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Synthesis and photodynamic properties of amphiphilic A_3B-phthalocyanine derivatives bearing *N*-heterocycles as potential cationic phototherapeutic agents

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ABSTRACT

Unsymmetrically substituted A_3B-phthalocyanine derivatives bearing annulated 6-membered *N*-heterocycles, pyridine and pyrazine rings, were synthesized by the ring expansion reaction of boron (III) subphthalocyanine chloride. A geometrically constrained subphthalocyanine unit was reacted with a range of 1,2-phthalonitrile derivatives in presence of 8-diazabicyclo[5.4.0]undec-7-ene to form the A_3B-phthalocyanine. The reactions were carried out in DMSO/1-chloronaphthalene at 130–140 °C for 15 h. This synthetic strategy resulted in phthalocyanines in 37–42% yield that required only simple purification. Annulated 6-membered *N*-heterocycles were methylated to obtain cationic Zn(II)phthalocyanine derivatives. The spectroscopic and photodynamic properties of these photosensitizers were studied in *N*,*N*-dimethylformamide solution. Antifungal activity photoinduced by these compounds was evaluated in *Candida albicans* cellular suspensions. The results indicate that amphiphilic cationic phthalocyanines represent interesting agents with potential applications in photodynamic inactivation of microorganisms.

1. Introduction

Unsubstituted phthalocyanines and many substituted examples are highly symmetrical compounds. A great number of unique properties arise from their electronic delocalization, which makes these compounds valuable in different application fields [1]. However, the intrinsic symmetry of the molecule sometimes represents a limitation for many purposes. Thus, the possibility of designing and synthesizing unsymmetrical compounds with substituents located at specific positions may facilitate enhanced applications of phthalocyanines [2].

In recent years, cationic phthalocyanines have shown important applications as sensitizers to photoinduce direct inactivation of multidrug resistant microorganisms [3,4]. The positive charge on the photosensitizer molecule appears to promote a tight electrostatic interaction with the microbial cells. This association increases the efficiency of the photoinactivation processes [5]. Also, the combination of hydrophobic and hydrophilic substituents in the sensitizer structure results in an intramolecular polarity axis, which can facilitate membrane penetration and produces a better accumulation in subcellular compartments, enhancing the effective photosensitization [6]. Thus, novel photosensitizer structures were designed to be amphiphilic in nature [4]. Previous studies have shown that the presence of one or more cationic charges greatly increases the antimicrobial photoinactivation efficacy of porphyrin-based photosensitizers. The charge increases the association of the photosensitizer with pathogen membranes, whereas the hydrophobic character increases association with or penetration into the lipid components of the membrane. In general, increasing the number of cationic charges and lowering the hydrophobicity tended to increase the PDI efficiency against bacteria and yeast [7,8].

The design of amphiphilic photosensitizer architecture requires the formation of phthalocyanines bearing one different (B) and three identical (A) isoindole subunits (A_3B type). Different strategies have been employed for the preparation of these low-symmetry derivatives. Statistical condensation is the most widely used strategy to prepare A_3B-phthalocyanines. This non-selective method is based on the reaction of two differently substituted phthalonitriles and it affords a mixture of six compounds. This way demands the use of chromatographic techniques for the isolation of the desired macrocycle and the yields of desired products usually are very low. In contrast, a selective approach to prepare A_3B-phthalocyanines involves ring expansion reaction of subphthalocyanines [9–11]. Good yields have been obtained when the subphthalocyanine derivative is treated with phthalonitriles in the

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presence of a strong base, such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and a metal salt [12,13].

In previous studies, we have investigated the photodynamic activity of phthalocyanine derivatives in different media and *in vitro* on human carcinoma cells and microorganisms [14–16]. In particular, the photoinactivation capacity of a cationic Zn(II) tetramethyltetrapyridinoporphyrazinium salt was compared with that of a non-charged Zn(II) tetrapyridinoporphyrazine, both *in vitro* using human red blood (HRB) cells and a typical Gram-negative bacterium *Escherichia coli*. Both phthalocyanines produce similar photohemolysis of HRB cells. However, only the cationic phthalocyanine induces significant photoinactivation of Gram-negative bacteria [15].

