Supporting Information:

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Fluorogenic Substrates for Phospholipase D and Phospholipase C

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Running Title: Fluorogenic PLD Substrates

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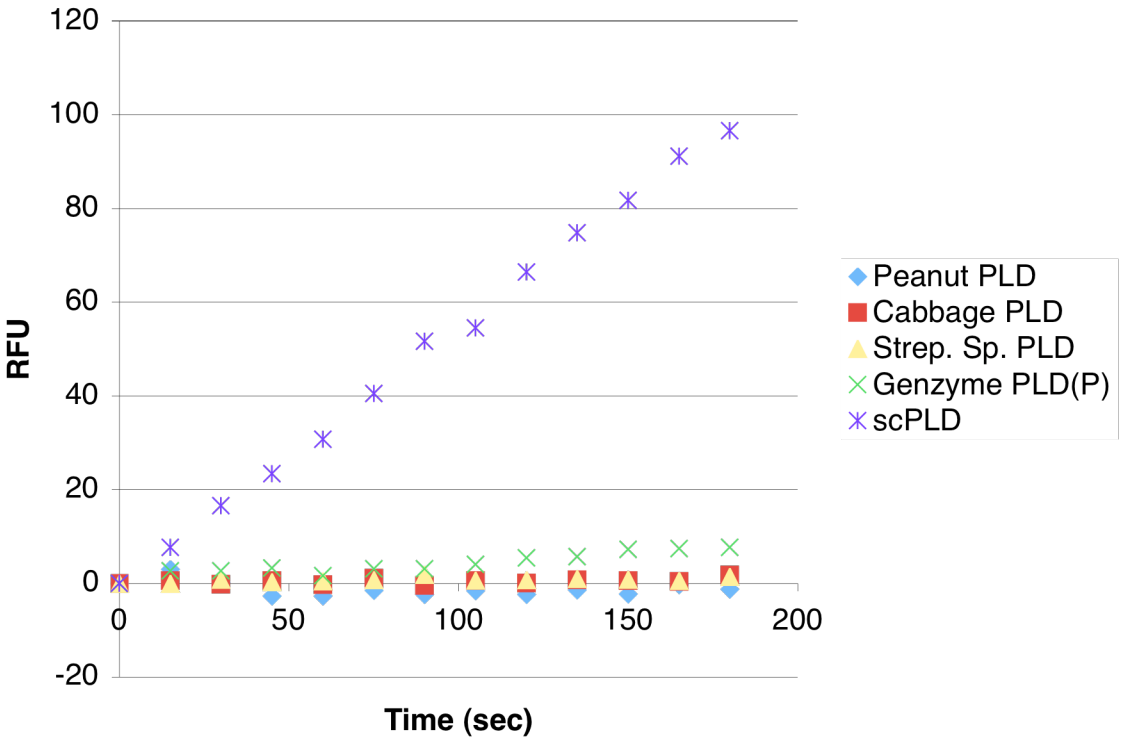
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RFU
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Genzyme PLD(P)
scPLD

Figure 2

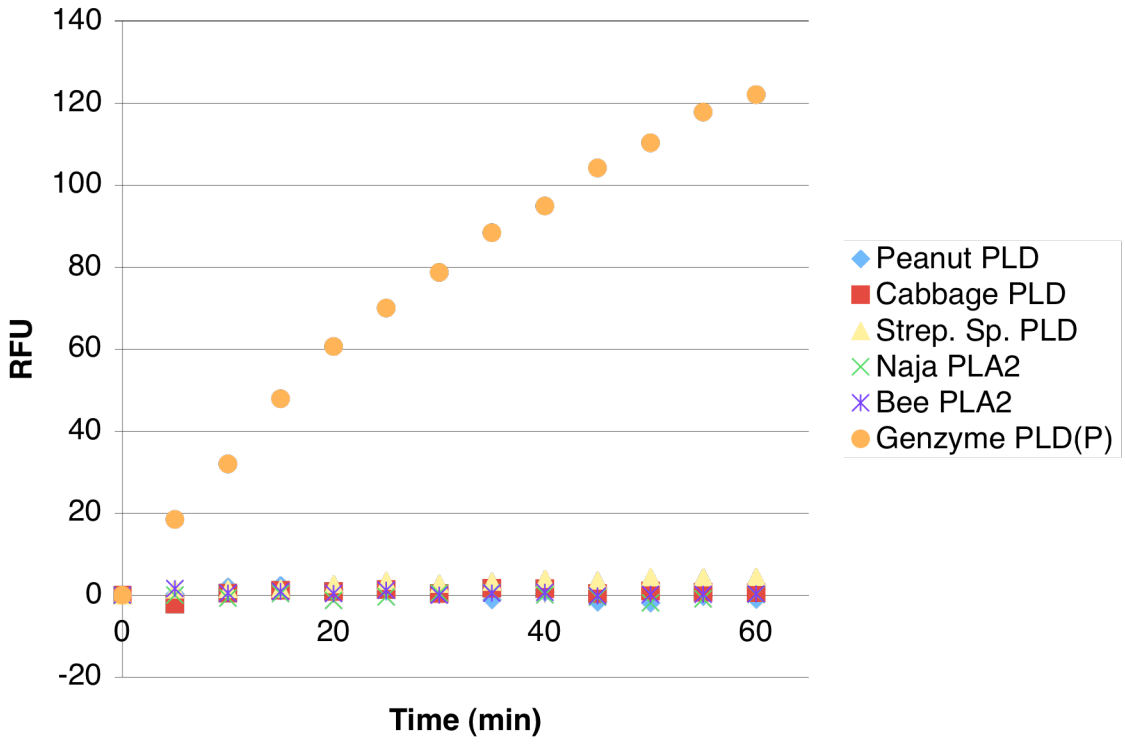
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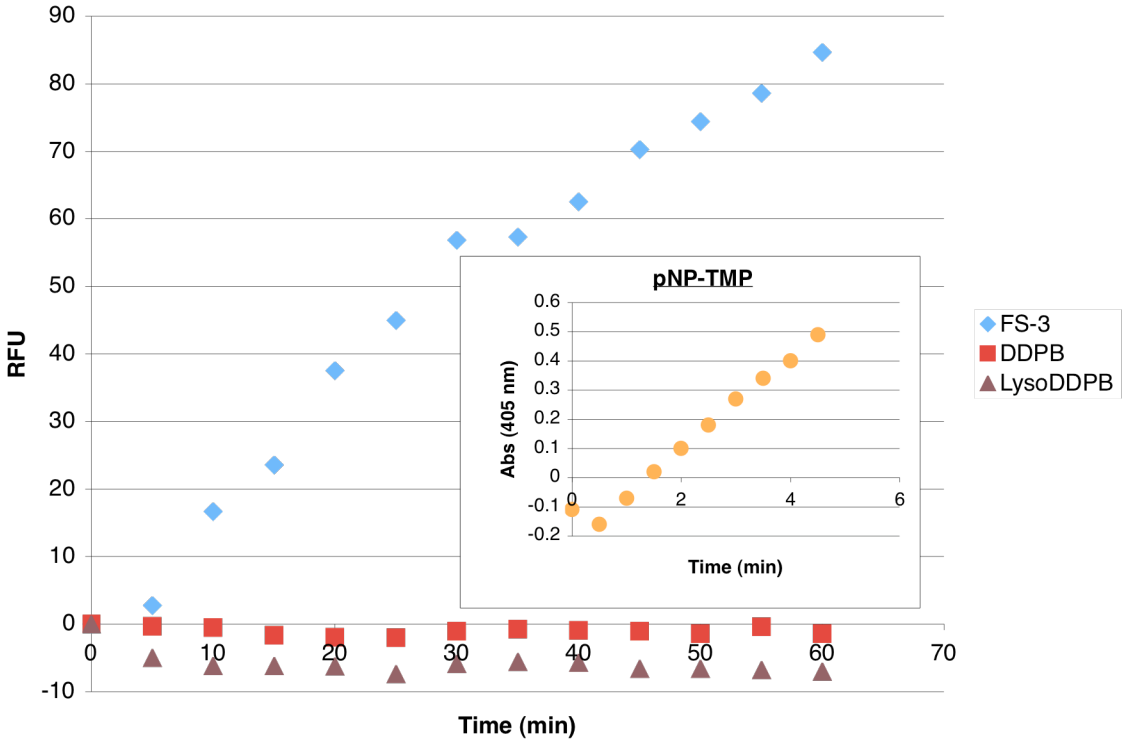
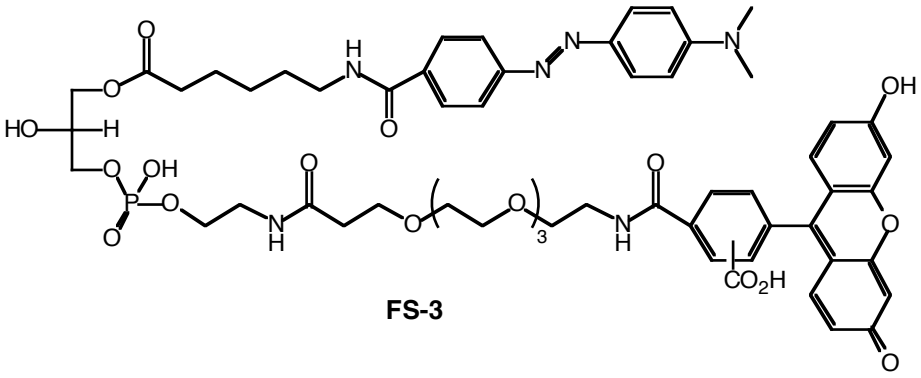
Figure 3





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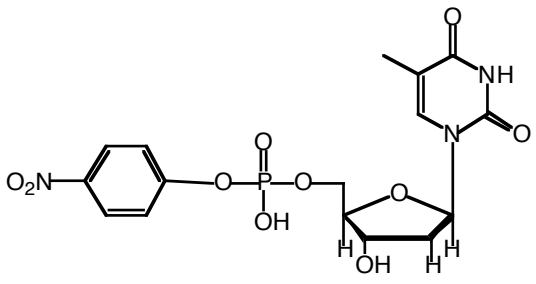


*p*NP-TMP

Figure 4

EXPERIMENTAL METHODS

Synthesis of 6-(p-methyl red) aminohexanoic acid. The sodium salt of *p*–methyl red (Acros) was converted to its acid form by treatment with 2 M HCl, followed by lyophilization. The acid form (6.8 g, 25 mmol) was then combined with NHS (*N*–hydroxysuccinimide, 4.4 g, 38 mmol) and EDCI (*N*–(3–Dimethylaminopropyl)–*N'*–ethylcarbodiimide hydrochloride, 7.3 g, 38 mmol) in DMF (60 mL). After stirring overnight, the reaction was concentrated under vacuum and washed (H_2O) to give the NHS–ester of *p*–methyl red (**3**). Compound **3** (226 mg, 0.62 mmol) was further reacted with 6–aminocaproic acid (123 mg, 0.94 mmol) in a 2:1 solution of DMF:H_2O containing 7% DMAP. After 24 h, the reaction was acidified with 3 M HCl, then extracted with 10% MeOH in CH_2Cl_2. Solvents were removed *in vacuo* and the product was precipitated from EtOH/H_2O as a deep red solid (200 mg, 77% yield, two steps). δ_H(400 MHz, $CDCl_3$): 7.87–7.77 (m, 6H), 6.74–6.69 (d, J = 9.2 Hz, 2H), 3.41–3.33 (t, J = 7.0 Hz, 2H), 3.06 (s, 6H), 2.32–2.25 (t, J = 7.4 Hz, 2H), 1.67–1.56 (m, 4H), 1.44–1.34 (m, 2H). HRMS (ESI): *m/z* 383.2088; calcd: 383.2083 $(M+H)^+$.

*Synthesis of 1-O-(6-(p-methyl red)-aminohexanoyl)-sn-glyceryl phosphatidylcholine (**2**)*. CDI (Carbonyldiimidazole, 35 mg, 0.22 mmol) was added to 6-(p-methyl red) aminohexanoic acid (53 mg, 0.14 mmol) stirring in 2 mL $CHCl_3$. After one hour, another 18 mg (0.11 mmol) CDI was added until the acid was completely consumed. After another hour stirring, the solvents were removed from the reaction mixture by rotary evaporator. The residue was dissolved in DMF (2 mL) and cooled in an ice bath. 36 mg (0.14 mmol) *sn*-glycero-3-phosphocholine (**1**, Bachem) and 42 μL (0.28 mmol) DBU were added and the reaction was allowed to slowly warm to 4 °C.

After 48 hours at 4 °C, the DMF was removed under vacuum and the residue passed through a SiO_2 (Flash Chromatography ASTM 230–400 Silica Gel) column using 65:25:4 CH_2Cl_2:MeOH:H_2O (23 mg, 26% yield). δ_H(d-DMSO): 8.03 (d, J= 8.4 Hz, 2H, dabcyl), 7.83 (d, J= 8.8 Hz, 2H, dabcyl), 7.82 (d, J= 8.4 Hz, 2H, dabcyl), 6.84 (d, J= 9.6 Hz, 2H, dabcyl), 4.14-4.05 (br m, 2H), 4.05-3.94 (m, 2H), 3.80-3.75 (br m, 1H), 3.74-3.68 (br m, 2H), 3.60-3.54 (br m, 2H), 3.32-3.24 (br m, 2H), 3.15 (s, 9H), 3.07 (s, 6H, dabcyl), 2.33 (t, J= 7.2 Hz, 2H), 1.63-1.51 (br m, 4H), 1.40-1.29 (br m, 2H). δ_C(101 MHz, d-DMSO): 173.6, 166.2, 154.5, 153.5, 143.3, 135.6, 129.0, 125.8, 122.1, 112.2, 68.9, 66.6, 66.0, 65.8, 59.2, 59.1, 53.8, 34.1, 29.5, 26.6, 24.9. δ_P(162 MHz, d-DMSO): 1.0. HRMS (MALDI): *m/z* 622.3011; calcd: 622.3006 (M^+).

