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The Synthesis and Enzymatic Incorporation of Sialic Acid Derivatives for use as Tools to Study the Structure, Activity, and Inhibition of Glycoproteins and other Glycoconjugates

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Abstract—Methods have been developed for the enzymatic synthesis of complex carbohydrates and glycoproteins containing in the sialic acid moiety the heavy metal mercury or the transition-state analog phosphonate of the influenza C 9-*O*-acetyl-neuraminic acid esterase-catalyzed reaction. 5-Acetamido-3,5-dideoxy-9-methylphosphono-β-D-*glycero*-D-*galacto*-nonulopyranosidonic acid (**1**), 5-acetamido-3,5-dideoxy-9-methylphosphono-2-propyl-α-D-*glycero*-D-*galacto*-nonulopyranosidonic acid triethylammonium salt (**2**), and 5-acetamido-9-thiomethylmercuric-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-nonulopyranosidonic acid (**3**) were synthesized. Compounds **1** and **2** are proposed transition state inhibitors of an esterase vital for the binding and infection of influenza C. Compound **3** was enzymatically incorporated into an oligosaccharide and a non-natural glycoprotein for use as an aid in the structure determination of these compounds by X-ray crystallography.

Introduction

Sialic acid terminated oligosaccharides are involved in a wide variety of necessary and vital biological functions[1] such as modulators in cell adhesion,[2] receptors for virus infection,[3] ligands for animal and plant lectins,[4,5] and the regulation of uptake and clearance of glycoconjugates to the liver.[6] The importance of sialic acid in these roles and the ubiquity of it in nature lead to the theory that incorporation of variants of sialic acid might be a useful method by which to investigate the structure and function of some of these interactions. To this end, we wish to report two different applications of this methodology.

A prime example of a biological process which relies on a displayed sialic acid is the binding of the influenza virus to the cell surface. Infection by this virus is mediated by an attachment process involving a receptor-binding (haemagglutinin) and a receptor-destroying activity. In the case of influenza A and B viruses, the receptor is sialic acid and the receptor-destroying enzyme is neuraminidase, a glycosidase.[7,8] In contrast, the influenza C virus haemagglutinin utilizes 9-*O*-acetyl-*N*-acetylneuraminic acid as its receptor,[9] and the receptor-destroying enzyme was found to be 9-*O*-acetylneuraminic acid esterase,[10–12] a serine enzyme.[13,14] Various benzoxazinones, isocoumarins, boronic acids and trifluoromethyl ketones were tested and only the isocoumarins were found to effectively inhibit this enzyme.[15] Unfortunately, these are irreversible inhibitors that also affect many other serine proteases. Several 9-substituted sialic acid analogs have been reported and displayed K_is in the millimolar range.[16,17] We report here an expedient synthesis of a potentially specific phosphonate transition-state analogue inhibitor of this esterase.

A second example is related to the structure of carbohydrate in glycoproteins. Much research has been centered on the functions of the glycoconjugates mentioned above. However due to the mobility of oligosaccharides the structures are difficult to study. The field of glycobiology would significantly benefit from an increase in

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3-D X-ray structural information. Although some insight on the mechanism of action of glycosyltransferases has been published,[18–23] to date, there is no known X-ray crystal structures for any of these enzymes. This structural information would aid in the elucidation of the mechanism of action as well as for rational drug design. In addition, several methods have been devised to analyze the primary structure of oligosaccharides chains on glycoproteins.[24] However, the 3-D structure of the carbohydrate coating of these proteins has been more difficult to obtain.[25,26] This information is a prerequisite in understanding the structure and function relationship of these various carbohydrate arrangements.

2-α-Thiomercuric analogs of sialic acids were shown to be useful derivatives yielding isomorphous derivatives for X-ray crystal structure determination of pertussin toxin[27] and 9-*O*-acetylsialic acid esterase from influenza C virus.[28] However, attachment of the mercury atom to the 2-position prevents its incorporation of this heavy metal derivative into oligosaccharide and glycoproteins. Thus, we decided to synthesize the analogue **3** shown in Figure 1, its subsequent enzymatic incorporation into the oligosaccharide sialyl Le^x and a novel glycoform of RNase.

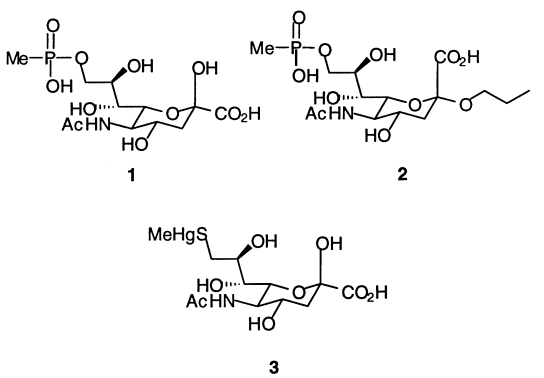
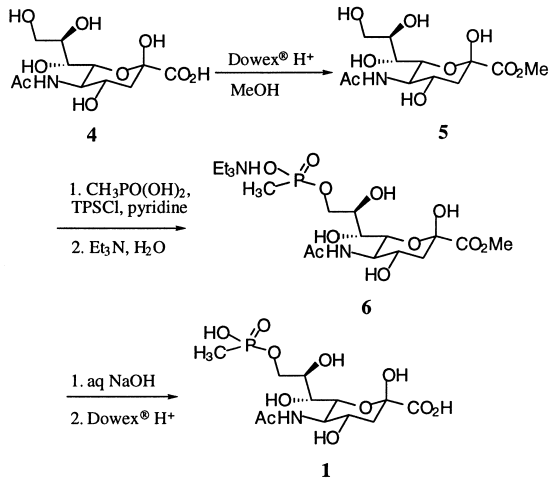


Figure 1. Sialic acid derivatives synthesized.

Results and Discussion

Synthesis of 9-Me-phosphonate sialic acid

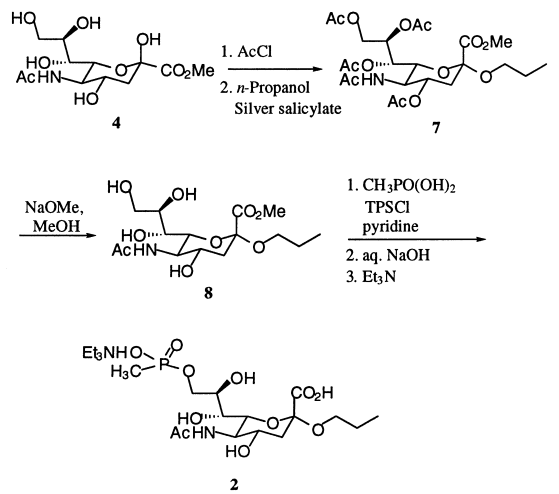
As shown in Scheme 1, *N*-acetylneuraminic acid (**4**) was first protected by esterification with methanol in the presence of an acidic ion exchange resin.[29] Selective reaction of the primary hydroxyl group with a mixed anhydride between methylphosphonic acid and 2,4,6-triisopropylbenzene sulfonyl chloride afforded the phosphonate salt **6**, along with the C-8 phosphorylated species. These two positional isomers were then separated by chromatography. Saponification of the ester and subsequent protonation with an acidic ion exchange



Scheme 1. Synthesis of 9-methyl phosphonate sialic acid (**1**).

resin afforded the desired free acid 5-acetamido-3,5-dideoxy-9-methylphosphono-β-D-*glycero*-D-*galacto*-nonulopyranosidonic acid (**1**).

