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Synthesis and Biological Evaluation of Methoxyphenyl Porphyrin Derivatives as Potential Photodynamic Agents

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Abstract—A new *meso*-2,4,6-trimethoxyphenyl porphyrin covalently linked to a 2′,6′-dinitro-4′-trifluoromethylphenyl group by an amine bond **5** and its metal complex with Cd(II) **6** was prepared. The photodynamic activities of **5** and **6** were evaluated in vitro on Hep-2 cells. A considerable increase in the photocytotoxic effect was found for **6**, which has higher singlet molecular oxygen, $O_2(^1\Delta_g)$, production.

Introduction

The tetrapyrrolic macrocycles play highly diverse roles in biological systems.[1] One of more recent and promising applications of porphyrins in medicine is in the detection and cure of tumors.[2,3] Photodynamic therapy (PDT) is based on the principle that a photosensitizer become concentrated in tumor cells, upon subsequent irradiation with visible light in the presence of oxygen, specifically inactive neoplastic cells.[4,5]

Basically, two types of reactions can occur after photoactivation of the photosensitizer. One involves the generation of free radicals (type I photochemical reaction) and the other, the production of singlet molecular oxygen, $O_2(^1\Delta_g)$ (type II), is the main species responsible for cell inactivation.[5] Evidence favors the role of the type II process in cells, although the photodynamic process of the sensitizers on neoplastic tissues is still not well understood.[6]

Recently, several porphyrin derivatives covalently linked to actives molecules have been synthesized with a potential use in the treatment of tumors.[7–11] Studies of triplet state and in biological media carried out with a series of 5,10,15,20-tetrakis (methoxyphenyl) porphyrins have shown that these synthetic porphyrins are effective photosensitizers which can be used as model compounds to investigate the theoretical and instrumental aspects of PDT.[12–14]

This paper reports a convenient procedure for the synthesis of a new porphyrin dyad **5**, which contain three *meso*-2,4,6-trimethoxyphenyl groups on the porphyrin ring. The macrocycle is covalently attached to a 2′,6′-dinitro-4′-trifluorophenyl group by an amine bond. The synthesis of this type of compound requires access to asymmetrically substituted porphyrins.[11,15] Thus, porphyrins bearing two different types of *meso*-substituents can be prepared by a binary mixed aldehyde condensation.[16] This approach is statistical in nature and usually a multiple porphyrin products are obtained. Usually, six porphyrins are formed; the workup is not simple because of the tar present and the yields are very poor.[15] More direct approaches to porphyrins bearing three identical *meso*-phenyl substituents and one different are provided by condensation of dipyrromethane with aldehyde.[15,17] By application of this method, 5-(4-acetaminophenyl)-10,15,20-tris(2,4,6-trimethoxyphenyl) porphyrin (**3**) was obtained, which was hydrolyzed to give amino porphyrin **4**. Dyad **5** was obtained by means of a chloro-substitution reaction under two-phase catalysis conditions, reacting amino porphyrin **4** and 1-chloro-2,6-dinitro-4-trifluoromethylbencene. In this heterogeneous system, the presence of potassium hydroxide activates the amino group on the porphyrin **4** to aromatic nucleophilic substitution reactions.[18] This method provides a general procedure for the synthesis of porphyrin dyads bearing an amine linkage.

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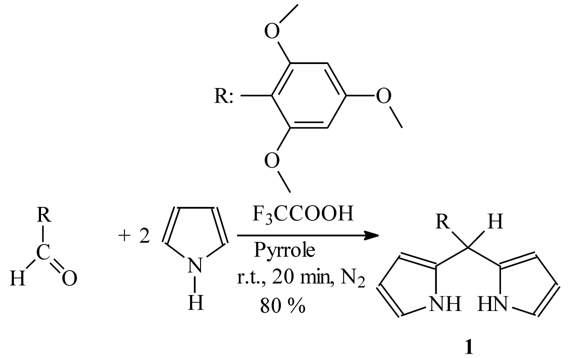
On the other hand, the spectroscopic properties and quantum yield of singlet molecular oxygen, $O_2(^1\Delta_g)$, production (Φ_Δ) of porphyrins can be largely changed by forming complexes with metals.[19–22] In particular, the metal complex with Cd(II) produces a considerable increase in Φ_Δ and a high absorption coefficient in the visible region of the spectrum, mainly in the phototherapeutic window (600–1000 nm). Taking into account these properties, the Φ_Δ value of dyad **5** was considerably increased by forming its metal complex with Cd(II) to form dyad **6**. Absorption and emission spectroscopic properties of the dyads were determined in pure solvent. The mediation of $O_2(^1\Delta_g)$ in the photooxidation mechanism was analyzed in tetrahydrofuran-bearing photo-oxidizable substrate. In the biological system, the response of Hep-2 human larynx-carcinoma cell line to cytotoxicity photo-induced by the porphyrins was evaluated. The survival of the irradiated cells, previously treated with the porphyrin, was dependent on both light dose and sensitizer photochemical properties. The effect shows a correlation between the $O_2(^1\Delta_g)$ production and the photodynamic activity upon Hep-2 cells. Therefore, the intermediacy of $O_2(^1\Delta_g)$ appears to be mainly responsible for the photocytotoxic activities of these porphyrins. The results contribute to understanding the photodynamic process yielded by these agents and the sensitivity of Hep-2 cells to this photodamage.

