Electronic Supporting Information

A sialosyl sulfonate as a potent inhibitor of influenza virus replication

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MOLECULAR MODELLING

Binding pose Prediction:

In order to visualize their potential binding conformations, octopyranosyl sulfonates **4** and **11**, and 2-deoxy-2-H-*N*-acetyl-β-neuraminic acid **2** were docked into the active site of influenza A virus N2 sialidase structure, PDB code 1inx, using Glide (Schrödinger®).[S1] Ligand minimization was carried out using the LigPrep module of Schrödinger® with OPLS3 force field. Protein Preparation Wizard[S2] was used to preprocess the raw X-ray structures: all water molecules were deleted and the complexes were minimized with the OPLS3 parameter set. During the process of 'Receptor Grid Generation', H-bond constraints were defined in order to restrict the possible binding modes in the active site. Standard-precision (SP) was applied in ligand docking using a 15.0 kcal/mol energy window for ring sampling and enhanced (4x) conformer generation to help overcome barriers of torsional rotation. The docking protocol was validated by re-docking octopyranosyl α-phosphonate **3** ('ePANA' [S3]), and the corresponding β-anomer ('aPANA' [S3]) into their initial N2 complex crystal structures (PDB codes: 1inx and 1inw, respectively) and calculating RMSD values, which were found to be 0.76 Å and 0.48 Å, respectively. Results were visualized using the Maestro interface of Schrödinger®. The manuscript Figure (Fig. 2) was prepared using AstexViewer.[S4]

Relative ranking of binding affinities:

Knowledge of the preferred binding orientations of the ligands was used as a starting point for MM-GBSA[S5] calculations. Prime[S5] from Schrödinger® was used to estimate relative binding affinities for the ligands **2**, **4**, and **11** (reported in kcal/mol) (Table S1, ΔΔG MM-GBSA). During the calculations, variable-dielectric generalized Born (VSGB) solvation model, OPLS3 force field and hierarchical sampling were applied. Within 10 Å from the ligands, protein residues were treated as flexible.

The MM-GBSA values (Table S1, column 4) suggested that the equatorial sulfonate **4** should be more potent than carboxylate analogue **2** and significantly more potent than the axial sulfonate isomer **11**. The predicted relative sialidase inhibitory potency of sialosyl sulfonates **4** and **11** was observed experimentally in the sialidase inhibition assay (see Figure S2, pg S12), while the predicted relative potency of **4**, **2**, and **11** was also seen in the viral infection assay, which probed for inhibition of viral sialidase activity during cell infection. As shown in Figure S1, the predicted relative free energies (ΔΔG MM-GBSA) strongly correlated with the experimental values from the infection assay [ΔRT ln(IC50)].

Table S1. Experimental vs calculated relative free energies of compounds **4**, **11**, and **2**.

Compound	IC_{50} (M) (Infection assay)	Δ RT ln(IC_{50}) [kcal/mol]	ΔΔG MM-GBSA [kcal/mol]
4	7.0E-07	-4.07	-4.03
11	5.0E-03*	1.65	2.34
2	5.5E-04	0.00	0.00

* Value extrapolated from infection assay results for **11** (see manuscript Figure 3)

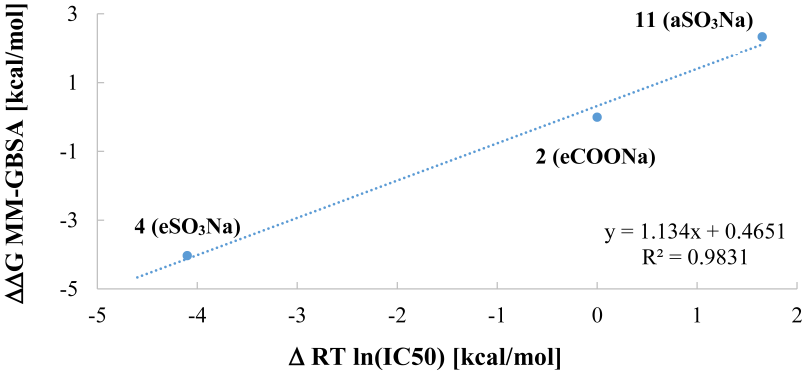


Figure S1. Predicted relative free energy (ΔΔG) by MM-GBSA plotted against experimental free energy based on IC_{50} measurements from the viral infection assay (see Table S1). Compound **2** was chosen as reference. The IC_{50} of **11** was extrapolated to be approximately 5 mM from the plot of the infection assay results measured by *in situ* ELISA (see manuscript Figure 3).

Acknowledgement:

The authors would like to thank Prof György Miklós Keserű and Dávid Bajusz (Medicinal Chemistry Research Group, Research Centre for Natural Sciences, Budapest, Hungary) for providing access to their HPC workstations and software collection (Schrödinger®).

COMPOUND SYNTHESIS AND CHARACTERIZATION

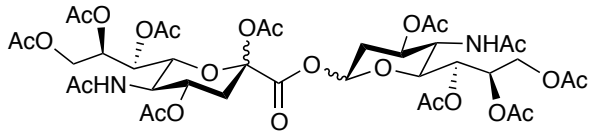
General methods and materials

Reactions were monitored using thin layer chromatography (TLC) on aluminium plates pre-coated with Silica Gel 60 F254 (E. Merck). Developed plates were observed under UV light at 254 nm and then visualized after application of a solution of H_2SO_4 in EtOH (5% v/v) and charring at ~200 °C. Flash chromatography was performed on Silica Gel 60 (0.040-0.063 mm) using AR grade solvents. In order to obtain better separation, TLC plates where run multiple times where indicated. High-Performance Liquid Chromatography (HPLC) was performed using an Agilent 1100-series instrument. RP-HPLC was carried-out on a Synergi Hydro-RP Phenomenex column (250 x10 mm). ^{1}H and ^{13}C, spectra were recorded using a Bruker Avance 400 MHz spectrometer. Data acquisition and processing were performed with Mestrenova software. For ^{1}H and ^{13}C spectra, chemical shifts are expressed as parts per million (ppm, δ) and are relative to the solvent used [$CDCl_3$: 7.26 (s) for ^{1}H; 77.16 (t) for ^{13}C; CD_3OD: 3.31 (pent) for ^{1}H; 49.0 (sept) for ^{13}C; D_2O 4.79 (s) for ^{1}H]. Multiplicities are denoted as s (singlet), d (doublet), t (triplet), q (quartet), pent (pentet), dd (doublet of doublets), ddd (doublet of doublet of doublets), td (triplet of doublets) and app (apparent). 2D NMR experiments were performed using ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) to confirm ^{1}H and ^{13}C assignments. Low-resolution mass spectra (LRMS) were recorded using a Bruker Daltonics Esquire 3000 ESI spectrometer, in positive or negative mode (as indicated). High-Resolution mass spectra (HRMS) were recorded on Bruker QTOF mass spectrometers using an ESI source (University of Debrecen, Department of Applied Chemistry, or Griffith University FTMS Facility, Eskitis Institute).

Microwave reactions were conducted using a CEM Discover® SP Explorer Hybrid-12 microwave system, in a pressure tube sealed with a teflon septum.

N-Acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-ulosonic acid, **5**) was obtained from Carbosynth UK. 5-Acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid (**1**)[S6] was prepared through base-catalysed deprotection of the corresponding peracetylated methyl ester.[S7] 5-Acetamido-2,6-anhydro-3,5-dideoxy-D-*erythro*-L-*gluco*-nononic acid (**2**) was synthesized according to literature procedures.[S8, S9]

4-Acetamido-3,6,7,8-tetra-*O*-acetyl-1-(1,4,7,8,9-penta-*O*-acetyl-*N*-acetyl-neuraminyl)-2,4-dideoxy-D-*glycero*-D-*galacto*-octopyranose (6) and **4-acetamido-2,4-dideoxy-1,3,6,7,8-penta-*O*-acetyl-D-*glycero*-D-*galacto*-octopyranose** (**7**)



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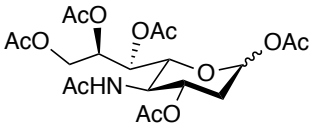

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A suspension of *N*-acetylneuraminic acid **5** (100 mg, 0.32 mmol) in pyridine (3.5 mL) and acetic anhydride (0.5 mL) was subjected to microwave irradiation for 15 min at 140 °C (300 W – dynamic cycle). The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The residual dark brown oil was dissolved in EtOAc washed successively with 1M aq. sol. of HCl, satd. aq. sol. of $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated. The crude material was purified by flash chromatography on silica gel ($CHCl_3$/acetone/MeOH 8:1.8:0.2) to afford a diastereomeric mixture of pseudo-disaccharide **6** (α/β 5:1) as a pale-yellow foam (15 mg, 10%; R_f 0.10 $CHCl_3$/acetone/MeOH 8:1.8:0.2) and an inseparable anomeric mixture of **7** (α/β 1:4) as a pale-yellow foam (122 mg, 80%; R_f 0.40 $CHCl_3$/acetone/MeOH 8:1.8:0.2).

In a similar manner, reaction on **5** (800 mg, 2.59 mmol) in pyridine (28 mL) and acetic anhydride (4.0 mL), afforded **6** (α/β 5:1; 143 mg, 12%) and **7** (α/β 1:4; 874 mg, 71%).

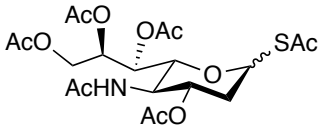
Note: The applied concentration and reagent ratios were found to be essential to minimize the formation of the pseudo-disaccharide and other side products.

