# Supplemental Material: Chemical Synthesis and cell-based experiments

## Trifunctional lipid derivatives: PE's mitochondrial interactome

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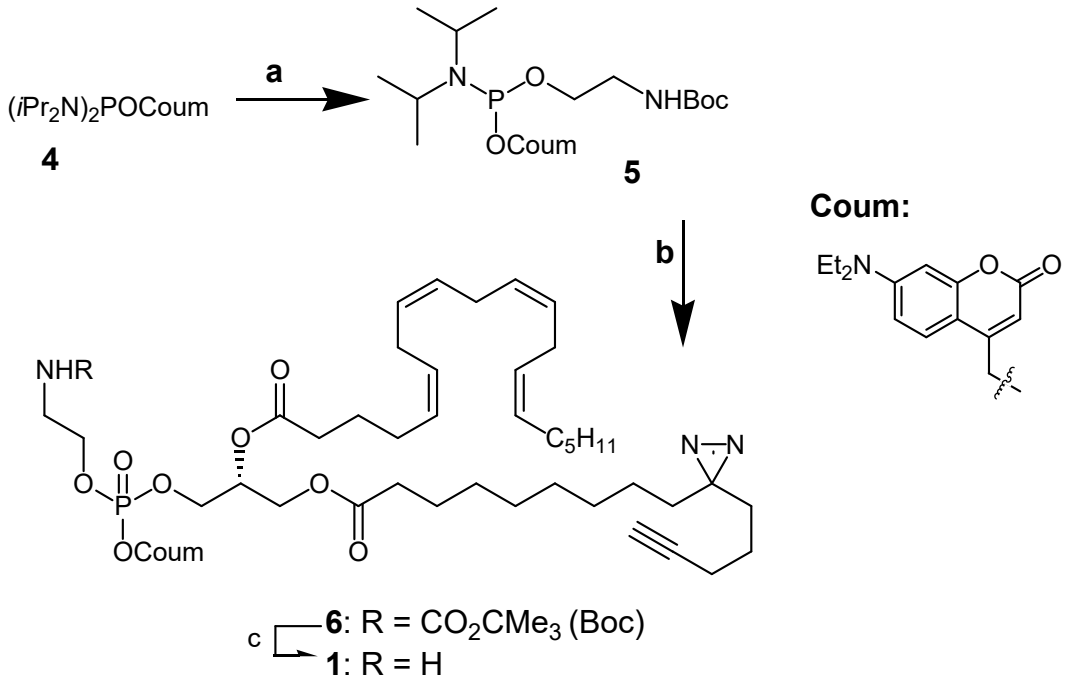
## Chemical Synthesis

### General procedures

All chemicals were obtained from commercial sources (Acros, Sigma, Alfa Aesar, TCI or Merck) and were used without further purification. Solvents for flash chromatography were from VWR and dry solvents were from Sigma or Acros. Deuterated solvents were obtained from Deutero GmbH, Karlsruhe, Germany. All reactions were carried out using dry solvents under inert atmosphere unless stated otherwise in the respective experimental procedure.
TLC was performed on pre-coated plates of silica gel (Merck, 60 F254) using UV light (254 or 366 nm) or a solution of phosphomolybdic acid in EtOH (10 g phosphomolybdic acid in 100 mL EtOH) for visualization. Preparative column chromatography was performed using silica gel 60 (grain size 0.04-0.063 mm) from Macherey-Nagel GmbH, Germany with a pressure of 1-1.5 bar. For RP flash column chromatography, LiChroprep® RP-18 material (Merck, grain size 0.040-0.063 mm) or LiChroprep® C18 (Macherey-Nagel, grain size 0.060-0.080 mm) was employed.
HPLC analysis was performed on a Knauer Smartline pump 1000 using a Knauer Smartline UV Detector 2500 with a LiChroCART® 250-5 mm cartridge (LiChrospher 100 RP18 (10 µm, Merck)), or a Shimadzu HPLC with Nucleodur column (Machery-Nagel). Preparative HPLC was performed using a Knauer K-1800 preparative pump with a K-2501 UV detector and a Merck Prepbar steel column (250 x 50 mm) filled with RP18 material (Merck, LiChrospher®, 220 g, 12 µm). For all HPLC experiments, the eluents were methanol-water mixtures unless stated otherwise; compositions are given in % methanol.
$^{1}$H-, $^{13}$C-, and $^{31}$P-NMR spectra were obtained on a 400 MHz Bruker UltraShield™ spectrometer. Chemical shifts of $^{1}$H- and $^{13}$C-NMR spectra are referenced to solvent resonances, $^{31}$P-NMR spectra are referenced to 85% phosphoric acid. *J* values are given in Hz and chemical shifts in ppm. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet; b, broad. $^{13}$C- and $^{31}$P-NMR spectra were broadband hydrogen decoupled. Mass spectra (ESI) were recorded using a Waters Micromass ZQ mass spectrometer or a HP Esquire-LC mass spectrometer. High-resolution mass spectra were recorded at the University of Heidelberg (ICR Apex-Qe instrument, ESI) or at OHSU (Orbitrap Eclipse, ESI).

**Synthesis of trifunctional phosphatidylethanolamine derivative (1)**




**Scheme S1.** Synthesis of trifunctional phosphatidyl ethanolamine **1** as trifluoroacetate. Reagents and conditions: a) 1*H*-tetrazole, $CH_2Cl_2$, 24°C, 1h, 89%; b) 1. diacylglycerol **3** [1], 1*H*-tetrazole, $CH_2Cl_2$, 24°C, 1 h; 2. $AcO_2H$/AcOH, $CH_2Cl_2$, -78-24°C, 1 h, 69% over two steps; c) $CF_3COOH$, $CH_2Cl_2$, 24°C, 1 h, 99%.

**(2-*tert*-Butylcarbamoylethyl)-(7-diethylamino-2-oxo-2*H*-chromen-4-ylmethyl) *N*,*N*-di*iso*propylphosphoramidite (5)**

A solution of *N*-Boc-aminoethanol (162.1 mg, 1 mmol) in MeCN (3 mL) was evaporated at 23°C/0.02 mbar. Under argon, a 1*H*-tetrazole solution in MeCN (~0.45 M, 1.8 mL, 0.81 mmol) was added and volatiles were removed at 23°C/0.02 mbar. Under argon, a solution of phosphordiamidite **4** [2] (477.6 mg, 1 mmol) in anhydrous $CH_2Cl_2$ (5 mL) was added via a syringe. The reaction was stirred at 24°C for 1 h protected from light, then diluted with the eluent and concentrated under reduced pressure. The semi-solid residue was chromatographed on silica gel 60 that was previously deactivated with a mixture of the eluent (270 mL) and triethylamine (30 mL). Starting material **4** eluted as a yellow band with *n*-heptane:EtOAc:triethylamine 92:7:1. The product **5** eluted with *n*-heptane:EtOAc:triethylamine 30:19:1.

Yield: 410 mg (76.3%, 88.8% based on **4** consumed), $R_f$ *n*-heptane:EtOAc:triethylamine 19:30:1 = 0.63.
**4** (67.6 mg white solid) was recovered.

$^1$H NMR (400 MHz, CDCl3) δ = 7.30 (d, *J*=9.0, 1H, H-5), 6.54 (dd, *J*=9.0, 2.6, 1H, H-6), 6.48 (d, *J*=2.5, 1H, H-8), 6.24 (s, 1H, H-3), 4.92 (bs, 1H, NH), 4.85 – 4.66 (m, 2H, 4-$CH_2$), 3.82 – 3.55 (m, 4H, $OCH_2$, $N(CH(CH_3)_2)$, 3.38 (q, *J*=7.1, 4H, $N(CH_2CH_3)_2$), 3.35 – 3.20 (m, 2H, $NHCH_2$), 1.41 (s, 9H, *t*Bu), 1.32 – 1.06 (m, 18H, $N(CH_2CH_3)_2$, 2 x $CH(CH_3)_2$).

$^{31}$P NMR (162 MHz, CDCl3) δ = 149.15 (s, 1P).

$^{13}$C NMR (101 MHz, $CDCl_3$) δ = 162.25, 156.10, 155.86, 152.81, 152.74, 150.39, 124.34, 108.42, 106.28, 106.11, 97.67 (s, $C(CH_3)_3$), 79.18, 77.44, 77.12, 76.81, 62.86 (d, $J_{CP}$=16.7, $OCH_2$), 61.39 (d, $J_{CP}$=19.2, $OCH_2$), 44.68 (s, $N(CH_2CH_3)_2$, 43.14 (d, $J_{CP}$=12.4, 2 x NCH), 41.72, 41.65, 28.36 (s, $C(CH_3)_3$), 24.82 – 24.46 (m, $N(CH(CH_3)_2)_2$), 12.40 (s, $N(CH_2CH_3)_2$).

HRMS of **5** (ICR Apex-Qe, ESI positive): m/z found 538.3050, calculated for $C_{27}H_{45}N_3O_6P^+$, 538.3040, $[M+H]^+$; m/z found 560.2867, calculated for $C_{27}H_{45}N_3NaO_6P^+$, 560.2860, $[M+Na]^+$, intensity 22.3%; m/z found 1097.5846, calculated for $C_{54}H_{88}N_6NaO_{12}P_2^+$, 1097.5828, $[2M+Na]^+$, intensity 100.0%

**(2-*tert*-Butylcarbamoylethyl)-(7-diethylamino-2-oxo-2*H*-chromen-4-ylmethyl)-(2-*O*-arachidonyl-1-*O*-(3'(4''-pentyn-1''-yl))-*H*-diazirine-3'-octanoyl)-*sn*-glycero) phosphate, *P*-diasteromeric mixture (6)**

Freshly prepared **3** [1] (230 mg, 368 µmol) in EtOAc:*n*-heptane 2:1 (5 mL) was evaporated under reduced pressure and dried at 24°C/0.03 mbar. Under argon, 1*H*-tetrazole solution in MeCN (~0.45 M, 2 mL, 900 µmol) was added and volatiles were removed at 23°C/0.02 mbar. Under argon, a solution of phosphoramidite **5** (296 mg, 551 µmol) in anhydrous $CH_2Cl_2$ (6 mL) was added via syringe. After stirring at 23°C for 1 h the mixture was diluted with $CH_2Cl_2$ (14 mL) and cooled in a dry ice/acetone bath. Under argon and with stirring, peracetic acid solution (85.8 µL, 670 µmol) was added. The cooling bath was removed and the reaction was allowed to warm to 23°C. $^{31}$P NMR after 20 min indicated complete reaction. After 1 h, volatiles were removed under reduced pressure and the residue obtained was subjected to RP chromatography on a column of LiChroprep RP18 (40-63 µm, 17 x 3 cm, ~100 g) with 88-98% MeCN.

Yield: 270 mg (69.0%) yellow oil, $t_R$ 100% MeOH = 4.3 min, 95% MeOH = 18.8 min, 90% MeCN >18 min.

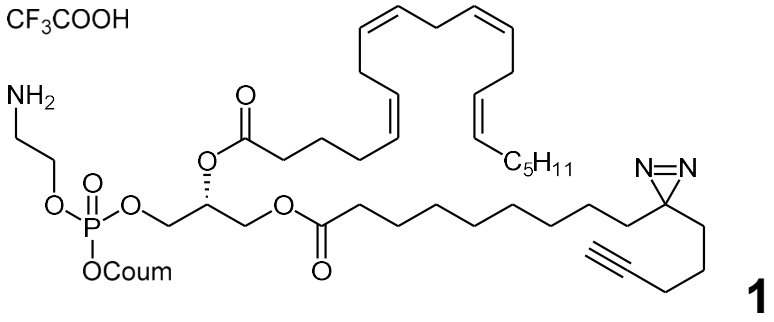
$^1$H NMR (400 MHz, CDCl3) δ = 7.28 – 7.23 (m, 1H, H-5), 6.56 (dd, *J*=9.0, 2.4, 1H, H-6), 6.49 (d, *J*=2.5, 1H, H-8), 6.16 (ad, 1H, H-3), 5.42 – 5.27 (m, 8H, 4 x CH=CH), 5.28 – 5.21 (m, 1H), 5.20 – 5.14 (m, 2H, 4-$CH_2$), 5.13 – 5.03 (m, 1H), 4.37 – 4.07 (m, 6H, $OCH_2CH_2$), 3.40 (aq, *J*=7.0, 6H, $N(CH_2CH_3)_2$, $NHCH_2$), 2.87 – 2.71 (m, 6H, 3 x $CH_2(CHCH)_2$), 2.40 – 2.22 (m, 4H), 2.18 – 1.97 (m, 6H), 1.94 (t, *J*=2.6, 1H, CCH), 1.77 – 1.62 (m, 2H), 1.62 – 1.51 (m, 2H), 1.50 – 1.43 (m, 2H), 1.41 (s, 9H, *t*Bu), 1.37 – 1.13 (m, 24H), 1.12 – 0.97 (m, 2H), 0.86 (t, J=6.9, 3H, ω-$CH_3$).

$^{31}$P NMR (162 MHz, CDCl3) δ = -0.88 (s, 1P).

