Supporting Information

Development of Oligomeric Mannose-6-phosphonate Conjugates for Targeted Protein Degradation

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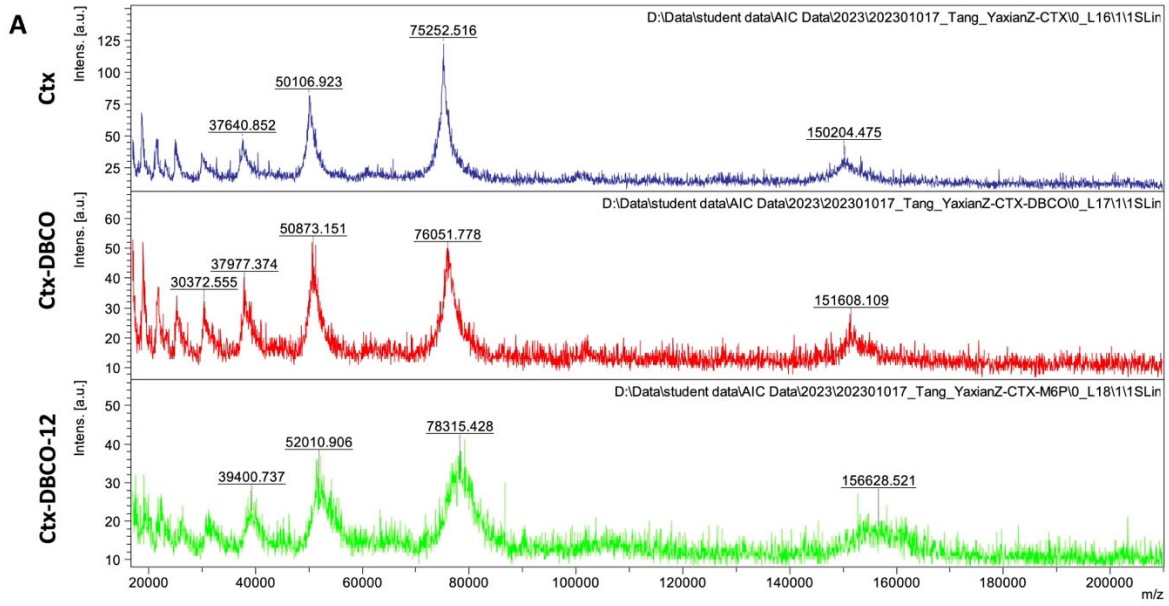
Supplemental Figures

A



B

	M^{2+} (from spectrum) in Da	Mass of conjugate in Da	Mass Difference (Da)	Mass Conjugate (Da)	Number of labeling per antibody
Ctx	75252.52	150505.04	------		-----
Ctx-DBCO	76051.78	152103.56	1598.52	799	3.35
Ctx-DBCO-12	78315.43	157030.86	4927.3	2264	2.04

Figure S1. MALDI-OTF-MS Characterization of antibody-based degrader. **A)** MALDI analysis of Ctx (top), Ctx-DBCO following incorporation of the DBCO motif (middle), and Ctx-DBCO-12 following the incorporation of conjugate 12 on to the DBCO modified Ctx (bottom). **B)** Table showing calculation of conjugation ratio. The first column gives the relevant M^{2+} peaks from the spectrum in A. The second and third column give step-wise calculation to yield number of labeing per antibody in the final column. Analysis showed an average incorporation of 3.35 DBCO per Ctx antibody, and an average incorporation of 2.04 Conjugate 12 per Ctx-DBCO.

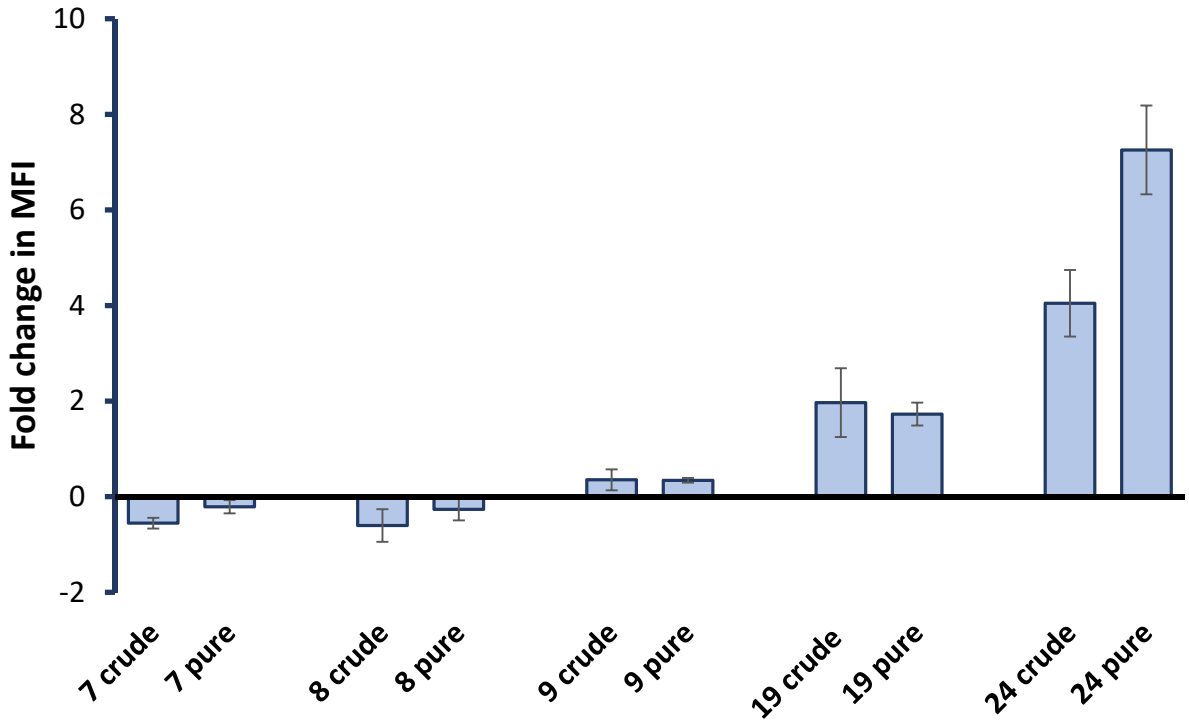


Figure S2. Fold change in MFI for crude and pure conjugates. Each conjugate **7**, **8**, **9**, **19**, and **24** was purified and both the crude and pure versions were tested for uptake using the NA-650 assay. All conjugates except **24** showed similar uptake between crude and pure, with pure **24** showing observable higher uptake compared to crude **24**.

Material and General Methods

Safety Statement

No unexpected or unusually high safety hazards were encountered.

Cell culture

Huh7 cells were cultured in T75 flasks and maintained in low-glucose DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 1% L-glutamine and 1% penicillin/streptomycin under 5 % CO2 at 37 °C.

NeutrAvidin uptake experiments for M6P-biotin

Cells were seeded at 35,000 cells per well in 100µL complete culture media in 96-well cell culture plates. The next day, the medium was replaced followed by the sequential addition of 500 nM NA-650 and 10 uM M6P-biotin. The cells were incubated at 37 °C for 24 h and then washed twice with PBS to removed extracellular NA-650. The uptake in 96-well plate was determined by measuring the fluorescent intensity at 650 nm excitation/680 nm emission using the Synergy H1 microplate reader. Data was acquired using Gen5 software.

Antibody labeling and preparation of Ctx-DBCO-12

To label the antibody with M6P, 100μL of the Cetuximab (Ctx) in PBS was mixed with DBCO-NHS at 1:25 molar ratio and incubated overnight at room temperature on a rotator, followed by filtration with 500μL of PBS for 5 times using 10kDa Amicon Centrifugal Filter. After determining the concentration by BCA assay, the DBCO-tethered Ctx was mixed with 25 equivalence M6P-azido and incubated overnight at room temperature on a rotator, followed by filtration with 500μL of PBS for 5 times using 10kDa Amicon Centrifugal Filter.

MALDI-MS

α-Cyano-4-hydroxycinnamic acid (HCCA) was dissolved in 50% Acetonitrile/H2O to give a 10 mg/mL solution as the matrix solution. The sample was absorbed on Omix C4 pipette tips, washed by 0.1% TFA for three times and then eluted with 20 μL 75% Acetonitrile/H2O. 1 μL sample solution and 1 μL HCCA solution were spotted on the MALDI target plate and mixed thoroughly before the spot was allowed to dry under room temperature. MALDI-MS spectra were acquired on Bruker UltraFlex MALDI-TOF/TOF mass spectrometer operated in linear positive ion mode. Masses were calculated from windowed raw data in Sigmaplot 13.0 by fitting to gaussian curves, with constant baseline as an additional free parameter. Parameter starting values were the default values of the program, and were automatically iterated 200 times to obtain fits. Plots were made in Origin 2020, where high-frequency noise was removed using 100 points windowed FFT filter.

EGFR degradation analysis

Hela were seeded at 1x10^5 cells per well in a 24-well plate. Various concentrations of M6P-bearing Cetuximab as indicated in the figures were added into the well containing 500 μL complete media. Samples were collected at different time points for western blot analysis.

Western blotting

Cells were lysed with 1X RIPA lysis buffer containing 25 mM Tris, pH 7−8, 150 mM NaCl, 0.1% (w/ v) sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1% (v/ v) Triton X-100, protease inhibitor cocktail (Roche, one tablet per 10 mL) and 1 mM phenylmethylsulfonyl fluoride] on ice for 10 min. The lysates were then centrifuged at 14,000G at 4 °C for 15 min and the supernatant was collected followed by measuring the protein concentration using BCA assay. Lysates were adjusted to the equal amount before mixed with the 4x Laemmli Loading Dye and heated at 95−100 °C for 5 min. After cooling down, samples were loaded onto 7.5% SDS−polyacrylamide gel electrophoresis and transferred to PVDF membrane. The membrane was first blocked in 5% (w/v) nonfat milk in the TBS-T washing buffer (137 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween) and then incubated with primary antibodies at 4 °C overnight. After 3 washes with TBST, the membrane was incubated with secondary HRP-linked antibodies for 1 h, and then washed 3 times with TBST. Then the membrane was incubated in the Clarity ECL substrate for 3- 5 min before acquiring the immunoblot by ChemiDoc MP Imaging Systems.

General experimental protocols:

All reactions in non-aqueous media were conducted under a positive pressure of dry argon in glassware that had been dried in oven prior to use unless noted otherwise. Anhydrous solutions

of reaction mixtures were transferred via an oven dried syringe or cannula. All solvents were dried prior to use unless noted otherwise. All reagents were of commercial grade and used as received unless otherwise noted. Thin layer chromatography was performed using precoated silica gel plates (EMD Chemical Inc. 60, F254). Flash column chromatography was performed with silica gel (Silicycle, 40-63μm). Infrared spectra (IR) were obtained on a Bruker Equinox 55 Spectrophotometer. ^{1}H, ^{13}C, and ^{31}P nuclear magnetic resonance spectra (NMR) were obtained on a Varian Unity-Inova 400 MHz or 500 MHz recorded in ppm (δ) downfield of TMS (δ = 0) in $CDCl_3$ unless noted otherwise. Signal splitting patterns were described as singlet (s), doublet (d), triplet (t), quartet (q), quintet (quint), or multiplet (m), with coupling constants (J) in hertz. High resolution mass spectra (HRMS) were performed by Analytical Instrument Center at the School of Pharmacy or Department of Chemistry on an Electron Spray Injection (ESI) mass spectrometer. Optical rotations were measured on a Perkin-Elmer 241 Polarimeter. HPLC purification of peptides and conjugates was done on a Shimadzu LC-8A preparative liquid chromatograph with a XBridge Prep, C18, 5μm, 19x250mm column. LCMS analysis was done using an Agilent Technologies 1290 Infinity II system with an Agilent Zorbax, 3.5μm, C18, 4.6x50mm column. Absorbance was measured at 210nm, 254nm, and 280nm using an Agilent Technologies 6120 Quadrupole mass spectrometer.

General procedure for solid phase peptide synthesis:

All the peptides used for conjugate formation were synthesized using an Fmoc protection strategy. A Rink amide MBHA resin (0.77meq/g; ~125 mg starting for each peptide) was used in all cases, with a 20% piperidine in DMF solution used to cleave Fmoc, and DIC/HOBt chemistry used for all conjugations. Peptides were washed with DCM and DMF in-between all steps, and Ninhydrin testing was done to measure conjugation completion. The consecutive steps were followed: 1) Deprotection of Fmoc using 20% piperidine for 10 minutes, wash with DMF twice, then additional deprotection using piperidine for 10 minutes; 2) Wash with DMF five times, then wash with DCM twice. Check cleavage using Ninhydrin test, then wash twice with DMF; 3) Coupling of the appropriate amino acid was done by dissolving the Fmoc protected amino acid (3eq, 0.277 mmol) in DMF (4mL) with HOBt (6eq, 0.544 mmol) and DIC (6eq, 0.544 mmol), and then shaken at room temperature for 2-5 hours; 4) Wash with DMF twice, then check coupling with Ninhydrin test. Once the main peptide had been synthesized and while still on resin, each was capped with a biotin or azido group. For biotin, the coupling involved dissolving D-biotin (2eq, 0.185 mmol) in DMF (4 mL) with HOBt (6 eq, 0.544 mol), HBTU (6 eq, 0.544 mmol), and DIEA (10 eq, 0.924 mmol) and shaking for 2-5 hours. For azido, the coupling involved dissolving azidoacetic acid NHS ester (3eq, 0.288 mmol) in DMF (4mL) with DIPEA (3.1eq, 0.298 mmol). The Fmoc amino acids used in the synthesis were Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Ser(tBu)-OH, and Fmoc-Cys(Trt)-OH.

General procedure for peptide cleavage from the resin and deprotection

Following completion of peptide synthesis, the peptides were cleaved from the resin and deprotected using a custom cleavage cocktail (TFA/DTT/TIS | 95/2.5/2.5; 4mL total volume). The cleaved peptides were then precipitated into cold ether, centrifuged to pellet, redissolved into water, and lyophilized. Following lyophilization, the peptides were dissolved in a small amount (1-2 mL) of water/acetonitrile and purified via HPLC. The pure peptides were checked via LCMS

(20-100% MeOH, 4 min run) for purity, then redissolved in water and lyophilized again to a powder.

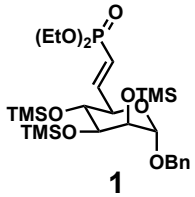
General procedure for thiol-ene conjugation (procedure 1):
The generalized thiol-ene reaction for glycopeptide formation is as follows. Pure deprotected peptide (5 mg) was dissolved in an approximately 0.4 mL mixture of water and methanol (specific ratio determined by each peptide). The synthesized mannose-6-phosphonate, compound 6 (2 eq per cysteine residue on peptide), was then added with DPAP (0.1 eq) to the reaction mixture and the mixture was stirred. The solution was then flushed thoroughly with argon gas (2-3 times), then exposed to UV-365 and encased in foil to increase exposure. After 1 hour, the reaction was checked by LCMS for completion, then filtered via syringe tip, diluted with water, and lyophilized to yield pure glycoconjugate as a white solid.

Purification of Conjugates:
Crude conjugates following lyophilization were purified via HPLC. Following, the pure conjugate was lyophilized and redissolved into a concentration solution and checked by LCMS for purity (20-100% MeOH, 4 min run).

Detailed Synthetic Procedures:

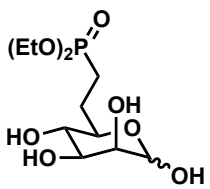
Synthesis of M6Pn and precursors:

Synthesis of 1:
Compound 1 was synthesized in six steps from 1,2,3,4,6-penta-*O*-acetyl-D-mannopyranose using the same conditions previously reported.[1] All characterization data for precursors matched with those found in literature. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26 – 7.09 (m, 5H), 6.80 – 6.64 (m, 1H), 5.99 – 5.84 (m, 1H), 4.59 – 4.44 (m, 2H), 4.34 (d, *J* = 12.2 Hz, 1H), 4.04 – 3.88 (m, 4H), 3.96 (s, 1H), 3.72 – 3.62 (m, 2H), 3.56 (td, *J* = 9.6, 1.3 Hz, 1H), 1.25 – 1.17 (m, 5H), 1.21 – 1.08 (m, 1H), 0.03 (s, 9H), 0.00 (s, 9H), -0.03 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 148.6, 148.5, 137.2, 128.1, 127.3, 127.2, 117.6, 115.8, 99.9, 77.0, 76.9, 76.7, 76.4, 73.2, 72.9, 72.5, 71.1, 68.7, 61.4, 61.3, 16.1, 16.0, 0.4, 0.3, 0.0.



1

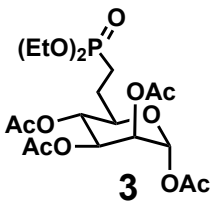
Synthesis of 2:
Compound 1 (2.1 mmol, 1.3 g) was dissolved in methanol (52 mL) and flushed with argon. Following, Palladium 10 wt.% (dry) on carbon powder (wet) catalyst (1.3 g, 1:1 starting material mass) and glacial acetic acid (20 eq, 2.4 mL) was added to the reaction mixture. The mixture was then cycled between vacuum and flushing with H_2 gas several times before leaving the reaction under H_2 gas for 24 hr. The reaction mixture was then filtered over celite, and the filtrate was concentrated under vacuum. The crude product was then purified via flash



2

column chromatography (DCM: MeOH | 10:1 → 5:1 → 1:1) to yield **2** as a light yellow oil in 73% yield (1.5 mmol, 480 mg, anomeric mixture). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.94 (s, 1H), 5.17 (s, 1H), 4.97 (s, 3H), 4.87 (s, 0H), 4.79 – 4.69 (m, 1H), 4.08 (pq, *J* = 7.3, 2.3 Hz, 5H), 3.96 (s, 1H), 3.89 – 3.82 (m, 1H), 3.75 (s, 1H), 3.54 (s, 1H), 3.18 (s, 1H), 3.12 (s, 1H), 2.16 – 2.04 (m, 2H), 1.31 (t, *J* = 7.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 94.4, 77.4, 77.2, 77.0, 76.7, 71.7, 71.4, 71.3, 70.8, 70.1, 62.1, 62.0, 53.4, 24.14, 21.6, 20.2, 16.4.

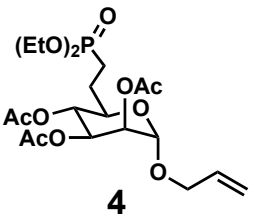
Synthesis of 3:



3

Compound 2 (0.23 mmol, 71 mg) was dissolved in ethyl acetate (15 mL) and placed under argon gas while stirring. Next, pyridine (10 eq, 0.18 mL) and acetic anhydride (5 eq, 0.11 mL) were added, followed by DMAP (7.3 mg). The reaction mixture was then stirred and monitored for 2 hours. The reaction mixture was then diluted with ethyl acetate up to 50 mL, then washed twice with 50 mL of a 1 M HCl solution and once with 50 mL of a brine solution. The resulting solution was then dried with magnesium sulfate and concentrated to yield **3** (0.21 mmol, 101 mg) in 93% yield. The crude product was used directly with no further purification. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.85 (d, *J* = 1.9 Hz, 1H), 5.15 (dd, *J* = 10.0, 3.5 Hz, 1H), 5.09 (dd, *J* = 3.5, 1.9 Hz, 1H), 5.03 – 4.89 (m, 1H), 4.01 – 3.83 (m, 4H), 3.73 (ddd, *J* = 10.3, 8.1, 2.6 Hz, 1H), 2.06 (d, *J* = 6.1 Hz, 1H), 2.00 (s, 4H), 1.96 – 1.86 (m, 6H), 1.84 (d, *J* = 2.1 Hz, 3H), 1.83 – 1.47 (m, 2H), 1.15 (t, *J* = 7.0 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 169.8, 168.3, 90.5, 90.4, 77.4, 77.2, 77.0, 76.7, 71.5, 71.3, 70.9, 68.9, 68.6, 68.5, 68.4, 61.8, 61.7, 61.6, 46.7, 24.2, 21.4, 20.9, 20.8, 20.7, 20.6, 19.9, 16.5, 16.4, 8.7.

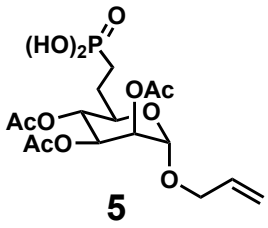
Synthesis of 4*:*



4

Compound 3 (0.22 mmol, 105 mg) was dissolved in dry DCM (1.5 mL) with allyl alcohol (2 eq, 0.03 mL). The mixture was then flushed with argon gas three times, then placed in an ice bath and brought to 0 °C while stirring. While still on the ice bath, BF_3•OEt_2 (5 eq, 0.13 mL) was added dropwise. Following, the reaction mixture was brought to room temperature and monitored for 22.5 hours. The reaction mixture was then dried under vacuum before diluting with 50 mL of ethyl acetate, then washed 50 mL of a sodium bicarb solution three times. The resulting solution was dried with magnesium sulfate, then dried again under vacuum. The crude product was purified via flash column chromatography (DCM: MeOH | 1:0 → 25:1) to yield **4** (0.18 mmol, 56 mg) as a clear oil in 54% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.81 (ddt, *J* = 15.5, 10.9, 5.6 Hz, 1H), 5.33 – 5.12 (m, 5H), 5.03 (t, *J* = 9.9 Hz, 1H), 4.73 (d, *J* = 1.7 Hz, 1H), 4.12 – 3.99 (m, 5H), 3.92 (ddt, *J* = 12.9, 6.2, 1.5 Hz, 1H), 3.77 – 3.67 (m, 1H), 2.08 (s, 3H), 1.98 (s, 3H), 1.92 (s, 3H), 1.89 – 1.76 (m, 1H), 1.76 – 1.58 (m, 2H), 1.26 (td, *J* = 7.1, 1.1 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 133.0, 118.2, 96.4, 77.3, 77.2, 77.0, 76.7, 69.8, 69.7, 69.5, 69.2, 68.4, 61.7, 29.7, 24.4, 20.9, 20.8, 20.7, 16.5.

