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Mono-(L)-aspartylchlorin-e_6†‡

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ABSTRACT

Mono-(L)-aspartylchlorin-e_6 (also known as Talaporfin, NPe6, MACE, and most recently LS-11) is a potent sensitizer for photodynamic therapy that is currently undergoing clinical trials. Using a combination of unambiguous partial synthesis from pheophytin-a and methyl pheophorbide-a, NMR spectroscopy, and single crystal X-ray diffraction, the structure of mono-(L)-aspartylchlorin-e_6 is definitively shown to be the isomer in which the aspartyl residue is attached at the 15^2-side chain position. This conclusion is contrary to earlier assumptions, but affirms the conclusions of a study based on NMR spectroscopy; a rationale for the unique formation of the 15^2-aspartyl derivative is proposed.

INTRODUCTION

Mono-(L)-aspartylchlorin-e_6, a chlorophyll *a* derivative—also known as Talaporfin, NPe6, MACE and (most recently) LS-11—is a second-generation photosensitizer in advanced-stage clinical trials for oncologic applications of photodynamic therapy (PDT). For convenience, and to stay abreast with current literature, in this paper we shall use the acronym LS-11 (though this is a fairly recent commercial moniker). PDT is a binary cancer therapy that relies on the selective uptake of a photosensitizer in tumor tissues, followed by generation of singlet oxygen and other cytotoxic species upon irradiation with light of appropriate wavelength (1–3). In addition to necrosis (as the result of oxidative damage) some porphyrins induce apoptosis (programmed cell death) particularly at low light doses (4–7). Singlet oxygen is considered to be the main cytotoxic species generated in PDT. It has a limited range of diffusion within tissue and it readily reacts with a variety of electron-rich biomolecules, such as unsaturated lipids, amino acids and DNA, at the site of its generation.

Once limited to the treatment of superficial skin dysplasias, PDT is now utilized in broader applications. Four photosensitizing drugs have been approved in Canada, the United States and/or in the European Union for the treatment of various malignancies, including cervical cancer, bladder cancer and cancers of the head and neck. Endoscopic light delivery has made the irradiation of hollow structures possible allowing PDT of advanced and early lung cancer, superficial gastric cancer and esophageal cancer. PDT has also benefited from technological advances in fiber optics, which has made possible precise interstitial light delivery to almost any internal tumor site in the body, including large buried tumors that would normally require extensive surgery for treatment (8–11).

In 1975, Dougherty demonstrated that HpD could selectively destroy tumors upon irradiation (12). In 1983, a purer form of HpD, now commercially known as Photofrin® (porfimer sodium), was developed. Photofrin® received FDA approval in the United States in 1995 and is currently approved in more than 40 countries. Although Photofrin® has been shown to be efficacious in the treatment of many cancer types, it has some undesirable properties, such low absorption of light within the "therapeutic window" (600–800 nm) and it is not rapidly cleared from skin, causing residual patient photosensitivity. LS-11 is a so-called second-generation photosensitizer currently in advanced-stage clinical trials for oncologic PDT applications. As a chlorin (*i.e.* a 17,18 dihydroporphyrin), LS-11 has a characteristic strong absorption at 666 nm (solvent dependent) which allows for greater depth-of-light penetration and increased photon utilization than Photofrin®. Upon irradiation LS-11 has been shown to give high yields of cytotoxic singlet oxygen (13). Additionally LS-11 shows rapid clearance from normal tissue and in a direct comparison of LS-11with Photofrin® in the PDT treatment of cholangiocarcinoma, LS-11 was superior to Photofrin® at reducing tumor volume, inhibiting tumor regrowth, increasing depth of tissue injury (by 67%) and decreasing the troublesome side effect of cutaneous photosensitization (14). Furthermore LS-11 has increased stability and amphiphilicity compared with synthetic chlorins, such as temoporfin [5,10,15,20-tetra(*meta*-hydroxyphenyl)chlorin].

LS-11 is prepared commercially by coupling of chlorin-e_6 (**1**) with aspartic acid (15); a mono-aspartyl derivative is formed, and ^{1}H-NMR spectroscopy of the corresponding tetramethyl ester (Fig. 1) shows the product to be at least (and almost certainly greater than) 95% of one pure regioisomer, apparently uncontaminated with either of the two other potential regioisomers so far as ^{1}H-NMR spectroscopy is concerned. This is somewhat surprising as chlorin-e_6 (**1**) possesses no less than three carboxylic acid functional groups, all in principle able to undergo amino- acid coupling, and no carboxylic protecting groups were used in the synthesis. This scenario notwithstanding, because of the isolation of only one pure regioisomer, chemical intuition suggested that it must be

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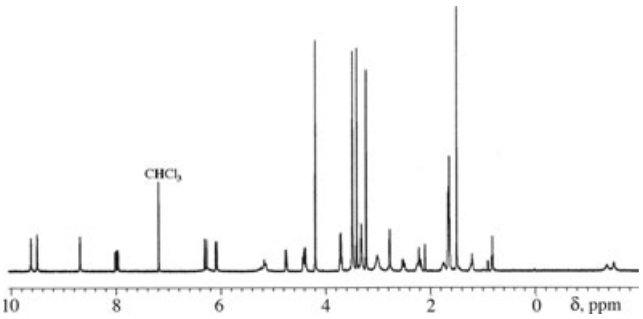
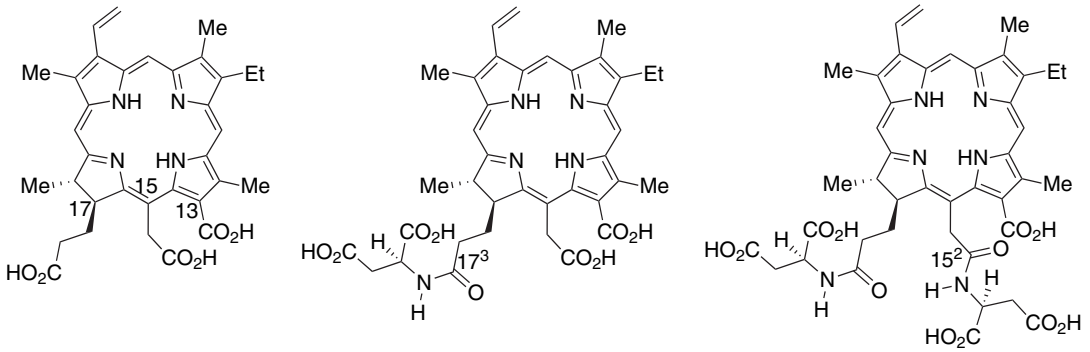


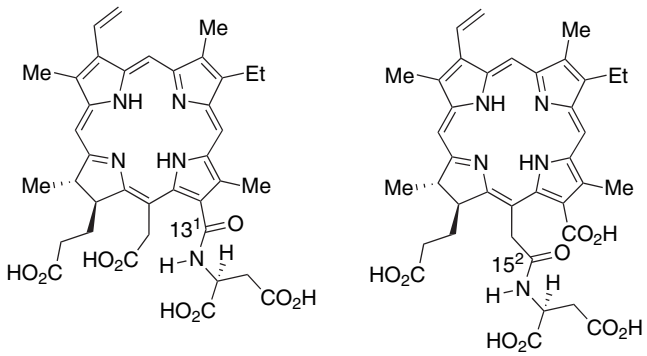
Figure 1. ^{1}H-NMR spectrum, in $CDCl_3$, of "authentic" LS-11 tetramethyl ester. This compound was prepared from a commercial sample by treatment with diazomethane.

the 17^3-aspartyl compound (**2**); the propionic side chain is the most reactive (compared with the acetic and formic analogues) and is also the least sterically encumbered of the three possible amino- acid conjugation sites. This structural assignment was assumed in virtually all academic publications.

