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Original article

Synthesis and comparative photodynamic properties of two isosteric alkyl substituted zinc(II) phthalocyanines

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The synthesis and photophysical parameters of two novel isosteric cationic zinc(II) phthalocyanines: 2,9(10),16(17),23(24)-tetrakis[(N-butyl-N-methylammoniumethylsulfanyl]phthalocyaninatozinc(II) tetraiodide (**6**) and 2,9(10),16(17),23(24)-tetrakis[(N-dibutyl-N-methylammonium)ethoxy]phthalocyaninatozinc(II) tetraiodide (**7**) were investigated. Maximum absorption values were 686.5 nm and 678 nm for **6** and **7**, respectively, whereas singlet molecular oxygen generation was 0.42 and 0.67, respectively. The photodynamic effect and the cellular uptake of both phthalocyanines were evaluated on human nasopharynx KB carcinoma cells. After light exposure, phthalocyanine **6** showed a higher cytotoxic activity than **7.** In addition, a higher intracellular uptake of **6** and a preferential localization within lysosomes were demonstrated. The production of a greater amount of reactive oxygen species after phthalocyanine **6** irradiation would be responsible for its potent phototoxic action on KB cells.

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1. Introduction

Phthalocyanines (Pcs) are important dyes used in medicinal field as promising candidates for photodynamic therapy (PDT) in cancer treatment [1]. This technology is based on the light excitation of a photosensitizer which induces a localized oxidative damage within the cells by formation of highly reactive oxygen species, the most important of which is singlet oxygen. Many photo-activable molecules have been synthesized. These photo-activable molecules include porphyrins, chlorines and more recently phthalocyanines, which present a strong light absorption at wavelengths around 670 nm and are therefore well-adapted to the optical window required for PDT applications [2]. It has been reported that peripheral substituents [3–5], the nature of the central metal ion [6,7] as well as the axial ligand coordinated to the metal center [8] of phthalocyanines, change their physical, chemical, and biological properties. Zinc(II) phthalocyaninates showing improved photophysical properties have been developed during the last years [9]. In addition, attempts have been made both to increase the uptake of the dye by the targeted cells and to improve subcellular localization within the cells.

In a recent study we stated that a sulfur-linked cationic dye, named: 2,9(10),16(17),23(24)-tetrakis[(2-trimethylammonium) ethylsulfanyl]phthalocyaninatozinc(II) tetraiodide (**Pc13**) was the most active of four sensitizers assayed showing a singlet oxygen quantum yield of 0.58 and a higher bathochromic shift of 10 nm for the Q-band than the isosteric oxygen-linked cationic aliphatic phthalocyanine [10]. Our photobiological assays using KB cells indicated that the above-mentioned dye partially localized in lysosomes, inducing apoptosis after photodynamic treatment. It has also been reported, that oxygen-linked phthalocyanines with a long aliphatic side chain at the quaternary nitrogen of the pyridine or the amino-groups are photodynamically more efficient than those which have only one methyl group as substituents [11].

On the basis of these results, we increased the lipophilicity of two novel isosteric zinc(II) phthalocyanines as compared with their analogs: 2,9(10),16(17),23(24)-tetrakis[(2-trimethylammonium)ethoxy]phthalocyaninatozinc(II) tetraiodide (**Pc11**) and **Pc13** [10], in order to obtain a better photodynamic behavior. Thus, 2,9(10),16(17),23(24)-tetrakis[(N-butyl-N-methylammonium)ethylsulfanyl]phthalocyanin natozinc(II) tetraiodide (**6**) and 2,9(10),16(17),23(24)-tetrakis[(N-dibutyl-N-methylammonium)ethoxy]phthalocyaninatozinc(II) tetraiodide (**7**) were synthesized and their photophysical parameters and

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photobiological potentials were evaluated on human nasopharynx KB carcinoma cells.

2. Results and discussion

2.1. Chemistry

The synthesis of phthalocyanines **4**–**6** is depicted in Scheme 1. The sequence begins with the reaction of the commercially available 4-nitrophthalonitrile with 2-(butylamino)-ethanethiol to give 4-[2-(N-butylamino)ethylsulfanyl]phthalonitrile (**2**). The reaction of compound **2** with butyryl chloride in anhydrous pyridine at room temperature afforded 4-[2-(N-butyryl-N-butylamino)ethylsulfanyl]phthalonitrile (**3**). Phthalocyanine **4** was readily prepared by cyclotetramerization of phthalonitrile **3** using 1,8-diazabicyclo [5,4,0]undec-7-ene (DBU) and zinc acetate. We have recently demonstrated that zinc(II)phthalocyanine derivatives carrying amido substituents in the macrocycle are effectively reduced by diborane to the corresponding amino-phthalocyanines under relatively mild conditions in good yields [12,13]. Reduction of **4** with diborane in tetrahydrofuran gave phthalocyanine **5** in 79% yields. Cationic phthalocyanine **6** was obtained in 67% yield by treatment of **5** at 60 °C with methyl iodide in methylene chloride.

Intermediates **2**–**3** and dyes **4**–**6** were characterized by IR and ^{1}H NMR spectroscopy and ESI-TOF mass spectroscopy. 2,9(10),16(17), 23(24)-Tetrakis[(2-dibuty-2-methylammonium)ethoxy]phthalocyaninatozinc(II) tetraiodide (**7**) (Fig. 1) was also characterized as described above, while the synthesis of precursors were achieved as reported elsewhere [14].

With regard to the solubility of phthalocyanines **4**–**5** they are soluble in almost all organic solvents, while cationic derivatives **6** and **7** are fully soluble in methanol and N,N-dimethylformamide.

2.2. Photophysical parameters

The UV–vis absorption spectra of phthalocyanines **4**–**6** showed a Soret band of 357 nm, 354 nm, and 374 nm and a Q band at 687 nm, 685 nm, and 686.5 nm, respectively. Phthalocyanine **7** exhibited a Soret band of 351 nm, and a Q band at 678 nm. A bathochromic shift of 8.5 nm was observed for the Q-bands of cationic phthalocyanine when oxygen and sulfur were alternatively present. The cationic dimethylaminosulfanyl derivative **6** presented higher molar absorption coefficient than cationic dimethylaminoethoxy analog **7** a property that improved skin light penetration. Typical fluorescence emission spectra of zinc phthalocyanines were also observed (Fig. 2).

