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Syntheses and PDT activity of new mono- and di-conjugated derivatives of chlorin e_6

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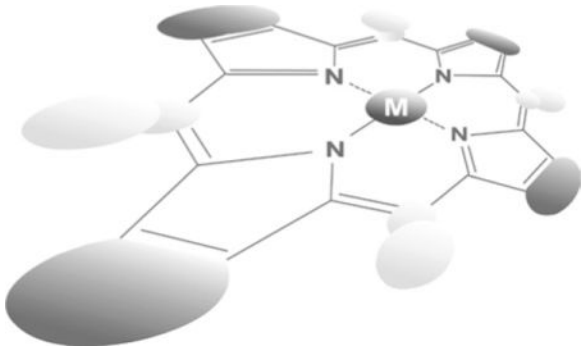
Abstract

Syntheses of three new chlorin e_6 conjugates for PDT of tumors are reported. One of the new compounds **17** is conjugated with lysine at the 13^1-position, but the others are mono-conjugated **14** or diconjugated **15** with the non-amino acid species ethanolamine. Cellular experiments with the three new compounds and previously synthesized non-amino acid 15^2-conjugates (**7**–**10**), 13^1-monoconjugates **14**, **16**, and a 13^1,15^2-diconjugate **12** are reported. *In vitro* cytotoxicity experiments show that the 13^1-conjugates are more toxic than the 15^2-conjugates, and the most toxic derivative (dark- and photo-toxicity) is the 13^1-ethylenediamine conjugate **11**. The most useful PDT photosentitizers appear to be the ethanolamine derivatives, conjugated at the 15^2- and the 13^1,15^2-positions; these show high phototoxicity but relatively low dark toxicity compared with **11**, and also the highest dark/photo cytotoxicity ratios.

Graphical abstract



Keywords

photodynamic therapy; chlorin e_6; syntheses; ethanolamine; ethylene diamine; *in vitro* cellular studies

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Dedicated to Professor Claudio Ercolani on the occasion of his 80th birthday

Supporting information

^{1}H and ^{13}C NMR spectra for compounds **12**, **14**, **17**–**19** (Figs S1–S7), dark- and photo-toxicity for compounds **1**, **7**–**10**, **14**, **15** and **17** (Figs S8–S12), and cellular uptake and subcellular localization details for compounds **1** and **17** (Figs S13–S15, Table S1), are given in the supplementary material. This material is available free of charge *via* the Internet at http://www.worldscinet.com/jpp/jpp.shtml.

INTRODUCTION

Photodynamic therapy (PDT) depends upon the selective uptake of a photosensitizer into tumor tissues, followed by production of singlet oxygen or other toxic species upon irradiation with light of a wavelength that can be absorbed by the sensitizer [1–3]. A first-generation FDA-approved sensitizer, Photofrin® (porfimer sodium), has been developed commercially and used in more than 40 countries. This photosensitizer suffers from issues associated with minimal absorption of light within the preferred "therapeutic window" (600–850 nm) and its prolonged retention in skin tissue, causing patient photosensitivity that can last for weeks [4]. New, so-called "second generation" photosensitizers, such as mono-(L)-aspartylchlorin e_6, (NPe6, Talaporfin, LS-11) have more recently been used in PDT clinical trials. NPe6 is prepared from the tricarboxylic acid chlorin e_6 (**1**) by treatment with aspartic acid salts in the presence of a base and a standard peptide coupling agent (*e.g.* DCC). NPe6 possesses a typical chlorin-type absorption spectrum with λ_{max} at about 660 nm; studies show that, upon irradiation, it can produce cytotoxic singlet oxygen in high yields. Compared with Photofrin, it also clears rapidly from normal tissues [4, 5].

Chlorin e_6 (**1**) (Chart 1) possesses three chemically distinct carboxylic acid functions that can be used for selective conjugation purposes. These are a formic carboxylate substituent at position-13, an acetic carboxylate at position-15, and a longer chain propionate substituent at position-17. The NPe6 patent identifies this amino acid conjugate as the 17^3-aspartyl derivative of chlorin e_6 (**2**) [6]. In 1998 a study primarily involving 2D NMR spectroscopy claimed that NPe6 is in fact the 15^2-regioisomer **3** of chlorin e_6 (**1**) [7]. This revelation was largely ignored, possibly because the 15-acetic side chain appeared to be of reduced reactivity toward amidation compared with the unhindered and more aliphatic propionic side chain at position-17. Conjugations with chlorin e_6 continued to be reported as taking place at the 17-position. At this point, our group reported the unambiguous syntheses of the three regioisomers **2**–**4** of NPe6, as their easily purified and fully characterized tetramethyl esters. This work showed conclusively that NPe6 is indeed the 15^2-regioisomer **3** [8], as proposed by Gomi *et al.* [7]. A variety of other conjugations were also shown to occur preferentially at the 15^2 position [9], and an X-ray structure of the tetramethyl ester prepared from authentic commercial NPe6, definitively placed the aspartic acid residue at the 15^2 position [8].

A hypothesis was advanced [8] that the 13^1: 15^2-anhydride **5** of chlorin e_6 was a key intermediate in the aspartic acid conjugation reaction, produced by a DCC-mediated dehydration reaction between the 13^1 and 15^2 carboxylic acids prior to involvement of the aspartic acid; subsequent reaction of the nucleophilic amino acid at the more electrophilic and reactive aliphatic 15^2-carbonyl rather than the aromatic 13^1-nuclear carbonyl of the anhydride affords the conjugate **3**. More recently, we have definitively identified the anhydride **5** as the critical intermediate that controls the regiochemistry of the overall reaction [12]; this was done by isolation and full characterization of the methyl ester **6** of anhydride **5**, as well as by reporting a single crystal X-ray structure of **6**.

