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Supporting Information

Sequential Bioorthogonal Dual Strategy: ManNAl and SiaNAl as distinct tools to unravel sialic acids metabolic pathways

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Note added after first publication: This Supplementary Information file replaces that originally published on 5th January 2016. In this version the previously omitted NMR, IR spectra and additional references are added.

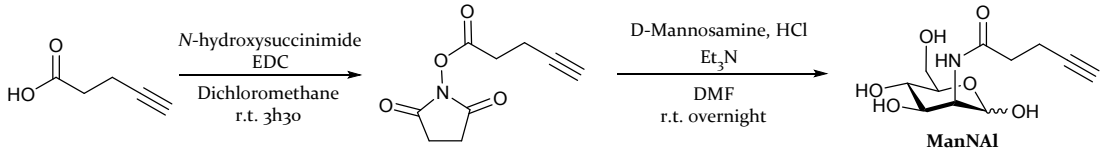
General methods and materials

Chemicals reagents were purchased from Sigma Aldrich, TCI and Carbosynth and were used with no further purification. Anti-TGN46 and anti-EEA1 antibodies were from BD Biosciences (Franklin lakes, NJ, USA). Anti LAMP2 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Reactions were monitored by TLC on pre-coated TLC sheets ALUGRAM XtraSil from Macherey-Nagel. Detection was under UV light (254 nm) or by revelation with a solution of phosphomolybdic acid or a solution of vanillin / H_2SO_4.

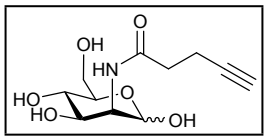
Flash chromatography was performed on Interchim Puriflash 430. Silica column were purchased from Interchim (30 µm) or Macherey-Nagel (50 µm). Final products were freeze-dried before being characterized. ^{1}H NMR (1D, 2D) and ^{13}C NMR were recorded on a Bruker advanced DPX 300 MHz bearing a BBFO probe. NMR spectra were recorded in $CDCl_3$ or D_2O purchased from Eurisotop, chemical shifts are given in ppm relative to solvent peaks and coupling constants (J) are reported in hertz. MALDI-TOF experiments were performed on a 4800 Proteomics Analyzer mass spectrometer (Applied Biosystems, Framingham, MA, USA) in reflectron positive mode. Dihydroxybenzoïc acid (DHB) was used as a matrix. Infrared spectra were recorded on a MIRacle10 (Shimadzu) with an ATR probe. Melting points were measured with SMP30 (Stuart).

Synthetic procedures

Scheme S1. Synthesis of ManNAl



Synthesis of *N*-(4-pentynoyl) mannosamine (ManNAl)[1,2]



4-pent-ynoïc acid (502 mg, 5.1 mmol, 1 eq.) was solved into CH_2Cl_2 (25 mL). N-hydroxysuccinimide (674 mg, 5.8 mmol, 1.14 eq.) and EDC or 1-ethyl (3-dimethylaminopropyl)carbodiimide (1.96 g, 10.2 mmol, 2eq.) were slowly added. The mixture was stirred for 3h30 at room temperature. The end of reaction was confirmed by TLC (cyclohexane/AcOEt ; 70 : 30). The solution was then washed four times with $KHSO_4$ (aqueous solution 2.8%). The recombinated organic layers were dried over sodium sulfate, filtered and then concentrated under reduced pressure to give the succinimidyl ester as a white solid (875.3 mg, 4.48 mmol, 88 %). The product was used in the next step without further purification.

The coupling reaction with Mannosamine hydrochloride was conducted under nitrogen atmosphere. Hydrochloride D-Mannosamine (759 mg, 3.52 mmol, 1 eq.) and the succinimidyl ester from previous step (688 mg, 3.52 mmol, 1 eq.) were solved into DMF (25 mL). Triethylamine (1.4 mL, 10.77 mmol, 3 eq.) was slowly added. The mixture was stirred overnight. DMF was then removed under reduced pressure and the crude product was purified by flash chromatography (silica column, 40 g, 30 µm) with an elution by CH_2Cl_2 / EtOAc / MeOH ; 45 : 45 : 10). Solvents were then eliminated to yield ManNAl as a white solid (830 mg, 3.34 mmol, 95 %).

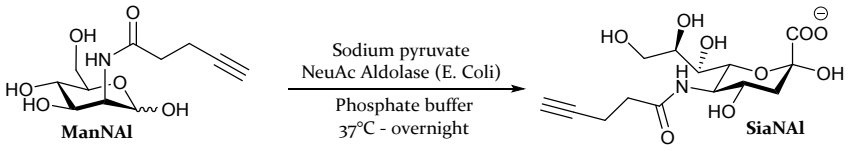
NMR: Mixture of anomers (α / β ≈ 60% / 40%). **α** ^{1}H NMR (300 MHz, D_2O): δ = 5.00 (d, *J*=1.4, 1H, $\mathbf{H_1}$), 4.23 (dd, *J*=4.7, 1.4, 1H, $\mathbf{H_2}$), 3.93 (dd, *J*=9.8, 4.7, 1H, $\mathbf{H_3}$), 3.80 – 3.62 (m, 3H, $\mathbf{H_5}$ **&** $\mathbf{H_6}$), 3.50 (t, *J*=9.6, 1H, $\mathbf{H_4}$), 2.58 – 2.32 (m, 4H, $\mathbf{H_8}$ **&** $\mathbf{H_9}$), 2.26 (t, *J*=2.3, 1H, $\mathbf{H_{11}}$). ^{13}C NMR (75 MHz, D_2O): δ = 175.26 ($\mathbf{C_7}$), 93.17 ($\mathbf{C_1}$), 83.43 ($\mathbf{C_{10}}$), 71.94 ($\mathbf{C_5}$), 70.14 ($\mathbf{C_{11}}$), 68.73 ($\mathbf{C_3}$), 66.72 ($\mathbf{C_4}$), 60.36 ($\mathbf{C_6}$), 53.15 ($\mathbf{C_2}$), 34.10 ($\mathbf{C_8}$), 14.40 ($\mathbf{C_9}$). **β** ^{1}H NMR (300 MHz, D_2O): δ = 4.90 (d, *J*=1.6, 1H, $\mathbf{H_1}$), 4.35 (dd, *J*=4.4, 1.4, 1H, $\mathbf{H_2}$), 3.82 – 3.61 (m, 3H, $\mathbf{H_3}$ **&** $\mathbf{H_6}$), 3.40 (t, *J*=9.8, 1H, $\mathbf{H_4}$), 3.29 (ddd, *J*=9.9, 4.8, 2.3, 1H, $\mathbf{H_5}$), 2.53 – 2.31 (m, 4H, $\mathbf{H_7}$ **&** $\mathbf{H_8}$), 2.26 (t, *J*=2.3, 1H, $\mathbf{H_{11}}$). ^{13}C NMR (75 MHz, D_2O): δ = 175.98 ($\mathbf{C_7}$), 92.84 ($\mathbf{C_1}$), 83.85 ($\mathbf{C_{10}}$), 76.28 ($\mathbf{C_5}$), 71.96 ($\mathbf{C_3}$), 69.99 ($\mathbf{C_{11}}$), 66.45 ($\mathbf{C_4}$), 60.36 ($\mathbf{C_6}$), 53.98 ($\mathbf{C_2}$), 34.25 ($\mathbf{C_8}$), 14.26 ($\mathbf{C_9}$).

