Supporting Information

Design and Synthesis of Phospholipase C and A_2-Activatable Near Infrared Fluorescent Smart Probes

Anatoliy V. Popov,*[,†] Theresa M. Mawn, [†] Soungkyoo Kim, [†] Gang Zheng, [†,‡] and E. James Delikatny [†]

[†]*University of Pennsylvania, Department of Radiology, Philadelphia PA, 19104, USA*

[‡]*University of Toronto, Department of Medical Biophysics, Toronto ON, M5G 1L7, Canada*

*E-mail: avpopov@mail.med.upenn.edu

Table of Contents

List of abbreviations	S2
General Information	S4
Synthesis	S6
^{1}H NMR Spectra	S14
Enzyme mediated cleavage	S21
References	S21

List of abbreviations

BHQ-3 carboxylic acid – black hole quencher-3, 3-diethylamino-5-phenylphenazium-7-diazobenzene-4''-(*N*-methyl)-*N*-butyric acid

BHQ-3^{+}-SU PF_6^{-} – succinimidyl ester of BHQ-3 carboxylic acid, hexafluorophosphate

BOC – *tert*-butoxycarbonyl

Cho – choline

DCM – dichloromethane, CH_2Cl_2

DMAP – 4-dimethylaminopyridine

EDC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride salt

Etn – ethanolamine

$H_2N(CH_2)_{11}CO_2H$ – λ-aminolauric acid

$H_2N(CH_2)_5CO_2H$ – ε-aminocaproic acid

Invitrogen PED6 – *N*-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt

Invitrogen B77101 – 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine

MALDI-TOF – Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

N-BOC *Lyso* PtdEtn – 1-palmytoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(*tert*-butoxycarbonyl) (sodium salt)

NHS – *N*-hydroxysuccinimide

NIR – near infrared

NIRF – near infrared fluorophore

PC – phosphocholine

PC-PLC – phosphatidylcholine-specific phospholipase C

PC-PLD – phosphatidylcholine-specific phospholipase D

PE – phosphoethanolamine

PI-PLC – phosphatidylinositol-specific phospholipase C

PL – phospholipase

PLs – phospholipases

PtdCho – phosphatidylcholine

PtdEtn – phosphatidylethanolamine

PtdGro – phosphatidylglycerol

PtdIns – phosphatidylinositol

PtdSer – phosphatidylserine

Pyro – pyropheophorbide *a*

Pyro-SU – succinimidyl ester of pyropheophorbide *a*

PyroC_{12} acid – λ-Pyropheophorbideamidolauric acid or 17^3-deoxy-17^3-(α-carbhydroxyundecylene-λ- amino)pyropheophorbide *a*

PyroC_{12}-PtdEtn – 1-palmitoyl-2-(λ-pyropheophorbideamidolauroyl)-*sn*-glycero-3-phosphoethanolamine

PyroC_{12}-PtdEtn-BHQ – 1-palmitoyl-2-(λ-pyropheophorbideamidolauroyl)-*sn*-glycero-3-phosphoethanolamide of BHQ-3 carboxylic acid

PyroC_6 acid – ε-Pyropheophorbideamidocaproic acid or 17^3-deoxy-17^3-(α-carbhydroxypentylene-ε-amino)pyropheophorbide *a*

PyroC_6-PyroC_6-PtdCho – 1,2-bis(ε-pyropheophorbideamidocaproyl)-*sn*-glycero-3-phosphocholine

Pyro-PtdEtn – 1-palmitoyl-2-pyropheophorbide-*sn*-glycero-3-phosphoethanolamine

Pyro-PtdEtn-BHQ – 1-palmitoyl-2-pyropheophorbide-*sn*-glycero-3-phosphoethanolamide of BHQ-3 carboxylic acid

SMase – sphingomyelinase

sPLA_2 – secretory phospholipase A_2

TEA, Et_3N – triethylamine

TFA – trifluoroacetic acid

TLC – thin layer chromatography

I. General Information

Dry solvents were purchased from ACROS Organics. Regular solvents and Celite were purchased from Fisher Scientific. *Spirulina Pacifica* algae (the starting material for Pyropheophorbide *a*) was purchased from Cyanotech Corporation, Kailua-Kona, HI, USA. *N*-BOC *Lyso* PtdEtn (N-Boc16:0 Lyso PE) was purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA. BHQ-3 carboxylic acid succinimidyl ester hexafluorophosphate was purchased from Bioresearch Technologies, Novato, CA, USA. Other reagents/reactants were purchased from Sigma-Aldrich and used without further purification. Silica Gel Standard Grade (230x450 mesh) was purchased from Sorbent Technologies, Atlanta, GA, USA. Thin Layer Chromatography Plates, Partsil® PK6F, Silica Gel 60 Å, 20x20 cm, were purchased from Whatman, washed with EtAc-MeOH (60:40, v/v) and baked at 150 °C overnight before use. All chemical reactions with pyropheophorbide *a* and its derivatives were carried out in the dark under dry Ar. ^{1}H NMR spectra were recorded using a Bruker DMX 360 MHz spectrometer. MALDI-TOF mass-spectra were recorded with an Applied Biosystems Voyager DE Mass Spectrometer using a positive mode ionization and CHCA (α-Cyano-4-hydroxycinnamic Acid) or HABA (2-(4-hydroxyphenylazo)benzoic acid) matrix. Time-dependent release of fluorescence was measured using a SpectraMax M5 fluorescent plate reader.

Table 1. Phospholipases

PL	Company	Catalog №	CAS №	Source	E.C. №	Enzyme Specificity
PLD	Sigma	P0065	9001-87-0	*Streptomyces chromofuscus*	3.1.4.4	Hydrolyzes the phosphate bonds of phospholipids, lysophospholipids and sphingomyelin to give the corresponding phosphatidic acid [a] . Prefers PtdCho and PtdEtn to PtdSer, PtdIns or PtdGro (*1*).
SMase	Sigma	S7651	9031-54-3	*Bacillus cereus*	3.1.4.12	Highly specific. Generates ceramide from sphingomyelin. Activity enhanced by the presence of PtdEtn or cholesterol and inhibited by PtdCho (*2*).
PI-PLC	Sigma	P5542	37288-19-0	*Bacillus cereus*	4.6.1.13	Highly specific. Cleaves PtdIns and phosphorylated derivatives (*3*). Does not cleave PtdCho, PtdEtn or Sphingomyelin (*4, 5*).
$sPLA_2$ IB porcine	Sigma	P6534	9001-84-7	Porcine pancreas	3.1.1.4	Acts preferentially on anionic (PtdGro, PtdEtn and PtdSer) compared to charge-neutral PtdCho phospholipid vesicles (*6-9*).
$sPLA_2$ IB bovine	Sigma	P8913	9001-84-7	Bovine pancreas	3.1.1.4	See above
$sPLA_2$ III	Cayman Chemical	60500	9001-84-7	Bee venom	3.1.1.4	Preferred substrates are PtdCho, PtdEtn and their plasmalogen analogues. PtdIns and PtdSer are also hydrolyzed ([a] and (*10*)).
$sPLA_2$ V [b]	Cayman Chemical	10009563	9001-84-7	Human recombinant	3.1.1.4	Hydrolyzes both anionic phospholipids and PtdCho (*11-13*).

[a] manufacturer's product information
[b] Accession № NP_000920 (provided by Cayman Chemical)

Phospholipases were dissolved in Tris buffer (50 mM Tris-HCl, pH 7.4) and stored in aliquots at -20 °C.

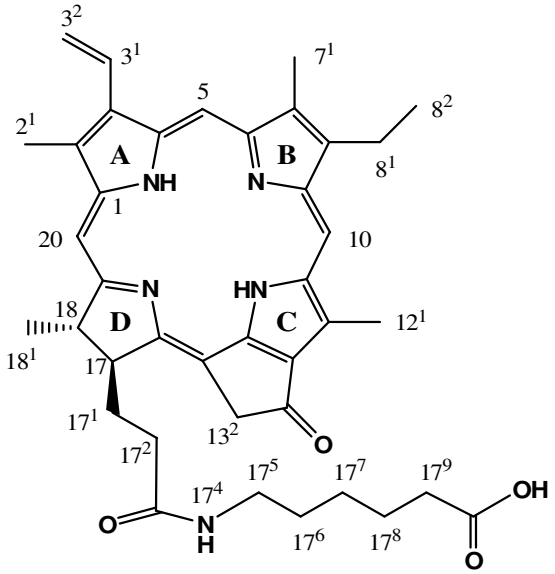
II. Synthesis

Pyropheophorbide *a* (**1**) was prepared from *Spirulina Pacifica* algae according to procedure (*14*).

