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Water-soluble 1,3-diphenylisobenzofuran derivatives. Synthesis and evaluation as singlet molecular oxygen acceptors for biological systems

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Abstract

Two isomeric water-soluble 1,3-diphenylisobenzofuran derivatives, containing an *m*- or *p*-trimethylammonium iodide group in one of the phenyl substituents (***m*-4** and ***p*-4** respectively), were prepared and characterized, and their ability to quench $O_2(^1\Delta_g)$ was evaluated in both homogeneous aqueous media and dioctadecyldimethylammonium chloride (DODAC) vesicles. The solubility in water of each isomer was higher than 2×10^{-4} M and the respective $O_2(^1\Delta_g)$ total quenching rate constants (k_q) were $(2.3\pm0.2)\times10^9$ and $(1.1\pm0.1)\times10^9$ M^{-1} s^{-1}. The reactive pathway fraction for isomer ***m*-4** in D_2O was 0.72 ± 0.10, a value obtained by comparison with unsubstituted 1,3-diphenylisobenzofuran in the system cetyltrimethylammonium chloride–D_2O. Both quenchers ***m*-4** and ***p*-4** can be used with $O_2(^1\Delta_g)$ generators, e.g. thionine and methylene blue, but not with anionic dyes, such as rose bengal, because of the formation of ground state, ion pair complexes. When methylene blue and isomer ***p*-4** were separated by a DODAC bilayer, the quencher was consumed with zero-order kinetics, but the consumption rate was much smaller than in the absence of the bilayer. A rough estimate indicates that the quencher ***p*-4** reacts with approximately 25% of the generated $O_2(^1\Delta_g)$ escaping from the vesicle.

Keywords: 1,3-Diphenylisobenzofuran derivatives; Singlet molecular oxygen acceptors

1. Introduction

Singlet molecular oxygen ($O_2(^1\Delta_g)$) plays an important role in a number of processes of biological interest, including those involved in the photoinduced damage of tissues and the phototherapy of cancer [1–3]. The quantitative analysis of these events requires an understanding of the reactivity of $O_2(^1\Delta_g)$ towards different acceptors in systems in which gradients can occur in both the local concentration of $O_2(^1\Delta_g)$ and its reactivity. Therefore it is of interest to study the influence of the characteristics of the lipid bilayers on the reactivity of the acceptors incorporated into them, as well as the relationship between the acceptor localization and its vulnerability to $O_2(^1\Delta_g)$.

The unambiguous detection of $O_2(^1\Delta_g)$ in biological processes using acceptors or quenchers is a difficult task as the best compounds for use are insoluble in water or non-specific [4,5]. It is usually necessary to perform complementary experimental tests in order to eliminate the participation of OH^-, O_2^- and H_2O_2. Other methods, such as emission analysis and the study of the influence of 1,4-diazabicyclo[2.2.2]octane (DABCO) and D_2O, can help. The use of fast response detectors with near-IR sensitivity allows the direct detection of the presence of $O_2(^1\Delta_g)$ by analysing its weak emission at 1268 nm [6]. This ultrasensitive method is very important for the analysis of $O_2(^1\Delta_g)$ in water solutions of biological systems [7]. With this technique, Khan et al. [8] detected, for the first time, the $O_2(^1\Delta_g)$ emission from an enzymatic reaction. However, chemical trapping with water-soluble acceptors remains the easiest method for current laboratory work: rubrene-2,3,8,9-tetracarboxylate [9], cholesterol [10], guanosine [11] and salts of anthracene-9,10-di(ethanesulphonic acid) [12] have been suggested as water-soluble traps, with some limitations.

The ability of $O_2(^1\Delta_g)$ to permeate lipid bilayers can be studied using a water-soluble acceptor with zero-order kinetics for the quenching of all $O_2(^1\Delta_g)$ entering or leaving the bilayer. In a previous study [13], a probe which did not reach zero-order kinetics was used with this purpose, but yielded much less information than expected, because the interpre-

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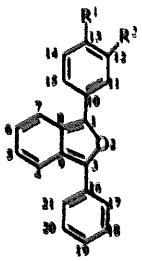
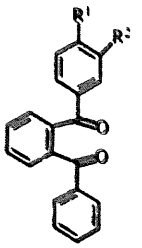
tation of the results was based on differences between stationary states. In order to allow for use in microheterogeneous aqueous systems, the desirable acceptor must be charged, positively if possible, so as to be rejected by the biological membrane.

One of the best (if not the best) singlet oxygen acceptors in organic solvents is 1,3-diphenylisobenzofuran (DPBF, **1**; Scheme 1). Its sensitized or auto-sensitized oxygenation gives rise to an unstable endoperoxide (ozonide) produced by [4+2] cycloaddition of $O_2(^1\Delta_g)$ to the diene system in the heterocycle. This endoperoxide has been isolated working at low temperature, and forms *o*-dibenzoylbenzene in solution at room temperature [14,15]. The disappearance of DPBF and the formation of *o*-dibenzoylbenzene have been used as diagnostic tests for the presence of $O_2(^1\Delta_g)$ in both chemical and biological processes [16] for the following reasons: (a) DPBF reacts rapidly with $O_2(^1\Delta_g)$ (rate constants of 7×10^8 M^{-1} s^{-1} in benzene at 25 °C [17] and 8×10^8 M^{-1} s^{-1} in methanol [18]); (b) DPBF does not react with ground state (triplet) molecular oxygen nor with superoxide anion; (c) the only reaction of DPBF with $O_2(^1\Delta_g)$ is chemical [19]. However, it must be pointed out that DPBF can be converted into *o*-dibenzoylbenzene by dark oxidation with some oxidizing agents or by auto-oxidation [20,21]. The disappearance of DPBF in solution (λ_{max} in methanol, 410 nm; $\epsilon = 22\,200$ M^{-1} cm^{-1}) can be easily monitored by absorption spectroscopy.

