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Photodynamic inactivation of *Penicillium chrysogenum* conidia by cationic porphyrins†

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This work reports the photophysical and biological evaluation of five cationic porphyrins as photosensitizers (PS) for the photodynamic inactivation (PDI) of *Penicillium chrysogenum* conidia. Two different cationic porphyrin groups were synthesized from 5,10,15,20-tetrakis(4-pyridyl)porphyrin and 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin. The photostability and singlet oxygen generation studies showed that these molecules are photostable and efficient singlet oxygen generators. PDI experiments of *P. chrysogenum* conidia conducted with 50 μmol L^{-1} of photosensitiser under white light at a fluence rate of 200 mW cm^{-2} over 20 min showed that the most effective PS caused a 4.1 log reduction in the concentration of viable conidia. The present results show that porphyrins **1a** and **1b** are more efficient PSs than porphyrin **2a** while porphyrins **1c** and **2b** show no inactivation of *P. chrysogenum*. It is also clear that the effectiveness of the molecule as PS for antifungal PDI is strongly related with the porphyrin substituent groups, and consequently their solubility in physiological media. The average amount of PS adsorbed per viable conidium was a determining factor in the photoinactivation efficiency and varied between the different studied PSs. Cationic PSs **1a** and **1b** might be promising anti-fungal PDI agents with potential applications in phytosanitation, biofilm control, bioremediation, and wastewater treatment.

Introduction

During the last two decades, fungi have been increasingly recognized as agents of biodeterioration and relevant pathogens to plants and animals causing significant economic losses in agriculture and industry.[1] The mold *Penicillium chrysogenum* (formerly *P. notatum*) is widely found on food and in indoor environments.[2] Like many species of *Penicillium*, this fungus reproduces by forming spores. In particular, conidia are asexual spores that form at the apex of a specialized aerial hypha and often accumulate protective pigments. These spores, resistant to desiccation and favourable to dispersion, allow *P. chysogenum* to survive in extreme environmental conditions for long periods of time. Fungal spores are challenging in terms of antimicrobial chemotherapy because they are protected by a rigid cell wall mainly composed of insoluble polysaccharide polymers, like chitin, β- and α-glucans and glycoproteins.[3,4] The surface of conidia is covered with a layer of regularly arranged fibres, known as the rodlet layer, in which a group of extremely hydrophobic proteins (hydrophobins) is present,[5] which favours air buoyancy and dispersion of the conidia by air currents.[6] Antifungal therapy relies on a small number of drugs in comparison to antibacterial treatments. The fact that antifungal treatments are necessarily prolonged and often expensive has renewed the interest on the search for new antifungal strategies.[7] Antimicrobial photodynamic therapy or photodynamic inactivation (PDI) has already demonstrated to be an interesting alternative for the inactivation of microorganisms.[8–10] This therapy uses three non-toxic elements (light, oxygen and a photosensitizer (PS)) which lead to the formation of highly cytotoxic reactive oxygen species (ROS), resulting in lethal oxidative damage.[11–13] Cationic porphyrins proved to be effective against viruses,[14,15] gram-negative and gram-positive bacteria,[16–18] *Bacillus cereus* endospores,[19] *Candida albicans* cells,[7] dermatophyte *Trichophyton rubrum*[20,21] and protozoa.[22] Photosensitization of fungal spores with phenothiazine dyes has been demonstrated[23] and endogenous porphyrins have also been successfully used for the photoinactivation of the yeast *Candida albicans.*[24] However, studies on the use of porphyrins for the inactivation of conidia of other filamentous fungi[25] and for non-pharmacological

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applications are scarce.[26–28] The photoinactivation efficiency of cationic porphyrins against several groups of microorganisms is highly dependent on the structure of the PS molecules. The 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl)porphyrin (**1a**) has already been tested in several photoinactivation studies and has presented good PDI results.[14,18,19] In this family of PSs, the size of the alkyl chains[29,30] and the use of membrane disrupting conjugate agents[31] have been described as crucial for the magnitude of microbial photoinactivation.

In fungal spores, the uptake process of a drug is affected by the hydrophobicity of the the spores' cover and by the presence of charged groups.[3,32] Following the PS distribution, it may remain mostly adsorbed on the surface of the spore or hyphae, or may be distributed to sub-cellular targets.[32] Fungi, as eukaryotic organisms, present a higher diversity of sub-cellular targets than prokaryotic cells, requiring a multi-hit process involving more than one molecular target.[3]

The aim of this work was to investigate the efficiency of two cationic porphyrin families, **1** and **2** (Fig. 1), with four pyridinium groups with different lengths of hydrocarbons chains, on the photoinactivation of conidia of the filamentous fungus *P. chrysogenum*. This filamentous fungus was used as a model conidia-producing filamentous fungus because it is widely distributed in nature and is associated with low biological risk during laboratory handling.

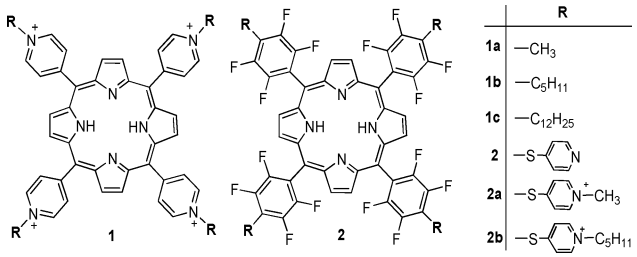

Fig. 1 Representation of the structure of the synthesized porphyrins used for the photoinactivation studies of *P. chrysogenum* conidia.

Experimental

Porphyrin synthesis and characterization

Six porphyrins, hereafter identified as **1a, 1b, 1c, 2, 2a** and **2b**, were synthesized according to the procedures described below, using adequate reagents purchased from Sigma–Aldrich. Porphyrin **2** was only synthesized as a precursor for the synthesis of porphyrins **2a** and **2b**.^{1}H, ^{19}F and ^{13}C solution NMR spectra were recorded using either a BrukerAvance 300 spectrometer at 300.13, 282.38 and 75.47, respectively, or a BrukerAvance 500 at 125.77 MHz for ^{13}C. DMSO-d_6 was used as solvent (except when otherwise indicated) and tetramethylsilane (TMS) as internal reference. The chemical shifts are expressed in δ (ppm) and the coupling constants (J) in Hz. MALDI mass spectra were recorded on a 4800 Maldi-TOF/TOF Analyser and ESI mass spectra on a Micromass Q-Tof 2 (Micromass, Manchester, UK). The UV-vis spectra were recorded on an UV-vis 2501PC. Flash chromatography was carried out on silica gel (Merck, 35–70 mesh). Analytical TLC was carried out on precoated sheets with silica gel (0.2 mm thick, Merck).