General procedure for synthesis of *N*-Pyropheophorbide substituted ω-amino acids (PyroC$_6$ and PyroC$_{12}$ acids)

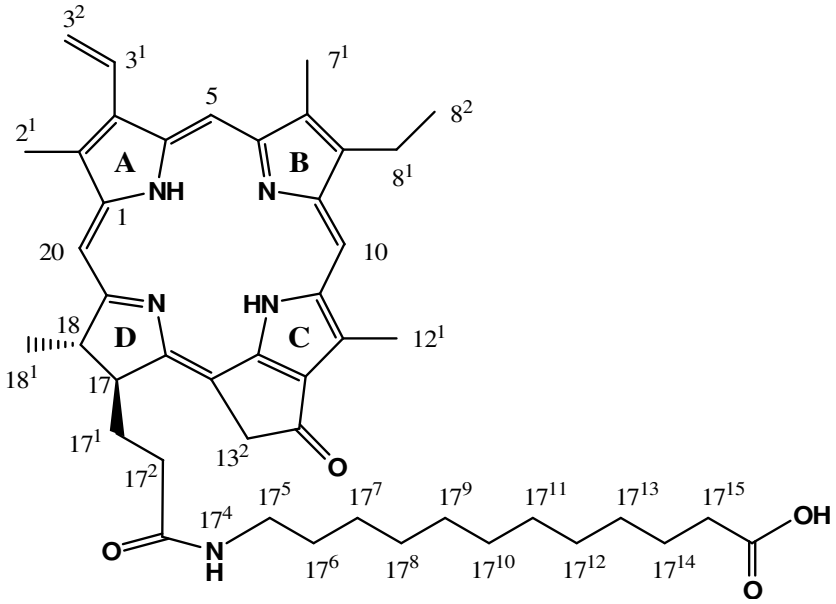
A 500 mL round bottom flask was charged with pyropheophorbide acid (**1**) (0.38 mmol), NHS (0.38 mmol), EDC (0.38 mmol), DMAP (23.3 mg. 0.19 mmol) and 200 ml of dry DCM. The reaction mixture was stirred in dark under Ar for 3 h until Pyropheophorbide acid was converted completely into its succinimidyl ester (TLC $CHCl_3$/MeOH = 5/1, v/v). Then $H_2N(CH_2)_nCO_2H$ (n = 5, 11, 0.38 mmol) and dry pyridine (25 ml) were added. The second reaction was carried out for 48 h until complete conversion of Pyro-SU. The solvents were then evaporated; the solid residue was dissolved in 100 ml of DCM, rinsed twice with 2% HCl, then water. The product was isolated by column chromatography on silica gel using (DCM-ethyl acetate (0-100%), then ethyl acetate-MeOH (0-40%)). Isolated yields are 75-85%.

ε-Pyropheophorbideamidocaproic acid or 17^3-deoxy-17^3-(α-carbhydroxypentylene-ε-amino)pyropheophorbide *a*, PyroC$_6$ acid (8)



Yield 185 mg, 75%. ^{1}H NMR (360 MHz, $CDCl_3$, CD_3OD δ ppm): 9.14, 9.02 and 8.38 (each s, 1H, 5-*H*, 10-*H* and 20-*H*); 7.74 (dd, J=11.5 Hz, J=17.6 Hz, 1H, 3^1-C*H*=CH_2); 6.09 (d, J=17.6 Hz, 1H, *trans*-3^2-CH=CH*H*); 5.99 (d, J=11.5 Hz, 1H, *cis*-3^2-CH=C*H*H); 5.03 (AB, A=5.11, B=4.96, J_{AB}=20.2 Hz, 2H, 13^2-CH_2); 4.32 (q, J=8.1 Hz, 1H, 18-*H*); 4.12 (dm, 8.7 Hz, 1H, 17-*H*); 3.45-3.32 m 5H, 3.27-3.12 m 5H and 2.94 s 3H (2^1-CH_3, 12^1-CH_3, 7^1-CH_3, 8^1-CH_2, 17^5-CH_2); 2.61-2.39 m 2H and 2.31-2.08 m 4H (17^2-CH_2, 17^9-CH_2, 17^1-CH_2); 1.77-1.40 m 10H (18^1-CH_3, 8^2-CH_3, 17^6-CH_2, 17^8-CH_2); 1-36-1.21 (m, 2H, 17^7-CH_2). MALDI-TOF, *m/z*: $(M+Na)^+$ 670.41, calculated for $C_{39}H_{45}N_5NaO_4$ 670.34.

λ-Pyropheophorbideamidolauric acid or 17^3-deoxy-17^3-(α-carbhydroxyundecylene-λ-amino)pyropheophorbide *a*, PyroC$_{12}$ acid (5)

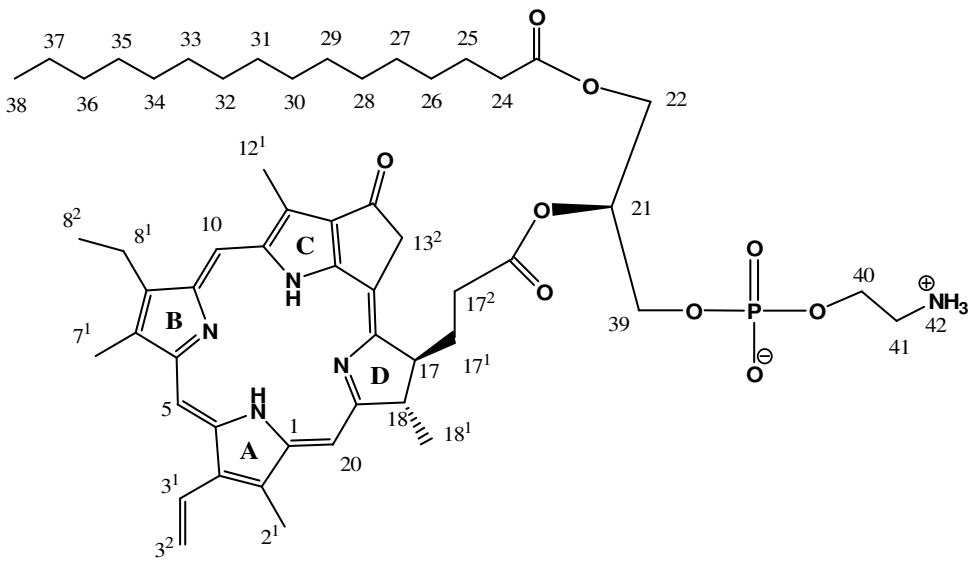


Yield 236 mg, 85%. ^{1}H NMR (360 MHz, $CDCl_3$, CD_3OD δ ppm): 9.17, 9.06 and 8.41 (each s, 1H, 5-*H*, 10-*H* and 20-*H*); 7.77 (dd, J=11.5 Hz, J=17.6 Hz, 1H, 3^1-C*H*=CH_2); 6.11 (d, J=17.6 Hz, 1H, *trans*-3^2-CH=CH*H*); 6.02 (d, J=11.5 Hz, 1H, *cis*-3^2-CH=C*H*H); 5.06 (AB, A=5.14, B=4.99, J_{AB}=20.2 Hz, 2H, 13^2-CH_2); 4.36 (q, J=8.1 Hz, 1H, 18-*H*); 4.15 (dm, 8.7 Hz, 1H, 17-*H*); 3.51-3.35 m 5H, 3.30-3.14 m 5H and 2.98 s 3H (2^1-CH_3, 12^1-CH_3, 7^1-CH_3, 8^1-CH_2, 17^5-CH_2); 2.67-2.41 m 2H and 2.35-2.08 m 4H (17^2-CH_2, 17^{15}-CH_2, 17^1-CH_2); 1.79-1.46 m 10H (18^1-CH_3, 8^2-CH_3, 17^6-CH_2, 17^{14}-CH_2); 1.44-1.12 (m, 14H, 17^7-17^{13} 7xCH_2). MALDI-TOF, *m/z*: $(M+Na)^+$ 754.51, calculated for $C_{45}H_{57}N_5NaO_4$ 754.43.

General procedure for synthesis of 2-pyropheophorbide *a* (with and without C_{12} spacer) substituted 1-palmitoyl-*sn*-glycero-3-phosphoethanolamines

A 200 mL dry flask was charged with *N*-BOC *Lyso* PtdEtn (0.087 mmol), a Pyro-containing acid (0.087 mmol), EDC (0.130 mmol), DMAP (0.043 mmol) and DCM (70 mL). The conversion of Pyro-acid was monitored by TLC ($CHCl_3$/MeOH = 4/2). After 72 h the reaction mixture was diluted with hexanes (30 mL) and passed through a small column with Celite to eliminate non-soluble by-products. After evaporation of solvents, the solid was dissolved in a small amount of DCM and moved into a dry 100 mL flask. After solvent evaporation the residue was dried under high vacuum overnight. Then dry DCM (25 mL) was added, the flask was cooled until -20 °C and TFA (5 mL) was added. The BOC-deprotection reaction was carried out at 0 °C 4 h. Following that, dry toluene (20 mL) was added (to avoid TFA concentrating under evaporation) and volatiles were removed under vacuum. The solid residue was treated with 5% solution of Et_3N in DCM (30 mL) to neutralize traces of TFA. After liquids evaporation the residue was dried under high vacuum overnight. This residue was then dissolved in dry DCM and put onto 6 preparative TLC plates. Preparative TLC (20% MeOH in $CHCl_3$) resulted in the Pyro-PtdEtn derivative as a dark green amorphous solid.