$^{13}$C NMR (101 MHz, CDCl3) δ = 173.14, 172.60, 172.57, 161.54, 156.24, 155.75, 150.67, 148.92, 148.84, 130.45, 128.98, 128.69, 128.57, 128.26, 128.05, 127.80, 127.50, 124.23, 108.76, 106.35, 105.45, 97.84 (s, $C(CH_3)_3$), 83.41, 79.64, 77.44, 77.12, 76.80, 69.36, 69.29, 68.91, 67.71, 65.94, 64.80, 61.41, 44.79 (s, $N(CH_2CH_3)_2$), 40.84, 33.87, 33.45, 32.78, 31.77, 31.48, 29.28, 29.15, 29.08, 29.06, 28.97, 28.38, 28.31 (s, $C(CH_3)_3$), 27.18, 26.41, 25.58, 24.71, 24.63, 23.77, 22.72, 22.54, 17.91, 14.07, 12.39 (s, $N(CH_2CH_3)_2$).

HR-MS of **6** (ICR Apex-Qe, ESI positive): m/z found 1077.6246, calculated for $C_{59}H_{90}N_4O_{12}P^+$ 1077.6287 $[M+H]^+$, intensity 8.5%; m/z found 1099.6108, calculated for $C_{59}H_{89}N_4NaO_{12}P^+$, 1099.6107 $[M+Na]^+$, intensity 100.0%.

**(2-Aminoethyl)-(7-diethylamino-2-oxo-2*H*-chromen-4-ylmethyl) (2-*O*-arachidonyl-1-*O*-(3'(4"-pentyn-1"-yl))-*H*-diazirine-3'-octanoyl)-*sn*-glycero)phosphate trifluoroacetate, *P* diastereomeric mixture (1)**




**1**

TFA (0.5 mL, 6.5 mmol) was added to a solution of **6** (67 mg, 62 µmol) in DCM (4.5 mL) under argon atmosphere. After 10 min TLC indicated almost complete consumption of the starting material. After 1 h the reaction was diluted with EtOAc (10 mL) and volatiles were removed under reduced pressure. This process was repeated two times. Finally, the yellow oil obtained was dissolved in MeOH:EtOAc (1:1, 20 mL) and volatiles were removed under reduced pressure and the crude salt was dried at 24 °C/0.014 mbar.

Yield: 67 mg (99%) yellow oil, $t_R$ 95% MeOH 50 mM TEAF = 11.5 min; $R_f$ EtOAc:MeOH 9:1 = 0.38.

$^1$H NMR (400 MHz, $CDCl_3$) δ= 13.42 (bs, 2H), 8.19 (bs, 3H), 7.41 (d, *J*=9.0, 1H, H-5), 6.89 (d, *J*=9.0, 1H, H-6), 6.82 (s, 1H, H-8), 6.34 (s, 1H, H-3), 5.50 – 5.20 (m, 11H, 4 x CH=CH, 4-$CH_2$), 4.48 (bs, 2H, $OCH_2CH_2$), 4.40 – 4.10 (m, 4H), 3.60 – 3.32 (m, 6H, $N(CH_2CH_3)_2$, $NCH_2CH_2$), 2.90 – 2.72 (m, 6H, 3 x $CH_2(CH=CH)_2$), 2.41 – 2.24 (m, 4H), 2.20 – 2.13 (m, 2H), 2.13 – 2.00 (m, 4H), 1.96 (t, *J*=2.6, 1H, CCH), 1.76 – 1.62 (m, 2H), 1.62 – 1.52 (m, 2H), 1.42 – 1.12 (m, 20H), 1.19 (t, *J*=7.2, 6H, $N(CH_2CH_3)_2$), 0.89 (t, *J*=6.8, 3H, ω-$CH_3$).

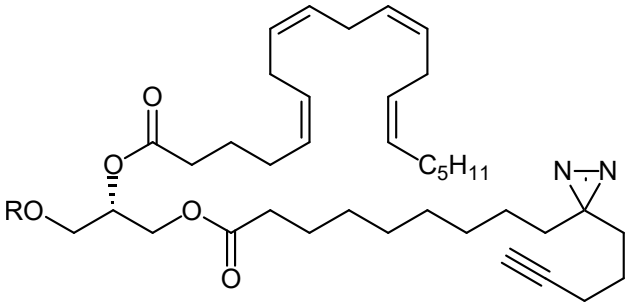
$^{31}$P NMR (162 MHz, $CDCl_3$) δ = -2.16 (s, 0.5P, dia-1), -2.23 (s, 0.5P, dia-2).

$^{13}$C NMR (101 MHz, $CDCl_3$) δ = 173.58, 173.56, 172.99, 172.92, 162.04, 160.78 (q, $J_{CF}$=38.5, $CF_3CO$), 155.42, 149.38, 149.30, 149.29, 147.85, 130.50, 129.02, 128.61, 128.32, 128.01, 127.78, 127.49, 124.86, 115.52 (q, $J_{CF}$=290.3, $CF_3$), 112.23, 109.03, 109.01, 107.77, 107.75, 101.69, 83.43, 77.37, 77.25, 77.05, 76.73, 69.36, 69.31, 68.89, 66.73, 66.68, 65.19, 65.17, 64.69, 64.66, 61.48, 47.51, 40.27, 40.22, 40.20, 33.84, 33.43, 32.77, 31.78, 31.49, 29.30, 29.18, 29.09, 29.06, 28.97, 28.43, 27.19, 26.37, 25.60, 25.57, 25.55, 24.69, 24.61, 23.78, 22.73, 22.55, 17.92, 14.05 (s, ω-$CH_3$), 11.64 (s, $N(CH_2CH_3)_2$).

$^{19}$F NMR (377 MHz, $CDCl_3$) δ = -75.95.

HR-MS (ICR Apex-Qe, ESI positive): m/z found 977.5779, calculated for $C_{54}H_{82}N_4O_{10}P^+$ 977.5763 $[M+H]^+$, intensity 100.0%; m/z found 999.5592, calculated for $C_{54}H_{81}N_4NaO_{10}P^+$, 999.5582 $[M+Na]^+$, intensity 6.9%.

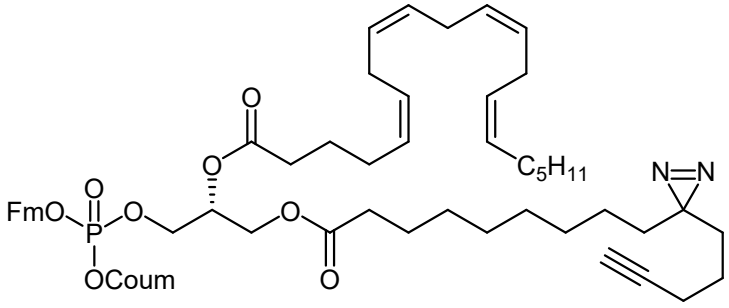
**Synthesis of a trifunctional phosphatidic acid derivative (2)**




**a** **3:** R = H
**b** **7:** R = P(O)(OCoum)(OFm)
**c** **8:** R = P(O)(OCoum)(OH)
**2:** R = P(O)(Coum)($OCH_2OAc$)

Reagents and conditions: a) 1. *i*$Pr_2$NP(OCoum)(OFm) [3,4], 1*H*-tetrazole, $CH_2Cl_2$, 23°C, 2 h; 2. $AcO_2H$/AcOH, -78-22°C, 1 h, 99% over two steps; b) $Me_2$NEt, MeCN, 23°C, 1 h; c) AcOCH2Br, MeCN, 24°C, 6 h, 34% over two steps.

**Synthesis of 2-*O*-arachidonyl-1-*O*-(3′(4″-pentyn-1″-yl)-*H*-diazirine-3′-octanoyl)-3-*O*-(7-diethylamino-2-oxo-2*H*-chromen-4-ylmethyl)-(9*H*-fluoren-9-ylmethyl)-*sn*-glycero phosphate, *P*-diasteromeric mixture (7)**




**7**

**The starting diacylglycerol (3) was prepared as previously described** [1, 2].
Diacylglycerol **3** (215 mg, 344 µmol) in toluene (10 mL) and a 1*H*-tetrazole solution in MeCN (~0.45 M, 1.2 mL, 540 µmol) were mixed and evaporated at 24 °C/0.006 mbar. Under an argon atmosphere, a solution of phosphoramidite *i*$Pr_2$NP(OCoum)(OFm) [3, 4] (222 mg, 370 µmol) in anhydrous dichloromethane (5 mL) was added with stirring. After 2 h, the mixture was diluted with 30 mL dichloromethane and cooled in a dry ice/acetone bath. Peracetic acid solution (47 µL, 370 µmol) was added and the cooling bath was removed. After 1 h, volatiles were removed under reduced pressure. $^{31}$P NMR indicated complete oxidation. The crude fully protected PA derivative **7** was purified by preparative HPLC on RP-18 silica (MeOH:EtOAc 19:1).

Yield: 379 mg (99.0%) red oil, $t_R$ MeOH:EtOAc 4:1 = 3.2 min, 9:1 = 4.3 min

$^1$H NMR (400 MHz, $CDCl_3$) δ = 7.76 (t, *J*=7.2, 2H), 7.62 (d, *J*=7.5, 2H), 7.41 (dd, *J*=17.3, 7.5, 2H), 7.33 (t, *J*=7.5, 2H), 7.18 (dd, *J*=8.9, 2.9, 1H), 6.56 (d, *J*=9.0, 1H), 6.53 (d, *J*=2.4, 1H), 6.11 (s, 1H), 5.49 – 5.27 (m, 8H), 5.21 (s, 1H), 5.08 – 4.87 (m, 2H), 4.49 (d, *J*=4.2, 2H), 4.27 (dd, *J*=12.9, 8.7, 2H), 4.20 – 3.99 (m, 4H), 3.44 (q, *J*=7.1, 4H), 2.96 – 2.68 (m, 6H), 2.30 (td, *J*=7.5, 3.2, 4H), 2.18 (td, *J*=6.9,

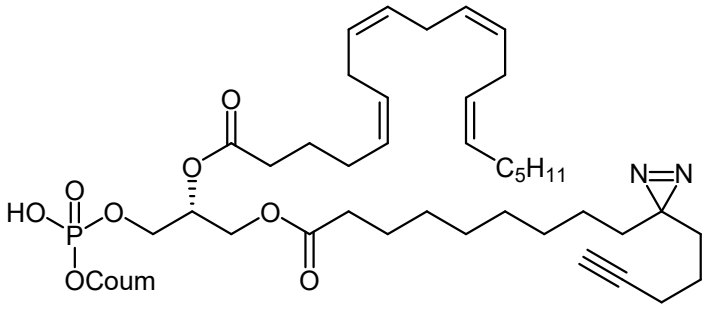
2.6, 2H), 2.14 – 2.01 (m, 5H), 1.97 (t, *J*=2.6, 1H), 1.65 (dd, *J*=22.6, 15.5, 5H), 1.54 – 1.44 (m, 2H), 1.44 – 1.15 (m, 27H), 1.08 (s, 2H), 0.91 (t, *J*=6.8, 3H).

$^{31}$P NMR (162 MHz, $CDCl_3$) δ = -1.40 (broad).

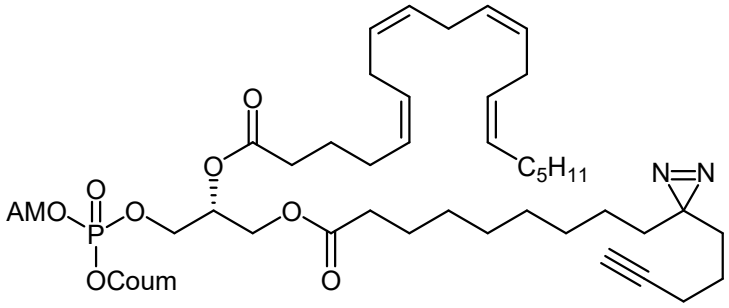
$^{13}$C NMR (101 MHz, $CDCl_3$) δ = 173.07, 172.48, 161.52, 156.24, 150.70, 148.85, 148.77, 142.78, 142.76, 142.74, 141.41, 130.46, 128.95, 128.71, 128.58, 128.26, 128.07, 128.04, 127.83, 127.53, 127.24, 124.98, 124.93, 124.92, 124.30, 120.10, 108.60, 106.45, 105.45, 97.76, 83.43, 77.46, 77.14, 76.82, 69.75, 69.69, 69.68, 69.32, 69.24, 68.93, 65.81, 65.76, 64.65, 64.61, 61.39, 60.35, 47.97, 47.90, 44.73, 33.87, 33.44, 32.80, 31.80, 31.49, 29.30, 29.17, 29.09, 29.07, 28.98, 28.38, 27.20, 26.42, 25.63, 25.61, 25.58, 24.73, 24.64, 23.78, 22.74, 22.56, 17.93, 14.08, 12.43.

MS of **7** (No ICR Apex-Qe, ESI positive mode): m/z found 1113.3, calculated for $C_{66}H_{88}N_3O_{10}P^+$ 1112.61236 $[M+H]^+$; m/z found 1135.2, calculated for $C_{66}H_{87}N_3NaO_{10}P^+$ 1134.59430 $[M+Na]^+$

**Synthesis of 2-*O*-arachidonyl-1-*O*-(3′(4′′-pentyn-1′′-yl)-*H*-diazirine-3′-octanoyl)-3-*O*-(acetoxymethyl)-(7-diethylamino-2-oxo-2*H*-chromen-4-ylmethyl)-*sn*-glycero phosphate, *P*-diastereomeric mixture (2)**




**8**




**2**

A solution of **7** (91 mg, 82 µmol) in MeCN (0.5 mL) and *N*,*N*-dimethylethylamine (0.5 mL, 4.6 mmol) was stirred at 23 °C for 1 h under argon. A sample of this solution gave the expected mass for the deprotected compound (**8**, ESI negative). Volatiles were removed at 24 °C/0.01 mbar. To the residue was added dry MeCN (1.0 mL), *N*,*N* -di*iso*propylethylamine (87 µL, 500 µmol) and bromomethyl acetate (34 µL, 347 µmol) under an argon atmosphere. The mixture was stirred at 24 °C for 6 h, protected from light. The reaction mixture was diluted with DMF (2.5 mL) and volatiles were removed at 23°C/0.004 mbar. Preparative HPLC (95-100% MeOH) afforded the pure compound.