m/z: calculated: $[M + Na]^+$ = 282.247 ; measured: 282.041.

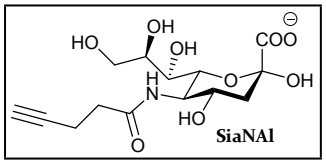
m.p.: 36.5 - 39 °C.

Combustible elemental analysis: calculated C, 50.96; H, 6.61; N, 5.40; measured C, 51.02; H, 6.58; N, 5.38.

Scheme S2. Synthesis of SiaNAl



Synthesis of *N*-4-pentynoylneuraminic acid (SiaNAl)[3]



ManNAl, (60 mg, 0.23 mmol, 1 eq.), sodium pyruvate (46 mg, 0.345 mmol, 2 eq.) and Neuraminic5Ac aldolase (Sigma Aldrich EC 4.1.3.3) from *E. Coli* K12 (5 units dissolved in 0.1 mL) were added into 600 µL of phosphate buffer (KH_2PO_4, 10.3 mM, K_2HPO_4, 52.8 mM and $MgCl_2$ 20 mM, pH – 7.5) into a reaction tube which was incubated at 37.5°C with moderate shaking (140

rpm) for 18 hours. The reaction completion was monitored by TLC (propan-1-ol / 25% Ammonia / H_2O ; 6 : 1 : 2.5). Reaction was quenched by the addition of 30 mL of water. The product was purified on an anion exchange resin (Bio-Rad AG1X2) activated with NH_4HCO_3 0.1M. The sample was loaded on the column which was washed with H_2O (5 CV). Elution was performed with aqueous NH_4HCO_3 (0.05M, 5 CV) and then NH_4HCO_3 (0.2M, 5 CV). Fractions were detected by TLC (same elution as for the reaction monitoring) combined and freeze dried. After being solved into H_2O (1mL), sample was passed through a gel filtration column (P2) and then freeze dried again. SiaNAl was obtained as white powder (73 mg, 0.21 mmol, 91 %).

NMR: ^{1}H NMR (600 MHz, D_2O): δ = 3.96 – 3.86 (m, 2H, **H_4 & H_9**), 3.83 (t, *J*=10.1, 1H, **H_5**), 3.72 (dd, *J*=11.9, 2.7, 1H, **H_9**), 3.64 (ddd, *J*=9.2, 6.7, 2.7, 1H, **H_8**), 3.51 (d, *J*=9.2, 1H, **H_7**), 3.49 – 3.43 (m, 1H, **$H_{9'}$**), 2.47 – 2.34 (m, 4H, **H_{11} & H_{12}**), 2.28 (d, *J*=2.0, 1H, **H_{13}**), 2.10 (dd, *J*=13.0, 4.9, 1H, **H_{3eq}**), 1.70 (dd, *J*=12.7, 11.7, 1H, **H_{3ax}**). ^{13}C NMR (75 MHz, D_2O): δ 175.43 (**C_1**), 172.91 (**C_{10}**), 95.16 (**C_2**), 83.54 (**C_{13}**), 70.40 (**C_{14}**), 70.37 (**C_8**), 70.23 (**C_6**), 68.29 (**C_7**), 66.45 (**C_4**), 63.21 (**C_9**), 52.04 (**C_5**), 38.79 (**C_3**), 34.67 (**C_{11}**), 14.53 (**C_{12}**).
m/z: calculated: $[M]^+$ = 346.312 ; measured: 346.017
m.p.: 26 – 28.5 °C
Combustible elemental analysis: calculated C, 48.41; H, 6.09; N, 4.04; measured C, 48.87 H, 5.89; N, 4.12.

BTTAA was synthesized as previously described by Peng Wu *et al.*[4]

Imaging experiments

Cell culture

Primary skin fibroblasts and sialin deficient patient cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Lonza) at 37°C in humidity saturated 5% CO_2 atmosphere.

Metabolic labeling with alkyne tagged analogs

Fibroblasts were grown overnight on glass coverslips (12 mm diameter). Medium was then changed with pre-warmed medium containing 500 µM of modified sugar (SiaNAl or ManNAl). The labeling was stopped at the different time points mentioned by fixing the cells with 4% paraformaldehyde (PAF). Cells were then permeabilized in 0.5% Triton X-100 for 10 min. Permeabilized cells were then incubated with 100 µL/coverslip of a freshly prepared click solution (K_2HPO_4, 100 mM ; Sodium ascorbate, 2.5 mM ; $CuSO_4$, 150 µM ; BTTAA, 300 µM, AzidoFluor 545, 10 µM). CuAAC was performed during 45 min, in the dark, at room temperature with gentle shaking. After 2h saturation in blocking buffer (0.2 % gelatin, 1% BSA and 2% normal goat serum (Invitrogen) in PBS), fixed cells were incubated at room temperature for 1h with primary antibodies (Molecular Probes) diluted at 1/100 in blocking buffer. Finally, cells were incubated at room temperature for 1h with Alexa 488-, Alexa 568-, and Alexa 700-conjugated secondary antibodies (Molecular Probes) diluted at 1/600 in blocking buffer.

