Photosensitizer-peptoid conjugates for photoinactivation of gram-negative bacteria: structure-activity relationship and mechanistic studies

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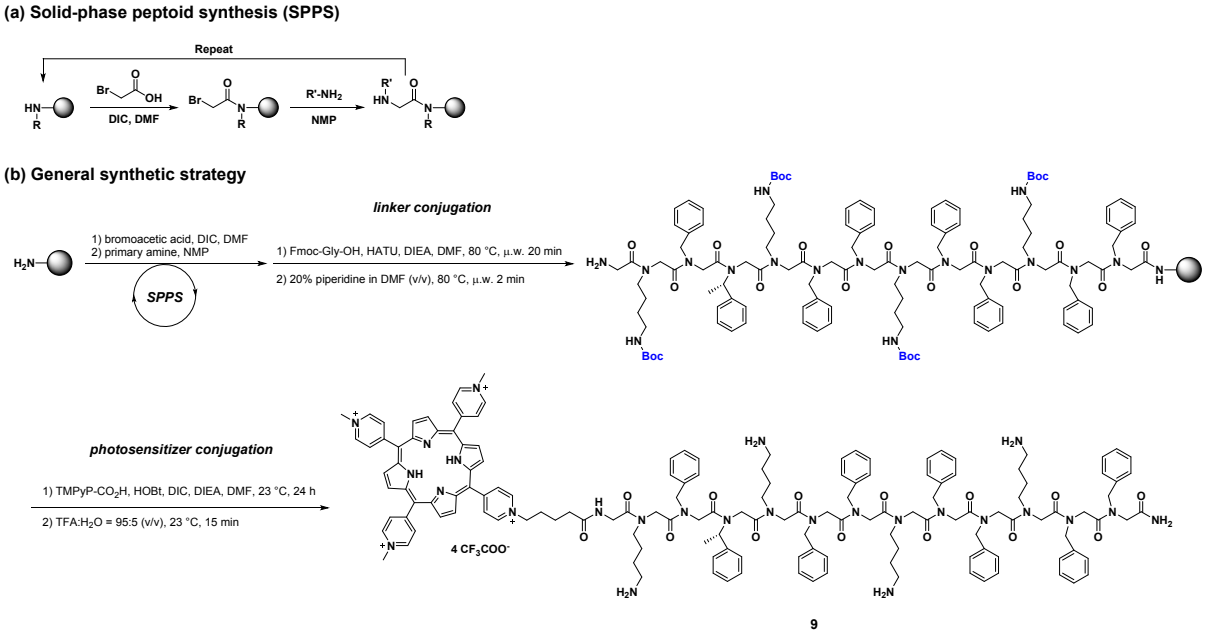

Scheme S1. (a) Peptoid submonomer synthesis. (b) General synthetic strategy used to prepare PsPC **1**–**11**. Synthesis of **9** is shown as a representative.

Synthesis of photosensitizers

5-(1-(4-(Ethoxycarbonyl)butyl)pyridinium-4-yl)-10,15,20-tripyridylporphyrin bromide (TPyP-CO_2Et)[1]

5,10,15,20-Tetrapyridylporphyrin (TPyP; 1.26 g, 2.04 mmol) and ethyl-5-bromovalerate (4.98 g, 23.82 mmol, 3.77 mL) were charged and dissolved in 25% (v/v) ethanol (EtOH) in $CHCl_3$ (400 mL). The solution was refluxed for 6 days. The crude product was purified using a silica gel column with 30% (v/v) EtOH/CH_2Cl_2 to yield the desired product as a purple solid (0.82 g, 54%; **Figure S8**). ^{1}H NMR (400 MHz, $CDCl_3$): 9.61 (d, 2H, *J*=6.0 Hz), 9.08 (m, 6H), 8.96 (s, 3H), 8.86 (m, 5H), 8.16 (m, 6H), 5.28 (t, 2H, *J*=7.2 Hz), 4.20 (q, 2H, *J*=7.2 Hz), 2.59 (t, 2H, *J*=6.8 Hz), 2.49 (m, 2H), 2.01 (m, 2H), 1.31 (t, 3H, *J*=7.2 Hz), -2.89 (s, 2H); ESI-MS m/e: $[M]^+$ calcd. for $C_{47}H_{39}N_8O_2$, 747.32; found, 747.1.

5-(1-(4-(Carboxylic acid)butyl)pyridinium-4-yl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tetra(trifluoroacetate) (TMPyP-CO_2H)[1]

TPyP-CO_2Et (0.32 g, 0.43 mmol) was dissolved in DMF (24 mL), and iodomethane (2.08 g, 14.67 mmol, 0.91 mL) was added to the solution. The mixture was stirred for 4 h at 42 ℃. The solvent was removed under reduced pressure, and the remaining solid was redissolved in aqueous HCl solution (1.0 M, 24 mL). The solution was refluxed for 1 h. Subsequent purification was performed according to the method reported by Giuntini and co-workers.[2] The heated solution was allowed to cool to room temperature and diluted with water (56 mL). Then, 10% (w/w) sodium hexafluorophosphate in water (10 mL) was added to the solution to induce precipitation of the desired product. The suspension was centrifuged (3000 rpm, 5 min), and the remaining solid was dissolved in acetone (80 mL). The resulting mixture was treated with 10% (w/w) tetrabutylammonium chloride in acetone (13 mL) and successively centrifuged (3000 rpm, 5 min) to precipitate the product. The compound was further purified by

recrystallization from methanol (MeOH)/diethyl ether and subsequently with a preparative HPLC system (0.29 g, 88%; **Figure S9**). ^{1}H NMR (400 MHz, DMSO-d_6): 9.60 (d, 2H, *J*=6.8 Hz), 9.52 (d, 6H, *J*=6.8 Hz), 9.10 (m, 16H), 4.99 (t, 2H, *J*=6.8 Hz), 4.74 (s, 9H), 2.31 (m, 2H), 1.87 (m, 2H), -3.10 (s, 2H); ESI-MS m/e: $[M]^{4+}$ calcd. for $C_{48}H_{44}N_8O_2$, 764.36; found, 381.1.

5-(4-Nitrophenyl)-10,15,20-triphenylporphyrin (TPP-NO_2)[3]

A 50 mL round-bottom flask was charged with 5,10,15,20-tetraphenylporphyrin (TPP; 1.00 g, 1.63 mmol) and TFA (25 mL). The solution was stirred for 10 min, and sodium nitrite (0.20 g, 2.93 mmol) was added to the solution. The reaction mixture was stirred for 3 min at room temperature and directly poured into ice-cold water (300 mL). Sodium bicarbonate was slowly added to the mixture for quenching, and the product was extracted with CH_2Cl_2 (500 mL). The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure to obtain the desired product with a quantitative yield (1.08 g).

5-(4-Aminophenyl)-10,15,20-triphenylporphyrin (TPP-NH_2)[3]

A 1 L round-bottom flask was charged with TPP-NO_2 (1.08 g, 1.63 mmol), and the solid was dissolved in 25% (v/v) MeOH in CH_2Cl_2 (250 mL). The solution was treated with 5% Pd/C (0.20 g), and sodium borohydride (0.62 g, 16.30 mmol) was added to initiate the reaction. The mixture was stirred overnight at room temperature. The reaction was quenched by the addition of water (500 mL), and the mixture was filtered through Celite to remove residual Pd/C. The product was extracted with CH_2Cl_2 (300 mL) and washed with water (300 mL). The organic layer was dried over magnesium sulfate, and the solvent was removed under reduced pressure. Purification was performed using a silica gel column with 40% (v/v) *n*-hexane/CH_2Cl_2 to yield the desired product as a purple solid (0.44 g, 43% over two steps; **Figure S10**). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.94 (d, 2H, *J*=4.4 Hz), 8.84 (m, 6H), 8.22 (m,

6H), 8.00 (m, 2H), 7.76 (m, 9H), 7.07 (m, 2H), 4.03 (s, 2H), -2.74 (s, 2H); ESI-MS m/e: $[M+H]^+$ calcd. for $C_{44}H_{32}N_5$, 630.27; found, 630.2.

