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Synthesis and properties of 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl] chlorin as potential broad-spectrum antimicrobial photosensitizers

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ABSTRACT

A novel 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]chlorin (TAPC) was synthesized by reduction of the corresponding porphyrin TAPP with *p*-toluenesulfonhydrazide, followed by selective oxidation with *o*-chloranil. Spectroscopic properties and the photodynamic activity of these photosensitizers were compared in *N*,*N*-dimethylformamide. An increase in the absorption band at 650 nm was found for the chlorin derivative with respect to TAPP. These photosensitizers emit red fluorescence with quantum yields of 0.15. Both compounds were able to photosensitize singlet molecular oxygen with quantum yields of about 0.5. Also, the formation of superoxide anion radical was detected in the presence of TAPC or TAPP and NADH. Photodynamic inactivation was investigated on a Gram-positive bacterium *Staphylococcus aureus*, a Gram-negative bacterium *Escherichia coli* and a fungal yeast *Candida albicans* cells. *In vitro* experiments showed that TAPC or TAPP were rapidly bound to microbial cells at short incubation periods. These photosensitizers, without intrinsic positive charges, contain four basic amino groups. These substituents can be protonated at physiological pH, increasing the interaction with the cell envelopment. Photosensitized inactivation improved with an increase of both photosensitizer concentrations and irradiation times. After 15 min irradiation, a 7 log reduction of *S. aureus* was found for treated with 1 μM photosensitizer. Similar result was obtained with *E. coli* after using 5 μM photosensitizer and 30 min irradiation. Also, the last conditions produced a decrease of 5 log in *C. albicans* cells. Therefore, TAPC was highly effective as a broad-spectrum antimicrobial photosensitizer.

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1. Introduction

The use of antibiotics to destroy selectively microorganisms suggests one of the most revolutionary progresses made in scientific medicine. However, the advent of multiple drug resistance in microbes has posed new challenge to researchers [1]. Well known resistance carriers with high clinical impact include the Gram-positive organism *Staphylococcus aureus*. In contrast to this bacteria that is usually still treatable with newer alternative antibacterial drugs, some Gram-negative bacteria, especially *Escherichia coli*, have developed resistance to most or all available antibiotics [2]. Also, fungal infections have increased significantly in recent years [3]. *Candida albicans* is known as an opportunistic fungal microorganism that can cause skin infections. Inadequate dosing of antifungal agents has contributed to treatment failure and the emergence of resistance [4]. The scientists are now evaluating alternatives for combating infectious diseases [1]. Thus, photodynamic inactivation (PDI) of microorganisms represents an interesting alternative to eradication of microorganisms [5]. PDI involves the addition of a phototherapeutic agent, which is rapidly bound to cells. The aerobic irradiation of the infection with visible light produces highly reactive oxygen species (ROS) that react with a variety of biomolecules. These reactions induce a loss of biological functionality leading to cell inactivation [6]. Therefore, PDI may be a promising treatment modality for several localized infections [5].

In this sense, discovery of an appropriate photosensitizer is decisive in improving the efficacy of PDI. A large number of potential photosensitizers have been proposed for different microorganisms [6,7]. The properties of chlorins often satisfy the demands of a good photosensitizer, and therefore they are strong candidates for better photosensitizers. In some cases, *in vivo* applications of porphyrins are hampered by wavelengths with limited tissue penetration. However, tetrapyrrolic macrocycles can be converted to the corresponding chlorin derivatives, which show an intense absorption band at 650 nm and high singlet molecular oxygen, $O_2(^1\Delta_g)$, generation. Thus, for example chlorins are highly effective to produce photohemolysis of human red blood cells in comparison with its porphyrin homologs [8]. Moreover, chlorin e6 has been used as a second-generation photosensitizer for photodynamic

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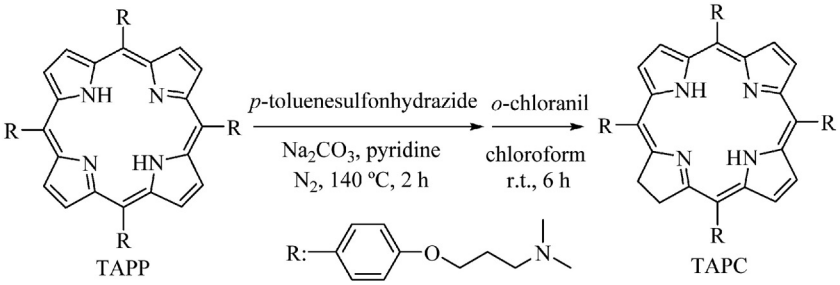

Scheme 1. Synthesis of TAPC from TAPP.

therapy of neoplastic diseases [9]. In particular, chlorin e6 was covalently conjugated to polycation chains to efficiently inactivate bacterial and yeast cells [10]. Also, a synthetic cationic chlorin and bacteriochlorin can be regarded as promising for the treatment of bacterial infections under red light [11,12].

In this study, 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]chlorin (TAPC) was obtained from the corresponding porphyrin derivative TAPP. This chlorin contains four basic amino groups in the periphery of the macrocycle, which can be protonated at physiological pH [13]. In previous investigations, it was demonstrated that this intrinsically non-charged porphyrin TAPP represents an interesting photosensitizer in aqueous solutions [14]. Therefore, we are interested in comparing the spectroscopic properties and photodynamic activity of TAPC with those of TAPP in solution. Moreover, the PDI efficacy of TAPC and TAPP was evaluated against a Gram-positive *S. aureus*, a Gram-negative *E. coli* and a yeast *C. albicans*. The results were used to establish conditions for the eradication of microorganisms mediated by these photosensitizers.

