

## Supporting Information

### Determinants of undesired $\alpha$ 2-6-sialoside formation by PmST1 M144D

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**General Information:** All reagents and solvents were purchased from Sigma-Aldrich, Canada, and were used as received.  $\text{CDCl}_3$  (chloroform D) and  $\text{D}_2\text{O}$  (deuterium oxide) were purchased from Deutero GmbH. Reactions were carried out in Eppendorf Flex-tubes. All reagents were purchased from commercial sources and were used without further. All reactions were carried out at 37 °C in shaking incubator and were monitored by TLC on Silica Gel 60 F<sub>254</sub> (0.25 mm, E. Merck). Spots were detected under UV light ( $\lambda$  max = 254 nm) or by charring with 10%  $\text{H}_2\text{SO}_4$  in EtOH. All column chromatography was performed on LH20. NMR experiments were conducted on a Varian 500 MHz instrument in the Chemistry NMR Facility, University of Alberta. For  $^1\text{H}$  spectra, chemical shifts are expressed as parts per million (ppm,  $\delta$ ) and are relative to the solvent used.  $^1\text{H}$  chemical shifts are reported relative to the deuterated solvent peak to HOD (4.78,  $\text{D}_2\text{O}$ ).  $^1\text{H}$  data were reported as though they were first order. Coupling constants (J) are reported in Hz and apparent multiplicities were described in standard abbreviations as singlet (s), doublet (d), doublet of doublets (dd), doublet of doublet of doublets (ddd), triplet (t), broad singlet (bs) and multiplet (m).

**CMP-Neu5Ac (5-acetamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid)**

Neu5Ac was purchased from Sigma Aldrich, and CMP-Neu5Ac was synthesized according to literature procedure (1).

### **Procedure for synthesis of $\alpha$ 2-3 sialoside compounds 1-4**

Acceptors (1 eq, 15 mmol) (Gal- $\beta$ (1 $\rightarrow$ 4)-6-O-sulfo- GlcNAc- $\beta$ -ethyl-NHCbz, LacNAc- $\beta$ -ethyl-NHCbz, LacNAc- $\beta$ -ethyl-N<sub>3</sub>, and LacNAc) and CMP-Neu5Ac (1.5 eq) were dissolved in Tris-HCl buffer (pH 8.5) containing 20 mM MgCl<sub>2</sub>. Recombinant shrimp alkaline phosphatase (rSAP, NEB; 3  $\mu$ L) and CST-06 (0.1 mg/mL) were added to the reaction. The reaction tube was incubated overnight at 37 °C. Reaction progress was monitored by thin-layer chromatography (TLC) using a solvent system of iPrOH:NH<sub>4</sub>OH:H<sub>2</sub>O (7:2:1, v/v/v). Upon completion, reaction was quenched by a 4-fold dilution with cold absolute ethanol and incubated at -20 °C for 1 hour to precipitate the enzymes. The precipitated proteins were removed by centrifugation (3700  $\times$  g, 15 minutes, 4 °C). The resulting supernatant was carefully collected and evaporated under reduced pressure. The crude residues were resuspended in water and purified by size-exclusion chromatography on an LH20 column equilibrated with MeOH:H<sub>2</sub>O (1:1), producing  $\alpha$ 2-3 sialoside compounds **1-4**.

### **Procedure for synthesis of $\alpha$ 2-6 sialoside compounds 5-8 (2)**

Acceptors (1 eq, 15 mmol) (Gal- $\beta$ (1 $\rightarrow$ 4)-6-O-sulfo- GlcNAc- $\beta$ -ethyl-NHCbz, LacNAc- $\beta$ -ethyl-NHCbz, LacNAc- $\beta$ -ethyl-N<sub>3</sub>, and LacNAc) and CMP-Neu5Ac (1.5 eq) were dissolved in Tris-HCl buffer (pH 8.5) containing 20 mM MgCl<sub>2</sub>. Pd2,6ST (0.1 mg/mL) were added to the reaction. The reaction tube was incubated overnight at 37 °C and the reaction progress was monitored by thin-layer chromatography (TLC) using a solvent system of iPrOH:NH<sub>4</sub>OH:H<sub>2</sub>O (7:2:1, v/v/v). Upon completion, reaction was quenched by a 4-fold dilution with cold absolute ethanol and incubated at -20 °C for 1 hour to precipitate the enzymes. The precipitated proteins were removed by centrifugation (3700  $\times$  g, 15 minutes, 4 °C). The resulting supernatant was collected and evaporated under reduced pressure. The crude residues were resuspended in water and

purified by size-exclusion chromatography on an LH20 column equilibrated with MeOH:H<sub>2</sub>O (1:1), producing  $\alpha$ 2-6 sialoside compounds **5-8**.

