Supporting Information

Investigation on the substrate specificity and *N*-substitution tolerance of PseF in catalytic transformation of pseudaminic acids to CMP-Pse derivatives

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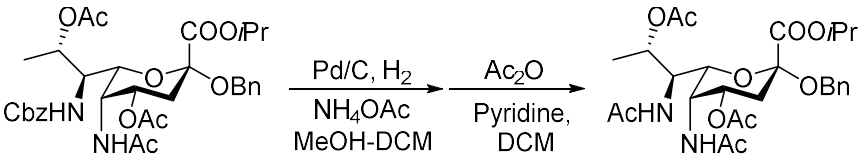
General Information

Commercially available reagents were used without further purification, unless otherwise stated. Anhydrous solvents were prepared from AR grade solvents following the standard methods (DCM and MeCN). Analytical thin layer chromatography (TLC) was performed on silica gel 60-F254 pre-coated on glass plates (E. Merck), with detection by fluorescence and/or or by staining with Hanessian's stain [5% (*w*/*v*) ammonium molybdate, 1% (*w*/*v*) cerium(II) sulfate and 10% (*v*/*v*) sulfuric acid in water] or sulfuric acid stain [10% (*v*/*v*) sulfuric acid in ethanol]. Normal phase column chromatography was performed on silica gel (230-400 mesh, Davisil).

^{1}H and ^{13}C NMR spectra were recorded on Advance DRX Bruker 400 and 500 MHz spectrometers at 25 °C. ^{31}P NMR spectra were recorded on Advance DRX Bruker 500 MHz spectrometer at 25 °C. Two-dimensional NMR spectra were recorded on Advance DRX Bruker 500 MHz spectrometers at 25 °C. Chemical shifts for ^{1}H NMR are reported in parts per million (δ) relative to the respective residual peaks ($CDCl_3$: δ 7.26 in ^{1}H and 77.16 in ^{13}C NMR; D_2O: δ 4.79 in ^{1}H NMR). The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet. Coupling constants (*J*) are reported in Hertz (Hz). High-resolution mass spectrometry was performed on a Waters Micromass Q-Tof Premier Mass Spectrometer.

Synthesis of pseudaminic acid analogues

Isopropyl (2*R*,4*S*,5*S*,6*S*)-5-acetamido-6-((1*S*,2*S*)-1-acetamido-2-acetoxypropyl)-4-acetoxy-2-(benzyloxy)tetrahydro-2*H*-pyran-2-carboxylate (S1):



13 **S1**

The *N*5-acetyl Pse compound **13** (20 mg, 0.030 mmol, 1.0 equiv), Pd/C (10% Pd on activated carbon, 6 mg) and NH_4OAc (4.8 mg, 0.060 mmol, 2.0 equiv) were dissolved in the

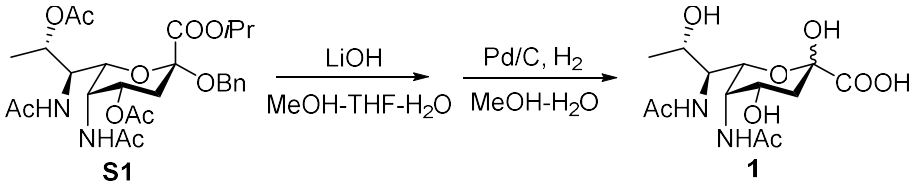
mixture of MeOH (1 mL) and DCM (1 mL). This mixture was stirred under 1 atm H_2 atmosphere for 1 h, then filtered through celite to remove the related Pd/C. The filtrate was concentrated under vacuum, and the residue was dissolved in DCM (3 mL). Then Ac_2O (0.5mL, excess amount) and pyridine (1 mL) were added. The mixture was stirred at r.t. overnight, and then concentrated under vacuum. The residue was dissolved in EtOAc (30 mL), and the solution was washed with 1 M aq. HCl, sat. aq. $NaHCO_3$ and brine sequentially. The organic phase was dried over anhydrous sodium sulphate, and the solvent was removed under vacuum. The residue was purified by silica gel column chromatography using DCM : MeOH = 15 : 1 as eluent. The product **S1** was collected as colourless solid (15 mg, 88%), which showed the same ^1H and ^{13}C spectra as our former report.[1]

^1H NMR (400 MHz, $CDCl_3$) δ = 7.35 (m, 5H, ArH), 6.12 (d, *J* = 9.4 Hz, 1H, NH), 5.99 (d, *J* = 10.6 Hz, 1H, NH), 5.32 (dd, *J* = 6.7, 2.8 Hz, 1H, H-8), 5.09 (p, *J* = 6.3 Hz, 1H, $(CH_3)_2CH$), 4.84 (d, *J* = 10.9 Hz, 1H, $PhCH_2$), 4.79 (dd, *J* = 8.6, 4.6 Hz, 1H, H-4), 4.54 (ddd, *J* = 14.3, 8.5, 3.3 Hz, 2H, $PhCH_2$), 4.40 (d, *J* = 11.0 Hz, 1H, H-6)), 4.09 (dd, *J* = 10.0, 1.9 Hz, 1H, H-6), 2.61 – 2.42 (m, 1H, H-3e), 2.04 (s, 3H, CH_3CO), 2.02 (s, 6H, 2*CH_3CO), 1.93 (s, 3H, CH_3CO), 1.88 (t, *J* = 13.0 Hz, 1H, H-3a), 1.37 (d, *J* = 6.6 Hz, 3H, H-9), 1.29 (m, 6H, $(CH_3)_2CH$).

^{13}C NMR (126 MHz, $CDCl_3$): δ = 171.6, 170.7, 170.7, 170.4, 167.5, 136.8, 128.6, 128.4, 128.3, 99.5, 72.6, 70.6, 70.2, 67.8, 67.1, 50.8, 45.6, 33.4, 23.4, 23.3, 21.9, 21.9, 21.4, 21.1, 14.3.

ESI^+ HRMS (m/z): calcd for $C_{27}H_{38}N_2O_{10}Na^+$ $(M + Na)^+$: 573.2419, found: 573.2395.

(4*S*,5*S*,6*S*)-5-acetamido-6-((1*S*,2*S*)-1-acetamido-2-hydroxypropyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid (1):



$LiOH-H_2O$ (12.6 mg, 0.30 mmol, 10 equiv) was dissolved in H_2O (0.3 mL), and this solution was added to the solution of S1 (17 mg, 0.030 mmol, 1.0 equiv) in MeOH (1.2 mL) and THF (0.3 mL). The mixture was stirred at r.t. for 48 h. When finished, the reaction was neutralized by DOWEX 50 H^+ resin. After filtration, the solvent was removed under vacuum,

and the residue was used in the next step without purification.

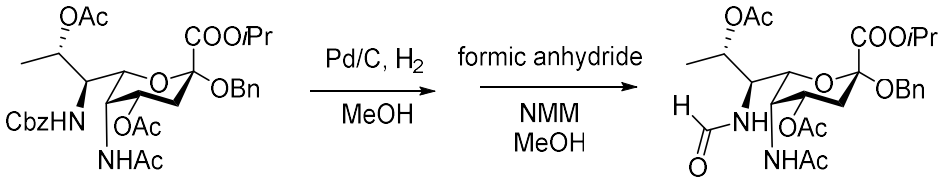
Pd/C (10% Pd on activated carbon, 10 mg) was added to the above residue, followed by MeOH (2 mL) and H_2O (0.8 mL). The mixture was stirred under 1 atm H_2 atmosphere for 4 h. After filtration, the solvent was removed under vacuum, and the residue was further purified by BioGel P2 column using water as eluent. The product **1** was obtained after lyophilization as white solid (6.7 mg, 79%), which showed the same ^{1}H and ^{13}C NMR spectra as our former report.[1]

^{1}H NMR (400 MHz, D_2O) δ = 8.57 (d, *J* = 9.9 Hz, 1H, NH), 4.22 (br, 1H), 4.17 – 4.05 (m, 3H, H-5; H-7; H-8), 3.99 (dd, *J* = 10.3, 1.8 Hz, 1H, H-6), 1.98 (s, 3H, C*H*$_3$CO), 1.95 (s, 3H, C*H*$_3$CO), 1.88 (d, *J* = 6.4 Hz, 1H, H-3e), 1.75 (t, *J* = 12.7 Hz, 1H, H-3a), 1.07 (d, *J* = 6.3 Hz, 3H, H-9).

^{13}C NMR (101 MHz, D_2O): δ = 176.4, 174.6, 173.7, 96.4, 69.9, 66.7, 65.1, 52.9, 48.8, 34.8, 22.0, 21.8, 15.2.

ESI$^+$ HRMS (m/z): calcd for $C_{13}H_{23}N_2O_8^+$ $(M + H)^+$: 335.1449, found: 335.1453.

Isopropyl (2R,4S,5S,6S)-5-acetamido-4-acetoxy-6-((1S,2S)-2-acetoxy-1-formamidopropyl)-2-(benzyloxy)tetrahydro-2*H*-pyran-2-carboxylate (16):



13 16

Intermediate **13** (32 mg, 0.050 mmol, 1.0 equiv) and Pd/C (10% Pd on activated carbon, 6 mg) were mixed in MeOH (2 mL) and DCM (2 mL). The mixture was stirred under 1 atm H_2 atmosphere for 1 h, then was filtered through celite to remove Pd/C. The filtrate was cooled to -40 °C, followed by the addition of NMM (0.4 mL, excess amount) and freshly prepared formic anhydride (~2.0 mmol, excess amount) solution in ether. After being stirred at -20 °C overnight, the mixture was dissolved in EtOAc (50 mL), and the solution was washed with 1 M aq. HCl, sat. aq. $NaHCO_3$ and brine sequentially. The organic phase was dried over anhydrous sodium sulphate, and the solvent was removed under vacuum. The residue was purified by silica gel column chromatography using DCM : MeOH = 40 : 1 to 10 : 1 as eluent. The desired product

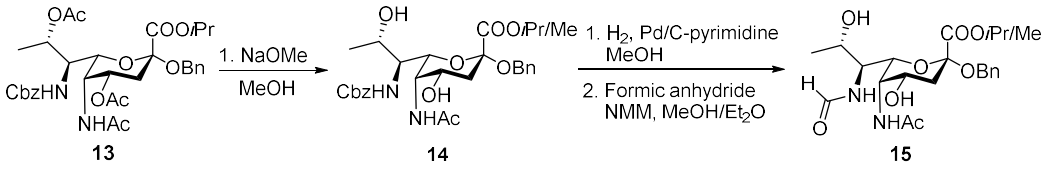
16 was collected as colourless solid (6.7 mg, 25%).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 8.02 (d, *J* = 1.7 Hz, 1H), 7.39 – 7.27 (m, 5H), 7.17 – 7.09 (m, 1H), 6.15 (d, *J* = 9.4 Hz, 1H), 5.44 (dd, *J* = 6.7, 2.9 Hz, 1H), 5.09 (p, *J* = 6.3 Hz, 1H), 4.97 (dt, *J* = 13.2, 4.4 Hz, 1H), 4.89 (d, *J* = 11.2 Hz, 1H), 4.67 (td, *J* = 10.6, 2.9 Hz, 1H), 4.57 – 4.50 (m, 1H), 4.44 (d, *J* = 11.1 Hz, 1H), 4.17 (dd, *J* = 10.5, 2.0 Hz, 1H), 2.53 (dd, *J* = 13.1, 4.8 Hz, 1H), 2.01 (s, 3H), 1.99 (s, 3H), 1.97 (s, 3H), 1.87 (t, *J* = 13.2 Hz, 1H), 1.38 (d, *J* = 6.5 Hz, 3H), 1.26 (m, 6H).

^{13}C NMR (126 MHz, $CDCl_3$) δ = 172.0, 170.8, 170.7, 167.0, 162.0, 136.9, 128.5, 128.3, 128.0, 99.0, 77.2, 71.8, 70.1, 69.4, 67.9, 66.7, 48.6, 45.3, 33.3, 29.7, 23.3, 21.7, 21.7, 21.3, 21.0, 13.4.

ESI$^+$ HRMS (m/z): calcd for $C_{26}H_{36}N_2NaO_{10}^+$ $(M + Na)^+$: 559.2262, found: 559.2275.

(4*S*,5*S*,6*S*)-5-acetamido-6-((1*S*,2*S*)-1-formamido-2-hydroxypropyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid (15):



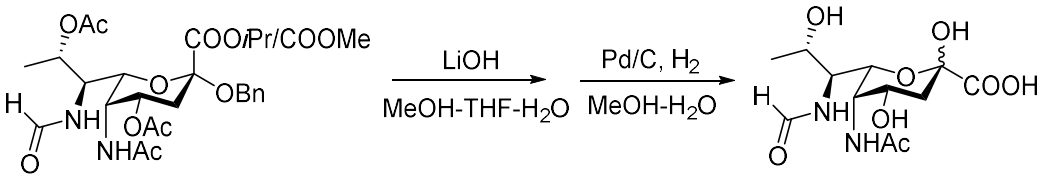
13 **14** **15**

Intermediate **13** (32 mg, 0.050 mmol, 1.0 equiv) was dissolved in MeOH (32 mL), followed by addition of 30% (*w*/*w*) NaOMe (29 mg, 0.16 mmol, 6.4 equiv) to form the 5 mM final concentration of NaOMe. The mixture was stirred at r.t. for 56 h until full conversion, then the reaction was neutralized by DOWEX 50 H$^+$ resin. After filtration, the solvent was evaporated under vacuum, and the residue containing **14** was used in the next step without purification. The exchange of isopropyl ester to methyl ester was observed in variable extent (isopropyl ester : methyl ester ~ 5 : 1 – 10 : 1). Since both types of esters will be hydrolyzed in the final saponification step, separation was not attempted.

Pd/C (10% Pd on activated carbon, 6 mg) that pre-poisoned with pyrimidine (6 μL) was added to the above residue, followed by the addition of MeOH (10 mL). The mixture was stirred under 1 atm H_2 atmosphere for 8 h, and the progress was monitored by UPLC-MS. When finished, the mixture was filtered through celite to remove the catalyst. The filtrate was cooled to -20 °C, followed by the addition of NMM (0.2 mL, excess amount) and freshly prepared

formic anhydride (~0.5 mmol, excess amount) solution in ether. After being stirred at -20 ºC overnight, the solvent was removed under vacuum. The residue was purified by silica gel column chromatography using DCM : MeOH = 30 : 1 to 10 : 1 as eluent. The product **15** was collected as white foam (11.3 mg, 50%). The variable methyl ester/isopropyl ester ratio of product **15** led to complicated NMR spectra. The mixture was directly subjected to the next saponification step without separation and characterization.

(4*S*,5*S*,6*S*)-5-acetamido-6-((1*S*,2*S*)-1-formamido-2-hydroxypropyl)-2,4-dihydroxytetrahydro-2*H*-pyran-2-carboxylic acid (2):



15 **2**

LiOH-H_2O (6.7 mg, 0.16 mmol, 10 equiv) was dissolved in H_2O (0.3 mL), and the solution was added to the mixture of **15** (9 mg, 0.016 mmol, 1.0 equiv), MeOH (1.2 mL) and THF (0.3 mL). The mixture was stirred at r.t. for 48 h. When finished, the reaction was neutralized by DOWEX 50 H^+ resin. After filtration, the solvent was removed under vacuum, and the residue was used in the next step without purification.

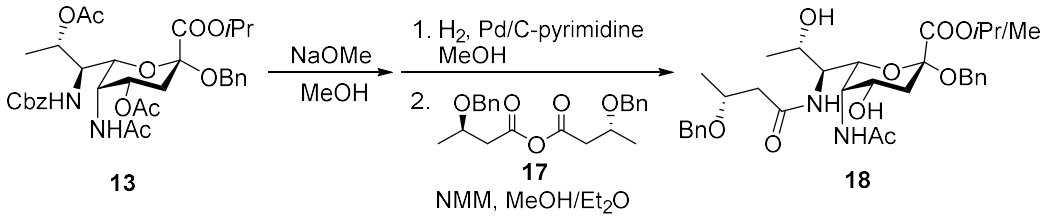
Pd/C (10% Pd on activated carbon, 6 mg) was added to the above residue, followed by MeOH (2 mL) and H_2O (0.8 mL). Mixture was stirred under 1 atm H_2 atmosphere for 4 h. After filtration, the solvent was removed under vacuum, and the residue was further purified by BioGel P2 column using water as eluent. The product **2** was obtained after lyophilization as white solid (3.8 mg, 79%).

^{1}H NMR (400 MHz, D_2O) δ = 7.97 (s, 1H), 4.22 – 4.16 (m, 2H), 4.15 – 4.09 (m, 1H), 4.07 – 3.99 (m, 2H), 3.51 (ddt, *J* = 6.3, 4.0, 2.1 Hz, 1H), 1.90 (s, 3H), 1.70 (dd, *J* = 13.3, 12.2 Hz, 1H), 1.51 – 1.44 (m, 1H), 1.01 (d, *J* = 6.6 Hz, 3H).

^{13}C NMR (101 MHz, D_2O) δ = 174.9, 173.0, 164.1, 95.4, 70.0, 66.3, 64.7, 61.5, 51.4, 48.6, 34.2, 27.8, 22.0, 15.2.

ESI$^-$ HRMS (m/z): calcd for $C_{12}H_{19}N_2O_8^-$ (M - H)$^-$: 319.1147, found: 319.1045.

Isopropyl (2*R*,4*S*,5*S*,6*S*)-5-acetamido-2-(benzyloxy)-6-((1*S*,2*S*)-1-((*R*)-3-(benzyloxy)butanamido)-2-hydroxypropyl)-4-hydroxytetrahydro-2*H*-pyran-2-carboxylate (18):



Intermediate **13** (32 mg, 0.050 mmol, 1.0 equiv) was dissolved in MeOH (32 mL), followed by addition of 30% (*w*/*w*) NaOMe (29 mg, 0.16 mmol, 6.4 equiv) to form the 5 mM final concentration of NaOMe. The mixture was stirred at r.t. for 56 h, then was neutralized by DOWEX 50 H^+ resin. After filtration, the solvent was removed under vacuum, and the residue containing **14** was used in the next step without purification. The same as the synthesis of **2**, the methyl ester/isopropyl ester exchange (isopropyl ester : methyl ester ~ 5 : 1 – 10 : 1) was observed, and the inseparable mixture was directly used in the following transformations.

The pre-poisoned Pd/C catalyst was prepared as follows. The commercial Pd/C (10% Pd on activated carbon, 200 mg) was added to round bottom flask under argon, followed by anhydrous MeOH (10 mL). To this mixture, pyrimidine (200 μL) was added. The mixture was stirred at room temperature under argon for 4 h. The solvent was removed via filtration, and the collected poisoned Pd/C was thoroughly washed with anhydrous MeOH and dried under vacuum overnight.

Pd/C (10% Pd on activated carbon, 6 mg) that pre-poisoned with pyrimidine (6 μL) was added to the above residue followed by addition of MeOH (10 mL). The mixture was stirred under 1 atm H_2 atmosphere for 8 h, and the progress was monitored by UPLC-MS. When finished, the mixture was filtered through celite to remove the catalyst. The filtrate was cooled to -20 °C, followed by the addition of NMM (0.2 mL, excess amount) and freshly prepared anhydride **17** (~0.5 mmol, excess amount) solution in ether. After being stirred at -20 °C overnight, the solvent was removed under vacuum. The residue was purified by silica gel column chromatography using DCM : MeOH = 30 : 1 to 10 : 1 as eluent. The product was **18**

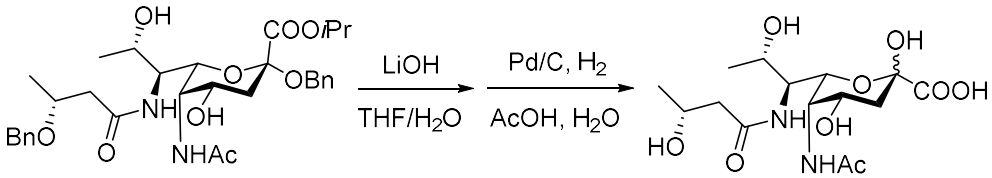
collected as white foam (17.4 mg, 58%). The isopropyl ester product was isolated here for characterization.

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.44 – 7.17 (m, 10H), 6.75 (d, *J* = 9.9 Hz, 1H), 6.16 (d, *J* = 8.4 Hz, 1H), 5.06 (p, *J* = 6.3 Hz, 1H), 4.74 (d, *J* = 11.2 Hz, 1H), 4.62 (d, *J* = 11.1 Hz, 1H), 4.40 (dd, *J* = 15.7, 11.1 Hz, 2H), 4.17 (dd, *J* = 9.6, 3.9 Hz, 2H), 4.00 (td, *J* = 8.3, 4.5 Hz, 2H), 3.83 (d, *J* = 9.7 Hz, 1H), 3.65 (dd, *J* = 12.4, 4.0 Hz, 1H), 3.08 (q, *J* = 7.3 Hz, 1H), 2.57 (dd, *J* = 13.5, 4.8 Hz, 1H), 2.41 (dd, *J* = 17.3, 6.1 Hz, 2H), 2.02 (s, 3H), 1.74 (t, *J* = 12.8 Hz, 1H), 1.37 (d, *J* = 7.3 Hz, 1H), 1.31 (d, *J* = 6.3 Hz, 3H), 1.26 (d, *J* = 4.8 Hz, 9H).

^{13}C NMR (126 MHz, $CDCl_3$) δ = 173.6, 171.5, 168.1, 138.1, 136.7, 128.5, 128.5, 128.1, 127.9, 127.9, 99.9, 73.4, 72.6, 70.7, 70.7, 70.0, 67.9, 66.8, 53.5, 53.4, 49.6, 43.9, 35.8, 29.7, 23.3, 21.8, 21.7, 19.5, 19.5.

ESI$^+$ HRMS (m/z): calcd for $C_{32}H_{44}N_2NaO_9^+$ $(M + Na)^+$: 623.2939, found: 623.2954.

(4*S*,5*S*,6*S*)-5-acetamido-2,4-dihydroxy-6-((1*S*,2*S*)-2-hydroxy-1-((*R*)-3 hydroxybutanamido)propyl) tetrahydro-2*H*-pyran-2-carboxylic acid (3):



18 **3**

LiOH-H_2O (9.2 mg, 0.22 mmol, 20 equiv) was dissolved in H_2O (0.2 mL) and added to the solution of intermediate **18** (6.8 mg, 0.011 mmol, 1.0 equiv) in MeOH (0.8 mL) and THF (0.2 mL). The mixture was stirred at r.t. for 70 h. When finished, the reaction was neutralized by DOWEX 50 H^+ resin. After filtration, the solvent was removed under vacuum, and the residue was used in the next step without purification.

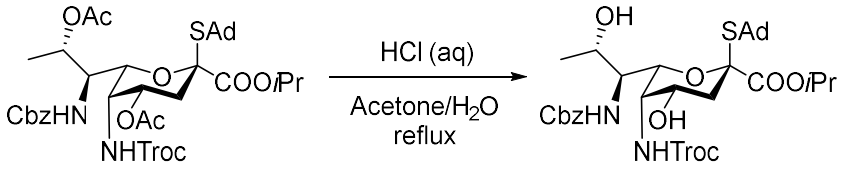
Pd/C (10% Pd on activated carbon, 6 mg) was added to the above residue, followed by MeOH (1.5 mL) and H_2O (1.5 mL). The mixture was stirred under 1 atm H_2 atmosphere for 4 h. After filtration, the solvent was removed under vacuum, and the residue was further purified by BioGel P2 column using water as eluent. The product **3** was obtained after lyophilization as white solid (3.4 mg, 82%).

^{1}H NMR (500 MHz, D_2O) δ = 4.25 – 4.11 (m, 5H), 4.03 (dd, *J* = 10.5, 1.8 Hz, 1H), 2.41 – 2.36 (m, 2H), 2.01 (s, 3H), 1.95 – 1.90 (m, 1H), 1.78 (dd, *J* = 13.3, 12.3 Hz, 1H), 1.24 (d, *J* = 6.3 Hz, 3H), 1.11 (d, *J* = 6.5 Hz, 3H).

^{13}C NMR (125 MHz, D_2O) δ = 176.4, 174.6, 173.7, 96.5, 70.0, 67.0, 65.3, 65.0, 53.0, 48.9, 45.1, 34.8, 22.1, 21.9, 15.4.

ESI$^-$ HRMS (m/z): calcd for $C_{15}H_{25}N_2O_9^-$ (M - H)$^-$: 377.1565, found: 377.1433.

