Supplemental information

Protected *N*-Acetyl Muramic Acid Probes Improve Bacterial Peptidoglycan Incorporation via Metabolic Labeling

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Table of Contents:

I. General Information: Materials and Methods

Materials. All reagents were purchased from Sigma Aldrich, Fisher Scientific, Alfa Aesar, Chem-Impex, Click Chemistry Tools, or Oakwood Chemical and used without further purification. IRA H^+ resin was rinsed with either methanol or water depending on solvent used in reaction. NMR solvents were purchased from Cambridge Isotope Laboratories, Inc.

General Procedures. Unless otherwise noted, all reactions were performed in oven- or flame-dried glassware under an atmosphere of nitrogen equipped with rubber septa, and magnetic stirring. All solvents were anhydrous and transferred via stainless steel syringe. Reactions were monitored by electrospray ionization liquid chromatography-mass spectrometry (ESI LC-MS) and thin layer chromatography (TLC) in which glass plates coated with silica gel (250 µM, Silica Gel HL, Sorbent Technology) were used and visualized with shortwave 254 nm UV light and/or developed upon heating with *p*-anisaldehyde (PAA). Flash chromatography was carried out on silica gel (60 Å, 40-63 µM, Sorbent Technologies). Preparative HPLC purification was performed on a Waters 2767 Sample Manager with HPLC and SQD2 MS using a Sunfire® Prep C18 OBD 5 µM 19x100 mm or 4.6 x 50 mm columns.

Instrumentation. All NMR spectra were recorded on Bruker AV 400 MHz, AV Neo 600 and AV III 600 MHz NMR spectrometers. Proton chemical shifts were recorded in parts per million (ppm) on the δ scale, downfield from tetramethylsilane and referenced from an internal standard of the residual protium in the NMR solvents. Assignments are made for the major species; full spectrum for all new compounds are shown. Data for ^{13}C NMR were reported in ppm downfield from tetramethylsilane and referenced based on the chemical shift from the carbon resonances of the solvent. Hydroxyl protons were exchanged with D_2O or acetic acid-d_4. NMR data were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad), coupling constant in Hz, integration, and assignment based on two dimensional COSY, HSQC and HMBC. High-resolution mass spectra (HR-MS), ESI mode, were obtained on a Thermo Q-Exactive Orbitrap at the Mass Spectroscopy Facility at the Department of Chemistry, University of Delaware. Confocal microcopy images were taken a Zeiss LSM900 instrument with Airyscan 2 and with Plan-Apochromat 63X/1.40 Oil DIC M27 objective. Growth curves were performed using the Eppendorf BioSpectrometer (140 minutes) and Tecan Spark Plate Reader- serial number: 2006012422 (6 hours).

II. Growth Curve Assays

Growth Curve Assay using multi-channel UV-Vis (140 minutes)

Overnight pre-cultured *E. coli ΔMurQ-KU* [S3] cells were inoculated into fresh LB medium supplemented with kanamycin and chloramphenicol and were incubated shaking at 37°C until the OD_{600nm} was approximately 0.600. 1 mL of cell culture was incubated in 5 mL sterilized tubes. 600 µM or 60 µM sugar substrates **10**, **12a**, or **12** (0.1 M stock in mq H_2O) , 1mM IPTG and 200 µg/mL fosfomycin were added into experimental samples, and water was used as a negative control. Three biological replicates samples were used for each study. Cells were incubated at 37 °C, cell samples were removed every 20 min, and OD_{600nm} was measured (Eppendorf 6136) for 140 min in total. Cell growth curves were formulated with Origin 2019.

Growth Curve Assay with Plate Reader (6 hours)

Overnight pre-cultured *E. coli ΔMurQ-KU* [S3] cells were inoculated into fresh LB medium supplemented with kanamycin and chloramphenicol and were incubated shaking at 37°C until the OD_{600nm} was approximately 0.200. 4 mL of overnight cells were added to a new sterile tube and 1mM IPTG and 200 µg/mL fosfomycin were added. 100 µL of cell solution was added into each well of a white, clear bottom 96 well plate in triplicate for each probe concentration. *N*-acetyl muramic acid (NAM) probes were added at various concentrations from 0 mM to 6 mM, and a different plate was run for each probe, with the NAM serving as the common control. Plates were incubated at 37°C in plate reader where they were shaken for 2 min, scanned at 600 nm, and then repeated every 20 mins for 6 hours. Cell growth curves were formulated with Origin 2019.

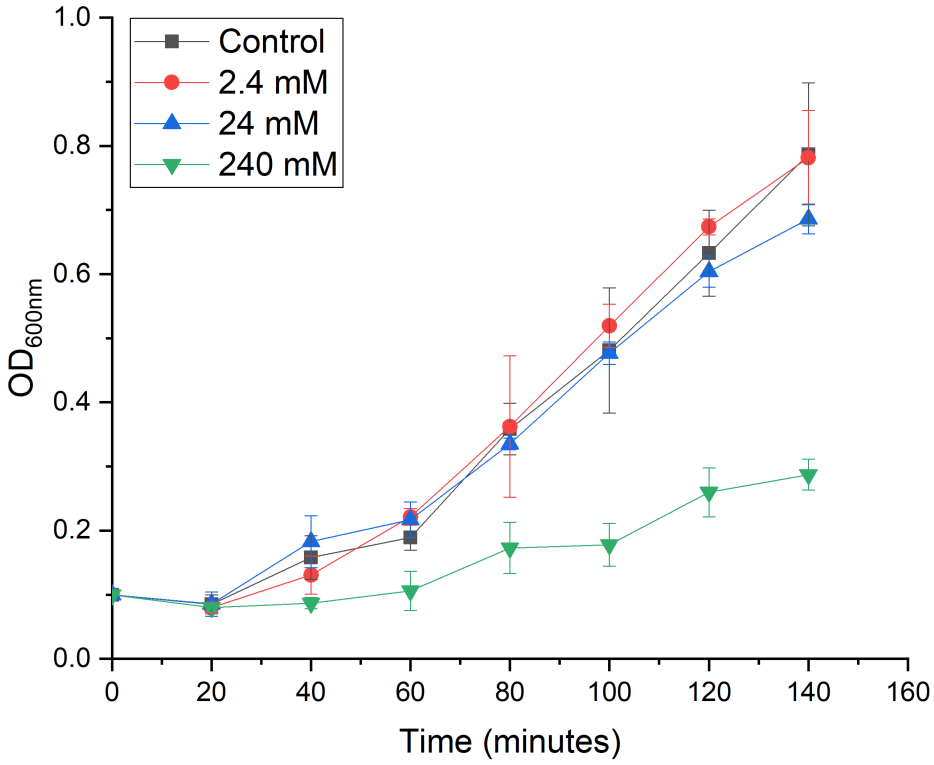


Figure S1: Growth curve of treatment of *E. coli ΔMurQ-KU* cells with sodium acetate. Removal of acetates at 6 mM probe or less will not affect cell growth rate. Control cells are untreated *E. coli ΔMurQ-KU cells.* Error bars represent the standard deviation of three biological replicates from one trial. The graph is representative of the average of triplicates from one of the three biological experiment replicates.

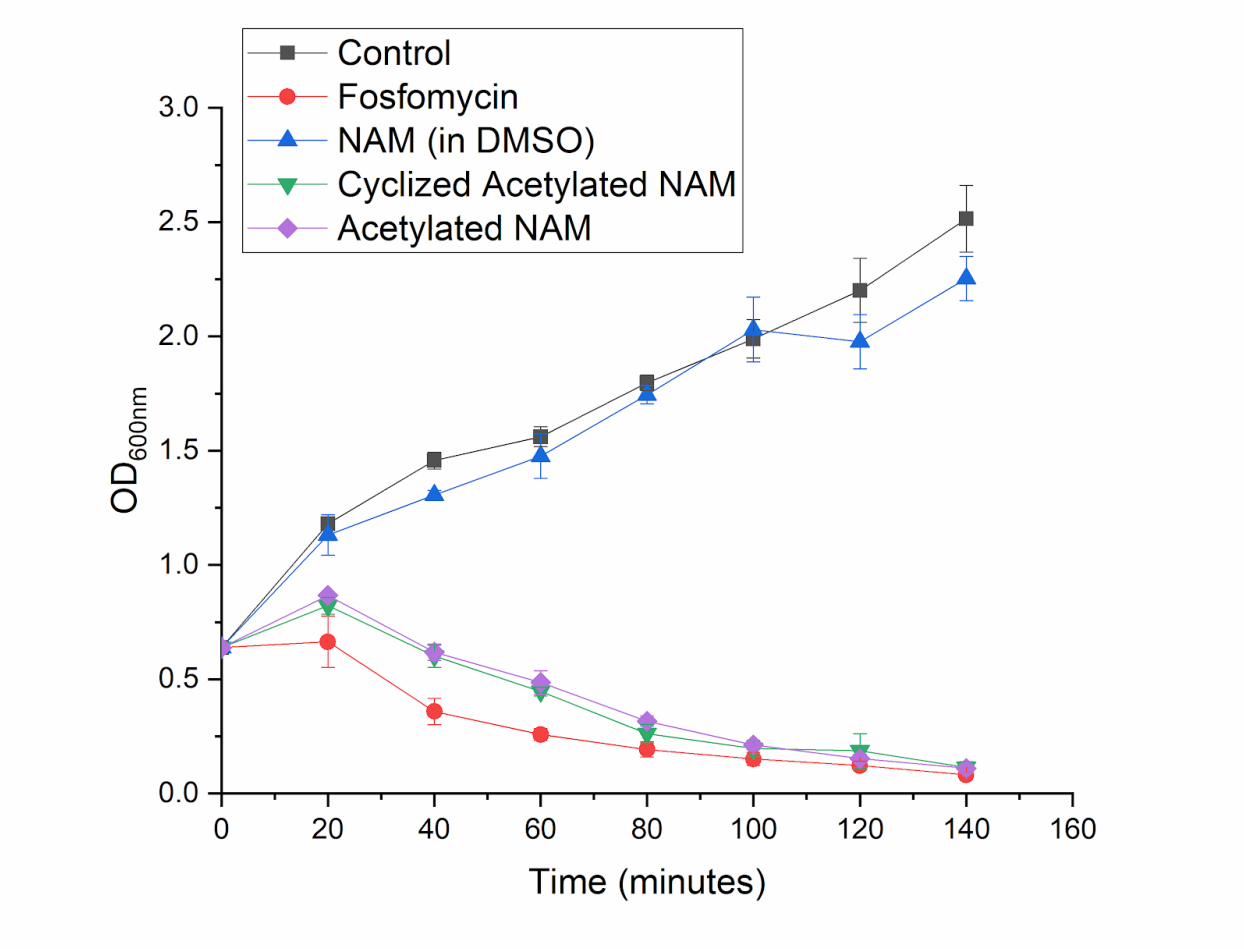


Figure S2: Cyclized (**12a**) and acetylated (**12**) compounds are not incorporated into the cell. As acetylated compounds are only soluble in DMSO, we ensured that the DMSO solvent did not stymie growth by using a NAM DMSO control. Control cells are untreated *E. coli ΔMurQ-KU* cells and Fosfomycin sample was treated with lethal dose of fosfomycin but no NAM (**10**) as a negative control (red). All other samples are treated with a lethal dose of fosfomycin and their respective compound. Error bars represent the standard deviation of three biological replicates from one trial. The graph is representative of the average of triplicates from one of the three biological experiment replicates.

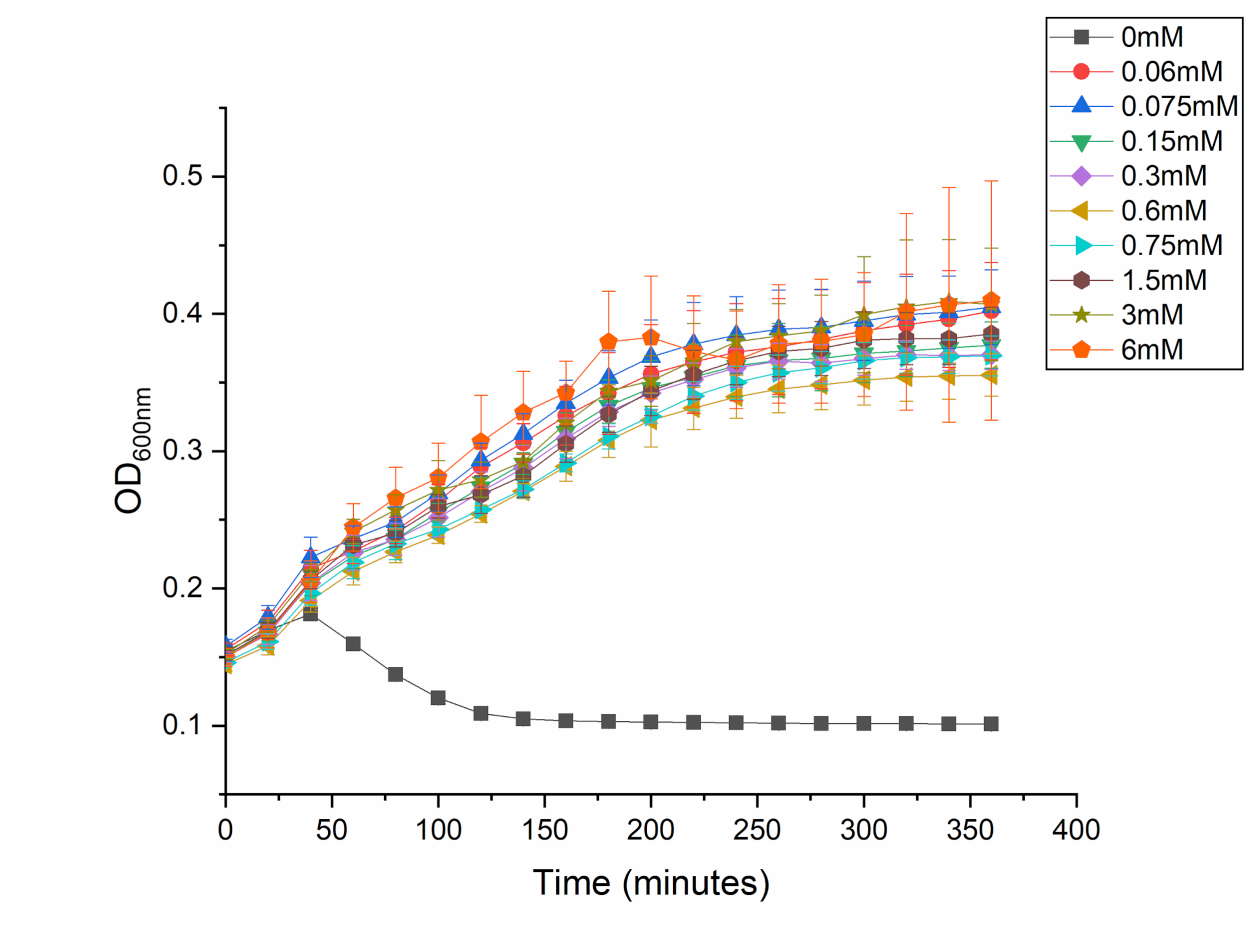


Figure S3: Incorporation of NAM (**10**) probe at various concentrations. NAM (**10**) is incorporated at all concentrations tested. All samples are treated with a lethal dose of fosfomycin and their respective compound at the concentration listed. Error bars represent the standard deviation of three biological replicates from one trial. The graph is representative of the average of triplicates from one of the three biological experiment replicates.

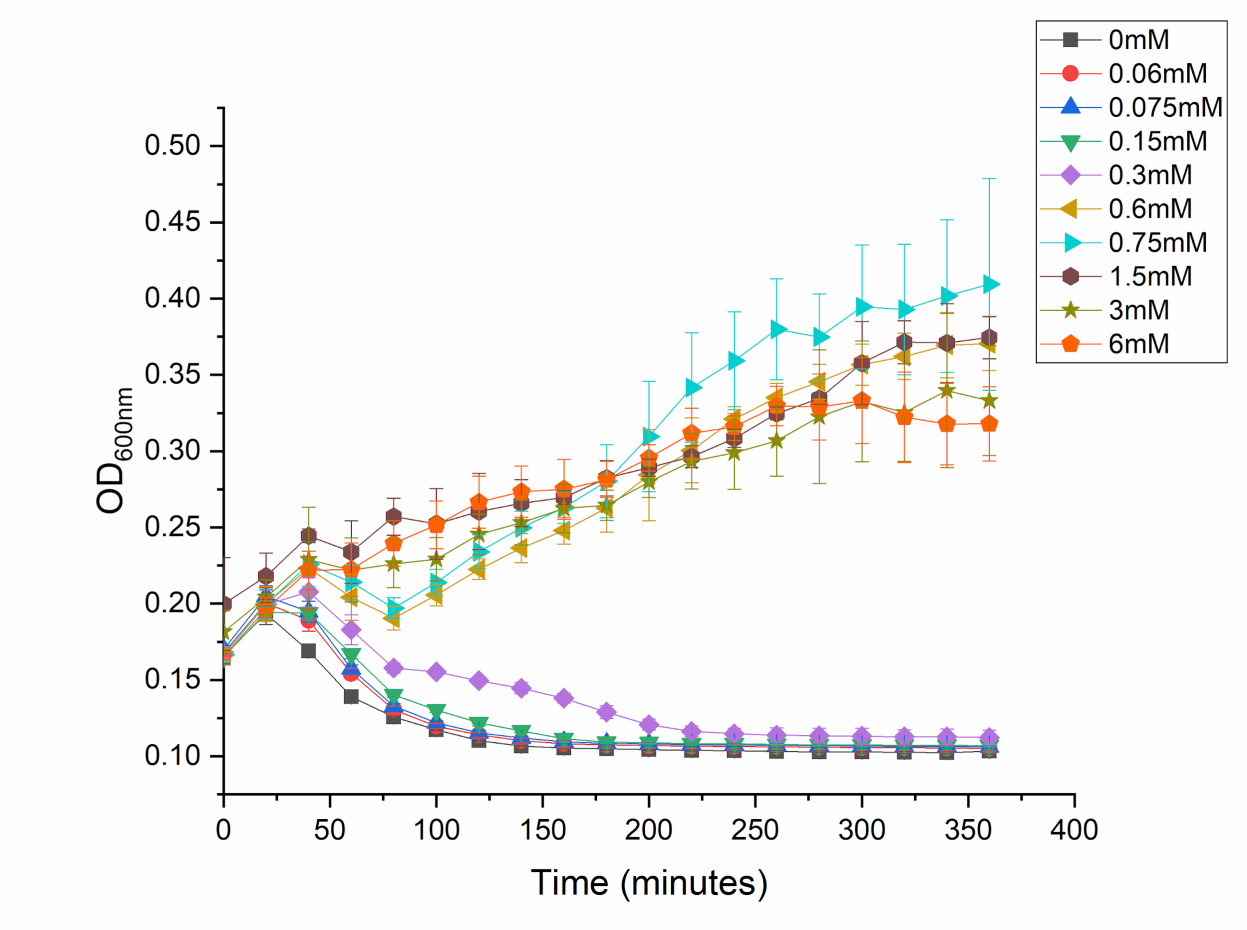


Figure S4: Incorporation of AzNAM probe (**7**) at various concentrations. AzNAM (**7**) recovers cell growth at concentrations 600 μM and above. All samples are treated with a lethal dose of fosfomycin and their respective compound at the concentration listed. Error bars represent the standard deviation of three biological replicates from one trial. The graph is representative of the average of triplicates from one of the three biological experiment replicates.

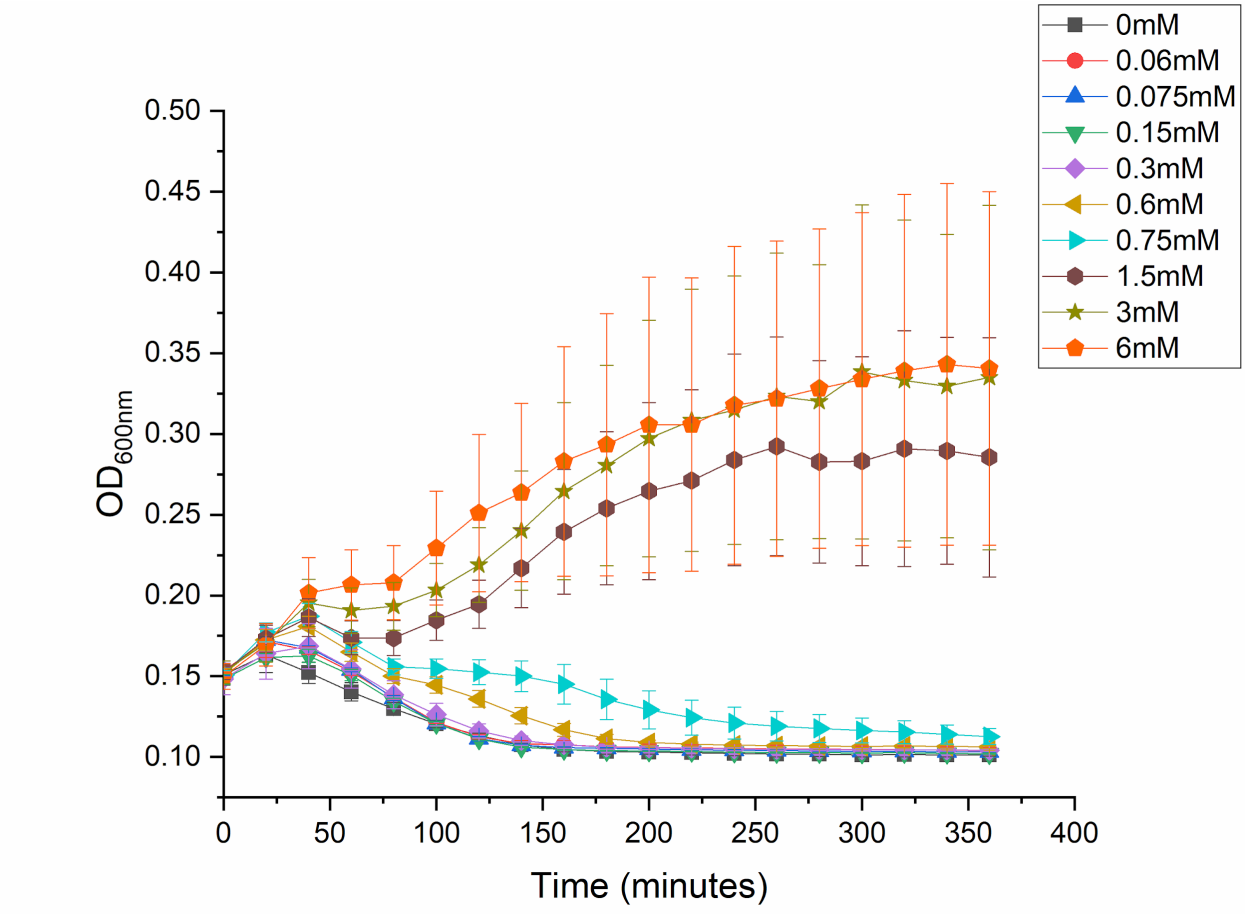


Figure S5: Incorporation of AlkNAM probe (**9a**) at various concentrations. AlkNAM (**9a**) recovers cell growth at concentrations 1.5 mM and above. All samples are treated with a lethal dose of fosfomycin and their respective compound at the concentration listed. Error bars represent the standard deviation of three biological replicates from one trial. The graph is representative of the average of triplicates from one of the three biological experiment replicates.

III. Bacterial Growth, Bioorthogonal Cell Labeling Procedures, and Microscopy

Overnight pre-cultured *E. coli ΔMurQ-KU* cells[S3] were inoculated into fresh LB medium supplemented with kanamycin and chloramphenicol and were incubated shaking at 37°C until the OD_{600nm} was approximately 0.600. 1.2 mL of cells were collected by centrifugation at 8,000 rpm for 5 min at room temperature. *E. coli ΔMurQ-KU* were resuspended in 200 µL LB medium. NAM probes at various concentrations (6 mM, 600 µM, and 60 µM final concentration; 0.1M stock in mqH_2O), 200 µg/mL fosfomycin and 1 mM IPTG were added to the cell samples. Cells were incubated at 37°C for 1 hour. Cells were then collected (10,000 rpm, 2 min) and washed with 600 µL 1 x PBS buffer twice. After the wash, cells were resuspended in 1xPBS buffer and fixed at room temperature with 4% paraformaldehyde in 1 x PBS for 20 min. The cells were washed two more times with 200 µL 1 x PBS. Cells were resuspended in 200 µL 1 x PBS to prepare for the click reaction. To the bioorthogonally tagged bacterial cells was sequentially added 1 mM $CuSO_4$ solution, 140 µM BTTAA (Click Chemistry Tools), 1.2 mM freshly prepared (+)- sodium (L) ascorbate (Sigma-Aldrich), and 7.5µM Alk488 (Sigma Aldrich). Cells

were incubated at room temperature on the shaker for 30 min in the dark. Cells were washed 2 times with 600 µL 1 x PBS then 3 washes with 200 µL 1 x PBS with the second wash standing at room temperature for 30 min. The cells were resuspended in 100 µL 1 x PBS. 15µL of cells were added to pre-treated coverslips for microscopy preparation. The rest of the cells were stored in 4°C until flow cytometry analysis.

Confocal Microscopy

Slides with cell samples were imaged with Airyscan Confocal Microscopy. Images were taken on a Zeiss LSM 900 microscope with Airyscan 2 and Plan-Apochromat 63x/1.4 Oil differential interference contrast (DIC) M27 objective. Excitation of Alk488 were achieved with 488 nm, 0.2% laser excitations. Scan mode was frame, and scan mode was bidirectional. Airyscan detector was used (GaAsP-PMT). Program Carl Zeiss ZEN 2012 was used to process the raw data to construct the SIM images. Processing and filtering settings were kept constant and image intensity was preserved with the raw image scale option in Zen 2012. Two-dimensional (2D) Airyscan images were generated with Zen 2012. Scale bars were made with the line measurement tool function.

Flow Cytometry

Flow cytometry was performed on ACEA Novocyte Flow Cytometer. Samples were briefly vortexed before each run. 100,000 cell counts were collected for each sample and were analyzed in triplicate, and fluorescence intensities (height) were generated and overlaid.

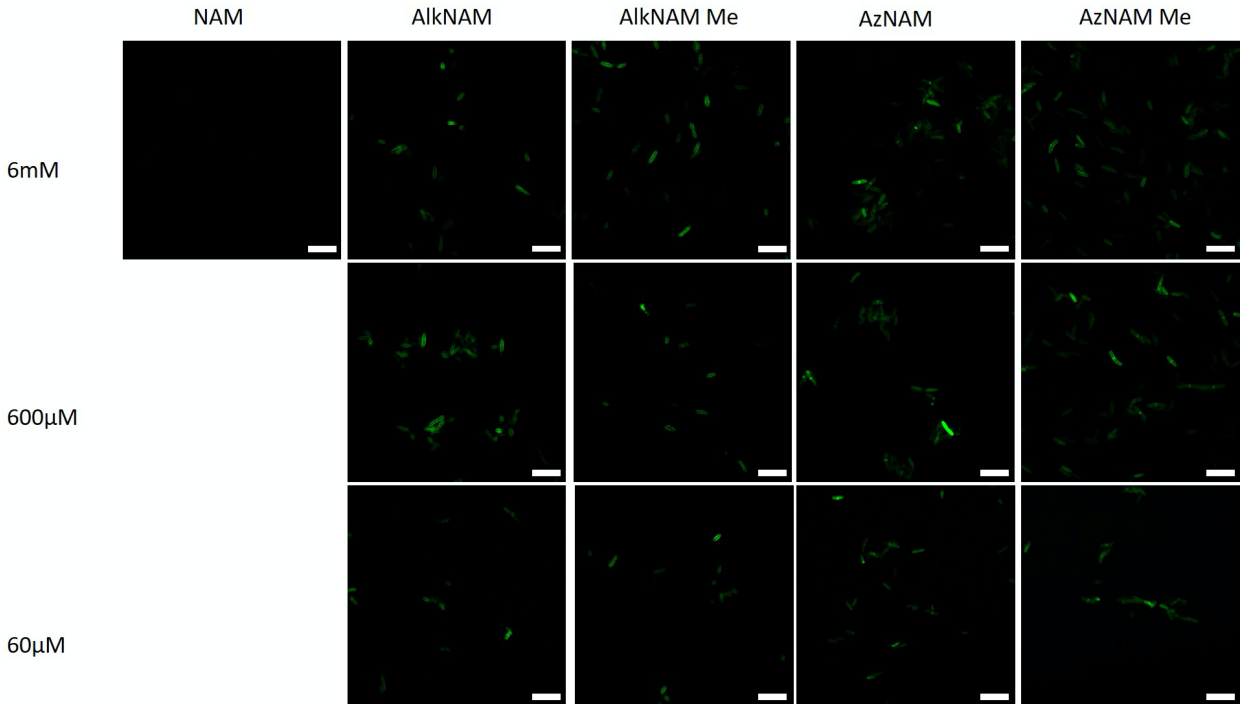


Figure S6: Confocal microscopy images of *E. coli ΔMurQ-KU* bacteria remodeled with NAM (**10**), AlkNAM (**9a**), AlkNAM methyl ester (**9**), AzNAM (**7**), and AzNAM methyl ester (**8**) compounds. *E. coli*

ΔMurQ-KU cells were remodeled with probe of varying concentrations (6mM, 600µM, or 60µM) and labeled with fluorophore (green) using copper (I) catalyzed azide alkyne cycloadditions. Images were prepared in Zen Blue and are representative of at least three fields of view per sample with three biological replicates. Scale bars = 10 µm

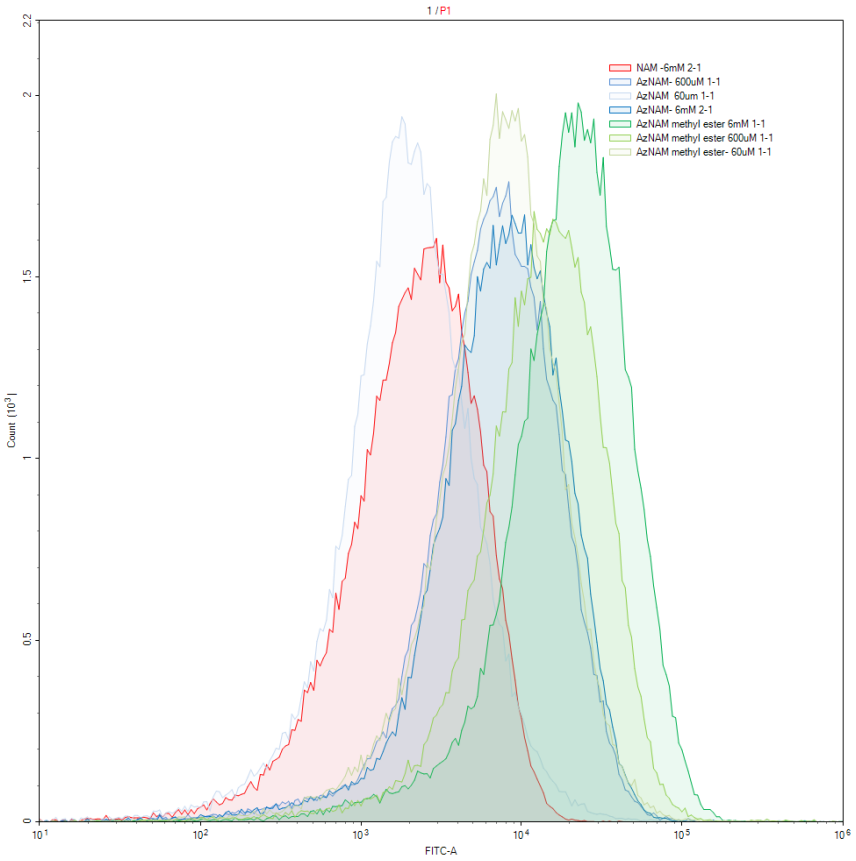


Figure S7: Flow cytometry verification of incorporation of AzNAM (**7**) and AzNAM methyl ester (**8**) compounds into the cell wall. Data shown is representative of experiments performed in technical duplicate and biological triplicate.

