Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Copper-Responsive Liposomes for Triggered Cargo Release Employing a Piconilamide–Lipid Conjugate

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General Experimental

Reagents and solvents were generally purchased from Acros, Sigma–Aldrich, or Fisher Scientific and used without further purification. PC (L-α-phosphatidylcholine, mixed isomers from chicken eggs), PA (L-α -phosphatidic acid sodium salt from chicken eggs), DOPE (1,2-dioleoyl-*sn-glycero*-3-phosphoethanolamine), DOTAP(1,2-dioleoyl-3-trimethylammonium-propane) and DPPC (1,2-dipalmitoyl-*sn-glycero*-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL). Dry solvents were obtained from a Pure solvent delivery system purchased from Innovative Technology; Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies. NMR spectra were obtained using Varian 500 MHz and/or Bruker 500 MHz spectrometers. Mass spectra were obtained with JEOL DART-AccuTOF and Waters Synapt G2-Si mass spectrometers (Milford, MA). Liposome extruder and poly-carbonate membranes were obtained from Avestin (Ottawa, Canada). Ultrapure water was purified via a Millipore water system (≥18 MW·cm triple water purification system). Osmotic pressure measurements were done with a OsmoTECH-Single-Sample Micro-Osmometer (Advanced Instruments, Norwood, MA). Small quantities (<5mg) were weighed on a OHRUS analytical-grade mass balance. Fluorescence studies were performed using a Cary Eclipse Fluorescence Spectrophotometer from Agilent Technologies. Plots. Plots were generated using Origin Pro 2022_SR1. Error bars indicate standard error over triplicate experiments.

Synthesis

*(2R)-3-((Hydroxy(2-(picolinamido)ethoxy)phosphoryl)oxy)propane-1,2-diyl dioleate (**1**).* Picolinic acid (0.017 g, 0.134 mmol) and hydroxybenzotriazole (HOBt) (0.025 g, 0.134 mmol) were added to a dried 50 mL round bottom flask under argon atmosphere. Dry DMF (3 mL) was then added to the flask via a syringe. DOPE (0.050 g, 0.0672 mmol; dissolved in chloroform, obtained from Avanti Polar Lipids) was added to the flask and the mixture was stirred at 0°C for 5 minutes. After

adding 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (0.026 g, 0.134 mmol) and *N*,*N*-diisopropylethylamine (DIEA) (0.029 mL, 0.168 mmol) to the reaction mixture, ice bath was removed, and the reaction was allowed to stir overnight at room temperature. The reaction was diluted with chloroform (20 mL) and the organic phase was washed five times with water (25 mL) and twice with brine (25 mL). Upon drying with Na_2SO_4, the crude mixture was filtered and concentrated under reduced pressure. Column chromatography using silica as stationary phase and gradient elution from 100% chloroform to 20% methanol-chloroform afforded **1** (0.030 g, 0.035 mmol, 53% yield) as a pale-yellow oil. ^{1}H NMR (500 MHz, 20% CD_3OD-$CDCl_3$) δ 8.43 (d, *J* = 3.8 Hz, 1H), 8.02 (d, *J* = 7.8 Hz, 1H), 7.76 (td, *J* = 7.8, 1.7 Hz, 1H), 7.34 (td, *J* = 7.7, 4.8 Hz, 1H), 5.21 (m, *J* = 5.2 Hz, 4H), 5.07 (dq, *J* = 8.9, 5.2 Hz, 1H), 4.21 (dd, *J* = 12.0, 3.3 Hz, 1H), 3.99 (dd, *J* = 12.1, 6.7 Hz, 1H), 3.94 – 3.83 (m, 5H), 3.58 (t, *J* = 5.7 Hz, 2H), 2.15 (td, *J* = 7.6, 2.4 Hz, 4H), 1.88 (q, *J* = 6.5 Hz, 8H), 1.45 (t, *J* = 7.3 Hz, 5H), 1.07-1.23 (m, 46H), 0.75 (t, *J* = 6.8 Hz, 7H).^{13}C NMR (126 MHz, 20% CD_3OD-$CDCl_3$) δ 173.70, 173.32, 165.21, 149.17, 148.26, 137.54, 129.96, 129.58, 126.54, 122.27, 70.22, 64.20, 63.60, 62.46, 49.31, 49.14, 48.97, 48.80, 48.63, 48.46, 48.29, 39.96, 34.08, 33.94, 31.82, 29.66, 29.60, 29.43, 29.22, 29.14, 29.06, 29.00, 27.12, 27.08, 24.78, 24.74, 22.57, 13.90. ^{31}P NMR (202 MHz, 20% CD_3OD-$CDCl_3$) δ -2.94. ESI-MS $[M+H]^+$ calcd for $C_{47}H_{81}N_2O_9P$: 849.5680, found: 849.5732.

*4-(((tert-Butyldimethylsilyl)oxy)methyl)phenol (**3**).* 4-(Hydroxymethyl)phenol (1 g, 8.064 mmol) was added to a 100 mL argon (Ar) protected round bottom flask. The solution was cooled to 0°C after addition of 15 mL dry DMF. This was followed by addition of *tert*-butyldimethylsilyl chloride (TBDMS-Cl) (1.46 g, 9.67 mmol) and imidazole (0.658 g, 9.67 mmol). The reaction mixture was stirred at room temperature for 2 hours. Upon completion, the reaction mix was diluted with 15 mL diethyl ether and the organic layer was washed with saturated ammonium chloride solution (40 mL) and brine (40 mL). After drying with Na_2SO_4, the crude was filtered and concentrated under reduced pressure. It was then loaded onto a silica column and purified using gradient elution from 100% hexanes to 20% ethyl acetate-hexanes. The product **3** (1.3563 g, 5.695 mmol, 70% yield) was obtained as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.06 (d, *J* = 8.4 Hz, 2H), 6.63 (d, *J* = 8.5 Hz, 2H), 5.80 (s, 1H), 4.56 (s, 2H), 0.84 (s, 9H), 0.00 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 154.80, 133.14, 128.05, 115.28, 65.05, 26.05, 18.51, -5.10. DART-MS $[M+H]^+$ calcd for $C_{13}H_{22}O_2Si$: 239.1467, found: 239.1338.

*4-(((tert-Butyldimethylsilyl)oxy)methyl)phenyl picolinate (**4**).* Compound **3** (0.2 g, 0.839 mmol) was added to a 50 mL Ar(g) protected flask. 5mL dry DCM was added to dissolve **3** followed by addition of picolinic acid (0.113 g, 0.9236 mmol), EDC (0.217 g, 1.133 mmol) and DMAP (0.0256 g, 0.209 mmol). The reaction mixture was left to stir overnight and poured into 1N HCl (20 mL) upon completion. The crude was then extracted into DCM (30 mL) and washed thrice with 30 mL water and once with 30 mL brine, dried using Na_2SO_4, filtered, and concentrated under reduced pressure. A silica column packed with hexanes was used to obtain pure **4** (0.256 g, 0.7452 mmol, 89% yield) using a gradient elution from 100% hexanes- 50% ethyl acetate-hexanes. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (d, *J* = 3.9 Hz, 1H), 8.26 (d, *J* = 7.8 Hz, 1H), 7.95 – 7.84 (m, 1H), 7.53 (d, *J* = 7.5, 4.8 Hz, 1H), 7.38 (d, *J* = 8.2 Hz, 2H), 7.21 (d, *J* = 8.4 Hz, 2H), 4.75 (s, 2H), 0.94 (s, 9H), 0.10 (s, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.05, 150.19, 149.82, 147.65, 139.44, 137.28, 127.46, 127.15,

125.92, 121.46, 64.51, 26.04, 18.50, -5.16. DART-MS $[M+H]^+$ calcd for $C_{19}H_{25}NO_3Si$: 344.1682, found: 344.1741.

*4-(Hydroxymethyl)phenyl picolinate (**5**).* Methanol (10 mL) was added to a round-bottom flask containing compound **4** (0.256 g, 0.7452 mmol) followed by addition of 0.1 mL concentrated HCl solution. The reaction mixture was stirred at room temperature for 30 minutes. Upon completion, saturated sodium bicarbonate (20 mL) was poured into the reaction mixture followed by extraction into 30 mL DCM. The organic layer was then washed thrice with 30 mL water and once with 30 mL brine. The organic layer containing **5** was then dried using Na_2SO_4, filtered, and concentrated under reduced pressure. Compound **5** was obtained in a 76% yield (0.134 g, 0.584 mmol) and was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$) δ 8.55 (d, *J* = 4.2 Hz, 1H), 7.99 (d, *J* = 7.8 Hz, 1H), 7.76 (td, *J* = 7.8, 1.6 Hz, 1H), 7.43 – 7.36 (m, 1H), 7.04 (d, *J* = 8.4 Hz, 2H), 6.65 (d, *J* = 8.4 Hz, 2H), 3.92 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$) δ 165.44, 156.11, 149.36, 147.28, 137.45, 131.96, 128.60, 127.22, 125.14, 115.03, 64.21. DART-MS $[M+H]^+$ calcd for $C_{13}H_{11}NO_3$: 230.0817., found: 239.0903.

*4-((((4-Nitrophenoxy)carbonyl)oxy)methyl)phenyl picolinate (**6**).* Dry DCM (5 mL) was added to an Ar (g) protected flask containing **5** (0.1 g, 0.436 mmol) at 0°C. This was followed by addition of 4-nitrophenyl chloroformate (0.132 g, 0.655 mmol) and triethylamine (0.121 mL, 0.873 mmol). After stirring at 0°C for 5 minutes, the ice bath was removed, and the reaction mixture was left to stir overnight. Upon completion, the crude reaction mixture was diluted with 10 mL DCM, washed five times with 35 mL saturated sodium bicarbonate, twice with 35 mL water and once with 35 mL brine. After drying with Na_2SO_4, filtration and concentration, the crude was subjected to silica column chromatography using 100% hexanes to 50% ethyl acetate -hexanes. Compound **6** was obtained in 48% yield (0.0820 g, 0.208 mmol). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.85 (d, 1H), 8.30 – 8.23 (m, 3H), 7.92 (td, *J* = 7.8, 1.7 Hz, 1H), 7.57 (dd, *J* = 7.1, 5.3 Hz, 1H), 7.54 – 7.49 (m, 2H), 7.42 – 7.36 (m, 2H), 7.34 – 7.28 (m, 2H), 5.31 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$) δ 163.84, 155.58, 152.52, 151.50, 150.27, 147.32, 145.53, 137.39, 132.24, 130.21, 127.69, 126.05, 125.41, 122.26, 121.90, 70.34. DART-MS $[M+H]^+$ calcd for $C_{20}H_{14}N_2O_7$: 395.0879, found: 395.0861.

