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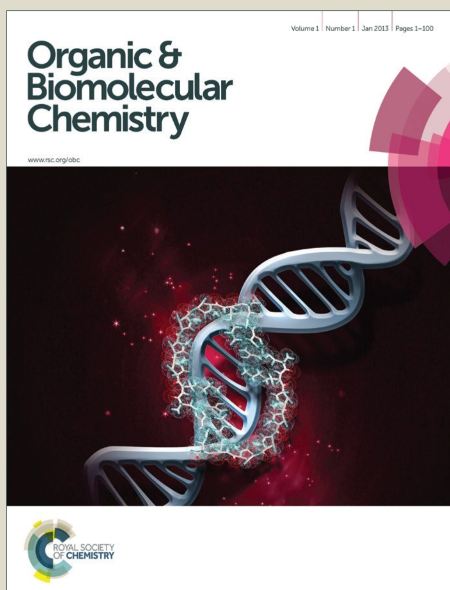

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Syntheses and Cellular Investigations of Di(aspartate) and Aspartate-lysine Chlorin e_6 Conjugates

R. G. Waruna Jinadasa, Zehua Zhou, M. Graça H. Vicente and Kevin M. Smith*

Chlorin e_6 is a tricarboxylic acid degradation product of chlorophyll a. Four chlorin e_6 bis(amino acid) conjugates were regioselectively synthesized bearing two aspartate conjugates in the 13^1,17^3- and 15^2,17^3-positions, or at the 13^1,15^2 via an ethylene diamine linker. One additional conjugate bearing two different amino acids, lysine at 13^1 via an ethylene diamine linker and an aspartate at 15^2 via a β-alanine linker was also synthesized. The cytotoxicity and uptake of four di(amino acid) chlorin e_6 conjugates were investigated in human HEp2 cells, and compared with chlorin e_6. The most cytotoxic and most taken up conjugates were the zwitterionic 13^1,15^2-disubstituted conjugates **28** and **33**; these also localized in multiple organelles. In contrast, the tetra-anionic 13^1,17^3- and 15^2,17^3-di-aspartyl chlorin e_6 conjugates **12** and **13** showed low dark cytoxicity and lower phototoxicity compared with chlorin e_6.

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Electronic Supplementary Information (ESI) available: Copies of ^{1}H NMR spectra, ^{13}C NMR spectra, dark toxicity curves, and subcellular localizations for chlorin e_6 (21 pages). See DOI: 10.1039/x0xx00000x

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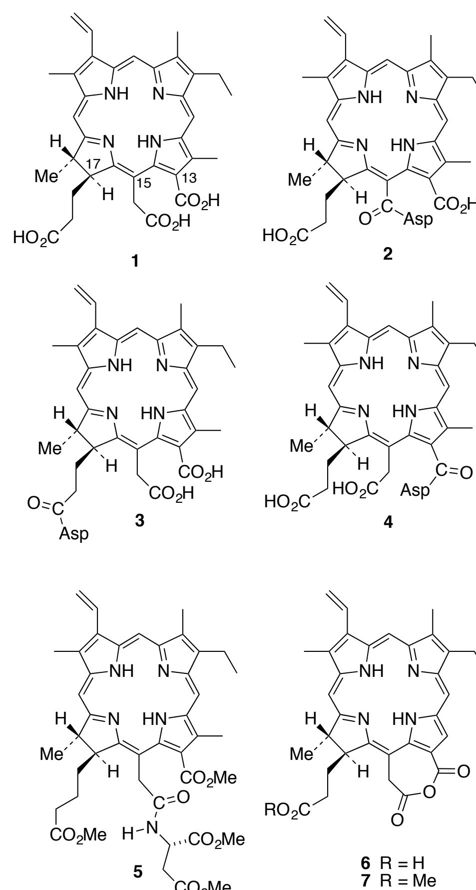
Introduction

Photodynamic therapy (PDT) is special form of cancer photochemotherapy involving a photosensitizer and light. Selective uptake of a photosensitizer into tumor cells followed by irradiation with laser light within the “therapeutic window” (λ_{max} 600-800 nm) produces singlet oxygen and other reactive oxygen species (ROS).[1-5] Singlet oxygen, in particular, readily reacts with nearby local biomolecules (unsaturated lipids, amino acids and DNA) to cause destruction of apparatus within the cell. Because of limited diffusion of singlet oxygen through tissue, the destructive PDT effects are mainly localized to the photosensitizer-containing cells, thus minimizing the possibility of damage to normal cells near the tumor. Two essentials for the success of PDT treatment are: (i) the tumor-targeting ability of the photosensitizer, and (ii) the wavelength of the light required to activate the photosensitizing drug. Researchers aim for minimal “dark” toxicity, a high quantum yield of triplet state formation upon excitation, high selectivity for tumor cells compared with normal cells, reasonably rapid clearance from normal tissues, and a strong absorption peak between 600 and 800 nm to facilitate maximum light penetration through tumor and normal tissues.

Photofrin is a porphyrin-based photosensitizer mixture that was developed and approved in several countries for the PDT of melanoma, lung, digestive tract, genitourinary tract and for treatment of Barrett’s esophagus.[3-6] It suffers from the fact that it is ineffective at wavelengths above 630 nm, and that it clears from tissues so slowly that patients can suffer from residual skin photosensitivity for weeks after treatment. Thus, a number of so-called second generation tetrapyrrole photosensitizers have been invented, these usually possessing strong red-shifted absorption maxima at the red end of the therapeutic window. Examples are tetra(meta-hydroxyphenyl)chlorin, (mTHPC or Temoporfin), 2-(1-hexyloxyethyl)-2-devinyl-pyropheophorbide a (HPPH or Photochlor), mono-L-aspartylchlorin e_6 (LS-11, or Talaporfin) and a phthalocyanine called “Pc4”.[3-6] Of these, chlorins (dihydroporphyrins) have received special attention for PDT because of their intense absorptions above 640 nm. Indeed, chlorophyll a derivatives are amphiphilic tetrapyrroles that have been extensively investigated and shown to exhibit low dark toxicities while at the same time able to generate cytotoxic ROS upon irradiation with light.[7-9] Both HPPH and Talaporfin are in advanced-stage clinical trials for oncologic PDT treatment;[10-13] they exhibit superior PDT activity, and are rapidly cleared from tissue such that residual photosensitivity in patients is minimized compared with Photofrin.



Chlorin e_6 (**1**) is a particularly interesting chlorophyll *a* derivative because it possesses no less than three chemically different carboxylic acid functions (that could potentially be differentially functionalized with biomolecules): a nuclear aromatic acid in position 13^1, a nuclear acetic acid in position 15^2, and a propionic side-chain acid that is truly aliphatic, in position 17^3. Conjugations of peptides, sugars, lipoproteins, polyethylene glycols, and polyamines with chlorin e_6 have been reported.[14-22] Amino acid conjugations, in particular, have been shown to improve the PDT effects of tetrapyrrole compounds, and the type and position on the macrocyclic core can have a major impact upon efficacy in PDT. Talaporfin (**2**) was originally formulated in 1987 as the 17^3-aspartyl derivative (**3**) of chlorin

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e_6. Gomi et al.[23] used spectroscopic techniques (mostly) to propose that Talaporfin was in fact (**2**), not (**3**), but this work was almost universally ignored until our own group confirmed by total synthesis of the three possible isomers (**2**-**4**) (and comparison with authentic material), that (**2**) was indeed the correct structure for Talaporfin.[24] A definitive X-ray structure of the tetramethyl ester (**5**) of Talaporfin finally ended all conjecture.[24] It was proposed that the anhydride (**6**) is an intermediate in the formation of (**2**) whereby the aspartic acid is not involved in the first step of the reaction. The anhydride (**6**) is formed by dehydration using only DCC or EDC, and then the aspartic acid nucleophile opens the anhydride ring by attacking the more electrophilic aliphatic carbonyl at position 15^2.[24] This point of view was solidified by isolation of the anhydride, a single crystal X-ray structure of the anhydride methyl ester (**7**), and the demonstration that ring-opening of the anhydride with eight different nucleophiles resulted only in formation of the 15^2-conjugate (**8**).[25] Bis-conjugation was shown to be possible (at 17^3 and 15^2) in one case,[25] or when two equivalents of DCC or EDC were used,[26] but at no time was the 17^3-conjugate the sole product of the conjugation.

Making use of the differential conjugation methods developed in our earlier work,[24] regioselective amino acid conjugates at the 17^3, 15^2 and 13^1 positions of chlorin e_6 were synthesized[27] to evaluate the effect *in vitro* of the conjugation site and structure of the amino acid on the PDT efficacy of the conjugates, using human carcinoma HEp2 cells. Syntheses of the 17^3 and 13^1 aspartyl regioisomers of mono-L-aspartylchlorin e_6 were developed, along with the corresponding cationic lysine derivatives (which would be expected to show strong interactions with anionic biomolecules and membranes, resulting in enhanced PDT effectiveness.[28] In addition, molecules with an ethylene diamine or β-alanine spacer group between the macrocycle and the amino acid were also synthesized. Based on *in vitro* biological evaluations, it was concluded that the site of amino acid conjugation, rather than the nature of the amino acid conjugated, was the major determinant of phototoxicity.[27] 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, was 13^1-aspartylchlorin e_6. Unfortunately, poor solubility of these molecules was one of the major drawbacks in the cellular studies, and some of the sensitizer molecules tended to self-aggregate easily, resulting in lower cell uptake. In the present work, in order to overcome these shortcomings, we report the syntheses and biological evaluations of a new series of chlorin e_6 derivatives that possess two amino acids in a single chlorin e_6 molecule (Figure 1).

Kimani et al.[19] recently reported the synthesis of mono-, di- and tri-PEGylated chorin e_6 photosensitizers with tri(ethylene glycol) attached at the three carboxylic acid positions in chlorin e_6.[19] These were tested for solubility and hydrolytic stability, as well as phototoxicity, cell uptake and localization in ovarian OVCAR-5 cancer cells. Their results showed that increasing the number of PEG groups increased the water solubility and cellular uptake of the compounds. Computational studies also indicated that the PEG groups prevent formation of aggregates by π-π stacking, by wrapping onto the chlorin ring. Most significantly an increased number of PEG groups increased the phototoxicity, and cellular uptake. We therefore anticipated that an increase in the overall charge of the molecule and resulting amphiphilicity, might increase the water solubility[29,30] and concomitantly decrease the self-aggregation in biological media.[31] Our hypothesis was that a higher number of charged substituents would increase both the phototoxicity and the cellular uptake. To test this hypothesis all three possible regioisomers of diamino acid chlorin e_6 derivatives were synthesized (Figure 1). It was found from our previous study, that the position of the amino acid plays a vital role in determining the conformation of the molecule.[27] Therefore new synthetic routes were developed to prepare all three regioisomers. Both the 15^2,17^3- and 13^1,17^3-di(amino acid) derivatives were synthesized starting from chlorin e_6 (**1**) and the 13^1,15^2-diamino derivatives were synthesized from pheophytin a. Thus, the aims of the present work were to determine: (i) the degree to which di-(amino acid)-chlorin e_6 derivatives were taken up and localized in cancer cells, (ii) the main site(s) of intracellular accumulation, and (iii) whether the position and charge of the amino acid substituents influences the dark and photo-toxicity.