Compound **2** was synthesized according to Scheme 2. Treatment of **4** with acetyl chloride followed by glycosylation with *n*-propanol, using silver salicylate as the catalyst,[30–32] afforded **7** which was deacetylated using catalytic sodium methoxide in methanol. Compound **8** was then phosphorylated, as mentioned above, and an unseparable mixture of C-8 and C-9 phosphorylated species were obtained. Saponification of the mixture and subsequent purification by preparative TLC afforded the desired 5-acetamido-3,5-dideoxy-9-methylphosphono-2-propyl-α-D-*glycero*-D-*galacto*-nonulopyranosidonic acid triethylammonium salt (**2**).



Scheme 2. Synthesis of 2-propyl-9-methyl phosphonate sialic acid (**2**).

The inhibition constant of **1** was determined to be approximately 2 mM, using a *p*-nitrophenyl acetate based assay described previously.[17] This is similar to other 9-substituted sialic acid analogs.[16] The possibility exists that the phosphonate moiety is not properly situated in the active site for effective inhibition by this transition state analogue. The stereochemistry at the anomeric position, which is different from the natural glycoside substrates, could also preclude proper alignment of this inhibitor. The synthesis of *O*-glycosylated analogue **2** was accomplished to verify this however, it showed no improvment in inhibition. It is likely that the sialic acid portion contributes only weakly to the binding and that the rest of the oligosaccharide chain of the natural substrates is largely responsible for binding. It is also conceivable that the haemagglutinin binding could activate the esterase binding conformation.

Incorporation of **1** into a asialoglycoprotein was attempted using a coupled enzyme system of CMP-sialic acid synthase and α-2,3 sialyltransferase (system is discussed in greater detail below). It was found that **1** was not a substrate for CMP-sialic acid synthase. We had reported previously that a 9-dimethylphosphonate derivitized sialic acid was not accepted by the synthase;[33] however, it is known that many bulky 9-derivatives, including fluorescent probes, are substrates of the enzyme.[34–37] These results lead us to investigate the inhibition of CMP-sialic acid synthase by **1**. Inhibition was determined by the thiobarbituric acid assay for the production of CMP-sialic acid in the presence of varying concentrations of **1**. The data shown in Figure 2 indicates that **1** is not an effective inhibitor of sialic acid synthase. The lack of inhibition or acceptance as a substrate indicates a lack of binding of **1** in the of CMP-sialic acid synthase active site.

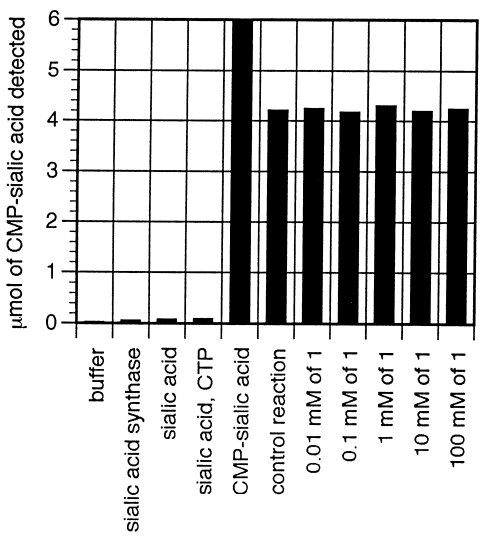


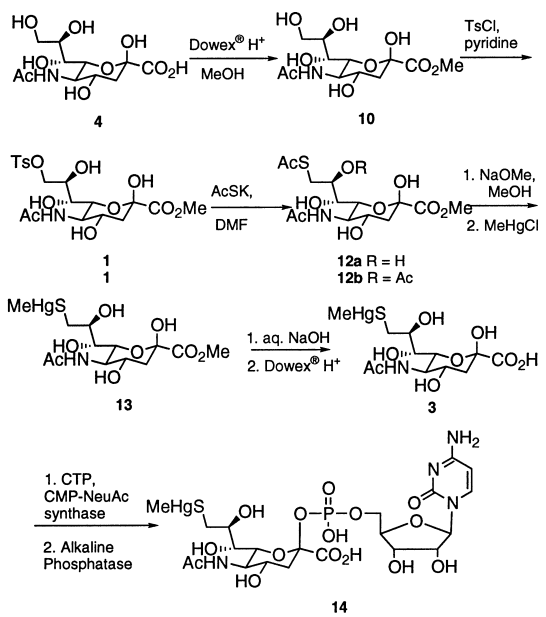
Figure 2. Determination of the concentration of CMP-sialic acid as detected by the thiobarbituric acid assay. Lack of inhibition of CMP-sialic synthase by **1** is demonstrated by the identical CMP-sialic acid concentrations generated in the CMP-sialic acid synthase catalyzed reactions containing 0 mM **1** (control reaction) and 0.01–100 mM **1**.

Synthesis of 9-MeHgS-sialic acid

As shown in Scheme 3, *N*-acetylneuraminic acid (**4**) was first protected by esterification with methanol in the presence of an acidic ion exchange resin.[29] Selective tosylation of the primary hydroxyl group[38] followed by displacement with potassium thioacetate gave the desired thioacetate **12a** and the 8-*O*-acetylated compound **12b** in a 2:1 mixture. Both these compounds could be converted to the same product **13** by deacetylation with sodium methoxide in methanol followed by reaction with methyl mercury (II) chloride. The methyl ester was then saponified and the resulting *N*-acetylneuraminic acid analogue 5-acetamido-9-thiomethylmercuric-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-nonulopyranosidonic acid (**3**).

Enzymatic incorporation of 9-MeHgS-sialic acid

Compound **3** was converted enzymatically to the desired CMP-(5-acetamido-9-thiomethylmercuric-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-nonulopyranosidonic acid) **14** by reaction with CTP and CMP-NeuAc synthase. The choice of a chemoenzymatic synthesis was necessary since the chemical synthesis of the required

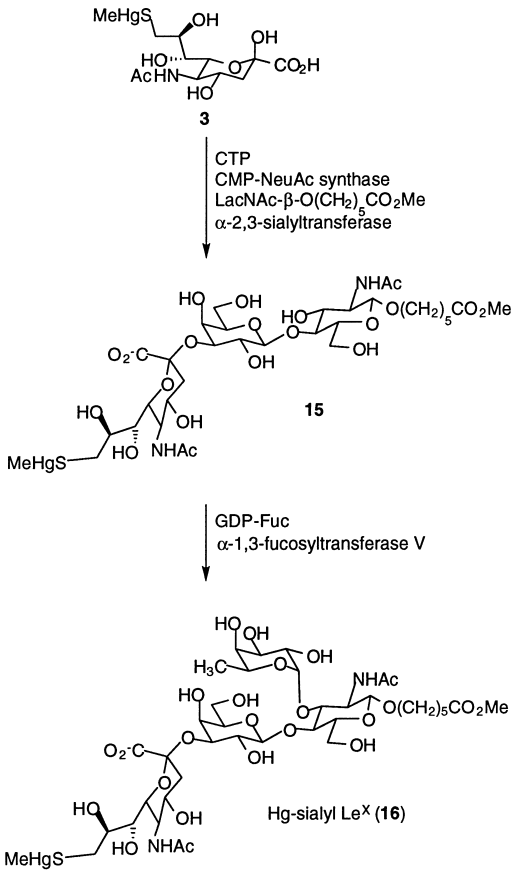


Scheme 3. Chemo-enzymatic synthesis of **14**.