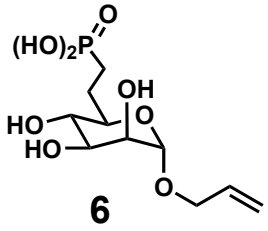
Synthesis of 5:



5

Compound 4 (0.092 mmol, 44 mg) was dissolved in dry acetonitrile (0.5 mL) with pyridine (18 eq, 0.13 mL). The reaction mixture was then placed under argon gas, following which TMSBr (10 eq, 0.12 mL) was added dropwise. The reaction mixture was then stirred and monitored for 1.5 hours. Following, the mixture was diluted with methanol (0.5 mL) and stirred for 30 min. The solution was then dried under vacuum, and the resulting powder was dissolved in ethyl acetate and sonicated to ensure dissolution. The mixture was then vacuum filtered, and the filtrate was dried under vacuum to yield compound **5**(0.083 mmol, 35 mg) as a white powder in 90% yield. ^{1}H NMR (400 MHz, MeOD) δ 5.96 (dddd, *J* = 17.3, 10.4, 6.1, 5.3 Hz, 1H), 5.33 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.28 – 5.18 (m, 3H), 5.08 (t, *J* = 9.7 Hz, 1H), 4.85 (d, *J* = 1.4 Hz, 1H), 4.22 (ddt, *J* = 12.9, 5.2, 1.5 Hz, 1H), 4.13 – 4.00 (m, 1H), 3.84 (ddd, *J* = 10.0, 8.0, 2.4 Hz, 1H), 2.12 (s, 3H), 2.05 (s, 3H), 1.95 (s, 3H), 2.01 – 1.84 (m, 1H), 1.79 – 1.66 (m, 1H), 1.40 – 1.19 (m, 2H), 0.98 – 0.86 (m, 1H). ^{13}C NMR (101 MHz, MeOD) δ 170.3, 170.2, 146.7, 139.8, 133.4, 116.7, 96.4, 69.7, 69.6, 69.5, 68.9, 68.0, 48.2, 48.0, 47.8, 47.6, 47.4, 47.2, 47.0, 24.7, 19.2.

Synthesis of 6:

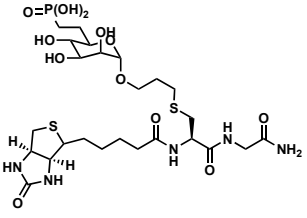


6

Compound 5 (0.068 mmol, 29 mg) was dissolved in methanol (0.5 mL). To this, a 0.5 M solution of NaOMe in methanol was added until the pH of the mixture was approximately 10 (approximately 0.3 mL of the solution was added). The reaction mixture was stirred and monitored for 2 hours. Following, the reaction solution was diluted with methanol (5 mL) and neutralized using a Dowex-50 resin, then filtered via vacuum twice and dried under vacuum. The resulting solid was then dissolved in water and lyophilized to yield **6** (0.067 mmol, 20 mg) as a golden oil in quantitative yield. Optical rotation: $[\alpha]_D^{21}$ = +57.2 (c = 0.40, MeOH). ^{1}H NMR (400 MHz, MeOD) δ 5.93 (ddd, *J* = 22.4, 10.7, 5.5 Hz, 1H), 5.29 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.17 (dd, *J* = 10.4, 1.6 Hz, 1H), 4.75 (d, *J* = 1.6 Hz, 1H), 4.17 (ddt, *J* = 13.1, 5.1, 1.5 Hz, 1H), 3.98 (ddt, *J* = 13.1, 5.9, 1.4 Hz, 1H), 3.81 (dd, *J* = 3.4, 1.7 Hz, 1H), 3.66 (dd, *J* = 9.0, 3.4 Hz, 1H), 3.49 – 3.43 (m, 2H), 3.34 (s, 1H), 2.23 – 1.93 (m, 3H), 1.84 – 1.63 (m, 3H). ^{13}C NMR (101 MHz, MeOD) δ 134.1, 115.9, 99.3, 72.1, 71.9, 71.1, 70.8, 70.6, 67.5, 48.5, 48.2, 48.0, 47.8, 47.6, 47.4, 47.2, 47.0, 24.7, 23.4, 22.0. ^{31}P NMR (MeOD, 162 MHz): δ 30.7. HRMS (ESI) for $C_{10}H_{19}O_8P$ $[M+H]^+$ 299.0890 (Calc.), found 299.0890. IR (neat): ν 3165, 2355, 2347, 2325, 1725, 1691, 1658, 1630, 1549, 1513, 989.

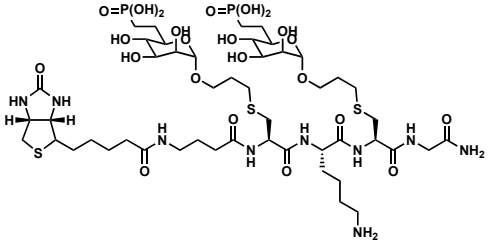
Synthesis of conjugates:

Synthesis of 7:



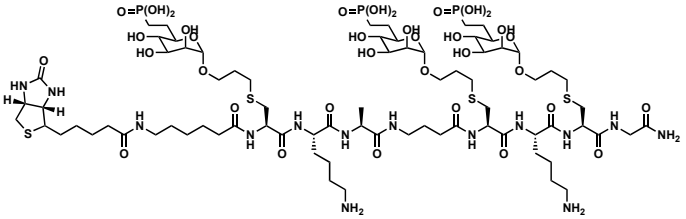
Compound 7 (0.011 mmol, 7.01 mg) was obtained in 71% purity using procedure 1 with a methanol to water solvent ratio of 2:1. LCMS: Rt= 1.25 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{25}H_{44}N_5O_{12}PS_2$ $[M+H]^+$ 702.2238 (Calc.), found 702.2246. Further purification was done using the outlined purification of conjugates.

Synthesis of 8:



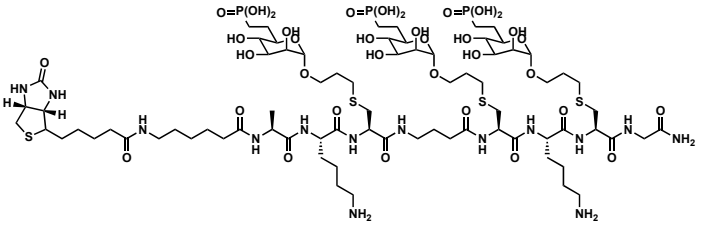
Compound 8 (0.0081 mmol, 13.2 mg) was obtained in 76% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.28 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{48}H_{87}N_9O_{23}P_2S_3$ $[M+H]^+$ 1316.4625 (Calc.), found 1316.4662. Further purification was done using the outlined purification of conjugates.

Synthesis of 9:



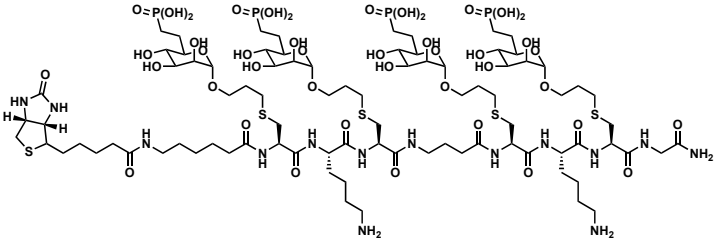
Compound 9 (0.0051 mmol, 10.2 mg) was obtained in 80% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.66 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{76}H_{139}N_{14}O_{35}P_3S_4$ $[M+2H]^{2+}$ 1015.3884 (Calc.), found 1015.3928. Further purification was done using the outlined purification of conjugates.

Synthesis of 10:



Compound 10 (0.0042 mmol, 8.1 mg) was obtained in 79% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.70 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{76}H_{139}N_{14}O_{35}P_3S_4$ $[M+2H]^{2+}$ 1015.3884 (Calc.), found 1015.3921.

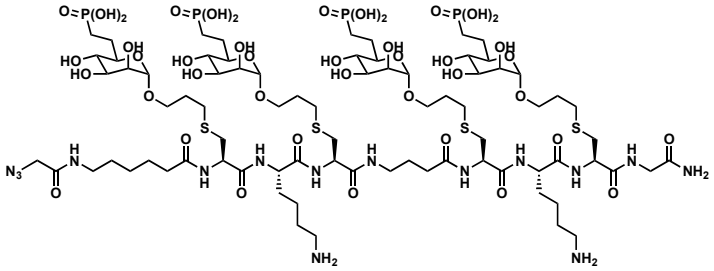
Synthesis of 11:



Compound 11 (0.0035 mmol, 8.3 mg) was obtained in 81% purity using procedure 1 with a methanol to water solvent ratio of 2:1. LCMS: Rt= 1.68 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{86}H_{158}N_{14}O_{43}P_4S_5$ $[M+3H]^{3+}$ 787.2793 (Calc.), found 787.2806.

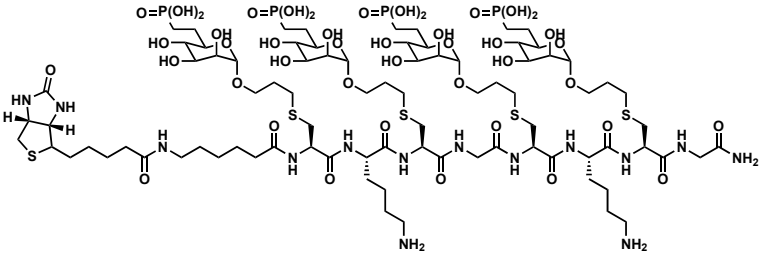
Synthesis of 12:

Compound 12 (0.0049 mmol, 11.1 mg) was obtained in 80% purity using procedure 1 with a methanol to water solvent ratio of 2:1. LCMS: Rt= 1.69 (20-100% MeOH, 5 min run); HRMS (ESI) for $C_{78}H_{145}N_{15}O_{42}P_4S_4$ $[M+2H]^{2+}$ 1108.8825 (Calc.), found 1108.8829. Further purification was done using the outlined purification of conjugates.



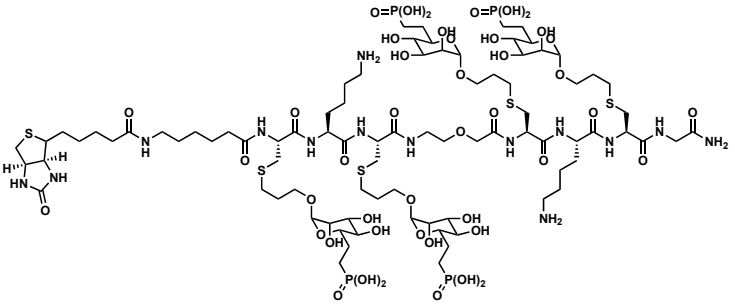
Synthesis of 13:

Compound 13 (0.0045 mmol, 10.5 mg) was obtained in 78% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.67 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{84}H_{154}N_{14}O_{43}P_4S_5$ $[M+2H]^{2+}$ 1166.3997 (Calc.), found 1166.4015.



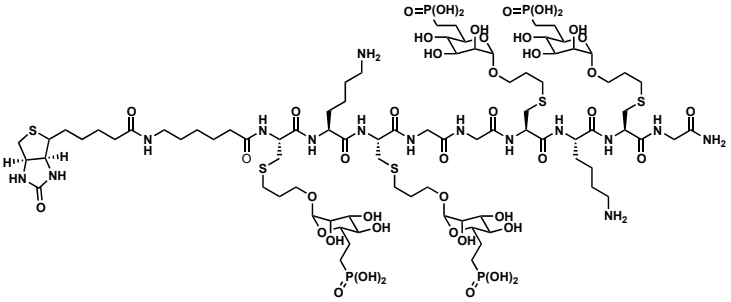
Synthesis of 14:

Compound 14 (0.0036 mmol, 8.6 mg) was obtained in 84% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.66 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{86}H_{158}N_{14}O_{44}P_4S_5$ $[M+3H]^{3+}$ 792.6110 (Calc.), found 792.6119.



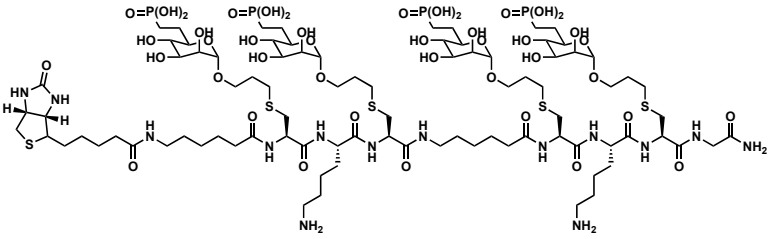
Synthesis of 15:

Compound 15 (0.0042 mmol, 10.0 mg) was obtained in 81% purity using procedure 1 with a methanol to water solvent ratio of 2:1. LCMS: Rt= 2.15 (20-100% MeOH, 5 min run); HRMS (ESI) for $C_{86}H_{157}N_{15}O_{44}P_4S_5$ $[M+3H]^{3+}$ 796.9427 (Calc.), found 796.9447.



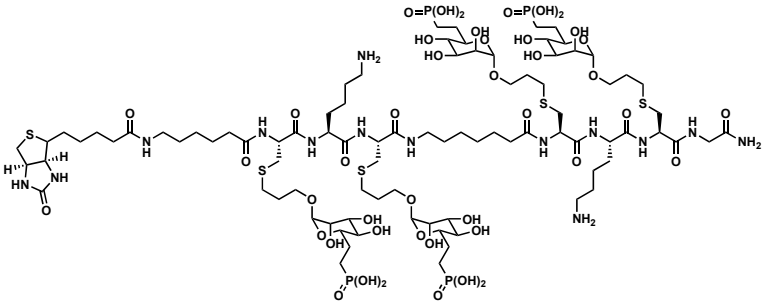
Synthesis of 16:

Compound 16 (0.0042 mmol, 10.0 mg) was obtained in 82% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.73 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{88}H_{162}N_{14}O_{43}P_4S_5$ $[M+2H]^{2+}$ 1194.4310 (Calc.), found 1194.4356.



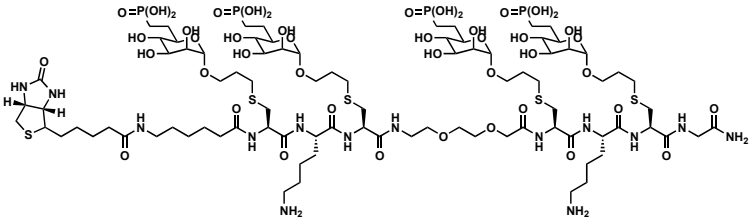
Synthesis of 17:

Compound 17 (0.0043 mmol, 10.3 mg) was obtained in 79% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.88 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{89}H_{164}N_{14}O_{43}P_4S_5$ $[M+2H]^{2+}$ 1201.4388 (Calc.), found 1201.4430.



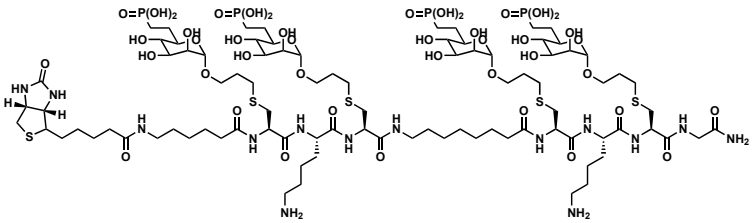
Synthesis of 18:

Compound 18 (0.0076 mmol, 18.4 mg) was obtained in 84% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 1.72 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{88}H_{162}N_{14}O_{45}P_4S_5$ $[M+2H]^{2+}$ 1210.4259 (Calc.), found 1210.4294.



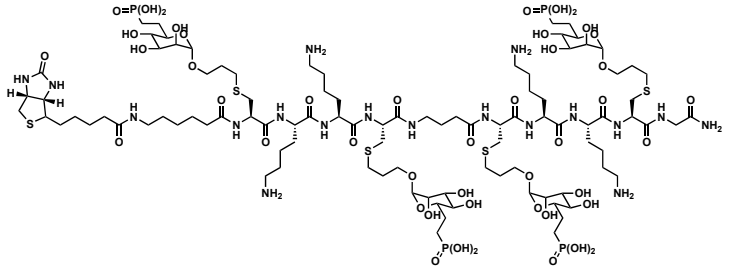
Synthesis of 19:

Compound 19 (0.0047 mmol, 11.4 mg) was obtained in 74% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 2.04 (20-100% MeOH, 4 min run); HRMS (ESI) for $C_{90}H_{166}N_{14}O_{43}P_4S_5$ $[M+2H]^{2+}$ 1208.4466 (Calc.), found 1208.4514. Further purification was done using the outlined purification of conjugates.



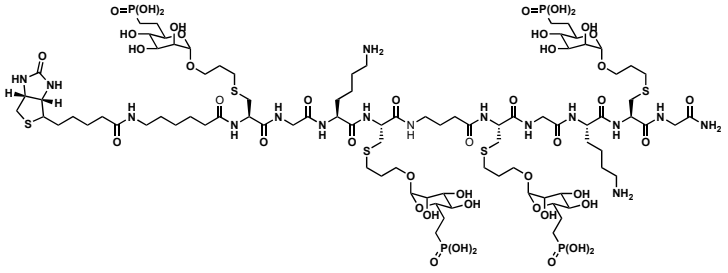
Synthesis of 20:

Compound 20 (0.0035 mmol, 9.2 mg) was obtained in 78% purity using procedure 1 with a methanol to water solvent ratio of 2:1. LCMS: Rt= 2.05 (20-100% MeOH, 5 min run); HRMS (ESI) for $C_{98}H_{182}N_{18}O_{45}P_4S_5$ $[M+3H]^{3+}$ 872.6760 (Calc.), found 872.6797.



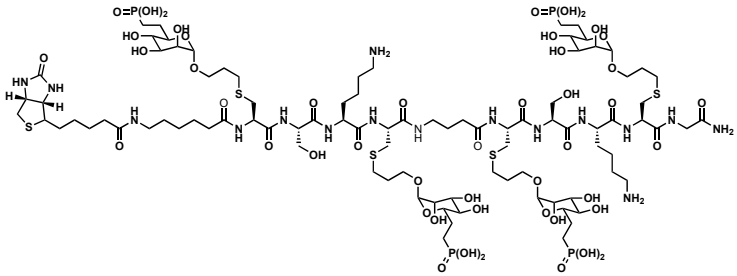
Synthesis of 21:

Compound 21 (0.0039 mmol, 9.7 mg) was obtained in 76% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 2.24 (20-100% MeOH, 5 min run); HRMS (ESI) for $C_{90}H_{164}N_{16}O_{45}P_4S_5$ $[M+3H]^{3+}$ 825.2936 (Calc.), found 825.2970.



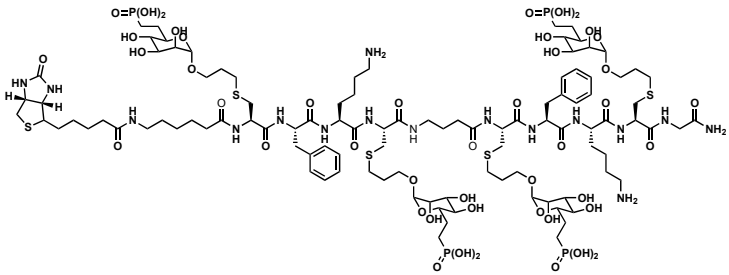
Synthesis of 22:

Compound 22 (0.0037 mmol, 9.4 mg) was obtained in 79% purity using procedure 1 with a methanol to water solvent ratio of 3:1. LCMS: Rt= 2.21 (20-100% MeOH, 5 min run); HRMS (ESI) for $C_{92}H_{168}N_{16}O_{47}P_4S_5$ $[M+3H]^{3+}$ 845.3007 (Calc.), found 845.3032.

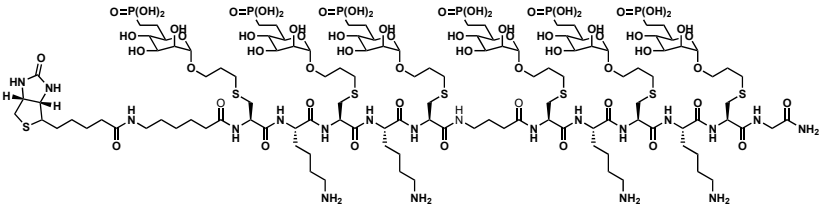


Synthesis of 23:

Compound 23 (0.0034 mmol, 9.0 mg) was obtained in 83% purity using procedure 1 with a methanol to water solvent ratio of 1:1. LCMS: Rt= 2.87 (20-100% MeOH, 5 min run); HRMS (ESI) for $C_{104}H_{176}N_{16}O_{45}P_4S_5$ $[M+3H]^{3+}$ 885.3250 (Calc.), found 885.3274.



Synthesis of 24:



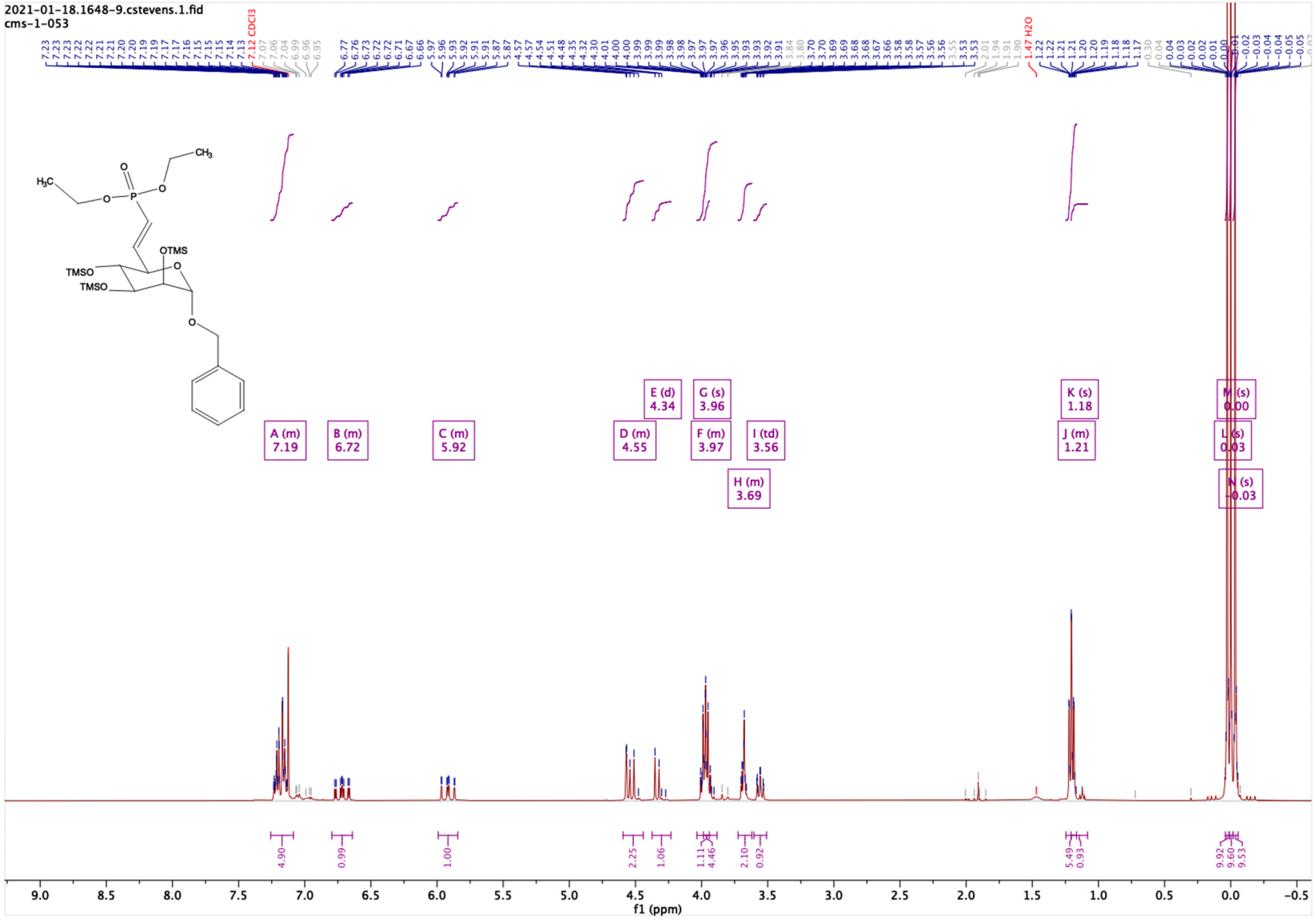
Compound 24 (0.0031 mmol, 10.6 mg) was obtained in 72% purity using procedure 1 with a methanol to water solvent ratio of 3:1. LCMS: Rt= 1.60 (20-100% MeOH, 5 min run); HRMS (ESI) for $C_{124}H_{230}N_{20}O_{63}P_6S_7$ $[M+4H]^{4+}$ 855.3043 (Calc.), found 855.3062. Further purification was done using the outlined purification of conjugates.