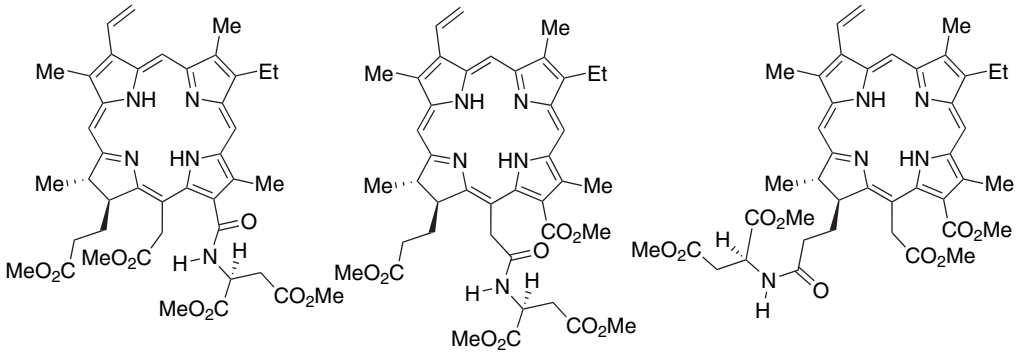
A patent search therefore identifies LS-11 (at that time "NPe6" or Talaporfin) as the 17^3-aspartyl derivative (**2**), though the option for admixture with other regioisomers was left open in the patent itself (15). Subsequent to the initial patent, we had also synthesized and biologically studied "NPe6" (to which we gave the acronym MACE in order to differentiate the "commercial" product from our "rogue academic" material), as well as an over-reacted di-(L)-aspartylchlorin-e_6 ("DACE") that was correctly identified as the 17^3,15^2-diaspartyl compound (**3**) (16). Herein we report unambiguous syntheses of 13^1- (**4**), 15^2- (**5**) and 17^3- (**2**) aspartyl regioisomers of LS-11, as their tetramethyl esters (**6**)–(**8**), respectively. We also report the X-ray crystal structure of the tetramethyl ester (**7**) from commercially obtained LS-11; these data conclusively establish LS-11 as the 15^2-aspartyl regioisomer (**5**). Key improvements in the synthesis of



(**1**) (**2**) (**3**)



(**4**) (**5**)



(**6**) (**7**) (**8**)

15^2-LS-11 (**5**) are also reported, and a rationalization of the unique formation of **5** in the amino- acid coupling reaction between chlorin-e_6 and (L)-aspartic acid is presented. Each LS-11 regioisomer is fully characterized by spectroscopic methods.

MATERIALS AND METHODS

General. Unless otherwise indicated, all commercially available starting materials were used directly without further purification. Reactions under anhydrous conditions were performed in dried and distilled solvents under an argon atmosphere. All reactions were monitored by TLC using Sorbent Technologies 0.25 mm silica gel plates with or without UV indicator (60 F-254). Silica gel Sorbent Technologies 32–63 μm was used for flash column chromatography. ^{1}H NMR were obtained using a Bruker AVANCE DRX-500 MHz or ARX-300 MHz spectrometer. Chemical shifts (δ) are given in p.p.m. relative to internal chloroform. Mass spectra were obtained using a Bruker ProFLEX III MALDI-TOF mass spectrometer and CCA as the matrix. *Spirulina pacifica* alga was purchased as a spray-dried powder from Cyanotech, Hawaii.

X-ray crystallography. Diffraction data were collected at T = 110 K on a Nonius KappaCCD diffractometer equipped with MoKα radiation (λ = 0.71073 Å) and an Oxford Cryostream cooler. Crystal data for **7**: Dark brown needles, $C_{42}H_{49}N_5O_9{\cdot}1.5\ CH_3OH{\cdot}H_2O$, M_r = 833.94, orthorhombic space group $P2_12_12_1$, a = 10.837(2), b = 17.363(4), c = 46.918(12) Å, V = 8828(3) Å^3, Z = 8, ρ_{calcd} = 1.255 gcm^{-3}, μ = 0.091 mm^{-1}, 24873 measured data, R = 0.105 ($F^2 > 2\sigma$), Rw = 0.267 for 6337 unique data (3850 observed) having $\theta < 22.4°$, and 638 refined parameters. The crystal was a very weak scatterer even at low temperature, and it was not possible to refine the carbon atoms anisotropically. The asymmetric unit contains two chlorin molecules. The absolute configuration could not be determined directly from the X-ray data, but was assigned from the known configuration of the (L)-aspartyl substituent, and was confirmed based on the known (17S,18S) configuration of the chlorophyll derivative.

*Isolation of pheophytin-a (**9**) from S. pacifica alga*. Approximately 700 g of dried *S. pacifica* alga was wetted with acetone and subsequently slurried with 4 L of liquid nitrogen in a resistant 2 gallon bucket to form a frozen slush. This slush was allowed to sit for 1 h after which more liquid nitrogen was added and was allowed to sit overnight protected from light. The alga was then transferred to a 4 L reaction vessel and 2 L of acetone was added. The vessel was fitted with a Fisher jumbo mechanical stirrer with a 46 cm impeller shaft and a three-neck lid was clamped to the vessel. The reaction was heated to reflux under argon with mechanical stirring for 3 h. The supernatant was then filtered through Whatman No. 1 paper on a Buchner funnel and more acetone was added to the solid. The extraction and filtration process was repeated twice. The green filtrates were combined and evaporated and then purified by flash column chromatography on silica gel. Elution first with dichloromethane removed the fast-running yellow carotenoid band. Then elution with 80:20 dichloromethane/ethyl acetate eluted the major blue-grey pheophytin-a (**9**) band (3.9 g of $C_{56}H_{77}N_4O_4$ from 700 g alga [extraction yield without Fisher Jumbo mechanical stirrer: 500 mg from 700 g alga]). UV–Vis (CH_2Cl_2): λ_{max} (ε/M^{-1} cm^{-1}) 668 nm (44,600), 611 (8600), 538 (9710), 507 (10,800), 414 (106,000); Mass Spectra (MALDI): m/z 872 (M + H)$^+$; ^{1}H-NMR ($CDCl_3$ 300 MHz): δ 9.50 (1H, s), 9.35 (1H, s), 8.57 (1H, s), 8.0 (1H, m), 6.28 (1H, m), 6.26 (1H, s), 6.18 (1H, m), 4.48 (1H, m), 4.21 (1H, m), 3.88 (3H, s), 3.64 (3H, s), 3.60 (1H, q, J = 7.5 Hz), 3.40 (3H, s), 3.20 (3H, s), 2.63 (1H, m), 2.34 (1H, m) 1.74(3H, d, J = 7.5 Hz), 1.61 (3H, t, J = 7.5 Hz) Phytyl: 5.13 (1H, m), 4.50 (1H, m), 1.90 (2H, m), 1.56 (3H, m), 1.0–1.3 (2H, m), 0.85 (6H, m) 0.71 (6H, m). *Note*: ^{1}H-NMR shows the presence of equilibrium amounts of so-called pheophytin-a′ (17), the 13^2 epimer (diastereomer) of pheophytin-a (18-S, 17-S, 13^2-S instead of 18-S, 17-S, 13^2-R). This epimer is not a nuisance and need not be separated because the 13^2 chiral center is absent in chlorin-e_6 derivatives.

*Pheophorbide-a (**12**)*. Pheophytin-a (**9**),(500 mg, 0.57 mmol) was selectively hydrolyzed to the 17^3-carboxylic acid derivative (**12**) without affecting the 13^1-methoxycarbonyl group by stirring pheophytin-a in 75 mL of degassed TFA/H_2O (80:20) at 0°C for 1 h (16). The reaction mixture was poured into 500 mL of H_2O and extracted with $CHCl_3$. The extract was washed three times with H_2O and twice with 10% sodium bicarbonate, then dried over anhydrous sodium sulfate. Evaporation of the solvent provided a brown residue that was purified *via* silica gel column chromatography, eluting with 40% ethyl acetate in dichloromethane. Alternatively the residue can be purified *via* a column of powdered confectioner's sugar (3–8 cm × 30 cm columns, elution with 10% acetone in CCl_4). Yield 315 mg (93%) of **12**, $C_{35}H_{36}N_4O_5$ was obtained: mp 191–195°C [lit. mp (18) 190–200°C]. UV–Vis (CH_2Cl_2): λ_{max} (ε/M^{-1} cm^{-1}) 675 nm (46,800), 616 (12,100), 542 (13,500), 511 (13,000), 416 (102,400) Mass Spectra (MALDI): m/z 593 (M + H)$^+$; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.51 (1H, s), 9.37 (1H, s), 8.59 (1H, s), 7.97 (1H, m), 6.28 (1H, m), 6.26 (1H, s), 6.18 (1H, m), 4.43 (1H, m), 4.16 (1H, m), 3.84 (3H, s), 3.67 (3H, s), 3.63 (1H, q, J = 7.5 Hz), 3.40 (3H, s), 3.21 (3H, s), 2.54 (1H, m), 2.21 (1H, m), 1.74 (3H, d, J = 7.5 Hz), 1.62 (3H, t, J = 7.5 Hz). ^{13}C-NMR ($CDCl_3$): 189.6, 172.4, 171.4, 171.0, 169.6, 161.2, 156.6, 152.2, 149.7, 145.2, 142.1, 137.9, 136.7, 136.3, 132.0, 129.1, 128.1, 122.8, 105.3, 104.4, 97.6, 94.0, 64.7, 52.8, 52.7, 51.9, 51.1, 50.1, 48.2, 35.8, 32.6, 29.9, 23.1, 19.5, 17.4, 12.1, 12.1, 11.3.