Results and Discussion

Synthesis

The 5-(4-aminophenyl)-10,15,20-tris(2,4,6-trimethoxyphenyl)porphyrin (**4**) was synthesized from dipyrromethane derivate and the appropriate aldehydes (Scheme 2).

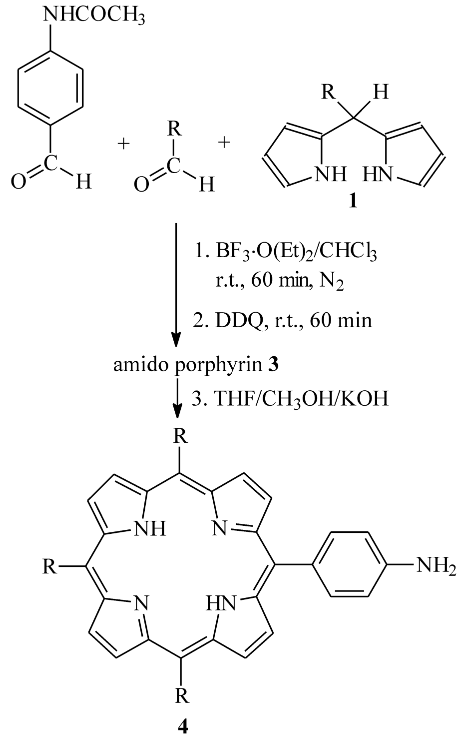
Dipyrromethane formation. Aldehyde and pyrrole undergo acid-catalyzed condensation at room temperature.[15,17,23] The condensation of 2,4,6-trimethoxybenzaldehyde with a large excess of pyrrole (1:45 aldehyde/pyrrole mol ratio) catalyzed by trifluoroacetic acid affords *meso*-2,4,6-trimethoxyphenyldipyrromethane (**1**) (Scheme 1). The reaction mixture was stirred for 20 min at room temperature resulting in complete disappearance of aldehyde. In this reaction condition, pyrrole serves as the reactant in excess and as the solvent for the reaction, giving direct formation of dipyrromethane (Scheme 1). The brown crude solution was washed with dilute aqueous NaOH. The dipyrromethane **1** was isolated yielded 80% by flash chromatography on silica gel in a mildly basic medium, using *n*-hexane/ethyl acetate/triethylamine (80/20/1) as eluent. Triethylamine (≅1%) was added to prevent decomposition of the dipyrromethane on silica column, which is slightly acidic.[15,17] Dipyrromethane **1** is stable in the purified form upon storage at 0 °C in nitrogen atmosphere and absence of light. Performing high purity dipyrromethane **1** is essential for its application in the synthesis of substituted porphyrin.



Scheme 1.

Porphyrin formation. Scheme 2 shows the condensation of dipyrromethane **1** with 2,4,6-trimethoxybenzaldehyde and 4-acetamidobenzaldehyde to form porphyrins. The reaction was performed using catalytic among of $BF_3O(C_2H_5)_2$ and chloroform as solvent at room temperature. The reaction mixture was subject to oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). Two porphyrins were easily separated by flash chromatography, the first less polar moving band corresponding to *meso*-tetrakis(2,4,6-trimethoxyphenyl) porphyrin (**2**) and the second 5-(4-acetamidophenyl)-10,15,20-tris(2,4,6-trimethoxyphenyl) porphyrin (**3**). The desired amide porphyrin **3** was obtained in 16% yield. Finally, basic hydrolysis of amide porphyrin **3** with aqueous KOH in mixture THF/methanol (2:1) affords 78% of 5-(4-aminophenyl)-10,15,20-tris-(2,4,6-trimethoxyphenyl) porphyrin (**4**).

