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A non-singlet oxygen mediated reaction photoinduced by phenalenone, a universal reference for singlet oxygen sensitization†

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Photosensitized oxidations involve the generation of radicals (type I mechanism) and/or the production of singlet molecular oxygen ($^1O_2$) (type II mechanism). Phenalenone (PN) is one of the well-known type II photosensitizers and is widely used as a $^1O_2$ reference sensitizer. In this work, we have studied the interaction of tryptophan (Trp) with the excited states of PN, the production of $^1O_2$ in the presence of Trp, the formation of radicals and the kinetics of the photodegradation of Trp under different experimental conditions. In contrast to what is usually assumed, we have proven that PN is also able to act as a type I photosensitizer. Moreover, we have demonstrated that the predominant mechanism of the photosensitization of Trp by PN involves an electron transfer initiated-process.

Introduction

A photosensitized reaction is defined as a photochemical alteration occurring in one molecular entity as a result of initial absorption of radiation by another molecular entity called a photosensitizer.[1] The biological and medical importance of photosensitized reactions is mostly related to their participation in processes involved in the generation of skin cancer[2,3] and to several applications in disinfection[4,5] and photodynamic therapy (PDT) for the treatment of cancer and other pathologies.[6,7]

The chemical changes in biological components resulting from photosensitized reactions can take place through different mechanisms. Energy transfer from the triplet state of the photosensitizer can generate excited states in the target molecule.[8] Photosensitized oxidations also contribute to biological damage induced by UV radiation. These processes involve the generation of radicals (type I), *e.g.*, *via* electron transfer or hydrogen abstraction, and/or the production of singlet molecular oxygen ($O_2(^1\Delta_g)$, denoted throughout as $^1O_2$) (type II).[9]

Singlet oxygen is an important oxidizing intermediate in chemical processes and one of the main reactive oxygen species responsible for the damaging effects of light on biological systems (photodynamic effects).[10,11] Photosensitization is primarily responsible for the production of $^1O_2$ *in vivo*.[12] In this process, $^1O_2$ is most often produced by energy transfer from the excited triplet state of a sensitizer to dissolved molecular oxygen (O_2). Currently, it is accepted that the photosensitization of proteins occurs mainly through the reactions of $^1O_2$ with tryptophan (Trp), tyrosine, histidine, methionine and cysteine side chains.[13]

Several compounds are used as model photosensitizers for the study of $^1O_2$-mediated reactions. In this context, 1*H*-phenalen-1-one (1*H*-benzonaphthen-1-one or 7-perinaphthenone, denoted throughout as phenalenone (PN)), is a unique photosensitizer due to its quantum yield of $^1O_2$ production (Φ_Δ, defined as the number of $^1O_2$ molecules produced per absorbed quantum of light) close to unity.[14–16] In addition, PN is photostable in most solvents and has a very low ability to deactivate $^1O_2$. These properties have made PN one of the well-known $^1O_2$ photosensitizers.[17] In fact, it is widely used as a reference for the determination of Φ_Δ values and for investigating oxidations of biological compounds by $^1O_2$.

PN is an aromatic ketone whose chemical structure and absorption spectrum are shown in Fig. 1. This compound is present in the environment[18] and some plants produce secondary metabolites containing its skeleton[19] as a response to pathogen attacks. These phenalenone derivatives would act as $^1O_2$-photosensitizers able to use solar energy for defense.[20] The PN photodynamic activity and that of its derivatives have been demonstrated early,[21,22] and it has recently been used as standard for singlet oxygen-mediated damage to DNA[23] and oxidation of amino acids[24] and proteins.[25]

A study using electron paramagnetic resonance (EPR) analysis revealed in the 1960's that radicals are formed when solutions of PN in 2-propanol were exposed to UV radiation

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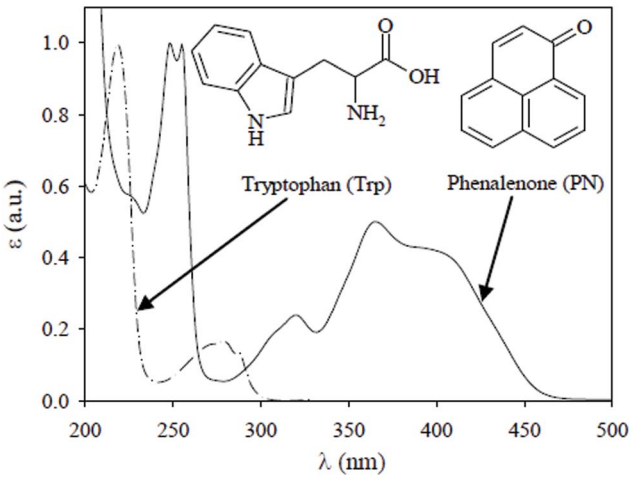

Fig. 1 Molecular structure of PN and Trp, and the corresponding absorption spectra in air-equilibrated aqueous solutions; solid line: PN; dashed-dotted lines: Trp.

(λ > 300 nm).[26] Later, it was demonstrated that, despite low quantum yields, significant photochemical decomposition of PN occurred under steady-state irradiation in air equilibrated 1,4-dioxane and *N*,*N*′-dimethylacetamide, mainly due to hydrogen abstraction from the solvent by its triplet excited state.[27] In 2004, Flors and Nonell demonstrated that the triplet excited state of PN is quenched by the typical electron donor 1,4-diazabicyclo-[2.2.2]octane (DABCO) with a rate constant approaching the diffusion control limit.[28] Moreover, in the same work evidence of the formation of the PN radical anion ($PN^{\bullet -}$) was provided.

Despite the information mentioned in the previous paragraph, to the best of our knowledge, no study on the capability of PN to act as a photosensitizer through electron transfer-initiated reactions has been published up-to now and PN is systematically used as a purely type II photosensitizer. Therefore, we investigated the mechanism of the oxidation of Trp photosensitized by PN. We chose Trp as an oxidizable substrate because it is a compound able to be photooxidized through both mechanisms, it is highly soluble in water and may be easily quantified by chromatographic methods. In this work, we have studied the interaction of Trp with the excited states of PN, the production of $^1O_2$ in the presence of Trp, the formation of radicals and the kinetics of the photodegradation of Trp under different experimental conditions.