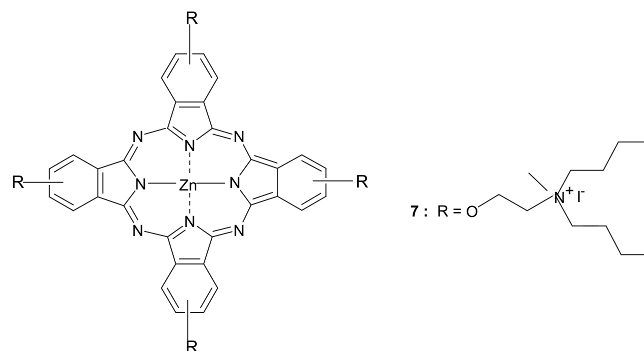
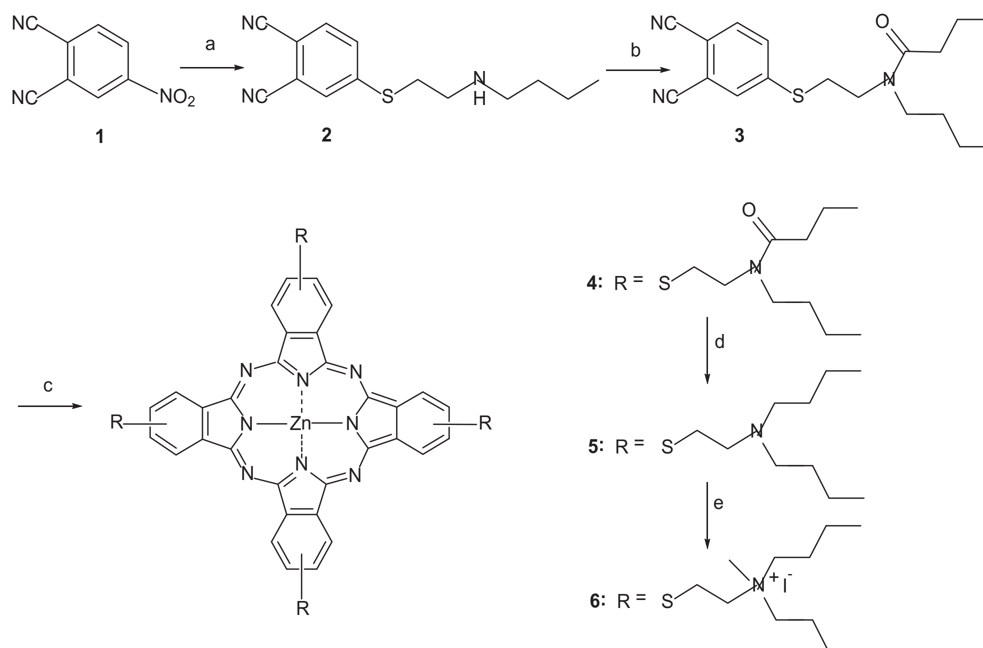


Fig. 1. Chemical structure of phthalocyanine **7**.

When excited at 610 nm, compounds **4**–**7** showed fluorescence emission spectra typical of zinc phthalocyanines at a concentration of 10^{-6} M in tetrahydrofuran for dyes **4**–**5** and in N,N-dimethylformamide for **6**–**7.** The emission maxima occurred at 694 nm, 690.5 nm, 695 nm and 687.5 nm for **4**–**7**, respectively.

The quantum yield of singlet oxygen production (Φ_Δ) and fluorescence quantum yields (Φ_F) of **4**–**7** are listed in Table 1. Sample absorbances were kept as low as possible (10^{-6} M) to obtain measurable values of quantum yield of singlet oxygen production; values obtained for cationic dyes were similar, indicating, however, that at this concentration dyes are aggregated [15].

It has been reported that cationic phthalocyanines show Φ_Δ values in the range of 0.01–0.15. However, these dyes are highly



Scheme 1. Synthetic route to phthalocyanine **6**. Reagents and conditions. a) $HSCH_2CH_2NH(CH_2)_3CH_3$, DMF, K_2CO_3, r.t., 24 h, 61%; b) $ClCO(CH_2)_2CH_3$, Py/CH_2Cl_2, r.t., 48 h, 79%; c) $Zn(AcO)_2$, DBU, 160 °C, 5 min, 57%; d) B_2H_6, THF, r.t., 48 h, 79%; CH_3I, CH_2Cl_2, 60 °C, 48 h, 67%.

