Supporting Information

7-Fluorosialyl glycosides are hydrolysis resistant but readily assembled by sialyltransferases providing easy access to more metabolically stable glycoproteins

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ONLINE METHODS

Chemical and chemoenzymatic synthesis

General

All chemicals were of analytical grade purchased from the Sigma-Aldrich company, unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed on aluminium-backed sheets of Silica Gel $60F_{254}$ (E. Merck) of thickness 0.2 mm. The plates were visualised using UV light (254 nm) and/or by exposure to 10% ammonium molybdate (2 M in H_2SO_4) followed by charring. Flash column chromatography was carried out using Merck Kieselgel 60 (230-400 mesh). Ion-exchange chromatography was performed using Dowex® 1X2-200 resin (formate form) using an ammonium formate gradient (50 mM to 1 M). Reverse-phase silica gel chromatography was performed using Waters Sep-Pak® C18 cartridges using a water/acetonitrile gradient as specified. Size exclusion chromatography was performed using Bio-Rad Bio-Gel® P-2 column (22 mm x 80 cm, 100-200 mesh), eluting with ammonium formate buffer (20 mM, pH 8.1) at a rate of 10 mL/hour. HPLC was performed using Waters 600 multi-solvent delivery system (buffer A is acetonitrile, buffer B is 20 mM ammonium formate, pH 8.1) and Waters 2996 photodiode array detector. Reverse phase silica gel HPLC was performed with a Phenomenex Jupiter C18 reverse-phase column (10 x 250 mm) at a rate of 4 mL/min and a linear gradient (0-10 % buffer A over 40 minutes) and regular phase silica gel HPLC was performed with TSKgel Amide-80 column (21.5 x 300 mm) at a rate of 6 mL/min and a linear gradient (20-70 % buffer B over 40 minutes).

Proton and carbon NMR spectra were recorded on Bruker Avance 600inv Fourier Transform spectrometer fitted with a TCI-Z cryoprobe or Bruker Avance 400inv Fourier Transform spectrometer fitted with 5 mm BBI-Z probe. Fluorine and phosphorous NMR spectra were recorded on Bruker Avance 300 fitted with a 5 mm QNP probe. All spectra are recorded using an internal deuterium lock and are referenced internally using the residual solvent peak. Carbon and proton chemical shifts are quoted in parts per million (ppm) downfield of tetramethylsilane, fluorine chemical shifts are quoted downfield of trifluoroacetic acid, phosphorus chemical shifts are quoted downfield of aqueous phosphoric acid. ^{1}H, ^{13}C, ^{19}F and ^{31}P chemical shifts are rounded to the nearest 0.1 ppm. Coupling constants (*J*) are given in Hertz (Hz) and are quoted to the nearest 0.5 Hz. Carbon NMR spectra were performed with broadband proton decoupling and were recorded with DEPT. ^{1}H-NMR experiments performed in D_2O solvent were recorded

with a water suppression protocol. Complex ^{1}H- and ^{13}C-spectra were assigned on the basis of ^{1}H, COSY, ^{13}C, DEPT, ^{1}H-^{13}C HMQC and ^{1}H-^{13}C HMBC as relevant.

Mass spectra were recorded on a Waters/Micromass LCT using electrospray ionisation (ESI) and recorded using Time-Of-Flight (TOF) method using methanol or 20% water in acetonitrile as solvent.

General synthesis of 7-deoxy-7-fluoro sialic acids - 4-Deoxy-4-modified GlcNAc derivatives[1] (1 eq.) and sodium pyruvate (5 eq.) were dissolved in water to give a final sugar concentration of 100 mM. The GlcNAc/ManNAc epimerase (0.2 U/μmol) and sialic acid aldolase (2 U/μmol) were added and the mixture was incubated at 37 °C for 48 hours. The mixture was centrifuged (10,000 rpm) for 5 minutes and the supernatant was loaded directly onto a pre-equilibrated (50 mM ammonium formate) ion-exchange column (Dowex® 1X2-200 resin, formate form). The column was washed (20 mL, 50 mM ammonium formate) to elute any starting materials. The products were eluted from the column (20 mL, 1 M, ammonium formate), and crude product-containing fractions, as identified by TLC (mobile phase of ethyl acetate/methanol/water/acetic acid in a 4:2:1:0.1 ratio), were pooled and lyophilized. Purification was achieved by regular-phase HPLC, using TSKgel Amide-80 column (21.5 x 300 mm), an MeCN/20 mM ammonium formate pH 8.1 buffer at a rate of 6 mL/min and a linear gradient (20-70% over 40 minutes), with the products eluting at around 45% MeCN. The product-containing fractions were identified by UV (*N*-acetyl absorbs at 198 nm) and TLC before being pooled and lyophilised to yield pure product.

General syntheses of CMP 7-deoxy-7-fluoro sialic acids - 7-Deoxy-7-fluoro sialic acid derivative (1 eq.) and CTP disodium salt (1.05 eq.) were dissolved in Tris buffer (100 mM, pH 8.5) containing magnesium chloride (20 mM) and DTT (0.1 mM) to give a final sialic acid concentration of 15 mM. CMP-sialic acid synthetase (1 U/μmol) and inorganic pyrophosphatase (1 U/mmol) were added and the mixture was tumbled at ambient temperature. The pH of the solution was checked regularly and aqueous sodium hydroxide solution (1 M) was added as appropriate to keep the pH constant. On completion of reaction, as observed by TLC analysis (ethyl acetate/methanol/water/ concentrated ammonia solution in a 4:3:2:1 ratio mobile phase), the mixture was cooled to -80 °C. Once it had thawed it was filtered (0.44 μm) and incubated with alkaline phosphatase (20 U/mmol) for 10 minutes. The mixture was filtered (0.44 μm) again and loaded directly onto an ion-exchange column (Dowex® 1X2-200 resin, formate form) that

had been pre-equilibrated with ammonium formate (50 mM). After an initial wash using the same buffer, a stepped gradient (50 mM to 1 M) was performed and product-containing fractions were identified by TLC, pooled and lyophilised. This crude product was dissolved in the minimum volume of buffer (20 mM ammonium formate buffer, pH 8.1) and loaded onto a size exclusion column (Bio-Rad Bio-Gel® P-2 column, 22 mm x 80 cm, 100-200 mesh). The column was run at 10 mL/hour and fractions were collected every 15 minutes. Product-containing fractions were identified by TLC analysis, pooled and lyophilised.

Synthesis of 7-deoxy-7-fluoro sialyl galactosides -The aryl galactoside acceptor (1.2 eq.) was incubated with the CMP 7-modified sialic acid (1.0 eq.) donor in HEPES (50 mM, pH 7.5) buffered solution containing manganese chloride (10 mM) in the presence of CjST1 (65 μM) and alkaline phosphatase (300 U/mL) at room temperature. On completion of reaction, as observed by TLC (ethyl acetate/methanol/water, in a 7:2:1 ratio mobile phase), the mixture was filtered (0.44 μm) and loaded onto a pre-equilibrated Waters SepPak C18 (2 g) column, washed with water (10 column volumes) and products eluted with 5% acetonitrile in water. Product-containing fractions were identified by TLC, pooled and lyophilised. Further purification was performed using a Phenomenex Jupiter C18 reverse-phase column (10 x 250 mm) at a rate of 4 mL/min and a linear gradient (0-10% MeCN/20 mM ammonium formate pH 8.1 over 40 minutes), with products eluting at around 7% MeCN.

5-Acetamido-3,5,7-trideoxy-7-fluoro-D-glycero-D-galacto-2-nonulopyranosidic acid ammonium salt (4)

4-Deoxy-4-fluoro GlcNAc **1** (220 mg, 0.94 mmol)[1] and sodium pyruvate (519 mg, 4.72 mmol) were combined in buffered solution (total volume = 9.4 mL) as described in General Synthesis. The 7-deoxy-7-fluoro sialic acid (266 mg, 0.86 mmol, 91%) was obtained as a white solid with identical physical data to that previously reported.[2]

5-Acetamido-3,5,7-trideoxy-7,7-difluoro-D-glycero-D-galacto-2-nonulopyranosidic acid ammonium salt (6)

4-Deoxy-4,4-difluoro GlcNAc **3** (100 mg, 0.41 mmol)[1] and sodium pyruvate (225 mg, 2.05 mmol) were combined in buffered solution (total volume = 4.1 mL) in the procedure described in

General Synthesis. The 7-deoxy-7,7-difluoro sialic acid (68 mg, 0.21 mmol, 51%) was obtained as a white solid as a 2:1 mixture of β:α anomers.

δ_H (400 MHz D_2O) 1.75 (2 H, dd, *J* 13.5 and 12.5, α- and β-H3ax), 1.83 (1 H, dd, *J* 13.5 and 5.0, β-H3eq), 1.88 (3 H, s, α-Ac), 1.92 (3 H, s, β-Ac), 2.08 (1 H, dd, *J* 13.5 and 4.5, α-H3eq), 3.54 (1 H, dd, *J* 12.0 and 7.5, α-H9a), 3.56 (1 H, dd, *J* 12.0 and 7.5, β-H9a), 3.73 (1 H, dd, *J* 12.0 and 3.0, β-H9b), 3.75 (1 H, ddd, *J* 12.0, 2.5 and 1.5, α-H9b), 3.92-3.84 (3 H, m, α-H4, α-H5 and β-H8), 4.01 (1 H, ddd, *J* 23.0, 7.5 and 3.0, α-H8), 4.10 (1 H, ddd, *J* 12.0, 5.0 and 4.0, β-H4), 4.14 (1 H, ddd, *J* 21.5, 9.0 and 3.0, α-H6), 4.40 (1 H, dd, *J* 18.5 and 3.5, β-H6), 4.44 (1 H, d, *J* 4.0, β-H5);

δ_C (100 MHz D_2O) 21.4 (β-CO*Me*), 21.6 (α-CO*Me*), 34.3 (β-C3), 38.4 (α-C3), 46.8 (β-C5), 50.2 (α-C5), 59.3 (α- and β-C9), 64.4 (β-C4), 66.6 (α-C4), 66.8 (dd, *J* 33.5 and 20.5, β-C6), 67.6 (dd, *J* 28.5 and 24.0, α-C6), 68.8 (dd, *J* 31.0 and 23.0, α-C8), 69.0 (dd, *J* 28.5 and 22.0, β-C8), 95.9 (α-C2), 96.3 (β-C2), 120.4 (dd, *J* 254.5 and 248.0, β-C7), 121.5 (dd, *J* 249.0 and 252.5, α-C7), 173.8 (CO), 174.3 (CO), 175.1 (CO), 175.3 (CO);

δ_F (282 MHz D_2O) -121.0 (1 F, ddd, *J* 262.5, 21.5 and 1.5, α-7a), -122.9 (1 F, ddd, *J* 262.5, 23.0 and 4.5, α-7b), -123.0 (1 F, ddd, *J* 263.0, 18.5 and 7.0, β-7a), -124.1 (1 F, ddd, *J* 263.0, 17.5 and 3.5, β-7b);

HRMS calc for $C_{11}H_{16}NO_8F_2$ (M-H) is 328.0844, found 328.0848 (+1.2 ppm).

