Supplementary Information For
Fluorogenic sialic acid glycosides for quantification of sialidase activity upon unnatural substrates.

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General Experimental Details.

All reactions were performed in open air unless otherwise stated. Flash column chromatography was performed on Whatman silica gel, 230-400 mesh. Analytical and preparative thin layer chromatography was carried out on EMD silica gel 60 F_{254} plates. Products were visualized using UV and by staining with either ninhydrin stain or *p*-anisaldehyde stain. NMR spectra were recorded on a Bruker Advance III NMR spectrometer at 500 MHz for ^{1}H NMR, 125 MHz for ^{13}C-NMR, and 470 MHz for ^{19}F-NMR. Mass spectra were recorded using a Finnegan LTQ ESI-MS with an additional APCI source. UV/Vis spectra were measured using a Nanodrop UV/Vis spectrophotometer. High-resolution mass spectra (HRMS) were obtained at the MIT Department of Chemistry Instrumentation Facility. Fluorescence readings were taken on an Infinite M200 microtiter plate reader (Tecan AG, Mannedorf, Switzerland). High-performance liquid chromatography was performed on Vydac C-18 columns on a Hitachi HPLC system.

Reagents and Enzymes. Methanol, dichloromethane and toluene were dried on an Innovative Technologies PureSolv 400 solvent purifier. NMR solvents were obtained from Cambridge Isotope Labs (Andover, MA). Mannosamine hydrochloride was purchased from LC Scientific (Concord, Ontario), Boc anhydride and HBTU were purchased from Santa Cruz Biotech (Santa Cruz, CA). *N*-Acetylneuraminic acid aldolase (EC 4.1.3.3) was purchased from Nacalai USA, Inc. (Tokyo, Japan). *N*-Acetylneuraminic acid, 2,3-dehydro-2-deoxy-*N*-aceyltneuraminic acid (DANA) and neuraminidase from *C. perfringens* [EC 3.2.1.18] were purchased from Sigma-Aldrich (St. Louis, Missouri.) All other chemicals and cell culture supplies were purchased from Sigma-Aldrich and Fisher Scientific.

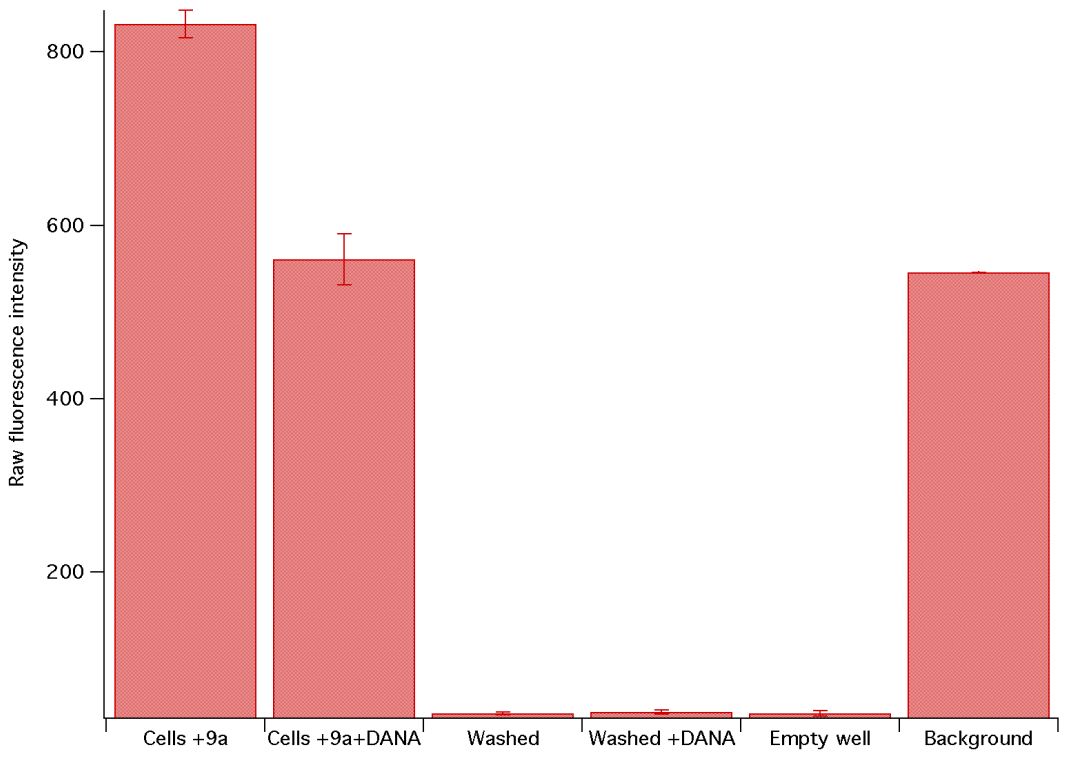

Figure S1. Cell permeability of HL60 to 4-methylumbelliferyl glycosides. Cells were treated with 125 μM of the corresponding glycoside and incubated for 2 h before thorough washing and lysis. Fluorescence was read with excitation wavelength of 365 nm and emission wavelength of 450 nm. Background reading corresponds to 125 μM of a corresponding glycoside in buffer without cells.