Experimental section

General

Tryptophan (Trp) (Sigma Chemical Co) was of the highest purity available (>98%) and was used without further purification. 1*H*-Phenalen-1-one (PN) (perinaphthenone, Merck) was purified as indicated in ref. 14. Other chemicals were also provided by Sigma.

Measurements of pH were performed with a pH-meter PHM220 (Radiometer Copenhagen) combined with a pH electrode pHC2011-8 (Radiometer Analytical). The pH of the aqueous solutions was adjusted by adding very small aliquots (a few μL) of concentrated (0.1–2 M) HCl or NaOH solutions using a micropipette. In experiments using D_2O as a solvent, D_2O (>99.9%; Sigma-Aldrich), DCl (99.5%; Aldrich) in D_2O, and NaOD (Euriso-Top CEA, France) in D_2O were employed.

Singlet oxygen ($^1O_2$) studies

The experiments were carried out at room temperature using D_2O as a solvent since the lifetime of $^1O_2$ (τ_Δ) is much longer in D_2O than in H_2O.[29,30]

Steady-state experiments, equipment 1. The experiments were performed using a NIR PMT Module H10330-45 (Hamamatsu) coupled to a single-photon-counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon).[31] The sample solution (0.8 mL) in a quartz cell (1 cm × 0.4 cm) was irradiated with a CW 450W Xenon source through an excitation monochromator (FL-1004 with a 1200 groove per mm, 330 nm blaze grating). The luminescence in the near-infrared (NIR) region, after passing through an emission monochromator (FL-1004 with a 600 groove per mm, 1000 nm blaze grating), was detected at 90° with respect to the incident beam using the NIR PMT and counted by a FluoroHub-B module. Corrected emission spectra obtained by excitation at 365 nm were recorded between 950 and 1400 nm, and the total integrated $^1O_2$ phosphorescence intensities (I_P) were calculated by integration of the emission band centered at *ca.* 1270 nm.

Steady-state experiments, equipment 2. Singlet oxygen measurements upon continuous monochromatic excitation of the sensitizer were also performed on a custom-built equipment described elsewhere.[32,33] Briefly, the solutions in a quartz cell (1 cm × 1 cm) were irradiated with a Xe–Hg arc lamp (1 kW) through a water filter, focusing optics and a monochromator. The $^1O_2$ emission was collected at right angle with respect to the incident beam using a mirror, chopped, and after passing through a focusing lens and appropriate filters (cut-off at 1000 nm, interference at 1270 nm) was detected using a cooled NIR photomultiplier (Hamamatsu R5509 PMT), a lock-in amplifier and an oscilloscope.

Time-resolved experiments. A phosphorimeter (FL-1040), including a pulsed xenon lamp (0.05–25 Hz, 3 μs at full-width half-maximum) and gating electronics to control the size and temporal displacement of the detection windows, was used. The other optical devices were the same as those described in "Steady-state experiments, equipment 1". In this case the emission at 1270 nm was registered as a function of time.

Steady-state irradiation

Irradiation set-up. Aqueous solutions containing Trp and PN were irradiated in 1 cm path length quartz cells at room temperature with a Rayonet RPR lamp emitting at 350 nm (bandwidth ~20 nm) (Southern N. E. Ultraviolet Co.). The experiments were performed in the presence and absence of dissolved O_2. Experiments with air-equilibrated solutions were performed in open quartz cells without bubbling, whereas argon and oxygen-saturated solutions were obtained by bubbling for 20 min with the corresponding gas (Linde, purity > 99.998%), previously water saturated.

Actinometry. Aberchrome 540 (Aberchromics Ltd) was used as an actinometer for the measurements of the incident photon flux ($q_{p,0}$) at the excitation wavelength. A value of 1.4 ($\pm$0.1) $\times$ 10^{-5} einstein L^{-1} s^{-1} was obtained for $q_{p,0}$ at 350 nm in the irradiation setup described in the previous paragraph. The method for the determination of $q_{p,0}$ has been described in detail elsewhere.[34,35] Values of the photon flux absorbed ($q_{p,a}$) were calculated from $q_{p,0}$ according to the Lambert–Beer law ($q_{p,a} = q_{p,0}(1-10^{-A})$, where A is the absorbance of the reactant at the excitation wavelength).

UV/Visible spectrophotometry. Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer, using quartz cells of 1 cm optical path length. The absorption spectra of the solutions were recorded at regular intervals of irradiation time.

High-performance liquid chromatography. A high-performance liquid chromatograph Prominence from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP and photodiode array detector SPD-M20A) was employed for monitoring the reaction. A Synergi Polar-RP column (ether-linked phenyl phase with polar end capping, 150 $\times$ 4.6 mm, 4 μm, Phenomenex) was used for product separation. Aqueous solutions of $AcNH_4$ (10 mM, pH = 6.5) were used as mobile phase.

Determination of O_2 concentration. The O_2 consumption during irradiation was measured with an O_2-selective electrode (Consort c932). The solutions and the electrode were placed in a closed glass cell of 130 mL.

Detection and quantification of H_2O_2. H_2O_2 was determined by its reaction with 4-aminophenazone and phenol catalyzed by the enzyme peroxidase to yield 4-(*p*-benzoquinone monoimino)-phenazone, which is detected by its absorbance in the visible region.[36,37] This assay has high sensitivity and specificity due to the intense absorbance of the product at 505 nm and the enzymatic catalysis, respectively. The reactants were purchased from Wiener Laboratorios SAIC (cholesterol kit). Briefly, 500 μL of irradiated solution was added to 600 μL of reagent. The absorbance of the resulting mixture at 505 nm was measured after 30 min at room temperature, under conditions of reduced environmental light, using the reagent as a blank. Aqueous H_2O_2 solutions prepared from commercial standards were employed for obtaining the corresponding calibration curves.

Laser flash photolysis

The lifetimes of the PN triplet excited state in Ar- and O_2-saturated and in air-equilibrated D_2O solutions were determined by laser flash photolysis. A Nd:YAG laser (GCR 130-1, pulse width 9 ns, 355 nm) was used for sample irradiation. The absorbance of PN at 355 nm was 0.5. The monitoring system consisted of a 150 W pulsed Xe arc lamp, a R928 photomultiplier and a 05-109 Spectra Kinetics Applied Photophysics monochromator. Signals were digitized by a HP54522A oscilloscope. Samples were irradiated in quartz cells; solutions were over-oxygenated or deoxygenated by continuous bubbling with O_2 or Ar.

