Supplementary data for

Modified GM3 gangliosides produced by metabolic oligosaccharide engineering

Authors: Chad M. Whitman, Fan Yang, and Jennifer J. Kohler

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General

Chemical

All chemicals were used as received from commercial suppliers without further purification. 1-hydroxybenzotriazole hydrate was purchased from AnaSpec. *N*-butyric anhydride, phenylacetic acid and propionic anhydride were purchased from TCI America. All other chemicals were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise noted. Reaction progress was monitored by analytical thin layer chromatography (TLC) on silica gel 60 F_{254} glass backed plates (Fisher) and stained with ceric ammonium molybdate or bromocresol green. Flash column chromatography was carried out with silica gel 60 (particle size 40-63 μm, EMD Chemicals). All ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian 500 MHz spectrometer and are reported in δ ppm scale. ^{1}H-NMR spectra were referenced to D_2O (4.80 ppm) or $CDCl_3$ (7.26 ppm). ^{13}C-NMR spectra were referenced to $CDCl_3$ (77.23 ppm). ESI-MS data were collected at the UT Southwestern Medical Center Protein Chemistry Technology Center. All acetylated sugars were prepared as 10 mM stock solutions in ethanol. The purity of acetylated sugars was confirmed by HPLC analysis.

Cell culture and flow cytometry

RPMI 1640 with 2 mM L-glutamine, α-minimum Eagle's medium with glutamine, ribonucleosides, and deoxyribonucleosides, Opti-MEM, fetal calf serum, penicillin/streptomycin, dPBS, PBS (pH = 7.4), FITC-streptavidin, aminooxy-biotin, and propidium iodide were purchased from Invitrogen. Nutridoma SP and BSA Fraction V were purchased from Roche Applied Science. Aniline was purchased from Sigma-Aldrich. Glycerol was purchased from Fisher Scientific. SNA-FITC was purchased from EY Labs. MAA-biotin (B-1265) was purchased from Vector Labs. DTAF-streptavidin was purchased from Jackson Immunoresearch. Cell counting was performed on the Invitrogen Countess Automated Cell Counter. Flow cytometry experiments were performed on a BD Biosciences FACSCaliber flow cytometer

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Synthesis of *N*-acetylmannosamine (ManNAc) analogs

Synthesis of Ac_4ManNAc

Ac_4ManNAc was synthesized as described.[1] Briefly, to a solution of *N*-acetylmannosamine (301.7 mg, 1.36 mmol) in pyridine (16.4 mL, 204 mmol), acetic anhydride (4.72 mL, 54 mmol) was added and stirred overnight on ice. The reaction mixture was diluted by CH_2Cl_2 and washed successively by 1.0 M HCl, saturated sodium bicarbonate, and brine. The organic layer was dried over magnesium sulfate and evaporated *in vacuo*. The residue was purified by flash chromatography (hexanes:ethyl acetate gradient = 5:1, 3:1, 1:1) to afford Ac_4ManNAc (282 mg, 53%, mixture of anomers). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.65 (3H, s), 2.02 (3H, s), 2.07 (3H, s), 2.11 (3H, s), 2.18 (3H, s), 4.10 (1H, dd, J = 2.3, 12.5 Hz), 4.28 (1H, t, J = 3.7 Hz), 4.78 (1H, ddd, J = 1.6, 3.9, 9.1 Hz), 5.06 (1H, d, 4.0), 5.13 (1H, t, J = 9.8 Hz), 5.33 (1H, d, J = 4.5 Hz), 5.79 (1H, d, J = 9.0 Hz), 5.86 (1H, d, J = 1.6 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 20.88, 20.90, 20.92, 20.96, 20.97, 21.01, 21.08, 23.56, 23.65, 49.52, 49.74, 62.20, 65.41, 65.62, 68.99, 70.29, 71.56, 73.68, 90.86, 91.90, 168.34, 168.55, 169.92, 169.93, 170.23, 170.32, 170.73, 170.74, 170.82. ESI-MS for $C_{16}H_{23}NO_{10}$ [M], calculated for 389.13, found 389.12. ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra and evidence of purity (HPLC) are presented on pages 11-14.

Synthesis of Ac_5ManNGc

Monoacetylated ManNGc was synthesized as previously reported.[2] Briefly, to a solution of mannosamine hydrochloride (216 mg, 1.00 mmol) and sodium bicarbonate (1.68 g, 20 mmol) in water (8.6 mL, 480 mmol) chilled on ice, acetoxyacetyl chloride (537 μL, 5.00 mmol) was added dropwise and the reaction was stirred for 3 hours on ice, monitoring reaction progress by TLC (ethyl acetate:acetic acid:water = 3:2:1) using ninhydrin to detect unreacted starting material and orcinol-sulfuric acid to detect sugars. After filtering through Celite in a Pasteur pipette, the filtrate was neutralized with 1.0 M HCl (pH ~ 7, added dropwise). The resulting mixture was concentrated *in vacuo*, roughly purified by flash chromatography (ethyl acetate:isopropanol:water = 27:8:4), and used directly to synthesize the fully acetylated product, Ac_5ManNGc. Acetylation of monoacetylated ManNGc was performed by the same procedure described in the synthesis of Ac_4ManNAc and afforded Ac_5ManNGc (159 mg, 36%, mixture of anomers). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.97 (3H, s), 2.03 (3H, s), 2.07 (3H, s), 2.15 (3H, s), 2.18 (3H, s), 4.03 (1H, dd, J = 1.9, 12.6 Hz), 4.23 (1H, d, J = 4.7 Hz), 4.58 (2H, s), 4.65 (1H, ddd, J = 1.9, 4.4, 9.0 Hz), 5.04 (1H, d, J = 3.8 Hz), 5.14 (1H, t, J = 10.3 Hz), 5.30 (1H, d, J = 4.3 Hz), 6.01 (1H, d, J = 1.3 Hz), 6.37 (1H, d, J = 9.2 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 20.84, 20.86, 20.87, 20.87, 20.89, 20.91, 20.96, 21.06, 49.16, 49.61, 61.96, 62.04, 63.25, 63.30, 65.15, 65.26, 68.99, 70.31, 71.40, 73.63, 90.62, 91.63, 167.41, 168.00, 168.32, 168.53, 169.53, 169.58, 169.75, 169.79, 170.29, 170.32, 170.61, 170.62. ESI-MS for $C_{18}H_{25}NO_{12}$ [M], calculated for 447.14, found 447.15. ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra and evidence of purity (HPLC) are presented on pages 15-18.

Synthesis of Ac_4ManNProp

ManNProp was synthesized as previously reported.[3] Briefly, to a solution of mannosamine hydrochloride (300 mg, 1.39 mmol) in MeOH (20 mL) and 3.0 M NaOH (0.5 mL), propionic anhydride (1.0 mL, 7.80 mmol) was added dropwise on ice while stirring for several hours, monitoring reaction progress by TLC. After completion, 1.0 M HCl was added dropwise to neutralize the solution (pH ~ 7). After the solvent was evaporated *in vacuo*, dried with several washes of toluene, filtered with cotton in Pasteur pipette, and evaporated under vacuum. The resulting mixture was roughly purified by flash chromatography (CH_2Cl_2:MeOH = 1:0, 10:1, 4:1) and used directly to synthesize the acetylated product, Ac_4ManNProp. Acetylation of ManNProp was performed by the same procedure described in the synthesis of Ac_4ManNAc, affording Ac_4ManNDAz (284 mg, 51%, mixture of anomers). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.19 (3H, t, J = 7.9 Hz), 2.00 (3H, s), 2.07 (3H, s), 2.11 (3H, s), 2.19 (3H, s), 2.34 (2H, m), 4.06 (1H, dd, J = 1.4, 12.0 Hz), 4.28 (1H, t, J = 4.8 Hz), 4.80 (1H, ddd, J = 1.6, 4.4, 9.2 Hz), 5.06 (1H, d, J = 4.0 Hz), 5.18 (1H, t, J = 10.3 Hz), 5.22 (1H, d, J = 4.5 Hz), 5.65 (1H, d, J = 9.3 Hz), 6.04 (1H, d, J = 4.8 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 9.92, 10.11, 20.88, 20.90, 20.92, 20.94, 20.95, 20.99, 21.09, 29.84, 29.99, 49.28, 49.54, 53.64, 54.65, 62.07, 62.19, 65.40, 65.58, 69.06, 70.26, 71.56, 73.62, 90.88, 91.93, 168.36, 168.53, 169.90, 170.22, 170.29, 170.72, 173.94, 174.59. ESI-MS for $C_{17}H_{25}NO_{10}$ $[M-H]^-$, calculated for 402.15, found 402.15. ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra and evidence of purity (HPLC) are presented on pages 19-21.

Synthesis of Ac_4ManNBut

ManNBut was synthesized as previously reported.[3] Briefly, to a solution of mannosamine hydrochloride (300 mg, 1.39 mmol) in MeOH (5.0 mL) and 3.0 M NaOH (0.5 mL), butyric anhydride (1.0 mL, 6.13 mmol) was added dropwise on ice while stirring for several hours, monitoring reaction progress by TLC. After completion, 1.0 M HCl was added dropwise to

neutralize the solution (pH ~ 7). After the solvent was evaporated *in vacuo*, dried with several washes of toluene, filtered with cotton in Pasteur pipette, and evaporated under vacuum. The resulting mixture was roughly purified by flash chromatography (CH_2Cl_2:MeOH = 1:0, 10:1, 4:1) and used directly to synthesize the acetylated product, Ac_4ManNBut. The acetylated of ManNBut was performed by the same procedure described in the synthesis of Ac_4ManNAc, affording Ac_4ManNBut (266 mg, 45%, mixture of anomers). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.00 (3H, t, J = 7.3 Hz), 1.71 (2H, m), 2.01 (3H, s), 2.07 (3H, s), 2.11 (3H, s), 2.19 (3H, s), 2.25 (2H, t, J = 7.5 Hz), 4.06 (1H, dd, J = 4.9 Hz), 4.28 (1H, t, J = 4.8 Hz), 4.68 (1H, ddd, J = 4.6, 9.5, 12.6 Hz), 5.06 (1H, d, J = 4.0 Hz), 5.18 (1H, t, J = 10.3 Hz), 5.33 (1H, d, J = 4.4 Hz), 5.65 (1H, d, J = 9.4 Hz), 6.04 (1H, s). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 13.72, 13.82, 19.3, 19.49, 20.88, 20.90, 20.93, 20.95, 21.10, 38.73, 38.90, 49.24, 49.56, 62.06, 62.89, 65.37, 65.55, 69.09, 70.29, 71.60, 73.65, 90.84, 91.94, 168.37, 168.51, 169.88, 170.22, 170.72, 173.16, 173.80. ESI-MS for $C_{18}H_{27}NO_{10}$ $[M-H]^-$, calculated for 416.16, found 416.17. ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra and evidence of purity (HPLC) are presented on pages 22-26.

Synthesis of Ac_4ManNPhAc

To a solution of phenylacetic acid (136 mg, 1.00 mmol), mannosamine hydrochloride (216 mg, 1.00 mmol) and triethylamine (280 μL, 2.00 mmol) in MeOH (10 mL), 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (388 mg, 2.00 mmol) was added. The reaction mixture was stirred on ice for 10 min, followed by stirring at room temperature overnight. The resulting mixture was concentrated *in vacuo*, roughly purified by flash chromatography (CH_2Cl_2:MeOH = 1:0, 10:1, 4:1), and used directly to synthesize the acetylated product, Ac_4ManNPhAc. Acetylation of ManNPhAc was performed by the same procedure described in the synthesis of Ac_4ManNAc, affording Ac_4ManNPhAc (117 mg, 25%, mixture of anomers). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.94 (3H, s), 2.01 (3H, s), 2.04 (3H, s), 2.16 (3H, s), 3.99 (1H, ddd, J = 2.2, 6.0, 13.0 Hz), 4.06 (1H, dd, J = 2.7, 9.7 Hz), 4.66 (1H, ddd, J = 4.3, 9.4, 13.7 Hz), 4.97 (2H, s), 4.99 (1H, t, J = 5.6 Hz), 5.02 (1H, d, J = 3.7 Hz), 5.28 (1H, d, J = 4.4 Hz), 5.63 (1H, t, J = 9.0 Hz), 5.96 (1H, d, J = 1.6 Hz), 7.34 (3H, m), 7.42 (2H, m). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 20.81, 20.83, 20.84, 20.86, 20.89, 20.92, 21.04, 43.80, 43.99, 49.43, 49.46, 61.94, 61.96, 65.19, 65.24, 69.21, 70.16, 71.35, 73.37, 90.58, 91.71, 127.63, 127.78, 129.22, 129.37, 129.41, 129.49, 134.48, 168.33, 168.36, 169.65, 169.70, 170.20, 170.24, 170.68, 170.70, 171.35. ESI-MS for $C_{22}H_{27}NO_{10}$ [M], calculated for 465.16, found 465.16. ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra and evidence of purity (HPLC) are presented on pages 27-30.

Synthesis of Ac_4ManNAz

ManNAz was synthesized as previously reported.[4] Briefly, to a solution of azidoacetic acid (360 mg, 3.00 mmol), mannosamine hydrochloride (432 mg, 2.00 mmol) and triethylamine (560 μL, 4.00 mmol) in MeOH (20 mL), 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (766 mg, 4.00 mmol) and 1-hydroxybenzotriazole hydrate (270 mg, 2.00 mmol) were added. The reaction mixture was stirred on ice for 10 min, followed by stirring at room temperature overnight. The resulting mixture was concentrated *in vacuo*, roughly purified by flash chromatography (CH_2Cl_2:MeOH = 1:0, 5:1, 3:1), and used directly to synthesize the acetylated product, Ac_4ManNAz. Acetylation of ManNAz was performed by the same procedure described in the synthesis of Ac_4ManNAc, affording Ac_4ManNAz (437 mg, 50%, mixture of anomers). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.96 (3H, s), 2.03 (3H, s), 2.08 (3H, s), 2.15 (3H, s), 4.02 (2H, s), 4.09 (1H, ddd, J = 2.0, 8.0, 14.4 Hz), 4.20 (1H, d, J = 1.8 Hz), 4.58 (1H, ddd, J = 4.2, 9.3, 13.5

Hz), 5.04 (1h, d, J = 3.9 Hz), 5.19 (1H, t, J = 10.1 Hz), 5.20 (1H, d, J = 4.3 Hz), 6.01 (1H, s), 6.65 (1H, d, J = 18.1 Hz). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 20.80, 20.84, 20.87, 20.91, 20.95, 20.98, 21.06, 49.44, 49.90, 52.55, 52.74, 61.84, 61.93, 65.08, 65.27, 69.02, 70.41, 71.62, 73.55, 90.42, 91.47, 166.94, 167.52, 168.30, 168.54, 169.76, 170.30, 170.35, 170.73. ESI-MS for $C_{16}H_{22}N_4O_{10}$ [M], calculated for 430.13, found 430.14. ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra and evidence of purity (HPLC) are presented on pages 31-34.

Synthesis of Ac_4ManNDAz

ManNDAz was synthesized as previously reported.[5] Briefly, to a solution of 4,4-azo-pentanoic acid[6] (128 mg, 1.00 mmol), mannosamine hydrochloride (216 mg, 1.00 mmol) and triethylamine (278 μL, 2.00 mmol) in MeOH (10 mL), 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (383 mg, 2.00 mmol) and 1-hydroxybenzotriazole hydrate (135 mg, 1.00 mmol) were added. The reaction mixture was stirred on ice for 10 min, followed by stirring at room temperature overnight. The resulting mixture was concentrated *in vacuo*, roughly purified by flash chromatography (CH_2Cl_2:MeOH gradient = 1:0, 10:1, 4:1), and used directly to synthesize the acetylated product, Ac_4ManNDAz. Acetylation of ManNDAz was performed by the same procedure described in the synthesis of Ac_4ManNAc, affording Ac_4ManNDAz (84 mg, 28% over two steps, mixture of anomers). ^{1}H-NMR (500 MHz, $CDCl_3$): δ 1.06 (3H, s), 1.81 (2H, m), 2.02 (3H, s), 2.07 (3H, s), 2.12 (3H, s), 2.19 (3H, s), 4.05 (2H, s), 4.09 (1H, ddd, J = 6.2, 18, 30.5 Hz), 4.29 (1H, t, J = 4.5 Hz), 4.78 (1H, dd, J = 2.3, 8.2 Hz), 5.06 (1H, d, J = 4.0 Hz), 5.20 (1H, t, J = 9.8 Hz), 5.32 (1H, d, J = 4.4 Hz), 5.80 (1H, d, J = 9.0 Hz), 6.04 (1H, s). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 20.18, 20.19, 20.85, 20.88, 20.91, 20.95, 20.97, 21.08, 25.50 25.54, 29.94, 30.05, 30.71, 30.84, 49.55, 49.76, 62.02, 62.15, 65.32, 69.06, 70.32, 71.57, 73.67, 90.80, 91.79, 168.33, 168.53, 169.82, 169.91, 170.20, 170.28, 170.75, 170.78, 171.62, 172.14. ESI-MS for $C_{19}H_{27}N_3O_{10}$ [M], calculated for 457.17, found 457.16. ^{1}H-NMR, ^{13}C-NMR, and ESI-MS spectra and evidence of purity (HPLC) are presented on pages 35-38.

Cell culture

General

BJAB K20 and K88 cells were cultured in RPMI 1640 with 2 mM L-glutamine containing 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C, 5% CO_2 in a water-saturated environment. Cells were diluted to 2.5 x 10^5 cells/mL and passaged after 48 hr. Typically, cell densities were maintained between 2.5 x 10^5 cells/mL and 2.0 x 10^6 cells/mL. Cell viability was analyzed using Trypan blue dye staining with the Countess Automated Cell Counter instrument.

wt CHO and CHO *Lec3* cells were cultured maintained in α-minimum Eagle's medium with glutamine, ribonucleosides, and deoxyribonucleosides containing 10% fetal calf serum at 37 °C, 5% CO_2 in a water-saturated environment. Cells were diluted to 2.5 x 10^4 cells/mL and passaged after 72 hr. Typically, cell densities were maintained between 2.5 x 10^4 cells/mL and 2.0 x 10^6 cells/mL.

Addition of ManNAc analogs

Initially, CHO and CHO *Lec3* cells were diluted to 5.0 x 10^4 cells/ml and cultured for 24 hr. Cells were washed 3 times with PBS, then cultured with OptiMEM media for serum-free exposure. Using 10 mM ethanol stocks of each sugar, ethanol or acetylated sugar was added to

each plate while swirling to achieve a final concentration of 100 μM. After culturing cells with the appropriate monosaccharide for 48 hr, cells were removed from the plate by exposure to trypsin for 5 minutes, counted, and harvested as described for each experiment.

To achieve serum-free conditions for BJAB K20 and K88 cells, the cells were grown in RPMI 1640 with 2 mM L-glutamine containing 1X Nutridoma SP, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured for two passages at 2.5 x 10^5 cells/ml in media for 72 hours at a time before supplementation with monosaccharides. Prior to the addition of cells to a tissue culture plates, acetylated sugar or ethanol were added and the ethanol was pre-evaporated. After cell counting, BJAB cells were diluted to 2.5 x 10^5 cells/mL in serum-free media for each monosaccharide condition. After 72 hr, cells were counted and harvested.

Flow cytometry

Lectin binding

Cells were resuspended at 1.875 x 10^6 cells/mL in PBS and aliquoted into a v-bottom 96-well plate in 200 μL volumes. Analyses were performed in triplicate with three separate cultures prepared for each condition. Cells were washed 3 times with 200 μL of 0.1% BSA/PBS and centrifuged at 650*g*, 4 °C for 4 min. For MAA binding experiments, cells were incubated with 50 μL of 10 μg/ml of MAA-biotin in 0.1% BSA/PBS for 30 minutes on ice. After washing 3 times with 200 μL of 0.1% BSA/PBS, cells were incubated with 50 μL of 20 μg/ml of streptavidin-FITC for 30 min on ice. After washing 3 times with 200 μL of 0.1% BSA/PBS, cells were resuspended in 400 μL of 0.1% BSA/PBS. Propidium iodide, used to identify dead cells, was added at a final concentration of 50 μg/mL to each tube before analysis. The cells were analyzed using a FACSCaliber flow cytometer. Live cells (10,000 cells/sample) were identified by their forward scatter versus side scatter plot; all propidium iodide positive cells (dead cells) were excluded from analysis. FITC fluorescence was measured on the FL-1 channel of the instrument.

For SNA binding experiments, the cells were incubated with 10 μg/ml of SNA-FITC in 0.1% BSA/PBS for 30 min on ice. After washing 3 times with 0.1% BSA/PBS, cells were resuspended in 400 μL of 0.1% BSA/PBS. Propidium iodide, used to identify dead cells versus live cells, was added at 50 μg/mL into each tube before analysis. Flow cytometry analysis was performed as described for MAA.

