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ARTICLE

# Photobiological characteristics of chlorophyll a derivatives as microbial PDT agents

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Chlorin-e6 (**chl-e6)** and a hydrogenated derivative (**chl-e6H)** were semi-synthesized, and their photophysical properties and photodynamic activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* evaluated. Methyl pheophorbide-a (**Mepheo-a**) was obtained from *S. maxima* using methanolic extraction with acid catalysis ($CH_3OH/H_2SO_4$). Chlorin-e6 was prepared from **Mepheo-a** by basic hydrolysis with $H_2O$/acetone and NaOH. Hydrogenated Chlorin-e6 was synthesized by a similar procedure starting from the hydrogenated methyl pheophorbide-a (**Mepheo-aH**). Photophysical studies were performed in order to determine the singlet oxygen quantum yield of **chl-e6H** which is higher than **chl-e6**. The microorganism inactivation of **chl-e6** and **chl-e6H** was investigated at two concentrations and three fluence levels. Both **chl-e6** and **chl-e6H** showed microorganism inactivation against Gram-positive bacteria and a fungus.

## Introduction

Photodynamic therapy (PDT) is a combination of a photosensitizer agent, light and molecular oxygen to cause cell death. During the irradiation, the photosensitizer present in the target area is excited, and induces a local toxicity, that can cause oxidative cellular damage.[1] Cell death can be induced through two types of mechanism: type I, when a photo-induced electron transfer from the excited photosensitizer (PS) to biomolecules is observed, producing active free radicals; and type II, when the triplet oxygen is excited and results in the production of the highly reactive singlet oxygen species.[2]

Some photosensitizers (PSs) have been clinically approved and applied for the local treatment of diseases such as cancers and infected lesions. The increasing appearance of resistant microorganism strains makes the development of new techniques for microbial control extremely important, and PDT is one of the most promising techniques for these treatments.[3,4,5,6,7,8,9] Although distinct PSs have been reported against microorganisms there are several challenges for widespread use of PDT, and the development of more appropriate PSs with a better photodynamic efficiency is necessary.[10,11]

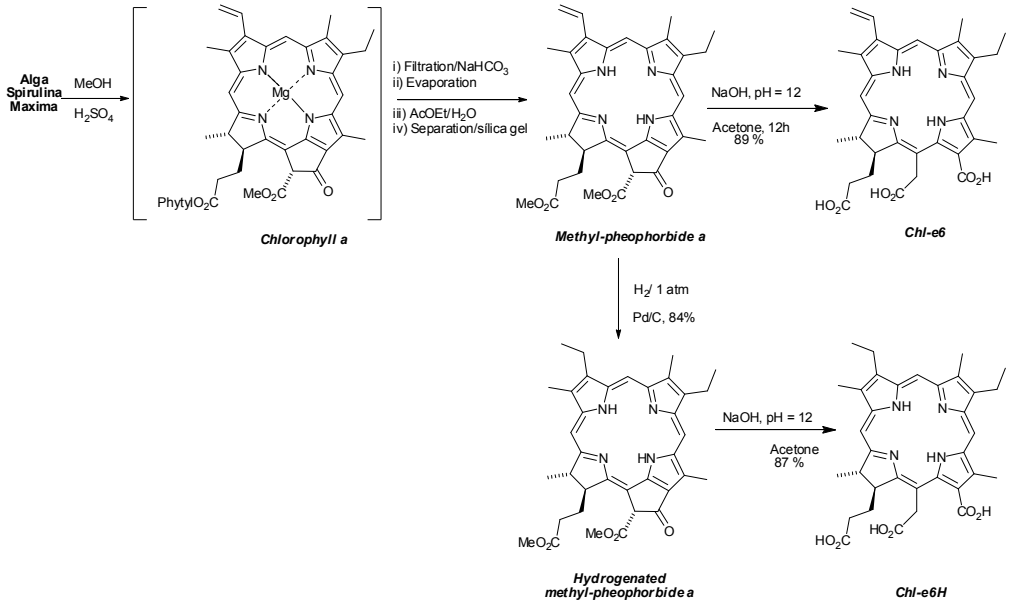
The chemistry of porphyrins, chlorins and their derivatives has been extensively studied since the discovery of their potential application as PSs in photodynamic therapy.[12,13,14] Some PSs can be easily prepared by semi-synthesis using abundant natural starting materials, such as the chlorophylls. The use of natural pigments is well recommended, and one of the main reasons is their lower cost when compared to the synthetic PSs.[15] Natural chlorophylls and derivatives are biosynthetically related to protoporphyrin IX present in higher organisms. Consequently, the biocompatibility of natural related PSs is expected, mainly in terms of pharmacokinetic clearance. Chlorophylls can also be used as starting materials for the synthesis of functionalized chlorins, since some pharmacophoric groups can be introduced to improve the photodynamic activity. Chlorophylls constitute useful templates for use as PSs considering their photophysical and photobiological proprieties, and their high level of functionalization that permits chemical transformations.[16] In general, chlorophylls derivatives are good candidates for photodynamic inactivation, because they have a better selectivity in microorganisms compared to mammalian cell, especially under low incubation times. [4,17,18]

In this study, we synthesized a **chl-e6** and a hydrogenated derivative **chl-e6H** from chlorophyll *a*,[19] and compare their photophysical properties, and their photodynamic efficiencies against bacteria and a fungus.