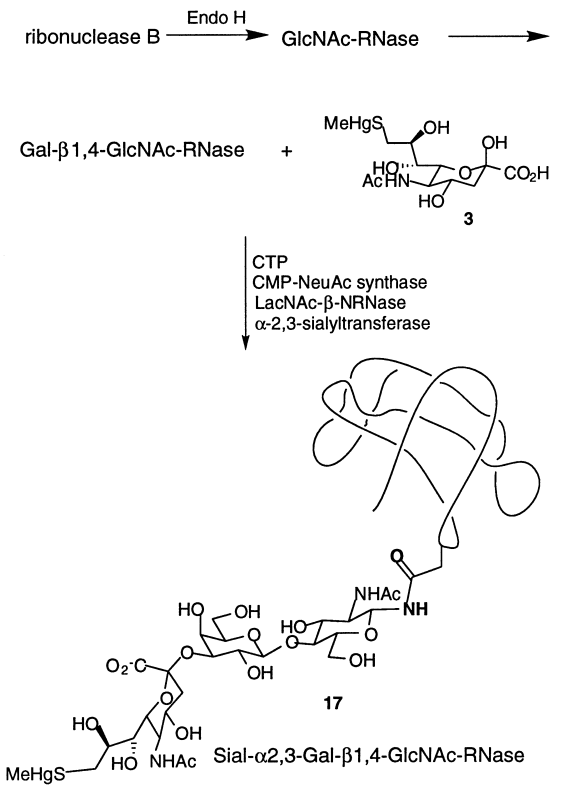
sugar-nucleotides requires strong Lewis acid conditions which are not compatible with the presence of a sulfur–mercury bond. As anticipated, the mercury atom-containing sialic acid was accepted by the synthase to give (**14**). This reaction took approximately the same amount of time, according to TLC, as with the natural substrate NeuAc. Alkaline phosphatase was used to simplify the purification which was accomplished by ion exchange chromatography. Unfortunately, the purification step afforded somewhat low yields of the desired compound **14** due to the volatility of mercury-containing organic compounds. The loss occurred during the removal of the ammonium bicarbonate by lyophilization. The X-ray structure determination of α-2,3-sialyltransferase complexed with **14** is currently in progress.

The incorporation of Hg into the oligosaccharide sialyl Le^X is shown in Scheme 4. In this example the sugar nucleotide was generated in situ to overcome the isolation problems. Therefore, the reaction of **3** with CTP and CMP-NeuAc synthase afforded the sugar nucleotide **14**, which was then reacted with LacNAc-β-$O(CH_2)_5CO_2Me$[39] and α-2,3-sialyltransferase to give the desired 9-MeHgS-NeuAc-α-2,3-LacNAc-β-$O(CH_2)_5CO_2Me$ (**15**). Compound **15** was then fucosylated using GDP-Fuc and α-1,3-fucosyltransferase V to give the desired Hg-sialyl Le^X (**16**). The isolation of **15** and **16** was confirmed by mass spectrometry and NMR spectroscopy, by comparison with the reported complete assignment of NeuAc-α-2,3-LacNAc and sialyl Le^X glycosides.[40–42] This Hg-sialyl Le^X should prove to be helpful in the elucidation of the X-ray structure of selectin–sialyl Le^X complexes.

The usefulness of this methodology was also demonstrated by enzymatically transferring **3** into a non-natural glycoprotein, LacNAc-RNase[43] to afford 9-MeHgS-NeuAc-α-2,3-LacNAc-β-RNase (**17**) as confirmed by MALDI mass spectrometry (Scheme 5). Figure 3 indicates the computer generated structure of the mercury labeled glycoprotein. Work is in progress to determine the X-ray structure of this glycoform to investigate the effect of carbohydrate on glycoprotein structure. The incorporation of the mercury labeled sialic acid into a



Scheme 4. Enzymatic synthesis of a sialyl Le^X derivative with a covalently labeled mercury at the sialic acid residue.



Scheme 5. Enzymatic synthesis of a glycoprotein with a well defined carbohydrate covalently labeled with mercury at the sialic acid residue. For the synthesis of Gal-β1,4-GlcNAc-RNase see ref 43.

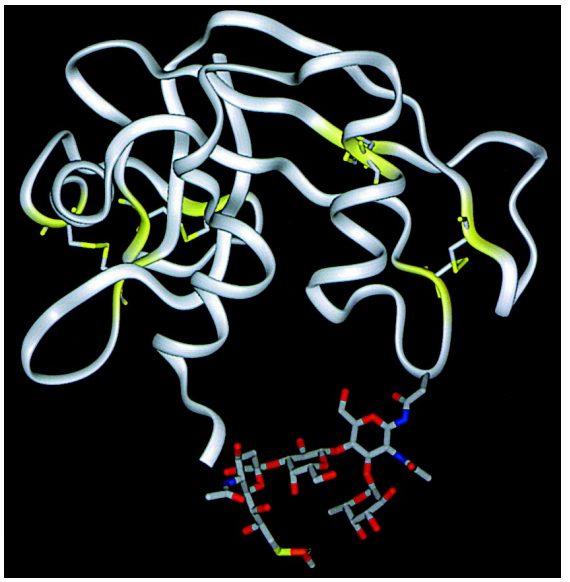
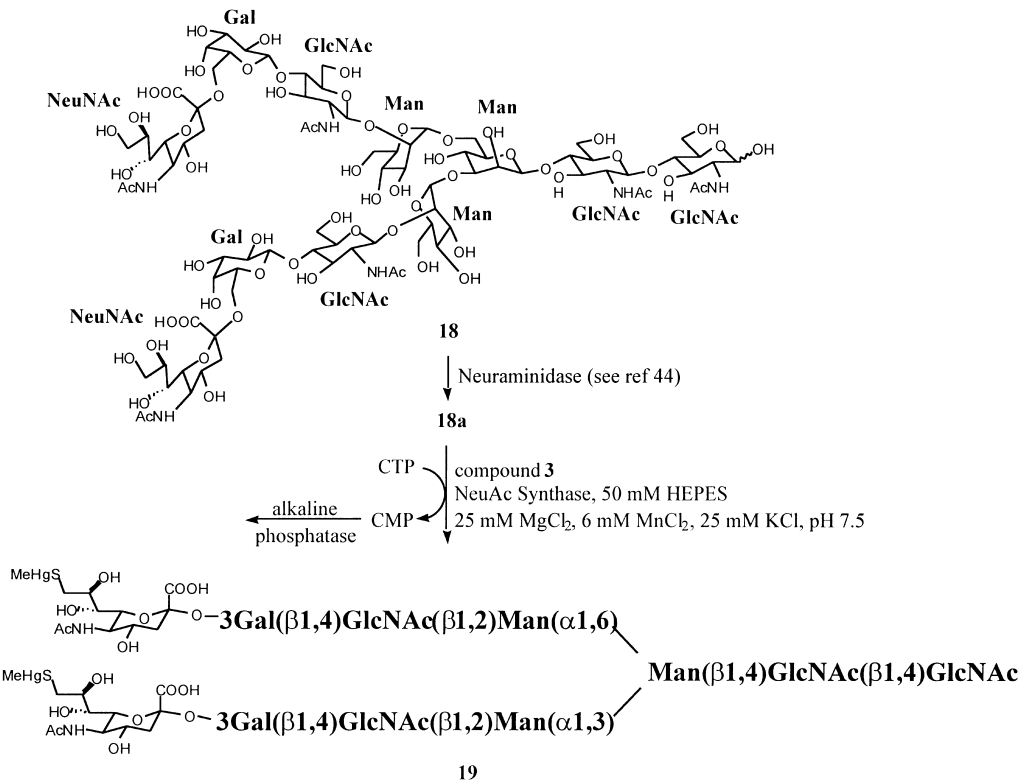