Yield: 28 mg (34.0%) as a yellow oil, $t_R$ 100% MeOH = 3.9 min

**NMR 2:**

$^1$H NMR (400 MHz, $CDCl_3$) δ = 7.28 – 7.21 (m, 1H), 6.59 (dd, *J*=9.0, 2.5, 1H), 6.53 (d, *J*=2.5, 1H), 6.21 (s, 1H), 5.68 (dd, *J*=13.4, 2.6, 2H), 5.49 – 5.31 (m, 4H), 5.28 (dd, *J*=9.7, 4.9, 1H), 5.22 (d, *J*=6.7,

1H), 4.28 (dddd, *J*=34.2, 17.5, 9.9, 4.6, 2H), 3.43 (q, *J*=7.1, 4H), 2.83 (dd, *J*=14.7, 5.3, 3H), 2.35 (dt, *J*=19.2, 7.7, 2H), 2.23 – 2.00 (m, 5H), 1.96 (t, *J*=2.6, 1H), 1.72 (dd, *J*=14.8, 7.3, 1H), 1.66 – 1.54 (m, 1H), 1.50 (dd, *J*=9.4, 6.5, 1H), 1.44 – 1.13 (m, 13H), 1.08 (s, 1H), 0.90 (t, *J*=6.8, 2H).

$^{31}$P NMR (162 MHz, $CDCl_3$) δ = -2.60, -2.68.

$^{13}$C NMR (101 MHz, $CDCl_3$) δ = 173.15, 172.59, 169.14, 161.57, 156.27, 150.72, 148.68, 148.59, 130.50, 129.00, 128.73, 128.61, 128.29, 128.09, 127.83, 127.53, 124.16, 108.78, 106.44, 105.43, 97.90, 83.45, 82.76, 82.71, 77.35, 77.04, 76.72, 69.26, 69.18, 68.89, 66.20, 66.14, 65.03, 64.99, 61.39, 61.36, 44.81, 33.90, 33.48, 32.82, 31.81, 31.51, 29.32, 29.19, 29.11, 29.09, 29.01, 28.42, 27.21, 26.45, 25.63, 25.61, 25.60, 24.74, 24.66, 23.79, 22.74, 22.57, 20.64, 17.95, 14.08, 12.41.

MS of **8** (No ICR Apex-Qe, ESI negative mode): *m/z* found 932.9, calculated for $C_{52}H_{75}N_3O_{10}P^-$ 932.51956 $[M-H]^-$

MS of **2** (No ICR Apex-Qe, ESI positive mode): *m/z* found 1008.1, calculated for $C_{55}H_{81}N_3O_{12}P^+$ 1006.55524 $[M+H]^+$; *m/z* found 1029.1, calculated for $C_{55}H_{80}N_3NaO_{12}P^+$ 1028.53718 $[M+Na]^+$

HR-MS (Orbitrap Eclipse, ESI positive): m/z found 1006.5586, calculated for $C_{55}H_{81}N_3O_{12}P^+$ 1006.5558 $[M+H]^+$. Mass error = +0.0028 daltons = +2.78 ppm

## Cell Culture

The Hela Kyoto cell line (female) was kindly provided by R. Pepperkok (European Molecular Biology Laboratory, Germany). Hela Kyoto (passage 15-35) were grown in high glucose DMEM (41965-039, Life Technologies) supplied with 10% fetal bovine serum (10270098, Life Technologies) at 37 °C and 5% $CO_2$.

## Antibodies and Chemicals

- Anti-calreticulin, rabbit (Invitrogen 3501S)
- Anti-Tom20, mouse (Santa Cruz, sc-17764)
- Anti-GM130, rabbit (Cell Signaling, D6B1)
- Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed secondary antibody, Alexa Fluor Plus 488 (Invitrogen, A32731)
- Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed secondary antibody, Alexa Fluor Plus 647 (Invitrogen A32728)
- For proteomics, the kit Click Chemistry Tools (Cat No 1235) was used following the standard manufacturer's protocol.
- For microscopy, the kit Click-&-Go® Plus 568 Imaging Kit (Vector Labs, CCT-1318) was used following the standard manufacturer's protocol

## Cell-based Experiments

## Live cell uncaging of TF-probes

HeLa cells were grown to 70% density in an 8-well Labtek dish. Each well was washed two times with DMEM free of FBS and loaded with 250 µL of a 5 µM caged lipid solution. Fluorescence images of the coumarin were captured on an inverted dual scanner confocal laser scanning microscope (Olympus Fluoview 1200) with a 63× oil objective using excitation at 405 nm for coumarin imaging and simultaneous excitation at 375 nm for uncaging.

## Analysis of Trifunctional Lipids by Thin-Layer Chromatography

HeLa cells were seeded in 6 cm dishes and grown to confluence. The cells were loaded with the lipid derivatives (10 µM) in FBS free media and allowed to sit on cells for 30 min at 37 °C prior to uncaging. Trifunctional probes were uncaged using a lamp with narrow band 400 nm (NailStar LED lamp, Amazon, ASIN B01286DTFQ). After the probe media was removed and replaced with normal complete media, dishes were exposed to 400 nm light to uncage the probe and either immediately processed or returned to the incubator for the indicated amount of time to allow metabolization. For the lipid extraction, dishes were rinsed with ice-cold PBS, and scraped in 300 µL of ice-cold PBS. The scraped cells were transferred into a glass tube to which 600 µL of methanol was added at room temperature. Samples were vortexed for 10 seconds and then, 150 µL of chloroform was added and samples were vortexed for 10 seconds again. Samples were centrifuged at 3000 x g for 10 min. The supernatants were transferred to fresh tubes, to which 300 µL of chloroform and 600 µL of 0.1% (v/v) aqueous

acetic acid were successively added. Following vortex mixing, samples were left at -20 °C over-night to allow for phase separation. The next day the lower phases were transferred into fresh tubes and dried under a stream of nitrogen. Lipids were labeled with the fluorogenic 3-azido-7-hydroxycoumarin dye prior to TLC analysis. The dried lipid extracts were re-dissolved in 8 µL chloroform, to which 30 µL was added from a copper-click master mix containing 5 µL of 10 mM 3-azido-7-hydroxycoumarin in acetonitrile, 100 µL of 10 mM tetrakis(acetonitrile)copper(I) tetrafluoroborate in acetonitrile, and 400 µL of ethanol. The reaction was allowed to proceed for 3 hours at 37 °C, and then extracts were once more dried under a stream of nitrogen. *TLC* Extracted lipids were redissolved in 10 µL chloroform and plated on 10 × 10 cm HPTLC silica 60 glass plates without F254 fluorophore. Lipids were resolved by a two-step system: first using chloroform/methanol/water/acetic acid 65:25:4:1 for 6 cm, then drying, and finally using hexane/ethyl acetate 1:1 for 9 cm. Fluorescently labeled lipids were visualized by using the SYBR Green channel of a BioRad Chemidoc Touch Imaging System. *TLC/CMS* bands with enough signal intensity (PE) were subjected to analysis by TLC-MS using an Advion Plate Express Compact Mass Spectrometer.

**Subcellular Visualization of Lipids by Confocal Microscopy**

HeLa cells were seeded on coverslips in 24-well plates and grown to about 70% confluence. The cells were washed with DMEM and loaded with the lipid derivatives (5 µM) in FBS free media and allowed to sit on cells for 30 min at 37 °C prior to uncaging. Dishes were exposed to 400 nm light to uncage the probe and returned to the incubator for varying amounts of time to allow for metabolism. Then, they were exposed to 350 nm to photocross-link using a lamp with narrow band 350 nm (NailStar 36 W UV lamp, Amazon, ASIN B00R4M0TI0), and immediately fixed by washing twice with PBS, then left in methanol for 20 min. To remove non-crosslinked probes, cells were washed once with or chloroform/methanol/ammonium hydroxide (10:55:0.75) and then the coverslips were transferred to a new 24-well plates and washed three times with PBS to remove organic solvent. 250 µL of a click mix was added in each well (Click-&-Go Plus 568 Imaging Kit). The reaction was allowed to proceed for 30 min in the dark. Click mix was removed, cells were washed twice with PBS, and blocking buffer (3% BSA in PBS) was added. Cells were blocked for 1 h before the addition of primary antibodies. All primary antibodies were diluted 1:250 in blocking buffer and left on cells, with rocking, overnight at 4 °C. The next day, primary antibodies were removed, cells were washed four times with PBS, and fluorescently tagged secondary antibodies were added, either A488 antirabbit or A647 antimouse, 1:1000 dilution in blocking buffer, for 1 h at RT, with rocking. Secondary antibodies were removed, cells were washed four times with PBS, and DAPI (1:1000) was added for 5 min. Cells were imaged on a dual scanner confocal microscope Olympus Fluoview 1200, using a 63x (oil) objective or a Zeiss LSM 980 airyscan equipped with a 63x objective. Image resolution was improved down to 0.1 µm using joint deconvolution algorithm from the Zeiss software. Pearson's correlation coefficients between the lipid signal and the signal for each organelle marker were calculated using an ImageJ pipeline running the plugin JaCoP. Each image was cropped to exclude any extracellular space or the nucleus. For each channel, the thresholding algorithm IsoData was used to generate mask in order to remove background pixels. Individual cells were selected based on regions of intensity of the lipid signal, and coefficients were calculated within each cell.

**Isolation of Protein–Lipid Complexes**

HeLa cells were seeded in 10 cm dishes and grown to confluency. Cells were loaded with 10 µM of the lipid probes in DMEM overnight. The cells were subjected to photocross-linking directly after uncaging. After photo-cross-linking, cells were washed with PBS and scraped

into PBS. Cells were pelleted by centrifugation (500*g* for 5 min) and the supernatant was discarded.
Cells fractions were then isolated using the MinElute plasma membrane protein isolation kit (Invent Biotechnologies, Cat No SM-005). The total membrane fraction (resuspended in PBS:0.5% Triton X-100) for each sample were incubated with 200 µl prewashed picolyl-azide agarose beads (Click Chemistry Tools, Cat No 1235, Component A) in presence of 1 mM final CuSO4, 100 µM BTTAA and 1 mM sodium ascorbate to catalyze the click reaction (RT for 1 h agitating). The beads were then extensively washed in disposable PD-10 columns (full column volume of each buffer): 3x with PBS, 3x with 50mM ammonium-bicarbonate, 3x 50 mM ammonium-bicarbonate 3M urea and 3x with 50 mM ammonium-bicarbonate to remove unspecific binders. The beads were then transferred to a clean Eppendorf tube, spun down and resuspended in 500 µL 50 mM ammonium-bicarbonate/3 M urea. Bound proteins were reduced via the addition of 5 mM final TCEP and incubated at 55°C with orbital shaking for 30 min. Proteins were then alkylated with 10 mM iodoacetamide (final concentration) for 20 min, incubated at RT in the dark, followed by the addition of 20 mM final DTT for 10 min at RT with shaking. The beads were precipitated vie centrifugation and the supernatant was removed from the beads. The beads were resuspended in 150µl 50 mM ammonium-bicarbonate/2M urea. 3 µg of MS grade trypsin was added and incubated overnight at 37°C with vigorous shaking to prevent the beads from settling. In the morning 1µg fresh trypsin was added and incubated for additional 2h. The supernatant was saved and beads washed once with 250 µl 50 mM ammonium-bicarbonate/2M urea that was added to the previous supernatant containing digested peptides. The total supernatant containing the digested peptides was acidified with 0.5% TFA (final concentration). The peptides were purified on a C18 desalting column, and dried.

**Identification of Proteins by LC-MS/MS**

Dried peptides were shipped to the EMBL proteomics core facility, where they were TMT-labeled using the TMT-16-plex system and analyzed by LC-MS/MS on an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). Peptides were separated using an Ultimate 3000 nano RSLC system (Dionex) equipped with a trapping cartridge (Precolumn C18 PepMap100, 5 mm, 300 µm i.d., 5 µm, 100 Å) and an analytical column (Acclaim PepMap 100. 75 × 50 cm C18, 3 mm, 100 Å) connected to a nanospray-Flex ion source. The peptides were loaded onto the trap column at 30 µL per min using solvent A (0.1% formic acid) and eluted using a gradient from 2 to 80% Solvent B (0.1% formic acid in acetonitrile) over 2 h at 0.3 µL per min (all solvents were of LC-MS grade). The Orbitrap Fusion Lumos was operated in positive ion mode with a spray voltage of 2.2 kV and capillary temperature of 275 °C. Full scan MS spectra with a mass range of 375–1500 m/z were acquired in profile mode using a resolution of 120,000 with a maximum injection time of 50 ms, AGC operated in standard mode, and an RF lens setting of 30%. Fragmentation was triggered for 3 s cycle time for peptide-like features with charge states of 2–7 on the MS scan (data-dependent acquisition). Precursors were isolated using the quadrupole with a window of 0.7 m/z and fragmented with a normalized collision energy of 34%. Fragment mass spectra were acquired in profile mode and a resolution of 30,000. The maximum injection time was set to 94 ms and AGC target to custom. The dynamic exclusion was set to 60 s.

