Global Metabolic Inhibitors of Sialyl- and Fucosyltransferases

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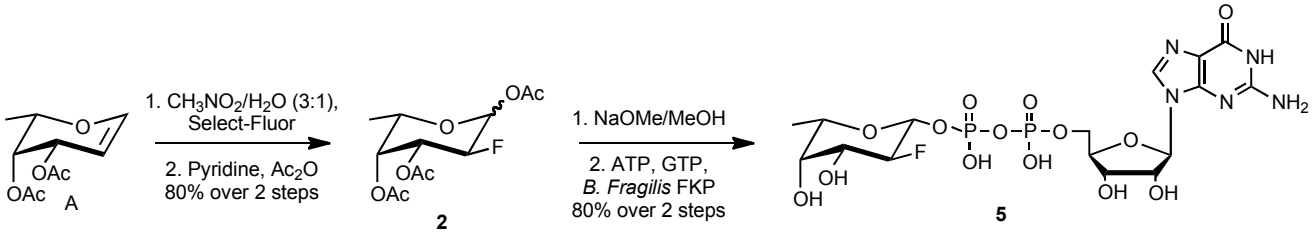
General Methods for Synthesis. All chemicals were obtained from Sigma Aldrich or Acros Chemical Companies unless otherwise specified. Tetraacetyl-Fucose (**1**), GDP-Fucose (**4**), and CMP-NeuAc (**10**) were from Sigma Aldrich. Compound (**7**) was synthesized as previously described[1]. C_{18} columns were obtained from Waters Corp. Reactions were monitored by thin-layer chromatography on 60F pre-coated TLC plates (EMD Chemicals, Inc.). 5-Carboxy Fluorescein NHS-Ester was from ChemPep (Wellington, FL). Compounds were visualized by UV light and/or dipping in 5% sulfuric acid in EtOH followed by charring on a hot plate. NMR spectra were obtained on a Bruker AV-400 MHz or Bruker DRX-600 MHz instrument at 25°C. Spectra obtained in D_2O were referenced to external acetone (^1H δ 2.225 and ^{13}C δ 29.9) or $CFCl_3$ (^{19}F δ 0.0). ^{19}F NMR taken in $CDCl_3$ were referenced to internal $CFCl_3$ signal. ESI-TOF high-accuracy mass spectrometry was recorded with an LC MSD TOF (Agilent Technologies). Silica gel column chromatography was performed with 60-200 mesh silica gel under medium pressure. Gel filtration was carried out with Bio-Gel P-2 resin (Bio Rad) with solvents degassed prior to use. The *B. Fragilis* FKP, *N. Meningitidis* CMP-NeuAc Synthetase, UDP-Glc Epimerase/Galβ1,4Transferase, and PmST1 α2,3 Sialyltransferase enzymes were prepared as previously described[2-5]. Calf Intestinal Alkaline Phosphatase was from New England Biolabs.



Supplementary Scheme 1 **|** Synthesis of Ac_3-2F-Fucose (**2**) and GDP-2F-Fucose (**5**).

Synthesis of Compound 2

The published procedure of Burkart et al.[6] describing the synthesis of (**2**) was modified due to substantial by-product formation as reported using DMF/H_2O as the reaction solvent. Instead, 3,4-diacetyl fucal[7] (**A**) (32.8 g, 153.1 mmol) was dissolved in CH_3NO_2 (600 ml) and H_2O (200 ml) was added to the reaction flask. Select-Fluor (81.4 g, 230.0 mmol) was then added in four portions and the reaction was left to proceed overnight with vigorous stirring. The next day the reaction was diluted in 1L of CH_2Cl_2, extracted with H_2O (2 x 300 mls), brine (1 x 300 mls), and dried over Na_2SO_4 to obtain 33.2 g of an off white solid. This was then dissolved in pyridine (200 mls), cooled to 0ºC, and acetic anhydride (37.6 mls, 398 mmols) was added dropwise over 15 minutes. The reaction was then left to warm to room temperature and proceed overnight before the solvent was evaporated to dryness. The residue was redissolved in 1.5 L of EtOAc, extracted with 5% citric acid (3 x 300 mls), sat. aq. $NaHCO_3$ (1 x 300 ml), brine (1 x 300 ml), and dried over Na_2SO_4. The solvent was evaporated to dryness and the product was further purified by silica gel chromatography eluting with 20% EtOAc in Hexanes (v/v). 35.8 g (80% over two steps) was obtained as a light yellow, viscous oil which slowly solidified over time.

2F-Peracetyl-Fucose (Compound 2)

^1H NMR (400 MHz, $CDCl_3$) α-Anomer, δ 6.45 (d, J = 4.0 Hz, 1H, H-1), 5.47 – 5.40 (m, 1H, H-3), 5.38 (dd, J = 3.5, 3.5, 1.3 Hz, 1H, H-4), 4.90 (ddd, J = 49.4, 10.2, 4.0 Hz, 1H, H-2), 4.30 – 4.23 (m, 1H, H-5), 2.19 (6H), 2.08 (s, 3H), 1.17 (d, J = 6.5 Hz, 3H, H-6).

^{13}C NMR (151 MHz, $CDCl_3$) α-Anomer, δ 171.18, 170.96, 169.95, 90.96 (d, J = 22.7 Hz, C1), 85.07 (d, J = 191.8 Hz, C2), 71.91 (d, J = 7.4 Hz, C4), 69.44 (d, J = 18.1 Hz, C3), 67.97 (C5), 21.78, 21.53, 21.4, 16.59 (C6).

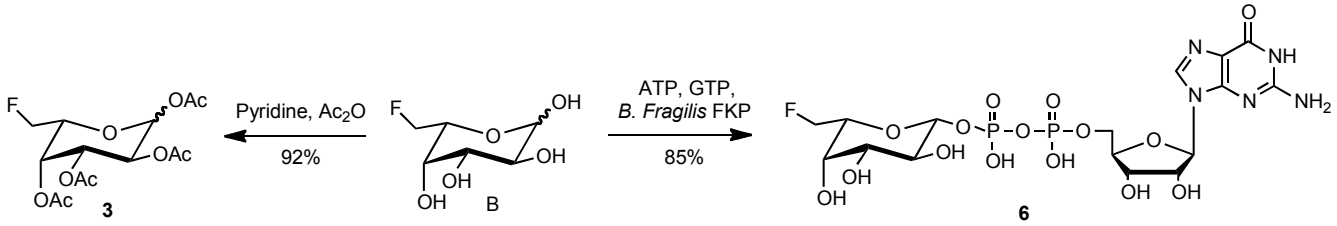
^{19}F NMR (376.5 MHz, $CFCl_3/CDCl_3$) α-Anomer, δ -210.10 (s).

^{1}H NMR (400 MHz, $CDCl_3$) β-Anomer, δ 5.79 (dd, J = 8.0, 4.1 Hz, 1H, H-1), 5.34 – 5.30 (m, 1H, H-4), 5.19 (ddd, J = 13.3, 9.8, 3.6 Hz, 1H, H-3), 4.65 (ddd, J = 51.8, 9.8, 8.0 Hz, 1H, H-2), 4.00 (dddd, J = 6.4, 6.4, 6.4, 1.1 Hz, 1H, H-5), 2.21 (s, 3H), 2.19 (s, 3H), 2.08 (s, 3H), 1.24 (d, J = 6.4 Hz, 3H, H-6).

^{13}C NMR (151 MHz, $CDCl_3$) β-Anomer, δ 171.15, 170.72, 169.87, 92.44 (d, J = 24.2 Hz, C1), 87.63 (d, J = 187.2 Hz, C2), 72.19 (d, J = 18.1 Hz, C3), 71.42 (d, J = 8.2 Hz, C4), 71.14 (C5), 21.72, 21.45, 21.40, 16.62 (C6).

^{19}F NMR (376.5 MHz, $CFCl_3/CDCl_3$) α-Anomer, δ -209.13 (s).

HRMS: $C_{12}H_{17}FO_7$, $[M+Na]^+$ Expected = 315.0856, Found = 315.0859



Supplementary Scheme 2 **|** Synthesis of Ac_4-6F-Fucose (**3**) and GDP-6F-Fucose (**6**).

Synthesis of Compound 3

6F-Fucose[8] (**B**) (90 mg, 0.49 mmol) was dissolved in pyridine (10 ml), cooled to 0ºC, and acetic anhydride (412 µl, 4.39 mmol) was added dropwise over a period of 5 min. The reaction was left to proceed overnight and then the solvent was evaporated, the residue diluted in 100 ml EtOAc and extracted with 5% citric acid (3 x 20 ml), sat. aq. $NaHCO_3$ (1 x 20 ml), brine (1 x 20 ml), and dried over Na2SO4. The product was further purified by silica gel chromatography eluting with 9:1 Toluene:EtOAc (v/v) to afford the product as a yellow oil (160 mgs, 92% yield).

6F-Peracetyl Fucose (Compound 3)

^{1}H NMR (400 MHz, $CDCl_3$) α-Anomer δ 6.43 (broad s, 1H, H-1), 5.58 (d, J = 1.0 Hz, 1H, H-4), 5.40 – 5.35 (m, 2H, H-3 and H-2), 4.60 – 4.33 (m, 3H, H-5, H-6, H-6'), 2.19 (s, 3H), 2.18 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$) α-Anomer δ 170.92, 170.89, 170.73, 169.73, 90.47 (C1), 81.56 (d, J = 171.84 Hz, C6), 70.33 (d, J = 23.56 Hz, C5), 68.28 (d, J = 5.44 Hz, C4), 68.11 (C3), 67.22 (C2), 21.72, 21.48, 21.42, 21.38.

^{19}F NMR (376.5 MHz, $CFCl_3/CDCl_3$) α-Anomer δ -232.33 (s)

^{1}H NMR (400 MHz, $CDCl_3$) β-Anomer δ 5.75 (d, J = 8.3 Hz, 1H, H-1), 5.51 (d, J = 2.9 Hz, 1H, H-4), 5.40 – 5.35 (m, 1H, H-2), 5.12 (dd, J = 10.4, 3.4 Hz, 1H, H-3), 4.60 – 4.33 (m, 2H, H-6, H-6'), 4.15 – 4.08 (m, 1H, H-5), 2.19 (s, 3H), 2.14 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$) β-Anomer δ 170.86, 170.75, 170.22, 169.77, 92.97 (C1), 81.10 (d, J = 172.74 Hz, C6), 73.05 (d, J = 24.01, C5), 71.59 (C3), 68.60 (C2), 67.59 (d, J = 5.13 Hz, C4), 21.626, 21.487, 21.440, 21.381.

^{19}F NMR (376.5 MHz, $CFCl_3/CDCl_3$) β-Anomer δ -232.18 (s)

HRMS: $C_{14}H_{19}FO_9$, $[M+Na]^+$ Expected = 373.0905, Found = 373.0898

Synthesis of Compound 5

Compound **2** (63 mg, 216 µmol) was dissolved in MeOH (3 mls) and NaOMe (0.1 M in MeOH) was added dropwise until pH ~ 10 was achieved. The reaction was left to proceed at room temperature for four hours before it was neutralized with Amberlite resin and filtered. The solvent was evaporated to afford 37 mg of 2F-Fucose as an off white solid (quantitative yield).

2F-Fucose (26 mgs, 156 µmol), ATP (113 mgs, 203.5 µmol), and GTP (113 mgs, 203.5 µmol) were dissolved in 8 mls of 100 mM Tris, 20 mM $MgCl_2$, 20 mM $MnCl_2$, pH 7.5. The *B. Fragilis* FKP enzyme[2] (~5 mgs) was then added and the reaction was allowed to proceed for 8 hrs at 37°C before calf intestinal alkaline phosphatase (100 U) was added to digest the phosphate groups of the nucleotide impurities (to aid in purification). After an 8 hr incubation the reaction was centrifuged and the supernatant was lyophilized. This was then purified on a Bio Gel P-2 column (2.5 x 100 cm) eluting with 125 mM NH_4CO_3. Fractions containing the product were pooled, lyophilized, redissolved in H_2O and passed through a C18 column (2g, C18 Waters) eluting with H_2O. Lyophilization afforded 74 mgs (125 µmols, 80% yield) of a white powder.

2F-GDP-Fucose (Compound 5)

^{1}H NMR (600 MHz, D_2O) δ 7.98 (s, 1H), 5.79 (d, J = 6.4 Hz, 1H), 5.03 (ddd, J = 8.1, 8.1, 3.6 Hz, 1H, Fucose-H1), 4.68 (m, 1H, under water signal) 4.39 (dd, J = 5.1, 3.2 Hz, 1H), 4.22 (ddd 52.2 Hz, 9.5, 7.7 Hz, 1H, Fucose-H2), 4.22-4.20 (m, 1H), 4.09 – 4.04 (m, 2H), 3.77 (ddd, J = 14.1, 9.6, 3.6 Hz, 1H), 3.67 (ddd, J = 6.7, 6.7, 6.7 Hz, 1H), 3.63 (dd, J = 3.1, 3.1 Hz, 1H), 1.08 (d, J = 6.5 Hz, 3H).

^{13}C NMR (151 MHz, D_2O) δ 159.81, 154.75, 152.67, 138.57, 117.13, 96.21 (dd J = 24.1, 4.8 Hz), 92.30 (dd J = 182.3, 7.8 Hz), 87.54, 84.73 (d, J = 9.3 Hz), 74.23, 72.51(d, J = 3.7 Hz), 72.48 (d, J = 3.3 Hz), 72.02 (d, J = 17.0 Hz), 71.31, 66.09 (d, J = 5.5 Hz), 15.96.

^{19}F NMR (376.5 MHz, D_2O) δ -207.98

HRMS: $C_{16}H_{24}FN_5O_{14}P_2$, $[M+H]^+$ Expected = 592.0852, Found = 592.0838

Synthesis of Compound 6

This compound was synthesized from 6F-Fucose using the *B. Fragilis* FKP enzyme, in an analogous manner as above, and as previously reported[2].

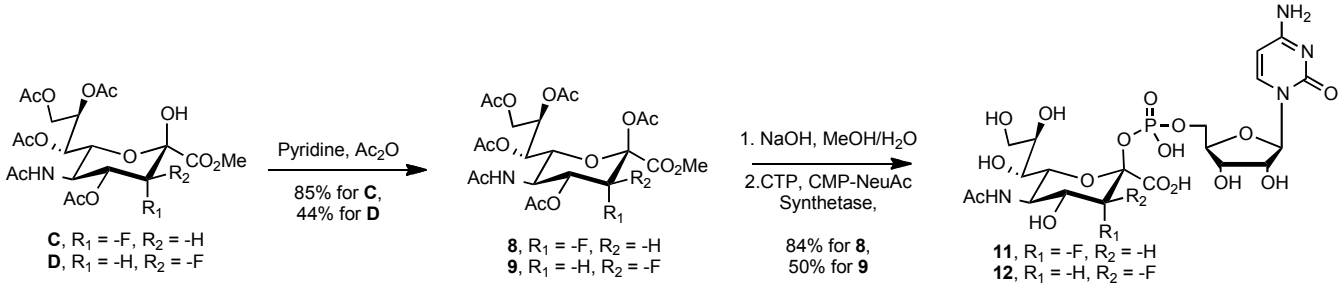
6F-GDP-Fucose (Compound 6)

^{1}H NMR (600 MHz, D_2O) δ 7.97 (s, 1H), 5.79 (d, J = 6.3 Hz, 1H), 4.84 (dd, J = 8.1, 8.1 Hz, 1H), 4.66 (1H under water signal), 4.55 – 4.51 (m, 1H), 4.44-4.43 (m, 1H), 4.39 (dd, J = 5.2, 3.2 Hz, 1H), 4.23 – 4.18 (m, 1H), 4.09 – 4.04 (m, 2H), 3.86 – 3.80 (m, 2H), 3.55 (dd, J = 10.0, 3.4 Hz, 1H), 3.48 (dd, J = 10.0, 7.7 Hz, 1H).

^{13}C NMR (151 MHz, D_2O) δ 159.81, 154.75, 152.67, 138.46, 117.05, 99.21 (d, J = 6.0 Hz), 87.49, 84.65 (d, J = 9.2 Hz), 83.33 (d, J = 164.5 Hz), 74.34 (d, J = 21.1 Hz), 74.33, 72.80, 71.88 (d, J = 8.1 Hz), 71.27, 68.83 (d, J = 6.5 Hz), 66.12 (d, J = 5.8 Hz).

^{19}F NMR (376.5 MHz, D_2O) δ -230.07 (s)

HRMS: $C_{16}H_{24}FN_5O_{15}P_2$, $[M+H]^+$ Expected = 608.0801, Found = 608.0806



C, R_1 = -F, R_2 = -H
D, R_1 = -H, R_2 = -F

8, R_1 = -F, R_2 = -H
9, R_1 = -H, R_2 = -F

11, R_1 = -F, R_2 = -H
12, R_1 = -H, R_2 = -F

Supplementary Scheme 3 **|** Synthesis of Ac_5-3F-Ax-NeuAc-Me (**8**), Ac_5-3F-Ax-NeuAc-Me (**9**), CMP-3F-Ax-NeuAc (**11**), and CMP-3F-Eq-NeuAc (**12**)

Synthesis of Compounds 8 and 9

Compounds **C** and **D** were synthesized as previously described[6] with the exception (as above) that CH_3NO_2/H_2O was used as the reaction solvent for the fluorination reaction. **C** (3.96 g, 7.77 mmol) was dissolved in pyridine (100 mls), cooled to 0°C, and acetic anhydride (1.46 mls, 15.5 mmol) was then added over 5 min. The reaction was left to proceed overnight before the solvent was evaporated. The residue was redissolved in EtOAc (500 mls), extracted with 5% citric acid (3 x 150 mls), sat. aq. $NaHCO_3$ (2x 150 mls), and brine (1 x 150 mls). After drying over Na_2SO_4, the organic layer was evaporated and the product was recrystallized from EtOAc/Hexanes to yield 3.63 g (85% yield).

3F-Ax-Peracetyl NeuAc (Compound 8)

^{1}H NMR (600 MHz, $CDCl_3$) δ 5.54 (dd, J = 27.9, 10.9 Hz, 1H), 5.39 (d, J = 9.0 Hz, 1H), 5.34 (d, J = 4.2 Hz, 1H), 5.12 (broad s, 1H), 4.93 (d, J = 49.1 Hz, 1H), 4.55 (d, J = 12.4 Hz, 1H), 4.24 (d, J = 10.5 Hz, 1H), 4.21 – 4.10 (m, 2H), 3.83 (s, 3H), 2.17 (s, 3H), 2.16 (s, 3H), 2.11 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.91 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 171.51, 171.43, 171.40, 171.13 (2C), 167.95, 165.95, 95.99 (d, J = 29.0 Hz, C2), 87.82 (d, J = 185.4 Hz, C3), 72.69, 72.12, 69.93, (d, J = 17.1 Hz, C4), 68.81, 62.92, 54.38, 46.61, 24.17, 21.75, 21.67, 21.62, 21.53, 21.41.