Synthesis of 1-O-(6-(p-methyl red)-amino-hexanoyl)-2-O-(12-Boc-amino-dodecanoyl)-sn-glyceryl phosphatidylcholine. 12-Boc-amino-dodecanoic acid (139 mg, 0.44 mg), TPSNT[36] (161 mg, 0.42 mmol), and methylimidazole (65 μL, 0.82 mmol) were combined in 4 mL distilled CH_2Cl_2 under Ar. After 40 min., the resulting solution was transferred to 85 mg (0.14 mmol) **2** stirring in 5 mL CH_2Cl_2. After 24 hours at room T, the solvent was removed and the product purified from SiO_2 with 65:25:4 CH_2Cl_2:MeOH:H_2O (98 mg, 78% yield). δ_H(d-DMSO): 8.04 (d, J= 8.4 Hz, 2H, dabcyl), 7.84-7.78 (m, 4H, dabcyl), 6.85 (d, J= 9.2 Hz, 2H, dabcyl), 5.12-5.05 (br m, 1H), 4.30 (dd, J= 12, 3.2 Hz, 1H), 4.12 (dd, J= 12, 6.4 Hz, 1H), 4.10-4.03 (br m, 2H), 3.82-3.75 (br m, 2H), 3.58-3.53 (br m, 2H), 3.31-3.23 (br m, 2H), 3.15 (s, 9H), 3.08 (s, 6H, dabcyl), 2.91-2.83 (br m, 2H), 2.31 (t, J= 6.8 Hz, 2H), 2.27 (t, J= 7.6 Hz, 2H), 1.62-1.44 (br m, 6H), 1.39-1.28 (br m, 4H), 1.36 (s, 9H), 1.26-1.15 (br m, 14 H). δ_C(d-DMSO): 173.3, 173.1, 166.1, 156.2, 154.5, 153.5, 143.3, 135.6, 129.0, 125.7, 122.1, 112.2, 77.9, 71.2,

66.1, 63.2, 63.0, 59.1, 59.0, 55.6, 53.8, 34.2, 34.0, 30.2, 29.7, 29.6, 29.6, 29.4, 29.1, 28.9, 27.0, 26.6, 25.1. δ_P(d-DMSO): -0.44. HRMS (MALDI): *m/z* 919.5327; calcd: 919.5310 (M^+).

*Synthesis of 1-O-(6-(p-methyl red)-amino-hexanoyl)-2-O-(12-(p-methyl red)-amino-dodecanoyl)-sn-glyceryl phosphatidylcholine (**4**).* A solution of 2-*O*-boc-protected PC intermediate (42 mg, 0.05 mmol) in 5 mL CH_2Cl_2 was treated with 0.5 mL TFA. After 1 hour, solvents were completely removed by vacuum and the residue was dissolved in 5 mL DMF. TEAB buffer (4 mL, 1 M, pH 8.4) and **3** (26 mg, 0.07 mmol) were added and the resulting solution was stirred overnight. Product **4** was collected from SiO_2 using a step gradient: a) 20% MeOH in CH_2Cl_2; b) 70:20:2 CH_2Cl_2:MeOH:H_2O; c) 65:25:4 CH_2Cl_2:MeOH:H_2O (37 mg, 76% yield). δ_H($CDCl_3$:CD_3OD, 1:1): 7.89 (d, J= 8.8 Hz, 2H, dabcyl), 7.87 (d, J= 8.4 Hz, 2H, dabcyl), 7.85-7.77 (m, 8H, dabcyl), 6.73 (d, J= 9.6 Hz, 4H, dabcyl), 5.23-5.16 (br m, 1H), 4.40 (dd, J= 12, 3.4 Hz, 1H), 4.25-4.18 (br m, 2H), 4.13 (dd, J= 12, 6.6 Hz, 1H), 3.97 (t, J= 6.5 Hz, 2H), 3.59-3.54 (br m, 2H), 3.41-3.32 (br m, 4H), 3.17 (s, 9H), 3.07 (s, 6H, dabcyl), 3.06 (s, 6H, dabcyl), 2.33 (t, J= 7.6 Hz, 2H), 2.30 (t, J= 7.2 Hz, 2H), 1.70-1.52 (br m, 6H), 1.45-1.36 (br m, 2H), 1.38-1.20 (br m, 16 H). δ_C($CDCl_3$:CD_3OD, 1:1): 173.9, 173.7, 168.4, 168.4, 155.1, 155.1, 153.2, 143.6, 134.9, 134.8, 128.2, 128.2, 125.5, 122.1, 111.6, 70.5, 66.5, 63.6, 62.7, 59.2, 59.2, 54.0, 40.3, 40.0, 34.2, 33.9, 30.5, 29.6, 29.5, 29.5, 29.4, 29.1, 27.2, 26.5, 25.0, 24.6. δ_P($CDCl_3$:CD_3OD, 1:1): 0.44. HRMS (MALDI): *m/z* 1070.5856; calcd: 1070.5844 (M^+).

*(3-dimethylamino-propyl)-carbamic acid-1,1-dimethylethyl ester (**5**).* 3-dimethylamino-propylamine (1 mL, 8 mmol) was dissolved in 1 M NaOH (27 mL). Di-

tert-butyl dicarbonate (4 mL, 19 mmol) was added to the stirring solution. After 1.5 hours, the reaction was extracted twice with CH_2Cl_2. The organic layer was washed with brine and then stripped of solvent under vacuum. The product was of sufficient purity for use directly in the next step (598 mg, 37% yield). $\delta_H(CD_3OD)$: 3.05 (t, J= 7.0 Hz, 2H), 2.36-2.31 (m, 2H), 2.23 (s, 6H), 1.65 (tt, J= 7.6, 7.0, 2H), 1.43 (s, 9H). $\delta_C(CD_3OD)$: 157.3, 78.6, 56.9, 44.3, 38.4, 27.7, 27.5. HRMS (MALDI): *m/z* 203.17555; calcd: 203.1760 (M^+).

*(3-(1,1-dimethylethoxycarbonyl)amino-propyl)-(2-hydroxyethyl)-dimethyl-ammonium bromide (**6**)*. Boc-protected 3-dimethylamino-propylamine (178 mg, 0.88 mmol) was dissolved in 3 mL DMF. DIPEA (153 μL, 0.88 mmol), then 2-bromoethanol (63 μL, 0.88 mmol) were added and the reaction was stirred under Ar. The reaction was stirred overnight at 50 °C, at which time the volatile components were removed under vacuum. The residue was macerated in acetone and filtered through Celite to give solid product, **5**, which was nearly pure by NMR and used directly in the ensuing step (214 mg, 98% yield). $\delta_H(CD_3OD)$: 4.03-3.97 (br m, 2H), 3.58-3.53 (br m, 2H), 3.53-3.45 (br m, 2H), 3.22 (s, 6H), 3.15 (t, J= 6.6, 2H), 2.05-1.96 (br m, 2H), 1.43 (s, 9H). $\delta_C(CD_3OD)$: 157.2, 79.1, 65.5, 63.4, 51.5, 51.4, 51.4, 37.3, 27.7, 23.3. HRMS (MALDI): *m/z* 247.2005; calcd: 247.2022 (M^+).

*Synthesis of 1-O-(6-(p-methyl red)-amino-hexanoyl)-2-O-(12-(p-methyl red)-amino-dodecanoyl)-sn-glyceryl-N-(3-Boc-amino-propyl)-N,N-dimethyl-phosphatidylethanolamine (**7**)*. Compound **4** (17 mg, 0.02 mmol) was dissolved in $CHCl_3$ (3 mL) with a > 20-fold molar excess of **6** (151 mg, 0.46 mmol). *Streptomyces Sp.* PLD(P) (Genzyme) (1.3 mg, ca. 400 U.) was added in 0.5 mL buffer (0.2 M NaOAc, 0.08

M $CaCl_2$, pH 5.4), and the biphasic solution was allowed to stir at 45 °C. After 1 hour, another 0.7 mg PLD (ca. 200 U.) was added in 0.2 mL buffer, and after an additional 2 hours, another 0.5 mg PLD (ca. 150 U.) was added in 0.3 mL buffer, resulting in almost complete consumption of **4**. The reaction mixture was diluted with AcCN and evaporated to dryness. The residue was purified from SiO_2 using a step gradient: a) 10% MeOH:CH_2Cl_2; b) 20% MeOH:CH_2Cl_2; c) 65:25:4 CH_2Cl_2;MeOH:H_2O (9.5 mg, 50% yield). δ_H(d-DMSO): 8.01 (d, J= 8.6 Hz, 2H, dabcyl), 7.99 (d, J= 8.6 Hz, 2H, dabcyl), 7.84-7.76 (m, 8H, dabcyl), 6.84 (d, J= 9.2 Hz, 2H, dabcyl), 6.83 (d, J= 9.2 Hz, 2H, dabcyl), 5.13-5.05 (br m, 1H), 4.30 (dd, J= 12, 3.2 Hz, 1H), 4.12 (dd, J= 12, 6.4 Hz, 1H), 4.10-4.03 (br m, 2H), 3.84-3.77 (br m, 2H), 3.57-3.50 (br m, 2H), 3.40-3.33 (br m, 2H), 3.31-3.21 (m, 4H), 3.09 (s, 6H), 3.08 (s, 6H, dabcyl), 3.07 (s, 6H, dabcyl), 3.02-2.94 (br m, 2H), 2.34-2.23 (m, 4H), 1.89-1.78 (br m, 2H), 1.61-1.45 (br m, 6H), 1.45-1.18 (br m, 18H), 1.38 (s, 9H). δ_C(d-DMSO, selected peaks): 166.0, 129.0, 122.1, 112.3, 95.4, 31.4, 29.5, 29.2, 28.9, 1.4, 0.8. δ_P(d-DMSO): -1.0. HRMS (ESI): *m/z* 607.34284; calcd: 607.34288 $(M+H)^{++}$.

*Synthesis of 1-O-(6-(p-methyl red)-amino-hexanoyl)-2-O-(12-(p-methyl red)-amino-dodecanoyl)-sn-glyceryl-N-(3-(5-BODIPY-pentanoyl)-amino-propyl)-N,N-dimethyl-phosphatidylethanolamine (**DDPB**).* Compound **7** (4 mg, 3.3 μmol) was dissolved in $CHCl_3$ (2 mL), to which was added 0.2 mL TFA. After 15-20 min. the solvents were removed completely under vacuum. The resulting red film was dissolved in TEAB buffer (3 mL, 1 M, pH 8.3) and treated with 1.8 mg (4.3 μmol) C_5-BODIPY, SE in 0.7 mL DMF. After 2 hours, more C_5-BODIPY, SE (1.1 mg, 2.6 μmol) in 0.8 mL DMF was added. After 1 hour, the solvents were removed by vacuum and the residue

purified on SiO_2 with a step gradient: a) 20% MeOH:CH_2Cl_2; b) 25% MeOH:CH_2Cl_2; c) 65:25:4 CH_2Cl_2;MeOH:H_2O (3.0 mg, 64% yield). δ_H(d-DMSO): 8.70 (br s, 1H, amide-NH), 8.57 (br s, 1H, amide-NH), 8.41 (br s, 1H, amide-NH), 8.04-7.95 (br m, 4H, dabcyl), 7.85-7.75 (br m, 8H, dabcyl), 7.6-7.64 (br m, 1H, BODIPY), 7.13-7.07 (br m, 1H, BODIPY), 6.87-6.80 (br m, 4H, dabcyl), 6.44-6.38 (br m, 1H, BODIPY), 6.28-6.25 (br m, 1H, BODIPY), 5.12-5.04 (br m, 1H), 4.32-4.23 (br m, 1H), 4.15-4.07 (br m, 1H), 4.07-3.98 (br m, 2H), 3.83-3.74 (br m, 2H), 3.53-3.47 (br m, 2H), 3.30-3.21 (br m, 4H), 3.18-3.10 (br m, 2H), 3.05 (br m, 18H), 2.87-2.78, 2.45 (br s, 3H, BODIPY), 2.34-2.23 (br m, 4H), 2.24 (br s, 3H, BODIPY), 2.20-2.12 (br m, 2H), 2.12-2.06 (br m, 2H), 1.93-1.82 (br m, 2H), 1.70-1.43 (br m, 12H), 1.40-1.15 (br m, 18H). δ_C(d-DMSO, selected peaks): 134.8, 128.2, 125.5, 122.1, 111.6, 110.0, 40.0. δ_P(d-DMSO): 0.11. HRMS (ESI): *m/z* 708.38732; calcd: 708.38732 $(M+H)^{++}$.

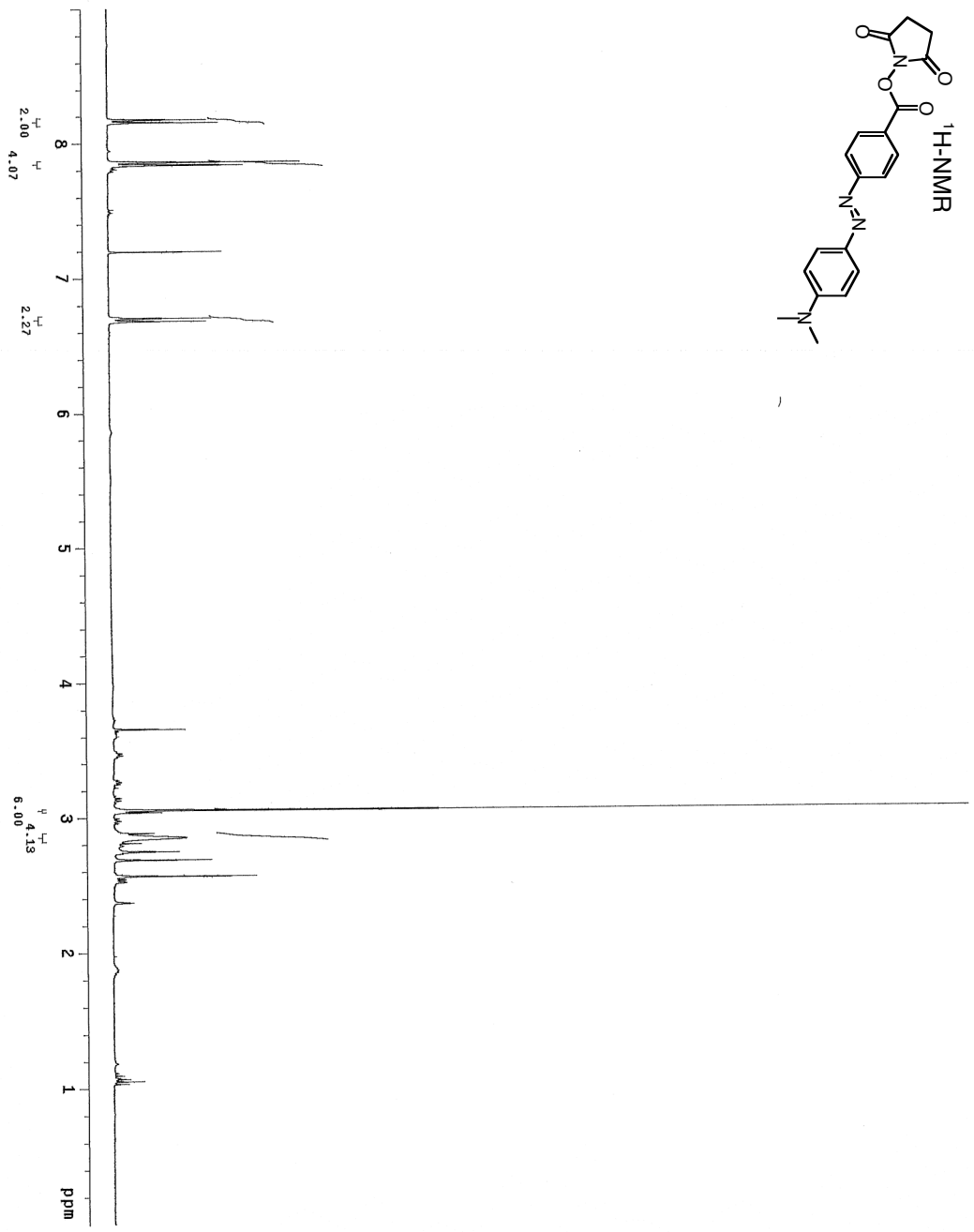
*Synthesis of 1-O-(6-(p-methyl red)-amino-hexanoyl)-sn-glyceryl-N-(3-(5-BODIPY-pentanoyl)-amino-propyl)-N,N-dimethyl-phosphatidylethanolamine (**lysoDDPB**)*. Tris buffer (0.1 M) at pH 8.9 containing $CaCl_2$ (0.1 M) and 0.05% NaN_3 was used to prepare a 1 U/μL stock solution of *Naja mossambica moss*. PLA_2. **DDPB** (2 mg, 1.4 μmol) dissolved in MeOH (0.4 mL) was treated with 400 μL of the PLA_2 stock solution and allowed to stir at RT for 1.5 h. Solvents were then removed *in vacuo* and the residue applied to a column of SiO_2 and eluted with 65:25:4 CH_2Cl_2:MeOH:H_2O (65:25:4). **LysoDDPB** (1.3 mg, 96% yield) was collected as a red solid. δ_H(d-DMSO): 8.71-8.65 (br s, 1H, amide-NH), 8.31-8.23 (br s, 1H, amide-NH), 8.03-7.96 (d, J= 8.0 Hz, 2H, dabcyl), 7.85-7.77 (br m, 4h, dabcyl), 7.69-7.65 (br, 1H, BODIPY), 7.12-7.08 (br, 1H, BODIPY), 6.89-6.82 (d, J= 8.0 Hz, 2H, dabcyl), 6.44-6.38 (br, 1H, BODIPY), 6.30-