The differential reactivity of the three carboxylate side chains in chlorin e_6 (**1**) was exploited by syntheses and cellular investigations of 17^3-, 15^2- and 13^1-conjugates with aspartic acid

and lysine [9]. The three different regioisomers were synthesized in good yields. Experiments using human carcinoma HEp2 cells showed that the 15^2-lysyl regioisomers accumulated best within cells, but the most phototoxic conjugates were the 13^1-conjugates. The main factor influencing the biological activity of any individual compound appeared to be the amino acid conjugation site, with the 17^3-conjugates (identified in the patent [6] as being the structure of, for example, NPe_6) being the least active sensitizers. These studies were followed up with new syntheses and cellular studies of di-aspartate and aspartate-lysine chlorin e_6 conjugates [13]. Regioselective synthetic procedures to four chlorin e_6 bis(amino acid) conjugates were developed for bis-conjugates bearing two aspartate residues in the 13^1,17^3- and 15^2,17^3-positions, as well as at the 13^1,15^2 sites connected *via* an ethylene diamine insert. A fourth conjugate featuring two different amino acids, namely lysine at 13^1 through an ethylene diamine linker and an aspartate residue at 15^2 through a β-alanine linker was also synthesized. The dark- and photo-cytotoxicity and uptake of these four bis(amino acid) chlorin e_6 conjugates as well as chlorin e_6 itself were measured using human HEp2 cells. The best conjugates were the 13^1,15^2-disubstituted conjugates, which were shown to be well taken up and which also localized in multiple organelles. In comparison, any bis-conjugate bearing an amino acid at position 17^3 (*i.e.* the 13^1,17^3-and 15^2,17^3-di-aspartyl conjugates) showed low dark cytoxicity but lower phototoxicity properties compared with chlorin e_6 itself. Indeed, all of the bis-conjugates synthesized were inferior as PDT sensitizers compared with the corresponding mono-conjugates [13].

Our attention therefore moved back toward mono-conjugates of chlorin e_6. In particular, four non-amino acid 15^2-conjugates (**7**–**10**) of chlorin e_6 were synthesized [12], as well as a bis-conjuate with ethylenediamine [13]. In the present paper we report the syntheses of three additional mono-conjugates and of one bis-conjugate (for comparison purposes), and also report the results of cellular studies designed to assess their potential as effective PDT agents.

RESULTS AND DISCUSSION

Selection and syntheses of non-amino acid monoconjugates

Four non-amino acid 15^2-mono-conjugated chlorin e_6 derivatives **7**–**10** [12], one 13^1-conjugate **11** and one 13^1,15^2-bis-conjugate **12** [9] that had previously been reported were selected for cellular investigations; these syntheses were accomplished using the anhydride **6** as an intermediate for **7**–**10**, or from methyl pheophorbide a (**13**) for **11** and **12** (Scheme 2). Initial *in vitro* biological evaluations of these compounds indicated that the 15^2-ethanolamine conjugate **10** was particularly promising, and based on our earlier conclusions that 13^1-conjugates are significantly more PDT-active than the 15^2-conjugates, we also proceded to synthesize the analogous 13^1-ethanolamine conjugate **14**. This compound, **14**, was obtained in 48% yield by ring-opening of methyl pheophorbide a (**13**) with 18 equivalents of ethanolamine in anhydrous ethyl acetate at room temperature for 24 h (Scheme 2).

Finally, for completeness and to correlate results with the bis-conjugated derivatives reported earlier, the 13^1,15^2-bis-ethanolamine conjugate **15** was also synthesized (Scheme 3). This bis-conjugate was synthesized by firstly treating methyl pheophorbide a (**13**) with 1.5

equivalents of ethanolamine in refluxing toluene, until TLC indicated disappearance of the starting material, to give *N*-(2-hydroxyethyl)-acetamide pheophorbide a (**16**) in 82% yield. Then excess ethanolamine (5.0 equivalents) was added with stirring for 19 h at 40 °C to accomplish cleavage of the isocyclic ring and provide the 13^1,15^2-(2-hydroxylethyl)amide-17^3-methyl ester chlorin e_6 (**15**) in 47% yield (Scheme 3).

Synthesis of 13^1-lysylchlorin e_6 trimethyl ester (17)

Our previous monoconjugation syntheses and cellular experiments [9] did not include a lysyl mono-conjugate **17** in which the lysyl residue was directly attached to the chlorin e_6 core; in the previously reported 13^1-lysyl conjugate [9] the lysine was attached *via* an ethylene diamine spacer. An attempt was made to synthesize the proposed chlorin e_6 derivative using the one-step nucleophilic attack of either of the lysine differentially mono-protected nitrogens upon the 13^1-carbonyl of methyl pheophorbide a [14]. This procedure failed to yield a useful product under a variety of solvent conditions. We therefore turned our attention to a route (Scheme 4) involving amino acid coupling to the 13^1-carboxylic acid function in **18**. The dimethyl ester **18** was prepared by treatment of chlorin e_6 (**1**) with methanol in sulfuric acid; under these acidic conditions [8] the imine nitrogens of chlorin e_6 (**1**) are protonated, thereby inactivating the electronically conjugated 13^1-carboxyl group toward protonation, a key step in the esterification process. A quantitative yield of **18** was obtained. Treatment of **18** with Boc-protected lysine methyl ester hydrochloride in presence of HOBt and TBTU [9] gave 13^1-mono-(Boc)lysyl-chlorin e_6 trimethyl ester (**19**), and this was deprotected with TFA to give 13^1-mono-lysylchlorin e_6 trimethyl ester (**17**), in a yield of 33% from chlorin e_6 (**1**).

Cellular studies

Cytotoxicity—The dark and phototoxicity (~1.5 J/cm^2 light dose) of chlorin e_6 (**1**) and its derivatives were evaluated in human carcinoma HEp2 cells using a CellTiter Blue assay, up to 100 μM concentrations. The results obtained are shown in Table 1 and Figs S1 and S2 of the Supporting information. It is clear from our results that the 15^2-substituted conjugates have much lower dark cytotoxicity (IC_{50} > 100 μM) compared with the conjugates bearing a substituent on the 13^1-position. The 13^1-ethylenediamine, 13^1-ethanolamine, and the 13^1-lysine conjugates **11**, **14** and **17**, showed the highest dark cytotoxicity, with calculated IC_{50} values of 7, 36 and 34 μM, respectively (Table 1). The 13^1,15^2-disubstituted conjugates **12** and **15** were also found to be moderately cytotoxic with IC_{50} values of 45 and 59 μM, respectively. Upon exposure to a low light dose (~1.5 J/cm^2) the most phototoxic dyes were generally again the 13^1-substituted conjugates, particularly the cationic 13^1-ethylenediamine-chlorin e_6 **11** with a calculated IC_{50} value of 0.23 μM, followed by the 13^1-ethanolamine, 13^1-lysine, and 13^1,15^2-bis-ethanolamine conjugates **14**, **15**, and **17**. For example, the substitution at the 15^2-position *vs.* the 13^1-position with an ethanolamine group, as in **10** and **14**, resulted in a 2–3 fold decrease in both dark and phototoxicity. On the other hand, the introduction of hydrophobic groups at the 15^2-position of chlorin e_6 further lowers the phototoxicity, particularly for the 15^2-benzyl derivative **7**, which showed the lowest phototoxicity of all compounds in this series, even lower than that for chlorin e_6 (**1**). These results are in agreement with our previous observations on the dark/photo cytotoxicity