6: NMR data for the major anomer (α) are given. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.79 – 2.18 (35H, m, NHCOC*H*$_3$ × 2, OHCOC*H*$_3$ × 9, H-2ax, H-3'ax), 2.30 (1H, ddd, $J_{2eq,2ax}$ = 12.4 Hz, $J_{2eq,3}$ = 4.8 Hz, $J_{2eq,1}$ = 2.0 Hz, H-2eq), 2.42 (1H, dd, $J_{3'eq,3'ax}$ = 13.5 Hz, $J_{3'eq,4'}$ = 5.0 Hz, H-3'eq), 3.79 (1H, dd, $J_{5,4}$ = 10.4 Hz, $J_{5,6}$ = 2.3 Hz, H-5), 3.97 – 4.15 (5H, m, H-4, H-5', H-6', H-8a, H-9'a), 4.26 (1H, dd, $J_{8b,8a}$ = 12.5 Hz, $J_{8b,7}$ = 2.6 Hz, H-8b), 4.43 (1H, dd, $J_{9'b,9'a}$ = 12.3 Hz, $J_{9'b,8'}$ = 2.4 Hz, H-9'b), 4.92 (1H, ddd, $J_{8',9'a}$ = 6.8 Hz, $J_{8',7'}$ = 4.0 Hz, $J_{8',9'b}$ = 2.4 Hz, H-8'), 4.96 – 5.08 (2H, m, H-3, H-7), 5.23 (1H, td, $J_{4',5'} \approx J_{4',3'ax} \approx$ 11.0 Hz, $J_{4',3'eq}$ = 5.0 Hz, H-4'), 5.33 (1H, dd, $J_{7',8'}$ = 4.0 Hz, $J_{7',6'}$ = 2.3 Hz, H-7'), 5.37 (1H, dd, $J_{6,7}$ = 6.9 Hz, $J_{6,5}$ = 2.3 Hz, H-6), 5.63 (1H, dd, $J_{1,2ax}$ = 10.3 Hz, $J_{1,2eq}$ = 2.0 Hz, H-1α), 5.78 (1H, d, $J_{NH,4}$ = 10.1 Hz, N*H*), 5.88 (1H, d, $J_{NH',5'}$ = 10.1 Hz, N*H*). ^{13}C NMR (101 MHz, $CDCl_3$) δ 20.6, 20.7, 20.8, 20.8, 20.9, 20.9, 21.0 (OCO*C*H$_3$ × 9), 23.2 (NHCO*C*H$_3$ × 2), 34.9 (C-2), 36.1 (C-3'), 49.0, 49.1 (C-4, C-5'), 62.05, 62.09 (C-9', C-8), 67.2 (C-6), 68.1 (C-7'), 68.2 (C-4'), 70.1 (C-7), 70.2 (C-3), 72.0 (C-8'), 73.0 (C-6'), 74.0 (C-5), 92.9 (C-1), 96.7 (C-2'), 164.7 (C-1'), 168.3,

170.2, 170.3, 170.5, 170.6, 170.7, 170.9, 171.0 (NH*C*OCH$_3$ × 2, O*C*OCH$_3$ × 9). LRMS (ESI): *m/z* 957.3 [(M+Na)$^+$, 100%]. HRMS *m/z* calcd for [$C_{39}H_{54}N_2O_{24}$+Na]$^+$: 957.296, Found: 957.265. In the ^{1}H NMR spectrum of **6**, H-5' was assigned at δ 3.97-415 (5H, m) and not at 4.96 ppm as previously reported.[S10]

7: NMR data for the major anomer (β) are given. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.90 (3H, s, NHCOC*H$_3$*), 2.00 – 2.16 (17H, m, H-2ax, H-2eq, OCOC*H$_3$* × 5), 3.99 – 4.13 (3H, m, H-4, H-5, H-8a), 4.34 (1H, dd, $J_{8a,8b}$ = 12.4 Hz, $J_{8a,7}$ = 2.7 Hz), 5.16 – 5.32 (3H, m, H-3, H-6, H-7), 6.28 (1H, d, $J_{1,2ax}$ = 3.3 Hz, H-1). ^{13}C NMR (101 MHz, $CDCl_3$) δ 20.6, 20.9, 21.0, 21.1, 21.1 (OCO*C*H$_3$ × 5), 23.3 (NHCO*C*H$_3$), 34.4 (C-2), 50.1 (C-4), 62.3 (C-8), 67.8 (C-6), 68.6 (C-3), 70.4 (C-7), 71.4 (C-5), 91.3 (C-1), 168.8, 169.9, 170.2, 170.3, 170.7, 171.4 (NH*C*OCH$_3$, O*C*OCH$_3$ × 5). LRMS (ESI): *m/z* 498.3 [(M+Na)$^+$, 100%]. The ^{1}H NMR data was in accordance with that previously reported.[S11, S12]

4-Acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-1-*S*-acetyl-1-thio-D-*glycero*-D-*galacto*-octopyranose (8)



8 (α/β 1:1)

To a solution of **7** (100 mg, 0.21 mmol) in anhydrous acetonitrile (2 mL) under argon were added at room temperature thioacetic acid (23 μL, 0.32 mmol) and $BF_3{\cdot}Et_2O$ (78 μL, 0.63 mmol). The reaction mixture was stirred under inert atmosphere at room temperature for 12 h, after which time, the solution was diluted with EtOAc, washed with satd. aq. sol. $NaHCO_3$ and brine, dried over $MgSO_4$ and filtered. The solvent was removed under reduced pressure and the crude residue was co-evaporated three times with toluene, then purified by flash chromatography on silica gel (EtOAc) to give **8** (83 mg, 80%) as an inseparable anomeric mixture (α/β 1:1). R_f 0.54, 0.61 ($CHCl_3$/acetone 8:2, TLC plate run 3 times).

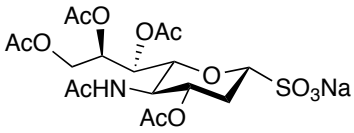
In a similar manner, reaction on **7** (1.0 g, 2.1 mmol) in anhydrous acetonitrile (20 mL), with thioacetic acid (230 μL, 3.2 mmol) and $BF_3{\cdot}Et_2O$ (780 μL, 6.3 mmol), for 2 h at room temperature, afforded **8** (887 mg, 86%) as an inseparable anomeric mixture (α/β 1:1).

Implementing the above method for the synthesis of **8** from **7**, compound mixture **6** (100 mg, 0.11 mmol) was converted into **8** (43 mg, 80%; α/β 1:1).

^{1}H NMR (400 MHz, $CDCl_3$) δ 1.88, 1.89 (6H, 2 × s, NHCOC*H$_3$* × 2), 1.98 – 2.31 (28H, m), 2.33, 2.37 (6H, 2 × s, SCOC*H$_3$* × 2), 3.77 (1H, dd, *J* = 10.4 Hz, *J* = 2.3 Hz), 3.91 (1H, dd, *J* =

10.5 Hz, J = 1.8 Hz), 3.95 – 4.05 (3H, m), 4.15 – 4.39 (4H, m), 4.63 (1H, d, J = 8.5 Hz), 4.72 (1H, dd, J = 12.6 Hz, J = 2.4 Hz), 4.95 – 5.15 (5H, m), 5.20 (1H, dd, J = 12.4 Hz, J = 2.0 Hz, H-1α), 5.28 – 5.38 (2H, m), 5.42 – 5.52 (2H, m), 6.13 (1H, d, J = 5.2 Hz, H-1β), 6.25 – 6.37 (2H, m). ^{13}C NMR (101 MHz, $CDCl_3$) δ 20.6, 20.9, 20.9, 20.9, 21.0, 21.1 (OCO*C*H$_3$ × 8), 23.2, 23.3 (HNCO*C*H$_3$ × 2), 30.7, 31.3 (SCO*C*H$_3$ × 2), 33.2, 35.6 (C-2 × 2), 48.4, 50.2 (C-4 × 2), 61.5, 62.2 (C-8 × 2), 67.9, 69.8, 70.0, 70.4, 72.0, 72.6, 76.4, 77.5 (C-3 × 2, C-5 × 2, C-6 × 2, C-7 × 2), 77.3 (C-1α), 79.2 (C-1β), 168.0, 169.2, 169.6, 169.9, 170.2, 170.4, 170.5, 170.7, 170.7, 171.1 (HN*C*OCH$_3$ × 2, O*C*OCH$_3$ × 8), 191.6, 192.4 (S*C*OCH$_3$ × 2). HRMS *m/z* calcd for $[C_{20}H_{29}NO_{11}S+Na]^+$: 514.136; Found: 514.133.

(4-Acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-D-*glycero*-α-D-*galacto*-octopyranosyl)sulfonic acid, sodium salt (**9**) and (**4-acetamido-2,4-dideoxy-3,6,7,8-tetra-*O*-acetyl-D-*glycero*-β-D-*galacto*-octopyranosyl)sulfonic acid, sodium salt** (**10**)



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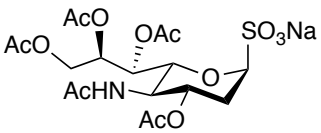

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NOTE: Reactions and subsequent operations involving peracids and peroxy compounds should be run behind a safety shield. Dilution of the reaction solution with water should be done before concentration. Removal of residual peroxides should be validated using a test for peroxides.[S13]

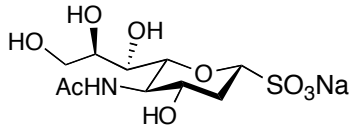
To a solution of **8** (1.17 g, 2.38 mmol) in glacial acetic acid (30 mL) was added NaOAc (390 mg, 4.76 mmol). Hydrogen peroxide (35% w/v - 9.5 mL, 95.2 mmol) was then added slowly to the solution. The solution was stirred at 50 °C for 6 h, after which time it was diluted with H_2O (40 mL), concentrated, and co-evaporated with H_2O (3 times), then with toluene (3 times), maintaining the rotary evaporator heating bath below 40 °C. The resulting crude mixture was purified by flash chromatography on silica gel (solid loading with Celite® - DCM/MeOH 85:15) to give an inseparable mixture of **9** and **10** as a white solid (679 mg, 55%; α/β 1:1). R_f 0.13 (DCM/MeOH 85:15). The anomeric mixture was then separated by RP-HPLC. RP-HPLC purification conditions: gradient elution in aqueous MeCN (1%→7%) at a flow rate of 3 mL/min and a column temperature of 36 °C: retention time 12.7 min for **9** and retention time 14.9 min for **10**.