Porphyrin dyad. Coupling of amine porphyrin **4** and 1-chloro-2,6-dinitro-4-trifluorometilbenzene by amine



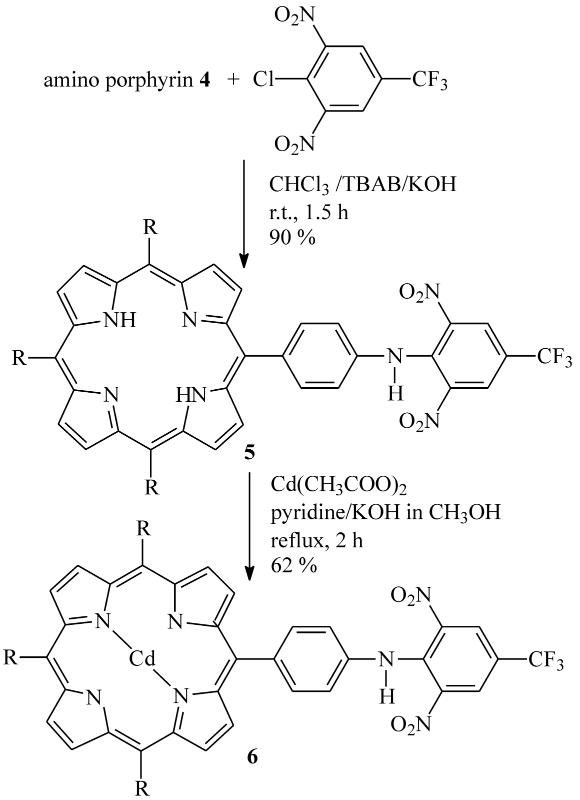
Scheme 2.

bond allows formation of new dyads **5**, as shown in Scheme 3. To synthesize the dyad **5**, the nucleophylic aromatic substitution reaction was performed under two phase catalytic conditions, using tetrabutylammonium bromide (TBAB) as catalyst. When the reactants were mixed in homogeneous media (chloroform) without potassium hydroxide, no reaction was observed after 2 h of stirring at room temperature. However, when the solid potassium hydroxide was added, the reaction takes place, and after 1.5 h 90% of the dyad **5** is formed. The basic medium activates the $-NH_2$ group to nucleophilic reaction with chloro aromatic substrates.[18]

Finally, dyad **5** was treated with cadmium acetate in basic medium to afford metal complex Cd(II), dyad **6**, in 62% of yield (Scheme 3).

Spectroscopic studies and singlet molecular oxygen production

The absorption spectrum of porphyrin **2**, dyads **5** and **6**, in dichloromethane are summarized in Table 1. These porphyrins show the typical Soret and *Q*-bands characteristic of free-base and Cd(II) metalloporphyrins (Table 1).[21] The relative intensity of the *Q*-bands for **2** and **5**, show an *etio*-type spectrum ($\epsilon_{VI} > \epsilon_{III} > \epsilon_{II} > \epsilon_{I}$).[11,24] Also, as can be observed in Table 1, dyad **6** presents a value of ϵ higher than **2** and **5** at $\lambda > 600$ nm.



Scheme 3.

The steady-state fluorescence emission spectra of these porphyrins present two maxima, as shown in Table 1. The same values of λ_{em} were obtained exciting the sample at the wavelength of maximum absorption of the Soret and the *Q*-bands. The two bands are characteristic for similar porphyrins and they have been assigned to *Q*(0-0) and *Q*(0-1) transitions.[19] A small Stokes shift is expected for tetraphenylporphyrin derivatives indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state.[19] The fluorescence quantum yields (ϕ_F) of the porphyrins were calculated by steady state comparative method using TPP as a reference.[25,26] The values of $\phi_F = 0.053 \pm 0.002$, 0.032 ± 0.002 and 0.011 ± 0.001 were obtained in tetrahydrofuran for porphyrin **2**, dyads **5** and **6**, respectively. These results are consistent with the obtained for similar metalloporphyrins.[19]