Figure 3. Computer generated structure of **17** based on the known X-ray structure of ribonuclease A[43] and the known solution conformation of the trisaccharide sialyl-*N*-acetyl lactosamine.[40] The structure is colored acccording to atom type: carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow; mercury, orange.

glycoprotein should provide a useful tool in the structure determination of the glycoconjugate by X-ray crystallography and should be applicable to any glycoconjugate accepted by the synthase and transferase enzymes. Finally, the enzymatic method can also be used to remodel glycoconjugates. As illustrated in Scheme 6, a natural dimeric sugar chain isolated from chicken egg can be remodeled via removal of the terminal sialic acid by neuraminidase,[44] followed by introduction of the 9-mercuryl sialic acid derivative catalysed by α-2,3-sialyl transferase with altered glycosidic linkage. This method should be applicable to glycoprotein remodeling as shown in Scheme 5.

Conclusion

We have shown the synthesis of several 9-substituted sialic acids including 5-acetamido-3,5-dideoxy-9-methylphosphono - β - D - *glycero* - D - *galacto* - nonulopyranosidonic acid (**1**) and 5-acetamido-9-thiomethylmercuric-3,5,9-trideoxy-β-D-*glycero*-D-*galacto*-nonulopyranosidonic acid (**3**). In addition, compound **3** has in turn been incorporated enzymatically into both an oligosaccharide and a glycoprotein for use as an aid in X-ray crystallographic determination of the carbohydrate



Scheme 6. Enzymatic remodeling of a natural dimeric sialyl lactosamine dimer via neuraminidase and sialyltransferase reaction to incorporate the 9-mercuryl-sialic acid to the dimeric oligosaccharide with altered glycosidic linkage.

structure. The method developed for the synthesis of glycoproteins with well defined carbohydrate structure should help address an important question in glycobiology regarding the effect of glycosylation on glycoprotein structure and function.

Experimental

General methods

All processes involving air or moisture sensitive reactants were conducted under an atmosphere of dry argon using oven-dried glassware. The reagents used were purchased from Sigma and were of the highest purity available. The solvents were reagent grade and used as supplied unless otherwise mentioned. Solvent evaporation was performed under reduced pressure below 30 °C using a Büchi rotary evaporator, followed by evacuation (< 0.1 mm Hg) to constant sample weight except for the mercury-containing intermediates which were dried under high vacuum for only 1 h. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE instrument under fast atom bombardment (FAB) conditions unless otherwise noted. ^{1}H NMR spectra were obtained at 400 or 500 MHz and ^{13}C NMR at 100 or 125 MHz on a Bruker AMX-400 or 500 instrument. ^{1}H NMR chemical shifts are reported in parts per million (ppm) downfield relative to tetramethylsilane (TMS) using the solvent resonance as the reference: CD_3OD δ 3.30, HOD δ 4.63; ^{13}C NMR chemical shifts are reported relative to CD_3OD δ 49.0, and dioxane δ 66.5 for D_2O samples. Sialic acid was obtained from Taiyo Kagaku Co., Ltd (Japan). CMP-Neuraminic acid synthase, α-2,3-sialyltransferase and α-1,3-fucosyltransferase were a gift from the Cytel Corporation (San Diego, CA).

Methyl 5-acetamido-3,5-dideoxy-β-D-glycero-D-galacto-nonulopyranosonate (5). *N*-Acetylneuraminic acid (**4**) (3.09 g, 10 mmol) was added to a mixture of dry Dowex® 50W-X4 (H^+, 1 g) and anhydrous MeOH (300 mL) while stirring. The resulting mixture was then stirred at 20 °C for 39 h. The resin was then filtered over Celite® and the resulting solution concentrated on the rotovap. The residue was then flash chromatographed through a short silica gel column with a mixture of CH_2Cl_2 and MeOH (3/1) to give **5** (2.88 g, 89%) as a white solid: (TLC R_f = 0.27, CH_2Cl_2:MeOH, 3:1), ^{1}H NMR (CD_3OD) δ 4.03 (1H, ddd, J = 11.2, 10.1 and 4.9 Hz), 3.99 (1H, dd, J = 10.5 and 1.5 Hz), 3.82–3.77 (2H, m), 3.77 (3H, s), 3.69 (1H, ddd, J = 9.1, 5.7, and 2.8 Hz), 3.61 (1H, dd, J = 11.2 and 5.7 Hz), 3.47 (1H, dd, J = 9.2 and 1.4 Hz), 2.21 (1H, dd, J = 12.9 and 4.9 Hz), 2.01 (3H, s), 1.88 (1H, dd, J = 12.9 and 11.4 Hz); ^{13}C NMR (CD_3OD) δ 175.09, 171.76, 96.64, 72.03, 71.60, 70.13, 67.82, 64.79, 54.27, 53.17, 40.65, 22.69.

Methyl 5-acetamido-3,5-dideoxy-9-methylphosphono-β-D-glycero-D-galacto-nonulopyranosonate triethylammonium salt (6). To a mixture of methylphosphonic acid (211 mg, 2.20 mmol) and compound **5** (647 mg, 2.00 mmol) in anhydrous pyridine (10 mL) at 0 °C under Ar was added 2,4,6-triisopropylbenzene sulfonyl chloride (787 mg, 2.6 mmol). Three other portions of TPSCl (3×182 mg, 1.80 mmol) were then added at 15 min intervals. The reaction mixture was then stirred at 0 °C for 18 h and triethylamine (1.5 mL) was added, followed by water (5 mL). The resulting mixture was then evaporated under high vacuum and flash chromatographed on silica gel with a mixture of $CHCl_3$ and MeOH (5:1 to 2:1) containing Et_3N (1%) to give recovered **5** (124 mg, 19%), methyl 5-acetamido-3,5-dideoxy-8-methylphosphono-β-D-*glycero*-D-*galacto*-nonulopyranosonate triethylammonium salt (236 mg, 23%) and **6** (458 mg, 46%) as a light brownish amorphous solid: (TLC R_f = 0.11, $CHCl_3$/MeOH, 3/1 containing 1% Et_3N), ^{1}H NMR (CD_3OD) δ 4.11 (1H, ddd, J = 11.3, 7.6 and 2.5 Hz), 4.05–3.96 (2H, m), 4.03 (1H, dd, J = 10.5 and 1.5 Hz), 3.83 (1H, dd, J = 10.3 and 10.2 Hz), 3.79–3.72 (1H, m), 3.76 (3H, s), 3.58 (1H, dd, J = 9.2 and 1.3 Hz), 3.16 (6H, q, J = 7.3 Hz), 2.19 (1H, dd, J = 12.8 and 4.9 Hz), 2.00 (3H, s), 1.87 (1H, dd, J = 12.8 and 11.4 Hz), 1.29 (9H, t, J = 7.3 Hz), 1.26 (3H, d, J = 16.5 Hz); ^{13}C NMR (CD_3OD) ° 174.89, 171.82, 96.69, 71.92, 71.00 (d, J = 5.2 Hz), 69.48, 68.05, 66.81 (d, J = 5.5 Hz), 54.30, 53.08, 47.54, 40.74, 22.75, 12.26 (d, J = 138.0 Hz), 9.25.

