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The photodynamic activity of a novel porphyrin derivative bearing a fluconazole structure in different media and against *Candida albicans*

S. Jimena Mora, M. Paula Cormick, M. Elisa Milanesio, Edgardo N. Durantini*

Departamento de Química, Facultad de Ciencias Exactas Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal Nro. 3, X5804BYA Río Cuarto, Córdoba, Argentina

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ABSTRACT

The spectroscopic properties and photodynamic activity of a novel porphyrin covalently linked to an antifungal fluconazole structure were investigated in DMF and different biomimetic systems, results being compared with those obtained for the non-fluconazole homologoue. Absorption and fluorescence studies indicated that the tetrapyrrolic macrocycle retained its individual spectroscopic properties. Photosensitization ability was first evaluated using 9,10-dimethylanthracene; in microheterogenic media, the porphyrin interacted with sodium bis(2-ethylhexyl)sulfosuccinate reverse micelles and also β-cyclodextrin, photosensitized decomposition of L-tryptophan being observed in these systems. The *in vitro* photodynamic activity of the photosensitizers when associated with β-cyclodextrin, as tested against *Candida albicans*, revealed that growth delays imparted by fluconazole alone and the unsubstituted porphyrin were almost additive when the two compounds were used together. However, when the fluconazole was covalently linked to a porphyrin nucleus and exposed to light, reduction in growth delay efficacy was observed relative to the mixed system.

1. Introduction

Candidiasis is the most common oral fungal infection in man and is manifest in various clinical guises ranging from pseudomembraneous, erythematous and hyperplastic variants to more recently described linear gingival erythema associated with HIV infection [1]. All forms of oral candidiasis are considered opportunistic as a multiplicity of predisposing factors facilitates conversion of commensal *Candida* into a parasitic existence. As a consequence of the increasing prevalence of other compromised patient groups in the community, common endocrine disorders such as diabetes mellitus as well as nutritional deficiencies have contributed to the resurgence of oral candidiasis as a relatively common affliction. There is now ample evidence to indicate that prophylactic use of azole agents such as fluconazole and clotrimazole, absorbed either fully or partially through the gastrointestinal tract, reduce clinical signs of oral candidiasis in cancer patients on chemotherapy [2]. Fluconazole is commonly used to prevent oropharyngeal and oesophageal candidiasis in immune-compromised patients and in the treatment of upper gastrointestinal candidiasis in neutropenic patients with cancer [1]. It is also effective in reducing oropharyngeal colonization of *Candida albicans* in patients with leukemia and bone marrow transplants [3]. In contrast, the widespread use of topical and systemic antifungal agents as treatments for oral candidiasis has resulted in resistance in *C. albicans* that appears to increase proportionally with the extension of previous exposure to antifungal drugs. Moreover, because of the fungistatic rather than the fungicidal effect of azoles, host defences are essential for eradicating the infection. Thus, in immune-suppressed patients, the use of azole agents to treat oral candidiasis can be ineffective [4–6].

Hence, it is necessary to develop alternative therapies for the treatment of candidiasis [7]. A promising modality is photodynamic inactivation (PDI) of microorganisms, which uses a combination of light, a photosensitizer and oxygen to achieve a cytotoxic effect in the cells [8,9]. In the PDI process, the photosensitizer excited state can react with molecules from its direct environment by electron or hydrogen transfer, leading to the production of radicals (type I reaction), or the photosensitizer can transfer its energy to oxygen, generating the highly reactive singlet molecular oxygen, $O_2(^1\Delta_g)$ (type II reaction) [10]. Both pathways can impart cell damage that inactivates the microorganism.

Interest in this field and the need for new phototherapeutic agents with improved characteristics is high, owing to the large variety of therapeutic applications. In previous studies, a Zn(II) phthalocyanine derivative containing four fluconazole structures (ZnPcF) was evaluated as an antifungal agent [11]. Azoles are

* Corresponding author. Tel.: +54 358 4676157; fax: +54 358 4676233.
E-mail address: edurantini@exa.unrc.edu.ar (E.N. Durantini).

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antifungal agents that inhibit the activity of cytochrome $P450_{14DM}$ of the fungi and contain an imidazolic ring, which is involved in the observed pharmaceutic activity [12–14]. One of the problems that affects the sensitizing ability of the large p-conjugated phthalocyanine structures is aggregation, which present an efficient non-radiative energy relaxation pathway, thereby diminishing the triplet-state population and the $O_2(^1\Delta_g)$ quantum yield. Therefore, the formation of aggregation precludes photodynamic activity.