5,10,15,20-Tetrakis(*N*-methylpyridinium-4-yl)porphyrin tetraiodide (1a). Excess of iodomethane (4 mL, 64.3 mmol) was added to a suspension of 5,10,15,20-tetrakis(4-pyridyl)porphyrin (100 mg, 162 μmol) in dry DMF (20 mL). The reaction mixture was maintained under stirring for 5 h at 40 °C in a closed flask. After this period, the mixture was cooled and the product precipitated with diethyl ether. The precipitate was filtered and washed with diethyl ether. The solid was then taken in acetone/water (1 : 1) and, after concentration, it was again re-precipitated. The methylated product was filtered, washed with acetone and dried under vacuum, to yield a brown-reddish powder (180 mg, 94%). ^{1}H NMR: δ –3.12 (s, 2H, NH), 4.73 (s, 12H, CH_3), 9.00 (d, J = 6.5, 8H, Py-*o*-H), 9.22 (s, 8H, β-H), 9.49 (d, J = 6.5, 8H, Py-*m*-H). UV-Vis (DMSO) λ_{max}. nm (ε): 425 (5.46), 516 (4.30), 550 (3.78), 588 (3.86), 644 (3.34). ESI-MS m/z: 169.6 (M^{4+}), 226.1 (M^{3+}).

5,10,15,20-Tetrakis(*N*-pentylpyridinium-4-yl)porphyrin tetraiodide (1b). Excess of 1-iodopentane (4 mL, 30.3 mmol) was added to a suspension of 5,10,15,20-tetrakis(4-pyridyl)porphyrin (50 mg, 80.9 μmol) in DMF (10 mL). The reaction mixture was kept under stirring for 12 h at 100 °C. After this period, the mixture was cooled and the product precipitated with diethyl ether. The precipitate was filtered and washed with diethyl ether. The residue was then taken in methanol and, after concentration; it was re-precipitated by addition of diethyl ether. The alkylated product was filtered, washed with diethyl ether and dried under vacuum, to yield a brown-reddish powder (85 mg, 75%). ^{1}H NMR: δ –3.10 (s, 2H, NH), 1.04 (t, J = 7.0, 12H, CH_3), 1.50–1.63 [m, 16H, $CH_2CH_2(C\underline{H}_2)_2CH_3$], 2.28–2.33 [m, 8H, $CH_2C\underline{H}_2(CH_2)_2CH_3$], 4.96 (t, J = 7.3, 8H, $C\underline{H}_2CH_2(CH_2)_2CH_3$), 9.03 (d, J = 6.3, 8H, Py-*o*-H), 9.26 (s, 8H, β-H), 9.58 (d, J = 6.3, 8H, Py-*m*-H). UV-Vis (DMSO) λ_{max}.nm (ε): 427 (5.47), 517 (4.30), 551 (3.83), 589 (3.88), 644 (3.39). ESI-MS m/z: 300.9 (M^{3+}), 451.3 (M^{2+}).

5,10,15,20-Tetrakis(*N*-dodecilpyridinium-4-yl)porphyrintetrabromide (1c). Excess of 1-bromododecane (0.5 mL, 2.08 mmol, 51.4 equiv.) was added to a suspension of 5,10,15,20-tetrakis(4-pyridyl)porphyrin (25 mg, 40.5 μmol) in DMF (10 mL). The reaction mixture was kept under stirring for 12 h at reflux (160 °C). After this period, the mixture was cooled and the product precipitated with diethyl ether. The precipitate was filtered and washed with diethyl ether and chloroform. The residue was then taken in chloroform/methanol (85 : 15) and, after concentration, it was precipitated in acetone. The obtained solid was filtered, washed with acetone and dried under vacuum, to yield a reddish powder (45.7 mg, 70%). ^{1}H NMR: δ –3.11 (s, 2H, NH), 0.86 (t, J = 6.9, 12H, CH_3), 1.27–1.60 (m, 72H, $CH_2CH_2(C\underline{H}_2)_9CH_3$), 2.28 (m, 8H, $CH_2C\underline{H}_2(CH_2)_9CH_3$), 4.96 (t, J = 6.9, 8H, $\underline{CH}_2CH_2(CH_2)_9CH_3$), 9.03 (d, J = 6.4, 8H, Py-*o*-H), 9.24 (s, 8H, β-H), 9.59 (d, J = 6.4, 8H, Py-*m*-H). UV-Vis (DMSO) λ_{max}.nm (ε): 427 (5.44), 517 (4.35), 550 (3.93), 589 (3.95), 643 (3.53). ESI-MS m/z: 323.8 (M^{4+}), 431.7 (M^{3+}), 648.6 [$(M+2H)^{2+}$].