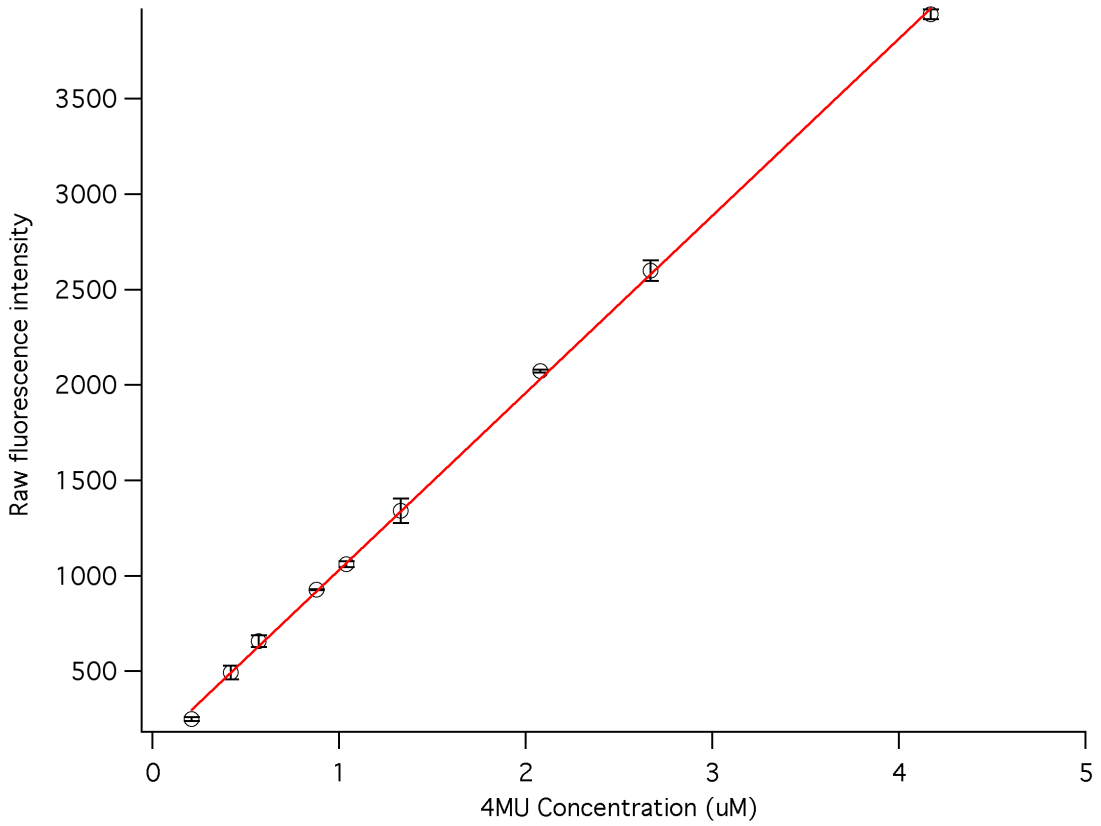

Figure S2. Standard curve generated from solutions of 4-methylumbelliferone in assay phosphate buffer for enzyme activity quantification. One unit of sialidase is the amount required to release one nmol of 4-MU per million cells.

Cell Culture Conditions. The human promyelocytic leukemia cell line HL60 (ATCC catalog no. CCL-240) was cultured continuously as a suspension culture in RPMI 1640 culture medium supplemented with 20% fetal bovine serum. Cells were maintained under a 5% CO_2 atmosphere at 37°C in a cell culture incubator. Cell counts for each population of cells were determined by Trypan Blue exclusion with a hemocytometer.

Determination of sialidase activity by fluorogenic sialic acids. Soluble purified bacterial sialidase or endogenous HL60 sialidases[1] were assayed as described elsewhere. Whole HL60 cells were removed from suspension culture and seeded into 2 mL Eppendorf tubes at a density of 1-2 x 10^6 cells/mL in 200 µL of buffer

(0.05 M NaOAc, pH 4.4). In the case of bacterial sialidase, 0.05 mU of bacterial enzyme was suspended in the same sodium acetate buffer. Samples were provided 125 μM **9a**-**9f** and allowed to incubate at 37°C for 1 or 2 h. Soluble neuraminidase from *C. perfringens* served as a positive control. Samples received 0.1 mM 2,3-dehydroxy-2-deoxy-neuraminic acid (DANA) in some cases as a negative control. After the reaction is complete, all samples quickly received 1000 μL of a quenching buffer (0.133 M glycine, 0.06 M NaCl, 0.083 Na_2CO_3, pH = 10.7) and an aliquot immediately taken for fluorometric determination of released 4-methylumbelliferone (4MU) at excitation wavelength 365 and emission wavelength 450 using a Tecan plate reader. The concentration of 4MU and units of sialidases activity were determined by subtracting the fluorescence reading of **9a** alone in buffer from the cell replicate readings and comparing the result to a standard curve generated from solutions of free 4-methylumbelliferone. The relative fluorescence intensity is determined by subtracting the background fluorescence sample from the cell samples, and normalizing the sample to the fluorescence of **9a.**

Cell Permeability Assay

HL60 cells were removed from suspension culture and seeded into 2 mL Eppendorf tubes at a density of 1-2 x 10^6 cells/mL in 200 μL of NaOAc buffer (0.05 M s NaOAc, pH 4.4). Samples were provided 125 μM **9a** and allowed to incubate at 37°C for 2 h. Soluble neuraminidase from *C. perfringens* served as a positive control. Samples received 100 μM 2,3-dehydroxy-2-deoxy-neuraminic acid (DANA) in some cases as a negative control. After 2 h, half of the sample received 500 μL of a quenching buffer (0.133 M glycine, 0.06 M NaCl, 0.083 Na_2CO_3, pH = 10.7) and fluorescence read, exciting at 365 nm and reading emission at 450 nm. The remaining cells were centrifuged gently at 100 *x g* for 3 min and the supernatant removed. Cells were gently washed three times with PBS, and then resuspended in a NaOAc buffer containing 1% Triton-100X and allowed to react for 10 min at 4 °C. The cells were quenched with 500 μL quenching buffer and the amount of 4-methylumbelliferone (4MU) released from cell interiors was quantified at excitation wavelength 365 and emission wavelength 450 using a microtiter plate reader. The concentration of 4MU

and units of sialidases activity were determined by subtracting the fluorescence reading of **9a** alone in buffer from the cell replicate readings and comparing the result to a standard curve generated from solutions of 4-methylumbelliferone.

Synthesis of Compounds 2-9.

