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Synthesis and Cellular Investigations of the 131-Lysyl Derivative of Chlorin e6

Stewart Wynn Humble

Synthesis and Cellular Investigations of the 13^1-Lysyl Derivative of Chlorin e_6

by

Stewart Wynn Humble

Undergraduate Honors Thesis under the direction of

Dr. Kevin M. Smith

Department of Chemistry

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& Agricultural and Mechanical College
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DEDICATION

I would like to dedicate this Thesis to my family, for their unwavering support throughout my college career at LSU. My time here has truly flown by, but without support from my loved ones, I could not wish to have accomplished what I have.

Thank You Mom, Dad, Blake, and Paw Paw.

ACKNOWLEDGEMENTS

I would like to thank: Dr. Kevin M. Smith and Dr. Graça Vicente for allowing me to become a part of their research group, to which I owe all of my success as an upper division Honors College student researcher…they have allowed me to transform the idea of an Honors College thesis into a reality; Waruna Jinadasa for all of his help in making my project as a success…I could not have a better graduate student to look up to and work with; Timsy Uppal and Dinesh Bhupathiraju for setting a great example in the lab, guiding me through the Thesis writing process, and all of the great advice; David Jones II for his continued advice, support, and friendship; Dean Nancy L. Clark and Dr. William Worger for exposing me to new and challenging topics, both at home and abroad in South Africa; Dr. Drew Lamonica Arms for her never-ending support and advice, both as a professor and mentor; Dr. Mark “Doc” Dochterman for being a role-model and leader, as well as challenging me to take the Honors College experience to a new level; Dr. Granger Babcock for always being there for advice and allowing me to make my Honors College experience truly unique; Coach Sam Nader for giving me the opportunity to chase the dream of becoming a Long Snapper for the LSU Football Team; Mrs. Cindy Segher for her advice about life, school, and résumés….; Jeremy Joiner for his advice, leadership, and dedication to the students of the Honors College;

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PERSONAL MESSAGE

Dedicating myself to participating in the Upper Division Honors Program has allowed me to strive for goals that I would not have originally thought possible. When I entered the program, I had little to no research experience, but wanted to reach out for new opportunities. Overall, my most enjoyable experiences from the program have been through the interactions with the outstanding faculty and graduate students here at LSU. I have gained many new friendships that will most certainly last a lifetime, and have been mentored by some of the finest researchers there are in the field of developing photodynamic cancer treatments. However, one of my fondest memories occurred after completing my biological studies. After assessing the initial data, I realized for the first time that the compound I had synthesized was actually successful at eliminating cancer cells; it was a very rewarding feeling.

Whether dealing with my research, academic success, passion for service, or my leadership, I am most proud of the recognition I received from one of my closest friends. He said, “Stewart, you have the Tiger inside of you brother, and nothing can stop you.” That confidence meant so much to me: having the “Tiger” inside, to me, means being passionate about what this university is and will always represent…. the “Tiger” represents passion and courage, determination and excellence, leadership and resolve, and intelligence and selflessness. For me, the “Tiger” is deep inside; it’s in my blood, and part of my DNA. It is who I am, what I am, and what this university means to me; it is my strength in this world, and why I will always stand strongly and say,

“Forever L-S-U.”

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GLOSSARY OF ABBREVIATIONS

Boc = tert-Butyloxycarbonyl

Ce_6 = Chlorin e_6

DCC = *N,N′*-Dicyclohexylcarbodiimide

DCM = Dichloromethane, CH_2Cl_2

DIEA = *N*,*N*-Diisopropylethylamine

DME = Dimethyl ester

DMAP = 4-Dimethylaminopyridine

Et = Ethyl, $–CH_2CH_3$

HOBt = Hydroxybenzotriazole

Lys-Ce_6TME = 13^1-Lysyl Chlorin e_6 Trimethyl Ester

Me = Methyl, –CH3

MeOH = Methanol, CH_3OH

NMR = Nuclear Magnetic Resonance

PDT = Photodynamic Therapy

TBTU = *N,N,N′,N′*-Tetramethyl-*O*-(benzotriazol-1-yl)uronium tetrafluoroborate

TEA = Triethylamine, $(CH_3CH_2)_3N$

TFA = Trifluoracetic Acid, CF_3CO_2H

TLC = Thin Layer Chromatography

TME = Trimethyl Ester

Synthesis and Cellular Investigations of the 13^1-Lysyl Derivative of Chlorin e_6

ABSTRACT

Compared with porphyrins, chlorophyll derivatives absorb longer wavelength light that penetrates further into the tissues of the body than does shortwave light, providing the opportunity to treat large tumors. These derivatives also have the ability to accumulate in tumor tissues. Through organic synthesis, one can modify the amphiphilic characteristics of the molecule by introducing certain polar amino acids. Use of positively charged amino acids (e.g. lysine) also aids localization into the most sensitive portions of the cell such as the nucleus and mitochondria. There are three carboxylic acid chains within the selected chlorophyll molecule, chlorin e_6, allowing for formation of three regioisomers that can give different biological effects. This Thesis describes the regioselective synthesis of the 13^1-lysyl-derivative of chlorin e_6, as well as the cellular uptake, subcellular localization, and photophysical properties of the compound as a photosensitizer. Increasing the tumor selectivity of chlorophyll derivatives is the main goal of my research.

CHAPTER 1: INTRODUCTION

The therapeutic use of light began as far back as 1900 when scientists reported that the combination of acridine orange and light could destroy living organisms.[1] Soon after, it was noted that tumor tissue was inherently more fluorescent than healthy tissue, which indicates the aggregation of conjugated molecules. Between 1940 and 1960, Figge et al. administered natural porphyrins to patients and tumor-bearing animals in an attempt to more accurately detect tumor tissue by fluorescence.[2] During the 1960s Winkelman used synthetic porphyrins to detect tumor tissue.[3] Throughout the 20th century several attempts were made to treat tumor tissue with photosensitizing agents. However, these experiments mainly included non-porphyrin photosensitizers. Once porphyrin photosensitizers were examined, they were found to be efficient singlet oxygen generators and have absorption maxima in the red portion of the electromagnetic spectrum.[1]

Photodynamic therapy, or PDT, is a method for cancer treatment that uses a photosensitizer and visible light to produce reactive oxygen species that will destroy malignant cells. PDT is currently used in several countries for the treatment of several types of cancer, including skin, mouth, esophageal, lung, and bladder tumors.[1, 4] The selectivity and overall effectiveness of the PDT treatment depends upon both the tumor-targeting ability of the photosensitizer and the light used to activate it. Current efforts are aimed to produce a photosensitizer that possesses several key traits ideal to the method of PDT. These traits include being a single compound, having increased absorbance in the red region of visible light, giving a high quantum yield of triplet formation, having good cytotoxic oxygen species generation, showing increased

selectivity for malignant tissue over normal tissue, and exhibiting low dark toxicity.[4b, 5] Synthesizing a photosensitizer that possesses a majority of these traits will give scientists an edge in the research for cancer treatment. Furthermore, chlorophyll derivatives have been shown to possess a unique ability to accumulate in malignant tissues. This natural phenomenon can be harnessed along with the capability of the molecule to act as a photosensitizer in order to produce toxic singlet oxygen molecules with the aid of laser radiation in photodynamic therapy.[4a]

Every photosensitizer must include a chromophore to absorb light and generate the singlet state oxygen necessary to destroy targeted cells. Additionally, there may be amino acids, peptide chains, or other molecules attached to the photosensitizer to increase the selectivity towards the tumor cells over normal cells, and to improve its amphiphilicity.[1, 4a] Indeed, these photosensitizer attachments help to determine the overall amphiphilicity of the molecule, which is extremely important in regards to cellular uptake. The photosensitizer itself is responsible for the overall color of the molecule when it absorbs certain wavelengths of visible light and transmits or reflects others. This ability gives it the dual function of creating the singlet oxygen, as well as providing scientists with the ability to track and identify the molecule within a cell.[1] The activating light for PDT is most often generated by lasers, or in some cases by fluorescent light sources. Lasers are used because they produce highly coherent monochromatic light that can be efficiently channeled through specific delivery devices, such as fiber-optic cables within an endoscope.[1, 5]

Photosensitizers in photodynamic therapy allow for the transfer and translation of light energy into type II photoreactions. In clinical practice, photosensitizers arise from three families—porphyrins, chlorophylls, and dyes.[5] All clinically successful photosensitizers have the ability to target specific tissues and reliably activate at a high enough light wavelength useful for therapy.[5] Their ability to fluoresce and visualize the tumor is a further advantage. However, photosensitizers that have been developed from each family have unique properties and have so far been minimally exploited in some clinical settings.[1, 5]

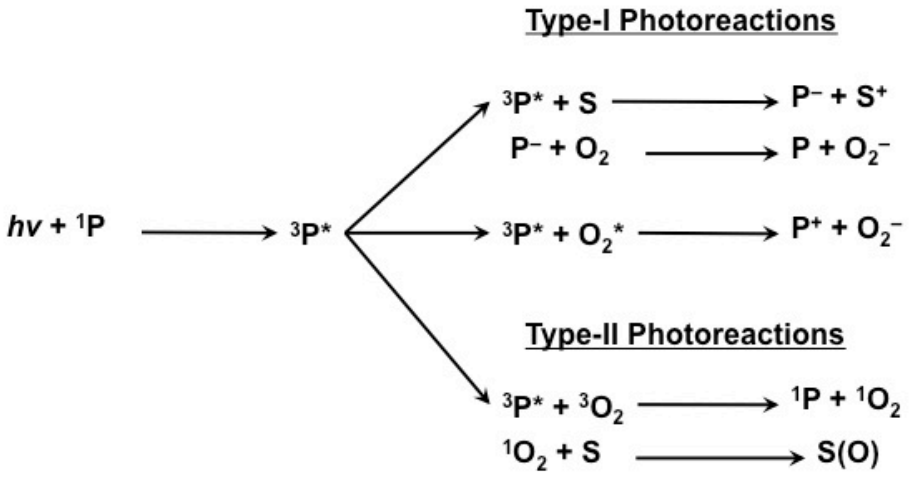


Figure 1.1: Type-I and Type-II photoreactions, where ^{1}P is a photosensitizer in a singlet ground state, $^{3}P^{*}$ is a photosensitizer in a triplet excited state, and S is a substrate molecule.