Isopropyl (2*R*,4*S*,5*S*,6*S*)-2-(((3*R*)-adamantan-1-yl)thio)-6-((1*S*,2*S*)-1-(((benzyloxy)carbonyl)amino)-2-hydroxypropyl)-4-hydroxy-5-(((2,2,2-trichloroethoxy)carbonyl)amino)tetrahydro-2*H*-pyran-2-carboxylate (19):



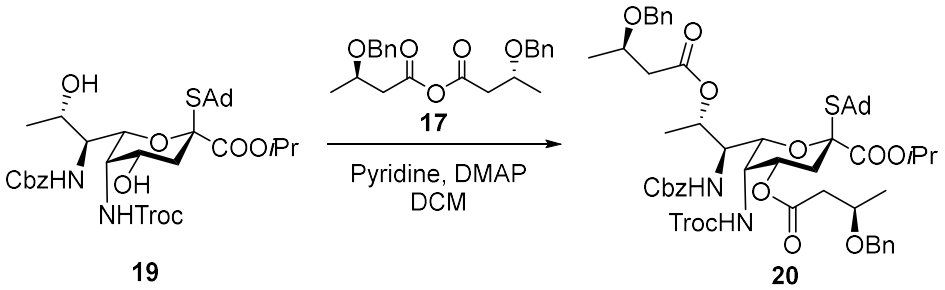
11 **19**

To a stirred solution of intermediate **11** [2](42 mg, 0.050 mmol, 1.0 equiv) in acetone (3 mL), HCl solution (prepared from 37% HCl 0.3 mL and H_2O 1.1 mL) was added. The mixture was refluxed for 1 h, then more H_2O (1.0 mL) was added. The mixture was further refluxed for 24 h. Acetone was removed under vacuum, then the water phase was extracted with EtOAc (2 × 50 mL). The organic phase was washed with sat. aq. $NaHCO_3$ and brine and dried over anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was purified by silica gel flash chromatography using *n*-hexane : EtOAc = 2 : 1 as eluent. Product **19** was collected as colorless solid (31 mg, 82%).

^{1}H NMR (400 MHz, CD_3CN) δ = 7.46 – 7.28 (m, 5H), 6.19 (d, *J* = 10.1 Hz, 1H), 5.62 (d, *J* = 9.6 Hz, 1H), 5.13 – 5.07 (m, 1H), 5.06 (s, 2H), 4.79 – 4.71 (m, 2H), 4.54 (dd, *J* = 9.5, 1.9 Hz, 1H), 4.15 (d, *J* = 10.1 Hz, 2H), 4.04 (s, 1H), 3.93 (s, 1H), 3.72 – 3.62 (m, 1H), 3.19 (d, *J* = 5.1 Hz, 1H), 2.61 (s, 1H), 2.11 (dd, *J* = 14.0, 4.6 Hz, 1H), 2.02 – 1.94 (m, 9H), 1.84 (dd, *J* = 14.1, 12.1 Hz, 1H), 1.70 (m, 6H), 1.33 (dd, *J* = 6.3, 1.9 Hz, 6H), 1.10 (d, *J* = 6.2 Hz, 3H).

^{13}C NMR (126 MHz, CD_3CN) δ = 170.5, 157.1, 156.5, 138.3, 129.4, 128.8, 128.7, 87.5, 75.3, 74.2, 70.9, 69.3, 67.0, 65.7, 57.2, 55.2, 52.4, 50.8, 44.2, 38.3, 36.7, 30.8, 29.7, 22.0, 21.8, 19.7.

Isopropyl (2*R*,4*S*,5*S*,6*S*)-2-(((3*R*)-adamantan-1-yl)thio)-4-(((*R*)-3-(benzyloxy)butanoyl)oxy)-6-((5*S*,6*S*,10*R*)-6,10-dimethyl-3,8-dioxo-1,12-diphenyl-2,7,11-trioxa-4-azadodecan-5-yl)-5-(((2,2,2-trichloroethoxy)carbonyl)amino)tetrahydro-2*H*-pyran-2-carboxylate:



To a stirred solution of (*R*)-3-(benzyloxy)butanoic acid (0.30 g, 1.5 mmol, 2.0 equiv) in anhydrous DCM (4 mL), DCC (150 mg, 3.0 mmol, 1.0equiv) was added at 0 °C. The mixture was stirred at 0 °C for 3 h. The solvent was removed under vacuum, and the residue was redissolved in anhydrous ether followed by filtration to remove DCU. The solvent was removed under vacuum, and the crude anhydride intermediate **17** was used in the next step without purification.

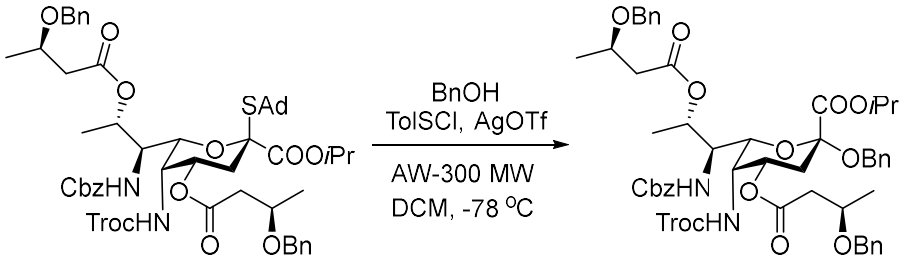
Intermediate **19** (75.2 mg, 0.10 mmol, 1.0 equiv) was dissolved in anhydrous DCM (5 mL), followed by the addition of the freshly prepared anhydride **17** (0.75 mmol, 7.5 equiv), anhydrous pyridine (1 mL) and DMAP (4 mg, 0.030 mmol, 0.3 equiv). The mixture was stirred at r.t. overnight. The mixture was diluted with EtOAc. The organic phase was washed with 1 M aq. HCl, sat. aq. $NaHCO_3$ and brine. The organic phase was dried over anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was purified by silica gel flash chromatography using *n*-hexane : EtOAc = 4 : 1 as eluent. Product **20** was collected as colorless syrup (78 mg, 72%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.52 – 7.08 (m, 15H), 5.53 (d, *J* = 9.9 Hz, 1H), 5.25 (dt, *J* = 12.6, 4.7 Hz, 2H), 5.16 – 5.04 (m, 2H), 4.99 (d, *J* = 12.2 Hz, 1H), 4.90 (dd, *J* = 11.4, 7.2 Hz, 2H), 4.67 – 4.40 (m, 6H), 4.37 – 4.24 (m, 2H), 4.01 (ddd, *J* = 11.1, 6.5, 4.0 Hz, 2H), 2.65 – 2.27 (m, 5H), 2.07 – 1.88 (m, 10H), 1.66 (s, 6H), 1.35 – 1.22 (m, 15H).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.6, 169.1, 155.7, 154.6, 138.6, 138.6, 136.3, 128.5, 128.4,

128.4, 128.1, 128.1, 127.68, 127.65, 127.5, 95.7, 86.4, 77.3, 74.6, 72.1, 72.0, 71.0, 70.9, 70.2, 70.2, 70.1, 67.8, 67.1, 53.7, 50.6, 48.6, 43.8, 41.9, 36.0, 29.9, 29.7, 21.8, 21.7, 19.9, 19.8, 15.2.

Isopropyl (2*R*,4*S*,5*S*,6*S*)-2-(benzyloxy)-4-(((*R*)-3-(benzyloxy)butanoyl)oxy)-6-((5*S*,6*S*,10*R*)-6,10-dimethyl-3,8-dioxo-1,12-diphenyl-2,7,11-trioxa-4-azadodecan-5-yl)-5-(((2,2,2-trichloroethoxy)carbonyl)amino)tetrahydro-2*H*-pyran-2-carboxylate (21):



20 **21**

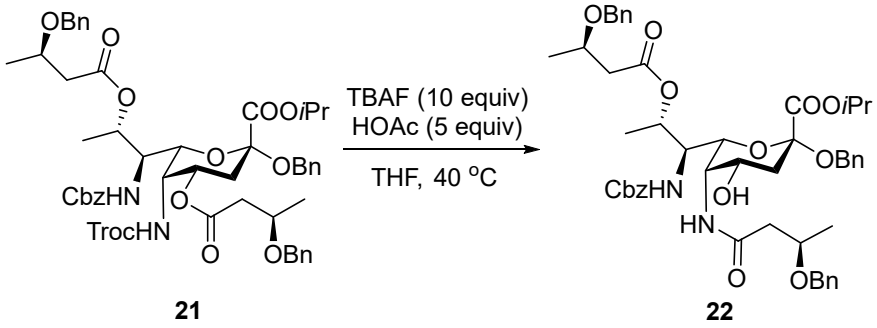
To a flame-dried Schlenk tube, the flame-dried AW-300 molecular sieves (200 mg) were added under argon, followed by BnOH (10.4 μL, 0.10 mmol, 2.0 equiv), Pse donor **20** (58 mg, 0.050 mmol, 1.0 equiv), and anhydrous DCM (2.0 mL, freshly prepared). The mixture was stirred at r.t. for 1 h, then was cooled to −78 °C. AgOTf (38.5 mg, 0.15 mmol, 3.0 equiv) was added under argon, followed by *p*-toluenesulfenyl chloride (22 μL, 0.15 mmol, 3.0 equiv) in dropwise manner. The mixture was stirred at −78 °C for 4 h and quenched by addition of Et_3N. After filtration and aqueous workup, the product was purified by silica gel column chromatography using *n*-hexane : EtOAc = from 6 : 1 to 3 : 1 *v*/*v* as eluent. Only the β anomer product **21** was collected as colorless foam (44.7 mg, 82%).

^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.40 – 7.15 (m, 20H), 5.40 (dt, *J* = 9.8, 8.2 Hz, 2H), 5.08 (dd, *J* = 12.1, 5.7 Hz, 2H), 4.89 – 4.77 (m, 3H), 4.60 – 4.36 (m, 6H), 4.33 – 4.20 (m, 2H), 4.06 – 3.88 (m, 3H), 2.65 – 2.50 (m, 3H), 2.40 (td, *J* = 15.4, 5.3 Hz, 2H), 1.92 (t, *J* = 13.2 Hz, 1H), 1.38 (d, *J* = 6.6 Hz, 3H), 1.31 (d, *J* = 6.3 Hz, 3H), 1.27 – 1.21 (m, 9H).

^{13}C NMR (126 MHz, $CDCl_3$) δ = 170.8, 170.7, 167.4, 155.8, 154.8, 138.7, 136.7, 136.5, 128.6, 128.5, 128.4, 128.2, 128.1, 128.1, 127.7, 127.6, 127.5, 99.4, 95.7, 77.3, 74.6, 72.8, 72.1, 71.9, 71.0, 70.9, 70.6, 69.8, 68.0, 67.1, 67.0, 52.8, 48.0, 42.0, 41.9, 33.3, 29.7, 21.8, 21.7, 19.8, 13.9.

Isopropyl (2*R*,4*S*,5*S*,6*R*)-2-(benzyloxy)-5-((*R*)-3-(benzyloxy)butanamido)-6-((5*S*,6*S*,10*R*)-

6,10-dimethyl-3,8-dioxo-1,12-diphenyl-2,7,11-trioxa-4-azadodecan-5-yl)-4-hydroxytetrahydro-2*H*-pyran-2-carboxylate (22):



21 **22**

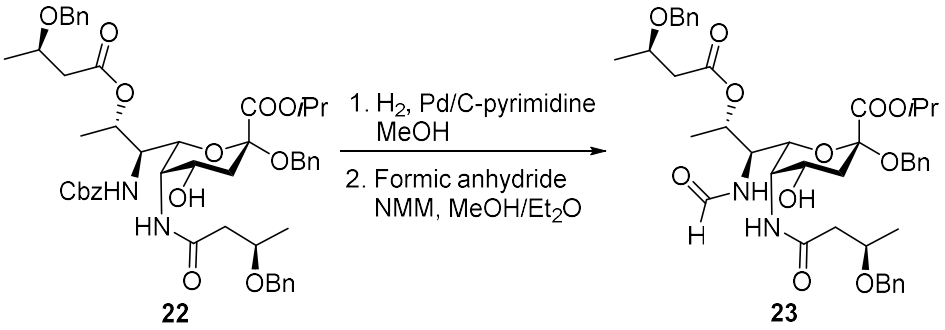
In a 1.5 mL Eppendorf tube, TBAF (1.0 M solution in THF, 0.5 mL, 0.50 mmol, 10.0 equiv) and HOAc (0.015 mL, 0.25 mmol, 5.0 equiv) were added. This mixture was then added to the solution of **21** (52 mg, 0.050 mmol, 1.0 equiv) in THF (1.0 mL). The final concentration of the TBAF was controlled at 0.33 M. After being heated at 40 °C for 48 h, the mixture was diluted with EtOAC (30 mL). This solution was washed with sat. aq. $NaHCO_3$ and H_2O and dried over anhydrous sodium sulfate. The solvent was removed under vacuum, and the residue was purified by silica gel column chromatography using *n*-hexane : EtOAc = 1 : 2 *v*/*v* as eluent. The product **22** was collected as colorless syrup (32 mg, 74%), together with recovered starting material **21** (3 mg, 6%).

^{1}H NMR (500 MHz, CD_3CN) δ = 7.37 – 7.28 (m, 21H), 6.75 (d, *J* = 9.4 Hz, 1H), 5.76 (d, *J* = 10.3 Hz, 1H), 5.41 (qd, *J* = 6.7, 3.2 Hz, 1H), 5.07 (dd, *J* = 12.4, 5.9 Hz, 2H), 4.96 (d, *J* = 12.6 Hz, 1H), 4.78 (d, *J* = 11.3 Hz, 2H), 4.52 – 4.40 (m, 7H), 4.29 (td, *J* = 10.4, 3.0 Hz, 2H), 4.00 – 3.71 (m, 6H), 2.49 – 2.23 (m, 6H), 1.71 (t, *J* = 12.9 Hz, 1H), 1.38 (d, *J* = 6.5 Hz, 1H), 1.32 (d, *J* = 6.6 Hz, 3H), 1.29 – 1.26 (m, 6H), 1.19 (dd, *J* = 11.2, 6.2 Hz, 6H).

^{13}C NMR (126 MHz, CD_3CN) δ = 173.7, 171.4, 168.8, 157.1, 140.2, 140.1, 138.5, 138.4, 129.4, 129.3, 129.1, 129.1, 129.0, 128.7, 128.7, 128.6, 128.5, 128.3, 128.2, 118.3, 100.5, 73.6, 73.2, 73.0, 71.2, 71.1, 71.0, 70.6, 67.7, 67.0, 67.0, 53.7, 49.2, 44.2, 42.9, 36.8, 21.98, 21.95, 20.5, 20.1, 14.0.

Isopropyl (2*R*,4*S*,5*S*,6*S*)-2-(benzyloxy)-5-((*R*)-3-(benzyloxy)butanamido)-6-((1*S*,2*S*)-2-(((*R*)-3-(benzyloxy)butanoyl)oxy)-1-formamidopropyl)-4-hydroxytetrahydro-2*H*-pyran-

2-carboxylate (23):

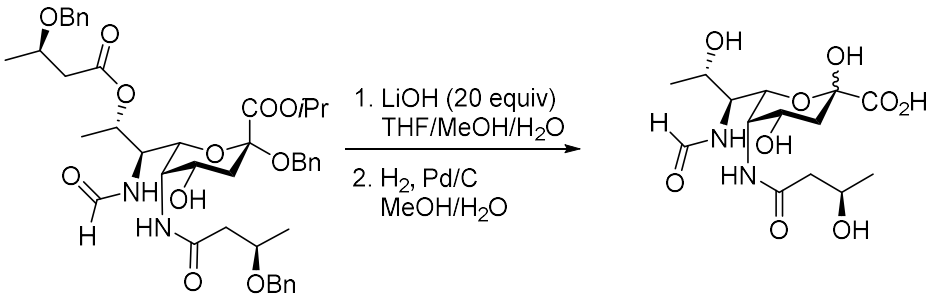


Pd/C (10% Pd on activated carbon, 6 mg) that pre-poisoned with pyrimidine (6 μL) was added to the intermediate **22** (20 mg, 0.023 mmol, 1.0 equiv) followed by MeOH (5 mL). The mixture was stirred under 1 atm H_2 atmosphere for 6 h, and the progress was monitored by UPLC-MS. When finished, the mixture was filtered through celite to remove the catalyst. The filtrate was cooled to -20 ºC, then NMM (0.2 mL, excess amount) and freshly prepared formic anhydride (~0.5 mmol, excess amount) solution in ether were added. After being stirred at -20 ºC overnight, the solvent was removed under vacuum. The residue was purified by silica gel column chromatography using DCM : MeOH = 30 : 1 to 10 : 1 as eluent. The product **23** was collected as white foam (4.2 mg, 24%).

^{1}H NMR (500 MHz, CD_3CN) δ = 8.04 (s, 1H), 7.39 – 7.25 (m, 15H), 6.75 (dd, *J* = 9.6, 5.5 Hz, 1H), 6.59 (d, *J* = 10.4 Hz, 1H), 5.41 (ddd, *J* = 10.9, 6.7, 2.9 Hz, 1H), 5.11 (p, *J* = 6.3 Hz, 1H), 4.80 (dd, *J* = 11.3, 7.2 Hz, 1H), 4.69 – 4.41 (m, 7H), 4.33 (dt, *J* = 9.5, 2.9 Hz, 1H), 4.06 – 3.98 (m, 1H), 3.95 (dddd, *J* = 13.2, 9.2, 5.0, 2.3 Hz, 2H), 3.85 – 3.70 (m, 2H), 2.58 – 2.35 (m, 5H), 2.25 – 2.20 (m, 1H), 1.97 (p, *J* = 2.5 Hz, 1H), 1.78 – 1.66 (m, 2H), 1.36 – 1.29 (m, 9H), 1.25 – 1.20 (m, 6H).

^{13}C NMR (126 MHz, CD_3CN) δ = 173.7, 171.4, 168.7, 161.7, 129.2, 129.2, 129.1, 129.1, 129.1, 129.0, 128.9, 128.6, 128.54, 128.53, 128.48, 128.42, 128.2, 128.1, 100.4, 73.5, 73.1, 72.9, 71.2, 71.1, 70.9, 70.9, 70.4, 67.7, 66.9, 49.9, 49.0, 44.2, 42.9, 36.8, 21.9, 21.9, 21.8, 21.8, 20.3, 20.0, 14.0.

(4*S*,5*S*,6*S*)-6-((1*S*,2*S*)-1-formamido-2-hydroxypropyl)-2,4-dihydroxy-5-((*R*)-3-hydroxybutanamido)tetrahydro-2*H*-pyran-2-carboxylic acid (4):



23 **4**

LiOH-H_2O (6.6 mg, 0.15 mmol, 20 equiv) was dissolved in H_2O (0.2 mL) and added to the solution of **23** (6.0 mg, 0.008 mmol, 1.0 equiv) in MeOH (0.8 mL) and THF (0.2 mL). The mixture was stirred at r.t. for 70 h. When finished, the reaction was neutralized by DOWEX 50 H^+ resin. After filtration, the solvent was removed under vacuum, and the residue was used in the next step without purification.

Pd/C (10% Pd on activated carbon, 3 mg) was added to the above residue, followed by MeOH (1.0 mL) and H_2O (1.0 mL). The mixture was stirred under 1 atm H_2 atmosphere for 4 h. After filtration, the solvent was removed under vacuum, and the residue was further purified by BioGel P2 column using water as eluent. The product **4** was obtained after lyophilization as white solid (2.2 mg, 76%).

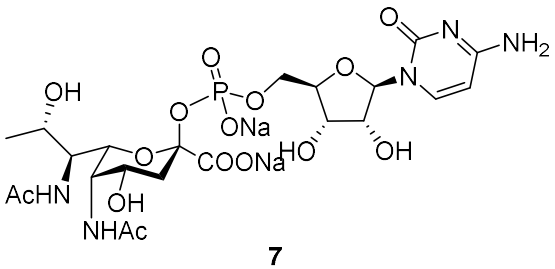
^{1}H NMR (500 MHz, D_2O) δ = 8.09 (s, 1H), 4.37 – 4.13 (m, 5H), 4.09 – 3.98 (m, 1H), 2.55 – 2.40 (m, 2H), 1.98 – 1.91 (m, 1H), 1.80 (t, *J* = 12.8 Hz, 1H), 1.25 (d, *J* = 6.3 Hz, 3H), 1.10 (dd, *J* = 10.9, 6.5 Hz, 3H).

^{13}C NMR (126 MHz, D_2O) δ = 176.3, 174.7, 164.0, 96.5, 69.9, 66.45, 65.41, 65.0, 51.5, 48.8, 44.8, 34.7, 22.0, 15.1.

ESI$^-$ HRMS (m/z): calcd for $C_{14}H_{23}N_2O_9^-$ (M - H)$^-$: 363.1409, found: 363.1269.

Enzymatic synthesis of CMP-Pse analogues catalyzed by PseF

CMP-Pse5Ac7Ac (7):



7

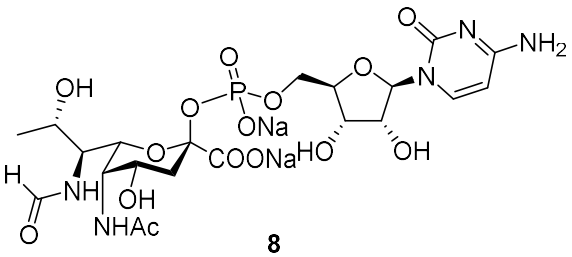
A reaction mixture containing 0.9 mM Pse5Ac7Ac **1** (0.88 mg, 2.6 μmol), 0.1 mg mL^{-1} PseF, 6 mM CTP and 20 mM $MgCl_2$ in 30 mM sodium phosphate pH 7.4 were incubated at 37 °C for 24 h. Equal volume EtOH was added, and the mixture was stored at -20 °C for 2 h to precipitate enzymes, followed by centrifugation (8000×rpm, 1 h, 4 °C). The mixture was lyophilized, followed by resuspending in water for manual Bio-Gel P-2 resin column. 2 mL fractions were collected for 8 h and checked via negative ESI-MS before lyophilization to collect CMP-Pse5Ac7Ac **7** as a white foam (1.2 mg, 72%). The characterization data of the synthetic product were the same as the reported ones.[4]

^{1}H NMR (500 MHz, D_2O) δ = 8.02 (d, *J* = 7.6 Hz, 1H), 6.14 (d, *J* = 7.6 Hz, 1H), 5.99 (d, *J* = 4.1 Hz, 1H), 4.36 – 4.32 (m, 2H), 4.31 – 4.27 (m, 2H), 4.24 (tt, *J* = 7.5, 3.1 Hz, 4H), 4.11 (dd, *J* = 6.6, 5.1 Hz, 1H), 4.02 (dd, *J* = 10.1, 4.9 Hz, 1H), 2.27 – 2.16 (m, 1H), 1.99 (s, 3H), 1.96 (s, 3H), 1.61 (ddd, *J* = 13.5, 12.1, 5.3 Hz, 1H), 1.20 (d, *J* = 6.3 Hz, 3H).

^{13}C NMR (126 MHz, D_2O) δ = 174.6, 174.0, 173.3, 164.8, 157.8, 141.9, 100.1, 96.5, 89.2, 82.99, 82.92, 74.2, 72.7, 69.3, 68.7, 64.9, 53.8, 48.8, 36.0, 22.0, 21.9, 17.3.

ESI$^-$ HRMS (m/z): calcd for $C_{22}H_{33}N_5O_{15}P^-$ $(M - H)^-$: 638.1746, found: 638.1444.

CMP-Pse5Ac7Fo (8):



8

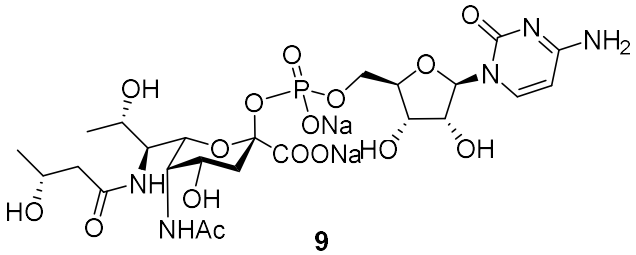
A reaction mixture containing 0.9 mM Pse5Ac7Fo **2** (0.85 mg, 2.7 μmol), 0.1 mg mL^{-1} PseF, 6 mM CTP and 20 mM $MgCl_2$ in 30 mM sodium phosphate pH 7.4 were incubated at 37 °C for 24 h. Equal volume EtOH was added, and the mixture was stored at -20 °C for 2 h to precipitate enzymes, followed by centrifugation (8000×rpm, 1 h, 4 °C). The mixture was lyophilized, followed by resuspending in water for manual Bio-Gel P-2 resin column. 2 mL fractions were collected for 8 h and checked via negative ESI-MS before lyophilization to collect CMP-Pse5Ac7Fo **8** as a white foam (1.2 mg, 72%).

