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Original Contribution

Tryptophan oxidation photosensitized by pterin

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ABSTRACT

Pterins are normal components of cells and they have been previously identified as good photosensitizers under UV-A irradiation, inducing DNA damage and oxidation of nucleotides. In this work, we have investigated the ability of pterin (Ptr), the parent compound of oxidized pterins, to photosensitize the oxidation of another class of biomolecules, amino acids, using tryptophan (Trp) as a model compound. Irradiation of Ptr in the UV-A spectral range (350 nm) in aerated aqueous solutions containing Trp led to the consumption of the latter, whereas the Ptr concentration remained unchanged. Concomitantly, hydrogen peroxide (H_2O_2) was produced. Although Ptr is a singlet oxygen ($^1O_2$) sensitizer, the degradation of Trp was inhibited in O_2-saturated solutions, indicating that a $^1O_2$-mediated process (type II oxidation) was not an important pathway leading to Trp oxidation. By combining different analytical techniques, we could establish that a type I photooxidation was the prevailing mechanism, initiated by an electron transfer from the Trp molecule to the Ptr triplet excited state, yielding the corresponding radical ions ($Trp^{\bullet+}$/Trp(-H)$^{\bullet}$ and $Ptr^{\bullet-}$). The Trp reaction products that could be identified by UPLC-mass spectrometry are in agreement with this conclusion.

Introduction

Proteins have been shown to represent important targets for photodamage under UV and visible irradiation [1]. Among the amino acids, tryptophan (Trp) is particularly susceptible to a variety of oxidizing agents. UV-A (320–400 nm) and visible radiation induce oxidation reactions through photosensitized processes. These processes involve excitation of the photosensitizers to yield singlet and triplet excited states. The latter having longer lifetimes may undergo bimolecular reactions more efficiently and, in particular, may transfer energy to molecular O_2, forming the reactive singlet oxygen ($^1O_2(^1\Delta_g)$ denoted as $^1O_2$) responsible for type II photooxidation reactions [2]. Electron transfer involving excited triplet states and different substrates may also initiate oxidation reactions (type I photooxidation).

Pterins, heterocyclic compounds widespread in biological systems, have been identified as photosensitizers. Under UV-A excitation (320–400 nm), pterins can fluoresce, undergo photooxidation, and generate reactive oxygen species (ROS) [3]. In the presence of oxygen, pterin (Ptr), the parent and unsubstituted compound of oxidized pterins, acts as a photosensitizer through both type I [4] and type II mechanisms [5]. Moreover, pterin photoinduces DNA damage [6,7] and the oxidation of nucleotides, such as 2′-deoxyguanosine 5′-monophosphate [8] and 2′-deoxyadenine 5′-monophosphate [9].

Pterins are present in human epidermis. In particular, 5,6,7,8-tetrahydrobiopterin (H_4Bip) is an essential cofactor in the hydroxylation of the aromatic amino acids [10] and participates in the regulation of melanin biosynthesis [11]. Several dihydro and tetrahydropterins are involved in the metabolism of H_4Bip and, hence, also present in human skin [12]. Vitiligo is a skin disorder that affects an estimated 1% of the world population and is characterized by the acquired loss of constitutional pigmentation manifesting as white macules and patches [13]. In this disease, the H_4Bip metabolism is altered [14] and the protection against UV radiation fails due to the lack of melanin, the main pigment of skin. Patients suffering from vitiligo express a characteristic fluorescence in their white skin patches upon Wood's light examination due to the presence of oxidized pterins [11]. Several studies have reported that the concentration of pterins in diseased skin cells is more than one order of magnitude higher than in healthy cells. In

Abbreviations: DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; PBN, α-(4-pyridyl-1-oxide) *N*-*t*-butylnitrone; NH_4OAc, ammonium acetate Ptr, pterin; ROS, reactive oxygen species; SOD, superoxide dismutase.

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the affected tissues micromolar concentrations of pterins have been determined; e.g., in human keratinocytes and cell cultures from suction blister roofs the concentration of total biopterin was determined to be in the range 4–93 μM (41–950 pmol/mg of protein), depending on the cell type [11]; concentrations higher than 35 μM (360 pmol/mg of protein) were determined for 6-carboxypterin [15]. In addition, fluorescence studies have determined that the concentration of pterins is not homogeneous inside the cells. Therefore the local concentrations could be even much higher than those noted above.

In a recent publication, it has been suggested that in the skin of patients affected by vitiligo, Trp is oxidized, since its concentration is depleted and known oxidation products are present in high concentrations [16]. However, tryptophan hydroxylase activity is undetectable, indicating that, in this case, the oxidation of Trp is a nonenzymatic process. The Trp oxidation may imply different mechanistic pathways such as direct UV-B excitation or photosensitization processes.

To evaluate the capability of Ptr to photosensitize the degradation of Trp, aqueous solutions containing both compounds were exposed to UV-A irradiation (320–400 nm) under different experimental conditions. In this spectral region, Ptr absorbs radiation, whereas Trp does not (Fig. 1). Most of the experiments were performed in the pH range 5.5–6.0, so that more than 99% of the pterin ($pK_a = 7.9$) [17] was in the acid form, the predominant form at physiological pH. The photochemical reactions were followed by UV/visible spectrophotometry, HPLC, and an enzymatic method for H_2O_2 determination. Radical intermediates were investigated by electronic paramagnetic resonance (EPR) and reaction products were characterized by UPLC-mass spectrometry.

Experimental

General

Pterin (purity > 99%, Schircks Laboratories, Switzerland and Sigma-Aldrich) was used without further purification after checking for impurities by HPLC. Tryptophan and ammonium acetate (NH_4OAc) (Sigma Chemical Co.) were of the highest purity available (> 98%) and were used without further purification. Methanol (MeOH) and KI were purchased from J. T. Baker and Sigma, respectively. Other chemicals were from Sigma Chemical Co. Solutions were prepared dissolving Ptr and Trp in water. The final pH of the solutions was adjusted by adding drops of HCl or NaOH solutions (0.1–0.2 M) with a micropipette. The ionic strength was ca. 10^{-3} M in all experiments. Concentration ranges used for the experiments were 50–100 and 50–300 μM for Ptr and Trp, respectively.

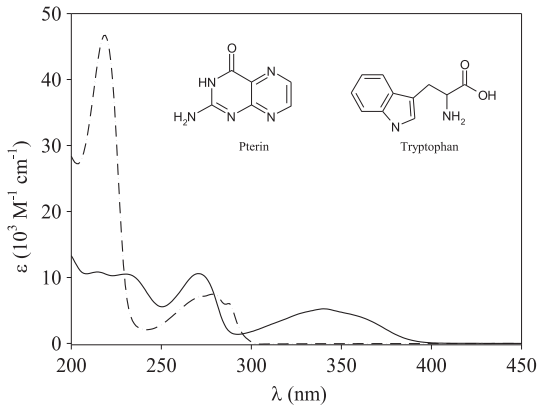


Fig. 1. Molecular structure of Ptr and Trp, and the corresponding absorption spectra in air-equilibrated aqueous solutions; solid line, acid form of Ptr (pH 5.5); dashed–dotted lines, Trp.

Steady-state irradiation

Irradiation setup

Aqueous solutions containing Ptr and Trp (pH 5.5) were irradiated in 1 cm path length quartz cells at room temperature with Rayonet RPR3500 lamps with emission centered at 350 nm (Southern N.E. Ultraviolet Co.). The spectral discrimination was achieved using filters with bandwidths (fwhm) of ~20 nm. The experiments were performed in the presence and in the absence of O_2. Oxygen-free solutions were obtained by bubbling with Ar for 20 min. The measurements were carried out under conditions of reduced environmental light.