Electron paramagnetic resonance-spin trapping experiments

EPR spectra were collected on a Bruker ESP 500E spectrometer. Samples were irradiated with a Rayonet RPR3500 lamp. The following instrumental settings were employed for the measurements: microwave power: 20 mW; field modulation amplitude: 0.1 mT; field modulation frequency: 100 kHz; microwave frequency: 9.77 GHz.

EPR-spin trapping detection. Nitrones are common reagents for the detection and identification of transient radicals due to their ability to form persistent radical adducts that are detectable and finger printable by EPR spectroscopy.[38,39] In our experiments, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) from Sigma was used as spin trap.[40] Samples (1 mL) contained PN ($Abs_{(365\ nm)} = 0.8$), 3 $\times$ 10^{-4} M Trp, buffer Tris/HCl, pH = 7.0 and DMPO (5 $\times$ 10^{-4} M). The irradiation was performed in air-equilibrated and O_2-saturated solutions. Samples in quartz flat cells were exposed to UV-A irradiation (Rayonet RPR lamp, *vide supra*) at room temperature. EPR spectra were recorded at various irradiation times.

Results and discussion

Quenching of $^1O_2$ and of the triplet excited state of PN by Trp

The photosensitized formation of singlet oxygen ($^1O_2$) by 1*H*-phenalen-1-one (PN) involves energy transfer from the triplet excited state of PN (^{3}PN*) to dissolved molecular oxygen ($^3O_2$) (reactions (1) and (2)).

$$PN \xrightarrow{h\nu} {}^1PN^* \xrightarrow{k_{ISC}} {}^3PN^* \quad (1)$$

$$^3PN^* + {}^3O_2 \xrightarrow{k_{et}} PN + {}^1O_2 \quad (2)$$

$^1O_2$ relaxes to its ground state ($^3O_2$) through solvent induced radiationless and radiative pathways (reactions (3) and (4)). It may also be deactivated by a physical quencher (reaction (5)) and/or oxidize an acceptor molecule (reaction (6)).

$$^1O_2 \xrightarrow{k_d^\Delta} {}^3O_2 \quad (3)$$

$$^1O_2 \xrightarrow{k_e^\Delta} {}^3O_2 + h\nu'' \quad (4)$$

$$Q + {}^1O_2 \xrightarrow{k_q^\Delta} Q + {}^3O_2 \quad (5)$$

$$Q + {}^1O_2 \xrightarrow{k_r^\Delta} QO_2 \quad (6)$$

Steady-state and time resolved analyses of $^1O_2$ were performed irradiating PN at 365 nm in D_2O in the presence of various tryptophan (Trp) concentrations and registering the phosphorescence emission of $^1O_2$ in the near-infrared (NIR) region. As can be inferred from the corresponding absorption spectra (Fig. 1), under these experimental conditions, PN was excited whereas Trp did not absorb radiation.

Time-resolved singlet oxygen measurements

In time-resolved experiments, first-order kinetics were observed for the decays of the $^1O_2$ emission, following PN excitation in the presence of different concentrations of Trp. The corresponding $^1O_2$ lifetimes (τ_Δ) were calculated by fitting the emission decay with eqn (7):

$$S(t) = S_i\, e^{-t/\tau_\Delta} \quad (7)$$

where $S(t)$ is the signal registered by the NIR detector (proportional to the $^1O_2$ concentration at a given time t) and S_i is the pre-exponential factor (proportional to the initial $^1O_2$ concentration ($t = 0$, end of flash)). It should be noted that eqn (7) is only valid if the $^1O_2$ lifetime (τ_Δ) is much longer than that of the sensitizer triplet excited state (τ_T) under the experimental conditions used.[41] This is the case for PN in air-equilibrated D_2O solutions: τ_T was measured by laser flash photolysis and found to be approx. 1.7 μs, compared to a τ_Δ value of 59 (±2) μs measured in this work, in good agreement with literature values.[30,31]

In the presence of a quencher (Q), τ_Δ is given by:

$$\tau_\Delta = 1/(k_d^\Delta + k_t^\Delta[Q]) \quad (8)$$

where k_d^Δ ($= 1/\tau_\Delta^0$) is the rate constant of $^1O_2$ deactivation by the solvent (s^{-1}, with $k_e^\Delta \ll k_d^\Delta$, in most solvents (reactions (3) and (4))[42,43] and τ_Δ^0 is the $^1O_2$ lifetime in the absence of Q), k_t^Δ (L mol^{-1} s^{-1}) is the rate constant of $^1O_2$ total (physical and chemical) quenching by Q ($k_t^\Delta = k_r^\Delta + k_q^\Delta$, reactions (5) and (6)). It should be noted that there is no significant $^1O_2$ quenching by the sensitizer itself in the case of PN in the range of concentrations generally used (k_t^Δ [PN] $\ll k_d^\Delta$).[15]

The analysis showed that τ_Δ decreased as a function of the Trp concentration (Fig. 2). This result was expected since Trp is able to deactivate $^1O_2$ by physical quenching (reaction (5)) and to trap $^1O_2$ by chemical reaction (reaction (6)).[44]

According to eqn (8), $^1O_2$ quenching by Trp (Q) should follow a linear Stern–Volmer behavior (eqn (9)).

$$\tau_\Delta^0/\tau_\Delta = 1 + \tau_\Delta^0 k_t^\Delta[Trp] \quad (9)$$

Fig. 2 shows that the corresponding plot (eqn (9)) was linear with a slope $K_{SV} = 2690$ L mol^{-1}, and a value of 4.4 (±0.4) × 10^7 L mol^{-1} s^{-1} was obtained for k_t^Δ by Trp, which is of the same order of magnitude as a previously published value (3.2 × 10^7 L mol^{-1} s^{-1}).[44]

The emission signal at $t = 0$ (S_i, eqn (7)) and, consequently, the amount of $^1O_2$ initially produced by energy transfer from ^{3}PN* to $^3O_2$ (reaction (2)), decreased with the increase of the Trp concentration (Fig. 2a). This fact reveals that, besides quenching by dissolved $^3O_2$ (reaction (2)) and deactivation by intersystem crossing to the ground state (reaction (10)), ^{3}PN* may be quenched by Trp (reactions (11) and (12)). Note that the quenching of the singlet excited state of PN (^{1}PN*) by Trp would also result in a decrease of the ^{3}PN* concentration and consequently in a decrease in the $^1O_2$ production. However, the quenching of ^{1}PN* by Trp can be discarded at the concentrations of Trp used because the lifetime of ^{1}PN* is very short, due to the efficient intersystem crossing to yield ^{3}PN*.[45]

$$^3PN^* \xrightarrow{k_d^T} PN \quad (10)$$

$$^3PN^* + Trp \xrightarrow{k_q^T} PN + Trp \quad (11)$$

$$^3PN^* + Trp \xrightarrow{k_r^T} Products \quad (12)$$

Under these conditions, ^{3}PN* quenching by Trp competes with energy transfer from ^{3}PN* to $^3O_2$ (reaction (2)), and therefore the efficiency of energy transfer (ϕ_{et}) and thus the quantum yield of $^1O_2$ production (Φ_Δ) decreases as the Trp concentration increases (eqn (13)).