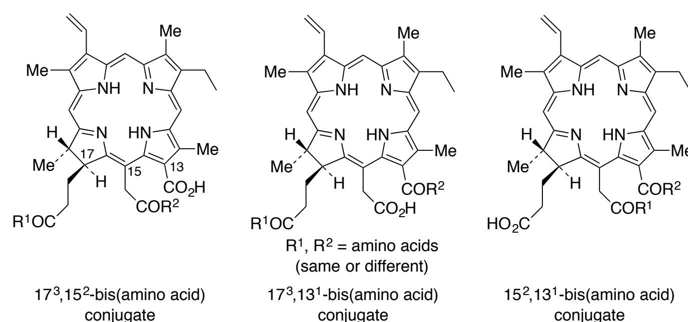

Figure 1: All possible bis-amino acid regioisomers of chlorin e_6

Results and Discussion

Synthesis of 15^2,17^3-diaspartylchlorin e_6 derivative

A major side product in the synthesis of 15^2-mono-aspartylchlorin e_6 derivative (**2**) was the 15^2,17^3-diaspartylchlorin e_6 derivative **8**,[26] the yield of which can be increased by increasing the number of equivalents of coupling reagent and aspartic acid. According to our proposed mechanism,[24] DCC and DMAP activated the acetic side chain in **1** and formed the anhydride intermediate **6**. The excess coupling reagent can further activate the propionic acid side chain to form the *O*-acylisourea anhydride intermediate **9** (Scheme 1).[25]

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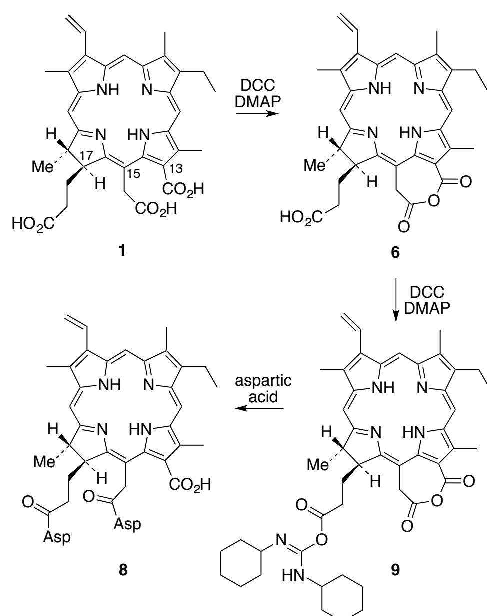
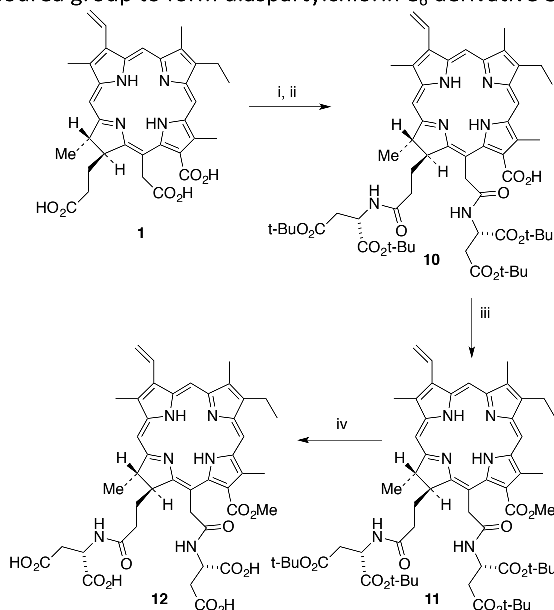


Scheme 1. Proposed intermediates in the formation of $15^2,17^3$ di-aspartic acid derivative **8**.

Thereafter, the free amine group of the amino acid can attack both carbonyl groups in intermediate **9**, one in the anhydride ring and the other one in the *O*-acylisourea group. Mechanistic studies revealed the nucleophile first attacks the more reactive 15^2 position of the anhydride ring instead of the 13^1 position, regardless of the size or nucleophilicity of the molecule. Then the second equivalent can react with the *O*-acylisourea group to form diaspartylchlorin e_6 derivative **8**.



Scheme 2. Synthesis of $15^2,17^3$-diaspartylchlorin e_6 methyl ester (**12**).(Reagents: i. 3 equiv. DCC, DMAP, CH_2Cl_2, 2 h; ii. 3 equiv. Asp di-t-Bu.HCl, DIEA, rt, 24 h; iii. CH_2N_2, CH_2Cl_2, 5 min, 30% (3 steps); iv. TFA/CH_2Cl_2 (1:3), thioanisole, 6 h, rt, 88%.)

The detailed synthetic route to $15^2,17^3$-diaspartylchlorin e_6 methyl ester (**12**) is shown in Scheme 2. It was possible to obtain diaspartyl methyl ester **11** in 33% overall yield from chlorin e_6 (**1**). The optimal yield of diaspartyl chlorin e_6 **12** (26%) was obtained upon activation of chlorin e_6 with three equivalents of DCC and DMAP in CH_2Cl_2 at room temperature for two hours followed by coupling with 2.5 equivalents of di-*tert*-butyl protected aspartic acid for 24 hours. Less than three equivalents of DCC and aspartic acid tended to form significant amounts of the 15^2-monoaspartic acid conjugate as a side product. When the number of equivalents of either DCC or aspartic acid was increased, the yield of diaspartyl chlorin e_6 **10** improved, but it was not possible to prevent the formation of mono-aspartyl conjugate as a side product. Longer reaction times also helped to increase the yield of diaspartyl derivative **10**. After confirming the formation of *tert*-butyl protected diaspartyl chorin e_6 **10** by TLC and mass spectrometry, freshly prepared diazomethane gas was bubbled through the mixture for five minutes to convert the remaining free acid groups into methyl esters. This reaction was monitored by TLC. It was possible to see two new spots in the TLC plate. A brighter spot with lower R_F value belonged to the desired product $15^2,17^3$-diaspartylchlorin e_6 **11** and the second spot had a higher R_F value similar to that of 15^2-monoaspartlychlorin e_6 methyl ester. Purification was achieved via silica gel column chromatography and the identity of the esterified product **11** was confirmed using ^1H NMR and mass spectrometry. ^1H NMR spectroscopy showed four singlets between 3.0 and 4.5 ppm each integrating to three protons. Of the four peaks, three belong to the methyl groups directly connected to the macrocycle and the remaining peak belongs to the 13^1 methyl ester group. The most downfield singlet at δ 4.25 confirmed the formation of the 13^1 methyl ester because this peak is unique to the methyl group on the formic (13-) side chain of the trimethyl ester of chlorin e_6 (**1**). This observation confirmed that aspartic acid coupled with the acetic (15-) and propionic (17-) side chains of the molecule.

In the final step, all four *tert*-butyl esters in molecule **11** were removed to give **12** in a TFA/CH_2Cl_2 mixture for six hours at room temperature. TFA and CH_2Cl_2 were first removed and the product was freeze-dried by dissolving it in a water/acetonitrile mixture. Once the sample was neutralized the color changed from purple to dark green. The identity of product **12** was confirmed by ^1H NMR, ^{13}C NMR and mass spectrometry.

Synthesis of $13^1,17^3$-diaspartylchlorin e_6 derivative

There are two possible routes to synthesize the $13^1,17^3$-chlorin e_6 conjugate **13**: 1) coupling of the first amino acid to the propionic acid chain of pheophorbide a (**14**) and subsequent isocyclic ring opening of pheophorbide **15** with ethylene diamine to give **16**, followed by coupling of the carboxylate of a second amino acid to the free amine group of the resulting chlorin derivative, to give **17** (Scheme 3), or 2) selective esterification of the acetic acid side chain (15^2 ester) of chlorin e_6 (**1**) followed by

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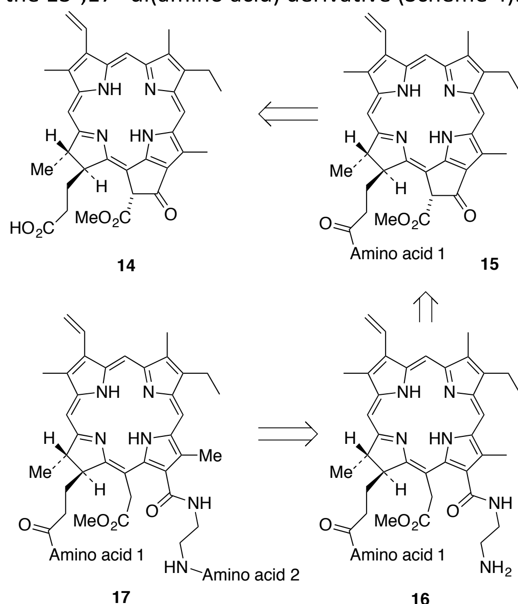
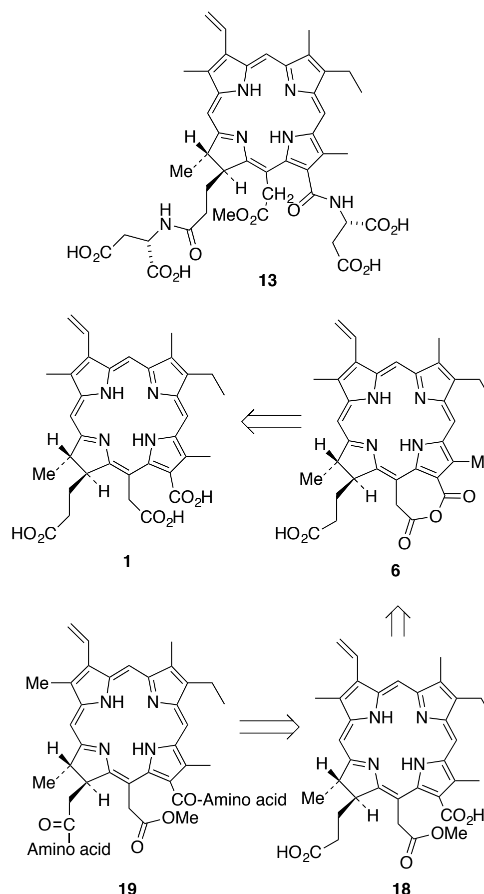
coupling with two equivalents of an identical amino acid to afford the $13^1,17^3$-di(amino acid) derivative (Scheme 4).



Scheme 3. Retrosynthetic route to $13^1,17^3$-di(amino acid) derivatives, Method 1.

In the first method, pheophorbide a (**14**) can be obtained by selective hydrolysis of the phytyl ester group of pheophytin a (obtained from *Spirulina maxima* or *S. pacifica* alga[32]) using a procedure developed by Wasielewski and Svec.[33] Then the amino acid can be coupled to the propionic side chain to obtain the pheophorbide derivative **15**. The pheophorbide isocyclic ring in **15** can be cleaved, using ethylene diamine,[34] to provide **16** bearing a tethered free amine group able to couple with the carboxylic group of a second (potentially different) amino acid.



Scheme 4. Retrosynthetic route to $13^1,17^3$-di(amino acid) derivatives, Method 2

The key step in the second method (Scheme 4) is the selective protection of the acetic acid side chain of chlorin e_6 (**1**) in the presence of the formic and propionic carboxylic side chains. From the previous work, it was found that the acetic acid group of chlorin e_6 can be activated selectively by forming a cyclic anhydride. Chlorin e_6 was activated using one equivalent of DCC and DMAP to form the anhydride intermediate (**6**).[24] Formation of cyclic anhydride **6** can be confirmed by using UV-Vis spectroscopy. Then freshly prepared 0.5 M sodium methoxide was added until the color of the reaction mixture changed from brown-purple to dark green. Mass spectroscopy confirmed the formation of desired product **18**. Purification of di-acid **18** was challenging due to its high polarity. After work-up it was possible to obtain a ^{1}H NMR spectrum of the crude product and this was clear enough to identify the unique peak for the 15^2 methyl ester, and indicated it was sufficiently pure for the next step. Appearance of a new peak at 3.7 ppm for 3 protons provided evidence that esterification took place uniquely at the acetic acid side chain. Then *tert*-butyl-protected aspartic acid was coupled with crude 15^2-chlorin e_6 monomethyl ester (**18**). The optimal yield was obtained with HOBt and TBTU as coupling reagents at room temperature for 48 hours.[35] This reaction was monitored by TLC. The product was purified via

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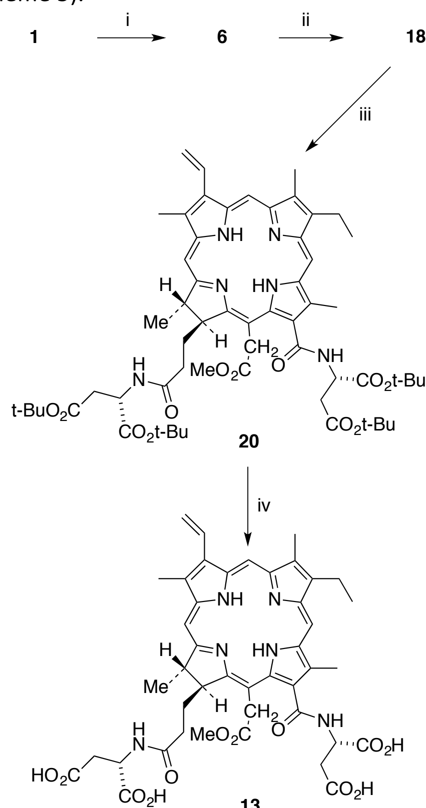
silica gel column chromatography and the first moving band with 5% methanol/DCM was collected. The structure of product **20** was confirmed by mass and ^{1}H NMR spectroscopy. Purified *tert*-butyl protected diaspartyl chlorin e_6 **20** was treated with TFA/CH_2Cl_2 mixture for six hours at room temperature to deprotect all four carboxylic acid groups. Pure compound **13** was obtained after removal of TFA. The final product, $13^1,17^3$-diaspartyl chlorin e_6 **13**, was obtained in 28% yield over four steps (Scheme 5).



Scheme 5. Synthesis of $13^1,17^3$-diaspartyl chlorin e_6 methyl ester (**13**). (Reagents: i. DCC, DMAP, CH_2Cl_2, 1 h; ii. 0.5 M NaOMe/MeOH, 0 °C, 99% (2 steps); iii. HOBt, TBTU, DMF, L-Asp di-t-Bu.HCl, DIEA, CH_2Cl_2, rt, 48 h, 48%; iv. TFA/CH_2Cl_2, thioanisole, 6 h, rt, 53%.).