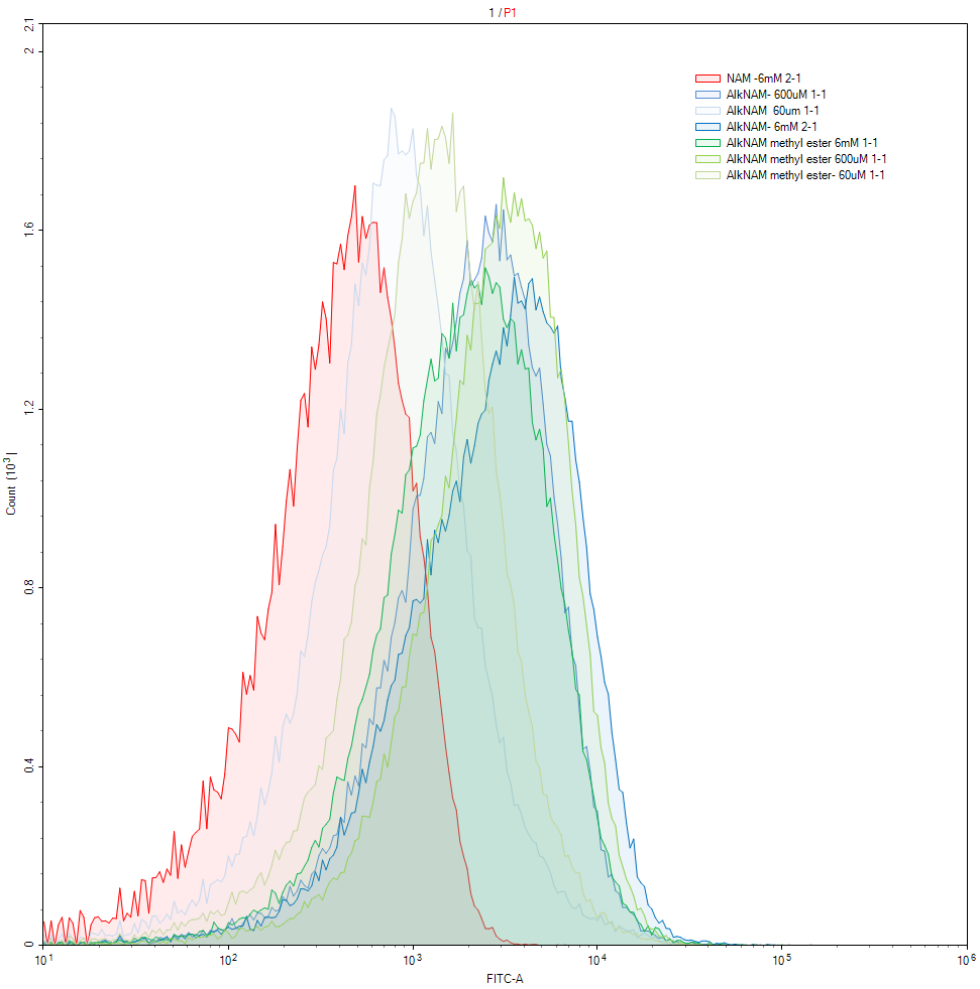


Figure S8: Flow cytometry verification of incorporation of AlkNAM (**9a**) and AlkNAM methyl ester (**9**) compounds into the cell wall. Data shown is representative of experiments performed in technical duplicate and biological triplicate.

IV. Mass Spectrometry Incorporation Assay

Overnight pre-cultured *E. coli ΔMurQ-KU* cells were inoculated into 33.3 µL of fresh LB medium supplemented with kanamycin and chloramphenicol and were incubated shaking at 37°C until the OD_{600nm} was approximately 0.600. Cells were collected by centrifugation at 3,800G for 5 min at room temperature. *E. coli ΔMurQ-KU* were resuspended in 5 mL LB medium. NAM sugars **8** and **9** at (6 mM final concentration), 200 µg/mL fosfomycin and 1 mM IPTG were added to the cell samples. Cells were incubated at 37°C for 1 hour. Cells were then collected (3,800G, 5 min) and washed with 10 mL of 1xPBS buffer twice. After the wash, cells were resuspended in 2.5 mL of digestion buffer (250 µL of 1 M Tris pH 7.9, 25 µL of 5M NaCl, 20 µL of 0.5 EDTA, 4.75 mL of mq water). Cells were incubated shaking at 37°C. 20 µL of freshly prepared lysozyme was added every 12 hours for 3 days (a total of 6 additions). Cells were added to Amicon Ultra 3K Filter Devices and centrifuged at 3,800G for 30mins. The flowthrough was collected, frozen, and lyophilized. The sample was dissolved in minimal amount of DI water (20µL) and loaded onto an Acquity UPLC BEH C18 column 2.1 × 50 mm (Waters) using a

Dionex UHPLC coupled to a Q-Exactive Orbitrap (Thermo Fisher Scientific). Elute with following gradient:

0.5 mL/min − 1 linear gradient starting from 0% A to 50% B in 4 min.
Eluent A was 0.1% formic acid in water
Eluent B was 0.1% formic acid in acetonitrile.
The absorbance of the eluting peaks was measured at 505 nm and further subjected high-resolution mass analysis on the Q-Exactive.
All data were processed and analyzed on a Thermo Xcalibur Qual Browser.

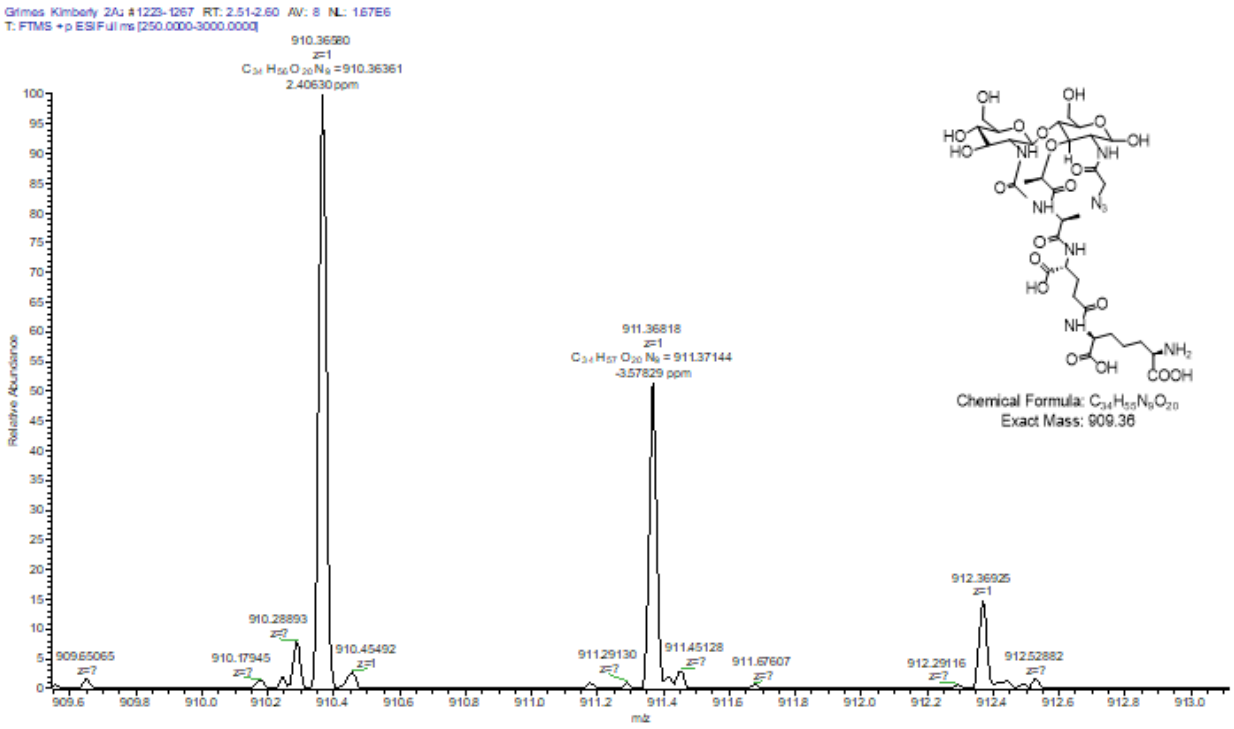


Figure S9: Mass spectrometry verification of incorporation of 2AzNAM methyl ester into the cell wall.

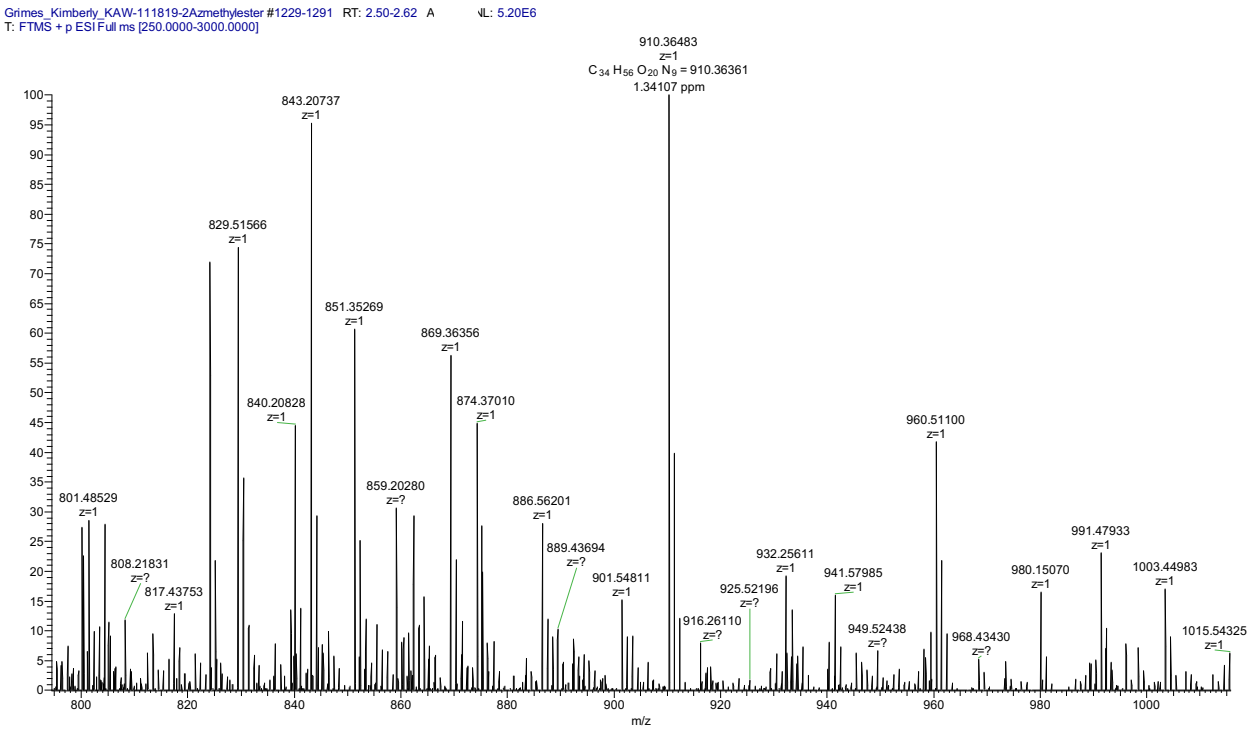


Figure S10: Zoomed out view of mass spectrometry verification of incorporation of 2AzNAM methyl ester into the cell wall.

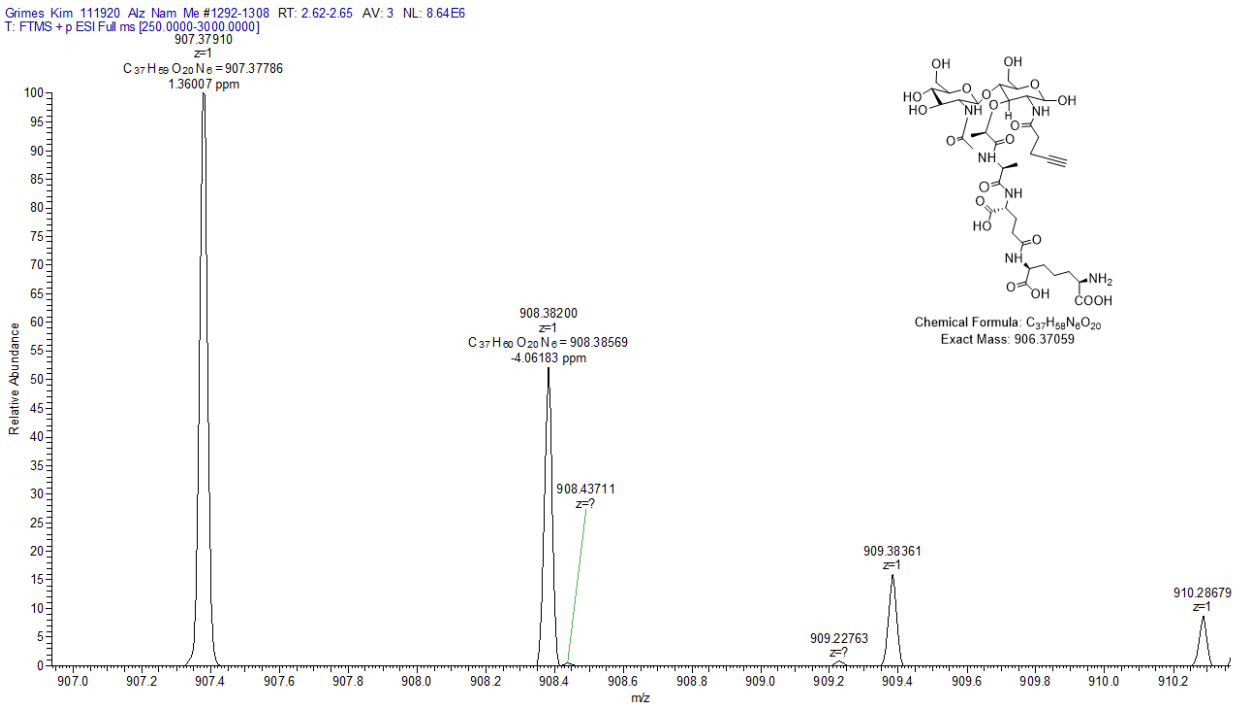


Figure S11: Mass spectrometry verification of incorporation of 2AlkNAM methyl ester into the cell wall.

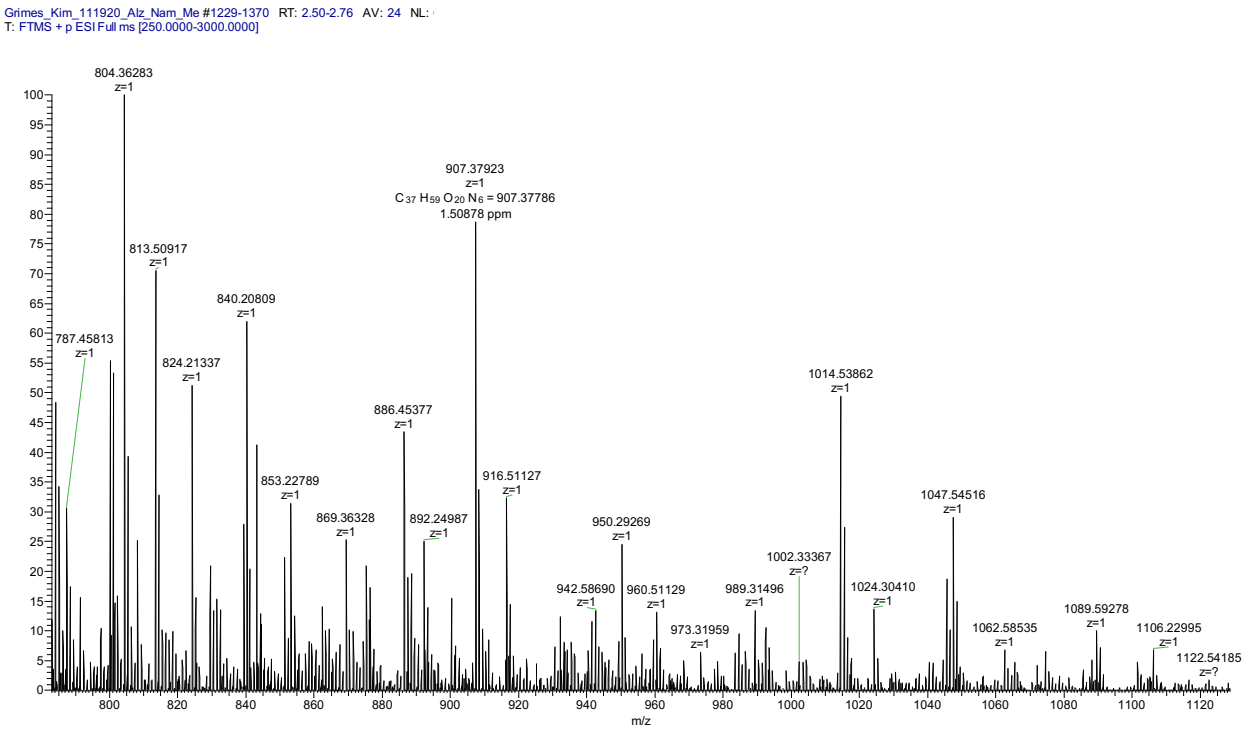


Figure S12: Zoomed out view of mass spectrometry verification of incorporation of 2AlkNAM methyl ester into the cell wall.

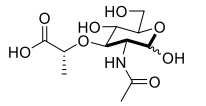
V. Bacterial Lysis and Mass Spectrometry Probe Incorporation Protocol

Overnight pre-cultured *E. coli ΔMurQ-KU* cells (5 mL) were collected by centrifugation at 3,800G for 5 min at room temperature. *E. coli ΔMurQ-KU* were resuspended in lysis buffer (250 µL of 1 M Tris pH 7.9, 25 µL of 5 M NaCl, 20 µL of 0.5 EDTA, 4.75 mL of mq water) and placed on ice for 1 hour. Cells were sonicated for 6 cycles (30 seconds sonication, 30 seconds on ice). NAM sugars **9, 10,** and **12** at (600 µM final concentration) were added to the cell samples. Cells were incubated at 37°C for 1 hour (the same time as an incubation for cell wall labeling). Cells were then added to Amicon Ultra 3K Filter Devices and centrifuged at 3,800G for 30 mins. The flowthrough was collected, frozen, and lyophilized. The sample was dissolved in minimal amount of DI water (20 µL) and loaded onto an Acquity UPLC BEH C18 column 2.1 × 50 mm (Waters) using a Dionex UHPLC coupled to a Q-Exactive Orbitrap (Thermo Fisher Scientific). Elute with following gradient:

0.5 mL/min − 1 linear gradient starting from 0% A to 50% B in 4 min.
Eluent A was 0.1% formic acid in water
Eluent B was 0.1% formic acid in acetonitrile.
The absorbance of the eluting peaks was measured at 505 nm and further subjected high-resolution mass analysis on the Q-Exactive.
All data were processed and analyzed on a Thermo Xcalibur Qual Browser.

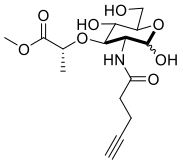
Results and Chromatograms/HRMS Spectra of Identified Fragments:

NAM



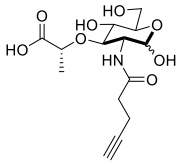
Chemical Formula: $C_{11}H_{19}NO_8$
Exact Mass: 293.11107

AlkNAM methyl ester



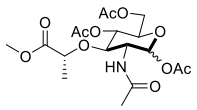
Chemical Formula: $C_{15}H_{23}NO_8$
Exact Mass: 345.14237

AlkNAM



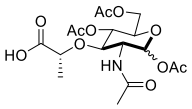
Chemical Formula: $C_{14}H_{21}NO_8$
Exact Mass: 331.12672

Peracetylated NAM methyl ester



Chemical Formula: $C_{18}H_{27}NO_{11}$
Exact Mass: 433.15841

Peracetylated NAM



Chemical Formula: $C_{17}H_{25}NO_{11}$
Exact Mass: 419.14276

SI Table 1:

Entry	Starting fragment	Possible fragment products	Exact Mass	Expected [M+H]	Observed [M+H]	ppm	Retention Time (RT)
1	NAM	NAM	293.11107	294.11834	294.11762	2.47475	0.84-1.02
2	AlkNAM methyl ester	AlkNAM methyl ester	345.14237	346.14964	Not observed	Not observed	0-6
2	AlkNAM methyl ester	AlkNAM	331.12672	332.13399	332.13352	-1.43299	2.29-2.51
3	Peracetylated NAM methyl ester	Peracetylated NAM methyl ester	433.15841	434.16569	434.16535	-0.77921	3.58-4.53
3	Peracetylated NAM methyl ester	Peracetylated NAM	419.14276	420.15004	420.14912	-2.17746	3.40-3.54
3	Peracetylated NAM methyl ester	NAM	293.11107	294.11834	Not observed	Not observed	0-6

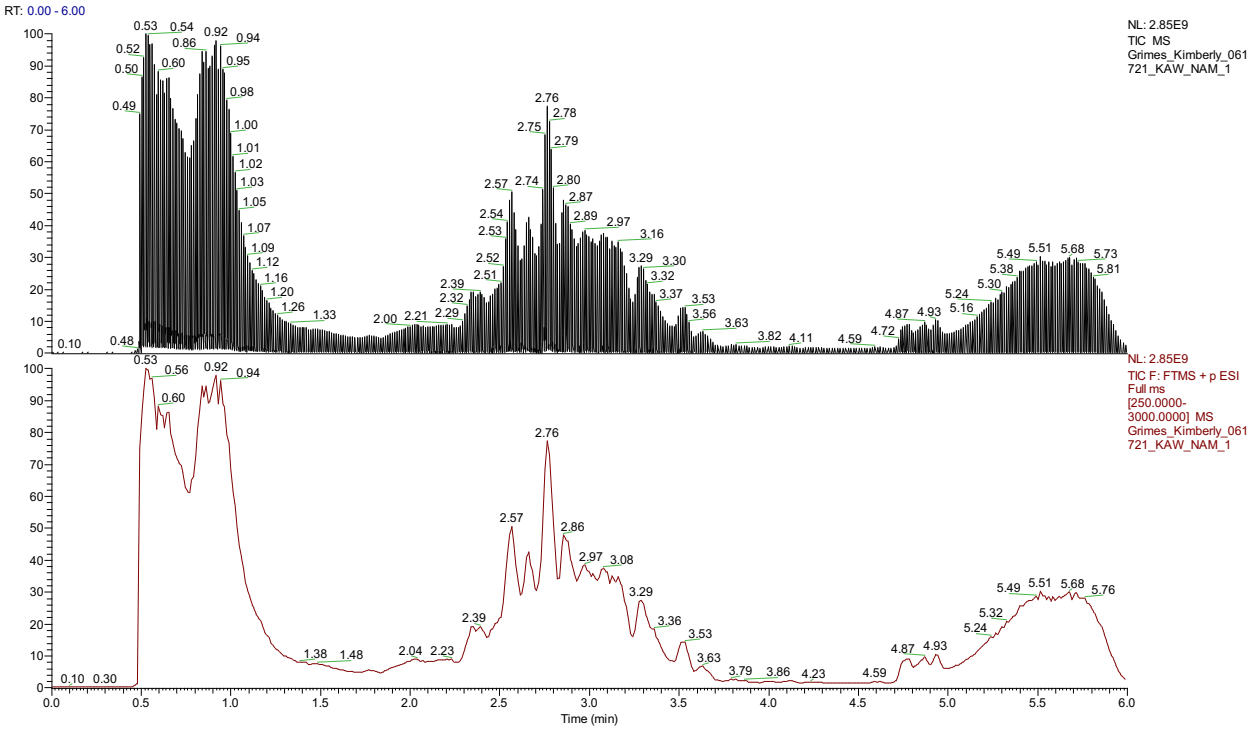


Figure S13: Total ion chromatogram of Sample Entry 1- NAM starting material; NAM product

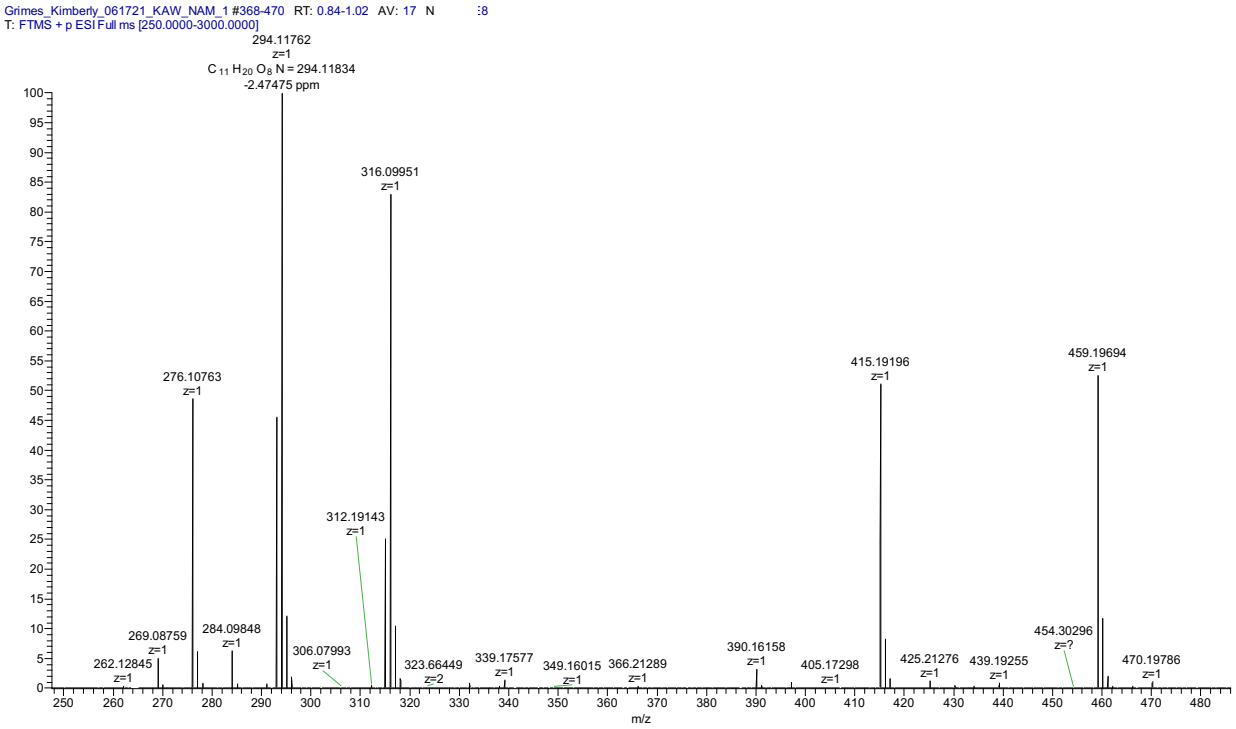


Figure S14: HRMS of Sample Entry 1- NAM starting material; NAM product

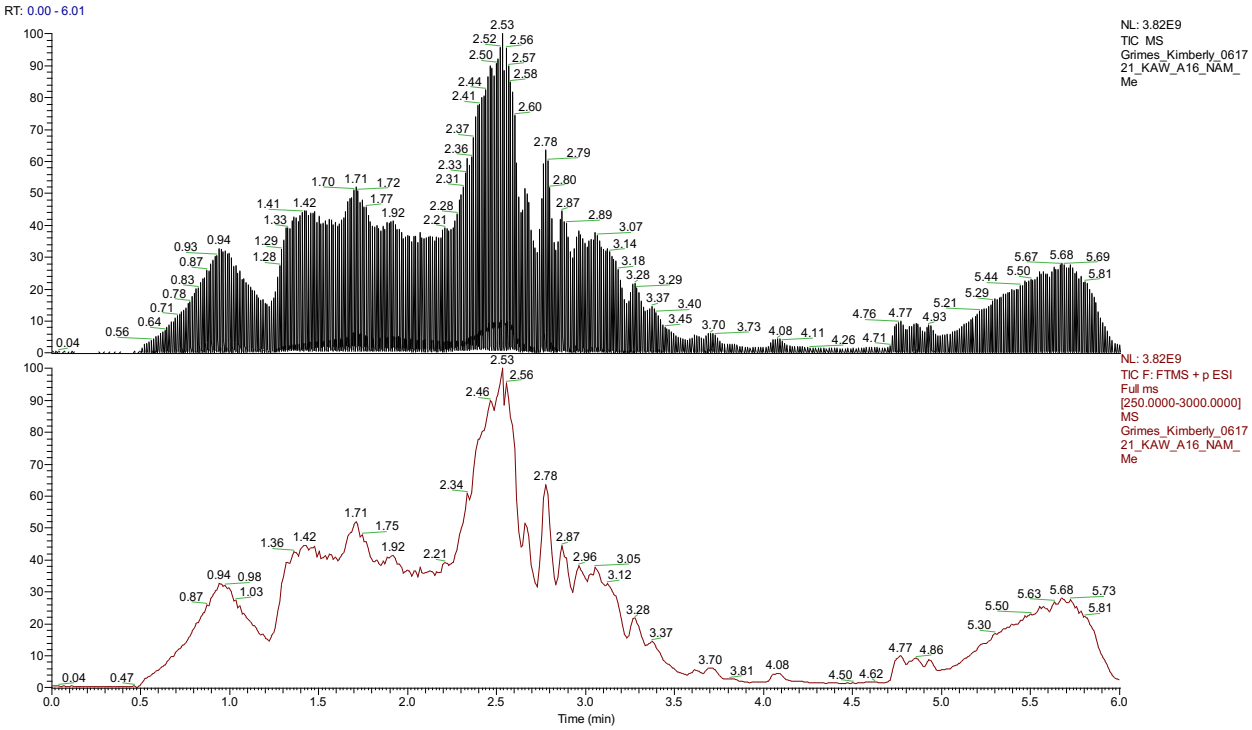


Figure S15: Total ion chromatogram of Sample Entry 2-AlkNAM methyl ester starting material; AlkNAM product

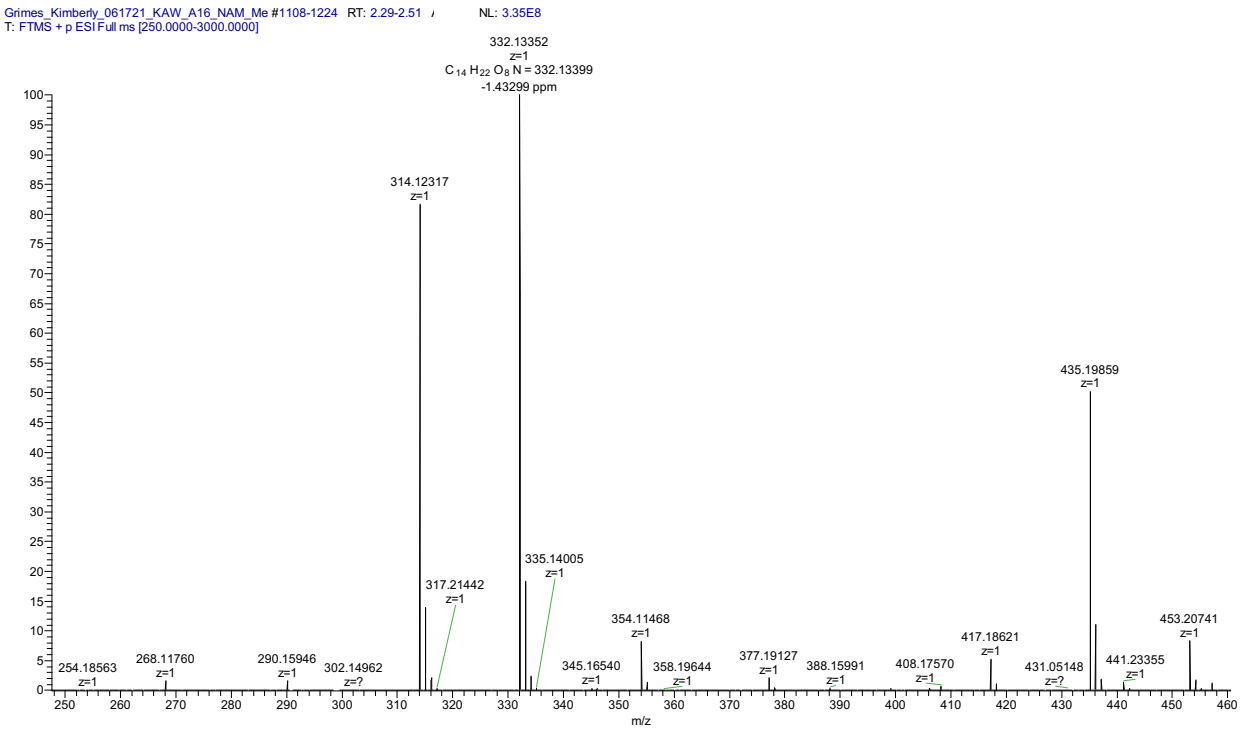


Figure S16: HRMS of Sample Entry 2- AlkNAM methyl ester starting material; AlkNAM product