## Results and discussion

### Semi-synthesis

For our studies, chlorin-e6 (**chl-e6)** and the hydrogenated derivative (**chl-e6H)** were used as substrates. First, methyl pheophorbide-*a* (**Mepheo-a**) was obtained from *S. maxima* in 0.8% yield using methanolic extraction with acid catalysis ($CH_3OH/H_2SO_4$) and purification. The hydrogenated methyl pheophorbide-*a* (**Mepheo-aH**) was prepared from (**Mepheo-a**) by hydrogenation with a palladium catalyst (Pd/C 10%) in 84% yield. The chlorin-e6 (**chl-e6**) and hydrogenated derivative (**chl-e6H**) were obtained after basic hydrolysis with NaOH in water and acetone (Scheme 1).




**Scheme 1:** Synthesis of **chl-e6** and **chl-e6H**.

The products were characterized by $^1$H-NMR, HRMS and UV-Vis spectra, as described in the experimental section and supporting information (Figures S1-S12).

### UV-Vis spectra

The compounds **chl-e6** and **chl-e6H** showed typical UV-Vis absorption spectra for chlorins, with a main band around 350-450 nm (Soret), and Q-bands around 600-670 nm (Figure 1). When excited at 405 nm the chlorins **chl-e6** and **chl-e6H** showed fluorescence emission at 670 and 655 nm, respectively (Figure 1).

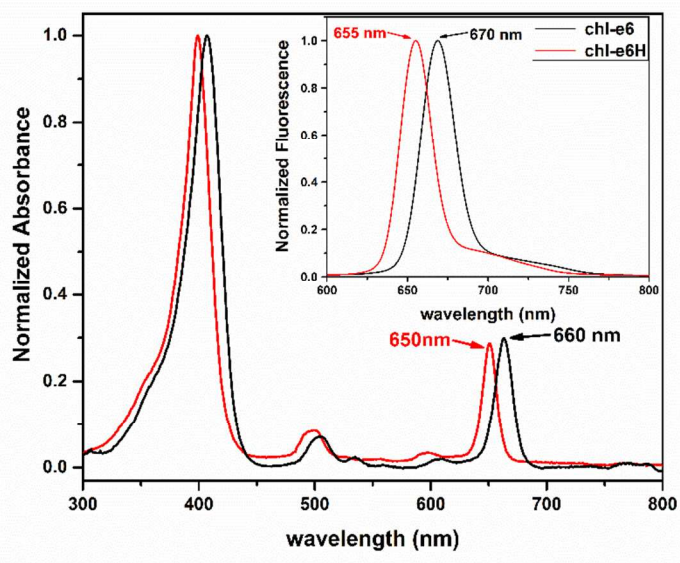



**Figure 1:** Normalized absorption spectra for compounds **chl-e6** and **chl-e6H** in DMSO. Insert: Normalized fluorescence emission spectra in DMSO with excitation at 405 nm for compounds **chl-e6** and **chl-e6H**.

The absorbance spectra of **chl-e6** and **chl-e6H** in ethyl acetate are showed in the supporting information (Figures S11-S12).

### Singlet oxygen quantum yields

The singlet oxygen quantum yield ($\Phi_\Delta$) measurements were carried out using the experimental procedure described in the literature, wherein the singlet oxygen quantum yield is determined relatively to a standard photosensitizer with a well-known value of $\Phi_\Delta$ in a proper solvent.[6,20,21,22] Here, the $\Phi_\Delta$ values for **chl-e6** and **chl-e6H** were determined in ethanol using methylene blue (**MB**) as standard photosensitizer and DPBF as chemical quencher. Equation (1) was used for the calculations of the $\Phi_\Delta$ values:

$$\Phi_\Delta = \Phi_\Delta^{Std} \frac{k}{k^{Std}} \frac{I_{abs}^{Std}}{I_{abs}} \qquad (1)$$

Where $\Phi_\Delta^{Std}$ is the singlet oxygen quantum yield for the standard (0.49 for methylene blue in ethanol)[20]. $k$ and $k^{Std}$ are the DPBF photobleaching rate constants in the presence of the respective sample and $I_a$ and $I_a^{Std}$ are the rates of photon absorption at the irradiation wavelength of 660 nm for the samples and standard, respectively. The ratio can be obtained from equation 2. [22]

$$\frac{I_a^{Std}}{I_\alpha} = \frac{1-10^{-A_{660}^{std}}}{1-10^{-A_{660}}} \qquad (2)$$

Where $A_{660}^{std}$ and $A_{660}$are respectively, the absorption of the standard (methylene blue) and sample at 660 nm.

The decrease of DPBF absorbance for which first order kinetics were observed upon irradiation (irradiated for 5 second periods) was monitored using a UV-Vis spectrophotometer (Figures S13-S15). The decrease of DPBF absorbance, for which the first order kinetics was observed, is shown in Figure 2.