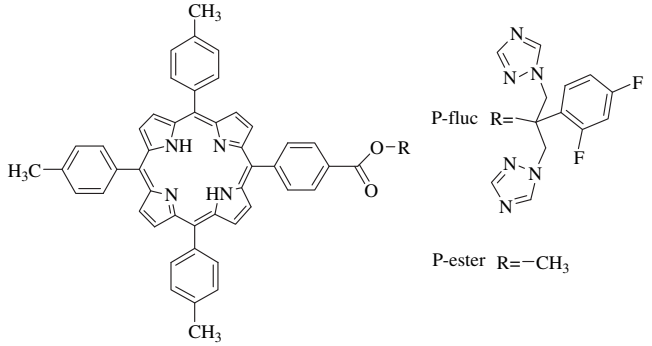
Microheterogeneous systems such as reverse micelles can disaggregate both water-soluble and water-insoluble compounds, simulating a biomimetic microenvironment [15,16]. Cyclodextrins are water-soluble compounds that form inclusion host–guest complexes with a variety of guest molecules which are bound by hydrophobic forces and H-bonding. On account of the hydrophobic interior of cyclodextrins, even hydrophobic substances can be transferred to aqueous solutions. The photophysical properties of cyclodextrin-bound porphyrins have been detected in the case of monomeric species confined within a cyclodextrin cavity and separated from bulk solution [17–19].

This work concerns the spectroscopic characteristics and photodynamic activity of a novel porphyrin derivative covalently linked to a fluconazole (P-fluc, Scheme 1) in different media. The properties of P-fluc were compared with those of the fluconazole-free homolohue (P-ester, Scheme 1). The photoinactivation efficiency of the sensitizers were evaluated against *C. albicans* in cellular suspension, providing information that can be used to establish conditions for the eradication of yeast cells growing in liquid medium and irradiated with visible light.

2. Materials and methods

2.1. General

UV–visible absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. FT-IR spectra were recorded on a Nicolet Impact 400. Proton nuclear magnetic resonance spectra were recorded on a FT-NMR Bruker Advance 200 spectrometer at 200 MHz. Mass Spectra were taken with a Varian Matt 312 operating in EI mode at 70 eV. Cultures absorption was determinate at 660 nm in a Barnstead Turner SP-830 (Dubuque, IA, USA) spectrophotometer. The visible light source used was a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. A wavelength range between 350 and 800 nm was selected by optical filters. The light intensity at the treatment site was 90 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA).



Scheme 1. Molecular structures of the porphyrin derivatives.

All the chemicals were obtained from Aldrich (Milwaukee, WI, USA) were used without further purification. Sodium bis(2-ethylhexyl)sulfosuccinate (AOT) from Sigma (St. Louis, MO, USA) was dried under vacuum. β-Ciclodextrina (β-CD) was purchased from Aldrich. Silica gel thin-layer chromatography (TLC) plates 250 microns from Analtech (Newark, DE, USA) were used. Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from a Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Sensitizers

5,10,15,20-Tetrakis(phenyl)porphyrin (TPP) from Aldrich was used as received. 5-(4-Carboxymethylphenyl)-10,15,20-tris(4-methylphenyl)porphyrin (P-ester) and 5-(4-carboxyphenyl)-10,15,20-tris (4-methylphenyl)porphyrin (P-acid) were synthesized as previously described [20]. 5-[(4-((2-(2,4-Difluorophenyl)-1,3-di(1*H*-1,2,4-triazol-1-yl)propan-2-yloxy)carbonyl)phenyl)]-10,15,20-tris(4-methylphenyl)porphyrin (P-fluc) was prepared by dissolving P-acid (25 mg, 0.0036 mmol) and fluconazole (11 mg, 0.0036 mmol) in 5 mL of dry dichloromethane. This mixture was stirred under argon for 15 min and then dicyclohexylcarbodiimide (DCC, 7 mg, 0.0035 mmol), 4-(dimethylamino)pyridine (DMAP, 4 mg, 0.0035), 1-hydroxybenzotriazole (BtOH, 7 mg, 0.0053 mmol) and triethylamine (TEA, 7 μL, 0.0053 mmol) were added. The reaction mixture was stirred for 5 h at room temperature and the ensuing solution was treated with water (10 mL) and the reaction mixture extracted using three portions of chloroform (10 mL each). The organic solvent was removed under reduced pressure and evaporated to dryness and flash column chromatography (silica gel, dichloromethane) afforded 24 mg of pure P-fluc (68%). TLC (sílica gel) R_f (dichloromethane) = 0.63. ^{1}H NMR ($CDCl_3$, TMS) δ [ppm] −2.73 (br, 2H, pirrol N–H), 2.74 (s, 9H, Ar–CH_3), 4.54–4.70 (4H), 7.02 (m, 1H), 7.20 (m, 2H), 7.60 (d, 6H, J = 7.8 Hz, 5,10,20-Ar 3,5-H), 7.72 (s, 2H), 7.85 (d, 2H, J = 8.0 Hz, 20-Ar 2,6-H), 8,13 (d, 6H, J = 7.8 Hz, 5,10,20-Ar 2,6-H), 8.20 (d, 2H, J = 8.0 Hz, 20-Ar 3,5-H), 8.24 (s, 2H), 8.85–8.93 (brs, 8H, pyrrole). FT-IR (KBr) cm^{-1} 2964, 2934, 2852, 1782, 1620, 1510, 1380, 1235, 966, 794, 743, 610. MS [*m*/*z*] 988 (M^+) (988.3793 calculated for $C_{61}H_{46}F_2N_{10}O_2$).

2.3. Spectroscopic studies

Absorption and fluorescence spectra were recorded at 25.0 ± 0.5 °C using 1 cm path length quartz cells. The fluorescence quantum yield (Φ_F) of porphyrins were calculated by comparison of the area below the corrected emission spectrum in *N*,*N*-dimethylformamide (DMF) with that of TPP as a reference (Φ_F = 0.12) [21]. Absorbances of sample and reference were matched at the excitation wavelength (515 nm) and the areas of the emission spectra were integrated in the range 600–800 nm.