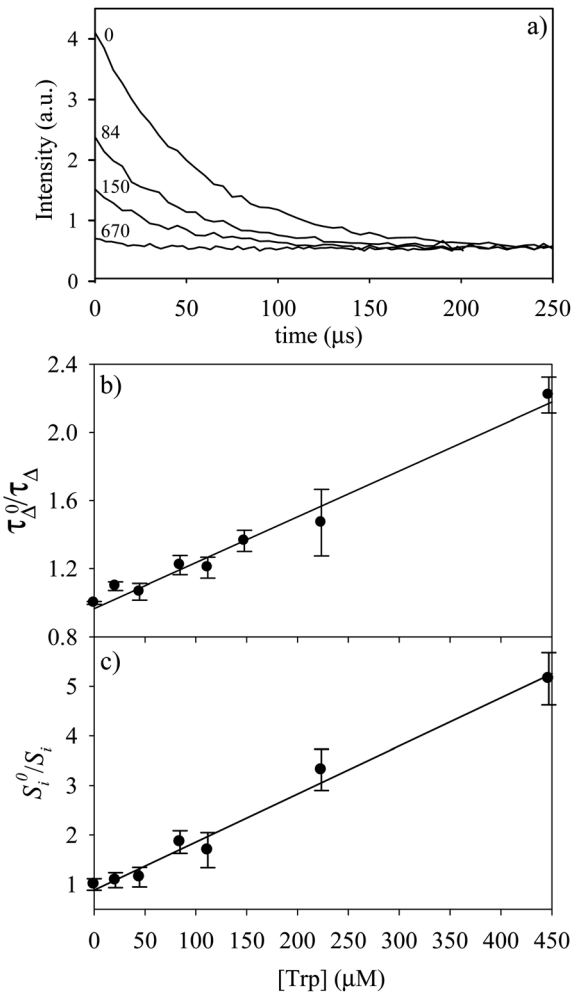

Fig. 2 Time-resolved $^1O_2$ experiments: quenching of $^1O_2$ emission by Trp. Experiments performed in D_2O solutions containing PN and Trp at pD 6.2; excitation wavelength 365 nm, A (365 nm) = 1.07. (a) Selected NIR emission decays in the absence and in the presence of various concentrations of Trp that appear above each decay (μM); (b) Stern–Volmer plot of the $^1O_2$ lifetimes (τ_Δ), calculated analyzing the NIR $^1O_2$ luminescence decays (eqn (7)); (c) Stern–Volmer plot of the initial $^1O_2$ concentration, estimated through the pre-exponential factor (S_i, eqn (7)).

$$\Phi_\Delta = \Phi_T \phi_{et} = \Phi_T \frac{k_{et}[^3O_2]}{(k_d^T + k_{et}[^3O_2] + k_t^T[Trp])} \quad (13)$$

where Φ_T is the quantum yield of ^{3}PN* formation, k_d^T (s^{-1}) is the rate constant of ^{3}PN* deactivation in the absence of quencher (reaction (10)), k_{et} (L mol^{-1} s^{-1}) is the rate constant of the energy transfer from ^{3}PN* to $^3O_2$ (reaction (2)) and k_t^T (L mol^{-1} s^{-1}) is the rate constant of ^{3}PN* total quenching by Trp ($k_t^T = k_q^T + k_r^T$, reactions (11) and (12)).

The ratio of the Φ_Δ values in the absence and in the presence of Trp is thus given by:

$$\frac{\Phi_\Delta^0}{\Phi_\Delta} = \frac{\phi_{et}^0}{\phi_{et}} = 1 + \tau_T^0 k_t^T[Trp] \quad (14)$$

where τ_T^0 is the lifetime of ^{3}PN* in the absence of Trp in the air-equilibrated solution ($\tau_T^0 = \frac{1}{k_d^T + k_{et}[^3O_2]}$). Since the initial $^1O_2$ emission signal (S_i, eqn (7)) is proportional to the initial $^1O_2$ concentration, and therefore to Φ_Δ, the quenching efficiency of ^{3}PN* by Trp may be evaluated from the Stern–Volmer analysis of the variation of S_i as a function of the Trp concentration (eqn (14), with $S_i^0/S_i = \Phi_\Delta^0/\Phi_\Delta$).

In agreement with eqn (14), the Stern–Volmer plot of S_i^0/S_i *vs.* [Trp] was linear (Fig. 2c). Taking into account the τ_T value measured in air-equilibrated D_2O solution (1.7 μs), calculation of k_t^T from the slope of the Stern–Volmer plot (eqn (14)), $K_{SV} = \tau_T^0 k_t^T = 9900$ L mol^{-1} yielded a value of 6 (±1) × 10^9 L mol^{-1} s^{-1}. This value is higher than the rate constant of ^{3}PN* quenching by $^3O_2$ measured in this work by laser flash photolysis (k_{et} = 2.0 (±0.2) × 10^9 L mol^{-1} s^{-1}).

Steady-state singlet oxygen measurements

In another set of experiments, the emission of $^1O_2$ was recorded at a constant PN concentration and different concentrations of Trp (0 to 400 μM) at pD 6.2. The measurements were carried out in independent experiments using equipment 1 and 2, described in the Experimental section. Emission spectra registered with equipment 1 showed a strong decrease in the $^1O_2$ phosphorescence with the increase of the Trp concentration, the wavelength of the emission maximum remaining unchanged (Inset Fig. 3).