*L-Aspartic acid dimethyl ester pheophorbide-a (**13**)*. Pheophorbide-a (**12**), (300 mg; 0.506 mmol; 1.0 eq) was dissolved in 175 mL dry dichloromethane and allowed to stir under argon. In a separate flask, (L)-aspartic acid dimethyl ester hydrochloride (120 mg, 0.607 mmol; 1.2 eq) and 0.11 mL of diisopropylethylamine (0.607 mmol; 1.2 eq) were dissolved in 30 mL of dichloromethane with sonication. Dicyclohexylcarbodiimide (DCC; 104 mg; 0.506 mmol; 1.0 eq) and dimethylaminopyridine (DMAP; 20 mg; 0.16 mmol; 0.32 eq) were added to the pheophorbide-a solution and the reaction mixture was allowed to stir for 5 min. To the pheophorbide-a and DCC/DMAP solution was added the above 30 mL dichloromethane solution containing the dissolved (L)-aspartic acid dimethyl ester hydrochloride and the diisopropylethylamine. (*Note*: The order of addition of reagents is important.) The solution was stirred under argon for 3 h. The reaction mixture was then washed with water and brine, dried over sodium sulfate, filtered and evaporated. The crude mixture was dissolved in 60 mL of acetonitirile and this was placed in an ice bath and allowed to cool for 1 h to precipitate dicyclohexylurea (DCU). The solution was filtered and then evaporated. A mobile phase was prepared by allowing a flask of acetronitrile to sit in an ice bath for 1 h. To eliminate any trace amounts of DCU, the crude product was then filtered over a silica plug with cold acetonitrile as the mobile phase. The product was eluted from the silica plug and evaporated. It was then re-dissolved in a minimal amount of 30% ethyl acetate/dichloromethane and purified on a silica gel column eluting with the same mobile phase. After the major brown band was eluted from the column the solvent was evaporated to give 354 mg, 95% of the title compound (**13**), $C_{41}H_{45}N_5O_8$: mp 173–178°C. UV–Vis (dichloromethane): λ_{max} (ε/M^{-1} cm^{-1}) 667 nm (41,000), 611 (11,200), 535 (12,300), 505 (13,300), 413 (90,000); Mass Spectra (MALDI): m/z 737 (M + H)$^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 9.50 (1H, s), 9.38 (1H, s), 8.57 (1H, s), 7.98 (1H, dd, J = 11.6, 17.8 Hz), 6.28 (1H, J = 17.8, 1.3 Hz), 6.25 (1H, s), 6.17 (1H, dd, J = 11.6, 1.3 Hz), 4.71 (1H, m), 4.47 (1H, m), 4.23 (1H, m), 3.85 (3H, s), 3.68 (3H, s), 3.65 (3H, s), 3.63 (1H q, J = 7.6 Hz), 3.47 (3H, s), 3.39 (3H, s), 3.21 (3H, s), 2.81 (1H, m) 2.63 (1H, m), 2.52 (1H, m), 2.30 (1H, m), 1.92 (1H, m), 1.81 (3H, d, J = 7.3 Hz), 1.67 (3H, t, J = 7.6 Hz); ^{13}C-NMR ($CDCl_3$): δ 189.6, 172.6, 171.7, 171.5, 171.3, 170.93, 169.6, 162.1, 149.8, 145.0, 142.1, 137.9, 136.6, 136.3, 136.9, 132.0, 129.5, 129.1, 129.0 122.9, 105.5, 104.4, 97.5, 94.0, 64.7, 53.4, 52.8, 52.6, 51.8, 51.1, 50.1, 48.2, 35.7, 32.5, 29.8, 23.1, 19.4, 17.3, 12.1, 12.1 11.2.

*17^3-Mono-(L)-aspartylchlorin-e_6 tetramethyl ester (**8**)*. (L)-Aspartic acid dimethyl ester pheophorbide-a (**13**) (100 mg, 0.135 mmol) was dissolved in dry methanol and stirred under argon for 10 min. Sodium methoxide (0.27 mL of a 0.5 *M* solution) was added and the reaction mixture was allowed to stir at 0°C for 1 h. The reaction was monitored by UV–Vis spectroscopy. The solution turned from brown to green as the isocyclic ring opened. The reaction mixture was then poured into water. The mixture was extracted with dichloromethane and the organic layer was washed with water, dried over sodium sulfate and then evaporated. The residue was dissolved in 2% methanol/dichloromethane and purified on a silica gel plug with the same mobile phase. The solvent was evaporated and 100 mg (97%) of **8**, $C_{42}H_{49}N_5O_9$ was obtained: mp 158–161°C. UV–Vis (dichloromethane): λ_{max} (ε/M^{-1} cm^{-1}) 660 nm (34,600), 608 (6700), 530 (7200), 500 (13,400),

404 (109,400); Mass Spectra (MALDI): *m/z* 768 $(M + H)^+$ HRMS requires 767.87; found 767.947; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 9.61 (1H, s), 9.48 (1H, s), 8.67 (1H, s), 7.98 (1H, *J* = 11.6, 17.8 Hz), 6.29 (1H, dd, *J* = 17.8, 1.3 Hz), 6.06 (1H, br s), 6.06 (1H, dd, *J* = 11.6, 1.3 Hz), 5.22 (2H, s), 4.74 (1H, m), 4.37 (2H, m), 4.19 (3H, s), 3.68 (3H, s), 3.71 (2H, q, *J* = 7.6 Hz), 3.59 (3H, s), 3.49 (3H, s), 3.45 (3H, s), 3.40 (3H, s), 3.22 (3H, s), 2.81 (1H, m), 2.59 (1H, m), 2.21 (2H, m), 1.81 (1H, m) 1.66 (3H, d, *J* = 5.2 Hz), 1.61 (3H, t, *J* = 7.6 Hz), −1.34 (s), −1.48 (s); Anal. Calcd for $C_{42}H_{49}N_5O_9$: C, 65.69, H, 6.43, N, 9.12. Found: C, 65.30, H, 6.47, N, 8.95.

*Methyl pheophorbide-a (**10**). Method 1*: Algal extract was treated with 5% sulfuric acid in methanol (degassed by bubbling with argon) for 12.5 h at room temperature under argon and protected from light. It was diluted with dichloromethane, washed with water and then with 10% saturated aqueous sodium bicarbonate. The aqueous layer was dried over sodium sulfate, filtered and then evaporated. Recrystallization of the residue from dichloromethane and methanol gave the title product.

Method 2: Pheophorbide-a (**12**) (100 mg; 0.169 mmol), was treated with excess ethereal diazomethane. Argon was flushed through the flask and the solution was evaporated and recrystallized from dichloromethane and methanol to quantitatively give 102 mg of the product (**10**), $C_{36}H_{38}N_4O_5$; mp 218–122°C [lit. mp (20) 224–226°C]. UV–Vis (dichloromethane): λ_{max} (ε/M^{-1} cm^{-1}) 668 nm (40,700), 610 (8100), 560 (3200), 538 (9400), 506 (10,400), 412 (93,400); ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.50 (1H, s), 9.36 (1H, s), 8.57 (1H, s) 8.0 (1H, m), 6.29 (1H, m), 6.26 (1H, s), 6.16 (1H, m), 4.46 (1H, m), 4.20 (1H, m), 3.88 (3H, s), 3.70 (2H, q, *J* = 7.6 Hz), 3.68 (3H, s), 3.57 (3H, s), 3.41 (3H, s), 3.25 (3H, s), 2.63 (1H, m), 2.32 (1H, m), 2.52 (1H, m), 2.2 (1H, m), 1.81 (3H, d, *J* = 7.3 Hz) 1.69 (3H, t, *J* = 7.6 Hz), 0.53 and −1.67 (2H, br, s).