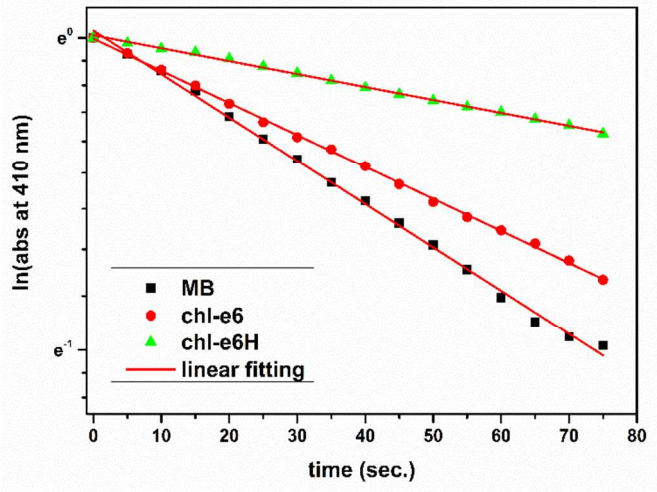



**Figure 2**: Absorbance of DPBF at 410 nm as a function of irradiation time for **MB**, **chl-e6H** and **chl-e6H**.

The measured values of $\Phi_\Delta$ for **chl-e6** and **chl-e6H** were $\Phi_\Delta$= 0.64 and 0.79, respectively. The singlet oxygen quantum yield for **chl-e6** corroborates with the literature.[20] The singlet oxygen quantum yield of **chl-e6H** was higher than for **chl-e6**, probably because the **chl-e6H** was reduced at the very reactive vinylic group. Actually, the **chl-e6H** was designed for this purpose, aiming for the decrease of photobleaching.

**Photobleaching studies**

Photobleaching is the degradation of fluorescent molecules of PS after irradiation, caused by interaction with oxidative species. This is an important parameter to be determined, because stability under light irradiation represents a fundamental characteristic for a good PS. [23] All the protocols described here were performed in ethyl acetate. **Chl-e6** and **chl-e6H** present high stability under light exposure, showing an absorbance decrease at 400 nm and 395 nm of 0.004 and 0.003, respectively, after 10 irradiations periods of 1 min, at 63.7 mW/cm$^2$ (Figures S16 and S17). For irradiance at 191 mW/cm$^2$ and 6 irradiance periods of 3 min, photobleaching was observed, with absorbance decreases of 0.13 and 0.05 for **chl-e6** and **chl-e6H**, respectively (Figures S18 and S19). The photodegradation comparison for the two chlorins is presented in Figure 3, where it is evident that **chl-e6** showed a higher photodegradation than **chl-e6H** under the same irradiation conditions. Using these comparative photobleaching analyses, **chl-e6H** shows a higher stability than **chl-e6**.

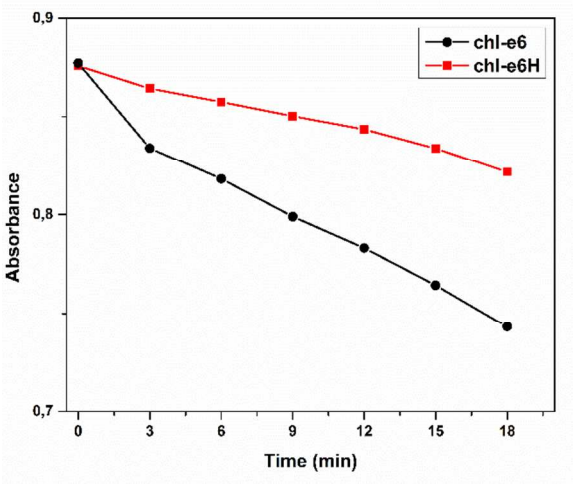



**Figure 3:** Photodegradation of chlorins **chl-e6** and **chl-e6H**.

The data demonstrate that the chemical modification performed in **chl-e6** resulted in a remarkable improvement in terms of its potential as a PDT photosensitizer.

Photobleaching is sometimes used as an indirect measurement of singlet oxygen generation, however this strategy can yield mistakes since different porphyrinoids may present different reactivities with singlet oxygen. In our case, we have performed a strategic hydrogenation on of the exocyclic double bond of **chl-e6**, in order to obtain a lower photodegradation in **chl-e6H**. It important to mention that the exocyclic double bond in **chl-e6** is more reactive than the other conjugated double bonds in the chlorin core structure.

**Photodynamic inactivation of microorganisms**

Our main objective here was to compare the effective PDT activity of **chl-e6** and **chl-e6H** against pathogenic microorganisms. For this purpose, a Gram-positive (*Staphylococcus aureus*) and a Gram-negative (*Escherichia coli)* bacteria, and a fungus (*Candida albicans)* were selected. The PDT activity was investigated under different PS concentrations and fluence. As usually performed, the chlorins were incubated in the microorganism culture for 20 min and then irradiated.

*Staphylococcus aureus* was selected due to its high prevalence in wounds and in hospital infections.[24,25,26] In Figure 4, the results of two concentrations (1 and 10 µg/mL) of both photosensitizers (**chl-e6** and **chl-e6H**) and three fluences at 660 nm (15, 30 and 45 J/cm$^2$) are shown. At the concentration of 1 and 10 µg/mL under 15 J/cm$^2$, a reduction of 5-logs was achieved with both PSs. At 1 and 10 µg/mL in 30 and 45 J/cm$^2$ both chlorins **chl-e6** and **chl-e6H** showed complete inactivation of *Staphylococcus aureus* (Figure 4).

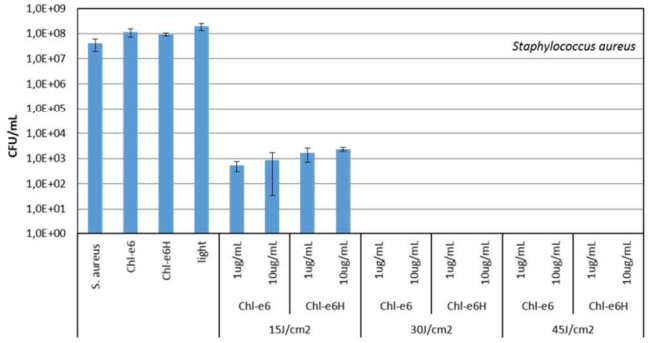



**Figure 4**: PDT effect of **chl-e6** and **chl-e6H** against *Staphylococcus aureus*. At the groups with none bar, 30 or less colony forming units per millilitre were counted.