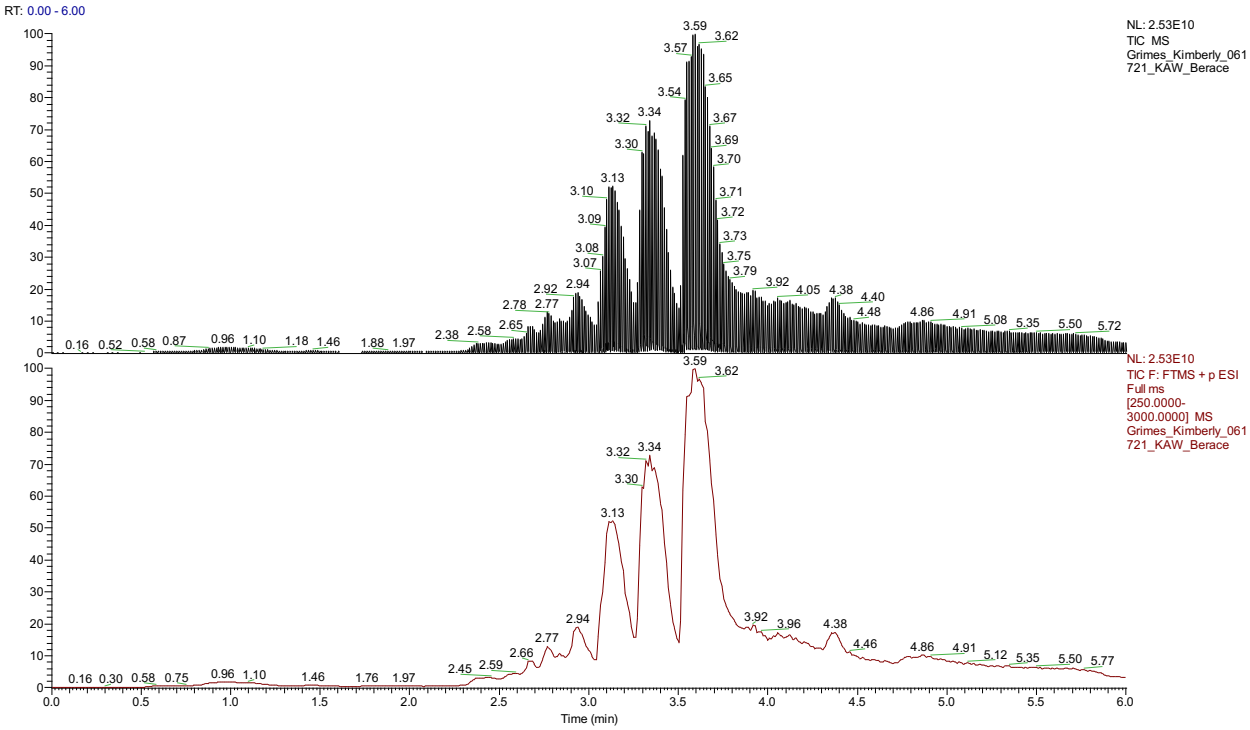


Figure S17: Total ion chromatogram of Sample Entry 3-Peracetylated NAM methyl ester starting material

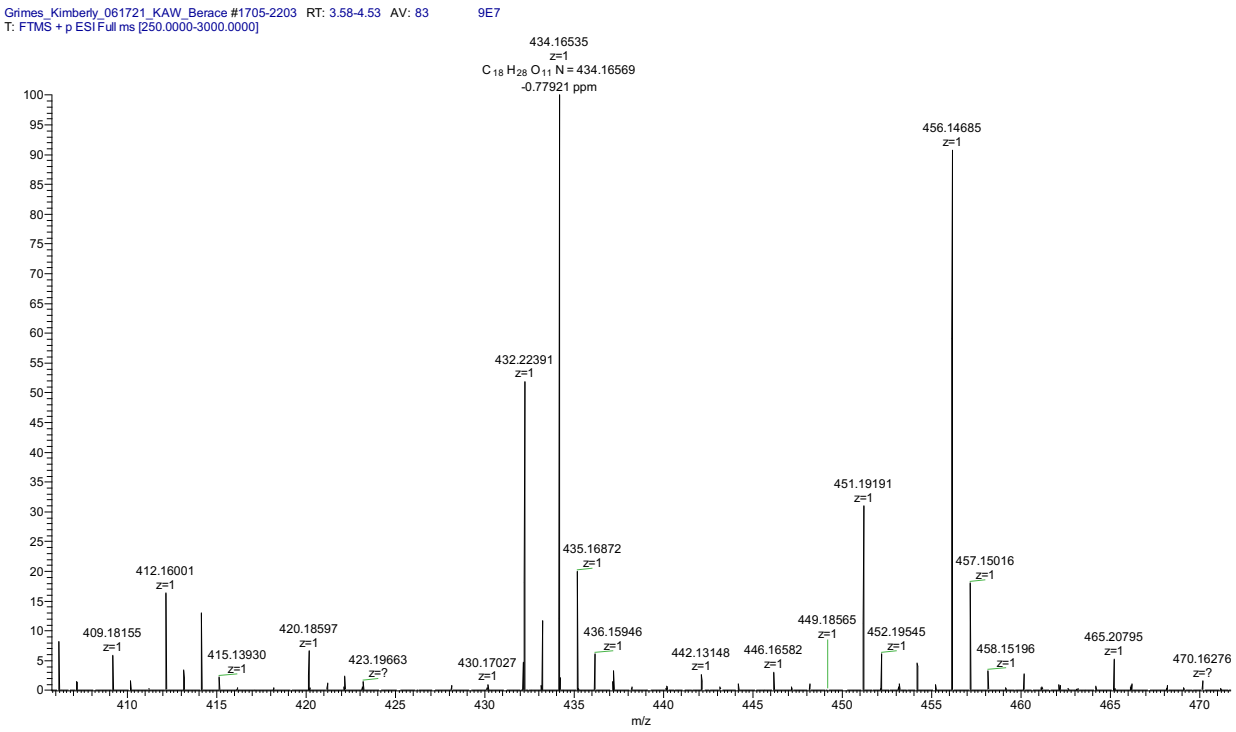


Figure S18: HRMS of Sample Entry 3- Peracetylated NAM methyl ester starting material; peracetylated NAM methyl ester product

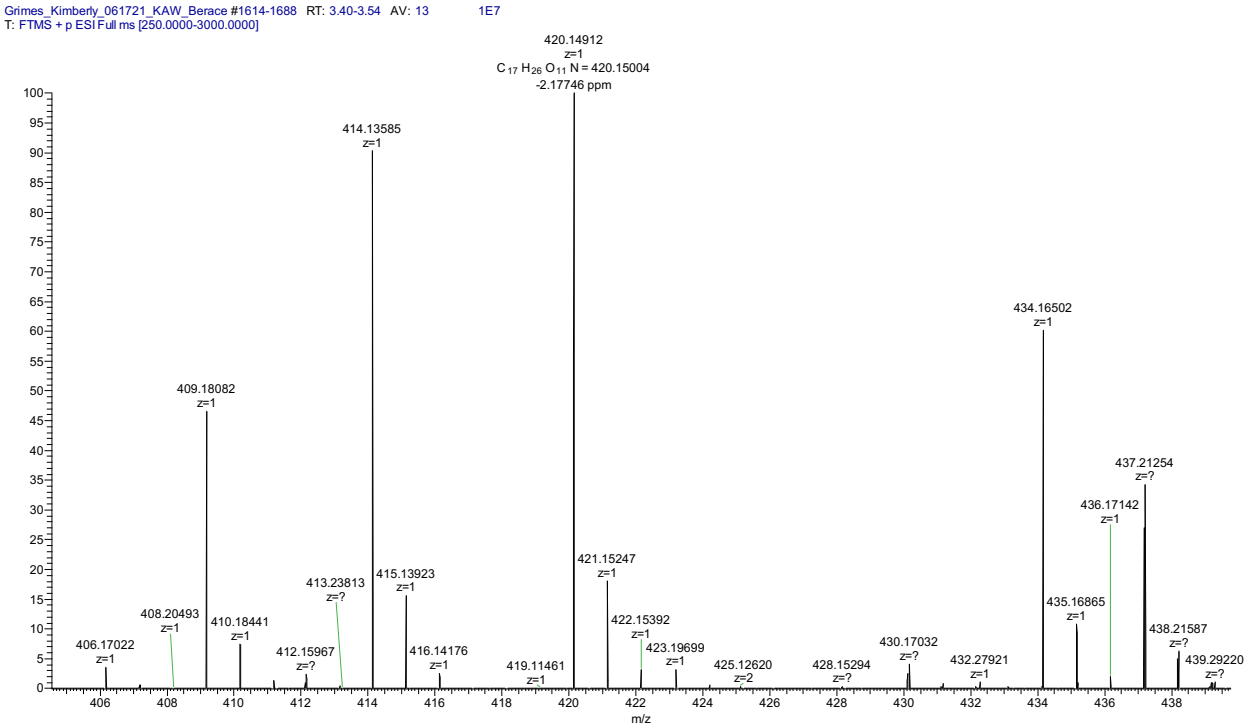
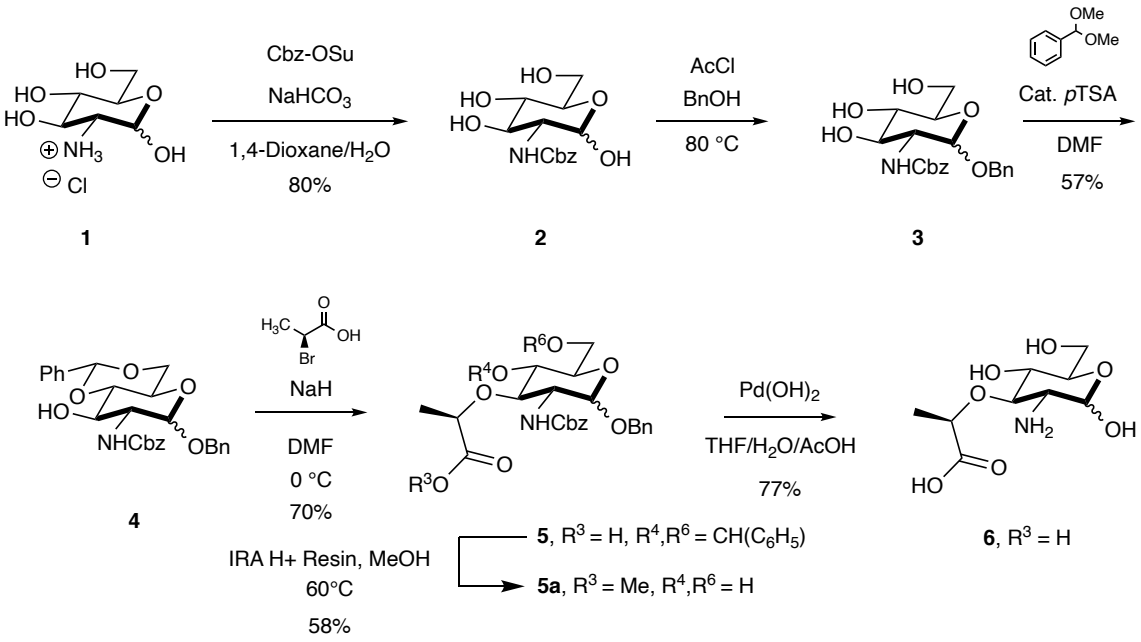


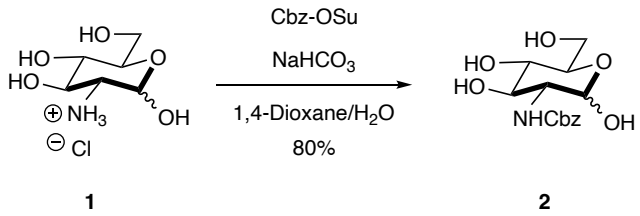
Figure S19: HRMS of Sample Entry 3- Peracetylated NAM methyl ester starting material; peracetylated NAM product

VI. Synthesis of NAM Probes

A. Preparation of 2-Amino Muramic Acid Derivatives



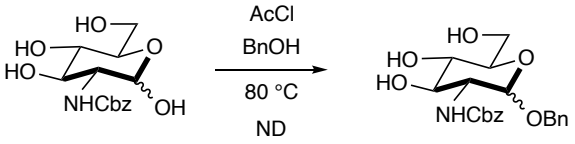
Scheme S1



Scheme S2

benzyl ((3*R*,4*R*,5*S*,6*R*)-2,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yl)carbamate (**2**): To D-(+)-glucosamine **1** (14.995 g, 69.540 mmol, 1.0 eq) dissolved in 1:1 THF:H_2O (348 mL) was added sodium bicarbonate (10.250 g, 122.01 mmol, 1.8 eq). The solution was stirred vigorously for 10 min and Cbz-OSu (20.833 g, 83.593 mmol, 1.2 eq) was subsequently added. After 16 h, TLC (20% MeOH/EtOAc; PAA) indicated that the reaction was complete. The reaction mixture, a frothy yellowish-white solution, was concentrated under reduced pressure. The crude material was recrystallized in water and the resulting white solid was then collected by vacuum filtration with washes of ice-cold water. The solid was then dried under vacuum, yielding a white powder 17.52 g (80%).^{1}H NMR (600 MHz, DMSO): (Anomers 1.00α : 0.79β) δ 7.42 – 7.33 (m, 5H, aromatic), 7.33 – 7.28 (m, 1H, aromatic), 6.90 (d, *J* = 8.3 Hz, 1H, N-Hα), 5.10 – 4.99 (m, 2H, Cbz benzyl methylene), 4.97 (d, *J* = 3.5 Hz, 1 H, α-H1), 3.63 – 3.54 (m, 2H, α-H6, α-H5), 3.51 – 3.45 (m, 2H, α-H3, α-H6'), 3.36 – 3.27 (m, 2H, α-H2), 3.15 – 3.06 (m, 1H, α-H4). ^{13}C NMR (151 MHz, DMSO) δ 172.07, 156.18, 156.11, 137.33, 137.16, 128.35, 128.32, 128.20,

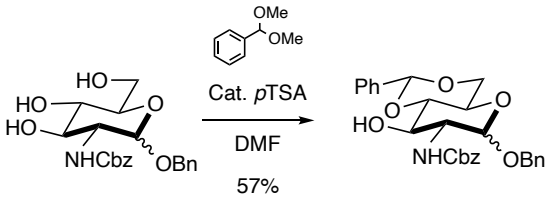
127.80, 127.77, 127.42, 95.50, 90.69, 76.79, 74.24, 72.06, 71.02, 70.89, 70.28, 65.40, 65.25, 65.05, 61.21, 61.11, 58.78, 56.39. HRMS (ESI-Pos) *m/z* calculated for $C_{14}H_{19}NO_7$ (313.11615): Found 314.12304 $[M+H]^+$



2 **3**

Scheme S3

Benzyl((2*R*,4a*R*,7*R*,8*R*,8a*S*)-6-(benzyloxy)-8-hydroxy-2-phenylhexahydropyrano[3,2-*d*][1,3]dioxin-7-yl)carbamate (**3**): **2** (16.778 g, 53.5508 mmol, 1.0 eq) was suspended in anhydrous benzyl alcohol (100 mL, 6 mL/g). To the solution acetyl chloride (3.80 mL, 53.4488 mmol, 1.0 eq) was slowly added over 10 minutes. The reaction mixture was stirred vigorously at 80 °C. Additional AcCl (1.0 mL, 14.0655 mmol) was added after 1 h to reacidify the pH and drive the reaction. After 7 h, TLC (10% MeOH/DCM; PAA) indicated the reaction was complete, and the reaction was removed from the heat source to come to ambient temperature. The reaction was then quenched by pouring the reaction solution into a solution of Et_3N (9.4 mL, 67.514 mmol) in hexanes (1000 mL) stirring at 0 °C for 1 h. The resulting white solid was collected by vacuum filtration and rinsed with cold EtOH. The filtrate was concentrated and purified with a short flash column (5 cm H x 8 cm D; 0% → 5% → 10% MeOH/DCM). Fractions containing the product were collected and an excess of hexanes was added to precipitate out the product. The white solid was collected by vacuum filtration with washes of cold EtOH. The product was used in the next reaction without further isolation. HRMS (ESI-Pos) *m/z* calculated for $C_{21}H_{25}NO_7$ (403.16310): Found 404.17003 $[M+H]^+$

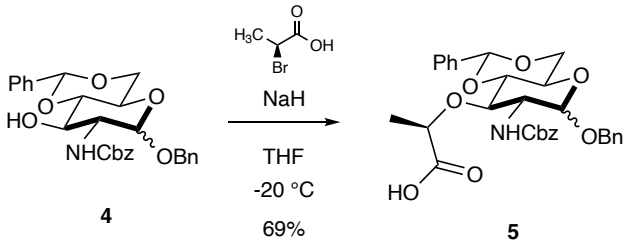


3 **4**

Scheme S4

(benzyl((3*R*,4*R*,5*S*,6*R*)-2-(benzyloxy)-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yl)carbamate) **(4)**: **3** (16.198 g, 40.1507 mmol, 1.0 eq) and *p*-toluenesulfonic acid monohydrate (1.5282 g, 8.0339 mmol, 0.2 eq) were dissolved in DMF (167 mL) and $PhCH(OMe)_2$ (20 mL, 124.8439 mmol, 3.1 eq) was added. The reaction flask was fitted with a vacuum adaptor and the mixture was stirred under nitrogen and light vacuum at 60 °C. At 1 h, additional $PhCH(OMe)_2$ (20 mL, 124.8439 mmol, 3.1 eq) was added and stirring was continued. At 2.5 h, TLC (40% EtOAc/Hexanes; PAA) indicated that the reaction was complete. The reaction mixture was brought to ambient temperature and an excess amount of satd. $NaHCO_3$ was added. The reaction mixture was stirred for 1 h, collected by vacuum filtration, washed with copious amounts of H_2O, and then diethyl ether. The material was then cleaned by refluxing

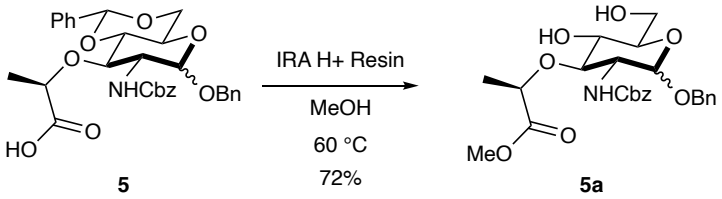
in EtOH and then collecting by vacuum filtration with rinses of hot EtOH in order to get rid of impurities. The product was stirred in water for 1 h, filtered, and washed with water followed by ether in order to get rid of any remaining salt. The product was left under vacuum to dry. A white solid (15.029 g, 57% over 2 steps) was obtained. ^{1}H NMR (600 MHz, DMSO): (Anomers 1.04α : 0.33β) δ 7.48 – 7.41 (m, 3H, aromatic), 7.42 – 7.18 (m, 17 H, aromatic), 5.63 – 5.59 (m, 1H, α 4,6-benzylidene C-H), 5.13 – 4.94 (m, 3H, Cbz benzyl methylene), 4.87 (d, *J* = 3.7 Hz, 1H, α-H1), 4.70 (d, *J* = 12.6 Hz, 1H, α-OBn benzylic H), 4.49 (d, *J* = 12.6 Hz, 1H, α-OBn benzylic H'), 4.14 (dd, *J* = 9.7, 4.6 Hz, 1H, α-H6), 3.79 – 3.71 (m, 2H, α-H6', α-H3), 3.70 – 3.64 (m, 1H, α-H5), 3.59 (dd, *J* = 10.3, 3.8 Hz, 1H, α-H2), 3.54 – 3.47 (m, 1H, α-H4). ^{13}C NMR (151 MHz, DMSO) δ 156.25, 137.90, 137.81, 137.76, 137.30, 137.15, 128.99, 128.46, 128.44, 128.30, 128.26, 128.14, 127.90, 127.85, 127.78, 127.74, 127.60, 127.58, 127.51, 127.18, 126.48, 126.46, 101.85, 100.97, 100.79, 97.20, 81.96, 81.30, 70.29, 68.64, 68.07, 67.93, 67.20, 66.14, 65.48, 65.30, 62.88, 58.07, 56.44, 56.35. HRMS (ESI-Pos) *m*/*z* calculated for $C_{28}H_{29}NO_7$ (491.19440): Found 492.20122 $[M+H]^+$



Scheme S5

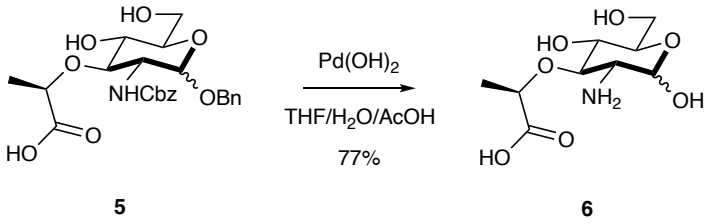
(*R*)-2-(((2*R*,4a*R*,7*R*,8*R*,8a*S*)-6-(benzyloxy)-7-(((benzyloxy)carbonyl)amino)-2-phenylhexahydropyrano[3,2-*d*][1,3]dioxin-8-yl)oxy)propanoic acid (**5**): **4** (5.0012 g, 10.1965 mmol, 1.0 eq) was suspended in THF (170 mL) and the reaction flask was cooled to -20°C, using an isopropanol-dry ice bath. NaH (2.1515 g—in 60 wt % in mineral oil, 1.2909 g, 53.7922 mmol, 5.3 eq) was added slowly to the reaction mixture. After 0.5 h, (S)-(-)-2-Bromopropionic acid (2.5 mL, 27.7160 mmol, 2.7 eq) in THF (36.0 mL) was added dropwise over 0.5 h. At 3 h, an additional portion of (S)-(-)-2-Bromopropionic acid (2.0 mL, 22.1728 mmol, 2.2 eq) was slowly added followed by NaH (2.1449 g--in 60% mineral oil, 1.2869 g, 53.6272 mmol, 5.3 eq). The reaction was then removed from the isopropanol-dry ice bath, and slowly brought to ambient temperature for 0.75 h. At 4.5 h, water was slowly added to quench the reaction. The reaction was quenched with H_2O and then the reaction was diluted with EtOAc. The organic layer was washed 3x with H_2O. The organic layer was then washed with 1N HCl (acidifying it to~ pH 3), dried over Na_2SO_4-, filtered, and concentrated. The crude product was purified by column chromatography (2% → 5% MeOH/DCM w/ 0.01% AcOH). The product was washed with water and then ether yielding a white solid (3.9543 g, 69%). ^{1}H NMR (600 MHz, DMSO): (Anomers 1.00α : 0.70β) α δ 7.49 – 7.11 (m, 23H, aromatic), 5.70 (s, 1H, α 4,6-benzylidene C-H), 5.16 – 4.97 (m, 3H, α-H1, Cbz benzyl methylene), 4.79 (d, *J* = 12.4 Hz, 1H, α-OBn benzylic H), 4.55 (d, *J* = 12.4 Hz, 1H, α-OBn benzylic H'), 4.31 – 4.24 (m, 2H, α-lactyl C-H, α-H6), 3.88 – 3.73 (m, 5H, α-H3, α-H6'), 3.73 – 3.65 (m, 1H, α-H5), 3.64 – 3.59 (m, 1H, α-H2), 3.50 – 3.44 (m, 2H, α-H4), 3.41 – 3.28 (m, 5H, α-lactyl CH_3). ^{13}C NMR (151 MHz, DMSO) δ 175.30, 173.85, 156.04, 155.86, 137.74, 137.62, 137.57, 137.36, 137.05, 128.85, 128.81, 128.40, 128.35, 128.28, 128.26, 128.20, 128.17, 127.80, 127.62, 127.57, 127.50, 127.43, 127.30, 127.15, 125.86, 125.85, 101.57, 100.33, 100.07, 96.77, 81.59, 81.01, 77.44, 74.83, 74.69, 74.49,

70.20, 68.98, 67.86, 67.79, 65.64, 65.45, 65.20, 62.90, 56.59, 55.10, 18.82, 18.57. HRMS (ESI-Pos) *m*/*z* calculated for $C_{31}H_{33}NO_9$ (563.21553): Found 564.22233 $[M+H]^+$



Scheme S6

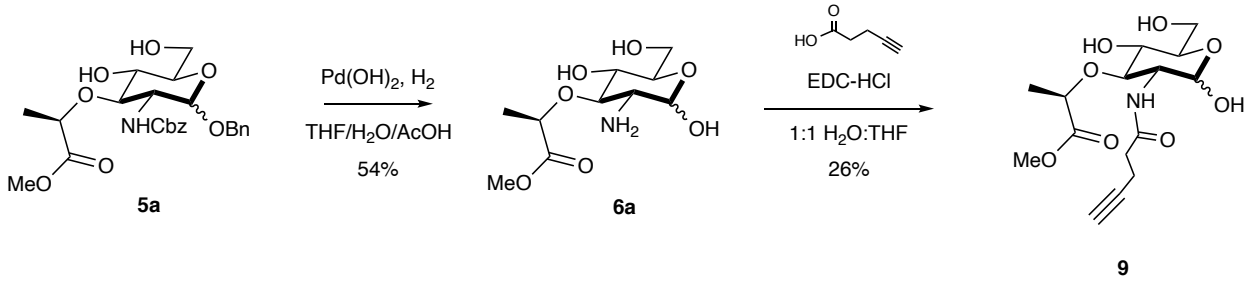
(*R*)-methyl-2-(((3*R*,4*R*,5*S*,6*R*)-3-acetamido-2,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanoate **(5a)**: **5** (0.3174 g, 0.5632 mmol) was dissolved in MeOH (11 mL) and heated to 60℃. IRA H^+ resin was added and the reaction was stirred for 10.5 h under reflux with a condenser attached. Upon completion, the reaction was cooled to room temperature, the resin was removed by filtration, and the solvent was removed under reduced pressure. The mixture was azeotroped 3 times with toluene to remove excess benzaldehyde and purified by column chromatography with 3% → 5% EtOH/DCM to give **5a** (0.198 g, 72%). ^{1}H NMR (600 MHz, MeOD): (α Anomers only) δ 7.39 – 7.32 (m, 5H, aromatic), 7.32 – 7.22 (m, 4H, aromatic), 7.20 – 7.15 (m, 1H, aromatic), 5.14 – 4.98 (m, 3H, Cbz benzyl methylene, α-H1), 4.71 (d, *J* = 12.1 Hz, 1H, α-OBn benzylic H), 4.65 (q, *J* = 6.9 Hz, 1H, α-lactyl C-H), 4.49 (d, *J* = 12.1 Hz, 1H, α-OBn benzylic H'), 3.82 – 3.75 (m, 1H, α-H6), 3.74 – 3.65 (m, 4H, α-H6', methyl ester CH3), 3.65 – 3.60 (m, 2H, α-H5, α-H3), 3.60 – 3.55 (m, 1H, α-H2), 3.55 – 3.47 (m, 1H, α-H4), 1.38 (d, *J* = 7.0 Hz, 3H, α-lactyl CH3). ^{13}C NMR (151 MHz, MeOD) δ 176.65, 158.52, 158.48, 138.89, 138.29, 129.46, 129.40, 129.36, 129.16, 129.05, 128.94, 128.87, 128.80, 128.75, 128.74, 97.46, 79.76, 79.73, 76.78, 74.24, 72.68, 70.24, 67.44, 62.37, 56.44, 56.36, 52.66, 19.21. HRMS (ESI-Pos) *m*/*z* calculated for $C_{25}H_{31}NO_9$ (489.19988): Found 490.20488 $[M+H]^+$



Scheme S7

(*R*)-2-(((3*R*,4*R*,5*S*,6*R*)-3-amino-2,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanoic acid (**6**): **5** (0.5002 g, 0.8872 mmol, 1 eq) was suspended in 14:5:1 THF:H_2O:AcOH (88.6 mL). $Pd(OH)_2$ (0.9341 g--20% wt, 0.1868 g, 1.3303 mmol, 1.5 eq) was added and the reaction solution was evacuated under vacuum 3 times prior and placed under an atmosphere of H_2 (H_2 balloon). The solution was stirred vigorously until complete as indicated by mass spectrometry. At 48 h, the reaction was quenched by filtering the black solution through a thin pad of packed celite with washes of water. The clear, colorless filtrate was then concentrated and purified by column chromatography (normal phase SiO_2; 1:1 iPrOH:EtOAc → 4:4:1 iPrOH:EtOAc:H_2O → 4:4:3 iPrOH:EtOAc:H_2O), yielding a white

solid (0.171 g, 77%) after lyophilization. ^{1}H NMR (600 MHz, D_2O): (Anomers 1.00α : 0.71β) δ 5.39 (d, *J* = 3.7 Hz, 1H, α-H1), 4.34 – 4.22 (m, 2H, α lactyl C-H), 4.16 (q, *J* = 7.0 Hz, 0.37H, lactic acid C-H), 3.89 – 3.77 (m, 4H, α-H6), 3.74 (d, *J* = 5.2 Hz, 1H, α-H6'), 3.70 – 3.61 (m, 3H, α-H3), 3.56 – 3.47 (m, 3H, α-H4), 3.45 (ddd, *J* = 9.6, 5.8, 2.1 Hz, 1H, α-H5), 3.24 (dd, *J* = 10.6, 3.7 Hz, 1H, α-H2), 1.87 (s, 0.14H, lactic acid CH3),1.39 – 1.32 (m, 6H, α lactyl CH_3).^{13}C NMR (151 MHz, D_2O) δ 181.74, 181.65, 93.08, 89.03, 79.97, 79.23, 79.00, 78.68, 78.27, 75.96, 75.76, 71.43, 70.58, 70.34, 70.31, 69.96, 63.06, 60.87, 60.46, 60.31, 60.13, 58.90, 55.78, 53.85, 53.50, 22.42, 19.17, 18.95, 18.88. HRMS aligns with previously published data. [S3]

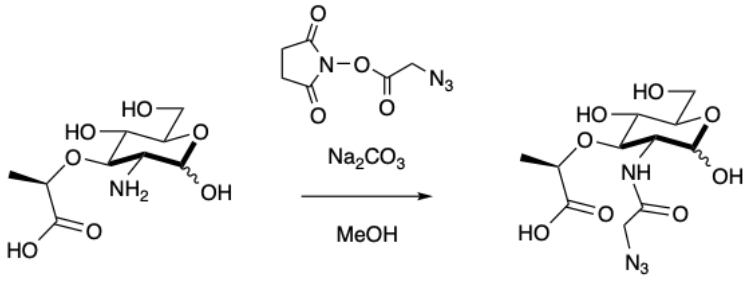


Scheme S8

(*R*)-methyl 2-(((3*R*,4*R*,5*S*,6*R*)-3-amino-2,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanoate (**6a**): **5a** (0.1143 g, 0.2337 mmol) was suspended in 24:71:6 THF:H_2O:AcOH (42.5 mL). $Pd(OH)_2$ (0.2300 g--20% wt, calculate 0.0460 g, 0.3276 mmol) was added and the reaction solution was evacuated under vacuum 3 times prior and placed under an atmosphere of H_2 (H_2 balloon). The solution was stirred vigorously until complete as indicated by mass spectrometry. The reaction was quenched by filtering the black solution through a thin pad of packed celite with washes of water. The filtrate was condensed and purified by column chromatography (1:1 EtOAc:iPrOH → 4:4:1 EtOAc:iPrOH:H_2O). The appropriate fractions were combined and lyophilized to give a yielding a yellowish white solid (0.1272 g, 54%). HRMS (ESI-Neg) *m*/*z* calculated for $C_{10}H_{19}NO_7$ (265.11615): Found 264.10797 $[M-H]^-$

(*R*)-methyl-2-(((3*R*,4*R*,5*S*,6*R*)-2,5-dihydroxy-6-(hydroxymethyl)-3-(pent-4-ynamido)tetrahydro-2*H*-pyran-4-yl)oxy)propanoate (**9)**: 4-pentynoic acid (12 mg, 0.1224 mmol, 1.1 eq) and EDC-HCl (26 mg, 0.1354 mmol, 1.2 eq) were stirred in THF (0.7 mL) for 0.5 h at rt. An aqueous solution (0.7 mL) of **6a** (30 mg, 0.1132 mmol, 1 eq) was added and the resulting reaction mixture was stirred for 1 h. The aqueous phase was washed with DCM (3 x 1 mL) and then lyophilized. The crude was purified by column chromatography with 0% → 20% MeOH/EtOAc and the appropriate fractions were condensed to give a colorless oil. This oil was dissolved in water and lyophilized to give a white solid (11 mg, 26%). ^{1}H NMR (600 MHz, MeOD): (Anomers 1.00α : 0.28β) δ 5.22 (d, *J* = 3.2 Hz, 1H, α-H1), 4.60 (q, *J* = 7.0 Hz, 1H, α lactyl C-H), 4.54 (q, *J* = 8.2 Hz), 4.50 (d, *J* = 7.68 Hz), 3.68 (s, 3H, methyl ester CH_3), 3.67-3.64 (m, 2H, α-H4, α-H6), 3.60-3.57 (m, H2, α-H6'), 3.55-3.52 (m, 1H, α-H3), 3.40 (t, *J* = 9.8 Hz, 1H, α-H5), 2.38 (m, methylene adjacent to amide and methylene adjacent to alkyne), 2.14 (bs, 1H, alkyne C-H), 1.30 (d, *J* = 7.0 Hz, 3H, α lactyl CH_3), 1.27 (d, *J* = 6.9 Hz). ^{13}C NMR (151 MHz, MeOD) δ 175.82, 172.66, 96.04,

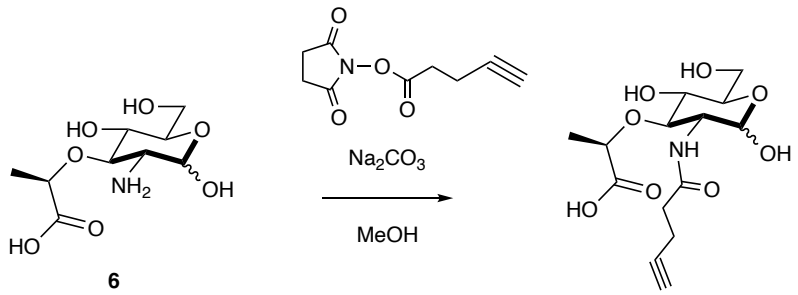
90.52, 82.12, 80.97, 77.82, 76.50, 75.30, 72.02, 71.58, 71.19, 68.75, 61.08, 56.60, 54.17, 51.33, 34.90, 17.85, 14.09. HRMS (ESI-Pos) *m/z* calculated for $C_{15}H_{23}NO_8$ (345.14237): Found 346.14939 $[M+H]^+$



6 7

Scheme S9

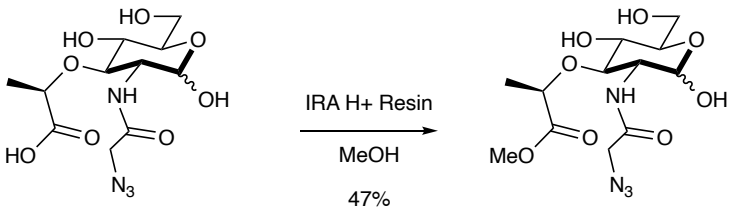
(*R*)-5-amino-4-((*S*)-2-((*R*)-2-(((3*R*,4*R*,5*S*,6*R*)-2,5-dihydroxy-3-(2-hydroxyacetamido)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanamido)propanamido)-5-oxopentanoic acid **(7)**: 2-Azide-NAM was synthesized according to literature precedent[S3] from **18** with the NMR data aligning with the previously published assignments.



