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Thymidine radical formation via one-electron transfer oxidation photoinduced by pterin: Mechanism and products characterization

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ABSTRACT

UV-A radiation (320–400 nm), recognized as a class I carcinogen, induces damage to the DNA molecule and its components through different mechanisms. Pterin derivatives are involved in various biological functions, including enzymatic processes, and it has been demonstrated that oxidized pterins may act as photosensitizers. In particular, they accumulate in the skin of patients suffering from vitiligo, a chronic depigmentation disorder. We have investigated the ability of pterin (Ptr), the parent compound of oxidized pterins, to photosensitize the degradation of the pyrimidine nucleotide thymidine 5′-monophosphate (dTMP) in aqueous solutions under UV-A irradiation. Although thymine is less reactive than purine nucleobases, our results showed that Ptr is able to photoinduce the degradation of dTMP and that the process is initiated by an electron transfer from the nucleotide to the triplet excited state of Ptr. In the presence of molecular oxygen, the photochemical process leads to the oxidation of dTMP, whereas Ptr is not consumed. In the absence of oxygen, both compounds are consumed to yield a product in which the pterin moiety is covalently linked to the thymine. This compound retains some of the spectroscopic properties of Ptr, such as absorbance in the UV-A region and fluorescence properties.

1. Introduction

A photosensitized reaction is defined as a photochemical alteration occurring in one molecular entity as a result of the initial absorption of radiation by another molecular entity called 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]. Most of the solar UV energy incident on the Earth surface corresponds to UV-A radiation (320–400 nm), which is not significantly absorbed by DNA, but penetrates deeper in human skin than the more energetic UV-B radiation (280–320 nm). UV-A radiation acts indirectly through photosensitized reactions and is now recognized as a class I carcinogen [3]. Moreover, epidemiological evidence has shown that exposure of humans to artificial UV-A radiation (*i.e.* sun lamps and tanning beds) is a major risk factor for melanoma induction [4–6]. Photosensitization is also important in relation to several applications in disinfection [7,8] and photodynamic therapy (PDT), a medical procedure for the treatment of aged-related macular degeneration, cancer and other pathologies whereby undesired tissue (*e.g.* tumor) can be destroyed *in situ* [9,10].

The chemical changes in biological components resulting from photosensitized reactions can take place through different mechanisms. Photosensitized oxidations 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) [11]. In particular, $^1O_2$ is one of the main reactive oxygen species (ROS) responsible for the damaging effects of light on biological systems (photodynamic effects) and plays a key role in the mechanism of cell death in PDT [12–14]. This ROS reacts selectively with guanine components at the exclusion of other nucleobases and the 2-deoxyribose moiety [15,16]. On the other hand, all the nucleobases may undergo one-electron oxidation, with corresponding ionization potentials in the following order: guanine < adenine < cytosine ~ thymine [17–19]. It is accepted that whereas ionizing radiation is able to ionize all of the four main DNA bases with similar efficiency, most type I photosensitizers produce damage only in guanine base [16]. In addition, hole transfer has been shown to occur in double-stranded DNA [20–22], principally from pyrimidine and adenine radical

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cations to guanine, which acts as a sink. As a consequence, the amount of modified pyrimidine bases in a DNA molecule is lower than the total amount of pyrimidine bases that underwent electron transfer.

It is well documented that direct excitation of pyrimidine bases by absorption of UV photons gives rise to cyclobutane pyrimidine dimers with a predominance of thymine dimers [23,24]. But there is a lack of information about reactions photoinduced by endogenous and exogenous photosensitizers that lead to the ionization of pyrimidine bases. However, the photooxidation of the thymine moiety in different substrates via a type I mechanism has been proven using menadione [25] and benzophenone [26]. In these studies, two pathways leading to different products are proposed: the formation of a 5-(uracilyl) methyl radical ($-CH_2\bullet$) derived from the methyl group of the thymine moiety and the hydration of the thymine radical cation.

Pterins are present in the human epidermis because 5,6,7,8-tetrahydrobiopterin (H_4Bip) is an essential cofactor in the hydroxylation of the aromatic amino acids [27] and participates in the regulation of melanin biosynthesis [28]. Several dihydro and tetrahydropterins are involved in the metabolism of H_4Bip and, hence, are also present in the human skin [29]. Vitiligo is a skin disorder [30] in which the protection against UV radiation fails due to the lack of melanin, the main pigment of skin. In addition, the H_4Bip metabolism is altered [31] and unconjugated oxidized or aromatic pterins accumulate in the affected tissues. These compounds are photochemically reactive in aqueous solution and, upon UV-A excitation, can fluoresce, undergo photooxidation to produce different photoproducts, generate ROS, such as $^1O_2$, and photosensitize the oxidation of biomolecules [32]. Therefore, the photochemistry of pterins and, especially their photosensitizing properties, are of particular interest for the study of this disease.

In the late 1990s it was reported that oxidized pterins are efficient $^1O_2$ photosensitizers [33,34], and that UV-A excitation of pterins induces DNA damage [35]. Later studies provided additional evidence on the photosensitizing capability of pterins to degrade DNA, but contradictory mechanisms were proposed [36,37]. In the context of our investigations on the photosensitizing properties of pterins in aqueous solutions, we have previously demonstrated that pterin (Ptr), the parent unsubstituted compound of oxidized pterins (Fig. 1), and the vitiligo-related pterin derivatives (biopterin, formylpterin and carboxypterin) are efficient photosensitizers inducing the degradation of purine nucleotides (2′-deoxyguanosine 5′-monophosphate (dGMP) [38–40] and 2′-deoxyadenosine 5′-monophosphate (dAMP) [41,42]). In the case of the oxidation of dAMP and dGMP photoinduced by the neutral form of pterins (neutral and acidic media), the predominant mechanism is type I and involves an initial electron transfer from the nucleotide to the triplet excited state of pterins. On the other hand, in alkaline media, where monoanionic forms of pterins are present, the main mechanism for the photosensitized oxidation of dGMP involves $^1O_2$ as the reactive intermediate.

In the present work we report our investigations on the Ptr-photosensitized degradation of thymine in aqueous solutions under UV-A radiation, using the pyrimidine nucleotide thymidine 5′-monophosphate (dTMP, Fig. 1). This compound is highly soluble in water and may be quantified by chromatographic methods. The main objectives of this work were to evaluate the capability of pterins to photoinduce chemical changes in thymine and elucidate the mechanisms involved. We have evaluated the role of molecular oxygen (O_2), and analyzed the products formed under different experimental conditions. The potential biological implications of the results and the proposed mechanisms are discussed.

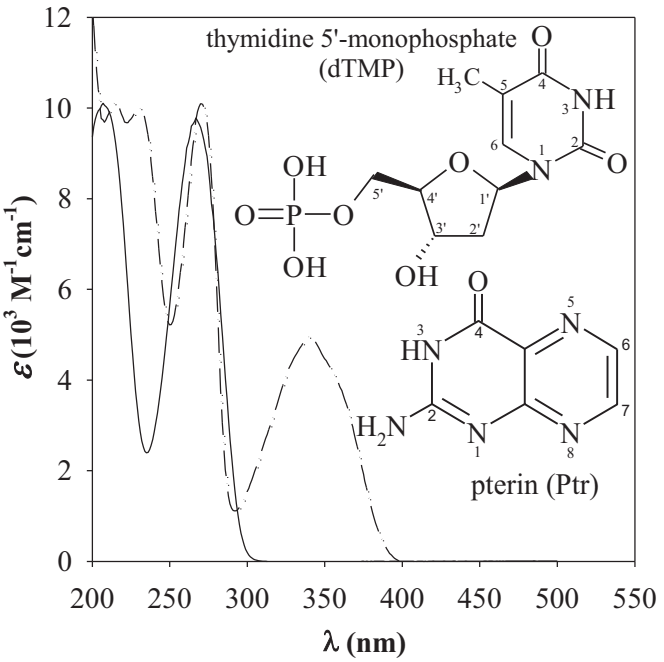


Fig. 1. Molecular structures of Ptr and dTMP, and corresponding absorption spectra in air-equilibrated aqueous solutions at pH 5.5; solid line: dTMP; dashed line: Ptr.

2. Materials and methods

2.1. General

Pterin (Ptr) was purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification. Thymidine 5′-monophosphate (dTMP), formic acid, KI (purity >99%), superoxide dismutase (SOD) from bovine erythrocytes (lyophilized powder, ≥95% biuret, ≥3000 units per mg of protein) and other chemicals were provided by Sigma-Aldrich and used without further purification.

All the experiments were carried out in aqueous solutions and the pH measurements were performed with a pH-meter sensION+pH31 GLP combined with a pH electrode 5010T (Hach). 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.

2.2. Steady-state irradiation

Aqueous solutions containing Ptr and dTMP were irradiated in 1 × 0.4 cm fluorescence quartz cells at room temperature, using 3 Rayonet 3500RPR lamps emitting at 350 nm (bandwidth ≈ 20 nm) (Southern N.E. Ultraviolet Co.). In most of the experiments the Ptr concentration was in the micromolar range because in diseased skin cells such concentrations of pterins have been determined [43]. The concentration of dTMP was about 1 mM, which is much higher than that found in biological systems, but is necessary to obtain products in suitable amounts for their analysis.

The experiments were performed in the presence and absence of dissolved O_2 in the solutions. Experiments with air-equilibrated solutions were performed in open quartz cells without bubbling during irradiation to prevent light scattering. However, to avoid that the consumption of O_2 might lead to hypoxic conditions, the irradiation was interrupted every 30 min and the sample was bubbled with air for 10 min. Argon and oxygen-saturated solutions were obtained by bubbling for 20 min with these gases (Linde,

purity > 99.998%), previously saturated in water. In the case of oxygen-saturated solutions, the same procedure as that explained for air was carried out to ensure a high enough concentration of the gas.

2.3. Analysis of irradiated solutions

2.3.1. UV–vis spectrophotometry

Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer, using quartz cells of 0.4 cm optical path length. The absorption spectra of the solutions were recorded at regular intervals of irradiation time.

2.3.2. High-performance liquid chromatography

A high-performance liquid chromatography equipment Prominence from Shimadzu (solvent delivery module LC-20AT, online degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP, photodiode array (PDA) detector SPD-M20A and fluorescence (FL) detector RF-20A) was employed for monitoring the reaction. A Synergi Polar-RP column (ether-linked phenyl phase with polar endcapping, 150 × 4.6 mm, 4 μm, Phenomenex) was used for product separation. A solution of 25 mM formic acid (pH = 3.2) was used as mobile phase.

