Naturally-derived porphyrin and chlorin photosensitizers for photodynamic therapy

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NATURALLY-DERIVED PORPHYRIN
AND CHLORIN PHOTOSENSITIZERS
FOR PHOTODYNAMIC THERAPY

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Chemistry

By
Jodie Angela Hargus
B.S., Louisiana State University, 2001
December 2005
Dedication

For Sheridan Simon, Kathrine Havens, Cordia Havens, Marjorie Hargus, Louis Hargus II, Raymond Muro, Madeleine Clay and Margaret Johnson, loved ones I have lost to cancer.
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GLOSSARY OF ABBREVIATIONS

Bchl = Bacteriochlorin

Bu' = Butyl-C(CH₃)₃

Chl = Chlorophyll

COSY = Correlated spectroscopy. Two-dimensional NMR experiments relating two independent parameters (e.g. ¹H chemical shifts to ¹³C shifts).

DCM = Dichloromethane CH₂Cl₂

Dme = Dimethyl ester

Et = Ethyl -CH₂CH₃

HMBC = Heteronuclear Multiple Bond Correlation. Inverse detected ¹H – ¹³C long-range correlation experiment.

HPLC = High Performance Liquid Chromatography

HRMS = High Resolution Mass Spectrometry

HSQC = Heteronuclear Single Quantum Coherence. Inverse detected ¹H – ¹³C one bond correlation experiment.

LHC = Light Harvesting Complex

Me = Methyl-CH₃

MeOH = Methanol, CH₃OH.

NMR = Nuclear Magnetic Resonance

Oac = Acetate -OCOCH₃

OAC = Oxalyl Chloride

PDT = Photodynamic Therapy

Pheid = Pheophorbide

PS = Photosystem

TBAB = Tetrabutylammonium Bromide
**TEA** = Triethylamine (CH\textsubscript{3}CH\textsubscript{2})\textsubscript{3}N

**TFA** = Trifluoracetic Acid CF\textsubscript{3}CO\textsubscript{2}H

**TLC** = Thin Layer Chromatography

**TME** = Tetramethy Ester

**TMS** = Tetramethylsilane

**TOCSY** = Total Correlation Spectroscopy. 2D COSY method with net magnetization transfer extending throughout coupled spin groups. All mutually coupled spins show cross peaks.

## ABBREVIATIONS WITH SELECTED STRUCTURES

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Structure Description</th>
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<tbody>
<tr>
<td>DMF</td>
<td>N,N dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
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<tr>
<td></td>
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<td>-------</td>
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<tr>
<td><strong>EDC</strong></td>
<td>1-[3-{(Dimethylamino)propyl}] -3ethylcarbodiimide HCl</td>
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<td><strong>HBTU</strong></td>
<td>O, benzotriazol 1-yl- N, N, N', N' tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td></td>
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<tr>
<td><strong>AA</strong></td>
<td>L- Aspartic Acid</td>
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<tr>
<td><strong>Lys</strong></td>
<td>L- Lysine</td>
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<tr>
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<tr>
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In oncologic applications of photodynamic therapy (PDT), the discriminating localization of porphyrin-type compounds in solid tumors is exploited for the selective ablation of neoplastic tissue with minimal destruction and irritation to normal tissue.

PDT is a locoregional, binary cancer therapy in which a photosensitizer—light-activated drug—absorbs light of an appropriate wavelength and excites to the singlet state. This photosensitizer in the excited singlet state can undergo an internal transition to the excited triplet state, a relatively long-lived and high-energy species that transfers its excess energy to molecular oxygen. Molecular oxygen subsequently excites from the stable triplet state to the highly reactive singlet state. With no spin-state restriction, singlet oxygen is cytotoxic, readily reacting with electron-rich biomolecules such as unsaturated lipids, amino acids and DNA consequently destroying the tumor cell. Singlet oxygen has a limited range of diffusion. Therefore, the site of its generation is also the site of initial damage.

Mono-L-aspartyl chlorin e₆, a chlorophyll a derivative also known as talaporfirin and subsequently referred to here as NPe₆, is a 2nd-generation photosensitizer currently in advanced-stage clinical trials for PDT. NPe₆ is obtained by transesterification of the phytol ester group of chlorophyll a with a methyl ester group to form pheophorbide a. Subsequent isocyclic ring opening forms chlorin e₆ trimethyl ester. Alkaline hydrolysis of the methyl esters and then activation and coupling to a protected aspartic acid followed by deprotections yields NPe₆. The structural elucidation of NPe₆ has been performed employing a classical methodology of an unambiguous synthesis used adjunctively with modern NMR techniques. The synthesis of NPe₆ has been made more efficient via the optimization of the isocyclic ring opening and coupling reaction. Natural reactivities of chlorophyll a derivatives have been
exploited to synthesize two regiosomers of NPe₆ for biological property investigation. A novel route to a 17³ chlorin e₃ derivative has been generated.

Because to date no chlorin photosensitizers have received FDA approval in the United States, various amino-acid porphyrin conjugates specifically PPIX conjugates have been synthesized and their preliminary biological evaluation, which demonstrates that subtle differences in structure can correlate to huge differences in function, is described.
1.1 Photodynamic Therapy

In oncologic applications of photodynamic therapy (PDT), the discriminating localization of porphyrin-type compounds in solid tumors is exploited for the selective ablation of neoplastic tissue with minimal destruction and irritation to normal tissue. PDT is a locoregional, binary cancer therapy in which a photosensitizer—a light-activated drug—absorbs light of an appropriate wavelength resulting in its excitation to the singlet state. This photosensitizer in the excited singlet state can undergo an internal transition to the excited triplet state, a relatively long-lived and high-energy species that transfers its excess energy to molecular oxygen. Molecular oxygen subsequently excites from the stable triplet state to the highly reactive singlet state. With no spin-state restriction, singlet oxygen is cytotoxic, readily reacting with electron-rich biomolecules such as unsaturated lipids, amino acids and DNA, consequently destroying the tumor cell. Singlet oxygen has a limited range of diffusion. Therefore, the site of its generation is also the site of initial damage as seen in Equation 1.1 and Figure 1.1.

Equation 1.1 Singlet oxygen generation via photosensitization. P = Porphyrin; ISC = Intersystem Crossing.

Chemical sensitization with light and acridine was first observed with a paramecium in 1900 by Raab. In 1903, using eosin and sunlight in the treatment of a number of human
Figure 1.1 Simplified Jablonski diagram showing porphyrin and oxygen singlet and triplet state. P=Porphyrin; * = electronically excited state; 0 = ground state; 1 = singlet excited state; 3 = triplet excited state.
skin conditions, Jesionek and Tappeiner demonstrated the basic principle of PDT.\textsuperscript{5} See Figure 1.2. Although it can be used as a stand-alone treatment, PDT is also amenable to combination with other therapies. It can be used adjunctively with chemotherapy, radiotherapy or surgery.\textsuperscript{6}

![Acridine](image1)

![Eosin](image2)

\textbf{Figure 1.2} Early photosensitizers.

Recent studies suggest a synergistic effect is observed when PDT is used with radiotherapy: Tumor cell destruction occurs to a much greater extent than can be accounted for simply by the additive effect of PDT and radiotherapy.\textsuperscript{7}

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 USA and/or 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.\textsuperscript{8,9,10,11} Now that adequate optical technology is available, the expanded utility of PDT as a viable, broad-application treatment option for multiple types of
localized malignancies and pre-malignant diseases largely depends upon improvement of the biological properties of the photosensitizers employed.

1.2 Porphyrins

The potential of porphyrins as anti-tumor agents in oncology was first fully recognized in the 1960s with the development of hematoporphyrin derivative (HpD), which showed selective localization in solid tumors.\textsuperscript{12} Porphyrins, as illustrated in Figure 1.3, are tetrapyrrole macrocycles with 22 $\pi$ conjugated electrons, 18 $\pi$ of which are in any one delocalized pathway.\textsuperscript{13,14} Porphyrins obey Huckel’s rule of aromaticity. As a result of the extended conjugation, porphyrins are vibrantly colored compounds. The visible absorption spectrum shows an intense Soret band around 400 nm.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{porphyrin.png}
\caption{Porphyrin: 22 $\pi$ electron tetrapyrrole of which 18 $\pi$ are in any one delocalized pathway.}
\end{figure}

Porphyrs and other closely related tetrapyrrolic pigments occur widely in nature with significant roles in various biological processes. For example, heme, the iron (II) protoporphyrin-IX complex, is the prosthetic group in hemoglobins and myoglobins responsible for oxygen transport in red blood cells and oxygen storage in tissue. Heme, shown in Figure 1.4, is also found in the enzyme peroxidase that catalyzes the oxidation of substrates by hydrogen peroxide and the related enzyme catalase.
Figure 1.4 Heme, the iron (II) protoporphyrin-IX complex.

The most significant group of photosynthetic pigments are the chlorophylls, which are magnesium chelates of the closely related tetrapyrroles. They are present in all organisms that convert light energy into chemical energy. Chlorophylls are tetrpyrrolic pigments that can be of the porphyrin, chlorin or bacteriochlorin oxidation state. They are characterized by the presence of a central magnesium atom and a fifth isocyclic ring that is biosynthetically derived from the C-13 propionic acid side chain of protoporphyrin. The biological definition of chlorophyll varies slightly from this chemical characterization and includes those tetrpyrrolic pigments active in photosynthetic electron transport, such as pheophytin a the demetalated form of chlorophyll a. There are more than fifty naturally occurring chlorophylls.