5-Acetamido-3,5-dideoxy-9-methylphosphono-β-D-glycero-D-galactononulo-pyranosidonic acid (1). To a solution of the methyl ester **6** (103 mg, 205 μmol) in water (4 mL) was added an aqueous NaOH solution (1%), gradually, until the pH remained > 8. The solution was then stirred at 20 °C for 4 h and neutralized with Dowex® 50W-X4 (H^+) resin and subsequently passed through a short column of the same resin and eluted with water until the eluent was no more acidic. Lyophilization afforded **1** (78.0 mg, 98%) as a white solid: ^{1}H NMR (D_2O) δ 3.94 (1H, ddd, J = 11.0, 5.9 and 2.4 Hz), 3.88–3.80 (3H, m), 3.70 (1H, dd, J = 10.3 and 10.2 Hz), 3.67–3.63 (1H, m), 3.40 (1H, dd, J = 9.4 and 0.9 Hz), 2.08 (1H, dd, J = 13.1 and 4.9 Hz), 1.82 (3H, s), 1.65 (1H, dd, J = 13.0 and 11.6 Hz); ^{13}C NMR (D_2O) δ 174.68, 172.86, 95.03, 70.13, 68.50 (d, J = 7.3 Hz), 67.32, 66.38, 66.18 (d, J = 5.7 Hz), 51.88, 38.61, 21.88, 10.10 (d, J = 138.6 Hz); ^{31}P NMR (D_2O) δ 25.74. HRMS calcd for $C_{12}H_{23}NO_{11}P$ ($M+H^+$): 388.1009, found: 388.1012.

Methyl 5-acetamido-3,5-dideoxy-2-propyl-4,7,8,9-tetra-acetyl-α-D-glycero-D-galacto-nonulopyranosidonate (7). Compound **4** (1.00 g, 3.09 mmol) was stirred with AcCl (10 mL) at room temperature in a pressure tube for 24 h. The reaction mixture was then concentrated and the residue was coevaporated with dry toluene (3×5 mL).

The residue was then dissolved in anhydrous *n*-propanol (30 mL), under Ar, and silver salicylate (1.14 g, 4.64 mmol) was added to the stirred reaction mixture. The suspension was stirred at room temperature in the dark for 30 min. The precipitate was filtered off on Celite® and washed with $CHCl_3$ (~20 mL). The combined filtrate and washings were then concentrated on the rotovap. A solution of the residue in $CHCl_3$ (125 mL) was washed with ice-cold 5% $NaHCO_3$ (2×75 mL), 5% $Na_2S_2O_3$ (75 mL) and water (2×75 mL), dried ($MgSO_4$), filtered, and evaporated. The residue was then flash chromatographed with a mixture of hexanes and AcOEt (1:2) to give **7** (1.18 g, 72%) as a white solid: (TLC R_f = 0.16, hexanes/AcOEt, 1/2), ^{1}H NMR ($CDCl_3$) δ 5.37 (1H, ddd, J = 8.2, 5.6 and 2.7 Hz), 5.30 (1H, dd, J = 8.2 and 1.9 Hz), 5.12 (1H, bd, J = 9.5 Hz), 4.81 (1H, ddd, J = 12.4, 9.9 and 4.6 Hz), 4.29 (1H, dd, J = 12.5 and 2.7 Hz), 4.10–4.01 (2H, m), 3.77 (3H, s), 3.70 (1H, dt, J = 9.3 and 6.4 Hz), 3.15 (1H, dt, J = 9.3 and 6.7 Hz), 2.56 (1H, dd, J = 12.8 and 4.6 Hz), 2.12 (3H, s), 2.11 (3H, s), 2.02 (3H, s), 2.00 (3H, s), 1.93 (1H, t, J = 12.6 Hz), 1.86 (3H, s), 1.54 (2H, m), 0.88 (3H, t, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 171.04, 170.67, 170.18, 170.15, 170.04, 168.54, 98.72, 72.41, 69.14, 68.65, 67.34, 66.62, 62.34, 52.62, 49.44, 38.06, 23.20, 22.85, 21.10, 20.86, 20.83, 20.77, 10.35.

Methyl 5-acetamido-3,5-dideoxy-2-propyl-α-D-glycero-D-galactononulo-pyranosidonate (8). Compound **7** was dissolved in anhydrous MeOH (10 mL) and NaOMe (50 μL of 25% wt. in MeOH, 0.22 mmol) was added under Ar and the resulting mixture was stirred for 19 h at room temperature. The reaction mixture was then neutralized with Dowex® 50W-X4 (H^+, prewashed with MeOH) and stirring continued for 20 min. Filtration over a small bed of the same resin and concentration gave **8** (597 mg, 75%) as a white solid: (TLC R_f = 0.36, CH_2Cl_2/MeOH, 5/1), ^{1}H NMR (CD_3OD) δ 3.85–3.81 (2H, m), 3.83 (3H, s), 3.77–3.71 (2H, m), 3.65–3.58 (2H, m), 3.54 (1H, dd, J = 10.4 and 1.8 Hz), 3.50 (1H, dd, J = 8.7 and 1.7 Hz), 3.22–3.27 (1H, m), 2.67 (1H, dd, J = 12.8 and 4.7 Hz), 1.99 (3H, s), 1.72 (1H, dd, J = 12.7 and 12.0 Hz), 1.53 (2H, m), 0.89 (3H, t, J = 7.4 Hz); ^{13}C NMR (CD_3OD) δ 175.25, 171.22, 100.16, 74.86, 72.54, 70.15, 68.53, 66.85, 64.64, 53.84, 53.35, 41.75, 23.95, 22.66, 10.78.

5-Acetamido-3,5-dideoxy-9-methylphosphono-2-propyl-α-D-glycero-D-galactononulo-pyranosidonic acid triethylammonium salt (2). Compound **8** (209 mg, 0.572 mmol) was phosphorylated as described above for the synthesis of **6** and purification by flash chromatography with a mixture of $CHCl_3$ and MeOH (10/1 to 3/1), containing Et_3N (1%), gave recovered starting material (61 mg, 29%) and an unseparable mixture of the C-8 and C-9 phosphorylated species (210 mg, 67%). Saponification of the mixture (92 mg, 0.17 mmol) gave a quantitative yield of the two products and purification of 20 mg by preparative TLC using a mixture of AcOEt and MeOH (1:2, containing 1% Et_3N) gave 5-acetamido-3,5-dideoxy-8-methylphosphono-2-propyl-α-D-*glycero*-D-*galacto*-nonulopyranosidonic acid triethylammonium salt (5 mg, 25%) and **2** (10 mg, 50%) as a white solid: (TLC R_f = 0.28, AcOEt/MeOH, 1/2 containing 1% Et_3N), ^{1}H NMR (CD_3OD) δ 4.11 (1H, dd, J = 8.7 and 5.5 Hz), 3.98–3.91 (2H, m), 3.73–3.55 (5H, m), 3.40 (1H, dt, J = 9.2 and 6.9 Hz), 3.13 (6H, q, J = 7.3 Hz), 2.81 (1H, dd, J = 12.3 and 4.3 Hz), 1.99 (3H, s), 1.59–1.49 (3H, m), 1.27 (9H, t, J = 7.3 Hz), 1.26 (3H, d, J = 16.0 Hz), 0.88 (3H, t, J = 7.5 Hz); ^{13}C NMR (CD_3OD) δ 175.25, 174.59, 101.86, 74.10, 72.25 (1C, d, J = 7.0 Hz), 69.88, 66.84, 66.62 (1C, d, J = 3.8 Hz), 60.14, 54.18, 47.50, 42.82, 24.19, 22.65, 12.21 (1C, d, J = 136.7 Hz), 10.90, 9.33; ^{31}P NMR (CD_3OD) δ 26.26. HRMS calcd for $C_{15}H_{28}NNaO_{11}P$ ($M + Na^+$): 452.1298, found: 452.1313.

