Published in final edited form as:

J Med Chem. 2011 November 10; 54(21): 7464–7476. doi:10.1021/jm2005139.

Syntheses and Cellular Investigations of 17³-, 15²- and 13¹-Amino Acid Derivatives of Chlorin e₆¹

R. G. Waruna Jinadasa, Xiaoke Hu, M. Graça H. Vicente, and Kevin M. Smith*

Louisiana State University, Department of Chemistry, Baton Rouge LA, 70803, USA.

Abstract

A series of amino acid conjugates of chlorin e₆, containing lysine or aspartic acid residues in positions 17³, 15² or 13¹ of the macrocycle were synthesized and investigated as photosensitizers for photodynamic therapy of tumors. All three regioisomers were synthesized in good yields and in 5 steps or less from pheophytin *a* (**1**). In vitro investigations using human carcinoma HEP2 cells show that the 15²-lysyl regioisomers accumulate the most within cells, and the most phototoxic are the 13¹ regioisomers. The main determinant of biological efficacy appears to be the conjugation site, probably because of molecular conformation. Molecular modeling investigations reveal that the 17³-substituted chlorin e₆ conjugates are L-shaped, the 15² and 13¹ regioisomers assume extended conformations, and the 13¹ derivatives are nearly linear. It is hypothesized that the 13¹-aspartylchlorin e₆ conjugate may be a more efficient photosensitizer for PDT than the commercial currently used 15² derivative.

Introduction

Photodynamic therapy (PDT) is a binary cancer therapy that relies on the selective uptake of a photosensitizer into cancer cells followed by irradiation with red light, which produces singlet oxygen and other reactive oxygen species.^{1–4} Highly cytotoxic singlet oxygen readily reacts with electron-rich biomolecules in its vicinity, such as unsaturated lipids, amino acids and DNA.⁵ Due to the known limited diffusion of singlet oxygen through tissues the PDT effects are largely localized to the photosensitizer-containing cells, thus reducing potential damage to normal cells in the vicinity of the tumor. The selectivity of the PDT treatment depends both on the tumor-targeting ability of the photosensitizer and the light used to activate it. An ideal photosensitizer should have minimal toxicity in the dark, a high quantum yield of triplet state formation in the presence of light, high selectivity for tumor cells over normal cells, rapid clearance from normal tissues, and a strong absorption peak within the “therapeutic window” (600–800 nm) for optimal light penetration through tissue.

Commercial hematoporphyrin derivative is a porphyrin-based photosensitizer that has been commercially developed and approved in several countries for the PDT treatment of melanoma, early and advanced stage cancer of the lung, digestive tract, genitourinary tract

¹Abbreviations: PS, photosensitizer; PDT, photodynamic therapy; DMF, dimethylformamide; DMSO, dimethylsulfoxide; THF, tetrahydrofuran; DME, dimethyl ester; TME, trimethyl ester; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, 1,3-dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; DIEA, *N,N*-diisopropylethylamine; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; DMAP, 4-dimethylaminopyridine; HOBt, 1-hydroxybenzotriazole; PBS, phosphate buffered saline; FBS, fetal bovine serum; MEM, modified eagle medium; ER, endoplasmic reticulum.

*To whom correspondence should be addressed. Phone: 225-578-7442. Fax: 225-578-3458. kmsmith@lsu.edu.

Supporting Information Available: Normalized absorption spectra, energy minimized conformations in water, time-dependent uptake, and subcellular localization microscopy figures (S1–S10). Proton NMR spectra for compounds **2–13** and other synthetic intermediates, carbon-13 NMR shifts, carbon-13 NMR spectra (21 pages). This material is available free of charge via the internet at <http://pubs.acs.org>.

and Barret's esophagus.³⁻⁶ However, it has only a weak absorption within the therapeutic window and often induces skin photosensitivity in patients. For these reasons, several second generation photosensitizers bearing stronger and red-shifted absorption within the therapeutic window have been investigated in PDT; these include tetra(meta-hydroxyphenyl)chlorin, (mTHPC), HPPH 2-[1-hexyloxyethyl]-2-devinylpyropheophorbide a (HPPH), lutetium(III) texaphyrin (LuTex), mono-L-aspartylchlorin e₆, and phthalocyanine 4 (Pc4).³⁻⁶ Chlorins (dihydroporphyrins) are particularly promising photosensitizers for PDT because of their intense absorptions above 640 nm; in particular chlorophyll a derivatives are inherently amphiphilic macrocycles that have been extensively investigated and shown to have low dark toxicities while being able to effectively generate singlet oxygen upon light activation.⁷⁻⁹ Among these, HPPH and mono-L-aspartylchlorin e₆ have been extensively investigated and are currently in advanced-stage clinical trials for oncologic PDT applications;¹⁰⁻¹³ both of these chlorophyll a based compounds have shown superior PDT activity as well as rapid clearance from normal tissue and decreased patient photosensitivity compared with commercial hematoporphyrin derivative.

Chlorophyll a derivatives of the chlorin e₆ series possess three carboxylic side chains that can be derivatized in multiple ways to produce novel amphiphilic photosensitizers for PDT investigations. For example, their conjugation to peptides, sugars, lipoproteins and polyamines have been reported.¹⁴⁻¹⁹ In particular, amino acid residues have been found to improve the biological effects of porphyrin-based compounds, and their nature and position about the macrocycle can have a significant impact on PDT efficacy.^{5,20,21} In the present study we synthesized and investigated 17³, 15² and 13¹ amino acid-conjugates of chlorin e₆ (Figure 1), to evaluate the effect of the nature, conjugation site and position of the amino acid on the PDT efficacy of the conjugates *in vitro*, using human carcinoma HEp2 cells. The structure of mono-L-aspartylchlorin e₆, bearing the aspartyl residue in position 15² (rather than in 17³ as initially proposed) has recently been established.²² This study led us to design new syntheses of the 17³ and 13¹ aspartyl regioisomers of mono-L-aspartylchlorin e₆, as well as investigate the corresponding cationic lysine derivatives, which could potentially show stronger interactions with negatively charged biological molecules and plasma membranes, and consequently enhanced PDT efficacy.²³ Furthermore, we investigated the introduction of a spacer group, ethylene diamine, and of metalation, via insertion of palladium(II). The photosensitizing properties of porphyrin derivatives are known to be modulated by inner coordinated metal ions and associated axial ligands.²⁴⁻²⁶ In particular, Pd(II)-complexes have been shown to be efficient generators of singlet oxygen, and the palladium(II)-bacteriopheophorbide is currently being evaluated in clinical trials for the treatment of prostate cancer.^{27,28} The work described herein indicates that the site of amino acid conjugation, rather than the presence of Pd(II) and even the nature of amino acid, is the major determinant of phototoxicity.

Results and Discussion

1. Syntheses

The synthetic route to the 17³-lysyl conjugate of chlorin e₆ (**3**) is shown in Scheme 1. Pheophytin a (**1**) was obtained by extraction from *Spirulina pacifica* alga, an ideal source for chlorophyll a because of the complete absence of chlorophyll b. The selective hydrolysis of the phytol ester group of pheophytin a (**1**) using aqueous TFA produced pheophorbide a in high yield, without affecting the β -keto ester of the isocyclic ring.²⁹ This ring serves as a natural protecting group for the 13¹- and 15²-positions during the coupling reactions. Selective hydrolysis of the phytol ester produced a free 17³-carboxylic acid group which was activated with DCC/DMAP and coupled with H-lysine (OtBu) methyl ester to form the lysine (OtBu) methyl ester pheophorbide a (**2**) in 52% yield.³² ¹H NMR spectroscopy demonstrated the existence of the isocyclic ring and new peaks for the lysine residue.

Subsequent isocyclic ring-opening with sodium methoxide³¹ in THF produced the 17³-lysyl(Boc) chlorin e₆ TME in high yield (89%); deprotection of the amine group of the lysine side chain with TFA in CH₂Cl₂ followed, and yielded the 17³-lysylchlorin e₆ (**3**) in 30% overall yield from pheophytin *a* (**1**).

In order to synthesize the 15² and 13¹ amino acid derivatives of chlorin e₆, pheophytin *a* (**1**) was converted into methyl pheophorbide *a* (**4**) by transesterification of the ester group using sulfuric acid in methanol, as previously reported,³¹ followed by isocyclic ring-opening, as shown in Scheme 1. An optimized yield of 98% of chlorin e₆ trimethyl ester was obtained when 1.1 equiv of freshly prepared sodium methoxide in THF were used in the ring-opening reaction. Chlorin e₆ (**5**) was obtained by hydrolysis of the three methyl esters using an excess of LiI in ethyl acetate under reflux conditions for 24 hours.³³ Alternative hydrolysis under basic conditions using NaOH and LiOH gave lower yields of chlorin e₆. Under acidic conditions the 13¹-carboxylic acid of chlorin e₆ is deactivated³⁴ allowing for the selective methylation of the 15²- and 17³-carboxylic acids with 5% H₂SO₄/MeOH to give chlorin e₆ dimethyl ester (**6**) in quantitative yield.²² Chlorin e₆ (**5**) was used as starting material for the preparation of the 15² amino acid derivatives of chlorin e₆ (as shown in Scheme 2), whereas the chlorin (**6**) and methyl pheophorbide *a* (**4**) were used to prepare the 13¹ amino acid derivatives of chlorin e₆ (as shown in Schemes 3 and 4).

We have previously shown²² that the 15²-carboxylic acid of chlorin e₆ is the most reactive, regardless of the coupling reagent employed. Therefore, upon activation of chlorin e₆ (**5**) with one equiv of DCC/DMAP, followed by addition of protected amino acid, reaction with diazomethane³⁵ and deprotection under acidic conditions, the 15²-aspartyl (**7a**) and -lysyl (**7b**) derivatives of chlorin e₆ were obtained in overall 36% and 56% yields, respectively (Scheme 2). The use of excess DCC significantly reduced the yields obtained due to the formation of the 17³,15²-di-amino acid conjugate as a side product.