5,10,15,20-Tetrakis[2,3,5,6-tetrafluoro-4-(4-pyridylsulfanyl)-phenyl]porphyrin (2). 4-Mercaptopyridine (47 mg, 4.1 eq.) and diethylamine (1 mL) were added to a solution of 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin (**$TPPF_{20}$**) (100 mg; 0.1 mmol) in dry DMF (5 mL). This mixture was kept under stirring for 1 h, under a nitrogen atmosphere, at r.t. After

evaporation of the DMF under reduced pressure, the residue was subjected to flash chromatography using dichloromethane and 2% methanol in dichloromethane as eluents. The fraction containing porphyrin **2** (120 mg, 90%) was concentrated and the porphyrin was crystallized from 98 : 2 dichloromethane : methanol/hexane. ^{1}H NMR: δ −3.08 (s, 2H, NH), 7.73 (d, J = 5.0, 8H, Ar-*o*-H), 8.64 (d, J = 5.0, 8H, Ar-*m*-H), 9.59 (s, 8H, β-H). ^{19}F NMR: δ −160.95 (dd, J = 11.3 and 25.4, 8F, Ar-*o*-F), −156.04 (dd, J = 11.3 and 25.4, 8F, Ar-*m*-F). ^{13}C NMR: δ 104.0, 109.9 (t, J = 20.9, C_6F_4S-C1), 121.1, 122.0 (t, J = 19.7, C_6F_4S-C4), 144.7, 145.8 and 147.7 (2dd, J = 14.8 and 110.6, C_6F_4S-C2 and -C3), 150.1. UV-Vis (DMSO) λ_{max}.nm (ε): 415 (5.48), 507 (4.34), 581(3.87), 633 (3.59). MALDI-MS m/z: 1339.0 [$(M+H)^+$].

5,10,15,20-Tetrakis[2,3,5,6-tetrafluoro-4-(*N*-methylpyridinium-4-ylsulfanyl)phenyl]porphyrin tetra-iodide (2a). Iodomethane (3 mL, 48.1 mmol) was added to a suspension of 5,10,15,20-tetrakis[2,3,5,6-tetrafluoro-4-(4-pyridylsulfanyl)phenyl] porphyrin (**2**) (75 mg, 56.0 μmol) in DMF (15 mL). The reaction mixture was stirred for 24 h at 40 °C. Afterwards, the mixture was cooled and the product precipitated with diethyl ether. The precipitate was filtered and washed with diethyl ether. The product was retaken in acetone/methanol and distilled water, and re-precipitated twice, first from acetone and then from methanol. The methylated porphyrin **2a** was filtered, washed with methanol and dried under vacuum to yield a brown-reddish powder (96 mg, 90%). ^{1}H NMR: δ −3.08 (s, 2H, NH), 4.36 (12H, CH_3), 8.46 (d, J = 5.5, 8H, Py-*o*-H), 8.96 (d, J = 5.5, 8H, Py-*m*-H), 9.65 (s, 8H, β-H). ^{19}F NMR: δ −160.29 (dd, J = 9.9 and 28.2, 8F, Ar-*o*-F), −155.35 (dd, J = 9.9 and 28.2, 8F, Ar-*m*-F). ^{13}C NMR: δ 47.3 (s, CH_3), 103.8, 107.8 (t, J = 20.8, C_6F_4S-C1), 123.1 (t, J = 19.2, C_6F_4S-C4), 123.5, 144.9, 145.9 and 147.8 (2dd, J = 15.0 and 101.9, C_6F_4S-C2 and C3), 156.4. UV-Vis (DMSO) λ_{max}.nm (ε): 416 (5.50), 507 (4.37), 581 (3.95), 632 (3.40). ESI-MS m/z: 349.6 (M^{4+}), 508.4 [$(M+I)^{3+}$], 826.1 [$(M+2I)^{2+}$].

5,10,15,20-Tetrakis[2,3,5,6-tetrafluoro-4-(*N*-pentylpyridinium-4-ylsulfanyl)phenyl]porphyrin tetra-iodide (2b). 1-Iodopentane (1.5 mL, 11.4 mmol) was added to a suspension of **2** (15 mg, 11.2 μmol) in DMF (5 mL). The reaction mixture was kept under stirring for 10 h at 100 °C. The reaction workup was similar to the used for porphyrin **2a**, yielding a brown-reddish powder (17 mg, 71%). ^{1}H NMR: δ −3.08 (s, 2H, NH), 0.90–0.99 (m, 12H, CH_3), 1.35–1.42 [m, 16H, $CH_2CH_2(C\underline{H}_2)_2CH_3$], 1.99 [t, J = 7.1, 8H, $CH_2C\underline{H}_2(CH_2)_2CH_3$], 4.60 (t, J = 6.0, 8H, $C\underline{H}_2CH_2(CH_2)_2CH_3$), 8.47 (d, J = 6.8, 8H, Py-*o*-H), 9.05 (d, J = 6.8, 8H, Py-*m*-H), 9.63 (s, 8H, β-H). ^{19}F NMR: δ −160.21 (dd, J = 11.3 and 26.1, 8F, Ar-*o*-F), −155.23 (dd, J = 11.3 and 26.1, 8F, Ar-*m*-F). ^{13}C NMR: δ 13.8 (CH_3), 21.7, 27.6, 30.3 (pentyl-C2-C4), 60.0 (pentyl-C1), 103.8, 107.7 (t, J = 20.8, C_6F_4S-C1), 123.2 (t, J = 19.2, C_6F_4S-C4), 123.9, 145.9 and 147.8 (2dd, J = 14.7 and 104.7, C_6F_4S-C2 and C3), 157.0. UV-Vis (DMSO) λ_{max}. nm (ε): 414 (5.54), 506 (4.39), 581 (3.98). ESI-MS m/z: 405.6 (M^{4+}), 583.1 [$(M+I)^{3+}$], 938.7 [$(M+H+2I)^{2+}$].

Photosensitizer stock solutions

Stock solutions of photosensitizers to be used in photophysical and biological studies were prepared in dimethylsulfoxide (DMSO), at a concentration of 500 μmol L^{-1}, and diluted to the final concentration in water, DMF/H_2O (9 : 1) or phosphate buffered saline (PBS, 4 g of NaCl, 0.1 g of KCl, 0.72 g of Na_2HPO_4, 0.12 g of KH_2PO_4 to a final volume of 500 mL and pH 7.4 ± 0,2), depending on the experiments.

Light source

All the irradiation experiments were performed under white light from a compatible fibre optic probe (400–800 nm) attached to a 250 W quartz/halogen lamp (LumaCare®, USA, model LC122) at a fluence rate of 200 mW cm^{-2}.

Porphyrin solubility

Porphyrin solubility in DMSO and PBS was assessed by UV-visible spectroscopy. Several concentrations, between 1.0 and 13.0 μmol L^{-1}, obtained by the addition of several aliquots of each porphyrin stock solution, were analysed. The intensity of the Soret band *versus* porphyrin concentration was plotted to determine if the solutions follows the Beer–Lambert law at these concentrations (see Fig. SI-1 and SI-2†).

