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Dyes and Pigments 77 (2008) 229–237
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Synthesis and antibacterial photosensitizing properties of a novel tricationic subphthalocyanine derivative

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Received 15 February 2007; received in revised form 5 May 2007; accepted 11 May 2007
Available online 2 June 2007

Abstract

A novel chloro[2,9,16(2,9,17)-trikis-4-(*N*-methylpyridyloxy)]subphthalocyaninnato boron(III) iodide (SubOPc^{3+}) was synthesized by boron trichloride-induced cyclotrimerization of 4-(4-pyridyloxy)phthalonitrile in 1-chloronaphthalene. Exhaustive methylation of SubOPc with methyl iodide yielded the tricationic SubOPc^{3+}. The spectroscopic and photodynamic properties of the SubOPc^{3+} were compared with those of both the uncharged SubOPc and the subphthalocyanine (SubPc). The cationic SubOPc^{3+} aggregates in various solvents and is partially dissolved as a monomer in aqueous sodium dodecyl sulfate; photodynamic inactivation was strongly dependent on the medium. The photodynamic inactivation imparted by the subphthalocyanines was investigated *in vitro* on the Gram-negative bacterium, *Escherichia coli*. SubOPc^{3+} rapidly bound to *E. coli* cells reaching a value of ~2 nmol/10^6 cells after 5 min of incubation with 4 μM of sensitizer in the dark. Photosensitized inactivation of *E. coli* cellular suspensions by SubOPc^{3+} produced a ~2.5 log decrease of cell survival (99.7% of cellular inactivation). The growth of *E. coli* cells was completely arrested when cultures were exposed to 8 μM of cationic subphthalocyanine and irradiated, whereas a negligible effect was found for the non-charged SubOPc. Also, SubOPc^{3+} was able to inactivate *E. coli* cells immobilized on agar surfaces.

Keywords: Subphthalocyanine; Photosensitizer; Bacteria; *Escherichia coli*; Photodynamic inactivation

1. Introduction

Subphthalocyanines are composed of three diiminoisoindole rings N-fused around a boron core. Their chemical and physical properties, which result from their 14 π-electron aromatic core and nonplanar cone-shaped structure, make them attractive compounds [1,2]. The compounds are synthesized by cyclotrimerization of phthalonitrile precursors in the presence of a boron trihalide [3,4]. Symmetrically substituted phthalonitriles produce a single subphthalocyanine, while asymmetric phthalonitriles yield a mixture of two subphthalocyanine regioisomers with C_1 and C_3 symmetries [5]. When electronic or steric interactions are not influencing the regioisomeric distribution, the ratio C_3:C_1 follows a statistical distribution 1:3 [1].

UV–visible absorption spectra of subphthalocyanines are comparable to those of phthalocyanines in that both show Soret and Q-bands. However, in the case of subphthalocyanines, the absorption spectrum tends to show both a Soret band (~300 nm) and a Q-band (~560 nm), which are shifted to shorter wavelengths than those of phthalocyanines. This effect occurs as a consequence of the decrease in the π-conjugation system [6]; also, subphthalocyanines are fluorescent and have long triplet excited state lifetimes that efficiently produce singlet molecular oxygen, $O_2(^1\Delta_g)$ [6–8]. Thus, subphthalocyanines are interesting candidates for use in photosensitization processes, especially in situations where absorption in the red part of the spectra is not required. This can be the case of pathogenic microorganisms grown *in vivo* as localized foci of infection, on skin or on accessible mucous membrane, which can be treated by photodynamic inactivation (PDI)

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doi:10.1016/j.dyepig.2007.05.008

[9,10]. This approach has recently been proposed as an alternative antibacterial therapy for the treatment of pathogenic bacteria [11–13]. PDI is based on the administration of a photosensitizer, which is preferentially accumulated in the microbial cells; subsequent irradiation with visible light, in the presence of oxygen, produces cell damage that inactivates the microorganisms [14].

In most cases, while Gram-positive bacteria are susceptible to the photosensitizing action of a variety of sensitizers, Gram-negative bacteria exhibit a remarkable resistance to negatively charged or neutral agents [12]. This resistance has been ascribed to the presence of a highly organized outer membrane that hinders the interaction of the photosensitizer with the cytoplasmic membrane and intercepts the photogenerated reactive species [15–18]. However, studies with cationic photosensitizer derivatives have shown that these compounds can cause direct photoinactivation of Gram-negative bacteria even in the absence of additives [19–24].

In this paper we evaluate, for first time, the photodynamic activity of subphthalocyanine derivatives *in vitro* on *Escherichia coli* cells. Thus, a novel chloro[2,9,16(2,9,17)-trikis-4-(*N*-methylpyridyloxy)]subphthalocyaninnato boron(III) iodide ($SubOPc^{3+}$) was synthesized and its spectroscopic and photodynamic properties were compared with the homologous, non-charged chloro[2,9,16(2,9,17)-trikis-4-(pyridyloxy)] subphthalocyaninnato boron(III) (SubOPc) as well as the subphthalocyanine (SubPc). The results indicate that $SubOPc^{3+}$ is an interesting agent with potential application in the PDI of bacteria.

2. Materials and methods

2.1. General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer and on a Spex FluoroMax fluorometer, respectively. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a FT-NMR Bruker Avance DPX400 multinuclear spectrometer at 400 MHz. Mass spectra were taken with a Varian Matt 312 operating in EI mode at 70 eV. FAB mass spectra were taken with a ZAB-SEQ Micromass equipment. All the chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Solvents (GR grade) from Merck were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

2.2. Sensitizers

Subphthalocyanine (SubPc) was purchased from Aldrich. 4-(4-Pyridyloxy)phthalonitrile was synthetized from 4-nitrophthalonitrile and 4-hydroxypyridine in the presence of dry potassium carbonate as previously reported [25]. According to the synthetic procedure, the subphthalocyanine macrocycle is obtained as a mixture of the corresponding regioisomers.

2.2.1. Chloro[2,9,16(2,9,17)-trikis-4-(pyridyloxy)]-subphthalocyaninnato boron(III) (SubOPc)

A solution of 4-(4-pyridyloxy)phthalonitrile (183 mg, 0.81 mmol) and boron trichloride (410 μL, 0.41 mmol, 1 M in heptane) in 6 mL of 1-chloronaphthalene was stirred for 10 min under an argon atmosphere. The reaction mixture was heated to 250 °C for 10 min and then cooled to room temperature after which the product was purified by flash chromatography (silica gel, dichloromethane/methanol 5%) and recrystallized from dichloromethane/cyclohexane to yield 37 mg (19%) of the pure SubOPc. ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 6.51 (6H), 7.75 (6H), 7.93 (6H), 8.60 (6H). MS [*m*/*z*] 709 (M^+) (709.1549 calculated for $C_{39}H_{21}BClN_9O_3$). Anal. Calcd. C 65.98, H 2.98, N 17.76; found C 65.90, H 2.93, N 17.85.