2. Materials and methods

2.1. General

Proton nuclear magnetic resonance spectra were taken on a FT-NMR Bruker Avance DPX400 spectrometer at 400 MHz. Mass spectra were taken with a Bruker micrO-TOF-QII (Bruker Daltonics, MA, USA) equipped with an atmospheric pressure photoionization (APPI) source. UV–visible absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). Fluorescence spectra were performed on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). Cell growth was measured with a Turner SP-830 spectrophotometer (Dubuque, IA, USA). Fluence rates were obtained with a Radiometer Laser Mate-Q (Coherent, Santa Clara, CA, USA). Chemicals from Aldrich (Milwaukee, WI, USA) were used as received. Thin-layer chromatography (TLC) was carried out on the silica gel plates (250 μm) from Analtech (Newark, DE, USA).

2.2. Photosensitizers

5,10,15,20-Tetrakis(4-methoxyphenyl)porphyrin (TMPP) was purchased from Aldrich. 5,10,15,20-Tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (TAPP) was obtained as previously described [15]. 5,10,15,20-Tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]chlorin (TAPC) was synthetized by dissolving TAPP (29 mg, 0.028 mmol) and Na_2CO_3 (0.85 g, 0.008 mol) in 20 mL pyridine. The mixture was purged with argon for 15 min. Then, a solution of de *p*-toluenesulfonhydrazide in pyridine (4 mL, 0.4 M) was added and the reaction mixture was stirred at 140 °C for 2 h. The formation of chlorin and bacteriochlorin derivatives was detected by UV–visible spectroscopy following the appearance of the bands at 650 and 740 nm, respectively. The solution was cooled at room temperature and treated with 40 mL toluene/water (1:3). The organic phase was separated and washed with a saturated solution of $NaHCO_3$ in water. The organic solvent was evaporated, the solid was redissolved in 10 mL chloroform and treated with *o*-chloranil (17 mg, 0.069 mmol) for 6 h at room temperature. The reaction was monitored by TLC and UV–visible spectroscopy until the disappearance of the bacteriochlorin absorption band at 740 nm. The product was purified by flash column chromatography (silica gel, chloroform/triethylamine (TEA) 2%/methanol 10–30% gradient) yielded 25 mg (87%) of the pure TAPC. TLC analysis (chloroform/methanol 15%/TEA 2%) R_f = 0.62. ^{1}HNMR ($CDCl_3$, TMS) δ [ppm] −1.46 (bs, 2H, NH-pyrrole), 1.98 (m, 8H, $-CH_2$-), 2.28 (s, 24H, $-N(CH_3)_2$), 2.50 (t, 8H, J = 6.5 Hz, $-CH_2$-N), 4.13 (s, 4H, CH-pyrrole), 4.27 (t, 8H, J = 7.0 Hz, $-OCH_2$-), 7.18 (d, 4H, J = 8.4 Hz, Ph), 7.19 (d, 4H, J = 8.4 Hz, Ph), 7.74 (d, 4H, J = 8.6 Hz, Ph), 7.98 (d, 4H, J = 8.6 Hz, Ph), 8.17 (d, 2H, J = 4.7, CH-pyrrole), 8.41 (s, 2H, CH-pyrrole), 8.56 (d, 2H, J = 4.7 Hz, CH-pyrrole). APPI-MS [m/z] 1021.6068 $(M + H)^+$ (1020,5990 calculated for $C_{64}H_{76}N_8O_4$).

2.3. Spectroscopic studies

UV–visible absorption and fluorescence spectra were recorded in a quartz cell of 1 cm path length at 25.0 ± 0.5 °C. The fluorescence quantum yield (Φ_F) of the photosensitizers was determined by comparison of the area below the corrected emission spectrum in DMF. TMPP was used as a reference ($\Phi_F = 0.14$) in DMF [16]. The absorbance of the sample and reference (<0.05) were matched at the excitation wavelength (522 nm) and the areas of the emission spectra were integrated in the range 600–800 nm.

2.4. Partition coefficient measurements

1-Octanol/water partition coefficients (P_{ow} = [photosensitizer]$_o$/[photosensitizer]$_w$) were determined at 25 °C using equal volumes of water (0.5 mL) and 1-octanol (0.5 mL). A solution of each

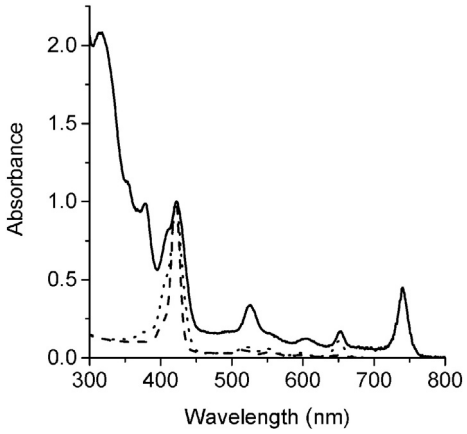

Fig. 1. Absorption spectra of reaction mixtures (solid line), TAPP (dashed line) and TAPC (dotted line) in DMF.

photosensitizer (~50 μM) was stirred in the thermostat after the equilibrium was reached (2 h). An aliquot (100 μL) of aqueous and organic phases were dissolved in 2 mL of DMF and the final photosensitizer concentrations were determined by absorption spectroscopy [17].

2.5. Steady state photolysis

Solutions of 9,10-dimethylanthracene (DMA, 35 μM) and photosensitizer in DMF were irradiated in 1 cm path length quartz cells (2 mL) with monochromatic light at $\lambda_{irr} = 515$ nm (photosensitizer absorption 0.1 at 515 nm). The light source was a Cole–Parmer illuminator 41720-series (Cole–Parmer, Vernon Hills, IL, USA) with a 150 W halogen lamp through a high intensity grating monochromator (Photon Technology Instrument, Birmingham, NJ, USA). The light fluence rate was measured given a value of 0.35 mW/cm^2. The photooxidation rate of DMA was studied by following the decrease of the absorbance (A) at $\lambda_{max} =$ 378 nm. The observed rate constants (k_{obs}) were determined by a linear least-squares fit of the semilogarithmic plot of Ln A_0/A *vs.* time. Quantum yields of $O_2(^1\Delta_g)$ production (Φ_Δ) were calculated comparing the k_{obs} for the corresponding photosensitizer with that for TAPP, which was used as a reference ($\Phi_\Delta = 0.53$) [14]. Determinations of k_{obs} values for the sample and reference under the same conditions afforded Φ_Δ by direct comparison of the slopes in the linear region of the plots.