Fluorescence quantum yields

The fluorescence quantum yields (Φ_F) of the porphyrin derivatives in DMF were calculated by comparison of the area below the corrected emission spectrum (600–750 nm range) with that of *meso*-tetraphenylporphyrin (TPP). TPP was used as fluorescence standard (λ_{exc} = 580 nm) with Φ_F = 0.11 in DMF.[33] In all cases, the absorbance of the sample and reference solutions was kept at 0.02 at the excitation wavelength. Quantum yields were calculated according equation SI-1.†

Porphyrin photostability

An aliquot of each porphyrin stock solution (500 μmol L^{-1} in DMSO) was diluted in 2 mL of PBS (**1c** was diluted in PBS with 5% DMSO), transferred to a glass cuvette (absorption around 1) and irradiated at room temperature under the same conditions used in the biological assays, under gentle magnetic stirring, for different periods of time. UV-visible spectroscopy assessed the intensity of the Soret band at different intervals of time and the photostability was expressed as I_t/I_0 (%) (I_t = intensity of the band at given time of irradiation, I_0 = intensity of the band before irradiation). Similar assays were performed in the dark to account for the effect of aggregation as a source of light-independent decay.

Singlet oxygen generation

Stock solutions of each porphyrin derivative at 500 μmol L^{-1} in DMSO and a stock solution of 3-diphenylisobenzofuran (DPBF) at 10 mmol L^{-1} in DMF/H_2O (9 : 1) were prepared. A solution of the porphyrin (0.5 μmol L^{-1}) and 3-diphenylisobenzofuran (DPBF, 50 μmol L^{-1}) in DMF/H_2O (9 : 1) were irradiated in a glass cuvette (2 mL), at room temperature and under gentle magnetic stirring, for different periods of time, with white light filtered through a cut-off filter for wavelengths <540 nm, at a fluence rate of 9.0 mW cm^{-2}. The absorption decay of DPBF at 415 nm was measured at irradiation intervals of time up to 20 min. The production of singlet oxygen was evaluated qualitatively through the DPBF, a singlet oxygen quencher.[15] The percentage of the DPBF absorption decay, proportional to the production of $^1O_2$,

was assessed by the difference between the initial absorbance and the absorbance after a given period of irradiation.

Preparation of conidia stock suspension

A *Penicillium chrysogenum* strain isolated at the University of Aveiro and identified by 18S rDNA sequencing (Alves, personal communication) was selected as non-pathogenic model strain and cultivated in Potato Dextrose Agar (PDA, Merck). Cultures were incubated at 25 °C for approximately one week until the development of a dense mycelium. The cultures were then removed from incubation, sealed with Parafilm® and kept at 4 °C. For the preparation of conidia suspensions, a volume of 5 mL of PBS (pH 7.4 ± 0.2) was added to the surface of each culture plate and the mycelium was gently scrubbed with a sterilized glass spreader in order to release the conidia. The suspension was collected in a sterilized beaker and filtered through sterilized cotton gauze, to remove hyphae and detrital material. The absence of hyphae from the filtered suspension was visually inspected by light microscopy (Laborlux K, LeitzWetzlar, Germany). The conidia suspension was centrifuged for 5 min at 11 000 *g* (Eppendorf Microcentrifuge 5414). The supernatant was discarded and the pellet was re-suspended in 2 mL PBS stored at −18 °C. To determine the concentration of conidia in the initial suspension, an aliquot was serially diluted in PBS and spread-plated on Rose Bengal Caf Agar (RBCA, Liofilchem). Colonies were counted after 5 days of incubation at 25 °C and the concentration of conidia was expressed as CFU per millilitre of suspension.

PDI experimental setup

A first series of experiments using **1a**, was conducted to determine the most suitable concentration for comparative photoinactivation assays. Aliquots of conidia suspension (100 μL) were transferred to 6-well plates and PBS was added up to a final volume of 5 mL. Different volumes of the stock solution of **1a** were added in order to obtain final concentrations of 10, 25, 50, 75 and 100 μmol L^{-1} in a final volume of 5 mL fulfilled with PBS. The resulting mixtures were kept under stirring, on melting ice to prevent heating during irradiation. To reduce the self-shading effect associated with the intense colour of the PS solutions, a pre-exposure treatment was also tested. For that, conidia suspensions were pre-incubated in the dark for 3 h with PS **1a** (100 μmol L^{-1}) at 37 °C with stirring. After this period, the unbound PS was removed by centrifugation, conidia were re-suspended in PBS and the irradiation was conducted as previously described.

In the assays performed with the other PS, the photoinactivation of *P. chrysogenum* conidia was studied only with the concentration of 50 μmol L^{-1},which corresponded to the maximum photoinactivation observed with **1a**, with a pre-incubation period of 30 min in the dark. Two controls were included in each irradiation experiment: a light control (LC) submitted to the same irradiation conditions as the samples but without PS, and a dark control (DC) containing the PS at the highest concentration, but was kept in the dark. Because of the low solubility in PBS, the solutions of porphyrin **1c** were in PBS with 5% of DMSO and the corresponding light (LC + DMSO 5%) and dark (DC **1c** + DMSO 5%) controls were included.

All treatments consisted of 20 min continuous irradiation. Samples of 100 μL were collected in the beginning of irradiation and every 5 min, serially diluted in PBS and spread-plated on RBCA for the determination of the concentration of viable spores. Colonies formed after 48 h of incubation at 25 °C were counted in the most convenient dilution. The average value of the triplicates was used as an estimate of the concentration of viable conidia in the suspension and expressed as CFU mL^{-1}. Two independent assays were conducted for each PS, being the profile of inactivation of different photosensitizers constructed with the average and the standard deviation of the obtained results. Data were represented in survival curves plotted as logarithm of the concentration of viable conidia (log CFU mL^{-1}) *versus* irradiation time (min).