2.3.3. Mass spectrometry analysis

The liquid chromatography equipment coupled to mass spectrometry (LC/MS) system consisted of an UPLC chromatograph (ACQUITY UPLC from Waters) coupled to a quadrupole time-of-flight mass spectrometer (Xevo G2-QTof-MS from Waters) (UPLC-QTof-MS). UPLC analyses were performed using an Acquity UPLC BEH C18 (1.7 μm; 2.1 × 50 mm) column (Waters), and isocratic elution with 25 mM formic acid (pH = 3.2) at a flow rate of 0.2 mL min^{-1}. The mass spectrometer was operated in the negative ion mode. Therefore the samples were injected into the chromatograph, the components were separated and then the mass spectra were registered for each peak of the corresponding chromatograms. In addition, mass chromatograms, *i.e.* representations of mass spectrometry data as chromatograms (the x-axis representing time and the y-axis signal intensity), were registered using different scan ranges.

2.4. Electron paramagnetic resonance-spin trapping experiments

Electron paramagnetic resonance (EPR) experiments were performed in order to detect the dTMP radical cation. EPR spectra were collected on a Bruker ESP 500E spectrometer. Samples were irradiated with Rayonet RPR3500 lamps. 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.

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 [44,45]. In our experiments, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) from Sigma was used as the spin trap [46]. Samples (1 mL) contained 180 μM Ptr, 1.3 mM dTMP, buffer Tris/HCl (pH 7.0), and DMPO (50 mM). The O_2-free solutions were irradiated at room temperature in sealable quartz cells of 0.4 cm optical path length. EPR spectra were recorded every minute since the beginning of the irradiation up to 15 min.

2.5. Fluorescence measurements

Steady-state and time-resolved fluorescence measurements were performed at room temperature using a single-photon-counting equipment FL3 TCSPC-SP (Horiba Jobin Yvon), described elsewhere [47].

For a given solution, the decay curve was recorded at multiple emission wavelengths to construct the time-resolved emission spectra (TRES), 3D data set of counts *versus* time and *versus* wavelength. The Global Analysis of TRES, a fit calculation (up to 5 exponentials) performed globally on up to 100 separate decay curves, was carried out using the DAS6 Fluorescence Decay Analysis software.

2.6. Singlet oxygen ($^1O_2$) detection

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 [48,49]. The sample solution (0.8 mL) in a quartz cell (1 cm × 0.4 cm) was irradiated with a pulsed LED source (SpectraLED, maximum emission at 560 nm, light pulse duration 200 μs). The $^1O_2$ emission at 1270 nm was registered and analyzed using the equipment described elsewhere [40].

3. Results and discussion

3.1. Photosensitization of dTMP by pterin in air-equilibrated solutions

The first aim of this work was to find out if upon UV-A irradiation pterin (Ptr) was able to photoinduce damage, not only in the purine nucleotides as previously published [38–42], but also in the pyrimidine nucleotides which are less susceptible to oxidation, partly due to their higher redox potential. Therefore air-equilibrated aqueous solutions containing Ptr and thymidine 5′-monophosphate (dTMP) were exposed to UV-A radiation (350 nm) for different periods of time. The pH range (5.5–6.0) was chosen so that Ptr was present at more than 99% in its acid form (pKa 7.9). The corresponding absorption spectra of Ptr and dTMP (Fig. 1) show that only Ptr was excited under these experimental conditions. The samples were analyzed by UV–vis spectrophotometry and HPLC using the absorbance detector (HPLC-PDA, Section 2). The concentration of dTMP decreased significantly as a function of the irradiation time, whereas the decrease of the Ptr concentration was very slow (Fig. 2, dTMP (•) and Ptr (▾)). Irradiation of Ptr solutions in the absence of dTMP showed that the consumption of Ptr was identical, within experimental error, to that registered in the presence of the nucleotide (Fig. 2, Ptr (Δ)). Consequently, the consumption of the photosensitizer was due to its own photolysis. Additional control experiments showed that no consumption of the nucleotide was detected in dTMP solutions irradiated in the absence of Ptr (Fig. 2, dTMP (○)), thus excluding the possibility of product formation by spurious direct excitation of dTMP. Moreover, dTMP degradation was not observed in solutions containing Ptr and dTMP that were kept in the dark. The products formed in solutions containing Ptr and dTMP under irradiation were analyzed by HPLC and mass spectrometry (*vide infra*).

3.2. Photosensitization of dTMP by pterin in O_2-free solutions

In the experiments carried out in the absence of O_2, under otherwise the same experimental conditions, the dTMP concentration also decreased as a function of irradiation time (Fig. 3). However, the rate of consumption of dTMP was slower than that observed under aerobic conditions (Fig. 2). In addition, a significant consumption of Ptr was also observed (Fig. 3) and such consumption cannot be attributed to the photochemistry of Ptr itself since, as previously reported, this compound is photostable in O_2-free solutions [32]. Moreover, a control experiment carried out in the absence of dTMP

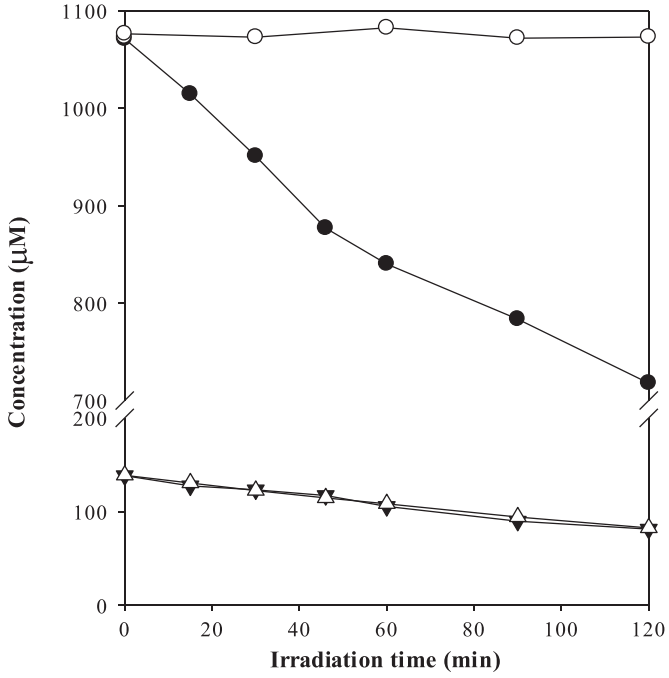


Fig. 2. Time-evolution of the dTMP (•) and Ptr (▾) concentrations under UV-A irradiation of air-equilibrated aqueous solutions (pH 5.5). Control experiments: (○) evolution of the dTMP concentration in the absence of Ptr; (Δ) evolution of the Ptr concentration in the absence of dTMP. Concentrations determined by HPLC analysis ($[Ptr]_0 = 140$ μM), ($[dTMP]_0 = 1170$ μM).

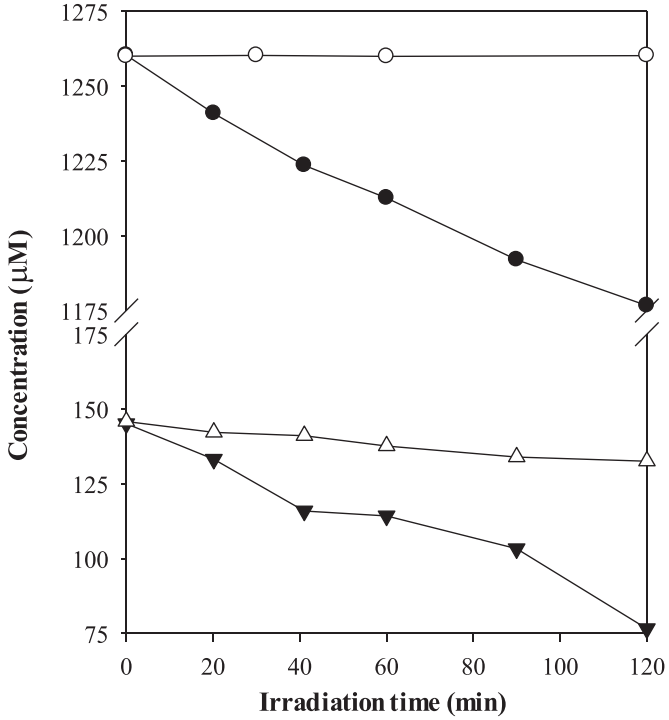


Fig. 3. Time-evolution of the dTMP (•) and Ptr (▾) concentrations under UV-A irradiation of O_2-free aqueous solutions (pH 5.5). Control experiments: (○) evolution of the dTMP concentration in the absence of Ptr; (Δ) evolution of the Ptr concentration in the absence of dTMP. Concentrations were determined by HPLC analysis ($[Ptr]_0 = 140$ μM), $[dTMP]_0 = 1260$ μM).

showed that the decrease of the Ptr concentration was negligible in the time window used (Fig. 3). Interestingly, the rate of dTMP consumption (-0.7 ± 0.1 μM min^{-1}) for the experiment shown in Fig. 3 was equal to the rate of Ptr consumption within experimental error (-0.6 ± 0.1 μM min^{-1}), a result that suggests a photochemical reaction with a 1:1 stoichiometry.

The results presented in this section were surprising since the degradation of purine nucleotides photosensitized by Ptr and other pterin derivatives does not take place under anaerobic conditions [38–42]. The radical ions formed by electron transfer from the purine nucleotide (dGMP or dAMP) to the triplet excited state of Ptr (^{3}Ptr*) (Reaction (1)) undergo an efficient back electron transfer (Reaction (2)):

$$^3P\,tr^* + dGMP/dAMP \rightarrow Ptr^{\bullet -} + dGMP^{\bullet +}/dAMP^{\bullet +} \quad (1)$$

$$Ptr^{\bullet -} + dGMP^{\bullet +}/dAMP^{\bullet +} \rightarrow Ptr + dGMP/dAMP \quad (2)$$

The fact that the consumption of dTMP was faster in aerobic than in anaerobic media suggested that back electron transfer took place in the absence of O_2, but additional reactions consuming dTMP also occurred.

Moreover, other studies demonstrated that molecular oxygen is necessary for the degradation of free amino acids photosensitized by Ptr [50,51]. This is therefore the first time that a photosensitized process consuming Ptr is described. Hence, it is clear that photosensitization of dTMP might involve a mechanism different from those previously reported that deserves a more detailed analysis.

3.3. Participation of the triplet excited state of pterin

The triplet excited states of pterins are reactive species that initiate all the photosensitized processes reported in the literature so far (Section 1). Therefore it is straightforward to assume that this is also the case for the photosensitization of dTMP. A series of experiments was conducted to confirm this hypothesis.