^{1}H NMR (500 MHz, D_2O) δ = 8.05 (s, 1H), 8.02 (d, *J* = 7.5 Hz, 1H), 6.14 (d, *J* = 7.6 Hz, 1H), 6.00 (d, *J* = 4.3 Hz, 1H), 4.36 – 4.32 (m, 2H), 4.31 – 4.28 (m, 2H), 4.27 – 4.21 (m, 4H), 4.20 – 4.11 (m, 2H), 2.27 – 2.18 (m, 1H), 2.00 (s, 3H), 1.62 (td, *J* = 12.8, 5.3 Hz, 1H), 1.22 (d, *J* = 6.3 Hz, 3H).

^{31}P NMR (202 MHz, D_2O) δ = -5.14.

ESI$^-$ HRMS (m/z): calcd for $C_{21}H_{31}N_5O_{15}P^-$ $(M - H)^-$: 624.1560, found: 624.1274.

CMP- Pse5Ac7(3$_R$Hb) (9):



9

A reaction mixture containing 0.9 mM Pse5Ac7$_R$Hb **3** (0.87 mg, 2.3 μmol), 0.1 mg mL^{-1} PseF, 6 mM CTP and 20 mM $MgCl_2$ in 30 mM sodium phosphate pH 7.4 were incubated at 37 °C for 24 h. Equal volume EtOH was added, and the mixture was stored at -20 °C for 2 h to precipitate enzymes, followed by centrifugation (8000×rpm, 1 h, 4 °C). The mixture was lyophilized, followed by resuspending in water for manual Bio-Gel P-2 resin column. 2 mL fractions were collected for 8 h and checked via negative ESI-MS before lyophilization to collect CMP-Pse5Ac7(3$_R$Hb) **9** as a white foam (1.1 mg, 70%).

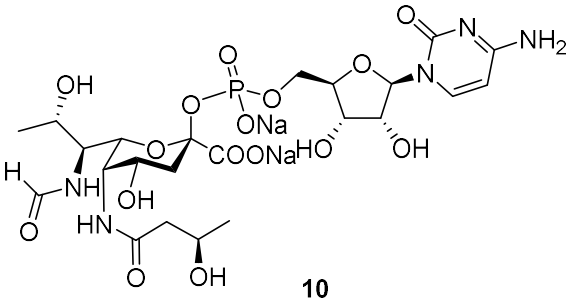
^{1}H NMR (500 MHz, D_2O) δ = 8.01 (d, *J* = 7.6 Hz, 1H), 6.13 (d, *J* = 7.5 Hz, 1H), 6.00 (d, *J* = 4.2 Hz, 1H), 4.36 – 4.31 (m, 2H), 4.30 – 4.20 (m, 5H), 4.20 – 4.16 (m, 1H), 4.12 (dd, *J* = 6.5, 5.1 Hz, 1H), 4.06 (dd, *J* = 10.0, 5.0 Hz, 1H), 2.36 (d, *J* = 2.9 Hz, 1H), 2.35 (s, 1H), 2.23 (d, *J* =

4.7 Hz, 1H), 1.99 (s, 3H), 1.60 (td, *J* = 12.8, 5.3 Hz, 1H), 1.24 (d, *J* = 6.3 Hz, 3H), 1.21 (d, *J* = 6.5 Hz, 3H).

^{31}P NMR (202 MHz, D_2O) δ = -5.06.

ESI$^-$ HRMS (m/z): calcd for $C_{24}H_{37}N_5O_{16}P^-$ (M - H)$^-$: 682.1978, found: 682.1647.

CMP-Pse5(3$_R$Hb)7Fo (10):



10

A reaction mixture containing 0.9 mM Pse5(3$_R$Hb)7Fo **4** (0.90 mg, 2.5 μmol), 0.1 mg mL^{-1} PseF, 6 mM CTP and 20 mM $MgCl_2$ in 30 mM sodium phosphate pH 7.4 were incubated at 37 °C for 24 h. Equal volume EtOH was added, and the mixture was stored at -20 °C for 2 h to precipitate enzymes, followed by centrifugation (8000×rpm, 1 h, 4 °C). The mixture was lyophilized, followed by resuspending in water for manual Bio-Gel P-2 resin column. 2 mL fractions were collected for 8 h and checked via negative ESI-MS before lyophilization to collect CMP-Pse5(3$_R$Hb)7Fo **10** as a white foam (0.40 mg, 24%).

^{1}H NMR (500 MHz, D_2O) δ = 8.05 (s, 1H), 8.01 (d, *J* = 7.5 Hz, 1H), 6.13 (d, *J* = 7.6 Hz, 1H), 5.99 (d, *J* = 4.0 Hz, 1H), 4.33 (dd, *J* = 6.2, 4.3 Hz, 3H), 4.25 (dd, *J* = 9.2, 4.3 Hz, 4H), 4.17 – 4.14 (m, 1H), 2.52 – 2.37 (m, 2H), 2.27 – 2.17 (m, 1H), 1.63 (td, *J* = 12.8, 5.2 Hz, 1H), 1.24 (d, *J* = 6.3 Hz, 3H), 1.22 (d, *J* = 6.3 Hz, 3H).

^{31}P NMR (202 MHz, D_2O) δ = -5.18.

ESI$^-$ HRMS (m/z): calcd for $C_{23}H_{35}N_5O_{16}P^-$ (M - H)$^-$: 668.1822 found: 668.1801.

Kinetics analysis of PseF catalyzed enzymatic reaction

UPLC analysis method

UPLC analysis were quantified with a Glycan BEH Amide 1.7 μm particle 2.1 × 100 mm column (Waters, ACQUITY Premier). Separation of CMP-Pse from CTP used the following method with acetonitrile as eluent A and 0.1 % (w/v) aq. ammonium acetate solution as eluent B. The column chromatography method was 10% to 40% B over 8 minutes, flow 0.4 mL/min. The injection volume was 1.0 μL each sample. Two repeats in parallel for each concentration and the absorbance average was used for the standard fitting curve.

Cause cytidine 5'-monophosphate (CMP) showed enormous UV absorption intensity than Pse itself, only the standard curve of CMP-Pse5Ac7Ac was assayed to represent all the CMP-Pse derivatives.

Table. S1 Calculation of varying concentration CMP-Pse5Ac7Ac for UV absorption

c (mg/mL)	***n*** (nmol)	absorption1	absorption2	absorption average
0.05	0.0785	640000	610000	625000
0.1	0.157	1150000	1100000	1125000
0.2	0.314	1890000	2380000	2135000
0.3	0.471	3380000	3540000	3460000
0.4	0.628	4640000	4670000	4655000
0.5	0.785	5550000	5620000	5585000
0.7	1.099	7660000	7650000	7655000
0.9	1.413	10000000	9960000	9980000

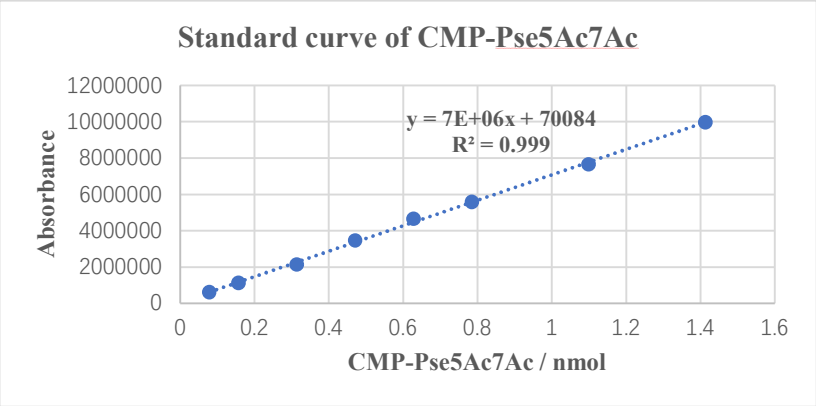


Fig. S1 Standard curve of CMP-Pse5Ac7Ac. Pure CMP-Pse5Ac7Ac was isolated from the previous enzymatic reaction.

As showed in Fig. S2, 15 samples were prepared with three repeats in parallel at 0, 0.25, 0.5, 1 and 5 mins. Once the reaction time was reached, the reaction mixture was quenched with 60 μL ACN immediately, followed by centrifuging (4000 rpm) at 4 °C for 10 mins to remove PseF. Please note that 0 min indicated MeCN was added before PseF. Then samples were ready for HILIC column chromatography via the above-mentioned UPLC analysis method.

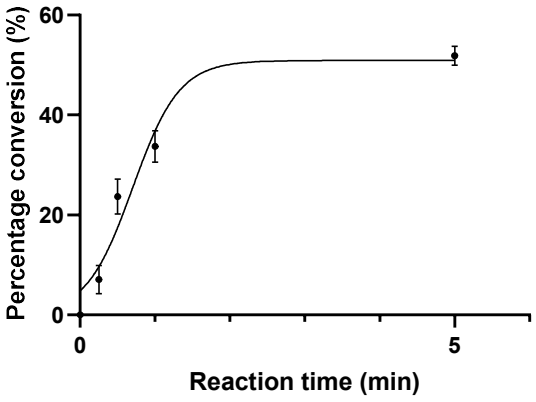


Fig. S2 CMP-Pse5Ac7Ac percentage conversion (%) at varying reaction time. To a final volume at 60 μL, PseF (3.8 μM) was assayed using Pse5Ac7Ac (0.9 mM, 18 μg), CTP (2 mM) as substrates in the presence of $MgCl_2$ (20 mM) and sodium phosphate (30 mM, pH 7.4) at 37 °C for 0, 0.25, 0.5, 1, 5 mins, respectively. The error bars were derived from three parallel replicates of each experiment.

To a final volume at 60 μL, PseF (2.27 μM) was assayed using Pse5Ac7Ac (0.9 mM, 18 μg), CTP (2 mM), $MgCl_2$ (20 mM) and sodium phosphate (30 mM, pH ranging from 4 to 11) at 37 °C for 5 minutes. Three repeats in parallel for each pH. Once the reaction time was reached, the reaction mixture was quenched with 60 μL ACN immediately, followed by centrifuging (4000 rpm) at 4 °C for 10 mins to remove PseF. Then samples were ready for HILIC column chromatography via the above-mentioned UPLC analysis method. As showed in Fig. S3, it indicated PseF activity was optimal at pH 9.0. A big loss of activity was observed when the pH was below 7.0. The activity was completely deactivated at pH 5.0 or lower. A decrease of activity was observed at basic conditions when pH was above 11.0. This pH tolerance characteristic should be noted for further PseF involved enzymatic reactions.

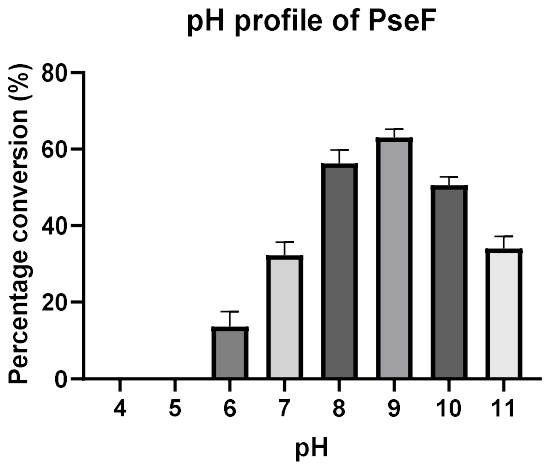


Fig. S3 pH profile of PseF. To a final volume at 60 μL, PseF (2.27 μM) was assayed using Pse5Ac7Ac (0.9 mM, 18 μg), CTP (2 mM) as substrates in the presence of $MgCl_2$ (20 mM) and sodium phosphate (30 mM, pH ranging from 4 to 11) at 37 °C for 5 minutes. The error bars were derived from three parallel replicates of each experiment.

To a final volume at 60 μL, PseF (2.27 μM) was assayed using Pse5Ac7Ac (0.9 mM, 18μg), CTP (2 mM), $MgCl_2$ (20 mM) and sodium phosphate (30 mM, pH 7.4) at 4, 25 and 37 °C. Three repeats in parallel for each temperature. Once the reaction time was reached, the reaction mixture was quenched with 60 μL ACN immediately, followed by centrifuging (4000 rpm) at

4 °C for 10 mins to remove PseF. Then samples were ready for HILIC column chromatography via the above-mentioned UPLC analysis method. As showed in Fig. S4, the result indicated that PseF activity was decreased as temperature decreased. 37 °C was the optimal condition here. But to our surprise, even half of the activity was observed at 4 °C, which indicated the extension of reaction time may achieve high conversion at 4 °C. This showed huge potential in recycling use of PseF at the immobilized phase. And that will be another story in the future.

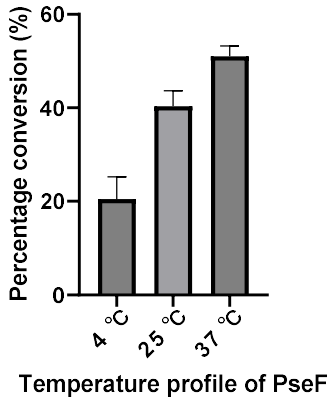


Fig. S4 Temperature profile of PseF. To a final volume at 60 μL, PseF (2.27 μM) was assayed using Pse5Ac7Ac (0.9 mM, 18 μg), CTP (2 mM) as substrates in the presence of $MgCl_2$ (20 mM) and sodium phosphate (30 mM, pH 7.4) at 4, 25 and 37 °C, respectively, for 5 minutes. The error bars were derived from three parallel replicates of each experiment.

To keep a suitable reaction rate, a fixed concentration of Pse5Ac7Ac (0.9 mM, a little bigger than K_m (Pse5Ac7Ac) in **Tab. 1** was chosen. To a final volume at 60 μL, PseF (2.27 μM) was assayed using a fixed concentration of Pse5Ac7Ac (0.9 mM, 18 μg), $MgCl_2$ (20 mM), sodium phosphate (30 mM, pH 7.4) and varying CTP concentrations (ranging from 0.4 to 6 mM) at 37 °C for 15 seconds. Once the reaction time was reached, the reaction mixture was quenched with 60 μL MeCN immediately, followed by centrifuging (4000 rpm) at 4 °C for 10 mins to remove PseF. Then samples were ready for HILIC column chromatography via the above-mentioned UPLC analysis method. The Michaelis-Menten best fit values showed that V_{max} = 9.836 ± 0.274 μM/s; K_m = 0.6295 ± 0.065 mM; k_{cat} = 4.333 ± 0.121 /s; k_{cat}/K_m = 6.883

/(s·mM). Fig. S5 showed that $c_{(CTP)}$ was a key factor that played an important role in the enzymatic reaction, further experiment will be better with $c_{(CTP)}$ higher than 0.6295 ± 0.065 mM.

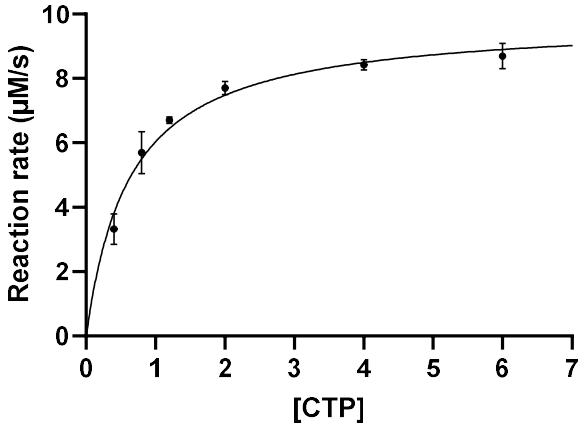


Fig. S5 PseF kinetic assay results at varying CTP concentrations. To a final volume of 60 μL, PseF (2.27 μM) was assayed using a fixed concentration of Pse5Ac7Ac (0.9 mM, 18 μg) and varying CTP concentrations (ranging from 0.4 to 6 mM) in the presence of $MgCl_2$ (20 mM) and sodium phosphate (30 mM, pH 7.4) at 37 °C for 15 seconds. The error bars were derived from three parallel replicates of each experiment. Michaelis-Menten best fit values: V_{max}= 9.836 ± 0.274 μM/s; K_m= 0.6295 ± 0.065 mM; k_{cat}= 4.333 ± 0.121 /s; k_{cat}/K_m= 6.883 /(s·mM).

Cloning, expression, and purification of PseF

The full-length *pseF* gene was amplified via PCR from *Acinetobacter baumannii* clinical isolate strain 00.191 (Ab2), referencing to our former publication (Andolina et al., *ACS Chem. Biol.* **2018**, *13*, 3030-3037).[3] The genome assembly is GCF_003072095.1. The PCR was conducted using forward primer (5'-CGGGATCCATGCAACTGGCGGTTATT-3') and reverse primer (5'- CCGCTCGAGTTATACATCTAATAGTAGTTGTGCC-3'). The PCR product was DNA gel electrophoresis confirmed and purified. The *pse*F gene fragment containing *Sac*I/*Bam*HI restriction sites was cloned into plasmid vector pET15b which contains the T7 promoter. The recombinant plasmid was transformed into *E. coli* DH5α and further transformed into *E. coli* BL21 for expression of the protein.

The overnight culture of BL21 cell was inoculated into 1 liter of Luria Broth (LB)

containing 100 μg/mL ampicillin and incubated at 37°C with shaking at 220 rpm until an optical density of 0.6 at 600 nm (OD_{600}) was reached. The expression of the protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM and incubated at 16°C for 16 hours. The cells were harvested by centrifugation at 12,000 x g for 10 minutes and resuspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 250 mM NaCl, 5 mM imidazole) and then broken by sonication. The soluble fraction was loaded onto Ni-nitrilotriacetic acid (NTA) column, rinsed with 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 30 mM imidazole, and finally eluted with 25 mM Tris-HCl, pH 8.0, 250 mM NaCl, and 250 mM imidazole. The eluted proteins were concentrated using the Amicon Ultra-15 (nominal molecular weight limit [NMWL] = 30 000) centrifugal filter device. Purification under optimized conditions afforded 25 mg L^{-1} expressed PseF protein. The purity of the protein was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Figure S6.

***pseF* gene sequence**

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ACATTAAAGACTTCTACGGTAAGCCCATGATTGCGTGGTCTATAGAAGCAGCACTG
AAAAGTGAATGTTTTGACGAGGTCTGGGTCTCGACTGATGATCAAGAAATTGCAG
ATATTGCAGTTAAGTTTGGAGCGAAAGTTCCATTTCTGCGACCAGCTCATTTGTCTG
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TTACAGGGACAATCGCCAGACTTTGTTTGTTGTTTATATGCCACTGCGCCGTTTGTA
CAGGTTGATGATCTGAAAACTGGGTTACAGCTATTACAATCTCATCCACTAGATTAT
GCTTTTAGTGCAACGACTTTTGCTTTCCCGATTCAAAGAGCTATTAAATTGAATGAG
GATGGTAATGTTGAAATGTTCCAGCCAGAAAACTTTAATAAGCGTTCTCAGGACTT
TGAAGAAGCATGGCACGATGCAGGGCAATTCTATTGGGGGACAGCAAAGGCATGG
CTAGAGAAATCTGTGATTTTTTCAAATAGATCACGGATTGTAGAGCTTCCTAGAAC
ACGGGTTCAGGATATTGATACTCCTGAAGATTGGCATCGTGCAGAGTTGATGGCAC
AACTACTATTAGATGTATAA

PseF protein sequence

MQLAVIPARGGSKRIPRKNIKDFYGKPMIAWSIEAALKSECFDEVWVSTDDQEIADIAV

KFGAKVPFLRPAHLSDDYATTADVMQHATQAFAQLQGQSPDFVCCLYATAPFVQVDD

LKTGLQLLQSHPLDYAFSATTFAFPIQRAIKLNEDGNVEMFQPENFNKRSQDFEEAWH

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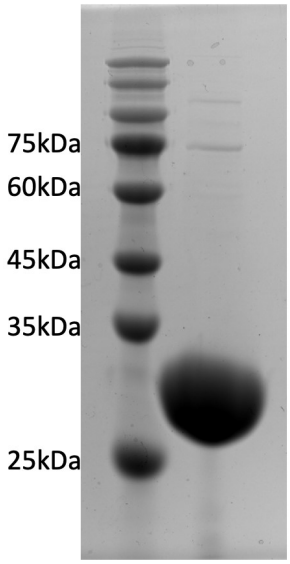

Fig. S6 SDS-PAGE analysis of the purified enzyme. Lane 1, protein marker; Lane 2, PseF protein (26.1 kDa).

References:

1. H. Liu, Y. Zhang, R. Wei, G. Andolina and X. Li, Total synthesis of Pseudomonas aeruginosa, 1244 pilin glycan via de novo synthesis of pseudaminic acid, *J. Am. Chem. Soc.*, 2017, **139**, 13420.
2. R. Wei, H. Liu, A. H. Tang, R. J. Payne and X. Li, A solution to chemical pseudaminylation via a bimodal glycosyl donor for highly stereocontrolled α- and β-glycosylation, *Org. Lett.*, 2019, **21**, 3584.
3. G. Andolina, R. Wei, H. Liu, Q. Zhang, X. Yang, H. Cao, S. Chen, A. Yan, X. D. Li and X. Li, Metabolic labeling of pseudaminic acid-containing glycans on bacterial surfaces. *ACS. Chem. Biol.*, 2018, **13**, 3030.

4. H. S. Chidwick, E. K. P. Flack, T. Keenan, J. Walton, G. H. Thomas and M. A. Fascione, *Sci. Rep.*, 2021, **11**, 4756.