2.6. Detection of superoxide anion radical

The nitro blue tetrazolium (NBT) was carried out using 0.2 mM NBT, 0.5 mM NADH and photosensitizer (absorbance 0.3 at Soret band) in 2 mL of DMF/water (9/1) [18,19]. Control experiments were performed in the absence of NBT, NADH or photosensitizer. Samples were irradiated in 1 cm path length quartz cells under aerobic condition with the Cole–Parmer illuminator 41720 source described above through a 2.5 cm glass cuvette filled with water (44 mW/cm^2). The decomposition of NBT was examined by following the increase of the absorbance at $\lambda = 560$ nm.

2.7. Microorganisms and growth conditions

The microorganisms used in this study were the strains of *S. aureus* ATCC 25923, *E. coli* (EC7) and *C. albicans* (PC31), which were previously characterized and identified [20,21]. Microbial cells were grown aerobically in sterile condition overnight at 37 °C in 4 mL tryptic soy or Sabouraud (Britania, Buenos Aires, Argentina) broths for cultures of bacteria or yeast, respectively. An aliquot (60 μL) of the bacterial culture was aseptically transferred to 4 mL of fresh tryptic soy broth and incubated at 37 °C to exponential phase of growth (absorbance 0.7 at 660 nm). Cells were centrifuged (3000 rpm for 15 min) and re-suspended in equal amount of 10 mM phosphate-buffered saline (PBS, pH = 7.4) solution, corresponding to ~10^8 colony forming units (CFU)/mL. After overnight cultures of *C. albicans*, cells were harvested by centrifugation (3000 rpm for 15 min) and re-suspended in PBS. Yeast cells (absorbance 0.5 at 650 nm) were diluted 1:4 in PBS to obtain ~10^6 CFU/mL. After each assay, cell suspensions were serially diluted 10-fold in PBS. From each dilution 20 μL aliquots were streaked horizontally on tryptic soy or Sabouraud agar plates in sextuplicate. Also, cell suspensions were quantified by the spread plate counting method in triplicate for estimating the survival rate of microorganisms. Both techniques showed no significant difference in the results. Viable microbial cells were monitored and the number of CFU was determined after ~24 h (bacteria) or ~48 h (*C. albicans*) incubation at 37 °C in the dark.

2.8. Photosensitizer binding to microbial cells

Photosensitizer was added from a stock solution (~0.5 mM) in DMF. Microbial cell suspensions (2 mL, ~10^8 CFU/mL bacteria and ~10^6 CFU/mL yeast) in PBS were incubated with 5 μM photosensitizer for different times (2–30 min) in the dark at 37 °C in Pyrex culture tubes (13 × 100 mm). Cell suspensions were centrifuged at 3000 rpm for 10 min. Pellets were re-suspended in 2 mL of 2% aqueous SDS, incubated overnight at 4 °C and sonicated for 30 min. The concentration of photosensitizer in the supernatant was determined by spectrofluorimetry (TAPC: $\lambda_{exc} = 420$ nm, $\lambda_{em} = 660$ nm; TAPP: $\lambda_{exc} = 420$ nm, $\lambda_{em} =$ 650 nm). The fluorescence intensities of each sample were referred to

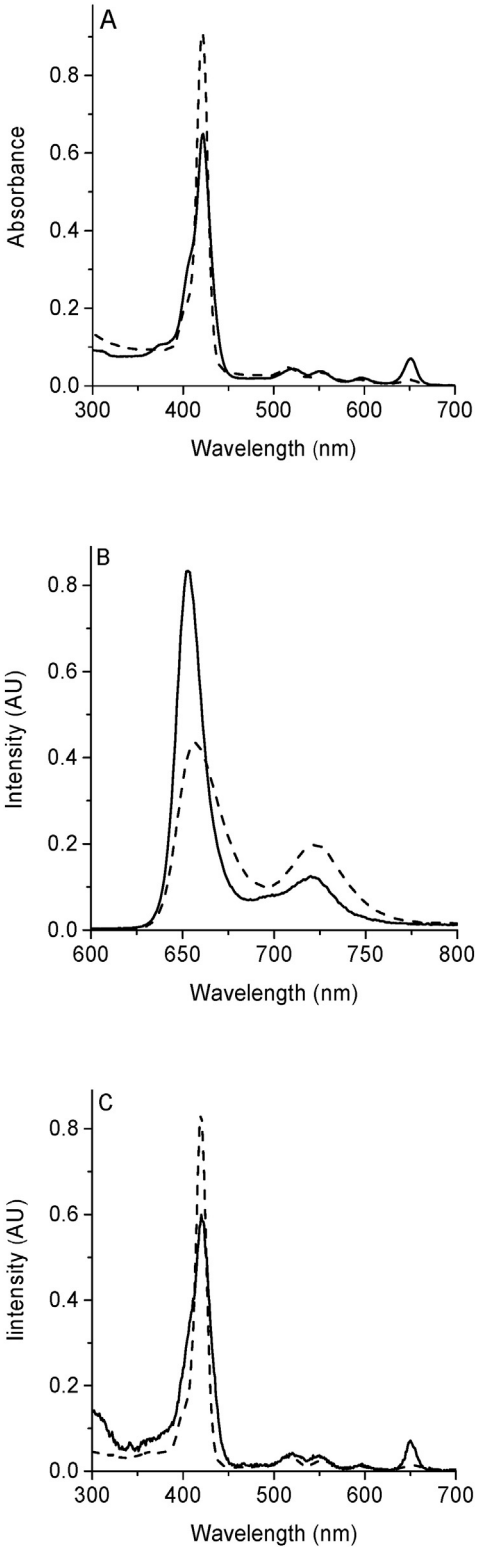

Fig. 2. (A) Absorption, (B) fluorescence emission ($\lambda_{exc} = 520$ nm) and (C) excitation ($\lambda_{em} = 721$ nm) spectra of TAPC (solid line) and TAPP (dashed line) in DMF.