**MS Data Analysis**

Acquired data were analyzed using IsobarQuant [5] and Mascot V2.4 (Matrix Science) using a reverse UniProt FASTA Homo sapiens database (UP000005640 from May 2016) including common contaminants. The following modifications were taken into account:

Carbamidomethyl (C, fixed), TMT16plex (K, fixed), Acetyl (N-term, variable), Oxidation (M, variable) and TMT16plex (N-term, variable). The mass error tolerance for full scan MS spectra was set to 10 ppm and for MS/MS spectra to 0.02 Da. A maximum of 2 missed cleavages were allowed. A minimum of 2 unique peptides with a peptide length of at least seven amino acids and a false discovery rate below 0.01 were required on the peptide and protein level[6]. only proteins which were identified in two out of two mass spec runs were kept. Log2 transformed raw TMT reporter ion intensities ('signal_sum' columns) were first cleaned for batch effects using limma[6] and further normalized using vsn (variance stabilization normalization[7]. Different normalization coefficients were estimated for +UV and -UV conditions in order to maintain for the abundance difference. Proteins were tested for differential expression using the limma package. The replicate information was added as a factor in the design matrix given as an argument to the 'lmFit' function of limma. A protein was annotated as a hit with a false discovery rate (fdr) smaller 5 % and a fold-change of at least 100 % and as a candidate with a fdr below 20 % and a fold-change of at least 50 %.

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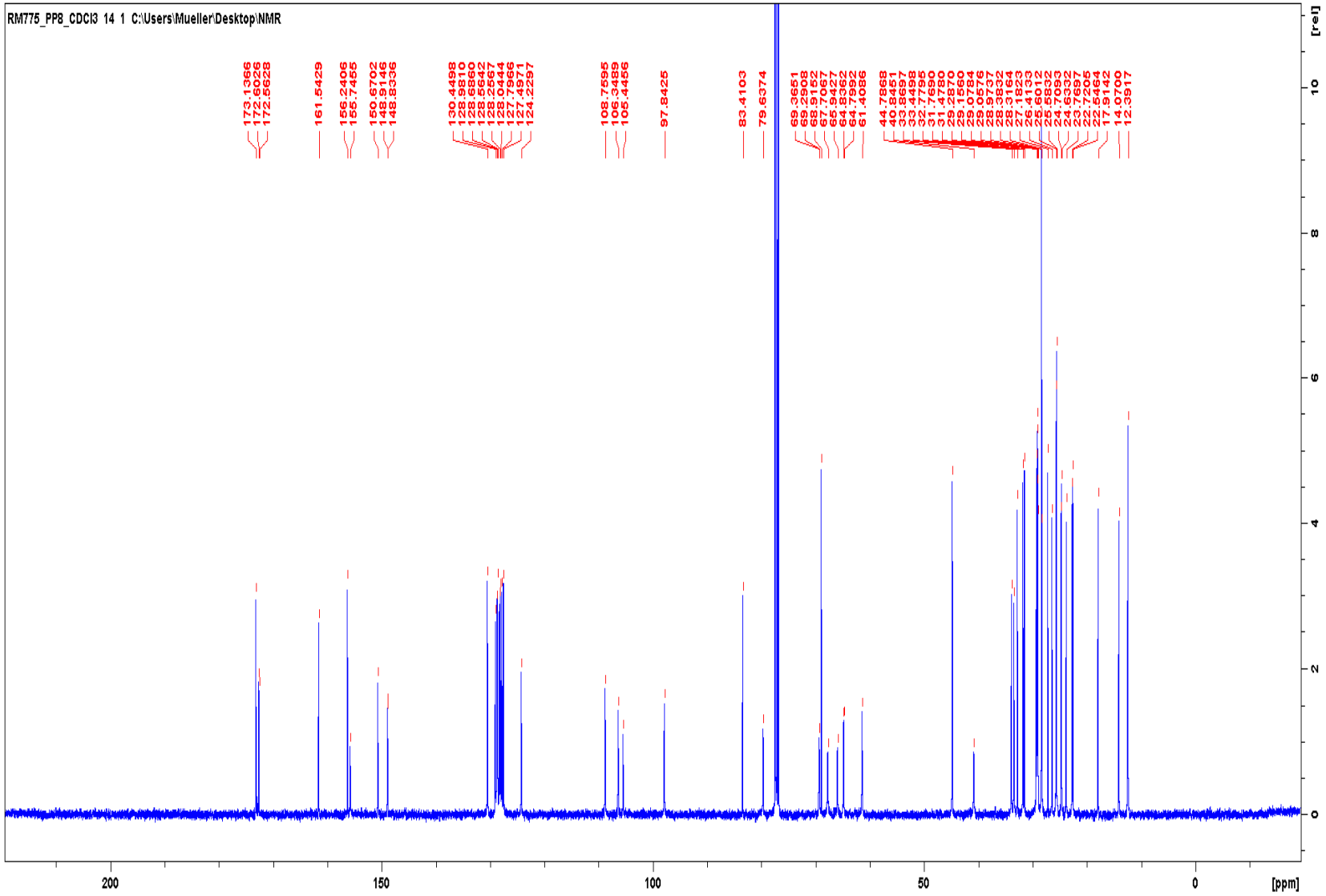
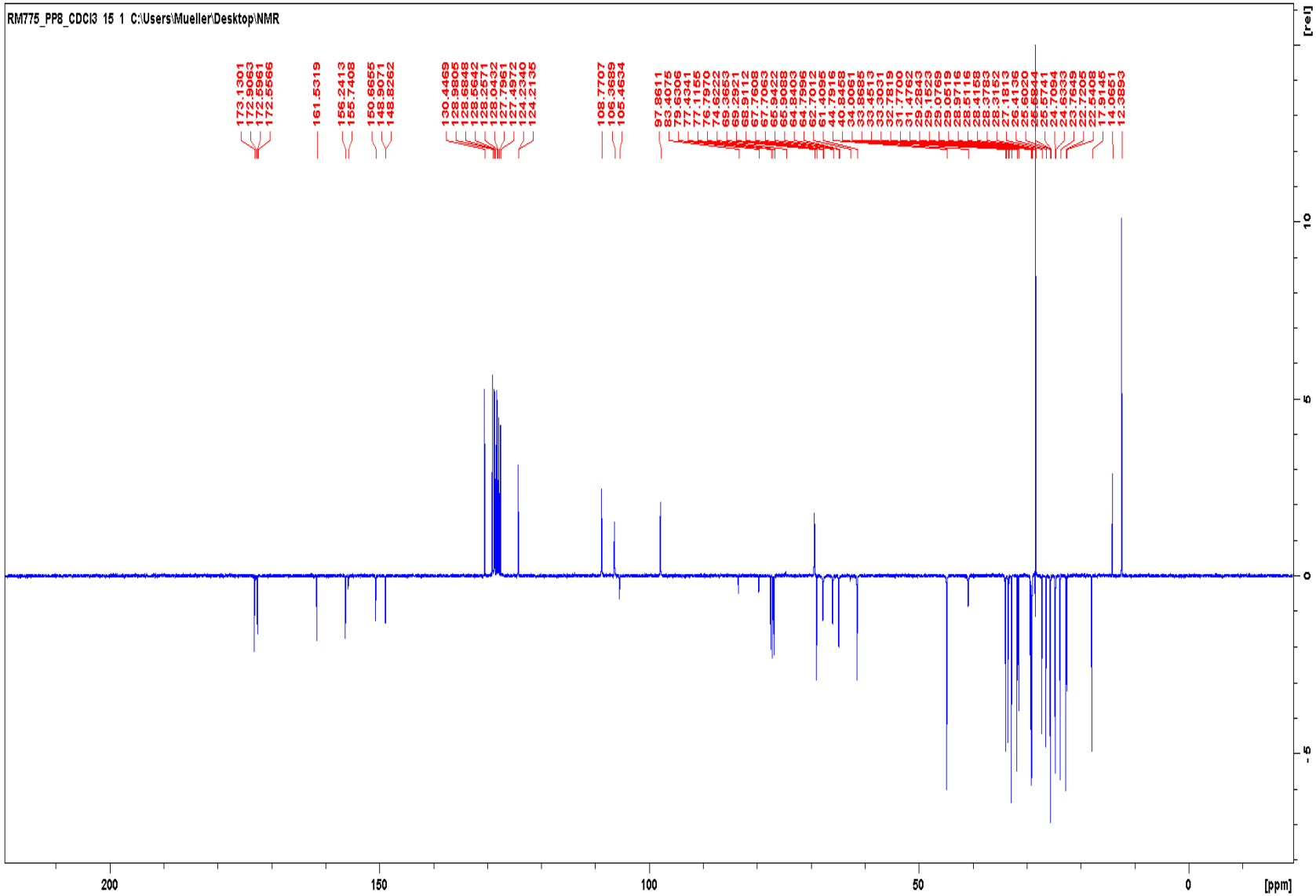