6.24 (br, 1H, BODIPY), 4.11-4.01 (br, 2H), 4.01-3.86 (br m, 2H), 3.78-3.72 (br m, 1H), 3.73-3.60 (br m, 2H), 3.44-3.16 (br H_2O peak), 3.16-3.10 (br m, 2H), 3.08 (s, 6H, dabcyl), 3.06 (s, 6H), 2.93-2.80 (br m, 4H), 2.44 (s, 3H, BODIPY), 2.35-2.28 (br m, 2H), 2.24 (s, 3H, BODIPY), 2.19-2.11 (br m, 2H), 1.95-1.79 (br m, 4H), 1.70-1.46 (br m, 8H), 1.39-1.27 (br m, 2H). δ_p(d-DMSO): 1.3. HRMS (MALDI): *m/z* 967.4820; calcd: 967.4830 (M^+).

*Assay procedure for **DDPB** and **lysoDDPB** in mixed micelles.* **DDPB** or **lysoDDPB** (2.5 μg) was sonicated into a solution of Triton X-100 (reduced, 2.5 mM) to give mixed micelles 0.2 (wt)% in **DDPB** or **lysoDDPB**. For scPLD (BIOMOL), 0.1 M Tris, 0.1 M $CaCl_2$, 0.05% NaN_3, pH 8 was used. 20 mM HEPES, 10 mM $CaCl_2$, 150 mM NaCl, pH 7.4 was the buffer for assays with *B. Cereus* PC-PLC (Sigma); the same buffer at pH 8.3 was also used to assay *B. Cereus* PI-PLC (Sigma) and the PLA_2 from bee and *Naja Mossambica mossambica* venoms (Sigma). A pH 5.6 buffer (0.2 M NaOAc, 80 mM $CaCl_2$) was used with PLD from cabbage (Sigma), *Steptomyces sp.* (BIOMOL), and peanut (Sigma), and Genzyme PLD(P). Mixed micelle solutions (95 μL) were placed in a black 96-well plate and treated with 5 μL of the listed enzyme solutions to give a final enzyme concentration of 1 U/well. In the case of the FBS assay, a 1:1 ratio (50 μL each) of FBS (Atlanta Biologicals, Inc.) to mixed micelle solution was used. Fluorescence was monitored (Ex 500 nm/Em 530 nm) every 15 s over 3 min to 1 h at 37 °C. Results are plotted as RFU (relative fluorescence units) versus time.

Assay procedure with p*NP-TMP and FS-3.* A 2.5 mM solution of *p*NP-TMP (Sigma) was prepared in 0.1 M Tris, 0.1 M $CaCl_2$, 0.05% NaN_3, pH 8.9. 20 μL of this solution was added to 80 μL FBS and monitored at room temperature for the time period