6 9a

Scheme S10

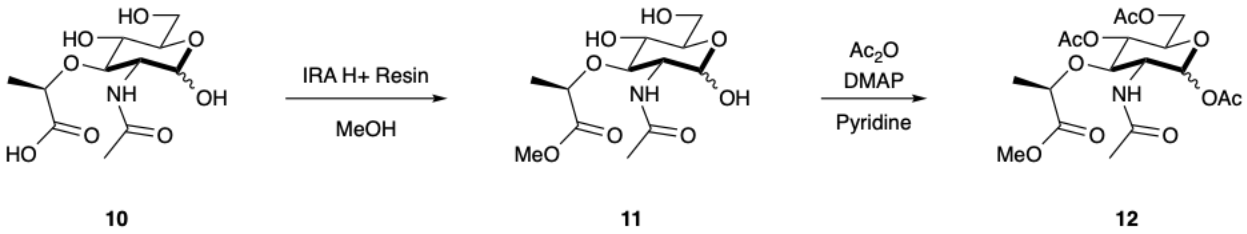
(2R)-2-(((3R,4R,5S,6R)-2,5-dihydroxy-6-(hydroxymethyl)-3-(pent-4- ynamido)tetrahydro-2H-pyran-4-yl)oxy)propanoic acid (3). Preparation 2,5- dioxopyrrolidin-1-yl pent-4-ynoate **(9a)**: 2-Alkyne-NAM was synthesized according to literature precedent[S3] from **18** with the NMR data aligning with the previously published assignments.



7 8

Scheme S11

R)-2-(((3*R*,4*R*,5*S*,6*R*)-3-(2-azidoacetamido)-2,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanoic acid **(8):** 2-Azide NAM **7** (32.3mg, 0.0967 mmol, 1 eq) was dissolved in Methanol to a final concentration of 0.1 M. 2 spatula scoops of IRA H^+ Resin were added to the mixture to bring the pH < 2. The solution was allowed to stir at room temperature for 8 to 12 hours. The resin was filtered by vacuum filtration and the methanol was evaporated under reduced pressure using no heat. The crude was purified by column chromatography with a gradient of 1:1 (EtOAc:IPA) to 4:4:1 (EtOAc:IPA:H_2O) and lyophilized to yield an off-white solid (6.1 mg, 18% yield). Starting material was also recovered (15.2 mg, 47% recovery). ^{1}H NMR (600 MHz, MeOD) (Anomers 1.00α : 0.11β) δ 5.31 (d, *J* = 3.2 Hz, 1H, α-H1), 4.70 (q, *J* = 7.0 Hz, 1H, α-lactyl C-H), 3.95 (d, *J* = 1.7 Hz, 2H, CH2 next to Azide α), 3.78-3.75 (m, 5H, -OMe α, α-H6, α-H6'), 3.76-3.74 (m, 2H, α-H2, α-H5), 3.69 (m, 1H, α-H4), 3.52 (t, *J* = 9.2 Hz, 1H, α-H3), 1.39 (d, *J* = 7.0 Hz, 3H, α-lactyl CH3). ^{13}C NMR (151 MHz, MeOD) δ 176.91, 176.04, 171.16, 170.43, 96.68, 91.80, 82.72, 79.15, 76.93, 76.75, 73.38, 72.96, 72.58, 62.48, 62.30, 57.80, 55.49, 53.36, 53.27, 53.23, 52.73, 52.56, 19.33, 19.18. HRMS (ESI-Pos) *m*/*z* calculated for $C_{12}H_{20}N_4O_8$ (348.12812): Found 349.13477 $[M+H]^+$



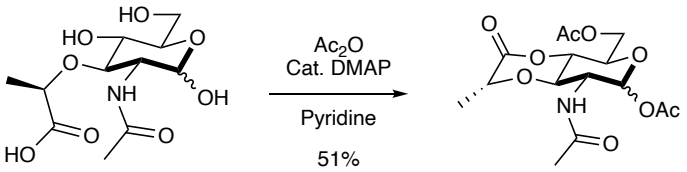
10 11 12

Overall yield: 31%

Scheme S12

(*R*)-2-(((3*R*,4*R*,5*S*,6*R*)-3-acetamido-2,5-diacetoxy-6-(acetoxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanoic acid (**12)**: **10** To MurNAc (0.1006 g, 0.3430 mmol) was dissolved in MeOH (3 mL), and IRA H^+ resin was added. The reaction mixture was stirred at room temperature for 3.75 h. After mass spec indicated conversion to the product, the reaction was filtered and then azeotroped 3 times with

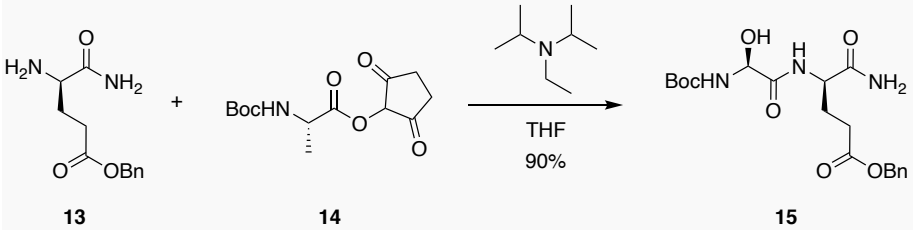
toluene. The clear, colorless residue **11** was suspended in pyridine (1.05 mL) and DMAP (0.0088 g, 0.0720 mmol) and acetic anhydride (0.20 mL, 2.0178 mmol) were added. The reaction was stirred for 2.5 h, and the reaction was then azeotroped 3 times with toluene. The crude material was purified by column chromatography (2.0:8.0:2.8 EtOAc:DCM:EtOH), yielding a clear, colorless residue (0.046 g, 31% over 2 steps). ^{1}H NMR (600 MHz, $CDCl_3$): (Anomers 1.00α : 0.16β) δ 7.75 (s, 1H, α-NH peak), 6.88 (d, J = 6.9 Hz), 6.52 (d, J = 3.3 Hz, 1H, α-H1), 5.68 (d, J = 8.6 Hz), 5.15 (dd, J = 10.2, 9.2 Hz, 1H, α-H4), 5.08 (t, J = 9.4 Hz), 4.28 (q, J = 7.1 Hz, 1H, α lactyl C-H), 4.23 (d, J = 7.0 Hz), 4.17 (dd, J = 12.4, 4.3 Hz, 1H, α-H6), 4.00 (ddd, J = 11.1, 4.6, 2.8 Hz, 2H, α-H2, α-H6'), 3.86 (ddd, J = 10.2, 4.3, 2.4 Hz, 1H, α-H5), 3.82 – 3.79 (m, 4H, α-H3, lactyl CH3) 2.11 (d, J = 2.4 Hz, 7H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 175.36, 174.47, 171.68, 171.35, 170.98, 170.94, 169.70, 169.24, 169.14, 168.74, 93.30, 90.33, 75.45, 75.30, 75.28, 73.02, 71.66, 71.32, 70.18, 61.91, 61.84, 60.52, 54.34, 53.17, 52.73, 52.52, 23.50, 23.08, 21.14, 21.00, 20.97, 20.94, 20.89, 20.86, 18.92, 18.85. HRMS (ESI-Pos) m/z calculated for $C_{18}H_{27}NO_{11}$ (433.15841): Found 434.16555 $[M+H]^+$



10 **12a**

Scheme S13

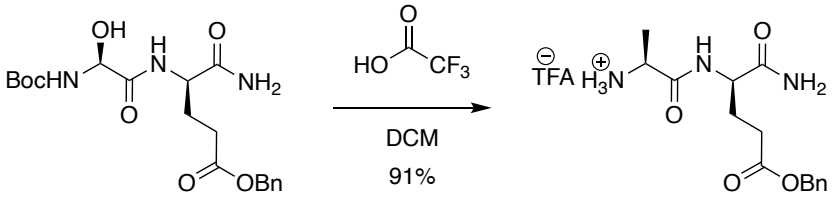
(2*R*,4a*S*,5*R*,8*R*,8a*R*)-8-acetamido-5-(acetoxymethyl)-2-methyl-3-oxohexahydro-2*H*-pyrano[3,4-*b*][1,4]dioxin-7-yl acetate **12a**: **10** (0.0605g, 0.2063 mmol) in pyridine (0.550 mL) was added acetic anhydride (0.12 mL, 1.270 mmol), and the reaction solution was stirred vigorously for ~2.75 h. The clear, colorless reaction solution was concentrated and then coevaporated with toluene three times. The crude material was purified by column chromatography with 60% → 80% → 100% EtOAc/Hexane to give **12a** (0.044 g, 51%). ^{1}H NMR (600 MHz, Chloroform-*d*): (Anomers 1.00α : 0.96β) δ 6.20 (d, J = 3.6 Hz, 1H, α-H1α), 6.09 (d, J = 8.4 Hz, 1H, NH), 4.73 (q, J = 7.0 Hz, 1H, lactyl α H α anomer), 4.63 – 4.39 (m, 3H, α-H4, α-H2, α-H6'), 4.23 (dd, J = 12.4, 4.2 Hz, 1H, α-H6), 4.05 – 3.98 (m, 1H, α-H5), 3.98 – 3.78 (m, 1H, α-H3), 2.18 (s, 3H, OAc H1), 2.10 (s, 1H, OAc H6), 2.03 (s, 3H, NAc), 1.54 (d, J = 7.0 Hz, 3H, lactyl α CH3 α anomer). ^{13}C NMR (151 MHz, $CDCl_3$) δ 171.19, 170.64, 170.43, 170.34, 169.27, 168.99, 168.87, 168.49, 92.15, 90.72, 74.58, 71.89, 71.14, 71.06, 70.88, 69.26, 69.03, 61.34, 61.07, 60.40, 53.06, 50.20, 23.26, 23.11, 21.04, 20.88, 20.85, 20.74, 20.70, 19.13, 17.80, 17.71, 14.17. HRMS (ESI-Pos) m/z calculated for $C_{15}H_{21}NO_9$ (359.12163): Found 360.12815 $[M+H]^+$



13 14 15

Scheme S14

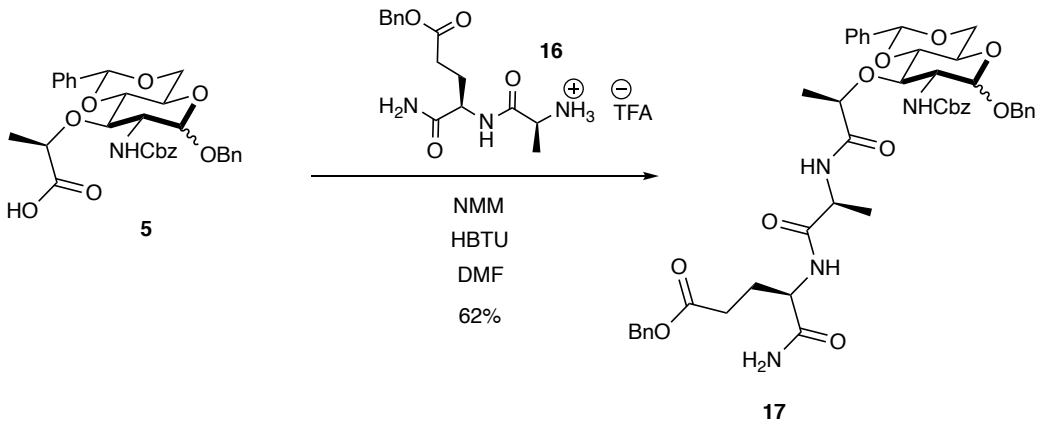
(*R*)-benzyl 5-amino-4-((*S*)-2-((*tert*-butoxycarbonyl)amino)propanamido)-5-oxopentanoate (**15)**: **15** was synthesized according to literature precedent[S1]. A white solid **15** (2.641 g, 90%) was obtained. ^{1}H NMR (600 MHz, MeOD) δ 7.42 – 7.25 (m, 6H, aromatic), 5.12 (d, *J* = 1.6 Hz, 2H, OBn benzyl methylene), 4.38 (dd, *J* = 9.7, 4.5 Hz, 1H, isoglutamine α C-H), 4.01 (q, *J* = 7.1 Hz, 1H, alanine α C-H), 2.46 (t, *J* = 7.6 Hz, 2H, isoglutamine β methylene), 2.33 – 2.15 (m, 1H, isoglutamine γ-C-H), 1.97 – 1.88 (m, 1H, isoglutamine γ-C-H'), 1.42 (s, 9H, Boc t-butyl CH3), 1.28 (d, *J* = 7.2 Hz, 3H, alanine methyl).^{13}C NMR (151 MHz, MeOD) δ 176.29, 176.20, 174.16, 137.55, 129.52, 129.23, 129.18, 80.74, 67.40, 53.47, 52.06, 31.38, 28.70, 28.03, 17.68.



15 16

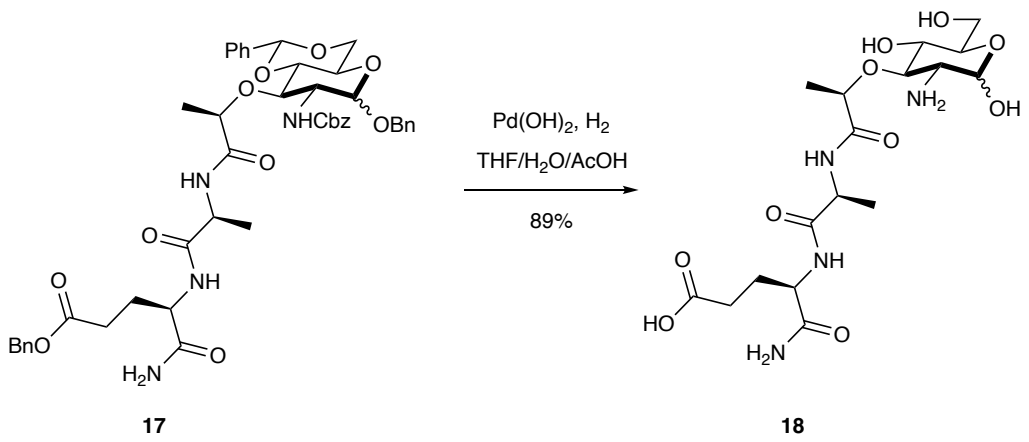
Scheme S15

(*R*)-5-amino-4-((*S*)-2-((*tert*-butoxycarbonyl)amino)propanamido)-5-oxopentanoic acid **(16)**: **15** The dipeptide (1.2321 g, 3.0257 mmol) was suspended in anhydrous 1:3 DCM:TFA (22 mL) was added. The solution was stirred for 6 h and then coevaporated 2 times with toluene under reduced pressure. The crude material was purified by trituration with ether and then dried under reduced pressure, yielding a white foam (1.136 g, 91%). ^{1}H NMR (600 MHz, MeOD) δ 7.42 – 7.26 (m, 5H, aromatic), 5.13 (s, 2H, OBn benzyl methylene), 4.41 (dd, *J* = 9.1, 5.1 Hz, 1H, alanine C-H), 3.96 (q, *J* = 7.1 Hz, 1H, isoglutamine α C-H), 2.48 (t, *J* = 7.6 Hz, 2H, isoglutamine β methylene), 2.19 (m, 1H, isoglutamine γ C-H), 1.97 (ddt, *J* = 14.3, 9.1, 7.2 Hz, 1H, isoglutamine γ-C-H'), 1.48 (d, *J* = 7.0 Hz, 3H, alanine methyl).^{13}C NMR (151 MHz, MeOD) δ 175.78, 173.99, 171.17, 137.48, 129.57, 129.27, 129.26, 67.52, 53.71, 50.31, 31.35, 28.35, 17.64.



Scheme S16

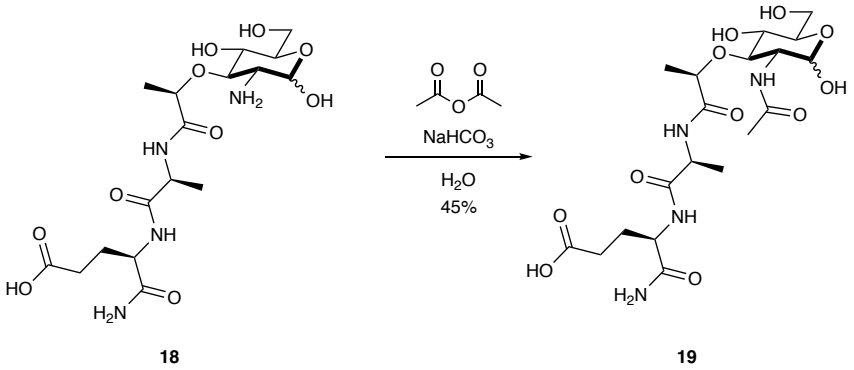
(*R*)-benzyl-5-amino-4-((*S*)-2-((*R*)-2-(((2*R*,4a*R*,7*R*,8*R*,8a*S*)-6-(benzyloxy)-7-(((benzyloxy)carbonyl)amino)-2-phenylhexahydropyrano[3,2-*d*][1,3]dioxin-8-yl)oxy)propanamido)propanamido)-5-oxopentanoate (**17**): **5** (0.1035 g, 0.1836 mmol) was dissolved in anhydrous DMF (2.6 mL). HBTU (0.1746 g, 0.4604 mmol) and NMM (50.0 μL, 0.0544 mmol) were added and the reaction stirred for 15 min. The dipeptide (0.0721 g, 0.2346 mmol) was then added to the reaction. Additional NMM (1.01 mL, 1.098 mmol) was added. After ~16 h, the reaction was quenched with H_2O (4 mL) and the solution was diluted with $CHCl_3$. The solution was then washed sequentially with the following: 1 N HCl, Satd. $NaHCO_3$, and brine. The organic layer was dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The crude material was purified by column chromatography (0% → 1% → 2% → 3% MeOH/DCM) yielding a white residue (0.098 g, 62%). ^{1}H NMR (600 MHz, $CDCl_3$): (α Anomers only) δ 7.47 – 7.41 (m, 2H, aromatic), 7.39 – 7.23 (m, 15H, aromatic), 7.18 (s, 1H, NH peak), 7.11 (s, 1H, NH peak), 6.89 (s, 1H,), 5.56 (s, 1H, α 4,6-benzylidene C-H), 5.44 (s, 1H, NH peak), 5.19 – 5.00 (m, 2H, Cbz benzyl methylene), 4.94 (d, *J* = 3.9 Hz, 1H, H1), 4.70 (d, *J* = 11.7 Hz, 1H, OBn benzylic H), 4.48 (d, *J* = 11.7 Hz, 1H, OBn benzylic H'), 4.40 (td, *J* = 8.2, 4.3 Hz, 1H, isoglutamine α C-H), 4.24 (dd, *J* = 10.2, 4.7 Hz, 1H, H6), 4.18 – 4.07 (m, 1H, alanine C-H), 4.05 – 3.99 (m, 1H, H2), 3.93 – 3.81 (m, 2H, lactyl C-H, H5), 3.79 – 3.70 (m, 2H, H6', H3), 3.70 – 3.62 (m, 1H, H4), 2.63 – 2.51 (m, 1H, isoglutamine γ C-H), 2.48 – 2.37 (m, 1H, isoglutamine γ C-H'), 2.19 – 2.10 (m, 1H, isoglutamine β C-H), 2.07 – 1.95 (m, 1H, isoglutamine β C-H'), 1.65 (s, water), 1.35 (d, *J* = 6.8 Hz, 3H, lactyl CH3), 1.29 (d, *J* = 7.0 Hz, 3H, alanine methyl). ^{13}C NMR (151 MHz, $CDCl_3$) δ 174.19, 173.38, 172.13, 137.08, 136.61, 135.60, 129.26, 128.85, 128.80, 128.76, 128.57, 128.50, 128.48, 128.34, 128.19, 128.18, 126.05, 101.67, 97.69, 78.46, 78.17, 70.25, 68.94, 67.42, 66.87, 63.14, 52.83, 50.02, 30.87, 26.34, 19.53, 16.60, 0.15. HRMS (ESI-Pos) *m*/*z* calculated for $C_{46}H_{52}N_4O_{12}$ (852.35818): Found 853.36383 $[M+H]^+$



17 **18**

Scheme S17

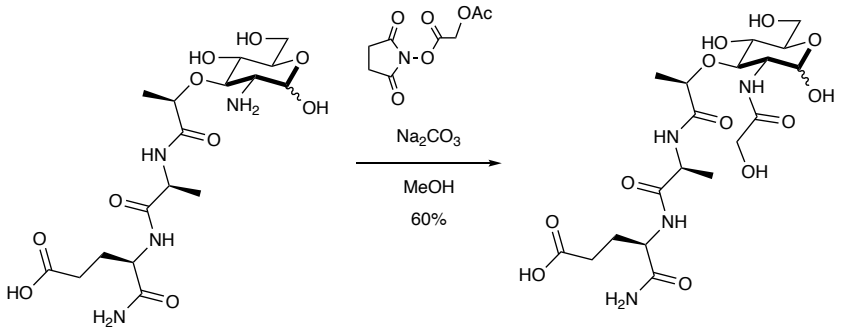
(*R*)-5-amino-4-((*S*)-2-((*R*)-2-(((3*R*,4*R*,5*S*,6*R*)-3-amino-2,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanamido)propanamido)-5-oxopentanoic acid **(18)**: **17** (0.0504 g, 0.0591 mmol) was dissolved in 14:5:1 THF:H_2O:AcOH (6.16 mL). $Pd(OH)_2$ (20 wt % --- 0.0175 g, 0.01245 mmol) was added and the flask was placed under H_2 (g). After 28 h, the black solution was filtered through a syringe filter with washes of H_2O and concentrated under reduced pressure. The crude material was purified via C18 column (100% H_2O) and concentrated under reduced pressure yielding a white solid (23.8 mg, 89%). ^{1}H NMR (600 MHz, D_2O): (Anomers 1.00α : 0.83β) δ 5.45 (d, *J* = 3.7 Hz, 1H, α-H1), 4.97 (d, *J* = 8.3 Hz, 1H, β-H1), 4.86 – 4.68 (m, 1H, alanine C-H), 4.42 – 4.35 (m, H, lactyl C-H), 4.33 – 4.29 (m, 2H, isoglutamine α C-H), 3.96 – 3.89 (m, 2H, α-H6), 3.89 – 3.87 (m, 0H), 3.87 – 3.84 (m, 1H), 3.83 – 3.73 (m, 3H, α-H3, α-H6), 3.71 – 3.62 (m, 2H, α-H4), 3.56 – 3.51 (m, 1H, α-H5), 3.35 (dd, *J* = 10.5, 3.7 Hz, 1H, α-H2), 3.06 (t, *J* = 9.0 Hz, 1H, β-H1), 2.47 – 2.33 (m, 2H, isoglutamine *γ* methylene), 2.22 – 2.11 (m, 1H, isoglutamine β C-H), 2.07 – 1.97 (m, 3H, isoglutamine β C-H', acetic acid CH3), 1.50 – 1.39 (m, 9H, alanine CH3, α and β lactyl CH3).^{13}C NMR (151 MHz, D_2O) δ 176.41, 175.91, 175.38, 92.69, 88.90, 78.63, 76.84, 76.42, 76.15, 75.89, 71.40, 70.57, 70.31, 60.21, 60.02, 55.71, 53.70, 53.60, 53.45, 50.01, 49.83, 27.14, 27.03, 19.09, 18.93, 16.58, 16.47.



18 **19**

Scheme S18

(*R*)-4-((*S*)-2-((*R*)-2-(((3*R*,4*R*,5*S*,6*R*)-3-acetamido-2,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanamido)propanamido)-5-amino-5-oxopentanoic acid **(19)**: *N*-acetyl MDP was synthesized from **18** according to literature precedent[S2] and purified by normal phase column chromatography (4:4:1 EtOAc:iPrOH:H_2O) to yield a white solid (12.4 mg, 45%). The NMR data aligned with previously published assignments.

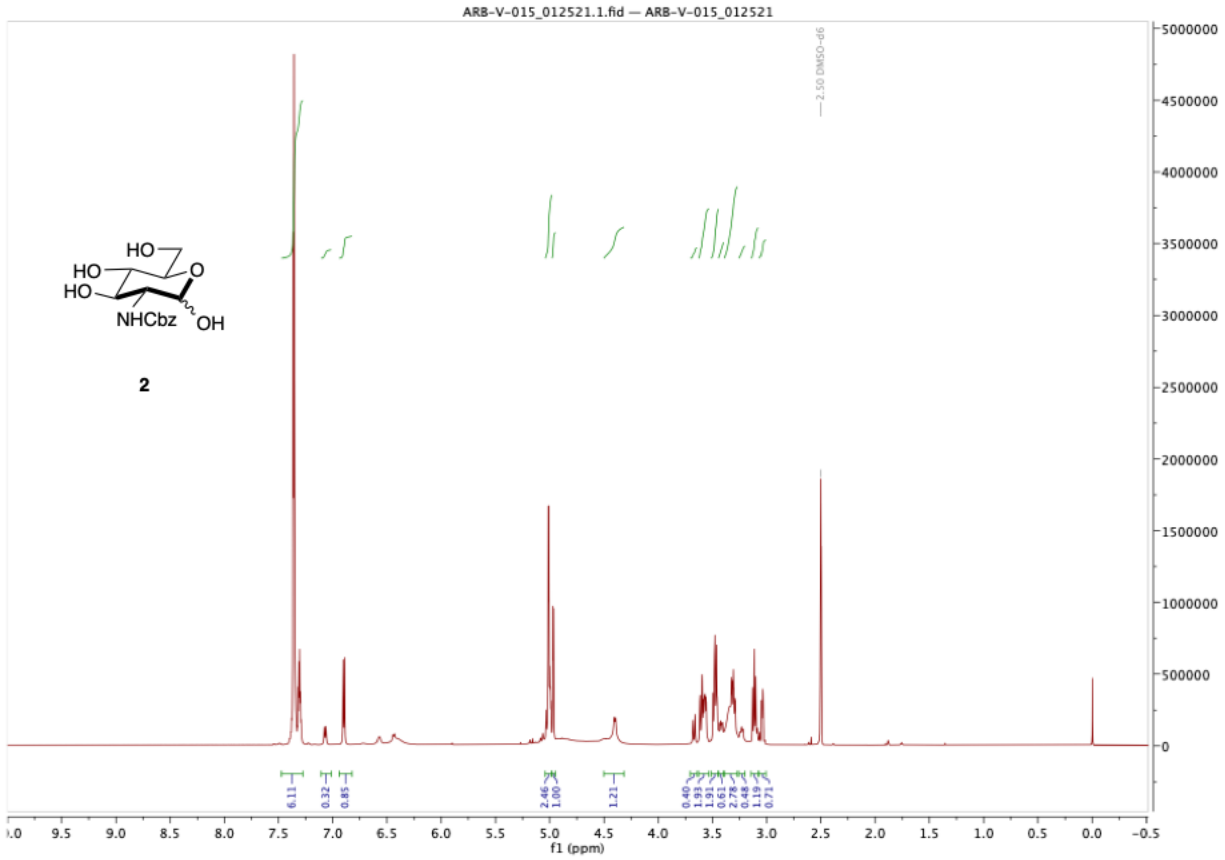


18 20

Scheme S19

(*R*)-5-amino-4-((*S*)-2-((*R*)-2-(((3*R*,4*R*,5*S*,6*R*)-2,5-dihydroxy-3-(2-hydroxyacetamido)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-4-yl)oxy)propanamido)propanamido)-5-oxopentanoic acid **(20)**: *N*-glycolyl MDP was synthesized according to literature precedent[S2] from **18** with the NMR data aligning with the previously published assignments. A white solid (17.1 mg, 60%) was obtained.