of chlorin e_6 amino acid derivatives, showing that the 13^1-substituted conjugates are in general the most cytotoxic [13]. This could in part be due to the preferentially linear molecular conformations of the 13^1-derivatives, which might facilitate interactions and binding to biological substrates that enhance their cytotoxicity. On the other hand, the cationic derivatives **11**, **12** and **17** all showed high cytotoxicity; for example, the substitution with an ethylenediamine group at the 13^1-position (as in **11**) with ethanolamine (as in **14**) also resulted in a 2–5 fold decrease in both dark and phototoxicity. The combination of high dark/photo cytotoxicity ratio, resulting from relatively low dark cytotoxicity but potent phototoxicity, observed for the 15^2-ethanolamine and the 13^1,15^2-bis-ethanolamine conjugates **10** and **15** makes them the most promising compounds for PDT among this series. Although the 13^1-substituted derivatives **11**, **14** and **17** show high phototoxicity, their high dark cytotoxicies decrease their usefulness as PDT agents (see SI).

Time-dependent cellular uptake—The results obtained for the time-dependent uptake of chlorin e_6 **1** and six of its derivatives, including the most promising photosensitizers **10** and **15**, at a concentration of 10 μM in human HEp2 cells are shown in Fig. 1. All derivatives accumulated within cells to a larger extent than chlorin e_6, (**1**), at all time points investigated. The ethanolamine substituted derivatives **10**, **14** and **15** accumulated the fastest in the first 1–2 h, after which a plateau was reached and slower uptake was observed at times > 4 h, particularly for **14** and **15**. At 24 h the propyl derivative **8** showed the highest cellular uptake, about 20-fold that of chlorin e_6, followed by **9** and **10** (1- and 14-fold, respectively), indicating that these 15^2-substituted derivatives are efficiently taken up by cells. The 13^1-ethanolamine and 13^1,15^2-bis-ethanolamine derivatives **14** and **15** accumulated rapidly at short times and at 24 h showed about 12- and 8-fold higher uptake than chlorin e_6. These results indicate that the mono-substituted chlorin e_6 derivatives are more efficiently taken up by cells compared with the di-substituted derivatives. The benzyl ester derivative **7** showed the lowest uptake at 24 h, although still about 4-fold higher than for chlorin e_6.

Figure 2 shows the fluorescence of select chlorin e_6 derivatives in HEp2 cells after 6 h incubation. All compounds efficiently accumulated within the cells in multiple organelles, including the lysosomes and the Golgi apparatus, as previously observed for amino acid derivatives of chlorin e_6 [13]. In addition, the 13^1-substituted compounds **11** and **12** appear to localize to a small extent in cell mitochondria, and this might be one of the causes of the observed higher dark and phototoxicities for these derivatives.

EXPERIMENTAL

General

All air and moisture sensitive reactions were performed in dried and distilled solvents under an argon atmosphere. All solvents and reagents were purchased from commercial sources, unless otherwise stated. Silica gel 60 (230 × 400 mesh, Sorbent Technologies) was used for column chromatography. Analytical thin-layer chromatography (TLC) was carried out using polyester backed TLC plates 254 (precoated, 200 μm) from Sorbent Technologies. NMR spectra were recorded on an AV-400 spectrometer (400 MHz for ^{1}H, 100 MHz for ^{13}C). The chemical shifts are reported in δ ppm using the following partially deuterated solvents as

internal references: CD_2Cl_2 5.32 ppm (^{1}H), 54 ppm (^{13}C); *d*-DMSO 2.49 ppm (^{1}H), 39.5 ppm (^{13}C); *d*-CH_3OH 4.78 ppm (^{1}H), 49.0 ppm (^{13}C); $CDCl_3$ 7.26 ppm (^{1}H), 77.16 ppm (^{13}C); $(CH_3)_2CO$ 2.50 ppm (^{1}H), 29.84 ppm (^{13}C). Electronic absorption spectra were measured on an Agilent 8453 UV-vis spectrophotometer. Mass spectra were obtained on a Bruker Omniflex MALDI Time-of-Flight Mass Spectrometer. All compounds synthesized were purified and isolated in ≥95% purity, as evidenced by analytical TLC in at least two solvent systems, and confirmed by the absence of extraneous tetrapyrrole resonances in ^{1}H- and ^{13}C-NMR spectra. Conjugates **7**–**10** [12], **11** [9] and **12** [13] were prepared according to appropriate literature procedures and were authenticated by comparison with the literature data.

Synthetic procedures

13^1-(2-Hydroxyethyl)amide-15^2,17^3-dimethyl ester chlorin e$_6$ (14)—Methyl pheophorbide a (**13**) (50 mg, 0.082 mmol) was dissolved in dry EtOAc (3 mL) and stirred under argon for 10 min. Then 0.091 mL of ethanolamine (0.091 mL, 1.510 mmol, 25.0 equiv.) was added to the solution and the mixture was stirred for 24 h. The reaction was monitored by UV-vis spectrophotometry. The mixture was evaporated and the crude product was chromatographed on a silica gel column eluted with 50% acetone/CH_2Cl_2 to afford 26.6 mg, 0.040 mmol, 48% yield of 13^1-(2-hydroxyethyl)amide-15^2,17^3-dimethyl ester chlorin e$_6$ (**14**) ($C_{38}H_{45}N_5O_6$); mp 110–115 °C. UV-vis (MeOH): λ_{max}, nm (ε) 399 (95,400), 499 (10,600), 526 (3,900), 556 (2,800), 607 (4,900), 661 (34,500). ^{1}H NMR [$(CD_3)_2CO$, 400 MHz]: δ, ppm 9.82 (s, 1H), 9.77 (s, 1H), 9.12 (s, 1H), 8.26 (dd, *J* = 17.80, 11.52 Hz, 1H), 8.10 (br s, 1H), 6.43 (d, *J* = 17.84 Hz, 1H), 6.16 (d, *J* = 11.60 Hz, 1H), 5.67 (d, *J* = 19.16 Hz, 1H), 5.39 (d, *J* = 19.16 Hz, 1H), 4.67 (q, *J* = 7.12 Hz, 1H), 4.52 (d, *J* = 9.52, 1H), 4.09 (m, 1H), 4.02 (t, *J* = 5.72 Hz, 2H), 3.94 (m, 1H), 3.75 (s, 3H), 3.60 (s, 3H), 3.58 (s, 3H), 3.54 (s, 3H), 3.33 (s, 3H), 2.70 (m, 1H), 2.31 (m, 2H), 1.80 (m, 1H), 1.72 (d, *J* = 6.96, 3H), 1.71 (t, *J* = 6.56, 3H), 1.60 (s, 1H), 1.29 (s, 1H), −1.58 (br s, 1H), −1.89 (br s, 1H). ^{13}C NMR [$(CD_3)_2CO$, 100 MHz]: δ, ppm 174.0, 170.2, 169.9, 168.7, 154.6, 150.0, 145.4, 139.3, 137.0, 136.4, 135.8, 135.2, 135.0, 130.9, 130.8, 130.3, 121.9, 104.1, 101.7, 99.4, 94.8, 69.7, 61.9, 55.5, 54.1, 52.3, 51.7, 49.7, 44.1, 38.1, 23.5, 19.9, 18.1, 12.3, 11.9, 11.2. HRMS (MALDI-TOF): *m/z* 668.329 $[M + H]^+$, calcd. for $C_{38}H_{46}N_5O_6$ 668.345.