The corresponding Stern–Volmer plot of the emission intensity (I_p) (I_p^0/I_p *vs.* [Trp]) was not linear. The same result was observed for the signals registered using equipment 2 (Fig. 3). This behavior is again a consequence of quenching of both $^1O_2$ and ^{3}PN* by Trp. Indeed, under continuous irradiation, I_p is proportional to the steady-state concentration of $^1O_2$ ($[^1O_2]_{ss}$). Taking into account the reactions involved in the production and quenching of $^1O_2$ (eqn (1)–(6)) and applying the quasi-stationary hypothesis to the concentrations of excited states, $[^1O_2]_{ss}$ may be expressed as:

$$[^1O_2]_{ss} = q_{p,a}\Phi_\Delta\tau_\Delta \quad (15)$$

where $q_{p,a}$ (einstein L^{-1} s^{-1}) is the photon flux absorbed by the sensitizer (see Experimental section).

In a given solvent and at a constant concentration of the sensitizer PN (photon flux absorbed by PN constant), a Stern–Volmer analysis of the quenching of I_p by Trp leads to the following relation by combining eqn (9), (14) and (15):

$$\begin{aligned} I_p^0/I_p &= [^1O_2]_{ss}{}^0/[^1O_2]_{ss} = (\Phi_\Delta^0\tau_\Delta^0)/(\Phi_\Delta\tau_\Delta) \\ &= (1 + \tau_\Delta^0 k_t^\Delta[\mathrm{Trp}])\,(1 + \tau_T^0 k_t^T[\mathrm{Trp}]) \end{aligned} \quad (16)$$

where the superscript "0" refers to the values in the absence of Trp in air-equilibrated solutions.

The plot of I_p^0/I_p *vs.* [Trp] can be fitted with a second order polynomial, in agreement with eqn (16). Using the values of the various constants determined by time-resolved measurements, a reasonable fit of the non linear Stern–Volmer plot (I_p^0/I_p) could be obtained (Fig. 3).

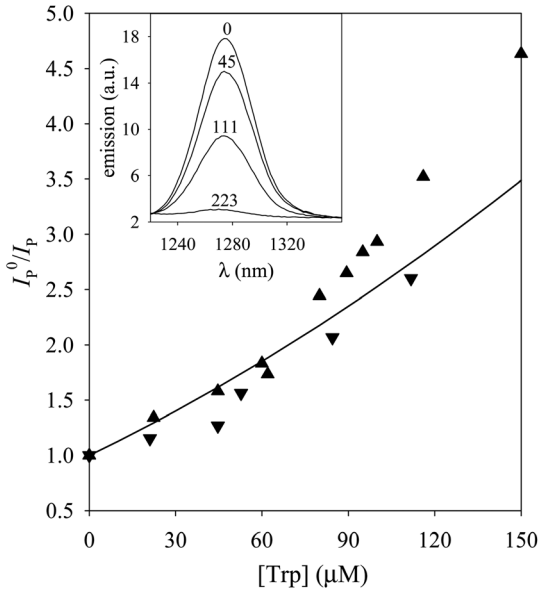

Fig. 3 Steady-state $^1O_2$ experiments. Quenching of the emission of $^1O_2$ by Trp. Experiments performed in D_2O solutions containing PN and Trp at pD 6.2. Stern–Volmer plot of the phosphorescence intensities (I_P); excitation wavelength 365 nm, experiments performed using equipments 1 (▼) and 2 (▲), described in the Experimental section, solid line: fit using eqn (16) and the parameters obtained in time-resolved experiments (Fig. 2). Inset: selected NIR emission spectra registered in the absence and in the presence of Trp at various concentrations (corresponding values indicated above each spectrum (μM)).

Taking into account the results presented in this section, we can conclude that Trp is able to interact with both $^1O_2$ and ^{3}PN*. Moreover the quenching of ^{3}PN* is more efficient and, subsequently, occurs at lower Trp concentrations.

Evidence for the involvement of an electron transfer process

Singlet oxygen studies demonstrated that Trp quenches ^{3}PN*, but no information about the nature of that interaction was provided. As mentioned above the quenching may consist in a physical process (reaction (11)) and/or a chemical reaction (reaction (12)). If the latter takes place, the process might involve an electron transfer to yield the corresponding radical ion pair (reaction (17)). The Trp radical cation may deprotonate to the tryptophanyl radical (Trp(–H)$^\bullet$) (pKa = 4.1) (reaction (18)).[46] It is well-established that O_2 readily quenches an organic radical anion to produce the superoxide anion ($O_2^{\bullet-}$) (reaction (19)).[47,48]

$$^3\mathrm{PN}^* + \mathrm{Trp} \xrightarrow{k_r^T} \mathrm{PN}^{\bullet-} + \mathrm{Trp}^{\bullet+} \quad (17)$$

$$\mathrm{Trp}^{\bullet+} \rightleftarrows \mathrm{Trp}(-\mathrm{H})^\bullet + \mathrm{H}^+ \quad (18)$$

$$\mathrm{PN}^{\bullet-} + \mathrm{O_2} \rightarrow \mathrm{PN} + \mathrm{O_2}^{\bullet-} \quad (19)$$

The free energy change (ΔG) of reaction (17) can be estimated with eqn (20):[1]

$$\Delta G = F\,[E_{(\mathrm{Trp}^{\bullet+}/\mathrm{Trp})} - E_{(\mathrm{PN/PN}^{\bullet-})} - (e_o{}^2/\varepsilon R_{\mathrm{D^+A^-}})] - \Delta E_{0,0} \quad (20)$$

where $E_{(Trp^{\bullet+}/Trp)}$ and $E_{(PN/PN^{\bullet-})}$ are the standard electron potentials of electron donor and acceptor, respectively. These values have already been reported for Trp ($E_{(Trp^{\bullet+}/Trp)} = 1.015$ V *vs.* NHE[49]) and PN ($E_{(PN/PN^{\bullet-})} = -0.86$ V *vs.* NHE[28]). $\Delta E_{0,0}$ is the energy of the triplet excited state of PN and has been estimated from its phosphorescence spectra ($\Delta E_T = 185$ kJ mol^{-1}).[15,17] The term $e_o^2/\varepsilon R_{D^+A^-}$ is the solvation energy of an ion pair D^+A^- and can be ignored in the case of strong polar solvents. The calculated ΔG value was -4.1 kJ mol^{-1}, thus indicating that electron transfer from Trp to the triplet excited state of ^{3}PN* can spontaneously occur.