The aerobic irradiation with monochromatic light ($\lambda = 515$ nm) of photosensitizer in tetrahydrofuran was performed in the presence of 9,10-dimethylanthracene (DMA). This substrate quenches $O_2(^1\Delta_g)$ by exclusively chemical reaction.[27] A time-dependent decrease in the DMA concentration was observed by following a decrease in its absorbance. The photo-process follows first-order kinetics with respect to DMA concentration. Figure 1 shows the semi-logarithmic plots describing the progress of the reaction for DMA, using different photosensitizers. From these plots the values of the observed rate constant (k_{obs}) were calculate for DMA. The results of k_{obs} were $(9.34 \pm 0.05) \times 10^{-5}$, $(7.35 \pm 0.04) \times 10^{-5}$, $(3.62 \pm 0.04) \times 10^{-5}$ and $(7.68 \pm 0.05) \times 10^{-5}$ for TPP, porphyrin **2**, dyad **5** and **6**, respectively. The quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) was calculated from the slopes of the plots for the porphyrin compared with the corresponding slope obtained for the reference (TPP). The values of Φ_Δ in tetrahydrofuran were 0.49, 0.24 and 0.51 for **2**, **5** and **6**, respectively. As can be expected, a considerable increase in Φ_Δ was obtained by forming a metal complex of dyad **5** with Cd(II).[19,20]

Studies in vitro on Hep-2 cells

Since these porphyrins are water-insoluble, they were added to the cell cultures from a liposomal solution of L,D-α-dipalmitoyl phosphatidylethanolamina bearing 20% mols of cholesterol. The use of phospholipid lipo-

Table 1. Absorption and fluorescence data for porphyrin **2** and dyads **5** and **6**

Porphyrin	Absorption λ_{max} (nm) (ε [$M^{-1}cm^{-1}$])[a]					Fluorescence λ_{max}[b]	
2	418 (179,490)	512 (10,190)	545 (3350)	592 (3150)	646 (940)	650	719
Dyad **5**	420 (216,950)	515 (14,270)	550 (5770)	592 (5110)	650 (2470)	652	719
CdDyad **6**	435 (226,320)	570 (12,920)	610 (6620)			624	668

[a]Dichloromethane.
[b]Tetrahydrofuran.

somes as vehicles to transport hydrophobic photosensitizers could constitute an advantage for its uses in PDT, since liposomes optimizes the release of lipophilic sensitizer to LDL.[28,29]

Cell toxicity induced by the photosensitizers was first analyzed in dark condition. The Hep-2 cells were treated with different concentration of the sensitizer for 24 h at 37 °C. The result are shown in Figure 2. No toxicity in terms of cell survival (measured by microscopy in the presence of TB) was detected at 0.1 and 1 μM of dyad **5** and **6**, while similar cytotoxicity was found incubating the cells with 10 μM of these dyads. However, porphyrin **2** was significantly toxic at every evaluated concentration (Fig. 2). Therefore, dyad **5** and **6** where selected for the photodynamic studies in biological medium. After the combined treatment of cells with 1 μM of the dyad for 24 h, the culture plates were irradiated with visible light. The irradiation system used in these studies is described in experimental section. The corresponding survival curve for each photosensitizer is shown in Figure 3. As expected, Hep-2 cell inactivation depends on the light dose employed. After an irradiation of ~50 J/cm^2, the survival fractions were 0.52 and 0.16 for cell line treated with dyad **5** and its Cd(II) complex **6**, respectively.

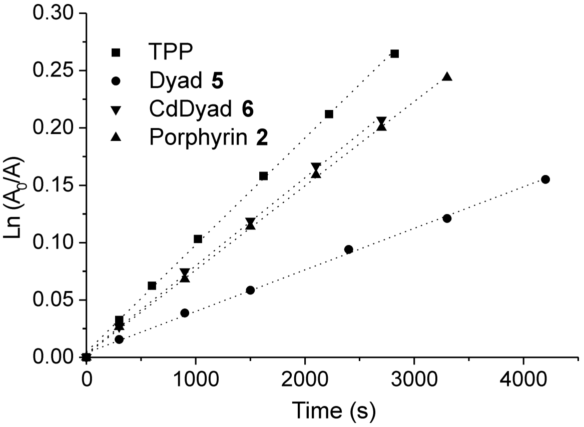


Figure 1. First-order plots for the photooxidation of DMA (45 μM) photosensitized by TPP (■), dyad **5** (●), Cddyad **6** (▼) and porphyrin **2** (▲) in tetrahydrofuran.

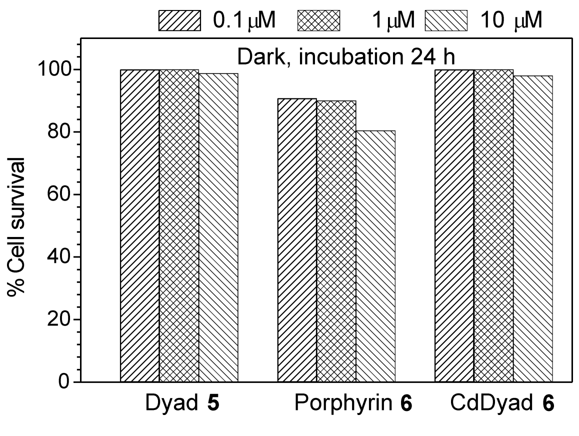


Figure 2. Cytotoxic effect of the photosensitizers on Hep-2 cells incubated with different concentration of porphyrin for 24 h at 37 °C under dark. Values represent mean ± standard deviation of three separate experiments.