Synthesis of $13^1,15^2$-di(amino acid) chlorin e_6 derivatives

Previous studies have revealed that the formic and acetic acid derivatives of chlorin e_6 are more potent photosensitizers than the propionic acid conjugates.[27] Thus it was assumed that the $13^1,15^2$-di(amino acid) derivatives may show more potent photosensitizing ability in this di(amino acid) series compared to the two other regioisomers (Figure 1). Therefore two different amino acid conjugates of the $13^1,15^2$ regioisomer were synthesized.

The main challenge was to protect the propionic side chain in the presence of the two other carboxyl groups. There is no known chemical method to selectively methylate the propionic side chain of chlorin e_6 without methylating the acetic side chain. Esterification with methanol under acidic conditions will form the $15^2,17^3$-chlorin e_6 dimethyl ester.[36] Diazomethane will methylate all three carboxylic groups to form chlorin e_6 trimethyl ester.[37] But in the recent literature there is a reported chemo-selective aminolysis of the β-keto ester of methyl pheophorbide a (**21**).[38-40] This opens up a new route to synthesize $13^1,15^2$ chlorin e_6 derivative from methyl pheophorbide a (**21**).

Previously, Shinoda and Osuka reported a facile transesterification of the β-keto ester of methyl pheophorbide a.[41] These authors were able to introduce various alcohols and steroid groups in the presence of 2-chloro-1-methylpyridinium iodide (CMPI) and of 4-(*N*,*N*-dimethylamino)pyridine (DMAP) to the 13^2-position of pheophorbide. Haavikko and coworkers also reported a selective aminolysis of the β-keto ester of the methyl pheophorbide a (**21**).[38] They used various primary, secondary and aromatic amines for the aminolysis of the β-keto ester. It is known that aminolysis of the β-keto ester is usually facile compared to inactivated esters.[42,43] In the previous literature the formation of β-enaminoesters by reaction between secondary amines and β-ketoesters at low temperatures is described.[44] It was noticed that at higher temperatures they tend to form substantial amounts of the corresponding β-ketoamide along with the expected enamino-ester.

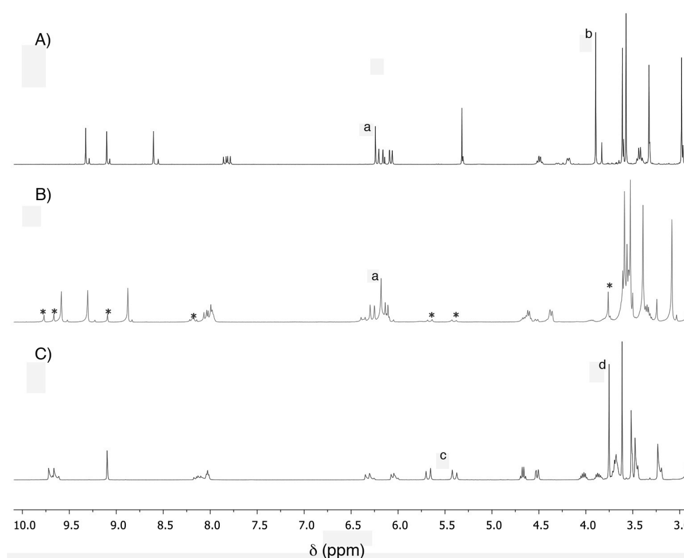


Figure 2. ^{1}H NMR spectral comparison of: A) methyl pheoporbide a (**21**); B) β-ketoamide **22** (*contains 10% of ring-opened product **24**); and C) 13^1-ethylene-diaminyl-chlorin e_6 dimethyl ester **24**. Assignments: a, 13^2 CH; b, 13^3 CO_2CH_3; c, 15^1 CH_2; d, 15^2 CO_2CH_3.

We have observed the same behavior with pheophorbide a.[27] In our previous work,[25] it was possible to open the isocylic ring of pheophorbide a (by cleaving the β-keto ester) using ethylene diamine at room temperature. It was noticed that at higher temperatures (90 °C in toluene) the reaction with ethylene diamine tended to form two major products (two new very close spots in TLC). Both products were isolated using preparative TLC and characterized by ^{1}H NMR and mass spectroscopy. One of the products still clearly shows the unique peak for the

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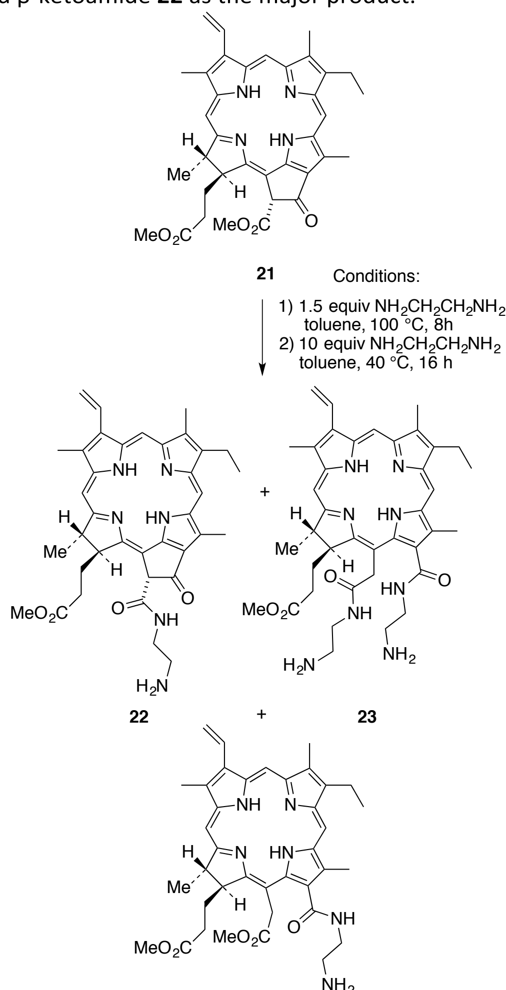
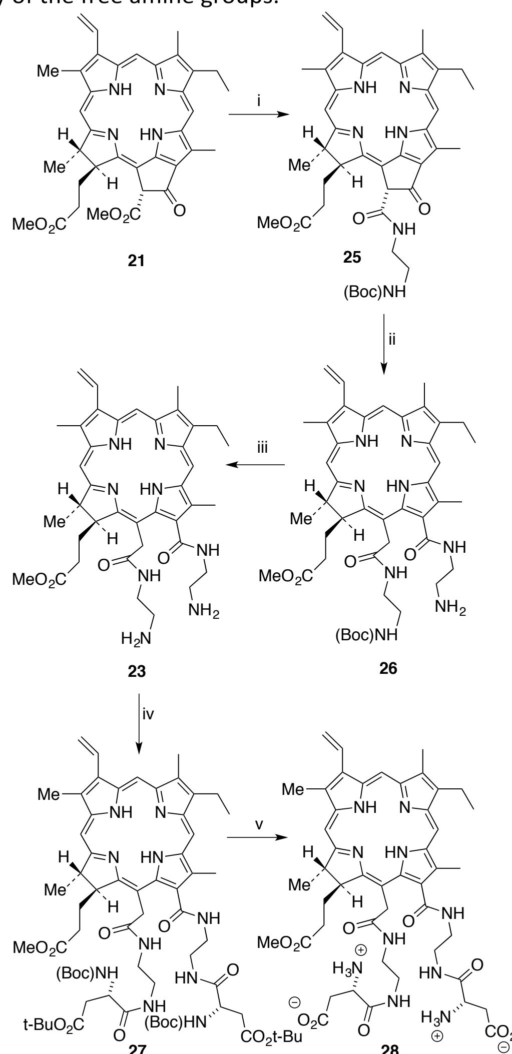
pheophorbide isocylic ring: the proton appears as a sharp singlet at δ 6.26 (Figure 2). The disappearance of the peak for the methyl group of the β-ketoester and new peaks for the ethylene diamine methylene groups confirmed the formation of β-ketoamide **22**. The second product was identified as the expected chlorin e_6 derivative **23**. As reported in the literature, temperature and the concentration of the amine play vital roles in deciding the major product.[38] A large excess of amine and mild heating always produced the classical ring-opened product **23**, and a stoichiometric amount of amine in refluxing toluene yielded β-ketoamide **22** as the major product.



Scheme 6. Attempted synthesis of $13^1,15^2$-bis-ethylenediamine substituted chlorin e_6 derivative **23**

The first attempt to introduce the ethylene diamine to both ester and keto groups of methyl pheophorbide **21** to form the $13^1,15^2$-disubstituted chlorin e_6 derivative **23** was unsuccessful. Both β-keto-ester aminolysis and ring-opening reactions were attempted in one pot. First methyl pheophorbide **21** and ethylene diamine were refluxed in toluene until disappearance of the starting material, as monitored by TLC. Then excess amine was added and the mixture was stirred at 40 °C for 12 hours to complete the ring cleavage reaction. Unfortunately, this process resulted in a mixture of compounds, including the desired product **23**. Mass spectrometry revealed three major compounds (**22**-**24**) in this mixture, as shown in Scheme 6. Purification of these compounds was challenging due to the high polarity of the free amine groups.



Scheme 7. Synthesis of $13^1,15^2$-di(ethylenediaminylaspartyl)chlorin e_6 **28**, (Reagents: i. 1.5 equiv (Boc)$NHCH_2CH_2NH_2$, toluene, 100 °C, 12-16 h, 65%; ii. 10 equiv $NH_2CH_2CH_2NH_2$, toluene, 45 °C, 12 h, 64%; iii. TFA, CH_2Cl_2 1:3, 6 h, rt, 88%; iv. Boc-Asp(O-tBu)OH, DIEA, CH_2Cl_2, TBTU, DMF, 40%; iv. TFA/CH_2Cl_2 1:3, 6 h, rt, 70%.)

It was decided to use half-protected ethylene diamine to simplify the purification process. As indicated by TLC, aminolysis of the β-keto-ester of methyl pheophorbide a (**21**) with protected ethylene diamine was completed after 12 hours. But the second step ring-cleavage was unsuccessful with Boc-protected ethylene diamine, possibly due to steric bulkiness of the amine. Thus, product **25** was isolated and purified before going on to the ring cleavage reaction. As it was possible to

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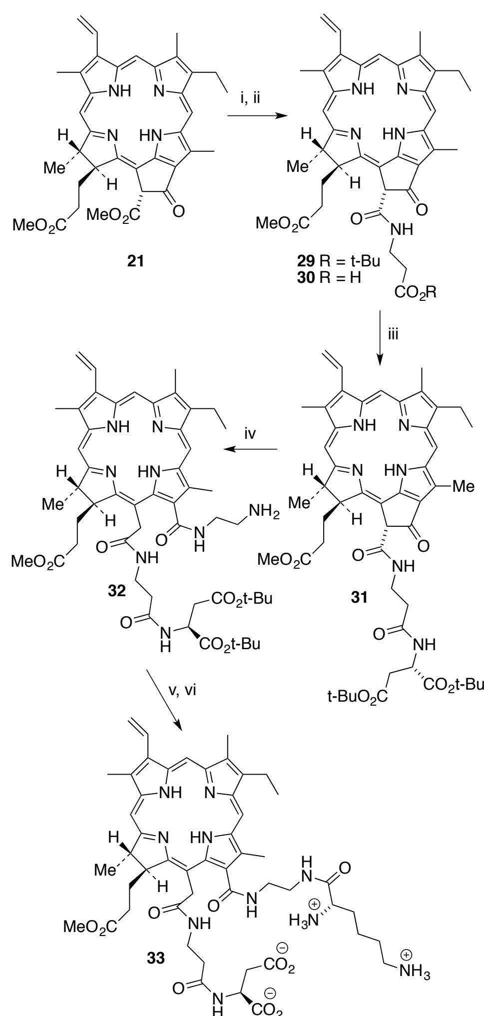
achieve the isocyclic ring cleavage using free ethylene diamine, unprotected ethylene diamine was used for the second step and the diethylene diamine substituted product **26** was obtained. Product **26** was purified using a short silica gel column and its identity was confirmed using mass and ^{1}H NMR spectroscopy. Boc deprotection was achieved using TFA to obtain the free amines (**23**) for the subsequent coupling reaction. Coupling with protected aspartic acid, to give **27** followed by deprotection produced the desired product **28**, in 15% yield over 5 steps from methyl pheophorbide a (**21**) (Scheme 7).

Advantage was taken of a previous route to introduce two types of amino acids to the 13^1 and 15^2 positions. Protected β-alanine was used for the aminolysis reaction of **21** as it greatly reduces the unwanted ring-opened product and creates a carboxylic end to couple aspartic acid through its amino group. Subsequent aminolysis with protected β-alanine, to give **29**, followed by deprotection of the *tert*-butyl ester provided free acid **30**. Its identity was confirmed by mass spectrometry. Free acid **30** was activated and coupled with protected aspartic acid to obtain pheophorbide derivative **31**. This was purified and characterized by ^{1}H NMR and mass spectroscopy. A classical ring-opening reaction was performed with excess ethylene diamine in toluene at 40 °C to provide the chlorin e_6 derivative **32**. The reaction was monitored using UV-Vis spectroscopy, and the color changed from dark green to bright green during isocyclic ring-opening. ^{1}H NMR spectroscopy shows the new peaks for ethylene diamine and mass spectroscopy confirmed the identity of the product. Then Boc-protected lysine was coupled with the free amine group of chlorin e_6 derivative **32** to produce the desired 13^1-ethylenediaminyl-lysyl-15^2-β-alanylaspartylchlorin e_6 *tert*-butyl methyl ester. This product was purified using silica gel chromatography. Finally, global deprotection with TFA in CH_2Cl_2 produced the final product **33** in 9% yield over six steps starting from methyl pheophorbide a (**21**) (Scheme 8).