Imaging

Immunostaining and fluorescent proteins were detected through an inverted Leica TCS-SP_5 confocal microscope. Pictures were taken by using Leica Application suite Advanced Fluorescence (LAS AF) software (Leica Microsystems Wetzlar, Germany). For comparison purposes, each picture has been taken under the same settings. For quantification, we used the Leica TCS-SP_5 intensity plotting tool that provides relative fluorescence intensities in different collection channels over a region of interest (ROI). Plot of fluorescence intensity in a ROI corresponding to the Golgi region has been performed for each cell. For image analysis, three different field of two independent experiments were examined. Around 100 cells have been quantified. The LAS AF pictures were then exported in TIFF format and processed with Adobe Photoshop 7.0.

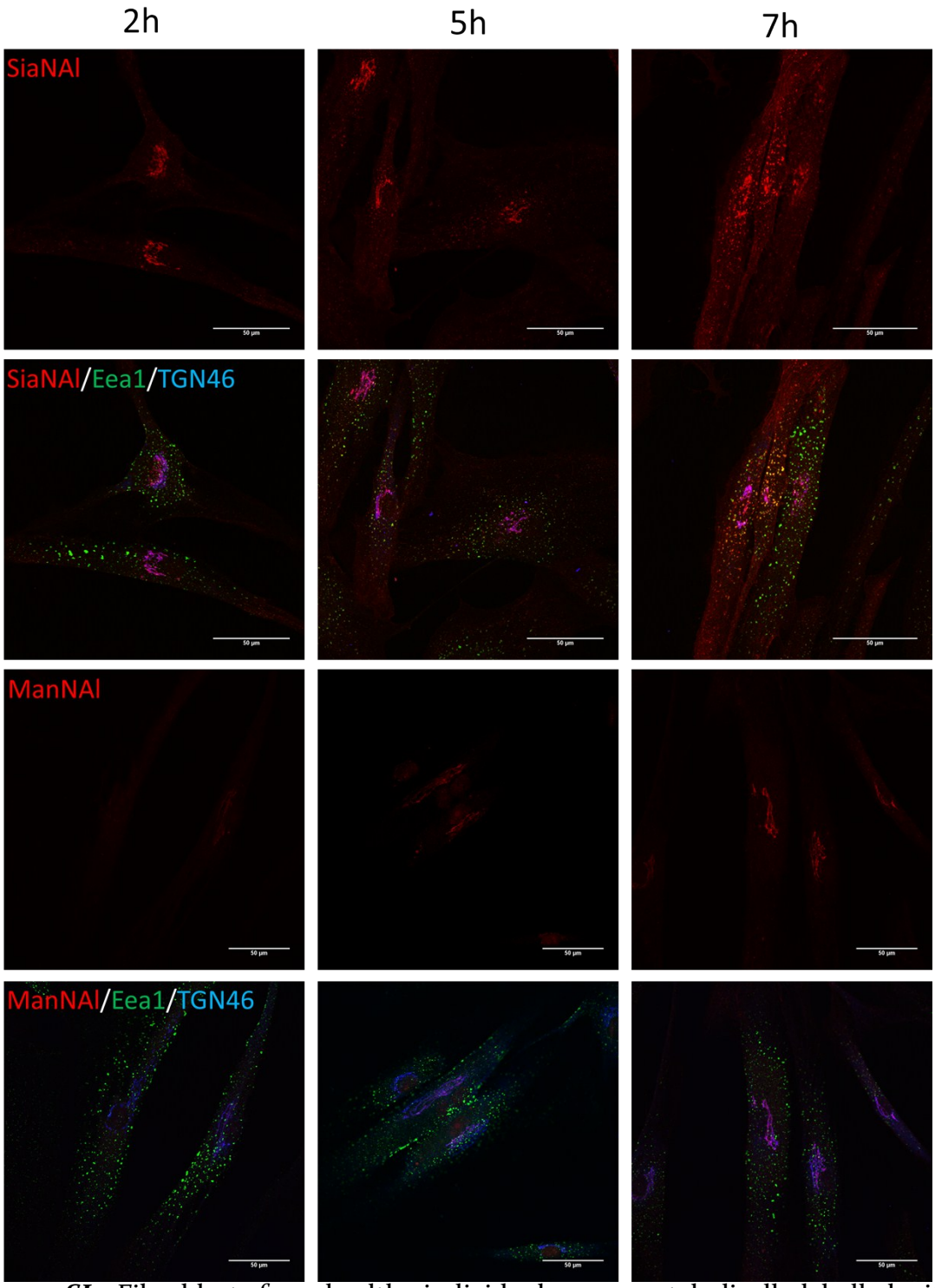


Figure SI1. Fibroblasts from healthy individuals were metabolically labelled with 500 µM of SiaNAl or ManNAl for 3, 6 and 8h respectively and stained with AzidoFluor 545 fluorescent probe (sialic acid into glycoconjugates in red) or antibodies against a late Golgi marker (TGN46 in blue) and an early endosomal marker (EEA1 in green). Staining was then visualized using confocal microscopy.