5-Acetamido-3,5,7-trideoxy-2-(hydrogen 5'-cytidylate)-7-fluoro-D-glycero-β-D-galacto-2-nonulopyranosidic acid diammonium salt (7)

Acid **4** (120 mg, 0.39 mmol) was combined with cytidine triphosphate (214 mg, 0.41 mmol) and dithiothreitol (1 mg) in a buffered solution (total volume of 24.4 mL) with CMP-sialic acid synthetase and inorganic pyrophosphatase in the procedure described in General Synthesis. After purification the CMP 7-deoxy-7-fluoro conjugate **7** was obtained as a white solid (207 mg, 0.34 mmol, 86%).

δ_H (400 MHz D_2O) 1.55 (1 H, ddd, *J* 13.0, 11.5 and 5.5, H3ax), 1.94 (3 H, s, Ac), 2.41 (1 H, dd, *J* 13.0 and 4.5, H3eq), 3.55 (1 H, ddd, *J* 12.5, 5.0 and 2.0, H9a'), 3.71 (1 H, dt, *J* 12.5 and 3.0,

H9b'), 3.76-4.04 (9 H, m), 4.33 (1 H, dd, *J* 45.5 and 9.5, H7'), 5.86 (1 H, d, *J* 4.5, H1), 6.00 (1 H, d, *J* 7.5, *m*-Ar), 7.85 (1 H, d, *J* 7.5, *p*Ar);

δ_P (162 MHz D_2O) -4.3 (s);

δ_F (282 MHz D_2O) -206.7 (ddd, *J* 45.5, 29.5 and 2.0);

HRMS calc for $C_{20}H_{29}N_4O_{15}FP$ (M-H) is 615.1351, found 615.1343 (-1.3 ppm).

5-Acetamido-3,5,7-trideoxy-2-(hydrogen 5'-cytidylate)-7,7-difluoro-D-glycero-β-D-galacto-2-nonulopyranosidic acid diammonium salt (8)

Acid **6** (9.8 mg, 29.8 μmol) was combined with cytidine triphosphate (17.3 mg, 32.8 μmol) and dithiothreitol (1 mg) in a buffered solution (total volume of 1.99 mL) with CMP-sialic acid synthetase and inorganic pyrophosphatase in the procedure described in General Synthesis. After purification the CMP 7-deoxy-7,7-difluoro conjugate **8** was obtained as a white solid (12.0 mg, 18.5 μmol, 62%).

δ_H (400 MHz D_2O) 1.56 (1 H, ddd, *J* 13.0, 11.0 and 5.5, H3ax), 1.87 (3 H, s, Ac), 2.39 (1 H, dd, *J* 13.0 and 3.0, H3eq), 3.25 (1 H, dd *J* 12.0 and 8.0, H9a), 3.75 (1 H, dt, *J* 12.0 and 2.0, H9b), 3.88-4.28 (9 H, m), 5.85 (1 H, d, *J* 4.5, H1), 5.99 (1 H, d, *J* 7.5, *m*-Ar), 7.86 (1 H, d, *J* 7.5, *p*-Ar);

δ_P (162 MHz D_2O) -4.5 (s);

δ_F (282 MHz D_2O) -121.7 (1 F, dd, *J* 260.5 and 21.5, F7a), -125.4 (1 F, dd, *J* 260.5 and 25.5, F7b);

HRMS calc for $C_{20}H_{28}N_4O_{15}F_2P$ (M-H) is 633.1257, found 633.1265 (+1.3 ppm).

***ortho*-Nitrophenyl (5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 →3)-β-D-galactopyranoside (10)**

ortho-Nitrophenyl-β-D-galactopyranoside (7.5 mg, 25.0 μmol) was combined with CMP-sialic acid (19.1 mg, 30.0 μmol) in a buffered solution (total volume of 5.0 mL) with CjST1 and alkaline phosphatase according to the procedure described in General Synthesis. After isolation and purification the *disaccharide* **10** was obtained as a white solid (11.1 mg, 18.7 μmol, 75%).

δ_H (400 MHz D_2O) 1.69 (1 H, t, *J* 12.0, H3''ax), 1.90 (3 H, s, CO*Me*), 2.65 (1 H, dd, *J* 12.0 and 4.5, H3''eq), 3.45 (1 H, dd, *J* 7.0 and 2.0, H7''), 3.50 (1 H, dd, *J* 11.5 and 5.5, H9a''), 3.51 (1 H,

dd, *J* 10.0 and 1.5, H6''), 3.61-3.64 (2 H, m, H6a' and H6b'), 3.67-3.79 (5 H, m, H2', H5', H5'', H8'', H9b''), 3.90 (1 H, d, *J* 3.0, H4'), 4.09 (1 H, dd, *J* 9.5 and 3.0, H3'), 5.15 (1 H, d, *J* 8.0, H1'), 7.13 (1 H, t, *J* 7.5, H4-Ar), 7.31 (1 H, d, *J* 8.5, H6-Ar), 7.56 (1 H, dd, *J* 7.5 and 2.0, H5-Ar), 7.81 (1 H, dd, 8.5 and 2.0, H3-Ar);

δ_C (100 MHz D_2O) 22.2 (CO*Me*), 39.8 (C3''), 51.9 (C5''), 60.8 (C6'), 62.8 (C9''), 67.5 (C4'), 68.3 (C7''), 68.5 (C4''), 68.9 (C2'), 72.0 (C8''), 73.0 (C6''), 75.6 (C5'), 75.6 (C3'), 100.1 (C2''), 101.0 (C1'), 117.7 (C6-Ar), 123.2 (C4-Ar), 125.8 (C3-Ar), 135.3 (C5-Ar), 140.1 (C2-Ar), 149.6 (C1-Ar), 174.0 (CO), 175.2 (CO);

HRMS calc for $C_{23}H_{32}N_2O_{16}Na$ (M+Na) is 615.1650, found 615.1638 (-1.9 ppm).

ortho-Nitrophenyl (5-Acetamido-3,5,7-trideoxy-7-fluoro-D-_glycero_-α-D-_galacto_-non-2-ulopyranosylonic acid)-(2 →3)-β-D-galactopyranoside (11)

ortho-Nitrophenyl β-D-galactopyranoside (5.7 mg, 18.8 μmol) was combined with CMP 7-deoxy-7-fluoro-sialic acid **7** (10.2 mg, 15.7 μmol) in a buffered solution (total volume of 3.14 mL) with CjST1 and alkaline phosphatase according to the procedure described in General Synthesis. After isolation and purification the *disaccharide* **11** was obtained as a white solid (6.0 mg, 10.0 μmol, 64%).

δ_H (600 MHz D_2O) 1.75 (1 H, t, *J* 12.5, H3''ax), 1.92 (3 H, s, CO*Me*), 2.66 (1 H, dd, *J* 12.5 and 4.5, H3''eq), 3.56 (1 H, ddd, *J* 12.5, 6.5 and 1.5, H9''a), 3.59 (1 H, ddd, *J* 12.5, 10.0 and 4.5, H4''), 3.64-3.67 (2 H, m, H6'a and H6'b), 3.71 (1 H, dd, *J* 29.0 and 10.5, H6''), 3.72 (1 H, dt, *J* 12.5 and 2.5, H9''b), 3.78-3.80 (1 H, m, H5'), 3.79 (1 H, dd, *J* 10.0 and 8.0, H2'), 3.82 (1 H, t, *J* 10.0, H5''), 3.93 (1 H, d, *J* 3.0, H4'), 3.97 (1 H, dtd, *J* 8.5, 6.5 and 2.5, H8''), 4.10 (1 H, dd, *J* 10.0 and 3.0, H3'), 4.39 (1 H, dd, *J* 48.0 and 9.0, H7''), 5.19 (1 H, d, *J* 8.0, H1'), 7.16 (1 H, t, *J* 8.0, H4-Ar), 7.33 (1 H, d, *J* 8.5, H6-Ar), 7.56 (1 H, ddd, *J* 8.5, 8.0 and 1.5, H5-Ar), 7.84 (1 H, dd, 8.0 and 1.5, H3-Ar);

δ_C (150 MHz D_2O) 21.6 (CO*Me*), 38.8 (C3''), 50.6 (C5''), 60.0 (C6'), 61.4 (C9''), 67.0 (C4'), 67.6 (C4''), 68.2 (C2'), 68.4 (d, *J* 27.5, C8''), 71.1 (d, *J* 18.5, C6''), 74.9 (C5'), 75.3 (C3'), 88.0 (d, *J* 180.0, C7''), 99.8 (C2''), 100.3 (C1'), 116.9 (C6-Ar), 122.5 (C4-Ar), 125.1 (C3-Ar), 134.7 (C5-Ar), 139.3 (C2-Ar), 149.0 (C1-Ar), 173.0 (CO), 174.2 (CO);

δ_F (282 MHz D_2O) -208.5 (ddd, *J* 48.0, 29.0 and 6.5);

HRMS calc for $C_{23}H_{30}N_2O_{15}F$ (M-H) is 593.1630, found 593.1635 (+0.8 ppm).

4-Methylumbelliferyl (5-acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 →3)-β-D-galactopyranoside (12)

4-Methylumbelliferyl β-D-galactopyranoside (3.4 mg, 10.1 μmol) was dissolved in DMSO (50 μL) and carefully diluted into a buffered solution (total volume of 1.68 mL) containing CMP-sialic acid (6.5 mg, 10.1 μmol) with CjST1 and alkaline phosphatase according to the procedure described in **General Synthesis**. After isolation and purification the *disaccharide* **12** was obtained as a white solid (4.1 mg, 6.6 μmol, 65%), with identical physical data to those previously reported.[3]

4-Methylumbelliferyl (5-acetamido-3,5,7-trideoxy-7-fluoro-D-*glycero*-α-D-*galacto*-non-2-ulopyranosylonic acid)-(2 →3)-β-D-galactopyranoside (13)

4-Methylumbelliferyl β-D-galactopyranoside (3.5 mg, 10.5 μmol) was dissolved in DMSO (50 μL) and carefully diluted into a buffered solution (total volume of 1.75 mL) containing CMP 7-deoxy-7-fluoro-sialic acid **7** (5.6 mg, 8.7 μmol) with CjST1 and alkaline phosphatase according to the procedure described in **General Synthesis**. After isolation and purification the *disaccharide* **20** was obtained as a white solid (2.8 mg, 4.4 μmol, 51%)

δ_H (600 MHz D_2O) 1.77 (1 H, t, *J* 12.5, H3''ax), 1.93 (3 H, s, CO*Me*), 2.35 (3 H, s, Ar-*Me*), 2.67 (1 H, dd, *J* 12.5 and 4.5, H3''eq), 3.56 (1 H, dd, *J* 11.5 and 5..5, H9a''), 3.61 (1 H, ddd, *J* 12.5, 11.0 and 4.5, H4''), 3.66-3.69 (2 H, m, H6a' and H6b'), 3.72 (1 H, dd, *J* 11.5 and 2.0, H9b''), 3.73 (1 H, dd, *J* 29.0 and 10.5 H6''), 3.79 (1 H, dd, *J* 10.0 and 8.0, H2'), 3.81-3.85 (2 H, m, H5' and H5''), 3.96 (1 H, d, *J* 3.0, H4'), 3.99 (1 H, dddd, *J* 9.0, 7.0, 5.5 and 2.5, H8''), 4.14 (1 H, dd, *J* 10.0 and 3.0, H3'), 4.40 (1 H, dd, *J* 46.5 and 9.0, H7''), 5.17 (1 H, d, *J* 8.0, H1'), 6.17 (1 H, s, H3-Ar), 7.02 (1 H, d, *J* 2.0, H9-Ar), 7.05 (1 H, dd, *J* 9.0 and 2.0, H7-Ar), 7.65 (1 H, d, *J* 9.0, H6-Ar);

δ_C (150 MHz D_2O) 18.4 (Ar-*Me*), 22.5 (CO*Me*), 39.7 (C3''), 51.6 (C5''), 61.0 (C6'), 62.4 (C9''), 67.9 (C4'), 68.5 (C4''), 69.2 (C2'), 69.4 (d, *J* 27.5, C8''), 72.1 (d, *J* 17.0, C6''), 75.7 (C5'), 76.2 (C3'), 88.9 (d, *J* 180.0, C7'') 100.3 (C1'), 100.6 (C2''), 104.0 (C9-Ar), 111.7 (C3-Ar), 114.3 (C7-Ar), 115.8 (C5-Ar), 127.1 (C6-Ar), 154.4 (C10-Ar), 156.8 (C4-Ar), 159.8 (C8-Ar), 165.2 (C2-Ar), 173.8 (CO), 175.2 (CO);

δ_F (282 MHz D_2O) -208.6 (ddd, *J* 46.5, 29.0 and 7.0);

HRMS calc for $C_{27}H_{33}NO_{15}F$ (M-H) is 630.1834, found 630.1818 (-2.6 ppm).