indicated at 405 nm. FS-3 (kindly donated by Echelon Biosciences, Inc.) was dissolved in 50 mM Tris, 50 mM $CaCl_2$, 10 mM $MgCl_2$, pH 8 to give a 25 μM solution. 20 μL of this solution was mixed with 80 μL FBS and monitored for the time period indicated at 37 °C and $\lambda_{Ex}/\lambda_{Em}$ = 484/520 nm.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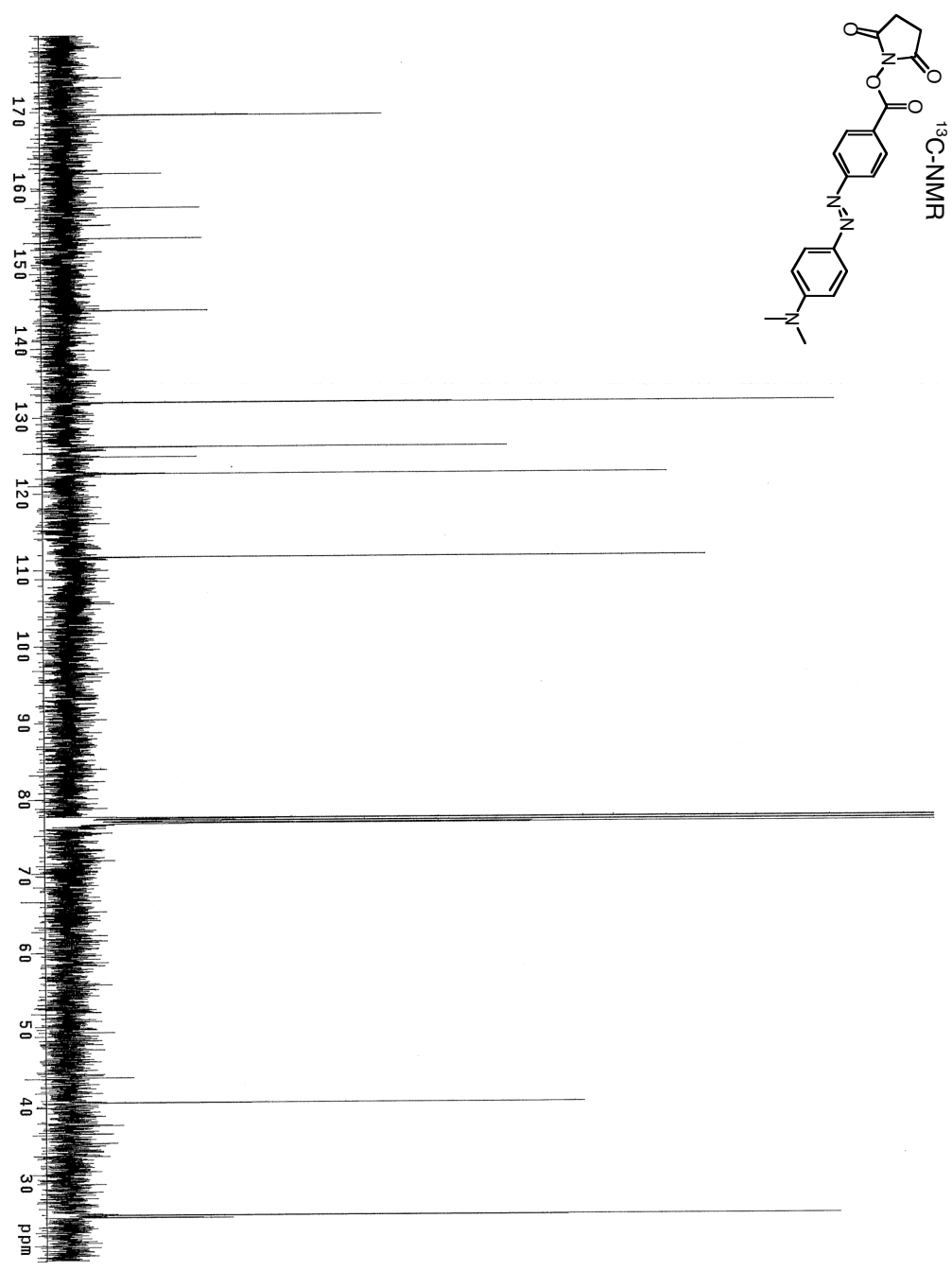
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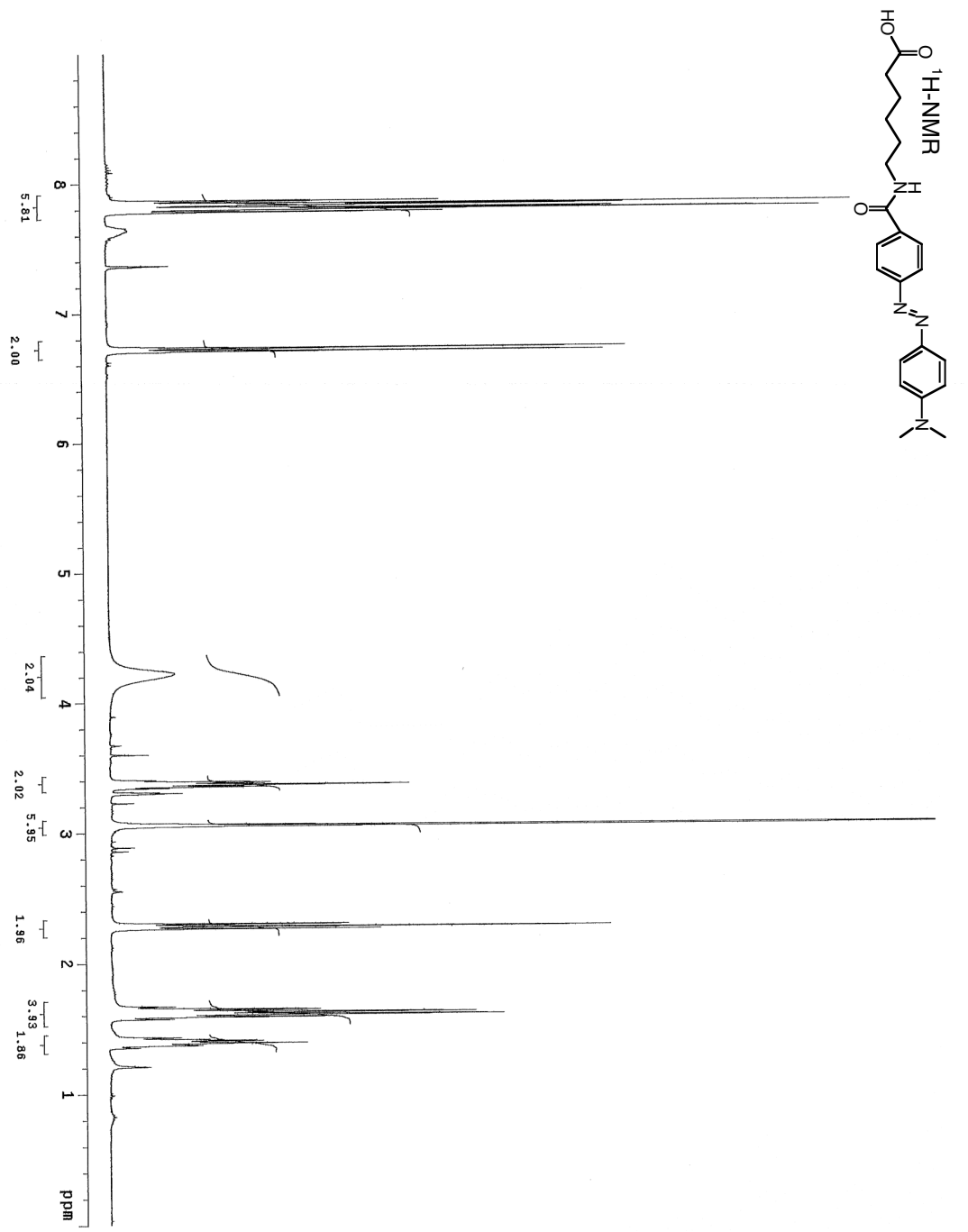