Insertion of palladium into the chlorin ring was attempted before the deprotection step to simplify the purification process; however low yields of the target metallochlorins were obtained. Therefore, palladium insertion was performed after deprotection, using either palladium(II) acetate or palladium(II) chloride. Quantitative yields for the metal insertion reaction were obtained using 1.2 equiv of palladium(II) acetate in THF at 40 °C, to produce the corresponding Pd(II) complexes (**8**). As expected, significant blue shifts in the UV-Vis spectrum along with ¹H NMR and mass spectrometry confirmed the insertion of palladium. Heavy metal atoms in the core of the tetrapyrroles have been shown to facilitate intersystem crossing and increase the production of singlet oxygen due to the inner heavy atom effect.^{36,37}

Two routes were used to synthesize the 13¹-amino acid conjugates: (1) Selective esterification of 17³- and 15²- carboxylic acids of chlorin e₆ to produce the monocarboxylic acid (**6**) followed by coupling³⁴ (Scheme 3), and (2) isocyclic ring-opening of methyl pheophorbide *a* (**4**) with a nucleophile (Scheme 4). The 13¹-carboxylic acid of chlorin (**6**) was activated using HOBt/TBTU under basic conditions³⁸ and coupled with L-aspartic di(tert)butyl ester. With DCC/DMAP a lower yield was obtained for the coupling reaction. Subsequent deprotection of both carboxylic acids in the aspartic chain provided (**9**) in 57% overall yield from chlorin e₆ (Scheme 3). Coupling of the 13¹-carboxylic acid of chlorin (**6**) with β-alanine tert-butyl ester followed by deprotection using TFA gave the 13¹-β-alanylchlorin e₆ dimethyl ester (**10**) in 64% overall yield. Aspartic di(tert)butyl ester was then coupled under similar conditions, followed by removal of the tert-butyl protecting group to give (**11**) in 54% yield from (**10**). β-Alanine provides a 4-atom link between the aspartic acid and chlorin e₆ dimethyl ester and allows for direct comparison with the lysine derivative **13** (see Scheme 4). The use of a linker significantly increased the yield of the

coupling reaction (from 66 to 87%) and it can also affect the biological properties of the photosensitizer.^{20,39}

The nucleophilic ring-opening of the isocyclic ring in pheophorbide *a* with ethylenediamine and ethanolamine has already been previously reported.^{25,40} We took advantage of this high yielding isocyclic ring-opening reaction to develop a potentially high yielding synthetic route to novel chlorin *e*₆ photosensitizers, directly from pheophytin *a* (**1**) in four steps. Nucleophilic ring-opening of the isocyclic ring with ethylenediamine produced molecule (**12**) in 91% yield. Subsequent coupling with protected lysine followed by deprotection gave the desired product (**13**) in four steps and in 55% overall yield from pheophytin *a* (**1**). No absorption quenching of the chlorin macrocycle was observed upon conjugation with the amino acid lysine in comparison with the aspartic acid derivative (see Figure S1 of the Supporting Information).

2. Molecular Modeling

Conformation analyses for 17³-, 15²- and 13¹-lysylchlorin *e*₆ derivatives **3**, **7b** and **13** were performed in both the gas phase and in water at the HF/6-31G level. These calculations were based on the atom coordinates from the X-ray structure of 15²-aspartylchlorin *e*₆ tetramethyl ester.²² The minimum energy conformations found for compounds **3**, **7b** and **13** in the gas phase, shown in Figure 2a–c, respectively, were very similar to those found in water phase (Figure S2 of the Supporting Information). In addition, the conformation of a mono-cationic 13¹-lysylchlorin *e*₆ derivative without a linker was also investigated in the gas phase (Figure 2d). As expected, the lysine residue in the 17³-lysyl derivative **3** is nearly perpendicular to the macrocyclic plane (Figure 2a), while in the 15²-lysyl derivative **7b** it forms approximately a 120° angle (Figure 2b). On the other hand the lysine residue extends away from the macrocycle in the 13¹-lysyl derivatives, with and without the short spacer (Figures 2c,d). Consequently, in the L-shape conformation of the 17³-lysylchlorin *e*₆ derivative, the amino acid shelters one face of the chlorin ring, while in the case of the 15²- and 13¹-lysyl derivatives, it extends away from the macrocycle resulting in a nearly linear conformation for the 13¹ derivatives. The use of a short linker and the presence of two (rather than one) positive charges, as a result from conjugation to the C-terminus rather than the N-terminus of the amino acid, do not appear to have a significant effect upon the preferred conformation; the main determinant of molecule conformation is the site of substitution. While the minimum energy conformations found in water were similar to those in the gas phase, it is possible that in water intermolecular forces (such as π - π stacking) predominate over the intramolecular interactions, in particular for the linear 13¹-lysylchlorin *e*₆ derivatives.

3. Cell Culture Studies

3.1. Time-dependent Cellular Uptake—The results obtained for the time-dependent uptake of chlorin *e*₆ and its derivatives at a concentration of 10 μ M in human HEp2 cells are shown in Figure 3 (also see Figure S3 of the Supporting Information). All amino acid conjugates of chlorin *e*₆ were readily taken up by cells and showed uptake kinetics similar to those of unconjugated chlorin *e*₆. Interestingly, the 15²-lysylchlorin *e*₆ derivatives **7b** and its palladium(II) complex **8b** accumulated to a much higher extent than all other compounds, at all time points studied. In comparison with chlorin *e*₆, the lysyl derivatives **7b** and **8b** showed 18-fold and 4-fold higher cellular uptake, respectively, after 24 h. The observed high uptake for derivatives **7b** and **8b** is probably due to the lysine residue in position 15² since the corresponding aspartyl derivatives **7a** and **8a** accumulated to a significantly lower extent within cells. Presumably, the stronger interactions between the positively charged lysine derivatives (compared with the corresponding aspartyl derivatives), with the negatively charged plasma membrane leads to enhanced cellular uptake.^{20,23} On the other

hand a lysine residue in position 17³, as in derivative **3**, showed a dramatic decrease in cellular uptake compared with the same residue at position 15², suggesting that the molecule conformation plays an important role on the mechanism of cellular uptake. Indeed the extended conformation of the 15²-lysylchlorin e₆ derivative **7b** (Figure 2b) might be the most favored for penetration across the plasma membrane, compared with the L-shape and linear conformations of the 17³- and 13¹-lysyl derivatives (Figures 2a,c), respectively. The presence of a chelated palladium ion, as well as the more linear structure of the 13¹-lysyl substituent might lead to enhanced π - π stacking of the macrocycles, thereby decreasing cellular uptake.

3.2. Cytotoxicity—The dark cytotoxicity and phototoxicity of chlorin e₆ and its derivatives was evaluated in HEp2 cells exposed to increasing concentrations of each compound up to 400 μ M; the results are shown in Figures 4 and 5, respectively, and are summarized in Table 1. Chlorin e₆ and its lysyl derivatives **3**, **7b** and **8b** were found to be non-toxic in the dark up to the highest concentration (400 μ M) investigated. All other amino acid derivatives showed very low dark cytotoxicities, with IC₅₀ > 320 μ M except for the 13¹-chlorin e₆ derivatives **9** and **13**, which showed IC₅₀ of 285 and 268 μ M, respectively. However, upon exposure to a low light dose (1 J/cm²), all chlorin e₆ derivatives were found to be highly toxic to HEp2 cells (Figure 3). The most phototoxic were the 13¹-chlorin e₆ derivatives **9**, **11** and **13**, with estimated IC₅₀ values of 0.61, 0.82 and 1.34 μ M, respectively. Among these, the most promising aspartyl derivatives for PDT applications are compounds **9** and **11**, because they have the highest dark cytotoxicity/phototoxicity ratio, > 466:1. The presence of the β -alanine spacer between the 13¹ carbonyl group and the aspartic acid residue seems to have only a small effect, slightly decreasing compound cytotoxicity. On the other hand the 15²-aspartylchlorin e₆ derivative **7a** was less phototoxic than its 13¹ regioisomer **9** by approximately 7-fold, and the introduction of palladium(II) further reduced its phototoxicity. The positively charged 17³- and 15²-lysylchlorin e₆ derivatives **3** and **7b** were the least phototoxic, and the introduction of palladium increased the phototoxicity of the 15² derivative by about 10-fold. These results show for the first time that the phototoxicity of amphiphilic conjugates of chlorin e₆ depends mainly on the site of conjugation, probably as a result of its effect on molecular conformation; the nature of the amino acid, the molecule overall charge, and the presence of palladium(II) also affect phototoxicity, but apparently to a smaller extent. Our results suggest that the most extended, nearly linear conformation of the 13¹ regioisomers facilitates binding to specific biological substrates, enhancing their toxic effect.

3.3. Intracellular Localization—The preferential sites of subcellular localization of this series of chlorin e₆ derivatives were evaluated by fluorescence microscopy, upon exposure of HEp2 cells to 10 μ M compound concentrations for 18 h. Figure S4 of the Supporting Information shows the fluorescence pattern observed for all compounds and Table 2 summarizes their main sites of subcellular localization. Overlay experiments using the organelle specific fluorescence probes BoDIPY Ceramide (Golgi), LysoSensor Green (Lysosomes), MitoTracker Green (mitochondria), and ER tracker Blue/White fluorescence (ER) were conducted to evaluate the preferential sites of compound localization, as seen in Figures 6, 7 and 8 for the most phototoxic 13¹-regioisomers, respectively, and Figures S5–S10 of the Supporting Information. All chlorin e₆ derivatives **3**, **7a**, **7b**, **8a**, **8b**, **9**, **11** and **13** were found to localize in the lysosomes and the ER (Table 2). This is not surprising, since the structurally-related 15²-aspartylchlorin e₆ is a known lysosomal localizer, and HPPH (2-[1-hexyloxyethyl]-2-devinylpyropheophorbide *a*) localizes preferentially in the ER.⁴¹ Photodamage to ER and/or lysosomes has been shown to lead to activation of apoptotic pathways.^{41–43} Furthermore, all the 13¹-chlorin e₆ regioisomers **9**, **11** and **13** were also found in Golgi, and in addition the most phototoxic derivative **9** also localized in

mitochondria; presumably, the photodamage effect to multiple organelles caused by the 13^1 derivatives can trigger various apoptotic pathways, leading to more effective cell destruction. The multiple sites of intracellular localization observed for the 13^1 -chlorin e_6 derivatives might again be due to their linear conformations that facilitate their binding to various intracellular sites.

Conclusions

A series of eight chlorin e_6 derivatives, conjugated with either aspartic acid or lysine residues at positions 17^3 , 15^2 or 13^1 of the chlorin macrocycle have been synthesized in good yields from pheophytin *a* (**1**). In comparison with chlorin e_6 (**5**), all amino acid derivatives readily accumulated within human HEp2 cells, and in particular the 15^2 -lysyl derivatives were taken up by cells to a significantly higher extent than were all other regioisomers. On the other hand, the metal-free 15^2 -lysylchlorin e_6 derivative (**7b**) showed the *least* phototoxicity, followed by the 17^3 -lysyl regioisomer (**3**); insertion of palladium into 15^2 -lysychlorin e_6 (**7b**) increased toxicity by approximately 10-fold. However, the most phototoxic compounds were found to be the 13^1 -regioisomers, bearing either an aspartic acid or a lysine residue directly conjugated to position-13 of the chlorin macrocycle, or connected via a β -alanine or ethylene diamine spacer. The most phototoxic compound of this series, and the most promising for PDT applications, is 13^1 -aspartylchlorin e_6 (**9**). Molecular modeling calculations show that the 13^1 -regioisomers assume extended, nearly linear conformations that might facilitate their binding to multiple intracellular components, and subsequent photodamage to multiple cellular sites. Therefore, the site of amino acid conjugation at the chlorin e_6 macrocycle is a major determinant of compound phototoxicity, and we hypothesize that the 13^1 -regioisomer of mono-L-aspartylchlorin e_6 (**9**) might be a more efficient photosensitizer for PDT than is the commercial regioisomer (15^2 -mono-L-aspartylchlorin e_6).

