Title: Quantifying Siglec-Sialylated Ligand Interactions: A versatile ^{19}F-T_2 CPMG Filtered Competitive NMR Displacement Assay

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SUPPORTING MATERIALS AND METHODS

Synthesis of diF α2,6SLN

N-difluoroacetyl-D-mannosamine (1): Difluoroacetic anhydride (70 μL, 0.56 mmol) and triethylamine (120 μL, 0.84 mmol) were added at 0ºC to a solution of commercially available mannosamine (50 mg, 0.28 mmol) in MeOH (2 mL). After stirring overnight at room temperature the crude was neutralized with amberlite, evaporated, and purified by silica gel column chromatography DCM:MeOH (8:2) to provide a mixture of alpha and beta anomers (α 60%, β 40%) of compound **1** (52 mg, 0.20 mmol, 72%).

^{1}H NMR (500 MHz, D_2O) data for α anomer: δ 6.20 (t, *J* = 53.2 Hz, 1H, CHF_2), 5.19 (s, 1H, H-1), 4.42 (d, *J* = 4.6 Hz, 1H, H-2), 4.11 (dd, *J* = 9.9, 4.6 Hz, 1H, H-3), 3.95 – 3.77 (m, 3H, H-6, H-5), 3.65 (t, *J* = 9.9 Hz, 1H, H-4); data for β anomer: δ 6.24 (t, *J* = 53.6 Hz, 1H, CHF*2*), 5.10 (s, 1H, H-1), 4.55 (d, *J* = 4.4 Hz, 1H, H-2), 3.95 – 3.77 (m, 4H, H-6, H-5, H-3), 3.56 (t, *J* = 9.9 Hz, 1H, H-4). ESI MS: m/z: calcd for $C_8H_{13}F_2NO_6Na$: 280.1; found: 280.1 $[M + Na]^+$.

N-(difluoroacetyl)-α-Neuraminic acid (2): To a solution of compound **1** (52 mg, 0.20 mmol) in a buffer containing 100 mM Tris pH 7.5, 20 mM $MgCl_2$, sodium pyruvate (107 mg, 0.97 mmol) and sialic acid aldolase (0.5 U) were added. The reaction was placed at 37°C and agitated at 220 rpm overnight until analytical HPLC shows full conversion. The enzyme was removed by heating and centrifuging, and the crude was purified using a carbon-based cartridge and a cotton column to obtain pure compound **2** (56 mg, 0.16 mmol, 80%).

^{1}H NMR (500 MHz, D_2O) δ 6.19 (t, *J* = 53.6 Hz, 1H, CHF_2), 4.16 – 4.09 (m, 2H, H-4, H-6), 4.04 (m, 1H, H-5), 3.84 (dd, *J* = 11.8, 2.7 Hz, 1H, H-9), 3.76 (ddd, *J* = 9.3, 6.5, 2.6 Hz, 1H, H-8), 3.63 (m, 1H, H-9), 3.49 (dd, *J* = 9.2, 1.2 Hz, 1H, H-7), 2.24 (dd, *J* = 12.8, 4.9 Hz, 1H, H-3eq), 1.85 (t, *J* = 13.0 Hz, 1H, H-3ax). ESI MS: m/z: calcd for $C_{11}H_{17}F_2NO_9Na$: 368.1; found: 368.1 $[M + Na]^+$.

Cytidine-5′-Monophosphate-N-(difluoroacetyl)-β-Neuraminic acid (3): Cytidine triphosphate (100 mg, 0.21 mmol) was added to a solution of compound X (56 mg, 0.16 mmol) in a buffer containing 100 mM Tris, 20 mM $MgCl_2$, and the pH was adjusted to 8.5. CMP-neuraminic acid synthetase (0.5 U) was added and the reaction was stirred at 220 rpm and 37 ºC overnight. The enzyme was removed by heating and centrifuging and the crude was purified, first using a carbon-based cartridge and then on a preparative HPLC with a HILIC column to obtain **3** (44 mg, 0.07 mmol, 42%).

^{1}H NMR (500 MHz, D_2O) δ 8.00 (dd, *J* = 7.7, 3.6 Hz, 1H, CH=CH), 6.19 (t, *J* = 53.2 Hz, 1H, CHF_2), 6.15 (d, *J* = 7.7 Hz, 1H, CH=CH), 5.98 (d, *J* = 4.4 Hz, 1H, CH), 4.37 – 4.29 (m, 3H, H-6, 2 x CH), 4.27 – 4.23 (m, 3H, CH2, CH), 4.20 – 4.14 (m, 1H, H-4), 4.07 (t, *J* = 10.3 Hz, 1H, H-5), 3.94 (ddd, *J* = 9.4, 6.7, 2.5 Hz, 1H, H-8), 3.88 (dd, *J* = 11.8, 2.5 Hz, 1H, H-9), 3.61 (dd, *J* = 11.9, 6.6 Hz, 1H, H-9), 3.44 (dd, *J* = 9.5, 1.1 Hz, 1H, H-7), 2.52 (dd, *J* = 12.6, 4.1 Hz, 1H, H-3eq), 1.67 (m, 1H, H-3ax). ESI MS: m/z: calcd for $C_{20}H_{31}F_2N_4O_{16}PNa$: 675.1; found: 675.1 $[M + Na]^+$.

α-N-(difluoroacetyl)-neuraminyl-(2-6)-β-D-galactosyl-(1-4)-N-acetyl-β-D-glucosamine (diF α2,6SLN): N-Acetyl lactosamine (5 mg, 0.013 mmol) and donor **3** (13 mg, 0.020 mmol, 1.5 eq per acceptor added) were dissolved at a final acceptor concentration of 100 mM in a Tris buffer solution (100 mM, pH 7.5) containing MgCl2 (20 mM) and BSA (0.1% wt/wt). *Photobacterium damselae* α2,6 sialyltransferase (10 μg per μmol acceptor) and CIAP (10 U/μL) were added, and the reaction mixture was incubated for 4 hours at 37 ºC with moderate shaking. The enzyme was removed by heating and centrifuging and the crude was purified, first using a carbon-based cartridge, then on a preparative HPLC with a HILIC column and finally on a biogel P2 column to obtain **diF α2,6SLN** (4 mg, 0.006 mmol, 43%).

^{1}H NMR (500 MHz, D_2O) δ 6.15 (t, *J* = 53.6 Hz, 1H, CF_2H), 5.19 (d, *J* = 2.4 Hz, 1H, H-1GlcNAcα), 4.74 (m, 1H, H-1GlcNAcβ), 4.45 (d, *J* = 8.0 Hz, 1H, H-1Gal), 4.05 – 3.79 (m, 11H, H-5Neu5Ac, H-2GlcNAcα, H-6GlcNAc, H-9Neu5Ac, H-6Gal, H-4Gal, H-5GlcNAcα, H-3GlcNAcβ, H-8Neu5Ac, H-5Gal), 3.71 (m, 12H, H-2GlcNAcβ, H-9Neu5Ac, H-4Neu5Ac, H-6Neu5Ac, H-5GlcNAcβ, H-3Gal, H-3GlcNAcα, H-4GlcNAc), 3.54 (ddd, *J* = 10.3, 5.2, 3.0 Hz, 5H, H-6Gal, H-7Neu5Ac, H-2Gal), 2.68 (dd,

J = 12.4, 4.7 Hz, 1H, H-3Neu5Ac eq), 2.07 (s, 3H, CH3), 1.74 (t, *J* = 12.2 Hz, 1H, H-3Neu5Ac ax). ESI MS: m/z: calcd for $C_{25}H_{40}F_2N_2O_{19}Na$: 733.2; found: 733.2 $[M + Na]^+$.

Synthesis of sTn derivative (17)

1,3,4,6-Tetra-O-acetyl-2-deoxy-2-trifluoroacetamido-α,β-Dgalactopyranose (5).[49] To a solution of D-galactosamine hydrochloride (10.5 g, 48.7 mmol) in dry MeOH (60 mL), MeONa 0.5M in MeOH (117 mL, 58.4 mmol) was added and it was stirred for 20 minutes at room temperature. Trifluoroacetic anhydride (7.2 mL, 51.1 mmol) and triethylamine (6.8 mL, 48.7 mmol) were added at 0 ºC, and the reaction was stirred for 30 minutes at 0 ºC and overnight at room temperature. The crude was evaporated, and the resulting solid product was used in the next step without further purification. Acetic anhydride (75 mL, 793 mmol) and DMAP (3.2 g, 23.4 mmol) were added to a solution of the galactosamine in pyridine (150 mL), at 0 ºC, and the solution was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate, washed with HCl 1M, $NaHCO_3$ sat. and NaCl sat., dried over anhydrous $MgSO_4$, filtered, evaporated, and dried in vacuum. Flash column chromatography in hexane/ethyl acetate (1:1) gave a mixture of α, β anomers of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-trifluoroacetamido-D-galactopyranose **5** (15.3 g, 34.5 mmol, 71%).

^{1}H-NMR (500 MHz, Chloroform-d) data for α anomer: δ 6.42 (bs, 1H, NH), 6.28 (d, *J* = 3.27 Hz, 1H, H-1), 5.47 (dd, *J* = 3.2, 1.4 Hz, 1H, H-4), 5.29 (dd, *J* = 11.4, 3.2 Hz, 1H, H-3), 4.68 (m, 1H, H-2), 4.28–4.03 (m, 3H, H-5, H-6a, H-6b), 2.18–2.00 (m, 12H, CH3); data for β anomer: δ 6.76 (bs, 1H, NH), 5.76 (d, *J* = 8.8 Hz, 1H, H-1), 5.41 (dd, *J* = 3.4, 1.2 Hz, 1H, H-4), 5.17 (dd, *J* = 11.3, 3.3 Hz, 1H, H-3), 4.49 (m, 1H, H-2), 4.28–4.03 (m, 3H, H-5, H-6a, H-6b), 2.18–2.00 (m, 12H, CH_3). ESI MS: m/z: calcd for $C_{16}H_{20}F_3NO_{10}Na$: 466.1; found: 466.1 $[M + Na]^+$.

p-Tolyl 3,4,6-tri-O-acetyl-2-deoxy-2-N-trifluoroacetamido-1-thio-β-D-galactopyranoside (6).[50] Compound **5** (15.3 g, 34.5 mmol) and 4-methylbenzenethiol (4.7 g, 38.1 mmol) were dissolved in dry DCM (100 mL), followed by slow addition of boron trifluoride etherate (14.6 mL, 118.3 mmol) at 0 ^{O}C. The reaction was stirred at room temperature overnight under argon. Upon completion, the reaction was washed with NaOH 1M, concentrated and purified using flash column chromatography (Hex:EtOAc, 7:3) to afford the product **6** (7.2 g, 14.2 mmol, 41%).

^{1}H NMR (500 MHz, Chloroform-d) δ 7.39 (d, *J* = 8.1 Hz, 2H, Ar), 7.11 (d, *J* = 8.1 Hz, 2H, Ar), 6.94 (bs, 1H, NH), 5.37 (dd, *J* = 3.3, 1.1 Hz, 1H, H-4), 5.20 (dd, *J* = 10.8, 3.3 Hz, 1H, H-3), 4.81 (d, *J* = 10.4 Hz, 1H, H-1), 4.27 – 4.05 (m, 3H, H-6, H-2), 3.93 (m, 1H, H-5), 2.33 (s, 3H, CH_3-Ar), 2.11 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.95 (s, 3H, CH_3). ESI MS: m/z: calcd for $C_{21}H_{24}F_3NO_8S$: 530.1; found: 530.1 $[M + H]^+$.

Phenylmethyl *N*-[5-[[3,4,6-tri-*O*-acetyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (7). Donor **6** (2.0 g, 3.95 mmol), benzyl (5-hydroxypentyl)carbamate[51] (1.4 g, 5.90 mmol) and activated 5 Å powdered molecular sieves (2.0 g/mmol) were dissolved in anhydrous dichloromethane (90 mL) under argon atmosphere and then cooled to -40 ºC followed by the addition of N-iodosuccinimide (2.12 g, 9.5 mmol) and trifluoromethanesulfonic acid (0.35 mL, 3.95 mmol). The reaction mixture was stirred at 40 ºC for 30 minutes until the total conversion of the donor in TLC [EtOAc/Hex (1:1)]. Afterwards, the reaction was quenched with triethylamine and warmed to room temperature. The mixture was diluted with DCM, filtered through celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography EtOAc/Hex (1:1) to provide compound **7** as a white solid (1.4 g, 2.3 mmol, 57%).

^{1}H NMR (500 MHz, Chloroform-d) δ 7.59 (bs, 1H, NH), 7.35 – 7.25 (m, 5H, Ar), 5.34 (d, *J* = 3.4 Hz, 1H, H-4), 5.24 (m, 1H, H-3), 5.07 (m, 2H, CH_2-Ar), 4.62 (d, *J* = 8.3 Hz, 1H, H-1), 4.13 (m, 3H, H-6, H-2), 3.88 (m, 2H, H-5, OCH_2), 3.43 (m, 1H, OCH_2), 3.12 (m, 2H, $NHCH_2$), 2.13 (s, 3H, CH_3), 2.02 (s, 3H, CH_3), 1.96 (s, 3H, CH_3), 1.59 – 1.43 (m, 4H, CH_2) 1.34 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{27}H_{35}F_3N_2O_{11}$: 621.2; found: 621.2 $[M + H]^+$.

Phenylmethyl *N*-[5-[[2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (8). To a solution of **7** (1.3 g, 2.1 mmol) in anhydrous methanol (11 mL) and under an argon atmosphere, 0.5 M sodium methoxide solution was added (1.26 mL, 0.63 mmol) at 0 ºC. The mixture was stirred for 30 minutes at room temperature followed by treatment with amberlite resin. Then, it was filtered and concentrated to provide compound **8** without further purification.