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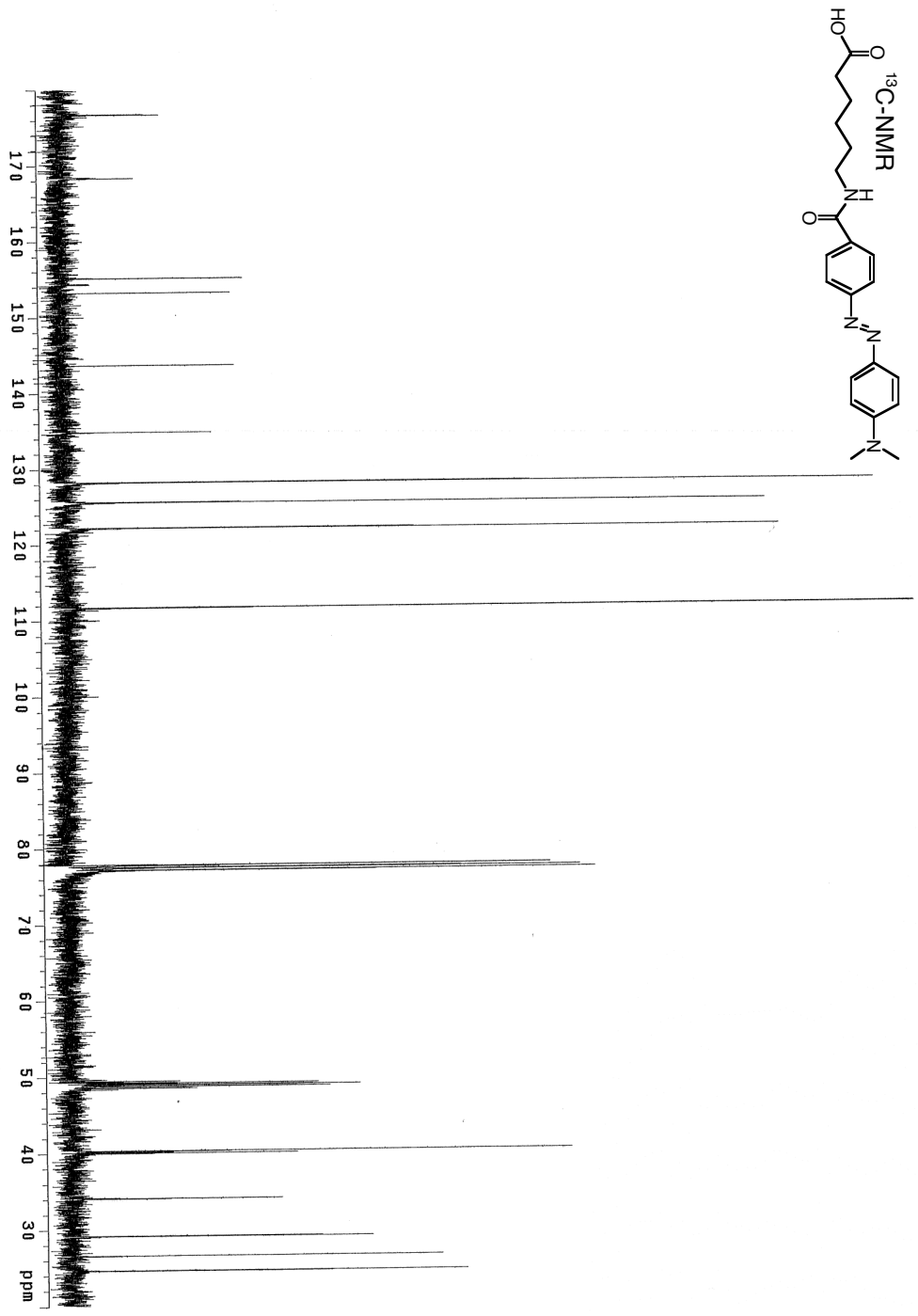
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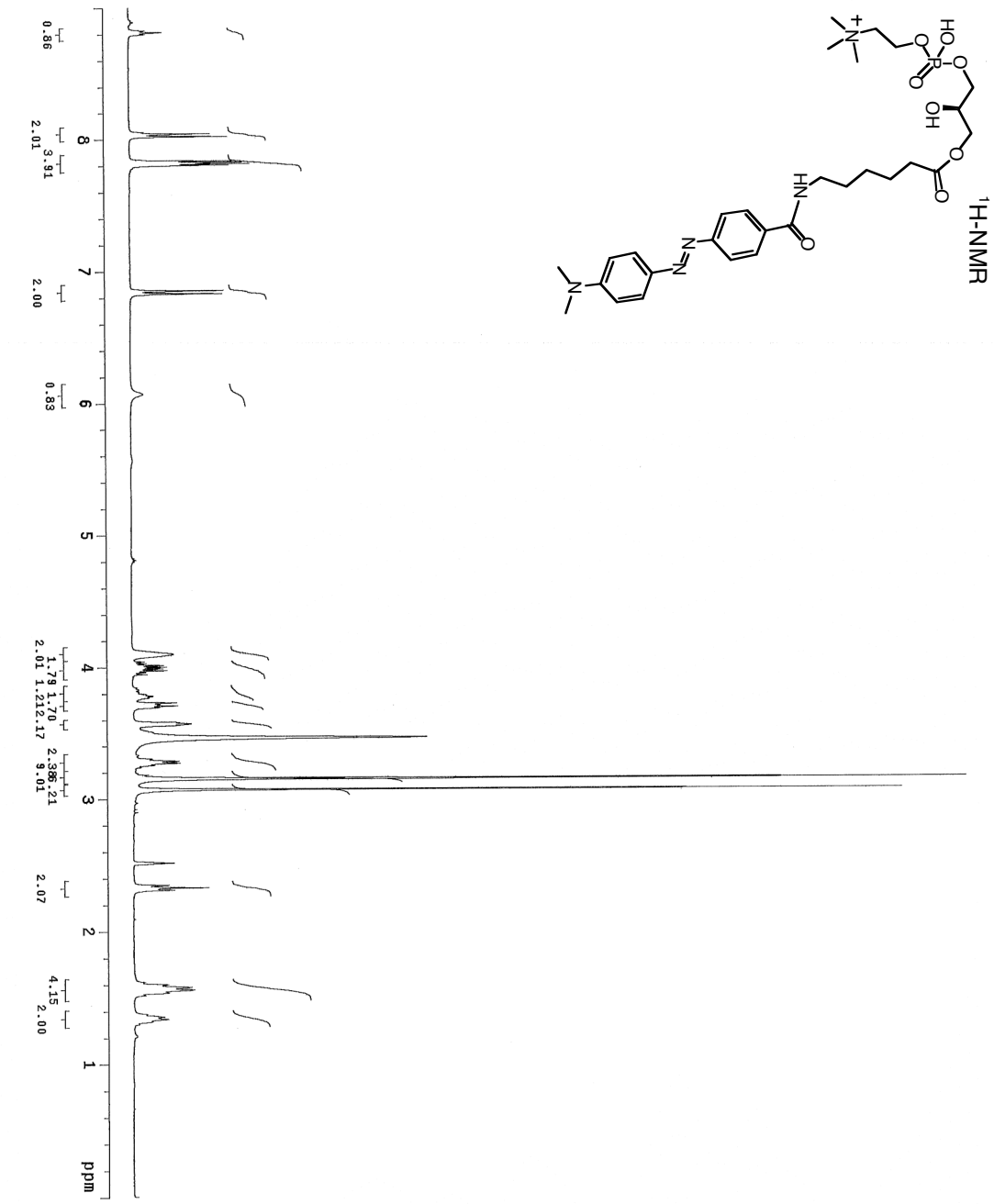
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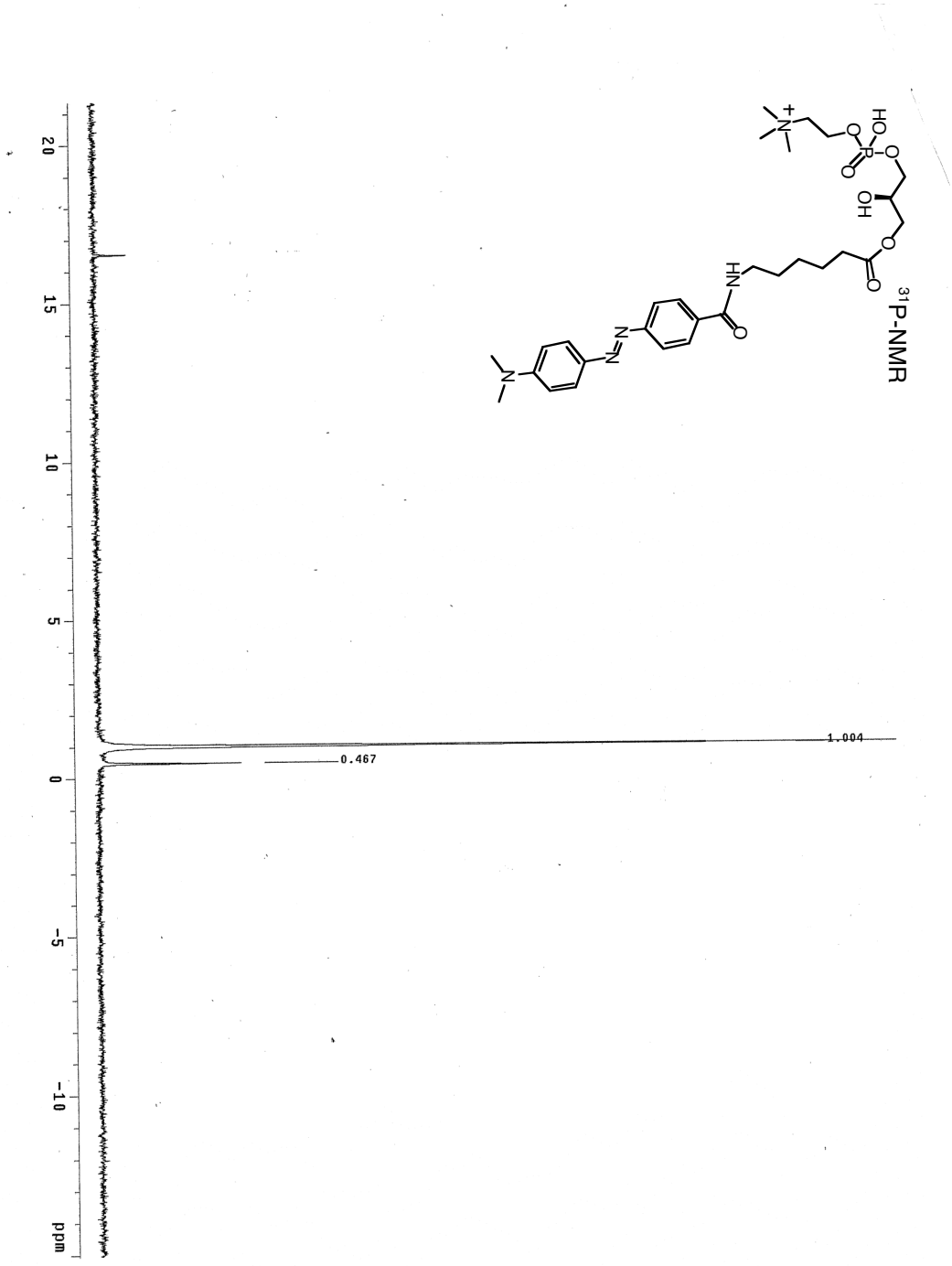
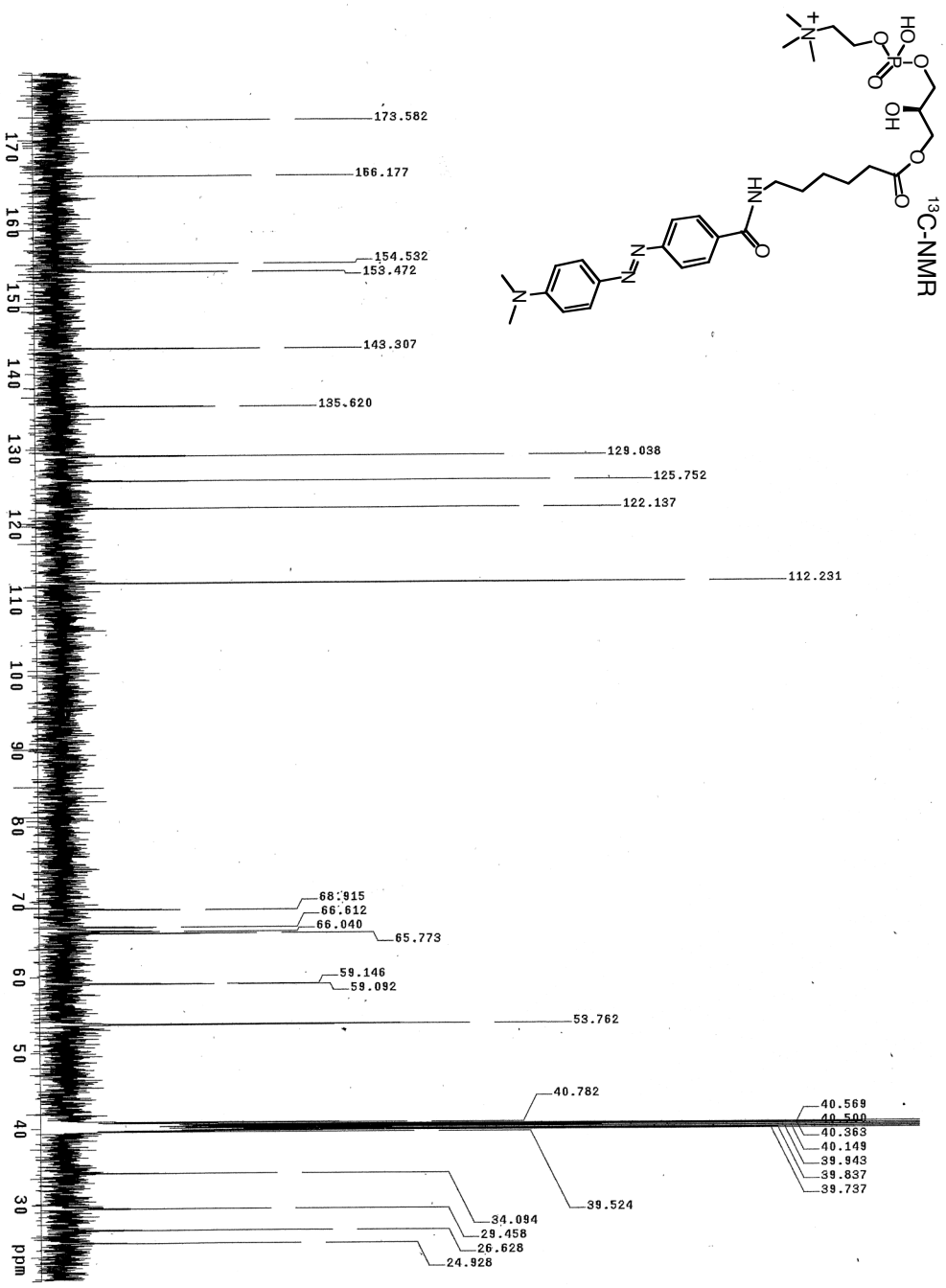
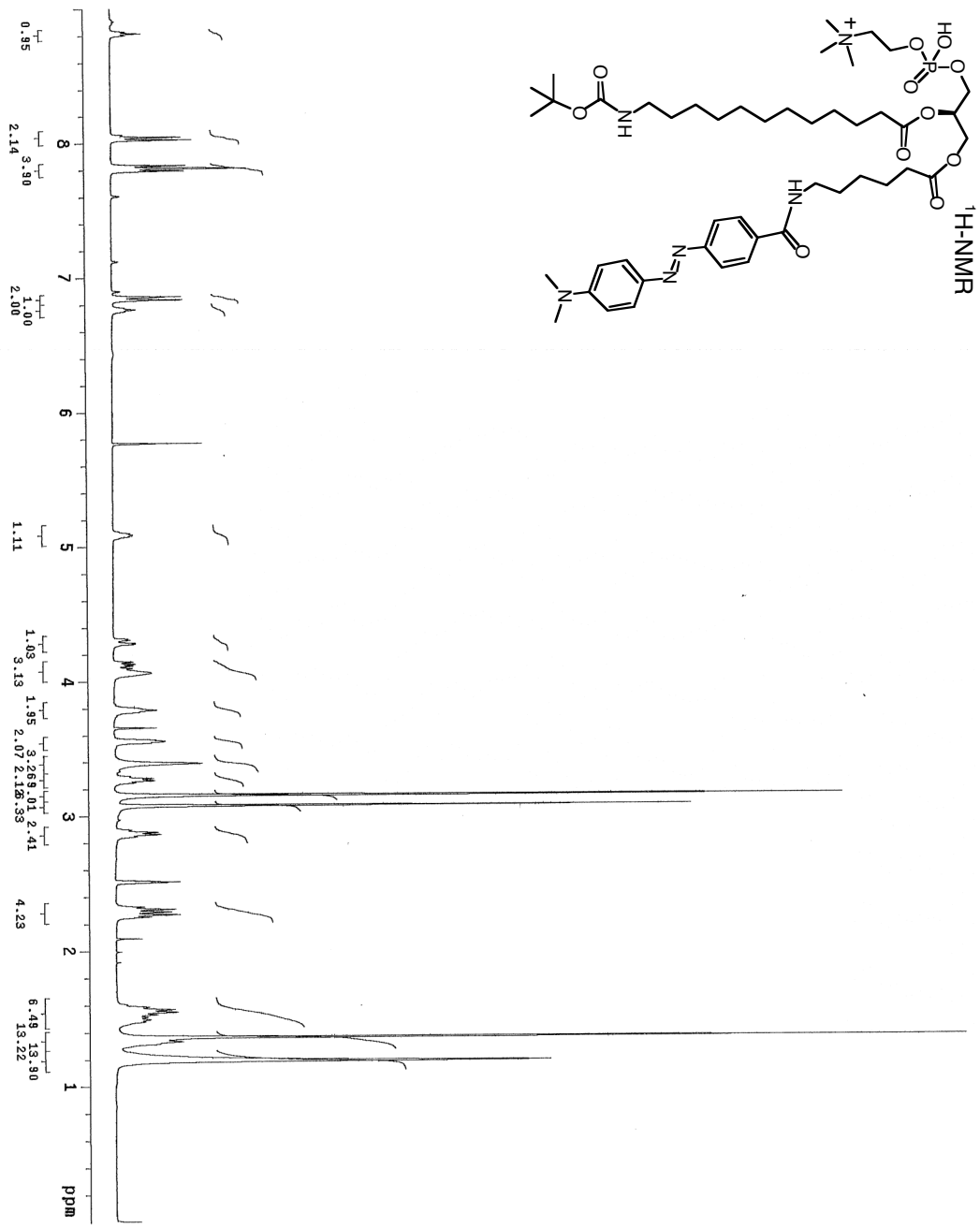
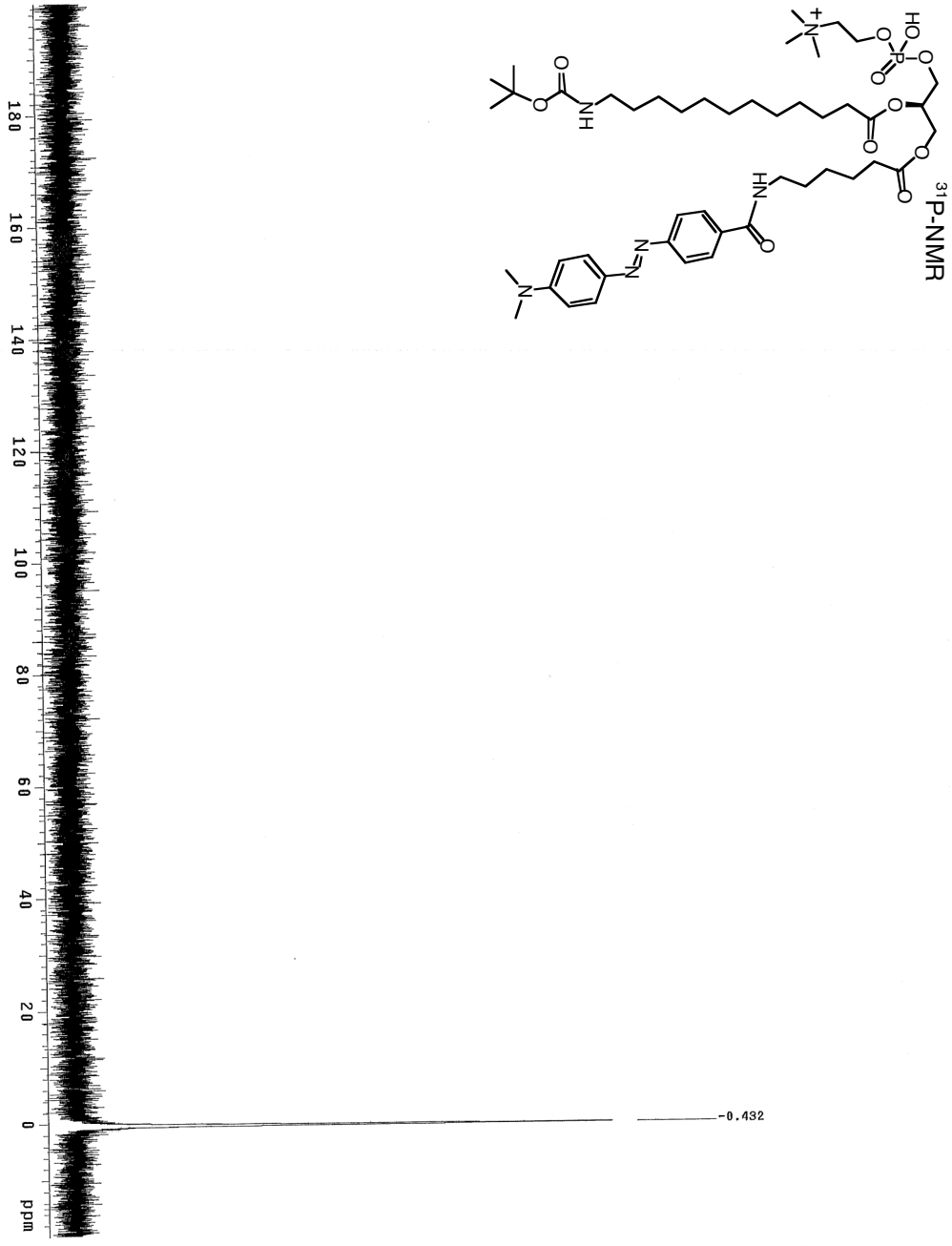
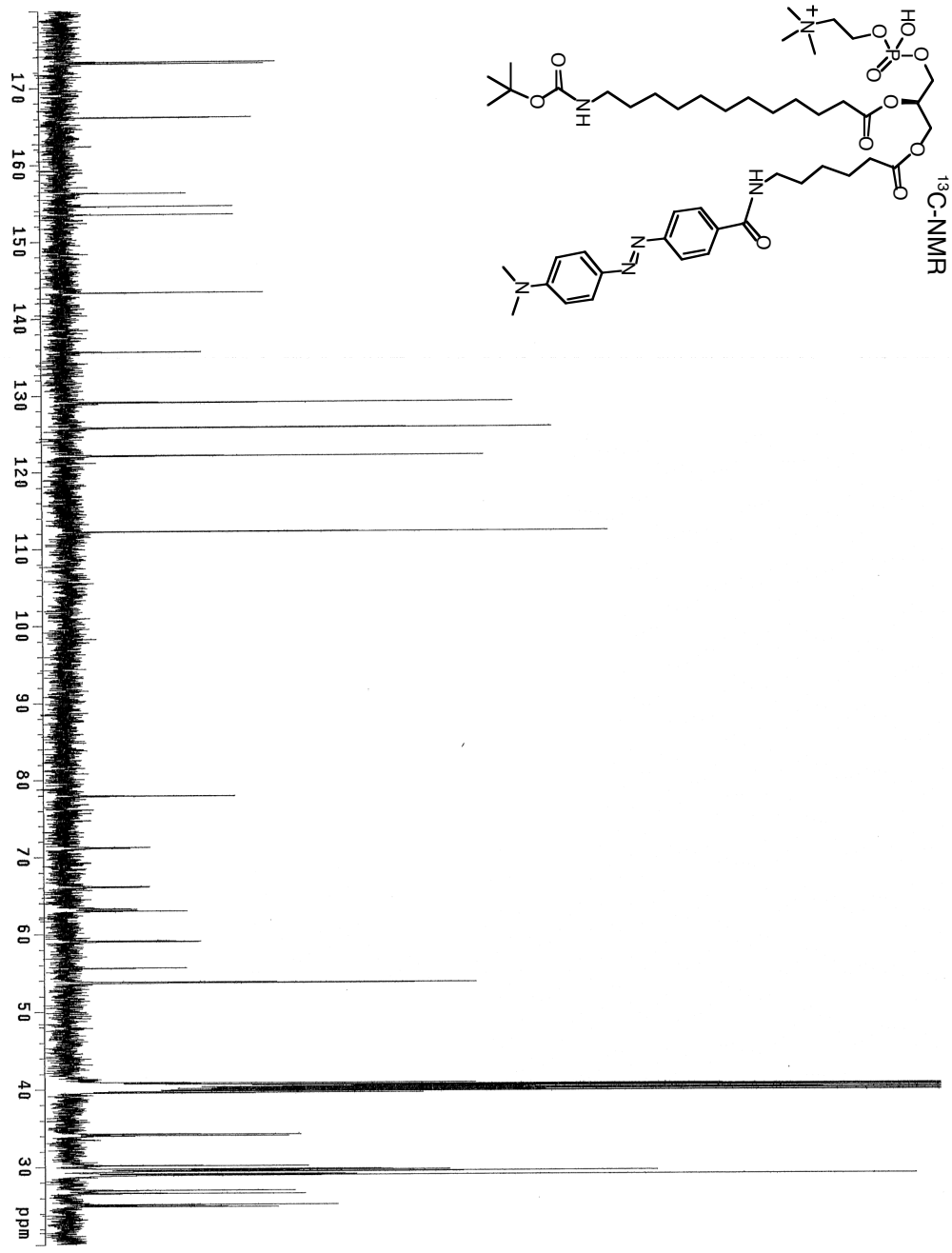
ppm