Actinometry

Aberchrome 540 (Aberchromics Ltd.), the anhydride form of the (*E*)-*R*-(2,5-dimethyl-3-furylethylidene)(isopropylidene)-succinic acid, was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength. The method for the determination of P_0 has been described in detail elsewhere [18,19]. Values of the photon flux absorbed (P_a) were calculated from P_0 ($P_0^{350} = 5.12 \times 10^{-4}$ einstein L^{-1} min^{-1}) according to the Lambert–Beer law ($P_a = P_0\ (1-10^{-A})$, where A is the absorbance of the sensitizer at the excitation wavelength).

UV/visible analysis

UV-visible absorption spectra were registered on a Shimadzu UV-1800 spectrophotometer. Measurements were made in quartz cells of 0.4 and 1 cm optical path length.

High performance liquid chromatography (HPLC)

A Prominence equipment from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, oven CTO-10AS VP, and photodiode array detector SPD-M20A) was used to monitor and quantify the reactants and the photoproducts. Separation was performed on a Sinergy Polar-RP column (150 × 4.6 mm, 5 μm; Phenomenex) using as mobile phase solutions containing 10 mM NH_4OAc aqueous solution (pH 6.8). HPLC runs were monitored by UV/vis spectroscopy at different wavelengths.

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 [20,21]. 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.

In all cases in which H_2O_2 was detected and quantified using the technique described in the previous paragraph, controls with catalase, the enzyme that catalyzes specifically the decomposition of H_2O_2 to H_2O and O_2, were also carried out. Catalase was added after irradiation and before mixing the analyzed solution with the reactants. Thus, the absence of absorbance at 505 nm in these controls confirmed the formation of H_2O_2 in the studied reactions.

Electron paramagnetic resonance-spin trapping experiments

EPR experiments were performed in order to detect the Trp radical cation ($Trp^{\bullet+}$). 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 fingerprintable by EPR spectroscopy [22,23]. In our experiments, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and α-(4-pyridyl-1-oxide) *N*-*t*-butylnitrone (PBN) from Sigma were used as spin traps [24].

Samples (1 ml) contained 1×10^{-4} M Ptr, 3×10^{-4} M Trp, buffer Tris/HCl, pH 7.0, and one spin trap: DMPO (50 mM) or PBN (3 mM). The irradiation was performed in O_2-free solutions. Samples were irradiated in quartz flat cells at room temperature. EPR spectra were recorded every minute since the beginning of the irradiation up to 15 min.

Mass spectrometry analysis

The LC/MS system was equipped with an UPLC chromatograph (UHPLC Ultimate 3000 RS Dionex) and a triple quadrupole mass spectrometer (Q TRAP Applied Biosystems). UPLC analyses were performed using an Acquity UPLC BEH C18 (1.7 μm; 2.1 × 50 mm) column (150 mm, Waters), and isocratic elution with 0.1% formic acid at a flow rate of 0.6 mL min^{-1}. The mass spectrometer was equipped with an electrospray ion (ESI) source (turbo ion spray (TIS)) and was operated in both positive and negative ion modes. Nitrogen served as auxiliary, collision gas, and nebulizer gas. The nitrogen temperature of the TIS source was 450 °C and the declustering potential (DP) 30 V. The detection was scan mode with a step size of 0.1 atomic mass unit (amu) and a scan range of 150–500 amu. Mass chromatograms, i.e., representations of mass spectrometry data as chromatograms (the *x*-axis represents time and the *y*-axis represents signal intensity), were registered using different scan ranges.

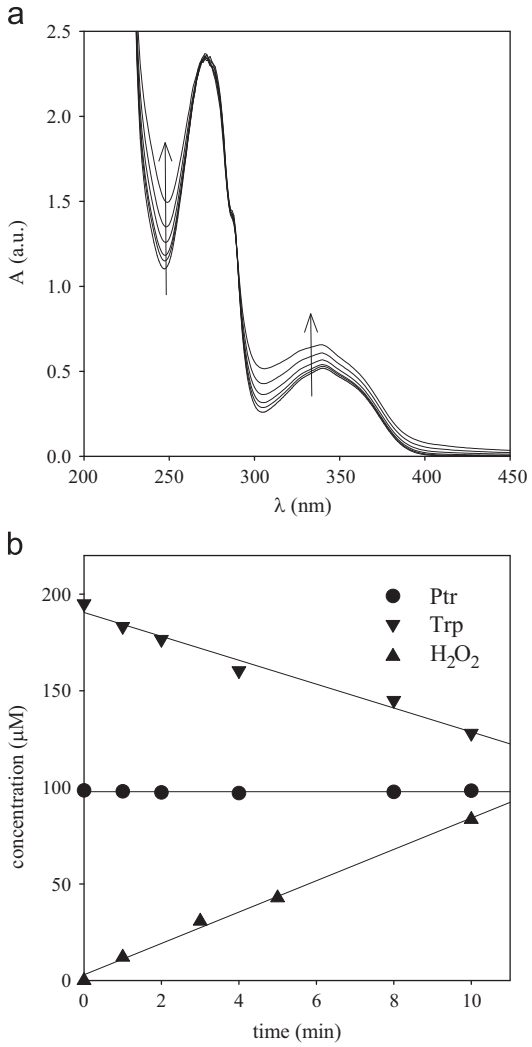


Fig. 2. (a) Time evolution of the absorption spectra of air-equilibrated solutions of Trp and Ptr irradiated under UV-A irradiation. Spectra were recorded every 10 min, optical path length = 1 cm. Arrows indicate the changes observed at different wavelengths. (b) Evolution of the Trp, Ptr, and H_2O_2 concentrations in aqueous solutions as a function of irradiation time in air-equilibrated solutions. λ_{EXC} = 350 nm; $[Ptr]_0$ = 100 μM, $[Trp]_0$ ~200 μM, pH 5.5.

Results

Irradiation of solutions containing Ptr and Trp

Air-equilibrated solutions containing Ptr (~100 μM) and Trp (~200 μM) were exposed to UV-A (350 nm) radiation for different periods of time. The experiments were performed in the pH range 5.5–6.0, where Ptr is present at more than 99% in its acid form (pK_a 7.9, [17] Fig. 1).

Significant changes in the absorption spectra of the solutions containing Ptr and Trp were registered during irradiation (Fig. 2). The concentration profiles of Ptr and Trp were determined by HPLC. A decrease of the Trp concentration was observed as a function of the irradiation time, whereas the Ptr concentration did not change in the analyzed time window.

H_2O_2 was found to be generated and its concentration increased as a function of the irradiation time at an initial rate of production of the same order of magnitude as the initial rate of Trp consumption. Trp might be oxidized to intermediate organic hydroperoxides. However, these compounds are not stable and rapidly decay to yield more stable compounds [25]. To discard potential interferences in the determination of H_2O_2 caused by organic hydroperoxides, the assay was carried out immediately after interrupting the irradiation and after 1 h to ensure that no organic hydroperoxide remained in the solutions. The same H_2O_2 concentration was obtained under both conditions. This result suggests that hydroperoxides, if present in significant concentrations, were not detected by our analytical method. Finally, in additional control experiments, the elimination of H_2O_2 by catalase resulted in the absence of absorbance at 505 nm (Experimental), thus confirming that the species detected and quantified by the test used was only H_2O_2.