13^1,15^2-Bis(2-hydroxyethyl)amide-chlorin e$_6$ 17^3-methyl ester (15)—The *N*-(2-hydroxyethyl)acetamide pheophorbide a (**16**; 38.0 mg, 0.060 mmol, 1.0 equiv.) was dissolved in toluene (5 mL) and ethanolamine (0.018 mL, 0.299 mmol, 5.0 equiv.) was added. The reaction mixture was heated at 40 °C overnight. After the reaction was complete by TLC, the solvent was removed and the residue was dissolved in CH_2Cl_2 and washed with 5% aqueous citric acid to remove excess amine, followed by a wash with brine. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 50:50 acetone/CH_2Cl_2 and purified *via* silica gel column chromatography to afford 13^1,15^2-bis(2-hydroxyethyl)amide-chlorin e$_6$ 17^3-methyl ester (**15**, $C_{39}H_{41}N_6O_6$, 19.7 mg, 0.028 μMol, 47%); mp 135–140 °C. UV-vis (MeOH): λ_{max}, nm (e) 399 (97,900), 499 (8,800), 527 (2,000), 556 (600), 608 (2,500), 661 (33,700). ^{1}H NMR (acetone-d_6, 400 MHz): δ, ppm 9.79 (s, 1H), 9.76 (s, 1H), 9.12 (s, 1H), 8.37 (br. s, 1H), 8.25 (dd, *J* = 17.8, 11.6 Hz, 1H), 6.97 (br. s, 1H), 6.42 (d, *J* = 17.8, 1H), 6.15 (d, *J* = 11.5 Hz, 1H), 5.55 (d, *J* =

18.5 Hz, 1H), 5.19 (d, *J* = 17.9 Hz, 1H), 4.69–4.58 (m, 2H), 4.15 (s, 1H), 4.02 (br s, 2H), 3.95–3.88 (m, 1H), 3.84–3.76 (m, 4H), 3.31 (s, 3H), 2.81 (s, 3H), 2.72–2.65 (m, 1H), 2.60 (s, 3H), 2.37–2.27 (m, 2H), 1.74 (d, *J* = 7.2 Hz, 3H), 1.70 (t, *J* = 7.5 Hz, 3H), −1.61 (s, 1H), −1.96 (s, 1H). ^{13}C NMR [$(CD_3)_2CO$, 100 MHz]: δ, ppm 174.0, 170.3, 170.1, 168.9, 154.7, 150.0, 145.5, 139.3, 137.0, 136.2, 136.0, 135.2, 135.0, 131.0, 130.9, 130.3, 122.0, 104.2, 101.7, 99.4, 94.9, 69.7, 61.7, 61.3, 55.5, 54.0, 51.7, 44.3, 43.2, 40.6, 23.3, 19.9, 18.1, 12.3, 11.9, 11.3. MS (MALDI-TOF): *m/z* 697.333 $[M + H]^+$, calcd. for $C_{39}H_{49}N_6O_6$ 697.364.

15^2-[*N*-(2-Hydroxyethyl)acetamide]-pheophorbide a (16)—Methyl pheophorbide a (**13**) (51.3 mg, 0.085 mmol, 1.0 equiv.) was dissolved in dry toluene (5 mL) and the mixture was heated to 100 °C under nitrogen. Then, ethanolamine (0.008 mL, 0.127 mmol, 1.5 equiv.) was added. The reaction mixture was allowed to stir at 100 °C for 19 h while monitoring by TLC. Then the solvent was removed and the residue was dissolved in CH_2Cl_2 and washed with 5% aqueous citric acid, followed by water and brine. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 50% acetone/CH_2Cl_2 and purified *via* silica gel column chromatography with the same mobile phase to afford 15^2-[*N*-(2-hydroxyethyl) acetamide]-pheophorbide a (**16**, $C_{37}H_{41}N_5O_5$, 44.0 mg, 0.069 mmol, 82%). UV-vis (MeOH): λ_{max}, nm (ε) 399 (79,800), 500 (5,700), 531 (900), 556 (500), 608 (1,400), 663 (27,400). ^{1}H NMR (acetone-d_6, 400 MHz): δ, ppm 9.71 (s, 1H), 9.44 (s, 1H), 8.90 (s, 1H), 8.16–7.95 (m, 2H), 6.34 (d, *J* = 17.8 Hz, 1H), 6.24 (s, 1H), 6.17 (d, *J* = 11.5, 1H), 4.64–4.59 (m, 1H), 4.44 (d, *J* = 9.3, 1H), 3.69–3.65 (m, 2H), 3.63 (s, 3H), 3.53 (s, 3H), 3.43 (d, *J* = 5.4 Hz, 3H), 3.18 (s, 3H), 2.72–2.64 (m, 2H), 2.42–2.37 (m, 1H), 2.27–2.20 (m, 1H), 1.83 (*d*, *J* = 7.3 Hz, 3H), 1.72–1.68 (m, 1H), 1.64 (t, *J* = 7.5 Hz, 3H), −1.60–1.86 (m, 2H). ^{13}C NMR [$(CD_3)_2CO$, 100 MHz]: δ, ppm 174.3, 173.3, 169.5, 163.4, 155.6, 151.5, 150.4, 145.6, 142.3, 138.6, 136.8, 136.6, 136.3, 132.6, 130.3, 129.9, 122.8, 107.8, 104.7, 97.6, 94.5, 66.9, 62.1, 55.6, 52.2, 51.8, 50.9, 43.6, 23.5, 19.5, 17.7, 12.1, 11.9, 10.9. MS (MALDI-TOF): *m/z* 636.239 $[M + H]^+$, calcd. for $C_{37}H_{42}N_5O_5$ 636.319.