^{1}H NMR (500 MHz, MeOD) δ 7.38 – 7.25 (m, 5H, Ar), 5.08 (s, 2H, CH_2Ar), 4.43 (d, *J* = 8.4 Hz, 1H, H-1), 4.00 (dd, *J* = 10.8, 8.4 Hz, 1H, H-2), 3.92 – 3.83 (m, 2H, H-4, OCH_2), 3.76 (m, 1H, H-6), 3.69 (dd, *J* = 10.8, 3.3 Hz, 1H, H-3), 3.56 – 3.43 (m, 3H, H-6, H-5, OCH_2), 3.09 (m, 2H, $NHCH_2$), 1.60 – 1.44 (m, 4H, CH_2), 1.35 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{21}H_{29}F_3N_2O_8Na$: 517.2; found: 517.2 $[M + Na]^+$.

Phenylmethyl *N*-[5-[[4,6-O-benzylidene-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (9). To a solution of **8** (1.6 g, 3.2 mmol) in acetonitrile (10 mL), benzaldehyde dimethyl acetal (850 µL, 5.7 mmol) and CSA (0.15 g, 0.65 mmol) were added and the reaction was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate, washed with $NaHCO_3$ sat. and NaCl sat., dried over anhydrous $MgSO_4$, filtered, evaporated, and dried in vacuum. The residue was purified by silica gel column chromatography EtOAc/Hex (1:1) to provide compound **9** (1.14 g, 1.96 mmol, 61%).

^{1}H NMR (500 MHz, MeOD) δ 7.57 (m, 2H, Ar), 7.39 – 7.26 (m, 8H, Ar), 5.64 (s, 1H, CHAr), 5.08 (s, 2H, CH_2Ar), 4.54 (d, *J* = 8.3 Hz, 1H, H-1), 4.24 – 4.13 (m, 3H, H-4, H-6), 4.07 (dd, *J* = 11.0, 8.3 Hz, 1H, H-2), 3.93 – 3.85 (m, 2H, H-3, OCH_2), 3.56 (m, 1H, H-5), 3.49 (m, 1H, OCH_2), 3.09 (m, 2H, $NHCH_2$), 1.59 (m, 2H, CH_2), 1.50 (m, 2H, CH_2), 1.38 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{28}H_{33}F_3N_2O_8$: 583.2; found: 583.2 $[M + H]^+$.

Phenylmethyl *N*-[5-[[4,6-O-benzylidene-3-O-benzyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (10). To a solution of **9** (400 mg, 0.69 mmol) in dry dichloromethane (1 mL) and pyridine (1 mL), benzoyl chloride (100 µL, 0.83 mmol) was added at 0 ºC. The reaction was stirred 2 hours at 0 ºC upon completion by TLC. The crude was evaporated and purified by flash column chromatography EtOAc/Hex (1:1) to afford compound **10** (430 mg, 0.63 mmol, 91%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.02 (m, 2H, Ar), 7.57 (t, *J* = 7.4 Hz, 1H, Ar), 7.50 (dd, *J* = 6.8, 2.8 Hz, 2H, Ar), 7.42 (t, *J* = 7.6 Hz, 2H, Ar), 7.39 – 7.28 (m, 8H, Ar), 6.94 (bs, 1H, NH), 5.56 (s, 1H, CHAr), 5.51 (d, *J* = 9.6 Hz, 1H, H-3), 5.09 (m, 2H, CH_2Ar), 4.83 (d, *J* = 8.1 Hz, 1H, H-1), 4.50 (d, *J* = 3.5 Hz, 1H, H-4), 4.39 (m, 2H, H-6, H-2), 4.11 (d, *J* = 12.4 Hz, 1H, H-6), 3.99 (m, 1H, OCH_2), 3.56 (bs, 1H, H-5), 3.49 (m, 1H, OCH_2), 3.17 (m, 2H, $NHCH_2$), 1.62 (m, 2H, CH_2), 1.52 (m, 2H, CH_2), 1.39 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{35}H_{37}F_3N_2O_9$: 686.2; found: 686.2 $[M + H]^+$.

Phenylmethyl *N*-[5-[[3-O-benzyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (11). Compound **10** (300 mg, 0.44 mmol) was dissolved in 40 mL of 80% acetic acid and heated to 50 ºC for 2 hours. Once complete, the reaction was diluted with dichloromethane, washed with $NaHCO_3$, brine and dried over anhydrous $MgSO_4$. The concentrated material was chromatographed (Hex:EtOAc, 2:3) to yield the desired compound **11** (250 mg, 0.42 mmol, 95%).

^{1}H NMR (500 MHz, Acetone) δ 8.40 (bs, 1H, NH), 7.87 (m, 2H, Ar), 7.51 (m, 1H, Ar), 7.37 (m, 2H, Ar), 7.18 (m, 5H, Ar), 6.17 (bs, 1H, NH), 5.04 (dd, *J* = 11.1, 3.1 Hz, 1H, H-3), 4.93 (d, *J* = 8.4 Hz, 2H, CH_2Ar), 4.65 (d, 1H, H-1), 4.47 (m, 1H, H-2), 4.21 (m, 1H, H-4), 3.82 – 3.66 (m, 3H, H-6, OCH_2), 3.56 (t, 1H, H-5), 3.42 (m, 1H, OCH_2), 3.00 (m, 2H, $NHCH_2$), 1.46 (m, 2H, CH_2), 1.39 (m, 2H, CH_2), 1.25 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{28}H_{33}F_3N_2O_9$: 599.2; found: 599.2 $[M + H]^+$.

Methyl-7,8,9-tri-O-acetyl-5-N,4-O-carbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[3-O-benzyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (13). Donor **12**[52] (50 mg, 0.086 mmol), acceptor **11** (56 mg, 0.093 mmol) and activated 5 Å powdered molecular sieves (2.0 g/mmol) were dissolved in a mixture of anhydrous dichloromethane and anhydrous acetonitrile (1:1; 4 mL) under argon atmosphere and then cooled to -70 ºC followed by the addition of N-iodosuccinimide (50 mg, 0.22 mmol) and trifluoromethanesulfonic acid (3.0 µL, 0.03 mmol). The reaction mixture was stirred at -70 ºC for 30 minutes until the total conversion of the donor in TLC. Afterwards, the reaction was quenched with triethylamine and warmed to room temperature. The mixture was diluted with DCM, filtered through celite, washed with 20 % aqueous $Na_2S_2O_3$ solution, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography EtOAc/Hex (3:2) to provide compound **13** (70 mg, 0.07 mmol, 77%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.02 – 7.96 (m, 2H, Ar), 7.53 (t, *J* = 7.3 Hz, 1H, Ar), 7.40 (t, *J* = 7.8 Hz, 2H, Ar), 7.36 – 7.27 (m, 5H, Ar), 5.53 – 5.39 (m, 3H, H-3 GalN, H-7 Neu5Ac, H-8 Neu5Ac), 5.07 (s, 2H, CH_2Ar), 4.74 (dd, *J* = 12.1, 2.6 Hz, 1H, H-9 Neu5Ac), 4.65 (d, *J* = 8.4 Hz, 1H, H-1g GalN, 4.58 (dd, *J* = 9.4, 1.2 Hz, 1H, H-6 Neu5Ac), 4.50 (dd, *J* = 19.4, 9.6 Hz, 1H, H-2 GalN), 4.34 (d, *J* = 3.2 Hz, 1H, H-4 GalN), 4.06 – 3.88 (m, 5H, H-5 GalN, H-4 Neu5Ac, H-9 Neu5Ac, H-6 GalN, OCH_2), 3.84 (s, 3H, CH_3), 3.68 (dt, *J* = 11.2, 6.3 Hz, 1H, H-5 Neu5Ac, 3.52 – 3.43 (m, 2H, OCH_3, H-6 GalN), 3.13 (m, 2H, $NHCH_2$), 2.87 (dd, *J* = 12.1, 3.4 Hz, 1H, H-3eq Neu5Ac), 2.49 (s, 3H, CH_3), 2.17 (s, 6H, CH_3), 2.10 (m, 1H, H-3ax Neu5Ac), 1.95 (s, 3H, CH_3), 1.62 – 1.44 (m, 4H, CH_2), 1.35 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{47}H_{56}F_3N_3O_{21}$: 1056.3; found: 1056.4 $[M + H]^+$.

Methyl-5-N-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2-deoxy-2-N-trifluoroacetamido-β-D-galactopyranosyl]oxy]penthyl]carbamate (14). To a solution of **13** (70 mg, 0.07 mmol) in anhydrous methanol (1 mL) and under an argon atmosphere, 0.5 M sodium methoxide solution was added (100 µL, 0.05 mmol) at 0 ºC. The mixture was stirred 2 hours at room temperature followed by treatment with amberlite resin. Then, it was filtered, concentrated, and purified by a C18 cartridge to provide compound **14** (50 mg, 0.06 mmol, 66%).

^{1}H NMR (500 MHz, MeOD) δ 7.39 – 7.26 (m, 5H, Ar), 5.09 (m, 2H, CH_2Ar), 4.59 (s, 1H, NH), 4.42 (d, *J* = 8.4 Hz, 1H, H-1 GalN), 3.99 (dt, *J* = 13.1, 6.6 Hz, 1H, H-2 GalN), 3.92 (m, 1H, H-6 GalN), 3.89 – 3.59 (m, 14H, CH_3, H-6 GalN, H-5 GalN, H-4 GalN, H-3 GalN, H-9 Neu5Ac, H-8 Neu5Ac, H-6 Neu5Ac, H-5 Neu5Ac, H-4 Neu5Ac, OCH_2), 3.52 (m, 1H, H-7 Neu5Ac), 3.46 (m, 1H, OCH_2), 3.09 (m, 2H, $NHCH_2$), 2.69 (dd, *J* = 12.8, 4.7 Hz, 1H, H-3eq Neu5Ac), 2.03 (s, 3H, CH_3), 1.77 (t, *J* = 12.3 Hz, 1H, H-3ax Neu5Ac), 1.60 – 1.44 (m, 4H, CH_2), 1.37 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{33}H_{48}F_3N_3O_{16}$: 799.3; found: 799.3 $[M + H]^+$.

5-N-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2- deoxy-2-N-β-D-galactopyranosyl]oxy]penthyl]carbamate (15). A solution of compound **14** (50 mg, 0.06 mmol) and potassium carbonate (3 mg, 0.02 mmol) in water was stirred overnight at room temperature. After 2 hours it was concentrated and purified by a C18 cartridge to provide compound **15** (40 mg, 0.058 mmol, 87%).

^{1}H NMR (500 MHz, D_2O) δ 7.42 (m, 5H, Ar), 5.08 (s, 2H, CH_2Ar), 4.24 (d, *J* = 8.1 Hz, 1H, H-1 GalN), 3.96 – 3.54 (m, 13H, H-4 GalN, H-5 GalN, H-6 GalN, H-4 Neu5Ac, H-5 Neu5Ac, H-6 Neu5Ac, H-7 Neu5Ac, H-8 Neu5Ac, H-9 Neu5Ac, OCH_2), 3.48 (dd, *J* = 10.4, 3.3 Hz, 1H, H-3 GalN), 3.11 (t, *J* = 6.5 Hz, 2H, $NHCH_2$), 2.80 (m, 1H, H-2 GalN), 2.73 (dd, *J* = 12.4, 4.6 Hz, 1H, H-3eq Neu5Ac), 2.03 (s, 3H, CH_3), 1.67 (t, *J* = 12.1 Hz, 1H, H-3ax Neu5Ac), 1.61 (m, 2H, CH_2), 1.49 (m, 2H, CH_2), 1.35 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{30}H_{47}N_3O_{15}Na$: 712.3; found: 712.3 $[M + Na]^+$.