2.2.2. Chloro[2,9,16(2,9,17)-trikis-4-(N-methylpyridyloxy)]-subphthalocyaninnato boron(III) iodide ($SubOPc^{3+}$)

A mixture of SubOPc (15 mg, 0.021 mmol) and 4 mL of methyl iodide in 4 mL of *N*,*N*-dimethylformamide (DMF) was stirred for 72 h at 70 °C. The solvents were removed under vacuum and the solid was re-suspended in cyclohexane and then filtered to yield 22 mg (92%) of $SubOPc^{3+}$. ^{1}H NMR (DMSO-d_6, TMS) δ [ppm] 4.21–4.26 (9H), 7.80 (6H), 7.94 (6H), 8.62 (6H), 8.93 (6H). MS [*m*/*z*] 754 (M^+-3I) (754.2253 calculated for $C_{42}H_{30}BClN_9O_3$). Anal. Calcd. C 44.42, H 2.66, N 11.10; found C 44.49, H 2.73, N 11.16.

2.3. Spectroscopic studies

Spectra were recorded using 1 cm path length quartz cells at 25.0 ± 0.5 °C. The fluorescence quantum yield (ϕ_F) of subphthalocyanines was calculated by comparison of the area below the corrected emission spectrum with that of cresyl violet perchlorate as a fluorescence standard ($\phi_F = 0.54$ in methanol), exciting at $\lambda_{exc} = 530$ nm [26]. The intensities were corrected for refraction index differences between methanol and DMF [27].

2.4. Steady-state photolysis

Solutions of 9,10-dimethylanthracene (DMA, 35 μM, 2 mL) in different media and photosensitizer were irradiated in quartz cells with monochromatic light at $\lambda_{irr} = 570$ nm (sensitizer absorbance 0.1) from a 75 W high-pressure Xe lamp through a high intensity grating monochromator, Photon Technology Instrument [28]. The light intensity was determined as 0.7 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). The kinetics of photooxidation were studied by following the decrease of the absorbance (A) at $\lambda_{max} = 378$ nm for DMA. 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 singlet molecular oxygen, $O_2(^1\Delta_g)$, production by the photosensitizers. SubPc was used as the standard ($\Phi_\Delta = 0.61$) [1]. Measurements of the sample and reference under the same

conditions afforded Φ_Δ for sensitizers by direct comparison of the slopes in the linear region of the plots [29]. All the experiment were performed at 25.0 ± 0.5 °C. The pooled standard deviation of the kinetic data, using different prepared samples, was less than 5%.

2.5. Bacterial strain and preparation of cultures

E. coli strain EC7 recovered from clinical urogenital material [23] was grown aerobically at 37 °C in 30% w/v tryptic soy (TS) broth overnight. Aliquots (~40 μL) of this culture were aseptically transferred to 4 mL of fresh medium (30% w/v TS broth) and incubated at 37 °C to mid logarithmic phase (absorbance ~ 0.6 at 660 nm). Cells in the logarithmic phase of growth were harvested by centrifugation of the broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0). The cells were then diluted 1/1000 in PBS, corresponding to ~10^6 colony forming units (CFU)/mL. In all the experiments, 2 mL of the cell suspensions in glass culture tubes (13 × 100 mm) were used and the sensitizer was added from a stock solution of sensitizer (4.5×10^{-4} M) in DMF. Viable bacteria were monitored and their number was calculated by counting the number of colony forming units after appropriate dilution on TS agar plates [23]. Bacterial cultures grown under the same conditions with and without photosensitizers kept in the dark as well as illuminated cultures without sensitizer served as controls.

2.6. Sensitizer binding to bacterial cells

Suspensions of *E. coli* (2 mL, ~10^6 CFU/mL) in PBS were incubated in dark at 37 °C with 4 μM of sensitizer for different times. The cultures were centrifuged (3000 rpm for 15 min) and the cell pellets were then re-suspended in 2 mL of DMF/water (10%)/HCl (1.2 mM), incubated overnight at 4 °C and sonicated for 30 min [23]. The concentration of sensitizer in the supernatant was measured spectrofluorimetrically ($\lambda_{exc} = 545$ nm, $\lambda_{em} = 578$ nm and 579 nm for SubOPc and $SubOPc^{+3}$, respectively). The fluorescence values for each sample refer to the total number of bacteria contained in the suspension. The concentration of the sensitizer in this sample was estimated by comparison with a calibration curve obtained using standard solutions of the sensitizer in DMF/water (10%)/HCl (1.2 mM) ([sensitizer] ~ 0.05–1.0 μM).

2.7. Photosensitized inactivation of bacteria cells in PBS suspension

Cell suspensions of *E. coli* (2 mL, ~10^6 CFU/mL) in PBS were incubated with 4 μM of sensitizer for 30 min in the dark at 37 °C. The cultures were then exposed for different times to visible light provided by a 150 W lamp housed in a Novamat 130 AF slide projector filtered through a 2.5 cm glass cuvette filled with water to absorb heat. The wavelength range of 350–800 nm was selected by optical filters [30]. The light intensity at the treatment site was 75 mW/cm^2 (Radiometer Laser Mate-Q, Coherent). Both control and irradiated cell suspensions were serially diluted with PBS, then plated in triplicate on TS agar and the number of colonies formed after 18–24 h incubation at 37 °C was counted.

2.8. Growth delay experiment

E. coli cells were grown overnight as described above. A portion (60 μL) of this culture was transferred to 20 mL of fresh TS broth (10%) medium and the ensuing suspension was homogenized and 2 mL aliquots were incubated with 8 μM of sensitizer at 37 °C. The culture grown was measured by turbidity at 660 nm using a Tuner SP-830 spectrophotometer [23]; the flasks were then irradiated with visible light at 37 °C, as described above.

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

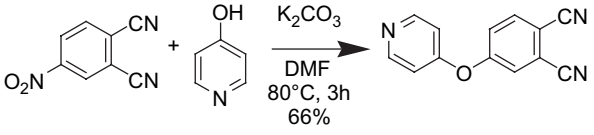
2.9. Photosensitized inactivation of bacteria cells on agar surface

TS agar plates (5 cm diameter) were spread with 8 nmol of sensitizer on an area of ~0.6 cm^2 from a solution 4.5×10^{-4} M in DMF. The plates were then incubated for 15 min at 37 °C and were plated with the suspension of *E. coli* (~10^9 CFU/mL) in PBS. The plates were incubated at 37 °C for 30 min in the dark after which time, the plates were irradiated as described above for 1 h and incubated for 18–24 h at 37 °C in dark. Plates both with and without photosensitizers were kept in the dark and plates without sensitizer that had been irradiated were used as controls. Each experiment was repeated separately three times.