13^1-monolysyl-chlorin e$_6$ trimethyl ester (17)—In a round bottom flask, chlorin e_6 dimethyl ester [8] (**18**, 102 mg, 0.165 mmol) was dissolved in dry CH_2Cl_2. A mixture of HOBt (24 mg, 0.18 mmol), TBTU (58 mg, 0.18 mmol), and DIEA (0.02 mL) in DMF was added to the mixture and allowed to stir for 30 min. H-Lys(Boc)-OMe hydrochloride (60 mg, 0.21 mmol) and DIEA (0.02 mL) were mixed in CH_2Cl_2 and added to the reaction mixture. The mixture was allowed to stir for approximately 48 h and monitored using TLC. Then, it was diluted with DCM, washed with 5.0% citric acid, followed by a wash with water then with brine. The mixture was then dried over Na_2SO_4 and evaporated. The residue was dissolved in 5.0% acetone/CH_2Cl_2 and purified *via* silica column chromatography with the same mobile phase, to give the Boc-protected derivative (**19**). ^{1}H NMR $(CD_3)_2CO$: δ, ppm 9.75 (s, 1H), 9.71 (s, 1H), 9.12 (s, 1H), 8.51 (d, 1H), 8.20 (dd, 1H), 6.38 (d, 1H), 6.11 (d, 1H), 6.00 (br s, 1H), 5.75 (d, 1H), 5.24 (d, 1H), 4.95 (q, 1H), 4.67 (q, 1H), 4.49 (d, 1H), 3.97 (s, 3H), 3.74 (s, 3H), 3.73 (q, 2H), 3.64 (s, 3H), 3.61 (s, 3H), 3.51 (s, 3H), 3.27 (s, 3H), 3.16 (q, 2H), 2.72 (m, 1H), 2.30 (m, 2H), 2.10 (m, 1H), 1.72 (d, 3H), 1.70 (m, 5H), 1.68 (t, 3H), 1.46 (m, 1H), 1.28 (s, 9H), −1.60 (s, 1H), −1.90 (s, 1H). In a round bottom flask, the Boc-protected chlorin e_6 derivative (**19**) was dissolved in 5.0 mL of dry DCM in an ice bath

under argon. Thioanisole (0.01 mL) and TFA (5.0 mL) were added and stirred overnight. Then, the reaction mixture was diluted with CH_2Cl_2 and washed with water and 10% $NaHCO_3$ then dried over Na_2SO_4. The solvent was removed through rotary evaporation. The residue was dissolved in 10% acetone/CH_2Cl_2 and purified *via* silica column chromatography with the same mobile phase. This produced the title compound (**17**) in overall 33% yield (43.5 mg, 0.057 mmol) starting from chlorin e_6 (**1**). ^{1}H NMR $(CD_3)_2CO$: d, ppm 9.75 (s, 1H), 9.70 (s, 1H), 9.11 (s, 1H), 8.60 (d, 1H), 8.19 (dd, 1H), 6.37 (d, 1H), 6.10 (d, 1H), 5.75 (d, 1H), 5.24 (d, 1H), 4.95 (br s, 1H), 4.67 (q, 1H), 4.49 (d, 1H), 3.96 (s, 3H), 3.74 (s, 3H), 3.73 (q, 2H), 3.63 (s, 3H), 3.61 (s, 3H), 3.50 (s, 3H), 3.26 (s, 3H), 2.76 (m, 3H), 2.38 (m, 4H), 1.75 (m, 5H) 1.72 (d, 3H), 1.68 (t, 3H), 1.47 (m, 1H), 1.28 (m, 1H) −1.60 (s, 1H), −1.90 (s, 1H). MS (MALDI-TOF): *m/z* 767.437 $[M + H]^+$, calcd. for $C_{43}H_{55}N_6O_7$ 767.414.

Cell studies

The HEp2 cell line used in this study was purchased from ATCC. The cells were cultured in the medium (DMEM: AMEM = 1:1) containing 10% FBS and 1% antibiotic (Penicillin-streptomycin), and sub-cultured twice weekly to maintain sub-confluent stocks. All reagents and culture medium were purchased from Life Technologies. A 32 mM compound stock solution was prepared by dissolving the compound in DMSO (Sigma-Aldrich). All compound working solutions were prepared by diluting the 32 mM stock solution with culture medium.

Dark toxicity—HEp2 cells were plated in a Costar 96-well plate (BD biosciences) 15000 cells per well and grown until the cell was getting 100% confluence. Subsequently, the cell was treated with the compound concentration of 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0 μM, five repetitions for each concentration, and then incubated overnight at 37 °C. After 20–24 h incubation, the compound was removed by washing cells with 1X PBS and replaced with the media containing 20% cell titer blue. The cells were incubated for an additional 4 h at 37 °C. The viable cells is measured fluorescently at 570/615 nm using a FluoStar Optima micro-plate reader (BMG LABTECH). The dark toxicity was expressed in terms of the percentage of viable cells.

Phototoxicity—HEp2 cells were placed in a 96-well plate as above. Whenever the cell get 100% confluence it was treated with compound at the concentration range of 20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, and 0 μM for 24 h at 37 °C. After 24 h treatment, the loading media was removed. The cells were washed with media, and then refill fresh media. The cells were exposed to a 600 W halogen lamp light source filtered with a water filter and a beam turning mirror 200 nm–3 μM (Newport) for 20 min. The total light dose was approximately 1.5 J/cm^2. After exposed to light, the cell was returned back to incubator for another 24 h. The medium was removed and replaced with the media containing 20% cell titer blue. The cells were incubated for an additional 4 h. The viable cells is measured fluorescently at 570/615 nm using a FluoStar Optima micro-plate reader. As for the dark toxicity, the phototoxicity was expressed in terms of the percentage of viable cells.