^{19}F NMR (376.5 MHz, $CFCl_3/CDCl_3$) δ -209.37 (s).

HRMS: $C_{22}H_{30}FNO_{14}$, [M+H]+ Expected = 552.1723, Found = 552.1733

Compound **D** (272 mg, 534 µmol) was dissolved in pyridine (20 mls) and Ac_2O (125 µl, 1.34 mmol) was added. After an overnight reaction, the reaction had proceeded ~50% as judged by TLC, so more Ac_2O was added (125 µl, 1.34 mmol), but no further reaction proceeded. The solvent was evaporated and the residue was coevaporated 2x with toluene before loading onto a silica gel column. The starting material was eluted with 30% Hexanes/EtOAc and the product eluted with 15% Hexanes/EtOAc. 130 mgs (44% yield) of a white foam was obtained.

3F-Eq-Peracetyl NeuAc (Compound 9)

^{1}H NMR (400 MHz, $CDCl_3$) δ 5.54-5.46 (m, 2H), 5.36 (dd, J = 4.8, 2.4 Hz, 1H), 5.06-5.03 (m, 1H), 4.64 (dd, J = 49, 9.4 Hz, 1H), 4.52 (dd, J = 12.4, 2.8 Hz, 1H), 4.24 (dd J = 10.6, 10.6 Hz, 1H), 4.15 (dd, J = 12.8. 6.4 Hz, 1H). 4.13 (dd, J = 11, 2.4 Hz, 1H), 3.87 (s, 3H). 2.26 (s, 3H), 2.17 (s, 3H), 2.12 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 1.92 (s, 3H).

^{13}C NMR (151 MHz, $CDCl_3$) δ 171.84, 171.44, 171.26, 171.06, 171.01, 168.50, 163.2 (d, J = 2.0 Hz), 96.09 (d, J = 18.7 Hz), 88.32 (d, J = 204.3 Hz), 72.75, 72.36, 71.49, 71.36, 62.63, 54.45, 49.51 (d, J = 6.2 Hz), 23.94, 21.81, 21.64, 21.63, 21.60, 21.53.

^{19}F NMR (376.5 MHz, $CDCl_3/CFCl_3$) δ -199.79 (s)

HRMS: $C_{22}H_{30}FNO_{14}$, $[M+H]^+$ Expected = 552.1723, Found = 552.1736

Compound **8** (14.8 mg, 26.8 µmol) was dissolved in 3 mls in a 0.1 M NaOH solution in 19:1 MeOH/H_2O (v/v). Deprotection was left to proceed overnight before the reaction was neutralized with Amberlite resin and filtered through a cotton plug. The MeOH was removed under reduced pressure and the remaining water was lyophilized. The residue, along with CTP (24.9 mg, 45.6 µmol), was dissolved in 2 mls of 100 mM Tris, 20 mM $MgCl_2$, pH 9.0 to which CMP-NeuAc-Synthetase (25 U) from *N. Meningitidis* was added. The reaction was left to proceed at 37°C and was complete within a few hours. Calf intenstinal alkaline phosphatase (100 U) was added to digest excess nucleotides before the reaction was centrifuged and the supernatant lyophilized. The product was purified on a Bio Gel P-2 column (2.5 x 100 cm) running in 125 mM NH_4CO_3. Fractions containing the product were pooled, lyophilized, redissolved in H_2O and passed through a C18 column (2g, Waters) eluting in H_2O. After lyophilization, 13.3 mg of a white solid was obtained (84% yield over two steps).

CMP-3F-Ax-NeuAc (Compound 11)

^{1}H NMR (600 MHz, D_2O) δ 7.92 (d, J = 7.7 Hz, 1H), 6.06 (d, J = 7.7 Hz, 1H), 5.83 (d, J = 3.8 Hz, 1H), 4.78 (dd, J = 48.5, 2.2 Hz, 1H), 4.21 – 4.11 (m, 5H), 4.09-3.97 (m, 3H), 3.84 (ddd, J = 9.3, 6.7, 2.3 Hz, 1H), 3.76 (dd, J = 11.8, 2.3 Hz, 1H), 3.50 (dd, J = 11.8, 6.6 Hz, 1H), 3.33 (d, J = 9.7 Hz, 1H), 1.92 (s, 3H).

^{13}C NMR (151 MHz, D_2O) δ 175.56, 172.09, 163.58, 154.13, 143.74, 98.82 (d, J = 30.7 Hz), 96.74, 91.18 (dd, J = 175.2, 14.8 Hz), 89.96, 83.92 (d, J = 8.0 Hz), 75.14, 72.50, 70.50, 70.02, 69.50, 68.77 (d, J = 18.1 Hz), 65.84 (d, J = 5.5 Hz), 63.68, 47.65 (d, J = 2.6 Hz), 22.87.

^{19}F NMR (375 MHz, D_2O) δ -208.17 (s).

HRMS: $C_{20}H_{30}FN_4O_{16}P$, $[M+H]^+$ Expected = 633.1451, Found = 633.1457.

Compound 12 was synthesized analogously as above for **Compound 11**, however, it was noted that far longer reaction times and 10x more enzyme were required to get the reaction to proceed to a substantial degree (~75% completion after 72 hrs). After identical purification, ~50% isolated yield was obtained.

CMP-3F-Eq-NeuAc (Compound 12)

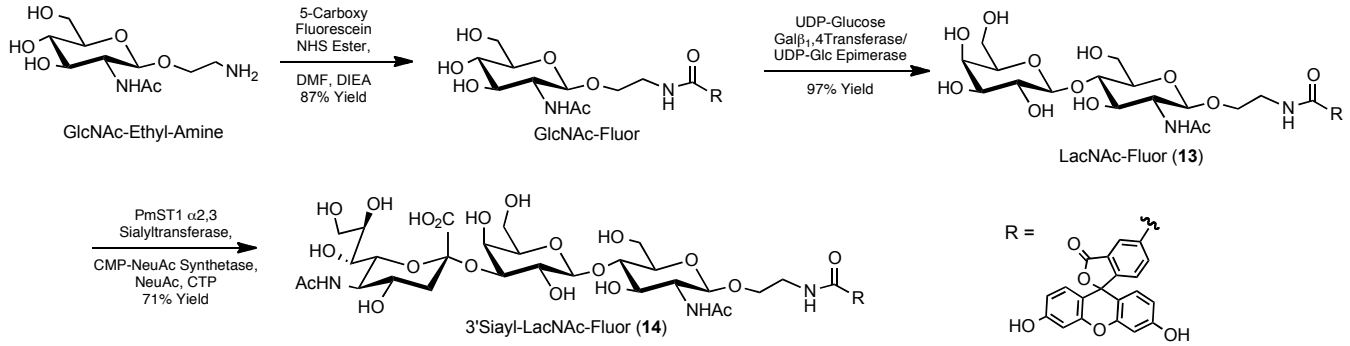
^{1}H NMR (600 MHz, D_2O) δ 7.98 (d, J = 7.8 Hz, 1H), 6.08 (d, J = 7.8 Hz, 1H), 5.83 (d, J = 4.3 Hz, 1H), 4.23 (dd, J = 4.8, 4.8 Hz, 1H), 4.20 (dd, J = 4.8, 4.8 Hz, 1H), 4.19 – 4.12 (m, 4H), 4.08 – 3.96 (m, 3H), 3.78 – 3.71 (m, 2H), 3.47 (dd, J = 11.7, 6.5 Hz, 1H), 3.30 (dd, J = 9.5, 0.8 Hz, 1H), 1.91 (s, 3H).

^{13}C NMR (151 MHz, D_2O) δ 175.34, 172.27, 165.32, 156.43, 143.05, 98.40 (dd, J = 17.7, 7.4 Hz), 97.13, 92.70 (dd, J = 194.8, 9.3 Hz), 89.91, 83.90 (d, J = 8.0 Hz), 75.08, 72.10, 70.56, 70.44, 70.13, 69.34, 65.69 (d, J = 5.6 Hz), 63.65, 51.16 (d, J = 7.5 Hz), 22.82.

^{19}F NMR (376.5 MHz, D_2O) δ -199.80 (s).

HRMS: $C_{20}H_{30}FN_4O_{16}P$, $[M+H]^+$ Expected = 633.1451, Found = 633.1456

Synthesis of LacNac-Fluor (13) and 3'Sialyl-LacNAc-Fluor (14)



Supplementary Scheme 4 **|** LacNAc-Fluor (**13**) and 3'Sialyl-LacNAc-Fluor (**14**).

GlcNAc-Ethyl-Amine[9] (50 mg, 189 μmol) and 5-Carboxy-Fluorescein-NHS-Ester (125.4 mg, 264 μmol) were added to an oven dried round-bottom flask and DMF (10 mls) and DIEA (0.5 ml) were added and the reaction was allowed to proceed overnight at room temperature. The solvent was evaporated and the product was purified on Iatrobeads (Shell-USA, Fredericksburg, VA) with 50:3:3:2 (EtOAc:CH_3OH:AcOH:H_2O) (v/v) as the eluent. After evaporation, 103 mgs of an orange solid (GlcNAc-Fluor) was obtained (87%).

GlcNAc-Fluor (5.6 mg, 9 μmol) and UDP-Glucose (6.6 mg, 11.65 μmol) were dissolved in 1 ml of 100 mM Sodium Cacodylate, 20 mM $MnCl_2$, and 20 mM $MgCl_2$ pH 7.5. To this was added 0.1 U of the UDP-Glucose Epimerase / Galβ1,4Transferase fusion protein[4]. The rxn was left to proceed for 18 hrs at room temperature and then was purified by loading onto a Waters C18 Sep-Pak column (2g). The column was washed with water and the product was then eluted with 50% MeOH/H_2O. After evaporation of the MeOH, the residual H_2O was lyophilized to obtain LacNAc-Fluor (**13**), 6.9 mgs of a yellow solid (97% yield).

LacNac-Fluor (13)

^{1}H NMR (600 MHz, D_2O/MeOD) δ 8.41 (d, *J* = 1.2 Hz, 1H), 8.12 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.33 (d, *J* = 8.0 Hz, 1H), 6.93 (dd, *J* = 8.9, 6.6 Hz, 2H), 6.79 (d, *J* = 2.2 Hz, 2H), 6.71 (dd, *J* = 9.0, 1.7 Hz, 2H), 4.58 (d, *J* = 8.4 Hz, 1H), 4.45 (d, *J* = 7.7 Hz, 1H), 4.08 (ddd, *J* = 10.4, 5.1, 5.1 Hz, 1H), 3.95 (dd, *J* = 11.8, 1.6 Hz, 1H), 3.91 (d, *J* = 3.3 Hz, 1H), 3.89 – 3.66 (m, 10H), 3.64 (dd, *J* = 9.9, 3.3 Hz, 1H), 3.59 (m, 1H), 3.55 (dd, *J* = 9.9, 7.8 Hz, 1H), 1.87 (s, 3H).

^{13}C NMR (151 MHz, D_2O/MeOD) δ 175.36, 172.73, 170.06, 156.67, 137.18, 133.08, 131.84 (2C), 131.81, 129.01, 127.50, 118.39 (2C), 113.77, 104.62 (2C), 104.42, 102.69, 80.19, 76.97, 76.41, 74.26, 74.01, 72.60, 70.20, 69.45, 62.57, 61.66, 56.65, 41.43, 23.49.

Note: Some of the fluorescein quartenary carbons are missing even after overnight ^{13}C scans.

HRMS: $C_{37}H_{40}N_2O_{17}$, $[M+H]^+$ Expected = 785.2405, Found = 785.2406

LacNAc-Fluor (**13**) (20 mg, 25.5 μmol), CTP (18 mg, 33 μmol), and NeuAc (10 mg, 33 umol) were dissolved in 4 mls of 100 mM Tris, 20 mM $MgCl_2$ pH 9.0 to which CMP-NeuAc Synthetase[3] (2.3 U) and PmST1 α2,3 Sialyltransferase[5] (0.23 U) were added. The rxn was left to proceed for 2 hrs at which time TLC indicated the reaction was complete. The reaction mixture was lyophilized, redissolved in a minimal amount of water, and purified on a Bio-Gel P2 column (0.625 x 42.5 cm) running in 125 mM NH_4CO_3. After 2 rounds of purification, 19.4 mgs of 3'Sialyl-LacNAc-Fluor **(14)** was obtained as an orange solid (71% yield).

3'Sialyl-LacNAc-Fluor (14)

^1H NMR (600 MHz, D_2O) δ 8.24 (s, 1H), 7.98-7.92 (m, 1H), 7.30 – 7.23 (m, 1H), 7.00 – 6.89 (m, 2H), 6.78-6.69 (m, 2H), 6.68-6.60 (m, 2H), 4.46 (d, J = 8.3 Hz, 1H), 4.40 (d, J = 7.8 Hz, 1H), 4.00-3.92 (m, 2H), 3.84 (d, J = 10.8 Hz, 1H), 3.81 (d, J = 3.0 Hz, 1H), 3.78 – 3.67 (m, 5H), 3.67 – 3.36 (m, 14H), 2.62 (dd, J = 12.4, 4.6 Hz, 1H), 1.89 (s, 3H), 1.78 – 1.54 (m, 4H, NHAc and NeuAc-H3-Ax).

^{13}C NMR (151 MHz, D_2O) δ 175.79, 175.06, 174.68, 172.78, 169.93, 156.89, 136.21,135.82 131.71 (2C), 131.37, 129.45, 127.35, 119.00 (2C), 114.16, 103.77 (2C), 103.36, 101.88, 100.59, 79.06, 76.24, 75.94, 75.56, 73.67 , 73.16, 72.53, 70.18, 69.16, 68.86, 68.79, 68.26, 63.34, 63.25, 61.81, 60.83, 55.83, 52.46, 40.65, 40.39, 39.49, 22.82 (2C).

Note: Some of the fluorescein quartenary carbons are missing even after overnight ^{13}C scans.

HRMS: $C_{48}H_{57}N_3O_{25}$, $[M+H]^+$ Expected = 1076.3354 , Found = 1076.3364

Supplementary Methods

Mass Spectrometry Analysis

Glycan profiling – Snap frozen HL-60 cells (~2x10^8 cells) were treated as described previously[10]. Briefly all samples were subjected to homogenization using a 130-watt Vibra-Cell ultrasonic processor (VC 130 PB, Sonics & Materials) within a sound-abating enclosure in extraction buffer (25 mM Tris, 150 mM NaCl, 5 mM EDTA, and 1% CHAPS at pH 7.4). The samples were then subjected to reduction in 4 M guanidine HCl (Pierce) containing 2 mg/ml dithiothreitol, carboxymethylation, and trypsin digestion, and the digested glycoproteins were purified by C_{18}-Sep-Pak (Waters Corp., Hertfordshire, UK). N-linked glycans were released by peptide:N-glycosidase F (EC 3.5.1.52, Roche Applied Science) digestion, whereas O-linked glycans were released by reductive elimination. N- and O-linked glycans were then permethylated using the sodium hydroxide procedure, and finally, the permethylated N- and O-linked glycans were purified by C_{18}-Sep-Pak.

All permethylated samples were dissolved in 10 μl of methanol, and 1 μl of dissolved sample was premixed with 1 μl of matrix (for MS, 20 mg/ml 2,5-dihydroxybenzoic acid in 70% (v/v) aqueous methanol; for MS/MS, 20 mg/ml 3,4-diaminobenzophenone in 75% (v/v) aqueous acetonitrile). The mixture was then spotted onto a target plate (2x0.5 μl), and dried under vacuum. MS data were acquired using a Voyager-DE STR MALDI-TOF (Applied Biosystems, Darmstadt, Germany). MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. The collision energy was set to 1 kV, and argon was used as collision gas. The 4700 calibration standard kit, Calmix (Applied Biosystems), was used as the external calibrant for the MS mode of both instruments, and [Glu1] fibrinopeptide B human (Sigma) was used as an external calibrant for the MS/MS mode of the MALDI-TOF/TOF instrument.

The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The spectra were subjected to manual assignment and annotation with the aid of the glycobioinformatics tool, GlycoWorkBench[11]. The proposed assignments for the selected peaks were based on ^{12}C isotopic composition together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS experiments.

Endo-β-galactosidase digestion - Endo-β-galactosidase (*Escherichia freundii*, EC 3.2.1.103, Glyco) digestion was carried as described previously[12]. Briefly, released N-linked glycans were dissolved in 200 μl of sodium acetate buffer (37 °C, pH 5.8). 20 milliunits of enzyme were added to the sample for 48 h with a fresh aliquot of the enzyme added after 24 h.