Experimental Section

1. Chemistry

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 $\times$ 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 μ m) from Sorbent Technologies. NMR spectra were recorded on an AV-400 spectrometer (400 MHz for ^1H , 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 (^1H), 54 ppm (^{13}C); d-DMSO 2.49 ppm (^1H), 39.5 ppm (^{13}C); d- CH_3OH 4.78 ppm (^1H), 49.0 ppm (^{13}C); CDCl_3 7.26 ppm (^1H), 77.16 ppm (^{13}C); $(\text{CH}_3)_2\text{CO}$ 2.50 ppm (^1H), 29.84 ppm (^{13}C). Electronic absorption spectra were measured on an Agilent 8453 UV/Vis spectrophotometer. Mass spectra were obtained on a Bruker Omnimflex MALDI Time-of-Flight Mass Spectrometer. *Spirulina pacifica* alga was purchased as a spray-dried powder from Cyanotech, Hawaii. Pheophytin *a* (**1**) was extracted from *Spirulina pacifica* alga as previously published and its spectroscopic characterization agreed with the published data.²⁹ All compounds synthesized were purified and isolated in $\geq 95\%$ purity, as evidenced by analytical TLC in at least two solvent systems, and confirmed by the absence of extraneous tetrapyrrole resonances in ^1H - and ^{13}C -NMR spectra. Energy minimization of all compounds was performed in the framework of Hartree Fock. The restricted-Hartree Fock functional was used at the 6-31G level. All structures were optimized without any symmetry constraints. The solvent effects were accounted for using the Polarizable Continuum Model (PCM). All calculations were performed using the Gaussian 09 program package.

¹⁷3-Lysyl(Boc)OMe-pheophorbide a (2)—Pheophytin *a* (**1**) (250 mg, 0.29 mmol) was selectively hydrolyzed to the ¹⁷3 carboxylic acid without affecting the ¹³1 carbomethoxy group, as previously reported.³¹ After evaporation of solvent, 155 mg (0.26 mmol), 90% of pheophorbide *a* (C₃₅H₃₆N₄O₅) was obtained. The spectroscopic data obtained for the compound agreed with published data. Pheophorbide *a*, (100 mg; 0.169 mmol) was dissolved in 10 ml of CH₂Cl₂. Then DCC (42 mg) and DMAP (10 mg) were added and left it for 15 min. H-Lys(Boc)OMe•HCl (60 mg) and DIEA (0.035 ml) were dissolved in 5 ml of CH₂Cl₂, added to the reaction mixture and stirred for 4 h. [Note: this reaction is favored by dilute conditions; concentrated conditions favor the formation of the anhydride (bisphorbide), and will slow the reaction]. The reaction mixture was washed with 10% citric acid, water and brine, dried over anhydrous Na₂SO₄ and purified on a silica gel column (10% acetone in CH₂Cl₂). After the major brown band was eluted from the column, the solvent was evaporated and the solid was dissolved in ethyl acetate and filtered (DCC precipitated in ethyl acetate.) Evaporation of the filtered ethyl acetate gave 74 mg, 0.089 mmol, 52% yield of lysyl(Boc)OMe-pheophorbide *a* (C₄₇H₅₈N₆O₈), ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.12 (s, 1H), 8.71 (s, 1H), 8.70 (s, 1H), 7.64 (dd, *J* = 17.8, 11.63 Hz, 1H), 7.20 (d, *J* = 7.2 Hz, 1H), 6.33 (s, 1H), 6.02 (d, *J* = 17.85 Hz, 1H), 5.93 (d, *J* = 11.61 Hz, 1H), 5.77 (s, 1H), 4.65 (m, 1H), 4.41 (m, 1H), 4.21 (m, 1H), 3.91 (s, 3H), 3.59 (s, 3H), 3.47 (s, 3H), 3.19 (s, 3H) 3.09 (q, *J* = 7.5 Hz, 2H), 2.92 (3H, s), 2.83 (m, 2H), 2.66 (m, 1H), 2.48 (m, 1H), 2.25 (m, 1H), 1.85-1.62 (br, 4H), 1.86 (d, *J* = 7.24, 3H), 1.37 (t, *J* = 7.58, 3H), 1.43 (m, 2H), 1.26 (s, 9H), -1.95 (s, 1H), -2.23 (s, 1H).

¹⁷3-Lysyl-chlorin e₆ TME (3)—Compound **2** (74 mg, 0.089 mmol) was dissolved in dry 2:1 THF/MeOH (10 ml) and stirred under argon for 10 min. Sodium methoxide (0.17 mL of a 0.5 M solution) was added and the reaction was allowed to stir at 0 °C for 1 h. The reaction was followed using spectrophotometry. The solution turned from brown to green as the isocyclic ring opens. The reaction mixture was then poured into water. The mixture was extracted with CH₂Cl₂ and the organic layer was washed with water and 5% citric acid, dried over anhydrous Na₂SO₄ and then evaporated. The residue was dissolved in 2% MeOH/CH₂Cl₂ and purified on a silica gel plug with the same mobile phase. The solvent was evaporated and 68 mg (0.078 mmol), 89% yield of ¹⁷3-lysyl(Boc)chlorin e₆ TME (C₄₈H₆₂N₆O₉) was obtained. ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.80 (s, 1H), 9.65 (s, 1H), 9.03 (s, 1H), 8.19 (dd, *J* = 17.87, 11.58 Hz, 1H), 7.44 (d, *J* = 7.6 Hz, 1H), 6.40 (d, *J* = 17.87, 1.33 Hz, 1H), 6.13 (d, *J* = 11.61, 1.36 Hz, 1H), 5.89 (s, 1H), 5.38 (m, 2H), 4.63 (q, *J* = 7.22 Hz, 1H), 4.55 (dd, *J* = 10.34, 1.95 Hz, 1H), 4.44 (m, 1H), 4.25 (s, 3H), 3.78 (q, *J* = 7.67, 2H) 3.75 (s, 3H), 3.69 (s, 3H), 3.56 (s, 3H) 3.49 (s, 3H), 3.27 (3H, s), 3.00 (m, 2H), 2.66 (m, 1H), 2.33 (m, 1H), 2.20 (m, 1H), 1.85-1.62 (br, 4H), 1.78 (d, *J* = 7.24, 3H), 1.69 (t, *J* = 7.58, 3H), 1.43 (m, 2H), 1.32 (s, 9H), -1.32 (s, 1H), -1.52 (s, 1H). Lysyl(Boc)chlorin e₆ TME (68mg, 0.078 mmol) was dissolved in 2 ml of dry CH₂Cl₂ in an ice bath under argon. Thioanisole (0.057 mmol) and 1 ml of TFA were added and the reaction mixture was allowed to stir overnight. The reaction mixture was evaporated several times with diethyl ether to remove TFA. The resulting precipitate was washed several times with diethyl ether to remove thioanisole. The precipitate was dissolved in CH₂Cl₂ and washed three times with H₂O and once with 10% NaHCO₃ to remove last traces of TFA. The organic layer was dried over anhydrous Na₂SO₄. Solvent was evaporated and 43 mg (0.056 mmol), 72% yield of ¹⁷3-lysylchlorin e₆ TME (C₄₃H₅₄N₆O₇) was obtained. UV-Vis (acetone): λ_{max} (ε/M⁻¹cm⁻¹) 664 (65,000), 608 (4871), 528 (4461), 500 (16,100), 400 (213,000); ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.79 (s, 1H), 9.63 (s, 1H), 9.03 (s, 1H), 8.17 (dd, *J* = 17.87, 11.58 Hz, 1H), 7.59 (d, *J* = 7.6 Hz, 1H), 6.38 (d, *J* = 17.87, 1.33 Hz, 1H), 6.12 (d, *J* = 11.61, 1.36 Hz, 1H), 5.37 (m, 2H), 4.63 (q, *J* = 7.22 Hz, 1H), 4.55 (dd, *J* = 10.34, 1.95 Hz, 1H), 4.44 (m, 1H), 4.25 (s, 3H), 3.76 (m, 2H) 3.75 (s, 3H), 3.69 (s, 3H), 3.55 (s, 3H) 3.48 (s, 3H), 3.25 (3H, s), 3.09 (m, 2H), 2.67 (m, 1H), 2.30 (m, 1H), 2.20 (m, 1H), 1.85-1.62 (br, 3H), 1.78 (d, *J* =

7.24, 3H), 1.68 (t, $J = 7.58$, 3H), 1.51 (m, 2H), 1.39 (m, 2H), -1.33 (s, 1H), -1.53 (s, 1H). HRMS (MALDI-TOF) m/z 767.442 $[M+H]^+$, calcd. for $C_{43}H_{55}N_6O_7$ 767.413.

Chlorin e₆ (5)—Chlorin e₆ TME was prepared as previously published and its spectroscopic characterization agreed fully with the published data.²² Chlorin e₆ TME (50 mg, 0.078 mmol) was dissolved in anhydrous ethyl acetate (10 ml) under argon. Lithium iodide (124 mg, 0.94 mmol) was added. The reaction mixture was refluxed for 48 h under argon. The reaction mixture was diluted with water and adjusted to pH 3 with aqueous citric acid and then washed with CH_2Cl_2 . The solution was evaporated, re-dissolved in acetone and evaporated several times. The solid was washed with water and then dried under vacuum. The residue was dissolved in methanol and purified on a Sephadex LH-20 column to yield 10 mg, 0.017 mmol, 21% yield of chlorin e₆ ($C_{34}H_{36}N_4O_6$). UV-Vis (MeOH): λ_{max} ($\epsilon/M^{-1}cm^{-1}$) 666 (45,271), 610 (8,706), 558 (7,835), 530 (9,721), 502 (15,525), 402 (145,100); MS MALDI: m/z 597 ($M + H$)⁺; ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.73 (s, 1H), 9.51 (s, 1H), 9.04 (s, 1H), 8.05 (dd, $J = 17.86$, 11.61 Hz, 1H), 6.28 (dd, $J = 17.85$, 1.26 Hz, 1H), 6.02 (dd, $J = 11.57$, 1.37 Hz, 1H), 5.60 (d, $J = 18.94$ Hz, 1H), 5.40 (d, $J = 18.94$ Hz, 1H), 4.65 (m, 1H), 4.55 (m, 1H), 3.64 (m, 2H), 3.63 (s, 3H), 3.42 (s, 3H), 3.15 (s, 3H), 2.72 (m, 1H), 2.35 (m, 1H), 2.26 (m, 1H), 2.06 (m, 1H), 1.75 (d, $J = 7.5$ Hz, 3H), 1.64 (t, $J = 7.5$ Hz, 3H), -1.6 (s, 2H).