To investigate further if an electron transfer process takes place when solutions containing PN and Trp are exposed to UV-A irradiation, electron paramagnetic resonance (EPR) experiments were performed in the presence of a spin trap (5,5-dimethyl-1-pyrroline *N*-oxide, DMPO).

No EPR signal was registered after irradiation of an air-equilibrated solutions (pH 7.0) containing PN (A (365 nm) = 0.8), Trp (3×10^{-4} M) and DMPO (5×10^{-2} M) (Fig. 4a). However, the irradiation of the same solution, previously bubbled with N_2, led to the immediate formation of an EPR signal, which increased with the irradiation time (Fig. 4). The solution containing DMPO as a spin trap presents a six line spectrum having hyperfine coupling constants of $a_N = 15.8$ G, $a_H = 23.8$ G ($g = 2.0060$). This EPR spectrum corresponds to a carbon-centered radical.[50] Moreover, our values match well those reported for the radical resulting from the one-electron oxidation of Trp by $Br_2^{\bullet-}$ under anaerobic conditions,[51] which allows us to assign the EPR spectrum registered in our reaction system to a Trp radical.

To confirm that the registered EPR spectra correspond to a radical generated in the photosensitized process, several control experiments were carried out. First, no EPR signal was detected in solutions containing PN, Trp and DMPO before irradiation (solutions were kept in the dark for 30 minutes). In addition, no EPR signal could be recorded after irradiation of solutions containing: (i) Trp and DMPO; (ii) PN and DMPO; (iii) only DMPO.

Therefore, the results presented in this section demonstrate that upon UV-A irradiation aqueous solutions containing PN and Trp produce radicals, in particular Trp radicals, which in turn demonstrate that electron transfer takes place from Trp to excited PN. As mentioned in the Introduction, the formation of PN radicals upon excitation in the presence of the electron donor DABCO was reported.[28] However, it is worth mentioning that DABCO is more easily oxidized than Trp ($E_{(DABCO^{\bullet+}/DABCO)} < E_{(Trp^{\bullet+}/Trp)}$). Therefore, it can be concluded that ^{3}PN* is able to oxidize not only typical organic electron donors, but also oxidizable biomolecules.

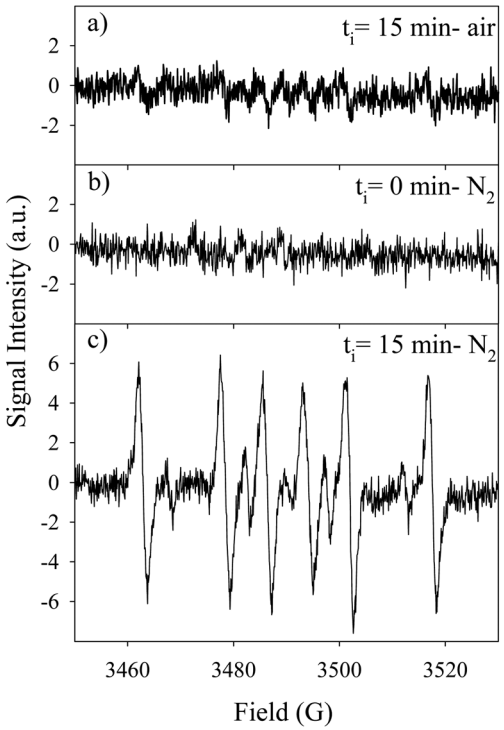

Fig. 4 EPR experiments performed in aqueous solutions containing PN (A (365 nm) = 0.8), Trp (3×10^{-4} M) and DMPO (5×10^{-2} M) as spin trap; excitation wavelength 350 nm, buffer Tris/HCl, pH = 7.0. (a) Signal registered after 15 minutes of irradiation in air-equilibrated solutions; (b) signal registered in a N_2-saturated solution before irradiation; (c) signal registered in a N_2-saturated solution after 15 minutes of irradiation.

Oxidation of Trp photosensitized by PN

The efficiency of Trp oxidation photosensitized by PN was investigated under different experimental conditions. Air-equilibrated aqueous solutions containing the photosensitizer and the substrate were exposed to UV-A (350 nm) radiation for various periods of time. From the corresponding absorption spectra (Fig. 1), it can be inferred that only PN was excited. The samples were analyzed by UV-visible spectrophotometry, HPLC and the concentration of H_2O_2 produced was measured (see Experimental section). Significant changes in the absorption spectra of the solutions were registered after irradiation (Fig. 1S, ESI†) and the concentration profile of Trp, determined by HPLC, showed a decrease of the Trp concentration as a function of irradiation time (Fig. 5). In addition, H_2O_2 was generated and its concentration increased during irradiation (Fig. 5). The detected H_2O_2 would then be the product of the spontaneous disproportionation of $O_2^{\bullet-}$ with its conjugated acid, the hydroperoxyl radical ($HO_2^{\bullet}$) (reactions (21) and (22)).[52]

$$H_3O^+ + O_2^{\bullet-} \rightleftarrows HO_2^{\bullet} + H_2O \quad (21)$$

$$HO_2^{\bullet} + O_2^{\bullet-} \rightarrow HO_2^- + O_2 \quad (22)$$

To better elucidate the role of O_2 in the photosensitization of Trp by PN, photolysis experiments were carried out in deoxygenated solutions and the results were compared with those performed in air-equilibrated solutions (Fig. 5). HPLC measurements showed that, in the experiments lacking oxygen, the Trp concentration did not decrease significantly, within experimental error. Control experiments were also carried out on Trp (100 μM) solutions (pH 5.5) in the absence of PN. As

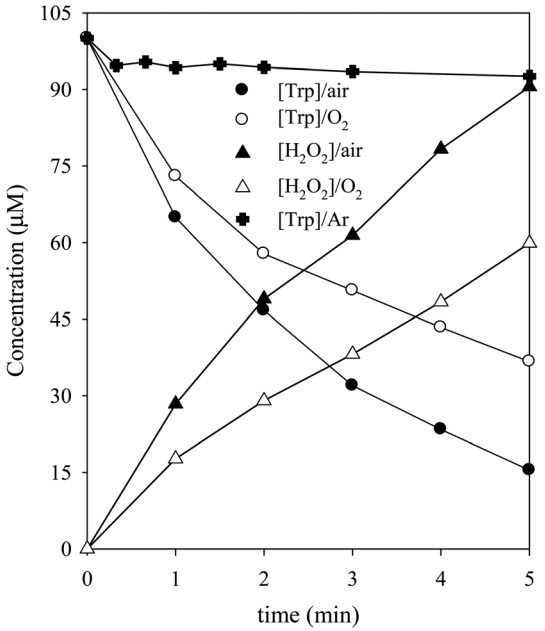

Fig. 5 Evolution of the Trp and H_2O_2 concentrations in air-equilibrated, O_2-saturated and Ar-saturated aqueous solutions (pH 5.5) of PN (A (350 nm) = 0.5) and Trp (100 μM) exposed to UV-A radiation (350 nm) as a function of elapsed time.