While the process of photodynamic therapy may seem complex, the photochemistry behind the technique is actually quite simple. When light of the appropriate wavelength shines onto the chromophore of the photosensitizer, the molecule becomes excited to an excited singlet state. The photosensitizer can relax back to the ground state by emitting a fluorescent photon or switch to excited triplet states through intersystem crossing. From triplet-excited states, the photosensitizer can

relax back to the ground state by emitting a phosphorescent photon or transferring energy to another molecule. In oxygenated environments the photosensitizer readily transfers its energy to ground state, or triplet, molecular oxygen to produce singlet oxygen, which can form adducts with organic substrates. Singlet oxygen, the predominant cytotoxic agent produced during PDT is a highly reactive form of oxygen that is produced by inverting the spin of one of the outermost electrons.[1] Photochemical reactions of this type are known as Type-II photoreactions and are characterized by a dependence on the oxygen concentration. The Jablonski diagram below does a superb job showing the various modes of excitation and relaxation in a chromophore: (A) excitation; (B) fluorescence; (C) intersystem crossing; (D) phosphorescence; (E) non-radiative transfer of energy to singlet oxygen; (F) substrate oxidation by singlet oxygen; (G) internal conversion.[1]

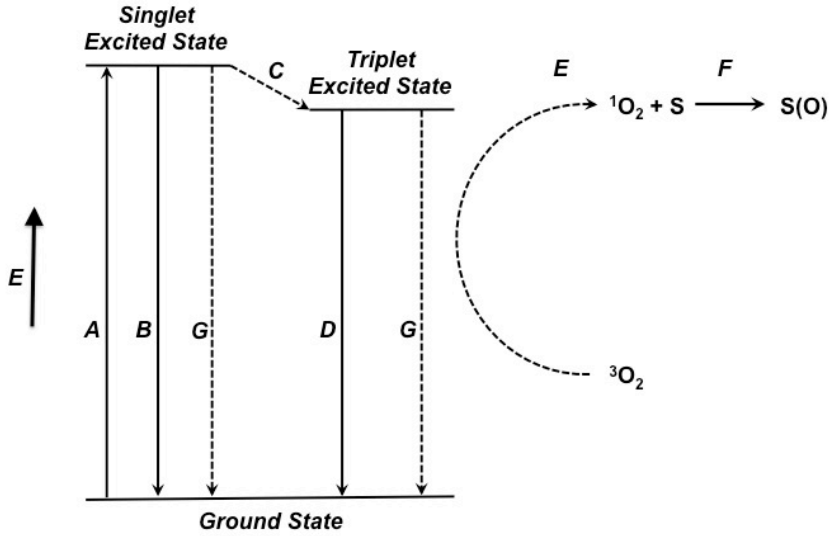


Figure 1.2: The photochemical reaction that generates singlet oxygen, the cytotoxic agent in Photodynamic Therapy, can be clearly represented by the Jablonski diagram.

In order for photodynamic therapy to be successful and efficient, it is best to target the most sensitive parts of diseased cells. More specifically, several key molecules, organelles, and tissues have been identified as primary targets for photosensitizers in photodynamic therapy. Some of these include vasculature, nucleus, lysosomes, mitochondria, and cellular membranes, with some organelles being more critical to the cell's existence, such as the mitochondria.[1, 6]

Researchers have shown that a significant percentage of tumor cells in vivo die after PDT treatment because vascular shutdown and hemorrhage starves them of oxygen and nutrients.[1, 7] Henderson et al. have demonstrated that vascular shutdown and inflammation killed tumor cells in vitro, with vascular destruction occurring to a greater extent in vivo. Hemorrhaging was found to starve tumor cells of oxygen and nutrients, and the destruction of the vasculature was crucial to reducing the survivability the tumor cells. As a side effect to vascular destruction, the inflammatory response observed after tissue injury or infection promoted cell death. Thus inflammatory agents have been examined as mediators of the PDT response.[1] There are also several direct cell effects that can be seen from treatment through photodynamic therapy. Previously, PDT was thought to destroy tissues primarily by damaging cells that had accumulated enough photosensitizer and received enough light to produce lethal amounts of singlet oxygen, but as research continues, further methods of tumor cell destruction are being realized.

Mechanisms similar to the vascular shutdown may be stimulating an intracellular process known as apoptosis, or programmed cell death, within tumor cells. More specifically, some apoptotic pathways involve specific plasma membrane death

receptors that can initiate some of these pathways involving mitochondria, lysosomes, and the ER. Subsequent photoactivation of an organelle-localized photosensitizer can similarly cause the release of unique proapoptotic proteins that play essential roles in apoptotic signaling.[6b, 8]

The nucleus, lysosomes, and mitochondria have all been identified as potential targets in association with photodynamic therapy. DNA is within the cells of all organisms, so as a macromolecule in the cell, it may be susceptible to PDT treatments when the nucleus is a target. More specifically, modification and mutation of nucleotides within DNA, along with damage to DNA repair mechanisms from PDT could ultimately kill the tumor cells.[9] Damage to guanine nucleotides was the most common mutation, and DNA damage induced by PDT in cells with inefficient DNA-damage repair mechanisms led to decreased survivability. However, the same research groups compared the DNA damage caused by PDT to X-ray induced DNA damages, finding much less DNA damage from PDT treatment than from X-ray treatment. The researchers showed that X-ray treatment produced 80% more strand breaks, 5% more sister-chromatid exchanges, and more chromatid aberrations than PDT treatment. Additionally, PDT-induced strand breaks were repaired more efficiently than those caused by X-ray irradiation.[9]

Lysosomal damage is another important focus for photodynamic therapy treatments. Many photosensitizers can be observed within the cell by fluorescence microscopy, and have been seen to localize at cytosolic targets such as the Golgi apparatus, endoplasmic reticulum (ER), mitochondria, lysosomes, and cellular membranes.[6] Lysosomes are a major localization organelle for photosensitizers

because of their characteristics within the cell, but they may be useful in regards to PDT due to their function. Researchers have demonstrated that lysosomal disruption was not directly detrimental to the cell. However, upon disruption, apoptotic pathways of the lysosome are initiated that may assist in the further degradation of cellular components and adversely effect cell processes.[1, 6b]

One of the most important targets for photodynamic therapy may be the mitochondria of tumor cells. Because of their importance to the cell, through signaling mechanisms and energy production, damage to the mitochondria would be detrimental to any tumor cell. One research group used HpD-PDT, or hematoporphyrin-derivative photodynamic therapy, to inhibit several mitochondrial proteins in vivo.[1, 10] Malate dehydrogenase, succinate dehydrogenase, and cytochrome c oxidase are required to maintain the electrochemical gradient across the inner-mitochondrial membrane and were all partially inhibited. The group also examined the functioning of several cytosolic proteins such as glucose phosphate isomerase, pyruvate kinase, and lactate dehydrogenase. Their findings suggested that cytosolic proteins might not be as susceptible to photochemical oxidation as membrane bound proteins. Conclusions were based on the theory that the singlet-oxygen lifetime is drastically shortened in a reducing aqueous environment.[1, 10] Additionally, photodynamic damage to mitochondrial proteins is thought to be the primary cause in a chain of events leading to the breakdown of the electron transport chain, disruption of the mitochondrial-membrane potential, and overall mitochondrial breakdown.[1]

From a strategic standpoint, efficiency is key when killing tumor cells, and lysing a cell will instantly cause it to cease its function. By damaging the plasma membrane of

a tumor cell, the potential to destroy the tumor is drastically increased. Therefore, employing photodynamic therapy to cellular membranes is a promising tactic in the battle against cancer. While the specific targeting of the plasma membrane will be a tough area of focus in future research, some photosensitizers have been found to localize to the cellular membranes and the membranes of organelles. For example, PDT can cause lysosomal rupture, small ruptures in the plasma membrane, and increase the flip-flop rate of membranes lipids.[1] These ruptures may cause slight leakage of potassium ions and small molecules from cells, but these events usually occur after protein damage. Even though PDT treatments for membrane lipids may not be tremendously lethal to a cell, membranes may still have an important role in PDT. Specifically, the oxidation of lipids that lie within certain membranes may not kill the cell in a direct fashion, but they serve as a trigger for potential signals of a transduction pathway that could cause a cell to run through an apoptotic mechanism.[1]

Presently, effort has been put into designing, synthesizing, and characterizing a chlorophyll-derived photosensitizer for photodynamic therapy. Ample research has been focused on developing effective drug delivery systems for the preparation of chlorins as potential photosensitizers for PDT, with investigations of fluorescence kinetics in cells and overall efficiency in PDT. Compared with porphyrins, chlorophyll derivatives absorb longer wavelength light that penetrates further into the tissues of the body than does shortwave light, providing the opportunity to treat large tumors. These derivatives also have the ability to accumulate in tumor tissues.[4a] Through organic synthesis, one can modify the amphiphilic characteristics of the molecule by introducing certain polar amino acids, thereby enhancing their effect as photosensitizers.

Amphiphilicity is a measure of the extent to which a molecule can behave in both a polar and nonpolar way, giving these molecules the ability to dissolve in the blood and also to pass through hydrophobic cell membranes. The use of positively charged amino acids (e.g. lysine) also aids localization into the most sensitive portions of the cell such as the nucleus and mitochondria.[4a, 6] Chlorophyll-a derivatives of the chlorin e_6 series have three carboxylic side chains (at positions 13^1, 15^2, and 17^3), which provide a wide range of opportunities for synthesis of novel amphiphilic photosensitizers. This also allows for formation of three regioisomers that can give different biological effects.[4a]

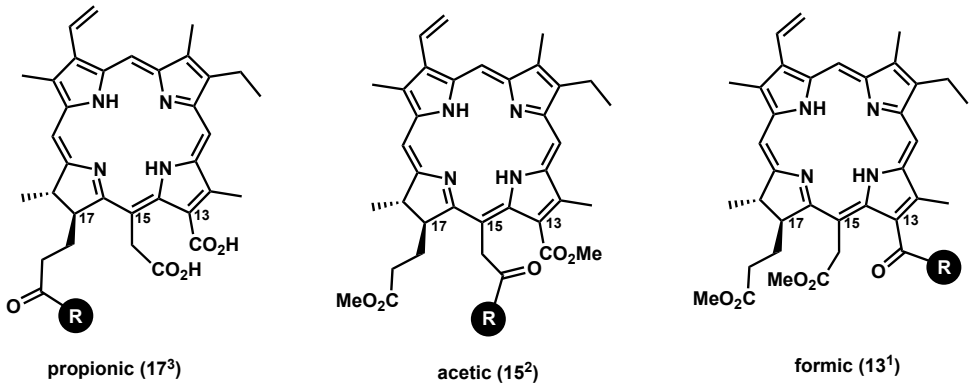


Figure 1.3: Chlorin e_6 Conjugates

The principal motivation behind this project originated from a series of discoveries from previous research with NPe6, a second-generation photosensitizer in advanced stage clinical trials for oncologic applications of photodynamic therapy (PDT). According to the 1987 patent, NPe6—also known as mono-(L)-aspartylchlorin-e_6—was believed to have a single aspartyl derivative on the propionic position (17^3) of chlorin e_6.[11] However, the proposed structure of NPe6 was challenged when the Japanese group Gomi et al. showed that NPe6 actually existed as the acetic conjugate (15^2). [12]