Chlorin e₆ DME (6)—Chlorin e₆ DME was prepared as previously published and its spectroscopic characterization agreed with the published data.²²

15²-Aspartylchlorin e₆ DME (7a)—Chlorin e₆ (100 mg, 0.168 mmol) was dissolved in dry CH_2Cl_2 . DCC (35 mg) and DMAP (9 mg) were added and allowed to stir until completely dissolved. After 3 h, H-Asp(OtBu)₂•HCl (47.2 mg) and DIEA (0.029 ml) were mixed in CH_2Cl_2 and added to the reaction mixture. The reaction was allowed to stir overnight. The mixture was diluted with CH_2Cl_2 and then washed with 5% aqueous citric acid, followed by washing with brine and water. It was dried over anhydrous Na₂SO₄ and then evaporated. The residue was dissolved in CH_2Cl_2 and treated with ethereal diazomethane. The residue was dissolved in 2% MeOH/ CH_2Cl_2 and purified via silica gel column chromatography using the same mobile phase to afford 65 mg, 0.076 mmol; 45% yield of 15²-monoaspartylchlorin e₆ di(tert)butyl dimethyl ester ($C_{48}H_{61}N_5O_9$). MS (MALDI) m/z 853 ($M + H$)⁺; ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.64 (s, 1H), 9.32 (s, 1H), 9.02 (s, 1H), 7.81 (dd, $J = 11.58$, 17.85 Hz, 1H), 7.00 (s, 1H), 6.09 (dd, $J = 17.87$, 1.26 Hz, 1H), 5.85 (dd, $J = 11.60$, 1.29 Hz, 1H), 5.38 (s, 2H), 4.6 (br, 3H), 4.32 (s, 3H), 3.62 (s, 3H), 3.54 (s, 3H), 3.52 (q, $J = 7.3$ Hz, 2H), 3.30 (s, 3H), 3.02 (s, 3H), 2.78 (m, 3H), 2.44 (m, 2H), 1.87 (m, 1H), 1.65 (d, $J = 7.3$ Hz, 3H), 1.58 (t, $J = 7.7$ Hz, 3H), 1.26 (s, 9H), 1.16 (s, 9H), -1.42 (s, 1H), -1.53 (s, 1H). The 15²-Aspartylchlorin e₆ di(tert)butyl dimethyl ester (65 mg, 0.076 mmol) was dissolved in 2 ml of dry CH_2Cl_2 in an ice bath under argon. Thioanisole (0.006 ml) and 2 ml of TFA were added and the reaction mixture was allowed to stir overnight. The reaction mixture was evaporated several times with diethyl ether to remove TFA. The resulting precipitate was washed several times with diethyl ether to remove thioanisole and then dissolved in CH_2Cl_2 and washed three times with H₂O and once with 10% NaHCO₃ to remove TFA. The organic layer was washed with citric acid until all precipitate re-dissolved in the organic layer. The organic layer was dried over anhydrous Na₂SO₄. Solvent was evaporated and 44 mg, 0.06 mmol; 79% yield of 15²-aspartylchlorin e₆ DME was obtained ($C_{40}H_{45}N_5O_9$). UV-Vis (acetone) λ_{max} ($\epsilon/M^{-1}cm^{-1}$) 664 (48,400), 609 (6,000), 560 (2,000), 529 (6,280), 500 (14,160), 402 (151,800); MS (MALDI) m/z 841 ($M + H$)⁺; ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.76 (s, 1H), 9.58 (s, 1H), 9.04 (s, 1H), 8.10 (dd, $J = 17.85$, 11.59 Hz, 1H), 6.33 (dd, $J = 17.87$, 0.98 Hz, 1H), 6.07 (dd, $J = 11.61$, 1.09 Hz, 1H), 5.32 (m, 2H), 4.84 (m, 1H), 4.60 (m, 2H), 4.26 (s, 3H), 3.72 (q, $J =$

7.3 Hz, 2H), 3.57 (s, 3H), 3.54 (s, 3H), 3.45 (s, 3H), 3.21 (m, 3H), 2.95 (dd, $J = 16.85, 5.53$ Hz, 1H), 2.88 (dd, $J = 16.85, 5.09$ Hz, 1H), 2.68 (m, 1H), 2.32 (m, 2H), 1.78 (m, 1H), 1.74 (d, $J = 7.3$ Hz, 3H), 1.66 (t, $J = 7.7$ Hz, 3H), -1.39 (s, 1H), -1.58 (s, 1H). HRMS (MALDI-TOF) m/z 740.376 $[M+H]^+$, calcd. for $C_{40}H_{46}N_5O_9$ 740.330

¹⁵2-Lysylchlorin e₆ TME (7b)—Chlorin e₆ (75 mg, 0.12 mmol) was dissolved in dry CH_2Cl_2 (10 ml). DCC (31.3 mg) and DMAP (11 mg) were added and allowed to stir until completely dissolved. After 3 h, H-lysyl(Boc)OMe•HCl (45 mg) and DIEA (0.022 ml) were mixed in CH_2Cl_2 and added to the reaction mixture. The reaction was allowed to stir overnight. It was then diluted with CH_2Cl_2 and washed with 5% aqueous citric acid, followed by a wash with brine and water. It was dried over anhydrous Na_2SO_4 and then evaporated to dryness. The residue was dissolved in CH_2Cl_2 and treated with excess ethereal diazomethane. The residue was dissolved in 12% acetone/ CH_2Cl_2 and purified via silica gel column chromatography with the same mobile phase to afford 84 mg, 0.097 mmol; 77% yield of ¹⁵2-lysyl(Boc)chlorin e₆ TME ($C_{48}H_{62}N_6O_9$). MS (MALDI) m/z 868 ($M + H$)⁺; ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.77 (s, 1H), 9.55 (s, 1H), 9.04 (s, 1H), 8.08 (dd, $J = 17.87, 11.58$ Hz, 1H), 7.07 (m, 1H), 6.31 (dd, $J = 17.87, 1.33$ Hz, 1H), 6.06 (dd, $J = 11.61, 1.36$ Hz, 1H), 5.87 (s, 1H), 5.32 (s, 2H), 4.65 (q, $J = 7.22$ Hz, 1H), 4.60 (dd, $J = 10.34, 1.95$ Hz, 1H), 4.49 (m, 1H), 4.26 (s, 3H), 3.71 (q, $J = 7.67$, 2H), 3.57 (s, 3H), 3.54 (s, 3H), 3.47 (s, 3H), 3.44 (s, 3H), 3.19 (3H, s), 2.97 (m, 2H), 2.71 (m, 1H), 2.37 (m, 2H), 1.80 (m, 3H), 1.77 (d, $J = 7.24$, 3H), 1.66 (t, $J = 7.58$, 3H), 1.39 (s, 9H), 1.27 (m, 2H), -1.35 (s, 1H), -1.56 (s, 1H). The ¹⁵2-lysyl(Boc)chlorin e₆ TME (84 mg, 0.097 mmol) was dissolved in 3 ml of dry CH_2Cl_2 in an ice bath under argon. Thioanisole (0.01 ml) and 1 ml of TFA were added and the reaction mixture was allowed to stir overnight. The reaction mixture was evaporated several times with diethyl ether to remove residual TFA. The resulting precipitate was washed several times with diethyl ether to remove thioanisole. Then the precipitate was dissolved in CH_2Cl_2 and washed three times with H₂O and once with 10% NaHCO₃ to remove TFA. The organic layer was washed with citric acid until the precipitate is re-dissolved in the organic layer. The organic layer was dried over anhydrous Na_2SO_4 and then was evaporated to give 56 mg, 0.071 mmol; 73% yield of ¹⁵2-lysylchlorin e₆ TME ($C_{43}H_{54}N_6O_7$). UV-Vis (acetone) λ_{max} ($\epsilon/M^{-1}cm^{-1}$) 664 (57,800), 608 (4,600), 529 (4,900), 500 (14,800), 400 (187,200); ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.68 (s, 1H), 9.42 (s, 1H), 9.02 (s, 1H), 7.95 (dd, $J = 17.87, 11.58$ Hz, 1H), 7.12 (d, $J = 6.8$ Hz, 1H), 6.20 (dd, $J = 17.87, 1.33$ Hz, 1H), 5.95 (dd, $J = 11.61, 1.36$ Hz, 1H), 5.35 (s, 2H), 4.65 (q, $J = 7.22$ Hz, 1H), 4.61 (dd, $J = 10.34, 1.95$ Hz, 1H), 4.53 (m, 1H), 4.27 (s, 3H), 3.59 (m, 2H), 3.55 (s, 6H), 3.48 (s, 3H), 3.37 (s, 3H), 3.09 (3H, s), 2.99 (m, 3H), 2.73 (m, 1H), 2.38 (m, 2H), 1.80 (m, 3H), 1.78 (d, $J = 7.24$, 3H), 1.61 (t, $J = 7.58$, 3H), 1.43 (m, 2H), -1.40 (s, 1H), -1.57 (s, 1H). HRMS (MALDI-TOF) m/z 767.399 $[M+H]^+$, calcd. for $C_{43}H_{55}N_6O_7$ 767.413

Palladium(II) ¹⁵2-Aspartylchlorin e₆ DME (8a)—¹⁵2-Aspartylchlorin e₆ DME (44 mg, 0.06 mmol) was dissolved in 2 ml of dry THF. Palladium(II) acetate (14.2 mg, 0.063 mmol) was dissolved in THF and added to the reaction vessel and allowed to stir at 40 °C for 3 h. The reaction was followed by spectrophotometry. The solution turned from green to bluish green as the complex formed. After evaporation of solvent, the residue was dissolved in methanol and purified via Sephadex LH-20 chromatography using the same mobile phase to afford 50 mg, 0.059 mmol; 98% yield of palladium ¹⁵2-lysylchlorin e₆ TME ($C_{40}H_{43}N_5O_9Pd$). UV-Vis (acetone): λ_{max} ($\epsilon/M^{-1}cm^{-1}$) 619 (31,240), 579 (6,210), 489 (4,035), 393 (44,160); MS (MALDI) m/z 845 ($M + H$)⁺; ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.53 (s, 1H), 9.54 (s, 1H), 8.93 (s, 1H), 8.03 (dd, $J = 17.82, 11.54$ Hz, 1H), 6.17 (d, $J = 17.79$ Hz, 1H), 6.00 (dd, $J = 11.61$, 1H), 5.19 (d, $J = 18.77$ Hz, 1H), 5.04 (d, $J = 18.59$ Hz, 1H), 4.92 (m, 1H), 4.63 (m, 2H), 4.20 (s, 3H), 3.62 (s, 3H), 3.57 (m, 2H), 3.57 (s, 3H), 3.37 (s, 3H), 3.07 (m, 3H), 2.68 (m, 2H), 2.38 (m, 1H), 2.12 (m, 1H), 1.78 (m, 2H), 1.74 (d, $J = 7.3$

Hz, 3H), 1.50 (t, $J = 7.7$ Hz, 3H). HRMS (MALDI-TOF) m/z 843.109 $[M]^+$, calcd. for $C_{40}H_{43}N_5O_9Pd$ 843.210.

Palladium(II) 15²-Lysylchlorin e₆ TME (8b)—15²-Lysylchlorin e₆ TME (56 mg) was dissolved in 5 ml of dry THF. Palladium(II) acetate (53 mg) was dissolved in THF and added to the reaction vessel and stirred at 60 °C for 3 h. The reaction was followed by spectrophotometry. The solution turned from green to bluish green as the complex formed. After evaporation of solvent, the residue was dissolved in methanol and purified via Sephadex LH-20 chromatography using the same mobile phase to afford 61 mg, 0.07 mmol; 99% yield of palladium(II) 15²-lysylchlorin e₆ TME ($C_{43}H_{53}N_6O_7Pd$). UV-Vis (acetone): λ_{max} ($\epsilon/M^{-1}cm^{-1}$) 620 (97,520), 581 (14,500), 490 (7,600), 394 (140,900); ¹H NMR [(CD₃)₂CO, 400 MHz] all the peaks were broad due to partial paramagnetic nature of the compound. HRMS (MALDI-TOF) m/z 872.337 $[M+2H]^+$, 767.450 $[M+H-Pd]^+$ Calcd. for $C_{43}H_{54}N_6O_7Pd$ 871.301, $C_{43}H_{55}N_6O_7$ 767.413.