Scheme 8. Synthesis of 13^1,15^2-di(amino acid) derivative **33**. (Reagents: i. 1.5 equiv $NH_2CH_2CH_2CO_2$t-Bu, toluene, 100 °C, 12-16 h, 54%; ii. TFA, CH_2Cl_2 1:3, 6 h, rt, 99%; iii. L-Asp di-t-Bu.HCl, DIEA, CH_2Cl_2, HOBt, TBTU, DMF, 59%; iv. 10 equiv $NH_2CH_2CH_2NH_2$, toluene, 45 °C, 12 h, 58%; v. Boc-Lys(Boc)-OH, DCHA, DIEA, CH_2Cl_2, HOBt, TBTU, DMF; vi. TFA, CH_2Cl_2 1:3, 6 h, rt, 88%; iv. Boc-Asp(O-t-Bu)OH, DIEA, CH_2Cl_2, TBTU, DMF, 40%; iv. TFA/CH_2Cl_2 1:3, 12 h, rt, 48% (2 steps).

Cell Culture Studies

Cytotoxicity. The dark cytotoxicity and phototoxicity (~1.5 J/cm^2) of chlorin e_6 (**1**) and its di(amino acid) conjugated derivatives **12**, **13**, **28** and **33** were evaluated in HEp2 cells using a Cell Titer Blue assay. Significant differences were observed in the dark and phototoxicity of the di(amino acid) conjugates, as shown in Table 1. These differences are attributed to both the sites of conjugation (13^1,15^2 or 13^1,17^3 or 15^2,17^3) and the overall charge and amphiphilic character of the chlorin e_6 derivatives. While conjugation of two aspartate groups via their α-amine groups gave tetra-anionic conjugates **12** and **13**, the conjugation of two aspartates via the carboxylate groups gave zwitterionic **28**. On the other hand compound **33** bearing an aspartate conjugated via the α-amine group and a lysine conjugated via the carboxylate group, is also zwitterionic.

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In comparison with chlorin e_6 (**1**), the zwitterionic $13^1,15^2$-disubstituted conjugates **28** and **33** showed higher phototoxicity while the tetra-anionic **12** and **13** showed lower phototoxicity. These results are in agreement with our previous observations that 13^1- and 15^2-mono(amino acid) chlorin e_6 derivatives tend to show higher phototoxicities than the corresponding 17^3 derivatives.[27] This might in part be due to the different molecular conformations conferred by 13^1, 15^2 or 17^3 substitutions; while the 17^3 derivatives tend to assume L-shape conformations with the side group positioned perpendicular, or folded over the macrocycle, the 15^2 and in particular the 13^1 derivatives tend to assume linear conformations with the side groups extending away from the macrocycle.[27] Such linear conformations might favor binding to specific intracellular components, inducing higher phototoxicity. However, the zwitterionic $13^1,15^2$ conjugates also showed about 5-fold higher dark cytotoxicity than chlorin e_6 and the tetra-anionic conjugates, and lower dark/photo cytotoxicity ratio. The observed higher dark and photo cytotoxicities of conjugates **28** and **33** might in part be due to their higher cellular uptake (see below).

Table 1. Dark and phototoxicity (~ 1.5 J/cm^2) for chlorin e_6 and its di-amino acid derivatives in HEp2 cells, using a Cell Titer Blue assay

Compound	Dark toxicity (IC_{50}, μM)	Phototoxicity (IC_{50}, μM)	Ratio
Chlorin e_6 (**1**)	350	14.5	24.1
$15^2,17^3$-di(Asp)Ce$_6$ MME (**12**)	>400	35	>11
$13^1,17^3$-di(Asp)Ce$_6$ MME (**13**)	305	26	11.7
$13^1,15^2$-di(EDAsp)Ce$_6$ MME (**28**)	70	11	6.4
13^1-EDLys-15^2-β-AlaAspCe$_6$ MME (**33**)	65	9.1	7.2

Time-dependent Cellular Uptake. The results obtained for the time-dependent uptake of chlorin e_6 and its di(amino acid) derivatives at a concentration of 10 μM in human carcinoma HEp2 cells are shown in Figure 3. Conjugates **12** and **13** showed similar uptake to chlorin e_6, probably due to their low interaction and low permeability across the cell membrane as a result of their negative charge. In addition to phosphate groups, the plasma membranes of tumor cells generally contain higher net negative charge compared with normal cells because of over-expression of polysialic acid residues.[45] On the other hand, the zwitterionic conjugates **28** and **33**, showed much higher cellular uptake at all time points investigated, and after 24 h about 4-fold and 6-fold higher cellular uptake were observed for **28** and **33** than chlorin e_6, respectively. Although the $13^1,15^2$-substituted conjugates **28** and **33** were found to have similar cytotoxicities (see Table 1), conjugate **33** bearing two different amino acid residues, lysine and aspartate, had significant higher uptake than **28** at all times investigated, maybe due to its higher amphiphilicity conferred by substitution with two different amino acids.

The preferential sites of subcellular localization of the di(amino acid) conjugates of chlorin e_6 were evaluated by fluorescence microscopy, and the results are shown in Table 2 and Figures 4-7. The organelle specific probes BODIPY Ceramide (Golgi), LysoSensor Green (Lysosomes), MitoTracker Green (mitochondria), and ER tracker Blue/White fluorescence (ER) were used in the overlay experiments. All conjugates localized in the lysosomes and the Golgi apparatus, and to a smaller extent in the ER. In addition, conjugate **33** bearing lysine and aspartate residues, was also observed in mitochondria, and this might in part be responsible for its higher dark and phototoxicities. These results are in agreement with the known preferential localization of LS-11 (15^2-aspartyl chlorin e_6) in the lysosomes,[46] and with our previous observations of 13^1, 15^2 and 17^3 mono(amino acid) conjugates of chlorin e_6 localizing in lysosomes, ER and Golgi.[27]

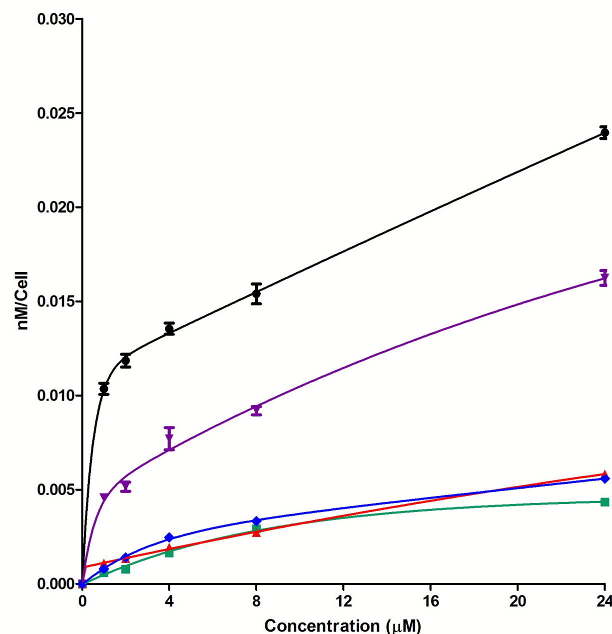

Figure 3. Time-dependent uptake of chlorin e_6 (**1**, green) and its derivatives $15^2,17^3$-di(Asp)Ce$_6$ MME (**12**, blue), $13^1,17^3$-di(Asp)Ce$_6$ MME (**13**, red), $13^1,15^2$-di(EDAsp)Ce$_6$ MME (**28**, purple) and 13^1-EDLys-15^2-β-AlaAspCe$_6$ MME (**33**, black), at 10 μM by HEp2 cells.

Table 2. Major and minor subcellular sites of localization for chlorin e_6 and its di-amino acid derivatives in HEp2 cells

Compound	Lyso	ER	Golgi	Mito
Chlorin e_6 (**1**)	+	++	-	-
$15^2,17^3$-di(Asp)Ce$_6$ MME (**12**)	+	+	+++	-
$13^1,17^3$-di(Asp)Ce$_6$ MME (**13**)	+++	+	+++	-
$13^1,15^2$-di(EDAsp)Ce$_6$ MME (**28**)	+++	+	+++	-
13^1-EDLys-15^2-β-AlaAspCe$_6$ MME (**33**)	+++	+	+++	+

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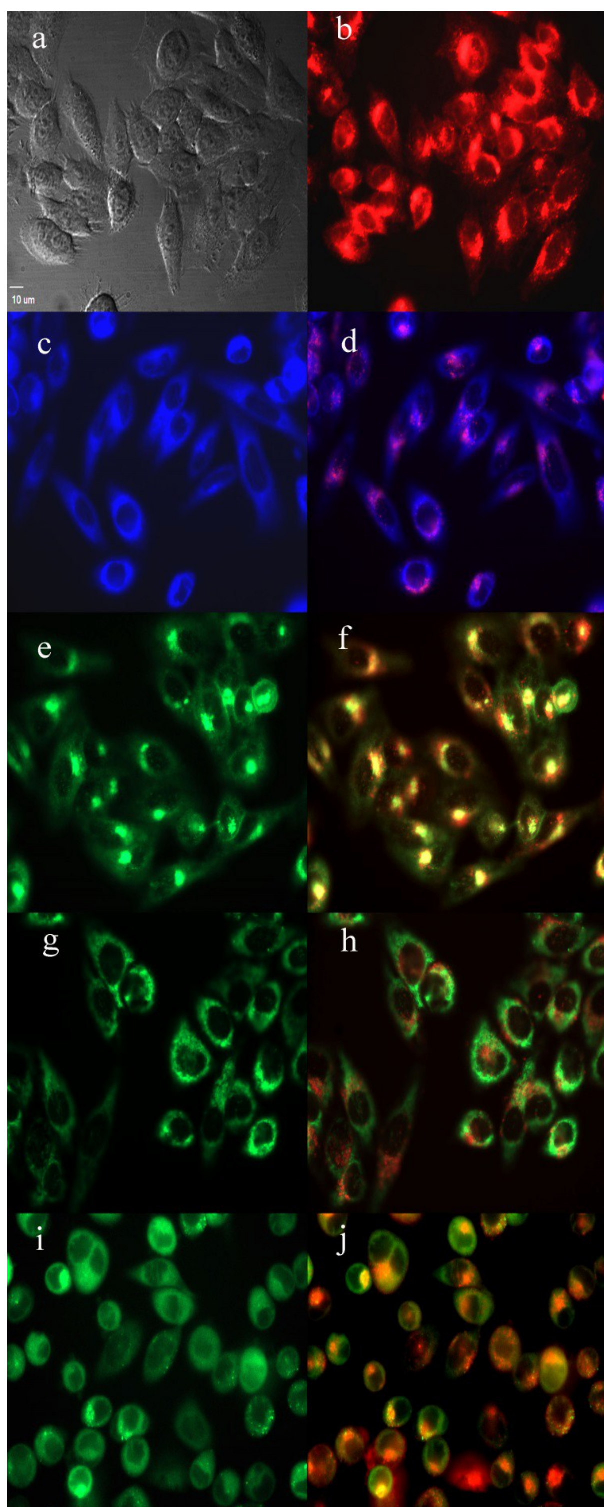

Figure 4. Subcellular localization of 15^2,17^3-di(aspartate) chlorin e_6 **12** in HEp2 cells at 10 µM for 6h, (a) phase contrast, (b) overlay of **12** and phase contrast, (c) ER Tracker Blue, (d) overlay of **12** and ER Tracker Blue, (e) BODIPY Ceramide, (f) overlay of **12** and BODIPY Ceramide, (g) MitoTracker Green, (h) overlay of **12** and MitoTracker Green, (i) LysoSensor Green, (j) overlay of **12** and LysoSensor Green. Scale bar: 10 µm.