In order to take advantage of the well-known ability of DPBF as a singlet oxygen acceptor and, at the same time, to make its use in aqueous solution viable, we report the synthesis of two water-soluble DPBF derivatives containing an *m*- or *p*-trimethylammonium iodide group in one of the phenyl substituents (***m*-4** and ***p*-4** respectively) and their evaluation as $O_2(^1\Delta_g)$ quenchers. We also include preliminary results on the application of these water-soluble acceptors to the determination of the capacity of $O_2(^1\Delta_g)$ to permeate lipid membranes.



	R^1	R^2
1 (DPBF)	H	H
p-2	NO_2	H
m-2	H	NO_2
p-3	NH_2	H
m-3	H	NH_2
p-4	$N^+Me_3I^-$	H
m-4	H	$N^+Me_3I^-$



	R^1	R^2
p-5	NO_2	H
m-5	H	NO_2

Scheme 1.

2. Experimental details

2.1. General

Analytical thin layer chromatography (TLC) was carried out on Merck 60 F_{254} silica gel plates (0.2 mm layer); flash column chromatography was performed on SDS (France) silica gel 60 (230–400 mesh). Melting points (not corrected) were determined on a Kofler-Reichert micro-hot stage apparatus. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were run on Varian-Unity (300 MHz) and Varian-Gemini (200.6 MHz) spectrometers. IR spectra were obtained on a Perkin–Elmer 681 or a Fourier transform Nicolet instrument. UV–visible spectra were recorded on a Perkin–Elmer Lambda 5 spectrophotometer. Mass spectra (MS) were recorded on a Hewlett-Packard 5988-A instrument using: (a) fast atom bombardment (FAB) analysis with a Capillaritron Frasor, Xe as inert gas and either 1,4-dithiothreitol/dithioerithritol or nitrobenzyl alcohol as matrix; (b) direct insertion probes chemical ionization (DIP-CI), with NH_3 as reagent gas. Fluorescence spectra were recorded on a Perkin–Elmer LS 50 spectrofluorometer using air-saturated solutions with excitation at the absorption maximum (maximum absorbance, 0.1). All spectroscopic measurements were performed at room temperature.

2.2. Materials

All solvents used in the synthesis, absorption and fluorescence measurements were of reagent grade quality, and were purified further by the usual procedures [22]. DPBF and rose bengal (RB) (both from Aldrich) were used as received. Methylene blue hydrochloride (MB) (Bayer) was dried in an oven at 100 °C to constant weight; λ_{max}(water) = 664 nm, $\epsilon = 64\,800$ M^{-1} cm^{-1}, quantum yield of $O_2(^1\Delta_g)$ production (Φ_Δ) in water = 0.39 [23]. Thionine hydrochloride (TH) (Panreac) was purified by washing with boiling ethyl acetate in a Soxhlet for several days [24]. The resulting product showed maximum absorption in water at 598 nm ($\epsilon = 42\,400$ M^{-1} cm^{-1}) and Φ_Δ in dimethylformamide = 0.71. Bilirubin IXα (BR) (Janssen) was used as received. Its extinction coefficient in water–dimethylsulphoxide (DMSO) (3:2, v/v) was estimated as 49 100 M^{-1} cm^{-1} by the formula $\epsilon_{mix} = [\epsilon_{water} \times$ (molar fraction of water) $+ \epsilon_{DMSO} \times$ (molar fraction of DMSO)].

2.3. Chemical synthesis

Due to the high sensitivity of DPBF and the derivatives described to photoinduced oxidation, reactions and sample manipulations were carried out in red dim light and under argon.

2.3.1. 1-(p-Nitrophenyl)-3-phenylisobenzofuran (p-2) and its m isomer (m-2)

These compounds were prepared by a modification of a previously described procedure [14] as follows. DPBF (0.9 g, 3.3 mmol) was added with stirring to an ice-cooled solution of $NaNO_3$ (0.34 g) in concentrated H_2SO_4 (135 ml). After 5 min, the solution was poured onto ice and the red precipitate formed was extracted with CH_2Cl_2. The organic solution was successively washed with water, 5% aqueous $NaHCO_3$ and water, dried (Na_2SO_4) and the solvent was vacuum evaporated. TLC analysis (silica gel, hexane–CH_2Cl_2 (2:1)) of the isolated mixture showed three spots with R_f values of 0.6, 0.5 and 0.1. The component with lower R_f was separated by washing with refluxing methanol. It was identified as a mixture of the two dibenzoylbenzene derivatives ***p*-5** and ***m*-5** (250 mg, 27%). The other two products were separated by column chromatography (silica gel; hexane–CH_2Cl_2, gradient from 10:1 to 2:1), yielding ***p*-2** as a deep red solid (345 mg, 33%) (melting point (m.p.) = 165 °C) and ***m*-2** as a red solid (308 mg, 29%) (m.p. = 162–165 °C) (Ref. [14] gives m.p. = 147–148 °C).

2.3.2. 1-(p-Aminophenyl)-3-phenylisobenzofuran (p-3) and its m isomer (m-3)

A solution of the corresponding nitro derivative ***p*-2** or ***m*-2** (40 mg, 0.13 mmol) in 96% ethanol (2 ml) was warmed at 50 °C in a flask equipped with a condenser. Ethanol-wetted 10% Pd/C (catalyst) (a few milligrams), hydrazine hydrate (three drops) and catalyst (a few milligrams) were successively added with stirring at the same temperature. After refluxing for 1 h, the catalyst was separated by filtration through Celite, the filtered solution was boiled for 5 min and warm water (10 ml) was added. The reaction mixture was extracted with CH_2Cl_2, and the subsequent work-up yielded a crude product which was purified by flash chromatography (silica gel, gradient of eluents from hexane–CH_2Cl_2 (1:1) to CH_2Cl_2–methanol (10:1), producing the corresponding purified amines ***p*-3** (37 mg, yield approximately 100%) as an orange solid (m.p. = 80–110 °C (decomp.)) and ***m*-3** (22 mg, 61% yield) as a yellow oil.