13¹-Aspartylchlorin e₆ DME (9)—Chlorin e₆ dimethyl ester (55 mg, 0.088 mmol) was dissolved in dry CH₂Cl₂. A mixture of HOBT (12 mg), TBTU (29 mg) and DIEA (0.017 ml) in DMF was added and the mixture was allowed to stir for 30 min. H-Asp(OtBu)₂•HCl (60 mg) and DIEA (0.033 ml) were mixed in CH₂Cl₂ and added to this reaction mixture. The mixture was stirred overnight. It was diluted with CH₂Cl₂ and then washed with 5% aqueous citric acid, followed by a wash with brine and water. It was dried over anhydrous Na₂SO₄ and then evaporated. The residue was dissolved in 5% acetone/CH₂Cl₂ and purified via silica gel column chromatography using the same mobile phase to afford 50 mg, 0.058 mmol; 66% yield of 13¹-aspartylchlorin e₆ di(tert)butyl dimethyl ester ($C_{48}H_{61}N_5O_9$). MS (MALDI) m/z 852 (M + H)⁺; ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.79 (s, 1H), 9.70 (s, 1H), 9.10 (s, 1H), 8.39 (d, $J = 7.93$ Hz, 1H), 8.19 (dd, $J = 11.58, 17.85$ Hz, 1H), 6.37 (dd, $J = 17.85, 1.23$ Hz, 1H), 6.10 (dd, $J = 11.87, 1.26$ Hz, 1H), 5.71 (d, $J = 18.95$ Hz, 1H), 5.30 (m, 2H), 4.67 (q, $J = 7.22$ Hz, 1H), 4.49 (dd, $J = 10.34, 1.95$ Hz, 1H), 3.76 (q, $J = 7.3$ Hz, 2H), 3.74 (s, 3H), 3.64 (s, 3H), 3.61 (s, 3H), 3.50 (s, 3H), 3.27 (s, 3H), 3.16 (dd, $J = 5.8, 0.78$ Hz, 2H), 2.73 (m, 1H), 2.32 (m, 2H), 1.79 (m, 1H), 1.69 (m, 6H), 1.64 (s, 9H), 1.53 (s, 9H), -1.57 (s, 1H), -1.85 (s, 1H). The 13¹-Aspartylchlorin e₆ di(tert)butyl dimethyl ester (50 mg, 0.058 mmol) was dissolved in 2 ml of dry CH₂Cl₂ in a ice bath under argon. Thioanisole (0.005 ml) and 2 ml of TFA were added and the reaction mixture was stirred overnight. The reaction mixture was evaporated several times with diethyl ether to remove residual TFA. The resulting precipitate was washed several times with diethyl ether to remove thioanisole. Then the precipitate was dissolved in CH₂Cl₂ and washed three times with H₂O and once with 10% NaHCO₃ to remove TFA. The organic layer was dried over anhydrous Na₂SO₄ and then evaporated to give 38 mg, 0.051 mmol; 88% yield of 13¹-aspartylchlorin e₆ DME ($C_{40}H_{45}N_5O_9$). UV-Vis (acetone): λ_{max} ($\epsilon/M^{-1}cm^{-1}$) 663 (126,800), 607 (9,000), 528 (7,450), 500 (31,540), 399 (385,800); ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.79 (s, 1H), 9.70 (s, 1H), 9.12 (s, 1H), 8.50 (d, $J = 7.93$ Hz, 1H), 8.19 (dd, $J = 11.58, 17.85$ Hz, 1H), 6.37 (d, $J = 18.85$ Hz, 1H), 6.10 (d, $J = 11.87$ Hz, 1H), 5.71 (d, $J = 18.95$ Hz, 1H), 5.45 (dd, $J = 13.76, 5.80$ Hz, 1H), 5.30 (d, $J = 18.93$ Hz, 1H), 4.67 (q, $J = 7.22$ Hz, 1H), 4.50 (dd, $J = 10.34, 1.95$ Hz, 1H), 3.76 (q, $J = 7.3$ Hz, 2H), 3.71 (s, 3H), 3.63 (s, 3H), 3.60 (s, 3H), 3.51 (s, 3H), 3.31 (dd, $J = 5.76, 3.47$ Hz, 2H), 3.26 (s, 3H), 2.70 (m, 2H), 2.31 (m, 2H), 1.79 (m, 1H), 1.71 (d, $J = 7.24, 3H$), 1.67 (t, $J = 7.58, 3H$), -1.57 (br s, 1H), -1.87 (s, 1H). HRMS (MALDI-TOF) m/z 740.344 $[M+H]^+$, Calcd. for $C_{40}H_{46}N_5O_9$ 740.330

13¹- β -Alanylchlorin e₆ DME (10)—Chlorin e₆ DME (55 mg, 0.088 mmol) was dissolved in dry CH₂Cl₂. A mixture of HOBT (12 mg), TBTU (29 mg) and DIEA (0.017 ml) in DMF was added and the mixture was stirred for 30 min. β -Alanyl(OtBu)•HCl (18 mg) and DIEA (0.017 ml) were mixed in CH₂Cl₂ and added to the reaction mixture. The mixture

was then allowed to stir for overnight before being diluted with CH_2Cl_2 and then washed with 5% aqueous citric acid, followed by a wash with brine and water. It was dried over anhydrous Na_2SO_4 and then evaporated to dryness. The residue was dissolved in 5% acetone/ CH_2Cl_2 and purified via silica gel column chromatography using the same mobile phase to afford 45 mg, 0.06 mmol; 68% yield of 13^1 - β -alanylchlorin e_6 (*tert*)butyl dimethyl ester ($\text{C}_{43}\text{H}_{53}\text{N}_5\text{O}_7$). ^1H NMR [$(\text{CD}_3)_2\text{CO}$, 400 MHz]: δ 9.70 (s, 1H), 9.63 (s, 1H), 9.01 (s, 1H), 8.17 (t, $J = 5.71$ Hz, 1H), 8.11 (dd, $J = 11.58, 17.85$ Hz, 1H), 6.31 (d, $J = 18.85$ Hz, 1H), 6.04 (d, $J = 11.87$ Hz, 1H), 5.65 (d, $J = 18.95$ Hz, 1H), 5.38 (d, $J = 19.06$ Hz, 1H), 4.66 (q, $J = 7.22$ Hz, 1H), 4.52 (dd, $J = 10.34, 1.95$ Hz, 1H), 4.02 (m, 1H), 3.09 (m, 1H), 3.77 (s, 3H), 3.68 (q, $J = 7.3$ Hz, 2H), 3.61 (s, 3H), 3.51 (s, 3H), 3.46 (s, 3H), 3.21 (s, 3H), 2.71 (m, 2H), 2.31 (m, 2H), 1.79 (m, 1H), 1.72 (d, $J = 7.24$, 3H), 1.65 (t, $J = 7.58$, 3H), 1.54 (s, 9H), -1.62 (s, 1H), -1.91 (s, 1H). The 13^1 - β -alanylchlorin e_6 (*tert*)butyl dimethyl ester (45 mg, 0.059 mmol) was dissolved in 1.5 ml of dry CH_2Cl_2 in a ice bath under argon. Thioanisole (0.005 ml) and 1.5 ml of TFA were added and the reaction mixture was stirred overnight before being diluted with CH_2Cl_2 and washed three times with H_2O and once with 10% NaHCO_3 . The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give 39 mg, 0.056 mmol; 95% yield of 13^1 - β -alanylchlorin e_6 DME ($\text{C}_{39}\text{H}_{45}\text{N}_5\text{O}_7$). ^1H NMR [$(\text{CD}_3)_2\text{CO}$, 400 MHz]: δ 9.74 (s, 1H), 9.72 (s, 1H), 9.10 (s, 1H), 8.22 (dd, $J = 11.58, 17.85$ Hz, 1H), 8.15 (t, $J = 5.71$ Hz, 1H), 6.39 (d, $J = 18.85$ Hz, 1H), 6.12 (d, $J = 11.87$ Hz, 1H), 5.65 (d, $J = 18.95$ Hz, 1H), 5.37 (d, $J = 19.06$ Hz, 1H), 4.66 (q, $J = 7.22$ Hz, 1H), 4.52 (dd, $J = 10.34, 1.95$ Hz, 1H), 4.07 (m, 1H), 3.95 (m, 1H), 3.77 (s, 3H), 3.68 (q, $J = 7.3$ Hz, 2H), 3.61 (s, 3H), 3.53 (s, 3H), 3.52 (s, 3H), 3.28 (s, 3H), 2.69 (m, 2H), 2.31 (m, 2H), 1.79 (m, 1H), 1.72 (d, $J = 7.24$, 3H), 1.65 (t, $J = 7.58$, 3H), -1.62 (s, 1H), -1.91 (s, 1H).

13^1 - β -Alanyl-aspartylchlorin e_6 DME (11)—Chlorin e_6 DME (39 mg, 0.056 mmol) was dissolved in dry CH_2Cl_2 . A mixture of HOBT (8 mg), TBTU (18 mg) and DIEA (0.017 ml) in DMF was added and stirred for 30 min. H-Asp(OtBu) $_2$ •HCl (40 mg) and DIEA (0.025 ml) were mixed in CH_2Cl_2 and added to the reaction mixture. The mixture was stirred overnight before being diluted with CH_2Cl_2 and then washed with 5% aqueous citric acid, followed by a wash with brine and water. It was dried over anhydrous Na_2SO_4 and then evaporated. The residue was dissolved in 10% acetone/ CH_2Cl_2 and purified via silica gel column chromatography using the same mobile phase to afford 45 mg, 0.048 mmol; 87% yield of 13^1 - β -alanyl-aspartylchlorin e_6 di(*tert*)butyl dimethyl ester. ^1H NMR [$(\text{CD}_3)_2\text{CO}$, 400 MHz]: δ 9.74 (s, 1H), 9.70 (s, 1H), 9.10 (s, 1H), 8.19 (dd, $J = 11.58, 17.85$ Hz, 1H), 8.11 (t, $J = 5.66$ Hz, 1H), 7.64 (d, $J = 8.11$ Hz, 1H), 6.37 (d, $J = 19.18$ Hz, 1H), 6.10 (d, $J = 12.94$ Hz, 1H), 5.66 (d, $J = 19.03$ Hz, 1H), 5.38 (d, $J = 19.09$ Hz, 1H), 4.77 (dt, $J = 8.14, 5.74$ Hz, 1H), 4.66 (q, $J = 7.22$ Hz, 1H), 4.52 (dd, $J = 10.34, 1.95$ Hz, 1H), 4.07 (m, 1H), 3.95 (m, 1H), 3.75 (s, 3H), 3.76 (q, $J = 7.3$ Hz, 2H), 3.60 (s, 3H), 3.53 (s, 3H), 3.50 (s, 3H), 3.27 (s, 3H), 2.80 (d, $J = 2.57$ Hz, 1H), 2.79 (d, $J = 3.06$ Hz, 1H), 2.31 (m, 2H), 1.79 (m, 1H), 1.71 (d, $J = 7.24$, 3H), 1.68 (t, $J = 7.58$, 3H), 1.43 (s, 9H), 1.42 (s, 9H), -1.61 (s, 1H), -1.91 (s, 1H). The 13^1 - β -alanyl-aspartylchlorin e_6 di(*tert*)butyl dimethyl ester (45 mg, 0.048 mmol) was dissolved in 2 ml of dry CH_2Cl_2 in a ice bath under argon. Thioanisole (0.004 ml) and 2 ml of TFA were added and the reaction mixture was stirred overnight before being evaporated several times with diethyl ether to remove residual TFA. The resulting precipitate was washed several times with diethyl ether to remove more residual thioanisole. The precipitate was dissolved in CH_2Cl_2 and washed three times with H_2O and once with 10% NaHCO_3 to remove TFA. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give 38 mg, 0.046 mmol; 97% yield of 13^1 -aspartylchlorin e_6 DME ($\text{C}_{43}\text{H}_{50}\text{N}_6\text{O}_{10}$). UV-Vis (acetone): λ_{max} ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$) 663 (77,125), 607 (3,621), 528 (1,831), 500 (17,200), 399 (237,100); ^1H NMR [$(\text{CD}_3)_2\text{CO}$, 400 MHz]: δ 9.72 (s, 1H), 9.70 (s, 1H), 9.10 (s, 1H), 8.20 (dd, $J = 11.58, 17.85$ Hz, 1H), 8.11 (br