9: ^{1}H NMR (400 MHz, CD_3OD) δ 1.85 (3H, s, NHCOC*H*$_3$), 1.89 (1H, app q, $J_{2ax,1} \approx J_{2ax,2eq} \approx J_{2ax,3} \approx$ 12.5 Hz, H-2ax), 2.00, 2.00, 2.05, 2.07 (12H, 4 × s, OCOC*H*$_3$ × 4), 2.49 (1H, ddd, $J_{2eq,2ax}$ = 12.5 Hz, $J_{2eq,3}$ = 5.1 Hz, $J_{2eq,1}$ = 1.9 Hz, H-2eq), 3.82 (1H, dd, $J_{5,4}$ = 10.3 Hz, $J_{5,6}$ = 2.1

Hz, H-5), 3.98 (1H, app t, $J_{4,5} \approx J_{4,3} \approx 10.3$ Hz, H-4), 4.20 (1H, dd, $J_{8a,8b}$ =12.4 Hz, $J_{8a,7}$ = 6.2 Hz, H-8a), 4.27 (1H, dd, $J_{1,2ax}$ = 12.5 Hz, $J_{1,2eq}$ = 1.9 Hz, H-1), 4.52 (1H, dd, $J_{8b,8a}$ = 12.4 Hz, $J_{8b,7}$ = 2.7 Hz, H-8b), 5.00 (1H, ddd, $J_{3,2ax}$ = 12.5 Hz, $J_{3,4}$ = 10.3 Hz, $J_{3,2eq}$ = 5.1 Hz, H-3), 5.31 (1H, td, $J_{7,8a}$ = 6.2 Hz $J_{7,6}$ = 6.2 Hz, $J_{7,8b}$ = 2.7 Hz, H-7), 5.40 (1H, dd, $J_{6,7}$ = 6.2 Hz, $J_{6,5}$ = 2.1 Hz, H-6). ^{13}C NMR (101 MHz, CD_3OD) δ 20.7, 20.8, 20.8, 20.9 (OCO*C*H$_3$ × 4), 22.7 (HNCO*C*H$_3$), 33.6 (C-2), 50.5 (C-4), 63.3 (C-8), 69.2 (C-6), 72.2 (C-7), 73.2 (C-3), 78.4 (C-5), 87.2 (C-1), 171.7, 171.9, 172.0, 172.5, 173.4 (HN*C*OCH$_3$, O*C*OCH$_3$ × 4). HRMS *m/z* calcd for $[C_{18}H_{26}NNaO_{13}S+Na]^+$: 542.092; Found: 542.087; *m/z* calcd for $[C_{18}H_{26}NO_{13}S]^-$: 496.1130; Found: 496.1108.

10: ^{1}H NMR (400 MHz, CD_3OD) δ 1.85 (3H, s, NHCOC*H*$_3$), 1.99 (1H, ddd, $J_{2ax,2eq}$ = 13.6 Hz, $J_{2ax,3}$ = 10.3 Hz, $J_{2ax,1}$ = 6.8 Hz, H-2ax), 1.98, 2.00, 2.07, 2.09 (12H, 4 × s, OCOC*H*$_3$ × 4), 2.69 (1H, dd, $J_{2eq,2ax}$ =13.6 Hz, $J_{2eq,3}$ = 5.6 Hz, H-2eq), 3.95 (1H, app t, $J_{4,5} \approx J_{4,3} \approx 10.3$ Hz, H-4), 4.11 (1H, dd, $J_{8a,8b}$ = 12.4 Hz, $J_{8a,7}$ = 5.3 Hz, H-8a), 4.42 (1H, dd, $J_{8b,8a}$ = 12.4 Hz, $J_{8b,7}$ = 2.8 Hz, H-8b), 4.67 (1H, d, $J_{1,2ax}$ = 6.8 Hz, H-1), 4.76 (1H, dd, $J_{5,4}$ = 10.3 Hz, $J_{5,6}$ = 2.0 Hz, H-5), 5.25 (1H, ddd, $J_{7,6}$ = 8.0 Hz, $J_{7,8a}$ = 5.3 Hz, $J_{7,8b}$ = 2.8 Hz, H-7), 5.38 (1H, dd, $J_{6,7}$ = 8.0 Hz, $J_{6,5}$ = 2.0 Hz, H-6), 5.55 (1H, td, $J_{3,4} \approx J_{3,2ax} \approx 10.3$ Hz, $J_{3,2eq}$ = 5.6 Hz, H-3). ^{13}C NMR (101 MHz, CD_3OD) δ 20.7, 20.8, 20.9, 21.3 (OCO*C*H$_3$ × 4), 22.6 (HNCO*C*H$_3$), 31.4 (C-2), 50.6 (C-4), 63.1 (C-8), 69.0 (C-6), 70.8 (C-7), 71.0 (C-3), 72.8 (C-5), 85.7 (C-1), 171.6, 172.0, 172.4, 172.6, 173.4 (HN*C*OCH$_3$, O*C*OCH$_3$ × 4). HRMS *m/z* calcd for $[C_{18}H_{26}NNaO_{13}S+Na]^+$: 542.092; Found: 542.087; *m/z* calcd for $[C_{18}H_{26}NO_{13}S]^-$: 496.1130; Found: 496.1108.

(4-Acetamido-2,4-dideoxy-D-*glycero*-α-D-*galacto*-octopyranosyl)sulfonic acid (4), sodium salt

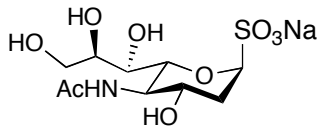


4

To a solution of **9** (100 mg, 0.19 mmol) in anhydrous methanol under argon at 0 ºC, was added dropwise 1 M methanolic sol. of NaOMe until the pH of the reaction mixture reached 10-11. The solution was stirred for 12 h, while the temperature was raised gradually to room temperature. The mixture was then acidified with Amberlite® IR-120 (H^+) resin (to pH = 3-4), filtered and the solution was concentrated under reduced pressure to give **4** (68 mg, 100%). RP-HPLC characterisation: isocratic elution in aqueous MeCN (0.1%) at a flow rate

of 2.5 mL/min and a column temperature of 30 °C: retention time 5.4 min. Sulfonate **4** was found to be stable as a solid or in aqueous solution, stored at –20 °C, for at least eight months. **4**: ^{1}H NMR (400 MHz, D_2O) δ 1.67 – 1.85 (1H, m, H-2ax), 2.05 (3H, s, NHCOC*H*$_3$), 2.49 (1H, ddd, $J_{2eq,2ax}$ = 12.3 Hz, $J_{2eq,3}$ = 4.1 Hz, $J_{2eq,1}$ = 2.1 Hz, H-2eq), 3.49 (1H, dd, $J_{6,7}$ = 9.2 Hz, $J_{6,5}$ = 0.9 Hz, H-6), 3.62 (1H, dd, $J_{8a,8b}$ = 12.0 Hz, $J_{8a,7}$ = 6.4 Hz, H-8a), 3.73 (1H, dd, $J_{5,4}$ = 10.0 Hz, $J_{5,6}$ = 0.9 Hz, H-5), 3.80 – 3.95 (4H, m, H-3, H-4, H-7, H-8b), 4.46 (1H, dd, $J_{1,2ax}$ = 11.8 Hz, $J_{1,2eq}$ = 2.1 Hz, H-1). ^{13}C NMR (101 MHz, D_2O) δ 22.1 (HNCO*C*H$_3$), 34.1 (C-2), 51.9 (C-4), 63.2 (C-8), 68.3 (C-6), 69.3 (C-4), 70.0 (C-7), 76.5 (C-5), 85.3 (C-1), 174.8 (HN*C*OCH$_3$). LRMS (ESI): *m/z* 327.9 [(M-Na)$^-$, 100%]. HRMS *m/z* calcd for [$C_{10}H_{18}NO_9S$]$^-$: 328.0702; Found: 328.0700.

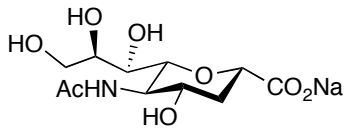
(4-Acetamido-2,4-dideoxy-D-*glycero*-β-D-*galacto*-octopyranosyl)sulfonic acid (11), sodium salt



11

To a solution of **10** (100 mg, 0.19 mmol) in anhydrous methanol under argon at 0 ºC, was added dropwise 1 M methanolic sol. of NaOMe until the pH of the reaction mixture reached 10-11. The solution was stirred for 12 h, while the temperature was raised gradually to room temperature. The mixture was then acidified with Amberlite® IR-120 (H^+) resin (to pH = 3-4), filtered and the solution was concentrated under reduced pressure to give **11** (68 mg, 100%). RP-HPLC characterisation: isocratic elution in aqueous MeCN (0.1%) at a flow rate of 2.5 mL/min and a column temperature of 30 °C: retention time 5.2 min. Sulfonate **11** was found to be stable as a solid or in aqueous solution, stored at –20 °C, for at least eight months. **11**: ^{1}H NMR (400 MHz, D_2O) δ 2.00 – 2.10 (1H, m, H-2ax), 2.06 (3H, s, NHCOC*H*$_3$), 2.62 (1H, dd, $J_{2eq,2ax}$ = 14.4 Hz, $J_{2eq,3}$ = 5.2 Hz, H-2eq), 3.53 (1H, d, $J_{6,7}$ = 8.8 Hz, H-6), 3.63 (1H, dd, $J_{8a,8b}$ = 11.9 Hz, $J_{8a,7}$ = 6.6 Hz, H-8a), 3.82 – 3.96 (3H, m, H-4, H-7, H-8b), 4.25 – 4.36 (2H, m, H-3, H-5), 4.87 (1H, d, $J_{1,2ax}$ = 6.8 Hz, H-1). ^{13}C NMR (101 MHz, D_2O) δ 22.0 (HNCO*C*H$_3$), 31.9 (C-2), 52.0 (C-4), 63.1 (C-8), 66.1 (C-3), 68.4 (C-6), 70.6 (C-7), 72.7 (C-5), 85.2 (C-1), 174.9 (HN*C*OCH$_3$). LRMS (ESI): *m/z* 327.9 [(M-Na)$^-$, 100%].

