UV-A (320–400 nm) and UV-B (280–320 nm) radiation causes damage to DNA and other biomolecules through reactions induced by different endogenous or exogenous photosensitizers. Lumazines are heterocyclic compounds present in biological systems as biosynthetic precursors and/or products of metabolic degradation. The parent and unsubstituted compound called lumazine (pteridine-2,4(1,3H)-dione; Lum) is able to act as photosensitizer through electron transfer-initiated oxidations. To get further insight into the mechanisms involved, we have studied in detail the oxidation of 2′-deoxyadenosine 5′-monophosphate (dAMP) photosensitized by Lum in aqueous solution. After UV-A or UV-B excitation of Lum and formation of its triplet excited state (3Lum*), three reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state, energy transfer to O2, and electron transfer between dAMP and 3Lum* yielding the corresponding pair of radical ions (Lum∑− and dAMP∑+). In the following step, the electron transfer from Lum∑− to O2 regenerates Lum and forms the superoxide anion (O2∑−), which undergoes disproportionation into H2O2 and O2. Finally dAMP∑+ participates in subsequent reactions to yield products.

**Introduction**

Within the pteridine family, pterins are those compounds derived from 2-aminopteridine-4(3H)-one (pterin; denoted Ptr), whereas lumazines are derived from pteridine-2,4(1,3H)-dione (lumazine; denoted Lum). Lumazines are present in living systems as the main products of the metabolic degradation of reduced pterins. In addition, 6,7-dimethyl-8-ribityllumazine is the biosynthetic precursor of riboflavin (vitamin B2). Although Lum presents different acid–base equilibria in aqueous solutions, the only relevant equilibrium at physiological pH involves the neutral form and the monoanion (Fig. 1), with a pKₐ value of 7.95. The absorption spectra of both acid and base forms, although quite different, have intense bands in the UV-B (280–320 nm) and UV-A (320–400 nm) spectral regions (Fig. 1).

Solar radiation induces modifications to genomic DNA and is implicated in the generation of human skin cancers. UV radiation at wavelengths lower than 300 nm damages DNA as a result of the direct excitation of the nucleobases. On the other hand, although nucleobases absorb very weakly above 300 nm, both UV-B and UV-A radiation can induce modifications to DNA through photosensitized reactions. This indirect action may be mediated by endogenous or exogenous sensitizers. The chemical changes in DNA and its components resulting from photosensitized reactions can take place through different mechanisms. Energy transfer from the triplet excited state of the photosensitizer to pyrimidine bases leads to the formation of pyrimidine dimers. Photosensitized oxidations also contribute to DNA damage induced by UV-A and UV-B radiation. These processes involve the generation of radicals (type I mechanism), e.g., via electron transfer or hydrogen...
abstraction, and/or the production of singlet oxygen (\(^1\text{O}_2\)) (type II mechanism).

Ptr and Lum are able to act as sensitizers in photoinduced oxidations through both type I and type II mechanisms. Although Lum is a relatively efficient \(^1\text{O}_2\) photosensitizer, studies performed on its conjugated acid (the perhydroxyl radical, HO\(_2\)) and its quenching by O\(_2\) have not been studied so far. Another interaction of Lum triplet state with nucleotides and the kinetics of dissolved O\(_2\) in time-resolved experiments and carried out analysis on O\(_2\).

In these studies, carried out in aqueous solutions under UV steady-state irradiation, consumption of a given nucleotide and O\(_2\), production of H\(_2\)O\(_2\), and constant concentration of Lum during irradiation were observed. Interestingly, it was also found that the rate of nucleotide disappearance was lower in O\(_2\)-saturated solutions than in air-equilibrated solutions. Since quenching of the triplet state of Lum by O\(_2\) has already been reported, this result was attributed to a competition between two pathways involving the Lum triplet state: energy transfer to O\(_2\) and electron transfer from the nucleotide. However, to the best of our knowledge, the interaction of Lum triplet state with nucleotides and the kinetics of its quenching by O\(_2\) have not been studied so far. Another interesting finding reported in the mentioned studies consists in the effect of superoxide dismutase (SOD), an enzyme that catalyzes the disproportionation of the superoxide radical anion (O\(_2^−\)) and its conjugated acid (the perhydroxyl radical, HO\(_2^−\)) into H\(_2\)O\(_2\) and O\(_2\). The photosensitized oxidations were faster in the presence of SOD than in its absence, thus suggesting the participation of O\(_2^−\) in the mechanism, that still remains to be demonstrated.

In this work, we present a study aimed at establishing a general mechanism for the electron transfer-initiated oxidations of biomolecules photosensitized by Lum. dAMP was chosen as a model substrate for several reasons: (i) if a given photosensitizer produces \(^1\text{O}_2\), its presence in the medium does not interfere with the analysis of the electron transfer process because adenine is not oxidized by this reactive oxygen species; (ii) dAMP is highly soluble in H\(_2\)O; (iii) it is easily quantified by chromatographic methods and (iv) some general features of the Lum-dAMP system are already known (vide supra). In this work, we have investigated the formation of O\(_2^−\) as a product of the photosensitized reaction, studied the quenching of the Lum triplet state by dAMP and dissolved O\(_2\) in time-resolved experiments and carried out analysis of photoproducts.