2.3.3. 4-(3-Phenylisobenzofuryl)phenyl trimethylammonium iodide (p-4) and its m isomer (m-4)

A solution of the corresponding amine ***p*-3** or ***m*-3** (126 mg, 0.44 mmol), K_2CO_3 (500 mg) and excess methyl iodide (1 ml) in dry acetone (10 ml) was stirred at room temperature for 24 h under argon. The precipitated yellow solid was filtered, washed with acetone and extracted with CH_2Cl_2. The solution was again filtered and the solvent was vacuum evaporated, yielding the corresponding ammonium salts ***p*-4** (65 mg, 32% yield) (m.p. = 177–181 °C) and ***m*-4** (76 mg, yield 37%) (m.p. = 175–178 °C) as yellow solids. They were used without further purification.

The relevant spectroscopic data of the compounds are given in the Appendix.

2.4. Determination of total $O_2(^1\Delta_g)$ quenching rate constants of p-4 and m-4 in aqueous solution

Aqueous solutions containing the quencher ***p*-4** or ***m*-4** (20–200 μM) and the dye TH (10 μM) in Pyrex cuvettes (path length, 10 cm) were bubbled with water-saturated oxygen for 15 min and irradiated with light from a 200 W medium-pressure mercury lamp which was successively filtered through a cylindrical Pyrex cuvette (50 mm × 19 mm internal diameter) containing water and a 570 nm interference filter (Oriel). The spectra of the solutions do not change in the dark or on irradiation under an inert atmosphere for at least 20 min, the maximum irradiation time used. The total quenching rate constants (k_q) were deduced from the absorbance decreases of the solutions at 410 nm, for less than 20% reaction (Fig. 1) [23b]. The reaction rates of each quencher with $O_2(^1\Delta_g)$ (v) were deduced from the slopes of the plots of the absorbance vs. irradiation time (Fig. 2). The slope/intercept ratio of the straight line obtained by plotting $1/v$ vs. the reciprocal of the initial quencher concentration (Fig. 3) is equal to the ratio k_d/k_q, where k_d is the $O_2(^1\Delta_g)$ unimolecular decay rate constant in the absence of quencher (2.5×10^5 s^{-1} in water [25]). The deduced k_q values for ***p*-4** and ***m*-4** were $(1.1 \pm 0.1) \times 10^9$ and $(2.3 \pm 0.2) \times 10^9$ M^{-1} s^{-1} respectively.

The related dye MB can also be used instead of TH. The spectrum of an aqueous solution of MB (10 μM) plus any isomer **4** (40 μM) does not change in the dark, and shows

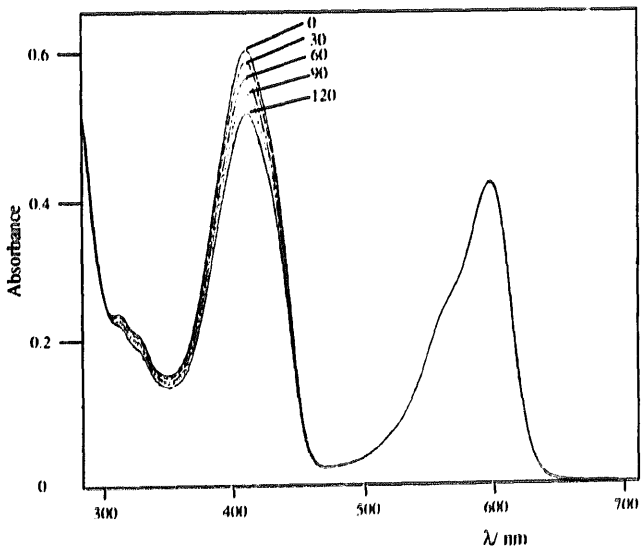


Fig. 1. Spectral changes during the photo-oxidation of the quencher ***m*-4** (40 μM) sensitized by TH (40 μM) in aqueous solution. Reaction times in seconds.

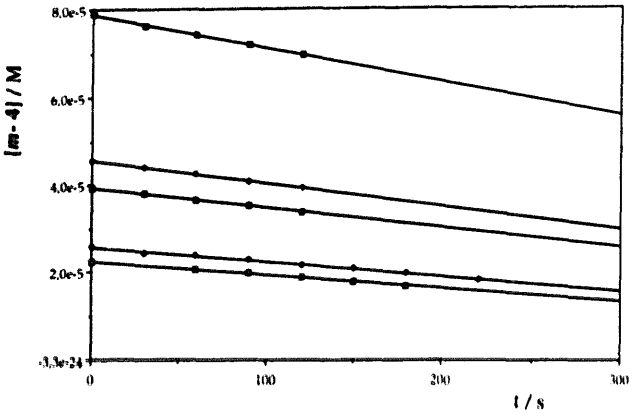


Fig. 2. Concentration changes during the photo-oxidation of aqueous solutions of the quencher *m*-4 at different initial concentrations, sensitized by TH (40 μM).

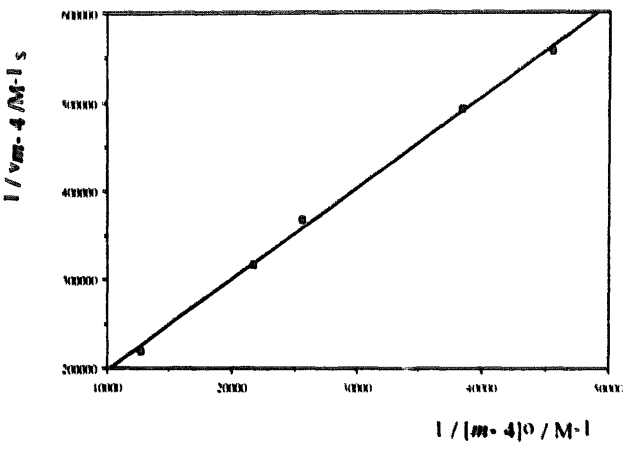


Fig. 3. Plot of the reciprocal of the initial rates of *m*-4 photo- oxidation (v) vs. the reciprocal of the initial quencher concentration, sensitized by TH (40 μM) in aqueous solution.

only small absorbance decreases at 410 nm (always lower than 10%) when irradiated for 20 min under argon.