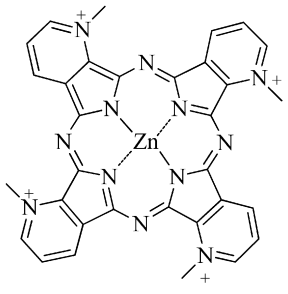
One of the main problems that affect the sensitizing ability of the phthalocyanines is the aggregation tendency in biological media due to large π conjugated systems [17]. In this way, efficient photosensitization ability was found for a highly water soluble zinc(II) tetramethyltetrapyridino[2,3-*b*:2′,3′-*g*:2″,3″-*l*:2‴,3‴-*q*]porphyrazinium salt (ZnTM2,3PyPz, Scheme 1), investigated in aqueous homogeneous solution and in biomimetic reverse micelle systems bearing photooxidizable biological substrates [18].

In the present study, we report the synthesis of novel unsymmetrically substituted A_3B-phthalocyanine derivatives bearing annulated 6-membered *N*-heterocycles, pyridine and pyrazine rings. Thus, boron(III) subphthalocyanine chloride (SubPc, A_3) was reacted with 1,2-phthalonitrile derivatives (B) in presence of DBU and Zn(II) acetate to form the A_3B-phthalocyanines. This approach produces selectively A_3B-phthalocyanines with different substitution patterns, which are precursors of amphiphilic unsymmetrically substituted cationic phthalocyanines with potential applications in the photodynamic inactivation of microorganisms.

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 fluorimeter, respectively. Proton nuclear magnetic resonance spectra were recorded on a FT-NMR Bruker Advance 200 spectrometer at 200 MHz. Mass Spectra were taken with a Bruker MicroQTOFII (Bruker Daltonics, MA, USA), equipped with an ESI source. Culture absorption was determinate at 550 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 30 mW/cm^2 (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA).



ZnTM2,3PyPz

Scheme 1. Structure of zinc(II) tetramethyltetrapyridino[2,3-*b*:2′,3′-*g*:2″,3″-*l*:2‴,3‴-*q*]porphyrazinium.

All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. 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. Synthesis of phthalocyanines

Zinc(II)tetramethyltetrapyridino[2,3-*b*:2′,3′-*g*:2″,3″-*l*:2‴,3‴-*q*] porphyrazinium methylsulphate (ZnTM2,3PyPz) was synthesized as previously described [19].

2.2.1. General procedure for A_3B-phthalocyanine formation

A solution of phthalonitrile derivate (0.19 mmol) and DBU (7 μL, 0.07 mmol) in DMSO/1-chloronaphthalene (4 mL, 5:1) was heated to 130 °C. Then, a suspension of SubPc (43 μg, 0.10 mmol) and zinc(II) acetate dihydrate (22 mg, 0.10 mmol) in DMSO/1-chloronaphthalene (2 mL, 5:1) was added drop-wise to the heated mixture over a period of 1 h. The reaction was kept at 130–140 °C for 15 h. The reaction mixture was cooled to room temperature and precipitated with water (50 mL). The solid was separated by centrifugation and washed with cyclohexane. Zinc(II) tribenzo[*b*:*g*:*l*]pyrido[3,4-*q*]porphyrazine, ZnPc **1**, yield 39%, green solid, melting point >300 °C, ESI-MS [*m*/*z*] 578.0748 $(M + H)^+$ (577.0742 calculated for $C_{31}H_{15}N_9Zn$), spectroscopy data agree with those previously reported [20]. Zinc(II) tribenzo[*b*:*g*:*l*]pyrazino[2,3-*q*]porphyrazine, ZnPc **2**, yield 37%, green solid, melting point >300 °C, ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 8.18–8.22 (6H), 8.80–9.40 (8H), ESI-MS [*m*/*z*] 579.0691 $(M + H)^+$ (578.0694 calculated for $C_{30}H_{14}N_{10}Zn$). Zinc(II) tribenzo[*b*:*g*:*l*]-17,18-dimethylpyrazino[2,3-*q*]porphyrazine, ZnPc **3**, yield 34%, green solid, melting point >300 °C, ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 8.18–8.24 (6H), 8.85–9.39 (6H), ESI-MS [*m*/*z*] 607.1010 $(M + H)^+$ (606.1007 calculated for $C_{32}H_{18}N_{10}Zn$).

