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Photophysical, photochemical and antibacterial photosensitizing properties of a novel octacationic Zn(II)-phthalocyanine

Anna Segalla,[a] Claudio D. Borsarelli,†[b] Silvia E. Braslavsky,[b] John D. Spikes,[c] Gabrio Roncucci,[d] Donata Dei,[d] Giacomo Chiti,[d] Giulio Jori *[a] and Elena Reddi [a]

[a] *Department of Biology, University of Padova, I-35131 Padova, Italy*
[b] *Max-Planck-Institut für Strahlenchemie, Postfach 10 13 65, Mülheim an der Ruhr, D-45713, Germany*
[c] *Department of Biology, University of Utah, Salt Lake City, Utah 84112, USA*
[d] *Molteni Farmaceutici, I-50018 Scandicci, Firenze, Italy*

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A novel Zn(II)-phthalocyanine (**1**), peripherally substituted with four bis(*N*,*N*,*N*-trimethyl)amino-2-propyloxy groups prepared by chemical synthesis, is shown to be an efficient photodynamic sensitizer with a quantum yield of 0.6 for singlet oxygen generation in neat water, which is reduced to about 0.3 in phosphate-buffered saline. The physicochemical properties of **1** in both the ground and the electronically excited states strongly depend on the nature of the medium; in particular, aggregation of **1** was favoured by polar media of high ionic strength. Compound **1** exhibited an appreciable affinity for a typical Gram-positive bacterium (*Staphylococcus aureus*) and a typical Gram-negative bacterium (*Escherichia coli*). Both bacterial strains were extensively inactivated upon 5 min-irradiation with 675 nm light in the presence of 1 μM photosensitizer, even though the binding of **1** to the two bacterial cells appears to occur according to different pathways. In particular, *E. coli* cells underwent initial photodamage at the level of specific proteins in the outer wall, thus promoting the penetration of the photosensitizer to the cytoplasmic membrane where some enzymes critical for cell survival were inactivated.

Introduction

Phthalocyanines and their analogues (*e.g.* naphthalocyanines) show great potential as phototherapeutic agents for the treatment of a variety of oncological and non-oncological diseases.[1,2] These diversified applications are at least in part a consequence of the numerous approaches which are available for the insertion of specific functionalities into the phthalocyanine molecule,[3] hence for imparting predetermined physicochemical properties (*e.g.* desired levels of hydrophobicity or hydrophilicity). The chemical structure of phthalocyanines can be modified by the introduction of substituents in the peripheral positions of the tetraazaisoindole macrocycle, as well as by the coordination of metal ions with the central nitrogen atoms and the addition of axial ligands in the fifth and sixth coordinative positions of such ions.[4,5] These changes can strongly modulate the photophysical properties of phthalocyanines[6] and affect their interaction with cells and tissues, thereby leading to different photobiological effects.[7]

Previous investigations showed that some Zn(II)-phthalocyanines (ZnPcs) can efficiently photosensitize the inactivation of various microbial pathogens.[8–10] The importance of this class of compounds as antimicrobial photosensitizers was further enhanced by the observation that the presence of positively charged functional groups allows an extensive photoinduced killing also of Gram-negative bacterial cells,[11] which are usually resistant to the action of non-cationic porphyrinoid photosensitizers.[12]

Inasmuch as photodynamic therapy (PDT) is a very promising recent development for the treatment of infectious diseases,[13] it is undoubtedly of interest to define the relationship between the chemical structure of phthalocyanines and their efficiency as photosensitizers for the inactivation of selected microbial strains. In this paper, we describe the photophysical, photochemical, and photobiological properties of a novel octacationic Zn-phthalocyanine (**1**), which exhibits a number of interesting characteristics for a prospective therapeutic use and a remarkable phototoxicity against some highly infectious bacteria.

Materials and methods

Chemicals

Octacationic Zn(II)-phthalocyanine, {2(3),9(10),16(17),23(24)-tetrakis[1,3-bis(*N*,*N*,*N*-trimethylammonium)-2-propyloxy]-phthalocyaninato}zinc(II) iodide (**1**, Fig. 1) was prepared as a

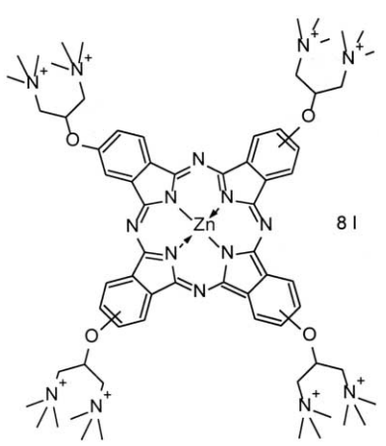

Fig. 1 Chemical structure of {2(3),9(10),16(17),23(24)-tetrakis[1,3-bis(*N*,*N*,*N*-trimethylammonium)-2-propyloxy]phthalocyaninato}zinc(II) iodide (**1**).

† *Present address*: Departamento de Ciencias Básicas, Universidad de Santiago del Estero, Santiago del Estero, 4200 Argentina.

mixture of four regioisomers by chemical synthesis according to a procedure described elsewhere.[14] Overall, the four isomers account for more than 98% as shown by NMR and mass spectroscopic analysis.[14] The molar absorption coefficient of **1** was found to be 1.7×10^5 M^{-1} cm^{-1} at 677 nm in dimethylformamide: spectroscopic titration studies showed that the solutions of **1** closely follow the Beer–Lambert law up to a *ca.* 0.1 mM concentration in this solvent, indicating that **1** exists in a purely monomeric state. Similar observations were made in water solutions.

The solutions of **1** were made up in either re-distilled water (pH = 5.0–5.5) or 10 mM phosphate-buffered saline (PBS) at pH = 7.4, containing 2.7 mM KCl and 140 mM NaCl.