Determination of inhibition of influenza C haemagglutinin binding. The activity of 9-*O*-acetylsialic acid esterase was measured by the hydrolysis of *p*-nitrophenyl acetate. The reactions were performed in 0.5 mL of sodium phosphate buffer (pH 7.4, 100 mM) containing sodium chloride (50 mM) and the bromelain-released glycoprotein of influenza C virus.[44] The reactions were started by adding the *p*-nitrophenyl acetate from 25 or 100 mM stock solutions in acetonitrile followed by sample inversion. The change in absorption was detected at 400 nm. Activity was measured at substrate concentrations of 0.125, 0.25, 0.5, 1.0 and 2.0 mM. The data obtained for a given concentration of substrate was corrected for background hydrolysis of the substrate in buffer. Using these conditions, the IC_{50} of both **1** and **9** were determined to be > 2 mM. Due to the weakness of their inhibition no detailed kinetic analysis was performed.

Determination of inhibition of CMP-sialic acid synthase by compound 1. A 50 mM Tris buffer with 5.5 mM $MgCl_2$ and 6 mM $MnCl_2$ at pH 9.0. Five reactions were set up. All reactions had 5.5 mM sialic acid and 10 mM CTP. The five reactions had the following concentrations of **1**: 100 mM, 10 mM, 1 mM, 0.1 mM, and 10 nM. Reactions were started by the addition of 0.4 U of CMP-sialic acid synthase and allowed to proceed for 30 min at 37 °C. Reactions were the cooled to room temperature and 50 μL of 2 M $NaBH_3$ were added. After 15 min. at room temperature the reactions were cooled to 0 °C and 50 μL of H_3PO_4 was added and the reactions were keep an additional 5 min at 0 °C. The reactions were then warmed to 37 °C for 10 min. After cooling back to room temperature, 50 μL of 1 M $NaIO_4$ was added and the reaction were keep at room temperature for 10 min. 400 μL of 2 M $NaAsO_2$/2 N HCl was added and the tubes were agitated until the brown

color disappeared. The entire content of the tube was then transfered to a tube containing 1 mL of 4% thiobarbituric acid/0.5 M $NaSO_4$. This solution was heated at 100 °C for 15 min and then cooled to room temperature. The resulting solution was extracted 1:1 with cyclohexanone. The red upper layer was analyzed at 549 nm.

Methyl 5-acetamido-3,5-dideoxy-β-D-glycero-D-galacto-nonulopyranosonate (10). *N*-Acetylneuraminic acid **4** (3.09 g, 10 mmol) was added to a mixture of dry Dowex® 50W-X4 (H^+, 1 g) and anhydrous MeOH (300 mL) while stirring. The resulting mixture was then stirred at 20 °C for 39 h. The resin was then filtered over Celite® and the resulting solution concentrated on the rotovap. The residue was then flash chromatographed through a short silica gel column with a mixture of CH_2Cl_2 and MeOH (3:1) to give **10** (2.88 g, 89%) as a white solid: (TLC R_f = 0.27, CH_2Cl_2/MeOH, 3/1), ^{1}H NMR (CD_3OD) δ 4.03 (1H, ddd, J = 11.2, 10.1 and 4.9 Hz), 3.99 (1H, dd, J = 10.5 and 1.5 Hz), 3.82–3.77 (2H, m), 3.77 (3H, s), 3.69 (1H, ddd, J = 9.1, 5.7, and 2.8 Hz), 3.61 (1H, dd, J = 11.2 and 5.0 Hz), 3.47 (1H, dd, J = 9.2 and 1.4 Hz), 2.21 (1H, dd, J = 12.9 and 4.9 Hz), 2.01 (3H, s), 1.88 (1H, dd, J = 12.9 and 11.4 Hz); ^{13}C NMR (CD_3OD) δ 175.09, 171.76, 96.64, 72.03, 71.60, 70.13, 67.82, 64.79, 54.27, 53.17, 40.65, 22.69.

Methyl-5-acetamido-3,5-dideoxy-9-*O*-(*p*-toluenesulfonyl)-β-D-glycero-D-galacto-nonulo-pyranosonate (11). To a solution of **10** at 0 °C, *p*-toluenesulfonyl chloride (0.744 g, 3.90 mmol) was added in portions to a solution of methyl 5-acetamido-3,5-dideoxy-β-D-glycero-D-galacto-nonulopyranosonate (0.970 g, 3.00 μmol) in anhydrous pyridine (11 mL) over 1 h. The reaction mixture was then stirred at 0 °C for 12 h. The solvent was evaporated and the residue purified by flash chromatography on silica gel by using a mixture of CH_2Cl_2 and MeOH (10/1) to give **11** (1.01 g, 71%) as a white solid: (TLC R_f = 0.23, CH_2Cl_2/MeOH, 10/1); ^{1}H NMR (CD_3OD) δ 7.78 (2H, d, J = 8.3 Hz), 7.43 (2H, d, J = 8.2 Hz), 4.26 (1H, dd, J = 10.0 and 2.2 Hz), 4.03 (1H, dd, J = 10.0 and 6.1 Hz), 4.00 (1H, ddd, J = 11.3, 10.1 and 4.9 Hz), 3.92 (1H, dd, J = 10.5 and 1.4 Hz), 3.84 (1H, ddd, J = 9.1, 6.0, and 2.2 Hz), 3.75 (3H, s), 3.73 (1H, dd, J = 10.7 and 10.3 Hz), 3.42 (1H, dd, J = 9.1 and 1.3 Hz), 2.45 (3H, s), 2.19 (1H, dd, J = 12.9 and 4.9 Hz), 2.00 (3H, s), 1.85 (1H, dd, J = 12.9 and 11.5 Hz); ^{13}C NMR (CD_3OD) δ 175.17, 171.62, 146.40, 134.11, 131.03 (2C), 129.07 (2C), 96.57, 73.73, 71.75, 69.90, 69.20, 67.64, 54.21, 53.21, 40.65, 22.67, 21.59.