## References:

1. Yu H, Yu H, Karpel R, Chen X. Chemoenzymatic synthesis of CMP-sialic acid derivatives by a one-pot two-enzyme system: comparison of substrate flexibility of three microbial CMP-sialic acid synthetases. *Bioorg Med Chem*. 2004;12(24):6427–35.
2. Yu H, Chokhawala HA, Huang S, Chen X. One-pot three-enzyme chemoenzymatic approach to the synthesis of sialosides containing natural and non-natural functionalities. *Nature Protocols*. 2007;1(5):2485–92.

## Characterization Data

**2-(Benzyloxycarbonylaminoethyl)-5-acetamido-3,5-dideoxy-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosylonate-(2 $\rightarrow$ 3)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)-2-N-acetyl-2-deoxy-(6-O-sulfo)- $\beta$ -D-glucopyranoside (1) (Neu5Ac- $\alpha$ 2-3-Gal-6-O-sulfo-GlcNAc- $\beta$ -Ethyl-NHCbz):** white solid; yield 72%. <sup>1</sup>H-NMR (500 MHz, D<sub>2</sub>O)  $\delta$  = 7.36-7.48 (m, 5H), 5.16 (m, 2H), 4.59 (d, *J* = 8.0 Hz, 1H), 4.52 (d, *J* = 8.0 Hz, 1H), 4.39 (d, *J* = 11.0 Hz, 1H), 4.30 (d, *J* = 9.5 Hz, 1H), 4.11 (dd, *J* = 3, 10 Hz, 1H), 3.82-3.84 (m, 5H), 3.52-3.78 (m, 14H), 3.31 (bs, 2H), 2.75 (dd, *J* = 4.5, 12.5 Hz, 1H), 2.20 (s, 3H), 2.18 (s, 3H), 1.80 (t, *J* = 12 Hz, 1H).

**2-(Benzyloxycarbonylaminoethyl)-5-acetamido-3,5-dideoxy-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosylonate-(2-3)- $\beta$ -Dgalactopyranosyl-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside (2) (Neu5Ac- $\alpha$ 2-3-LacNAc- $\beta$ -Ethyl-NH-Cbz):** white solid; yield 68%. <sup>1</sup>H-NMR (500 MHz, D<sub>2</sub>O)  $\delta$  = 7.36-7.42 (m, 5H), 5.32 (m, 2), 4.53 (d, *J* = 5 Hz, 1H), 4.50 (d, *J* = 7.7, 1H), 4.12 (dd, *J* = 9.1, 13.3 Hz, 1H), 3.81-3.94 (m, 5H), 3.60-3.72 (m, 11H), 3.52-3.62 (m, 3H), 3.26–3.36 (bs, 2H), 2.75 (dd, *J* = 4.9, 11.9 Hz, 1H), 2.03 (s, 3H), 1.96 (s, 3H), 1.80 (t, *J* = 11.9 Hz, 1H).

**2-Azidoethylyl 5-acetamido-3,5-dideoxy-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosylonate-(2-3)- $\beta$ -Dgalactopyranosyl-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside (3) (Neu5Ac- $\alpha$ 2-3-LacNAc- $\beta$ -Ethyl-N<sub>3</sub>):** white solid; yield 52%. <sup>1</sup>H-NMR (500 MHz, D<sub>2</sub>O)  $\delta$  = 4.41 (dd, *J* = 8,

15 Hz, 1H), 4.10 (d,  $J = 7.5$ , 1H), 3.81-4.05 (m, 4H), 3.81-3.92 (m, 5H), 3.66-3.79 (m, 7H), 3.54-3.68 (m, 8H), 2.75 (dd,  $J = 4.5$ , 12 Hz, 1H), 2.03 (s, 6H), 1.78 (t,  $J = 12$  Hz, 1H).

***N*-acetylneuraminosyl $\alpha$ 2-3-*O*-D-galactopyranosyl $\beta$ (1-4)-2-(acetylamino)-2-deoxy- $\beta$ -D-glucopyranose (4) (Neu5Ac- $\alpha$ 2-3LacNAc):** white solid; yield 79%.  $^1\text{H-NMR}$  (500 MHz  $\text{D}_2\text{O}$ )  $\delta =$  4.52 (d,  $J = 7.5$  Hz, 1H), 4.12 (d,  $J = 7.5$ , 1H), 3.81-4.05 (m, 9H), 3.52-3.78 (m, 11H), 2.75 (dd,  $J = 3.5$ , 12.5 Hz, 1H), 2.04 (s, 6H), 1.78 (t,  $J = 12.5$  Hz, 1H).