Time-dependent cellular uptake—As described before, HEp2 cells were placed in 96 well plates, and grown for overnight, and then treated by adding 10 μM working solution 100 μL/well at different time periods of 0, 1, 2, 4, 8, and 24 h. The loading medium was removed at the end of the treatments. The cells were washed with 1X PBS, and solubilized by adding 0.25% Triton X-100 in 1X PBS. A compound standard curve, 10 μM, 5 μM, 2.5 μM, 1.25 μM, 0.625 μM, 0.3125 μM, was made by diluting 400 μM compound solution with 0.25% Triton X-100 (Sigma-Aldrich) in 1X PBS. A cell standard curve was prepared using 10000, 20000, 40000, 60000, 80000, and 100000 cells per well. The cells were quantified by CyQuant Cell Proliferation Assay (Life Technologies). The compound and cell number were determined using a FluoStar Optima micro-plate reader with wavelengths 680/700 nm for compounds and 485/520 nm for cells, respectively. Cellular uptake was expressed in terms of nM compound per cell.

Microscopy—The subcellular fluorescence experiments were performed by culturing the cells in a 6-well plate (Celltreat) with 10 μM concentration compound solution at 37 °C for 6 h. Afterwards, the medium was removed and cells were washed with 1X PBS three times. Images were acquired using a Leica DMRXA2 microscope with a water immersion objective and DAPI, GFP, and TRITC filter cubes (Chroma Technologies).

CONCLUSION

A series of chlorin e_6 conjugates bearing one or two substituents on the 13^1 and/or 15^2 positions were synthesized and investigated for their cellular uptake, intracellular localization and toxicity using human HEp2 cells. All compounds efficiently accumulated within cells to a larger extent than chlorin e_6, particularly the 15^2-substituted derivatives, about 4–20 fold higher than chlorin e_6 at 24 h. The *in vitro* cytotoxicity experiments reveal that the 13^1-conjugates are much more toxic than the 15^2-conjugates, with the most toxic derivative being the 13^1-ethylenediamine conjugate **11**. The most useful PDT photosentitizers appear to be the ethanolamine derivatives, **14** and **15**, which are conjugated at the 15^2-and the 13^1,15^2-positions due to their high phototoxicity but low dark toxicity compared with **11**.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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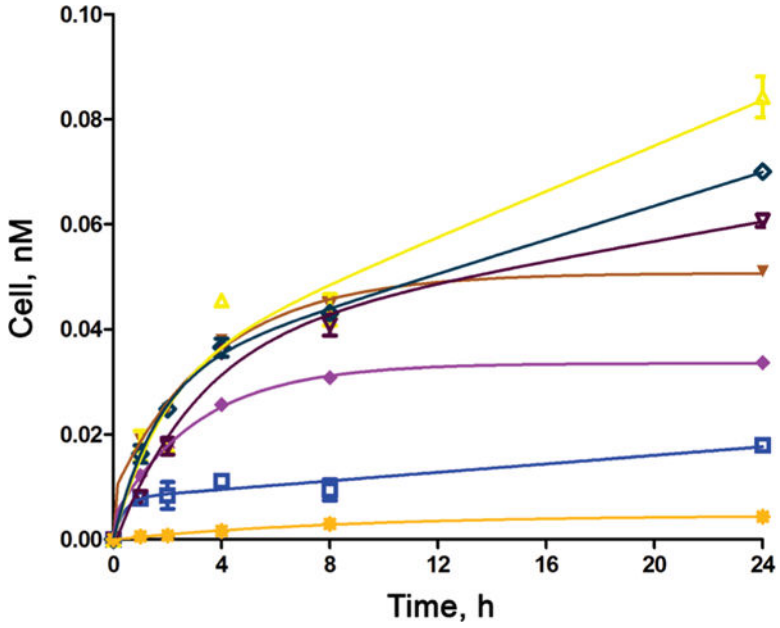


Fig. 1.

Time-dependent uptake of chlorin e_6 (**1**, orange) and its derivatives **7** (blue), **8** (yellow), **9** (red), **10** (green), **14** (brown) and **15** (purple) at 10 μM by HEp2 cells

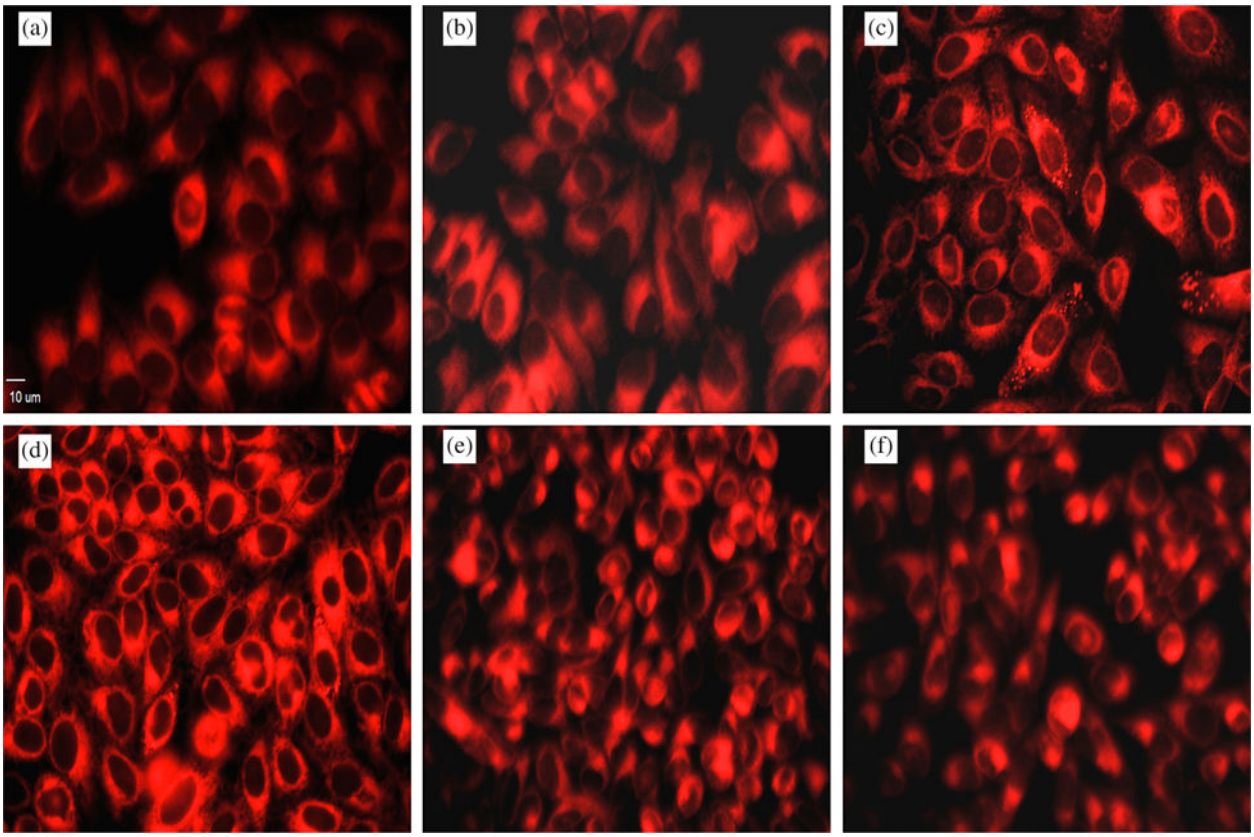
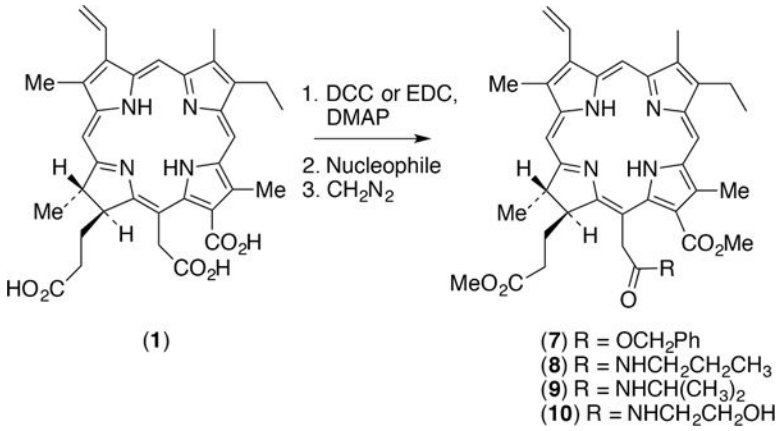