$^{13}$C-NMR ($CDCl_3$) of fully protected TF-PE **6**

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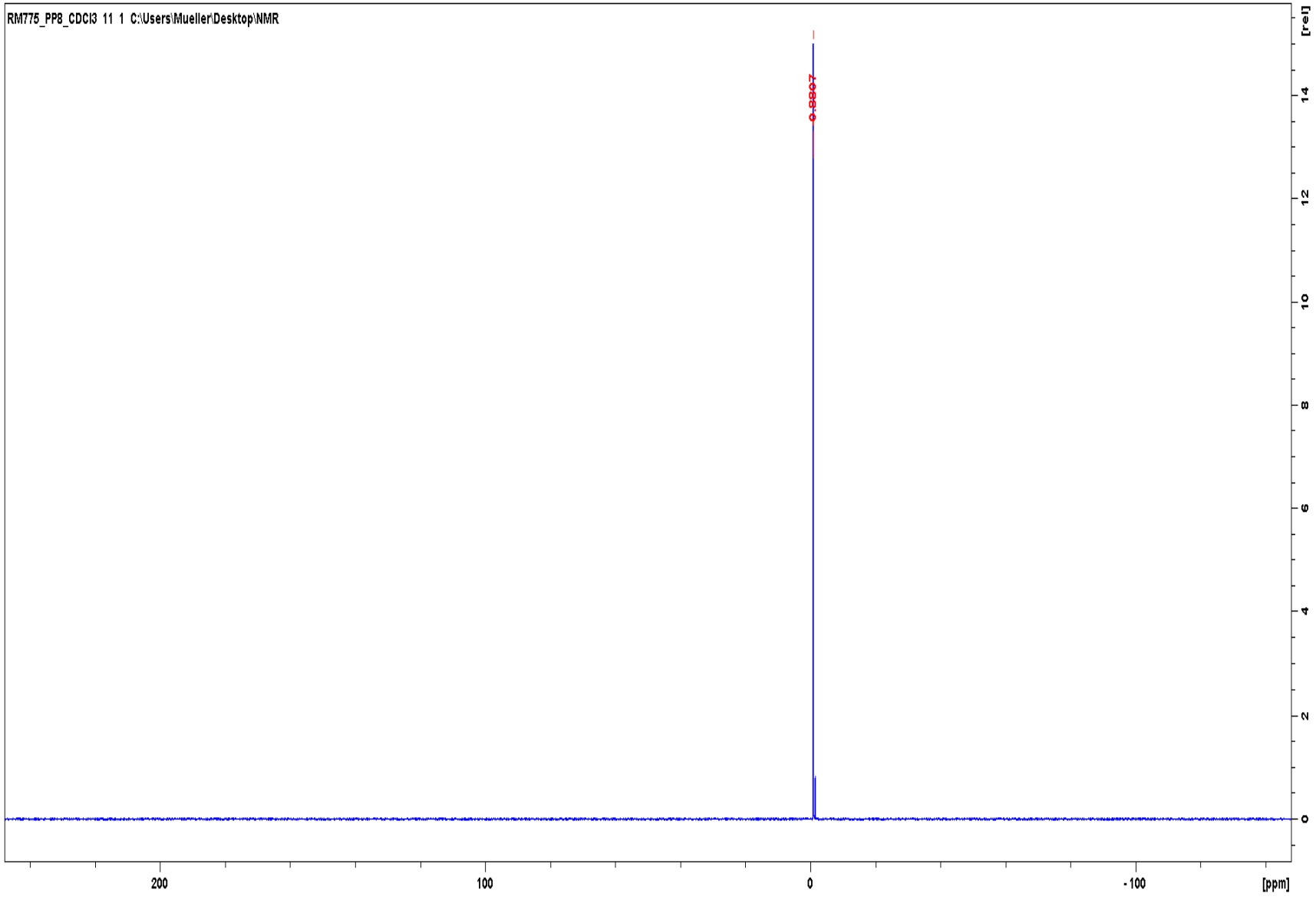
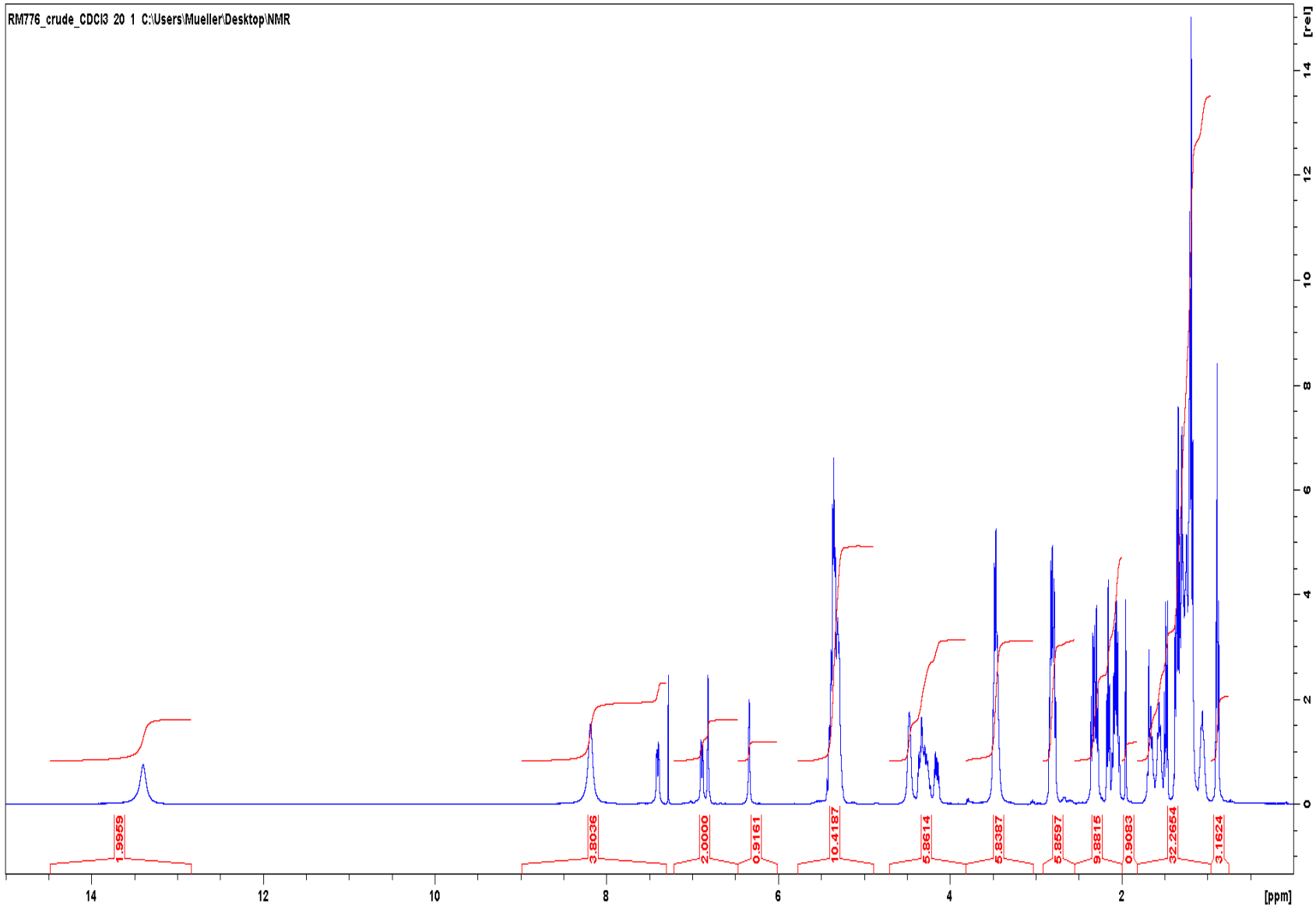
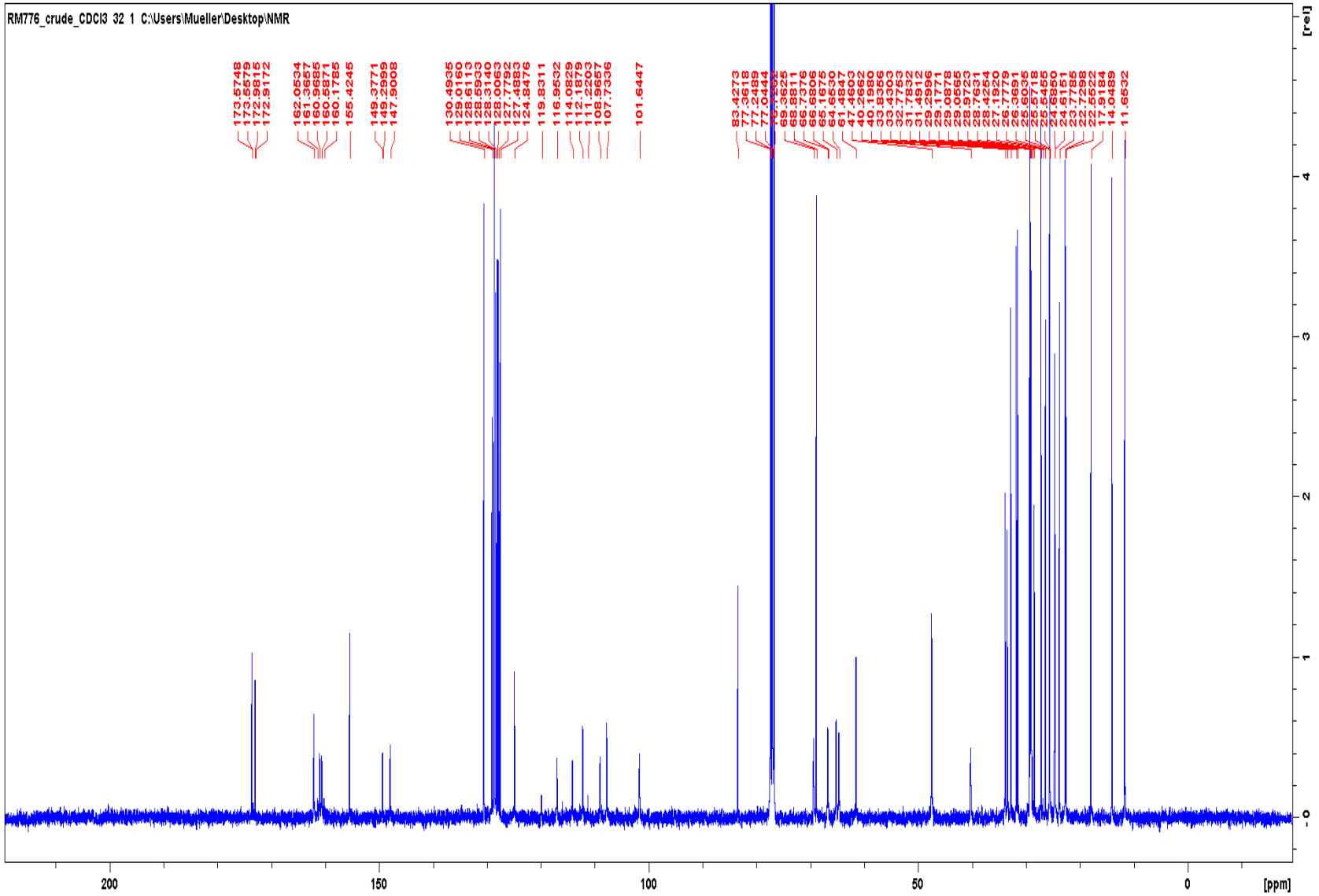



$^{31}$P-NMR ($CDCl_3$) of fully protected TF-PE **6**




$^{1}$H-NMR ($CDCl_3$) of TF-PE **1**

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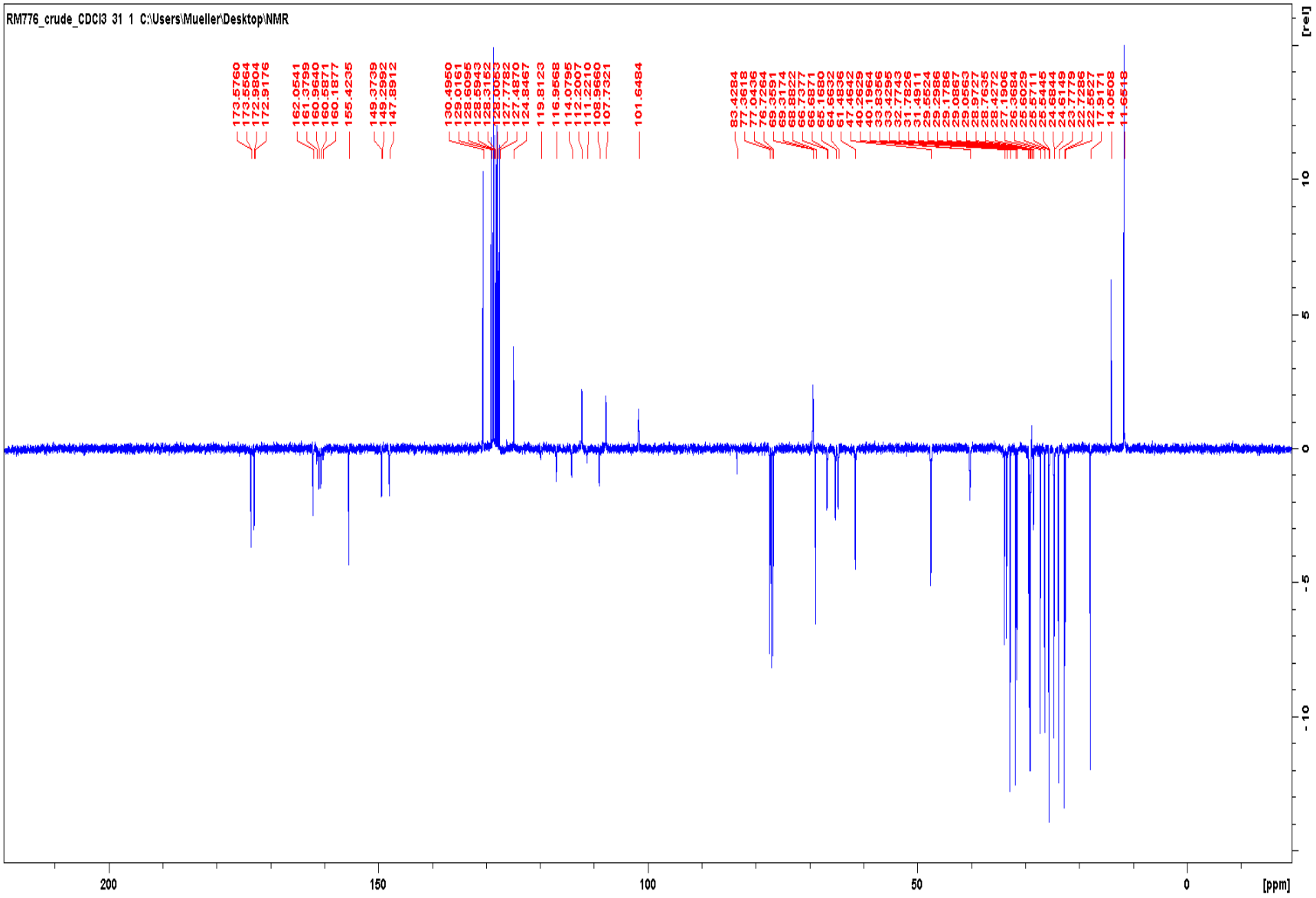


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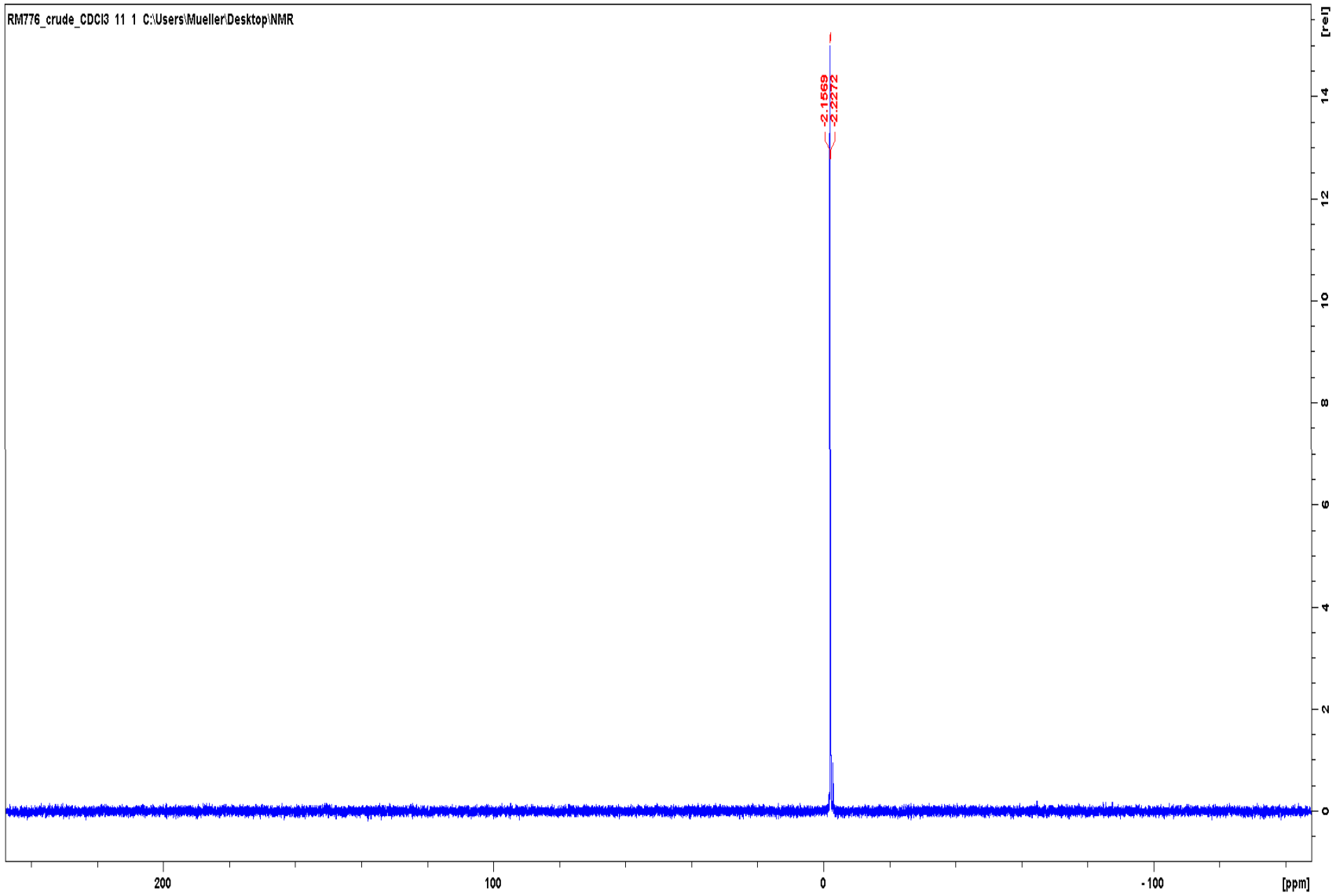