**2-(Benzyloxycarbonylaminoethyl)-5-acetamido-3,5-dideoxy-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosylonate-(2 $\rightarrow$ 6)- $\beta$ -D-galactopyranosyl-(1 $\rightarrow$ 4)-2-*N*-acetyl-2-deoxy-(6-*O*-sulfo)- $\beta$ -D-glucopyranoside (5) (Neu5Ac- $\alpha$ 2-6-Gal- $\beta$ (1 $\rightarrow$ 4)-6-*O*-sulfo-GlcNAc- $\beta$ -Ethyl-NHCbz):** white solid; yield 73%.  $^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ )  $\delta =$  7.38-7.48 (m, 5H), 5.36 (m, 2H), 4.55 (d,  $J = 8.0$  Hz, 1H), 4.46 (d,  $J = 8.0$  Hz, 1H), 4.40 (d,  $J = 11.0$  Hz, 1H), 4.25 (dd,  $J = 5$ , 11.0 Hz, 1H), 3.98 (t,  $J = 9.5$  Hz, 1H), 3.85-3.94 (m, 4H), 3.75-3.84 (m, 3H), 3.61-3.75 (m, 9H), 3.49-3.58 (m, 3H), 3.31 (bs, 2H), 2.65 (dd,  $J = 5.0$ , 12.5 Hz, 1H), 2.10 (s, 3H), 1.98 (s, 3H), 1.70 (t,  $J = 12$  Hz, 1H).

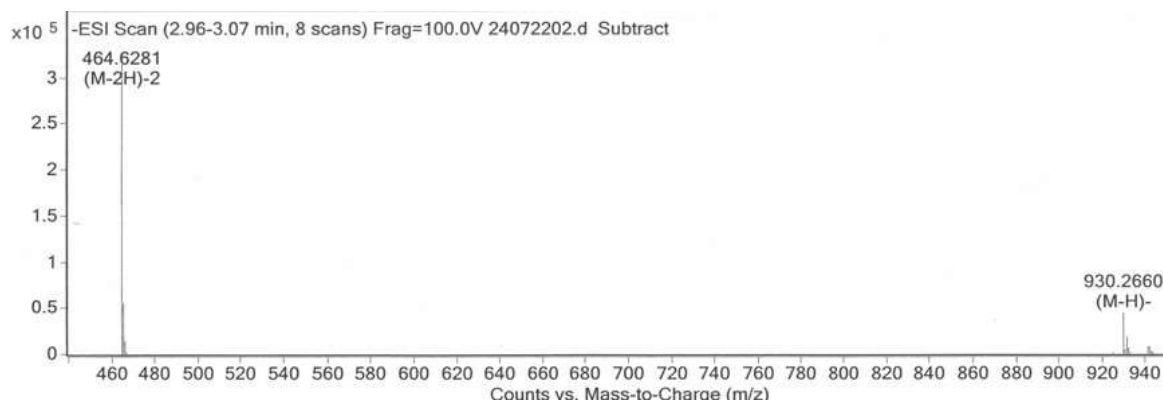
**2-(Benzyloxycarbonylaminoethyl)-5-acetamido-3,5-dideoxy-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosylonate-(2-6)- $\beta$ -Dgalactopyranosyl-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside (6) (Neu5Ac- $\alpha$ 2-6-LacNAc- $\beta$ -Ethyl-NHCbz):** white solid; yield 59%.  $^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ )  $\delta =$  7.38-7.46 (m, 5H), 5.31 (m, 2), 4.53 (d,  $J = 5$  Hz, 1H), 3.84-3.98 (m, 5H), 3.75-3.81 (m, 3H), 3.56-3.73 (m, 10H), 3.51-3.54 (m, 3H), 3.30 (bs, 2H), 2.65 (dd,  $J = 4.9$ , 11.9 Hz, 1H), 2.05 (s, 3H), 1.98 (s, 3H), 1.69 (t,  $J = 11.9$  Hz, 1H).

**2-Azidoethyl 5-acetamido-3,5-dideoxy-D-glycero- $\alpha$ -D-galacto-2-nonulopyranosylonate-(2-6)- $\beta$ -Dgalactopyranosyl-(1 $\rightarrow$ 4)-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside (7) (Neu5Ac- $\alpha$ 2-6-LacNAc- $\beta$ -Ethyl- $\text{N}_3$ ):** white solid; yield 77%.  $^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ )  $\delta =$  4.60 (dd,  $J = 8$ , 15 Hz,