Chlorophyll a is present in all organisms capable of oxygenic photosynthesis. It functions as the primary photosynthetic pigment acting as the primary donor in the reaction center of photosystem I and photosystem II and also functions in light-harvesting in the antenna complexes of oxygenic organisms. Chlorophyll b is present in an approximate ratio of 1:3 with chlorophyll a and functions as a light-harvesting pigment. See Figure 1.5. The C-20 terpenoid alcohol esterifying the C-17 propionic acid side chain is phytol. The term phytol refers to the carbon chain itself: (2E)-(7R, 11R), -3, 7, 11, 15-tetramethyl hexadecenyl. The
Phytyl chain is the most common terpene chain of the chlorophylls. There are other chains, however, such as farnesyl (2 E, 6E)-3, 7, 11 trimethyl-2, 6, 10 dodecatrienyln. See Figure 1.6.

![Chlorophyll a and Chlorophyll b](image)

**Figure 1.5** Chlorophyll b varies from chlorophyll a by the presence of a 7-formyl group instead of a 7 methyl substituent.

![Phytyl, Farnesyl, and Geranylgeranyl](image)

**Figure 1.6** The long terpene chain esterified at the C-17 chain is phytyl for chlorophyll a and chlorophyll b.
In 1884, Nencki isolated the first pure porphyrin by preparing hematoporphyrin hydrochloride directly from isolated heme.\textsuperscript{15} In 1912, Kuster first proposed the structure of porphyrins as four pyrrole units linked by four methine bridges.\textsuperscript{16} This structure was later confirmed in 1926 when Fisher synthesized etioporphyrin-I\textsuperscript{17} thereby demonstrating that the aromatic structure initially proposed by Kuster was correct. See Figure 1.7 and Figure 1.8. Woodward accomplished the first total synthesis of chlorophyll \textit{a} in 1960.\textsuperscript{18}

In 1975, Dougherty demonstrated HpD\textsuperscript{19} could selectively destroy tumors upon irradiation.\textsuperscript{20} In 1983, a purer form of HpD, now commercially known as Photofrin\textsuperscript{®} (porfimer sodium), was developed. Photofrin\textsuperscript{®} received FDA approval in the United States in 1995 and is now also approved in more than 40 countries. Although Photofrin\textsuperscript{®} has been shown to be efficacious in the treatment of many cancer types, it has many undesirable properties.

Porphyrrins absorb strongly around 500 nm where depth-of-light penetration is weak due to interference with the absorption of other tissue chromophores, such as hemoglobin in the blood. Photofrin\textsuperscript{®} is not rapidly cleared from normal tissue and exists as a complex mixture of oligomers. These properties inhibit its generalized use in oncology. Additionally, side effects such as prolonged skin photosensitivity can make its application inconvenient.

![Tetrapyrrole macrocycle](image)

\textbf{Figure 1.7} Kuster was the first to propose that porphyrins were tetrapyrrole macrocycles.
Figure 1.8 Early porphyrins synthesized by Nencki and Fisher.

1.3 NPe₆

Development of 2nd-generation photosensitizer candidates has focused on improving the photophysical and pharmacokinetic properties of potential photosensitizers to increase the efficacy and expand the utility of PDT, while simultaneously obviating negative side effects of the currently approved treatments. Mono-L-aspartyl chlorin e₆ (1), which is also known as talaporfin and subsequently referred to here as NPe₆²¹, is a 2nd-generation photosensitizer currently in advanced-stage clinical trials for oncologic PDT applications. As a chlorin—17,18 dihydroporphyrin—NPe₆ has characteristic longer wavelength absorption at 666 nm, which allows for greater depth-of-light penetration and increased photon utilization than Photofrin.²² See Figure 1.9, Figure 1.10 and Figure 1.11.

NPe₆ is more specifically an aspartic acid conjugate of chlorin e₆. Upon irradiation, NPe₆ gives good yields of long-lived triplets (lifetimes, 500 to 800 μs) and therefore gives high yields of cytotoxic singlet oxygen.²² Additionally, NPe₆ shows rapid clearance from normal tissue. In a direct comparison of NPe₆ with Photofrin in PDT of cholangiocarcinoma, NPe₆ 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.\textsuperscript{23}

\textbf{Figure 1.9} Chlorin: 20$\pi$ electron tetrapyrrole of which 18$\pi$ are in any one localized pathway.

\textbf{Figure 1.10} Mono L-aspartyl chlorin also known as talaporfin and NPe\textsubscript{6}.

As a chlorophyll-$\alpha$ derivative, NPe\textsubscript{6} also possesses additional qualities that rival even other chlorin photosensitizers. Compared with synthetic chlorins such as temoporfin\textsuperscript{24} NPe\textsubscript{6} has increased stability since synthetic chlorins can readily oxidize back to porphyrins. See Figure 1.12. Chlorophyll-$\alpha$ derivatives have increased stability because they possess unusual structural characteristics not easily accessible with present synthetic methodologies.\textsuperscript{25,26} Stability is especially significant in binary treatment modalities because degradation products absorb light outside the laser window consequently making treatment ineffectual.
Figure 1.11 UV-Vis Spectra of Porphyrin and Chlorin.
Amphiphilicity, another favorable trait, has been shown to improve the effectiveness of photosensitizers. Chlorophyll-\(\alpha\) derivatives of the chlorin e\(_6\) series possess three carboxylic side chains, making them ideal substrates for synthesis of novel amphiphilic photosensitizers.

A recent study discovered that small differences in photosensitizer structure, including regioisomerism, can correlate to huge differences in function, such as subcellular localization. Photosensitizer subcellular localization has recently been demonstrated to be a factor in the mode of cell damage (i.e. necrosis vs. apoptosis) and therefore helps determine PDT efficiency.

The success of NPe\(_6\) as a photosensitizer warranted optimization of its synthesis. The data suggesting that subtle differences in structure can improve biological properties warranted generation of its regioisomers (2; 3) for comparison. Therefore, in addition to describing the structural elucidation of NPe\(_6\), Chapter 2 of this manuscript also describes an improved overall synthesis of NPe\(_6\) from chlorophyll \(\alpha\) and the creation of novel, selective synthetic routes to the generation of its regioisomers in order to compare the biological properties. See Scheme 1.1. Of note, one synthetic step — isocyclic ring opening — shared by
Scheme 1.1 Synthetic scheme showing generation of three NPe₆ isomers from chlorophyll a. Each isomer shares one intermediate step, which has been optimized. The route to the 17³ NPe₆ is novel and high yielding — 86% overall from chlorophyll a.
all three regioisomers has been made highly efficient. These new synthetic methodologies are now being employed to generate novel chlorin conjugates to study how variation of peripheral substituents such as other amino acids and other bioactive molecules affect biological properties such as uptake, cytotoxicity and subcellular localization. We are systematically evaluating the effects of substituent charge, position and size on these biological properties to better understand how to improve photosensitizer activity and therefore help increase the efficiency and expand the utility of PDT.

Because to date NPe₆ is still in advanced-stage clinical trials and no chlorin photosensitizers have received approval in the United States, Chapter 3 is devoted to the synthesis of various amino-acid porphyrin conjugates—specifically PPIX conjugates—for biological property investigation, as shown in Scheme 1.2. Preliminary biological evaluation has demonstrated that once again subtle differences in structure can correlate to huge differences in function.

Scheme 1.2 Synthesis of PPIX derivatives. Chapter 3 describes synthesis where R = Lysine and R = Glutamate and includes the preliminary biological properties of these compounds.
1.4 References


19 HpD is a derivative of hematoporphyrin, which is prepared by treating acetylated hematoporphyrin with alkali. HpD is a complex mixture of porphyrins.


21 NPe<sub>6</sub> was named for Nippon Chemical Company, which owns the patent.


24 Temoporfin (meta-tetrahydroxyphenylchlorin) is employed in PDT of the head and neck.


27 See Ref. 1.


CHAPTER 2
CHLOROPHYLL A DERIVATIVES:
STRUCTURAL ELUCIDATION AND EFFICIENT SYNTHESIS
OF MONO-L-ASPARTYL CHLORIN E₆ AND ITS CONSTITUTIONAL ISOMERS

2.1 Introduction

Mono-L-aspartyl chlorin e₆, also known as talaporfin and subsequently referred to here as NPe₆, is a 2nd-generation photosensitizer in advanced-stage clinical trials for oncoligic applications of photodynamic therapy (PDT). NPe₆ is a chlorophyll a derivative. NPe₆ is obtained by transesterification of the phytol ester group with a methyl ester group to form methyl pheophorbide a (4). Subsequent isocyclic ring opening forms chlorin e₆ trimethyl ester (5). Alkaline hydrolysis of the methyl esters and then activation and coupling to a protected aspartic acid followed by deprotection yields NPe₆. See Scheme 2.1.