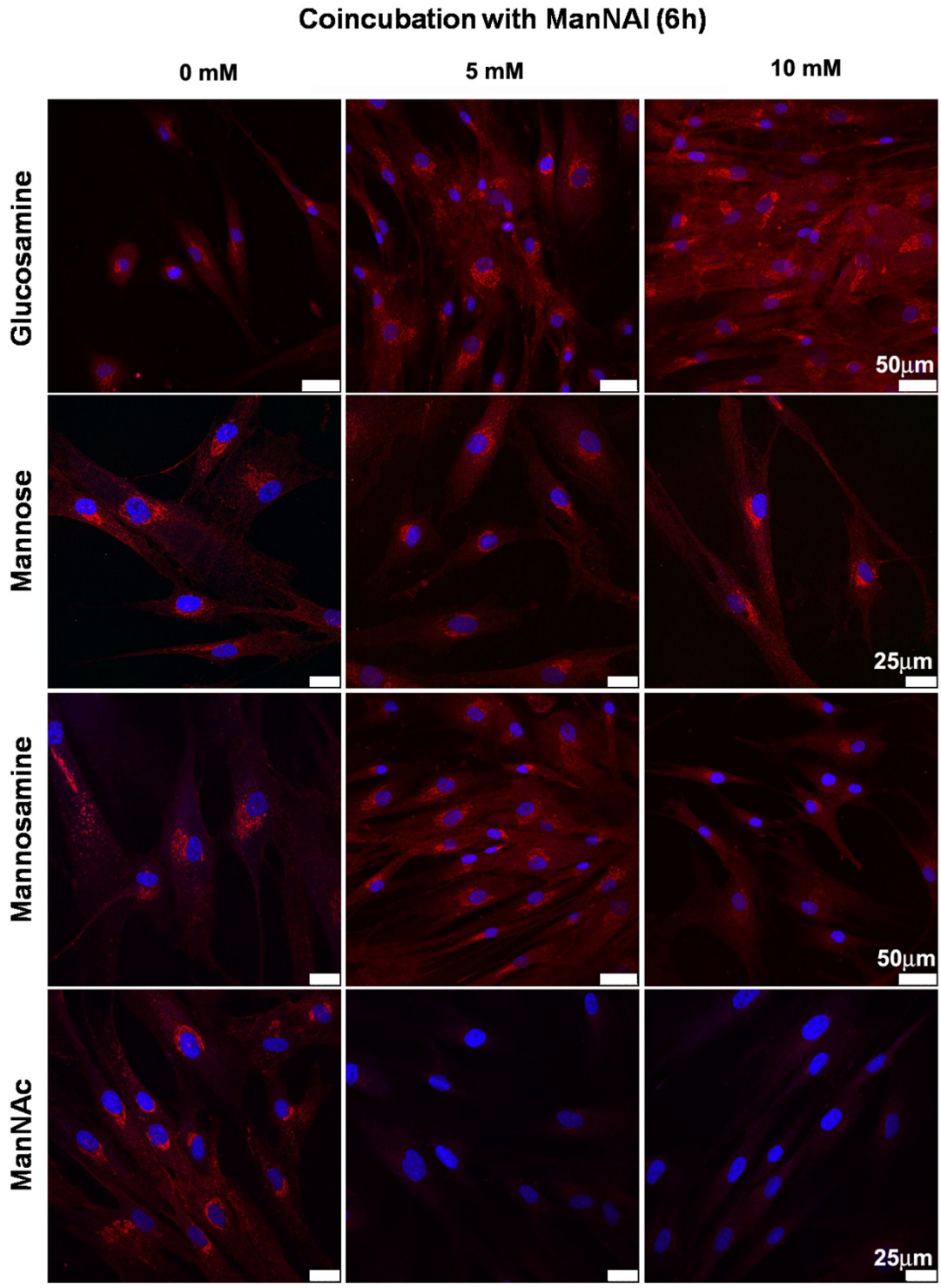


Figure SI2. Fibroblasts from healthy individuals were metabolically labeled for 6h with 500 µM of ManNAl in absence (0mM) or in presence of 5mM or 10mM of glucosamine, mannose, mannosamine and N-acetylmannosamine respectively. Cells were stained with AzidoFluor 545 fluorescent probe (sialic acid into glycoconjugates in red) and visualized by confocal microscopy.

Quantification of sialic acids

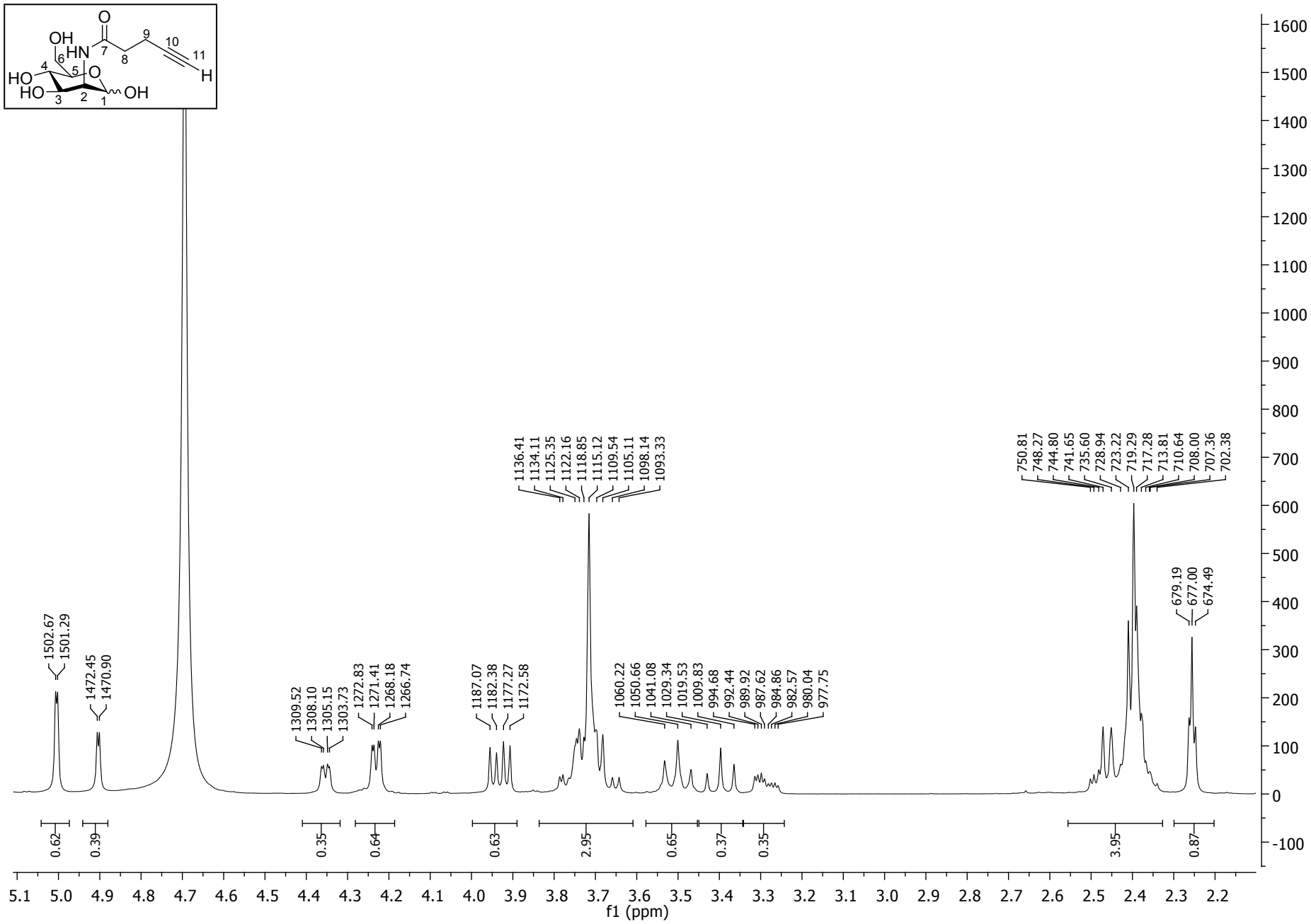
DMB derivatization[5]