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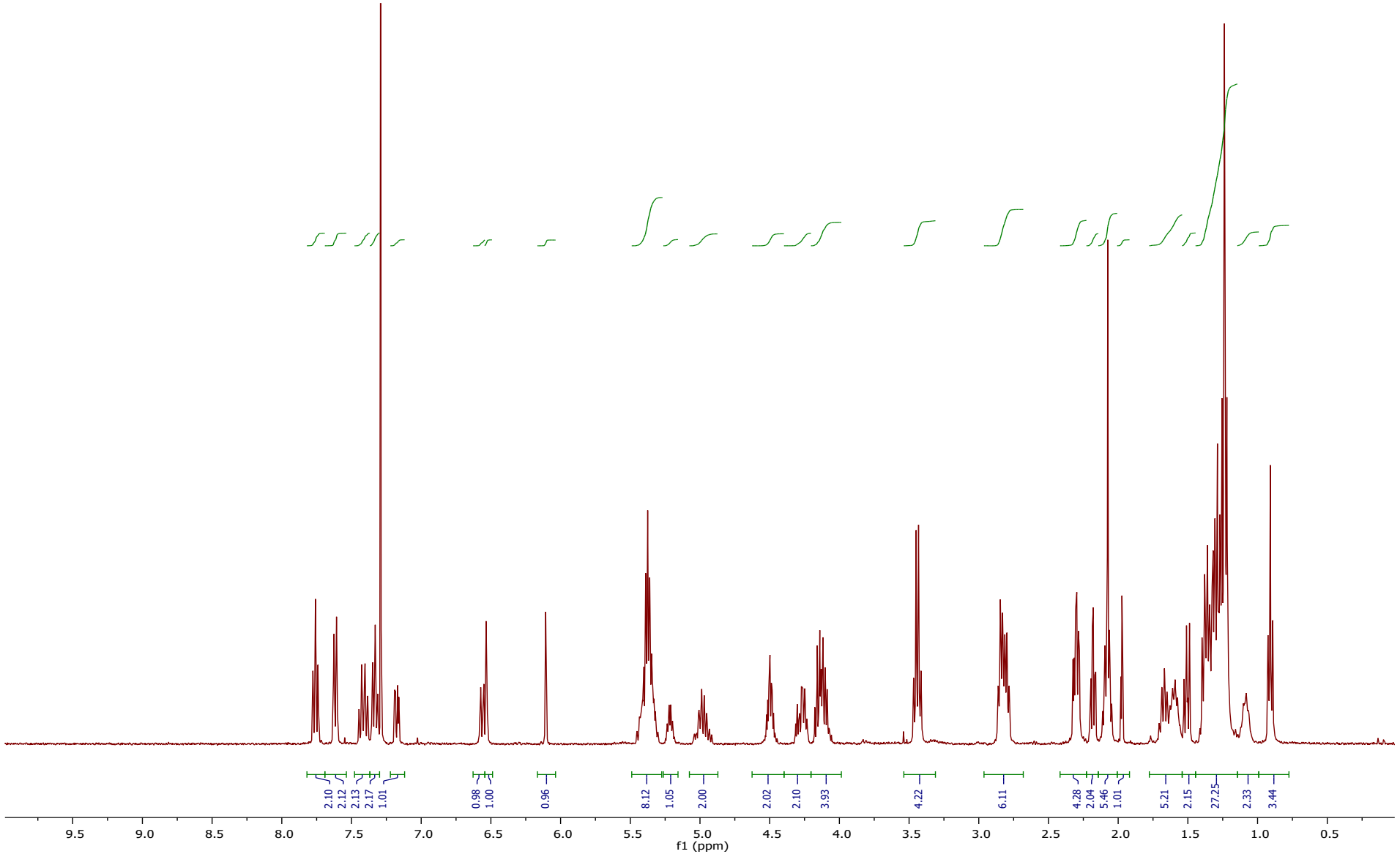
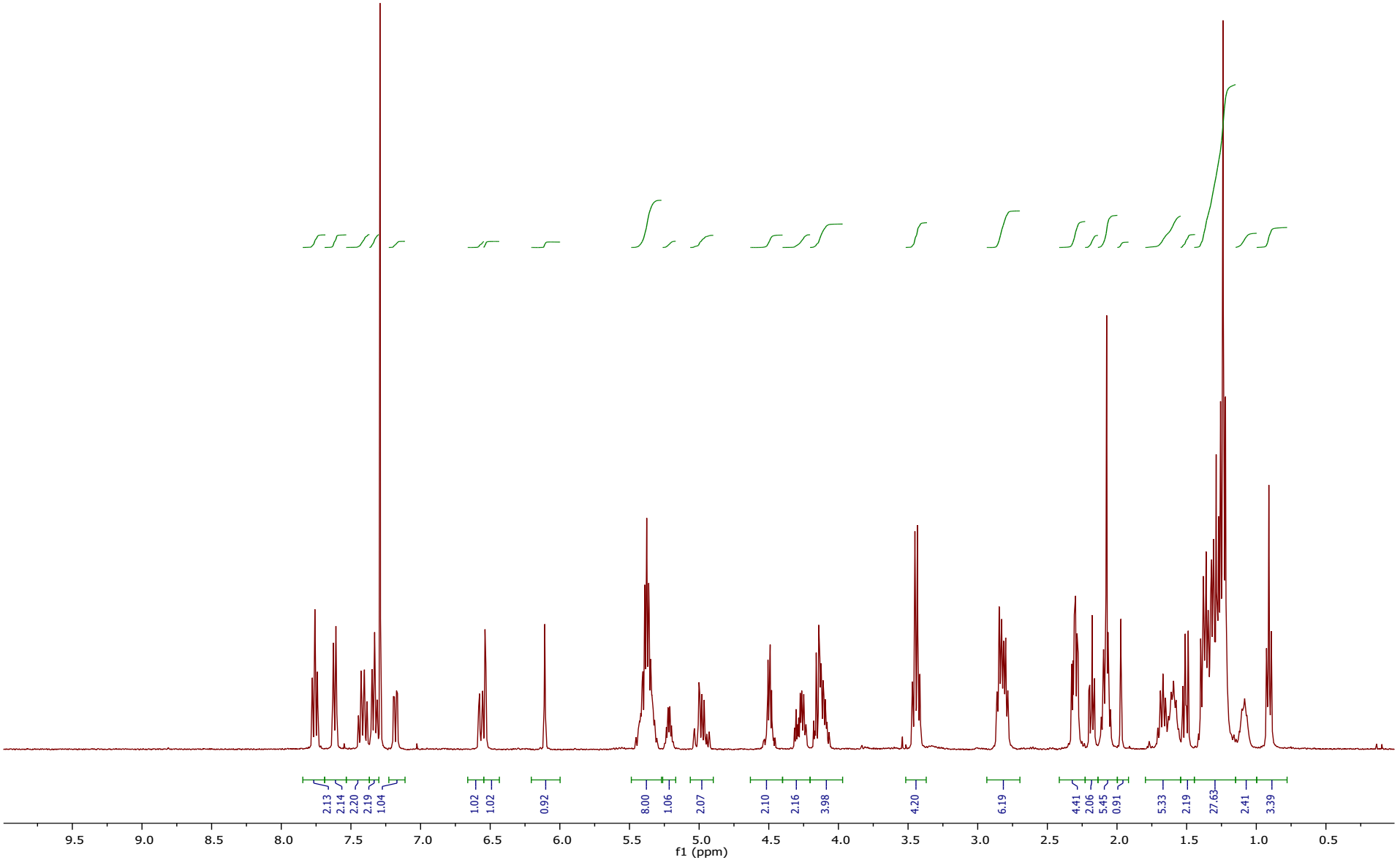
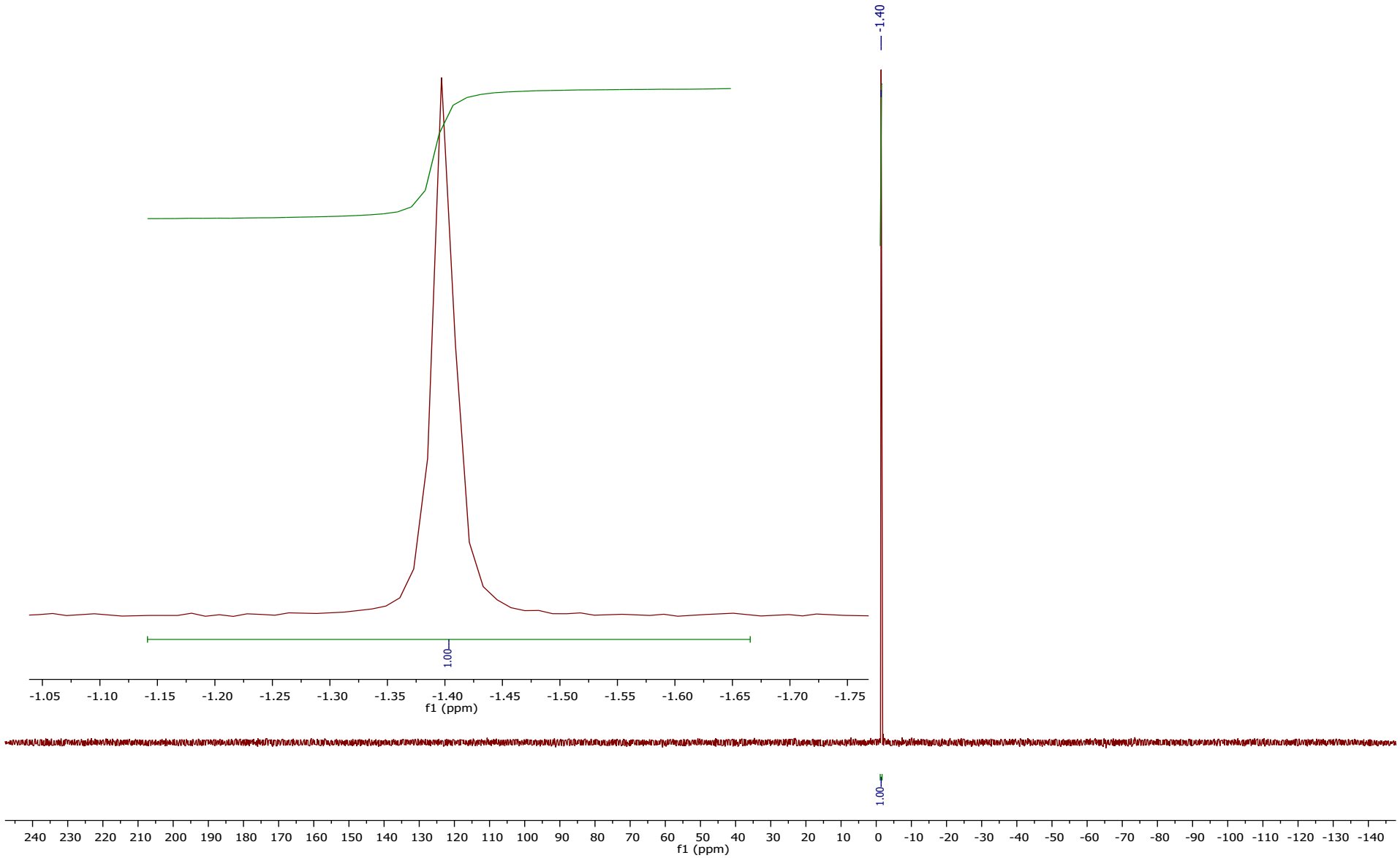