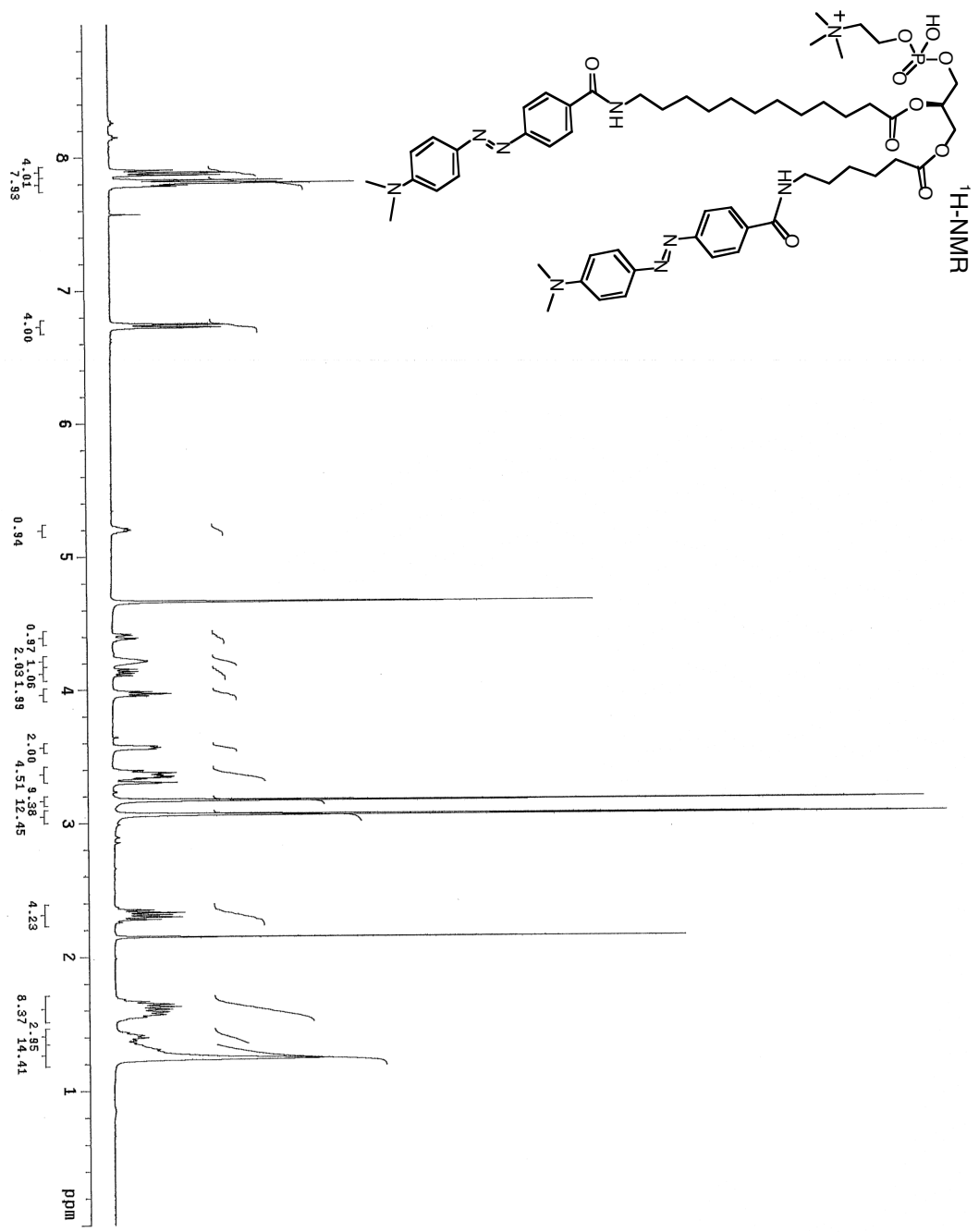

31P-NMR
1.004
0.467
20
15
10
5
0
-5
-10
ppm

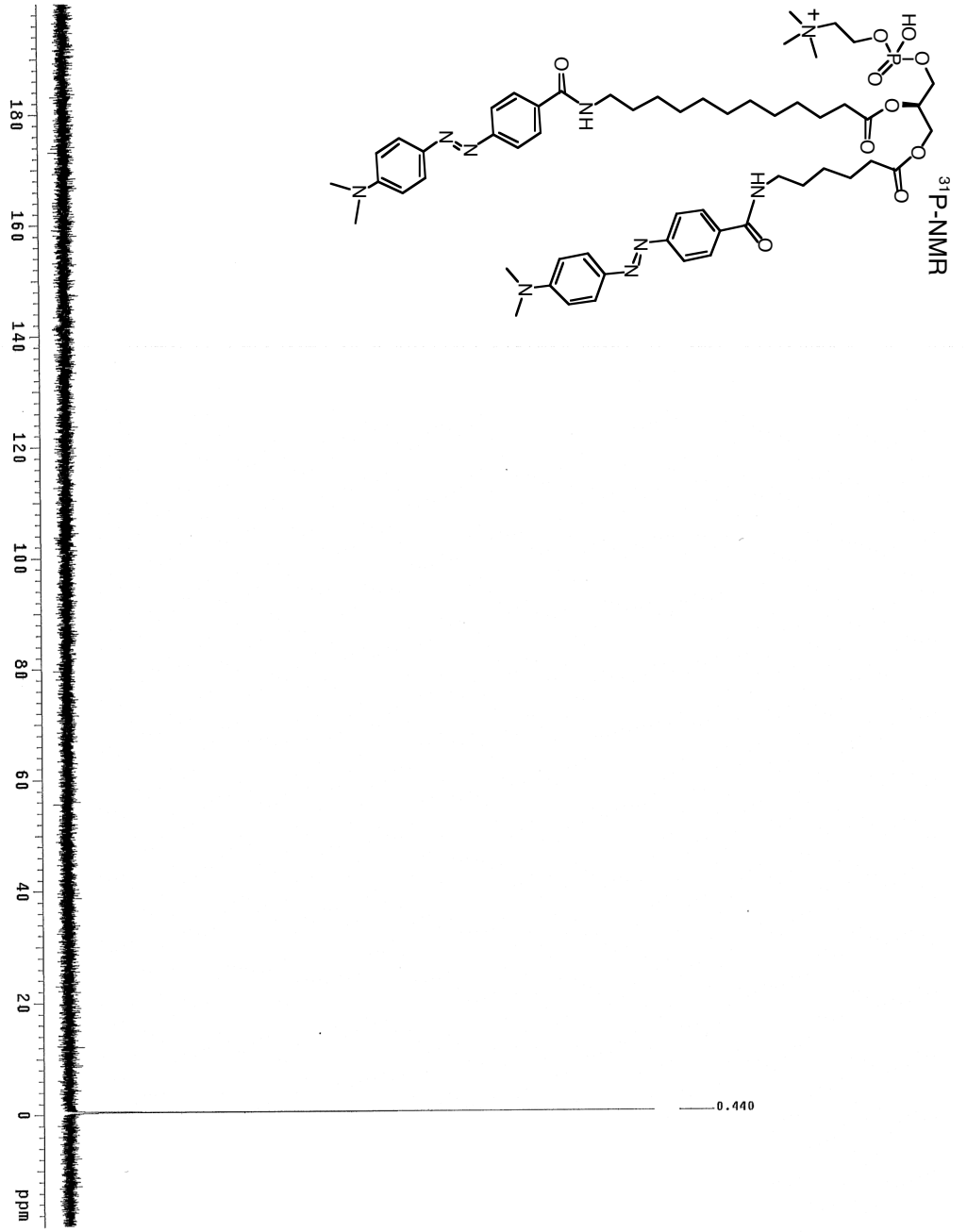

173.582
166.177
154.532
153.472
143.307
135.620
129.038
125.752
122.137
112.231
68.915
66.612
66.040
65.773
59.146
59.092
53.762
40.782
40.569
40.500
40.363
40.149
39.943
39.837
39.737
39.524
34.094
29.458
26.628
24.928
ppm
13C-NMR