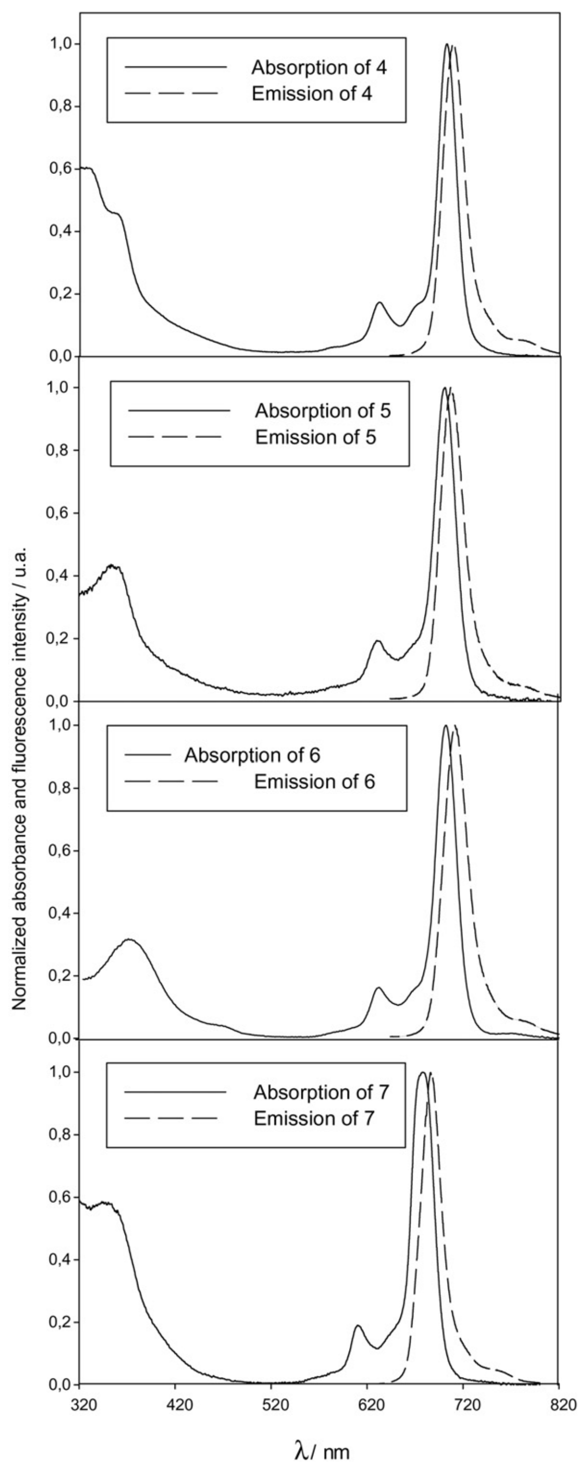


Fig. 2. Absorption and fluorescence spectra of **4** and **5** 10^{-6} M in THF, and **6** and **7** 10^{-6} M in DMF.

Table 1
Photophysical parameters obtained for phthalocyanines **4**–**5** in THF and **6-7** in DMF.

Pc	Solvent	Q-band λ_{max}/nm	ε_{max} M^{-1} cm^{-1}	Φ_F	Φ_Δ
4	THF	687	2.7×10^4	0.23 ± 0.02	0.47 ± 0.03
5	THF	685	1.6×10^4	0.21 ± 0.02	0.44 ± 0.03
6	DMF	686.5	2.7×10^4	0.13 ± 0.01	0.42 ± 0.03
7	DMF	678	1.6×10^3	0.14 ± 0.01	0.67 ± 0.02

phototoxic against different cell lines such as: human HEp2 cells [9], MCF-7c3 cells [16], and HT29 human colon adenocarcinoma cells [17]. Also, amphiphilic zinc(II) phthalocyanines substituted with 2-(dimethylamino)ethylthio moieties, whose Φ_Δ values are in the range of 0.19–0.29, have shown their efficacy against HepG2 human hepatocarcinoma cells and HT29 and T84 human colon adenocarcinoma cells [18]. Since photosensitizers **6**–**7** exhibited higher values of Φ_Δ than the above-mentioned phthalocyanines, a more efficient effect for photobiological purposes could be expected.

2.3. Photocytotoxicity studies

The effect of different concentrations of phthalocyanines **6** and **7** on KB cell survival was evaluated in the dark and upon exposure to a light dose of 4.7 J cm^{-2}, 1.96 mW cm^{-2} by using the MTT assay. Dark cytotoxicity was observed when cells were exposed to concentrations ≥10 or 20 μM of compounds **6** or **7**, respectively. After irradiation, **6** and **7** were found to be cytotoxic, and IC_{50} values from three independent experiments (mean ± SE) were 1.45 ± 1 μM and 10.5 ± 2 μM, respectively (Fig. 3). According to these results, **6** was more potent after irradiation than **7** and showed a greater cytotoxicity in the presence of light than in its absence.

We have previously demonstrated that KB cells incubated with the sulfur-linked cationic phthalocyanine 2,9(10),16(17),23(24)-tetrakis [(2-trimethylammonium)ethylsulfanyl]phthalocyaninatozinc(II) tetraiodide (**Pc13**) showed an IC_{50} value of 2.7 ± 0.6 μM after irradiation, and that treatment of cells either upon exposure to light or in the dark with the oxygen-linked cationic phthalocyanine 2,9(10),16(17),23(24)-tetrakis[(2-trimethylammonium)ethoxy]phthalocyaninatozinc(II) tetraiodide had no effect on cell proliferation [10]. Thus, sulfur-linked

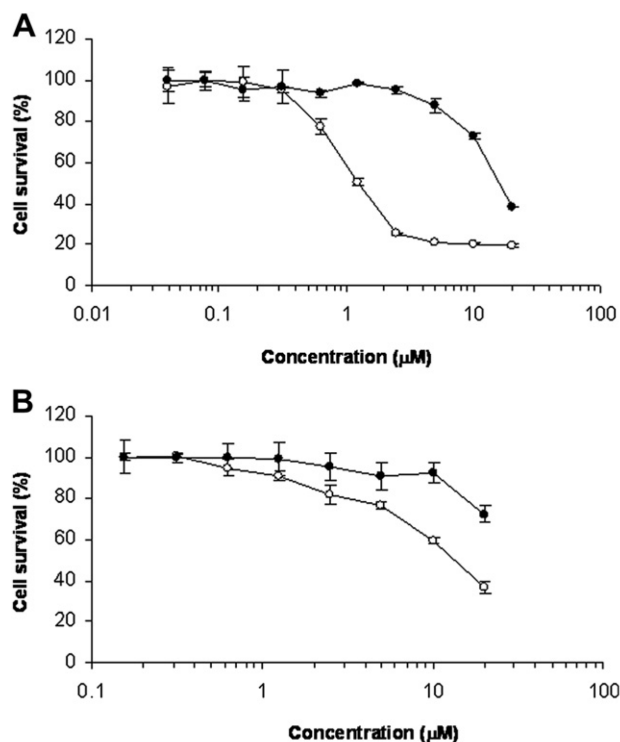


Fig. 3. Effect of phthalocyanines **6** and **7** on KB cell viability. Different concentrations of **6** (A) and **7** (B) were incubated with KB cells in the dark (●) or exposed to a light dose of 4.7 J cm^{-2} (○). The MTT cytotoxicity assay was carried out 24 h after the treatment, as described under Materials and methods. Results are expressed as the percentage of cell growth obtained in the absence of phthalocyanines (control) and represent the mean ± S.E. of three different experiments.

cationic phthalocyanines behave as more efficient phototoxic agents than oxygen-linked phthalocyanines.