**Experimental**

**Chemicals**

Lumazine (Lum) was purchased from Schircks Laboratories (Jona, Switzerland) and used without further purification. Superoxide dismutase (SOD, bovine erythrocytes), 5,5-dimethyl-1-pyrrrole-N-oxide (DMPO), tris(hydroxymethyl)aminomethane (TRIS), 2′-deoxyadenosine 5′-monophosphate (dAMP), and ammonium acetate (NH\(_4\)OAc) were provided by Sigma. DMPO was purified by activated carbon and stored at −20°C before use.

The pH of the aqueous solutions was adjusted by adding drops of HCl or NaOH solutions from a micropipette. The concentrations of the acid and base used for this purpose ranged from 0.1 M to 2 M. The ionic strength was approximately 10\(^−3\) M in all the experiments.

Electron paramagnetic resonance: spin trapping experiments

**Electron paramagnetic resonance (EPR) set-up.** EPR spectra were collected on a Bruker ESP 500E spectrometer. Samples were irradiated directly inside the microwave cavity of the spectrometer using a 150 W Oriel Hg lamp (Palaiseau, France). The light was delivered via an optical fiber to the grid of the cavity. Under these conditions, the samples were irradiated with polychromatic light. 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 of superoxide anion (O\(_2^−\)).** 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. In our experiments, DMPO was used as a spin trap.

The samples used (500 μL) contained Lum (10\(^−4\) M) and DMPO (5 × 10\(^−2\) M) in an aqueous buffer of 1 mM TRIS/HCl and 0.5 mM NaCl (pH 7.2). Samples were transferred in quartz flat cells and irradiated in the EPR cavity at room temperature. EPR spectra were recorded at different times since the beginning of the irradiation and until a plateau was observed in the EPR signal intensity (after 3 to 4 min under our experimental conditions). To confirm the formation of O\(_2^−\) upon irradiation of the samples, similar EPR-spin trapping experiments were performed in the presence of SOD (22 U mL\(^−1\)).

**Laser flash photolysis**

**Time-resolved absorption experiments.** Time-resolved absorption experiments were performed as described previously. Briefly, the frequency-tripled output (355 nm) of a Quanta-Ray GCR 230 Nd:YAG laser operating at the repetition rate of 10 Hz was used as the excitation source (pulse fwhm ~ 5 ns). Transient species thus produced were monitored using the spectrally-resolved output of a steady-state Xe lamp. To increase the signal-to-noise ratio, data from ~250 independent laser pulses were typically averaged.

**Singlet oxygen experiments.** Samples were irradiated with the laser described in the previous paragraph. \(^1\text{O}_2\), was monitored in time-resolved experiments via its phosphorescence at 1270 nm using a 77 K Ge detector (EOL-817-P North Coast, Santa Clara, CA). The detector output was monitored using a digital oscilloscope (Lecroy 9410; Chestnut Ridge, NY), and acquired by a computer for storage and analysis. To improve signal-to-noise ratios, data recorded from 10 to 100 independent laser pulses were generally averaged.

**Steady-state irradiation**

**UV irradiation.** Solutions containing Lum and dAMP were irradiated in 1 cm path length quartz cells at room temperature with Rayonet RPR lamps (Southern N.E. Ultraviolet Co.) with emission centered at 300 or 350 nm (bandwidth (fwhm) ~ 20 nm). These differences in irradiation wavelength allow a better overlap between lamp emission and pH-dependent absorption spectra of Lum (Fig. 1). To avoid irradiation below 300 nm, where the nucleotide absorbs (Fig. 1), a cutoff filter was placed between the
lamp emitting at 300 nm and the cell. The experiments were performed in open cells with air-equilibrated aqueous solutions under stirring. The measurements were carried out under conditions of reduced environmental light.

**High performance liquid chromatography (HPLC).** An HPLC chromatograph Prominance from Shimadzu was employed for monitoring the reaction. A Synergi Polar-RP column (150 × 4.6 mm, 4 µm, Phenomenex) was used for product separation. 10 mM NH4OAc aqueous solution (pH 6.8) was used as the mobile phase. HPLC runs were monitored by UV/visible spectroscopy at different wavelengths.

**Detection and quantification of H2O2.** H2O2 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. 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 S.A.I.C. (Cholesterol Kit). Briefly, 500 µL of irradiated solution were added to 600 µL of reagent. The absorbance at 505 nm of the resulting mixture was measured after 30 min at room temperature, using the reagent as a blank. Aqueous H2O2 solutions prepared from commercial standards were employed for obtaining the corresponding calibration curves.