Periodate oxidation and aniline-catalyzed oxime ligation (PAL)

Cells were resuspended at 1.0 x 10^6 cells/mL in PBS and aliquoted into a v-bottom 96-well plate in 200 μL amounts. Three separate cultures were prepared for each condition. Cells were washed 2 times with PBS (pH = 7.4) and centrifuged at 650*g*, 4 °C for 4 min. Cells were resuspended in 200 μL 1.0 mM $NaIO_4$/PBS (pH = 7.4) and incubated for 30 min on ice. After incubation, 50 μL of 5.0 mM glycerol/PBS (pH = 7.4) was added and incubated for 30 min on ice to quench the oxidation reaction. Cells were pelleted by centrifugation at 2,000 rpm, 4 °C for 4 min to remove the supernatant and resuspended with 200 μL of 5.0% FBS/PBS (pH = 6.7). Cells were washed 2 times with 5.0% FBS/PBS (pH = 6.7) and centrifuged at 650*g*, 4 °C for 4 min. After washing, cells were resuspended in 200 μL of 0.1 mM aminooxy-biotin, 10.0 mM aniline 5.0 % FBS/PBS (pH = 6.7) and incubated for 90 min on ice. After incubation, cells were

washed 3 times with 5.0% FBS/PBS (pH = 6.7) and centrifuged at 650*g*, 4 °C for 4 min. Cells were resuspended in 200 μL of 3.2 μg/mL DTAF-Streptavidin 5.0% FBS/PBS for 30 min on ice. Cells were washed 3 times with 5.0% FBS/PBS (pH = 6.7) and centrifuged at 650*g*, 4 °C for 4 min. After washing 3 times with 0.1% BSA/PBS, cells were resuspended in 400 μL of 5.0% FBS/PBS (pH = 6.7). Cells were analyzed using a FACSCaliber Flow Cytometer. Live cells (10,000 cells/sample) were identified by their forward scatter versus side scatter plot. DTAF fluorescence was measured on the FL-1 channel of the instrument.

Ganglioside extraction

Total lipid extraction

After counting, cells were harvested by centrifugation at 220*g* for 5 min in 50 mL conical tubes. To ensure that the results reflected incorporation levels, equal numbers of cells were analyzed for each experimental condition: total cells obtained ranged between 3.0-4.0 x 10^7 cells for CHO/CHO *Lec3* analysis and 1.0-1.2 x 10^8 cells for BJAB K88/K20 analysis. Cell pellets were stored at -80 °C overnight before proceeding to ganglioside extraction.

Cell pellets were thawed to room temperature, resuspended with 300 μL of ddH_2O (W), and dounced 50 times with a tissue grinder. With a glass Pasteur pipette and a 2 mL rubber bulb, the cell lysate suspension was transferred into a 4 mL glass vial containing 800 μL of methanol (M), already stirring. 400 μL of chloroform (C) was added to the vial and the mixture was stirred thoroughly for 2 hr at room temperature. Samples were covered in foil to prevent exposure to light. After stirring, the mixture was transferred by Pasteur pipette into a 13 x 100 mm glass culture tube and centrifuged at 2800*g* for 10 min at 30 °C. The supernatant (containing the total lipid extract) was transferred by Pasteur pipette to a new 4 mL glass vial. Solvent was removed by evaporation under N_2 gas.

Phospholipid extraction

The dried total lipid extract was resuspended with 800 μL butanol and 1200 μL diisopropyl ether and sonicated in a water bath for 10 min. The resuspended lipids were then transferred into a 13 x 100 mm glass culture tube. To extract undesired phospholipids from the mixture, 1 mL of 50 mM NaCl was added to the tube and mixed vigorously by pipetting up and down repeatedly with a glass Pasteur pipette. The mixture was then centrifuged at 2800*g* for 10 min at 30 °C to separate the two phases. Using a Pasteur pipette, the organic phase (top layer) was carefully removed. The mixture was then extracted two more times using the same ratio of butanol and diisopropyl ether.

SepPak purification

After the final extraction, the remaining lipid mixture was loaded onto a SepPak tC18 column, 0.3g size. The column was first pre-treated with three 2 mL washes of C/M/W (2:43:55) followed by two 2 mL washes of C/M (1:1) and ending with three more 2 mL washes of C/M/W (2:43:55). After loading the sample, the column was washed three times with 2 mL of C/M/W (2:43:55) followed by three 2 mL washes of C/M (1:1) to desalt the sample and remove unwanted contaminants. Elution of gangliosides was achieved using 2 mL 100% methanol. Ganglioside extracts were then transferred into a new 4 mL glass vial and dried under N_2.

Ganglioside analysis

HPTLC analysis

Extracted ganglioside samples were redissolved with 30 μL C/M/W (2:1:0.1) and resolved on HPTLC plates. Ganglioside standards (Matreya) were loaded in adjacent lanes to provide mobility references. Gangliosides were separated using chloroform:methanol:0.2% $CaCl_{2(aq)}$ (80:45:10) as the running buffer. HPTLC plates were first pre-run before loading 10 μL of ganglioside extract. After thoroughly drying the plate in a fume hood, gangliosides were detected by resorcinol staining (0.1% resorcinol, 0.04% $CuSO_4$ in hydrochloric acid:water [4:1]). Plates imaged using an Alpha Innotech FluorChem HD2. Contrast and brightness factors for an entire image were adjusted using Adobe Photoshop.

Mass Spectrometry

Dried ganglioside extracts were analyzed by the Complex Carbohydrate Research Center at the University of Georgia. To measure the intact masses of extracted gangliosides, MALDI-TOF-MS was performed. Samples were crystallized onto a MALDI plate with trihydroxyacetophenone monohydrate (THAP) as a matrix. Analysis of gangliosides was performed in the negative ion mode using a Bruker microflex instrument. Spectra for BJAB cell extracts (unsupplemented K88, K20 supplemented with Ac_4ManNAc, K20 supplemented with Ac_5ManNGc, K20 supplemented with Ac_4ManNAz, and K20 supplemented with Ac_4ManNDAz) are presented on pages 39-43. The data are also tabulated below, along with proposed structural assignments. Spectra for CHO cell extracts (unsupplemented wt, *Lec3* supplemented with Ac_4ManNAc, *Lec3* supplemented with Ac_5ManNGc, and *Lec3* supplemented with Ac_4ManNAz) are presented on pages 44-47. The data are also tabulated below, along with proposed structural assignments.

Tabulated mass spectrometry data

BJAB K88, unsupplemented

Observed Mass	Peak Identity	Ceramide composition
1153.9	GM3-NeuAc	d18:1/16:0
1238.2	GM3-NeuAc	d18:1/22:0
1264.3	GM3-NeuAc	d18:1/24:0
1519.9	GM1-NeuAc	d18:1/16:0
1630.2	GM1-NeuAc	d18:1/24:0

BJAB K20 + Ac_4ManNAc

Observed Mass	Peak Identity	Ceramide composition
1154.0	GM3-NeuAc	d18:1/16:0
1238.3	GM3-NeuAc	d18:1/22:0
1264.3	GM3-NeuAc	d18:1/24:0
1519.8	GM1-NeuAc	d18:1/16:0
1630.3	GM1-NeuAc	d18:1/24:0

BJAB K20 + Ac_5ManNGc

Observed Mass	Peak Identity	Ceramide composition
1170.1	GM3-NeuGc	d18:1/16:0
1254.4	GM3-NeuGc	d18:1/22:0

1280.4	GM3-NeuGc	d18:1/24:0
1536.0	GM1-NeuGc	d18:1/16:0
1646.4	GM1-NeuGc	d18:1/24:0

BJAB K20 + Ac_4ManNAz

Observed Mass	Peak Identity	Ceramide composition
1195.1	GM3-SiaNAz	d18:1/16:0
1223.2	GM3-SiaNAz	d18:1/18:0
1279.4	GM3-SiaNAz	d18:1/22:0
1305.5	GM3-SiaNAz	d18:1/24:0
1520.0	GM1-NeuAc	d18:1/16:0
1561.1	GM1-SiaNAz	d18:1/16:0
1671.4	GM1-SiaNAz	d18:1/24:0

BJAB K20 + Ac_4ManNDAz

Observed Mass	Peak Identity	Ceramide composition
1222.2	GM3-SiaNDAz	d18:1/16:0
1306.4	GM3-SiaNDAz	d18:1/22:0
1332.5	GM3-SiaNDAz	d18:1/24:0
1588.1	GM1-SiaNDAz	d18:1/16:0
1670.3	GM1-SiaNDAz	d18:1/22:0
1698.5	GM1-SiaNDAz	d18:1/24:0

CHO *Lec3*, no supplement

Observed Mass	Peak Identity	Ceramide composition
1153.9	GM3-NeuAc	d18:1/16:0
1266.3	GM3-NeuAc	d18:1/24:0

CHO *Lec3* + Ac_4ManNAc

Observed Mass	Peak Identity	Ceramide composition
1153.9	GM3-NeuAc	d18:1/16:0
1238.2	GM3-NeuAc	d18:1/22:0
1266.3	GM3-NeuAc	d18:1/24:0

CHO *Lec3* + Ac_5ManNGc

Observed Mass	Peak Identity	Ceramide composition
1170.0	GM3-NeuGc	d18:1/16:0
1254.3	GM3-NeuGc	d18:1/22:0
1282.4	GM3-NeuGc	d18:1/24:0

CHO *Lec3* + Ac_4ManNAz

Observed Mass	Peak Identity	Ceramide composition
1195.1	GM3-SiaNAz	d18:1/16:0
1307.5	GM3-SiaNAz	d18:1/24:0

References

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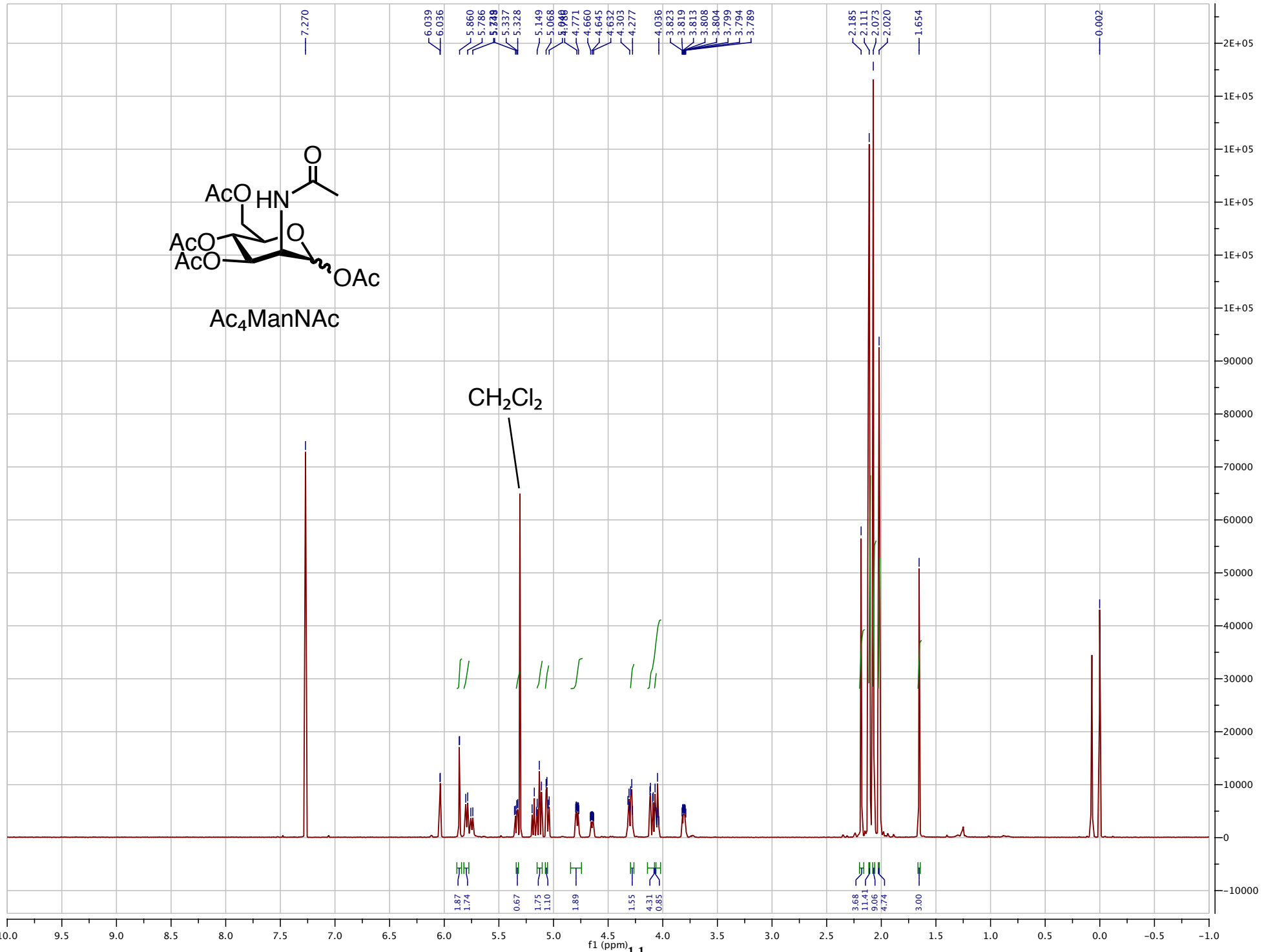
3. Pan, Y.; Ayani, T.; Nadas, J.; Wen, S.; Guo, Z., Accessibility of *N*-acyl-D-mannosamines to N-acetyl-D-neuraminic acid aldolase. *Carbohydr. Res.* 2004, *339* (12), 2091-100.

4. Luchansky, S. J.; Hang, H. C.; Saxon, E.; Grunwell, J. R.; Yu, C.; Dube, D. H.; Bertozzi, C. R., Constructing azide-labeled cell surfaces using polysaccharide biosynthetic pathways. *Methods Enzymol.* 2003, *362*, 249-72.

5. Tanaka, Y.; Kohler, J. J., Photoactivatable crosslinking sugars for capturing glycoprotein interactions. *J. Am. Chem. Soc.* 2008, *130* (11), 3278-9.

6. Church, R. F. R.; Weiss, M. J., Diazirines 2. Synthesis and properties of small functionalized diazirine molecules - Some observations on reaction of a diaziridine with iodine-iodide ion system. *J. Org. Chem.* 1970, *35* (8), 2465-.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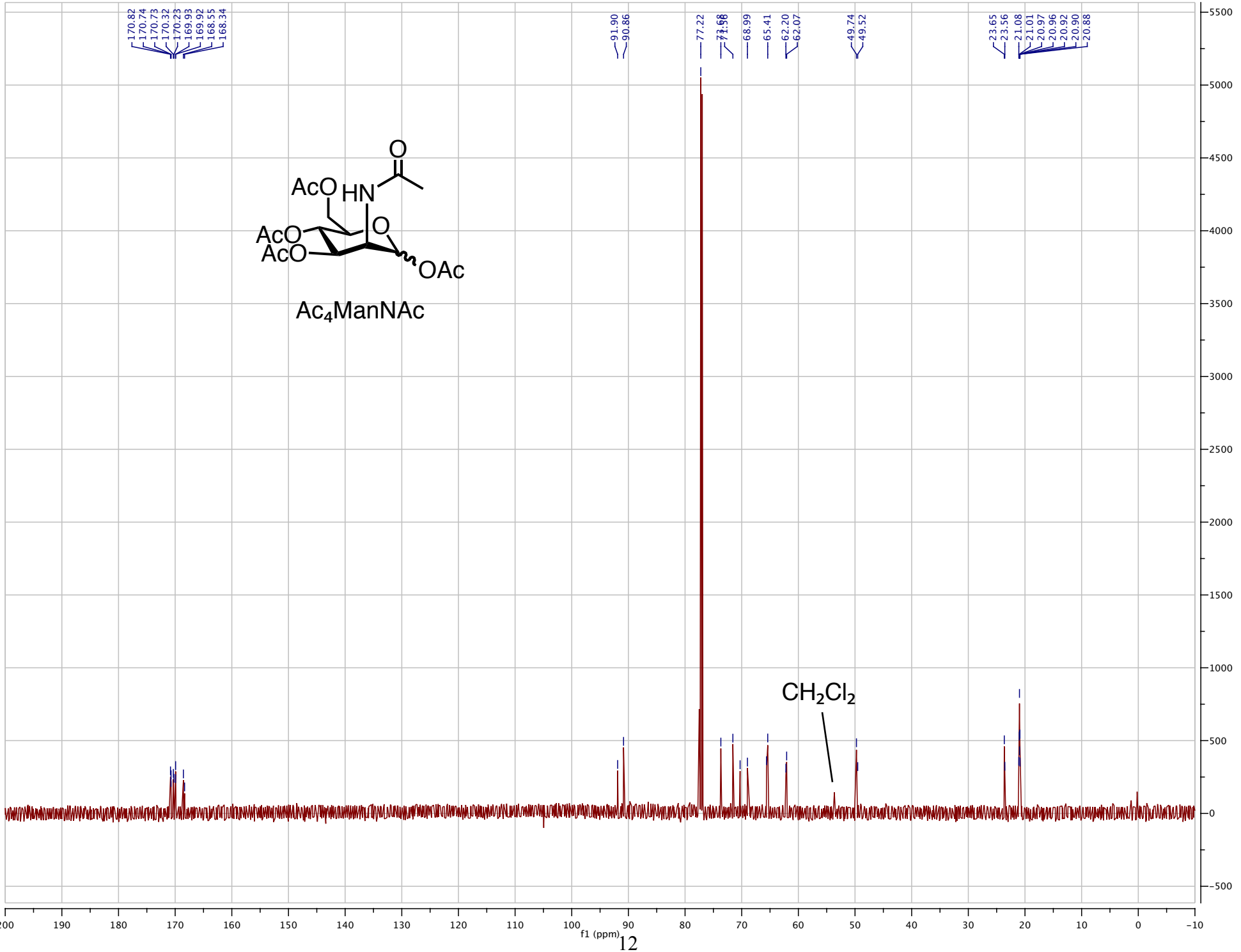
4.660
4.645
4.632
4.303
4.277
4.036
3.823
3.819
3.813
3.808
3.804
3.799
3.794
3.789
2.185
2.111
2.073
2.020
1.654
0.002
AcO
HN
O
AcO
AcO
O
OAc
Ac4ManNAc
CH2Cl2
1.87
1.74
0.67
1.75
1.10
1.89
1.55
4.31
0.85
3.68
11.41
9.06
4.74
3.00
f1 (ppm)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170.23
169.93
169.92
168.55
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90.86
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77.16
77.00
76.78
71.98
68.99
65.41
62.20
62.07
49.74
49.52
23.65
23.56
21.08
21.01
20.97
20.96
20.92
20.90
20.88
AcO
HN
O
AcO
AcO
O
OAc
Ac4ManNAc
CH2Cl2
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
-500
200
190
180
170
160
150
140
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20
10
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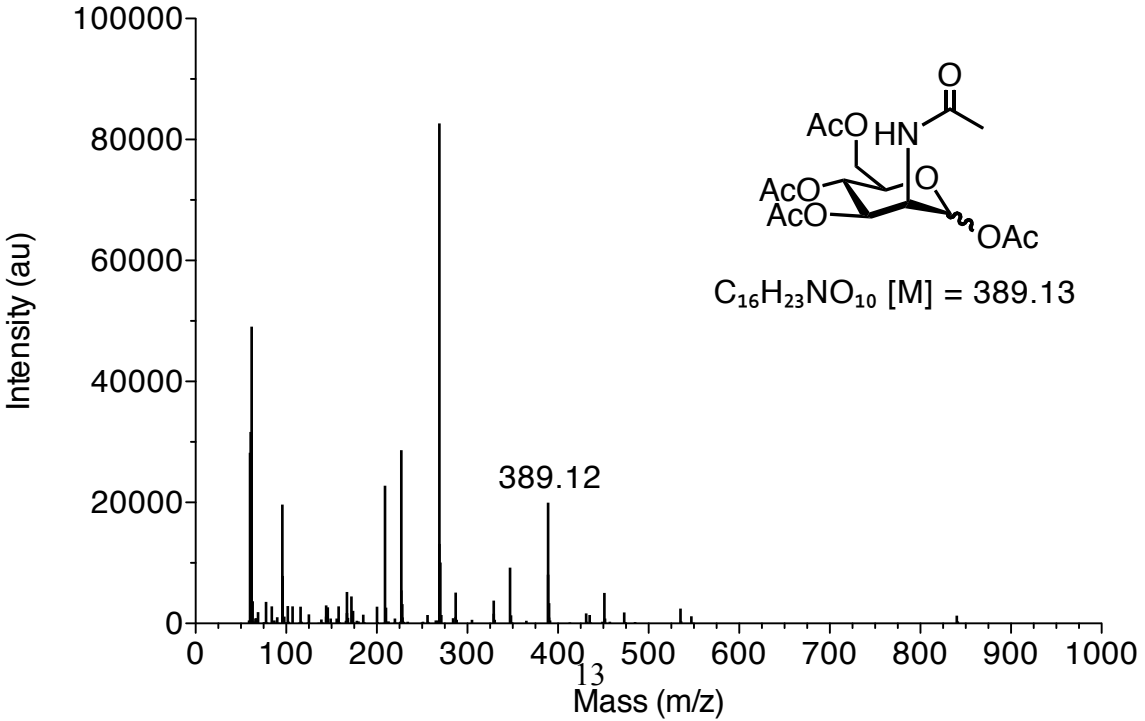
f1 (ppm)