Cetyltrimethylammonium bromide (CTAB) and sodium dodecylsulfate (SDS) were purchased from Merck AG, while catalase and superoxide dismutase (SOD) were the products of Calbiochem. Furfuryl alcohol (FA) was a spectroscopic grade product of Aldrich. *N*-Acetyl-L-tryptophanamide (NATA) was supplied by Sigma Chemical Co. All other reagents and solvents were commercially available products of at least analytical grade and were used without further purification.

Spectroscopic and photophysical studies

Absorption spectra of **1** in the various solvent systems were recorded by means of either a Shimadzu UV-2102PC or a Perkin Elmer UV/VIS Lambda 2 spectrophotometer. The fluorescence emission spectra were measured at room temperature (*ca.* 25 °C) by means of either a Perkin Elmer MPF-66 or a computer-controlled Spex-Fluorolog spectrofluorimeter; the measurements were performed in air- or oxygen-saturated solutions, as well as in solutions deoxygenated by extensive nitrogen bubbling.

The fluorescence quantum yield of **1** was determined by comparing its emission spectrum in the 600–800 nm wavelength range with that of 2,7,12,17-tetra-*n*-propylporphycene in toluene, which was used as a reference standard with Φ_F = 0.38.[15] Corrections were made for the difference in the refractive index of the solvents. The absorbance of **1** and reference solutions (*ca.* 0.08) were matched within 3% at the excitation wavelength of 370 nm. The fluorescence lifetime of **1** was measured in aqueous and dimethylformamide (DMF) solutions at room temperature by using the single photon counting technique;[16] the excitation and emission (detection) wavelengths were 354 and 650 nm, respectively.

The triplet energy level, E_T, of **1** was determined in degassed (three freeze–pump–thaw cycles) DMF and aqueous solutions at 25 °C by means of a home-built near-IR steady-state emission spectrophotometer.[17]

The triplet–triplet absorption spectrum of **1** was determined in deoxygenated DMF solutions by means of laser flash photolysis with optical detection; the excitation wavelength was 660 nm. The transient spectrum was built by using the zero-time extrapolated value of the absorbance decay curve at each wavelength. The triplet quantum yields (Φ_T) were determined by using laser-induced optoacoustic spectroscopy (LIOAS) in DMF and aqueous deoxygenated solutions. The triplet state of **1** is long-lived (τ > 100 μs, see Results section), *i.e.*, its lifetime is markedly longer than the heat integration time (1.5 μs) in LIOAS; therefore, it acts as an energy-storing species. Under these conditions, the amplitude of the LIOAS signals can be used directly to determine the fraction of energy released as heat (a) upon generation of the triplet state. Evans blue was utilized as a calorimetric reference; the absorbance of **1** and Evans blue were matched at the excitation wavelength (660 nm).

Singlet molecular oxygen, $O_2(^1\Delta_g)$, formation following pulsed laser excitation of **1** was detected by monitoring the typical luminescence emission of $O_2(^1\Delta_g)$ at 1270 nm with a nitrogen-cooled Ge diode as a near-IR detector. The experimental set up was described elsewhere.[17,18] The $O_2(^1\Delta_g)$ luminescence decay was measured in oxygen-saturated DMF, water, and D_2O solutions and the quantum yield (Φ_Δ) of $O_2(^1\Delta_g)$ production was calculated by comparing the $O_2(^1\Delta_g)$ emission signal extrapolated to zero time after the laser pulse (640 nm) to that of an absorbance matched solution in the same solvent of 5,10,15,20-tetrakis(4-sulfonatophenyl)porphine ($TSPP^{4-}$) used as a reference.[19]

Photobleaching studies with 1

Kinetic studies of **1** photobleaching were performed in both air-saturated and degassed water solutions, as well as in DMF and PBS aqueous media containing various additives. Typically, 7–10 μM solutions of **1** were irradiated with 675 nm light from a diode laser operated at a fluence rate of 200 mW cm^{-2}. In a few experiments, the fluence-rate was lowered by interposing a 32% transmittance neutral filter between the light source and the 1 cm-quartz cuvette containing the sample. The photobleaching quantum yields, defined as [initial rate of **1** disappearance]/[initial rate of photon absorption], were measured by irradiation of 6 μM solutions of **1** in the various solvent systems; a 500 W quartz halogen lamp equipped with an interference filter at 676 nm (17 nm-bandwidth at 50% of peak transmission) was used as the light source. The fluence-rate at the cuvette containing compound **1** was 16 mW cm^{-2}. The accumulation of hydrogen peroxide in the illuminated aqueous solutions of **1** was tested by injecting 50 μl of a catalase suspension containing 4×10^4 units ml^{-1} into 4 ml of the reaction mixture in a vessel connected to an oxygen electrode and measuring the amount of oxygen evolved.

Photosensitized oxidation of furfuryl alcohol and *N*-acetyl-L-tryptophanamide

The photosensitizing activity of **1** with model substrates was evaluated by following the photooxidation of furfuryl alcohol (FA) and *N*-acetyl-L-tryptophanamide (NATA). Typically, 2 mM FA and 5 μM solutions of **1** in high purity water, pH 5.1, or in PBS were irradiated at 25 °C by using the same apparatus as that for the determination of the photobleaching quantum yield. The oxygen electrode system adopted for recording the oxygen uptake was the same as previously described.[20] In another set of experiments, 5 μM NATA and 1 μM solutions of **1** in DMF or in PBS were irradiated with 675 nm light at 25 °C and at 200 mW cm^{-2}. The decrease in the NATA concentration as a function of the irradiation time was followed by measuring the intensity of the 290 nm-excited fluorescence emission spectrum (300–400 nm) typical of the tryptophanyl moiety. The details of the experimental procedure were published in a previous paper.[21]

Bacterial strains

Escherichia coli strain O4 and *Staphylococcus aureus* MRSA strain 110 were grown aerobically at 37 °C in brain heart infusion broth (Difco, Detroit, MI, USA). The cells in the stationary phase of growth were harvested by centrifugation of broth cultures (2000*g* for 15 min), washed twice with 10 mM PBS and diluted in the same buffer to an optical density of 0.7 at 650 nm, corresponding to 10^8 –10^9 cells ml^{-1}.