In another set of experiments, solutions containing Ptr (~100 μM) and Trp (~200 μM), previously bubbled with Ar, were irradiated. No significant changes were observed in the absorption spectra of the solutions after more than 80 min of irradiation. HPLC measurements showed that, in these experiments lacking oxygen, the Trp concentration did not decrease. Accordingly, no photoproducts could be detected. In addition, control experiments showed that reactions between Ptr and Trp in the dark could be discarded. Moreover, no chemical modification of the amino acid could be detected when Trp (200 μM) solutions were irradiated at 350 nm in the absence of Ptr, thus excluding spurious effects of light absorption by Trp.

Results presented so far (consumption of Trp, production of H_2O_2, and constant concentration of Ptr during the reaction) clearly demonstrate that Ptr photosensitizes the oxidation of Trp under UV-A irradiation in the presence of O_2. To the best of our knowledge, this is the first time that photosensitization of an amino acid by a pterin is reported.

Mechanistic analysis

Effect of the addition of potassium iodide and Ptr triplet excited state involvement

It has been previously suggested that iodide (I^-) at micromolar concentrations is an efficient and selective quencher of the triplet excited states of pterins [26,27]. Therefore, photosensitization experiments were carried out in air-equilibrated aqueous solutions containing Trp and Ptr at pH 5.5 in the presence of 400 μM KI. The results revealed that, under these conditions, the rate of Trp consumption was slower than in the absence of I^- (Fig. 3). Likewise, the rate of H_2O_2 production was slower in the presence of I^- than in its absence.

The effect observed cannot be due to a quenching of the singlet excited state of pterin because at a concentration of 400 μM, I^- does not quench the Ptr fluorescence, as previously reported [26]. The interaction between I^- and $^1O_2$ is negligible under the current experimental conditions, since the k $[I^-] \ll k_d$ (k, bimolecular rate constant for the interaction of $^1O_2$ with I^- [28]; k_d, rate constant of $^1O_2$ deactivation by the solvent, see below). In a control experiment, a solution containing I^- and H_2O_2, both at micromolar concentrations, was kept in the dark for 30 min, the time required for H_2O_2 determination. In this control no consumption of H_2O_2 was detected. Thus, the inhibition of the photosensitized degradation of Trp in the presence of I^- (Fig. 3), strongly suggests the participation of the triplet excited state of Ptr.

Effect of the concentration of molecular oxygen and contribution of singlet oxygen

In order to check the effect of the O_2 concentration, a new set of experiments was performed in O_2-saturated solutions at pH 5.5. Concentration profiles clearly showed that the rates of Ptr-sensitized Trp disappearance and H_2O_2 formation were greater in air-equilibrated than in O_2-saturated solutions (Fig. 3). Since $^1O_2$ is formed by energy transfer from the triplet state of the sensitizer to dissolved O_2 (Reactions (1)–(4)), these results suggest that oxidation of Trp by $^1O_2$ (Reaction (5)) should not be the main mechanism.

$$\mathrm{Ptr} \longrightarrow {}^1\mathrm{Ptr}^* \quad (1)$$

$$^1\mathrm{Ptr}^* \xrightarrow{\mathrm{ISC}} {}^3\mathrm{Ptr}^* \quad (2)$$

$$^3\mathrm{Ptr}^* \longrightarrow \mathrm{Ptr} \quad (3)$$

$$^3\mathrm{Ptr}^* + {}^3\mathrm{O}_2 \longrightarrow \mathrm{Ptr} + {}^1\mathrm{O}_2 \qquad \Phi_\Delta \quad (4)$$

$$\mathrm{Trp} + {}^1\mathrm{O}_2 \xrightarrow{k_r} \mathrm{Trp(ox)} \quad (5)$$

$$\mathrm{Trp} + {}^1\mathrm{O}_2 \xrightarrow{k_q} \mathrm{Trp} + {}^3\mathrm{O}_2 \quad (6)$$

Considering the results at different O_2 concentrations, experiments were performed in D_2O where the $^1O_2$ lifetime (τ_Δ) is much longer than in H_2O (ca. 60 and 4 μs, respectively) [28,29]. If $^1O_2$-mediated oxidation of Trp (Reaction (5)) would be dominant, Trp consumption should be much faster in D_2O than in H_2O. The experiments showed, however, that the rate of Trp consumption was only slightly higher in D_2O (Fig. 4).

Taking into account a previously reported value of the rate constant of the chemical reaction (k_r) between $^1O_2$ and Trp [30], ($k_r = 1.3 \times 10^7\ M^{-1}\ s^{-1}$), the contribution of $^1O_2$ to the photosensitized oxidation of Trp by Ptr can be evaluated by comparing the experimental initial rate of Trp consumption to the initial rate of the reaction between $^1O_2$ and Trp calculated from Eqs. (7) and (8) [28].

$$-(\mathrm{d[Trp]/dt}) = k_r\,[\mathrm{Trp}]\,[^1\mathrm{O}_2] \quad (7)$$

The steady-state concentration of $^1O_2$ during irradiation of a solution containing Ptr and Trp is given by

$$[^1\mathrm{O}_2] = P_a\,\Phi_\Delta / (k_d + k_t^{\mathrm{Ptr}}\,[\mathrm{Ptr}] + k_t^{\mathrm{Trp}}\,[\mathrm{Trp}]) \quad (8)$$

where P_a (einstein $L^{-1}\ s^{-1}$) is the photon flux absorbed by Ptr and Φ_Δ the quantum yield of $^1O_2$ production by Ptr ($\Phi_\Delta = 0.18 \pm 0.02$, at pD 5.5) [31], respectively; k_d (s^{-1}) is the rate constant of $^1O_2$ deactivation by the solvent ($=1/\tau\Delta$); the overall (physical and reactive) rate constants of $^1O_2$ quenching ($k_t = k_q + k_r$) by Ptr and Trp are $k_t^{\mathrm{Ptr}} = 2.9 \times 10^6\ M^{-1}\ s^{-1}$ [32] and $k_t^{\mathrm{Trp}} = 3.2 \times 10^7\ M^{-1}\ s^{-1}$ [30]. The initial rate of the reaction between $^1O_2$ and Trp, $(\mathrm{d[Trp]/dt})_{\mathrm{calc}}$, for a given experiment was calculated using Eqs. (7) and (8), and compared to the corresponding initial rate of Trp consumption experimentally determined by HPLC analysis, $(-\mathrm{d[Trp]/dt})_{\mathrm{exp}}$ (Table 1).

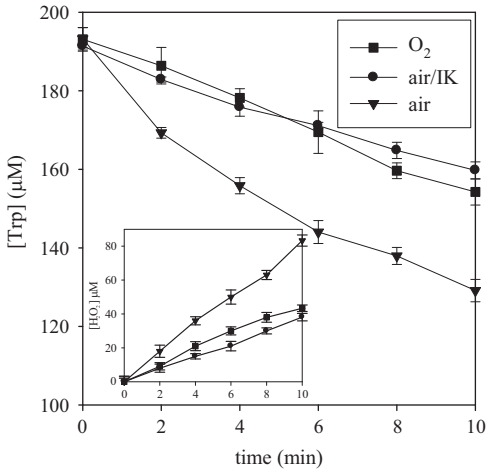


Fig. 3. Evolution of the Trp and H_2O_2 concentrations in aqueous solutions under UV-A irradiation as a function of time. Experiments were performed in quadruplicate in air-equilibrated solutions in the absence of KI (▾) and in the presence of KI (●), and in O_2 equilibrated solutions (■), $\lambda_{EXC} = 350$ nm, pH 5.5; $[Ptr]_0 \sim 100$ μM, $[Trp]_0 \sim 200$ μM. Error bars indicate one standard deviation.