2.5. Determination of the chemical $O_2(^1\Delta_g)$ quenching rate constant of isomers *m*-4 and *p*-4

In one approach, the kinetics of consumption of *m*-4 in D_2O solution by reaction with singlet oxygen were compared with the kinetics of consumption of DPBF in the same solvent, incorporated into cetyltrimethylammonium chloride (CTAC) micelles (0.1 M in surfactant), employing in both cases MB as sensitizer. The light from a W filament lamp filtered through a yellow glass filter (cutting out light at wavelengths shorter than 450 nm) was used for irradiation. The consumption of DPBF and *m*-4 was followed by the decrease in absorption of the sample measured at 418 nm (DPBF) and 410 nm (*m*-4). No significant bleaching of the sensitizer was observed during irradiation.

In another approach, an alternative procedure was used, adapted from the competitive method reported previously [26]. Two oxygen-saturated solutions in water–DMSO (3:2, v/v), one containing the dye TH (10 μM) plus *p*-4 (5 μM) and the other with the same dye concentration plus BR (5 μM), were irradiated as described above for the determination of k_q. Quencher disappearances were deduced from the absorbance decreases at 410 and 453 nm respectively. From the ratio of the respective quenching rates $v_{p\text{-}4}/v_{BR}$ (equal to 2.5), obtained for less than 20% reaction, the chemical quenching rate constant for the acceptor *p*-4 ($k_c^{p\text{-}4}$) can be deduced as follows. The rate of disappearance of any quencher Q (v_Q) is given by expression (1), where v_Δ is the rate of $O_2(^1\Delta_g)$ generation and $[Q]^0$ is the initial quencher concentration

$$v_Q = v_\Delta k_c[Q]^0/\{(k_c+k_p)[Q]^0+k_d\} \quad (1)$$

In the case of two quenchers such as *p*-4 and BR, the ratio of the rates $v_{p\text{-}4}/v_{BR}$ can be expressed as

$$\frac{v_{p\text{-}4}}{v_{BR}} = \frac{(k_c^{p\text{-}4}[p\text{-}4]^0[\{(k_c^{BR}+k_p^{BR})[BR]^0\}+k_d])}{(k_c^{BR}[BR]^0[\{(k_c^{p\text{-}4}+k_p^{p\text{-}4})[p\text{-}4]^0\}+k_d])} \quad (2)$$

where k_p is the physical quenching rate constant. Under our experimental conditions, the initial concentration of each quencher was the same ($[p\text{-}4]^0 = [BR]^0 = 5$ μM), and a $k_c^{p\text{-}4}$ value of 0.55×10^9 M^{-1} s^{-1} was deduced from expression (2); hence the deduced $k_p^{p\text{-}4}$ value is 0.55×10^9 M^{-1} s^{-1}, i.e. the difference between $k_q^{p\text{-}4}$ and $k_c^{p\text{-}4}$.

In this calculation, two assumptions were made: (a) the values of k_p^{BR} and k_c^{BR} in the 3:2 water–DMSO mixture are the same as in $CHCl_3$–methanol (9:1, v/v) (1.08×10^9 and 0.22×10^9 M^{-1} s^{-1} respectively [26]); (b) k_d in the same water–DMSO mixture is the same as in water (2.5×10^5 s^{-1} [25]). However, a value of k_d of 5.2×10^4 s^{-1} has been reported for the decay rate constant of singlet oxygen in DMSO [27]. When this value is used to evaluate k_d in 3:2 water–DMSO using the known k_d value in each solvent and the mole fraction χ of each solvent in the mixture, with the expression $k_d^{mixture} = \chi^{water}k_d^{water} + \chi^{DMSO}k_d^{DMSO}$, a k_d value of 2.21×10^5 s^{-1} is obtained, i.e. only about 10% lower than the described value of k_d^{water}. Indeed, the precise value of k_d (2.5×10^5 vs. 2.21×10^5 s^{-1}) employed is not critical because, at the initial concentrations of *p*-4 and BR used, the terms $(k_c^{BR}+k_p^{BR})[BR]^0$ and $(k_c^{p\text{-}4}+k_p^{p\text{-}4})[p\text{-}4]^0$ in expression (2) are two orders of magnitude lower than k_d, and therefore an approximate form of Eq. (2) is $v_{p\text{-}4}/v_{BR} \approx k_c^{p\text{-}4}/k_c^{BR}$, i.e. the ratio of the observed rates is roughly independent of k_d.

2.6. Evaluation of *p*-4 as an $O_2(^1\Delta_g)$ quencher in a vesicular medium

The vesicles were prepared by injecting a 20 mM $CHCl_3$ solution of dioctadecyldimethylammonium chloride (DODAC) (5 ml; flow rate, 0.27 ml min^{-1}) onto a 0.77 mM aqueous solution of the dye MB (10 ml) at 73 °C and under a nitrogen atmosphere. The residual MB in the aqueous phase was removed by passing the mixture through a Sephadex G-25 column (flow rate, 0.4 ml min^{-1}) at 14 °C. Acceptor *p*-4