*(2R)-3-((Hydroxy(2-((((4-(picolinoyloxy)benzyl)oxy)carbonyl)amino)ethoxy)phosphoryl)oxy)propane-1,2-diyl dioleate (**2**).* DOPE (0.058 g, 0.078 mmol) and **6** (0.039 g, 0.100 mmol) were combined in a 50 mL RBF under an Ar (g) atmosphere. Ethyl acetate (3 mL) was then added to dissolve the starting materials at 0 °C. After adding triethylamine (28.1 µL, 0.201 mmol), the reaction was stirred overnight. The aqueous layer was extracted thrice with 30 mL chloroform. The combined organic layer was washed five times with 40 mL water and once with 40 mL brine. The collected organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The resulting crude was subjected to silica gel column chromatography. Gradient elution from 100% chloroform to 20% MeOH in chloroform afforded the compound **2** as a clear oil (0.006g, 0.006 mmol, 8% yield). ^{1}H NMR (500 MHz, 20% CD_3OD-$CDCl_3$) δ 8.75 (d, *J* = 4.4 Hz, 1H), 8.23 (d, *J* = 7.8 Hz, 1H), 7.91 (td, *J* = 7.8, 1.7 Hz, 1H), 7.56 (dd, *J* = 7.1, 5.3 Hz, 1H), 7.37 (d, *J* = 8.4 Hz, 2H), 7.19 (d, *J* = 8.4 Hz, 2H), 5.32 – 5.27 (m, 4H), 5.13 (d, *J* = 23.4 Hz, 1H), 5.05 (s, 2H), 4.31 (dd, *J* = 12.1, 3.1 Hz, 1H), 4.09 (dd, *J* = 12.1, 6.7 Hz, 1H), 3.91 – 3.82 (m, 6H), 3.34 (d, *J* = 7.0 Hz, 2H), 2.24 (q, *J* = 6.8 Hz, 4H), 1.95 (q, *J* =

6.4 Hz, 8H), 1.53 (s, 4H), 1.22 (d, *J* = 13.3 Hz, 40H), 0.81 (q, *J* = 6.1 Hz, 6H). ^{31}P NMR (202 MHz, 20% CD_3OD-$CDCl_3$) δ -2.42. ESI-MS $[M+H]^+$.

Liposome experiments

Preparation of liposomes encapsulating Carboxyfluorescein (CF). Stock solutions for lipid **1**, bulk lipids were prepared in methanol/chloroform mix and stored in a -20°C freezer. Adequate volumes of each stock solution were then pipetted into clean, dry vials to obtain the desired lipid percentages at a specific lipid content (5 mM). The solvents were evaporated under nitrogen stream and the resulting lipid film was further dried under vacuum for at least two more hours. A 50 mM solution of CF in 20 mM Tris buffer, pH 7.4 was prepared (pH was adjusted back to 7.4 with addition of 1 M sodium hydroxide). The resulting lipid film was then hydrated with the 50 mM CF stock solution in a 60ºC water bath for 4 sets of 15 mins with vigorous vortexing after each set. This was followed by ten freeze-thaw cycles, alternating between a dry ice-acetone bath and a 60ºC water bath. The solutions were then extruded through a 200 nm polycarbonate membrane for 31 passes using a LiposoFast extruder (Avestin, Inc.). Finally, the unencapsulated dye was removed with a size exclusion column (SEC). Sephadex G-50 used for SEC was pre-equilibrated with 20 mM Tris buffer (pH 7.4, osmotic pressure adjusted to 188 $mOsmkg^{-1}$ by addition of solid NaCl) for at least one hour prior to use. A micro-column was used for separation. Fractions were collected every 1 mL, and the yellowish-brown fraction was usually the liposome solution with the quenched CF dye compared to fluorescent green appearance of the dye in unquenched state. This was further confirmed by adding Triton X- 100 after which an increase in fluorescence intensity was observed depicting the release of the encapsulated dye.

CF release assays with copper addition. A 80 µL aliquot of the prepared liposome solution was added into a sub-micro quartz cuvette. Corresponding amounts of 200 mM copper chloride solution in Tris buffer pH 7.4 were then added to the cuvette Fluorescence intensity was then measured over time (excitation wavelength=492 nm, excitation slit=2.5 nm, emission slit=2.5 nm). After completion, 1 µL of an aqueous 10% Triton X-100 solution was added to trigger complete release. Control experiments were also done by adding Tris buffer into liposomes containing **1** or adding copper into liposomes without **1**. When processing the data, fluorescence intensities at 525 nm were selected and fluorescence increases were reported as a percentage of the fluorescence after triton X-100 treatment for each sample. Experiments were run at least 3 times each with different batches of liposomes, and averaged data were reported with error bars showing standard error.

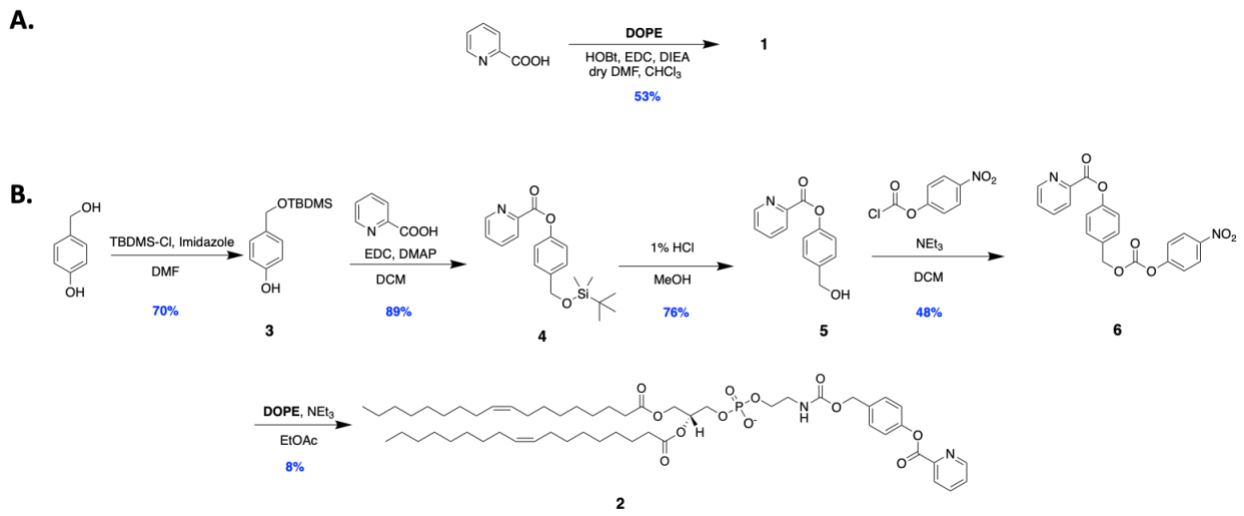
Dynamic Light Scattering (DLS) analysis of particle sizes before and after copper addition. DLS measurements were carried out with a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW laser operating at l = 633 nm. Samples were prepared by diluting the liposome solutions before or after adding copper by 10x with Tris buffer. All samples were determined at a scattering angle of 173° at 20°C. The experiments were run in triplicates and data reported as average with error bars depicting standard error.

Selectivity studies for various metal ions. Stock solutions of different metals (200 mM) were prepared in Tris buffer {$CuCl_2.2H_2O$, $CoCl_2.6H_2O$, $NiCl_2.6H_2O$, $Zn(OAc)_2$, $MnSO_4.H_2O$, $CaCl_2.2H_2O$, $Mg(OAc)_2.4H_2O$, NaCl, KCl, and $Cu(MeCN)_4(PF_6)$ }. A 80 µL of the prepared liposome solution was added into a sub-micro quartz cuvette. 0.4 µL of prepared stock solutions in Tris buffer were then added to the cuvette. Fluorescence intensities of the resulting solution were then measured after a 2-hour incubation period at room temperature (excitation wavelength = 492 nm, excitation slit = 2.5 nm, emission slit = 2.5 nm). The same liposome solution was used for one set of comparison studies using each of the described metals. Intensities at 525 nm were selected when processing the data and reported as percentage of the fluorescence after triton X-100 treatment for each sample. Experiments were run in triplicate sets with a different batch of liposome used each time and averaged data were reported with error bars showing standard error.

NMR analysis of free lipid 1 after treatment with copper chloride. For analysis of free lipid, compound **1** was dissolved in a mixture of $CDCl_3/CD_3OD/D_2O$=65/25/4 (v/v) at a concentration of 1.15 mM. A 0.6 mL solution was transferred into an NMR tube for analysis. After taking an initial spectrum, 1 µL of 200 mM copper chloride dissolved in tris buffer was added and NMR spectra were recorded over time and analyzed by MestReNova.

Mass spectrometry analysis of free lipid 1 after copper treatment. For free lipid **1**, 0.50 µL of a 200 mM copper chloride solution in Tris buffer was added into 200 µL of a 1 mM stock solution lipid **1** in 20% methanol/chloroform and incubated for 2 hours. The mixture was next diluted to 1 mL total volume with methanol before being subjected to the mass spectrometer. The final concentration was ~200 µM. ESI positive mode was used for detection.

Liposome stability test. CF leakage assays were carried out with a Cary Eclipse Fluorescence Spectrophotometer and DLS measurements were done on a Malvern Zetasizer Nano ZS instrument equipped with a 4.0 mW laser operating at l = 633 nm. The stability of liposomes was tracked by taking fluorescence and DLS measurements every 24 h. When not taking measurements, liposomes were stored at 4 °C. Experiments were conducted in triplicate, and averaged data were reported with error bars denoting standard error.



Scheme S1. Synthesis of target copper-responsive lipids **A) 1** and **B) 2**.

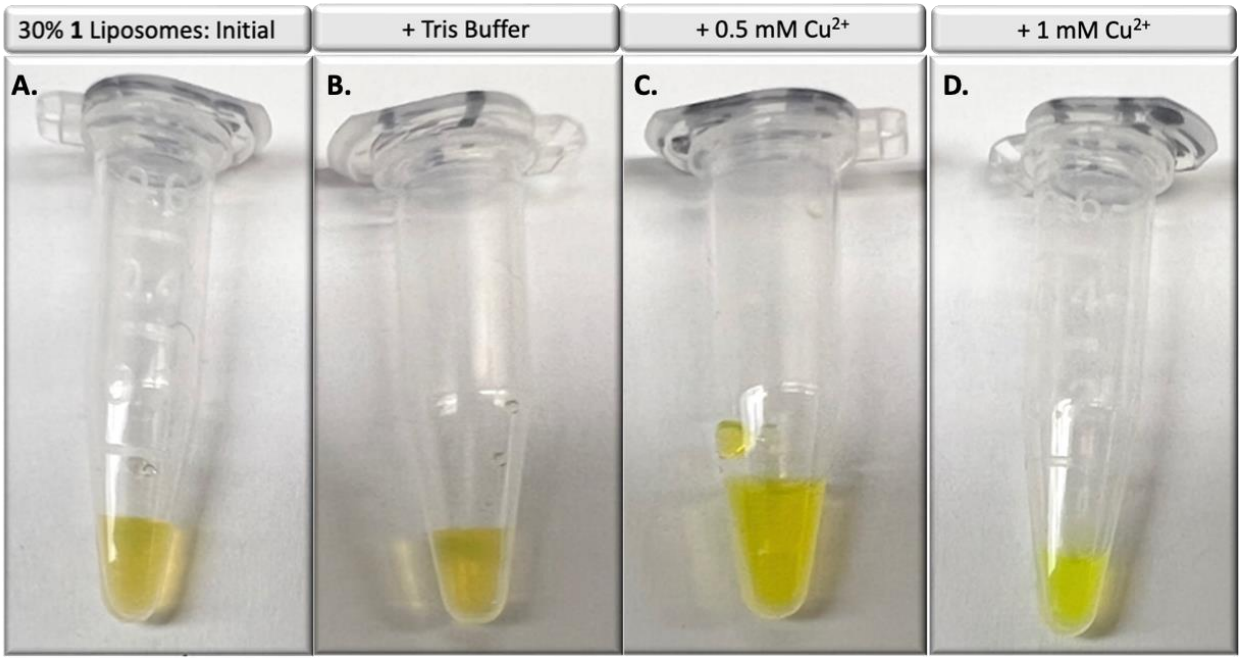


Figure S1. Reaction mix containing liposome solution and various concentrations of copper to suggest dye leakage visually. **(A)** The first Eppendorf containing 30% **1** liposomes before copper addition yellowish-brown in color; **(B)** There is no apparent change in the appearance of liposomes upon addition of tris buffer; and the color of the reaction mix changes to a more fluorescent green with addition of **(C)** 0.5mM Cu^{2+} and **(D)** 1mM Cu^{2+}.