Kinetics

Hydrolysis rate of CMP 7-modified sialic acids, by ^{1}H-NMR - CMP 7-modified sialic acid was dissolved in deuterated phosphate buffer (10 mM, pH 7.2, 700 µL) containing sodium chloride (100 mM) to give a final concentration of 2 mM. The mixture was equilibrated to 60 °C for 10 minutes in the NMR spectrometer before spectra were acquired (32 scans each using water suppression pulse sequence) at 5 or 15 minute time intervals. The intensity of the signals corresponding to the $H3_{eq}$ and $H3_{ax}$ ring protons, and the H4-proton of the cytosine ring were measured and normalized to the intensity of the total H5-cytosine peak. The decays of the signals from each of these three protons were fitted to single exponentials to derive rate constants and these three values were averaged.

Hydrolysis of 7-modified sialyl-galactose conjugates, by ^{1}H-NMR - The 4-methylumbelliferyl -7-modified sialyl-galactoside conjugates **12** or **13** were dissolved in a mixture of D_2O and hydrochloric acid (25 mM final concentration of HCl, 700 µL) containing sodium chloride (250 mM) to give a final substrate concentration of 2 mM. The mixture was equilibrated at 60 °C for 10 minutes in the NMR spectrometer before spectra were acquired (32 scans each using water suppression pulse sequence) at 5 or 15 minute time intervals. The intensities of the signals corresponding to the anomeric proton of the galactose (H1') and the H3-equatorial proton ($H3''_{eq}$) of the sialic acid were measured and normalized to the total H4-coumarin peak. The decays of the signal from each of these three protons were fitted to single exponentials to derive rate constants and these three values were averaged.

CMP release kinetic assay for sialyltransferases – Performed as described.[4] CjST1 kinetics were performed at 37 °C in HEPES pH 7.5 buffer (20 mM) containing sodium chloride (50 mM), manganese chloride (10 mM) and magnesium chloride (10 mM) at substrate concentrations from 0.1 to 2 mM. PsST1 kinetic analyses were performed at 37 °C in HEPES pH 8.5 buffer (20 mM) containing sodium chloride (50 mM) and magnesium chloride (20 mM) at substrate concentrations from 0.02 to 1 mM. These data were analysed using GraFit from Erithacus software and errors quoted are those derived from fits to the data.

Neuraminidase kinetic assay - Performed using a *β*-galactosidase coupled assay by a similar method to that previously described.[5,6] The *C. perfingens* assays were performed at 37 °C, in pH 6.7 phosphate buffer (20 mM) containing sodium chloride (50 mM), magnesium chloride (2 mM) and BSA (0.5 mg/ml). The *T. rangeli* assays were performed at 30 °C, in phosphate pH 6.0 buffer (20 mM) containing sodium chloride (50 mM), magnesium chloride (2 mM) and BSA (0.5 mg/mL). The *influenza* assays were performed at 25 °C in pH 5.9 Tris buffer (50 mM) containing calcium chloride (20 mM) and BSA (0.5 mg/mL). A continuous UV assay, using a Varian Cary-4000 UV-vis spectrophotometer, was used to monitor the release of the *ortho*-nitrophenolate at 410 nm (ε = 3800 $M^{-1}cm^{-1}$). In brief, the substrate (α2,3-SA/7FSA-Gal-oNP, 0.1 to 2 mM) was incubated for 10 minutes in the desired buffer (190 µL total volume) containing β-galactosidase (0.04 Units), before the assay was initiated by the addition of a small (<10 µL) volume of the sialidase (variable concentrations).

Catalytic activities of the catalytic domains of NedA and human NEU2 were measured with α2,3-SA/7FSA-Gal-MU as a substrate in an endpoint kinetic assay. α2,3-SA/7FSA-Gal-MU (0.125-4 mM) was pre-incubated with β-galactosidase (bovine, 5 u/mL) and 1 mg/mL BSA in 100 mM sodium phosphate buffer pH 6.5 until galactoside depletion was complete. Kinetics were initiated by addition of sialidase and reaction was quenched at 4-5 different time points (within 10-15 min) by adding 4 µL reaction mix to 56 µL quenching buffer (0.133 M glycine, 0.17 M Na_2CO_3, 60 mM NaCl, pH 10). Samples were measured at 450 nm and resultant data fitted to the Michaelis Menten equation.

All kinetic data were analysed using GraFit from Erithacus software and errors quoted are those derived from fits to the data.

Protein preparations

All sialyltransferases, sialidases, porcine AGE, and CMP-sialic acid synthetase used herein were produced in house as described below. The influenza H1N9 membrane paste was a kind gift from Dr. Martin Petric. All other enzymes were obtained from Sigma Aldrich.

His_6-AGE

Protein was expressed in BL21 Tuner cells grown in TB supplemented with 100 µg/mL kanamycin at 25 °C while shaking at 180 rpm, induced with 0.5 mM IPTG and incubated overnight. Cells were pelleted, resuspended in 20 mM Tris, 150 mM NaCl pH 8.3 and frozen at -20 °C. After thawing cells were homogenized twice using a French press (16000 psi on ice) and lysate was cleared and filtered. 20 mM imidazole were added and protein was purified by using a HisTrap (5 mL) column with a linear gradient of 20 mM to 500 mM imidazole in 20 mM Tris, 300 mM NaCl, pH 8.1. Protein was concentrated and 10% glycerol was added as a cryopreservative. Stored at -20 °C.

Δ32-CjST2

Protein was expressed in *E. coli* BL21 grown in LB medium supplemented with 50 µg/mL kanamycin at 22 °C, 230 rpm. Protein expression was induced with 250 µM IPTG at OD 0.7 and cells were harvested after 14.5 h and resuspended in 20 mM Tris, 150 mM NaCl pH 8.3. All purification steps were carried out at 4 °C. Cells were homogenized three times, DNase I was added and chilled on ice for 1 h. Lysate was cleared and filtered through a 0.22 µm membrane, and then loaded onto a pre-equilibrated Ni-NTA column. The column was washed with 50 mM imidazole in lysis buffer and eluted with a gradient from 50 mM to 400 mM imidazole (in the same buffer) over 20 min. Fractions were checked by SDS-PAGE and fractions containing the protein were pooled. Combined eluate was extensively dialysed against 20 mM Tris, 150 mM NaCl, 100 mM sucrose at 4°C overnight and concentrated with an Amicon10 kDa cut-off. 10% glycerol was added to the protein solution and stored at -20 °C.

human Neu2-MBP

Protein was expressed in *E. coli* Origami2 (DE3) grown in LB medium supplemented with ampicillin (100 µg/mL) and streptomycin (25 µg/mL) at 30 °C. Cells were grown to an OD600 0.32 before induction with 0.4 mM IPTG. Temperature was reduced to 20 °C and incubated for 15 h while shaking at 230 rpm. Cells were harvested and pellet was frozen at -80 °C. Note: All subsequent steps were performed on ice. Pellet was resuspended in 20 mM Tris, 150 mM NaCl, pH 7.8 and homogenized twice and incubated with DNase I for 1 h. After centrifugation cleared

supernatant was loaded onto a pre-equilibrated amylose column continuously for 60 min, washed with 10 column volumes loading buffer containing 300 mM NaCl and eluted with wash buffer supplemented with 20 mM maltose. Eluate was concentrated with Vivaspin filter devices (30 kDa cut-off) and loaded onto a Superdex 200 SEC. Pure fractions were pooled, concentrated and 10% glycerol was added before freezing at -20 °C.

His_6-MBP-NedA

Protein was expressed *E. coli* BL21(DE3) in LB medium containing 50 µg/mL kanamycin at 30 °C and 190 rpm. Protein production was induced by 300 µM IPTG at OD600 0.95. Cells were harvested after 6 h and pellets were frozen at -20 °C. After thawing, cells were resuspended in 20 mM sodium phosphate buffer containing 500 mM NaCl, pH 7.4. Suspension was homogenized once and benzonase (Millipore) was added. The lysate was incubated for 0.5 h at room temperature and then cleared by centrifugation. The supernatant supplemented with 20 mM imidazole was loaded onto a HisTrap FF column (bed volume 1 mL, GE Healthcare) using a peristaltic pump. Followed by washing with 20 mM sodium phosphate, 500 mM NaCl, 50 mM imidazole pH 7.4. elution was performed with a gradient from 50 mM imidazole to 500 mM imidazole in 25 min. Peak fractions were pooled and concentrated (Amicon ultrafiltration device, Merck Millipore, molecular weight cutoff 10 kDa), followed by gel filtration (Sephadex 75) in 20 mM Tris, 500 mM NaCl, pH 7.4. Peak fractions were concentrated and flash frozen in aliquots in liquid nitrogen for storage at -70 °C.

His6-NedA

The His-tagged catalytic domain of NedA was expressed in *E. coli* BL21(DE3) grown in autoinduction medium[7] containing 100 µg/mL kanamycin. Cells were grown for 6 h at 30 °C followed by 18 °C for 1.5 d. Cells were harvested and the frozen at -70 °C. The pellet was thawed and resuspended in 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.4. Cells were lysed by sonication and benzonase were added. After 30 min incubation at RT, insoluble material was removed by centrifugation and imidazole (from a 1.5 M stock solution adjusted to pH 7.4; final concentration 20 mM) was added to the supernatant. For nickel affinity chromatography, the supernatant was loaded onto a HisTrap FF column (GE Healthcare, bed volume 1 mL) using a peristaltic pump followed by washing with 10 mL 20 mM sodium phosphate, 500 mM sodium chloride, pH 7.4 using the peristaltic pump. Elution was performed on an AKTA FPLC system with a gradient from 20 mM to 500 mM imidazole over 30 min. Peak fractions were concentrated using an Amicon ultrafiltration device (Merck Millipore, Molecular

Weight Cutoff 10 kDa) and further purified using Superdex 200 size exclusion chromatography (120 mL column volume, mobile phase: 20 mM HEPES, 500 mM NaCl, pH 7.4, flow rate 1 mL/min). Fractions were analyzed by SDS-page and fractions containing the protein were pooled and concentrated. The protein was then aliquotted and flash frozen in liquid nitrogen for long-term storage at -70 °C.