5-(4-Carboxypentylaminophenyl)-10,15,20-triphenylporphyrin (TPP-ahx-NH_2)

TPP-NH_2 (29.5 mg, 0.047 mmol) was dissolved in CH_2Cl_2 (7 mL). Then, the solution was mixed with Boc-6-aminohexyl-OH (21.7 mg, 0.094 mmol), EDC (18.0 mg, 0.094 mmol), 1-hydroxybenzotriazole (HOBt; 12.7 mg, 0.094 mmol), and DIPEA (36.3 mg, 0.281 mmol, 0.05 mL). The resulting mixture was stirred overnight at room temperature and washed with water (50 mL). The organic layer was dried over sodium sulfate, and the solvent was removed under reduced pressure. Purification was performed using a silica gel column with 20% (v/v) EtOH/CH_2Cl_2 to yield TPP-Ahx-NH-Boc (37.0 mg, 93%). TPP-Ahx-NH-Boc (37.0 mg, 0.044 mmol) was dissolved in CH_2Cl_2 (1.8 mL), and the solution was mixed with TFA (0.2 mL) for Boc deprotection. The mixture was stirred for 15 min at room temperature. The reaction was quenched by the addition of saturated aqueous sodium bicarbonate solution (10 mL), and the product was washed with water (100 mL). The organic layer was extracted with CH_2Cl_2 (100 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, and the product was obtained as a purple solid (29.3 mg, 90%; **Figure S11**). ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.69 (s, 1H), 8.90 (m, 2H), 8.83 (m, 5H), 8.23 (m, 6H), 8.13 (s, 4H), 7.83 (m, 10H), 2.83 (t, 2H, *J*=8.0 Hz), 1.97 (m, 2H), 1.72 (m, 4H), 1.47 (m, 2H), -2.91 (s, 2H); ESI-MS m/e: $[M+H]^+$ calcd. for $C_{50}H_{42}N_6O$, 742.34; found, 742.1.

Chlorin e6 17^3,15^2-dimethyl ester (Ce6-DME)[4]

Chlorin e6 (100.0 mg, 0.17 mmol) was dissolved in 5% (v/v) sulfuric acid in MeOH (60 mL), and the solution was stirred overnight at room temperature. The reaction was quenched by the addition of ice-cold saturated sodium bicarbonate solution (100 mL). The crude product

was extracted with CH_2Cl_2 and purified using a silica gel column with 6% (v/v) MeOH/CH_2Cl_2, yielding a dark green solid (103.6 mg, 99%; **Figure S12**). ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.55 (d, 2H, *J*=24.0 Hz), 8.70 (s, 1H), 8.05 (dd, 1H, *J*=11.2 Hz), 6.35 (d, 1H, *J*=17.6 Hz), 6.14 (d, 1H, *J*=11.6 Hz), 5.62 (d, 1H, *J*=18.4 Hz), 5.27 (d, 1H, *J*=17.2 Hz), 4.36 (m, 2H), 3.77 (s, 3H), 3.67 (m, 2H), 3.59 (s, 3H), 3.52 (s, 3H), 3.45 (s, 3H), 3.26 (s, 3H), 2.45 (m, 1H), 2.05 (m, 3H), 1.72 (d, 3H, *J*=6.4 Hz), 1.64 (t, 3H, *J*=7.6 Hz), -1.35 (s, 1H); ESI-MS m/e: $[M+H]^+$ calcd. for $C_{36}H_{41}N_4O_6$, 625.30; found, 625.3.

Synthetic procedure for the photosensitizer-NHS ester

A 25 mL round-bottom flask was charged with *N*-hydroxysuccinimide (22.6 mg, 0.196 mmol), DCC (40.5 mg, 0.196 mmol), CH_2Cl_2 (4 mL), photosensitizer (i.e., photochlor (50.0 mg, 0.078 mmol), verteporfin (56.1 mg, 0.078 mmol), or Ce6-DME (48.7 mg, 0.078 mmol)). The reaction was initiated by the addition of pyridine (15.5 mg, 0.196 mmol, 0.016 mL) and DMAP (0.5 mg, 5 mol%), and the solution was stirred overnight at room temperature. The crude product was purified using a silica gel column with 25% (v/v) EtOAc/CH_2Cl_2.

Photochlor-NHS ester

Yield: 33.7 mg (58%); ESI-MS m/e: $[M+H]^+$ calcd. for $C_{43}H_{52}N_5O_6$, 734.39; found, 734.4.

Verteporfin-NHS ester

Yield: 22.9 mg (66%); ESI-MS m/e: $[M+H]^+$ calcd. for $C_{45}H_{46}N_5O_{10}$, 816.32; found, 816.3.

Chlorin e6 17^3,15^2-dimethyl-13^1-NHS ester

Yield: 51.4 mg (91%); ESI-MS m/e: $[M+H]^+$ calcd. for $C_{40}H_{44}N_5O_8$, 722.32; found, 722.2.

Synthetic procedure for peptoids

Peptoid synthetic protocol

Peptoids were synthesized by a microwave-assisted solid-phase submonomer synthesis method.[5] The reactions were run in a cartridge (Applied Separations, Allentown, PA, USA, 25 mL) assembled with a filter (Applied Separations, 20 micron) and accelerated by a CEM MARS 230/60 microwave reaction system (CEM Corp., Matthews, NC, USA) using a fiber optic temperature probe and magnetic stirrer under atmospheric pressure. The resin (0.25 mmol, 0.48 g) was swelled in DMF for 20 min and was treated twice with 20% (v/v) piperidine in DMF (5 min and 20 min, 10 mL each) for Fmoc deprotection. Bromoacetylation was performed by the addition of bromoacetic acid (6.25 mmol, 1.2 M in DMF, 5.21 mL) and DIC (0.70 g, 5.63 mmol, 0.87 mL). The mixture was stirred in a microwave oven (300 W max power, 35 ℃, ramp 30 s, hold 1 min) and washed with CH_2Cl_2 and DMF. Amine displacement was carried out by the addition of (*S*)-(-)-α-methylbenzylamine (*N*spe, 3.75 mmol, 1.0 M in NMP, 3.75 mL), benzylamine (*N*pm, 3.75 mmol, 1.0 M in NMP, 3.75 mL), or mono-Boc protected 1,4-diaminobutane (*N*Lys(Boc), 3.75 mmol, 1.0 M in NMP, 3.75 mL) to the bromoacetylated peptoids. The mixture was stirred in a microwave oven (300 W max power, 80 ℃, ramp 2 min, hold 90 s) and washed with CH_2Cl_2 and DMF. Bromoacetylation and amine displacement were repeated until the desired sequence was obtained. Peptoids were cleaved from the resin with 95% (v/v) TFA/CH_2Cl_2 for 30 min at room temperature. The cleavage reaction solution was filtered using solid-phase extraction (SPE) cartridges with a 20 μ PE frit (Applied Separations, Allentown, PA, USA), and the volatiles were removed by a stream of nitrogen. The crude peptoid was dissolved in 50% (v/v) H_2O/MeCN for further purification steps.

General conditions for the peptide coupling reactions between Fmoc-protected linkers and resin-bound peptoids

A resin-bound peptoid (0.016 mmol) was swelled in DMF (3 mL) for 20 min, and the solvent was drained. The resin-containing cartridge was charged with linker (i.e., Fmoc-Gly-OH (14.3 mg, 0.048 mmol) or Fmoc-*N*-amido-dPEG®$_4$-acid (23.4 mg, 0.048 mmol)), DIPEA (16.5 mg, 0.128 mmol, 22 μL), HATU (18.3 mg, 0.048 mmol), and DMF (1 mL). The mixture was stirred in a microwave oven (300 W max power, 80 ℃, ramp 2 min, hold 18 min). The resin was then successively washed with CH_2Cl_2 and DMF. For Fmoc deprotection, 20% (v/v) piperidine in DMF was added to the cartridge, followed by stirring in a microwave oven (300 W max power, 75 ℃, ramp 2 min, hold 2 min). The resin was washed with CH_2Cl_2 and DMF.