Phthalocyanine binding to bacterial cells

To determine the amount of photosensitizer associated with the cells, suspensions of *E. coli* and *S. aureus* were incubated in the dark for 5 min with different concentrations of **1**, centrifuged (2000*g* for 15 min) and then washed with PBS. The recovery of cell-bound phthalocyanine after different washing steps (0, 1, and 3) was estimated spectrophotofluorimetrically. Toward this aim the cell pellets obtained by centrifugation were resuspended in 2% aqueous SDS, incubated overnight, and diluted in the same solvent; the fluorescence emitted by **1** (630–800 nm) was

measured after excitation at 610 nm. The concentration of **1** was calculated by measuring the intensities of the emission peaks and interpolating the data on a calibration plot obtained with known amounts of **1** in 2% SDS. The protein content of the samples was assayed by the method of bicinchoninic acid (BCA)[22] and used to express the recovery as nmoles of **1** (mg of protein)$^{-1}$.

Photosensitized inactivation of bacterial cells

Five ml of cell suspensions were incubated with the appropriate concentrations of **1** for 5 min in the dark at room temperature and then irradiated for different times with 675 nm light at a fluence rate of 100 mW cm^{-2}. The light power entering the irradiated solution was 154 mW. During irradiation the bacterial suspensions were magnetically stirred, air-equilibrated, and maintained at 37 °C by circulating water. For the experiments under anoxic conditions the cell suspensions were degassed by evaporating half of the total buffer volume by means of a vacuum pump, incubated with **1**, and irradiated in an Atmos-bag (Sigma, St. Louis, Mo, USA) filled with N_2. Control experiments were carried out without illumination in the absence and in the presence of **1**. Control and irradiated cell suspensions were serially diluted with PBS; each dilution was plated on brain heart agar and the number of colonies formed after 18–24 h incubation at 37 °C was counted.

Enzymic activity assays

The activity of different enzymes localized in the cytoplasmic membrane of *E. coli* was measured in lysed spheroplasts obtained by resuspending photosensitized and untreated cells in Tris-HCl 0.05 M buffer at pH = 6.8 containing 0.01 M EDTA, 0.3 M saccarose and lysozyme (1 mg ml^{-1}). After 1 h incubation at 37 °C the cells were centrifuged at 2000*g* for 10 min. The pellet was resuspended in water and the spheroplasts were lysed by sonication in an ice bath.

NADH dehydrogenase activity was measured according to Marriott *et al.*[23] by following the oxidation of NADH spectrophotometrically at 340 nm in the presence of $K_3Fe(CN)_6$ as electron acceptor.

Lactate dehydrogenase and succinate dehydrogenase activities were measured by the methods described by Santos *et al.*[24] and Osborne *et al.*,[25] respectively.

The protein content of the samples was measured by the BCA method; the activities of the enzymes are expressed as percentage of the specific activity measured in untreated cells.

Protein electrophoresis

Outer membrane proteins of *E. coli* cells were obtained as described by Rapp *et al.*[26] About 20 μg of proteins were loaded in a 12% polyacrylamide gel with 2% aqueous SDS for electrophoretic analysis.[27] Proteins in the gel were stained with Coomassie brilliant blue and destained in methanol–acetic acid–water (4 : 3 : 33, v/v/v).

Results

Ground state properties of 1

The absorption spectrum of 1 μM **1** in DMF is shown in Fig. 2a over the 300–800 nm wavelength range. An essentially identical absorption spectrum is obtained for **1** in neat water, where this phthalocyanine is readily soluble. The spectrum exhibits three bands peaking at 348, 610, and 677 nm: the position of the maxima and the relative intensities of the bands are typical of monomeric phthalocyanines.[28] This conclusion is supported by the observation that the shape of the absorption spectrum is essentially unchanged over the 1–20 μM range of **1** concentration. A somewhat different picture is obtained when **1** is

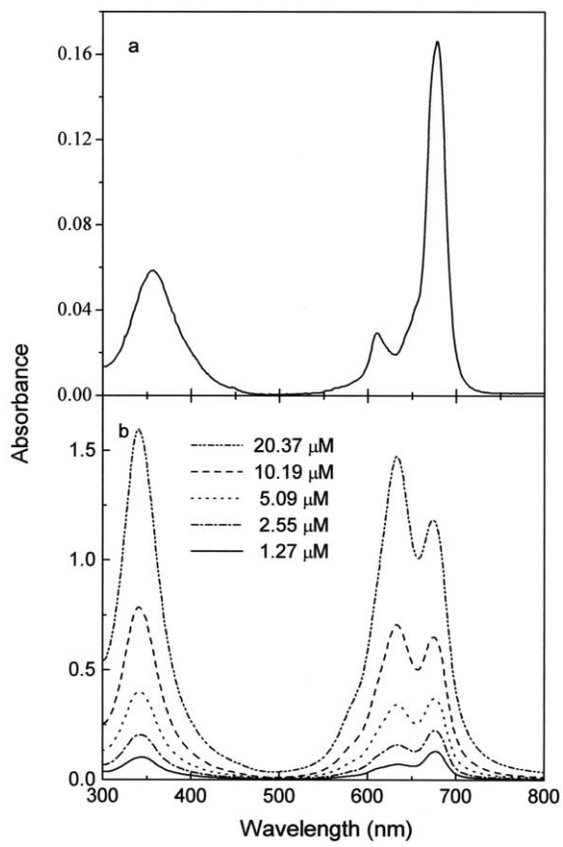

Fig. 2 (a) Typical near-UV–visible absorption spectrum of 1 μM **1** in *N*,*N*-dimethylformamide (DMF) solution. (b) Effect of **1** concentration on the near-UV–visible absorption spectrum in phosphate-buffered saline (PBS, pH = 7.4).