2.2.2. General procedure for A_3B-phthalocyanine methylation

A solution of A_3B-phthalocyanine (20 mg) and dimethyl sulphate (1 mL) was heated to 120 °C with stirring under argon atmosphere for 18 h. The mixture was cooled and the product was precipitated with ether. The solid was separated by centrifugation and washed with ether. Zinc(II) tribenzo[*b*:*g*:*l*]-*N*-methylpyridino [3,4-*q*]porphyrazinium, ZnPc **4**, yield 92%, green solid, melting point >200 °C, ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 4.54 (3H), 8.18–8.21 (6H), 9.07 (1H), 9.20 (1H), 9.29–9.35 (6H), 10.4 (1H), ESI-MS [*m*/*z*] 592.0968 $(M)^+$ (592.0971 calculated for $C_{32}H_{18}N_9Zn$). Zinc (II) tribenzo[*b*:*g*:*l*]-*N*,*N*-dimethylpyrazino[2,3-*q*]porphyrazinium, ZnPc **5**, yield 90%, green solid, melting point >200 °C, ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 4.62 (6H), 7.89 (2H), 8.19–8.21 (6H), 9.30–9.36 (6H), ESI-MS [*m*/*z*] 608.1150 (M^+) (608.1153 calculated for $C_{32}H_{20}N_{10}Zn$). Zinc(II) tribenzo[*b*:*g*:*l*]-*N*,*N*-dimethyl–17,18-dimethylpyrazino[2,3-*q*]porphyrazinium, ZnPc **6**, yield 95%, green solid, melting point >200 °C, ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 4.40 (6H), 8.17–8.20 (6H), 9.30–9.39 (6H), ESI-MS [*m*/*z*] 636.1473 (M^+) (636.1466 calculated for $C_{34}H_{24}N_{10}Zn$).

2.3. Partition coefficient measurements

1-Octanol/water partition coefficients (*P*) were determined at 25 °C using equal volumes of water (1 mL) and 1-octanol (1 mL). Typically, a solution of each phthalocyanine (~10 μM) was stirred in the thermostat after the equilibrium was reached (~6 h). An aliquot (100 μL) of aqueous and organic phases were dissolved in 2 mL of *N*,*N*-dimethylformamide (DMF) and the final phthalocyanine concentration determined by absorption spectroscopy [15].