**Mass spectrometry analysis**

An LC/MS system, equipped with an HPLC chromatograph (Agilent 1100) and a triple quadrupole mass spectrometer (Q TRAP Applied Biosystems), were employed. HPLC analyses were performed by using an X-bridge column (150 mm, Waters), and isocratic elution with 100% formic acid (0.1%) at a flow rate of 0.3 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 units (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.

**Results and discussion**

**Production of O2− photoinduced by Lum**

In previous EPR-spin trapping studies, it was shown that when air-equilibrated aqueous solutions containing Ptr were irradiated, O2− was formed, even in the absence of substrates that could act as electron donors. The process responsible for the O2− is initiated by an electron transfer between Ptr in its ground state and electronically excited Ptr to yield the corresponding pair of radical ions (Ptr+ and Ptr−). In a second step O2− is formed by the reduction of the dissolved O2 by Ptr−, which is a common reaction of organic radical anions.

To characterize the formation of O2− upon photolysis of Lum, EPR analyses were performed in the presence of a spin trap (DMPO). The irradiation of a solution (pH 7.2) containing Lum (10⁻⁴ M) and DMPO (5 × 10⁻² M) led to the immediate formation of an EPR signal, which increased with the irradiation time (Fig. 2a–2c). The intensity of the EPR signal reached a maximum after 4 min of irradiation (Fig. 2c). The EPR spectrum was characterized by hyperfine coupling constants aH = 14.2 G, aH = 11.2 G, aN = 1.25 G, corresponding to the adduct DMPO-OOH. This result suggests that DMPO has trapped O2−. Control experiments were carried out in the absence of Lum and, as expected, no signal corresponding to the adduct DMPO-OOH was registered. To confirm the involvement of the O2− in the formation of the DMPO-OOH adduct, similar experiments were performed in the presence of SOD (Experimental section). The addition of this enzyme caused an important decrease of the EPR signal (Fig. 2d). Therefore, these results indicate that UV irradiation of Lum in aqueous solution induces the production of O2−.

![Fig. 2](image-url)
in the presence of SOD was much higher than that measured in its absence (Fig. 3). This result is in agreement with the data obtained from EPR–spin trapping experiments, but also implies the existence of recombination reactions that consume most O$_2$$^\cdot$ formed in the absence of SOD. In addition, results showed that the rate of Lum consumption was higher in the presence of SOD than in its absence. This fact may be interpreted as follows: SOD, by removing O$_2$$^\cdot$, prevents back electron transfer from O$_2$$^\cdot$ to Lum$^+$ and thus stimulates Lum consumption.

In the presence of O$_2$, photosensitized production of ¹O$_2$ (reaction 4') by Lum has been demonstrated with pH sensitive quantum yields of 0.44 and 0.08 in air-equilibrated acidic and alkaline aqueous solutions, respectively. It was also shown that Lum is poorly reactive with O$_2$.

Superoxide anion (O$_2$$^\cdot$) may be produced by direct electron transfer from ¹Lum$^*$ to O$_2$ (reaction 4') or by electron transfer from Lum$^-$ (reaction 9) formed through reaction 5. If O$_2$$^\cdot$ were formed predominantly by the former pathway (reaction 4'), the relative efficiency of the production of O$_2$$^\cdot$ (and of H$_2$O$_2$ resulting from O$_2$$^\cdot$ disproportionation, reaction 11, see below) would be expressed by:

\[ k_q [O_2]/(k_4^{\text{red}} [O_2] + k_5 [\text{Lum}] + k_6), \]

with \( k_q^{\text{red}} = k_4 + k_5 + k_6 \).

This efficiency should then increase when the O$_2$ concentration increases (or remain unchanged if \( k_q^{\text{red}} [O_2] \gg k_5 [\text{Lum}] + k_6 \)). However, the experimental evidence shows that the rate of H$_2$O$_2$ formation decreases with the O$_2$ concentration (Fig. 3). Therefore reaction 5 should be the predominant initiating step in the O$_2$$^\cdot$ formation, in agreement with the expression giving the relative efficiency of this reaction \((k_5 [\text{Lum}] / (k_4^{\text{red}} [O_2] + k_5 [\text{Lum}] + k_6))\).