2.4. Steady state photolysis

Solutions of 9,10-dimethylanthracene (DMA, 35 μM) and photosensitizer (λ = 515 nm, absorbance 0.1) in DMF (2 mL) were irradiated in quartz cuvettes with monochromatic light at λ = 515 nm from a 75 W high-pressure Xe lamp through a high intensity grating monochromator (Photon Technology Instrument). The fluence rate was determined as 1.58 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). The kinetics of DMA photooxidation were studied by following the decrease of the absorbance (*A*) at λ_{max} = 378 nm. Photooxidation of DMA was also used to determine $O_2(^1\Delta_g)$ production by the photosensitizers [22]. TPP was used as a reference (Φ_Δ = 0.62) [21]. Measurements of the sample and reference under the same conditions afforded Φ_Δ for porphyrins by direct comparison of the slopes in the linear region of the plots.

The studies in the presence of Trp (20 μM) were performed irradiating the samples with visible light (photosensitizer absorption at *Soret* band 0.1). The kinetics of Trp decomposition were studied by following the decrease of the fluorescence intensity (F) at $\lambda = 350$ nm. The Trp fluorescence was excited by 290 nm light. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of ln A_0/A *vs.* time for DMA and Ln F_0/F *vs.* time for Trp. 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 10%.

2.5. Studies in reverse micelles

Studies in reverse micelles were performed using a stock solution of (AOT) 0.1 M, which was prepared by weighing and dilution in *n*-heptane. The addition of water to the corresponding solution was performed using a calibrated microsyringe. The amount of water present in the system was expressed as the molar ratio between water and the AOT present in the reverse micelle ($W_0 = [H_2O]/[AOT]$). In all experiments, $W_0 = 10$ was used. The mixtures were sonicated for about 10 s to obtain perfectly clear micellar system [29]. The binding constants, $K_{AOT} = [\text{porphyrin}_b]/[\text{porphyrin}_f][AOT]$ (where the terms $[\text{porphyrin}_b]$ and $[\text{porphyrin}_f]$ refer to the concentration of bound and free porphyrin, respectively, and [AOT] is the total surfactant concentration) were calculated by using the Ketelaar's equation from the spectral changes at the Soret band upon varying the AOT concentration [23]:

$$\frac{1}{A - A_{Hp}} = \frac{1}{(\varepsilon_b - \varepsilon_{Hp})[\text{porphyrin}]_0} + \frac{1}{(\varepsilon_b - \varepsilon_{Hp})[\text{porphyrin}]_0 K_{AOT}[AOT]} \quad (1)$$

where $[\text{porphyrin}]_0$ is the initial concentration of the porphyrin, A is the absorbance at different [AOT], A_{Hp} is the absorbance in *n*-heptane, ε_b and ε_{Hp} are the molar absorptivity for the porphyrin bound to the interface and in the organic medium, respectively. Plotting the left-hand side term of Equation (1) *vs.* 1/[AOT], the value of K_{AOT} is obtained from the intercept to slope ratio [23]. Decomposition of substrates was made as explained above in homogenic solution.

2.6. Interaction of porphyrin derivatives with β-CD

A stock solution of β-CD (0.025 M) was prepared by dissolving appropriate amount of solid material in of water. Aliquot of this solution was transferred into 2 mL of a solution of porphyrin in water (0.2 μM) using a calibrated microsyringe. The mixed solution was thoroughly shaken. After allowed to equilibrate for 5 min, the fluorescence emission spectra were taken with an excitation wavelength at Soret band. The equilibrium binding constants ($K_{\beta\text{-CD}}$) were determined by measuring the fluorescence intensities of porphyrin at wavelength of maximum emission as a function of different β-CD concentrations. Because a large excess of β-CD relative to porphyrin was employed, it was assumed that [β-CD] >> [porphyrin] and therefore the data were analyzed by curve fitting method using Equation (2) [24]:

$$I = \frac{I_0 + I_\infty K_{\beta-CD}[\beta - CD]_0^n}{1 + K_{\beta-CD}[\beta - CD]_0^n} \quad (2)$$

where I_0 and I denote the fluorescence intensity of porphyrin in the absence and in the presence of excess of β-CD, respectively; $[\beta\text{-CD}]_0$ is the analytical concentration of β-CD and n is the stoichiometric coefficient of β-CD in the formation of inclusion complexes. Photooxidation of Trp was performed as described above in DMF using 0.2 μM porphyrin.