2.4. Spectroscopic studies

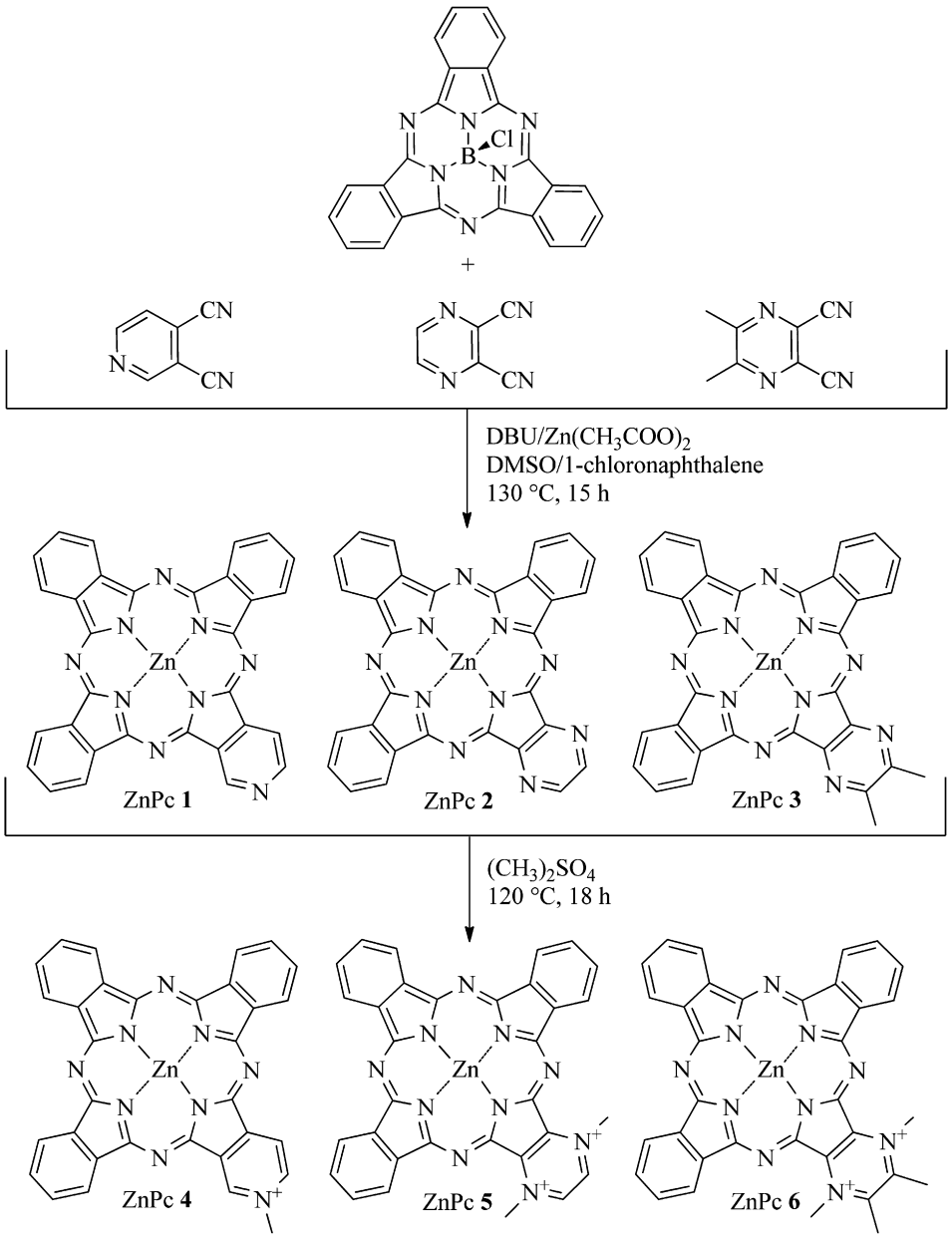
Absorption and fluorescence spectra were recorded at 25.0 ± 0.5 °C using 1 cm path length quartz cells. The fluorescence quantum yield (Φ_F) of phthalocyanines was calculated by comparison of the area below the corrected emission spectrum in DMF with that of Zn(II) phthalocyanine (ZnPc) as a reference ($\Phi_F = 0.28$) [16]. Absorbances of sample and reference were matched at the excitation wavelength (605 nm) and the areas of the emission spectra were integrated in the range 600–800 nm.

2.5. Steady-state photolysis

Solutions of 9,10-dimethylanthracene (DMA, 35 μM) and photosensitizer in different media were irradiated in 1 cm path length quartz cells (2 mL) with monochromatic light at $\lambda_{irr} = 670$ nm (sensitizer absorbance 0.2), from a 75 W high-pressure Xe lamp through a high intensity grating monochromator (Photon Technology Instrument, Birmingham, NJ, USA). The light fluence rate was determined as 1.5 mW/cm^2. The kinetics of DMA photooxidation were studied by following the decrease of the absorbance (A) at $\lambda_{max} = 378$ nm. The observed rate constants (k_{obs}) were obtained by a linear least-squares fit of the semilogarithmic plot of Ln A_0/A *vs.* time. Photooxidation of DMA was used to determine $O_2(^1\Delta_g)$ production by the photosensitizer. ZnPc ($\Phi_\Delta = 0.56$) was used as a reference in DMF [21]. Measurements of the sample and reference under the same conditions afforded Φ_Δ for phthalocyanines 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 10%.

2.6. Microorganism and growth conditions

The strain of *Candida albicans* PC31, recovered from human skin lesion, was previously characterized and identified [7]. Yeast was



Scheme 2. Synthesis of phthalocyanine derivatives **1**–**6**.

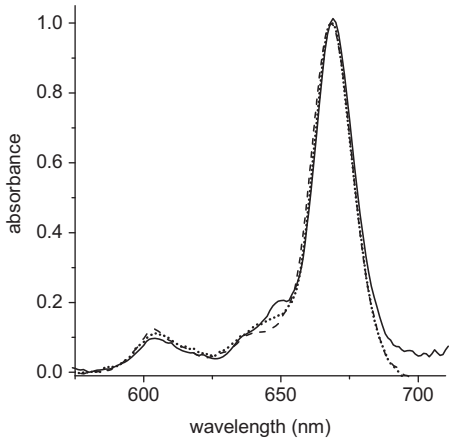


Fig. 1. Absorption spectra of ZnPc **4** (solid line) ZnPc **5** (dashed line) and ZnPc **6** (dotted line) in DMF.

grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (3 mL) at 37 °C to stationary phase. An aliquot of this culture (1 mL) was dissolved in 3 mL Sabouraud broth. Then, 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 $\sim 10^7$ colony forming units (CFU)/mL.