5-N-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2- deoxy-2-N-acetyl-β-D-galactopyranosyl]oxy]penthyl]carbamate (16). To a solution of compound **15** (30 mg, 0.04 mmol) in a 0.5M solution of $NaHCO_3$ (2 mL), Ac_2O (51.3 µL, 0.50 mmol)

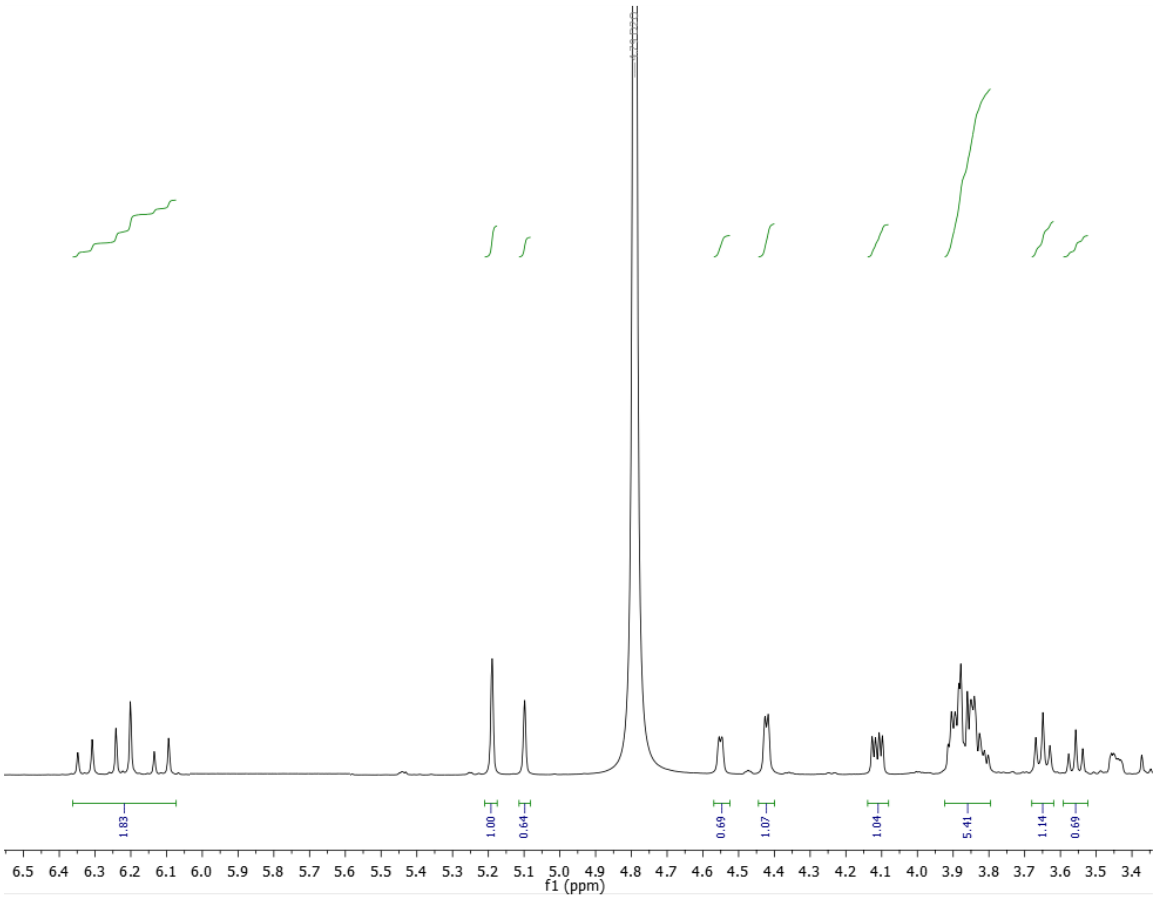
was added slowly at 0 ºC. The reaction was stirred for 1 hour and then evaporated. The resulting solid was purified by a C18 cartridge to obtain **16** (13 mg, 0.018 mmol, 41%).

^{1}H NMR (500 MHz, D2O) δ 7.49-7.37 (m, 5H, Ar), 5.10 (s, 2H, CH_2Ar), 4.41 (d, *J* = 8.5 Hz, 1H, H-1 GalN), 3.98 – 3.92 (m, 2H, H-4 GalN, H-9 Neu5Ac), 3.91 – 3.80 (m, 4H, H-2 Neu5Ac, H-6 Neu5Ac, H-8 Neu5Ac, OCH_2), 3.77 – 3.61 (m, 7H, H-5 Neu5Ac, H-6 GalN, H-9 Neu5Ac, H-4 Neu5Ac, H-3 GalN, H-5 Neu5Ac, H-6 Neu5Ac), 3.61 – 3.53 (m, 2H, H-7 Neu5Ac, OCH_2), 3.11 (t, *J* = 6.8 Hz, 2H, $NHCH_2$), 2.73 (dd, *J* = 12.4, 4.8 Hz, 1H, H-3eq Neu5Ac), 2.04 (s, 3H, CH_3), 2.00 (s, 3H, CH_3), 1.69 (t, *J* = 12.2 Hz, 1H, H-3ax Neu5Ac), 1.54 (m, 2H, CH_2), 1.48 (m, 2H, CH_2), 1.31 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{32}H_{49}N_3O_{16}Na$: 670.3; found: 670.3 $[M + Na]^+$.

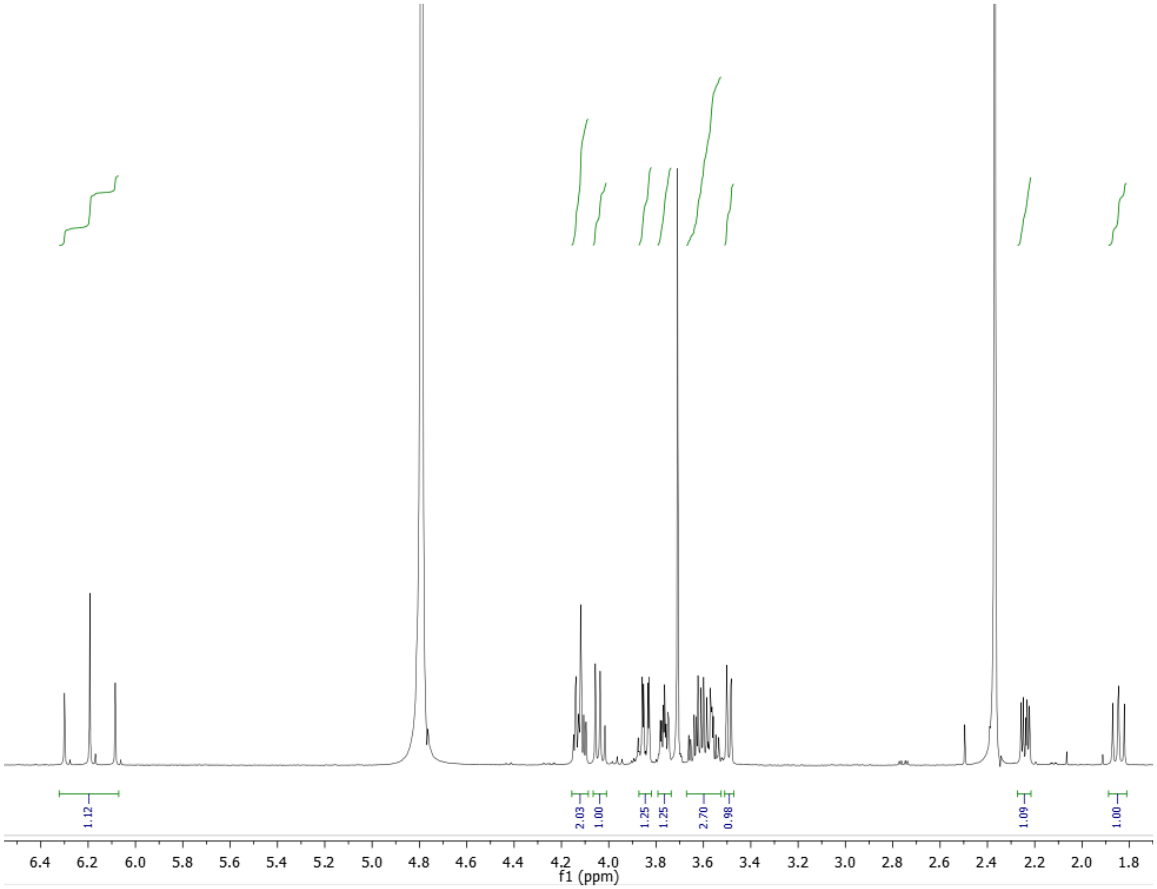
3-aminopropyl-3,5-dideoxy-D-glycero-*α*-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2- deoxy-2-N-acetyl-β-D-galactopyranoside (17). Compound **16** (13 mg, 0.018 mmol) was dissolved in MeOH (1.5 mL), followed by the addition of trifluoroacetic acid (2 µL). Then, palladium on active carbon (1.5 mg, 10 %) was added making sure everything had been poured into the solution. The reaction mixture was purged with a hydrogen-containing balloon. The reaction was left stirring for 2 hours and then evaporated. The resulting solid was purified by a C18 cartridge to obtain **17** (6.7 mg, 0.011 mmol, 64%).

^{1}H NMR (500 MHz, D_2O) δ 4.33 (d, *J* = 8.4 Hz, 1H, H-1 GalN), 3.93 – 3.70 (m, 7H, H-4 GalN, H-9 Neu5Ac, H-6 GalN, H-2 GalN, H-5 Neu5Ac, H-8 Neu5Ac, OCH_2), 3.70 – 3.47 (m, 8H, H-5 GalN, H-6 Neu5Ac, H-4 Neu5Ac, H-3 GalN, H-7 Neu5Ac, OCH_2, H-6 GalN, H-9 Neu5Ac), 2.91 (t, *J* = 7.2 Hz, 2H, $NHCH_2$), 2.64 (dd, *J* = 12.4, 4.7 Hz, 1H, H-3eq Neu5Ac), 1.95 (s, 3H, CH_3), 1.94 (s, 3H, CH_3), 1.64 – 1.55 (m, 3H, CH_2, H-3ax Neu5Ac), 1.50 (m, 2H, CH_2), 1.32 (m, 2H, CH_2). ESI MS: m/z: calcd for $C_{24}H_{43}N_3O_{14}Na$: 598.3; found: 598.3 $[M + Na]^+$.

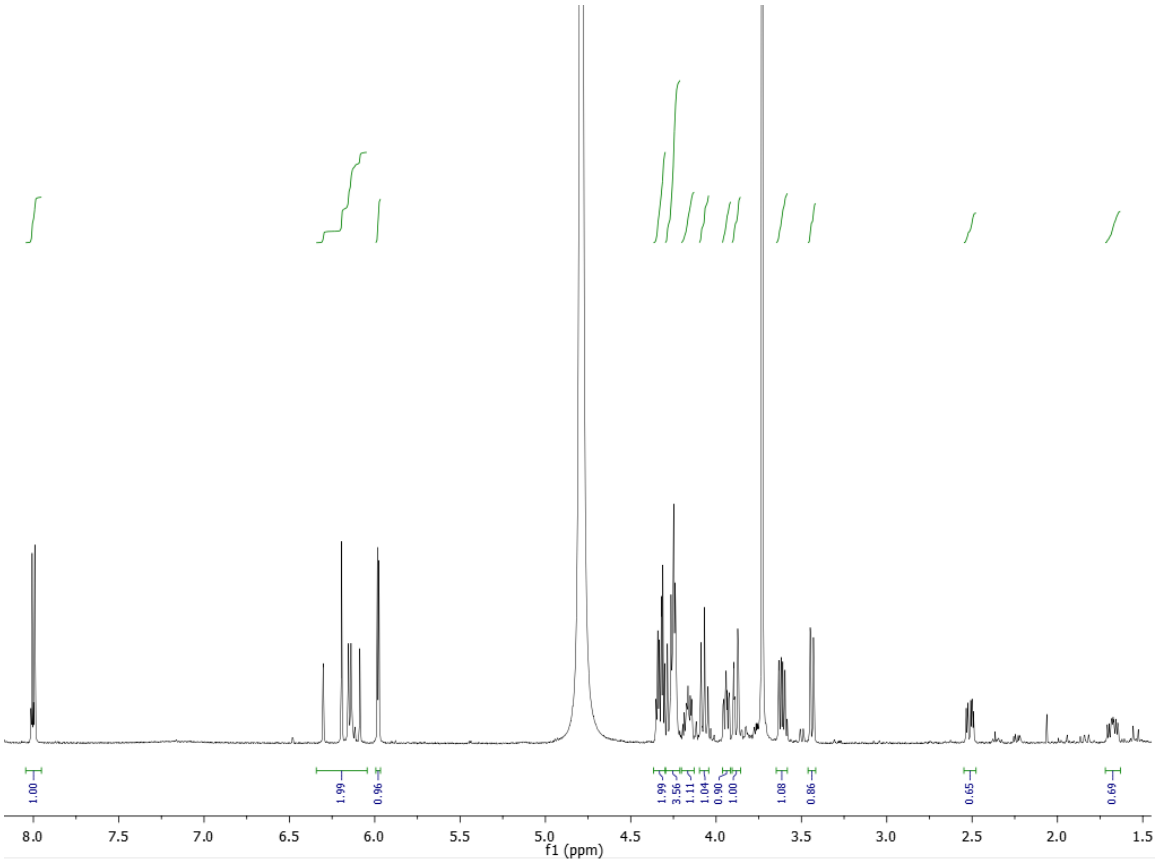
N-difluoroacetyl-D-mannosamine (1)



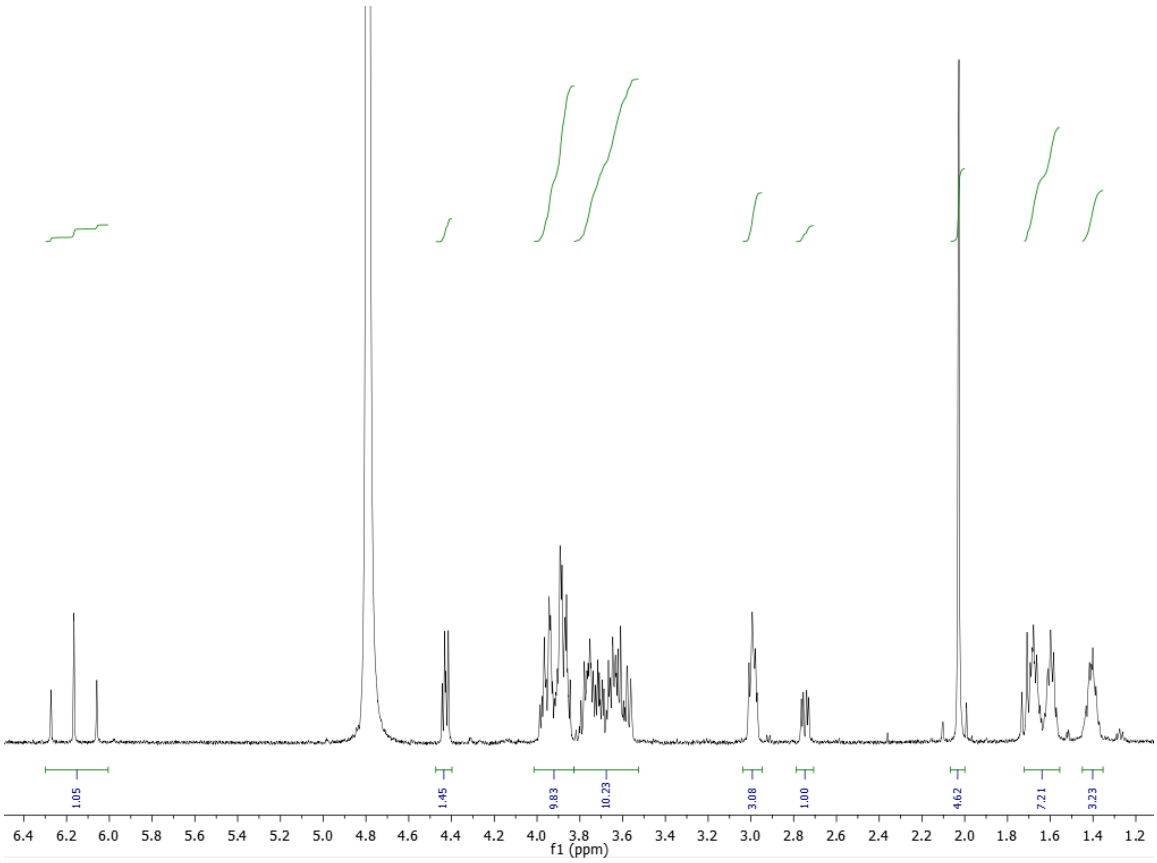
N-(difluoroacetyl)-α-Neuraminic acid (2)