*Chlorin e_6 trimethyl ester (**11**)*. Methyl pheophorbide-a (**10**) (102 mg, 0.168 mmol), was dissolved in dry methanol and stirred under argon for 10 min. Thereafter, 0.35 mL of a 0.5 *M* sodium methoxide solution was added to the solution and it was allowed to stir for 2 h at 0°C. The solution was diluted with H_2O and extracted with dichloromethane. The organic layer was dried with sodium sulfate, filtered and then evaporated. The solid obtained was dissolved in dichloromethane and chromatographed on a plug of neutral alumina (Brockmann grade III) with the same mobile phase. Chlorin-e_6 trimethyl ester (**11**) was eluted with dichloromethane. After evaporation, 105 mg (98%) of **11**, $C_{37}H_{42}N_4O_6$ was obtained: mp 206–210°C [lit. mp (21) 210°C]. UV–Vis (dichloromethane): λ_{max} (ε/M^{-1} cm^{-1}) 664 nm (49,600), 608 (9900), 530 (9900), 501 (17,600), 402 (154,200); Mass Spectra (MALDI): *m/z* 639 $(M + H)^+$; ^{1}H-NMR ($CDCl_3$, 500 MHz): δ 9.63 (1H, s), 9.45 (1H, s), 8.71 (1H, s), 7.93 (1H, dd, *J* = 11.4, 17.8), 6.25 (1H, dd, *J* = 17.8, 1.2 Hz), 6.03 (1H, dd, *J* = 11.4, 1.2 Hz), 5.30 (2H, d, *J* = 18.9 Hz), 4.43 (1H, m), 4.39 (1H, m) 4.24 (3H, s) 3.76 (3H, s), 3.68 (2H, q, *J* = 7.6 Hz), 3.56 (3H, s), 3.55 (3H, s), 3.39 (3H, s), 3.19 (3H, s), 2.55 (1H, m), 2.19 (2H, m), 1.75 (1H, m), 1.74 (3H, d, *J* = 7.4 Hz), 1.66 (3H, t, *J* = 7.6 Hz), −1.33 (1H, s), −1.47 (1H, s); ^{13}C-NMR ($CDCl_3$, 500 MHz instrument): δ 174, 173.1, 169.6, 169.6, 167, 154.8, 148.9, 144.8, 139.5, 136.4, 134.7, 135.9, 135.3, 135.4, 130.5, 129.3, 129.3, 123.3, 121.2, 102.2, 102.1, 99, 93.6, 53.0, 52.9, 52.1, 51.6, 49.4, 38.7, 29.1, 27.6, 22.9, 19.6, 17.7, 12.1, 12.0, 11.3.

*Chlorin-e_6 17^3,15^2-dimethyl ester (**14**)*. Chlorin-e_6 (**1**) (75 mg, 0.126 mmol) was dissolved in 5% sulfuric acid and methanol and allowed to stir protected from light, under argon overnight. The reaction was poured into cold saturated aqueous sodium bicarbonate and extracted twice with dichloromethane. The extract was washed twice with brine, dried over anhydrous sodium sulfate and filtered. The solvent was evaporated and re-dissolved in dichloromethane. It was then purified on a silica gel column. Elution with 6% methanol and dichloromethane gave a major green fraction that was collected. The solvent was removed to afford 75 mg (95%) of **14**, $C_{36}H_{40}N_4O_6$: mp 280–290°C. UV–Vis ($CHCl_3$): λ_{max} (ε/M^{-1} cm^{-1}) 666 nm (49,700), 610 (5900), 562 (2700), 523 (5900), 502 (13,200) 402 (143,400); Mass Spectra (MALDI): *m/z* 625 $(M + H)^+$; ^{1}H-NMR ($CDCl_3$, 300 MHz): δ 9.65 (1H, s), 9.52 (1H, s), 8.72 (1H, s), 8.06 (1H, m), 6.32 (1H, dd, *J* = 17.8, 1.2 Hz), 6.13 (1H, dd, *J* = 11.5, 1.2 Hz), 5.50 (1H *J* = 18.6 Hz), 5.23 (1H, d, *J* = 18.6 Hz), 4.45 (1H, m), 3.82 (3H, s), 3.76 (2H, q, *J* = 7.6 Hz), 3.60 (3H, s), 3.59 (3H, s), 3.46 (3H, s), 3.28 (3H, s), 1.69 and 2.12 (2H, m), 2.19 and 2.56 (2H, m), 1.81 (3H, d, *J* = 7.1 Hz), 1.64 (3H, t, *J* = 7.6 Hz).

*13^1-Mono-(L)-aspartylchlorin-e_6 tetramethyl ester (**6**)*. Chlorin-e_6 dimethyl ester (**14**) (75 mg, 0.120 mmol) was dissolved in dry dichloromethane with 1 mL triethylamine. O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU, 136 mg, 0.36 mmol) was added and the mixture was stirred until completely dissolved. Then aspartic acid dimethyl ester was added. The reaction mixture was heated under reflux for 6 h (or stirred overnight at room temperature), and after cooling it was diluted with dichloromethane and then washed with 5% aqueous citric acid, followed by a wash with brine and with water. It was dried over anhydrous sodium sulfate and then evaporated. The residue was dissolved in 2% methanol/dichloromethane and purified *via* silica gel column chromatography, with the same mobile phase, to yield 50 mg (51%) of **6**, $C_{42}H_{49}N_5O_9$: mp 159–163°C. UV–Vis (dichloromethane): λ_{max} (ε/M^{-1} cm^{-1}) 660 nm, 608, 558, 530, 500, 404; Mass Spectra (MALDI): *m/z* 768 $(M + H)^+$; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.70 (1H, s), 9.62 (1H, s), 8.78 (1H, s), 8.08 (1H, m), 6.35 (1H, dd, *J* = 17.8, 1.5 Hz), 6.12 (1H, dd, *J* = 11.5, 1.5 Hz), 5.56 (1H, d, *J* = 18.8 Hz), 5.48 (1H, *J* = 8.1, 4.6, 4.6 Hz) 5.39 d, *J* = 8.1), 4.45 (2H, m), 3.96 (3H, s), 3.77 (3H, s), 3.80 (2H, q, *J* = 7.7 Hz), 3.66 (3H, s), 3.62 (3H, s), 3.60 (3H, s), 3.60 (3H, s), 3.50 (3H, s), 3.33 (3H, s), 2.55–2.16 (4H m), 1.74 (m), 1.70 (3H, d *J* = 7.2 Hz), 1.68 (3H, t, *J* = 7.7 Hz), −1.47 (1H, s), −1.53 (1H, s).