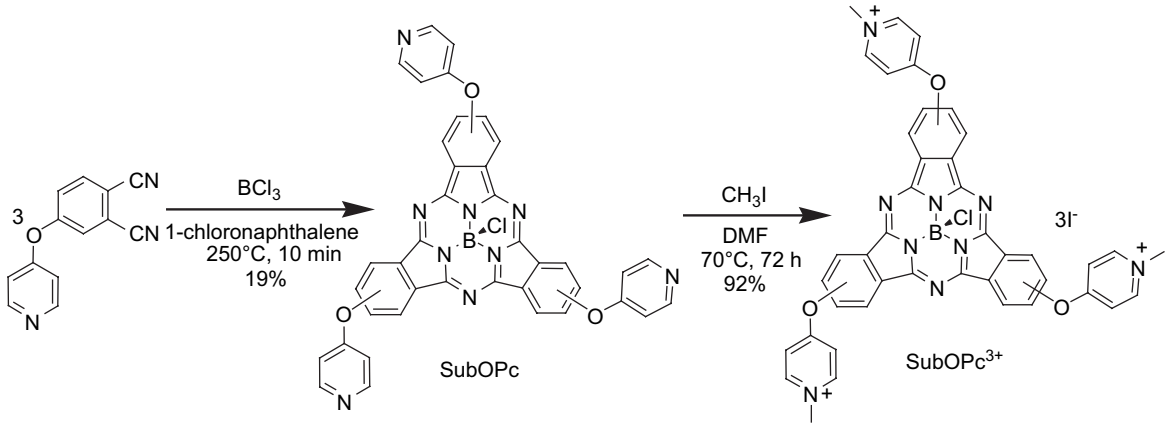
3. Results and discussion

3.1. Synthesis of subphthalocyanines

A three-step method was used to synthesize $SubOPc^{3+}$. Firstly, 4-(4-pyridyloxy)phthalonitrile was formed by the nucleophilic aromatic substitution reaction of 4-nitrophthalonitrile with 4-hydroxypyridine in the presence of K_2CO_3 (Scheme 1). The phthalonitrile derivative was isolated by flash chromatography in 66% yield. The boron trichloride-induced cyclotrimerization of 4-(4-pyridyloxy)phthalonitrile was performed in 1-chloronaphthalene (Scheme 2). After mixing, the reaction was heated to 250 °C for 10 min and then cooled to room temperature. The subphthalocyanine was isolated by flash chromatographic column (silica gel, dichloromethane/methanol 5%)



Scheme 1. Synthesis of 4-(4-pyridyloxy)phthalonitrile.



Scheme 2. Synthesis of trisubstituted subphthalocyanines SubOPc and SubOPc^{3+}.

and the product was re-crystallized from dichloromethane/cyclohexane to obtain SubOPc as a regioisomeric mixture in 19% yield. Cationic SubOPc^{3+} was obtained treating the SubOPc with an excess of methyl iodide at 70 °C for 72 h in DMF (Scheme 2). The exhaustive methylation produced SubOPc^{3+} in 92% yield.

3.2. Spectroscopic studies

The uncharged SubOPc was soluble as monomer in several organic solvents of middle polarity, such as dichloromethane and DMF, as indicated by the intense absorption Q-band around 570 nm. However, the spectrum of the tricationic SubOPc^{3+} in different media showed a low intensity, broadening Q-band. Aggregation of tricationic subphthalocyanine was observed in dichloromethane, dimethylsulphoxide, methanol, DMF, water and PBS. The monomeric form of SubOPc^{3+} increased in DMF/water (10%) moderately acidified with HCl (1.2 mM) (Fig. 1). However, as seen in Fig. 1, the fluorescence excitation spectrum of SubOPc^{3+} showed that even in this condition the cationic subphthalocyanine was not completely disaggregated. An increase in the Q-band of SubOPc^{3+} was observed in aqueous 2% w/v sodium dodecyl sulfate (SDS) (Fig. 1). The micellar system provides a more appropriate microenvironment where the sensitizer can be solubilized as a monomer. The spectroscopic characteristics of subphthalocyanines in acidified DMF/water are summarized in Table 1. The Q-band of C_δ substituted subphthalocyanines, such as SubOPc and SubOPc^{3+}, displays a small bathochromic shift (~3 nm) when compared with that of SubPc [1].

The steady-state fluorescence emission spectra of subphthalocyanines were performed in DMF/water (10%)/HCl (1.2 mM) (Fig. 2). The spectra showed one intense band at ~578 nm, which is characteristic of subphthalocyanines [1]. The fluorescence quantum yields (ϕ_F) are summarized in Table 1; a lower ϕ_F was found for SubOPc^{3+} probably due to its incomplete monomerization. Moreover, very low fluorescence emission was found in dichloromethane, dimethylsulphoxide, methanol, water and PBS showing that the tricationic phthalocyanine is poorly soluble as a monomer in these media.

In all cases, a small Stokes shift (~10 nm) was observed indicating that the spectroscopic energy was almost identical to the relaxed energy of the singlet state. Taking into account the energy of the 0–0 electronic transitions, the energy levels of the singlet excited states (E_s) were calculated (Table 1). These results are in agreement with those previously reported for this family of photosensitizers [1].

3.3. Photodynamic activity

9,10-Dimethylanthracene (DMA) was used to evaluate the ability of the sensitizers to produce $O_2(^1\Delta_g)$ as this substrate

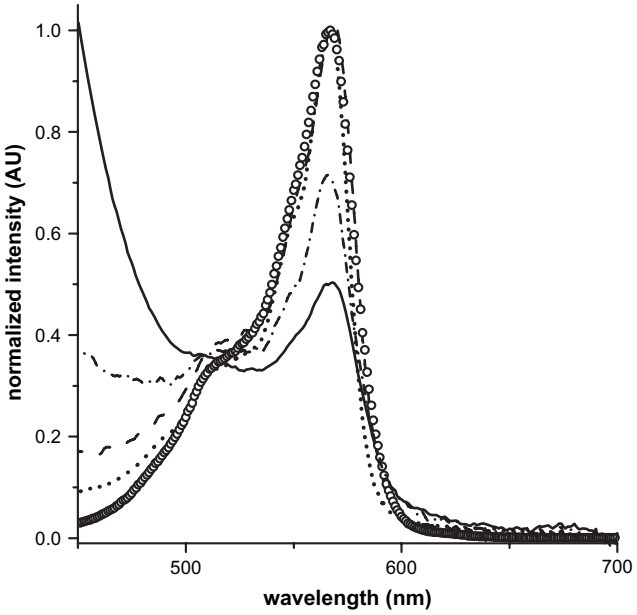


Fig. 1. Absorption spectra of SubOPc (dashed line), SubOPc^{3+} (solid line), SubPc (dotted line) in DMF/water (10% v/v)/HCl (1.2 mM) and SubOPc^{3+} (dashed dotted line) in SDS 2% w/v, and fluorescence excitation spectrum of SubOPc^{3+} (○, $\lambda_{em} = 625$ nm) in DMF/water (10% v/v)/HCl (1.2 mM).