Primary skin fibroblasts and sialin deficient cells were grown into T75 Flask. At confluence, cells were labelled with SiaNAl during 8h as previously described. Both primary skin and sialin deficient cells were also incubated without SiaNAl as negative controls. After incubation, all cells were scrapped with a rubber policeman in PBS. After being washed, cells were centrifuged at 3000 rpm and supernatant was removed. Cells were lysed with 100 µL ultrapure water and then centrifuged at 3000 rpm for 10 min. In supernatant were found soluble glycoproteins and free saccharides whereas in the pellet were contained membrane glycoproteins and glycolipids. Supernatant was removed, added to 900 µL of ethanol and kept overnight at -20°C in order to precipitate glycoproteins. After another centrifugation at 10000 rpm for 10 minutes, supernatant was removed and the pellet was pooled with the glycoproteins and glycolipids previously extracted. Then, this fraction was submitted to hydrolysis by addition of 100 µL of trifluoroacetic acid (TFA) 0.1 M during 2 h at 80 °C. All samples were then dried into a vacuum concentrator (Concentrator 5301, Eppendorf).

1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB) reaction solution (β-mercapto-ethanol, 1M ; $Na_2S_2O_4$, 0.018M, TFA 0.02 M and DMB, 7 mM in water) was prepared. Dried samples were treated with 50 µL of DMB reaction solution and 50 µL of TFA 0.01 M and then incubated for 2 h in the dark. Samples were then diluted before being analyzed by HPLC.