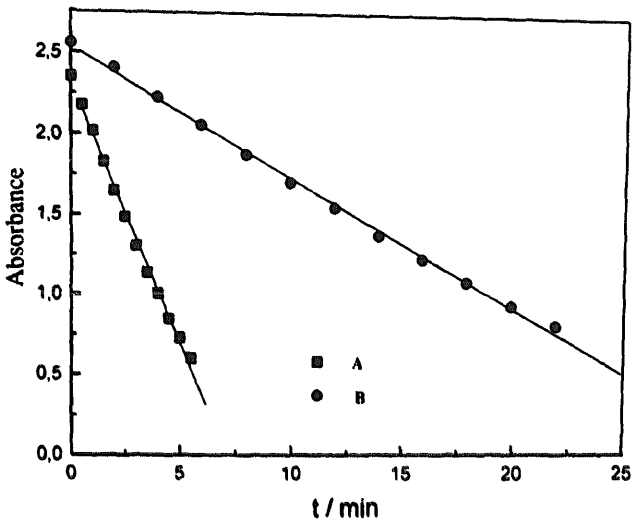


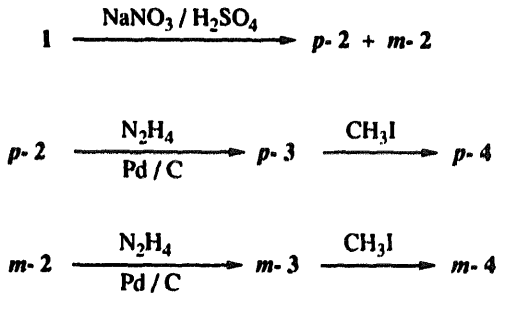
Fig. 4. Absorption changes during the photo-oxidation of *p*-4 sensitized by MB: (A) in aqueous solution; (B) in aqueous solution with the dye within DODAC vesicles.

was then added, and the solutions, with an approximate pH of 5.8 (no buffer was used), were irradiated with light from a W filament lamp, filtered through a 500 nm cut-off filter. Control experiments were carried out in the absence of vesicles, using an equivalent concentration of MB. In these experimental conditions, irradiation of the acceptor in the absence of MB, or in its presence but under an inert atmosphere, produced negligible spectral changes. The results are shown in Fig. 4.

3. Results and discussion

3.1. Synthesis of water-soluble DPBF derivatives

DPBF (1) and its derivatives have been obtained previously by the reduction of *o*-dibenzoylbenzenes [28–30]. However, the use of the direct nitration of DPBF to introduce an *m*-nitro group in one of the two phenyl substituents has also been described [14]. This reaction has been used in this study to obtain two water-soluble DPBF derivatives containing a trimethylammonium group, following the approach shown in Scheme 2, with an approximate overall yield of 10%.



Scheme 2.

Nitration of DPBF under the described conditions [14] gave rise to an approximate 1:1 mixture of *p*-nitro and *m*-nitro derivatives (*p*-2 and *m*-2 respectively) (62% overall yield), as well as the corresponding oxidation products *p*-5 and *m*-5 (27% overall yield). Pure isomers *p*-2 and *m*-2, isolated by column chromatography, were reduced to the corresponding amines *p*-3 and *m*-3 with hydrazine hydrate in the presence of Pd/C, as described for the reduction of other aromatic nitro compounds. Finally, exhaustive methylation of each amine [31,32] yielded the corresponding ammonium salts *p*-4 and *m*-4, whose solubility in water is at least 2×10^{-4} M, sufficient for use as $O_2(^1\Delta_g)$ quenchers in this medium.

3.2. Evaluation of *p*-4 and *m*-4 as $O_2(^1\Delta_g)$ quenchers in homogeneous aqueous solution

TH and MB were selected as photosensitizers for the production of $O_2(^1\Delta_g)$ in aqueous solution in the presence of acceptors *p*-4 or *m*-4 because solutions containing any dye–acceptor couple are stable in the dark or on visible irradiation in the absence of oxygen, at least under the experimental conditions used here. The total quenching rate constants k_q for both isomers were deduced from the time dependence of the acceptor disappearance, under steady state conditions [23b], when oxygen-saturated solutions of the mixtures TH–*p*-4 and TH–*m*-4 were irradiated with 570 nm light (see Section 2.4). The k_q values obtained for *p*-4 and *m*-4 (1.1×10^9 and 2.3×10^9 M^{-1} s^{-1} respectively) are similar to those previously reported for the parent compound DPBF in methanol or acetonitrile [27] ($(1.1\pm0.4)\times10^9$ M^{-1} s^{-1}), indicating that the presence of a trimethylammonium group in the DPBF molecule does not influence its behaviour as an $O_2(^1\Delta_g)$ quencher.

RB, eosin and other anionic dyes with high efficiencies as $O_2(^1\Delta_g)$ generators could not be employed due to the existence of ground state reactions with the quenchers *p*-4 and *m*-4. Thus the visible absorption maximum at 548 nm of a 3 μM aqueous solution of RB is shifted to 570 nm in the presence of excess (40 μM) of either isomer of 4. This is interpreted as the result of changes in the microenvironment of the polycyclic aromatic system of the dye by the formation of a dye–quencher ion pair complex, which is a well-known reaction in aqueous media between anionic dyes and non-symmetric quaternary ammonium compounds [33]. The reaction was not observed in methanol solution.

The involvement of $O_2(^1\Delta_g)$ in the sensitized photo-oxidation of *p*-4 and *m*-4 in the presence of TH was confirmed by irradiation of their D_2O solutions. Initial rates of acceptor disappearance were about nine times higher than those observed in H_2O. These are the expected increases resulting from the deuterium effect on the $O_2(^1\Delta_g)$ lifetime (Kearns effect), assuming that the physical and chemical quenching rate constants k_p and k_c for both quenchers, and the quantum yield of $O_2(^1\Delta_g)$ generation of TH, are the same in both solvents [34]. The photo-oxidation inhibition in the presence

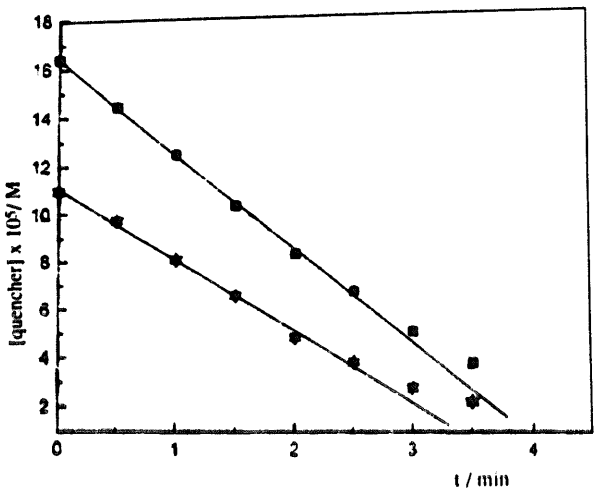


Fig. 5. Consumption of DPBF (squares) and *m*-4 (stars) during aerobic irradiation at 21 °C of solutions in D_2O in the presence of 2×10^{-5} M MB and, in the case of DPBF, 0.1 M CTAC.

of sodium azide, another classical method for checking the involvement of $O_2(^1\Delta_g)$, could not be employed, as dark reactions were observed with both isomers of 4.

