Photochemistry and Photobiology, 2010, 86: 1147–1153

A New Nonconjugated Naphthalene Derivative of *Meso*-tetra-(3-hydroxy)-phenyl-porphyrin as a Potential Sensitizer for Photodynamic Therapy

Pedro Silva[1], Sofia M. Fonseca[1], Cláudia T. Arranja[1], Hugh D. Burrows[1], Ana M. Urbano[2] and Abilio J. F. N. Sobral*[1]

[1]Departamento de Química, FCTUC, Universidade de Coimbra, Coimbra, Portugal
[2]Unidade de Química Física Molecular, Departamento de Ciências da Vida, FCTUC, Universidade de Coimbra, Coimbra, Portugal

Received 8 January 2010, accepted 5 May 2010, DOI: 10.1111/j.1751-1097.2010.00764.x

ABSTRACT

A new 5,10,15,20-tetra-(phenoxy-3-carbonyl-1-amino-naphthyl)-porphyrin was prepared by an isocyanate condensation reaction and its photophysical properties fully evaluated, both in terms of photostability and singlet oxygen production. It shows considerably enhanced photostability when compared with the parent 5,10,15,20-tetra-(3-hydroxy-phenyl)-porphyrin, with the photodegradation quantum yields for T(NAF)PP and T(OH)PP being 4.65×10^{-4} and 5.19×10^{-3}, respectively. Its photodynamic effect in human carcinoma HT-29 cells was evaluated. The new porphyrin showed good properties as a sensitizer in photodynamic therapy with an *in vitro* cytotoxicity IC_{50} value of 6.80 μg mL^{-1} for a 24 h incubation. In addition to the potential of this compound, the synthetic route used provides possibilities of extension to a wide range of new sensitizers.

INTRODUCTION

Cancer is a major public health problem that affects millions of people worldwide. The techniques most commonly used in cancer treatment are surgery, chemotherapy and radiotherapy, but other techniques, like photodynamic therapy (PDT), are showing promising results in the treatment of certain types of cancer, in particular in the treatment of melanomas, oesophageal and retinal cancers (1–4).

The PDT approach consists in the administration of a photosensitizer that becomes concentrated in tumor cells. Upon light absorption, the photosensitizer undergoes excitation to the singlet state (S1) and subsequently, by intersystem crossing, forms the triplet excited state (T1). The photosensitizer triplet state can then either participate in a one-electron oxidation-reduction reaction (Type I photoprocess) with a neighboring molecule, producing free radical intermediates that may react with oxygen to generate various reactive oxygen radical species, or can transfer energy to ground state oxygen (Type II photoprocess), generating the highly cytotoxic singlet molecular oxygen, that may destroy tumor cells, where the sensitizer accumulates preferentially (5,6).

*Corresponding author email: asobral@ci.uc.pt (Abilio J. F. N. Sobral)

PDT is one of the most important applications of porphyrins and their derivatives. These molecules have shown excellent properties as photosensitizers that make them ideal candidates for medical applications in PDT, with some already being in clinical use (7). The importance of porphyrins and related compounds as therapeutic agents in PDT has increased significantly over the last few years. They have high affinity and toxicity for tumor tissues, high photostability, photodynamic activity and intense absorption in a region where biological tissues are relatively transparent (600–800 nm) (8,9). Recently, studies have shown the potential of *meso*-tetraphenylporphyrin (TPP) derivatives for use in PDT, because of their high selectivity in tumoral tissues (10). The clinically approved Foscan®, a light sensitive drug that contains temoporfin (5,10,15,20-tetra-(3-hydroxy-phenyl)-chlorin) is an example. Naphthyl-isocyanate (NAF) is an aromatic compound with numerous applications in the chemical industry, in particular for polymer synthesis. In addition, recent studies showed its potential application in medicine as a therapeutic agent. In fact, *in vitro* studies showed that NAF participates actively in the formation of reactive oxygen species, having a toxic effect on cells (11,12). Following our previous work on medicinal chemistry (13–15) and PDT (16,17), we have designed a new macrocyclic molecule with the expectation that, by connecting both porphyrin and NAF characteristics, we may obtain a good PDT effect, through the possibility of two different modes of excitation, arising from the porphyrin macrocycle and the peripheral naphthyl groups, therefore improving the therapeutic response for the same molar doses. As with the related meso tetra-(4-(*N*-methyl-*N*-9-anthracenyl-methyl)-sulfamoylphenyl)-porphyrin (18), both singlet and triplet energy transfer pathways exist from the naphthalene moieties to the porphyrin. In addition, the naphthyl groups may act to stabilize the system with respect to photodegradation.

MATERIALS AND METHODS

Reagents. All reagents used in the synthesis of the new porphyrin were synthesis grade, purchased from commercial sources and used as received. Solvents were purified by standard methods before use. Most reagents used in cell culture, such as RPMI-1640, the penicillin/streptomycin mixture, Trypan Blue and 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT), were obtained from Sigma-Aldrich. Fetal calf serum (FCS) was obtained from Invitrogen.