2.4. Cellular uptake of phthalocyanine **6** and **7** in KB cells

KB cells were incubated under the same experimental conditions with 5 μM concentrations of **6** and **7** for 24 h in the dark. The relative uptake was quantified by comparison of images taken by confocal microscopy under identical settings. Cytosolic localization of both phthalocyanines with a typical red fluorescence emission was observed by exciting at 633 nm and detecting the emission fluorescence at wavelengths >660 nm (Fig. 4A). A significant difference in fluorescence intensities was shown for both compounds, being the relative integrated intensities of fluorescence images for phthalocyanine **6** and **7** approximately 2:1 (Fig. 4B), according to estimations performed with the Image-Pro Plus software. Taking into consideration that fluorescence quantum yields are similar for both phthalocyanines **6** and **7**, these results suggest that the intracellular uptake of the sulfur-linked cationic phthalocyanines is higher than that of the oxygen-linked phthalocyanines. It is known that bioisosterism is a strategy in medicinal chemistry which changes the physicochemical properties of a substance [19]. The bioisosteric replacement of oxygen by sulfur increased phthalocyanine cellular uptake due to the improvement of phthalocyanine **6** amphiphilicity.

2.5. Intracellular production of reactive oxygen species (ROS)

It is known that, upon illumination, a photosensitizer is excited from the ground state to an excited state, generating free radicals and reactive oxygen species (ROS), which are responsible for oxidative damage and cell death [20]. In order to evaluate the formation of ROS after irradiation, KB cells pre-treated with phthalocyanines **6** and **7**, were loaded with the probe 2′,7′-dichlorfluorescein-diacetate (DCFH-DA). After diffusing into cells, DCFH-DA is deacetylated by esterases and is then oxidized to the fluorescent 2′,7′-dichlorfluorescein (DCF) in the presence of ROS (mainly hydrogen peroxide and lipid hydroperoxides). As shown in Fig. 5, a significant increase in ROS levels was observed for both phthalocyanines in a concentration-dependent manner. However, the amount of ROS produced by **6** was higher than that generated by **7** for all the concentrations probed, being required a 5-fold increase in phthalocyanine **7** concentration with respect to phthalocyanine **6** to obtain a similar oxidative response.

2.6. Intracellular localization of phthalocyanine **6**

The subcellular localization of phthalocyanine **6** was evaluated by confocal microscopy after incubating KB cells for 24 h in the dark and staining them with fluorescent dyes for specific organelles. The

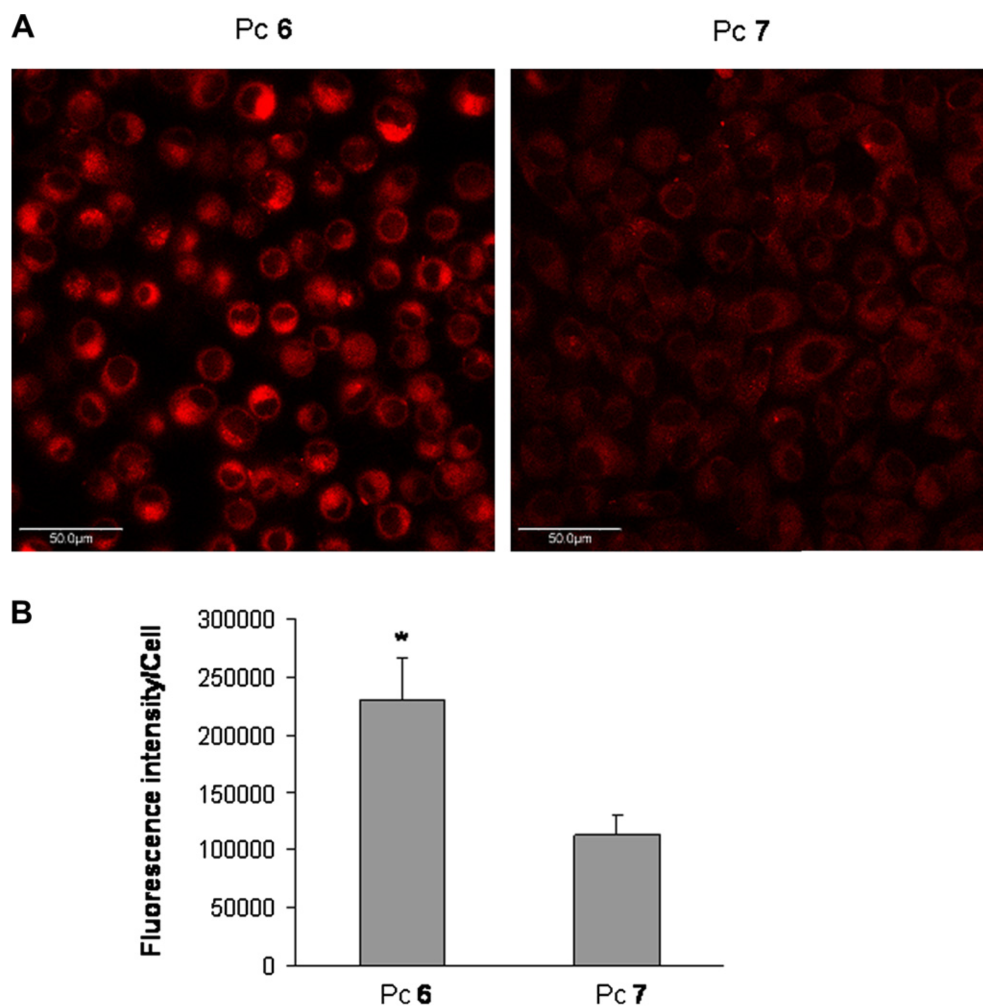


Fig. 4. Cellular uptake of phthalocyanines **6** and **7**. (A) After incubating KB cells for 24 h in the dark with a 5 μM concentration of Pc 6 or Pc 7, the intracellular localization of both phthalocyanines was visualized by confocal microscopy. Representative images are shown (B). Integrated intensities of fluorescence were estimated with the Image-Pro Plus software. Results represent the mean ± S.E. of three different experiments. Statistical significance, obtained with Student's *t*-test, is indicated by $^{*}p < 0.0005$. Scale bar 50 μm.