Reaction 10 (recombination of the Lum radical cation, or its deprotonated form, with O$_2$$^\cdot$) explains the low quantum yields of Lum consumption \( (\Phi_{\text{Lum}} = (2 \pm 1) \times 10^{-2} \text{ at pH 5.5}) \) and of H$_2$O$_2$ production under aerobic conditions (Fig. 3). Alternatively, O$_2$$^\cdot$ disproportionation with its conjugated acid HO$_2$$^\cdot$ (represented by the overall reaction 11) leads to the formation of H$_2$O$_2$. Whereas reaction 10 is predominant and reaction 11 is negligible in the absence of SOD, the latter reaction becomes significant in its presence and H$_2$O$_2$ is produced in larger amounts. Finally, reactions of Lum$^+$ or Lum$^-$ with O$_2$ and/or the solvent, schematically represented by reaction 12, lead to the oxidation of Lum.

\[ \text{Lum}^{\cdot\cdot} \rightarrow ¹\text{Lum}^* \]  

(1)

\[ ¹\text{Lum}^* \rightarrow ¹\text{Lum}^* \]  

(2)

\[ ¹\text{Lum}^* \rightarrow \text{Lum} \]  

(3)

\[ ³\text{Lum}^* + 1\text{O}_2 \rightarrow \text{Lum} + \text{O}_2 \]  

(4)

\[ ³\text{Lum}^* + ¹\text{O}_2 \rightarrow \text{Lum} + ¹\text{O}_2 \]  

(4')

\[ ³\text{Lum}^* + ³\text{O}_2 \rightarrow \text{Lum}^\cdot + ³\text{O}_2 \]  

(4'')

\[ ³\text{Lum}^* + \text{Lum} \rightarrow \text{Lum}^\cdot + \text{Lum}^\cdot \]  

(5)

\[ \text{Lum}^\cdot + \text{H}^+ \Leftrightarrow \text{Lum(H)}^\cdot \]  

(6)

\[ \text{Lum}^\cdot + \text{H}^+ \Leftrightarrow \text{Lum(H)}^\cdot + \text{H}^+ \]  

(7)

\[ \text{Lum(H)}^\cdot + \text{Lum(H)}^\cdot \rightarrow 2\text{Lum} \]  

(8)

\[ \text{Lum(H)}^\cdot + \text{Lum(H)}^\cdot + \text{O}_2 \rightarrow \text{Lum} + \text{HO}_2^\cdot/\text{O}_2^\cdot \]  

(9)

\[ \text{Lum(H)}^\cdot + \text{Lum(H)}^\cdot + \text{O}_2 \rightarrow \text{Lum} + \text{HO}_2^\cdot/\text{O}_2^\cdot \]  

(10)

\[ 2\text{H}^+ + 2\text{O}_2^\cdot \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]  

(11)

To better understand the mechanism of O$_2$$^\cdot$ production in the absence of electron donors, a series of irradiation experiments were performed in O$_2$-saturated solutions. In photolysis experiments carried out in the absence, as well as in the presence of SOD, the increase in the O$_2$ concentration caused a significant decrease in the rate of H$_2$O$_2$ production (Fig. 3). This behavior is an indirect evidence of the participation of Lum triplet excited state in the formation of H$_2$O$_2$ (air-equilibrated and O$_2$-saturated solutions). In photolysis experiments performed in O$_2$-saturated solutions. In photolysis experiments performed in O$_2$-saturated solutions. In photolysis experiments performed in O$_2$-saturated solutions. In photolysis experiments performed in O$_2$-saturated solutions. In photolysis experiments performed in O$_2$-saturated solutions. In photolysis experiments performed in O$_2$-saturated solutions.
Lum(−H) + O₂ → Lum(ox) \hspace{1cm} (12)

Production of O₂^− photoinduced by Lum in the presence of dAMP

In contrast to the results obtained with solutions containing only Lum (Fig. 3), in the presence of nucleotides, such as dAMP or dGMP, the photosensitizer, under UV-A or UV-B irradiation, generates significant amounts of H₂O₂, comparable to the consumption of nucleotides.\(^\text{14,15}\) This fact, in the context of an electron transfer-initiated mechanism, suggests the formation of O₂^− in the photosensitized process. Therefore, to investigate this point further, EPR-spin trapping experiments were performed in the presence of dAMP and compared to those carried out in its absence. For different irradiation times, the signals corresponding to the DMPO-OOH adduct obtained in the solutions containing dAMP (200 µM) were much more intense than those registered in its absence (Fig. 2e–2g). To the best of our knowledge, this is the first time that direct evidence is shown in favor of the formation of O₂^− in the oxidation of a nucleotide photosensitized by a pteridine derivative.

It was reported that no photosensitization of dAMP by Lum takes place in the absence of O₂ and that in air-equilibrated solutions the dAMP consumption is faster in the presence of SOD.\(^\text{15}\) Thus, this result shows again that recombination reactions consuming O₂^− and recovering dAMP are taking place in the absence of SOD. Therefore, taking into account these previous results and those obtained in EPR experiments in this work, reactions 13 to 17 may be proposed to complete the reaction scheme (reactions 1 to 12), in the presence of dAMP. Reaction 13 is an additional and the predominant source of Lum^\text{−}, which, in turn, increases the O₂^− production, via reaction 9, explaining the increase in the DMPO-OOH EPR signal in the presence of dAMP (Fig. 2). Depending on the pH, the dAMP radical cation may deprotonate (reaction 14). The recombination between Lum^\text{−}, and the dAMP radical/radical cation (reactions 15 and 15') may explain the absence of dAMP photosensitization by Lum under anaerobic conditions. Finally, dAMP radicals can react with O₂^− to recover the substrate (reactions 16 and 16') or yield products (reaction 17).