The Gram-negative bacteria *E. coli* is pathogenic to humans and other animals. Due to the previous reported higher resistance of Gram-negative species [27, 28] the PS concentration and light fluences were increased, when compared to the Gram-positive *S. aureus*. Figure 5 shows the results for *E. coli*, where even higher concentrations of both PSs and higher fluences were not effective for an effective inactivation. A PS concentration of 400 and 500 µg/mL and a fluence of 60 J/cm$^2$ only presented a slight microorganism reduction, and **chl-e6H** was more active for these bacteria at these conditions (Figure 5).

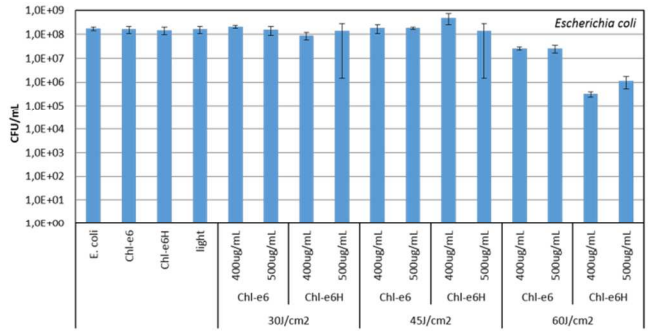



**Figure 5**: PDT effect of **chl-e6** and **chl-e6H** against *Escherichia coli*.

In previous studies with porphyrinoid photosensitizers, Gram-positive bacteria were inhibited after irradiations, but the activity against Gram-negative bacteria was limited.[29, 30, 31] In this study, we have obtained a low activity of **chl-e6** and **chl-e6H** against a Gram-negative microorganism, and higher activity in a Gram-positive microorganism.

Previous studies show that is easier to inactivate Gram-positive than Gram-negative bacteria, which can be explained by the electronic charges of the cell walls. Gram-positive bacteria present a cell wall where the photosensitizers (neutral, anionic and cationic) can easily diffuse. Whereas Gram-negative bacteria have lipopolysaccharides present in their cell membrane that allows the diffusion of hydrophilic dissolved particles.[32] Gram-negative bacteria are relatively impermeable to neutral or anionic photosensitizers due to their highly negatively charged surface.[33] However, the cationic chlorins appear to be quite effective for such microorganisms.[34,35,36]

The PDT inactivation experiments with *Candida albicans* were also performed to evaluate the effect of these PSs in a fungus cell, as it has a different cell wall when compared to bacteria.[37] We evaluated the PDT activity of both PSs at 20 and 30 µg/mL under the fluences of 15, 30 and 45 J/cm$^2$.

The results for *Candida albicans* are shown in Figure 6. The best results were observed at 20 and 30 µg/mL for both **chl-e6** and **chl-e6H**, using 30 and 45 J/cm$^2$, showing a complete microorganism inhibition. For a PS concentration of 20 and 30 µg/mL at 15J/cm$^2$, a 3-log reduction was achieved. Previous studies has reported the inactivation of *Candida albicans* with PDT using Photogem,[37] which presents the same complete inhibition with 50 µg/mL and fluence of 18 J/cm$^2$.

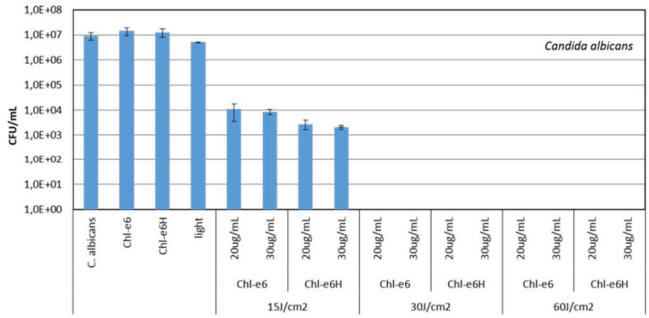



**Figure 6:** PDT effect of **chl-e6** and **chl-e6H** against *Candida albicans.* No bar corresponds to the groups that did not show a value correspond to the CFU counted less than 30 colonies per millilitre.

Both chlorins are not completely soluble in water, so they were initially dissolved in DMSO, and the final PS concentration was obtained with further water dilution. For *C. albicans* and *Staphylococcus aureus* the final DMSO concentration is lower than 1% and for *E. coli*. is lower than 2.5%. However, no significant dark toxicity was observed for all PS concentrations tested. In addition, no microorganism reduction was achieved with the cultures treated only with light.

Recent articles report that DMSO has an effect on the cell membrane of microorganisms causing their progressive thinning, followed by pore formation and loss of the membrane integrity.[38,39,40] These membrane alterations improve the penetration of PSs in the microorganism cell.

**Photosensitizer uptake**

The uptake of the photosensitizer in the microorganisms was observed through fluorescence confocal microscopy images. For *C. albicans* (Figure 7) and for *S. aureus* (Figure 8) no qualitatively difference was observed between **chl-e6** and **chl-e6H.** For *E. coli* (Figure 9), **chl-e6H** appears to show more interaction, and this may explain the higher inactivation activity observed at Figure 5. The **chl-e6H** probably has a better interaction with this microorganism.