The rate constant k_q for a quencher includes the physical and chemical quenching rate constants k_p and k_c. In the case of the parent compound DPBF, it has been reported [18,19,23a] that k_c is at least ten times higher than k_p in all solvents considered. In this work, the contribution of the chemical reaction to the total $O_2(^1\Delta_g)$ quenching by isomer *m*-4 was deduced by two different methods. In one method, the kinetics of consumption of *m*-4 in solution by singlet oxygen were compared with the kinetics of consumption of DPBF incorporated into micelles. D_2O was employed as solvent in order to increase the singlet oxygen lifetime, allowing its total trapping by the quenchers. Under these conditions, zero-order kinetics were obtained (Fig. 5) for both *m*-4 in solution and DPBF incorporated into CTAC micelles. The micelles were introduced in order to allow solubilization of DPBF in the aqueous phase. At the concentrations employed, the mean occupation number of DPBF in the micelles was below unity, avoiding its consumption in a chain reaction [35].

Under conditions of zero-order kinetics, the quantum chemical yield of *m*-4 ($\Phi_{m\text{-}4}$) can be obtained directly by

$$\Phi_{m\text{-}4} = \Phi_{DPBF}(d[m\text{-}4]/dt)/(d[DPFB]/dt) \quad (3)$$

This relationship assumes that the bleaching of both *m*-4 and DPBF is a zero-order process, and that the singlet oxygen production rate is the same in both systems. The latter condition was achieved by working at the same sensitizer concentration and employing micelles from which the dye is excluded due to coulombic repulsion. The data given in Fig. 5, together with a value of unity for DPBF consumption in the micellar assembly, indicate that the reactive pathway fraction is 0.72 ± 0.10, i.e. that *m*-4 can be used as a good chemical quencher for $O_2(^1\Delta_g)$.

In another procedure, we compared, under identical experimental conditions, the quenching kinetics of *p*-4 and BR (a quencher with known k_p and k_c values of the same order as those expected for *p*-4 (see Section 2.5)). However, the BR solubility in water is lower than 10^{-7} M (at pH 7.4 and an ionic strength of 0.1 M [36]), so that a 3:2 water–DMSO mixture was used. In this medium, we estimated a k_c/k_q ratio of 0.5, i.e. 50% of the $O_2(^1\Delta_g)$ quenching by *p*-4 occurs via a photochemical process. The discrepancy between this value and that obtained in the previous, more reliable, procedure (72%) must be due to the estimated k_p^{BR} and k_c^{BR} values used here (see Section 2.5).

3.3. Evaluation of *p*-4 as an $O_2(^1\Delta_g)$ quencher in a vesicular aqueous medium

Previous studies on $O_2(^1\Delta_g)$ processes and dynamics in aqueous micellar systems have been reported by Rodgers and coworkers [37].

In our case, the ability of $O_2(^1\Delta_g)$ to permeate lipid bilayers in an aqueous medium was determined in a series of experiments in which $O_2(^1\Delta_g)$ was produced inside DODAC vesicles by photosensitization with the dye MB. The disappearance with time of the quencher *p*-4, situated in the external aqueous phase, can be used as a measure of the efficiency of $O_2(^1\Delta_g)$ to permeate the membrane vesicle, assuming the same percentage of chemical reaction with the external reagent in both the presence and absence of vesicles. The results shown in Fig. 4 indicate that, although the dye and *p*-4 are separated by the membrane, the quencher is consumed with zero-order kinetics. This provides proof of $O_2(^1\Delta_g)$ migration through the membrane towards the external medium. However, the consumption rate is only approximately 25% of the rate observed during similar photo-oxidation, but in the absence of vesicles. This could be used as an argument to demonstrate that the quencher does not penetrate the vesicle, and that about 25% of the $O_2(^1\Delta_g)$ generated within the liposome pool escapes to the external medium. This value is only a rough estimate for the following reasons: (a) alternative mechanisms of quencher consumption can take place when dye and quencher are not separated by the bilayer; (b) the rate of consumption may be influenced by the formation of MB dimers at the high local concentrations present inside the vesicles. A more detailed study of the $O_2(^1\Delta_g)$ fraction escaping from the vesicle, as well as its dependence on temperature and the presence of additives, is underway.

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5. Appendix

5.1. Spectroscopic data of the synthesized compounds

Nitrocompound p-2. ^{1}H-NMR ($CDCl_3$, δ, ppm, J, Hz): 8.24 (2xdd, 2H, H_{12}, H_{14}; $J=9$ and 1.9), 7.95 (2xdd, 2H, H_{11}, H_{15}, $J=9$ and 1.9), 7.92 (dd, 2H, H_{17}, H_{21}, $J=8.6$ and 1.5), 7.86 (d, 1H, H_7 or H_4, $J=8.6$), 7.79 (d, 1H, H_4 or H_7, $J=8.6$), 7.49 (dt, 2H, H_{18}, H_{20}, $J=7.8$, 8.6 and 1.5), 7.35 (tt, 1H, H_{19}, $J=7.8$ and 1.5), 7.14 (ddd, 1H, H_6 or H_5, $J=8.6$, 6.6 and 1.5), 7.07 (ddd, 1H, H_5 or H_6, $J=8.6$, 6.6 and 1.5). ^{13}C-NMR $CDCl_3$, δ, ppm): 146.9 (C_{13}); 145.1 (C_8); 141.1 (C_9); 137.0 (C_1); 130.7 (C_3); 129.1 (C_{18}, C_{20}); 128.1 (C_{19}); 127.3, 125.6 (C_4, C_7 or C_5, C_6); 125.4 (C_{17}, C_{21}); 124.9 (C_{10}); 124.5 (C_{11}, C_{15}); 123.8 (C_{12}, C_{14}); 122.6 (C_{16}); 120.8, 119.5 (C_5, C_6 or C_4, C_7). IR (KBr, ν, cm^{-1}): 1496, 1330 (NO_2). UV–Vis (CH_2Cl_2), λ_{max}, nm (ϵ, M^{-1} cm^{-1}): 476 (25,000). MS (DIP–CI–NH_3) m/z: 316 (M+1), 333 (M+18). Fluorescence (CH_2Cl_2): λ_{exc} (nm) = 476; λ_{em} (nm) = 458, 482.