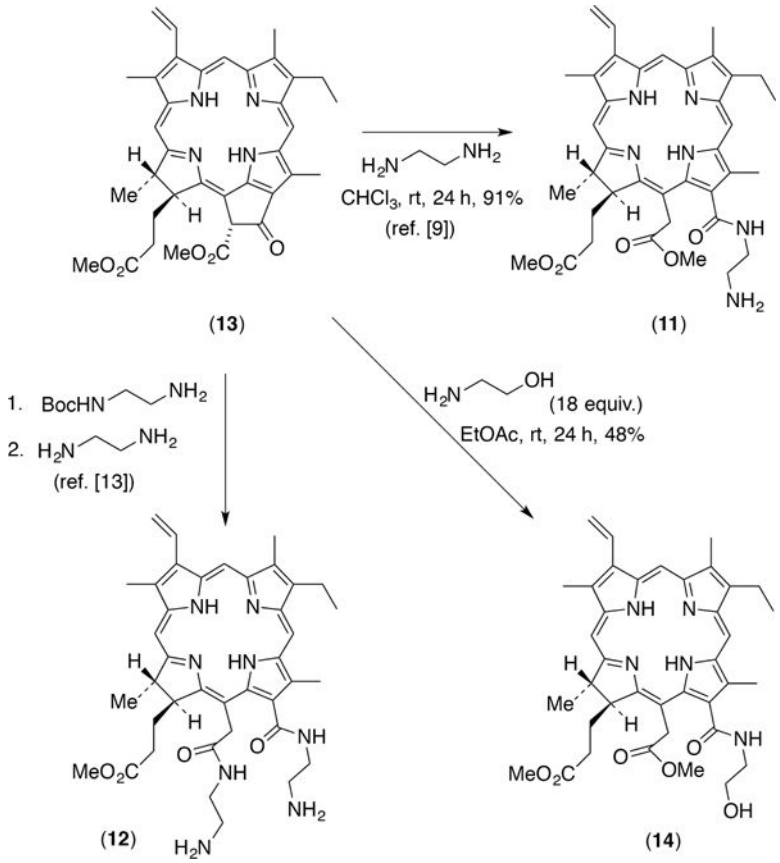
Fig. 2.

The fluorescence of select chlorin e_6 derivatives in HEp2 cells at 10 μM for 6 h; (a) 15^2-(Bz)Ce_6 DME (**7**), (b) 15^2-(^{n}Pr)Ce_6 DME (**8**), (c) 15^2-(^{i}Pr)Ce_6 DME (**9**), (d) 15^2-(EA)Ce_6 DME (**10**), (e) 13^1-(EDA)Ce_6 DME (**11**), and (f) 13^1,15^2-di(EDA)Ce_6 MME (**12**). Scale bar: 10 μM



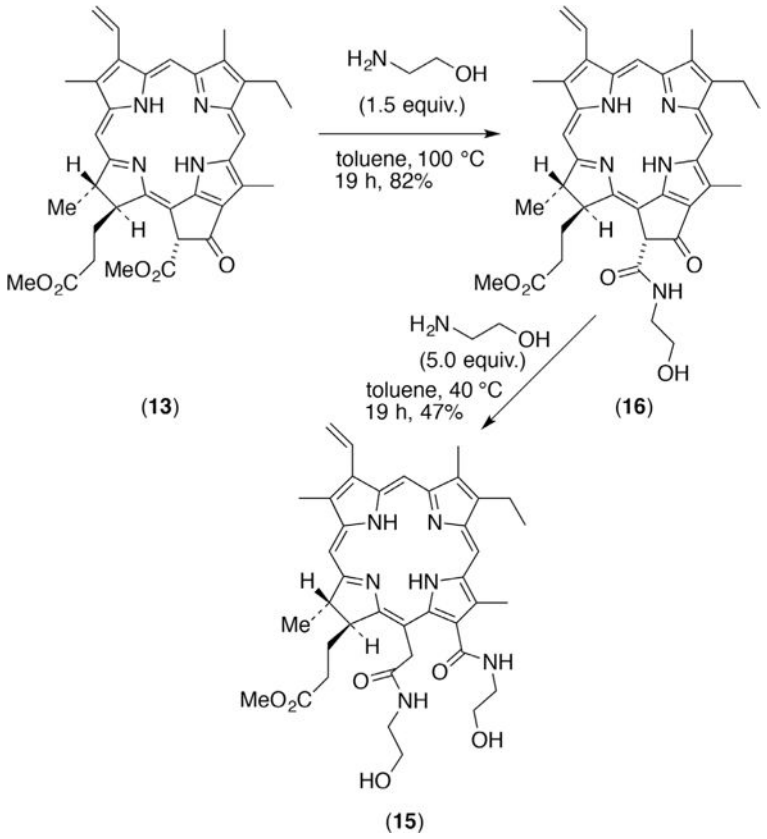
Scheme 1.