On the other hand, when the cell cultures do not treated with the sensitizer were irradiated with visible light, no significant lethality was found (Fig. 3, control). Therefore, the cell mortality obtained after irradiation of the cultures treated with the porphyrin is due to the photosensitization effect of the agent produced by visible light. Also, the dyad uptake for the Hep-2 cell was analyzed by fluorescence. A similar value of ~0.003 μmol/10^6 cells was found for these agents.

The results of cytotoxicity (Fig. 3) show higher cell mortality with an increase of the O$_2$($^1\Delta_g$) production by the porphyrins. From the cell inactivation curves, the irradiation time required to inactivate 50% of cell population (t_{50}) were obtained (48 and 12 min for **5** and **6**, respectively). Taking into account the light intensity at the treatment site (see experimental section), the corresponding light dose (D$_{50}$) were calculated given values of 52 and 13 J/cm^2. Under these conditions, the cytotoxic effect considerably is increases with dyad **6**, correlating with the sensitizer production of O$_2$($^1\Delta_g$).

In conclusion, the following two basic steps were used sequentially in the synthesis: (1) *meso*-(2,4,6-trimethoxyphenyl) dipyrromethane was formed from correspondent benzaldehyde derivative and pyrrole catalyzed by acid; (2) condensation of dipyrromethane with appropriate benzaldehydes yields the desired asymmetric porphyrins **3**, with an appreciable yields of 16%. The resultant amido porphyrins **3** can be hydrolyzed to amine porphyrin **4** by heat in tetrahydrofuran/methanol/KOH medium. This macrocycle was covalently attached to a phenyl group bearing electron-withdrawing –NO$_2$ and –CF$_3$ groups by amine bond. Two-phase catalysis conditions allow performing the amine linkage with high yield due to the nucleophilic activation of the –NH$_2$ group. The presence of methoxy groups appears to be beneficial from the standpoint of tumor localization.[30] For this reason, extra methoxy

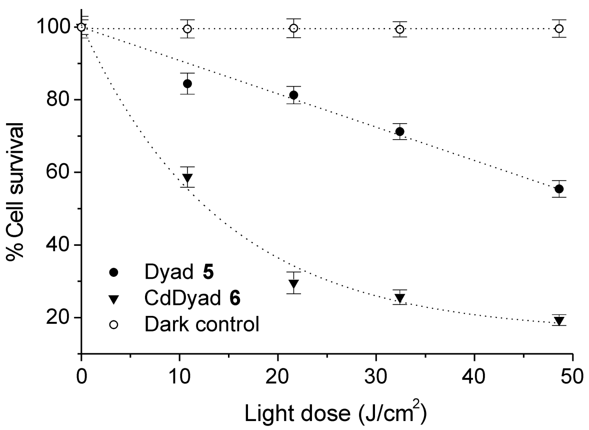


Figure 3. Inactivation of the Hep-2 cells incubated with 1 μM of dyad for 24 h and exposed to different light doses with visible light; (●) dyad **5**; (▼) Cddyad **6**; (○) corresponding controls in dark conditions. Values represent meanstandard deviation of three separate experiments.

groups were included in the porphyrin moiety of this dyad. On the other hand, a lipophilic trifluoromethyl group gives an amphiphilic character of this agent. The influence of the trifluoromethyl group in biologically active molecules is often associated with the increased lipophilicity that this substituent imparts.[31] This present strategy may be easily used for preparation of other similar dyad derivatives. The applications of these ways of reactions to the synthesis of potential phototherapeutic agents could be of considerable interest for PDT. In biological medium, the behavior of these photosensitizers indicated that the photodynamic effect correlates with the $O_2(^1\Delta_g)$ production, and therefore, these dyads appear to perform its photocytotoxic activities on Hep-2 cells through a mechanism which involves mainly intermediacy of $O_2(^1\Delta_g)$.