VII. ^{1}H NMR and ^{13}C NMR Spectra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0.85
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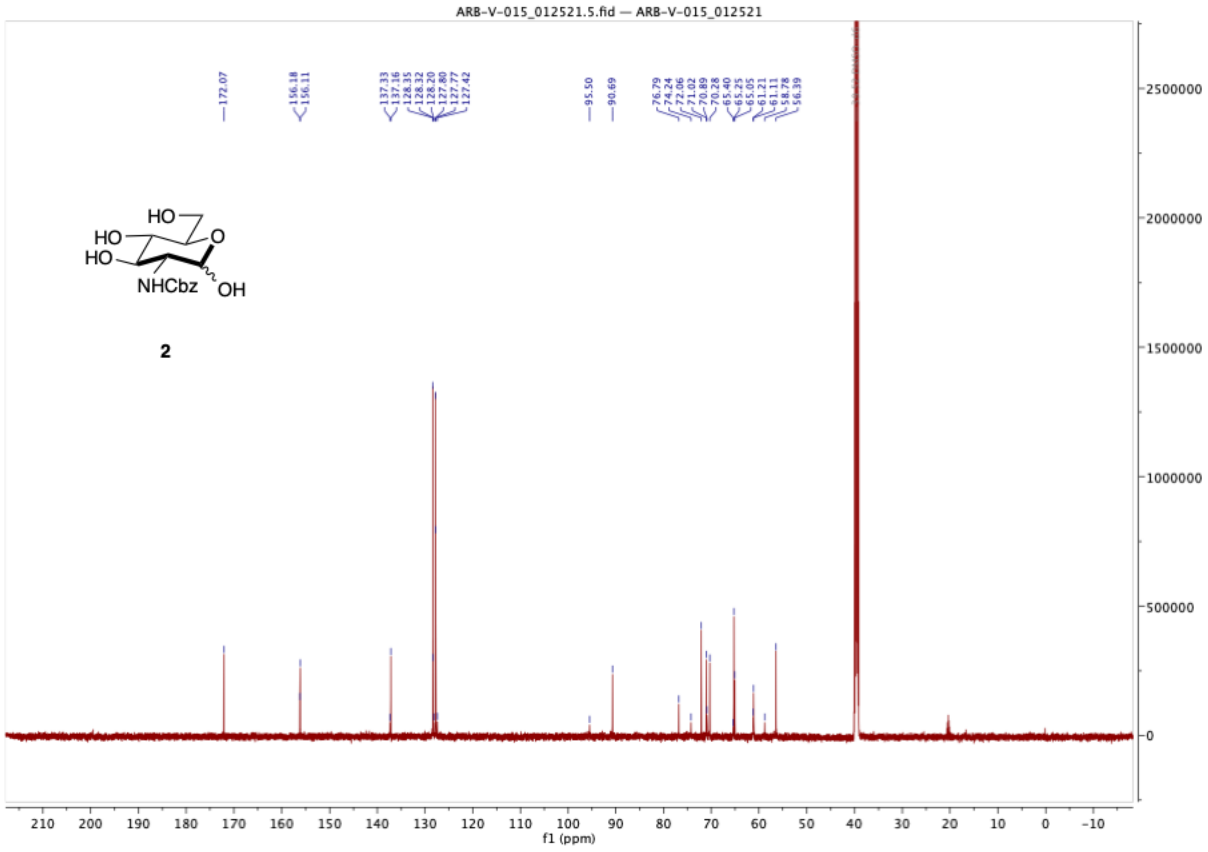
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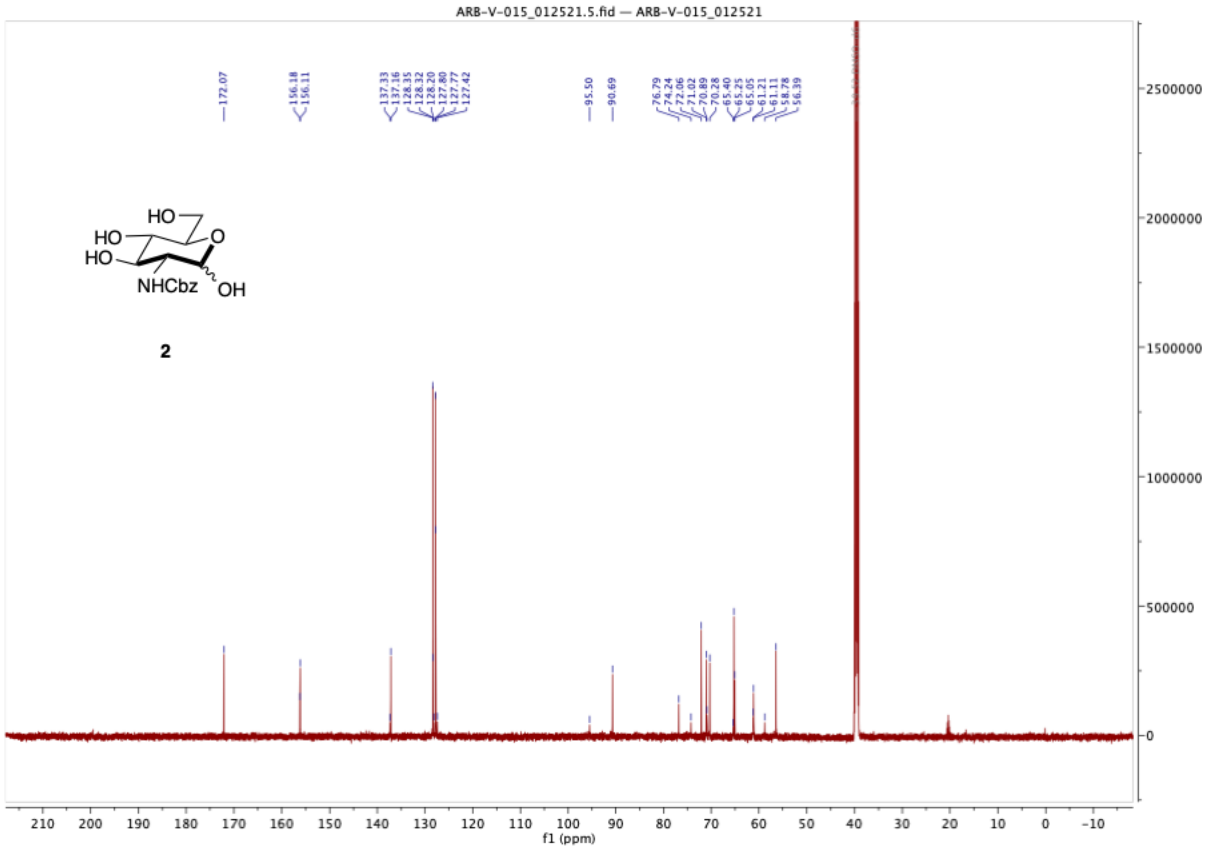
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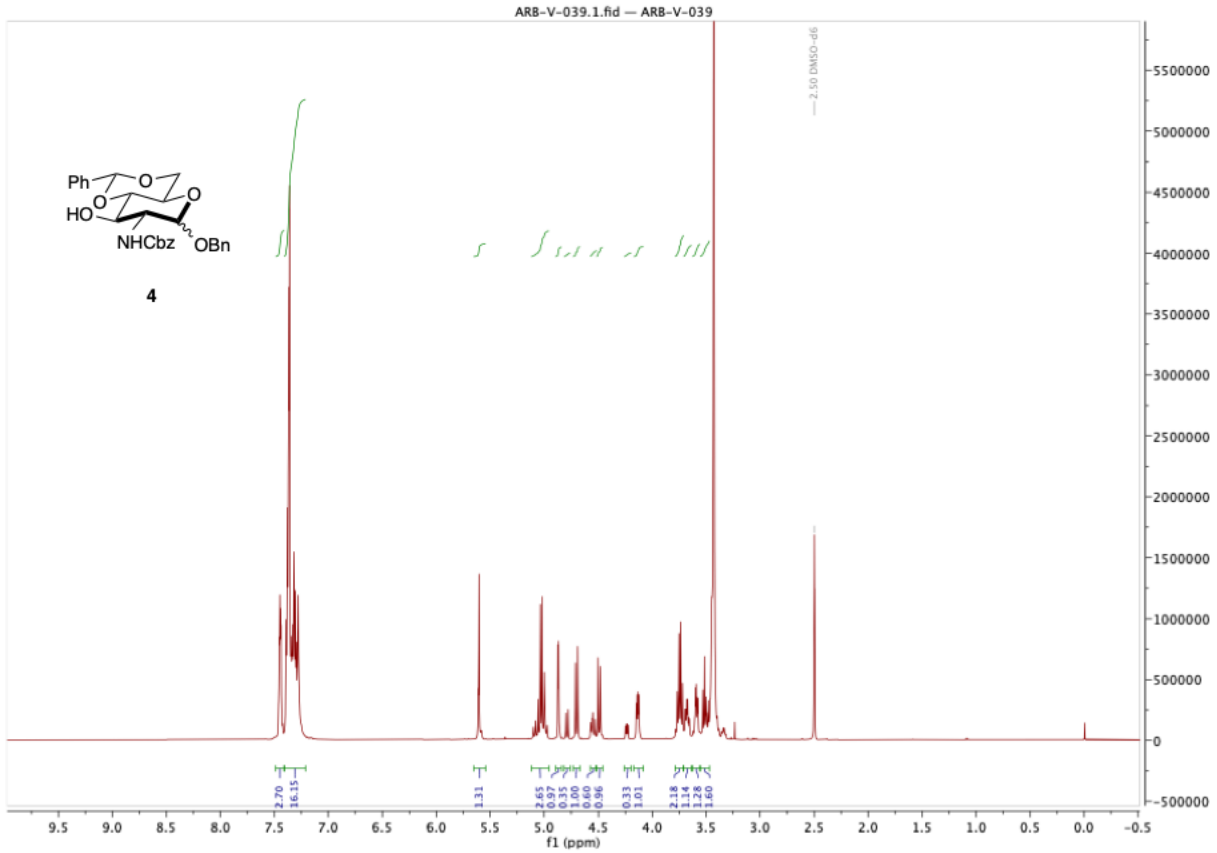
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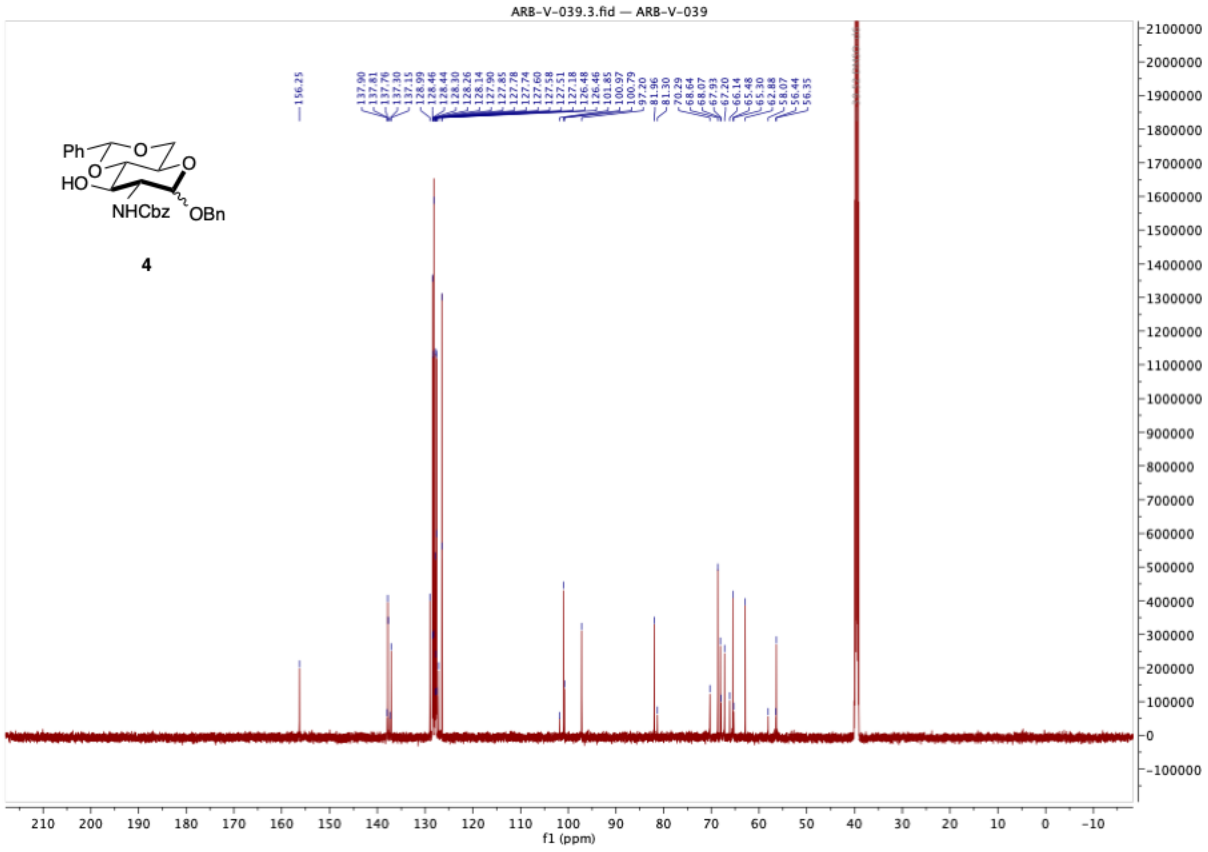
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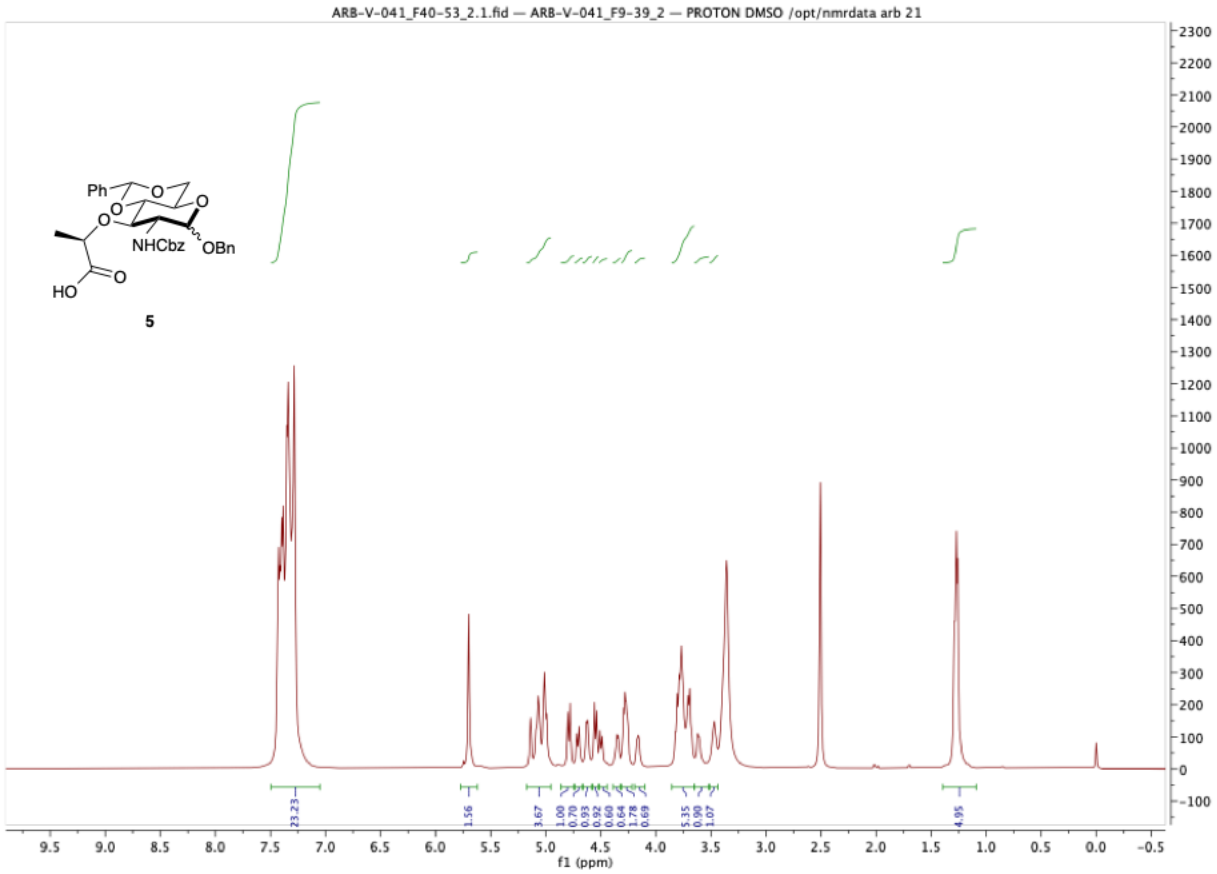
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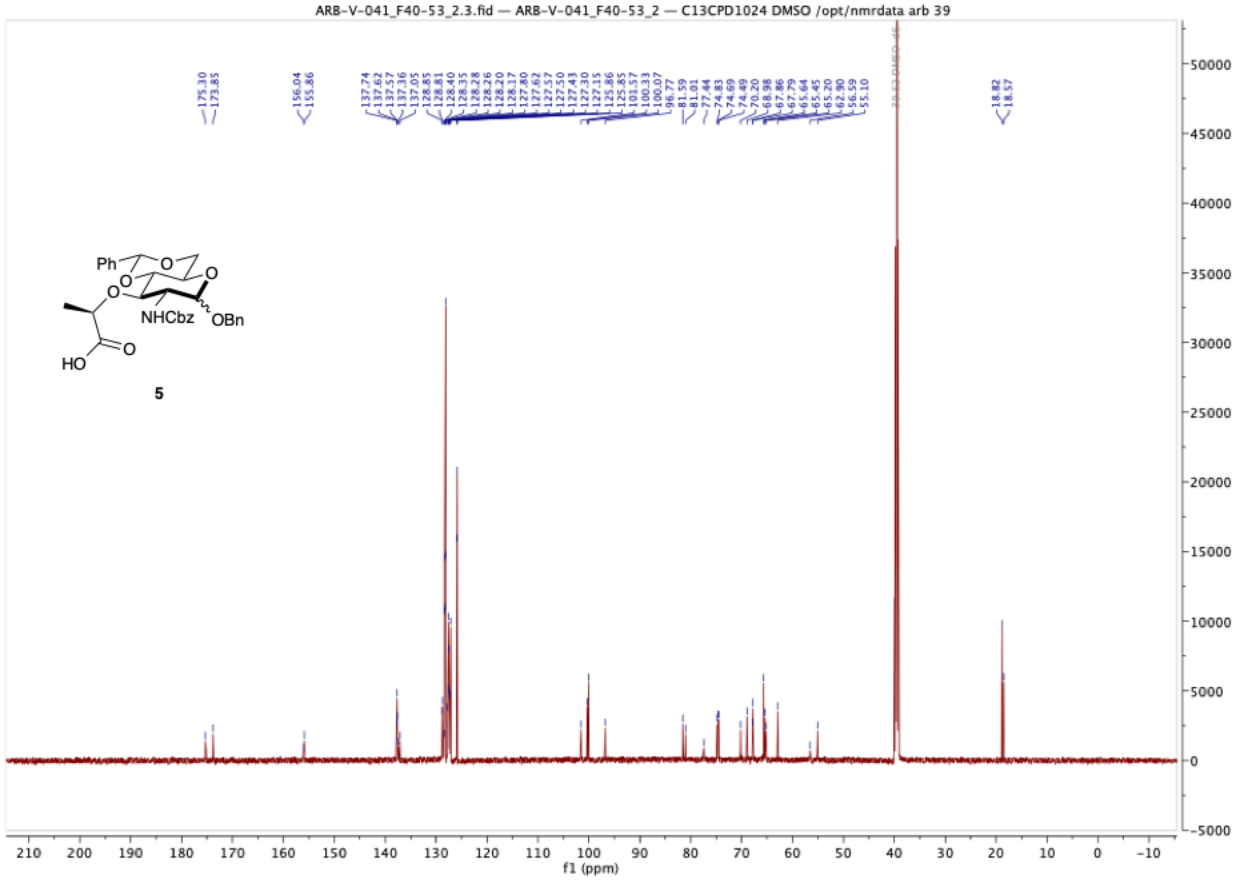
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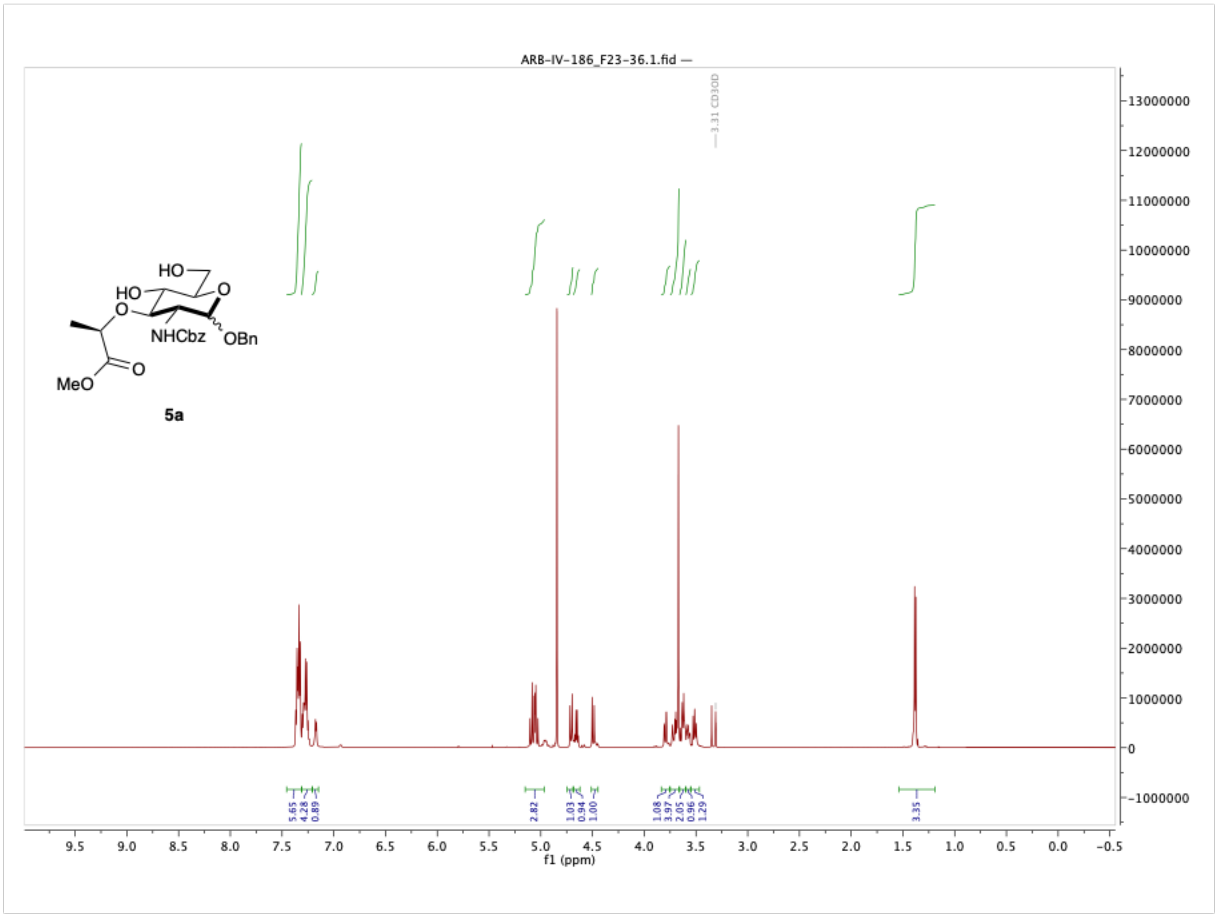
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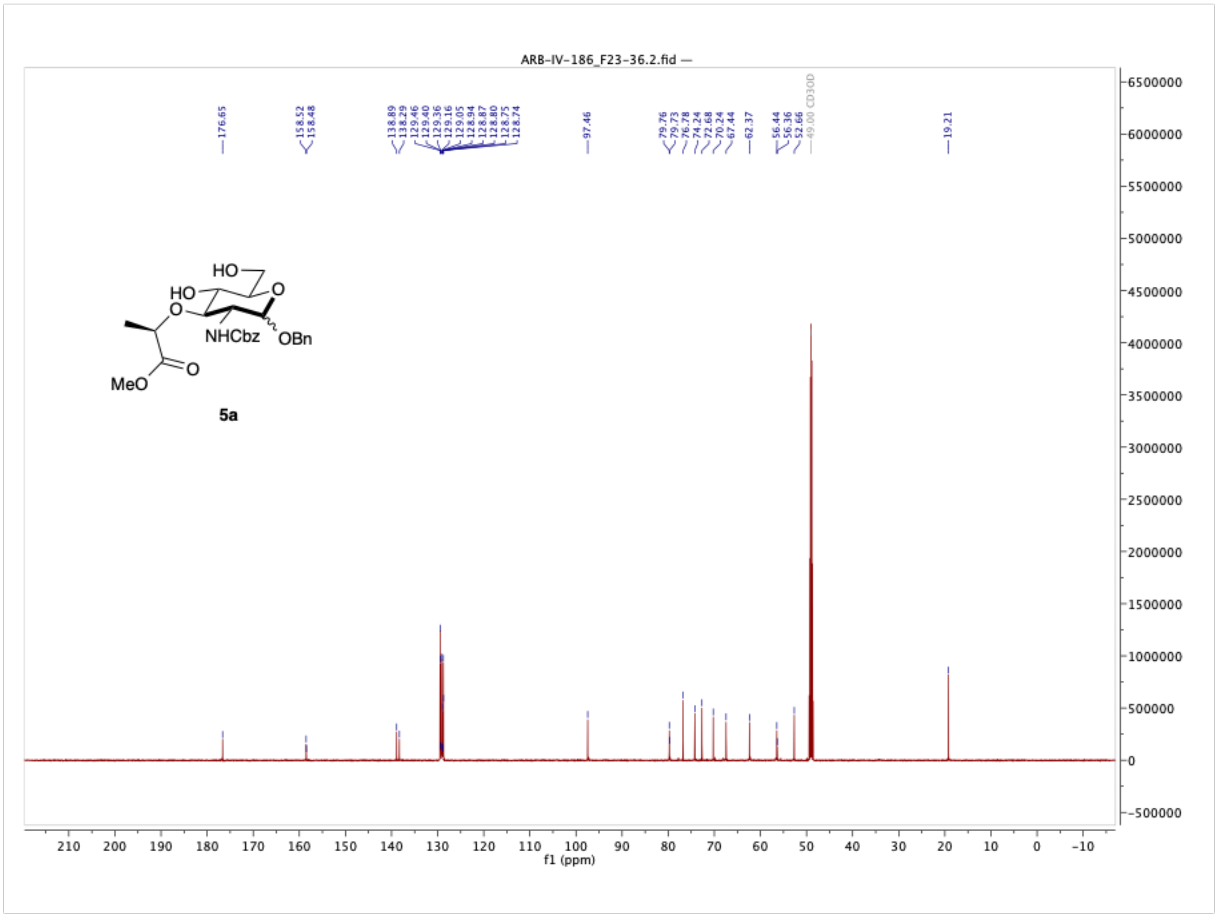
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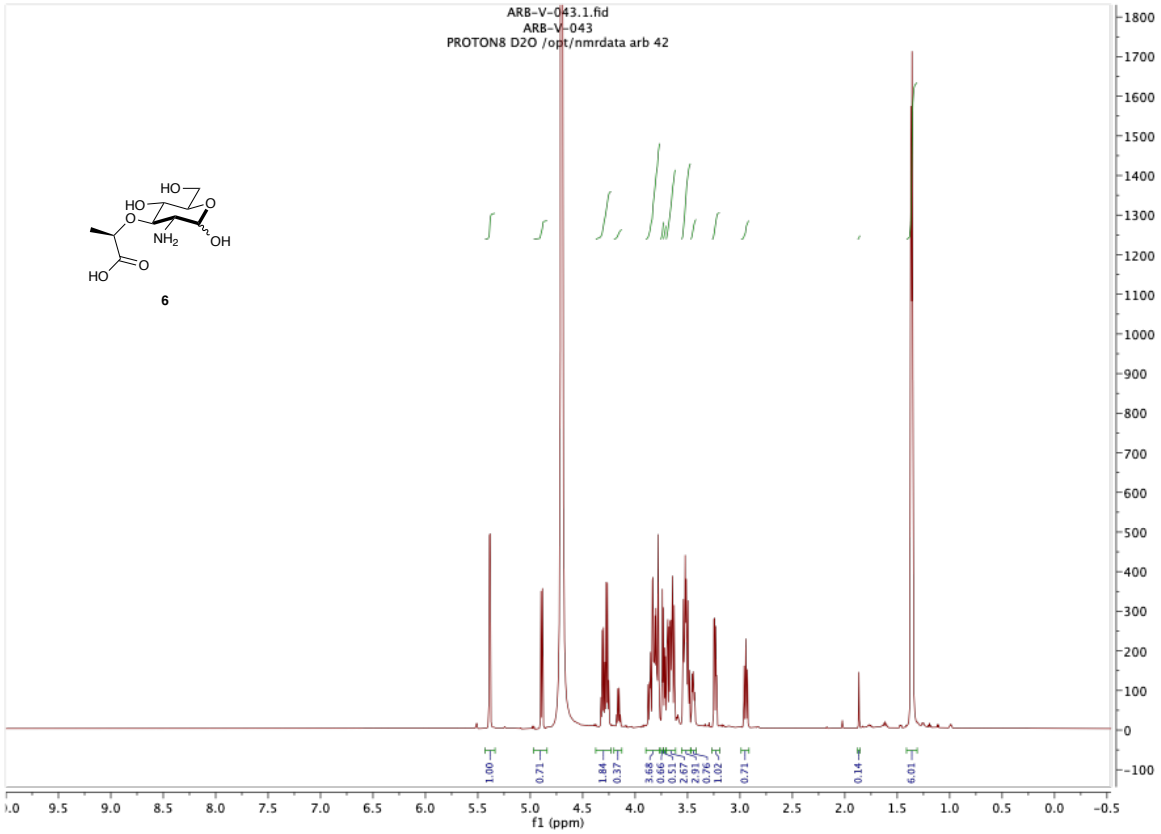
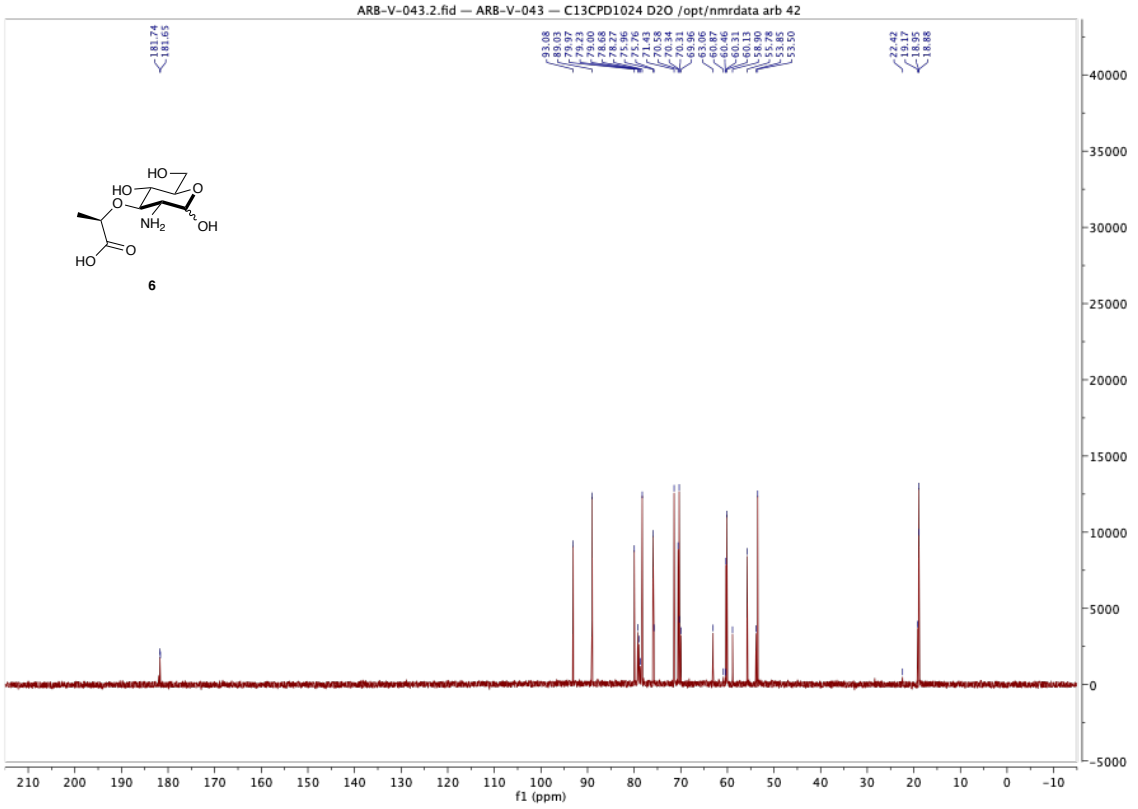
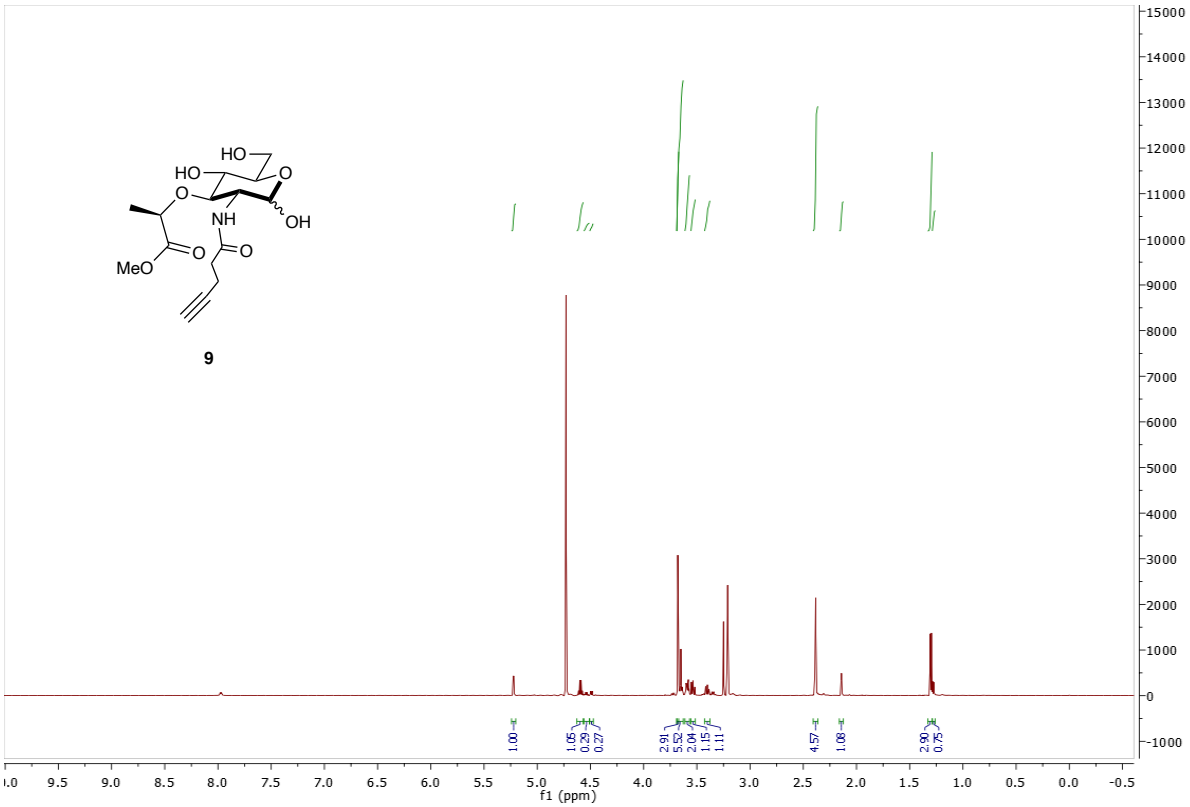
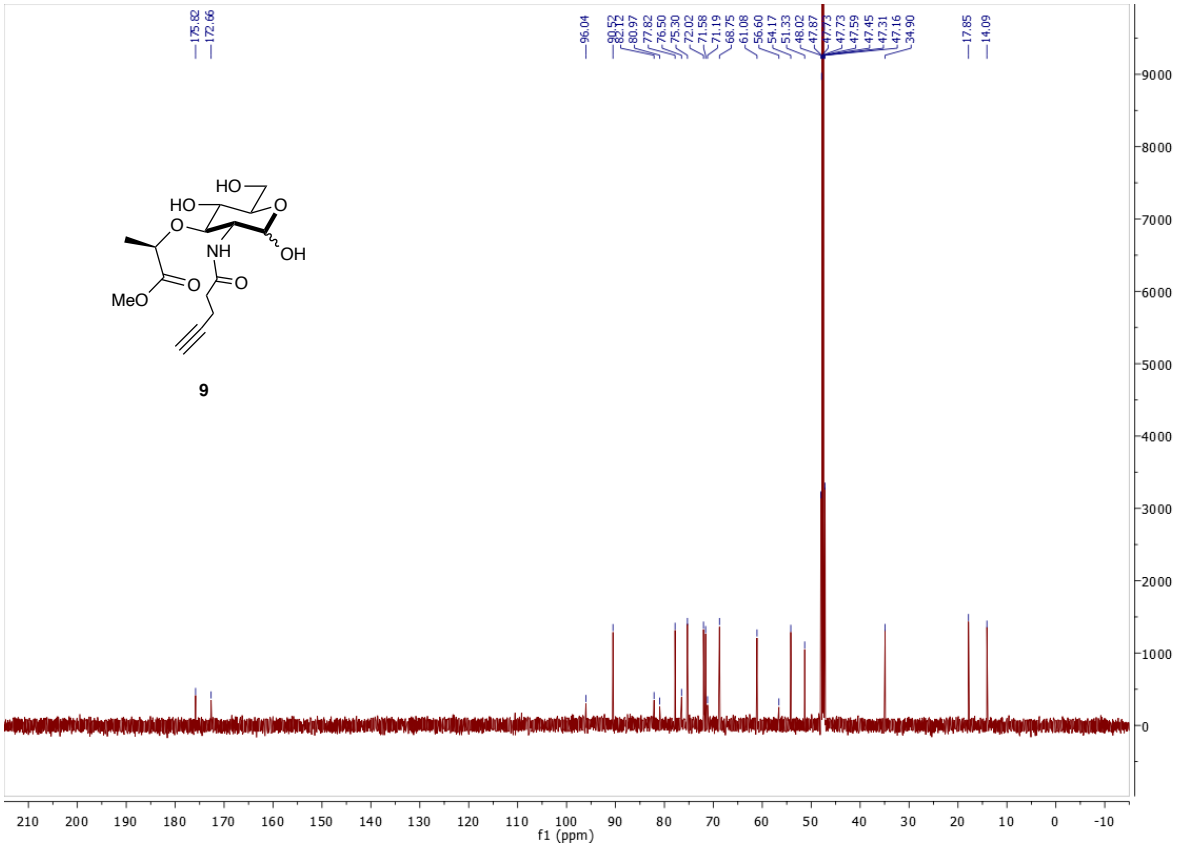
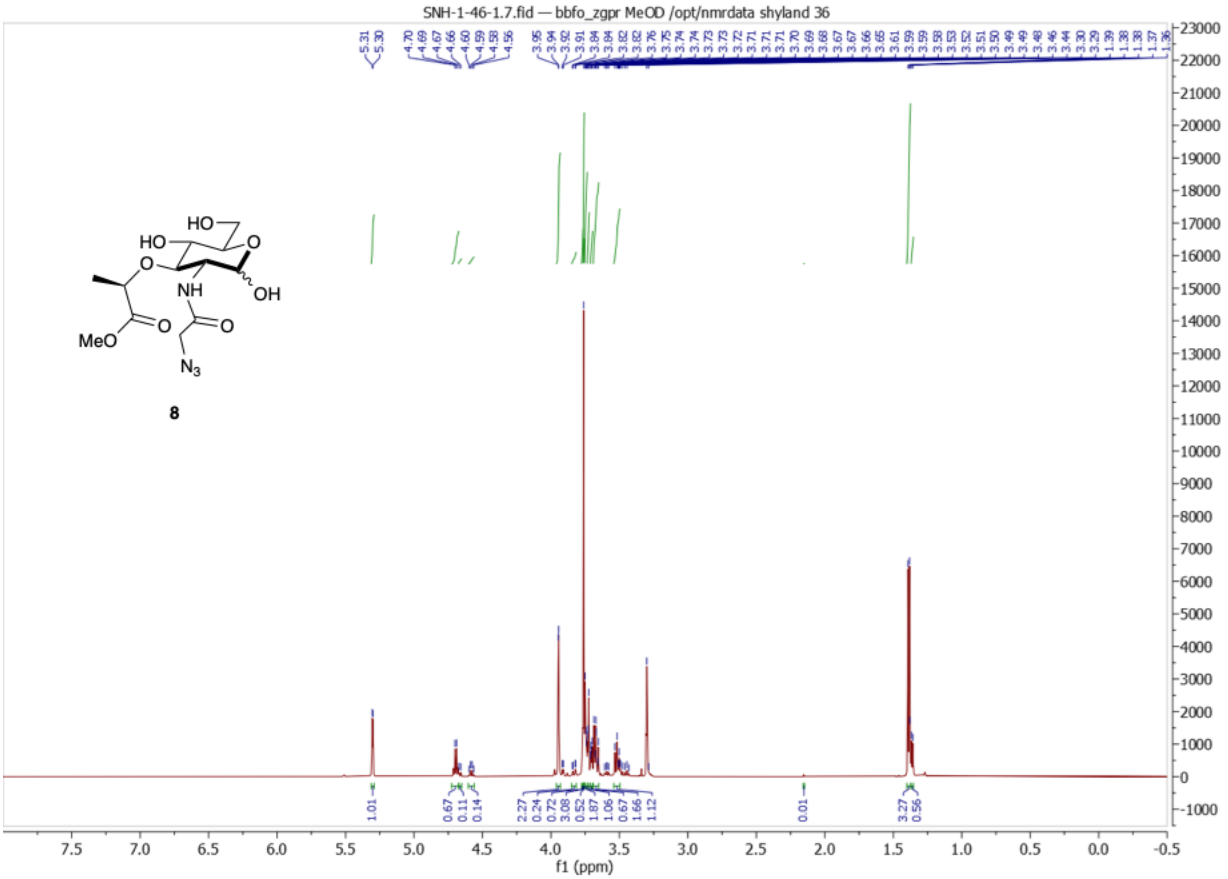
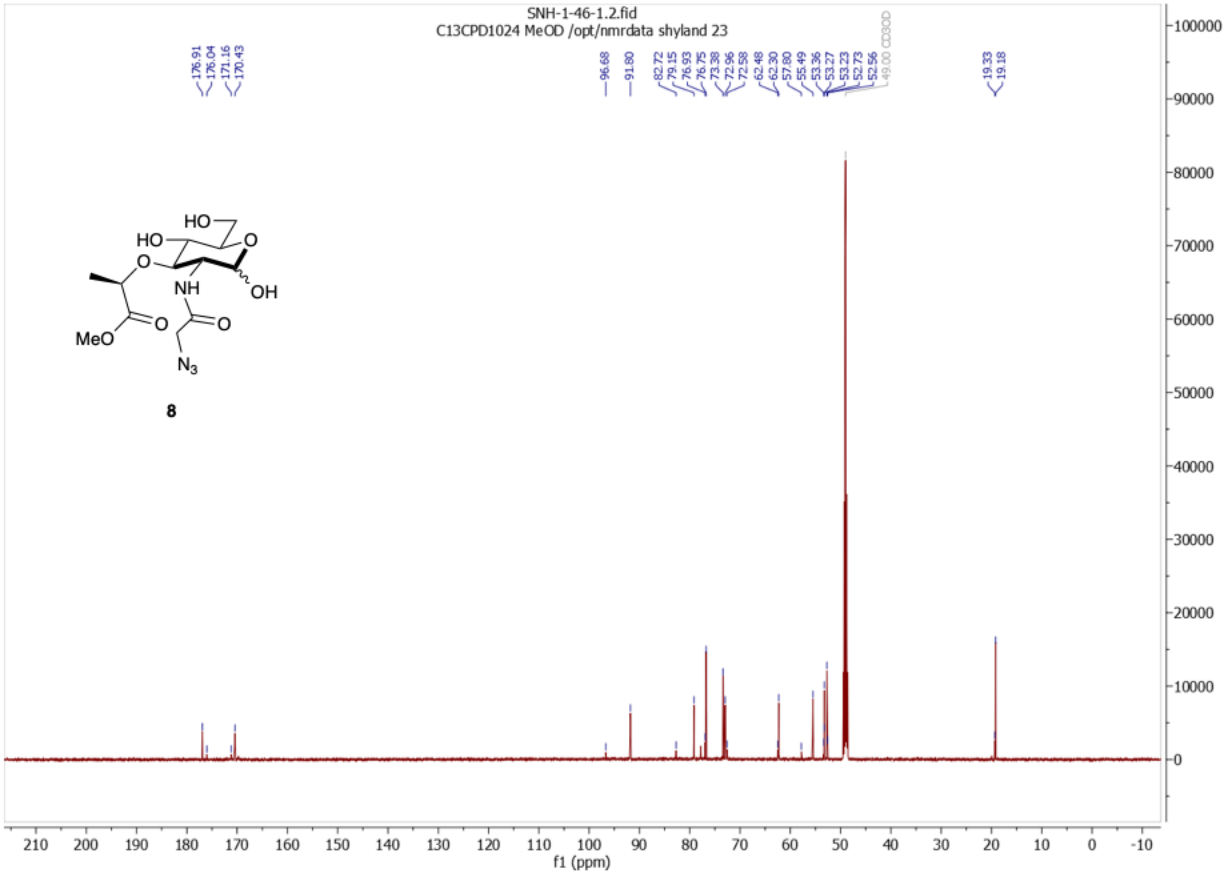