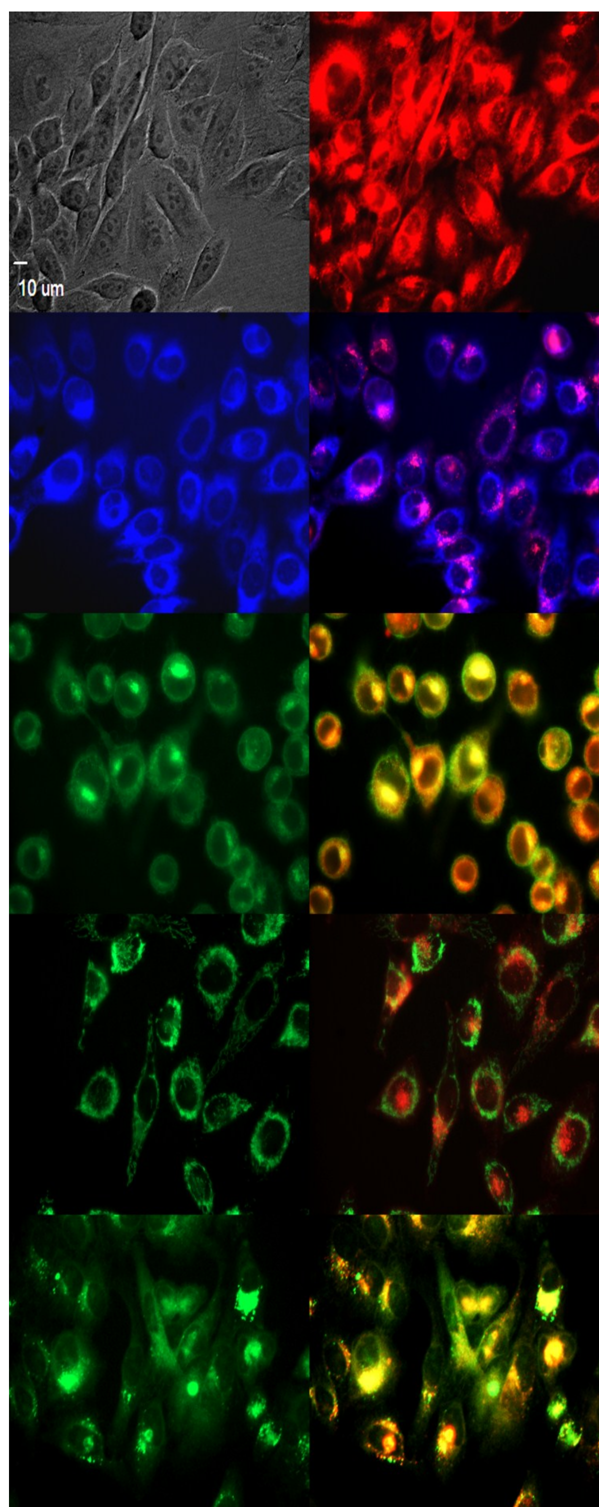

Figure 5. Subcellular localization of 15^2,17^3-di(aspartate) chlorin e_6 **13** in HEp2 cells at 10 µM for 6h, (a) phase contrast, (b) overlay of **13** and phase contrast, (c) ER Tracker Blue, (d) overlay of **13** and ER Tracker Blue, (e) BODIPY Ceramide, (f) overlay of **13** and BODIPY Ceramide, (g) MitoTracker Green, (h) overlay of **13** and MitoTracker Green, (i) LysoSensor Green, (j) overlay of **13** and LysoSensor Green. Scale bar: 10 µm.

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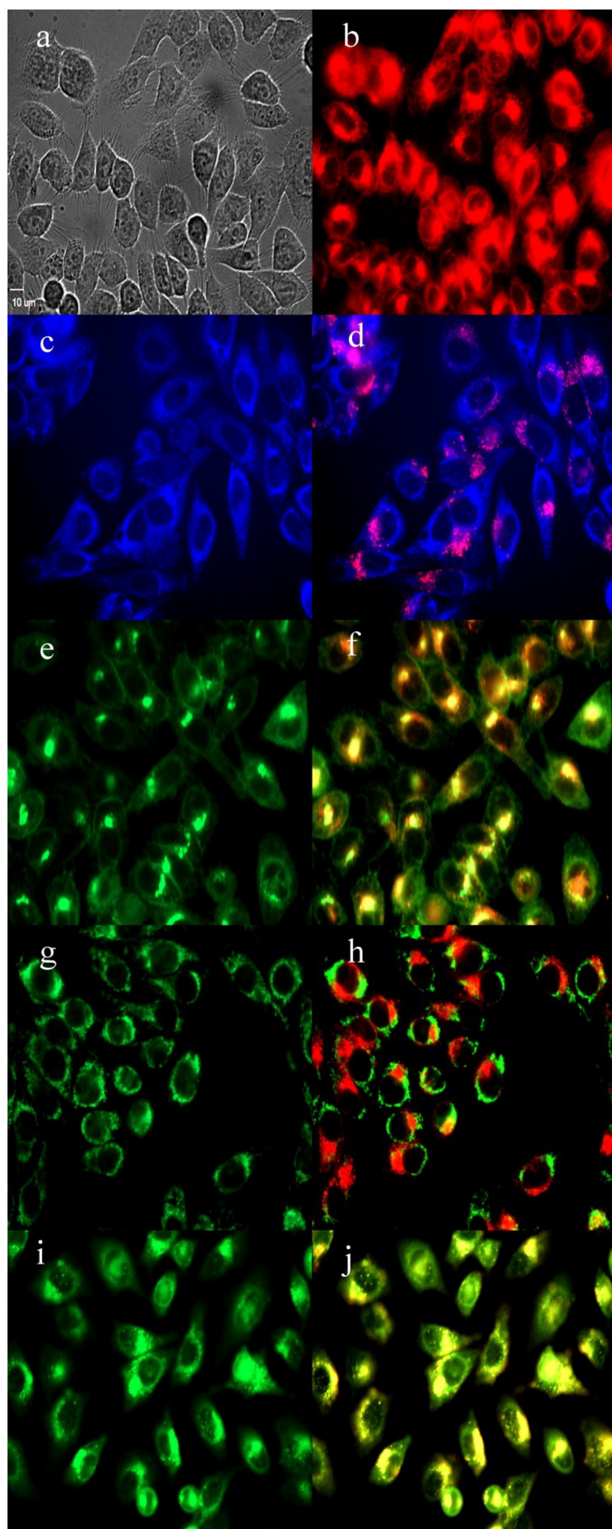

Figure 6. Subcellular localization of 15^2,17^3-di(aspartate) chlorin e_6 **28** in HEp2 cells at 10 μM for 6h, (a) phase contrast, (b) overlay of **28** and phase contrast, (c) ER Tracker Blue, (d) overlay of **28** and ER Tracker Blue, (e) BODIPY Ceramide, (f) overlay of **28** and BODIPY Ceramide, (g) MitoTracker Green, (h) overlay of **28** and MitoTracker Green, (i) LysoSensor Green, (j) overlay of **28** and LysoSensor Green. Scale bar: 10 μm.

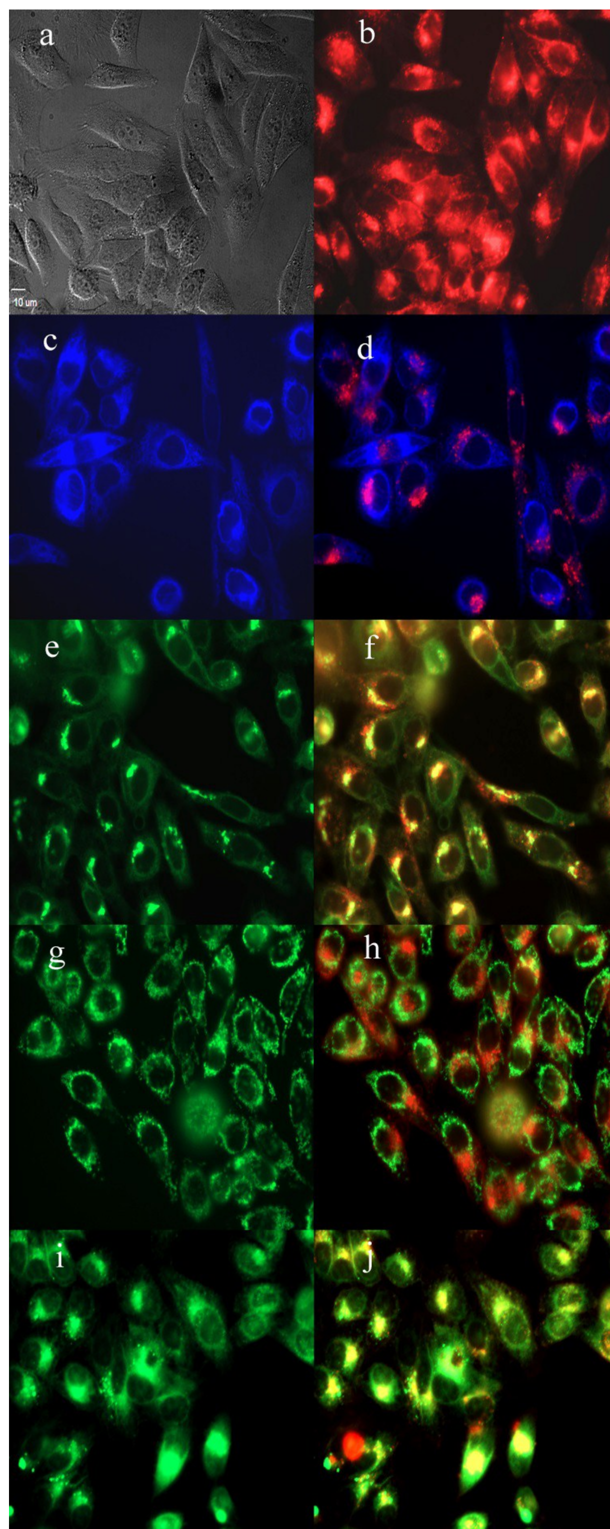

Figure 7. Subcellular localization of 15^2,17^3-di(aspartate) chlorin e_6 **33** in HEp2 cells at 10 μM for 6h, (a) phase contrast, (b) overlay of **33** and phase contrast, (c) ER Tracker Blue, (d) overlay of **33** and ER Tracker Blue, (e) BODIPY Ceramide, (f) overlay of **33** and BODIPY Ceramide, (g) MitoTracker Green, (h) overlay of **33** and MitoTracker Green, (i) LysoSensor Green, (j) overlay of **33** and LysoSensor Green. Scale bar: 10 μm.

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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. *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.[34] 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.

Synthetic procedures and characterization

15^2,17^3-Diaspartylchlorin e_6 tetra(*tert*-butyl)-monomethyl ester (11). Chlorin e_6 (1, 100 mg, 0.168 mmol) was dissolved in dry CH_2Cl_2 (7 mL) and DIEA (0.06 mL, 0.34 mmol) was added. A mixture of DCC (105 mg, 0.51 mmol) and DMAP (163 mg, 0.51 mmol) in CH_2Cl_2 (8 mL) was added and the mixture was allowed to stir for 2 h. Then aspartic acid di(tert)butyl ester hydrochloride (125 mg, 0.445 mmol) and DIEA (0.075 ml) were mixed in CH_2Cl_2 (2 ml) and added to the reaction mixture. The solution was allowed to stir overnight at room temperature and after 12 h it was treated with excess ethereal diazomethane. Then the mixture was diluted with CH_2Cl_2 and washed with 5% aqueous citric acid, followed by a wash with brine and finally water. It was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 5% methanol/CH_2Cl_2 and purified via silica gel column chromatography with the same mobile phase to afford 15^2,17^3-diaspartylchlorin e_6 tera(*tert*-butyl)-monomethyl ester (**11** , $C_{59}H_{80}N_6O_{12}$, 54 mg, 0.051 mmol, 30% yield); UV-Vis (acetone): λ_{max} (ε/$mM^{-1}cm^{-1}$) 664 nm (52), 608 (3.9), 528 (3.6), 500 (13), 400 (170); ^{1}H NMR (acetone-*d_6*, 400 MHz): δ 9.80 (s, 1H), 9.63 (s, 1H), 9.06 (s, 1H), 8.15 (dd, *J* = 17.9, 11.6 Hz, 1H), 7.37 (d, *J* = 7.9 Hz, 1H), 7.23 (d, *J* = 8.0 Hz, 1H), 6.37 (dd, *J* = 17.9, 1.5 Hz, 1H), 6.10 (dd, *J* = 11.6, 1.4 Hz, 1H), 5.37 (d, *J* = 12.0 Hz, 2H), 4.67 (s, 2H), 4.26 (s, 3H), 3.77 (d, *J* = 7.6 Hz, 2H), 3.57 (s, 3H), 3.48 (s, 4H), 3.25 (s, 3H), 2.83 – 2.63 (m, 3H), 2.52 – 2.28 (m, 2H), 2.14 (s, 2H), 1.89 – 1.79 (m, 2H), 1.78 (d, *J* = 7.1 Hz, 3H), 1.69 (t, *J* = 7.6 Hz, 3H), 1.43 (s, 9H), 1.35 (s, 9H), 1.26 (s, 9H), 1.16 (s, 9H), -1.36 (s, 1H), -1.57 (s, 1H); MS (MALDI-TOF) *m/z* 1065.591 $[M+H]^+$, calcd. for $C_{59}H_{81}N_6O_{12}$ 1065.591.