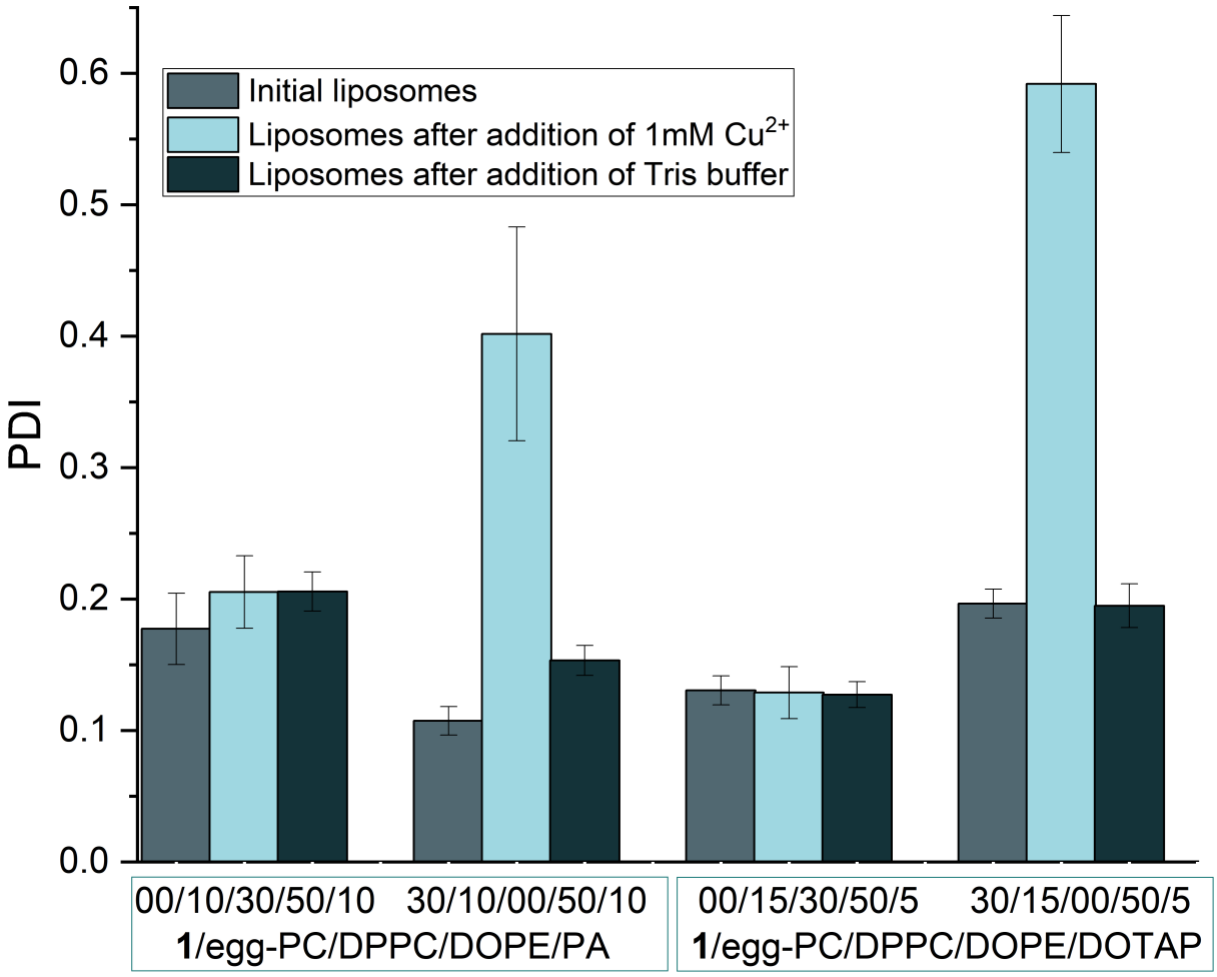


Figure S2. Polydispersity indices (PDIs) for 0-30% **1** liposomes upon addition of 0 or 1 mM copper chloride. An increase in PDI was observed for 30% **1** liposomes after a 2-hour incubation with copper chloride, indicating change in liposome size and morphology. Minimal change was observed for addition of copper to 0% **1** liposomes. Error bars represent standard error over triplicate measurements.

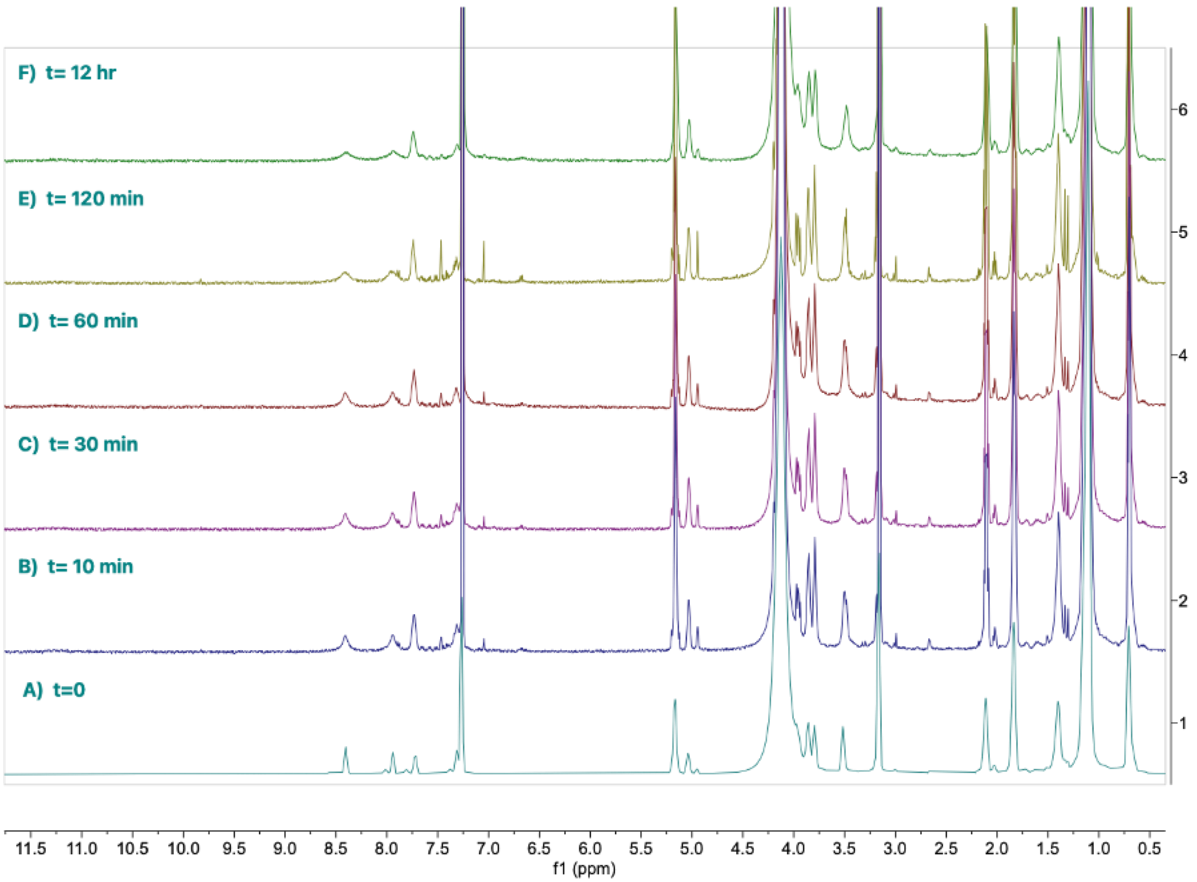


Figure S3. Time-dependent NMR analysis of compound **1** in $CDCl_3/CD_3OD/D_2O$ (65/25/4) upon addition of 0.3mM copper chloride. Trace A: Lipid **1** before copper treatment. Traces B-F. Lipid **1** after treatment with copper chloride, over time.

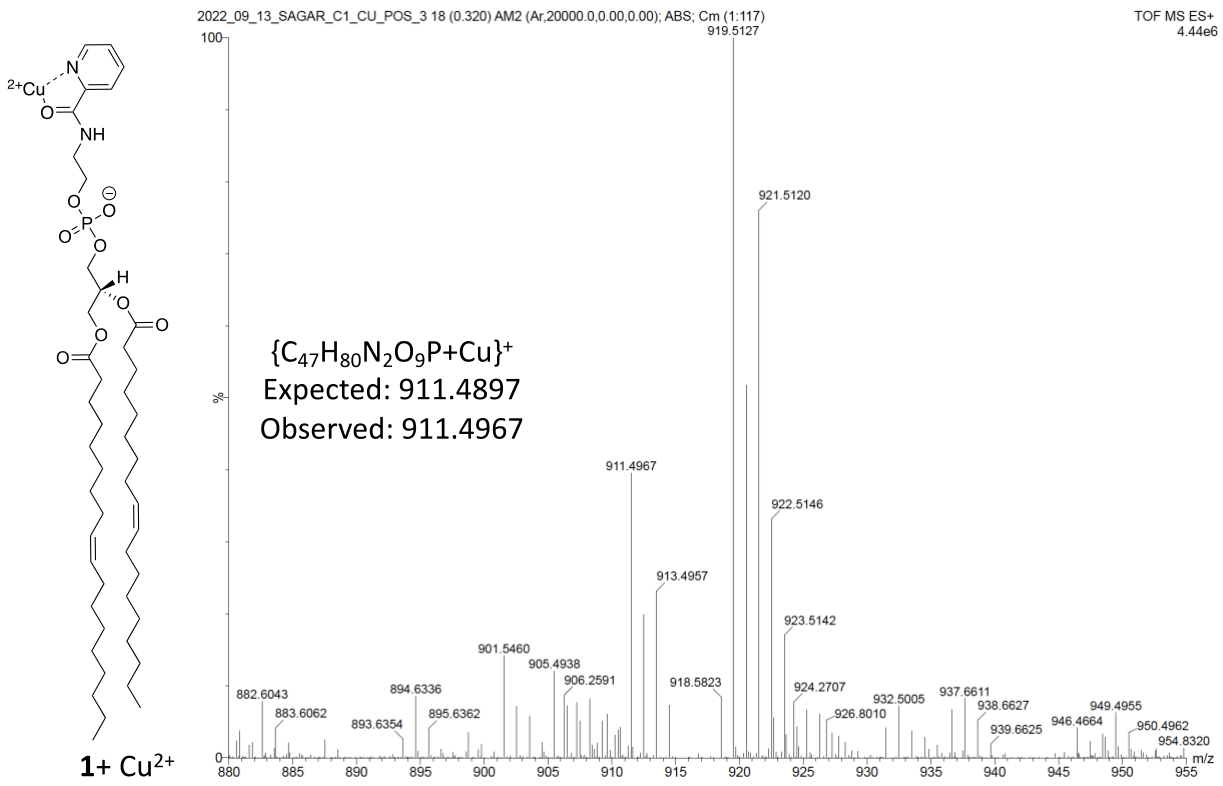


Figure S4. Mass spectrum of **1**-Cu^{2+} complex detected from MS experiments following copper treatment.