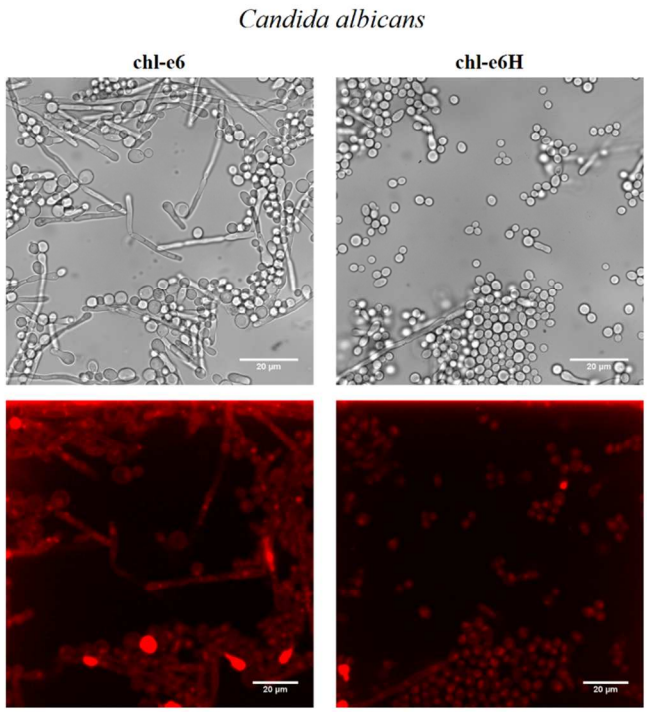



**Figure 7:** Optical microscopy of *Candida albicans* culture. *Upper:* Brightfield image; *Lower*: Fluorescence image of the cells treated with **chl-6** *(left)* **chl-e6H** *(right)*. Scale Bar: 20µm.

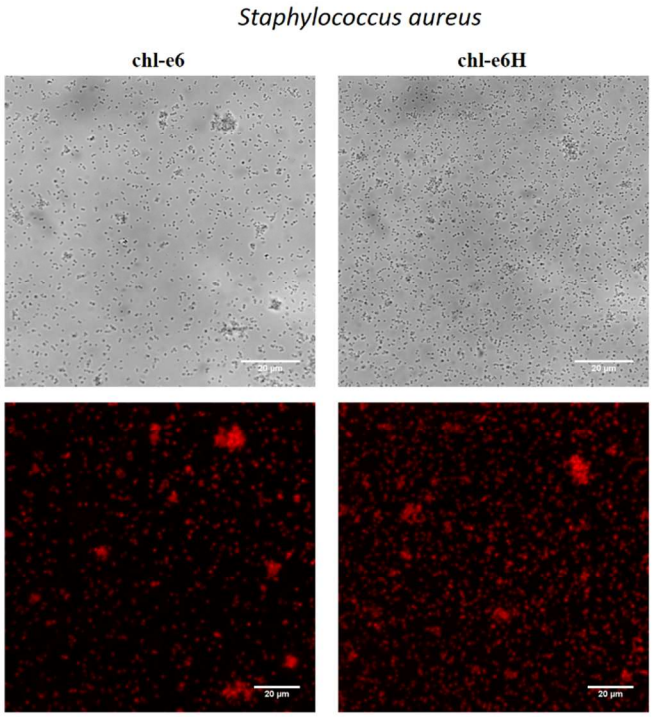



**Figure 8:** Optical microscopy of *Staphylococcus aureus* culture. *Upper:* Brightfield image; *Lower*: Fluorescence image of the cells treated with **chl-6** *(left)* **chl-e6H** *(right)*. Scale Bar: 20µm.

In general, photosensitizers have different behaviours in Gram-negative bacteria when compared to Gram-positive bacteria and fungus. The interaction between PSs and microorganisms plays an important role at the final response, and this aspect needs to be further investigated to understand the mechanism of PDT. For example, results of a cationic galactoporphyrin against *Pseudomonas sp*. (Gram-negative bacteria) was shown by Almeida and Cunha's groups. These cationic porphyrins were the most efficient PSs against *Pseudomonas sp*. with reduction of 5-6 log numbers, after 15 min of irradiation.[41] This group also showed that the number of positive charges in the porphyrins structure resulted in different effects on the photoinactivation of Gram-positive and Gram-negative bacteria. For example Tri-Py$^{+}$-Me-PF (tri-cationic porphyrin) reduced 99.999% of microorganisms.[42] Previous results also showed that the cationic nanomagnet porphyrin hybrids can be considered highly efficient for the photoinactivation of Gram-negative bacteria (*E. coli*). [43]