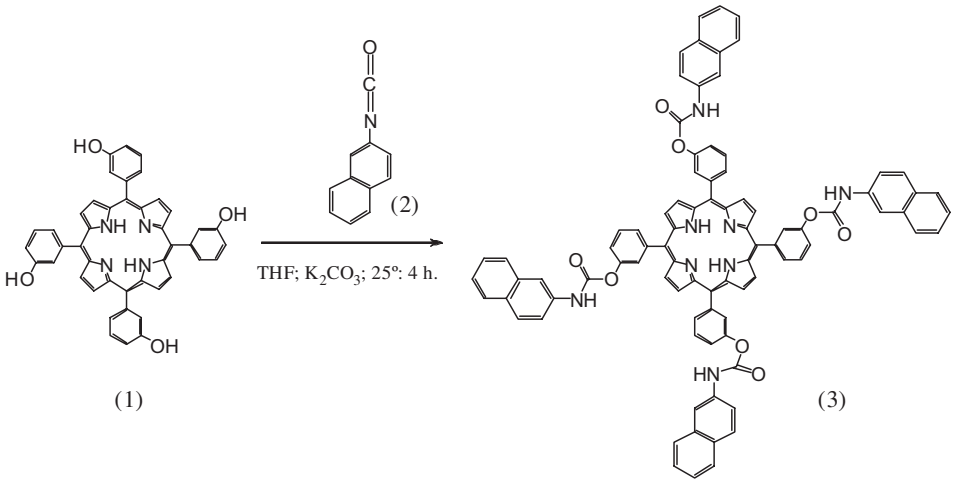

Figure 1. Scheme of the synthesis of 5,10,15,20-tetra-(phenoxy-3-carbonyl-1-amino-naphthyl)-porphyrin (**3**) from 5,10,15,20-tetra-(3-hydroxyphenyl)-porphyrin (**1**) and naphthylisocyanate (**2**).

Methods. Reaction progress was monitored by UV–visible (UV–Vis) absorption spectroscopy, using a Jasco V-530 spectrometer, and thin-layer chromatography (TLC) on silica gel 60 F_{254} (Merck) with detection by UV–Vis. Silica gel H (Fluka) was used for preparative TLC. NMR experiments were carried out on a Bruker-AC200 instrument and recorded at 500 MHz. Mass spectra (ESI/MALDI/TOF+) were measured on a Varian VG7070C spectrometer.

Synthesis. The new derivative of 5,10,15,20-tetra-(3-hydroxyphenyl)-porphyrin (T(OH)PP) was obtained from the reaction of this porphyrin with NAF (Fig. 1). The parent T(OH)PP used as starting material was synthesized in our laboratory by the Rothemund/Adler/Longo method (16) and gave spectroscopic data in full agreement with the literature (19). The synthesis of 5,10,15,20-tetra-(phenoxy-3-carbonyl-1-amino-naphthyl)-porphyrin (T(NAF)PP) was carried out in tetrahydrofuran (12 mL) in the presence of K_2CO_3 (3 g, 0.02 mol) using T(OH)PP (55 mg, 0.08 mmol) and NAF (54 mg, 0.32 mmol), following a standard procedure for the general alcohol/isocyanate coupling (20).

Preparative TLC (SiO_2/CH_2Cl_2) and precipitation in *n*-hexane, to remove remnants of NAF, allowed the isolation of the T(NAF)PP (0.02 mmol, 25%). ^{1}H-NMR ($CDCl_3$/TMS): δ 9.15 (s, 4H, aminocarbonyl group), 7.80–8.29 (m, 28H, naphthyl group), 7.19–7.67 (m, 16H, meso porphyrin phenyl groups), 6.44 (s, 8H, β-pyrrolic positions of porphyrin), −2.10 (s, 2H, NH inner pyrrole hydrogens). MS calculated for $C_{88}H_{58}N_8O_8$ (M^+) 1355.45, found 1354.80 (the mass spectrum of T(NAF)PP can be found in Fig. S1 of the Supporting Information).

Photophysical studies. Toluene was used as solvent in the photophysical characterization of T(NAF)PP. The ground-state absorption and fluorescence spectra were recorded at room temperature with a Shimadzu UV-2100 spectrophotometer and a Horiba-Jobin-Yvon-SPEX Fluorolog 3-22 spectrofluorometer, respectively. Fluorescence spectra were corrected for the wavelength response of the system. Fluorescence quantum yields were determined using 5,10,15,20-tetraphenylporphyrin (TPP) in toluene (Φ_F = 0.11) as standard (21).

Flash photolysis experiments were performed with an Applied Photophysics LKS.60 flash kinetic spectrometer pumped by the third harmonic (355 nm) of a Nd:YAG laser (Spectra-Physics Quanta Ray GCR 130). The monitoring light was produced by a 150 W pulsed Xe lamp. Signals were detected with a Hamamatsu IP28 photomultiplier and transient spectra were obtained by monitoring the absorbance change at 10 nm intervals over the 300–700 nm range.

Singlet oxygen ($^1\Delta_g$) yields and lifetimes were obtained by direct measurement of the phosphorescence at 1270 nm using an adaptation of the LKS.60 spectrometer. The singlet oxygen emission at room temperature was detected using a Hamamatsu R5509-42 photomultiplier, cooled to 193 K in a liquid nitrogen chamber (Products for Research, model PC176TSCE005), following excitation (355 nm) from a Nd:YAG laser of an aerated solution of the porphyrin ($A_{355\ nm}$ = 0.25) in toluene. The modification of the spectrometer involved the interposition of a Melles Griot dielectric mirror (08MLQ005/345) that reflects more than 99.5% of the incident light in the 610–860 nm range, and a Schott RG665 filter. A 600 line diffraction grating was mounted in place of the standard spectrometer one to extend spectral response to the infrared. The filters employed are essential in eliminating from the infrared signal all the first harmonic contributions of the sensitizer emission in the 500–800 nm range.