^{1}H-NMR
ppm

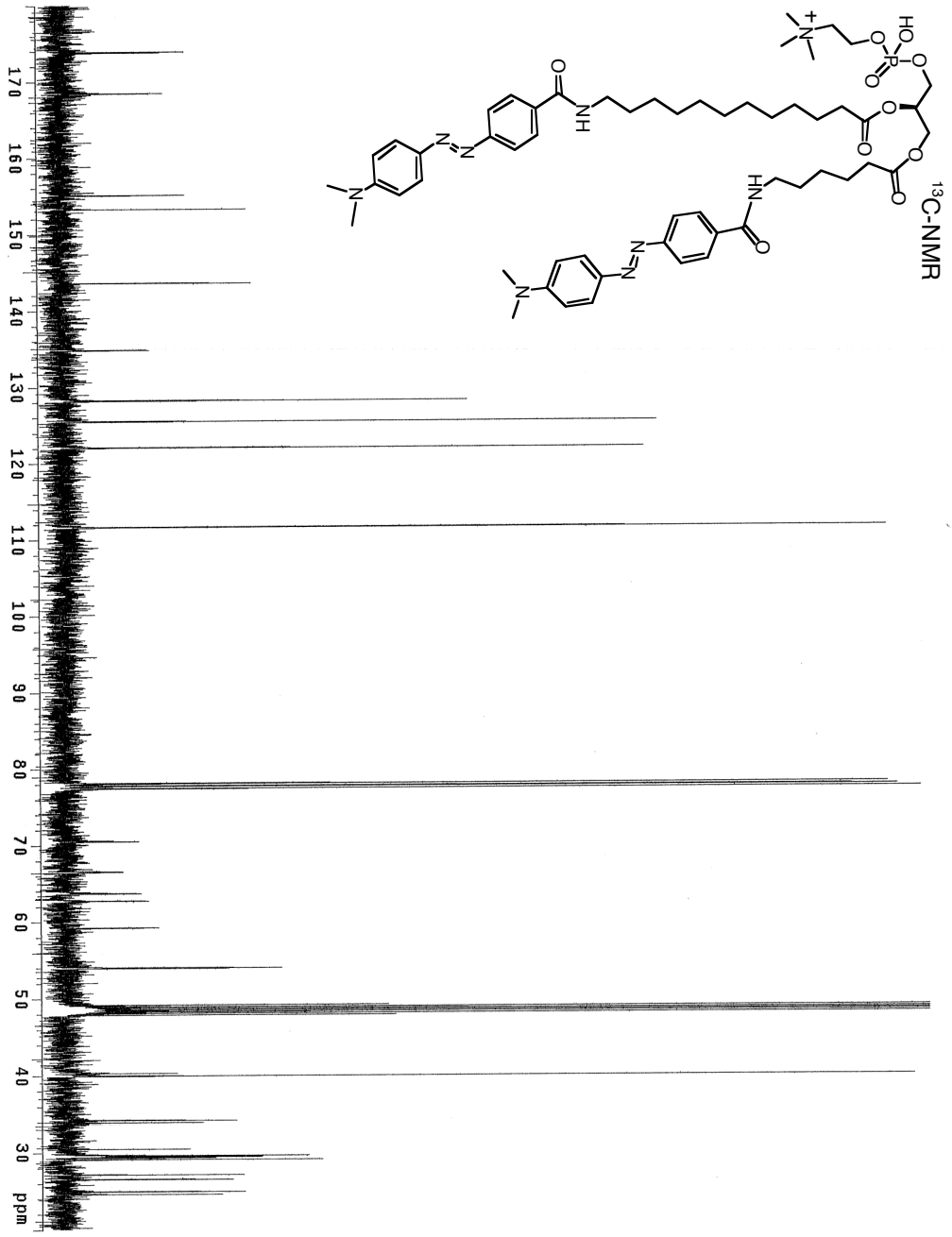

31P-NMR
-0.432
180
160
140
120
100
80
60
40
20
0
ppm

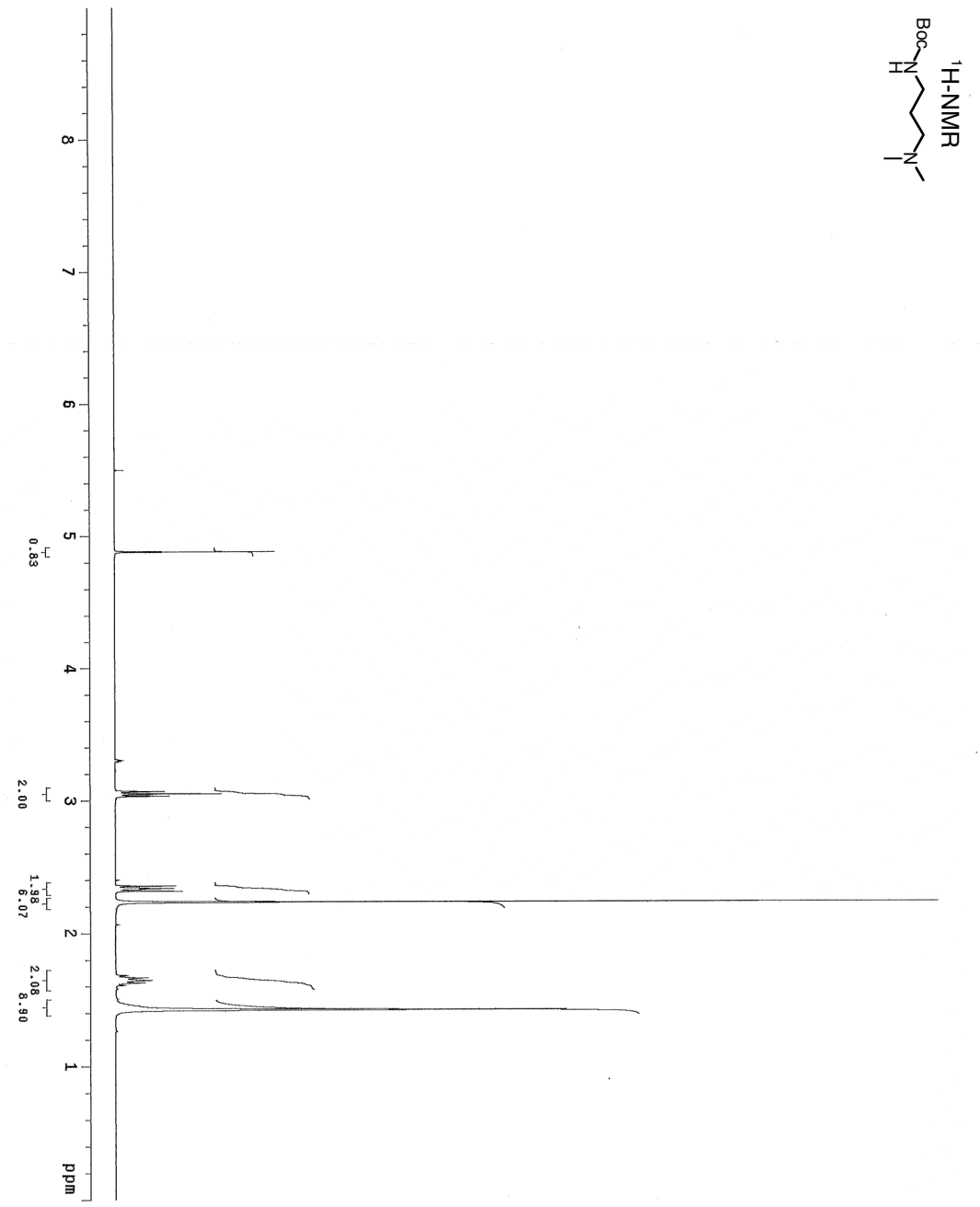

^{13}C-NMR
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

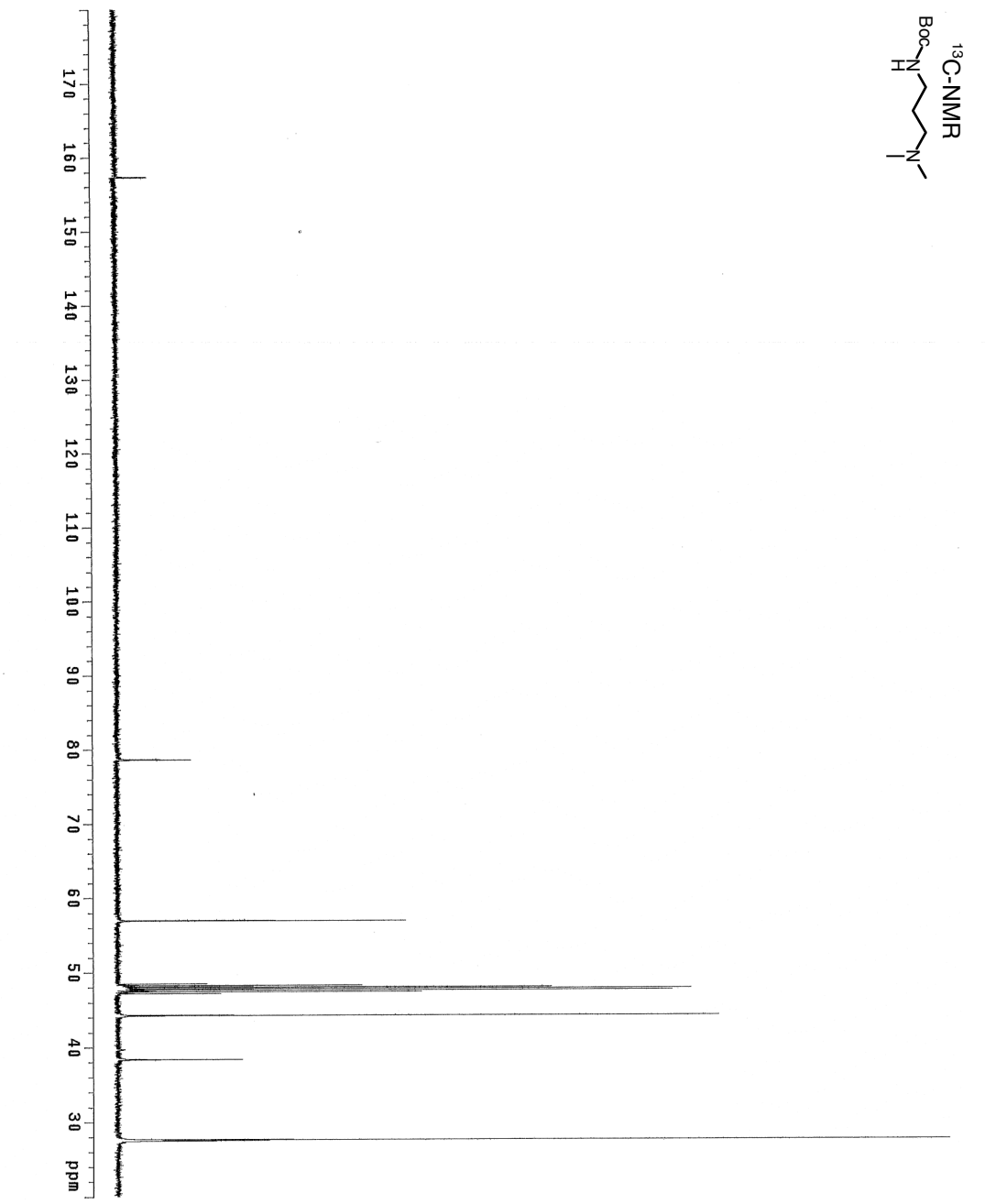

^{1}H-NMR
ppm
8
7
6
5
4
3
2
1
4.01
7.93
4.00
0.94
0.97
1.06
2.03
1.99
2.00
4.51
9.38
12.45
4.23
2.95
8.37
14.41