It has been previously demonstrated that iodide (I^-) at micromolar concentrations is an efficient and selective quencher of triplet excited states of pterins [47,52]. The results of photosensitization experiments carried out in air-equilibrated and O_2-free solutions containing dTMP and Ptr in the presence of KI (300 μM) confirmed that, in both cases, the rate of dTMP consumption was slower in the presence of I^- than in its absence (Fig. 4). In addition, under anaerobic conditions, where a decrease of the Ptr concentration was observed upon irradiation (Fig. 3), I^- led to an inhibition of the consumption of the photosensitizer as well (Fig. 4b). Thus, the inhibition of the photosensitized degradation of dTMP by I^- strongly suggests that the process takes place via a purely dynamic mechanism initiated by ^{3}Ptr*.

3.4. Mechanistic pathways: energy transfer and singlet oxygen sensitization

Three types of mechanisms might be responsible for the observed photosensitized process: triplet-triplet energy transfer (TTET) from the photosensitizer (Ptr) to the nucleotide, and type I and/or type II photosensitization (Section 1). The three types of mechanisms are, in general, initiated by the triplet excited state of the photosensitizer.

A triplet-triplet energy transfer between the donor (^{3}Ptr*) and the acceptor (dTMP) may be discarded as the energies of the triplet excited states of Ptr (E_T^{Ptr}) and dTMP (E_T^{dTMP}) have been estimated to be 243 kJ mol^{-1} and 310 kJ mol^{-1}, respectively [53–55]. Since E_T^{Ptr} is much lower than E_T^{dTMP}, such an endothermic energy transfer would be highly inefficient and the TTET process can be ruled out [56].

A type II mechanism might not be relevant since it is accepted that thymine does not react significantly with $^1O_2$ [15,16]. Nevertheless, to the best of our knowledge, the rate constant of the chemical reaction (k_r) between $^1O_2$ and dTMP (Reaction (3)) and

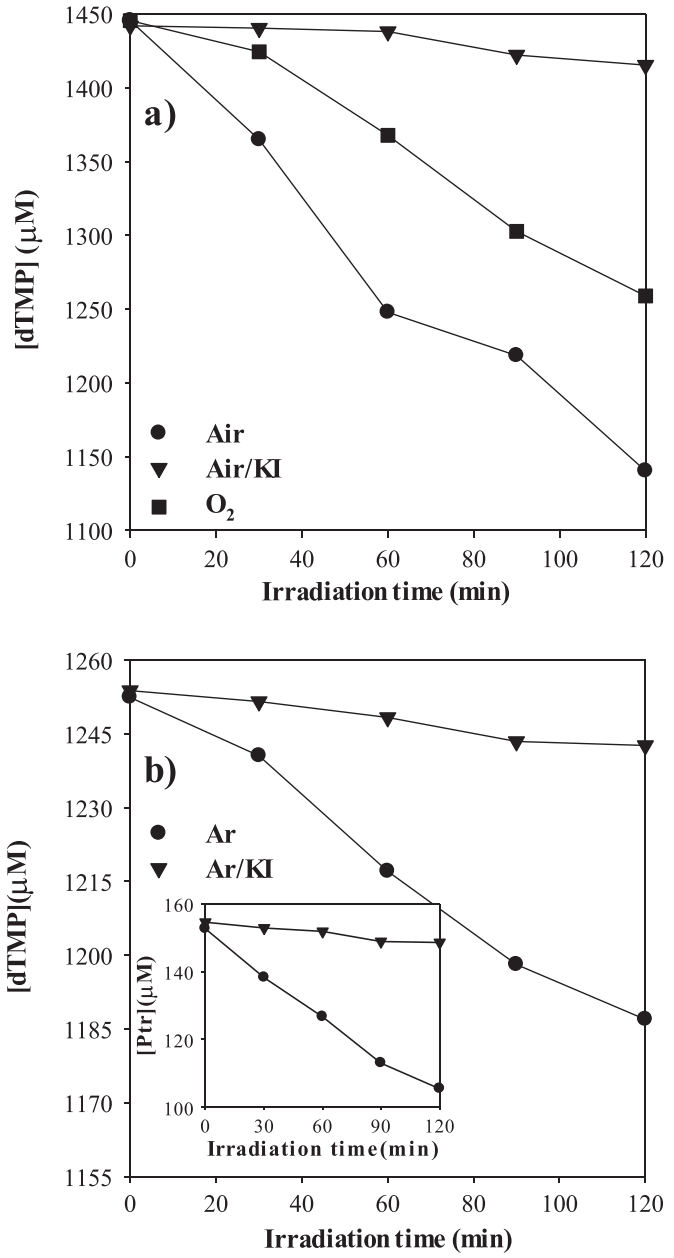


Fig. 4. a) Time-evolution of the dTMP concentration under UV-A irradiation of Ptr: i) in air-equilibrated solutions in the absence (•) and in the presence of KI 300 μM (▾), and ii) in O_2-saturated solutions (■); $[dTMP]_0 = 1445$ μM, $[Ptr]_0 = 150$ μM. b) Time-evolution of the dTMP and Ptr (inset) concentrations under UV-A irradiation of Ar-saturated solutions in the absence (•) and in the presence of KI 300 μM (▾); $[dTMP]_0 = 1250$ μM, $[Ptr]_0 = 155$ μM. Concentrations were determined by HPLC analysis, pH = 5.5.

the rate constant of $^1O_2$ physical quenching (k_q) by dTMP (Reaction (4)) are not known.

$$dTMP + {}^1O_2 \xrightarrow{k_r} dTMPO_2 \quad (3)$$

$$dTMP + {}^1O_2 \xrightarrow{k_q} dTMP + {}^3O_2 \quad (4)$$

Therefore time-resolved experiments in D_2O were performed using rose bengal (RB) as a standard $^1O_2$ photosensitizer (quantum yield of $^1O_2$ production in aqueous solutions: $\Phi_\Delta = 0.75$ [57,58]). The rate constant of $^1O_2$ total quenching by dTMP ($k_t = k_r + k_q$, Reactions (3) and (4)) was determined from the Stern-Volmer analysis of the quenching of the characteristic $^1O_2$ emission in the NIR spectral region by dTMP (see ESI for details, Fig. S1). A k_t value of $7\ (\pm 1) \times 10^4\ M^{-1}\ s^{-1}$ was obtained. This value is about 250 times lower than that reported for dGMP ($1.7\ (\pm 0.1) \times 10^7\ M^{-1}\ s^{-1}$) [38].

The method for the assessment of the role of $^1O_2$ in the photosensitized oxidation of nucleotides has been described in detail elsewhere [38]. Briefly, for a given experiment, the initial rate of oxidation of dTMP by $^1O_2$ $(d[dTMP]/dt)_{i,\Delta}$ can be calculated using (Eq. (5)).

$$\left(\frac{d[dTMP]}{dt}\right)_{i,\Delta} = -k_r[{}^1O_2]_{SS}[dTMP]_i \quad (5)$$

where $[^1O_2]_{ss}$ is the steady-state concentration of $^1O_2$ that depends on the photon flux absorbed by the sensitizer, on its Φ_Δ (0.18 in the case of Ptr [32]) and on the value of τ_Δ under the experimental conditions used.

The initial rate $(d[dTMP]/dt)_{i,\Delta}$ was calculated considering that $k_r = k_t$ (maximum possible value of k_r, if physical quenching is negligible). As expected, results obtained for different initial dTMP and Ptr concentrations showed that, in all cases, the calculated rate $(d[dTMP]/dt)_{i,\Delta}$ was negligible in comparison to the experimental initial rate of dTMP consumption determined by HPLC analysis, thus indicating that a contribution of $^1O_2$ can be discarded, which is in total agreement with previous works that demonstrated that thymine does not react with $^1O_2$ [15,16].

3.5. Electron transfer mechanism: formation of superoxide anion and organic radicals

A predominant mechanism *via* electron transfer (Type I) would be in agreement with previous evidence on the capability of Ptr to photosensitize the oxidation of other nucleotides and amino acids through this type of mechanism [59]. In this case, the photosensitized oxidation would be initiated by an electron transfer from dTMP to the triplet excited state of Ptr (^{3}Ptr*) to form the Ptr radical anion ($Ptr^{\bullet -}$) and the dTMP radical cation ($dTMP^{\bullet +}$) (Reaction (6)). The latter may deprotonate to the corresponding neutral radical ($dTMP(-H)^\bullet$) (Reaction (7)).

$$^3P\ tr^* + dTMP \rightarrow Ptr^{\bullet -} + dTMP^{\bullet +} \quad (6)$$

$$dTMP^{\bullet +} \rightleftarrows dTMP(-H)^\bullet + H^+ \quad (7)$$

The lifetime of ^{3}Ptr* (τ_T) is 3.9 μs and 1.4 μs in the absence of O_2 and in air-equilibrated solutions, respectively [40]. The rate constant of quenching of ^{3}Ptr* by dGMP is close to the diffusion controlled limit and it can be assumed that the corresponding rate constant of quenching by dTMP should be of the same order of magnitude. Therefore the rate of quenching of ^{3}Ptr* by dTMP should be significant in our reaction system and Reaction (6) should take place if the electron transfer process were spontaneous.

The feasibility of this electron transfer process can be evaluated by estimating the free energy change (ΔG) of the reaction using Eq. (8) [60]:

$$\Delta G(eV) = \left[E_{(dTMP+\bullet/dTMP)} - E_{(Ptr/Ptr^{-\bullet})} - \left(e_o^2/4\Pi\epsilon R_{D+A^-}\right)\right] - \Delta E_{0,0} \quad (8)$$

where $E_{(dTMP+\bullet/dTMP)}$ and $E_{(Ptr/Ptr^{-\bullet})}$ are the standard electron potentials of electron donor and acceptor, respectively. These values have already been reported for dTMP ($E_{(dTMP+\bullet/dTMP)} = 1.21$V *vs.* NHE [61]) and Ptr ($E_{(Ptr/Ptr^{-\bullet})} = -0.55$V *vs.* NHE [53]). $\Delta E_{0,0}$ is the energy of the triplet excited state of Ptr ($E_T^{Ptr} = 2.52$ eV [53]); the term $e_o^2/4\Pi\varepsilon R_{D+A^-}$ 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 $-100.4\ J\ mol^{-1}$, thus indicating that electron transfer from dTMP to ^{3}Ptr* can spontaneously occur.

To investigate the formation of dTMP(–H)$^\bullet$ (pKa < 4) [62] in the irradiated solutions, a series of EPR experiments was performed in the presence of the spin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), which reacts with a variety of organic radicals and reactive oxygen species to form stable radical adducts [63], with characteristic EPR spectra.