$Ac_4ManNAc$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60000
40000
20000
0
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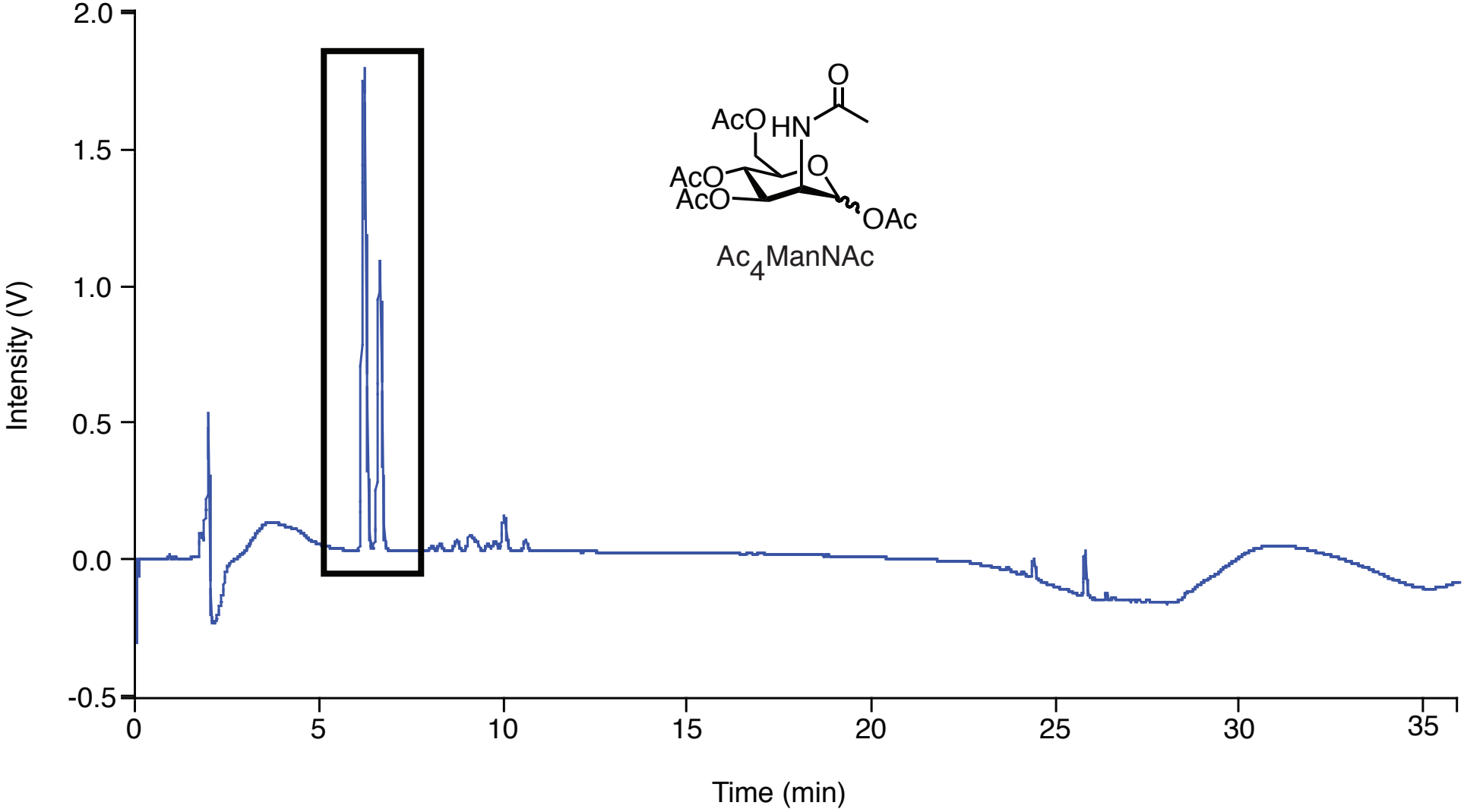
0
100
200
300
400
500
600
700
800
900
1000
Mass (m/z)
AcO
HN
O
AcO
AcO
O
OAc
C16H23NO10 [M] = 389.13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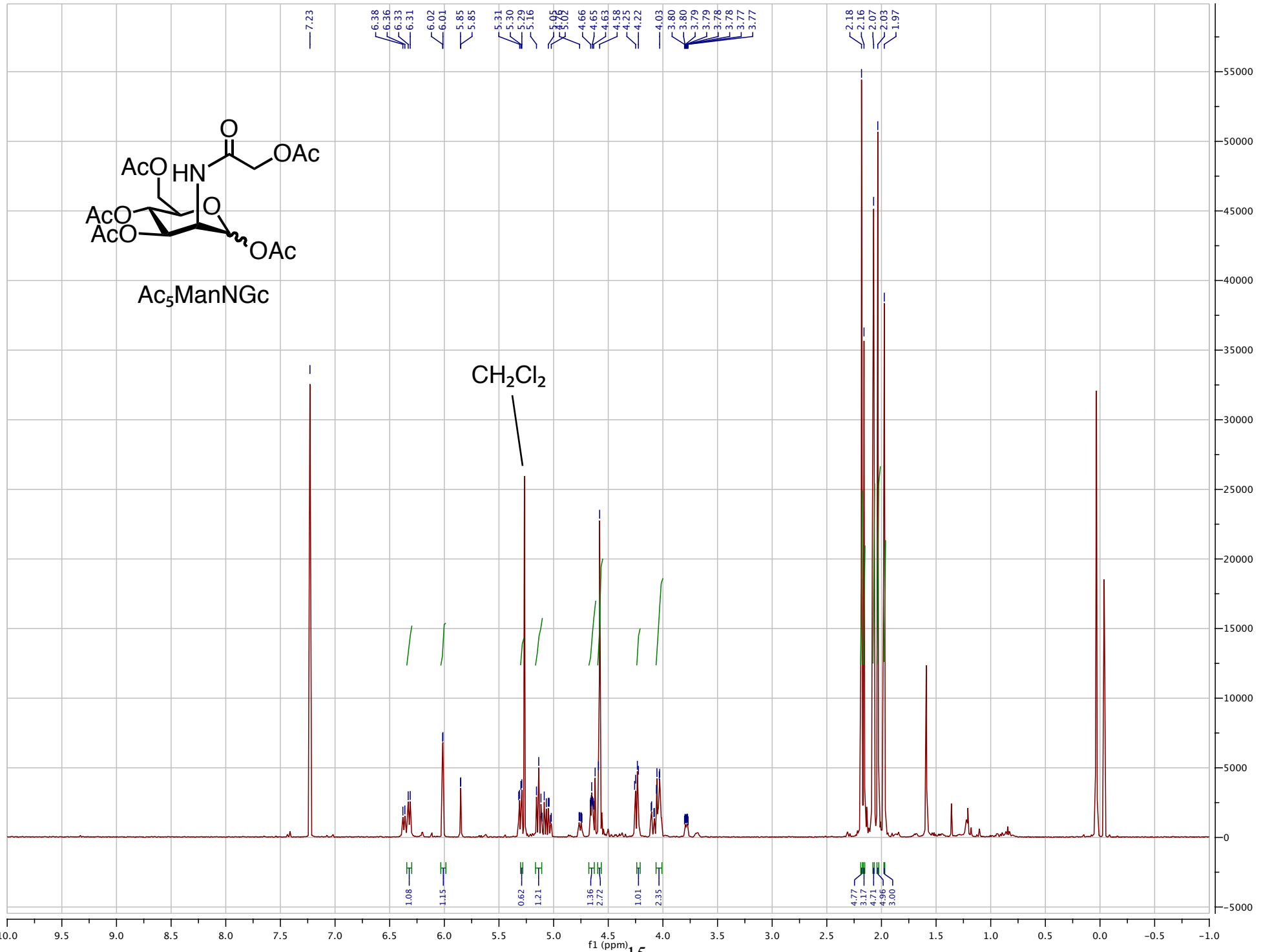
1.0
0.5
0.0
-0.5
Intensity (V)
0
5
10
15
20
25
30
35
Time (min)
AcO
HN
O
AcO
O
AcO
OAc
Ac4ManNAc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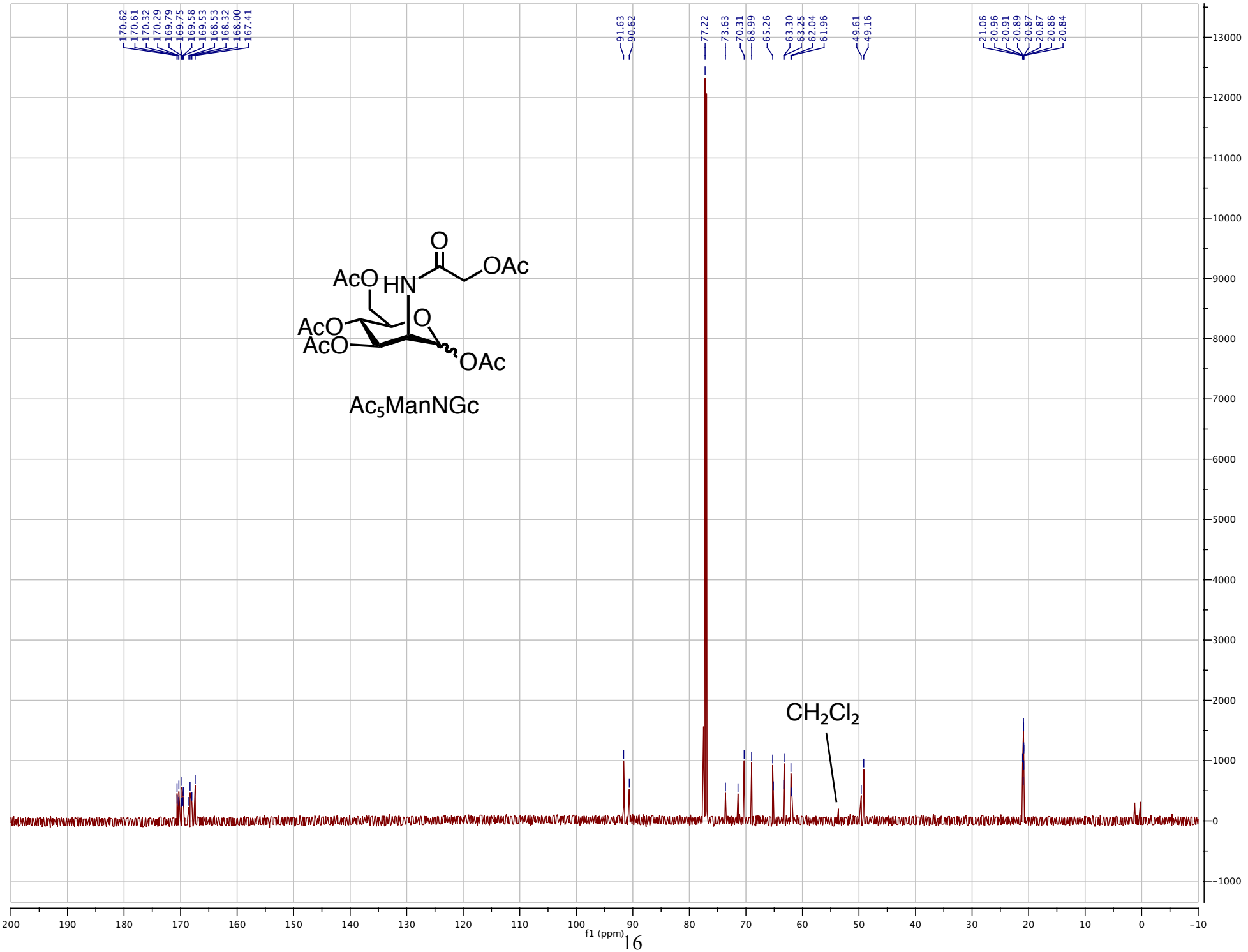
HN
O
OAc
AcO
O
AcO
OAc
Ac5ManNGc
CH2Cl2
f1 (ppm)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169.58
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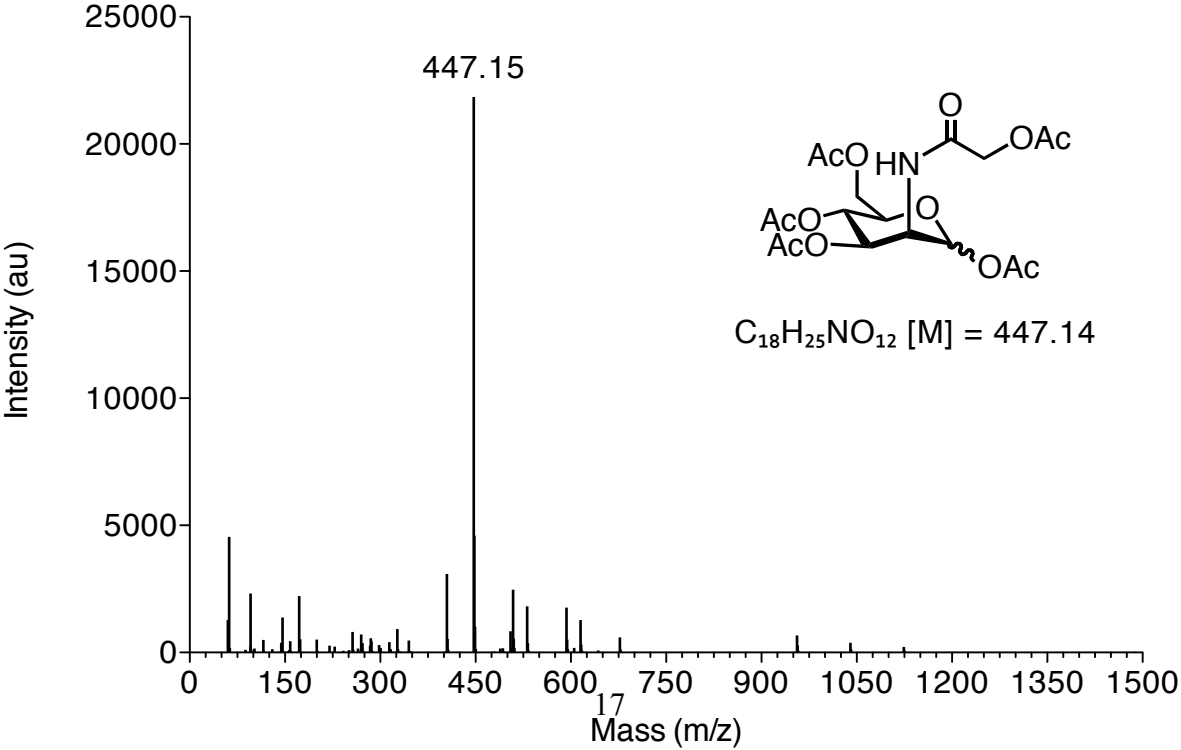
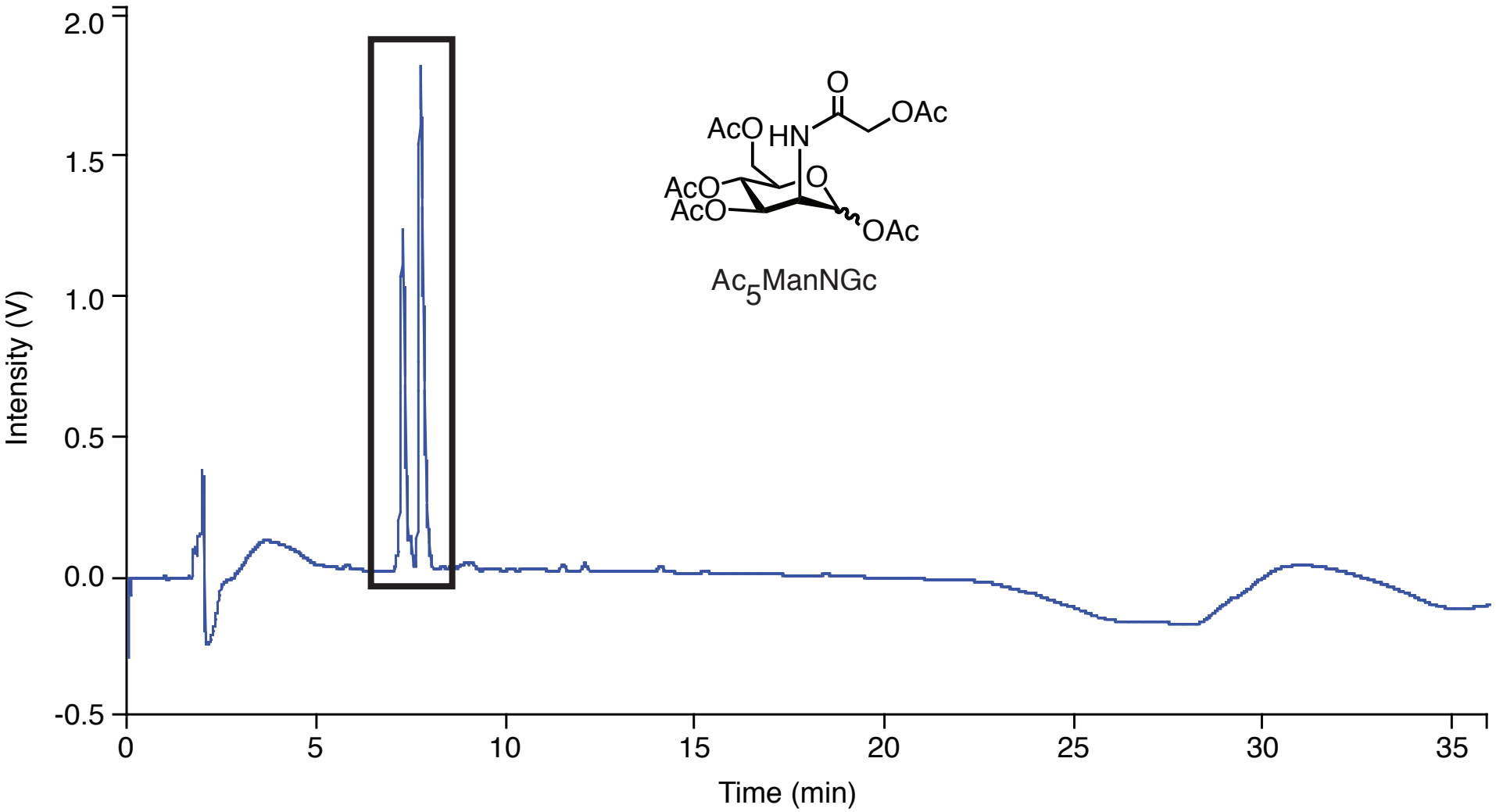
168.53
168.32
168.00
167.41
91.63
90.62
77.22
73.63
70.31
68.99
65.26
63.30
63.25
62.04
61.96
49.61
49.16
21.06
20.96
20.91
20.89
20.87
20.87
20.86
20.84
AcO
HN
O
OAc
AcO
AcO
O
OAc
Ac5ManNGc
CH2Cl2
f1 (ppm)