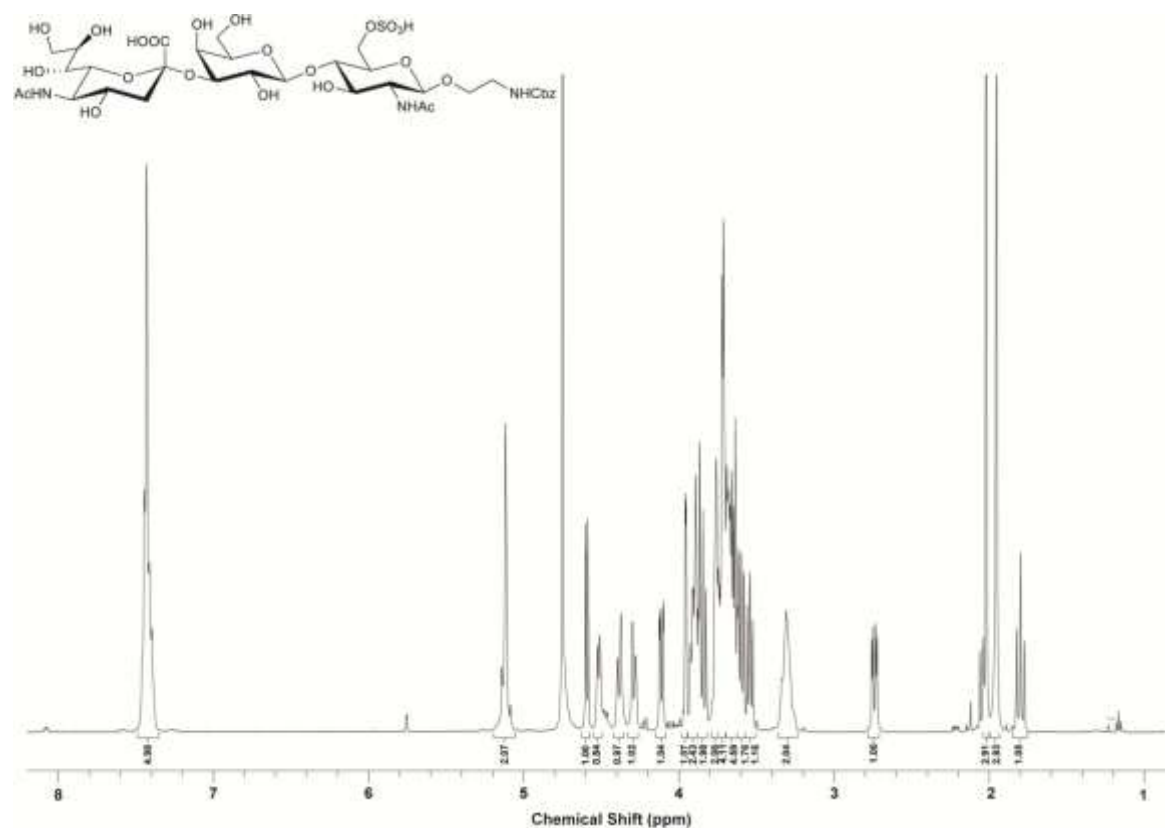
1H), 4.42 (d,  $J = 7.5$ , 1H), 3.81-4.05 (m, 4H), 3.94-4.20 (m, 5H), 3.58-3.92 (m, 10H), 3.48-3.51 (m, 5H), 2.65 (dd,  $J = 4.5$ , 12 Hz, 1H), 2.40 (s, 3H), 2.03 (s, 3H), 1.68 (t,  $J = 12.5$  Hz, 1H).

***N*-acetylneuraminosyl $\alpha$ 2-6-*O*-D-galactopyranosyl $\beta$ (1-4)-2-(acetylamino)-2-deoxy- $\beta$ -D-glucopyranose (8) (Neu5Ac- $\alpha$ 2-6-LacNAc):** white solid; yield 75%.  $^1\text{H-NMR}$  (500 MHz,  $\text{D}_2\text{O}$ )  $\delta$  = 4.52 (d,  $J = 7.5$  Hz, 1H), 4.45 (d,  $J = 7.5$ , 1H), 3.80-4.02 (m, 9H), 3.49-3.75 (m, 11H), 2.68 (dd,  $J = 3.5$ , 12.0 Hz, 1H), 2.04 (s, 6H), 1.70 (t,  $J = 12.0$ Hz, 1H).

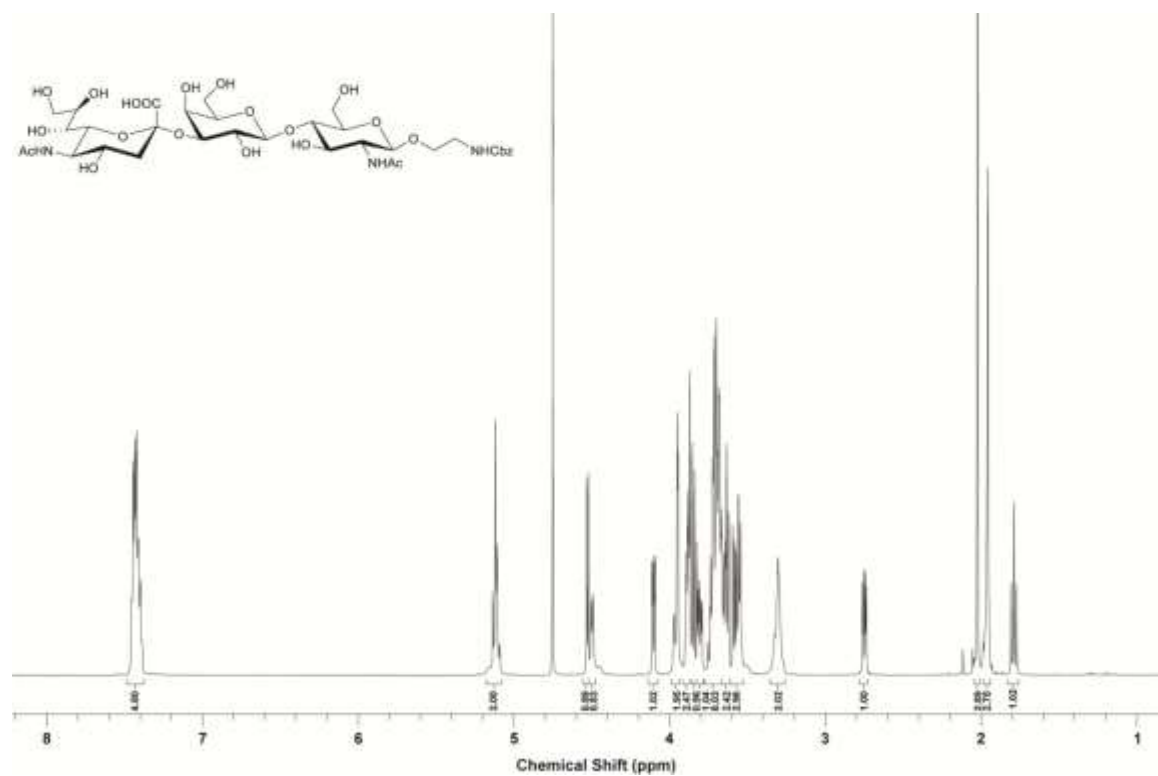
### Copies of $^1\text{H}$ NMR and mass spectra