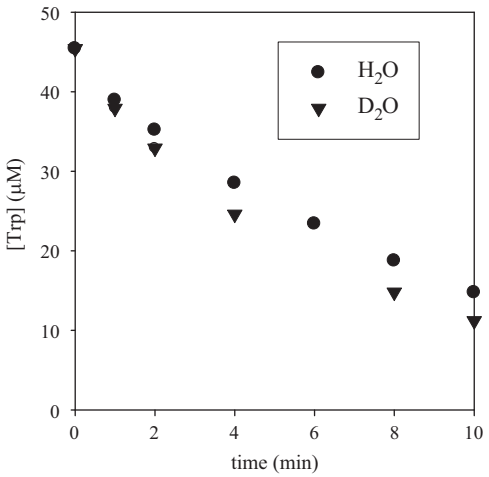


Fig. 4. Evolution of the Trp concentration in H_2O (●) and D_2O (▾) solutions under UV-A irradiation as a function of time. $\lambda_{EXC} = 350$ nm, pH 5.5; $[Ptr]_0 \sim 100$ μM, $[Trp]_0 \sim 50$ μM.

The results obtained reveal that: (i) the experimental rate hardly increases in D_2O compared to H_2O (Table 1); (ii) in both H_2O and D_2O, the expected rate of Trp consumption due to oxidation by $^1O_2$ is slower than the experimental rates, confirming that a type II photosensitization process via $^1O_2$ should play only a minor role and is not the dominant mechanism of Trp oxidation.

Evidence for the involvement of the tryptophanyl radical and the superoxide anion

From the above kinetic analysis, a predominant mechanism via electron transfer (type I) may be postulated, in agreement with previous evidence on the capability of Ptr to photosensitize the oxidation of biological substrates through this type of mechanism [4]. In this case, the photosensitized oxidation would start with an electron transfer from Trp to the triplet excited state of Ptr to form the Trp radical cation (Reaction (9)) that may deprotonate to the tryptophanyl radical ($Trp(-H)^{\bullet}$) (Reaction (10)) [33]. It is well established that, in a typical type I process, ground state O_2 will readily quench an organic radical anion to produce the superoxide anion ($O_2^{\bullet-}$) (Reaction (11)) [34,35]. The detected H_2O_2 (vide supra) would then be the product of the spontaneous disproportionation of $O_2^{\bullet-}$ with its conjugated acid, the hydroperoxyl radical ($HO_2^{\bullet}$) (Reactions (12) and (13)) [36].

$$^3Ptr^* + Trp \longrightarrow Ptr^{\bullet-} + Trp^{\bullet+} \quad (9)$$

$$Trp^{\bullet+} \longrightarrow Trp(-H)^{\bullet} + H^+ \quad (10)$$

$$Ptr^{\bullet-} + O_2 \longrightarrow Ptr + O_2^{\bullet-} \quad (11)$$

$$H_3O^+ + O_2^{\bullet-} \longrightarrow HO_2^{\bullet} + H_2O \quad (12)$$

$$HO_2^{\bullet} + O_2^{\bullet-} \longrightarrow HO_2^- + O_2 \quad (13)$$

Table 1
Experimental initial rates of Trp consumption during irradiation of Ptr in aqueous solutions (pH or pD 5.5) and initial rates of the reaction between $^1O_2$ and Trp calculated according to Eqs. (7) and (8).

Solvent	Experimental $-(d[Trp]/dt)_0$	Calculated $-(d[Trp]/dt)_0$ (for reaction with $^1O_2$)
H_2O	5.1×10^{-6} M min^{-1}	0.3×10^{-6} M min^{-1}
D_2O	6.3×10^{-6} M min^{-1}	2.0×10^{-6} M min^{-1}

To investigate the formation of $Trp(-H)^{\bullet}$ ($pK_a=4.1$) [33] in the irradiated solutions, EPR analyses were performed in the presence of spin traps. DMPO and PBN were used in this work, since the carbon-centered tryptophanyl radical adducts formed with DMPO and PBN have been previously characterized [37]. The irradiation of a solution (pH 7.0) containing Ptr (1×10^{-4} M), Trp (3×10^{-4} M), and DMPO (5×10^{-2} M) or PBN (3×10^{-3} M) (in the absence of O_2) led to the immediate formation of an EPR signal, which increased with the irradiation time.

The solution containing DMPO as a spin trap presents a six line spectrum (Fig. 5a) having hyperfine coupling constants of $a_N=15.8$ G, $a_H=23.8$ G ($g=2.0060$), whereas the solution containing PBN also shows a six line spectrum (triplet of doublets, Fig. 6a) having hyperfine coupling constants of $a_N=15.8$ G, $a_H=2.5$ G ($g=2.0060$). Both spectra correspond to a carbon-centered radical [38]. Moreover, our values match well with those reported for the radical resulting from the one-electron oxidation of Trp by $Br_2^{\bullet-}$ under anaerobic conditions [37], which allows us to assign the EPR spectra registered in our reaction system to a Trp radical. In addition, in another work on Trp oxidation, in which

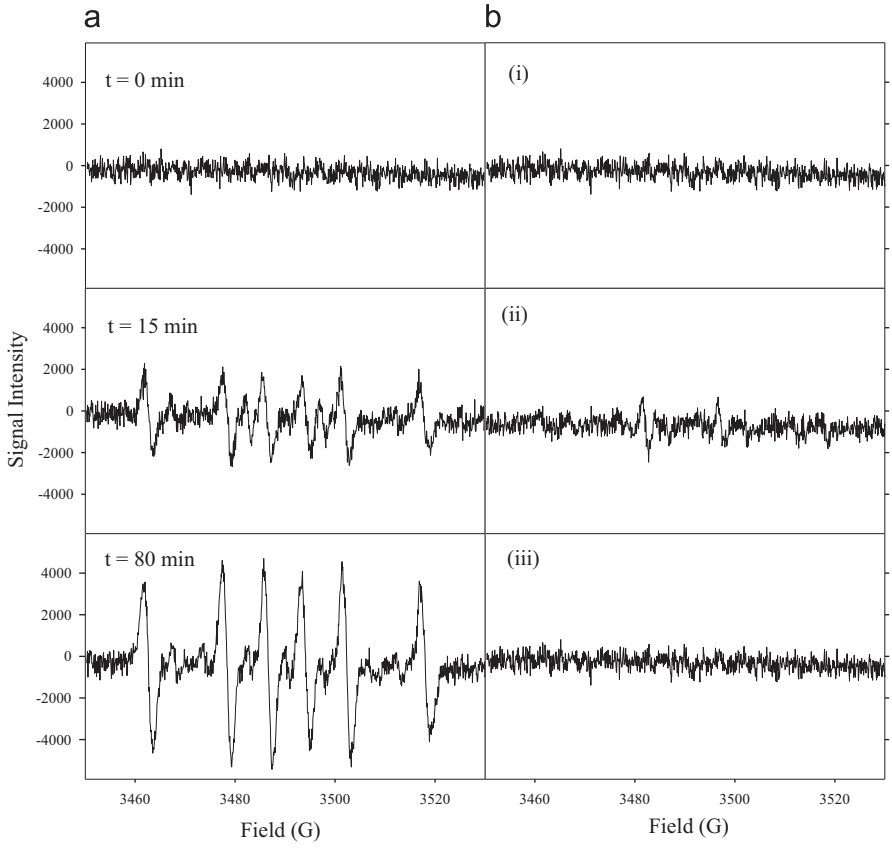


Fig. 5. EPR experiments performed in Ar-equilibrated solutions (buffer Tris/HCl, pH 7.0), using DMPO (5×10^{-2} M) as spin trap. (a) Signals registered at different irradiation times. $\lambda_{EXC}=350$ nm, $[Ptr]_0=100$ μM; $[Trp]_0=300$ μM; (b) Controls experiments, signal registered after 60 min of irradiation. (i) $[Ptr]_0=0$ μM; $[Trp]_0=300$ μM; (ii) $[Ptr]_0=100$ μM; $[Trp]_0=0$ μM; (iii) $[Ptr]_0=0$ μM; $[Trp]_0=0$ μM.