Historically, NPe₆ has had a great amount of ambiguity associated with its structure. Its patent filed in 1987 claimed NPe₆ was probably a mixture of regioisomers.¹ Because purity is assured via its isolation by HPLC, academic papers published since 1987 assumed NPe₆ was the 17₃ regioisomer. In 1998, a 2D NMR study published in Heterocycles claimed NPe₆ was actually the 15₃ regioisomer.² This paper was not unanimously accepted by the porphyrin community because these studies were performed on the carboxylate salts therefore requiring dissolution in D₂O where chlorin aggregation can seriously complicate NMR analysis.³ Also, the result was counterintuitive from a mechanistic perspective. The structure remained a matter of conjecture.

The mechanism allows for ambiguity because no protecting group strategies are employed in its synthesis and three carboxylic acids exist at the chlorin’s periphery available for activation and coupling. The reaction is a classic carboxylic acid activation with DCC as
Scheme 2.1: Synthetic route of NPe₆ from chlorophyll a.
the coupling agent, proceeding via a nucleophilic addition subsequent elimination mechanism. See Figure 2.1.

![Mechanism of carboxylic acid coupling to an amine with DCC coupling reagent.](image)

**Figure 2.1** Mechanism of carboxylic acid coupling to an amine with DCC coupling reagent.

According to the nature of the mechanism, it is therefore reasonable to assume that the aliphatic 17\(^3\) carboxylic acid, which is farthest from the aromatic macrocycle and therefore less susceptible to the deactivating affects of the ring, would be the most reactive site. Additionally, the priopionic side chain’s situation above the plane of the macrocycle makes it sterically less inhibited. The 13\(^1\) carboxylic acid is deactivated because it is attached directly to the aromatic macrocycle and the 15\(^2\) acid presumably suffers from steric restraints. See Figure 2.2. Neither was considered serious competition for the 17\(^3\) carboxylic acid.

When X-ray crystallography attempts failed at structural elucidation of NPe\(_6\), a classical methodology of unambiguous synthesis was employed adjunctively with 1D NMR and 2D NMR (COSY,TOCSY, HMBC, HSQC) analysis. However, these NMR studies were performed on the methyl esters enabling increased organic solubility, which allowed acquisition of monomic spectra. Concurrently, another study reported small changes in
Believed to NPe6 in advanced-staged clinical trials

17 side chain
- lies above the tetrapyrrole plane
- believed to be the least hindered of the side carboxylic acid side chains

Figure 2.2: NPe₆ Regioisomers.
structure, including regioisomerism, could correlate to huge differences in function, such as subcellular localization.\(^4\) Subcellular localization has been demonstrated to be a key factor in mode of tumor cell damage (i.e. necrosis vs. apoptosis) thereby effecting PDT efficacy.\(^5\) Therefore efficient generation of the regioisomers of NPe\(_6\) was warranted for the comparison of biological properties and the elucidation of the structure/function relationship. Methyl ester protecting groups were consistently utilized to simplify spectra analysis.

### 2.2 Unambiguous \(17^3\) NPe\(_6\) Synthesis

A new selective route was required for NPe\(_6\) synthesis. The demetalated version of chlorophyll \(a\) is pheophytin \(a\) (7). See Figure 2.3. It is the significant compound obtained from extraction of *Spirulina Maxima*. *Spirulina Maxima* is the ideal chlorophyll \(a\) source for NPe\(_6\) studies because it contains only chlorophyll \(a\) and no chlorophyll \(b\), greatly simplifying algal extract purification.

![Pheophytin a](image)

**Figure 2.3** Pheophytin \(a\).

The chemistry of pheophytin and the pheophorbides is dictated by the presence of a \(\beta\) keto ester on ring \(E\), the isocyclic ring. (This ring is biosynthetically derived from the C-13
propionic acid side chain of protoporphyrin.) The ring is susceptible to enolization,\textsuperscript{6} decarboxymethylation,\textsuperscript{7} nucleophilic attack\textsuperscript{8} and an autoxidation reaction known to chlorophyll chemists as “allomerization” See Figure 2.4.\textsuperscript{9,10} Because of the extremely reactive nature of the β keto ester ring, synthetic modifications immediately following extraction usually involve either intentional decarboxymethylation or ring opening.

![Diagram of side reactions of isocyclic ring.](image)

**Figure 2.4** Side reactions of isocyclic ring.

Wasielewski and Svec demonstrated that the phytol ester group of pheophytin \textit{a} (7) could be selectively hydrolyzed to form pheophorbide \textit{a} (8) in high yield (<92%) with degassed TFA/H\textsubscript{2}O (80:20) at 0° for 1 hour without touching the β keto ester of the isocyclic ring.\textsuperscript{11} See Figure 2.5. The feasibility of partial hydrolysis could allow for a unique protecting group strategy. If integrity of the isocyclic ring and β keto ester could be maintained throughout coupling conditions, this ring could serve as a natural protecting group during the coupling of the free 17\textsuperscript{3} carboxylic acid group to aspartic acid dimethyl ester to form aspartic acid dimethyl ester pheophorbide \textit{a} (9). Subsequent ring opening with methoxide would yield
17$^3\ $NPe$_6$ TME (10) unambiguously. See Figure 2.5. Classical ring opening conditions are to treat the pheophorbide with an excess of sodium methoxide in THF.$^{12}$ To minimize lost product because of partial hydrolysis of the ester groups, the solution is treated with diazomethane before chromatography.$^{13}$ Yields are highly variable (from 30% to 60%) under these conditions, but starting material is recoverable.

An unambiguous synthetic route could clarify any confusion associated with the structure of NPe$_6$. For structural elucidation purposes, however, rigorous proof of the presence of the isocyclic ring and ß keto ester group in steps one and two would have to be provided. Degradation of the ß keto ester and/or ring would invalidate the entire structural elucidation process.

The large $\pi$ system of the chlorin macrocycle produces an induced ring current that causes peripheral protons in the plane of the macrocycle to be deshielded. For example, the central NH protons are significantly deshielded and appear upfield of TMS. Protons situated above and below the plane of the macrocycle are significantly shielded. For pheophytin $a$ (7), the $meso$ protons 20, 5 and 10 resonate at $\delta$ 9.35, $\delta$ 9.50 and $\delta$ 8.57. In addition to the characteristic chlorin peaks of the meso protons and the central nitrogen protons (among others), the significant peaks in this structural elucidation process are the peaks that demonstrate the presence of the isocyclic ring and ß keto ester. The diagnostic peaks then for pheophorbide $a$ (8), and aspartic acid dme pheophorbide $a$ (9) are the 13$^2$ proton $\delta$ 6.26 (in CDCl$_3$) and the 13$^3$ carboxy methyl group $\delta$ 3.84 (in CDCl$_3$). Degradation products are easily recognized via $^1$H NMR. The 13$^2$ hydroxy degradation product, for example, is missing the characteristic 13$^2$ proton.
Figure 2.5 Unambiguous synthetic route for the generation of $17^3\text{NPe}_6$ TME.
Partial hydrolysis of the phytyl group with respect to the $13^3$ carboxy methyl group proceeded as predicted from Wasielewski and Svec’s report. Pheophorbide $a$ (8) was obtained with a 93% overall yield. The $^1$H NMR shows the spectrum of the starting material pheophytin $a$ (7). The $^1$H NMR of pheophorbide $a$ (8) shows that the ester and isocyclic ring have survived the first step. See Figure 2.6 and Figure 2.7.

The second step requires the coupling of pheophorbide $a$ (8) with aspartic acid dimethyl ester. The coupling reagent DCC was employed with DMAP as a catalyst. The reaction was complete within 2 hours and proceeded with a typical coupling yield of 95%. Figure 2.8 shows the isocyclic ring has survived the second step as well. It should be noted that coupling with modern reagents such as HBTU did not work as the isocyclic ring did not survive.

Step three, the subsequent ring opening step, was easy to follow via UV-Vis. Compounds of the chlorin $e_6$ series, i.e. a class of chlorophyll $a$ derivatives without the isocyclic ring, have a sharper soret band when compared with the pheophorbides. The reaction quickly turned from brown to green upon adding sodium in methanol. The UV-Vis ($\lambda_{\text{max}}$ 660, 608, 558, 530, 500, 404) obtained was identical to authentic NPe$_6$ TME. See Figure 2.9.