For biotin labeling, TCEP at a final concentration of 20 µg/mL was added to 1 mg of protein in 330 µL 20 mM HEPES, 500 mM NaCl, pH 7.4 and incubated on ice for 15 min. Biotin-PEG_{11}-maleimide (Thermo Scientific, 0.5 mg dissolved in 10 µL DMF) were added and the reaction was incubated at 4 °C for 19 h. The reaction mixture was diluted with the same buffer to 1 mL and the protein was purified on a PD-10 desalting column (GE Healthcare). Eluate was concentrated using a 10 kDa MWCO Amicon ultrafitration device and precipitated protein was removed by centrifugation. Protein was flash frozen in aliquots and stored at -70 °C. Later performed intact mass spectrometry indicated that the protein was not biotinylated, and the bead interaction might have occurred non-specifically.

For immobilization, Streptavidin beads (Streptavidin Separopore, bioWORLD, 500 µL of slurry per 100 µL of NedA) were equilibrated with 20 mM Hepes, 500 mM sodium chloride, pH 7.0 and blocked with 2.5 mg/mL Prolastin C at 4 °C overnight. NedA-Biotin was added and the beads were rotated at 4 °C for 6 h followed by washing in flow with a peristaltic pump at a flow rate of 0.5 mL/min with the following buffers; 3 h 2 M urea, 0.1 % SDS, 20 mM sodium phosphate pH 7.0; 20 min 20 mM sodium phosphate pH 6.5; overnight 1 M urea, 2 M sodium chloride, 1% Triton X-100; 10% glycerol pH 7.0; 1 h 20 mM sodium phosphate pH 6.5. Sialidase activity on the beads was confirmed with the plate activity assay described below (Section “Glycan engineering of A1AT”).

CMP-sialic acid synthetase *(NSY-05)*

Protein was expressed in LB medium at 37 °C, 180 rpm. After induction with 0.5 mM IPTG at OD600 of 0.8, cells were grown for 5 h. Cells were harvested and resuspended in 10 mM Tris pH 8.2. DNase I was added and cells were homogenized once. Suspension was spun at 14000x g for 30 min at 4 °C and protein was precipitated with saturated ammonium sulfate solution on ice 30-50% ammonium sulfate fractions were pooled and resuspended in buffer and concentrated with Amicon filtration devices (10 kDa cut-off). Protein was further purified by

anion exchange chromatography (HiTrap Q, column 5 ml) in two runs. Protein was eluted with 100-300 mM NaCl in 10 mM Tris, pH 8.1 and pure fractions were pooled.

NanA-His_6

Protein was expressed in BL21(DE3) grown in 2YT medium, supplemented with 100 µg/mL ampicillin, at 30 °C. After induction with 0.2 mM IPTG at OD600 1.04 cells were allowed to produce protein overnight, before they were harvested and frozen. After thawing the pellet and resuspending in 20 mM sodium phosphate buffer, 500 mM NaCl, pH 7.5, the suspension was homogenized once and DNase I was added. After 1 h, the suspension was stored for further processing. Purification was done by affinity chromatography with a HisTrap 5 mL FF column, eluting the protein with a linear gradient of 20 mM to 400 mM imidazole in lysis buffer over 18 min. Fractions were analyzed by SDS-PAGE and pure were pooled and concentrated. Final protein stocks contained 10% glycerol and were stored at -20 °C until further use.

rhST6Gal1

Protein was expressed in *E. coli* Origami 2 cells grown in LB medium supplemented with 100 µg/mL ampicillin and 0.5% (w/v) glucose. Overnight culture was grown at 30 °C and main culture was inoculated and grown at 30 °C. The temperature was lowered to 25 °C after reaching the log-phase. Protein production was induced with 0.5 mM IPTG at OD600 0.55 and proceeded overnight. Cells were harvested and resuspended in 20 mM Tris, 150 mM NaCl, pH 7.7 and homogenized once at 15000 psi. 5 mM β-mercaptoethanol and 0.35 g Celite 545 per 1 g wet cell mass were added to suspension, cleared and incubated with benzonase. After second clearing step, solution was filtered and supernatant was loaded on a pre-packed and equilibrated amylose resin column. Column was extensively washed with buffer and protein was eluted with lysis buffer containing 20 mM maltose. Fractions were analysed by SDS-PAGE and pure fractions were combined. After concentration via Amicon 10 kDa, protein was loaded onto a Sephadex 75 column and eluted with 20 mM Tris, 150 mM NaCl, pH 7.6, concentrated and stored at 4 °C until further use. Note: protein needs to be prepared freshly because it steadily loses activity over a short time period.

PmST1 wt and mut#6

Protein was expressed in BL21(DE3) grown in TB medium, supplemented with 50 µg/mL kanamycin, at 30 °C. After induction with 0.5 mM IPTG at OD600 0.9, cells were cooled down to 20°C and incubated overnight. Cells were harvested and resuspended in 20 mM sodium phosphate buffer, 150 mM NaCl, pH 8.3. Cells were homogenized twice on ice, treated with DNaseI for 30 min at rt and supernatant was cleared by centrifugation. Protein was purified with a HisTrap 5 mL FF column, eluting the protein with a linear gradient of 20 mM to 500 mM imidazole. Fractions were analyzed by SDS-PAGE and pure fractions were pooled and concentrated and desalted and polished using a Superdex 200 SEC column. Final protein fractions were pooled, concentrated and stored with 10% glycerol at -20 °C until further use.

pST3Gal1

pST3Gal1- MBP-pST3Gal1 was expressed and purified as described in Rakic et al.[8] with minor modifications. Briefly, the pET28-His6-MBP-□45pST3Gal1 construct was transformed into in Shuffle™ T7 Express *E. coli* and grown in 2YT media + 50 µg/mL at 30 °C until OD600 ~0.6. The cultures were then transferred to 20 °C and induced with 0.5 mM IPTG for 16 h. The MBP-pST3Gal1 was subsequently purified by HisTrapFF and HiTrapQ chromatography. Fractions were pooled and concentrated to 2.8 mg/mL as determined by A280 measurement.

PsST

E. coli AD202 expressing this protein were grown in 2YT media supplemented with 100 µg/mL ampicillin at 30 °C until the culture reached an OD600 of 0.8-1. At this point, the culture was cooled in an ice water bath and IPTG was added to a final concentration of 0.4 mM. Growth was continued for 16 h at 20 °C. Cells were harvested by centrifugation and the pellet was frozen at -70 °C. Upon thawing, cells were resuspended in 20 mM Tris, 150 mM NaCl, pH 7.4 and lysed by sonication. After adding benzonase (Millipore) and incubation at room temperature for 0.5 h, cell fragments were removed by centrifugation. The supernatant was loaded onto pre-equilibrated amylose resin (NEB). The column was washed with five column volumes of in 20 mM Tris, 150 mM NaCl, pH 7.4 and the protein eluted with two column volumes of the same buffer supplemented with 20 mM maltose. The eluate was concentrated (Amicon ultrafiltration device, Merck Millipore, Molecular Weight Cut Off 10 kDa) and the concentrate was loaded onto a Superdex 200 size exclusion chromatography column with a bed volume of 120 mL. Separation was performed in the 20 mM Tris, 150 mM NaCl, pH 7.4 at 1 ml/min. Peak fractions

containing the protein were identified by SDS-PAGE, pooled, and concentrated. The purified protein was stored at 4 °C.

Glycan engineering of interferon α2b

*Preparation of TAg-IFNα2b**

TAg modified GB1-hIFNα2b* (TAg-IFNα2b*), with a sequon-optimized O-glycosylation site, was prepared as described in Du et al (Du et al., 2019). Briefly, Origami™ 2(DE3) E. coli strain harboring the pET21b-GB1-hIFNα2b* and O-glycosylation operon OGO-2 was grown in LB + 100 µg/mL ampicillin + 30 µg/mL chloramphenicol at 37 °C until OD600 ~0.6. The cultures were then transferred to 18 °C and induced with 0.5 mM IPTG for 16 h. GB1-hIFNα2b* was subsequently purified by HisTrapFF chromatography and buffer-exchanged into 20 mM Tris pH 7.5, 150 mM NaCl and concentrated to 23 mg/mL as determined by A280 measurement.

*Sialylation of TAg-IFNα2b**

TAg-IFNα2b* sialylation with 7OHSA or 7FSA was achieved using an MBP fusion protein of porcine ST3Gal1 (MBP-pST3Gal1). Reactions were set up using TAg-IFNα2b* (5 mg/mL) in 50 mM Tris pH 7.5, 10 mM MgCl2, 2 mM CMP-OHSA/7FSA along with 0.11 mg/mL MBP-pST3Gal1 at 30 °C for 20 h. A control reaction was also set up omitting the sialic acid donor. The reaction mixtures were then loaded onto an amylose column to remove the MBP-pST3Gal1 and the flowthrough and washes were pooled, concentrated and exchanged into 100 mM ammonium bicarbonate pH 8.

Intact protein mass spectrometry

Samples were diluted to 2.5 ng/uL in 0.1% formic acid + 5% acetonitrile and subjected to intact mass analysis. Samples (5 µL per injection) were subjected to liquid chromatography with coupled electrospray mass spectrometry (Waters nanoACQUITY UPLC with Waters Xevo G2 qTOF mass spectrometer) equipped with a Zorbax 300SB-C8 column (Agilent) and eluted using a gradient of 5% to 90% acetonitrile (0.1% formic acid). The protein elution peak was integrated

and deconvolution from the multiple charged species was performed using MaxEnt1 as part of Waters MassLynx 4.1.

Glycan engineering of A1AT

Preparation of asialo-A1AT

A1-antitrypsin was bought as commercially available Prolastin C. Protein batches were desialylated by incubation with 10-40 µg/mL His6-MBP-NedAcat per 10 mg Prolastin C in 20 mM sodium phosphate pH 6.8, 1x cOmplete protease inhibitor cocktail EDTA-free (Pierce) and gently shaken for 16 h at 37 °C. Reaction mixture was diluted 1:2 with water prior to loading onto pre-equilibrated amylose resin (1 mL resin bed per 10 mg of A1AT; 10 mM sodium phosphate buffer pH 6.2) for the removal of the sialidase. Reaction mixture was passed through by gravity flow, resin was washed and combined with flow through. Combined solution was loaded onto a pre-equilibrated anion exchange Q column (buffer A: 10 mM sodium phosphate buffer pH 6.2, 5 mL bed volume), washed and eluted with a gradient buffer B (100 mM sodium phosphate pH 6.2) to remove any residual sialidase. Collected fractions were checked for protein via SDS-PAGE and protein was combined and concentrated via ultrafiltration (Amicon devices, 10 kDa cut-off). Concentrated protein was further purified on a Sephadex 75 and pooled fractions were concentrated again (“asialo-A1AT”). Buffer was exchanged to 200 mM Tris pH 8, with a final protein concentration of around 20 mg/mL.

Alternatively, His6-NedAcat immobilized on Streptavidin beads was used for desialylation of Prolastin (10 mg/mL) by rotation at 37 °C for 24 h in 20 mM sodium phosphate pH 6.8 with 1x cOmplete protease inhibitor and 3.6 mM methionine. Monobasic sodium phosphate was used to adjust pH to 6.2 and two consecutive Q FastFlow anion exchange columns (as above) were used to remove leaked sialidase. The final product was concentrated, and buffer exchanged to 200 mM Tris pH 8 using ultrafiltration (Amicon, 3 kDa MWCO). Sialidase activity tests were performed at all stages of the sialidase removal to track and ensure complete removal.