Experimental

General

UV–visible and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. ^{1}HNMR spectra was recorded on a Varian Gemini spectrometer at 300 MHz. Mass spectra were taken with a Varian Matt 312 operating in EI mode at 70 eV and a Vestec Laser Tec Research Instrument by laser desorption time-of-flight mass spectroscopy. Uniplate Silica gel GHLF 250 microns thin layer chromatography plates from Analtech and silica gel 200–400 Mesh for column chromatography from Aldrich were used.

All the chemicals from Aldrich were used without further purification. D,L-α-Dipalmitoyl phosphatidylethanolamine from Sigma was used in liposome preparation. Solvents from Merck (GR grade) were distilled and storage over 4 Å molecule sieves and sodium bicarbonate. Tetrahydrofuran was distilled from lithium aluminum hydride under an argon atmosphere.

Synthesis

***meso*-(2,4,6-Trimethoxylphenyl)dipyrrometane (1).** A solution of 2,4,6-trimethoxybenzaldehyde (1.76 g, 9 mmol) and pyrrole (25.0 mL, 360 mmol) was degassed by bubbling with nitrogen for 15 min. Then trifluoroacetic acid (139 μL, 1.8 mmol) was added. The solution was stirred for 20 min at room temperature, at which point no starting aldehyde was shown by TLC analysis (cyclohexane/ethyl acetate/triethylamine 80:20:1). The mixture was diluted with dichloromethane (100 mL), washed with aqueous 0.1 M NaOH (50 mL) and then washed with water. The organic phase was dried with $MgSO_4$, filtered and the solvent was removed under reduced pressure. The unreacted pyrrole was removed by vacuum distillation at room temperature. The product was purified by flash chromatography (silica gel, cyclohexane/ethyl acetate/triethylamine 80:20:1) yielded 2.25 g (80%) of the pure dipyrrometane 1. TLC (silica gel) R_f (cyclohexane/ethyl acetate/triethylamine 80:20:1) = 0.25. ^{1}H NMR ($CDCl_3$, TMS) δ (ppm) 3.73 (s, 6H, Ar–OCH_3), 3.81 (s, 3H, Ar–OCH_3), 5.91 (m, 2H, pyrrole-H), 6.07 (s, 1H, meso-H), 6.10 (q, 2H, pyrrole-H), 6.20 (s, 2H, Ar 3,5-H), 6.63 (m, 2H, pyrrole-H); 8.47 (s, brs, 2H, pyrrole NH). MS (*m*/*z*) 312 (M^+) (312.1475 calcd for $C_{18}H_{20}N_2O_3$). Anal. calcd C 69.21, H 6.45, N 8.97; found C 69.52, H 6.28, N 8.80.

5-(4-Aminophenyl)-10,15,20-tris(2,4,6-trimethoxylphenyl) porphyrin (4). A solution of 2,4,6-trimethoxylbenzaldehyde (0.57 g, 2.91 mmol), 4-acetamidobenzaldehyde (0.66 g, 4.07 mmol) and *meso*-(2,4,6-trimethoxylphenyl)dipyrrometane **1** (2.00 g, 4.76 mmol) in 500 mL of chloroform was purged with nitrogen for 15 min. Then $BF_3O(C_2H_5)_2$ (2.0 mmol, 0.80 mL of 2.5 M stock solution in chloroform) was added. The solution was stirred for 60 min at room temperature. Then, DDQ (1.02 g, 4.50 mmol) was added and the mixture was stirred for an additional 1 h at room temperature. The solvent was removed under reduced pressure and flash column chromatography (silica gel, dichloromethane/methanol gradient) yielded 438 mg (16%) of the pure selected 5-(4-acetamidophenyl)-10,15,20-tris(2,4,6-trimethoxylphenyl) porphyrin **3** as second porphyrin moving band. TLC analysis (dichloromethane/methanol 5%) R_f 0.45. MS (*m*/*z*) 941 (M^+) (941.3638 calculated for $C_{55}H_{51}N_5O_{10}$). To a solution of amido-porphyrin **3** (200 mg, 0.21 mmol) in 40 mL of THF was added 40 mL of saturated solution of KOH in methanol. The reaction mixture was stirred under a nitrogen atmosphere for 18 h at 60 °C. Then, the mixture was diluted with dichloromethane and washed with aqueous sodium carbonate. The organic solvent was evaporated under reduced pressure. Flash column chromatography (silica gel, dichloromethane/methanol 0.5%) afforded 147 mg 78% of the desired 5-(4-aminophenyl)-10,15,20-tris-(2,4,6-methoxylphenyl) porphyrin **4**. TLC (silica gel) R_f (dichloromethane/methanol 5%) = 0.80. ^{1}H NMR ($CDCl_3$, TMS) δ (ppm) −2.70 (brs, 2H, pyrrole N–H), 3.88 (s, brs, 2H, Ar–NH_2), 4.09 (s, 24H, Ar–OCH_3), 4.17 (s, 9H, Ar–OCH_3), 6.55 (s, 6H, 10,15,20-Ar 3,5-H) 7.05 (d, 2H, *J* = 8.3 Hz, 5-Ar 3,5-H), 7.97 (d, 2H, *J* = 8.3 Hz, 5-Ar 2,6-H); 8.75–8.95 (m, 8H, pyrrole). MS *m*/*z* 899 (M^+) (899.3533 calcd for $C_{53}H_{49}N_5O_9$). Anal. calcd C 70.73, H 5.49, N 7.78; found C 70.38, H 5.70, N 7.64.