Nucleotide Sugar Analysis

Extraction of nucleotide sugars

Treated cells (2.2 x 10^8 cells) were added to a dounce-type homogenizer tube with water and homogenized at least 10 times. Each homogenized sample was transferred into a screw-cap glass tube, added with 75 μg internal standard (GDP-glucose), and added with chloroform:methanol to achieve a chloroform:methanol:water ratio of 4:8:3. Lipids and nucleotide sugars were extracted by gentle agitation for 2 hr and the cells were sedimented thereafter at 2000 rpm for 20 min. The supernatants were transferred into another glass tubes and dried under a stream of nitrogen gas. Each dried extract was dissolved with nanopure H_2O, loaded into a C18 sep pak cartridge, rinsed with nanopure H_2O and at the same time eluting the nucleotide sugars. The eluates were lyophilized subsequently. Finally, the extracts were redissolved with 40 mM phosphate buffer (pH 9.1); passed through spin filters (10k cutoff membrane) and analyzed by HPAEC-UV

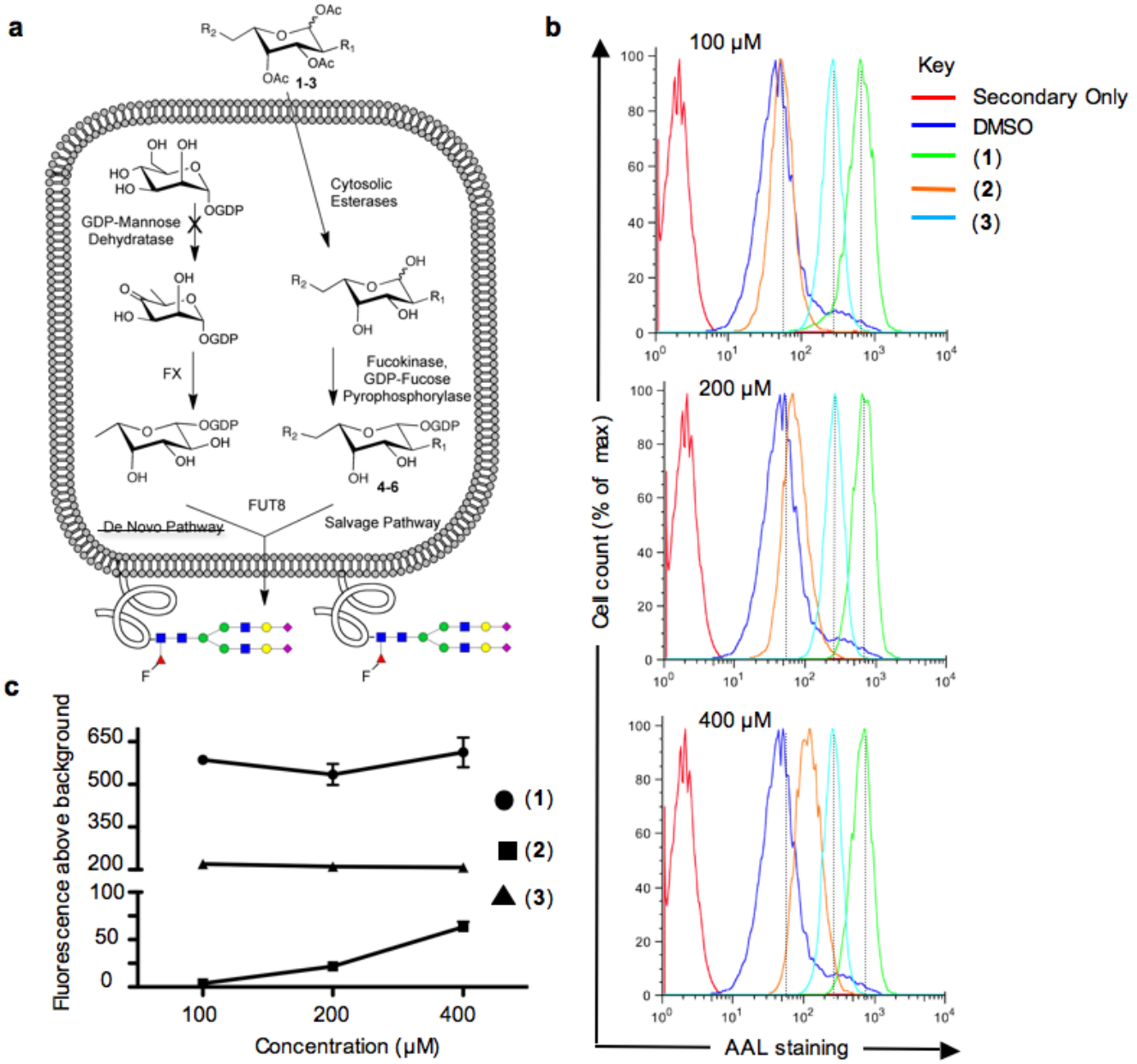
Analysis of nucleotide sugars by HPAEC-UV

A 15-µL volume of each filtered cell extract was directly (without dilution or concentration) and manually injected into an HPLC. The HPLC was equipped with GP50 gradient pump for the delivery of mobile phase eluents, 1.0 mM NaOH and 1.0 M Na acetate in 1.0 mM NaOH. The nucleotide sugars were separated by CarboPac PA1 column (4 x 250 mm), which was interfaced to an AD25 absorbance detector at 260 nm wavelength. Each sample/extract was injected three times into the analytical instrument.

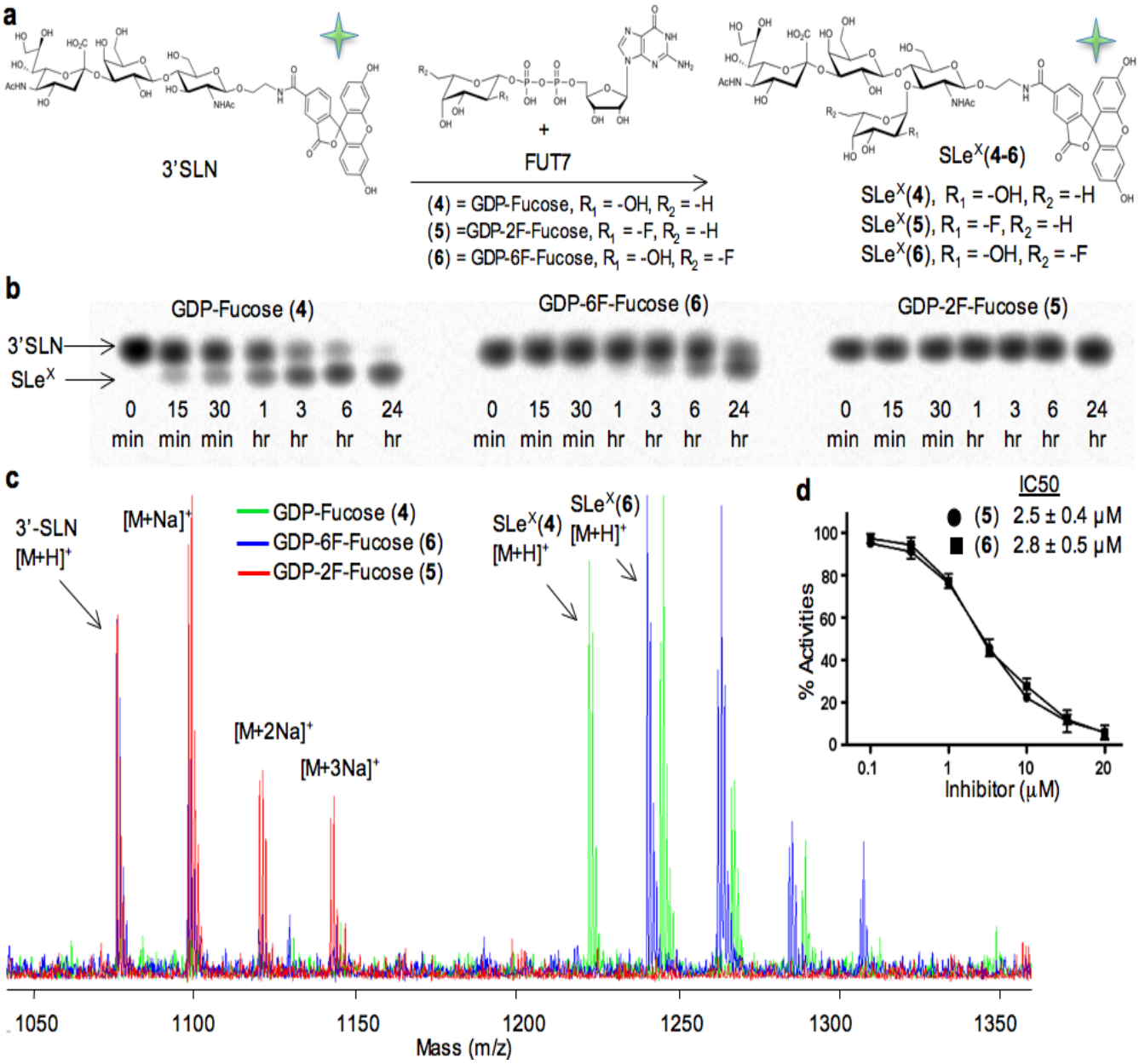
A mixture of CMP-NeuAc, 3F CMP-NeuAc, GDP-Fuc, and 2F GDP-Fuc standards (50 µg/standard) was prepared. Also, an equal amount of GDP-glucose was included in the standard mixture as internal standard to determine the response factor for each nucleotide sugar residue.

Supplementary Results

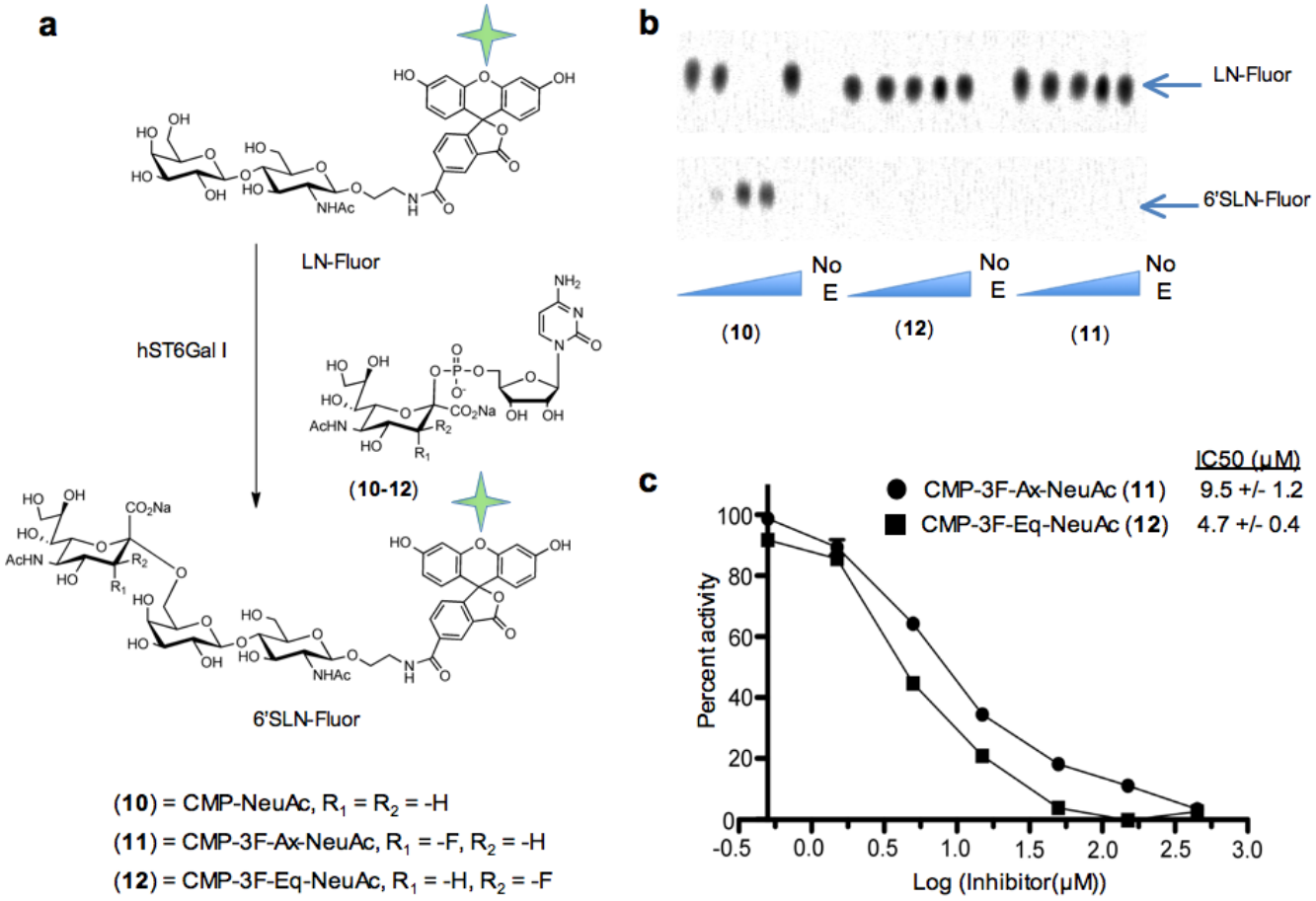
Supplementary Figure 1 – Analysis of 2F-Fucose and 6F-Fucose incorporation into N-linked glycans by FUT8. (**a**) Lec13 CHO cells, with a mutation in the endogenous GDP-Mannose Dehydratase enzyme, are deficient in *de novo* GDP-Fucose biosynthesis. By supplying exogenous fucose (or fucose analogs), however, GDP-Fucose (or GDP-Fucose analogs) can be produced via a salvage pathway and utilized as substrates by FUT8. Thus, to test if GDP-2F-Fucose and GDP-6F-Fucose are indeed utilized as slow substrates by FUT8, compounds (**1-3**) (100, 200, or 400 μM) were incubated with Lec13 cells for 48 hrs prior to analyzing cell surface fucose with biotinylated-AAL and flow cytometry (**b-c**). The results show that both GDP-2F-Fucose and GDP-6F-Fucose are slow substrates, but suggest that GDP-2F-Fucose has a much higher K_M than GDP-6F-as judged by the dose-response at high concentrations of the respective prodrugs.



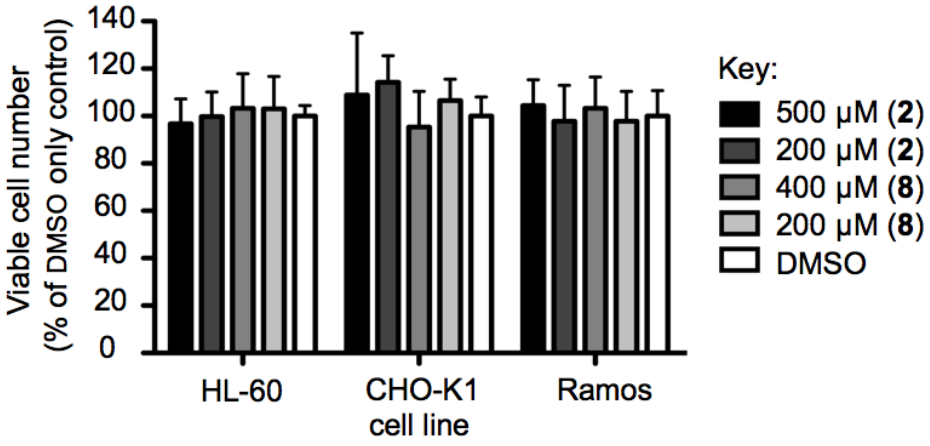
Supplementary Figure 2 – GDP-6F-Fucose (**6**) is utilized as a slow substrate by FUT7, while GDP-2F-Fucose (**5**) is not detectably transferred. (**a**) Substrates and set-up of the *in vitro* FUT7 assay. (**b**) 3'-Sialyl-LacNAc-Fluor (3'SLN) (500 μM) was incubated with GDP-Fucose (**4**), GDP-2F-Fucose (**5**), or GDP-6F-Fucose (**6**) (600 μM) and human FUT7 in 50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$, pH 7.5 at 37°C. After incubation for various lengths of time, 1 μl was spotted onto a TLC plate which was developed with a 6:3:3:2 (EtOAc:CH_3OH:AcOH:H_2O) (v/v) solvent system followed by imaging using a Typhoon Fluorescence Imager (GE Healthcare). (**c**) After a 24 hr incubation, the reactions shown in (**b**) were analyzed by MALDI-TOF MS to confirm the utilization of (**4**) and (**6**) as substrates and demonstrate that (**5**) is not utilized to any detectable extent. (**d**) A previously reported, fluorescence polarization-based inhibition assay[13] was utilized to demonstrate that both (**5**) and (**6**) can effectively inhibit the transfer of a fluorescent fucose analog to fetuin.



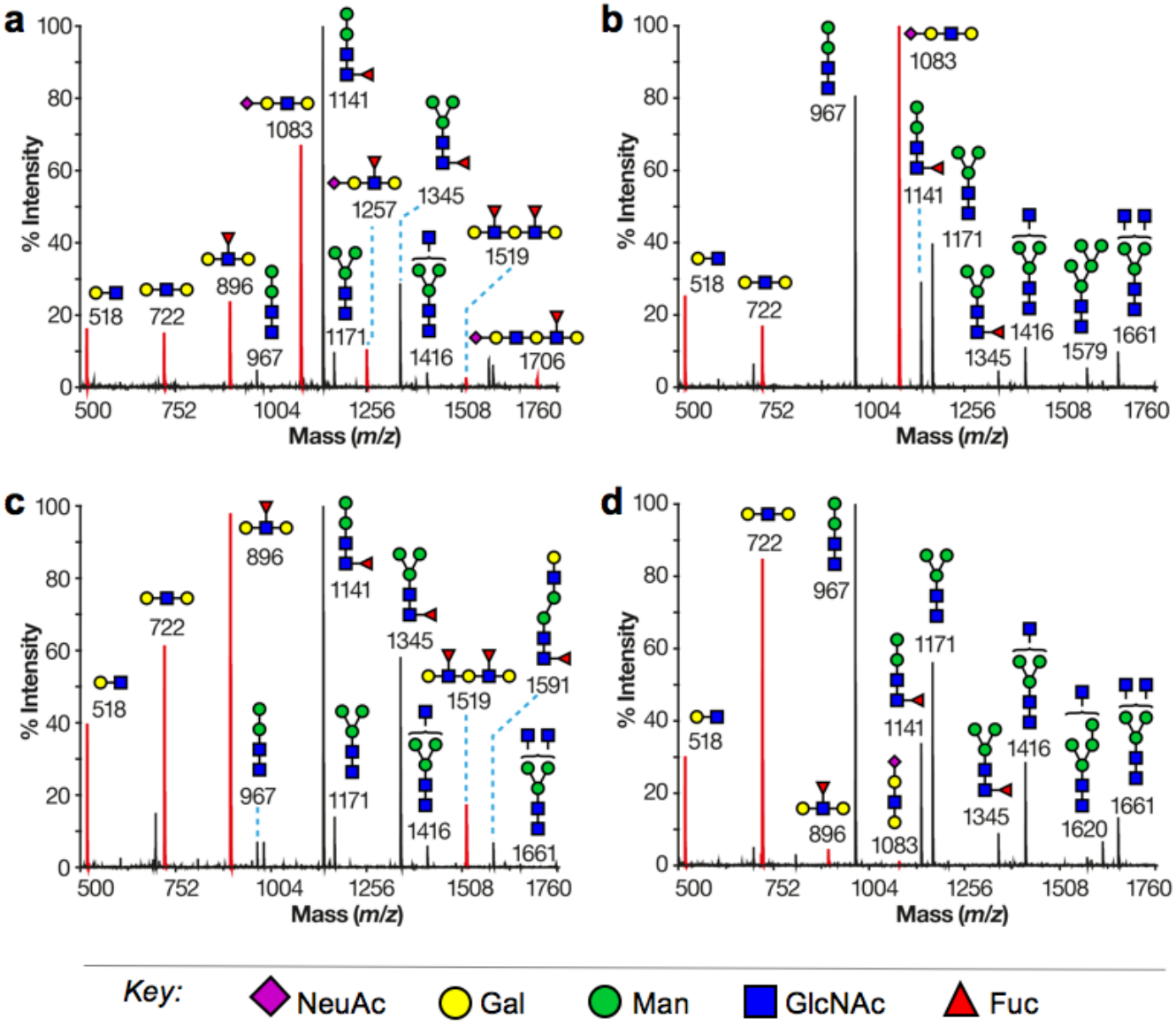
Supplementary Figure 3 – CMP-3F-Ax-NeuAc (**11**) and CMP-3F-Eq-NeuAc (**12**) are both i*n vitro* inhibitors of hST6Gal I and not utilized as appreciably slow substrates. (**a**) To analyze if (**11**) or (**12**) are utilized as substrates by hST6Gal I, a simple TLC assay was conducted where reaction progress can be monitored by analyzing disappearance of the fluorescent acceptor (LN-Fluor) and emergence of the fluorescent product (6'-SLN-Fluor). (**b**) Reactions conditions utilized: 0.5 mM LN-Fluor, 0.6 mM of the corresponding donor substrate (**10-12**), 30 mM Sodium Cacodylate pH 6.5, 30 mM NaCl and hST6Gal I at 910 mU/ml, 91 mU/ml, 9.1 mU/ml, 0.91 mU/ml, or 0 mU/ml. 20 μl reactions were run for 1 hr (**b**) or 12 hr (not shown), and then 1 μl was spotted onto a TLC plate, dried, and the starting material and product separated with 6:3:3:2 eluant (EtOAc:MeOH:AcOH:H_2O). After drying, the TLC plates were scanned on a Typhoon Imager (GE Healthcare). Only in the case of the natural substrate (**10**) is there any appreciable product formed. (c) Utilizing a fluorescence polarization inhibitor assay[13], both (**11**) and (**12**) were validated as in vitro inhibitors with IC50 values in the low micromolar range.