Nitrocompound m-2. ^{1}H-NMR ($CDCl_3$, δ, ppm, J, Hz): 8.63 (t, 1H, H_{11}, $J=1.8$ and 2.6), 8.12 (ddd, 1H, H_{13} or H_{15}, $J=8.25$, 1.8 and 1.3), 8.01 (ddd, 1H, H_{15} or H_{13}, $J=8.25$, 2.6 and 1.3), 7.89 (dd, 2H, H_{17}, H_{21}, $J=8.6$ and 1.5), 7.80 (dd, 1H, H_7 or H_4, $J=8.6$ and 1.5), 7.76 (dd, 1H, H_4 or H_7, $J=8.6$ and 1.5), 7.55 (t, 1H, H_{14}, $J=8.25$), 7.48 (dt, 2H, H_{18}, H_{20}, $J=7.8$ and 8.6), 7.32 (tt, 1H, H_{19}, $J=7.8$ and 1.5), 7.61 (ddd, 1H, H_6 or H_5, $J=8.6$, 6.6 and 1.5), 7.01 (ddd, 1H, H_5 or H_6, $J=8.6$, 6.6 and 1.5). ^{13}C-NMR ($CDCl_3$, δ, ppm): 148.8 (C_{12}); 145.4 (C_8); 140.7 (C_9); 132.9 (C_1); 130.9 (C_3); 129.9 (C_{14}); 129.4 (C_{15}); 129.0 (C_{18}, C_{20}); 127.7 (C_{19}); 126.6, 125.3 (C_4, C_7 or C_5, C_6); 125.1 (C_{17}, C_{21}); 123.4 (C_{10}); 122.1 (C_{16}); 120.6 (C_{13}); 120.5, 118.6 (C_6, C_6 or C_4, C_7); 119.2 (C_{11}). IR (KBr, ν, cm^{-1}): 1517, 1339 (NO_2), 1453, 748, 663. UV–Vis (CH_2Cl_2), λ_{max}, nm (ϵ, M^{-1} cm^{-1}): 414 (20,600). MS (DIP–CI–NH_3) m/z: 316 (M+1), 333 (M+18). Fluorescence (CH_2Cl_2): λ_{exc} (nm) = 414; λ_{em} (nm) = 459, 481.

Amine p-3. ^{1}H-NMR ($CDCl_3$, δ, ppm, J, Hz, Hz): 7.90 (d, 2H, H_{17}, H_{21}, $J=8.25$), 7.79 (d, 1H, H_4 or H_7, $J=8.4$), 7.75 (d, 1H, H_7 or H_4, $J=8.4$ Hz), 7.74 (d, 2H, H_{11}, H_{15}, $J=8.25$), 7.45 (t, 2H, H_{18}, H_{20}, $J=8.25$ Hz), 7.26 (t, 1H, H_{19}, $J=8.25$), 6.97 (dd, 1H, H_6 or H_5, $J=8.4$ and 6.4), 6.91 (dd, 1H, H_5 or H_6, $J=8.4$ and 6.4), 6.77 (d, 2H, H_{12}, H_{14}, $J=8.25$), 3.8 (broad, -NH_2). ^{13}C-NMR ($CDCl_3$, δ, ppm): 145.6 (C_{13}); 144.7 (C_{16}); 142.0 (C_{10}); 131.9 (C_3); 131.6 (C_1); 128.8 (C_{18}, C_{20}); 126.3 (C_{11}, C_{15}); 126.2 (C_{19}); 125.1, 124.0 (C_5, C_6 or C_4, C_7); 124.3 (C_{17}, C_{21}); 122.0 (C_8, C_9); 120.5, 119.9 (C_4, C_7 or C_5, C_6); 115.4 (C_{12}, C_{14}). IR (KBr, ν, cm^{-1}): 3445, 3881 (-NH_2), 1488, 756, 692. UV–Vis (CH_2Cl_2), λ_{max}, nm (ϵ, M^{-1} cm^{-1}): 428 (13,600). MS (DIP–CI–NH_3) m/z: 286 (M+1). Fluorescence (CH_2Cl_2): λ_{exc} (nm) = 428; λ_{em} (nm) = 460, 483.

Amine m-3. ^{1}H-NMR ($CDCl_3$, δ, ppm, J, Hz): 7.94 (d, 2H, H_{17}, H_{21}, $J=8.4$), 7.85 (2xd, 2H, H_7, H_4, $J=7.84$), 7.48 (t, 2H, H_{18}, H_{20}, $J=8.4$), 7.37 (d, 1H, H_{15} or H_{13}), 7.30 (t, 1H, H_{19}, $J=8.4$), 7.30–7.20 (2H, H_{11}, H_{14}), 7.01 (m, 2H, H_5, H_6), 6.64 (d, 1H, H_{13} or H_{15}), 3.8 (broad, -NH_2). ^{13}C-NMR ($CDCl_3$, δ, ppm): 146.8 (C_{12}); 144.8 (C_{10}); 143.3 (C_{16}); 132.4 (C_3); 130.6 (C_1); 129.7 (C_{14}); 128.8 (C_{18}, C_{20}); 126.7 (C_{19}); 125.0, 124.9 (C_5, C_6 or C_4, C_7); 124.7 (C_{17}, C_{21}); 122.0 (C_8, C_9); 120.3, 120.0 (C_4, C_7 or C_5, C_6); 115.4 (C_{11} or C_{13}); 114.0 (C_{13} or C_{11}); 111.1 (C_{15}). IR (KBr, ν, cm^{-1}): 3445, 3375 (-NH_2), 1488, 763, 685. UV–Vis (CH_2Cl_2), λ_{max}, nm (ϵ, M^{-1} cm^{-1}): 416 (18,900). MS (DIP–CI–NH_3) m/z: 286 (M+1). Fluorescence (CH_2Cl_2): λ_{exc} (nm) = 416; λ_{em} (nm) = 463, 485.