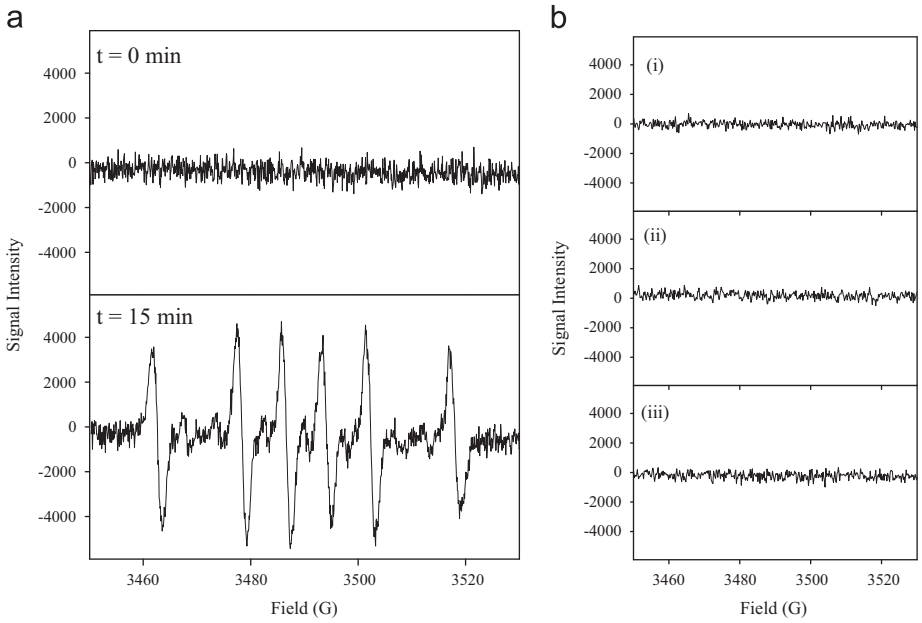


Fig. 6. EPR experiments performed in Ar-equilibrated solutions (buffer Tris/HCl, pH 7.0), using PBN (3×10^{-3} M) as spin trap. (a) Signals registered at different irradiation times. λ_{EXC}=350 nm, $[Ptr]_0$=100 μM; $[Trp]_0$=300 μM; (b) Controls experiments, signal registered after 15 min of irradiation. (i) $[Ptr]_0$=0 μM; $[Trp]_0$=300 μM; (ii) $[Ptr]_0$=100 μM; $[Trp]_0$=0 μM; (iii) $[Ptr]_0$=0 μM; $[Trp]_0$=0 μM.

radicals from Trp hydroperoxide derivatives were formed, similar EPR signals were reported [25].

To confirm that the registered EPR spectra correspond to a radical generated in the photosensitized processes, several control experiments were carried out. First, no EPR signal was detected in solutions containing Ptr, Trp, and a given spin trap before irradiation (solutions were kept in the dark for 30 min). In addition, no EPR signal could be registered after irradiation of solutions containing: (i) Trp and a given spin trap; (ii) Ptr and a given spin trap; (iii) only the spin trap (Figs. 5b and 6b).

Further experiments were carried out in the presence of superoxide dismutase (SOD), an enzyme that catalyzes the conversion of $O_2^{\bullet -}$ into H_2O_2 and O_2 [39]. The data showed an increase in the rate of Trp consumption when SOD was present in the solution (Fig. 7). These results are discussed in the following section.

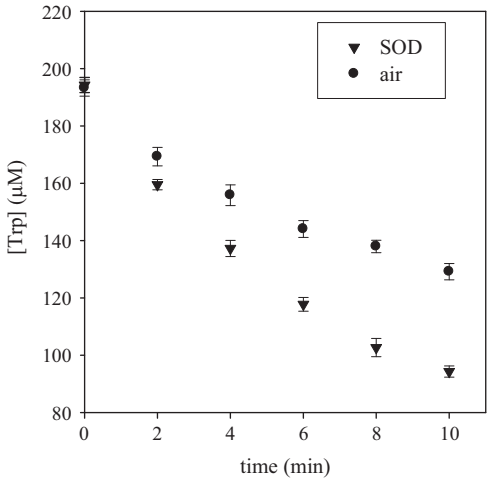


Fig. 7. Evolution of the Trp concentration in aqueous solutions under UV-A irradiation as a function of time. Experiments were performed in quadruplicate in the absence (•) and in the presence (▾) of SOD (50 U/ml). λ_{EXC}=350 nm, pH 5.5; $[Ptr]_0 \sim 100$ μM, $[Trp]_0 \sim 200$ μM. Error bars indicate one standard deviation.

Evidence in favor of a predominant electron transfer mechanism

Taking into account the results presented so far, we propose that the Ptr-sensitized oxygenation/oxidation of Trp involves the following reactions steps: excitation of Ptr leads to the formation of the reactive triplet excited state ^{3}Ptr* by intersystem crossing from the singlet excited state (Reactions (1) and (2)). Besides intersystem crossing to the ground state (Reaction (3)), two reaction pathways compete for the deactivation of ^{3}Ptr*: energy transfer to molecular oxygen leading to the regeneration of Ptr and the formation of $^1O_2$ (Reaction (4), type II mechanism), and electron transfer between Trp and ^{3}Ptr* yielding the corresponding radical ions, $Ptr^{\bullet -}$ and $Trp^{\bullet +}$ (Reaction (9), type I mechanism). The relative rates of these two latter reactions control the dominant character (type I or type II) of the Trp oxidation process. Under the assumption that Trp consumption results mainly from a type I process, an increase in O_2 concentration by a factor of 5 (O_2-saturated vs air-equilibrated solutions) would decrease significantly the proportion of ^{3}Ptr* reacting with the Trp by electron transfer (Reaction (9)). Consequently, the rate of Trp consumption should also be much slower, as observed (Fig. 3).