The photostability of the porphyrins T(NAF)PP and T(OH)PP was evaluated by determining their photodegradation quantum yields using azobenzene as actinometer. Light of wavelength 420 nm was used and was selected from the 450 W xenon arc lamp of a Horiba-Jobin-Ivon SPEX Fluorolog 3-22 spectrometer using a monochromator. Actinometry for quantum yield measurements used a solution of azobenzene in cyclohexane taking the value 0.40 for the quantum yield of its *cis–trans* photoisomerization on excitation at 420 nm (22). Quantum yields were determined by monitoring the change in absorbance as a function of irradiation time at the appropriate analytical wavelengths (444 nm for azobenzene and 514 nm for the porphyrins), using the expression:

$$\frac{\Phi_1}{\Phi_2} = \frac{dA_1/dt}{dA_2/dt}\frac{\varepsilon_2}{\varepsilon_1}\frac{\eta_1^2}{\eta_2^2}$$

where Φ_1 is the quantum yield of *cis–trans* photoisomerization of azobenzene, Φ_2 is the quantum yield of photodegradation of the porphyrins, dA_n/dt are the respective slopes of plots of absorbance as a function of irradiation time, ε_1 is the difference between the molar absorption coefficients of the *cis* and *trans* isomers of azobenzene (1140 and 490 $м^{-1}$ cm^{-1}, respectively, at 436 nm), ε_2 is the molar absorption coefficient of the porphyrins (2.02×10^4 and 2.06×10^4 $м^{-1}$ cm^{-1} at 514 nm, for T(NAF)PP and T(OH)PP, respectively) and η_1 and η_2 are the refractive indexes of cyclohexane and toluene, respectively.

Cell culture. Cells from the HT-29 cell line were used throughout this study. This epithelial-like adherent human cell line from a primary colorectal adenocarcinoma was obtained from the American Type Culture Collection (ATCC; Manassas, VA; ATCC no. HTB-38). Cells were cultured as monolayers at 37°C in a humidified atmosphere of 95% air:5% CO_2, in RPMI-1640 medium, supplemented with 10% FCS, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. Cells were always in the logarithmic phase of growth, with a duplication time of 24 h.

Porphyrin treatment. Porphyrins were dissolved in dimethyl sulfoxide (DMSO) and the resulting solutions (1 μg mL^{-1}) were sterilized through a 0.2 μm filter before use. To allow for proper cell attachment, the porphyrin solutions were only added to the cultures in complete medium 24 h after seeding. The volume of porphyrin solution added

was always ≤5% of the total volume and the final concentration of DMSO was always 0.5% (vol/vol). Control cultures, established and processed in parallel, received the same amount of the addition vehicle (DMSO) as the treated cultures did. Treatments were always carried out in the dark.

Cytotoxicity screening. For the initial cytotoxicity (dark toxicity) screening, cultures were subjected to a 24 h treatment with the specified concentration of the porphyrins under study. The effect of each treatment on the viability of the cultures was determined immediately after the treatment by Trypan Blue dye exclusion, which distinguishes between live and dead cells based on the integrity of their plasma membrane. Briefly, cells were seeded in 6-well plates at 20 000 cells cm^{-2} in 5 mL of medium. Triplicate wells were used for each condition. At the end of each incubation, cell counts were performed on cell suspensions using a hemocytometer.

Irradiation. For the determination of the photodynamic activity of the different porphyrins under study, cultures were irradiated using, as light source, a Reflecta Diamator slide projector (AGFA) equipped with a 150 W low voltage halogen lamp (Xenophot 64640 HLX; OSRAM) and a cutoff filter that only permitted the transmission of radiation with wavelengths longer than 600 nm (Fig. S2). The light was also filtered through a water layer for heat absorption. The fluence rate at the treatment site was 9.8 mW cm^{-2}, as determined using a Coherent® power meter (model 212; head sensitivity 0.351 mA mW^{-1} at 633 nm). Cells were exposed to this light intensity for different periods (0–30 min), corresponding to total light doses of 0–17.6 J cm^{-2}. For each independent experiment, porphyrin-treated cultures and the corresponding control cultures were irradiated simultaneously.

Determination of the photodynamic activity. The photodynamic activity of the different porphyrins was assessed in terms of their effects on the dehydrogenase activity (a viability measure) of the treated cultures, using the MTT colorimetric assay (23). Cells were seeded in 24-well plates, at a cell density of 20 000 cells cm^{-2}, in 1 mL of medium. Triplicate wells were used for each condition. At the end of the 24 h treatment, the porphyrin-containing medium was removed, the monolayers washed with PBS and incubated in 1 mL of PBS. Cultures were then irradiated for the specified times as just described. Afterward, the PBS solution was removed and 1 mL of porphyrin-free fresh medium was added to the cultures, which were returned to the incubator for a further 24 h. At the end of this incubation, the medium was removed and 0.5 mL of a 0.5 mg mL^{-1} MTT solution was added. After 3 h incubation, the MTT solution was aspirated and the insoluble formazan crystals that formed were dissolved in an equal volume (0.5 mL) of a 0.1 M HCl solution in isopropanol. Absorbance values at 570 nm (A_{570}) were then measured. For each irradiation time, control cultures, which were treated with the addition vehicle only, were processed in exactly the same way. The linearity between the MTT assay response (A_{570}) and the number of viable cells in culture was confirmed under the experimental conditions employed.

Statistical analysis. The statistical significance of the differences from the control was assessed using one-way ANOVA followed by Dunnett's posttest.