Copies of NMR Spectra

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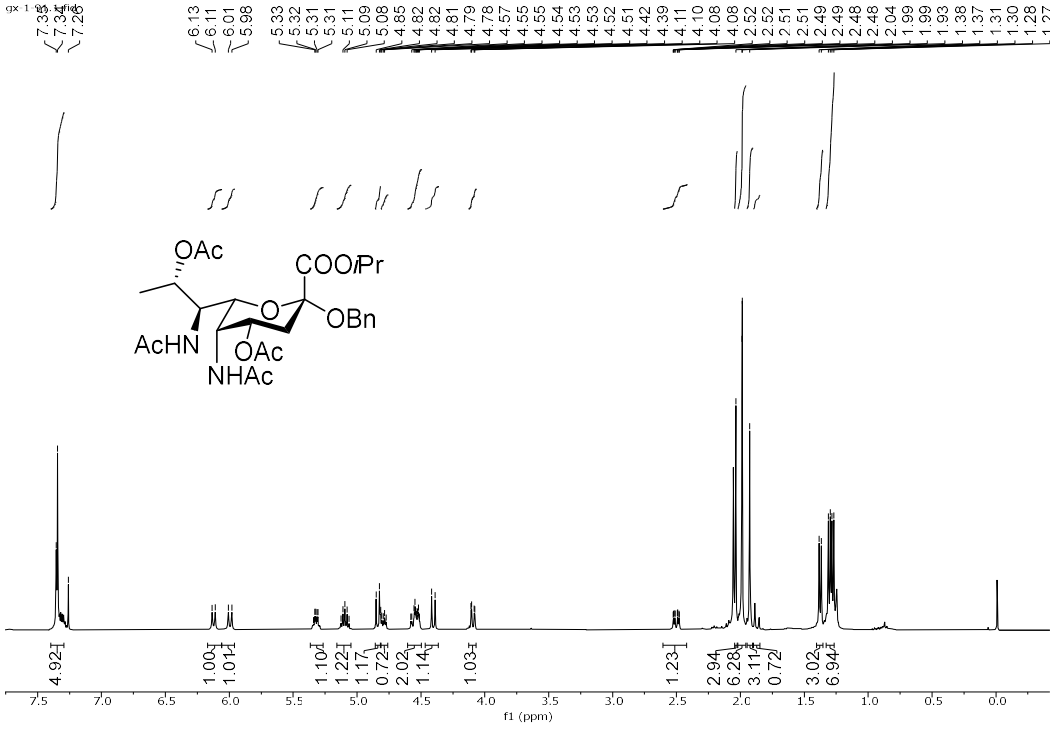

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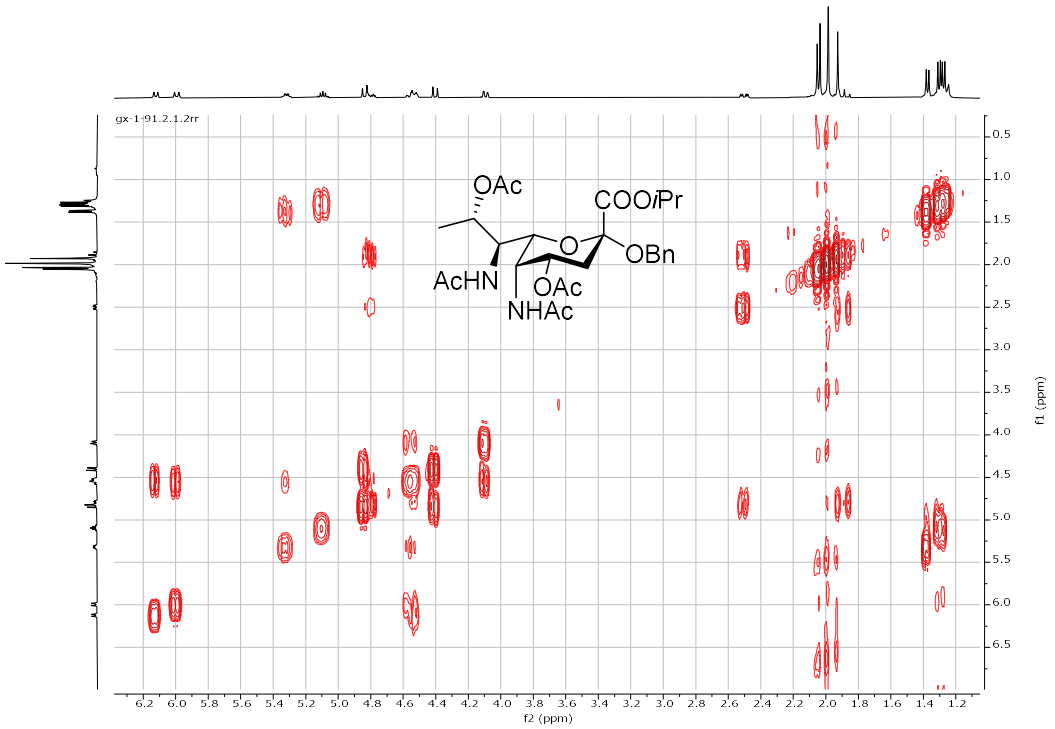

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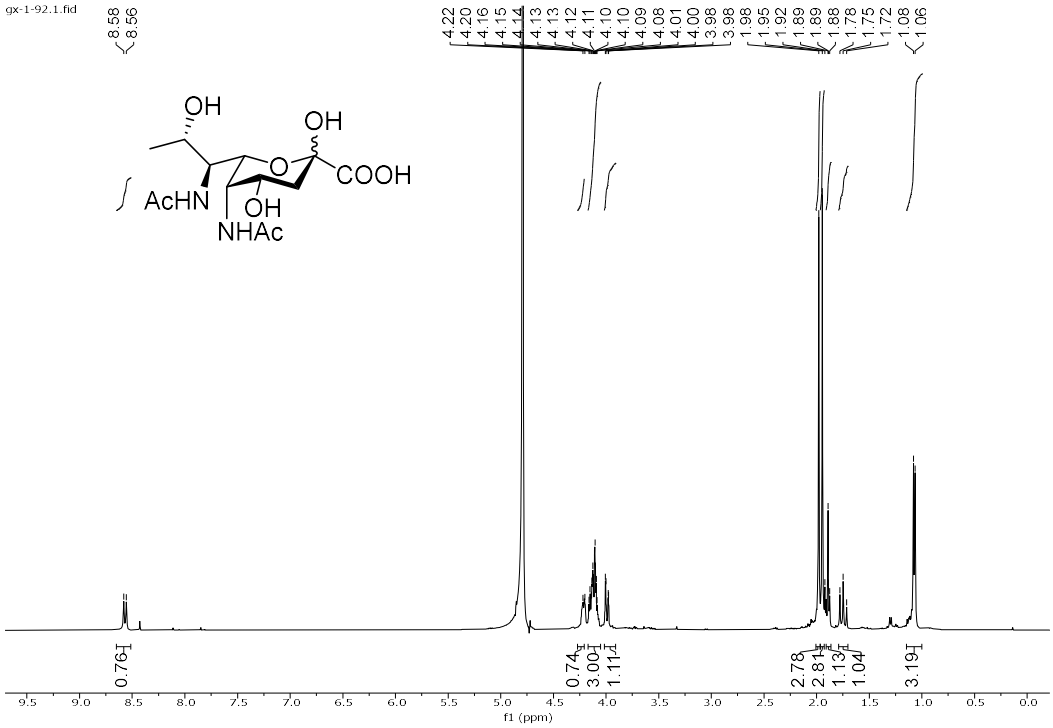

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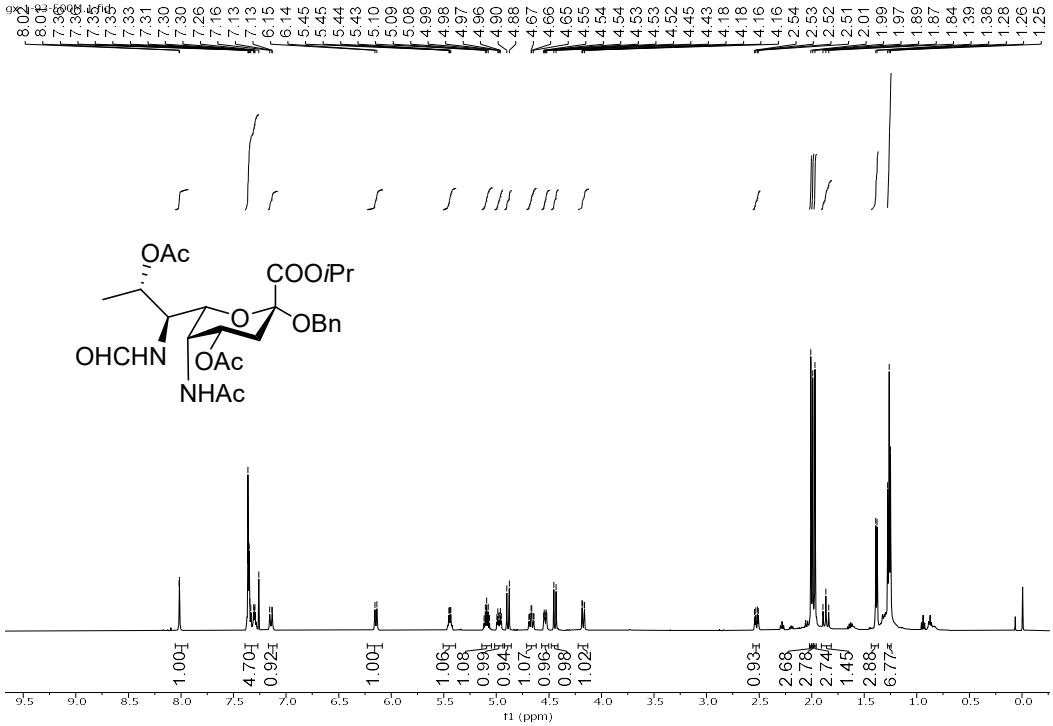

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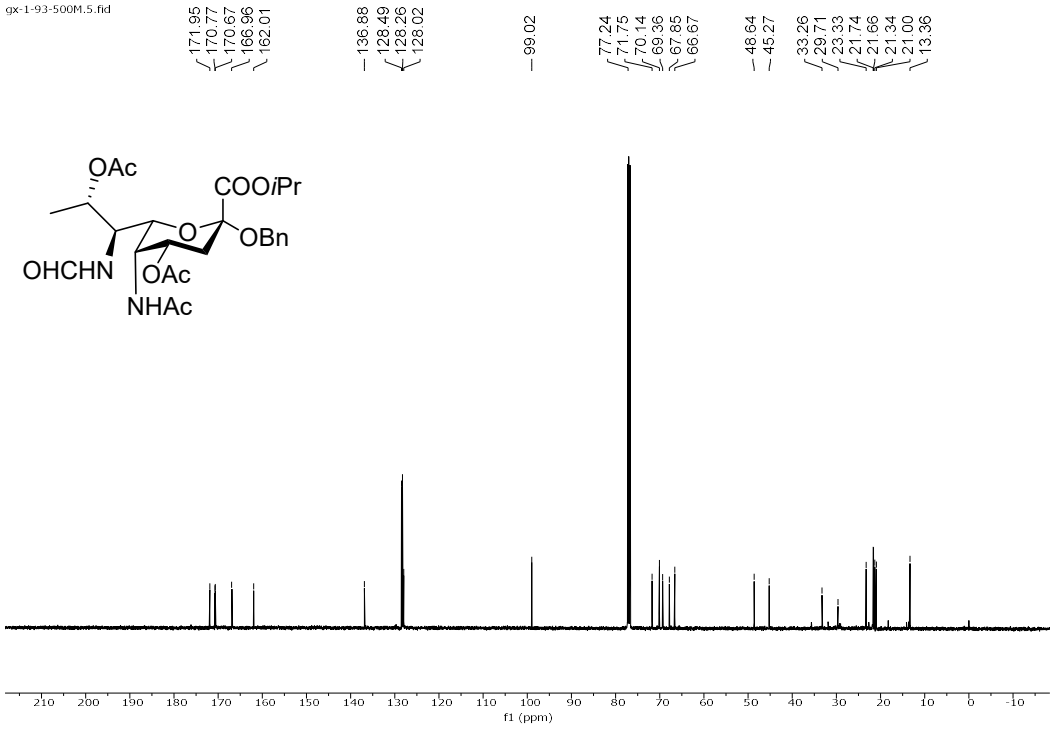

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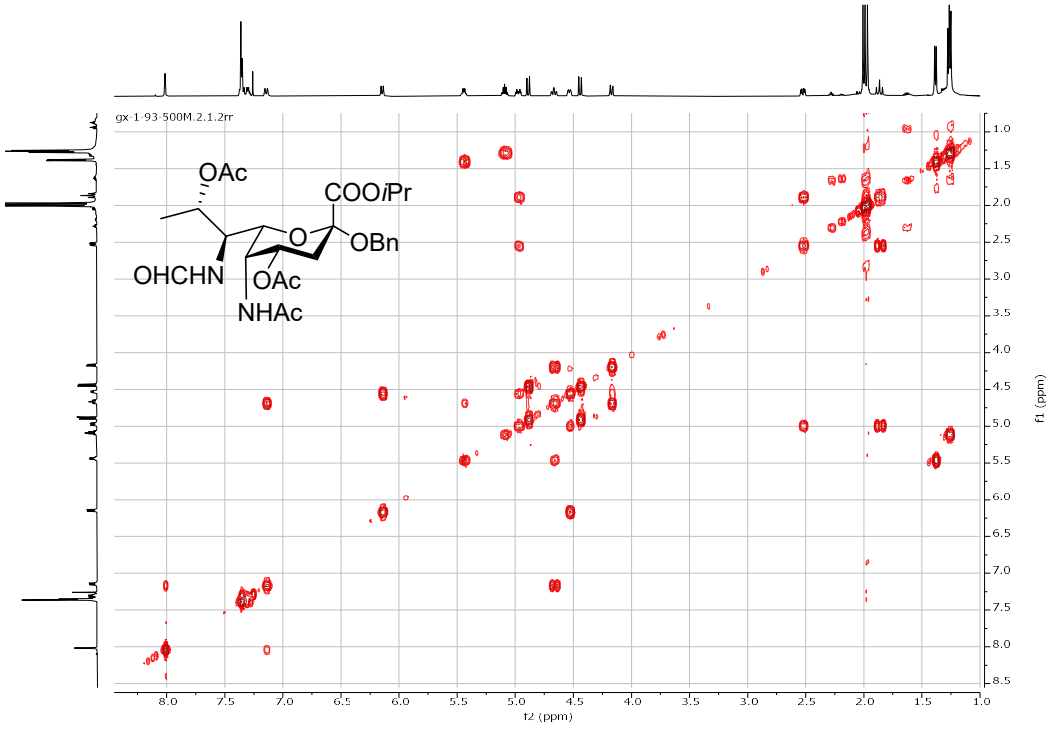

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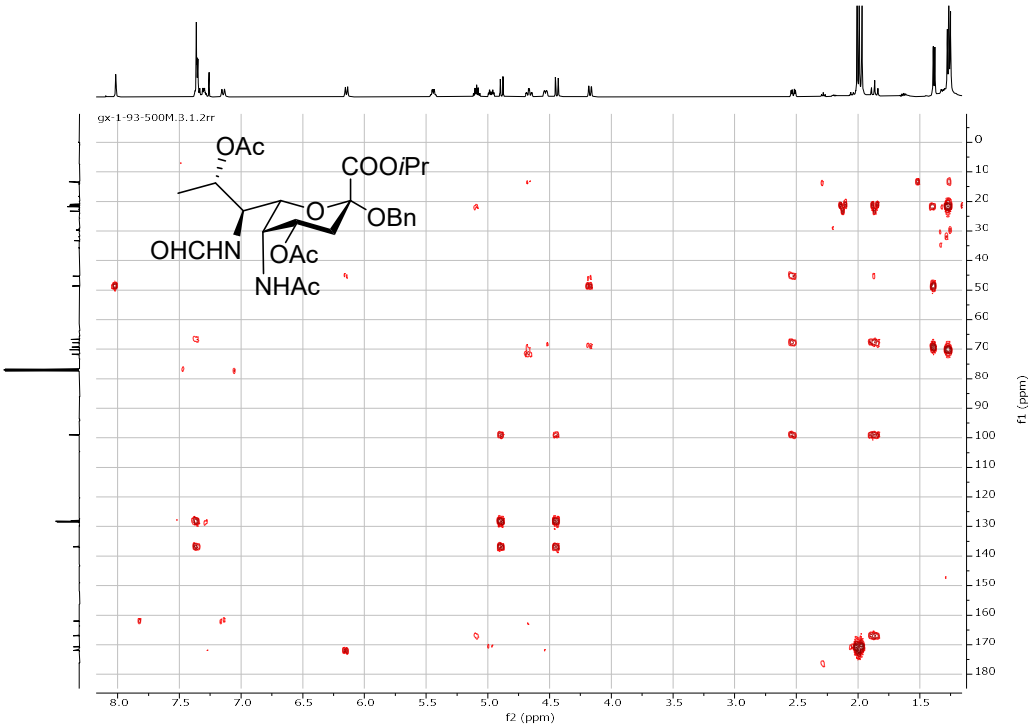

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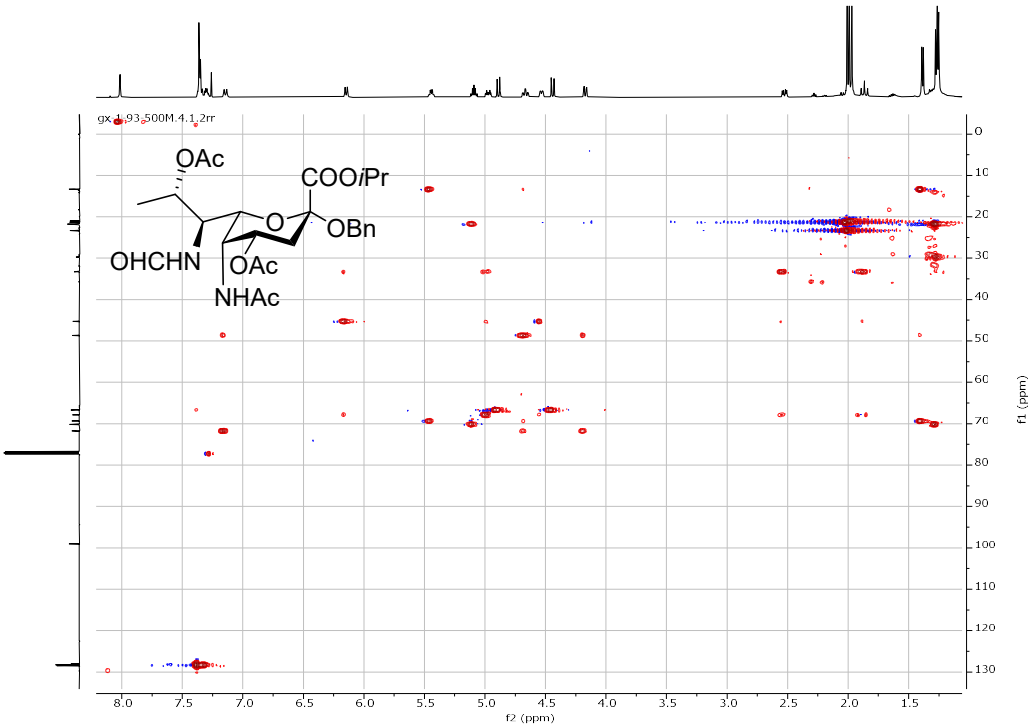

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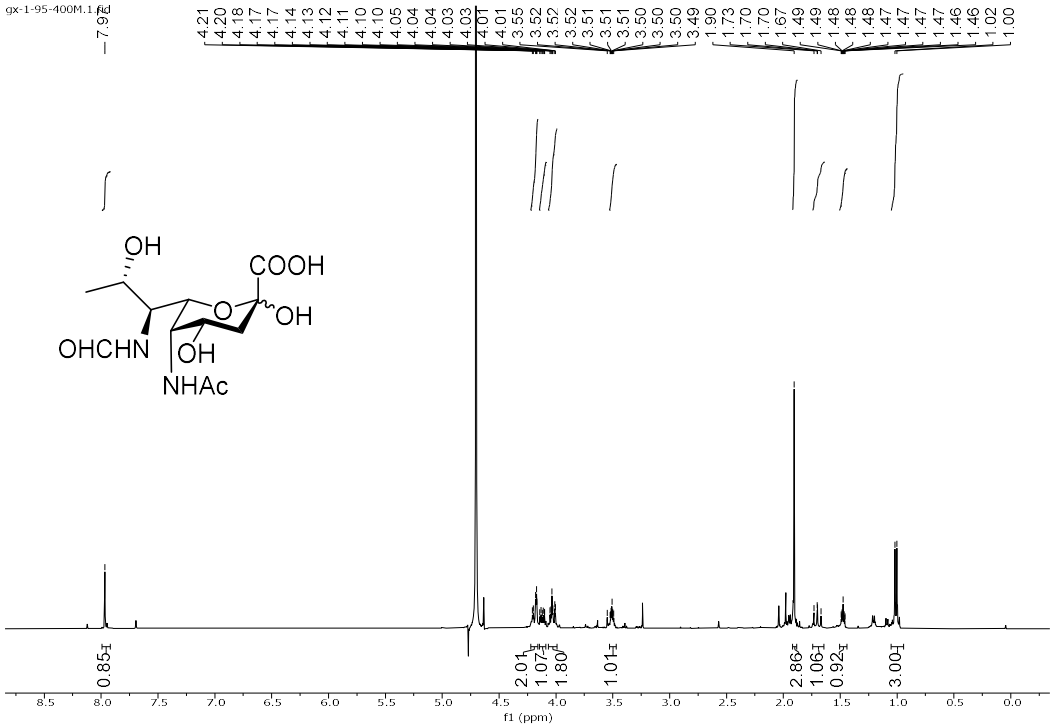

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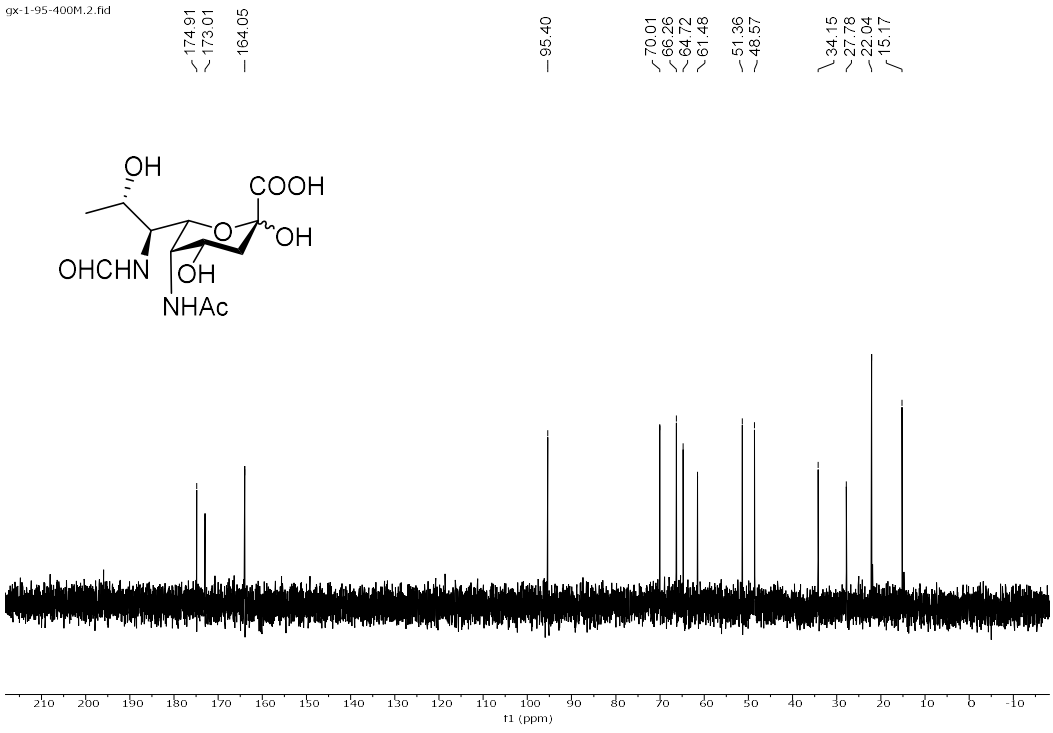

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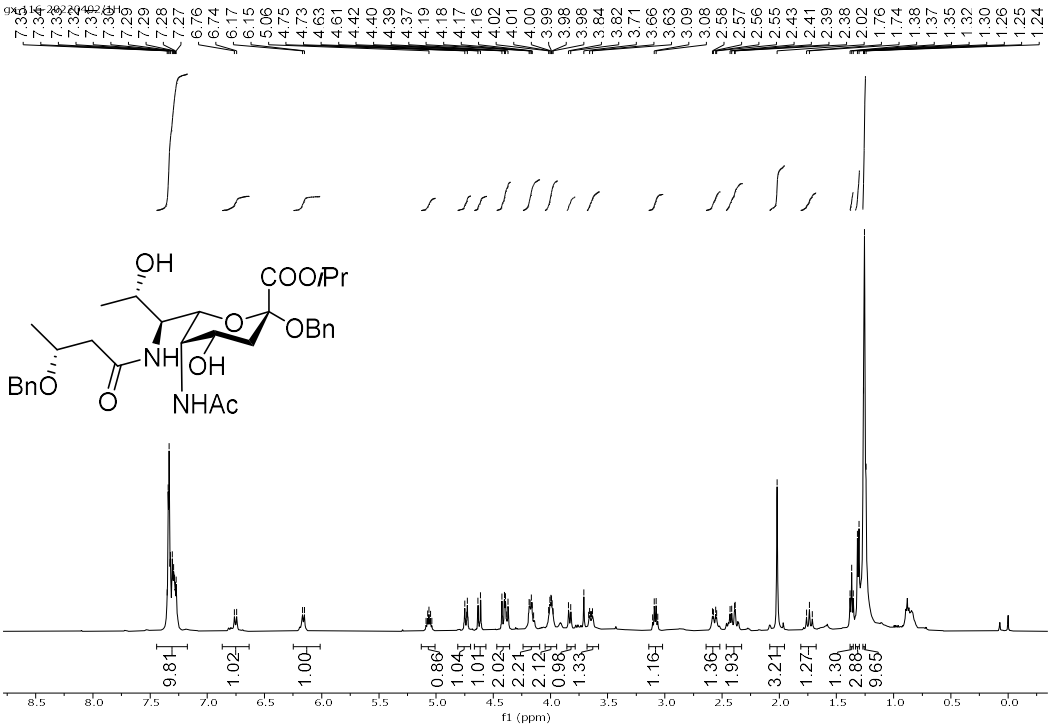