In the absence of O_2, the radical ions $Ptr^{\bullet -}$ and $Trp^{\bullet +}$/Trp(-H)$^{\bullet}$ formed in the electron transfer step (Reaction (9)) recombine efficiently (Reaction (14)), which explains the absence of Trp consumption. Nevertheless, addition of a spin trap allows the detection of the $Trp^{\bullet +}$ or, most likely, its deprotonated (Trp(-H)$^{\bullet}$) form by EPR (see above). In the presence of O_2, the electron transfer from $Ptr^{\bullet -}$ to O_2 regenerates Ptr and forms $O_2^{\bullet -}$ (Reaction (11)). This radical anion may disproportionate with its conjugated acid $HO_2^{\bullet}$ to form H_2O_2 (summarized by Reactions (12) and (13)) but may also react with $Trp^{\bullet +}$/Trp(-H)$^{\bullet}$ to regenerate Trp (Reaction (15)). Addition of SOD accelerates superoxide disproportionation (see above) and, therefore fast elimination of $O_2^{\bullet -}$ through this pathway prevents regeneration of Trp via Reaction (15). As a consequence, in the presence of SOD, enhancement of Trp consumption is observed experimentally (Fig. 7). Finally a group of processes, represented schematically by Reaction (16) and that may include reactions of $Trp^{\bullet +}$/Trp(-H)$^{\bullet}$ with O_2 and H_2O, leads to the oxidation of Trp and consumption of O_2.

$$Ptr^{\bullet-} + Trp^{\bullet+} / Trp(-H)^{\bullet} \longrightarrow Ptr + Trp \quad (14)$$

$$Trp^{\bullet+} / Trp(-H)^{\bullet} + O_2^{\bullet-} / HO_2^{\bullet} \longrightarrow Trp + O_2 \quad (15)$$

$$Trp^{\bullet+} / Trp(-H)^{\bullet} \xrightarrow{O_2 / H_2O} Trp(ox) \quad (16)$$

We have shown previously that ^{3}Ptr* oxidizes dGMP (2′-deoxyguanosine 5′-monophosphate) and dAMP (2′-deoxyadenosine 5′-monophosphate) via an electron transfer process leading to the formation of the corresponding dGMP and dAMP radical cations [8,9]. Since Trp has a lower standard redox potential (E(Trp$^{\bullet+}$/Trp) = 1.015 V vs NHE at pH 7 [40]) than that of dAMP (E(dAMP$^{\bullet+}$/dAMP) = 1.44 V vs NHE [41]) and dGMP (1.33 V, [42]), the electron transfer from Trp to the ^{3}Ptr* should be even more thermodynamically favored.

Based on the results presented so far and on the analysis developed in this section, we can conclude that the oxidation of Trp photosensitized by Ptr takes place via a predominant electron transfer mechanism and that singlet oxygen plays only a minor role. This conclusion raises the question of the generally accepted main pathway of a $^1O_2$-mediated photosensitized damage to proteins [1]. In the case of sensitization of Trp by Ptr, the process takes place mainly through an electron transfer-initiated mechanism, although Ptr is a relatively efficient $^1O_2$ sensitizer (Φ_Δ = 0.18, [31]). Therefore, one might infer that more attention should be paid to radical pathways involving oxidation of amino acids.

Analysis of photoproducts by mass spectrometry

To complete the present study a qualitative analysis of photoproducts was carried out. Aqueous solutions containing Trp and Ptr at pH 5.5 were analyzed by UPLC coupled to ESI mass spectrometry, before and after irradiation. The mass analysis was carried out in both positive and negative ion modes (ESI$^+$ and ESI$^-$, respectively). As expected, the signals corresponding to the intact molecular ions of Trp and Ptr as [M+H]$^+$ species at m/z 205 and 164 Da, respectively, were observed before irradiation. Solutions were analyzed at different irradiation times and several photoproducts were detected, both in positive and in negative mode. Analysis of the ESI mass chromatogram revealed that the molecular weights of the main products were 129, 145, 189, 220, and 236 g mol^{-1}.

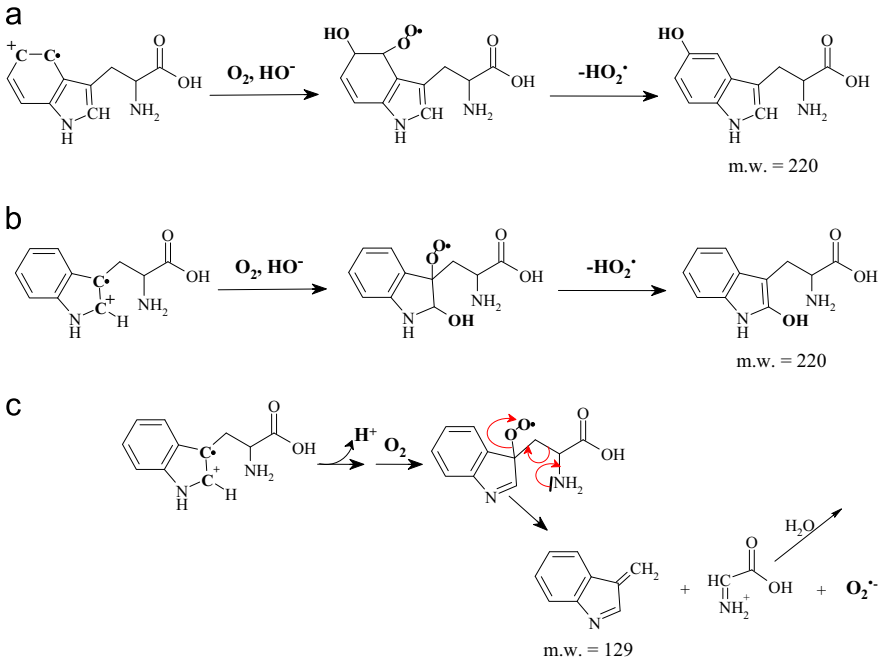
The molecular weight of 220 g mol^{-1} (signals at m/z 219 and 221 in ESI$^-$ and ESI$^+$ modes, respectively) corresponds to hydroxytryptophan (HO-Trp), which has been identified in the skin of patients affected by vitiligo [16]. Assuming a type I mechanism, formation of HO-Trp may be explained by addition of hydroxide anion (hydration) and of O_2 on the cationic and radical centers of Trp$^{\bullet+}$, respectively. The charges on Trp$^{\bullet+}$ are delocalizated on different sites of the molecule and several hydroxylated products (on the aromatic or pyrrole rings) may be formed. Examples of such reaction pathways are given in Schemes 1a and 1b. It should be noted that such products were not found in the reaction with singlet oxygen [43].

The molecular weight 129 g mol^{-1} (signal at m/z 130 in ESI$^+$ mode) may be assigned to a compound resulting from a Grob-like fragmentation [44,45] with a C–C bond cleavage in the tryptophanyl peroxide radical (obtained by deprotonation of Trp$^{\bullet+}$ followed by O_2 trapping of the radical) (Scheme 1c). This compound was previously observed in Trp solutions irradiated at 266 nm, as a consequence of a C–C cleavage of the Trp radical cation [46], and, to the best of our knowledge, is reported for the first time in a photosensitized reaction.

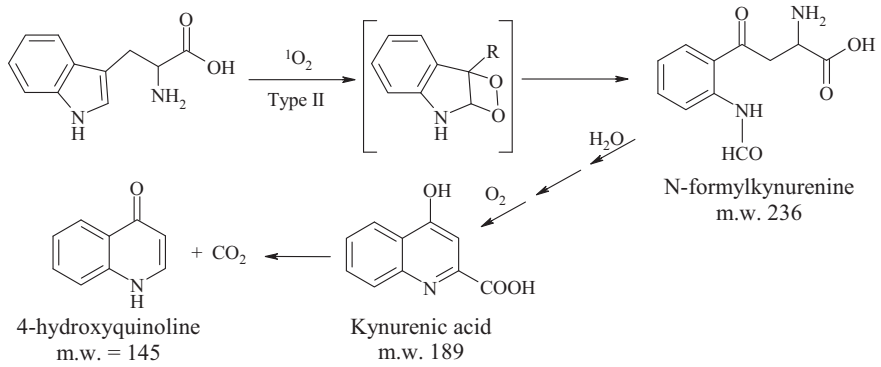
The mass 236 g mol^{-1} (signal at m/z 237 in ESI$^+$ mode) may be attributed to *N*-formylkynurenine (NFK), a product of the reaction of Trp with singlet oxygen (type II mechanism, Scheme 2), already identified by several authors [47]. Similarly the mass 189 g mol^{-1} (signals at m/z 188 and 190 in ESI$^-$ and ESI$^+$ modes, respectively) was previously attributed to kynurenic acid (KA), formed by several reactions steps from NFK. The mass 145 g mol^{-1} (signals at m/z 144 and 146 in ESI$^-$ and ESI$^+$ modes, respectively) corresponds to the decarboxylation product of KA, which may be formed by photolysis of KA, a colored pigment absorbing at 350 nm [43].