References

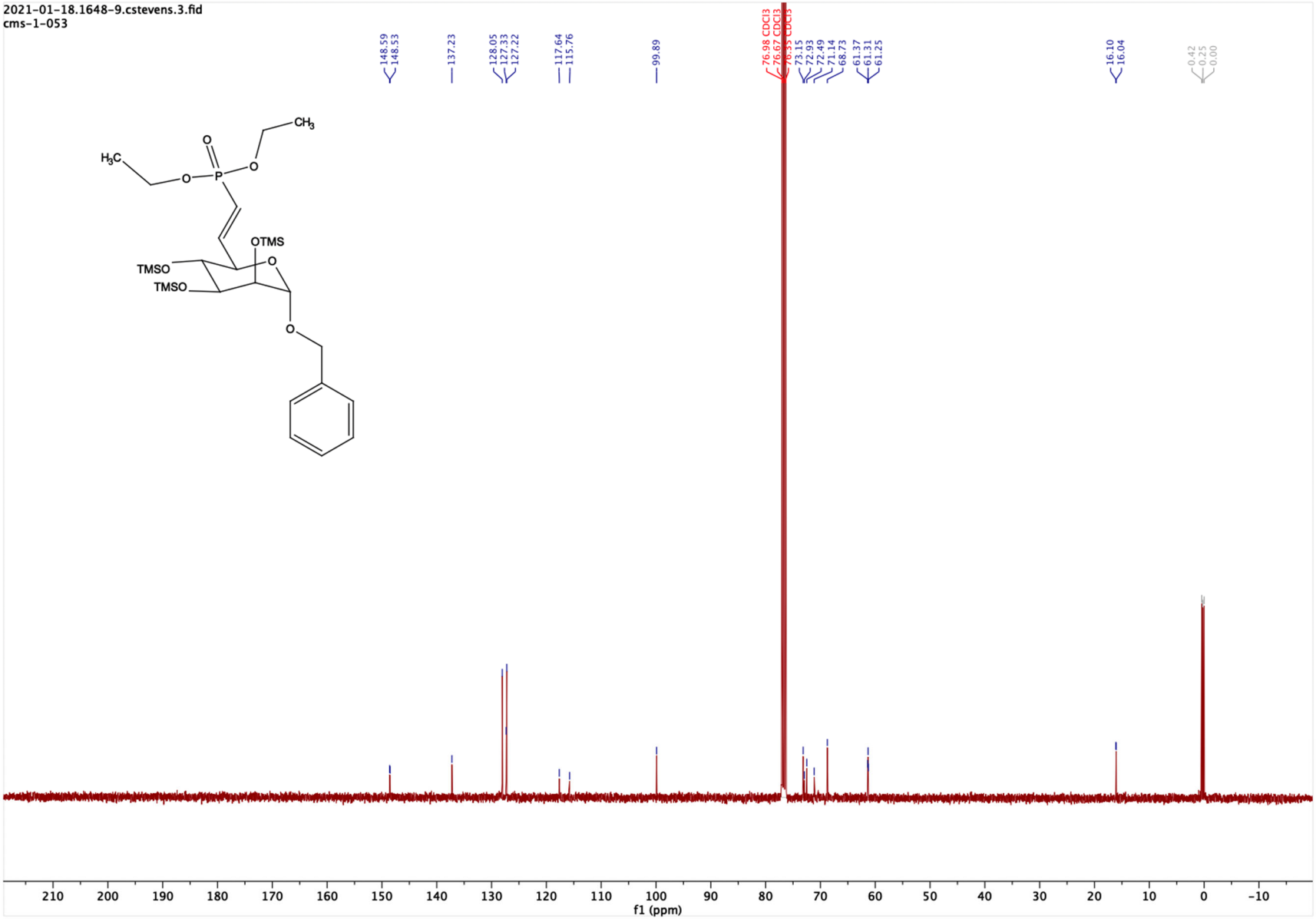
[1] S. M. Banik, K. Pedram, S. Wisnovsky, G. Ahn, N. M. Riley, C. R. Bertozzi, *Nature* **2020**, *584*, 291–297.

Spectra

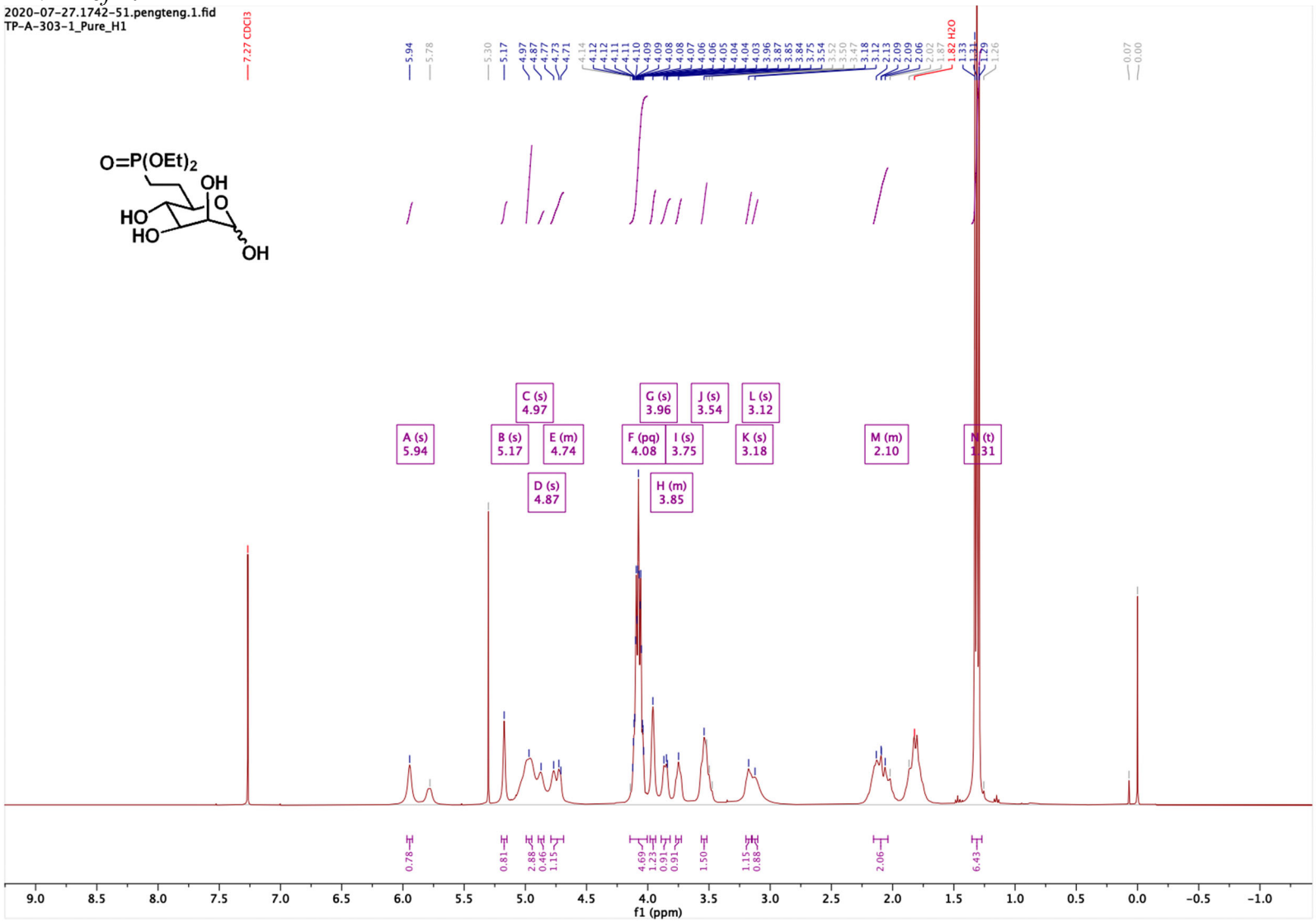
^{1}H NMR of ***1:***



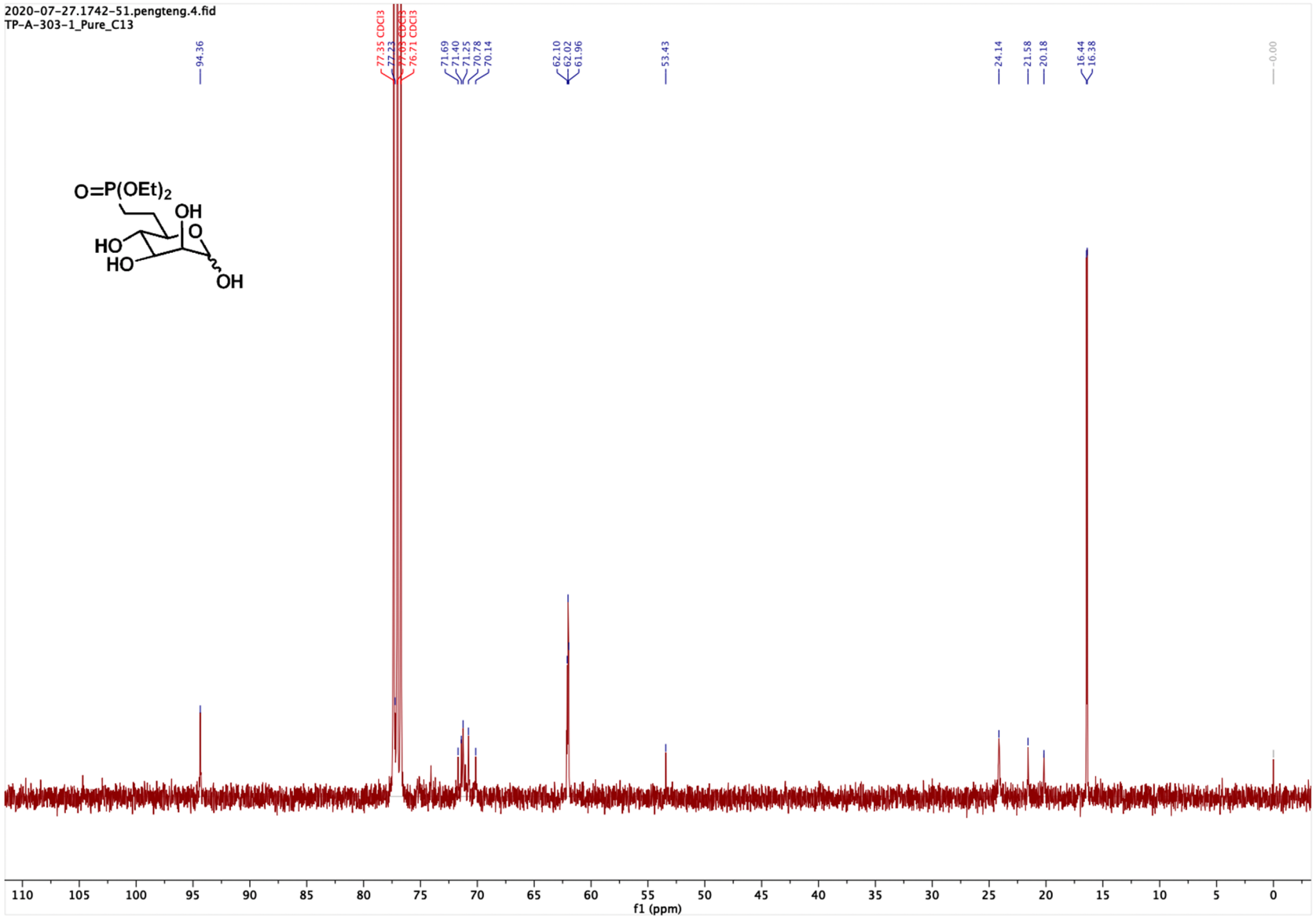
*^{13}C NMR of **1**:*


2021-01-18.1648-9.cstevens.3.fid
cms-1-053
148.59
148.53
137.23
128.05
127.33
127.22
117.64
115.76
99.89
76.98 CDCl3
76.67 CDCl3
76.35 CDCl3
73.15
72.93
72.49
71.14
68.73
61.37
61.31
61.25
16.10
16.04
0.42
0.25
0.00
OTMS
TMSO
TMSO
f1 (ppm)

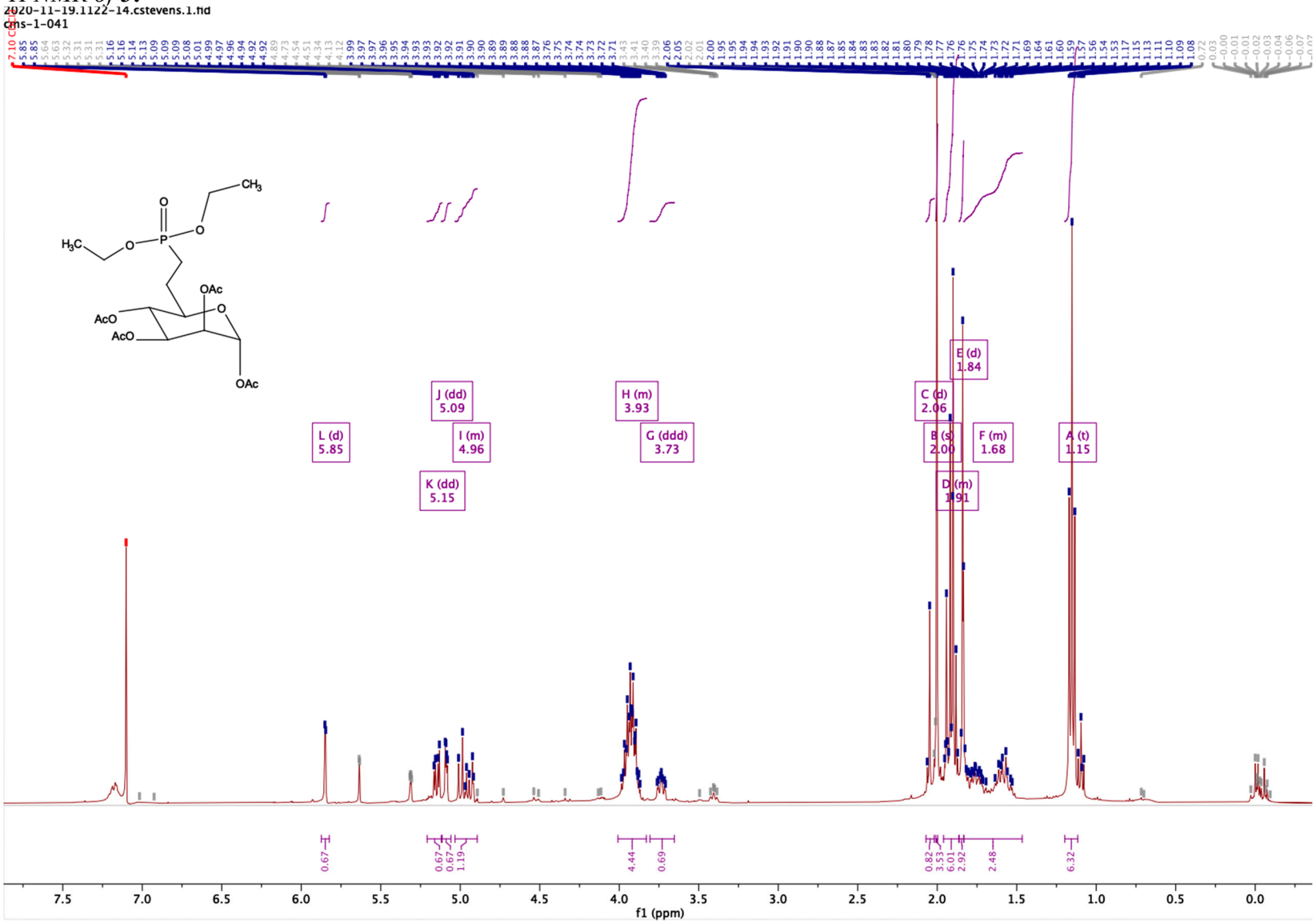
^{1}H NMR of ***2*****:**


2020-07-27.1742-51.pengteng.1.fid
TP-A-303-1_Pure_H1
7.27 CDCl3
1.82 H2O
O=P(OEt)2
OH
HO
HO
O
OH
A (s) 5.94
B (s) 5.17
C (s) 4.97
D (s) 4.87
E (m) 4.74
F (pq) 4.08
G (s) 3.96
H (m) 3.85
I (s) 3.75
J (s) 3.54
K (s) 3.18
L (s) 3.12
M (m) 2.10
N (t) 1.31
f1 (ppm)

*^{13}C NMR of **2**:*


2020-07-27.1742-51.pengteng.4.fid
TP-A-303-1_Pure_C13
94.36
77.35 CDCl3
77.23
77.03 CDCl3
76.71 CDCl3
71.69
71.40
71.25
70.78
70.14
62.10
62.02
61.96
53.43
24.14
21.58
20.18
16.44
16.38
0.00
O=P(OEt)2
OH
O
HO
HO
OH
f1 (ppm)

^{1}H NMR of ***3:***


2020-11-19.1122-14.cstevens.1.fid
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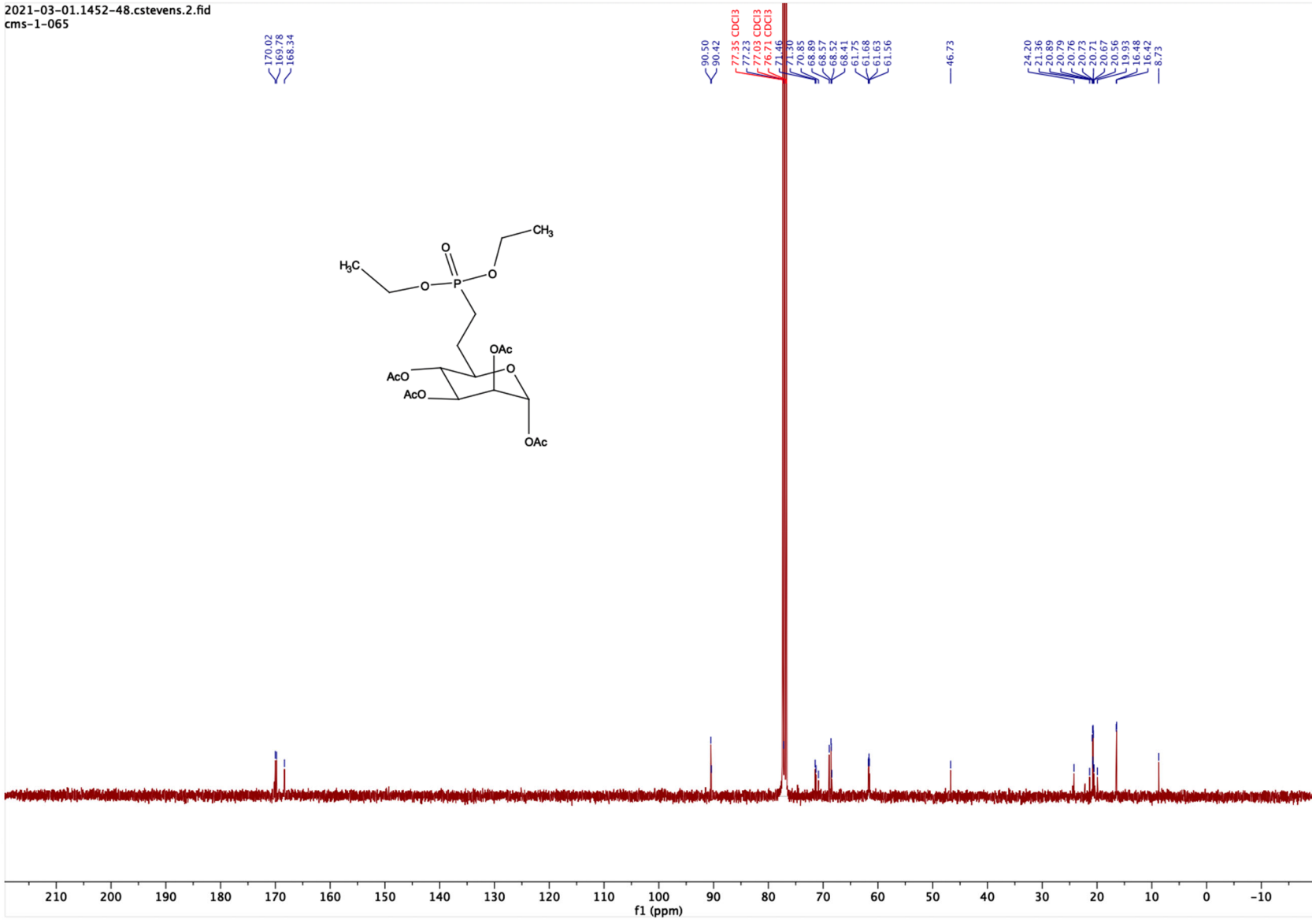
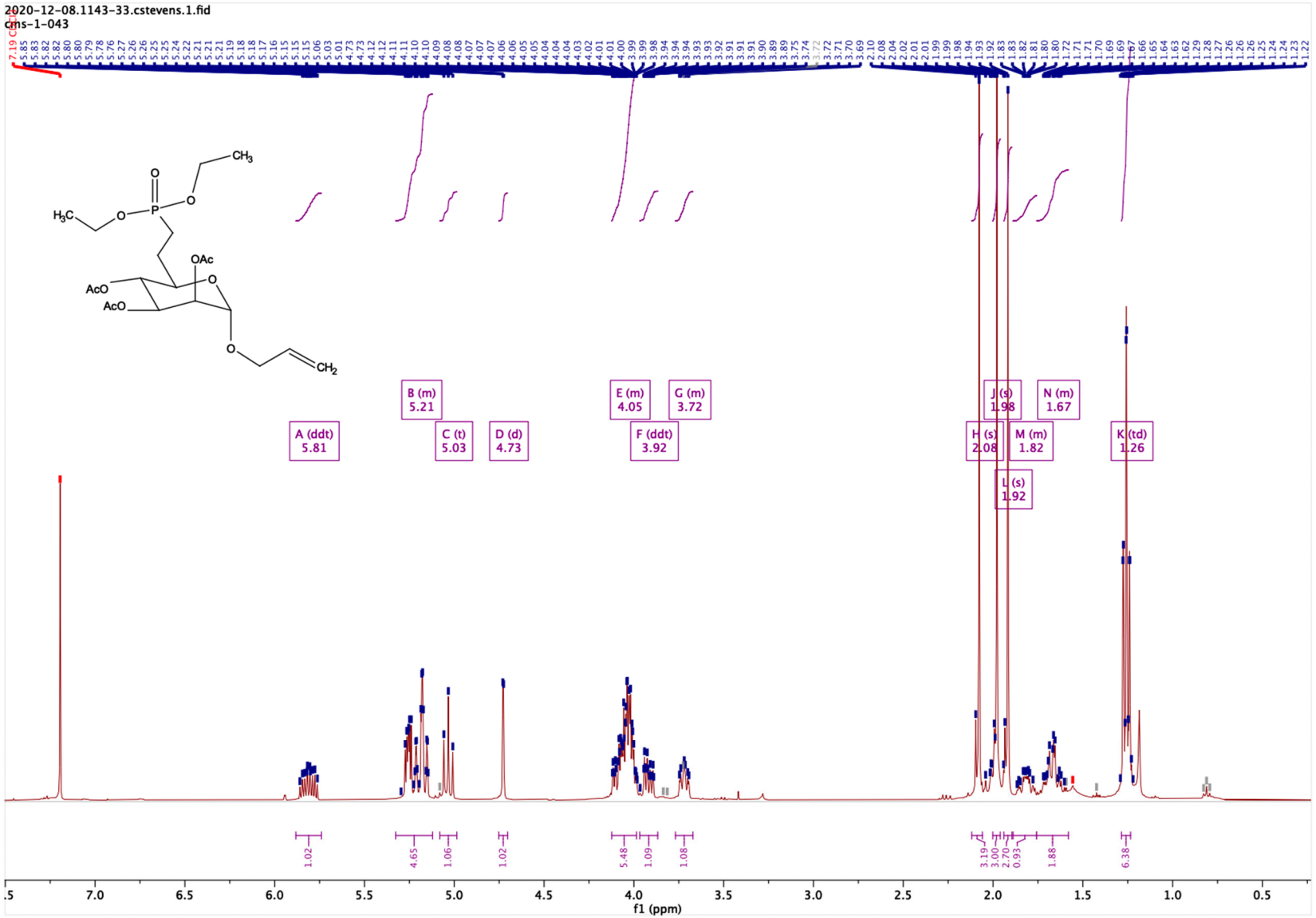
L (d) 5.85
J (dd) 5.09
I (m) 4.96
K (dd) 5.15
H (m) 3.93
G (ddd) 3.73
E (d) 1.84
C (d) 2.06
B (s) 2.00
F (m) 1.68
D (m) 1.91
A (t) 1.15
f1 (ppm)