15^2,17^3-Diaspartylchlorin e_6 methyl ester (12). 15^2,17^3-Diaspartylchlorin e_6 tetra(*tert*-butyl)-monomethyl ester (11, 54 mg, 0.051 mmol) was dissolved in 2 mL of dry CH_2Cl_2 in an ice bath under argon and TFA (1 mL) was added and the reaction mixture was allowed to stir overnight. The solvent was rotevaporated several times with diethyl ether to remove residual TFA. The residue was washed with CH_2Cl_2 several times. The final product was re-dissolved in a water/acetonitrile mixture and freeze dried to afford 15^2,17^3-diaspartylchlorin e_6 methyl ester (**12**, $C_{43}H_{48}N_6O_{12}$, 38 mg, 0.045 mmol, 88% yield); UV-Vis (MeOH): λ_{max} (ε/$mM^{-1}cm^{-1}$) 661 nm (71.6), 607 (11), 527 (9.3), 499 (22.4), 399 (172.3); ^{1}H NMR (acetone-*d_6*, 400 MHz): δ 9.88 (s, 1H), 9.66 (s, 1H), 9.21 (s, 1H), 8.11 (dd, *J* = 17.8, 11.6 Hz, 1H), 6.35 (d, *J* = 17.8 Hz, 1H), 6.14 (d, *J* = 11.6 Hz, 1H), 5.35 (t, *J* = 22.7 Hz, 2H), 4.81 (br. s, 1H), 4.65 (br. s, 1H), 4.63 (br. S, 1H), 4.25 (s, 3H), 3.70 (d, *J* = 7.9 Hz, 2H), 3.54 (s, 3H), 3.46 (s, 3H), 3.18 (s, 3H), 3.06 – 2.60 (m, 5H), 2.27 (m, 2H), 1.81 (d, *J* = 7.1 Hz, 3H), 1.55 (t, *J* = 7.4 Hz, 3H). 2.00 – 1.50 (m, 4H) 1.25 (m, 2H); ^{13}C NMR (acetone-*d_6*, 101 MHz) δ 193.12, 192.77, 191.95, 191.88, 191.84, 191.04, 188.72, 168.55, 164.14, 163.25, 160.42, 158.10, 157.50, 155.68, 155.26, 155.20, 151.92, 150.02, 149.04, 145.83, 142.64, 137.66, 134.79, 124.88, 121.07, 117.59, 116.00, 73.49, 73.03, 69.43, 69.24, 68.92, 59.56, 55.70, 53.44, 52.59, 50.75, 42.86, 39.10, 36.79, 31.75, 31.58, 30.41. MS (MALDI-TOF) *m/z* 840.451 $[M]^+$, calcd. for $C_{43}H_{49}N_6O_{12}$ 840.333

13^1,17^3-Diaspartylchlorin e_6 tetra(tert-butyl)-monomethyl ester (20). Chlorin e_6 (1, 100 mg, 0.168 mmol) was dissolved in methanol (5 mL). DCC (35 mg, 0.17 mmol) and DMAP (21 mg, 0.17 mmol) were added and the mixture was stirred until the anhydride intermediate was observed in the TLC. After 1 h, freshly prepared sodium methoxide (0.34 mL of a 0.5 M solution) was added into the reaction mixture dropwise until the color changed from brown to light green. The reaction was monitored by UV-Vis spectroscopy. The solution changed from brown to light green as the anhydride ring opened. The mixture was diluted with ethyl acetate and then washed with 5% aqueous citric acid, followed by a wash with brine and finally with water. It was dried over anhydrous Na_2SO_4 to afford chlorin e_6 15^2-methyl ester (**18**, $C_{35}H_{39}N_4O_6$, 101 mg, 0.165 mmol, 100% yield). Purification was challenging due to the polarity induced by the two free acid groups; thus, the crude product was subjected to the next reaction without purification. A ^{1}H NMR spectrum of the crude product confirmed the methylated acetic side chain. [MS (MALDI-TOF) *m/z* 633.311 $[M+Na]^+$, calcd. for $C_{35}H_{39}N_4O_6$ 633.269]. Chlorin e_6 monomethyl ester (**18**, 101 mg, 0.165 mmol) was dissolved in dry DMF (5 mL). A mixture of HOBt (46 mg, 0.34 mmol), TBTU (109 mg, 0.34 mmol) and DIEA (0.06 ml, 0.34

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mmol) in DMF (5 ml) was added and the mixture was allowed to stir for 30 min at room temperature. Then aspartic acid di-tert-butyl ester hydrochloride (125 mg, 0.445 mmol) and DIEA (0.08 ml, 0.445 mmol) were mixed in CH_2Cl_2 (2 mL) and added to the reaction mixture. The solution was allowed to stir for 48-72 h until formation of the desired product was confirmed by TLC. Then the reaction mixture was washed with 5% aqueous citric acid, followed by a wash with brine and then water. The organic phase was dried over anhydrous Na_2SO_4 and solvent was evaporated. The residue was dissolved in 5% methanol/CH_2Cl_2 and purified via chromatography on a short silica gel column with the same mobile phase to afford 15^2,17^3-diaspartylchlorin e_6 tetra(tert-butyl)-monomethyl ester (**20**, $C_{59}H_{80}N_6O_{12}$, 85 mg, 0.079 mmol, 48% yield); UV-Vis (acetone): λ_{max} (ε/mM^{-1}cm^{-1}) 663 nm (54), 607 (3.8), 528 (3.1), 500 (13), 399 (165); ^{1}H NMR (acetone-d_6, 400 MHz): δ 9.79 (d, *J* = 6.1 Hz, 1H), 9.65 (s, 1H), 9.10 (s, 1H), 8.42 (d, *J* = 7.9 Hz, 1H), 8.12 (dd, *J* = 17.8, 11.6 Hz, 1H), 7.29 (d, *J* = 8.2 Hz, 1H), 6.32 (d, *J* = 17.8 Hz, 1H), 6.06 (d, *J* = 11.4 Hz, 1H), 5.73 (d, *J* = 18.9 Hz, 1H), 5.50 – 5.21 (m, 2H), 4.76 – 4.61 (m, 2H), 4.61 – 4.48 (m, 1H), 3.76 (s, 3H), 3.74 – 3.68 (m, 1H), 3.65 (s, 3H), 3.47 (s, 3H), 3.42 (s, 1H), 3.22 (s, 3H), 3.20 – 3.11 (m, 2H), 2.66 (d, *J* = 5.2 Hz, 2H), 2.46 – 2.27 (m, 1H), 2.24 – 2.14 (m, 1H), 1.83 – 1.72 (m, 2H), 1.66 (s, 9H), 1.55 (s, 9H), 1.41 (s, 9H), 1.35 (s, 9H), 1.72-1.2 (m, 6H), -1.66 (1H), -1.85 (1H); MS (MALDI-TOF) *m/z* 1065.681 [M+H]$^+$, calcd. for $C_{59}H_{81}N_6O_{12}$ 1065.591.

13^1,17^3-Diaspartylchlorin e_6 methyl ester (13). 15^2,17^3-Diaspartylchlorin e_6 tetra(*tert*)butyl methyl ester (20, 50 mg, 0.047 mmol) was dissolved in 2 mL of dry CH_2Cl_2 in an ice bath under argon. TFA (1 mL) was added and the reaction mixture was allowed to stir overnight. The mixture was rotavaporated several times with diethyl ether to remove residual TFA. The residue was dissolved in water/acetonitrile mixture and freeze dried to obtain 13^1,17^3-diaspartylchlorin e_6 methyl ester (**13**, $C_{43}H_{48}N_6O_{12}$, 21 mg, 0.025 mmol, 53%). UV-Vis (MeOH): λ_{max} (ε/mM^{-1}cm^{-1}) 662 nm (75.3), 607 (23), 529 (10), 500 (8.3), 400 (165.2); ^{1}H NMR (MeOD, 400 MHz) δ 10.07 (s, 1H), 9.86 (s, 1H), 9.45 (s, 1H), 8.06 (dd, *J* = 17.3, 11.9 Hz, 1H), 6.30 (d, *J* = 17.8 Hz, 1H), 6.24 (d, *J* = 11.4 Hz, 1H), 5.62 (d, *J* = 18.9 Hz, 1H), 5.28 (d, *J* = 18.3 Hz, 2H), 4.78 – 4.76 (m, 2H), 4.76 - 4.56 (m, 3H), 3.80 (s, 3H), 3.59 (s, 3H), 3.52 (s, 3H), 3.21 (s, 3H), 2.87 – 2.69 (m, 2H), 2.68 – 2.47 (m, 1H), 2.21 (d, *J* = 19.9 Hz, 2H), 1.94 – 1.72 (m, 4H), 1.59 (d, *J* = 6.9 Hz, 4H), 1.30 – 1.01 (m, 4H). ^{13}C NMR (MeOD, 101 MHz) δ 175.33, 174.90, 174.72, 174.02, 173.88, 173.83, 173.66, 169.35, 143.94, 142.95, 142.47, 140.57, 140.42, 138.22, 138.01, 136.12, 135.46, 135.25, 133.96, 132.61, 131.29, 129.47, 124.69, 107.02, 100.12, 98.81, 97.77, 54.94, 53.05, 51.25, 50.55, 38.89, 36.86, 34.68, 33.77, 26.68, 26.02, 23.70, 20.00, 16.98, 12.22, 10.91, 9.11. MS (MALDI-TOF) *m/z* 840.532 [M]$^+$, calcd. for $C_{43}H_{49}N_6O_{12}$ 840.333.

Ethylenediaminyl(boc) pheophorbide a (25). Methyl pheophorbide a (21, 100 mg, 0.165 mmol) was dissolved in dry toluene (20 ml) and the mixture was heated to 100 ^{o}C under nitrogen. Then, mono-boc protected ethylene diamine (32 mg, 0.20 mmol) was added. The reaction mixture was allowed to stir overnight at 100 ^{o}C 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. It was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 3% methanol/CH_2Cl_2 and purified via silica gel column chromatography with the same mobile phase to afford ethylenediaminyl(boc) pheophorbide a (**25**, $C_{42}H_{50}N_6O_6$, 80 mg, 0.109 mmol, 65%); UV-Vis (DCM): λ_{max} (ε/mM^{-1}cm^{-1}) 667 nm (46), 609 (6.5), 535 (8), 505 (10), 413 (99); ^{1}H NMR (acetone-d_6, 400 MHz) δ 9.58 (s, 1H), 9.30 (s, 1H), 8.87 (s, 1H), 8.13 – 7.92 (m, 2H), 6.27 (dd, *J* = 17.9, 1.5 Hz, 1H), 6.18 (s, 1H), 6.12 (dd, *J* = 11.5, 1.4 Hz, 1H), 6.16 (m, 1H), 4.77 – 4.55 (m, 1H), 4.37 (dt, *J* = 9.3, 2.5 Hz, 1H), 3.59 (s, 3H), 3.52 (s, 3H), 3.42 – 3.36 (m, 2H), 3.39 (s, 3H), 3.35 (d, *J* = 6.5 Hz, 3H), 3.08 (s, 3H), 2.74 – 2.57 (m, 2H), 2.46 – 2.33 (m, 1H), 2.31 – 2.19 (m, 1H), 1.84 (d, *J* = 7.3 Hz, 3H), 1.76 – 1.64 (m, 1H), 1.58 (t, *J* = 7.6 Hz, 3H), 1.35 (s, 9H), -1.77 – -2.11 (m, 2H); MS (MALDI-TOF) *m/z* 735.561 [M+H]$^+$, calcd. for $C_{42}H_{51}N_6O_6$ 735.387

15^2-Ethylenediaminyl(boc)-13^1-ethylenediaminylchlorin e_6 methyl ester (26). Ethylenediaminyl(boc) pheophorbide-*a* (25, 80 mg, 0.109 mmol) was dissolved in toluene (10 mL) and ethylene diamine (30 mg, 0.5 mmol) was added. The reaction mixture was heated at 40 ^{o}C overnight. Progress was monitored by TLC and UV-Vis spectrometry. 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 5% methanol/CH_2Cl_2 and eluted from a silica gel column with the same mobile phase. Then the methanol percentage of the mobile phase was gradually increased up to 20% to elute the pure product from the column. The solvent was evaporated and the residue was re-dissolved in 5% acetone/CH_2Cl_2 and filtered to remove silica from the sample. After evaporation of the solvent pure 15^2-ethylenediaminyl(boc)-13^1-ethylenediaminylchlorin e_6 methyl ester was obtained (26, $C_{44}H_{58}N_8O_6$, 55 mg, 0.069 mmol, 64%); UV-Vis (acetone): λ_{max} (ε/mM^{-1}cm^{-1}) 663 nm (55), 607 (2.6), 528 (1.3), 500 (12), 399 (170); ^{1}H NMR (acetone-d_6, 400 MHz) δ 9.74 (s, 1H), 9.73 (s, 1H), 9.13 (s, 1H), 8.36 (br. s, 1H), 8.23 (dd, *J* = 17.8, 11.5 Hz, 1H), 7.46 (br. s, 1H), 6.64 (br. s, 1H), 6.40 (dd, *J* = 17.9, 1.5 Hz, 1H), 6.12 (dd, *J* = 11.7, 1.5 Hz, 1H), 5.55 (d, *J* = 18.0 Hz, 1H), 5.09 (d, *J* = 9.3 Hz, 1H), 4.76 – 4.51 (m, 2H), 4.28 (s, 1H), 4.01 – 3.83 (m, 1H), 3.80 – 3.67 (m, 4H), 3.58 (s, 3H), 3.54 (s, 3H), 3.52 (s, 3H), 3.29 (s, 3H), 3.25 – 3.16 (m, 2H), 3.08 (dd, *J* = 9.1, 4.9 Hz, 1H), 2.78 – 2.64 (m, 1H), 2.43 – 2.21 (m, 2H), 2.00 (d, *J* = 8.0 Hz, 1H), 1.76 (d, *J* = 7.1 Hz, 3H), 1.68 (t, *J* = 7.6 Hz, 3H), 1.20 (s, 9H), 0.97 (m, 3H), -1.66 (s, 1H), -2.02 (s, 1H); MS (MALDI-TOF) *m/z* 795.651 [M+H]$^+$, calcd. for $C_{44}H_{59}N_8O_6$ 795.456