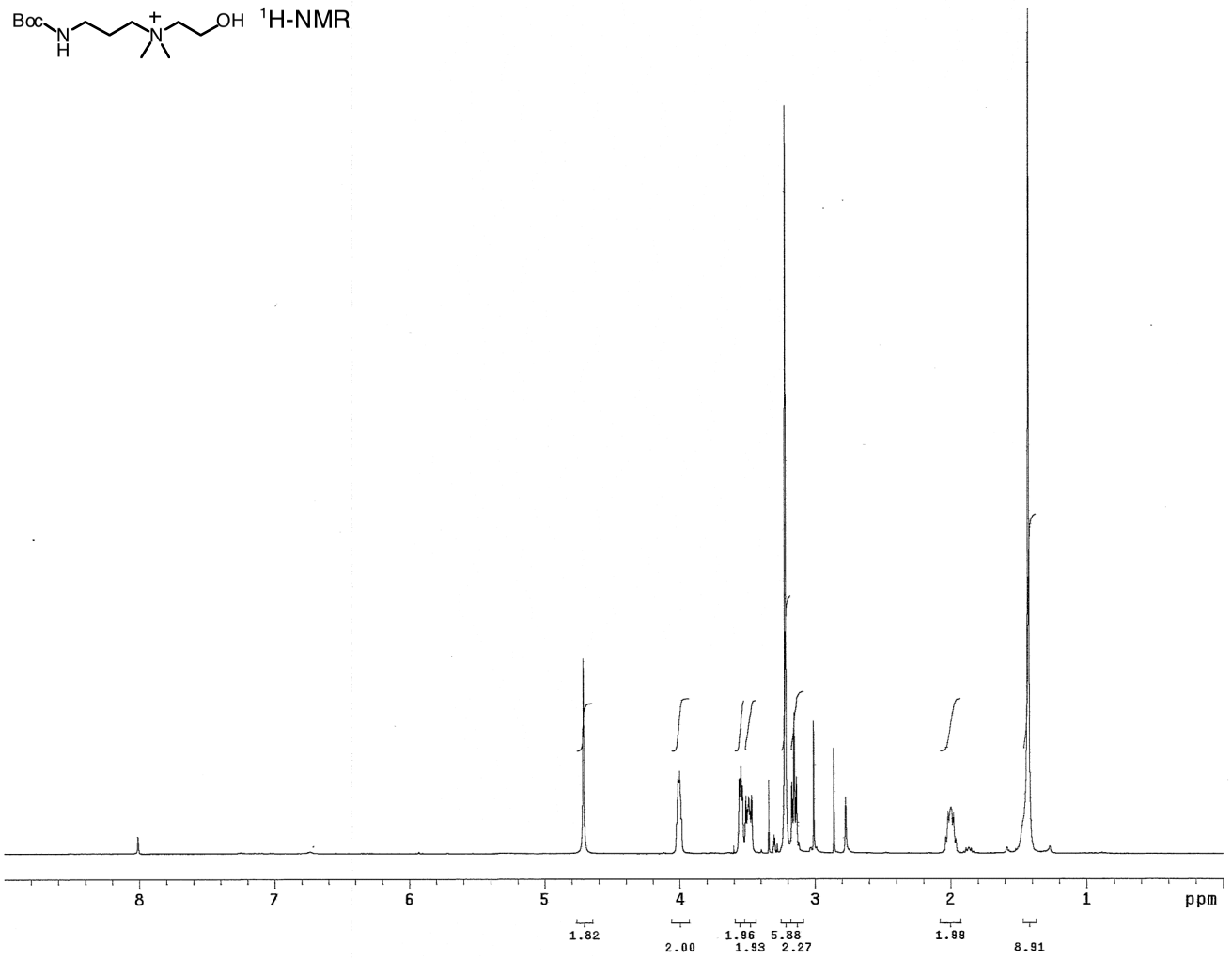


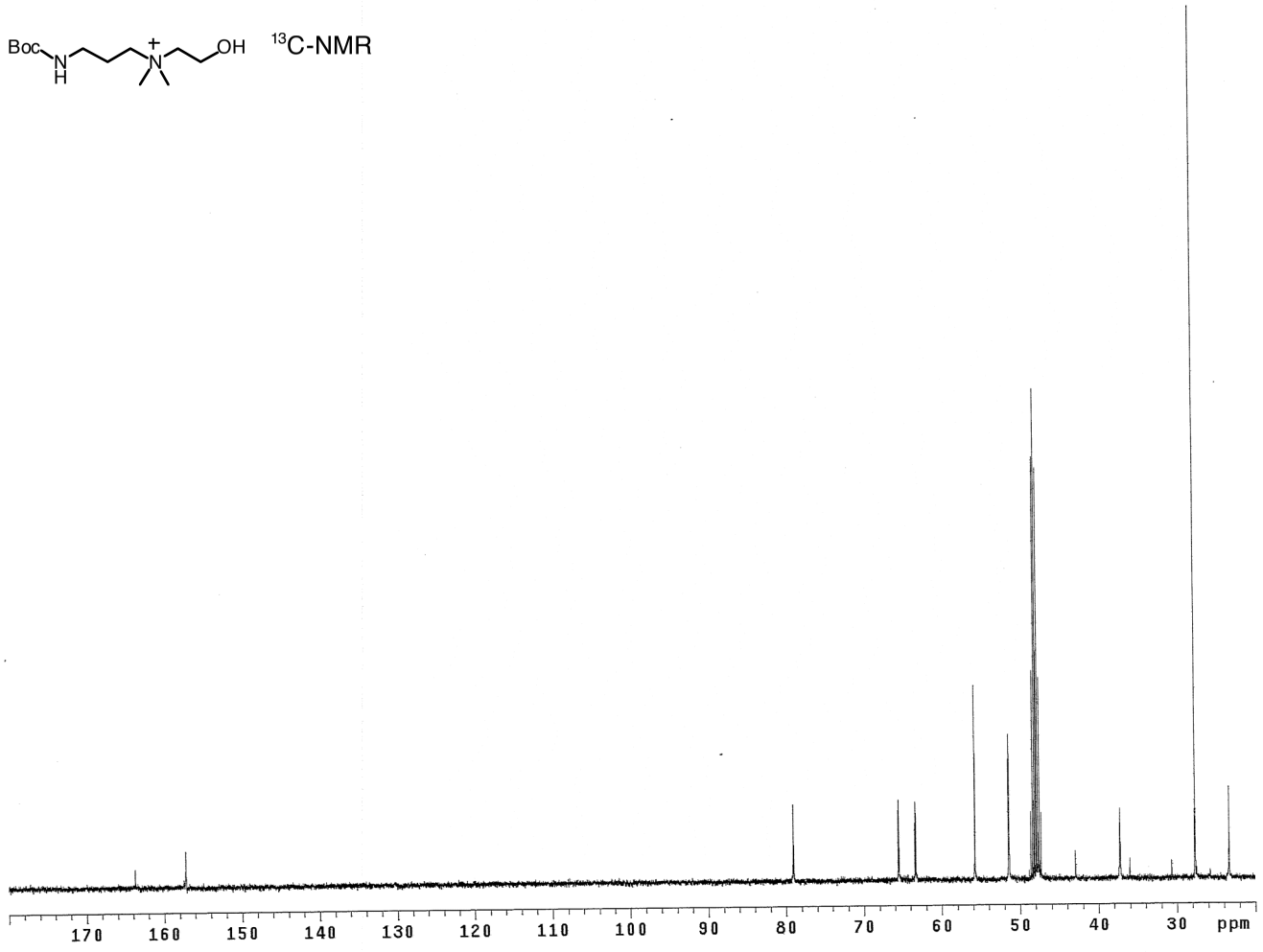
^{31}P-NMR

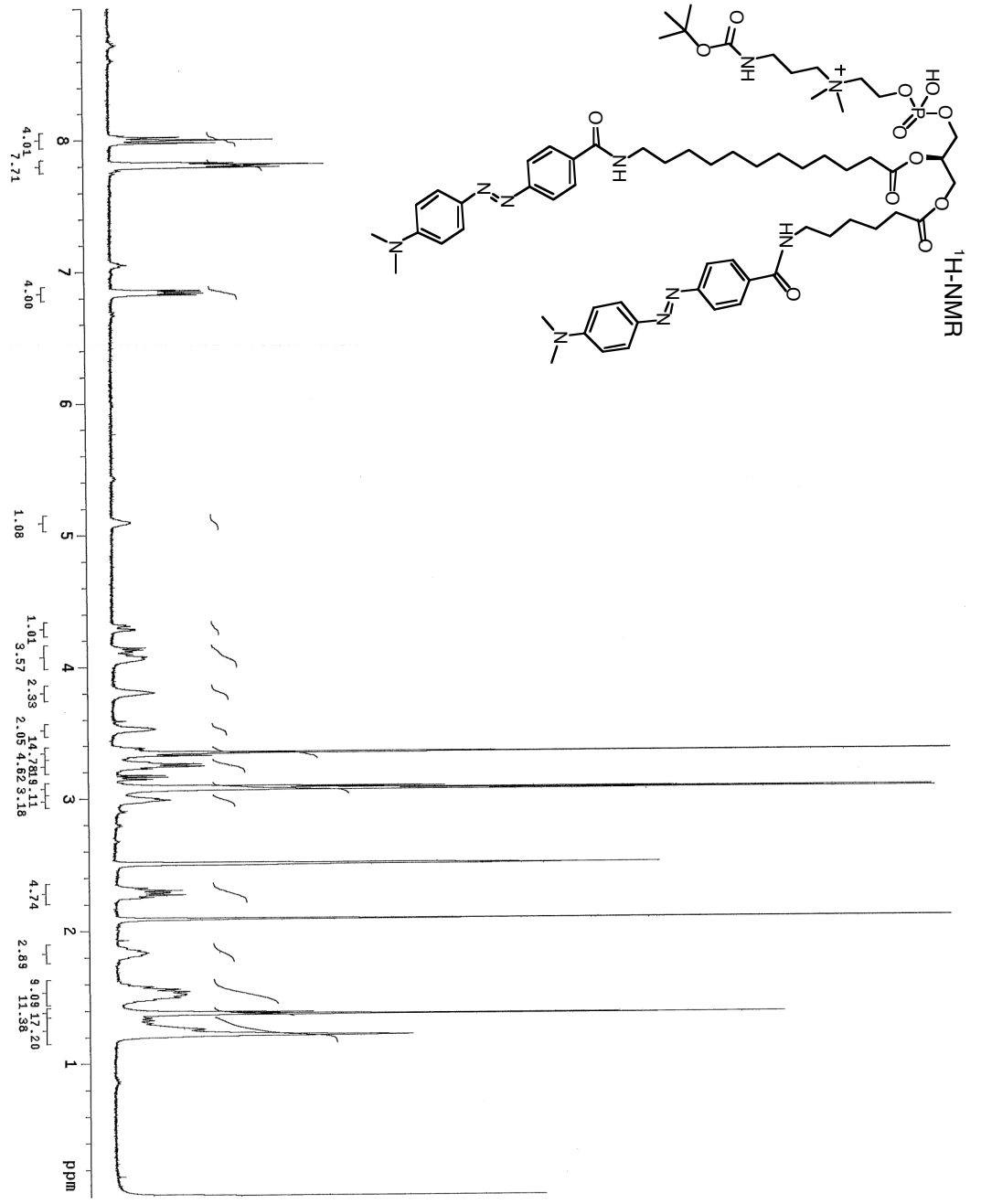

170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm
^{13}C-NMR

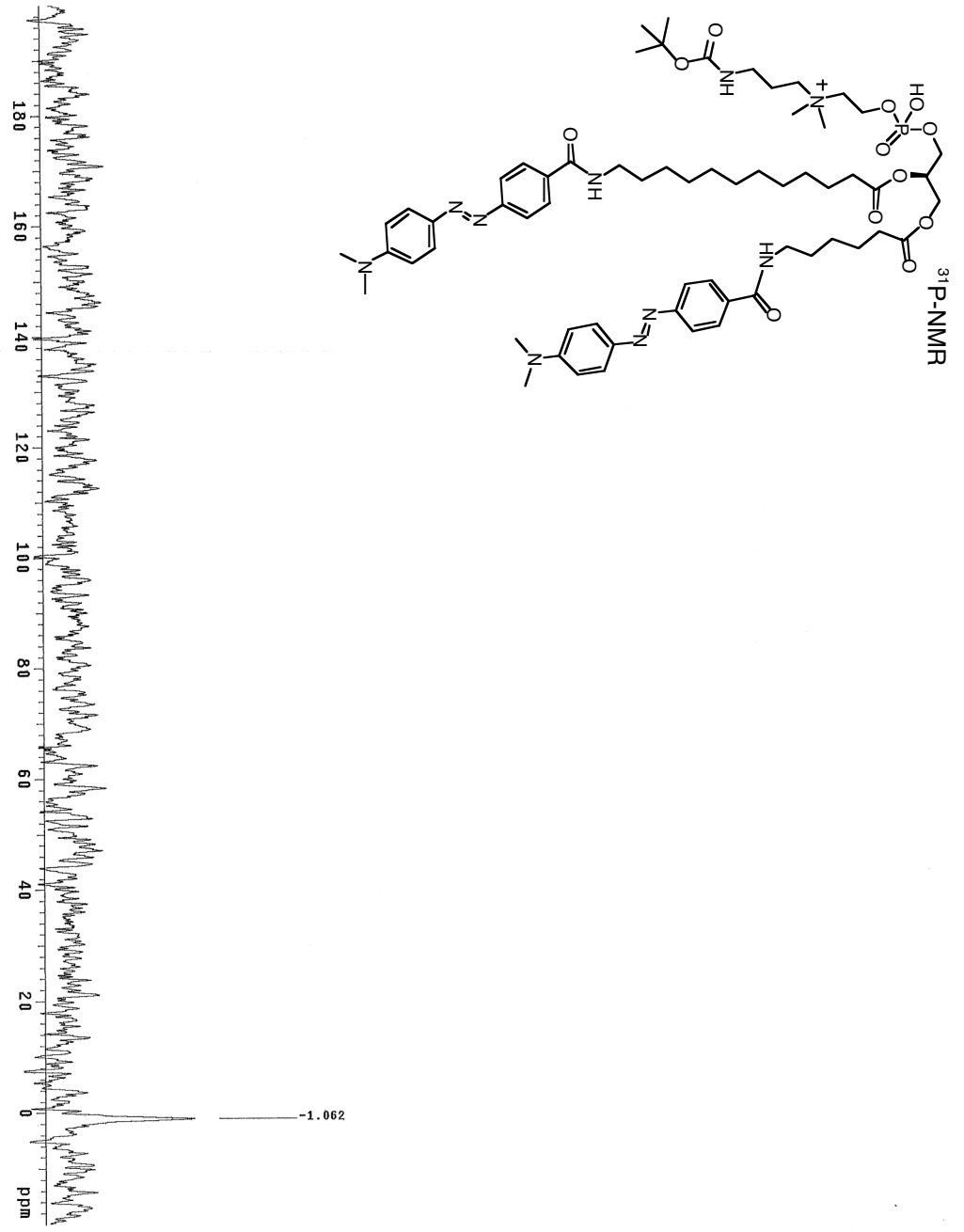

1H-NMR
Boc
N
H
N
N
8
7
6
5
4
3
2
1
ppm
0.83
2.00
1.98
6.07
2.08
8.90

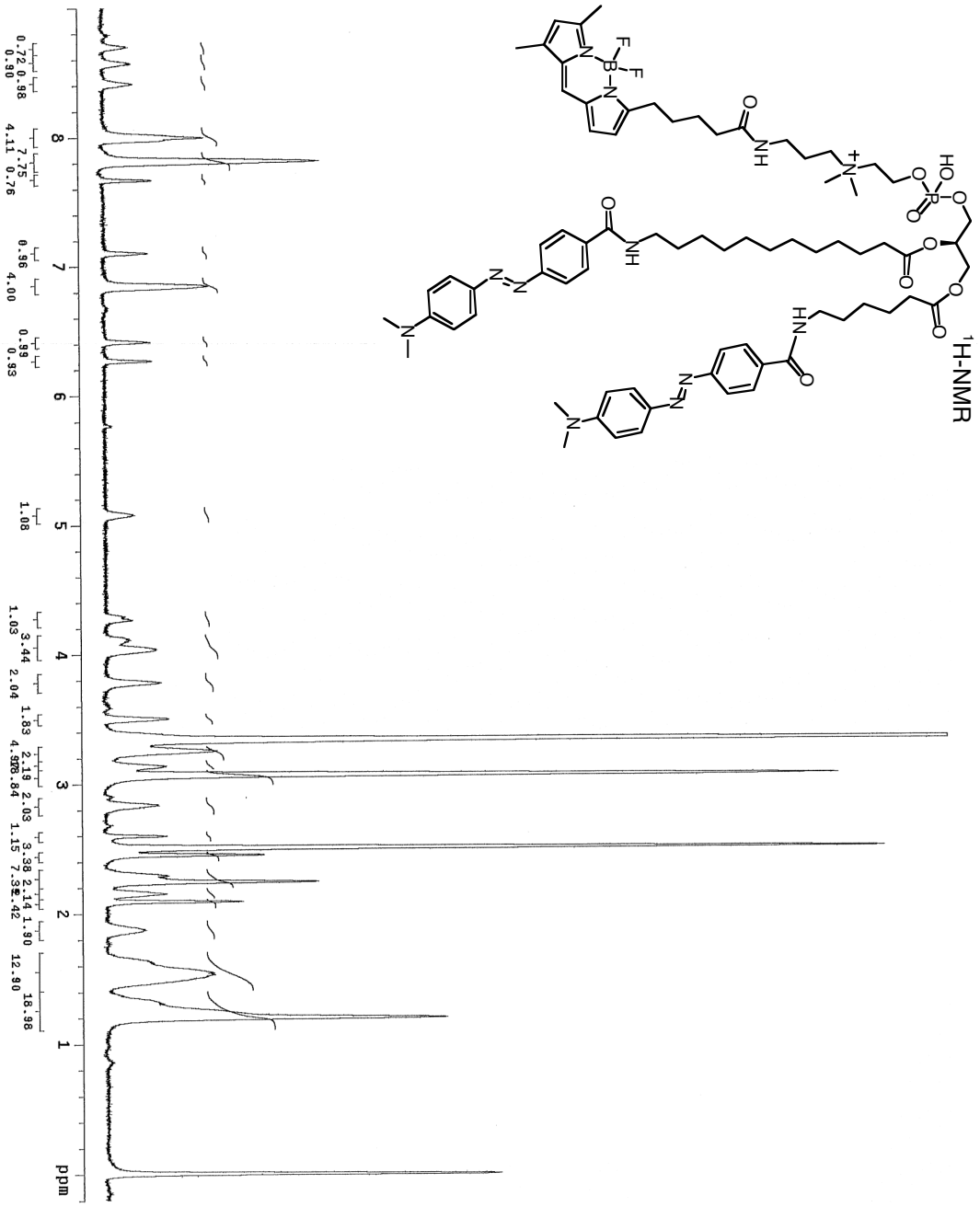

13C-NMR
Boc
N
H
N
N
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

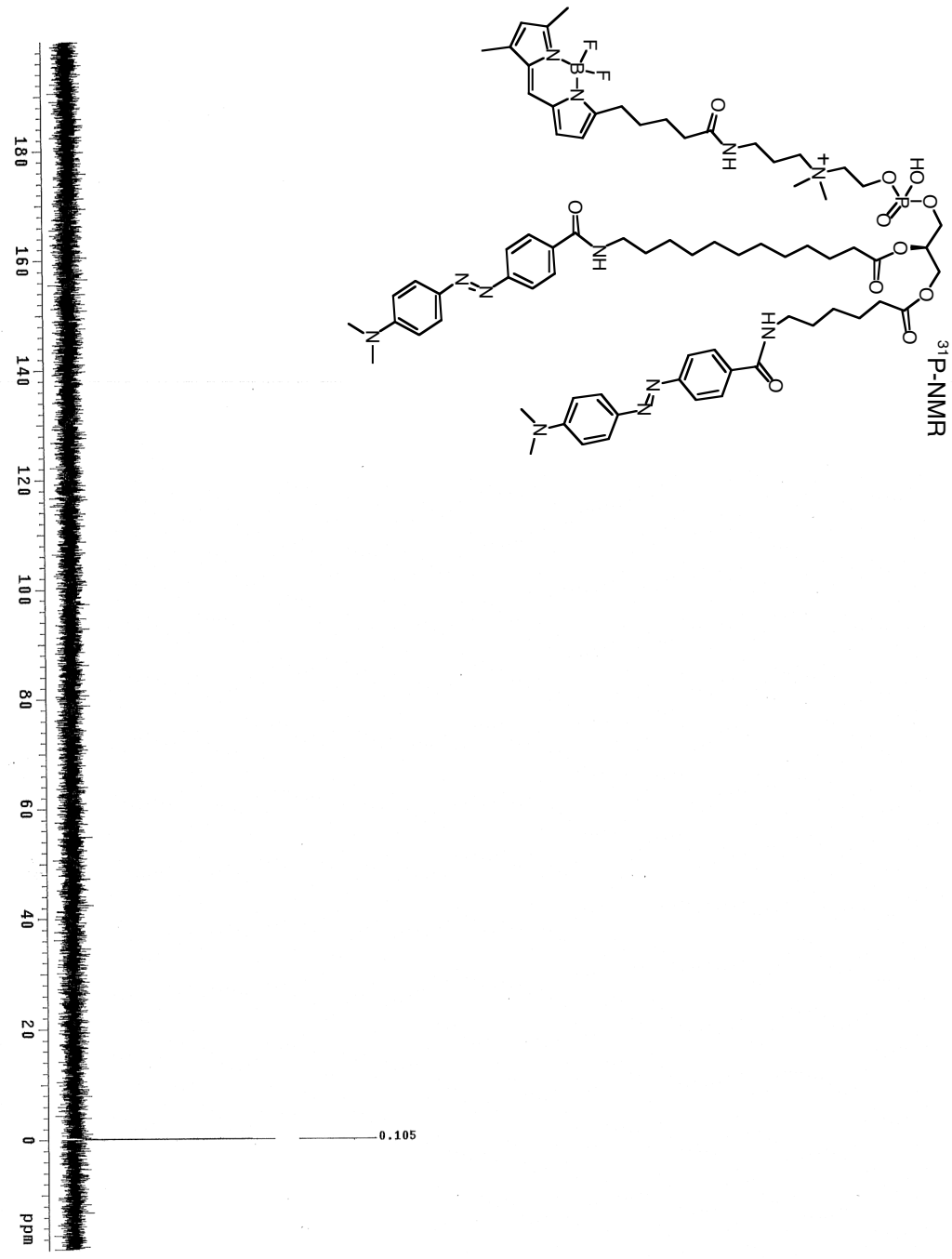

Boc
N
H
+
N
OH
1H-NMR
8
7
6
5
4
3
2
1
ppm
1.82
2.00
1.96
1.93
5.88
2.27
1.99
8.91


Boc
N
H
+
N
OH
13C-NMR
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
ppm

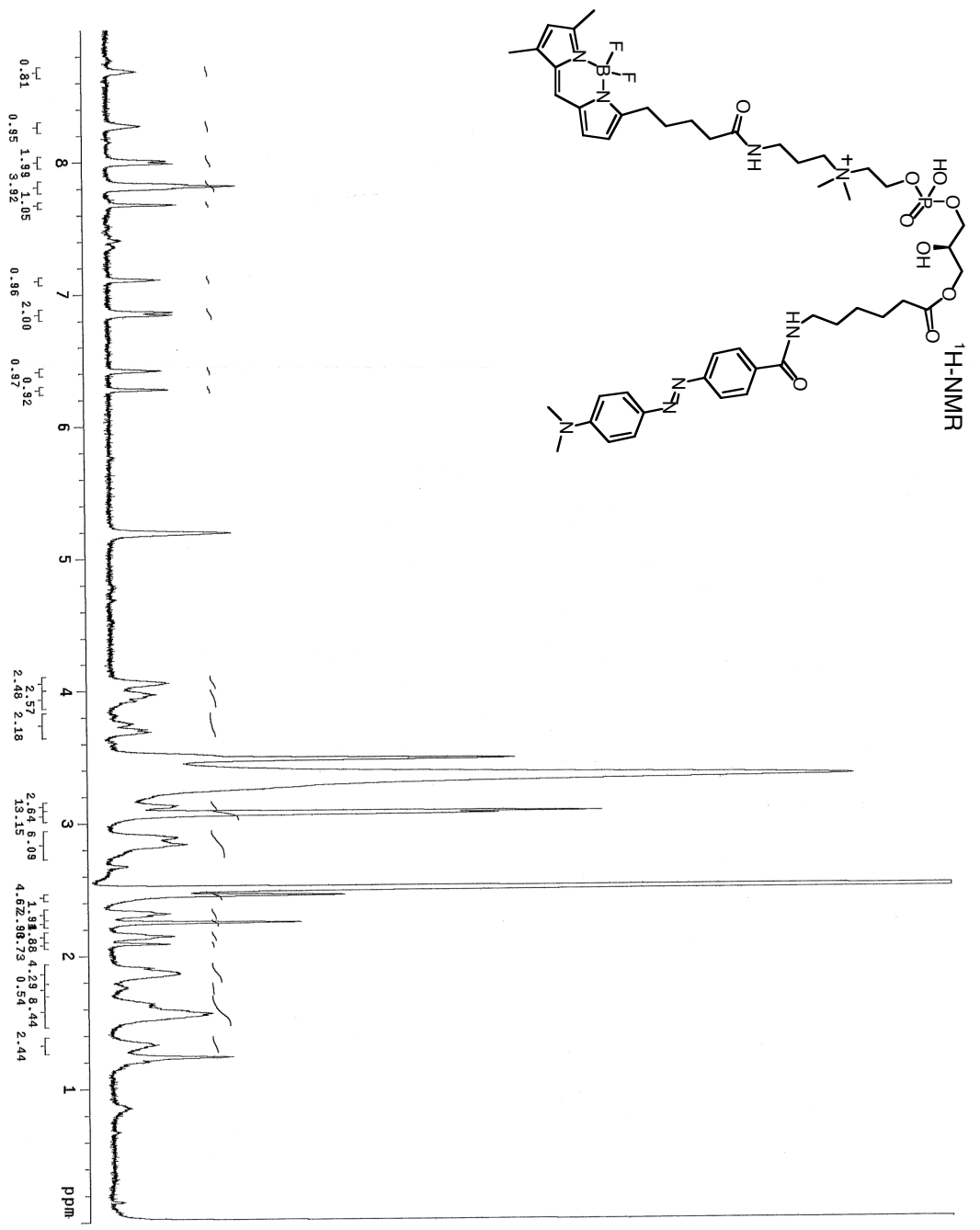

^{1}H-NMR
ppm

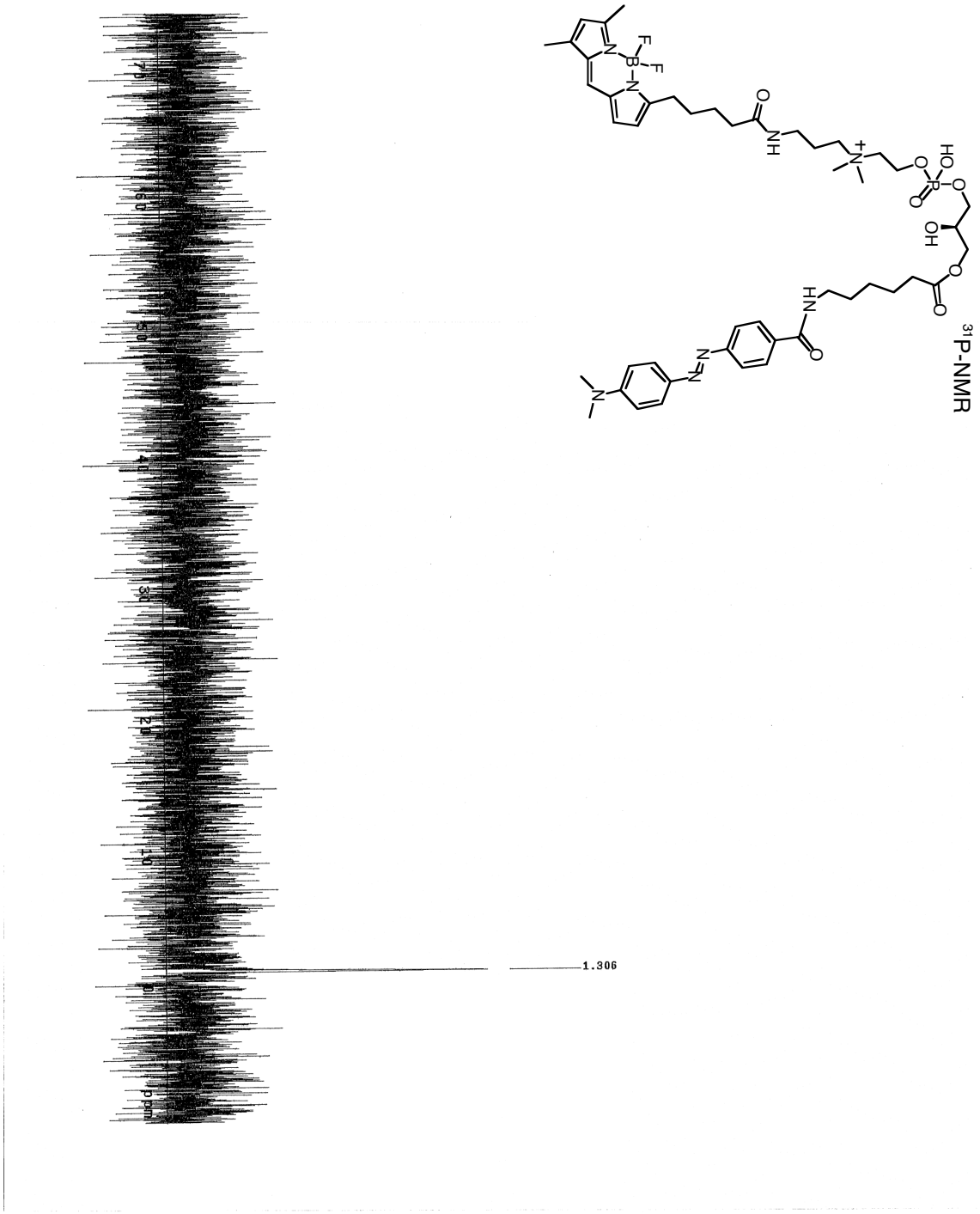

180
160
140
120
100
80
60
40
20
0
ppm
-1.062
^{31}P-NMR


^{1}H-NMR
ppm



^{31}P-NMR


^{1}H-NMR
ppm


1.306
31P-NMR