Synthesis of *N*-*tert*-butoxycarbonyl-D-mannosamine (2). To a solution of mannosamine hydrochoride **1** (0.5579 g, 2.587 mmol) in 40% EtOH in H_2O (12.9 mL) was added sodium bicarbonate (0.6443 g, 7.669 mmol). To this was added a solution of boc anhydride (0.6317 g, 2.894 mmol) in ethanol (1.22 mL). After sonication for 3 h, the reaction was concentrated *in vacuo* concentrated to remove ethanol, resulting in a yellow slurry. The material was diluted with water (5 mL) and washed with hexanes (3 X 30 mL). The aqueous layer was extracted (2 x 20 mL) with 1-butanol and the butanol layers combined and washed (2 x 4 mL) with H_2O. The organic layer was concentrated *in vacuo* and the clear, colorless film obtained was dissolved in H_2O and lyophilized to give *N*-*tert*-butoxycarbonyl-D-mannosamine (513.6 mg, 71%) with no further purification as a slightly yellow amorphous solid. The NMR spectra of this compound were identical to those previously reported.[2]

Synthesis of 5-*tert*-butyloxycarbonylamino-3,5-dideoxy-D-glycero-α,β-D-galaco-2-nonulsonic acid (3). To a solution of *N*-*tert*-butoxycarbonyl-D-mannosamine **2** (370.5 mg, 1.328 mmol) in 17.075 mL phosphate buffer (0.05M KH_2PO_4, 0.01M dithiothreitol, pH 7.2) was added 1.0 M sodium pyruvate in phosphate buffer (5.308 mL) and 30U of *N*-acetyl neuraminic acid aldolase. The reaction shook gently for 5 days at 37 °C. The reaction mixture was passed through a 3,000 MWCO Millipore YM-3 filter assembly at 3,000 *x g* for 6 h and the filtrate lyophilized. The crude material (1.6448 g) was taken to the next step with no further purification. The NMR spectra of this compound were identical to those previously reported.[2]

Synthesis of methyl 5-*tert*-butyloxycarbonylamino-3,5-dideoxy-D-glycero-α,β-D-galaco-2-nonulsopyranosonate (4). Crude material containing 5-*tert*-butyloxycarbonylamino-3,5-dideoxy-D-glycero-α,β-D-galaco-2-nonulsonic acid **3** (0.8275, 0.6678 mmol) was triturated with anhydrous MeOH multiple times and the filtrate dried under high vacuum overnight. The residue was dissolved in anhydrous MeOH (20 mL) to which freshly-washed Amberlite IR-120 H^+ resin (0.16 g) had been added to adjust pH to 4. The resin was collected by filtration and the filtrate concentrated *in vacuo* overnight to be taken to the next step with no further purification. The NMR spectra of this compound were identical to those previously reported.[2]

Synthesis of methyl 2,4,7,8,9-penta-O-acetyl-5-*tert*-butyloxycarbonylamino-3,5-dideoxy-D-glycero-α,β-D-galaco-2-nonulsopyranosonate (5). Crude methyl 5-*tert*-butyloxycarbonylamino-3,5-dideoxy-D-glycero-α,β-D-galaco-2-nonulsopyranosonate **4** (0.3261 g, 0.8551 mmol) was suspended in anhydrous pyridine (2mL). To this, acetic anhydride (1mL) was added directly to the reaction flask and the reaction stirred at room temperature for 48h. The reaction mixture was concentrated *in vacuo* and the crude material was purified by flash chromatography (EtOAc/hexanes) to afford the final product **5** (143.3 mg, 36% over three steps) as a pale yellow syrup. The NMR spectra of this compound were identical to those previously reported.[2]

Synthesis of methyl 3,5-dideoxy-D-glycero-α,β-D-galaco-2-nonulsopyranosonate amino glycoside (6). To a solution of **5** (68.8 mg, 0.1163 mmol) in dry CH_2Cl_2 (2 mL) at 3°C was added trifluoroacetic acid (1 mL) and the reaction stirred for 2 h. The resulting material was concentrated *in vacuo* overnight, yielding a red oil, and was used immediately with no further purification (84.4 mg, quant.)

General Procedure for HBTU-mediated acylation (7b-7f). To a solution of methyl 3,5-dideoxy-D-glycero-α,β-D-galaco-2-nonulsopyranosonate amino

glycoside **6** (0.1638 mmol) in DMF (0.2 mL) was added DIEA (0.059 mL). To this was added a solution of HBTU (0.3027 mmol, 1.8 eq.), a corresponding carboxylic acid (2 eq.) and DIEA (0.059 mL) in DMF (0.2 mL). The solution stirred for 2 h. The crude mixture was purified by flash chromatography (EtOAc/hexanes) to afford the desired product.

General procedure for synthesis of 4-methylumbelliferyl (4MU) glycosides (8a-f). Formation of the 4MU glycosides of derivitized sialic acids followed a two-step procedure previously reported for *N*-acetyl neuraminic acid glycosides.[3,4] Sugars **7a-7f** (0.0496 mmol) were dissolved in a mixture of glacial AcOH (0.53 mL) and Ac_2O (0.26 mL) and the reaction brought to 0 °C. Once cold, HCl (g) was bubbled through the reaction mixture until saturated (by weight) and the reaction was tightly stoppered and left to stir at 4 °C for 48 h. The reaction mixture was quickly removed via syringe to a dry, clean flask and dried thoroughly under high vacuum to be used immediately.

The resulting residue was dissolved in dry DMF (0.42 mL) and 4-methylumbelliferone sodium salt[5] (2.4 mmol) was added. The reaction stirred shielded from light and under argon for 15 h. The crude material was dissolved in CH_2Cl_2 and extracted 5 times with water until the aqueous layer appeared colorless. The organic layer was dried with $MgSO_4$ and concentrated *in vacuo* overnight, yielding glycosides **8a-8f**.

General procedure for saponification of 4MU glycosides (9a-f). In darkness, glycosides **8a-8f** (0.0422 mmol) were dissolved in a solution 0.035M NaOMe in dry methanol (1 mL) to adjust the pH to 8. After 1 h, the reaction mixture was concentrated in darkness to yield a slightly cloudy syrup. This was dissolved in H_2O (6.8 mL) and 2 M NaOH added dropwise to adjust the pH to 11. This mixture stirred overnight in darkness. After, Amberlite IR-120 H^+ resin was added to the reaction mixture to bring the pH to 6-7 and the resin filtered away. The filtrate was lyophilized, dissolved in water, and purified by HPLC to yield final glycosides **9a-9f**.