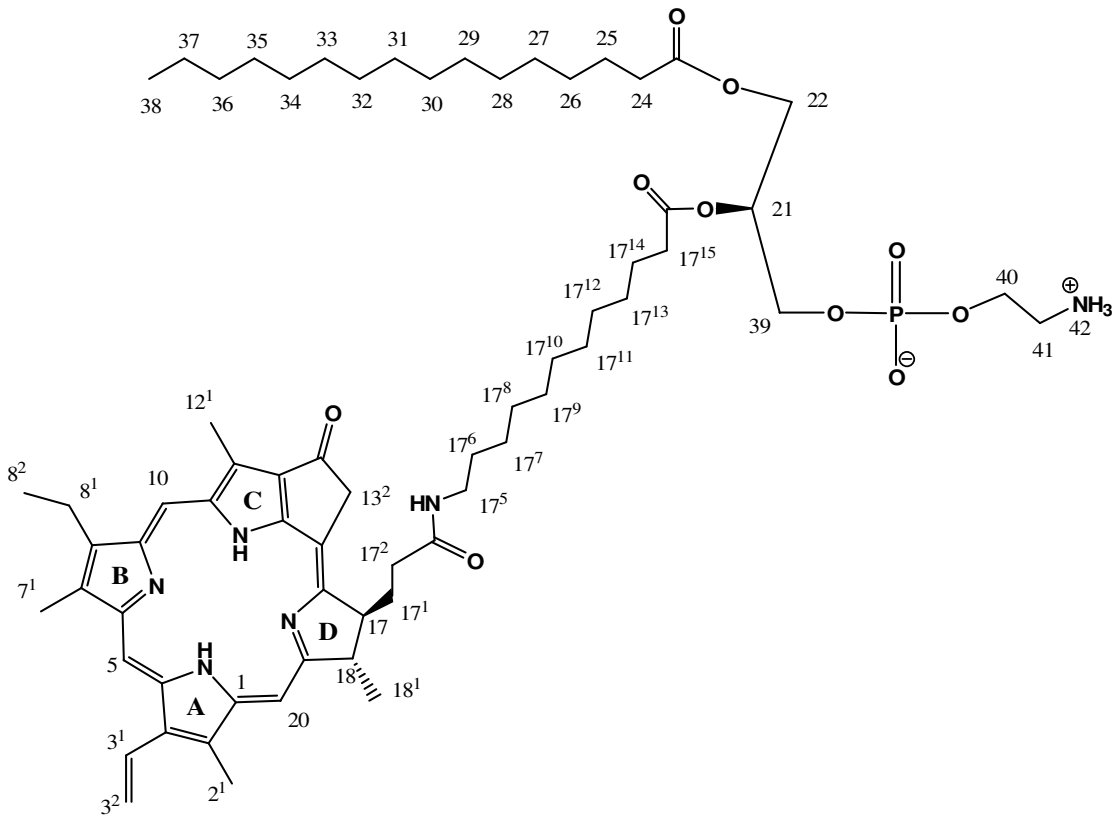
1-palmitoyl-2-pyropheophorbide-*sn*-glycero-3-phosphoethanolamine, Pyro-PtdEtn (3)



Yield 17 mg, 20%. R_f = 0.40 ($CHCl_3$/CH_3OH = 4/1, v/v). ^{1}H NMR (360 MHz, $CDCl_3$/CD_3OD, δ ppm): 9.37, 9.28 and 8.50 (each s, 1H, 5-*H*, 10-*H* and 20-*H*); 7.93 (dd, J=11.5 Hz, J=17.6 Hz, 1H, 3^1-C*H*=CH_2); 6.24 (d, J=17.6 Hz, 1H, *trans*-3^2-CH=CH*H*); 6.13 (d, J=11.5 Hz, 1H, *cis*-3^2-

CH=C*H*H); 5.40-5.01 m 3H (21-*H*, 13^2-CH_2); 4.45 m 2H and 4.32-3.94 m 6H (18-H, 17-H, 22-CH_2, 39-CH_2, 40-CH_2); 3.71-3.51 m 5H, 3.47-3.29 m 5H and 3.18 s 3H (2^1-CH_3, 12^1-CH_3, 7^1-CH_3, 8^1-CH_2, 41-CH_2); 3.12-2.53 m 2H and 2.41-2.14 m 4H (17^2-CH_2, 24-CH_2, 17^1-CH_2); 1.85-1.56 m 8H and 1.45-1.14 m 24H (18^1-CH_3, 8^2-CH_3, 25-37 13xCH_2); 1.05-0.87 (m, 3H, 38-CH_3). MALDI-TOF, *m/z*: $(M+Na)^+$ 992.69, calculated for $C_{54}H_{76}N_5NaO_9P$ 992.53.

1-palmitoyl-2-(λ-pyropheophorbideamidolauroyl)-*sn*-glycero-3-phosphoethanolamine, PyroC$_{12}$-PtdEtn (6)



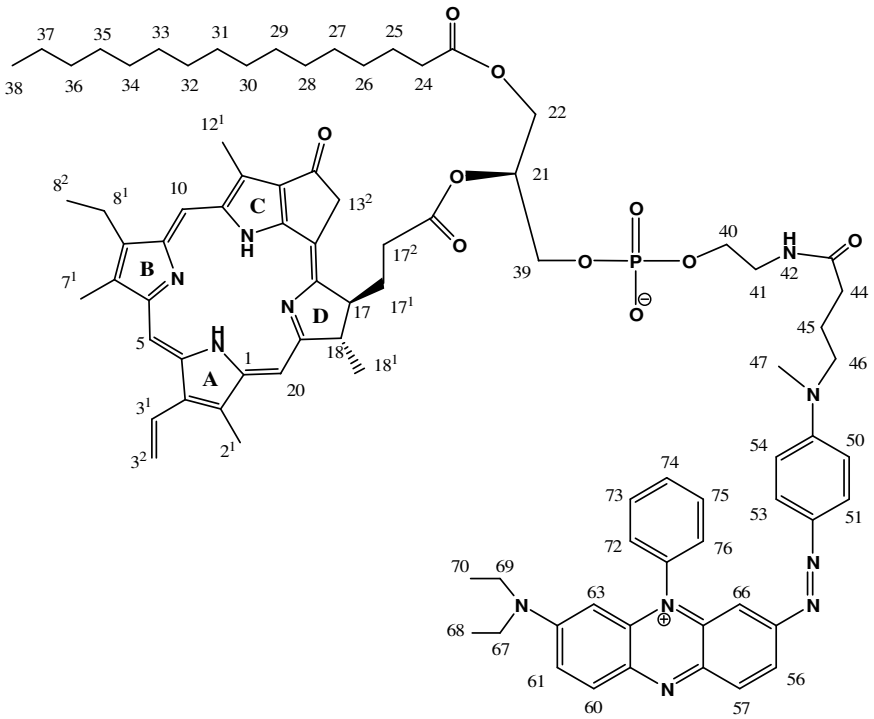
Yield 18 mg, 18%. R_f = 0.25 ($CHCl_3$/CH_3OH = 4/1, v/v). ^{1}H NMR (360 MHz, $CDCl_3$/CD_3OD, CD_2Cl_2, δ ppm): 9.18, 9.07 and 8.42 (each s, 1H, 5-*H*, 10-*H* and 20-*H*); 7.79 (dd, J=11.5 Hz, J=17.6 Hz, 1H, 3^1-C*H*=CH_2); 6.13 (d, J=17.6 Hz, 1H, *trans*-3^2-CH=C*H*H); 6.04 (d, J=11.5 Hz, 1H, *cis*-3^2-CH=C*H*H); 5.18-4.93 m 3H (21-*H*, 13^2-CH_2, overlapped partially with CD_2Cl_2); 4.60-3.96 m 8H (18-*H*, 17-*H*, 22-CH_2, 39-CH_2, 40-CH_2); 3.55-2.91 m, m and s 15H, (2^1-CH_3, 12^1-CH_3, 7^1-CH_3, 8^1-CH_2, 17^5-CH_2, 41-CH_2); 2.64-2.11 m 8H (17^2-CH_2, 24-CH_2, 17^1-CH_2, 17^{15}-

CH_2); 1.85-1.14 m 50H (18^1-CH_3, 8^2-CH_3, 25-37 and 17^6-17^{14} 22xCH_2); 1.06-0.90 (m, 3H, 38-CH_3). MALDI-TOF, *m/z*: $(M+Na)^+$ 1189.86, calculated for $C_{66}H_{99}N_6NaO_{10}P$ 1189.71.

General procedure for synthesis of Pyro-PtdEtn-BHQ and PyroC_{12}-PtdEtn-BHQ

A 100 mL flask was loaded with a Pyro-containing PtdEtn (**3** or **6**) (0.013 mmol), BHQ-3$^+$-SU PF_6^- (0.013 mmol) and 50 mL of dry DCM. After dissolution 1 drop of Et_3N was added. The reaction was carried out for 4 h. Volatiles were removed in a rotary evaporator and the residue was dried under high vacuum overnight. The residue was dissolved in $CHCl_3$ and put onto 4 preparative TLC plates. Thin layer preparative chromatography gave in result the final product as a dark sea-green amorphous solid.