Photosensitizer binding

Three sets of conidia suspensions prepared in PBS were incubated in the dark at 37 °C in the presence of 50 μmol L^{-1} of each of the PS. After 30 and 60 min of incubation, unbound photosensitizer was removed out of the suspension by centrifugation for 5 min at 11 000 rpm (Eppendorf Microcentrifuge 5414). In order to evaluate the strength of the attachment of the PS to the biological material, two series of aliquots were performed: one of the sets of conidia was digested immediately after centrifugation and the other was further washed with PBS prior to digestion. For the digestion, the pellets were resuspended in 1 mL of a digestion solution containing NaOH (0.1 mol L^{-1}, Fluka) and 1% SDS (Merck) and incubated at room temperature for at least 24 h or until the solution turned clear. In the case of compounds **1c** and **2b** that have lower solubility in water, the pellets were re-suspended in a digestion solution containing NaOH (0.1 mol L^{-1}), 1% SDS and 5% DMSO. The fluorescence of the extracts was measured on a FluoroMax3, slit 2 nm. The excitation wavelengths were: 426 nm (**1a**), 433 nm (**1b**), 399 nm (**1c**), 415 nm (**2a**) and 412 nm (**2b**). The range for emission was 600 to 750 nm. The measured fluorescence intensity allowed the determination of the corresponding concentration by interpolation with a calibration plot built with known concentrations of each PS using the digestion solution as solvent. Parallel aliquots of the conidia suspensions incubated in the presence of the PS were serially diluted and spread-plated in RBCA for the determination of the concentration of viable conidia (CFU mL^{-1}). The adsorption value (PS CFU^{-1}) was calculated according to the literature.[34] Each adsorption assay was performed in triplicate (independent assays).

PS targeting FM1-43 labeling and imaging

Conidia cells (10^8 CFU mL^{-1}) were incubated with each PS, as described in adsorption assay. After the incubation period, each sample was centrifuged (12 000 *g*, 2 min), and conidia were washed twice with PBS, in order to remove unbound PS. Conidia were fixed with 4% paraformaldehyde in PBS for 20 min at rt. Cells were permeabilized for 30 min in 0.1% Triton X-100 (Merck) in PBS, pH 7.4 at 50 °C and then stained with the membrane marker FM1-43 (25 μmol L^{-1}, Molecular Probes, Invitrogen) for 1 h at 37 °C. Images of PS and FM1-43 fluorescence were acquired with a confocal microscope (Zeiss LSM 710). The preparation was excited at 488 nm and light emitted above 493 nm was collected for

analysis of FM1-43. For analysis of the PS, each preparation was excited at 633 nm and emitted light was collected above 650 nm.

Statistical analysis

Statistical analysis was performed by using SPSS 15.0 for Windows (SPSS Inc, USA). The significance of the PDT effect of each porphyrin derivative and of the irradiation time on conidial cells inactivation was assessed by two-way univariate analysis of variance (ANOVA) model with Bonferroni post-hoc test. A value of $p < 0.05$ was considered significant.

Results

Porphyrin synthesis and characterization

Porphyrins **1a**, **1b** and **1c** were obtained in high yields from the direct *N*-alkylation of 5,10,15,20-tetrakis(4-pyridyl)porphyrin using an excess of the corresponding haloalkane according to a previous reported procedure.[31]

Another possibility to easily create a large variety of PS, including large combinatorial libraries, is the peripheral substitution of the *para* fluorine atoms of the commercial available porphyrin platform, 5,10,15,20-tetrakis(2,3,4,5,6-pentafluorophenyl)porphyrin (**TPPF$_{20}$**).[35–37] The initial results prompted the preparation of a new series of porphyrin pyridinium derivatives (porphyrins **2a** and **2b**) based on **TPPF$_{20}$** with different pyridine groups (Fig. 1).

NMR, UV-Vis and mass spectrometry confirmed the structure of all porphyrins. The ^{1}H NMR spectra of families **1** and **2** clearly show two distinct regions: the signals at the lower field are due to the resonances of the protons of the porphyrin core and of the pyridinium groups, and the ones at the higher field are due to the resonance of the aliphatic chains. The ^{19}F NMR spectrum of porphyrin **2** confirmed the substitution of the four *para*-fluorine atoms by the mercaptopyridine, by the disappearance of the signal corresponding to the resonances of all four *para*-fluorine atoms. The resonances of the *ortho*- and *meta*-fluorine atoms appear as two double doublets (J = 11.3 and 25.4 Hz) at δ –160.95 and –156.04 ppm, respectively. The cationic derivatives **2a** and **2b** show almost the same ^{19}F NMR features as presented by the precursor **2**. The mass spectrometry results confirmed all proposed structures.

Porphyrin solubility

The solubility of the porphyrins in DMSO and PBS was assessed by UV-visible spectroscopy. Several concentrations, between 1.0 and 13.0 μmol L^{-1}were analysed. The intensity of the Soret band *versus* porphyrin concentration was plotted in a graphic for linear regression, in order to determine if these concentrations follow the Beer–Lambert law (Fig. SI-1 and -2†). The normalized UV-Vis spectra in DMSO and in PBS of all studied PSs are summarized in Fig. 2. An increase in the length of the alkyl chain did not significantly change the ground state spectrum of the PS. Fig. 2b clearly shows that PBS solution of **1c** and **2b** have a sharper Soret band than observed in DMSO, suggesting aggregation phenomena in PBS. The UV-vis spectra of **1c** and **2b**, did not follow the Beer–Lambert law, indicating aggregation in PBS (see Fig. SI-1 and -2†) even at much lower concentrations than the 50 μmol L^{-1}used on the PDI studies.