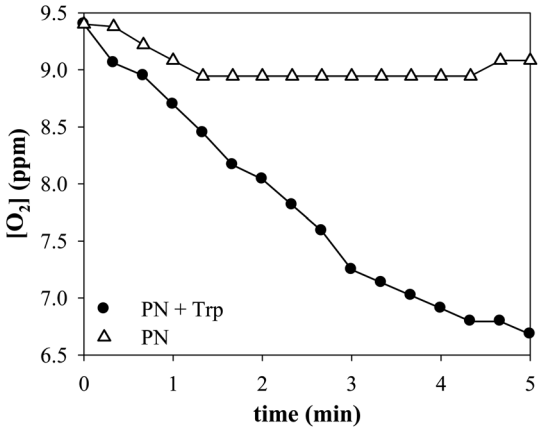

Fig. 6 Evolution of the O_2 concentration in irradiated solutions (pH = 5.5) containing Trp (400 μM) and PN (A (350 nm) = 0.50) as a function of time (●). Control experiment: irradiation of a solution of PN (A (350 nm) = 0.5) at pH 5.5 in the absence of Trp (Δ).

expected, no consumption of the amino acid was detected, thus excluding the possibility of product formation by spurious direct excitation of Trp.

In experiments performed with a different optical geometry, an O_2-selective electrode (Experimental section) was used to monitor the decrease of the O_2 concentration upon irradiation of air-equilibrated solutions containing Trp (400 μM) and PN (A (350 nm) = 0.5) at pH 5.5 (Fig. 6). Control experiments in the absence of Trp, showed that the consumption of O_2 resulting from the photolysis of PN itself was negligible in comparison with that observed in the presence of Trp (Fig. 6).

Mechanistic analysis

The results presented so far clearly demonstrate that PN photosensitizes the oxidation of Trp under UV-A irradiation in air-equilibrated aqueous solutions. Taking into account that: (i) the process needs O_2 to take place, (ii) PN is accepted as a pure type II photosensitizer due to its very high efficiency of $^1O_2$ production upon UV-A irradiation (see Introduction) and (iii) it has been previously demonstrated that Trp is easily oxidized by $^1O_2$,[44] it may be assumed that the photooxidation of Trp is a $^1O_2$-mediated reaction. However, in the previous sections it was also demonstrated that ^{3}PN* is quenched by Trp and that Trp radicals are formed in a process photosensitized by PN. Does the electron transfer process lead to a significant consumption of Trp or is it negligible compared to $^1O_2$-mediated oxidation? This question cannot be answered considering the results shown so far. Therefore additional experiments were carried out to explore this point.

In another set of photolysis experiments, O_2-saturated solutions were irradiated and the results were compared with those performed in air-equilibrated solutions. The concentration profiles obtained by HPLC clearly showed that the rate of Trp disappearance and the rate of H_2O_2 production were greater in air-equilibrated than in O_2-saturated solutions (Fig. 5). These results are not consistent with a process wherein Trp would be consumed only by reaction with $^1O_2$. However, we have shown (section "Quenching of $^1O_2$ and of the triplet excited state of PN by Trp") that ^{3}PN* is also quenched by Trp, this process competing with quenching of ^{3}PN* by O_2 to yield $^1O_2$ (reaction (2)). In oxygen-saturated solutions, the latter reaction competes more efficiently with ^{3}PN* quenching by Trp than in air-equilibrated solution and the proportion of ^{3}PN* reacting with Trp is smaller. Consequently, the rate of Trp consumption is also slower (Fig. 5), since the rate constant of reaction (2) (k_{et}) is lower than that of ^{3}PN* quenching by Trp (k_t^T, reactions (11) and (12)).

Calculation of Φ_Δ in air-equilibrated solutions using eqn (14) yields values of 0.49 and 0.20 at Trp concentrations of 100 μM and 400 μM, respectively, a large decrease compared to the value reported in the absence of quencher ($\Phi_\Delta^0 = 0.97$).[14,15] Subsequently, if PN would oxidize Trp only *via* a type II mechanism and Trp acted as a pure physical quencher of ^{3}PN*, a reduced consumption of the amino acid should be observed when the Trp concentration increases. Therefore, the experiment corresponding to Fig. 5 was repeated, but at a higher Trp concentration (photon flux absorbed by PN remained the same). As shown in Fig. 7, Trp was still consumed in this experiment and the rate of its consumption ($(d[Trp]/dt)_{i,exp}$, Table 1) was faster than that registered at lower Trp concentration (Fig. 5), confirming that PN is able to photosensitize the oxidation of Trp *via* a type I mechanism (reaction (17)).