Conclusions

The photosensitizing properties of Ptr, the parent compound of oxidized pterins, toward nucleotides and DNA in aerated aqueous



Scheme 1. Examples of reaction pathways for tryptophan hydroxylation (HO-Trp) (a and b) and fragmentation (c), photosensitized by pterin through an electron transfer mechanism (type I) in aerated aqueous solutions (initial formation of the radical cation, Trp$^{\bullet+}$, through Reaction (9)).



Scheme 2. Possible pathways for the oxidation of Trp by singlet oxygen (type II mechanism), leading to the intermediate formation of *N*-formylkynurenine yielding products of molecular weights 236, 189 and 145 g mol^{-1} detected in this work.

solutions have been previously reported. Ptr was shown to act as a photosensitizer through both electron transfer (type I) and singlet oxygen (type II)-mediated processes. In this work, we investigated for the first time the mechanism of the degradation of an amino acid (Trp) photoinduced by the acid form of Ptr, the predominant form at physiological pH, in aqueous solution at pH 5.5.

We have shown that the presence of molecular oxygen was required to observe significant changes in the reaction system. Excitation of Ptr at 350 nm in aerated aqueous solutions led to the consumption of the amino acid, whereas the photosensitizer (Ptr) concentration remained unchanged, within experimental error. As Ptr is a singlet oxygen sensitizer, albeit with a moderate quantum yield (Φ_Δ=0.18 at pD 5.5), it was expected that type II oxidation should be an important pathway leading to Trp oxidation. However, mechanistic analysis, including effects of KI addition, O_2 concentration, solvent deuteriation, addition of superoxide dismutase (SOD), EPR measurements, showed that the predominant pathway for Trp consumption involved an electron transfer process from the Trp molecule to the Ptr triplet excited state. In this mechanism, formation of the corresponding radical ions ($Ptr^{\bullet-}$ and $Trp^{\bullet+}$/ $Trp(-H)^{\bullet}$) was followed by electron transfer from $Ptr^{\bullet-}$ to O_2, regenerating Ptr and producing $O_2^{\bullet-}$. The latter is known to disproportionate with its conjugated acid ($HO_2^{\bullet}$) to form H_2O_2, that was actually detected in quasi-stoichiometric amounts relative to the Trp consumption. Analysis of photoproducts confirmed the presence of compounds that could not be formed through singlet oxygen-mediated Trp oxidation. Potential pathways for their formation from the Trp radical or its deprotonated form are proposed to involved O_2 and hydroxide anions, as well as fragmentation of the Trp side chain.

It should be noted that formation of H_2O_2 during Trp oxidation sensitized by Ptr may have some relevance from a medical point of view, since pterins and H_2O_2 accumulate in the skin of patients affected by vitiligo, a pathological disorder where the protection against UV-A is failing due to the absence of melanin.

Acknowledgments

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References

[1] Pattison, D. I.; Rahmanto, A. S.; Davies, M. J. Photo-oxidation of proteins. *Photochem. Photobiol. Sci.* **11:**38–53; 2012.
[2] Foote, C. S. Definition of Type I and Type II photosensitized oxidation. *Photochem. Photobiol.* **54:**659; 1991.
[3] Lorente, C.; Thomas, A. H. Photophysics and photochemistry of pterins in aqueous solution. *Acc. Chem. Res.* **39:**395–402; 2006.
[4] Lorente, C.; Petroselli, G.; Dántola, M. L.; Oliveros, E.; Thomas, A. H. Electron transfer initiated reactions photoinduced by pterins. *Pteridines* **22:**111–119; 2011.
[5] Oliveros, E.; Dántola, M. L.; Vignoni, M.; Thomas, A. H.; Lorente, C. Production and quenching of reactive oxygen species by pterin derivatives, an intriguing class of biomolecules. *Pure Appl. Chem.* **83:**801–811; 2011.
[6] Ito, K.; Kawanishi, S. Photoinduced hydroxylation of deoxyguanosine in DNA by pterins: sequence specificity and mechanism. *Biochemistry* **36:**1774–1781; 1997.
[7] Offer, T.; Ames, B. N.; Bailey, S. W.; Sabens, E. A.; Nozawa, M.; Ayling, J. E. 5-Methyltetrahydrofolate inhibits photosensitization reactions and strand breaks in DNA. *FASEB J.* **21:**2101–2107; 2007.
[8] Petroselli, G.; Dántola, M. L.; Cabrerizo, F. M.; Capparelli, A. L.; Lorente, C.; Oliveros, E.; Thomas, A. H. Oxidation of 2′-deoxyguanosine 5′-monophosphate photoinduced by pterin: type I versus type II mechanism. *J. Am. Chem. Soc.* **130:**3001–3011; 2008.
[9] Petroselli, G.; Erra-Balsells, R.; Cabrerizo, F. M.; Lorente, C.; Capparelli, A. L.; Braun, A. M.; Oliveros, E.; Thomas, A. H. Photosensitization of 2′-deoxyadenosine-5′-monophosphate by pterin. *Org. Biomol. Chem.* **5:**2792–2799; 2007.
[10] Ziegler, I. Production of pteridines during hematopoiesis and T-lymphocyte proliferation: potential participation in the control of cytokine signal transmission. *Med. Res. Rev.* **10:**95–114; 1990.
[11] Schallreuter, K. U.; Wood, J. M.; Pittelkow, M. R.; Gütlich, M.; Lemke, K. R.; Rödl, W.; Swanson, N. N.; Hitzemann, K.; Ziegler, I. Regulation of melanin biosynthesis in the human epidermis by tetrahydrobiopterin. *Science* **263:**1444–1446; 1994.
[12] Nichol, C. A.; Smith, G. K.; Duch, D. S. Biosynthesis and metabolism of tetrahydrobiopterin and molybdopterin. *Annu. Rev. Biochem.* **54:**729–764; 1985.
[13] Glassman, S. J. Vitiligo, reactive oxygen species and T-cells. *Clin. Sci.* **120:**99—120; 2011.
[14] Schallreuter, K. U.; Moore, J.; Wood, J. M.; Beazley, W. D.; Peters, E. M.; Marles, L. K.; Behrens-Williams, S. C.; Dummer, R.; Blau, N.; Thöny, B. Epidermal H_2O_2 accumulation alters tetrahydrobiopterin (6BH4) recycling in vitiligo: identification of a general mechanism in regulation of all 6BH4-dependent processes? *J. Invest. Dermatol.* **116:**167–174; 2001.
[15] Rokos, H.; Beazley, W. D.; Schallreuter, K. U. Oxidative stress in vitiligo: photooxidation of pterins produces H2O2 and pterin-6-carboxylic acid. *Biochem. Biophys. Res. Commun.* **292:**805–811; 2002.