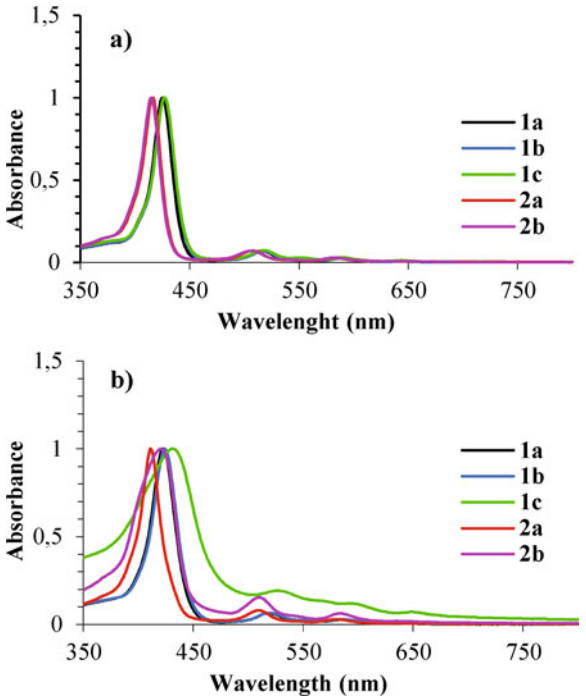

Fig. 2 Normalized absorption spectra of porphyrins **1a, 1b, 1c, 2a** and **2b** in DMSO (a) and PBS (b).

Fluorescence quantum yields

In order to follow the distribution of the PS in the conidial cell, the fluorescence quantum yield in DMF was evaluated (Table 1). Compounds **1b** and **1c** presented higher fluorescence quantum yield than compound **1a**. Compounds **2a** and **2b** showed lower fluorescence quantum yields.

Photostability of the synthesized porphyrins

The results of the photostability assays are summarized in Table 2. In general, with the exception of porphyrin **1c** that shows lower photostability (82% of the initial fluorescence after 30 min of irradiation), there were no pronounced changes in the absorbance height, indicating that the porphyrins were photostable under the conditions used on PDI assays.

Table 1 Fluorescence quantum yields (Φ) of porphyrin derivatives in DMF

Porphyrin	TPP	**1a**	**1b**	**1c**	**2a**	**2b**
Φ	0.11	0.02	0.04	0.04	0.01	0.01

Table 2 Photostability of the porphyrins after irradiation with white light (200 mW cm^{-2}) for different periods of time

	Irradiation time (min)					
Porphyrins	0	2	4	10	15	30
1a	100	92	92	91	91	89
1b	100	100	99	99	99	97
1c	100	97	91	88	86	82
2a	100	98	96	94	93	91
2b	100	97	95	93	92	91

Singlet oxygen generation

The ability of these PSs to generate singlet oxygen was qualitatively evaluated following the photooxidation of DPBF (Fig. 3). These results confirm that all the compounds are good singlet oxygen generators, causing a decay of DPBF absorption within 20 min when irradiated with light at a fluence of 9.0 mW cm^{-2}. The decay caused by family **1** PSs is higher than that caused by the family **2**, indicating a higher singlet oxygen rate production of the former family.

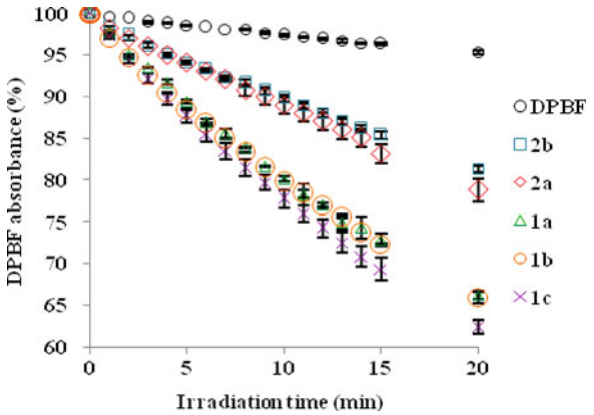

Fig. 3 Singlet oxygen generation by porphyrins **1a, 1b, 1c, 2a** and **2b** assessed by the relative photooxidation of DPBF (50 μmol L^{-1}) in DMF/H_2O (9 : 1) upon irradiation with white light filtered through a cut-off filter for wavelengths <540 nm (9.0 mW cm^{-2}) with or without PS (0.5 μmol L^{-1}). Values correspond to the average of two independent experiments. Error bars represent standard errors of the means.

Photodynamic inactivation of *Penicillium chrysogenum* conidia

The results of the preliminary PDI experiments of *P. chrysogenum* conidia with different concentrations of porphyrin **1a** are represented in Fig. 4. The independent assays of each concentration did not show significant differences (ANOVA, $p > 0.05$). A reduction of approximately 4.0 logs was observed in the presence of 50 μmol L^{-1} of **1a**, after 20 min of irradiation. Experiments conducted with 100 μmol L^{-1}, but in which a pre-exposure step was followed by the removal of the PS solution by centrifugation, did not show an improvement in the photoinactivation efficiency (data not shown). As expected, porphyrin **1a** did not exhibit significant toxicity in the absence of light (dark control) at the highest tested concentration (100 μmol L^{-1}). Direct inactivation by light in the absence of porphyrin (light control) was undetected within 20 min of irradiation.

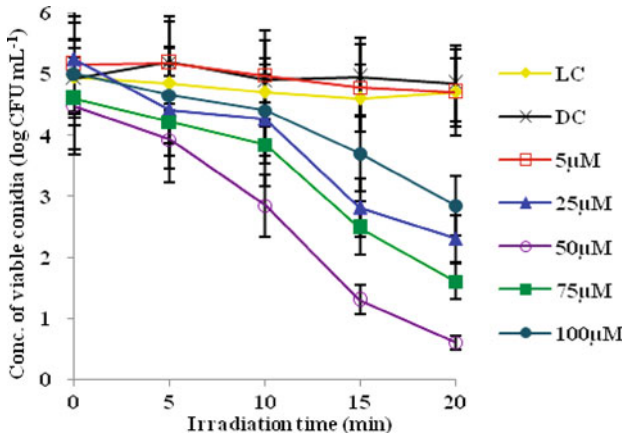

Fig. 4 Survival of *P. chrysogenum* conidia during PDI experiments with 5, 25, 50, 75 and 100 μmol L^{-1} of porphyrin **1a**, and irradiation with white light at a fluence rate of 200 mW cm^{-2}. Values correspond to the average of two independent experiments. Error bars represent standard deviation. Lines were used to connect the data points.