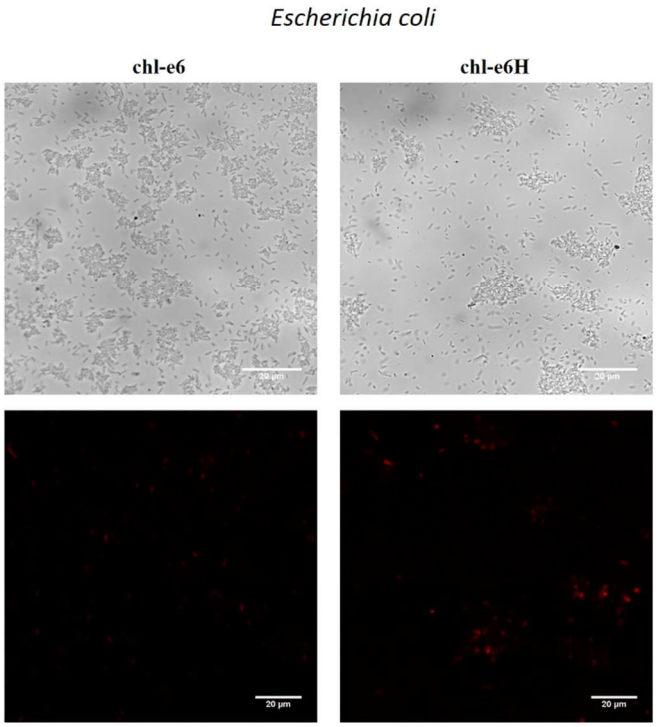



**Figure 9:** Optical microscopy of *against Escherichia coli* culture. *Upper:* Brightfield image; *Lower*: Fluorescence image of the cells treated with **chl-6** *(left)* **chl-e6H** *(right)*. Scale Bar: 20µm.

## Experimental

All reagents and starting materials were purchased from commercial sources, and used as received or purified when necessary. The NMR analyses were performed on a Bruker Avance 400 spectrometer at 400.15 MHz and 100.13 MHz for $^{1}H$ and $^{13}C$ respectively. The chemical shifts are expressed in parts per million (ppm) and the coupling constants (*J*) in Hertz (Hz).Tetramethylsilane was used as internal reference.

Flash chromatography was carried out using silica gel (230-400 mesh). UV-Vis analyses were performed using a double beam spectrometer LAMBDA 25 (Perkin Elmer) (Figures S11-S14).The Laser source for photobleaching measurements was a diode laser at 660 nm (FTC 500, OPTO, São Carlos, Brazil) in the PDT/iPDT mode. *Spirulina maxima alga* was purchased as powder from Pharma Nostra (Rio de Janeiro, Brazil).

The laser used for the singlet oxygen quantum yield measurements was a diode laser emitting at 660 nm; Eagle Heron® (Quantum Tech, São Carlos, São Paulo, Brazil), and the UV−Vis equipment for these specific measurements was a CARY 50 UV-VIS spectrophotometer (Varian, Germany). For confocal images an Zeiss confocal microscope (LSM780) was used. HRMS were obtained using the ESI-TOF (Waters Xevo G2-S QTof).

### Experimental procedures

**Isolation of methyl pheophorbide-a from *Spirulina maxima alga* (Mepheo-a):** Dried *Spirulina maxima* alga (300 g) was treated with a 5% methanolic solution of $H_2SO_4$ (1.5 L) for 48 h at room temperature, and with protection from light. This mixture was filtered and the filtrate washed with methanol (900 mL) and ethyl acetate (900 mL). The organic phases were combined and evaporated under reduced pressure. The crude residue was dissolved in 200g of crushed ice, and additionally refrigerated at 0°C.The residue was slowly neutralized with solid sodium bicarbonate, and placed on a silica gel plug. All the chlorophylls derivatives were retained on the plug, and the residual proteins and peptides were eluted with water until the disappearance of the pale yellow color. The green-pigments were eluted with ethyl acetate (900 mL) and the organic phase was additionally washed with water (3 x500mL), dried over sodium sulfate and filtered. After solvent evaporation under reduced pressure, the methyl pheophorbide-*a* was purified by silica gel flash chromatography using toluene:ethyl acetate (9:1) as eluent. Recrystallization from dichloromethane and methanol yielded methyl pheophorbide-*a* (**Mepheo-a**) (2.4 g, 3.9 mmols, 0.8% from natural alga) [44] **UV–Vis** (dichloromethane): λmax (nm): 666, 609, 534, 505, 409. **$^{1}$H-NMR** ($CDCl_3$, 400 MHz) δ: 9.45 (s, 1H), 9.29 (s, 1H), 8.55 (s, 1H), 7.93 (dd, *J*= 11.6; *J* = 17.8 Hz, 1H), 6.26 (s, 1H), 6.24 (dd, *J* = 1.5 and *J* = 17.8 Hz, 1H ), 6.15 (dd, *J* =1.5; *J* = 17.8 Hz, 1H), 4.48-4.43 (m, 1H), 4.21-4.19 (m, 1H), 3.88 (s, 3H), 3.67 (s, 3H), 3.60 (q, *J* = 7.7 Hz, 2H), 3.58 (s, 3H), 3.37 (s, 3H), 3.16 (s, 3H), 2.68 – 2.48 (m, 2H), 2.36-2.20 (m, 2H), 1.81 (d, *J* = 7.3 Hz, 3H), 1.66 (t, *J* = 7.6 Hz, 3H), 0.51 (br. s, 1H), -1.67 (br. s, 1H). **$^{13}$C-NMR** ($CDCl_3$, 100 MHz) δ : 192.0, 174.6, 172.4, 169.4, 162.8, 155.8, 151.0, 150.0, 145.3, 142.2, 137.9, 136.7, 136.3, 136.2, 131.9, 129.0, 128.9, 127.8, 122.8, 104.5, 104.4, 97.5, 93.3, 65.9, 65.0, 62.2, 52.1, 51.7, 50.2, 30.7, 30.0, 23.1, 19.3, 17.3, 12.0, 11.1.