RESULTS AND DISCUSSION

Photophysical studies

The porphyrin T(NAF)PP shows typical spectroscopic features of free-base porphyrins, *e.g.* an intense Soret band in the violet range of the visible region and four broad Q-bands in the 500–650 nm region. The absorption spectrum is shown in Fig. 2. The molar absorption coefficients were calculated from the Beer–Lambert law using solutions with concentrations in the range 10^{-7}–10^{-5} M. We found no evidence for aggregation at these concentrations, as good linear plots ($r \geq 0.999$) that pass through the origin were obtained. The steady-state fluorescence emission spectrum (Fig. S3) presents two maxima which we assign to Q(0–0) and Q(0–1) transitions on the basis of comparison with other porphyrin systems (24,25). The relevant data from the absorption and fluorescence spectra are

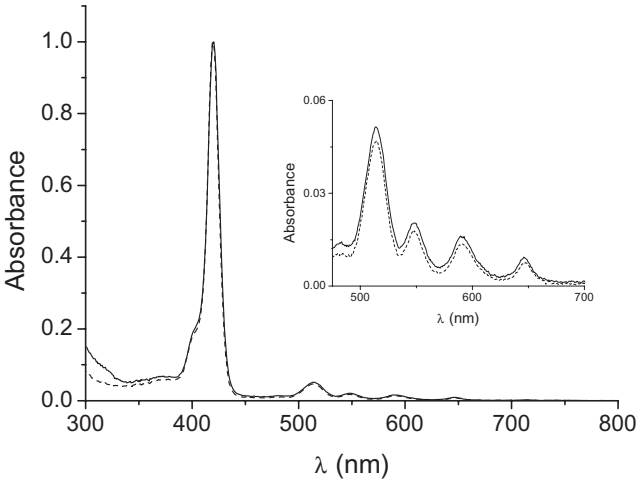

Figure 2. Normalized absorption spectrum of T(NAF)PP (solid line) in toluene solution. The absorption spectrum of T(OH)PP in toluene (dashed line) is also presented for comparison.

summarized in Table 1. The fluorescence quantum yield (Φ_F) was calculated by steady-state comparative method using TPP in toluene ($\Phi_F = 0.11$) as standard (21), and was obtained by comparison between the integrated emission spectra of optically matched deaerated solutions of T(NAF)PP and the reference (TPP). The value obtained was slightly lower than that found with T(OH)PP (0.12) (25). The spectroscopic singlet-state energy (E_S) was obtained from the intersection of the normalized absorption and fluorescence spectra.

Triplet-singlet difference absorption spectra of T(NAF)PP were obtained by laser flash photolysis, and presented the typical absorption band at ~450 nm and the ground state bleaching bands (Fig. 3). The triplet molar absorption coefficient (ε_T) was calculated by the singlet depletion method (26,27). The triplet formation quantum yield (Φ_T) was obtained by the comparative technique using TPP as standard ($\varepsilon_T = 6.6 \times 10^4$ M^{-1} cm^{-1} at 440 nm, $\Phi_T = 0.82$) (28,29). The triplet-state lifetimes were measured at 450 nm in the presence (air-equilibrated samples) and absence of oxygen, and from these the bimolecular rate constant (k_q) for the quenching of the triplet state of T(NAF)PP by molecular oxygen ($^3O_2$) was calculated using the concentration of oxygen in toluene (1.81×10^{-3} M) (30):

$$k_q = (1/\tau_{O_2} - 1/\tau_{N_2})/[O_2]$$

The values obtained for the lifetimes and the quenching rate constant by molecular oxygen are presented in Table 2. The value of k_q is in the range typically observed for quenching of triplet states by molecular oxygen leading to singlet oxygen generation (31,32).

The singlet oxygen quantum yield (Φ_Δ) was obtained by comparing the intensity of singlet oxygen emission at 1270 nm in an air-equilibrated sample of T(NAF)PP against the intensity obtained from an optically matched sample of a reference sensitizer. The singlet oxygen lifetime under these conditions was 29.8 ± 1.0 μs, which is in agreement with the literature value in toluene (33). The aromatic ketone 1*H*-phenalen-1-one (also called phenalenone and perinaphthenone), one of the most efficient singlet oxygen sensitizers, was

Table 1. Absorption and fluorescence data for the T(NAF)PP porphyrin in deaerated toluene solution.

	Absorption, λ_{max} [nm] (ε [M^{-1} cm^{-1}])					Fluorescence, λ_{max} [nm]			
	$Q_x(0–0)$	$Q_x(1–0)$	$Q_y(0–0)$	$Q_y(1–0)$	B(0–0)	Q(0–0)	Q(0–1)	Φ_F*	E_S [kJ mol^{-1}]
T(NAF)PP	646 (3.65×10^3)	590 (6.38×10^3)	548 (8.19×10^3)	514 (2.02×10^4)	420 (4.02×10^5)	652	718	0.086	201.2

*The estimated uncertainty is 10%.

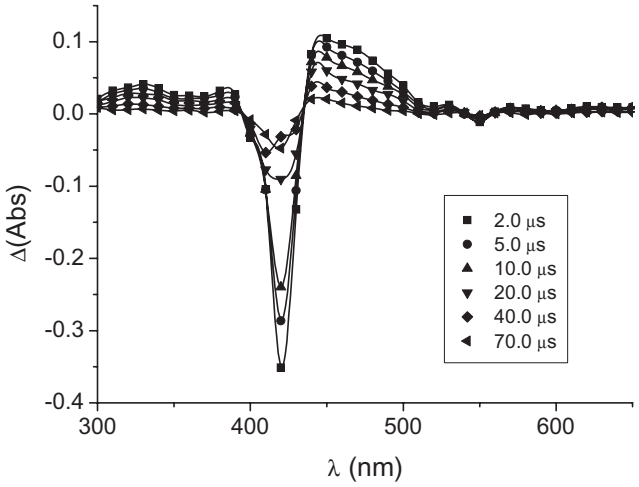

Figure 3. Triplet-singlet difference absorption spectra observed at various times following laser excitation at 355 nm of a deaerated solution of T(NAF)PP in toluene.