Comparison of the photosensitizing potential of PSs with different structures used a fixed concentration of 50 μmol L^{-1}, at which **1a** is the most efficient. Differences between the independent assays performed with each PS were not significant (ANOVA, $p > 0.05$) and the average values are presented in Fig. 5. Significant differences between the initial concentrations of viable conidia and the concentrations estimated during irradiation were detected for all molecules and for all irradiation periods (ANOVA, $p < 0.05$). Porphyrins **1a** and **1b** caused the highest inactivation of *P. chrysogenum* conidia (reductions of 4.1 and 3.4 log, respectively) and the differences in the results obtained with these two compounds are not significant (ANOVA, $p > 0.05$). Porphyrin **1c** caused a significantly smaller (ANOVA, $p < 0.05$) decrease (0.7 log) in the

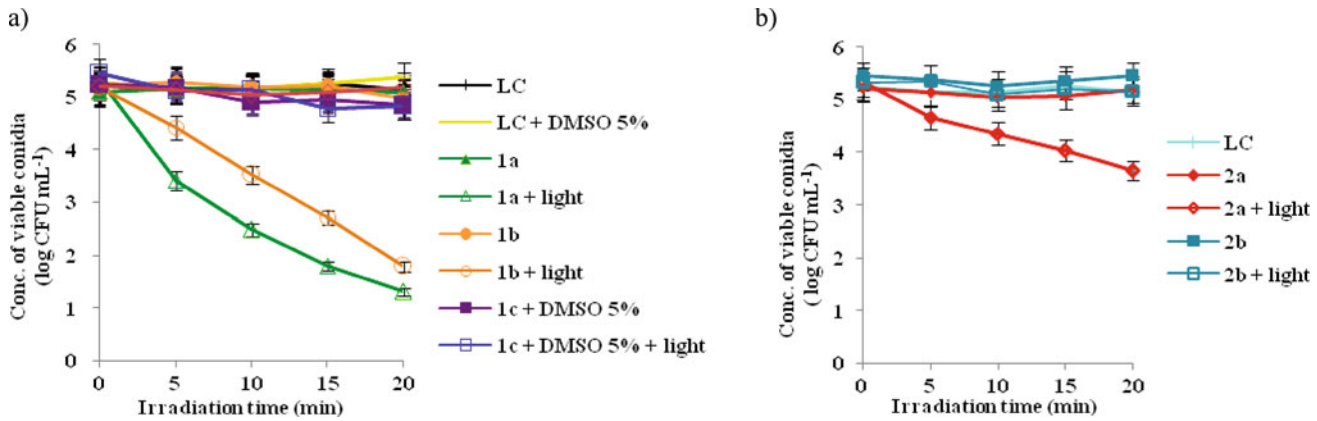

Fig. 5 Survival of *P. chrysogenum* conidia during PDI experiments with 50 μmol L^{-1} of porphyrins **1** (a) and**2** (b) with white light irradiation at a fluence rate of 200 mW cm^{-2}. Values correspond to the average of two independent experiments. Error bars represent standard deviation. Lines were used to connect the data points.

concentration of viable conidia, when compared to porphyrins **1a** and **1b**.

Porphyrin **2a** (1.7 log reduction) was less effective than **1a** and **1b** (ANOVA, $p < 0.05$), and porphyrin **2b** showed the lowest inactivation effect (0.16 log reduction) (ANOVA, $p < 0.05$) when compared to the others PSs.

Binding of the PS to *Penicillium chrysogenum* conidia

The amount of PS adsorbed to *P. chrysogenum* conidia in the presence of 50 μmol L^{-1} of the different cationic porphyrins is summarized in Fig. 6. Compounds **1c** and **2b** presented the highest amounts of retention in conidial material, with average values of 2.09×10^6 and 2.37×10^6 molecules CFU^{-1}, respectively. In compounds **1a** and **1b**, there was not a direct relation between the length of the N-alkyl substituents and the amount of PS retained, but in family **2**, molecules with longer N-alkyl chains showed higher affinity to the conidia. The amount of porphyrin taken up by the conidia increased with incubation time and washing caused a reduction on the average amount of PS.

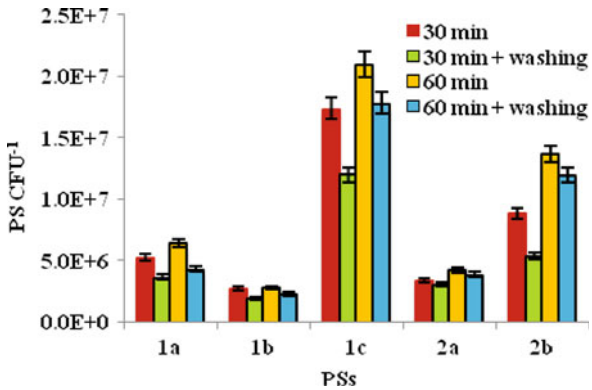

Fig. 6 Adsorption of the tested porphyrins to *P. chrysogenum* conidia after incubation in the dark for 30 and 60 min at 37 °C with 50 μmol L^{-1} of each PS. Error bars represent the standard deviation.

PS uptake, FM1-43 labelling and imaging

To investigate the cellular uptake and subcellular localization of the PS studied, confocal immunofluorescence microscopy was employed (Fig. 7). FM1-43 was used as a membrane cell marker. Family **1** (**1a** and **1b**) showed a more uniform distribution within the cell, while **2a** seems to accumulate preferentially at the cell membrane. Compounds **1c** and **2b** seem to agglomerate on the cell membrane, probably as a consequence of their tendency to aggregate in PBS.