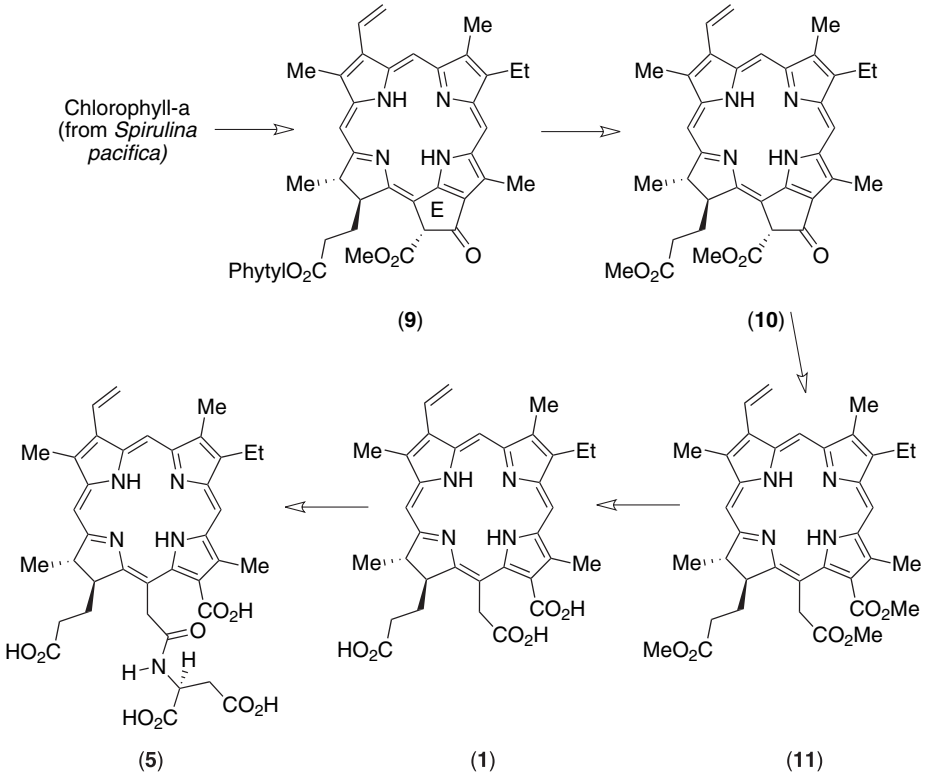
*15^2-Mono-(L)-aspartylchlorin-e_6 tetramethyl ester (**7**)*. Chlorin-e_6 (**1**) (75 mg, 0.126 mmol) was dissolved in dry dichloromethane with 1 mL triethylamine. HBTU (57 mg, 0.15 mmol) was added and stirred until completely dissolved. Then (L)-aspartic acid dimethyl ester hydrochloride was added. The reaction mixture was allowed to stir for 2 h. The mixture was diluted with dichloromethane and then washed with 5% aqueous citric acid, followed by a wash with brine and with water. It was dried over anhydrous sodium sulfate and then evaporated. The residue was dissolved in dichloromethane and treated with excess ethereal diazomethane. The residue was dissolved in 2% methanol/dichloromethane and purified *via* silica gel column chromatography with the same mobile phase to afford 60 mg (61%) of **7**, $C_{42}H_{49}N_5O_9$: mp 157–160°C. UV–Vis (dichloromethane): λ_{max} (ε/M^{-1} cm^{-1}) 660, 608, 558, 530, 500, 404; Mass Spectra (MALDI) *m/z* 768 $(M + H)^+$; ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 9.62 (1H, s), 9.50 (1H, s), 8.69 (1H, s), 7.95 (1H, m), 6.30 (1H, dd, *J* = 17.8, 1.5 Hz), 6.07 (1H, dd, *J* = 11.5, 1.5 Hz), 5.22 (2H, br), 4.76 (1H, ddd, *J* = 8.1, 4.9, 4.4 Hz), 4.40 (1H, m), 4.19 (3H, s), 3.71 (2H, q, *J* = 7.3 Hz), 3.50 (3H, s), 3.49 (3H, s), 3.40 (3H, s), 3.31 (3H, s), 3.22 (3H, s), 3.01 (3H, s), 2.78 (2H, dd, *J* = 16.8, 4.4 Hz) 2.56 and 2.21 (2H, m), 1.66 (2H, m), 1.65 (3H, d, *J* = 7.3 Hz), 1.61 (3H, t, *J* = 7.7 Hz), −1.34 (1H, s), −1.48 (1H, s).

RESULTS AND DISCUSSION

Synthesis of 15^2-LS-11 (5)

15^2-LS-11 tetramethyl ester (**7**) was obtained by transesterification of the phytyl ester group of pheophytin-a (**9**) [*i.e.* demetalated chlorophyll *a* obtained by extraction from *S. pacifica* alga], to form methyl pheophorbide-a (**10**). Subsequent isocyclic ring-opening and treatment with diazomethane formed chlorin-e_6 trimethyl ester (**11**). Alkaline hydrolysis of the methyl esters yielded chlorin-e_6 (**1**). Activation and coupling of chlorin-e_6 to di-*tert*-butyl-protected aspartic acid followed by deprotection with TFA yielded 15^2-LS-11 (**5**) (Scheme 1).

Classical isocyclic ring-opening conditions require treatment of methyl pheophorbide-a (**10**) with an excess of sodium methoxide in tetrahydrofuran (19). To minimize loss of product due to partial hydrolysis of the ester groups, the solution is subsequently treated with diazomethane (prior to chromatography) (19). Yields are extremely variable (30–60%) under these conditions, but starting material is recoverable and can be recycled. An improved ring-opening synthesis was reported in 1980; chlorin-e_6 trimethyl ester (**11**) was obtained in 80% yield when chlorin-e_6 (**1**) was treated with 0.5% KOH



Scheme 1. Synthesis of 15^2-LS-11 (**5**) from chlorophyll *a*.

in methanol. Side-products were chlorin-e_6 mono and dimethyl esters (22).

The isocyclic ring-opening reaction of methyl pheophorbide-a (**10**) is a reversed-Dieckmann condensation. As with most condensations, there exists an equilibrium between the starting material and the product. The Dieckmann condensation (23) is driven forward to completion by an irreversible deprotonation step thereby requiring excess base to drive the reaction toward the Dieckmann product. Consequently, if the synthetic objective is the *reversed*-Dieckmann product, less base is an ally. We determined that only a catalytic amount of base is required if the reaction is performed in methanol.

The ring-opening reaction of methyl pheophorbide-a (**10**), however, must proceed at a rate competitive with other side-reactions of the ring (including decarboxymethylation [17], enolization [24] and an auto-oxidation reaction known to as "allomerization" [25]). Varying concentrations of catalytic amounts of base were utilized. All proceeded with superior yields to standard protocol, but they were highly variable and erratic. An optimized yield of 98% of chlorin-e_6 trimethyl ester (**11**) was consistently obtained with 2.6 equivalents of sodium methoxide in methanol at room temperature. As a key intermediate in the multitude of derivatizations of chlorophyll *a*, chlorin-e_6 trimethyl ester (**11**) is a significant compound (26). It should be noted that the complementary reaction, isocyclic ring closure, proceeds in high yield when the nonnucleophilic *tert*-butoxide is used (27).

The final steps in the synthesis of 15^2-LS-11 (**5**) require the coupling of chlorin-e_6 with a protected aspartic acid (though the commercial process utilizes unprotected (L)-aspartic acid), followed by acidic deprotection. With three free carboxyl groups at the periphery, selectivity is *apparently* observed (but see later) for activation, nucleophilic addition and subsequent elimination at the 15^2-carboxylic acid. This selectivity is observed regardless of the coupling reagent employed, *i.e.* carbodiimide, uronium/guanidium salts or acid chloride. Selectivity is lost in the presence of protic solvents. Optimal yields (63%) of mono-(L)-aspartylchlorin-e_6 (**5**) are obtained with the water-soluble coupling reagent HBTU. As mentioned earlier, the di-aspartic acid conjugate is a side-product formed regardless of the coupling reagent utilized. Recently Taima *et al.* reported synthetic conditions using oxalyl chloride for activation and coupling of all three carboxyl groups of chlorin-e_6 (28).

Synthesis of 17^3-LS-11 tetramethyl ester (8)

A novel route to the 17^3-regioisomer of the biomedically significant LS-11 was initially visualized for two purposes: (1) structural elucidation of LS-11 *via* the classical methodology of unambiguous synthesis to be used in conjunction with spectroscopic characterization, and (2) development of a potentially high-yielding route to a chlorin-e_6 photosensitizer.

Historically, LS-11 has had a large amount of ambiguity associated with its structure. The patent (15) filed in 1987 claimed LS-11 ("NPe_6" or Talaporfin at the time) was possibly a mixture of the 13^1-, 15^2- and 17^3-regioisomers of mono-(L)-asparty1chlorin-e_6, but this was not borne out by ^{1}H-NMR spectroscopy (Fig. 1). Because purity is assured *via* its isolation by HPLC, academic papers published since 1987 assumed LS-11 was the 17^3-regioisomer (**2**). It was assumed to be the 17^3-regioisomer for two main reasons: (1) the 17-propionic side chain is farthest from the aromatic macrocycle and is therefore less susceptible to the electronic deactivating affects of the

chlorin ring, and (2) its situation at the extreme periphery of the molecule, and above the plane of the macrocycle, makes it sterically less congested toward attack by coupling agents and amino acids.