Table 1
Spectroscopic and photodynamic properties of TAPP and TAPC in DMF.

Photosensitizer	ε^{Soret}[a]	ε^{QI}[a]	E_s (eV)[b]	Φ_F[c]	k_{obs}^{DMA} (s^{-1})[d]	Φ_Δ[e]
TAPP	1.64×10^5	2.9×10^3	1.90	0.15 ± 0.02	$(3.1 \pm 0.1) \times 10^{-4}$	0.53 ± 0.02
TAPC	1.15×10^5	12.5×10^3	1.90	0.15 ± 0.02	$(3.2 \pm 0.1) \times 10^{-4}$	0.54 ± 0.02

[a] Molar absorption coefficient (L mol^{-1} cm^{-1}).
[b] Energy levels of the singlet excited stated.
[c] Fluorescence quantum yield.
[d] Observed rate constants for the photooxidation reaction of DMA.
[e] Quantum yield of $O_2(^1\Delta_g)$ production.

the total number of cells. The concentration of the photosensitizer in the solution was calculated by comparison with a calibration curve obtained with standard solutions (0.05–0.2 μM) of the photosensitizer in 2% SDS. Microscopic observations and photographs were performed using a Zeiss Axiophot (Carl Zeiss, Oberkochen, Germany) fluorescence microscope equipped with a HBO 100 W mercury lamp. Images were captured using an AxioCam HRc camera and subsequently processed using AxioVision Rel. 4.3 software. Fluorescence images of TAPC in microbial cells were observed using a DBP 406/23 + 530/45, DFT 435 + 570, DBP 467/30 + 618/75 filter (Carl Zeiss).

2.9. Photosensitized inactivation of microorganisms

Cell suspensions were previously treated as described above with different photosensitizer concentrations (1–5 μM) for 30 min in the dark at 37 °C. Then, 200 μL of each cell suspension was transferred to the 96-well microtiter plates (Deltalab, Barcelona, Spain). Cells were exposed for different time intervals (2–15 min for *S. aureus* and 2–30 min for *E. coli* and *C. albicans*) to visible light. Plates were irradiated with a Novamat 130 AF (Braun Photo Technik, Nürnberg, Germany) slide projector containing a 150 W lamp. A 2.5 cm glass cuvette filled with water was used to remove the heat from the lamp. A wavelength range between 350 and 800 nm was selected by optical filters (90 mW/cm^2). Cell viability was determined as described above.

2.10. Controls and statistical analysis

Control experiments were performed in the presence and absence of photosensitizer in the dark and in the absence of photosensitizer with cells irradiated. The amount of DMF (<1% *v*/v) used in each experiment was not toxic to microbial cells. Three values were obtained per each condition and each experiment was repeated separately three times. The unpaired *t*-test was used to establish the significance of differences between groups. Differences between means were tested for significance by one-way ANOVA. Results were considered statistically significant with a confidence level of 95% ($p < 0.05$). Data were represented as the mean ± standard deviation of each group.

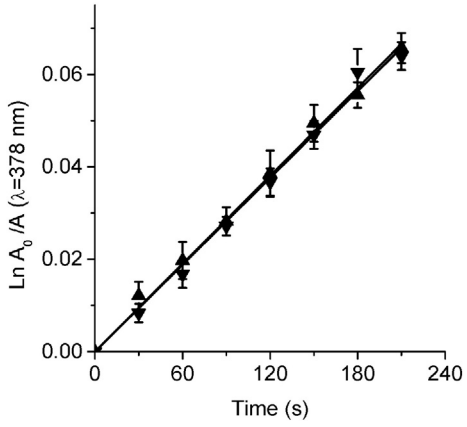

Fig. 3. First-order plots for the photooxidation of DMA photosensitized by TAPC (▲), TAPP (▼) and TMPP (■) in DMF.

3. Results

3.1. Synthesis of TAPC

A chlorin derivative TAPC was synthesized from the analogous porphyrin TAPP, as shown in Scheme 1. TAPP was treated with *p*-toluenesulfonhydrazide to produce the reduction of the tetrapyrrolic macrocycle. This procedure generated a mixture of TAPC and the corresponding bacteriochlorin. The reaction was maintained until the relationship between the absorbance of the bacteriochlorin band at 740 nm and chlorin Soret band 421 nm remained constant. Fig. 1 shows the absorption changes at the beginning and end of the reaction. After that, bacteriochlorin derivative was selectively oxidized with *o*-chloranil to obtain TAPC. The reaction mixture in chloroform was stirred until the disappearance of the bacteriochlorin absorption band at 740 nm. Using this approach, TAPC was obtained in a yield of 87%. This new synthetic chlorin contains four amino groups linked through an aliphatic spacer to the macrocycle.

3.2. Absorption and fluorescence spectroscopic characterization

Fig. 2A shows the absorption spectra of TAPC and TAPP in DMF. Both spectra are characterized by an intense Soret absorption band at 421 nm and four Q-bands in the visible region between 500 and 700 nm. The free-base porphyrin has the characteristic spectra of the *etio* type [16]. In contrast to TAPP, the Q-bands intensities of TAPC follow the sequence $\varepsilon_I > \varepsilon_{VI} \cong \varepsilon_{III} > \varepsilon_{II}$. Molar absorption coefficient values are summarized in

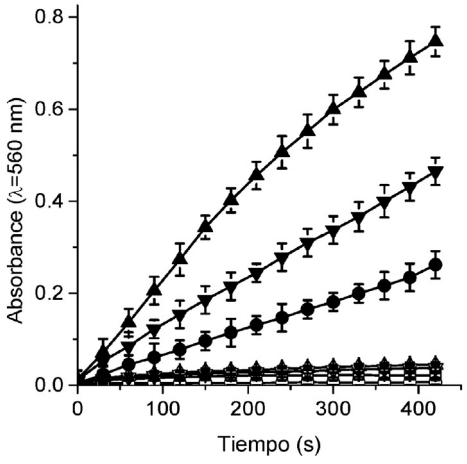

Fig. 4. Time course of $O_2^{\bullet-}$ generation detected by the NBT method as an increase in the absorption at 560 nm in DMF. Samples contain: TAPC, NTB and NADH (▲); TAPP, NTB and NADH (▼); TAPC and NTB (△); TAPP and NBT (▽); TAPC and NADH (□); TAPP and NADH (○); NADH and NBT (●) in DMF; [NBT] = 0.2 mM and [NADH] = 0.5 mM.