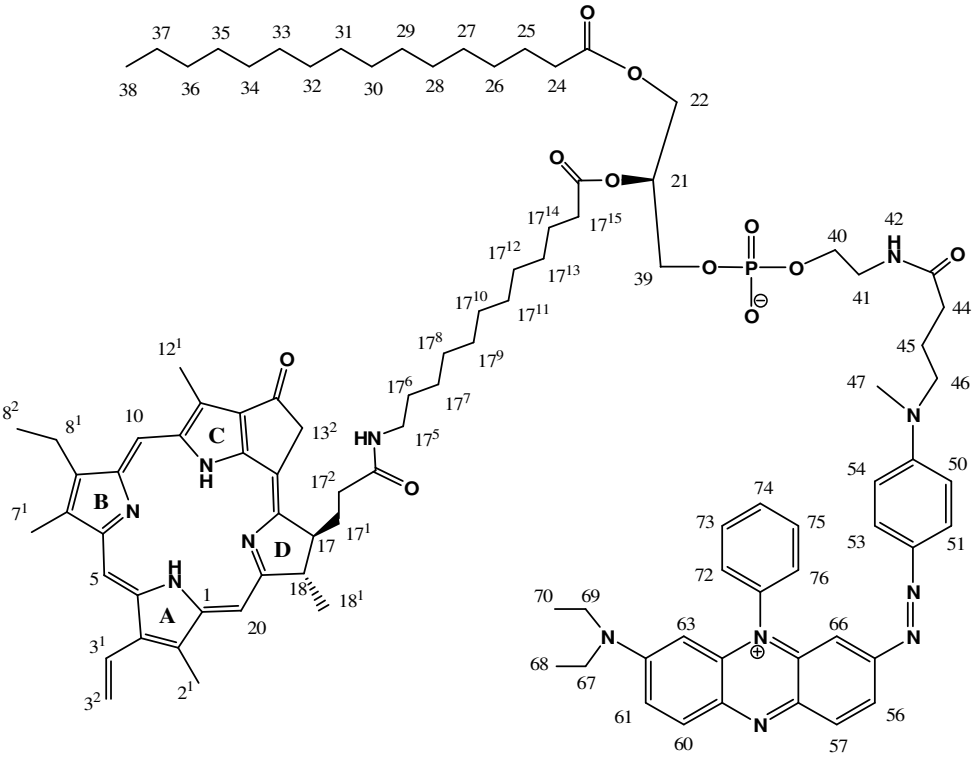
1-palmitoyl-2-pyropheophorbide-*sn*-glycero-3-phosphoethanolamide of BHQ-3 carboxylic acid, Pyro-PtdEtn-BHQ (4)



Yield 14.5 mg, 75%. R_f = 0.6 ($CHCl_3$/CH_3OH = 5/1, v/v). ^{1}H NMR (360 MHz, $CDCl_3$, CD_3OD, δ ppm): 9.39, 9.29 and 8.54 (each s, 1H, 5-*H*, 10-*H* and 20-*H*); 8.09-6.46 m 16H (50-*H*, 51-*H*, 53-*H*, 54-*H*, 56-*H*, 57-*H*, 60-*H*, 61-*H*, 63-*H*, 72-76 5x*H*, 3^1-C*H*=CH_2, overlapped partially with $CDCl_3$); 6.26 (d, J=17.6 Hz, 1H, *trans*-3^2-CH=CH*H*); 6.15 (d, J=11.5 Hz, 1H, *cis*-3^2-CH=C*H*H); 5.33-5.05 m 3H (21-*H*, 13^2-CH_2); 4.51-3.89 (m, 8H, 18-*H*, 17-*H*, 22-CH_2, 39-CH_2, 40-CH_2);

3.70-3.08 m 19H and 2.90 s 3H (2^1-CH_3, 12^1-CH_3, 7^1-CH_3, 47-CH_3 8^1-CH_2, 41-CH_2, 46-CH_2, 67-CH_2, 69-CH_2); 2.74-2.14 (m, 8H, 17^2-CH_2, 24-CH_2, 17^1-CH_2, 44-CH_2); 1.96-1.10 (m, 40H, 18^1-CH_3, 8^2-CH_3, 68-CH_3, 70-CH_3, 25-37 and 46- 14xCH_2); 1.04-0.86 (m, 3H, 38-CH_3). MALDI-TOF, *m/z*: $(M+Na)^+$ 1520.93, calculated for $C_{87}H_{108}N_{11}NaO_{10}P$ 1520.79.

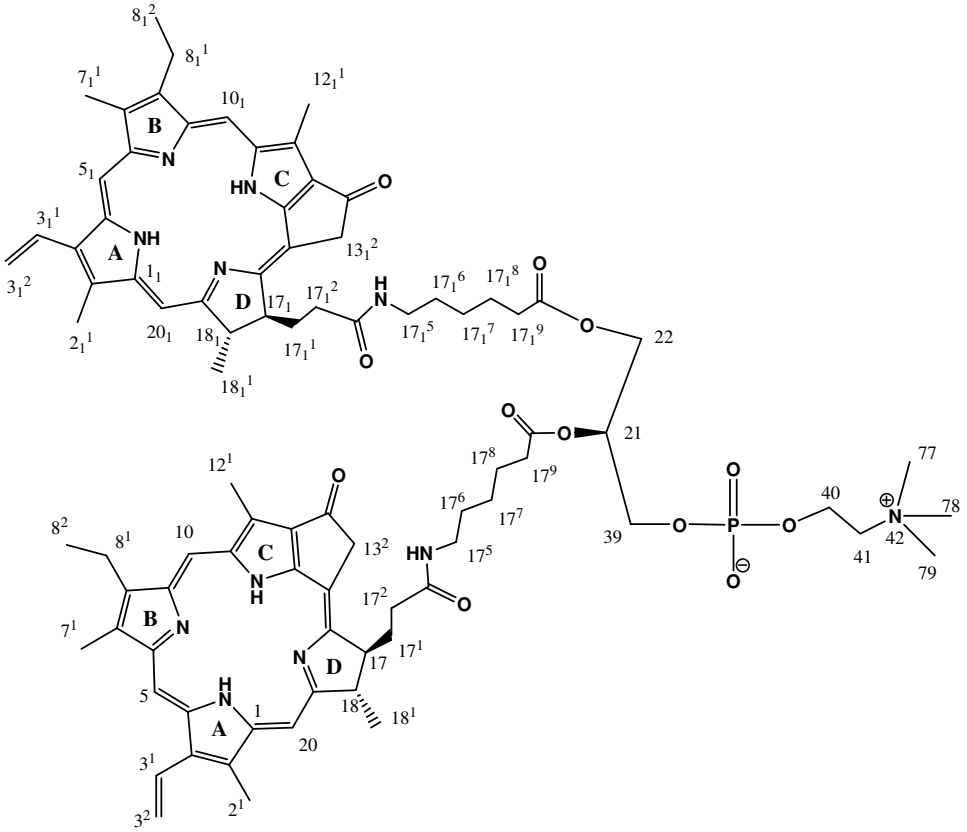
1-palmitoyl-2-(λ-pyropheophorbideamidolauroyl)-*sn*-glycero-3-phosphoethanolamide of BHQ-3 carboxylic acid, PyroC_{12}-PtdEtn-BHQ (7)



Yield 12.5 mg, 57%. R_f = 0.3 ($CHCl_3$/CH_3OH = 5/1, v/v). ^{1}H NMR (360 MHz, $CDCl_3$,CD_3OD, CD_2Cl_2, δ ppm): 9.19, 9.09 and 8.44 (each s, 1H, 5-*H*, 10-*H* and 20-*H*); 8.06-6.52 m 16H (50-*H*, 51-*H*, 53-*H*, 54-*H*, 56-*H*, 57-*H*, 60-*H*, 61-*H*, 63-*H*, 72-76 5x*H*, 3^1-C*H*=CH_2, overlapped partially with $CDCl_3$); 6.14 (d, J=17.6 Hz, 1H, *trans*-3^2-CH=CH*H*); 6.05 (d, J=11.5 Hz, 1H, *cis*-3^2-CH=C*H*H); 5.24-4.93 m 3H (21-*H*, 13^2-CH_2, overlapped partially with CD_2Cl_2); 4.65-3.94 (m, 8H, 18-*H*, 17-*H*, 22-CH_2, 39-CH_2, 40-CH_2); 3.59-3.13 m 18H, 2.98 s 3H and 2.90 s 3H (2^1-CH_3, 12^1-CH_3, 7^1-CH_3, 47-CH_3, 8^1-CH_2, 17^5-CH_2, 41-CH_2, 46-CH_2, 67-CH_2, 69-CH_2); 2.64-2.10 (m, 10H, 17^2-CH_2, 24-CH_2, 17^1-CH_2, 17^{15}-CH_2, 44-CH_2); 1.93-1.08 (m, 58H, 18^1-CH_3, 8^2-CH_3, 68-

CH_3, 70-CH_3, 25-37, 46- and 17^6-17^{14} 23xCH_2); 1.05-0.95 (m, 3H, 38-CH_3). MALDI-TOF, *m/z*: $(M+Na)^+$ 1718.14, calculated for $C_{99}H_{131}N_{12}NaO_{11}P$ 1717.97.

