Photosensitization of 2′-deoxyadenosine-5′-monophosphate by pterin†

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UV-A radiation (320–400 nm) induces damages to the DNA molecule and its components through photosensitized reactions. Pterins, heterocyclic compounds widespread in biological systems, participate in relevant biological processes and are able to act as photosensitizers. We have investigated the photosensitization of 2′-deoxyadenosine-5′-monophosphate (dAMP) by pterin (PT) in aqueous solution under UV-A radiation. The effect of pH was evaluated, the participation of oxygen was investigated and the products analyzed. Kinetic studies revealed that the reactivity of dAMP towards singlet oxygen ($^1O_2$) is very low and that this reactive oxygen species does not participate in the mechanism of photosensitization, although it is produced by PT upon UV-A excitation. In contrast, analysis of irradiated solutions by means of electrospray ionization mass spectrometry strongly suggested that 8-oxo-7,8-dihydro-2′-deoxyadenosine-5′-monophosphate (8-oxo-dAMP) was produced, indicating that the photosensitized oxidation takes place *via* a type I mechanism (electron transfer).

Introduction

Solar radiation is known to be mutagenic and carcinogenic and is implicated in the induction of human skin cancers.[1,2] Damage to DNA results from direct excitation of the nucleobases by UV-B radiation (280–320 nm), and, although nucleobases absorb very weakly above 320 nm, from photosensitized reactions induced by UV-A radiation (320–400 nm).[3,4] This indirect action may be mediated by endogeneous or exogeneous sensitizers.

The chemical changes to DNA and its components *via* photosensitized reactions can take place through different mechanisms. Energy transfer from the triplet state of the photosensitizer to pyrimidine bases leads to the formation of pyrimidine dimers.[4–6] However, the main processes responsible for DNA damage induced by UV-A radiation involve an electron transfer or hydrogen abstraction (type I) and/or the production of singlet molecular oxygen ($^1O_2$) (type II).[7] The nucleobases are the preferential DNA substrates of type I oxidation.[3] Although guanine is the main target because of its low ionization potential[8] and is the only DNA component that significantly reacts with $^1O_2$,[9] adenine is also a target in type I sensitized oxidations, being more reactive than pyrimidine bases. The study of the photoinduced oxidation of adenine in isolated and cellular DNA is difficult due to the drawbacks of the analytical methods used to assess the oxidative damage. In addition, it has been demonstrated that hole transfer occurs in double-stranded DNA from adenine radical to guanine.[10–12] As a consequence, the amount of modified adenine bases in a DNA molecule is lower than the total amount of adenine bases that underwent electron-transfer. This problem may be avoided by working with adenine in the absence of guanine, *i.e.* using nucleosides, nucleotides or oligonucleotides containing only adenine as a base.

Pterins, heterocyclic compounds widespread in biological systems, are derived from 2-aminopteridin-4(1*H*)-one or pterin (PT) (Fig. 1). Several pterin derivatives participate in important biological processes such as the synthesis of amino acids[13] and

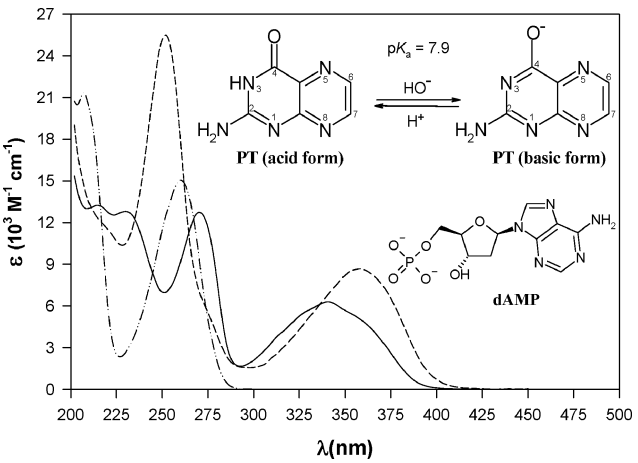

Fig. 1 Molecular structure of PT and dAMP, and the corresponding absorption spectra in air-equilibrated aqueous solutions; solid line: acid form of PT (pH = 5.5); dashed line: basic form of PT (pH = 10.5); dashed-dotted lines: dAMP.

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nucleobases,[14] nitric oxide metabolism[15] and the activation of cell-mediated immune responses.[16] Pterins behave as weak acids in aqueous solutions, the dominant equilibrium at pH > 5 involving an amide group (acid form) and a phenolate group (basic form)[17] (Fig. 1, pK_a = 7.9 for PT[18]). The participation of pterins in photobiological processes has been suggested or demonstrated in the past decade, and interest in the photochemistry and photophysics of these compounds has subsequently increased.[19] Under UV-A excitation, these biomolecules can fluoresce, undergo photooxidation to produce different photoproducts and generate reactive oxygen species such as $^1O_2$.[20] Interestingly, some pterin derivatives (*e.g.* biopterin, 6-formylpterin, 6-carboxypterin) accumulate in the skin of patients affected by vitiligo, a depigmentation disorder, where protection against UV radiation fails due to the lack of melanin.[21,22] Kawanishi and Ito demonstrated for the first time in 1997[23] that pterins are also able to photoinduce DNA damage. In a later study, cleavage of plasmid pUC18 photosensitized by PT was demonstrated.[24] Finally, a more recent investigation supported the hypothesis that pterins are able to damage DNA through a type I photosensitized oxidation.[25]

In this work, we report the photosensitization of 2′-deoxyadenosine-5′-monophosphate (dAMP) by PT in aqueous solution under UV-A radiation (Fig. 1). We have evaluated the effect of pH, investigated the participation of oxygen and analyzed the products. Mechanistic aspects and the biological implications of the results obtained are discussed. To the best of our knowledge, the photosensitization of isolated nucleotides by pterins has not been investigated so far. Moreover, the photoinduced chemical modification of adenine by pterins has not been reported in oligonucleotides nor in DNA molecules.