$Ac_5ManNGc$



$C_{18}H_{25}NO_{12}$ [M] = 447.14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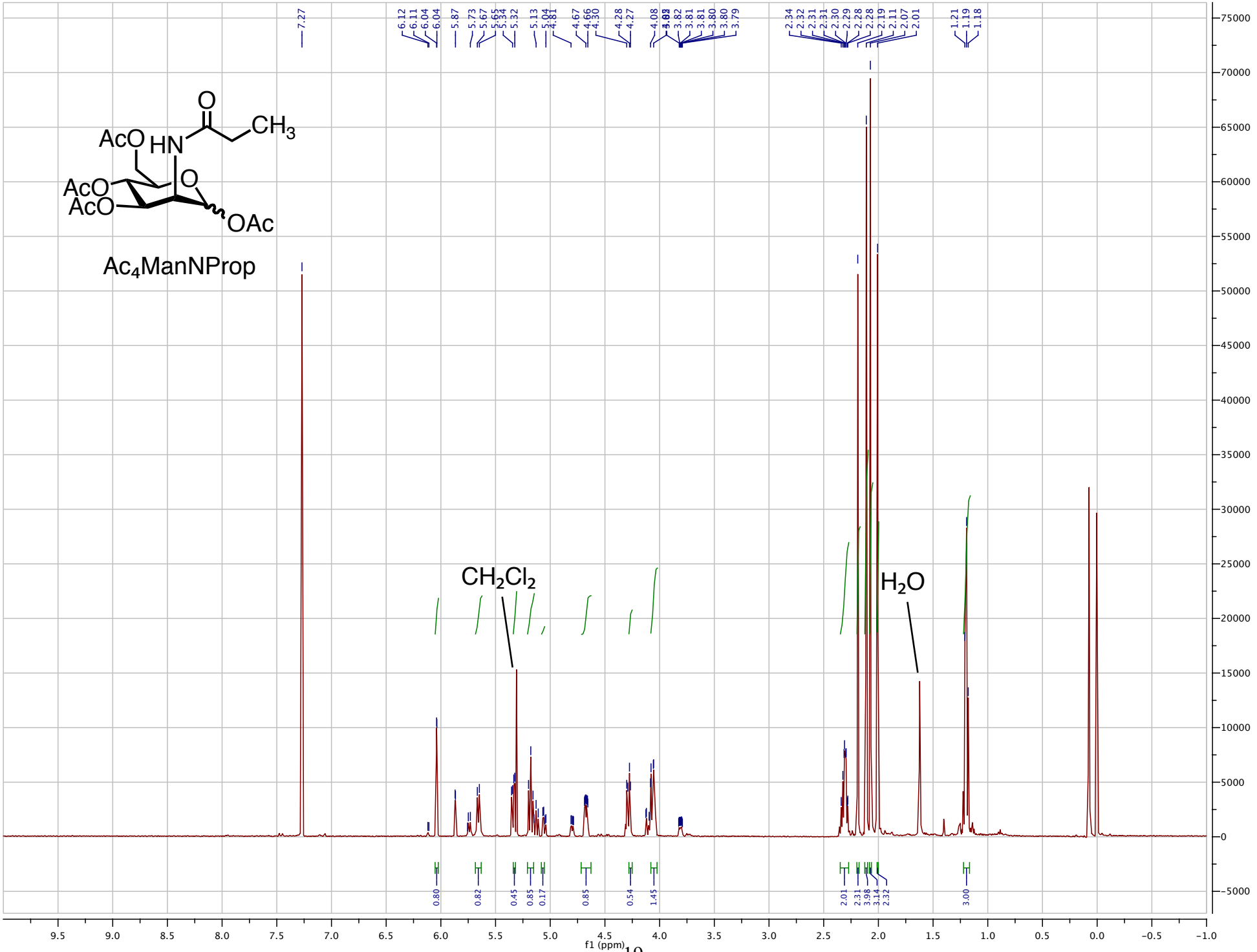
1.5
1.0
0.5
0.0
-0.5
Intensity (V)
0
5
10
15
20
25
30
35
Time (min)
AcO
HN
O
OAc
AcO
AcO
O
OAc
Ac5ManNGc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HN
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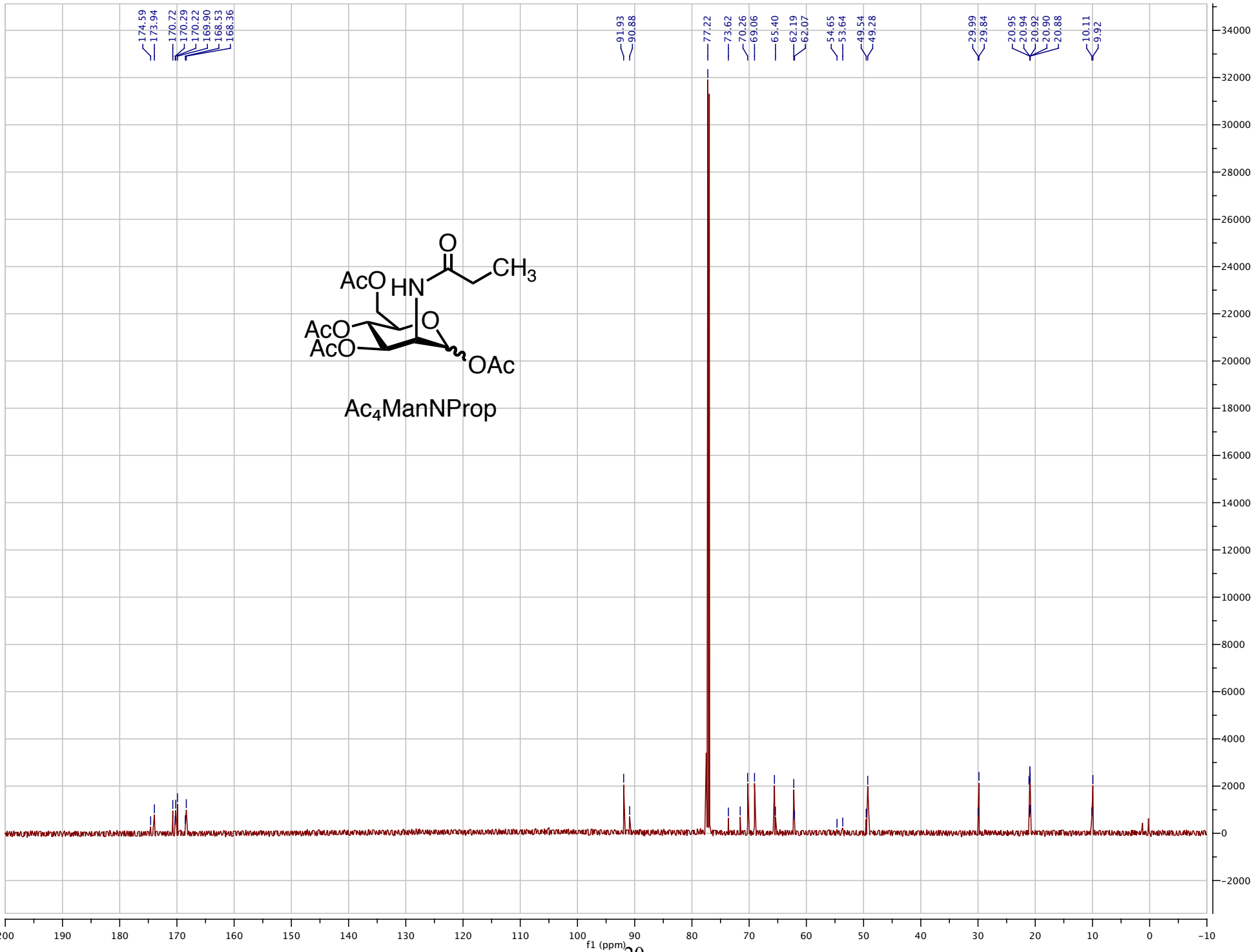
CH3
AcO
O
AcO
OAc
Ac4ManNProp
CH2Cl2
H2O
f1 (ppm)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170.22
169.90
168.53
168.36
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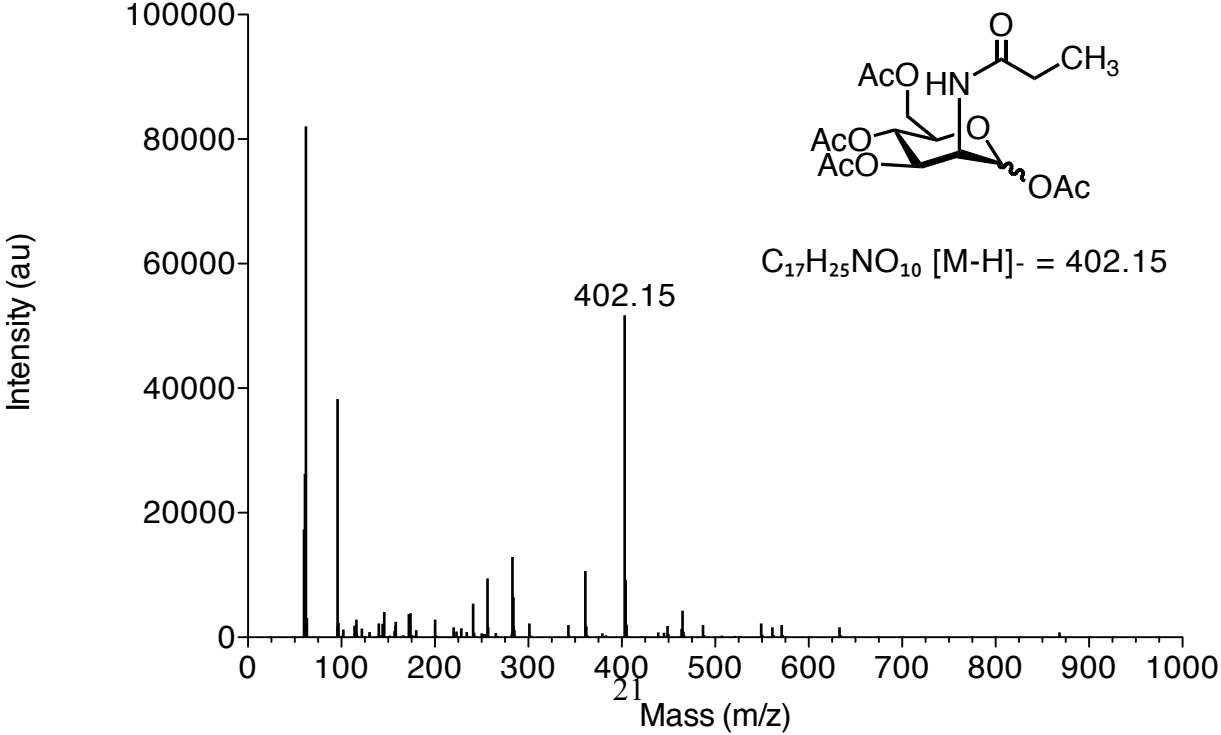
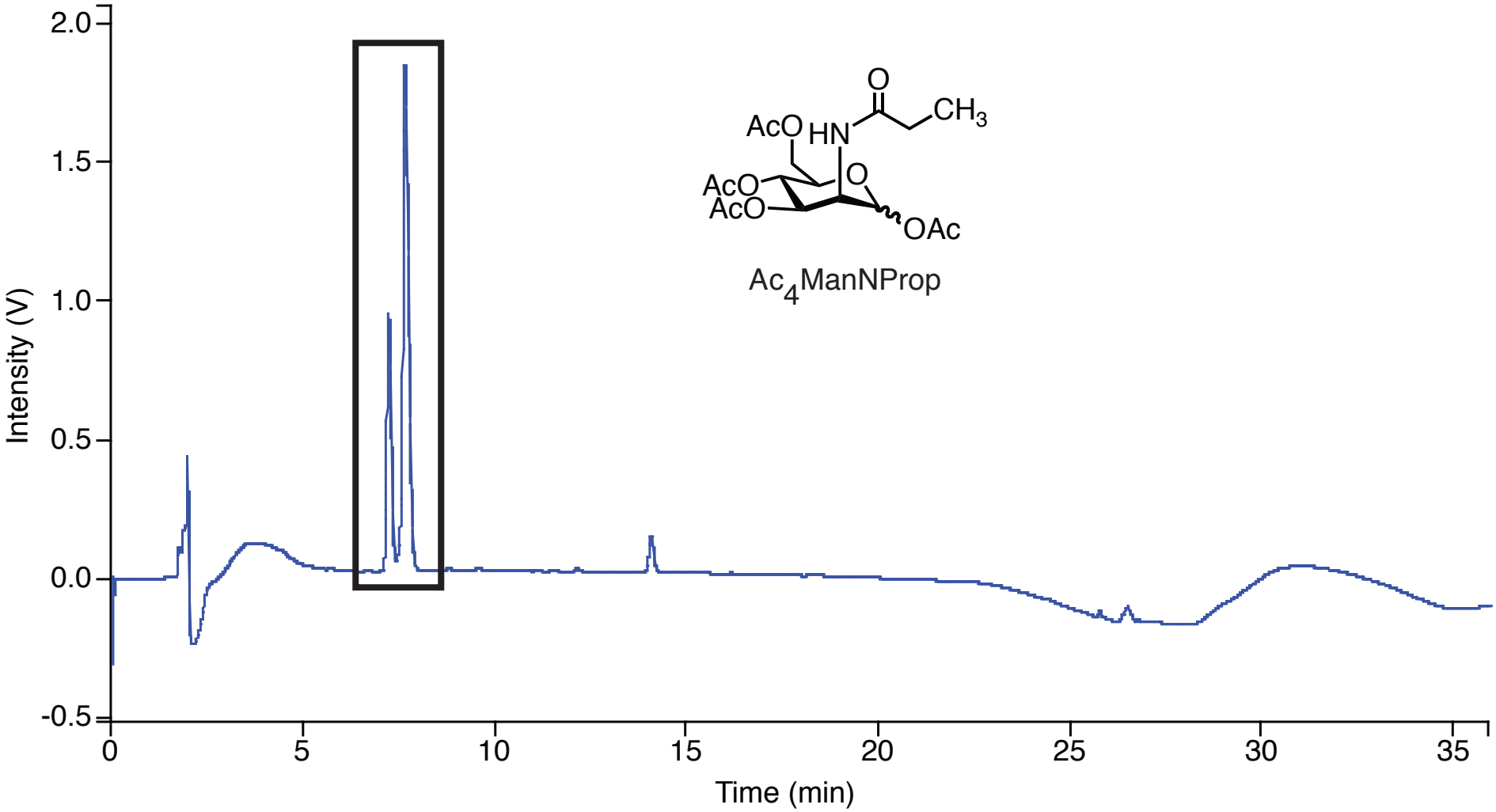
90.88
77.22
73.62
70.26
69.06
65.40
62.19
62.07
54.65
53.64
49.54
49.28
29.99
29.84
20.95
20.94
20.92
20.90
20.88
10.11
9.92
AcO
HN
O
CH3
AcO
AcO
O
OAc
Ac4ManNProp
f1 (ppm)

Ac_4ManNProp

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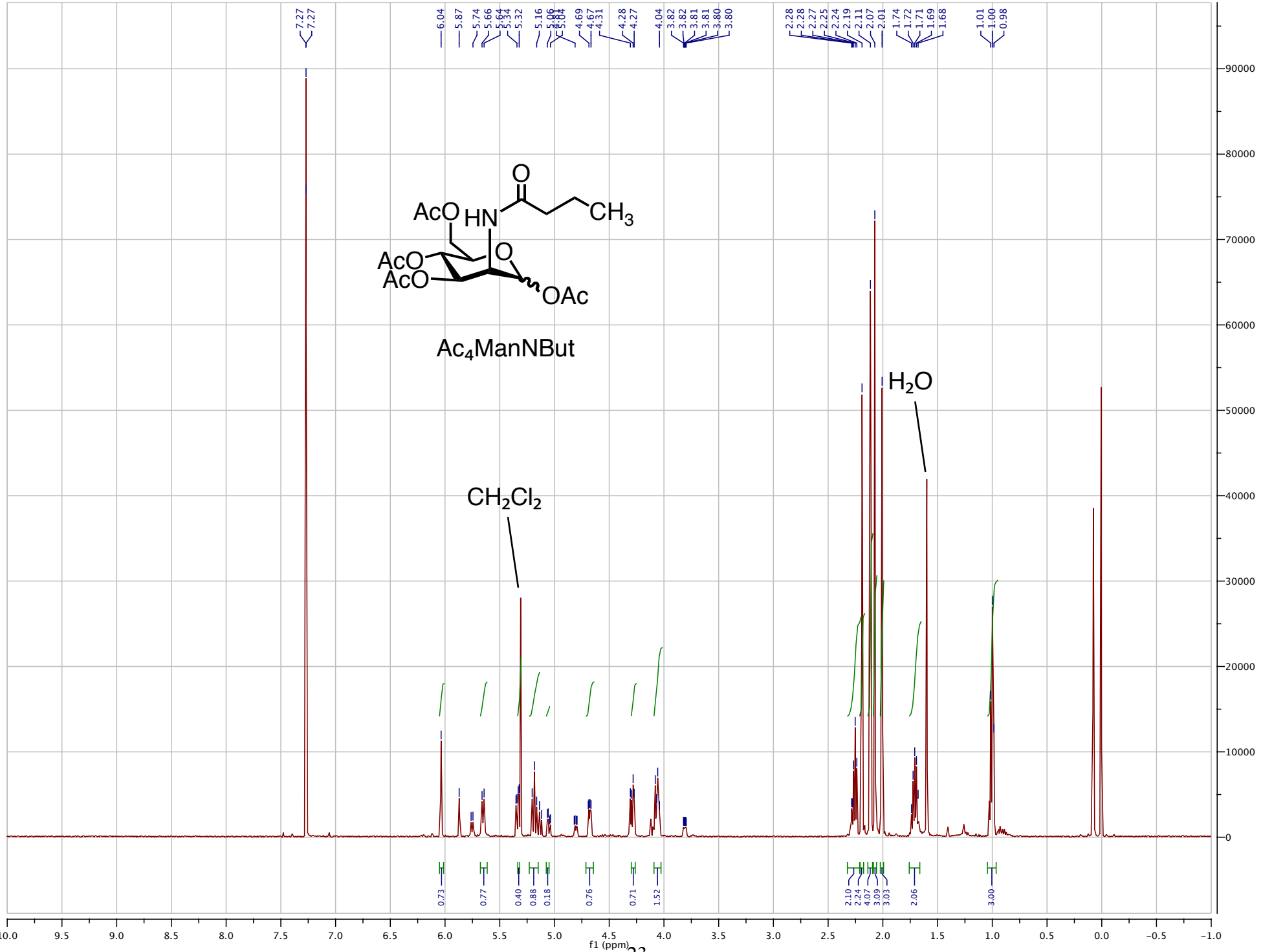
1.5
1.0
0.5
0.0
-0.5
Intensity (V)
0
5
10
15
20
25
30
35
Time (min)
AcO
HN
O
CH3
AcO
O
AcO
OAc
Ac4ManNProp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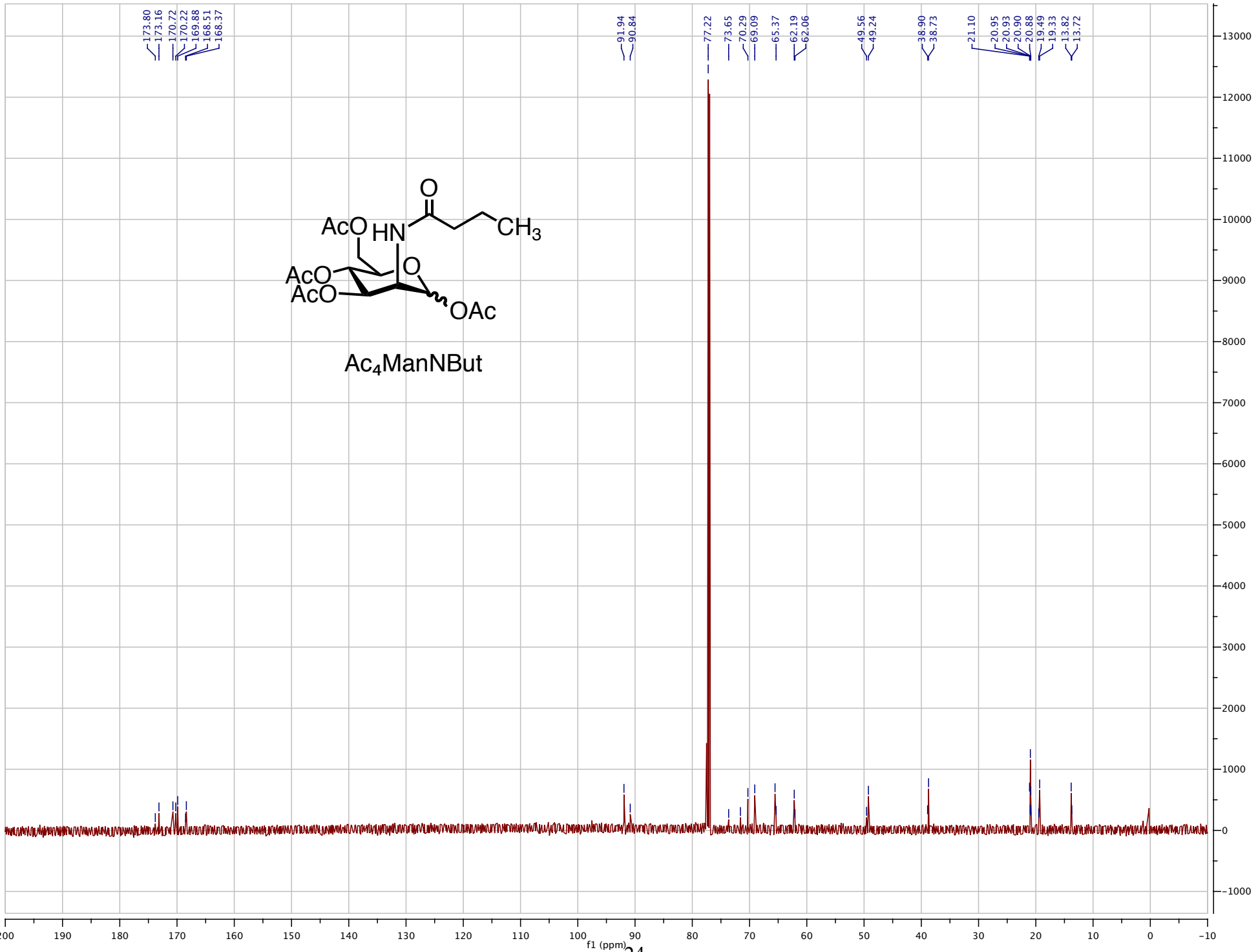
HN
O
CH3
AcO
O
AcO
OAc
Ac4ManNBut
H2O
CH2Cl2
f1 (ppm)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170.22
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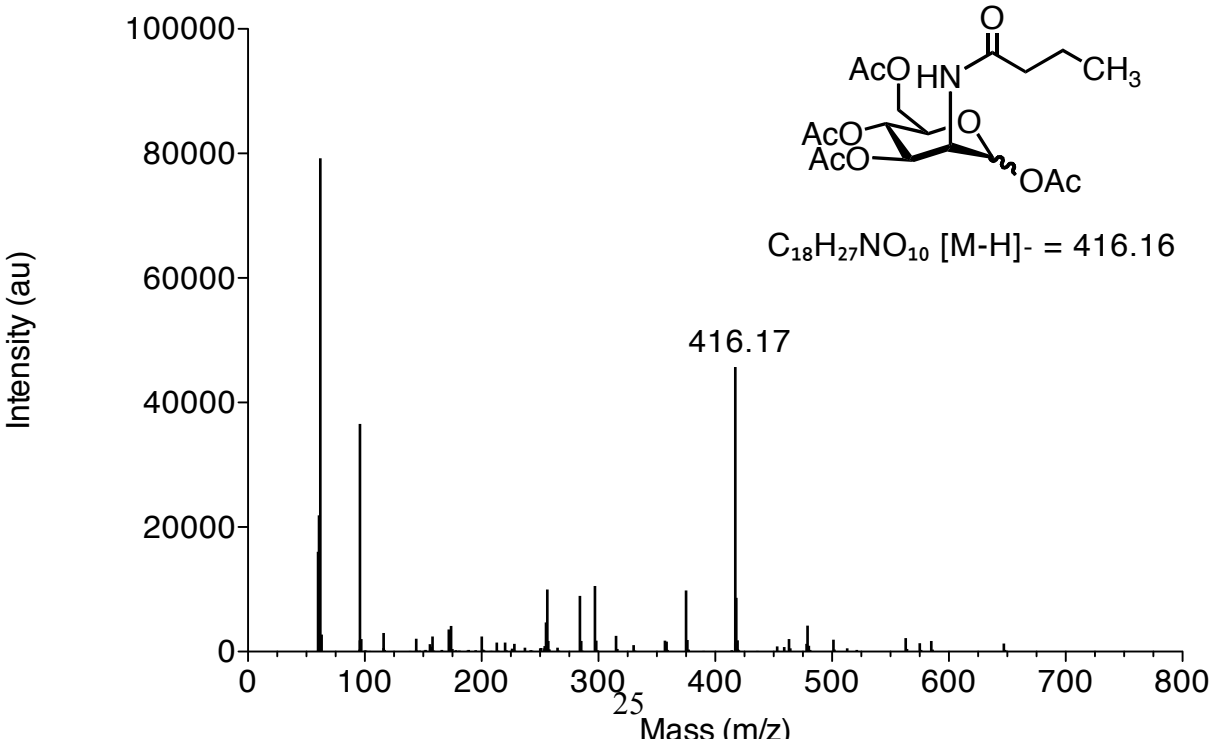
168.37
91.94
90.84
77.22
73.65
70.29
69.09
65.37
62.19
62.06
49.56
49.24
38.90
38.73
21.10
20.95
20.93
20.90
20.88
19.49
19.33
13.82
13.72
AcO
HN
O
CH3
AcO
AcO
O
OAc
f1 (ppm)