Table 1. Moreover, sharp absorption bands were obtained indicating that these photosensitizers were mainly dissolved as monomer in DMF.

The steady-state fluorescence emission spectra of TAPC and TAPP were studied in DMF. As shown in Fig. 2B, spectra present two bands around 650 and 725 nm, which are characteristic of chlorin and porphyrin derivatives [16,22]. These bands have been assigned to $Q_x(0–0)$ and $Q_x(0–1)$ transitions. Stokes shifts were calculated from the intersection of the absorption and fluorescence spectra of the $Q_x(0–0)$ band. Both photosensitizers exhibited small fluorescence Stokes shifts of 2 and 7 nm for TAPP and TAPC, respectively. Also, taking into account the energy of the 0–0 electronic transitions the energy levels of the singlet excited stated (E_s) were calculated (Table 1) from the intersection of the normalized absorption and fluorescence spectra. Fluorescence quantum yields (Φ_F) of these compounds were determined by comparing with TMPP as a reference (Table 1). The fluorescence emission was used to determine the amount of photosensitizer bound to microbial cells. Also, the fluorescence excitation spectra of TAPC and TAPP were recorded in DMF (Fig. 2C), following the emission at 721 nm. Comparing Fig. 2A and C, it can be observed that the excitation spectra of these photosensitizers are similar to the absorption spectra. Therefore, TAPC and TAPP were mainly dissolved as monomers in this organic solvent.

3.3. Log P_{OW} measurements

Partition coefficients of TAPC and TAPP between *n*-octanol/water were determined evaluated at 25 °C. This parameter can be used to evaluate the interaction of photosensitizers with biological systems [23]. Values of log P_{ow} of 1.13 and 1.67 were obtained for TAPC and TAPP, respectively. The lipophilic character was slightly larger for TAPP than that of chlorin analogous.

3.4. Photooxidation of DMA

Photodynamic activity mediated by TAPC and TAPP was first studied in the presence of DMA under aerobic condition in DMF. Photooxidation of this substrate was observed by the decay in the absorption at 378 nm. The results showed a first-order kinetic behavior (Fig. 3). From these plots, the values of the observed rate constant (k_{obs}^{DMA}) were calculated (Table 1). The kinetic data of DMA decomposition were used to estimate the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) since this compound quenches $O_2(^1\Delta_g)$ by chemical reaction [24]. Similar values of Φ_Δ were obtained for TAPC and TAPP in DMF (Table 1). The generation of $O_2(^1\Delta_g)$ by TAPC was consistent with those previously reported for these types of photosensitizers [25].

3.5. Photodecomposition of NBT

The reduction of NBT to diformazan was examined following the increase in absorption at 560 nm (Fig. 4) [18]. Changes in the absorption were monitored as a function of time after irradiation of samples with visible light under different conditions. For both photosensitizers, reduction of NBT by $O_2^{\bullet-}$ was negligible in the absence of NADH. An increase in NBT decomposition was found in the presence of TAPC and NADH with respect to solution without the photosensitizer derivative. Also, diformazan formation was observed in solution containing TAPP and NADH. Therefore, there is a contribution of the photodynamic activity mediated by TAPC or TAPP in the diformazan production in the presence of NADH.

3.6. Binding of photosensitizers to microbial cells

The ability of TAPC and TAPP to bind to microbial cells was determined in *S. aureus*, *E. coli* and *C. albicans* suspensions in PBS. Cell densities of ~10^8 CFU/mL and ~10^6 CFU/mL were used for bacteria and yeast, respectively. Cells were treated with 5 μM photosensitizer for different periods in the dark at 37 °C. The photosensitizer bound to the cells was quantified by fluorescence. The amount of photosensitizer recovered from cells after each incubation time is showed in Fig. 5. TAPC and TAPP were rapidly bound to cells, reaching high binding values after 2 min incubation. For *S. aureus*, the behavior of both macrocycles was very similar and the amount of recovered molecules was ~0.06 nmol/10^8 cells. In the case of *E. coli*, the binding of TAPC was similar to that obtained in *S. aureus*. The uptake of TAPP by *E. coli* cells was slightly lower than the chlorin derivative reaching a value of ~0.05 nmol/10^8 cells. When *C. albicans* cells were incubated with 5 μM photosensitizer, the quantity of TAPC recovery was 0.9 nmol/10^6 cells, while TAPP achieved a value of 1.1 nmol/10^6 cells. Moreover, the amount of cell-bound photosensitizer was not appreciably changed incubating the yeast cells for longer times. These results reflect the high