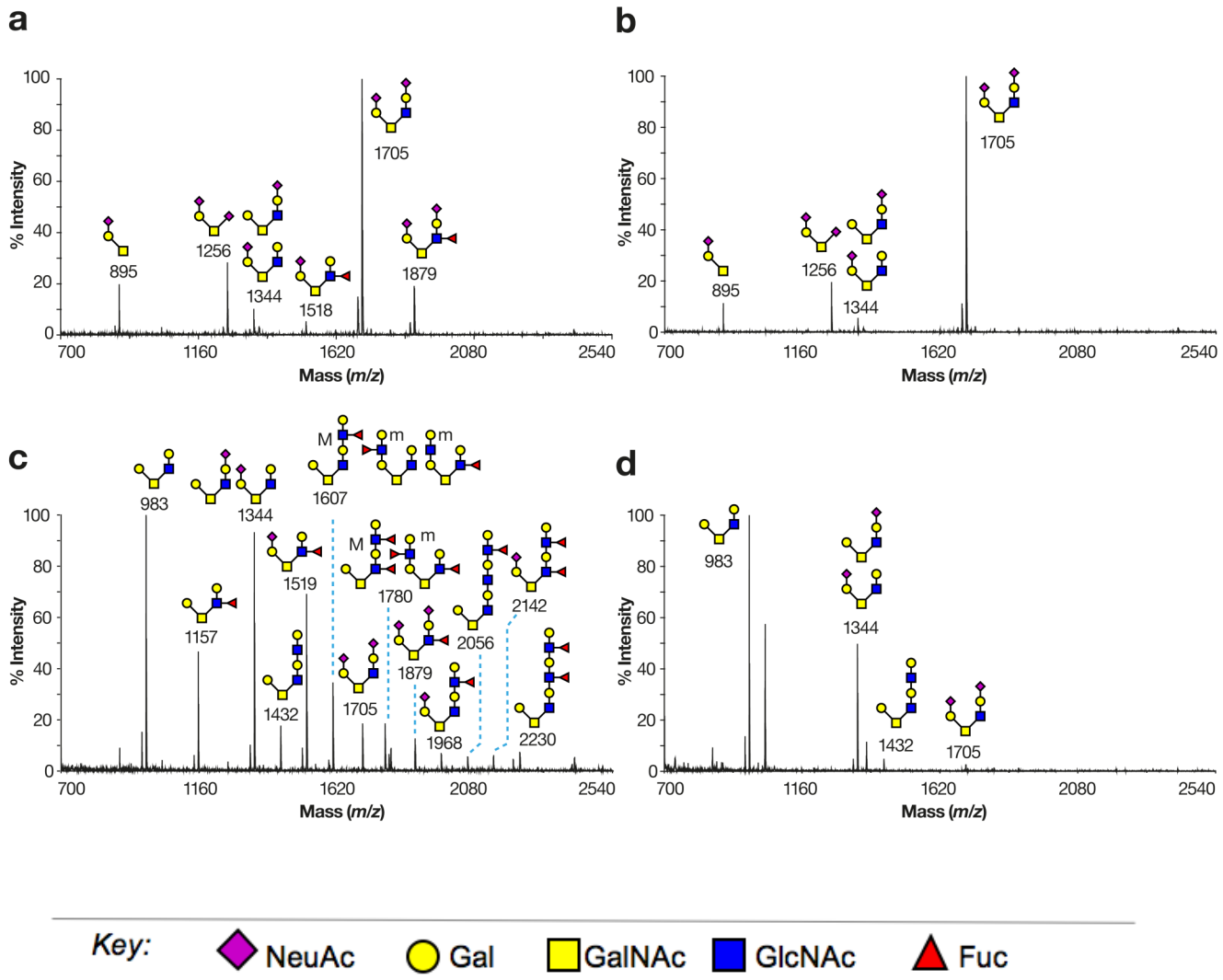
Supplementary Figure 4 – The best ST and FUT inhibitors are not cytotoxic at concentrations up to 400-500 μM. 20,000 HL-60, CHO-K1, or Ramos cells were plated into wells of a 96-well plate, the plate was centrifuged, the media removed, and fresh media containing inhibitors or DMSO was added (100 μl). After 72 hrs, 20 μl of CellTiter 96 (Promega) was added to each well, the plate was placed at 37°C for 1-2 hrs, and the absorbance at 540 nm was read. Cells treated with DMSO only were used to establish 100% cell viability, while cells treated with Triton X-100 (1% final) 5 min prior to addition of the CellTiter reagent were used as 0% cell viability. The data presented is the average of 3 independent experiments each carried out in triplicate (n=9).



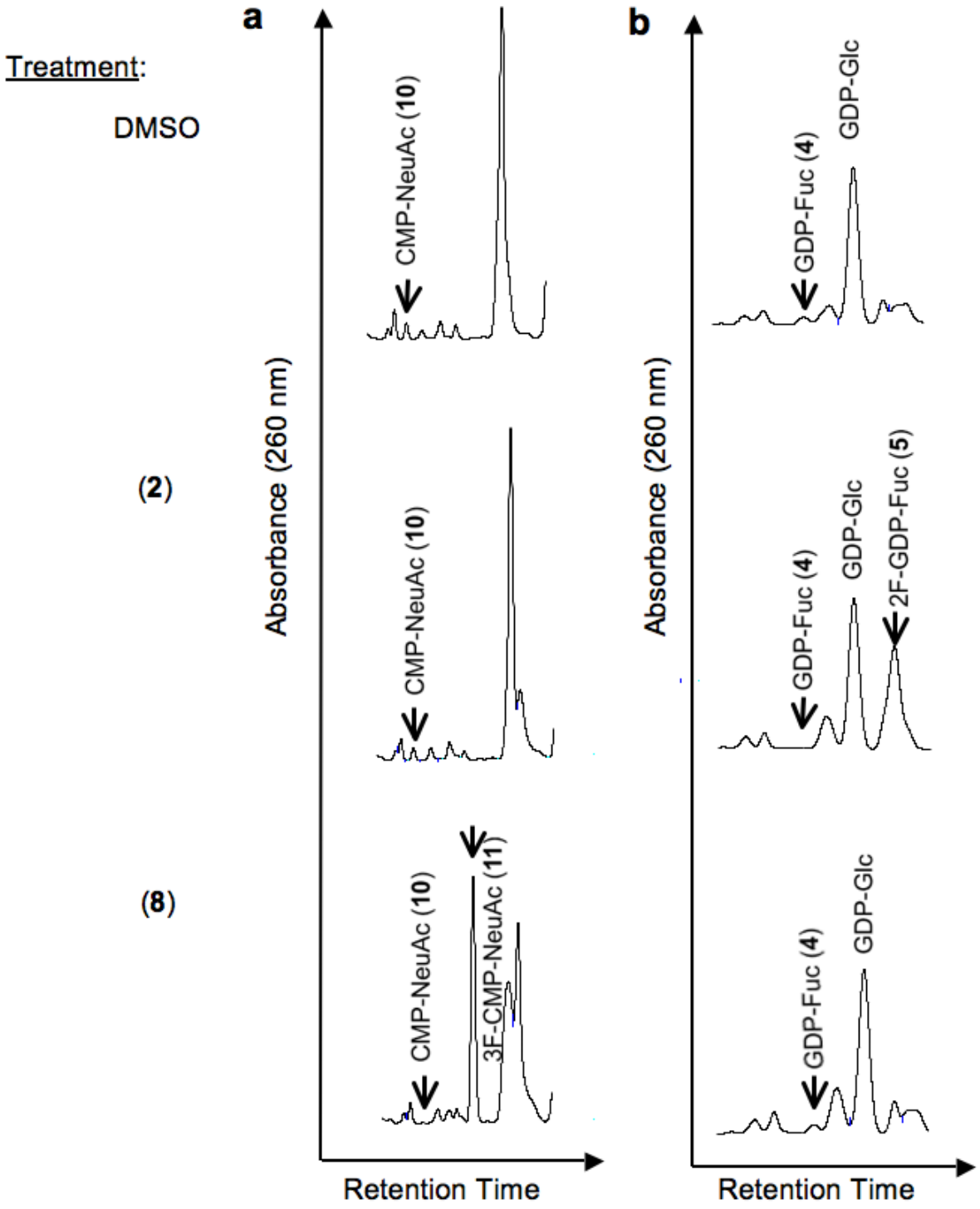
Supplementary Figure 5 – Low mass MALDI-TOF MS spectra derived from endo-β-galactosidase digestion from HL-60 cells treated for 7 days with (**a**) DMSO Only, (**b**) 200 μM (**2**), (**c**) 200 μM (**8**), or (**d**) 200 μM each of (**2**) and (**8**). All molecular ions are $[M+Na]^+$. Putative structures are based on composition, tandem MS, and biosynthetic knowledge. Structures that show sugars outside a bracket have not been unequivocally defined. Note in (**a**) the presence of undecorated *N*-acetyllactosamine units (*m/z* 722), the fucosylated (*m/z* 896), sialylated (*m/z* 1083) and SLe^X (*m/z* 1257) terminal epitopes; in (**b**) the absence of fucosylated terminal epitopes and the increase of sialylated epitope at *m/z* 1083; in (**c**) the absence of sialylated epitopes and the increase of fucosylated epitopes at *m/z* 896 and 1519; in (**d**) the minor abundance of fucosylated and sialylated epitopes at *m/z* 896 and 1083 respectively and the increase of undecorated *N*-acetyllactosamine units at *m/z* 722.



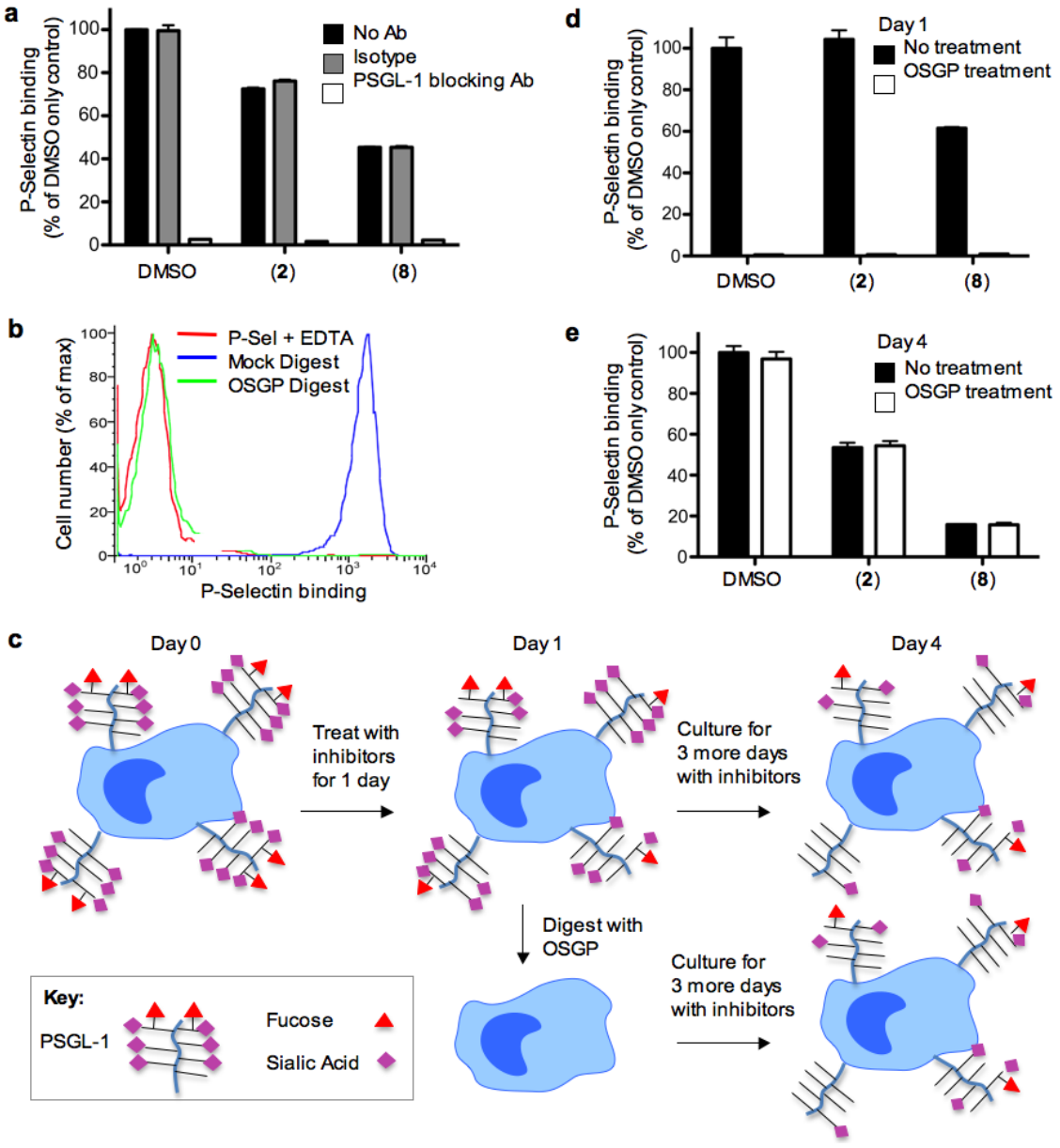
Supplementary Figure 6 – MALDI-TOF MS analysis of O-linked glycans from HL-60 cells treated for 7 days with (**a**) DMSO Only, (**b**) 200 μM (**2**), (**c**) 200 μM (**8**), or (**d**) 200 μM each of (**2**) and (**8**). All molecular ions are $[M+Na]^+$. Putative structures are based on composition, tandem MS, and biosynthetic knowledge. Structures that show sugars outside a bracket have not been unequivocally defined.



Supplementary Figure 7. HPAEC-UV Analysis of Natural and Unnatural Nucleotide Sugars Levels. HL-60 cells were treated for 7 days with DMSO, 200 μM 2F-Fucose (**2**), or 200 μM 3F-Ax-NeuAc (**8**). Nucleotide sugars were extracted (**Supplementary Methods**) and subjected to High Performance Anion Exchange Chromatography (**HPAEC-UV**). Representative Chromatograms of the CMP-NeuAc/3F_{ax}-CMP-NeuAc (**a**) and the GDP-Fucose/2F-GDP-Fucose (**b**) elution regions are shown.



Supplementary Figure 8 – Residual P-selectin binding to HL-60 cells after treatment with ST and FUT inhibitors is entirely PSGL-1 dependent and is not due to slow PSGL-1 recycling. (**a**) HL-60 cells were treated with 200 μM of inhibitors for three days prior to staining with human P-selectin. Cells were left untreated or blocked for 30 mins on ice with 1 μg/ml of the KPL-1 blocking antibody or isotype control. (**b**) P-selectin binding can be entirely abolished by pre-treatment with O-Sialoglycoprotease (OSGP), which degrades PSGL-1 and other sialylated mucins. (**c**) Experimental set-up to assess if remaining PSGL-1 sialylation and fucosylation is due to slow receptor turnover or due to incomplete inhibition by the the prodrugs (**2**) and (**8**). By removing PSGL-1 on the cell surface at day one, any that appears on the cell surface after three more days will be due to *de novo* synthesis in the presence of the inhibitors. (**d**) P-selectin binding of HL-60 cells treated for 1 day with DMSO only, 200 μM (**2**), or 200 μM (**8**) and then subjected to mock digest or OSGP treatment. (**e**) The six sets of cells from (**d**) were then recultured for three days with fresh media containing DMSO only, 200 μM (**2**), or 200 μM (**8**) and assessed for P-selectin binding on day 4. Control experiments (not shown) show that SLe^X expression is down to ~1% of the control with both inhibitors at this time point. In each case all experiments are normalized to DMSO only control and represent one of two independent experiments carried out in triplicate.



Supplementary Video 1 – Rolling Velocity Measurement of control (DMSO) treated HL-60 cells on E-Selectin at 3 dynes/cm^2

Supplementary Video 2 – Rolling Velocity Measurement of 2F-Fuc (2) treated HL-60 cells on E-Selectin at 3 dynes/cm^2

Supplementary Video 3 – Rolling Velocity Measurement of 3F$_{ax}$-NeuAc (8) treated HL-60 cells on E-Selectin at 3 dynes/cm^2

Supplementary Video 4 – Rolling Velocity Measurement of control (DMSO) treated HL-60 cells on P-Selectin at 3 dynes/cm^2

Supplementary Video 5 – Rolling Velocity Measurement of 2F-Fuc (2) treated HL-60 cells on P-Selectin at 3 dynes/cm^2

Supplementary Video 6 – Rolling Velocity Measurement of 3F$_{ax}$-NeuAc (8) treated HL-60 cells on P-Selectin at 3 dynes/cm^2

Supplementary Video 7 –Tethering Analysis of Control (DMSO) treated HL-60 cells on E-Selectin. Cells were allowed to settle briefly (<10 sec) and then flow was initiated at a shear stress of 1 dyne/cm^2. The shear stress was then increased in 1 dyne/cm^2 increments up to 6 dyne/cm^2.

Supplementary Video 8 –Tethering Analysis of 2F-Fuc (2) treated HL-60 cells on E-Selectin. Cells were allowed to settle briefly (<10 sec) and then flow was initiated at a shear stress of 1 dyne/cm^2. The shear stress was then increased in 1 dyne/cm^2 increments up to 6 dyne/cm^2.