Results

The first aim of this work was to find out if pterin was able to photosensitize 2′-deoxyadenosine-5′-monophosphate (dAMP) in aqueous solutions under UV-A irradiation. Therefore air-equilibrated solutions containing PT (50 μM) and dAMP (100–1000 μM) were irradiated at 350 nm during different times (10 min to 240 min). Under these experimental conditions, PT was excited, whereas dAMP did not absorb radiation. The corresponding absorption spectra are shown in Fig. 1. In order to avoid interferences between the acid and the basic forms of PT, the experiments were performed in the pH ranges 5.0–5.8, where PT is present at more than 99% in its acid form, and 10.2–10.7, where PT is present at more than 99% in the basic form. The photochemical reactions were followed by UV–visible spectrophotometry and HPLC. Control experiments showed that no reaction occurred between PT and dAMP in the dark and that, as expected, the dAMP molecule was stable under irradiation at 350 nm in the absence of PT.

In acidic media (pH = 5.5), significant changes in the absorption spectra of the solutions were observed after irradiation. These changes are illustrated in Fig. S1 (ESI†), where isosbestic points at *ca.* 220, 240 and 280 nm can be observed. Under the same pH conditions, the concentration profiles of PT and dAMP were determined by HPLC (Fig. 2). A decrease of the dAMP concentration was observed as a function of irradiation time, whereas the PT concentration did not change in the analyzed time-window. These results show that in the studied process dAMP was chemically modified by UV-A irradiation of PT. To the best of our knowledge, this is the first time that evidence of photosensitization of dAMP (isolated or included in a nucleic acid molecule) by a pterin has been reported.

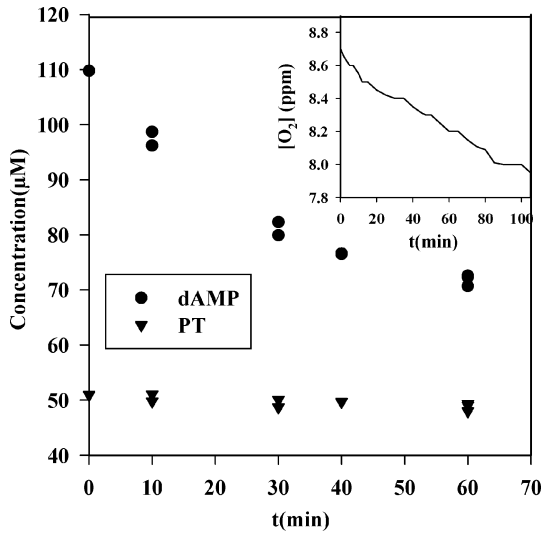


Fig. 2 Evolution of dAMP and PT concentrations in air-equilibrated aqueous solutions under UV-A irradiation (350 nm) as a function of time (pH = 5.5.; concentrations were determined by HPLC analysis). Inset: evolution of O_2 concentration in an irradiated solution containing PT (94 μM) and dAMP (380 μM) at pH 5.6 as a function of irradiation time.

In contrast, no evidence of a photochemical reaction induced by the basic form of PT was observed. No spectral changes and no decrease of the dAMP concentration were detected in experiments carried out at pH = 10.5. Photosensitization was not observed even at relatively high concentration of dAMP (1 mM) and long irradiation times (more than 2 hours). These results show that, whereas the acid form of PT is able to photosensitize and induce chemical modification of the nucleotide dAMP, this is not the case for the basic form of PT.

The role of oxygen

Solutions containing PT (50 μM) and dAMP (100 μM), previously purged with Ar, were irradiated. No significant changes were observed in the absorption spectra of the solutions after more than 100 min of irradiation in acidic (pH 5.0–5.5) or in alkaline (pH 10.2–10.6) media.

The evolution of the O_2 concentration during the irradiation of air-equilibrated solutions was monitored using an oxygen electrode in a closed cell. In acidic solutions containing dAMP and PT the O_2 concentration decreased as a function of irradiation time (Fig. 2). This result strongly suggests that the process described in the previous section consists in the oxidation of dAMP. In contrast, no significant O_2 consumption was observed in alkaline media. This observation supports the hypothesis that only the acid form of PT acts as a photosensitizer.

In several experiments performed at pH 5.5, the O_2 concentration was monitored with the oxygen electrode and the concentration of dAMP was measured by HPLC, before and after irradiation. The relationship between O_2 and dAMP consumptions ($\Delta[O_2]/\Delta[dAMP]$) was calculated for different irradiation times

and a value of 2.4 ± 0.3 was obtained, thus suggesting that the stoichiometry of the process is not simple (*vide infra*).

The formation of H_2O_2 was investigated in acidic solutions (pH 5.5) containing PT and dAMP irradiated (λ_{ex} = 350 nm) in the presence of O_2. Series of experiments were performed at different initial concentrations of PT and dAMP. H_2O_2 was found to be generated and its concentration increased as a function of irradiation time. The rate of H_2O_2 generated was of the same order of magnitude as the rate of dAMP consumption for the different experiments carried out. In alkaline medium, no H_2O_2 was detected even after 2 hours of irradiation. This result is in agreement with the absence of O_2 consumption in this medium as indicated above.

Singlet oxygen quenching by dAMP

It has been reported that adenine does not significantly react with $^1O_2$.[9] However, taking into account that PT is a rather good $^1O_2$ sensitizer under UV-A irradiation in aqueous solutions (quantum yield of $^1O_2$ production: Φ_Δ = 0.18–0.27 depending on the pH[20]) and the lack of reports on the interaction of $^1O_2$ with dAMP, we have determined the rate constant of the chemical reaction between the two species, as well as that of $^1O_2$ physical quenching by the studied nucleotide. The experiments have been performed in acidic media (pD ~ 5.5) because the photosensitization of dAMP by PT is only observed under such conditions.

Singlet oxygen was produced by photosensitization, using the standard 1*H*-phenalen-1-one (PHE)[26,27] as a sensitizer. In this process, $^1O_2$ is generated by energy transfer from the excited triplet state of the sensitizer (^{3}Sens*) to dissolved molecular oxygen (eqn (1)).