2.7. Photosensitized inactivation of C. albicans

The cells were appropriately diluted to obtain $\sim 10^6$ CFU/mL in PBS. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used and the sensitizer was added from a stock solution ~0.5 mM in DMF. Cellular suspensions of *C. albicans* were incubated with 10 μM phthalocyanine for 30 min in the dark at 37 °C. After that, the cultures were exposed to visible light using the irradiation system described above for different time intervals. Cell suspensions were serially diluted with PBS, each solution was plated in triplicate on Sabouraud agar and the number of colonies formed after ~48 h incubation at 37 °C was counted.

2.8. Growth delay of C. albicans

Cultures of *C. albicans* cells were grown overnight as described above. 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 with 5 μM of sensitizer at 37 °C. The culture grown was measured by turbidity at 550 nm. Then the flasks were irradiated with visible light at 37 °C, as described above.

In all cases, control experiments were carried out without illumination in the absence and in the presence of photosensitizer. Each experiment was repeated separately three times.

3. Results and discussion

3.1. Synthesis of A_3B-phthalocyanines

The A_3B-phthalocyanine derivatives were synthesized by ring expansion of SubPc with 1,2-phthalonitrile derivatives (Scheme 2). The reaction was carried out in the presence of DBU and zinc(II) acetate dihydrate in DMSO/1-chloronaphthalene. First, the SubPc was reacted with 3,4-pyridinedicarbonitrile to obtain ZnPc **1** in 42% yield. The SubPc was also ring expanded with pyrazine-2,3-dicarbonitrile and 5,6-dimethylpyrazine-2,3-dicarbonitrile to afford ZnPc **2** (40%) and ZnPc **3** (37%), respectively.

In this procedure, the SubPc was used as starting material for the formation of unsymmetrically substituted phthalocyanines by ring expansion employing substituted phthalonitriles. In previous studies, the reaction of SubPc with substituted diiminoisoindolenines in the presence of a zinc salt resulted in higher percentages of tetrasubstituted phthalocyanines because diiminoisoindolenines undergo cyclotetramerization with zinc salts [12]. However, the amount of the monosubstituted compounds in the mixtures was substantially increased by the use of 1,2-benzene- or 2,3-naphthalene-dicarbonitrile instead of 1,3-diiminoisoindolenines. This method has advantages over statistical condensation of two differently substituted phthalonitriles derivatives because the latter results in a complex mixture of products.

The structure of ZnPc **1** was the precursor of an unsymmetrical A_3B monocationic phthalonitrile, while ZnPc **2** and **3** were used to produce dicationic phthalocyanines by methylation of annulated 6-membered *N*-heterocycles [15]. Thus, cationic phthalocyanines **4**–**6** were obtained by treating the corresponding ZnPc **1**–**3** with dimethyl sulphate for 18 h at 120 °C (Scheme 2). The exhaustive methylation produces ZnPc **4**–**6** with high yields (90–95%).

ZnPc **4**–**6** contain three identical isoindole units and one pyridinium or pyrazinediium ring. To evaluate the effect produced by this distribution of different polarity groups, the *n*-octanol/water partition coefficients of phthalocyanines (*P*) were determined at 25 °C ($P = [\text{phthalocyanine}]_o/[\text{phthalocyanine}]_w$). This parameter is used to estimate the interaction of photosensitizers with biological systems. It is known that the *n*-octanol/water system mimics rather accurately the water/membrane interface. Thus, Log *P* has been extensively utilized to predict the relative tendency of compounds to interact with biological membranes [22,23]. The results showed values of Log *P* of 0.29, 0.34 and 0.81 for ZnPc **4**, **5** and **6**, respectively. Photosensitizers with these Log *P* values have shown a high intracellular accumulation in human tumour cell lines and the uptake mainly occurs by passive diffusion through the membrane [24].