Supplementary Video 9 –Tethering Analysis of 3F$_{ax}$-Fuc (8) treated HL-60 cells on E-Selectin. Cells were allowed to settle briefly (<10 sec) and then flow was initiated at a shear stress of 1 dyne/cm^2. The shear stress was then increased in 1 dyne/cm^2 increments up to 6 dyne/cm^2.

Supplementary Video 10 –Tethering Analysis of Control (DMSO) treated HL-60 cells on P-Selectin. Cells were allowed to settle briefly (<10 sec) and then flow was initiated at a shear stress of 1 dyne/cm^2. The shear stress was then increased in 1 dyne/cm^2 increments up to 6 dyne/cm^2.

Supplementary Video 11 –Tethering Analysis of 2F-Fuc (2) treated HL-60 cells on P-Selectin. Cells were allowed to settle briefly (<10 sec) and then flow was initiated at a shear stress of 1 dyne/cm^2. The shear stress was then increased in 1 dyne/cm^2 increments up to 6 dyne/cm^2.

Supplementary Video 12 –Tethering Analysis of 3F$_{ax}$-Fuc (8) treated HL-60 cells on P-Selectin. Cells were allowed to settle briefly (<10 sec) and then flow was initiated at a shear stress of 1 dyne/cm^2. The shear stress was then increased in 1 dyne/cm^2 increments up to 6 dyne/cm^2.

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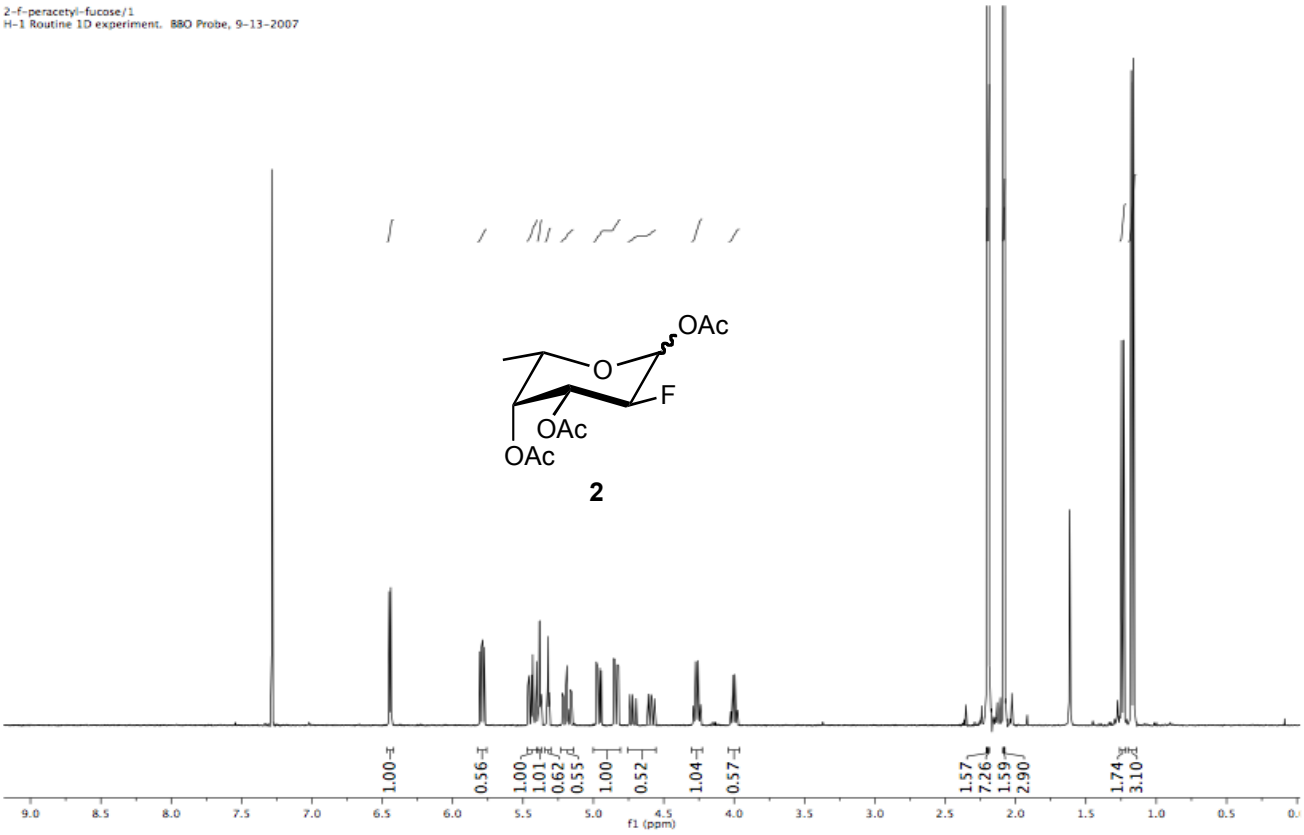
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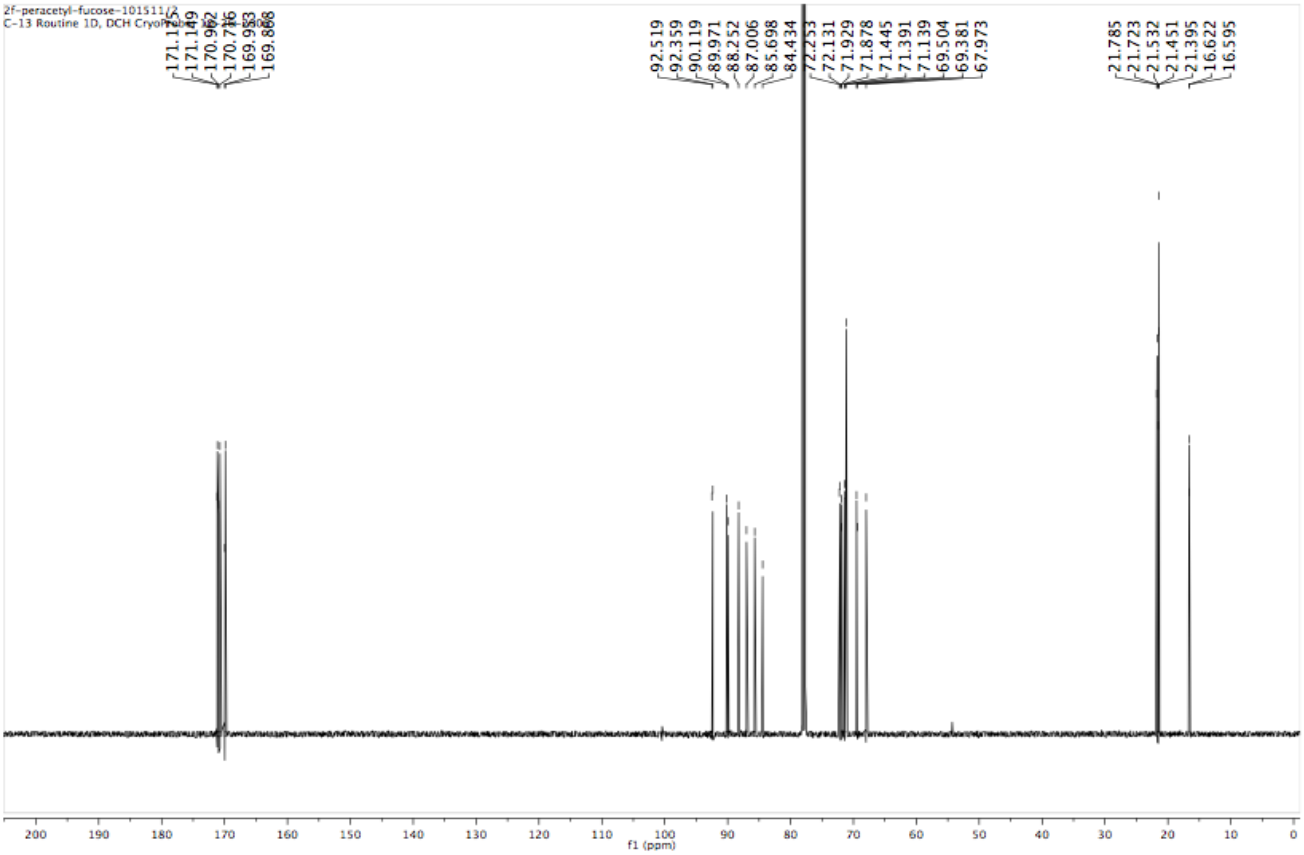
f1 (ppm)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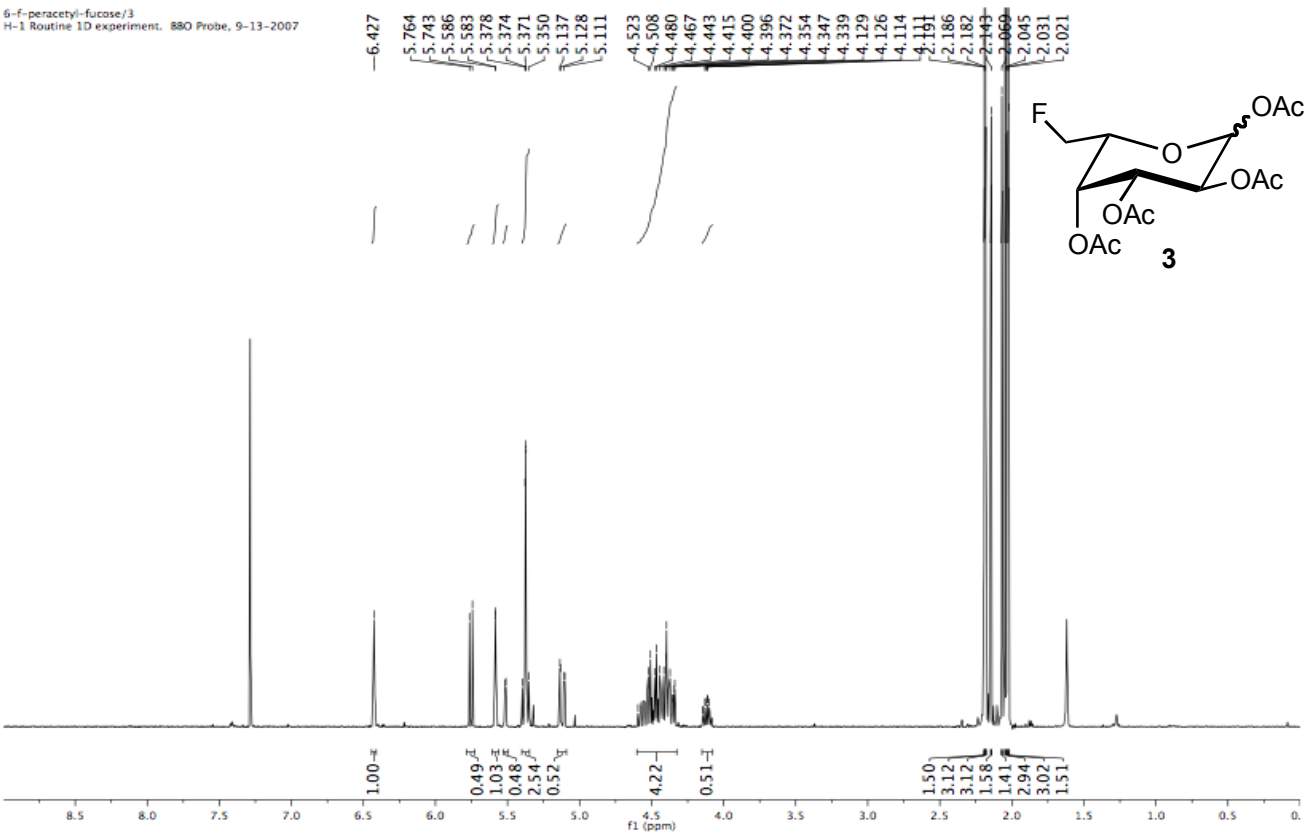
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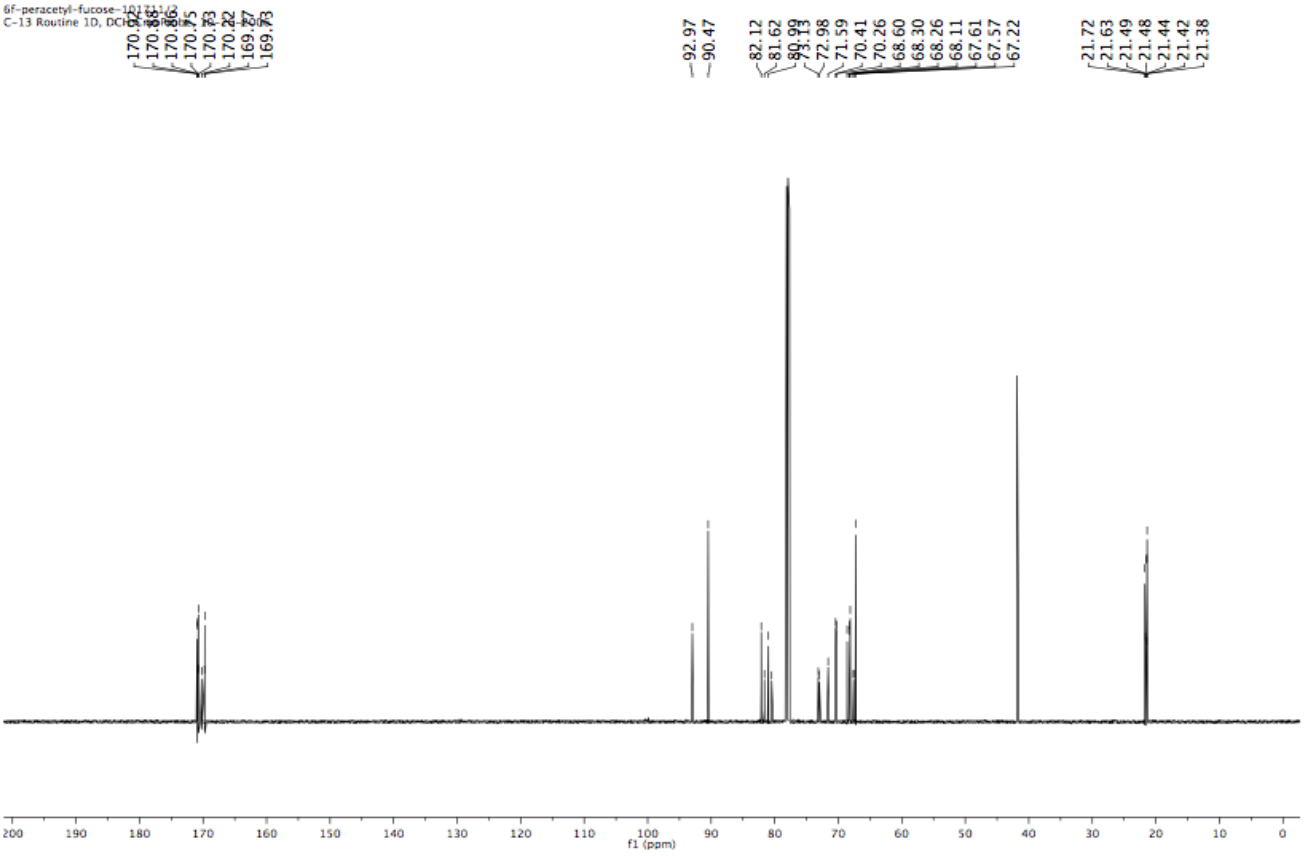
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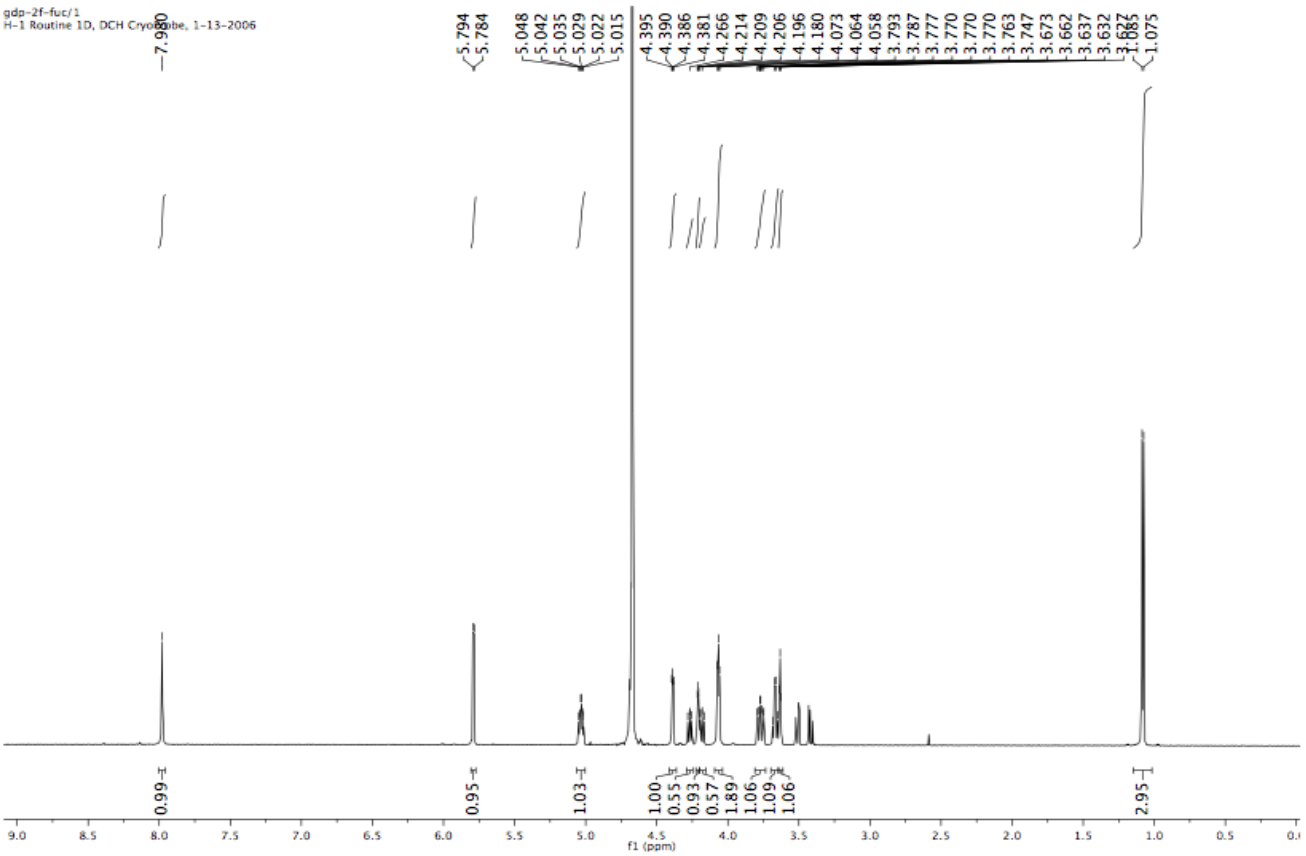
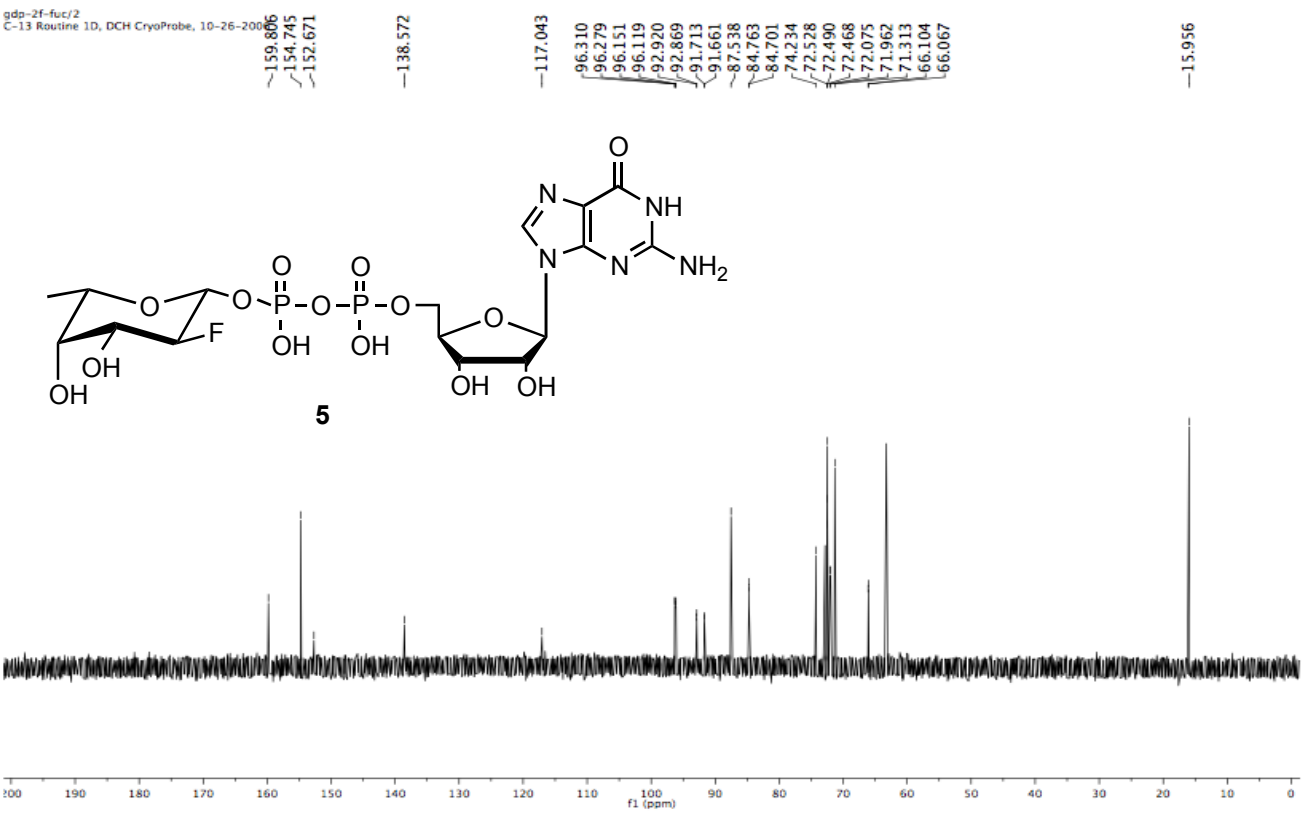
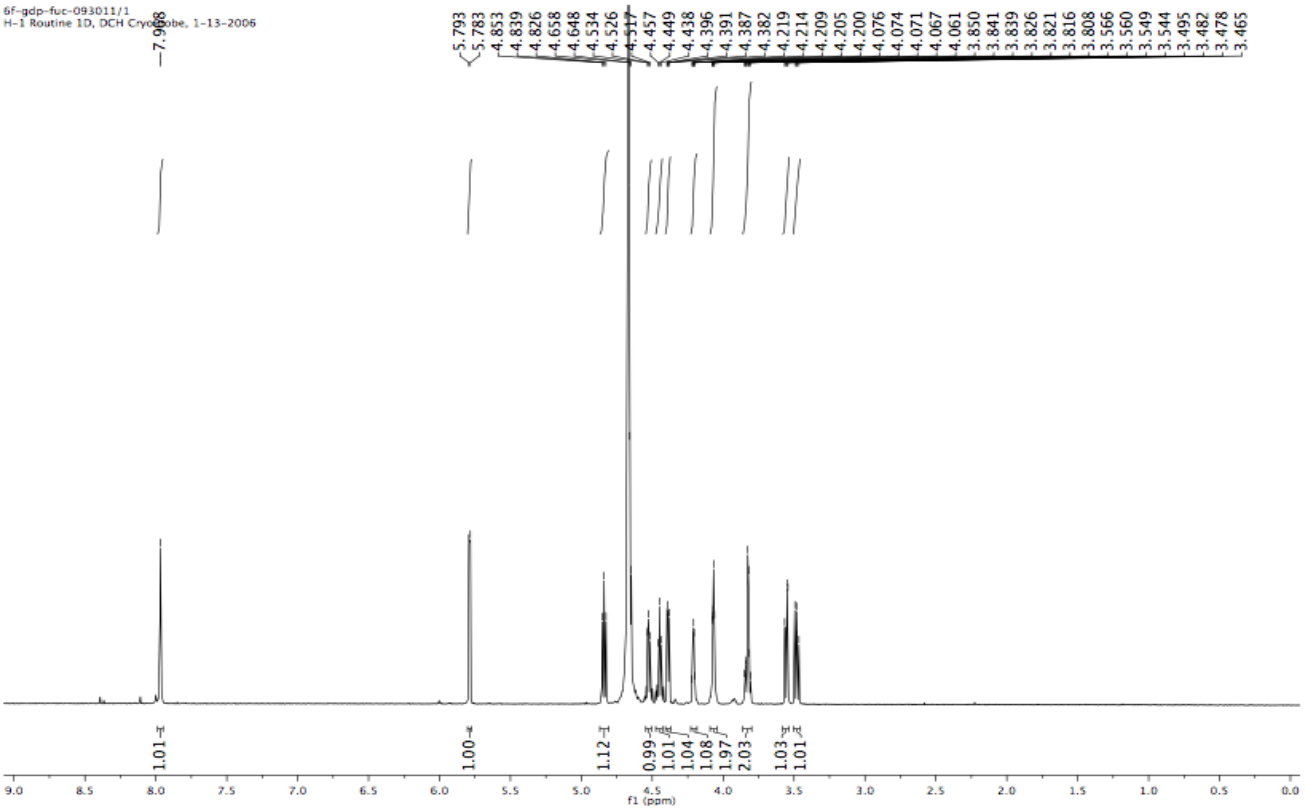
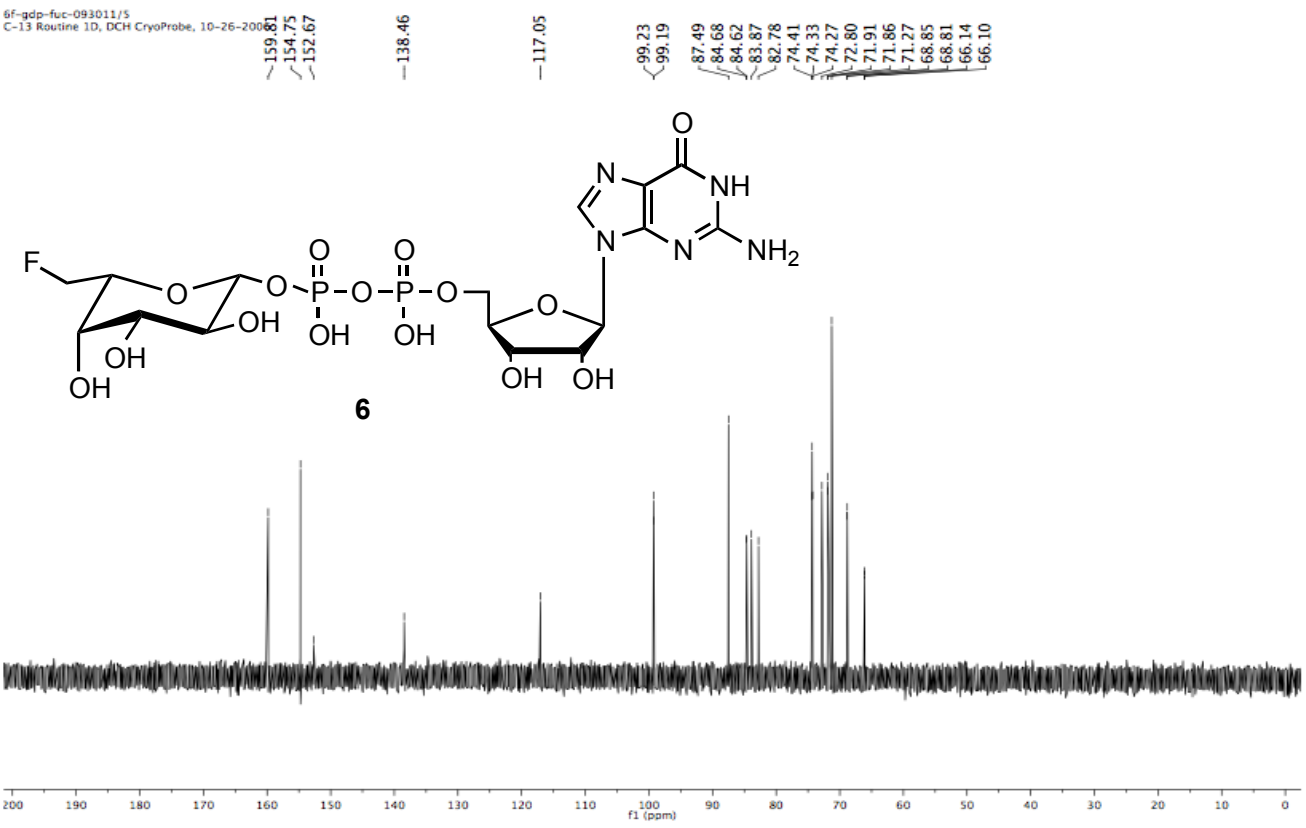
21.38
f1 (ppm)