5-Acetamido-2,6-anhydro-3,5-dideoxy-D-*erythro*-L-*gluco*-nononic acid (2), sodium salt



2

Compound **2** was prepared following a reported procedure.[S8] RP-HPLC purification conditions: isocratic elution in aqueous MeCN (0.3% + 0.05%TFA) at a flow rate of 2.9 mL/min and a column temperature of 33 °C: retention time 5.79 min.

^{1}H NMR (400 MHz, D_2O) δ 1.67 (1H, app q, $J_{3ax,3eq} \approx J_{3ax,2} \approx J_{3ax,4} \approx$ 12.2 Hz, H-3ax), 2.05 (3H, s, NHCOC*H*$_3$), 2.43 (ddd, $J_{3eq,3ax}$ = 12.2 Hz, $J_{3eq,4}$ = 4.4 Hz, $J_{3eq,2}$ = 1.9 Hz, H-3eq), 3.51 (1H, d, J = 9.2 Hz, H-7), 3.59 – 3.70 (2H, m, H-6, H-9a), 3.77 – 3.97 (4H, m, H-4, H-5, H-8, H-9b), 4.28 (1H, dd, $J_{2,3ax}$ = 12.2 Hz, $J_{2,3eq}$ = 1.9 Hz, H-2). ^{13}C NMR (101 MHz, D_2O) δ 22.0 (HNCO*C*H$_3$), 35.9 (C-3), 52.0 (C-5), 63.1 (C-9), 68.3 (C-7), 69.7 (C-4), 70.2 (C-8), 73.5 (C-2), 75.7 (C-6), 174.7, 174.8 (C-1, HN*C*OCH$_3$). LRMS (ESI): *m/z* 292 [(M-H)$^-$, 100%]. The NMR data was in accordance with that previously reported.[S8, S9]

INFLUENZA VIRUS REPLICATION ASSAY

Cells and virus: Madin-Darby canine kidney (MDCK) cells were maintained in EMEM supplemented with 5% FBS and penicillin/streptomycin at 37 °C, 5% CO_2. Influenza A virus (IAV) strain H3N2 (A/Perth/16/2009) obtained from the American Type Culture Collection (ATCC) was passaged in MDCK cells in un-supplemented EMEM containing 1 μg/mL of TPCK-treated trypsin (infection medium) at 35 °C, 5% CO_2.

Cell-based assays: Confluent MDCK cells in 96-well plates were overlaid with 350 plaque forming units of IAV per well in infection medium without trypsin and incubated for 1 h at 35 °C, 5% CO_2, shaking gently every 15 min. The inoculum was subsequently removed and replaced with 50 μL of drug dilutions in infection medium containing trypsin. The plates were incubated for 24 h at 35 °C, 5% CO_2. Infection was measured using an *in situ* enzyme-linked immunosorbent assay (ELISA).

***In situ* ELISA:** For immunostaining of IAV-infected cells, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature and washed 3 x 4 min with PBS.

To permeabilize cells and inhibit endogenous peroxidases, they were treated with 1% IGEPAL and 0.3% H_2O_2 in PBS for 20 min at 37 °C. After an additional washing step, they were incubated with a 1:1000 dilution of primary anti-IAV nucleoprotein (NP) antibody (mouse, Bio-Rad ref.: MCA400) in PBS/5% skim milk for 30 min at 37 °C. Cells were washed 3 x 4 min with PBS/0.02% Tween-20, incubated with a 1:3000 dilution of secondary goat anti-Mouse IgG(H+L)-HRP-conjugated antibody (Bio-Rad, ref: 170–6516) in PBS/5% skim milk for 30 min at 37 °C and washed another 3 x 4 min with PBS/0.02% Tween-20. Antibody complexes were detected by addition of 100 µL of BD OptEIA TMB substrate (BD Biosciences, San Jose, CA) to each well and reactions were stopped by addition of 50 µL of 0.6 M H_2SO_4 in PBS. The absorbance at 450 nm in each well was measured using a BioRad X-Mark Microplate Absorbance Spectrophotometer. The average absorbance value from the negative control of infection was subtracted from all wells and the resulting absorbance values were normalized to the average value of the positive control of infection. Results were expressed as percentage of control. IC_{50} values were determined by non-linear regression analysis using the software GraphPad Prism v.6 (GraphPad Software, La Jolla, California).

INFLUENZA VIRUS SIALIDASE INHIBITION ASSAY

Inhibition of influenza A virus sialidase was quantitatively assessed using a modification of the fluorometric sialidase assay of Potier *et al.*,[S14] with the fluorogenic substrate 4-methylumbelliferyl *N*-acetyl-α-D-neuraminic acid[S15] (MUN) and purified viral particles as the source of enzyme. To obtain virus for sialidase inhibition assays, clarified supernatants of infection were loaded onto a 30% sucrose cushion in GNTE buffer (glycine 200 mM, NaCl 200 mM, Tris-HCl 20 mM, EDTA 2 mM, pH 7.4) and ultracentrifuged in a SW 32.1 Ti rotor for 3 h at 4 °C, 32,000 x g. The resulting virus pellet was resuspended in GNTE buffer for subsequent use in sialidase inhibition assays.

Sialidase inhibition assays were carried out on ice in black 96-well plates, in triplicates and in a reaction volume of 10 µL. Virus, test compounds and MUN were diluted separately in assay buffer (50 mM sodium acetate, 6 mM $CaCl_2$, pH 5.5). Test compound dilutions were incubated with an equal volume of virus dilution at room temperature for 20 min. A volume of 6 µL of virus/compound dilution was subsequently pipetted into each replicate well, before the addition of 4 µL of MUN (0.1 mM final). A volume of 150 µL of stop buffer (0.25 M glycine, pH 10) was immediately added to negative control wells to stop the reaction, and the

plate was spun briefly to combine all components. Reactions were incubated for 20 min at 37 °C, with vigorous orbital agitation at 900 rpm. They were then stopped by addition of 150 µL of stop buffer. Fluorescence was read in a Tecan Infinite 200 Pro (Tecan Trading AG, Switzerland) fluorescence reader, at excitation and emission parameters of 355 nm and 460 nm, respectively. The background fluorescence of the reaction (negative control wells) was subtracted from all wells and enzyme activities were expressed as percentage of the positive control (reactions treated with assay buffer in place of compound). Fifty percent inhibitory concentrations (IC_{50}) (see Figure S2) were calculated from non-linear regression analysis using GraphPad Prism v.6 (GraphPad Software, La Jolla, California, USA).

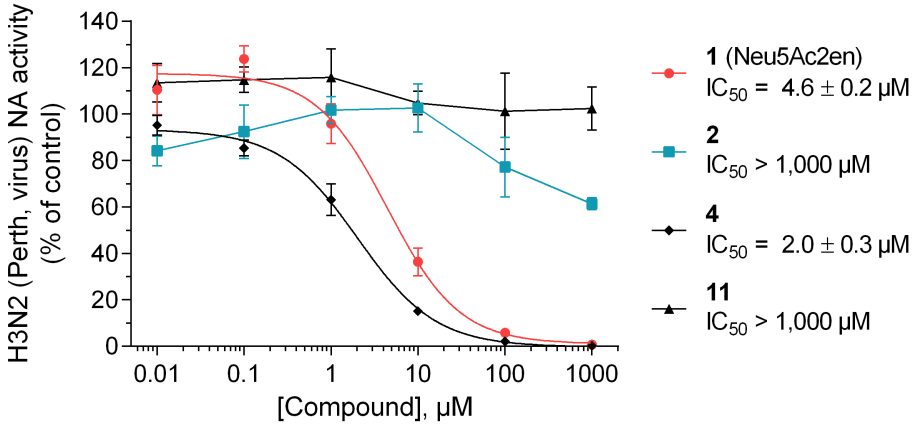


Figure S2. Inhibition of influenza A virus (IAV) H3N2 sialidase (NA) activity, using whole virus particles. Dose-dependent inhibition of IAV A/Perth/16/2009 H3N2 sialidase activity by Neu5Ac2en (**1**), and compounds **2**, **4** and **11** using a fluorometric assay. Data-points represent the mean of three independent experiments, each run in triplicate, ± SEM. IC_{50} values are the mean IC_{50} of three independent experiments, ± SEM.