Characterization Data

4-Methylcoumarin-7-yl 5-acetamido-3,5-dideoxy-a-D-glycero-D-galacto-2-nonulopyranosidonic acid (9a): Buff amorphous solid (83%). ^{1}H NMR (500 MHz, D_2O): δ 7.69 – 7.64 (m, 1H), 7.11 – 7.08 (m, 2H), 6.21 (s, 1H, vinyl H), 3.99 (dd, J = 10.5, 1.4 Hz, 1H), 3.86 (t, J = 10.1 Hz, 1H), 3.80 – 3.75 (m, 2H), 3.70 (m, 1H), 3.54 (dd, J = 12.09, 6.2 Hz, 1H), 3.50 (dd, J = 9.23, 1.3 Hz, 1H), 2.77 (dd, J = 12.64, 4.7 Hz, 1H, H-3_{eq}), 2.37 (s, 3H, coumarin Me), 1.95 (s, 3H, *N*-Ac), 1.91 (t, J = 12.22 Hz, 1H, 3-H_{ax}); ^{13}C NMR (125 MHz, D_2O): δ 177.2, 172.7, 164.2, 158.3, 153.5, 126.2, 117.5, 113.5, 111.8, 110.0, 108.0, 73.7, 71.7, 68.3, 67.9, 62.8, 51.8, 40.8, 22.1, 18.0; HRMS (ESI) m/z: calcd for $C_{21}H_{25}NO_{11}$ (M-H) 466.1335, found 466.1367.

4-Methylcoumarin-7-yl 5-trifluorobutamido-3,5-dideoxy-a-D-glycero-D-galacto-2-nonulopyranosidonic acid (9b): White amorphous solid (63%). ^{1}H NMR (500 MHz, D_2O): δ 7.66 – 7.64 (m, 1H), 7.16 – 7.04 (m, 2H), 6.21 (s, 1H, vinyl H), 4.01 (dd, J = 10.5, 1.4 Hz, 1H), 3.88 (t, J = 10.1 Hz, 1H), 3.80 – 3.75 (m, 2H), 3.70 (m, 1H), 3.52 (dd, J = 12.09, 6.2 Hz, 1H), 3.47 (dd, J = 9.23, 1.3 Hz, 1H), 2.77 (dd, J = 12.64, 4.7 Hz, 1H, H-3_{eq}), 2.53 – 2.39 (m, 4H), 2.37 (s, 3H, coumarin Me), 1.90 (t, J = 12.22 Hz, 1H, 3-H_{ax}); ^{13}C NMR (125 MHz, D_2O): δ 179.8, 173.0, 170.0, 157.3, 153.8, 151.1, 126.2, 117.5, 116.3, 111.8, 109.9, 108.0, 75.6, 73.6, 72.8, 71.7, 68.4, 67.8, 62.8, 61.8, 40.9, 28.5, 18.0; HRMS (ESI) m/z: calcd for $C_{23}H_{26}F_3NO_{11}$ (M-H) 548.1385, found 548.1385.

4-Methylcoumarin-7-yl 5-fluoroacetamido-3,5-dideoxy-a-D-glycero-D-galacto-2-nonulopyranosidonic acid (9c): White amorphous solid (19%). ^{1}H NMR (500 MHz, D_2O): δ 7.66 – 7.64 (m, 1H), 7.10 – 7.05 (m, 2H), 6.18 (s, 1H, vinyl H), 4.76 (d, J = 48 Hz, 2H), 4.08 (m, 1H), 3.97 (t, J = 10.1 Hz, 1H), 3.78 – 3.73 (m, 3H), 3.52 – 3.49 (m, 2H), 2.76 (dd, J = 12.7, 4.5 Hz, 1H), 3.47 (dd, J = 9.23, 1.3 Hz, 1H), 2.77 (dd, J = 12.64, 4.7 Hz, 1H, H-3_{eq}), 2.525 – 2.389 (m, 4H), 2.34 (s, 3H, coumarin Me), 1.90 (t, J = 12.1 Hz, 1H, 3-H_{ax}); ^{13}C NMR (125 MHz, D_2O): δ 187.3, 172.3, 164.7, 157.2, 157.2,

156.3, 126.2, 117.5, 116.3, 111.7, 109.2, 108.0, 79.8, 71.7, 68.1, 67.6, 62.7, 51.3, 40.8, 17.9; HRMS (ESI) m/z: calcd for $C_{21}H_{24}FNO_{11}$ (M-H) 485.1339, found 484.1263.

4-Methylcoumarin-7-yl 5-isobutamido-3,5-dideoxy-a-D-glycero-D-galacto-2-nonulopyranosidonic acid (9d): Slightly yellow amorphous solid (88%). ^{1}H NMR (500 MHz, D_2O): δ 7.64 – 7.61 (m, 1H), 7.08 – 7.05 (m, 2H), 6.19 (s, 1H, vinyl H), 3.96 (dd, J = 10.4, 1.2 Hz, 1H), 3.81 (t, J = 10.1 Hz, 1H), 3.83 – 3.65 (m, 3H), 3.50 (dd, J = 11.5, 6.0 Hz, 1H), 3.42 (dd, J = 9.0, 1.0 Hz, 1H), 2.75 (dd, J = 12.5, 4.4 Hz, 1H, H-3_{eq}), 2.42 (septet, J = 7.0 Hz, 1H), 2.34 (s, 3H, coumarin Me), 1.90 (dd, J = 12.3 Hz, 1H, 3-H_{ax}), 0.992 (dd, J = 6.9, 2.4 Hz, 6H); ^{13}C NMR (125 MHz, D_2O): δ 182.3, 172.8, 164.8, 157.3, 156.4, 153.5, 126.2, 117.6, 116.4, 111.8, 108.1, 102.7, 73.7, 71.7, 68.5, 67.7, 62.8, 51.6, 41.1, 35.3, 19.0, 18.5, 18.0; HRMS (ESI) m/z: calcd for $C_{23}H_{29}NO_{11}$ (M-H) 494.1668, found 494.1686.