The experiments were performed under Ar (bubbled for 20 min before irradiation) to avoid the interference of the superoxide anion ($O_2^{\bullet-}$) in the investigation of the dTMP radical. Indeed, it has been reported that an aqueous solution of Ptr exposed to UV-A produces $O_2^{\bullet-}$ [64]. The process involves an electron transfer between Ptr in its ground state and ^{3}Ptr* leading to the corresponding pair of radical ions (Ptr$^{\bullet+}$ and Ptr$^{\bullet-}$) (Reaction (9)), followed by electron transfer from Ptr$^{\bullet-}$ to O_2 and superoxide formation (Reaction (10)). DMPO reacts very efficiently with $O_2^{\bullet-}$ to form the adduct DMPO$^\bullet$ – OOH (Reaction (11)), which decays to the more stable adduct DMPO$^\bullet$ – OH with a characteristic EPR spectrum [65].

$$Ptr^* + Ptr \rightarrow Ptr^{\bullet+} + Ptr^{\bullet-} \quad (9)$$

$$Ptr^{\bullet-} + O_2 \rightarrow Ptr + O_2^{\bullet-} \quad (10)$$

$$DMPO + O_2^{\bullet-} + H^+ \rightarrow DMPO^\bullet - OOH \quad (11)$$

Before irradiation, no EPR signal was detected in Ar-bubbled solutions (pH 7.2) containing Ptr (180 μM) and DMPO (50 mM) in the absence of nucleotide (Fig. 5a). However, irradiation led to the immediate formation of an EPR signal (Fig. 5b), characterized by hyperfine coupling constants $A_N = A_H = 14.9$ G and corresponding to the adduct DMPO$^\bullet$ – OH [65]. This result shows that, despite Ar bubbling, some $O_2^{\bullet-}$ was formed due to the remaining O_2.

A more complex EPR signal consisting of two superimposed EPR spectra was registered after irradiation of solutions bubbled with Ar and containing Ptr (180 μM), dTMP (1.3 mM) and DMPO (50 mM) (Fig. 5c and d). One of the EPR spectra corresponds to the adduct DMPO$^\bullet$ – OH, which was expected considering the results of the control carried out in the absence of the nucleotide (Fig. 5b). The other spectrum was composed of six lines (Fig. 5d) having hyperfine coupling constants of $a_N = 16.3$ G, $a_H = 23.7$ G (g = 2.0060), which is typical of a DMPO adduct obtained from a carbon-centered radical. Moreover, the recorded spectrum is identical, within experimental error, to that reported for an adduct DMPO$^\bullet$ – thymidine [66].

Therefore, we have demonstrated that an electron transfer from the nucleotide to ^{3}Ptr* is thermodynamically feasible and that, in fact, dTMP radicals are formed when a solution containing both Ptr and the nucleotide is irradiated. Hence, it can be concluded that the process observed takes place via a type I mechanism and is initiated by Reaction (6).

Comparative photolysis experiments carried out in air-equilibrated and O_2-saturated solutions clearly showed that the rate of dTMP disappearance was much greater in the former than in the latter solutions (Fig. 4a), which apparently contradicts the fact that the photosensitized reaction is faster in air-equilibrated than in Ar-saturated solutions (*vide supra*). It has been demonstrated that, in contrast to the singlet excited state of Ptr (^{1}Ptr*), its triplet excited state (^{3}Ptr*) is efficiently quenched by O_2 [32,40]. Therefore the decrease in the rate of nucleotide consumption in O_2-saturated solutions may be interpreted as the result of the competition between the reaction of ^{3}Ptr* with dTMP and its quenching by O_2. The overall behavior can be interpreted assuming that there is an optimal concentration of O_2 for the rate of the dTMP consumption: lack of O_2 favors back electron transfer to recover dTMP and high

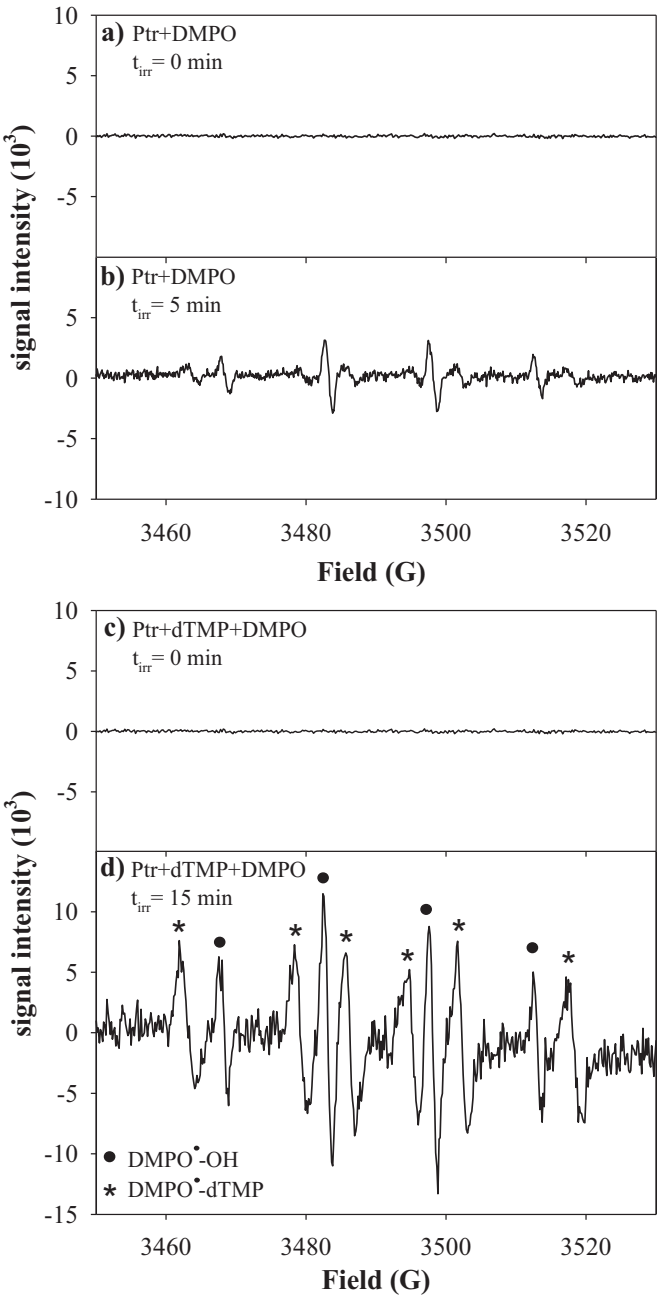


Fig. 5. EPR experiments performed in Ar-bubbled solutions (buffer Tris/HCl, pH = 7.2), using DMPO (50 mM) as a spin trap. Signals registered in solutions of Ptr (180 μM) before (a) and after 5 min of UV-A irradiation (b). Signals registered in solutions of Ptr (180 μM) and dTMP (1.3 mM) before (c) and after 15 min of UV-A irradiation (d).

O_2 concentration quenches ^{3}Ptr* decreasing the efficiency of electron transfer.

As mentioned above, $O_2^{\bullet-}$ is formed in our reaction system in the presence of O_2 (Reaction (10)). In the case of dGMP, it has been reported that $O_2^{\bullet-}$ reacts with oxidizing guanine radical very fast according to two main competitive mechanisms: chemical repair with the restoration of the guanine through electron transfer (Reaction (12)) [67,68] and addition, leading to the predominant formation of 2,5-diamino-4H-imidazolone (Iz) (Iz 5′-monophosphate (IzMP) for guanine in nucleotides) (Reaction (13)) [69,70].

$$dGMP(-H)^\bullet + O_2^{\bullet-}/HO_2^\bullet \rightarrow dGMP + O_2 \quad (12)$$

$$dGMP(-H)^{\bullet} + O_2^{\bullet-}/HO_2 \rightarrow IzMP \quad (13)$$

$$dGMP^{\bullet+} + HO^- \rightarrow dGMP(ox) \quad (14)$$

Due to the former process (Reaction (12)) and secondary oxidation pathways (*e.g.* Reaction (14)), the oxidation of guanine photosensitized by Ptr is faster in the presence of superoxide dismutase (SOD), an enzyme that catalyzes the conversion of $O_2^{\bullet-}$ into H_2O_2 and O_2 [71], than in its absence. To investigate the participation of $O_2^{\bullet-}$ in the mechanism of photosensitization of dTMP, we performed experiments in the presence of SOD. The data showed that the rate of dTMP consumption was not affected when SOD was present in the solution (ESI, Fig. S2). These results indicate that $O_2^{\bullet-}$ does not react significantly with $dTMP^{\bullet+}/dTMP(-H)^{\bullet}$ and, hence, is not involved in the photosensitized process.

3.6. Analysis of the photoproducts formed in the presence of O_2

Solutions containing Ptr and dTMP, before and after irradiation, were analyzed by HPLC-PDA and HPLC coupled to the fluorescence detector (HPLC-FL) (Section 2.3.2). Several photoproducts were detected by the former technique with retention times (t_r) lower and higher than that corresponding to the intact nucleotide. None of the products absorbed above 320 nm. Analysis with the fluorescence detector showed that the only fluorescent compound in the treated solutions, when excited at 340 nm, was the photosensitizer, indicating that no fluorescent products were formed. This point is relevant in the context of the results that will be presented in the next section.

To characterize the photoproducts a qualitative analysis was carried out by means of UPLC coupled to mass spectrometry (UPLC-QTof-MS, see Section 2.3.3). The solutions containing dTMP and Ptr at pH 5.5 were analyzed in both positive and negative ion modes (ESI^+ and ESI^-, respectively). As expected, the signals corresponding to the intact molecular ion of Ptr as $[M+H]^+$ and $[M-H]^-$ species at m/z 164.1 Da and 162.1 Da, respectively, were observed. However, in the case of dTMP, the resolution was much better in ESI^- than in ESI^+ mode. Therefore all the results presented in this section correspond to mass spectrometry analysis carried out in the former mode. In this way, the signal corresponding to the intact molecular ion of dTMP as $[M-H]^-$ species at m/z 321.05 Da was registered for the analysis of the dTMP peak.

The MS/MS spectrum in the ESI^- mode of dTMP was recorded (Fig. 6) and the observed fragmentation was in agreement with previous results obtained using soft ionization MS methods [72]. Briefly, the loss of the thymine (Thy) base is a prominent reaction and occurs *via* a 1,2-elimination, yielding the base as a deprotonated anion ($[Thy-H]^-$, m/z=125.03 Da) and a fragment corresponding to the 2′-deoxyribose 5′-phosphate ($[M-Thy-2H]^-$, m/z=195.01 Da) (Fig. 6). Following the loss of base, the 3′-C–OH and 4′-C–H bonds are cleaved to yield the $[M-Thy-2H-H_2O]^-$ ion at *m/z* 177.00 Da. Finally, the typical species $[PO_3]^-$ and $[PH_2O_4]^-$ at *m/z* 78.96 and 96.97 Da were also detected.