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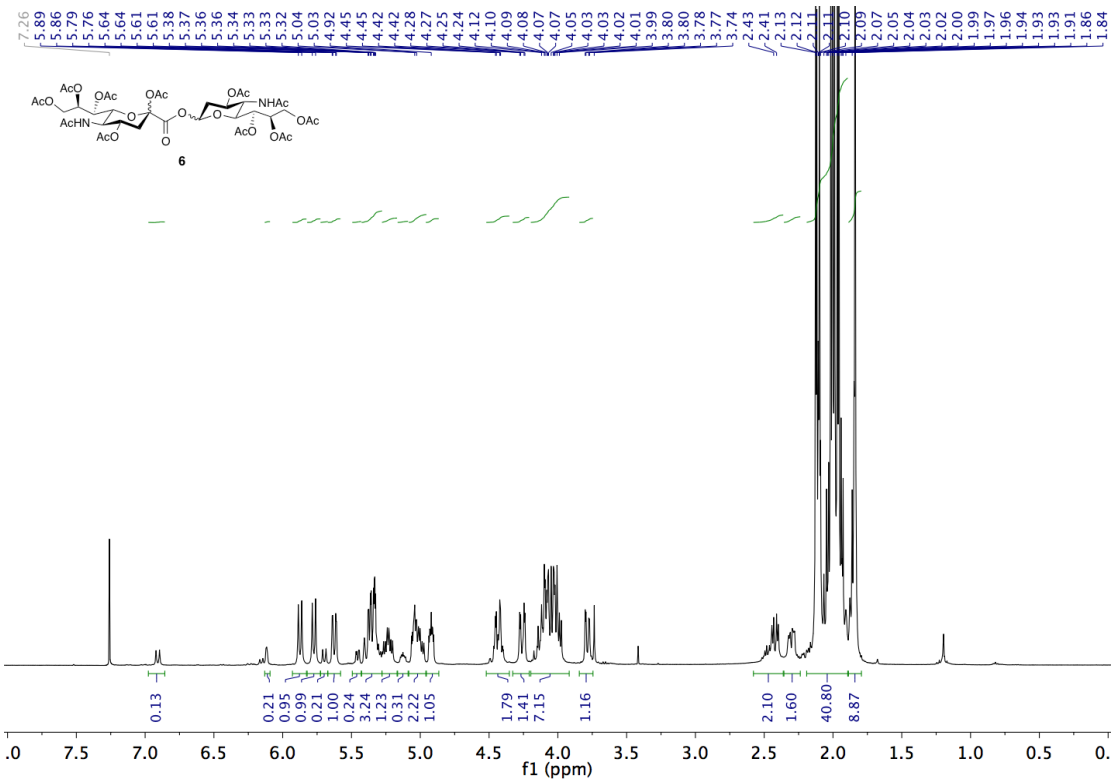
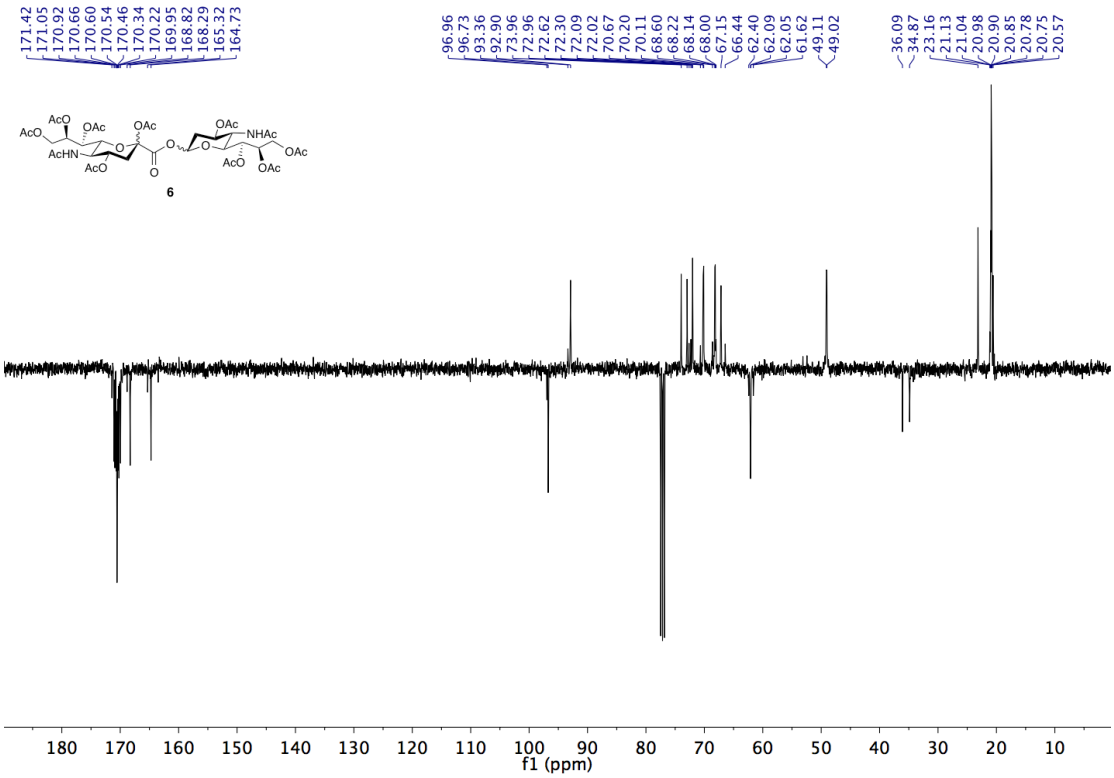
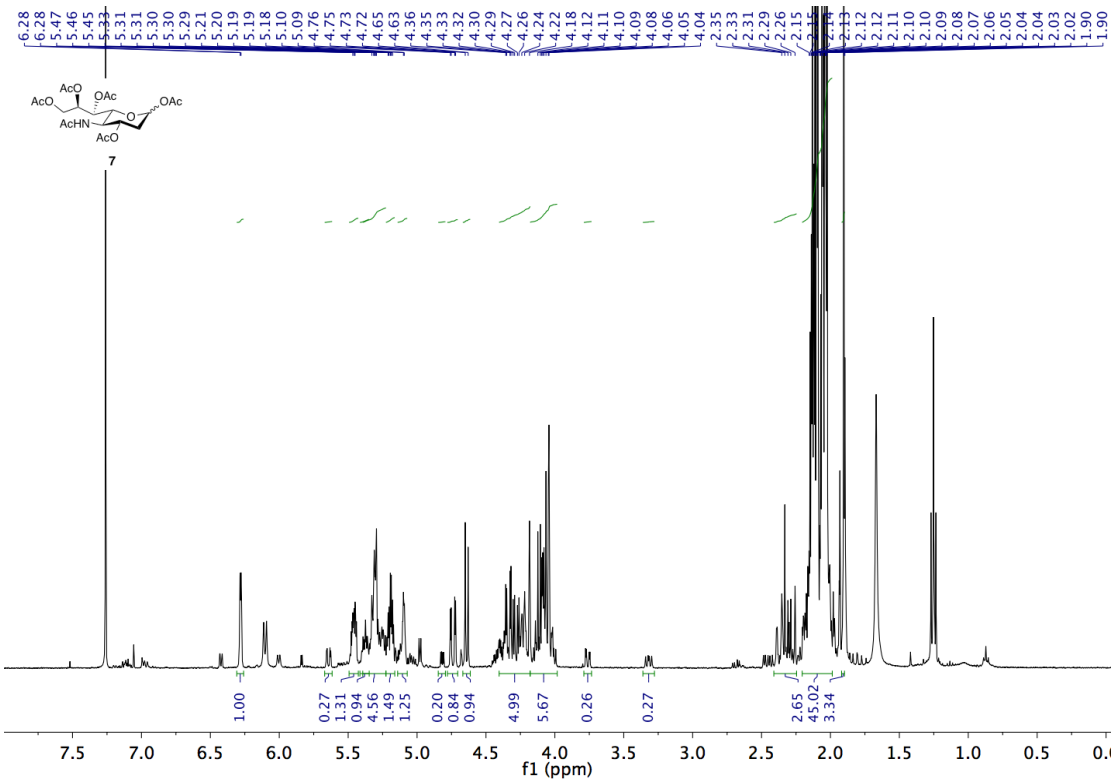
^{1}H NMR (400 MHz, $CDCl_3$) of **6** (α/β 5:1)



^{13}C NMR (101 MHz, $CDCl_3$) of **6** (α/β 5:1)



^{1}H NMR (400 MHz, $CDCl_3$) of **7** (α/β 1:4)



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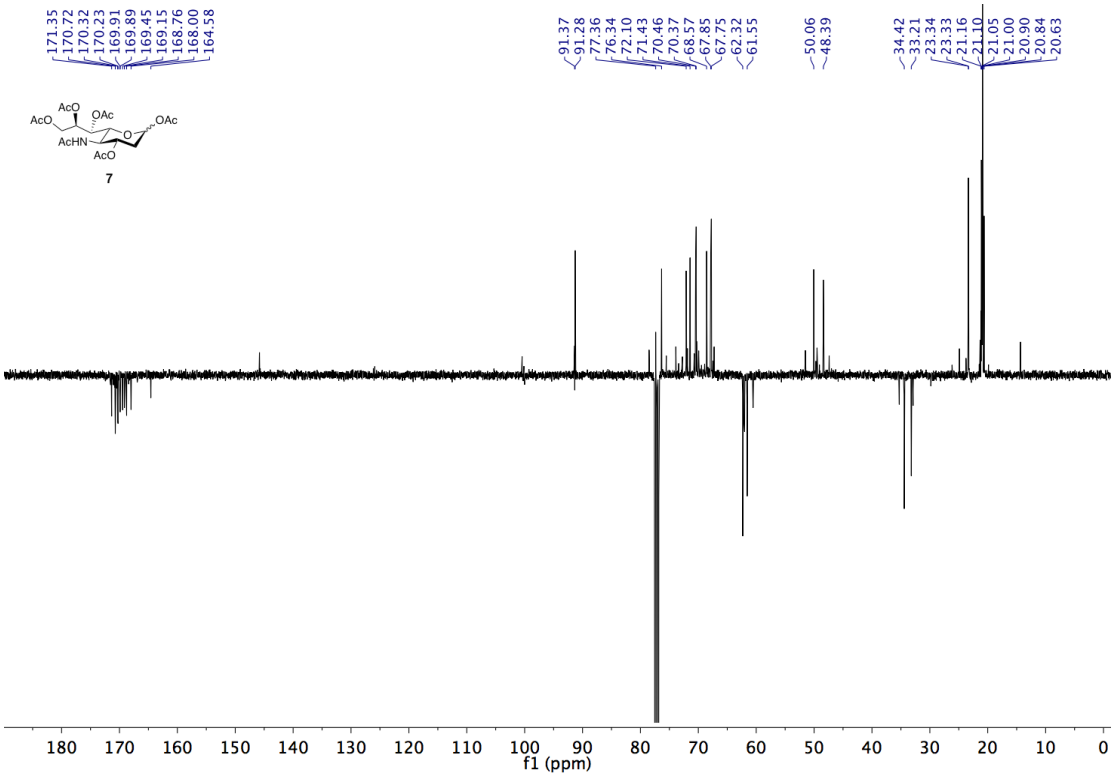
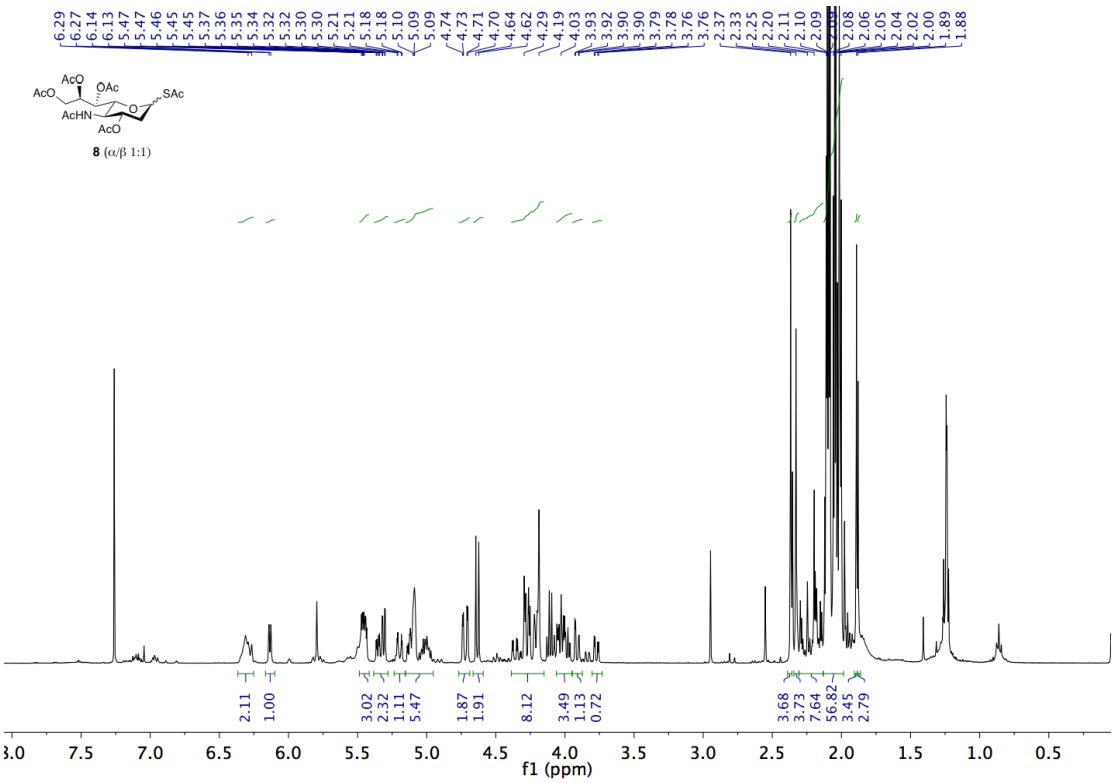