3.2. Spectroscopic studies

The absorption spectra of ZnPcs **4**–**6** in DMF are gathered in Fig. 1. The spectra show the typical *Soret* and *Q*-bands, characteristic of zinc(II) phthalocyanine derivatives [25]. A sharp absorption band

Table 1
Spectroscopic data, kinetic parameters (k_{obs}) for the photooxidation reaction of DMA and quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ)of ZnPcs **1**–**6** in DMF.

Phthalocyanine	λ_{max}^{abs} (nm)	λ_{max}^{em} (nm)	Φ_F	$k_{obs}^{DMA}(s^{-1})$	Φ_Δ[a]
ZnPc **1**	669	679	0.21 ± 0.01	$(0.80 \pm 0.04) \times 10^{-3}$	0.37 ± 0.03
ZnPc **2**	669	678	0.19 ± 0.01	$(1.10 \pm 0.06) \times 10^{-3}$	0.51 ± 0.04
ZnPc **3**	668	676	0.15 ± 0.01	$(0.43 \pm 0.03) \times 10^{-3}$	0.20 ± 0.02
ZnPc **4**	669	675	0.20 ± 0.01	$(0.46 \pm 0.02) \times 10^{-3}$	0.21 ± 0.02
ZnPc **5**	668	678	0.25 ± 0.01	$(1.01 \pm 0.07) \times 10^{-3}$	0.47 ± 0.03
ZnPc **6**	669	674	0.11 ± 0.01	$(0.60 \pm 0.05) \times 10^{-3}$	0.28 ± 0.02

[a] Using ZnPc as a reference $k_{obs}^{DMA} = (1.20 \pm 0.05) \times 10^{-3} s^{-1}$.

was obtained indicating that there is not aggregation of these phthalocyanines in this organic solvent. The spectroscopic properties of ZnPcs **1**–**6** are summarized in Table 1. In all cases, the shape of *Q*-bands was very similar to that found for ZnPc with only a small hypsochromic shift by ~2 nm [16]. In contrast, the *Q*-bands of ZnTM2,3PyPz is blue shifted by ~30 nm with respect to that of the ZnPc [18]. This indicates that the presence of one annulated 6-membered *N*-heterocycle, pyridine or pyrazine, has only a small perturbation effect on the electronic structure and symmetry of the main porphyrazine π-chromophore, which substantially retains its effective fourfold symmetry [26].

The steady-state fluorescence emission spectra of ZnPcs **4**–**6** were performed in DMF (Fig. 2). Similar results were obtained for ZnPcs **1**–**3** (Table 1). The spectra showed two bands in the red spectral region, which are characteristic for similar zinc(II) phthalocyanines [25]. The position of the maxima is independent on the excitation wavelength. The main emission band for all compounds is assigned to the 0–0 transition of the fluorescence. The fluorescence excitation spectra of these phthalocyanines coincide with the corresponding absorption spectra (Fig. 2 inset). A small Stokes' shift (~5–10 nm) was observed indicating that the spectroscopic energy is nearly identical to the relaxed energy of the singlet state. That suggests that only a minor geometric relaxation occurs in the first excited state. By comparison with ZnPc as a reference, the values of fluorescence quantum yields (Φ_F) were obtained in DMF (Table 1). These results are in agreement with those previously reported for similar phthalocyanines in different media [1].

3.3. Photodynamic properties

Photooxidation of DMA sensitized by phthalocyanine derivatives **1**–**6** was studied in DMF under aerobic conditions. Typical first-order kinetic plots of the DMA absorption at 378 nm with time describing the progress of the reaction are shown in Fig. 3. From these plots the values of the observed rate constant (k_{obs}) were obtained (Table 1). Under these conditions, DMA quenches $O_2(^1\Delta_g)$ exclusively by chemical reaction to form the corresponding endoperoxide. The decomposition of this substrate can be used as a method to evaluate the ability of the photosensitizers to produce $O_2(^1\Delta_g)$ in solution [27]. Thus, the quantum yield of $O_2(^1\Delta_g)$ production ($\Phi\Delta$) was calculated comparing the slope for the