*^{13}C NMR of **3**:*


2021-03-01.1452-48.cstevens.2.fid
cms-1-065
170.02
169.78
168.34
90.50
90.42
77.35 CDCl3
77.23
77.03 CDCl3
76.71 CDCl3
71.46
71.30
70.85
68.89
68.57
68.52
68.41
61.75
61.68
61.63
61.56
46.73
24.20
21.36
20.89
20.79
20.76
20.73
20.71
20.67
20.56
19.93
16.48
16.42
8.73
CH3
H3C
O
O
O
P
O
OAc
AcO
O
AcO
OAc
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)

[1]H NMR of ***4:***


2020-12-08.1143-33.cstevens.1.fid
cms-1-043
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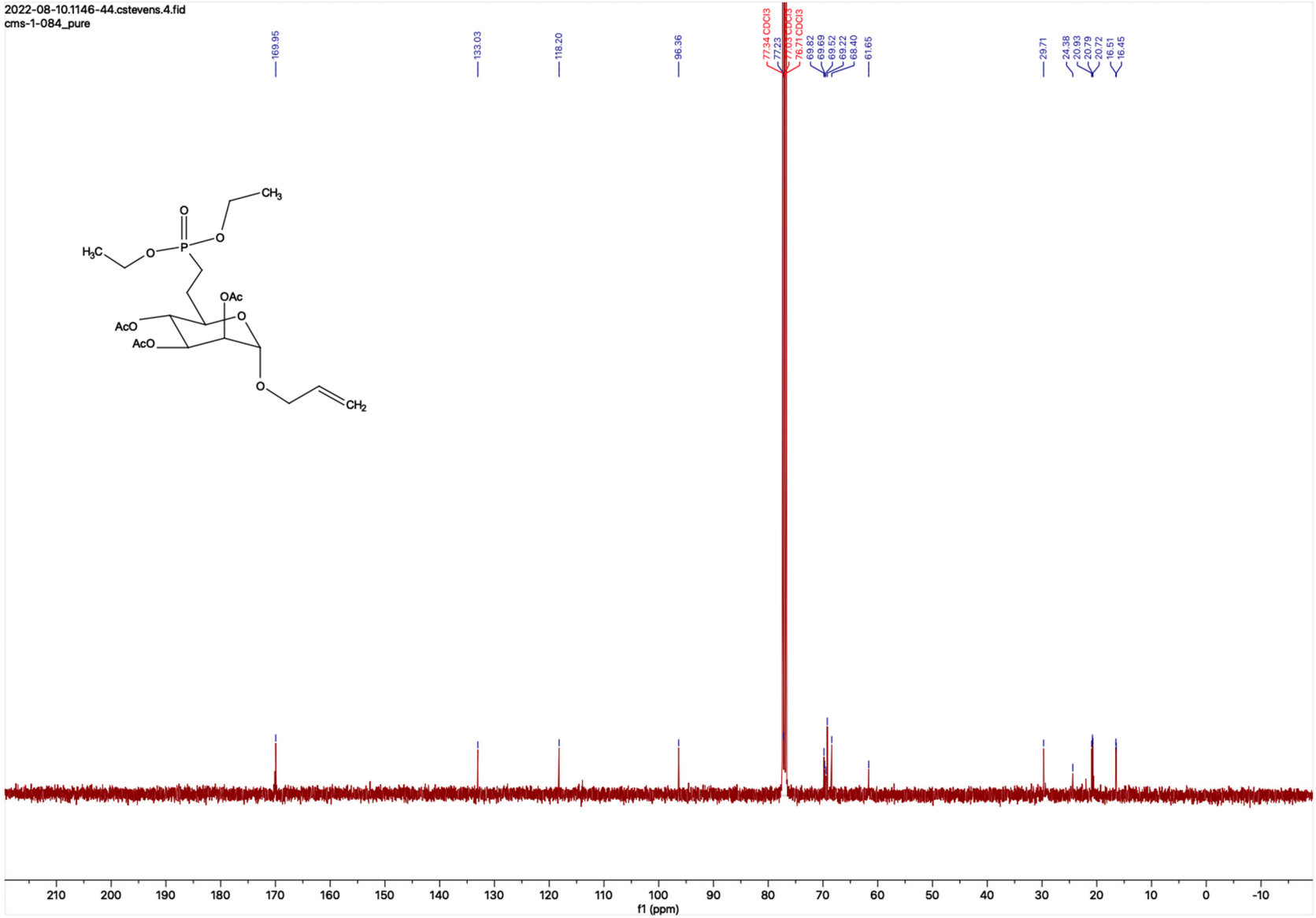
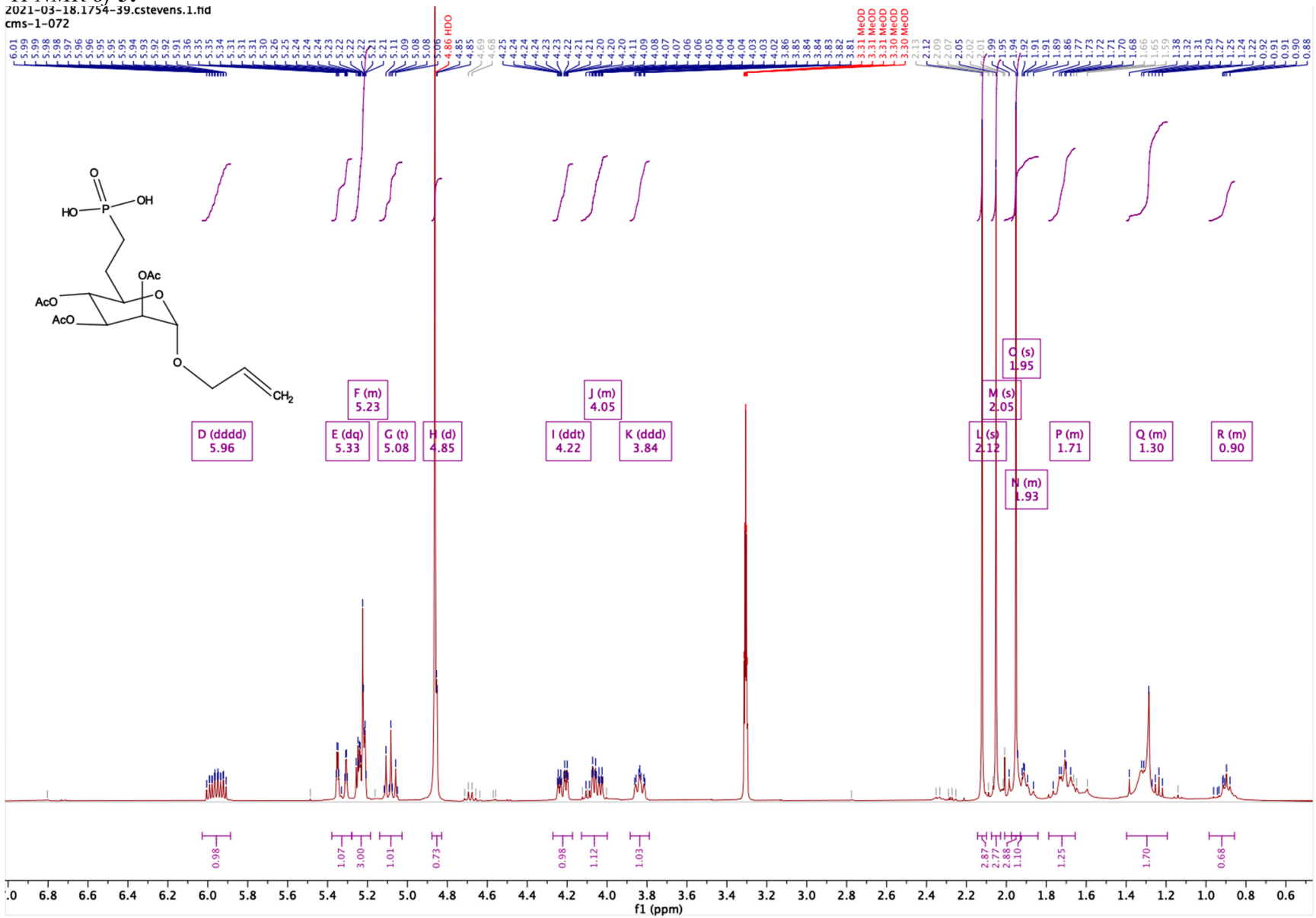
B (m) 5.21
C (t) 5.03
D (d) 4.73
E (m) 4.05
F (ddt) 3.92
G (m) 3.72
H (s) 2.08
I (s) 1.92
J (s) 1.98
K (td) 1.26
L (s) 1.92
M (m) 1.82
N (m) 1.67
1.02
4.65
1.06
1.02
5.48
1.09
1.08
3.19
3.00
2.70
0.93
1.88
6.38
f1 (ppm)

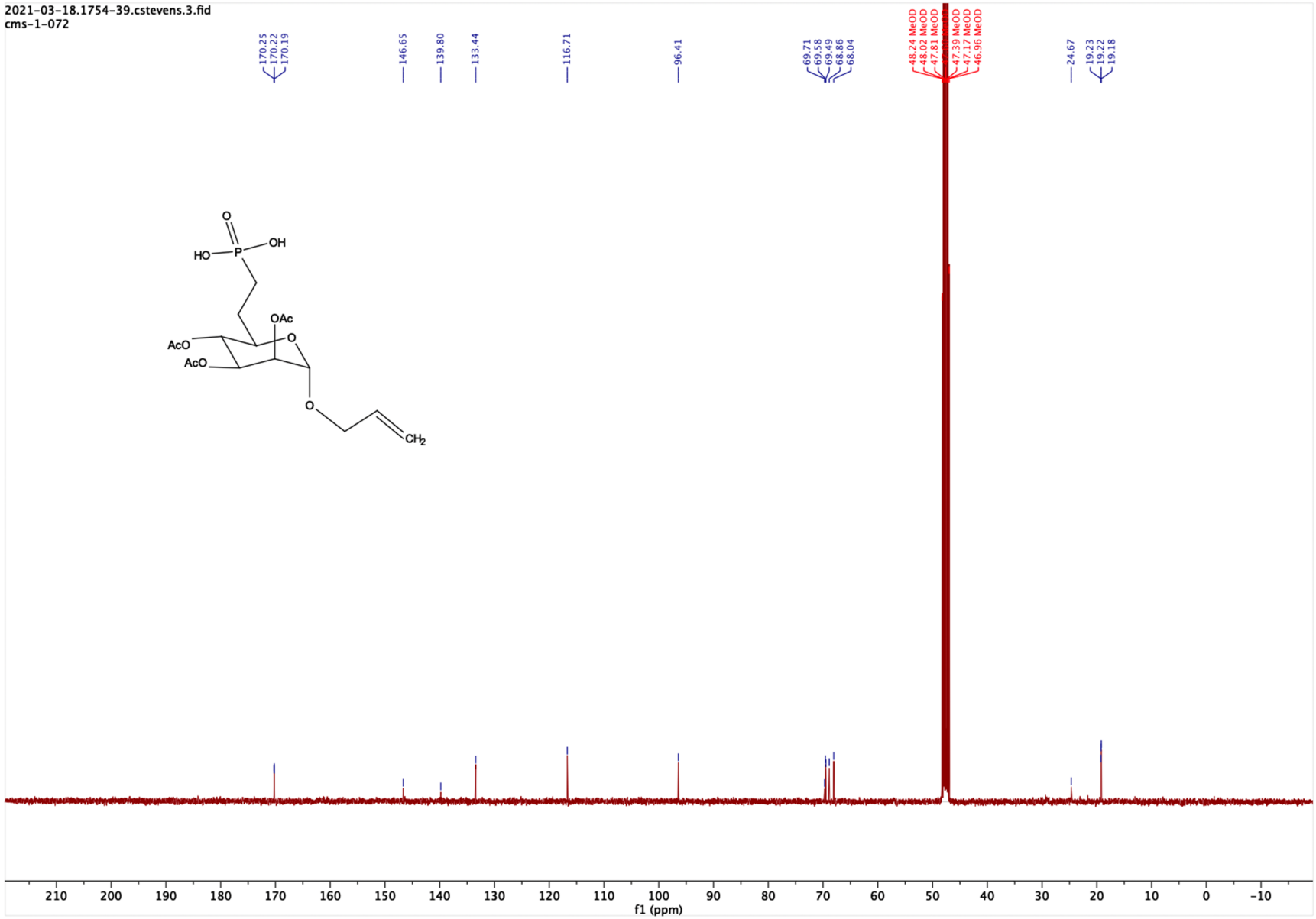
*^{13}C NMR of **4**:*


2022-08-10.1146-44.cstevens.4.fid
cms-1-084_pure
169.95
133.03
118.20
96.36
77.34 CDCl3
77.23
76.71 CDCl3
69.82
69.69
69.52
69.22
68.40
61.65
29.71
24.38
20.93
20.79
20.72
16.51
16.45
CH3
O
P
H3C
OAc
AcO
AcO
CH2
f1 (ppm)

^{1}H NMR of ***5:***


2021-03-18.1754-39.cstevens.1.fid
cms-1-072
HO
P
OH
O
OAc
AcO
AcO
O
O
CH2
D (dddd) 5.96
E (dq) 5.33
F (m) 5.23
G (t) 5.08
H (d) 4.85
I (ddt) 4.22
J (m) 4.05
K (ddd) 3.84
L (s) 2.12
M (s) 2.05
N (m) 1.93
O (s) 1.95
P (m) 1.71
Q (m) 1.30
R (m) 0.90
4.86 HDO
3.31 MeOD
3.31 MeOD
3.31 MeOD
3.30 MeOD
f1 (ppm)

^{13}C NMR of ***5****:*


2021-03-18.1754-39.cstevens.3.fid
cms-1-072
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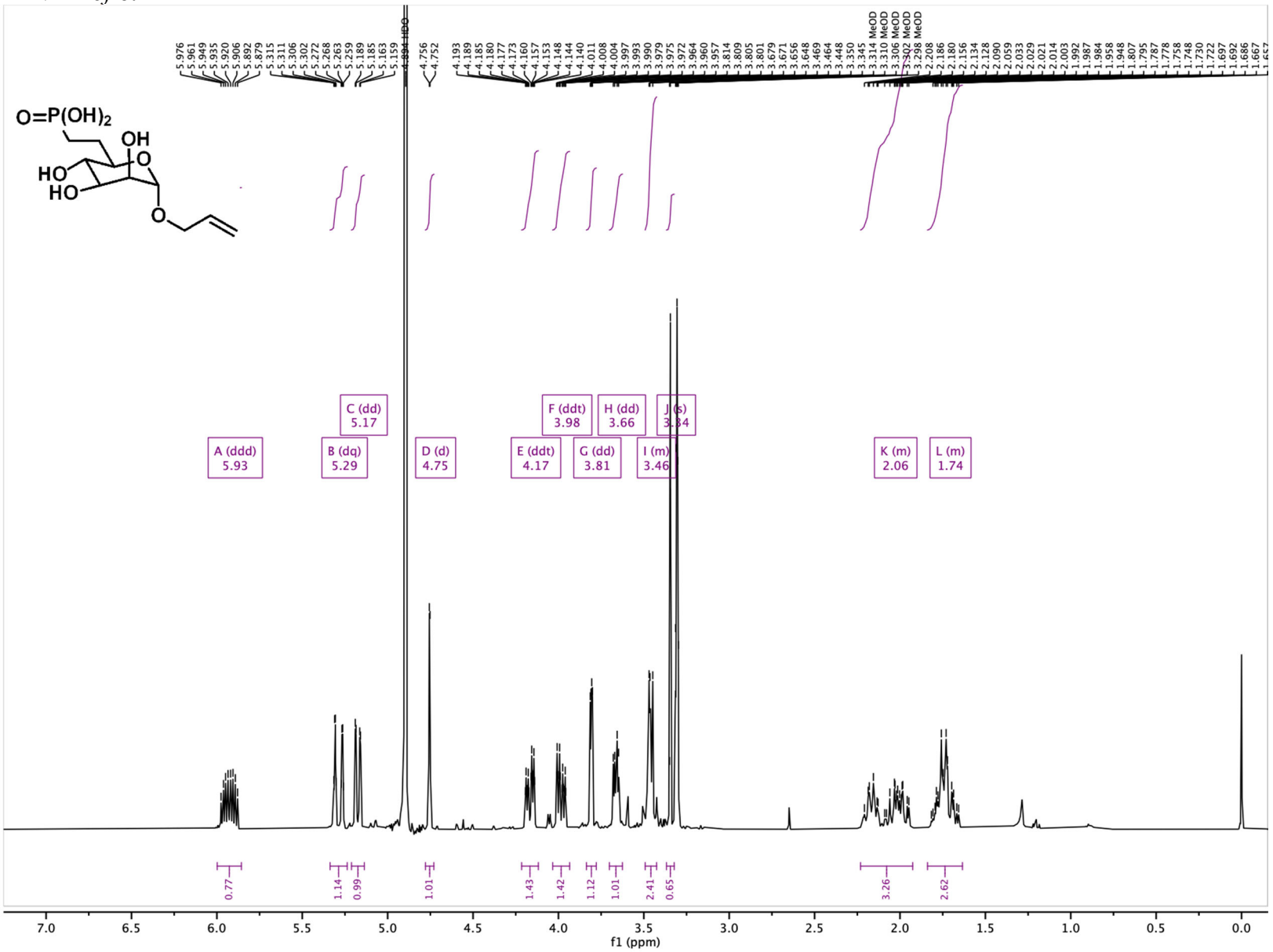
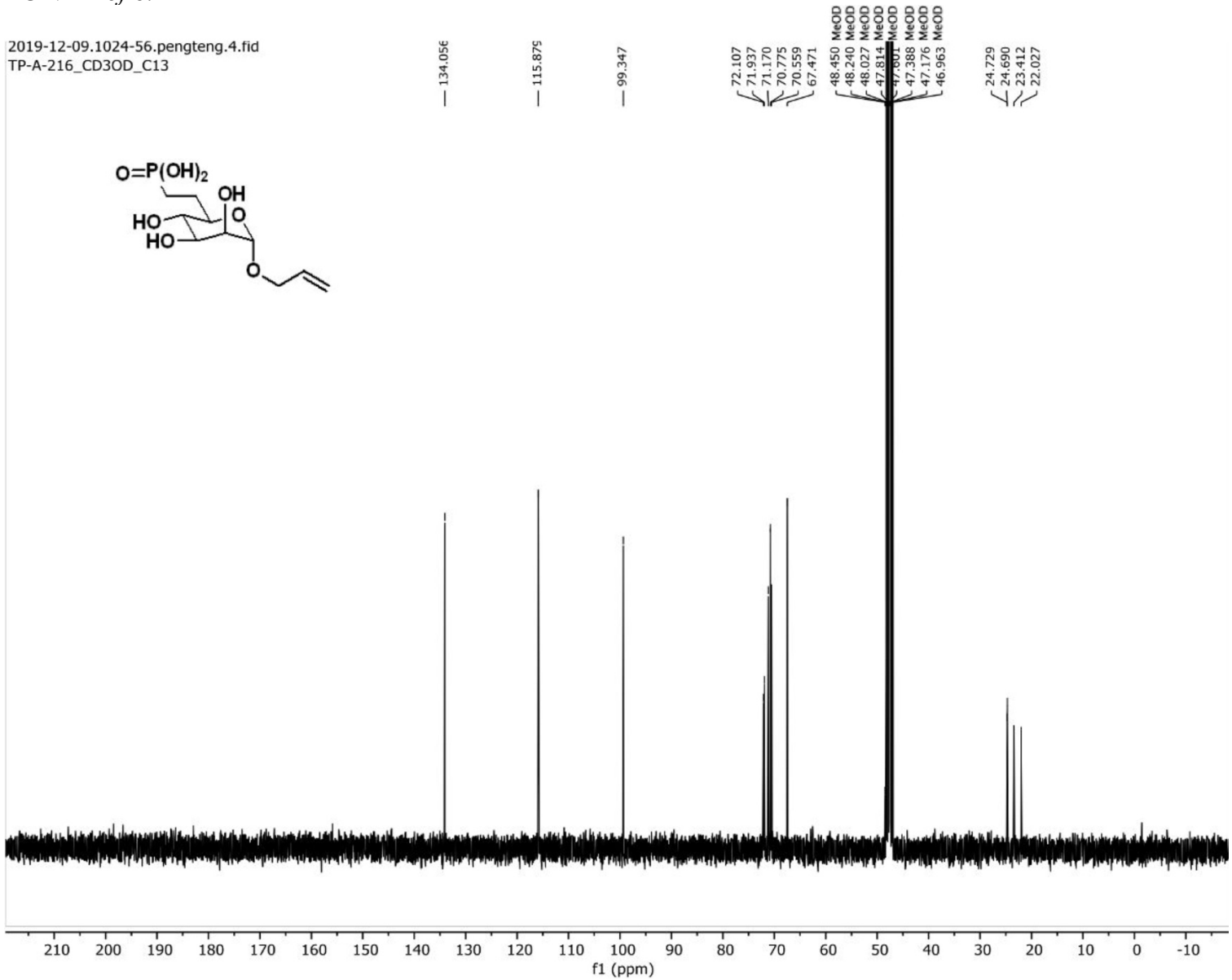
170.22
170.19
146.65
139.80
133.44
116.71
96.41
69.71
69.58
69.49
68.86
68.04
48.24 MeOD
48.02 MeOD
47.81 MeOD
47.39 MeOD
47.17 MeOD
46.96 MeOD
24.67
19.23
19.22
19.18
HO
P
OH
O
OAc
AcO
AcO
O
O
CH2
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