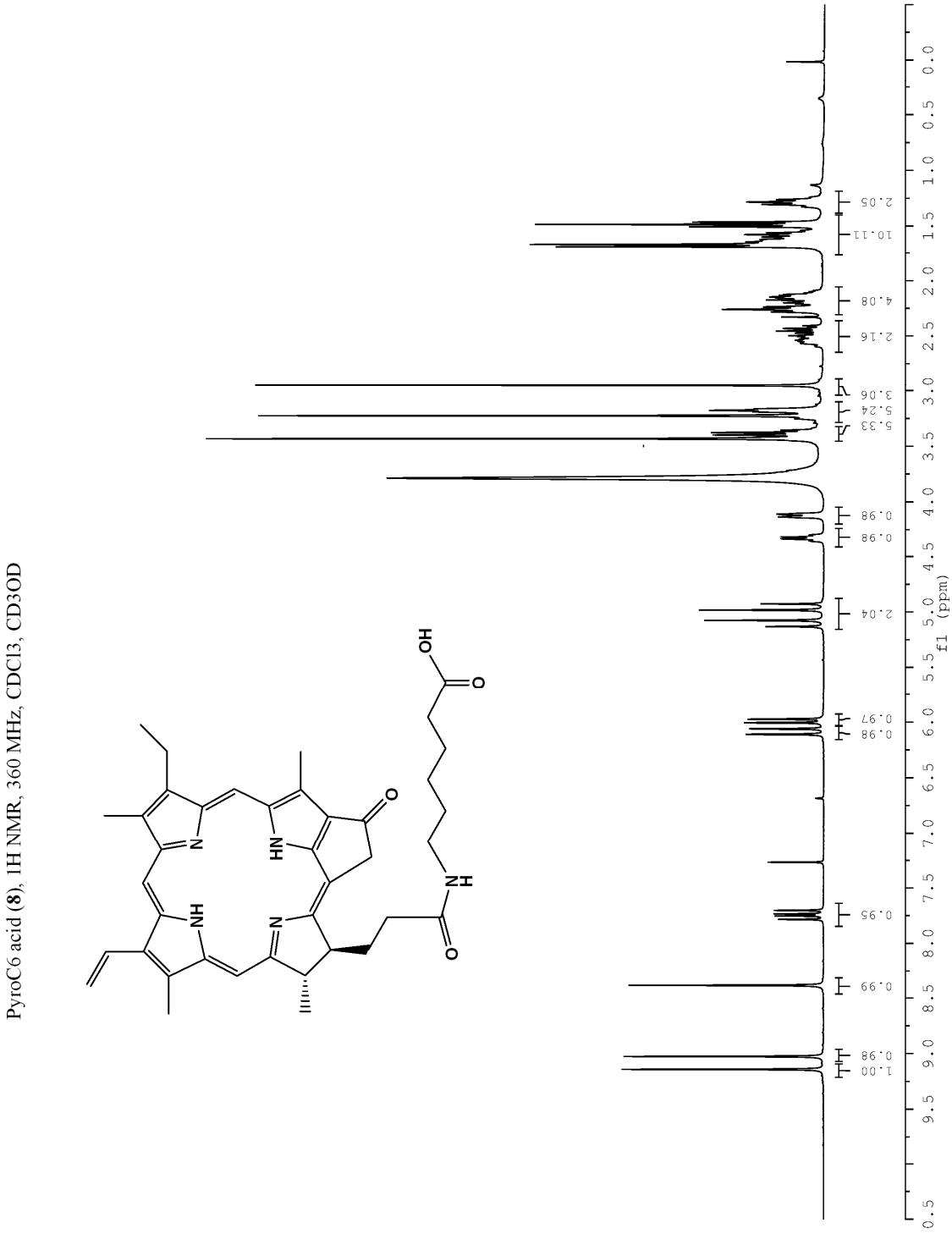
Synthesis of 1,2-bis(ε-pyropheophorbideamidocaproyl)-*sn*-glycero-3-phosphocholine, PyroC$_6$-PyroC$_6$-PtdCho (10)



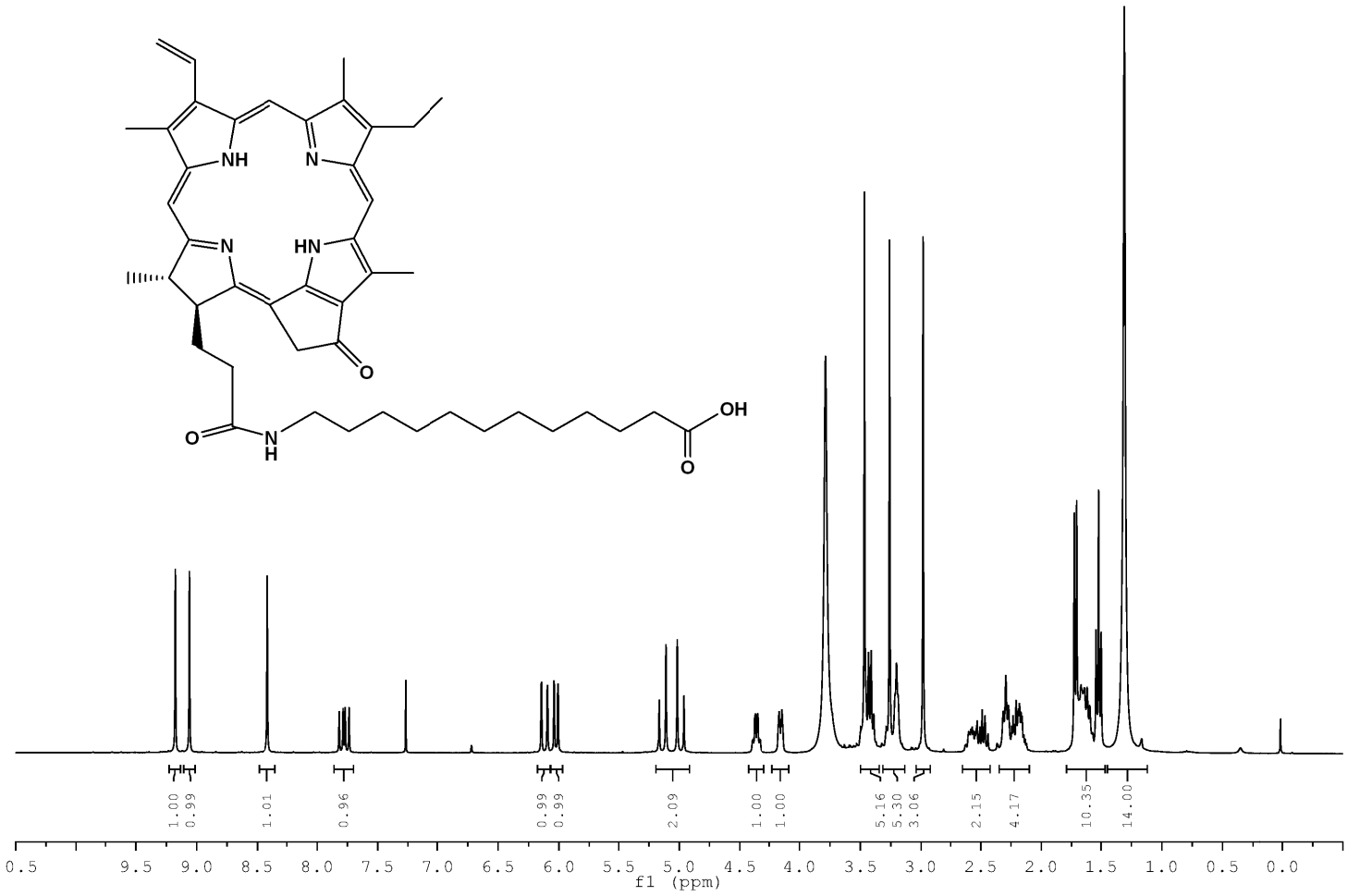
A 100 mL flask was loaded with *sn*-glycero-3-phosphocholine (**9**) (0.026 mmol), ε-Pyropheophorbideamidocaproic acid (**8**) (0.077 mmol), and 50 mL dry MeOH. The mixture was stirred 1h at rt. Then methanol was evaporated under low pressure and the resulting film was dried under high vacuum overnight. Next, EDC (0.077 mmol), DMAP (0.077 mmol) and dry DCM (50 mL) were added to the flask. The reaction mixture was stirred in dark under Ar at 40 °C for 85 h. After that the solution was rinsed with 0.5N HCl, and dried over Na_2SO_4. After solvent evaporation the mixture was dissolved in DCM and put onto three preparative TLC plates. Preparative TLC with $CHCl_3$/MeOH (3/1, v/v) resulted in a mixture of mono- and disubstituted *sn*-glycero-3-phosphocholines (R_f=0.1-0.2). The latter was separated a second time on TLC plates with $CHCl_3$/MeOH/H_2O (2/1/1, v/v/v). The target product (R_f=0.35,