Sialidase activity test

2 µL of sample were mixed with 2 µL of 10 mM 4-methylumbelliferyl N-acetyl-alpha-D-neuraminic acid (Carbosynth, diluted in water from 50 mM stock solution in DMF) and 16 µL 20

mM sodium phosphate buffer at pH 6.5. The reaction mixture was incubated at 37 °C. A time-dependent change in fluorescence was measured over one hour by removing 3 µL and adding those to 57 µL of stop solution (0.133 M glycine, 0.17 M sodium carbonate, 60 mM sodium chloride pH 10.6) in a dark 96 well half-area plate (Corning). Fluorescence of the released MU was determined at 365 nm excitation and 450 nm emission on a Biotek Synergy H1 plate reader.

Resialylation of A1AT

Asialo-A1AT resialylation with 7OHSA or 7FSA was achieved with an MBP fusion protein of an α2,6-ST from *Photobacterium* sp. JT-ISH-224 (PsST). In a typical reaction, 10 mg asialo-A1AT in 100 mM Tris, 1x c0mplete protease inhibitor cocktail, 20 mM MgCl2, 12 µM DANA (sialidase inhibitor), 5 mM CMP-OHSA/7FSA and 75 units alkaline phosphatase (Sigma) were incubated along with 100 µg/mL PsST at 37 °C for 48 h. Donor sugar and ST were added after 8 h and 24 h, respectively, and pH was carefully monitored over the course of the experiment. The reaction mixture was spun to remove any solids and processed as before on a Q column and a Sephadex 75 column, respectively (see above). Note: resialylated A1AT is retained longer on the Q column due to significantly more negative charges.

The desialylation/resialylation was followed by analytical glycan analysis deploying HPAEC-PAD and LC-MS.

Free glycan analysis.

N-Glycans were released with PNGase F (NEB, glycerol free) according to the manufacturer's protocol with minor changes. Briefly, a 150 µg glycoprotein sample was dried under vacuum and subsequently dissolved in 10 µL 0.1% SDS and incubated for 10 min at 95 °C. After cooling, 4 µL 1% NP-40, 2 µL glycobuffer, 3 µL water and 1 µL PNGase F were added to the solution and incubated for at least 4 h at 37 °C to allow complete N-glycan liberation. Reaction was stopped with 50 µL acetonitrile and sequentially purified over packed C18 (25 mg) and activated non-porous carbon (50 mg) material. Purified glycans were finally eluted with 30% acetonitrile in 0.1% TFA, dried under vacuum and freeze-dried from 1 mL water. Filtered and sonicated

samples were analysed with HPAEC-PAD (Thermo Scientific Dionex) using a P200 column according to manufacturer's recommendations, with 5 min isocratic elution with 10% B (1 M sodium hydroxide) followed by a gradient with constant 10% B and 0 to 50% D (1 M sodium acetate) with curve 7. Column regeneration and re-equilibration was performed with a 2 min pulse of 50% B and 50% D followed by constant 10% B for at least 6 min (Figure S1).

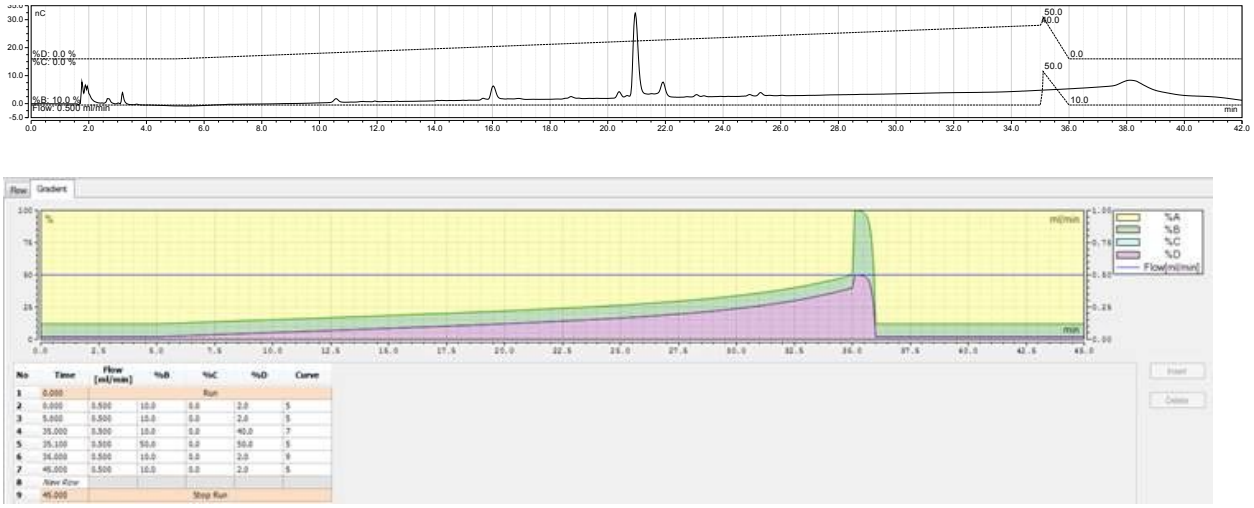

Figure S1: Gradients used for HPAEC-PAD N-glycan analysis

LS-MS analysis of released glycans

Analysis of the released glycans (see above) by LC-MS was performed on a Xevo G2S qTOF mass spectrometer attached to a nanoACQUITY UPLC (Waters Corporation). Separation of dissolved samples was performed on a Hypercarb column (Thermo Scientific) heated to 30 °C at a flow of 7 µL/min. A 5 min isocratic elution at 90% A (5 mM ammonium formate pH 5 with 5% acetonitrile), 10% B (acetonitrile with the addition of 5% 200 mM ammonium formate pH 5) was followed by a linear gradient to 30% B in 25 min. Signals were measured in positive ion mode. Results were evaluated using MassLynx 4.1 (Waters).

Protein Uptake Assay

Protein labeling

Protein samples (6-8 mg/mL) in 20 mM sodium phosphate pH 8 were mixed with 3 eq BODIPY-FL-NHS and incubated for 12 h at 4 °C while gently shaking. Reaction was quenched by adding 100 mM Tris pH 8 and further incubation for 4 h. Labelled proteins were separated from free dye by SEC processing using PD-10 columns and eluted with 20 mM Tris buffer pH 7.5 containing 150 mM NaCl. Finally, labelling efficiency was determined by OD measurements according to vendor recommendations.

Cell culture

1 x 10^6 HepG2 cells were seeded onto Biocoat Poly-D lysine coated, 12 mm, #1, coverslips (Corning) in 6-well plates the day prior to the assay. The next day, the medium was aspirated and cells were washed once with PBS. 10 uM of each test protein diluted in Opti-MEM was then added to each well and incubated for 3 hours in a tissue culture incubator at 37 °C to allow protein uptake to occur. After 3 h the protein solutions were aspirated from the wells and the cells were immediately washed twice with ice cold PBS to stop further reactions. Cells were fixed with 10% formalin for 10 min and the fixative was washed off with PBS 3x for 5 min. The coverslips were mounted onto glass slides using Prolong Gold Anti-fade with DAPI Mounting Medium and allowed to cure overnight. Three independent experiments were performed and each protein sample was done in duplicates for each experiment.

Microscopy Imaging and Analysis

Glass slides were imaged using an Olympus Fluoview FV1000 Confocal Microscope. A total of six images were captured per sample and one cell was randomly selected from each image for quantification and analysis. Brightfield images were taken to aid in the selection of cell periphery to determine cell area. Fluorescent images were taken using UV light (blue) to identify cells by their nucleus via DAPI and an Argon laser at 488nm (green) to determine fluorescent protein uptake inside cells. The Fiji (ImageJ) software (NIH) was used for image analysis. Using the software, the Corrected Total Cell Fluorescence (CTCF) = Integrated density – (Area of selected cell x Mean fluorescence of background reading) was used for comparisons between different protein samples, where integrated density is the product of area and mean fluorescent intensity. Fluorescence was also corrected by the Fluorescent Dye to Protein Ratio (F/P) Ratio. For statistical analysis, GraphPad Prism was used to compare the CTCF across samples using one way ANOVA and a post-hoc Bonferroni test.

Pharmacokinetic profile determination in CD-1 Mice

All animal procedures were approved by the National Research Council Human Health Therapeutics Research Centre animal care committee (ACC) in compliance with the Canadian Council of Animal Care (CCAC). Unmodified and modified proteins (A1AT, Asialo-A1AT, and A1AT resialylated with 7OHSA or 7FSA) were labeled with CF770 succinimidyl ester (Biotium Inc, Fremont, CA, USA) using methods recommended by the manufacturer. Labeling was optimized such that each protein had a dye/protein ratio ranging from 0.8-1.2. CF770-labeled proteins (6 mg/kg) were injected via the tail vein in CD-1 mice (n=3 per protein), followed by serial blood sampling (~50 µL) via the submandibular vein at select time points (10 min, 1 h, 3 h, 6 h, 24 h, 48 h, and 72 h). Collected blood was stored on ice in heparin-coated tubes until analysis. Blood samples were imaged in a black 96 well plate using an IVIS Lumina III (Perkin Elmer, Waltham, Massachusetts, United States) with an excitation wavelength of 740 nm and an emission wavelength of 790 nm. Fluorescent signals were converted into blood concentration values using standard curves performed in control blood samples spiked with known protein concentrations.

Pharmacokinetic parameters were estimated using Phoenix® WinNonlin® 8.2 software (Certara, Princeton, New Jersey, USA) from individual blood concentration-time profiles. A non-compartmental approach consistent with an intravenous bolus administration was applied using the linear/log trapezoidal method to estimate the area under the blood concentration vs time curve (AUC). The calculated parameters were: AUC from the start of dose administration to the last observed quantifiable concentration (AUC_{0-t}), AUC from the start of dose administration to infinity ($AUC_{0-\infty}$), terminal half-life ($T_{1/2\lambda z}$), observed maximum concentration (C_{max}), and apparent blood clearance (CL), which was determined from the equation $CL=Dose / AUC_{0-\infty}$.

SUPPORTING FIGURES

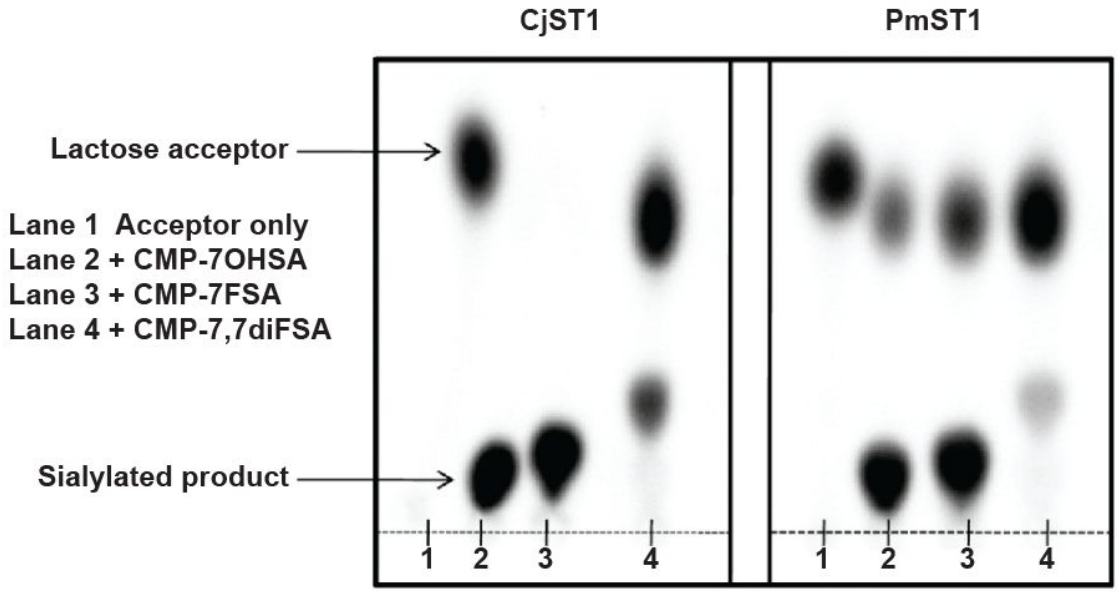

Figure S2: TLC analysis of sialyl transfer reaction of different sialic acid donors to BODIPY-lactose acceptor, catalyzed by CjST1 and PmST1.