General conditions for the peptide coupling reactions between photosensitizers and resin-bound peptoids

*Compounds **1** and **6**–**11**.*[6] The resin-bound peptoid (0.016 mmol) was suspended in DMF (3 mL) for 20 min, and the solvent was drained. The resin-containing cartridge was charged with TMPyP-CO_2H (36.7 mg, 0.048 mmol), HOBt (8.6 mg, 0.064 mmol), DIC (8.1 mg, 0.064 mmol, 10 μL), DIPEA (16.5 mg, 0.128 mmol, 22 μL), and DMF (1 mL). The mixture was stirred overnight at room temperature. The resin was washed successively with CH_2Cl_2 and DMF.

*Compound **2**.* The resin-bound peptoid (0.016 mmol) was suspended in DMF (3 mL) for 20 min, and the solvent was drained. The resin-containing cartridge was charged with carbonyldiimidazole (7.8 mg, 0.048 mmol) and DMF (1 mL). The mixture was stirred in a microwave oven (300 W max power, 95 ℃, ramp 2 min, hold 90 s). The resin was then successively washed with CH_2Cl_2 and DMF. For photosensitizer conjugation, the resin was treated with TPP-Ahx-NH_2 (35.6 mg, 0.048 mmol), DIPEA (6.2 mg, 0.048 mmol, 8 μL), and

DMF (1 mL). The mixture was stirred overnight at room temperature. The resin was successively washed with CH_2Cl_2 and DMF.

*Compounds **3**, **4**, and **5***. The resin-bound peptoid (0.016 mmol) was suspended in DMF (3 mL) for 20 min, and the solvent was drained. The resin was resuspended in CH_2Cl_2 (3 mL), and DIPEA (0.1 mL) was used for conditioning. After 2 min, the solution was drained, and the resin-containing cartridge was charged with the photosensitizer-NHS ester in CH_2Cl_2 (15 mM, 3 mL) and DIPEA (7.4 mg, 0.057 mmol, 10 μL). The reaction mixture was stirred overnight at room temperature. The resin was washed successively with CH_2Cl_2 and DMF.

HPLC purification

Analytical HPLC was conducted using a Waters reversed-phase HPLC system (Waters 2489 UV/visible detector, Waters 1525 Binary HPLC pump, Waters 2707 Autosampler, and Waters 5CH column oven) on a C18 column (SunFire C18, 4.6 × 250 mm, 5 μm) at 40 °C. A binary mobile phase system (A: deionized water + 0.1% TFA, B: MeCN + 0.1% TFA) was employed as follows: 5 min at 10% B, a linear gradient to 100% B over 25 min, and hold at 100% B for 10 min. The flow rate was 1 mL/min. The purity of the compound was monitored by measuring the absorbance at 220 nm. All the peptoids were purified on a Waters preparative HPLC system (Waters 2489 UV/visible detector, Waters 2545 Quaternary HPLC pump, Waters fraction collector III) with a C18 column (SunFire C18, 19 x 150 mm, 5 μm) at room temperature. The flow rate was set to 14 $mL \cdot min^{-1}$, and a binary mobile phase system (A: deionized water + 0.1% TFA, B: MeCN + 0.1% TFA) was used under the following conditions: 5 min at 10% B, a linear gradient to 100% B over 30 min, and hold at 100% B for 5 min. Sample elution was monitored by measuring the absorbance at 220 nm, and the purity of each fraction (>97%) was confirmed by analytical HPLC. ESI-MS analysis was carried out on an Agilent 1260 Infinity liquid chromatography system with an Agilent 6120 single quadrupole mass spectrometer.

Fractions containing the pure product were collected, lyophilized, and stored at −80 °C.

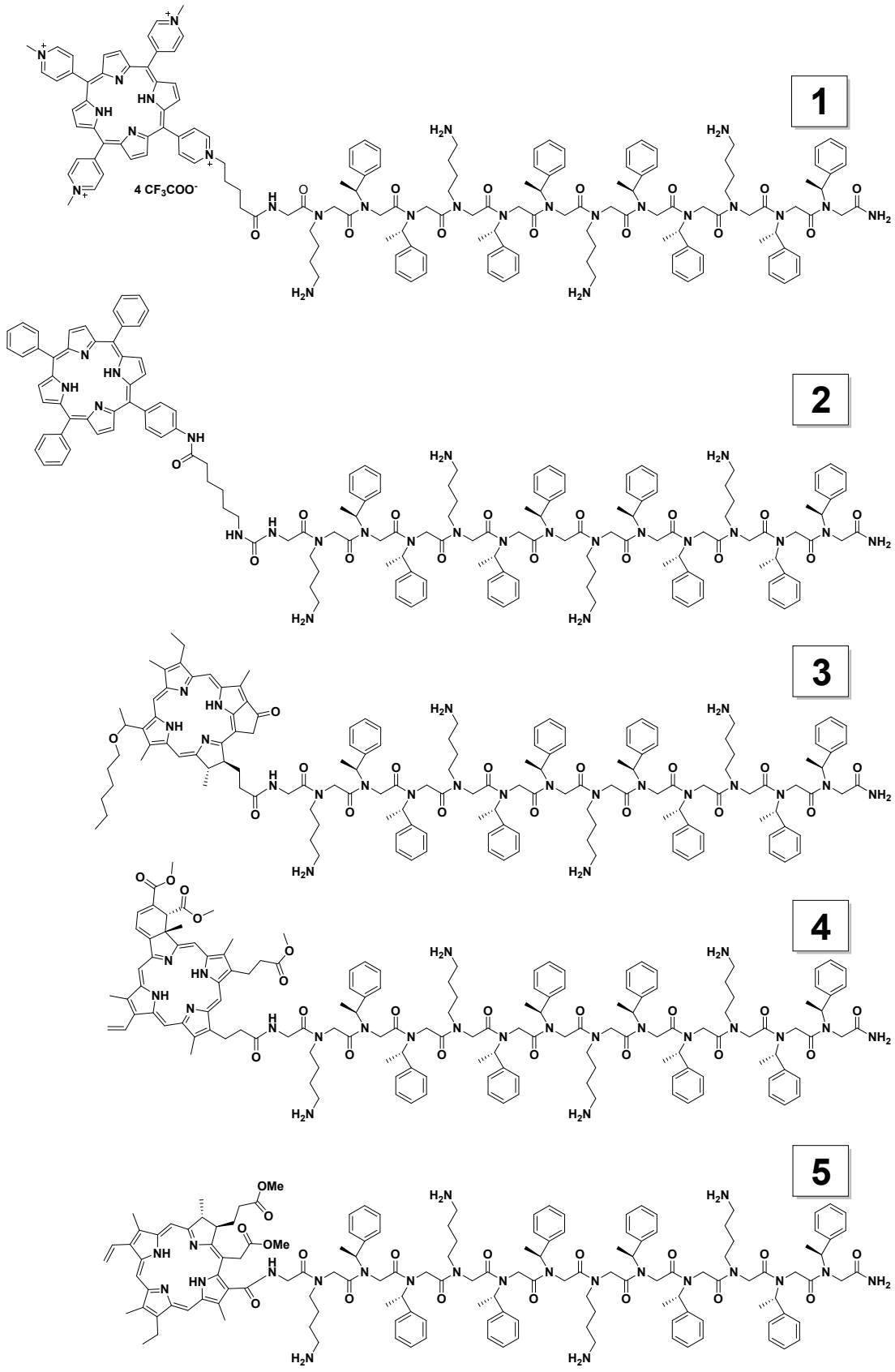

Figure S1 (continued). Structures of compounds **1**–**16**.