**Synthesis of hydrogenated methyl pheophorbide-a (Mepheo-aH):** Methyl pheophorbide-a (250 mg, 0.41 mmol) was dissolved in ethyl acetate (40 mL), and hydrogenated with 1 atm. of $H_2$ (the flask was equipped with a balloon filled with hydrogen) over Palladium/Carbon 10% catalyst (25 mg) for 2.5 h at room temperature. The catalyst was filtered off on celite and the solution was concentrated. The product was crystallized with dichloromethane and methanol. The **Mepheo-aH** was obtained in 84% yield (209 mg, 0.34 mmol).**UV–Vis** (dichloromethane): λmax (nm): 655, 599, 529, 500, 405. **$^{1}$H-NMR** ($CDCl_3$, 400 MHz) δ: 9.48 (s, 1H), 9.20 (s, 1H), 8.46 (s, 1H), 6.23 (s, 1H), 4.45-4.39 (m, 1H), 4.18-4.16 (m, 1H), 3.87 (s, 3H), 3.81 (q, *J* = 7.6 Hz , 2H), 3.67 (s, 3H), 3.69 (q, *J* = 7.6 Hz, 2H), 3.57 (s, 3H), 3.28 (s, 3H), 3.24 (s, 3H), 2.64 – 2.46 (m, 2H), 2.34-2.18 (m, 2H), 1.81 (d, *J* = 7.3 Hz, 3H), 1.73 (t, *J* = 7.6 Hz, 3H), 1.69 (t, *J* = 7.6 Hz, 3H), 0.70 (br. s, 1H), -1.54 (br. s, 1H). **$^{13}$C-NMR** ($CDCl_3$, 100 MHz) δ : 189.6, 173.3, 172.5, 169.7, 160.8, 155.7, 150.7, 149.7, 145.2, 142.9, 141.9, 137.6, 137.3, 135.9, 131.5, 128.7, 128.6, 105.0, 104.5, 96.3, 92.5, 64.4, 52.8, 51.6, 50.9, 50.2, 31.0, 29.8, 22.9, 19.4, 19.3,17.3 16.8, 12.1, 11.2, 10.9.

**Synthesis of chlorin-e6 and hydrogenated chlorin-e6:** The methyl pheophorbide-a (**Mepheo-a**), or hydrogenated methyl pheophorbide-a (**Mepheo-aH**) (100 mg, 0.167 mmol), were dissolved in degassed acetone (20 mL) under nitrogen. A degassed aqueous NaOH solution (1 mol.$L^{-1}$) was added until pH=12 was reached, and the reaction mixture was stirred under nitrogen for 12 h, yielding a solid product. This solid was washed with acetone to remove the remaining pheophorbide-*a*, and the product was dissolved in water for acidification with citric acid (to pH=3). The solid product was filtered and crystallized from dichloromethane and methanol. The chlorin-e6 (**chl-e6**) or hydrogenated chlorin-e6 (**chl-e6H**) were obtained in 89% (0.088 mg, 0.147 mmol) and 87% (0.086 mg, 0.143 mmol) yields respectively.

**Chl-e6:** $C_{34}H_{36}N_4O_6$ **UV–Vis** (dichloromethane): λmax, nm (log □): 665 (4.55), 610 (3.55), 532 (3.65), 501 (3.85), 404 (4.91). **$^{1}$H-NMR** [$(CD_3)_2CO$, 400 MHz]: δ: 9.84 (s, 1H), 9.68 (s, 1H), 9.08 (s, 1H), 8.20 (dd, *J* = 18.0 and 11.6 Hz, 1H), 6.42 (dd, *J* = 18.0 and 1.5 Hz, 1H), 6.14 (dd, *J* = 11.6 and 1.6 Hz, 1H), 5.60 (d, *J* = 18.9 Hz, 1H), 5.45 (d, *J* = 18.9 Hz, 1H), 4.68 (q, *J* = 7.0 Hz, 1H), 4.60 (dd, *J* = 10.7 and 2.4 Hz, 1H), 3.82 (q, *J* = 7.6 Hz, 2H), 3.65 (s, 3H), 3.52 (s, 3H), 3.29 (s, 3H), 2.80-2.72 (m, 2H), 2.43-2.29 (m, 2H),1.76 (d, *J* = 7.0 Hz, 3H), 1.71 (t, *J* = 7.6 Hz, 3H), -1.35 (br, s, 1H),-1.56 (br, s, 1H). **HRMS (ESI-TOF):** m/z calculated [M - H]$^-$ for **chl-e6** 595.2557; found 595.2561. Anal Calcd for $C_{34}H_{36}N_4O_6.H_2O$: C 66.43; H 6.23; N 9.11. Found C 66.29; H 6.04; N 8.79.

**Chl-e6H:** $C_{34}H_{38}N_4O_6$ **UV–Vis** (dichloromethane): λmax, nm (log □): 652 (4.58), 598 (3.74), 526 (3.70), 497 (4.04), 399 (5.10). **$^{1}$H-NMR** [$(CD_3)_2CO$, 400 MHz]: δ: 9.81 (s, 1H), 9.54 (s, 1H), 8.96 (s, 1H), 5.57 (d, *J* = 18.0 Hz,1H), 5.45 (d, *J* = 18.9 Hz, 1H), 4.64 (q, *J* = 7.0 Hz, 1H), 4.58 (dd, *J* = 10.3 and 2.4 Hz, 1H), 3.93 (q, *J* = 7.6 Hz, 2H), 3.84 - 3.78 (m, 2H), 3.64 (s, 3H), 3.40 (s, 3H), 3.29 (s, 3H), 2.78-2.70 (m, 2H), 2.41-2.27 (m, 2H), 1.76-1.69 (m, 9H), -1.34 (br, s, 1H), - 1.46 (br, s, 1H). **HRMS (ESI-TOF):** m/z calculated [M - H]$^-$ for **chl-e6H** 597.2713; found 597.2737