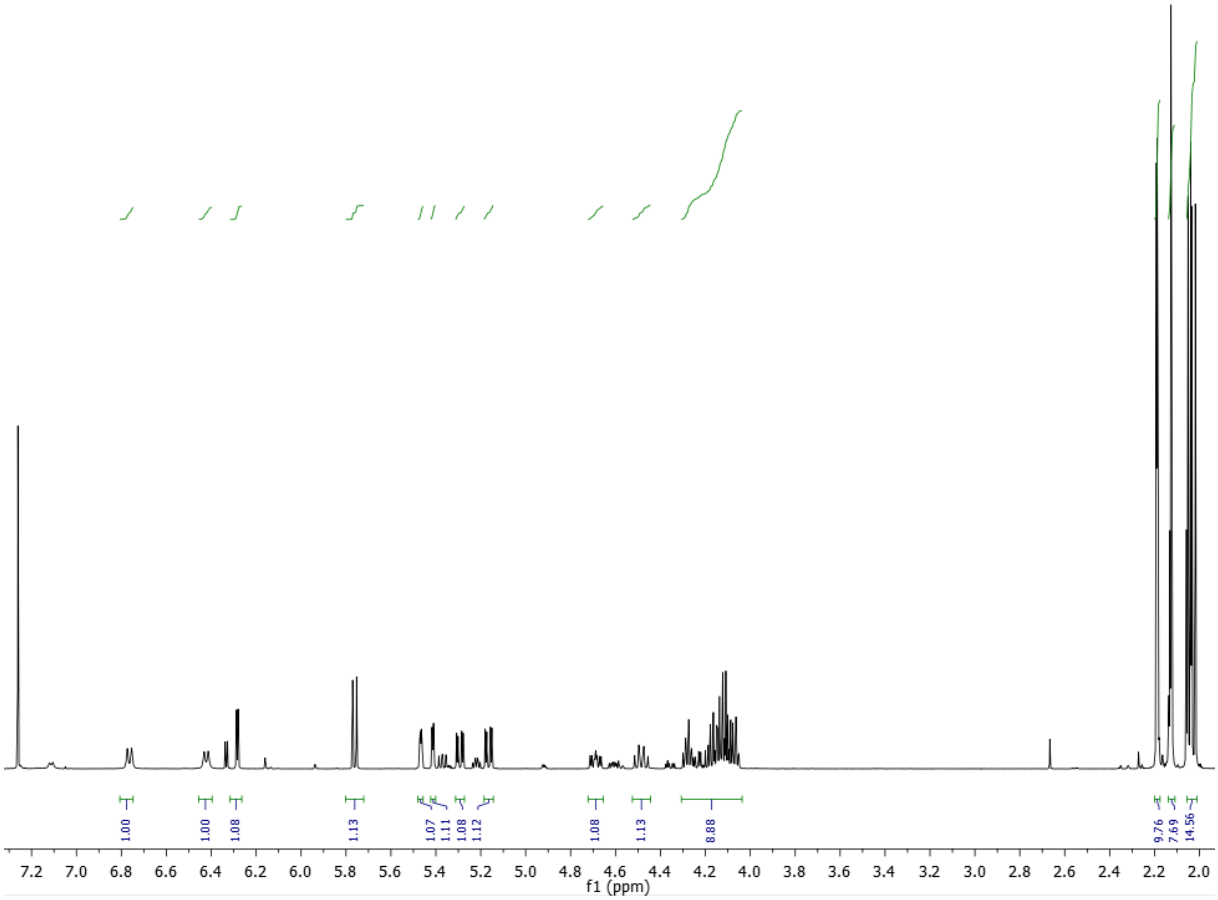
Cytidine-5′-Monophosphate-N-(difluoroacetyl)-β-Neuraminic acid (3)



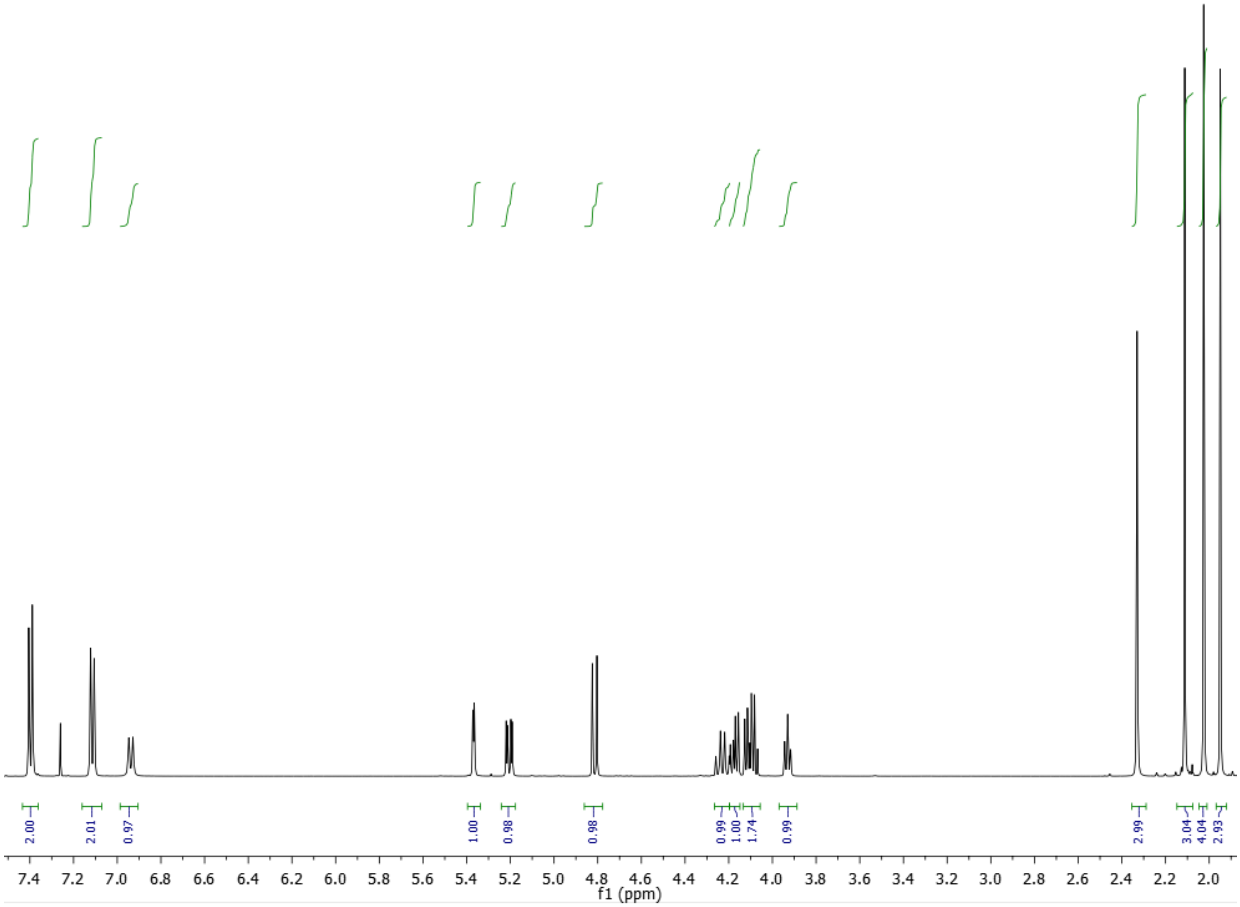
α-N-(difluoroacetyl)-neuraminyl-(2-6)-β-D-galactosyl-(1-4)-N-acetyl-β-D-glucosamine (diF α2,6SLN)



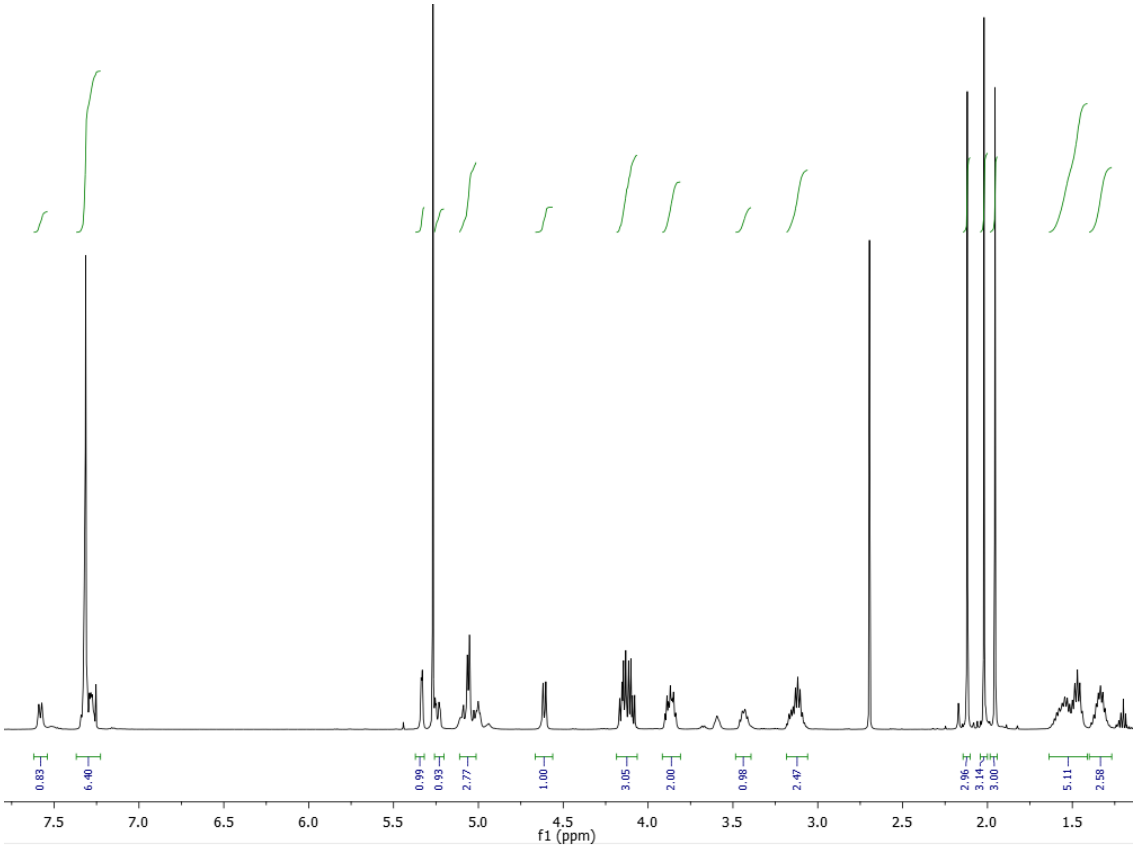
1,3,4,6-Tetra-O-acetyl-2-deoxy-2-trifluoroacetamido-α,β-Dgalactopyranose (5).