dissolved in PBS (Fig. 2b): as the phthalocyanine concentration is increased, the Q bands above 600 nm undergo a significant broadening and the 633 nm-peaking band becomes predominant over the 677 nm band. These spectral features are characteristic of aggregated phthalocyanines[28] and indicate that **1** exists as a mixture of monomeric and oligomeric species in PBS, whose relative weight is modulated by the phthalocyanine concentration. The differences between the spectra of **1** in neat water and saline solutions are not caused by differences in pH values; actually, adjusting the pH of the water solution (5.1–5.6) to about 7.4 through the addition of either NaOH or very small amounts of high pH PBS (final concentration <1 mM) have no detectable effect on the absorption spectrum of **1**. The partial aggregation of **1** in PBS is likely to be promoted by the large salt concentration (*ca.* 0.14 M). Thus, increasing the concentration of NaCl, NaI, or $CaCl_2$ in an aqueous solution of **1** is accompanied by the progressive broadening of the Q band and a decrease in the molar absorptivity of the longest wavelength band. The effect of the additional solute concentration on the latter process is shown in Fig. 3. The effect of $CaCl_2$ is more pronounced than that of NaCl due to the higher ionic strength, while NaI is the most effective salt out of those examined. Conversely, the addition of 1 M concentration of non-ionic compounds, such as urea and sucrose, has no appreciable influence on the spectral properties of **1**, thus confirming the observed effects of salts on the tendency of this phthalocyanine to aggregate.

The addition of 0.07 M SDS to a 5 μM solution of **1** in PBS converts the absorption spectrum to that typical of the monomer while no changes are observed in the presence of equivalent concentrations of CTAB. These observations are in agreement with previous findings showing that detergents induce the monomerization of porphyrins and phthalocyanines provided they do not carry the same type of charge.[29] Organic solvents also disrupt the hydrophobic and π–π interactions which

Table 1 Emission lifetimes, τ, and component contribution, A, for **1** in N_2- and O_2-saturated dimethylformamide (DMF) and in water solutions (λ_{exc} = 354 nm and λ_{em} = 650 nm). The percent contribution of each component is shown in brackets

	N_2	Air	O_2
DMF			
τ_1/ns	3.7 ± 0.2	3.6 ± 0.1	3.3 ± 0.1
A_1 (%)	0.16 ± 0.13 (31)	0.25 ± 0.11 (48)	0.25 ± 0.10 (47)
τ_2 /ns	2.3 ± 0.1	2.1 ± 0.1	1.9 ± 0.2
A_2 (%)	0.36 ± 0.10 (69)	0.27 ± 0.10 (52)	0.28 ± 0.10 (53)
H_2O			
τ_1/ns	3.3 ± 0.1	3.2 ± 0.1	—[a]
A_1 (%)	0.18 ± 0.02 (64)	0.32 ± 0.03 (54)	—[a]
τ_2/ns	1.3 ± 0.1	1.2 ± 0.1	—[a]
A_2 (%)	0.10 ± 0.01 (36)	0.28 ± 0.03 (46)	—[a]

[a] The values in water in the presence of oxygen are doubtful, since the sample was quickly bleached and it was not possible to average several signals.

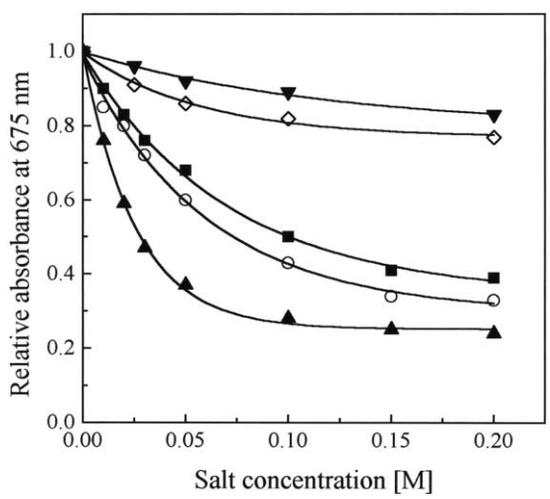

Fig. 3 Effect of different additives on the absorbance of 1 μM solution of **1** at 675 nm. (■), NaCl; (O), $CaCl_2$; (▲), NaI; (◊), sucrose; (▼), urea.

stabilize the aggregates of tetrapyrrolic derivatives[30] as shown by the absorption spectrum of **1** in DMF, again typical of the monomeric species (Fig. 2a).

Properties of the first excited singlet state of 1

The fluorescence emission spectrum of **1** shows a typical shape for phthalocyanines[28] with a peak at 693 nm in both water and PBS. Only marginal changes in the emission maximum are observed for **1** in DMF and in 0.07 M aqueous SDS. The emission quantum yield is 0.11 ± 0.01 in all the solvents. No effect on the fluorescence properties of **1** is caused by saturation of the solvent with nitrogen suggesting that oxygen has no quenching action on the lowest excited singlet state of **1**. In all cases, the fluorescence excitation spectrum is coincidental with the absorption spectrum of monomeric **1**.