To everyone's surprise, in 1998 a very thorough 2D NMR study was published claiming that LS-11 is actually the 15^2-regioisomer (**5**) (29). Unfortunately, the conclusions in this study were not universally accepted because the NMR experiments were performed in D_2O where chlorin aggregation can seriously complicate NMR chemical shifts and analysis (30). Additionally, the result was counterintuitive from a mechanistic perspective as the 15^2-carboxy group was assumed to be both more hindered and more susceptible to deactivation. The study also reported syntheses and spectroscopic evaluation of the 17^3- and 13^1-positional isomers of 15^2-LS-11 but in addition to being low-yielding, the synthetic routes were ambiguous. As a result, most studies since 1998 reported LS-11 to be either the 15^2-or the 17^3-aspartyl derivatives. The identity of LS-11 has remained a matter of conjecture and the distributors appear to have remained silent on the critically important structural issue raised by Gomi *et al.* (29).

The simple initial objective of our program, begun in 2003 as an undergraduate project, involved growing a crystal of the commercial material, or of a derivative produced without affecting the carbon skeleton or regiochemistry. Because, over a couple of years, satisfactory crystal growth was unsuccessful, we turned to the additional classical strategy of a parallel unambiguous synthesis, employed adjunctively with 1D and 2D NMR (COSY, TOCSY) analysis. These NMR studies (see later, Table 1) were performed on the methyl esters (**6**)–(**8**) of LS-11 enabling increased organic solubility, which allowed acquisition of monomeric (*i.e.* disaggregated) spectra.

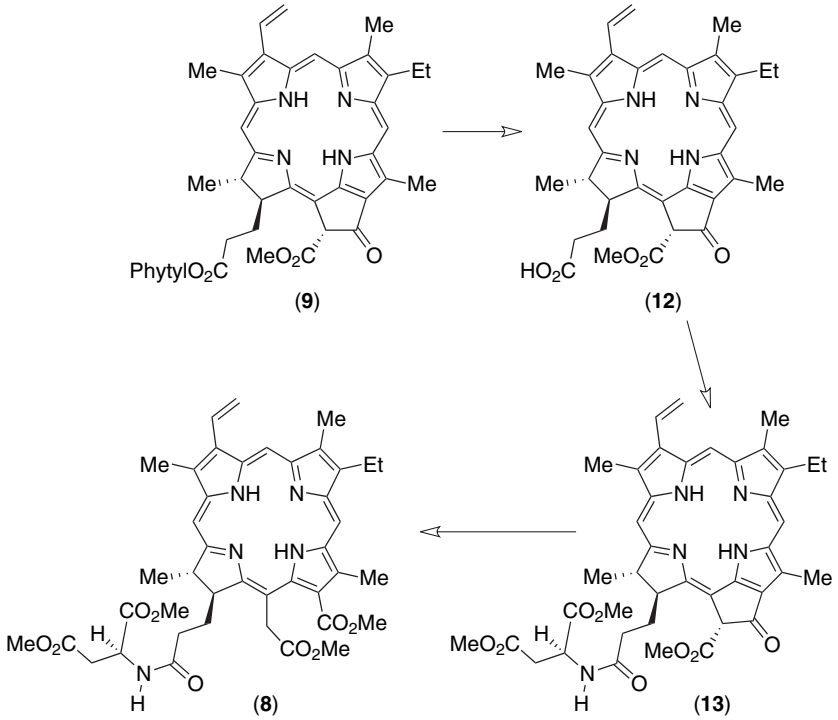
A selective route for 17^3-LS-11 synthesis required a new route in the degradation of chlorophyll *a*. The degradation chemistry of pheophytin-a (**9**, *i.e.* demetalated chlorophyll *a*) is dominated by the presence of a five-membered ß-ketoester ring E, the so-called isocyclic ring. Because of the extremely reactive nature of this ß-ketoester ring, synthetic modifications immediately following extraction usually involve either intentional demethoxycarbonylation (to give the so-called pyro-series of compounds) or isocyclic ring-opening.

The phytyl ester group of pheophytin-a (**9**) can be selectively hydrolyzed to form pheophorbide-a (**12**) in high yield without affecting the ß-ketoester of the isocyclic ring (31,32). The feasibility of partial hydrolysis thus allows for a new protecting group strategy. If integrity of the isocyclic ring and ß-ketoester could be maintained throughout coupling conditions, this ring could serve as a natural protecting group during the coupling of the free 17^3-carboxylic acid group to aspartic acid dimethyl ester. It was therefore necessary to determine whether or not the ring could remain intact during coupling to an amine and then subsequently opened to form a 17^3-chlorin-e_6 derivative (Scheme 2). Such a direct and unambiguous synthesis would not only offer a potentially high-yielding route to a chlorin e_6 derivative but would also assist in clarifying any confusion associated with the structure of LS-11.

Table 1. ^{1}H-NMR assignments* (500 MHz in $CDCl_3$) of chlorin-e_6 trimethyl ester (**11**), 15^2-LS-11 tetramethyl ester (**7**), 17^3-LS-11 tetramethyl ester (**8**) and 13^1-LS-11 tetramethyl ester (**6**).

	Chlorin-e_6 TME (**11**)	15^2-LS-11 TME (**7**)	17^3-LS-11 TME (**8**)	13^1-LS-11 TME (**6**)
10	9.63 s	9.72 s	9.61 s	9.73 s
5	9.45 s	9.55 s	9.48 s	9.65 s
20	8.71 s	8.81 s	8.67 s	8.81 s
3^1	7.93 (1H,dd, J = 11.4, 17.8 Hz)	8.0 (1H, dd, J = 11.7, 17.8 Hz)	7.98 (1H, dd, J = 11.6, 17.8 Hz)	8.10 (1H, dd, J = 11.7, 18.0 Hz)
3^2	6.25 (1H, dd, J = 17.8, 1.2 Hz); 6.03 (1H, dd, J = 11.4, 1.2 Hz)	6.32 (1H, dd, J = 17.8, 1.5 Hz); 6.12 (1H, dd, J = 11.7, 1.5 Hz)	6.29 (1H, dd, J = 17.8, 1.3 Hz), 6.06 (1H, dd, 11.6, 1.3 Hz)	6.37 (1H, dd, J = 18.0, 1.5 Hz); 6.16 (1H, dd, J = 11.7, 1.5 Hz)
15^1	5.30 (2H, J = 18.9 Hz)	5.33 (2H, m)	5.22 (2H, s)	5.59 (1H, d, J = 18.8 Hz); 5.24 (1H, d, J = 8.1 Hz)
17/18	4.39 (2H, m)	4.54 (2H, m)	4.37 (2H, m)	4.45 (2H, m)
13^2	4.24 (3H, s)	4.34 (3H, s)	4.19 (3H, s)	Absent
15^3	3.76 (3H, s)	Absent	3.68 (3H, s)	3.78 (3H, s)
8^1	3.68 (2H, q, J = 7.6 Hz)	3.77 (2H, q, J = 7.3 Hz)	3.71 (2H, q, J = 7.6 Hz)	3.80 (2H, q, J = 7.7 Hz)
17^4	3.55 (3H, s)	3.61 (3H, s)	Absent	3.63 (3H, s)
12^1	3.56 (3H, s)	3.61 (3H, s)	3.49 (3H, s)	3.63 (3H, s)
2^1	3.39 (3H, s)	3.47 (3H, s)	3.40 (3H, s)	3.50 (3H, s)
7^1	3.19 (3H, s)	3.28 (3H, s)	3.22 (3H, s)	3.33 (3H, s)
17^2	2.19 (2H, m)	2.20 (2H, m)	2.21 (2H, m)	2.21 (2H, m)
17^1	2.55 (1H, m), 1.75(1H, m)	2.68 (1H, m), 2.35 (1H, m)	1.81 (2H, m)	2.55 (1H, m), 1.84 (1H, m)
18^1	1.74 (3H, d, J = 7.4 Hz)	1.79 (3H, d, J = 7.3 Hz)	1.66 (3H, d, J = 5.2 Hz)	1.75 (3H, d, J = 7.1 Hz)
8^2	1.66 (3H, t, J = 7.6 Hz)	1.73 (3H, t, J = 7.7 Hz)	1.61 (3H, t, J = 7.6 Hz)	1.73 (3H, t, J = 7.7 Hz)
23	−1.33 (1H, s)	−1.26	−1.34	−1.56
21	−1.47 (1H, s)	−1.36	−1.48	−1.74
Aspartic acid				
aa1	Absent	4.9 (1H, m)	4.74 (1H, m)	5.49 (1H, m)
aa2	Absent	2.78 (2H, d, J = 5.0 Hz)	2.81 (1H, m), 2.59 (1H, m)	3.37 (2H, m)
aa OCH_3	Absent	3.44 (3H, s)	3.45 (3H, s)	3.67 (3H, s)
aa OCH_3	Absent	3.15 (3H, s)	3.59 (3H, s)	3.96 (3H, s)

*Assignments for the 17^2 and 17^1 protons are provisional and difficult to establish (34), even using 2D NMR.