Irradiated solutions were analyzed in the same way and the mass spectra corresponding to the chromatographic peaks of the photoproducts were registered. Although many products could be detected, three main compounds were registered, all of them having molecular weights higher than that corresponding to the intact nucleotide and could be attributed to the following species: $[M+O-H]^-$ at m/z=337.04 Da, $[M+O-3H]^-$ at m/z=335.03 Da and $[M+2O+H]^-$ at m/z=355.06 Da, with M=dTMP. Therefore these products will be named as P338, P336 and P356, respectively. It is worth mentioning that two well defined chromatographic peaks presented m/z=337.0450 Da (t_r=2.2 and 2.8 min), revealing that two isomeric products are formed with a molecular weight of 338 Da. To get more information on the structure of these products, their MS/MS spectra were registered and compared to that corresponding to dTMP.

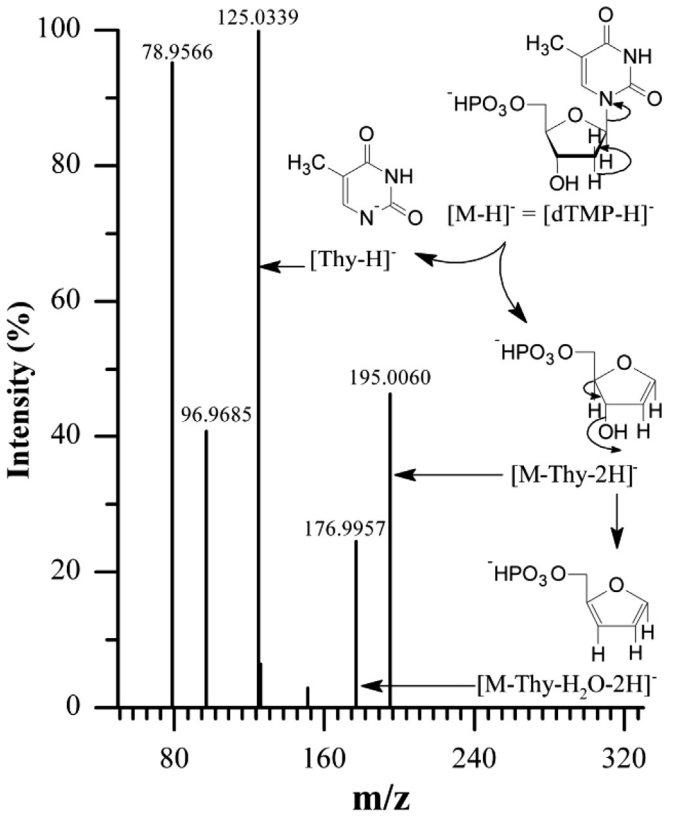


Fig. 6. MS/MS spectrum of dTMP recorded in ESI^- mode and fragmentation of dTMP via a 1,2-elimination reaction obtained using soft ionization MS methods. Arrows indicate the peaks corresponding to each fragment.

The MS/MS spectra of the two products P338 were similar, although not identical and a signal corresponding to Thy containing an additional oxygen atom in its structure was observed at *m/z* 141.03 Da ($[Thy+O-H]^-$) (Fig. 7). A peak at m/z 294.04 Da, resulting from the loss of a fragment CO-NH from the base, was registered. The typical fragments corresponding to the 2′-deoxyribose 5′-phosphate moiety, $[M-Thy-2H]^-$ at m/z 195.01 Da and $[M-Thy-2H-H_2O]^-$ at *m/z* 177.0 Da, and to $[PO_3]^-$ at *m/z* 79.0 and $[PH_2O_4]^-$ at *m/z* 97.0 Da, were also detected. Therefore, the oxidation took place on the Thy moiety and resulted in the incorporation of one oxygen atom. To the best of our knowledge, this is the first time that this type of products is described for the photosensitization of dTMP.

However, the oxidation of thymidine (Thd) photosensitized by benzophenone has been studied and 5-(hydroxymethyl)-2′-uridine (5-HmdUrd) has been reported as an oxidation product [26,56,73]. Similarly, in our case, one of the products with MW 338 Da might be the equivalent of 5-HmdUrd; *i.e.* 5-(hydroxymethyl)-2′-deoxyuridine 5′-monophosphate (5-HmdUMP), resulting from the oxidation of the Thy methyl group to hydroxymethyl. It has been reported that loss of H_2O from the hydroxymethyl group is a typical fragmentation of 5-HmdUrd [74]. The MS/MS spectrum of the product with t_r 2.8 min showed two peaks that were not registered for the product with t_r 2.2 min (Fig. 7). One of them is a small peak at m/z 319 Da ($[M-H-H_2O]^-$) and another one at m/z 276 Da ($[M-H-CONH-H_2O]^-$), that is, resulting from the loss of H_2O from the fragment at 294 Da. These results strongly suggest that the product P338 with t_r 2.8 min is 5-HmdUMP.

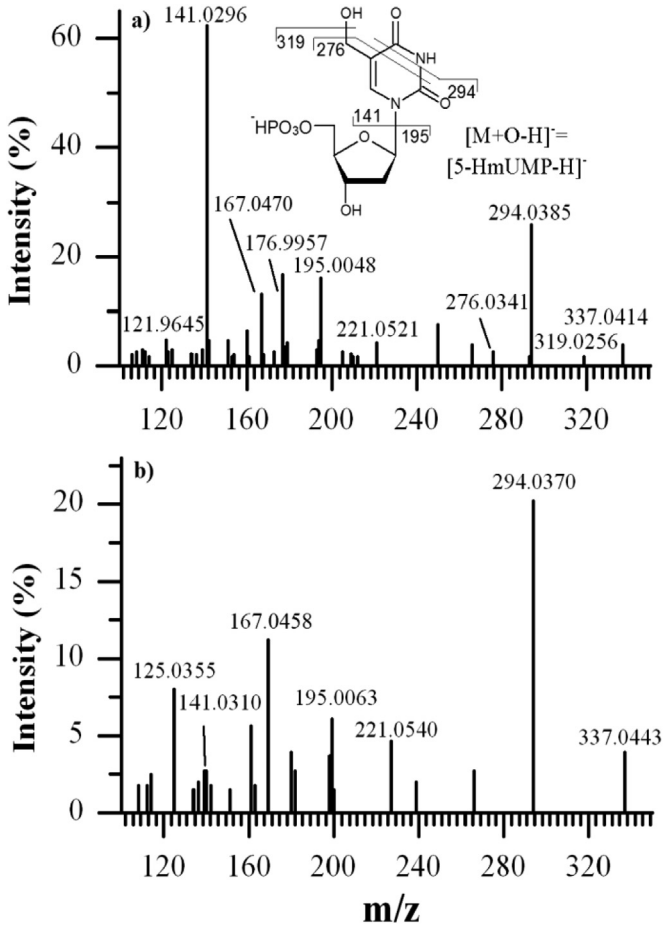


Fig. 7. MS/MS spectra recorded in ESI⁻ mode of the products P338 formed by Ptr photosensitization of dTMP in the presence of O_2. a) product with t_r=2.8 min; b) product with t_r=2.2 min. The chemical structure proposed for the former, 5-HmdUMP (5-(hydroxymethyl)-2′-deoxyuridine 5′-monophosphate), is depicted (M=dTMP).

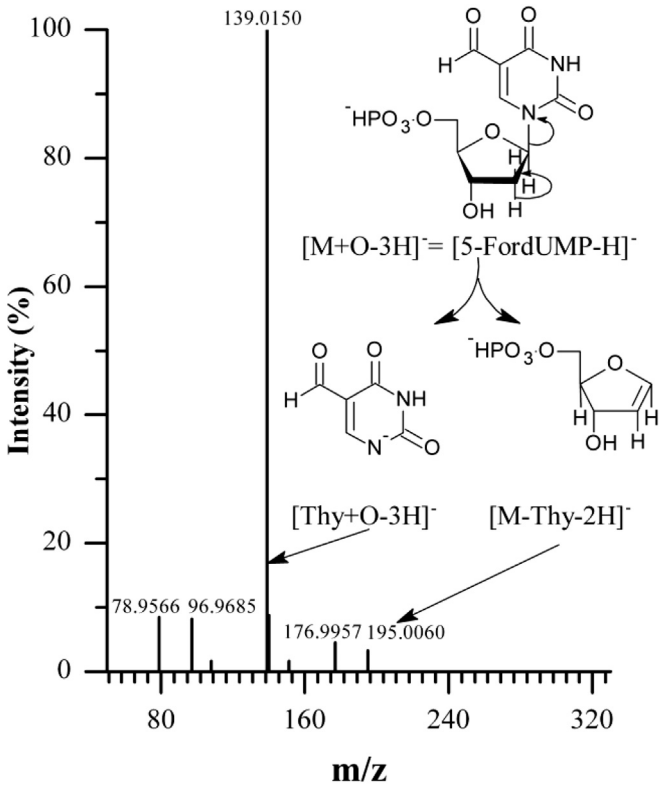


Fig. 8. MS/MS spectra recorded in ESI⁻ mode of the product P336 formed by Ptr photosensitization of dTMP in the presence of O_2. The chemical structure of the photoproduct proposed, 5-formyl-2′-deoxyuridine 5′-monophosphate (5-FordUMP), is depicted (M=dTMP).

Position 6 of the base moiety is also available for oxidation, and 6-hydroxythymidine 5′-monophophate (6-OHdTMP), or its keto tautomer (6-oxo-dTMP) containing the 6-oxo-thymine moiety (5-methylbarbituric acid), could be formed in the process. However, we can discard this product P338 with t_r 2.2 min is 6-oxo-dTMP because it has been reported that its fragmentation is quite different from that registered in our experiments [74]. The generation of 5-(hydroperoxymethyl)-2′-deoxyuridine 5′-monophosphate (5-HPmdUMP) might alternatively be considered since 5-(hydroperoxymethyl)-2′-deoxyuridine was reported to be formed in the photosensitization of Thd by 2-methyl-1,4-naphthoquinone, a type I photosensitizer, and partly decompose into 5-HmdUrd [75]. In our case, weak signals corresponding to a species $[M+2O-H]^-$ (m/z =353.04 Da) were registered in the MS spectra at t_r values coinciding with the second product P338. These results suggest that the hydroperoxide 5-HPmdUMP is very likely formed and the species assigned to a product P338 with t_r 2.2 min could have been produced by decomposition of 5-HPmdUMP. Since the amount of hydroperoxide present in the solution was quite low, no further significant changes could be detected in the composition of the irradiated solution kept in the dark.