The fluorescence decay of **1** in both water and DMF is satisfactorily fitted by a biexponential decay function, namely $I_{em}(t) = A_1 e^{-t/\tau 1} + A_2 e^{-t/\tau 2}$ where τ is the emission lifetime and A_i is the amplitude of the individual transients (see Table 1). Clearly, no important difference is detected between N_2-, air- and O_2-saturated solutions; however, the relative weight of the slower-decaying transient is somewhat larger in water than in DMF. In general, the fast-decaying species are ascribed to phthalocyanine aggregates.[31] However, this interpretation is unlikely to hold in this case since (a) the absorption spectra of **1** in DMF and in water indicate no detectable presence of aggregated species, and (b) the fluorescence intensity of **1** is linear with concentration over the 0.025–0.25 μM range.

Table 2 Triplet decay life times (μs) of 10 μM **1** measured at 490 nm in dimethylformamide (DMF) and water solutions following excitation at 670 nm

Medium	DMF	H_2O
N_2	320 ± 2	125 ± 3
Air	3.51 ± 0.10	0.90 ± 0.02
O_2	0.33 ± 0.01	0.14 ± 0.01

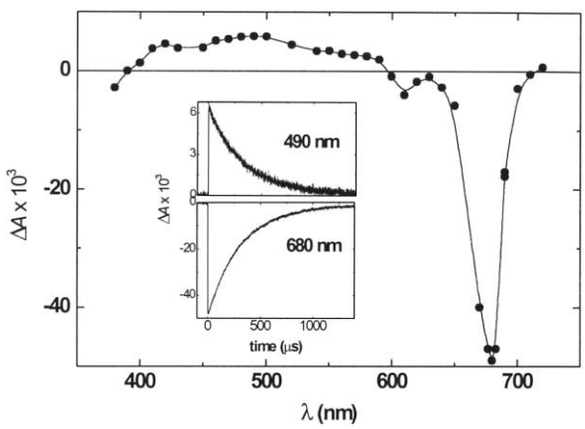

Fig. 4 Triplet–triplet absorption spectrum of **1** in degassed solutions of dimethylformamide immediately after the 660 nm laser pulse excitation. The transient spectrum was built by using the zero-time extrapolated value of the absorbance decay curve at each wavelength. *Inset*: absorption decay monitored at 490 and 680 nm.

Triplet state of 1 and singlet oxygen generation

The phosphorescence spectrum of **1** in degassed DMF and in water solutions at room temperature displays a maximum at 1110 nm which yields E_T = (108 ± 5) kJ mol^{-1}. The triplet–triplet absorption spectrum (Fig. 4) shows a peak around 490 nm, *i.e.* to the red of the Soret band, as is typical of most phthalocyanines.[31] The $\Delta\varepsilon_{T\text{-}T}$, as obtained by comparison with the bleaching at 680 nm, is 20790. The triplet lifetime of **1** (Table 2), of the order of a few hundred microseconds in N_2-saturated solutions, is strongly reduced in the presence of oxygen; the triplet quantum yield is larger than 0.8 in both DMF and in water and is markedly decreased in PBS, to the point that it cannot be measured with our flash photolysis equipment with a time resolution of several hundred nanoseconds.

The large Φ_T value in water and in DMF indicates that photoexcited **1** does not significantly return to the ground state *via* radiationless pathways since $\Phi_T + \Phi_{em} \cong 1$. Again, this behaviour is typical of phthalocyanines, where the electronic transitions of the tetraazaisoindole macrocycle are characterized by a negligible vibrational component, as suggested by the strong overlap between the 0–0 transition and the fluorescence emission.

The efficient quenching of **1** triplet by oxygen largely leads to the generation of $O_2(^1\Delta_g)$ as monitored from the luminescence decay at 1270 nm. Φ_Δ was found to be 0.6 ± 0.04 in DMF and 0.6 ± 0.03 in water. The lifetime of $O_2(^1\Delta_g)$ sensitized by **1** in PBS is strongly reduced to less than 1 μs; moreover, there is a strong bleaching of *ca.* 15% after excitation with 5 pulses of *ca.* 200 μJ each in this medium, which impairs signal averaging to reduce the noise. These two facts preclude the determination of Φ_Δ for **1** by luminescence emission in this medium.

Similar Φ_Δ values were independently obtained by following the **1**-sensitized photooxidation of FA in water. With this technique, the quantum yields of oxygen uptake are measured in the presence of increasing amounts of alcohol; at saturating alcohol concentrations, the $O_2(^1\Delta_g)$ yields are proportional to the quantum yields of oxygen uptake.[32] Using rose bengal as a standard (Φ_Δ = 0.75),[33] the Φ_Δ values of **1** were found to be 0.54

in water, 0.32 in PBS, and 0.52 in 0.07 M SDS (average of three determinations). The yield of oxygen uptake was 2.8-fold increased by replacing H_2O with D_2O and was inhibited by about 50% in the presence of 0.96 mM sodium azide: both of these results are in agreement with the involvement of $O_2(^1\Delta_g)$. On the other hand, the yields were not affected by the addition of catalase or superoxide dismutase suggesting that hydrogen peroxide and superoxide do not mediate FA photooxidation. However, hydrogen peroxide was accumulated in the reaction mixture with a yield of 0.46 mol H_2O_2/mol of O_2 consumed; in any case, **1** was not affected by 1 h exposure to 5% H_2O_2.

Photobleaching studies with 1

Several tetrapyrrolic derivatives are known to undergo an extensive photodegradation when exposed to visible light.[34] Compound **1** is also photobleached upon irradiation with selected visible wavelengths (see Experimental section for the irradiation protocol), whereas it appeared to be stable for prolonged storage in the dark in both aqueous and organic solvents. After 400 s illumination in air-equilibrated water the absorption of **1** in the red spectral region declines to zero, while a residual absorption is observed at 348 nm; the pattern of spectral changes suggests that the tetraazaisoindole macrocycle is irreversibly destroyed.