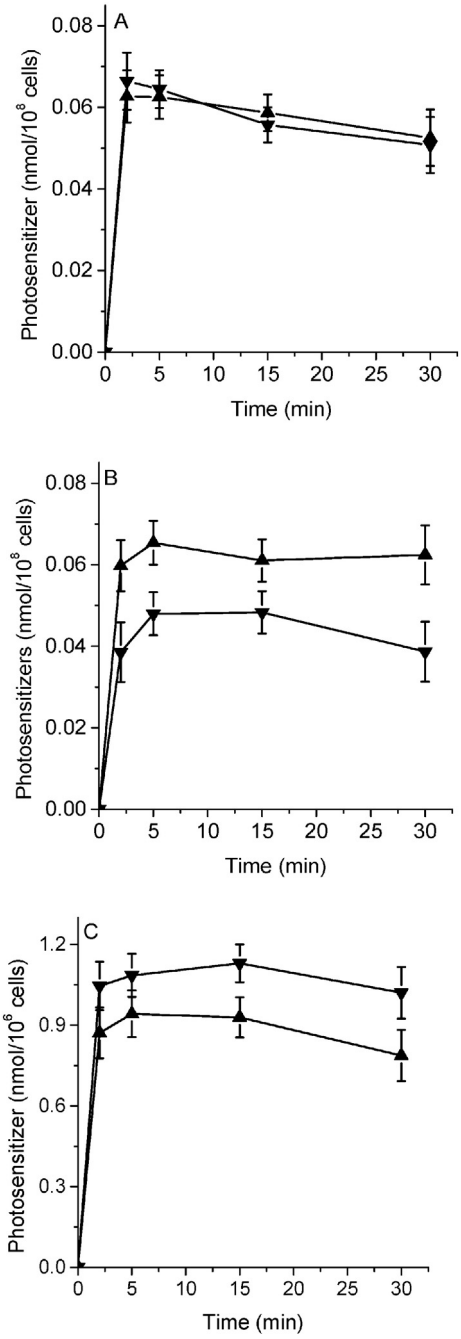

Fig. 5. Amount of TACP (▲) and TAPP (▼) recovered from (A) *S. aureus* (~10^8 CFU/mL), (B) *E. coli* (~10^8 CFU/mL) and (C) *C. albicans* (~10^6 CFU/mL) treated with 5 μM photosensitizer for different incubation times at 37 °C in the dark.

affinity between these two photosensitizers and the microbial cells. Moreover, the cellular localization of TAPC in microbial cells was investigated by fluorescence microscopy (Fig. 6). Images show that cells incubated with 5 μM TAPC in PBS for 30 min in the dark exhibited red fluorescence typical of chlorin derivatives.

3.7. Photosensitized inactivation of microorganisms

Photosensitized inactivation of *S. aureus*, *E. coli* and *C. albicans* was investigated in PBS cell suspensions incubated with different photosensitizer concentrations (1 and 5 μM). Survival curves are summarized in Fig. 7. No toxicity was found for the cells treated with these concentrations of photosensitizer for 30 min in the dark (result no shown). Moreover, the viability of microbial cells was not affected by irradiation without photosensitizer. Photoinactivation of microorganisms was dependent on photosensitizer concentrations and irradiation times. *S. aureus* (Fig. 7A) was the most susceptible strain, achieving a complete eradication after 15 min irradiation with 1 μM photosensitizer. Similar result was also obtained using 5 μM TAPC and a short irradiation time of 2 min. The difference between both photosensitizers was mainly noted at this period of irradiation. Moreover, 5 μM TAPC produced a photoinactivation of *S. aureus* similar to those found for TMPP (Fig. 7A). In contrast, *E. coli* cells were the most difficult to eradicate (Fig. 7B). A concentration of 1 μM photosensitizer produced a reduction of 5 log in the survival after 30 min irradiation. It was necessary 5 μM photosensitizer and 30 min irradiation to obtain complete eradication of *E. coli*. Under this condition, the photodynamic activity mediated by both photosensitizers was very similar. However, photoinactivation mediated by 5 μM TMPP only induced a 2.5 log decrease in *E. coli* survival after 15 min irradiation. On the other hand, Fig. 7C shows the photodynamic activity induced by TAPC and TAPP in *C. albicans*. At the lowest concentration used of TAPC (1 μM), the photodynamic effect yielded a ~ 5 log decrease in the cell viability after 30 min irradiation. For cells treated with 5 μM TAPC, a fast decrease of *C. albicans* survival (~5.5 log) was detected after 2 min irradiation with visible light. At short irradiation times there was a slightly higher photoinactivation activity of TAPC than TAPP. In contrast, photocytotoxic effect mediated by 5 μM TMPP produced a 3.2 log reduction in the survival of the yeast cells afters 15 min irradiation.

4. Discussion

Photosensitizers substituted by cationic groups have attracted significant interest because of their notable ability as phototherapeutic agents against several microorganisms [6]. The chlorin derivative TAPC was synthetized by reduction of TAPP with diimide followed by selective oxidation with *o*-chloranil. These two steps approach were previously used to obtain chlorins from symmetrically substituted porphyrins [22,26]. TAPC presents four basic amine groups in the periphery of the macrocycle [13]. These precursors of cationic centers are spaced from the chlorin macrocycle by an aliphatic chain, which provides a higher mobility of these substituents. These basis amine groups in the periphery can acquire positive charges favoring a better interaction with microbial cell envelope, depending on the medium in which the macrocycle is located [14]. Furthermore, the positive charges have a minimal influence on photophysical properties of the macrocycle due to the aliphatic spacer.

The chlorin derivative exhibited an increase in absorbance of the $Q_x(0–0)$ band, showing an intense absorption in the phototherapeutic window [8,22]. Also, TAPC showed two bands of red emission and it was equally fluorescent than TAPP. The results are consistent with those previously reported for similar molecular structures [16]. Small Stokes shift indicated that in TAPC the spectroscopic energy was similar to the relaxed energies of the singlet state. Thus, only minor structural changes occur between its ground and excited states, according to the rigid planar structure of the chlorin macrocycle.

The formation of ROS mediated by a photosensitizer can occur via two mechanisms [7]. After light activation of the photosensitizer, this can react with molecules from its direct environment by electron or hydrogen transfer, leading to the production of radicals (type I reaction), or it can transfer its energy to oxygen generating $O_2(^1\Delta_g)$ (type II reaction). Therefore, one important feature of the photosensitizers is to present high values of Φ_Δ. The production of $O_2(^1\Delta_g)$ of TAPC was very similar to that of TAPP in DMF. Also, the Φ_Δ of TAPC agrees with those previously reported for free-base chlorins [25]. On the other hand, the formation of $O_2^{\bullet-}$ through a type I photoreaction process mediated by TAPC was observed in DMF. Therefore, whereas $O_2(^1\Delta_g)$ can be generated effectively by photoexcited chlorin, it was found that $O_2^{\bullet-}$ was produced especially in the presence of a physiological concentration of a reductant, such as NADH. Moreover, the cellular microenvironment where the photosensitizer is located can produce important modifications in the photophysics of TAPC determined in solution. Thus, dependent on experimental conditions, both $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$ must be involved in the photodynamic activity. Furthermore, even if an excited photosensitizer reacts with a given substrate by type I photoprocess, the final result is also the oxidation of essential biomolecules. In a biological medium, both mechanisms can occur simultaneously and the ratio between the two processes is influenced by the photosensitizer, substrates and the nature of the environment [27]. It was previously observed that the killing of *C. albicans* cells by these cationic porphyrins and visible light irradiation seem to be mediated mainly by $O_2(^1\Delta_g)$ [28]. Although in a minor contribution, the participation of other active oxygen species could not be neglected particularly for *C. albicans* photoinactivated with 5,10,15,20-tetrakis(4-*N*,*N*,*N*-