t, $J = 5.66$ Hz, 1H), 7.64 (br d, $J = 8.11$ Hz, 1H), 6.32 (d, $J = 19.18$ Hz, 1H), 6.10 (d, $J = 12.94$ Hz, 1H), 5.63 (d, $J = 19.03$ Hz, 1H), 5.37 (d, $J = 19.09$ Hz, 1H), 4.92 (dt, $J = 8.14$, 5.74 Hz, 1H), 4.66 (q, $J = 7.22$ Hz, 1H), 4.51 (dd, $J = 10.34$, 1.95 Hz, 1H), 4.07 (m, 1H), 3.95 (m, 1H), 3.74 (s, 3H), 3.75 (q, $J = 7.3$ Hz, 2H), 3.60 (s, 3H), 3.51 (s, 3H), 3.50 (s, 3H), 3.25 (s, 3H), 2.96 (d, $J = 5.27$ Hz, 1H), 2.69 (d, $J = 3.06$ Hz, 1H), 2.31 (m, 2H), 1.79 (m, 1H), 1.71 (d, $J = 7.24$, 3H), 1.66 (t, $J = 7.58$, 3H), -1.61 (s, 1H), -1.91 (s, 1H). HRMS (MALDI-TOF) m/z 811.381 $[M+H]^+$, calcd. for $C_{43}H_{51}N_6O_{10}$ 811.367.

13^1 -Ethylenediaminylchlorin e₆ DME (12)—Methyl pheophorbide *a*, (100 mg, 0.164 mmol) was dissolved in dry $CHCl_3$ and stirred under argon for 10 min. 0.2 ml of ethylenediamine was added to the solution and the mixture stirred for 24 h. The reaction was monitored by spectrophotometry. The reaction mixture was evaporated and the residue was dissolved in 2.5% MeOH/ CH_2Cl_2 and then chromatographed on a short silica gel column using the same mobile phase to remove by-products, and then the product was eluted using 50% MeOH/ CH_2Cl_2 to afford 100 mg, 0.150 mmol: 91% yield of 13^1 -ethylenediaminylchlorin e₆ DME ($C_{38}H_{46}N_6O_5$). 1H NMR [$(CD_3)_2CO$, 400 MHz]: δ 9.69 (s, 1H), 9.63 (s, 1H), 9.09 (s, 1H), 8.09 (dd, $J = 17.78$, 11.58 Hz, 1H), 8.07 (br s, 1H), 6.29 (d, $J = 18.85$ Hz, 1H), 6.02 (d, $J = 11.87$ Hz, 1H), 5.67 (d, $J = 19.08$ Hz, 1H), 5.39 (d, $J = 19.11$ Hz, 1H), 4.66 (q, $J = 7.22$ Hz, 1H), 4.51 (dd, $J = 10.34$, 1.95 Hz, 1H), 4.01 (m, 1H), 3.87 (m, 1H), 3.75 (s, 3H), 3.66 (q, $J = 7.3$ Hz, 2H), 3.61 (s, 3H), 3.50 (s, 3H), 3.45 (s, 3H), 3.20 (s, 3H), 2.71 (m, 1H), 2.31 (m, 2H), 1.95 (br m, 1H), 1.80 (m, 1H), 1.72 (d, $J = 7.24$, 3H), 1.64 (t, $J = 7.58$, 3H), -1.64 (s, 1H), -1.93 (s, 1H). ^{13}C NMR [$(CD_3)_2CO$, 100 MHz] δ 173.1, 173.0, 169.2, 168.3, 167.7, 153.6, 149.1, 144.4, 138.1, 136.0, 135.3, 134.7, 133.9, 130.1, 129.8, 129.3, 120.9, 103.2, 100.7, 98.4, 93.9, 53.1, 51.4, 50.8, 50.2, 48.8, 41.2, 37.2, 30.7, 29.6, 22.6, 19.0, 17.2, 11.3, 11.0, 10.3. HRMS (MALDI-TOF) m/z 667.395 $[M+H]^+$, calcd. for $C_{38}H_{47}N_6O_5$ 667.361.

13^1 -Ethylenediaminyl-lysylchlorin e₆ DME (13)—(Boc)Lysine(Boc)OH (55 mg, 0.082 mmol) was dissolved in dry CH_2Cl_2 (10 ml). A mixture of HOBT (15 mg), TBTU (33 mg) and DIEA (0.025 ml) in DMF was added and then stirred for 1 h. 13^1 -Ethylenediaminylchlorin e₆ DME (50 mg, 0.075 mmol) was added to the reaction mixture, which was stirred overnight. The mixture was diluted with CH_2Cl_2 and then washed with 5% aqueous citric acid, followed by a wash with brine and water. It was dried over anhydrous Na_2SO_4 and then evaporated. The residue was dissolved in 2.5% MeOH/ CH_2Cl_2 and purified via silica gel column chromatography using the same mobile phase to afford 65 mg, 0.065 mmol; 78% yield of 13^1 -ethylenediaminyl(Boc)lysyl(Boc)chlorin e₆ DME ($C_{54}H_{74}N_8O_{10}$). 1H NMR [$(CD_3)_2CO$, 400 MHz]: δ 9.54 (s, 1H), 9.53 (s, 1H), 9.07 (s, 1H), 8.14 (br s, 1H), 7.94 (dd, $J = 17.78$, 11.58 Hz, 1H), 7.78 (br s, 1H), 6.18 (d, $J = 18.85$ Hz, 1H), 6.16 (br s, 1H), 5.93 (d, $J = 11.87$ Hz, 1H), 5.89 (br s, 1H), 5.63 (d, $J = 19.08$ Hz, 1H), 5.39 (d, $J = 19.11$ Hz, 1H), 4.67 (q, $J = 7.22$ Hz, 1H), 4.52 (dd, $J = 10.34$, 1.95 Hz, 1H), 4.17 (m, 1H), 3.85 (m, 1H), 3.75 (s, 3H), 3.66 (m, 3H), 3.61 (s, 3H), 3.52 (br q, $J = 7.14$ Hz, 2H), 3.41 (s, 3H), 3.39 (s, 3H), 3.11 (s, 3H), 3.00 (m, 2H), 2.71 (m, 1H), 2.32 (m, 2H), 1.95 (br m, 1H), 1.84 (m, 2H), 1.72 (d, $J = 7.24$, 3H), 1.67 (m, 1H), 1.59 (t, $J = 7.58$, 3H), 1.41 (m, 3H), 1.37 (s, 9H), 1.34 (s, 9H), -1.66 (s, 1H), -1.94 (s, 1H). The 13^1 -ethylenediaminyl(Boc)lysyl(Boc)-chlorin e₆ DME (65 mg, 0.065 mmol) was dissolved in 3 ml of dry CH_2Cl_2 in a ice bath under argon. Thioanisole (0.003 ml) and 2 ml of TFA were added and the reaction mixture was stirred overnight. The reaction mixture was evaporated several times with diethyl ether to remove TFA. The resulting precipitate was washed several times with diethyl ether to remove thioanisole. Then the precipitate was dissolved in CH_2Cl_2 and washed three times with H_2O and once with 10% $NaHCO_3$ to remove TFA. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to give 42 mg, 0.052 mmol; 81% yield of 13^1 -ethylenediaminyl-lysylchlorin e₆ DME

(C₄₄H₅₈N₈O₆). UV-Vis (acetone): λ_{max} ($\epsilon/\text{M}^{-1}\text{cm}^{-1}$) 663 (76,420), 607 (1810), 527 (657), 500 (14,560), 399 (242,100); ¹H NMR [(CD₃)₂CO, 400 MHz]: δ 9.76 (s, 1H), 9.69 (s, 1H), 9.10 (s, 1H), 8.29 (br s, 1H), 8.17 (dd, J = 17.78, 11.58 Hz, 1H), 7.86 (br s, 1H), 6.35 (d, J = 18.85 Hz, 1H), 6.07 (d, J = 11.87 Hz, 1H), 5.64 (d, J = 19.08 Hz, 1H), 5.40 (d, J = 19.11 Hz, 1H), 4.66 (q, J = 7.22 Hz, 1H), 4.52 (dd, J = 10.34, 1.95 Hz, 1H), 3.92 (m, 2H), 3.75 (br m, 5H), 3.74 (s, 3H), 3.60 (s, 3H), 3.53 (s, 3H), 3.49 (s, 3H), 3.26 (s, 3H), 2.96 (br t, 2H), 2.70 (m, 1H), 2.29 (m, 2H), 1.95 (br m, 1H), 1.84 (m, 2H), 1.72 (d, J = 7.24, 3H), 1.68 (t, J = 7.58, 3H), 1.45 (m, 2H), 1.31 (m, 2H), -1.60 (s, 1H), -1.89 (s, 1H). HRMS (MALDI-TOF) m/z 795.492 [M+H]⁺, calcd. for C₄₄H₅₉N₈O₆ 795.456.

2. Cell Studies

Human carcinoma HEp2 cells were maintained in a 50:50 mixture of DMEM:AMEM (Invitrogen) supplemented with 5% FBS (Invitrogen), 1% Primocin antibiotic (Invitrogen) in a humidified, 5% CO₂ incubator at 37 °C. The cells were sub-cultured twice weekly to maintain sub-confluent stocks. The 4th to 15th passage cells were used for all experiments.