Initially, few believed the results from the Japanese due to their use of dubious NMR spectroscopy in aqueous solvents. However, subsequent research from the Smith group at LSU, specifically from Jodie Hargus, unexpectedly proved the Japanese group correct.[13] Additionally, Hargus et al., with the help of Frank Fronczek, were able to produce the crystal structure of the tetramethyl ester of NPe6 using X-ray crystallography, definitively establishing the existence of the acetic conjugate (15^2) vs. the propionic conjugate (17^3).[13]

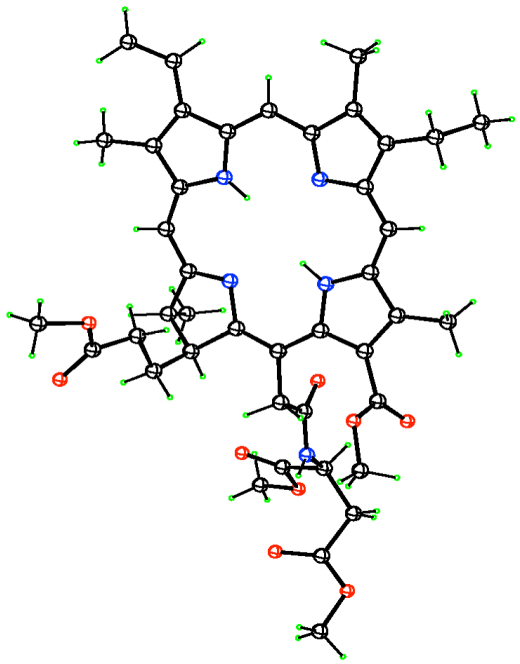


Figure 1.4: X-Ray Crystal Structure of NPe6; *Frank Fronczek, LSU*

Previous studies from Jinadasa et al. have demonstrated that formic acid conjugates (13^1) of chlorin e_6 are more effective photosensitizing agents compared to the acetic and propionic acid conjugates.[14] More specifically, the most phototoxic compounds in the study were found to be the 13^1-regioisomers, bearing either an aspartic acid directly conjugated to formic position of chlorin e_6 or a lysine residue

conjugated to the formic position with the use of a linker.[14] The most phototoxic compound of Jinadasa's series was 13^1-aspartylchlorin e_6, not NPe6 (the 15^2 isomer) or other derivatives, making it the most promising for PDT applications.[14] These results once again suggest that the 13^1 derivatives of chlorin e_6 may be the most potent of the series. The study's molecular modeling calculations show that the 13^1-regioisomers assumed nearly linear conformations, which may have facilitated diffusion through membranes and their binding to multiple intracellular components with subsequent photodamage to multiple cellular sites.[14] Therefore, the goal of my project is to synthesize a 13^1-compound with a directly attached lysine derivative in the belief that chlorin e_6 photosensitizers of the 13^1 series were more phototoxic than the other regioisomers, and lysine might drive localization to mitochondria. Investigating these effects and increasing the tumor selectivity of chlorophyll derivatives is the main purpose of my research.

CHAPTER 2: SYNTHESIS AND CHARACTERIZATION OF 13^1-LYSYL-CHLORIN-E_6

2.1: Introduction

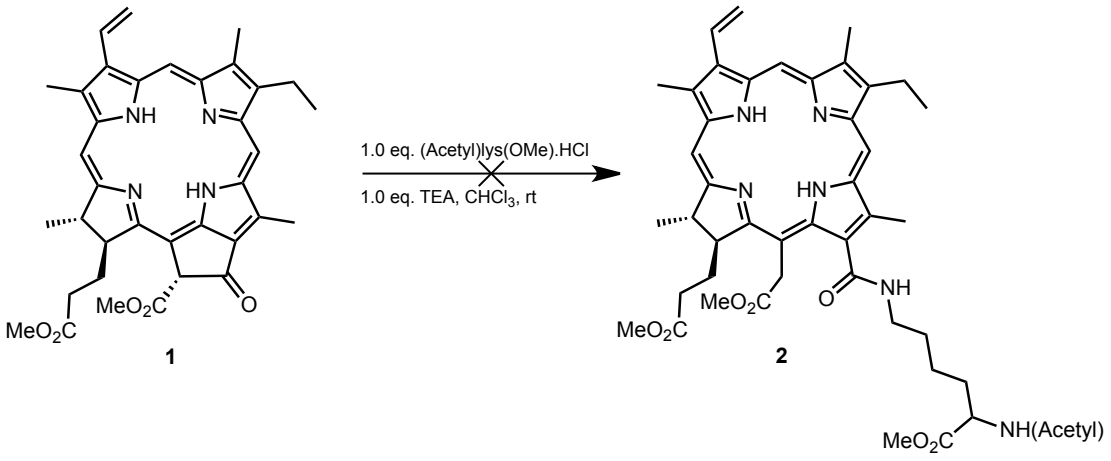
In present work, 13^1-mono-lysyl(OMe) chlorin e_6 trimethyl ester has been synthesized starting from chlorin e_6. From chlorin e_6, which was either purchased or prepared by degradation of pheophytin extracted from *Spirulina* alga, a selective esterification was performed to synthesize chlorin e_6 dimethyl ester, which was then coupled with protected lysine at the formic acid position, and was followed by the subsequent deprotection of the lysine Boc group to give the desired product. It is known that positively charged species interact best with mitochondria.[6] Therefore, positively charged amino acids are being utilized to specifically attack mitochondria in the chlorin e_6 conjugates of interest.

This initial synthesis and characterization will provide some new data that may help to identify more effective photosensitizers for future projects, with a promising outlook for further research. 2005 Chemistry Nobel Laureate Robert Grubbs has said – "If you plan a reaction and get the expected product, that is routine. If you plan a reaction and get an unexpected product, that is innovative". I am hoping for many unexpected products in current and future projects.

2.2: Synthesis of 13^1-mono-(acetyl)lysyl(OMe) Chlorin e_6 Trimethyl Ester

Initially, an attempt was made to synthesize the proposed chlorin e_6 derivative as 13^1-mono-(acetyl)lysyl(OMe) chlorin e_6 trimethyl ester through a simple one-step mechanism. The amino acid lysine was to be coupled to methyl pheophorbide-a (1) at

the more basic side chain amine, reacting through a ring-opening mechanism. To accomplish this, methyl pheophorbide-a (1) was treated with (Acetyl)lys(OMe).HCl in basic conditions in order to open the exocyclic ring to obtain the desired molecule 2. After 24 hours and 48 hours, the reaction was monitored by TLC and only the starting material, no new product, was observed at either checkpoint. Additionally, the reaction yielded no new product after 5 days, so a new approach was taken. The solvent was changed to DMF, in order to increase the solubility of the amino acid substituent. However, upon inspection of TLC 24 hours later, the reaction yielded no new product, and only the starting material was observed. The lysine derivative used is less sterically hindered at the side chain $–NH_2$ because it was left unprotected in order to select for a nucleophilic attack on the exocyclic ring; ethylene diamine readily accomplishes the ring-opening process, so it was puzzling as to why the amino side-chain of lysine would not do the same.[14-15] Presumably, the side chain amine was not sufficiently nucleophilic to open the cyclic ketone of the exocyclic ring under these conditions.



Scheme 2.1: Synthesis of 13[1]-mono-(acetyl)lysyl(OMe) chlorin e_6 trimethyl ester

2.3: Synthesis of 13^1-mono-lysyl(OMe) Chlorin e_6 Trimethyl Ester

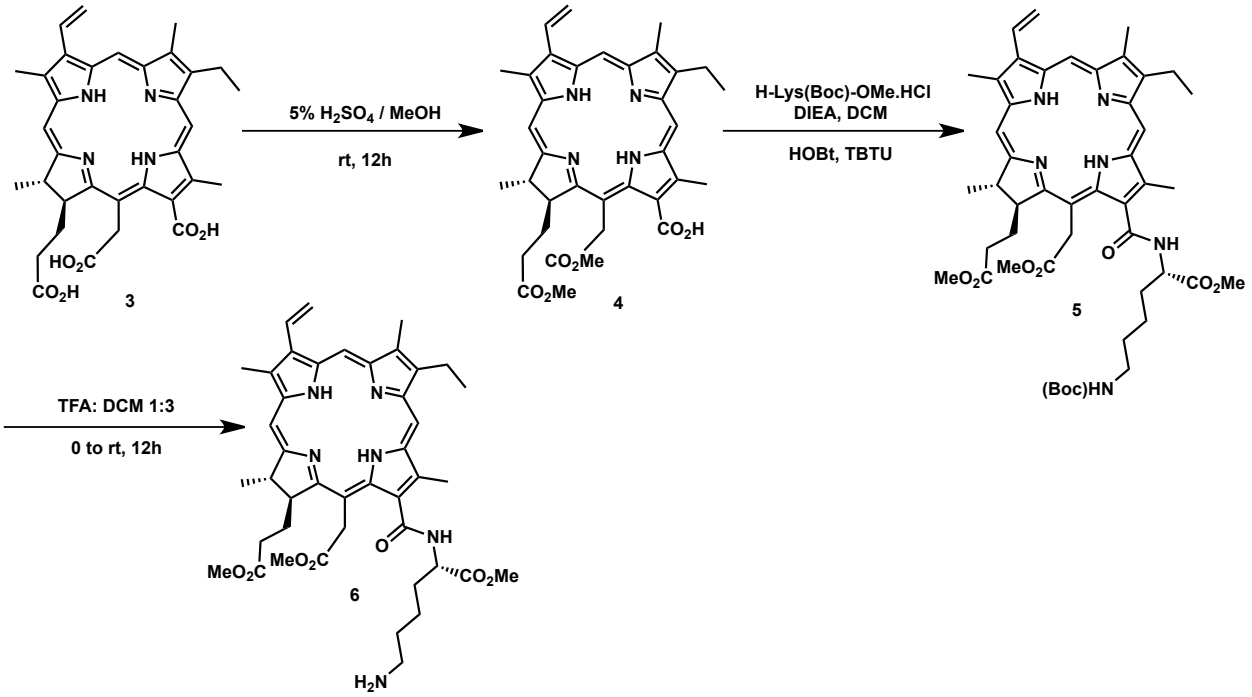
The synthesis of 13^1-mono-(acetyl)lysyl(OMe) chlorin e_6 trimethyl ester failed through the original method, therefore an entirely new reaction scheme was chosen to attach the lysine derivative to chlorin e_6. In order to accomplish this, two main aspects of the reaction were altered: the starting material was chlorin e_6 (3) (not methyl pheophorbide-a) and the amino acid was protected at the side chain (not the N-terminus). The advantage of starting from chlorin e_6 comes from its ability to selectively protect the 15^2 and 17^3 carboxyl groups, forming a dimethyl ester with a free 13^1 carboxylic group that can be used to couple an amino acid derivative.[16]