gdp-2f-fuc/1
H-1 Routine 1D, DCH Cryoprobe, 1-13-2006
f1 (ppm)

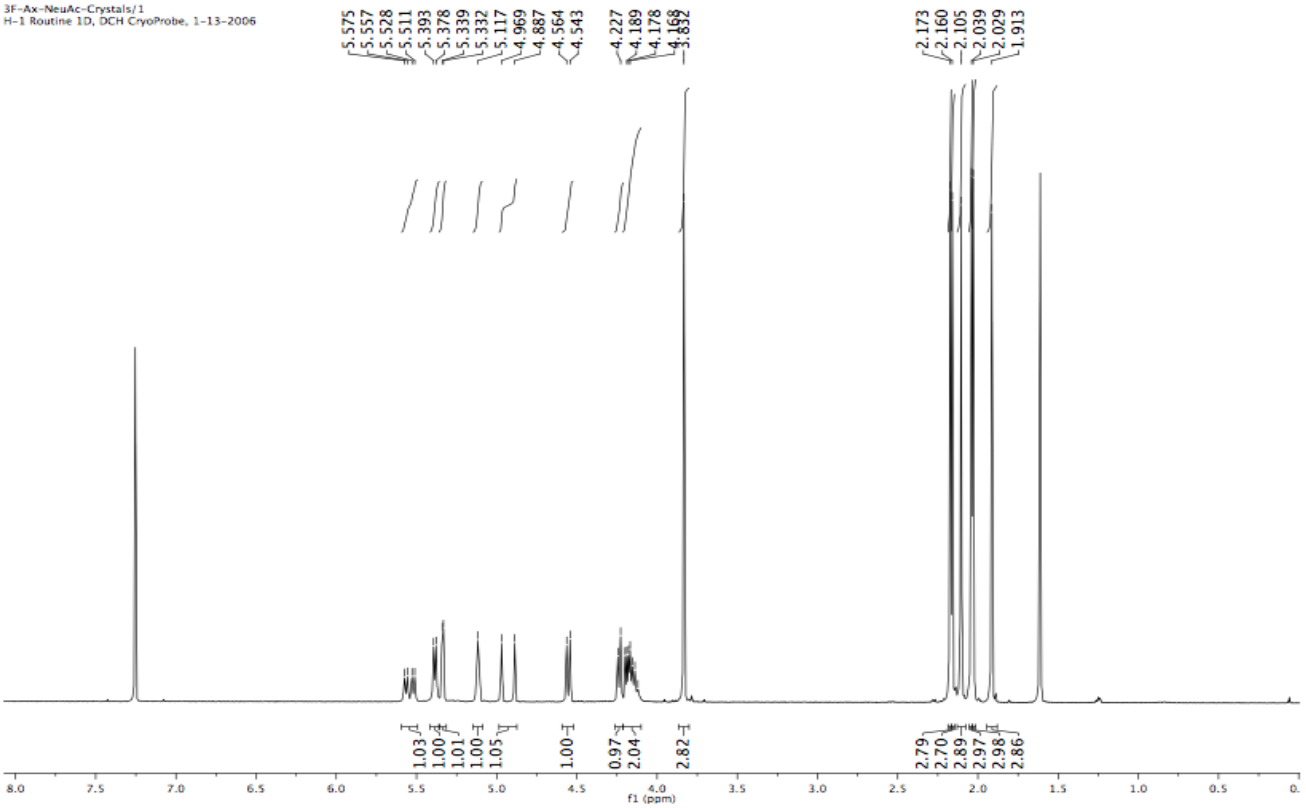

gdp-2f-fuc/2
C-13 Routine 1D, DCH CryoProbe, 10-26-2006
f1 (ppm)

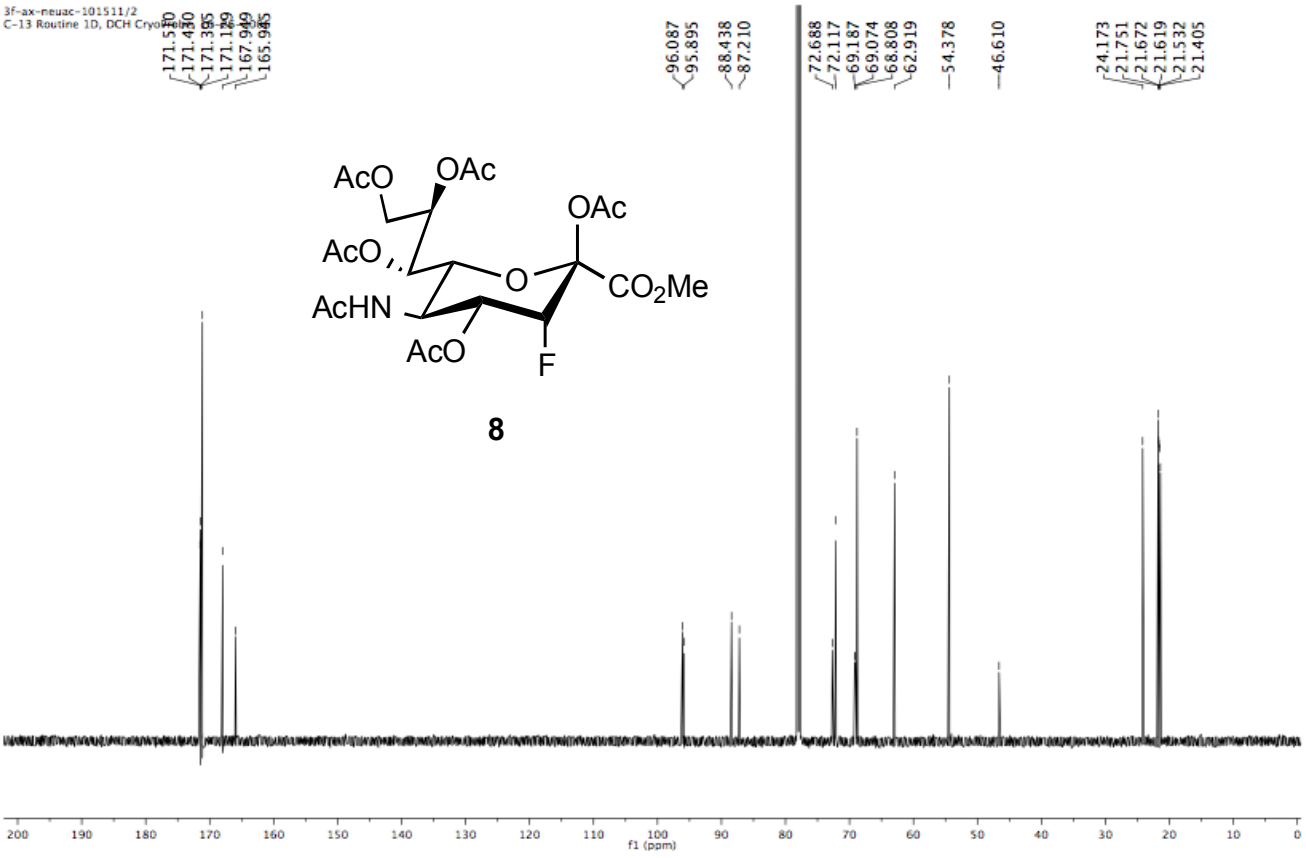
5


6f-gdp-fuc-093011/1
H-1 Routine 1D, DCH CryoProbe, 1-13-2006
f1 (ppm)


6f-gdp-fuc-093011/5
C-13 Routine 1D, DCH CryoProbe, 10-26-2006
f1 (ppm)