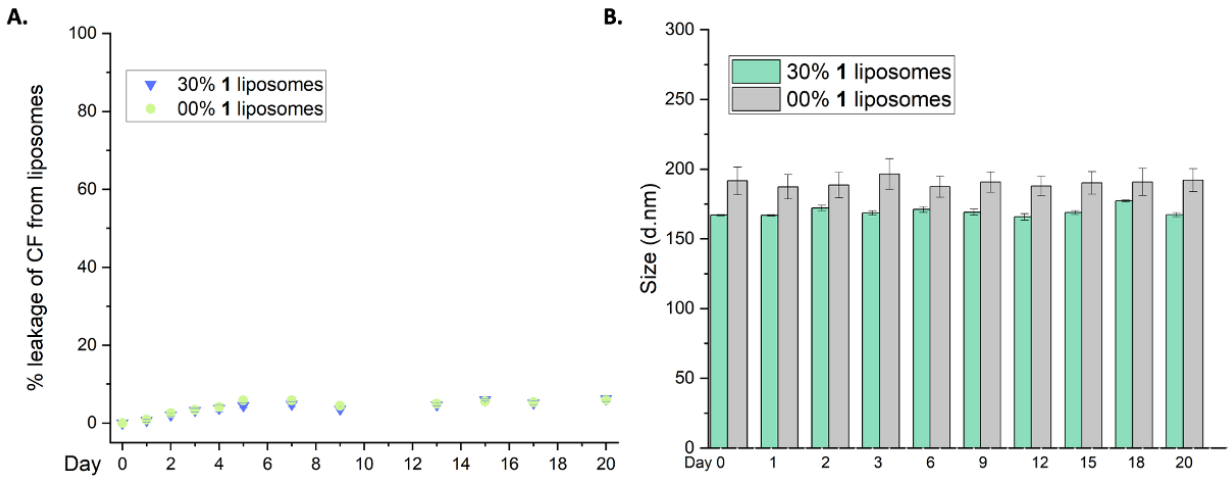
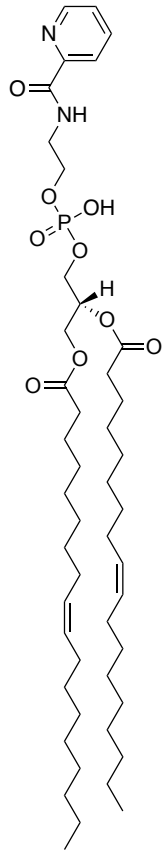


Figure S5. Liposome stability data over a period of 20 days depicted via (A) Fluorescence assay depicting CF leakage from 00-30% **1** liposomes (B) DLS measurements for 00-30% **1** liposomes. Error bars indicate standard error over triplicate measurements.



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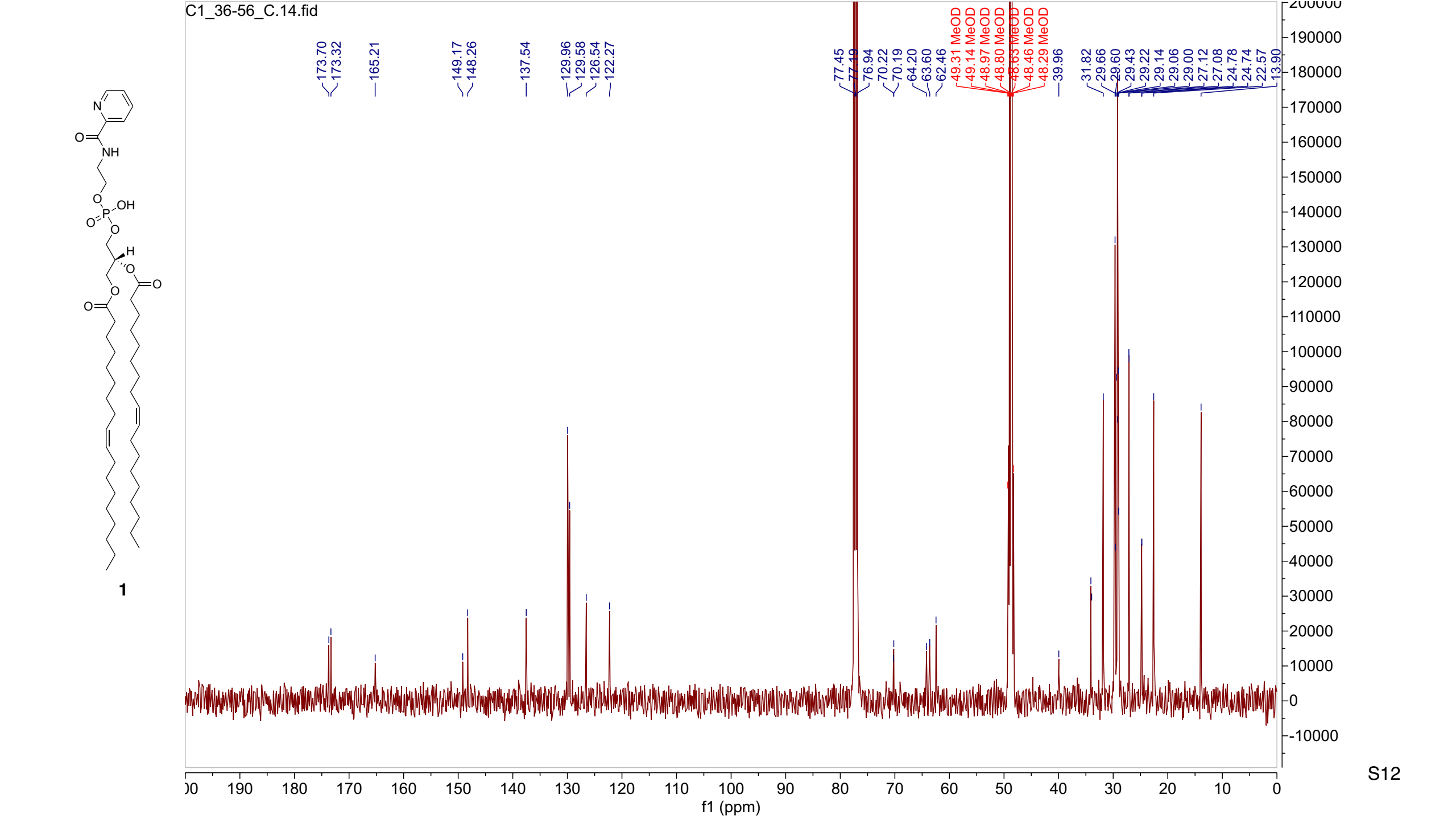
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70.22
70.19
64.20
63.60
62.46
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48.97 MeOD
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48.46 MeOD
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f1 (ppm)