2.7. Microorganism and growth conditions

Strain of *C. albicans* PC31, recovered from human skin lesion, was previously characterized and identified [25]. Cultures of *C. albicans* were grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (4 mL) at 37 °C to stationary phase. Cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0), corresponding to ~10^7 colony forming units (CFU)/mL. A portion (1 mL) of this culture was transfer to 20 mL of fresh Sabouraud broth medium. The suspension was homogenized and aliquots of 2 mL were incubated at 37 °C in Pirex brand culture tubes (13 × 100 mm) with: 10 μM P-fluc + 0.025 M β-CD, 10 μM P-ester + 0.025 M β-CD, 10 μM P-ester + 0.025 M β-CD + 10 μM fluconazole. The culture grown was measured by turbidity at 660 nm. Then the flasks were irradiated with visible light at 37 °C, using the irradiation system described above. In all cases, control experiments were carried out without illumination with 10 μM P-fluc + 0.025 M β-CD, 10 μM P-ester + 0.025 M β-CD, 10 μM P-ester + 0.025 M β-CD + 10 μM fluconazole, 10 μM fluconazole + 0.025 M β-CD and control culture untreated irradiated. Each experiment was repeated separately three times.

3. Results and discussion

3.1. Absorption and fluorescence spectroscopic studies

The absorption spectra of porphyrins in DMF are shown in Fig. 1A. These sensitizers showed the typical Soret band (~418 nm) and the four Q-bands (515–650 nm), characteristic of *meso*-tetraphenylporphyrin derivatives [26]. The relative intensities of the Q-bands for both porphyrins gave an *etio*-type spectrum ($\varepsilon_{VI} > \varepsilon_{III} > \varepsilon_{II} > \varepsilon_I$). Sharp absorption bands were obtained in every case indicating that these sensitizers are mainly dissolved as monomer in DMF. In contrast, these porphyrins show a very low intensity and broadening Soret band in PBS, indicating an extensive aggregation in aqueous solutions. The absorption spectrum of P-fluc is essentially identical to that of P-ester, which is consistent with only a weak interaction between the tetrapyrrolic macrocycle and fluconazole in the ground state and the two moieties retain their individual identities.

The steady-state fluorescence emission spectra of these porphyrins were analyzed in DMF (Fig. 1B). The two bands around 650 and 715 nm are characteristic for similar porphyrin. These bands have been assigned to Q(0–0) and Q(0–1) transitions [26,27]. The Stokes shift was for P-fluc and P-ester. A small Stokes shift (~2 nm) was found for these sensitizers indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state. The values of $\Phi_F = 0.15 \pm 0.01$ and 0.14 ± 0.01 were obtained for P-fluc and P-ester, respectively. The results are in agreement with those previously reported for *meso*-substituted porphyrins in different media [29]. These Φ_F are appropriate values for quantification and detection of the sensitizer in biological media.

3.2. Photosensitized decomposition of substrates in DMF

Photooxidation of DMA sensitized by porphyrins was studied in DMF under aerobic condition (Fig. 2A). From first-order kinetic plots of the DMA absorption at 378 nm with time the values of the observed rate constant (k_{obs}) were calculated (Table 1). As can be observed, both porphyrins photosensitize the decomposition of DMA with similar rates. Taking into account that DMA quenches $O_2(^1\Delta_g)$ exclusively by chemical reaction, it is used as a method to

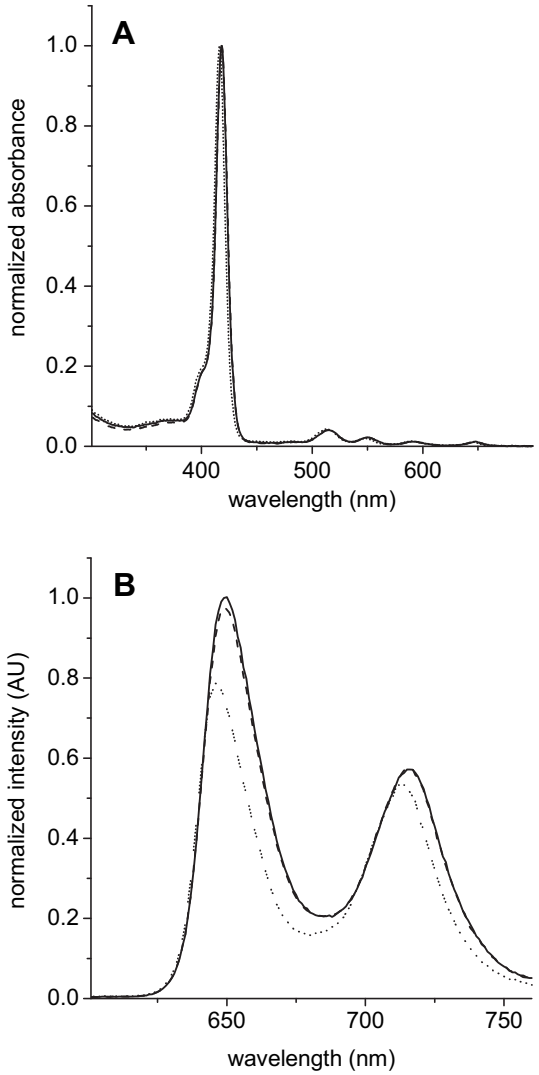


Fig. 1. (A) Absorption and (B) fluorescence emission spectra ($\lambda_{exc} = 515$ nm) of P-fluc (solid line), P-ester (dashed line) and TPP (dotted line) in DMF.