\[
\begin{align*}
3\text{Lum}^* + \text{dAMP} &\rightarrow \text{Lum}^− + \text{dAMP}^+ \hspace{1cm} (13) \\
3\text{Lum}^* + \text{dAMP} &\rightarrow \text{Lum} + \text{dAMP} \hspace{1cm} (13') \\
\text{dAMP}^+ &\rightleftharpoons \text{dAMP(−H)} + \text{H}^+ \hspace{1cm} (14) \\
\text{dAMP(−H)}^+ + \text{Lum}^− + \text{H}^+ &\rightarrow \text{dAMP} + \text{Lum} \hspace{1cm} (15) \\
\text{dAMP}^+ + \text{Lum}^− &\rightarrow \text{dAMP} + \text{Lum} \hspace{1cm} (15') \\
\text{dAMP(−H)}^+ + \text{O}_2^− + \text{H}^+ &\rightarrow \text{dAMP}^+ + \text{O}_2 \hspace{1cm} (16) \\
\text{dAMP}^+ + \text{O}_2^− &\rightarrow \text{dAMP} + \text{O}_2 \hspace{1cm} (16') \\
\text{dAMP}^+ / \text{dAMP(−H)}^+ + \text{H}_2\text{O} / \text{O}_2 &\rightarrow \text{dAMP(ox)} \hspace{1cm} (17)
\end{align*}
\]

Results shown so far suggest that the triplet excited state of Lum plays a key role in the photosensitized oxidation of dAMP. However, direct evidence for the interaction between the ³Lum* and the ground state of dAMP has yet to be presented. Dissolved O₂ also plays a key role and is consumed in this process, the rate of consumption of the nucleotide being lower in O₂-saturated than in air-equilibrated solutions. Moreover, comparison of the photosensitizing activity of Lum under different pH conditions revealed that the photoinduced oxidation of dAMP is much faster in acidic media, where Lum exists in its neutral form, than in alkaline media, where Lum exists as a monoanion (Fig. 1).\(^\text{15}\) Therefore in order to get more insight into the mechanism of the photosensitized oxidation of dAMP by Lum, the quenching of ³Lum* by O₂ and dAMP was investigated in both media by laser flash photolysis.

Quenching of Lum triplet excited state by O₂

Alkaline media. Laser flash excitation at 355 nm of de-aerated Lum solutions at pH 10.5 showed a strong transient absorption in the 400–600 nm spectral region. The absorption decays were clearly biexponential, with lifetimes of 2.1 (±0.5) ms and 15 (±3) ms (Fig. 4a). The short-lived transient could be assigned to ³Lum* based on the following results: (i) increase in its decay rate in the presence of O₂, (ii) lifetime in the absence of added quenchers (τ\(^\text{a}\)) comparable to those reported for the triplet states of other related heterocyclic compounds such as pterins.\(^\text{31,35}\) The long-lived
transient could be a radical produced from the triplet excited state (reactions 5 to 7).

We have determined the \( \tau_1 \) values as a function of the concentration of dissolved \( \text{O}_2 \) from the decay of the short-lived transient. The corresponding rate equation for the decrease in \( ^3\text{Lum}^* \) concentration is given by:

\[
-\frac{d[^3\text{Lum}^*]}{dt} = k_{\text{obs}}[^3\text{Lum}^*] = \left(1/\tau_1\right)[^3\text{Lum}^*]
\]  

(18)

where \( k_{\text{obs}} \) is the observed rate constant of the \( ^3\text{Lum}^* \) decay under the given experimental conditions (s\(^{-1}\)); in the absence of added quencher \( k_{\text{obs}} = k_3 + k_2[\text{Lum}] = 1/\tau_1; \) in the presence of \( \text{O}_2, k_{\text{obs}} = k_4[\text{O}_2] + 1/\tau_1 = 1/\tau_q. \)

The rate constant of quenching of \( ^3\text{Lum}^* \) by \( \text{O}_2 \) \((k_4^0)\) was calculated by a Stern–Volmer analysis of the \( \tau_1 \) values as a function of the concentration of \( \text{O}_2 \) according to eqn (19).

\[
1/\tau_q = 1/\tau_s^0 + k_4^0[\text{O}_2]
\]  

(19)

The corresponding Stern–Volmer plot at pH 10.5 is shown in Fig. 5a and a \( k_4^0 \) value of 1.2 (± 0.2) \( \times 10^8 \) M\(^{-1}\) s\(^{-1}\) was obtained.