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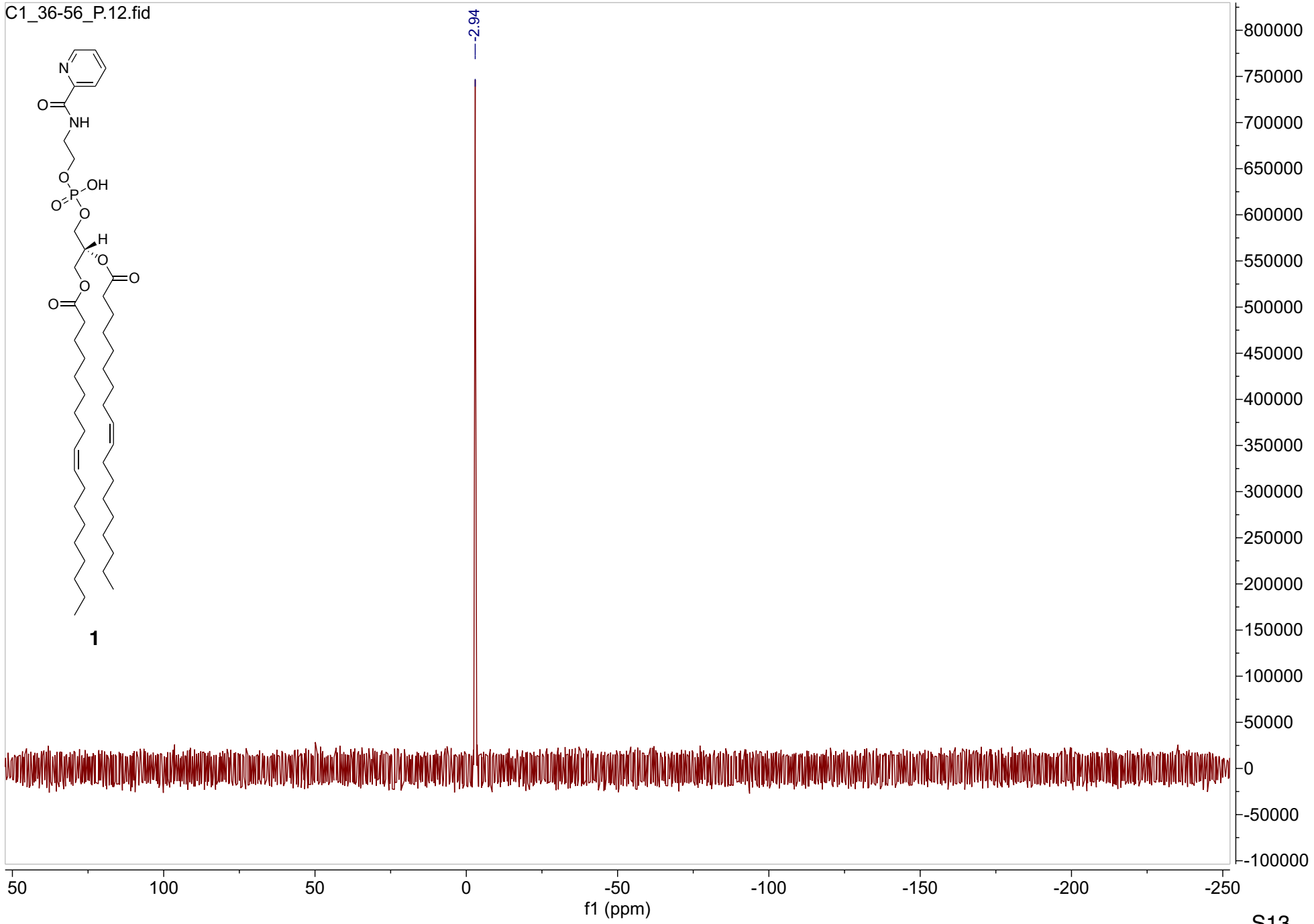
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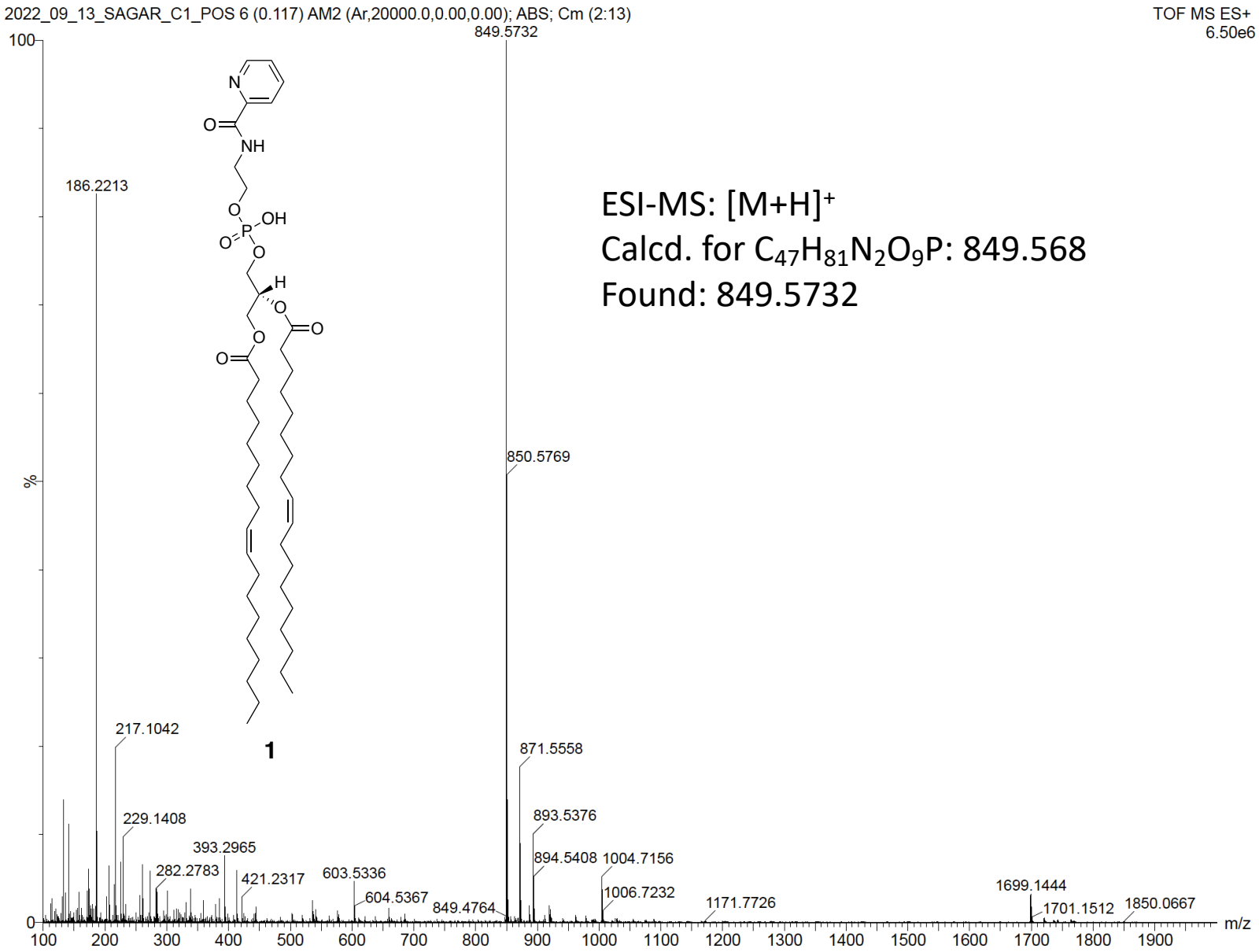
f1 (ppm)



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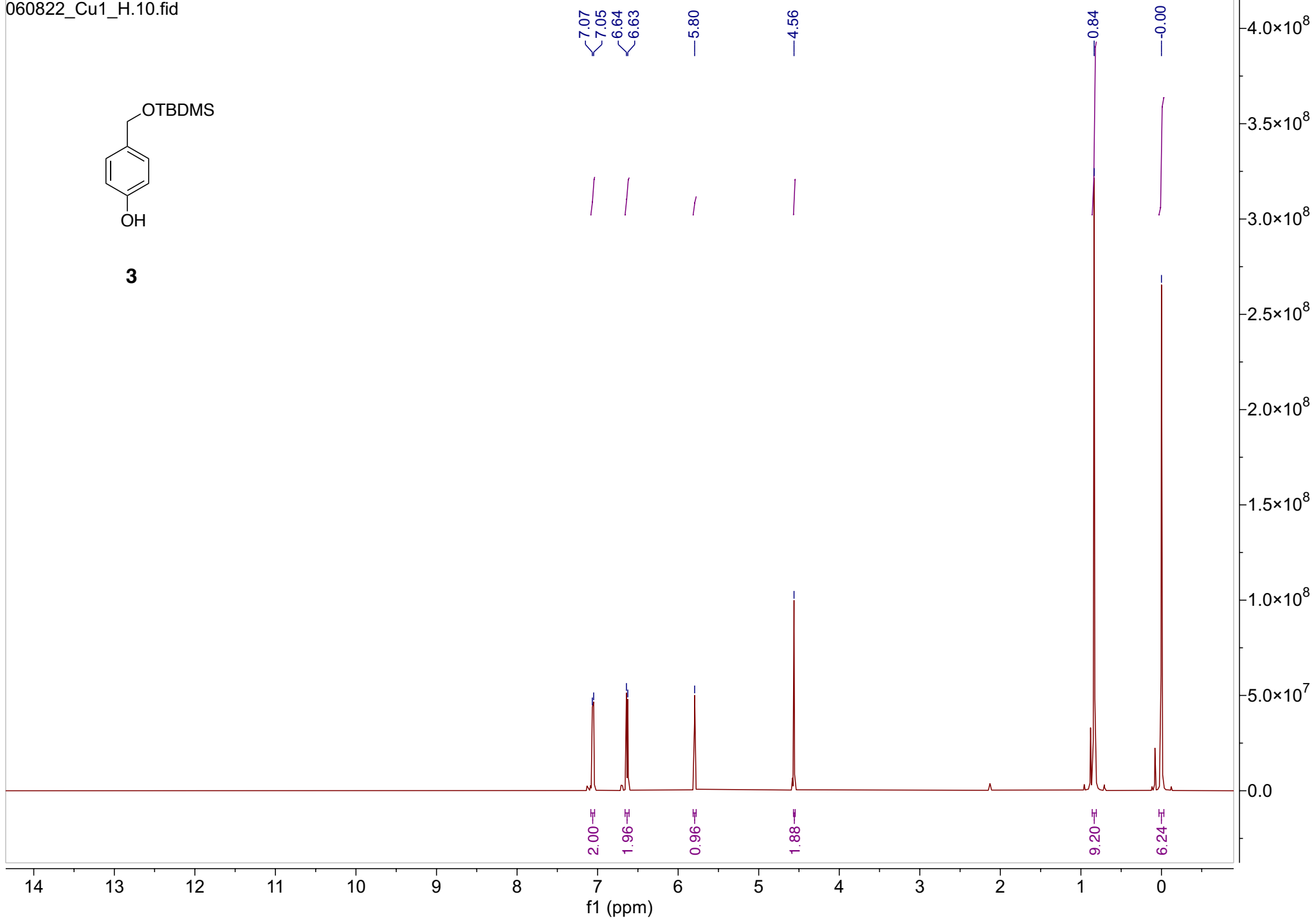
ESI-MS: $[M+H]^+$