The contribution of $^1O_2$ can be estimated considering the reported value of the rate constant of the chemical reaction between $^1O_2$ and Trp ($k_r^{Trp} = 1.3 \times 10^7$ M^{-1} s^{-1}).[44] The initial rate

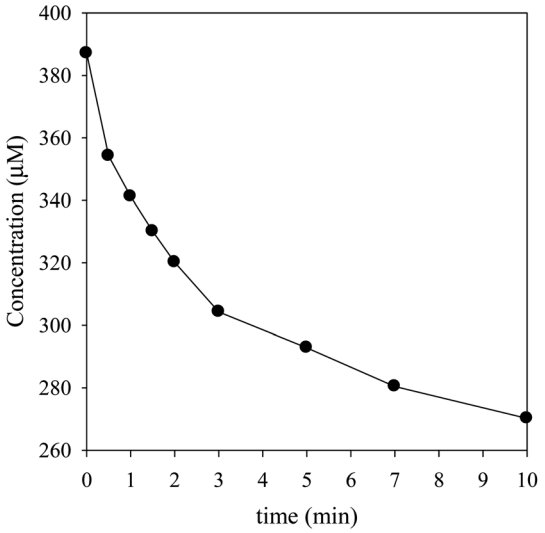

Fig. 7 Evolution of the Trp concentration in air-equilibrated aqueous solution (pH 5.5) of PN (A (350 nm) = 0.5) and Trp (390 μM) exposed to UV-A radiation (350 nm) as a function of elapsed time.

of the oxidation of Trp by $^1O_2$ ($(d[Trp]/dt)_{i,\Delta}$) was calculated using eqn (23):

$$\left(\frac{d[Trp]}{dt}\right)_{i,\Delta} = -k_r^{\Delta}[^1O_2]_{ss}[Trp]_i \quad (23)$$

Combining eqn (15), (16) and (23), $(d[Trp]/dt)_{i,\Delta}$ can be estimated as a function of the Trp concentration (eqn (24))

$$\left(\frac{d[Trp]}{dt}\right)_{i,\Delta} = -\frac{k_r^{\Delta} q_{p,a}\ \tau_{\Delta}^0\ \Phi_{\Delta}^0[Trp]_i}{(1+\tau_{\Delta}^0 k_t^{\Delta}[Trp]_i)(1+\tau_T^0 k_t^T[Trp]_i)} \quad (24)$$

In air-equilibrated solutions, the values of $(d[Trp]/dt)_{i,\Delta}$ calculated from eqn (24) were much lower than the initial rate of Trp consumption experimentally determined by HPLC analysis ($(d[Trp]/dt)_{i,exp}$, Table 1). This analysis clearly demonstrates that the oxidation of Trp by $^1O_2$ does not contribute significantly to the consumption of Trp, with calculated initial rates not exceeding 5% of the experimentally determined initial rates (Table 1). Therefore, the electron transfer process (reaction (17)) is largely dominant, at least in air-equilibrated solutions.

Therefore, taking into account all the results presented, the general mechanism summarized in Fig. 8 can be proposed. After UV-A excitation of PN and formation of its triplet excited state (^{3}PN*), three reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state,

Table 1 Initial rates of Trp consumption: experimental values $(d[Trp]/dt)_{i,exp}$ (air equilibrated aqueous solutions, pH 5.5, steady-state irradiation at 350 nm) and calculated values $(d[Trp]/dt)_{i,\Delta}$ due to the chemical reaction between $^1O_2$ and Trp[a]

$[Trp]_i$ (μM)	$(d[dTrp]/dt)_{i,\Delta}$ (10^{-7} M s^{-1})	$(d[Trp]/dt)_{i,exp}$ (10^{-7} M s^{-1})
100	0.22	4.3
390	0.33	7.5

[a] HPLC was used to determine $(d[Trp]/dt)_{i,exp}$, and values of $(d[Trp]/dt)_{i,\Delta}$ were calculated from eqn (24).

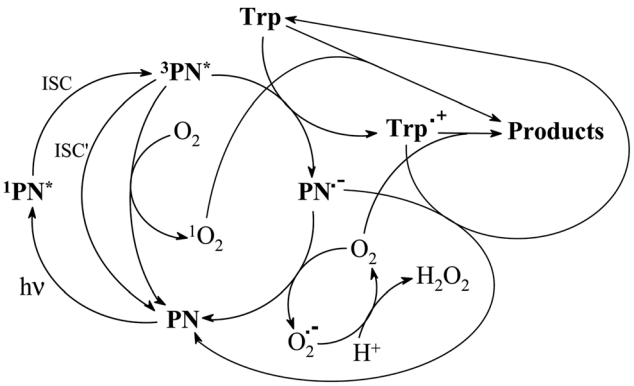

Fig. 8 Mechanism of the oxidation of tryptophan (Trp) photosensitized by phenalenone (PN).

energy transfer to O_2 leading to the regeneration of Ptr and the production of $^1O_2$, and electron transfer between Trp and ^{3}PN* yielding the corresponding pair of radical ions ($PN^{\bullet-}$ and $Trp^{\bullet+}$). In the following step, the electron transfer from $PN^{\bullet-}$ to O_2 regenerates PN and forms $O_2^{\bullet-}$, which undergoes disproportionation into H_2O_2 and O_2. Finally, the reaction of $Trp^{\bullet+}$/$Trp(-H)^{\bullet}$ with O_2 leads to the formation of products. In the absence of O_2 recombination of $PN^{\bullet-}$ and $Trp^{\bullet+}$ recovers the reactants, Fig. 8) and, therefore no consumption of the amino acid is observed under anaerobic conditions. Besides, in oxygen-saturated solutions the quenching of ^{3}PN* by O_2 competes with the electron transfer process and, consequently, the rate of Trp consumption is slower than in air-equilibrated solutions.

Conclusions

Although photosensitized oxidations may involve the generation of radicals (type I mechanism) and/or the production of singlet molecular oxygen ($^1O_2$) (type II mechanism), it is generally accepted that the photosensitization of proteins occurs mainly through the reactions of $^1O_2$ with tryptophan (Trp) and other amino acids. In this work, we have investigated the mechanism of the oxidation of Trp photosensitized by phenalenone (PN), one of the well-known type II photosensitizers, widely used as a $^1O_2$ reference sensitizer. We have studied the interaction of Trp with the excited states of PN, the production of $^1O_2$ in the presence of Trp, the formation of radicals and the kinetics of the photodegradation of Trp under different experimental conditions. From the results obtained, it could be established that $^1O_2$ played a minor role and that the predominant mechanism of the photosensitization of Trp by PN involved an electron transfer process (type I mechanism).

A photosensitized reaction is currently accepted to be a $^1O_2$-mediated oxidation (type II mechanism) if (i) the reaction needs O_2 to take place, (ii) the photosensitizer generates $^1O_2$ upon irradiation, (iii) the target molecule is sensitive to $^1O_2$. Although this assumption seems to be quite obvious and trivial, in this work we have presented evidence against it. The predominant type I mechanism of the oxidation of Trp photosensitized by PN,

which is systematically used as a purely type II photosensitizer, calls into question many reported photooxidation mechanisms and even the role of $^1O_2$ in processes involved in the photodynamic effects of a variety of photosensitizers in living systems.

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