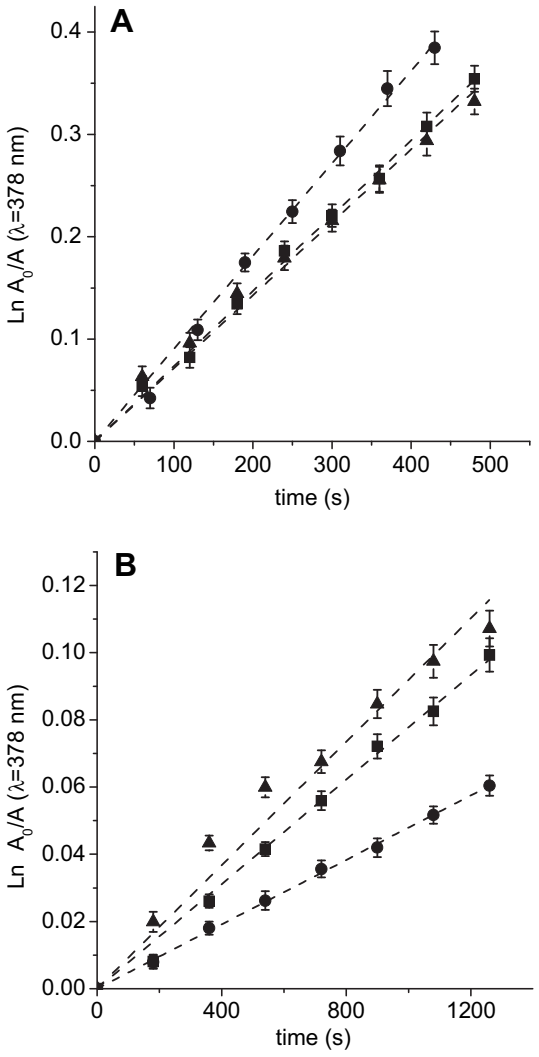


Fig. 2. First-order plots for the photooxidation of DMA (35 μM) photosensitized by P-fluc (▲), P-ester (■) and TPP (●) in (A) DMF and (B) *n*-heptane/AOT (0.1 M)/water ($W_0 = 10$) reverse micelles; $\lambda_{irr} = 515$ nm. Values represent mean ± standard deviation of three separate experiments.

evaluate the ability of the sensitizers to produce $O_2(^1\Delta_g)$ in solution [28]. Thus, the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) were calculated by comparing the slope for the porphyrin with that for the reference (TPP, $k_{obs} = (9.1 \pm 0.4) \times 10^{-4}$ s^{-1} in DMF) from the plots shown in Fig. 2A. The results showed values of $\Phi_\Delta = 0.46$ and 0.53 for P-Fluc and P-ester, respectively, which are quite reasonable values for free-base porphyrins in this solvent [22].

The amino acid Trp was used as a substrate model for the compounds of biological interest that can be potential targets of porphyrin photodynamic action. In general, this substrate can be efficiently photooxidized by both type I and type II reaction mechanisms [29]. The photoprocess follows first-order kinetics with respect to Trp concentration, as showed in Fig. 3A for [Trp] = 20 μM. From the plots in Fig. 3A, the values of the k_{obs}^{Trp} were calculated for Trp decomposition. The results in Table 1 indicate a higher k_{obs}^{Trp} value for the reaction sensitized by P-ester.

3.3. Solubilization and photodynamic activity in AOT reverse micelles

The solubilization and interaction of these porphyrins were spectroscopically analyzed in *n*-heptane/AOT (0.1 M)/water ($W_0 = 10$) reverse micelles. When the absorption spectra of porphyrins were studied at various AOT concentrations, an increase in the intensity of the Soret band was observed for P-fluc as the surfactant concentration increased. This effect is attributed to the interaction between the porphyrin and the micelle. Plotting the left-hand side term of Equation (1) *vs.* 1/[AOT], the value of the binding constant (K_{AOT}) was calculated from the ratio of slope and the intercept. Representative results are shown in Fig. 4 for P-fluc. The large value of

Table 1
Kinetic parameters, k_{obs} (s^{-1}), for the photooxidation reaction of DMA and Trp in different media.

Substrate	Media	P-fluc	P-ester
DMA[a]	DMF	$(7.1 \pm 0.3) \times 10^{-4}$	$(7.4 \pm 0.3) \times 10^{-4}$
DMA[a]	AOT[c]	$(8.0 \pm 0.2) \times 10^{-5}$	$(7.5 \pm 0.2) \times 10^{-5}$
Trp[b]	DMF	$(4.5 \pm 0.3) \times 10^{-5}$	$(6.5 \pm 0.3) \times 10^{-5}$
Trp[b]	AOT[c]	$(2.1 \pm 0.2) \times 10^{-5}$	$(2.2 \pm 0.2) \times 10^{-5}$
Trp[b]	β-CD[d]	$(2.0 \pm 0.3) \times 10^{-4}$	$(1.6 \pm 0.2) \times 10^{-4}$

[a] $\lambda_{irr} = 515$ nm.
[b] $\lambda_{irr} = 350$–800 nm.
[c] *n*-heptane/AOT (0.1 M)/water ($W_0 = 10$).
[d] [β-CD] = 0.025 M in water.