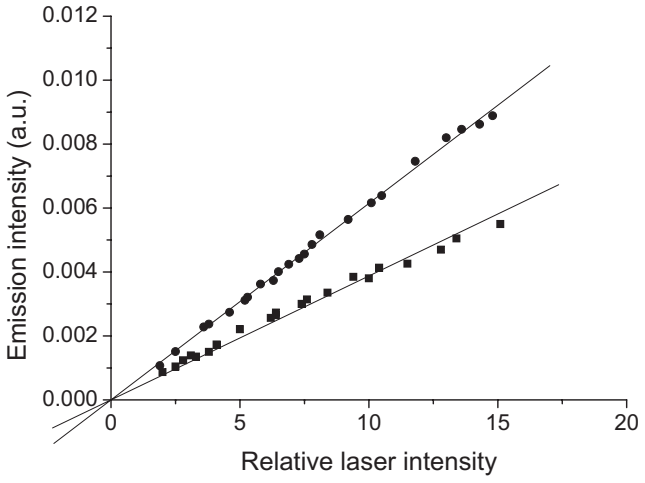

Figure 4. Singlet oxygen emission intensity (at 1270 nm) as a function of relative laser intensity: 1*H*-phenalen-1-one (circles) and T(NAF)PP (squares) in toluene solutions.

chosen as the reference sensitizer (Φ_Δ = 0.95) (34). Different laser intensities were employed and the quantum yield was determined from the ratio of the slopes obtained with T(NAF)PP and 1*H*-phenalen-1-one as standard (Fig. 4, Table 2). The singlet oxygen quantum yield value obtained for T(NAF)PP (0.60) is comparable to that of TPP (0.62), is somewhat higher than that found with T(OH)PP (0.46) (25) and almost twice the value for Photofrin II (0.32) (35). The value obtained for the fraction of triplets quenched by oxygen giving rise to singlet oxygen ($S_\Delta = \Phi_\Delta/\Phi_T$ = 0.79) is in agreement with the typical values found for porphyrins (0.76 ± 0.02) (36).

The photostability of the porphyrins T(NAF)PP and T(OH)PP was evaluated by monitoring the changes in their visible absorption spectra upon irradiation (λ_{ex} = 420 nm), using azobenzene actinometry to determine their photodegradation quantum yields (22). Figure S4a,b shows, respectively, the visible absorption spectra for the Q bands of T(NAF)PP and T(OH)PP for several irradiation times. It can be seen that with increasing irradiation time there are negligible changes in the visible absorption spectra of T(NAF)PP. However, for T(OH)PP an increase in absorbance is observed. Whilst we have not characterized this decomposition reaction, the fact that there is an increase in absorption over the whole spectrum and that this becomes more pronounced at shorter wavelengths strongly suggests that it is not due to reaction of the porphyrin moiety but instead arises from light scattering due to the formation of poorly soluble species. It is known that phenols react with singlet oxygen (37), and we feel it is probable the oxidation products of the hydroxyphenyl substituents react to produce oligomeric species. The photodegradation quantum yields for T(NAF)PP and T(OH)PP are 4.65×10^{-4} and 5.19×10^{-3}, respectively. Given the error limits, the value for T(NAF)PP is probably an upper limit. Although both compounds show reasonable photostability, we suggest that the carbonyl-1-amino-naphthyl groups are responsible for the increased photostability of T(NAF)PP.

Table 2. Triplet lifetimes for T(NAF)PP in deaerated and aerated toluene solutions, with respective oxygen quenching rate constant, triplet molar absorption coefficient, triplet and singlet oxygen quantum yields, and the fraction of triplets quenched by oxygen giving rise to singlet oxygen.

	$\tau_T(N_2)$ [μs]	$\tau_T(O_2)$ [ns]	$k_q(O_2)$ [M^{-1} s^{-1}]	ε_T [M^{-1} cm^{-1}]	Φ_T*	Φ_Δ*	S_Δ
T(NAF)PP	44.9 ± 1.0	320.6 ± 12.8	1.71×10^9	6.37×10^4	0.76	0.60	0.79

*The estimated uncertainty is 10%.

Photodynamic activity studies

The porphyrins T(OH)PP and T(NAF)PP are water-insoluble, but soluble in various organic solvents, including DMSO, which was chosen as the addition vehicle. For both control and porphyrin-treated cultures, the final concentration of DMSO in the medium was always 0.5% (vol/vol). At this concentration, the percentage of dead cells in culture did not exceed 2% and the total number of cells in culture was always ≥90% that of the control value for 24 h incubations (average values from two independent experiments that differed in less than 12%).

In order to choose porphyrin doses for the determination of the photodynamic activity that were not overtly cytotoxic, a preliminary dark toxicity screening was carried out with the porphyrin T(OH)PP. The results obtained in that screening are summarized in Fig. 5. From this dose–response curve, an *in vitro* cytotoxicity IC_{50} value (*i.e.* the concentration at which cell viability was inhibited by 50%) of 6.80 μg mL^{-1} (R^2 = 0.9554) was obtained for a 24 h incubation.