[1]H NMR of ***6:***


O=P(OH)2
OH
HO
HO
O
O
A (ddd) 5.93
B (dq) 5.29
C (dd) 5.17
D (d) 4.75
E (ddt) 4.17
F (ddt) 3.98
G (dd) 3.81
H (dd) 3.66
I (m) 3.46
J (s) 3.34
K (m) 2.06
L (m) 1.74
0.77
1.14
0.99
1.01
1.43
1.42
1.12
1.01
2.41
0.65
3.26
2.62
f1 (ppm)

*^{13}C NMR of **6**:*


2019-12-09.1024-56.pengteng.4.fid
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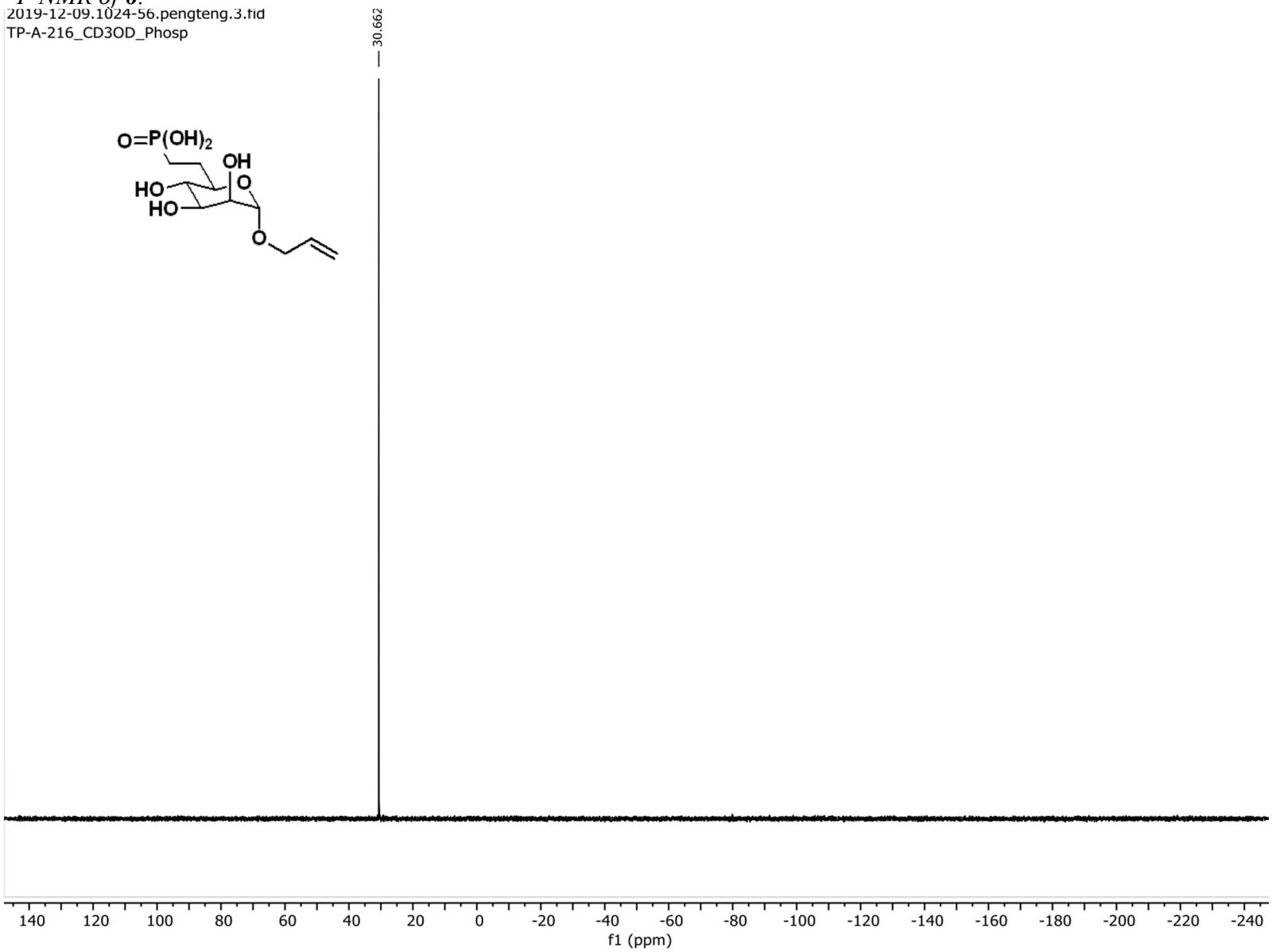
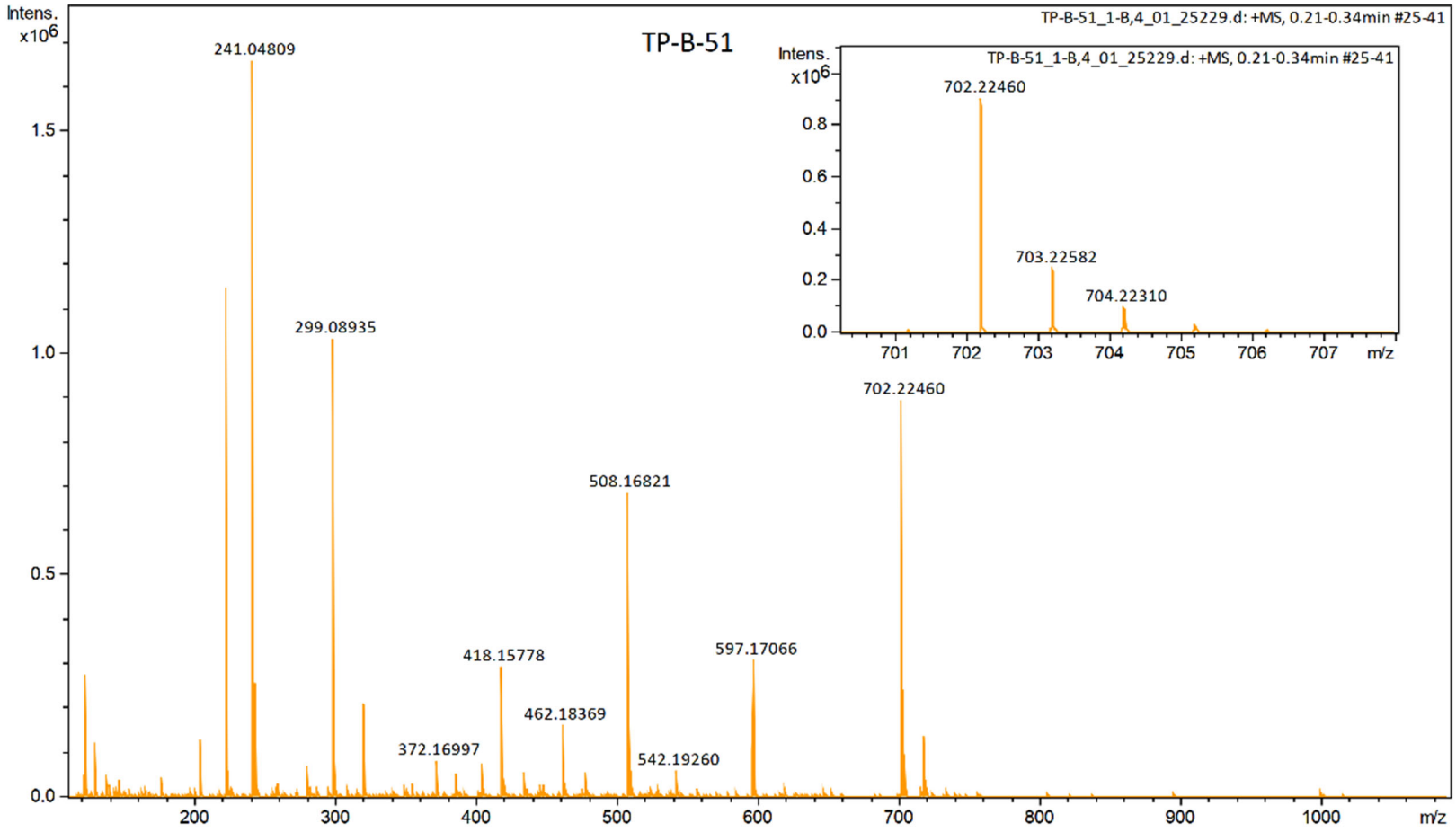
134.056
115.875
99.347
72.107
71.937
71.170
70.775
70.559
67.471
48.450 MeOD
48.240 MeOD
48.027 MeOD
47.814 MeOD
47.601 MeOD
47.388 MeOD
47.176 MeOD
46.963 MeOD
24.729
24.690
23.412
22.027
O=P(OH)2
OH
HO
HO
O
O
210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10
f1 (ppm)

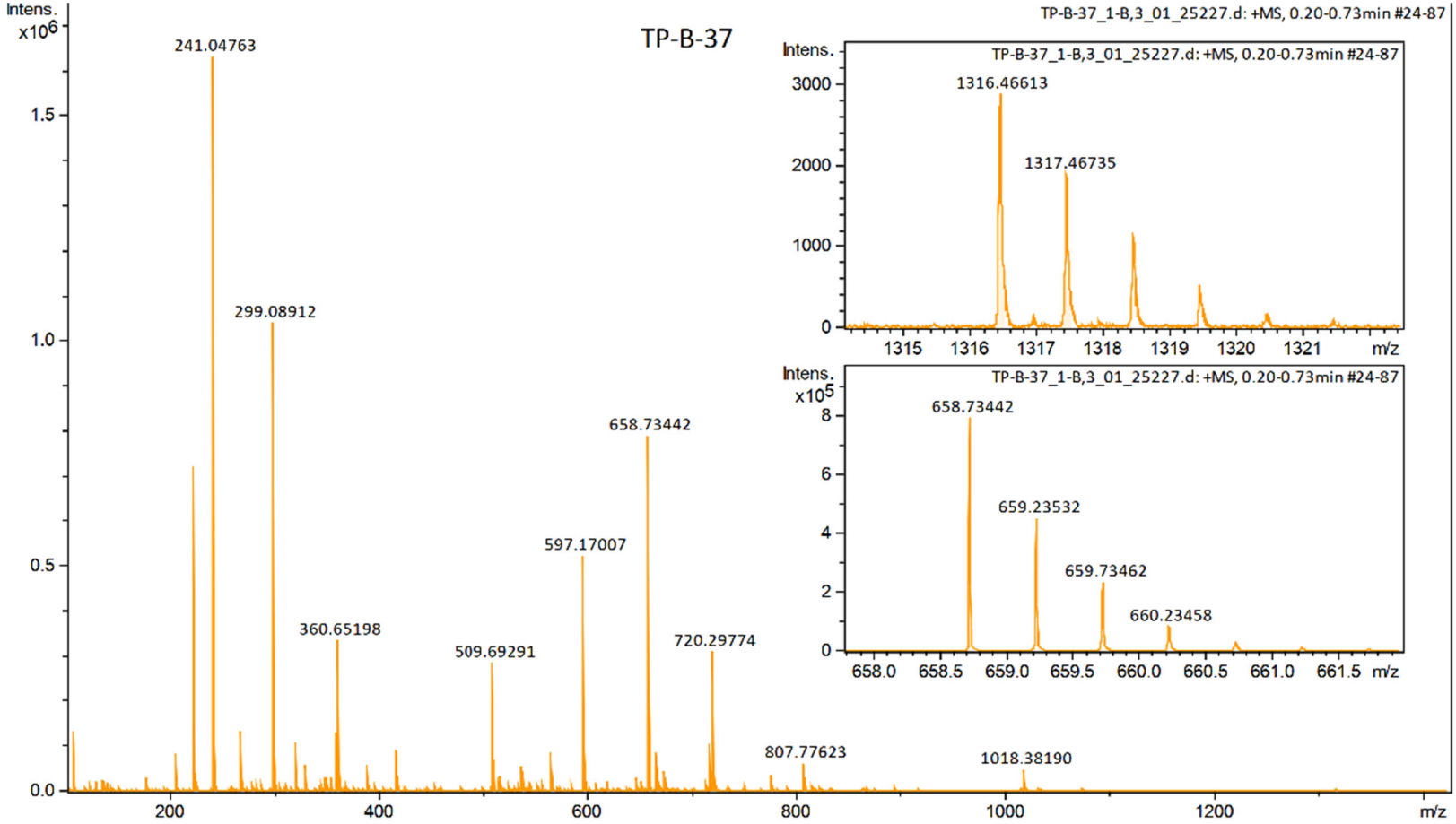
*^{31}P NMR of **6**:*


2019-12-09.1024-56.pengteng.3.fid
TP-A-216_CD3OD_Phosp
30.662
O=P(OH)2
OH
HO
HO
O
O
140
120
100
80
60
40
20
0
-20
-40
-60
-80
-100
-120
-140
-160
-180
-200
-220
-240
f1 (ppm)

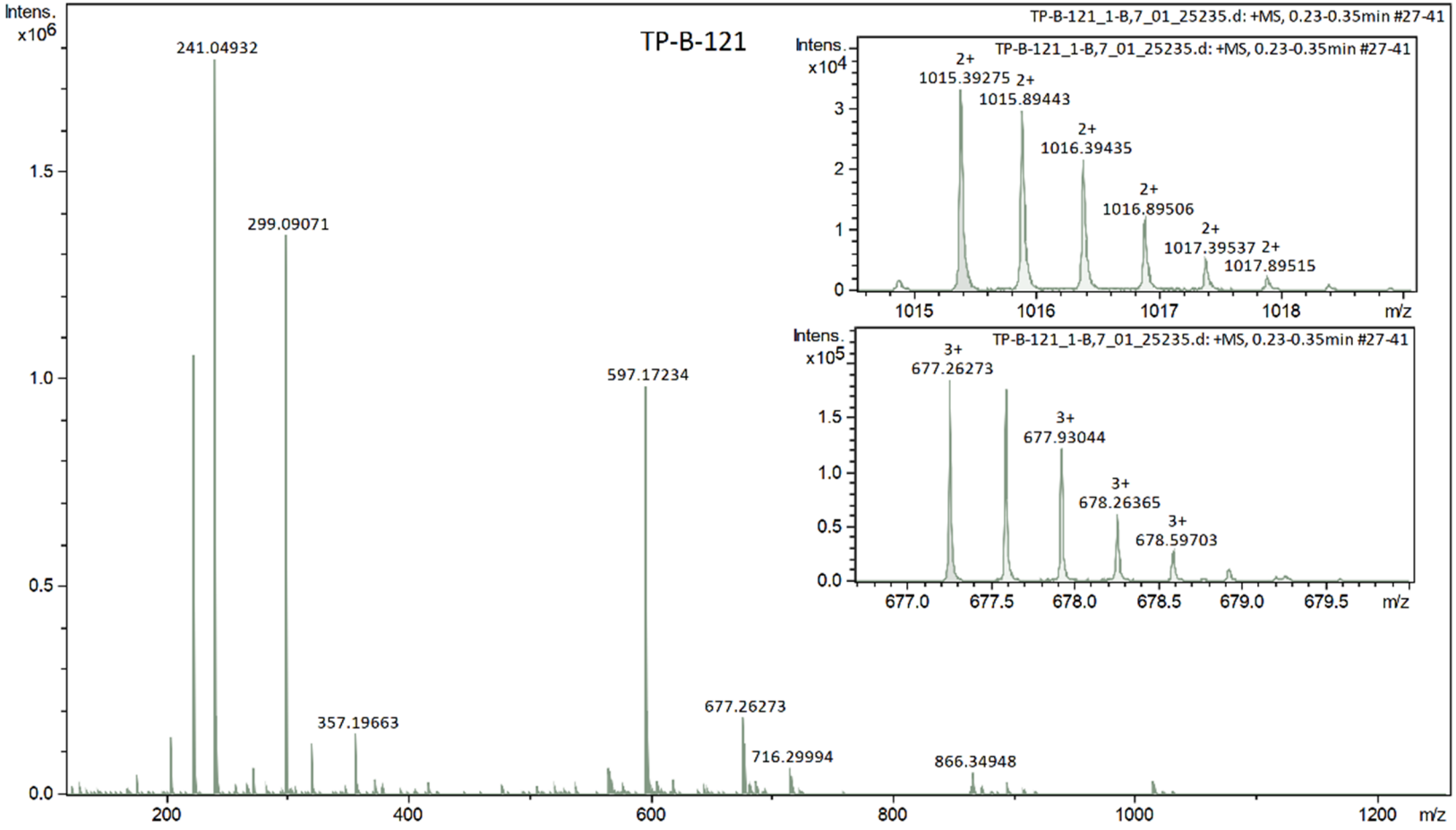
HRMS of **7:**


TP-B-51
TP-B-51_1-B,4_01_25229.d: +MS, 0.21-0.34min #25-41
Intens.
x10^6
241.04809
299.08935
508.16821
702.22460
418.15778
597.17066
462.18369
372.16997
542.19260
m/z
TP-B-51_1-B,4_01_25229.d: +MS, 0.21-0.34min #25-41
Intens.
x10^6
702.22460
703.22582
704.22310
m/z

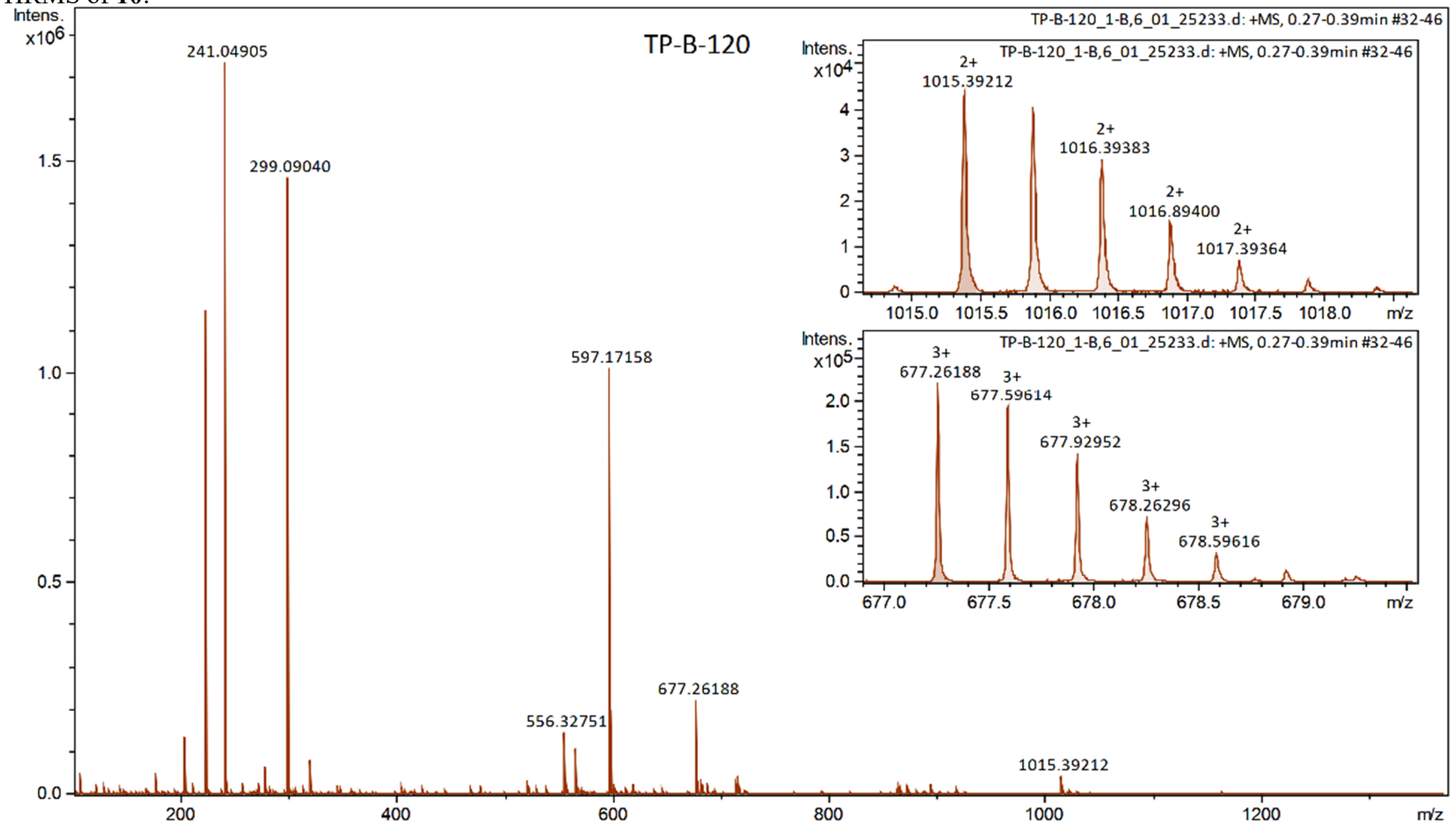
HRMS of **8**:


TP-B-37
Intens.
x10^6
241.04763
299.08912
360.65198
509.69291
597.17007
658.73442
720.29774
807.77623
1018.38190
m/z
TP-B-37_1-B,3_01_25227.d: +MS, 0.20-0.73min #24-87
Intens.
1316.46613
1317.46735
m/z
TP-B-37_1-B,3_01_25227.d: +MS, 0.20-0.73min #24-87
Intens.
x10^5
658.73442
659.23532
659.73462
660.23458
m/z

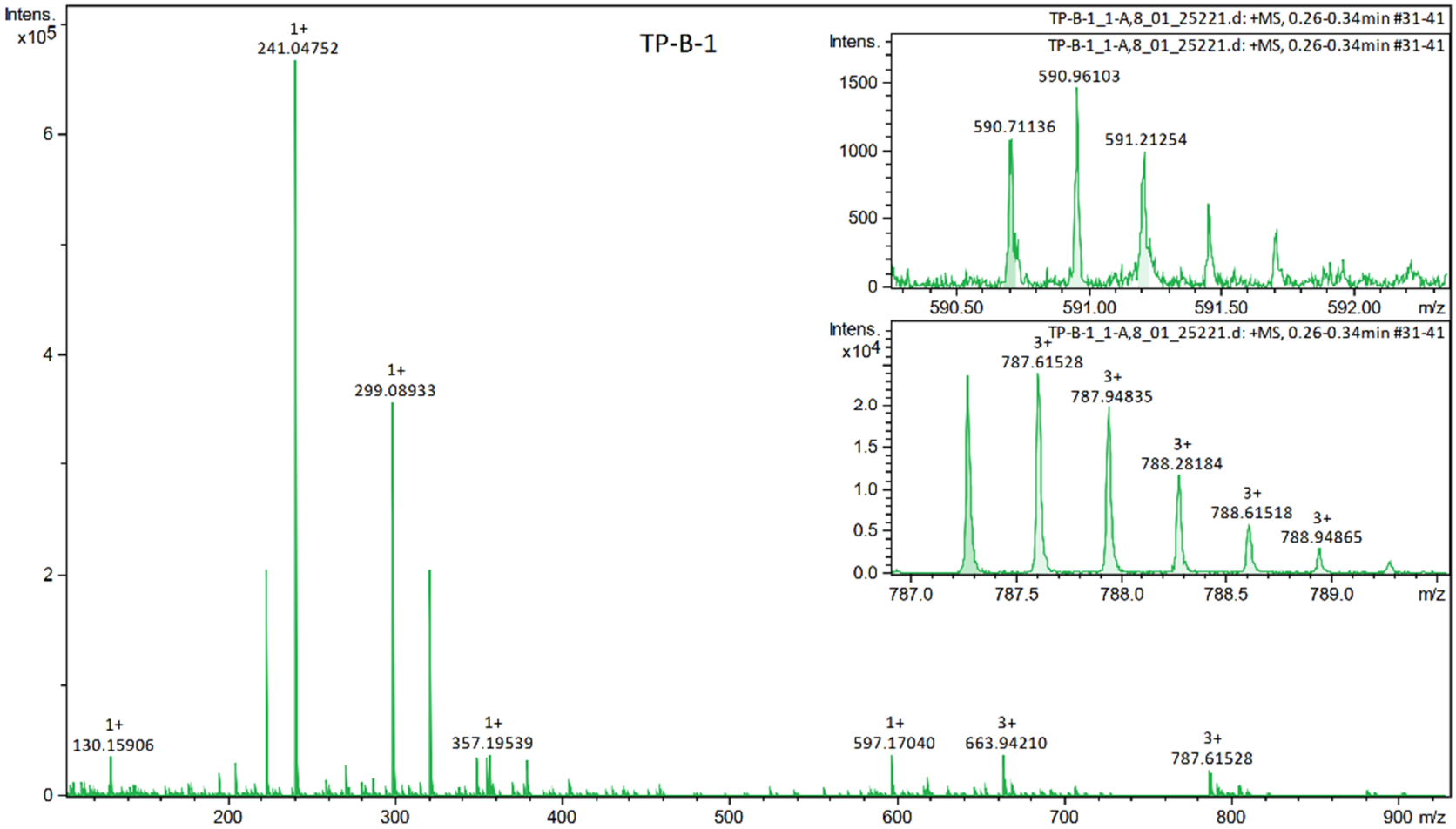
HRMS of **9**:


TP-B-121
TP-B-121_1-B,7_01_25235.d: +MS, 0.23-0.35min #27-41
Intens.
x10^6
241.04932
299.09071
357.19663
597.17234
677.26273
716.29994
866.34948
m/z
Intens.
x10^4
2+ 1015.39275
2+ 1015.89443
2+ 1016.39435
2+ 1016.89506
2+ 1017.39537
2+ 1017.89515
Intens.
x10^5
3+ 677.26273
3+ 677.93044
3+ 678.26365
3+ 678.59703

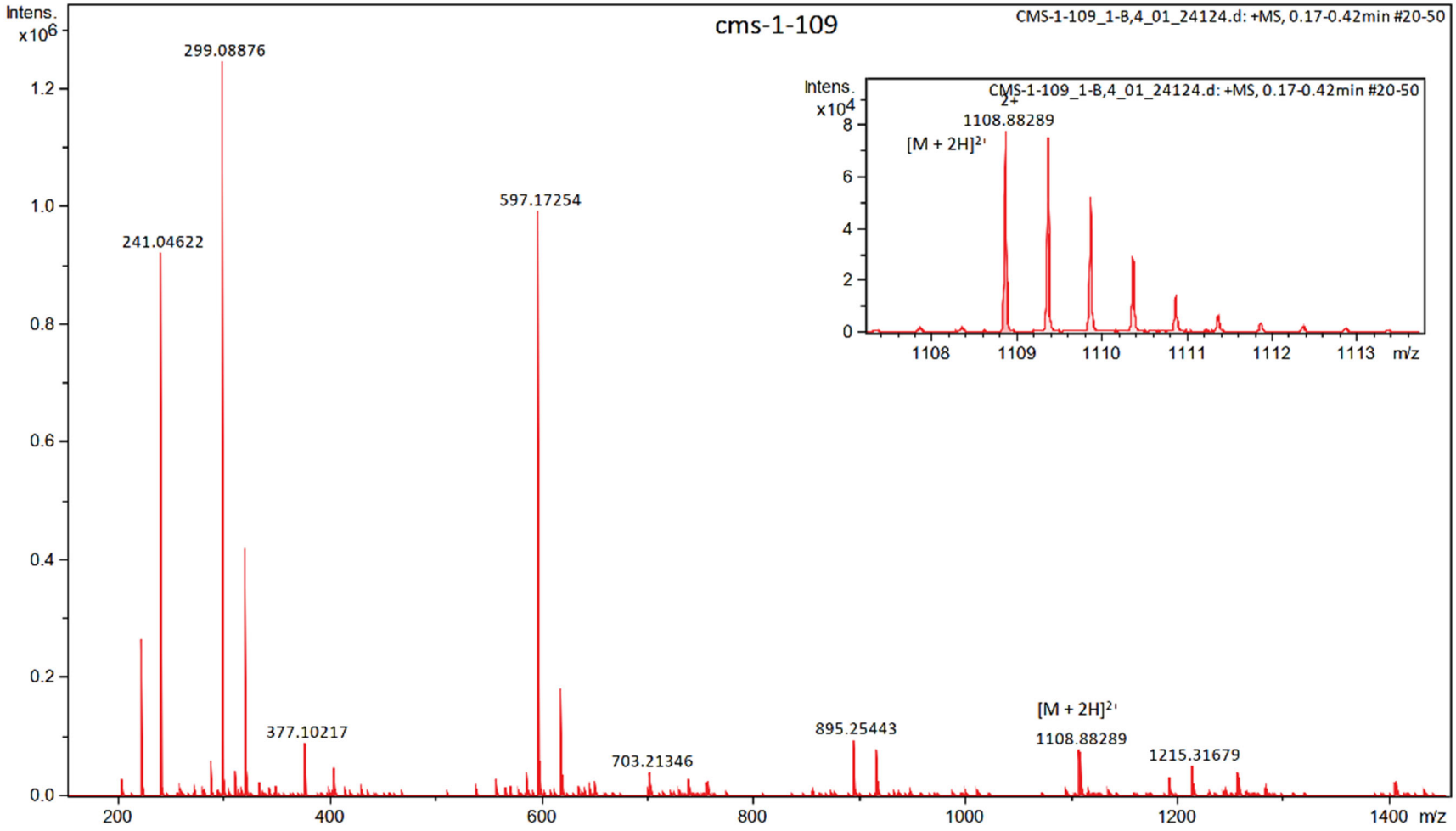
HRMS of **10**:


TP-B-120
Intens.
x10^6
241.04905
299.09040
597.17158
677.26188
556.32751
1015.39212
m/z
TP-B-120_1-B,6_01_25233.d: +MS, 0.27-0.39min #32-46
Intens.
x10^4
2+
1015.39212
2+
1016.39383
2+
1016.89400
2+
1017.39364
m/z
Intens.
x10^5
3+
677.26188
3+
677.59614
3+
677.92952
3+
678.26296
3+
678.59616
m/z

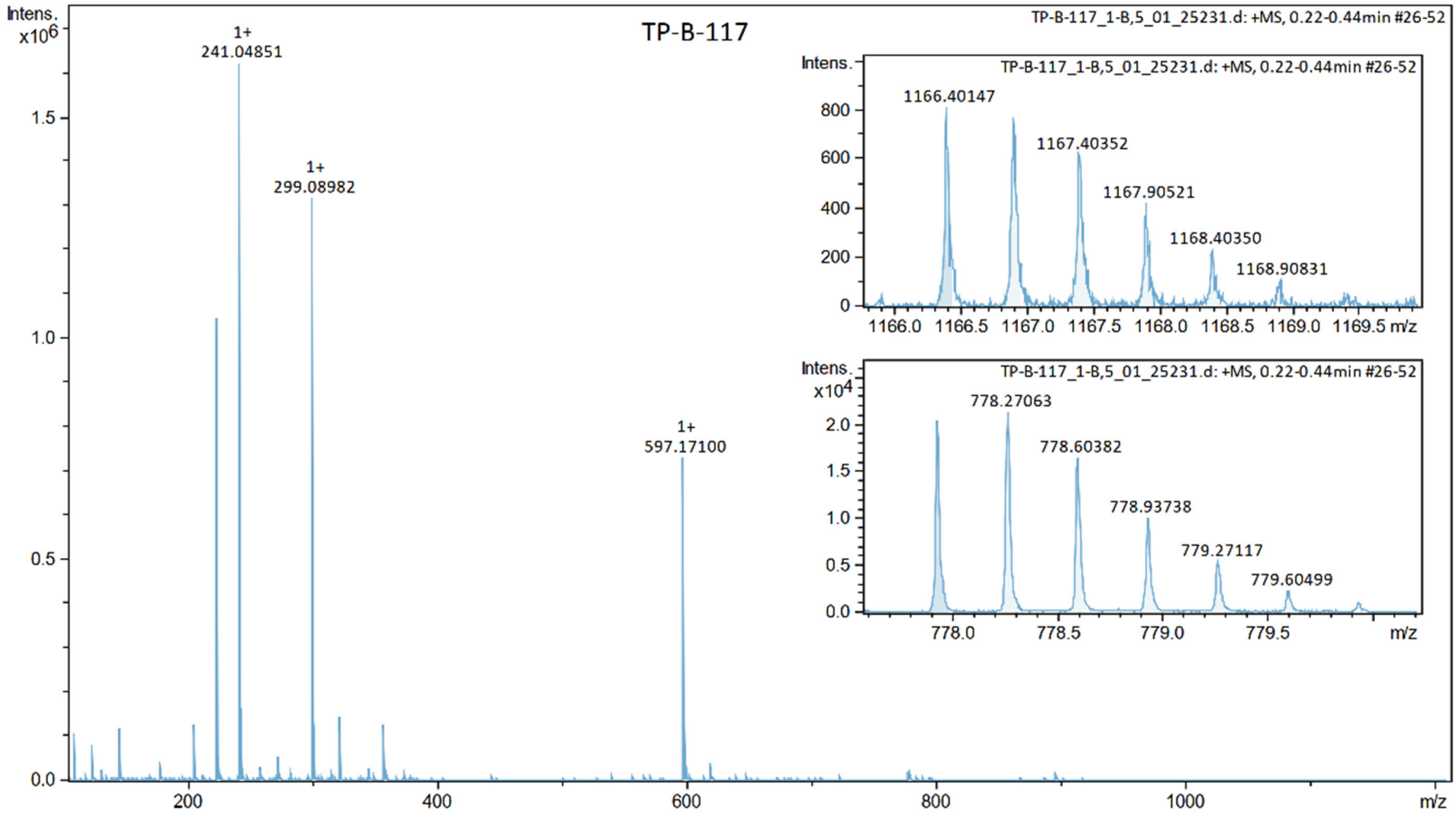
HRMS of **11**:


TP-B-1
Intens.
x10^5
1+
241.04752
1+
299.08933
1+
130.15906
1+
357.19539
1+
597.17040
3+
663.94210
3+
787.61528
200
300
400
500
600
700
800
900 m/z
TP-B-1_1-A,8_01_25221.d: +MS, 0.26-0.34min #31-41
Intens.
590.96103
590.71136
591.21254
590.50
591.00
591.50
592.00
m/z
Intens.
x10^4
3+
787.61528
3+
787.94835
3+
788.28184
3+
788.61518
3+
788.94865
787.0
787.5
788.0
788.5
789.0
m/z

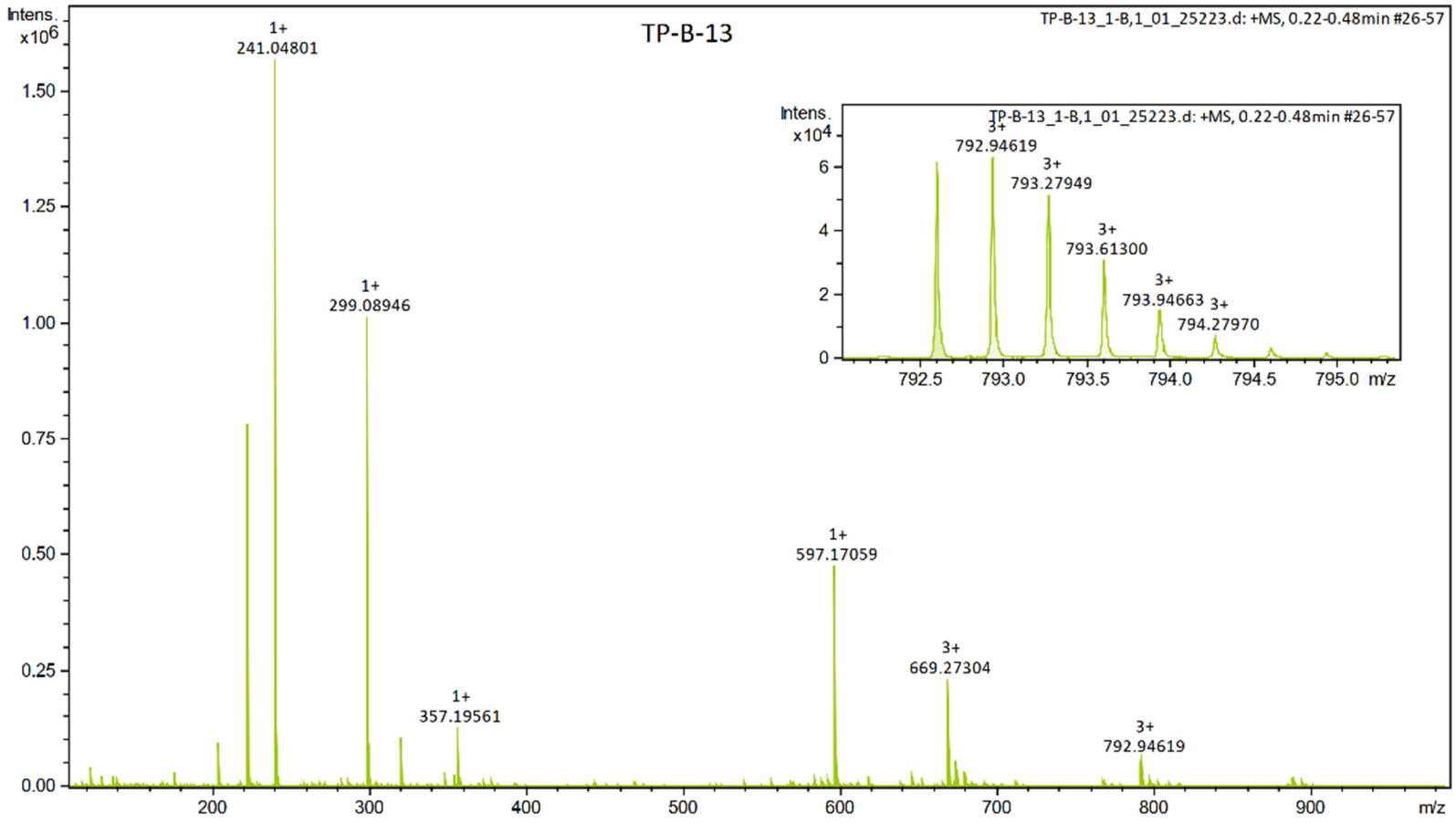
HRMS of **12**:


cms-1-109
CMS-1-109_1-B,4_01_24124.d: +MS, 0.17-0.42min #20-50
Intens.
x10^6
299.08876
241.04622
597.17254
377.10217
703.21346
895.25443
[M + 2H]2+
1108.88289
1215.31679
m/z
Intens.
x10^4
CMS-1-109_1-B,4_01_24124.d: +MS, 0.17-0.42min #20-50
[M + 2H]2+
1108.88289
m/z

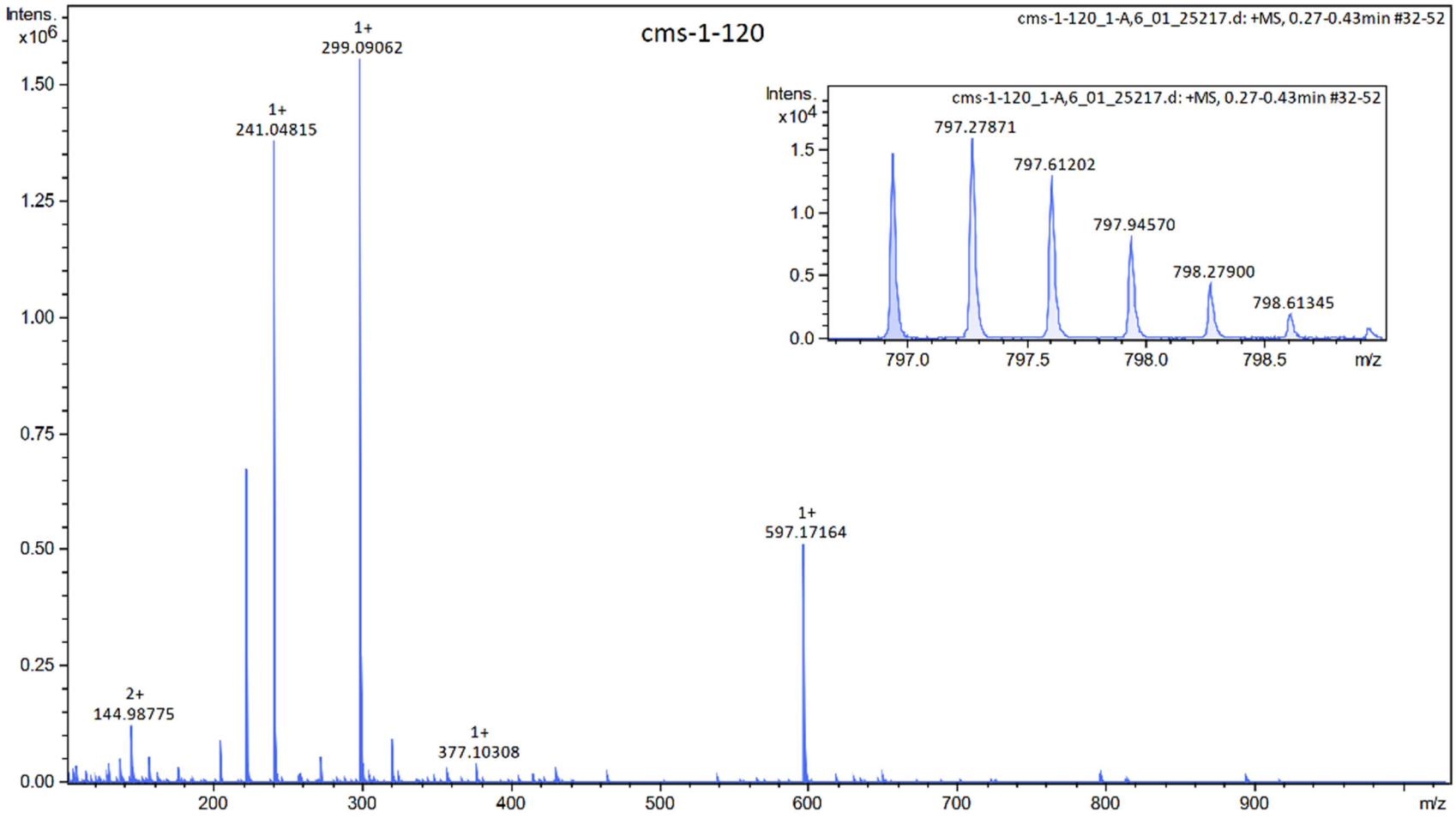
HRMS of **13**:


TP-B-117
TP-B-117_1-B,5_01_25231.d: +MS, 0.22-0.44min #26-52
Intens.
x10^6
1+
241.04851
1+
299.08982
1+
597.17100
m/z
TP-B-117_1-B,5_01_25231.d: +MS, 0.22-0.44min #26-52
1166.40147
1167.40352
1167.90521
1168.40350
1168.90831
m/z
TP-B-117_1-B,5_01_25231.d: +MS, 0.22-0.44min #26-52
x10^4
778.27063
778.60382
778.93738
779.27117
779.60499
m/z