ARB-V-043.1.fid
ARB-V-043
PROTON8 D2O /opt/nmrdata arb 42
HO
HO
O
O
NH2
OH
HO
O
6
1.00
0.71
1.84
0.37
3.68
0.66
0.51
2.67
2.91
0.76
1.02
0.71
0.14
6.01
f1 (ppm)

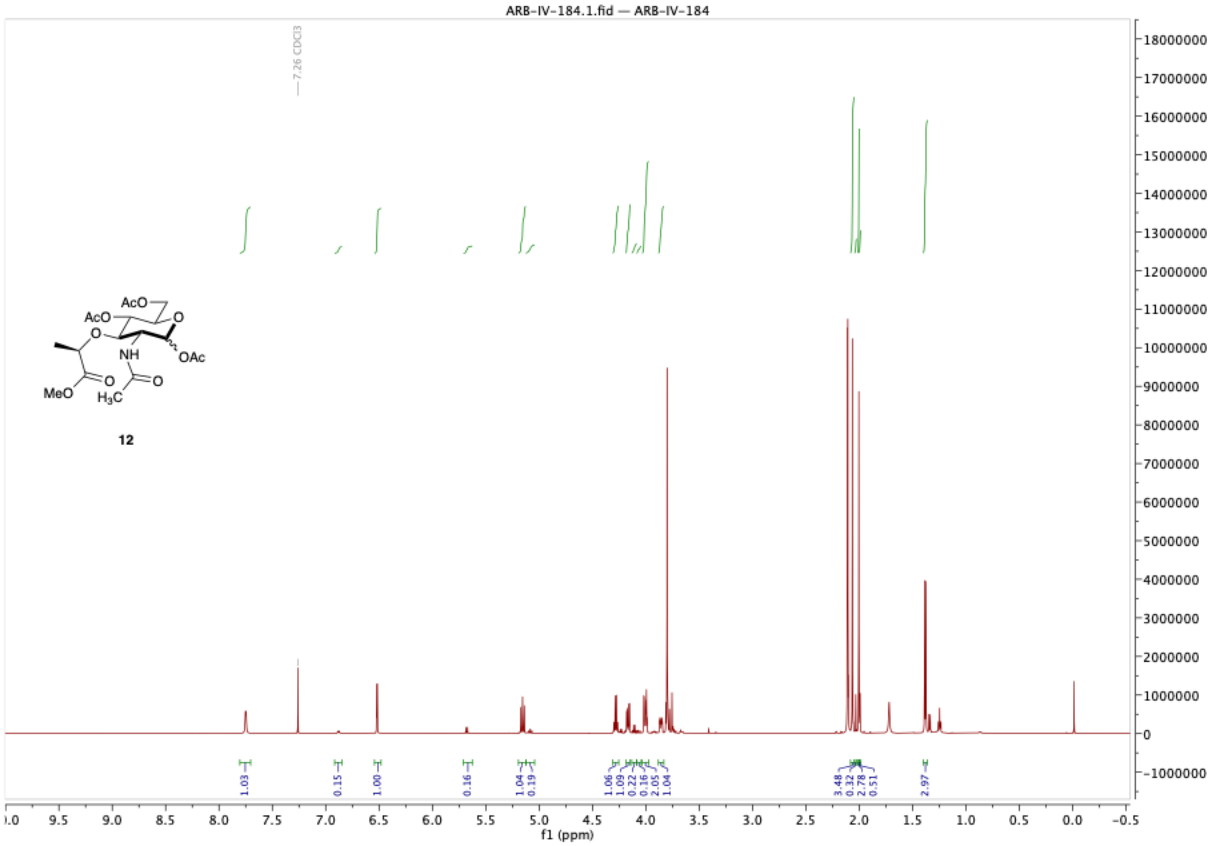

ARB-V-043.2.fid — ARB-V-043 — C13CPD1024 D2O /opt/nmrdata arb 42
181.74
181.65
93.08
89.03
79.97
79.23
79.00
78.68
78.27
75.96
75.76
71.43
70.58
70.34
70.31
69.96
63.06
60.87
60.46
60.31
60.13
58.90
58.78
53.85
53.50
22.42
19.17
18.95
18.88
HO
HO
O
O
NH2
OH
HO
O
6
40000
35000
30000
25000
20000
15000
10000
5000
0
-5000
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

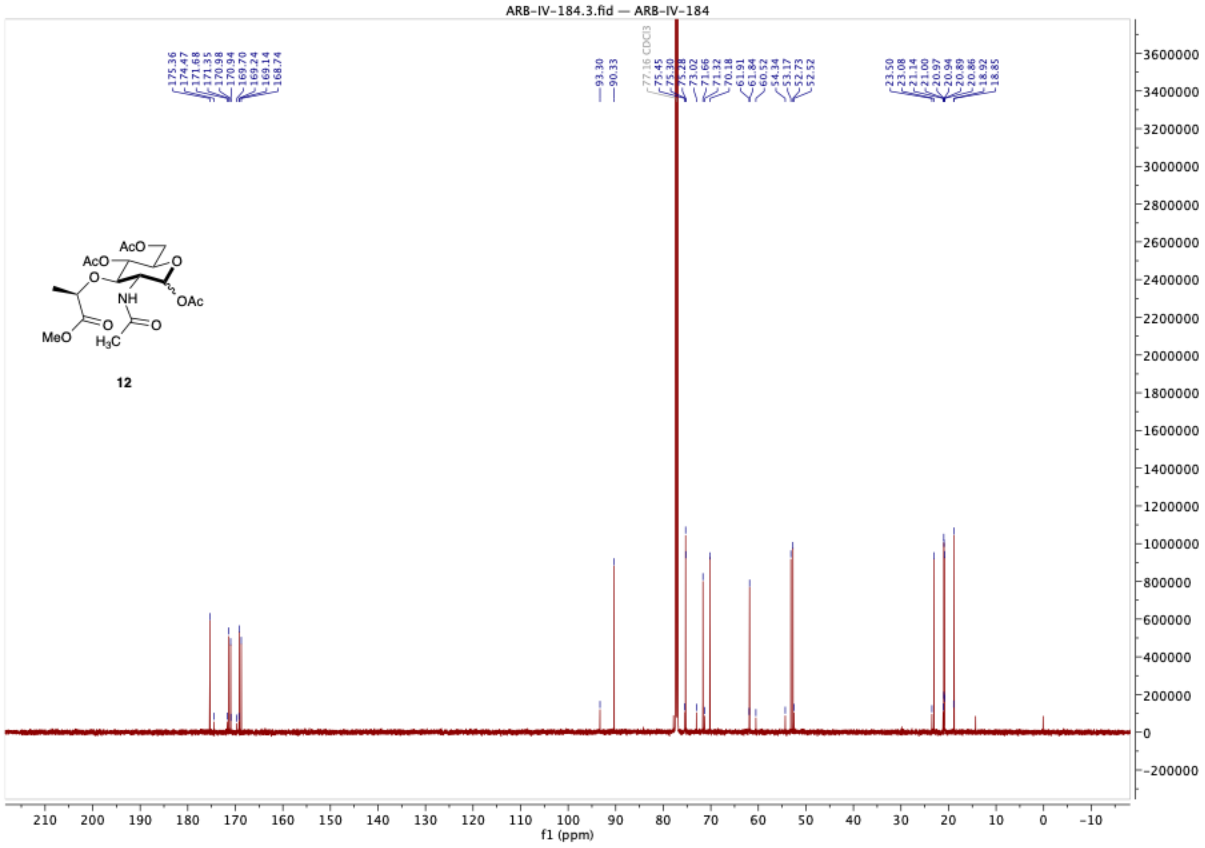

HO
HO
O
O
NH
OH
MeO
O
O
9
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-1000
1.00
1.05
0.29
0.27
2.91
5.52
2.04
1.15
1.11
4.57
1.08
2.90
0.75
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
f1 (ppm)

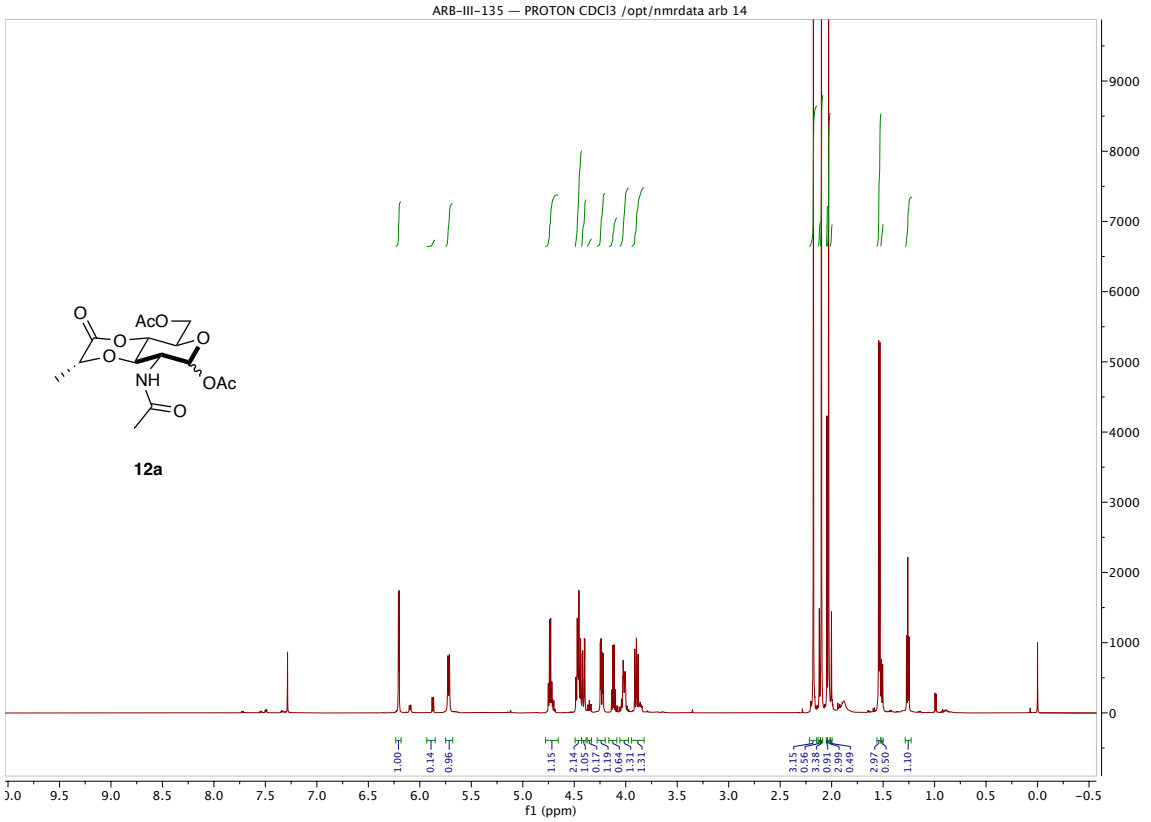

175.82
172.66
96.04
90.52
82.12
80.97
77.82
76.50
75.30
72.02
71.58
71.19
68.75
61.08
56.60
54.17
51.33
48.02
47.87
47.73
47.59
47.45
47.31
47.16
34.90
17.85
14.09
HO
HO
O
O
NH
OH
O
O
MeO
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

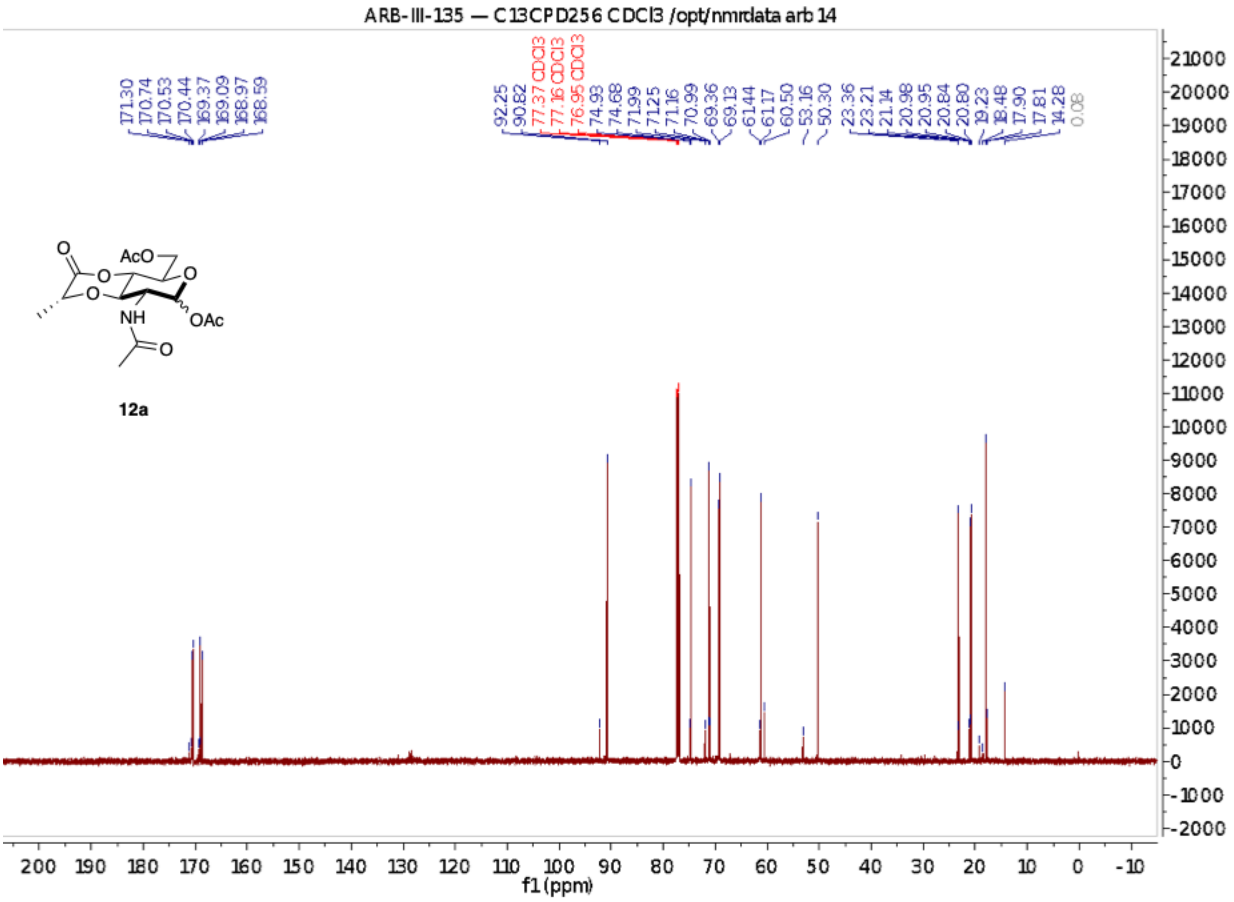

SNH-1-46-1.7.fid — bbfo_zgpr MeOD /opt/nmrdata shyland 36
HO
HO
O
O
NH
OH
MeO
O
O
N3
8
f1 (ppm)

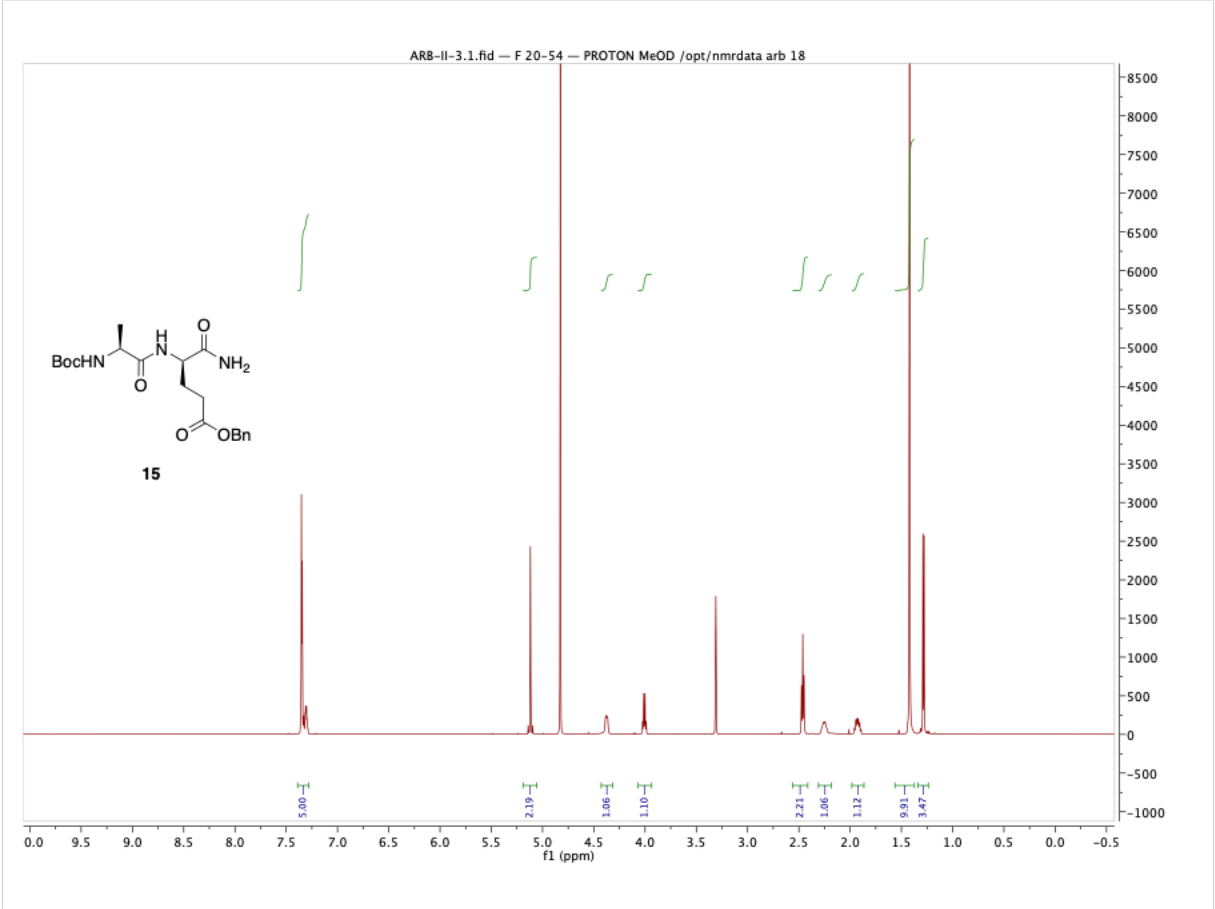

SNH-1-46-1.2.fid
C13CPD1024 MeOD /opt/nmrdata shyland 23
176.91
176.04
171.16
170.43
96.68
91.80
82.72
79.15
76.93
76.75
73.38
72.96
72.58
62.48
62.30
57.80
55.49
53.36
53.27
53.23
52.73
52.56
49.00 CD3OD
19.33
19.18
HO
HO
O
O
NH
OH
MeO
O
O
N3
8
f1 (ppm)

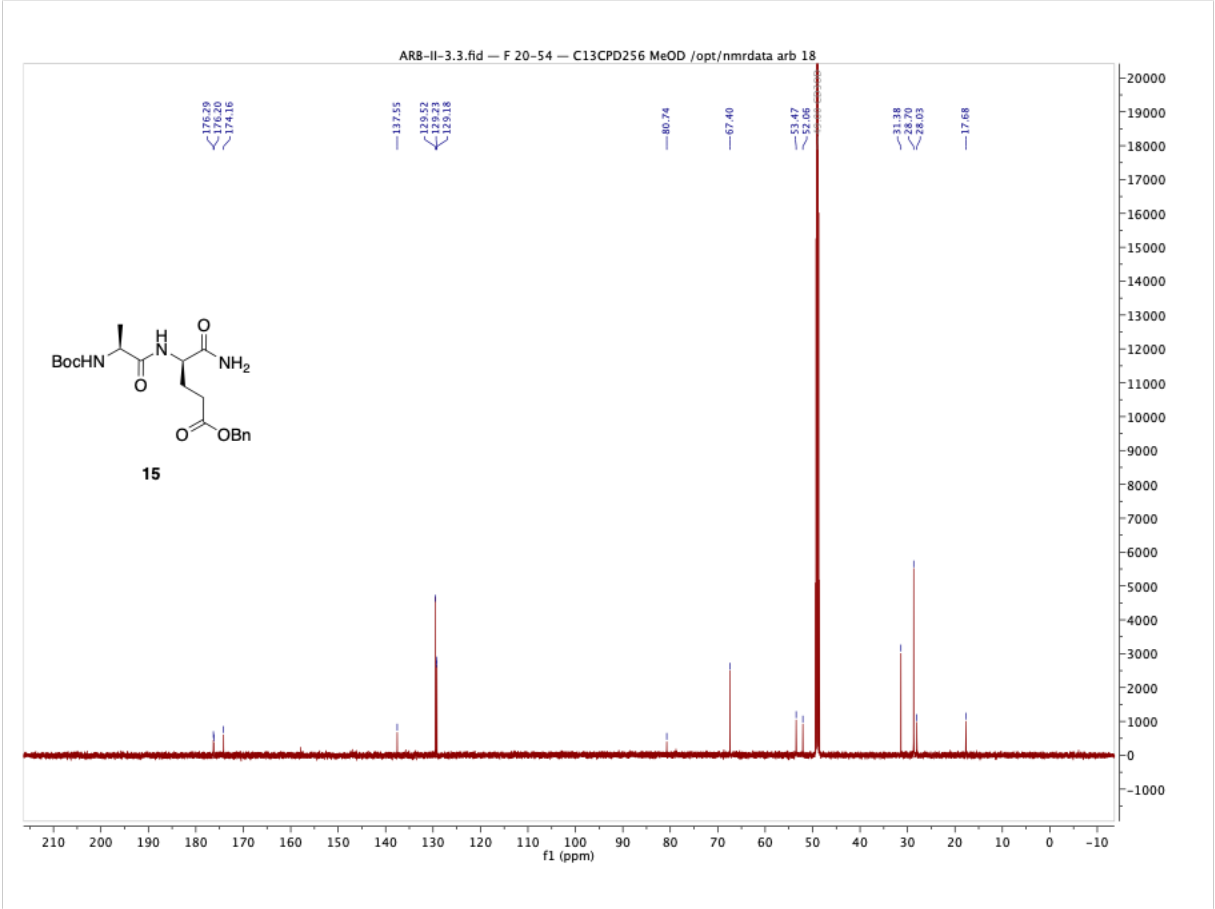

ARB-IV-184.1.fid — ARB-IV-184
7.26 CDCl3
AcO
AcO
O
O
NH
OAc
MeO
O
O
H3C
12
f1 (ppm)