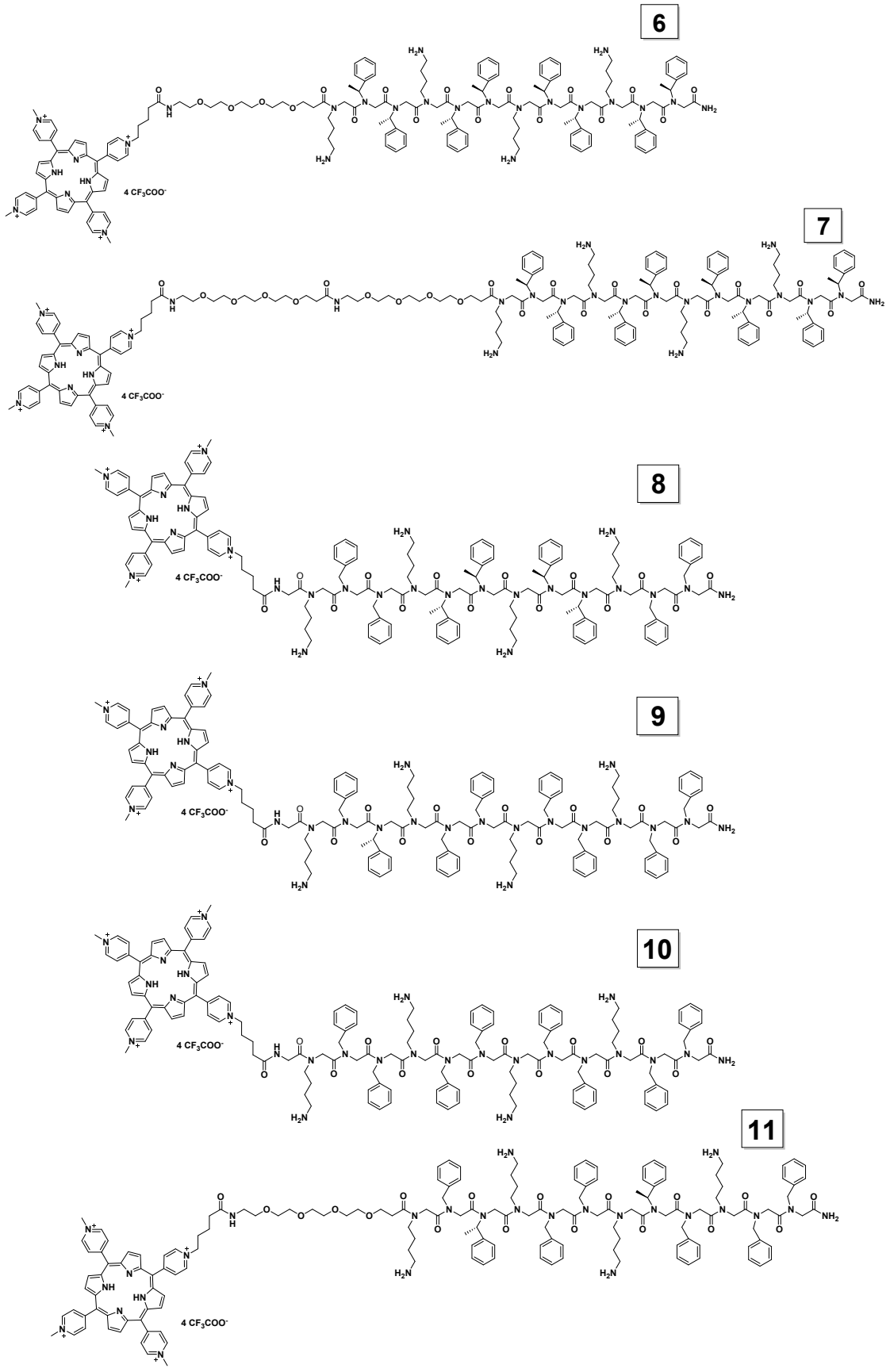

Figure S1 (continued). Structures of compounds **1**–**16**.

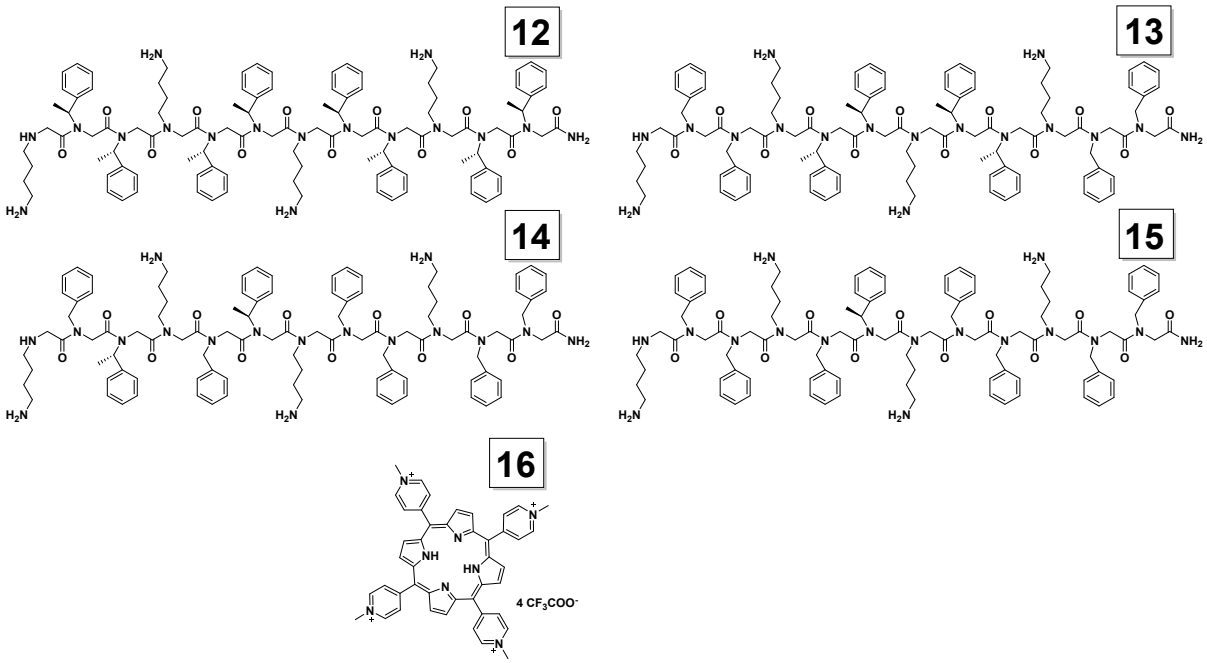

Figure S1. Structures of compounds **1**–**16**.