Calcd. for $C_{47}H_{81}N_2O_9P$: 849.568

Found: 849.5732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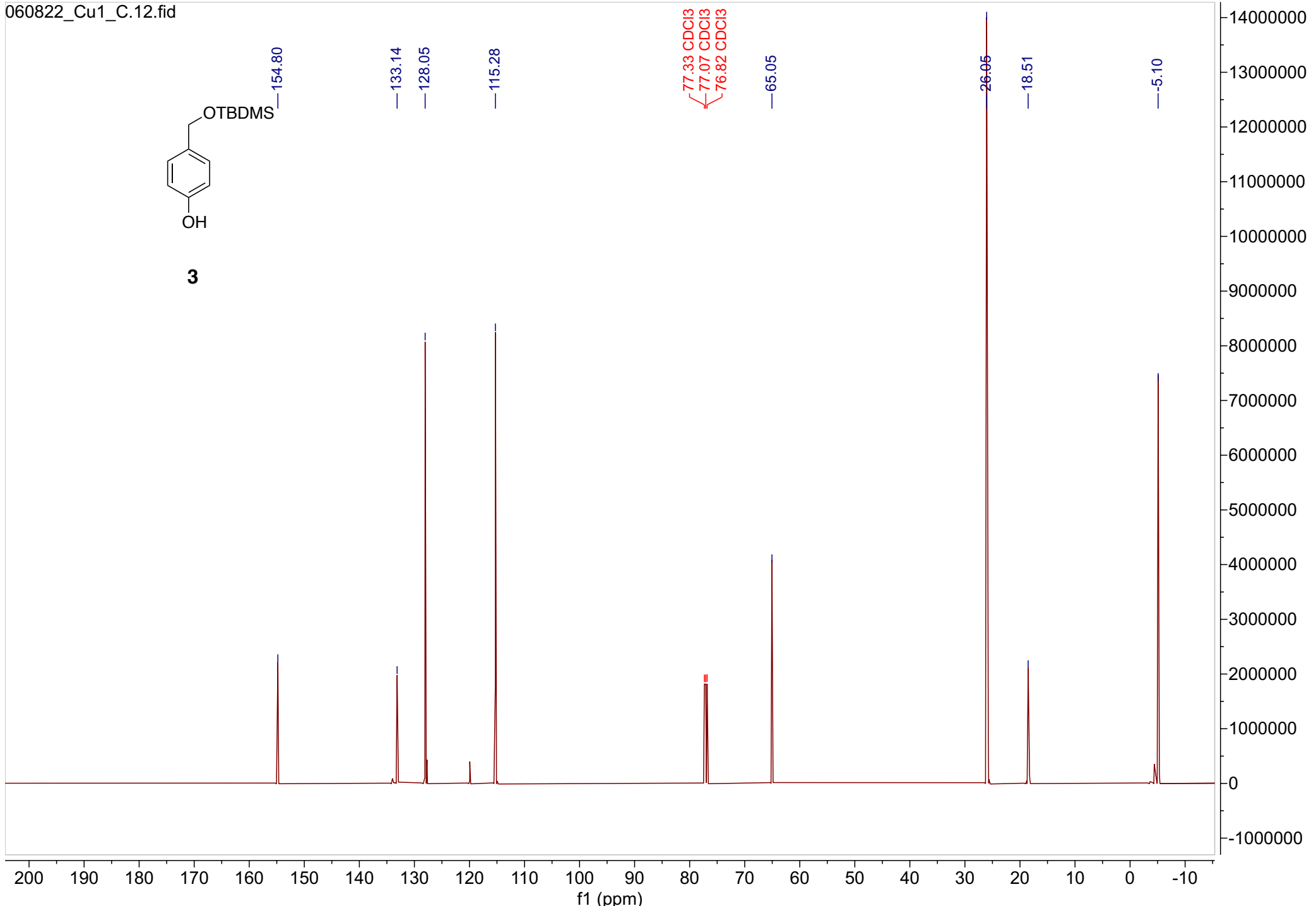
0
f1 (ppm)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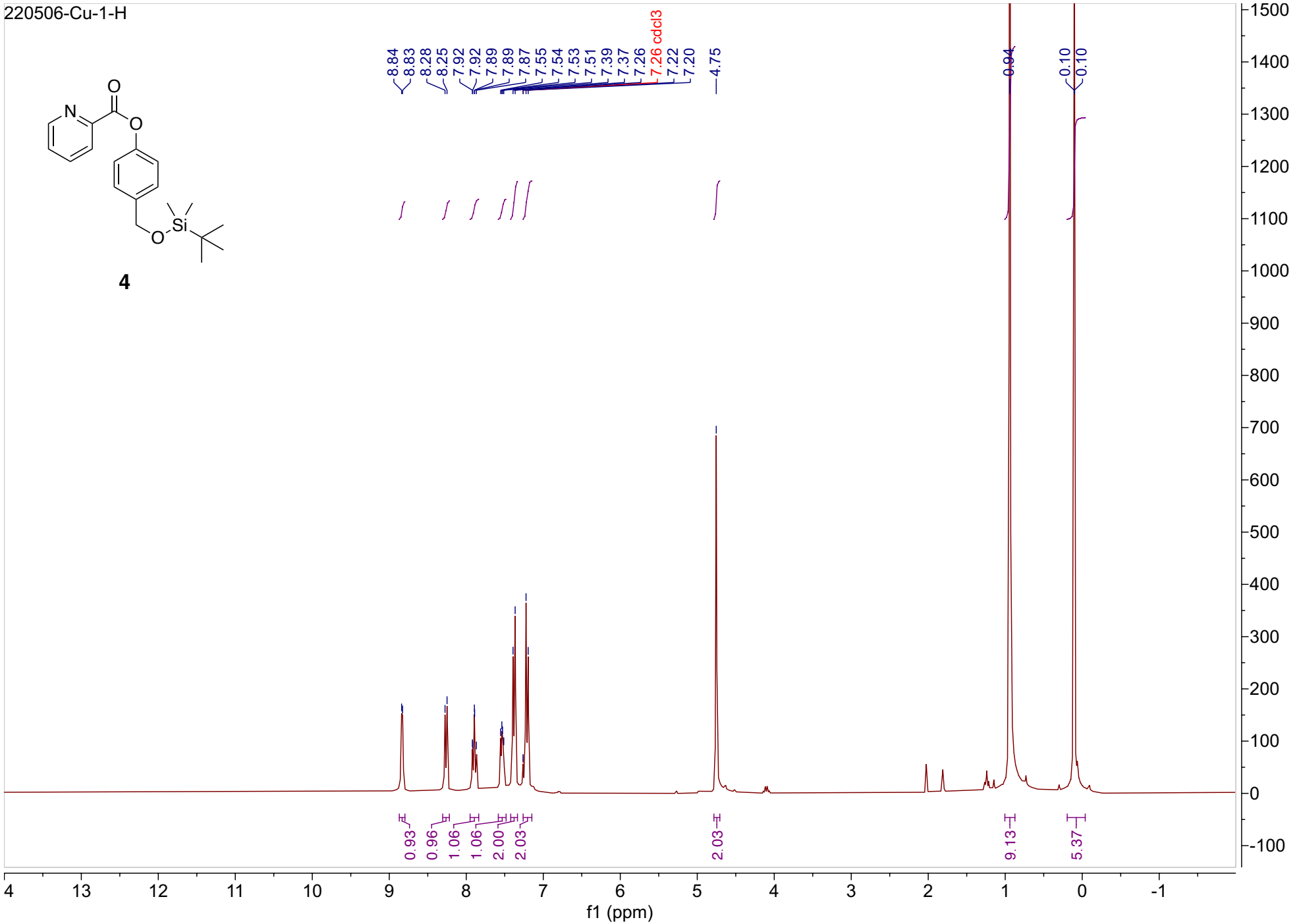
f1 (ppm)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7.89
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7.51