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

Singlet oxygen relaxes to its ground state ($^3O_2$) through deactivation by solvent molecules and a weak near-IR luminescence emission. If a substance (*e.g.* dAMP) in solution is able to trap or quench $^1O_2$, the chemical reaction (eqn (2)) and physical quenching (eqn (3)) must be considered.

$$Q + {}^1O_2 \xrightarrow{k_r} QO_2 \quad (2)$$

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

The rate constant of $^1O_2$ total quenching ($k_t = k_r + k_q$) by dAMP was determined by Stern–Volmer analysis of the $^1O_2$ near-IR luminescence quenching under continuous irradiation of the sensitizer (Experimental section, eqn (8)). The Stern–Volmer plot was linear within the range of concentrations used (Fig. 3). From the slope of this plot (K_{SV} = 25 ± 2 M^{-1}) and the $^1O_2$ lifetime (τ_Δ) in D_2O (62 μs),[28] a value of (4.1 ± 0.4) × 10^5 M^{-1} s^{-1} was calculated for k_t, at pD 5.5. This low value of k_t is of the same order of magnitude as others previously obtained in time-resolved experiments performed in D_2O using different $^1O_2$ sensitizers.[29,30]

Singlet oxygen quenching by dAMP using PT as a sensitizer

When the experiments for the determination of k_t were performed using PT as $^1O_2$ sensitizer under otherwise the same experimental conditions, the K_{SV} value was much higher ((1.21 ± 0.05) × 10^2 M^{-1}) (Fig. 3). This result shows that, in this case, the decrease

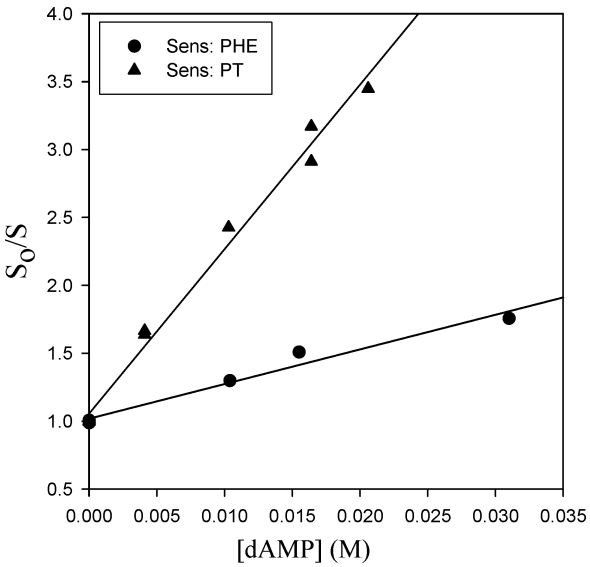


Fig. 3 Stern–Volmer plots of the quenching of $^1O_2$ near-infrared luminescence by dAMP in D_2O (Experimental section, PHE and PT were used as sensitizers, λ_{exc} = 367 nm).

of the $^1O_2$ emission as the dAMP concentration increased was not due to $^1O_2$ quenching by dAMP only. In order to explain such behavior, a quenching of the excited states, most likely of the triplet state, of PT by dAMP may be assumed: this process competes with energy transfer to molecular oxygen (eqn (1)), resulting in a lower production of $^1O_2$ and therefore in an apparently more efficient $^1O_2$ quenching when PT was used as a sensitizer. Electron transfer between excited states of pterins and guanine in DNA molecules has been already suggested,[23,25] and a similar reaction may be proposed for dAMP (eqn (4)).

$$^3\text{Sens}^* + \text{dAMP} \rightarrow \text{Sens}^{-\bullet} + \text{dAMP}^{+\bullet} \quad (4)$$

The free energy change (ΔG) of this process can be estimated with eqn (5):[31]

$$\Delta G\,(\text{eV}) = [E_{(\text{dAMP}+\bullet/\text{dAMP})} - E_{(\text{PT/PT}-\bullet)} - (e_o^2/\varepsilon R_{D+A-})] - \Delta E_{0,0} \quad (5)$$

where $E_{(\text{dAMP}+\bullet/\text{dAMP})}$ and $E_{(\text{PT/PT}-\bullet)}$ are the standard electron potentials of electron donor and acceptor, respectively. These values have already been reported for dAMP ($E_{(\text{dAMP}+\bullet/\text{dAMP})}$ = 1.44 V *vs.* NHE[32]) and PT ($E_{(\text{PT/PT}-\bullet)}$ = −0.55 V *vs.* NHE[33]). $\Delta E_{0,0}$ is the energy of the triplet excited state of PT and has been estimated from its phosphorescence spectra (ΔE_T = 2.52 eV[33]). The term $e_o^2/\varepsilon R_{D+A-}$ is the solvation energy of an ion pair D^+A^- and can be ignored in the case of strong polar solvents. The calculated ΔG value was −0.55 eV, thus indicating that electron transfer from dAMP to the triplet excited state of PT can spontaneously occur.

Rate constant of the chemical reaction between singlet oxygen and dAMP

The rate constant of the chemical reaction between $^1O_2$ and dAMP (k_r) was determined in D_2O solutions (pD = 5.5) from the analysis of dAMP disappearance (by HPLC) during photosensitized oxidation, using PHE as an $^1O_2$ sensitizer (Experimental section). The value obtained for k_r ((8 ± 3) × 10^3 M^{-1} s^{-1}) has a relatively large error due to very little consumption of dAMP (~4%), but

it is more than one order of magnitude lower than k_t ($(4.1 \pm 0.4) \times 10^5$ M^{-1} s^{-1}). Therefore, the chemical reaction between $^1O_2$ and dAMP (eqn (2)) is negligible in comparison with the physical quenching (eqn (3)).