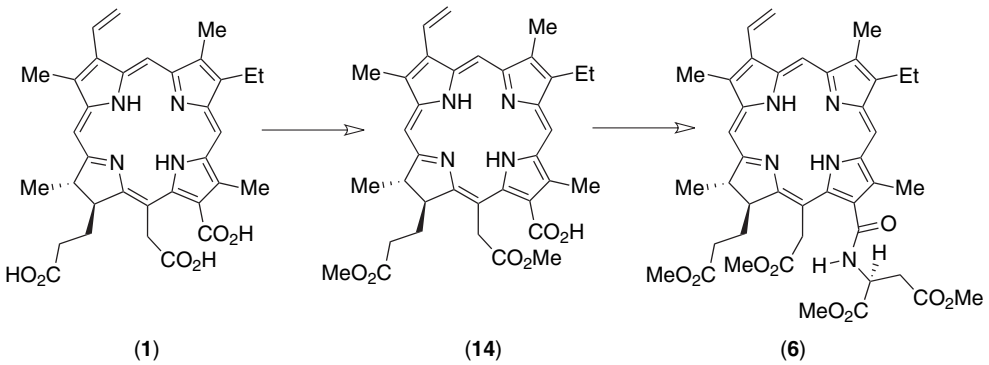


Scheme 2. Synthesis of 17^3-LS-11 tetramethyl ester (**8**) from pheophytin-a (**9**).

Partial hydrolysis of the phytyl group with respect to the 13^3-methoxycarbonyl group proceeded as predicted, and pheophorbide-a (**12**) was obtained in a 93% overall yield. ^{1}H-NMR spectroscopy demonstrated that the ester and isocyclic ring had survived this step. The second step required the coupling of pheophorbide-a (**12**) with aspartic acid dimethyl ester. The coupling reagent DCC was employed with DMAP as a catalyst. The reaction was complete within 2 h and proceeded reproducibly with a typical coupling yield of 95% of the conjugate (**13**). Again, ^{1}H-NMR spectroscopy demonstrated that the 13^3-ester and the isocyclic ring had survived. Using HBTU as the coupling agent was far less satisfactory, and the isocyclic ring did not survive. Isocyclic ring-opening in (**13**) with 2.6 equivalents of methoxide in methanol yielded the 17^3-LS-11 tetramethyl ester (**8**) in 97% yield. TOCSY spectroscopy (not shown) was employed to definitively identify some unassigned peaks in the 1D ^{1}H-NMR spectrum.

Synthesis of 13^1-LS-11 tetramethyl ester (6)

The 13^1-LS-11 tetramethyl ester (**6**) was also synthesized by a completely unambiguous route. Esterifications of carboxylic acids with H_2SO_4/MeOH are driven forward in high yield with an excess of methanol. Under acidic conditions, the inner nitrogen atoms of tetrapyrrolic macrocycles are fully protonated and the 13^1-carboxylic acid, attached directly to the conjugated chromophore, becomes severely deactivated; this permits selective methylation of the 15- and 17-side chain esters (33). Exploiting the pH sensitivity of the 13^1-side chain, the 17^3 and the 15^2 carboxylic acids of chlorin-e_6 (**1**) were selectively esterified (methyl esters) with 5%/H_2SO_4/MeOH to give (**14**) (Scheme 3). The remaining 13^1-carboxylic acid was then available for activation and coupling. Even under the basic conditions required for normal coupling, the 13^1-carboxylic acid was still slow to react and required heating for optimal yields of 13^1-LS-11 tetramethyl ester (**6**). The UV–Vis



Scheme 3. Synthesis of 13^1-LS-11 tetramethyl ester (**6**) from chlorin-e_6 (**1**).

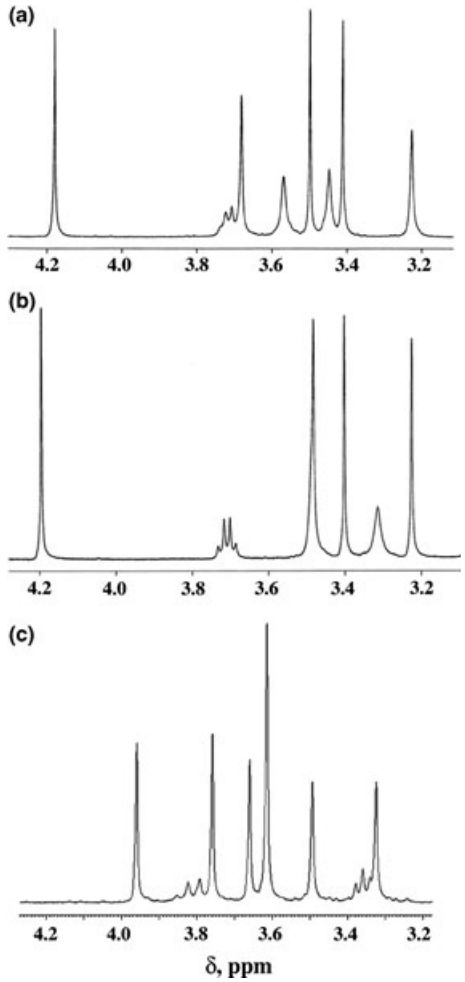


Figure 2. Stack-plotted ^{1}H-NMR spectra (3.2–4.2 ppm region only) in $CDCl_3$ of: (a) 17^3-LS-11 tetramethyl ester (**8**), (b) authentic LS-11 tetramethyl ester (*i.e.* the 15^2-aspartyl regioisomer, **7**) and (c) 13^1-LS-11 tetramethyl ester (**6**).

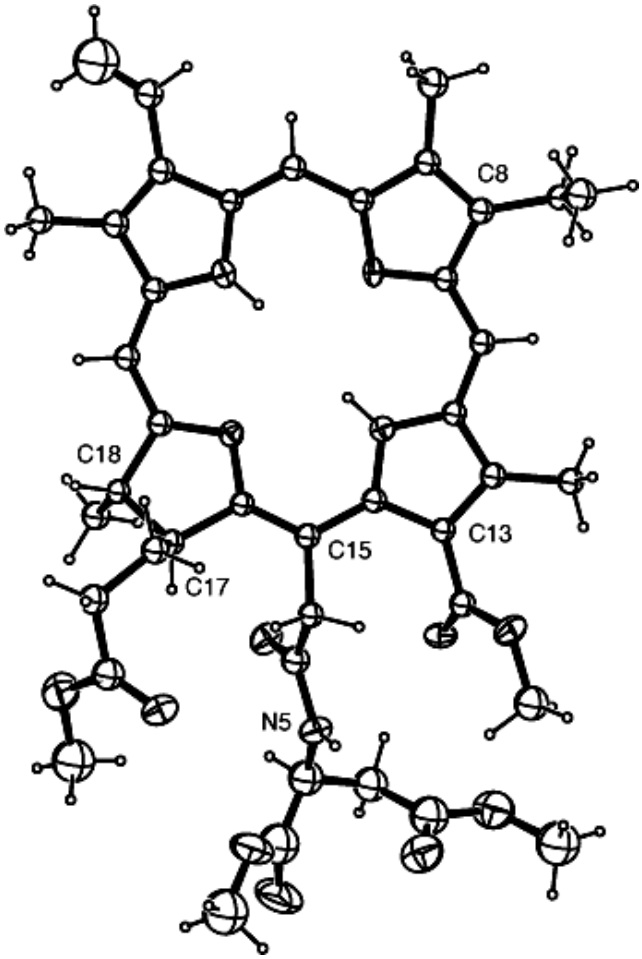


Figure 3. X-ray structure of authentic LS-11 tetramethyl ester (**7**), illustrating one of the two independent molecules with 30% ellipsoids.