**Figure S1.** ESI-MS spectrum of compound 1.

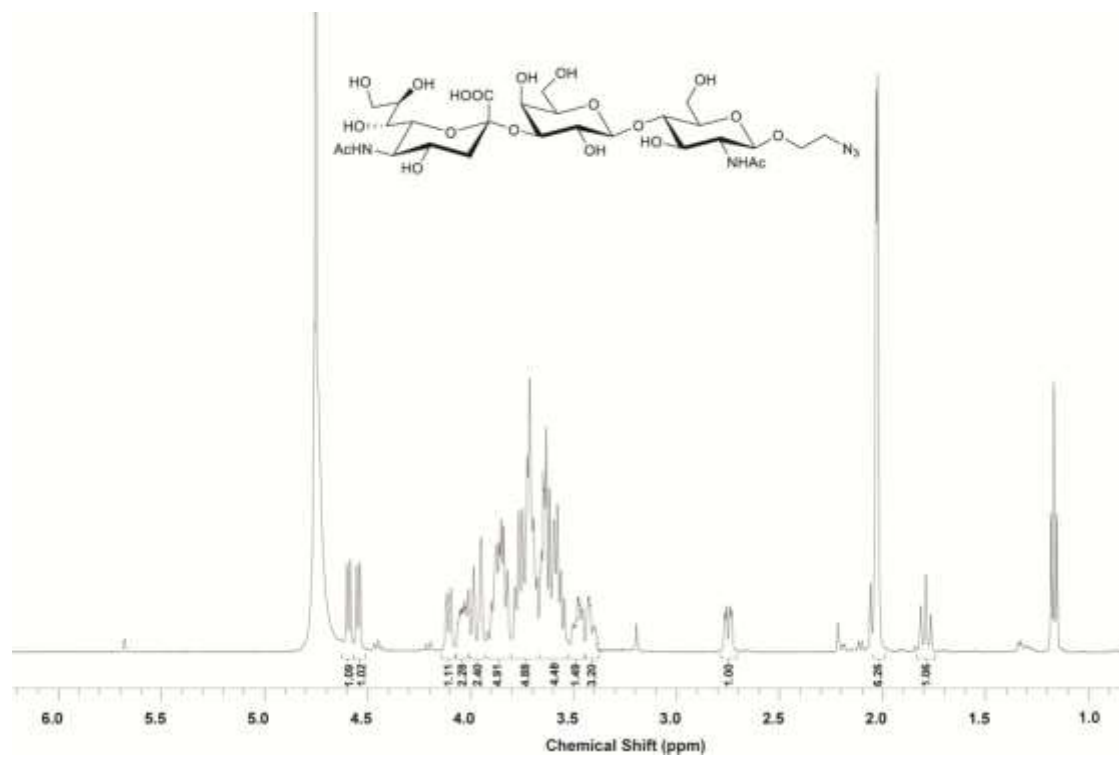


**Figure S2.** <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O) spectrum of compound 1.

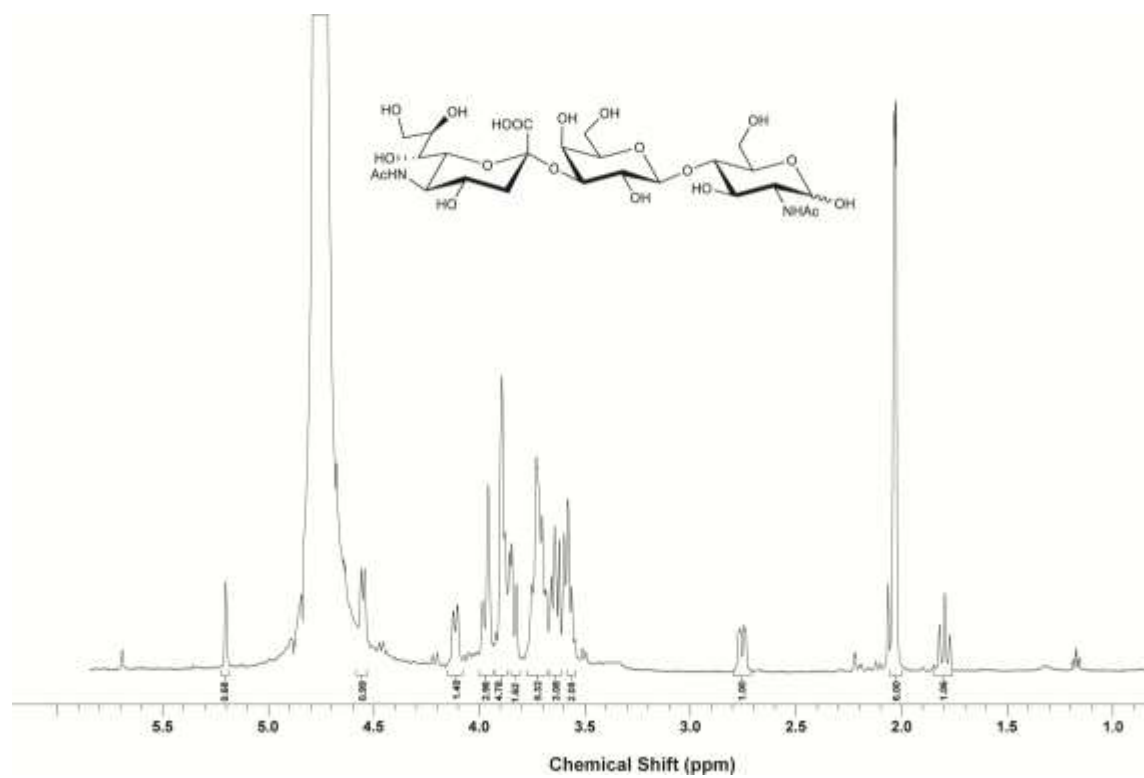


**Figure S3.** <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O) spectrum of compound 2.