The k_r value, determined for the first time in this study, is very low compared to values reported for other biological heterocyclic compounds, for instance, for pterins themselves.[34] The comparison of k_r of dAMP with that of dGMP ($(1.7 \pm 0.3) \times 10^7$ $M^{-1}s^{-1}$)[35] reveals that the latter nucleotide is much more reactive towards $^1O_2$ than the former, in agreement with other studies.[9]

Taking into account the value of k_r, the contribution of $^1O_2$ to the photosensitized oxidation of dAMP by PT can be evaluated by comparing the experimental initial rate of dAMP consumption to the initial rate of the reaction between $^1O_2$ and dAMP calculated from eqn (6).

$$-\mathrm{d[dAMP]/dt} = k_r\,[^1O_2]\,[\mathrm{dAMP}]_0 \quad (6)$$

The steady-state concentration of $^1O_2$ is given by eqn (7),

$$[^1O_2] = (P_a\Phi_\Delta)/(k_d + k_t\,[\mathrm{dAMP}]_0) \quad (7)$$

where P_a and Φ_Δ are, in this case, the photon flux absorbed by PT and the quantum yield of $^1O_2$ production by the acid form of PT ($\Phi_\Delta = 0.18$[20]), respectively; k_d is the rate constant of $^1O_2$ deactivation by the solvent ($1/\tau_\Delta$). Assuming that k_r in H_2O is similar to that determined in D_2O (no deuterium isotopic effect), a value of 3.3×10^{-4} μM min^{-1} was calculated for the initial rate of the reaction between $^1O_2$ and dAMP ($[\mathrm{dAMP}]_0 = 110$ μM), using eqn (6) and (7); this value is negligible in comparison with the experimental rate of dAMP consumption ((0.91 ± 0.08) μM min^{-1}). Therefore, these results indicate that $^1O_2$ is most probably not involved in the mechanism of the oxidation of dAMP photoinduced by PT.

To confirm this point, comparative photolysis experiments were performed in H_2O and D_2O: taking into account the longer $^1O_2$ lifetime in D_2O than in H_2O,[36] the photosensitized oxidation of dAMP should be faster in the deuterated solvent if $^1O_2$ would contribute significantly to the reaction. Air-equilibrated solutions containing PT (50 μM) and dAMP (155 μM) in H_2O and D_2O at pH and pD 5.7 and 5.9, respectively, were irradiated under otherwise identical conditions. Changes in the absorption spectra and evolution of the concentrations of PT, dAMP and H_2O_2 as a function of the irradiation time showed that the reaction was not faster in D_2O than in H_2O. On the contrary, slightly lower rates of dAMP disappearance and H_2O_2 formation were observed in D_2O ((0.37 ± 0.04) μM min^{-1} and (0.3 ± 0.1) μM min^{-1}, respectively) than in H_2O ((0.55 ± 0.06) μM min^{-1} and (0.5 ± 0.1) μM min^{-1}, respectively). The decrease in the rate of the process observed in D_2O cannot be easily explained and requires further investigation.

Mass spectrometry analysis

Electrospray ionization (ESI) mass spectra of irradiated and non-irradiated solutions containing dAMP and PT were registered and compared. The analysis was carried out in both positive and negative ion modes (ESI^+ and ESI^-, respectively). The signal corresponding to the intact molecular ion of dAMP as $[M - H]^-$ species at m/z 330.1 Da ($[M - H]^- = [\mathrm{dAMPO_4H}]^-$) and 352.1 Da ($[M - H]^- = [\mathrm{dAMPO_4Na}]^-$) were observed in ESI^- mode, together with new signals at m/z 328.1 and 346.1 Da (Fig. 4)

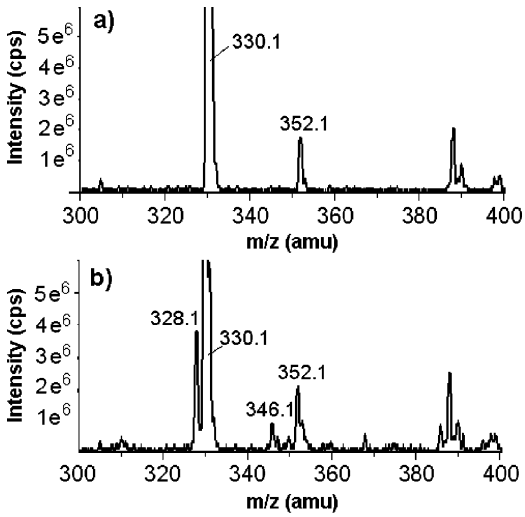


Fig. 4 Electrospray ionization mass spectra of solutions containing dAMP and PT. Analysis carried out in negative mode. Voltage = 50 V. Dilution 1 : 10 with 50 : 50 (v/v) MeOH–10 mM NH_4OAc in H_2O. (a) Non-irradiated solution. (b) Solution irradiated for 4 h at 367 nm.

after photosensitization by PT. In addition, in ESI^+ mode the intact molecular dAMP ion as $[M + H]^+$, and adducts $[M + Na]^+$ and $[M + 2Na]^+$ were detected at m/z 332.1 Da ($[M + H]^+ = [\mathrm{dAMPO_4H_3}]^+$), 354.0 Da ($[M + Na]^+ = [\mathrm{dAMPO_4NaH_2}]^+$) and 376.0 Da ($[M + 2Na]^+ = [\mathrm{dAMPO_4Na_2H}]^+$), together with new signals at 330.1, 347.9 and 352.2 Da (Fig. 5) after photosensitization by PT.