Enthusiasm over the success of the reaction was quickly quenched when the $^1$H NMR of the column purified compound was obtained. The methyl ester region of the spectra showed peaks doubled up. It is believed that under these harsh alkaline conditions of extreme excess of sodium methoxide, the aspartic acid dimethyl ester substituent racemized from the L (S) to the D (R) giving a mixture of diastereomers impossible to separate by gravity column
Figure 2.6 Proton NMR spectrum at 300 MHz in CDCl₃ of Pheophytin a.
Figure 2.7 Proton NMR spectrum at 300 MHz in CDCl₃ of Pheophorbide α (8). Red arrows show peaks of isocyclic ring: 13² proton (δ 6.26) and 13³ carboxy methyl group (δ 3.84).
Figure 2.8 Proton NMR spectrum at 500 MHz in CDCl$_3$ of Aspartic Acid Dimethyl Ester Pheophorbide $a$ (9). Red arrows indicate the presence of the isocyclic ring and $\beta$ keto ester: 13$^2$ proton ($\delta$ 6.26) and 13$^3$ carboxy methyl group ($\delta$ 3.85).
Figure 2.9 UV-Visable absorption spectra in CDCl$_3$. Top spectrum is of $17^3$NPe$_6$ TME. Bottom spectrum is of methylated NPe$_6$ currently in advanced-stage clinical trials.
chromatography. Amino acids, especially aspartic acid, are sensitive to racemization under alkaline conditions.\textsuperscript{14}

The next strategy was to decrease the amount of methoxide added and perform the reaction at 0\textdegree C (ring-opening conditions that should maintain the integrity of the aspartic acid dimethyl ester’s chiral center). When 1 equivalent of base was added, the reaction proceeded smoothly and within 3 hours only one spot was recognizable on silica TLC (2\% MeOH/DCM). The UV-Vis ($\lambda_{\text{max}}$ 660, 608, 558, 530, 500, 404) was identical to authentic NPe\textsubscript{6} TME.

No column was required for purification, only a quick silica plug was run to ensure purity and an incredibly unexpected yield of 97\% was obtained. The compound was shown to be pure via $^1\text{H}$ NMR. Additionally mass spectra and C, H, N analysis corroborated the compounds identity as 17\textsuperscript{3} NPe\textsubscript{6} TME.

The unexpected yield warranted a closer look at the reaction mechanism. The ring opening is a retro Dieckmann. The Dieckmann reaction is an intramolecular (ring closing) version of the Claisen condensation to form a five-membered ring with a $\beta$ keto ester. In the Dieckmann condensation—as with most condensations—there exists an equilibrium between the starting material and the product. Claisen condensation—and therefore Dieckmann condensations as well—are driven forward to completion by an irreversible deprotonation step. See Figure 2.10.

Excess base favors the Dieckmann product. Therefore, if the synthetic objective is the retrograde Dieckmann product, less base would be an ally. In fact, only a catalytic amount of base is required if the reaction is performed in methanol. After methoxide attacks the carbonyl
carbon the carbanion leaving group will rapidly reprotonate from the methanol, regenerating methoxide. See Figure 2.11.

![Diagram of mechanism](image)

**Figure 2.10** Claisen Condensation.\(^{15}\)

Varying concentrations of catalytic amounts of base were attempted. All proceeded with superior yields to standard protocol. But, they were highly variable and not precisely repeatable. It is assumed that the inner pyrrole nitrogens could have interrupted the catalytic cycle. The reaction will be tried with Mg metalated chlorins to test this theory. It is not surprising that entropy would favor the ring opening.\(^{16}\) The extent to which it is favored is surprising however. It should be noted that the forward Dieckmann proceeds with high yields (87%) when non-nucleophilic tert-butoxide is used.\(^{17}\)

![Diagram of mechanism](image)

**Figure 2.11** Mechanism of isocyclic ring opening reaction with methoxide as the nucleophile.
The success of the isocyclic ring opening of aspartic acid dme pheophorbide a (9) encouraged a similar attempt on the more widely utilized chlorin e₆ trimethyl ester. Chlorin e₆ trimethyl ester (5) is synthesized via the ring opening of methyl pheophorbide a (4). An optimized yield of 98% of chlorin e₆ trimethyl ester was obtained with 1.0 equivalent of sodium methoxide in methanol. See Figure 2.12.

\[ \text{Methyl Pheophorbide } a \quad \rightarrow \quad \text{Chlorin e₆ trimethyl ester} \]

Figure 2.12 Improved synthesis of Chlorin e₆ trimethyl ester.

The successful synthesis of 17³ NPe₆ TME (10) allowed comparison of spectra with authentic NPe₆ TME (11). NPe₆ was methylated with ethereal diazomethane with a disappointing yield. Miscibility was a problem. Although diazomethane is an outstanding reagent for the methylation of porphyrins and chlorins with two and even three peripheral carboxylic acid, methylation of four peripheral carboxylic acids causes serious solubility problems. Methylation under phase-transfer catalyzed conditions was tried with TBAB and methyl iodide. This alkylation technique was not useful. Although the phase-transfer reagent successfully transferred the chlorin from the aqueous to the organic layer where it could be methylated, it also catalyzed the methylation of the inner nitrogen atoms as well.
Upon initial inspection of $^1$H NMR of methylated authentic NPe$_6$ in comparison with the related structure, chlorin e$_6$ trimethyl ester (5), it was noticed that the spectrum of authentic NPe$_6$ TME was missing the singlet methyl ester peak that resonates at $\delta$ 3.73 in chlorin e$_6$ trimethyl ester (5). See Figure 2.13 and Figure 2.14. Literature values had tentatively assigned this signal to $15^2$ carboxy methyl group reasonably assuming that in chlorin e$_6$ trimethyl ester (5) the $15^2$ acetic ester would resonate between the $13^1$ formic ester peak at $\delta$ 4.24 and the $17^3$ propionic ester peak at $\delta$ 3.55.

Because this $3.50 - 4.24$ ppm region plays a critical role in the structural elucidation process we decided to more rigorously assign signals in chlorin e$_6$ trimethyl ester (5). This spectrum could be an invaluable reference when analyzing spectra of more complex chlorin e$_6$ derivatives. 2D NMR can be extremely valuable in assigning signals of compounds of known structure. Several 2D NMR studies were done—HSQC and HMBC—to definitively characterize this region. See Figure 2.15, Figure 2.16 and Figure 2.17. A typical spectrum of chlorin e$_6$ trimethyl ester (5) has multiple signals overlapping in the 3.2 and 4.24 ppm region. NMRs were obtained on a 500 MHz NMR to obtain better resolution of peaks between 3.2-4.24 ppm. These studies confirmed the tentative peak assignments.

The peak that corresponds to the $17^3$ methyl ester peak in the related chlorin e$_6$ trimethyl ester (5) is present in authentic methylated NPe$_6$. The presence of this methyl ester peak suggests an absence of the aspartic acid residue at the $17^3$ position in NPe$_6$ prior to methylation, strongly hinting that NPe$_6$ may be the $15^2$ regioisomer. However, the peaks resonate too closely together for this argument to be conclusive. For example, it could be argued that NPe$_6$ is structurally unique from chlorin e$_6$ and therefore the $15^2$ peak that resonates at $\delta$ 3.74 in chlorin e$_6$ may actually resonate at $\delta$ 3.55 in NPe$_6$. It was therefore
necessary to compare the spectra with the unambiguously synthesized $17^3 \text{NPe}_6 \text{TME}$ (10). TOCSY and COSY were employed to help definitively assign some signals. See Figure 2.18 and Figure 2.19.

When comparing the spectra of $17^3 \text{NPe}_6 \text{TME}$ and authentic $\text{NPe}_6 \text{TME}$ obtained at same concentrations (17 mg/0.5 mL) and on the same instrument (500 MHz spectrometer), it is clear that although the spectra are similar, there are key differences. See Table 1. Most notably, the absent authentic $\text{NPe}_6 \text{TME}$ ester peak that correlated to the $15^2$ acetic ester peak in chlorin $e_6$ trimethyl ester was present in $17^3 \text{NPe}_6$. The authentic $\text{NPe}_6$ ester peak that correlated to the $17^3$ propionic ester peak in chlorin $e_6$ trimethyl ester was absent in $17^3 \text{NPe}_6$. There are other minor differences in signals produced by the aspartic acid methyl esters as well. This study proves that the $\text{NPe}_6$ in advanced staged clinical trials is not the assumed $17^3 \text{NPe}_6$.

2.3 Synthesis of $13^1 \text{NPe}_6$

Under acidic conditions the inner nitrogen atoms of chlorin $e_6$ are fully protonated and the $13^1$ carboxylic acid becomes severely deactivated. Exploiting the pH sensitivity of the $13^1$ side chain, the $17^3$ and the $15^2$ carboxylic acids can be selectively methylated with 5% $\text{H}_2\text{SO}_4/\text{MeOH}$.

The $13^1$ chain is now available for activation and coupling. After neutralization, chlorin $e_6$ dimethyl ester (12) is readily purified via silica column chromatography. Even under the basic conditions required for coupling, the $13^1$ carboxylic acid is still unreactive and requires heating for optimal yields of $13^1 \text{NPe}_6 \text{TME}$. See Figure 2.20, Figure 2.21, Figure 2.22 and Figure 2.23.
Figure 2.13: Proton NMR spectrum at 500 MHz CDCl₃ of authentic NPe₆ TME (11).
Figure 2.14 Proton NMR spectrum at 500 MHz in CDCl$_3$ of chlorin e$_6$ trimethyl ester (5).
Figure 2.15 HSQC spectrum at 500 MHz in CDCl$_3$ of Chlorin e$_6$ trimethyl ester.
Figure 2.16 HMBC spectrum at 500 Mhz in CDCl$_3$ of Chlorin e$_6$ trimethyl ester.
Figure 2.17 Proton NMR spectrum at 500 MHz in CDCl$_3$ of $17^3$ NPe$_6$ TME.
Figure 2.18 COSY spectrum at 500 Mhz in CDCl$_3$ of $17^3$ NPe$_6$ TME.
Figure 2.19 TOCSY spectrum at 500 Mhz in CDCl$_3$ of 17$^3$ NPe$_6$ TME.
Table 1 ¹H NMR data 500 Mhz in CDCl₃

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Aspartic acid

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Figure 2.20 Proton NMR spectrum at 300 MHz in CDCl₃ of chlorin e₆ dimethyl ester.
Figure 2.21 Proton NMR spectrum at 250 MHz in CDCl$_3$ of 13$^1$ NPe$_6$ (13).
Figure 2.22 Generation of chlorin e₆ dimethyl ester (12) from chlorin e₆ (6).