HPLC

Analysis were performed on a Varian Prostar HPLC. 20 µL of sample was injected each time onto a Shodex C18 M4-E column (250 x 4.6 mm). The column was heated at 50°C during elution. Mobile was composed of two elution buffer: H_2O / Acetonitrile / MeOH (84 : 9 : 7) (A) and Acetonitrile / MeOH (1 : 1) (B). Elution method was the following: 100% A until 20 min, 100% A to 50% A until 28 minutes, 50% A during 5 min and then from 50% to 95% A in 1 min. A step at 95% A for 5 min and then back to 100% of A during 30 minutes for column equilibration. Derivatized sugars were detected by a ProStar Varian fluorescent detector (λ_{ex}: 372 nm / λ_{em}: 456 nm).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1266.74
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1182.38
1177.27
1172.58
1136.41
1134.11
1125.35
1122.16
1118.85
1115.12
1109.54
1105.11
1098.14
1093.33
1060.22
1050.66
1041.08
1029.34
1019.53
1009.83
994.68
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984.86
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980.04
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702.38
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677.00
674.49
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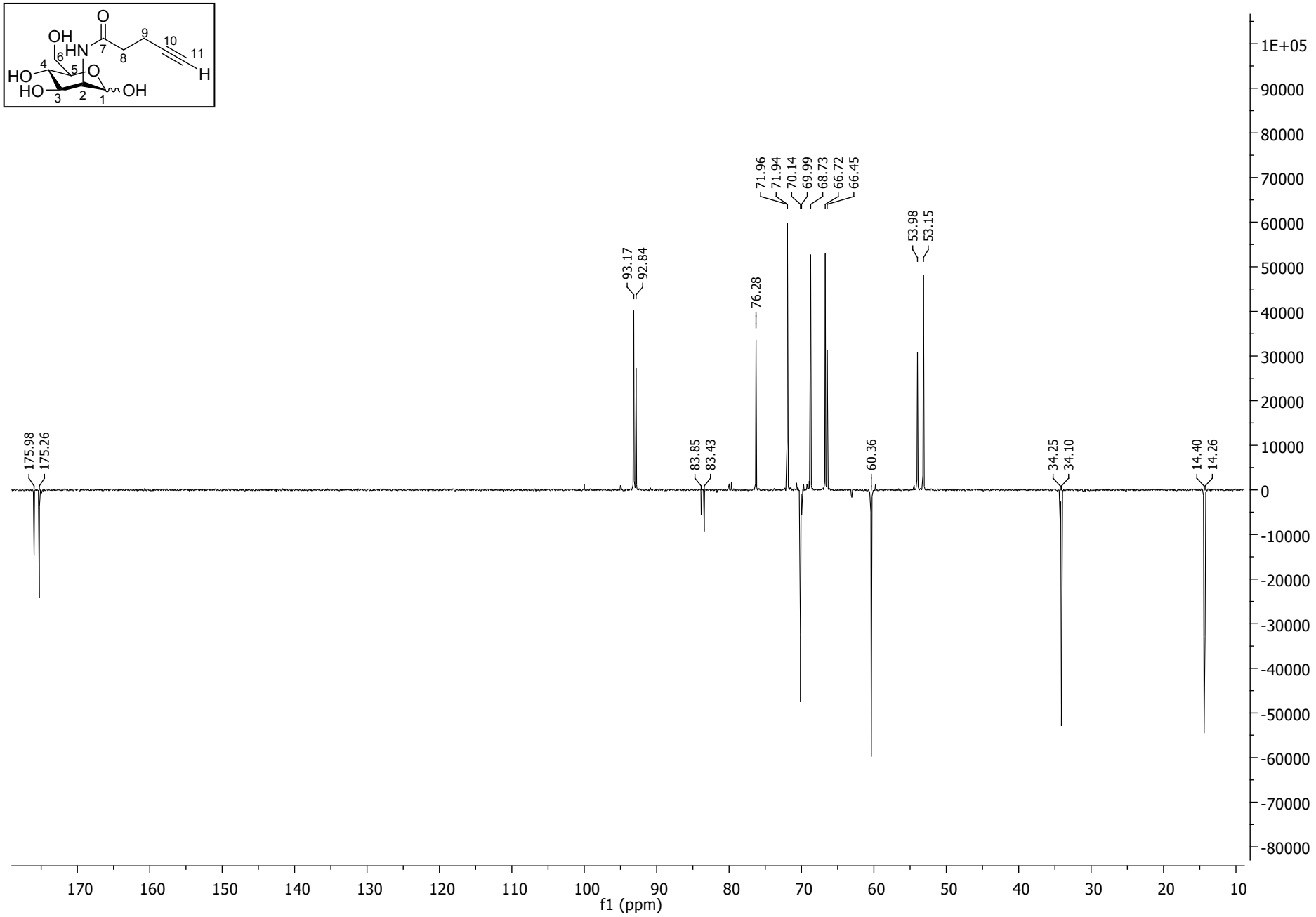
f1 (ppm)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71.94