The MS/MS spectrum of P336 did not show the typical fragment corresponding to the base at m/z=125.03 Da, and a signal corresponding to Thy containing an oxygen atom in its structure was observed at m/z 139.03 Da ($[Thy+O-3H]^-$) (Fig. 8). The fragments corresponding to the 2′-deoxyribose 5′-phosphate moiety, $[M-Thy-2H]^-$ at m/z 195.01 Da and $[M-Thy–2H-H_2O]^-$ at m/z 177.0 Da, and to phosphate, $[PO_3]^-$ at m/z 79.0 and and $[PH_2O_4]^-$ at m/z 97.0 Da, were detected. These results show that the oxidation takes place again in the Thy moiety. Taking into account that the increase in the m/z value of the fragment corresponding to the base was 14 Da and the previous characterization of the products of the photosensitized oxidation of Thd [26,56], we propose that P336 is 5-formyl-2′-deoxyuridine 5′-monophosphate (5-FordUMP) (Fig. 8).

Two products were found with molecular ions at m/z 355.06 Da (products P356), which suggests that two oxygen atoms were incorporated in the structure of dTMP. Products of photosensitized reactions containing two oxygen atoms in the Thy moiety have been reported [26,56]. The MS/MS spectra were identical for the two products P356 (Fig. 9). The comparison of these spectra with that corresponding to oxidation products of the Thy moiety reported for the treatment of oligodeoxynucleotides with osmium tetraoxide [76] suggests that these products are diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine 5′-monophophate (thymidine glycol 5′-monophophate, dTMPGly). Beside the phosphate fragments ($[PO_3]^-$ and $[PH_2O_4]^-$), four other peaks corresponding to the reported fragmentation of thymidine glycol (ThdGly) were observed at m/z values of 142.01 Da ($[Thy+2OH-NH_3-H]$), 194.02 Da ($[M-Thy+NH_2-H-H_2O]$), 212.03 Da ($[M-Thy+NH_2-H]$) and 238.01 Da ($[M-Thy+NCO-H]$) (Fig. 9).

Finally, two other products were detected in the MS spectra, with signals at m/z=371.05 Da and 325.04 Da, both of them having weak signals so that no suitable MS/MS spectra could be recorded. The former can be attributed to a species $[M+3O-3H]^-$ (M=dTMP), which may correspond to 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine 5′-monophosphate (5-HP-6-OHdTMP). The equivalent hydroperoxide derived from Thd has been also

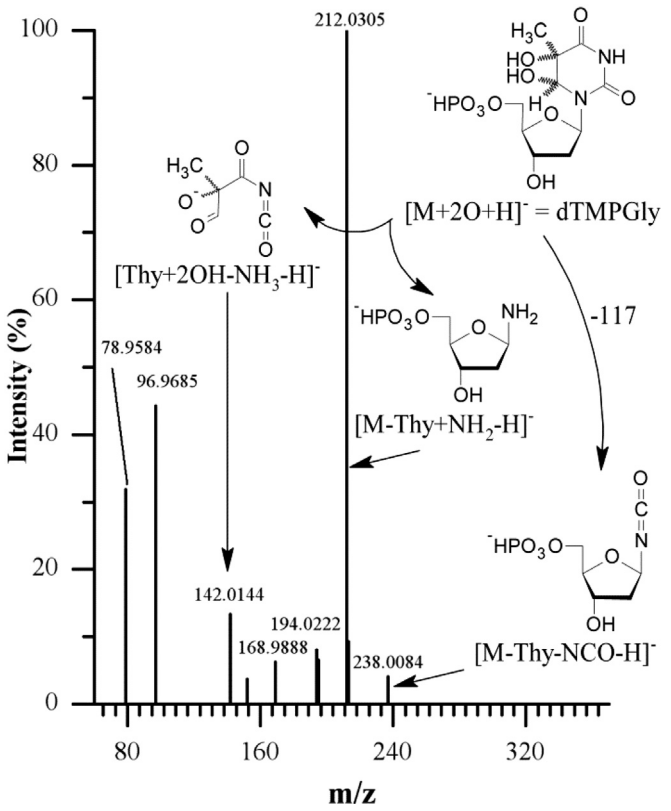


Fig. 9. MS/MS spectra recorded in ESI$^-$ mode of the product P356 formed by Ptr photosensitization of dTMP in the presence of O_2. The chemical structure of the photoproduct proposed is depicted (M=dTMP).

identified as a photoproduct of photosensitization of the nucleoside by 2-methyl-1,4-naphthoquinone [75], which partially decomposes to 5-hydroxy-5-methylhydantoin and ThdGly. The equivalent 1-(2-deoxy-beta-D-erythro-pentofuranosyl-5-phosphate)-5-hydroxy-5-methylhydantoin (5-OH-5MHMP) has a molecular weight of 326.04 Da and therefore the photoproduct with a signal at *m/z* 325.04 Da may be assigned to this compound.

The formation of the photoproducts found can be explained taking into account reactions of dTMP$^{\bullet+}$ previously reported [75,77,78]. The two main competitive reactions of dTMP$^{\bullet+}$ are deprotonation (Reaction (7)) and hydration (Reaction (15)) (Scheme 1).

$$dTMP^{\bullet+} + HO^- \rightarrow dTMP(OH)^{\bullet} \quad (15)$$

The former pathway leads mainly to the formation of the (5-uracilyl)-methyl radical, which traps oxygen on the methylene group, the resulting peroxyl radical yielding 5-HmdUMP and 5-FordUMP (Scheme 1), through tetroxide formation and decomposition [79,80]. Alternatively, reduction of the peroxyl radical leads to the corresponding hydroperoxide, 5-HPmdUMP, which can decompose into HmdUMP and 5-FordUMP (Scheme 1).

Hydration, in turn, leads predominantly to the 6-hydroxy-5,6-dihydrothym-5-yl radical that traps oxygen on position 5 [75,81] to yield the corresponding peroxyl radical, which by reduction gives rise to a group of diastereomers of the hydroperoxide 5-HP-6-HOdTMP (Scheme 1). These hydroperoxides decomposes to 5-HO-5MHMP and dTMPGly (Scheme 1) [75]. It has also been reported that the peroxyl radical yields 5-OH-5MHMP and dTMPGly, without 5-HP-6-OHdTMP as an intermediate (Scheme 1) [25,82].

3.7. Analysis of the photoproducts formed in the absence of O_2

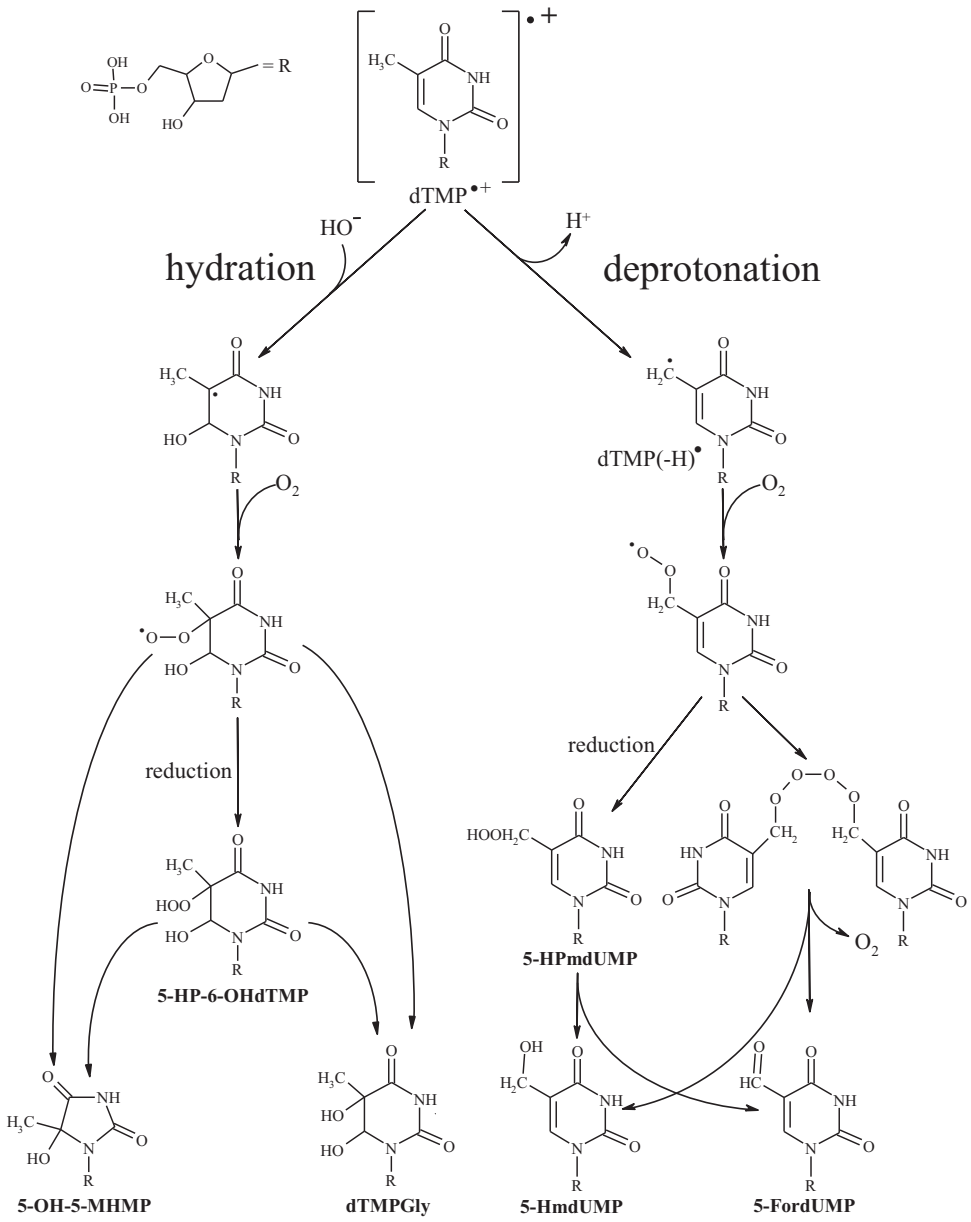
HPLC-PDA analysis of irradiated O_2-free solutions showed that a main product was formed, with a retention time (t_R) value higher than those corresponding to both dTMP and Ptr. Its absorption spectrum presented a band similar to the typical low-energy band of pterins (Fig. 10a). HPLC-FL analysis revealed that the main product found using HPLC-PDA was fluorescent and emitted at 450 nm when excited at 340 nm (Fig. 10b), which is compatible with the fluorescence properties of pterins. Considering the decrease in the Ptr concentration, these results suggested that the main product detected contains the Ptr moiety. The other chromatographic peaks observed could correspond to minor products formed under anaerobic conditions or products of the aerobic photosensitization at very low concentrations due to traces of remaining O_2. It is worth mentioning that non-fluorescent products absorbing below 300 nm, which is expected for dTMP degradation products, were not detected.