^{1}H NMR (400 MHz, $CDCl_3$) of **8** (α/β 1:1)



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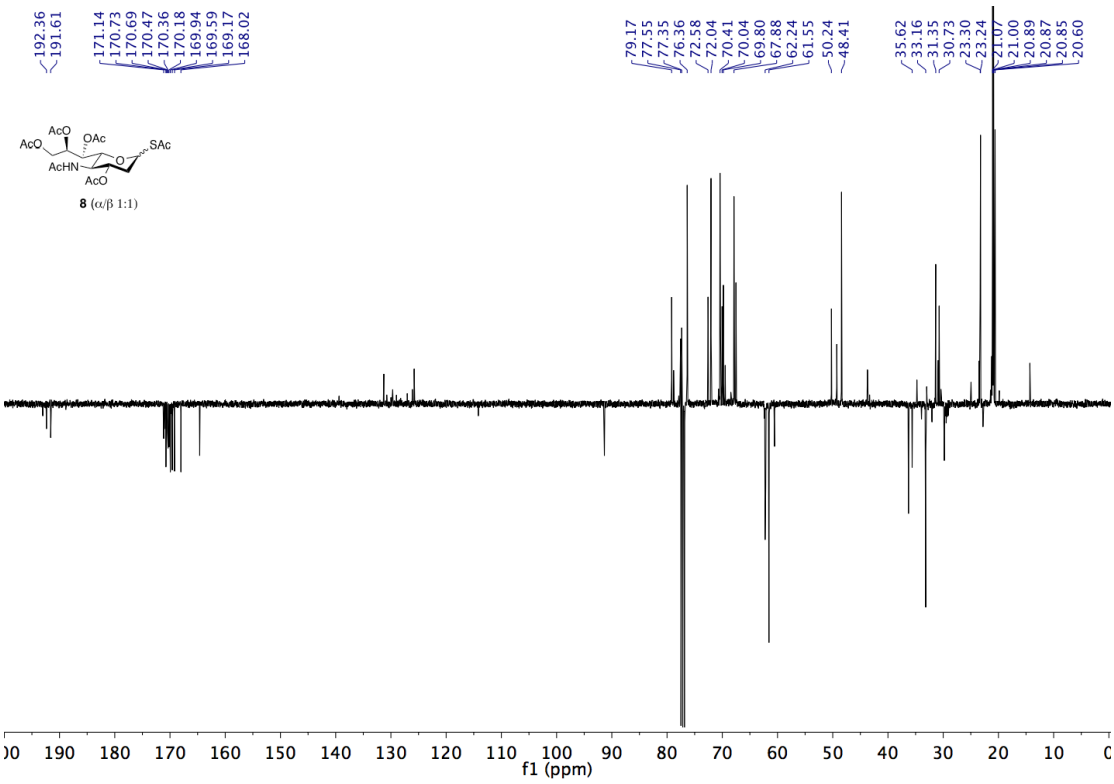
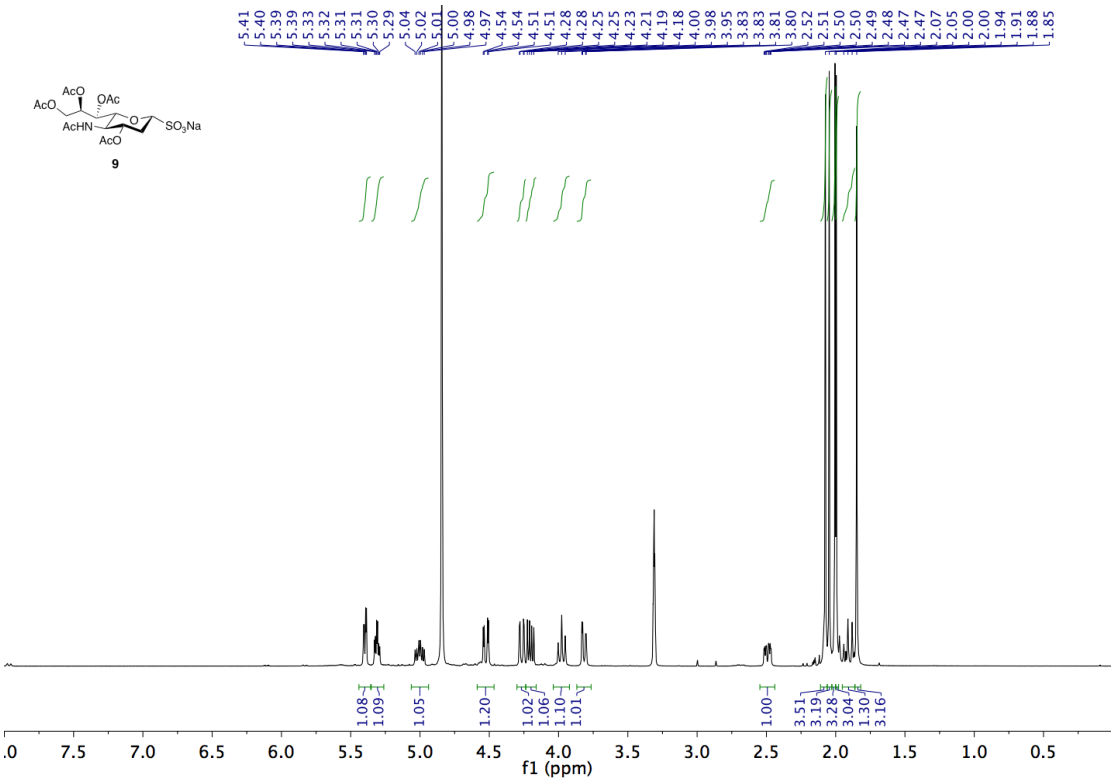


^{1}H NMR (400 MHz, CD_3OD) of **9**



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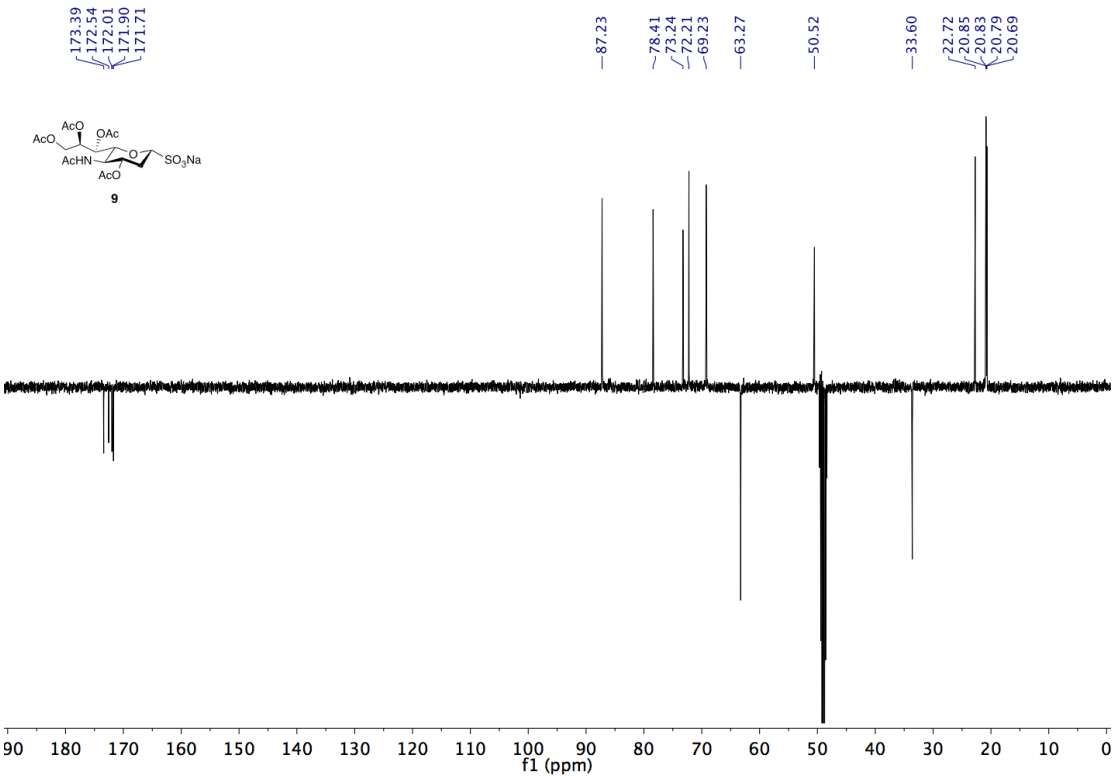