$CHCl_3$/MeOH/H_2O = 2/1/1, v/v/v) was isolated with the yield of 11% (4.3 mg) as an amorphous dark green solid. ^{1}H NMR (360 MHz, $CDCl_3$, CD_3OD, CD_2Cl_2, δ ppm): 9.36, 9.27, 9.16, 9.04, 8.49, and 8.40 (each s, 1H, 5-*H*, 10-*H*, 20-*H*, 5_1-*H*, 10_1-*H* and 20_1-*H*); 7.99-7.70 (m, 2H, 3^1-*H* and $3_1{}^1$-*H*), 6.30-5.98 (m, 4H, 3^2-C*H*$_2$ and $3_1{}^2$-C*H*$_2$), 5.33-4.93 (m, 5H, 21-*H*, 13^2-C*H*$_2$ and $13_1{}^2$-C*H*$_2$, overlapped partially with CD_2Cl_2); 4.52-3.90 (m, 10H, 18-*H*, 17-*H*, 18_1-*H*, 17_1-*H*, 22-C*H*$_2$, 39-C*H*$_2$, 40-C*H*$_2$); 3.67-2.93 (m, 37H, 2^1-C*H*$_3$, $2_1{}^1$-C*H*$_3$, 12^1-C*H*$_3$, $12_1{}^1$-C*H*$_3$, 7^1-C*H*$_3$, $7_1{}^1$-C*H*$_3$, 77-C*H*$_3$, 78-C*H*$_3$, 79-C*H*$_3$, 8^1-C*H*$_2$, $8_1{}^1$-C*H*$_2$, 17^5-C*H*$_2$, $17_1{}^5$-C*H*$_2$, 41-C*H*$_2$, overlapped partially with CD_3OD); 2.71-2.10 (m, 12H, 17^2-C*H*$_2$, 17^9-C*H*$_2$, 17^1-C*H*$_2$, $17_1{}^2$-C*H*$_2$, $17_1{}^9$-C*H*$_2$, $17_1{}^1$-C*H*$_2$); 1.83-1.46 (m, 20H, 18^1-C*H*$_3$, 8^2-C*H*$_3$, 17^6-C*H*$_2$, 17^8-C*H*$_2$, $18_1{}^1$-C*H*$_3$, $8_1{}^2$-C*H*$_3$, $17_1{}^6$-C*H*$_2$, $17_1{}^8$-C*H*$_2$); 1.36-1.20 (m, 4H, 17^7-C*H*$_2$, $17_1{}^7$-C*H*$_2$). MALDI-TOF, *m/z*: $(M+Na)^+$ 1538.92, calculated for $C_{86}H_{106}N_{11}NaO_{12}P$ 1538.77.

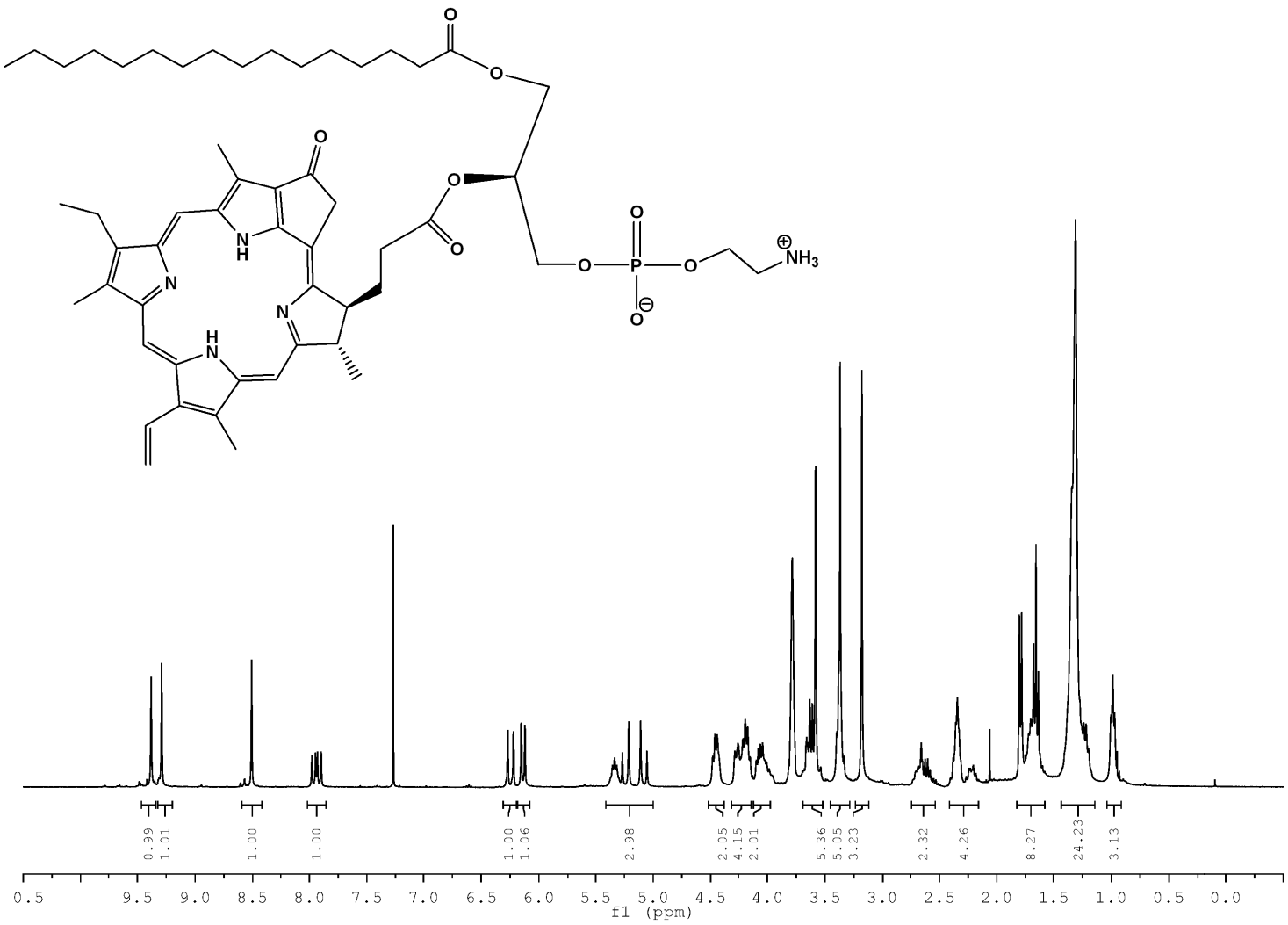
III. ^{1}H NMR Spectra



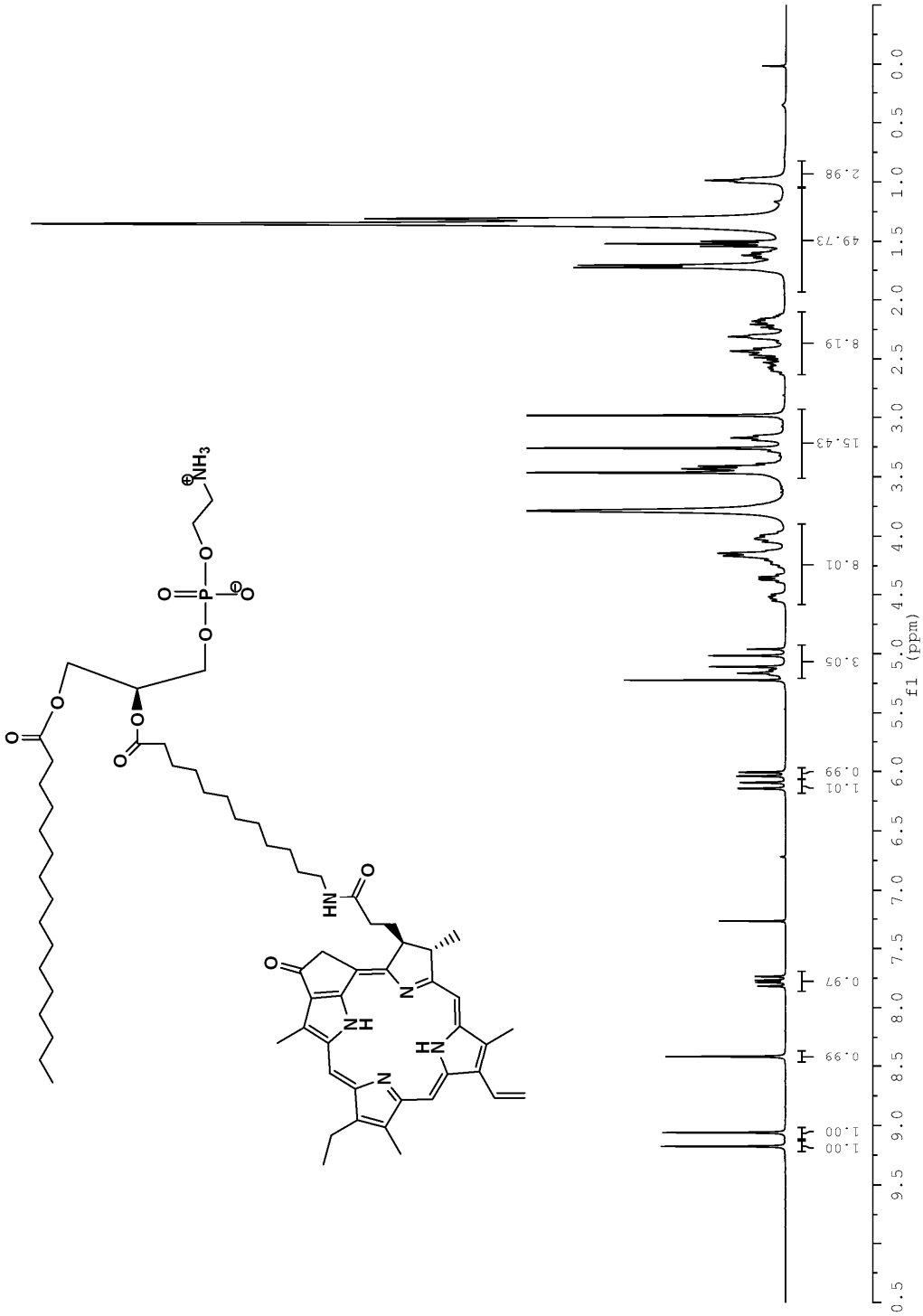
PyroC12 acid (**5**), 1H NMR, 360 MHz, CDCl3, CD3OD