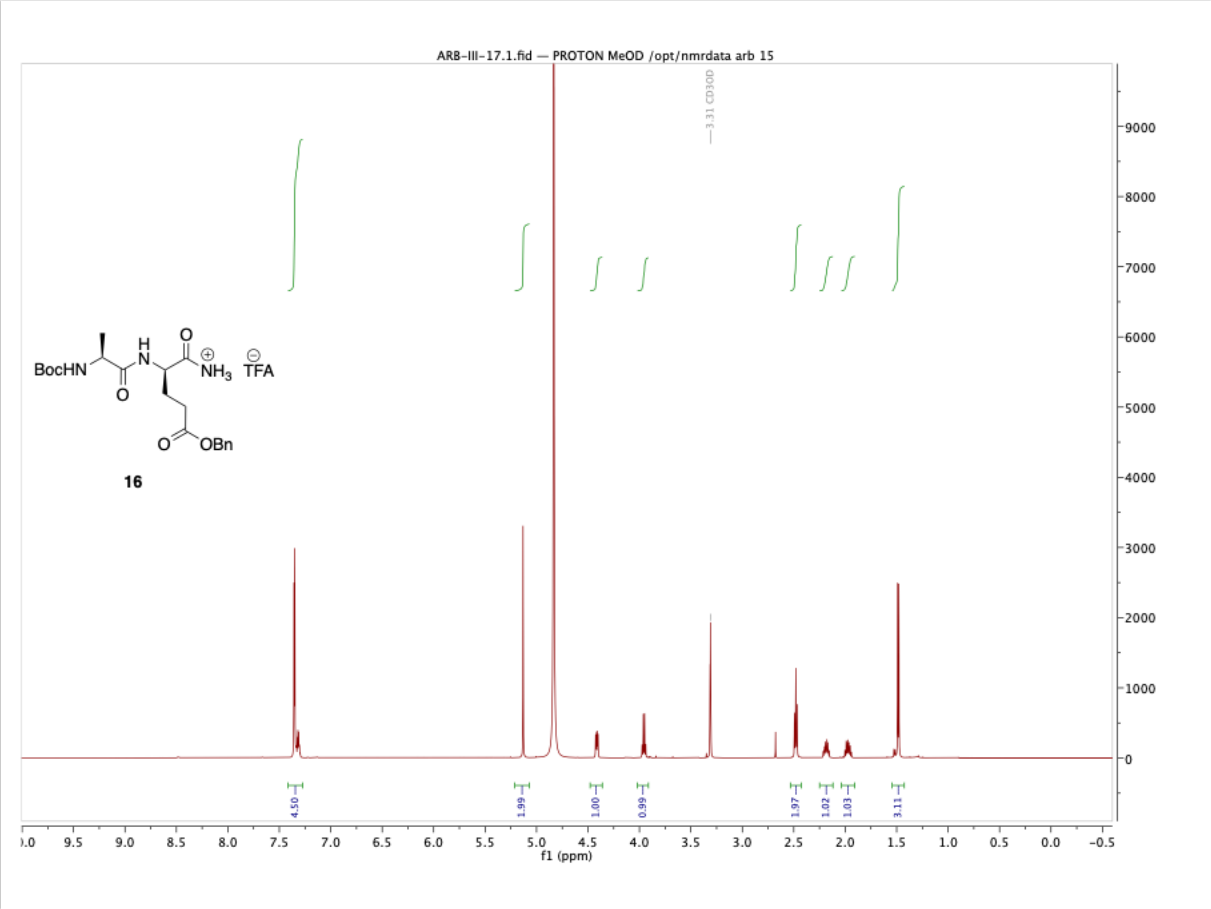

ARB-IV-184.3.fid — ARB-IV-184
AcO
AcO
O
O
NH
OAc
MeO
O
O
H3C
12
f1 (ppm)

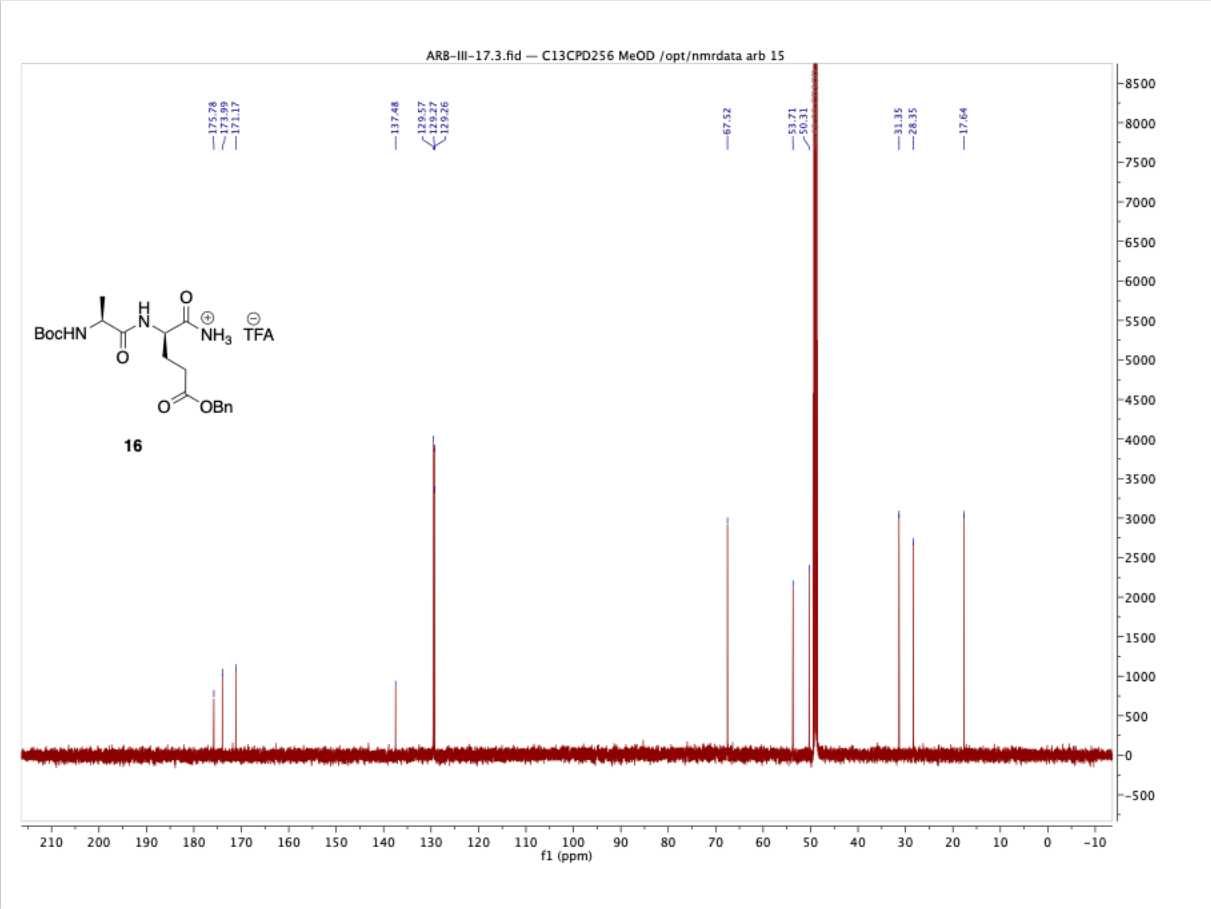

ARB-III-135 — PROTON CDCl3 /opt/nmrdata arb 14
AcO
O
O
O
O
NH
OAc
O
12a
f1 (ppm)

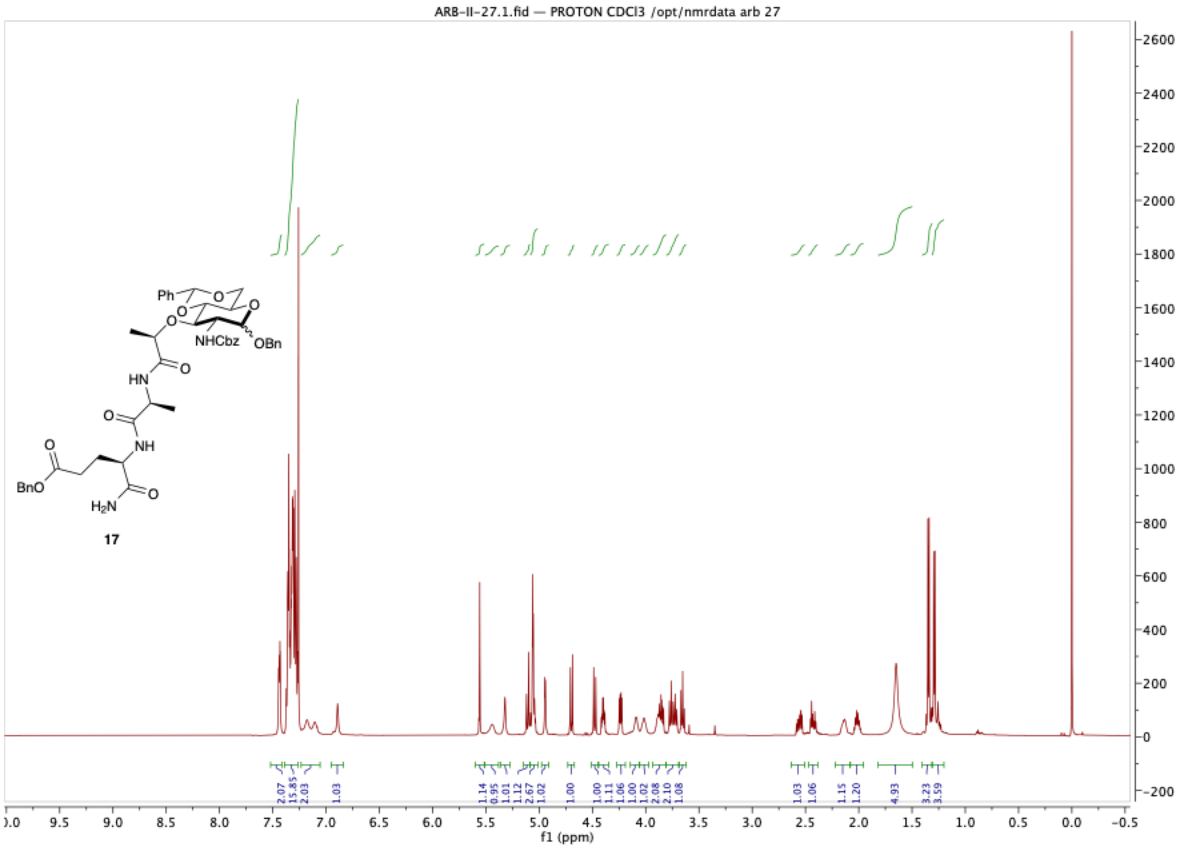

ARB-III-135 — C13CPD256 CDCl3 /opt/nmrdata arb 14
171.30
170.74
170.53
170.44
169.37
169.09
168.97
168.59
92.25
90.82
77.37 CDCl3
77.16 CDCl3
76.95 CDCl3
74.99
74.68
71.99
71.25
71.16
70.99
69.36
69.13
61.44
61.17
60.50
53.16
50.30
23.36
23.21
21.14
20.98
20.95
20.84
20.80
19.23
18.48
17.90
17.81
14.28
0.08
AcO
O
O
O
NH
OAc
O
12a
f1 (ppm)

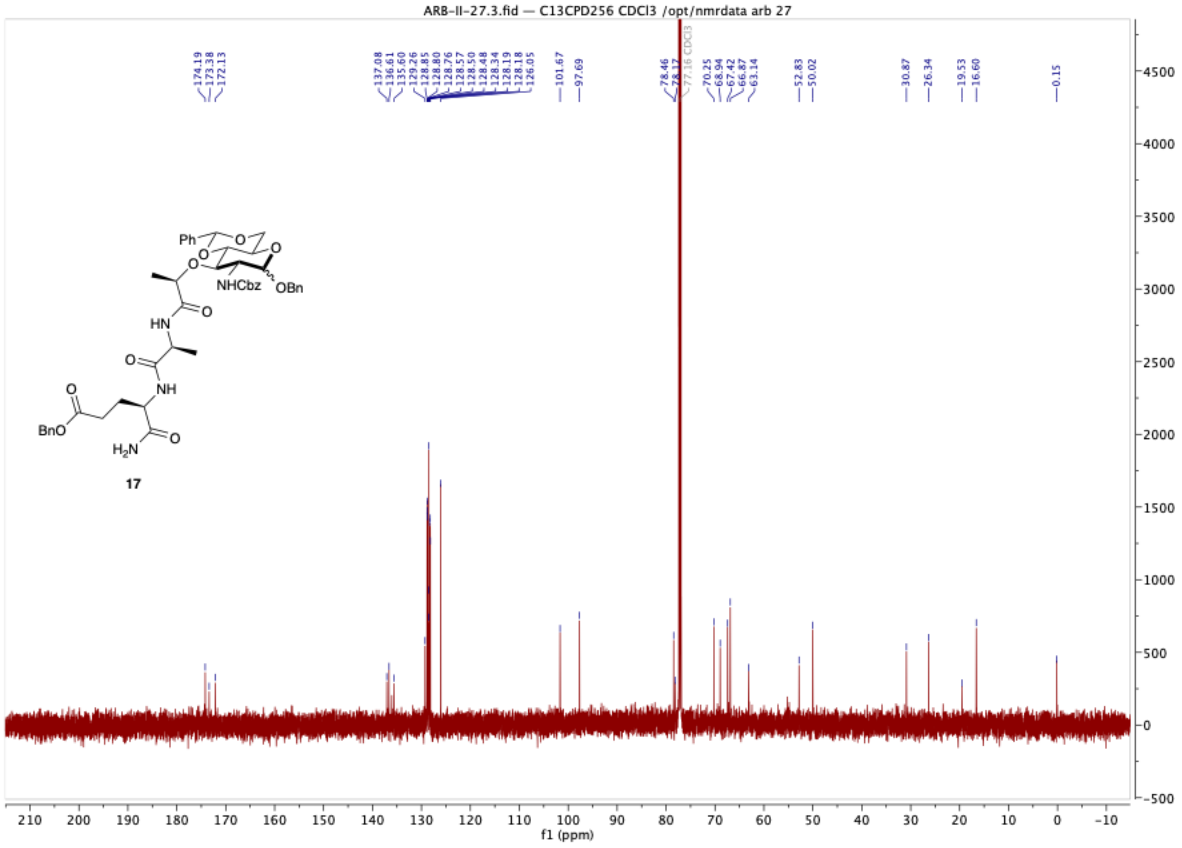

AR8-II-3.1.fid — F 20-54 — PROTON MeOD /opt/nmrdata arb 18
BocHN
H
N
O
O
NH2
O
O
OBn
15
5.00
2.19
1.06
1.10
2.21
1.06
1.12
9.91
3.47
f1 (ppm)

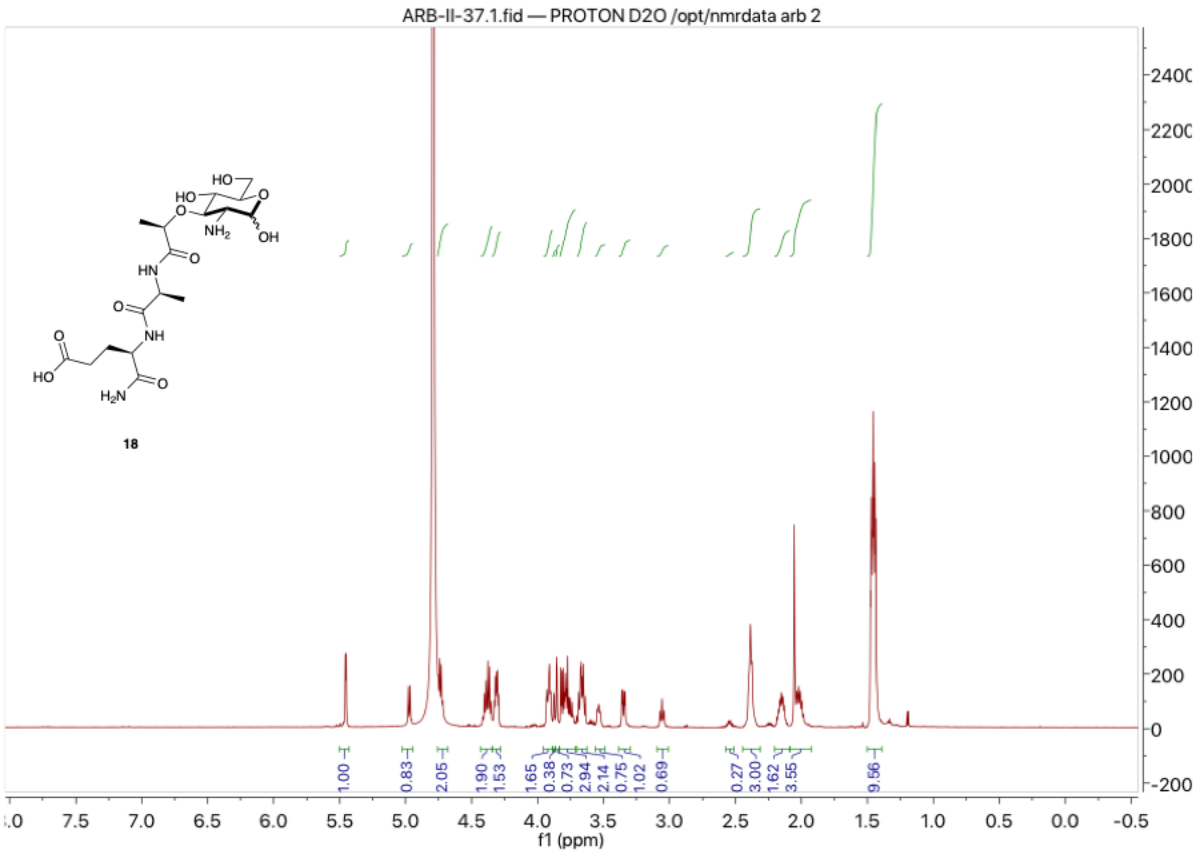

AR8-II-3.3.fid — F 20-54 — C13CPD256 MeOD /opt/nmrdata arb 18
176.29
176.20
174.16
137.55
129.52
129.23
129.18
80.74
67.40
53.47
52.06
31.38
28.70
28.03
17.68
BocHN
NH2
OBn
15
f1 (ppm)

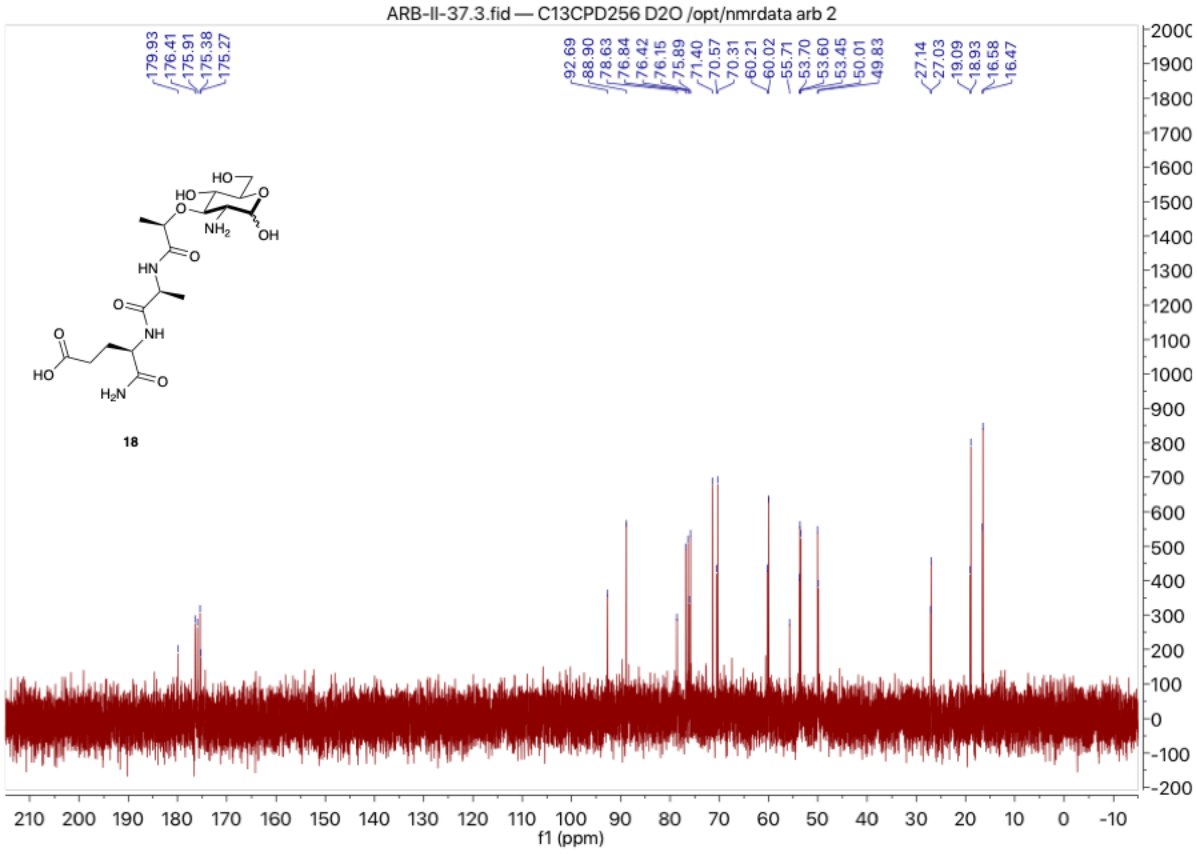

ARB-III-17.1.fid — PROTON MeOD /opt/nmrdata arb 15
3.31 CD3OD
BocHN
NH3
TFA
OBn
16
4.50
1.99
1.00
0.99
1.97
1.02
1.03
3.11
f1 (ppm)


AR8-III-17.3.fid — C13CPD256 MeOD /opt/nmrdata arb 15
175.28
173.69
171.17
137.48
129.57
129.27
129.26
67.52
53.71
50.31
31.35
28.35
17.64
BocHN
NH3
TFA
OBn
16
f1 (ppm)

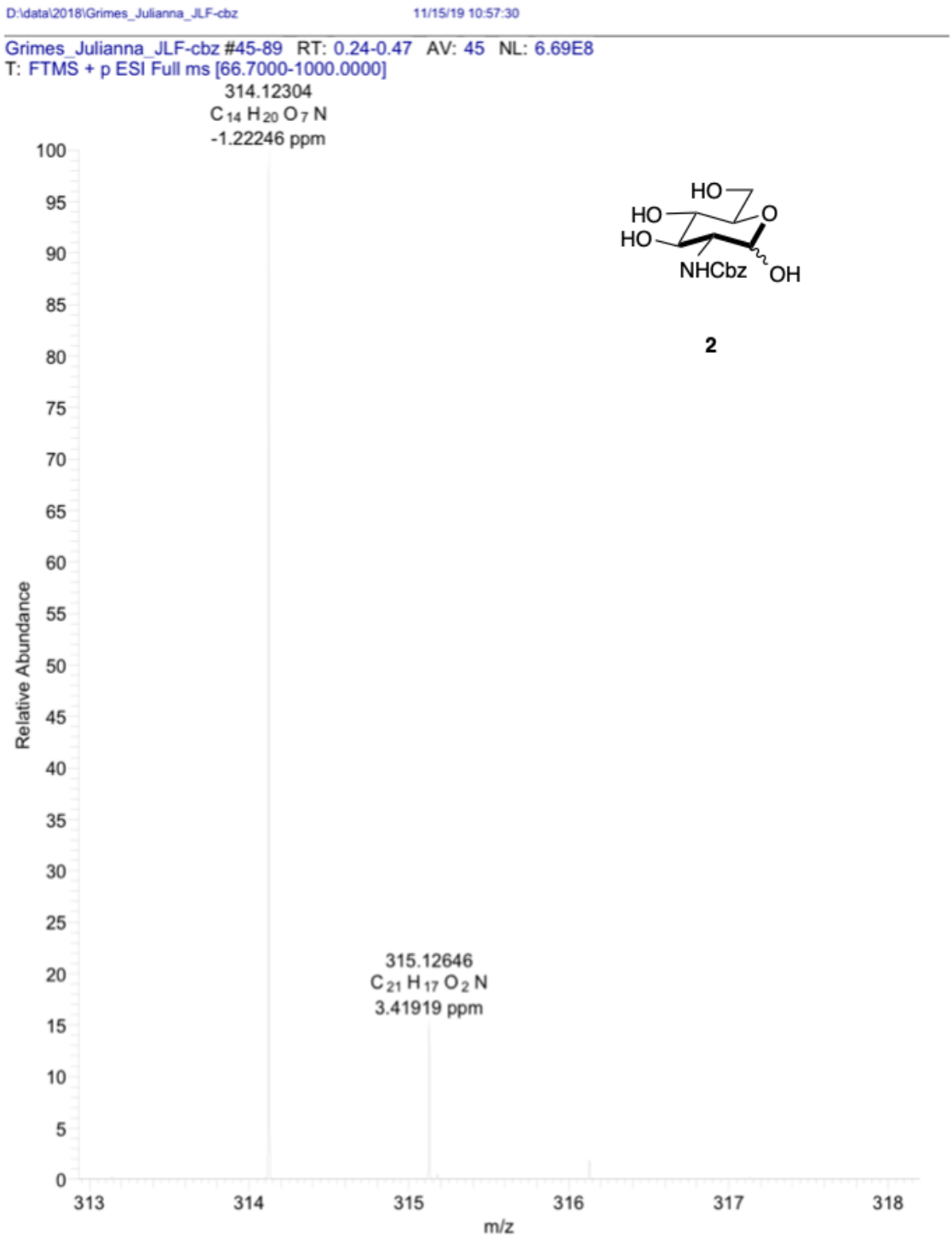

AR8-II-27.1.fid — PROTON CDCl3 /opt/nmrdata arb 27
Ph
O
O
O
NHCbz
OBn
HN
O
O
NH
BnO
O
O
H2N
17
f1 (ppm)

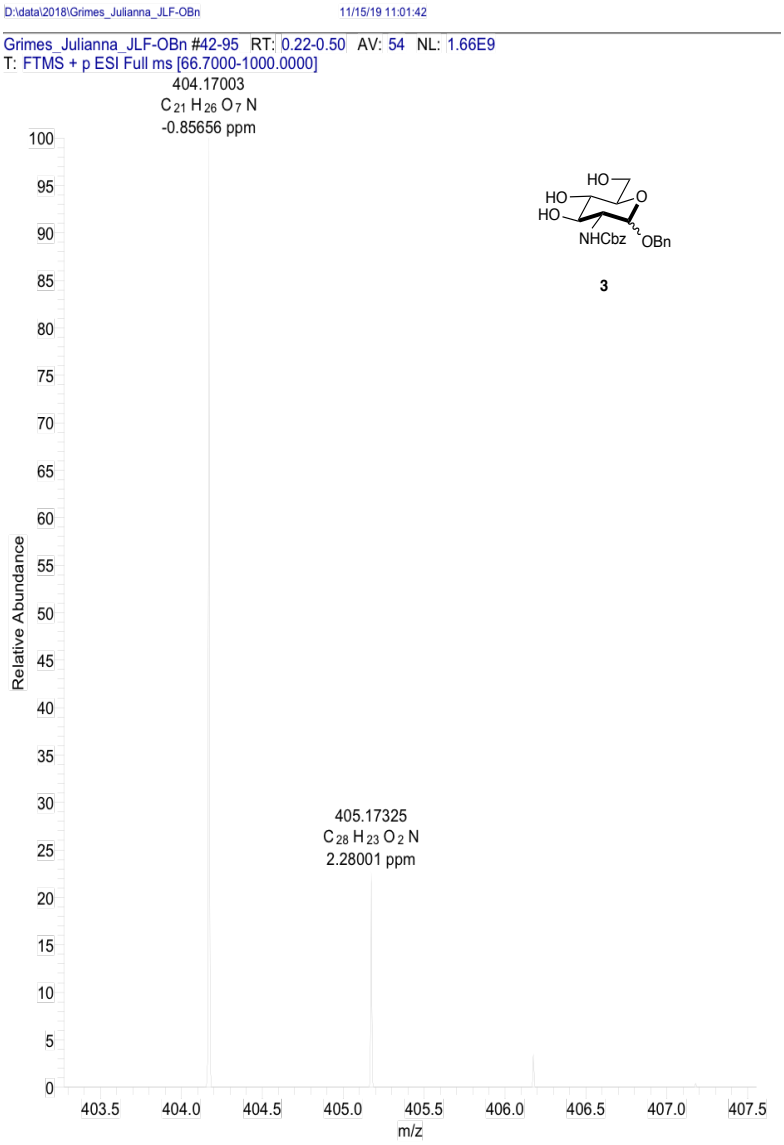

AR8-II-27.3.fid — C13CPD256 CDCl3 /opt/nmrdata arb 27
174.19
173.38
172.13
137.08
136.61
135.60
129.26
128.85
128.80
128.76
128.57
128.50
128.48
128.34
128.19
128.13
126.05
101.67
97.69
78.46
78.32
77.16 CDCl3
70.25
68.94
67.42
66.87
63.14
52.83
50.02
30.87
26.34
19.53
16.60
0.15
Ph
O
O
O
O
NHCbz
OBn
HN
O
O
NH
O
BnO
O
H_2N
O
17
f1 (ppm)

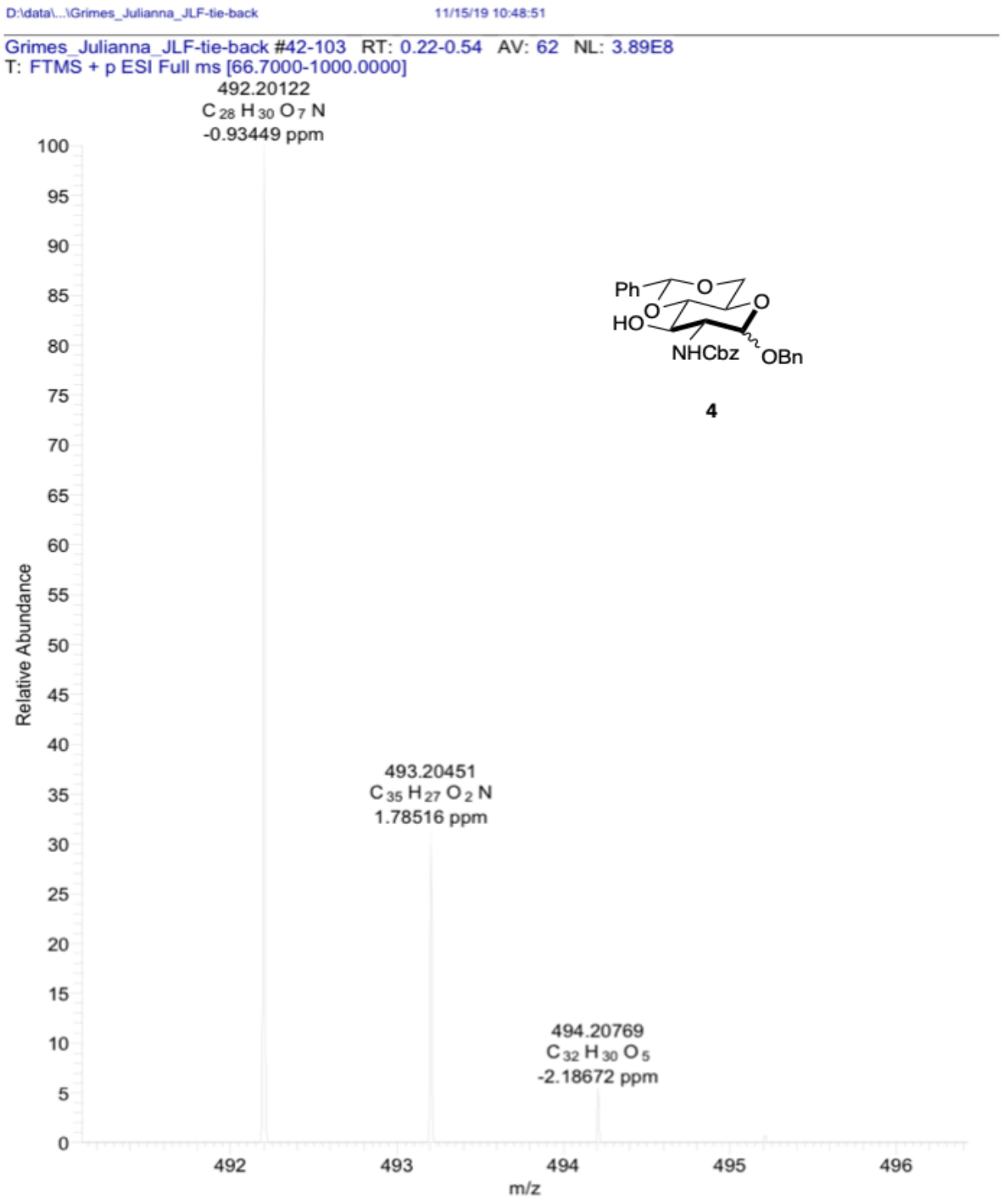

ARB-II-37.1.fid — PROTON D2O /opt/nmrdata arb 2
18
f1 (ppm)