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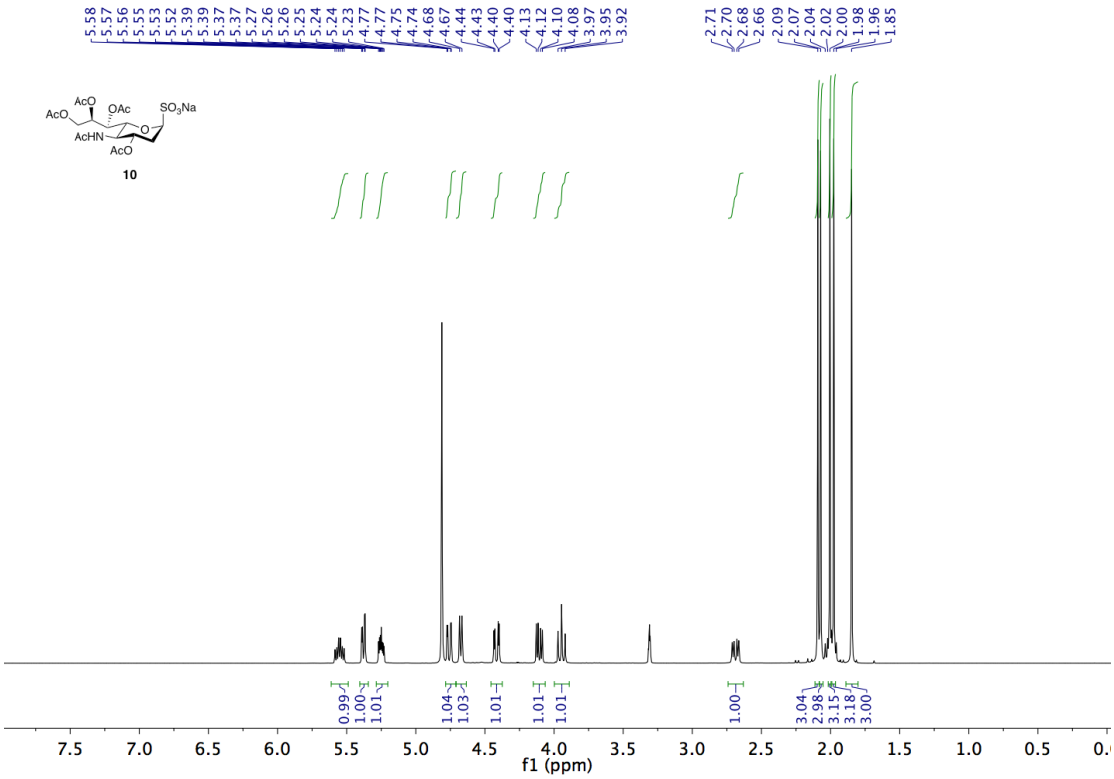

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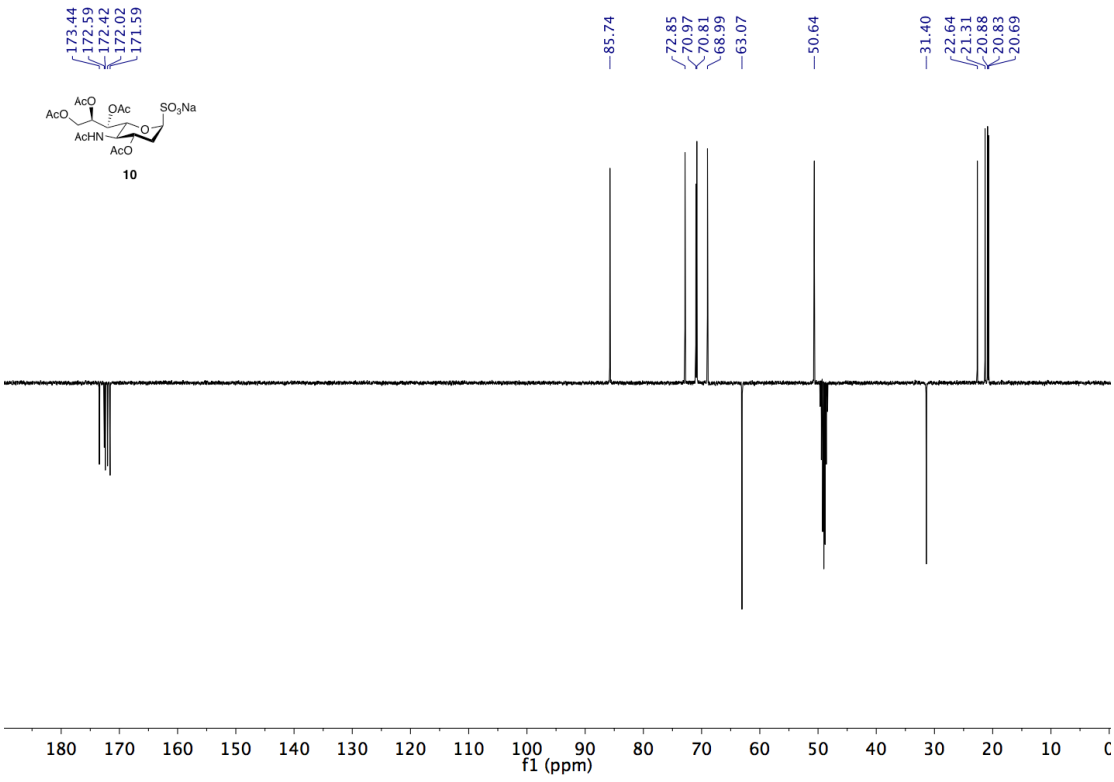
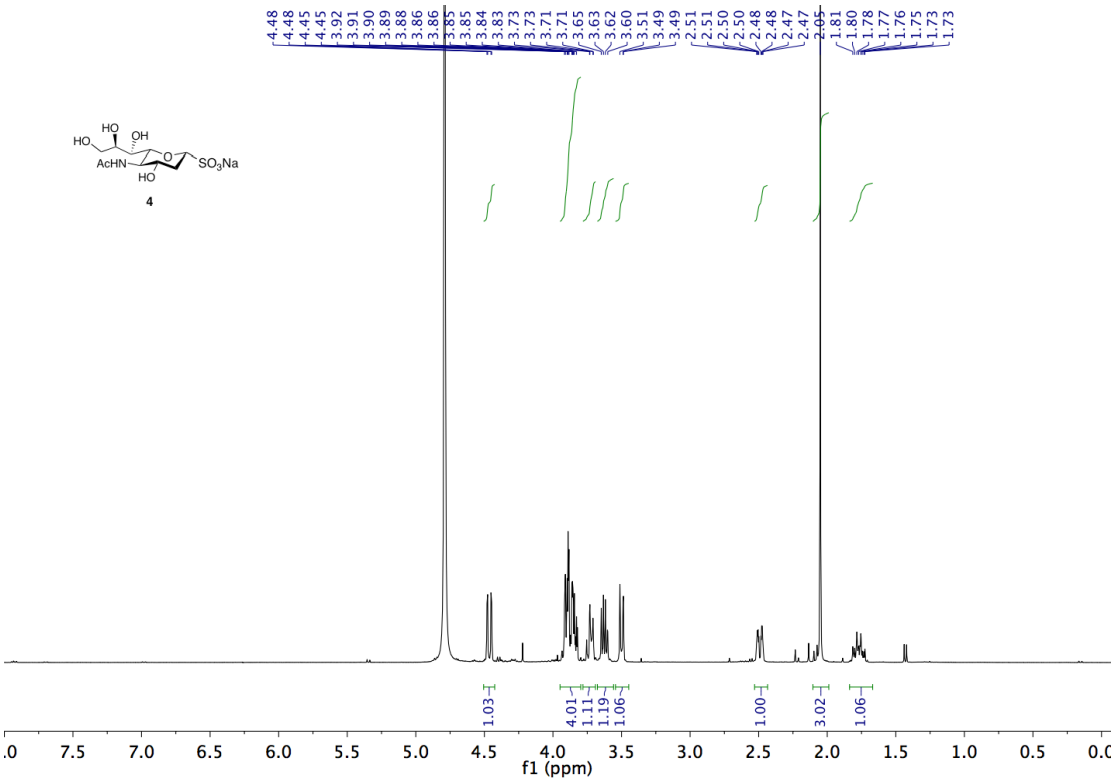