For a given set of experimental conditions, the \( \text{O}_2 \) concentration after the laser flash increased due to its formation by energy transfer from \( ^3\text{Lum}^* \) to dissolved \( \text{O}_2 \), reached a maximum value and decreased because of its relaxation to the ground state by both radiationless and radiative pathways (Fig. 4b). Quenching must also be considered if a substance present in the solution is able to deactivate or trap \( ^1\text{O}_2 \). These typical signals can be fitted with the following equation:

\[
I = A1\exp(-t/\tau_1) - A2\exp(-t/\tau_2)
\]  

(20)

where \( I \) is the signal registered by the NIR detector, which is proportional to the \( ^1\text{O}_2 \) concentration; \( A1 \) and \( A2 \) are pre-exponential factors, \( \tau_1 \) and \( \tau_2 \) are the lifetimes of \( ^1\text{O}_2 \) and of \( ^3\text{Lum}^* \), respectively, under the experimental conditions used.

In our case, \( \tau_1 \) was more than one order of magnitude longer than \( \tau_2 \), and it was calculated by fitting monoexponential decays \((I = A1\exp(-t/\tau_1)) \) obtained for time windows longer than 200 \( \mu s \). Then the average \( \tau_2 \) value obtained (63 ± 2 \( \mu s \)) was introduced in eqn (20) and \( \tau_2 \) was calculated for each \( \text{O}_2 \) concentration, analyzing signals within time windows shorter than 50 \( \mu s \). A Stern–Volmer analysis of the \( \tau_1 \) values as a function of the concentration of \( \text{O}_2 \) according to eqn (19) (Fig. 5b) gave a \( k_4^0 \) value of 7.0 (± 1) \( \times 10^8 \) M\(^{-1}\) s\(^{-1}\), about 40\% lower than in alkaline media. The value of \( \tau_2^0 \) in acidic media was estimated from the \( y \)-intercept of eqn (19) and a value of 2.6 (± 0.5) \( \mu s \) was obtained, which is close to that in alkaline media.

### Quenching of Lum triplet excited state by dAMP

**Alkaline media.** Laser flash photolysis experiments performed in the presence of dAMP at pH 10.5 (in the absence of \( \text{O}_2 \)) showed that this nucleotide does not quench \( ^3\text{Lum}^* \) up to a concentration of 680 \( \mu M \), i.e. the lifetime of the short-lived transient did not decrease with an increase in the quencher concentration (Fig. 6). Likewise, the lifetime of the long-lived transient did not change within the range of dAMP concentrations used. These results are in agreement with the fact that dAMP is not photosensitized by Lum in alkaline media\(^{14} \) and support the hypothesis that interaction of \( ^3\text{Lum}^* \) with the nucleotide is the first step of the photosensitized oxidation mechanism in acidic media.

**Acidic media.** As indicated in the previous section (Quenching of \( ^3\text{Lum}^* \) by \( \text{O}_2 \)), the transient absorption of \( ^3\text{Lum}^* \) could not be detected by laser flash photolysis in acidic media, and the quenching of \( ^3\text{Lum}^* \) by dAMP was also studied through the formation of \( ^1\text{O}_2 \) following the same procedure as described above. This analysis in air-equilibrated aqueous solutions (pD 5.5) showed that, under our experimental conditions, \( ^1\text{O}_2 \) was not quenched by dAMP, which was expected considering previous studies on quenching of \( ^1\text{O}_2 \) by nucleotides.\(^{12} \) The \( \tau_2 \) value (63 ± 2 \( \mu s \)) was introduced in eqn (20) and the \( \tau_2 \) value was calculated for each dAMP concentration. In the absence of dAMP, a \( \tau_2 \) of 1.6 (± 0.3) \( \mu s \) was obtained in air-equilibrated solutions.
Laser flash photolysis experiments: Stern–Volmer plots of the quenching of the Lum triplet state by dAMP (slope of the plots: $k_{q}^{\text{dAMP}}\tau_{T}$, with $\tau_{T}$ triplet lifetime in the absence of dAMP). Open triangles: experiments performed at pH 10.5 in O$_2$-free H$_2$O solutions ($\tau_{T}$ values were calculated analyzing $\Delta A$ vs. $t$ signals); excitation wavelength 355 nm, analysis wavelength 420 nm, $[\text{Lum}] = 220 \mu$M. Black triangles: experiments performed at pH 5.5 in air-equilibrated D$_2$O solutions ($\tau_{T}$ values were calculated by analyzing the near-infrared O$_2$ luminescence with eqn (19), see text); excitation wavelength 309 nm, $[\text{Lum}] = 80 \mu$M.

The rate constant of $^3$Lum* quenching by dAMP ($k_{q}^{\text{dAMP}} = k_{13} + k_{14}$) was calculated from the Stern–Volmer analysis of the data using eqn (21):

$$\frac{1}{\tau_{T}'} = \frac{1}{\tau_{T}} + k_{q}^{\text{dAMP}}[\text{dAMP}]$$

where $\tau_{T}'$ is the lifetime of $^3$Lum* in air-equilibrated solution in the absence of dAMP and $\tau_{T}$ is the lifetime with added dAMP ($=1/(k_{q}^{\text{dAMP}}[\text{dAMP}] + k_{q}^{\text{O}_2}[\text{O}_2] + 1/\tau_{T}^0)$).