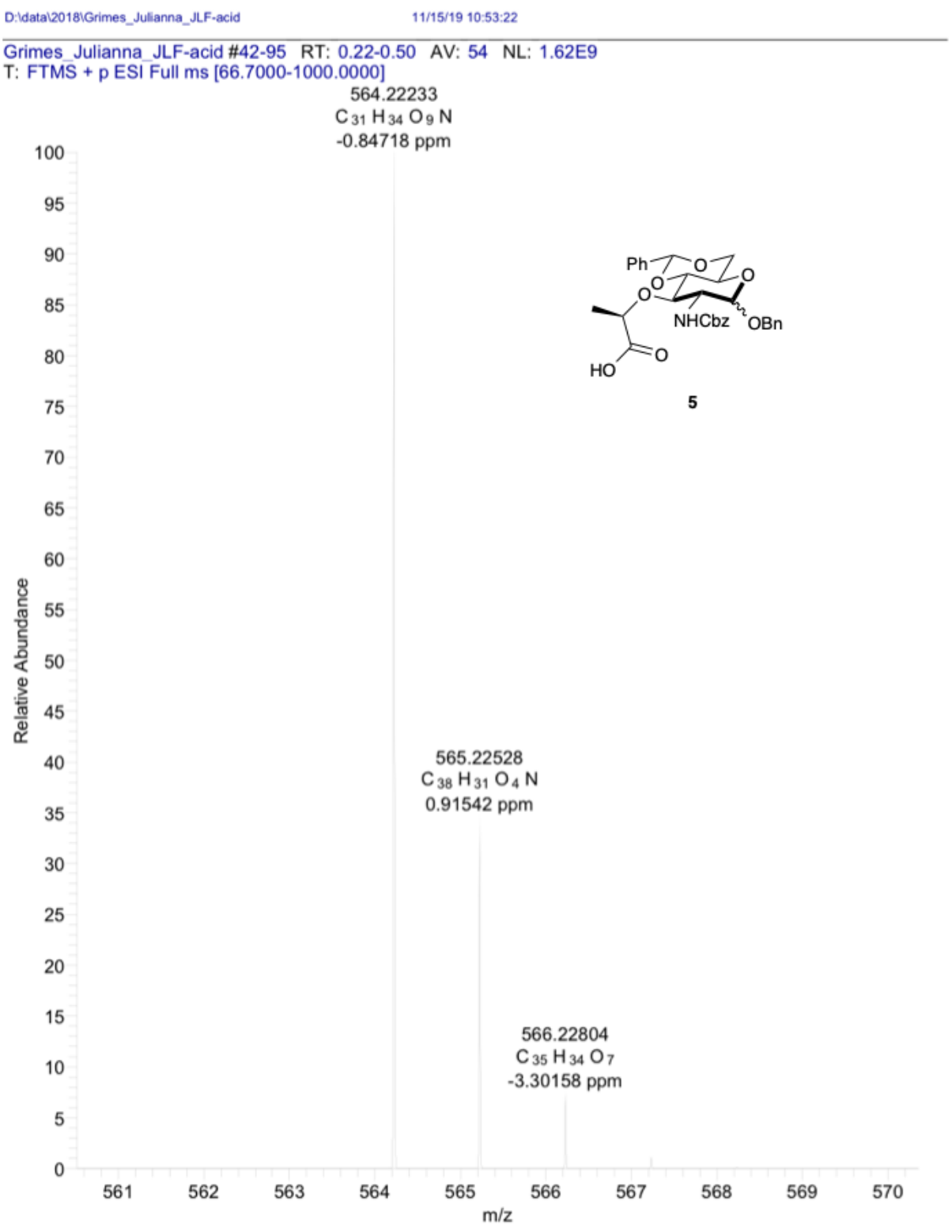

ARB-II-37.3.fid — C13CPD256 D2O /opt/nmrdata arb 2
179.93
176.41
175.91
175.38
175.27
92.69
88.90
78.63
76.84
76.42
76.15
75.89
71.40
70.57
70.31
60.21
60.02
55.71
53.70
53.60
53.45
50.01
49.83
27.14
27.03
19.09
18.93
16.58
16.47
18
f1 (ppm)

VIII. High Resolution Mass Spectrometry Spectra



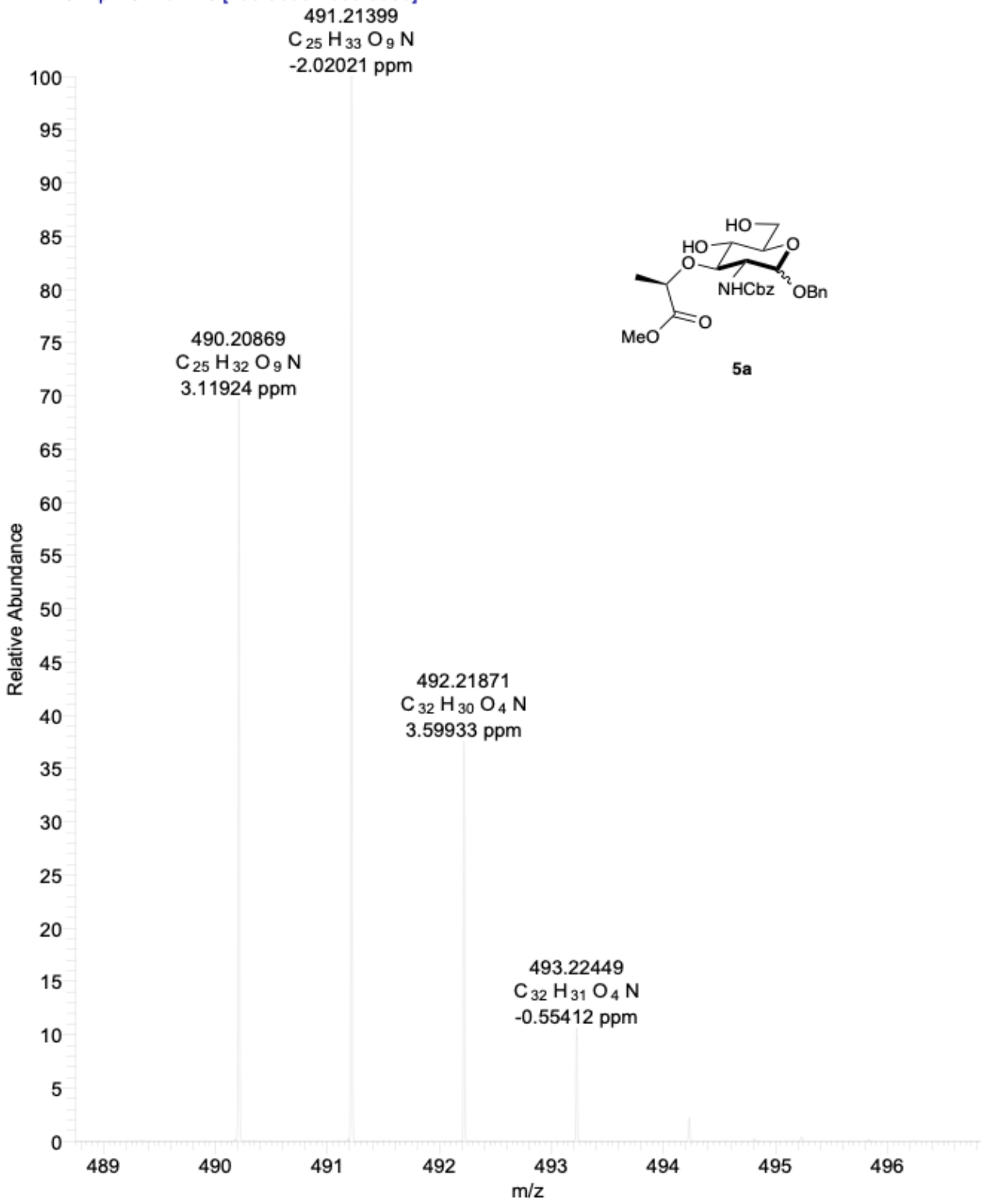

D:\data\2018\Grimes_Julianna_JLF-OBn 11/15/19 11:01:42
Grimes_Julianna_JLF-OBn #42-95 RT: 0.22-0.50 AV: 54 NL: 1.66E9
T: FTMS + p ESI Full ms [66.7000-1000.0000]
404.17003
C21 H26 O7 N
-0.85656 ppm
405.17325
C28 H23 O2 N
2.28001 ppm
Relative Abundance
m/z
HO
HO
HO
O
NHCbz
OBn
3


D:\data\...\Grimes_Julianna_JLF-tie-back
11/15/19 10:48:51
Grimes_Julianna_JLF-tie-back #42-103 RT: 0.22-0.54 AV: 62 NL: 3.89E8
T: FTMS + p ESI Full ms [66.7000-1000.0000]
492.20122
C 28 H 30 O 7 N
-0.93449 ppm
493.20451
C 35 H 27 O 2 N
1.78516 ppm
494.20769
C 32 H 30 O 5
-2.18672 ppm
Ph
O
O
HO
NHCbz
OBn
4
Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
492
493
494
495
496

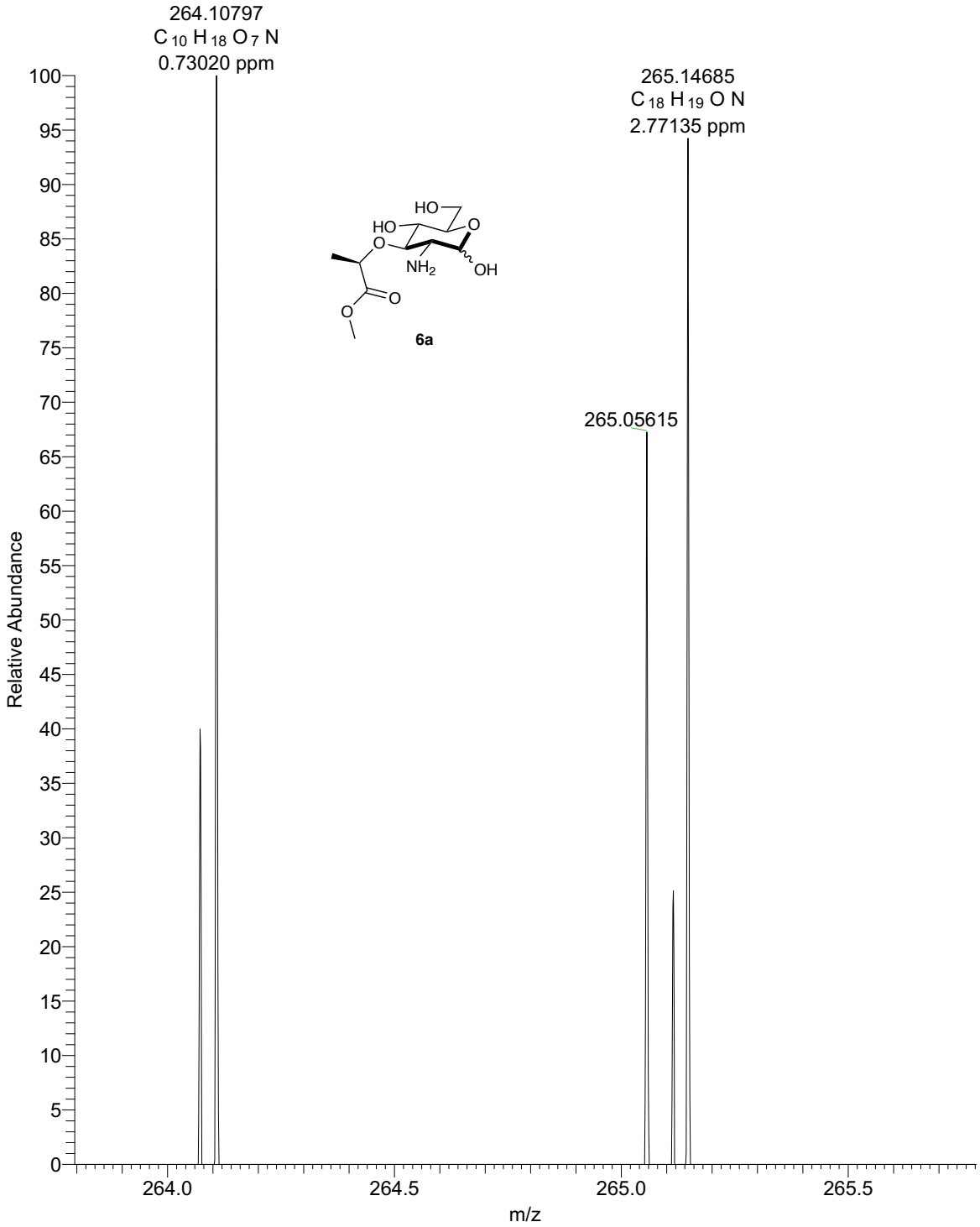

D:\data\2018\Grimes_Julianna_JLF-acid 11/15/19 10:53:22
Grimes_Julianna_JLF-acid #42-95 RT: 0.22-0.50 AV: 54 NL: 1.62E9
T: FTMS + p ESI Full ms [66.7000-1000.0000]
564.22233
C31 H34 O9 N
-0.84718 ppm
565.22528
C38 H31 O4 N
0.91542 ppm
566.22804
C35 H34 O7
-3.30158 ppm
Relative Abundance
m/z
Ph
O
O
O
O
NHCbz
OBn
HO
O
5

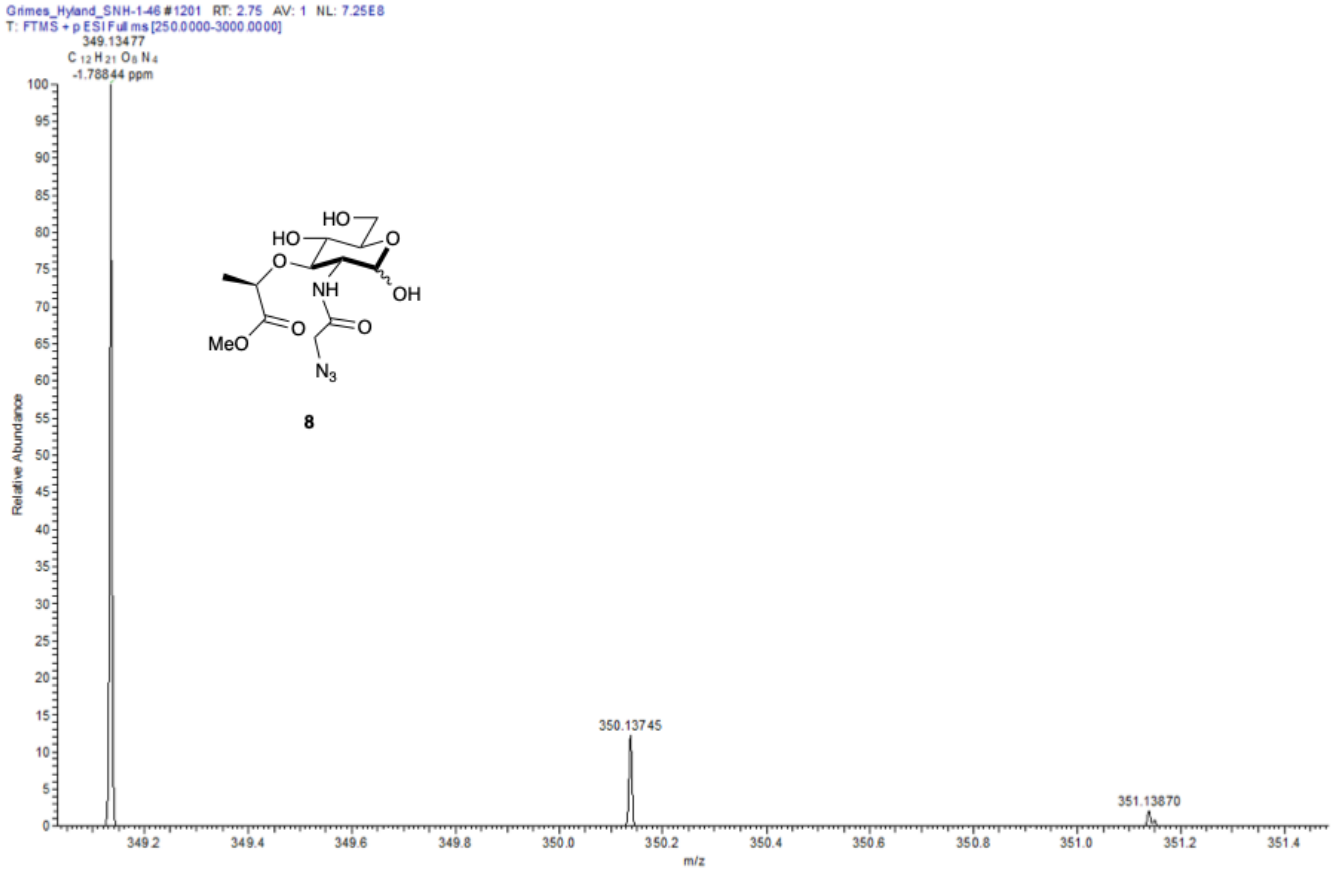
D:\data\2020\Grimes_Ashley_ARB-V-044 03/22/21 12:00:40

Grimes_Ashley_ARB-V-044 #50-107 RT: 0.26-0.56 AV: 58 NL: 6.67E7
T: FTMS + p ESI Full ms [100.0000-1500.0000]

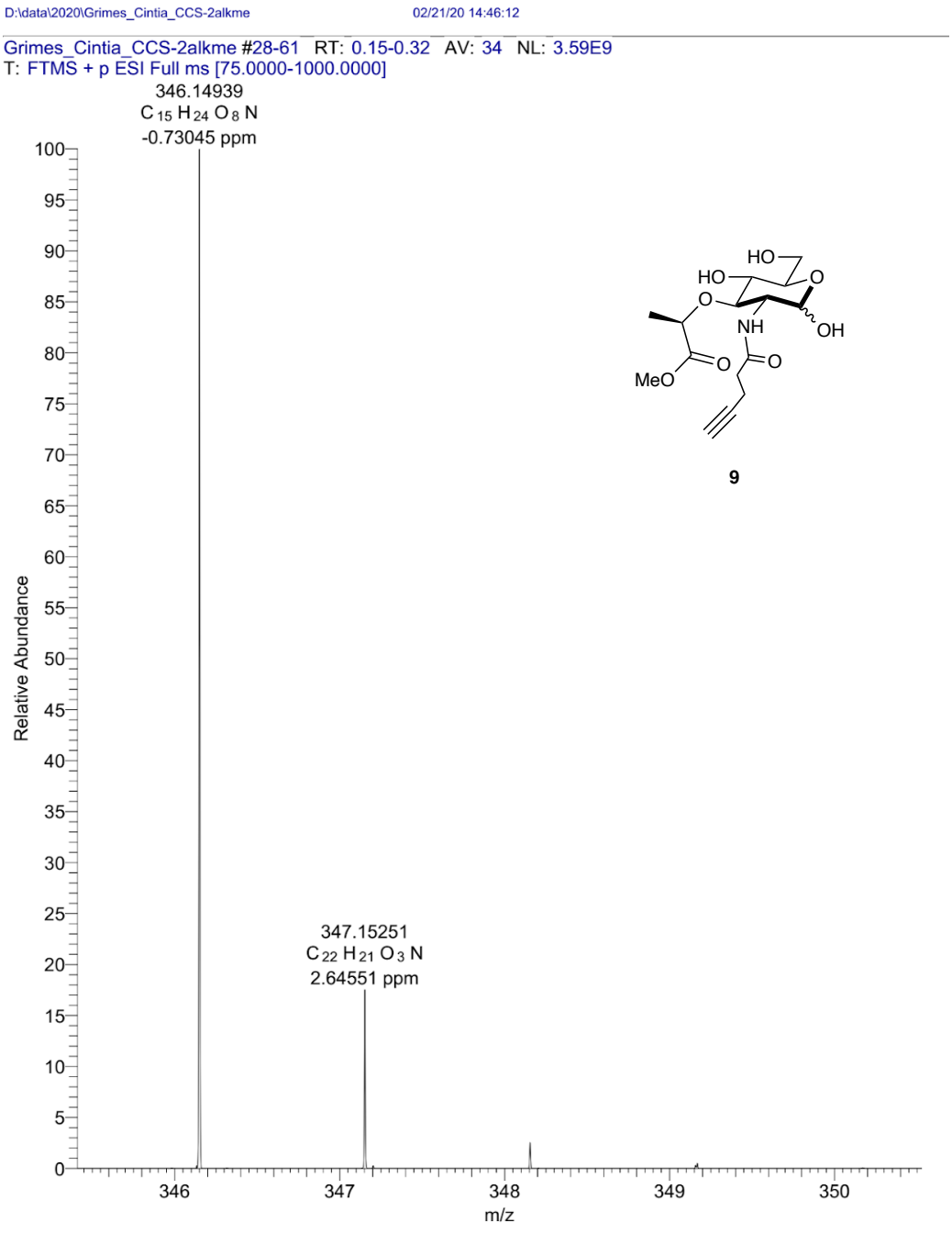


Grimes_Ashley_ARB-V-048_NEG #62 RT: 0.35 AV: 1 NL: 1.36E6
T: FTMS - p ESI Full ms [133.4000-2000.0000]

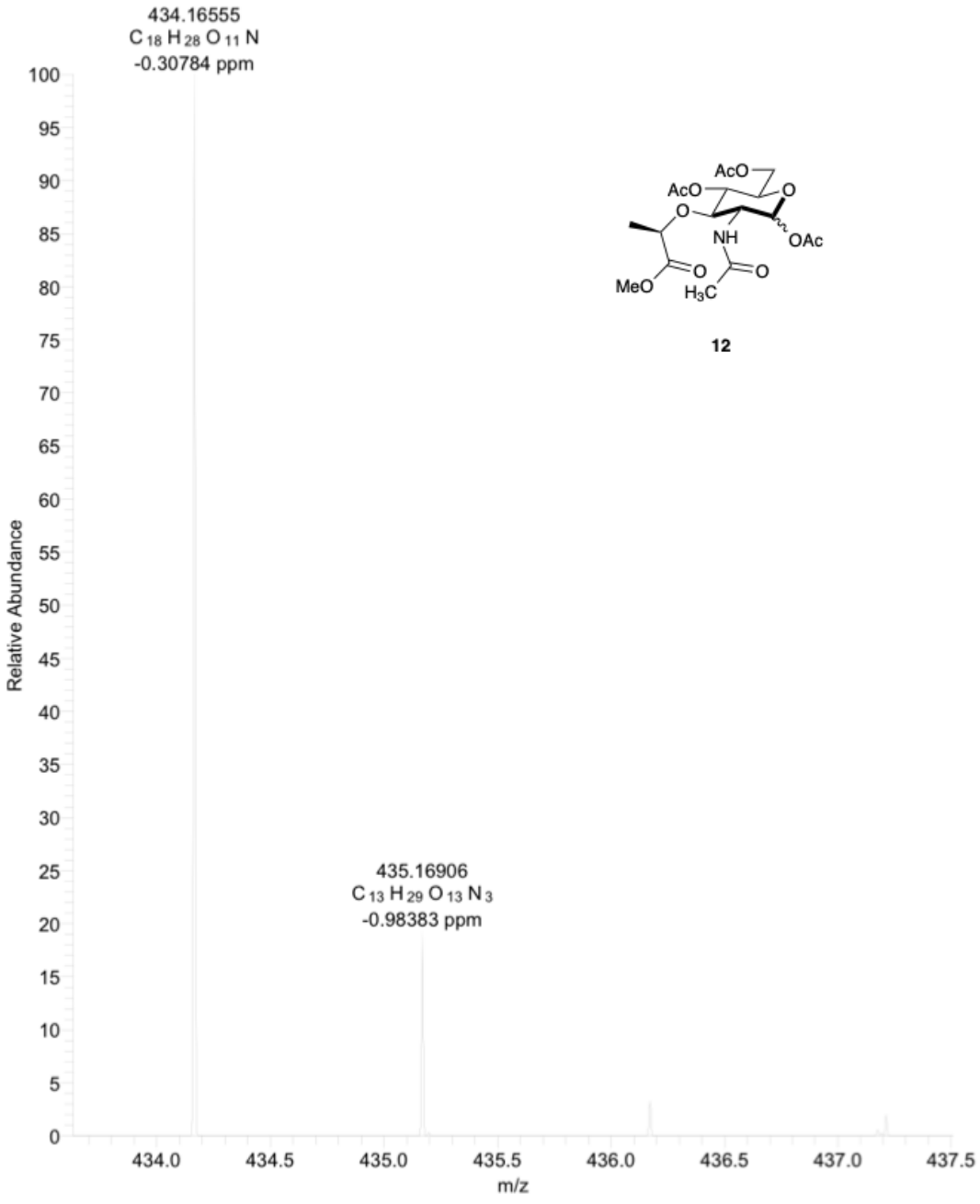



Grimes_Hyland_SNH-1-46 #1201 RT: 2.75 AV: 1 NL: 7.25E8
T: FTMS + p ESI Full ms [250.0000-3000.0000]
349.13477
C 12 H 21 O 8 N 4
-1.78844 ppm
Relative Abundance
m/z
350.13745
351.13870
HO
HO
O
O
NH
OH
MeO
O
O
N3

8


D:\data\2020\Grimes_Cintia_CCS-2alkme 02/21/20 14:46:12
Grimes_Cintia_CCS-2alkme #28-61 RT: 0.15-0.32 AV: 34 NL: 3.59E9
T: FTMS + p ESI Full ms [75.0000-1000.0000]
346.14939
C 15 H 24 O 8 N
-0.73045 ppm
347.15251
C 22 H 21 O 3 N
2.64551 ppm
Relative Abundance
m/z
HO
HO
O
O
NH
OH
O
O
MeO

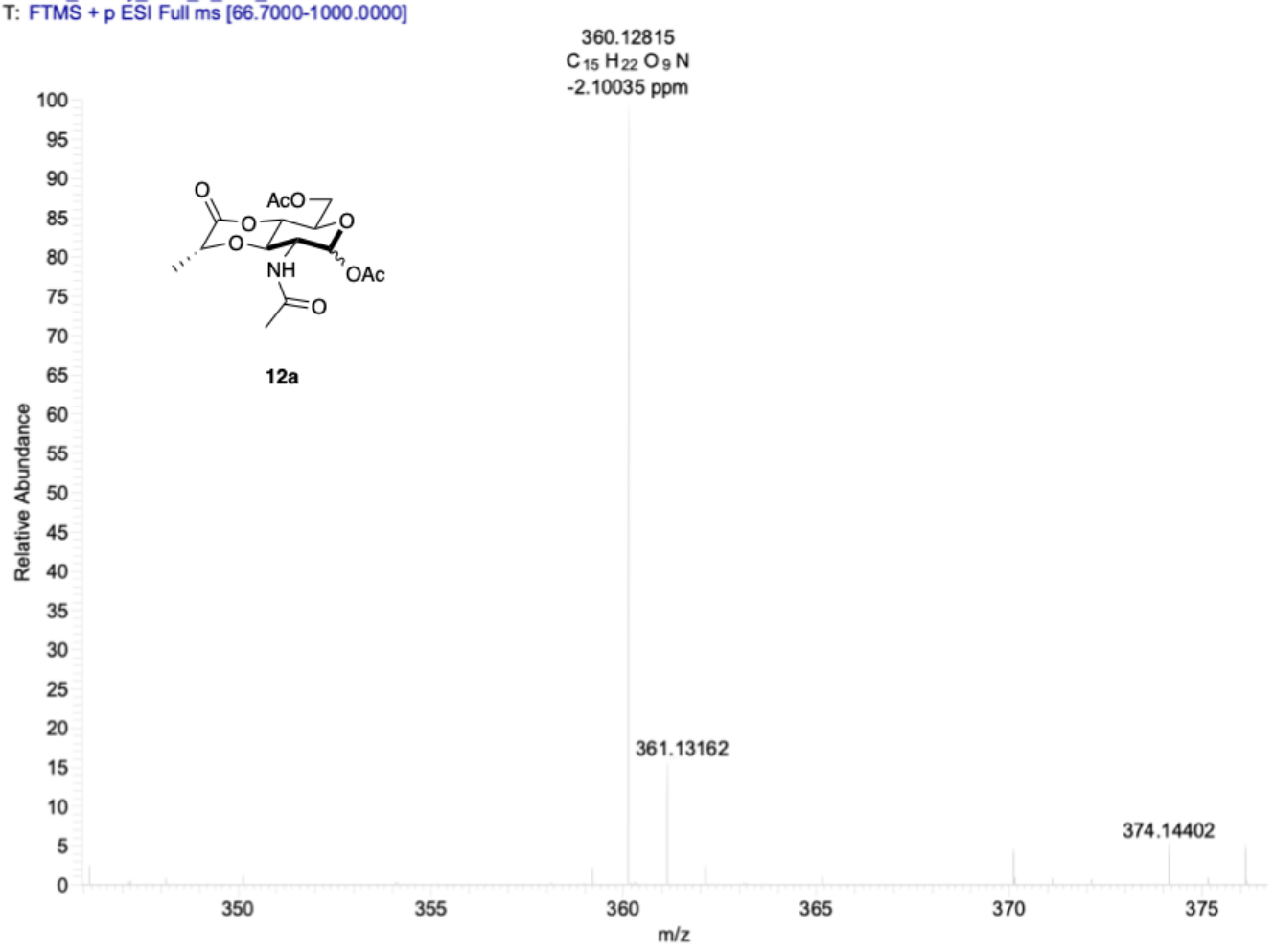
9

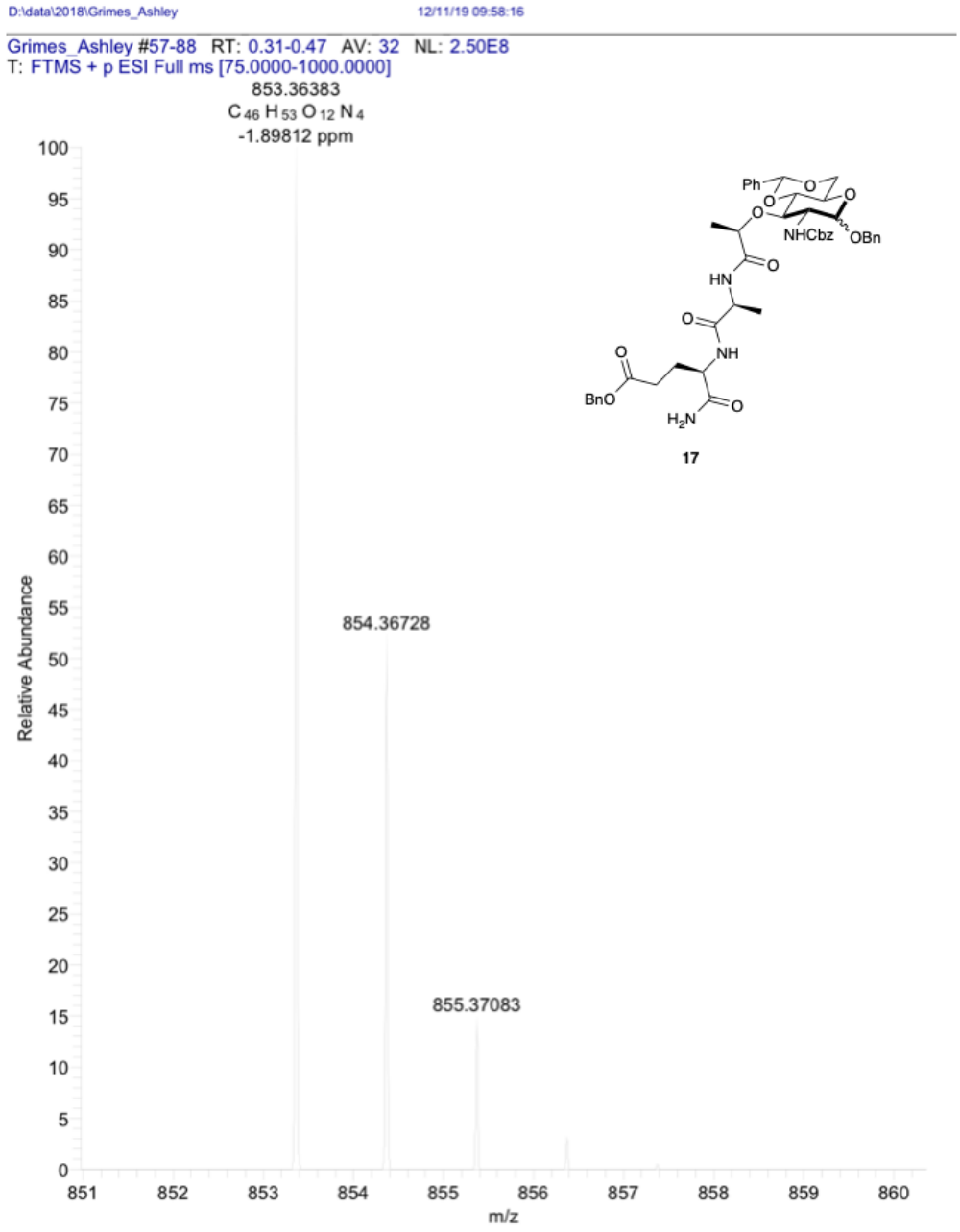

434.16555
C 18 H 28 O 11 N
-0.30784 ppm
435.16906
C 13 H 29 O 13 N 3
-0.98383 ppm
Relative Abundance
m/z
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
434.0
434.5
435.0
435.5
436.0
436.5
437.0
437.5
AcO
AcO
O
O
NH
OAc
MeO
O
O
H3C

12

Grimes_Ashley_ARB_iii_135_190617144439 06/17/19 14:44:39

Grimes_Ashley_ARB_iii_135_190617144439 #43-66 RT: 0.24-0.36 AV: 24 NL: 3.68E7
T: FTMS + p ESI Full ms [66.7000-1000.0000]




D:\data\2018\Grimes_Ashley
12/11/19 09:58:16
Grimes_Ashley #57-88 RT: 0.31-0.47 AV: 32 NL: 2.50E8
T: FTMS + p ESI Full ms [75.0000-1000.0000]
853.36383
C46 H53 O12 N4
-1.89812 ppm
854.36728
855.37083
Relative Abundance
m/z
Ph
O
NHCbz
OBn
HN
NH
BnO
H2N
17

IX. References

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