Synthesizing the chlorin e_6 dimethyl ester (4) is the first step in the reaction scheme, requiring the presence of methanol in acidic conditions. Under acidic conditions, the inner nitrogens of chlorin e_6 (3) will protonate, inactivating the 13^1 carboxyl group due to delocalization of the overall positive charge.[16] The characterization of the final product, chlorin e_6 dimethyl ester, was accomplished through ^{1}H-NMR, where two additional methyl peaks confirmed the selective esterification of the 15^2 and 17^3 carboxyl groups. A quantitative yield of the final product was obtained upon completion of this step in reaction scheme 2.2.

The second step in the synthesis of 13^1-mono-lysyl(OMe) chlorin e_6 trimethyl ester was a difficult and time consuming phase. This portion of the reaction scheme required the coupling of the lysine derivative onto chlorin e_6 dimethyl ester to form 13^1-mono-(Boc)lysyl(OMe) chlorin e_6 trimethyl ester (5). Referring to reaction scheme 2.2, molecule 4, chlorin e_6 dimethyl ester, was the starting material that would be coupled to the lysine derivative. In order to accomplish this, the coupling reagent DCC and DMAP

base were used in the initial reaction. The entire reaction was monitored by TLC and upon review at 24 and 48 hours, these reagents yielded no new product. After the initial coupling failed, scientific literature from our research group was reviewed to find another means of coupling the lysine derivative to chlorin e_e dimethyl ester. More specifically, Jinadasa et al. showed several examples of HOBT and TBTU as effective coupling reagents for similar 13^1 chlorin e_6 derivatives.[14] Once these reagents were incorporated into the reaction, the TLC results were showing a clear progression of the reaction within 24 hours, and subsequently the final product, 13^1-mono-(Boc)lysyl-(OMe) chlorin e_6 trimethyl ester (5), was obtained. The characterization of the final product was accomplished through ^{1}H-NMR, where the presence of the peak from the Boc group and the peaks associated with protected lysine confirmed the coupling of the lysine derivative to the 13^1 carboxyl group of chlorin e_6 dimethyl ester.

The Boc-protected side chain on the lysine derivative selected for the coupling reaction to occur at the N-terminus of the amino acid, preventing any competition with the side chain amine during coupling. In order to synthesize the final product of reaction scheme 2.2, the final step of the scheme must be performed to remove this protecting group. Deprotection of the Boc group on the side chain of the lysine derivative was relatively straightforward and required acidic conditions. This step ultimately produced the final product, 13^1-mono-lysyl(OMe) chlorin e_6 trimethyl ester (6), in an overall yield of approximately 33% starting from chlorin e_6 (4). The characterization of the final product was accomplished through H^1-NMR, where the absence of the peak from the Boc group confirmed that the lysine derivative had been successfully deprotected to form the final product, molecule 6 of reaction scheme 2.2.



Scheme 2.2: Synthesis of 13^1-mono-lysyl(OMe) chlorin e_6 trimethyl ester

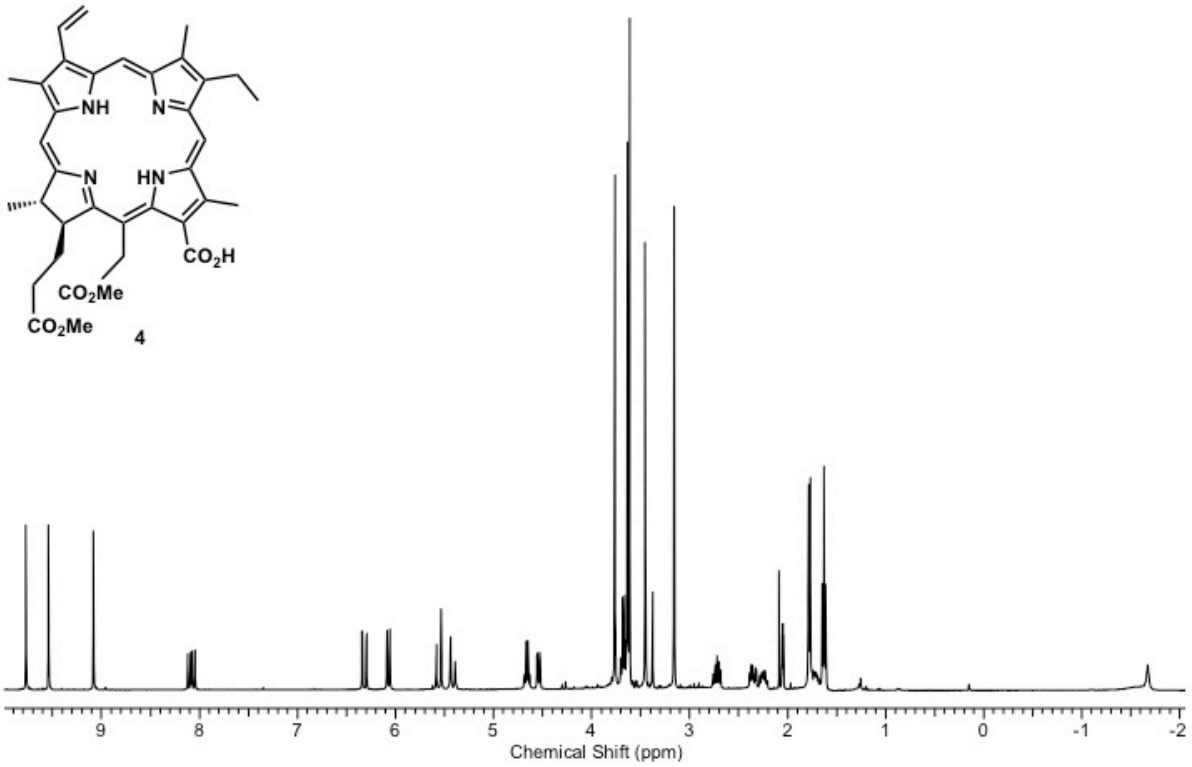


Figure 2.1: H^1-NMR Spectra of chlorin e_6 dimethyl ester (4)

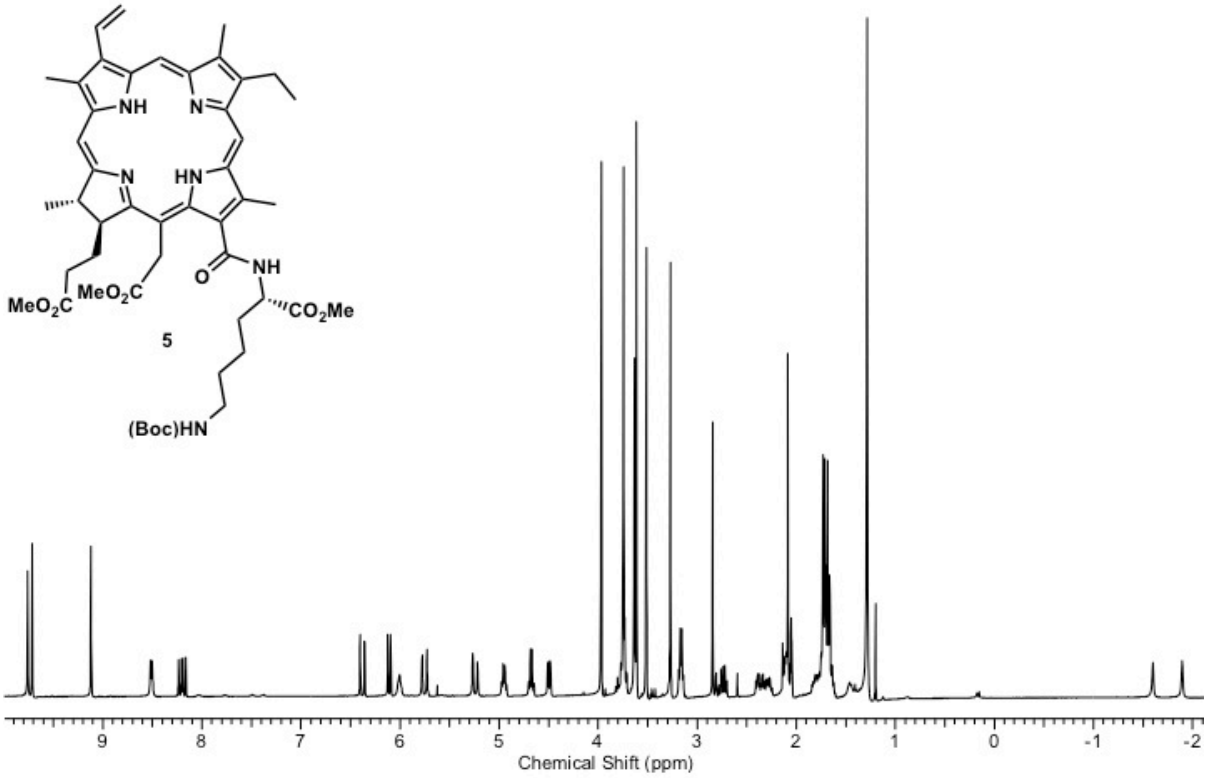


Figure 2.2: H^1-NMR Spectra of 13^1-mono-(Boc)lysyl-(OMe) chlorin e_6 trimethyl ester (5)