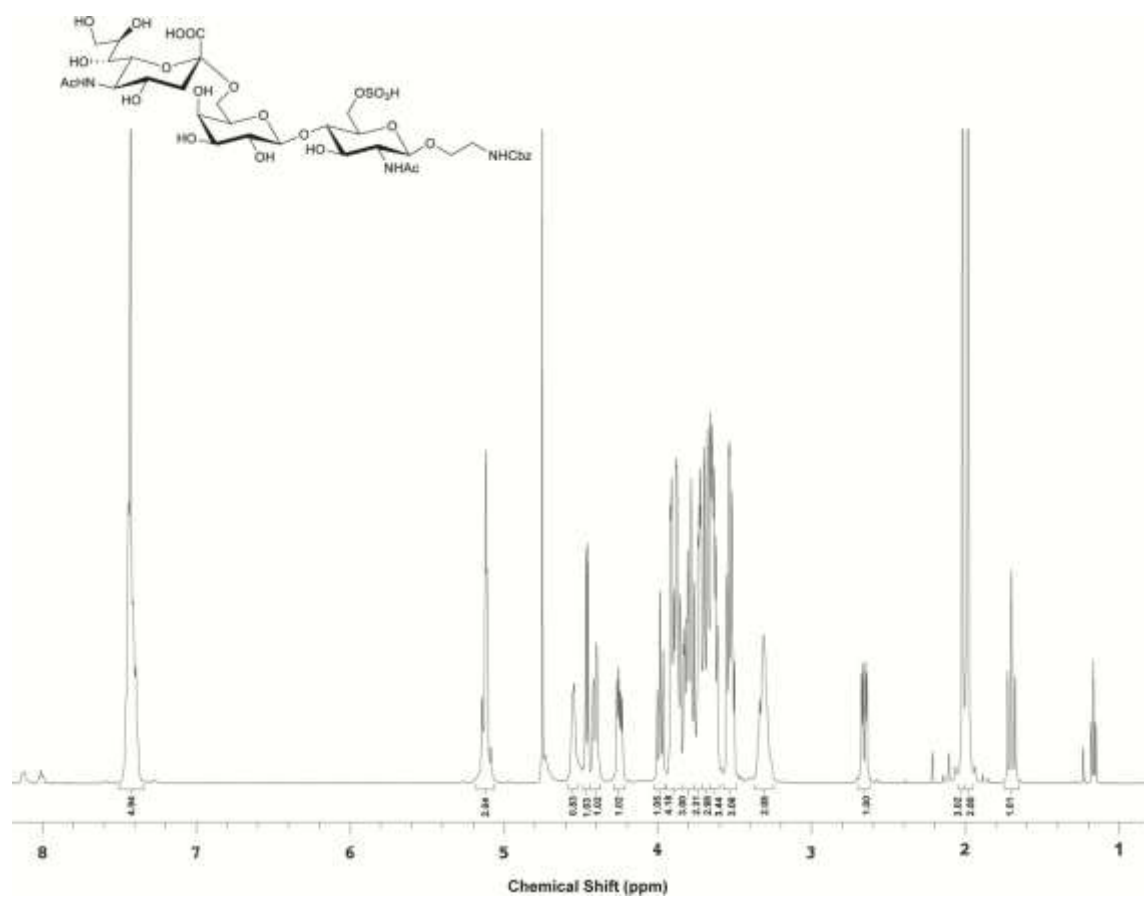




**Figure S4.** <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O) spectrum of compound **3**.

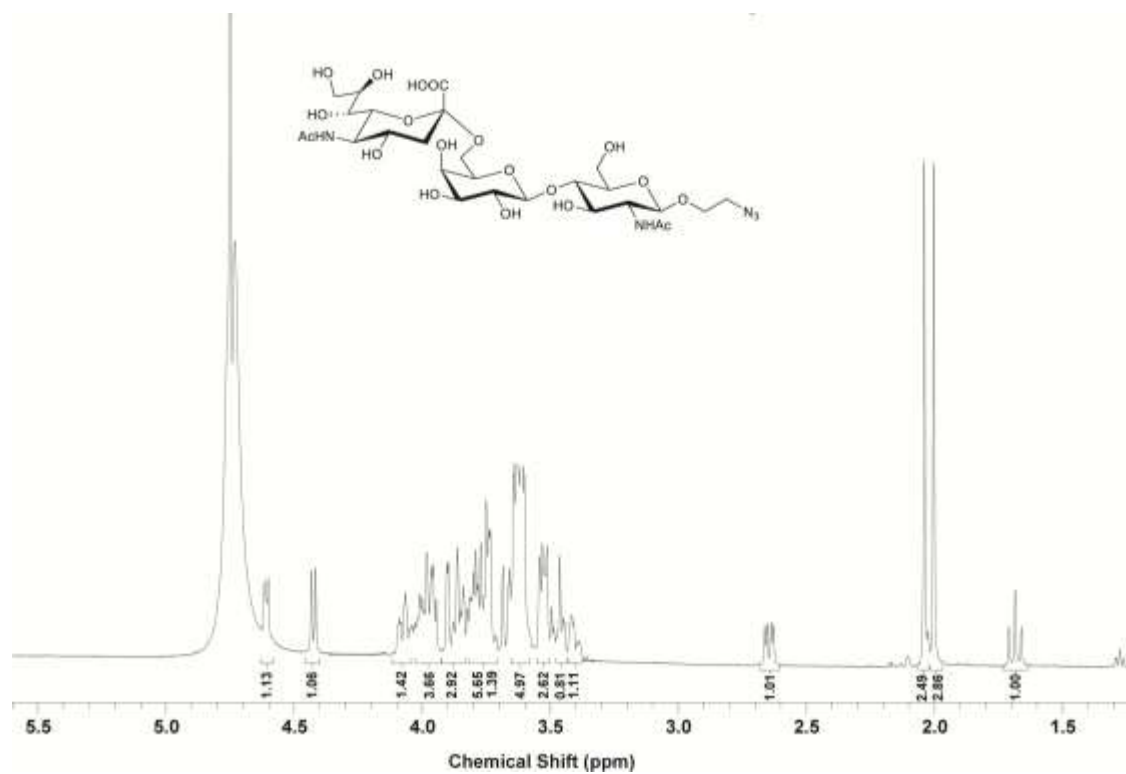


**Figure S5.**  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ ) spectrum of compound 4.