The absorption and emission properties of the irradiated solutions were analyzed and compared to those of solutions kept in the dark. The absorption spectrum of a solution containing dTMP (1255 μM) and Ptr (155 μM) at pH=5.5 almost did not change after 2 h of irradiation, although the Ptr concentration, determined by HPLC, decreased significantly (to 105.4 μM). This fact is in agreement with the HPLC-PDA analysis and confirms that the reaction leads to a product with spectral properties similar to those of Ptr itself.

In another set of experiments, excitation-emission matrices of irradiated and non-irradiated solutions were recorded after a 1:10 dilution (ESI, Fig. S3) and it was observed that irradiation led to a decrease in the overall emission of the solution. Spectra obtained by excitation into the low-energy pterin band showed that the emission intensity decreased, but the maximum remained unchanged (Fig. 11). This suggests that the photoproduct containing the pterin moiety presents an emission spectrum similar to Ptr, but with a lower fluorescence quantum yield.

Time-resolved experiments were carried out on irradiated and non-irradiated O_2-free solutions ($[Ptr]_0$= 15.5 μM, $[dTMP]_0$= 125.5 μM, pH 5.5). The study was performed by excitation at 341 nm and the corresponding fluorescence decays were recorded at 450 nm. First-order kinetics were observed for the decays before irradiation, with fluorescence lifetime (τ_F) of 8.4 ± 0.4 ns, which is in agreement with the reported τ_F of Ptr in aqueous solution at pH 5.5 [32]. In contrast, the emission decays of solutions irradiated for 2 h were clearly bi-exponential with a short-lived component, with a τ_F about 2 ns, and a long-lived component, with a τ_F value equal to that of Ptr, within experimental error (Fig. 12). To separate the emission of the two components, the remaining Ptr and the fluorescent photoproduct(s), time resolved emission spectra (TRES) were registered, by exciting at 341 nm and recording the corresponding decays in the wavelength range 360–560 nm. The global analysis of TRES also revealed the presence of two fluorescent components. The corresponding spectra were obtained from the corrected plots of the pre-exponential factors as a function of the wavelength (Fig. 12). The spectrum and the τ_F value of the long-lived component matched very well those previously published for Ptr [32], and, therefore, it was assigned to the remaining Ptr. A τ_F value of 2.0 ns was determined for the short-lived component and its spectrum was similar to that of Ptr. This component was assigned to the fluorescent product.

Analysis by means of UPLC-QTof-MS was carried out and a signal at *m/z* 482,08 Da was registered for the chromatographic peak of the main photoproduct. The *m/z* value found corresponds to the molecular ion of a compound bearing both the photosensitizer and the substrate moieties ([Ptr+dTMP–3H]$^-$). In the MS/MS spectrum of the product (Fig. 13), the typical fragments



Scheme 1. Pathways proposed for the formation of the photoproducts found upon irradiation of aqueous solutions of dTMP in the presence of Ptr and O_2.

corresponding to the 2′-deoxyribose 5′phosphate moiety at *m/z* 195.01 Da and 177.0 Da were observed, thus indicating that the chemical changes did not take place in this part of the molecule. The intensity of the typical fragment of the nucleobase at *m/z*=125.03 Da was almost negligible and no ion that could be assigned to the Ptr moiety was observed. However, an intense signal at *m/z* 286.07 was present, which can be attributed to a fragment bearing both the Ptr and the Thy moieties ($[Ptr+Thy-3H]^-$).

These results strongly suggest that after the electron transfer step (Reaction (6)), the radicals ions (or neutral radicals formed by protonation/deprotonation) combine to yield an adduct where the pterinic moiety is attached to the nucleobase (Reaction (16)).

$$Ptr^{\bullet-}/PtrH^{\bullet} + dTMP^{\bullet+}/dTMP(-H)^{\bullet} \rightarrow \quad \rightarrow (Ptr\text{–}dTMP)(-2H) \qquad (16)$$

Moreover, as indicated above, the analyzed product has absorption as well as emission spectra similar to those of Ptr, but with a lower fluorescence quantum yield. These findings are an additional indication that the chemical structure of Ptr is intact in the product and that the low fluorescence quantum yield is due to the attachment to the nucleotide that acts as internal quencher. Therefore positions 6 or 7 of the Ptr moiety (Fig. 1) are the most suitable for the link to dTMP. Taking into account that the analyzed product has a mass corresponding to Ptr-dTMP(-2H), that dTMP(−H)• may be stabilized on the methyl group of the base (methylene radical) and that no conjugation between the two ring systems was observed, we propose the chemical structure depicted in Fig. 13 for this product. A mechanism for its formation is presented in Scheme 2.

After the electron transfer step (Reaction (6)) in the absence of O_2, the coupling of the deprotonated form of $dTMP^{\bullet+}$ ($dTMP(-H)^{\bullet}$) neutral methylene radical derived from the methyl group of the thymine moiety, with the protonated radical anion of Ptr ($PtrH^{\bullet}$) may be proposed as the main reaction. Possible tautomeric forms for $PtrH^{\bullet}$ are C-centered radicals on the C-6 or C-7 position of the pterin moiety, as a result of the protonation of one of the N atoms in the pyrazine ring. Coupling of these radicals would yield a

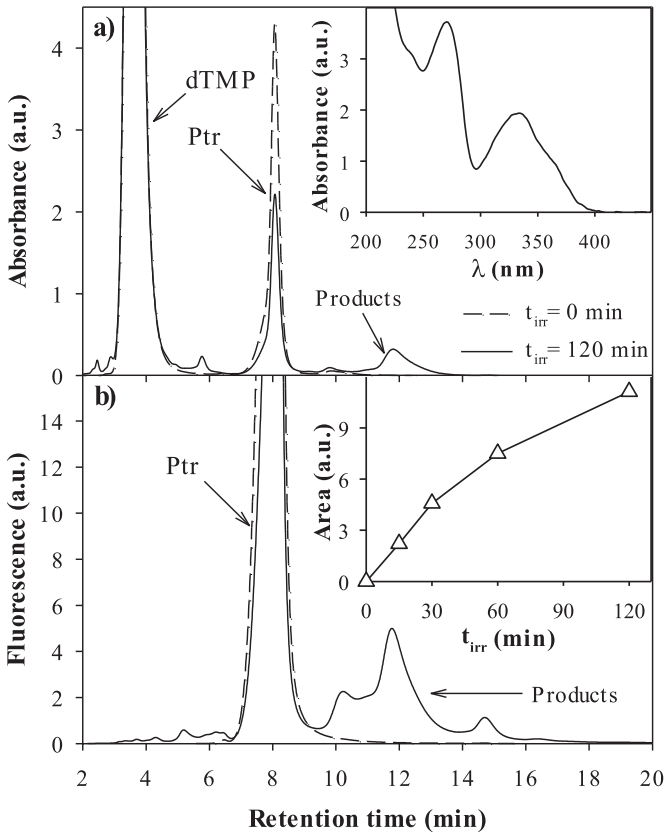


Fig. 10. a) Chromatograms obtained by HPLC-PDA analysis at 280 nm, before and after 2 h of irradiation. Inset: absorption spectrum of the main photoproduct. b) Chromatograms obtained by HPLC-FL analysis (λ_{exc}=340 nm, λ_{em}=450 nm) nm, after 2 h of irradiation. Inset: evolution of the peak area of the main photoproduct. $[Ptr]_0$=151 μM, $[dTMP]_0$=1600 μM, pH=5.5.

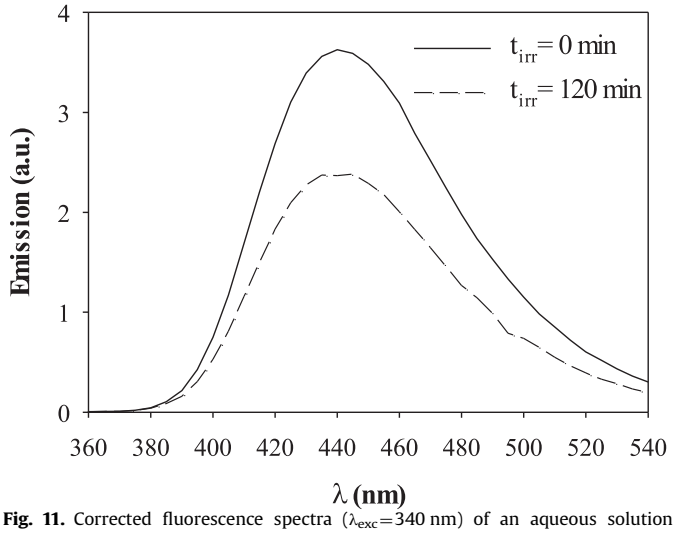


Fig. 11. Corrected fluorescence spectra (λ_{exc}=340 nm) of an aqueous solution (pH=5.5) of dTMP (125.5 μM) and Ptr (15.5 μM) before and after 2 h of irradiation.

primary adduct containing a dihydropterin moiety (H_2Ptr-dTMP(-H)) where the dTMP unit is linked through the CH_2 group to position 6 or 7 of the 5,6-(or 7,8-)dihydropyrazine ring (Scheme 2). This adduct has the same mass as the sum of the masses of Ptr and dTMP. Re-aromatization when the solution is exposed to air would yield the observed fluorescent product Ptr-

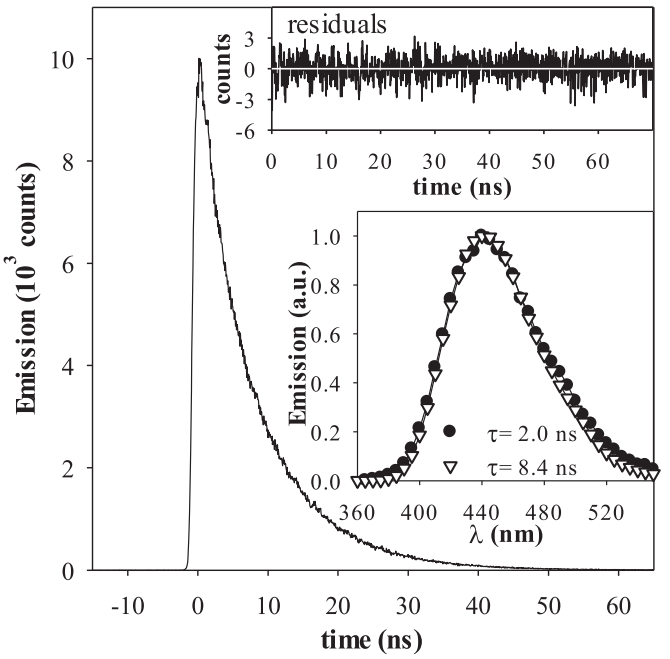


Fig. 12. Emission decay recorded at 450 nm (λ_{exc}=341 nm). Upper inset: residual analysis. Lower inset: Spectra obtained by global analysis of TRES for the short-lived and long-lived components (λ_{exc}=341 nm). $[dTMP]_0$=125.5 μM and $[Ptr]_0$=15.5 μM, Ar-saturated aqueous solutions at pH=5.5 irradiated for 2 h.