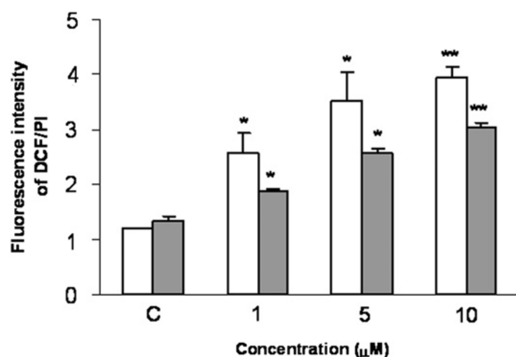


Fig. 5. Effect of phthalocyanines **6** and **7** on ROS generation in KB cells. KB cells (6×10^4 cells/well), pre-loaded with different concentrations of phthalocyanines **6** (white bar) and **7** (gray bar), were irradiated and then incubated at 37 °C for 30 min in the presence of 10 μM DCFH-DA. After washing the non-incorporated probe, cells were solubilized and DCF fluorescence was measured in a fluorometer. DNA content was estimated after incubating with a final concentration of 50 μM PI in the dark. Results are expressed as the ratio between DCF and PI fluorescence, and represent the mean ± S.E. of three different experiments. Statistical significance, obtained with Student's *t*-test, is indicated by $^{*}p < 0.005$, $^{**}p < 0.0001$.

lower cellular uptake and fluorescence emission obtained for phthalocyanine **7** made it difficult to assess its localization. The fluorescent images observed for phthalocyanine **6**, together with those obtained with specific green fluorescent probes for lysosomes and mitochondria are shown in Fig. 6. The photosensitizer **6** was found within cytosolic vesicles corresponding mainly to lysosomes, as yellow fluorescence signal was visualized from the overlay of the red fluorescence from **6** and the LysoTracker pattern (Fig. 6C). No evident colocalization was detected for **6** with the MitoTracker probe (Fig. 6F). We have previously reported a similar subcellular localization for the sulfur-linked cationic phthalocyanine **Pc13** [10]. Preferential lysosome localization has also been described for other cationic phthalocyanines or porphyrins, probably due to an endocytic uptake [9,21].

3. Conclusions

During our attempt to improve cellular uptake, we increased lipophilicity of novel zinc(II) phthalocyaninates by introducing butyl instead of ethyl chains in the periphery of the macrocycle. Therefore, isosteric zinc(II) phthalocyaninates **6** and **7** were synthesized and evaluated in cell cultures, using human nasopharynx carcinoma KB cells.

Cationic phthalocyanine **6** was obtained by quaternization of tertiary amino groups of phthalocyanine **5**, which was synthesized by the reduction of its precursor **4** with diborane in good yields. The stability of both the precursor (**4**) and the product (**5**) under these reaction conditions is noteworthy, as it shows their usefulness for the peripheral macrocyclic amido-group reduction. Dye **7** was obtained as described for **6**, whose precursors have been described elsewhere [14].

The photophysical studies indicated that **6** and **7** are efficient generators of singlet oxygen species. Although **7** presented a higher value of Φ_{Δ}, phthalocyanine **6** was photodynamically more active, due to its higher value of λ_{max}. Such bathochromic shift into the therapeutic window could be useful for biomedical applications such as tissue imaging and photodynamic therapy.

The photobiological studies showed a better phototoxic effect for the sulfur-linked cationic phthalocyanine **6** ($IC_{50} = 1.45 \pm 1$ μM) with respect to the oxygen-linked phthalocyanine **7** ($IC_{50} = 10.5 \pm 2$ μM). This result could be explained by the higher cellular uptake obtained for **6**, which was mainly localized within lysosomes. After

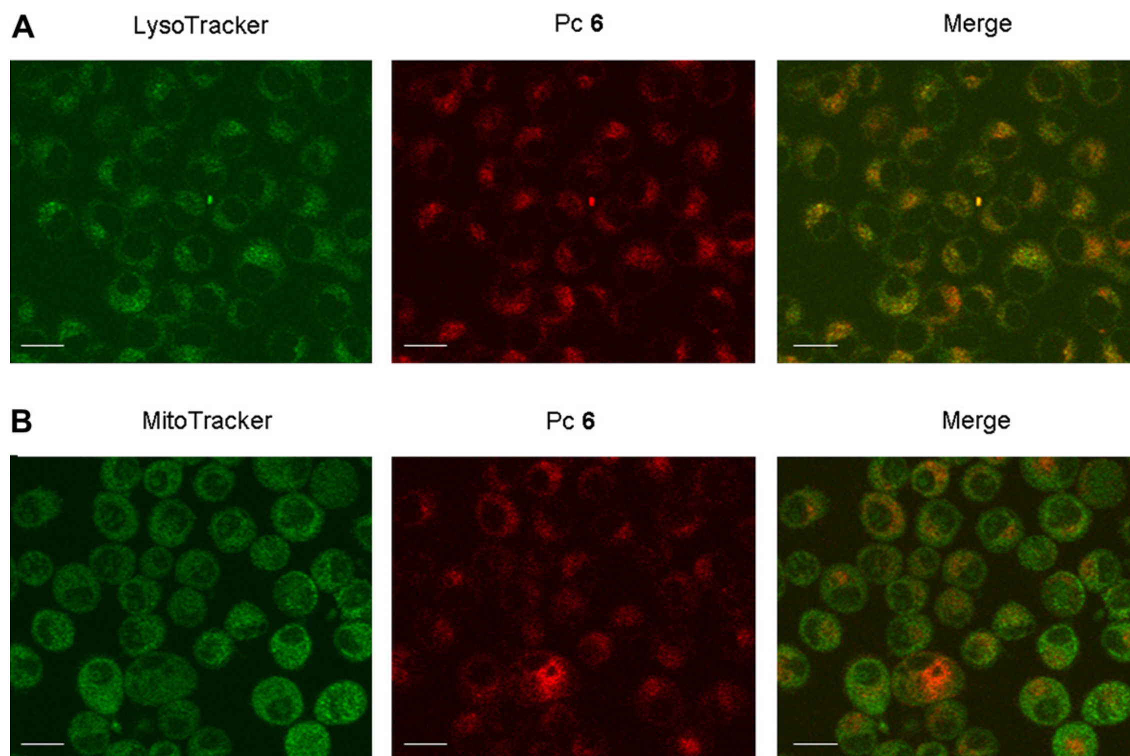


Fig. 6. Intracellular localization of phthalocyanine **6**. KB cells incubated with 5 μM of Pc 6 for 24 h were stained with LysoTracker Green DND-26 (75 nM, 30 min) (A) or MitoTracker Green FM (100 nM, 45 min) (B). Overlays of the red fluorescence corresponding to Pc 6 and the green fluorescence signal for lysosomes and mitochondria are shown. Scale bar 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

irradiation, the production of a greater amount of ROS by phthalocyanine **6** led to a more effective cell death. Besides, phthalocyanine **6** was more photoactive *in vitro* than **Pc13** [10], since the IC_{50} values obtained under similar experimental conditions were two-fold higher for **6** than for **Pc13** ($IC_{50} = 2.7 \pm 0.6$ μM) [10].