Two doses of relatively low cytotoxicity (0.5 and 1.0 μg mL^{-1}) were then chosen for the determination of the photodynamic activity of porphyrins T(OH)PP and T(NAF)PP. In the case of porphyrin T(NAF)PP, a statistically significant photodynamic activity (*i.e.* a cytotoxic activity upon irradiation statistically different from that obtained without irradiation) could only be observed for the longest light exposure and, even with this light dose, the effect was rather small (Fig. 6). However, an important finding arising from this study was that the insertion of naphthyl groups into the T(OH)PP molecule resulted in a significant decrease in dark cytotoxicity, therefore allowing for the use of higher porphyrin concentrations. Hence, a third dose (5.0 μg mL^{-1}) was included for T(NAF)PP and, this time, a significant photodynamic activity was observed. The results obtained are summarized in Fig. 6. As can be seen, both porphyrins exhibited photodynamic activities that, as expected, were dependent upon the concentration of the porphyrin and upon the light dose. In contrast to our initial expectations, under the experimental conditions used and for identical dark cytotoxicities, the results obtained do suggest a lower photodynamic activity for the new porphyrin than for the parent porphyrin.

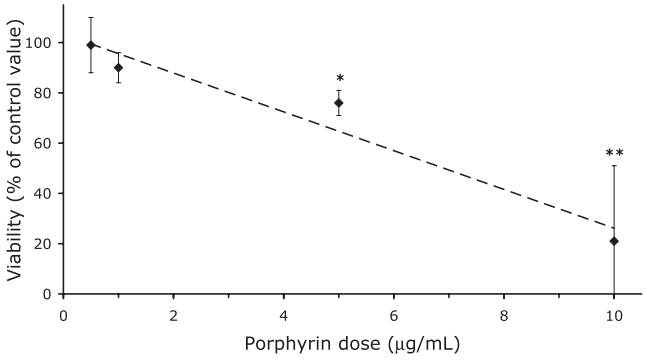

Figure 5. Viability of cultures of HT-29 cells after treatment with different concentrations of porphyrin T(OH)PP. For each culture, cell viability was determined using Trypan blue dye exclusion. Values are expressed as percentage of the corresponding control value, obtained with control cultures established and processed in parallel. Data represent the mean ± SD of at least three independent experiments. *P < 0.05, **P < 0.01, when compared with control value, with ANOVA followed by Dunnett's multiple comparison test.

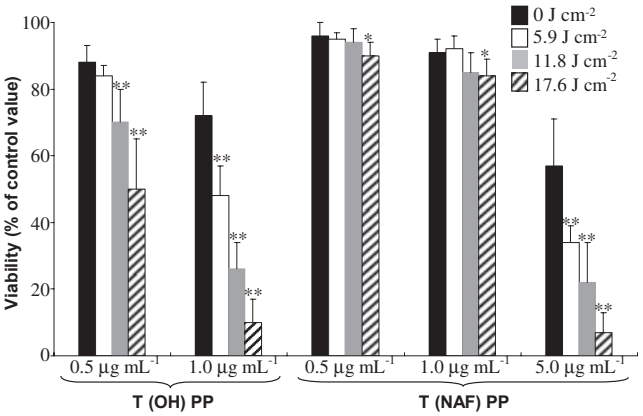

Figure 6. Photodynamic activity of porphyrins T(OH)PP and T(NAF)PP against cultures of HT-29 cells. The effect of the different porphyrin concentrations and light doses tested on the viability of the cultures was determined in terms of dehydrogenase activity using the MTT assay. Values are expressed as percentage of the corresponding control value, obtained with control cultures established and processed in parallel. Data represent the mean ± SD of at least four independent experiments. *P < 0.05, **P < 0.01, when compared with non-irradiated cultures, with ANOVA followed by Dunnett's multiple comparison test.

However, the new porphyrin was an active photosensitizer toward HT-29 cells, and although it is very difficult to establish direct comparisons between the results obtained in this *in vitro* photodynamic study and those described in the literature for other photosensitizers, due to variations in the experimental procedures (cell line, incubation time, light source, light dose, *etc.*) and in the dark toxicity values of the photosensitizers under comparison, the *in vitro* photocytotoxicity IC_{50} values for both porphyrins compare favorably with that of Photofrin. We note that, for a light dose of 5.9 J cm^{-2}, the values for T(OH)PP and T(NAF)PP were *ca* 1 μg mL^{-1} (1.5 μM) and < 5 μg mL^{-1} (< 3.7 μM), respectively, while that obtained for Photofrin under identical experimental conditions was 10 μM (38). Moreover, in the assessment of a given molecule as a potential sensitizer for PDT, parameters other than the photodynamic activity have to be taken into consideration, including photostability, which was higher for T(NAF)PP than for T(OH)PP.

CONCLUSIONS

The planned synthesis of the title compound was performed and the new porphyrin was isolated, fully characterized and its photodynamic properties were evaluated using cultures of HT-29 cells. The yield of triplet state and singlet oxygen formation was greater than that of T(OH)PP, possibly due to the involvement of energy transfer pathways. In addition, the compound showed markedly greater photostability than T(OH)PP. Taking into account the overall results of the present study, we conclude that the porphyrins tested demonstrate good potential as sensitizers for PDT, as they absorb light between 600 and 800 nm, have a low dark cytotoxicity and show phototoxicity at the same level as the clinically used PDT agents. In addition, the isocyanate condensation reaction used provides a new tool for the development of more efficient PDT drugs. Taken together, our observations suggest that the

synthetic approach described in the present study may represent a promising lead for the development of novel photosynthesizing agents.

Acknowledgements—The authors thank Dr. Francisco Gil (Departamento de Física, FCTUC, Universidade de Coimbra) for use of his power meter, and acknowledge financial support from FCT/FEDER/PTDC/AAC-CLI/098308/2008 (AJFNS), FCT/SFRH/BD/48269/2008 (CTA) and FCT/SFRH/BPD/34703/2007 (SMF).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Mass spectrum of T(NAF)PP (ESI/MALDI TOF+).

