqueous Triton X-100 solution (200 μL, 0.1%), and an aqueous solution of **13** (200 μL, 0.2 mM). To each reaction solution was added a 50-, 25-, 10-, 7.5-, 5-, 3-, or 2-μL aliquot of aqueous **1*S (5 mM). Water was then added to afford a final volume of 225 μL for all seven solutions. The reactions were initiated at 37 °C by the addition of α-2,3-ST (25 μL, 1 mU/μL), thus affording the following concentrations of reactants at the onset of the reaction: MES buffer, 200 mM; sodium chloride, 100 mM; disodium-EDTA, 0.5 mM; Triton X-100, 0.01%; **13**, 20 μM; **1*S***, 1000, 500, 200, 150, 100, 60, or 40 μM; α-2,3-ST, 100 mU/mL. Aliquots of 70 μL were removed at 4 and 8 h, frozen in liquid nitrogen, and stored frozen until analysis by HPLC. Formation of **14** was less than 15% at 1000 μM **1*S*** and ca. 1% at 40 μM **1*S***. Initial reaction velocities were obtained by fitting the data to $y = mx + b$, where y is the percent **14**, x is time, and m is the velocity. Velocities were then plotted against [**1*S***] and fitted to the equation $y = m1x/(m2 + x)$, where y is the velocity, x is [**1*S***], $m1$ is $[\alpha\text{-2,3-transferase}]_0$ (k_{cat}), and $m2$ is K_m.

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Supporting Information Available: Copies of representative ^{1}H and ^{31}P NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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