Syntheses of the 15^2-monoconjugates **7**–**10** from chlorin e_6 (**1**) *via* the anhydride **5**



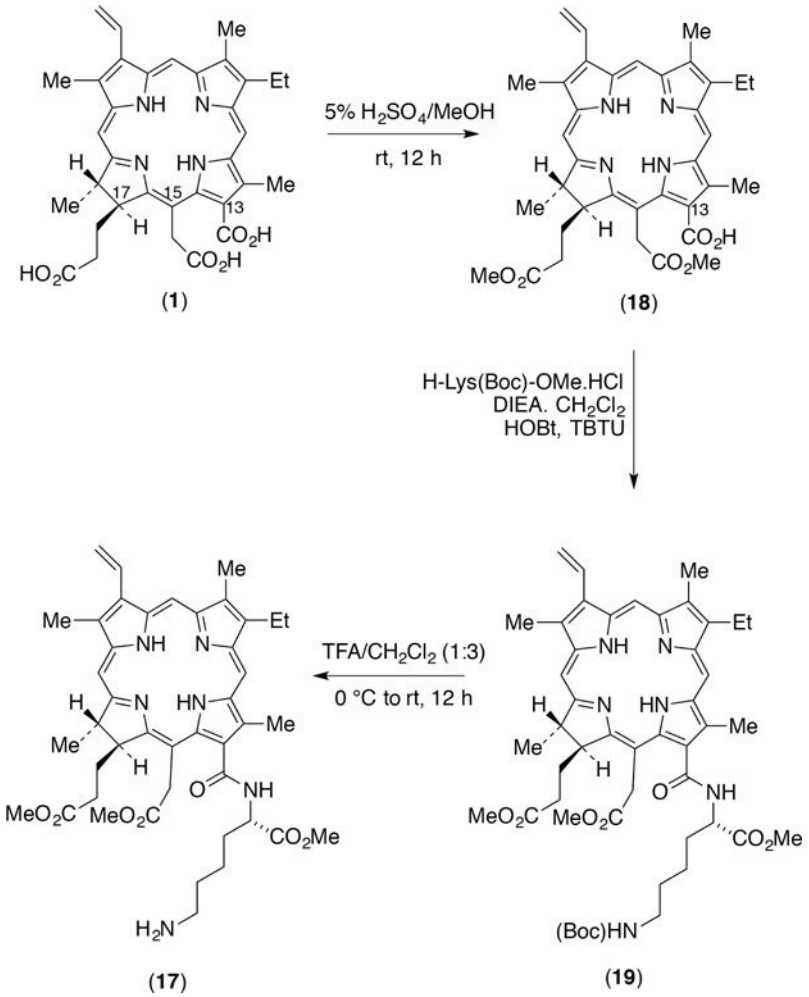
Scheme 2.

Synthesis of the 13^1-ethylenediamine- **11** [9], 13^1,15^2-ethylenediamine **12** [12] and 13^1-ethanolamine-conjugated ester derivatives **14** from methyl pheophorbide a (**13**)



Scheme 3.

Synthesis of 13^1,15^2-bis-ethanolamine conjugate (**15**) from methyl pheophorbide a (**13**)



Scheme 4.

Synthesis of 13^1-lysylchlorin e_6 trimethyl ester (**17**) from chlorin e_6 (**1**)

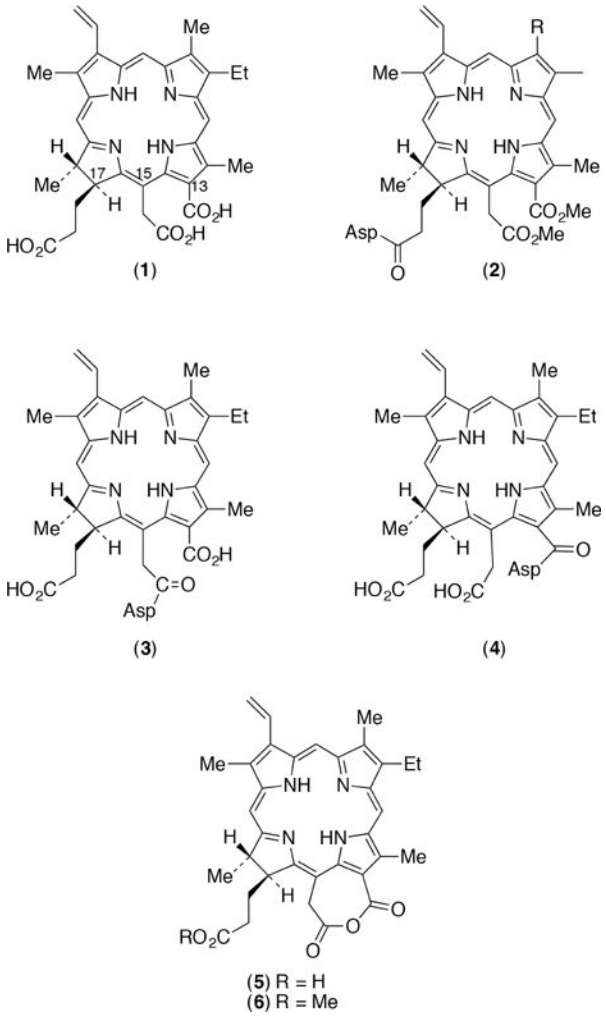


Chart 1.
Structures of key chlorin e_6 compounds

Table 1

Dark and phototoxicity (~1.5 J/cm^2) for chlorin e_6 and its derivatives in human HEp2 cells, using a Cell Titer Blue assay

Compound	Dark toxicity (IC_{50}, μM)	Phototoxicity (IC_{50}, μM)	Ratio
Chlorin e_6 (**1**)	>100	12.9	>8
15^2-(Bz)Ce_6 DME (**7**)	>100	>50	>2
15^2-(^{n}Pr)Ce_6 DME (**8**)	>100	1.54	>65
15^2-(^{i}Pr)Ce_6 DME (**9**)	>100	1.31	>76
15^2-(EA)Ce_6 DME (**10**)	>100	1.1	>91
13^1-(EDA)Ce_6 DME (**11**)	7	0.23	30
13^1,15^2-di(EDA)Ce_6 MME (**12**)	45	1.23	37
13^1-(EA)Ce_6 DME (**14**)	36	0.46	78
13^1,15^2-di(EA)Ce_6 DME (**15**)	59	0.31	190
13^1-LysCe_6 TME (**17**)	34	0.62	55