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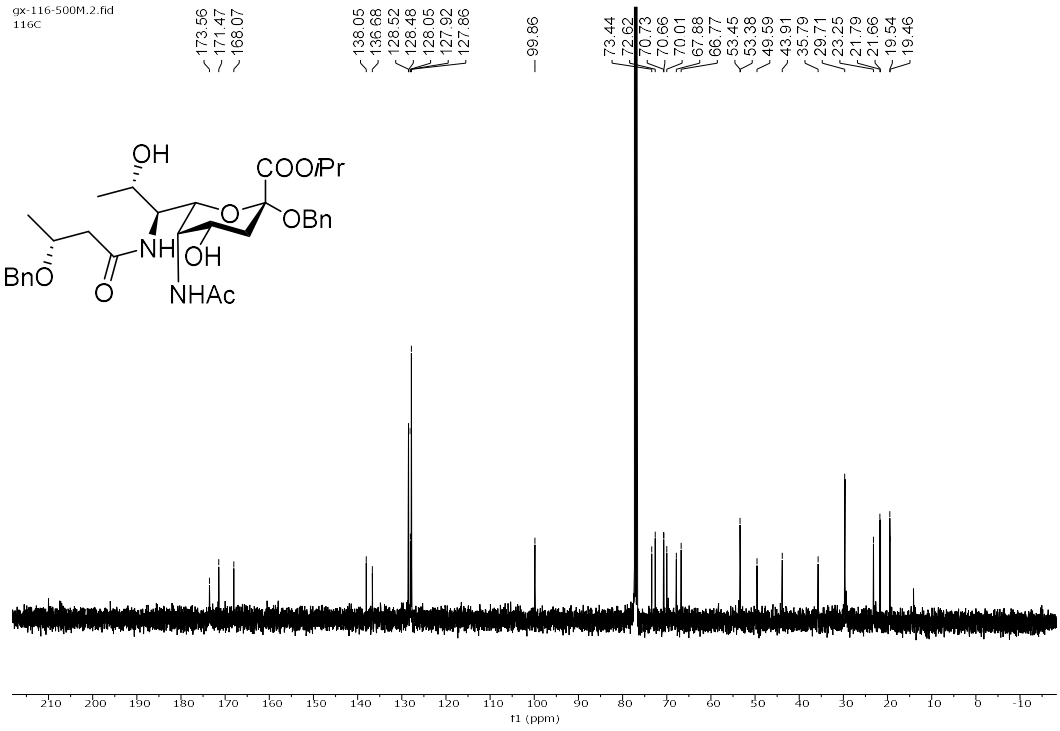

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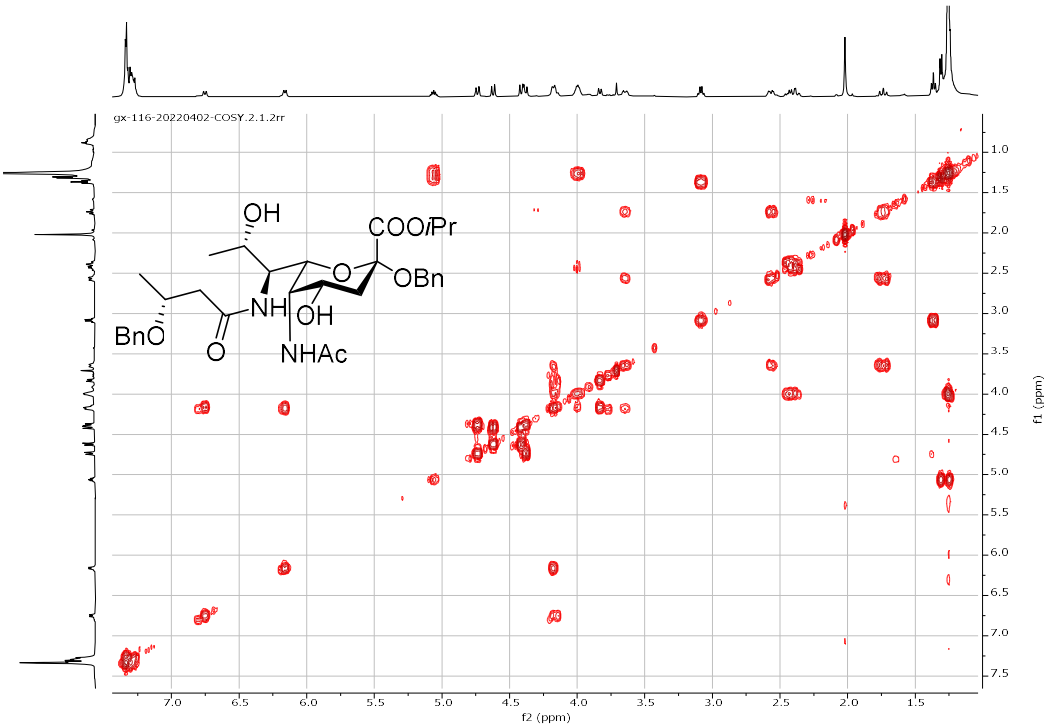

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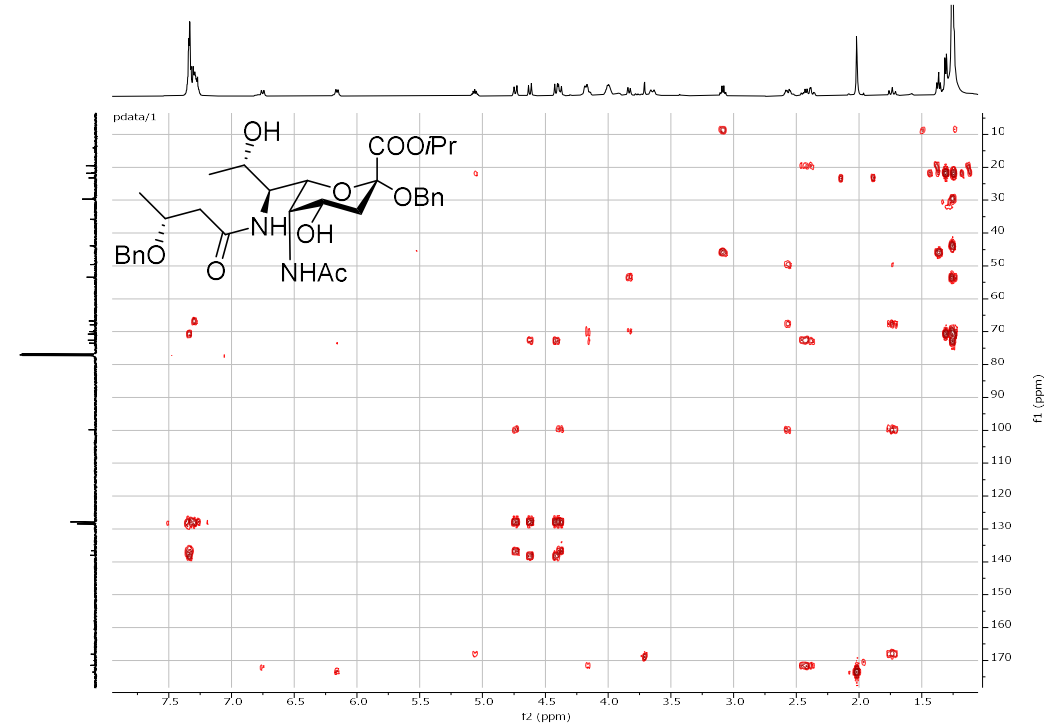

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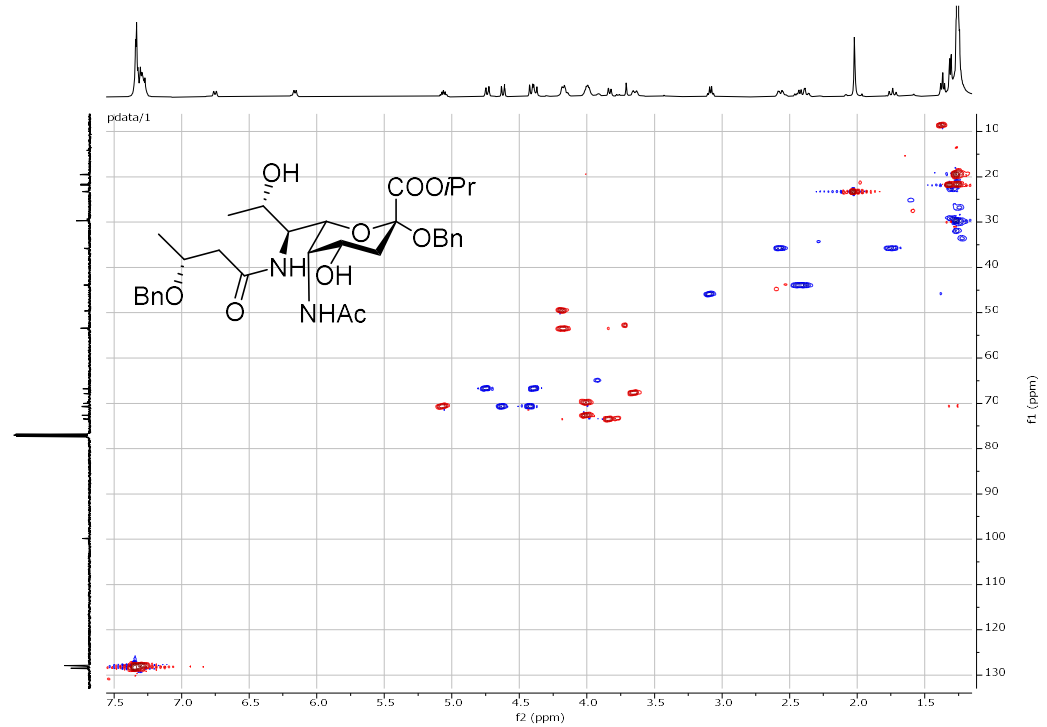

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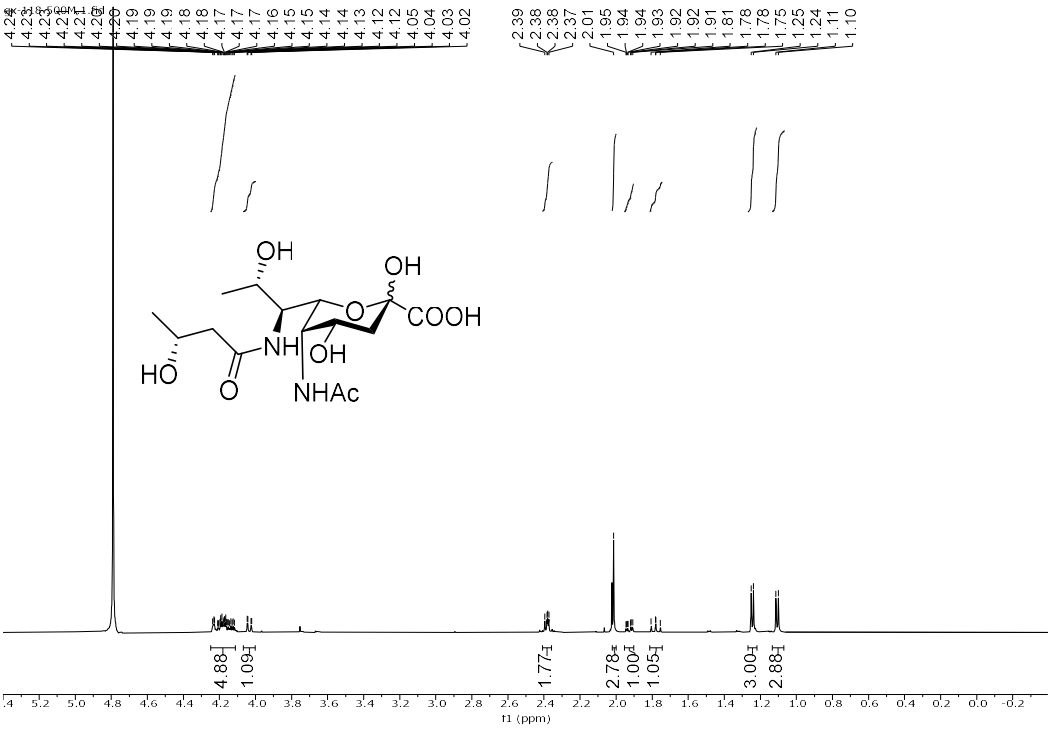

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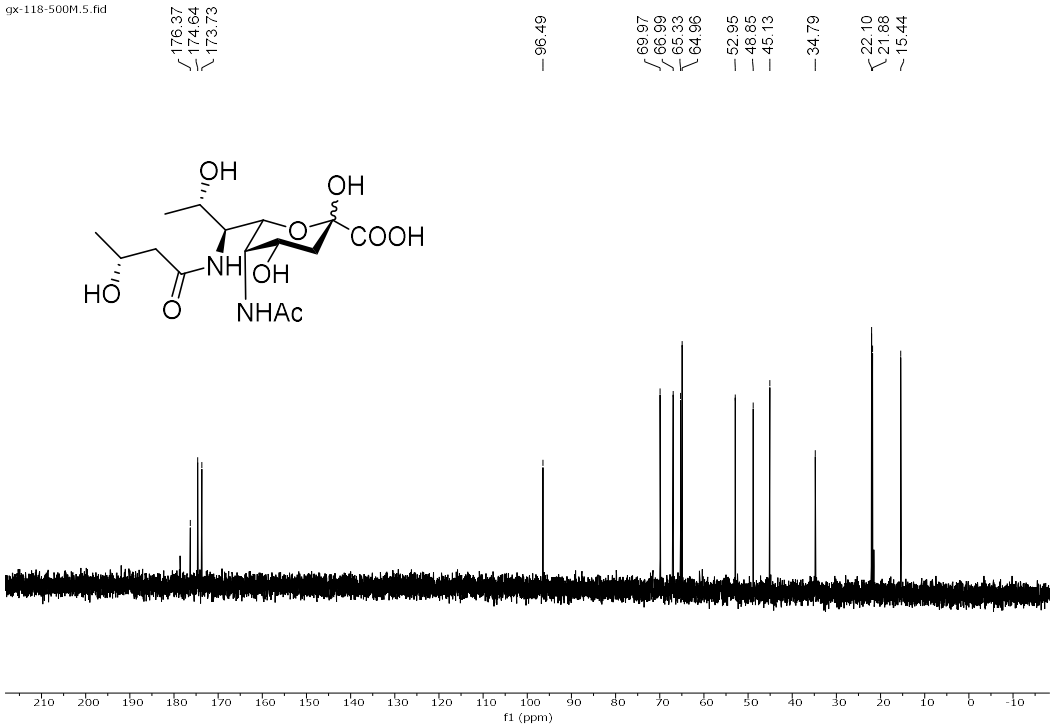

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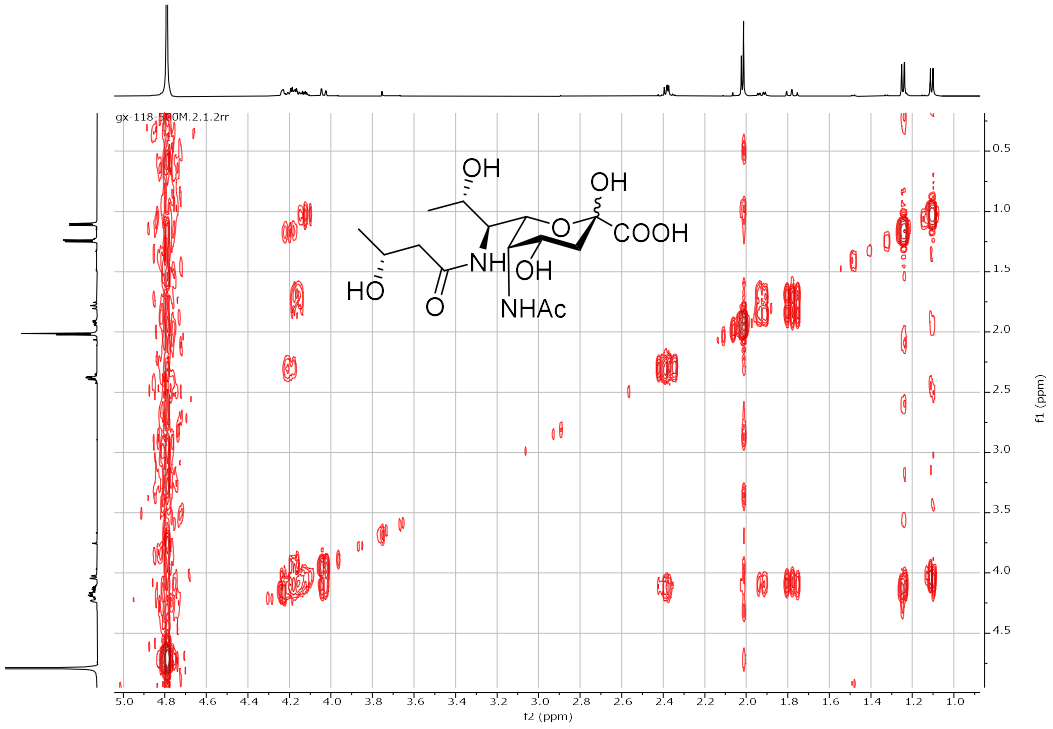

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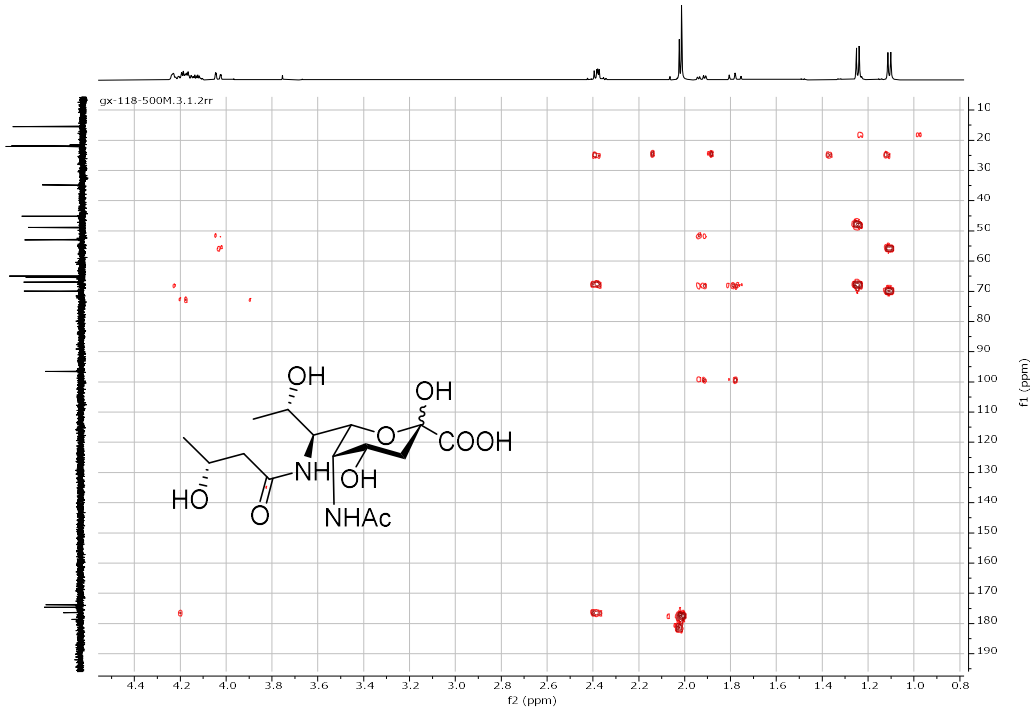

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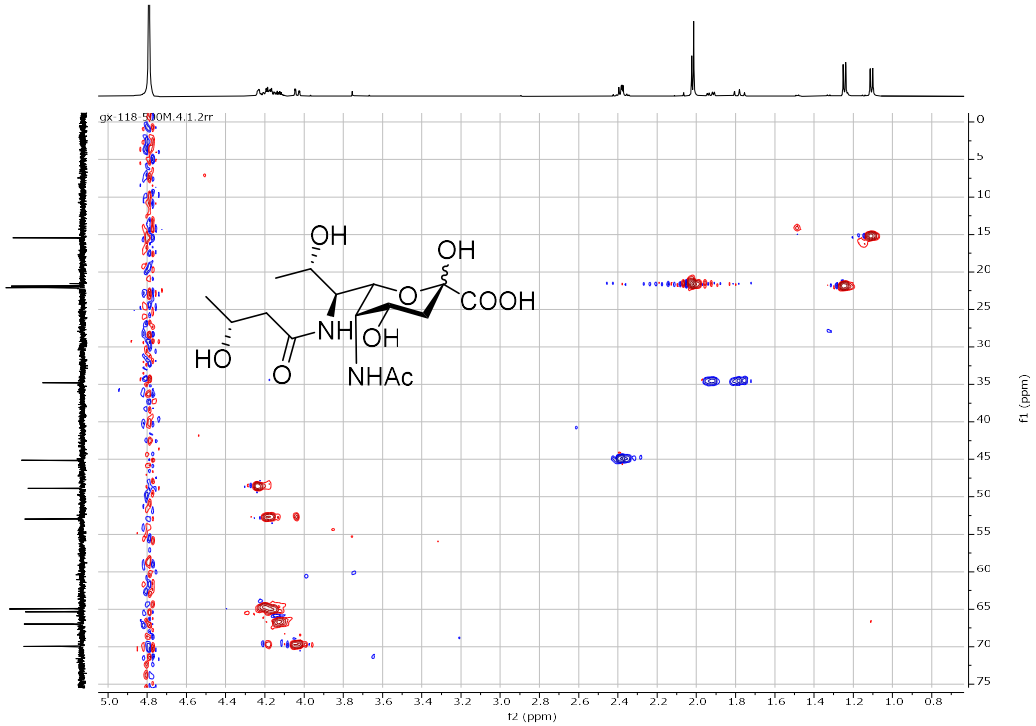

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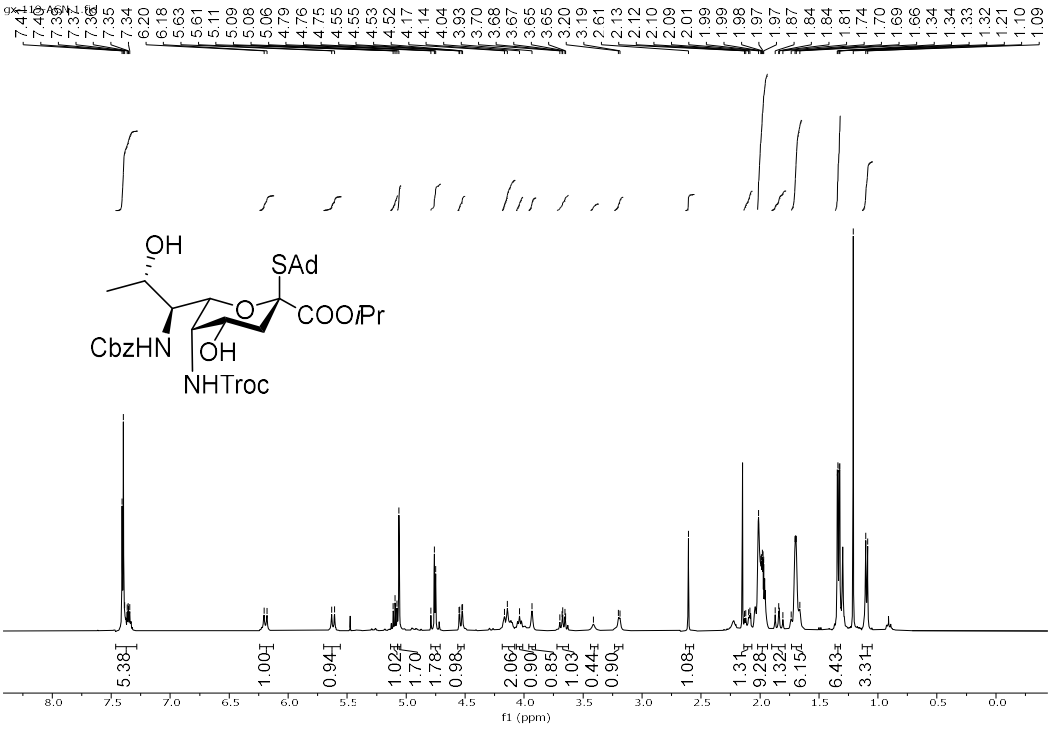