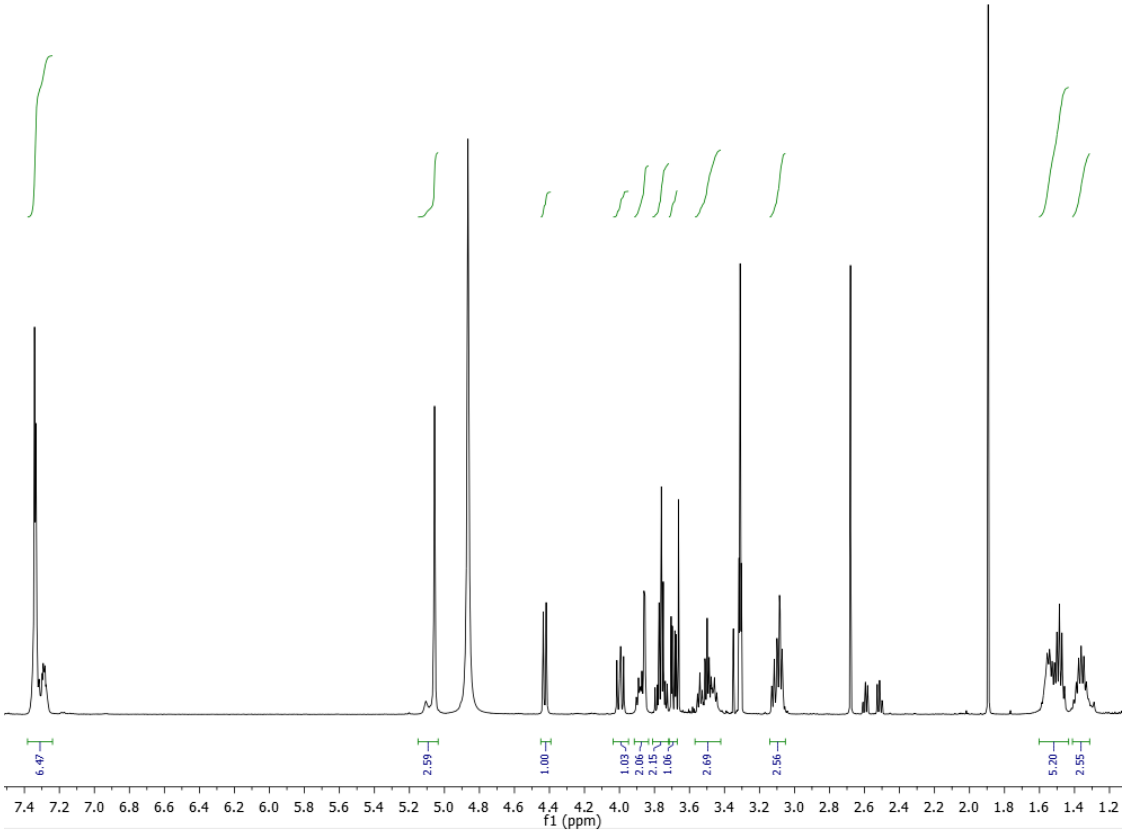
p-Tolyl 3,4,6-tri-O-acetyl-2-deoxy-2-N-trifluoroacetamido-1-thio-β-D-galactopyranoside (6)



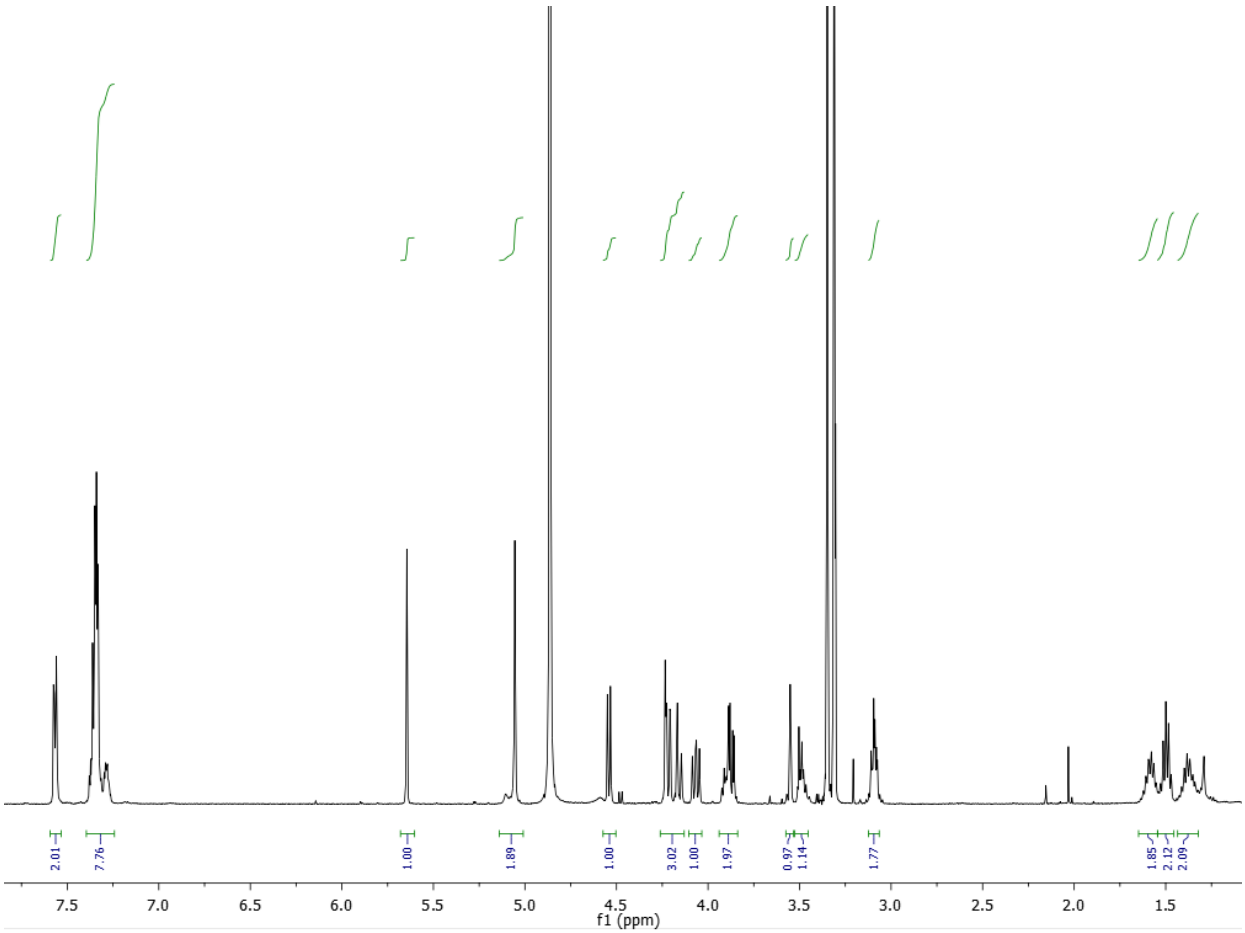
Phenylmethyl *N*-[5-[[3,4,6-tri-*O*-acetyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (7)



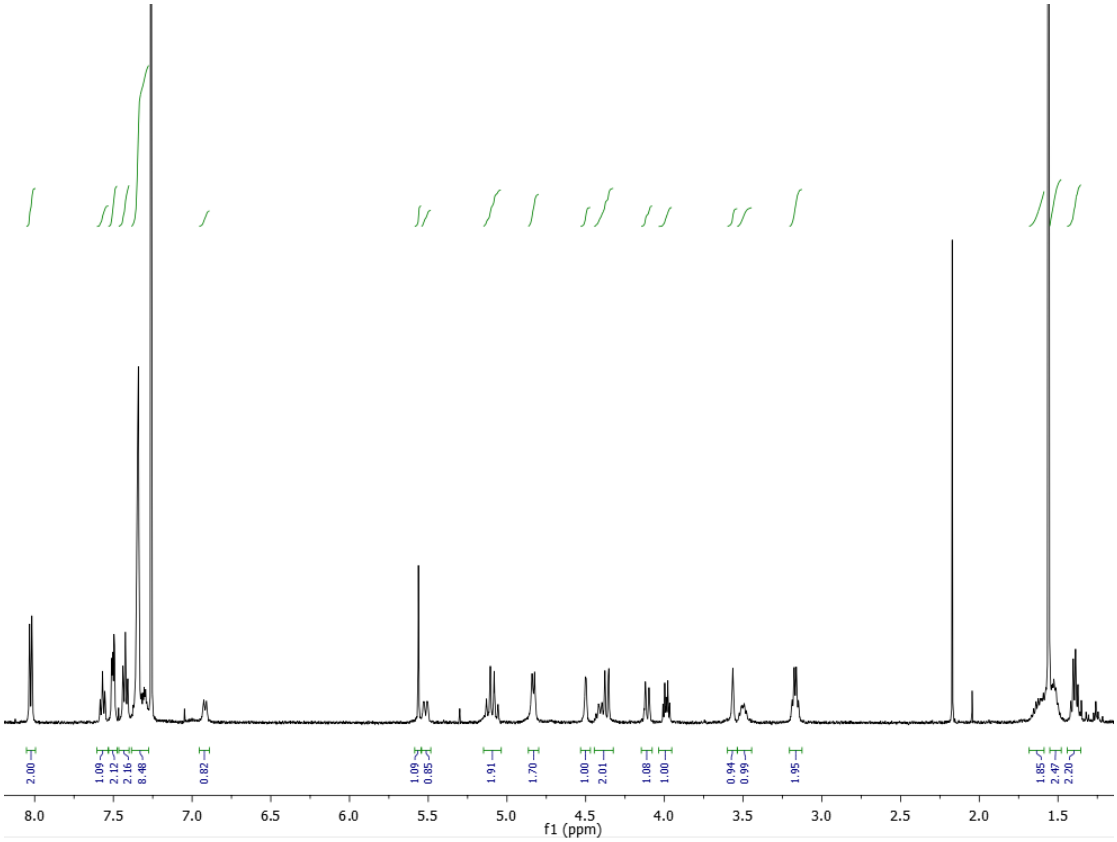
Phenylmethyl *N*-[5-[[2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (8).



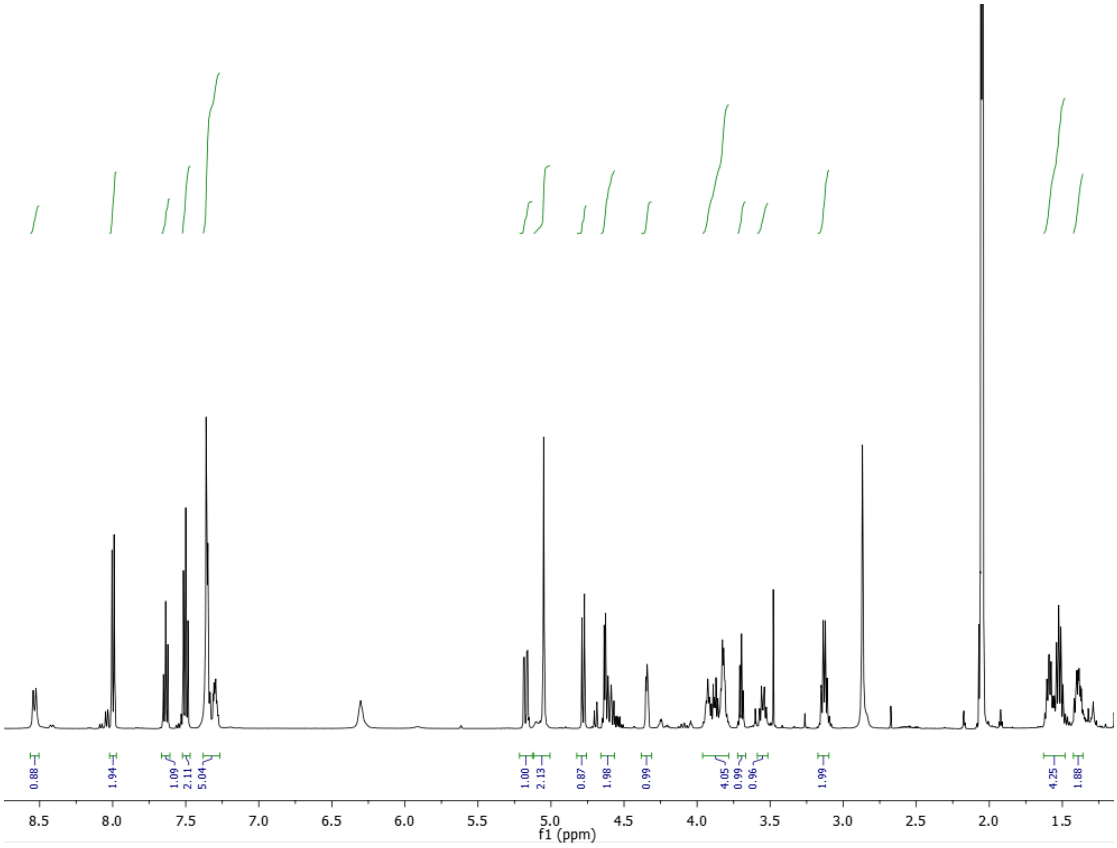
Phenylmethyl *N*-[5-[[4,6-O-benzylidene-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (9)



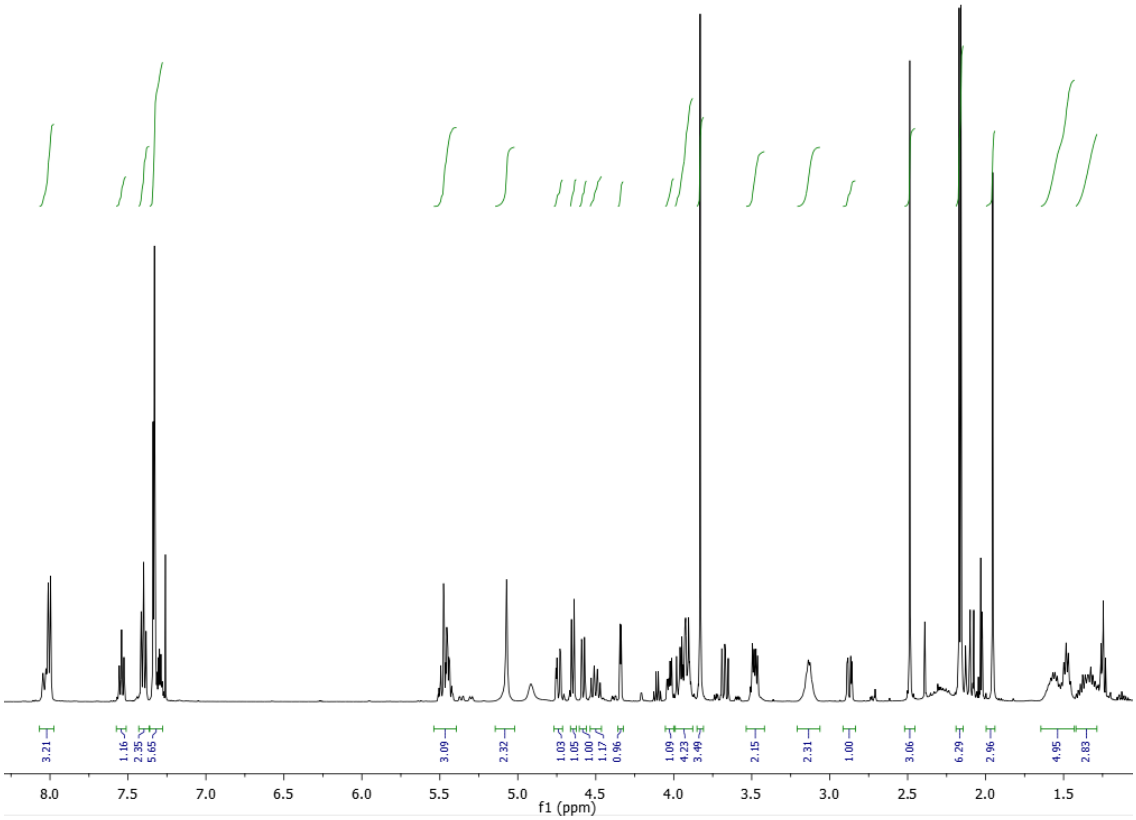
Phenylmethyl *N*-[5-[[4,6-O-benzylidene-3-O-benzyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (10)