The value of $k_{q}^{\text{dAMP}}$ obtained at pH 5.5 from the slope of the Stern–Volmer plot (Fig. 6) was $6 \pm 2 \times 10^7$ $\text{M}^{-1} \text{s}^{-1}$. This result provides direct evidence for the interaction of $^3$Lum* with dAMP, but also indicates that the quenching in acidic media is much more efficient than in alkaline media. This fact explains why the oxidation of dAMP photosensitized by Lum is much faster in acidic than in alkaline media, as previously reported.$^{15}$

The strong variation of $k_{q}^{\text{dAMP}}$ with the pH may be due to the differences in the charges of the molecules. (Neutral) and alkaline (monoanionic) forms of Lum have charges of 0 and −1, respectively (Fig. 1). Coulombic repulsion between alkaline forms of Lum and dAMP, molecule with negative charges in the phosphate moiety, very likely hinders the interaction necessary to allow the quenching process to proceed. Therefore, this repulsion, which does not exist for the acid form, may diminish the efficiency of the dynamic quenching via electron transfer and then makes inefficient the photosensitized chemical oxidation of the substrate.

Effect of O$_2$ concentration on the dAMP photosensitization

Knowing the values of $k_{q}^{\text{O}_2}$ and $k_{q}^{\text{dAMP}}$, the competition between the quenching of $^3$Lum* by dAMP and by O$_2$ can be analyzed for various experimental conditions, i.e. at different pH, and dAMP and O$_2$ concentrations under continuous irradiation of Lum.

Table 1 Lifetimes of $^3$Lum*, rate constants of its quenching by dAMP and O$_2$, and fraction of $^3$Lum* quenched by dAMP in acidic and alkaline aqueous solutions

| pH    | $\tau_{T}^0$ (µs) | $k_{q}^{\text{dAMP}}$ (M$^{-1}$ s$^{-1}$) | $k_{q}^{\text{O}_2}$ (M$^{-1}$ s$^{-1}$) | $f^{\text{dAMP}}$ |
|-------|-------------------|----------------------------------------|--------------------------------------|-----------------|
| 5.5   | 2.6 ± 0.5         | $6 \pm 2 \times 10^7$                  | $7 \pm 1 \times 10^6$                | 0.172 (air)     |
| 10.5  | 2.1 ± 0.5         | very small                             | 1.2 $(\pm 0.2) \times 10^3$         | 0.086 (O$_2$)   |

The fraction of $^3$Lum* quenched by dAMP under aerobic conditions ($f^{\text{dAMP}}$) is given by:

$$f^{\text{dAMP}} = \frac{k_{q}^{\text{dAMP}}[\text{dAMP}]/(k_{q}^{\text{dAMP}}[\text{dAMP}] + k_{q}^{\text{O}_2}[\text{O}_2] + 1/\tau_{T}^0)}$$

Values of $k_{q}^{\text{dAMP}}$, $k_{q}^{\text{O}_2}$ and $1/\tau_{T}^0$ were estimated for different experimental conditions; typically for those used in ref. 15 ([dAMP] $\sim$ 200 µM, pH 5.5), where the inhibition of the photosensitization of dAMP in O$_2$-saturated solutions was reported for the first time. Calculations show that the decrease of the $f^{\text{dAMP}}$ value is about a factor of 2 (Table 1) when the O$_2$ concentration increases from the value in air-equilibrated to that in O$_2$-saturated solutions. However, the decrease in the dAMP consumption observed experimentally is higher than that corresponding to a factor of 2. This result is related to the fact that quenching of $^3$Lum* by dAMP represents only the first step in a series of subsequent and competing reactions leading to dAMP consumption. The fraction of dAMP$^+$ formed by electron transfer (reaction 13) that undergoes a further transformation resulting in a permanent chemical change in the dAMP molecule has to be compared under air- and O$_2$-saturated conditions. Under the latter conditions, less dAMP$^+$ and Lum$^-$ are produced. However, higher O$_2$ concentration will result in a more efficient trapping of Lum$^-$ by O$_2$ (reaction 9) and therefore in a higher relative efficiency of O$_2^+$ production. Consequently, recombination reactions regenerating dAMP (reaction 16) should compete more favorably with the formation of oxidation products from the dAMP radicals (reaction 17). In the final balance, the effect of the O$_2$ concentration on dAMP consumption is higher than the effect expected considering only the efficiency of the first step of the process (quenching of $^3$Lum* by dAMP).