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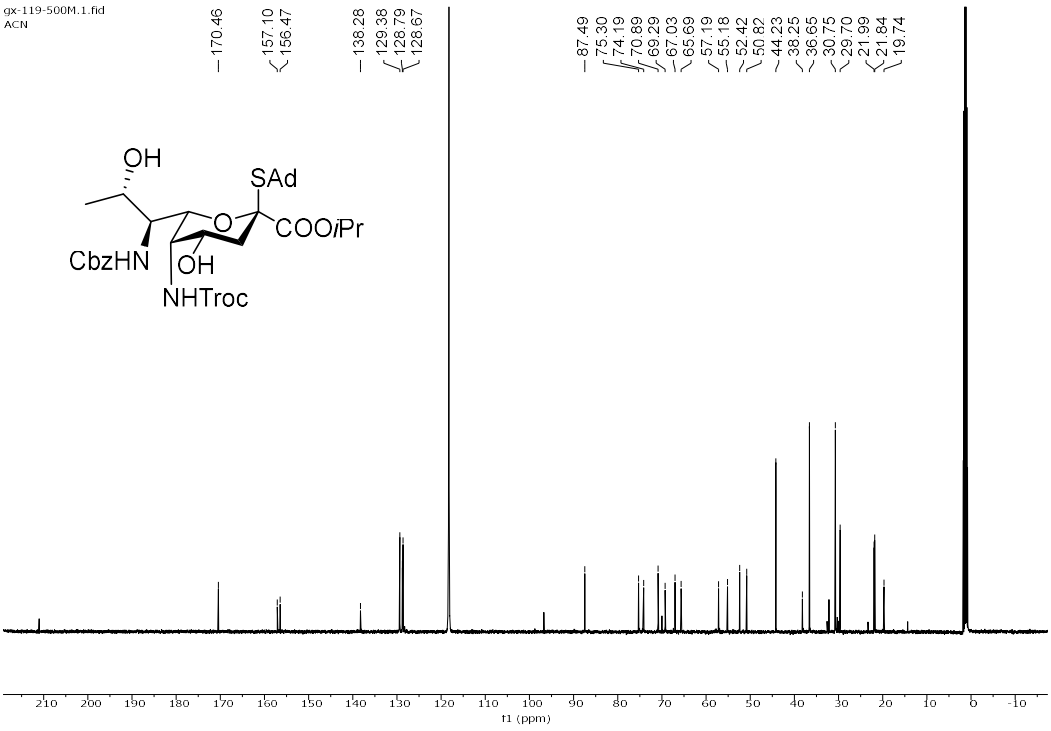

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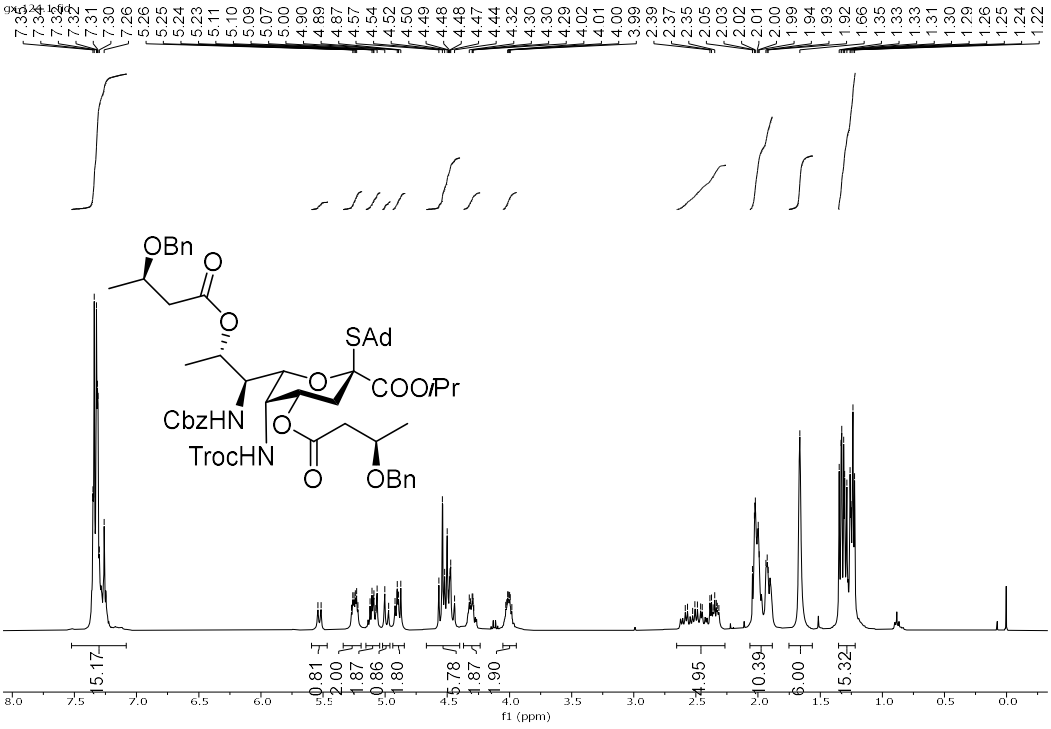

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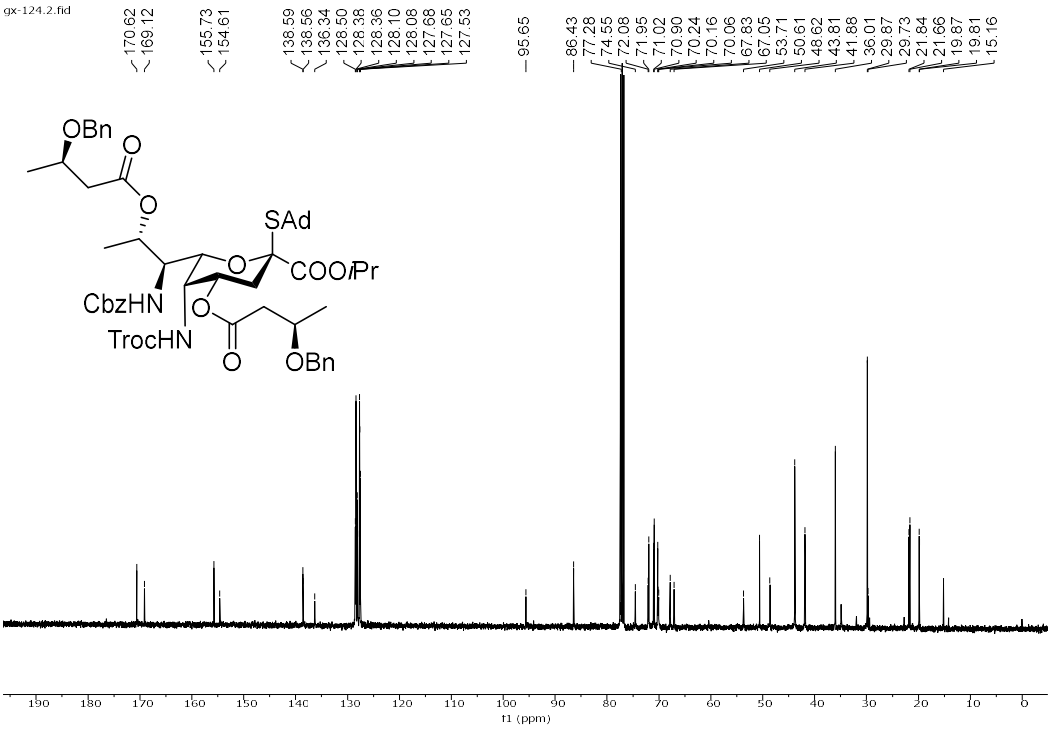

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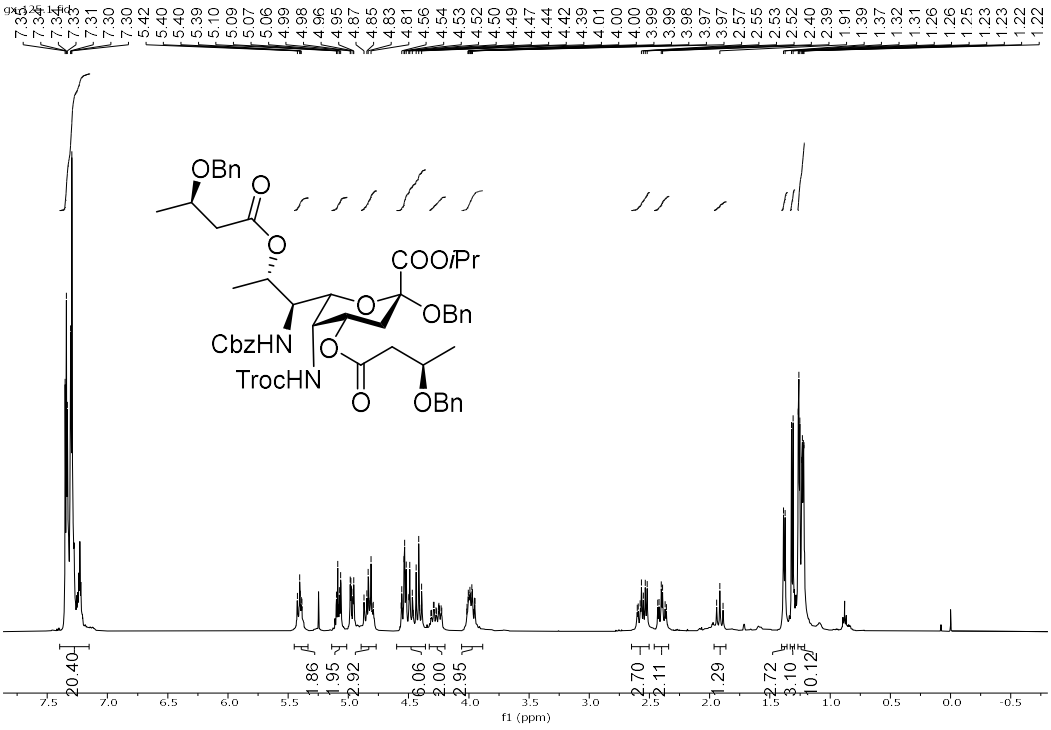

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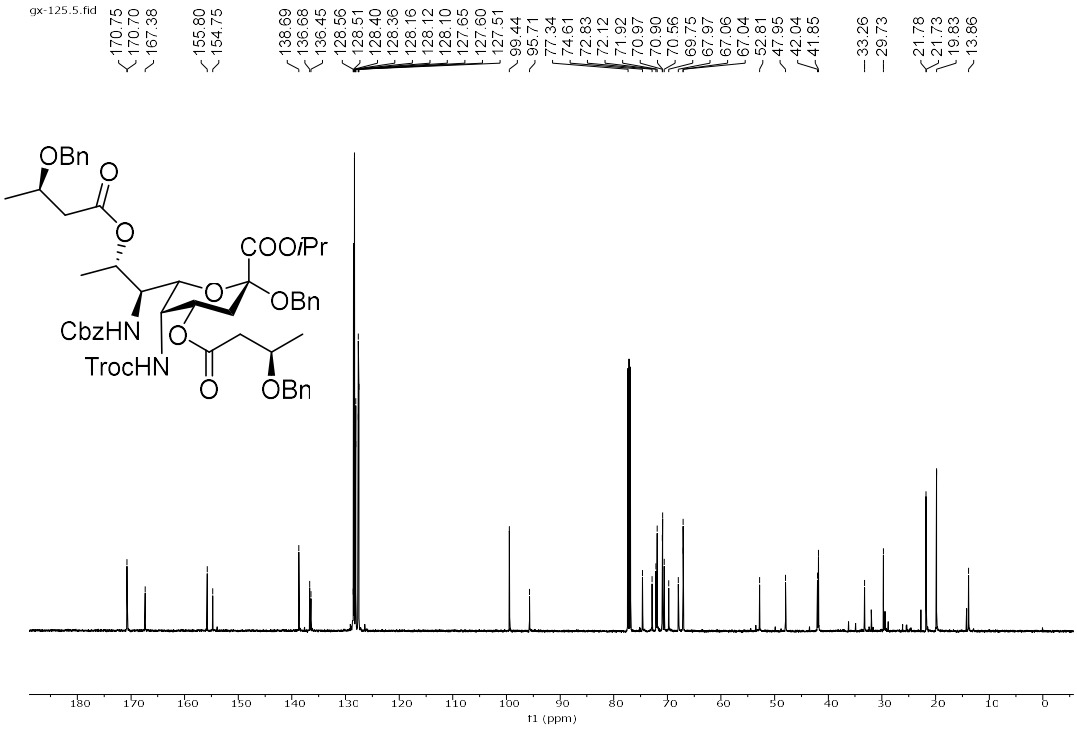

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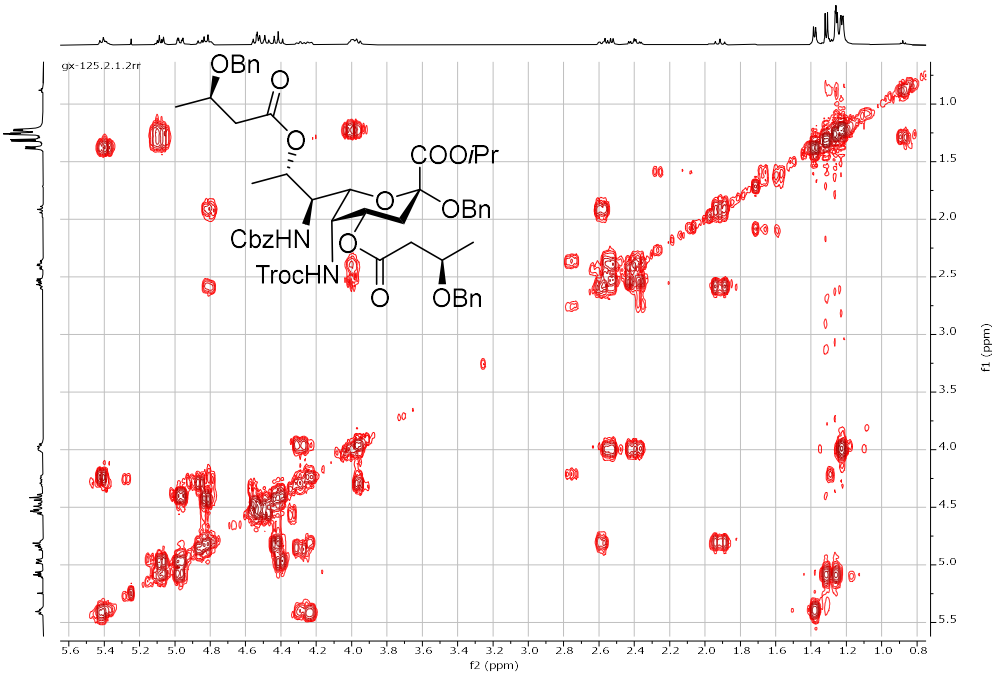

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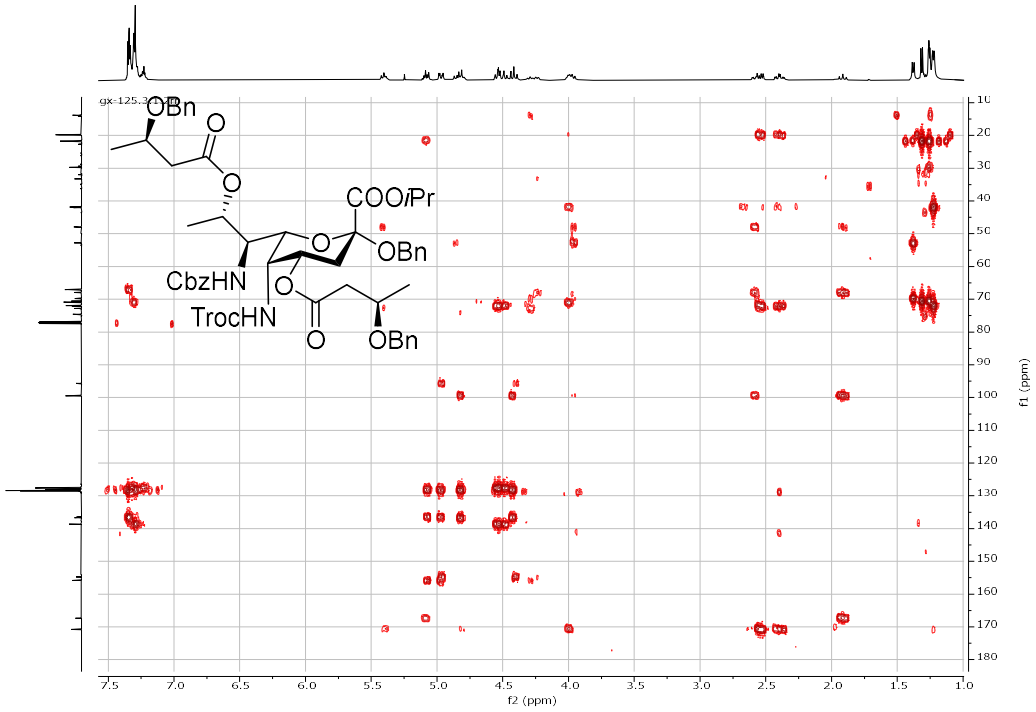

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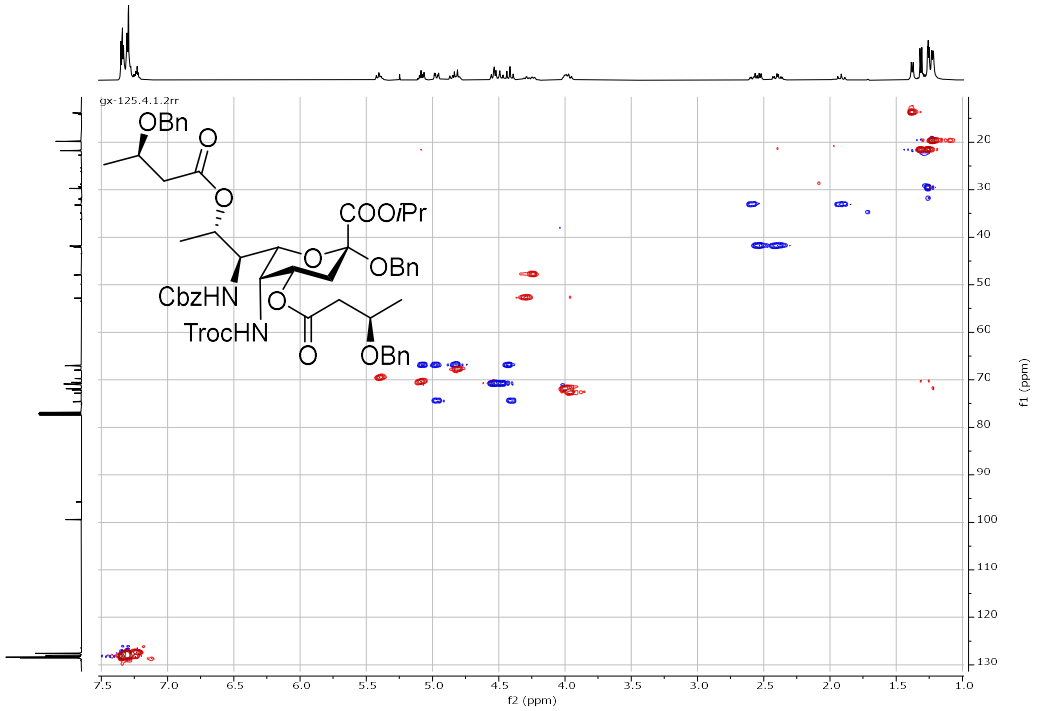
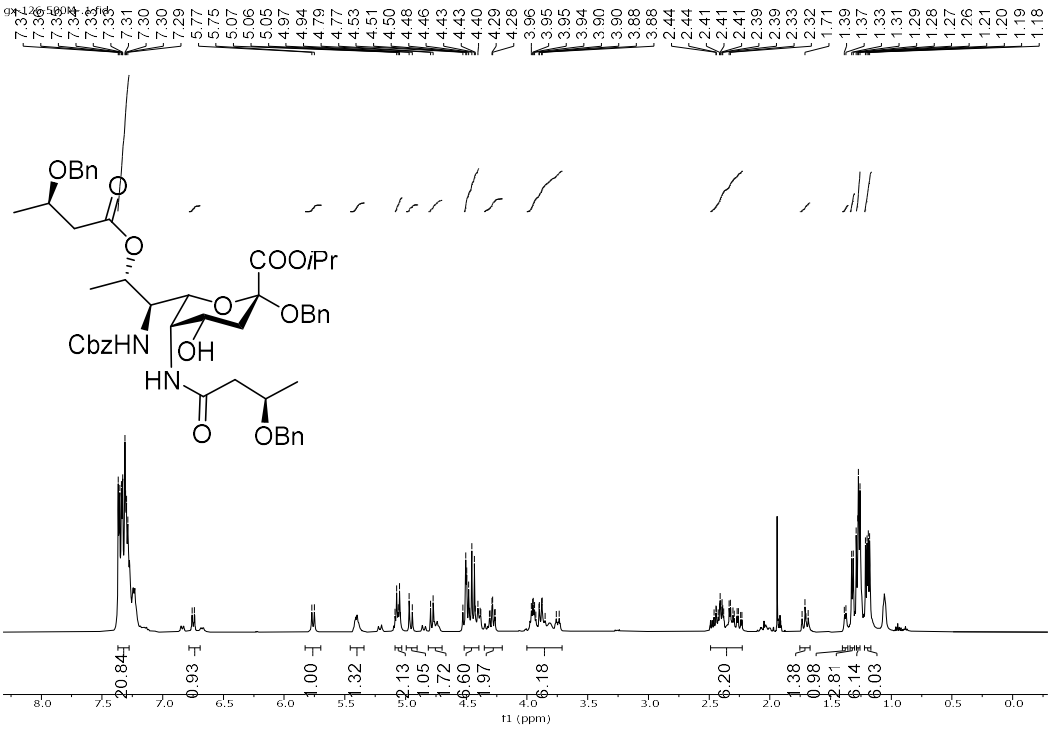
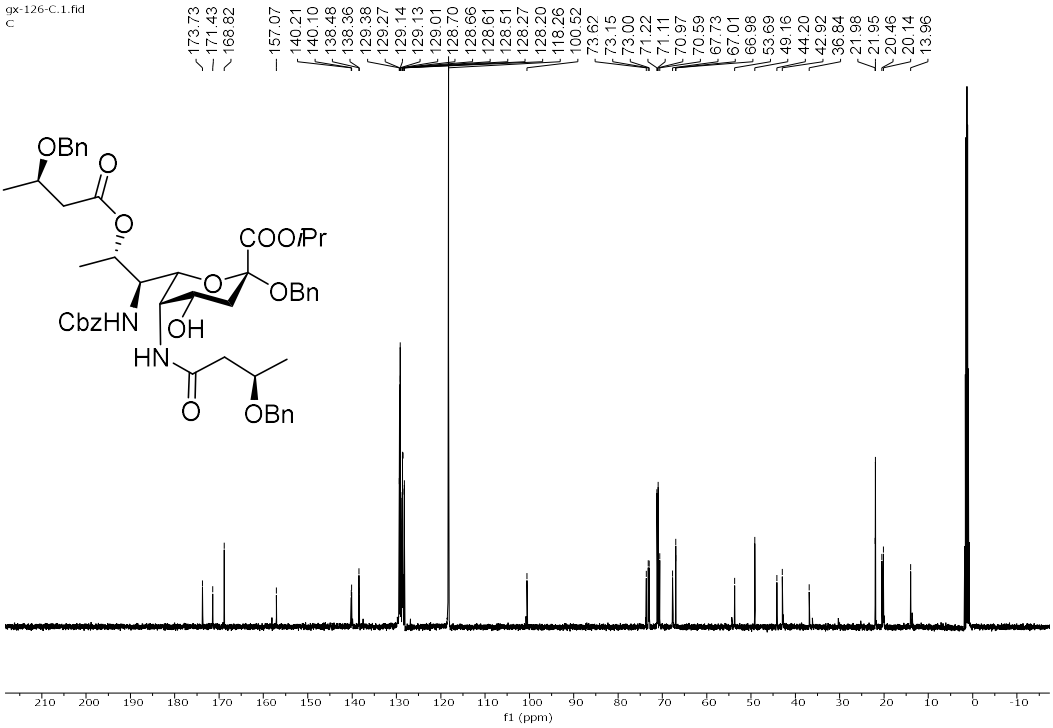