Table 1
Spectroscopic characteristics of subphthalocyanines in DMF/water (10%)/HCl (1.2 mM)

Subphthalocyanine	λ_{max}^{abs} (nm)[a]	λ_{max}^{em} (nm)[b]	ε ($M^{-1}\,cm^{-1}$)	ϕ_F	E_s (kJ mol^{-1})
SubPc	566	574	63,000[c]	0.18 ± 0.01[d]	210
SubOPc	569	578	63,500	0.14 ± 0.01	211
$SubOPc^{3+}$	569	579	31,000	0.10 ± 0.01	211

[a] Q-band.
[b] $\lambda_{exc} = 530$ nm.
[c] Benzene, Ref. [6].
[d] $\phi_F = 0.25$ (benzene), Ref. [6].

quenches $O_2(^1\Delta_g)$ exclusively by chemical reaction [28]. The photooxidative process was studied under aerobic condition in different media namely DMF, DMF/water (10%)/HCl (1.2 mM) and aqueous 2% w/v SDS. Fig. 3 shows representative results in acidified DMF/water. From first-order kinetic plots the values of the observed rate constant (k_{obs}) were calculated (Table 2). In DMF, the non-charged subphthalocyanines, SubPc and SubOPc, photodecompose DMA with identical rate, indicating that both sensitizers display comparable $O_2(^1\Delta_g)$ production in this solvent. Under similar conditions, DMA reaction was negligible using $SubOPc^{3+}$ as sensitizer since in DMF the cationic sensitizer is highly aggregated. Such aggregates provide an efficient nonradiative energy relaxation pathway, diminishing the triplet-state population and the $O_2(^1\Delta_g)$ quantum yield; thus, aggregation precludes photodynamic activity. The ability of the three sensitizers was compared in acidified DMF/water, under which conditions, the quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) was calculated comparing the slope for subphthalocyanines with

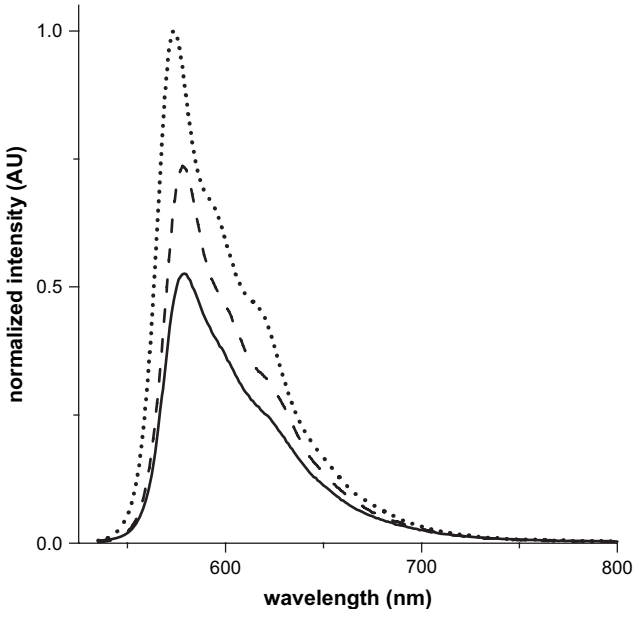


Fig. 2. Fluorescence emission spectra of SubOPc (dashed line), $SubOPc^{3+}$ (solid line) and SubPc (dotted line) in DMF/water (10% v/v)/HCl (1.2 mM), $\lambda_{exc} = 530$ nm.

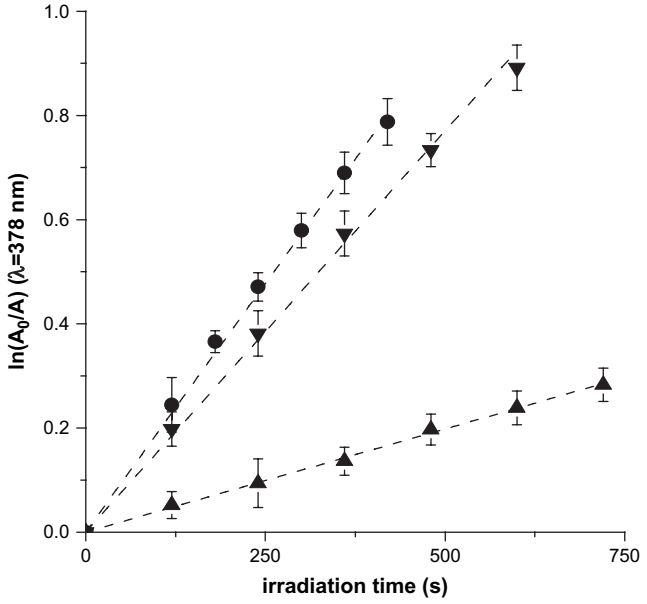


Fig. 3. First-order plots for the photooxidation of DMA (35 μM) in DMF/water (10% v/v)/HCl (1.2 mM) photosensitized by SubOPc (▼), $SubOPc^{3+}$ (▲) and SubPc (●), $\lambda_{irr} = 570$ nm. Values represent mean ± standard deviation of three separate experiments.

that obtained for the reference, SubPc. As can be seen in Table 2, the photodynamic effect of SubOPc remained relatively high in comparison with SubPc but the value of Φ_Δ was even low for $SubOPc^{3+}$. The reaction was also investigated in a microheterogeneous system formed by SDS micelles. In this medium, a higher rate of DMA decomposition was obtained using $SubOPc^{3+}$ as photosensitizer, while SubPc could not be evaluated due to its low solubility. These findings indicate that $O_2(^1\Delta_g)$ production depends on the medium in which the sensitizer is localized and diminishes, markedly, when the subphthalocyanine is aggregated.