5,10,15,20-tetrakis(2,4,6-trimethoxylphenyl) porphyrin (2). Porphyrin **2** was obtained as the first porphyrin-moving band in the flash column chromatography described above for porphyrin **3**. TLC (silica gel) R_f (dichloromethane/methanol 5%) = 0.82. ^{1}H NMR ($CDCl_3$, TMS) δ (ppm) −2.70 (brs, 2H, pyrrole N–H), 4.08 (s, 32H, Ar–OCH_3), 4.16 (s, 12H, Ar–OCH_3), 6.55 (s, 6H, 10,15,20-Ar-3,5-H); 8.75–8.95 (m, 8H, pyrrole). MS *m*/*z* 974 (M^+) (974.3740 calcd for $C_{56}H_{54}N_4O_{12}$). Anal. calcd C 68.98, H 5.58, N 5.75; found C 68.87, H 5.52, N 5.81.

5-[4-*N*-(*N*-2′,6′-dinitro-4′-trifluoromethylphenyl) aminophenyl]-10,15,20-tris(2,4,6-trimethoxyphenyl) porphyrin (dyad 5). Amino porphyrin **4** (50 mg, 0.056 mmol), 1-chloro-2,6-dinitro-4-trifluorometilbenzene (38 mg, 0.140 mmol) and tetrabutylammonium bromide (TBAB)

(45 mg, 0.140 mmol) were dissolved in 40 mL of chloroform. Then, 500 mg of potassium hydroxide was added and the mixture was stirred for 1.5 h at room temperature. The crude mixture was neutralized with hydrochloric acid (0.1 N) and then extracted with three portions of chloroform (30 mL each). The organic phase was dried with $MgSO_4$, filtered and the solvents removed under reduced pressure. Flash column chromatography (silica gel, chloroform/methanol gradient) afforded 57 mg (90%) of pure dyad **5**. TLC (silica gel) R_f (dichloromethane/methanol 3%) = 0.84 and R_f (dichloromethane) = 0.64. ^1H NMR ($CDCl_3$, TMS) δ (ppm) −2.76 (brs, 2H, pyrrole N–H); 4.10 (s, 24H, Ar–OCH_3); 4.19 (s, 9H, Ar–OCH_3); 6.57 (s, 6H, 10,15,20-Ar 3,5-H); 6.75 (d, 2H, J = 8.0 Hz, 5-Ar 3,5-H); 7.70 (d, 2H, J = 8.0 Hz, 5-Ar 2,6-H); 8.50 (s, 2H, Ar 3′,4′-H); 8.75–8.90 (m, 8H, pyrrole); 10.30 (s, brs, 1H,-NH); MS m/z 1133 (M^+) (1133.3421 calcd for $C_{60}H_{50}F_3N_7O_{13}$). Anal. calcd. C 63.55, H 4.44, N 8.65; found C 63.43, H 4.51, N 8.70.

Cadmium (II) 5-[4-*N*-(*N*-2′,6′-dinitro-4′-trifluoromethylphenyl) aminophenyl]-10,15,20-tris(2,4,6-trimethoxylphenyl) porphyrin (dyad 6). Dyad **5** (20 mg, 0.017 mmol) in pyridine (8 mL) and saturated potassium hydroxide in methanol (4 mL) was treated with cadmium acetate dihydrate (18 mg, 0.067 mmol). After 2 h reflux, the solution was cooled and treated with of water (30 mL). The organic phase was extracted with three portions of dichloromethane (15 mL each) and the solvents removed under vacuum to give 13 mg (62%) of pure Cd (II) porphyrin (**5**). MS m/z 1244 (M^+) (1243.7374 calcd for $C_{60}H_{48}F_3N_7O_{13}Cd$). Anal. calcd C 57.91, H 3.89, N 7.88; found C 57.98, H 3.75, N 7.80.