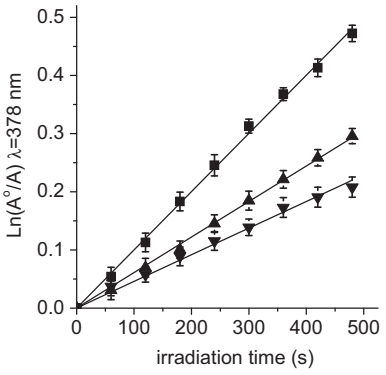


Fig. 3. First-order plots for the photooxidation of DMA (35 μM) photosensitized by ZnPc **4** (▼), ZnPc **5** (■) and ZnPc **6** (▲) in DMF; λ_{irr} = 670 nm. Values represent mean ± standard deviation of three separate experiments.

phthalocyanines with the corresponding slope obtained for the reference ZnPc. The results for Φ_Δ (Table 1) follow the order ZnPc **2** > **1** > **3** for non-charged phthalocyanines, while formation of $O_2(^1\Delta_g)$ of cationic sensitizers was ZnPc **5** > **6** > **4**. These are quite reasonable values for Zn(II) phthalocyanines in this solvent [28]. However, the values of Φ_Δ can significantly change in a different medium, diminishing when the sensitizer is partially aggregated. Also, the biological microenvironment of the sensitizer can induce important modifications in the photophysics of the phthalocyanine established in solution. In consequence, there are limitations to predict photodynamic efficiencies of sensitizers in biological systems on the basic of photophysical investigations in homogeneous solution.

3.4. Studies in vitro on C. albicans cells

The ability of cationic ZnPcs **4**–**6** to inactivate yeast cells was compared with that obtained for a tetracationic ZnTM2,3PyPz. After the treatment of *C. albicans* cells with 10 μM sensitizer for 30 min at 37 °C in dark, the cultures were irradiated with visible light. Control experiments showed that the viability of *C. albicans* was unaffected by illumination alone or by dark incubation with 10 μM of the photosensitizer for 30 min, indicating that the cell mortality

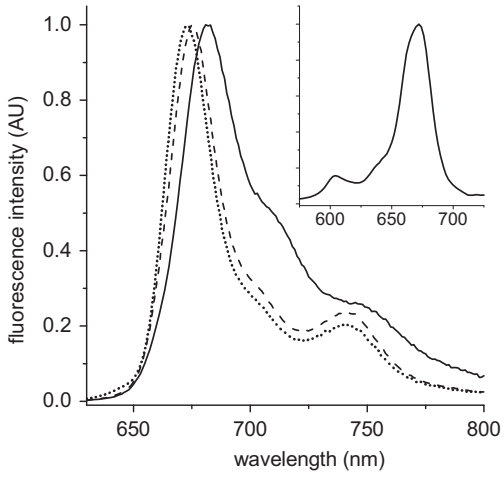


Fig. 2. Fluorescence emission spectra of ZnPc **4** (solid line) ZnPc **5** (dashed line) and ZnPc **6** (dotted line) in DMF, λ_{exc} = 605 nm. Inset: fluorescence excitation spectra of ZnPc **6**, λ_{em} = 750 nm.

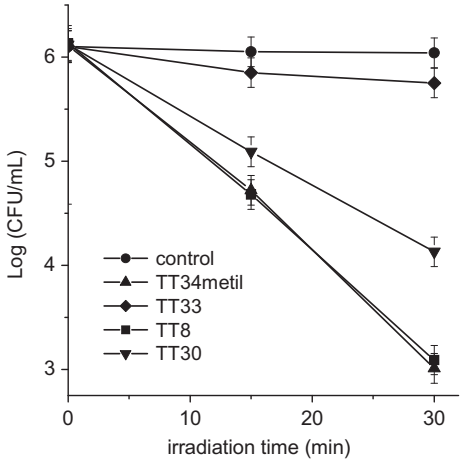


Fig. 4. Survival curves of *C. albicans* (~10^6 CFU/mL) incubated with 10 μM ZnPc **4** (▼), ZnPc **5** (■), ZnPc **6** (▲) and ZnTM2,3PyPz (◆) for 30 min at 37 °C in dark and exposed to visible light for different irradiation times. Control culture untreated with phthalocyanine and irradiated (●). Values represent mean ± standard deviation of three separate experiments.