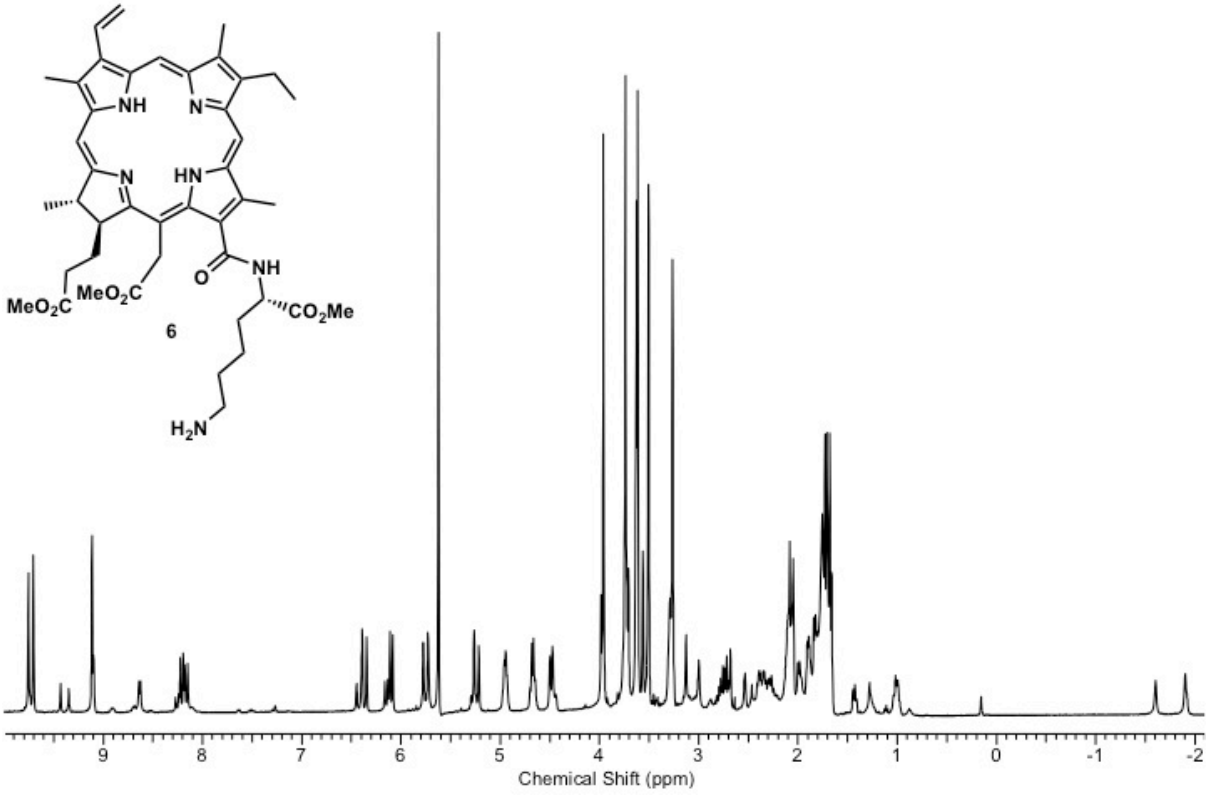


Figure 2.3: H^1-NMR Spectra of 13^1-mono-lysyl(OMe) chlorin e_6 trimethyl ester (6)

2.4: Suggestions for Future Work

In future work, some proposed objectives include the expansion of the project into a much broader study that will be able to compare the effectiveness of many separate 13^1 derivatives of chlorin e_6 for use as photosensitizers in PDT. More specifically, the 13^1-mono-lysyl derivative should be compared to poly-lysyl derivatives of chlorin e_6 such as the 13^1-tetra-lysyl and 13^1-octo-lysyl derivatives, as well as to 13^1-mono-arginyl, and to poly-arginyl derivatives of chlorin e_6 such as the 13^1-tetra-arginyl and 13^1-octo-arginyl derivatives.

2.5: Experimental for Chapter 2

Synthesis and characterization of chlorin e_6 dimethyl ester (4): In a round bottom flask, chlorin e_6 (3) (100 mg, 0.168 mmol) was dissolved in 5.0 mL of 5.0% sulfuric acid in methanol. The sample was allowed to stir overnight, protected from light and under nitrogen. Then, the reaction mixture was diluted with CH_2Cl_2 and poured into 5.0% aqueous $NaHCO_3$. A precipitate formed, so citric acid was added to bring the sample back into the organic layer. The organic layer was then washed with water and brine, dried over Na_2SO_4, and filtered. The solvent was removed through rotary evaporation and the final product chlorin e_6 dimethyl ester (4) was obtained in approximately 98% yield (102 mg, 0.165 mmol). ^{1}H NMR $(CD_3)_2CO$: δ_9.77 (s, 1H), 9.54 (s, 1H), 9.08 (s, 1H), 8.08 (dd, 1H), 6.32 (d, 1H), 6.07 (d, 1H), 5.56 (d, 1H), 5.41 (d, 1H), 4.66 (q, 1H), 4.54 (d, 1H), 3.76 (s, 3H), 3.67 (q, 2H), 3.63 (s, 3H), 3.61 (s, 3H), 3.45 (s, 3H), 3.16 (s, 3H), 2.72 (m, 1H), 2.35 (m, 2H), 1.78 (q, 3H), 1.73 (m, 1H), 1.63 (t, 3H), -1.67 (br s, 1H).

Synthesis and characterization of 13^1-mono-(Boc)lysyl-(OMe) chlorin e_6 trimethyl ester (5): In a round bottom flask, chlorin e_6 dimethyl ester (4) (102 mg, 0.165 mmol) was dissolved in dry CH_2Cl_2. A mixture of HOBt (24 mg, 0.18 mmol), TBTU (58 mg, 0.18 mmol), and DIEA (0.02 mL) in DMF was added to the mixture and allowed to stir for 30 minutes. H-Lys(Boc)-OMe Hydrochloride (60 mg, 0.21 mmol) and DIEA (0.02 mL) were mixed in CH_2Cl_2 and added to the reaction mixture. The mixture was allowed to stir for approximately 48 hours and monitored using TLC. Then, it was diluted with DCM, washed with 5.0% citric acid, followed by a wash with water then with brine. The mixture was then dried over Na_2SO_4 and evaporated. The residue was dissolved in 5.0% acetone/CH_2Cl_2 and purified via silica column chromatography with the same mobile phase. After the purification, we proceeded to the next step without measuring the yield due to difficulties with handling the compound. ^{1}H NMR $(CD_3)_2CO$: δ_9.75 (s, 1H), 9.71 (s, 1H), 9.12 (s, 1H), 8.51 (d, 1H), 8.20 (dd, 1H), 6.38 (d, 1H), 6.11 (d, 1H), 6.00 (br s, 1H), 5.75 (d, 1H), 5.24 (d, 1H), 4.95 (q, 1H), 4.67 (q, 1H), 4.49 (d, 1H), 3.97 (s, 3H), 3.74 (s, 3H), 3.73 (q, 2H), 3.64 (s, 3H), 3.61 (s, 3H), 3.51 (s, 3H), 3.27 (s, 3H), 3.16 (q, 2H), 2.72 (m, 1H), 2.30 (m, 2H), 2.10 (m, 1H), 1.72 (d, 3H), 1.70 (m, 5H), 1.68 (t, 3H), 1.46 (m, 1H), 1.28 (s, 9H), -1.60 (s, 1H), -1.90 (s, 1H).

Synthesis and characterization 13^1-mono-lysyl-(OMe) chlorin e_6 trimethyl ester (6): In a round bottom flask, 13^1-mono-(Boc)lysyl-(OMe) chlorin e_6 was dissolved in 5.0 mL of dry DCM in an ice bath under argon. Thioanisole (0.01 mL) and TFA (5.0 mL) were added and stirred over night. Then, the reaction mixture was diluted with CH_2Cl_2 and washed with water and 10% $NaHCO_3$ then dried over Na_2SO_4. The solvent was removed through rotary evaporation. The residue was dissolved in 10%

Acetone/CH_2Cl_2 and purified via silica column chromatography with the same mobile phase. This ultimately produced the final product, 13^1-mono-lysyl(OMe) chlorin e_6 trimethyl ester (6), in an overall yield of approximately 33% (43.5 mg, 0.057 mmol) starting from chlorin e_6 (4). ^{1}H NMR $(CD_3)_2CO$: δ_9.75 (s, 1H), 9.70 (s, 1H), 9.11 (s, 1H), 8.60 (d, 1H), 8.19 (dd, 1H), 6.37 (d, 1H), 6.10 (d, 1H), 5.75 (d, 1H), 5.24 (d, 1H), 4.95 (br s, 1H), 4.67 (q, 1H), 4.49 (d, 1H), 3.96 (s, 3H), 3.74 (s, 3H), 3.73 (q, 2H), 3.63 (s, 3H), 3.61 (s, 3H), 3.50 (s, 3H), 3.26 (s, 3H), 2.76 (m, 3H), 2.38 (m, 4H), 1.75 (m, 5H) 1.72 (d, 3H), , 1.68 (t, 3H), 1.47 (m, 1H), 1.28 (m, 1H) -1.60(s, 1H), -1.90(s, 1H).

CHAPTER 3: CELLULAR INVESTIGATIONS OF 13^1-LYSYL-CHLORIN-E_6

3.1: Introduction

Cell evolution studies have also been completed, where photo-cytotoxicity, dark-cytotoxicity, and cellular uptake evaluations for the synthesized chlorin e_6 derivative, as well as its intracellular localization (lysosomes, ER, Golgi, mitochondria, or nucleus) were investigated. Monitoring the cytotoxicity of the synthesized compounds in both light and dark environments is a good indicator for progress on this specific project because it will show the effectiveness of the chlorin e_6 derivative in regards to PDT. Additionally, time-dependent cellular uptake and fluorescence microscopy are two other important assays that will help to give information on the efficiency of this derivative as a photosensitizer. Ultimately, these studies will provide a better understanding of the underlying mechanisms involved in PDT, and give more insight into the effectiveness of 13^1 chlorin e_6 derivatives as photosensitizers.