$Ac_4ManNBut$

$Ac_4ManNBut$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40000
20000
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Intensity (au)
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200
300
400
500
600
700
800
Mass (m/z)
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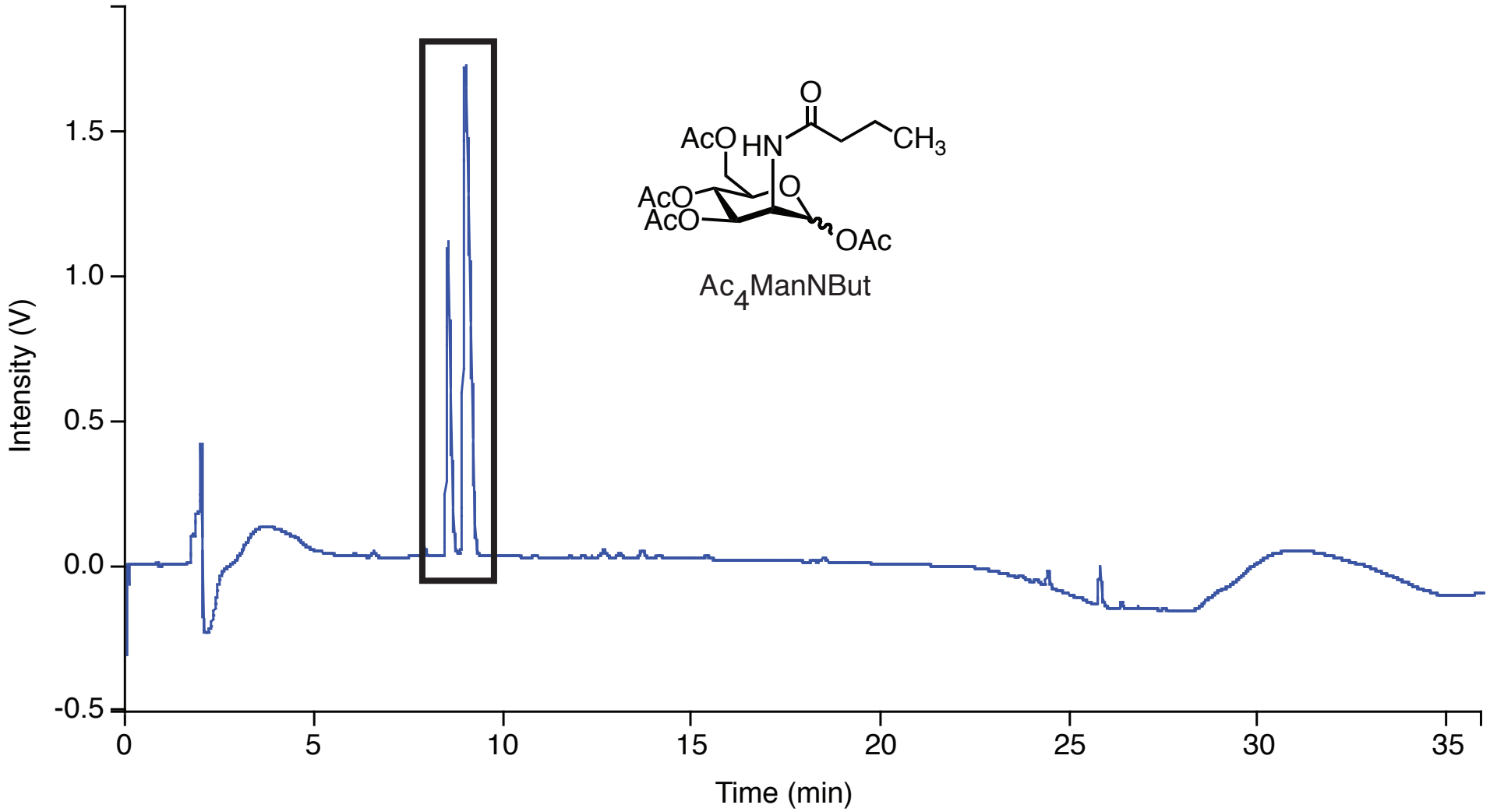
AcO
HN
O
CH3
AcO
AcO
O
OAc
C18H27NO10 [M-H]- = 416.16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0
5
10
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20
25
30
35
Time (min)
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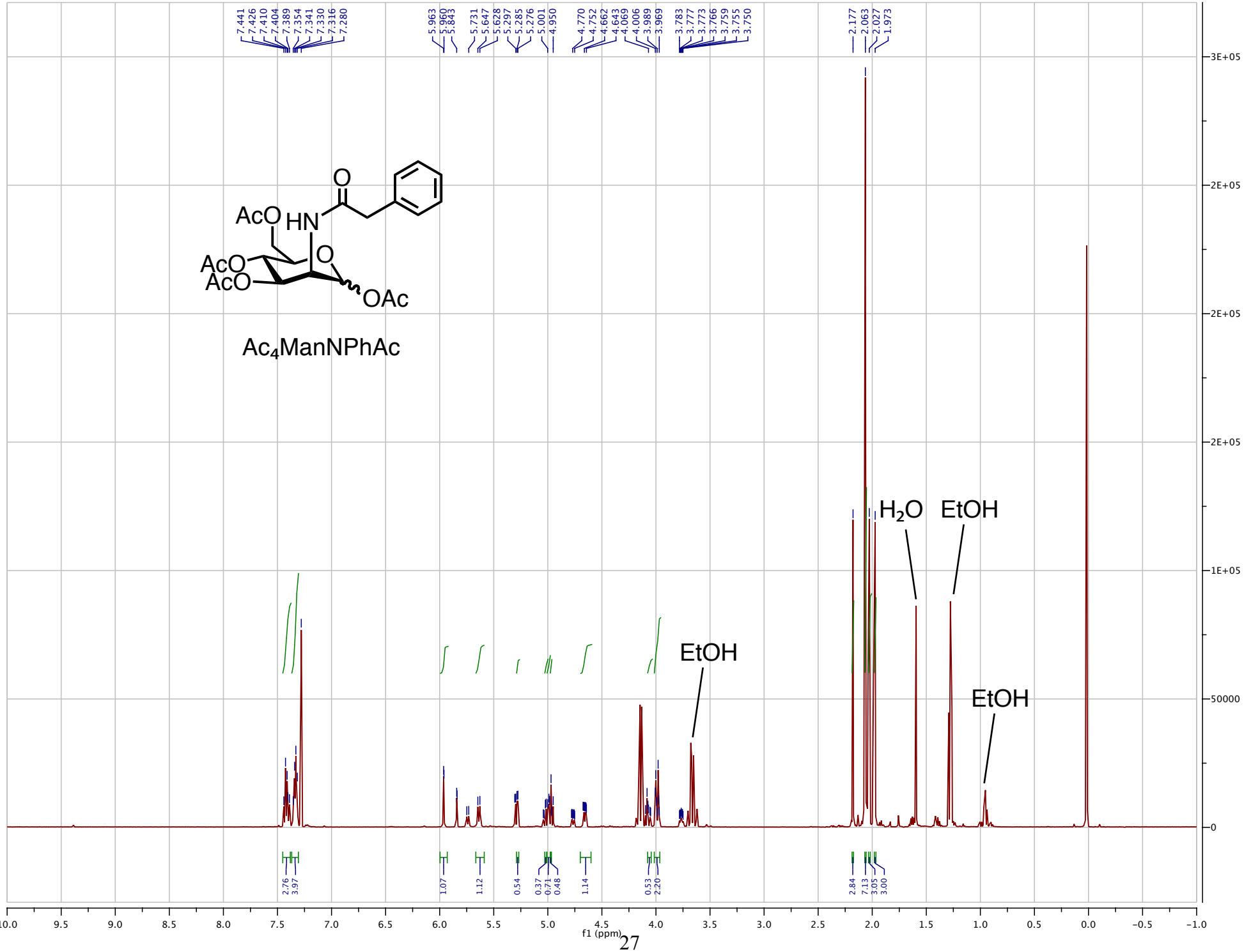
HN
O
CH3
AcO
AcO
O
OAc
Ac4ManNBut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3.773
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3.759
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2.177
2.063
2.027
1.973
AcO
HN
O
AcO
AcO
O
OAc
Ac4ManNPhAc
H2O
EtOH
EtOH
EtOH
3E+05
2E+05
2E+05
2E+05
1E+05
50000
0
2.76
3.97
1.07
1.12
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0.37
0.71
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1.14
0.53
2.20
2.84
7.13
3.05
3.00
10.0
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
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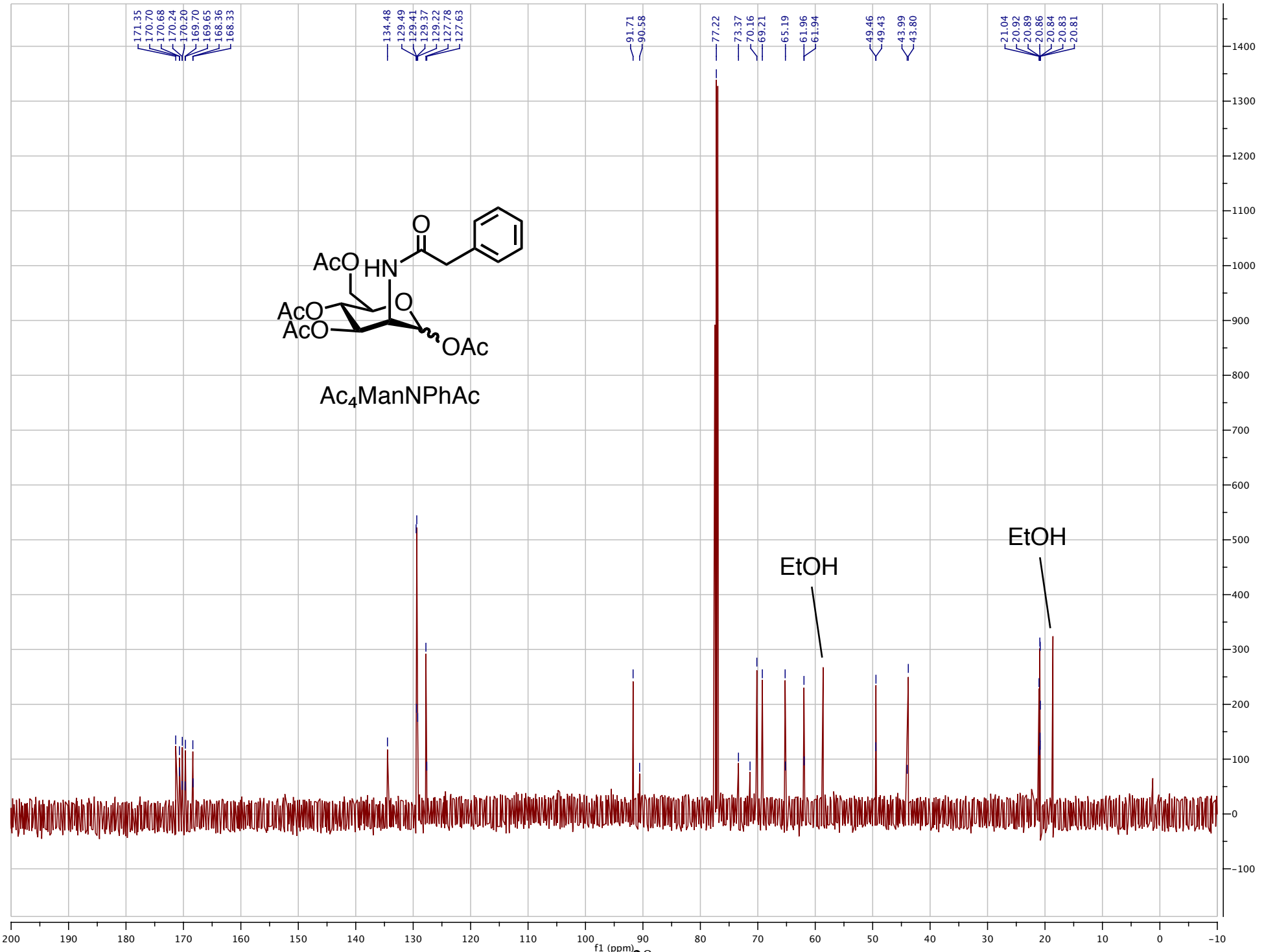
0.0
-0.5
-1.0
f1 (ppm)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170.20
169.70
169.65
168.36
168.33
134.48
129.49
129.41
129.37
129.22
127.78
127.63
91.71
90.58
77.22
73.37
70.16
69.21
65.19
61.96
61.94
49.46
49.43
43.99
43.80
21.04
20.92
20.89
20.86
20.84
20.83
20.81
AcO
HN
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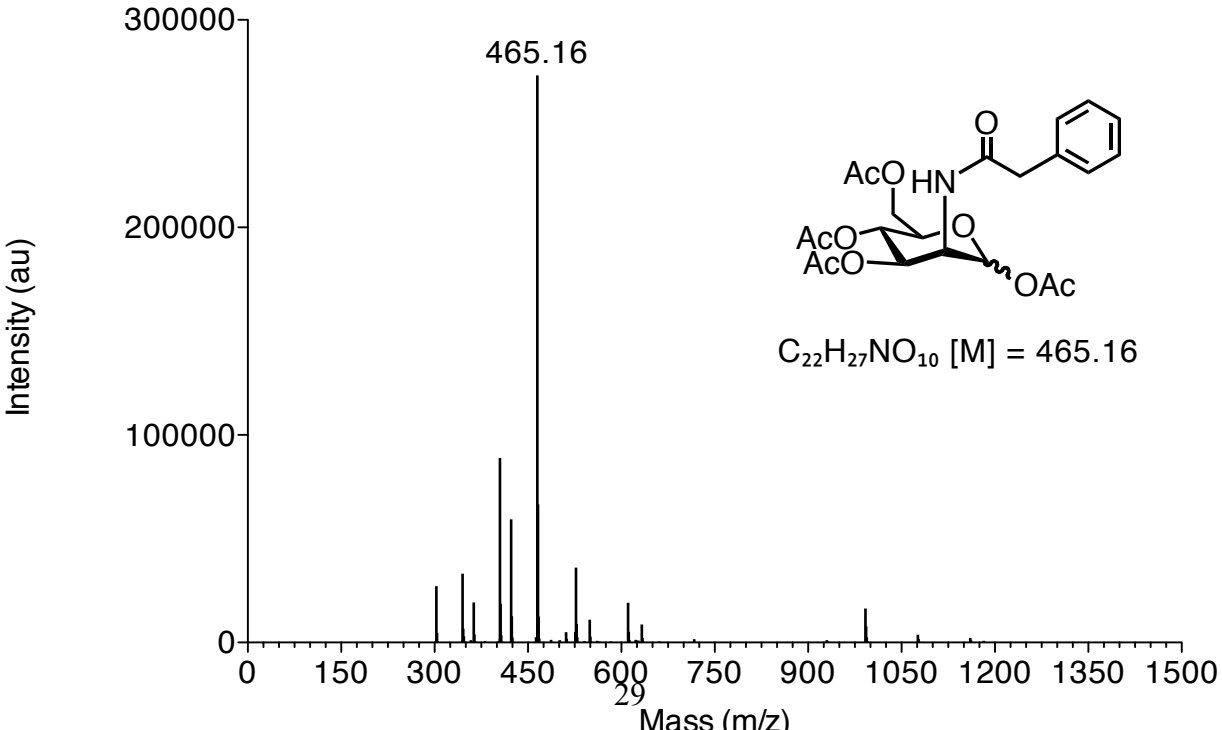
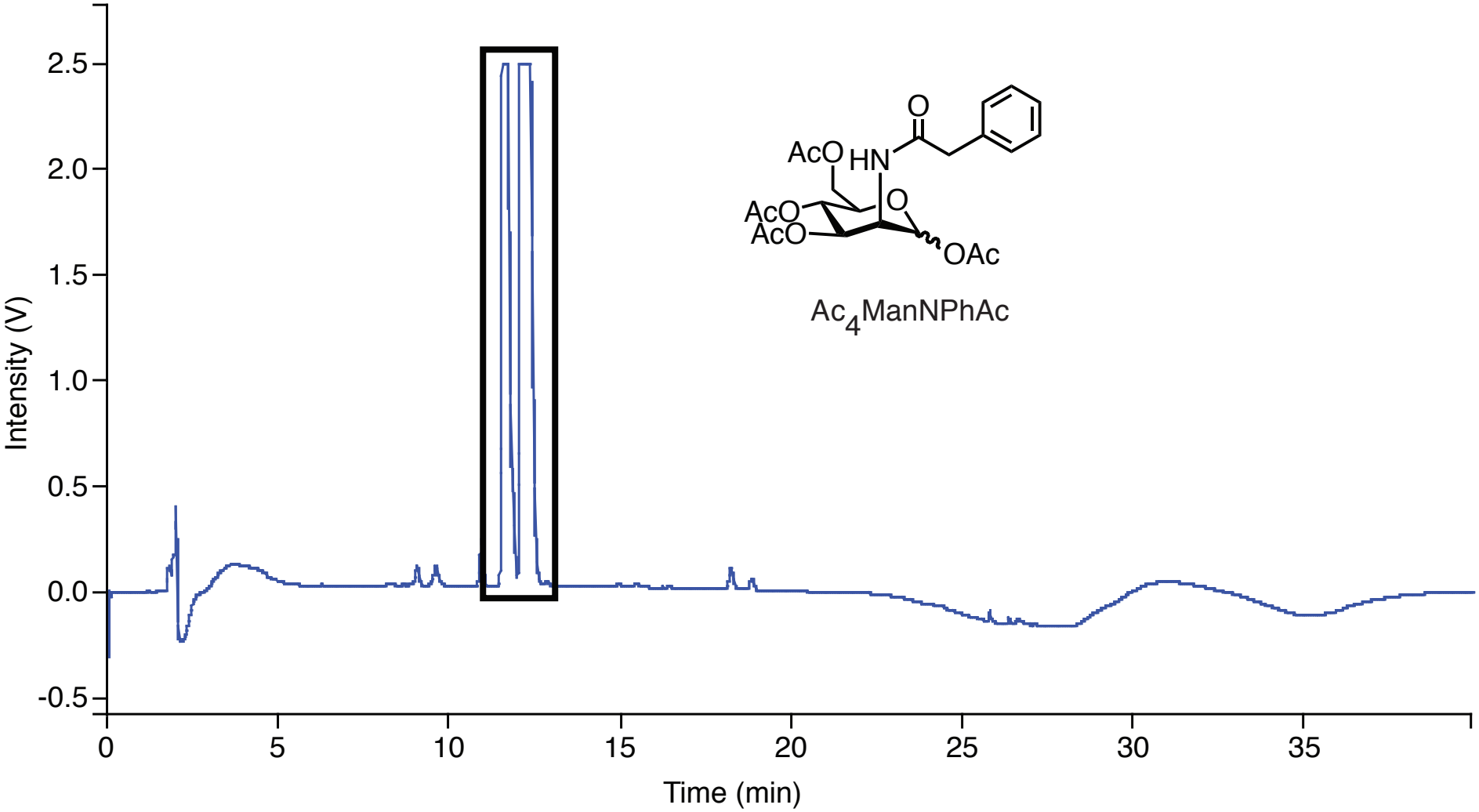
AcO
AcO
O
OAc
Ac4ManNPhAc
EtOH
EtOH
f1 (ppm)