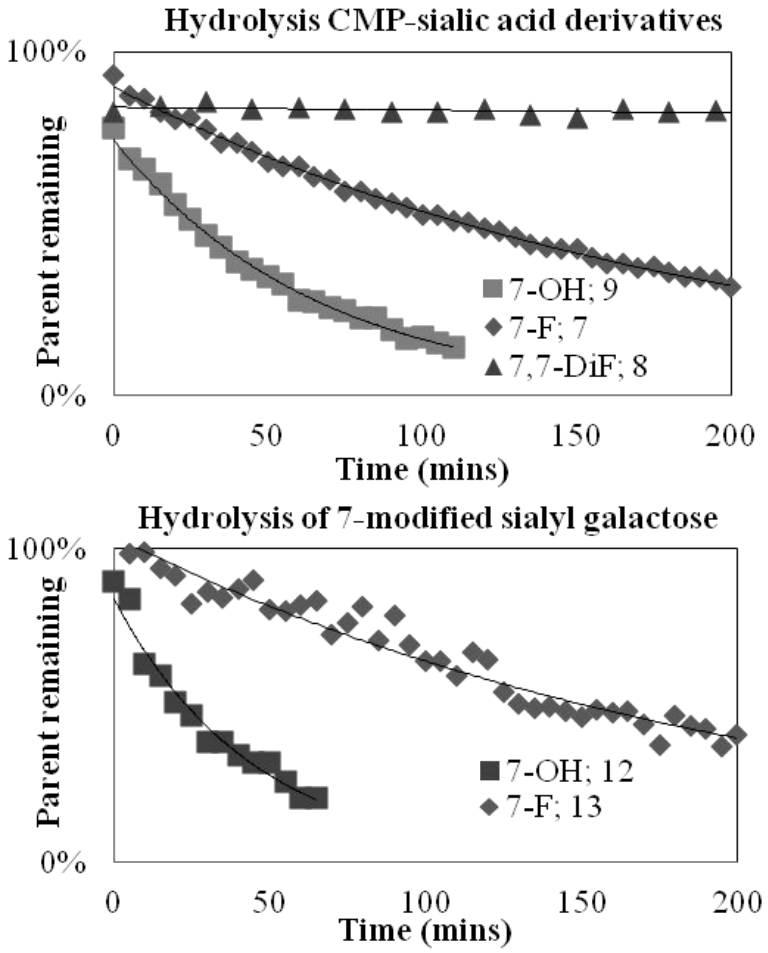

Figure S3: Measurement by ^{1}H-NMR, of rate of spontaneous hydrolysis of *Top*: CMP 7-modified sialic acids in 10 mM phosphate buffer, 100 mM NaCl, pH 7.2 and 60 °C and *Bottom*: 7-modified sialyl-galactose conjugates in 25 mM HCl and 60 °C.

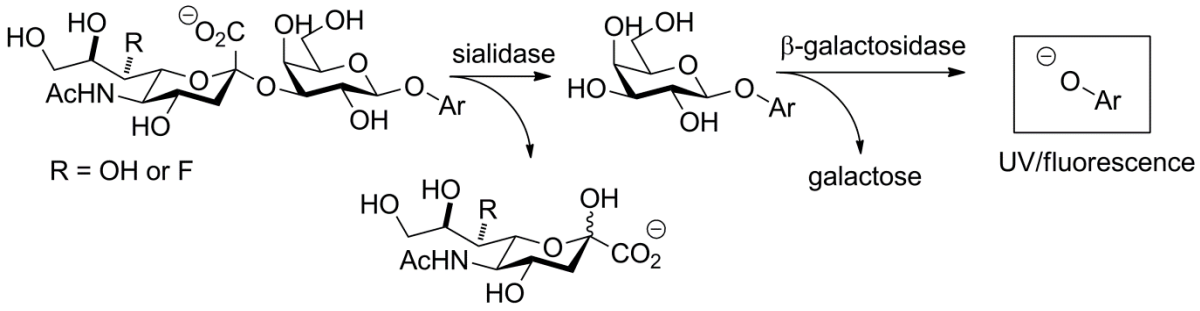

Figure S4: Overview of coupled kinetic assay.

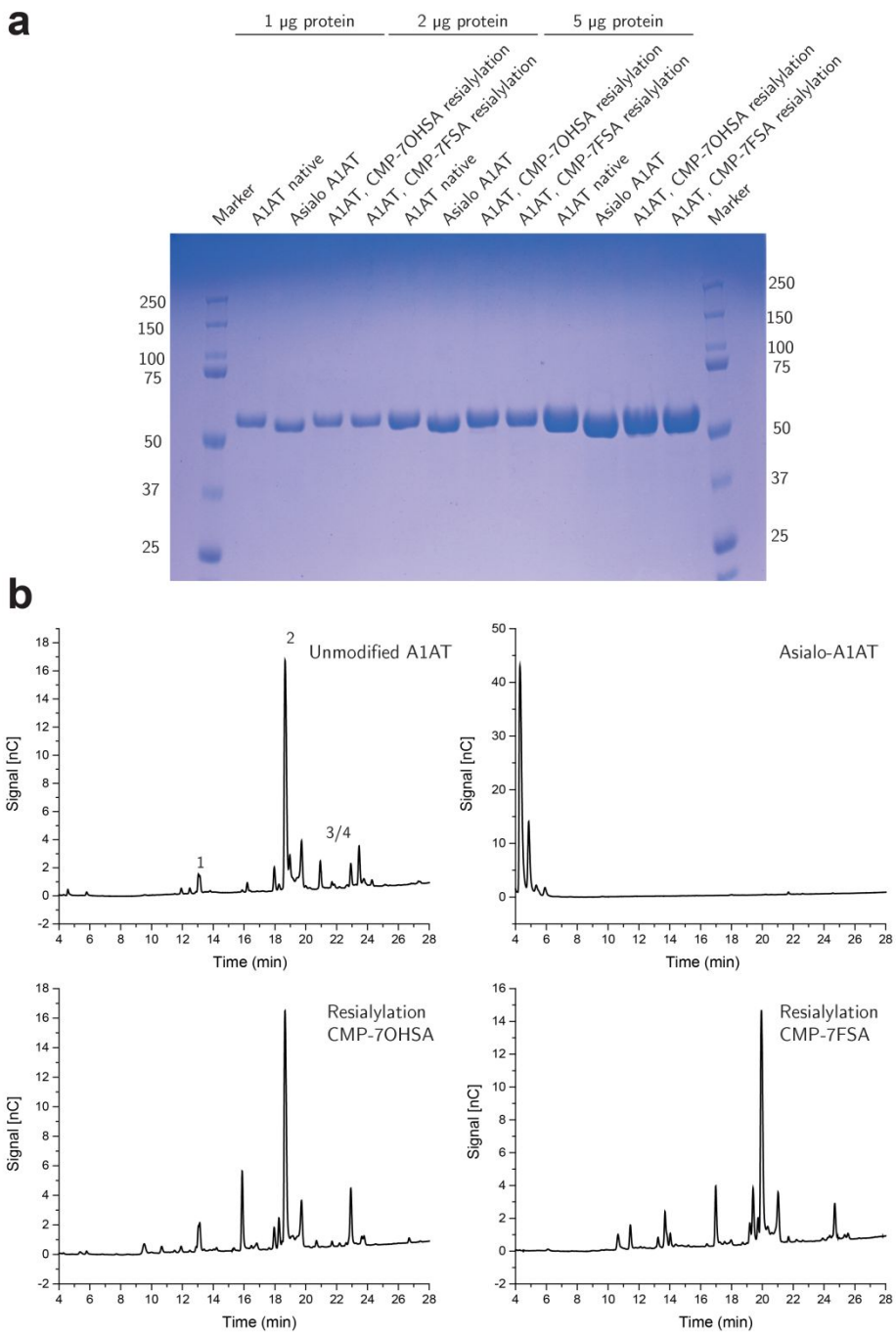

Figure S5: Protein characterization for in vivo studies by (a) SDS polyacrylamide gel electrophoresis for the intact glycoproteins and by (b) HPAEC-PAD analysis of N-glycans after cleavage off the protein. The numbers above the peaks indicate the number of (7F) sialic acids on the respective glycan. Interestingly, 7-fluorination caused a slight shift in retention times.

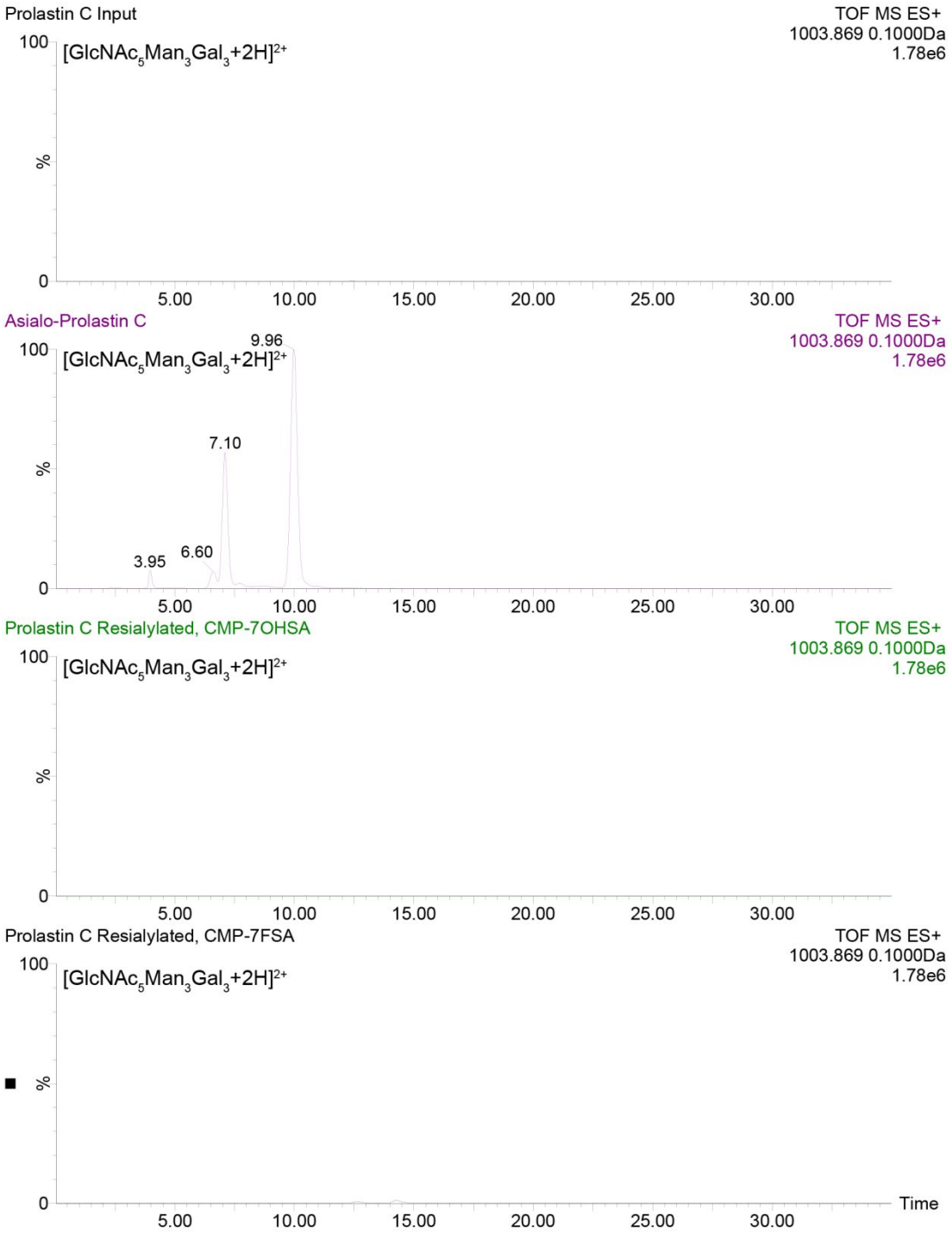

Figure S6: PGC-LC-MS analysis: Extracted ion chromatogram of triantennary N-glycan without attached sialic acids (no fucoses), two charges.