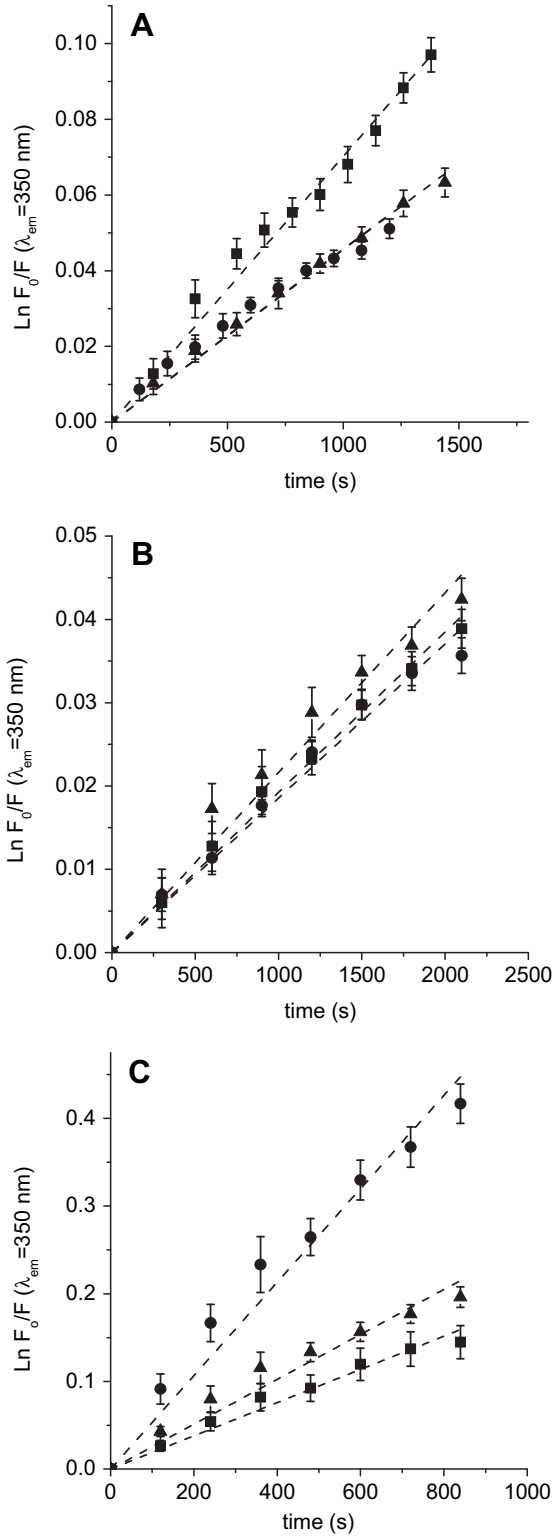


Fig. 3. First-order plots for the photooxidation of Trp (20 μM) photosensitized by P-fluc (▲), P-ester (■) and TPP (●) in (A) DMF, (B) *n*-heptane/AOT (0.1 M)/water ($W_0 = 10$) reverse micelles and (C) β-CD (0.025 M) irradiated with visible light. Values represent mean ± standard deviation of three separate experiments.

K_{AOT} = 3780 ± 300 M^{-1} found for the P-fluc indicates that this sensitizer is strongly associated with the micellar interface. On the other hand, the spectroscopic changes were not significant for P-ester and TPP, showing that both sensitizers does not interact significantly

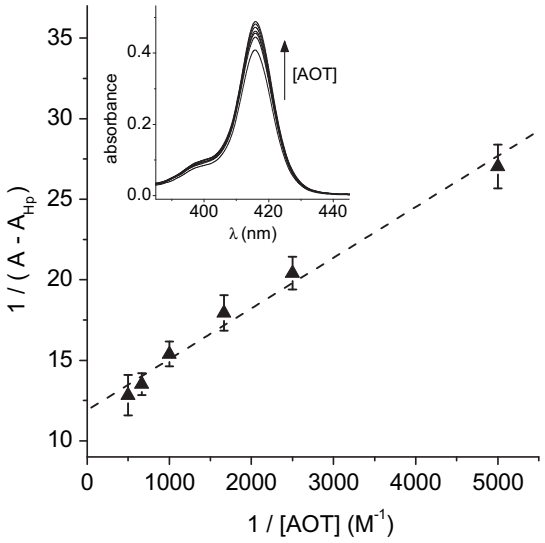


Fig. 4. Variation of $1/(A-A_{Hp})$ as a function of 1/[AOT] for P-fluc in *n*-heptane/AOT/water ($W_0 = 10$) reverse micelles (λ_{max} = 416 nm). Dashed line: linear regression fit by Equation (1). Inset: absorption spectra of P-fluc at different AOT concentrations (0.2–2.0 mM). Values represent mean ± standard deviation of three separate experiments.

with the micellar interface and it can be assumed that these porphyrins are mainly dissolved in the organic solvent.