3.2: Dark Toxicity

A side by side evaluation of the dark toxicity of chlorin e_6 (3) (Ce_6) and 13^1-mono-lysyl-(OMe) chlorin e_6 trimethyl ester (6) (13^1 Lys-Ce_6TME) was performed in HEp2 cells exposed to increasing concentrations of each compound up to 400 μM. Chlorin e_6 was found to be nontoxic in the dark up to the highest concentration investigated, making the IC50 value for chlorin e_6 greater than 400 μM (Figure 3.1). The synthesized 13^1-lysyl derivative of chlorin e_6 showed slight dark toxicity, with an IC50 value of approximately 80 μM (Figure 3.1). In regards to 13^1 Lys-Ce_6TME, high dark toxicity would prevent its

use as a photosensitizer. However, the concentrations at which the 13^1 Lys-Ce_6TME was found to be dark toxic to HEp2 cells is far from lower than the range of clinical concentrations. Further comparisons will be made with the results from phototoxicity concentrations in order to determine the quality of 13^1 Lys-Ce_6TME as a photosensitizer. Moreover, the ratio between the dark toxicity and the phototoxicity serves as a good baseline to compare one photosensitizer to another.

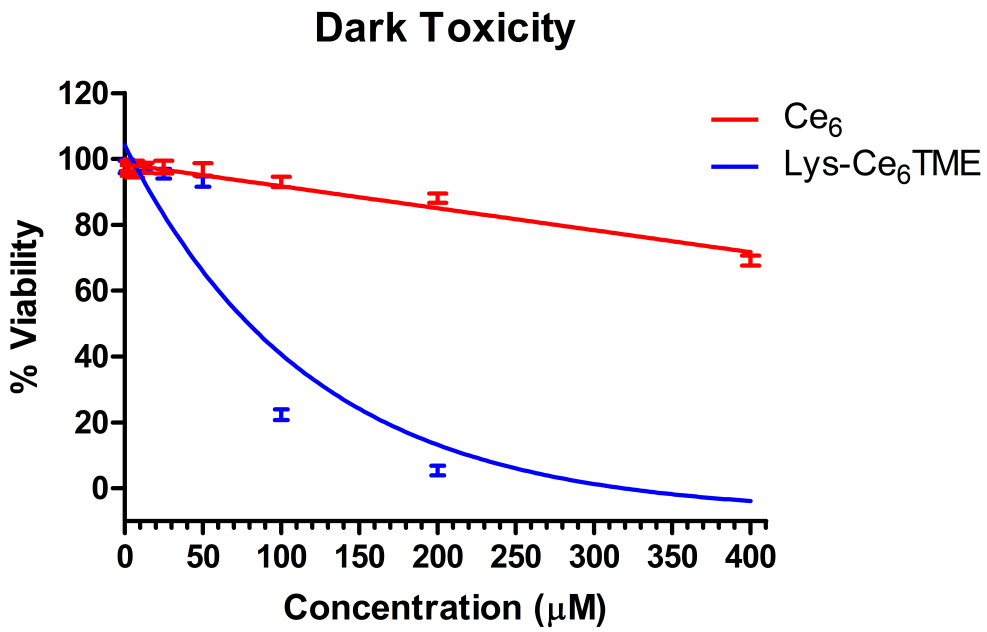


Figure 3.1: Dark Toxicity of Ce_6 (Red) and 13^1 Lys-Ce_6TME (Blue), 0-400 μM

3.3: Phototoxicity

A side by side evaluation of the phototoxicity of chlorin e_6 (3) (Ce_6) and 13^1-mono-lysyl-(OMe) chlorin e_6 trimethyl ester (6) (13^1 Lys-Ce_6TME) was performed in HEp2 cells exposed to increasing concentrations of each compound up to 100 μM. Figures 3.2 and 3.3 both illustrate the phototoxicity investigations of Ce_6 and 13^1 Lys-Ce_6TME, with Figure 3.2 showing the full view of concentrations used in the study and Figure 3.3 focusing on the range of concentrations between 0 and 15 μM. Figure 3.3

gives a much better representation of the differences in phototoxicity between Ce_6 and 13^1 Lys-Ce_6TME.

Overall, chlorin e_6 was found to be weakly phototoxic, with an IC50 value of approximately 30-40 μM (Figure 3.2). This concentration is higher than what would be provided in a clinical setting, making chlorin e_6 a poor photosensitizer. However, the synthesized 13^1-lysyl derivative of chlorin e_6 showed high phototoxicity, with an IC50 value of approximately 1.5 μM (Figure 3.2, 3.3). When comparing results from dark toxicity trials to the results of the phototoxicity trials, one can observe that at concentrations below where 13^1 Lys-Ce_6TME was dark toxic, the molecule worked well as a photosensitizer. For example, at lower concentrations, around 5 μM, 13^1 Lys-Ce_6TME killed approximately 96% of HEp2 cells, with only 3-4% HEp2 viability (Figure 3.2). Conversely, HEp2 cells had 95% viability when exposed to 13^1 Lys-Ce_6TME in the dark toxicity trials at similar concentrations (Figure 3.1).

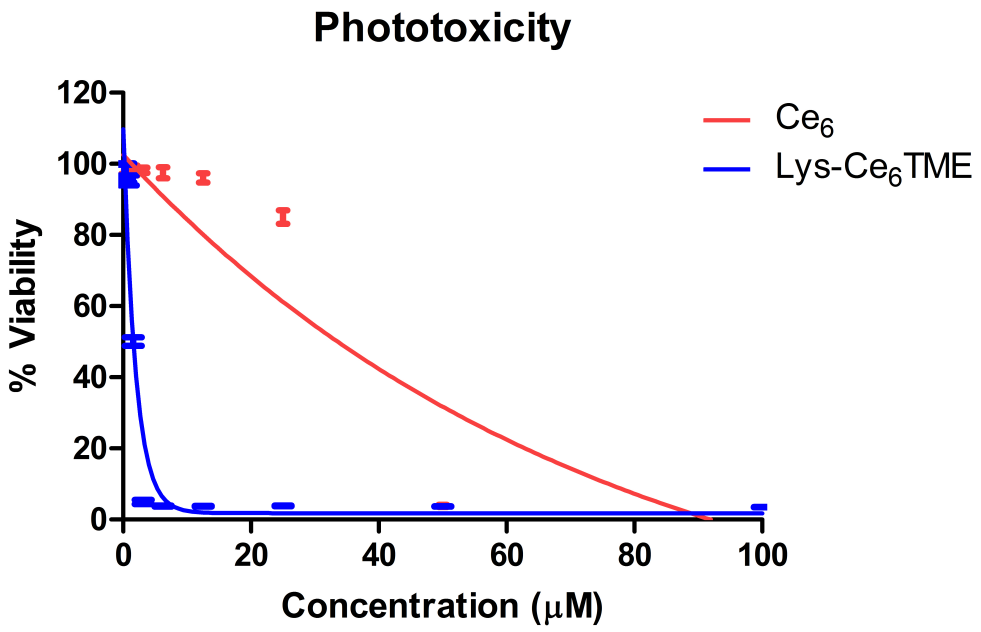


Figure 3.2: Phototoxicity of Ce_6 (Red) and Lys-Ce_6TME (Blue), 0-100 μM

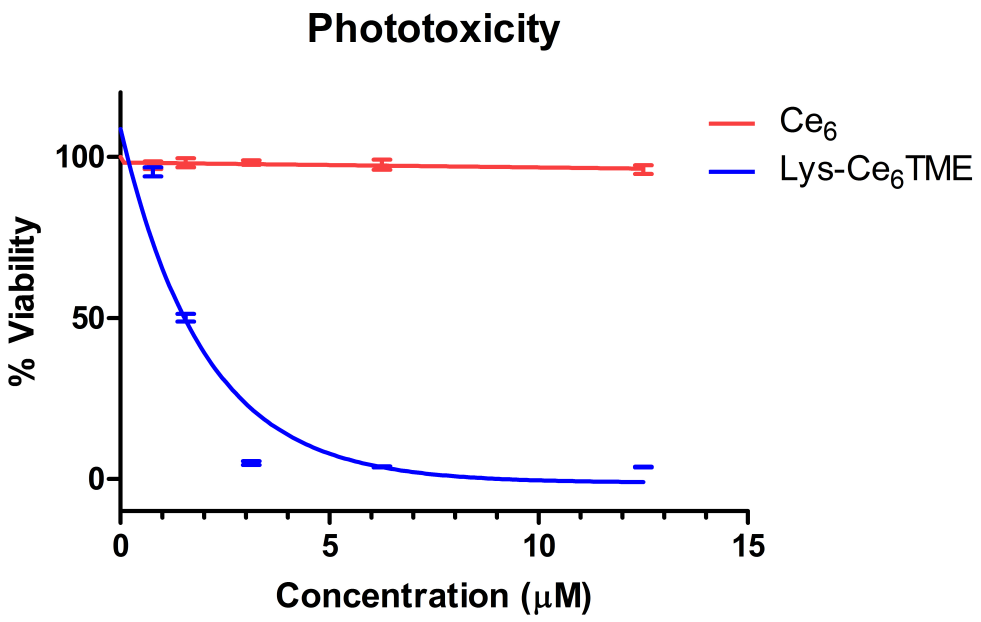


Figure 3.3: Phototoxicity of Ce_6 (Red) and Lys-Ce_6TME (Blue), 0-15 μM

3.4: Cellular Uptake

A side by side evaluation of the time-dependent cellular uptake of chlorin e_6 (3) (Ce_6) and 13^1-mono-lysyl-(OMe) chlorin e_6 trimethyl ester (6) (13^1 Lys-Ce_6TME) was performed in HEp2 cells exposed at concentration of 10 μM for each compound. Figure 3.4 illustrates the results obtained from the uptake trial for both Ce_6 and 13^1 Lys-Ce_6TME, where the HEp2 cells readily took up each compound. The amino acid conjugate of chlorin e_6, 13^1 Lys-Ce_6TME, showed uptake kinetics similar to those of unconjugated chlorin e_6, except to a lower extent than chlorin e_6. In fact, upon observing the results from the trial, Ce_6 was taken into the cells to nearly double the concentration of 13^1 Lys-Ce_6TME. These results are contrary to what we were expecting for a positively charged amino acid such as lysine. However, chlorin e_6 was in the tricarboxylic acid form, while 13^1 Lys-Ce_6TME was a trimethyl ester. Even though

esters are able to pass through lipophilic membranes more easily, they are harder to dissolve in more polar solvents that cells most like, which may be a part of the explanation as to why 13^1 Lys-Ce_6TME was unable to enter the HEp2 cells as efficiently as Ce_6. Additionally, previous studies with lysyl derivatives of chlorin e_6 showed markedly higher accumulation in HEp2 cells when compared to Ce_6.[14] For example, the 15^2-lysylchlorin e_6 derivatives accumulated to a much higher extent, nearly an 18-fold higher cellular uptake in comparison to chlorin e_6, after 24 h.[14] We originally expected stronger interactions between the positively charged lysine derivatives and the negatively charged plasma membrane, which would presumably lead to an enhanced cellular uptake. In the same trial by Jinadasa et al., the 17^3 lysyl derivative showed a dramatic decrease in cellular uptake compared to the 15^2 residue, leading them to believe that the conformation of the compound as a whole contributed to its ability for cellular accumulation.[14] Retrospectively, my own findings lead me to believe that the conformation of the 13^1 Lys-Ce_6TME derivative may play a key role in determining the cellular uptake of the compound. As in the Jinadasa study, linear conformations of the 13^1 derivatives may be causing enhanced π-π stacking of the macrocycles, which would decrease the overall cellular uptake of the compound.