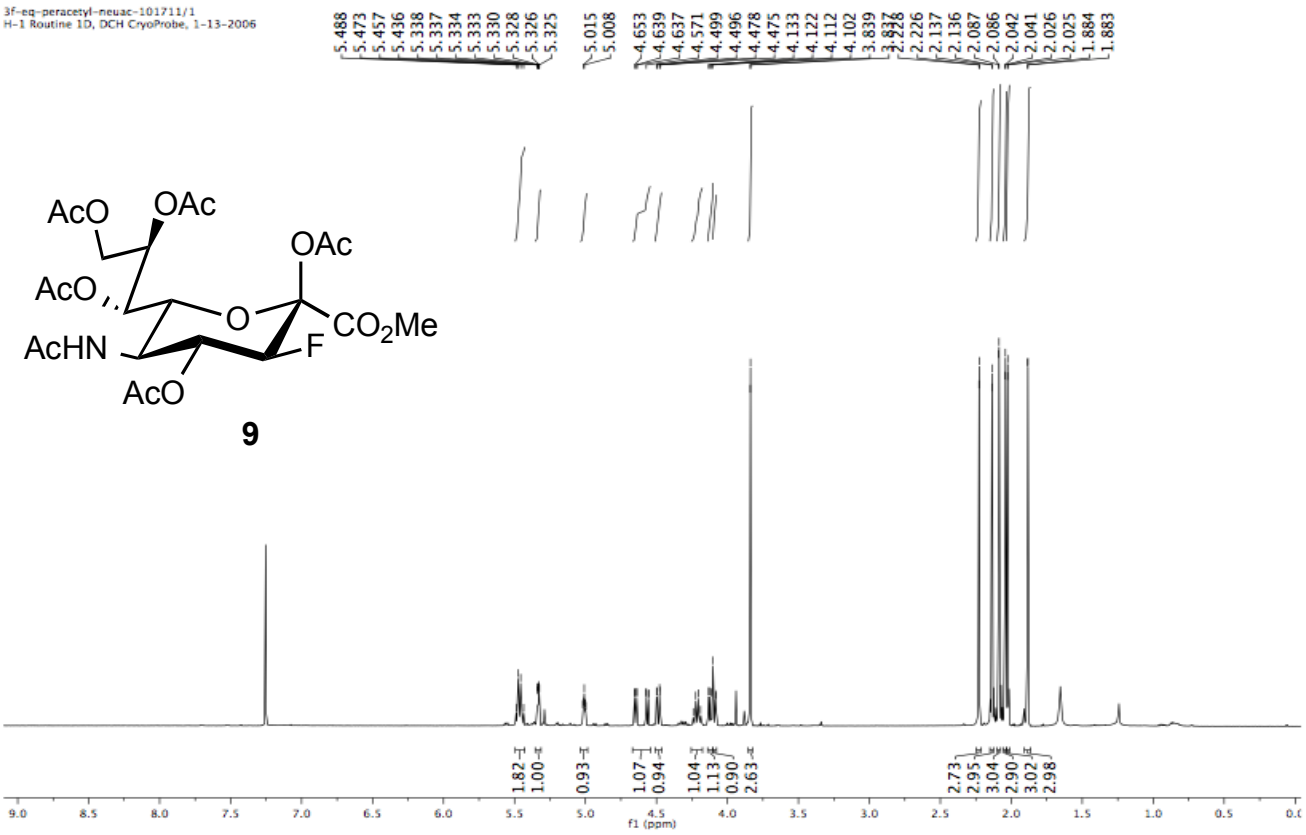
6

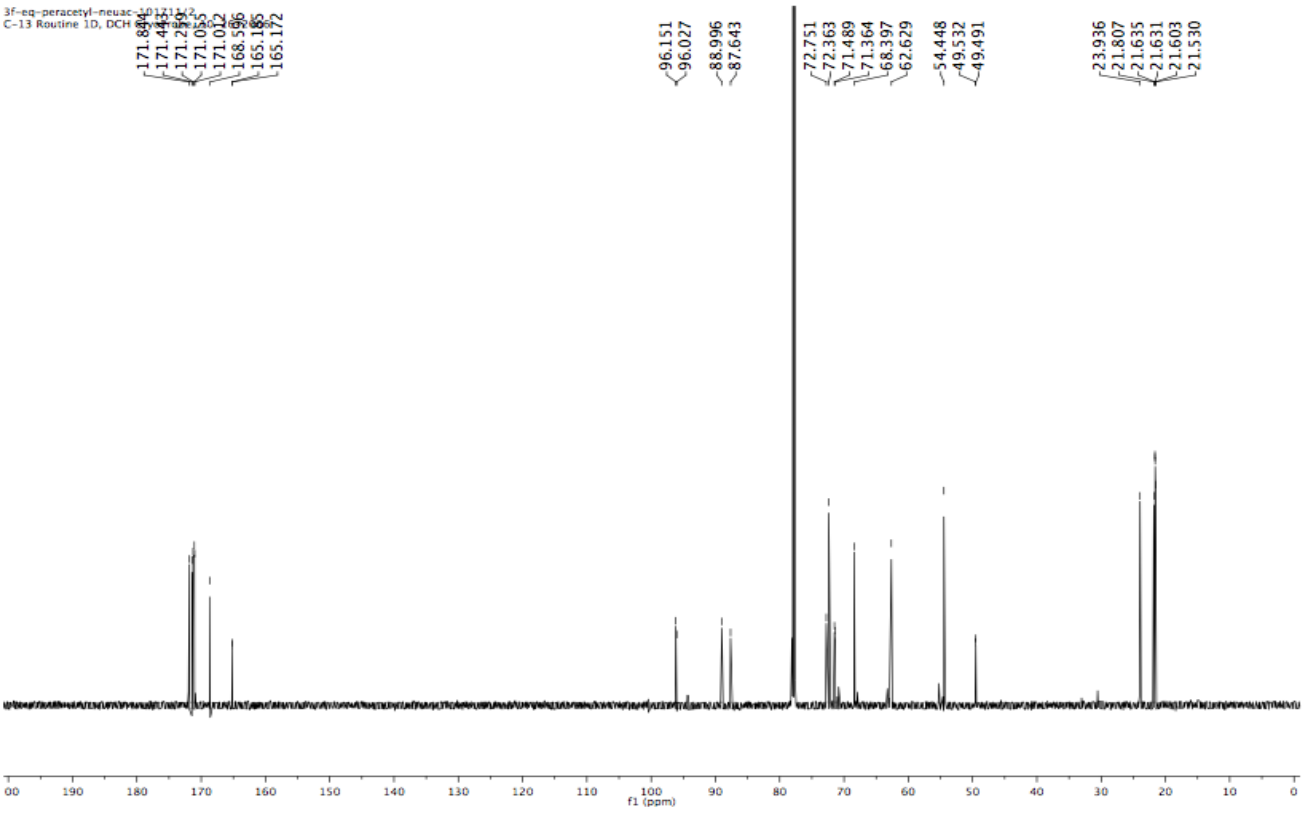

3F-Ax-NeuAc-Crystals/1
H-1 Routine 1D, DCH CryoProbe, 1-13-2006
f1 (ppm)

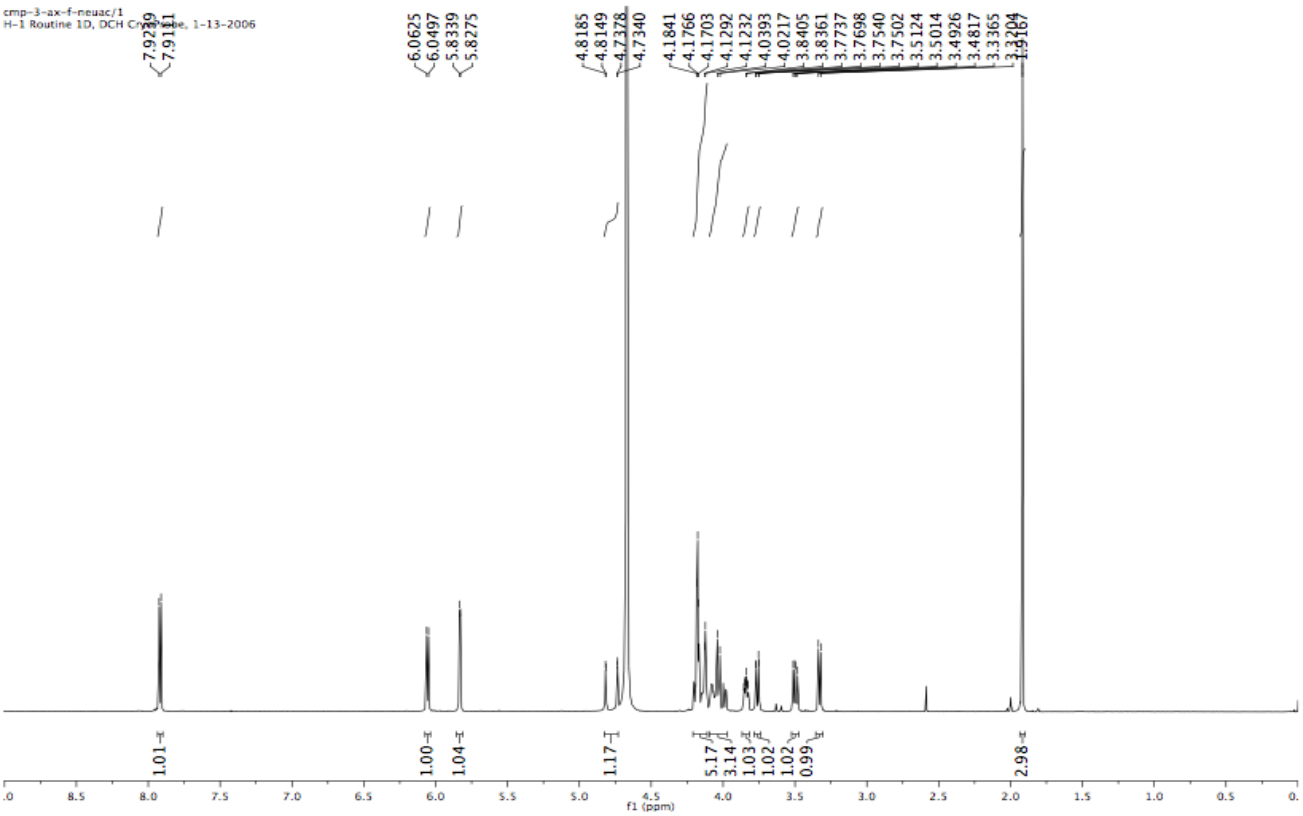

3f-ax-neuac-101511/2
C-13 Routine 1D, DCH CryoProbe
f1 (ppm)

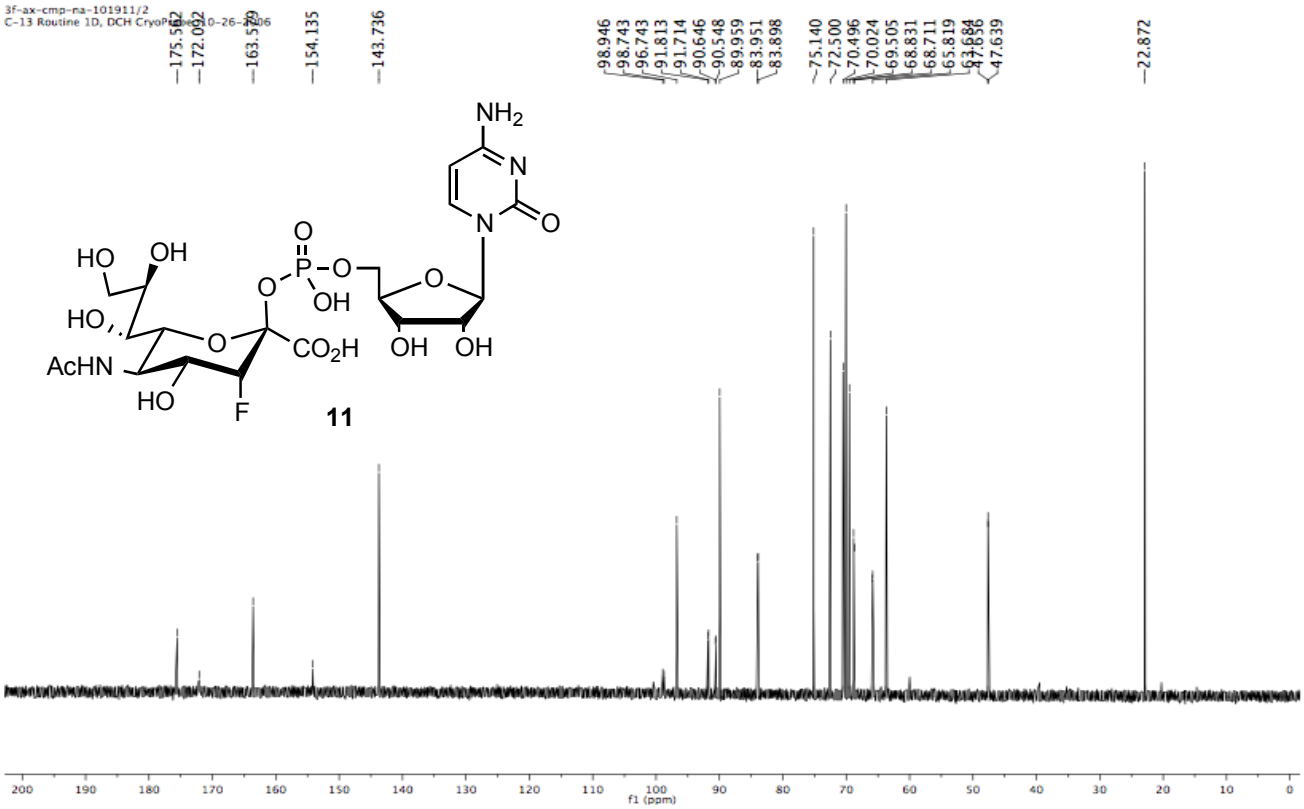
AcO
OAc
AcO
O
OAc
CO2Me
AcHN
AcO
F

8


3f-eq-peracetyl-neuac-101711/1
H-1 Routine 1D, DCH CryoProbe, 1-13-2006
AcO
OAc
AcO
OAc
O
CO2Me
AcHN
F
AcO
9
f1 (ppm)

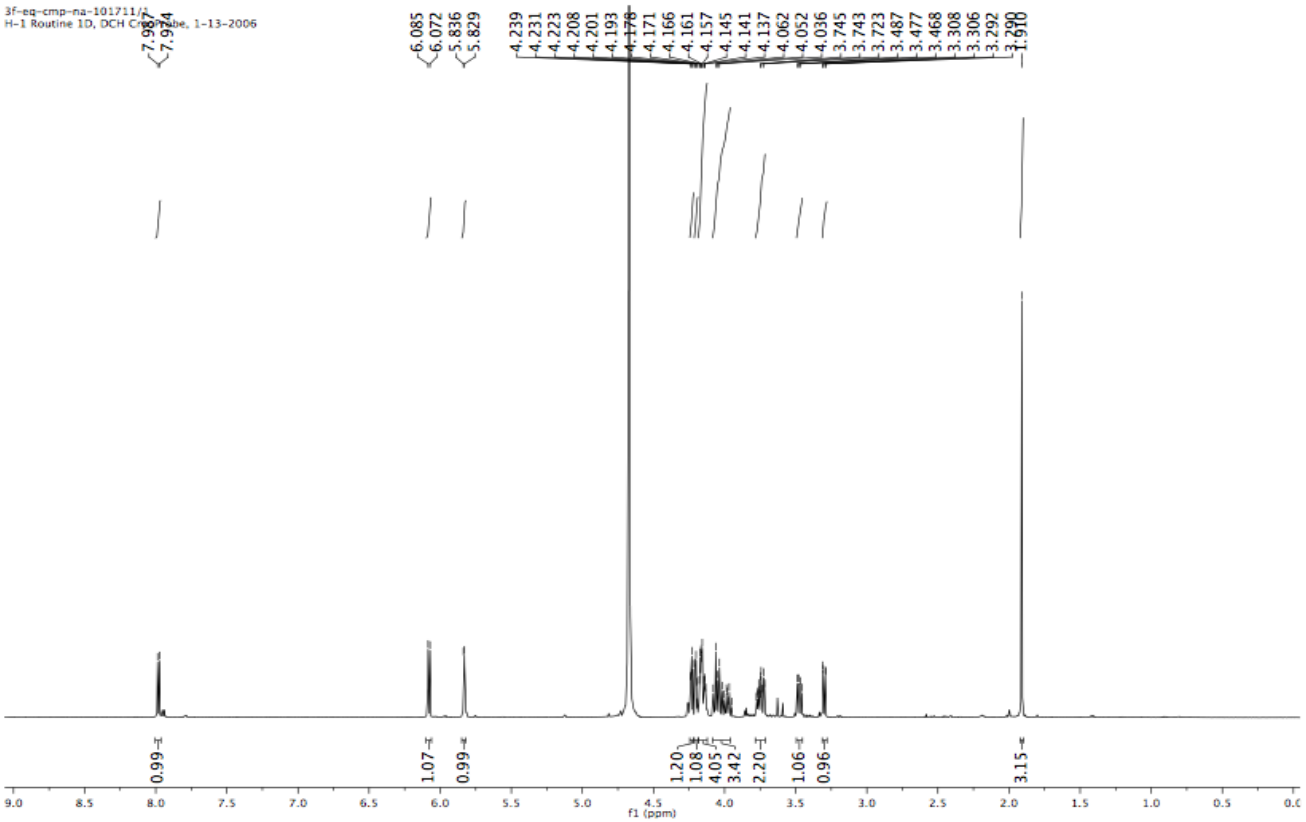

3f-eq-peracetyl-neuac-101711/2
C-13 Routine 1D, DCH
f1 (ppm)