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34.25
34.10
14.40
14.26
1E+05
90000
80000
70000
60000
50000
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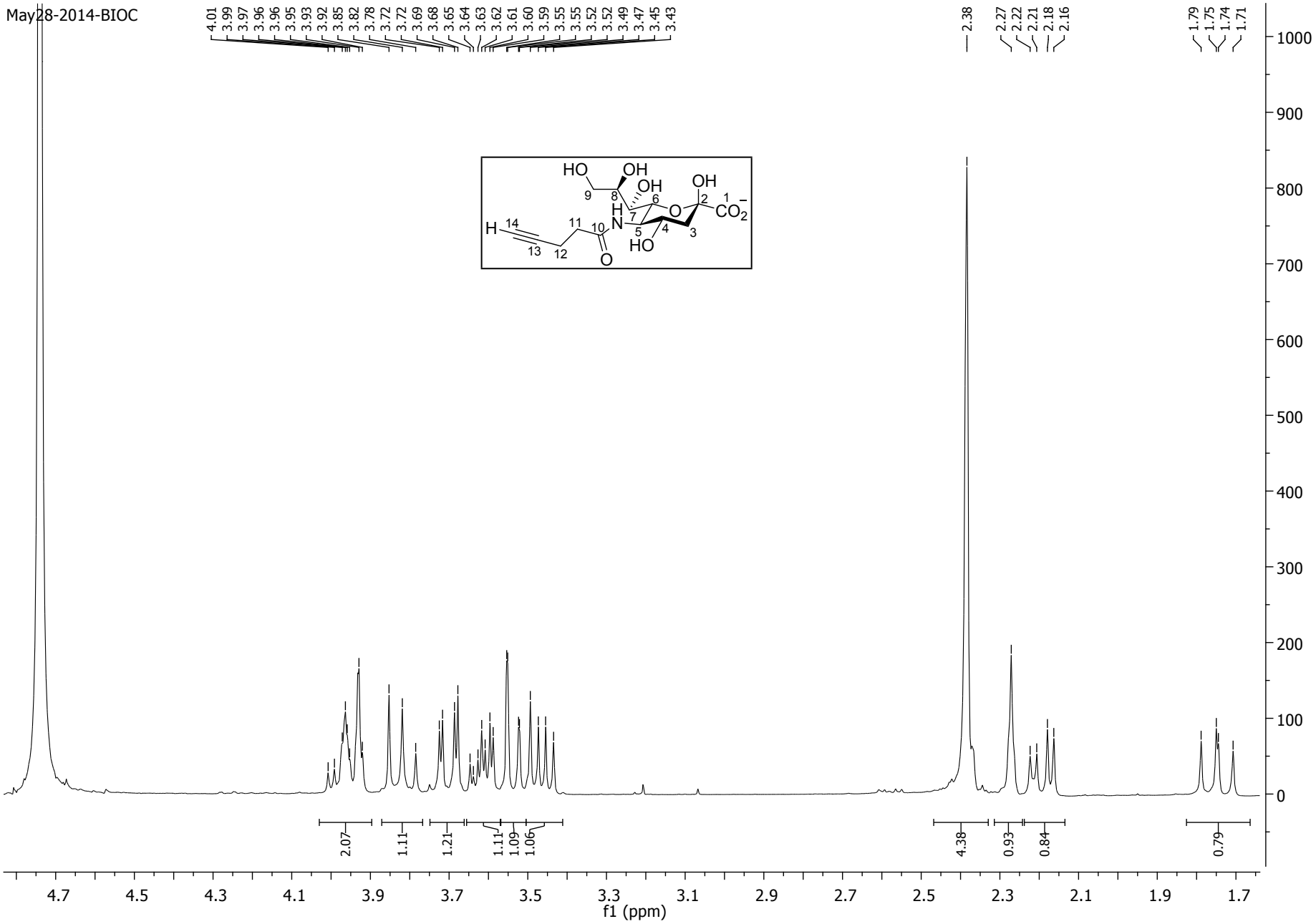
f1 (ppm)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3.64
3.63
3.62
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3.55
3.52
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3.49
3.47
3.45
3.43
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10
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4
3
H
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12
HO
O
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1.11
1.09
1.06
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0.84
0.79
4.7
4.5
4.3
4.1
3.9
3.7
3.5
3.3
3.1
2.9
2.7
2.5
2.3
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1.9
1.7
f1 (ppm)
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700
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400
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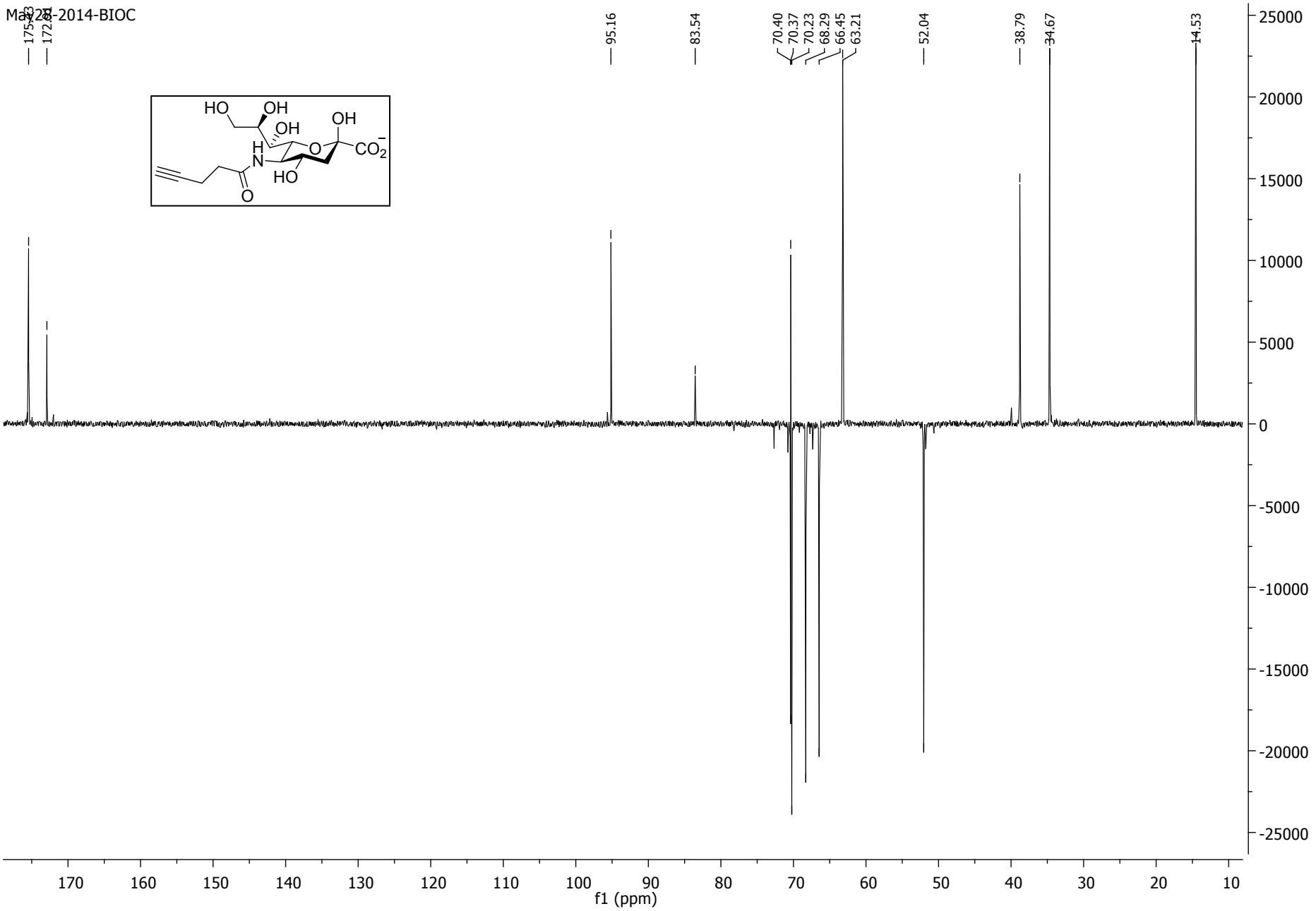
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34.67
14.53
HO
OH
OH
OH
H
N
O
CO2
HO
O
25000
20000
15000
10000
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-15000
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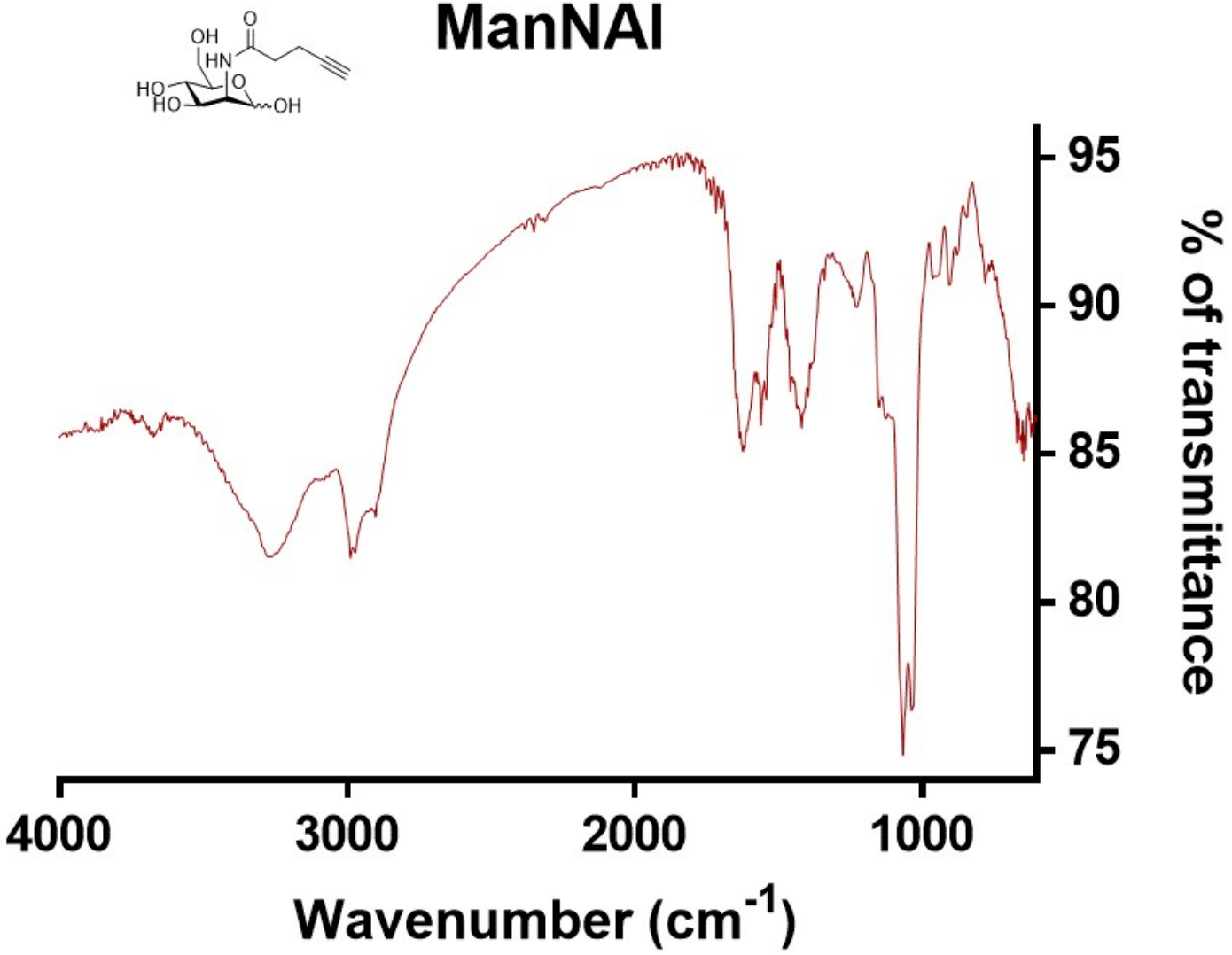
f1 (ppm)