Figure S2. Irradiation system used in the determination of the photodynamic activity of the different porphyrins under study. The slide projector was equipped with a cutoff filter that only permitted the transmission of radiation longer than 600 nm. For heat absorption, the light was filtered through a water layer.

Figure S3. Normalized fluorescence spectrum of T(NAF)PP (solid line) in toluene solution. The fluorescence spectrum of T(OH)PP in toluene (dashed line) is also presented for comparison.

Figure S4. Visible absorption spectra of the Q bands of T(NAF)PP (a) and T(OH)PP (b) (solid line) and for increasing irradiation times (1, 3, 5, 10, 15, 30 min).

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

REFERENCES

1. Epstein, J. H. (1990) Phototherapy and photochemotherapy. *N. Engl. J. Med.* **322**, 1149–1151.
2. Claydon, P. E. and R. Ackroyd (2004) Barrett's oesophagus and photodynamic therapy (PDT). *Photodiagn. Photodyn. Ther.* **1**, 203–209.
3. Ohnishi, Y., T. Yoshitomi, T. Murata, T. Sakamoto and T. Ishibashi (2002) Electron microscopic study of monkey retina after photodynamic treatment. *Med. Electron Microsc.* **35**, 46–52.
4. Maillard, P., B. Loock, D. S. Grierson, I. Laville, J. Blais, F. Doz, L. Desjardins, D. Carrez, J.-L. Guerquin-Kern and A. Croisy (2007) In vitro phototoxicity of glycoconjugated porphyrins and chlorins in colorectal adenocarcinoma (HT29) and retinoblastoma (Y79) cell lines. *Photodiagn. Photodyn. Ther.* **4**, 261–268.
5. Kalka, K., H. Merk and H. Mukhtar (2000) Photodynamic therapy in dermatology. *J. Am. Acad. Dermatol.* **42**, 389–416.
6. Pushpan, S. K., S. Venkatramant, V. G. Anand, J. Sankar, D. Parmeswaran, S. Ganesan and T. K. Chandrashekar (2002) Porphyrin in photodynamic therapy—A search for ideal photosensitizers. *Curr. Med. Chem.—Anti Cancer Agents* **2**, 187–207.
7. Bonnett, R. (1995) Photosensitizers of the porphyrin and phthalocyanine series for photodynamic therapy. *Chem. Soc. Rev.* **24**, 19–33.
8. Bonnett, R. (2000) *Chemical Aspects of Photodynamic Therapy*. Gordon and Breach Science Publishers, Amsterdam.
9. Roger, A., C. Kelty, N. Brown and M. Reed (2001) The history of photodetection and photodynamic therapy. *Photochem. Photobiol.* **74**, 656–669.
10. Berenbaum, M., R. Bonnett and E. Chevretton (1993) Selectivity of meso-tetra- (hydroxyphenyl) porphyrins and chlorins and of photofrin II in causing photodamage in tumour, skin, muscle and bladder. *Lasers Med. Sci.* **8**, 235–243.
11. Elms, J., P. N. Beckett, P. Griffin and A. D. Curran (2001) Mechanisms of isocyanate sensitisation. An in vitro approach. *Toxicol. In Vitro* **15**, 631–634.
12. Kevin, P. R., P. G. Penketh, K. Shyam and A. C. Sartorelli (2005) Differential inhibition of cellular glutathione reductase activity by isocyanates generated from the antitumor prodrugs Cloretazine™ and BCNU. *Biochem. Pharmacol.* **69**, 1463–1472.
13. Sobral, M. C. C. M., A. J. F. N. Sobral, J. T. Guthrie and M. H. Gil (2008) Ketotifen controlled release from cellulose acetate propionate and cellulose acetate butyrate membranes. *J. Mater. Sci. Mater. Med.* **19**, 677–682.
14. Silva, M. R., A. M. Beja, J. A. Paixão, A. J. F. N. Sobral, L. M. L. Cabral and A. M. d'. A. R. Gonsalves (2003) R_4^4 (30) rectangular rings in 2,5-dioxopiperazine-1,4-diacetic acid. *Acta Crystallogr., Sect. C: Cryst. Struct. Commun.* **59**, o562–o563.
15. Silva, M. R., A. M. Beja, J. A. Paixão, A. J. F. N. Sobral, L. M. L. Cabral, S. H. Lopes and A. M. d'A. R. Gonsalves (2002) Crystal structure of iminodiacetic acid monomethyl ester monohydrate, $C_5H_9NO_4{\cdot}H_2O$. *Z. Kristallogr. NCS* **217**, 535–536.
16. Sobral, A. J. F. N., S. Eléouet, N. Rousset, A. M. d'A. R. Gonsalves, O. Le Meur, L. Bourré and T. Patrice (2002) New sulfonamide and sulfonic ester porphyrins as sensitizers for photodynamic therapy. *J. Porphyr. Phthalocyanines* **6**, 456–462.
17. Santos, A., A. M. Rodrigues, A. J. F. N. Sobral, P. V. Monsanto, W. L. C. Vaz and M. J. Moreno (2009) Early events in photodynamic therapy: Chemical and physical changes in a POPC: cholesterol bilayer due to hematoporphyrin IX-mediated photosensitization. *Photochem. Photobiol.* **85**, 1409–1417.
18. Seixas de Melo, J., A. J. F. N. Sobral, A. M. d'A. Rocha Gonsalves and H. D. Burrows (2005) Singlet and triplet energy transfer in a bichromophoric system with anthracene covalently linked through sulfonamide to a *meso*-tetraphenylporphyrin. *J. Photochem. Photobiol. A Chem.*, **172**, 151–160.
19. Cretu, C., C. Bucovicean, I. Armeanu, A. M. Lacrama and E. Fagadar-Cosma (2008) Synthesis and spectroscopic characterization of meso-tetra (3-hydroxyphenyl)porphyrin. *Rev. de Chim.* **59**, 979–981.
20. Baker, J. W. and J. Gaunt (1949) The mechanism of the reaction of aryl isocyanates with alcohols and amines. Part II. The base-catalysed reaction of phenyl isocyanate with alcohols. *J. Chem. Soc.*, 9–18.
21. Murov, S. L., I. Carmichael and G. L. Hug (1993) *Handbook of Photochemistry*, 2nd edn. Marcel Dekker, Inc., New York.
22. Kuhn, H. J., S. E. Braslavsky and R. Schmidt (2004) Chemical actinometry (IUPAC Technical Report). *Pure Appl. Chem.* **76**, 2105–2146.
23. Carmichael, J., W. G. DeGraff, A. F. Gazdar, J. D. Minna and J. B. Mitchell (1987) Evaluation of tetrazolium-based semiautomated assay: Assessment of chemosensitivity testing. *Cancer Res.* **47**, 936–942.
24. Charlesworth, P., T. G. Truscott, D. Kessel, C. J. Medforth and K. M. Smith (1994) Photophysical studies of substituted porphyrins. *J. Chem. Soc. Faraday Trans.* **90**, 1073–1076.
25. Bonnett, R., P. Charlesworth, B. D. Djelal, S. Foley, D. J. McGarvey and T. G. Truscott (1999) Photophysical properties of 5,10,15,20-tetrakis(m-hydroxyphenyl)porphyrin (m-THPP), 5,10,15,20-tetrakis(m-hydroxyphenyl)chlorin (m-THPC) and 5,10,15,20-tetrakis(m-hydroxyphenyl)bacteriochlorin (m-THPBC): A comparative study. *J. Chem. Soc. Perkin Trans.* **2**, 325–328.
26. Bensasson, R. V., E. J. Land and T. G. Truscott (1993) *Excited States and Free Radicals in Biology and Medicine: Contributions from Flash Photolysis and Pulse Radiolysis*. Oxford University Press, Oxford.
27. Fonseca, S. M., J. Pina, L. G. Arnaut, J. Seixas de Melo, H. D. Burrows, N. Chattopadhyay, L. Alcácer, A. Charas, J. Morgado, A. P. Monkman, U. Asawapirom, U. Scherf, R. Edge and S. Navaratnam (2006) Triplet state and singlet oxygen formation in fluorene based alternating copolymers. *J. Phys. Chem. B* **110**, 8278–8283.
28. Tanielian, C. and C. Wolff (1995) Porphyrin-sensitized generation of singlet molecular oxygen: Comparison of steady-state and time-resolved methods. *J. Phys. Chem.* **99**, 9825–9830.