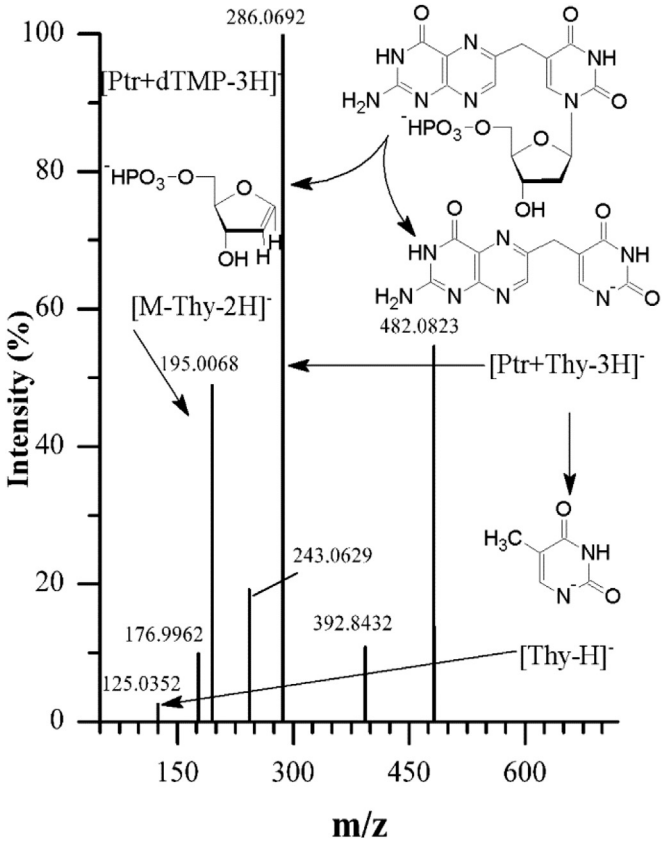
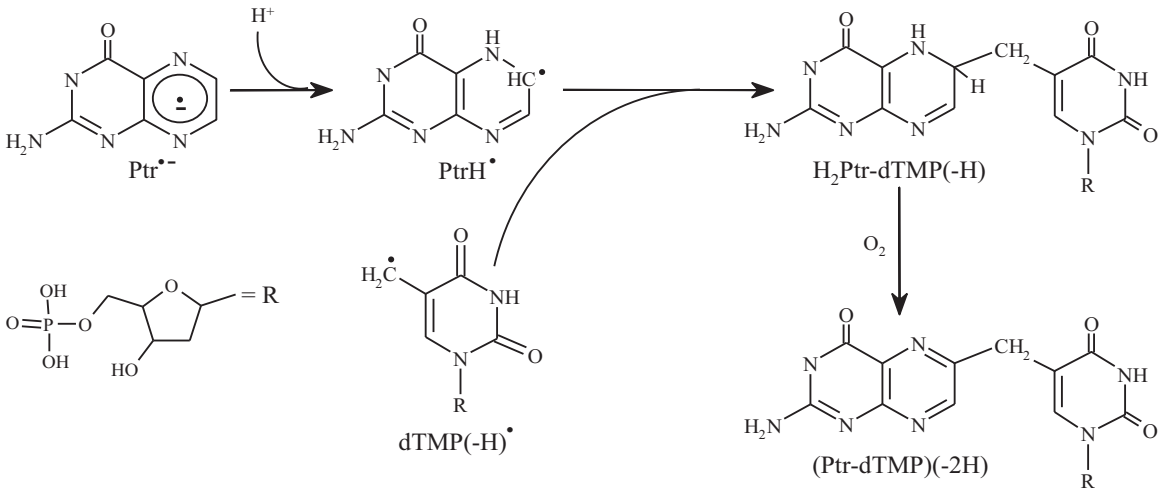


Fig. 13. MS/MS spectra recorded in ESI^- mode of the main product formed by Ptr photosensitization of dTMP in the absence of O_2. The proposed chemical structure of the product and its fragmentation are shown. Arrows indicate the peaks corresponding to each fragment.

dTMP(-2H) where the Ptr conjugated structure is regenerated. It should be noted that very low amounts of the coupling product Ptr-dTMP(-2H) have been detected in the presence of O_2.

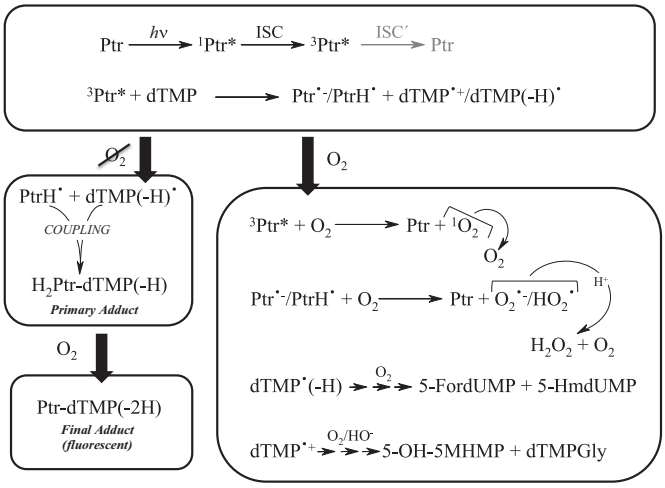


Scheme 2. Mechanism proposed for the formation of the coupling product Ptr-dTMP(-2H); note that the radical in PtrH· could also be on the C-7 position.

4. Conclusions

The degradation of the thymidine 5′-monophosphate (dTMP) photosensitized by pterin (Ptr), the parent compound of oxidized pterins, in aqueous solution under UV-A irradiation was investigated. The mechanism of the photodegradation, summarized in Scheme 3, depends on the presence of dissolved oxygen in the solution. After excitation of Ptr and formation of its triplet excited state (^{3}Ptr*), three reaction pathways compete for its deactivation: i) intersystem crossing to the singlet ground state, ii) energy transfer to O_2 leading to regeneration of Ptr and formation of $^1O_2$, and iii) electron transfer reaction from dTMP to ^{3}Ptr*, which leads to the formation of the Ptr radical anion ($Ptr^{\bullet-}$) and the dTMP radical cation ($dTMP^{\bullet+}$). The latter is in equilibrium with its deprotonated form ($dTMP(-H)^{\bullet}$) whereas the radical anion $Ptr^{\bullet-}$ may be protonated to yield $PtrH^{\bullet}$. Although Ptr produces $^1O_2$ under UV-A irradiation, we have shown that only the electron transfer pathway (iii) is responsible for the dTMP degradation, both under aerobic and anaerobic conditions. Further reactions of the dTMP and Ptr radical ions (or radicals) compete with back electron transfer from $Ptr^{\bullet-}$ to $dTMP^{\bullet+}$ (or eventually proton coupled electron transfer from $PtrH^{\bullet}$ to ($dTMP(-H)^{\bullet}$) to recover dTMP and Ptr.

Under aerobic conditions, the electron transfer from $Ptr^{\bullet-}/PtrH^{\bullet}$ to O_2 regenerates Ptr and yields $O_2^{\bullet-}/HO_2^{\bullet}$, which in turn disproportionate to H_2O_2. This reaction of O_2 with $Ptr^{\bullet-}/PtrH^{\bullet}$ prevents back electron transfer to $dTMP^{\bullet+}/dTMP(-H)^{\bullet}$ to regenerate dTMP, leading to the oxidation of the nucleotide, whereas Ptr is not consumed. Based on HPLC and mass spectrometry analyses and literature results on the photosensitized oxidation of thymidine (Thd), structures could be proposed for the dTMP oxidation products, taking into account the two main competitive reactions of $dTMP^{\bullet+}$: deprotonation (Reaction (7)) and hydration (Reaction (15)). Following hydration and trapping by O_2 of the resulting radical on the Thy moiety (Scheme 1), the following stable products may be formed: i) diastereoisomers of 5,6-dihydroxy-5,6-dihydrothymidine 5′-monophophate (thymidine glycol 5′-monophophate, dTMPGly), compounds with identical MS/MS spectra and incorporation of two O-atoms into the Thy moiety (MW 356 Da), ii) 1-(2-deoxy-beta-D-erythro-pentofuranosyl-5-phosphate)-5-hydroxy-5-methylhydantoin (5-OH-5MHMP, MW 326 Da) and iii) 5-hydroperoxy-6-hydroxy-5,6-dihydrothymidine 5′-monophosphate (5-HP-6-OHdTMP, MW 372 Da). Besides, trapping by O_2 of the (5-uracilyl)-methyl radical resulting from the deprotonation of $dTMP^{\bullet+}$ may explain the subsequent formation of 5-formyl-2′-deoxyuridine 5′-monophosphate (5-FordUMP, MW 336 Da), 5-(hydroxymethyl)-2′-deoxyuridine 5′-monophosphate (5-HmdUMP, MW 338 Da) and 5-(hydroperoxymethyl)-2′-deoxyuridine 5′-monophosphate (5-HPmdUMP, MW 354 Da).



Scheme 3. Main pathways in the mechanism of the degradation of dTMP photosensitized by Ptr.

In the absence of O_2, experimental evidence of the formation of an adduct where the pterinic moiety is attached to the nucleobase was obtained. In this case, coupling of the radical $PtrH^{\bullet}$ (C-centered radical on the C-6 or C-7 position of the pterin moiety) and ($dTMP(-H)^{\bullet}$) (neutral methylene radical) is the only pathway competing with back electron transfer to regenerate Ptr and dTMP (Scheme 3). Consequently, the consumption of dTMP is slower than in the presence of O_2, but Ptr is also consumed. When exposed to air, the adduct formed by the coupling reaction yields the observed product Ptr-dTMP(-2H), where the Ptr conjugated structure is regenerated. Consequently, Ptr-dTMP(-2H) has an absorption spectrum similar to that of Ptr, although its fluorescence efficiency is lower.

The results obtained in this work may be important from a biological point of view because they demonstrate that thymine, which is frequently assumed as a non-reactive nucleobase, can be damaged under UV-A radiation (a component of solar and artificial radiation), when compounds (*e.g.*, aromatic pterins) are present in the skin due to specific pathological conditions. Moreover, the photosensitized damages can take place at low O_2 concentration and even under anaerobic conditions. This fact should be

emphasized since the intracellular O_2 concentration in tissues is much lower than that corresponding to air-equilibrated aqueous solutions. Additionally, the spectroscopic properties of the adduct, in particular its fluorescence, might have some applications as fluorescent DNA probe. More studies are currently undertaken to find out if the Ptr moiety of the Ptr-dTMP(-2H) adduct is photochemically active and able to produce radicals and reactive oxygen species (ROS) upon irradiation.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.04.196.

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