[16] Schallreuter, K. U.; Salem, M. A. E. L.; Gibbons, N. C. J.; Martinez, A.; Slominski, R.; Lüdemann, J.; Hartmut, R. Blunted epidermal L-tryptophan metabolism in vitiligo affects immune response and ROS scavenging by Fenton chemistry, part 1. *FASEB J.* **26:**2457–2470; 2012.

[17] Monópoli, V. D.; Thomas, A. H.; Capparelli, A. L. Kinetics and equilibrium study of nickel(II) complexation by pterin and 6-carboxypterin. *Int. J. Chem. Kinet.* **32:**231–237; 2000.

[18] Braun, A. M.; Maurette, M. T.; Oliveros, E. *Photochemical technology*. Chichester, New York: Wiley; 1991. Chapt. 2.

[19] Kuhn, H. J.; Braslavsky, S. E.; Schmidt, R. Chemical actinometry. *Pure Appl. Chem.* **76:**2105–2146; 2004.

[20] Allain, C. C.; Poon, L. S.; Chan, C. S. G.; Richmond, W.; Fu, P. C. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **20:**470–475; 1974.

[21] Flegg, H. M. An investigation of the determination of serum cholesterol by an enzymatic method. *Ann. Clin. Biochem.* **10:**79–84; 1973.

[22] Janzen, E.; Spin, E. G. trapping. *Acc. Chem. Res.* **4:**31–40; 1971.

[23] Villamena, F. A.; Zweier, J. L. Detection of reactive oxygen and nitrogen species by EPR spin trapping. *Antioxid. Redox Signal.* **6:**619–629; 2004.

[24] Villamena, F. A. Superoxide radical anion adduct of 5,5-dimethyl-1-pyrroline *N*-oxide. 5. Thermodynamics and kinetics of unimolecular decomposition. *J. Phys. Chem. A* **113:**6398–6403; 2009.

[25] Gracanin, M. L.; Hawkins, C. L.; Pattison, D. I.; Davies, M. J. Singlet-oxygen-mediated amino acid and protein oxidation: formation of tryptophan peroxides and decomposition products. *Free Radic. Biol. Med.* **47:**92–102; 2009.

[26] Dántola, M. L.; Vignoni, M.; González, C.; Lorente, C.; Vicendo, P.; Oliveros, E.; Thomas, A. H. Electron-transfer processes induced by the triplet state of pterins in aqueous solutions. *Free Radic. Biol. Med.* **49:**1014–1022; 2010.

[27] Kritsky, M. S.; Lyudnikova, T. A.; Mironov, E. A.; Moskaleva, I. V. The UV radiation-driven reduction of pterins in aqueous solution. *J. Photochem. Photobiol. B* **39:**43–48; 1997.

[28] Wilkinson, F.; Helman, W. P.; Ross, A. B. 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; 1995.

[29] Egorov, S. Y.; Kamalov, V. F.; Koroteev, N. I.; Krasnovsky Jr A. A.; Toleutaev, B. N.; Zinukov, S. V. Rise and kinetics of photosensitized singlet oxygen luminescence in water. *Chem. Phys. Lett.* **163:**421–424; 1989.

[30] Michaeli, A.; Feitelson, J. Reactivity of singlet oxygen toward amino acids and peptides. *Photochem. Photobiol.* **59:**284–289; 1994.

[31] Thomas, A. H.; Lorente, C.; Capparelli, A. L.; Martínez, C. G.; Braun, A. M.; Oliveros, E. Singlet oxygen ($^1\Delta_g$) production by pterin derivatives in aqueous solutions. *Photochem. Photobiol. Sci.* **2:**245–250; 2003.

[32] Cabrerizo, F. M.; Dantola, M. L.; Petroselli, G.; Capparelli, A. L.; Thomas, A. H.; Braun, A. M.; Lorente, C.; Oliveros, E. Reactivity of conjugated and unconjugated pterins with singlet oxygen ($O_2(^1\Delta_g)$): physical quenching and chemical reaction. *Photochem. Photobiol.* **83:**526–534; 2007.

[33] Solar, S.; Gtoff, N.; Surdhar, P. S.; Armstrong, D. A.; Singh, A. Oxidation of tryptophan and N-methylindole by Ni, Brpc, and $(SCN)_2$' radicals in light and heavy-water solutions: a pulse radiolysis study. *J. Phys. Chem.* **95:**3639–3643; 1991.

[34] Hodgson, E. K.; Fridovich, I. The role of $O_2^{\bullet-}$ in the chemiluminescence of luminal. *Photochem. Photobiol.* **18:**451–455; 1973.

[35] Eriksen, J.; Foote, C. S.; Parker, T. L. Photosensitized oxygenation of alkenes and sulfides via a non-singlet-oxygen mechanism. *J. Am. Chem. Soc.* **99:**6455–6456; 1977.

[36] Bielski, B. H. J.; Cabelli, D. E.; Arudi, R. L.; Ross, A. B. Reactivity of HO2/O2$^{\bullet-}$ radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **14:**1041–1100; 1985.

[37] Candeias, L. P.; Wardman, P.; Mason, M. P. The reaction of oxygen with radicals from oxidation of tryptophan and indole-3-acetic acid. *Biophys. Chem.* **67:**229–237; 1997.

[38] Buetner, G. R. Spin trapping: ESR parameters of spin adducts. *Free Radic. Biol. Med.* **3:**259–303; 1997.

[39] Fridovich, I. Superoxide radicals, superoxide dismutases and the aerobic lifestyle. *Photochem. Photobiol.* **28:**733–741; 1978.

[40] Harriman, A. Further comments on the redox potentials of tryptophan and tyrosine. *J. Phys. Chem.* **91:**6102–6104; 1987.

[41] Fukuzumi, S.; Miyao, H.; Ohkubo, K.; Suenobu, T. Electron-transfer oxidation properties of DNA bases and DNA oligomers. *J. Phys. Chem. A* **109:**3285–3294; 2005.

[42] Jovanovic, S. V.; Simic, M. G. The DNA guanyl radical: kinetics and mechanisms of generation and repair. *Biochim. Biophys. Acta* **1008:**39–44; 1989.

[43] Catalfo, A.; Bracchitta, G.; De Guidi, G. Role of aromatic amino acid tryptophan UVA-photoproducts in the determination of drug photosensitization mechanism: a comparison between methylene blue and naproxen. *Photochem. Photobiol. Sci.* **8:**1467–1475; 2009.

[44] Grob, C. A.; Baumann, W. Die 1,4-Eliminierung unter Fragmentierung. *Helv. Chim. Acta* **38:**594–610; 1955.

[45] Oliveros, E.; Riviere, M.; Malrieu, J. P.; Teichteil, C. Theoretical exploration of the photochemical rearrangement of oxaziridines. *J. Am. Chem. Soc.* **101:**318–-322; 1979.

[46] Kang, H.; Dedonder-Lardeux, C.; Jouvet, C.; Martrenchard, S.; Grégoire, G.; Desfrançois, C.; Schermann, J. -P.; Barat, M.; Fayeton, J. A. Photo-induced dissociation of protonated tryptophan $TrpH^+$: a direct dissociation channel in the excited states controls the hydrogen atom loss. *Phys. Chem. Chem. Phys.* **6:**2628–2632; 2004.

[47] Ronsein, G. E.; Oliveira, M. C. B.; Miyamoto, S.; Medeiros, M. H. G.; Di Mascio, P. Tryptophan oxidation by singlet molecular oxygen [$O_2(^1\Delta_g)$]: mechanistic studies using ^{18}O-labeled hydroperoxides, mass spectrometry, and light emission measurements. *Chem. Res. Toxicol.* **21:**1271–1283; 2008. and the references therein.