2.1. Time-Dependent Cellular Uptake—The HEp2 cells were plated at 7500 cells per well in a Costar 96-well plate and allowed to grow for 48 h. Compound stock solutions were prepared at 32 mM in DMSO and Cremophor (10% of Cremophor in DMSO). Further dilution into the cells of the 96-well plate gave a final concentration of 10 μM with maximum DMSO concentration of 1% and Cremophor concentration of 0.1%. For the uptake test, the compounds were diluted to 20 μM (2 $\times$ stock solution) and added to the 96-well to give a final concentration of 10 μM at 0, 1, 2, 4, 8, 12 and 24 h interval. The uptake was terminated by removing the loading medium and washing the wells with 200 μL PBS buffer. Cells and compounds were then solubilized using 100 μL of 0.25% Triton X-100 in PBS buffer. The compound concentration was measured using intrinsic fluorescence as measured with a BMG FLUOstar plate reader equipped with a 355 nm excitation and a 650 nm emission filter. The cells were measured using a CyQuant Cell proliferation assay (Invitrogen) as per manufacturer's instructions, as previously reported.^{20,21,24}

2.2. Dark Cytotoxicity—The HEp2 cells were plated as described above for the uptake experiment. The compounds were diluted into media to give 400 μM solution concentrations. Two-fold serial dilutions were then prepared from 400 to 50 μM , and the cells were incubated for 18 h. The loading medium was removed and the cells were fed new medium, followed by 20 μL CellTiter blue per 100 μL medium, and incubated for 4 h. Cell toxicity was measured using Promega's CellTiter Blue Viability Assay Kit as per manufacturer's instructions; untreated cells were considered 100% viable and cells treated with 0.2% saponin as 0% viable, as previously reported.^{20,21,24}

2.3. Phototoxicity—The cells were prepared as described above with compound concentration range from 6.25–100 μM . After loading overnight, the medium was replaced with medium containing 50 mM HEPES pH 7.2. The cells were exposed to a 100 W halogen lamp filtered through a 610 nm long pass filter to provide approximately 1 Jcm^{-2} light dose. The cells were kept cool by filtering the IR radiation through 10 mm of water and placing the culture in an ice-water bath. After exposure to light for 20 min, the plate was incubated in the 37 °C, 5% CO₂ incubator overnight. Cell viability was then measured as described above using CellTiter blue Viability Assay Kit, as previously reported.^{20,21,24}

2.4. Microscopy—The cells were incubated in a glass bottom 6-well plate (MatTek) and allowed to grow for 48 h. The cells were then exposed to 10 μM of each compound for 18 h. Organelle tracers were obtained from Invitrogen and used at the following concentrations: LysoSensor Green 50 nM, MitoTracker Green 250 nM, ER Tracker Blue/white 100 nM, and

BODIPY FL C5 ceramide 1 mM. After 30 min incubation in the 37 °C, 5% CO₂ incubator, both the media and the organelle tracers were removed and washed with PBS buffer for 3 times. Images were acquired using a Leica DMRXA microscope with 40× NA 0.8 dip objective lens and DAPI, GFP and Texas Red filter cubes (Chroma Technologies).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institutes of Health, grant numbers CA132861 (KMS) and CA139385 (MGHV).

References

1. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbek M, Moan J, Peng Q. Photodynamic therapy. *J. Natl. Cancer Inst.* 1998; 90:889–905. [PubMed: 9637138]
2. Hahn SM, Glatstein E. The emergence of photodynamic therapy as a major modality in cancer treatment. *Rev. Contemp. Pharm.* 1999; 10:69–74.
3. Pandey RK. Recent advances in photodynamic therapy. *J. Porphyrins Phthalocyanines.* 2000; 4:368–373.
4. Brown SB, Brown EA, Walker I. The present and future role of photodynamic therapy in cancer treatment. *Lancet Oncol.* 2004; 5:497–508. [PubMed: 15288239]
5. Vicente MGH. Porphyrin-based Sensitizers in the Detection and Treatment of Cancer: Recent Progress. *Current Med. Chem., Anti-Cancer Agents.* 2001; 1:175–194.
6. Huang Z. A review of progress in clinical photodynamic therapy. *Tech. Cancer Res. Treatment.* 2005; 4:283–293.
7. Kostenicha GA, Zhuravkina IN, Zhavrid EA. Experimental grounds for using chlorin e₆ in the photodynamic therapy of malignant tumors. *J. Photochem. Photobiol. B: Biol.* 1994; 22:211–217.
8. Allison RR, Downie GH, Cuenca R, Hu X-H, Sibata CH, Childs CJ. Photosensitizers in clinical PDT. *Photodiagnosis and Photodynamic Therapy.* 2004; 1:27–42.
9. Kessel D. Determinants of photosensitization by mono-L-aspartyl chlorin e₆. *Photochem. Photobiol.* 2008; 49:447–452. [PubMed: 2727084]
10. Sunar U, Rohrbach D, Rigual N, Tracy E, Keymel K, Cooper MT, Baumann H, Henderson BH. Monitoring photobleaching and hemodynamic responses to HPPH-mediated photodynamic therapy of head and neck cancer: a case report. *Optics Express.* 2010; 18:14969–14978. [PubMed: 20639983]
11. (a) Usuda J, Ichinose S, Ishizumi T, Hayashi H, Ohtani K, Maehara S, Ono S, Kajiware N, Uchida O, Tsutsui H. Management of multiple primary lung cancer in patients with centrally located early cancer lesions. *J. Thoracic Oncology.* 2010; 5:62–68. (b) Usuda J, Ichinose S, Ishizumi T, Hayashi H, Ohtani K, Maehara S, Ono S, Honda H, Kajiware N, Uchida O. Outcome of Photodynamic Therapy Using NPe6 for Bronchogenic Carcinomas in Central Airways >1.0 cm in Diameter. *Clin. Cancer Res.* 2010; 16:2198–2204. [PubMed: 20332318]
12. Wang S, Bromley E, Xu L, Chen JC, Keltner L. Talaporfin sodium. *Expert Opinion on Pharmacotherapy.* 2010; 11:133–140. [PubMed: 20001435]
13. Bromley E, Briggs B, Keltner L, Wang S-S. Characterization of cutaneous photosensitivity in healthy volunteers receiving talaporfin sodium. *Photodermatology, Photoimmunology Photomedicine.* 2011; 27:85–89.
14. Akhlynina TV, Jans DA, Rosenkranz AA, Statsyuk NV, Balashova IY, Toth G, Pavo I, Rubin AB, Sobolev AS. Nuclear targeting of chlorin e₆ enhances its photosensitizing activity. *J. Biol. Chem.* 1997; 272:20328–20331. [PubMed: 9252335]

15. Soukos, Nikolaos S.; Hamblin, Michael R.; Hasan, T. The effect of charge on cellular uptake and phototoxicity of polylysine chlorin e_6 conjugates. *Photochem. Photobiol.* 1997; 65:723–729. [PubMed: 9114750]
16. Schmidt-Erfurth U, Diddens H, Birngruber R, Hasan T. Photodynamic targeting of human retinoblastoma cells using covalent low-density lipoprotein conjugates. *Brit. J. Cancer.* 1997; 75:54–61. [PubMed: 9000598]
17. Bisland SK, Singh D, Gariepy J. Potentiation of Chlorin e_6 Photodynamic Activity in Vitro with Peptide-Based Intracellular Vehicles. *Bioconjugate Chem.* 1999; 10:982–992.
18. Uzdensky AB, Dergacheva OY, Zhavoronkova AA, Reshetnikov AV, Ponomarev GV. Photodynamic effect of novel chlorin e_6 derivatives on a single nerve cell. *Life Sciences.* 2004; 74:2185–2197. [PubMed: 14969720]
19. Zheng X, Morgan J, Pandey SK, Chen Y, Tracy E, Baumann H, Missert JR, Batt C, Jackson J, Bellnier DA, Henderson BW, Pandey RK. Conjugation of 2-(1'-hexyloxyethyl)-2-devinylpyropheophorbide-a (HPPH) to carbohydrates changes its subcellular distribution and enhances photodynamic activity in Vivo. *J. Med. Chem.* 2009; 52:4306–4318. [PubMed: 19507863]
20. Sibrian-Vazquez M, Jensen TJ, Fronczek FR, Hammer RP, Vicente MGH. Synthesis and characterization of positively charged porphyrin-peptide conjugates. *Bioconjugate Chem.* 2005; 16:852–863.
21. Sibrian-Vazquez M, Jensen TJ, Vicente MGH. Influence of the number and distribution of NLS peptides on the photosensitizing activity of multimeric porphyrin-NLS. *Org. Biomol. Chem.* 2010; 8:1160–1172. [PubMed: 20165809]
22. Hargus JA, Fronczek FR, Vicente MGH, Smith KM. Mono-(L)-aspartylchlorin- e_6 . *Photochem. Photobiol.* 2007; 83:1006–1015. [PubMed: 17880493]
23. Jensen TJ, Vicente MGH, Luguya R, Norton J, Fronczek FR, Smith KM. Effect of overall charge and charge distribution on cellular uptake, distribution and phototoxicity of cationic porphyrins in HEp2 cells. *J. Photochem. Photobiol. B: Biol.* 2010; 100:100–111.
24. Josefsen LB, Boyle RW. Photodynamic therapy and development of metal-based photosensitizers. *Metal-Based Drugs.* 2008; 276109:1–24.
25. Ol'shevskaya VA, Savchenko AN, Zaitsev AV, Kononova EG, Petrovskii PV, Ramonova AA, VVT, Uvarov OV, Moisenovich MM, Kalinin VN, Shtil AA. Novel metal complexes of boronated chlorin e_6 for photodynamic therapy. *J. Organometal. Chem.* 2009; 694:1632–1637.
26. Obata M, Hirohara S, Tanaka R, Kinoshita I, Ohkubo K, Fukuzumi S, Tanihara M, Yano S. In vitro heavy-atom effect of palladium(II) and platinum(II) complexes of pyrrolidine-fused chlorin in photodynamic therapy. *J. Med. Chem.* 2009; 52:2747–2753. [PubMed: 19378972]
27. Tremblay T, Leroy S, Freitag L, Copin MC, Brun PH, Marquette C-H. Endobronchial phototoxicity of WST 09 (Tookad), a new fast-acting photosensitizer for photodynamic therapy: preclinical study in the pig. *Photochem. Photobiol.* 2003; 78:124–130. [PubMed: 12945579]
28. Trachtenberg J, Bogaards A, Weersink RA, Haider MA, Evans A, McCluskey SA, Scherz A, Gertner MR, Yue C, Appu S, Aprikian A, Savard J, Wilson BC, Elhilali M. Vascular targeted photodynamic therapy with palladium-bacteriopheophorbide photosensitizer for recurrent prostate cancer following definitive radiation therapy: assessment of safety and treatment response. *J. Urology.* 2007; 178:1974–1979.
29. Smith KM, Goff DA, Simpson DJ. Meso substitution of chlorophyll derivatives: direct route for transformation of bacteriopheophorbides d into bacteriopheophorbides c. *J. Am. Chem. Soc.* 1985; 107:4946–4954.
30. Kenner GW, McCombie SW, Smith KM. Separation and oxidative degradation of chlorophyll derivatives. *J. Chem. Soc., Perkin Trans. 1.* 1973:2517–2523.
31. Wasielewski MR, Svec WA. Synthesis of covalently linked dimeric derivatives of chlorophyll a, chlorophyll b and bacteriochlorophyll a. *J. Org. Chem.* 1980; 45:1969–1674.
32. Han S-Y, Kim Y-A. Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron.* 2004; 60:2447–2467.
33. Fisher JW, Trinkle KL. Iodide dealkylation of benzyl, PMB, PNB, and t-Butyl N-acyl amino acid esters via lithium ion coordination. *Tetrahedron Lett.* 1994; 35:2505–2508.