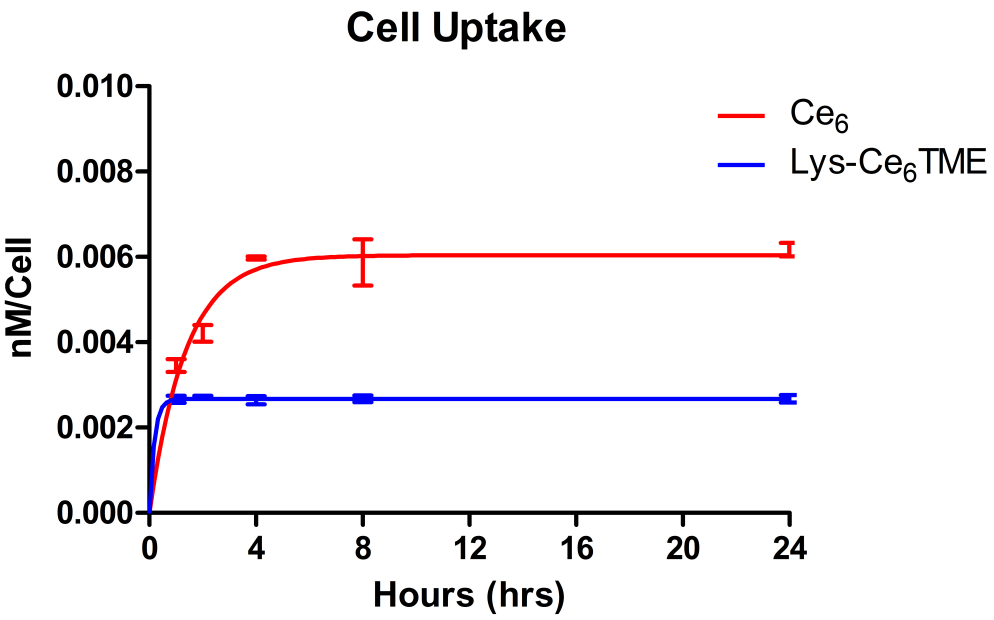


Figure 3.4: Cellular Uptake of Ce_6 (Red) and Lys-Ce_6TME (Blue), 0-24 hours

3.5: Intracellular Localization

A side by side evaluation of the preferential subcellular sites of localization for chlorin e_6 (3) (Ce_6) and 13^1-mono-lysyl-(OMe) chlorin e_6 trimethyl ester (6) (13^1 Lys-Ce_6TME) was performed in HEp2 cells exposed at concentration of 10 μM for each compound for a period of 18 h. Figures 3.5 and 3.6 illustrate the results obtained from the evaluation of fluorescence microscopy for both Ce_6 and 13^1 Lys-Ce_6TME, where the HEp2 cells readily took up each compound and their intracellular localizations can be observed. Also, Table 3.1 summarizes the following results from the intracellular localization trial.

Overlay experiments using the organelle specific fluorescence probes BODIPY Ceramide (Golgi), LysoSensor Green (lysosomes), MitoTracker Green (mitochondria), and ER Tracker Blue/White (ER) were conducted to evaluate the preferential sites of

compound localization, as seen in Figures 3.5 and 3.6. The major subcellular sites of localization for Ce_6 in HEp2 cells was found to be the ER, or endoplasmic reticulum, with the Golgi apparatus and lysosomes being minor sites of localization in comparison. Ce_6 did not seem to be associated with the mitochondria in the subcellular localization trial. The major subcellular sites of localization for 13^1 Lys-Ce_6TME in HEp2 cells were found to be the ER, or endoplasmic reticulum, and the Golgi apparatus. The results for the LysoSensor were not clear, but localization to the lysosome also appears likely. Sensitizer fluorescence in the nucleoli also appears to indicate nuclear localization, and this will be the subject of further studies. 13^1 Lys-Ce_6TME did not seem to be associated heavily with the mitochondria in the subcellular localization trial.

These results were not surprising because the high phototoxicity of 13^1 Lys-Ce_6TME is consistent with the findings of other studies where photodamage to the ER has been shown to lead to activation of apoptotic pathways. [6a, 6c] Additionally, 13^1 Lys-Ce_6TME associating with the mitochondria may have increased the overall photodamage effect. Presumably, localization to multiple organelles will more effectively trigger various apoptotic pathways, which would lead to more efficient cellular destruction. Jinadasa et al. hypothesized that the multiple sites of intracellular localization observed for 13^1-chlorin e_6 derivatives might be due to the linear conformation of the compound.[14] The conformation may help to facilitate their binding to various intracellular sites, despite its adverse effect on cellular uptake.[14]

Table 3.1: Summary of Major (++) and Minor (+) Subcellular Sites of Localization for Ce_6 and Lys-Ce_6TME in HEp2 Cells

Compound	ER	Mitochondria	Golgi	Lysosomes
Chlorin e_6 (Ce_6) (3)	++	—	+	+
13^1-LysChlorin e_6TME (Lys-Ce_6TME) (6)	++	—	++	+

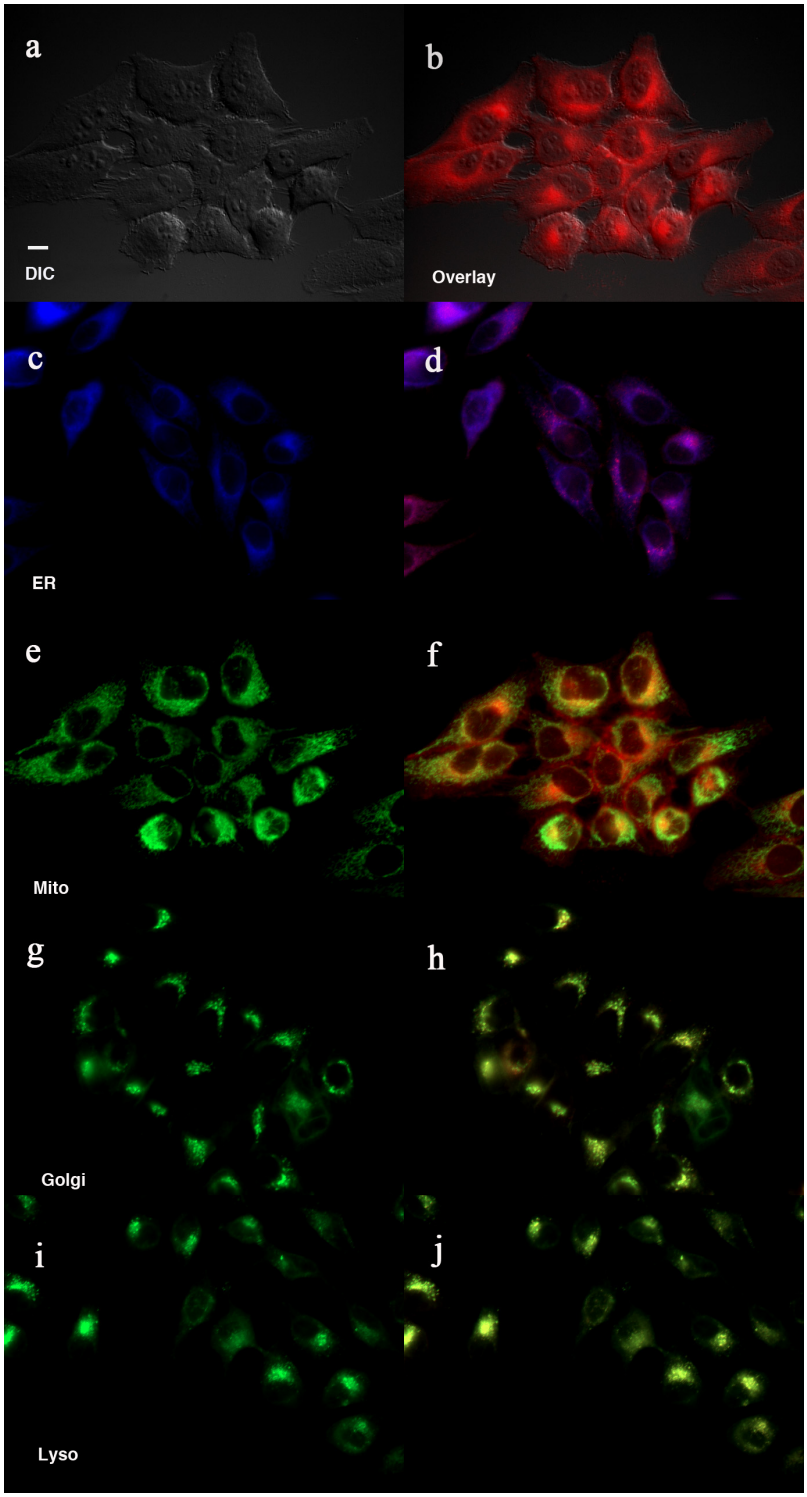


Figure 3.5: Subcellular localization of Ce_6 (3) in HEp-2 cells at 10 μM for 18 h (a) Differential Interference Microscopy (DIC), (b) overlay of Ce_6 and phase contrast, (c) ER Tracker Blue/White fluorescence (e) MitoTracker Green fluorescence, (g) BoDIPY Ceramide, (i) LysoSensor Green fluorescence, and (d,f,h,j) overlays of organelle tracers with compound fluorescence. Scale bar: 10 μm.