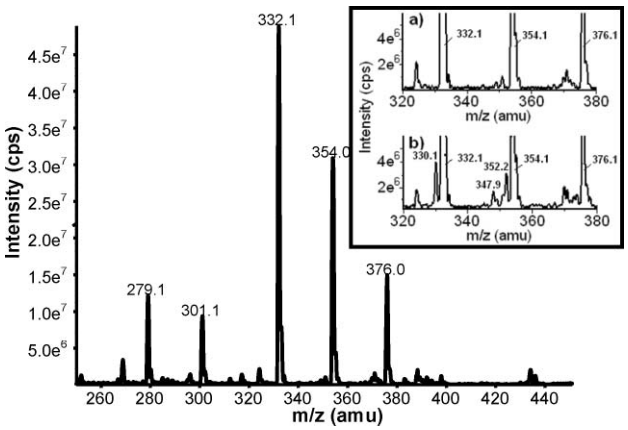
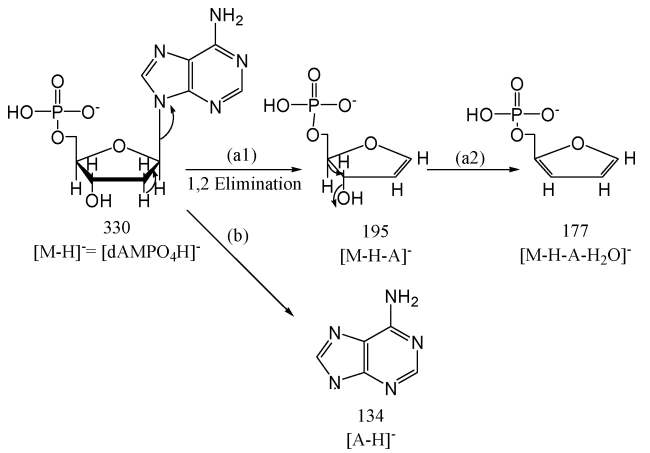


Fig. 5 Electrospray ionization mass spectra of a solution containing dAMP and PT. Analysis carried out in positive mode. Voltage = 50 V. Dilution 1 : 10 with 50 : 50 (v/v) MeOH–10 mM NH_4OAc in H_2O. Inset: (a) non-irradiated solution. (b) Solution irradiated for 4 h at 367 nm.

The new signals were observed in spectra corresponding to both 2 and 4 hours of irradiation and indicate the presence of, at least, two products (arbitrarily named P1 and P2). The molecular weight of product P1 is 347 (signals at m/z 346 and 348 in ESI^- and ESI^+ modes, respectively), whereas the molecular weight of product P2 is 329 (signals at m/z 328 and 330 in ESI^- and ESI^+ modes, respectively). The signal at m/z 352.2 observed in ESI^+ mode very likely corresponds to a Na-adduct of product P2.

The MS/MS spectra in the ESI^- mode of dAMP and the photoproducts were obtained and the fragmentations compared (Fig. 6(a), (b) and (c)). The fragmentation characterisation of oligonucleotides by using soft ionization MS methods, FAB and ESI, have been previously described. According to McLuckey *et al.*[37] the loss of the adenine (A) base is a prominent reaction and occurs *via* a 1,2-elimination reaction (Scheme 1). In negative ion mode, if the charge resides on base A, then the loss of the base as a deprotonated anion ($[A - H]^-$) is preferred (Fig. 6(a), signal at *m/z* 133.9 Da; RI 100%; Scheme 1, step (b)). Otherwise, the neutral base A is lost yielding the signal at *m/z* 195.0 Da (Scheme 1, step (a1)). Following the loss of base A, the 3′-C-OH and 4′-C-H bonds are cleaved to yield the $[M - H - A - H_2O]^-$ ion at *m/z* 177 Da (Scheme 1, step (a2)). The typical species $[PO_3]^-$ and $[PH_2O_4]^-$ at *m/z* 79 (78.9) and 97 Da were also detected (Fig. 6(a)).



Scheme 1 Fragmentation of dAMP *via* a 1,2-elimination reaction obtained using soft ionization MS methods. The peaks corresponding to each fragment are shown in Fig. 6(a).

The typical fragment corresponding to the base adenine at *m/z* = 134 Da, present in the MS/MS spectrum of dAMP as expected (Fig. 6(a)), is missing in the MS/MS spectrum of the product P1 (Fig. 6(b)) with a signal at *m/z* 346.1 Da shown in Fig. 4(b). The same result was observed in MS/MS spectra obtained at different voltages. Besides, a signal corresponding to adenine containing an oxygen atom in its structure was observed at *m/z* 150 Da (Fig. 6(b)). The analysis of this MS/MS spectrum shows the expected fragmentation pattern of the 8-oxo-7,8-dihydro-2′-deoxyadenosine-5′-monophosphate (8-oxo-dAMP),[38] which is detailed in Scheme 2. Thus, 8-oxo-dAMP is most probably one of the products (P1) formed during the photochemical process. As shown in Scheme 2 and in Fig. 6(b), for this compound, the loss of both the neutral and the charged oxo-base occurs yielding signals at *m/z* 195.1 Da and *m/z* 149.9 Da, respectively (Scheme 2, steps (a1) and (b)). The characteristic signal observed at *m/z* 230 Da can be rationalized, as is shown in Scheme 2 (step (c)), by a PO_3^- intramolecular rearrangement followed by the loss of the 8-phosphate-adenosine anion ($[8\text{-oxo-A-}PO_3H]^-$).

The identification of 8-oxo-dAMP as a product of the photosensitized oxidation of dAMP 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