**Singlet oxygen quantum yield measurement:** Singlet oxygen quantum yield measurements were carried out using the chemical trapping method. [6,20,21,22] Typically, a 2 mL portion of the respective PS solution, that contained diphenylisobenzofuran (DPBF), was irradiated at 660 nm. The $\Phi_\Delta$ values were obtained using the relative method with methylene blue as reference (standard), since this

molecule has an absorption band around 660 nm. To avoid chain reactions induced by DPBF in the presence of singlet oxygen, the concentration of DPBF was 50 μM in ethanol (absorbance ~ 2.0 at 410 nm). The photosensitizer stock solutions (**chl-e6** and **chl-e6H**) were prepared in ethanol (typically with absorbance ~ 0.40 at 410 nm). After the mixture of 2 mL of both solutions (DFBF and a PS), the quartz cuvette was irradiated with a 660 nm laser for 5 second periods. This procedure was performed in triplicate and the DPBF degradation was monitored by UV−Vis absorption spectra.

**Photobleaching studies:** Stock solutions of **chl-e6** and **chl-e6H** in ethyl acetate with an absorbance value around 0.8-0.9 were prepared. Two irradiation levels were used (661 nm); 63.7 mW/cm$^2$ for 10 min, and 1 min intervals; and 191 mW for 18 min, with 3 min intervals (the fiber was positioned at the wall of the cuvette). The absorbance spectra were monitored after each irradiation interval. [45, 46] These protocols were performed in triplicate.

***In vitro* experiment:** *Staphylococcus aureus* (American Type Culture Collection – ATCC 25923) and *Escherichia coli* (ATCC 25922) were grown in brain and heart infusion medium. *Candida albicans* (ATCC 10231) was grown in Sabouraud dextrose broth. For experimental purposes, the microorganism concentration was adjusted to $10^7$-$10^8$ cells/mL in sterile distilled water. 500 μL of each microorganism culture was added to a 24-wells plate with the PS. The PSs used in this study were **chl-e6** and **chl-e6H**. Solutions were prepared by dilution of PS powder (1mg for *Staphylococcus aureus* and *Candida albicans* or 4 mg for *Escherichia coli*) in 200 μL of DMSO (to dissolve the chlorins) and 900 μL of sterile water, and protected from light. Two concentrations of each PS were evaluated for each microorganism. The plates were kept in the dark at 37°C for 20 min.

A homemade LED equipment with emission centered at 660 nm was used to irradiate the culture plates. The irradiation was performed for 8, 16, 24 or 32 minutes, resulting in fluence levels of 15, 30, 45 or 60 J/cm$^2$, respectively. After the irradiation, 10-fold serial dilution was performed and cultured in agar plates. Colony forming units (CFUs) were determined 24 h after the experimental procedure. All experiments were performed three times.

The control group (no treatment) plates were maintained at room temperature for 32 minutes. The PS dark toxicity was evaluated using the same incubation time and with the plates covered by aluminum foil to avoid light exposure. The phototoxicity was determined with the irradiation of the cell plate with 45 J/cm$^2$ for *S. aureus* and *C. albicans* and 60 J/cm$^2$ for *E.coli*. Survival fractions (SF) were expressed as ratios of CFU of treated groups over the control group. The SF at 0 J/cm$^2$ gives a measure of the dark toxicity of the chlorins. It was counted colony-forming units between 30 and 300 units.

**Photosensitizer uptake:** Microorganisms were centrifuged for 7 minutes at 4000 rpm and then they were re-suspended and incubated with the sensitizer solution (**chl-e6** or **chl-e6H**) at a concentration of 150 μg/mL for 20 minutes at 37 °C. After this procedure the microorganisms were re-suspended in sterile distilled water, placed in a glass coverslip and imaged. This concentration of PS was chosen to obtain a high signal of fluorescence. Afterward, the images were acquired with a confocal microscope (LSM780 – Carl Zeiss, Germany) using a diode laser emitting at 405 nm for excitation. The fluorescence signal was collected in one channel from 630 to 670 nm for chlorins fluorescence (red color).

## Conclusions

In this study, we present the semi-synthesis, photophysical and photobiological properties of **chl-e6** and **chl-e6H**, as PDT sensitizers. As **chl-e6** presents a very reactive vinyl double bond, which may be reacting with the singlet oxygen formed during treatment with photodynamic therapy and decreasing the efficiency of the photosensitizer. We propose to make a hydrogenation (**chl-e6H**) of this double bond to make it more efficient.

Both compounds demonstrated efficiency against microbial cultures, completely inhibiting bacteria and fungus growth. From the photophysical point of view, we observed that the chemical modification proposed here was effective, since **chl-e6H** presented a higher singlet oxygen quantum yield and lower photobleaching. The photodynamic activity of **chl-e6** and **chl-e6H** in *Staphylococcus aureus* was almost the same. The hydrogenated derivative **chl-e6H** was more active than **chl-e6** for *Escherichia coli* and for *Candida albicans.* The **chl-e6H** seems to have more interaction with *E. coli*, resulting in a higher photoinactivation. Thus, the development of synthetic strategies is still needed to improve the efficiency for Gram-negative bacteria inactivation.

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## Notes and References

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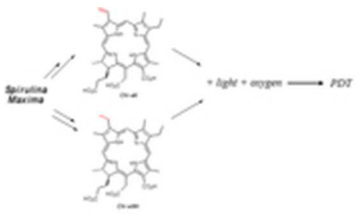


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