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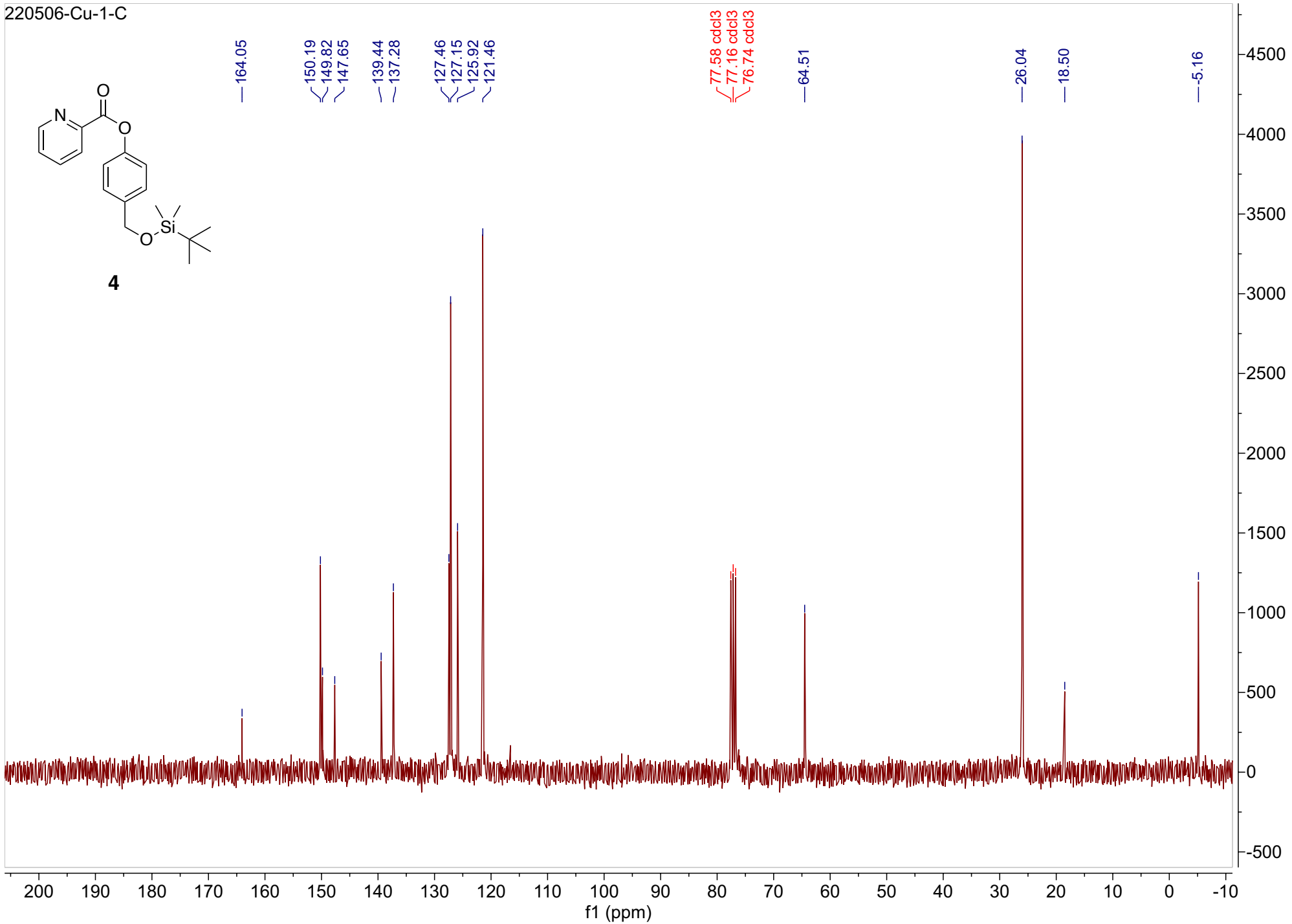
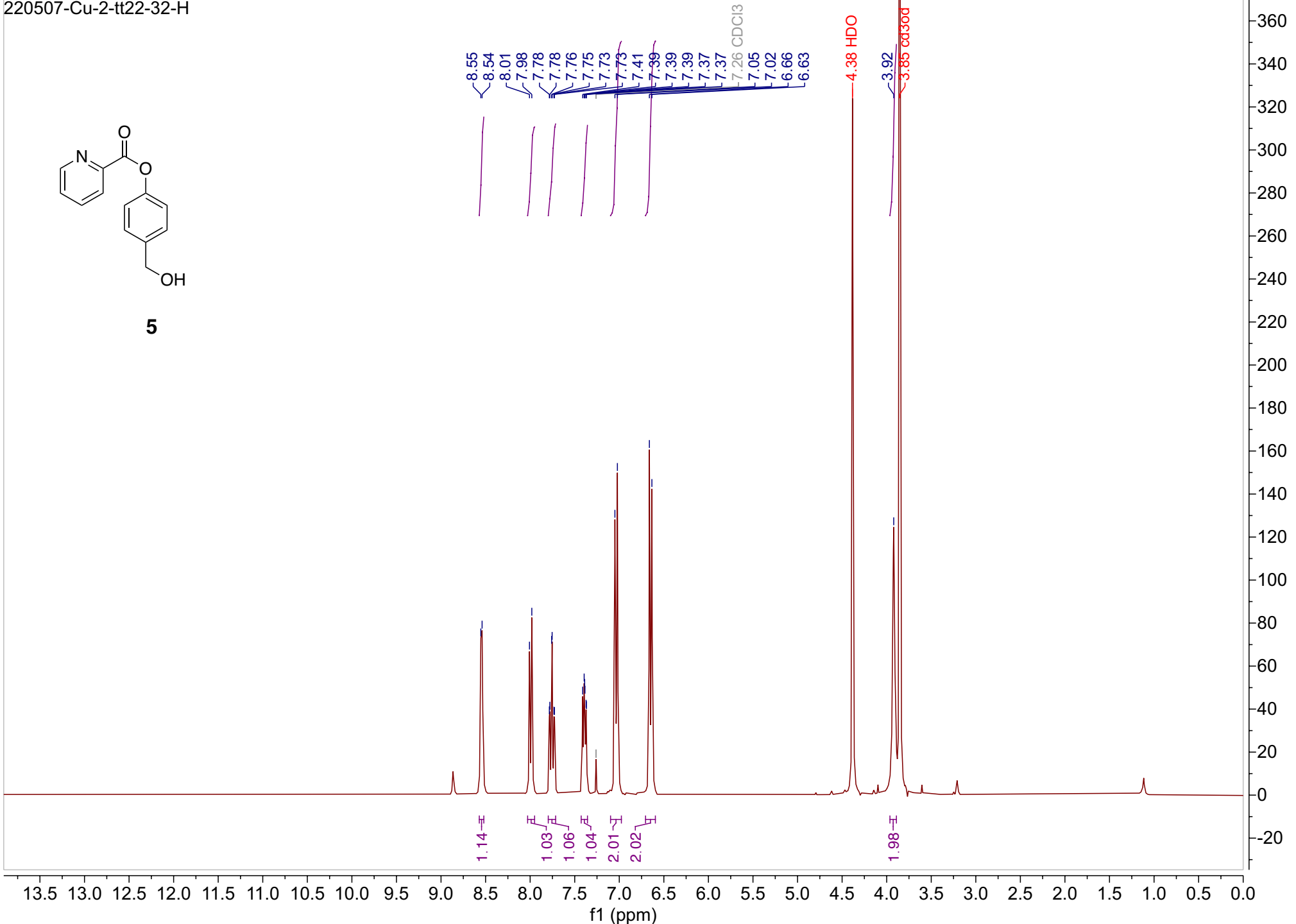
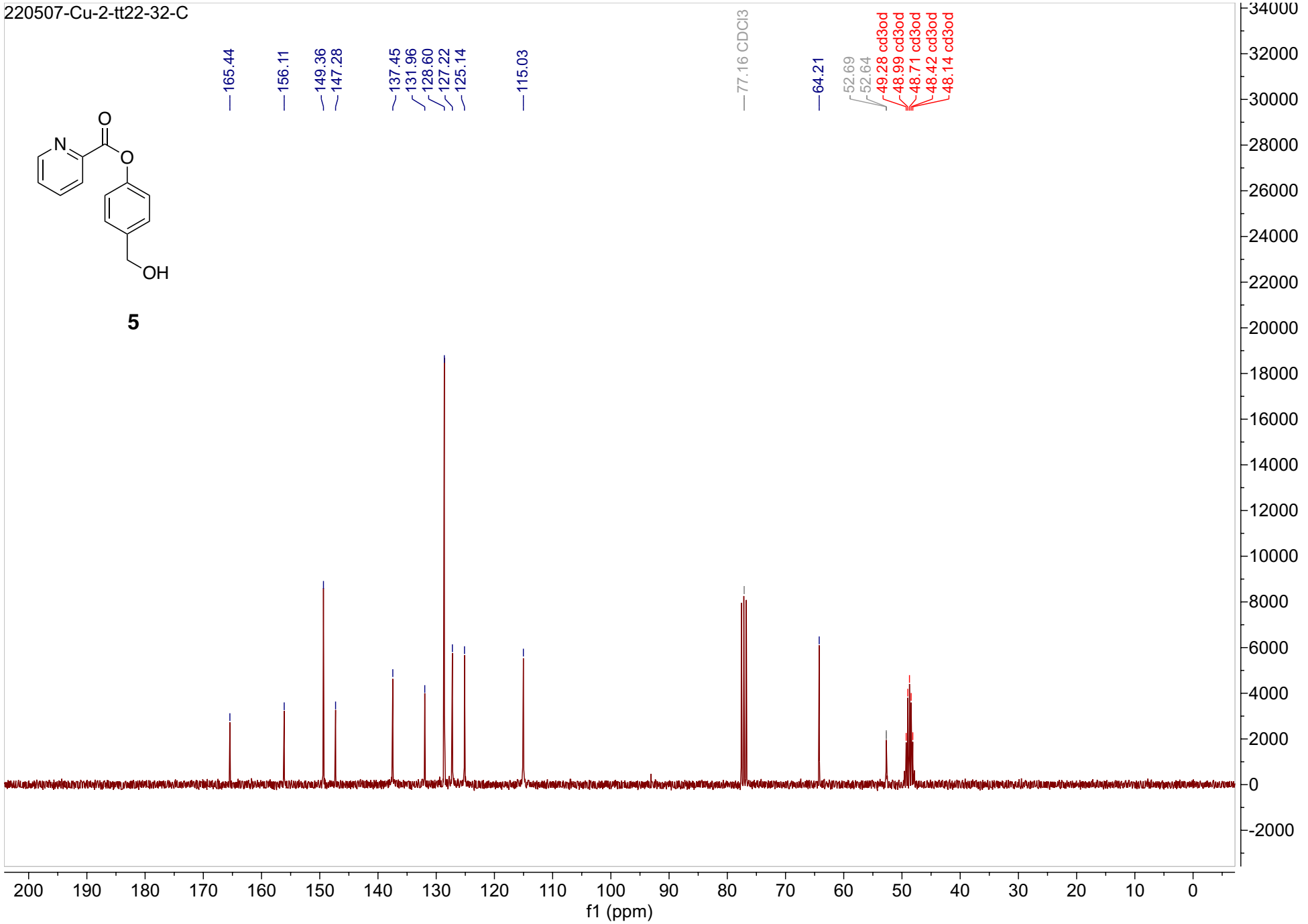
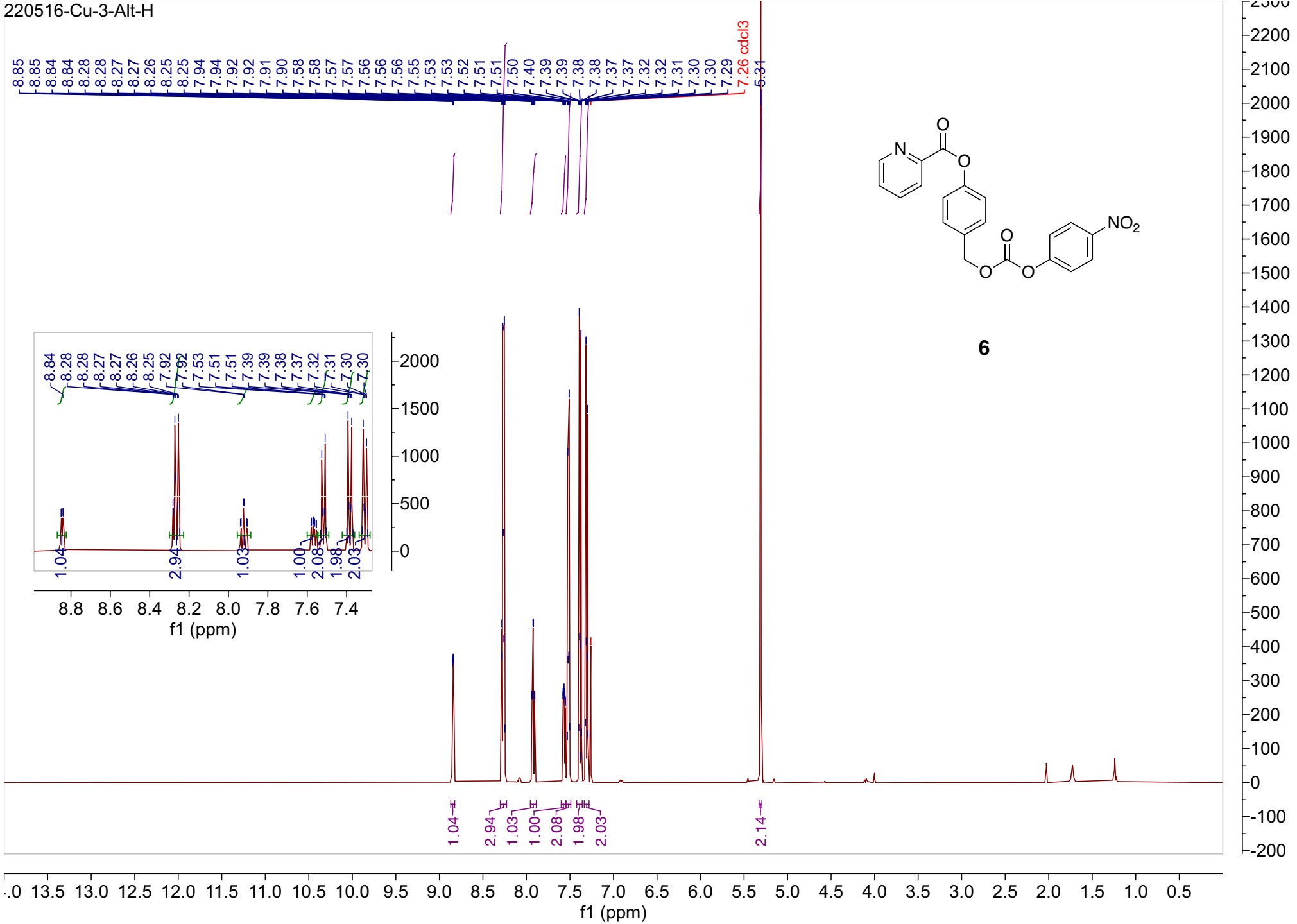
7.37
7.26
7.26 cdcl3
7.22
7.20
4.75
0.94
0.10
0.10
0.93
0.96
1.06
1.06
2.00
2.03
2.03
9.13
5.37
f1 (ppm)