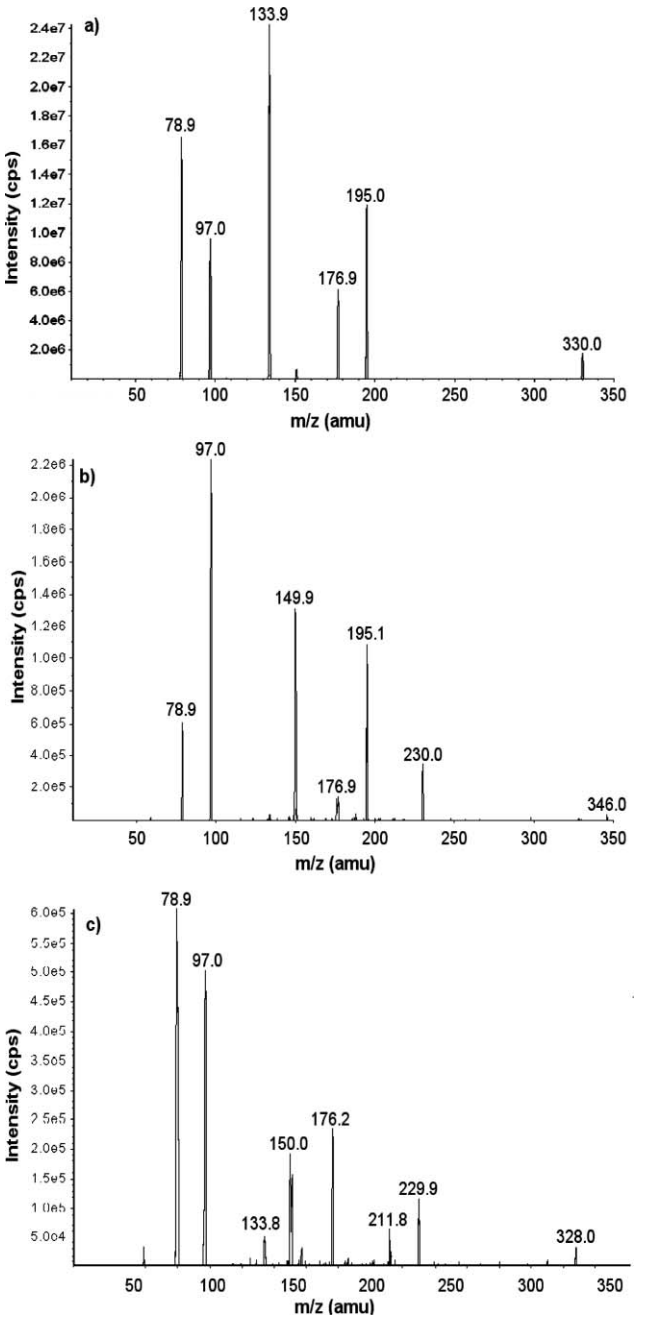
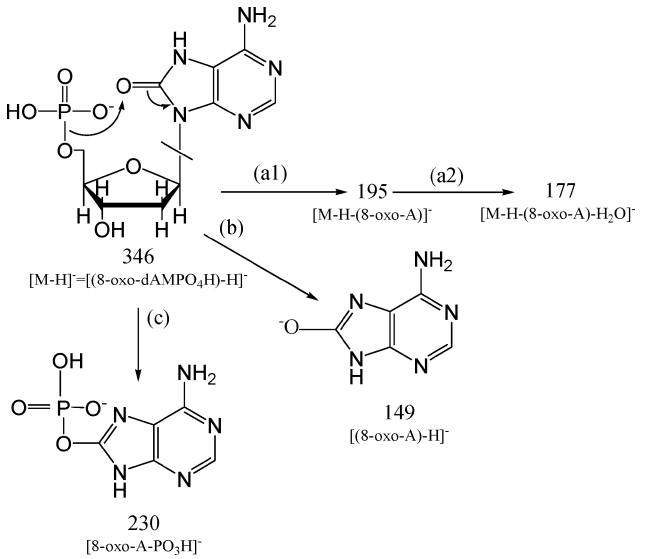


Fig. 6 MS/MS spectra obtained in ESI^- mode. (a) dAMP, (b) product P1 and (c) product P2.

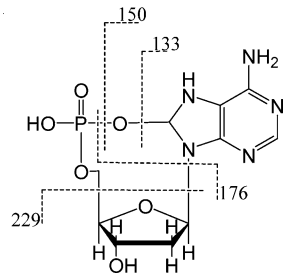
DNA *via* a type I mechanism.[8] Moreover, the efficient conversion of the radical cation of dAdo into 8-oxo-dAdo has been reported.[39] Therefore, the results obtained in ESI mass analysis support the hypothesis proposed in the previous section of an electron transfer from dAMP to excited PT (eqn (4)). The generated cation radical $dAMP^{+\bullet}$ may react further to yield 8-oxo-dAMP.

The corresponding MS/MS study for the product P2 is displayed in Fig. 6(c). The simultaneous presence of the fragments at *m/z* 133.8, 150.0, 176.2 and 229.9 Da suggests that a –OP(=O)(OH)O– bridge has been formed between the deoxyribose phosphate substituent and the C-8 of the adenine moiety, as shown in Scheme 3 (8-P-dAMP). In this Scheme, all the fragments



Scheme 2 Fragmentation of 8-oxo-dAMP observed in MS/MS analysis. The peaks corresponding to each fragment are shown in Fig. 6(b).

mentioned above (with corresponding signals shown in Fig. 6(c)) are indicated. The optimized geometry obtained by semiempirical AM1 calculations for the product 8-P-dAMP is shown in Scheme S1 (ESI†). The structure of this stable tetracyclic photoproduct detected in the reaction mixture can be compared with the rearranged intermediate proposed to explain the formation of the fragment observed at *m/z* 230 Da in the MS/MS analysis of 8-oxo-dAMP (see Fig. 6; Scheme 2, step (c) and Scheme 3).



Scheme 3 Proposed chemical structure and fragmentation of product P2 (M = 329).

These results indicate that 8-oxo-dAMP and the tetracyclic 8-P-dAMP are formed during the photosensitization of dAMP by pterin. 8-Oxo-dAMP and the tetracyclic compound 8-P-dAMP have a molecular weight of 347 and 329 Da, respectively. The full patterns of fragmentation obtained for each compound in the ESI^- mode (Fig. 6(b) and Fig. 6(c)) compared with that of dAMP (Fig. 6(a)) support these assignments.

Finally, it is interesting to mention that, although a previous LC-MS/MS analysis of 8-oxo-dAdo in ESI^+ mode has been reported,[37,38] only the molecular ion as $[M + H]^+$ ($m/z = 268$) and the 8-oxo-7,8-dihydro-adenosine fragment as $[A + H]^+$ ($m/z = 152$) due to the splitting of the *N*-glycosidic bond were detected. To the best of our knowledge, we describe in this work for the first time the full fragmentation patterns of dAMP and 8-oxo-dAMP in negative ion mode.