Methyl 5-acetamido-9-thioacetyl-3,5,9-trideoxy-β-D-glycero-D-galacto-nonulopyranosonate (12a) and methyl-5-acetamido-8-acetyl-9-thioacetyl-3,5,9-trideoxy-β-D-glycero-D-galacto-nonulopyranosonate (12b). A mixture of tosylate **11** (478 mg, 1.00 mmol) and potassium thioacetate (571 mg, 5.00 mmol) in anhydrous DMF (5 mL) was stirred at 20 °C for 16 h under Ar. The solvent was then evaporated and the residue flash chromatographed on silica gel with a mixture of CH_2Cl_2 and MeOH (10:1) to give a mixture of (298 mg, 2/1, 75%) as a yellowish amorphous solid: **12a**: (TLC R_f = 0.36, CH_2Cl_2/MeOH, 5/1), ^{1}H NMR (CD_3OD) δ 4.01 (1H, ddd, J = 11.3, 10.1 and 4.9 Hz), 3.99 (1H, dd, J = 10.5 and 1.4 Hz), 3.85–3.71 (2H, m), 3.77 (3H, s), 3.50 (1H, dd, J = 13.8 and 3.0 Hz), 3.35 (1H, dd, J = 8.4 and 1.4 Hz), 2.93 (1H, dd, J = 13.8 and 8.3 Hz), 2.32 (3H, s), 2.19 (1H, dd, J = 12.9 and 4.9 Hz), 2.01 (3H, s), 1.88 (1H, dd, J = 13.0 and 11.5 Hz); ^{13}C NMR (CD_3OD) δ 197.58, 172.11, 171.70, 96.59, 72.78, 71.85, 70.34, 67.76, 54.26, 53.23, 40.59, 34.90, 30.47, 22.70. **12b**: (TLC R_f = 0.46, CH_2Cl_2/MeOH, 5/1); ^{1}H NMR (CD_3OD) δ 5.02 (1H, ddd, J = 8.0, 5.7, and 3.1 Hz), 4.01–3.96 (1H, m), 3.85–3.72 (2H, m), 3.77 (3H, s), 3.68 (1H, bd, J = 7.9), 3.64 (1H, dd, J = 14.5 and 3.2 Hz), 3.09 (1H, dd, J = 14.5 and 5.7 Hz), 2.30 (3H, s), 2.18 (1H, dd, J = 12.8 and 4.9 Hz), 2.01 (3H, s), 1.97 (3H, s), 1.83 (1H, dd, J = 12.8 and 11.5 Hz); ^{13}C NMR (CD_3OD) δ 196.93, 174.87, 171.94, 171.62, 96.38, 72.31, 71.99, 69.84, 67.76, 54.20, 53.27, 41.04, 30.72, 30.36, 22.71, 21.01.

Methyl 5-acetamido-9-thiomethylmercuric-3,5,9-trideoxy-β-D-glycero-D-galacto-nonulopyranosonate (13). A solution of NaOMe in MeOH (32 μL of 25%, 0.139 mmol) was added to a solution of **12a,b** (53.0 mg, 0.139 mmol) in anhydrous MeOH (1 mL) at 20 °C under Ar. The resulting mixture was then stirred for 3 h and methyl mercury (II) chloride (34.9 mg, 0.139 mmol) was then added. After stirring for another 3 h, the solvent was evaporated and the residue was chromatographed on silica gel with a mixture of CH_2Cl_2 and MeOH (10/1) to give **13** (30 mg, 39%) as a white volatile solid: (TLC R_f = 0.17, CH_2Cl_2/MeOH, 10/1); ^{1}H NMR (CD_3OD) δ 4.02 (1H, ddd, J = 11.3, 10.1 and 5.0 Hz), 4.00 (1H, dd, J = 10.5 and 1.5 Hz), 3.79 (1H, dd, J = 10.2 and 10.0 Hz), 3.77 (3H, s), 3.66 (1H, ddd, J = 9.0, 7.6, and 2.6 Hz), 3.49 (1H, dd, J = 9.0 and 1.4 Hz), 3.36 (1H, dd, J = 13.6 and 2.6 Hz), 2.92 (1H, dd, J = 13.6 and 7.6 Hz), 2.20 (1H, dd, J = 12.9 and 4.9 Hz), 2.01 (3H, s), 1.90 (1H, dd, J = 12.9 and 11.4 Hz); ^{13}C NMR (CD_3OD) δ 175.06, 171.70, 96.70, 72.56, 72.27, 72.09, 67.83, 54.46, 53.17, 40.61, 32.21, 22.66, 9.63.

5-Acetamido-9-thiomethylmercuric-3,5,9-trideoxy-β-D-glycero-D-galacto-nonulopyranosidonic acid (3). To a solution of the methyl ester **13** (28.0 mg, 50.0 μmol) in pure water (2 mL) was added an aqueous NaOH solution (1%), gradually, until the pH remained >8. The mixture was then stirred at 20 °C for 8 h. Neutralization with Dowex® 50W-X4 (H^+), filtration and lyophilization gave **3** (26.0 mg, 95%) as a white volatile solid: ^{1}H NMR

(D_2O) δ 3.86 (1H, ddd, J=11.5, 10.0 and 4.8 Hz), 3.86 (1H, dd, J=10.4 and 1.0 Hz), 3.73 (1H, t, J=10.2 Hz), 3.53 (1H, m), 3.37 (1H, dd, J=9.1 and 0.8 Hz), 3.18 (1H, m), 2.75 (1H, m), 2.11 (1H, dd, J=13.0 and 4.8 Hz), 1.86 (3H, s), 1.68 (1H, dd, J=13.0 and 11.6 Hz); ^{13}C NMR (D_2O) δ 174.98, 174.65, 95.81, 71.43, 70.40, 70.24, 66.96, 52.22, 39.02, 30.43, 22.05, 8.74.

CMP-(5-Acetamido-9-thiomethylmercuric-3,5,9-trideoxy-β-D-glycero-D-galacto-nonulo-pyranosidonic acid) (14). Compound **3** (30 mg, 55.6 μmol) and CTP (34.5 mg, 61.2 μmol) were dissolved in 50 mM Tris pH 9 buffer (5 mL) containing 50 mM $MgCl_2$ (51.0 mg, 0.250 mmol). The reaction was then initiated by the addition of CMP-neuraminic acid synthase (25 μL, 1.125 U). The pH of the reaction was maintained at 9 by manual addition of 1 N NaOH. After 4 h, TLC showed complete reaction and alkaline phosphatase (3.8 mg, 10 U) was added, along with more $MgCl_2$ (51.0 mg, 0.250 mmol). The reaction mixture was then stirred for 3 h, filtered over Celite® and the cake washed with water (5 mL). The filtrate was then lyophilized and the resulting white solid was dissolved in a minimum of water and applied to an ion exchange resin Dowex® 1-X8 (HCO_2-) 200–400 mesh column (18×150 mm) and eluted with 50 mM NH_4HCO_3 (100 mL) followed by a gradient of 50 mM to 1M (200 mL) at 5 °C. The progress of the column was monitored by UV at 273 nm and the product containing fractions were combined and lyophilized to afford **14** as a white volatile solid (21 mg, 45%): (TLC R_f=0.16, n-BuOH/AcOH/H_2O, 5/3/2), ^{1}H NMR (D_2O) δ 7.76 (1H, d, J=7.6 Hz), 5.92 (1H, d, J=7.6 Hz), 5.77 (1H, d, J=4.5 Hz), 4.14–4.02 (5H, m), 3.95 (1H, bd, J=10.5 Hz), 3.85 (1H, ddd, J=11.0, 10.5 and 4.6 Hz), 3.74 (1H, t, J=10.3 Hz), 3.65 (1H, bt, J=7.8 Hz), 3.26 (1H, bd, J=9.3 Hz), 3.18 (1H, bd, J=13.4 Hz), 2.68 (1H, dd, J=13.4 and 8.1 Hz), 2.27 (1H, dd, J=13.3 and 4.7 Hz), 1.84 (3H, s), 1.44 (1H, ddd, J=13.3, 11.5 and 5.8 Hz), 0.49 (3H, s); ^{13}C NMR (D_2O) δ 174.57, 166.08, 160.27, 157.76, 141.62, 100.02, 96.63, 88.99, 82.96 (d, J=7.5 Hz), 74.30, 71.98, 70.95 (d, J=4.5 Hz), 70.29, 69.38, 66.77, 65.06 (d, J=5.4 Hz), 51.82, 40.99 (d, J=10.0 Hz), 29.58, 22.03, 8.57. MS (EI^-) calcd for $C_{21}H_{32}HgN_4O_{15}PS$ ($M-H^+$) 845, found 845.