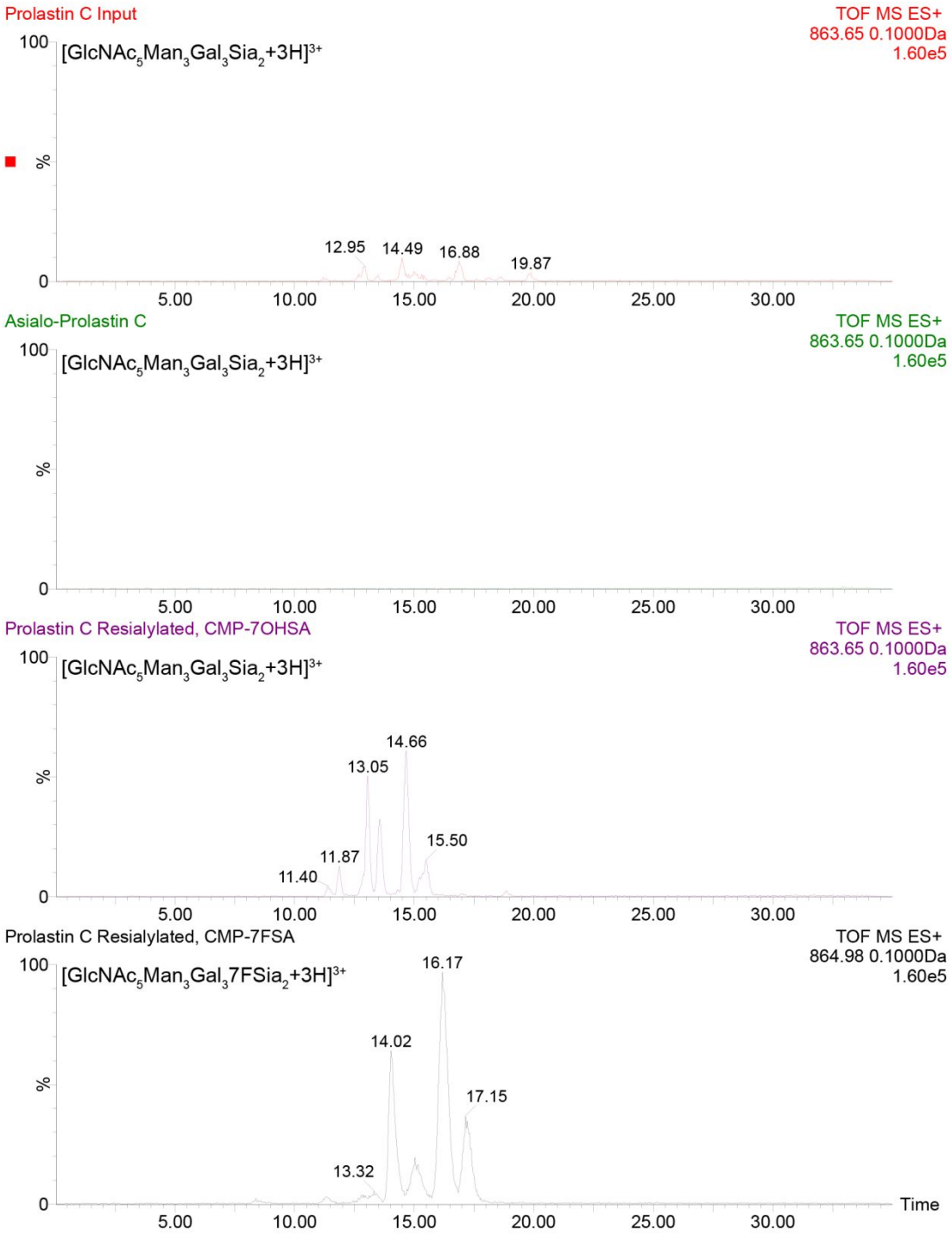

Figure S7: PGC-LC-MS analysis: Extracted ion chromatogram of triantennary N-glycan with two attached (7F) sialic acids (no fucoses), three charges. Interestingly, 7-fluorination caused a slight shift in retention times.

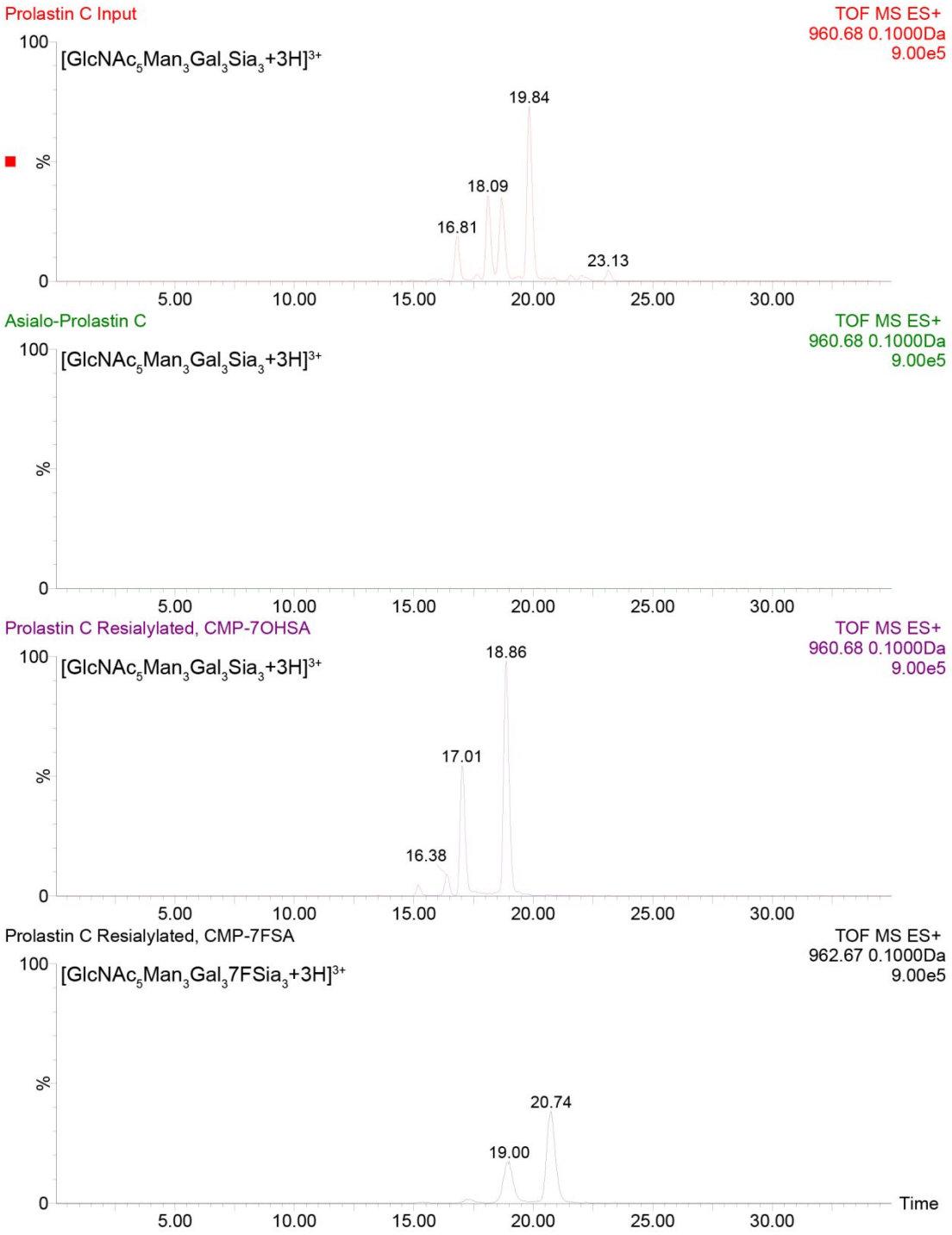

Figure S8: PGC-LC-MS analysis: Extracted ion chromatogram of triantennary N-glycan with three attached (7F) sialic acids (no fucoses), three charges. Interestingly, 7-fluorination caused a slight shift in retention times.