^{1}H NMR (400 MHz, D_2O) of **4**



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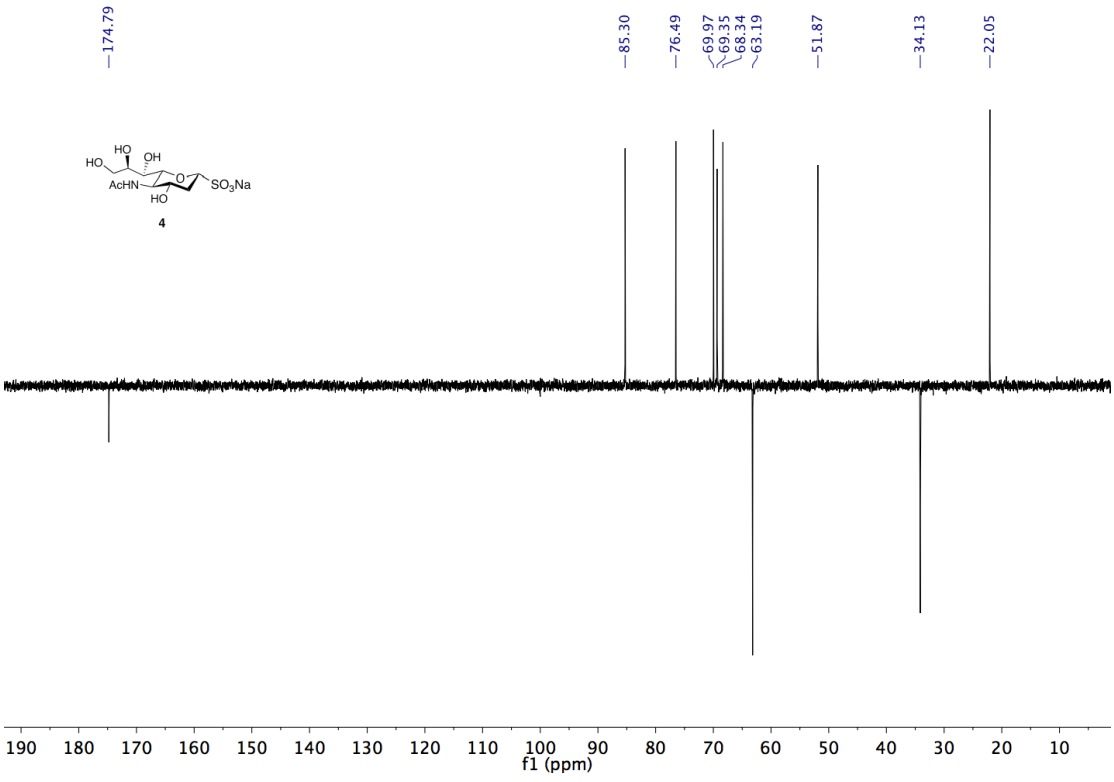

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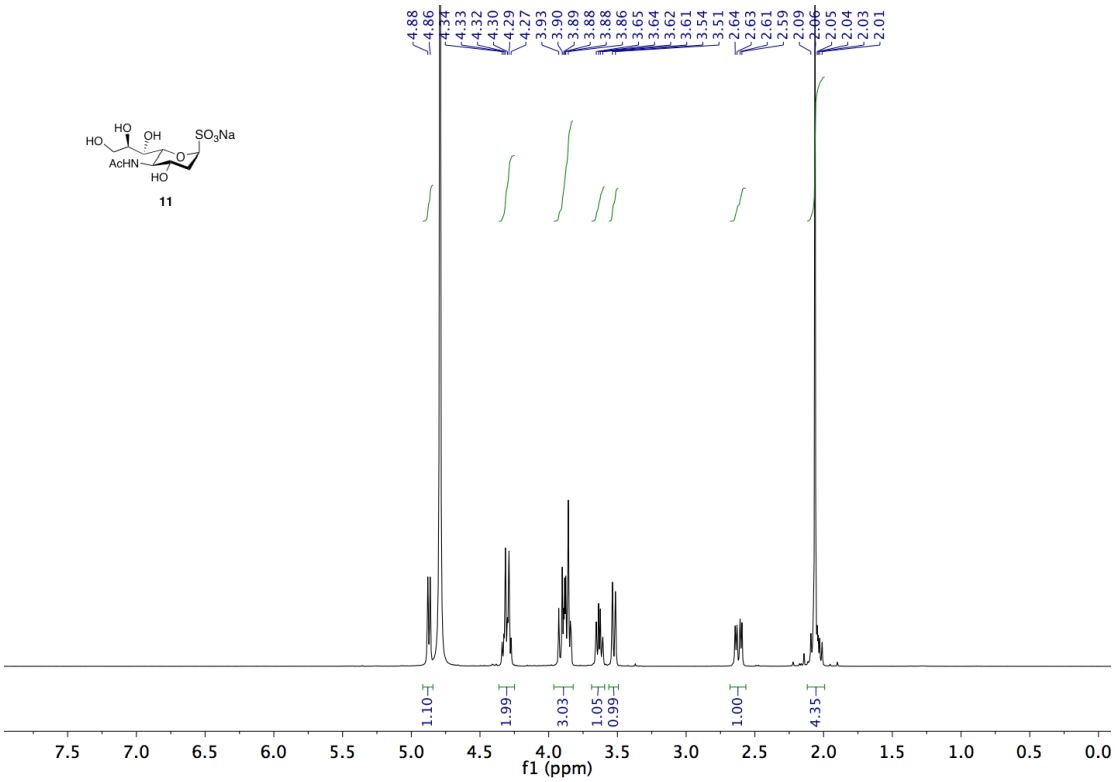

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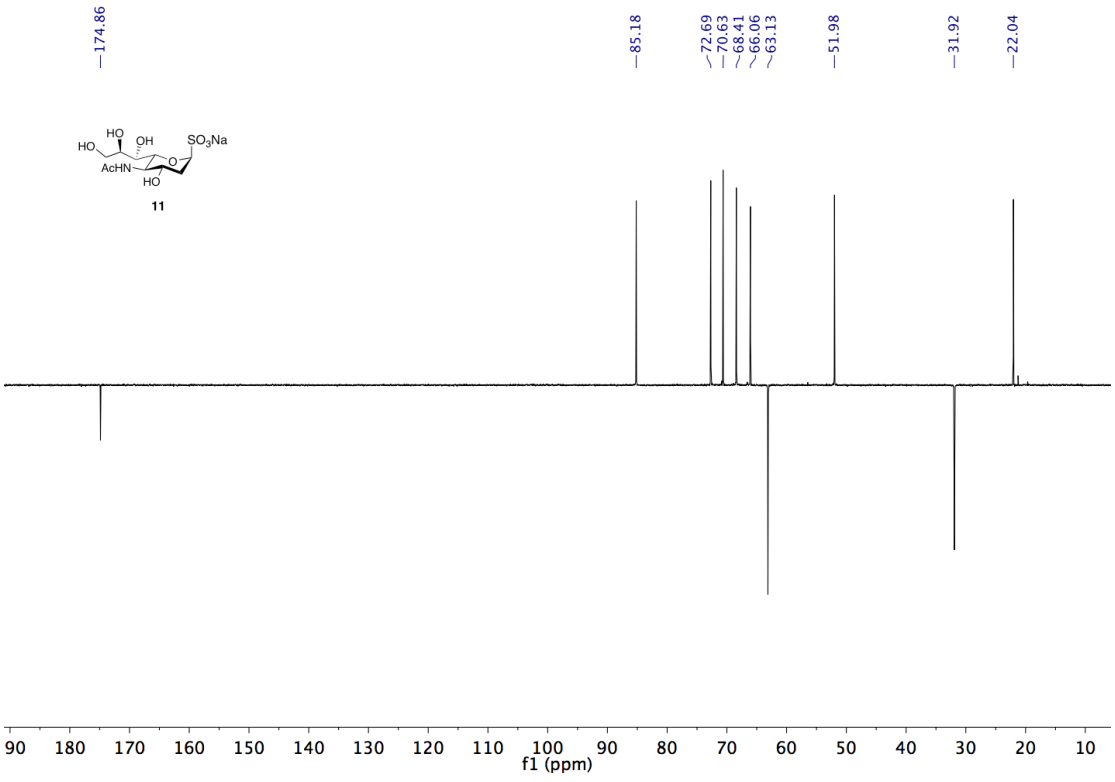
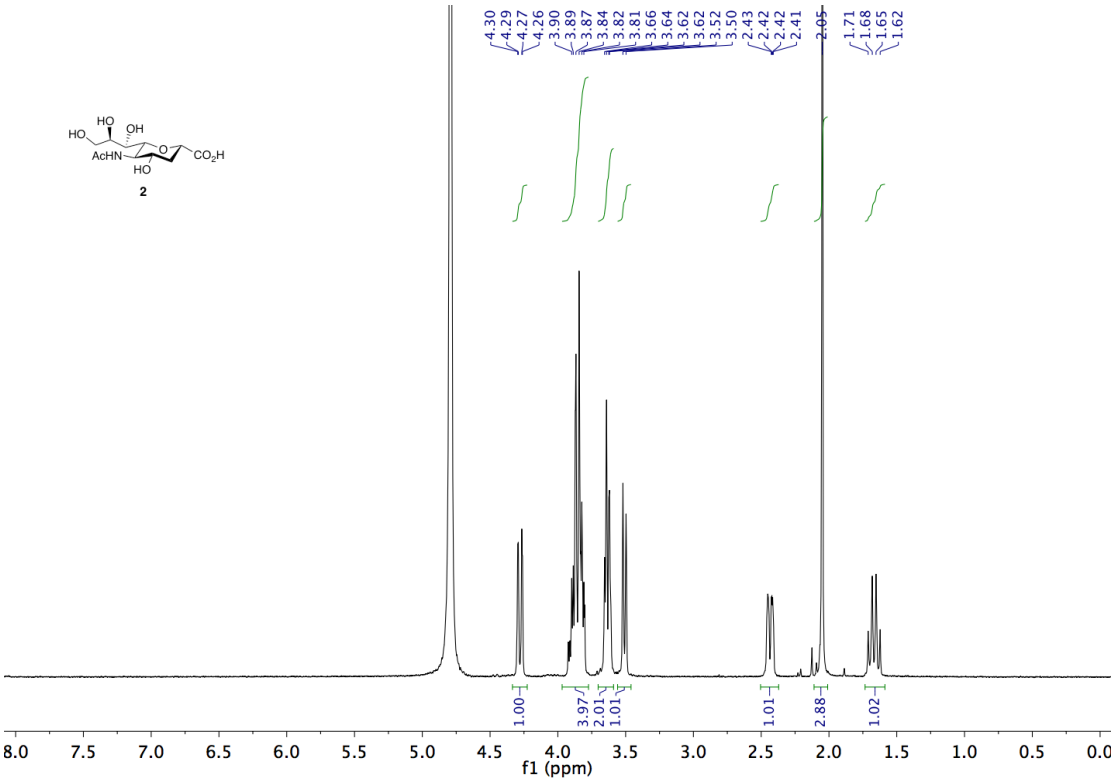


^{1}H NMR (400 MHz, D_2O) of **2**



^{13}C NMR (101 MHz, D_2O) of **2**