4-Methylcoumarin-7-yl 5-trifluoropropamido-3,5-dideoxy-a-D-glycero-D-galacto-2-nonulopyranosidonic acid (9e): Ashen amorphous solid (52%). ^{1}H NMR (500 MHz, D_2O): δ 7.71 – 7.60 (m, 1H), 7.12 – 7.06 (m, 2H), 6.20 (s, 1H, vinyl H), 4.01 - 3.80 (m, 2H), 3.80 - 3.68 (m, 3H), 3.56 - 3.47 (m, 2H), 3.26- 3.07 (m, 2H), 2.70 (dd, J = 12.65, 4.03 Hz, 1H, H-3_{eq}), 2.36 (s, 3H, coumarin Me), 1.91 (t, J = 14.16 Hz, 1H, 3-H_{ax}); ^{13}C NMR (125 MHz, D_2O): δ 179.8, 173.0, 169.9, 157.3, 153.8, 151.1, 126.2, 117.5, 116.3, 111.8, 109.9, 108.0, 75.6, 73.6, 72.8, 71.7, 68.4, 67.8, 62.8, 51.8, 40.9, 28.5, 18.0; HRMS (ESI) m/z: calcd for $C_{22}H_{24}F_3NO_{11}$ (M-H) 534.1229, found 534.1209.

4-Methylcoumarin-7-yl 5-glycolamido-3,5-dideoxy-a-D-glycero-D-galacto-2-nonulopyranosidonic acid (9f): Slightly yellow amorphous solid (38%). ^{1}H NMR (500 MHz, D_2O): δ 7.64 – 7.60 (m, 1H), 7.12 – 7.05 (m, 2H), 6.18 (s, 1H, vinyl H), 4.06 (m, 1H), 4.01 (s, 2H), 3.92 (t, J = 10.1 Hz, 1H), 3.81 – 3.70 (m, 3H), 3.51 (dd, J = 11.8, 5.9 Hz, 1H), 3.47 (m, 1H), 2.76 (dd, J = 12.6, 4.7 Hz, 1H, H-3_{eq}), 2.34 (s, 3H, coumarin Me), 1.89 (dd, J = 12.3 Hz, 1H, 3-H_{ax}); ^{13}C NMR (125 MHz, D_2O): δ 186.9, 172.7, 164.7, 157.2, 156.2, 153.4, 126.1, 117.4, 116.2, 111.7, 108.9, 108.0, 73.3, 71.6, 68.1, 67.5,

62.7, 60.9, 51.4, 40.8, 17.9; HRMS (ESI) m/z: calcd for $C_{21}H_{25}NO_{12}$ (M-H) 482.1304, found 482.1301.

References

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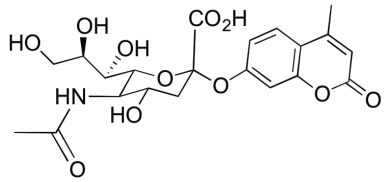
NMR Spectra

^{1}H NMR of compound **9a**



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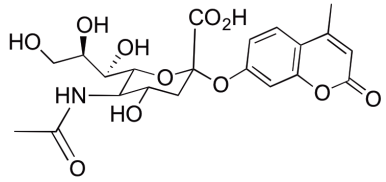

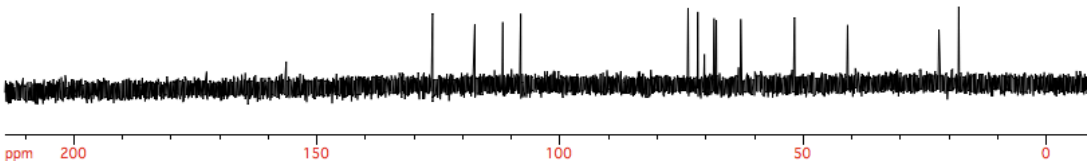

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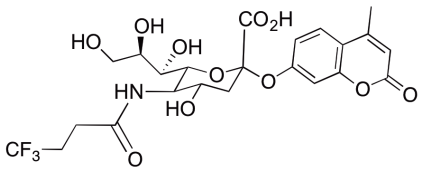