3.4. Binding of sensitizer to E. coli cells

Subphthalocyanine derivatives were evaluated as photodynamic agents *in vitro* using a typical Gram-negative bacterium, *E. coli*. Firstly, the cell toxicity induced by these photosensitizers was analyzed in the absence of light at different photosensitizer concentrations in PBS cellular suspension. When the cultures were treated with 1−10 μM of sensitizer for 30 min in the dark, no toxicity was detected for SubOPc. However, 10 μM of $SubOPc^{3+}$ was toxic to *E. coli* and the formation of colonies was not detected. Furthermore, 5 μM of $SubOPc^{3+}$ showed minor toxicity toward *E. coli* producing 32% of cellular inactivation. Finally, no toxicity was found when cell cultures were treated with 4 μM of $SubOPc^{3+}$ and therefore this concentration was selected for photodynamic *in vitro* studies using *E. coli* cells in PBS.

The capacity of these photosensitizers to bind to bacterial cells was compared after different incubation times of *E. coli* cultures using 4 μM of subphthalocyanine at 37 °C in

Table 2
Kinetic parameters (k_{obs}) and quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) of subphthalocyanines in different media

Subphthalocyanine	k_{obs} (s^{-1}) DMF	k_{obs} (s^{-1}) DMF/water[b]	Φ_Δ[b]	k_{obs} (s^{-1}) SDS[a]
SubPc	$(1.1 \pm 0.1) \times 10^{-3}$	$(1.9 \pm 0.2) \times 10^{-3}$	0.61[c]	–
SubOPc	$(1.1 \pm 0.1) \times 10^{-3}$	$(1.5 \pm 0.2) \times 10^{-3}$	0.48 ± 0.03	$(4.9 \pm 0.2) \times 10^{-4}$
$SubOPc^{3+}$	–	$(0.4 \pm 0.1) \times 10^{-3}$	0.13 ± 0.01	$(7.6 \pm 0.3) \times 10^{-4}$

[a] SDS 2% w/v.
[b] DMF/water (10%)/HCl (1.2 mM).
[c] Ref. [1].

the dark. The amounts of sensitizer associated with the cells are shown in Fig. 4 from which it is apparent that $SubOPc^{3+}$ reached the highest value of cell-bound sensitizer after a short time (~5 min). Prolonging the incubation time to 30 min did not impart an appreciable increase in the amount of photosensitizer bound to the *E. coli* cells. Under these conditions, the binding of cationic subphthalocyanine to *E. coli* cells reached a value of ~2 nmol/10^6 cells. This was not observed for SubOPc, whose binding to the *E. coli* cells achieved a value of ~0.5 nmol/10^6 cells.

The binding of $SubOPc^{3+}$ was also analyzed in *E. coli* cultures by fluorescence emission spectra. As can be observed in Fig. 5, a very low intensity around 575 nm was produced using 4 μM of subphthalocyanine in PBS. However, when $SubOPc^{3+}$ was added to a cellular suspension (10^6 cell/mL) in PBS solution, the band increased considerably. As the shape and intensity of the bands closely matched the emission spectrum of $SubOPc^{3+}$ in DMF/water (10% v/v)/HCl (1.2 mM), the cellular microenvironment, in which the $SubOPc^{3+}$ is localized, appears to be an appropriate medium to help to solubilize the cationic subphthalocyanine as a monomer.

3.5. Photosensitized inactivation of *E. coli* cells in PBS suspension

Suspensions of *E. coli* cells in PBS were treated with 4 μM of sensitizer for 30 min at 37 °C in dark and the cultures were then irradiated with visible light. Fig. 6 shows the survival of bacterial cells after different irradiation times. Control experiments demonstrated that the viability of *E. coli* was unaffected by illumination alone or by dark incubation with 4 μM of the photosensitizer for 30 min. Therefore, the cell mortality obtained after irradiation of the cultures treated with the subphthalocyanine can be attributed to the photosensitization effect of the agent produced by visible light.

The viability of *E. coli* cells after irradiation was dependent upon both light exposure level and the subphthalocyanine used in the treatment. As seen in Fig. 6, the *E. coli* cells are rapidly photoinactivated when the unwashed cultures treated with tricationic $SubOPc^{3+}$ are exposed to visible light. In particular, the tricationic $SubOPc^{3+}$ exhibits a photosensitizing activity causing a ~2.5 log decrease of cell survival, when the cultures are irradiated with a light fluence of 135 J/cm^2. These results represent a value greater than 99.7% of cellular inactivation. On the other hand, insignificant inactivation effect was found

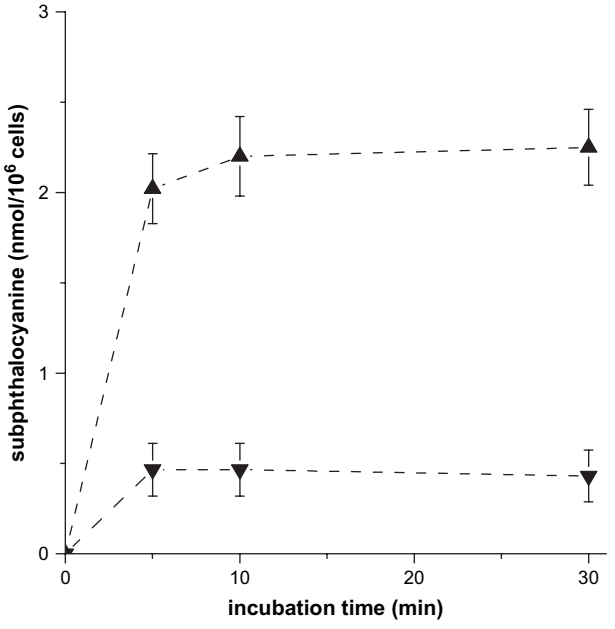


Fig. 4. Amount of SubOPc (▼) and $SubOPc^{3+}$ (▲) recovered from *E. coli* cells (~10^6 CFU/mL) treated with 4 μM of sensitizer for different incubation times at 37 °C in dark. Values represent mean ± standard deviation of three separate experiments.