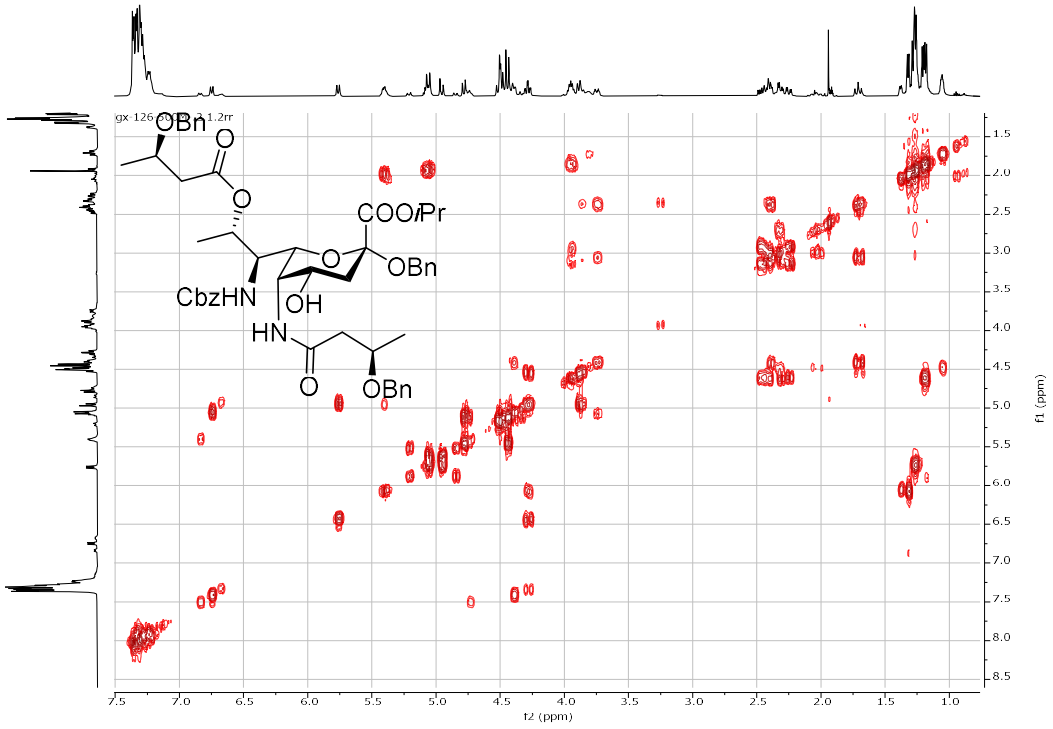
^{1}H NMR of compound 22



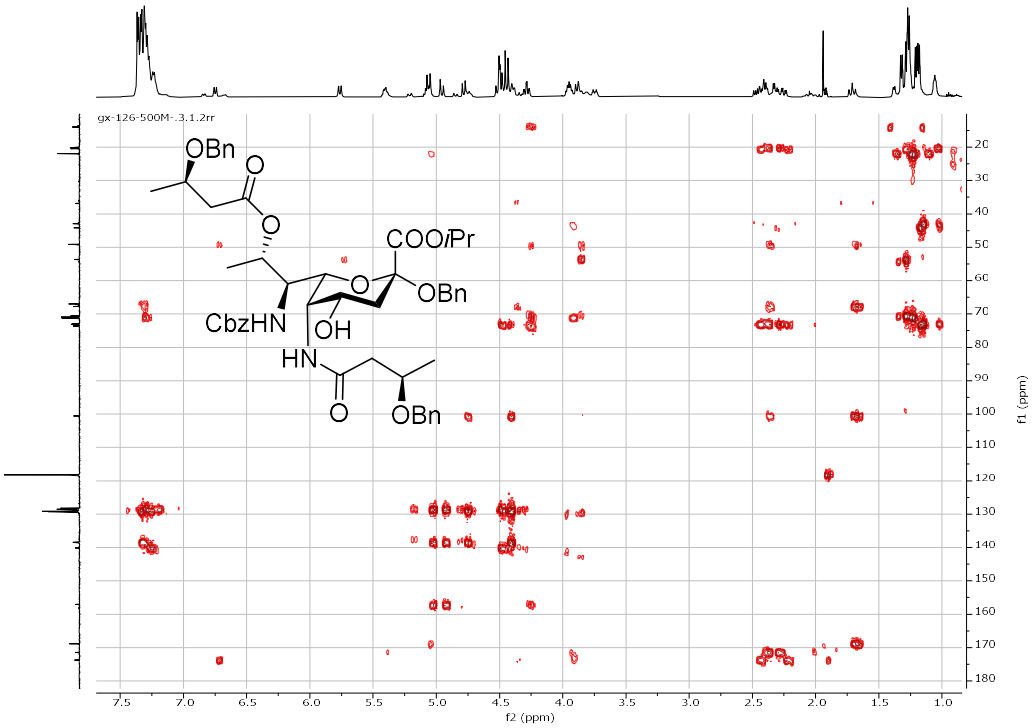
^{13}C NMR of compound 22



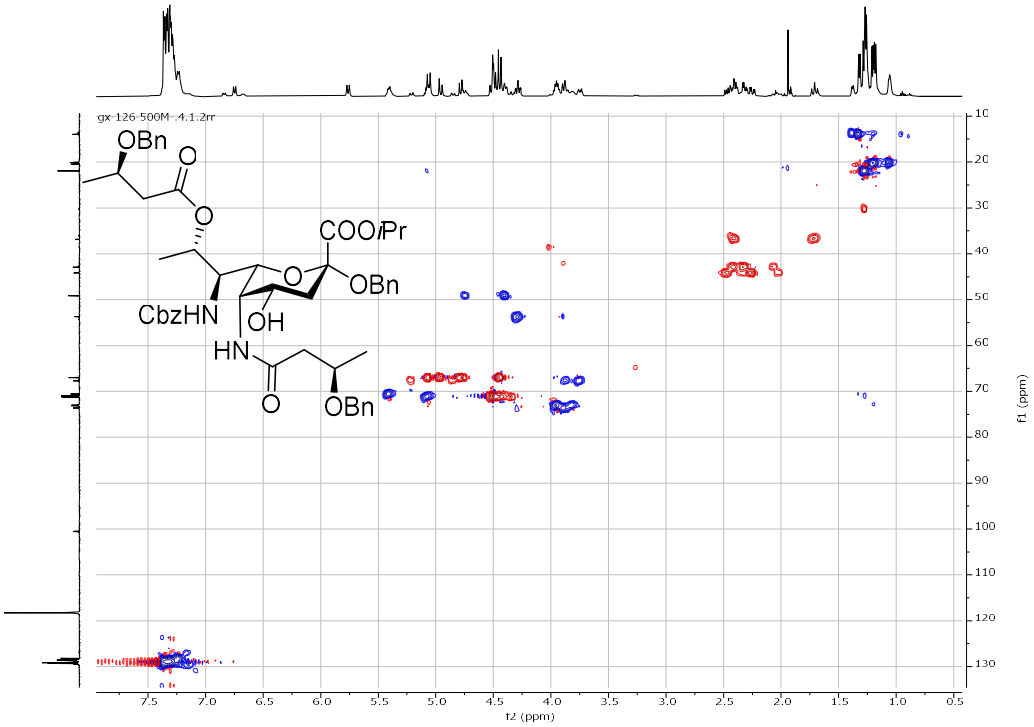
^{1}H - ^{1}H COSY of compound 22



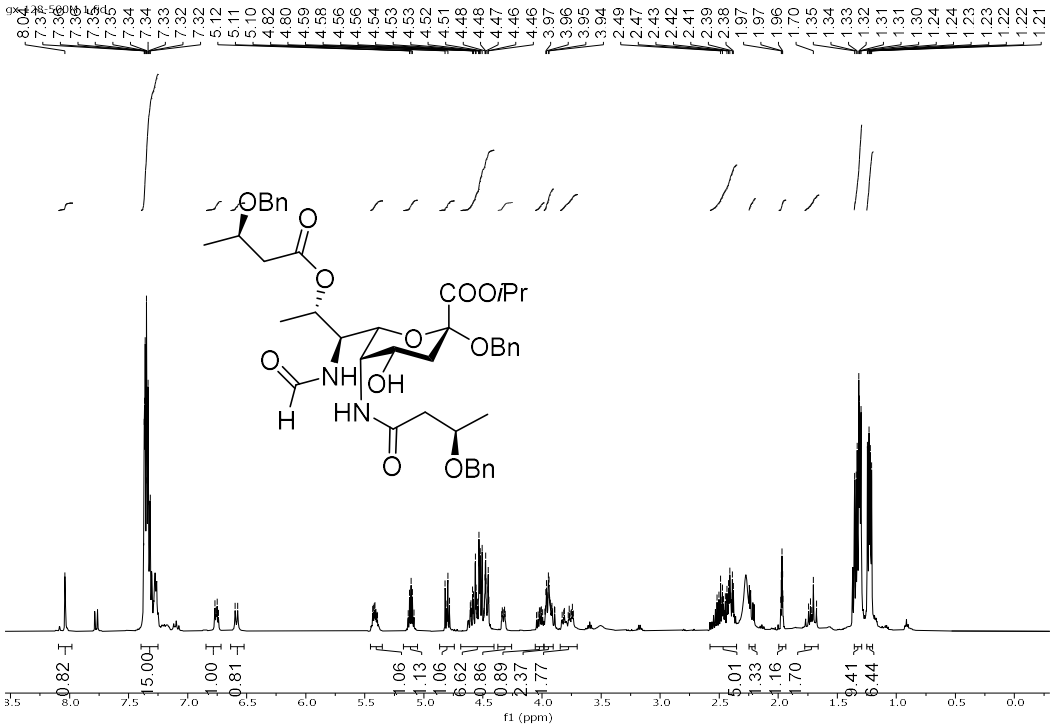
^{1}H - ^{13}C HMBC of compound 22



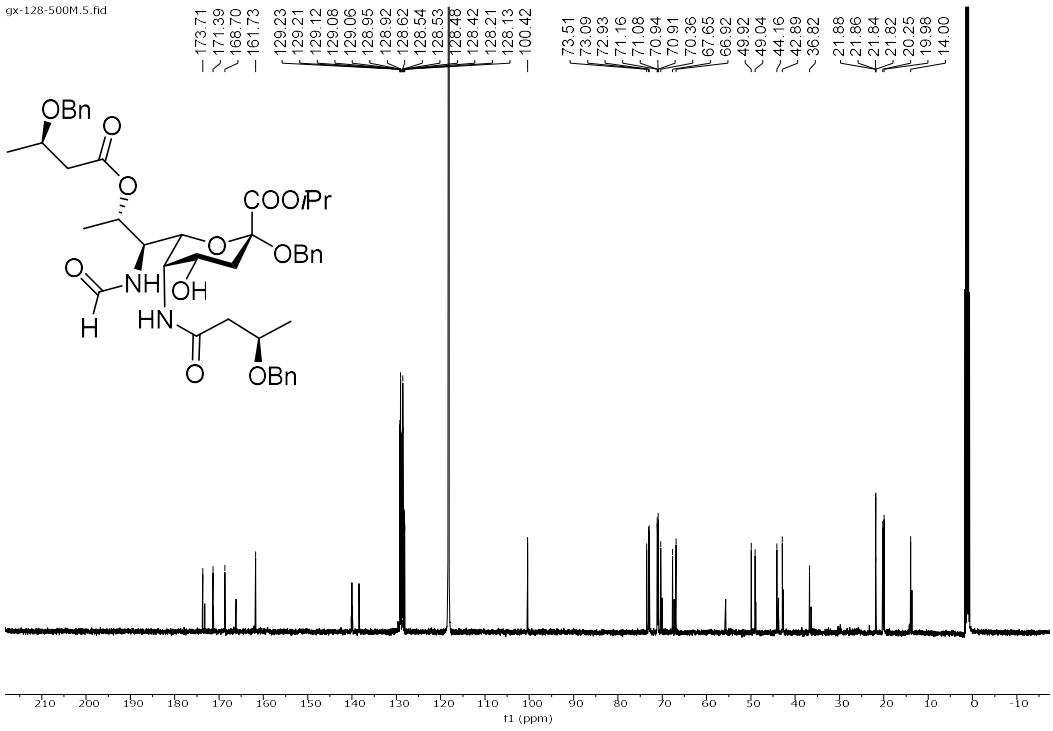
^{1}H - ^{13}C HSQC of compound 22



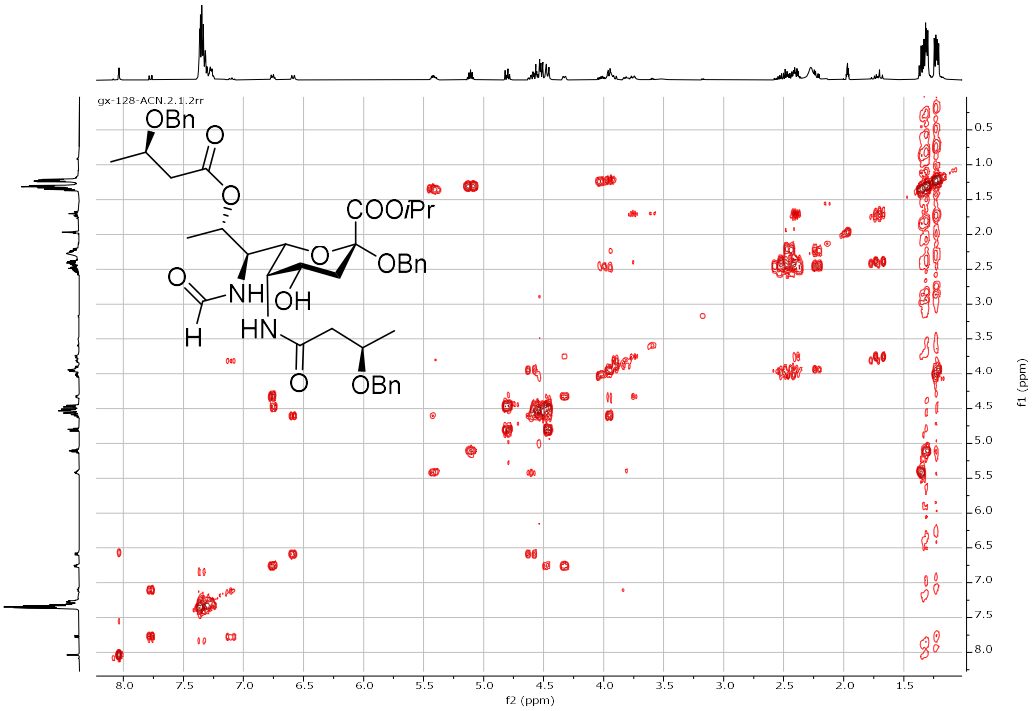
^{1}H NMR of compound 23



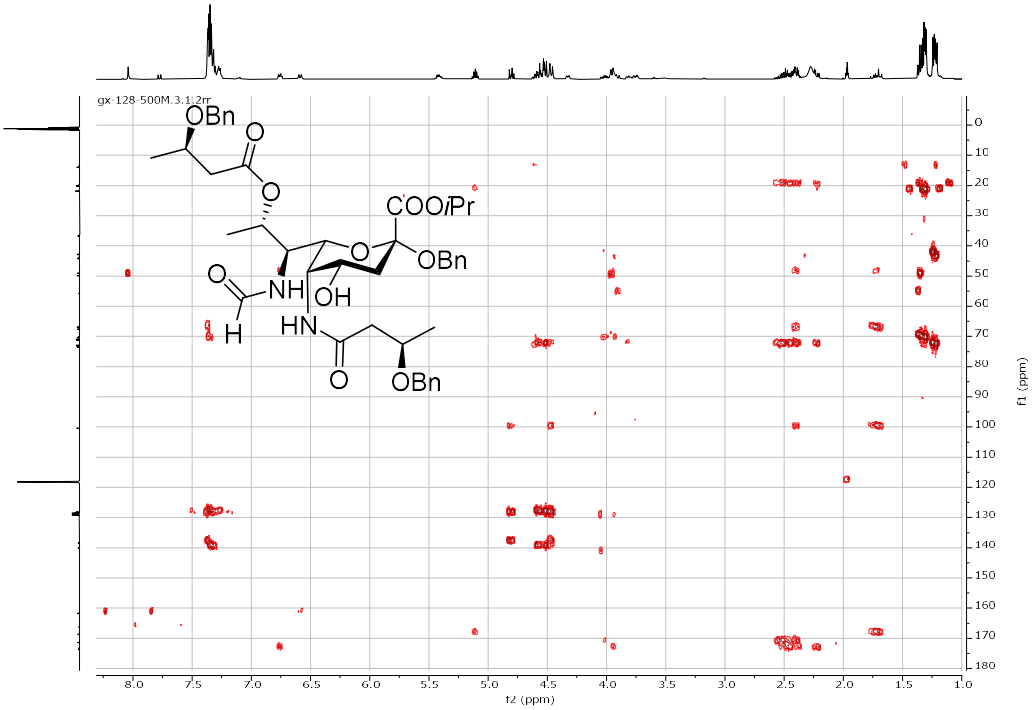
^{13}C NMR of compound 23



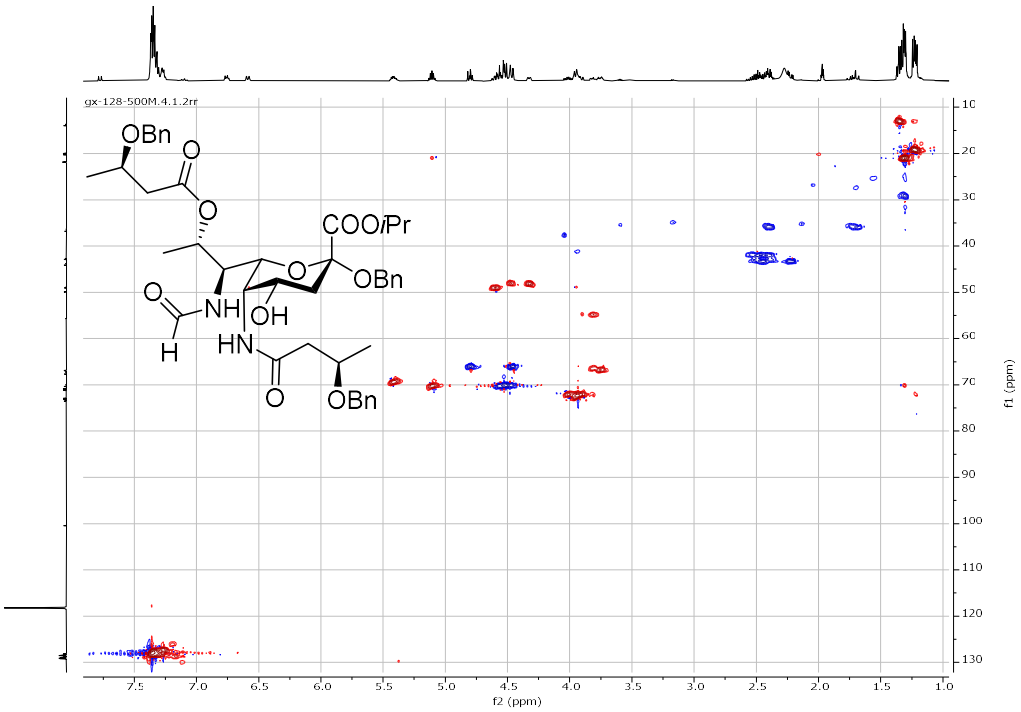
^{1}H - ^{1}H COSY of compound 23



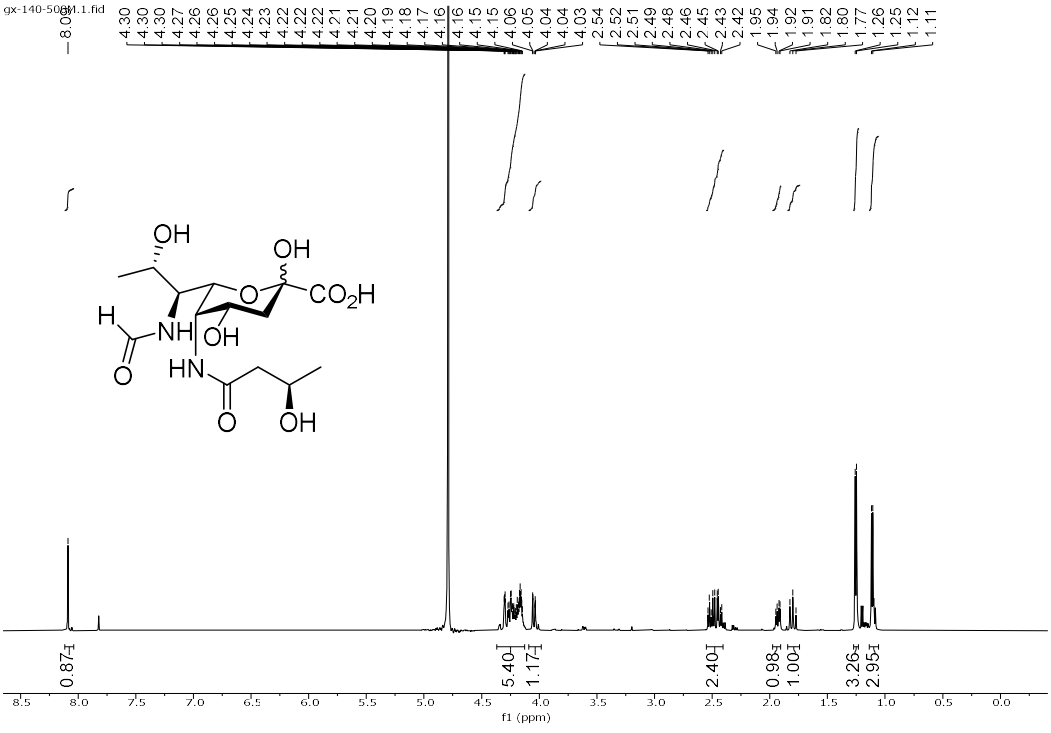
^{1}H - ^{13}C HMBC of compound 23



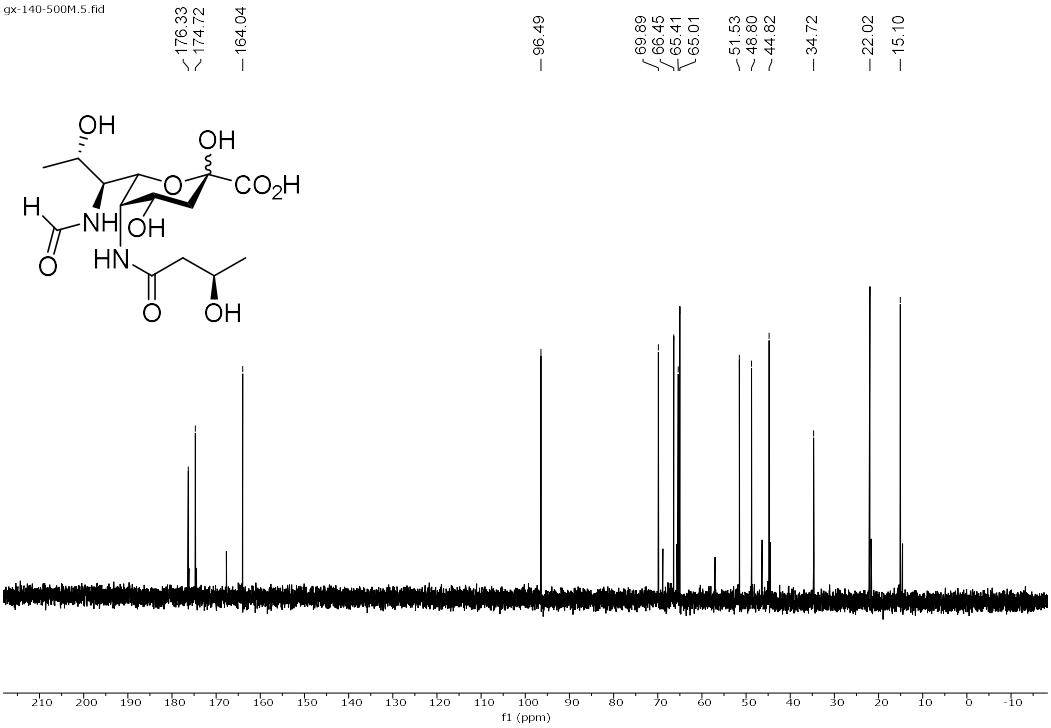
^{1}H - ^{13}C HSQC of compound 23



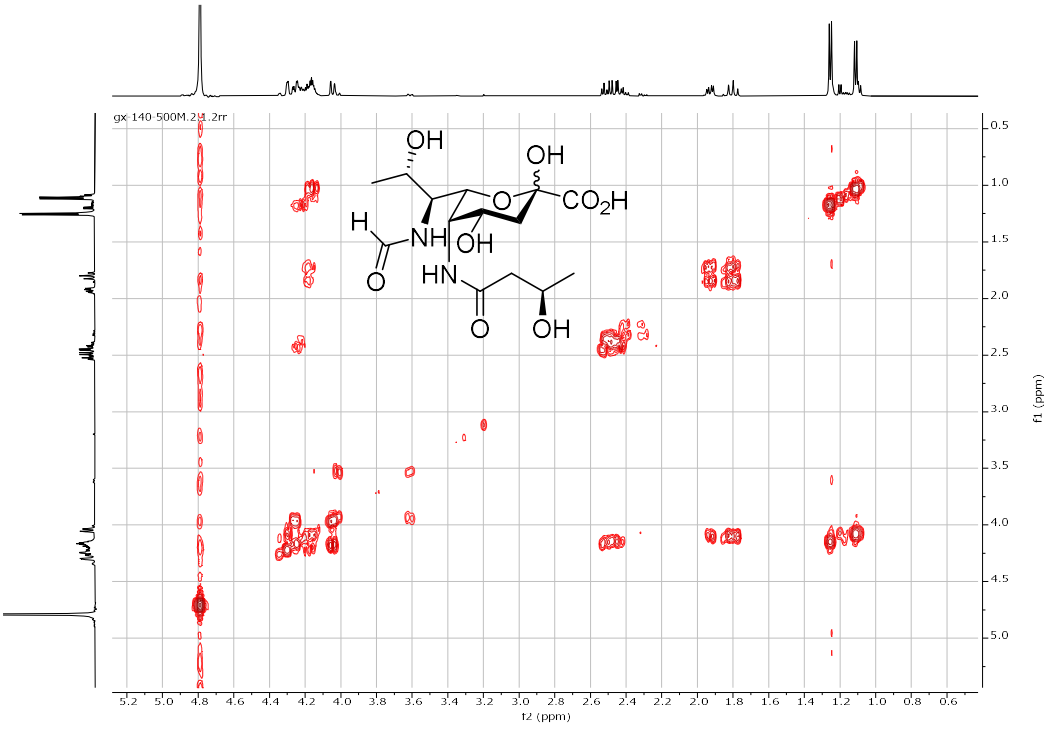
^{1}H NMR of compound 4



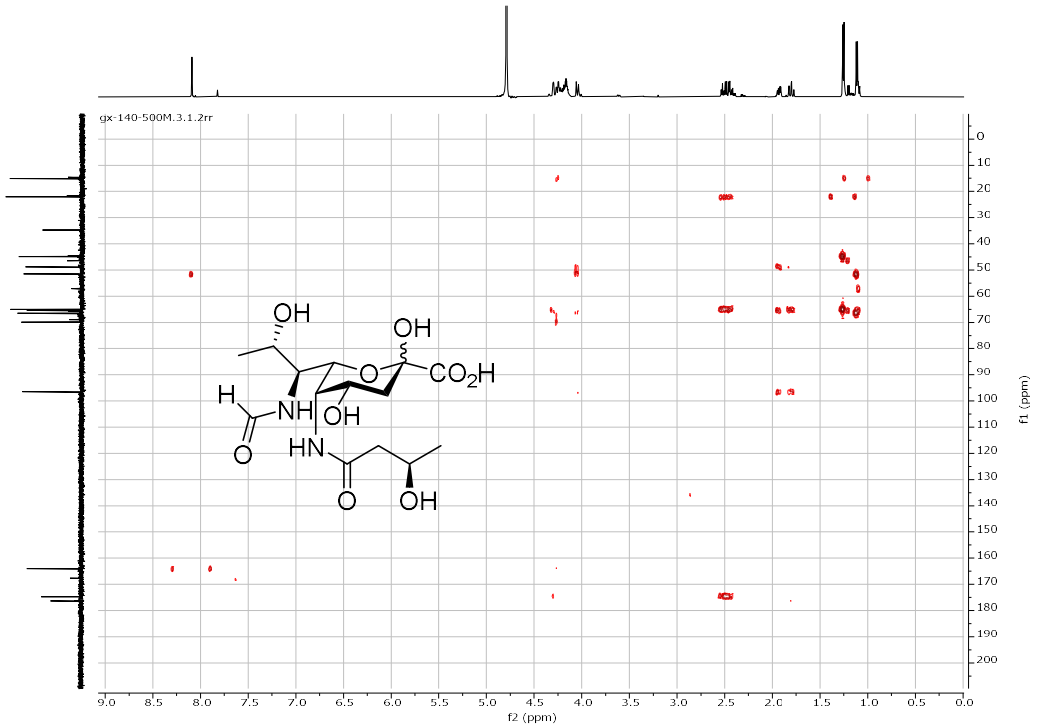