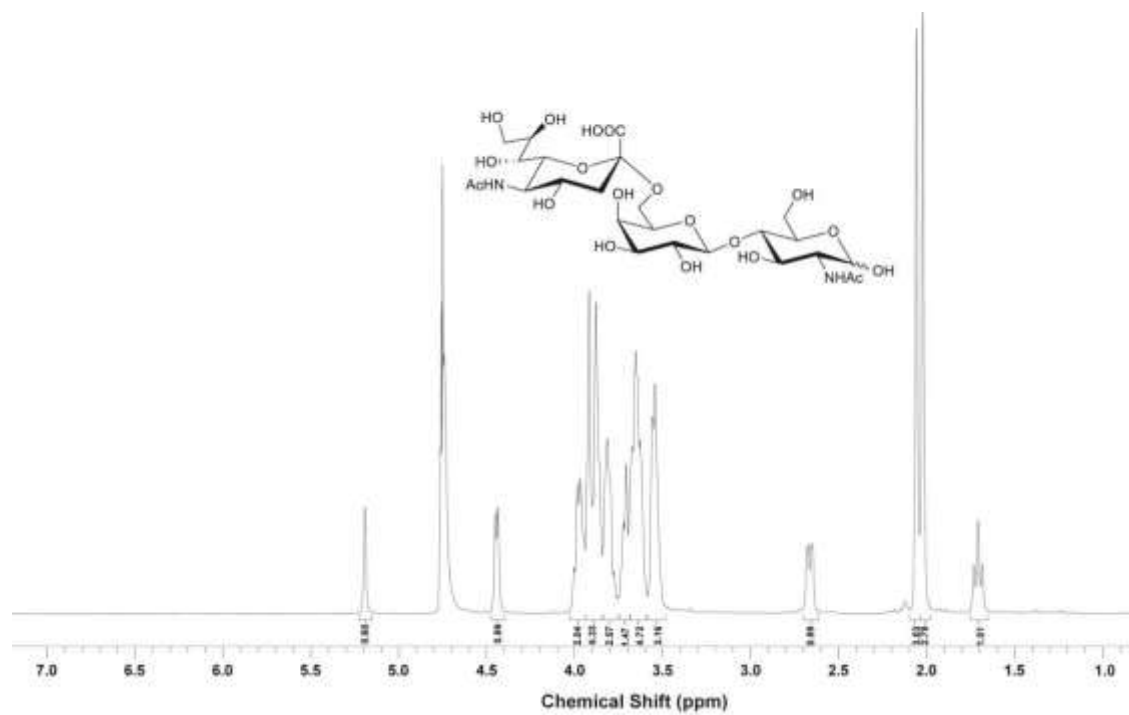


**Figure S6.**  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ ) spectrum of compound 5.



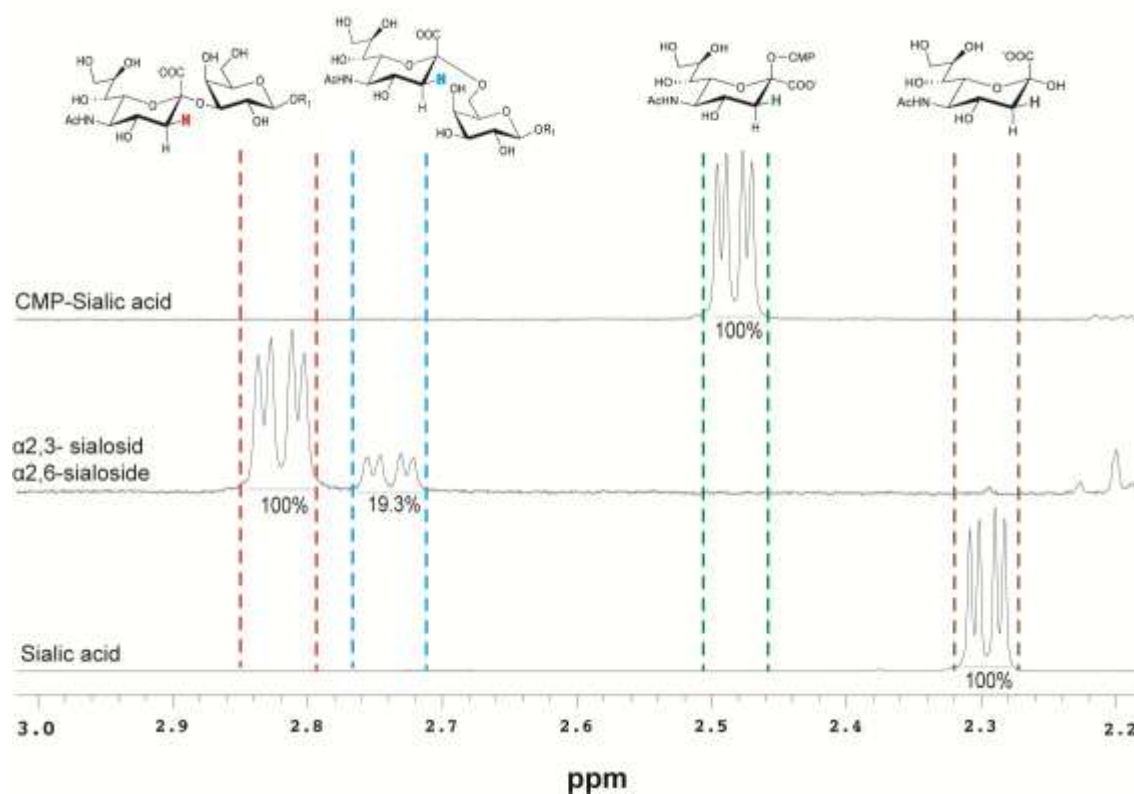


**Figure S8.**  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ ) spectrum of compound 7.



**Figure S9.**  $^1\text{H}$  NMR (500 MHz,  $\text{D}_2\text{O}$ ) spectrum of compound 8.

$R_1$ :  $\beta(1\rightarrow4)$ -6-O-sulfo-GlcNAc- $\beta$ -Ethyl-NHCbz



**Figure S10:** Stacked spectra of, bottom: Sialic acid, middle: mixture of  $\alpha$ 2-3 and  $\alpha$ 2-6-sulfated sialoside, and top: CMP-Sialic acid.