Summarizing, it is possible to consider a structure–activity relationship of alkylthio peripheral substituted zinc(II) phthalocyanines for photobiological purposes. Therefore, further photobiological studies, as well as search for improved phthalocyanine structures, are already in progress.

4. Experimental

4.1. Materials and methods

4.1.1. Synthesis of photosensitizers

Melting points were determined on an Electrothermal 9100 capillary melting point apparatus. ^{1}H NMR of precursors were recorded on a Bruker MSL 300 spectrometer while ^{1}H NMR of phthalocyanines on a Bruker AM 500. Intermediates ESI-TOF mass spectroscopy was determined with a ZQ Micromass spectrometer, while phthalocyanines ESI-TOF mass spectra were measured with an LTQ ion trap equipment. Electronic absorption spectra were determined with a Shimadzu UV-3101 PC spectrophotometer. Fluorescence spectra were monitored with a QuantaMaster Model QM-1 PTI spectrofluorometer. Infrared spectra were performed with a Perkin Elmer Spectrum One FT-IR spectrometer.

Chromatography columns were prepared with TLC Kiesegel (Merck), and Aluminum Oxide 90 standardized (Merck). N, N-dimethylformamide was dried over 3Ǻ molecular sieves during 72 h, then filtered and freshly distilled before utilization [22] Diphenylisobenzofuran (DPBF) as well as all reagents were provided by Sigma–Aldrich. 2,3,9,10,16,17,23,24-octakis[(N,N-dimethylaminoethylsulfanyl)]phthalocyaninatozinc(II) [4] was synthesized in our laboratory.

4.1.2. 4-[2-(N-butylamino)ethylsulfanyl]phthalonitrile (*2*)

A mixture of 4-nitrophthalonitrile (0.1 g, 0.58 mmol), 2-(butylamino)-ethanethiol (0.4 mL, 0.59 mmol), and K_2CO_3 (0.24 g, 1.74 mmol) in anhyd DMF (1 mL) was stirred for 24 h at r.t. under Ar and then poured into H_2O (30 mL) and extracted with CH_2Cl_2 (4 × 20 mL). The combined extracts were washed with H_2O (4 × 20 mL), dried (Na_2SO_4), and evaporated in vacuo. The solid residue was dissolved in a small volume of CH_2Cl_2-MeOH (9:1) and the solution was filtered through a silica gel column that was packed and pre-washed with the same solvent. Evaporation of the solvent gave highly viscous oil, yield: 0.091 (61%). IR (KBr, cm^{-1}): 3812, 3437, 3431, 3401, 3384, 3317, 3205, 3098, 3059, 2957, 2929, 2871, 2850, 2572, 2429, 2381, 2231 (CN), 1942, 1667, 1600, 1583, 1545, 1507, 1467, 1386, 1293, 1263, 1217, 1191, 1126, 1070, 872, 834, 738, 721, 650, 605, 598, 524, 497. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.92 (t, 3H, CH_3), 1.39 (m, 4H, CH_2), 2.64 (t, 2H, $NHCH_2$), 2.88 (s, 1H, NH), 2.96 (t, 2H, CH_2NH), 3.18 (t, 2H, SCH_2), 7.52–7.66 (m, 3H, Ar). ESI-TOF MS: *m*/*z* (%) [M^+] calcd. for: $C_{14}H_{17}N_3S$ 259.1143; found: $[M+H]^+$ 260.1000.

4.1.3. 4-[2-(N-butyryl-N-butylamino)ethylsulfanyl]phthalonitrile (*3*)

A solution of butyryl chloride (0.17 mL, 1.5 mmol) in anhyd CH_2Cl_2 (5 mL) was added dropwise to a stirred cold solution of amine **2** (0.070 g, 0.27 mmol) in anhyd pyridine (1.7 mL). The mixture was then stirred for 48 h at r. t. CH_2Cl_2 (30 mL) was added and the mixture was washed sequentially with 1 N HCl (4 × 20 mL) and H_2O (1 × 20 mL). The organic phase was washed again with 5% $NaHCO_3$ soln (2 × 20 mL) and H_2O (1 × 20 mL), then dried (Na_2SO_4) and evaporated to dryness in vacuo. The oil residue was dissolved in a small volume of CH_2Cl_2-MeOH (9:1) and the solution was filtered through a silica gel column packed and pre-washed with the same solvent. Evaporation of the solvent gave a viscous oil; yield: 0.070 g (79%). IR (KBr, cm^{-1}): 3812, 3444, 3414, 3267, 3165, 3095, 3058, 2964, 2936, 2931, 2874, 2734, 2675, 2568, 2344, 2231 (CN), 1942, 1743 (CO), 1692, 1638, 1583, 1545, 1465, 1422, 1378, 1261, 1210, 1192, 1163, 1126, 1094, 1072, 1028, 871, 834, 802, 753, 648, 599, 525, 496. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 0.95 (t, 3H, CH_3), 1.30 (m, 4H, CH_2), 1.67 (m, 2H, CH_2), 2.28 (t, 2H, $NCOCH_2$), 3.18 (s, 2H, SCH_2), 3.25(t, 2H, NCH_2), 3.50 (t, 2H, CH_2N), 7.68–7.87 (m, 3H, Ar). ESI-TOF MS: *m*/*z* (%) [M^+] calcd. for: $C_{18}H_{23}N_3SO$ 329.1562; found: $[M+H]^+$ 330.2000.