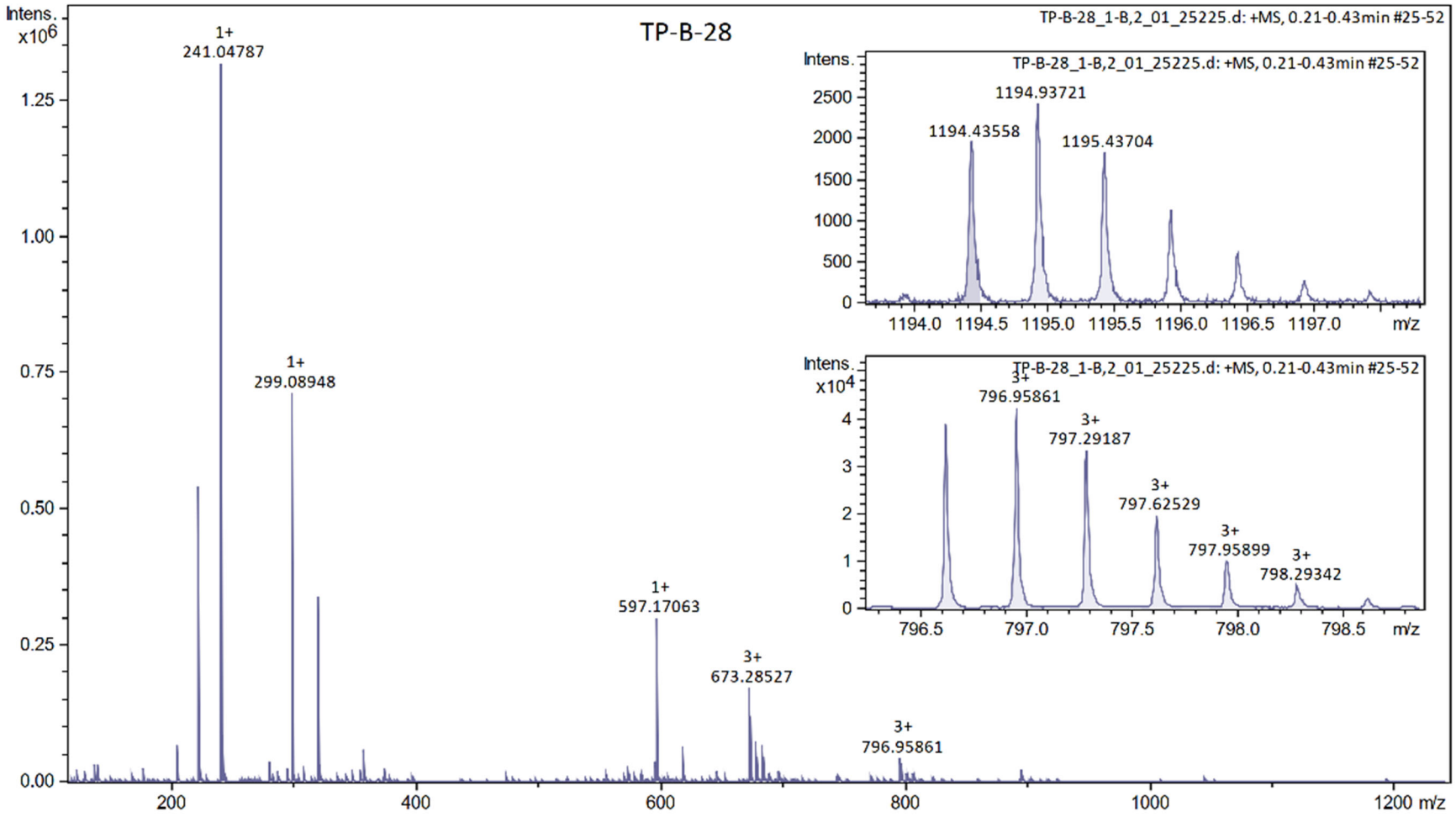
HRMS of **14**:


TP-B-13
TP-B-13_1-B,1_01_25223.d: +MS, 0.22-0.48min #26-57
Intens.
x10^6
1+
241.04801
1+
299.08946
1+
357.19561
1+
597.17059
3+
669.27304
3+
792.94619
Intens.
x10^4
3+
792.94619
3+
793.27949
3+
793.61300
3+
793.94663
3+
794.27970
m/z
m/z

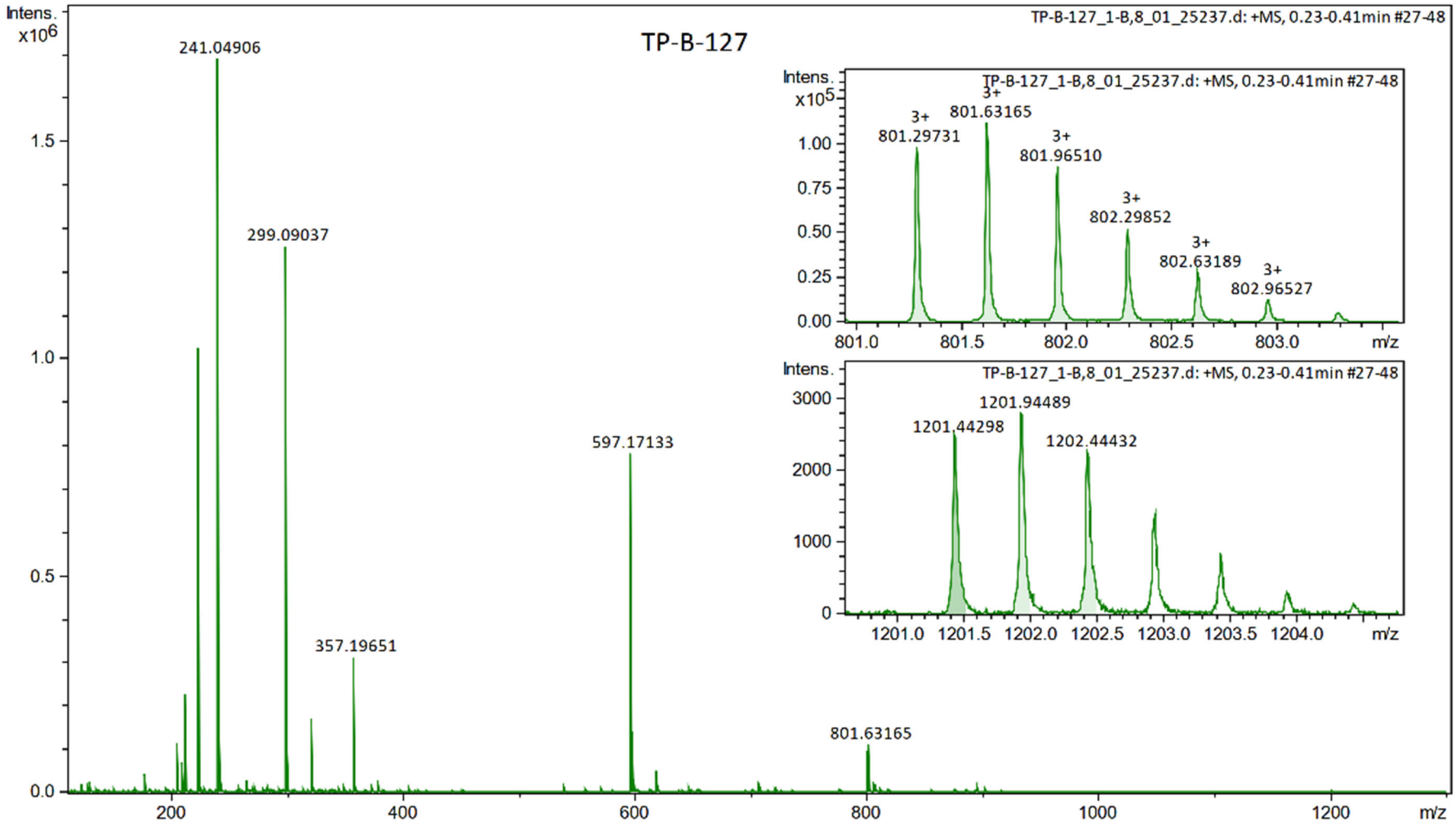
HRMS of **15**:


cms-1-120
cms-1-120_1-A,6_01_25217.d: +MS, 0.27-0.43min #32-52
Intens.
x10^6
1.50
1.25
1.00
0.75
0.50
0.25
0.00
1+
299.09062
1+
241.04815
1+
597.17164
2+
144.98775
1+
377.10308
200
300
400
500
600
700
800
900
m/z
Intens.
x10^4
1.5
1.0
0.5
0.0
797.27871
797.61202
797.94570
798.27900
798.61345
797.0
797.5
798.0
798.5
m/z

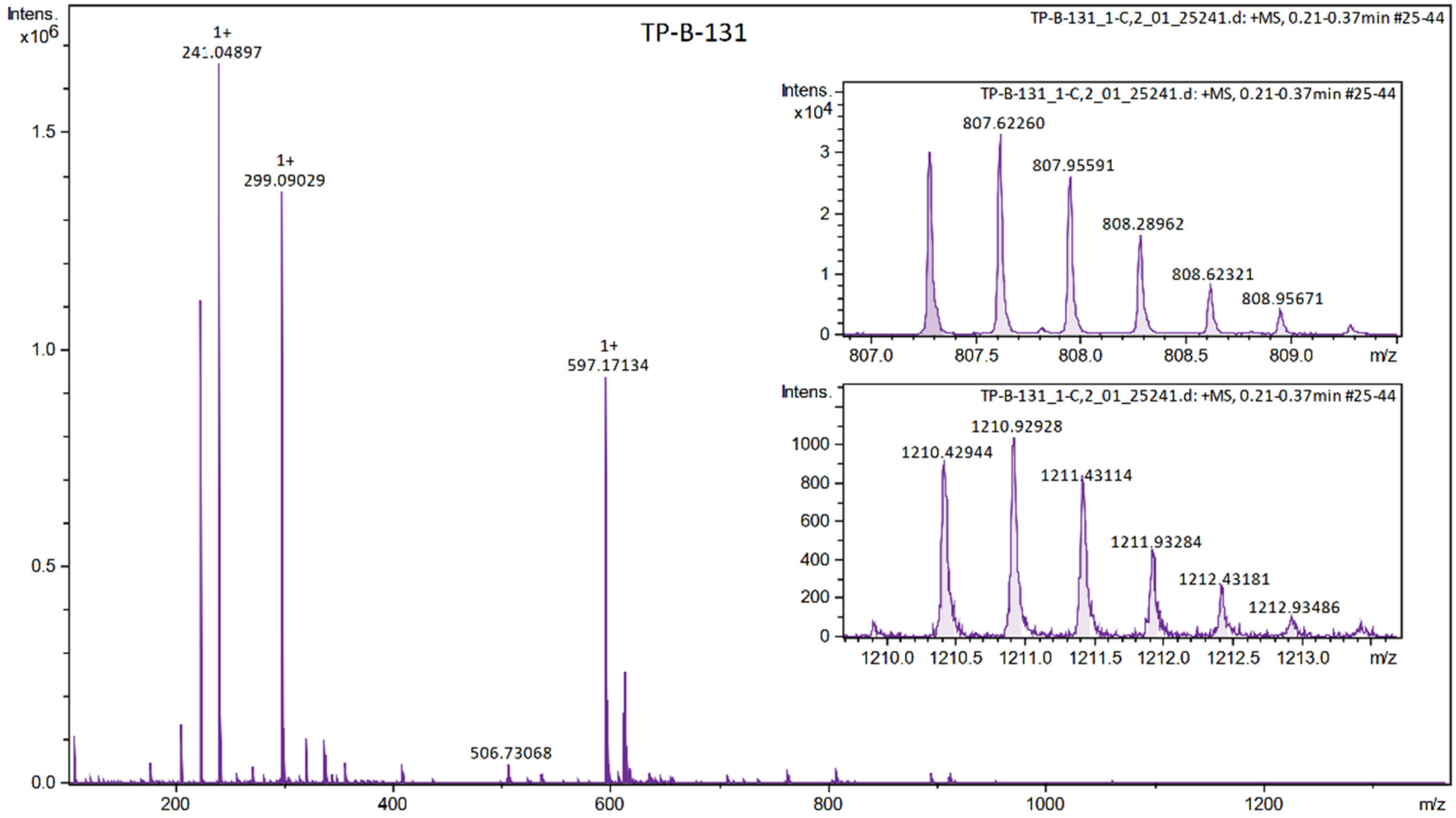
HRMS of **16**:


TP-B-28
TP-B-28_1-B,2_01_25225.d: +MS, 0.21-0.43min #25-52
Intens.
x10^6
1+
241.04787
1+
299.08948
1+
597.17063
3+
673.28527
3+
796.95861
Intens.
1194.93721
1194.43558
1195.43704
m/z
Intens.
x10^4
3+
796.95861
3+
797.29187
3+
797.62529
3+
797.95899
3+
798.29342
m/z
200
400
600
800
1000
1200 m/z

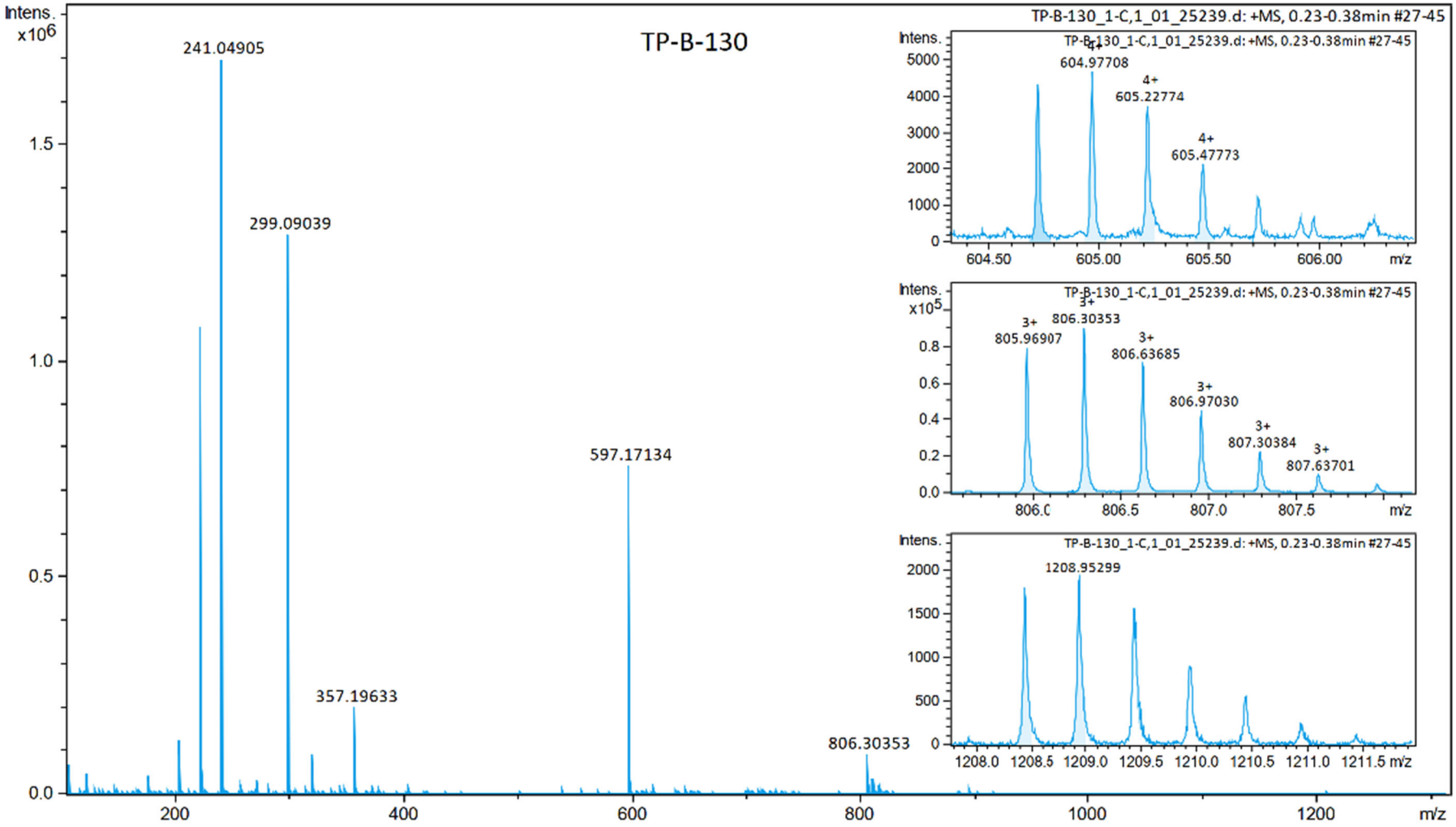
HRMS of **17**:


TP-B-127
TP-B-127_1-B,8_01_25237.d: +MS, 0.23-0.41min #27-48
Intens.
x10^6
241.04906
299.09037
357.19651
597.17133
801.63165
m/z
Intens.
x10^5
3+ 801.29731
3+ 801.63165
3+ 801.96510
3+ 802.29852
3+ 802.63189
3+ 802.96527
Intens.
1201.44298
1201.94489
1202.44432

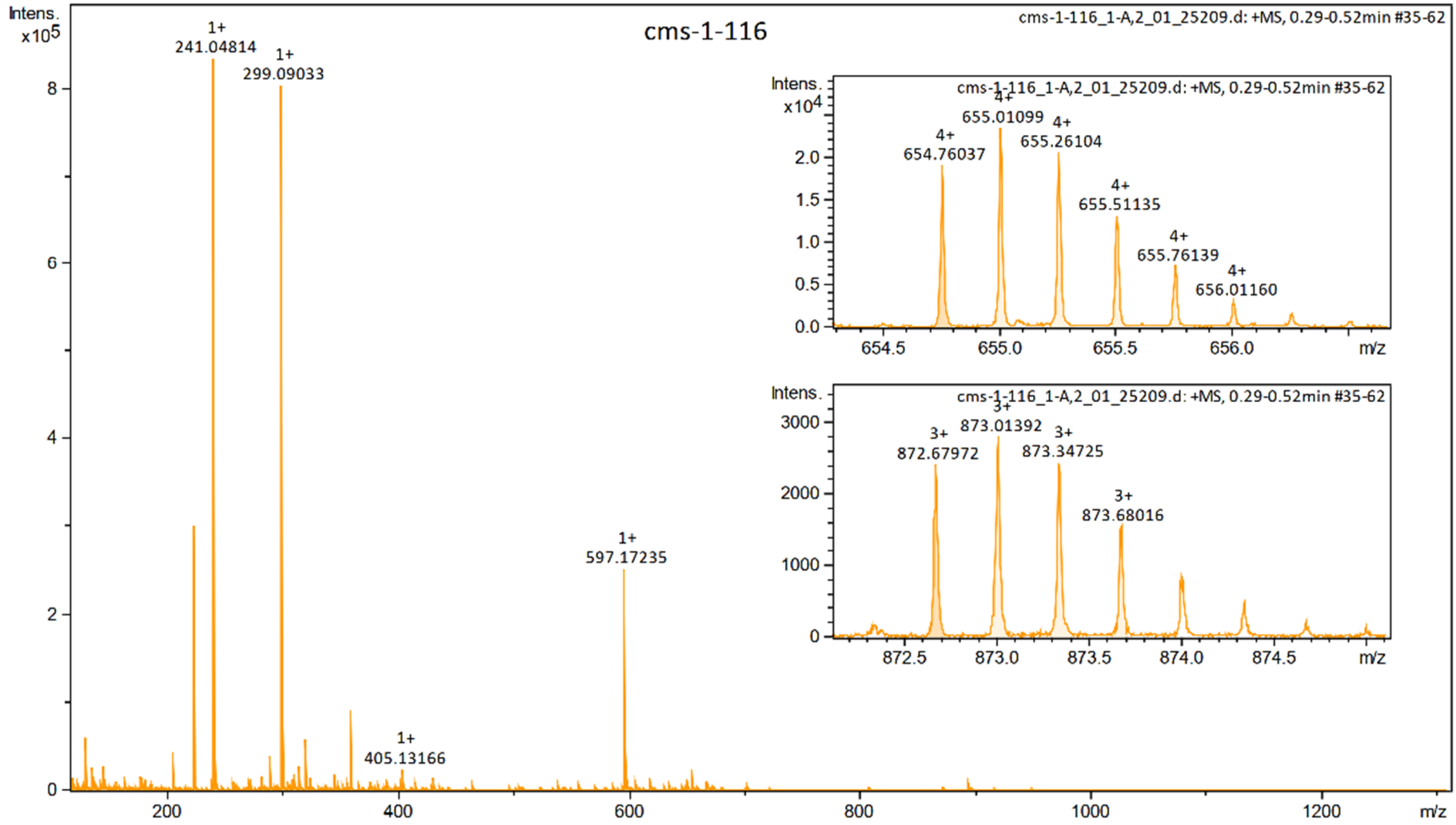
HRMS of **18**:


TP-B-131
TP-B-131_1-C,2_01_25241.d: +MS, 0.21-0.37min #25-44
Intens.
x10^6
1+
241.04897
1+
299.09029
1+
597.17134
506.73068
m/z
Intens.
x10^4
807.62260
807.95591
808.28962
808.62321
808.95671
Intens.
1210.92928
1210.42944
1211.43114
1211.93284
1212.43181
1212.93486