In all cases, the photobleaching kinetics of **1** follow a first-order law and the time-dependent decrease of absorbance at the absorption maximum is well fitted by a single-exponential decay function (Fig. 5). In particular, the photobleaching rate

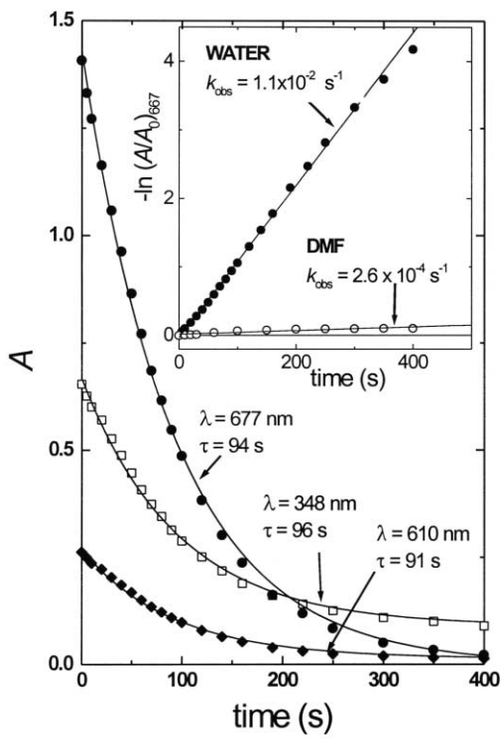

Fig. 5 Absorption changes at three different wavelengths *vs.* irradiation time at 675 nm for 1 μM solutions of **1** in air-saturated aqueous solutions at room temperature. Solid lines represent fits of the experimental data with a single exponential decay function. *Inset*: first order plots for the photobleaching of **1** in air-saturated water and dimethylformamide solutions monitored at 667 nm.

constant is appreciably larger in water (1.1×10^{-3} s^{-1}) than in DMF (2.6×10^{-4} s^{-1}).

It is worthwhile underlining that the yield of **1** photobleaching in water is unusually high, and is one to two orders of magnitude larger than that found for a variety of other phthalocyanines.[34] The yield is not significantly affected by the presence of 0.01 M sodium azide or the replacement of H_2O with D_2O, thus ruling out any major contribution of $O_2(^1\Delta_g)$ to the photobleaching process. Similarly, no effect is caused by the addition of catalase and superoxide dismutase, which suggests that neither hydrogen peroxide nor superoxide are involved. In fact, **1** is rapidly photobleached also after saturation of water with nitrogen (data not shown).

Photosensitized oxidation of *N*-acetyl-L-tryptophanamide by 1

Tryptophan represents a suitable substrate for testing the efficiency of photosensitizing agents since it is efficiently photooxidized by both type I (radicals) and type II (singlet oxygen) reaction mechanisms.[35]

Under our experimental conditions, the modification of NATA photosensitized by **1** in DMF occurs according to first-order kinetics. The rate constant of the photoprocess was found to be $(6.3 \pm 0.8) \times 10^4$ s^{-1} (average of three independent experiments). This value is close to that reported for the oxidation of tryptophan derivatives mediated by several photodynamic sensitizers.[36]

Binding of 1 to bacterial cells

The ability of **1** to bind to bacterial cells was studied by incubating for 5 min different phthalocyanine concentrations with Gram-negative (*E. coli*) and Gram-positive bacteria (*S. aureus*) (Fig. 6a and b). Preliminary experiments showed that prolonging the incubation time to 30 min has no appreciable effect on the amount of cell-bound phthalocyanine. A comparative analysis of the data of Fig. 6 clearly indicates that **1** exhibits a larger affinity for *S. aureus* than for *E. coli* cells. Actually, under all our experimental conditions, *S. aureus* binds markedly higher amounts of **1**. The fraction of tightly cell-bound phthalocyanine appears to be markedly influenced by

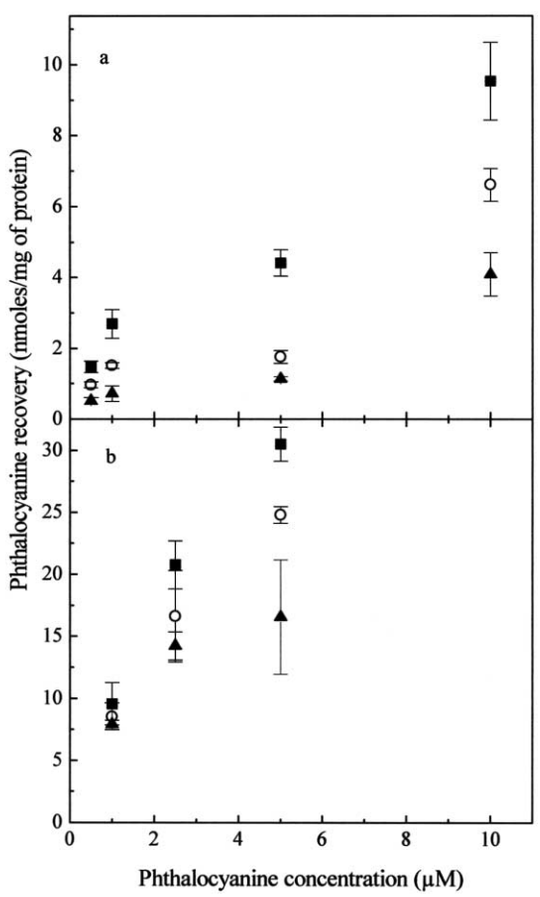

Fig. 6 Effect of **1** concentration on the recovery of **1** from (a) *E. coli* cells and (b) *S. aureus* cells, after 0 (■), 1 (○), and 3 (▲) washing steps. Incubation time: 5 min.

the concentration of compound **1** in the incubation medium: thus, repeated washing (up to 3 consecutive times) of phthalocyanine-loaded cells removes 60–80% of initially bound compound **1** in the case of *E. coli*, as compared with 25–50% removal in the case of *S. aureus*.