Figure 2.23 Coupling of aspartic acid dimethyl ester to chlorin e₆ dimethyl ester (12).

2.4 Optimization of 15² Synthesis

Surprisingly it is the 15² acetic ester group, which is more activated toward coupling. At this time the basis of regioselectivity is not know. It should be noted that regardless of coupling reagents used (i.e. DCC, EDC, HBTU, OAC), the 15² NPe₆ (1) is formed selectively.
The uronium/guanidium reagent HBTU, provides the greatest yield. Selectivity is lost when reactions of the carboxylic side chain require protic solvents. This finicky selectivity suggests that the $17^3$ group is tied up in inter or intramolecular interactions and therefore is not as competitive toward coupling in the absence of a protic medium.

![Figure 2.25](image)

**Figure 2.25** Synthesis of $15^2$NPe$_6$ (1) via coupling of chlorin e$_6$ (6) to aspartic acid dme.

### 2.5 Conclusion

Employing a classical methodology of unambiguous synthesis in conjunction with NMR spectroscopy the NPe$_6$ in advanced stage clinical trials has been conclusively shown to be the $15^2$NPe$_6$ regioisomer instead of the assumed $17^3$ NPe$_6$.

Natural reactivities of the chlorophyll $a$ derivatives have been exploited to generate selective routes for the synthesis of the three regioisomers of NPe$_6$. The route to the $17^3$ NPe$_6$ TME is exceptionally high yielding (86% overall yield from chlorophyll $a$) and to our knowledge represents a novel route to a $17^3$ chlorin e$_6$ derivative. Synthesis of chlorin e$_6$
trimethyl ester, a key step in the synthesis of both the $13^1$ NPe$_6$ and the $15^2$ NPe$_6$, has been made extremely efficient, with yields of 98%, therefore improving the synthesis of the $15^2$ NPe$_6$ in advanced-stage clinical trials.

2.6 Future Work

For comparison purposes, the three regioisomers of NPe$_6$ TME will be deprotected and submitted for biological evaluation. The route to the $17^3$ derivative will be extensively explored. For example, the route for the synthesis of $17^3$ derivatives with amide linkages has been successful, not surprising because the amide linkage is very stable. The incoming methoxide nucleophile, which is utilized in the ring opening, is less basic than the nitrogen leaving group therefore making the amide inert to nucleophilic addition. Could this reaction be generalized to include ester linkages? For example, could chemoselectivity be demonstrated for selective nucleophilic addition to the ketone carbonyl of the isocyclic ring instead of the carbonyl of a $17^3$ ester linkage, thereby increasing the synthetic utility of this route? These selective routes to the chlorin e$_6$ derivatives can be used as a model for the synthesis of novel conjugates.

2.7 Experimental to Chapter 2

Isolation of Pheophytin $a$ (7) from *Spirulina maxima*:

Approximately 700 g of dried *Spirulina maxima* algae 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 hour after which more liquid nitrogen was added and was allowed to sit overnight protected from light. The algae 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 3-neck lid was clamped to the vessel. The
reaction was heated to reflux under argon with mechanical stirring for 3 hours. The supernatant was then filtered through Whatman 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 DCM removed the fast-running yellow carotenoid band. Then elution with 80:20 DCM/Ethyl Acetate eluted the major blue-grey pheophytin-\(\alpha\) (7) band. 3.9 g \(\text{C}_{56}\text{H}_{77}\text{N}_{4}\text{O}_{4}\) from 700 g algae  (extraction yield without Fisher Jumbo mechanical stirrer: 500 mg from 700 g algae) UV-Vis (\(\text{CH}_2\text{Cl}_2\)): \(\lambda_{\text{max}} (\varepsilon/\text{M}^{-1}\text{cm}^{-1})\) 668 (44,600), 611 (8620), 538 (9710), 507 (10,800), 414 (106,000); Mass Spectra (MALDI): \(m/z\) 871 (M+H)+; \(^1\text{H}\) NMR (CDCl\(_3\) 300 MHz): \(\delta\) 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, \(J = 7.5\text{ Hz}\)), 3.40 (3H, s), 3.20 (3H, s), 2.63 (1H, m), 2.34 (1H, m) 1.74 (3H, d, \(J = 7.5\text{ Hz}\)), 1.61 (3H, t, \(J = 7.5\text{ Hz}\)) Phytol: 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: NMR shows minute amounts of the Pheophytin \(\alpha^\prime\), the 13\(^2\) epimer (diastereomer) of Pheophytin \(\alpha\) (18 S, 17 S 13\(^2\),S instead of 18 S, 17 S, 13\(^2\) R). If the objective is a 17\(^3\) chlorin derivative, this epimer is not a nuisance and should not be separated because this will needlessly diminish overall yield! (The sensitive chiral center is absent in the 17\(^3\) chlorin derivative.)

**Pheophorbide \(\alpha\) (8):**

Pheophytin \(\alpha\,(7, 500\text{ mg}, 0.57\text{ mmol})\) was selectively hydrolyzed to the 17\(^3\) carboxylic acid without affecting the 13\(^3\) carbomethoxy group via the Wasielewski and Svec procedure which requires stirring pheophytin \(\alpha\) (7) in 75 mL of degassed TFA/H\(_2\)O  80:20  at 0\(^\circ\) for 1 h\(^{11}\). The
reaction mixture was poured into 500 mL of H₂O and extracted with CHCl₃. The extract was washed three times with H₂O and twice with 10% NaHCO₃, then dried over anhydrous Na₂SO₄. Evaporation of solvent provided a brown residue that was purified via silica gel column chromatography with 40% Ethyl acetate in DCM. Alternatively the residue can be purified as reported by Wasielewski and Svec via a column of powdered confectioner’s sugar (3-8 cm x 30 cm columns, elution with 10% acetone in CCl₄) 311 mg; 93% of C₃₅H₃₆N₄O₅ was obtained. UV-Vis (CH₂Cl₂): λₘₐₓ (ε/M⁻¹cm⁻¹) 667 (55,200), 609 (7900), 535 (9470), 505 (12, 100), 413 (119, 200) Mass Spectra (MALDI): m/z 593 (M+H)+; 'H NMR (CDCl₃, 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)

Aspartic Acid Dimethyl Ester Pheophorbide a (9):

Pheophorbide a, (8, 100 mg; 0.169 mmol) was dissolved in 75 ml Dry CH₂Cl₂ and 1 mL TEA. DCC (40 mg, 0.194 mmol) and DMAP (25 mg, 0.2 mmol) and L-aspartic acid dimethyl ester (45 mg, 0.228 mmol) were added and the reaction was stirred for 2 hours. Note: this reaction is favored by dilute conditions. Concentrated conditions favors formation of the anhydride (bispeophorbide) and not the aspartic acid dimethyl ester pheophorbide a product. The reaction mixture was washed with water and brine, dried over sodium sulfate and then purified on a silica column (30% Ethyl Acetate in DCM) DCC is persistent. After the major brown band was eluted from the column, the solvent was evaporated and the solid was dissolved in 100% ethyl acetate and filtered. This step was repeated three times. (DCC precipitates in ethyl acetate.) Evaporation of ethyl acetate gave 118 mg, 95% of aspartic acid
dme pheophorbide a (9), C_{41}H_{45}N_{5}O_{8}. UV-Vis (CH_{2}Cl_{2}): \lambda_{\text{max}} (\epsilon/M^{-1} \text{cm}^{-1}) 667 (55,300), 611 (7890), 535 (9490), 505 (12, 100), 413 (119, 400); Mass Spectra (MALDI): m/z 737 (M+H)^{+}; 

\^1\text{H} NMR (CDCl_{3}, 500 MHz ): \delta 9.50 (1H, s), 9.38 (1H, s), 8.57 (1H, s), 8.0 (1H, m), 6.29 (1H, m), 6.26 (1H, s), 6.18 (1H, m), 4.71 (1H, m), 4.47 (1H, m), 3.85 (3H, s), 3.68 (3H, s), 3.65 (3H, s), 3.63 (1H q, J = 7.5 Hz), 3.47 (3H, s), 3.39 (3H, s), 3.21 (3H, s), 2.88 (1H, m) 2.65 (1H, m), 2.54 (1H, m), 2.21 (1H, m), 1.74 (3H, d, J = 7.5 Hz), 1.62 (3H, t, J =7.5 Hz); 

\^1\text{C} NMR (CDCl_{3}): \delta 189.6, 172.4, 171.7, 171.4, 171.0, 169.6, 161.2, 156.6, 152.2, 149.7, 145.2, 142.1, 137.9, 136.6, 136.7, 136.3, 132.0, 129.1, 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