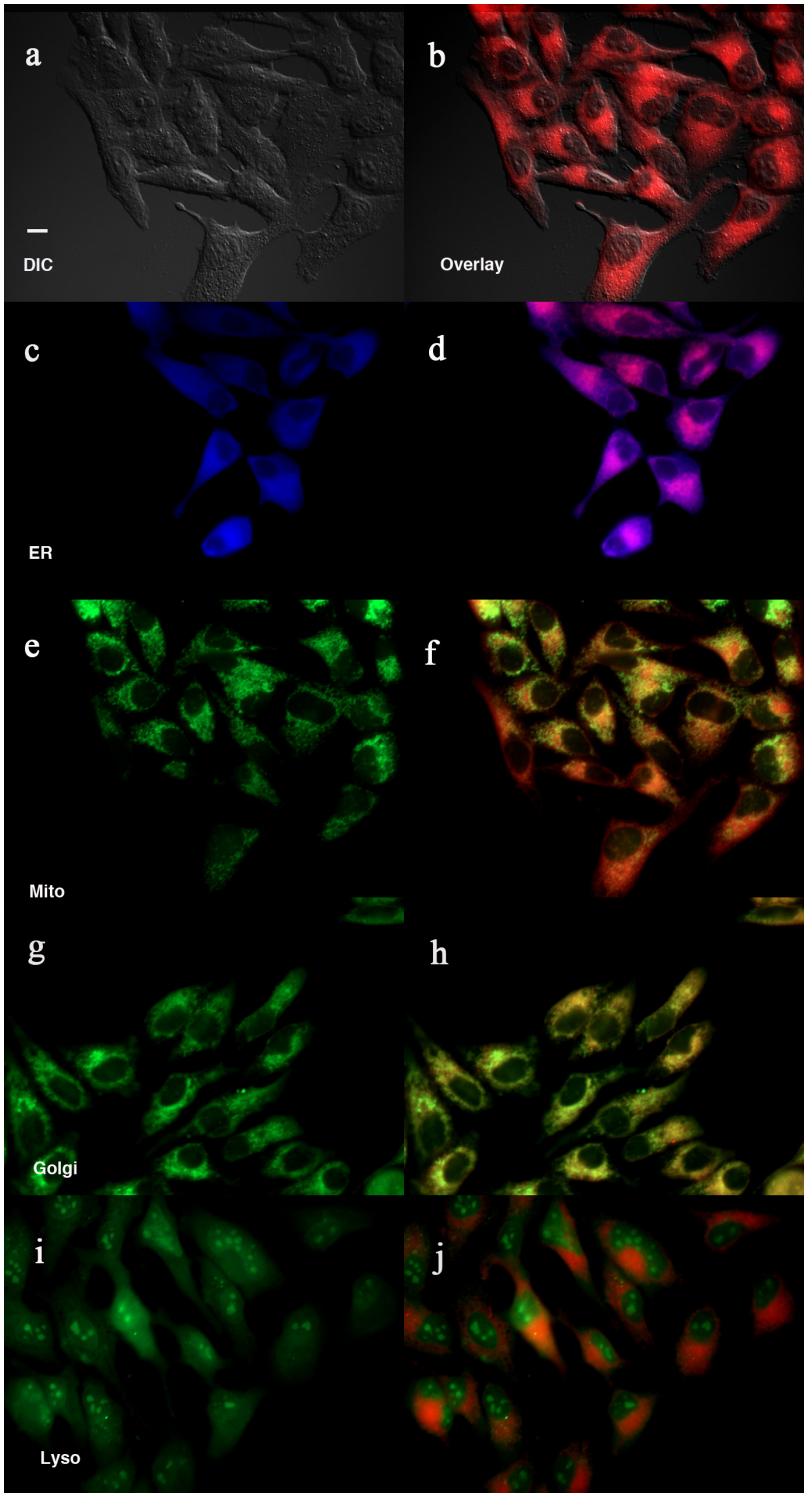


Figure 3.6: Subcellular localization of 13[1] Lys-Ce_6TME (6) in HEp-2 cells at 10 μM for 18 h (a) Differential Interference Microscopy (DIC), (b) overlay of Lys-Ce_6TME and phase contrast, (c) ER Tracker Blue/White fluorescence (e) MitoTracker Green fluorescence, (g) BoDIPY Ceramide, (i) LysoSensor Green fluorescence, and (d,f,h,j) overlays of organelle tracers with compound fluorescence. Scale bar: 10 μm.

3.6: Conclusions

Upon the successful synthesis and characterization of 13^1-mono-lysyl-(OMe) chlorin e_6 trimethyl ester (6) (Lys-Ce_6TME) from chlorin e_6 (3) (Ce_6), cellular evolution studies were completed to observe the possible application of Lys-Ce_6TME as a photosensitizer for photodynamic therapy. While the phototoxicity of Lys-Ce_6TME was found to be high, it is believed that the dark toxicity is holding back the compound from being considered an excellent photosensitizer. For example the ratio of dark toxicity to phototoxicity is approximately 53 for Lys-Ce_6TME and >10 for Ce_6, while other compounds like in the study from Jinadasa et al. boast ratios upwards of 400. These photosensitizers are just as phototoxic as Lys-Ce_6TME, but show little or no dark toxicity.

Another significant property of Lys-Ce_6TME is its conformation as a 13^1 derivative of chlorin e_6. After initial observations in the time-dependent cellular uptake and the subcellular localization trials, the conformation may not be beneficial for cellular uptake, but it may assist intracellularly with localization to many separate organelles, maximizing the potential for cellular destruction.

3.7: Suggestions for Future Work

In future work, cell evolution studies will be completed for the proposed expansion of the project, comparing the effectiveness of many separate 13^1 derivatives of chlorin e_6 for use as photosensitizers in PDT. More specifically, poly-lysyl derivatives of chlorin e_6 such as the 13^1-tetra-lysyl and 13^1-octo-lysyl derivatives, as well as to 13^1-mono-arginyl, and to poly-arginyl derivatives of chlorin e_6 such as the 13^1-tetra-arginyl and 13^1-octo-arginyl derivatives. The photo-cytotoxicity, dark-cytotoxicity, and cellular

uptake evaluations for the derivatives, as well as their intracellular localizations will be investigated. There are also plans to further investigate the observation of fluorescence in the nucleoli by incorporating a nuclear stain into the intracellular localization trials.

3.8: Experimental for Chapter 3

Dark Toxicity: HEp2 cells were placed at 13000 cells per well in Coastar 96 well plates and incubated for 48 h. The compounds were then diluted to 100 µM in the cell medium. Subsequently, two fold serial dilutions were prepared from 100 to 6.25 µM. If DMSO was required to dissolve the compound, a 1% DMSO concentration is maintained throughout the experiment and dilutions by replacing the growth medium with medium + DMSO before making the dilutions. A negative control (100% Viability) was set using cells that are not treated with compound, while a positive control (0% Viability) was set using cells treated with 100 µL of 0.4% saponin. The cells were then incubated overnight (20-24 h), after which the loading medium was removed and cells were fed medium containing 20 µL CellTiter Blue per 100 µL medium, then incubated for 4 h. The conversion of the substrate by viable cells was measured fluorescently at 640 nm.

Phototoxicity: HEp2 cells were placed at 13000 cells per well in Coastar 96 well plates and incubated for 48 h. The compounds were then diluted to 100 µM in the cell medium. Subsequently, two fold serial dilutions were prepared from 100 to 6.25 µM. If DMSO was required to dissolve the compound, a 1% DMSO concentration is maintained throughout the experiment and dilutions by replacing the growth medium with medium + DMSO before making the dilutions. A negative control (100% Viability)

was set using cells that are not treated with compound, while a positive control (0% Viability) was set using cells treated with 100 µL of 0.4% saponin. The cells were then incubated overnight (20-24 h), after which the loading medium was removed and cells were fed medium containing 50 mM HEPES (pH 7.4). The plate was then placed on a metal block in an ice water bath to keep cells cool while being exposed to 610nM LP filtered light from a Halogen lamp for 20 minutes. This corresponds to ≈1 J•cm^{-2}. The IR radiation was filtered using a 10 mm water layer contained in a Petri dish placed on top of the 96 well plate. The loading medium was then removed and cells were fed medium containing 20 µL CellTiter Blue per 100 µL medium and incubated for 4 h. The conversion of the substrate by viable cells was measured fluorescently at 640 nm.

Time-Dependent Cellular Uptake: HEp2 cells were plated at 13000 per well Costar 96 well dish and allowed to grow for 48 h, and the compounds were diluted to a 2x stock in medium. Then, an equal volume of 2x stock is added to triplicate wells containing cells (100 µL) to give final uptake concentration (10 uM), and the compounds were added so that all time points are finished at “0” i.e., 24 h, 8 h, 4 h, 2 h, 1 h, 0 h, all overlapping. The uptake is stopped by removing the loading medium and washing the cells with 200 µL of PBS, after which the cells and compound are then solubilized using 100 µL of 0.25% Triton X-100 in PBS. The compound concentration was read using FLUOstar plate reader using Ex/Em wavelengths (Excitation 355 nm/Emission 650 nm) for the compounds of study. A standard curve is also prepared using the compound in 0.25% Triton X-100 PBS. Cell numbers are determined by adding 100 µL of a 4x stock of CyQuant Cell proliferation assay reagent prepared in PBS by diluting 10 µL CyQuant per mL PBS. When added to the cells, this gives a 2x [CyQuant], which increases the

working range of the assay. A standard curve for cells must also be prepared using 10000, 20000, 40000, 60000, 80000, 100000, and 125000 cells.

Subcellular Localization and Microscopy: HEp2 cells were plated on Lab Tek II two chamber coverslips by trypsinizing a plate of cells and resuspending in 5 mL. Subsequently, 2 drops of cell suspension are added per chamber, and the cells are allowed to grow for 24-48 h. The compounds were then added at 10 μM and incubated overnight, and the organelle tracers were added concurrently with compound for the remainder of the incubation period: BODIPY Ceramide: 50 nM for 30 minutes, ER Tracker Green: 2 μM for 60 minutes, LysoSensor Green: 50nM for 30 minutes, Mito Tracker Green FM: 250nM for 30 minutes, and Hoechst 1 μg/mL for 5 minutes (for nuclei). The loading medium was then removed and the cells were washed 3 times with medium containing 50 mM HEPES (pH 7.4). The distribution of compound was determined using a Zeiss AxioVert 200M inverted fluorescent microscope fitted with standard Texas Red, FITC, DAPI and Cy5LP filter sets.

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