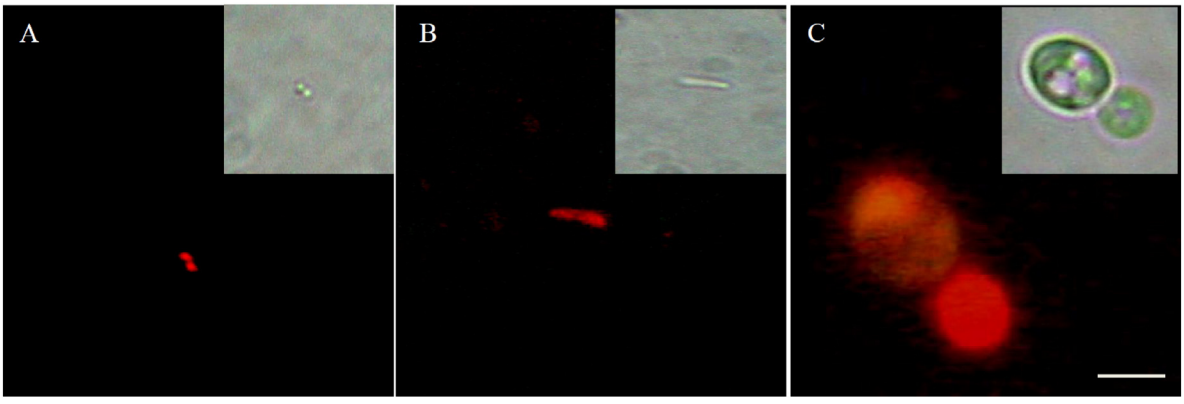

Fig. 6. Fluorescence microscopic observation of (A) *S. aureus*, (B) *E. coli* and (C) *C. albicans* incubated with 5 μM TAPC for 30 min at 37 °C in the dark. Inset: cells under bright field (100× microscope objective, scala bar 2 μm).

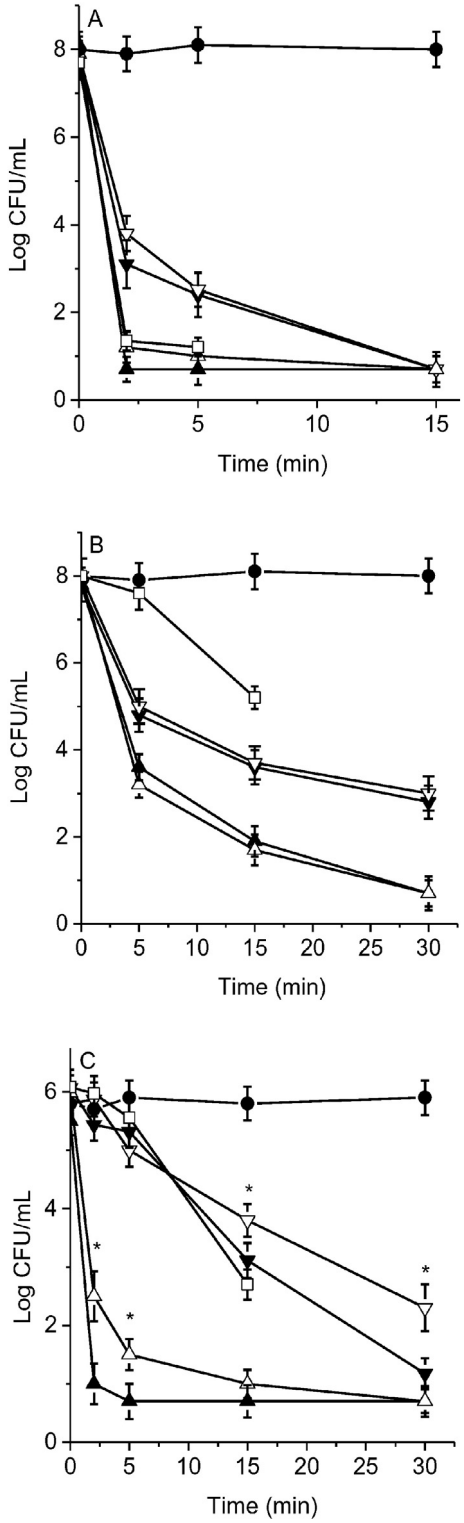

Fig. 7. Survival curves of (A) *S. aureus* (~10^8 CFU/mL), (B) *E. coli* (~10^8 CFU/mL) and (C) *C. albicans* (~10^6 CFU/mL) incubated with 1 μM (▼) and 5 μM (▲) TAPC and 1 μM (▽) and 5 μM (△) TAPP for 30 min at 37 °C in the dark and irradiated with visible light for different times. Control culture (●) of cells untreated with the photosensitizer and irradiated (*$p < 0.05$, compared with TAPP).

trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$). Studies in human red blood cells showed that the photodynamic activity of 5,10,15,20-tetrakis[3-(*N*-ethyl-*N*-methylcarbazoyl)]chlorin (TEMCC) was mediated by a contribution of type I and type II photooxidative mechanisms [8]. Also, both $O_2(^1\Delta_g)$ and $O_2^{\bullet-}$ must be involved in the observed photodynamic activity of 4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine-fused chlorins in human melanoma cell [29].