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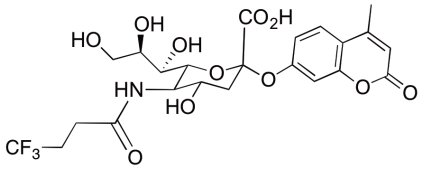
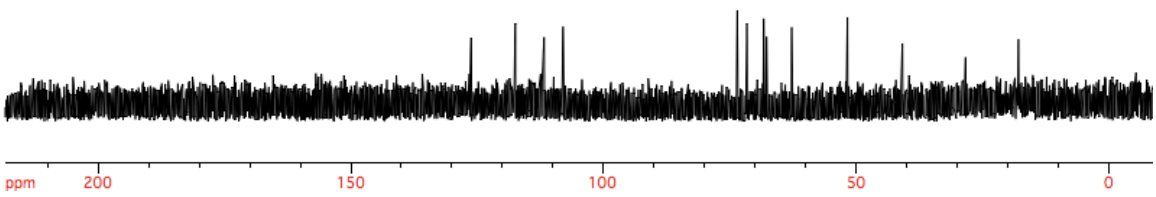
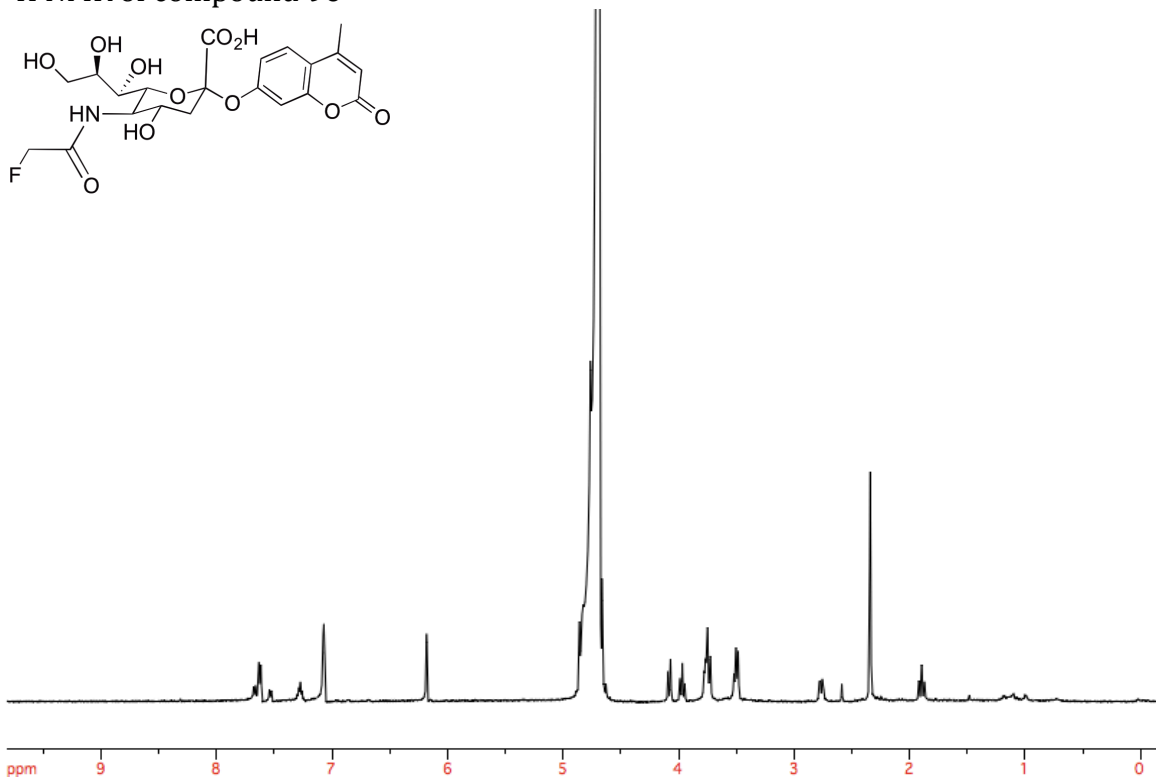
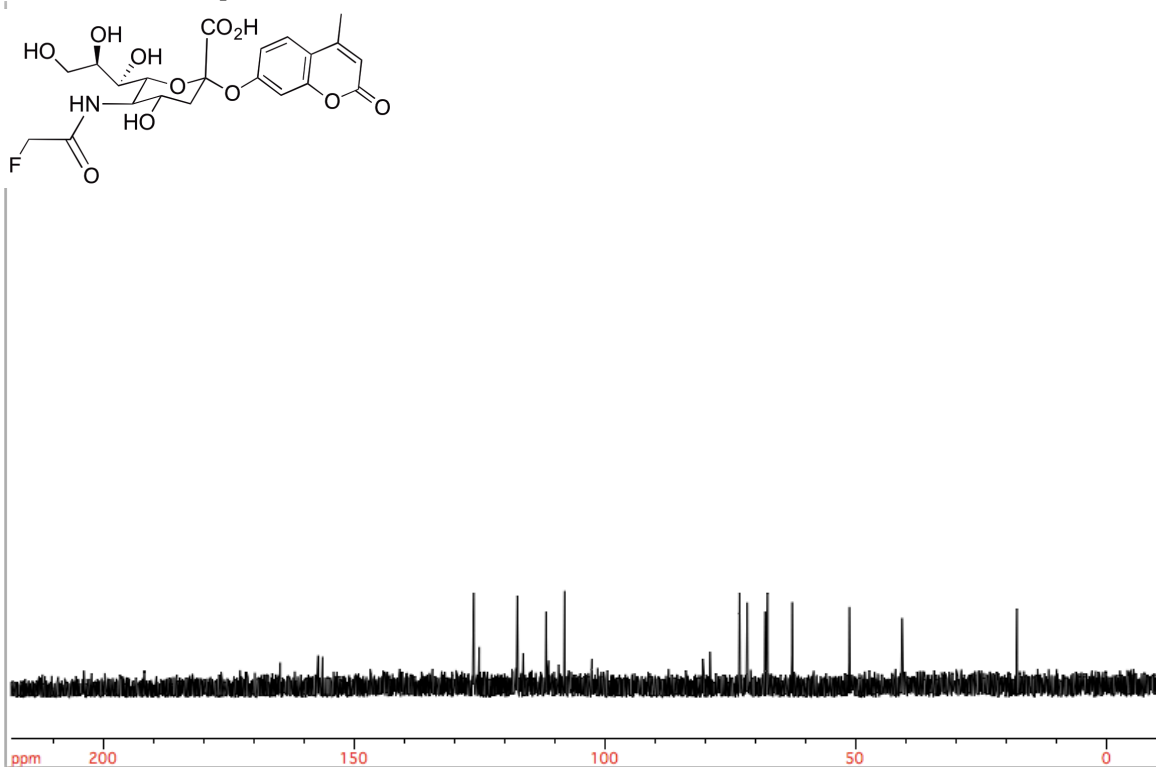