ManNAl
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HN
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HO
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95
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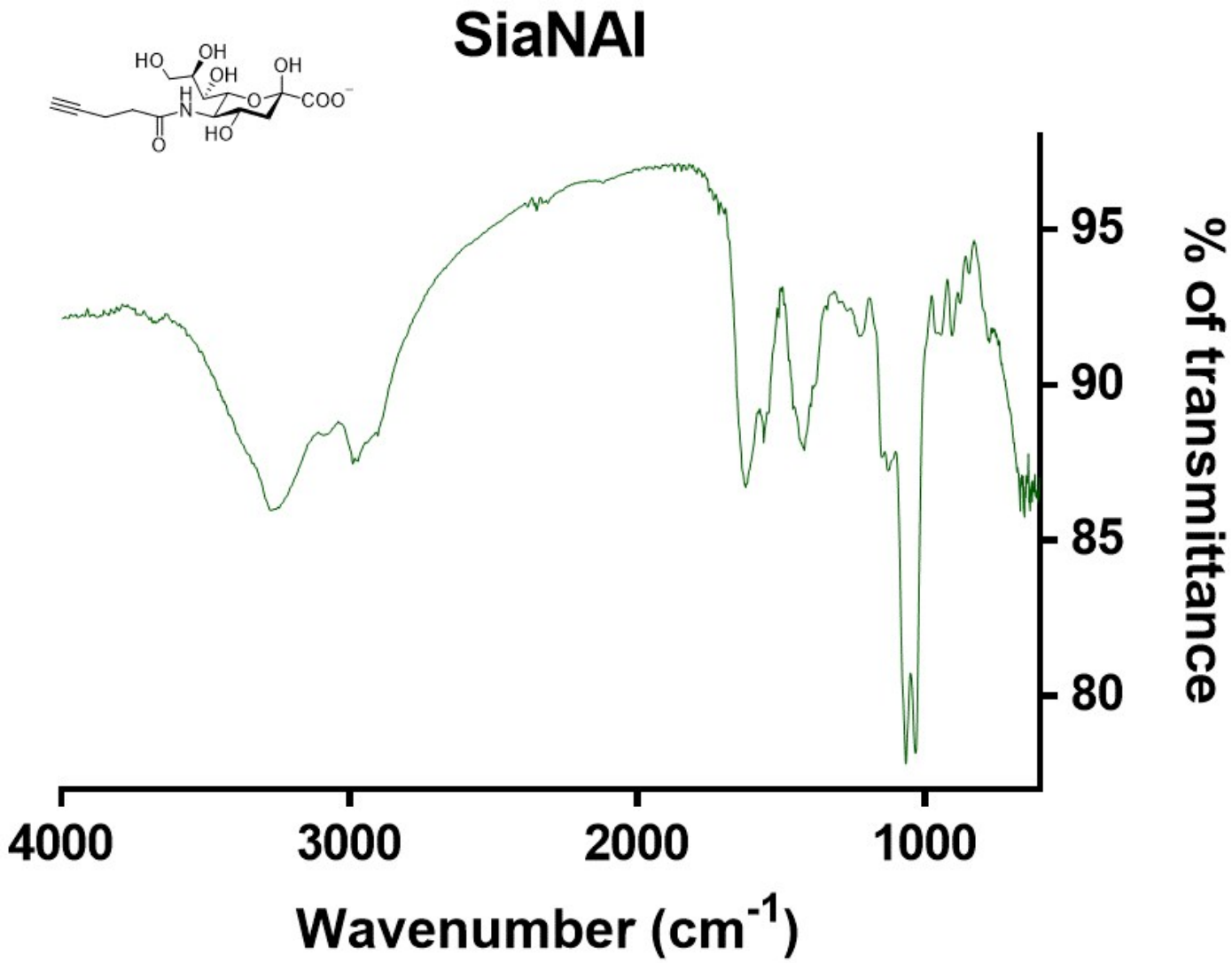
85
80
75
% of transmittance
4000
3000
2000
1000
Wavenumber (cm-1)


SiaNAl
HO
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OH
OH
H
N
O
COO⁻
HO
O
95
90
85
80
% of transmittance
4000
3000
2000
1000
Wavenumber (cm-1)

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