9-MeHgS-NeuAc-α-2,3-LacNAc-O(CH_2)$_5$$CO_2$Me (15). A solution of compound **14** (12.7 mg, 22.6 μmol), LacNAc-O(CH_2)$_5$$CO_2$Me (10.0 mg, 19.6 mmol) and CTP (10.9 mg, 19.6 mmol) in 100 mM HEPES pH 7.5 buffer (1 mL) containing 25 mM $MgCl_2$, 6 mM $MnCl_2$ and 25 mM KCl was adjusted to pH 7.5 with 1 N NaOH. The enzymes CMP-NeuAc synthase (4 μL of 45 U/mL, 0.18 U), α-2,3-sialyltransferase (40 μL of 6 U/mL, 0.24 U) and inorganic pyrophosphatase (40 μL, 4 U) were then added and the mixture was shaken under Ar at 37 °C for 1 day. More CTP (10.9 mg, 19.6 μmol) was then added and the reaction shaken for an additional day. Concentration and flash chromatographed with a mixture of AcOEt/MeOH, and 0.02% $CaCl_2$ (5/2/1) gave the desired **15** (20 mg, 99%): (TLC R_f=0.33, AcOEt/MeOH/0.02% aq $CaCl_2$, 5/2/1); ^{1}H NMR (CD_3OD) δ 4.45 (1H, d, J=7.8 Hz), 4.37 (1H, d, J=8.4 Hz), 4.03 (1H, dd, J=9.7 and 3.1 Hz), 3.95–3.83 (6H, m), 3.77–3.35 (16H, m), 3.64 (3H, s), 2.96 (1H, dd, J=13.3 and 6.8 Hz), 2.84 (1H, dd, J=12.1 and 3.5 Hz), 2.31 (2H, t, J=7.5 Hz), 2.00 (3H, s), 1.96 (3H, s), 1.72 (1H, t, J=11.9 Hz), 1.60 (2H, qt, J=7.6 Hz), 1.57–1.53 (2H, m), 1.40–1.34 (2H, m), 0.66 (3H, s); ^{13}C NMR (CD_3OD) δ 175.90, 175.44, 175.10, 173.47, 104.93, 102.79, 101.15, 81.09, 77.64, 77.09, 76.52, 75.11, 74.26, 73.36, 71.98, 70.78, 70.34, 69.42, 69.17, 69.09, 64.41, 62.77, 62.07, 56.62, 54.03, 51.92, 42.00, 34.79, 31.55, 30.25, 26.63, 25.72, 8.62; MS (EI^-) calcd for $C_{33}H_{55}HgN_2O_{20}S$ ($M-H^+$) 1033, found 1033.

Synthesis of 9-MeHgS-NeuAc-α-2,3-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-O(CH_2)$_5$$CO_2$Me (16). To a solution of 9-MeHgS-NeuAc-α-2,3-LacNAc-O(CH_2)$_5$$CO_2$Me (**15**) (3.0 mg, 2.9 mmol) in 100 mM MES buffer (500 mL) containing 15 mM $MnCl_2$ was added GDP-Fuc (2.2 mg, 2.9 μmol) and α-1,3-fucosyltransferase V (20 μL of 2.16 U/mL, 0.043 U). The resulting mixture was shaken at room temperature for 2 days under Ar. Alkaline phosphatase (1 μL of 1 U/mL, 1 U) and another portion of GDP-Fuc (2.2 mg, 2.9 μmol) and α-1,3-fucosyltransferase V (20 μL of 2.16 U/mL, 0.043 U) were then added and the mixture was shaken at 37 °C for 3 days. The reaction mixture was then evaporated and flash chromatographed with a mixture of AcOEt, MeOH, and 0.02% $CaCl_2$ (5/2/1). The fractions containing the product were then purified by size exclusion chromatography on biogel P-2 column (1.5×70 cm) and lyophilization of the desired fractions gave the desired **16** (2.0 mg, 58%): (TLC R_f=0.23, AcOEt/MeOH/0.02% aq $CaCl_2$, 5/2/1); ^{1}H NMR (D_2O) δ 4.92 (1H, d, J=4.0 Hz), 4.34 (2H, d, J=8.0 Hz), 3.89 (1H, dd, J=9.8 and 3.1 Hz), 3.84 (1H, d, J=10.2 Hz), 3.78–3.65 (9H, m), 3.60 (1H, d, J=2.8 Hz), 3.52–3.33 (10H, m), 3.51 (3H, s), 3.21 (1H, d, J=11.5 Hz), 2.75 (1H, dd, J=13.8 and 8.3 Hz), 2.59 (1H, dd, J=12.4 and 4.6 Hz), 2.21 (2H, t, J=7.5 Hz), 1.85 (3H, s), 1.84 (3H, s), 1.60 (1H, t, J=12.2 Hz), 1.43 (2H, qt, J=7.6 Hz), 1.40–1.36 (2H, m), 1.17–1.14 (2H, m), 0.99 (3H, d, J=6.6 Hz), 0.57 (3H, s); MS (EI^-) calcd for $C_{39}H_{65}HgN_2O_{24}S$ ($M-H^+$) 1179, found 1179.

Synthesis of 9-MeHgS-NeuAc-α-2,3-LacNAc-β-RNase (17). The procedure used was the same as for the synthesis of 9-MeHgS-Neu5Ac-α-2,3-LacNAc-O(CH_2)$_5$$CO_2$Me (**3**), but using LacNAc-RNase (5.2 mg, 0.37 mmol) as the substrate. Purification[43] gave the desired **17** (3.5 mg, 65%): MS (MALDI) calcd 14571; found 14572.

Synthesis of (9-MeHgS-NeuAc-α-2,3Gal-β-1,4GlcNAc-β-1,3Man-β-1,6)-(9-MeHgS-NeuAc-α-2,3Gal-β-1,4GlcNAc-β-1,3Man-β-1,3)Man-β-1,4GlcNAc-β-1,4GlcNAc (5). The reaction buffer consisting of 50 mM HEPES, 25 mM $MgCl_2$, 6 mM$MnCl_2$ and 25 mM KCl (pH 7.2) was used in making the following stock solutions: 55 mM CTP (25.9 mg/mL), 25 mM compound **3** (13.6 mg/mL) and 25 mM of the acceptor (Gal-β-1,4GlcNAc-β-1,3Man-β-1,6)-(2,3Gal-β-1,4GlcNAc-β-1,3Man-β-1,3)Man-β-1,4-GlcNAc-β-1,4GlcNAc **18a** (41 mg/mL), prepared from **18** as described previously.[44] To start the reaction 250 μL of the stock of **3** (6.25 μmol) and 250 μL of the CTP solution (13.8 μmol) were combined with 250 μL of the stock of **18a** (6.25 μmol) and an additional 168 μL of reaction buffer. Added to this was 20 μL of CMP-sialic acid synthase (20 U/mL), 60 μL of α2,3-sialyltransferase (2.3 U/mL) and 2 μL of alkaline phosphatase (10 U/mL) to give a total reaction volume of 1 m/L. The reaction was shaken at 37 °C for 1 day. A second aliquot of CTP, CMP-sialic acid synthase, sialyltransferase and alkaline phosphatase were added and the reaction shaken at 37 °C for a second day. The product **19** was isolated by size exclusion chromatography using Biogel P2 and lyophilized to dryness to give 14 mg of **19**, 84% yield. Mass analysis was consistent with the desired structure.

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