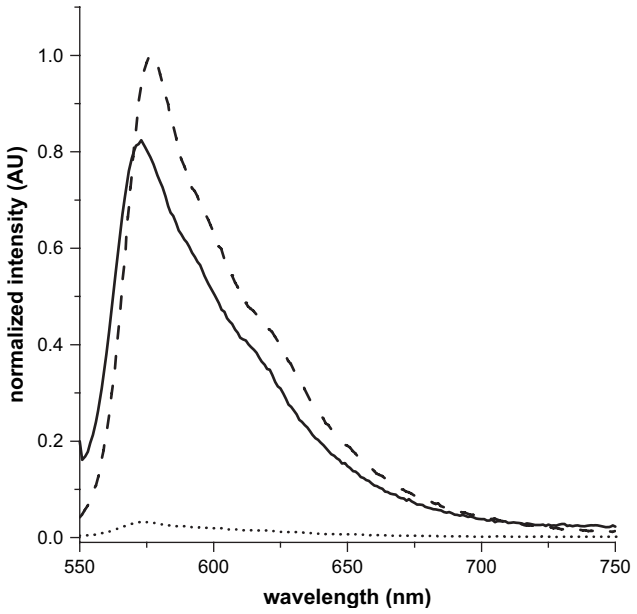


Fig. 5. Fluorescence emission spectra ($\lambda_{exc} = 530$ nm) of $SubOPc^{3+}$ (4 μM) in DMF/water (10% v/v)/HCl (1.2 mM) (dashed line), PBS (dotted line) and cellular suspension (10^6 cell/mL) in PBS (solid line).

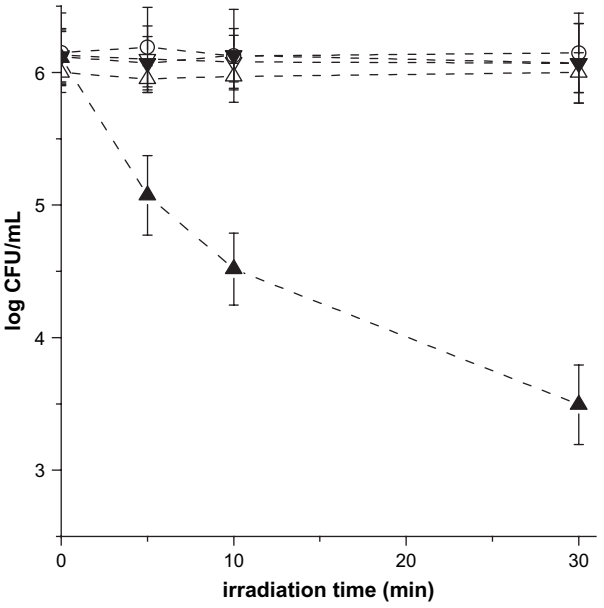


Fig. 6. Survival curves of *E. coli* cells ($\sim 10^6$ CFU/mL) incubated with 4 μM of SubOPc (▼) and $SubOPc^{3+}$ (▲) for 30 min at 37 °C in dark and exposed to visible light (75 mW/cm^2) for different irradiation times: control culture untreated (○), treated with 4 μM of SubOPc (▽) and $SubOPc^{3+}$ (△) in dark. Values represent mean ± standard deviation of three separate experiments.

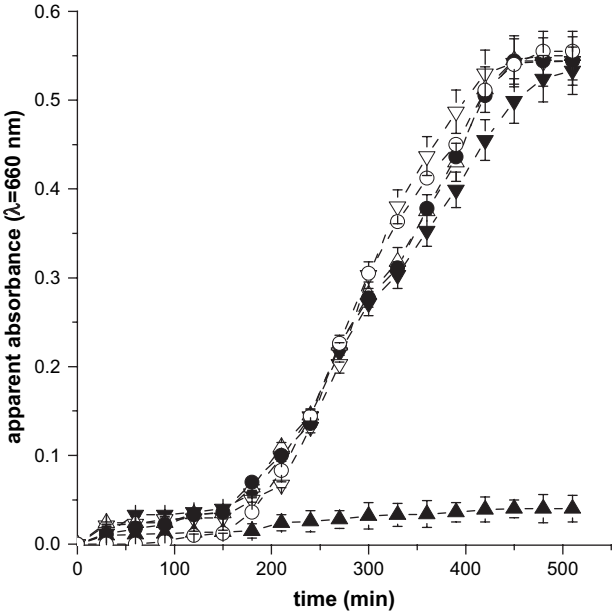


Fig. 7. Photosensitized growth delay curves of *E. coli* cells incubated with 8 μM of SubOPc (▼) and $SubOPc^{3+}$ (▲) and exposed to different irradiation times with visible light in 10% w/v TS broth at 37 °C: control culture untreated irradiated (●), untreated in dark (○), treated with 8 μM of SubOPc (▽) and $SubOPc^{3+}$ (△) in dark. Values represent mean ± standard deviation of three separate experiments.

for cultures treated with 4 μM of SubOPc still after 30 min of irradiation. This result is in agreement with that reported before for non-charged phthalocyanine derivatives, indicating that these non-cationic sensitizers are unsuccessful sensitizers for Gram-negative bacteria under these conditions [16,25]. The failure of SubOPc to produce any photosensitizing activity on the bacteria is possibly due to its low binding to them. In the case of $SubOPc^{3+}$, it seems to have a binding affinity for *E. coli* cells probably by electrostatic interaction with anionic functional groups at the cell surface [15]. Thus, the subphthalocyanine tightly bound is mainly involved in the photoinactivation of *E. coli*.

3.6. Photosensitized growth delay of E. coli cultures

In most *in vitro* studies, the photodynamic activity of the photosensitizers has been evaluated using PBS as the suspending medium for the bacterial cells. However, the consistency of the suspending medium can strongly influence the efficacy of antimicrobial PDI [31,32]. Therefore, experiments were performed to ensure that PDI of cells is still possible when the cultures were not under either starvation conditions or the potential damaging effects of phosphate buffer washing [16,25,31]. Thus, growth delay of *E. coli* cultures sensitized by subphthalocyanines was carried out in the medium (10% w/v TS broth). The cultures of *E. coli* in lag phase were treated with 8 μM of sensitizer and the flasks were irradiated with visible light at 37 °C. As can be seen from Fig. 7, growth was arrested when *E. coli* cultures were treated with $SubOPc^{3+}$ and illuminated. After irradiation in the presence of 8 μM of sensitizer $SubOPc^{3+}$, the cells no longer appeared to be growing as measured by turbidity at 660 nm. Furthermore, a minor effect in the growth delay was found for cells treated with 8 μM of SubOPc. In contrast, *E. coli* cells exposed to sensitizers in the dark or not treated with sensitizer and illuminated showed no growth delay compared with the controls. These results imply that the observed growth delay is due to the photoinactivation effect of the sensitizers on the cells [23]. Similar growth delay curves of *E. coli* were previously observed for cultures in the presence of 1−10 μg/mL of cationic zinc pyridinium phthalocyanine under similar experimental conditions [16]. Therefore, these findings indicate that tricationic subphthalocyanine is a very efficient sensitizer to arrest *E. coli* cells even under growth conditions of the cultures.