$Ac_4ManNPhAc$

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1.0
0.5
0.0
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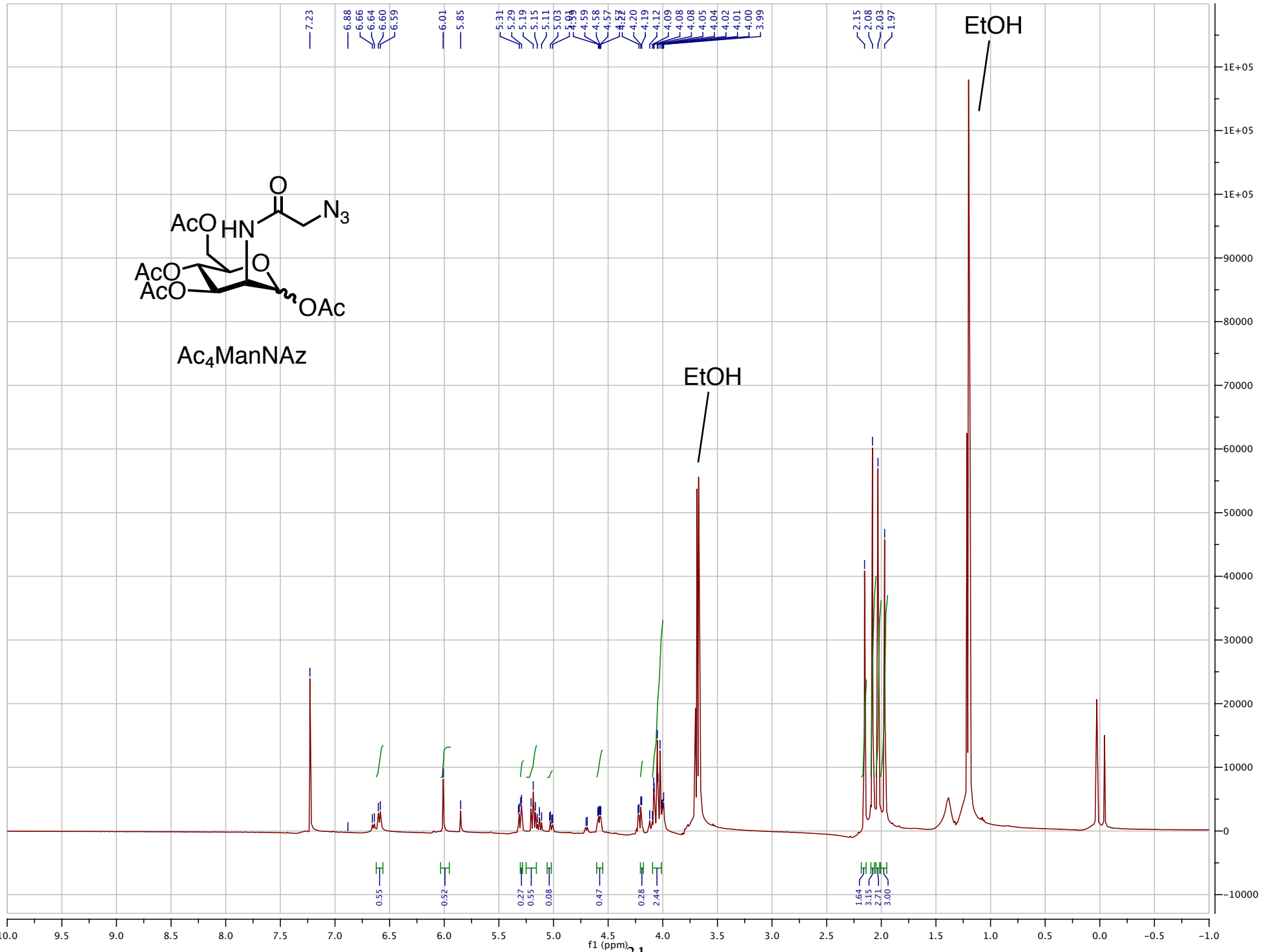
0
5
10
15
20
25
30
35
Time (min)
AcO
HN
O
AcO
AcO
O
OAc
Ac4ManNPhAc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5.15
5.11
5.03
4.59
4.58
4.57
4.20
4.19
4.12
4.09
4.08
4.08
4.05
4.04
4.02
4.01
4.00
3.99
2.15
2.08
2.03
1.97
EtOH
EtOH
AcO
HN
O
N3
AcO
AcO
O
OAc
Ac4ManNAz
0.55
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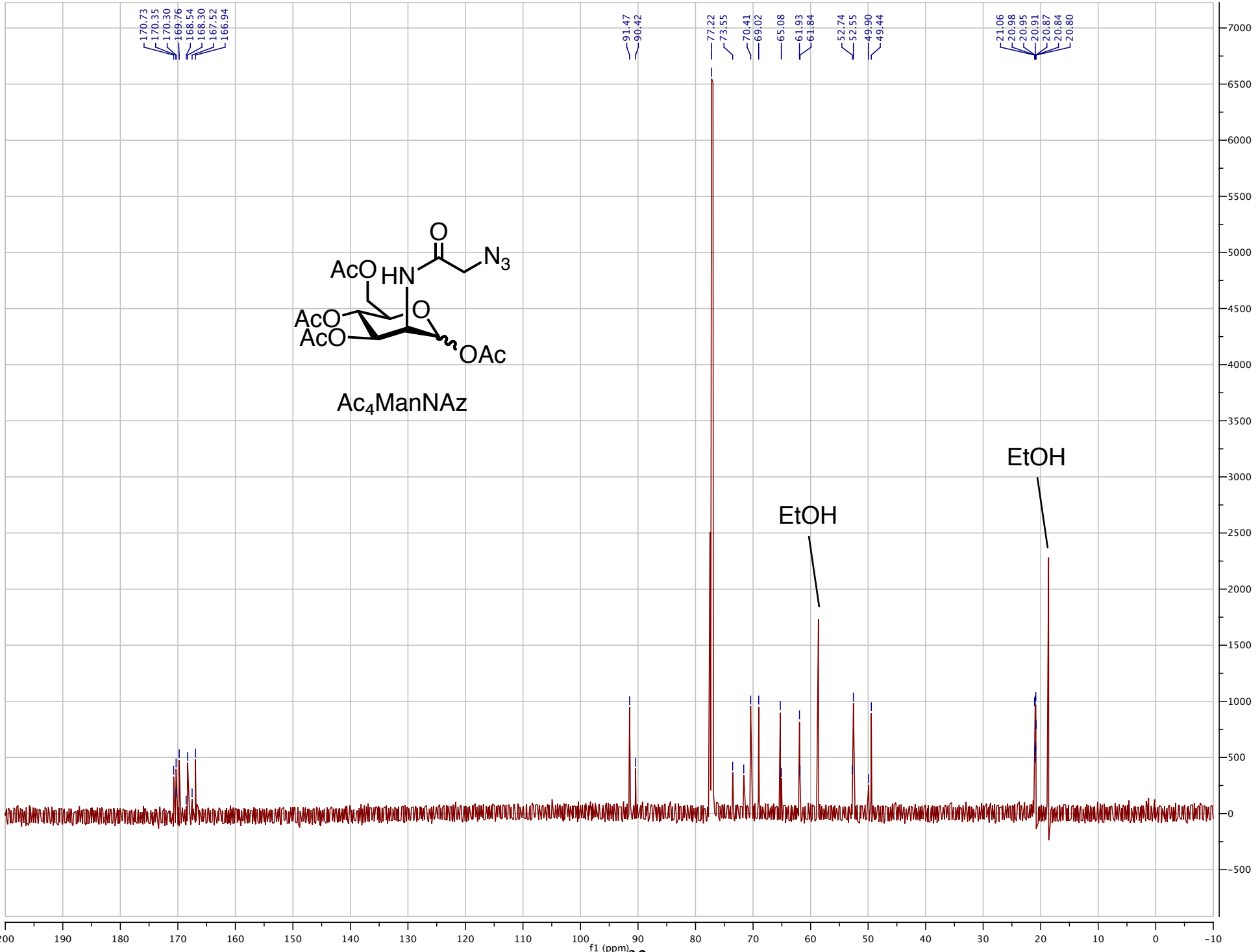
3.00
f1 (ppm)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168.54
168.30
167.52
166.94
91.47
90.42
77.22
73.55
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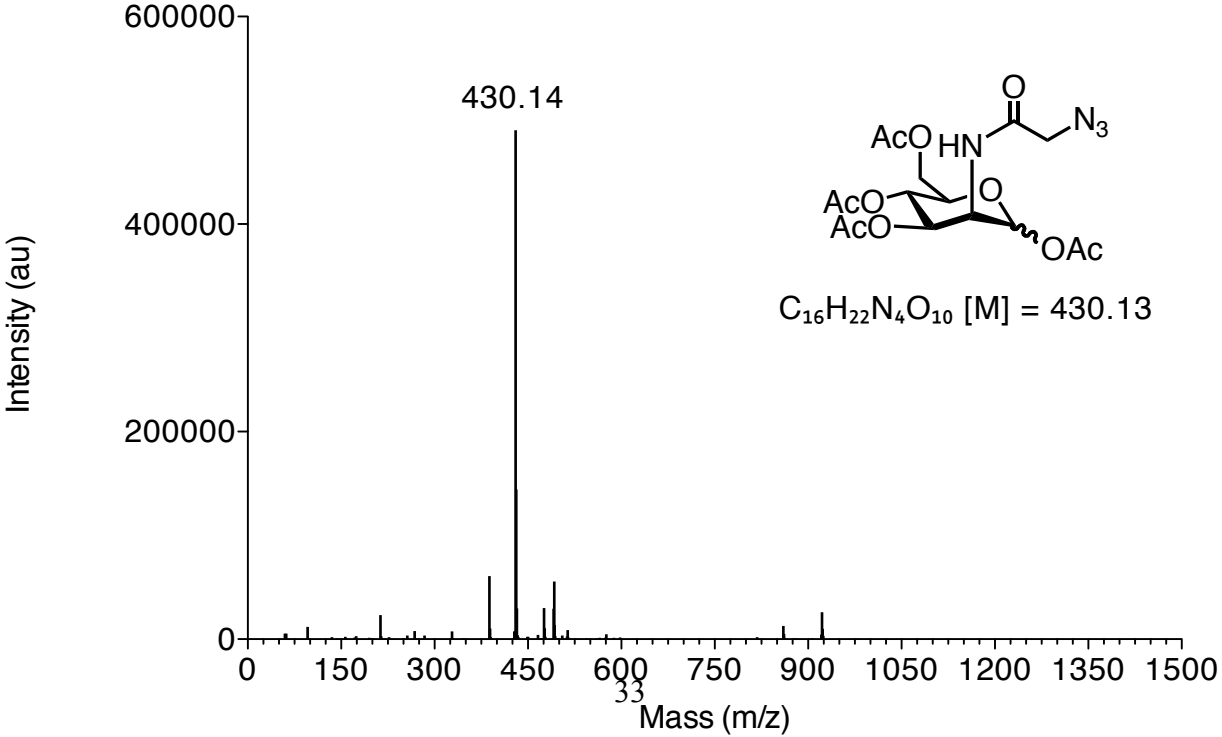
69.02
65.08
61.93
61.84
52.74
52.55
49.90
49.44
21.06
20.98
20.95
20.91
20.87
20.84
20.80
AcO
HN
O
N3
AcO
AcO
O
OAc
Ac4ManNAz
EtOH
EtOH
f1 (ppm)