4.1.4. 2,9(10),16(17),23(24)-Tetrakis[(N-butyryl-N-butylamino) ethylsulfanyl]phthalocyaninatozinc(II) (*4*)

A mixture of **3** (0.137 g, 0.42 mmol), anhyd $Zn(OAc)_2$ (0.139 g, 0.75 mmol), and DBU (0.14 mL, 0.94 mmol) was stirred and heated at 160 °C for 5 min under Ar. After cooling, it was treated with CH_2Cl_2 (5 mL) and centrifuged to eliminate the excess of $Zn(OAc)_2$. The organic solution was evaporated in vacuo leaving a blue-green residue, that was then dissolved in CH_2Cl_2 and filtered through a column with silica-gel packed and pre-washed with the same solvent. The title compound was eluted with CH_2Cl_2-MeOH (9.5:0.5). After evaporation in vacuo, the dye was obtained (0.082 g, 57% yield). IR (KBr, cm^{-1}): 3058, 2661, 2932, 2873, 2650, 2565, 2416, 2230, 1732 (CO), 1635, 1599, 1583, 1545, 1459, 1426, 1476, 1293, 1261, 1209, 1125, 1094, 1072, 1036, 978, 910, 871, 830, 760, 747, 649, 598, 524. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.79 (m, 24H, CH_3), 1.11 (m, 16H, CH_2CH_3), 1.45 (m, 8H, $\mathit{CH_2}CH_2CH_3$), 2.12 (t, 8H, $CO\mathit{CH_2}CH_2CH_3$), 3.07 (m, 8H, $\mathit{CH_2}CH_2CH_2CH_3$), 3.32 (br, 8H, CH_2N), 5.13 (br s, 8H, SCH_2), 7.53–7.70 (m, 12H, Ar). ESI-TOF MS: *m*/*z* (%) [M^+] cald for: $C_{72}H_{92}N_{12}O_4S_4Zn$ 1383.2300; found: [M^+] 1383.4700.

4.1.5. 2,9(10),16(17),23(24)-Tetrakis[(N,N-dibutylamino) ethylsulfanyl]phthalocyaninatozinc(II) (*5*)

$BF_3.Et_2O$ (25 mL) was slowly dropped into a suspension of $NaBH_4$ (7.5 g) in diglyme (25 mL) and the resulting B_2H_6 was bubbled through a suspension of **4** (0.100 g, 0.072 mmol) in anhyd THF (15 mL). The mixture was stirred for 48 h, poured into hexane and the blue-green precipitate was centrifuged, dried, and then applied to an Al_2O_3 column packed and pre-washed with toluene. After washing with toluene and CH_2Cl_2, the title compound was eluted with CH_2Cl_2-MeOH (8:2). After solvent evaporation, a green product was obtained. Yield: 0.076 g (79%).

IR (KBr, cm^{-1}): 3088, 3064, 3030, 2957, 2932, 2871, 2354, 2347, 2341, 2280, 2086, 1955, 1815, 1673, 1601, 1554, 1495, 1454, 1383, 1338, 1206, 1173, 1080, 1047, 1024, 910, 833, 770, 744, 735, 699, 596. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.72 (m, 24H, CH_3), 1.09 (br, 16H, $\mathit{CH_2}CH_3$), 1.52 (br, 16H, $\mathit{CH_2}CH_2CH_3$), 3.32 (br, 16H, $\mathit{CH_2}CH_2CH_2CH_3$), 4.54 (br s, 8H, CH_2N), 5.13 (br s, 8H, SCH_2), 7.19–7.21 (m, 12H, Ar). ESI-TOF MS: *m*/*z* (%) [M^+] cald for: $C_{72}H_{100}N_{12}S_4Zn$: 1327.2992; found: [M^+] 1327.3100.

4.1.6. 2,9(10),16(17),23(24)-Tetrakis[(N,N-dibutyl-N-methylammonium)ethylsulfanyl]phthalocyaninatozinc(II) tetraiodide (*6*)

MeI (12 mL, 0.2 mol) was added to a solution of phthalocyanine **5** (0.066 g, 0.05 mmol) in CH_2Cl_2 (15 mL) and the solution was stirred for 48 h at 60 °C. After cooling at r. t., the blue-green powder was centrifuged, suspended in CH_2Cl_2 (5 mL), and centrifuged again. Yield 0.063 g (67%).

IR (KBr, cm^{-1}): 3900, 3817, 3745, 3733, 2930, 2856, 2506, 2354, 2347, 2275, 2051, 1967, 1868, 1831, 1665, 1494, 1439, 1411, 1390, 1256, 1097, 1065, 662. ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) 0.99 (t, 24H, CH_3), 1.23 (br s, 16H, $\mathit{CH_2}CH_3$), 1.67 (br, 16H, $\mathit{CH_2}CH_2CH_3$), 3.66 (br, 16H, $\mathit{CH_2}CH_2CH_2CH_3$), 3.68 (br, 12H, CH_3), 3.69 (br, 8H,CH_2N),

4.68(br, 8H, SCH_2), 7.34–7.36 (m, 12H, Ar). ESI-TOF MS: m/z (%) [M^+] cald for: $C_{76}H_{112}N_{12}S_4ZnI_4$ 1895.0552; found: [M^+] 1895.0200.

4.1.7. 2,9(10),16(17),23(24)-Tetrakis[(N,N-dibutyl-N-methylammonium)ethoxy]phthalocyaninato(II) tetraiodide (**7**)

MeI (4 mL, 0.64 mmol) was added to a solution of tetrakis(N,N-dibutylaminoethoxy)phthalocyaninatozinc(II) [14] (0.020 g, 0.016 mmol) in CH_2Cl_2 (15 mL) and the solution was stirred for 48 h at 60 °C. After cooling to room temperature, the blue-green powder was centrifuged, suspended in CH_2Cl_2 (5 mL), and centrifuged again. Yield 0.018 g (62%).

IR (KBr, cm^{-1}): 3436, 2961, 2931, 2874, 2231, 1766, 1709, 1623, 1489, 1456, 1381, 1320, 1290, 1259, 1200, 1102, 887, 802, 748, 525. ^1H NMR (500 MHz, $CDCl_3$): δ (ppm) 1.6 (t, 24H, CH_3), 1.28 (br s, 16H, CH_2CH_3), 1.64 (br, 16H, $CH_2CH_2CH_3$), 3.45(m, 16H, $CH_2CH_2CH_2CH3$), 3.52 (br s, 12H, CH_3), 4.21 (br, 8H,CH_2N), 4.91(br, 8H, OCH_2), 7.48–7.85 (m, 12H, Ar). ESI-TOF MS: m/z (%) [M^+] cald for: $C_{76}H_{112}N_{12}O_4ZnI_4$ 1828.4398; found: [M^+] 1829.4400.