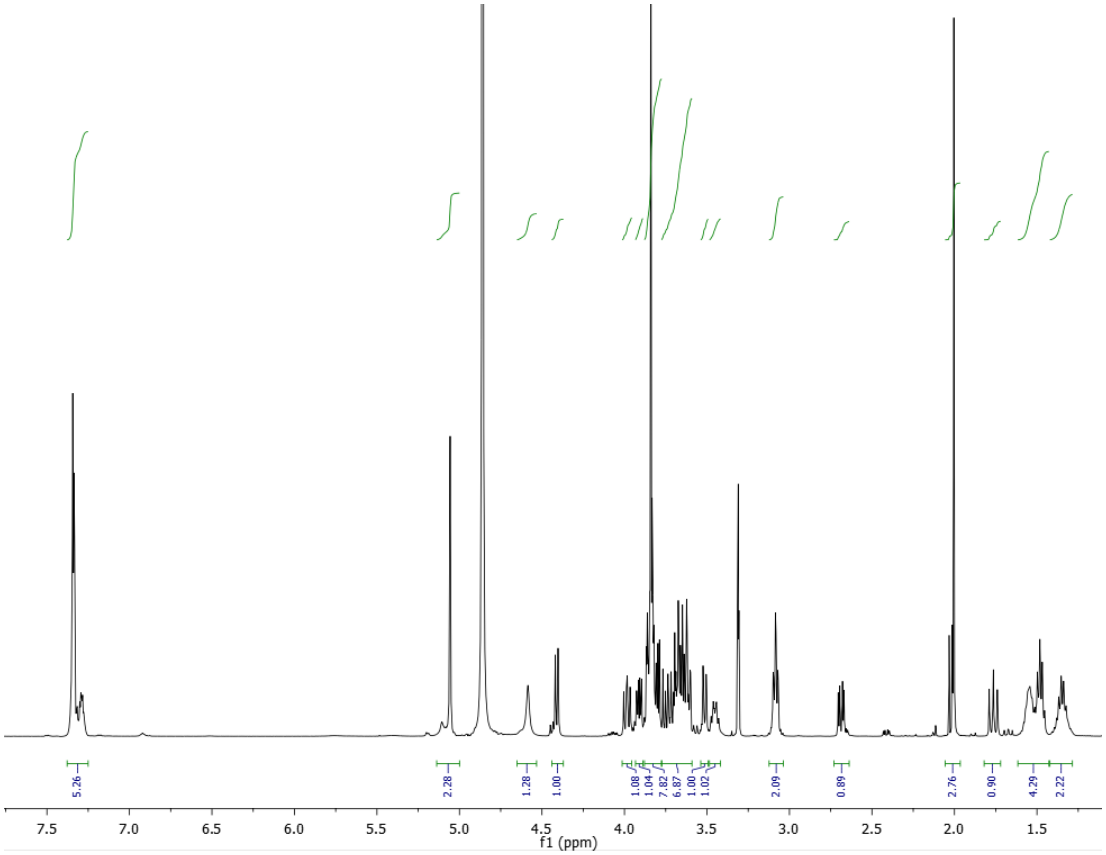
Phenylmethyl *N*-[5-[[3-O-benzyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (11).



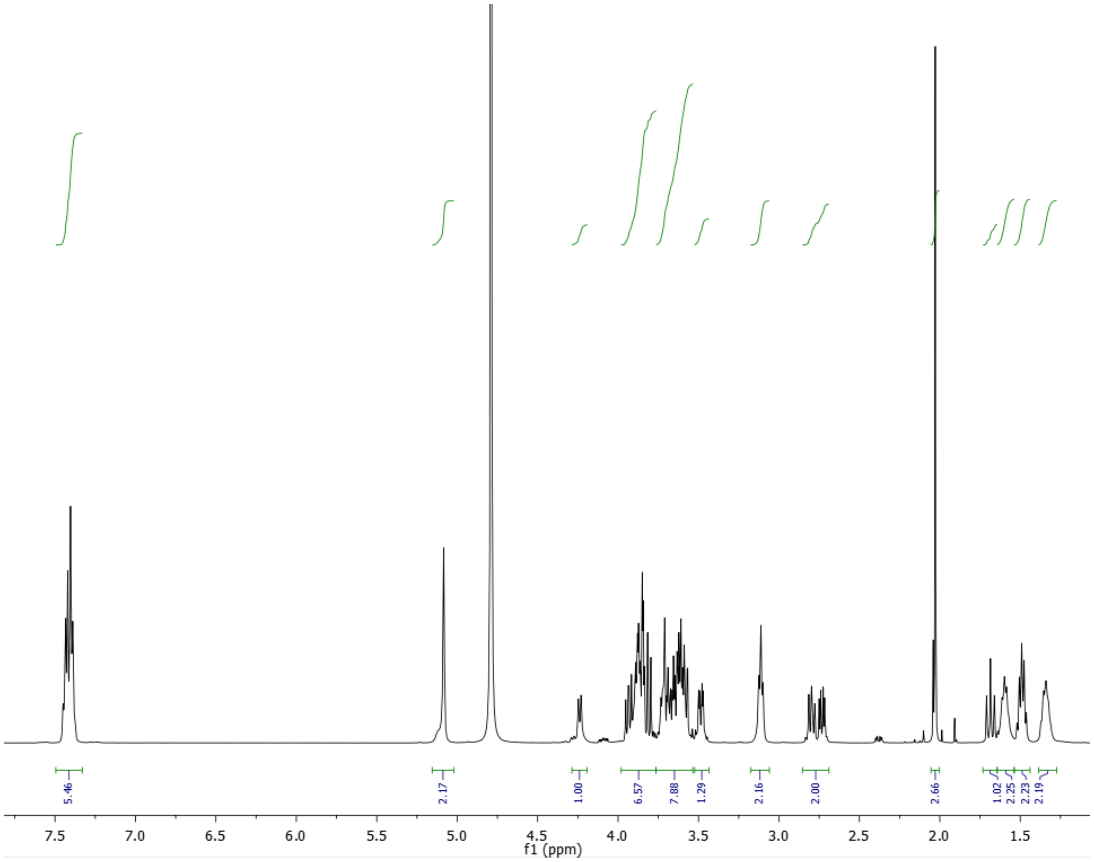
Methyl-7,8,9-tri-O-acetyl-5-N,4-O-carbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[3-O-benzyl-2- deoxy-2-N-trifluoroacetamido -β-D-galactopyranosyl]oxy]penthyl]carbamate (13)



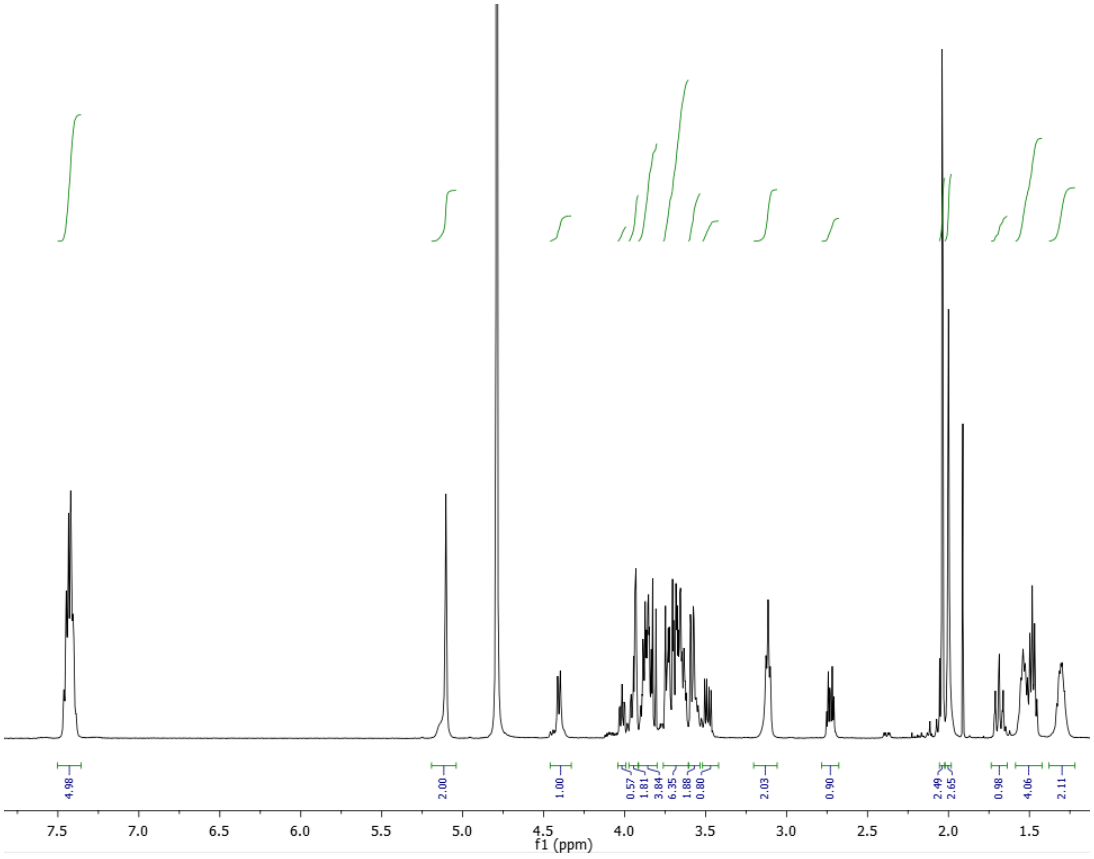
Methyl-5-N-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2-deoxy-2-N-trifluoroacetamido-β-D-galactopyranosyl]oxy]penthyl]carbamate (14)



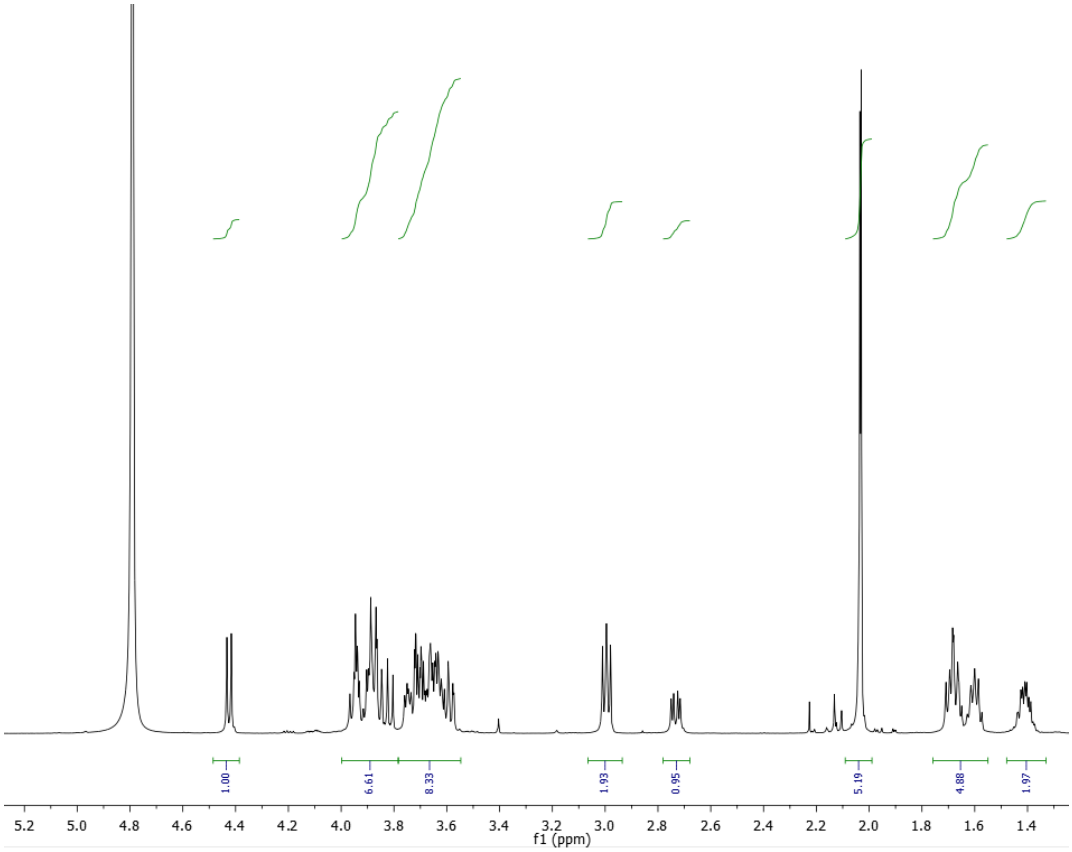
5-N-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2- deoxy-2-N-β-D-galactopyranosyl]oxy]penthyl]carbamate (15)