Conclusion

The photosensitization of 2′-deoxyadenosine-5′-monophosphate (dAMP) by pterin (PT) in aqueous solution under UV-A irradiation was investigated. We have shown that when an aerated solution containing dAMP and the acid form of PT was exposed to UV-A radiation the molecule of dAMP was consumed, whereas the photosensitizer (PT) concentration did not change significantly. During this process, O_2 was consumed and H_2O_2 was generated. To the best of our knowledge, this is the first time that evidence of photosensitized oxidation of dAMP by a pterin has been reported. In contrast, no evidence of a photochemical reaction induced by the basic form of PT was observed.

The rate constant of $^1O_2$ total quenching (k_t) by dAMP and the rate constant of the chemical reaction (k_r) between $^1O_2$ and dAMP in D_2O were determined and values of $(4.1 \pm 0.4) \times 10^5$ M^{-1} s^{-1} and $(8 \pm 3) \times 10^3$ M^{-1} s^{-1} were obtained, respectively. These values reveal that the reactivity of the studied nucleotide towards $^1O_2$ is very low. Calculations performed with these values and comparison of experiments carried out in H_2O and D_2O confirm that $^1O_2$ does not participate in the photosensitization of dAMP by PT.

The products of the studied process were analyzed by means of electrospray ionization (ESI) mass spectra. Two products with molecular weights of 347 and 329 (P1 and P2) were detected. MS/MS analysis strongly suggests that the former product is 8-oxo-7,8-dihydro-2′-deoxyadenosine-5′-monophosphate (8-oxo-dAMP), indicating that the photosensitized oxidation takes place *via* a type I mechanism. The pattern found in MS/MS spectra for P2 suggests that this compound contains a bridge formed between the deoxyribose phosphate substituent and the C-8 of the adenine moiety.

Finally, taking into account all the results shown in this work, the mechanism of the photosensitized oxidation of dAMP by PT can be summarized as follows:

$PT + h\nu \rightarrow PT^*$

$PT^* + dAMP \rightarrow PT^{-\bullet} + dAMP^{+\bullet}$

$PT^{-\bullet} + O_2 \rightarrow PT + O_2^{-\bullet}$

$O_2^{-\bullet} \rightarrow \rightarrow \rightarrow H_2O_2$

$dAMP^{+\bullet} + O_2^{-\bullet} \rightarrow$ 8-oxo-dAMP

The excitation of pterin is followed by an electron transfer reaction between dAMP and the pterin excited state, leading to the formation of the corresponding ion radicals ($PT^{-\bullet}$ and $dAMP^{+\bullet}$). In the following step, the electron transfer from $PT^{-\bullet}$ to O_2 regenerates the pterin (in agreement with the experimental results) and forms the superoxide anion. The latter may disproportionate with its conjugated acid $HO_2^\bullet$ to form H_2O_2 and react with the dAMP radical cation leading to the 8-oxo-dAMP.

Experimental

General

Pterin (PT) was purchased from Schircks Laboratories (Switzerland), and used without further purification. 2′-Deoxyadenosine-5′-monophosphate (dAMP) and ammonium acetate (NH_4OAc) were obtained from Sigma Chemical Co. Methanol (MeOH) was purchased from VWR. 1*H*-Phenalen-1-one (perinaphthenone, *Merck*) was purified as indicated in ref. 26. The pH of aqueous

solutions was adjusted by adding drops of 0.1–0.2 M aqueous NaOH or HCl solutions with a micropipette. The ionic strength was *ca.* 10^{-3} M in all experiments.

In experiments using D_2O as solvent, D_2O (>99.9%; *Euriso-top or Aldrich*), DCl (99.5%; *Aldrich*) in D_2O, and NaOD (*CEA*) in D_2O were employed.

For mass spectrometry analysis, the pH of samples was adjusted with a solution of NH_3 instead of NaOH in order to reduce the Na^+ concentration. Before analysis, samples were diluted with MeOH–10 mM NH_4OAc in H_2O (50 : 50 v/v).

Steady-state irradiation

Irradiation set-up. Aqueous solutions containing PT and dAMP were irradiated in 1 cm quartz cells at room temperature with a Rayonet RPR lamp emitting at 350 nm (Southern N.E. Ultraviolet Co.). The experiments were performed in the presence and in the absence of air. Oxygen-free solutions were obtained by bubbling with argon for 20 min.

Actinometry. Aberchrome 540 (Aberchromics Ltd.) was used as an actinometer for the measurements of the incident photon flux (P_0) at the excitation wavelength. Aberchrome 540 is the anhydride form of (*E*)α-(2,5-dimethyl-3-furylethylidene)-(isopropylidene)succinic acid which, under irradiation in the spectral range 316–366 nm, leads to a cyclized form. The reverse reaction to ring opening is induced by visible light. The method for the determination of P_0 has been described in detail elsewhere.[40] Values of the photon flux absorbed (P_a), were calculated from P_0 according to the Lambert–Beer law ($P_a = P_0\,(1–10^{-A})$, where A is the absorbance of the sensitizer at the excitation wavelength).

UV–VIS Analysis. Electronic absorption spectra were recorded on a Varian Cary-3 spectrophotometer. Measurements were made in quartz cells of 1 cm optical path length. The absorption spectra of the solutions were recorded at regular intervals of irradiation time, and the signals were averaged and smoothed with the Varian software. Experimental difference (ED) spectra were obtained by subtracting the spectrum at time $t = 0$ from the subsequent spectra recorded at different times t. Each ED spectrum was normalized yielding the normalized experimental difference (NED) spectrum.

High-performance liquid chromatography (HPLC). A System Gold HPLC setup (Beckman Instruments) was used to monitor and quantify the photosensitized reactions and photoproducts. A Pinnacle-II C18 column (250 × 4.6 mm, 5 mm; Restek) was used for product separation, eluting with a solution containing a mixture of 3% acetonitrile and 97% of a 20 mM potassium phosphate aqueous solution (pH 5.5). HPLC runs were monitored by UV–VIS spectroscopy at 260 nm.