3.7. Inactivation of E. coli cells on surfaces

The photodynamic activity of the photosensitizers to inactivate *E. coli* cells restricted on TS agar has been used to photoinactivate bacteria growing *in vivo* as localized foci of infection, on skin or on accessible mucous membrane [33,34]. These experiments were used to evaluate the photodynamic inactivation of *E. coli* cells, which were not initially treated with the sensitizer in solution. Thus, the cells were grown on TS agar surface containing the sensitizer in a small area. Firstly, 8 nmol of different subphthalocyanines were homogenously distributed over ~ 0.6 cm^2 on the TS agar and the plates were then spread with a suspension of *E. coli*, to allow a lawn of bacteria to be obtained. The cultures

were kept in the dark for 30 min at 37 °C, during which time, binding of the sensitizer to the *E. coli* cells can take place. Afterwards, the plates were irradiated with visible light for 3 h and incubated for 18–24 h at 37 °C in the dark. Characteristic results are shown in Fig. 8 from which it is evident that growth of the *E. coli* cells was not detected in the area which had been treated with $SubOPc^{3+}$, whereas negligible effect was observed in the area containing SubOPc. Also, modification of the cellular lawn was not observed for controls treated with sensitizer and kept in dark. Consequently, photodynamic inactivation was only found on the surface where $SubOPc^{3+}$ was located. This activity is probably due to a higher binding to cells of $SubOPc^{3+}$ as observed above in bacterial suspension.

4. Conclusions

A novel, tricationic subphthalocyanine was conveniently synthesized in three steps namely, nucleophilic aromatic substitution to produce 4-(4-pyridyloxy)phthalonitrile, boron trichloride-induced cyclotrimerization of 4-(4-pyridyloxy) phthalonitrile to provide SubOPc and the exhaustive methylation of SubOPc with methyl iodide to yield the tricationic $SubOPc^{3+}$ in which the cationic centers are isolated from the subphthalocyanine macrocycle ring by alcohoxy bonds which provide high mobility of the charges, thus facilitating interaction with the outer membrane of Gram-negative bacteria.

Owing to their cone-shaped structure, subphthalocyanines should not form aggregates in many solvents. However, the cationic $SubOPc^{3+}$ aggregates in various solvents and is partially dissolved as a monomer in SDS. Photodynamic studies in different media indicate that $O_2(^1\Delta_g)$ production sensitized by $SubOPc^{3+}$ depends on the medium in which it is located. Indeed, activity increases in a micellar, microheterogeneous system that contains regions that mimic a biological microenvironment. Consequently, photodynamic efficiency in biological systems is not directly predictable on the basis of photophysical investigations in solution [35]. Studies of PDI to evaluate the activity of these sensitizers on a Gram-negative bacterium *E. coli* were for first time investigated *in vitro*. Binding of $SubOPc^{3+}$ to cells takes place in a short period of incubation time reaching high values of intracellular concentration ($\sim$2 nmol/10^6 cells). Also, fluorescence studies in cellular suspension reveal that $SubOPc^{3+}$ is solubilised in a biological microenvironment, which apparently helps the monomerization of the sensitizer. Photosensitized inactivation of *E. coli* cellular suspensions by $SubOPc^{3+}$ exhibited $\sim$99.7% cellular inactivation after 30 min of irradiation. PDI was also established by growth delay experiments. The *E. coli* cells are rapidly photoinactivated in an environment free from the possible damaging effects of phosphate buffer washing. In terms of the ability of the photosensitizers to inactivate *E. coli* growing on agar surfaces, the photosensitizing effect of $SubOPc^{3+}$ remained high.

This work shows that cationic subphthalocyanines are interesting photosensitizers with useful antibacterial activity in the cases of both cellular suspension or on a surface.

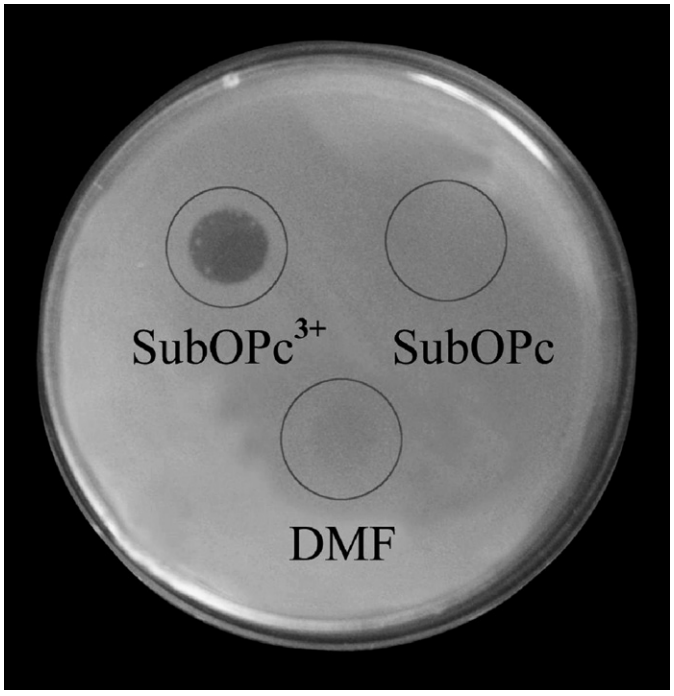


Fig. 8. Inactivation of *E. coli* cells on TS agar irradiated with visible light (75 mW/cm^2) for 1 h. The black circles indicate the area where 8 nmol of sensitizer was spread from a solution 4.5×10^{-4} M in DMF.

Acknowledgements

Authors thank Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) of Argentina, SECYT Universidad Nacional de Río Cuarto and Agencia Nacional de Promoción Científica y Técnológica (ANPCYT) of Argentina for financial support. E.N.D. is a Scientific Member of CONICET. M.B.S. thanks CONICET for a doctoral fellowship.

References

[1] Claeessens CG, González-Rodríguez D, Torres T. Subphtalocyanines: singular nonplanar aromatic compounds – synthesis, reactivity, and physical properties. Chem Rev 2002;102:835–53.
[2] Torres T. From subphthalocyanines to subporphyrins. Angew Chem Int Ed 2006;45:2834–7.
[3] Kudrevich SV, Gilbert S, van Lier JE. Syntheses of trisulfonated phthalocyanines and their derivatives using boron(III) subphthalocyanines as intermediates. J Org Chem 1996;61:5706–7.
[4] Claessens CG, González-Rodríguez D, del Rey B, Torres T, Mark G, Schuchmann H-P, et al. Highly efficient synthesis of chloro- and phenoxy-substituted subphthalocyanines. Eur J Org Chem 2003;2547–51.
[5] Claeessens CG, Torres T. Subphtalocyanine enantiomers: first resolution of a C3 aromatic compound by HPLC. Tetrahedron Lett 2000;41:6361–5.
[6] del Rey B, Keller U, Torres T, Rojo G, Agulló-López F, Nonell S, et al. Synthesis and nonlinear optical, photophysical, and electrochemical properties of subphthalocyanines. J Am Chem Soc 1998;120:12808–17.
[7] Ohno-Okumura E, Sakamoto K, Kato T, Hatano T, Fukui K, Karatsu T, et al. Synthesis of subphthalocyanine derivatives and their characterization. Dyes Pigments 2002;53:57–65.