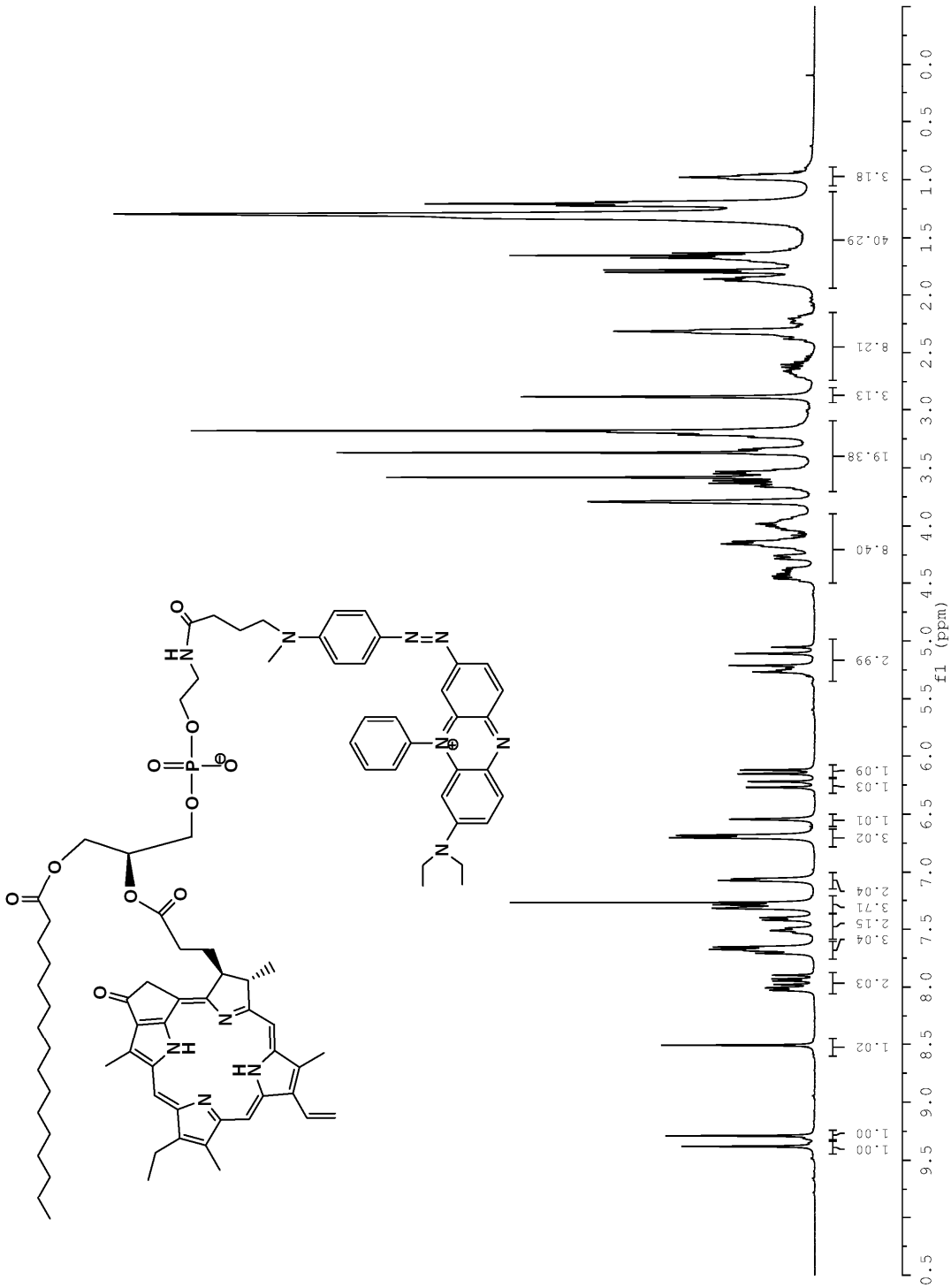
Pyro-PtdEtn (**3**), 1H NMR, 360 MHz, CDCl3, CD3OD



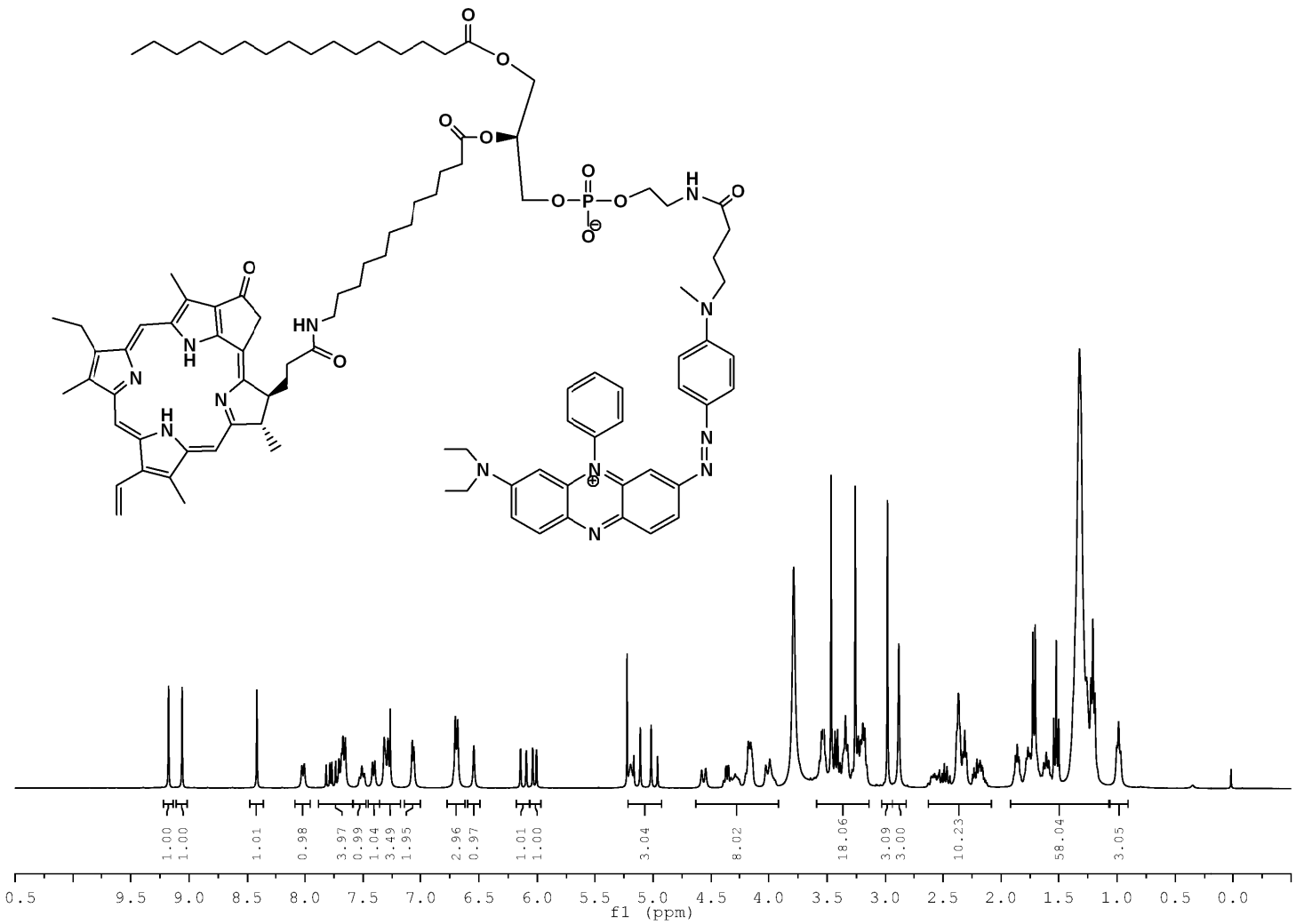
Pyro12-PtdEtn (**6**), 1H NMR, 360 MHz, CDCl3, CD3OD, CD2Cl2