Discussion

Cationic porphyrins have already proved to be more efficient against various microorganisms than the corresponding anionic and neutral ones.[14,16,18] In this work, new tetracationic porphyrins were synthesized and tested as to their ability to photoinactivate fungal conidia, regarded as more resistant biological structures. The effect of the length of the *N*-alkyl chain on the photoinactivation performance was assessed in two different porphyrin families. These novel porphyrins were compared to a well-known

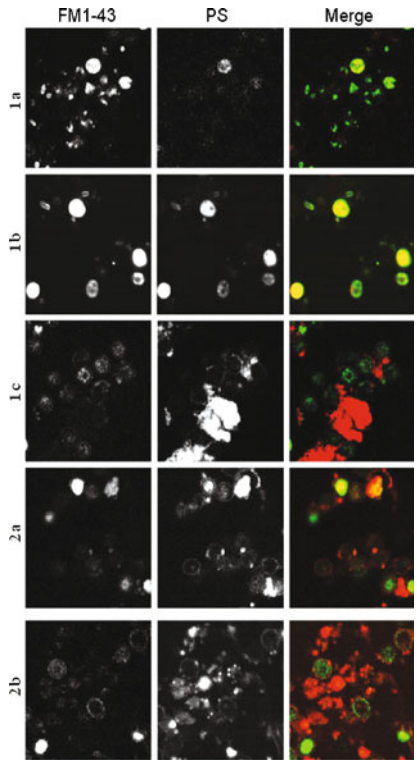

Fig. 7 Confocal fluorescence microscopy images of *P. chrysogenum* conidia, double stained with PS (**1a, 1b, 1c, 2a** and **2b**) and a cell marker FM1-43. The conidia were incubated with 50 μmol L^{-1} of each PS for 30 min at 37 °C, and the cell membranes were stained with FM1-43. Right panel shows the superimposed images from FM1-43 (green) and PS (red).

tetracationic porphyrin (**1a**), often used as model PS in studies of aPDT.[14] The two families of studied compounds show different peripheral substituents, which make them considerably different as to their physico-chemical properties. In the molecules that compose family **1**, the pyridinium moieties are directly linked on *meso* positions of the macrocycle core, and in case of family **2** the pyridinium moieties are linked to the tetrafluorophenyl groups.

In an overall analysis, none of the compounds showed significant toxicity in absence of light, as inferred from the stability of the counts of viable conidia in the dark controls, which were exposed to the highest tested concentrations of each PS. Family **1** showed to be more efficient than family **2** in the photoinactivation of *P. chrysogenum* conidia. This trend may be explained by the lower $^1O_2$ production of family **2** molecules, in comparison to family **1**. The best PS was the molecule with the shortest carbon chain (**1a**), suggesting that the increase of the *N*-alkyl length of all four alkyl chains does not improve the photodynamic efficiency. Instead, an increase of the carbon chain led to a reduction of the inactivation of conidia, in both families. In a similar study by Casteel and co-workers using compounds similar to those of family **1** but with different lengths of the carbon chains (C1, C4 and C8) the influence of the length of the alkyl chain could not be clearly established because the C1 molecule was more effective against hepatitis A virus than the C4 molecule and less effective that the C8.[38] Derivatives where one N-methyl group was replaced by a longer hydrocarbon chain[29] or when a pyridinium group was replaced by a phenyl carboxy-polylysine group,[31] showed a higher photosensitizing efficiency against *Staphylococcus aureus*

and *Escherichia coli*, mainly associated with their higher binding to bacterial cells.

In the previously mentioned work the modifications involved only one of the substituents whereas in the present work, all the four alkyl chains were changed in length leading to a much more drastic modification of the precursor molecules. Also, the nature of the fungal cell wall is very different form the wall of bacteria, complicating the comparison of results. The PS **1a** showed higher affinity to the conidial material than **1b**, and this might be the major determinant of the higher inactivation achieved with the former. Although **2a** was more taken up by conidia than **1b**, the higher $^1O_2$ production made the second one more effective in PDI experiments. The lower singlet oxygen production of sulphur containing family **2**, compared to family **1**, may be due to aggregation which is responsible for the reduction of singlet oxygen production by PS and/or by a self-scavenger of ROS. Several studies reporting that singlet oxygen can be effectively quenched by thiols, such as methionine.[39,40]

Compounds **1c** and **2b** also displayed unexpectedly high values of PS uptake, which are more likely an artefact associated to an aggregation process, observed in PBS. The confocal microscopy images reveal granulations in the surface of conidia and preferential accumulation at the cell surface which can also be related to the low water solubility of these molecules and their tendency to aggregate. Previous studies indicate that antifungal PDI is generally not dependent on surface-bound PS, but on the permeabilization of the cell membrane by reactive species produced by unbound PS molecules.[32] Hence, the aggregated molecules bound to the outer surface of the conidia may not induce lethal damage. The total amount of porphyrin detected in conidial materials in the binding assays corresponds to both the molecules that were attached into the spore surface and to the molecules that penetrated to the intracellular space after membrane permeabilization. The images obtained by confocal microscopy support this assumption because the most efficient photosensitizers were those better distributed within the conidia (**1a** and **1b**) whereas molecules of family **2** tend to be restricted to the surface of the conidia. In the confocal images, **1a** was less fluorescent that other molecules. This was interpreted as a lower fluorescence and not as a decreased affinity to the biological material, as confirmed by the lower fluorescence quantum yield of this molecule. The fluorescence of dye aggregates is often quenched.

The molecules **1a** and **1b** were identified as the most effective photosensitizers against fungal conidia. All the molecules showed similar photostability but family **1** compounds showed a slightly higher singlet oxygen generation and a better binding to the conidial material. The increase in the length of the *N*-alkyl chain from C1 to C5 improved the affinity of the PS to the target cell in compounds of family **1**, while C12 chain reduced solubility, favoured aggregation and consequently minor PDI efficiency. The observed low solubility for derivative **1c** discouraged the synthesis of the corresponding analogue **2c**.

Conclusions

This work demonstrates that tetracationic porphyrins can be considered as promising PS for the inactivation fungal conidia although the performance is highly variable between molecules with different *N*-alkyl chains. The size of the *N*-alkyl chain was shown to have a great influence on the photoinactivation efficiency (**1a** > **1b**), mainly by affecting the binding to cellular material and the solubility. In the molecules with the longest chains, photoinactivation was much less efficient probably as a consequence of PS aggregation (**1c,2b**). The higher singlet oxygen production, more efficient attachment to conidial material, lower aggregation in PBS and a more homogeneous distribution with the conidial cell are the most likely factors underlying the better performance of **1a** against *P. chrysogenum* conidia.

The results of this work confirm the potential of porphyrin derivatives for antifungal PDI in broad-field applications such as phytosanitation, biofilm control, bioremediation, and wastewater treatment to be envisaged, not excluding applications related to human health.

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