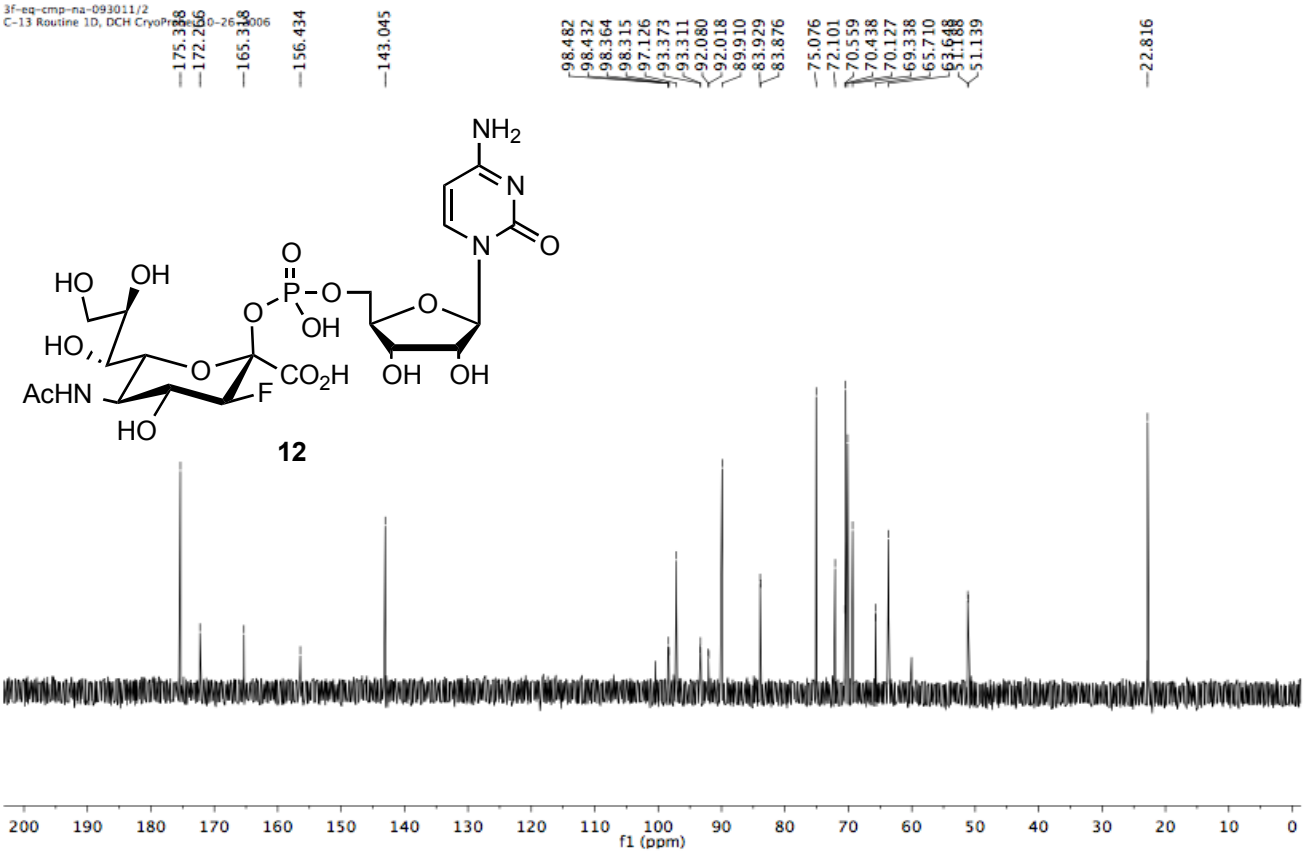

cmp-3-ax-f-neuac/1
H-1 Routine 1D, DCH CryoProbe, 1-13-2006
7.9239
7.9181
6.0625
6.0497
5.8339
5.8275
4.8185
4.8149
4.7378
4.7340
4.1841
4.1766
4.1703
4.1292
4.1232
4.0393
4.0217
3.8405
3.8361
3.7737
3.7698
3.7540
3.7502
3.5124
3.5014
3.4926
3.4817
3.3365
3.3204
2.1910
1.01
1.00
1.04
1.17
5.17
3.14
1.03
1.02
1.02
0.99
2.98
f1 (ppm)


3f-ax-cmp-na-101911/2
C-13 Routine 1D, DCH CryoProbe, 10-26-2006
175.582
172.052
163.579
154.135
143.736
98.946
98.743
96.743
91.813
91.714
90.646
90.548
89.959
83.951
83.898
75.140
72.500
70.496
70.024
69.505
68.831
68.711
65.819
63.684
47.658
47.639
22.872
f1 (ppm)

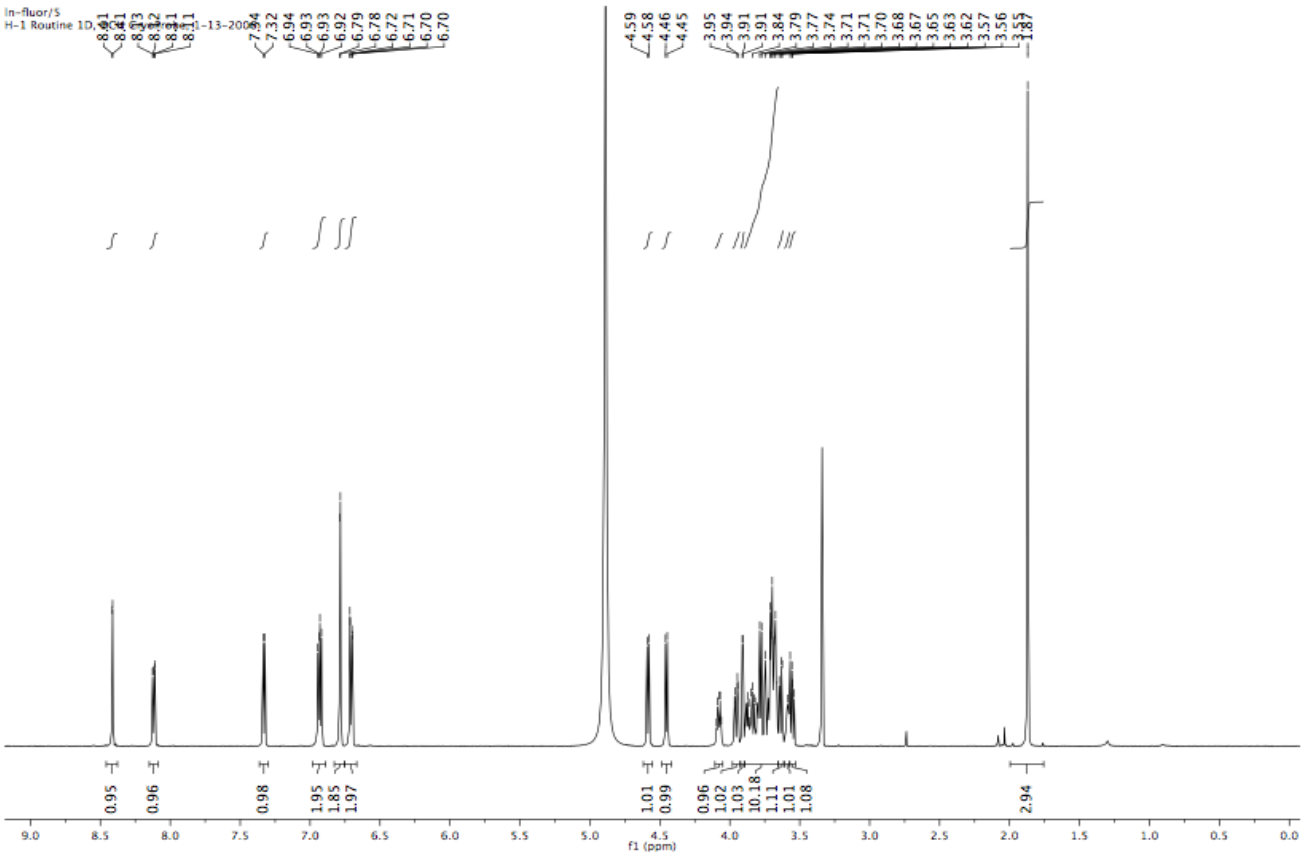
NH2
N
N
O
O
HO
OH
HO
O
P
O
O
OH
O
AcHN
CO2H
OH
OH
HO
F

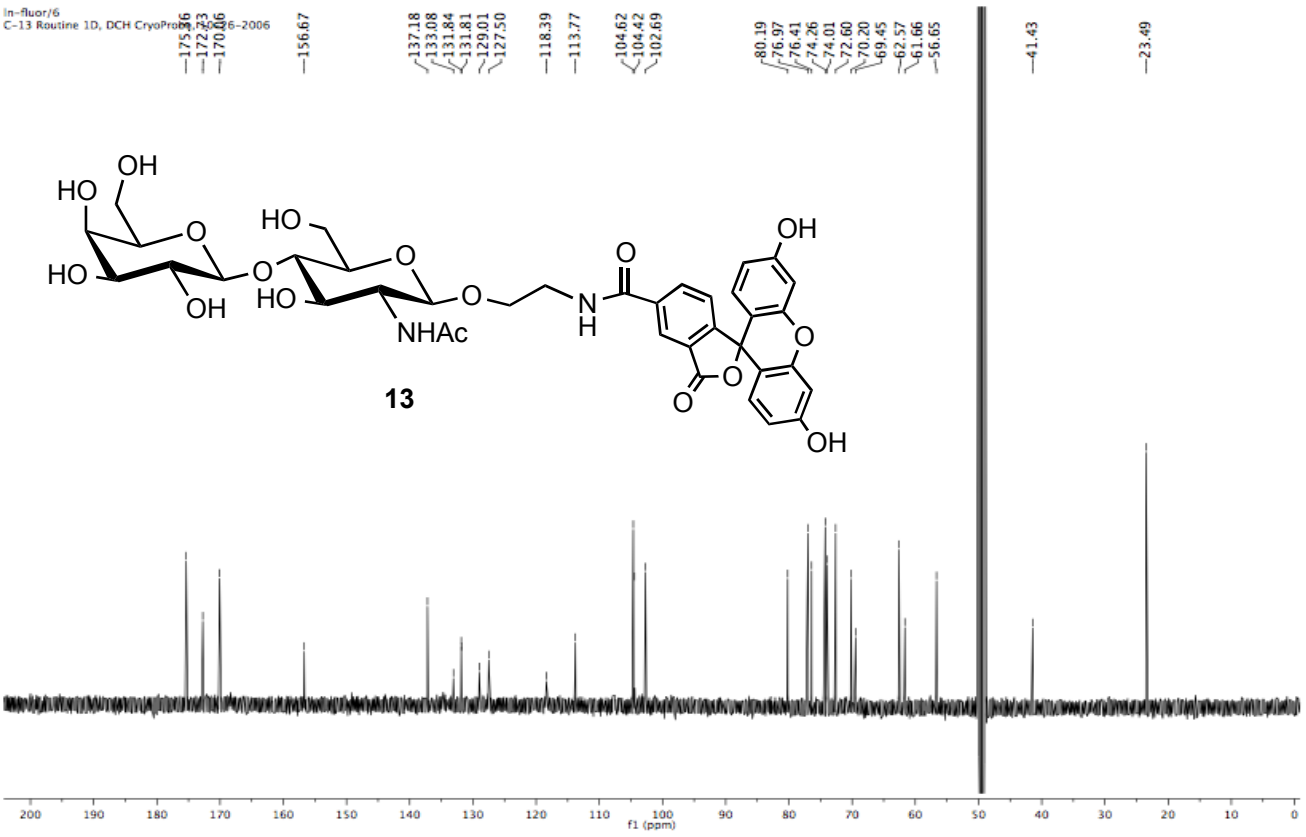
11





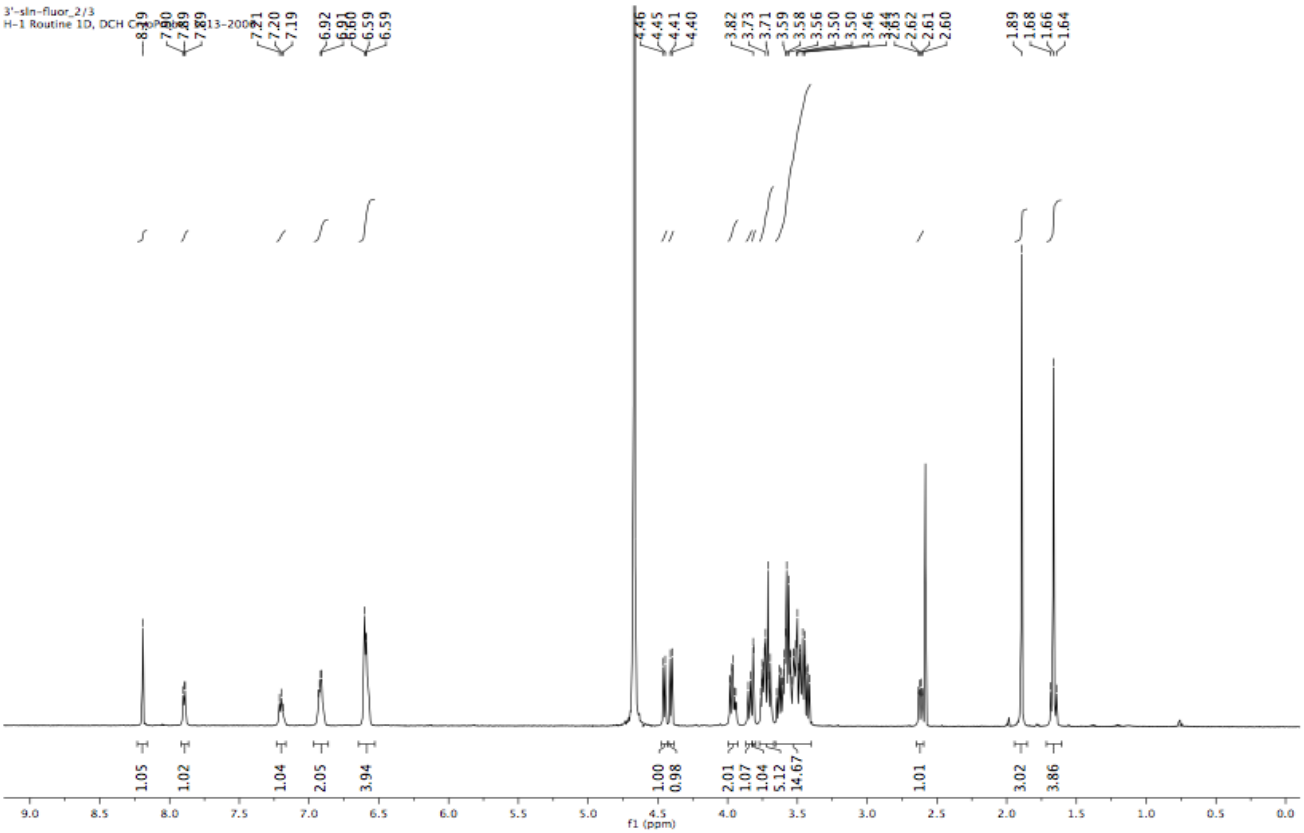
12

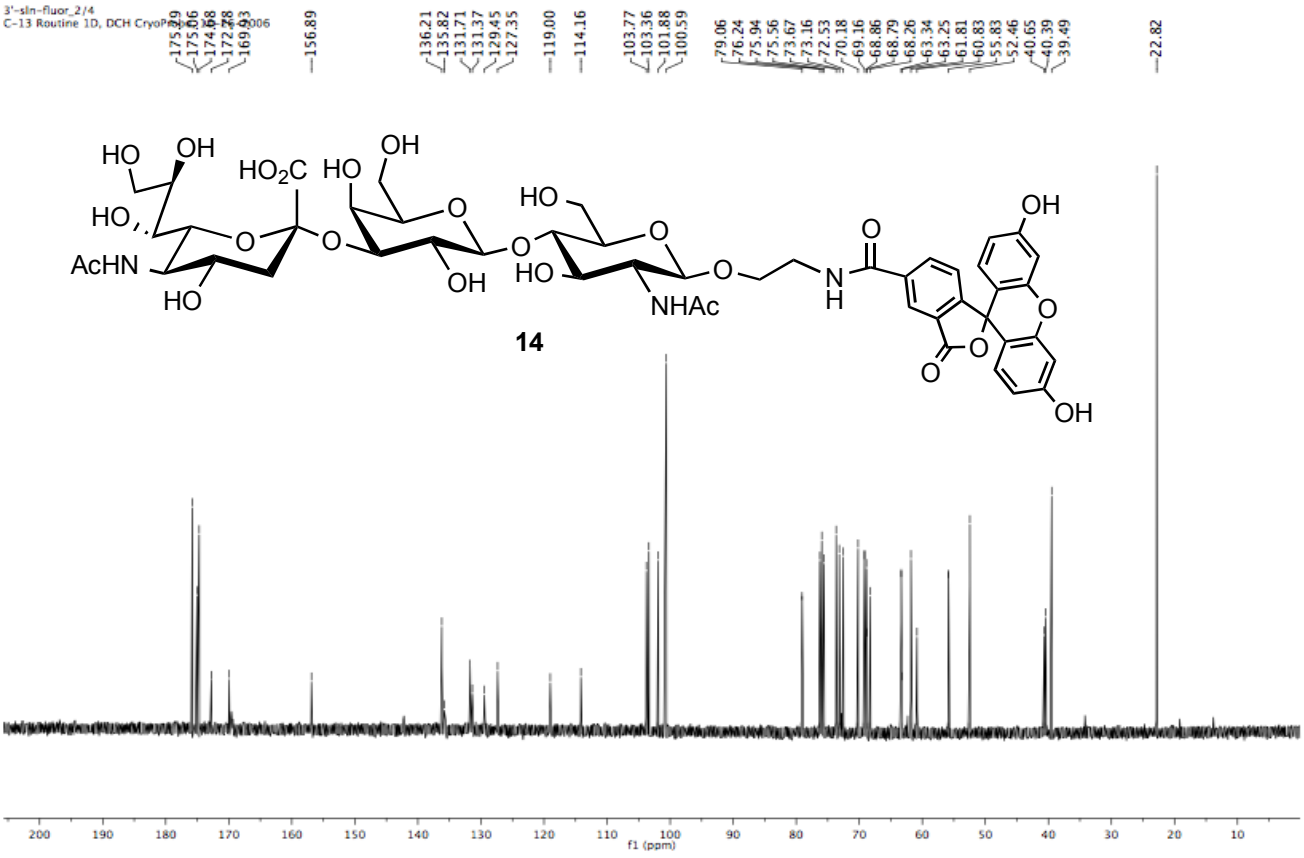

In-fluor/5
H-1 Routine 1D, PROTON 11-13-2006
f1 (ppm)


In-fluor/6
C-13 Routine 1D, DCH CryoProbe 11-13-2006
f1 (ppm)

OH
HO
O
HO
OH
HO
O
HO
O
NHAc
O
N
H
O
OH
O
O
O
OH

13


3'-sln-fluor_2/3
H-1 Routine 1D, DCH CryoPr
8.19
7.90
7.89
7.89
7.21
7.20
7.19
6.92
6.91
6.60
6.59
6.59
4.46
4.45
4.41
4.40
3.82
3.73
3.71
3.59
3.58
3.56
3.50
3.50
3.46
2.63
2.62
2.61
2.60
1.89
1.68
1.66
1.64
1.05
1.02
1.04
2.05
3.94
1.00
0.98
2.01
1.07
1.04
5.12
14.67
1.01
3.02
3.86
f1 (ppm)


3'-sln-fluor_2/4
C-13 Routine 1D, DCH CryoPr
175.29
175.06
174.68
172.28
169.93
156.89
136.21
135.82
131.71
131.37
129.45
127.35
119.00
114.16
103.77
103.36
101.88
100.59
79.06
76.24
75.94
75.56
73.67
73.16
72.53
70.18
69.16
68.86
68.79
68.26
63.34
63.25
61.81
60.83
55.83
52.46
40.65
40.39
39.49
22.82
f1 (ppm)

HO
OH
HO2C
HO
OH
HO
O
AcHN
HO
O
O
OH
HO
O
HO
O
NHAc
N
H
O
OH
O
O
O
OH

14