Irradiation

The light source used was a Kodak slide projector equipped with a 150 W lamp. The light was filtered through a 3 cm water layer to absorb heat and a glass filter. A wavelength range between 350 and 800 nm was selected by optical filters.[32] The light intensity at the treatment site was 18.0 mW/cm^2 (Radiometer Laser Mate-Q, Coherent).

Spectroscopic studies

Absorption spectra were recorded at 25.0 ± 0.5 °C using 1 cm path length cells. Wavelength maxima (λ_{max}) were measured by taking the mid-point between the two positions of spectrum where the absorbance of the band is equal to 0.9 A_{max}. The fluorescence quantum yield (ϕ_F) of porphyrins were calculated by comparison of the area below the corrected emission spectrum in tetrahydrofuran with that of tetraphenylporphyrin (TPP) as a fluorescence standard, exciting at λ_{ex} = 550 nm.[25] A value of $\phi_F = 0.10$ for TPP in tetrahydrofuran was calculated by the comparison with the fluorescence spectrum in toluene using $\phi_F = 0.11$[26] and taking into account the refractive index of the solvents.[25] Photooxidation 9,10-dimethylanthracene (DMA) was used to determine $O_2(^1\Delta_g)$ production by the photosensitizers. Solutions of DMA (45 µM) and photosensitizer (absorbance 0.1, λ = 515 nm) in tetrahydrofuran were irradiated with monochromatic light at λ = 515 nm. The DMA photooxidation was measured by the decrease of the absorbance at λ_{max} = 378 nm. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of Ln A_0/A versus time. TPP in tetrahydrofuran was used as the standard ($\Phi_\Delta = 0.62$).[33] Measurements of sample and reference under similar conditions afforded Φ_Δ for porphyrins **2**, dyads **5** and **6** by direct comparison of the slopes in the linear region of the plots. All the experiment were performed at 25.0 ± 0.5 °C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 5%.

Liposome preparation

The incorporation of the porphyrins into the phospholipid bilayer of the D,L-α-dipalmitoyl phosphatidylethanolamine was achieved by a modification of the ethanol injection procedure of Kremer et al.[34] Typically, 2 mL of a solution bearing 9.60 mM of phospholipid, 1.91 mM of cholesterol and 0.27 mM of porphyrin in ethanol-tetrahydrofuran binary mixture (1:1, vol/vol) was injected into 10 mL of phosphate-buffered saline (PBS) at 80 °C. The injection was performed at a speed of 50 µL/min with magnetic stirring.[28]

Cell culture

The Hep-2 human larynx-carcinoma cell line (Asociación Banco Argentino de Células, ABAC, Instituto Nacinal de Enfermedades Virales Humanas, Pergamino, Argentina) was maintained frozen in liquid nitrogen. The cells were grown as a monolayer employing Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 50 µg gentamycin as antibiotic. The cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere and the medium was changed daily. The cell line was routinely checked for the absence of mycoplasma contamination.

Cell photosensitization studies and quantification

An appropriate number of cells ($\sim 1 \times 10^6$ cells) were inoculated in 25 cm^2 culture flasks and incubated to obtain nearly confluent cell layers. Then, an appropriate amount of the porphyrin, incorporated into liposomes, was added to the culture flask bearing 5 mL of the medium. The cells were treated with sensitizer for 24 h in dark condition. Afterwards, the medium containing the photosensitizer was discarded. Cells were washed three times with medium and kept in 5 mL of it. The dishes were exposed for different time intervals to visible light. After each irradiation time, the viability of the cells was estimated by microscopy with trypan blue (TB) exclusion test using a Neubauer chamber counter.[32] The same procedure without irradiation was carried out for determining dark toxicity. The uptake was determined adding 1.0 mL of 4% sodium dodecyl sulphate (SDS, Merck) to 1 mL of cellular suspension. The mixture was incubated further for 15 min (in the dark and room temperature) and centrifuged at 9000 rpm for 30 min.

The concentration of sensitizer in the supernatant was measured by spectrofluorimetry in solution of 2% SDS in PBS. The fluorescence values obtained from each sample were referred to the total number of cells contained in the suspension. The concentration of the porphyrin in this sample was estimated by comparison with a calibration curve obtained with standard solutions of the sensitizer in 2% SDS ([sensitizer]$\sim$0.1–5 μM). Four culture flasks were used for each incubation time. Any experiments were compared with a culture control without photosensitizer.

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