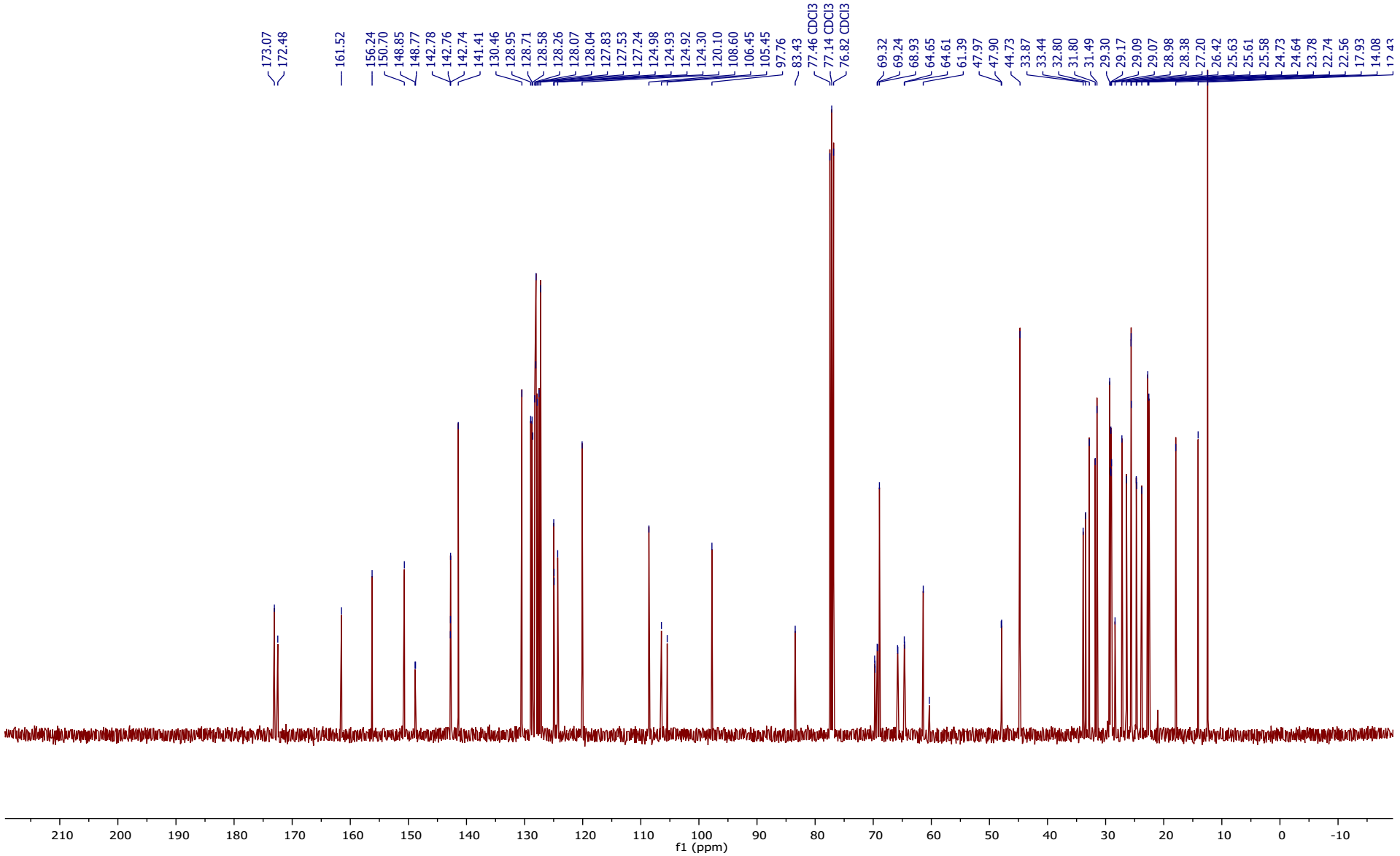

$^{1}$H NMR spectrum of TF-PA derivative **7** ($CDCl_3$, 400 MHz)



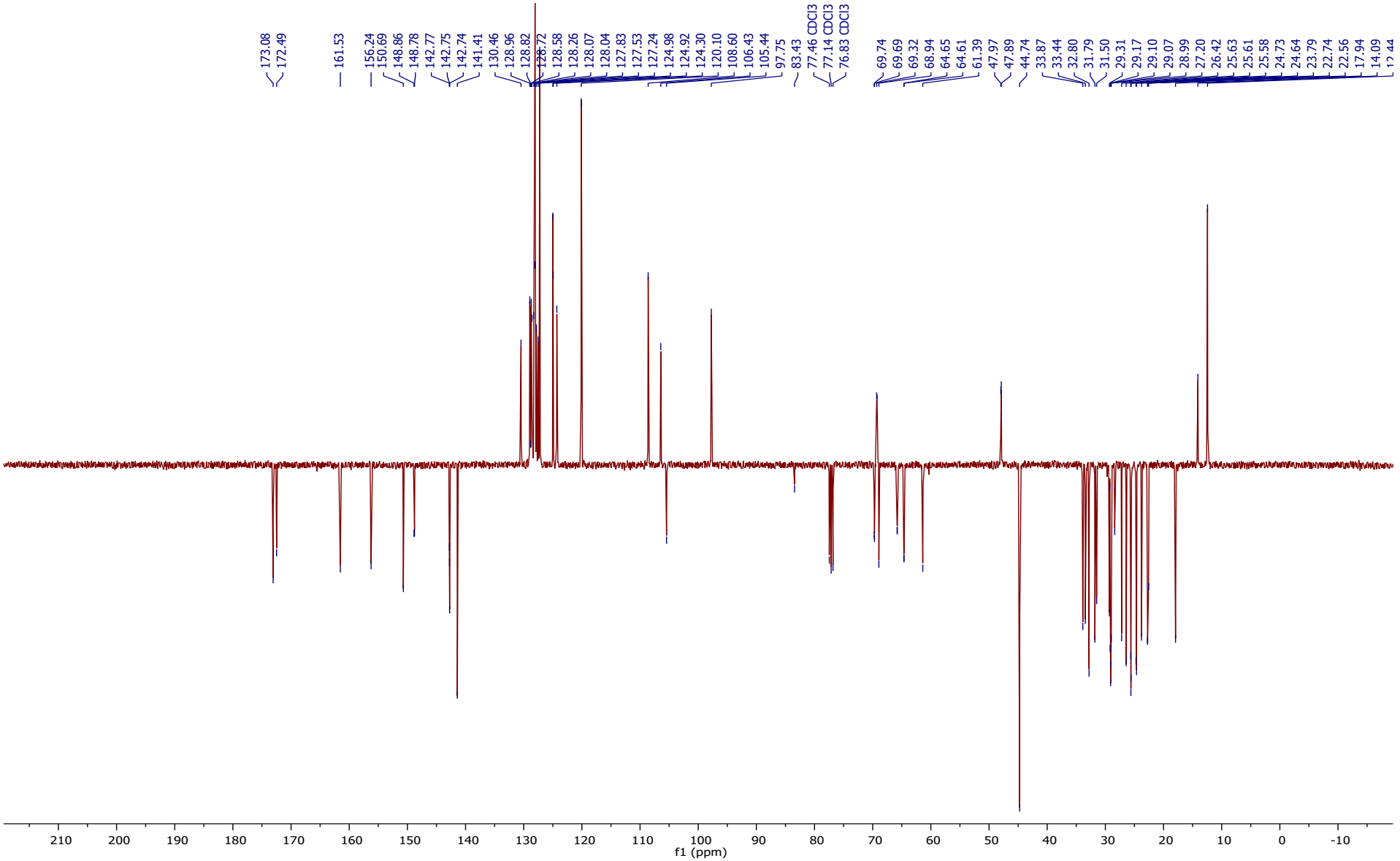

$^{1}H$ decoupled $^{31}P$ NMR spectrum of **7** ($CDCl_3$, 400 MHz)



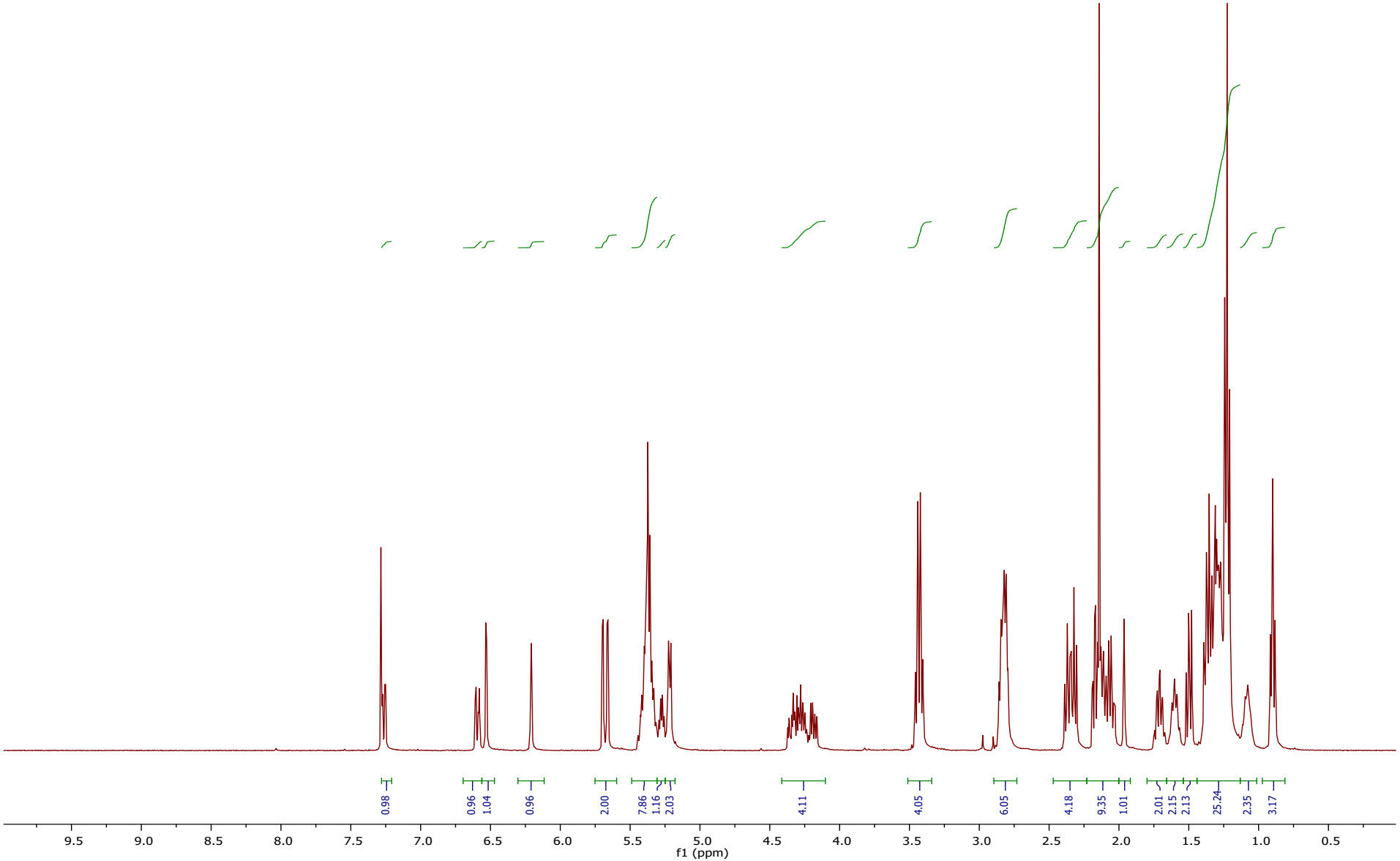

$^{31}$P NMR spectrum of **7** ($CDCl_3$, 162 MHz)



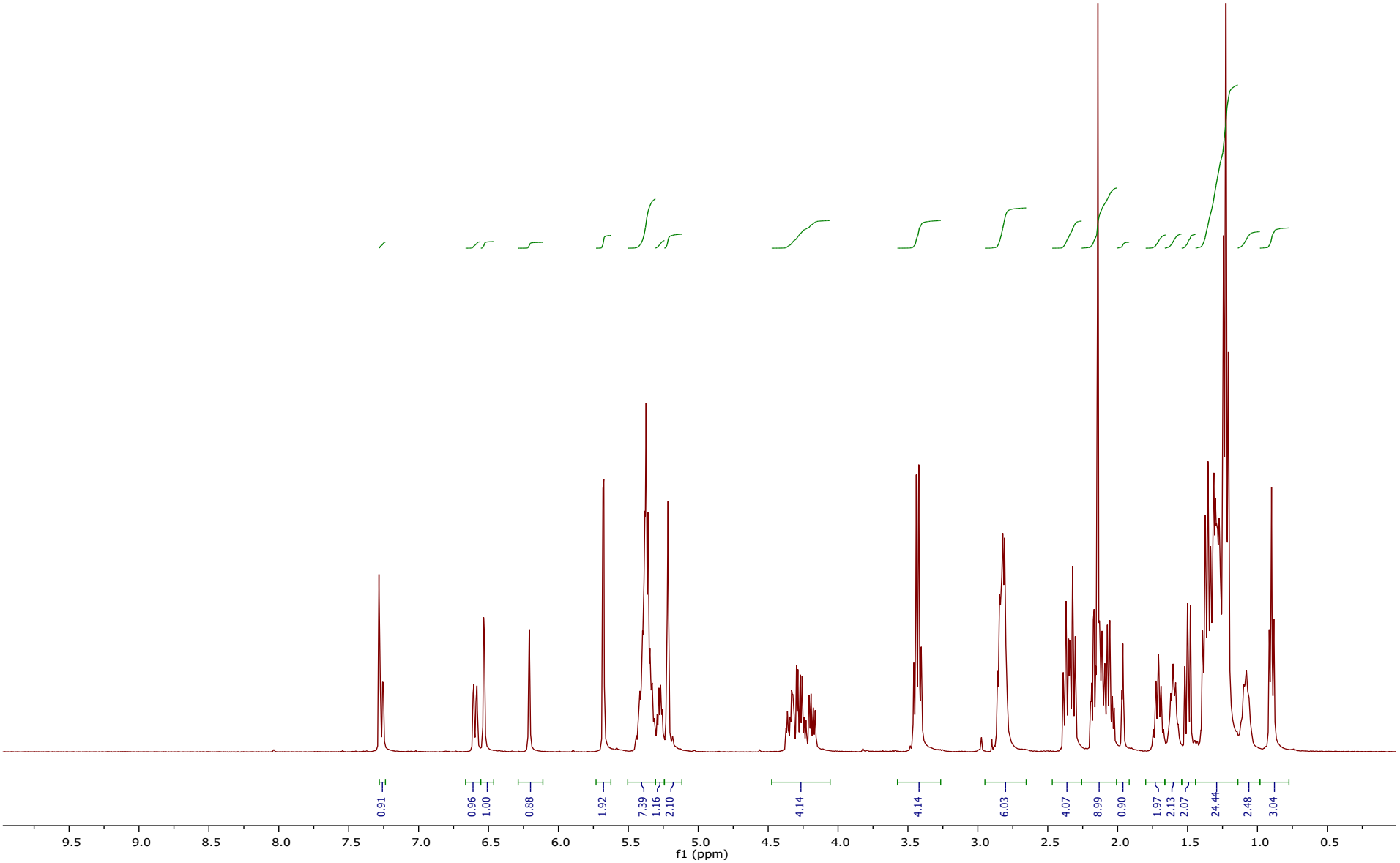

$^{13}$C NMR spectrum of **7** ($CDCl_3$, 101 MHz)