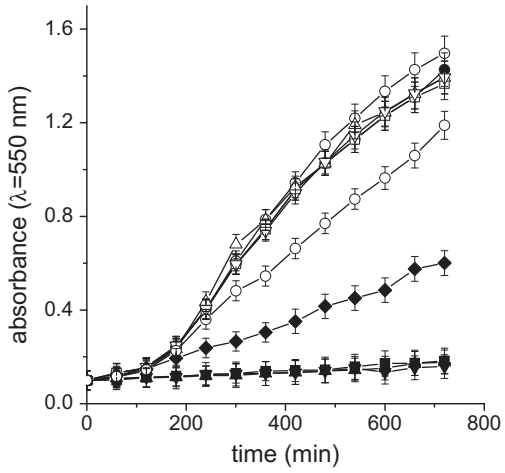


Fig. 5. Growth curves of *C. albicans* incubated with 10 μM ZnPc **6** (▲) and ZnTM2,3PyPz (◆) and exposed to different irradiation times with visible light in Sabouraud broth at 37 °C. Control cultures: cells treated 10 μM ZnPc **6** (△) in dark, cells treated 10 μM ZnTM2,3PyPz (◇) in dark, cells untreated with photosensitizer and irradiated (●) and in dark (○). Values represent mean ± standard deviation of three separate experiments.

obtained after irradiation of the cultures is produced by the photosensitization effect of phthalocyanines. The survival curves at different light exposure levels are shown in Fig. 4. As can be observed, the photoinactivation activities of cationic ZnPcs **5** and **6** are considerably higher than that found for ZnPc **4**. The photodynamic activity induced by ZnPc **6** produces a ~3 Log decrease of *C. albicans* cell survival, when the cultures are irradiated for 30 min. This decrease in cell survival represents ~99.94% of cell inactivation. Similar cytotoxicity results were observed for cultures sensitized by ZnPc **5**, while a ~2 Log decrease was found using monocationic ZnPc **4**. On the other hand, under these conditions a lower decrease in the cellular viability of *C. albicans* was found using tetracationic ZnTM2,3PyPz as the photosensitizer.

The photocytotoxic activity on the growth of *C. albicans* cultures sensitized by these phthalocyanines was achieved in Sabouraud medium to ensure that photosensitization was still possible when the cells were not under starvation conditions or the potential damaging effects of phosphate buffer washing. Thus, 10 μM sensitizer was added to fresh cultures of *C. albicans* reaching the Log phase and the flasks were irradiated with visible light at 37 °C. The effect induced by these phthalocyanines on growth of cells is shown in Fig. 5. Cells of *C. albicans* treated with ZnPcs **4**–**6** in the dark or not treated with sensitizer and illuminated showed no growth delay compared with control. On the contrary, growth was delayed when *C. albicans* cultures were treated with ZnPcs **4**–**6** and illuminated. After irradiation in the presence of sensitizer **4**–**6**, the cells no longer appeared to be growing as measured by turbidity at 550 nm. In the case of ZnTM2,3PyPz, this compound was toxic in the dark and the delay in cell growth increases when cultures are irradiated with visible light. However, the effect produced by ZnTM2,3PyPz is slower than those produced by amphiphilic phthalocyanines.

4. Conclusions

In summary, novel unsymmetrically substituted A_3B-phthalocyanine derivatives were synthesized by ring expansion reaction of SubPc. These macrocycles present a different pattern of substitution by annulations of 6-membered *N*-heterocycles, pyridine and pyrazine. To obtain A_3B-phthalocyanine, the geometrically constrained SubPc (A_3) was reacted with 1,2-phthalonitrile derivatives (B), in presence of DBU and zinc(II) acetate dihydrate. This approach produces selectively asymmetric type A_3B macrocycles in moderate yields. The annulated 6-membered *N*-heterocycles present in these compounds are precursors of cationic groups by methylation. The region of the phthalocyanine most distant from the charged cationic groups acts as the hydrophobic region, which can be used to obtain amphiphilic photosensitizers. Spectroscopic and photodynamic properties in solution indicate that cationic ZnPcs **4**–**6** are candidate agents to produce photocytotoxicity in biological media. Antifungal activity on *C. albicans* cells photoinduced by cationic ZnPcs **4**–**6** showed that the number of charges on the periphery of the macrocycle is important to increase the photoinactivation of yeasts. However, the activity decreases when a highly soluble tetracationic ZnTM2,3PyPz was used as sensitizer. Therefore, the results indicate that amphiphilic cationic phthalocyanines, such as **5** and **6**, represent interesting agents with potential applications in photodynamic inactivation of microorganisms.

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