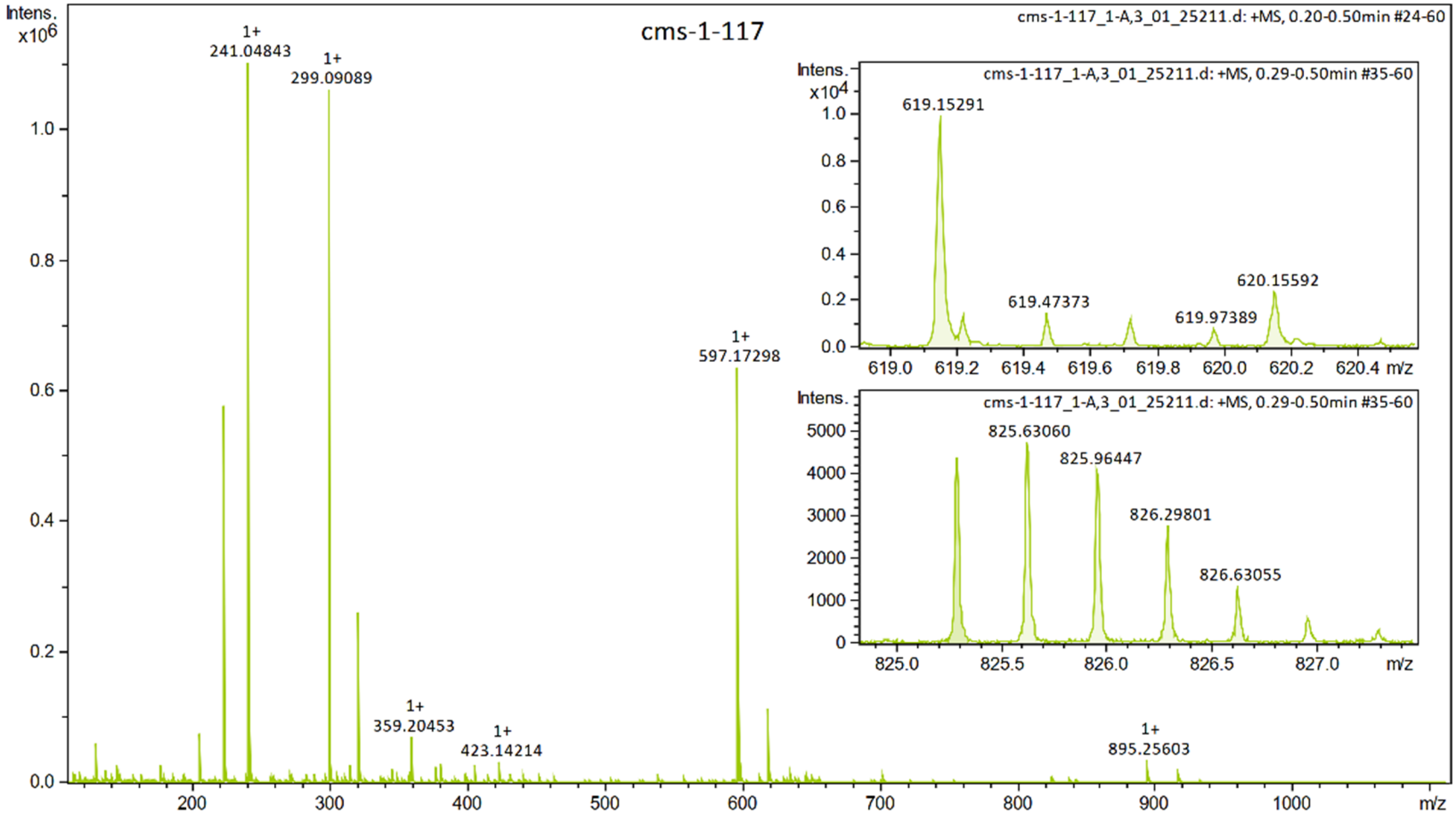
HRMS of **19**:


TP-B-130
TP-B-130_1-C,1_01_25239.d: +MS, 0.23-0.38min #27-45
Intens.
x10^6
241.04905
299.09039
357.19633
597.17134
806.30353
Intens.
5000
4000
3000
2000
1000
0
604.97708
4+
605.22774
4+
605.47773
4+
604.50
605.00
605.50
606.00
m/z
Intens.
x10^5
0.8
0.6
0.4
0.2
0.0
805.96907
3+
806.30353
3+
806.63685
3+
806.97030
3+
807.30384
3+
807.63701
3+
806.0
806.5
807.0
807.5
m/z
Intens.
2000
1500
1000
500
0
1208.95299
1208.0
1208.5
1209.0
1209.5
1210.0
1210.5
1211.0
1211.5
m/z
1.5
1.0
0.5
0.0
200
400
600
800
1000
1200
m/z

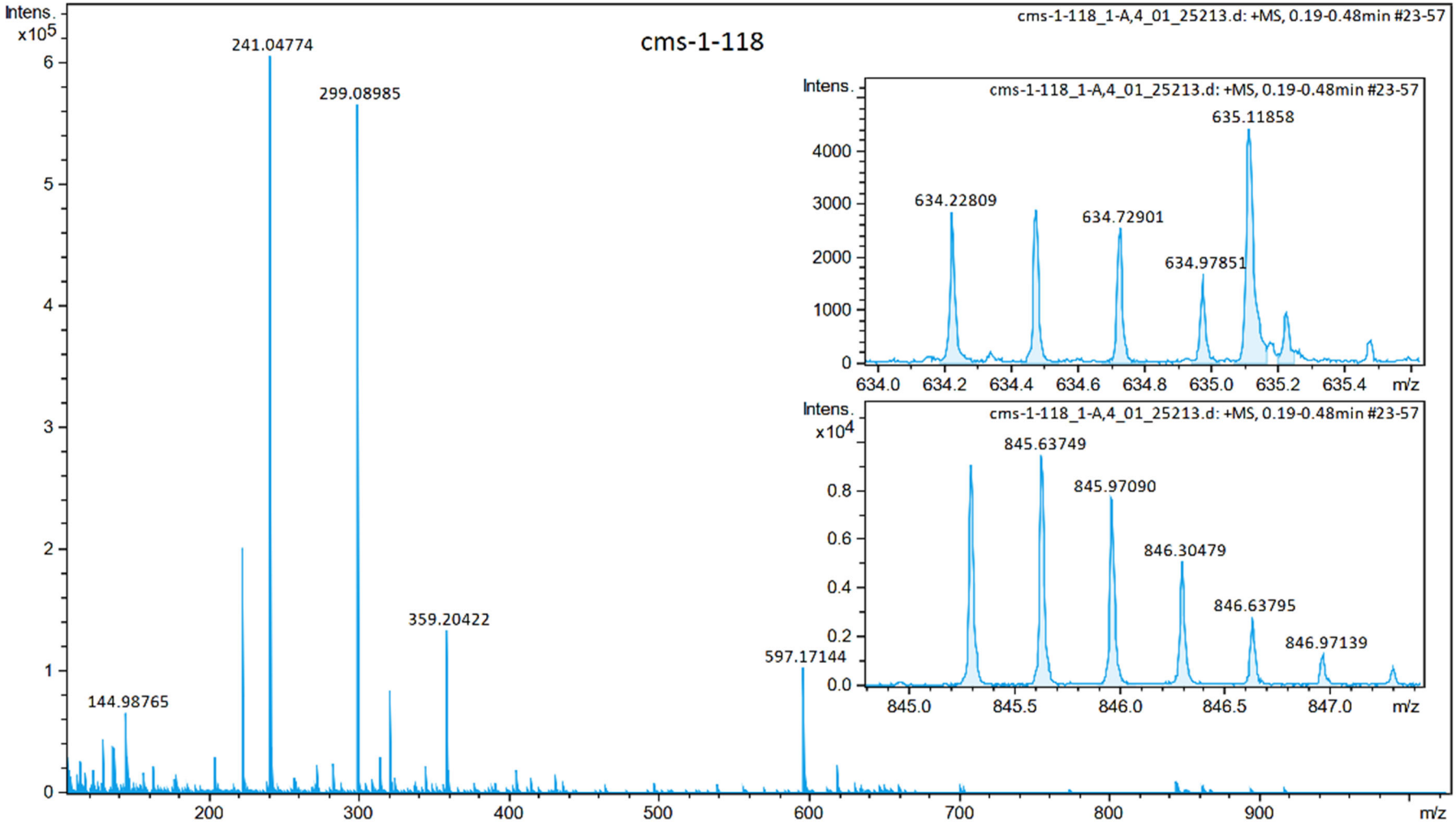
HRMS of **20**:


cms-1-116
cms-1-116_1-A,2_01_25209.d: +MS, 0.29-0.52min #35-62
Intens.
x10^5
1+
241.04814
1+
299.09033
1+
405.13166
1+
597.17235
m/z
cms-1-116_1-A,2_01_25209.d: +MS, 0.29-0.52min #35-62
Intens.
x10^4
4+
654.76037
4+
655.01099
4+
655.26104
4+
655.51135
4+
655.76139
4+
656.01160
m/z
cms-1-116_1-A,2_01_25209.d: +MS, 0.29-0.52min #35-62
Intens.
3+
872.67972
3+
873.01392
3+
873.34725
3+
873.68016
m/z

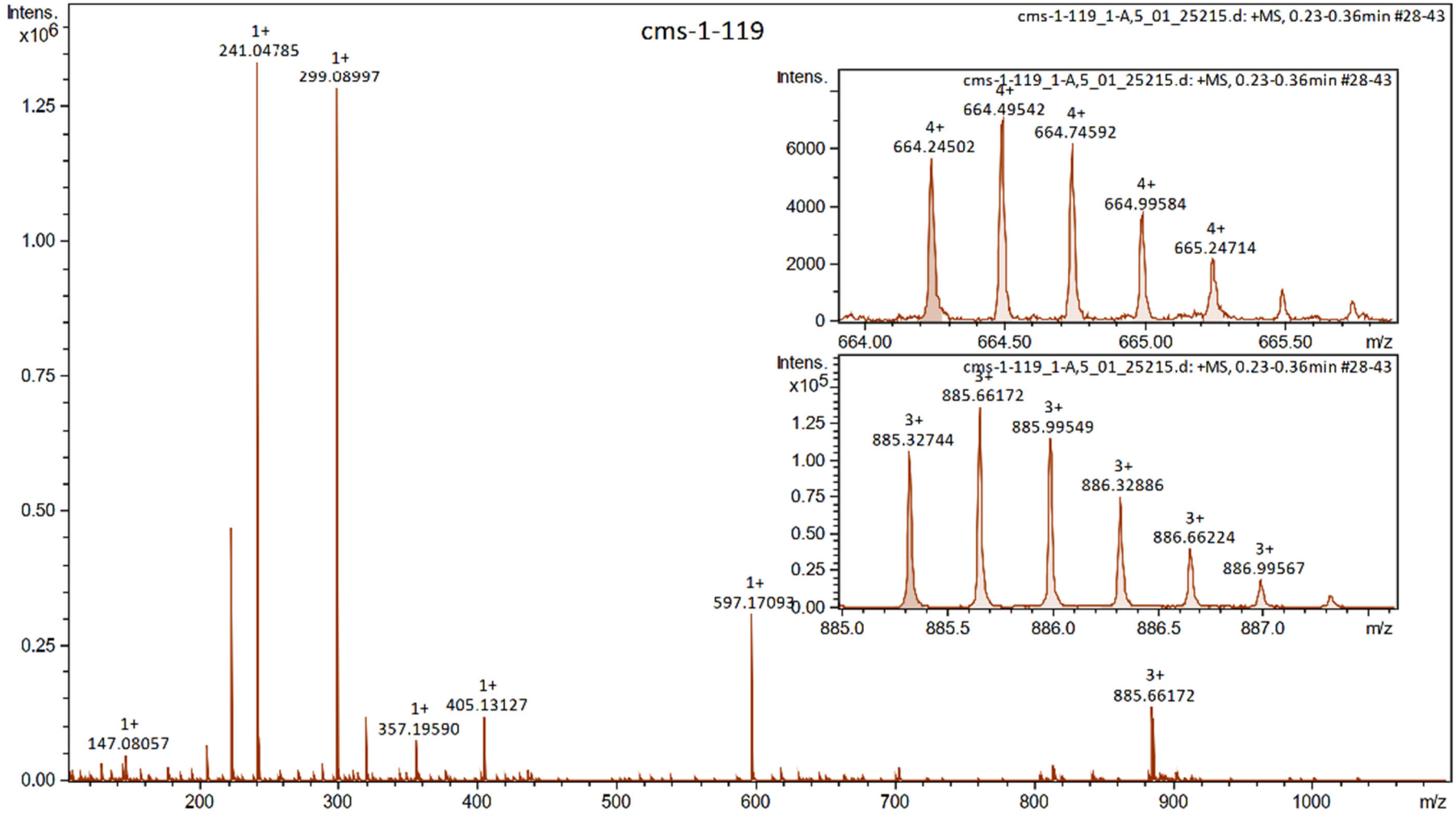
HRMS of **21**:


cms-1-117
cms-1-117_1-A,3_01_25211.d: +MS, 0.20-0.50min #24-60
Intens.
x10^6
1+
241.04843
1+
299.09089
1+
597.17298
1+
359.20453
1+
423.14214
1+
895.25603
m/z
cms-1-117_1-A,3_01_25211.d: +MS, 0.29-0.50min #35-60
Intens.
x10^4
619.15291
619.47373
619.97389
620.15592
m/z
cms-1-117_1-A,3_01_25211.d: +MS, 0.29-0.50min #35-60
Intens.
825.63060
825.96447
826.29801
826.63055
m/z

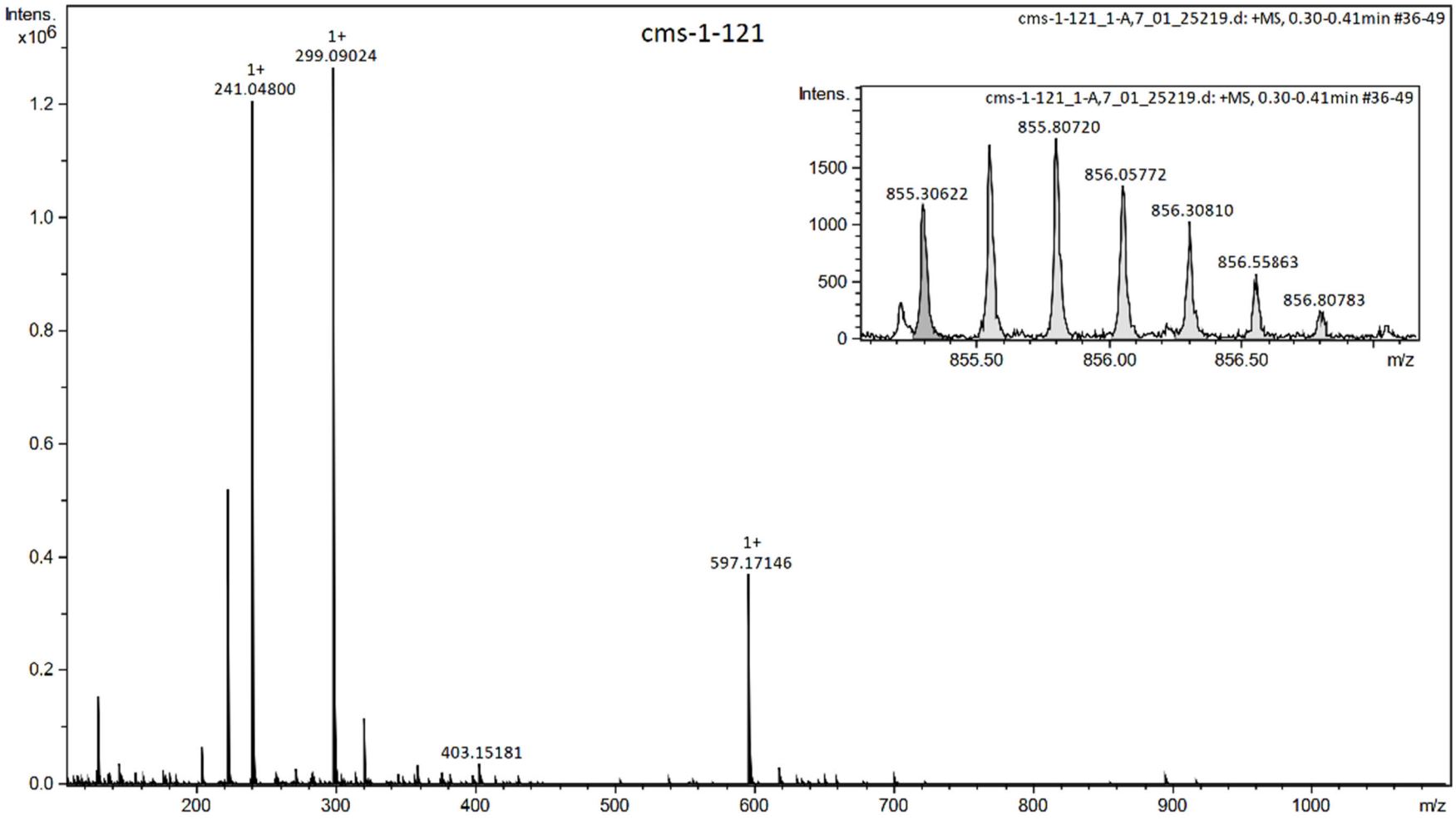
HRMS of **22**:


cms-1-118
cms-1-118_1-A,4_01_25213.d: +MS, 0.19-0.48min #23-57
Intens.
x10^5
241.04774
299.08985
359.20422
144.98765
597.17144
m/z
Intens.
635.11858
634.22809
634.72901
634.97851
m/z
Intens.
x10^4
845.63749
845.97090
846.30479
846.63795
846.97139
m/z

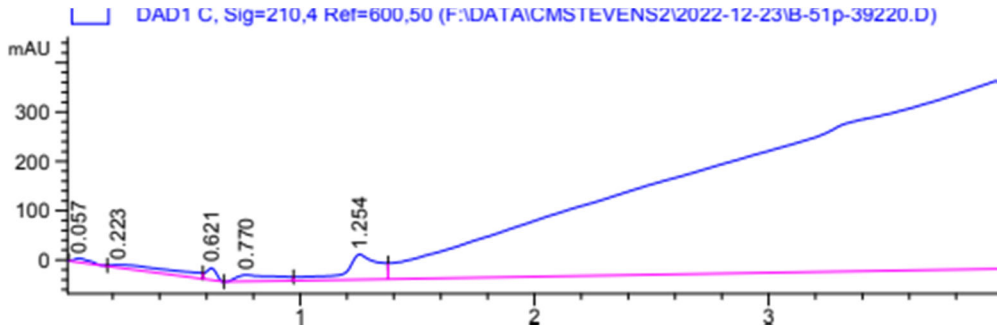
HRMS of **23**:


cms-1-119
cms-1-119_1-A,5_01_25215.d: +MS, 0.23-0.36min #28-43
Intens.
x10^6
1+
241.04785
1+
299.08997
1+
147.08057
1+
357.19590
1+
405.13127
1+
597.17093
3+
885.66172
m/z
4+
664.24502
4+
664.49542
4+
664.74592
4+
664.99584
4+
665.24714
x10^5
3+
885.32744
3+
885.66172
3+
885.99549
3+
886.32886
3+
886.66224
3+
886.99567

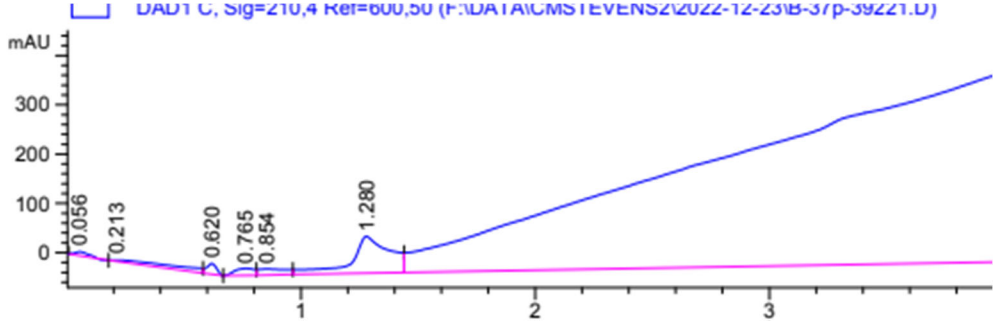
HRMS of **24**:


cms-1-121
cms-1-121_1-A,7_01_25219.d: +MS, 0.30-0.41min #36-49
Intens.
x10^6
1+
299.09024
1+
241.04800
1+
597.17146
403.15181
m/z
Intens.
855.80720
856.05772
855.30622
856.30810
856.55863
856.80783
855.50
856.00
856.50
m/z

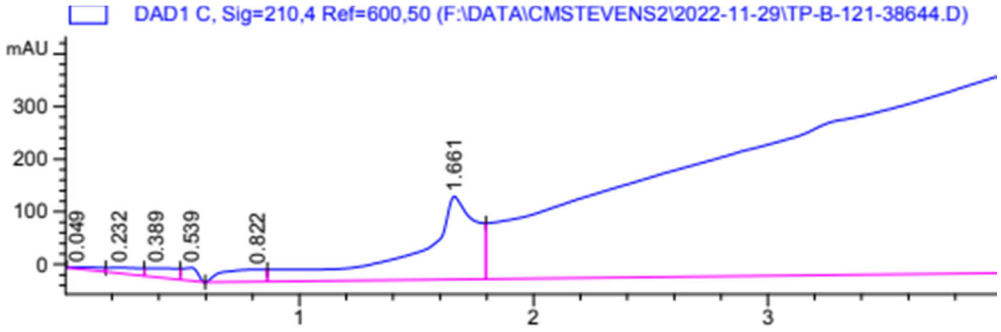
HPLC Profile of **7**



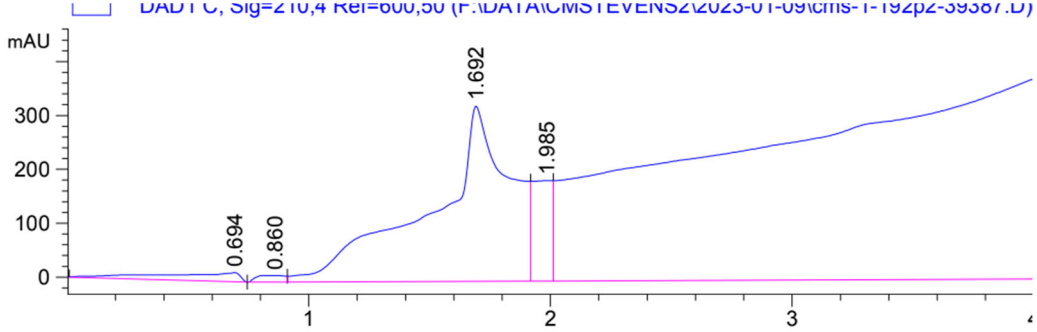
HPLC Profile of **8**



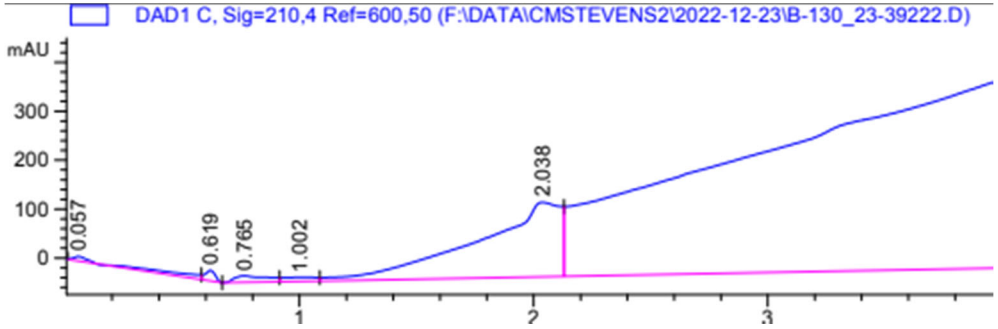
HPLC Profile of **9**



HPLC Profile of 12



HPLC Profile of **19**



HPLC Profile of **24**