[8] Xu S, Chen K, Tian H. A colorimetric and fluorescent chemodosimeter: fluoride ion sensing by an axial-substituted subphthalocyanine. J Mater Chem 2005;15:2676–80.
[9] O'Riordan K, Akilov OE, Hasan T. The potential for photodynamic therapy in the treatment of localized infections. Photodiag Photodyn Ther 2005;2:247–62.
[10] Durantini EN. Photodynamic inactivation of bacteria. Curr Bioact Comp 2006;2:127–42.
[11] Taylor PW, Stapleton PD, Luzio JP. New ways to treat bacterial infections. Drug Discov Today 2002;7:1086–91.
[12] Hamblin MR, Hasan T. Photodynamic therapy: a new antimicrobial approach to infectious disease? Photochem Photobiol Sci 2004;3: 436–50.
[13] Wainwright M. Photodynamic antimicrobial chemotherapy (PACT). J Antimicrob Chemother 1998;42:13–28.
[14] Jori G, Brown SB. Photosensitized inactivation of microorganisms. Photochem Photobiol Sci 2004;5:403–5.
[15] Merchat M, Spikes G, Bertoloni G, Jori G. Studies on the mechanism of bacteria photosensitization by *meso*-substituted cationic porphyrins. J Photochem Photobiol B Biol 1996;35:149–57.
[16] Minnock A, Vernon DI, Schofield J, Griffiths J, Parish JH, Brown SB. Mechanism of uptake of a cationic water-soluble pyridinium zinc phthalocyanine across the outer membrane of *Escherichia coli*. Antimicrob Agents Chemother 2000;44:522–7.
[17] Salmon-Divon M, Nitzan Y, Malik Z. Mechanistic aspect of *Escherichia coli* photodynamic inactivation by cationic tetra-meso(*N*-methylpyridyl)-porphine. Photochem Photobiol Sci 2004;3:423–9.
[18] Nitzan Y, Ashkenazi H. Photoinactivation of *Acinetobacter baumannii* and *Escherichia coli* B by cationic hydrophilic porphyrin at various light wavelengths. Curr Microbiol 2001;42:408–14.
[19] Merchat M, Bertoloni G, Giacomini P, Villanueva A, Jori G. *meso*-Substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. J Photochem Photobiol B Biol 1996;32:153–7.
[20] Reddi E, Ceccon M, Valduga G, Jori G, Bommer JC, Elisei F, et al. Photophysical properties and antibacterial activity of *meso*-substituted cationic porphyrin. Photochem Photobiol 2002;75:462–70.
[21] Minnock A, Vernon DI, Schofield J, Griffiths J, Parish JH, Brown SB. Photoinactivation of bacteria. Use of a cationic water-soluble zinc phthalocyanines to photoinactivate both Gram-negative and Gram-positive bacteria. J Photochem Photobiol B Biol 1996;32:159–64.
[22] Segalla A, Borsarelli CD, Braslavsky SE, Spikes JD, Roncucci G, Dei D, et al. Photophysical, photochemical and antibacterial photosensitizing properties of a novel octacationic Zn(II)-phthalocyanine. Photochem Photobiol Sci 2002;1:641–8.
[23] Lazzeri D, Rovera M, Pascual L, Durantini EN. Photodynamic studies and photoinactivation of *Escherichia coli* using *meso*-substituted cationic derivatives with asymmetric charge distribution. Photochem Photobiol 2004;80:286–93.
[24] Banfi S, Caruso E, Buccafurni L, Battini V, Zazzaron S, Barbieri P, et al. Antibacterial activity of tetraaryl-porphyrin photosensitizers: an *in vitro* study on Gram negative and Gram positive bacteria. J Photochem Photobiol B Biol 2006;85:28–38.
[25] Scalise I, Durantini EN. Synthesis, properties, and photodynamic inactivation of *Escherichia coli* using a cationic and noncharged Zn(II) pyridyloxyphthalocyanine derivatives. Bioorg Med Chem 2005;13: 3037–45.
[26] Magde D, Brannon JH, Cremers TL, Olmsted III J. Absolute luminescence yield of cresyl violet. A standard for red. J Phys Chem 1979;83:696–9.
[27] Demas JN, Crosby GA. The measurement of photoluminescence quantum yields. J Phys Chem 1971;75:991–1024.
[28] Milanesio ME, Alvarez MG, Yslas EI, Borsarelli CD, Silber JJ, Rivarola V, et al. Photodynamic studies of metallo 5,10,15,20-tetrakis(4-methoxyphenyl) porphyrin: photochemical characterization and biological consequences in a human carcinoma cell line. Photochem Photobiol 2001;74:14–21.
[29] Redmond RW, Gamlin JN. A compilation of singlet yields from biologically relevant molecules. Photochem Photobiol 1999;70:391–475.
[30] Spesia MB, Rovera M, Pascual L, Durantini EN. A new antibacterial approach using photodynamic inactivation. Chem Educator 2005;10:126–9.
[31] Nitzan Y, Balzam-Subakevitz A, Ashkenazi H. Eradication of *Acinebacter baumannii* by photosensitized agents *in vitro*. J Photochem Photobiol B Biol 1998;42:211–8.
[32] Lambrechts SAG, Aalders MCG, Verbraak FD, Lagerberg JWM, Dankert JB, Schuitmaker JJ. Effect of albumin on the photodynamic inactivation of microorganisms by a cationic porphyrin. J Photochem Photobiol B Biol 2005;79:51–7.
[33] Orenstein A, Klein D, Kopolovic J, Winkler E, Malik Z, Keller N, et al. The use of porphyrins for eradication of *Staphylococcus aureus* in burn wound infections. FEMS Immunol Med Microbiol 1998;19:307–14.
[34] Wood S, Nattress B, Kirkham J, Shore R, Brookes S, Griffiths J, et al. An *in vitro* study of the use of photodynamic therapy for the treatment of natural oral plaque biofilms formed *in vivo*. J Photochem Photobiol B Biol 1999;50:1–7.
[35] Aveline BM, Redmond RW. Can cellular phototoxicity be accurately predicted on the basic of sensitizer photophysics? Photochem Photobiol 1999;69:306–16.