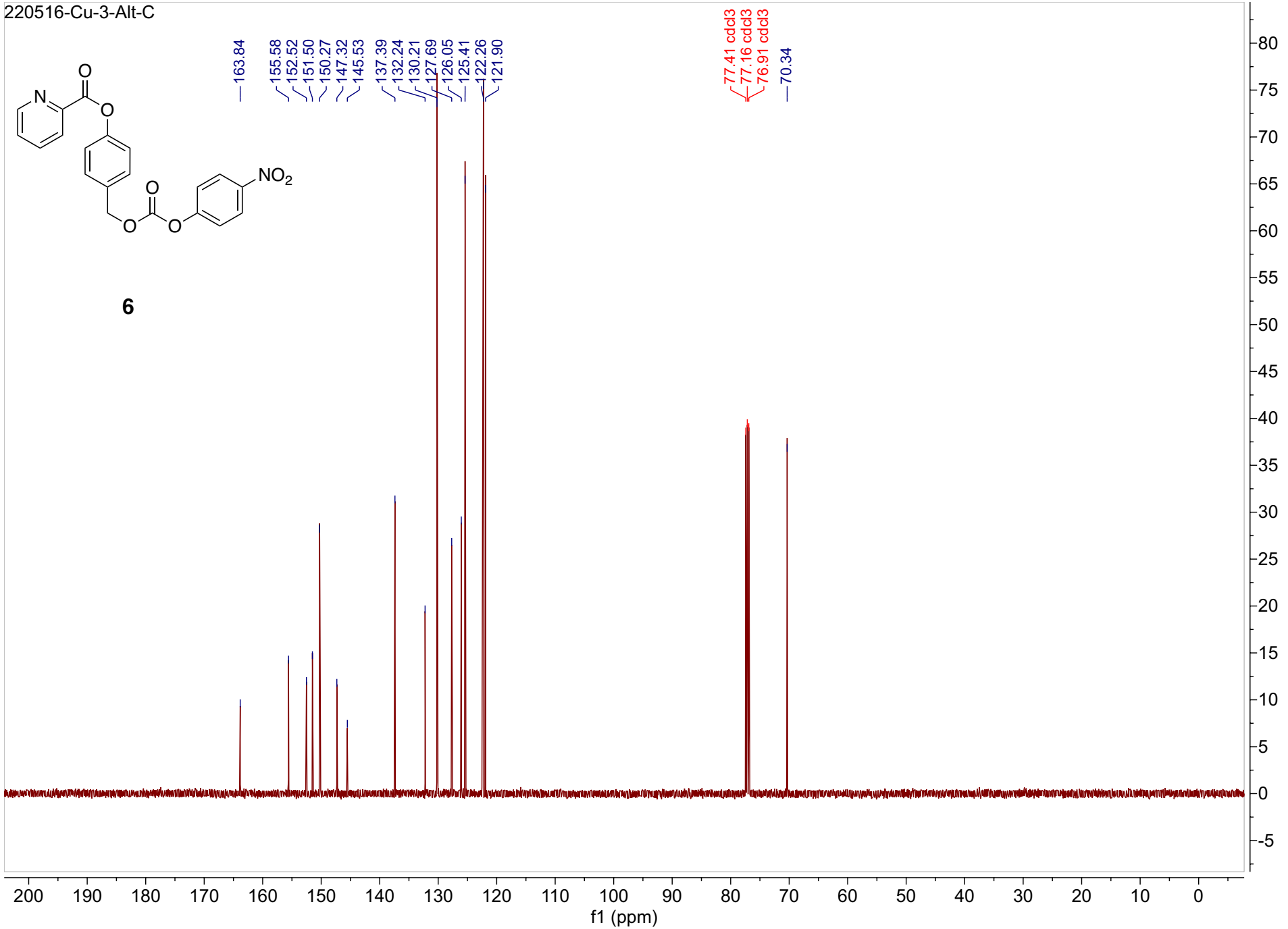

220506-Cu-1-C
164.05
150.19
149.82
147.65
139.44
137.28
127.46
127.15
125.92
121.46
77.58 cdcl3
77.16 cdcl3
76.74 cdcl3
64.51
26.04
18.50
-5.16
4
f1 (ppm)

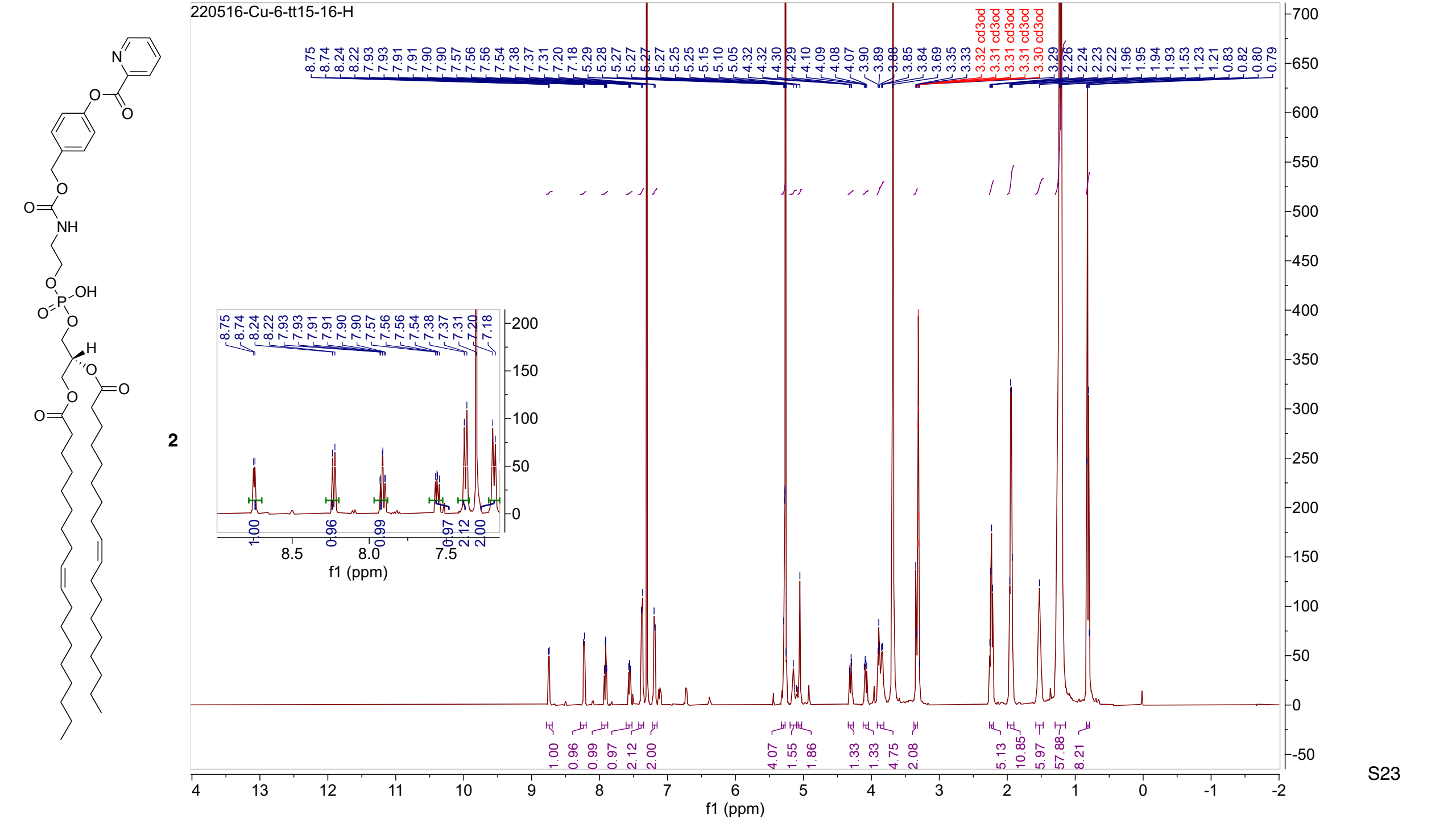

220507-Cu-2-tt22-32-H
8.55
8.54
8.01
7.98
7.78
7.78
7.76
7.75
7.73
7.73
7.41
7.39
7.39
7.39
7.37
7.37
7.26 CDCl3
7.05
7.02
6.66
6.63
4.38 HDO
3.92
3.85 cd3od
1.14
1.03
1.06
1.04
2.01
2.02
1.98
f1 (ppm)

5

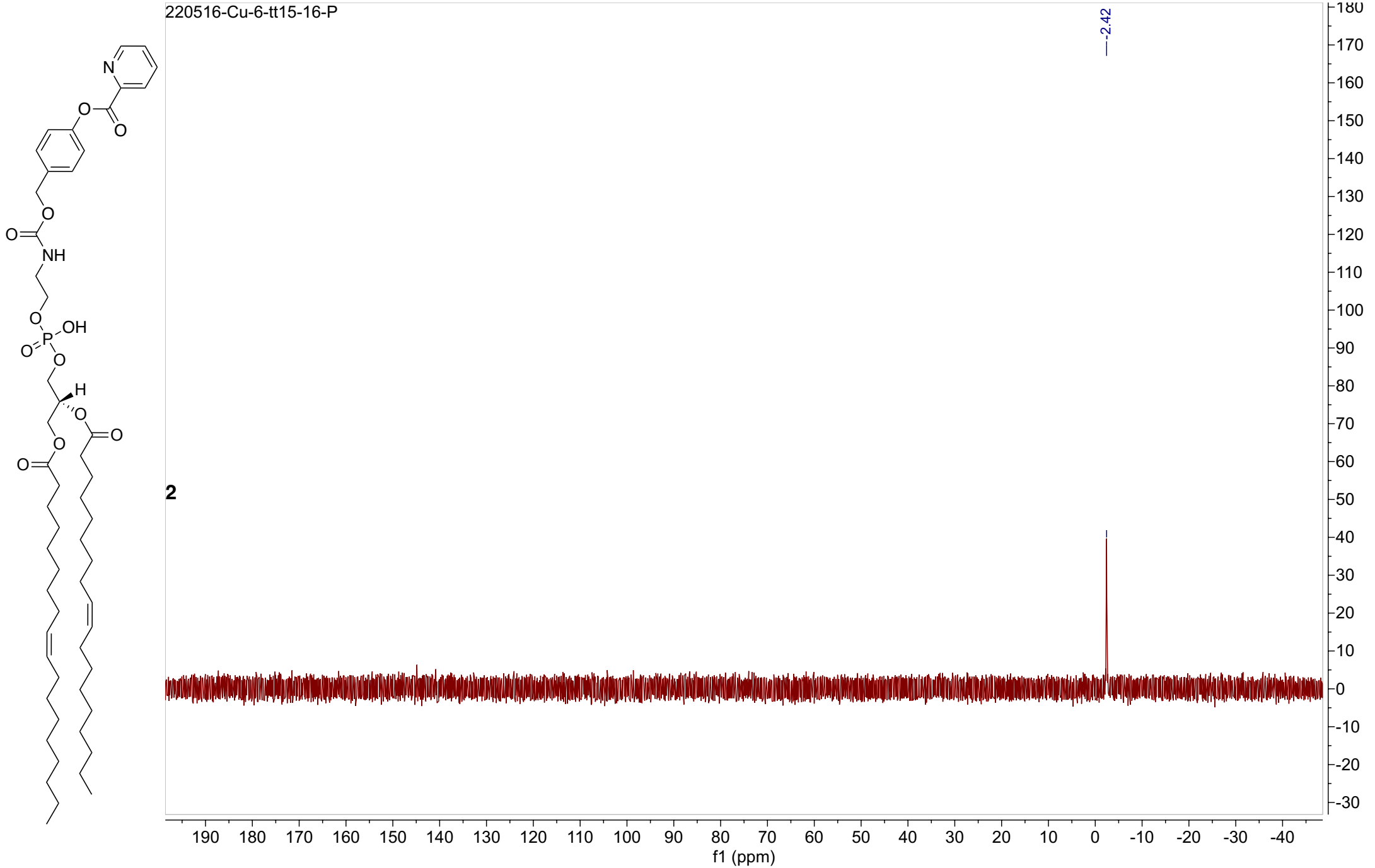

220507-Cu-2-tt22-32-C
165.44
156.11
149.36
147.28
137.45
131.96
128.60
127.22
125.14
115.03
77.16 CDCl3
64.21
52.69
52.64
49.28 cd3od
48.99 cd3od
48.71 cd3od
48.42 cd3od
48.14 cd3od
O
N
O
OH
5
f1 (ppm)


220516-Cu-3-Alt-H
7.26 cdcl3
5.31
6
f1 (ppm)


220516-Cu-3-Alt-C
163.84
155.58
152.52
151.50
150.27
147.32
145.53
137.39
132.24
130.21
127.69
126.05
125.41
122.26
121.90
77.41 cdcl3
77.16 cdcl3
76.91 cdcl3
70.34
O
N
O
O
O
O
O
NO2
6
f1 (ppm)



2


220516-Cu-6-tt15-16-P
-2.42
2
f1 (ppm)