13^1,15^2-Diethyleneaminylaspartylchlorin e_6 di(*tert*)butyl di(boc) methyl ester (27). 15^2-Ethylenediaminyl(boc)-13^1-ethylenediaminylchlorin e_6 methyl ester (26, 55 mg, 0.069 mmol)

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was dissolved in 3 mL of dry CH_2Cl_2 in an ice bath under argon and 1 m of TFA was added. Then the reaction mixture was allowed to stir overnight. The mixture was rotavaporated several times with diethyl ether to remove residual TFA. The residue was dissolved in water/acetonitrile mixture and freeze dried. Without any further purification the crude product was taken to the next step. (**23**, $C_{39}H_{50}N_8O_4$, 42 mg, 0.06 mmol, 88%), [MS (MALDI-TOF) *m/z* 695.445 $[M+H]^+$,calcd. for $C_{39}H_{51}N_8O_4$ 695.403]. (Boc)Asp(tBu)OH (70 mg, 0.24 mmol) was dissolved in dry DMF (5 mL). A mixture of HOBt (32 mg, 0.24 mmol), TBTU (77 mg, 0.24 mmol) and DIEA (0.05 ml, 0. mmol) in DMF (3 mL) was added and the mixture was allowed to stir for 1 h. $13^1,15^2$-Diethylenediaminylchlorin e_6 methyl ester (**23**, 42 mg, 0.060 mmol) was added to the reaction mixture and stirring was continued for 48 h. The mixture was diluted with CH_2Cl_2 and then washed with 5% aqueous citric acid, followed by washes with brine and water. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 6% MeOH/CH_2Cl_2 and purified via silica gel column chromatography using the same mobile phase to afford $13^1,15^2$-diethyleneaminyl-diaspartylchlorin e_6 di(*tert*)butyl di(boc) methyl ester (**27**, $C_{65}H_{92}N_{10}O_{14}$, 30 mg, 0.024 mmol, 40%); UV-Vis (acetone): λ_{max} (ε/mM^{-1}cm^{-1}) 663 nm (48), 607 (2.1), 528 (1.7), 500 (11), 399 (150); ^{1}H NMR (chloroform-*d*, 400 MHz) δ 9.68 (s, 1H), 9.61 (s, 1H), 8.79 (s, 1H), 8.07 (dd, *J* = 17.9, 11.5 Hz, 1H), 7.97 (d, *J* = 5.7 Hz, 1H), 7.74 (s, 1H), 6.34 (d, *J* = 17.8 Hz, 1H), 6.13 (d, *J* = 11.5 Hz, 1H), 5.81 (d, *J* = 8.3 Hz, 1H), 5.47 (d, *J* = 18.1 Hz, 1H), 5.28 – 4.88 (m, 2H), 4.67 – 4.30 (m, 3H), 4.10 – 3.68 (m, 10H), 3.65 – 3.12 (m, 5H), 3.54 (s, 3H), 3.48 (s, 3H), 3.31 (s, 3H), 2.93 – 2.69 (m, 2H), 2.67 – 2.53 (m, 1H), 2.23 (m, 3H), 2.04 (s, 1H), 1.90 – 1.55 (m, 7H), 1.31 (s, 18H), 1.14 (s, 18H), 0.94 – 0.80 (m, 1H), -1.62 (s, 1H), -1.80 (s, 1H); MS (MALDI-TOF) *m/z* 1237.651 $[M+H]^+$, calcd. for $C_{65}H_{93}N_{10}O_{14}$ 1237.687.

$13^1,15^2$-Diethyleneaminyl-diaspartylchlorin e_6 methyl ester (28). $13^1,15^2$-Diethyleneaminyl-diaspartylchlorin e_6 di(tert)butyl di(boc) methyl ester (**27**, 30 mg, 0.024 mmol) was dissolved in 2 mL of dry CH_2Cl_2 in an ice bath under argon. TFA (1 mL) was added and the reaction mixture was allowed to stir overnight. The reaction mixture was rotavaporated several times with diethyl ether to remove residual TFA. The residue was washed with CH_2Cl_2 several times. The final product was dissolved in water and freeze dried to obtain 13^1-15^2-diethyleneaminyl-diaspartylchlorin e_6 methyl ester (**28**, $C_{47}H_{60}N_{10}O_{10}$, 16 mg, 0.016 mmol, 70%). UV-Vis (MeOH): λ_{max} (ε/mM^{-1}cm^{-1}) 661 nm (74.7), 606 (8.4), 527 (6.2), 500 (20.5), 400 (190.2); ^{1}H NMR (methanol-d_4, 400 MHz) δ 10.18 (s, 1H), 10.03 (s, 1H), 9.47 (s, 1H), 8.20 (dd, *J* = 17.7, 11.6 Hz, 1H), 6.39 (d, *J* = 17.8 Hz, 1H), 6.29 (d, *J* = 11.5 Hz, 1H), 5.59 (d, *J* = 18.7 Hz, 1H), 5.42 (d, *J* = 18.7 Hz, 1H), 4.79 (d, *J* = 7.2 Hz, 1H), 4.54 (d, *J* = 10.8 Hz, 1H), 4.44 (ddd, *J* = 24.1, 8.5, 4.2 Hz, 1H), 4.25 – 4.05 (m, 1H), 4.03 – 3.83 (m, 4H), 3.74 (s, 3H), 3.67 (s, 3H), 3.56 (s, 3H), 3.49 (td, *J* = 10.6, 9.5, 4.6 Hz, 2H), 3.41 – 3.26 (m, 6H), 3.38 (s, 3H), 3.23 – 3.00 (m, 2H), 2.99 – 2.82 (m, 2H), 2.70 (dd, *J* = 17.9, 9.0 Hz, 1H), 2.59 – 2.47 (m, 1H), 2.46 – 2.31 (m, 1H), 1.91 – 1.51 (m, 3H), 1.83 (d, *J* = 7.1 Hz, 3H), 1.68 (t, *J* = 7.3 Hz, 3H), 1.29 (m, 3H); ^{13}C NMR (MeOD, 101 MHz) δ 174.42, 173.97, 173.75, 172.86, 171.55, 171.39, 169.05, 168.72, 168.05, 143.07, 142.13, 141.77, 139.03, 138.76, 137.66, 136.98, 135.23, 134.58, 132.81, 131.33, 130.49, 128.33, 123.54, 105.19, 99.20, 97.43, 97.06, 53.90, 51.08, 49.96, 49.13, 39.50, 39.30, 39.19, 38.70, 34.67, 34.46, 30.47, 29.65, 29.30, 22.83, 22.37, 18.95, 15.79, 11.06, 10.88, 9.85. MS (MALDI-TOF) *m/z* 947.559 $[M+Na]^+$, calcd. for $C_{47}H_{60}N_{10}NaO_{10}$ 947.439.

β-Alanylpheophorbide a *tert*-butyl methyl ester (29). Methyl pheophorbide a (21, 100 mg, 0.165 mmol) was dissolved in dry toluene (10 mL) and the mixture was heated to 100 °C under nitrogen. Then β-alanine(tBu).HCl (45 mg, 0.25 mmol) and DIEA (0.06 ml, 0.33 mmol) were added. The reaction mixture was allowed to stir overnight at 100 °C in an oil bath and was monitored 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 with brine. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 5% methanol/CH_2Cl_2 and purified via silica gel chromatography with the same mobile phase and to afford β-alanylpheoporbide a *tert*-butyl methyl ester (**29**, $C_{42}H_{49}N_5O_6$, 65 mg, 0.090 mmol, 54%); UV-Vis (DCM): λ_{max} (ε/mM^{-1}cm^{-1}) 667 nm (45), 609 (6.5), 535 (7.6), 505 (10.5), 412 (107); ^{1}H NMR (acetone-d_6, 400 MHz) δ 9.33, 9.23* (s, 1H), 8.99, 9.93* (s, 1H), 8.79, 8.77* (s, 1H), 7.97*, 7.92 (t, *J* = 6.0 Hz, 1H), 7.80 (m, 1H), 6.16, 6.13* (s, 1H), 6.07 (d, *J* = 9.7 Hz, 1H), 5.99 (dd, *J* = 11.6, 1.5 Hz, 1H), 4.65*, 4.57 (qd, *J* = 7.4, 2.0 Hz, 1H), 4.38 (tt, *J* = 9.7, 2.2 Hz, 1H), 3.68 (qd, *J* = 6.6, 2.3 Hz, 1H), 3.61 (q, *J*=6.4, 1H) 3.59*, 3.53 (s, 3H), 3.49 (s, 3H), 3.41 (s, 2H), 3.28*, 3.27 (s, 3H), 2.86*, 2.84 (s, 3H), 2.79 (m, 2H), 2.71 – 2.52 (m, 2H), 2.49 – 2.12 (m, 2H), 1.85 (d, *J* = 7.3 Hz, 3H), 1.64 (t, *J* = 7.3 Hz, 3H), 1.48*, 1.49 (s, 9H), -2.00 (s, 1H), -2.16 (s 1H).(* Minor 13^2 epimer); MS (MALDI-TOF) *m/z* 720.467 $[M+H]^+$, calcd. for $C_{42}H_{50}N_5O_6$ 720.368.

β-Alanylaspartylpheoporbide *a* di-*tert*butyl methyl ester (31). β-Alanylpheophorbide a *tert*-butyl methyl ester (**29**, 65 mg, 0.090 mmol) was dissolved in 2 mL of dry CH_2Cl_2 in an ice bath under argon. TFA (1 mL) was added and the reaction mixture was allowed to stir for 6 h. The reaction mixture was diluted with CH_2Cl_2 and washed with water and then with saturated sodium bicarbonate. This formed a precipitate while washing with sodium bicarbonate then citric acid solution was added until the precipitate dissolved in the organic phase. Then the solution was washed with brine and dried over anhydrous Na_2SO_4 to give β-alanylpheoporbide a methyl ester (**30**, $C_{38}H_{41}N_5O_6$, 60 mg, 0.09 mmol, 100%). [MS (MALDI-TOF) *m/z* 686.387 $[M+H]^+$, calcd. for $C_{38}H_{41}N_5NaO_6$ 686.295]. β-Alanylpheoporbide a methyl ester (**30**, 60 mg, 0.090 mmol) was dissolved in dry DMF (5 mL). A mixture of HOBt (18 mg, 0.135 mmol), TBTU (43 mg, 0.135) and DIEA (0.03 ml, 0.18 mmol) in DMF (3 mL) was added and the mixture was allowed to stir for 30 min. Then a mixture of aspartic acid di(tert)butyl ester hydrochloride (101 mg, 0.36

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mmol) and DIEA (0.06 ml, 0.36 mmol) in CH_2Cl_2 (3 mL) was added to the reaction mixture. The mixture was allowed to stir for 24 h. The mixture was diluted with CH_2Cl_2 and then washed with 5% aqueous citric acid, followed by with water and brine. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 5% methanol/CH_2Cl_2 and purified via silica column chromatography with the same mobile phase to afford β-alanyl aspartyl pheophorbide a di-tertbutyl methyl ester (**31**, $C_{50}H_{62}N_6O_9$, 48 mg, 0.053 mmol, 59%); UV-Vis (DCM): λ_{max} (ε/mM^{-1}cm^{-1}) 667 nm (41.5), 609 (5.5), 535 (7), 505 (10), 413 (103); ^{1}H NMR (acetone-*d*$_6$, 400 MHz) δ 9.06, 8.98* (s, 1H), 8.70, 8.59* (s, 1H), 8.69 (s, 1H), 8.2* 8.06 (t, *J* = 5.9 Hz, 1H), 7.70 – 7.45 (m, 2H), 6.17, 6.08* (s, 1H), 5.93 (dt, *J* = 17.7, 2.3 Hz, 1H), 5.85 (dd, *J* = 11.5, 1.4 Hz, 1H), 4.82*, 4.76 (dt, *J* = 8.3, 5.8 Hz, 1H), 4.64*, 4.55 (tt, *J* = 9.0, 4.5 Hz, 1H), 4.42*, 4.35 (dt, *J* = 9.7, 2.5 Hz, 1H), 3.77 (q, *J* = 6.5 Hz, 2H), 3.63*, 3.59 (s, 3H), 3.42, 3.31* (s, 3H), 3.14 (s, 3H), 3.05 (h, *J* = 6.8, 6.3 Hz, 3H), 2.78 (dd, *J* = 5.9, 4.7 Hz, 2H), 2.74 – 2.66 (m, 4H), 2.63 (s, 3H), 2.49 – 2.37 (m, 1H), 2.20 (td, *J* = 9.0, 3.3 Hz, 1H), 1.85, 1.65* (d, *J* = 7.3 Hz, 3H), 1.45 (s, 9H), 1.42 (s, 9H), 1.35 (t, *J* = 7.5 Hz, 2H), -2.17*, -2.33 (s, 2H).).(* Minor 13^2 epimer); MS (MALDI-TOF) *m/z* 890.461 [M]$^+$, calcd. for $C_{50}H_{63}N_6O_9$ 890.458.