\textbf{17}\^3\text{ Monoaspartyl Chlorin e\textsubscript{6} Tetramethyl Ester (10):}

Aspartic Acid Dimethyl Ester Pheophorbide a (9), (100 mg, 0.135 mmol) was dissolved in dry methanol and stirred under argon for 10 minutes. Sodium methoxide (0.27 mL of a 0.5 M solution) was added and the reaction was allowed to stir at 0°C for one hour. The reaction was followed by UV-Vis. The solution turns from brown to green as the isocyclic ring opens. The reaction mixture was then poured into water. The mixture was extracted with CH_{2}Cl_{2} and the organic layer was washed with water, dried over sodium sulfate and then evaporated. The residue was dissolved in 2% Methanol/DCM and purified on a silica plug with same mobile phase. Solvent was evaporated and 100 mg, 97% of C_{42}H_{49}N_{5}O_{9} (10) was obtained. UV-Vis (CH_{2}Cl_{2}): \lambda_{\text{max}} (\epsilon/M^{-1} \text{cm}^{-1}) 660, 608, 558, 530, 500, 404; Mass Spectra (MALDI): m/z 768 (M + H)^{+} HRMS requires 767.87 found 767.947; \^1\text{H} NMR (CDCl_{3}, 500 MHz ): \delta 9.61 (1H, s), 9.48 (1H, s), 8.67 (1H, s), 7.98 (1H, m), 6.30 (1H, dd, J = 17.8, 1.5), 6.0 (1H, br s), 6.06 (1H, dd, J = 11.5, 1.5), 5.22 (2H, br ), 4.74 (1H, ddd J = 8.1, 4.6, 4.6), 4.37 (q, J= 7.3), 4.19 (3H, s), 3.71 (3H, s), 3.70 (1H, q, J = 7.6 ) , 3.59 (3H, s), 3.49 (3H, s), 3.45 (3H, s), 3.40 (3H, s),
3.22 (3H, s), 2.81 (2H, dd, \( J = 17.2, 4.6 \)), 2.21 (m), 1.66 (3H, d, \( J = 7.3 \)), 1.61 (3H, t, \( J = 7.6 \)) -1.34 (s), -1.48 (s); \(^{13}\)C NMR (CDCl\(_3\)): \( \delta \) 174, 172.3, 171.5, 171.1, 169.8, 169.3, 166.7, 154.9, 148.6, 145.0, 139.5, 136.4, 135.9, 135.4, 135.3, 134.7, 130.4, 129.2, 129.1 123.5, 121.6, 102.3, 102.0, 98.4, 93.4, 52.9 52.8, 52.7, 51.9, 49.4, 48.1, 38.4, 35.8, 32.3, 30.1, 22.9, 19.5, 17.7, 12.0, 11.2; Elemental Analysis: Anal Calc for C\(_{42}\)H\(_{49}\)N\(_5\)O\(_9\) C, 65.69, H, 6.43, N, 9.12.

Found: C, 65.30, H, 6.47, N, 8.95

**17\(^3\) Monoaspartyl Chlorin e\(_6\) (2):**

Monoaspartyl Chlorin e\(_6\) tetramethyl ester (10) was dissolved in dry CH\(_2\)Cl\(_2\). LiI was added and allowed to stir for 1 hour. UV-Vis (acetone): \( \lambda_{\text{max}} (\varepsilon/M^{-1}cm^{-1}) \) 664 (45,400), 610 (4,600), 560 (2,000), 530 (4,900), 502 (12,300), 402 (135,200); Mass Spectra \( m/z \) 712 (M + H); \(^1\)H NMR (D\(_2\)O 300 MHz): \( \delta \) 9.30 (1H, s), 9.11 (1H, s), 8.0 (1H, m), 7.03 (1H, s), 7.03 (1H, m), 7.01 (1H, d, \( J = 17.8, 11.7 \) Hz), 5.49-5.51 (2H, m), 4.85 (1H, m), 3.64 (3H, s), 3.18 (3H, s), 2.30-2.68 (2H, m), 3.00 (2H, m), 2.76-2.68 (2H, m), 1.81 (3H, d \( J = 7.1 \) Hz). 1.23 (3H, t, \( J = 7.6 \) Hz)

**Methyl Pheophorbide a (4):**

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 CH\(_2\)Cl\(_2\), washed with water and then 10% saturated aqueous sodium bicarbonate. The aqueous layer was dried over sodium sulfate, filtered and then evaporated. Recrystallization of the residue from CH\(_2\)Cl\(_2\) and methanol gave the product.

Method 2: Pheophorbide a, (100 mg; 0.169 mmol), was treated with ethereal diazomethane. Argon was flushed through the flask and the solution was evaporated and recrystallized from CH\(_2\)Cl\(_2\) and Methanol to quantitatively give 102 mg the product, C\(_{36}\)H\(_{38}\)N\(_4\)O\(_5\) (4) UV-Vis (CH\(_2\)Cl\(_2\)): \( \lambda_{\text{max}} (\varepsilon/M^{-1}cm^{-1}) \) 668 (40,700), 610 (8100), 560 (3200), 538 (9400), 506 (10,400),
\[ \delta 9.50 \text{ (1H, s), 9.36 \text{ (1H, s), 8.57 \text{ (1H, s) 8.0 (1H, m), 6.29 (1H, m), 6.26 (1H, s), 6.16 (1H, m), 4.46 (1H, m), 4.20 (1H, m), 3.88 (3H, s), 3.70 (2H, q, J = 7.6), 3.68 (3H, s), 3.57 (3H, s), 3.41 (3H, s), 3.25 (3H, s), 2.63 (1H, m), 2.32 (1H, m), 2.52 (1H, m), 2.2 (1H, m), 1.81 (3H, d, J = 7.3) 1.69 (3H, t, J = 7.6), 0.53 and -1.67 (2H, br, s) } \]

**Chlorin e₈ Trimethyl Ester (5):**

Methyl Pheophorbide \( a \), \( 4, 102 \text{ mg, 0.168 mmol} \), was dissolved in dry methanol and stirred under argon for 10 minutes. 0.35 mL of a 0.5 M sodium methoxide solution was added to the solution and was allowed to stir for 2 hours at 0°C. The solution was diluted with H₂O and extracted with CH₂Cl₂. The organic layer was dried with sodium sulfate, filtered and then evaporated. The solid obtained was dissolved in CH₂Cl₂ and chromatographed on a plug of alumina (grade III) with the same mobile phase. Chlorin e₈ trimethyl ester eluted with CH₂Cl₂. After evaporation, 105 mg, 98% of C₃₇H₄₂N₄O₆ (5) was obtained. UV-Vis (CH₂Cl₂): \( \lambda_{\text{max}} (\epsilon/\text{M} \cdot \text{cm}^{-1}) 666 (43,700), 608 (4530), 558 (1740), 530 (4700), 500 (11,240), 404 (123,400); \) Mass Spectra (MALDI): m/z 639 (M+H)+; \( ^1\text{H NMR (CDCl}_3, 500 \text{ MHz): } \delta 9.63 \text{ (1H, s), 9.45 (1H, s), 8.71 (1H, s), 7.96 (1H, m), 6.27 (1H, dd, J = 17.8, 1.5 Hz), 6.04 (1H, dd, J = 11.5, 1.5 Hz), 5.37 (2H, d, J = 18.8 Hz), 5.21 (1H, br d, J = 18.8 Hz), 4.43 (2H, m), 4.24 (3H, s) 3.76 (3H, s), 3.74 (2H, q, J = 7.3), 3.56 (3H, s), 3.55 (3H, s), 3.39 (3H, s), 3.19 (3H, s), 2.56 (2H, m), 2.18 (2H, m), 1.75 (1H, m), 1.67 (3H, d, J = 7.3 Hz), 1.64 (3H, t, J = 7.7 Hz), -1.33 (1H, s), -1.47 (1H, s); \) \( ^{13}\text{C NMR (CDCl}_3, 500 \text{ MHz): } \delta 174, 173.1, 169.6, 169.6, 167, 154.8, 148.9, 144.8, 139.5, 136.4, 134.7, 135.9, 135.3, 135.4, 130.5, 129.3, 129.3, 123.3, 121.2, 102.2, 102.1, 99, 93.6, 53.0, 52.9, 52.1, 51.6, 49.4, 38.7, 29.1, 27.6, 22.9, 19.6, 17.7, 12.1, 12.0, 11.3 \)
**Chlorin e₆ (6):**

Chlorin e₆ trimethyl ester (5, 100 mg, 0.157 mmol) was dissolved in 25% aqueous DMF. KOH was added and the reaction stirred at 60° C for 12 hours. The reaction mixture was diluted with water and adjusted to pH 3 with aqueous citric acid and washed with CH₂Cl₂. The solution was evaporated and redissolved in acetone and evaporated several times. The solid was washed with water and then dried under vacuum. The residue was dissolved in methanol and purified on a Sephadex LH20 column to yield 40 mg, 43% of C₃₄H₃₆N₄O₆. This reaction is currently being optimized with LiI. UV-Vis (CH₃OH): \( \lambda_{\text{max}} \) (ε/M⁻¹cm⁻¹) 666 (45,271), 610 (8706), 558 (7835), 530 (9721), 502 (15,525), 402 (145,100); Mass Spectra MALDI: m/z 597 (M + H)⁺; ¹H NMR (CD₃)₂SO δ: 9.65 (1H, s), 9.57 (s, 1H), 8.80 (1H, m), 8.0 (1H, m), 6.40 (2H, dd, \( J = 17.1, 11.5 \) Hz), 5.40 (2H, m), 4.45 (2H, m), 3.78 (2H, q, \( J = 7.3 \)), 3.61 (3H, s), 3.25 (3H, s), 2.70-2.20 (4H, m), 1.78 (3H, t, \( J = 7.1 \) Hz), 1.72 (3H, d, \( J = 6.8 \)), -1.50 (1H, s), -1.72 (1H, s).