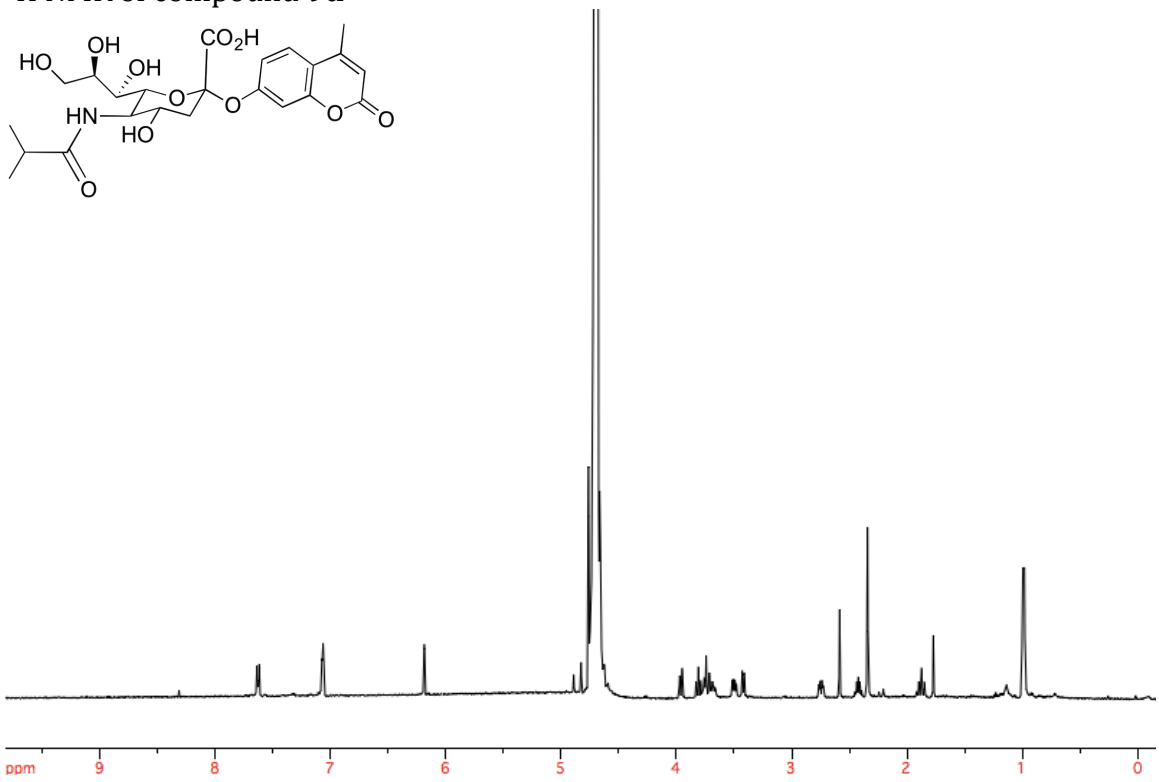
^{1}H NMR of compound **9c**



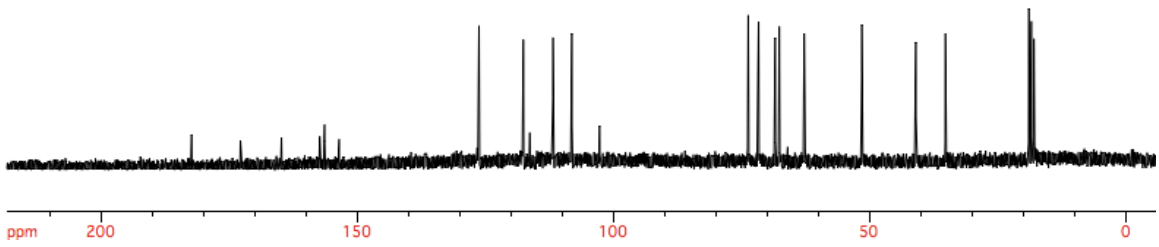
^{13}C NMR of compound **9c**



^{1}H NMR of compound **9d**



^{13}C NMR of compound **9d**