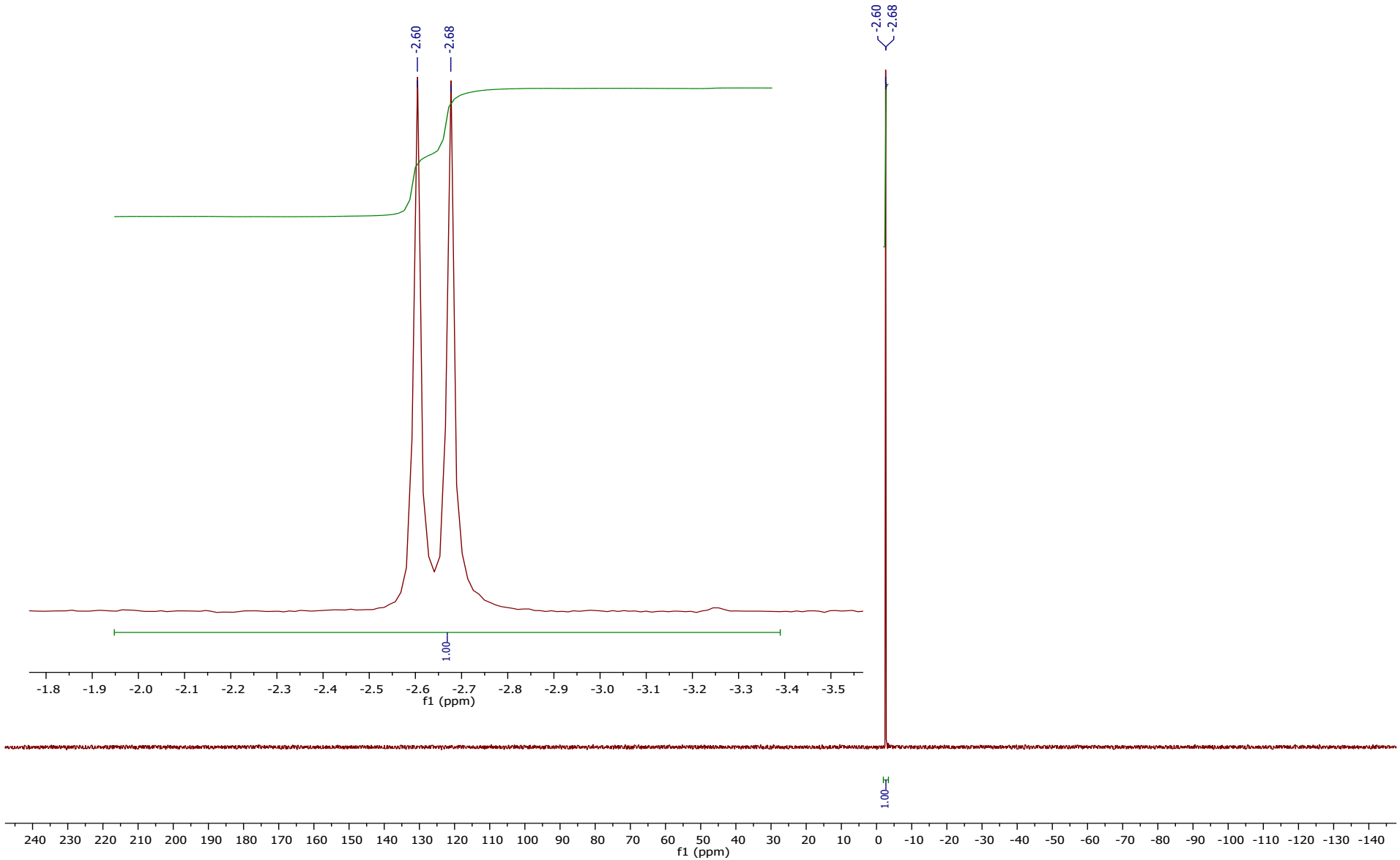

$^{13}$C NMR spectrum of **7** ($CDCl_3$, 101 MHz)



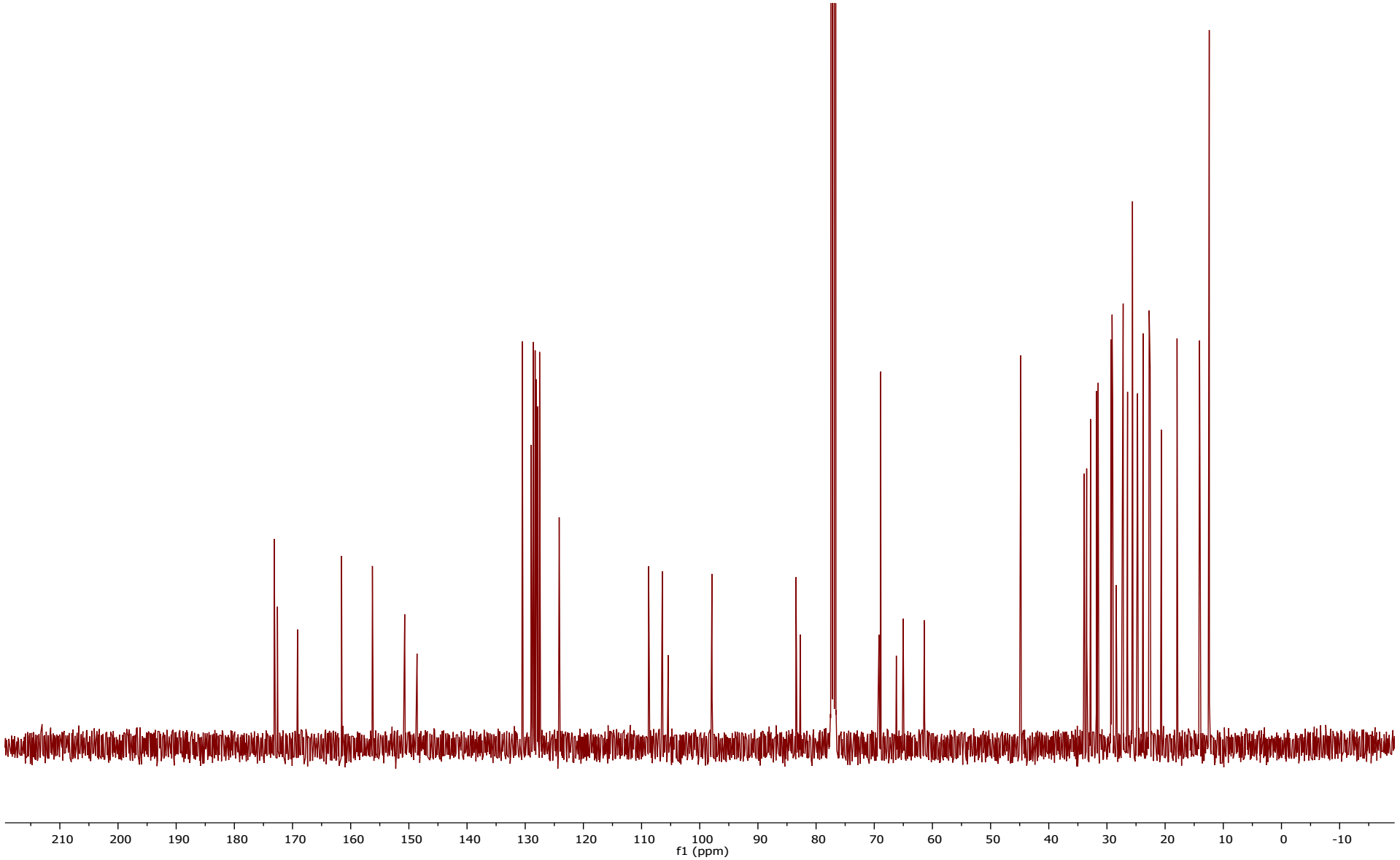

$^{1}$H NMR spectrum of TF-PA **2** ($CDCl_3$, 400 MHz)



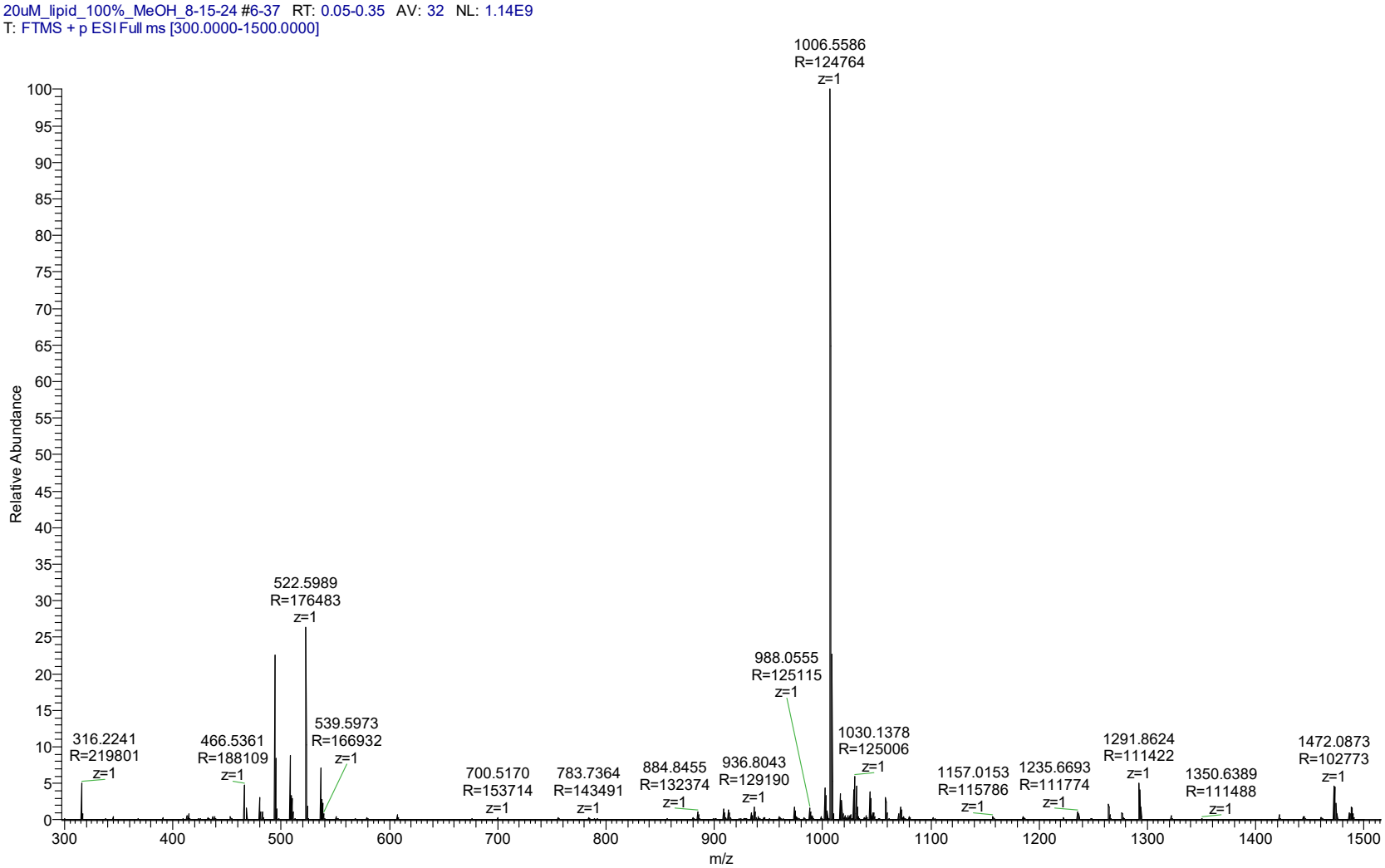

$^{1}H$ decoupled $^{31}P$ NMR spectrum of TF-PA **2** ($CDCl_3$, 400 MHz)




$^{31}$P NMR spectrum of TF-PA **2** ($CDCl_3$, 162 MHz)




$^{13}C$ NMR spectrum of TF-PA **2** ($CDCl_3$, 101 MHz)




High-resolution electrospray ionization (ESI) mass spectrum of TF-PA sample acquired in positive ion mode at a resolving power of 240,000. The sample (20 µM in 100% methanol) was infused at 5 µL/min. The protonated molecular ion peak at *m/z* = 1006.5558 is consistent with the expected mass for $C_{55}H_{81}N_3O_{12}P^+$.