$Ac_4ManNAz$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400000
200000
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0
150
300
450
600
750
900
1050
1200
1350
1500
Mass (m/z)
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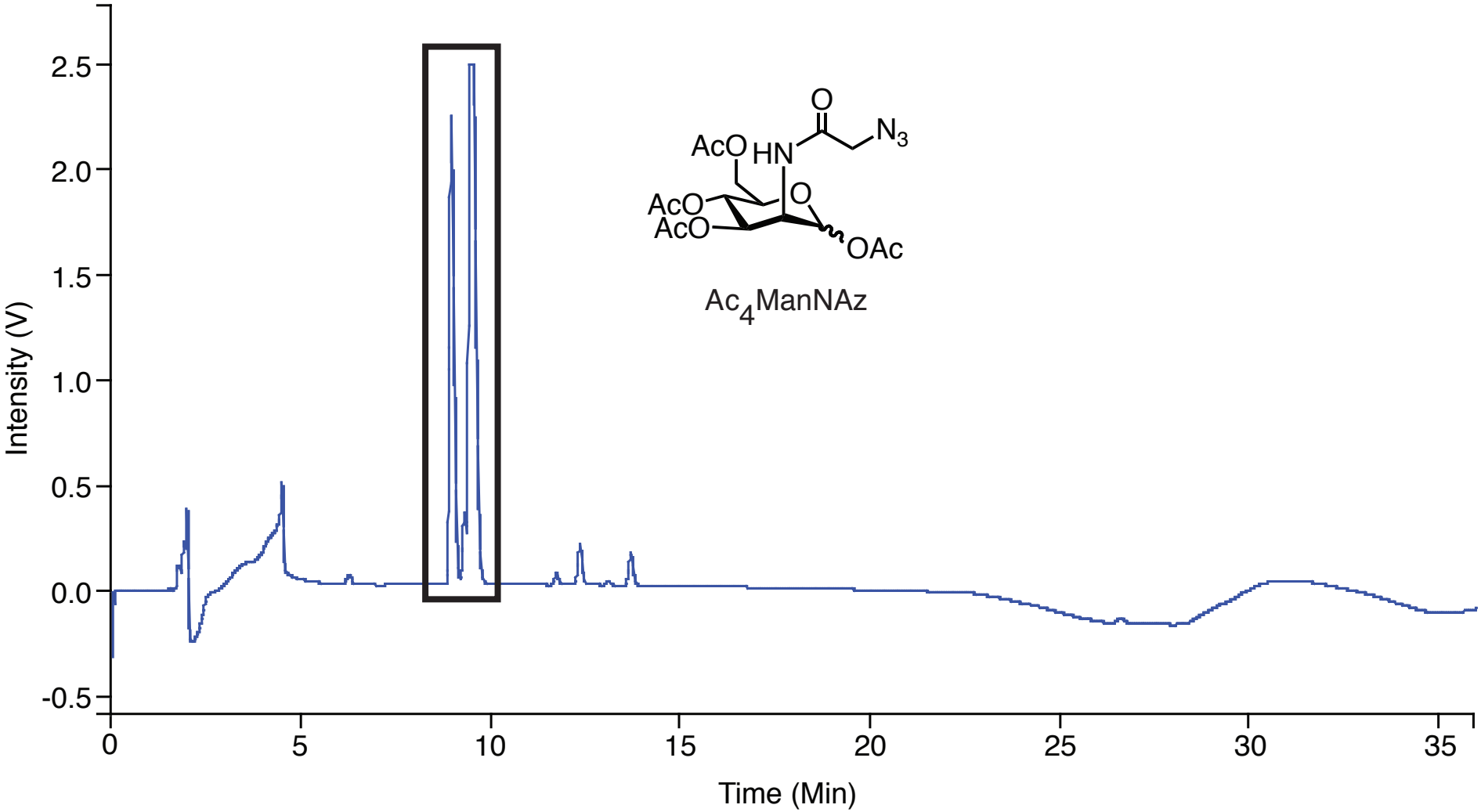
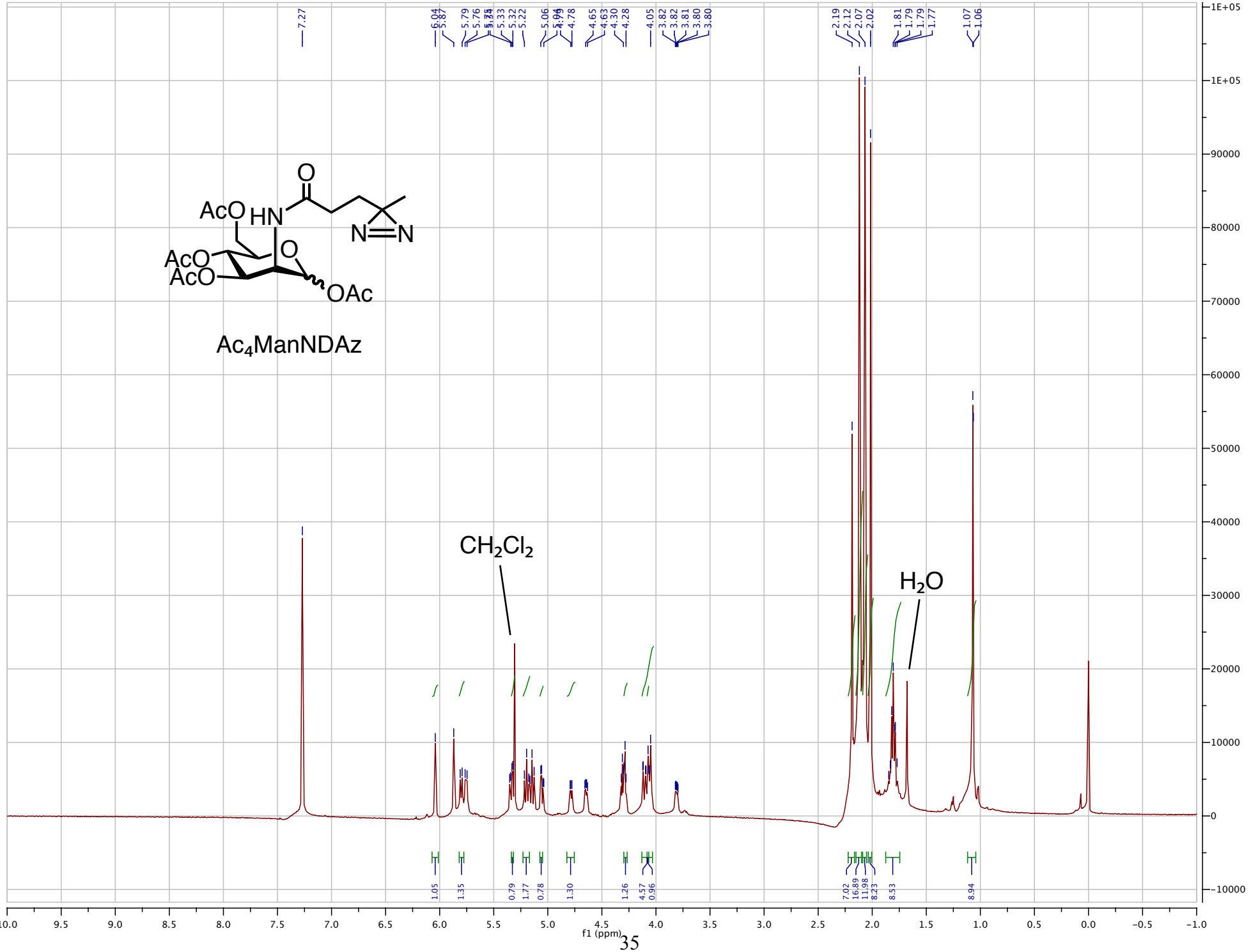
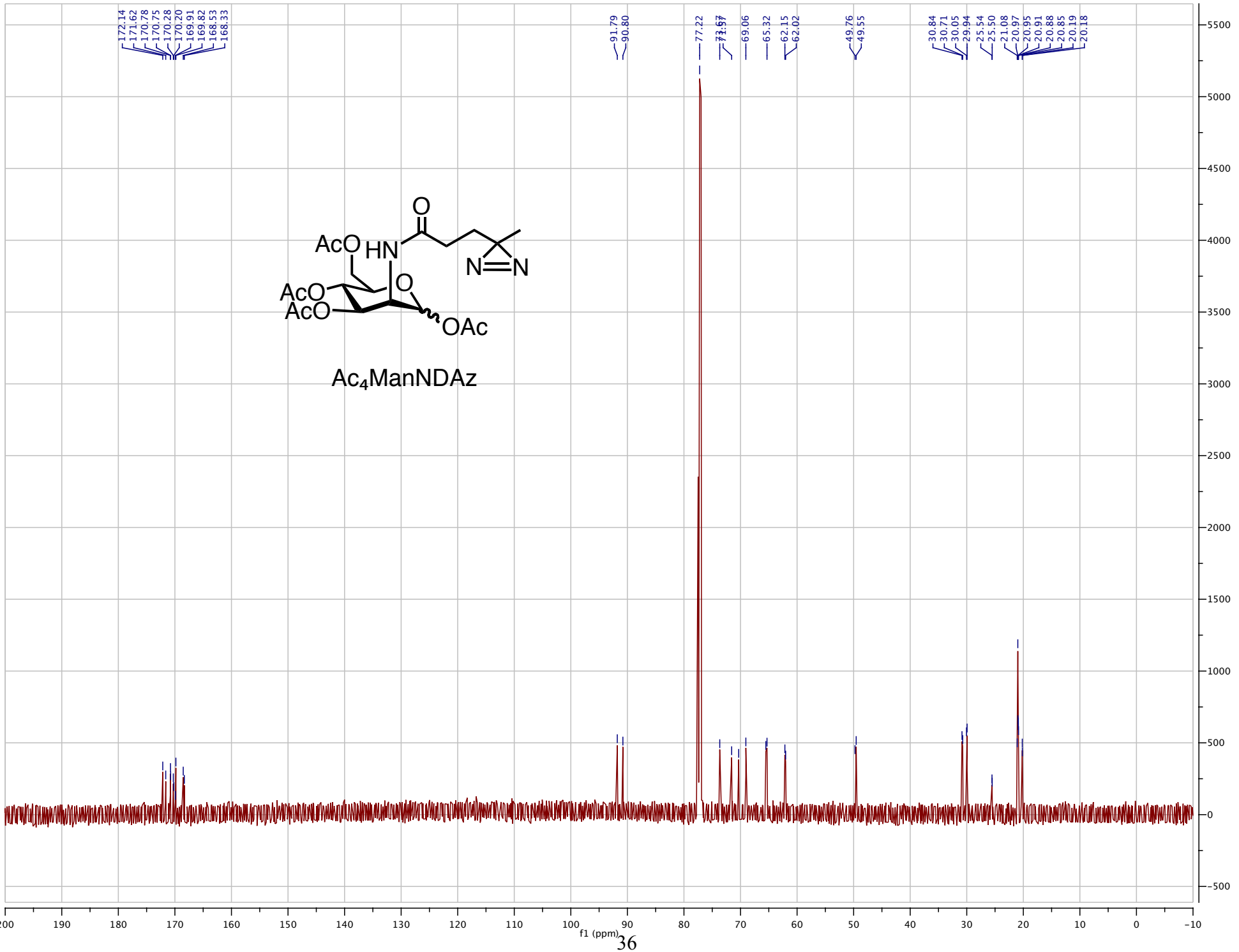
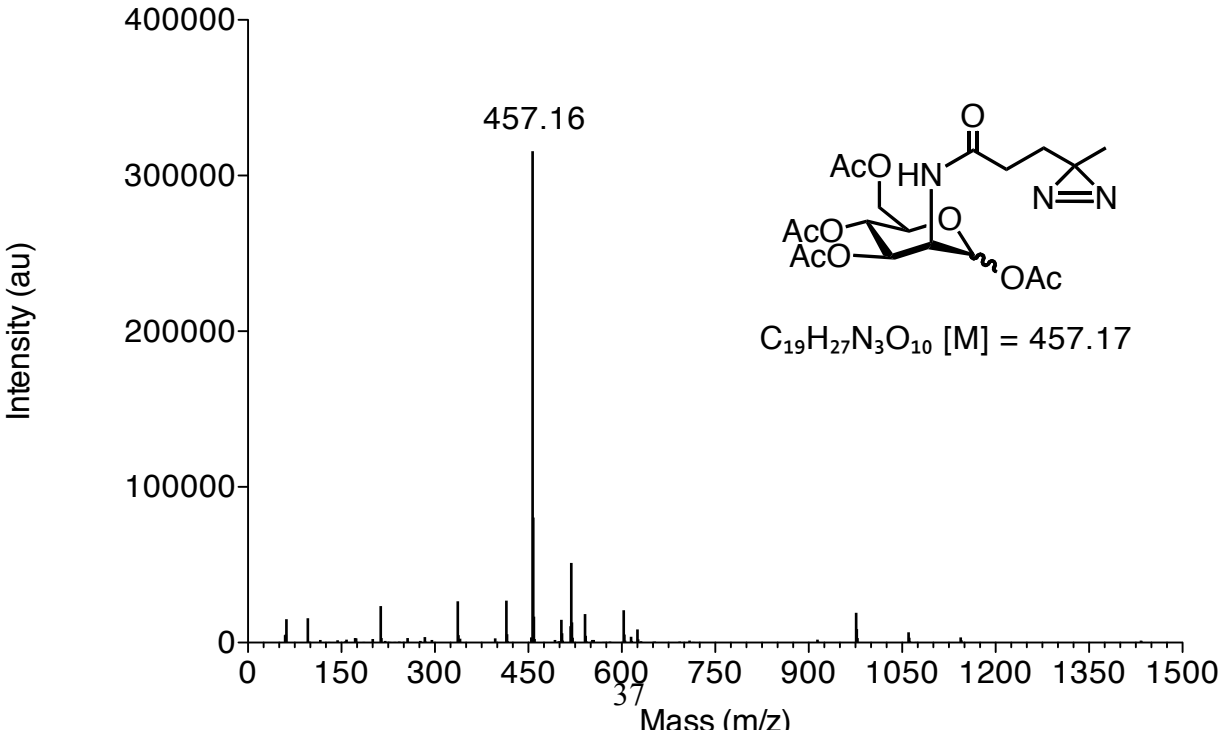
O
N3
AcO
AcO
O
OAc
C16H22N4O10 [M] = 430.13

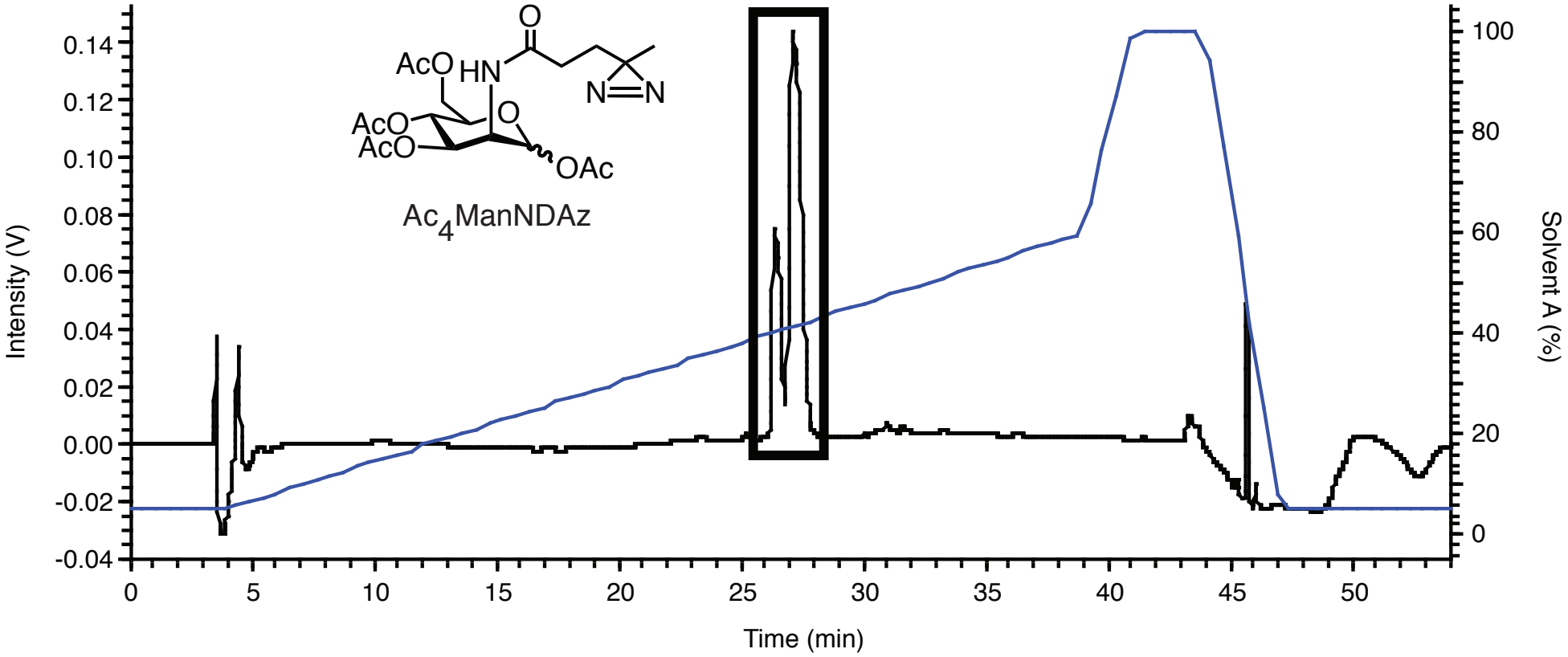

2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Intensity (V)
0
5
10
15
20
25
30
35
Time (Min)
AcO
HN
O
N3
AcO
AcO
O
OAc
Ac4ManNAz

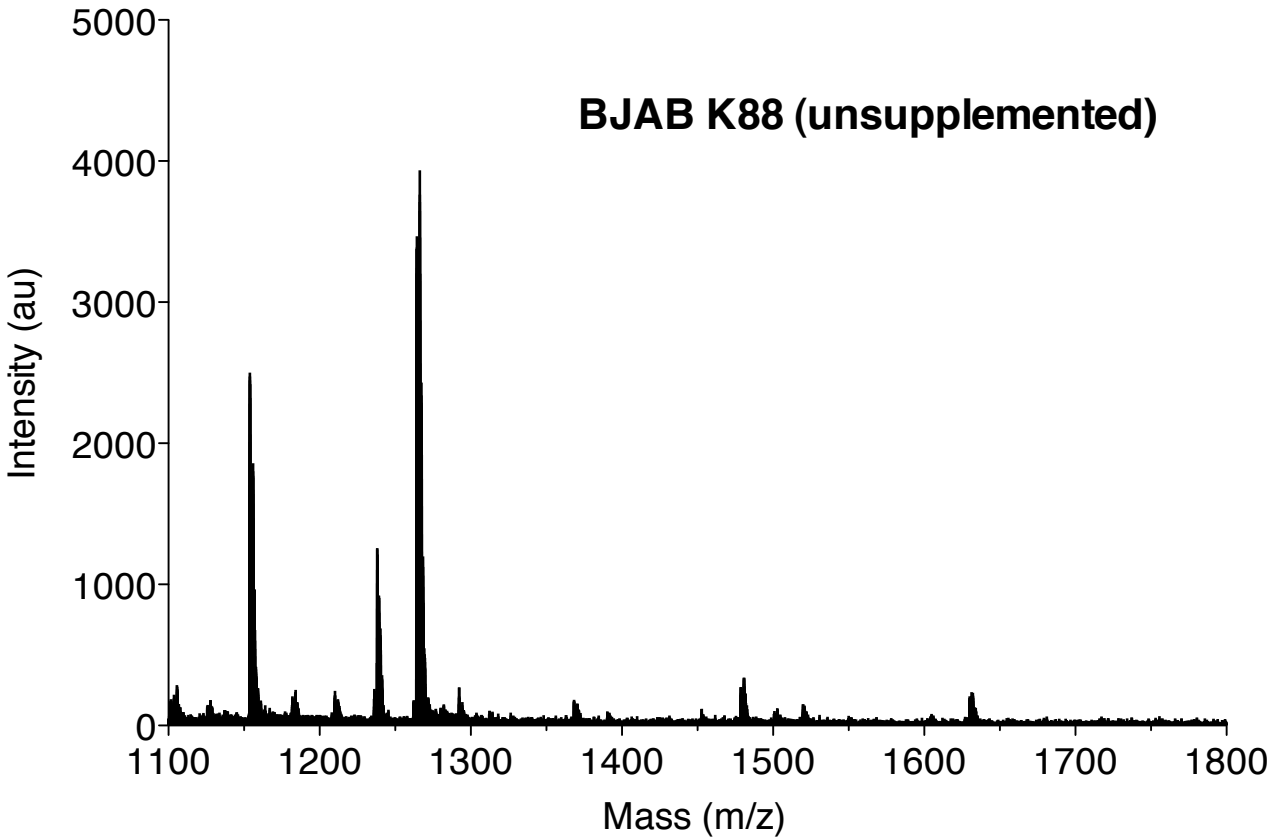

AcO
HN
O
N
N
AcO
O
AcO
OAc
Ac4ManNDAz
CH2Cl2
H2O
f1 (ppm)

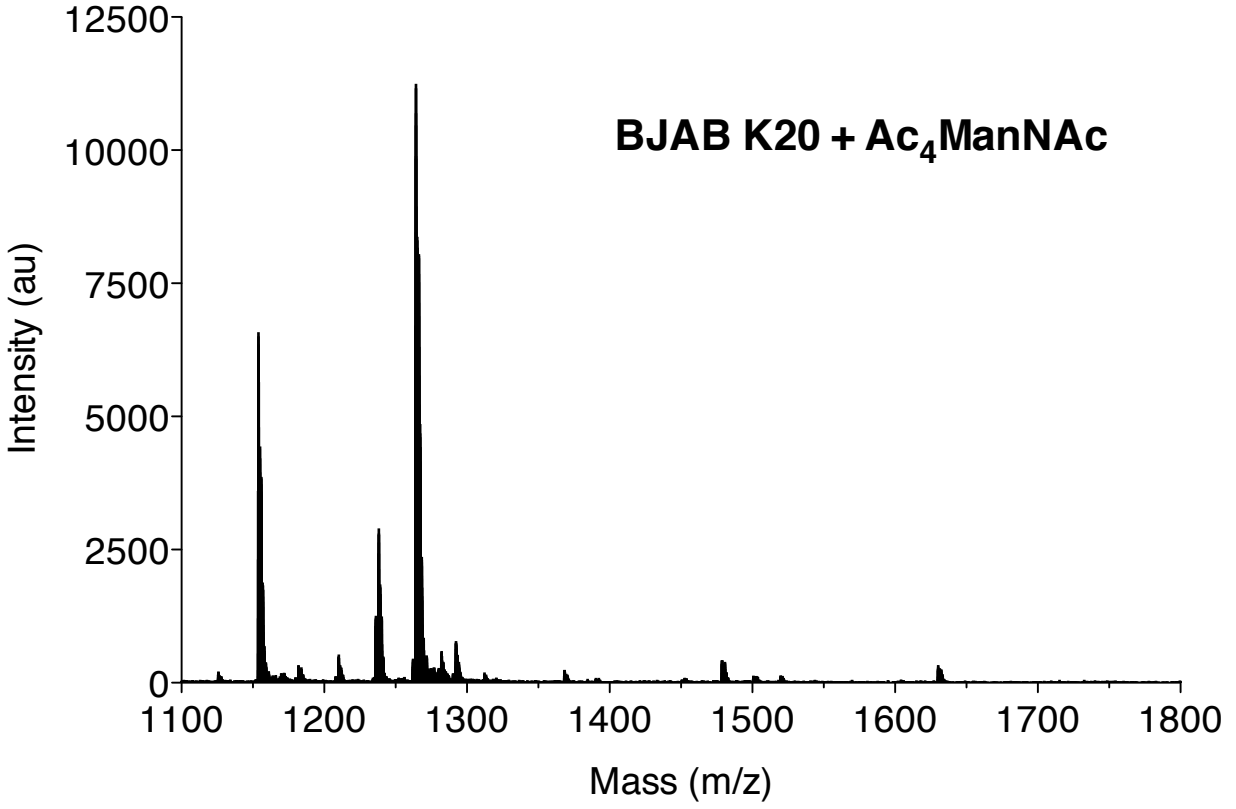

172.14
171.62
170.78
170.75
170.28
170.20
169.91
169.82
168.53
168.33
91.79
90.80
77.22
73.67
71.97
69.06
65.32
62.15
62.02
49.76
49.55
30.84
30.71
30.05
29.94
25.54
25.50
21.08
20.97
20.95
20.91
20.88
20.85
20.19
20.18
AcO
HN
O
N=N
AcO
AcO
O
OAc
Ac4ManNDAz
f1 (ppm)