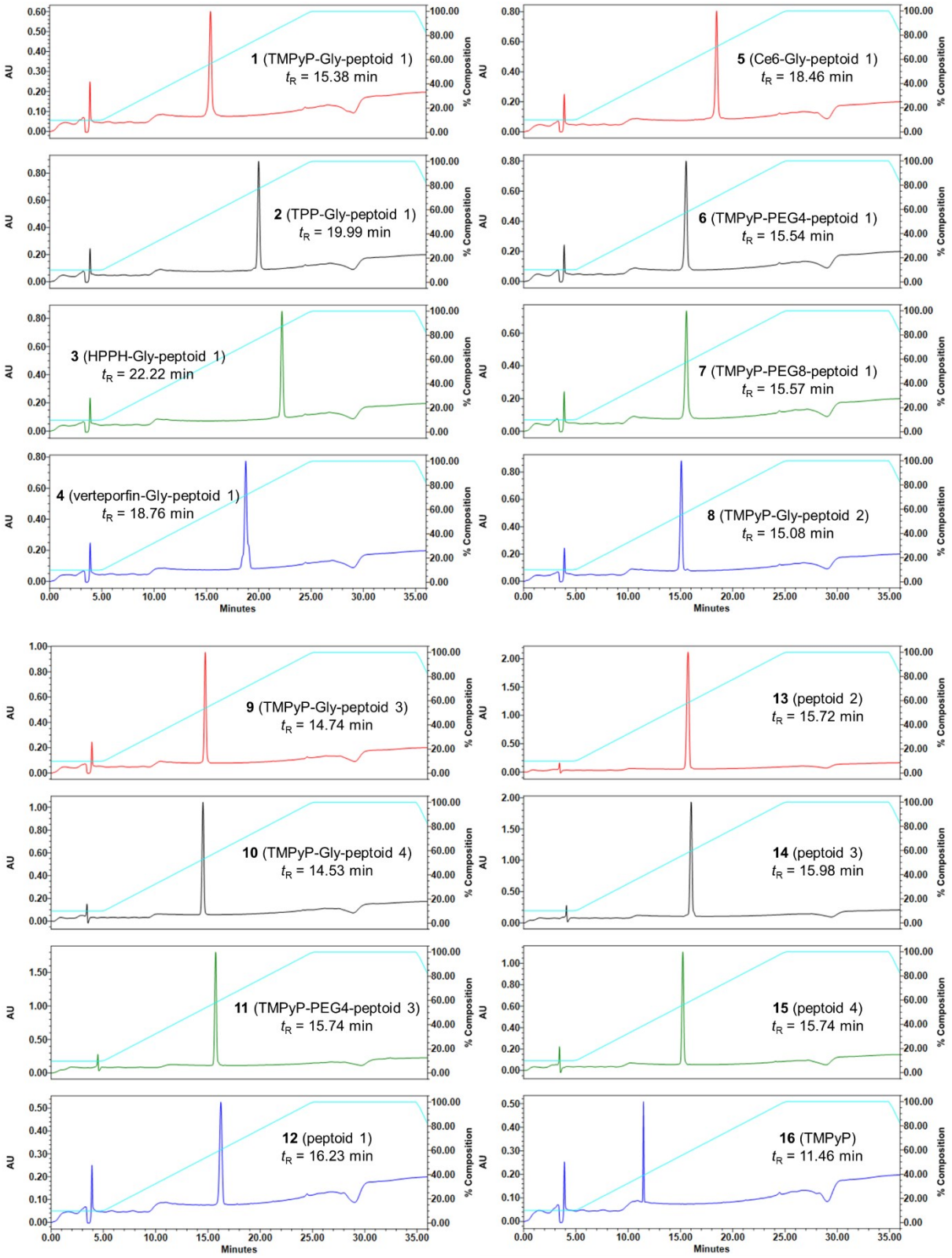

Figure S2. HPLC chromatograms of **1**–**16** monitored at 220 nm (>97% purity). The retention times (t_R) are shown.

Table S1. ESI-MS data of purified compounds **1**–**16**.[a]

compounds	mass calculated	mass observed
1	2621.45	655.7 $(M+H)^{4+}$, 873.6 $(M)^{3+}$
2	2643.42	882.4 $(M+3H)^{3+}$, 1323.6 $(M+2H)^{2+}$
3	2493.46	832.4 $(M+3H)^{3+}$, 1248.6 $(M+2H)^{2+}$
4	2575.39	859.8 $(M+3H)^{3+}$, 1289.6 $(M+2H)^{2+}$
5	2481.38	828.5 $(M+3H)^{3+}$, 1241.9 $(M+2H)^{2+}$
6	2811.57	937.3 $(M)^{3+}$
7	3058.71	1020.1 $(M+H)^{3+}$
8	2565.38	641.6 $(M+H)^{4+}$, 855.1 $(M)^{3+}$
9	2523.34	631.1 $(M+H)^{4+}$, 840.8 $(M)^{3+}$
10	2509.32	627.7 $(M+H)^{4+}$, 836.4 $(M)^{3+}$
11	2713.46	678.6 $(M+H)^{4+}$, 904.5 $(M)^{3+}$
12	1818.08	910.5 $(M+2H)^{2+}$, 1819.0 $(M+H)^{+}$
13	1762.02	881.9 $(M+2H)^{2+}$, 1763.4 $(M+H)^{+}$
14	1719.97	861.0 $(M+2H)^{2+}$, 1720.8 $(M+H)^{+}$
15	1705.95	854.1 $(M+2H)^{2+}$, 1706.7 $(M+H)^{+}$
16	678.32	338.1 $(M)^{2+}$

[a]Observed in ESI-MS. Observed masses are multiply charged species due to the detectable mass range of instrument.

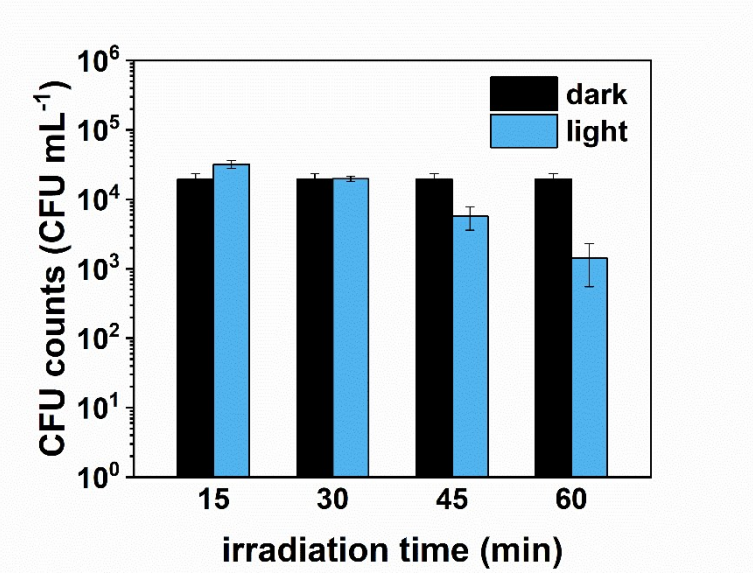

Figure S3. Effect of the blue-light (16.8 mW/cm^2) on the growth of *E. coli* (ATCC 25922). The cells in 96-well plate were irradiated for indicated time (15−60 min) or kept in the dark for the same time. Then, the cells were incubated at 37 °C overnight, and the number of colony-forming units (CFU) were counted. Data represent the mean (± s.d.) of three independent experiments.

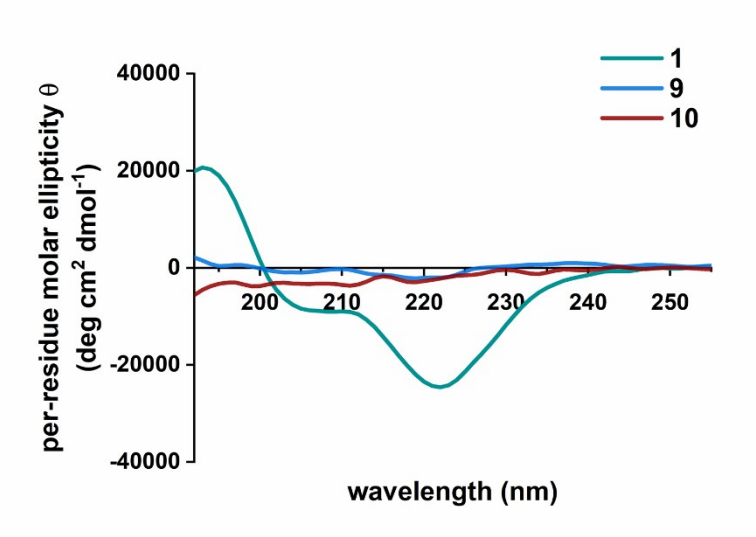

Figure S4. Circular dichroism (CD) spectra obtained for **1**, **9**, and **10** (50 μM) in 5 mM lipid vesicles (POPE:POPG=7:3) and 10 mM Tris-HCl buffer (pH 7.0; 20 ℃).