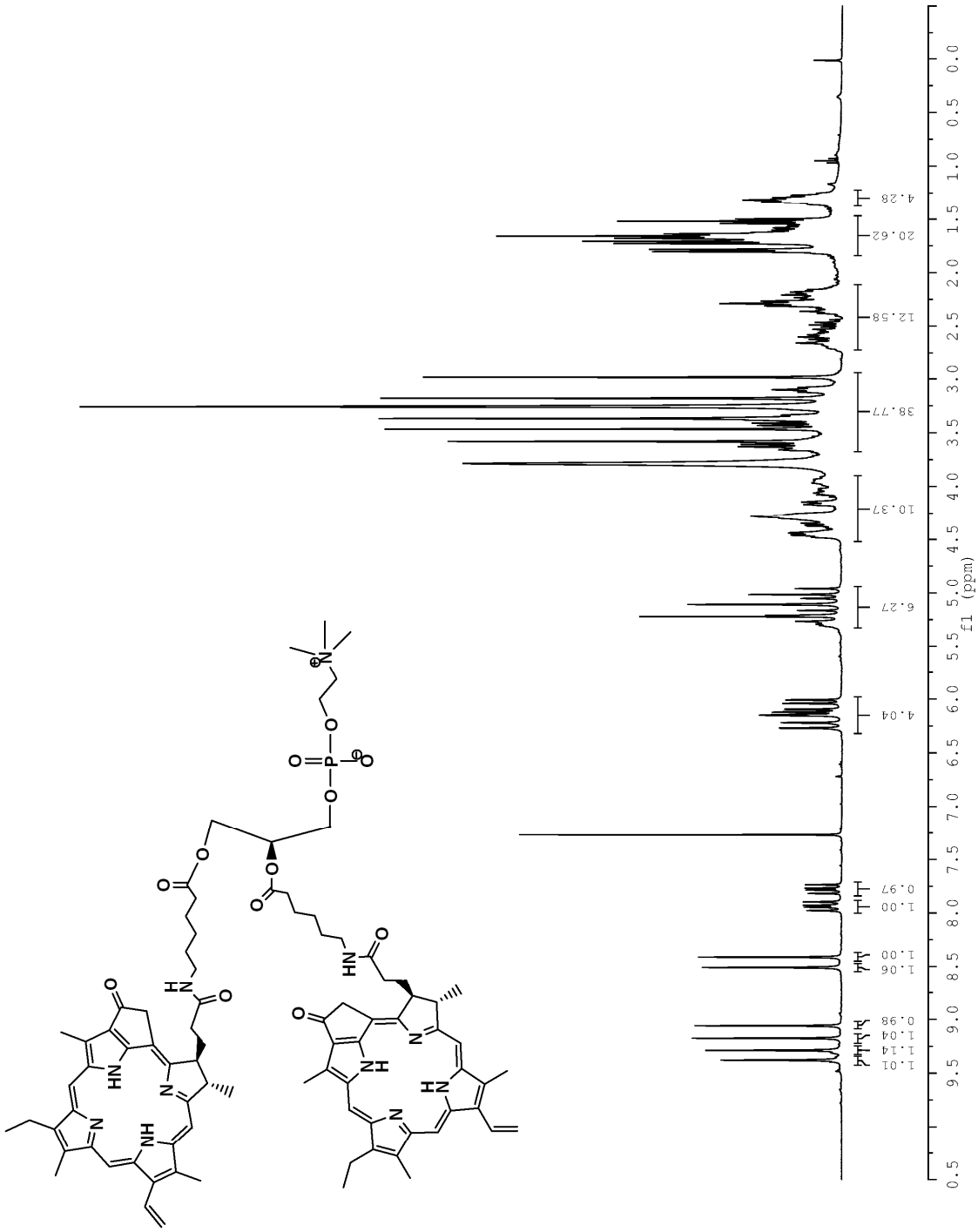
Pyro-PtdEtn-BHQ (**4**), 1H NMR, 360 MHz, CDCl3, CD3OD



Pyro12-PtdEtn-BHQ (7), 1H NMR, 360 MHz, CDCl3, CD3OD, CD2Cl2



PyroC6-PyroC6-PtdCho (**10**), 1H NMR, 360 MHz, CDCl3, CD3OD, CD2Cl2



IV. Enzyme mediated probe cleavage

The specificity of each probe to a series of phospholipases was determined by measuring fluorescence release in an *in vitro* assay. Probes were prepared in egg-phosphatidylcholine vesicle by adding fluorophores to a measured amount of egg-PtdCho in chloroform. Probe concentration was determined using the Beer-Lambert law by measuring optical density at 418 nm and using an extinction coefficient of 110,000 $M^{-1}cm^{-1}$. Chloroform was removed by evaporation, and the resulting film was resuspended in 50 mM Tris-HCl, pH 7.4, sonicated and vortexed until optically clear. The final concentration was 1 μM self-quenched phospholipid probe in 50 μM egg-yolk-PtdCho vesicles (i.e. mole fraction of 0.02). Lipid dispersions were aliquotted into 96-well plates at volumes of 100 μL. Reaction mixtures were incubated for 10 minutes at 37°C and reactions were started by addition of 10U of enzyme. The time-dependent release of fluorescence was measured using a Molecular Devices SpectraMax M5 fluorescent plate reader (λ_{Ex} 418 nm, λ_{Em} 675 nm for the Pyro-derivatives, λ_{Ex} 488 nm, λ_{Em} 530 nm for the BODIPY- derivatives).

V. References

(1) Yang, H., and Roberts, M. F. (2003) Phosphohydrolase and transphosphatidylation reactions of two Streptomyces phospholipase D enzymes: Covalent versus noncovalent catalysis. *Protein Sci. 12*, 2087-2098.

(2) Tomita, M., Sawada, H., Taguchi, R., and Ikezawa, H. (1987) The action of sphingomyelinase from Bacillus cereus on ATP-depleted bovine erythrocyte membranes and different lipid composition of liposomes. *Arch. Biochem. Biophys. 255*, 127-135.

(3) Ryan, M., Liu, T., Dahlquist, F. W., and Griffith, O. H. (2001) A Catalytic Diad Involved in Substrate-Assisted Catalysis: NMR Study of Hydrogen Bonding and Dynamics at the Active Site of Phosphatidylinositol-Specific Phospholipase C. *Biochemistry 40*, 9743-9750.

(4) Berg, O. G., Yu, B.-Z., Apitz-Castro, R. J., and Jain, M. K. (2004) Phosphatidylinositol-Specific Phospholipase C Forms Different Complexes with Monodisperse and Micellar Phosphatidylcholine. *Biochemistry 43*, 2080-2090.

(5) Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T., and Ohyabu, T. (1976) Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of Bacillus cereus. I. Purification, properties and phosphatase-releasing activity. *Biochim. Biophys. Acta, Lipids Lipid Metab. 450*, 154-164.

(6) Antikainen, N. M., Hergenrother, P. J., Harris, M. M., Corbett, W., and Martin, S. F. (2003) Altering Substrate Specificity of Phosphatidylcholine-Preferring Phospholipase C of Bacillus cereus by Random Mutagenesis of the Headgroup Binding Site. *Biochemistry 42*, 1603-1610.

(7) Benfield, A. P., Goodey, N. M., Phillips, L. T., and Martin, S. F. (2007) Structural studies examining the substrate specificity profiles of PC-PLCBc protein variants. *Arch. Biochem. Biophys. 460*, 41-47.
(8) Hergenrother, P. J., and Martin, S. F. (1997) Determination of the kinetic parameters for phospholipase C (Bacillus cereus) on different phospholipid substrates using a chromogenic assay based on the quantitation of inorganic phosphate. *Anal Biochem 251*, 45-49.
(9) Hergenrother, P. J., and Martin, S. F. (2001) Phosphatidylcholine-preferring phospholipase C from B. cereus. function, structure, and mechanism. *Top. Curr. Chem. 211*, 131-167.
(10) Kinkaid, A., and Wilton, D. C. (1991) Comparison of the catalytic properties of phospholipase A2 from pancreas and venom using a continuous fluorescence displacement assay. *Biochem. J. 278*, 843-848.
(11) Kudo, I., and Murakami, M. (2002) Phospholipase A2 enzymes. *Prostaglandins Other Lipid Mediators 68-69*, 3-58.
(12) Murakami, M., and Kudo, I. (2001) Diversity and regulatory functions of mammalian secretory phospholipase A2s. *Adv. Immunol. 77*, 163-194.
(13) Murakami, M., and Kudo, I. (2002) Lipid signaling. Phospholipase A2. *J. Biochem. 131*, 285-292.
(14) Zheng, G., Li, H., Zhang, M., Lund-Katz, S., Chance, B., and Glickson, J. D. (2002) Low-density lipoprotein reconstituted by pyropheophorbide cholesteryl oleate as target-specific photosensitizer. *Bioconjug Chem 13*, 392-396.