13^1-Ethylenediaminyl-15^2-β-alanylaspartylchlorin e$_6$ di-*tert*-butyl methyl ester (32). β-Alanylaspartylpeoporbide a di-*tert*-butyl methyl ester (**31**, 48 mg, 0.053 mmol) was dissolved in toluene (10 ml) and ethylenediamine (15 mg, 0.26 mmol) was added. The reaction mixture was heated at 40 °C for 24-36 h. It was monitored by TLC and UV-Vis spectroscopy. After reaction was complete as monitored 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 5% methanol/CH_2Cl_2 and chromatographed on a silica gel column with the same mobile phase. Then the methanol percentage of the mobile phase was gradually increased up to 20% to elute the pure product from the column. The solvent was evaporated and re-dissolved in 5% acetone/CH_2Cl_2 and filtered to remove silica from the sample. After evaporation of solvent 13^1-ethylenediaminyl-15^2-β-alanyl aspartylchlorin e$_6$ di-*tert*-butyl methyl ester was obtained (**32**, $C_{52}H_{70}N_8O_9$, 30 mg, 0.031 mmol, 58 %). UV-Vis (acetone): λ_{max} (ε/mM^{-1}cm^{-1}) 664 nm (51.5), 607 (2.5), 528 (2), 500 (11), 400 (160); It was not possible to obtain the ^{1}H NMR spectrum and so the crude product was subjected directly to next step. MS (MALDI-TOF) *m/z* 951.767 [M+H]$^+$, calcd. for $C_{52}H_{71}N_8O_9$ 951.534.

13^1-Ethylenediaminyllysinyl-15^2-β-alanylaspartylchlorin e$_6$ methyl ester (33) via 13^2-ethylenediaminyllysinyl-15^2-β-alanylaspartylchlorin e$_6$ di-*tert*-butyl di-(boc) methyl ester. Boc-Lys(Boc)OH.DCHA (61 mg, 0.116 mmol) was dissolved in dry DMF (5 mL). A mixture of HOBt (16 mg, 0.116 mmol), TBTU (37 mg, 0.116 mmol) and DIEA (0.024 ml, 0.14 mmol) in DMF (3 mL) was added and the mixture was stirred for 30 min. 13^1-Ethylenediaminyl-15^2-β-alanylaspartylchlorin e$_6$ di-tertbutyl methyl ester (**32**, 30 mg, 0.031 mmol) was added to the reaction mixture and it was stirred for 72 h. After the reaction was deemed complete by TLC, the mixture was diluted with CH_2Cl_2 and then washed with 10% sodium bicarbonate, 5% aqueous citric acid, then followed by washing with brine. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was evaporated. The residue was dissolved in 10% MeOH/CH_2Cl_2 and purified via silica gel column chromatography using the same mobile phase to afford 13^1-ethylenediaminyllysinyl-15^2-β-alanylaspartylchlorin e$_6$ di-tert-butyl di-tert-butyl carbamates methyl ester ($C_{68}H_{98}N_{10}O_{14}$, 24 mg, 0.018 mmol, 61%); UV-Vis (acetone): λ_{max} (ε/mM^{-1}cm^{-1}) 663 nm (43.5), 607 (3), 528 (2.5), 500 (10), 400 (136); ^{1}H NMR (acetone-*d*$_6$, 400 MHz) δ 9.67 (s, 1H), 9.65 (s, 1H), 9.10 (s, 1H), 8.71 (s, 1H), 8.26 (d, *J* = 5.5 Hz, 1H), 8.17 (dd, *J* = 17.8, 11.5 Hz, 1H), 7.05 (s, 2H), 6.34 (d, *J* = 17.8 Hz, 1H), 6.23 (d, *J* = 8.0 Hz, 1H), 6.07 (d, *J* = 11.5 Hz, 1H), 5.95 (s, 1H), 5.56 (d, *J* = 18.9 Hz, 1H), 5.22 (d, *J* = 19.2 Hz, 1H), 4.68 (q, *J* = 7.1 Hz, 1H), 4.54 (d, *J* = 9.6 Hz, 1H), 4.29 (q, *J* = 3.6 Hz, 1H), 3.98 – 3.62 (m, 9H), 3.57 (s, 3H), 3.48 (s, 6H), 3.25 (s, 3H), 3.02 (d, *J* = 6.0 Hz, 2H), 2.92 (s, 3H), 2.56 – 2.40 (m, 2H), 2.40 – 2.26 (m, 2H), 2.26 – 2.17 (m, 1H), 1.91 (dt, *J* = 23.0, 8.6 Hz, 2H), 1.74 (d, *J* = 7.1 Hz, 3H), 1.66 (t, *J* = 7.2 Hz, 3H), 1.57 – 1.44 (m, 4H), 1.37 (s, 18H), 1.34 (s, 18H), -1.65 (s, 1H), -1.94 (s, 1H); MS (MALDI-TOF) *m/z* 1279.775 [M+H]$^+$, calcd. for $C_{68}H_{99}N_{10}O_{14}$ 1279.734.

The abovementioned 13^1-ethylenediaminyllysinyl-15^2-β-alanylaspartylchlorin e$_6$ di-tertbutyl di-(boc) methyl ester (24 mg, 0.018 mmol) was dissolved in 2 mL of dry CH_2Cl_2 in an ice bath under argon. TFA (1 mL) was added and the reaction mixture was allowed to stir overnight. The mixture was rotavaporated several times with diethyl ether to remove TFA and the residue was washed with CH_2Cl_2 several times. The final product was dissolved in water and then freeze dried to obtain 13^1-ethylenediaminyllysinyl-15^2-β-alanylaspartylchlorin e$_6$ methyl ester (**33**, $C_{50}H_{66}N_{10}O_{10}$, 14 mg, 0.014 mmol; 79%). UV-Vis (MeOH): λ_{max} (ε/mM^{-1}cm^{-1}) 658 nm (18.9), 635 (67.8), 594 (14), 511 (10), 411 (150.8); ^{1}H NMR (400 MHz, MeOD) δ 9.96 (s, 1H), 9.70 (s, 1H), 9.42 (s, 1H), 7.98 (s, 2H), 6.39 – 6.01 (m, 3H), 5.57 (d, *J* = 18.5 Hz, 1H), 5.44 (d, *J* = 18.5 Hz, 1H), 4.80 (d, *J* = 6.3 Hz, 2H), 4.60 (d, *J* = 8.7 Hz, 1H), 4.36 (s, 1H), 4.17 (s, 1H), 4.01 (s, 1H), 3.95 – 3.79 (m, 3H), 3, 3.70 (s, 6H), 3.60 (s, 3H), 3.47 (s, 3H), 3.08 (m, 3H), 3.03 (m, 3H), 2.86 (d, *J* = 7.1 Hz, 1H), 2.78 (s, 1H), 2.60-2.32 (m, 5H), 2.21 – 1.98 (m, 4H), 1.83 (m, 3H), 1.67 (t, *J* = 6.7 Hz, 3H), 1.56 (br. s, 4H) 1.31 (m, 2H). ^{13}C NMR (MeOD, 101 MHz) δ 174.30, 173.45, 172.41, 171.99, 169.49, 169.40, 169.24, 143.12, 141.31, 138.38, 138.21, 136.42, 134.93, 134.49, 132.42, 130.83, 130.21, 128.13, 123.05, 121.13, 118.21, 115.30, 112.39, 109.05, 104.86, 99.38, 96.88, 53.87, 53.19, 51.07, 49.15, 48.73, 39.86, 39.15, 38.98, 37.50, 36.18, 35.26, 34.82, 30.76, 30.52, 29.83, 26.79, 22.43, 21.66, 18.72, 15.78, 11.06, 10.83, 9.62. MS (MALDI-TOF) *m/z* 967.602 [M+H]$^+$, calcd. for $C_{50}H_{67}N_{10}O_{10}$ 967.504

Cell Studies

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All reagents used in these studies were purchased from Invitrogen. The human HEp2 cells were obtained from ATCC and maintained in a 50:50 mixture of DMEM:AMEM supplemented with 5% FBS and 1% penicillin/streptomycin antibiotic. The cells were sub-cultured twice weekly to maintain sub-confluent stocks.

Time-Dependent 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 400 μM with maximum DMSO concentration of 1.95% and Cremophor concentration of 0.05%. Uptake was allowed to continue for 0, 1, 2, 4, 8, 12 and 24 h. The uptake was terminated by removing the loading medium and washing the wells once with PBS. 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 as per manufacturer's instructions.

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 and the cells were incubated overnight. Cell toxicity was measured using Promega's Cell Titer Blue viability assay[47] as per manufacturer's instructions, with untreated cells considered 100% viable and cells treated with 0.2% saponin as 0% viable.

The cells were prepared as described above with compound concentration range from 0-100 μM. After loading overnight, the medium was replaced with medium containing 50 mM HEPES pH 7.2. The cells were exposed to a 1000 W halogen lamp filtered through a 610 nm liquid filter to provide approximately 1.5 J/cm^2 light dose. The cells were kept cool by filtering the IR radiation through 10 mm of water and placing the culture in an Echotherm chilling plate (Torrey Pines Scientific, Inc.). After 20 min exposure to light, the plate was incubated overnight. Cell viability was then measured as described above.

In comparison with the previously investigated mono-amino acid derivatives of chlorin e6,[27] the di-amino acid conjugates appear to be less phototoxic than the 13^1- and the 15^2-aspartate derivatives (IC_{50} = 0.6 and 4.0 uM at 1 J/cm^2), probably due to the higher hydrophilicity of the di(amino acid) conjugates.

Microscopy: The Hep2 cells were plated in a 6-well plate and allowed to grow overnight. The cells were exposed to 10 μM of each compound, and then kept at 37 ^{0}C for 6 h in 5% CO_2 before adding the organelle tracer. The working concentrations of organelle tracers were as follows: LysoSensor Green 50 nM, MitoTracker Green 250 nM, ER Tracker Blue/white 100 nM, and BODIPY FL C5 Ceramide 50 nM. The organelle tracers were diluted in growing medium and the cells were incubated concurrently with the compound and the tracer for 30 min. The loading medium was removed and cells were washed with PBS 3 times before imaging. The images were acquired using a Leica DMRXA2 microscope with a water immersion objective and DAPI, GFP, and TRITC filter cubes (Chroma Technologies).

Conclusions

Previous work[27] has shown 13^1-monoconjugates of chlorin e_6 to be more potent PDT sensitizers (with regard to dark- and photo-toxicity) than the corresponding 15^2-monoconjugates. In the present work a series of chlorin e_6 di(amino acid) conjugates were regioselectively synthesized bearing two aspartates in the 13^1,17^3- and 15^2,17^3-positions, or at the 13^1,15^2 via an ethylene diamine linker. One conjugate bearing two different amino acids, lysine at 13^1 via an ethylene diamine linker and an aspartate at 15^2 via a β-alanine linker was also synthesized. The conjugation of aspartate residues via the α-amine groups gave anionic compounds while conjugation via the carboxylate gave a zwitterionic compound. The cytotoxicity and uptake of four di(amino acid) chlorin e_6 conjugates, two anionic and two zwitterionic, were investigated in human HEp2 cells, and compared with chlorin e_6. The most cytotoxic, IC_{50} (dark) = 65-70 μM and IC_{50} (light) = 9-11 μM, were the zwitterionic 13^1,15^2-disubstituted conjugates **28** and **33**; these were also the most taken up by cells and localized in multiple organelles. On the other hand, the tetra-anionic 13^1,17^3- and 15^2,17^3-di-aspartyl chlorin e6 **12** and **13** showed low dark cytoxicity and lower phototoxicity compared with chlorin e_6. These di(amino acid) derivatives of chlorin e_6 appear to be less phototoxic than previously investigated 13^1- and 15^2-mono aspartate chlorin e_6 derivatives,[27] maybe due to their increased hydrophilicity.

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