5-N-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2- deoxy-2-N-acetyl-β-D-galactopyranosyl]oxy]penthyl]carbamate (16)



3-aminopropyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate-(2→6)-phenylmethyl *N*-[5-[[2- deoxy-2-N-acetyl-β-D-galactopyranoside (17)



SUPPORTING FIGURES

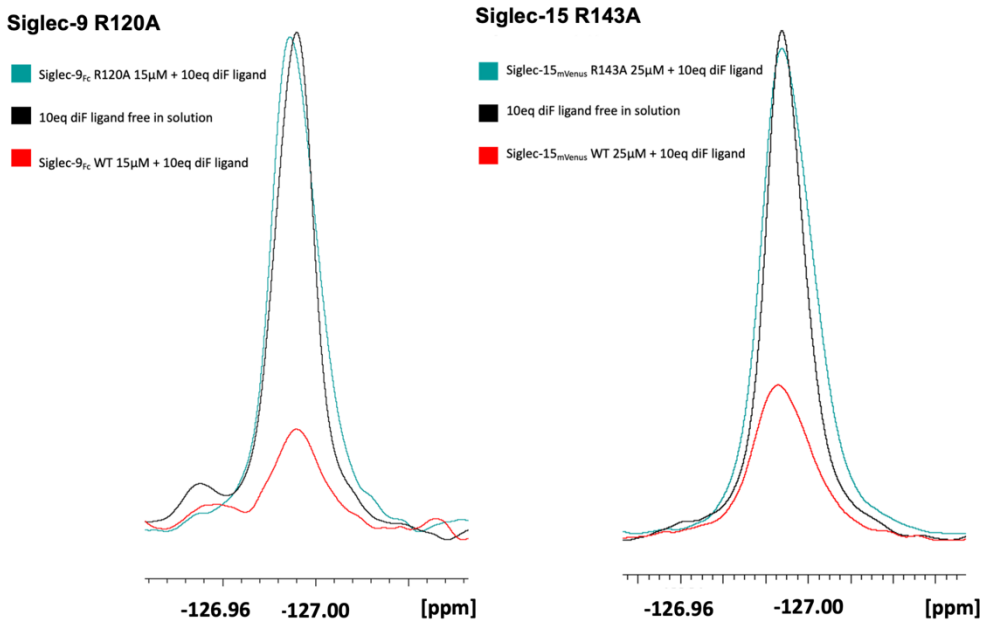


Figure S1. Absence of binding of diF α2,6SLN to Siglec-9 R120A and Siglec-15 R143A mutants. The spy molecule binds to Siglec-9 and Siglec-15 through their CRDs, as determined by the lack of intensity changes of its ^{19}F signal when mutant Siglecs without the canonical Arg are employed.

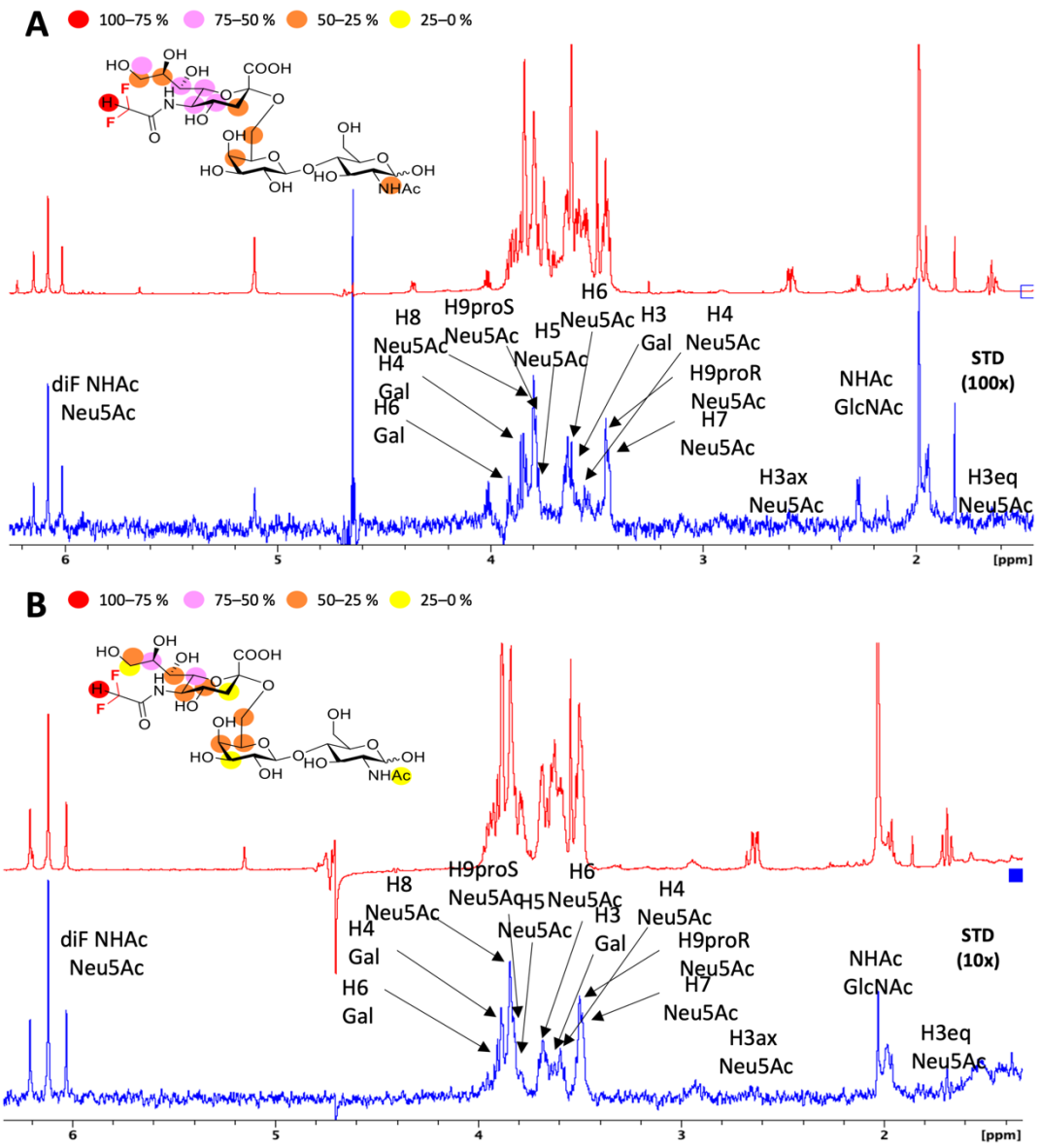


Figure S2. STD-NMR epitope mapping of diF α2,6SLN with Siglec-9 and Siglec-15. A) Reference off-resonance spectrum (red) and STD-NMR spectra with 100x amplification (blue) of Siglec-9 in the presence of the spy molecule (1: 40 Siglec-9: ligand molar ratio). The STD-based epitope mapping of the spy molecule is also shown. B) The analogous experiments for Siglec-15 (1: 17.6 molar ratio). The STD-based epitope mapping of the spy molecule is depicted. The relative STD response is colour coded according to the legend. The on-resonance frequency was set at 0.8 ppm and a saturation time of 2 s was employed.

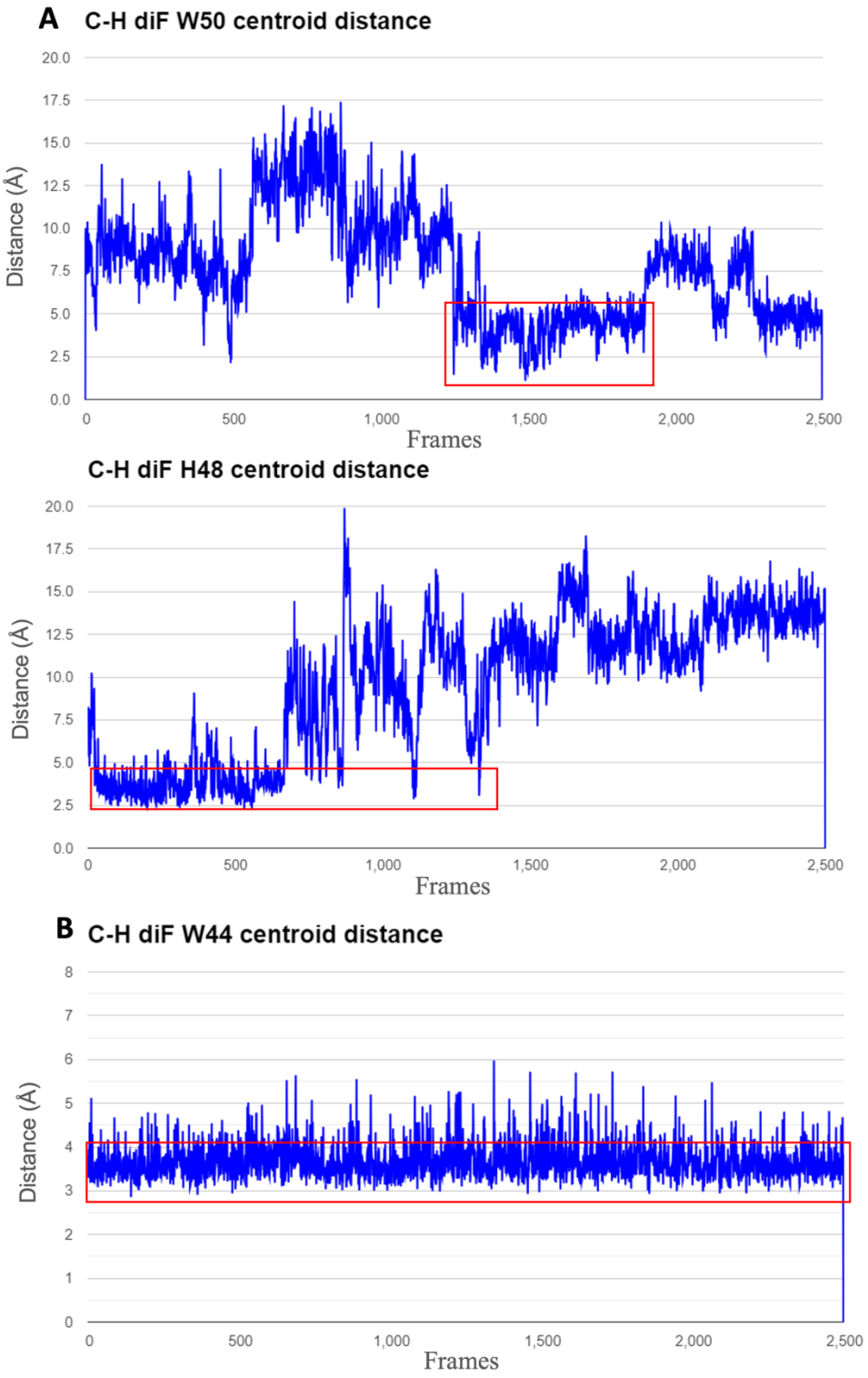


Figure S3. Establishment of CH-π interaction throughout the MD simulation. A. Distance between the H of the difluoro acetamide of diF α2,6SLN and W50 centroid (above), and centroid of H48 (below) in Siglec-9. **B**. Distance between the H of the difluoro acetamide of diF α2,6SLN and centroid of W55 indole side chain. The frames were CH-π interactions are established are highlighted within a red box.

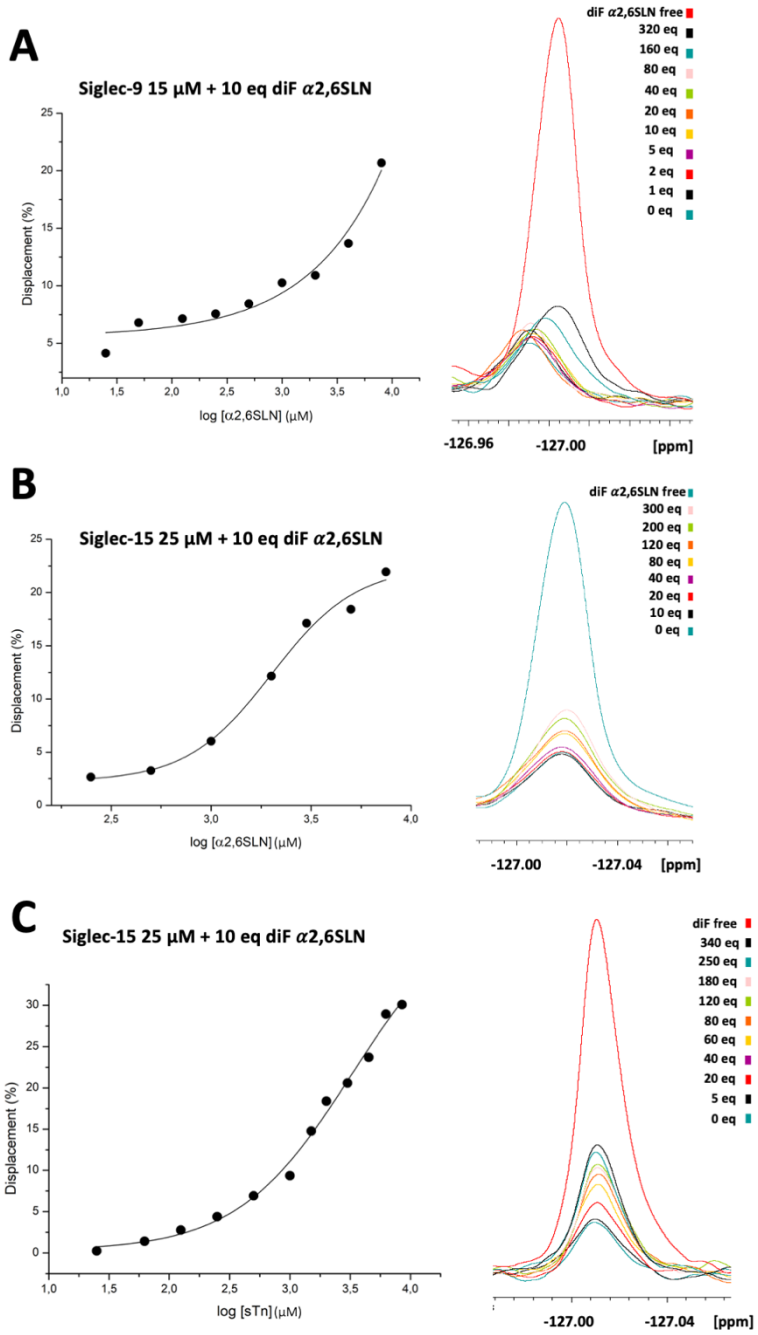


Figure S4. ^{19}F CPMG filtered NMR competitive displacement assays. A) Competition between 10 eq of diF α2,6SLN and increasing concentrations of α2,6SLN in the presence of Siglec-9 (15 µM). B) Competition between 10 eq of diF α2,6SLN and increasing concentrations of α2,6SLN in the with Siglec-15 (25 µM). C) Competition between 10 eq of spy molecule and siallyl Tn (sTn) in the with Siglec-15 25 µM.