34. Smith KM, Lewis MW. Partial synthesis of chlorophyll a from rhodochlorin. *Tetrahedron Lett.* 1981; 37:399–403.
35. Hudlicky M. An improved apparatus for the laboratory preparation of diazomethane. *J. Org. Chem.* 1980; 45:5377–5378.
36. Wiehe A, Stollberg H, Runge S, Paul A, Senge MO, Roder B. PDT-related photophysical properties of conformationally distorted palladium(II) porphyrins. *J. Porphyrins Phthalocyanines.* 2001; 5:853–860.
37. Romanova ZS, Deshayes K, Piotrowiak P. Remote intermolecular “heavy-atom effect”: spin-orbit coupling across the wall of a hemicarcerand. *J. Am. Chem. Soc.* 2001; 123:2444–2445. [PubMed: 11456902]
38. Meseguer B, Alonso-Diaz D, Griebenow N, Priv.-Doz TH, Waldmann H. Solid-phase synthesis and biological evaluation of a teleocidin library—discovery of a selective PKC δ down regulator. *Chem. Eur. J.* 2000; 6:3943–3957.
39. Shiah J-G, Sun Y, Peterson CM, Straight RC, Kopec J. Antitumor activity of *N*-(2-hydroxypropyl)methacrylamide copolymer-mesochlorin e₆ and adriamycin conjugates in combination treatments. *Clin. Cancer Res.* 2000; 6:1008–1015. [PubMed: 10741728]
40. Ol'shevskaya AV, Nikitina RG, Savchenko AN, Malshakova MV, Vinogradov AM, Golovina GV, Belykh DV, Kutchin AV, Kaplan MA, Kalinin VN, Kuzmin VA, Shtil AA. Novel boronated chlorin e₆ based photosensitizer: synthesis, binding to albumin and antitumor efficacy. *Bioorg. Med. Chem.* 2009; 17:1297–1306. [PubMed: 19121946]
41. Kessel D. Correlation between subcellular localization and photodynamic efficacy. *J. Porphyrins Phthalocyanines.* 2004; 8:1009–1014.
42. Wan Q, Liu L, Xing D, Chen Q. Bid is required in NPe6-PDT-induced apoptosis. *Photochem. Photobiol.* 2008; 84:250–257. [PubMed: 18173728]
43. Reiners JJ Jr, Caruso JA, Mathieu P, Chelladurai B, Yin X-M, Kessel D. Release of cytochrome c and activation of pro-caspase-9 following lysosomal photodamage involves bid cleavage. *Cell Death and Differentiation.* 2002; 9:934–944. [PubMed: 12181744]

propionic (17^3) acetic (15^2) formic (13^1)

Figure 1.
 17^3 , 15^2 and 13^1 regioisomers of “R”-substituted chlorin e₆.

Figure 2. Energy minimized conformations in gas phase for chlorin e_6 derivatives (a) [13¹ LysChlorin e_6 TME]⁺, (b) **13**, (c) **7b** and (d) **3**. Optimization by energy was carried out at HF/6-31G level.

Figure 3.

Time-dependent uptake of chlorin e_6 (5, black line) and its derivatives 17^3 -LysChlorin e_6 TME (3, brown line), 15^2 -AspChlorin e_6 DME (7a, light blue line), 15^2 -LysChlorin e_6 TME (7b, red line), 15^2 -AspPdChlorin e_6 DME (8a, green line), 15^2 -LysPdChlorin e_6 TME (8b, blue line), 13^1 -AspChlorin e_6 DME (9, maroon line), 13^1 - β AlaAspChlorin e_6 DME (11, purple line) and 13^1 -EDLysChlorin e_6 DME (13, pink line) at $10 \mu\text{M}$ by HEp2 cells.

Figure 4.

Dark toxicity of chlorin e_6 (5, black line) and its derivatives 17³-LysChlorin e_6 TME (3, brown line), 15²-AspChlorin e_6 DME (7a, light blue line), 15²-LysChlorin e_6 TME (7b, red line), 15²-AspPdChlorin e_6 DME (8a, green line), 15²-LysPdChlorin e_6 TME (8b, blue line), 13¹-AspChlorin e_6 DME (9, maroon line), 13¹- β AlaAspChlorin e_6 DME (11, purple line) and 13¹-EDLysChlorin e_6 DME (13, pink line) toward HEP2 cells using 1 J/cm² light dose and the Cell Titer Blue assay.

Figure 5. Phototoxicity of chlorin e_6 (5, black line) and its derivatives 17³-LysChlorin e_6 TME (3, brown line), 15²-AspChlorin e_6 DME (7a, light blue line), 15²-LysChlorin e_6 TME (7b, red line), 15²-AspPdChlorin e_6 DME (8a, green line), 15²-LysPdChlorin e_6 TME (8b, blue line), 13¹-AspChlorin e_6 DME (9, maroon line), 13¹- β AlaAspChlorin e_6 DME (11, purple line) and 13¹-EDLysChlorin e_6 DME (13, pink line) toward HEP2 cells using 1 J/cm² light dose and the Cell Titer Blue assay.

Figure 6. Subcellular localization of conjugate 13¹ AspCe₆DME (9) in HEp2 cells at 10 μM for 18 h (a) phase contrast, (b) overlay of conjugate 9 compound and phase contrast, (c) ER tracker Blue/White fluorescence (e) MitoTracker Green fluorescence, (g) BoDIPY Ceramide, (i) LysoSensor Green fluorescence, and (d, f, h, j) overlays of organelle tracers with compound fluorescence. Scale bar: 10 μm.

Figure 7. Subcellular localization of conjugate $13^1 \beta\text{-Ala-AspCe}_6\text{DME}$ (**11**) in HEp2 cells at $10 \mu\text{M}$ for 18 h (a) phase contrast, (b) overlay of conjugate **11** and phase contrast, (c) ER tracker Blue/White fluorescence (e) MitoTracker Green fluorescence, (g) BoDIPY Ceramide, (i) LysoSensor Green fluorescence, and (d, f, h, j) overlays of organelle tracers with compound fluorescence. Scale bar: $10 \mu\text{m}$.

Figure 8. Subcellular localization of conjugate 13^1 ED-LysCe₆DME (**13**) in HEP2 cells at 10 μ M for 18 h (a) phase contrast, (b) overlay of conjugate **11** and phase contrast, (c) ER tracker Blue/White fluorescence (e) MitoTracker Green fluorescence, (g) BoDIPY Ceramide, (i) LysoSensor Green fluorescence, and (d, f, h, j) overlays of organelle tracers with compound fluorescence. Scale bar: 10 μ m.

Scheme 1.

Reaction conditions: a) TFA:H₂O 4:1, 0 °C, 1 h, 90%; b) DCC, DMAP, H-Lys(Boc)-OMe•HCl, DIEA, CH₂Cl₂, rt, 14 h, 52%; c) NaOMe, THF, 0 °C, 4 h, 89%; d) TFA, CH₂Cl₂, thioanisole, 0 °C to rt, 12 h, 72%; e) 5% H₂SO₄/MeOH, rt, 12 h, 96%; f) NaOMe, THF, 0 °C, 4 h, 98%; g) 18 eq LiI, EtOH, reflux, 24 h, 21%; h) 5% H₂SO₄/MeOH, rt, 12 h, 99%.

Scheme 2.

Reaction conditions: a) DCC, DMAP, CH_2Cl_2 , rt, 2 h; H-Asp(Boc) $_2$ •HCl or H-Lys(Boc)-OMe•HCl, DIEA, CH_2Cl_2 , rt, 14 h; b) CH_2N_2 , CH_2Cl_2 , 30 min; c) TFA, CH_2Cl_2 , thioanisole, 0 °C to rt, 12 h (**7a**: 45%, **7b**: 77%); d) $\text{Pd}(\text{OAc})_2$, THF, 60 °C, 3 h (**8a**: 98%, **8b**: 99%).

Scheme 3.

Reaction conditions: a) L-Asp(OtBu)₂•HCl, DIEA, CH₂Cl₂, HOBt, TBTU, DMF, rt, 14 h, 66%; b) TFA, CH₂Cl₂, thioanisole, 0 °C to rt, 12 h, 88%; c) β-Ala(OtBu)•HCl, DIEA, CH₂Cl₂, HOBt, TBTU, DMF, rt, 12 h, 68%; d) TFA, CH₂Cl₂, thioanisole, 0 °C to rt, 12 h, 95%; e) L-Asp(OtBu)₂•HCl, DIEA, CH₂Cl₂, HOBt, TBTU, DMF, rt, 12 h, 87%; f) TFA, CH₂Cl₂, thioanisole, 0 °C to rt, 12 h, 97%.

Scheme 4.

Reaction conditions: a) NH₂C₂H₄NH₂, CHCl₃, rt, 24 h, 91%; b) (Boc)Lys(Boc)-OH, DIEA, CH₂Cl₂, HOBt, TBTU, DMF, rt, 12 h, 78%; c) TFA, CH₂Cl₂, thioanisole, 0 °C to rt, 12 h, 81%.

Table 1Cytotoxicity (HEp2 cells) for chlorin e₆ and its derivatives using the Cell Titer Blue assay

Compound	Dark toxicity (IC ₅₀ , μ M)	Phototoxicity (IC ₅₀ , μ M)	Ratio
Chlorin e ₆ (5)	>400	20.8	>19.2
17 ³ -LysChlorin e ₆ TME (3)	>400	26.2	>15.3
15 ² -AspChlorin e ₆ DME (7a)	373.1	4.0	93.4
15 ² -LysChlorin e ₆ TME (7b)	>400	28.8	>13.9
15 ² -AspPdChlorin e ₆ DME (8a)	324.8	16.7	19.4
15 ² -LysPdChlorin e ₆ TME (8b)	>400	3.3	>121.2
13 ¹ -AspChlorin e ₆ DME (9)	284.6	0.61	466.6
13 ¹ - β AlaAspChlorin e ₆ DME (11)	383.9	0.82	468.2
13 ¹ -EDLysChlorin e ₆ DME (13)	268.4	1.34	200.3

Table 2Major (++) and minor (+) subcellular sites of localization for chlorin e₆ and its derivatives in HEp2 cells

Compound	Lysosomes	ER	Golgi	Mitochondria
Chlorin e ₆ (5)	+	++	–	–
17 ³ -LysChlorin e ₆ TME (3)	+	++	+	–
15 ² -AspChlorin e ₆ DME (7a)	++	++	–	+
15 ² -LysChlorin e ₆ TME (7b)	++	++	+	–
15 ² -AspPdChlorin e ₆ DME (8a)	++	+	++	–
15 ² -LysPdChlorin e ₆ TME (8b)	+	++	–	–
13 ¹ -AspChlorin e ₆ DME (9)	+	++	++	++
13 ¹ -βAlaAspChlorin e ₆ DME (11)	++	++	++	–
13 ¹ -EDLysChlorin e ₆ DME (13)	++	++	++	–