Mass spectrometry analysis of photoproducts

Aqueous solutions containing dAMP and Lum at pH 5.5 were analyzed by HPLC 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). In non-irradiated solutions, as expected, the signals corresponding to the intact molecular ions of dAMP and Lum as [M + H]$^+$ species at m/z 332.2 Da and 165.2 Da were observed in ESI$^+$ mode. In addition, under the same conditions the adduct [M + Na]$^+$ was also detected at m/z 187.2 Da for Lum. Likewise, in ESI$^-$ mode the intact molecular ions of the photosensitizer and substrate as [M − H]$^-$ species were observed at m/z 163.1 Da and 329.9 Da, respectively.

Comparison of the areas of chromatographic peaks of irradiated and non-irradiated solutions revealed that whereas dAMP was consumed during the photolysis, Lum did not change its concentration. In addition, in irradiated solutions two photoproducts (arbitrarily named P1 and P2) were detected, the areas of the
corresponding peaks increasing with irradiation time. Analysis by ESI mass spectrometry revealed that the molecular weight of P1 is 347 (signals at m/z 346 and 348 in ESI− and ESI+ modes, respectively), whereas the molecular weight of P2 is 329 (signals at m/z 328 and 330 in ESI− and ESI+ modes, respectively).

The molecular weight of P1 corresponds to 8-oxo-7,8-dihydro-2′-deoxyadenosine 5′-monophosphate (8-oxo-dAMP, Scheme 1). This fact is important since 8-oxo-7,8-dihydro 2′-deoxyadenosine (8-oxo-dAdo) has been proposed as a product of the photosensitized oxidation of 2′-deoxyadenosine (dAdo) in DNA via a type I mechanism. Moreover, the efficient conversion of the radical cation of dAdo into 8-oxo-dAdo has been reported. Therefore, the results obtained in ESI mass analysis support the hypothesis proposed of an electron transfer from dAMP to excited Lum (reaction 13).

![Scheme 1](Image)

Finally, the molecular weight of P2 might correspond to a tetracyclic compound (8-P-dAMP) recently detected by ESI mass spectra of irradiated solutions containing dAMP and pterin (Ptr). Scheme 1 shows the chemical structure proposed for 8-P-dAMP, but more information is needed for a complete characterization of this compound. To the best of our knowledge this is the first time that separation of this compound (by HPLC, in our case) is carried out before mass spectrometry analysis, confirming that it is a product of the photosensitized reaction, instead of a compound formed during the ionization of the mass spectrometry analysis.

**Conclusions**

The mechanisms involved in electron transfer initiated oxidations of biomolecules photosensitized by lumazine (Lum), a compound belonging to a family of heterocycles present in biological systems, were studied. 2′-Deoxyadenosine 5′-monophosphate (dAMP) was used as a target because this nucleotide does not react significantly with singlet oxygen (1O2), reactive species produced by Lum under UV irradiation.

Using EPR and H2O2 analyses, we have shown, to the best of our knowledge for the first time, that 1O2− is produced during the oxidation of a nucleotide (dAMP) photosensitized by a pteridine derivative (Lum). Production of 1O2− occurs by electron transfer from Lum− to O2, and therefore requires as a prior step quenching of 1Lum* by dAMP to produce the radical ion pair (dAMP+/Lum−). Time-resolved transient absorption experiments and NIR analysis of 1O2 phosphorescence by laser flash photolysis provided evidence that dAMP does not quench 1Lum* in alkaline aqueous solutions, and subsequently Lum does not photosensitize dAMP oxidation in this medium. In acidic or neutral solutions, competitive quenching of 1Lum* by dAMP and O2 results in an apparent contradictory effect of the O2 concentration: O2 is required and consumed in the process, but the rate of consumption of the nucleotide is lower in O2-saturated than in air-equilibrated solutions. Two main products are formed by photosensitized oxidation of dAMP by Lum, as shown by HPLC coupled to mass spectrometry: 8-oxo-7,8-dihydro-2′-deoxyadenosine 5′-monophosphate (8-oxo-dAMP) and a second compound that might be 8-P-dAMP but is not yet fully characterized.

The mechanism involved in the photosensitized process is summarized in Scheme 2. After UV-A or UV-B excitation of Lum and formation of its triplet excited state (1Lum*), three reaction pathways compete for the deactivation of the latter: intersystem crossing to singlet ground state, energy transfer to O2 leading to the regeneration of Lum and the production of 1O2, and electron transfer between dAMP and 1Lum* yielding the corresponding pair of radical ions (Lum− and dAMP+). In the following step, the electron transfer from Lum− to O2 regenerates Lum and forms O2−. The latter undergoes disproportionation with its conjugated acid (HO2−) yielding H2O2 and O2, in competition with recombination reactions with dAMP+ (and/or deprotonated dAMP+) regenerating dAMP and O2 (reactions 16 and 16′). Finally, processes that may include reactions of dAMP+ with H2O and O2 lead to the formation of dAMP oxidation products.

![Scheme 2](Image)

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