Determination of the concentration of O_2. The O_2 consumption during irradiation was measured with an O_2-selective electrode (Orion 37-08-99). The solutions and the electrode were placed in a closed glass-cell of 130 ml.

Detection and quantification of H_2O_2. For the determination of H_2O_2, a Cholesterol Kit (Wiener Laboratorios S.A.I.C.) was used. H_2O_2 was quantified after reaction with 4-aminophenazone and phenol.[41,42] Briefly, 400 µl of irradiated solution were added to 1.8 ml 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 H_2O_2 solutions prepared from commercial standards were employed for obtaining the corresponding calibration curves.

Singlet oxygen ($^1O_2$) studies

Determination of the rate constants of $^1O_2$ total quenching (k_t) by dAMP. The rate constant of $^1O_2$ total quenching (k_t) by dAMP in acidic media (pD = 5.5) was determined by Stern–Volmer analysis of the $^1O_2$ luminescence quenching. The relationship between the ratio of the $^1O_2$ luminescence signals observed in the absence ($S_e°$) and in the presence (S_e) of quencher (Q: dAMP) and the quencher concentration is given by,

$$S_e°/S_e = 1 + K_{SV}\,[Q] \tag{8}$$

where K_{SV} is the Stern–Volmer constant. If Q interacts only with $^1O_2$, $K_{SV} = k_t\tau_\Delta$, where τ_Δ is the $^1O_2$ lifetime in the solvent used (D_2O) in the absence of Q, and the Stern–Volmer plot is linear.

The main features of the method have been described elsewhere.[20,43] Singlet oxygen was generated by photosensitization, using 1*H*-phenalen-1-one (PHE) ($\lambda_{ex} = 367$ nm, $\Phi_\Delta = 0.97$[26,27]) as a sensitizer. Because of the short $^1O_2$ lifetime (τ_Δ) in H_2O (3.8 µs), D_2O (where τ_Δ is much longer: 62 ± 3 µs) was used in all luminescence experiments.[36] Groups of experiments were carried out irradiating solutions of dAMP and PHE at 367 nm; at this wavelength, the investigated nucleotide does not absorb (Fig. 1). The solutions were irradiated in a 1 cm × 1 cm spectroscopic cell, under magnetic stirring, using a xenon/mercury arc (1 kW) through a water filter, focusing optics and a monochromator (ISA Jobin-Yvon B204, 6 nm bandwidth). The sensitizer (PHE) concentration was kept constant, whereas the dAMP concentration was varied within a series of experiments. A similar series of experiments were performed using PT as an $^1O_2$ sensitizer.

Determination of the rate constant of the chemical reaction between $^1O_2$ and dAMP (k_r). The rate of the sensitized photooxidation of dAMP was evaluated by following its disappearance by HPLC ($[dAMP]_0 = 100\,\mu M$). The main features of the method have already been described in detail.[28] Briefly, the rate of disappearance of a compound Q reacting with $^1O_2$ produced by sensitization is given by,

$$-d[Q]/dt = k_r\,[^1O_2]\,[Q] \tag{9}$$

If there is no interference by the oxidation product(s) and considering the steady-state approximation for the concentration of $^1O_2$, eqn (10) is obtained:

$$-d[Q]/dt = (P_a\Phi_\Delta k_r\,[Q])/(\,k_d + k_t\,[Q]) \tag{10}$$

where P_a (einstein L^{-1} s^{-1}) is the photon flux absorbed by the sensitizer, Φ_Δ is the quantum yield of $^1O_2$ production by the sensitizer and k_d is the rate constant of $^1O_2$ deactivation by the solvent ($1/\tau_\Delta$). In the case of dAMP (Q), $k_t\,[Q] << k_d$, and the conversion rate was very low (approx. 4%). As a consequence, the plot of [dAMP] *vs.* irradiation time was linear and, knowing P_a, Φ_Δ and k_d, k_r could be calculated from the slope of this plot ($(P_a\Phi_\Delta k_r\,[dAMP]_0)/k_d$).

For determining k_r, D_2O solutions (3 cm^3) containing dAMP (Q) and PHE as a sensitizer ($\lambda_{ex} = 367$ nm) were irradiated. The incident photon flux (P_0) at the wavelength of excitation of

the sensitizer was determined by actinometry (*vide supra*). The concentration of dAMP was determined by HPLC at different irradiation times. HPLC equipment (Hewlett Packard Series 1100) with a RP 18 LiChro CART 125-4 column was used for the determination of the dAMP concentration in this experiment. A solution containing a mixture of 2% acetonitrile and 98% of a 20 mM potassium phosphate aqueous solution (pH = 5.5) was used as eluent.

Comparison of continuous photolysis in H_2O and D_2O. Solutions of PT and dAMP were prepared in H_2O and D_2O. Couples of both types of solutions containing PT and dAMP at the same concentration were irradiated under identical experimental conditions. The effect of D_2O was evaluated by comparing results of UV–visible spectrophotometric analysis, HPLC analysis and determination of H_2O_2 concentration.

Mass spectrometry analysis

Irradiated and non-irradiated aqueous solutions of dAMP and PT were analyzed using mass spectrometry. The experiments were performed using an API 4000 Quadrupol mass spectrometer (Applied Biosystems) equipped with an electrospray ion (ESI) source (Turbo V(tm) Source, TurboIonSpray® probe, Applied Biosystems) operating in the positive and negative ion mode. N_2 was used as nebulizer, curtain, collision and auxiliary gas. Instrument control and data acquisition were carried out using the Analyst Software V 1.4 (Applied Biosystems). All parameter settings were optimized by flow injection experiments with standard solutions infused into the mass spectrometer using a syringe pump (Harvard Apparatus Inc) at an infusion flow rate of 10 μL min^{-1}. A mass calibration and optimization was performed using a polypropylene glycol standard solution from the manufacturer. Ionspray voltage was set to +4500 V and −4500 V, when the ESI source operated in positive and negative mode, respectively.

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