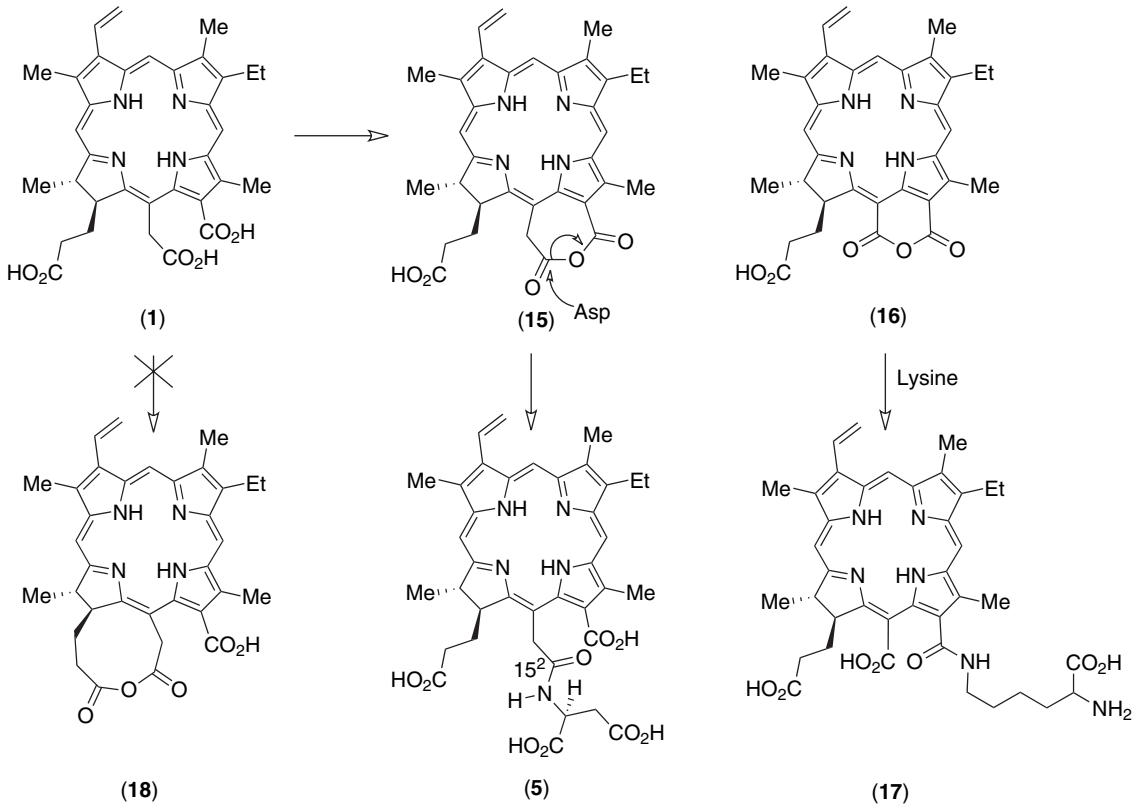
spectrum of **6** is identical with its 15^2- and 17^3-regioisomers, but once again the ^{1}H-NMR spectrum is definitively different (Table 1, Fig. 2). Figure 2 shows the stack-plotted 3.2–4.2 p.p.m. portions of the three LS-11 regioisomers, revealing the key peak absences that establish the structures of each; peak assignments are as listed in Table 1.

Comparisons with authentic 15^2-LS-11 tetramethyl ester (7)

Authentic LS-11 was obtained from Frontier Scientific and was esterified with diazomethane. The ^{1}H-NMR spectrum of this authentic tetramethyl ester (Fig. 1) was compared with that of the 17^3-LS-11 tetramethyl ester synthesized by way of the unambiguous route from pheophytin-a, and also with that of the synthetic 13^1-tetramethyl ester (**6**) obtained from chlorin-e_6 (**1**). Figure 2 shows the stack-plotted definitive 3.2–4.2 ppm portions of the spectra from the three LS-11 tetramethyl ester regioisomers (**6–8**), revealing key peak absences that unambiguously establish the structures of each; peak assignments can be found in Table 1, which also includes the spectrum, at 500 MHz, of chlorin-e_6 trimethyl ester (**11**). Although mass spectra and UV–Visible spectra (not shown) were identical for the three substances, the ^{1}H-NMR spectra are quite distinct (Table 1, Fig. 2).

X-ray crystal structure of authentic 15^2-LS-11 tetramethyl ester (7)

Throughout the course of the synthetic studies, multiple crystal-growth experiments were continually underway, but crystals of suitable quality remained elusive. We were comforted that few crystal structures of chlorophyll *a* derivatives (and to our knowledge none of the derivatives in the chlorin-e_6 series) have been reported. After 18 months, a crystal of sufficient quality emanated from liquid–liquid diffusion with methanol and dichloromethane. The molecular structure of one of the two independent molecules in the asymmetric unit is shown in Fig. 3. The two are chemically identical, differing in the conformations of the substituents at C8, C13, C15 and C17. Torsion angles about the chlorin-core-to-substituent bonds differ by 165(2)° at C8, 45(3)° at C13, 15(3)° at C15 and 64(2)° at C17. For neither molecule does the 24-atom chlorin core deviate greatly from planarity. In one molecule the mean deviation from coplanarity is 0.07 Å, and for the other molecule it is 0.19 Å. Unsurprisingly, the largest deviations,



Scheme 4. Rationale for unique formation of 15^2-LS-11 in the commercial process.

up to 0.69(1) Å, are in the dihydropyrrole portion of the molecule. There is no chlorin ring stacking in the crystal structure, and the (L)-aspartyl N-H groups, N5, do not form intermolecular hydrogen bonds to acceptors on other chlorin molecules, but rather to solvent molecules. The X-ray structure (CCDC 632562) contains the supplementary crystallographic data for this study in CIF format. These data can be obtained free of charge *via* www.ccdc.cam.ac.uk/conts/retrieving.html (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK; fax:(+44)1223-336-033; or deposit@ccdc.cam.ac.uk).

Rationale for the unique formation of the 15^2-LS-11 regioisomer (5) in the commercial synthesis

Based in the syntheses, spectroscopic and X-ray diffraction details discussed above, LS-11 has definitively been shown to be the 15^2-aspartyl derivative (**5**) and not: (1) a mixture of 13^1, 15^2 and 17^3 regioisomers as postulated in the patent (15), or (2) the 17^3-regioisomer (**2**) assumed in academic papers published since 1987. Furthermore, this study confirms conclusions from a substantially ignored study published in 1998 (29). With the certain knowledge that LS-11 is *not* the regioisomer (**2**) which would be intuitively predicted based on simple chemical reactivity, a solution to the riddle immediately becomes apparent, even obvious. The coupling reagent must be initially responsible for the formation of a seven-membered anhydride ring from two carboxylic acids of chlorin-e_6 (**1**) to give **15** (Scheme 4).

The aspartic acid next accomplishes a simple anhydride cleavage reaction at the 15^2-position (as expected), unaided by external coupling reagent, to give LS-11 (**5**). We have previously published on the ring-opening of the six-membered anhydride ring in purpurin-18 (**16**) to give amino acid conjugates such as (**17**) (35), and have described the biologic activity of these new PDT sensitizers (36). In the chlorin-e_6 case, the alternate, less stable, nine-membered anhydride (**18**) (which might eventually afford the 17^3-regioisomer [**2**] of LS-11), is presumably not formed because of its large ring-size and the enforced stereochemistry at the 17^1-position. Similar conclusions were reached by Xu and Pallenberg in an oral report presented at the 4th International Conference on Porphyrins and Phthalocyanines (ICPP-4; Rome, July 2006), though not in the Abstract of their paper (37).

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