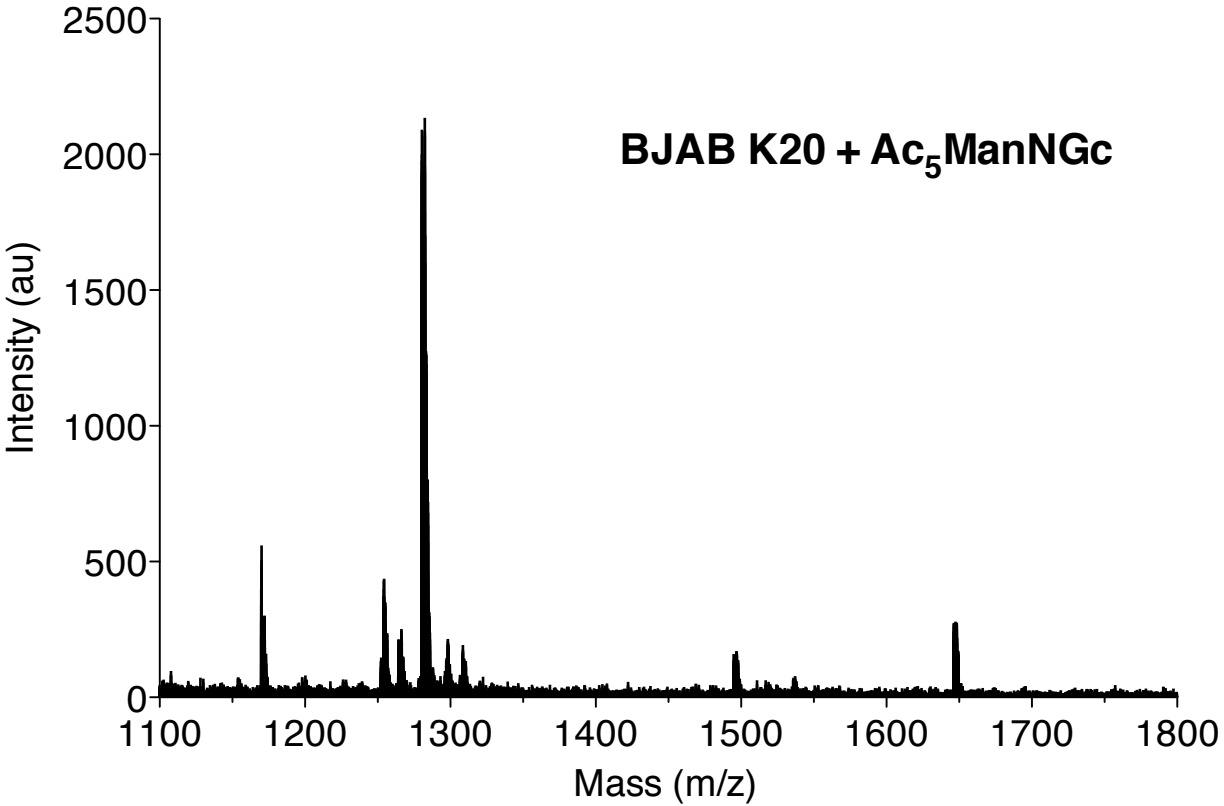
$Ac_4ManNDAz$

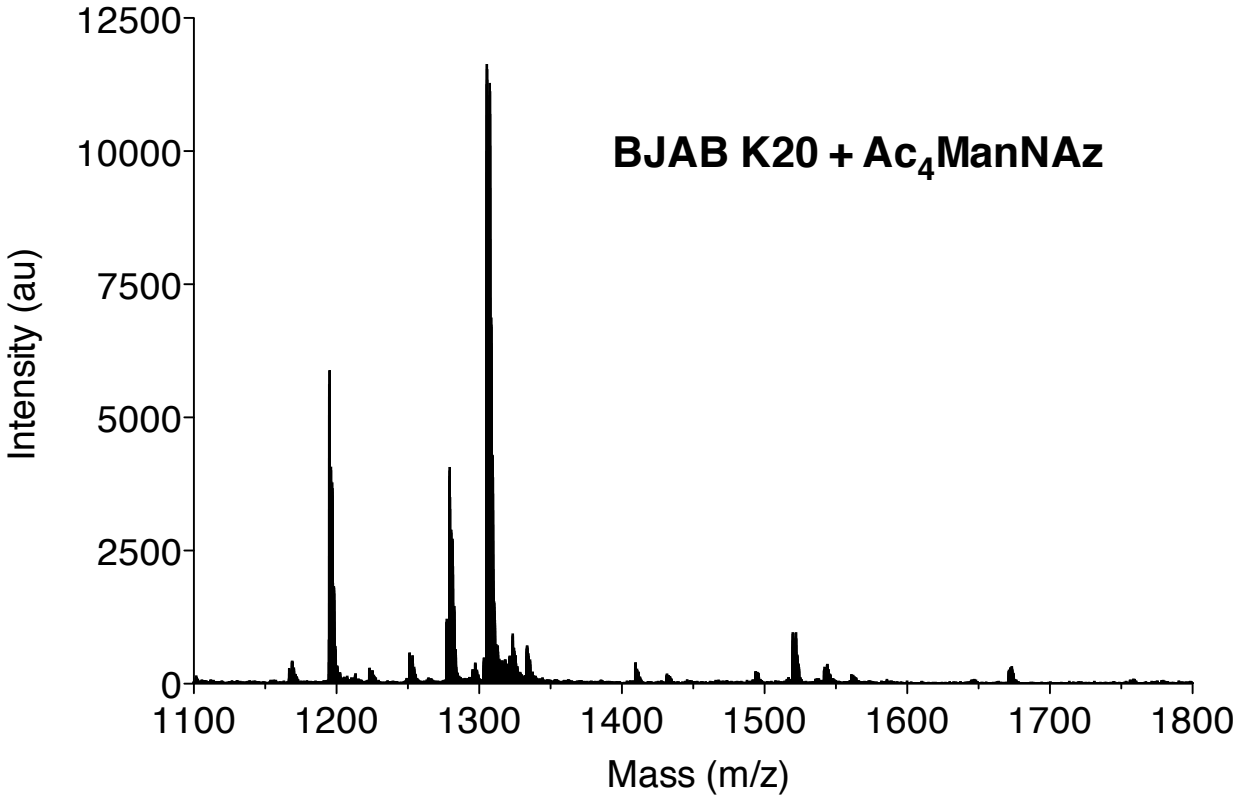

400000
300000
200000
100000
0
Intensity (au)
457.16
0
150
300
450
600
750
900
1050
1200
1350
1500
Mass (m/z)
AcO
HN
O
N
N
AcO
AcO
O
OAc
$C_{19}H_{27}N_3O_{10}$ [M] = 457.17

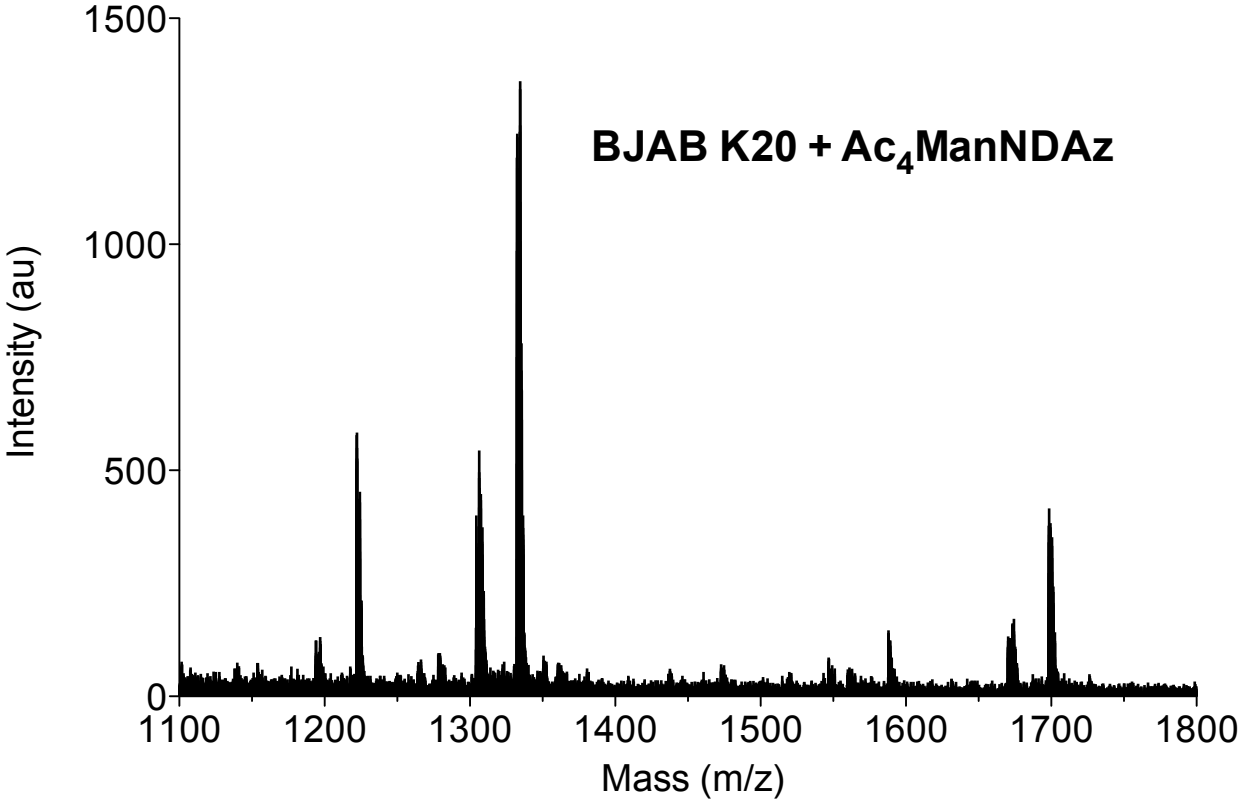

Intensity (V)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
-0.02
-0.04
0
5
10
15
20
25
30
35
40
45
50
Time (min)
100
80
60
40
20
0
Solvent A (%)
AcO
HN
O
N=N
AcO
AcO
O
OAc
Ac4ManNDAz

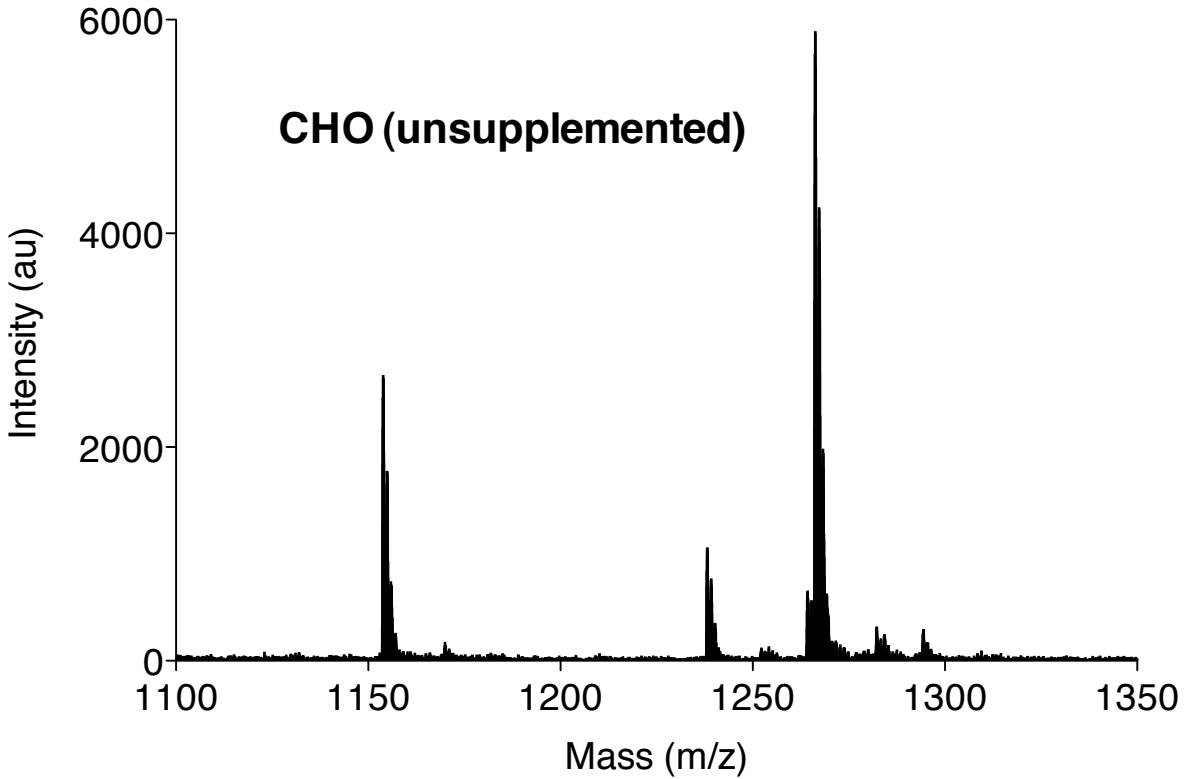

BJAB K88 (unsupplemented)
Intensity (au)
5000
4000
3000
2000
1000
0
1100
1200
1300
1400
1500
1600
1700
1800
Mass (m/z)

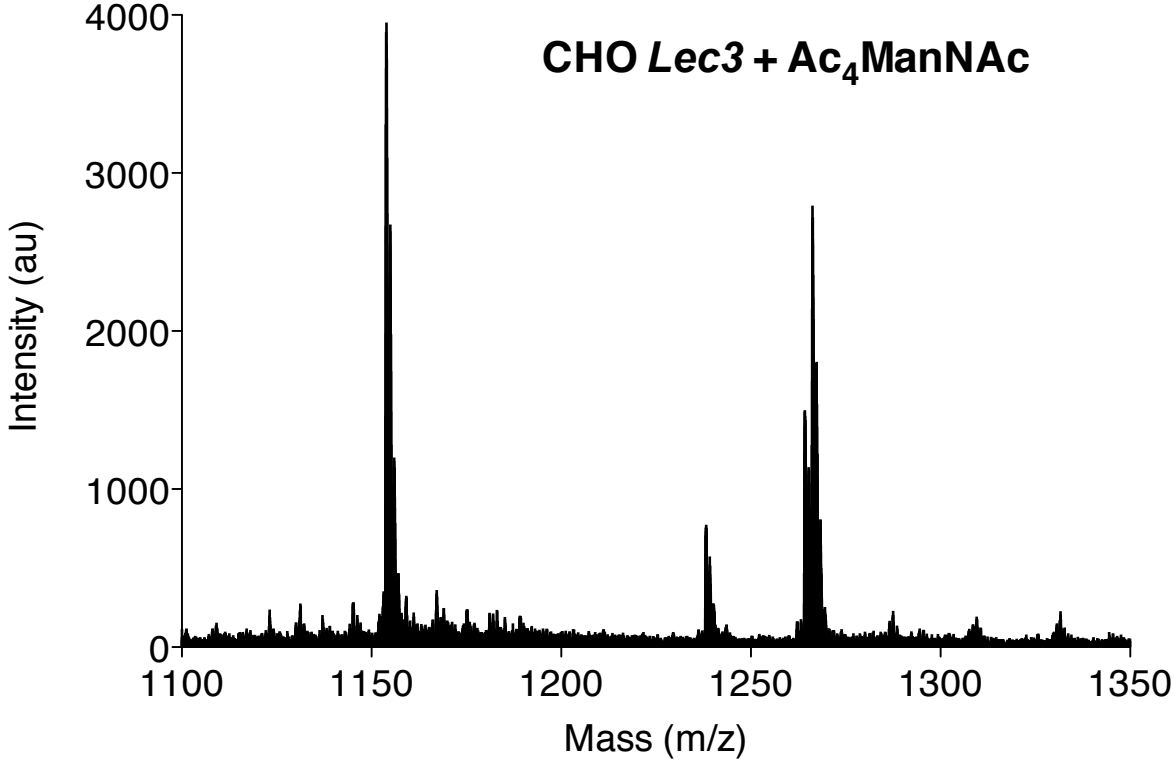

BJAB K20 + Ac4ManNAc
Intensity (au)
12500
10000
7500
5000
2500
0
1100
1200
1300
1400
1500
1600
1700
1800
Mass (m/z)

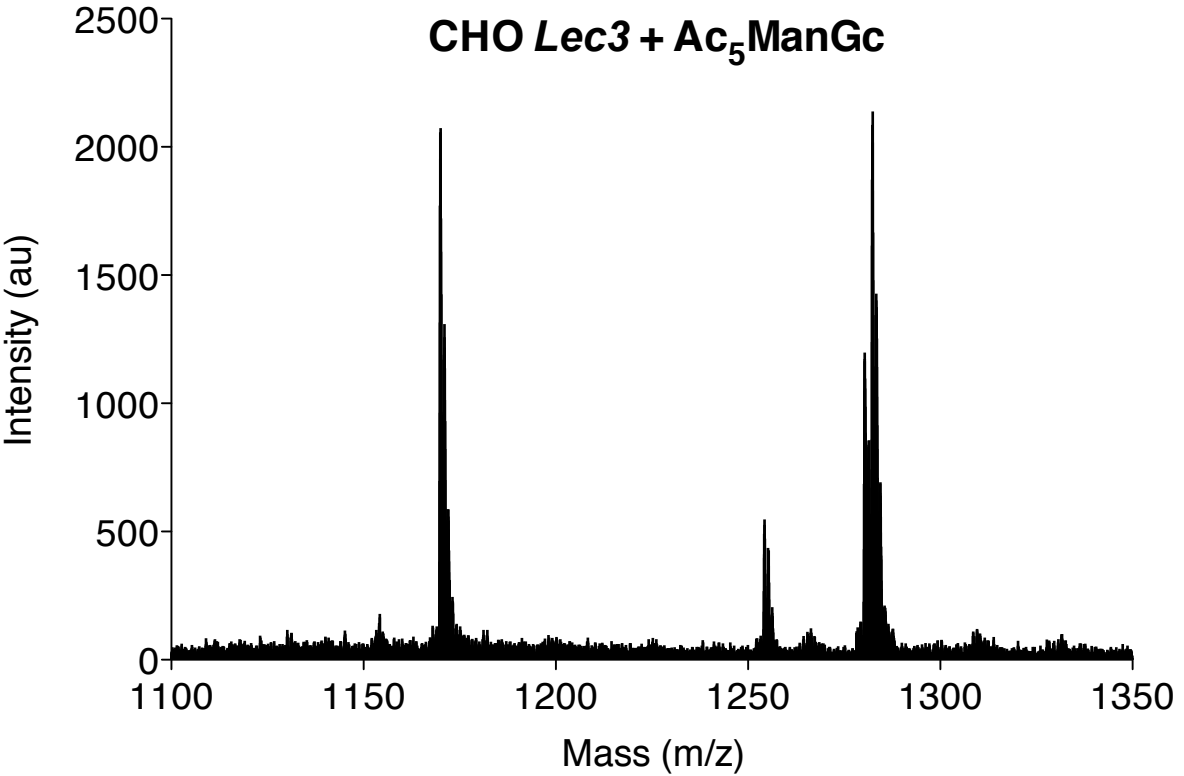

BJAB K20 + Ac5ManNGc
Intensity (au)
2500
2000
1500
1000
500
0
1100
1200
1300
1400
1500
1600
1700
1800
Mass (m/z)

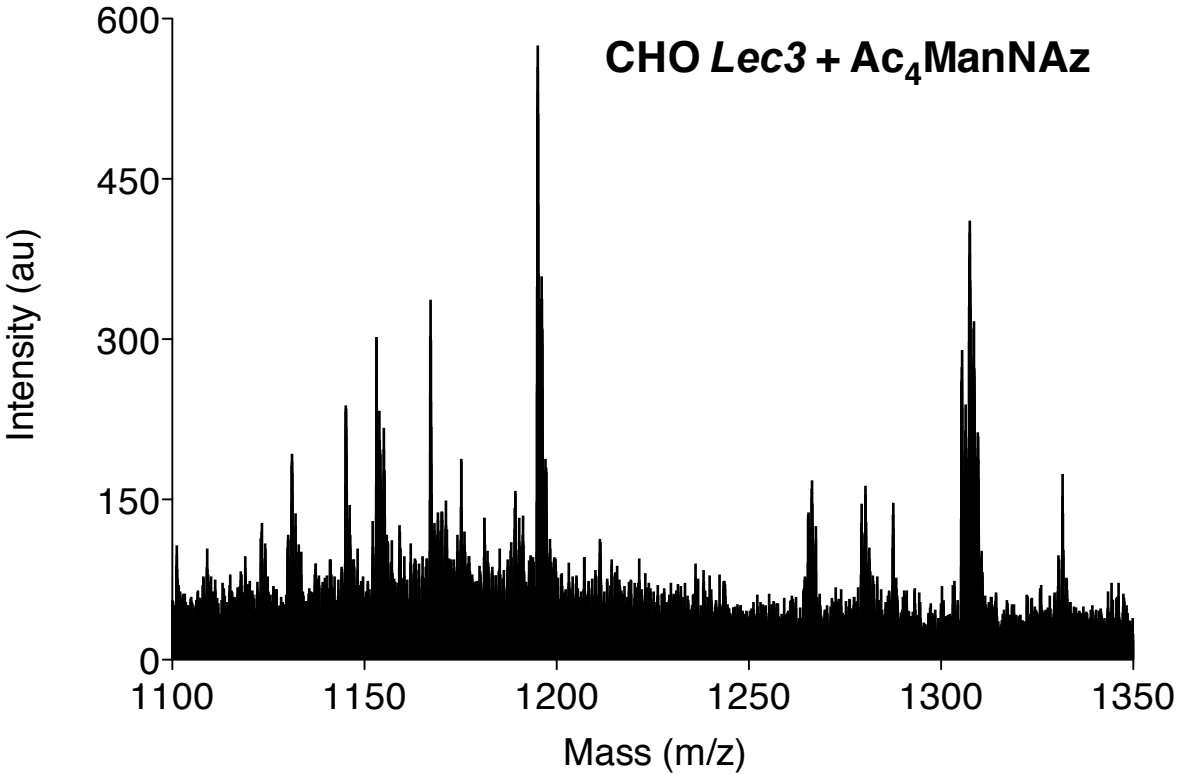

BJAB K20 + Ac4ManNAz
Intensity (au)
12500
10000
7500
5000
2500
0
1100
1200
1300
1400
1500
1600
1700
1800
Mass (m/z)


BJAB K20 + Ac4ManNDAz
Intensity (au)
1500
1000
500
0
1100
1200
1300
1400
1500
1600
1700
1800
Mass (m/z)


CHO (unsupplemented)
Intensity (au)
6000
4000
2000
0
1100
1150
1200
1250
1300
1350
Mass (m/z)


CHO *Lec3* + Ac_4ManNAc
Intensity (au)
4000
3000
2000
1000
0
1100
1150
1200
1250
1300
1350
Mass (m/z)


CHO Lec3 + Ac5ManGc
Intensity (au)
2500
2000
1500
1000
500
0
1100
1150
1200
1250
1300
1350
Mass (m/z)


CHO Lec3 + Ac4ManNAz
Intensity (au)
600
450
300
150
0
1100
1150
1200
1250
1300
1350
Mass (m/z)