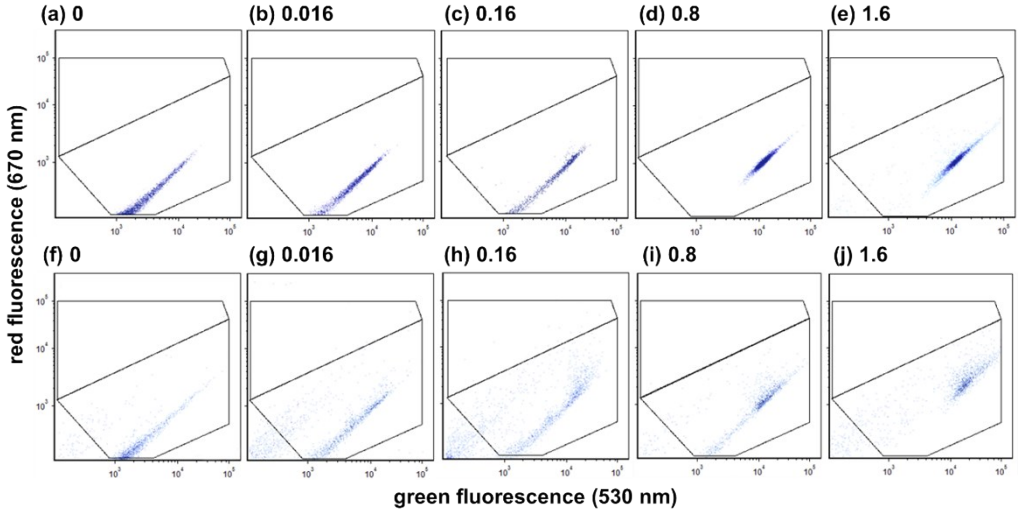

Figure S5. Flow-cytometric density plots (530 nm vs. 670 nm) for the total cell count (TCC) changes during aPDT against *E. coli* (ATCC 25922) as a function of molecular concentrations of **9** from zero to 1.6 μM. The samples were prepared with (a–e) or without (f–j) an additional washing step and stained with SGI (10 μL mL^{-1}) that binds to nucleic acids. The polygons illustrate gates depicting the region where properly SGI stained cells appear.

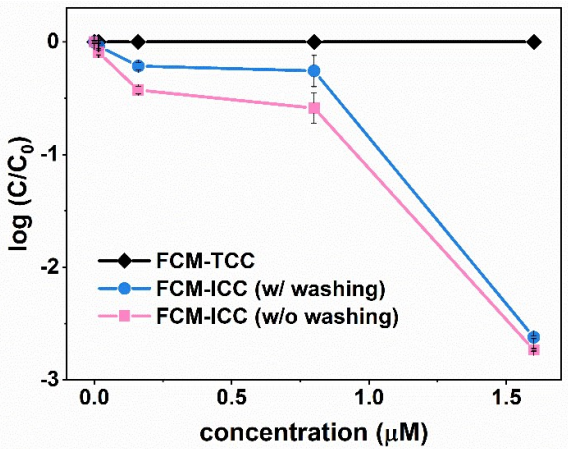

Figure S6. Changes in the logarithmic relative concentration of FCM-TCC (loss of cell integrity, SGI staining) and FCM-ICC (membrane damage, SGI/PI staining) during aPDT against *E. coli* (ATCC 25922) as a function of molecular concentrations of **9**. The samples were prepared with or without additional washing step. The symbols represent the measured data and the error bars represent one standard deviation from duplicate experiments.

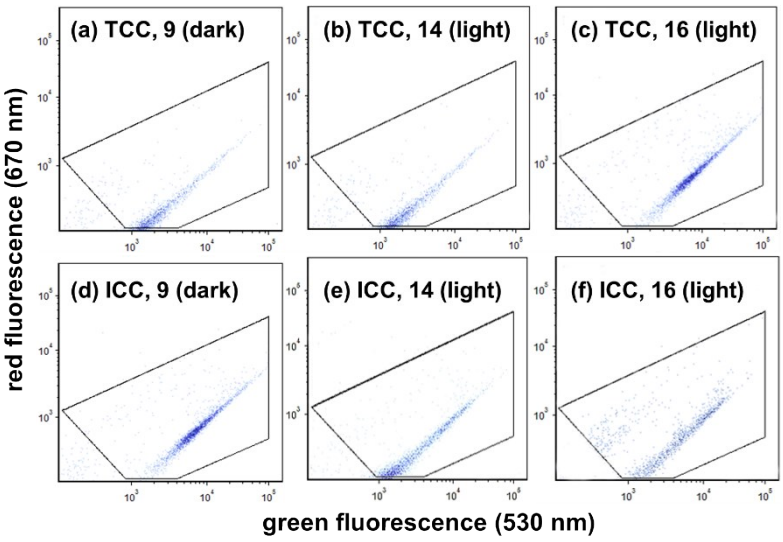

Figure S7. Flow-cytometric density plots (530 nm vs. 670 nm) for the total cell count (TCC; a–c) and intact cell count (ICC; d–f) changes against *E. coli* (ATCC 25922) in a fixed concentration of compounds (**9**, **14**, and **16**; 1.6 μM). (b–c and e–f) The samples were irradiated under blue light for 15 min (15.1 J·cm^{-2}). The samples were stained with (a–c) SGI (10 μL mL^{-1}) that binds to nucleic acids to assess the cell integrity or (d–f) SGI (10 μL mL^{-1})/PI (0.6 mM) to assess the membrane damage.

Cytotoxicity assay

Human lung cells (MRC-5) were purchased from the Korean Cell Line Bank and used as a model to study the phototoxicity of eukaryotic cells. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Welgene, Gyeongsan, South Korea) supplemented with 10% fetal bovine serum (FBS, Welgene), 38 units·mL^{-1} streptomycin, and 100 units·mL^{-1} penicillin G (Welgene). The cultures were maintained at 37 ℃ in a humidified atmosphere containing 5% CO_2. The photoinduced activity of compounds towards human lung cells (fibroblasts) was assessed using the same incubation and irradiation conditions as those used for bacteria. For these experiments, the cells were seeded in 96-well plates (1.3×10^4 cells/well) and allowed to attach and grow for 24 h in complete culture medium. Subsequently, the cells were incubated for 2 h in the dark with increasing concentrations of the compounds in culture medium with 10% FBS and irradiated with 15.1 J·cm^{-2} blue light. Then, the cells were returned to the incubator, and after 24 h, cell viability was measured with [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA). For the test, the cell medium was replaced with 180 μL of Opti-MEM and 20 μL of Cell Titer 96 reagent, and the wells were incubated for 3 h at 37 ℃. The MTS-formazan products in the viable cells were measured at 490 nm using a microplate reader, and the viability of the treated cells was expressed as a percentage of the absorbance of the control cells, which was taken as 100% viability.

Table S2. LC_{50} values of compounds for MRC-5 cell line.[a]

compounds	dark (μM)	light (μM)[b]
9	17.1	0.0016
14	>25	>0.050
16	>25	>0.050

[a] LC_{50}: Lethal concentrations causing 50% of cell death. The maximum concentration tested was 25 μM in the dark and 0.050 μM in the light conditions. Cell viability was measured via MTS assay in cells treated with compounds for 24 h, and LC_{50} values were derived from viability curves. [b] The samples were irradiated under the blue light for 15 min (15.1 J cm^{-2}).