^{13}C NMR of compound 4



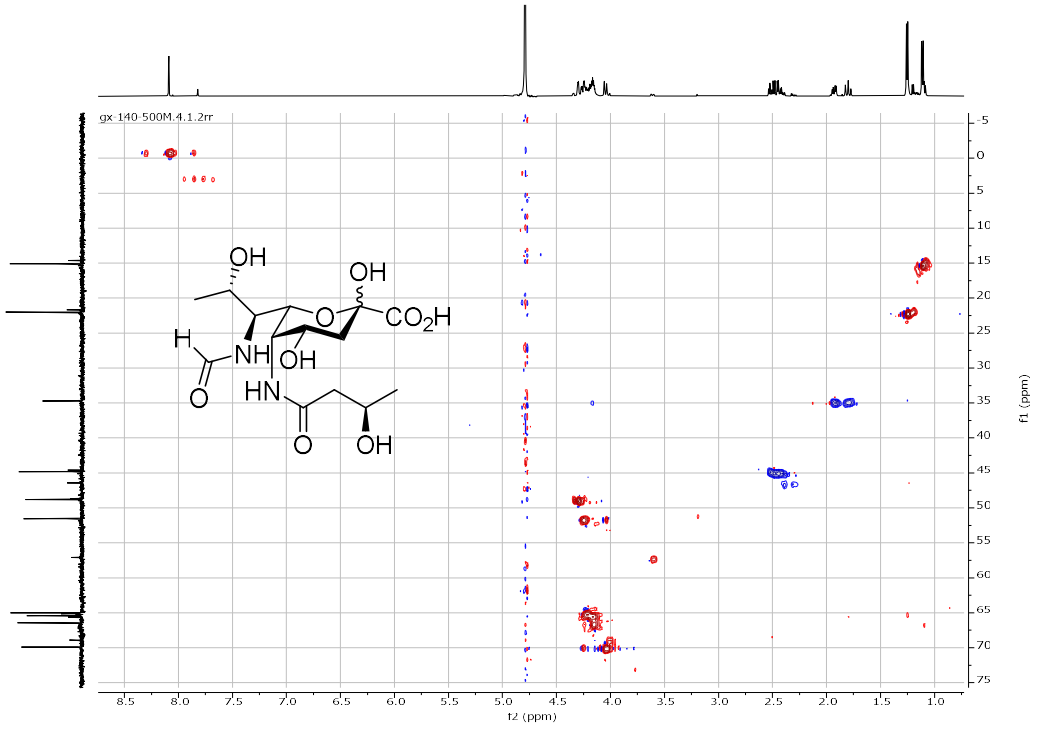
^{1}H - ^{1}H COSY of compound 4



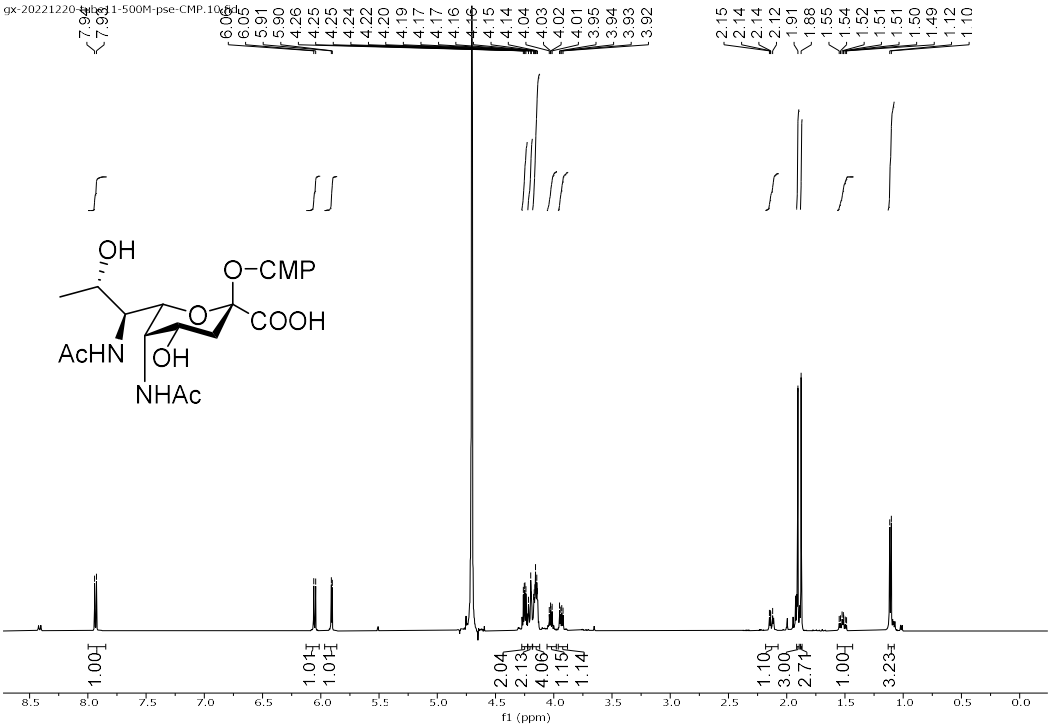
^{1}H - ^{13}C HMBC of compound 4



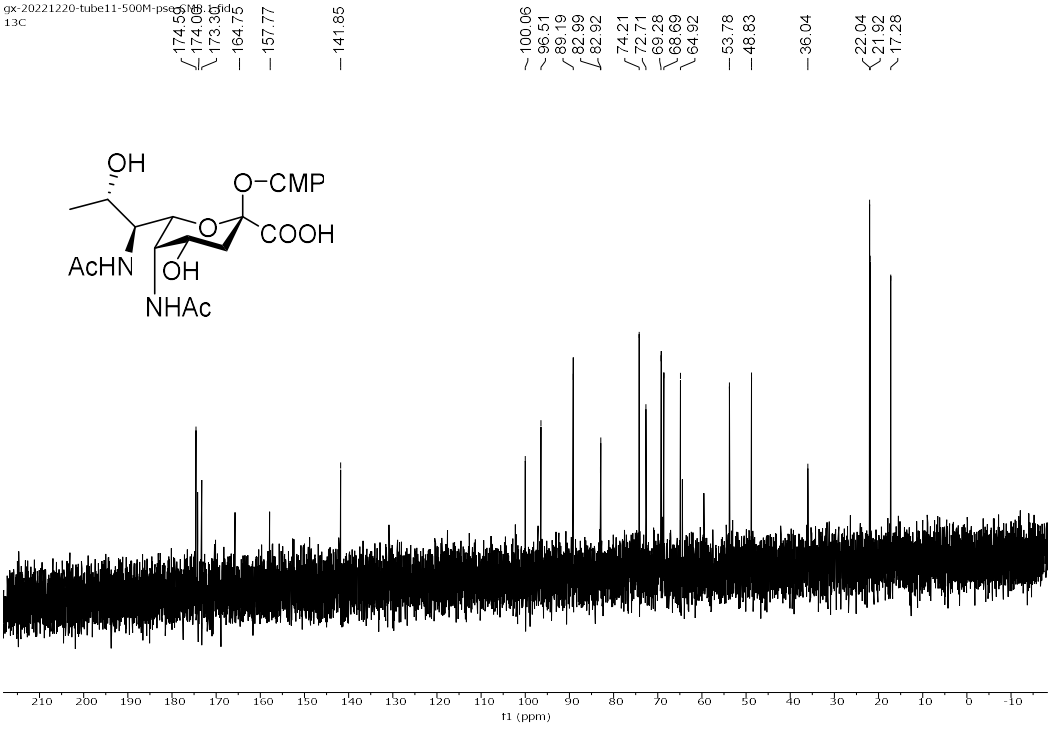
^{1}H - ^{13}C HSQC of compound 4



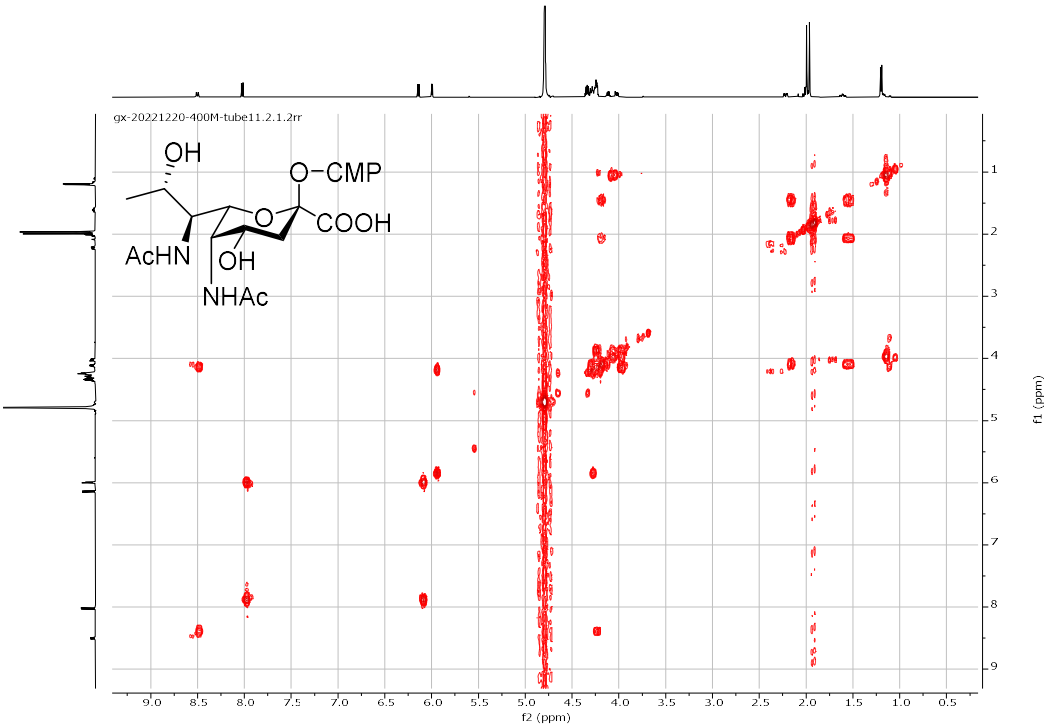
^{1}H NMR of compound CMP-Pse5Ac7Ac 7



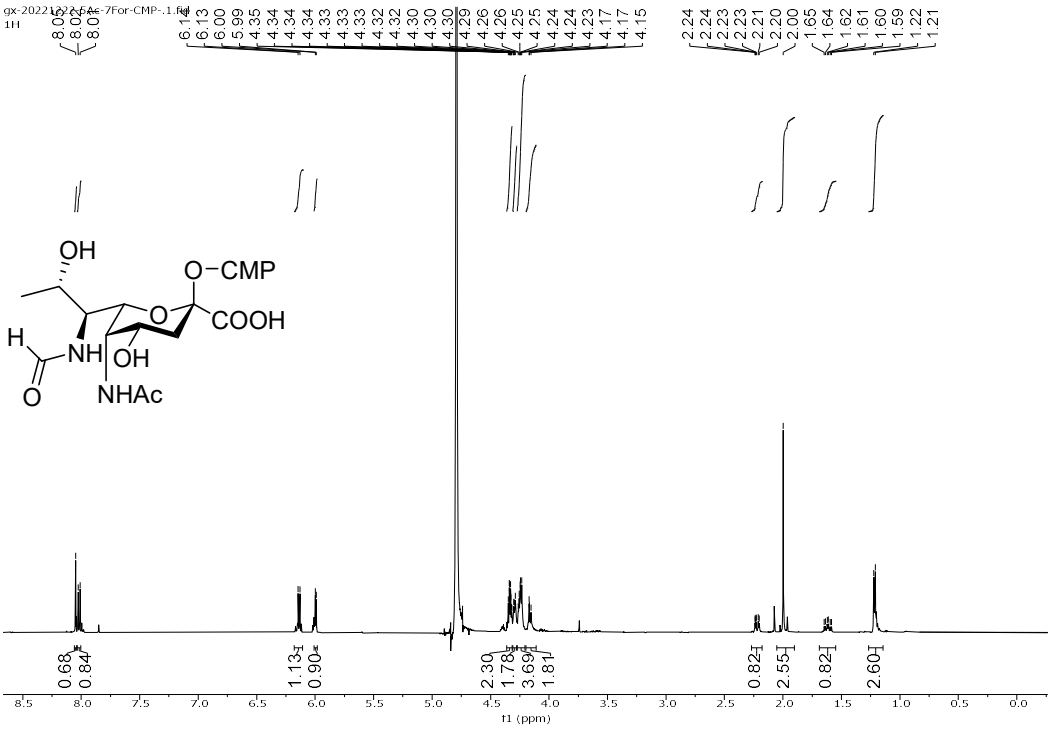
^{13}C NMR of compound CMP-Pse5Ac7Ac 7



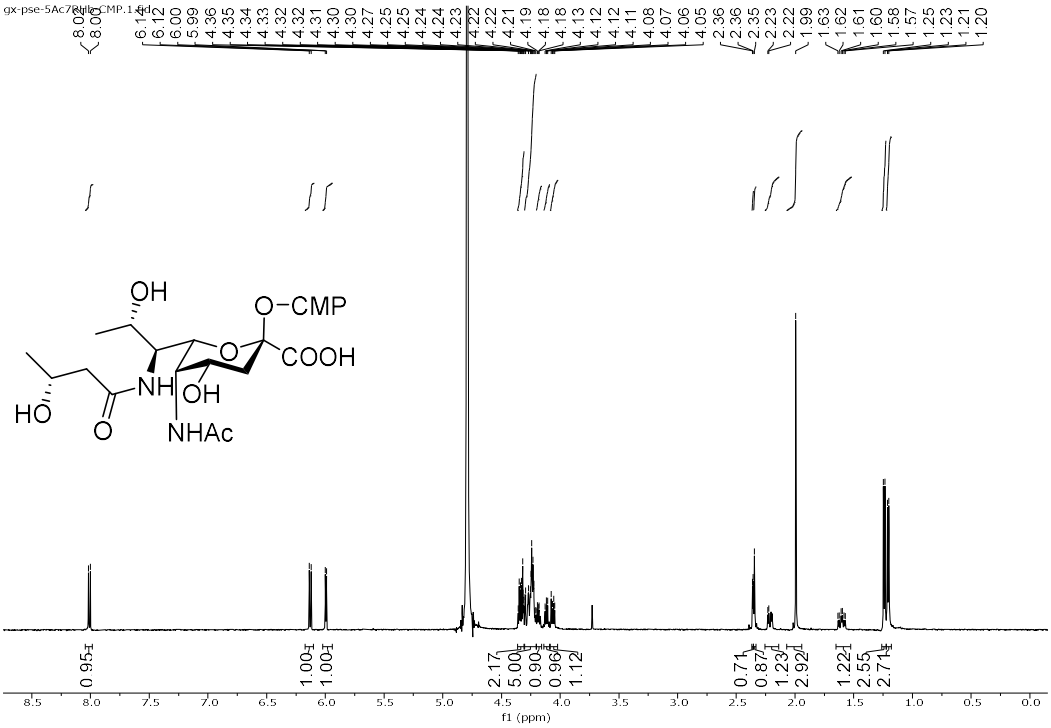
^{1}H - ^{1}H COSY of compound CMP-Pse5Ac7Ac 7



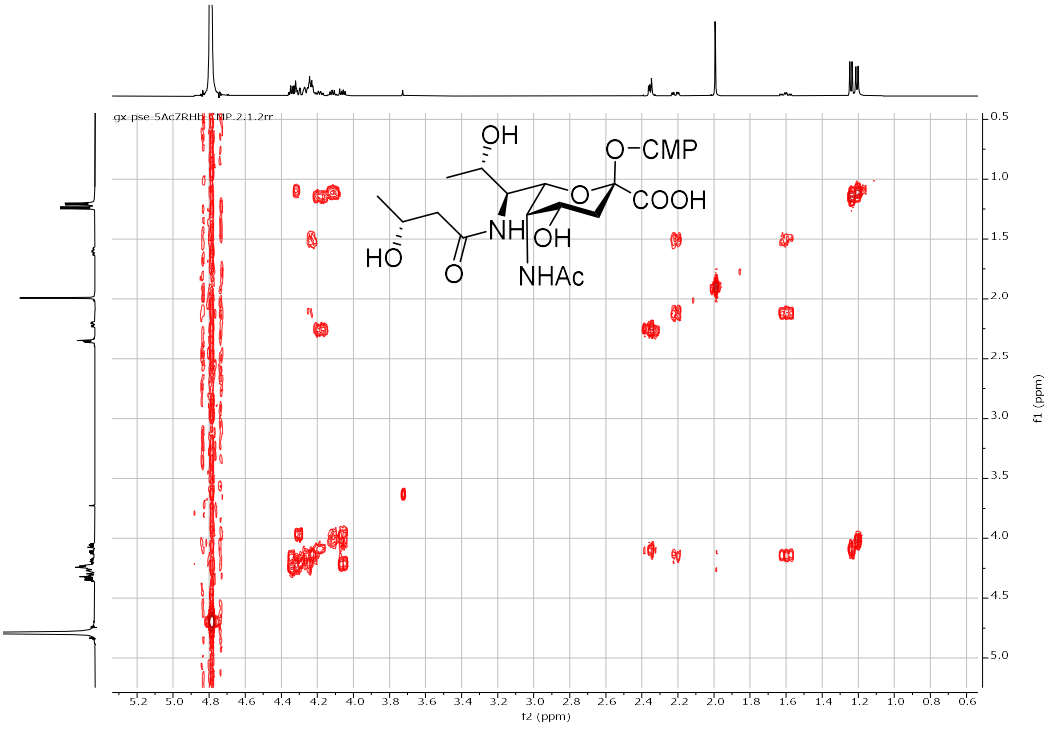
^{1}H NMR of compound CMP-Pse5Ac7Fo 8



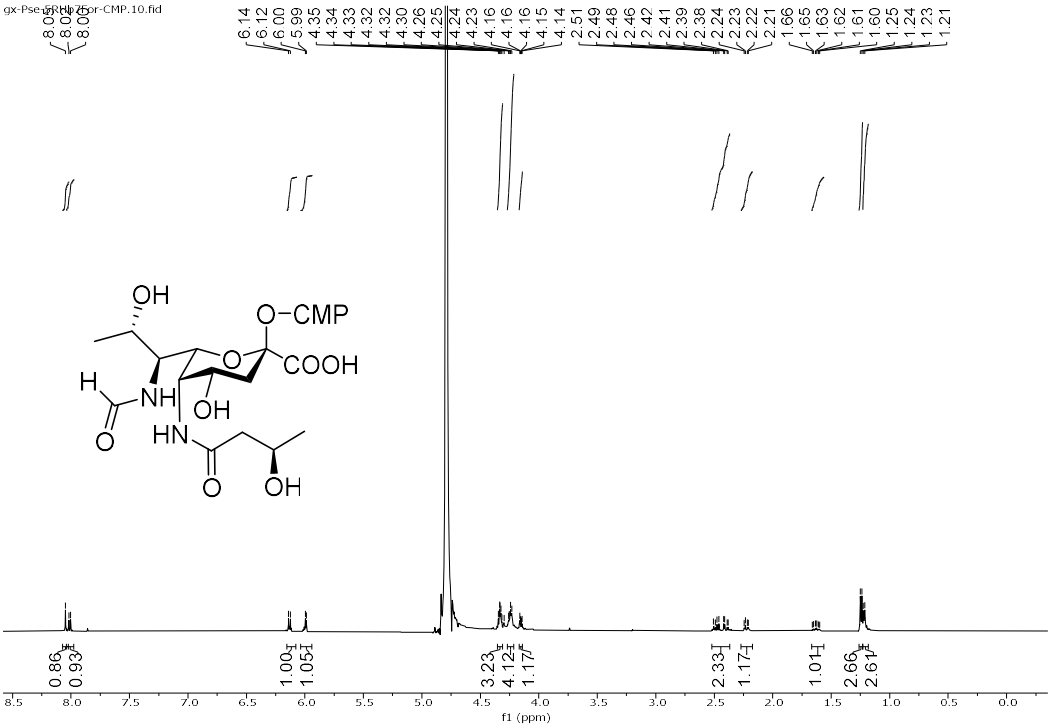
^{1}H NMR of compound CMP-Pse5Ac7(3$_R$Hb) 9



^{1}H - ^{1}H COSY of compound CMP-Pse5Ac7(3$_R$Hb) 9



^{1}H NMR of compound CMP-Pse5(3$_{R}$Hb)7Fo 10



^{1}H - ^{1}H COSY of compound CMP-Pse5(3$_{R}$Hb)7Fo 10