Irradiation of the AOT micelles containing DMA under aerobic conditions was performed in the presence of porphyrins. Because DMA is a non-polar compound, it is assumed that this substrate is mainly solubilized in the organic phase (*n*-heptane) of the micellar system [29]. In this microenvironment, the substrate reacts with the $O_2(^1\Delta_g)$ photosensitized by porphyrin. From the plots in Fig. 2B, the values of the k_{obs} were calculated for DMA in micelles. As can be observed in Table 1, the value of k_{obs} for P-fluc is slightly higher than that for P-ester. Under this condition, a value of $k_{obs} = (5.5 \pm 0.1) \times 10^{-5}\ s^{-1}$ was found for TPP. In contrast to the results in DMF, the P-fluc is the better photosensitizer to decompose DMA in micelles. On the other hand, the reaction rates of DMA sensitized by P-fluc or P-ester in DMF are faster than in the AOT micellar system. Similar behavior was previously observed in benzene/benzyl-*n*-hexadecyldimethyl ammonium chloride/water reverse micelles using porphyrins as sensitizers [23].

In the AOT system, Trp is exclusively solubilized in the polar side of the interface and it is oxidized by $O_2(^1\Delta_g)$ [29]. Under these conditions, photodecomposition of Trp follows the tendency showed in Fig. 3B. A similar behavior of P-fluc, P-ester and TPP ($k_{obs} = (1.8 \pm 0.2) \times 10^{-5}\ s^{-1}$) was found for the sensitized photooxidation of Trp in micelles. Also in this case, Trp decomposition is slower in AOT system than in DMF.

3.4. Interaction with β-CD and sensitized oxidation of Trp in water

Cyclodextrins are able to complex with a variety of molecules and especially water-insoluble organic compounds in aqueous solutions [18,19]. The effects of the addition of β-CD on the fluorescence properties of porphyrins were investigated in water. Addition of β-CD to an aqueous solution of porphyrin derivatives produces an increase in the overall emission intensity of porphyrin fluorescence compared to that observed in bulk water (Fig. 5 inset). These remarkable changes suggest an interaction of β-CD with porphyrin, whereby the porphyrin molecule preferentially binds within the relatively non-polar β-CD cavity. As shown in Fig. 5, the increase of the β-CD concentrations produces and important enhancement of the complex fluorescence intensity. Higher β-CD concentrations produce only small intensity variation. The experimental data were

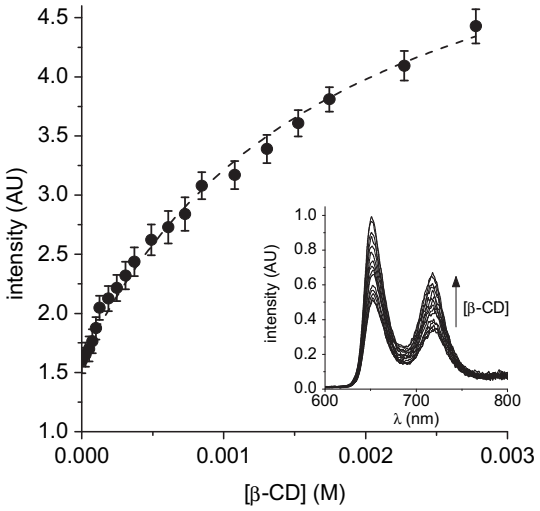


Fig. 5. Fluorescence intensity of P-fluc ($\lambda_{exc} = 420$ nm, $\lambda_{em} = 465$ nm) in the presence of different β-CD concentrations. Dashed line: curve regression fit by Equation (2), $n = 1$. Inset: emission spectra of P-fluc at different β-CD concentrations (0.025–2.8 mM). Values represent mean ± standard deviation of three separate experiments.

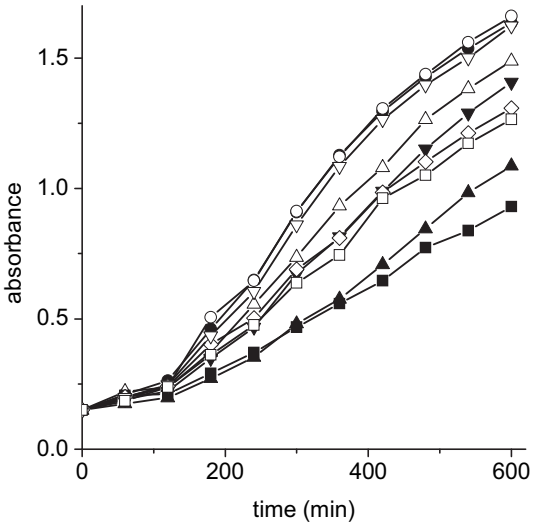


Fig. 6. Effect on growth of *C. albicans* cultures treated with: 10 μM P-fluc + 0.025 M β-CD and irradiated with visible light (▲), 10 μM P-ester + 0.025 M β-CD and irradiated with visible light (▼), 10 μM P-ester + 0.025 M β-CD + 10 μM fluconazole and irradiated with visible light (■), 10 μM P-fluc + 0.025 M β-CD in dark (△), 10 μM P-ester + 0.025 M β-CD in dark (▽), 10 μM P-ester + 0.025 M β-CD + 10 μM fluconazole in dark (□), 10 μM fluconazole + 0.025 M β-CD in dark (◇) in Saboreaoud broth at 37 °C. Control culture untreated irradiated (●), untreated + 0.025 M β-CD in dark (○). Values represent mean ± standard deviation of three separate experiments.

fitted by Equation (2), which provides the basic for the determination of stoichiometric ratio and binding constant ($K_{β\text{-CD}}$). The result shows in Fig. 5 indicates that the curve fit by Equation (2) with $n = 1$ is the best one to represent the experimental data; indicating a 1:1 complex ratio of β-CD to P-fluc in aqueous solution. Similar tendency was also observed for P-ester. The values of $K_{β\text{-CD}}$ calculated were $554 \pm 50\ M^{-1}$ for P-fluc and $1130 \pm 80\ M^{-1}$ for P-ester.