Photosensitized inactivation of bacterial cells

Preliminary control studies showed that the incubation of *S. aureus* and *E. coli* cells with 1 μM **1** for 5 min in the dark has no effect on cell survival. On the other hand, 675 nm-irradiation of the cells under such conditions causes a marked decrease in their survival. The irradiation of unwashed *S. aureus* cells induces an about 4 log decrease in cell survival already after 30 s irradiation (Fig. 7b). A much slower additional drop in cell

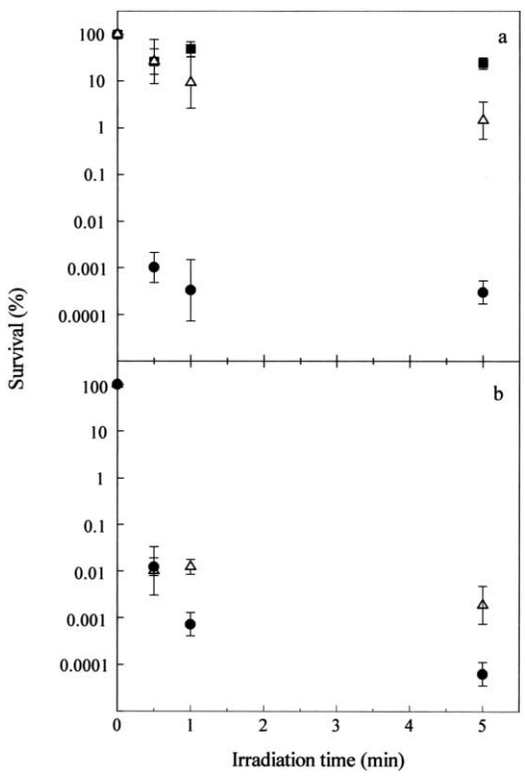

Fig. 7 Effect of the 675-nm irradiation time on the survival of (a) *E. coli* cells after 5 min incubation with 1 μM **1** after (●) 0 and (Δ) 3 washing steps, as well as after (■) no washing in the absence of O_2; (b) *S. aureus* cells after 5 min incubation with 1 μM of **1**; (■) no and (Δ) 3 washing steps.

survival is observed upon prolonging the irradiation time to 5 min. Only minor differences in the kinetics of cell photoinactivation are found for 2-fold washed cells after the same irradiation times.

E. coli cells show a similar loss of survival as *S. aureus*, when unwashed cells are exposed to 675 nm-light (Fig. 7a). On the other hand, a large difference in photosensitivity is observed when the *E. coli* cells are irradiated after three washing steps; only an about 2 log decrease in survival is observed after 5 min irradiation.

Moreover, when the irradiation is performed in the absence of oxygen, no decrease in survival of *E. coli* cells is observed even after 5 min light exposure. SDS-PAGE analysis of the *E. coli* outer membrane proteins (Fig. 8) shows no difference between control and dark-incubated samples; on the contrary, an evident alteration of the electrophoretic pattern is observed in the irradiated samples. In particular, some protein bands corresponding to molecular weights around 20 and 29 kDalton disappear already after 15 s irradiation; protein bands with molecular weights around 36 and <20 or >45 kDalton show a

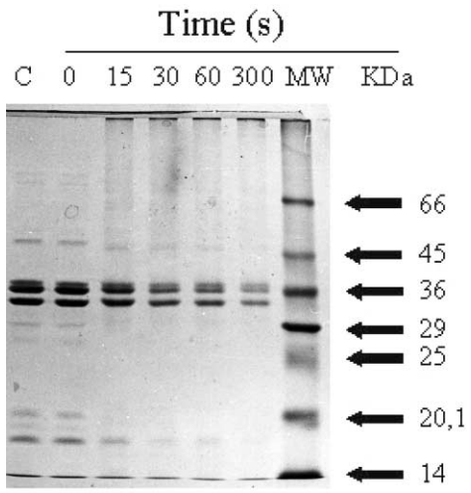

Fig. 8 SDS-PAGE of the outer membrane proteins of *E. coli* cells. Lane 1: proteins from control cells; lanes 2–6 : proteins from cells incubated for 5 min with 0.5 μM of **1** and irradiated for 0, 15, 30, 60, 300 s, respectively.

reduction of the staining intensity which progressively decreases with the irradiation time. In addition, high molecular weight products are observed as cross-linked material on the top of the gel in all the irradiated samples.

Lastly, the effect of the irradiation on selected enzymatic activities was studied in *E. coli* cells which had been incubated for 5 min with 0.5 μM **1**. Lactate dehydrogenase is rapidly inactivated by irradiation and a complete loss of the enzymic function is observed after 15 s irradiation (Fig. 9). The activities

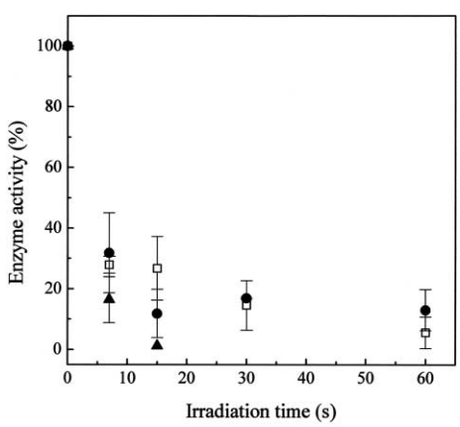

Fig. 9 Effect of the 675-nm irradiation time on the activity of (□) NADH dehydrogenase (●) succinate dehydrogenase, and (▲) lactate dehydrogenase in *E. coli* cells incubated for 5 min with 0.5 μM of **1**.

of both NADH and succinate dehydrogenases are also significantly decreased by photosensitization and are reduced to less than 10% after 1 min irradiation.