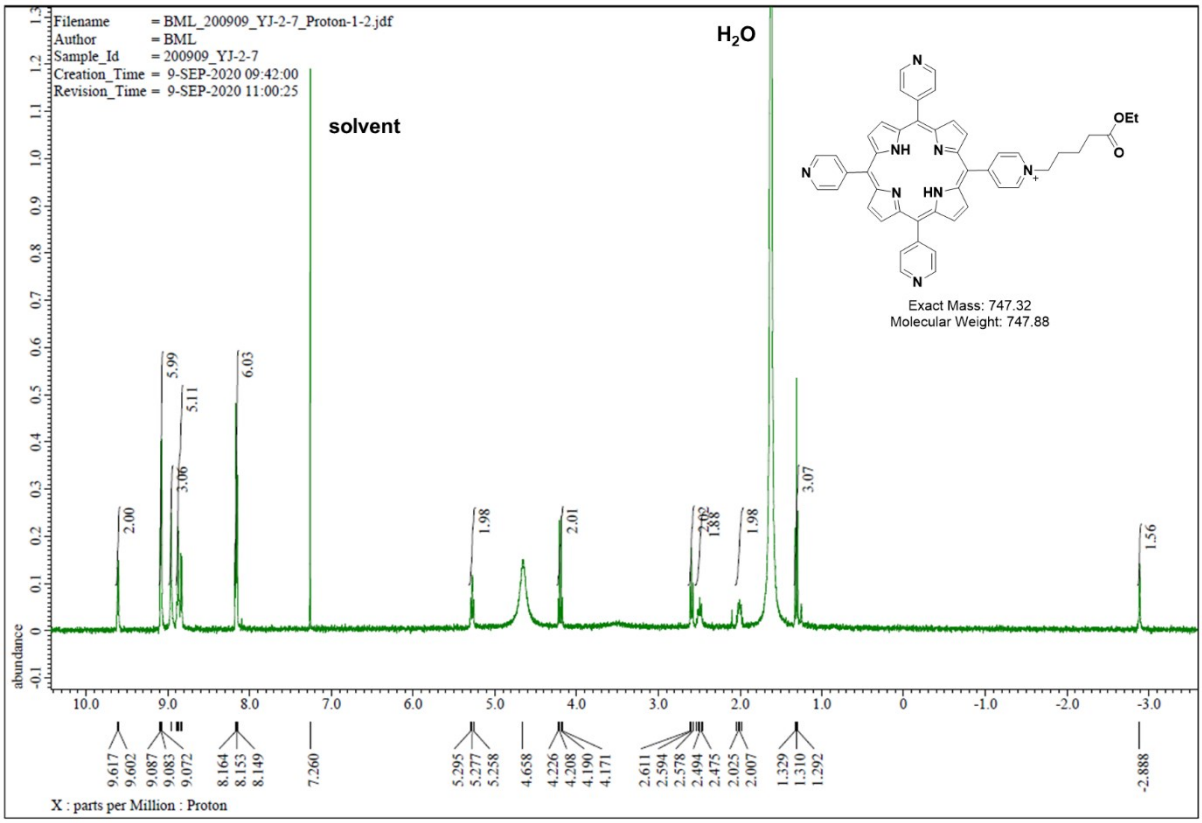

Figure S8. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of TPyP-CO_2Et.

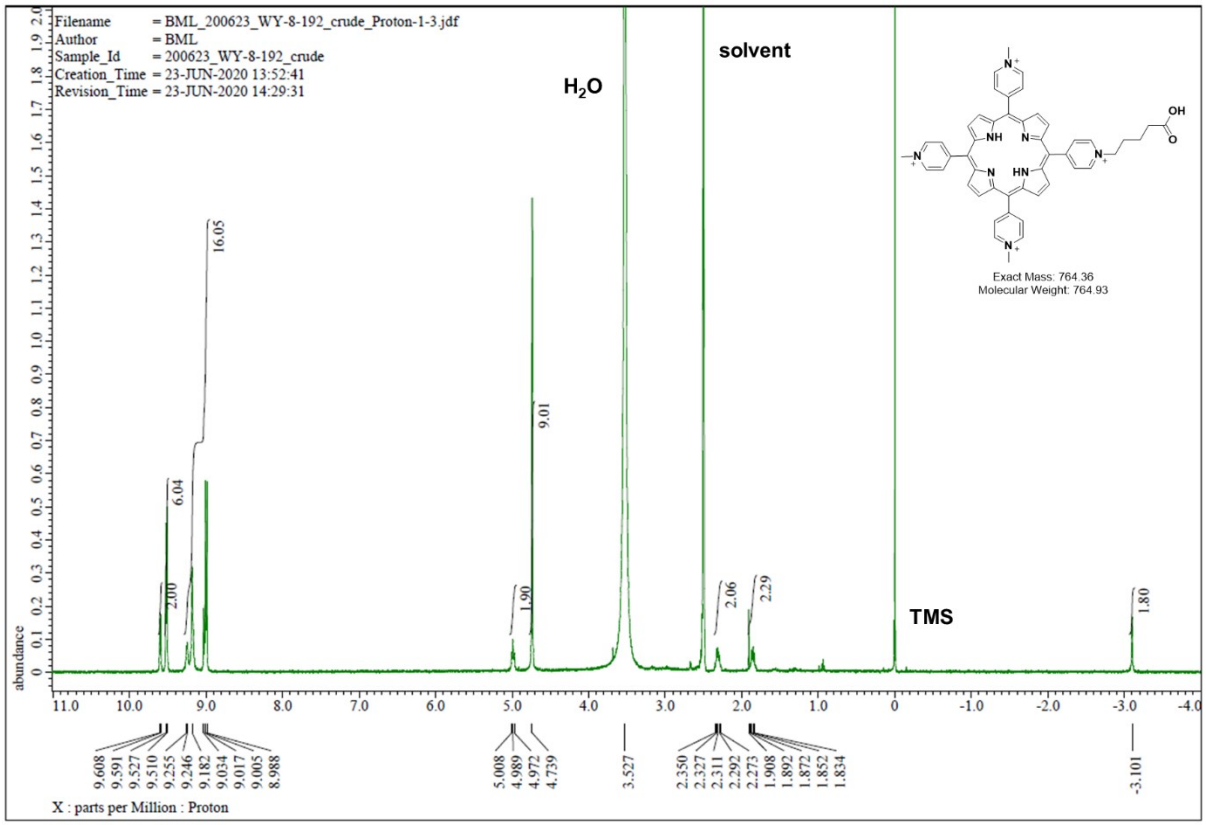

Figure S9. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of TMPyP-CO_2H.