**Chlorin e₆ dimethyl ester (12):**

Chlorin e₆ (6, 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 NaHCO₃ and extracted twice with CH₂Cl₂. The extract was washed twice with brine, dried over Na₂SO₄ and filtered. The solvent was evaporated and redissolved in CH₂Cl₂. It was then purified on a silica column. Development with 6% methanol and CH₂Cl₂ eluted the major green fraction which was collected. Solvent was removed to afford 75 mg, 95% of C₃₆H₄₀N₄O₆ (12). UV-Vis (CHCl₃): \( \lambda_{\text{max}} \) (ε/M⁻¹cm⁻¹) 666 (49,700), 610 (5,900), 562 (2,700), 523 (5,900), 502 (13,200) 402 (143,400); Mass Spectra (MALDI): m/z 625 (M + H)⁺;
\(^1\)H NMR (CDCl\(_3\), 300 MHz): \(\delta\) 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); \(^{13}\)C NMR (CDCl\(_3\), 300 MHz): \(\delta\) 174, 170.5, 168, 153.8, 147.7, 144.9, 140, 138, 137, 135.6, 135.1, 129, 122.4, 122.1, 102.5, 51.7, 52.3, 29.3, 38.9, 23, 18, 12.8, 11

13\(^1\) Monoaspartyl Chlorin e\(_6\) Tetramethyl Ester (13):

Chlorin e\(_6\), dimethyl ester (12, 75 mg, 0.120 mmol) was dissolved in dry CH\(_2\)Cl\(_2\) with 1 ml TEA. HBTU (136 mg, 0.36 mmol) was added and allowed to stir until completely dissolved. Then Aspartic Acid dimethyl ester was added. The reaction was allowed to stir overnight. The mixture was diluted with CH\(_2\)Cl\(_2\) and then washed with 5% aqueous citric acid, followed by a wash with brine and water. It was dried over sodium sulfate and then evaporated. The residue was dissolved in 2% Methanol/DCM and purified via silica column chromatography with the same mobile phase to yield C\(_{42}\)H\(_{49}\)N\(_5\)O\(_9\) UV-Vis (CH\(_2\)Cl\(_2\)): \(\lambda_{\text{max}}\) (\(\epsilon/M^{-1}\text{cm}^{-1}\)) 660, 608, 558, 530, 500, 404; Mass Spectra (MALDI): \(m/z\) 768 (M + H)+; \(^1\)H NMR (CDCl\(_3\), 300 MHz) \(\delta\) 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)
**152 Monoaspartyl Chlorin e6 Tetramethyl Ester (11):**

Chlorin e6 (6, 75 mg, 0.126 mmol) was dissolved in dry CH2Cl2 with 1 ml TEA. HBTU (57 mg, 0.15 mmol) was added and allowed to stir until completely dissolved. Then aspartic acid dimethyl ester hydrochloride was added. The reaction was allowed to stir for 2 hours. The mixture was diluted with CH2Cl2 and then washed with 5% aqueous citric acid, followed by a wash with brine and water. It was dried over sodium sulfate and then evaporated. Then it was dissolved in CH2Cl2 and treated with ethereal diazomethane. The residue was dissolved in 2% Methanol/DCM and purified via silica column chromatography with the same mobile phase to afford 60 mg, 61% of C42H49N5O9. UV-Vis (CH2Cl2): $\lambda_{\text{max}}$ ($\varepsilon$/M·cm$^{-1}$) 660, 608, 558, 530, 500, 404; Mass Spectra (MALDI) m/z 768 (M + H)$^+$; $^1$H NMR (CDCl3, 300 MHz ) $\delta$ 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)

**152 Monoaspartyl Chlorin e6 (1):**

152 Monoaspartyl Chlorin e6 Tetramethyl Ester was dissolved in dry DCM and LiI was added and allowed to stir. UV-Vis (acetone) $\lambda_{\text{max}}$ ($\varepsilon$/M·cm$^{-1}$) 664 (45,400), 610 (4,600), 560 (2,000), 530 (4,900), 502 (12,300), 402 (135,200); Mass Spectra m/z 712 (M + H)$^+$; $^1$H NMR (CD$_3$)$_2$SO: $\delta$ 9.56 (1H, s), 9.43 (1H, s), 8.68 (1H, s), 7.95-7.85 (1H, m), 7.10-7.04 (1H br s), 6.40 (1H, dd, $J = 17.8$, 11.6), 6.10 (1H, dd, $J = 11.6$, 1.4 Hz), 4.715-4.674 (1H, m), 4.70 (1H, m), 4.43 (1H, m), 3.59 (2H, m), 3.50 (3H, s), 3.30 (3H, s), 3.13 (3H, s), 2.78-2.68 (2H, m), 2.61-2.29 (2H, m), 1.70 (3H, t, $J = 7.3$ Hz), 1.63 (3H, d, $J = 7.3$ Hz) -1.903 (2H, s)
Generation of Diazomethane:

Assemble special diazomethane distillation equipment. Fill condenser with dry ice, then add acetone slowly until cold finger is 1/3 full. Add ethanol (95%, 10 ml) to a solution of KOH (5g) in H₂O (8 mL) in reaction vessel. Attach a 100 mL receiving flask with clear seal joint to condenser and cool receiver in ice bath. Provide an ether trap at the side arm. Cool trap with NaCl ice bath (33 g NaCl, 100g ice) Place separatory funnel over reaction vessel and charge funnel with solution of Diazald in ether. Warm reaction vessel to 65° C with H₂O bath add Diazald over a 20 minute period. Rate of distillation should equal rate of addition. Replenish cold finger with dry ice as necessary. When all Diazald has been consumed, slowly add 10 mL of ether until distillate is colorless. Neutralize any excess CH₂N₂ with acetic acid or on small scale, bubble argon through solution.

2.8 References


15 Crowe, W.E., Retrosynthesis; text to accompany lecture, Spring 2004, Louisiana State University.


CHAPTER 3

NATURAL PORPHYRIN DERIVATIVES:
AMINO ACID CONJUGATES OF PROTOPORPHYRIN IX

3.1 Introduction

This chapter explores the synthesis and preliminary biological property investigation of novel protoporphyrin IX (PPIX) amino acid conjugates as part of our ongoing research in the development of more efficacious naturally-derived photosensitizers for PDT. PPIX (14) is an ideal candidate for the synthesis of various novel photosensitizers because it has two peripheral carboxylic acids available for coupling. See Figure 3.1. Note in this investigation all amino acids conjugated to PPIX are proteogenic (i.e. L (S) isomer).

![Protoporphyrin IX](image)

**Figure 3.1** Protoporphyrin IX has two peripheral carboxylic acid groups available for coupling.

3.2 PPIX Dilysine and PPIX Diglutamate Synthesis

PPIX was coupled with tert-butyl ester protected glutamic acid. The PPIX diglutamate tetratertbutyl ester (15) was isolated and characterized. Subsequent deprotection with TFA yielded PPIX diglutamate (16). See Figure 3.2. In a separate coupling experiment, PPIX was conjugated to NBoc, t-butyl ester protected lysine. The intermediate (17) was isolated and
characterized. See Figure 3.3 and Figure 3.4. Subsequent deprotection with TFA yielded PPIX dilysine (18).

Figure 3.2 Coupling of PPIX to L-glutamic acid.
Figure 3.3 Coupling of PPIX (14) to lysine.
Figure 3.4 Representative proton NMR spectrum of a PPIX diamino acid conjugate. Spectrum at 500 MHz in CDCl₃ of PPIX dilysine [(Nbo)(OtBu)]₂
3.3 Preliminary Biological Property Investigation

The therapeutic effect of a photosensitizer is influenced by the amount of the drug incorporated by the cell, localization of the drug within the cell and the amount of cell kill. In an effort to expand the utility of PDT by improving the efficacy of the photosensitizers employed, we are screening our novel compounds by investigating key biological properties such as uptake, cytotoxicity and intracellular localization.

Variation of peripheral side chain from diglutamate to dilysine has a profound effect on both uptake and cytotoxicity. Figure 3.5 shows the time-dependent uptake by human epithelial cells (HEp2) of PPIX diglutamate (16) and PPIX dilysine (18) for a period of 24 hours. PPIX diglutamate’s (16) accumulation leveled at less than 0.01 μM/1000 cells after 2 hours, while PPIX dilysine (18) continued to accumulate even after 8 hours, ultimately reaching an intracellular concentration of 0.04 μM/1000 cells.