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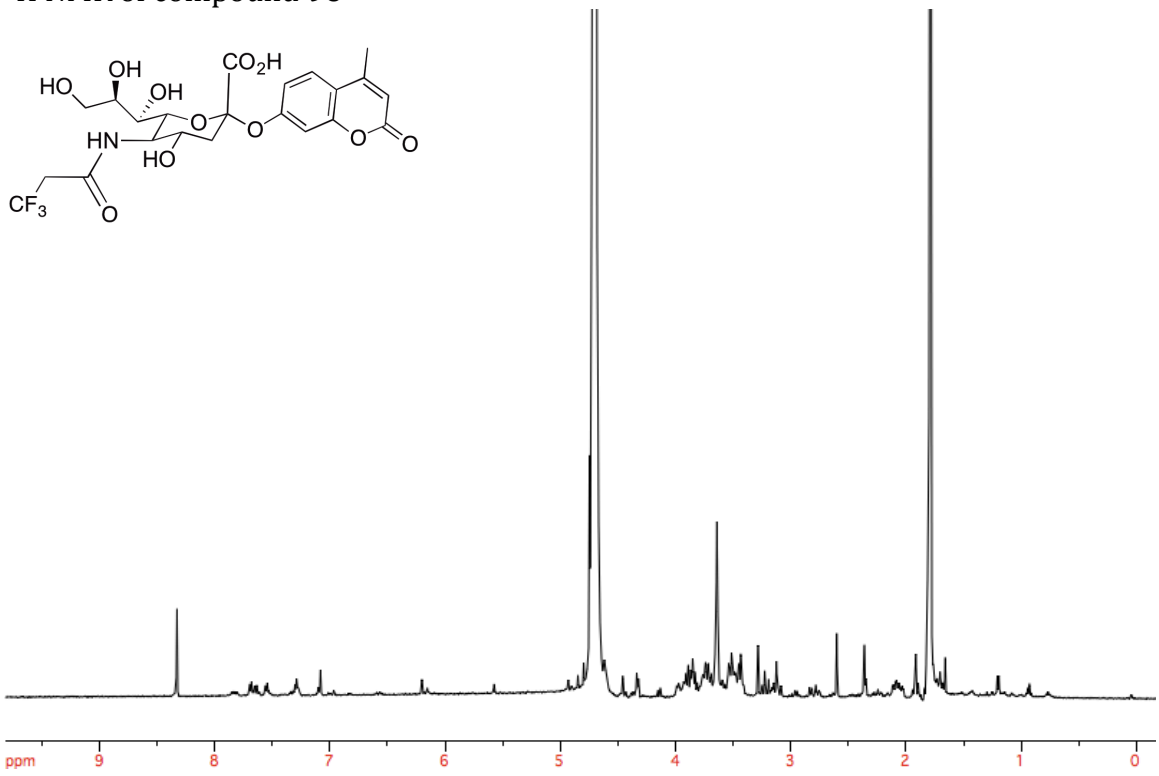
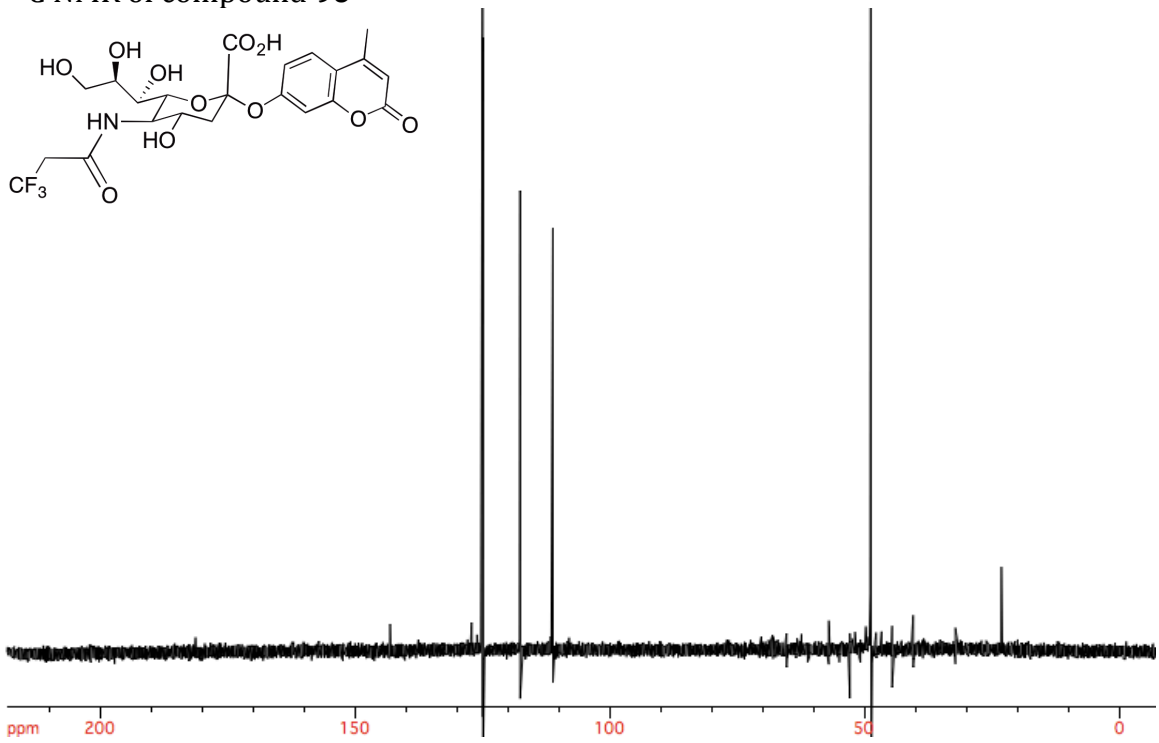
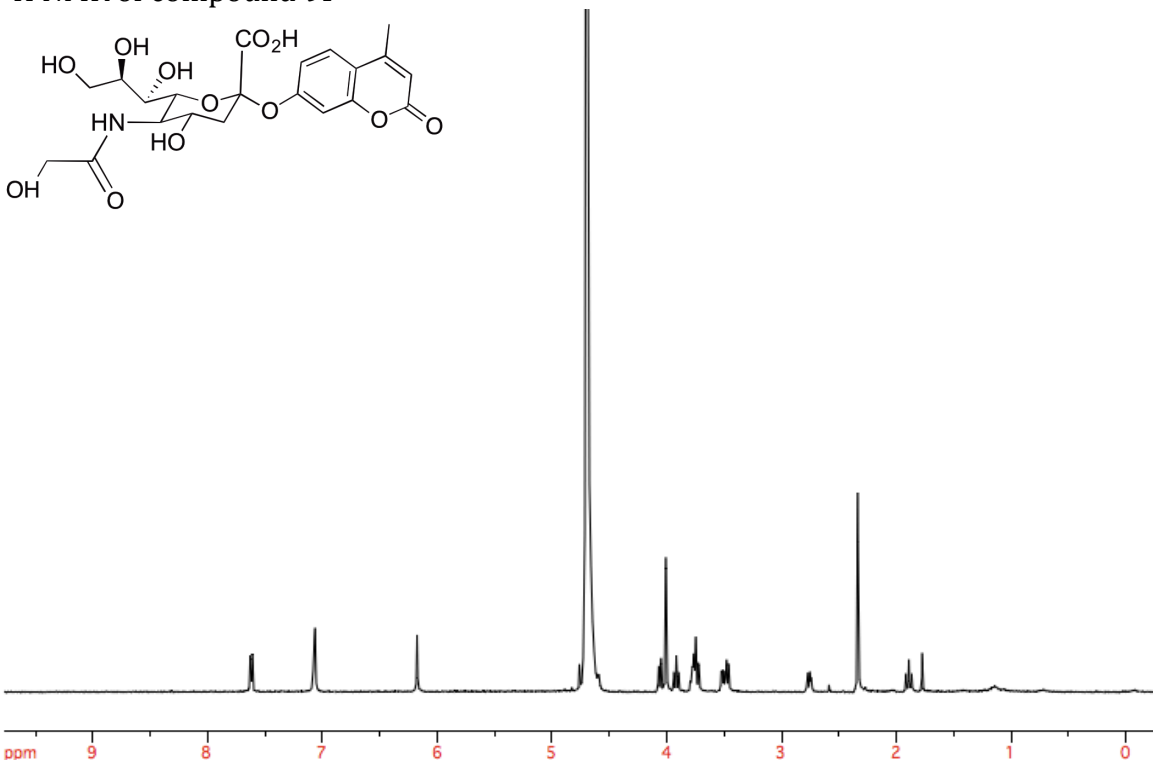


^{13}C NMR of compound **9e**



^{1}H NMR of compound **9f**



^{13}C NMR of compound **9f**