Discussion

Phthalocyanines are known as typical photodynamic sensitizers, namely they usually exhibit a significant photosensitizing activity only in the presence of oxygen, which is efficiently converted to $O_2(^1\Delta_g)$.[28,37] The present results show that **1** behaves in a similar manner as documented by several pieces of evidence including (a) the relatively high quantum yield for generation of $O_2(^1\Delta_g)$ (*ca.* 0.6 in water and SDS and 0.3 in PBS), (b) the first-order kinetics for the modification of two highly photosensitive substrates, such as FA and NATA, typical of most photodynamic processes,[35] and (c) the lack of any detectable photoinactivation of *E. coli* cells when the visible light irradiation is carried out in the absence of oxygen.

However, **1** shows some peculiar photophysical and photochemical properties. In particular, the behaviour of the ground and electronically excited states of this phthalocyanine appears to be markedly solvent dependent. Such a dependency is a consequence, at least in part, of the opposite effects caused on the one hand by the hydrophobic aromatic macrocycle, which promotes the formation of stacked phthalocyanine aggregates in polar media, and on the other hand by the eight positively charged ammonium groups, which impart a high degree of hydrophilicity to the molecule and counteract the aggregation process through electrostatic repulsion. In fact, the monomerization of **1** oligomers is favoured by the addition of DMF, as well as by the incorporation of **1** into SDS micelles, *i.e.* in media which disrupt the π–π and hydrophobic forces stabilizing the intermolecular interactions. It is especially interesting that the aggregation process of **1** is fostered by the increase in ionic strength of aqueous solutions, as shown by the absorption spectra in the presence of different salt concentrations. The nature of the counterion controls the extent of the overall process, since iodide ions promote the aggregation of **1** more efficiently than chloride, most likely through the formation of ion pairs stabilized by the polarizability of the former. Replacing Na^+ by Ca^{2+} brings about no appreciable difference: actually, no difference can be noted when using solutions of identical chloride concentration of the two different cations. In any case, a larger ionic strength should depress the photosensitizing activity of **1**, since Φ_Δ in PBS is significantly lower than that in neat water. This is probably due to the reported tendency of aggregates to shorten the lifetime of the electronically excited states of phthalocyanines.[37]

On the other hand, additional factors must be involved in the modulation of the photoproperties of **1**. This hypothesis is supported by two findings. (a) The presence of two emitting species (Table 1), whose relative weight is significantly different in H_2O and in DMF solutions, even though **1** is exclusively monomeric in both solvents; thus, the slow- and fast-decaying components could represent specific modes of solvation of **1**. The possible formation of water-bridged phthalocyanine dimers has been proposed in the past.[38] (b) The photobleaching rate of **1** is much larger in H_2O than in DMF, which suggests that the solvent controls the photoreactivity of **1**. Since photobleaching appears to be oxygen-independent, it is reasonable to suppose that the photoinduced degradation of the tetraazaisoindole macrocycle involves the participation of solvent molecules.

As a general conclusion, the above considerations underline the potentially strong effect of the microenvironment on the efficiency and mechanism of **1**-photosensitized processes. This aspect is of special interest for the application of **1** as a photosensitizer in complex biological systems, where the phthalocyanine can be partitioned among a variety of compartments with broadly different physicochemical features and biochemical composition. Thus, while $O_2(^1\Delta_g)$ is the main intermediate in **1**-photosensitized oxidation of model substrates such as FA, it cannot be taken for granted that other pathways are not operative under specific experimental conditions. The observed formation of hydrogen peroxide during FA photooxidation suggests that type I (radical) mechanisms may become important. Similar considerations can be used for the aggregated species of **1**, which are present in PBS solutions. Partitioning of **1** in the cells may cause, at least partially, disaggregation and maximise the *in situ* $O_2(^1\Delta_g)$ production.

A determining role of the photosensitizer binding site could be invoked to explain the very similar sensitivity of *E. coli* and *S. aureus* cells to visible light irradiation in the presence of **1** in spite of the markedly different amounts of phthalocyanine accumulated by the two cell types. That the mode of **1** localization differs in the Gram-positive and Gram-negative bacterial cells is clearly shown by the fast and extensive removal of **1** from *E. coli* cells upon washing with PBS as compared with its tight association with *S. aureus* cells. Previous investigations pointed out that the binding of cationic phthalocyanines with Gram-negative bacterial cells is driven by electrostatic interactions with anionic functional groups at the cell surface.[39] In fact, our electrophoretic analyses indicate that some protein molecules in the outer wall of phototreated *E. coli* cells are the target of the initial stages of the photoprocess, as shown by the disappearance of some protein bands already after 15 s irradiation. Such modifications are likely to induce an enhanced permeability of the wall, which is considered as the structural element responsible for the strong resistance of Gram-negative bacteria to photosensitization.[40] As a consequence, significant concentrations of **1** can reach the cytoplasmic membrane where endocellular sites, critical for *E. coli* survival, undergo an irreversible modification. Thus, the drop in *E. coli* survival parallels the photosensitized decrease in the activity of typical membrane enzymes (Figs. 8 and 9). Other hypotheses such as the direct interaction of **1** with the cytoplasmic membrane could also be invoked.

In any case, our data further confirm that cationic phthalocyanines are efficient photosensitizers for the inactivation of both Gram-positive and Gram-negative bacteria. Evidently, the unusually fast photobleaching of **1** does not preclude a photoinduced decrease of at least 5 log in the bacterial cell population (see Fig. 8). This represents a positive factor since the rapid disappearance of the phthalocyanine from the irradiated site minimizes the risk of a persistent photosensitivity, which would be an undesired side effect in case this technique is adopted as a phototherapeutic modality for the treatment of bacterial infections. The safety of the phototreatment is further supported by the observation that the photodegradation products of **1** exhibit no detectable toxic activity against both human fibroblasts and keratinocytes (data not shown).

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