To evaluate the possible modifications of the photodynamic activity of these porphyrins forming complexes with β-CD, the photooxidation of Trp was analyzed in water solution. As can be observed in Fig. 3C, irradiation of porphyrin associated to β-CD conduced to amino acid decomposition. The values of k_{obs} (Table 1) in this medium are even higher than those found in DMF and AOT micellar system. On the other hand, a negligible effect was found using porphyrins without β-CD in aqueous solution. This is an expected result because these sensitizers are highly aggregated in water showing a very low photoefficiency. Therefore, these results indicate that β-CD has ability to dissociate these porphyrins self-aggregates increasing the photodynamic action. In previous studies, inclusion complex between (2,3,6-tri-*O*-methyl)-*b*-cyclodextrin and 5,10,15,20-tetrakis(4-sulfonatophenyl)-porphyrin were used to lead to high yield oxidation of model biomolecules, such as L-methionine methyl ester and uracil. This supramolecular complex is also effective for phenol degradation in aqueous solution [30]. Furthermore, it has been shown that glucoconjugated photosensitizers are associated with the methyl-β-cyclodextrin, which exhibit high triplet lifetimes and singlet oxygen yields [31]. Therefore, porphyrins–cyclodextrins associations are effective to prevent porphyrins autoaggregation and exhibit reasonable singlet oxygen productions.

3.5. Photosensitized effect on growth of C. albicans cultures

According with the results observed above and since these porphyrins are water-insoluble, they were added to the cell cultures using β-CD as carries to improve the aqueous solubility. The cytotoxic activity on growth of *C. albicans* cultures sensitized by porphyrin derivatives was carried out in Sabouraud medium. Thus, 10 μM sensitizer in the presence of 0.025 M β-CD was added to fresh cultures of *C. albicans* reaching the log phase and the flasks were irradiated with visible light at 37 °C. The effects on growth of cells using different treatments are shown in Fig. 6. As can be observed, growth was arrested when *C. albicans* cultures were treated with 10 μM P-fluc. In dark, the toxicity induced by 10 μM P-ester was negligible, while 10 μM P-fluc produced a growth delay of cultures although somewhat smaller than that found for fluconazole. This means that the fluconazole moiety linked to the porphyrin keeps antifungal activity in dark. Moreover, the cytotoxic activity of P-fluc even increases when the cultures are irradiated with visible light. The last effect can be attributed to the photodynamic action produced by the photosensitizer, which enhances the inactivation of *C. albicans* cells. On the other hand, the cultures treated with 10 μM P-ester and irradiated showed a low cytotoxicity. However, the growth of fungal cells was higher suppressed in presence of 10 μM P-ester together with 10 μM fluconazole and irradiated. Under these conditions, a synergetic effect was observed due to the cytotoxic activity of fluconazole in dark accompanied by the photosensitization activity of P-ester.

4. Conclusions

This study provides information on the photodynamic effect of a porphyrin derivative containing a fluconazole unit in comparison with its homologue P-ester and the antifungi fluconazole. In DMF homogenic solution, both P-fluc and P-ester efficiently lead to the photosensitized decomposition of substrates, however this activity is loser in aqueous medium, where these porphyrins are mainly aggregated even at low concentration. Thus, the $O_2(^1\Delta_g)$ production can significantly change in different media, diminishing when the sensitizer is partially aggregated. Also, the photodynamic properties of the photosensitizer established in solution can be modified in the cellular microenvironment, limiting predict photodynamic efficiencies in biological systems. In a simple biomimetic system, P-fluc strongly interacts with the AOT reverse micelles and the photooxidation of substrates are similar to those produced by P-ester. Also, P-fluc and P-ester form complexes with β-CD and the solubilization of these water-insoluble sensitizers in aqueous solution is apparently accompanied by disruption of the non-covalent aggregates. *In vitro* studies were carried out to evaluate the photodynamic activity of these porphyrins against *C. albicans*.

In this way, β-CD was used as a vehicle to solubilize the water-insoluble sensitizers in the biological medium. The results indicate that fluconazole moiety in P-fluc retains antifungal action although somewhat less than fluconazole. In addition, cytotoxicity increases when the cultures treated with P-fluc are irradiated with visible light due to the photodynamic effect of the tetrapyrrolic macrocycle. However, it is evident that covalent linkage of the antifungal fluconazole to the photosensitizing porphyrin nucleus offers no advantages over simple mixing of the two species. A promissory additive action was found using a combined treatment with fluconazole and P-ester. Therefore, this investigation indicates that β-CD can be used as carrier of lipophilic porphyrins in the PDI of *C. albicans* and the treatment with either P-fluc or both P-ester and fluconazole provides antifungal activity in dark and the efficiency can be enhanced by irradiation due to the photodynamic inactivation.

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