In vitro experiments with microorganisms showed that TAPC or TAPP was rapidly bound to cells at short incubation period of 2 min. The formation of chlorin derivative produced only small changes in the lipophilic character of the macrocycle, as indicated by the log P_{ow} values. 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 [23,30]. However, the cell envelope of microorganisms represents a significant barrier to the binding of the photosensitizer and the number and distribution of charges may play a predominant role [21]. The cell-bound photosensitizer was very similar in *S. aureus*, while slightly higher amount of TAPC with respect to TAPP was found in *E. coli*. On the other hand, the quantity of TAPC bound to both bacteria was very similar. This behavior was also observed using polycationic conjugates between poly-L-lysine and chlorin e6 (pL-ce6) [31]. After 30 min incubation, *S. aureus* and *E. coli* took up comparable amounts of conjugates. The uptake of TAPC in *C. albicans* was a slightly lower than TAPP. The amount of these photosensitizers bound to yeast cells was similar to that found for $TMAP^{4+}$, which reached 1.4 nmol/10^6 cells, when the cell suspensions were incubated with 5 μM porphyrin [20]. Also, a similar value of uptake (1.7 nmol/10^6 cells) was found using 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin ($TMPyP^{4+}$) [32]. Thus, the binding of TAPC to *C. albicans* was comparable to those obtained for porphyrins with cationic groups directly linked to the macrocycle. Moreover, fluorescence microscopic images provided additional insight about the uptake of TAPC by cells. Red fluorescence in cells shows that TAPC has particularly high binding affinity for these microorganisms. Similar results were previously found for cationic porphyrins bound to *C. albicans* cells [33].

Photoinactivation of microorganisms mediated by TAPC and TAPP was compared using different photosensitizer concentrations and irradiation periods. Bacterial suspensions of *S. aureus* treated with 5 μM TAPC produced an over 7 log decrease in the viability after 2 min irradiation. These results represent a value greater than 99.9999% of cellular inactivation. Moreover, 1 μM of this chlorin exhibited a photosensitizing activity of 5 log units (99.999%). A slightly higher photoinactivation was found using TAPC than TAPP. Under these conditions, complete eradication was found after 30 min irradiation with both photosensitizers. Direct comparisons with other photosensitizers already described can be difficult due to different cell densities and irradiation systems. Under similar experimental condition, a decrease of 3 log in the survival was found using 1 μM polyethylenimine-chlorin(e6) conjugates [10]. Moreover, a pentacationic chlorin was effective to eradicate *S. aureus* at a concentration of 1 μM [11]. As expected due to the nature of the envelope of Gram-negative bacteria, it was more difficult to inactivate *E. coli* than *S. aureus* [6]. When the *E. coli* cells were incubated with 1 μM TAPC, an enhancement in the cell inactivation was found increasing the irradiation times, reaching a decrease of 5 log survival after 30 min irradiation. Photoinactivation of *E. coli* treated with 5 μM TAPC or TAPP produced over 7 log decrease after 30 min irradiation. These results represent a value greater than 99.9999% of cell inactivation. Also, the cytotoxic activity remained elevated during a shorter irradiation time of 5 min that produced 5 log decrease. The photocytotoxic effect for *E. coli* cells treated with TAPP was very similar to that of TAPC. Also, polycationic conjugates pL-ce6 was effective in photoinactivation of both Gram-positive and Gram-negative bacteria [31]. Susceptibility of *C. albicans* was intermediate between Gram-positive and Gram-negative bacteria. *C. albicans* cells treated with 1 μM TAPC produced a photoinactivation of 99.997% (~4.5 log decrease) after 30 min irradiation. Using 5 μM TAPC, the photoinactivation remained elevated (>99.999%) even to a short irradiation time of 5 min. The photocytotoxic affect mediated by TAPC was higher than those previously found using $TMAP^{4+}$ and $TMPyP^{4+}$ [21,32].

On the other hand, TMPP was used as a porphyrin model to examine the effect of the basic amine substituents on the macrocycle periphery of TAPP and TAPC. In *S. aureus*, photoinactivation induced by TMPP was very similar to that found for TAPP. Gram-positive bacteria are more susceptible to PDI and can be photoinactivated by neutral, anionic, or cationic photosensitizers [6]. However, the photodamage mediated by TMPP considerably decrease in *E. coli*. It is known the importance of positive charges on the photosensitizers to photoinactivate Gram-negative bacteria due to [7]. The different permeability barriers between Gram-positive and Gram-negative bacteria are mainly involved in the observed efficiency of these photosensitizers. Moreover, the difference in the photoinactivation was evident in *C. albicans* cells treated with TMPP. The presence of cationic charges is required for an efficient photokilling of *C. albicans* [21]. Fungal cell walls have a relatively thick layer of β-glucan and chitin that leads to a permeability barrier intermediate between Gram-positive and Gram-negative bacteria [34]. Thus, *S. aureus* cells were more susceptible to the photodynamic activity mediated by TAPC, while *E. coli* required longer irradiation or higher concentration to obtain a complete eradication. On the other hand, photoinactivation of *C. albicans* mediated by TAPC was possible even using a short irradiation periods.

5. Conclusions

A new chlorin TAPC substituted at the *meso* position by amino groups was synthesized from homologous porphyrin TAPP. Thus, the presence of four basic amine groups on the chlorin macrocycle could facilitate the interaction with the biological membrane. TAPC showed an increase in the absorption band at 650 nm respect to TAPP. Red fluorescence emission and $O_2(^1\Delta_g)$ production of TAPC was very similar to that of TAPP in DMF. Also, TAPC can produce the reduction of NBT to diformazan, in the presence of NADH. *In vitro* experiments showed that TAPC displayed strong binding to the microbial cells after a short period of incubation. TAPC was efficient photosensitizers to inactivate microorganisms in PBS suspensions. Although this photosensitizer is a non-charged agent, the amino groups can acquire a positive charge at physiological pH. Also, the mobility of cationic groups allows a strong interaction of the photosensitizer with the microbial cells. The results indicate that TAPC can contribute to improve the PDI of microorganisms mainly when penetration of light can be required in the antimicrobial treatment.

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