Ammonium salt p-4. ^{1}H-NMR ($CDCl_3$, δ, ppm, J, Hz): 8.10, 8.01 (2xd, AA'BB', 4H, H_{12}, H_{14}, H_{11}, H_{15}, $J=9.3$ in a 47 mM solution; singlet at 8.05 in the spectrum of a 16 mM solution; the shape and position of these signals strongly depend on the concentration), 7.93 (d, 2H, H_{17}, H_{21}, $J=7.5$), 7.86 (d, 1H, H_4 or H_7, $J=8.7$), 7.78 (d, 1H, H_7 or H_4, $J=8.7$), 7.50 (t, 2H, H_{18}, H_{20}, $J=7.5$), 7.34 (t, 1H, H_{19}, $J=7.5$), 7.13 (dd, 1H, H_6 or H_5, $J=8.7$ and 6.7), 7.06 (dd, 1H, H_5 or H_6, $J=8.7$ and 6.7), 4.05 (s, 9H, -NMe_3). ^{13}C-NMR (DMSO-d_6, δ, ppm): 145.2 (C_{13}), 144.5 (C_8); 141.0 (C_9); 131.8 (C_1); 130.3 (C_3); 129.3 (C_{18}, C_{20}); 127.8 (C_{19}); 126.8, 126.0 (C_4, C_7 or C_5, C_6); 125.2 (C_{17}, C_{21}); 124.9 (C_{11}, C_{15}); 122.8 (C_{16}); 121.6 (C_{10}); 121.5 (C_{12}, C_{14}); 120.3, 119.7 (C_5, C_6 or C_4, C_7); 56.4 (-NMe_3). IR (KBr, ν, cm^{-1}): 1599, 1495, 1451, 763, 685. UV–Vis, λ_{max}, nm (ϵ, M^{-1} cm^{-1}): (H_2O) 411 (18,000), (MeOH), 413 (26,100). MS (NBA/FAB$^+$): 328 (M^+–I). Fluorescence (H_2O): λ_{exc} (nm) = 411; λ_{em} (nm) = 460, 480.

Ammonium salt m-4. ^{1}H-NMR ($CDCl_3$, δ, ppm, J, Hz): 8.1 (s, 1H, H_{11}), 8.05 (d, 1H, H_{13}, $J=8.25$), 7.94 (d, 2H, H_{17}, H_{21}, $J=7.5$), 7.88 (d, 1H, H_7 or H_4, $J=8.7$), 7.81 (d, 1H, H_{15}, $J=8.25$), 7.80 (d, 1H, H_4 or H_7, $J=8.7$), 7.71 (t, H_{14}, $J=8.25$), 7.53 (t, 2H, H_{18}, H_{20}, $J=7.5$), 7.37 (t, 1H, H_{19}, $J=7.5$), 7.21 (dd, 1H, H_6 or H_5, $J=8.7$ and 6.7), 7.09 (dd, 1H, H_5 or H_6, $J=8.7$ and 6.7), 4.11 (s, 9H, -NMe_3). ^{13}C-NMR (DMSO-d_6, δ, ppm): 148.1 (C_{12}); 144.5 (C_8); 141.2 (C_9); 132.1 (C_1); 131.1 (C_{14}); 130.3 (C_3); 129.3 ($C_{18, C20}$); 127.8 (C_{19}); 126.7, 126.1 (C_4, C_7 or C_5, C_6); 125.7 (C_{15}); 124.9 (C_{17}, C_{21}); 122.7 (C_{16}); 121.6 (C_{10}); 120.3, 119.9 (C_5, C_6 or C_4, C_7); 118.7 (C_{13}); 115.8 (C_{11}); 61.6 (-NMe_3). IR (KBr, ν, cm^{-1}): 1595, 1481, 1453, 756, 670. UV–Vis, λ_{max}, nm (ϵ, M^{-1} cm^{-1}): (H_2O) 410 (16,000), (MeOH) 411 (24,400). MS (DTT/DTE/FAB$^+$): 328 (M^+–I). Fluorescence (H_2O): λ_{exc} (nm) = 410; λ_{em} (nm) = 463, 479.

Mixture of isomers p-5 and m-5. ^{13}C-NMR ($CDCl_3$, δ, ppm): 196.1 (C=O); 142.4 (C_{13}); 140.0 (C_9); 139.8 (C_9); 139.1 (C_{10}), 137.2 (C_{16}); 135.5 (C_5, C_6); 133.9 (C_{19}); 131.8–130.4 (C_{17}, C_{21}, C_4, C_7); 130.3 (C_{11} or C_{15}); 130.0 (C_{15} or C_{11}); 129.0 (C_{18}, C_{20}); 124.7 (C_{12} or C_{14}); 124.1 (C_{14} or C_{12}). IR (KBr, ν, cm^{-1}): 1662 (C=O), 1531, 1347 (NO_2), 1279 (C–O), 936, 711. UV–Vis (CH_2Cl_2), λ_{max}, nm (ϵ, M^{-1} cm^{-1}): 251 (23,200). MS (glycerol/FAB$^+$) m/z: 209 (100%), 226 (16%), 254 (30%), 332 (22%, M+1).

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