29. Harriman, A. (1980) Luminescence of porphyrins and metalloporphyrins. *J. Chem. Soc. Faraday Trans.* **1**(76), 1978–1985.
30. Darmanyan, A. P. and C. S. Foote (1993) Solvent effects on singlet oxygen yield from n,Pi* and Pi,Pi* triplet carbonyl compounds. *J. Phys. Chem.* **97**, 5032–5035.
31. Abdel-Shafi, A. A. and F. Wilkinson (2000) Charge transfer effects on the efficiency of singlet oxygen production following oxygen quenching of excited singlet and triplet states of aromatic hydrocarbons in acetonitrile. *J. Phys. Chem. A* **104**, 5747–5757.
32. Schmidt, R. (2006) Photosensitized generation of singlet oxygen. *Photochem. Photobiol.* **82**, 1161–1177.
33. Wilkinson, F. and J. G. Brummer (1981) Rate constants for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution. *J. Phys. Chem. Ref. Data* **10**, 809–999.
34. Flors, C. and S. Nonell (2001) On the phosphorescence of 1*H*-phenalen-1-one. *Helv. Chim. Acta* **84**, 2533–2539.
35. Kramer-Marek, G., C. Serpa, A. Szurko, M. Widel, A. Sochanik, M. Snietura, P. Kus, R. M. D. Nunes, L. G. Arnaut and A. Ratuszna (2006) Spectroscopic properties and photodynamic effects of new lipophilic porphyrin derivatives: Efficacy, localisation and cell death pathways. *J. Photochem. Photobiol. B, Biol.* **84**, 1–14.
36. McLean, A. J., D. J. McGarvey, T. G. Truscott, C. R. Lambert and E. J. Land (1990) Effect of oxygen-enhanced intersystem crossing on the observed efficiency of formation of singlet oxygen. *J. Chem. Soc. Faraday Trans.* **86**, 3075–3080.
37. Wilkinson, F., W. P. Helman and A. B. Ross (1995) Rate constants for the decay and reactions of the lowest electronically excited singlet state of molecular oxygen in solution. An expanded and revised compilation. *J. Phys. Chem. Ref. Data* **24**, 663–1021.
38. You, Y., S. L. Gibson, R. Hilf, S. R. Davies, A. R. Oseroff, I. Roy, T. Y. Ohulchanskyy, E. J. Bergey and M. R. Detty (2003) Water soluble, core-modified porphyrins. 3. Synthesis, photophysical properties, and in vitro studies of photosensitization, uptake, and localization with carboxylic acid-substituted derivatives. *J. Med. Chem.* **46**, 3734–3747.