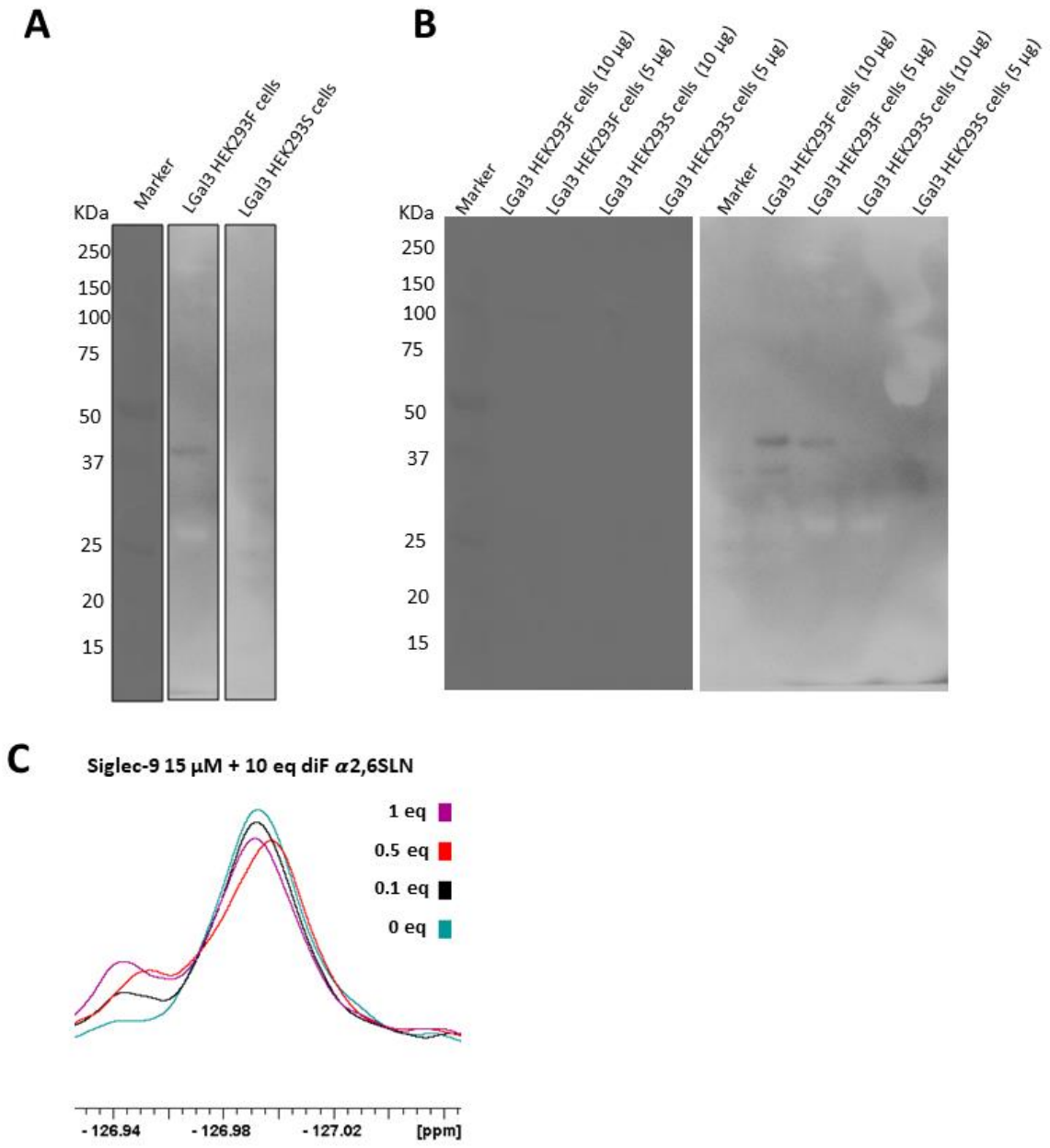


Figure S5. Expression of LGALS3BP glycoproteins in HEK293F and HEK293S cells. A) WB of LGALS3BP expressed in HEK293S and HEK293F cells using MAL II and incubating with streptavidin-HRP. B) Complete WB membranes developed with visible light (left) and chemiluminescent (right). C) ^{19}F T_2 NMR filtered competitive displacement assay between the fluorinated spy molecule and the LGALS3BP expressed on HEK293S cells with Siglec-9.

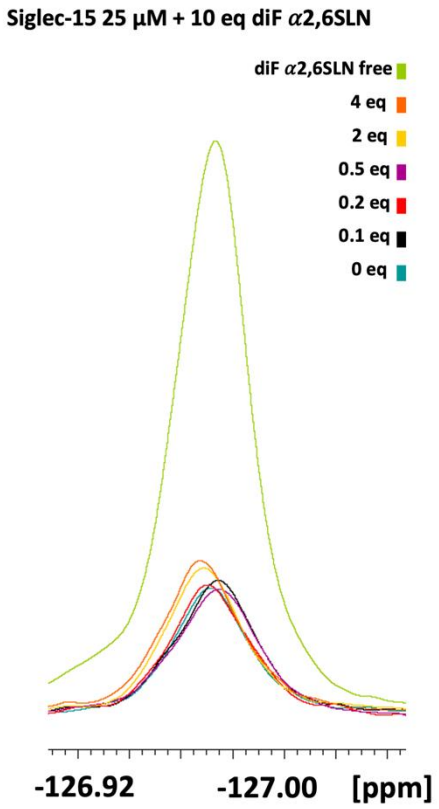


Figure S6. ^{19}F T_2 NMR filtered competitive displacement assay between the fluorinated spy molecule and the LGALS3BP expressed on HEK293F cells with Siglec-15.

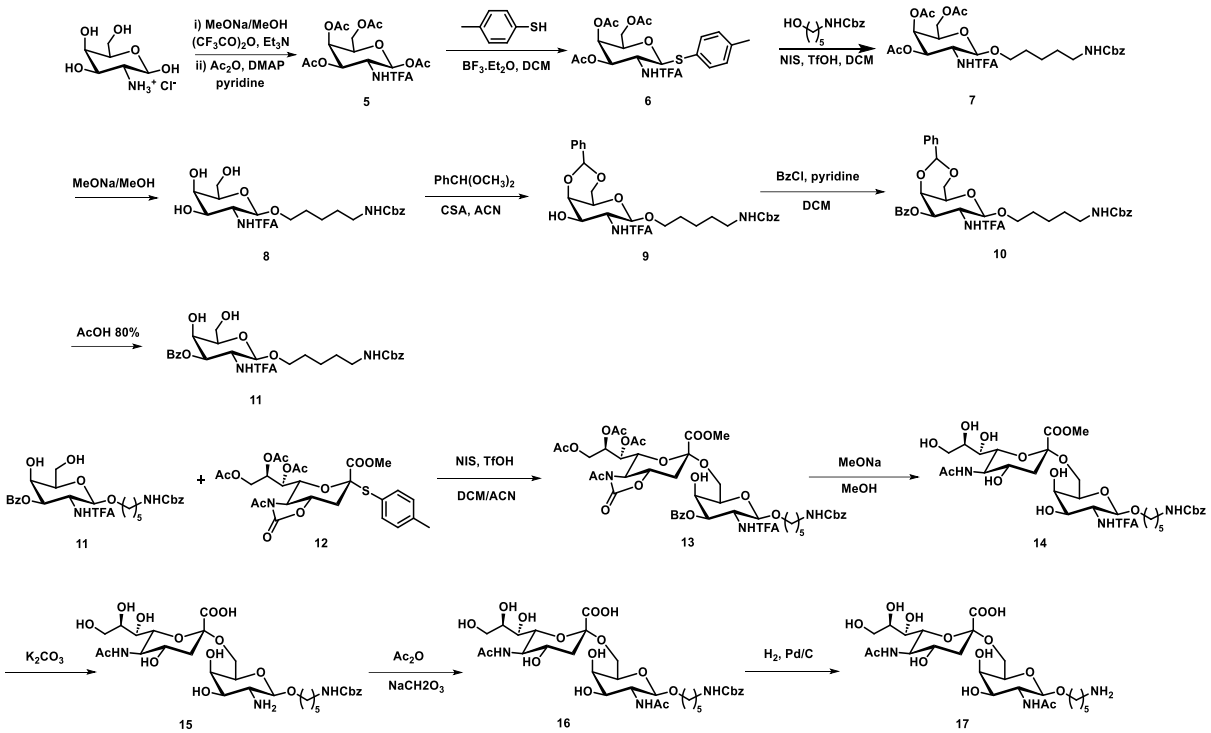


Figure S7. Scheme of the synthetic route for the preparation of the derivative sTn.

SUPPORTING TABLES

Table S1. STD-AF values of the difluoroacetamide group of diF α2,6SLN spy molecule obtained at different saturation times at increasing concentrations in the presence of 17.5 μM of Siglec-9.

Ligand concentration (μM)	**Saturation time (s)**			
	1.00	**2.00**	**4.00**	**6.00**
43.75	0.05	0.09	0.13	0.18
87.50	0.11	0.23	0.26	0.28
175.00	0.18	0.31	0.38	0.50
350.00	0.23	0.41	0.63	0.72
700.00	0.49	0.65	0.83	1.01

Table S2. Normalized STD-AF_0 values obtained by fitting the STD build-up curve from Siglec-9 and diF α2,6SLN.

Equivalents	**Concentration (μM)**	**STDmax**	**Ksat**	**STD-AF_0**
2.5	43.75	0.19	0.30	0.06
5	87.50	0.28	0.64	0.18
10	175.00	0.46	0.54	0.25
20	350.00	0.86	0.41	0.35
40	700.00	1.01	0.50	0.51

Table S3. STD-AF intensities of the diF α2,6SLN signal at different saturation times and concentrations of the ligand in the presence of 25 µM of Siglec-15.

Ligand concentration (µM)	**Saturation time (s)**			
	1.00	**2.00**	**4.00**	**6.00**
55	0.21	0.36	0.53	0.52
110	0.46	0.74	0.95	1.09
220	0.82	1.42	1.82	1.87
330	1.23	2.08	2.57	2.77
440	1.49	2.48	3.32	3.62

Table S4. Normalized STD-AF_0 values obtained by fitting the STD build-up curve from Siglec-15 and diF α2,6SLN.

Equivalents	**Concentration (µM)**	**STDmax**	**Ksat**	**STD-AF_0**
2.20	55	0.58	0.49	0.28
4.40	110	1.12	0.53	0.59
8.80	220	1.96	0.60	1.19
13.20	330	2.85	0.61	1.73
17.60	440	3.81	0.51	1.96

Table S5. STD-NMR-based epitope mapping for diF α2,6SLN in the presence of 17.5 µM Siglec-9_{Fc} (1:40 molar ratio). The STD-AF were measured using a 2 s saturation time and an interscan delay of 3 s.

Siglec-9_{Fc} + 40eq diF α2,6SLN	STD-AF	STD-AF(%)
diF NHAc Neu5Ac	0.69	100
H9proR Neu5Ac	0.4	57
H7Neu5Ac	0.39	56
H5Neu5Ac	0.37	54
H4 Neu5Ac	0.33	48
H8 Neu5Ac	0.33	48
NHAc GlcNAc	0.28	40
H3ax	0.28	40
H3eq	0.28	40
H9proS Neu5Ac	0.23	34
H4 Gal	0.2	28
H6Gal	0.2	28

Table S6. STD-NMR -based epitope mapping for diF α2,6SLN in the presence of 25 µM Siglec-15_{mVenus} (1:17.6 molar ratio). The STD-AF were measured using a 2 s saturation time and an interscan delay of 3 s.

Siglec-15_{mVenus} + 17.6 eq diF α2,6SLN	STD-AF	STD-AF(%)
diF NHAc Neu5Ac	2.6	100
H8 Neu5Ac	1.46	56
H5Neu5Ac	1.3	50
H7 Neu5Ac	1.09	42
H6Gal	1.09	42
H4 Neu5Ac	1.03	40
H4Gal	0.98	37
H9proR Neu5Ac	0.91	35
H3ax Neu5Ac	0.43	17
H3eq Neu5Ac	0.43	17
H3 Gal	0.52	20
H5Gal	0.64	25
H9proS Neu5Ac	0.65	25
NHAc GlcNAc	0.27	10