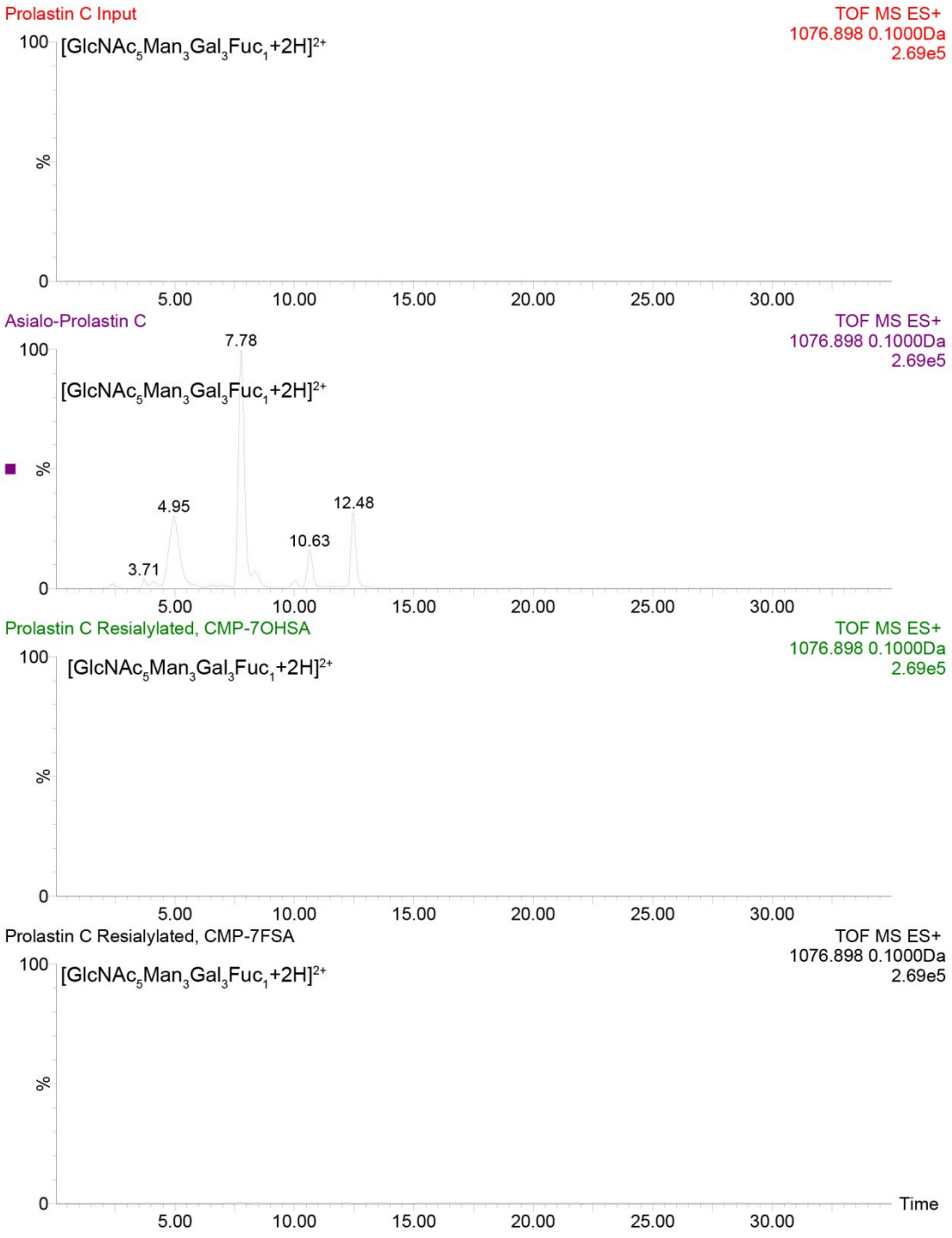

Figure S9: PGC-LC-MS analysis: Extracted ion chromatogram of triantennary N-glycan without attached sialic acids (one fucose), two charges.

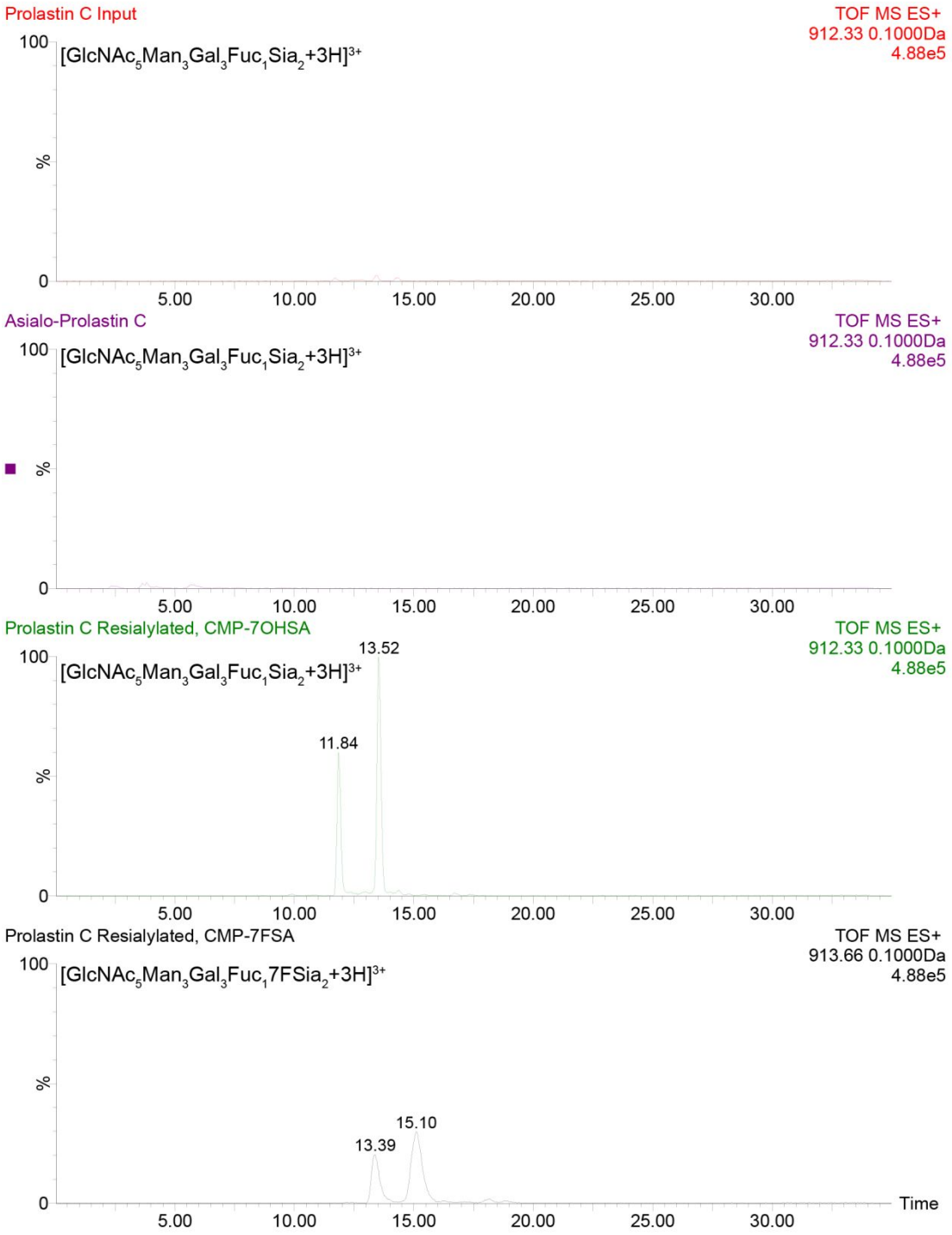

Figure S10: PGC-LC-MS analysis: Extracted ion chromatogram of triantennary N-glycan with two attached (7F) sialic acids (one fucose), three charges. Interestingly, 7-fluorination caused a slight shift in retention times.

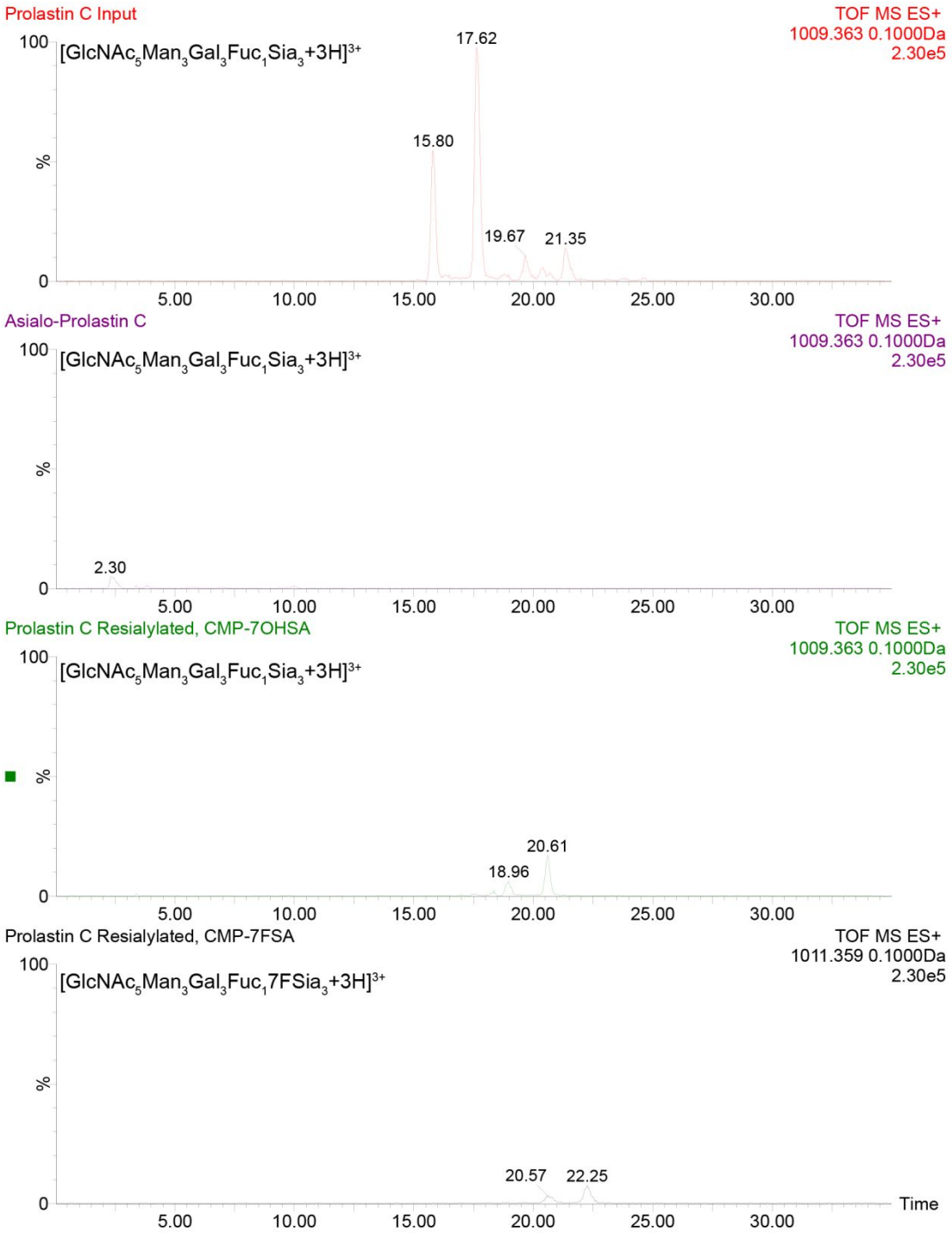

Figure S11: PGC-LC-MS analysis: Extracted ion chromatogram of triantennary N-glycan with three attached (7F) sialic acids (one fucose), three charges. Interestingly, 7-fluorination caused a slight shift in retention times.

SUPPORTING TABLES

Table S1. Pharmacokinetic parameter estimates of unmodified A1AT, Asialo A1AT, and A1AT resialylated with CMP-7OHSA or CMP-7FSA determined from non-compartmental analysis of blood concentration-time profiles following administration of a 6 mg/kg intravenous bolus to naïve mice (n=3). Results are shown as means ± SD.

		Unmodified and modified glycoproteins			
Parameter	**Unit**	Unmodified A1AT	Asialo A1AT	CMP-7OHSA Resialylation	CMP-7FSA Resialylation
C_{max}	μg/mL	28.2 ± 7.8	13.3 ± 2.9	17.6 ± 3.7	24.5 ± 4.7
$T_{1/2\lambda z}$	hr	13.0 ± 1.4	2.7 ± 0.9	4.6 ± 0.8	13.8 ± 1.1
$AUC_{0\text{-}\tau}$	μg*hr/mL	355 ± 58	8.2 ± 1.2	103 ± 40	227 ± 29
$AUC_{0-\infty}$	μg*hr/mL	363 ± 61	8.9 ± 1.3	116 ± 24	245 ± 27
CL	mL/hr/kg	16.8 ± 2.7	684 ±103	53.4 ± 12.3	24.6 ± 2.6

C_{max}: Observed peak concentration. $T_{1/2\lambda z}$: Terminal half-life. $AUC_{0\text{-}\tau}$: area under the blood concentration time curve from time 0 to last measured concentration. $AUC_{0-\infty}$: area under the blood concentration time curve from time 0 to infinity. CL: apparent clearance.

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