4.2. Photophysical parameters

Absorption and emission spectra were recorded at different concentrations using, a 10 × 10 mm quartz cuvette. All experiments were performed at room temperature. Phthalocyanines **4**–**5** were measured in THF and **6**–**7** in DMF. The emission spectra of **4**–**7** were collected at an excitation wavelength of 610 nm (Q-band) and recorded between 630 and 800 nm.

Fluorescence quantum yields (Φ_F) were determined by comparison with those of 2,3,9,10,16,17,23,24-octakis[(N,N-dimethylaminoethylsulfanyl)]phthalocyaninatozinc(II) ($\Phi_F = 0.26$ in THF and DMF) as a reference at $\lambda_{exc} = 610$ nm for **4**–**7** [4].

Standard chemical monitor bleaching rates were used to calculate the quantum yield of singlet oxygen generation rates [23,24]. For Φ_Δ studies in organic solvent, DPBF was used as a singlet-oxygen chemical quencher. To avoid chain reactions induced by DPBF in the presence of singlet oxygen, the absorbance of DPBF was kept under 1.9. DPBF decay at 410 nm was monitored. Polychromatic irradiation was performed by using a projector lamp (Philips 7748SEHJ, 24V-250W), and a cut-off filter at 610 nm (Schott, RG 610) and a water-filter were used to prevent ultraviolet and infrared radiation. Samples **4**–**7** and references 2,3,9,10,16,17,23,24-octakis[(N,N-thylaminoethylsulfanyl)]phthalocyaninatozinc(II) ($\Phi_\Delta = 0.69$ in THF and DMF) [4],were irradiated within the same wavelength interval $\lambda_1-\lambda_2$, and Φ_Δ was calculated according to Amore et al. [25].

4.3. Biological studies

4.3.1. Cells and culture conditions

Human nasopharynx carcinoma KB cells (ATCC CCL-17) were maintained in Minimum Essential Medium (MEM, Gibco BRL) containing 10% (v/v) fetal bovine serum (FBS, Gibco BRL), 2 mM L-glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 1 mM sodium pyruvate and 4 mM sodium bicarbonate, in a humidified atmosphere of 5% CO_2 at 37 °C.

4.3.2. Dark cytotoxicity and photocytotoxicity

KB cells were plated at a density of 1×10^4 cells/well in 96-well microplates and incubated overnight at 37 °C until 70–80% of confluence. Then, the culture medium was replaced by MEM containing 4% FBS and different concentrations of cationic phthalocyanines **6** and **7**, previously dissolved as 20 mM stock solutions in dimethyl sulfoxide (DMSO). After 24 h, compounds were removed and cells were exposed to a light dose of 4.7 J cm^{-2}, 1.96 mW cm^{-2}, with a 150 W halogen lamp equipped with a 10 mm water filter to maintain cells cool and attenuate IR radiation. In addition, a cut-off filter was used to bar wavelengths shorter than 630 nm. In parallel, non-irradiated cells were used to study dark cytotoxicity. Following treatment, cells were incubated for an additional 24 h period and cell viability was determined by means of the MTT reduction assay as described previously [10]. The absorbance (595 nm) was measured in a Biotrack II Microplate Reader (Amersham Biosciences).

4.3.3. Cellular uptake of phthalocyanines

KB cells (1×10^4 cells/well) were grown overnight at 37 °C on coverslips in cell culture dishes. Phthalocyanines **6** and **7**, dissolved at a 5 μM concentration in culture medium with 4% FBS, were incubated for 24 h in the dark. Then, cells were washed three times with phosphate-buffer saline (PBS) and fixed in 4% paraformaldehyde (PFA) solution for 10 min at room temperature. After washing three times with PBS, cells were visualized with a confocal microscopy Olympus FV 300. Phthalocyanines were excited at 633 nm and the emission was monitored at wavelengths >660 nm. In order to compare cellular uptake, the relative fluorescence intensity between images taken under identical microscopic conditions was calculated with the Image-Pro Plus program, version 5.1.2 (Media Cybernetics, Inc.).

4.3.4. Intracellular production of reactive oxygen species (ROS)

The endogenous ROS content was measured by using the probe DCFH-DA. Briefly, KB cells were plated at a density of 6×10^4 cells/well in 24-well microplates and incubated overnight at 37 °C until 70–80% of confluence. Then, the culture medium was replaced by MEM containing 4% FBS and 1, 5 and 10 μM of cationic phthalocyanines **6** and **7**. After 24 h, cells were irradiated as described above and washed three times with PBS at 37 °C. KB cells were then incubated at 37 °C for 30 min in the presence of 10 μM DCFH-DA, and the non-incorporated probe was eliminated by washing the cells twice with PBS. Cells were solubilized with Triton X-100 (0.1% v/v) in PBS, and the fluorescence of DCF was detected in a PerkinElmer LS55 Fluorometer (PerkinElmer Ltd., Beaconsfield, UK) using 488 nm excitation and 530 nm emission wavelengths. After 10 min of incubation with a final concentration of 50 μM propidium iodide (PI), DNA content was estimated from the fluorescence intensity of the DNA-PI complex at excitation and emission wavelengths of 538 and 590 nm, respectively. Results are expressed as the ratio between DCF and PI fluorescence.

4.3.5. Intracellular localization of phthalocyanine **6**

KB cells grown on coverslips were incubated with a 5 μM solution of compound **6** for 24 h at 37 °C in the dark. After removing the compound with PBS, cells were stained with the following fluorescent dyes for specific organelles diluted in the culture medium without FBS: LysoTracker Green DND-26 (75 nM, 30 min) was used to reveal lysosomes, and MitoTracker Green FM (100 nM, 45 min), to visualize mitochondria. Both organelle dyes were obtained from Invitrogen. After washing with PBS, coverslips were fixed for 10 min at room temperature with 4% PFA and cells were then examined by fluorescence with a confocal microscopy Olympus FV 300. Phthalocyanine **6** was excited as described above, and the organelles (lysosomes and mitochondria) were excited at 488 nm and green fluorescence was detected at 510–530 nm.

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