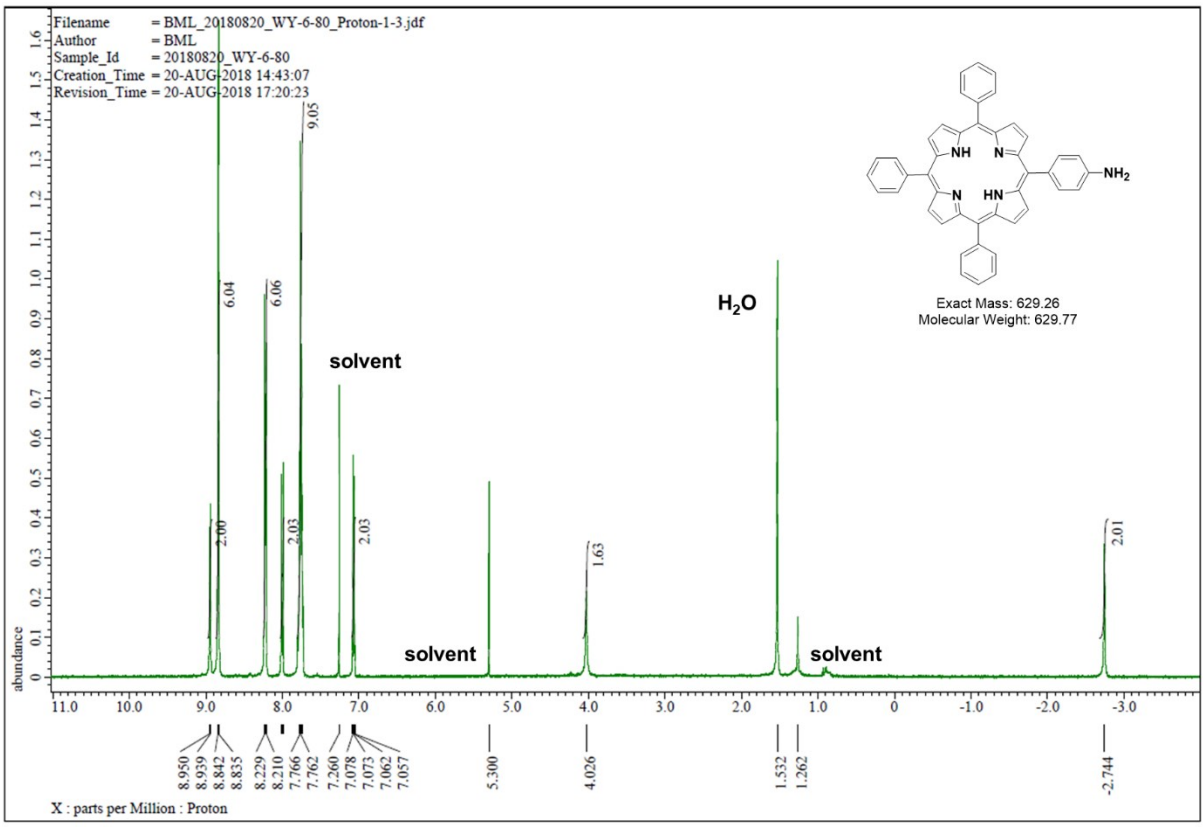

Figure S10. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of TPP-NH_2.

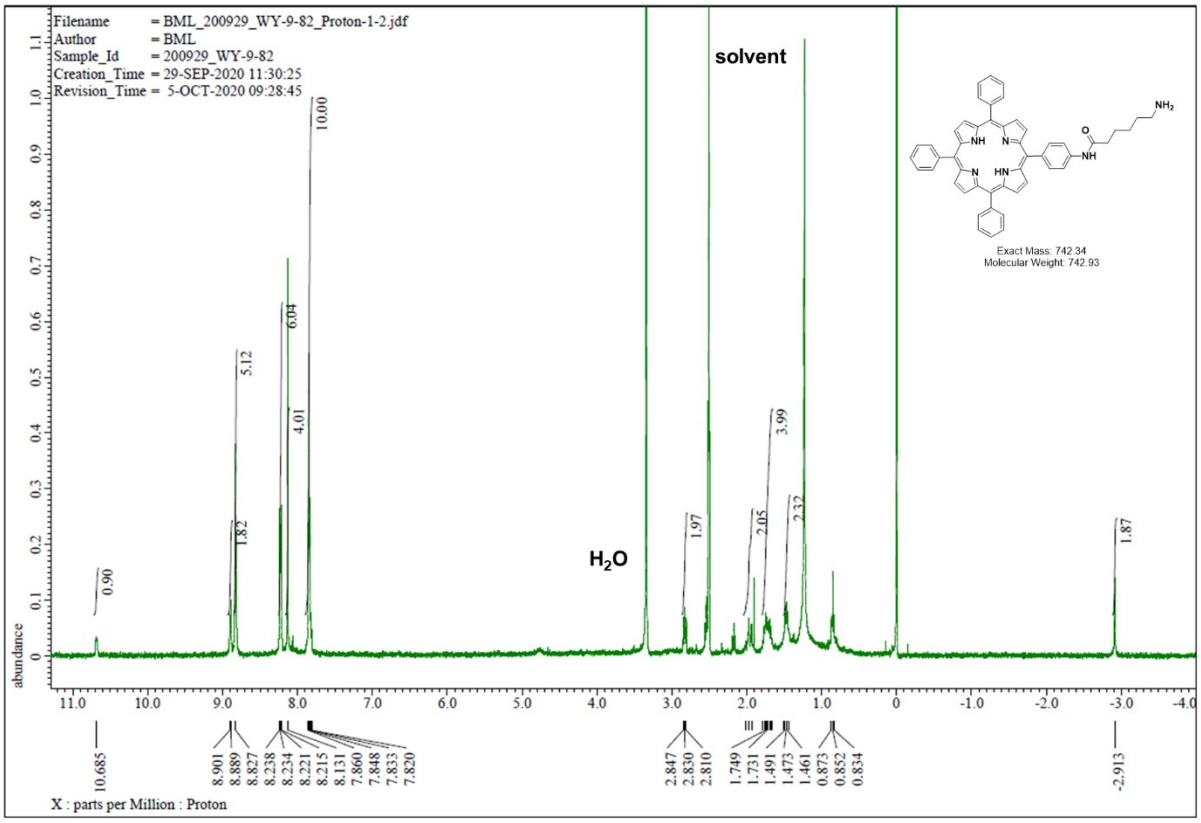

Figure S11. ^{1}H NMR (400 MHz, DMSO-d_6) spectrum of TPP-ahx-NH_2.

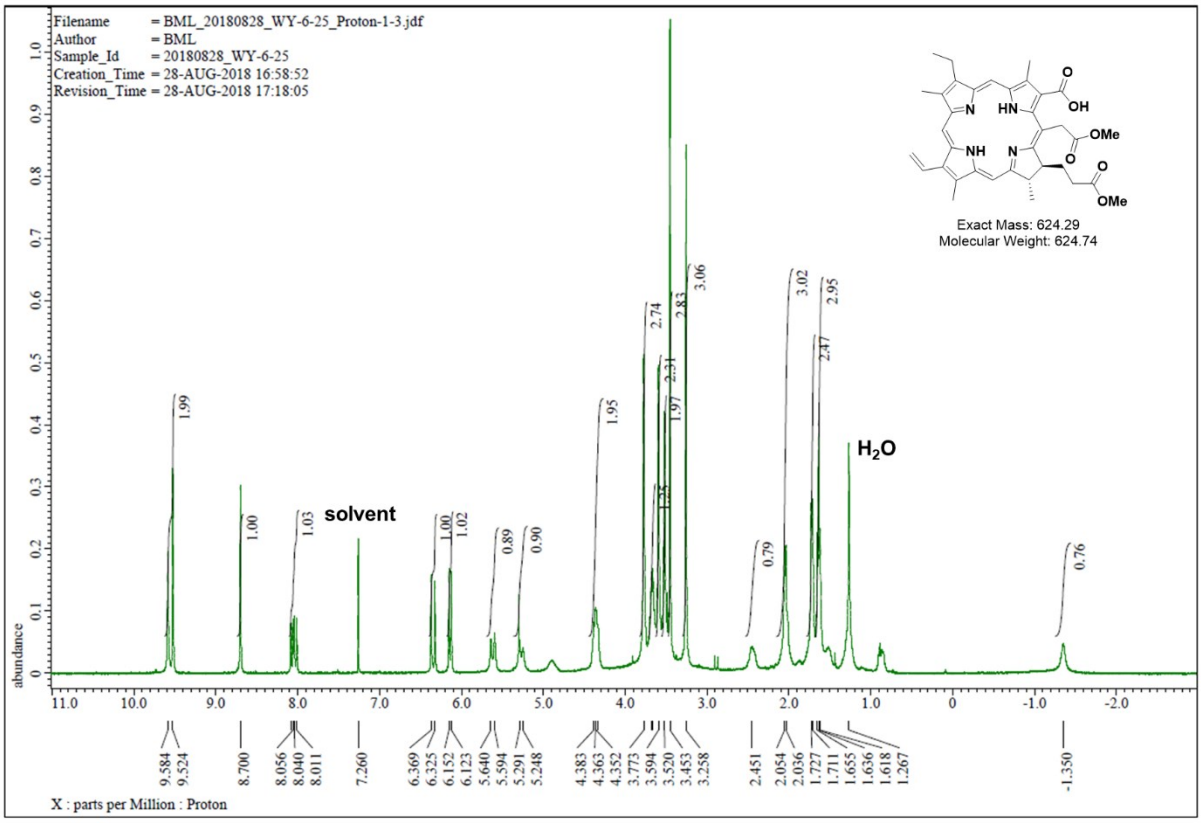

Figure S12. ^{1}H NMR (400 MHz, $CDCl_3$) spectrum of Ce6-DME.

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