Likewise, PPIX dilysine (18) was more cytotoxic than PPIX diglutamate (16), probably as a result of its greater accumulation within the cell. Whereas administration of PPIX diglutamate resulted in minimal cell death. The IC50 (inhibitory concentration 50) dose for PPIX dilysine was 110 μM. See Figure 3.6. Unlike uptake and cytotoxicity, however, both compounds exhibited the same intracellular localization behavior. Both PPIX dilysine (18) and PPIX diglutamate (16) localized in the lysosomes. See Figure 3.7.

3.4 Conclusion

Two PPIX conjugates have been synthesized. PPIX dilysine (18) and PPIX diglutamate (16) demonstrate how variation of peripheral substituent can effect biological properties such as uptake and cytotoxicity. PPIX dilysine (18) not only accumulated to a greater extent within HEp2 cells, it also resulted in superior cytotoxicity.
Figure 3.5: This figure shows time-dependent uptake for PPIX dilysine and PPIX diglutamate in HEp2 cells. PPIX dilysine shows greater uptake than PPIX diglutamate.
Figure 3.6: Cytotoxicity study of PPIX dilysine (18) and PPIX diglutamate (16). PPIX diglutamate (16) is nontoxic to HEp-2 cells regardless of concentration. PPIX dilysine (18) IC50 is 110 µM.
Figure 3.7: Intracellular localization of PPIX diglutamate (16) in HEp2 cells after 24 hour incubation. a) Phase Contrast h) Colocalization with lysosome observed.
3.5 Experimental

Synthesis of PPIX diglutamate tetratertbutyl ester (15):

PPIX (1, 100 mg, 0.178 mmol, 1 eq) was dissolved in approximately 75 ml of dry DCM. TEA (1 ml) was added. The solution is heated gently to 35°C to fully dissolve the PPIX. HBTU (202.5 mg, 0.531 mmol, 3 eq) was added and the solution was stirred vigorously for 5 minutes. Diterbutyl glutamate hydrochloride salt (158 mg, 0.51 mmol, 3 eq.) was added and the solution was stirred at 35 °C for about 20 minutes. Then it was stirred at room temperature for 6 hours. The mixture was washed with water three times. It was then dried over sodium sulfate and filtered. DCM was evaporated. The residue was redissolved in DCM and purified over Grade III alumina in DCM. Pure DCM was rinsed through the column to remove any residual amino acid (not coupled to the PPIX). Then with 1% MeOH and DCM the product PPIX diglutamate was eluted. (alternatively you can use silica 20% acetone and DCM but this is not recommended if you have a free amine group anywhere in your compound.) Yield 168 mg (90%) UV-VIs (CH₂Cl₂) λ<sub>max</sub> (ε/M⁻¹ cm⁻¹) 406, 505, 540, 578; Mass Spectra (MALDI) m/z 1046 (M+H)<sup>+</sup>; ¹H NMR: (CDCl₃ 300 MHz) δ 10.20 (1H, s), 10.18 (1H, s), 10.15 (1H, s), 10.06 (1H, s) 8.23 (2H, m), 6.41 (2H dd, J = 12 Hz, J = 2 Hz), 6.17 (2H, dd, J = 19, J = 2), 4.31 (6H, m), 3.72 (3H, s), 3.71 (3H, s), 3.64 (3H, s), 3.63 (3H, s), 3.19 (4H, m), 1.57-1.86 (8H, m), 1.03 (18H, s), 1.04 (18H, s), -3.5 (2H, br, s)

PPIX diglutamate (16):

PPIX diglutamate tetratertbutylester (15, 100 mg, 0.096 mmol) was deprotected via stirring in neat TFA for 10 minutes. TFA was evaporated. The residue was precipitated with ethyl ether. The precipitate was washed 5 times with ether and then let dry under vacuum. The precipitate was redissolved in methanol and purified on a Sephadex column (100% methanol). The
compound was eluted and the methanol was evaporated and dried under high vacuum. Yield is quantitative 78 mg 100%. UV-Vis (MeOH) $\lambda_{\text{max}}$ ($\epsilon$/$M^{-1}$ cm$^{-1}$) 415 (174,658), 545 (14,384), 583 (15,753); Mass Spectra (MALDI) $m/z$ 820 (M + H)$^+$; $^1$H NMR (CD$_3$)$_2$SO $\delta$ 12.3 (4H, br s), 10.29 (1H, s), 10.22 (1H, s), 10.20 (1H, s), 10.29 (1H, s), 8.22 (2H, m), 6.45 (2H dd, $J = 12$ Hz, $J = 2$ Hz), 6.20 (2H dd, $J = 19$, $J = 2$) 4.31 (6H, m), 3.72 (3H, s), 3.70 (3H, s), 3.60 (3H, s), 3.58 (3H, s), 3.14 (4H, m), 2.08 (4H, m), 2.06 (2H, m), 1.80 (2H m), -4.02 (2H, s)

**Synthesis of PPIX dilysine [(NBoc)(OtBu)]$_2$ (17):**

See procedure for synthesis of 15 Expect yields of 17 to be slightly lower than yields of 16. UV-VIs (CH$_2$Cl$_2$) $\lambda_{\text{max}}$ ($\epsilon$/$M^{-1}$ cm$^{-1}$) 406, 505, 540, 578 Mass Spectra (MALDI) $m/z$ 1132 (M+H)$^+$; $^1$H NMR: (CDCl$_3$, 300 MHz) $\delta$ 10.24 (1H, s), 10.20 (1H, s), 10.16 (1H, s), 10.07 (1H, s) 8.16 (2H, m), 7.02 (2H, br s), 6.42 (2H, dd $J = 11.5$ Hz, $J = 1.5$ Hz), 6.28 (2H, dd, $J = 17.5$ Hz, $J = 1.5$ Hz), 4.2-4.4 (6H, m), 3.74 (3H, s), 3.72 (3H, s), 3.67 (3H, s), 3.65 (3H, s), 3.2-3.4 (4H, m), 3.59 (4H, m), 3.16 (4H, m) 0-1.9 (8H, m) 1.22 (36 H, s)

**PPIX dilysine (18)**

See procedure for synthesis of 16 UV-VIs (CH$_2$Cl$_2$) $\lambda_{\text{max}}$ ($\epsilon$/$M^{-1}$ cm$^{-1}$) 415 (138,776), 545 (9524), 582 (10,204) Mass Spectra (MALDI) $m/z$ 1132 (M+H)$^+$ $^1$H NMR: (CDCl$_3$, 300 MHz) $\delta$ 10.43 (1H, s), 10.35 (1H, s), 10.30 (1H, s), 10.28 (1H, s), 8.26 (2H, m), 7.4 (6H, br s), 6.45 (2H dd, $J = 12$ Hz, $J = 2$ Hz), 6.20 (2H dd, $J = 19$, $J = 2$), 4.31 (6H, m), 3.81 (3H, s), 3.79 (3H, s), 3.68 (3H, s), 3.65 (3H, s), 3.16 (4H, m), 1.0-1.5 (18H, m)

**3.6 References**

1 Biological property investigation by Timothy Jensen.
APPENDIX A: PORPHYRIN NOMENCLATURE

Figure 1 Oxidation states of tetrapyrrole ring. A is a porphyrin. B is a chlorin (dihydroporphyrin). C is a bacteriochlorin (tetrahydrochlorin).
APPENDIX B: CHARACTERIZATION OF COMPOUNDS

Instrumental Analysis

**UV-Vis:** Electronic absorption spectra were measured using a Perkin-Elmer Lambda 35 UV-Vis spectrophotometer.

**MS:** MALDI: Matrix Assisted Laser Desorption/Ionization (LSU Mass Spectrometry facility).

**NMR:** $^1$H NMR spectra were recorded on a Bruker AC 250 MHz, Bruker ARX-300 MHz and Bruker AMX 500 MHz. $^{13}$C spectra were recorded on the Bruker AMX 500 MHz.
Deuterated solvents: CDCl$_3$: 7.26 ppm, (CD$_3$)$_2$SO: 2.54 ppm, CD$_3$OD: 3.34 ppm
Chemical shifts ($\delta$) are given in parts per million relative to tetramethylsilane (TMS, 0 ppm); multiplicities are indicated as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet) and m (multiplet).

**Elemental Analysis:** Midwest Microlab Indianapolis, Indiana.

Chromatographic Methods

**Column Chromatography:** Three types of packing material were employed: E. Merck neutral alumina (70-230 mesh), Merck silica gel 60 and Sephadex LH20.
Alumina was deactivated with with either 6% water (activity III) or 15% water (activity V) before use. Sephadex LH20 was purchased from Amersham Biosciences (Sweden) and was allowed to absorb methanol overnight prior to each use.

**Analytical TLC:** TLC was performed on Scientific Adsorbent Company Inc., silica or alumina gel plate.

Purification of Solvents and Reagents

**Dichloromethane:** distilled from calcium hydride
**Methanol:** distilled over magnesium turnings
**Tetrahydrofuran (THF):** distilled from Na/benzophenone
**Toluene:** distilled from Na/benzophenone
**Triethylamine:** stored over 4A molecular sieves
VITA

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